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Volume Editor: Stefan Schulz

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K. Dettner · L. Eberl · W. Francke · M. Givskov · P. Laurent
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In references *Topics in Current Chemistry* is abbreviated Top Curr Chem and is cited as a journal.

Visit the TCC content www.springerlink.com

Library of Congress Control Number: 2004115345

ISSN 0340-1022
ISBN 3-540-21308-2 **Springer Berlin Heidelberg New York**
DOI 10.1007/b83344

Springer is a part of Springer Science+Business Media
springeronline.com

Printed in Germany

Cover design: KünkelLopka, Heidelberg/design & production GmbH, Heidelberg
Typesetting: Fotosatz-Service Köhler GmbH, Würzburg

Printed on acid-free paper 02/3141/xv – 5 4 3 2 1 0

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Preface

Chemistry becomes particularly interesting when reaching out to other disciplines and this has been documented impressively over the last few decades by many cooperative efforts with biology. One of these fields which is now over forty years old is what most chemists know as pheromone research, started in 1959 by Butenandt with the identification of the first pheromone, bombykol. But pheromones are only part of the larger area of inter-individual chemical communication in general. This means of transportation of information is not used to a great extent by human beings, but vastly exploited by other living organisms. The research on these subject is part of Chemical Ecology, a discipline which tries to understand why secondary metabolites are produced by a certain organism and what their effects and functions are in an ecological perspective. This field goes beyond the normally anthropocentric view of traditional natural product research with its focus on application for human welfare.

To understand a given chemical communication system normally one needs a close and fruitful cooperation between chemists and biologists, making this field particularly interesting for many scientists. Chemists are involved in this research by identifying the compounds which provoke behavioral or physiological changes in the receiver, synthesizing them or their analogs, often in enantiomerically pure form, to prove a structure and to provide material for biological testing, working on the biosynthesis, and doing research on the large biomolecules which are needed for formation or processing of the exogenous signal molecules.

The terminology used in this research area is not well established in the chemical community; while the term pheromone is widely known, semiochemical is not. Nevertheless, chemicals used in the communication between individuals are correctly called semiochemicals; recently the equivalent term infochemicals was introduced. These compounds can be further divided into pheromones, compounds used in communication between individuals of the same species, while allelochemicals serve interspecific communication. Most pheromones are releasers, i.e. they provoke a behavioral change in the receiver. More rare are primers, which provoke physiological changes. Allelochemicals can be divided into kairomones, which are advantageous for the receiver, while the emitter benefits from allomones. Synomones are advantageous for both the emitter and the receiver.

Many different functions of pheromones have been found since Butenandt. Aggregation pheromones attract both sexes to a special location, while sex pheromones are offered by one sex only to attract or arouse the other one. Trail pheromones used by ants mark food trails and alarm pheromones change the state of alertness of conspecifics. These are only some of the functions pheromones can have, and similar different functions can be found in allelochemicals as well.

This two volume book tries to give an overview from a chemical perspective about the progress made during the last decade in semiochemical research. Synthesis, a key field of organic chemistry, is covered in many chapters, but the most innovative work is presented concisely in the first chapter by K. Mori, the focus of which is on the synthesis of pheromones, which is mostly target oriented and only rarely used to invent new methodology. Most work has been done on insect pheromones, which is reflected by the selected synthesis and the number of chapters devoted to insects in this book. This chapter is followed by a review on the lepidopteran pheromones by T. Ando. This order is the best investigated so far, primarily because of the great economic importance of moths and butterflies. Some species are ideally suited to serve as model organisms in studies going beyond the identification of pheromones to signal perception (see the chapter by Leal) and biosynthesis (see the chapter by Jurenka).

Pheromone identification is still difficult because the structure of unique compounds present in small amounts in mixtures of similar molecules has to be elucidated. This topic will be discussed in detail by Ando as well as by others, showing nicely the recent progress in analytical techniques. The following chapter by R. Jurenka deals with insect pheromone biosynthesis with special emphasis on lepidopteran pheromones and also covers genetic aspects. The subsequent chapter by C. Keeling et al. describes the hymenopteran semiochemicals (bees and ants), describing pheromones and allelochemicals. The hymenoptera add a certain flavor to the scene, because now the complexity of social insects with their many interactions comes into play, as well as the multi-level (multi-trophic) signals used by parasitoids.

The first volume ends with a chapter by G. Pohnert on chemical defence in the marine environment. Defense compounds, which can be regarded as allomones, are often, but not always, more complex than other semiochemicals and may have unique modes of action. The biological mechanisms are not always easy to unravel, which is shown by some of examples. The reader may be tempted to compare the chemical complexity with that of terrestrial insect defence, which can be found in the second volume chapter by D. Daloze and J.-C. Braekman. Insects thus do not only produce interesting pheromones, but also complex allelochemicals for their own protection.

The second volume starts with biochemistry and new insights into pheromone perception and transport by W. Leal. These findings show that specificity is not only achieved by uniquenss of compounds or blends, but also by the perceiving receptors and transport molecules. The following chapters on bugs and beetles by J. Millar and W. Francke and K. Dettner also cover

methods used for identification besides target-oriented synthetic approaches and discussion on the application of pheromones in insect control. The book will close with two chapters on highly complex and relatively simple organisms, namely mammals and bacteria. B. Burger points out the difficulty in working with behaviorally complex animals for establishing biological activity of certain compounds or mixtures. Furthermore, complexity can also be found in exocrine secretions of mammals which poses specific problems to the analytical chemist. P. Williams et al. explore chemical communication in bacteria. Microbiologists often use different terminology than zoologists, but from my point of view quorum-sensing-factors are still pheromones or at least semiochemicals. This exciting new field shows extensive progress and facilitates the application of biotechnological methods more easily than in more complex animals.

Hopefully the reader will get an overview of the recent work in the field after reading the chapters. Nevertheless, many exciting subjects have not been included, especially when recent reviews exist, as is the case for semiochemistry of arachnids (spiders and mites) or cockroaches. Further interesting subjects are pheromones of fish, reptiles, amphibians, algae, fungi, yeast, insect-plant interactions, etc. The research described here lays the foundation for further progress in the future, which will definitely benefit from the technological advances seen during the last years in chemistry and biology. A better understanding of the role and function of secondary metabolites may hopefuly be obtained.

Braunschweig, August 2004 Stefan Schulz

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Volume Editor: Stefan Schulz
ISBN 3-540-20828-3

Topics in Current Chemistry (2005) 240: 1–36
DOI 10.1007/b98314

Pheromone Reception

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Abstract Insects are analytical chemists *par excellence*. They perceive the world through semiochemicals with inordinate sensitivity. A male moth, for example, can detect a "scent of woman," i.e., a female-produced sex pheromone, even when the signal-to-noise ratio is very low. In a sense the antennae are "signal translators." The chemicals signals are "translated" into the language of the brain (nerve impulses or spikes) by an array of sensilla mainly located on the antennae. This information is conveyed to the brain for further processing. Chemical ecologists utilize insect antennae as biosensors for the identification of pheromones and other semiochemicals. The insect olfactory system is also highly selective, able to discriminate natural pheromones from molecules with minimal structural changes. In some cases, one stereoisomer functions as an attractant sex pheromone and its antipode is a behavioral antagonist (inhibitory signal). The specificity of the olfactory system seems to be achieved by two layers of filters. The first level of discrimination is determined by odorant-binding proteins (OBPs) that assist the hydrophobic pheromones to cross an aqueous barrier and reach their receptors. Both OBP and odorant receptor (OR) contribute to the specificity of the cell response and lead to the remarkable selectivity of the insect olfactory system. The members of the OBP-gene family, encoding the encapsulins, form a large group with olfactory and non-olfactory proteins. While the functions of many members of the family are yet to be determined, there is solid evidence for the mode of action of OBPs. Pheromones (and other semiochemicals) enter the sensillar lymph through pore tubules in the cuticle (sensillar wall), are solubilized upon being encapsulated by odorant-binding proteins, and transported to the olfactory receptors. Bound pheromone molecules are protected

from odorant-degrading enzymes. Upon interaction with negatively-charged sites at the dendritic membrane, the OBP-ligand complex undergoes a conformational change that leads to the ejection of pheromone. Direct activation of odorant receptors by odorant molecules initiates a cascade of events leading to the generation of spikes. Reverse chemical ecology is a new concept for the screening of attractants based on the binding ability of OBPs to test compounds.

Keywords Odorant-binding proteins · Odorant-degrading enzymes · Chiral discrimination · Encapsulins

1 Introduction

Insects perceive the world through small molecules which carry information (signature) for the recognition of potential mates, prey, and specific features of the environment, such as food sources, oviposition sites, etc. The information-carrying chemical compounds are referred to as semiochemicals, a generic term encompassing chemicals involved in intraspecific communications (pheromones) and interspecific interactions, such as kairomones (that give advantage to the receiver), and allomones (which benefit the sender). The entire olfactory process encompasses the perception of semiochemicals by a specialized apparatus in the periphery (normally the insect antennae; maxillary palpi in some cases), processing of signals in the antennal lobe, integration of these signals with other stimulus modalities in the protocerebrum, with ultimate translation into behavior (Fig. 1).

Because the chemical signals (semiochemicals) are normally produced in minute amounts and diluted in the environment with a complex mixture of chemical compounds derived from a myriad of sources, the olfactory system in insects evolved as a remarkably selective and sensitive system, which approaches the theoretical limit for a detector. For example, it has been estimated that the male silkworm moth is able to distinguish within 1 s 170 nerve impulses generated by the female silkworm moth's sex pheromone from 1700 spontaneous nervous impulses [1], thus, operating on a remarkably low S/N ratio!

In addition to sensitivity and selectivity, odor-oriented navigation in insects requires a dynamic process of signal deactivation (inactivation). While flying en route to a pheromone-emitting female (Fig. 2), males encounter pheromone molecules as intermittent signals comprised of short bursts of high flux separated by periods during which the flux is zero. The average duration of bursts of high flux is on the order of a millisecond and it decreases as the moth comes closer to the pheromone source [2]. Thus, a male moth has to detect rapidly and selectively minute amounts of pheromones buried in an "environmental mixture." Soon after the signal is detected, the pheromone detectors must be reset in a millisecond timescale so as to allow a sustained flight towards a pheromone source. In this chapter I provide a critical overview of our current under-

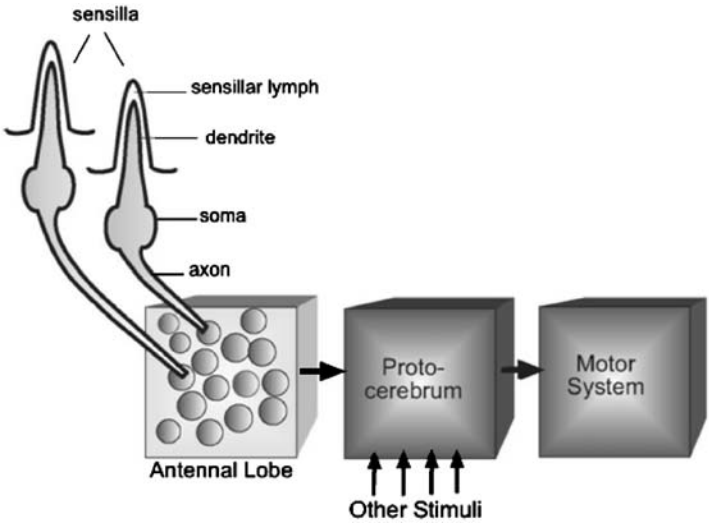

Fig. 1 Schematic view of the overall olfactory processing in insects. Pheromones and other semiochemicals are detected by specialized sensilla on the antennae, where the chemical signal is transduced into nervous activity. The olfactory receptor neurons in the semiochemical-detecting sensilla are connected directly to the antennal lobe. Here the semiochemical-derived electrical signals are processed and sent out (through projection neurons) to the protocerebrum. Olfactory information is then integrated with other stimulus modalities, a decision is made, and the motor system is told what to do

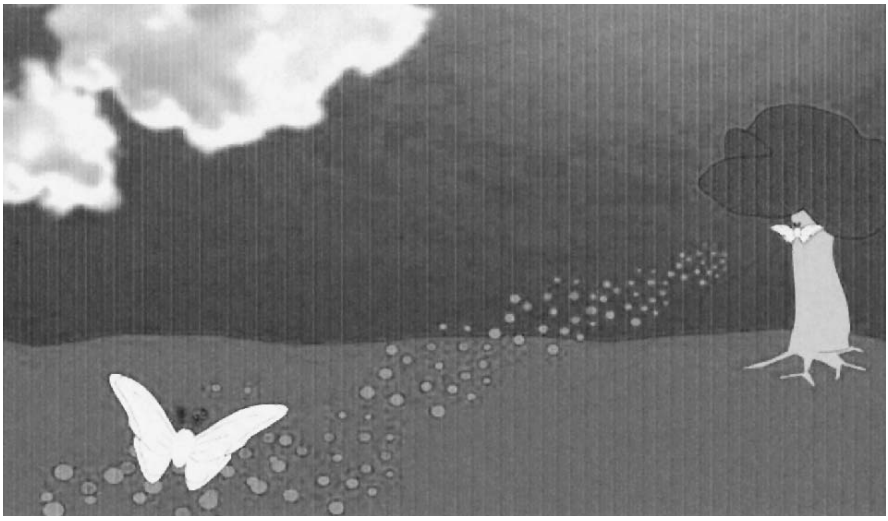

Fig. 2 Cartoon illustration of sex pheromone-mediated communication in insects. A female moth advertises her readiness to mate by emitting a chemical signal that permeates the air. Odorant-oriented navigation allows a male to pin-point the pheromone source

standing of olfactory mechanisms in insects, with emphasis on the molecular basis of pheromone reception.

2 Sensory Physiology

Largely, the insect detectors for pheromones and other semiochemicals are arrays of hair-like sensilla distributed over the surface of the antennae and palps. In some species, such as scarab beetles [3, 4] and the honeybee [5], semiochemicals are received by olfactory plates. The more ubiquitous hair-like sensilla typically consist of hollow cuticular hairs (10–400 µm long, 1–5 µm thick) innervated by one or several olfactory receptor cells (neurons) and three auxiliary cells [6].

The distal part of these receptor cells, the dendrites (0.1–0.5 µm in diameter), extend into the hair lumen (Fig. 3), whereas their axons are connected directly to the antennal lobes in the brain where they make the first synaptic contacts. In the giant silkmoth, *Antheraea polyphemus*, for example, each male

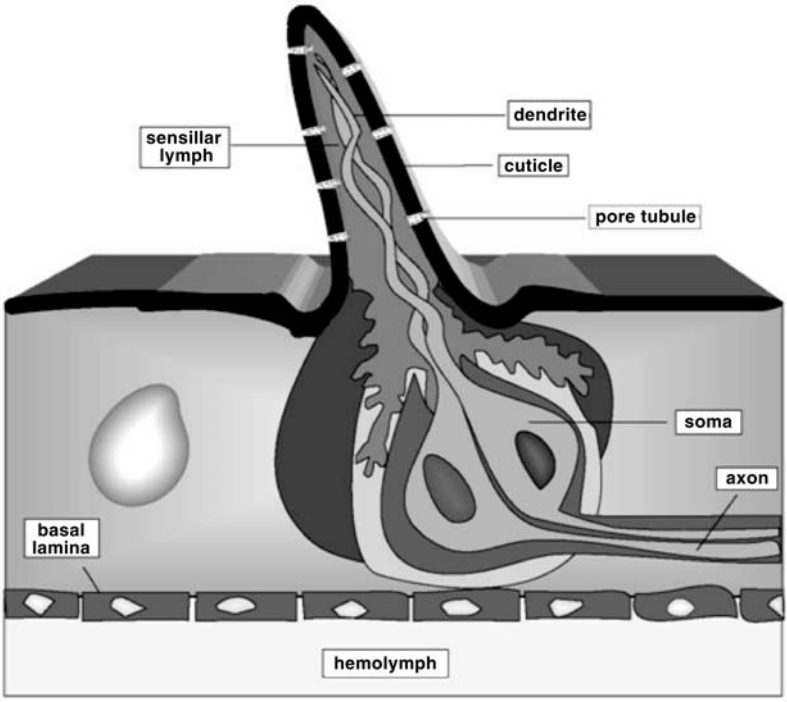

Fig. 3 Diagrammatic representation of a pheromone-detecting sensillum trichodeum of a moth antenna. Note the compartmentalization of the lymph and particularly its isolation from the hemolymph

antennae has ca. 60,000 pheromone-sensitive sensilla trichodea and 10,000 sensilla basiconica for the detection of other semiochemicals [7, 8]. On the other hand, females lack pheromone-detecting sensilla and have ca. 12,000 sensilla basiconica [9].

The first electrophysiological methods to study stimulus-response characteristics were developed along with the discovery of the first sex pheromones [10]. Upon interaction of pheromones and their receptors, the electrical conductance of the receptor cell membrane is modified producing a local depolarization, i.e., a receptor potential. Combined receptor potentials of many sensilla can be recorded in an electroantennogram (EAG). This is a simple approach to investigate stimulus-response characteristics, but requires pure chemicals. A powerful technique for the identification of pheromones, the gas chromatographic-electroanntenographic detection (GC-EAD) combines an EAG as a biological detector with a gas chromatograph (GC) for the separation of mixtures. The effluent from the GC column is split and sent towards a flame-ionization detector (FID) and an EAG, thus allowing the detection of stimuli "on the fly" from the GC. This "short-cut bioassay" allows the identification of minute chemical signals from highly contaminated samples (Fig. 4). This technique, widely applied in pheromone research, has also been utilized for the determination of the absolute configuration of pheromones, with stereoisomers being separated on a chiral phase capillary column [11].

The receptor potential, generated by interaction of pheromones and their receptors, spreads passively from the site of stimulation (somewhere in the

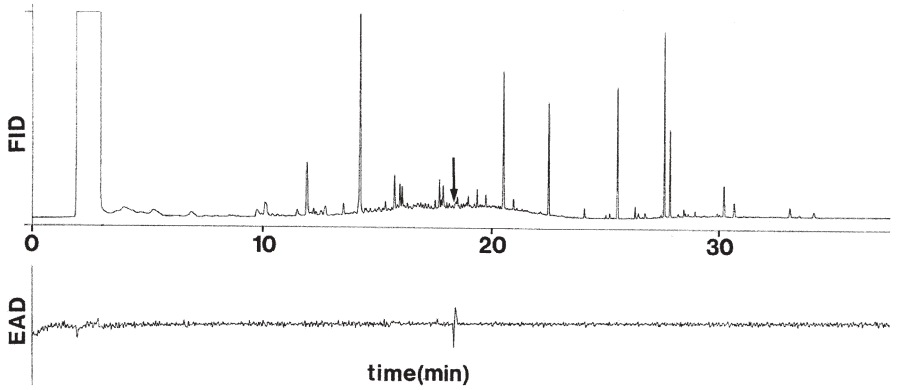

Fig. 4 Gas chromatographic traces of extracts from females of the pale brown chafer *Phyllopertha diversa* monitored by a conventional detector, flame-ionization detector (FID), and a biosensor, electroantennographic detector (EAD), using a male antenna as the sensing element. Although the peak of the sex pheromone (*arrow*) is hardly seen in the FID trace, its pheromonal activity was initially indicated by the strong EAD peak. Structural elucidation, followed by synthesis and behavioral studies lead to the identification of an unusual sex pheromone, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione [124]. It is unlikely that this minor compound would be fished out by a bioassay-oriented isolation procedure

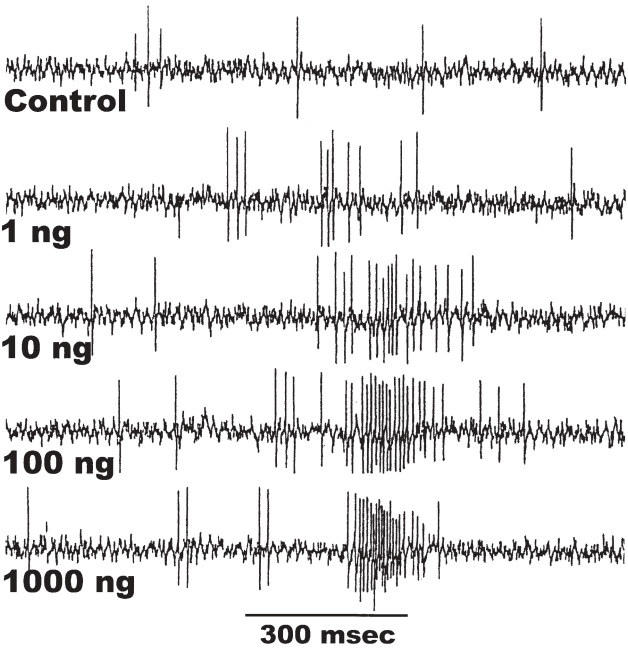

Fig. 5 Single sensillum recordings from the pheromone-detecting sensilla placodea on *P. diversa* male antennae. Note a dose-dependent increase in spike frequency after stimulus application for 300 ms (*bar*)

dendrite) towards an electrically-sensitive region (probably in the soma) where nerve impulses (spikes) are elicited [12] by the opening of voltage-dependent ion channels. Although intracellular recording of these nervous activities are technically difficult (if at all possible), olfactory sensilla allow extracellular recordings (Fig. 5), a technique called single sensillum recordings (SSR). As opposed to EAG, SSR represents the nervous activity generated by the neuron(s) innervating a single unit (sensillum) of the entire "compound nose." The number of olfactory receptor neurons (ORNs) in most olfactory sensilla ranges between two to five, but there are many exceptions, including sensilla placodea in wasps with as many as 140 ORNs [13]. Typically, multiple neurons in the same sensillum can be distinguished by different spike amplitudes, thus, allowing investigation of stimulus-response characteristics for each neuron.

Earlier experiments based on EAG and SSR highlighted the inordinate specificity and sensitivity of the insect olfactory system. While minimal structural modifications to pheromone molecules render them inactive [12], a single molecule of the native ligand is estimated to be sufficient to activate an olfactory neuron in male antennae [14]. The large number of detectors certainly contributes to the sensitivity of the olfactory system, but selectivity is a matter of

molecular recognition at the periphery. As described below, this remarkable selectivity of the insect olfactory system is likely to be achieved in two steps with odorant-binding proteins and odorant receptors participating as two "layers of filters."

3
Perireceptor Events in Insect Olfaction

Each sensillum in the insect antennae works as a "signal transducer" that responds to a specific chemical signal and "translates" it into the language of the brain, i.e., electrical signals. Interaction of pheromones and other chemical signals with their odorant receptors triggers a cascade of intracellular events called signal transduction (*sensu stricto*) which leads to nervous activity (spikes). Extracellular processes associated with the uptake, binding, transport, and release of the hydrophobic pheromones to their receptors as well as the post-interactive events related to inactivation of chemical signals are referred to as the "perireceptor events" [15] or early olfactory processing.

3.1
Odorant-Binding Proteins

In order to convey their message, pheromones and other semiochemicals must reach the dendritic surfaces of olfactory receptor neurons where the olfactory receptor proteins are located (Fig. 6). These odorant receptors are surrounded by an aqueous environment – the sensillar lymph. Although thin (1 µm), this aqueous layer is impenetrable for hydrophobic compounds per se. Thus, the transport through this barrier is assisted by odorant-binding proteins (OBPs). OBPs that are localized predominantly in pheromone-detecting sensilla with demonstrated ability to bind pheromones are referred to as pheromone-binding proteins (PBPs). Throughout this chapter the terms OBPs and PBPs are used as synonyms, although PBPs are OBPs which binds pheromones. PBPs are not only specific to antennae, but in some cases they occur mainly (if not only) in the sensillar lymph of male antennae. Strictly speaking, PBPs are not expressed in the sensillar cavity. They are expressed in auxiliary cells and secreted into the lumen; thus, the mature protein can be detected in the sensillar lymph. General odorant-binding proteins (GOBPs) are expressed in antennae of both sexes, or predominantly in female antennae, which are assumed to bind semiochemicals other than sex pheromones.

OBPs were initially identified in Lepidoptera and later isolated and/or cloned from various insect orders, namely, Coleoptera, Diptera, Hymenoptera, and Hemiptera ([16] and references therein). Recently, they have been identified from a primitive termite species [17], thus, suggesting that this gene family is distributed throughout the Neopteran orders. The three orders most

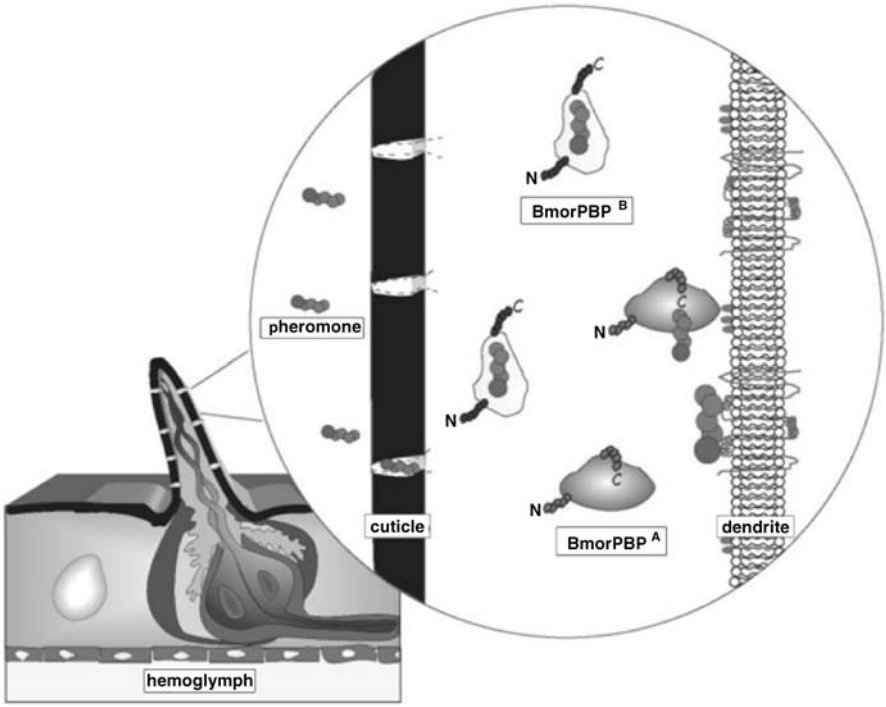

Fig. 6 Schematic representation of the proposed model for the mode of action of insect OBPs. Pheromones (and other semiochemicals) enter the sensillar lymph through pore tubules in the cuticle (sensillar wall), are solubilized upon being encapsulated by odorant-binding proteins, and transported to the olfactory receptors. Bound pheromone molecules are protected from odorant-degrading enzymes. Upon interaction with negatively-charged sites at the dendritic membrane, the OBP-ligand complex undergoes a conformational change that leads to the ejection of pheromone. In BmorPBP, this is achieved by the formation of a C-terminal α-helix in $BmorPBP^A$ that occupies the cavity that is the binding site in $BmorPBP^B$. In this model, the pheromone molecule (not the complex) activates the odorant receptor, thus, initiating a cascade of events leading to spike generation. As depicted in Fig. 1, the spikes travel through the axon to the antennal lobe

widely studied are the Lepidoptera (Saturniidae, Bombycidae, Sphingidae, Lymantridae, Tortricidae, and Pyralidae), Coleoptera mainly scarab beetles (Scarabaeidae), and Diptera (with the bulk of the literature focusing on *D. melanogaster*). In all rutelines (subfamily Rutelinae) investigated to date only one OBP has been found in each species, such as the Japanese beetle, *Popillia japonica*, the Osaka beetle, *Anomala osakana* [18], the Oriental beetle, *Exomala orientalis* [19], the cupreous chafer, *A. cuprea*, and *A. octiescostata* [20]. Binding data and homology suggest that the OBPs from these beetles are indeed PBPs. On the other hand, at least two OBPs have been identified in each melolonthine (subfamily Melolonthinae) species investigated, i.e., the pale

brown chafer, *Phyllopertha diversa* [21], the large black chafer, *Holotrichia parallela* and the yellowish elongate chafer, *Heptophylla picea* [22]. One of the two OBPs for each melolonthine species shows remarkable similarity to the pheromone-binding proteins from rutelines, whereas the second type of OBP forms a divergent group [20].

The literature describing the number of OBPs in different species is controversial with numbers ranging from 1 to 51 OBPs per species, but these values seem to be inaccurate. Even if a single OBP is involved in the detection of multiple compounds (see below), one would expect that the insect antennae possess multiple OBPs considering that insects can detect a number of physiologically relevant compounds (pheromones, flower scents, green leaf volatiles, other plant-derived compounds, etc.), which vary largely in their chemical structures. However, it is not clear how many proteins function as OBPs in insects. The discrepancy in the literature may be related to the method of "identification" of OBPs. Protein-based approaches are aimed at the isolation and identification of OBPs, followed by the cloning of the genes (or cDNAs) encoding these proteins. On the other hand, the gene-based approaches give little emphasis to expressed and functional proteins. While minor OBPs may be expressed at levels below the detection limits of the protein-based methods, the gene-based approach may lead to putative proteins which may not even be expressed in the sensillar lymph (of insect antennae). Another complication is that an identifying feature of insect OBPs, the six cysteine residues, is sometimes misleadingly used. The pheromone-binding proteins identified to date have six well-conserved cysteine residues, but this is not exclusive to OBPs and PBPs; insect defensins, for example, also have six well-conserved cysteine residues too. The spacing pattern between cysteine residues may indicate that a putative OBP belongs to the same OBP-gene family, but some members of this family may not be involved in olfaction [16]. The cysteine spacing pattern shows some variation when comparing OBPs from different insect orders (or different groups of OBPs), but they all have three residues between the second and the third Cys and eight residues between the fifth and the sixth Cys. Considering that the six cysteine residues play a pivotal role in the folding of pheromone-binding proteins [23–25], it is unlikely that other OBPs deduced from *Drosophila* genome sequence and having as many as 12-Cys residues [26] (Obp58b, Obp58c, Obp58d, Obp83c, Obp93a) would bind, transport, and release ligands in the same way as pheromone-binding proteins (like BmorPBP) do.

Out of the 51 deduced *Drosophila* OBPs, expression data is known only for 28 putative OBPs. Galindo and Smith used an elegant molecular approach to study expression of deduced *Drosophila* OBPs [27]. They fused several kilobases of upstream regulatory sequence for each OBP gene to a reporter gene encoding a nuclear-localized β-galactosidase. The transgenic flies carrying reporter constructs fused to each OBP promoter were stained for β-galactosidase activity [27]. Surprisingly, most members of the OBP-gene family were detected in various taste organs and olfactory tissues and some of them were expressed exclusively in taste organs. A caveat to their method is that the expression of the

proteins was not confirmed by immunocytochemistry using anti-OBP antibodies, thus not excluding completely the possibility that the reporter gene only, not the OBP genes, were expressed in some cases. Although it has been suggested that the *Drosophila* OBP-gene family comprises as many as 51 putative OBPs [26], only seven of them have been demonstrated to be expressed specifically in olfactory organs of *Drosophila* adults (antennae only or antennae and maxillary palpi): Obp19a, Obp57a, Obp69a (formerly named PBPRP-1), Obp83a (PBPRP-3, OS-F), Obp83b (OS-E), Obp84a (PBPRP-4), and Obp99d. Two other putative OBPs – Obp28a (PBPRP-5) and Obp76a (LUSH) – were detected in the antennae of adults as well as in larval chemosensory organs [27].

That LUSH functions as an odorant-binding protein was inferred from olfactory trap assays comparing wild-type adults with transgenic flies [28]. For this bioassay [29], traps are made of microfuge tubes and two pipette tips for each tube, one with the narrow end inserted into the severed end of the microfuge tube and the other placed as a sleeve in the opposite direction. Flies that are attracted to the lure (which is placed inside the microfuge tube) can get through the small aperture, but are unlikely to find a way out of the trap. A trap is placed inside a Petri dish (100 mm×20 mm) where ten adults are tested. These tests (performed during a period of time not specified in the original publications [28, 30]) showed no difference between wild-type and a LUSH-deficient mutant when a panel of 60 compounds was tested at low concentrations. However, there was a significant increase in the number of mutant flies in traps containing high concentrations of ethanol, propanol, and butanol. The high trapping at high concentrations of these alcohols could be due to increased attraction or a defect in avoidance. The authors supported the latter hypothesis because wild-type flies are less likely to be trapped in baits with an attractant (yeast extract) spiked with 25% ethanol. In other words, the so-called "avoidance to ethanol" would decrease the catches in traps baited with an attractant. To me these bioassays do not demonstrate conclusively that the increased trapping of *lush* mutant flies is due to a defect in avoidance rather than for an increased attraction to high concentrations of ethanol. Indeed the results suggest a decrease in trapping of wild-type flies in the yeast+25% ethanol traps as compared to yeast traps. The same tests, however, showed that the number of LUSH-deficient mutant flies caught in the yeast+25% ethanol traps were twice as much the number of flies captured either in traps baited only with yeast extract or those baited with ethanol only (see Fig. 3C in [30]). If this is due to avoidance to ethanol (rather than an attraction) why did the trapping of the *lush* mutant flies increase in the yeast-25% ethanol baits as compared to the baits with yeast alone? If they do not avoid ethanol at high concentrations, what is the explanation for the synergistic effect of ethanol and yeast extract? Last but not least, if flies are not attracted to ethanol why do they get through the ingenious device and get trapped? The inconsistency of these results may be derived from the design of the bioassay in which flies are subjected to still air and the only quantified observation is the end-product of the behavior (trapping). Also, there are no controls tested under identical conditions. Indeed, when flies

were tested with two-choice assay, the T-maze assay [31, 32], the *lush* mutant responded normally to ethanol not only at low but also at high concentrations [33]. It was observed, however, that the LUSH-deficient mutant lost attraction towards low concentrations of benzaldehyde while being repulsed by high concentrations, whereas the wild-type mutants showed attraction and repellency at low and high concentrations, respectively [33]. On the basis of these experiments, one cannot conclude that LUSH is involved in the binding, release, and delivery of either ethanol or benzaldehyde to olfactory receptors. A caveat to all bioassays utilizing benzaldehyde is the possible effect of benzoic acid. Typically, benzaldehyde is purchased from commercial sources and utilized without purification. It is, therefore, a mixture of at least benzaldehyde and benzoic acid (Fig. 7). Particularly when high doses are tested the amount of benzoic acid may be physiologically relevant.

Using a specific antibody, Shanbhag and collaborators [34] demonstrated that LUSH is expressed in sensilla trichodea of the *Drosophila* antennae along with two other putative odorant-binding proteins Obp83a (PBPRP-3, OS-F) and Obp83b (OS-E). When antennal sections of the LUSH-deficient mutant were la-

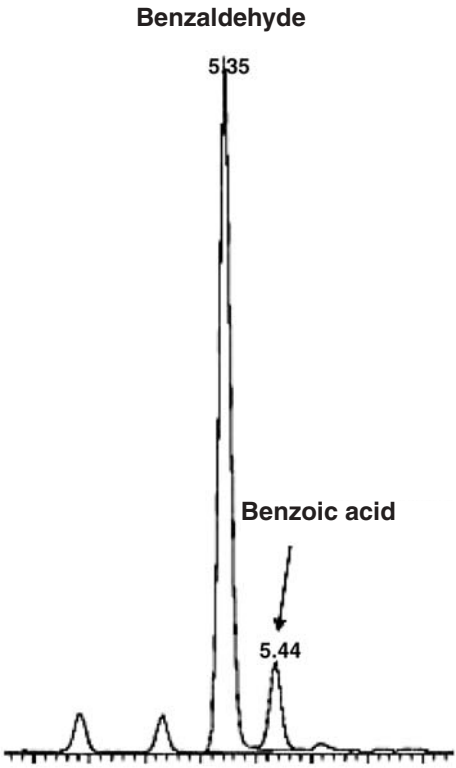

Fig. 7 GC-MS analysis data of a commercially available sample of benzaldehyde. Note the large peaks of impurities, particularly the considerable amount of benzoic acid

beled, they did not show any labeling with anti-LUSH, but showed normal staining with anti-Obp83a and anti-Obp83b [34]. Unfortunately, no electrophysiological data is available indicating that these sensilla are involved in the perception of benzaldehyde (or alcohol); it is known, however, that benzaldehyde and butanol are detected by sensilla coeloconica [35] and sensilla basiconica [36] and that the response for the whole antennae (EAG) recorded from *lush* mutant and wild-type flies were not different [28]. Also, there is no binding data supporting that LUSH binds benzaldehyde. Recently, the crystal structures of apo-LUSH was solved along with structures of LUSH bound to ethanol, propanol, and butanol [37], but there is no biochemical data indicating that LUSH binds to any ligand at physiologically relevant concentrations. Even if single sensillum recording experiments were to indicate that sensilla trichodea in *Drosophila* are involved in the detection of benzaldehyde or ethanol, one cannot make a clear-cut correlation between the defect of the *lush* mutant flies and the role of LUSH in olfaction. This is due to the co-expression of three putative odorant-binding proteins in these sensilla, namely, LUSH, OS-F, and OS-E [34].

In marked contrast to the ambiguous evidence for LUSH, there is growing evidence in the literature that other insect pheromone-binding proteins, such as, the PBP from the silkworm moth, *Bombyx mori* (BmorPBP), bind, solubilize, carry, and deliver pheromones to the pheromone receptors. (1) BmorPBP is predominantly expressed in the male antennae [38] and binds to bombykol, a cognate ligand [38] with some degree of specificity [39]. (2) BmorPBP is specifically localized in the long sensilla trichodea of males [40]. Females possess the same type of sensilla but rather than PBP they express a general odorant-binding protein. The long sensilla trichodea in male *B. mori* have been demonstrated to be the pheromone detectors [14], whereas in females they respond to benzoic acid and linalool [41]. (3) BmorPBP undergoes a pH-dependent conformational change [39, 42]. (4) The surfaces of dendrites are negatively-charged [43, 44], thus, generating localized low pH. (5) Evidence from structural biology (see below) demonstrates that the low pH (as expected near the surface of dendrites) triggers the formation of an additional C-terminal α-helix that fills the binding pocket thus leaving no room for pheromone in the binding cavity. (6) Binding assays showed that BmorPBP binds bombykol at the sensillar lymph pH but not at low pH as on the surface of dendrites [16].

3.1.1 Encapsulins, Members of the OBP-Gene Family

Insect OBPs are secretory proteins whose only posttranslational modification is the formation of three disulfide bridges [39, 45] from six cysteine residues. That six cysteine residues are well conserved in OBPs from species of the same order is a hallmark of these proteins. The disulfide links of OBPs in a few species have been determined by analytical methods, first in the OBPs from *B. mori* [45, 46]. As part of our attempt to get better insight into the structural biology of pheromone-binding proteins, we have determined the disulfide linkages

in recombinant and native BmorPBP [45]. The disulfide structures of the native PBP and GOBP-2 from *B. mori* were also identified by Scaloni and collaborators [46]. These OBPs showed the same cysteine pairing, i.e., Cys19-Cys54, Cys50-Cys108, and Cys97-Cys117. Similar disulfide structures were determined in the olfactory proteins from honeybee, *Apis mellifera*, ASP1 and ASP2 [47, 48] in the OBPs of the locust [49] and the paper wasp [50]. Therefore, the disulfide bridges of all OBPs analyzed to date show the profile of the first cysteine residue connected to the third one, the second linked to the fifth, and the fourth bound to the sixth, i.e., Cys(I)-Cys(III), Cys(II)-Cys(V), and Cys(IV)-Cys(VI). Another group of olfactory proteins, the chemosensory proteins (CSPs), differ from the six-cysteine-OBPs not only in the number of cysteine residues, but also in the function of the residues regarding the rigidity of their three-dimensional structures While in OBPs the three disulfide linkages play a pivotal role in the knitting together at least four of the helices (see below), the two disulfide bridges in CSPs close small loops involving residues 29 and 36 and 55 and 58 and, consequently, seem to have little rigidifying effect on the overall structure of CSPs [51].

Although the occurrence of six conserved cysteine residues, the spacing patterns of these residues, and possibly the pattern of disulfide structures are hallmarks of OBPs, the six-cysteine criterion alone is not sufficient to classify a certain protein as an olfactory protein [16]. It is important to demonstrate that an OBP is expressed only (or predominantly) in olfactory tissues. Evidence for their ability to bind odorants is also desirable, but *not sine qua non*. One of these criteria alone would not be enough to define a given protein as an OBP. For example, bovine serum albumin (BSA) binds to insect pheromones (Leal, unpublished data) and yet it is not an OBP because it not expressed in insect olfactory tissues. Conversely, a protein specific to antennae is not necessarily an OBP. There are other proteins that may be expressed in antennae but not in control tissues. Non-OBPs specifically accumulated in insect antennae have been previously detected (Ishida and Leal, unpublished data). Also, a glutathione-*S*-transferase has been reported to be expressed specifically in antennae of *M. sexta* [52].

The six conserved cysteine residues in a protein exhibiting the same pattern of cysteine spacing along with significant sequence similarity suggest that the protein may belong to the same structural (folding) family as PBPs and, consequently, infer that it may function in the same fashion. The assumption that such a protein is involved in olfaction, however, would be compromised if the protein was identified in non-olfactory tissues. Even if a non-olfactory protein has the same function as an OBP (carrier, for example), one has to keep in mind that the requirements for transport of hydrophobic ligands in non-olfactory tissues may not necessarily be as stringent as those for the fast delivery and inactivation of chemical signals.

Unfortunately, the term OBP has been rather imprecisely used in the literature. It sometimes refers to the olfactory function played by proteins, such as the pheromone-binding protein from BmorPBP. However, quite often OBP

refers to members of a gene-family, which may not be involved in olfaction. For example, a number of proteins with four conserved cysteine residues isolated from hemolymph of insects [53–55] are referred to as OBPs because of their sequence similarities and their conserved cysteine residues. Of particular note is the fact that the sensillar lymph (where OBPs assist in the transport of semiochemicals) is compartmentalized in olfactory tissues and completely isolated from the hemolymph by the epithelial cells, septate junctions between them, and basal membrane (Fig. 3). Indeed, the composition of the sensillar lymph is remarkably different from that of the hemolymph [56], particularly the unusual ion concentration (200 mmol/l K^+, 40 mmol/l Na^+), thus generating a transepithelial potential of +40 mV [6, 12, 56]. This compartmentalization is, therefore, the raison d'être for signal transduction. It is conceivable that these hemolymph proteins are part of a large family of carrier proteins that perform diverse functions in insects [55], but they are unlikely to be involved in any of the olfactory processes, particularly the perireceptor events.

The lack of a better term to separate the gene-family from the olfactory function performed by a few members of the family may be misleading. For example, Krieger and Ross [57] isolated two isoforms of a protein (GP-9) from the thorax of queens of the red imported fire ant, *Solenopsis invicta*, which has the same spacing pattern of six cysteine residues as observed in moth pheromone-binding proteins. Because the monogyne social form (colony having a single queen) and polygyne (multiple queens) form had only one (GP-9B) and two isoforms (GP-9B and GP-9b) of the protein, respectively, they suggest that these proteins may cause differences in worker's ability to recognize queens [57]. This work is widely referred to as "the first evidence for the direct involvement of PBPs in olfaction" [58]. Another citation is: "the two PBP alleles governing social behaviors suggest that different receptors might be activated by a specific PBP allele-social pheromone complex" [58]. The work by Krieger and Ross [57] lacks evidence that GP-9 either functions as a pheromone-binding protein or has any olfactory function. First, the protein was isolated from the thorax of queens; the existence of the protein in the sensillar lymph (where PBP functions) has never been demonstrated. Second, it is believed that workers detect a specific chemical signature related to by *Gp-9*^{b} gene in polygyne queens and thereby accept them, whereas all sexually mature queens lacking the same chemical signal are attacked and killed [59]. In other words, queens send off the signal that workers detect. If one is interested in "detection" of these semiochemicals, the olfactory system of workers (receivers) is to be investigated, not queens who are the emitters. Is it the lack of a "PBP" that makes them perceive a certain chemical signal? An elegant work [60] demonstrated that the monogyne queen emits a primer pheromone that makes the workers aggressive, i.e., the behavior is elicited because the workers can detect a certain primer pheromone, not because the monogyne workers are genetically impaired (anosmic) to some smell. In conclusion, Krieger and Ross work showed the existence of proteins from the OBP-gene family in the thorax of the red import fire ants, but there is no evidence for any chemosensory function, much less to explain differences in social behavior.

The field of insect olfaction could be devoid of such dogmas by the use of adequate terminology. Previously, I proposed that proteins of the *PBP*-gene family in general be named "encapsulins" [16]. As indicated by the structures of a hemolymph protein (GSP), THP12 [61], a pheromone-binding protein, BmorPBP [23–25], a chemosensory protein, MbraCSP6 [51], and a cockroach PBP [58], members of the *OBP*-gene family belong to the same structural family of helical proteins. In addition, their structures suggest that the olfactory and non-olfactory members of the *OBP*-gene family encapsulate hydrophobic ligands, with the ability to transport them in aqueous environments. The term "encapsulins" implies the common role of encapsulating small ligands. The encapsulin family would, therefore, encompass odorant-binding proteins (OBPs and PBPs), CSPs, and other non-olfactory proteins. The proposed terminology is not a replacement for pheromone-binding proteins, but rather would avoid mixing up function and gene family. Thus, all members of the PBP-gene family with no evidence for olfactory function (tissue specificity, binding ability and the like; see above) should be referred to as "encapsulins," not odorant-binding proteins.

3.1.2 Mechanism of Pheromone Binding and Release

3.1.2.1 The Pheromone-PBP Complex Model

In one of the earliest modes of action proposed for OBPs, Pelosi [62] hypothesized that – in analogy to a model of bacterial chemotaxis – OBPs not only solubilize specific pheromones, but trigger the olfactory receptors when bound to odorant molecules [62]. In a later version of the pheromone-PBP complex model, it was suggested that electrostatic and hydrophobic interactions from both the bound ligand and ligated protein are necessary and sufficient for receptor activation [63]. The notion that olfactory receptors are activated by interactions with pheromone-PBP complexes is not supported by recent findings. The structure of the BmorPBP-bombykol complex [23] showed the pheromone is completely buried inside the protein, thus, indicating that in the bound form it is highly unlikely that the ligand (pheromone) interacts directly with the pheromone receptor. Based solely on the structural biology of the BmorPBP-bombykol complex, one cannot refute Pelosi's model. However, recent electrophysiological evaluation of odorant receptors in a heterologous system suggest that ligand per se, not the complex, activates the odorant receptors. A putative odorant receptor from *Drosophila*, Or43a [64, 65], expressed in *Xenopus laevis* oocytes [66], was activated by four odorants, i.e., cyclohexanone, cyclohexanol, benzaldehyde, and benzyl alcohol [66] in the absence of *Drosophila* OBPs. This is in agreement with an earlier work showing that PBP was not necessary to obtain pheromone-dependent responses in cultured olfactory receptor neurons of *Manduca sexta* [67]. In the earlier case, however, the possibility that OBPs

have been produced in vitro and were present in cultured ORNs could not be excluded. The expression of a *Drosophila* odorant receptor in a heterologous system is very likely devoid of OBPs. In conclusion, the evidence that *Drosophila* receptors expressed in *Xenopus* oocytes responded to odorants in the absence of OBPs speak against the OBP-odorant complex model. However, OBPs are essential for the kinetics and sensitivity of the insect olfactory system (see below).

3.1.2.2 Conformational Changes of OBPs

My collaboration with structural biologists led to the serendipitous discovery of a pH-dependent conformational change in pheromone-binding proteins [39]. When Kurt Wüthrich and his co-workers analyzed by NMR our highly purified samples of ^{15}N- and ^{15}N,^{13}C-labeled BmorPBP, they were surprised with the number of "extra" peaks indicating inhomogeneity of the sample, possibly due to degradation or contamination. We were also surprised because, before sending the first samples to Zurich, we first analyzed the effect of lyophilization by chromatography, gel electrophoresis, mass spectrometry, circular dichroism (CD), etc. We found no evidence for degradation or any other changes in the samples before and after lyophilization, thus suggesting the samples were pure. The same was observed with the samples returned from Zurich; they showed "extra" peaks by NMR, but they were pure! A thorough investigation of the stability of the protein by various spectroscopic methods led to the conclusion that, although very stable, BmorPBP showed a pH-dependent conformational change. While the secondary structure of the protein was affected only slightly by changes in pH (as demonstrated by far-UV-CD), the tertiary structure (analyzed by near-UV-CD) exhibited a conformational transition between pH 6 and pH 5 [39]. It was somewhat intriguing that the protein kept its secondary structure but changed its tertiary structure at low pH. It became evident later that one helix is unfolded at low pH, whereas another flexibly disordered part of the molecule folds into an α-helix, thus maintaining the overall content of secondary structure (see below). pH titration using NMR showed that at pH below 4.9 there was a single form, whereas another form of the protein existed at pH above 6 [42]. We named these forms the "A" (BmorPBP^{A}) and "B" (BmorPBP^{B}) forms, respectively for "acid" and "basic" form. Note that strictly speaking at the bulk pH of the sensillar lymph (6.5) [56] the "B" form is not basic, but this was a rather simplified nomenclature. At the intermediate pH in the first NMR analysis the sample was a mixture of BmorPBP^{A} and BmorPBP^{B}, thus, explaining the "extra" peaks.

Conformational changes in BmorPBP were also studied in the presence of model membranes using CD spectroscopy. Conformational changes more pronounced than those observed at low pH were detected in the presence of anionic vesicles of dimyristoylphosphatidylglycerol (DMPG), whereas the effect of neutral phospholipids vesicles, dimyristoylphosphatidylcholine (DMPC) was

marginal [39]. The presence of a physiological concentration of KCl reduced the effect, but the interaction with negatively-charged membrane in the presence of KCl was still comparable to the effect of lowering the pH. The negatively-charged head groups of lipids in cell membranes give rise to an electrical surface potential, which in turn decreases the surface pH [68].

There is growing evidence in the literature that the pH-dependent conformational change in BmorPBP (and other PBPs) is physiologically relevant. Negatively-charged surface coats have been demonstrated on the pore tubules and dendritic membranes of olfactory hairs of male *A. polyphemus* by application of cation markers, such as lanthanum, ruthenium red, and cationized ferritin [43, 44]. As I pointed out earlier [69], as far as pheromone-binding proteins are concerned, the physiologically relevant pH is likely to be not only that of the sensillar lymph [56] (the bulk pH), but also the pH at the surface of dendrites (localized pH). It is yet to be determined whether the negatively-charged surface that may interact with odorant-binding proteins and promote conformational changes is a moiety from a glycoprotein, amino acid residues from membrane proteins like SNMPs [52, 70–72], or even an external site of olfactory receptors.

The kinetics of conformational changes is consistent with the fast kinetics of neuronal activities. Stopped-flow measurements of the pH-dependent conformational change in BmorPBP monitored by fluorescence showed that it has characteristics of first-order kinetics, with a rate constant, $k=184\pm6\ s^{-1}$ [16]. Thus, the time required for half of the conformation at the bulk pH to change into the conformation at lower pH (equivalent to the pH of a dendritic surface) is 3.8 msec. This half-time fits to a model of perireceptor events [73]. Also, the fast conformational change is consistent with the millisecond timescale for the dynamics of the olfactory system [14]. For example, males of *B. mori* respond to bombykol with wing vibration 100–500 ms after the onset of stimulation [74]. Moreover, the binding ability of odorant-binding protein is lost at low pH as demonstrated by fluorescence [39] and mass spectrometry [75] for BmorPBP and by calorimetric titration for an odorant-binding protein from the honeybee [47]. That BmorPBP binds bombykol at the bulk pH but not at the membrane-localized pH has been further demonstrated by a cold binding assay [16]. In addition, this binding assay showed that the loss of binding ability at low pH is not affected by the high salt concentrations, i.e., there is no binding of bombykol to BmorPBP at pH 5 either with 0, 170, or even 500 mmol/l of KCl [16]. The notion that the pH-dependent conformational change is a physiologically relevant mechanism for pheromone delivery (to olfactory receptors) is further substantiated by striking evidence from structural biology for an intramolecular mechanism of "occupation" of the binding site at low pH (see below).

3.1.2.3
Structures of OBPs and Encapsulins

In collaboration with Dr. Jon Clardy and Dr. Kurt Wüthrich we have studied the crystal and solution structures of BmorPBP bound to bombykol, unliganded at high and low pH. The crystal structure of the BmorPBP-bombykol complex (Fig. 8a) shows a roughly conical arrangement of six α-helices [23] remarkably similar to the NMR structure of the protein devoid of ligand (Fig. 8d) [25].

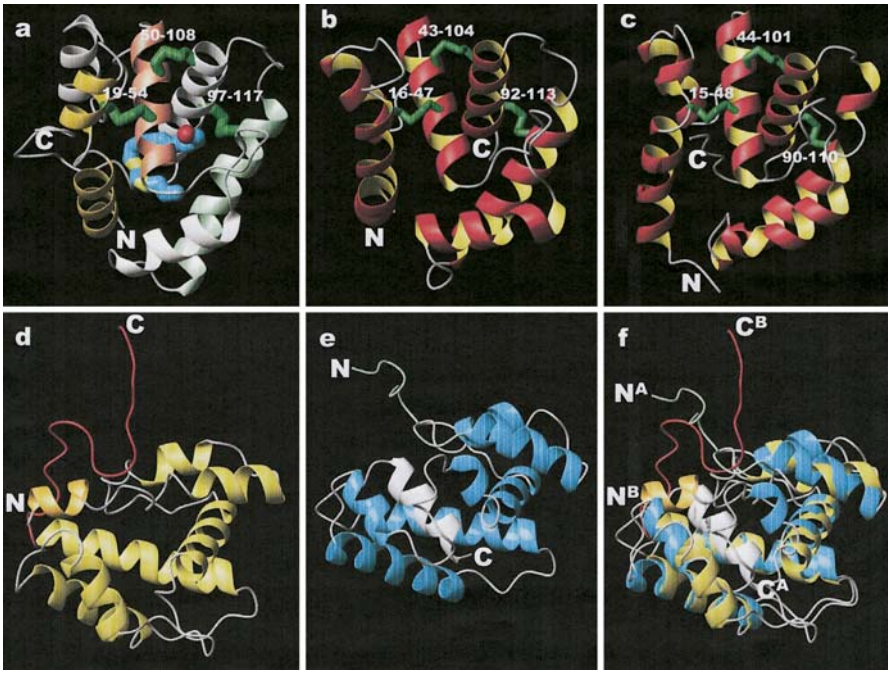

Fig. 8a–f Structures of proteins of the OBP-gene family prepared by using the program MOLMOL [125]. The N and C termini are denoted by N and C, respectively: **a** pheromone-binding protein of *B. mori* (BmorPBP) bound to bombykol, 10,12-(*E,Z*)-hexadecadienol. The polar end of the ligand is highlighted with the oxygen atom in *red*, whereas the double bonds are shown in *yellow*. Disulfide bridges (19–54, 50–108, and 97–117) are shown in *green*. Note the unstructured C-terminal as an extended conformation of the protein; **b** pheromone-binding protein of the cockroach *L. maderae*. Note the C-terminal α-helix; **c** LUSH, a putative odorant-binding protein from *D. melanogaster*. Note the unstructured C-terminus; **d**, **e** structures of BmorPBP (d) at the bulk high pH of the sensillar lymph ($BmorPBP^B$) and (e) at the localized low pH in the vicinity of the dendritic membrane ($BmorPBP^A$). At high pH, the C-terminal dodecapeptide (*red*) is unstructured, whereas the N-terminal segment (*gold*) forms a α-helix (d). At low pH, a new helix is formed (*gray*) and occupies the binding site, whereas the N-terminus (*green*) is unstructured (e); **f** superposition of the structures of $BmorPBP^B$ and $BmorPBP^A$

We kept the same nomenclature used in the initial work where the two segments of the interrupted N-terminal helix were named α1a and α1b and the loops linking the helices were named after the helix preceding them. For example, the loop following α1a is L1a, whereas the loop connecting helices α2 and α3 is L2. The most striking feature of the solution structure of $BmorPBP^B$ devoid of ligand is a hydrophobic cavity (occupied by bombykol in the solid state structure) with a volume of 272±17 $Å^3$, which is suitable to accommodate bombykol [25]. The preservation of the cavity in the absence of ligand is primarily due to the inherent rigidity of the disulfide structure linking a scaffold of four helices, namely α1b, α3a, α5, and α6 (Fig. 8a). Ab initio calculations indicated that reorganization of the binding cavity can be energetically expensive [76].

Utilizing recombinant protein expressed by another group, Oldham and colleagues [75] observed a possible noncovalent adduct generating an "extra" peak in the mass spectral analysis of BmorPBP. Later, they identified the contaminant as (*Z*)-11-octadecenoic acid (*cis*-vaccenic acid) and described a delipidation protocol [77]. They also suggest that the lipid is derived from *E. coli* and acquired by the protein during expression. Despite several attempts, we never found any contaminants in the BmorPBP samples prepared in my lab. Most likely the discrepancy between the findings of different labs is due to the different expression and purification protocols. In our case, NMR showed that the binding cavity is preserved in the absence of ligand [25].

The binding cavity of BmorPBP is formed by four antiparallel helices (α1, α4, α5, and α6) that converge to form the narrow end of the pocket, whereas the opposite end is capped by α3 (Fig. 8a). Bound bombykol has a roughly planar hook-shaped conformation and the outside (convex) part of bombykol interacts with numerous protein residues, whereas the inside (concave) part has fewer contacts. Interestingly, residues from all parts of the protein contribute to the binding cavity [23] that protects bombykol from the aqueous solvent. The solution structure showed that the binding cavity is lined with 21 hydrophobic side chains, namely, Met5 and Leu8 from the helix α1a, Phe12 from the loop L1a, Phe33, Tyr34, and Phe36 from L2, Ile52 from α3, Met61, Leu62 and Leu68 from L3, Ala73, Phe76, Ala77 from α4, Ala87, Leu90, Ile91, and Val94 from α5, and Trp110, Val114, Ala115, and Phe118 from α6 [25]. The cavity contained also four polar side chains of Asp32 from α2, Thr48 and Ser56 from α3, and Glu98 from α5. In the BmorPBP-bombykol complex, the hydroxyl group of bombykol forms a hydrogen bond with the side chain of Ser56 [23]. Ab initio calculations suggested that another hydrogen bond with Met61 may result in slightly stronger interaction [76]. The conjugated double bonds of bombykol are sandwiched by Phe12 and Phe118 with the aromatic rings parallel to the molecular plane of bombykol [23]. Bound bombykol is completely engulfed in BmorPBP, and the structure does not clearly indicate how the ligand enters or exits the binding cavity. The only part of the pheromone that is not surrounded by α helices is the hydroxyl end, which is covered by loop L3 [23]. As noted in the solution structure of $BmorPBP^B$, except for loop L2, the loops connecting the he-

lices contain numerous hydrogen bonds that help in the formation of well-ordered structures [25]. L3 is held together in an approximate antiparallel β-strand conformation by three hydrogen bonds (Gly66N-Asp63O, Asp63N-Asn67O, and His69N-Met61O), with additional interaction between the side chain of Asp63 and the backbone NH of Asn67. This loop is held in place by an interaction between the side chain NH of Leu68 and the side chain of Glu98. If this loop were not in place, the resulting opening would be adequate for bombykol to enter and egress [23]. Testing of this hypothesis is still underway.

The unliganded solution structure of BmorPBP at pH 4.5 ($BmorPBP^A$) showed remarkable conformational differences to the crystal structure of the BmorPBP-bombykol complex (Fig. 8e,f) [24]. The most pronounced differences are in the region of helix α1, which is N-terminally elongated in $BmorPBP^A$ (helix α1a in the BmorPBP-bombykol complex [23] and $BmorPBP^B$ [25]) and in the C-terminal helix α7, which is not present in $BmorPBP^B$ [25] and the complex [23]. The helices forming the bombykol-binding cavity in the complex and in $BmorPBP^B$ occur in close similar positions in $BmorPBP^A$ [24]. The most significant difference between the structures of BmorPBP-complex or $BmorPBP^B$ and the acidic form is the C-terminal helix (α7) in $BmorPBP^A$ which occupies a position that corresponds to the hydrophobic binding cavity in the crystal structure. The C-terminal dodecapeptide segment, which is an extended conformation and located on the protein surface at high pH forms a α-helix at low pH. This is one of the most remarkable conformational changes yet observed in receptor-ligand or enzyme-substrate binding, and leads to occupation of the binding site by an intramolecular mechanism triggered at low pH. The three histidine residues (His69, His70 and His95), forming a cluster at the end of loop L3 in $BmPBP^B$, are more widely separated in $BmorPBP^A$ [24]. This would reduce the charge repulsion resulting from histidine protonation at slightly acidic pH values and could thus destabilize the structure of the complex in favor of $BmorPBP^A$.

Recently, the structure of a pheromone-binding protein from the cockroach *Leucophaea maderae*, LmadPBP (Fig. 8b) has been solved by X-ray crystallography [58]. Despite the fact that LmadPBP and BmorPBP shared low amino acid identity (15%; similarity 22%) (Fig. 9), the two proteins present similar folds.

When compared to the structure of the BmorPBP-bombykol complex, the six helices have similar orientations; the three disulfide linkages knit together four of the helices in a similar pattern (Fig. 8b). The binding cavity is much smaller than the bombykol-binding cavity in $BmorPBP^B$ and in the complex structure; in LmadPBP the binding pocket is wide open to the bulk solvent. The conformations of LmadPBP unbound and bound to its pheromone (3-hydroxybutan-2-one) are very close [58], but these comparisons were made only at high pH values (>7) (for experimental details see [78]). That the bound and unbound structures are remarkably similar is also true for BmorPBP at high pH, but the acidic form is quite different from the basic form (see above). The major difference between BmorPBP and LmadPBP is that the cockroach

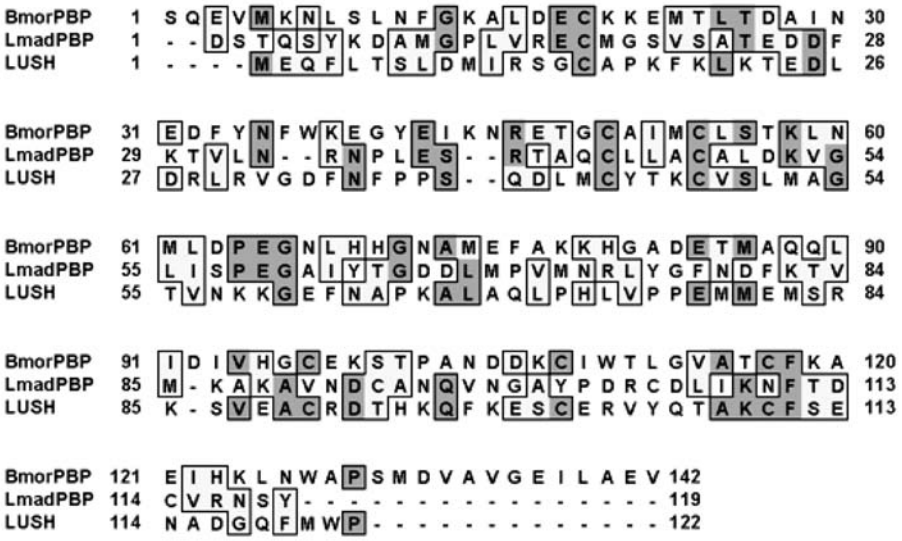

Fig. 9 Alignment of the amino acid sequences of pheromone-binding proteins from the silkworm moth *B. mori* and the cockroach *L. maderae*, BmorPBP and LmadPBP, respectively and a putative odorant-binding protein from *D. melanogaster*, LUSH. In LmadPBP and LUSH the N-terminal sequence of the mature proteins were predicted by cleaving signal peptides in silico [28, 79], whereas in BmorPBP this was confirmed by the sequence of the isolated protein [38]

protein is shorter by four residues at the N-terminus and 15 residues at the C-terminus. The authors suggest that due to the shorter C-terminus, LmadPBP would not undergo a pH-dependent conformational change [58] as observed for BmorPBP. It may be true that PBPs from insects of different orders have different "modes of action," but the evidence for the lack of a pH-dependent conformational change in LmadPBP is still missing. Although the shorter C-terminus indicates that a new helix may not be formed, this hypothesis can be tested only when the structure at low pH is determined. In their work, Lartigue and collaborators obtained solid state structures only at high pH [58]; thus, one cannot conclude what happens at low pH without experimental data.

One drawback of the cockroach PBP structure is that the recombinant protein was composed of 129 amino acids, with 11 of them (Met-Asp-Ile-Gly-Ile-Asn-Ser-Asp-Pro-Asn-Ser) not belonging to the native structure [79]. In their recombinant vector, pET-LmadPBP, the cockroach cDNA encoding the target protein was inserted away from the pelB leader sequence using an *Eco*RI recognition site. Thus, a long non-natural peptide at the N-terminus of the recombinant protein was added to the native sequence [79]. The possible effect of this extended N-terminal segment – although not discussed when the structure was reported [58] – may influence the folding of the protein. Note that in BmorPBP

the α1a helix unfolds at low pH. It is very unlikely that the conformational change in the native conformations of BmorPBP would be "visualized" if we had studied recombinant BmorPBPs having an addition N-terminal sequence. Therefore, we never use fusion tags such as His tags, GST tags, etc. in our recombinant proteins for structural studies. These non-native proteins may speed up the purification process, but may slow down our understanding of the physiology and molecular basis of insect olfaction.

The structure of LUSH, a putative odorant binding protein from *Drosophila* (see above), has just been solved [37]. In contrast to the recombinant proteins from *B. mori* and *L. maderae*, which were obtained by periplasmic expression, LUSH was obtained by cytosolic expression and refolding using a cysteine-cystine redox reaction [37]. In addition, recombinant LUSH had three additional amino acids at the N-terminus, Gly, Ser, and His, which were leftover after the removal of a His tag [37]. In LUSH (Fig. 8c), the C-terminus forms part of the alcohol-binding pocket, whereas helix α1 packs on the outside of the protein [37] and does not participate in the ligand-binding cavity as in BmorPBP-bombykol complex [23]. Based on these differences, it was suggested that the OBP family has at least two distinct structural isoforms [37]. Interestingly, crystal structures of LUSH at high and low pH values (6.5 and 4.6, respectively) showed alcohol in the binding pocket. Given the contradictory information regarding detection of alcohol by *lush* mutants, the lack of binding assays (see above), and the fact that the protein was incubated with extremely high concentration of alcohol (1%), it is difficult to interpret the physiological relevance of the occurrence of alcohol in the binding pocket at low pH. Artifacts may lead to compounds of little physiological significance being trapped in a binding pocket. The cavity of LmadPBP, for example, contains a ubiquitous glycerol molecule [58], which derives from the considerable amount of glycerol brought into the crystal for cryocooling. If LUSH functions as an odorant-binding protein, it may have a different mode of action. Of particular notice is the fact that LUSH is the only putative odorant-binding protein reported to date that is basic at the sensillar lymph pH. LUSH has a calculated pI of 8.28; thus, it is positively charged at the sensillar lymph pH (ca. 7). All other OBPs identified to date are acidic and they are considered to contribute to the anions in the sensillar lymph of which a minor fraction is covered by Cl^- [6]. In conclusion, the physiological function of LUSH is not yet clarified, despite the elegant structural biology studies [37]. It is clear, however, that despite the low sequence similarities (Fig. 9), BmorPBP, LmadPBP, and LUSH belong to the same structural family. Another insect protein of known structure is THP12 [61], a protein isolated from the hemolymph of the beetle *Tenebrio molitor* [55]. The overall folds of OBPs and THP12 are similar, but the latter is missing the N-terminal α1a helix. Similar to OBPs (above), four helixes are knitted together by two disulfide bridges. Because hemolymph is completely isolated from the sensillar lymph (see above), it is very unlikely that THP 12 has any olfactory function and, as such, it should be referred to as encapsulin rather than odorant-binding protein.

3.2 Mode of Action of OBPs

The following evidence based mainly on the pheromone-binding protein from *B. mori* strongly supports that OBPs uptake compounds entering the sensillar lymph through pore tubules, bind physiologically relevant ligands, encapsulate them, ferry these semiochemicals to the olfactory receptor, and deliver the chemical signal by a conformational change upon interaction with negatively charged sites in the dendrites; this model is depicted in Fig. 6. BmorPBP undergoes a pH-dependent conformational change [39, 42], binds bombykol at the sensillar lymph pH, but not at lower pH [16, 39]. Negatively-charged groups in cell membranes give rise to an electrical surface potential, which in turn decreases the surface pH [68]. In other words, a negatively-charged surface is equivalent to a low pH region (localized pH). The pH-dependent conformational change leads to a remarkable intramolecular "re-arrangement" in BmorPBP. At the bulk pH of the sensillar lymph, the C-terminus in BmorPBP (either bound to bombykol or unbound) is an extended conformation located on the protein surface [23, 25], whereas at low pH this C-terminal dodecapeptide segment forms a α helix that occupies the pheromone-binding cavity in the core of the protein [24]. The growing evidence from structural biology studies suggests that upon interaction with negatively-charged membrane (regions of low pH), the C-terminal helix takes over the binding pocket, thus, ejecting the pheromone out of the protein. Stopped-flow fluorescence measurements showed that this rapid conformational change is in the timescale of milliseconds [16]. Functional expression of an odorant receptor from *Drosophila* in *Xenopus laevis* oocytes [66], devoid of odorant-binding proteins, suggests that an odorant per se (not an OBP-odorant complex) can activate the receptor. The same experiments indicate that odorant-binding proteins are essential for the kinetics (and likely the specificity) of the olfactory system.

In this model, OBPs participate in the selective transport of pheromone and other semiochemicals to their olfactory receptors. The selectivity of the system is likely to be achieved by "layers of filters" [16], i.e., by the participation of compartmentalized OBPs and olfactory receptors. It seems that OBPs transport only a subset of compounds that reach the pore tubules. Some of these compounds may not bind to the receptors compartmentalized in the particular sensilla. The odorant receptors, on the other hand, are activated by a subset of compounds, as indicated by studies in *Drosophila*, showing that a single OR is activated by multiple compounds [66]. If some potential receptor ligand reaches the pore tubules but are not transported by OBPs, receptor firing is prevented because the receptors are "protected" by the sensillar lymph. In other words, even if neither OBPs nor odorant receptors (ORs) are extremely specific, the detectors (olfactory system) can show remarkable selectivity if they function in a two-step filter.

While engulfed in the binding cavity of an OBP, a pheromone (or other semiochemical) is not only solubilized, but also protected from odorant-degrading

enzymes (see below). Assisted by a protein, the pheromone is now transported through the sensillar lymph until it reaches certain negatively-charged sites on the surface of dendrites. The low pH at these sites triggers a conformational change of the OBP-odorant leading to the release of the ligand to the receptors. After stimulating the odorant receptor, the pheromone is inactivated or deactivated. Note that in this model OBPs are not merely carrier proteins, but they contribute to the specificity of the olfactory system. Also, they have evolved the ability to undergo a rapid pH-dependent conformational change for the fast delivery of ligands to the olfactory receptors, which contributes to the dynamics of the olfactory system.

3.3 Specificity of the Insect Olfactory System

The inordinate specificity of the insect olfactory system was highlighted in electrophysiological studies of pheromone perception. There is a body of evidence in the literature indicating that minimal structural modifications of pheromone molecules render them inactive, as demonstrated initially in the pheromone detectors in *B. mori* antennae [12]. Even the olfactory receptor neurons (detectors) for plant compounds in insect antennae, once called "generalists", have now been demonstrated to have remarkable specificity [20, 80–84]. In some cases, these specific detectors may respond when challenged with extremely high concentrations of other compounds. These responses may not be physiologically significant because insects will never encounter such high concentrations in the natural environment. When electrophysiological studies precede the discovery of physiological relevant semiochemicals (say pheromones), one tends to try high concentrations of test compounds and this may lead to the identification "non-specific" ORNs. Some ORNs in scarab beetle antennae were initially considered generalists, but are now known to be specific detectors for (*Z*)-3-hexenyl acetate [20, 80, 82, 83]. On the other hand, behavioral evidence that a certain compound has a physiological function (like a sex pheromone, for example) facilitates the discovery of specific ORNs. For that reason most of the evidence for the specificity of the olfactory system comes from studies on species of known pheromones. From an anthropomorphic perspective, stereochemical discrimination may be considered the ultimate refinement in the insect olfactory system. Scarab beetles, for example, can discriminate stereoisomers of a lactone pheromone and perceive one antipode as a sex pheromone and the other as a behavioral antagonist [18, 84, 85]. Interestingly, they perceive the two stereoisomers with two ORNs co-localized in the same sensilla [18] and respond differently if the stereoisomers are perceived either simultaneously or isolated by a few milliseconds [84].

Of notice is the case of *D. melanogaster*, a highly suitable model system for olfactory research given that it is an insect amenable to genetic manipulations, the complete genome has been sequenced, and the olfactory system is relatively simple, thus, allowing precise physiological measurements. *D. melanogaster*

possesses two olfactory organs, the antennae with ca. 1,200 ORNs and the maxillary palpi containing ca. 120 ORNs. These ORNs are compartmentalized in olfactory sensilla, which divide into morphologically distinct classes, including large basiconica, small basiconica, trichodea sensilla, and coeloconic sensilla [86]. The ORNs both in the antennae [87] and in the maxillary palpi [88] showed unique response spectra to a panel of tested compounds, ORN raising the question whether these sensilla are "generalists" or if the "key stimulus" for each has not yet been discovered. Recently, Stensmyr and collaborators [36] screened a large number of potential semiochemicals for *Drosophila* from food sources and conclude that "key stimuli" are detected by the fruitfly with high specificity at low concentration, but when the concentrations are increased the specificity decreases. One of the tested ORNs responded to ethyl hexanoate and methyl hexanoate with similar dose-response curves and threshold of 100 pg, whereas ethyl butyrate and butyl butyrate required 100-fold and 10,000-fold increase, respectively, in dose to produce any response [36]. That the *Drosophila* olfactory system is indeed specific to a physiological relevant "key stimulus" has been previously demonstrated [35]. Sensilla trichodea in the antennae responded in a dose-dependent manner to an aggregation pheromone, *cis*-vaccenyl acetate, but were not activated by 16 other compound tested, thus suggesting they are narrowly tuned to the pheromone [35]. In conclusion, the specificity (also the sensitivity and dynamics) of insect olfactory system may be a common feature, with the apparent exception of *Drosophila* where the "key stimuli" have yet to be discovered.

3.4 Odorant-Degrading Enzymes

In addition to sensitivity and discrimination, odor-oriented navigation requires a dynamic process of signal inactivation. While flying en route to a pheromone-emitting female (Fig. 2), males encounter pheromone molecules as intermittent signals comprised of short bursts of high flux separated by periods during which the flux is zero. The average duration of bursts of high flux of pheromones is on the millisecond scale and it decreases as the moth comes closer to the pheromone source [2]. Thus, a male moth has to detect selectively minute amounts of pheromones and reset the pheromone detectors (cells) on a millisecond timescale. The literature on the inactivation of chemical signals is dichotomous. One school favors the hypothesis that rapid inactivation of chemical signals is an enzymatic process regulated by pheromone-degrading enzymes, whereas the other school favors that preceding the "slow process of degradation" there is some molecular interaction of pheromones and other olfactory proteins. Based on an estimation of pheromone degradation in vitro, it has been hypothesized that fast inactivation of pheromones is achieved by pheromone-degrading enzymes [89]. However, the enzymatic degradation in vivo has been considered too slow (on a minute timescale) [90] to account for the fall of the receptor potential [73]. It has been suggested that the discrepancy

between data from in vivo and in vitro experiments is due to the involvement of PBPs that protect the pheromone from degradation [73].

If these pheromone-degrading enzymes are indeed involved in the fast inactivation of pheromone signals, they have a potential application in agriculture as their inhibitors could be used in insect pest management [91–94]. However, a rational approach for their design of environmentally-safe inhibitors requires full knowledge of the biological system. Specificity and selectivity of inhibitors can be dramatically improved upon design of new compounds, which fit not only into the binding pocket of pheromone-binding proteins, but also in the active site of pheromone-degrading enzymes. These compounds could then penetrate the sensillar lymph and inhibit the fast degradation of pheromone, thus disrupting chemical communication. Recent structural biology studies on pheromone-binding proteins already shed some light on specificity binding determinants [23], which may lay the foundation for the design of parapheromones developed based not on trial-and-error strategies, but rather on rational structure-activity relationships. Nevertheless, the complete lack of knowledge on the molecular structures of these pheromone-degrading enzymes prevents further progress in the rational design of inhibitors, parapheromones, and other semiochemical-based pest control strategies.

Hitherto, no pheromone-degrading enzymes has been isolated, identified and cloned. As with odorant receptors, the amount of protein is so low that isolation for protein identification is technically very difficult. In marked contrast to PBPs, which are expressed in the sensillar lymph in concentrations as high as 10 mmol/l [95], odorant-degrading enzymes are estimated to occur in concentrations at least four-order of magnitude below that of PBPs [96]. Thus, it has not been possible to date to generate large enough amounts of odorant-degrading enzymes (ODEs) for protein sequencing (by Edman degradation and/or mass spectrometry). It is possible, however, to isolate enough material for identification of olfactory enzymes involved in pheromone degradation. These studies require lower amounts of proteins and samples enriched in the enzymes, but not necessarily pure. For example, a sensillar esterase [89], partially isolated from *A. polyphemus*, was demonstrated to degrade the pheromone, 6,11-(*E*,*Z*)-hexadecadienyl acetate, by attacking the acetate group. Using a bioinformatics approach, we have recently cloned a cDNA encoding a male antennae-specific esterase in the same moth species [97]. It is yet to be demonstrated if the enzyme degrading the pheromone is the same as that encoded by the cDNA we have cloned. Similarly, Maibeche-Coisne and co-workers [98] have cloned the cDNA encoding a cytochrome P450 enzyme from *Mamestra brassicae*. On the other hand, we have demonstrated that the sex pheromone of the pale chafer, *Phyllopertha diversa*, 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione is rapidly degraded in vitro by a membrane-bound P450 [99]. Interestingly, the ability to degrade this unusual sex pheromone was not detected in 12 other species of scarab beetles. In addition, in *P. diversa* the enzymatic activity was restricted to male antennae, with no degradation observed in extracts from female antennae or control tissues.

The sex pheromone of the Japanese beetle, *Popillia japonica,* is a chiral compound, (*R*,*Z*)-5-(dec-1-enyl)oxacyclopentan-2-one ((*R*)-japonilure), whereas the other enantiomer ((*S*)-japonilure) is a behavioral antagonist that shuts down male response [100]. It seems that this chiral discrimination has evolved as part of the isolation mechanism between the Japanese beetle and the Osaka beetle (*A. osakana*) that share the same habitats in Japan [85]. Previously, it has been demonstrated that this chiral discrimination is not achieved by pheromone-binding proteins as the Japanese beetle possesses only one PBP (that binds to (*R*)- and (*S*)-japonilure) [18]. Studies on the degradation of radiolabeled enantiomers of japonilure by the Japanese beetle antennal enzyme(s) shed new light on chiral discrimination. Crude extracts of the Japanese beetle antennae showed a significant preference for the pheromone, (*R*)-japonilure, over the behavioral antagonist, (*S*)-japonilure (Fig. 10), whereas enzymes from non-sensory tissues (legs) showed no substrate specificity. These findings indicate that integumental esterases in leg tissues are not specific, but sensillar esterases may have evolved for the specific degradation of pheromones. Thus, I hypothesized that one stage of chiral specificity is

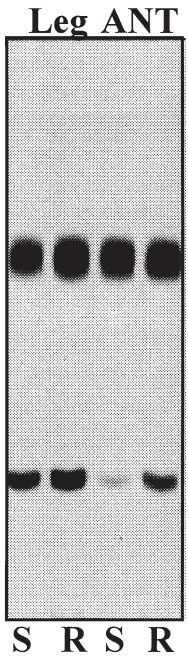

Fig. 10 TLC plate showing degradation of (*R*)- and (*S*)-japonilure (*upper spots*) by esterases from the legs (Leg) and antennae (Ant) of the Japanese beetle. The corresponding hydroxy-acids appear as *lower bands*. Note the slower degradation of the behavioral antagonist, (*S*)-japonilure, by sensillar esterase(s) from the antennae. Neither (*R*)- nor (*S*)-japonilure is degraded in control experiments (data not shown) under the same conditions, i.e., with the compounds incubated in buffer without Japanese beetle tissue extracts

achieved in the perireceptor events (early olfactory processing) by the pheromone-degrading enzymes as a mechanism of pheromone inactivation. Work is now in progress in my lab to test this hypothesis. Pheromone-degrading enzymes will be isolated, the cDNAs encoding these proteins will be cloned, and kinetics of degradation (of pheromone and behavioral antagonist) will be studied in a cell-free system with native and recombinant PDEs. Because the pheromone may be protected from degradation while bound to PBP [73], kinetics will be studied in the presence of the Japanese beetle PBP ([18] in a cell-free system mimicking the in vivo conditions. If these pheromone-degrading enzyme(s) is (are) fast enough (in the millisecond timescale) and isolated enzyme(s) show substrate-specificity the hypothesis will be supported.

4 Olfactory Receptors

That olfactory receptors in vertebrates are G-protein-coupled receptors (GPCRs) was suggested by early evidence that odorant receptors are membrane proteins [101, 102] and that cell-free preparations of rat olfactory cilia contain odorant-sensitive adenylyl cyclase, whose sensitivity depends on activation of a G protein [103]. The evidence was further substantiated by the cloning of a multigene family of vertebrate GPCRs [104]. Given the large body of evidence indicating that pheromone-dependent effects of secondary messengers, such as IP_3, cAMP, and cGMP (reviewed in [105]) have been observed in intact antennae and antennal homogenates, and that odorant receptors are also GPCRs [106], the cloning of vertebrate olfactory receptors prompted various groups to "fish" out insect pheromone receptor "homologs." Various approaches, including photoaffinity labeling, genetic mutants, radioligand bioassays, and PCR with primers designed on the basis of vertebrate GPCR sequences, were unrewarding [107]. With the sequence of the *Drosophila* genome about to be completed, two approaches led to the identification of the first insect odorant receptors. A bioinformatics approach that examines DNA databases for proteins that have a particular structure like the seven-transmembrane-domain of GPCRs led to several genes that could encode seven-transmembrane-domain proteins [64]. RT-PCR experiments showed that two of the genes were expressed specifically in *Drosophila* antennae. BLAST searches identified homologs of these genes, which were used to search for further homologs; a total of 16 genes were identified by this bioinformatics approach [64]. On the other hand, Vosshall and collaborators found a putative odorant receptor by a strategy designed to detect cDNA copies of mRNA present at extremely low frequencies in an mRNA population [65]. In situ hybridization revealed that the cDNA encoding the putative olfactory receptor anneals to ca. 15% of the 120 olfactory receptor neurons within the maxillary palpi but does not anneal with neurons in either the brain or antennae [65]. Searches of the then incomplete *Drosophila* sequence database led to 229 candidate genes, 11 of which encode

putative GPCRs with sequences similar to those obtained by the rare mRNA strategy. Completion of the genome sequence allowed extension of the odorant receptor family to 60 receptors, which is now predicted to consist of 62 odorant receptors [108].

Direct demonstration of the function of one member of the *Or* gene family (*Or43a*) was obtained by overexpression of the gene in the fly antennae [109], as well as by expression in a heterologous system, *Xenopus* oocytes [66]. The GAL4/UAS system was used to overexpress *Or43a* in the *Drosophila* antennae. In wild-type flies, Or43a expression is restricted to ca. 15 ORN at the distal edge of the third antennal segment, but in the transformed flies Or43a expression was drastically increased. In addition to the cells at the distal edge of the antennae, there were Or43a overexpressing cells in the transformed lines in a more proximal region that is covered mainly by large sensilla basiconica [109]. Electroantennogram experiments showed that the transformed flies showed (dose-dependent) increased responses to benzaldehyde as compared to wild-type flies, whereas ethyl acetate evoked similar responses in control and transformed lines at all concentrations [109]. In addition to benzaldehyde, EAG showed increased responses to cyclohexanol, cyclohexanone, and benzyl alcohol, thus, suggesting that Or43a is a "generalist" type of odorant receptor. That Or43a is a *Drosophila* odorant receptor was also suggested by two-electrode voltage-clamp recordings from *Xenopus* oocytes injected with Or43a (and Ga15) cRNA [66]. Again, benzaldehyde, cyclohexanol, cyclohexanone, and benzyl alcohol elicited responses, with current being developed at low micromolar concentrations, whereas eight other test compounds failed to activate Or43a [66]. Moreover, oocytes not injected with Or43a failed to respond to the four odorants (benzaldehyde, cyclohexanol, cyclohexanone, and benzyl alcohol) even at millimolar concentrations [66]. These two lines of evidence were the first demonstration that a *Drosophila Or* gene indeed functions as an olfactory receptor. Of particular note is the fact that in the heterologous system – devoid of odorant-binding proteins and odorant-degrading enzymes – the response to odorant was extremely slow compared to the dynamics of the *Drosophila* olfactory system. When *Xenopus* oocytes were stimulated (with cyclohexanol, for example) for as long as 15 s, it took as long as 2–5 s to develop inward currents [66]. By contrast, ORNs in *Drosophila* antennae when stimulated for 0.3–0.5 s generate slow potential and nerves impulses in less than 100 ms [87]. The slower response may be explained by the lack of other olfactory proteins, such as odorant-binding proteins. As previously discussed (see above), these proteins are essential for the detection of semiochemicals as they help in the transport of chemical signals through an aqueous environment while protecting the ligands from "deactivation." In the absence of OBPs in the *Xenopus* oocytes, the ligands were less soluble in water (as compared in the natural system), thus requiring a longer time to generate a threshold concentration at the receptor. In the natural insect system, stray semiochemicals (unbound odorants) in the sensillar lymph may never evoke neural activity as they are likely to be "deactivated" by aggressive odorant-degrading enzymes before reaching the receptors.

It has been a matter of considerable debate whether the remarkable selectivity of the insect olfactory system [12] is achieved by the specificity of pheromone-binding proteins or the odorant receptors. The expression of Or43a in transformed lines and a heterologous system suggests that this odorant receptor is "sloppy." OBPs, on the other hand, have been demonstrated to bind specifically when tested with a limited number of candidate ligands [39, 110–113], but lack specificity in various other cases [114]. I suggest that neither OBP nor OR specificity per se can account for the selectivity of the insect olfactory system, which is likely achieved by "layers of filters" (see above) [16]. The notion of a "dual layer of filters" is supported by the number of genes encoding OBPs and ORs. Even in *Drosophila*, with large numbers of putative OBPs [26] and ORs [108], the number of these olfactory proteins is much lower than the number of compounds insects can smell. Thus, it is not entirely surprising that neither OBPs nor ORs are specific. The specificity of the detectors must be achieved by a combinatorial process.

Putative odorant receptors were fished out from the sequenced genome of the malaria vector mosquito *Anopheles gambiae* by analyzing sequences similar to *Drosophila* ORs. Initially, five putative odorant receptors, AgamOR1-5, were identified [115]; RT-PCR analyses suggest that they are all expressed exclusively in olfactory tissues (antennae and maxillary palpi). Interestingly, one of the putative ORs, AgamOR1, was detected only in female antennae. Intriguingly, RT-PCR showed that AgamOR1 is down-regulated 12 h after a blood meal [115]. By contrast, levels of most OBP mRNAs in the same species remained the same 24 h after a blood meal [116]. Later, a bioinformatics-based approach to identify genes encoding putative transmembrane proteins led to the characterization of 79 candidate odorant receptors in *A. gambiae* [117]. As will be published in Nature, the Carlson's group demonstrated recently that AgamOR1 and AgamOR2 expressed in *D. melanogaster* respond to human odorants (John Carlson, personal communication), thus "de-orphanizing" two of the putative receptors.

The first putative odorant receptors in moths were identified by assessing a genome database of *Heliothis virescens* [118]. Following BLAST searches to identify sequences with significant similarity to *Drosophila* ORs, exon-specific probes of promising sequences were employed to screen antennal cDNA library [118]. RT-PCR results indicate that all nine HvirORs were mainly expressed in the antennae, with two of them (HvirOR7 and HvirOR9) being restricted to antennae [118]. Because they are not sex specific, it is unlikely that any of these ORs is a sex pheromone receptor. The search for pheromone receptors in *H. virescens* is somewhat limited by not having a complete genome given that the database was generated by a shot gun cloning strategy. Thus, one is limited to finding only genes that share significant sequence similarity to *Drosophila* ORs (Jürgen Krieger, personal communication). However, the use of low stringency screening may lead to other genes; this is the case of HvirOR9, which was obtained from HvirOR7.

Immunoelectron microscopy revealed localization of two *Drosophila* ORs, OR22a and OR22b, to the membranes of outer dendritic segments of ORNs. These neurons are housed in a subset of the large basiconic sensilla (LB-I) in the dorso-medial region of the antennae [119]. There are three types of basiconic sensilla in *Drosophila* antennae: ab1 housing four ORNs and ab2 and ab3 each with two ORNs. These sensilla can be distinguished by their response profiles to a panel of odorants tested by single sensillum recordings. To pinpoint the type of basiconic sensilla, strains of transgenic flies were generated in which the presumed promoters for OR22a/OR22b were used to drive expression of GAL4, which in turn drives expression of green fluorescence protein (GFP). Physiological recordings from the GFP-labeled sensilla led to the conclusion that both *22a-GAL4* and *22b-GAL4* drive expression in the ab3 sensillum. To pinpoint further the neuron in ab3 sensilla expressing OR22a/b, the *Or* promoter-*GAL4* constructs were used to drive the cell death gene *reaper* (*rpr*). Recordings from ab3 sensilla in flies engineered to lack *OR22a* (*OR22a-rpr*) did not show the large spike characteristic of ab3A neuron, whereas the small spikes of ab3B were present [119]. Interestingly, the ab3A neuron is also "silent" in the other genotype (*OR22b-rpr*), whereas the ab3B neuron in both genotypes responded to all of the odorants that elicit a response from a control line (*OR22a-GFP*). In conclusion, both *Or22a* and *Or22b* drivers direct expression in the ab3A neuron. Moreover, deletion of *Or22a* and *Or22b* (*Δhalo* mutant) showed an effect on the ab3A neuron similar to that observed in *rpr*-ablation experiments. Transformation rescue experiments demonstrate that rescue is provided only by those constructs containing an intact *Or22a* gene, suggesting that *Or22a* is necessary for rescue, whereas no rescue was provided by *Or22b* [119]. These results indicate that only *Or22a* is necessary for the electrophysiological responses obtained from ab3A with a panel of test compounds.

The *Δhalo* mutant with an empty neuron (ab3A) is an invaluable resource to test putative odorant receptors from flies and possibly other insect species. Indeed, a line designed to express another odorant receptor, *Or47a*, in ab3A neurons gave a different response spectrum as compared to the control lines. The response pattern of this transformed line was similar to that of the ab5B neurons, thus, suggesting that the *Drosophila* receptor *Or47a* is expressed in ab5B neurons [119]. It will be interesting to test the response of putative odorant receptors from other species and different orders to determine if/when the lack of odorant binding proteins from the same species would impair the olfactory function (for physiologically relevant odorants). It will be particularly exciting to test candidate pheromone receptors from moths when they become available. Note that in moths, scarab beetles, and other species of insects pheromone-detectors are narrowly tuned, whereas in *Drosophila* most of the detectors respond (to a panel of test compounds) with a broad spectrum.

5
Reverse Chemical Ecology

As discussed above, EAG and GC-EAD are invaluable tools in pheromone research. Characterization of pheromones from a mixture of compounds is tremendously simplified by using insect antennae as the sensing element either in EAD or in GC-EAD experiments. Although a compound eliciting electrophysiological response is not necessarily behaviorally active, the identification of EAD-active peaks expedites the process by leading to a few candidate compounds (whose biological function is confirmed by behavioral studies). The "molecular" equivalent of these electrophysiology-based approaches is the screening of potential attractants, pheromones, and repellents based on binding affinity to odorant-binding proteins. As with activity indicated by GC-EAD (and EAG) measurements, binding per se does not necessary imply a physiological function. Some test compound may be EAG-active without showing any pheromonal activity, i.e., there is a possibility of "false positives". However, compounds that do not bind (or are EAD inactive) can be eliminated from further behavioral tests. The protein-based screening of semiochemicals requires the full identification of odorant-binding proteins, cloning of the cDNAs (genes) encoding these OBPs, and expression of functional OBPs for binding assays. This "reverse chemical ecology" process is justified for cases in which semiochemicals are sorely needed, but bioassay-oriented approaches have failed. Conventional trial-and-error screenings in the field are too expensive and time-consuming [120]. Three years ago, I proposed the concept of OBP-based screening of mosquito attractants and repellents. Work is now in progress in my lab towards these goals; we have isolated OBPs from *Culex* species [121, 122], the principal vectors of West Nile Virus, and generated recombinant proteins for binding studies. The development of binding assays for throughput screening of candidate semiochemicals is underway. The concept of reverse chemical ecology is also aimed at the development of better lures for the Navel Orangeworm moth, *Amyelois transitella*. Hitherto, only one constituent of the sex pheromone (11,13-(*Z*,*Z*)-hexadecadienal) of this important agricultural pest has been identified [123] and better lures are highly desired for monitoring populations and applications in integrated pest management.

Protein-based assays are routinely used by the pharmaceutical industries for the development of new drugs, but their approach is largely based on receptor-drug interactions. Theoretically, screening of potential semiochemicals could be made by studying odorant receptor-ligand interactions. However, odorant receptors and putative odorant receptors are only known for species whose genome has been sequenced. Even for known ORs, such as *Drosophila* odorant receptors (see above), functional expression is technically very difficult. Thus, screening based on in vitro binding studies with receptors is as yet not technically feasible.

Acknowledgments I gratefully acknowledge the financial support of the NIH-National Institute of Allergy and Infectious Diseases (1U01AI058267-01), the National Science Foundation (NSF) (0234769), the United States Department of Agriculture (USDA) – National Initiative Competitive Grants Program (2003-35302), USDA-Animal and Plant Health Inspection Service (01-8500-0506-GR), USDA-ARS Chemical Affecting Behavior Lab (58-1275-1-042), the University of California Systemwide Mosquito Research Program, and the California Pistachio Commission. The collaborations of Kurt Wüthrich and Jon Clardy are gladly acknowledged. I benefited greatly from the work that my past and current undergraduate, graduate, postdoctoral, and visiting scientists carried out in my lab. I thank Tania Morgan, Göde Schüler, and Hubert Wojtasek for generating illustrations and experimental data for Figs. 1, 3, and 6, Fig. 2, and Fig. 10, respectively. I thank Yuko Ishida, Chris Bahr, John Gardner, Shinichi Tebayashi, Tania Morgan, Angela Chen, Karl-Ernst Kaissling, Paolo Pelosi, Wynand van der Goes van Naters, and Fred Damberger for their helpful suggestions to improve an earlier draft of the manuscript.

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Topics in Current Chemistry (2005) 240: 37–84
DOI 10.1007/b98315

Pheromones of True Bugs

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Abstract The Heteroptera, or true bugs, comprise a large and widely distributed group of sucking insects, with about 38,000 described species, some of which are important pests of crops. True bugs are characterized by well-developed scent glands, the contents of which provide an effective chemical defense against predation. Typical defensive compounds include short-chain alcohols, aldehydes, and esters, (*E*)-2-alkenals, 4-oxo-(*E*)-2-alkenals, alkanes, monoterpenes, and aromatic alcohols and aldehydes. The chemistry of bug pheromones is

more complex, and includes simple esters and monoterpenes, linear, monocyclic, and multicyclic sesquiterpenoids, and novel acetogenins. This chapter summarizes the identification and synthesis of these true bug semiochemicals. Overall, the identification and synthesis of chemicals produced by bugs has greatly outpaced our understanding of their specific roles as signals mediating bug behavior.

Keywords Heteroptera · Pheromone · Allomone · Semiochemical · Chemical defenses

1 Introduction

The Heteroptera, or true bugs, form one of two groups in the hemipteran suborder Prosorrhyncha [1, 2]. The group is large, with some 79 families and roughly 38,000 described species. Some of the more commonly known families include stink bugs, bed bugs, plant bugs, assassin bugs, and water striders. True bugs are found throughout the world in a wide variety of habitats, and include aquatic and even a few marine species. They are hemimetabolous with three life stages (egg, nymph, and adult), with the nymphal stages bearing some resemblance to the adults. All species have a tubular "beak" that is specialized for sucking fluids, and which is also used to inject salivary enzymes into food items to help in their predigestion and liquefaction. Most true bugs feed on plants, and some are serious pests of a wide variety of crops; as a whole, they have been ranked fourth among the most economically important groups of insects [3]. A minority of species are facultative or obligate predators, feeding on other arthropods, snails, and even small fish. Some species are beneficial insects that have found use in the biological control of pests. A few species, such as bed bugs and triatomine bugs, feed on vertebrate blood, and the latter species in particular are of major importance as vectors of human disease.

True bugs are miniature chemical factories characterized by well-developed scent glands, which usually occur in the abdomen in immature stages and the metathorax in adults [4]. The secretions from the scent glands are pungent irritants, and serve as effective chemical defenses. In fact, some species are so well protected chemically that they advertise their presence with bright colors (aposematism) so that predators, having once experienced their chemical arsenal, rapidly learn to avoid them. Because these defenses are very apparent, and the components are frequently produced in large amounts, their chemistry is quite well known [4, 5–9]. In some species (e.g., the Miridae, or plant bugs) chemicals from the scent glands may have a dual function, being used for defense in high doses, while also serving as pheromone components to attract members of the opposite sex at lower doses. In others (e.g., some species of phytophagous stink bugs, family Pentatomidae), the pheromones are entirely unrelated to the defensive chemicals in both their chemistry and function, and are produced in different tissues than the defensive compounds. However, the reader is strongly cautioned that in much of the published literature on

pheromones of true bugs, the distinction between the defensive and the pheromonal compounds has been blurred, with a number of compounds being labeled as pheromones with minimal or no bioassay data in support of their putative roles as intraspecific signals mediating sexual, aggregative, or other interactions between conspecific individuals.

2 Methods for Collection and Analysis of Semiochemicals

Several methods have been used to collect or extract pheromones from true bugs. As a preamble, preparation, analysis, and bioassay of bug pheromone extracts can be complicated by several factors. First, the defensive chemicals are usually present in orders of magnitude larger quantities than the pheromones, masking or overwhelming the pheromone components [9], and frequently render crude extracts unattractive to test insects in bioassays. Second, because of the diversity of insects represented within the true bugs, there is no clear pattern as to which sex produces and which sex responds to pheromones. In some species, one sex produces a pheromone that appears to attract both sexes (an aggregation pheromone) and even immatures. In others, one sex produces the pheromone, to which the other sex responds (a sex pheromone). However, even within a subfamily there may be no clear pattern of producing and responding sex. For example, males of the redshouldered stink bug *Thyanta pallidovirens* produce a pheromone that attracts exclusively females [10], whereas males of the southern green stink bug *Nezara viridula* produce a pheromone that attracts both sexes and even nymphs [11]. Bugs must also be in the correct physiological state to both produce and respond to pheromones. Whereas this seems self-evident, subtle changes in factors such as lighting and day length in laboratory colonies or in the field can trigger reproductive diapause in the longer-lived bugs, and once in this state, reproductive activities, including the production of pheromones, may cease.

Most of the methods that have been used to collect or extract pheromones from true bugs are analogous to methods used with insects in general, and will be summarized here only briefly. The interested reader is referred to several reviews [4, 12–14]. First, whole insects or body sections have been soaked in solvent (e.g., *N. viridula* [15]; *Campylomma verbasci* [16]). However, the value of whole body extracts is questionable because of their complexity, and the bulk of the compounds obtained may be unrelated to the pheromone components, hampering further investigation of the actual pheromone.

A somewhat better technique is to extract the dissected pheromone glands if their locations can be pinpointed, or if the glands are large enough, their secretions can be collected by puncturing the gland with a glass capillary [e.g., 17]. A general gland dissection procedure has been developed by J.R. Aldrich, in which the appendages are removed from an anesthetized bug, and the body is pinned ventral side down. The edges of the abdominal cuticle and the cuti-

cle at the junction between the thorax and abdomen are cut with fine scissors, and the cuticle is folded back with its various attached glands. The generally large metathoracic glands can then be dissected out of the thoracic cavity. However, this method may only be useful for bugs that produce pheromones in macroscopic glands. Furthermore, reconstructing active pheromone blends from the total gland contents can be complicated because of the presence of unrelated defensive compounds, and because the pheromone blend may actually be produced from more than one gland. In fact, the pheromones of some species appear to be produced from patches of unicellular glands that cannot be readily dissected and extracted [15, 18].

One of the best ways to obtain extracts of bug pheromones is to collect the emissions from live bugs held with food in glass aeration chambers by sweeping the chambers continuously with clean air and trapping the headspace volatiles on an adsorbent such as Super Q or activated charcoal, which is then extracted with solvent to recover the emitted compounds. The collected volatiles are representative of what undisturbed bugs actually release, aerations can be continued for days or even weeks, changing the collectors as required, and the bugs can be aerated on food, which enhances both longevity and pheromone production [e.g., 19, 20]. On the other hand, collectors can also be changed every hour or every few hours, so that the daily rhythm of pheromone production can be tracked. In all cases, dead bugs must be removed as soon as possible because they "leak" defensive compounds which contaminate the extracts (J.G.M., personal observation). However, aeration extracts are still not perfect because they are time averages, and if pheromone is produced only in short bursts, even aeration extracts may be misleading (e.g., *L. chinensis* [21]).

More recently, solid phase microextraction (SPME) [22] has been applied to the analysis of bug pheromones, using two techniques. In the first, headspace volatiles are trapped on the SPME fiber, analogous to trapping on SuperQ [e.g., 23]. Alternatively, if the source of the pheromone is known, the SPME fiber can be wiped on the cuticle to directly adsorb the compounds [24]. In either case, the fiber is then thermally desorbed directly into a GC or GC-MS. Whereas this method is excellent for analysis, with good recoveries, it does not provide a sample that can be used for bioassays or for isolation of an active compound.

All extract preparation and analysis methods have biases and potential weaknesses. For example, most of the methods described above recover polar, water-soluble compounds poorly if at all, very volatile compounds may be obscured by solvent peaks during analysis, or compounds may degrade during extraction or analysis (e.g., [25]).

Appropriate bioassays represent a critical component in the identification process of any biologically active compound. In most cases, simple end-point bioassays (e.g., the number of bugs caught in pheromone-baited traps) have been used to assess the activity of bug semiochemicals, but results from these types of bioassays may be misleading. For example, as discussed below, phytophagous stink bugs may be attracted towards a pheromone source, but not all the way into a trap, because over short ranges these bugs use vibrational sig-

nals instead of chemicals for orientation. At the other end of the scale, sophisticated bioassays such as computer-controlled servospheres that provide a detailed record of a walking bug's responses to semiochemical stimuli have been used. Gas chromatography coupled with electroantennogram detection, in which a bug antenna is used as a living detector to screen the GC effluent for bioactive compounds, has also proven useful with some species (e.g., [21]). Overall, space limitations preclude a detailed discussion of bioassay methods used with bugs, and the interested reader is referred to a recent review [13].

3 Bug Defensive Chemistry

A prominent characteristic of most true bugs is their use of defensive chemicals produced in specialized scent glands, usually found in the abdomen in immatures, and in the metathorax in adults. However, this pattern is not absolute; species that feed on poisonous plants from which they sequester toxic chemical defenses tend to have reduced or modified glands [8, 26–28]. Many of these species are also aposematic, vividly advertising their toxicity to would-be predators. The defensive chemistry of bugs has been the subject of a number of reviews [4, 6, 8, 9, 12, 29, 30] and will only be summarized here, with a focus on compounds with interesting or unusual chemistry.

The defensive chemicals produced by bugs de novo tend to be unremarkable, being small and simple compounds, with extensive sharing of compounds across species, genera, and even families. The identification of these compounds is facilitated both by their simple structures, and by the relatively large quantities in which they are produced. Thus, body washes or more comprehensive extractions of the volatiles from whole bugs are dominated by these compounds. In most cases, the structures are so simple that isolation is unnecessary; they can usually be identified from their mass spectra and retention times alone. Immatures typically produce mixtures of even-numbered unsaturated (*E*)-2-alkenals and 4-oxo-(*E*)-2-alkenals of six to ten carbons, and the constituents of the secretions change between nymphal stages [31–33]. The unusual 4-oxo-(*E*)-2-alkenals have never been reported from insects other than true bugs. Traces of the corresponding (*Z*)-isomers have also been found in bug extracts (e.g., [34]). Several multistep syntheses of these compounds have been reported (e.g., [35]), but the (*E*)-isomers can be most easily prepared in one step by reaction of 2-alkylfurans with *N*-bromosuccinimide and pyridine in THF/acetone/water at 0 °C (J.G. Millar, unpublished data). Both odd- and even-numbered straight-chain alkanes, particularly tridecane, are also common constituents of nymphal defenses. In contrast, defensive secretions of adults typically consist of mixtures of saturated or monounsaturated even-numbered alcohols and aldehydes of four to ten carbons, short-chain acids (C_2, C_4, and C_6, sometimes branched and/or unsaturated) and their esters with the above-mentioned alcohols, and straight-chain hydrocarbons (particularly tridecane).

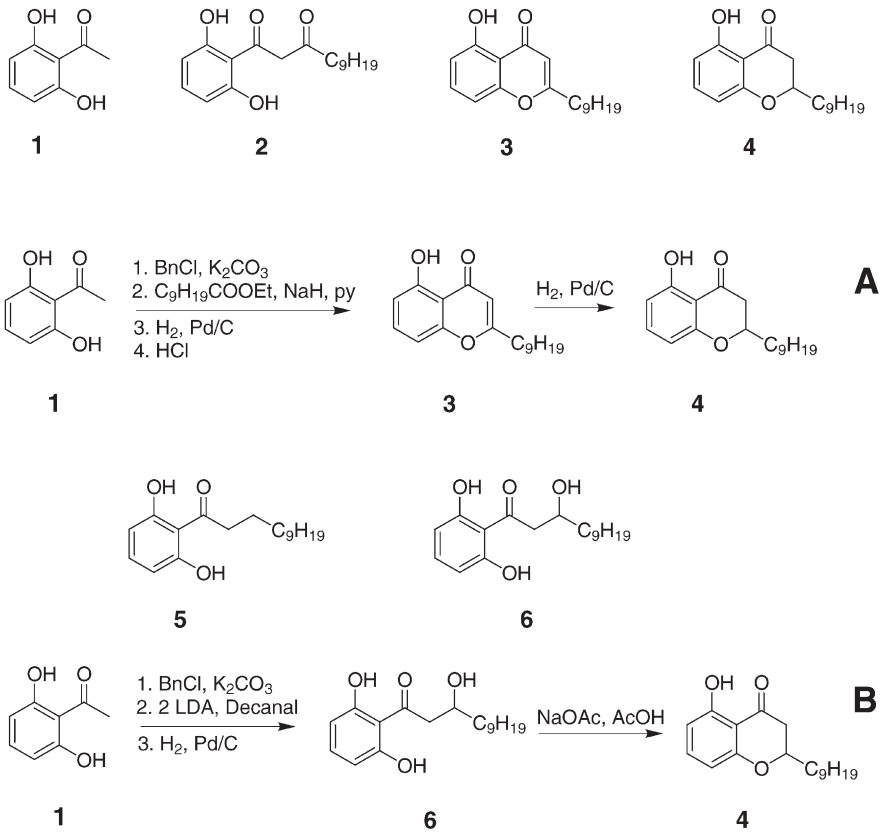
Adults of some species also produce 4-oxo-(*E*)-2-alkenals. Other types of simple compounds that have been found in the defensive secretions of true bugs include common terpenoids such as α- and β-pinenes, limonene, linalool, and *Z*,*E*-α-farnesene, and simple aromatic compounds such as benzyl alcohol, benzaldehyde, *p*-hydroxybenzaldehyde, methyl *p*-hydroxybenzoate, phenylethanol, and guaicol. In general, although a number of species may share particular components, each species does appear to produce its own particular blend. In at least one species, the blend of defensive compounds is reported to vary with season and/or diet [36].

Some species produce more interesting and unusual compounds in defensive glands. For example, *Jadera* spp. (Rhopalidae; scentless plant bug family) produce (4*S*,5*S*)-5-hydroxy-4-decanolide, *cis*- and *trans*-8-hydroxylinalool [27], and 4-methyl-2(5H)furanone [26]. They also sequester toxic cyanolipids as glucosides from their host plants [26]. The stink bug *Oechalia schellenbergi* (Pentatomidae) secretes a series of unusual terpenoids, including 8-hydroxygeranyl diacetate and analogs [37], whereas nymphs of *Corythuca cydoniae* and *Gargaphia solani* (both Tingidae, lace bug family) produce 2,6,10-trimethyl-10-hydroxydodeca-2,6,11-trien-1-al (nerolidol aldehyde), amongst other compounds [38]. (2*E*,6*E*)-Octadiene-1,8-dial and the corresponding diacetate are found in the MTG of another stink bug, *Eurydema ventrale* [39], and γ-butyrolactone was identified from the MTG of the stink bug *Aethus indicus* [40]. Several amines were reported from exocrine secretions of nymphs of the stink bug, *Cyclopelta siccifolia*, including diisopropylamine, o-isopropenyl aniline, and octadecylamine [41].

The aquatic belostomatid species *Abedus herberti* (giant water bug family) produces four pregnane-type steroids from its cephalic glands, the secretions of which are proposed to function as a defense against predation by fish [42]. Another aquatic species, *Plea minutissima* (Pleidae, pigmy backswimmer family), secretes hydrogen peroxide from its MTG glands, which it uses as an antimicrobial agent to prevent bacterial fouling of the hairs that hold its respiratory air bubble [43].

Aposematic species from two different families produce pungent pyrazines, presumably as an additional warning to potential predators of their toxicity. These include *Oncopeltus fasciatus* (Lygaeidae, seed bug family) that secretes 2-isobutyl-3-methoxypyrazine [28], and the stink bug *Murgantia histrionica* (Pentatomidae), that oozes froth containing 2-isobutyl- and 2-*sec*-butyl-3-methoxypyrazine when molested [39].

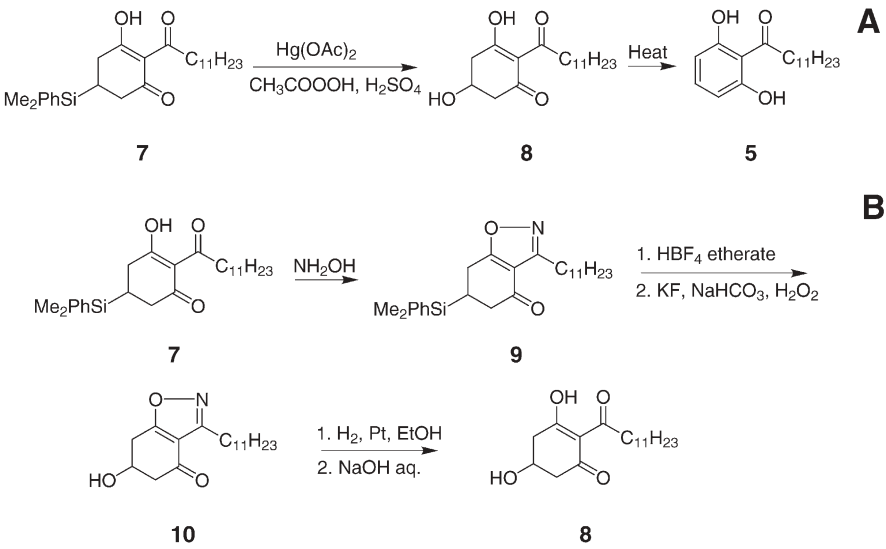
Probably the most interesting group of defensive compounds from true bugs are the unusual acetogenins secreted by nymphs and adults of lace bugs (Tingidae). These compounds are active against bacteria, fungi, and nematodes [44], and it has been proposed that they play a role in defense against predators [45, 46]. Thus, compounds from the azalea lace bug *Stephanitis pyrioides* included 2,6-dihydroxyacetophenone **1**, the diketone **2**, the related 5-hydroxy-2-nonylchromone **3** formed by cyclization and dehydration of **2**, and the chromanone **4** (Scheme 1) [47]. The latter compounds were readily synthesized



Scheme 1 Defensive compounds from lace bugs (Tingidae)

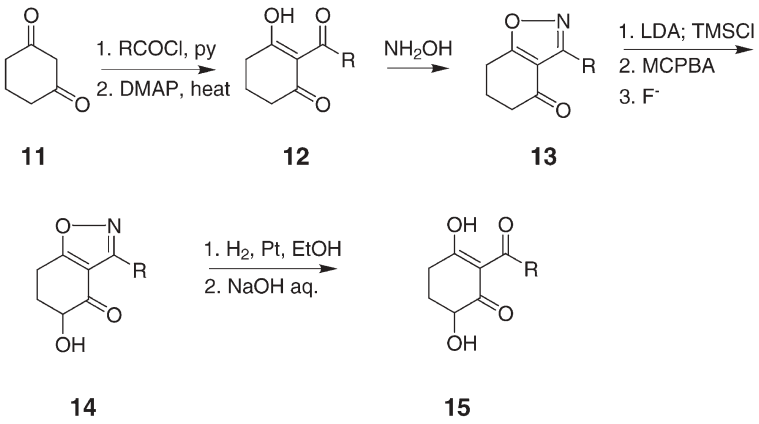
from **1** (Scheme 1A) [47]. The congeneric species *Stephanitis rhododendri* produced analogous diketones and chromones, with odd-numbered 7–17 carbon alkyl chains attached to the diketone and the corresponding chromones [48]. This species also produced analogs with a 2,4,6-trihydroxylation pattern, which were synthesized from 2,4,6-trihydroxyacetophenone as described above for the dihydroxy compounds, with the exception that two of the three hydroxyl groups were protected as *tert*-butyldimethylsilyl instead of benzyl ethers [48]. Extracts of the andromeda lace bug *S. takeyai*, contained the 2,6-dihydroxyacetophenone analog **5** and 5-hydroxy-2-nonylchromanone **4** (see above), and the related, thermally unstable ketoalcohol **6** in which the pyranone ring was not closed [46]. The latter compound was readily synthesized by reaction of the enolate of monoprotected 2,6-dihydroxy-acetophenone with decanal, and subsequent removal of the benzyl protecting group (Scheme 1B). Treatment with NaOAc/AcOH then effected the ring closure to form the chromanone **4**. This species also produced the 2-dodecanoyl-3,5-dihydroxycyclohex-2-ene-1-one **8** [46], the structure of which was confirmed by oxidative desilylation of

the known 5-phenyldimethylsilyl compound **7** [49], albeit in low yield (Scheme 2A). The synthesis was improved by protection of the 1,3-dicarbonyl system as an isoxazole (**9**), oxidative cleavage of the silyl group to give **10**, and removal of the isoxazole (Scheme 2B) [50].



Scheme 2 Defensive compounds from *Stephanitis* lace bug spp

Lace bugs in the genus *Corythucha* produce the isomeric 2-acyl-3,6-dihydroxy-2-cyclohexene-1-ones, with a different pattern of oxygenation [45, 51, 52]. These were synthesized from 1,3-cyclohexanedione **11** (Scheme 3), with key steps being acylation to give **12**, protection of the 1,3-dicarbonyl as the isox-



Scheme 3 Synthesis of lace bug chemical defense compounds

azole **13**, and MCPBA oxidation of the TMS enol ether derived from **13**. Reaction of the resulting epoxide with fluoride removed the TMS group, producing the key hydroxyketone intermediate **14**, which was then deprotected to give 2-acyl-3,6-dihydroxy-2-cyclohexene-1-one **15**. Analogs with double bonds in the side chain were selectively oxidized at the more electron-rich TMS enolate in preference to the sidechain alkene. Reduction of the sidechain alkene was avoided by cleavage of the isoxazole ring with P2 nickel ($NaBH_4/NiCl_2$ in DMF/THF, with excess 1-octene) instead of Pt and hydrogen [45, 52].

4
Bug Pheromones

In the sections that follow, the various bug families are dealt with in their commonly accepted taxonomic order [3]. It should be noted that pheromones have been described from less than half of the bug families. Even for those families in which pheromones are known, pheromone identifications have been carried out for only a few species, leaving a vast number of semiochemicals still to be discovered.

4.1
Stink Bugs (Pentatomidae)

When stink bug eggs hatch, the first instar nymphs cluster together in dense aggregations. The formation of these aggregations has been shown to be mediated by chemical cues [53], including 4-oxo-(*E*)-2-decenal [31–33], which is normally thought of as a defensive compound. Remarkably, the cues that mediate aggregation appear to be similar enough between species that nymphs of different species readily form heterospecific aggregations [33, 53], even in preference to conspecific groups [33]!

There are more pheromones known or suspected for adult pentatomid bugs than for any other bug family. However, a summary of pheromone chemistry within this group is confounded by several factors. First, a number of compounds have been labeled as pheromones or "putative pheromones" with little or no supporting biological data. Second, the pheromones are often only weakly attractive, making it difficult to verify the roles of compounds as bona fide biological signals. Third, mating behavior in phytophagous pentatomids actually consists of two distinct steps, with longer range orientation being mediated by pheromones, and shorter range orientation being controlled by species-specific, substrate-borne vibration signals produced by both sexes [54]. Although plant-feeding pentatomid bugs may be attracted to the vicinity of a pheromone lure, few bugs may be caught in pheromone-baited traps, which lack the vibrational signals needed for short-range attraction. The situation seems more straightforward for predatory pentatomid species in the subfamily Asopinae (soldier bugs), with evidence of strong attraction to

pheromones in some species. Thus, the predatory species will be discussed first.

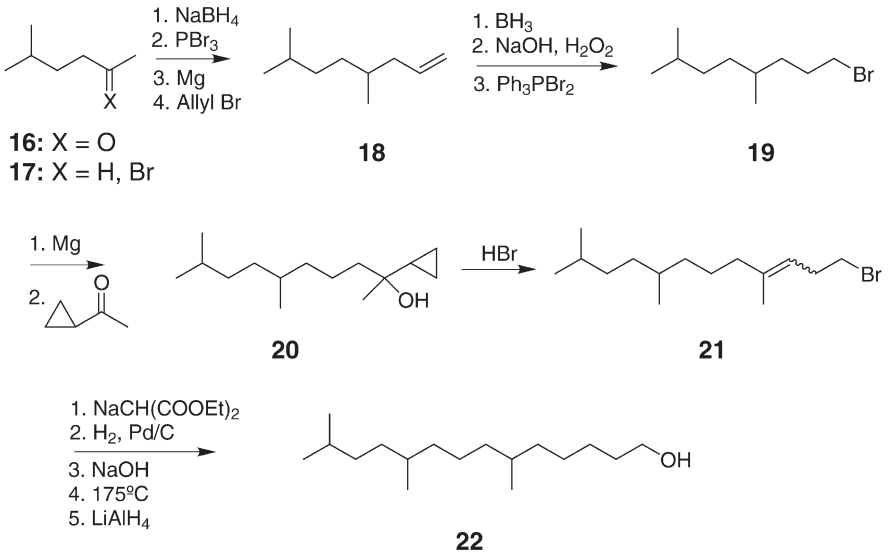
4.1.1 Podisus Species

The aggregation pheromone of the spined soldier bug *Podisus maculiventris* was the first attractant pheromone identified from a true bug. The attractive blend consists of three terpenes ((*R*)-α-terpineol, terpinen-4-ol, and linalool) in combination with benzyl alcohol and (*E*)-2-hexenal [55]. The compounds originate from the enlarged dorsal abdominal glands (DAGs) of males, and attract both sexes and nymphs [56]. Starved bugs responded most strongly [57], and it was suggested that males are the "pioneering sex", and that having found a good habitat, males produce pheromone to attract conspecifics [55]. Blends of (*E*)-2-hexenal, benzyl alcohol, and linalool were also attractive to both sexes of the congeneric species *Podisus fretus*, and were found in DAG extracts from males [58]. These and similar compounds, including (*E*)-2-hexenol, (*E*)-2-hexenyl tiglate, tiglyl aldehyde, benzyl tiglate, benzaldehyde, (*E*)-2-hexenyl benzoate, 9-hydroxy-2-nonanone, (*E*)-2-octenol, (*E*)-2-octenal, nonanol, (*Z*)-3-nonenol, 2-(4-hydroxyphenyl)ethanol, and *trans*-piperitol have been isolated from DAGs of males of four other *Podisus* spp. (*P. mucronatus*, *P. placidus*, *P. connexivus*, and *Podisus* new sp.) but their biological activity has not been tested [59].

4.1.2 Stiretrus, Perillus, Oplomus, and Mineus Species

Males of another group of asopine bugs, including *Stiretrus anchorago* [60, 61], *Perillus bioculatus* [60], *Oplomus servus* [60], *O. dichrous* [62], *Mineus strigipes* [62], and *Eocanthecona furcellata* [24] produce one or more of 6,10,13-trimethyltetradecanol **22**, the corresponding aldehyde, and the isovalerate ester from large sternal glands underlying pubescent patches on the abdomen. The alcohol, as a mixture of stereoisomers, attracted adults of both sexes and nymphs of *S. anchorago* close to but not into traps [61], suggesting that short-range signals were also important. However, no further tests of the biological activity of these compounds, with any of the species that produce them, have appeared in the primary literature. Hence, their possible roles as pheromones remains ambiguous.

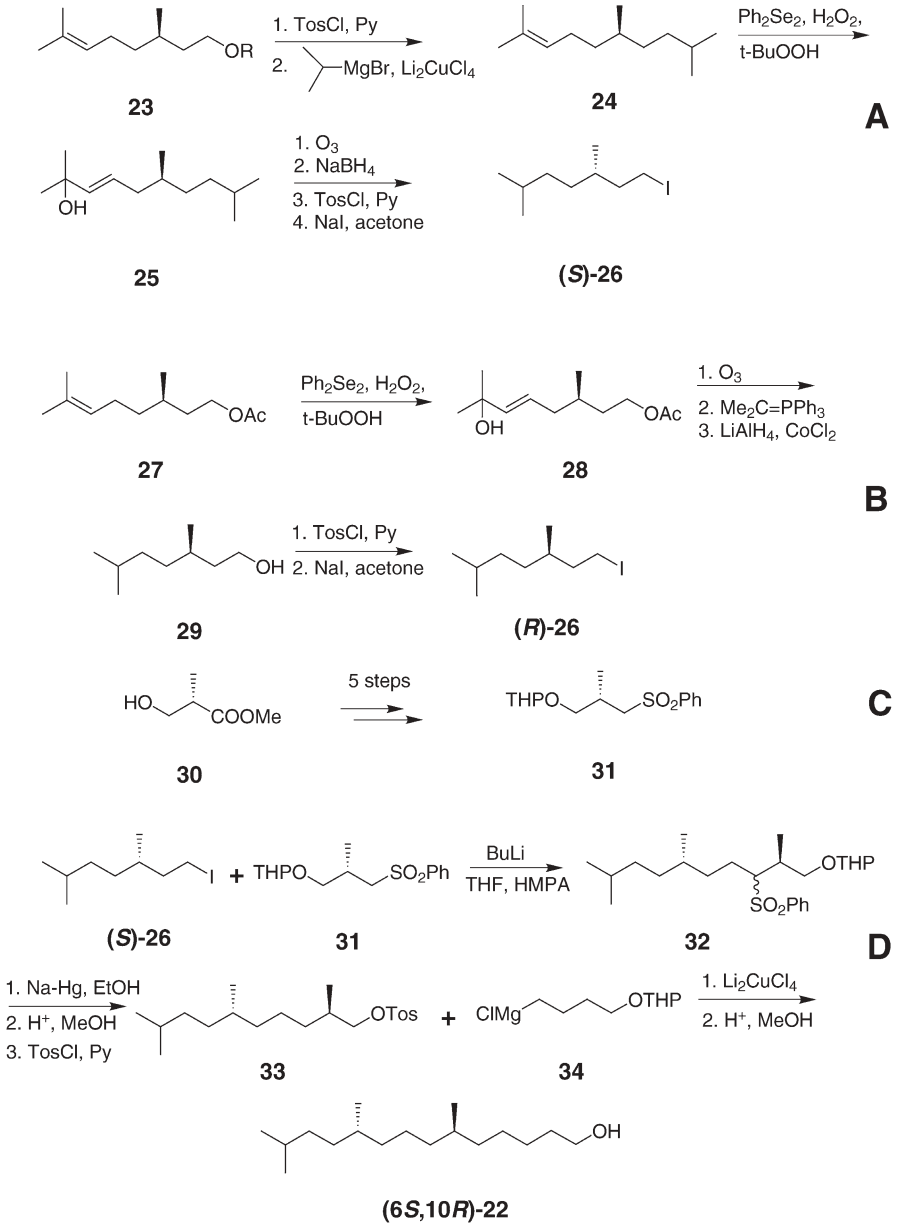
6,10,13-Trimethyltetradecanol **22**, while possibly of terpenoid origin, is characterized by a truncated isoprene unit on the terminus of the chain, so that there are only two rather than the normal three methylenes between the methyl groups. The first nonstereoselective synthesis of **22** began with methyl-branched 5-methyl-hexan-2-one **16**, with the two other methyl branches being introduced via reaction of a 2° Grignard reagent with an allyl halide and Julia rearrangement of methyl cyclopropanol respectively (Scheme 4) [61]. Thus, ke-



Scheme 4 Nonstereoselective synthesis of 6,10,13-trimethyltetradecanol [61]

tone **16** was reduced to the corresponding alcohol, converted to the bromide **17** and thence to the Grignard reagent. Reaction of this with allyl bromide introduced the second methyl branch, giving **18.** Further elaboration by hydroboration of the terminal alkene to an alcohol and conversion to the bromide **19,** formation of a Grignard reagent, and reaction of this with methylcyclopropyl ketone produced alcohol **20.** Ring opening and dehydration by treatment with HBr placed the third methyl group, giving bromide **21.** Two-carbon chain extension via malonic ester synthesis, followed by reduction of the alkene, decarboxylation, and reduction of the ester provided the desired product **22.**

Mori and Wu [63] developed syntheses of all four stereoisomers, using citronellol and methyl 3-hydroxy-2-methylpropionate enantiomers as the sources of the two chiral methyl groups (Scheme 5). A key step involved moving the double bond of citronellol one carbon closer to the chiral methyl group so that subsequent cleavage of the double bond would yield a difunctionalized unit with the correct chain length and a chiral methyl. Thus, tosylation of (*R*)-citronellol (**23**, R=H) followed by copper-catalyzed reaction with isopropyl magnesium bromide produced chiral hydrocarbon **24** (Scheme 5A). Oxidation with diphenyldiselenide, H_2O_2, and *tert*-butylhydroperoxide moved the double bond one position closer to the methyl group, producing allylic alcohol **25**, which was ozonized, reduced, and converted to iodide synthon (*S*)-**26**, containing two of the three methyl groups. In a clever twist (Scheme 5B), the other enantiomer of this synthon also was produced from the same starting material. Thus, (*R*)-citronellyl acetate **27** was oxidized as before, with a shift of the double bond to give **28**, followed by ozonolysis and chain extension of the resulting aldehyde by



Scheme 5 Synthesis of (6*S*,10*R*)-6,10,13-trimethyltetradecanol [63]

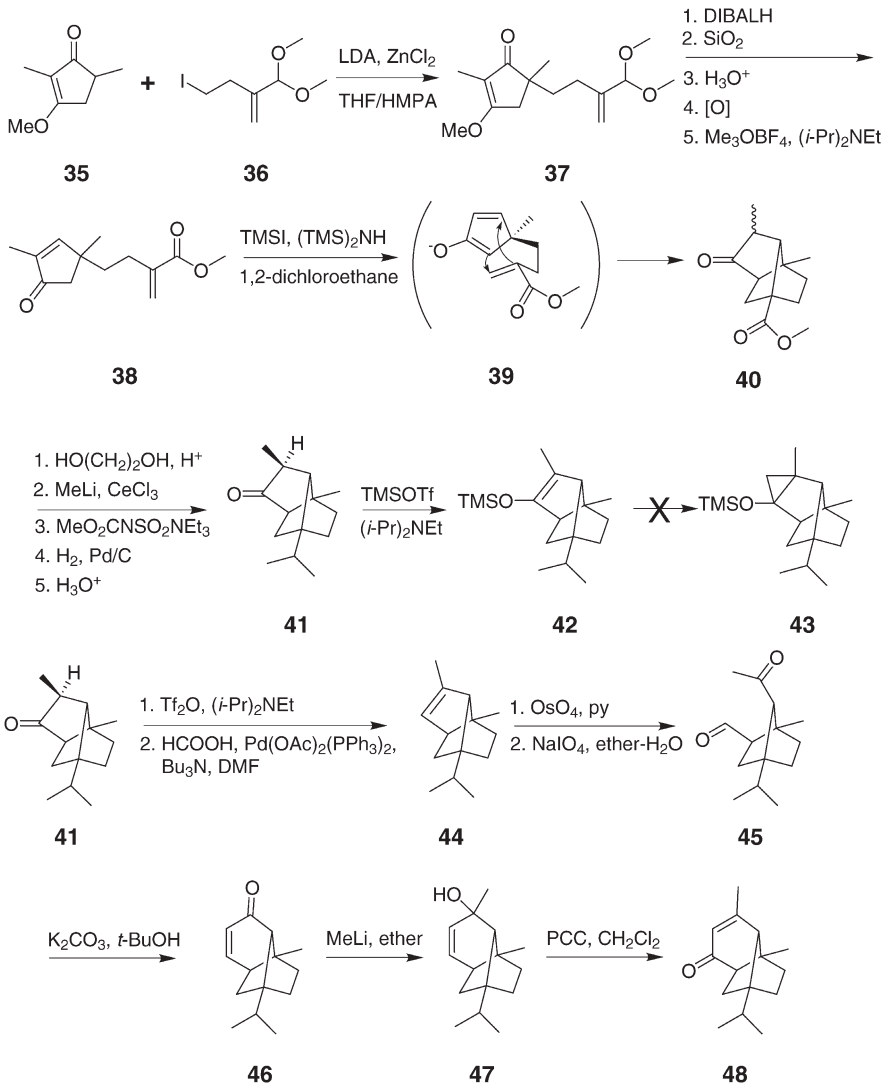
reaction with isopropylidenetriphenylphosphorane, then simultaneous reduction of the alkene and ester to give alcohol **29**. Straightforward conversion of the alcohol **29** to the iodide (*R*)-**26** completed this synthon.

The second chiral methyl group was derived from the enantiomers of methyl 3-hydroxy-2-methylpropionate **30** by the sequence: THP protection of the alcohol, reduction of the ester to the alcohol, tosylation, conversion to the iodide, and then to the phenylsulfone **31** (Scheme 5C). The anion of sulfone **31** was coupled with iodide **26** in THF/HMPA (Scheme 5D), placing the second chiral methyl group (**32**), followed by reductive removal of the sulfone group. Deprotection of the alcohol and tosylation to give **33**, followed by copper-catalyzed chain extension with the Grignard reagent prepared from THP-protected 4-chloro-1-butanol (**34**), and finally, removal of the THP, completed the synthesis of the (6*S*,10*R*)-**22**. The syntheses of the other stereoisomers were completed in analogous fashion by appropriate choice of the enantiomers of the two key chiral synthons. However, to date, the stereoisomer(s) which are actually produced by the various species of stink bugs that use this compound or its derivatives does not seem to have been determined.

4.1.3 Tynacantha Marginata

The asopine bug *Tynacantha marginata* represents a case in point of the premature designation of insect-produced compounds as pheromones. Males of this species produce a novel tricyclic sesquiterpenoid **48** from their "pheromone gland", which has been designated as a "putative sex pheromone" [64], apparently on the basis that it is produced only by males. No assessment of the biological activity of crude extracts from the bugs, the purified compound from the bugs, or the synthetic compounds (see below) has been reported in the primary literature.

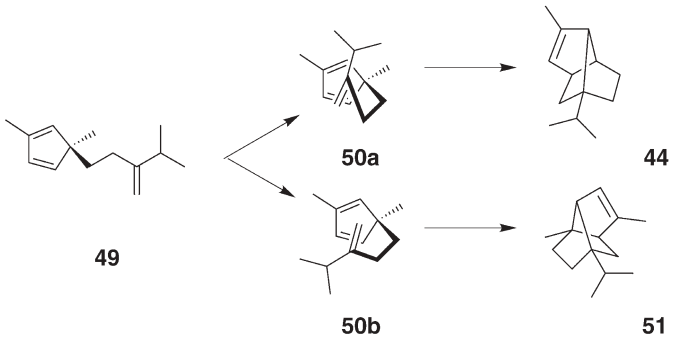
The structure of **48** was confirmed by synthesis of the racemate in 18 steps and 4.2% yield (Scheme 6) [65]. The key step was a tandem Michael cyclization that assembled a tricyclic skeleton from a substituted cyclopentenone. Thus, coupling of the zinc enolate of ketone **35** with iodide **36** gave ketone **37**. Straightforward functional group manipulations then produced key intermediate **38** for the cyclization. Treatment of **38** with trimethylsilyl iodide and bistrimethylsilylamine generated enolate **39**, which underwent two consecutive Michael additions to give tricyclic ketoester **40**. Protection of the ketone, followed by reaction of the methyl ester with MeLi and $CeCl_3$ gave the expected tertiary alcohol, which was dehydrated with Burgess' reagent and reduced to complete the construction of the isopropyl group. Aqueous acid then removed the ketal, providing ketone **41** ready for further manipulation. At this point, the synthetic scheme had called for ring expansion by cyclopropanation of TMS enol **42** to give **43**. However, all attempts at cyclopropanation failed. Consequently, the ring was expanded via opening and reclosure. Thus, ketone **41** was



Scheme 6 Synthesis of the racemate of a novel tricyclic sequiterpenoid produced by males of the stink bug *Tynacantha marginata* [65]

deoxygenated via the enol triflate to give alkene **44**, which was oxidatively cleaved to ketoaldehyde **45**. An aldol reaction then reclosed the third ring yielding enone **46**. Alkylation of **46** with MeLi in ether gave tertiary allylic alcohol **47**, which upon treatment with pyridinium chlorochromate in CH_2Cl_2 resulted in elimination of the tertiary alcohol and concomitant oxidation at the β-position to yield the desired enone product **48** as the racemate.

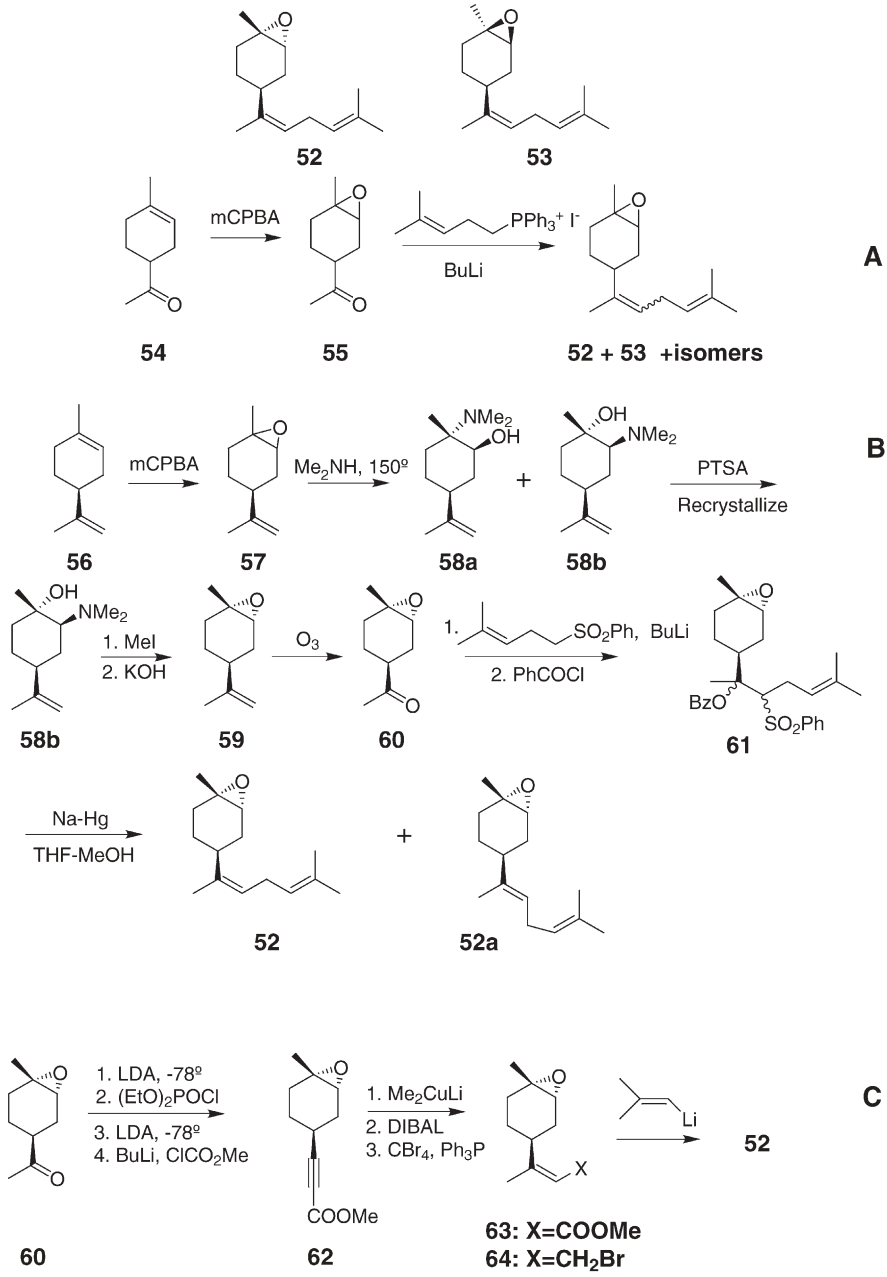
Elements of this synthesis were used as the foundation for a clever synthesis of both enantiomers from a single chiral precursor **49**, with a Diels-Alder reaction comprising the key step [64]. The critical concept was the recognition that the Diels-Alder reaction of **49** could proceed through two different transition states (Scheme 7), with the product from one transition state (**50a**) being the tricyclic alkene **44** which had been readily converted to the desired product **48** in the synthesis of the racemate (see above). The second transition state (**50b**) would provide intermediate **51** that, with slightly different synthetic manipulations, could be converted to the other enantiomer of **48**. The full synthesis has been described in Vol. 1, Chap. 1, and so will not be reiterated here.



Scheme 7 Diels-Alder reaction of a single precursor proceeds via different transition states that produce two isomeric intermediates, each of which can be converted to one of the enantiomers of the novel *Tynacantha marginata* sesquiterpenoid [64]

4.1.4 Nezara and Acrosternum Species

Nezara viridula is an important agricultural pest worldwide, with more than 200 known host plants. Following reports that sexually mature males attracted conspecifics [11, 66], the major pheromone components were identified approximately simultaneously by two groups [67, 68] as *trans*-*Z*-bisabolene epoxide ((*Z*)-(1′*S*,3′*R*,4′*S*)-(−)-2-(3′4′-epoxy-4′-methylcyclohexyl)-6-methylhepta-2,5-diene; *trans*-Z-BAE **52**, Scheme 8; the *trans* designation refers to the relationship between the epoxide and the pendant group on the ring), and the corresponding *cis*-isomer (*cis*-*Z*-BAE **53**, Scheme 8). Two compounds that were initially thought to be involved in the pheromone were later shown to be artifacts from dimerization of the defensive chemical 4-oxo-(*E*)-2-hexenal [17]. *Z*-α-Bisabolene, *E*-nerolidol, and nonadecane were also identified from male volatiles [68]. The chemicals are produced in unicellular glands that secrete onto the ventral abdominal cuticle [15, 18]. In laboratory bioassays, female bugs were attracted to extracts from males [18, 69], and to *trans*-*Z*-BAE, either alone



Scheme 8 Syntheses of *cis-Z-* and *trans-Z*-bisabolene epoxide pheromone components from *Nezara viridula* and other stink bug species, part 1 [67, 71, 76, 77]

or in combination with *cis-Z*-BAE, which was not attractive alone [70, 71]. In laboratory bioassays, only females were attracted to the synthetic pheromone [70], whereas field tests suggest that both sexes and nymphs were attracted to live males [11, 66]. Furthermore, bugs are rarely attracted into pheromone-baited traps [17] (also J.G.M. unpublished data), although they may indeed be attracted to the vicinity of traps. This graphically illustrates that mate location in this and other phytophagous pentatomids appears to be a two-step process, with longer range attraction being mediated by chemicals, but with critical, short-range orientation being mediated by substrate-borne vibrational signals [69]; reviewed in [54]. However, the two modes of signaling are linked, because males increase pheromone production when stimulated by vibrational signals of females [23].

The ratio of *trans-* to *cis-Z*-BAEs produced by male *N. viridula* is variable, and has been the subject of controversy. Initial reports suggested that the variation might be due to divergence in the blends produced by different geographic races of the insect [68, 71, 72]. However, analysis of extracts prepared from individuals revealed substantial intrapopulational variability [73]. More recently, a study of the time course of pheromone production by repeatedly sampling individuals using solid phase microextraction has confirmed that the blends produced by individuals within a population are variable, but that the blend ratio produced by a given individual remains constant throughout its life [74]. The importance of the *trans*:*cis* ratio remains unclear, and females apparently respond to a wide range of ratios [71].

Nezara antennata males produce similar volatile compounds, including *Z*-α-bisabolene, *trans*-Z-BAE, *cis*-Z-BAE, and *E*-nerolidol, as do males of four *Acrosternum* species [17, 72]. In three of these species (*A. aseadum*, *A. hilare*, and *A. marginatum*), the *trans*:*cis* ratio strongly favors *cis*-BAE, whereas in *A. pennsylvanicum*, the ratio is about equal. For all species except *A. hilare*, the absolute configurations of the compounds and their biological roles remain unknown.

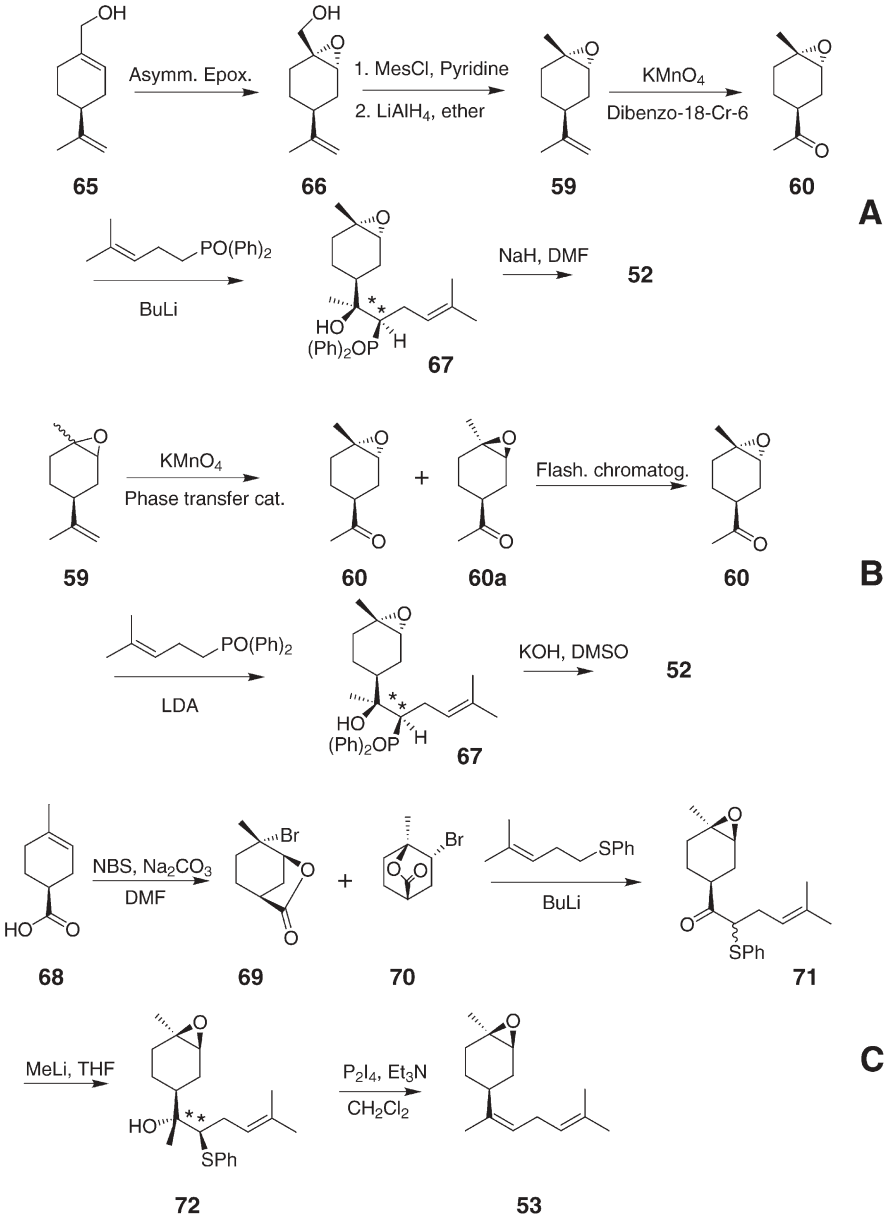
The pheromone chemistry of *A. hilare* has been studied in detail, using both laboratory (vertical Y-tube) and field bioassays [75]. In Y-tube bioassays, mature females were attracted by male odors, but males were not attractive to other males, and females were not attractive to either sex. Males produced (–)-*cis-Z*-BAE and (–)-*trans-Z*-BAE in a ~19:1 ratio, and other compounds in the extracts were not active. The 19:1 blend was more attractive to females than other ratios, and the individual components were not attractive, indicating that both compounds were required. Females were attracted to the synthetic blend in field cage trials [75], but as with most other phytophagous pentatomids, few bugs were caught in pheromone-baited traps (J.G. Millar and H.M. McBrien, unpublished data).

Although the BAEs can be produced from commercially available ketone **54** in two steps via epoxide **55** (Scheme 8A) [76], the resulting mixture of stereoisomers may be of little practical use because the insects appear to use specific blends of only two of the eight possible stereoisomers. However, the

production of multigram quantities of the pure BAE stereoisomers is not trivial, and several syntheses have been reported. The first synthesis commenced with readily available (+)- and (–)-limonene **56** (Scheme 9B) [67]. Thus, (–)-limonene **56** was regioselectively epoxidized to **57**, followed by opening of the epoxide ring with dimethylamine at 150 °C. The resulting mixture of amino-alcohols **58** was separated by recrystallization of the tosyl salts. Methylation of the amine and reclosure of the epoxide ring by treatment of the resulting trimethylamine salts with base gave the pure limonene oxide enantiomer (e.g., **59**). Each enantiomer was ozonized (**60**), then alkylated with the anion of phenyl (4-methyl-3-pentenyl)sulfone to give epoxide **61**. Reductive elimination of the sulfone moiety with Na-Hg amalgam then gave mixtures of the *Z* and *E* isomers **52** and **52a**, which were separable by HPLC. Whereas this synthesis controlled the relative stereochemistry of the pendant alkyl group and the epoxide ring, it did not control the stereochemistry of the trisubstituted alkene. A variation on this synthesis [71] proceeded via epoxidation of the trisubstituted double bond of (*S*)-(–)-limonene **56** followed by ozonolysis of the remaining double bond, separation of the resulting *cis*- and *trans*-epoxyketones, reaction of each ketone with the ylid from 4-methyl-3-pentenyl phosphonium iodide, and preparative GC separation of the resulting *Z* and *E* isomers.

A third synthesis [77] addressed the trisubstituted alkene problem (Scheme 9C). Thus, the epoxyketones **60** were prepared from commercial limonene oxide (a mixture of diastereomers, with the stereochemistry of the pendant isopropenyl group fixed) by ozonolysis, followed by chromatographic separation of the epoxyketones **60**. The ketone function was then converted to a terminal acetylene via a vinyl phosphate intermediate, the anion of which was then reacted with methyl chloroformate to produce the α,β-acetylenic ester **62**, setting the stage for stereocontrolled introduction of the key trisubstituted double bond by Michael reaction of the ester with dimethyllithium cuprate. DIBAL reduction of the ester **63**, conversion of the resulting alcohol to bromide **64**, and alkylation with 2-methyl-1-propenyl lithium at –78 °C in THF gave a single stereoisomer of **52**. Using this route, all four stereoisomers of **52** and **53** can be accessed in controlled fashion from one of the four readily available epoxyketone intermediates.

Further variations on the epoxyketone intermediate theme have been reported. In the first (Scheme 9A) [78], limonene oxide was prepared by Sharpless asymmetric epoxidation of commercial (*S*)-(–)- perillyl alcohol **65** followed by conversion of the alcohol **66** to the crystalline mesylate, recrystallization to remove stereoisomeric impurities, and reduction with $LiAlH_4$ to give (–)-limonene oxide **59**. This was converted to the key epoxyketone **60** by phase transfer catalyzed permanganate oxidation. Control of the trisubstituted alkene stereochemistry was achieved by reaction of the ketone with the anion from (4-methyl-3-pentenyl)diphenylphosphine oxide, yielding the isolable *erythro* adduct **67**, and the trisubstituted *E*-alkene **52a** from spontaneous elimination by the *threo* adduct. Treatment of the *erythro* adduct with NaH in DMF resulted



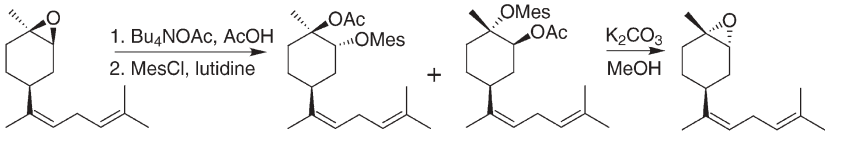
Scheme 9 Syntheses of *cis-Z*- and *trans-Z*-bisabolene epoxide pheromone components from *Nezara viridula* and other stink bug species, part 2 [78, 79, 81]

in stereospecific elimination of diphenylphosphinic acid to yield the *Z* stereoisomer **52.**

A short, practical synthesis capable of delivering several grams of any one of the four stereoisomers with a *Z*-alkene for field testing was developed by combining and optimizing elements of the above syntheses (Scheme 9B) [79]. Thus, chiral limonene oxide **59** was oxidized with permanganate to a chromatographically separable mixture of epoxyketones **60** and **60a.** Each pure epoxyketone was then reacted with the anion generated by treatment of (4-methyl-3-pentenyl)diphenylphosphine oxide with LDA (rather than BuLi, which resulted in low yields and epimerization), yielding the *erythro* adduct as a mixture of diastereomers **67.** These diastereomers were not separable by chromatography, but separated during recrystallization, as evidenced by two observed melting ranges. After extensive experimentation, the best conditions for the base-induced elimination from the purified *erythro* isomers were found to be rapid addition of powdered KOH to a DMSO solution of the *erythro* adducts at room temp, and quenching as soon as all the starting material was consumed, yielding **52** in quantities of several grams.

Epoxyketone **60** has also been prepared by hydroxyselenation of 4-acetyl-1-methylcyclohexene with phenylselenium chloride and water, oxidation of the selenide to selenoxide with buffered aqueous oxone, and elimination of the selenoxide in the same pot to provide the epoxide [80]. Control of the conditions was essential to prevent epimerization of the ketone. This route has little to recommend it given the expense and toxicity of the reagents, the moderate yield, and the problems with epimerization.

Kuwahara et al. [81] used a different approach, starting from chiral acid **68**, derived from an asymmetric Diels-Alder reaction. Bromolactonization gave a mixture of regioisomeric lactones **69** and **70** (Scheme 9C), which were reacted with the anion of phenyl (4-methyl-3-pentenyl)sulfide to open sequentially the lactone and close the epoxide, yielding a 5:3 mixture of stereoisomers **71.** Reaction of this mixture with methyllithium yielded a mixture of 2 pairs of *erythro* and *threo* isomers **72,** from which the *threo* isomers were isolated by chromatography. Base-induced *syn*-elimination from this pair of isomers would produce the undesired *E*-alkene, and so a stereospecific *anti*-elimination was carried out by treatment of the thioalcohols with P_2I_4 and Et_3N, producing *cis*-BAE **53** in 18% overall yield for the 4-step sequence from acid **68.** In a followup, the configuration of the epoxide ring of **53** was inverted to produce the other, isomeric component of the *N. viridula* pheromone blend, *trans*-BAE **52,** by the 3-step sequence of epoxide opening with tetrabutylammonium acetate in AcOH to produce a regioisomeric mixture of hydroxyacetates, mesylation of the alcohol function to give **73a** and **73b**, and sequential base hydrolysis of the acetate with reclosure of the epoxide with configuration inverted, yielding **52** (Scheme 10) [82].

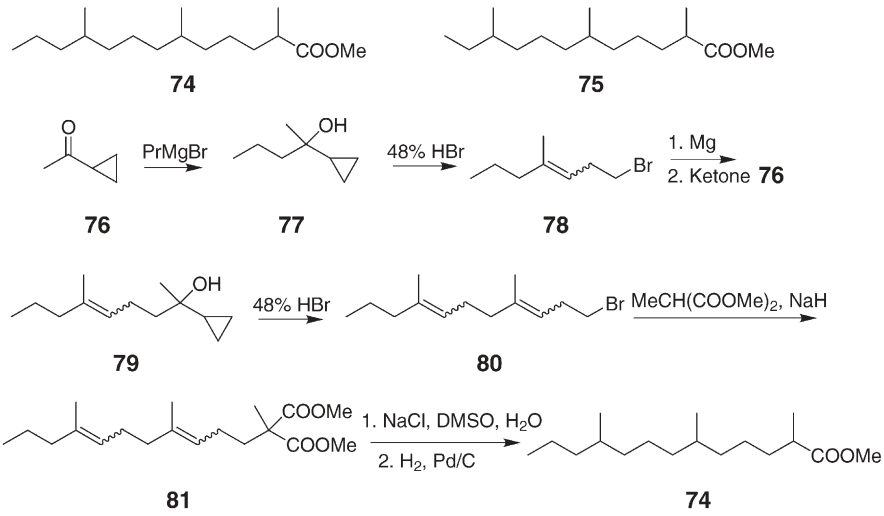


Scheme 10 Conversion of *cis-Z*-bisabolene epoxide to *trans-Z*-bisabolene epoxide

4.1.5 Euschistus Species

Pheromone components have been identified for several *Euschistus* species, many of which attack fruit and nut crops [1, 83]. Two general types of compounds are produced. Males of one group of species comprising *E. conspersus*, *E. tristigmus*, *E. politus*, *E. servus*, and *E. ictericus* produce methyl (2*E*, 4*Z*)-decadienoate (2*E*, 4*Z*-10:COOMe) as a major component [83], with 2*E*, 4*E*-10:COOMe being a minor component in all species except *E. tristigmus*. Other minor male-specific components included geranylacetone (*E. conspersus*, *E. tristigmus*, and *E. servus*), decanoic acid (*E. tristigmus*), and 2*Z*, 4*E*-10:COOMe. In field trials, traps baited with 2*E*, 4*Z*-10:COOMe caught a few *E. conspersus*, *E. tristigmus*, *E. politus*, and *E. servus* immatures and adults of both sexes, with additional bugs being clustered around the traps [83]. Minor male-specific components (decanoic acid for *E. tristigmus* and *E. politus*, geranylacetone for *E. servus*) did not synergise attraction [84], and for *E. conspersus*, isomeric purity of 2*E*, 4*Z*-10:COOMe does not appear to be critical for attraction (J.G. Millar and E. Cullen, unpublished data). Because of the tendency of bugs to cluster on plants close to pheromone-baited traps, the concept of using pheromone-baited "trap plants" has been developed for *E. conspersus* [85]. Thus, synthetic pheromone lures are attached to mullein plants, and the numbers of bugs attracted to the plants are counted, without actually using a trap. Approximately equal numbers of males and females were found on pheromone-baited plants [86]. 2*E*, 4*Z*-10:COOMe and/or the analogous ethyl ester are commercially available, and so their syntheses will not be discussed here.

In the second group of *Euschistus* spp., comprised of *E. heros* and *E. obscurus* [83, 87], male-specific volatiles of *E. heros* were dominated by methyl 2,6,10-trimethyltridecanoate (2,6,10–13:COOMe; **74**, Scheme 11), with 2,6,10–12:COOMe **75** and 2*E*, 4*Z*-10:COOMe being minor components. In *E. obscurus*, both 2,6,10–13:COOMe and 2*E*, 4*Z*-10:COOMe were major components, with traces of 2,6,10–12:COOMe and 2*E*, 4*E*-10:COOMe. The 12 and 13-carbon methyl esters have 8 possible stereoisomers, complicating their identification and synthesis (see below). Female *E. obscurus* were attracted to extracts from live males, but fractions of the extracts gave equivocal results [88]. Female *E. heros* were attracted to 10 μg of a synthetic mixture of all eight stereoisomers

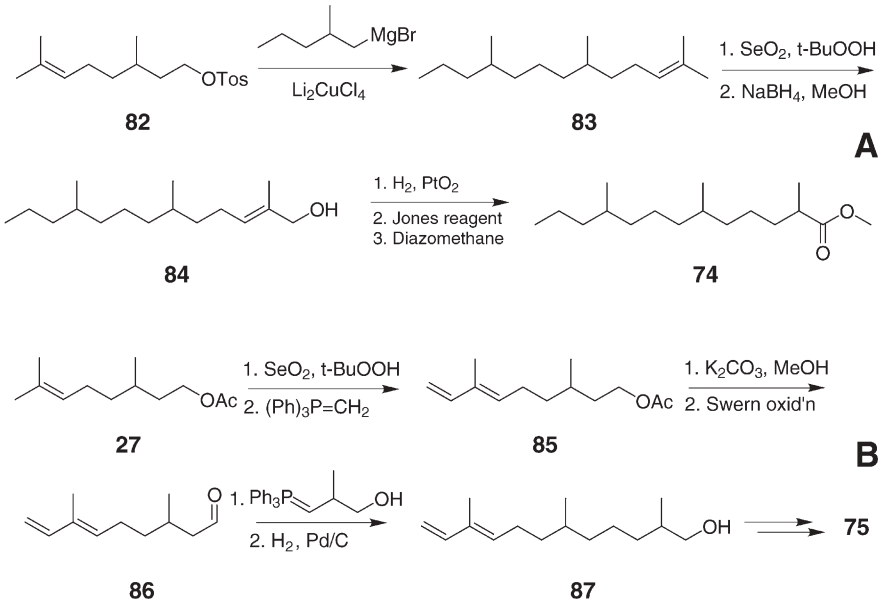


Scheme 11 First synthesis of methyl 2,6,10-trimethyltridecanoate as a mixture of stereoisomers [91]

of 2,6,10–13:COOMe in laboratory bioassays, with attraction being strongest during the latter half of the day [89]. In follow-up field trials with the stereoisomeric blend or with a blend of the 2*R*/*S*,6*S*,10*S*-13:COOMe isomers, a few *E. heros* were trapped [90], along with another pentatomid, *Piezodorus guildinii* (see below).

The first synthesis of methyl 2,6,10–13:COOMe **74** was carried out with no regard to stereochemistry to verify the basic carbon skeleton (Scheme 11) [91]. The Julia cyclopropane cleavage was used in two iterative steps to place two of the three methyl groups, similar to Kochansky's synthesis of 6,10,13-trimethyltetradecanol (see Scheme 4) [61]. Thus, Grignard reaction of cyclopropyl methyl ketone **76** with propyl magnesium bromide, followed by treatment of the resulting alcohol **77** with HBr, produced 1-bromo-4-methyl-3-heptene **78**. Conversion to the Grignard reagent, followed by reaction with cyclopropyl methyl ketone **76**, and treatment of the resulting alcohol **79** with HBr as before placed the second methyl group. The synthesis was completed by chain extension of bromide **80** with methyl dimethylmalonate to diester **81**, decarboxylation, and reduction of the alkene bonds. The resulting mixture of stereoisomers **74** was partially resolved (three peaks) on a Cyclodex B chiral GC column. The same sequence could be used to synthesize the methyl 2,6,10-trimethyldodecanoate homolog **75** by simply using ethyl instead of propyl magnesium bromide in the first step.

A second nonselective synthesis involved chain extension of the tosylate of (±)-citronellol (**82**) with 2-methylpentyl magnesium bromide and lithium tetrachlorocuprate catalysis to give the carbon skeleton **83** (Scheme 12A) [92]. Allylic oxidation with SeO_2 and *tert*-butylhydroperoxide, hydrogenation of the

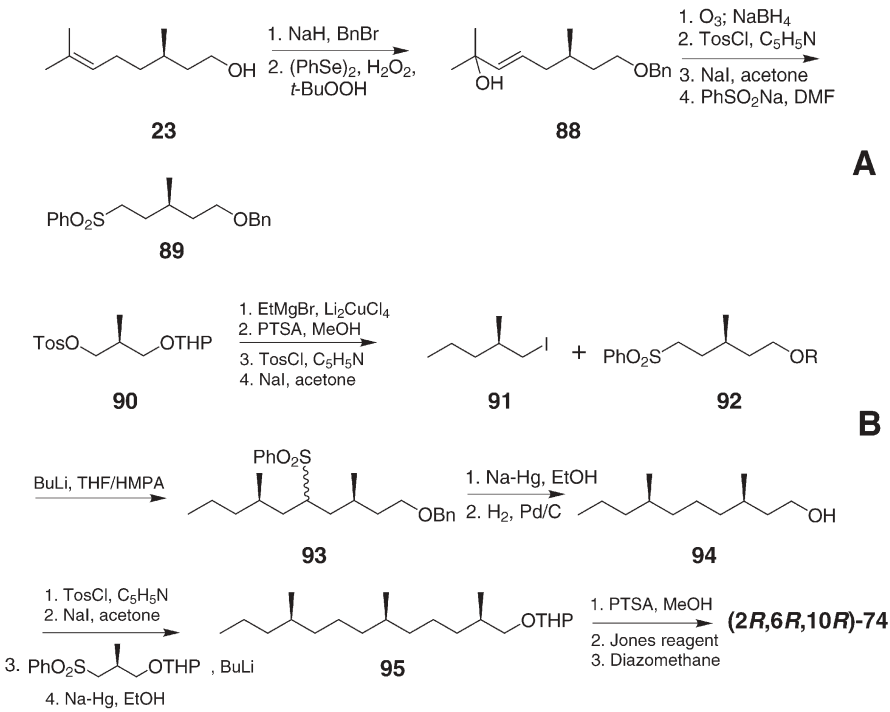


Scheme 12 Alternate syntheses of methyl 2,6,10-trimethyltridecanoate [92, 93]

resulting alkenol **84**, oxidation of the alcohol to the acid, and methylation with diazomethane gave the desired product **74** in six steps. A parallel synthesis of the homologous methyl 2,6,10-trimethyldodecanoate **75** was carried out by substituting 2-methylbutyl magnesium bromide in the chain extension step. The mixture of eight stereoisomers of the tridecanoate was attractive to female *Euschistus heros* in olfactometer bioassays, and the attraction appeared to be enhanced by addition of the dodecanoate homolog [92].

Zarbin et al. [93] published a second synthesis based on citronellol (Scheme 12B), reversing the modification of the ends of the chain. Thus, allylic oxidation of one of the methyl groups of citronellyl acetate **27** and one carbon chain extension via Wittig chemistry gave diene **85**, followed by hydrolysis and conversion of the alcohol at the opposite end of the chain to aldehyde **86**, and a second Wittig chain extension with the ylide from 3-hydroxy-2-methylpropyl triphenylphosphonium bromide to complete the carbon skeleton (**87**). Straightforward adjustment of functional groups completed the synthesis of **75**. This route was also amenable to the production of homologs by substitution of synthons of variable chain length in the first Wittig reaction step.

Stereospecific syntheses of the eight stereoisomers [94] used a variation of the methodology developed in Mori's previous synthesis of the stereoisomers of 6,10,13-trimethyltetradecanol (see Scheme 5) [91], using chiral synthons derived from commercially available enantiomers of citronellol and methyl 3-hydroxy-2-methylpropanoate, and the iterative series of steps outlined in Scheme 13A for one of the stereoisomers [94]. A key step involved moving the



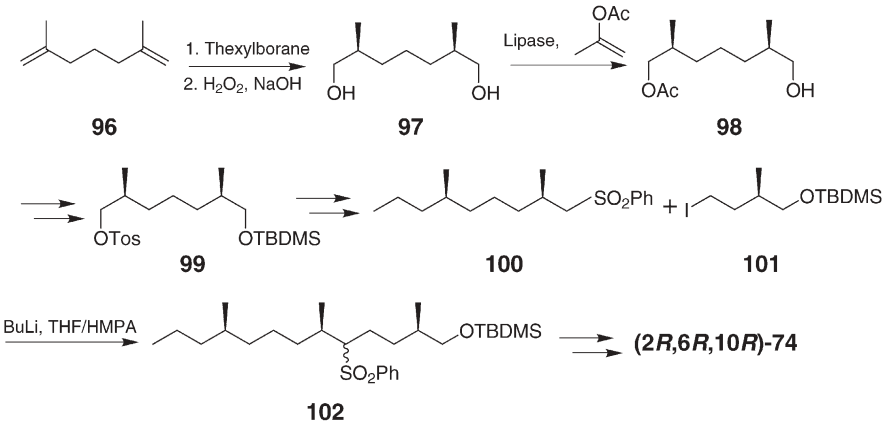
Scheme 13 Stereospecific synthesis of methyl (2*R*,6*R*,10*R*)-trimethyl-tridecanoate [94]

double bond of citronellol one carbon closer to the methyl group so that subsequent cleavage of the double bond would yield a 1,5-difunctionalized 5-carbon unit with a chiral methyl in the 3 position. Thus, benzyl protection of (*R*)-citronellol **23** followed by reaction with diphenyldiselenide, *tert*-butyl hydroperoxide, and hydrogen peroxide produced allylic alcohol **88**, which was ozonized and reduced to a monoprotected chiral diol. After conversion of the free alcohol to the iodide via the tosylate, the synthesis of the first building block was completed by conversion of the iodide to the phenylsulfone **89** by reaction with phenyl sulfinate in DMF. The second building block was constructed from methyl (2*S*)-3-hydroxy-2-methylpropanoate **30** as previously described (see Scheme 5).

The assembly of the building blocks began with Li_2CuCl_4-catalyzed reaction of tosylate **90** with ethyl magnesium bromide, removal of the protecting group, two-step conversion of the resulting alcohol to iodide **91**, reaction of this with the anion derived from the phenylsulfone building block **92** to give the coupling product **93**, removal of the phenylsulfone activating group by reduction with Na-Hg amalgam in EtOH, and finally removal of the benzyl protecting group. An analogous sequence of reactions on the resulting alcohol **94** then placed the third methyl group, giving **95**. The synthesis of (2*R*,6*R*,10*R*)-**74** was completed

by removal of the THP protecting group, oxidation to the acid, and methylation with diazomethane. Overall, mixing and matching the various synthons allowed access to each of the eight stereoisomers by the same sequences of steps.

Nakamura and Mori [95] developed another synthesis of the (2*R*,6*R*,10*R*)-isomer, in which two key steps were the facile synthesis of *meso*-2,6-dimethyl-1,7-heptanediol **97** by reaction of achiral 2,6-dimethylhepta-1,6-diene **96** with thexylborane, producing a 15:1 ratio of *meso* and racemic diols **97**, and chemoenzymatic desymmetrization of *meso*-**97** with isopropenyl acetate and commercial *Pseudomonas cepacia* lipase (Amano lipase PS30) in THF to give the chiral acetate **98** (Scheme 14) [96]. Manipulation of functional groups produced the tosylate **99.** Chain extension and functional group manipulation gave sulfone **100,** which was extended again with iodide **101** to give sulfone **102.** Further straightforward manipulations produced the (2*R*,6*R*,10*R*)-isomer **74** in 46% overall yield from acetate **98.**

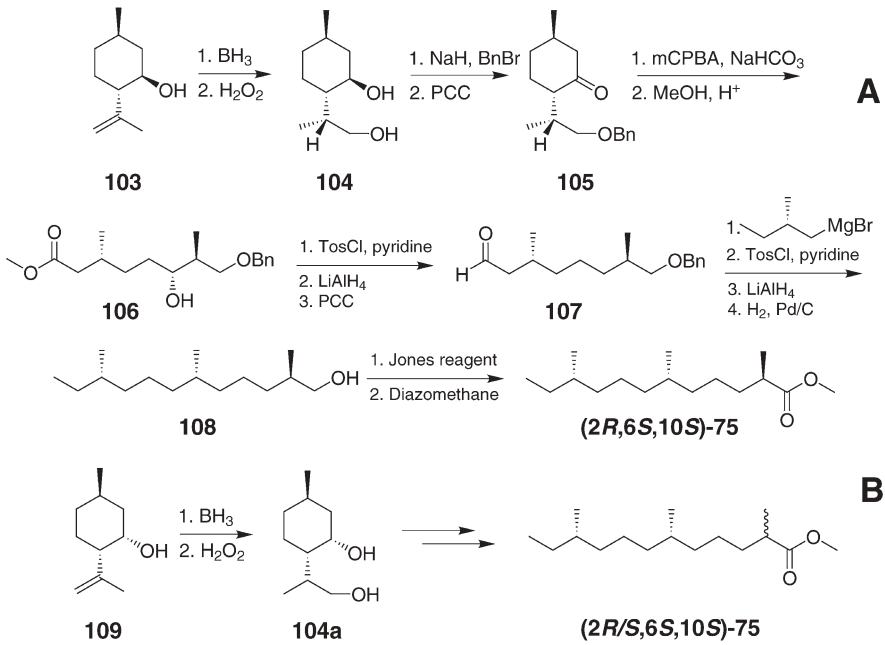


Scheme 14 Stereospecific synthesis of methyl (2*R*,6*R*,10*R*)-trimethyl-tridecanoate using a chemoenzymatic step to desymmetrize an achiral precursor [95]

Ferreira and Zarbin's syntheses [97] of two of the eight stereoisomers of the homologous 2,6,10–12:COOMe began with the chiral synthons (–)-isopulegol **103** and (+)-neo-isopulegol **109** (Scheme 15), and incorporated a third chiral synthon, (*S*)-(+)-1-bromo-2-methylbutane. In theory, these syntheses can be modified to produce any of the other isomers by combining the other enantiomers of these three synthons in appropriate combinations. Thus, (–)-isopulegol **103** was stereoselectively hydroborated to produce a separable 4:1 mixture of diastereomic diols, a factor that was crucial to this route (Scheme 15A). After purification, the primary alcohol of the major, desired isomer **104** was selectively protected, followed by oxidation of the 2° alcohol to the ketone **105**, Baeyer-Williger oxidation, and acid catalyzed opening of the lactone. Tosylation of the resulting hydroxyester **106** followed by $LiAlH_4$ reduction of the tosylate

and ester functions gave an alcohol with two chiral methyls now in place. The alcohol was oxidized to the aldehyde **107**, followed by Grignard reaction with chiral 2-methylbutyl magnesium bromide, placing the third chiral methyl group. The synthesis was completed by removal of the alcohol by tosylation and reduction as before, removal of the benzyl protecting group, oxidation of the resulting alcohol **108**, and finally, methylation with diazomethane to produce (2*R*,6*S*,10*S*)-2,6,10–12:COOMe **75**.

However, in the synthesis of the diastereomeric (2*S*,6*S*,10*S*)-enantiomer, the hydroboration of (+)-*neo*-isopulegol **109** was not as stereoselective, nor was it possible to separate the resulting diastereomeric alcohols **104a** (Scheme 15B). Thus, the final product was a 7:3 mixture of the (2*R*)- and (2*S*)-diastereomers of **75**.



Scheme 15 Stereoselective syntheses of two of the stereoisomers of methyl 2,6,10-trimethyldodecanoate [97]

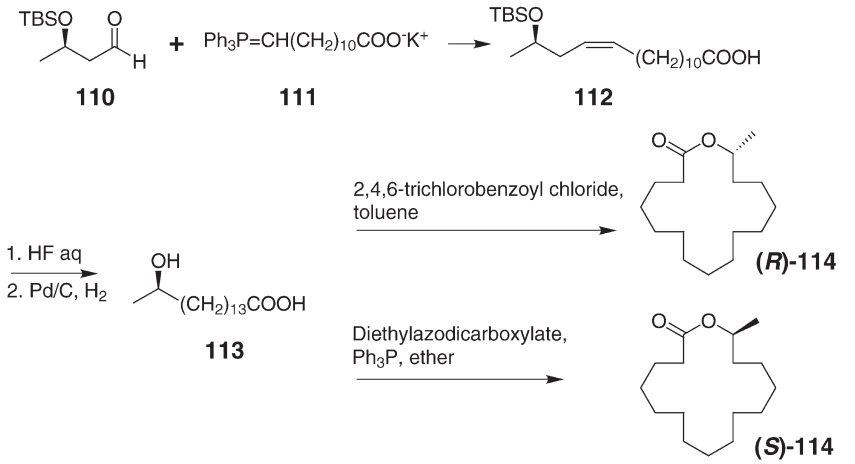
4.1.6 Piezodorus Species

Low numbers of *P. guildinii* were serendipitously attracted to pheromone traps baited with a mixture of stereoisomers of 2,6,10–13:COOMe **74** in a field test targetting *Euschistus heros* [90]. This compound, and the chain-shortened homolog, 2,6,10–12:COOMe **75**, were subsequently identified in headspace

volatiles from male bugs [98], but the biological roles of these compounds have not been delineated, nor have the absolute configurations of the insect-produced compounds been determined.

Following a report that male *P. hybneri* were attractive to both sexes [99], males were found to produce a mixture containing the sesquiterpene ß-sesquiphellandrene, (*R*)-15-hexadecanolide **114**, and methyl (*Z*)-8-hexadecenoate [100]. Odors from live males were attractive to adults of both sexes, with males also becoming sexually stimulated. Each component alone had some slight activity, with the 3-component blend being the best attractant in laboratory bioassays. The (*S*)-enantiomer of the macrolide lactone component was not inhibitory.

(*R*)-15-Hexadecanolide **114** was initially synthesized by asymmetric methylation of the hydrazone prepared from (*S*)-1-amino-2-(methoxymethyl)pyrrolidine (SAMP) and cylopentadecanone, followed by cleavage of the hydrazone and Baeyer-Williger oxidation of the ketone to produce the macrolide [100]. However, both the yield and the asymmetric induction were very poor. A linear route proved much more satisfactory (Scheme 16), particularly because both enantiomers could be produced from a single intermediate [101]. Thus, aldehyde **110**, prepared from ethyl (*R*)-3-hydroxybutyrate in two steps, was alkylated via Wittig reaction with the phosphorane **111**. Deprotection and reduction of the product **112** gave the free hydroxyacid **113**, which was then subjected to two different sets of macrolactonization conditions, the first (Yamaguchi conditions) proceeding with retention of configuration to provide (*R*)-**114**, and the second (Mitsunobu conditions) proceeding with inversion of configuration to provide the antipodal (*S*)-**114**.



Scheme 16 Synthesis of both enantiomers of 15-hexadecanolide from a single precursor via judicious choice of macrolactonization conditions

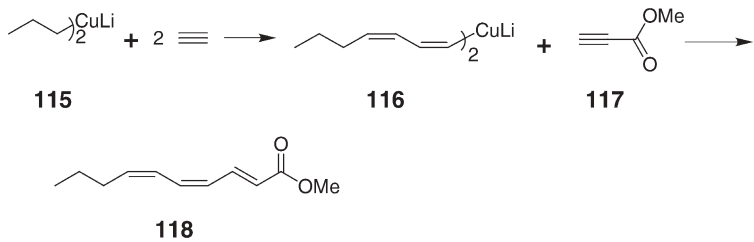
4.1.7
Plautia stali

Males of the brown-winged green bug *Plautia stali* attracted adults of both sexes [102], but the bugs were not sexually aroused, suggesting that the pheromone had an aggregative rather than a sexual function. The pheromone was identified from extracts of 180,000 bugs as methyl (2*E*, 4*E*, 6*Z*)-decatrienoate (2*E*, 4*E*, 6*Z*-10:COOMe) [103], a more unsaturated analog of 2*E*, 4*Z*-10:COOMe, a pheromone component for several *Euschistus* species (see above). A nonstereospecific synthesis of 2*E*, 4*E*, 6*Z*-10:COOMe based on Wittig chemistry has appeared in the patent literature [104]. The initial report of the identification indicated that the synthetic pheromone was attractive to both sexes, but no further information on the chemistry or biological activity of this compound has appeared in the general literature. Anecdotal reports suggest that it is being developed for insect management in Japan.

4.1.8
Thyanta Species

Almost simultaneously with the identification of 2*E*, 4*E*, 6*Z*-10:COOMe, a thermally unstable stereoisomer, 2*E*, 4*Z*, 6*Z*-10:COOMe **118**, was found to be a key component of the male-produced sex pheromone of *Thyanta pallidovirens*, along with the sesquiterpenes (+)-α-curcumene, (–)-β-sesquiphellandrene, and (–)-zingiberene **119** [10, 25]. 2*E*, 4*Z*, 6*Z*-10:COOMe was an essential component of the attractive blend, whereas any one, any two, or all three of the sesquiterpene components were equally effective as the other portion of the blend. None of the components were active alone. Pheromone blends attracted only females in both laboratory and field bioassays [10]. The same compounds are also produced by the congener *T. custator* [10] and other *Thyanta* spp. (J.G. Millar, unpublished data).

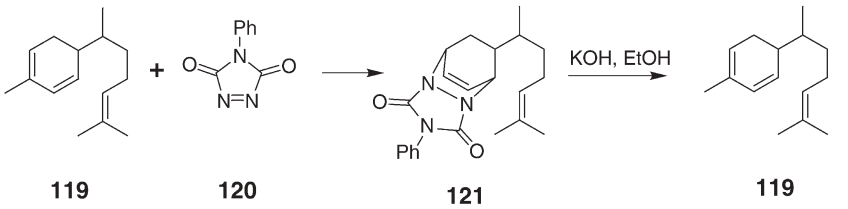
2*E*, 4*Z*, 6*Z*-10:COOMe was produced by a short and stereospecific one-pot synthesis (Scheme 17) [25], via sequential addition of dipropyllithium cuprate **115** to two equivalents of acetylene, followed by Michael addition of the resulting conjugated dienyl cuprate **116** with methyl propiolate **117**. The only sig-



Scheme 17 One-pot, stereospecific synthesis of methyl (2*E*, 4*Z*, 6*Z*)-decatrienoate [25]

nificant side product was the adduct from addition of only one equivalent of acetylene, which was readily removed by reverse phase flash chromatography. The synthesis proceeds equally well with the cuprate prepared from propylmagnesium bromide (J.G. Millar, unpublished data).

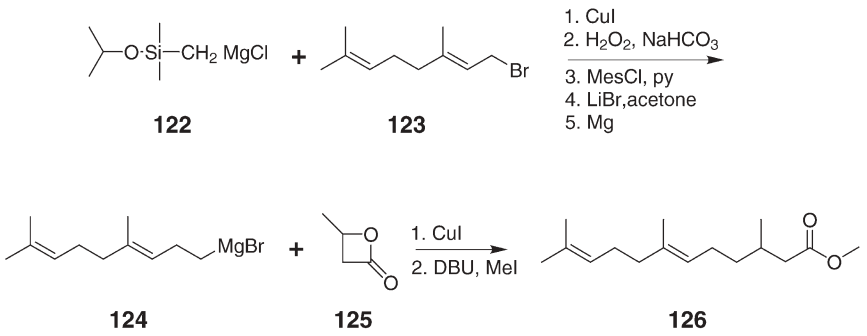
(–)-Zingiberene **119** was isolated from the sesquiterpene fraction of ginger oil by a derivatization-dederivatization sequence (Scheme 18) [105]. Thus, treatment of the crude fraction with the powerful dienophile phenyl 1,2,4-triazoline-3,5-dione (PTAD) **120** resulted in rapid Diels-Alder reaction of PTAD with the conjugated diene of zingiberene to produce the adduct **121**. Unreacted hydrocarbons and byproducts were readily removed by chromatography, following which the purified adduct was hydrolyzed to return (–)-zingiberene **119** in >99% purity. The other two sesquiterpene components, (–)-β-sesquiphellandrene and (+)-α-curcumene, were isolated from ginger oil by careful chromatography on silica gel [10].



Scheme 18 Separation of zingiberene from ginger oil using a selective and reversible Diels-Alder reaction with phenyl 1,2,4-triazoline-3,5-dione (PTAD) [105]

4.1.9 Chlorochroa Species

Male-produced pheromones have been identified, synthesized, and bioassayed for three *Chlorochroa* spp. *Chlorochroa ligata*, which are brown to black with red-orange margins, and *C. uhleri*, which are bright green, appear to use the same pheromone blend, composed of methyl (3*R*)-(*E*6)-2,3-dihydrofarnesoate **126**, and traces of methyl farnesoate and a chain-shortened homolog, methyl (*E*5)-2,6,10-trimethyl-5,9-undecadienoate [106]. Methyl farnesoate was synthesized by Wittig reaction of trimethylphosphonoacetate with geranyl acetone, and separation of the *Z* and *E* isomers by flash chromatography [106]. Racemic methyl dihydrofarnesoate **126** was synthesized by one-carbon chain extension of geranyl bromide **123** with Grignard reagent **122**. Oxidation of the resulting siloxane to the alcohol, conversion to the bromide, copper-catalyzed reaction of the Grignard reagent **124** prepared from the bromide with 2-methyl-β-propiolactone **125**, and methylation of the resulting acid gave racemic (*E*6)-2,3-dihydrofarnesoate **126** (Scheme 19) [107]. (3*R*)-**126** was prepared in one step in small amounts by stereoselective and regiospecific hydrogenation of methyl farnesoate with a chiral semicorrin catalyst [106]. The homolog, methyl (*E*5)-



122 123 124 125 126

Scheme 19 Synthesis of racemic methyl dihydrofarnesoate [107]

2,6,10-trimethyl-5,9-undecadienoate, was prepared by alkylation of the dianion of propionic acid with homogeranyl iodide and methylation [106].

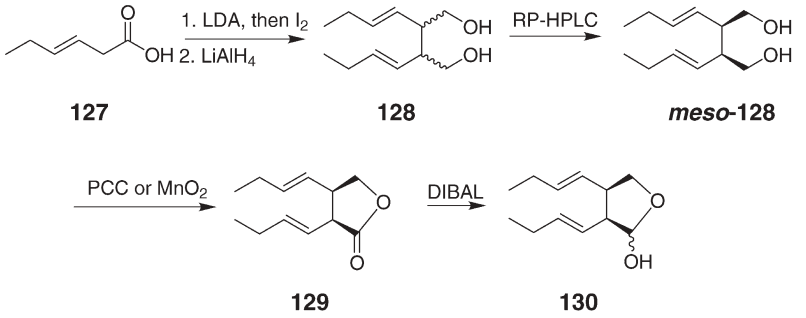
Chlorochroa sayi males produce a pheromone consisting primarily of methyl geranate, with trace amounts of methyl citronellate and methyl dihydrofarnesoate [107]. Methyl geranate is readily available in multigram quantities from fractional distillation of the commercially available mixture of methyl geranate and methyl nerate (J.G. Millar, unpublished data), or the two isomers can be separated chromatographically [107].

The biological activity of the synthetic pheromones of all three *Chlorochroa* species has been verified in bioassays [106, 107]. However, as with other species, bugs are attracted to the vicinity of pheromone-baited traps, but few bugs enter the traps.

4.1.10 Biprorulus bibax

Male spined citrus bugs, *Biprorulus bibax*, produce aggregation pheromones in enlarged sexually dimorphic DAGs, as occurs in some predacious pentatomids [8]. Male DAGs contained a hemiacetal, (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol (**130**, Scheme 20) and linalool, with lesser amounts of two farnesol isomers and nerolidol [108, 109]. In a field cage, females were attracted to the racemic synthetic hemiacetal alone, and to a blend (using racemic components) mimicking the male DAG extract [109]. Diapausing females were unresponsive. Bugs would not enter traps, but citrus trees containing traps and adjacent trees became heavily infested with bugs of both sexes and all reproductive stages. Further tests determined that (*E*)-2-hexenal also attracted reproductively active bugs [110].

The hemiacetal pheromone was first prepared in low yield as a mixture of isomers (Scheme 20) [108]. Thus, oxidative coupling of the dianion of (*E*)-3-hexenoic acid **127** and $LiAlH_4$ reduction of the resulting diacid produced a mixture of diols from which the *E*,*E-meso* isomer **128** could be separated. The *meso*

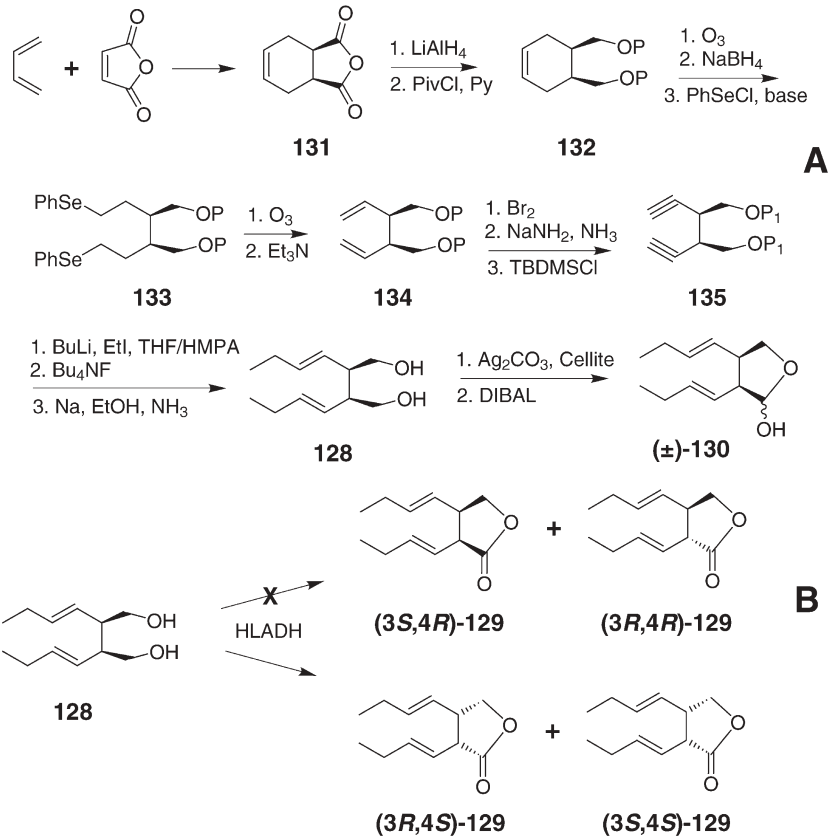


Scheme 20 Synthesis of racemic lactol pheromone component from *Biprorulus bibax* [108]

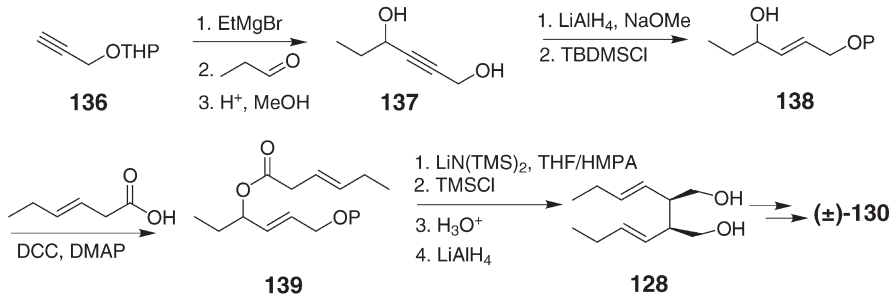
diol **128** was oxidized to furanone **129** with either pyridinium chlorochromate or MnO_2, followed by partial reduction to an anomeric mixture of lactols **130**.

A route to the racemate that could be adapted to production of the enantiomers was developed soon afterward [111], with the key chiral synthon being produced from *meso* diol **128** by desymmetrization with horse liver alcohol dehydrogenase (HLADH). The synthesis of the diol **128** began with a Diels-Alder reaction between butadiene and maleic anhydride to give anhydride **131**, which was then reduced to the diol and protected, giving **132** (Scheme 21A). Ozonolysis followed by reduction of the ozonide and selenation gave diselenide **133**, which was oxidized to the diselenoxide, followed by base-induced elimination to diene **134**. Bromination and elimination of four equivalents of HBr with strong base gave the diyne **135** (P_1=H), with concomitant removal of the benzyl protecting groups. Reprotection gave the bis-silylether (**135**, P_1=TBDMS), which was then converted to the *meso* diol in three steps by alkylation of the terminal acetylenes, deprotection of the alcohols, and stereoselective reduction to dienediol **128**. Oxidation to the lactone and partial reduction to the lactol (±)-**130** completed the synthesis of the racemic compound.

The key *meso*-diol **128** was then subjected to oxidation with HLADH (Scheme 21B), which stereospecifically oxidized the diol to a lactone. The resulting chiral compound proved to be identical to the insect-produced compound, and on the basis of the supposed "known" stereospecificity of HLADH, with further support from a positive Cotton effect in the CD spectrum as predicted by the Klyne lactone sector rule, the synthetic compound was initially assigned the (3*S*,4*R*)-configuration. However, during the development of a more efficient synthesis (9 steps, 35% yield, vs 13 steps and 4.6% yield in their first synthesis) [112], Mori and coworkers discovered that this assignment was erroneous. Thus, Mori's second synthesis was adapted to provide an unambiguous synthesis of one of the enantiomers. The new synthesis was first worked out for the racemate (Scheme 22). Thus, the Grignard reagent from THP-protected propynol **136** was reacted with propanal. Deprotection yielded the alkynediol **137**, which was stereospecifically reduced to the *E*-alkenediol.



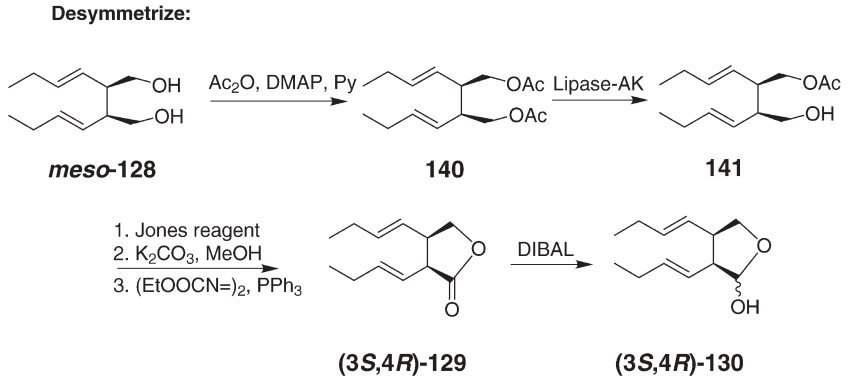
Scheme 21 Syntheses of racemic and chiral forms of the lactol pheromone of *Biprorulus bibax* [111]



Scheme 22 Synthesis of racemic lactol pheromone of *Biprorulus bibax* [112]

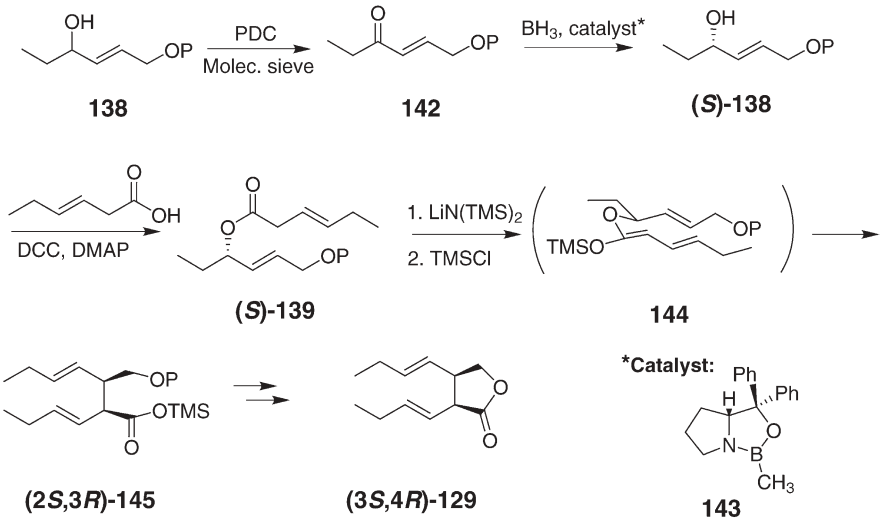
Selective protection of the primary alcohol gave **138** (P=TBDMS), which was then esterified with (*E*)-3-hexenoic acid to produce the key intermediate **139** for cyclization. Ireland ester-enolate Claisen rearrangement and hydrolysis produced a protected hydroxyacid, which, after reduction of the acid and deprotection of the alcohol, yielded *meso* diol **128** more quickly and efficiently than in the previous synthesis. The *meso* diol was then converted to the racemate of the lactol pheromone **130** as previously described.

With a good route to the key *meso* diol **128** in hand, the authors turned their attention to desymmetrization, using the known asymmetric hydrolysis of *meso* diacetates by Lipase AK (Scheme 23). The *meso* diol **128** was first converted to diacetate **140**, and then hydrolyzed with Lipase AK to cleave selectively one of the two acetates, producing chiral hydroxyester **141**. Oxidation, cleavage of the acetate, and lactonization yielded the (3*S*,4*R*) lactone **129**. The corresponding lactol (3*S*,4*R*)-**130** was found to be the enantiomer of the compound produced in the HLADH synthesis.



Scheme 23 Chemoenzymatic synthesis of the (3*S*,4*R*)-enantiomer of the lactol pheromone of *Biprorulus bibax* [112]

To conclusively prove the absolute configuration of the insect-produced compound, the authors then modified their second synthesis to produce one enantiomer of the lactol **130** of known configuration, by using a chiral intermediate in the Claisen rearrangement (Scheme 24). Thus, the racemic monoprotected diol **138** was oxidized to the ketone **142**, which was then enantioselectively reduced back to alcohol (*S*)-**138** using borane and Corey's oxazaborolidine catalyst **143**. The configuration was proven by conversion to a known compound. The synthesis was then executed as before, with the stereochemistry of the final product **145** being controlled by the chair conformation **144** adopted during the Claisen rearrangement. Carrying the rearrangement product through the final steps produced the lactone precursor (**129**) to the pheromone, with the (3*S*,4*R*) configuration.



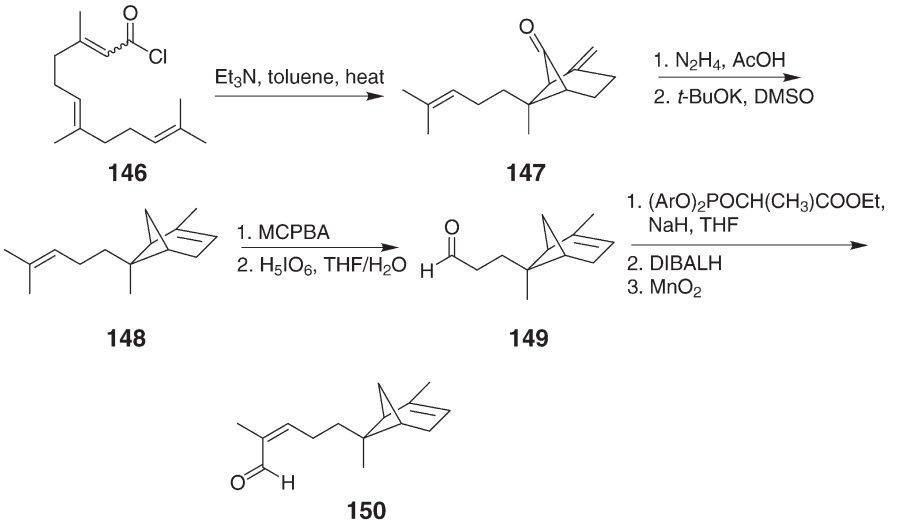
Scheme 24 Unambiguous chiral synthesis of the (3*S*,4*R*)-enantiomer of the lactol pheromone of *Biprorulus bibax* to confirm the absolute stereochemistry [112]

The authors then used a modification of their Lipase-AK route to produce the natural enantiomer, as described in detail in the chapter by Kenji Mori in this volume. Instead of using the enzyme to execute a stereoselective monohydrolysis of meso diacetate **140**, the enzyme was used to esterify selectively one of the hydroxy groups of *meso* diol **128**, resulting in the antipodal hydroxyester. After oxidation of the free hydroxyl to the acid, and recrystallization of its salt with (*R*)-1-naphthylethylamine, the purified acid was then carried through the remaining steps to furnish the chiral pheromone compound (see the chapter by Kenji Mori in this volume).

In one final improvement to the synthesis of the racemate, after it had been shown that even the unnatural enantiomer of the pheromone attracted bugs, the *Z*-isomer of diene ester **139** was used in the ester-enolate Claisen rearrangement, resulting in a slightly higher yield, and avoiding the use of HMPA as a solvent in that step [113].

4.1.11 Eysarcoris parvus

This Asian species is a major agricultural pest. The pheromone has been proposed to consist of three male-specific compounds, only one of which, (*Z*)-*exo*-α-bergamotenal **150**, has been reported in the literature [114]. The racemic compound was synthesized starting from farnesoic acid chloride **146** (Scheme 25) [114]. Thus, the vinyl ketene prepared from acid chloride **146** underwent 2+2 cycloaddition to give bicyclic ketone **147**. The ketone function was removed by reaction with hydrazine followed by treatment of the resulting hy-



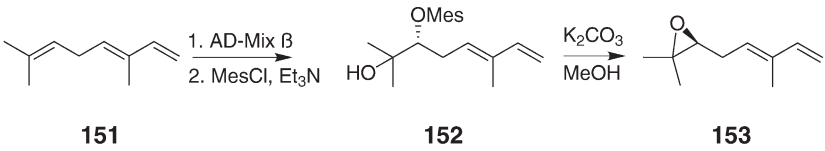
Scheme 25 Synthesis of (*Z*)-*exo*-α-bergamotenal, isolated from male *Eysarcoris parvus* stink bugs [114]

drazone with strong base (modified Wolff-Kishner conditions). Under the conditions used, the exocyclic double bond also migrated into the desired position in the ring, giving *exo*-α-bergamotene **148**. Regioselective epoxidation with *m*-CPBA, followed by cleavage of the epoxide with periodic acid gave aldehyde **149**. *Z*-selective Wittig reaction yielded an ester intermediate which was converted to the desired aldehyde **150** by DIBAL reduction and allylic oxidation with activated MnO_2.

4.2 Squash Bugs and Leaf-Footed Bugs (Coreidae)

Current knowledge of the pheromone chemistry of coreid bugs is fragmented. Several reports indicate that male-produced pheromones attract adults of both sexes of *Leptoglossus australis* [115, 116] and *L. occidentalis* [117, 118]. Male-specific compounds, including decanal, (3*R*)-(*E*)-nerolidol, *trans*-α-bergamotene, and several other unidentified sesquiterpenes have been reported from *L. phyllopus*, but their function remains unknown. Male-specific sesquiterpenes are also produced by *L. clypealis* (J.G. Millar, unpublished data). Male *L. clypealis* also secrete benzyl alcohol and guaiacol from ventral abdominal glands, which, when wiped on females' antennae, render the females receptive to male mating attempts [119]. (3*R*)-(*E*)-Nerolidol, *E*,*E*-α-farnesene, β-ocimene, linalool, *E*-β-farnesene, and two unidentified components were found in headspace and gland extracts of *Amblypelta lutescens*, and in limited field tests, a few adult bugs were observed within 30 cm of baited traps [120]. (3*R*)-(*E*)-Nerolidol was also found in male *A. nitida* [120]. The interesting com-

pound (3*R*,5*E*)-2,6-dimethyl-2,3-epoxy-octa-5,7-diene **153**, an oxygenated analog of β-ocimene, was also recovered from headspace extracts of male *A. nitida*. The racemate was readily prepared by *m*-CPBA oxidation of β-ocimene **151**, and the absolute configuration was determined by synthesis of one of the two enantiomers from β-ocimene by asymmetric dihydroxylation and selective mesylation to give mesylate **152**, and closure to give the epoxide (3*S*,5*E*)-**153**, which was in fact the unnatural enantiomer (Scheme 26) [121]. The biological function of this compound remains unknown.



Scheme 26 Synthesis of (3*R*,5*E*)-2,6-dimethyl-2,3-epoxy-octa-5,7-diene, isolated from male *Amblypelta nitida* [121]

Alarm pheromones, consisting of typical components of bug defensive secretions (e.g., simple aldehydes and esters), have been reported for *Leptoglossus zonatus* [122] and *Leptocorisa oratorius* [123].

4.3 Broad-Headed Bugs (Alydidae)

Pheromones have been identified or suggested for a few alydid species. Thus, reproductively active male *Riptortus clavatus* produce a pheromone that attracts adults of both sexes and 2nd instar nymphs [19, 20]. The pheromone, released only in the presence of food [20], consists of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate, and tetradecyl isobutyrate. Large doses of tetradecyl isobutyrate as a single component also attracted both sexes [124]. The related compound (*E*)-2-hexenyl hexanoate acted as an alarm pheromone, causing agitation and dispersal of both nymphs and adults [125]. Caged males of the congeneric species *R. linearis* also attract adults and 2nd instar nymphs, and caged females were reported to attract males, but the active compounds have not been identified [126]. (*E*)-2-Hexenyl (*Z*)-3-hexenoate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenol have been identified from gland and headspace extracts from *R. serripes* and *Mirperus scutellaris*, but the biological activity of these compounds has not been tested [120].

The pheromone of *Leptocorisa chinensis* illustrates the critical importance of the interplay between attractive and inhibitory chemicals [21]. Headspace extracts from males and females were qualitatively similar. From the eight compounds in headspace extracts that elicited strong antennal responses from males, four were discounted as being defensive chemicals or alarm pheromones. The remaining group of four chemicals, consisting of octanol,

octyl acetate, (*E*)-2-octenol, and (*E*)-2-octenyl acetate, was not attractive to males, nor were any subsets of three of the four components attractive. The active blend proved to be a 5:1 blend of (*E*)-2-octenyl acetate and octanol. As with *Trigonotylus caelestialium* [127] (see below), the mechanism by which the female regulates the production of a specific blend ratio, from a set of compounds that are produced by both males and females, remains unknown.

A subset of metathoracic gland components from female *Alydus eurinus* consisting of (*S*)-2-methylbutyl butyrate, (*E*)-2-methyl-2-butenyl butyrate, and butyl butyrate constitutes an attractant pheromone, with adults of both sexes and nymphs being attracted [128]. The racemate was as attractive as the (*S*)-enantiomer, indicating no inhibition by the "unnatural" enantiomer. Furthermore, the pheromone components were produced in the metathoracic glands, which also produce defensive compounds, again illustrating that female bugs must be able to regulate the production of specific subsets of components at will.

4.4 Seed Bugs (Lygaeidae)

Attractant pheromones were first reported for the seed bugs in 1997, from male *Tropidothorax cruciger* and *Neacoryphus bicrucis* [28]. For the former species, the attractive blend consisted of (*E*)-2,7-octadienyl acetate and (*E*)-2-octenyl acetate (1:10), whereas in the latter, the blend consisted of (2*E*, 4*E*)-hexadienyl acetate and phenethyl acetate (9:1). The compounds were produced in accessory glands associated with the metathoracic glands, and in olfactometer and field bioassays, adults of both sexes were attracted [28, 129]. In similar fashion, adults of *Oncopeltus fasciatus* and *Lygaeus kalmii* were attracted by male-produced blends of (*E*)-2-hexenyl acetate, (2*E*, 4*E*)-hexadienyl acetate, (*E*)-2-octenyl acetate, and (*E*)-2,7-octadienyl acetate [129]. For all of the above species, it was suggested that male pheromones play a role in the colonization of new habitats, with males finding high quality hosts, and then producing aggregation pheromones to attract conspecifics [129]. These compounds were also found in males of two other *Oncopeltus* spp., in which their biological function has not yet been tested [129].

However, in the seed bug family the lygaeid *Geocoris punctipes* is a predatory species. Females of this species release (*E*)-2-octenyl acetate, which, while not attractive per se, increases activity levels and stimulates searching behavior of males, with stimulated males dashing towards and investigating small moving objects [35].

4.5 Assassin and Thread-Legged Bugs (Reduviidae)

This family contains species in the genera *Triatoma*, *Rhodnius*, and *Panstrongylus* that are of major importance as vectors of Chagas disease, a debilitat-

ing disease afflicting approximately 16–18 million people in South and Central America [130]. These species have been studied extensively, and there is a large, confusing, and frequently contradictory literature on their chemical ecology, the subject of an excellent recent review [130]. Putative semiochemicals include sex and aggregation pheromones, and a "footprint factor" that promotes arrestment and aggregation. Ammonia and several other compounds in bug feces appear to have an attractant effect. Recently, short chain aldehydes collected from the headspace above mating pairs were suggested to be involved in attraction [131]. However, as succinctly summarized by Cruz-Lopez et al. [130], '...remarkably little is really understood about the chemical ecology of these famous haematophagous bugs. Much of the available evidence on the semiochemical behavior of this group remains conflicting'.

There is a single report of a male-produced aggregation pheromone for another reduviid, *Pristhesancus plagipennis* [132]. (*Z*)-3-Hexenyl (*R*)-2-hydroxy-3-methylbutyrate was isolated from adult males, and attracted both males and females in olfactometer and field cage bioassays. The racemate was not attractive, indicating that the (*S*)-enantiomer inhibited the response. The (*E*)-isomer also decreased attraction. Both enantiomers were synthesized by conversion of the commercially available enantiomers of valine to the corresponding 2-hydroxy-3-methylbutyric acid enantiomers with $NaNO_2$ in dilute H_2SO_4, and esterification with (*Z*)-3-hexenol [132].

4.6
Minute Pirate Bugs (Anthocoridae)

Possible pheromone components have been identified for a single species, *Orius insidiosus*. In a preliminary study, males were attracted to females, and (*E)*-2,7-octadienal, (*E*)-2,7-octadienoic acid, and (*E*)-2,9-decadienal were reported as female-specific compounds [133]. In a followup study, males were attracted in low numbers to a 3:10 blend of (*E*)-2,7-octadienal and (*E*)-2-octenal [134]. There is also preliminary evidence for a female-produced pheromone from *Orius sauteri* [135].

There is also one remarkable example of an anthocorid bug, *Elatophilus hebraicus*, using the pheromone ((2*E*,6*E*,8*E*)-5,7-dimethyl-2,6,8-decatrien-4-one) of its prey, the pine bast scale *Matsucoccus josephi*, as an aggregation kairomone and sexual stimulant [136, 137]. Adult bugs of both sexes are attracted by the host's pheromone, and bug mating activity is enhanced in the presence of the host's pheromone. This may be the first known example of the pheromone of one species being exploited as a "pseudopheromone" by a second species, rather than the latter developing its own pheromones.

4.7 Plant Bugs (Miridae)

The family Miridae is the largest heteropteran family, with ≈10,000 species [6], a number of which are serious agricultural pests. There is abundant evidence that mirids use attractant pheromones (reviewed in [13]), but despite their importance, the pheromones of only about ten species have been identified. These insects appear to fall into two groups. The first group (e.g., *Campylomma verbasci* and *Phytocoris* spp.) is characterized by pheromones that were straightforward to identify, whereas pheromones of the second group, including *Lygus* and *Lygocoris* spp., have proven to be remarkably intractible, despite decades of effort by numerous research groups. With the latter group, many insect-produced compounds have been identified, but it remains unclear whether the lack of response to extracts, fractions, or synthetic compounds is due to inhibition by defensive compounds, incomplete blends, incorrect blend ratios, or some other factor(s). It is also noteworthy that for the few pheromone blends that have been reported, in one species, all pheromone components are produced only by females, whereas in other species, the pheromone consists of female-specific compounds admixed with one or more compounds produced by both sexes, and in another species, all components of the pheromone are produced by both sexes, but only males are attracted!

For the few mirid pheromones that have been identified, all are female sex pheromones with quite simple chemistry, that attract males. The first to be identified was the sex pheromone of *Campylomma verbasci* [16], consisting of a 16:1 blend of the female-specific compounds butyl butyrate and (*E*)-2-butenyl butyrate. This has been developed for sampling *C. verbasci* in orchards [138, 139], and tested as a mating disruptant [140, 141]. More recently, sex attractant pheromones have been identified for three *Phytocoris* species, *P. relativus* (hexyl acetate and (*E*)-2-octenyl butyrate, 2:1) [142], *P. californicus* (hexyl acetate and (*E*)-2-octenyl acetate, 2:1) [143], and *P. difficilis* (hexyl acetate, (*E*)-2-hexenyl acetate, and (*E*)-2-octenyl acetate, 4:3:2) [144]. In all three cases, the compounds appear to be produced by the metathoracic glands, with hexyl acetate being produced by both sexes, whereas the other components were produced only by females. A fourth species, *P. breviusculus*, was weakly attracted to blends of hexyl acetate and (*E*)-2-octenyl acetate [144], but its actual pheromone remains to be identified. It is also interesting to note the interplay of attractants and interspecific antagonists: *P. californicus* responses to hexyl acetate and (*E*)-2-octenyl acetate were diminished by addition of (*E*)-2-octenyl butyrate (a component of sympatric *P. relativus* pheromone) [143], and both (*E*)-2-hexenyl acetate and (2*E*,4*E*)-hexadienyl acetate inhibited attraction of *P. breviusculus* males to attractive blends of hexyl acetate and (*E*)-2-octenyl acetate [144]. Furthermore, *P. difficilis* male-specific compounds (hexyl butyrate and (*E*)-2-hexenyl butyrate) inhibit responses to the female pheromone, suggesting that these compounds might deter other males from attempting copulation with a previously-mated female [145].

The sex pheromone of *Trigonotylus caelestialium* represents an interesting case because the three components of the blend (hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and octyl butyrate, ~10:5:1) are produced by both sexes, along with a number of similar compounds [127]. The female must be able to manipulate the blend ratio that she releases in order to be able to attract males with a subset of compounds that are otherwise shared by both males and females. In field trials, dose also appeared to be important, with doses in the low microgram range remaining attractive to males for several days [127].

A preliminary report has identified pheromone blends for two tropical species, *Distantiella theobroma* and *Suhlbergella singularis* [146]. Females of both species produce hexyl (*R*)-3-hydroxybutyrate and its (*E*)-2-butenoate ester (~1:2). In initial field trials, male *S. singularis* were attracted to the blend. Further work is in progress to conclusively identify and optimize blends for each species [146].

Several *Lygus* spp. are notorious pests of numerous crops [1] and pheromones for use in pest management programs would be immensely valuable. Although it is well established that female lygus bugs attract males, numerous efforts since the late 1960s have failed to identify pheromones for any *Lygus* species, possibly because of several factors. First, there are minimal differences in the profile of volatile chemicals released by males and females. Second, no research group has been able to produce consistently active extracts. To date, it remains unknown whether the activity of extracts from virgin females is being masked by inhibitors, or whether the extract preparation methods are failing to collect or even destroying the active components. Third, reliable laboratory bioassays have not yet been developed for *Lygus* spp.

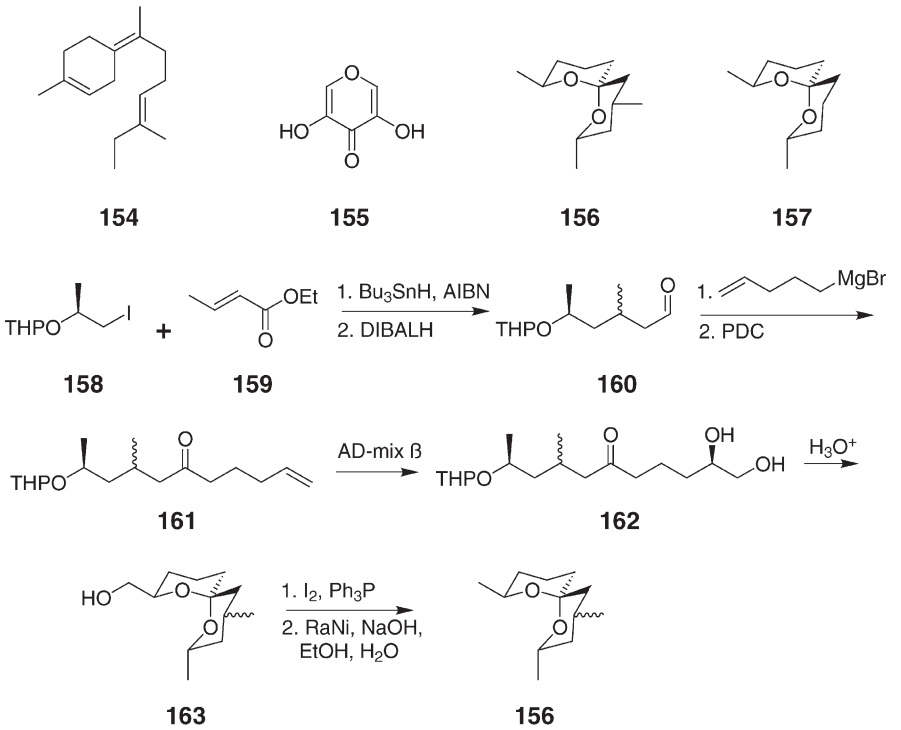
Electroantennogram studies have also proven fruitless in the search for Lygus bug pheromones. Antennae from males and females respond to numerous compounds in extracts from both sexes, and although some sex-specific differences have been noted in the magnitudes of antennal responses, no obvious pheromone candidates have emerged [147, 148]. Even with abundant evidence that females use a volatile sex attractant, *Lygus* spp. pheromones have resisted all attempts at identification for more than 30 years.

The pheromone blend of *Calocoris norvegicus* consists of at least three (and possibly more) components (hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-2-octenyl butyrate) (J.G. Millar and R.E. Rice, unpublished data), the first of which is abundant in both sexes, the second of which is predominantly in females, and the third of which is only female-produced. (*E*)-2-Octenyl butyrate is a key component of the pheromone, because lures that did not contain this compound were not attractive. Further delineation of the pheromone blend has been hindered by highly variable trap catches; within a single block, trap catches in traps baited with identical lures will frequently vary from zero to more than 100, confounding statistical analyses. Nevertheless, over several seasons, pheromone traps have caught thousands of males, and trap catches were species- and stage-specific, with a large preponderance of males (in high populations, apparently random catches of females occur).

The buckeye bug *Neurocolpus longirostrus* represents an analogous case. About 20 compounds have been identified from headspace collections from live insects of both sexes. After several seasons of iterative testing of blends, it appears that the female pheromone consists of the female-specific compound (*E*)-2-octenyl hexanoate, which is essential for attraction, mixed with hexyl hexanoate, (*E*)-2-hexenyl hexanoate, and possibly (*E*)-2-hexenal, all of which are produced by both sexes (J.G. Millar and R.E. Rice, unpublished data). However, as with *C. norvegicus*, trap catches were extremely variable. Nevertheless, these blends are close to the true pheromone blend; male bugs were observed trying to copulate with the pheromone lures!

4.8 Shield Bugs (Scutelleridae)

Several interesting compounds (Scheme 27) have been identified from insects in this family, but no pheromones have been conclusively demonstrated. For example, homo-γ-bisabolene **154** has been identified from male Sunn bugs, *Eurygaster integriceps*, but nothing is known of its biological activity [149].



Scheme 27 Compounds isolated from shield bugs, and synthesis of a spiroketal produced by the shield bug *Cantao parentum* [152]

Similarly, male *Tectoris diophthalmus* produce 3,5-dihydroxy-4-pyrone **155**, referred to as a "presumed sex pheromone" [150], but bioassay data to support this claim is lacking. A series of aldehydes, including nonanal, (*Z*)-4-nonenal, 8-nonenal, (*Z*)-4,8-nonadienal, and (*E*)-4,8-nonadienal were identified from the sexually dimorphic dorsal abdominal glands of *Sphaerocoris annulus*, but their function remains unknown [151]. Finally, dorsal abdominal glands of both male and female *Cantao parentum* yielded two spiroketals, **156** and **157**, the first report of spiroketals of any type from Heteroptera or the lower insect orders [152]. The major component was synthesized as a mixture of diastereomers as shown in Scheme 27. Thus, conjugate addition of iodide **158** to ethyl crotonate **159**, followed by DIBAL reduction gave aldehyde **160**. The aldehyde was reacted with 4-pentenyl magnesium bromide, and the resulting alcohol was oxidized to ketone **161**. Asymmetric dihydroxylation then played a pivotal role in introducing a chiral center into diol **162** that determined the stereochemistry in the spiroketal products **163**, obtained as a mixture of diastereomers at carbon 4. Straightforward removal of the primary alcohol from **163** completed the synthesis. Despite the novelty and interesting chemistry of spiroketal **156**, its biological function has not been reported.

4.9
Water Striders and Smaller Water Striders (Gerridae and Veliidae)

The gerrid genus *Halobates* includes a few species that are the only insects known to live on the open ocean [153]. The small, wingless females of *H. hawaiiensis* were attracted to extracts of males, which contained several male-specific lipids, including palmitic and oleic acids, but conclusive evidence that these compounds do indeed comprise the attractive pheromone is lacking. In similar fashion, females of the veliid species *Trochopus plumbus* were strongly attracted to live males and to extracts of males [154]. Aldehydes and carboxylic acids were identified from extracts, but no male-specific compounds were found. In both cases, the attractive compounds were suggested to spread along the water surface and attract females, rather than dispersing as an odor plume [153, 154].

5
Applications of Bug Pheromones

The extent to which heteropteran pheromones can be integrated into pest management programs must be considered on a case by case basis, weighing factors relating to both biology and economics. First, the strength of the response to pheromones is highly variable among species. Some species, such as mirids in the genus *Phytocoris*, are strongly attracted to synthetic pheromones, to the extent that pheromone-baited traps catch bugs when none can be found by other sampling methods [142]. Other species, such as phytophagous stink bugs,

appear to be only weakly attracted to their pheromones (see above), and attraction is further confounded by other types of signaling.

Second, many bug species are polyphagous and highly mobile, moving rapidly between crops and native vegetation. For these species, strategies such as pheromone-based mating disruption cannot be employed, both because the insects may not be in the crop when the disruptant is deployed, and because previously mated females can readily immigrate into the crop.

Third, some of the pheromone components are simple and cheap compounds such as straight-chain esters and aldehydes, that are readily available in bulk. Others fall into a middle ground, whereby multigram-scale synthesis to produce sufficient material for use as trap lures should be possible. However, the pheromone structures of a number of species appear to be of sufficient complexity that it is unlikely that they could be made in sufficient quantity and at affordable cost for widespread use in pheromone-based control programs.

Fourth, our understanding of the roles of semiochemicals in bug biology is rudimentary. It is clear that some species produce sex pheromones, with one sex producing and the other sex responding to the chemical(s) to bring the sexes together for mating. However, with other species in which adults of both sexes and even immatures appear to be attracted, the ultimate reason for the attraction remains speculative. Any assessment of the potential for incorporating bug pheromones into management programs would be greatly bolstered by more detailed knowledge of the role(s) of pheromones in the biology and life history of these insects.

Thus, as with many insect pheromones, a clear distinction must be made depending on the intended use of pheromones. Pheromone traps for sampling and quarantine purposes will almost certainly find a market for some species, whereas the potential for developing bug pheromones for control purposes appears more limited. Despite these limitations, some bug pheromones, such as those for *Euschistus conspersus* and *Plautia stali*, are moving forward to commercialization. Others, such as the pheromones of Lygus bugs, the "holy grail" of bug pheromones, would have enormous potential if they could be identified, due to the importance and ubiquity of Lygus bugs throughout agriculture worldwide.

6 Concluding Remarks

From the examples discussed above, it is clear that heteropteran species have unique and interesting chemistry. Given that much less than 1% of the number of known species have been examined, a wealth of novel compounds must remain to be discovered. The discovery process should become easier, for several reasons. First, as the sensitivity of analytical methods increases, it will be possible to identify ever-smaller quantities of novel compounds. This may provide the key to identifying the pheromones of some of the problem species. In par-

ticular, pheromone collections from live insects are by necessity time averages, and the particular blend that is attractive may be obscured or altered in a time-averaged blend, to the extent that the attractive blend is unrecognizable. It would be a tremendous advantage if collection periods could be shortened to a few minutes, so that collections were made only when an insect was producing pheromone and demonstrably attractive. A better knowledge of the sites of production of pheromone components, and specifically, how the release of specific blends of components is regulated, also would be enormously useful in helping to identify new pheromones.

Second, in tandem with better analytical methods, as our knowledge of bug defensive chemistry increases through the identification of compounds from an increasing number of species, it will become easier to discern potential pheromone components against the background of common defensive chemicals that are shared by multiple species.

Third, as alluded to at several points in this chapter, one of the biggest failures in the unravelling of the complexities of bug semiochemistry has been the lack of rigorous testing of the biological activities of new compounds; in fact, this chapter graphically demonstrates that the chemistry has far outstripped the biology. Premature designation of compounds as pheromones, in the absence of supporting data on biological activity, has further obfuscated the true function of many bug-produced compounds, and confused the overall picture of the role of semiochemicals in heteropteran communication. In other cases, compounds have been synthesized in quantities sufficient to verify structures, absolute configurations, and biological activities, but the followup work does not appear to have been done. This lack of coordination between biology and chemistry represents the most pernicious obstacle to further elucidation of heteropteran semiochemistry, and can only be overcome with multidisciplinary teams of chemists and behavioral biologists. The chemistry and biology of these remarkable animals is too complex to be tackled by one discipline alone.

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Topics in Current Chemistry (2005) 240: 85–166
DOI 10.1007/b98316

Chemical Signalling in Beetles

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Abstract This chapter reviews chemical structures of biologically active, volatile compounds in beetles. Techniques used for structure elucidation are briefly discussed as well as facts and speculations on the biosynthesis of target compounds. Syntheses of selected substances are cursorily presented. The order of sections follows taxonomic classifications. Depending on the biological significance of relevant compounds in certain taxa, the corresponding sections are again subdivided into "attractive compounds" (mostly intraspecifically active pheromones) and "defensive compounds" (mostly interspecifically active allomones).

Keywords Beetles · Attractive compounds · Pheromones · Defensive compounds · Biosynthesis · Identification techniques · Structure elucidation

1 Introduction

Beetles (Coleoptera) comprise the most species-rich insect order. About 350,000 species have been described today, about 10% of the estimated actual amount. Apart from open oceans, beetles are colonizing almost every habitat and are able

to cope with extreme climatic conditions. Their body size ranges from very small (some feather-winged beetles – Ptiliidae – show a body length below 0.1 mm) to gigantic (some scarabs – Scarabaeidae – and longhorn beetles – Cerambycidae – are up to 15–20 cm big), and some species truly look bizarre.

In beetles, the front pairs of wings are transformed into hardened wing covers under which the soft hind wings are folded when not in use. This hard cover provides protection which may be a major reason for the successful evolution of this largest order in the animal kingdom.

Beetles pass a holometabolous development with several larval instars, pupae (often poorly known), and completely transformed adults. Larvae show biting mouth parts and often possess abdominal cerci-like structures which are absent in adults. The usually short heads of adults may be elongated to form a snout (as in weevils). Antennae and legs, especially the tarsomeres, may vary strongly with species and are taxonomically useful.

A large number of phytophagous beetles is economically important as pests on crops, forests, and stored products, and they are vectors of fungi and viral plant diseases. On the other hand, many species have beneficial functions in the detritus cycle, and carnivorous species may feed on herbivorous insects.

Apart from optical, acoustical, and tactile cues, transfer of information by volatile compounds plays a pivotal role in the transfer of information between living beings. In insects, intra- and interspecific chemical communication is particularly widespread and important.

Similar to the beetles' global distribution as well as their very different appearance and habitats, the chemical structures that beetles use as signals and their biological significance are highly diverse. They comprise a wide range of volatiles including low boiling carboxylic acids, carbonyl compounds, alcohols as well as simple aromatic compounds, derivatives of amino acids, oxygen containing heterocycles showing several stereogenic centres, and (high boiling, unsaturated, branched) hydrocarbons.

Aiming at the development of integrated pest management systems, research on beetle pheromones has predominantly been carried out with economically important species. In contrast, investigations on the defence chemistry of beetles has largely been phenomenologically oriented. Pheromones of beetles have been listed [1] and presented in a more general context [2] as well as discussed under specific aspects including some biological background [3–7]. The evolution of chemical defence as well as compounds involved in defence chemistry have been reviewed [8, see also the chapter by Laurent et al., this volume]. Valuable data on defensive substances from insect eggs have recently been compiled [9].

Syntheses of pheromones have been comprehensively treated by Mori [10–14]. The role of synthesis in the research on semiochemicals, the importance of stereochemistry in chemical signalling, and the significant relations between enantiomeric composition and biological activity of chiral semiochemicals have been thoroughly discussed by Mori [15–17]. In the present context, presentation of pheromone synthesis plays a minor role; syntheses

leading to mixtures of stereoisomers or to non-natural stereoisomers are not included.

2
Isolation and Structure Elucidation

Techniques in isolation and structure elucidation of (volatile) semiochemicals from beetles are the same as in other insects. Problems are mainly due to the often very small amounts of target compounds, embedded in large amounts of non-active substances which form a kind of "cosmetic formulation" for the biologically active principle. Comprehensive reviews of analytical approaches have been published [18–20].

There is no optimal method for sample preparation. Solvent extraction of crushed insects is certainly a most "dirty" method, as extremely large amounts of body lipids are obtained as contaminants which may cause serious problems during further separation steps. Nevertheless, extraction of dissected glands and of faeces, frass or gnawings as well as short time surface washings are frequently used as standard operations. Headspace techniques using adsorption of emitted volatiles on charcoal or porapak etc. including closed loop stripping, cryo-focussing, and purge-and-trap may largely avoid contamination and greatly facilitate the detection of compounds that are truly released by the investigated organism. Solid sample injection (dissected glands, tissue [21]) and solid-phase-micro-extraction (SPME) [22] avoid any solvent; however, the sample is usually used up during one analytical run, and no derivatives of target components can be prepared (see below). The non-invasive SPME-technique has become almost routine in many labs [23, 24], as it is particularly suitable to follow continuously changes in the production and release of semiochemicals in the same animal depending on time, food, and other parameters.

Tracing biologically active compounds that are perceived by the insects' antennae may be greatly facilitated by on-line linking of gas chromatographic separation (GC) with electrophysiological detection (EAD) [25]. This technique combines high resolution at the separation site with high specificity and high sensitivity at the detector site [18, 20, 26].

Structure elucidation of semiochemicals by modern NMR-techniques (including HPLC/NMR) is often hampered by the very small amounts of available material and problems in the isolation of pure compounds from the complex mixtures they are embedded in. Thus, the combination of gas chromatography and mass spectrometry, GC/MS, is frequently the method of choice. Determination of the molecular mass of the target compound (by chemical ionisation) and its atomic composition (by high resolution mass spectrometry) as well as a careful use of MS-libraries (mass spectra of beetle pheromones and their fragmentation pattern have been described [27]) and gas chromatographic retention indices will certainly facilitate the identification procedure. In addition, the combination of gas chromatography with Fourier-transform infrared spec-

trometry (GC-/FT-IR) may provide important information. As the fragmentation of organic compounds does not always follow strict rules, the interpretation of mass spectra does not always unambiguously enable the deduction of a definite structure. Similarly, infrared spectroscopy provides information on the nature of functional groups rather than on carbon skeletons. Structure elucidation (especially in the case of entirely new compounds) will inevitably need proof through independent synthesis of the proposed structure and comparison of the analytical data of the synthetic compound with those of the natural product.

Microreactions followed by GC/MS-investigations of the reaction products may provide additional information on the chemical structures of target compounds [21, 22]. There are two major areas where micro reactions can be particularly helpful: investigations on the carbon skeleton of a target compound and on the nature of its functional groups. In contrast to NMR-investigations which largely base on the interpretation of signals caused by the influence of functional groups, structure assignment of carbon skeletons by mass spectrometry is limited and often not facilitated by functional groups. This is predominantly due to the formation of stable fragments formed upon α- or β-cleavage, while other signals are of very minor abundance. As a consequence, removal of functional groups or their transformation into groups that do not stabilize the charge may be advantageous. The following micro-reactions proved to be particularly successful in structure elucidation of semiochemicals.

1. Investigations on the carbon skeleton.

Information on the number of double bonds and the number of rings through:
– Removal of double bonds upon hydrogenation (including deuterium)

Location of double bonds through:
– Cleavage of double bonds upon ozonization
– Addition of thiomethyl groups to double bonds upon the reaction with dimethyl disulfide
– Diels-Alder reaction of conjugated double bonds with suitable dienophiles
– Replacement of carbonyl groups or hydroxy groups by hydrogen (including deuterium)
– Transformation of carboxylic acids, esters, alcohols, or ethers into nitriles [28] or other nitrogen containing derivatives [29]
– Investigations on the nature of functional groups

Gas chromatographic separation may be facilitated, and important structural information may be obtained upon the formation of:
– Silylation or (trifluoro)acetylation of hydroxy- or amino groups
– Derivatization of carbonyl groups (reduction or formation of *N,N*-dimethylhydrazones or oximes)
– Transformation of carboxyl groups (esterification or reduction).

The sensitivity of modern mass spectrometers enables subsequent employment of micro reactions even if the yields per step are only moderate. Here an

example: A methyl ketone showing a long carbon chain with several methyl branchings (as indicated by its retention index) was detected among a huge cluster of unseparable hydrocarbons. The mass spectrum of the ketone was strongly dominated by the fragment produced upon McLafferty-rearrangement and did not provide any hints about the branching points. Reduction of the ketone with lithium aluminium deuteride, followed by mesylation and a second reduction step with again lithium aluminium deuteride yielded a di-deuterated hydrocarbon with two deuteriums replacing the oxygen of the former carbonyl group. Its mass spectrum could be easily distinguished from the accompanying non-deuterated hydrocarbons, and its substitution pattern could be assigned according to the literature [30]. Despite the fact that hydride reduction of mesylates of secondary alcohols are proceeding with low yield, the three-step reaction sequence was successfully carried out with less than 50 ng of the target compound [31].

Investigations on the stereochemistry of chiral semiochemicals may be carried out by (gas) chromatographic separation of stereoisomers using chiral stationary phases, e.g. modified cyclodextrins [32]. Alternatively, formation of diastereomers (e.g. Mosher's ester or derivatives involving lactic acid etc.) may be followed by separation on conventional achiral stationary phases. Assignment of the absolute configuration of the natural product will again need comparison with an authentic (synthetic) reference sample.

Another approach ("biogenetic analyses") involves considerations of a reasonable biogenesis of a target compound. Reflections on relationships of components which belong to the same odour bouquet or which have already been known from related species may suggest structures to be expected and, thus, be helpful in the identification process.

3
Biosynthesis and Structural Principles

The biosynthesis and endocrine regulation of pheromone production in beetles has been reviewed [33, 34]. Nevertheless, some more general pathways will be briefly discussed here. As corresponding structures are widespread among insects [2], the examples shown here are selected mostly from taxa other than beetles. Structures representing beetle pheromones will be shown in the context of the discussion of the corresponding species.

Acetogenins. Acetogenins are produced upon chain elongation with activated acetate units (or malonate followed by loss of carbon dioxide). A simplified sketch of this sequence is given in Fig. 1. During the first steps, a Claisen-type condensation of two acyl precursors yields a β-ketoacyl intermediate **A**. Upon reduction to **B** and dehydration to **C**, followed by hydrogenation to **D** and hydrolysis, the chain elongated fatty acid **E** is produced. The next cycle will add another two carbons to the chain. Similarly, a reversed sequence leads to chain

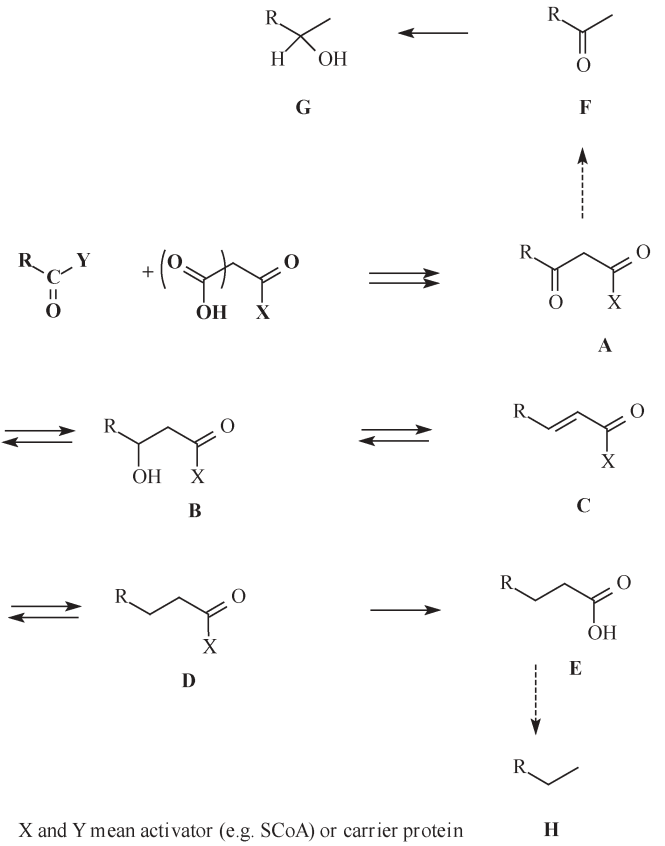


Fig. 1 Sketch of the biosynthesis of simple acetogenins

shortening. Further reactions like decarboxylation of the β-ketoacyl intermediate (obtained after hydrolysis) will yield a methyl ketone **F** which may be reduced to the corresponding (chiral) carbinol **G.** Acetogenins typically form long chain, unbranched compounds, which, according to the general principles of their biosyntheses, occur as characteristic rows of bishomologues with even numbers of carbon atoms (Fig. 1). Classical representatives are saturated fatty acids. Upon decarboxylation, even numbered fatty acids will yield uneven numbered hydrocarbons **H** which are particularly widespread constituents of insect cuticular lipids.

During chain formation, retention of double bonds or oxygen containing functional groups (as remnants of catabolic mechanisms) or introduction of functional groups in the course of secondary reactions (as a result of catabolic processes) may form complex molecules including cyclic and bicyclic structures [35]. Many beetle pheromones originate from the "acetate pool" [2]. The "classical" sex pheromones of moths are represented by straight chain unsat-

urated aldehydes, alcohols or corresponding acetates. In some cases, esters of fatty acids with short chain alcohols have been identified [36]. As shown in Fig. 1, chain elongation as well as chain shortening will pass β-ketoacyl units which, upon loss of the carboxyl carbon, will yield methyl ketones which may be reduced to chiral alcohols. Both classes of compounds have been identified as pheromones in moths [37] and beetles (see below).

Propanogenins and Related Compounds. When during chain formation along a polyketide route acetate units (or malonate) are replaced by propanoate (or methylmalonate), a distinct methyl branching in the final product will be the result. Already Chuman has pointed to close structure relations among a group of pheromones that formally involve propanoate units [38]. When the chain is exclusively formed by a formal condensation of propanoate, the chain will start with a straight chain C3-unit after which every second carbon will carry a methylene group (Fig. 2). During biosynthesis, some or all of the oxygens may be removed, leaving a polymethylated fatty acid like **I**, which has been found in the preen-gland wax of the domestic goose, *Anser a. f. domesticus* [39]. Four propanoate units (including removal of two oxygens) form the lactone **II**, invictolide, a pheromone component of several ant species [40]. The biosynthesis of this compound in *Camponotus* ants has been carefully followed up using isotope-labelling [41]. Formal condensation of five propanoate units and further derivatization is realized in the pyrone **III**, supellapyrone, the female produced sex pheromone of the cockroach *Supella longipalpa* [42].

According to Fig. 2, one of the steps in chain formation with propanoate will result in the formation of an α-methyl-β-ketoacyl moiety **A′**, which, similarly to an acetogenin (Fig. 1), may be converted to the acid precursor **D′**, via reduction to **B′** and dehydration to **C′**, followed by hydrogenation. Alternatively, after another two cycles, decarboxylation would provide an ethyl ketone like 4,6-dimethylnonan-3-one, **III**, a component of the pheromone bouquet of caddis flies, *Potamophylax* spp. [43].

Ethyl ketones may, in general, originate from the decarboxylation of an α-methyl-β-ketoacid as has been shown for 9-methyldecan-3-one, a volatile produced by the myxobacterium *Myxococcus xanthus* [44]. In contrast, a methylketone or a methyl carbinol moiety may originate from a β-ketoacid, produced during chain elongation with acetate (malonate etc.) instead of propanoate as the last step in chain formation (see above). However, an alternative mechanism, proven by isotope labelling, is oxidative decarboxylation of a polymethylated precursor formed from propanoate units [45] to yield compounds like **V**, the pheromone of the mite *Lardoglyphus konoi* [46] (compare structures **I** and **V**). The biosynthesis of the unique branched polyenes which make up the male-produced pheromones of sap beetles, *Carpophilus* spp. has been carefully studied by Bartelt and coworkers using isotope labelled precursors [47, 48]. The carbon skeleton of the tetraene **VI**, the major pheromone component of *C. hemipterus* and *C. brachypterus* is formally made up by an acetate unit as a starter and four propanoate units (the last of which is losing

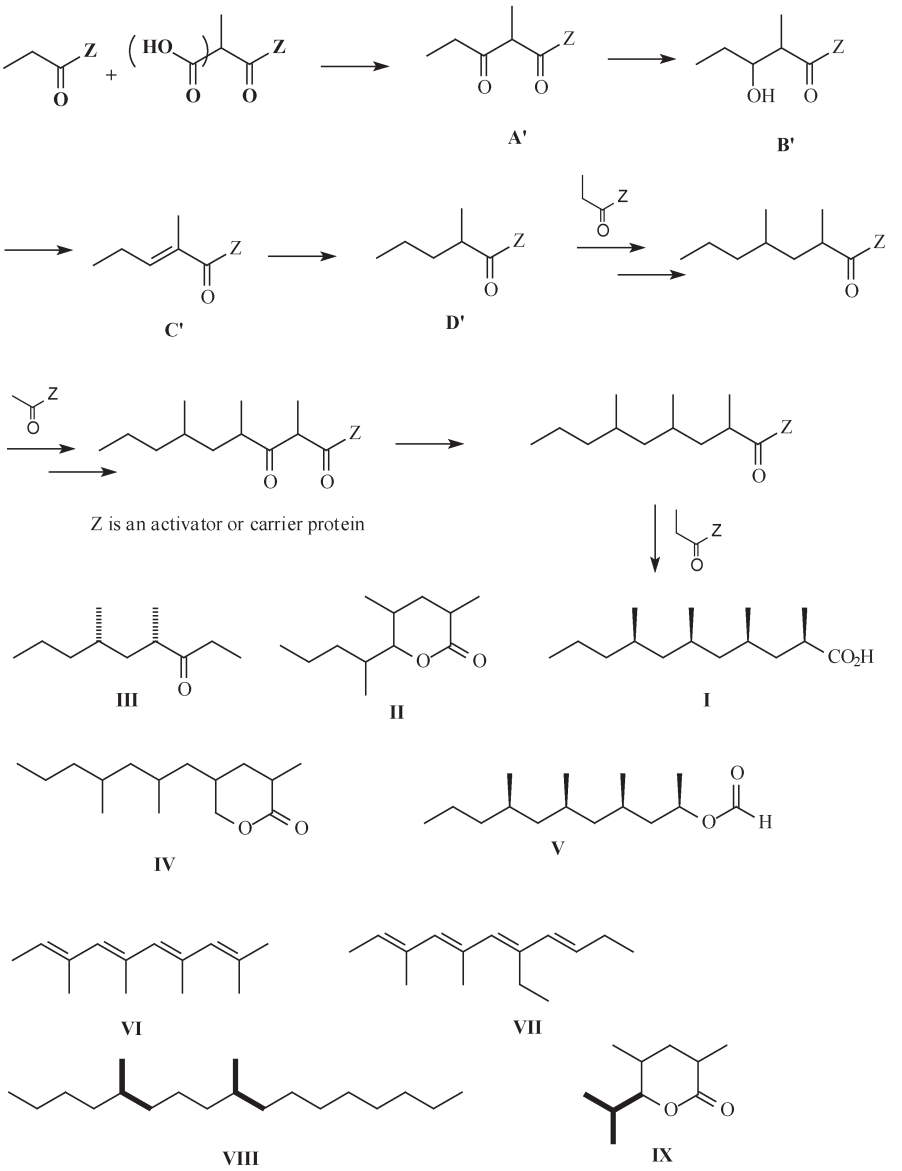


Fig. 2 Sketch of the biosynthesis of simple polyketides made up of propanoate units and some exemplary structures

carbon dioxide).The biosynthesis of the tetraene **VII**, major pheromone component of *C. lugubris*, again starts with acetate, but is continued with two propanoate and two butanoate units (again loss of one carbon through decarboxylation).

The system discussed here is highly versatile: reduction of carbonyl groups and elimination of water will yield (poly)unsaturated structures with characteristic 1,3-dimethyl branching (as in the sap beetles) which may be (partially) hydrogenated. Formation of ethyl branching along the chain through incorporation of butanoate has been described for insects [48] and marine natural products [49]. In mixed biosynthesis, chain elongation may include several acetate units, leading to an uneven number of methylene groups between the methyl branchings as in the hydrocarbon **VIII**, the sex pheromone of the leaf miner moth, *Leucoptera scitella* [50] (formal propanoate units in bold).Various building blocks, including amino acids, may serve as starters for branched compounds: valine may be involved in the biosynthesis of **IX** – possible starter unit in bold – a pheromone identified from a parasitic wasp [51]. Terminal branching has also been described to originate from a leucine starter [2, 44]. Consequently, isoleucine (or a sequence of acetate and propanoate) should give ante-iso branching. Finally, the formation of methyl branching provides stereoisomerism as another disposable variant in the formation of unique signals. Indeed, stereoisomeric composition of chiral compounds plays an important role in chemical communication.

Biogenetic principles involving propanoate as shown in Fig. 2 seem to be very widespread among insects [2]. Similar ways have been described for marine organisms a well as for microorganisms [44, 52–54]. It would be interesting to investigate whether insect volatiles showing polypropanoate structures are truly produced by the insects or whether they result from activities of yet unknown (endo)symbionts.

Isoprenoids. The biosynthesis of isoprenoids has been thoroughly investigated, particularly in plants [55]. The "classical" way involves the diphosphate of mevalonate, (*R*)-3,5-dihydroxy-3-methylpentanoate **a**, which is formed from three acetate units. Elimination of water and carbon dioxide yields 3-methyl-3-butenyl diphosphate **d**, which forms an equilibrium with 3-methyl-2-butenyl diphosphate **e** by the action of an isopentenyl diphosphate isomerase. Coupling of these two C_5 units yields geranyl diphosphate **f**, the parent compound of monoterpenes (Fig. 3). Apart from this "mevalonate" pathway, amino acids such as valine or leucine **b** may serve as starters for the formation of **d** and **e** (for a review see [56]). Another "non-mevalonate" pathway leading to monoterpenes has been discovered in eubacteria, green algae and higher plants [57–61]. Glyceraldehyde-3-phosphate and a C_2 unit derived from pyruvate decarboxylation are the precursors of **d** and **e** via a deoxy-D-xylulose **c** [62–65].

Almost all types of signals, from sex pheromones to highly potent defence substances, are found among the isoprenoids. Many of these compounds seem to be directly sequestered from plants or represent simple transformation

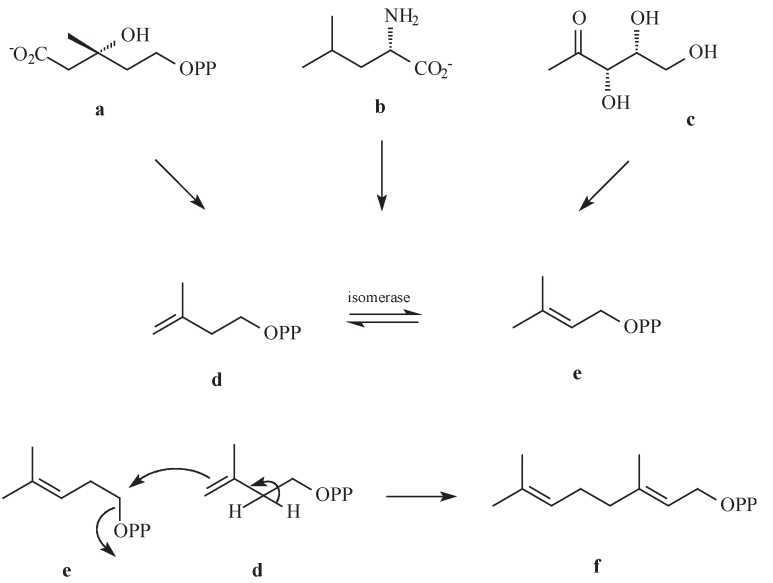


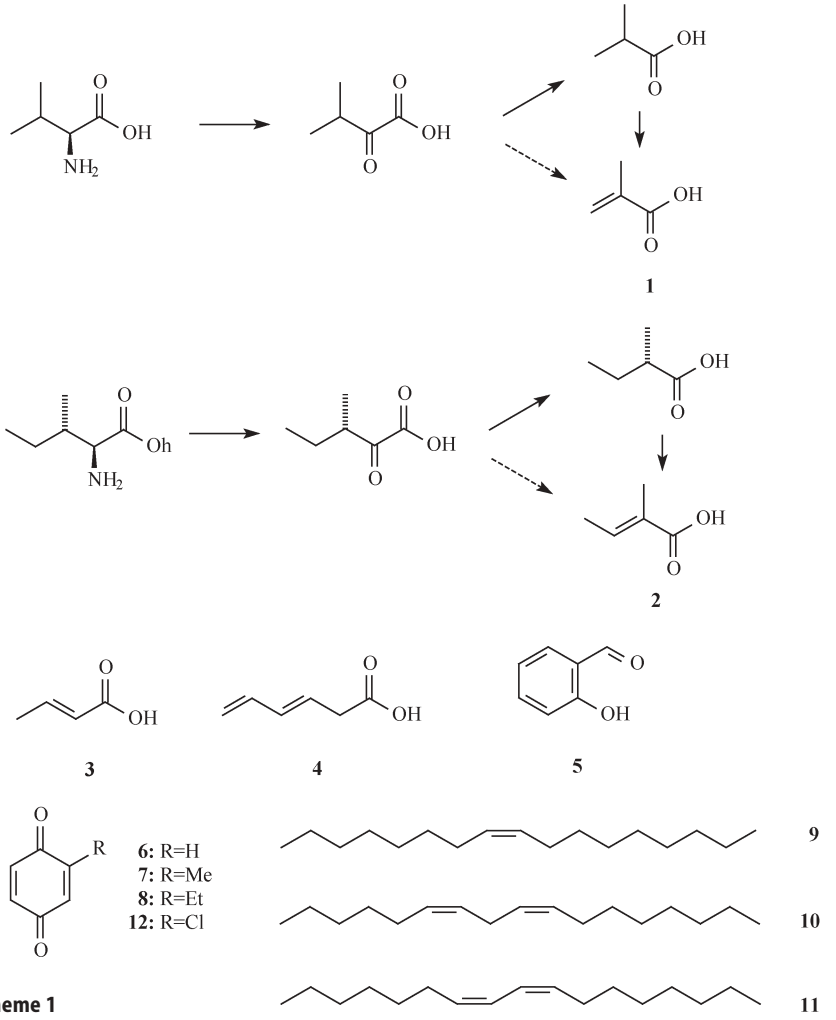
Fig. 3 Sketch of the biosynthesis of terpenoids

products thereof. It has been shown, however, that de novo syntheses may also take place [66–70]. De novo production of isoprenoids has been demonstrated in endothelial cells in the anterior mid gut of bark beetles [71]. Sometimes associated microorganisms play an important role in the production of terpenoids: they may be involved in de novo synthesis and also in secondary transformations of plant compounds [72, 73].

This chapter reviews the structures of beetle produced, intraspecifically active sex-attractants and aggregation pheromones as well as volatile substances that are used for interspecific signalling and for defence. In addition, some metabolites from microorganisms, which have been isolated from beetles and which might have a function in defence, are reported. Compounds identified in beetles but without a proven biological or physical function are mentioned only exceptionally. In the following review, the order of the sections has been arranged according to taxonomical classifications [74, 75]. In each family, relevant volatiles are grouped into subtopics "Attractive Compounds" and "Defence Compounds". In some taxa only defence compounds are known while in others only attractive compounds have been identified. As may be seen, in a given species attractants are frequently made up by only a few compounds while defence chemistry is represented by an array of compounds including solvents and surfactants.

4
Carabidae (Ground Beetles)

Defensive Compounds. The chemistry of pygidial glands has been studied in more than 350 species of ground beetles [8]. Since Blum's important compilation [76] data were reported from a lot of species including representatives of Cicindelidae (see Cicindelidae) and other Carabidae especially bombardier beetles from the Paussinae and Brachinitae families. In *Oodes americanus* (Oodini, Callistitae) a striking sexual dimorphism was revealed [77]: Whereas the unsaturated methacrylic acid **1**, tiglic acid **2**, and crotonic acid **3** are exclusively found in females, males only produce the corresponding saturated analogs. In other



Scheme 1

species such as *Pasimachus subsulcatus* no sexual differences in the carboxylic acid patterns were found [78, 79]. Obviously, these acids are derived from amino acids via the α-ketoacid intermediates [80, 81] (see Scheme 1). It seems probable that males lack the desaturases and that these secretions may also play a pheromonal role. The genera *Oodes* (Oodini) and *Moriosomus* (Morionini) also contain benzoic acid and (*E*)-2-octenoic acid as was found in water beetles [79, 82]. Hexanoic acid, (*E*)-3-hexenoic acid, (*E*)-3,5-hexadienoic acid **4**, and octenoic acids are typical acetogenins. Pygidial gland acids could be characterized as pentafluorobenzyl derivatives [80]. The production of formic acid, typical for many ground beetles, was studied in detail in *Galerita lecontei* [83], where the gland contains formic acid in amounts of up to 3% of the body mass, enough for more than six ejections. The secretory output of formic acid may reach as much as 5% of the gland volume per hour. Formic acid is probably produced from the amino acids L-serine and glycine, via N^5-formyltetrahydrofolate. The separated glands of *Helluomorphoides clairvillei* contain a mixture of compounds including carboxylic acids, aliphatic esters, and hydrocarbons [84]. *Oodes amaroides* (Oodini) secretes salicylic aldehyde **5** from its pygidial glands, while other species produce nonyl acetate, various other acetogenic acetates, formates, hexanoates and 2-pentadecanone.

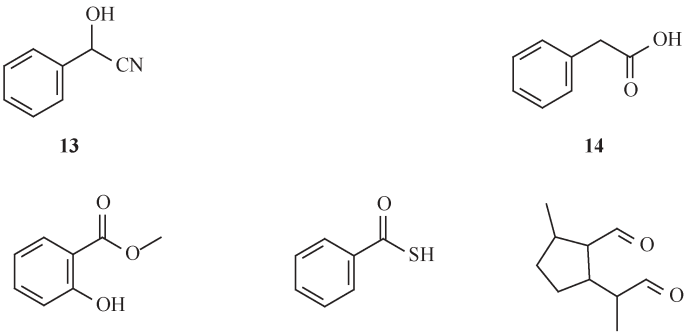
A detailed predator-prey analysis of the chemical relations between the carabid *Pasimachus subsulcatus* and the skink *Eumeces inexpectus* proved that the latter were repelled by constituents of the carabids' secretions, indicating that the beetles are chemically protected from attacks by the lizards [85].

In abdominal defensive glands of carabid beetles, two lineages exhibiting a bombarding mechanism can be observed: the brachinoid (Brachinini with worldwide 14 genera, Crepidogastrini) and the paussoid (Paussini, Ozaenini, Mystropomini, Metriini) lineage. Discharging mechanisms and aiming techniques vary between the two lines. Brachinini rotate their abdominal tips whereas Paussini use their elytral flanges. According to Eisner et al. [86] both groups are characterized by bicompartmented glands and a hot, audible discharge of quinones, i.e. 1,4-benzoquinone **6**, as well as 2-methyl-1,4-benzoquinone **7**, and 2-ethyl-1,4-benzoquinone **8**.

The ability to bombard either evolved only once in Carabidae or independently in both lineages. The glands contain 1,4-benzoquinones and various straight chain or methylbranched alkanes, alkenes and alkadienes such as pentadecane, (*Z*)-8-heptadecene **9** or (6*Z*,9*Z*)-6,9-heptadecadiene **10** and (7*Z*,9*Z*)-7,9-heptadecadiene **11**. In *Metrius contractus* the secretion contains small amounts of 2-chloro-1,4-benzoquinone **12**. As compared to alkanes, the slightly more polar alkenes are better solvents for the quinones and for spreading of the secretion over the beetles [87, 88]. The *Z,Z*-configuration of conjugated dienes of bombardier beetles seems to prohibit a Diels-Alder reaction of these "solvent components" with the active defence compounds, benzoquinones [89].

5
Cicindelidae (Tiger Beetles)

Defensive Compounds. Many tiger beetle species from several genera [90] (about 90 species: *Megacephala, Neocollyris, Odontocheila, Pentacomia, Cicindela*) release benzaldehyde and hydrogen cyanide which are produced from the cyanogenic precursor, mandelonitrile **13** (Scheme 2), which is probably synthesized de novo from phenylalanine. In addition, several species contain benzoic and phenyl acetic acid **14** (as in Hydradephaga and a few Carabidae), methyl salicylate **15** [91], thiobenzoic acid **16**, tridecane and pentadecane [90, 92], tetradecyl acetate, and hexadecyl acetate [90], heptadecanol [92] and even iridodial isomers **17**. As a whole, phylogenetic factors as evidenced by DNA-comparison may predominantly influence the pygidial gland chemistry pattern of tiger beetles [90, 93]. In *Cicindela*, aposematic coloration was restricted to a phylogenetic group producing large amounts of the benzaldehyde. Species previously thought to lack benzaldehyde were later shown to produce small but detectable amounts [92].



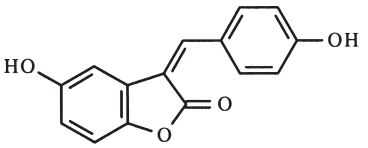
13 14 15 16 17

Scheme 2

6
Dytiscidae (Predaceous Diving Beetles)

Defensive Compounds. As all other terrestrial and aquatic adephagan beetles, dytiscids are characterized by paired pygidial glands which are found laterally behind the eighth abdominal tergites. Chemically the secretions are characterized either by phenylacetic acid (Hydroporinae, Noteridae, Haliplidae) or by benzoic acid, hydroxybenzaldehyde and related compounds (Dytiscinae, Colymbetinae; [8, 82]). The secretion exhibits a pronounced antimicrobial activity and protects from adhering bacteria and fungi [94]. Moreover, the beetle may modify the wettability of their body surface [82, 95].

Marginalin [96] **18** (Scheme 3), a yellow pigment from pygidial glands of *Dytiscus marginalis*, was found to fix solidly on a variety of supports. When



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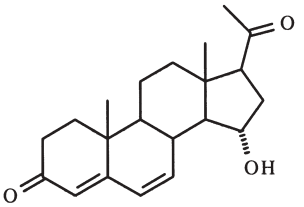

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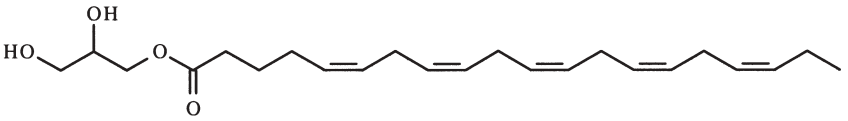

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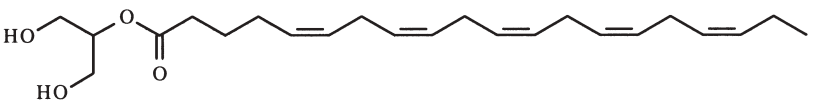

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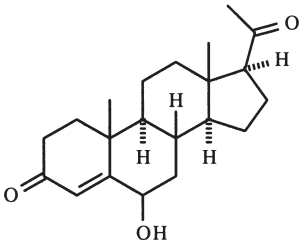

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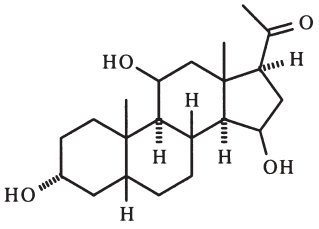

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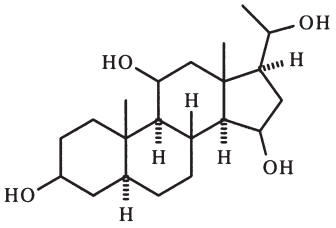

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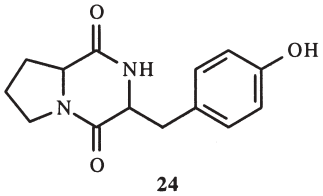

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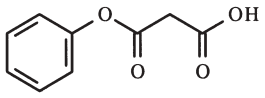

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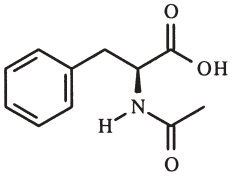

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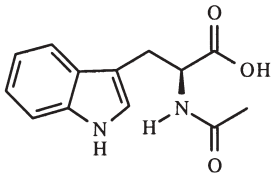

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Scheme 3

in contact with bacteria and fungi, marginalin may react with the proteins at the cell surface [97]. *Z*-Marginalin has been synthesized by base-catalysed condensation of *p*-hydroxybenzaldehyde with 2,5-dihydroxyphenylacetic acid [98].

Paired prothoracic defence glands opening behind the prothoracic margin are present in Dytiscidae and Hygrobiidae. The secretions are targeted against predatory vertebrates (esp. fish, amphibians) and contain both toxic anaesthetic and odorous substances [8]. Various steroids were found in high amounts. Several constituents are discussed in the chapter by Laurent et al., this volume. In addition, 15α-hydroxypregna-4,6-dien-3,20-dione **19** was identified in prothoracic defensive glands of *Agabus affinis* along with four 1- or 2- monoglycerides of a polyunsaturated fatty acid: 1- or 2-[(5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-icosatetraenoyl]glycerol and 1- or 2-[(5*Z*,8*Z*,11*Z*,14*Z*,18*Z*)-5,8,11,14,18-icosapentaenoyl]-glycerol **20a/20b**. Since the 2-acylated monoglycerides showed only a weak activity as feeding deterrents against minnows, their possible role as cannabimimetics needs to be investigated [99].

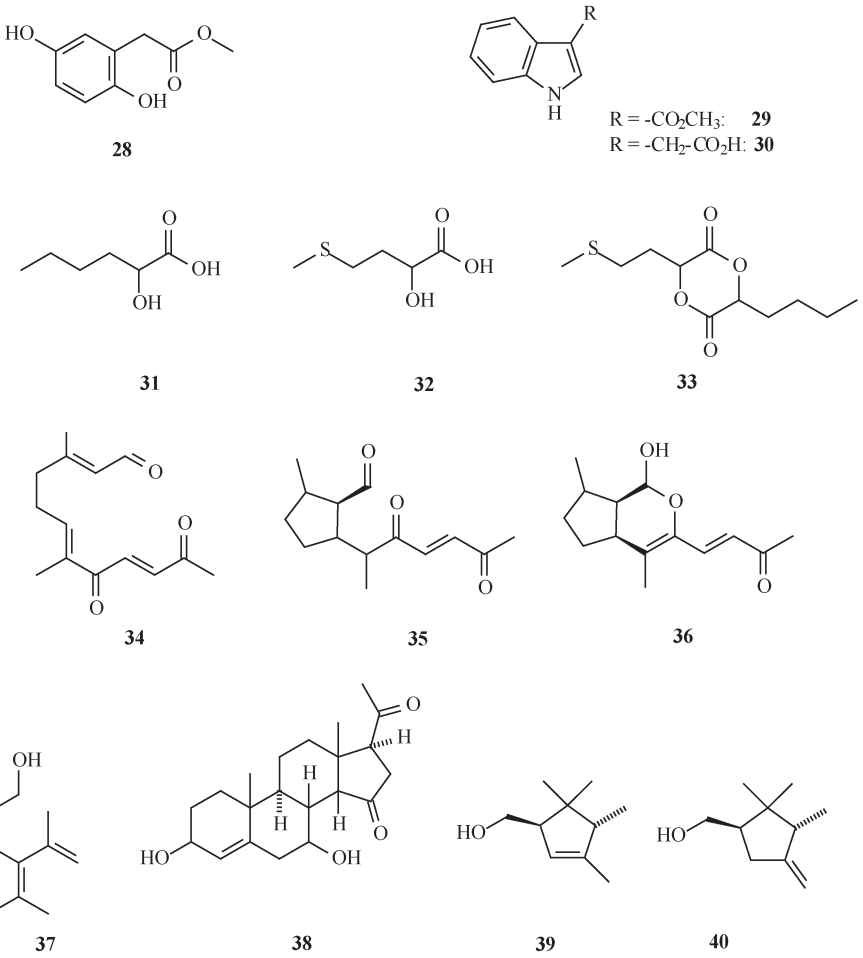
In the prothoracic gland secretion of *Agabus guttatus* testosterone and estradiol as well as nine higher oxygenated pregnane derivatives could be identified [100]: 3α-hydroxy-5β-pregnane-20-one, 3α,11β-dihydroxy-5β-pregnane-20-one, 5β-pregnane-20-one, 3β,20α-dihyroxypregn-5-ene, 6β-hydroxypregn-4-en-3,20-dione **21**, 3α,20α-dihydroxy-5α-pregnane, 3α,11β,15β-trihydroxy-5β-pregnane-20-one **22**, 16α,20β-dihydroxypregn-4-ene, 3β,11β,15β,20β-tetrahydroxy-5α-pregnane **23**, and 3β,11β,15α-trihydroxy-5α-pregnane-20one.

Since the predatory water beetles cannot biosynthesise the steroid skeleton de novo, steroidal precursors must be obtained from exogenous sources. *Bacillus*-strains, isolated from the foregut of the water beetle *Agabus affinis*, were tested for their ability to transform steroids [101]. After incubation with androst-4-en-3,17-dione two *Bacillus* strains produced 13 different transformation products. Hydroxylation took place at C6, C7, C11 and C14 resulting in the formation of 6β-, 7α-, 11α-, and 14α-hydroxyandrost-4-en-3,17-diones. After incubation with pregnenolone the two *Bacillus* strains produced a variety of steroids among which 7α-hydroxypregnenolone was the major product [102].

From the fore gut of *Laccophilus minutus*, a *Bacillus pumilus* strain was isolated which produced maculosin, the diketopiperazine formed from proline and tyrosine [103] **24**, phenyl malonate **25**, *N*-acetylphenylalanine **26**, *N*-acetyltryptophane **27** and 3,4-dihydroxybenzoic acid [103]. Maculosin which has also been isolated from several microorganisms and sponges shows phytotoxic and cytotoxic properties [103], 3,4-dihydroxybenzoic acid shows antioxidant properties and was already found in pygidial defensive glands of several dytiscid beetles.

7
Amphizoidae (Trout Stream Beetles)

Defensive Compounds. Pygidial glands of *Amphizoa lecontei* contain dimethyldisulfide, methyl *p*-hydroxybenzoate, methyl homogentisate **28** (Scheme 4), methyl indole-3-carboxylate **29**, and the pigment marginalin **18** [96]. Beetles may use the aromatic compounds as both antimicrobic and fungicide agents to keep their body surface clean, which may explain why they leave the water in order to distribute their pygidial gland secretion over the body surface [95].



Scheme 4

8
Noteridae (Burrowing Water Beetles)

Defensive Compounds. The sweetish smell of *Noterus* species is due to the presence of phenyl acetic acid **14** as the main constituent of the pygidial gland secretion. Furthermore, some additional aromatics and 3-indole acetic acid **30** could be identified [82, 104].

9
Hygrobiidae=Pelobiidae (Squeak Beetles)

Defensive Compounds. The pygidial gland secretion of *Hygrobia hermanni* contains unusual 2-hydroxy acids such as 2-hydroxyhexanoic **31** acid and 2-hydroxy-4-(methylthio)butanoic acid **32.** The compounds may form lactides **33**, which are the oxygen-analogues of diketopiperazins. Traces of benzoic acid and *p*-hydroxybenzaldehyde could be identified [104].

10
Haliplidae (Crawling Beetles)

Defensive Compounds. Crawling beetles of the genera *Haliplus* and *Brychius* contain pygidial gland secretions with phenyl acetic acid **14** as the main constituent [8]. Secretion grooming was observed which may serve for distributing the antimicrobics on the body surface and for modifying the wettability of the surface [82, 95].

11
Gyrinidae (Whirligig Beetles)

Defensive Compounds. In the stinking pygidial gland secretion of these beetles [8], 3-methylbutanal and the corresponding alcohol are present [8]. In addition, the secretions of *Gyrinus* and *Dineutes* contain the toxic sesquiterpenes, gyrinidal **34**, gyrinidione **35**, and gyrinidone **36** [105–108] (Scheme 4).

Captive fish, *Micropterus slamoides*, rejected both the beetle *Dineutes hornii* and mealworms after topical treatment with gyrinidal. The fish also exhibited an intensive and dose dependent oral flushing behaviour to get rid of gyrinidal [109].

Borg-Karlsson et al. [110] showed that the pygidial gland secretions of certain *Gyrinus* species may contain volatiles which act as intra- and interspecific alarm signals.

12
Silphidae (Carrion Beetles)

Defensive Compounds. Carrion beetles may spray defensive secretions from their anal region which are usually mixed with faecal material [8, 111]. Apart from ammonia, the material contains fatty acids, lavandulol **37**, and ketopregnanes such as 15β-hydroxyprogesterone [8, 111]. Two new pregnanes could be identified from *Silpha novaboracensis* [111]: 3α,7β-dihydroxy-14β-pregn-4-en-15,20-dione **38** (major defensive steroid) and 3α,7β,20-trihydroxy-14β-pregn-4-en-15-one (configuration at C-20 remains unassigned; minor constituent) [111]. Bioassays with the unusual cylopentanoid terpenes α- and β-necrodol **39,40** identified from *Necrodes surinamensis* [112], proved these compounds to be repellent for *Monomorium*-ants as well as topically irritant against the cockroach *Periplaneta americana* and the fly *Phormia regina*.

13
Staphylinidae (Rove Beetles)

Attractive Compounds. In contrast to defence chemistry, little is known about the pheromone systems of rove beetles.

In the sternal gland secretion of males of *Aleochara curtula*, 1-methylethyl (*Z*)-9-hexadecenoate was identified. The compound was attractive to males and females and acts as an aggregation pheromone [113].

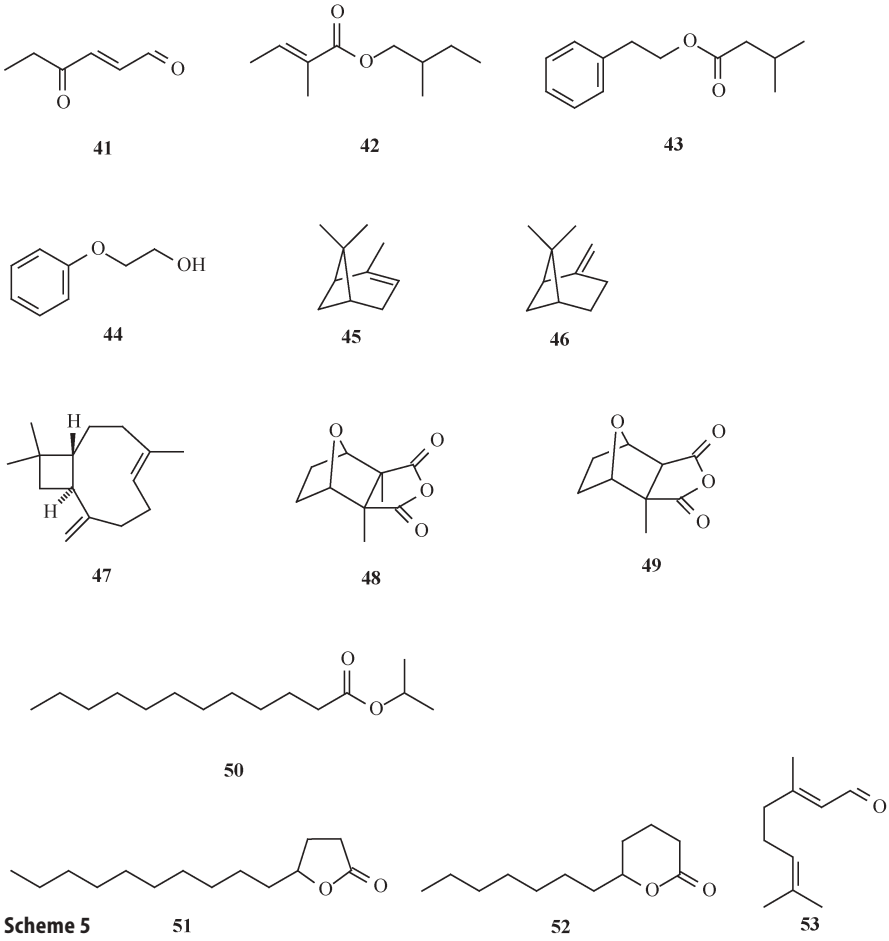
The female produced sex pheromone of *Aleochara curtula* has been described to consist of a mixture of (*Z*)-7-henicosene and (*Z*)-7-tricosene [114]. The same compounds are reported to be used by young males as a kind of camouflage to avoid aggression from older males. Similarly, chemical camouflage by using hydrocarbons plays a role in the relations between the myrmecophilous staphylinid beetle *Zyras cones* and the ant *Lasius fuliginosus*. The host worker ants never attack these beetles which show the same profiles of cuticular hydrocarbons as the ants [115].

The neotropical staphylenid *Leistrotrophus versicolor* use volatile compounds secreted from their abdominal tips to attract their prey, drosophilid and phorid flies [116]. The structures of the active compounds are yet unknown, however, it has been speculated that actinidine or other iridoids, typically found in the defensive gland which are located at the abdominal tips of these beetles, may be key components [117].

Defensive Compounds. Many data on chemical defences of rove beetles have been compiled by Dettner [118]. Recent taxonomic compilations indicate that this beetle family with its omaliine, oxyteline, tachyporine and staphylinine subgroups consists of about 60,000 species, worldwide [119]. Within all four groups, chemical defensive systems evolved independently, because free living rove bee-

tles have an usually soft unsclerotized abdomen which is completely unprotected from predatory attack.

Representatives of the subfamilies Omaliinae and Proteininae (*omaliine group*) possess an abdominal defensive gland reservoir that opens out between sternite 7 and 8 [120]. The multi-component mixtures contained in these glands are used for defence. In Omaliinae and Proteininae the secretion is characterized by mixtures of acids (e.g. 2-methylpropanoic acid, hexanoic acid, 2-octenoic acid, 2-methylbutanoic acid, 3-methylbutanoic acid, butyric acid, and tiglic acid), aldehydes ((*E*)-2-hexenal, heptanal, octanal, nonanal), ketoaldehydes such as 4-oxo-2-hexenal **41** (Scheme 5), 6-methyl-5-hepten-2-one, alcohols (octanol, (*E*)-2-hexen-1-ol, 2-methylbutan-1-ol), alkanes (nonadecane), esters (2-methylbutyl tiglate **42**, various propanoates, 2-hexenyl 3-methylbutanoate, 2-methylbutyl 2-methylbutanoate, octanoates, butanoates), and aromatic compounds (e.g. 2-phenethyl 3-methylbutanoate **43**). Unusual compounds are 2-



Scheme 5

phenoxyethanol **44** and benzonitrile as well as α-pinene **45**, β-pinene **46**, and β-caryophyllene **47**.

In species of the genera *Omalium, Lathrimaeum, Phyllodrepa, Eusphalerum, Phleonomus,* and *Proteinus* the secretions are characterized by acids, corresponding aldehydes and alcohols, ketones and the corresponding esters. In several *Eusphalerum*-species and *Anthophagus,* esters seem to be replaced by hydrocarbons.

Specimens of the pollen-feeding staphylinid beetle *Eusphalerum minutum* were found in cantharidin traps, which indicates that they are canthariphilous [121]. In addition, they contain small amounts of cantharidin **48**, which is accompanied by palasonin **49**. Palasonin has been previously only known from seeds and fruits of the Indian shrub *Butea frondosa* (Leguminaceae; [122]).

In the *oxyteline group*, considerable knowledge has accumulated concerning the morphology and chemistry of the paired 8/9 th tergite gland system within Oxytelinae and Pseudopsinae [118]. All the 1700 worldwide known species of Oxytelinae share this defensive gland which contains *p*-toluquinone **7** as the active principle (see Fig. 4). The solvents range from esters of 2-propanol

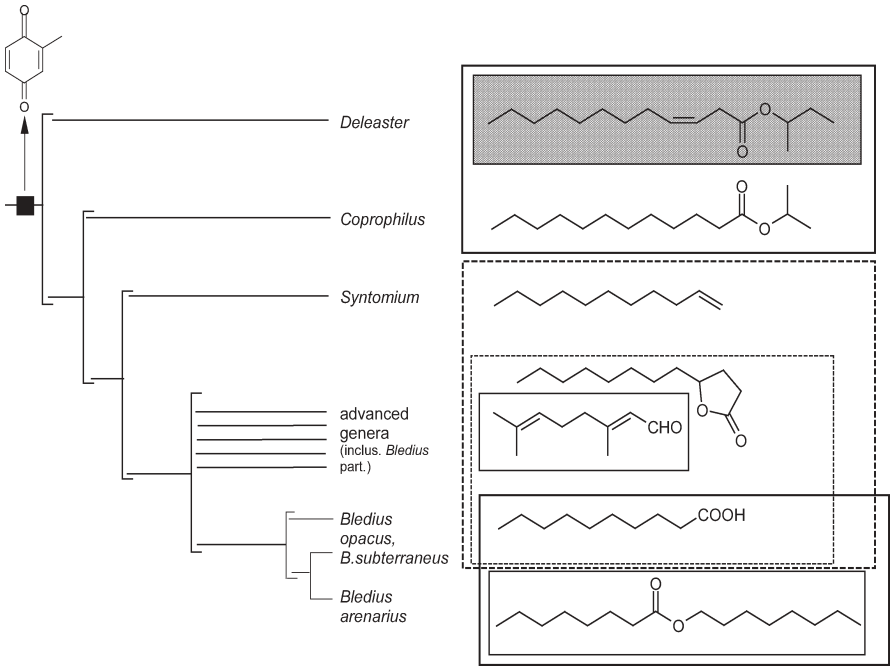


Fig. 4 Evolution of solvents and solvent-mixtures in the defensive secretion of Oxytelinae (Staphylinidae) beetles. The secretions of all worldwide investigated species are saturated with the toxic compound *p*-toluquinone (*left*). The topical irritancy of the mixtures is continuously increased from primitive to advanced taxa. The cladogram on the left side includes the most important primitive (*Deleaster, Coprophilus, Syntomium*) and several advanced genera [117, 122]

or 2-butanol (e.g. 1-methylethyl dodecanoate, **50**) in the primitive genera such as *Deleaster* and *Coprophilus* to 1-alkenes and γ-lactones (e.g. γ-tetradecalactone **51**) in more advanced genera including many *Bledius*-species (Fig. 4). Advanced species additionally produce δ-lactones (e.g. δ-dodecalactone **52**), citral (a mixture of geranial **53** and its *cis*-isomer, neral), various acetates and esters such as hexyl decanoate.

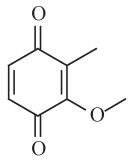
It is astonishing that *Bledius arenarius* represents the only species which does not fit into this concept, because its toluquinone is dissolved only in decanoic acid and octyl octanoate (Fig. 4). However, *B. opacus* and *B. subterraneus* keep an intermediate position because they secret both alkenes/lactones and acids which are also found in *B. arenarius* [123].

Interestingly, the defensive secretion of Oxytelinae is optimised by replacing mixtures of physicochemically similar solvents such as esters of 2-propanol or 2-butanol by mixtures of physicochemically different compounds as 1-alkenes and γ-lactones [87]. Only in the second case it is possible to vary both physicochemical and biological parameters of the mixture, and the topical irritancy of the quinone containing mixture against arthropod targets is significantly improved from primitive to advanced beetles due to a quasi-synergistic effect. Moreover, it was found that the beetles maintain a certain solvent ratio of about one part of lactone and five parts of alkenes. Through this defined mixture the beetles achieve an optimal topical efficiency and can, thus, reduce the amounts of the toxic quinone as was shown by the *Calliphora*-constriction test. Finally, the optimal response of target organisms is due to the fact that maximal amounts of the toxic quinone penetrate the lipophilic cuticle of the target arthropod organism [118]. It was shown that the abdominal gland secretion represents an optimal defence against predators [124] and that the solvent ratio of various *Bledius* species is optimally adapted to their natural targets such as earwigs, ants, flies, carabid beetles, and wading-birds [125].

With respect to the biosynthesis of the solvents it has been speculated on the basis of quantitative data and the identification of β,γ-unsaturated acids in primitive oxytelid beetles that pairs of 1-alkenes and γ-lactones are synthesized from corresponding 3-alkenoic acids by either lactonization or by decarboxylation [118].

Among aleocharine larvae (tachyporine group) two bark-inhabiting representatives of the genera *Leptusa* and *Bolitochara* were investigated [118, 126]. They possess an unpaired abdominal defensive gland reservoir with few polyploidous gland cells associated with the eighth abdominal tergite. Upon molestation, the larvae generate a toxic defensive secretion which is topically active. The secretions contain *p*-toluquinone **7** and 3-methoxytoluquinone **54** (Scheme 6) as active principles which are dissolved in ethyl esters, isopropyl esters, and alkanes. In addition, the antimicrobic benzyl propanoate **55** and methyl 4-hydroxybenzoate **56** were identified.

Apart from the primitive Deinopsini and Gymnusini, adult Aleocharinae show unpaired tergal glands situated between tergites 6 and 7 [127]. Up to now, chemical data of the topically active defensive secretions are available from



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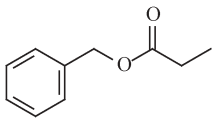

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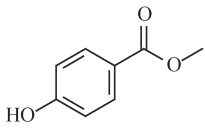

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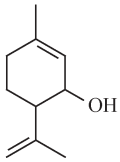

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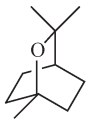

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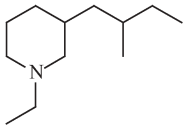

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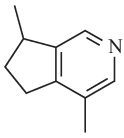

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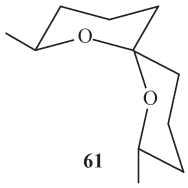

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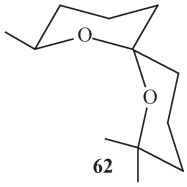

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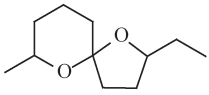

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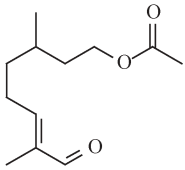

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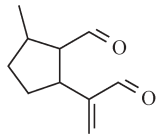

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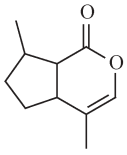

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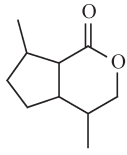

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Scheme 6

about 30 species. One group of species including representatives of Aleocharini, Myrmedoniini, Athetini, and Oxypodini contain hydrocarbons from nonane to heptadecane (undecane as the main component), aldehydes (decanal, dodecanal, tetradecanal, tetradec-5-enal, tetradec-5,8-dienal), short-chain fatty acids like isobutyric acid and isovaleric acid as well as esters such as dodecyl acetate as solvents for *p*-toluquinone **7**, the methoxyquinone **54** and sometimes 1,4-benzoquinone **6**. All quinones are accompanied by the corresponding hydroquinones. The genus *Dinarda* and Bolitocharini predominantly contain long-chain fatty acid esters (ethyl octadecanoate to ethyl octadecadienoate, and ethyl hexadecanoate; group 2) and isoamyl propionate whereas the third group is characterized by *p*-toluquinone and 2-heptanone (Placusini). Within the fourth group of aleocharine beetles (*Falagria*, *Autalia*) only aqueous alkylquinone-solutions could be recorded from small gland reservoirs.

The following chemically defended taxa belong to the staphylinine group: Steninae, Paederinae, Staphylininae, and Xantholininae, sometimes also Silphidae (see above) are incorporated in this group.

Adults of the Steninae possess paired eversible abdominal defensive gland reservoirs [119, 128]. When the beetles walk on the water surface the spreading secretion propels the beetle forward which represents an unique escape mechanism. The secretion contains isopiperitenole **57**, 1,8-cineole **58**, 6-methyl-5-hepten-2-one and the unique spreading alkaloid stenusine, *N*-ethyl-3-(2-methylbutyl)piperidine **59**. Natural stenusine was found to be a mixture of all four stereoisomers in a ratio of (*S, S*):(*S, R*):(*R, R*):(*R, S*)=43:40:13:4. An enantioselective synthesis of stenusine has been carried out via an Enders-approach [129].

Representatives of certain adult Paederinae (Paederus, Paederidus) possess a median complex gland which is situated at the front margin of the fourth sternite [130]. In the genus *Rugilus* even two glands are located at the front margin of sternites 4 and 5. The constituents of abdominal glands of *Paederus*/*Paederidus* have not been be fully elucidated but the presence of various alkenes seems probable. Whether the *Paederus*-glands are able to externalise the hemolymphtoxin pederin has to be investigated. Further data on the microbial-derived insect toxin pederin and the defensive chemistry of Paederinae can be found in the chapter by Laurent et al., this volume.

The paired defensive gland reservoirs of Staphylininae are situated between tergites 8/9 and may be everted upon molestation. Therefore, the secretion acts topically. The chemistry varies considerably between species. While Staphylinina use terpenoids as solvents for, e.g. iridodial 17, representatives of Philonthina produce a lot of acetates and hydrocarbons as solvents for actinidin **60** [8, 118].

Recordings from Staphylininae [115] include: 3-methylbutanal, the corresponding alcohol, and its acetate, various ketones such as 4-methyl-3-hexanone, 4-methyl-3-heptanone, 5-methyl-3-hexanone (and the corresponding alcohol), 2-heptanone, 6-methyl-2-heptanone, 6-methyl-5-hepten-2-one as well as methylcyclopentene and methylfuran. In addition, the secretions of *Ontholestes murinus* contain the spiroacetals (2*S*,6*R*,8*S*)-2,8-dimethyl-1,7-dioxaspiro[5,5]-

undecane, **61**, (in *Ontholestes tesselatus* largely racemic), (6*R*,8*S*)-2,2,8-trimethyl-1,7-dioxaspiro[5,5]-undecane **62**, and (*E*,*E*)- as well as (*Z, E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4,5]-decane **63** [131–133]. Further gland constituents are α-pinene **45**, neral and its E-isomer, geranial **53**, nerol, citronellol and esters such as (*E*)-8-oxocitronellyl acetate **64**, ethyl hexadecenoate, and ethyl octadecenoate. Several iridoids were reported, sometimes of unknown stereochemistry: actinidine **60** [119], various iridodial-isomers **17**, dolichodial **65**, nepetalactone **66** and dihydronepetalactone **67**.

There was proposed a detailed account of iridoid biosynthesis in rove beetles which resembles the biosynthesis in leaf beetle larvae but exhibits distinct stereochemical differences [134], see also the chapter by Laurent et al., this volume.

Within Quediini defensive glands are either present (*Algon*) or are lacking (*Quedius* [8, 118]). In the first case the paired glands contain hexanoic acid, hexanal, and (*E*)-2-hexenal which may be sprayed upon disturbance.

Representatives of Xantholininae possess an unpaired nonreversible anal gland reservoir at their abdominal tip. As already reported, the secretion contains iridodial, actinidin, terpenoid aldehydes, ketones, limonene, and isopulegol [8, 118].

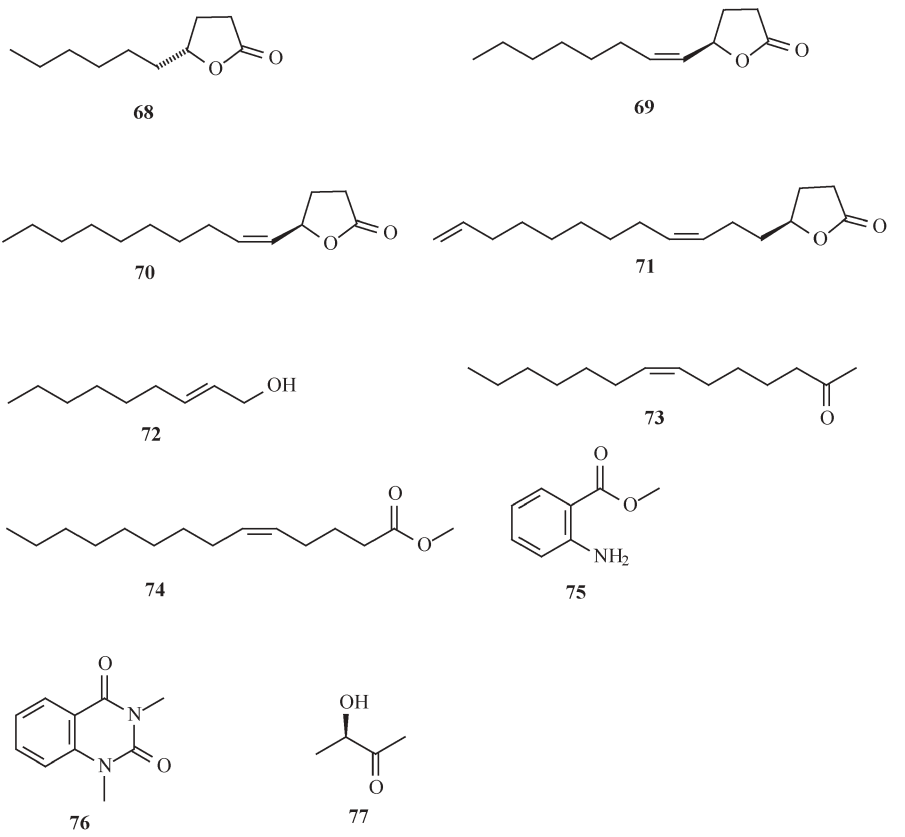
14
Scarabaeidae (Scarab Beetles, Chafers, Dung Beetles)

Attractive Compounds. Though the first report on the identification of a pheromone from a scarabaeid beetle dates back more than 30 years – phenol as an attractant for males of the gras grub beetle *Costelytra zealandica* [135] which turned out to be produced by beetle associated bacteria [136] – most of the pheromone structures known today have been elucidated during the last decade [3, 137, 138].

Pheromone chemistry in scarab beetles, chafers, and dung beetles covers a wide range of structures, including esters of amino acids and aromatics as well as branched and straight chain aliphatic compounds, among which a row of γ-lactones forms a most characteristic group.

The male released pheromone of *Osmoderma eremita* is (*R*)-5-hexyloxacyclopentan-2-one **68** [139] (Scheme 7). In contrast, in other scarab species, pheromones are mostly produced by females.

Females of several species use (*R*)-5-[(1*Z*)-1-octenyl]oxacyclopentan-2-one, buibuilactone **69** [140–144]. The first γ-lactone identified from a scarab beetle was (*R*)-5-[(*Z*)-1-decenyl]oxacyclopentan-2-one, japonilure **70**, the female produced sex pheromone of the Japanese beetle *Popillia japonica* [145]. Both **69** and **70** are components of specific blends of several species [140–143]. The Japanese beetle is extremely sensitive to the non-natural enantiomer of his pheromone: as little as 1% of the (*S*)-enantiomer inhibits the attractiveness of the pheromone [145]. With respect to species discrimination, this is particularly



Scheme 7

interesting on an evolutionary point of view. The closely related species *Anomala osakana* uses this very compound, (*S*)-5-[(*Z*)-1-decenyl]oxacyclopentan-2-one as the pheromone [146] and, in turn, the species is repelled by the pheromone of the Japanese beetle. For scarab beetles, olfactory discrimination of enantiomers at the level of odorant binding proteins as well as enantiomeric anosmia has been described [138, 147, 148]. The biosynthesis of those γ-lactones proceeds via an enantioselective 8-hydroxylation of fatty acids and chain shortening, followed by ring closure [149]. Another γ-lactone, (*R*)-5-[(*Z*)-dodeca-3,11-dienyl]oxacyclopentan-2-one **71** is the sex pheromone of the yellowish elongate chafer, *Heptophylla picea* [150, 151]. In this case, only the (*R*)-enantiomer showed attractiveness, while its activity was not inhibited by the presence of its antipode [152].

Several syntheses of optically active japonilure and related lactones involve enzyme-catalysed transformations [153]; however, recently, it has been efficiently prepared in high enantiomeric purity via boronic esters of 1,2-dicyclohexyl-1,2-ethanediol [154] (Fig. 5).

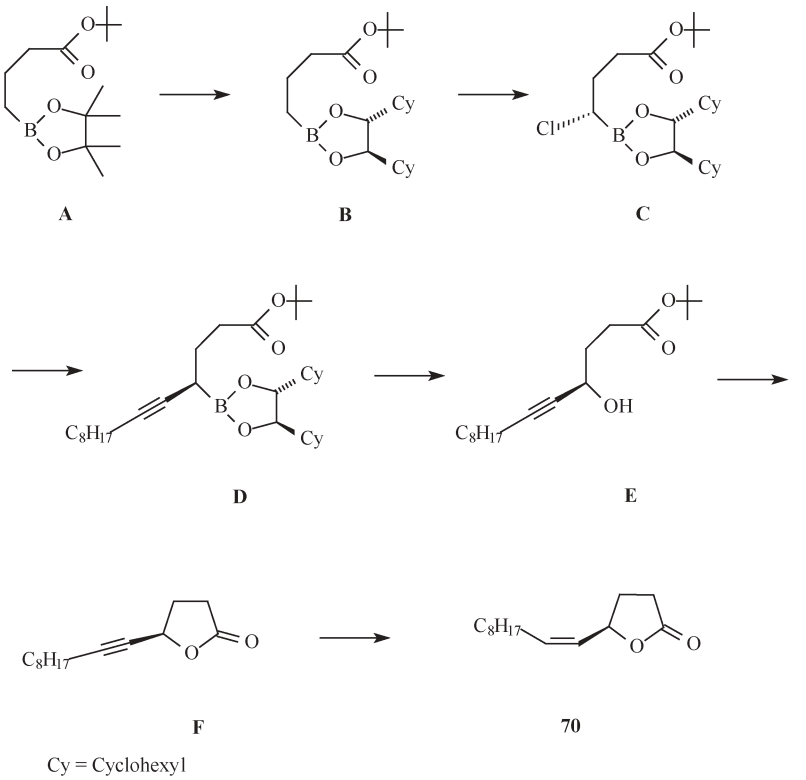


Fig. 5 Reaction scheme for the synthesis of optically active japanilure **70**

The boronic acid ester **B** was synthesized by transesterification of the corresponding pinacolester **A** with (1*R*,2*R*)-1,2-dicyclohexyl-1,2-dihydroxyethane. Stereoselective chlorination of **B** was carried out with (dichloromethyl) lithium and zinc chloride. Reaction of the obtained chloroboronic ester **C** with lithio 1-decyne followed by oxidation of the intermediate **D** with alkaline hydrogen peroxide afforded the propargylic alcohol **E**. Treatment with acid to saponify the *tert*-butyl ester moiety and to achieve ring closure, produced lactone **F**. Finally, Lindlar-hydrogenation provided japonilure **70** in an excellent yield and high enantiomeric purity.

In some species, (*E*)-2-nonenol **72** represents a second pheromone component along with the lactone **69** [141, 144], while in *Anomala schönfeldti* **72** is the only attractive component [155]. The alcohol **72**, the corresponding aldehyde, lactone **69** and methyl benzoate make up the pheromone of *Anomala albopilosa albopilosa* [144].

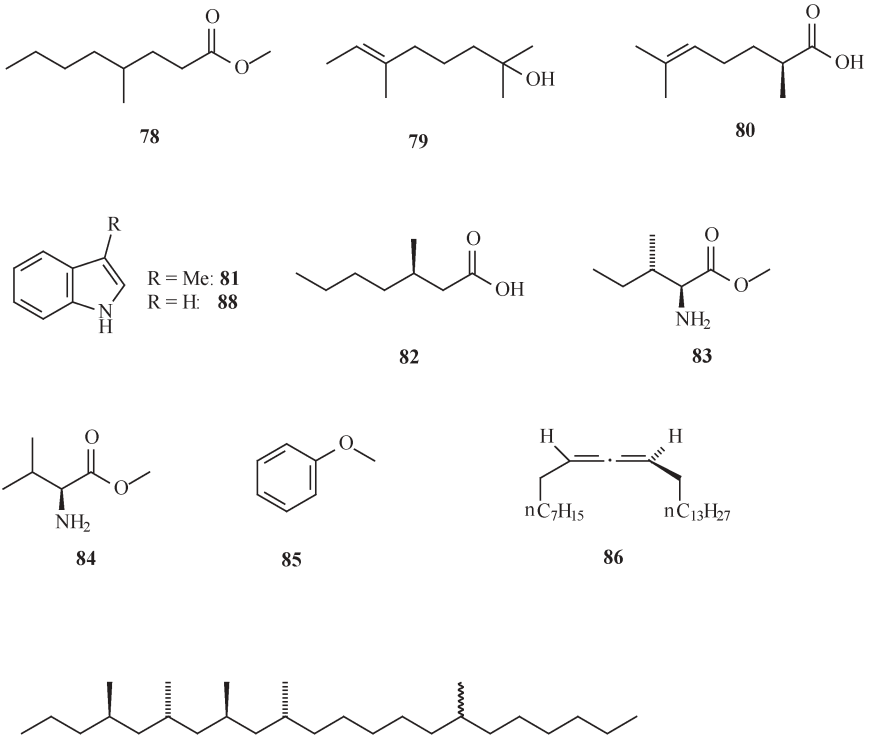
A cluster of straight chain aldehydes and methyl ketones was identified from airborne volatiles collected from females of *Hoplia equina*. While all compounds were perceived by the males' antennae, only tetradecane-2-one proved to be

attractive, and none of the other compounds enhanced its activity [156]. A mixture of (*Z*)- and (*E*)-7-tetradecen-2-one are components of the pheromone of the Oriental beetle, *Exomala orientalis* (=*Blitophertha orientalis*=*Phyllopertha orientalis*=*Anomala orientalis*) [157, 158]. The (*Z*)-isomer **73** proved to be attractive in the field. Its activity was neither synergized nor inhibited by the presence of the (*E*)-isomer. The pheromone of the soybean beetle, *Anomala rufocuprea* is methyl (*Z*)-5-tetradecenoate **74**, the biosynthesis of which may show a certain relationship to those of the γ-lactones [159].

Some scarab species are strongly attracted by plant volatiles which may optimise both host finding and/or mate finding. In the case of *Anomala rufocuprea* methyl anthranilate **75** was even more attractive to males than the female produced pheromone. In addition, it caught substantial amounts of females [160]. Interestingly, anthranilic acid has been described as the pheromone of the black chafer *Holotrichia loochooana loochooana* [161]. It should be noted that the unique pheromone of *Phyllopertha diversa* [162] 1,2,3,4-tetrahydro-1,3-dimehtylchinazolin-2,4-dione, **76**, shows the same substitution pattern at the benzene ring as anthranilic acid. *Phyllopertha diversa* displays specificity and sensitivity to so called green leaf volatiles as (*Z*)-3-hexen-1-ol, the corresponding acetate, and (*E*)-2-hexenal etc [163]. The forest cockchafer *Melolontha hippocastani* is strongly attracted to (*Z*)-3-hexen-1-ol released from damaged leaves [164] and so is the European cockchafer *Melolontha melolontha* [165]. Surprisingly, 1,4-benzoquinone **6**, a typical and widespread insect defence compound, is the female produced sex pheromone of the forest chafer [166]. The combined odour of green leaf volatiles and the quinone allows the males to discriminate sites where females feed from those with unspecific leaf damage.

The antennae of both males and females of the summer chafer *Amphimallon solstitiale* react well to green leaf volatiles [167]. Both sexes produce acetoin of high enantiomeric purity, and the corresponding 2,3-butanediols; however, females do not perceive these compounds. While (*R*)-acetoin **77** proved to be highly attractive to swarming males, neither the racemate nor the 2,3-butanediols showed a behaviour mediating capacity. The same set of small molecules was also found in other scarab beetles [168]. Males of the Melanesian rhinoceros beetle *Scapanes australis* also produce acetoin with high enantiomeric excess, along with 2-butanol as a second important component, showing an enantiomeric composition of (*R*):(*S*)=2:1. Racemic acetoin and racemic 2-butanol in a ratio of 5:90 proved to be highly attractive in the field [169].

Two related scarab species produce ethyl 4-methyloctanoate **78** (Scheme 8) as an aggregation pheromone: the African rhinoceros beetle *Oryctes monoceros* [170] and the coconut rhinoceros beetle *Oryctes rhinoceros* [171, 172]. The latter is readily attracted to the racemate. Its secretion was found to contain the free acid as well as ethyl 4-methylheptanoate [171]. Similarly to other cases, the attractiveness of ethyl 4-methyloctanoate is enhanced by host compounds, i.e. coconut wood [173]. The date palm fruit stalk borer, *Oryctes elegans*, uses 4-methyloctanoic acid as a male produced pheromone [174]. Structurally



78 79 80 82 83 84 85 86 87

Scheme 8

related compounds such as its ethyl ester **78**, the corresponding methyl ester or 4-methyloctanol and its acetate, which were found to be additionally present, did not increase the attractivity of the acid. However, addition of crushed date palm tissue dramatically increased trap catches.

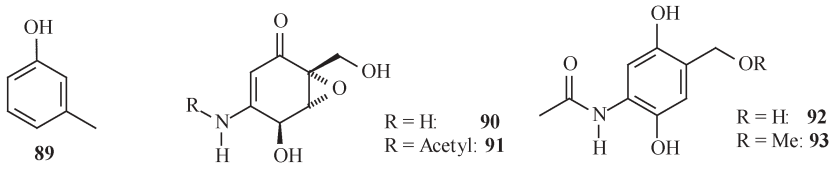
Dung beetles, *Kheper* species, use branched aliphatic compounds as semiochemicals: males of *Kheper subaeneus* produce (*E*)-2,6-dimethyl-6-octen-2-ol, (*E*)-subaeneol, **79.** The compound is active on the antennae of both males and females; (*S*)-(+)-2,6-dimethyl-5-heptenoic acid **80** is the main component of the secretion [175]. Earlier, this acid (absolute configuration not assigned) had been described as a volatile compound in a closely related species, *Kheper lamarcki*, along with hexadecanoic acid and skatole **81**. Three components of the abdominal sex-attracting secretion of male *Kheper nigroaeneus* are well perceived by males and females. Two of them could be identified to be (*R*)-(+)-3-methylheptanoic acid **82** and (the possibly tryptophane derived) skatole **81** [177]. Interspecific attraction in dung beetles has been described by Burger [178].

Females of several other species of scarab beetles use methyl esters of L-isoleucine **83** and L-valine **84** as sex pheromones [179, 180]. In *Phyllophaga elenans*, apart from **83**, the corresponding *N*-formyl- and *N*-acetyl derivatives have been

identified, however, these amides do not seem to play a role as intraspecific attractants [181]). In contrast to *Holotrichia parallela* that uses **83** as the pheromone [177], the related *Holotrichia consanguinea* and *Holotrichia reynaudi* use anisol [182, 183] **85**.

Recently, two new facets have been added to scarab chemistry. A suite of unusual $\Delta^{9,10}$-allenic hydrocarbons like **86** has been identified among the cuticular hydrocarbons from several Australian melolonthine scarab beetles [184]. Though very low-level components in the related cane beetle *Antitrogus parvulus*, the major cuticular hydrocarbons in this species proved to be oligomethyldocosanes like **87**. Only the relative configurations of these compounds could be determined [185]. Whether these interesting hydrocarbons have a function as pheromones needs to be established.

Defensive Compounds. Some species of dung beetles emit an odorous secretion when attacked by vertebrates. Representatives of the genus *Canthon* have two small glands on the posterior margin of the elytra and contain indole **88**, *m*-cresol **89** (Scheme 9), and phenol [186]. As a rule, species of this genus possess paired pygidial glands at sternite 8 which produce intensely smelling defence compounds [187, 188]. The dung beetle *Oniticellus egregius* flips onto its back, exhibits thanatosis, and releases a brown odorous fluid containing methyl salicylate **15** and 1,4-benzoquinone **6** from the lateral edges of the anterior abdominals segments [189].



Scheme 9

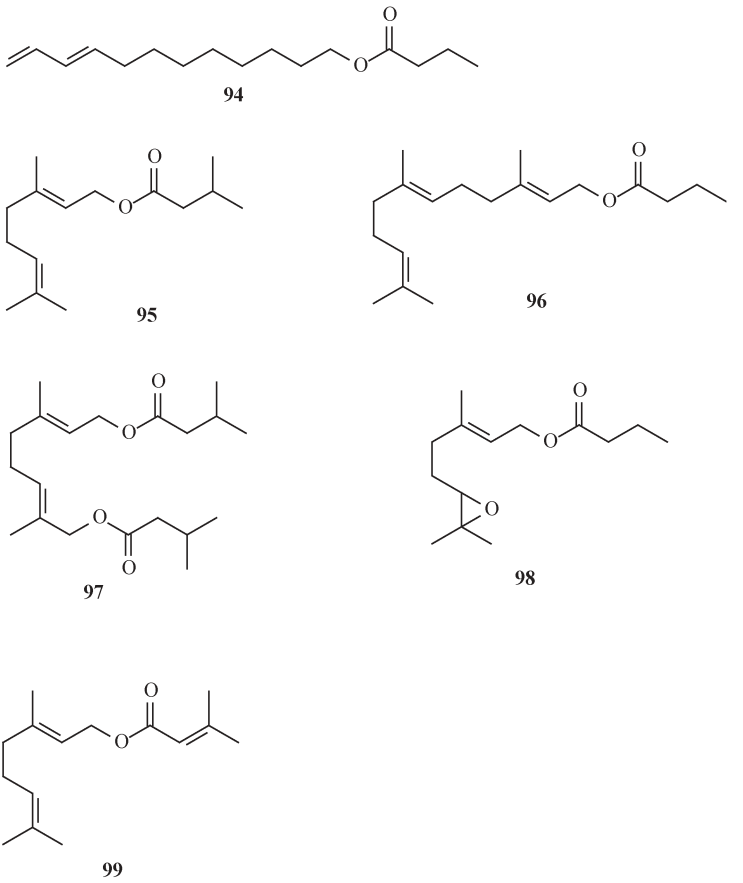
From the hind gut of *Cetonia aurata* an *Actinomyces* species was isolated which produces the new cytotoxic epoxy quinoles [190] named cetoniacytone A **90** and B **91**. In addition, the structurally related aromatic compounds 2,5-dihydroxy-4-hydroxymethylacetanilide **92** and 2,5-dihydroxy-4-methoxymethylacetanilide **93** were found in minor amounts.

15
Elateridae (Click Beetles)

Attractive Compounds. Larvae of several click beetle species (wire worms) can be serious pests in agriculture and forestry. In a few cases, sex pheromones produced in a female specific abdominal gland, have been identified.

The first biologically active compounds identified from click beetles were hexanoic acid and pentanoic acid from *Limonius canus* and *L. californicus*, respectively [191].

The structures of the female produced pheromones of the sugar cane wire worms *Melanotus sakishimensis* and *M. okinawensis* look much more like "conventional" moth pheromones: While in the latter species it simply is dodecyl acetate, *M. sakishimensis* uses a mixture of (*E*9,11)-dodecadienyl butyrate **94** (Scheme 10) and the corresponding hexanoate [192].



Scheme 10

In females of the genus *Agriotes,* several esters of acyclic terpenes have been identified as pheromone components. Typical examples are geranyl 3-methylbutyrate **95**, the first pheromone identified from an *Agriotis* species [193] or (*E*,*E*)-farnesyl butyrate **96**, which together with geranyl butyrate is the major component of the sex pheromone of *A. brevis* [194]. In *A. lineatus*, the activity

of the main component, geranyl octanoate, is strongly synergised by geranyl butyrate [194]. The main components in the bouquet of *A. obscurus* are geranyl hexanoate and geranyl octanoate [195–199].

In *Agriotes,* the biogenetic principle in the formation of unique blends of specific pheromone components seems to be based on the combination (esterification) of acyclic isoprenoid alcohols with short chain acids. Disposable variants in relevant structures are provided by the number of isoprene subunits (mono-, sesqui-, di-terpenes) and double-bond configurations at the terpene site as well as on chain length and methyl branching at the acid moieties [199]. Other features are the introduction of an additional oxygen at the terpene site forming either a second alcohol group followed by esterification as in **97** [200, 201] or an epoxide. Apart from geranyl butyrate, 6,7-epoxygeranyl butyrate (unknown stereochemistry) **98** is the second major component in the secretion of the abdominal gland of females of *A. sputator* [201]. The acids, representing substructures of terpene esters may be unsaturated as in geranyl 3-methyl-3-butenoate or geranyl 3-methyl-2-butenoate **99**, minor components in the abdominal secretion of *A. litigiosus*. The latter acids may represent *hemi*-terpenes. Mechanisms accounting for species specificity need to be clarified in some species.

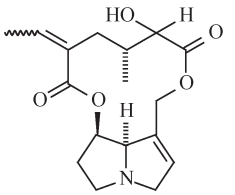
Defensive Compounds. *Agrypnus (=Lacon=Adelocera) murinus* possess paired abdominal defensive glands which are everted on molestation during thanatosis [8]. The four stink gland constituents are indole **88** dimethylsulfide, dimethyldisulfide, dimethyltrisulfide, and dimethyltetrasulfide.

16
Lampyridae (Lightningbugs or Fireflies)

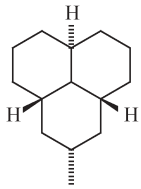
Defensive Compounds. The developmental stages of fireflies are poisonous due to the presence of steroidal pyrones called lucibufagins. Recently it became evident that exotic reptiles and amphibians from habitats without the poisonous fireflies, e.g. the Australian lizard *Pogona*, are killed immediately if they ingest just one firefly.

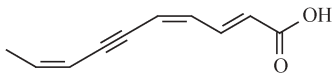
17
Cantharidae (Soldier Beetles)

Defensive Compounds. The aposematically coloured *Chauliognathus fallax* which feed on *Senecio brasiliensis* (Asteraceae) sequester the four pyrrolizidine alkaloids senecionine (**100** main compound), integerrimine (**101** main compound), retrorsine **102**, and usaramine **103** [203] (Scheme 11). Other *Chauliognathus*-species may contain either precoccinelline **104** and related alkaloids (*C. pulchelus*) or *Z*-dihydromatricaria acid **105** (*C. pennsylvanicus*).



(*Z*,*R*): **100**
(*E*,*R*): **101**
(*Z*,*S*): **102**
(*E*,*S*): **103**





Scheme 11 **104** **105**

18
Dermestidae (Skin Beetles)

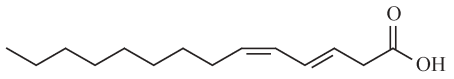
Attractive Compounds. Pheromones of dermestid beetles were among the first ones identified from insects. Almost all have been described as "one-component-systems", and re-investigations employing refined techniques, especially GC-EAD and sensitive GC-MS, may reveal the presence of additional and important compounds, which may lead to improved activity of synthetic lures, and under natural conditions may account for species specificity etc.

The female produced sex pheromone of the black carpet beetle, *Attagenus unicolor* (formerly called *A. megatoma* or *A. piceus*) has been identified as early as 1967 to be (3*E*,5Z)-3,5-tetradecadienoic acid, megatomoic acid **106** [204, 205] (Scheme 12). The (3*Z*)-isomer of megatomoic acid was found to be the major male attracting component in the female produced pheromone of A. *brunneus* (formerly *A. elongatulus*) [206].

Virgin females of the furniture carpet beetle, *Anthrenus flavipes*, produce (*Z*)-3-decenoic acid as a sex pheromone **107** [207]. In contrast, the female-produced sex pheromone of the varied carpet beetle *Anthrenus verbasci* is a two component mixture of (*Z*)-5-undecenoic acid **108** and its (E)-isomer [208]. Recent investigations showed the presence of additional electrophysiologically active components, however, no behaviour tests have been carried out [209].

The sex pheromone of the Guernsey carpet beetle, *Anthrenus sarnicus*, contains 1-decanol and its butyrate **109** in almost equal amounts [210].

Fatty acid esters also play a role in the communication system of the hide beetle, *Dermestes maculatus*. In a sex specific gland, situated at the ventral side of the fourth sternite, males produce a bouquet of isopropyl esters of fatty acids showing 12, 14, 16, and 18 carbon atoms [211]. Apart from the esters of the four saturated acids and isopropyl (*Z*)-hexadec-9-enoate as well as isopropyl oleate,



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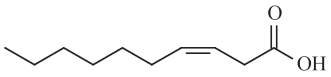

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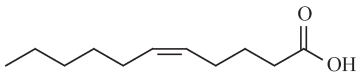

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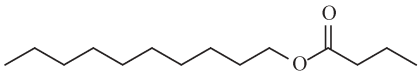
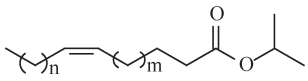
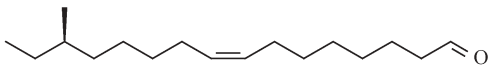


109



m = 1, 3, 5
n = 5, 3, 1

110



111

Scheme 12

esters of three dodecenoic acids **110** and three tetradecenoic acids, each showing (*Z*)-configured double bonds at positions 5, 7, and 9, make up a complex mixture [212, 213]. The unsaturated esters, especially the lower boiling ones, evoked high olfactory receptor potentials in *D. maculatus* but also in the related species *D. lardarius* and *D. ater*. Behaviour studies led to the conclusion that the gland secretion represents a male recognition signal releasing aggregation behaviour. The mixture of synthetic esters was found to be considerably less active than the natural secretion and, in fact, a reinvestigation revealed a much more complex composition showing the presence of several doubly unsaturated esters [214].

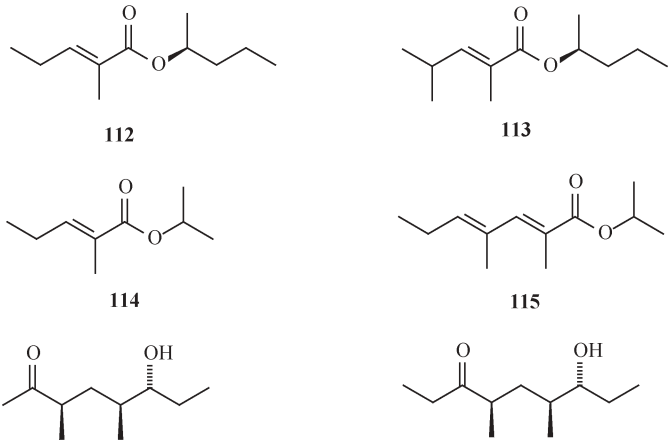
Structure elucidation of the female-produced sex pheromones of *Trogoderma* spp. has a rather confused history. Extracts of females of *T. inclusum* were shown to contain (*Z*)-14-methyl-8-hexadecenol and the methyl ester of the respective carboxylic acid [215]. The corresponding compounds showing (*E*)-configuration were shown to be present and behaviourally active in *T. inclusum* [216]. Finally, investigations of head space collections, obtained with live females, revealed the presence of 14-methyl-8-hexadecenal, an aldehyde

which was not detected in extracts of females. In *T. inclusum* and *T. variabile* this proved to be the (*Z*)-isomer **111** while in *T. glabrum* it was shown to be the (*E*)-isomer, and in *T. granarium* a ca. 9:1 (*Z*):(*E*)-mixture was found [217–220]. Because of its constant occurrence as a pheromone in *Trogoderma* species, 14-methyl-8-hexadecenal was termed trogodermal [221]. Determination of the absolute configuration of trogodermal was again accompanied by some confusion. The available amounts of naturally produced trogodermal were too small to determine its rotation value. Enantiomeric separation by enantio-selective gas chromatography was impossible. Even today trogodermal cannot be resolved on chiral columns as the stereogenic centre appears too far away from the functional group (ozonolysis and enantiomeric separation of the produced 6-methyloctanal may, however, be worth a trial). In contrast to the aldehyde, the alcohol could be isolated in sufficient amounts to measure its rotation value. Finally, Mori carried out unambiguous syntheses of both enantiomers of (*Z*)-14-methyl-8-hexadecenal via the corresponding alcohols [222]. Comparison of rotation values of the synthetic material with that of naturally occurring (*Z*)-14-methyl-8-hexadecenol showed that the beetle produced compound and the corresponding pheromone aldehyde keep the (*R*)-configuration in *T. granarium*. This was supported in bioassays where the (*S*)-enantiomer of trogodermal elicited a response at dosages 100–1000 times lower than the (*R*)-enantiomer [223]. Corresponding results were found during tests with *T. glabrum*, *T. inclusum*, and *T. variabile* [224]. During recent years, only very few syntheses of dermestid pheromones have been reported [225].

19
Bostrychidae (Powder-Post Beetles)

Attractive Compounds. Pheromones of three Bostrychid species have been identified. Males of the lesser grain borer, *Rhizopertha dominica*, produce (*S*)-1-methylbutyl (*E*)-2-methyl-2-pentenoate (dominicalure 1) **112** and (*S*)-1-methylbutyl (*E*)-2,4-dimethyl-2-pentenoate (dominicalure 2) **113** (Scheme 13). Both compounds induce aggregation of males and females; however, the mixture does not show synergistic effects [226]. Pheromone release and inter-male variation as well as effects of different hosts and the presence of conspecific females on pheromone production by males of *Rhizopertha* have been recently investigated [227, 228].

Similar to *R. dominica*, a two-component male produced pheromone accounts for the aggregation of both sexes of another Bostrychid, *Prostephanus truncatus*. 1-Methylethyl (*E*)-2-methyl-2-pentenoate (trunc-call 1, T1) **114** shows the same acid moiety as dominicalure 1 [229]. The second (slightly more active [230]) component proved to be 1-methylethyl (2*E*,4*E*)-2,4-dimethyl-2,4-heptadienoate (trunc-call 2, T2) **115**. In this species, synergistic effects of the two compounds have been reported [229]. The effect of age and sex on the response of walking *P. truncatus* to its pheromone has been investigated [230]. Inter-



112 **113**

114 **115**

116 **117**

Scheme 13

male variation in pheromone release [231], the effect of age and sex [232], as well as other factors influencing response to the aggregation pheromone have been described [233]. Obviously, apart from attracting both sexes, the male produced signal also acts as a sex pheromone and plays a role in sexual selection [234]. In contrast, males of a predator of grain borers, *Teretriosoma nigrescens*, while using the *Prostephanus* pheromone as a kairomone, were slightly more responsive than females [230].

The Bostrychid *Dinoderus bifoveolatus* is a serious pest on cassava, the dried roots of manioc. Again, this species shows male specific volatiles, two of which were found to produce intense signals in the antennae of conspecific males and females. The minor component proved to be (3*R*,5*S*,6*R*)-3,5-dimethyl-6-hydroxyoctan-2-one **116**, while the major one was shown to be its homologue, (4*R*,6*S*,7*R*)-4,6-dimethyl-7-hydroxy-nonan-3-one **117** [235].

As already pointed out by Chuman et al. [38] structures like **112, 114, 115,** and **117** are very likely biosynthesised from propanoate units, see Fig. 2. Three propanoate units (keeping one oxygen) would yield 2,4-dimethyl-5-hydroxyheptanoate, while a propanoate-stopper (and loss of carbon dioxide) would complete the formation of the ethylketone **117**. Correspondingly, an acetate-stopper would give rise to the formation of the methylketone **116.** In the biosynthesis of **113**, the starting unit contributing four carbon atoms and producing iso-branching, may well originate from an amino acid, e.g. valine.

As depicted in Fig. 6, syntheses of enantiomerically pure **116** and **117** have been carried out [236]. Lipase AK-catalysed asymmetric acetylation of *meso*-2,4-dimethyl-1,5-pentanediol **A** yielded (2*R*,4*S*)-5-acetoxy-2,4-dimethylpentanol **B**. Protection of the free hydroxy group as the *tert*-butyldimethylsilyl (TBS) ether, saponification of the acetate, and oxidation furnished the aldehyde **C.** Reaction of **C** with ethylmagnesium bromide gave a diastereomeric mixture of the corresponding secondary alcohols which could be resolved by asym-

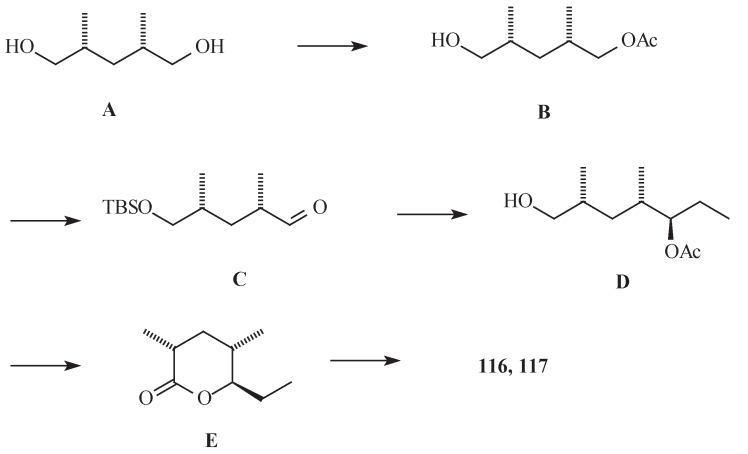


Fig. 6 Reaction scheme for the synthesis of pheromone components of male *Dinoderus bifoveolatus* **116** and **117**

metric acetylation with vinyl acetate and lipase PS-D (Amano). Chromatographic separation followed by deprotection at the primary alcohol side yielded **D.** After saponification of **D** to the corresponding diol, oxidation of the primary hydroxy group with tetra(*n*-propyl)ammonium perruthenate produced lactone **E.** Reaction of **E** with either methylmagnesium bromide or ethylmagnesium bromide gave the target compounds **116** or **117**, respectively.

First bioassays with synthetic compounds were highly promising [235]. Interestingly, the major component **117** is a stereoisomer of serricornin **118**, the sex pheromone of the cigarette beetle, *Lasioderma serricorne* (see below). The pheromone of this anobiid beetle shows, however, (4*S*,6*S*,7*S*)-configuration [38, 237, 238]. Whether such differences in the stereochemistry of pheromones may have played a role in species discrimination during earlier times when *Dinoderus* and *Lasioderma* may have lived in the same habitat, awaits further investigations (see also the above mentioned mutual agonistic-antagonistic activities of pheromones of the scarab beetles *Popillia japonica* and *Anomala osakana* [146]). The structural similarities between the Bostrychid pheromones and those of the Anobiidae (next section) may serve as a further proof for the close relationship between the two families.

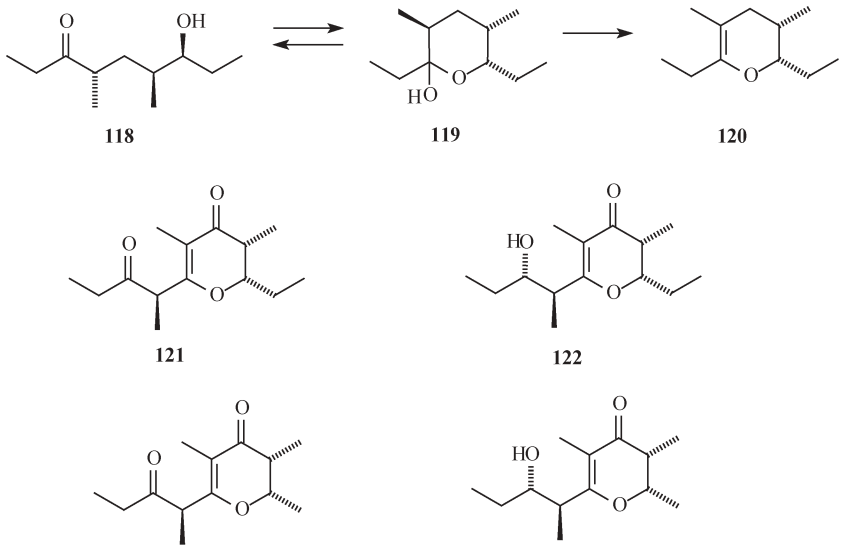
20
Anobiidae (Cigarette Beetles, Drugstore Beetles)

Attractive Compounds. Only little is known about the systems of chemical communication in anobiid beetles. Investigations have been mainly concerned with two economically important species, the cigarette beetle, *Lasioderma serricorne,* and the drugstore beetle, *Stegobium paniceum*.

(4*S*,6*S*,7*S*)-7-Hydroxy-4,6-dimethylnonan-3-one **118** (serricornin) is the female produced sex pheromone of the cigarette beetle [38, 237, 238]. The pheromone is produced in a female specific gland located at the second abdominal segment [239]. Serricornin forms a 1:3 equilibrium with its cyclic hemiacetal **119** [240, 241]. Its attractivity is strongly inhibited by the non-natural (4*S*,6*S*,7*R*)-diastereomer [242]. The dihydropyrane **120** representing the dehydrated **119** which had been described as highly attractive [243], proved to be biologically inactive after careful reinvestigation [244].

Additional compounds found in the pheromone gland of female cigarette beetles are (2*S*,3*R*,1′*R*)-2,3-dihyro-2-ethyl-3,5-dimethyl-6-(1′-methyl-2′-oxobutyl)-4*H*-pyran-4-one **121** (β-serricorone), its (1′*S*)-epimer (α-serricorone), and its reduction product, serricorole, **122** which shows (1′*S*,2′*S*)-configuration [245–247]. These compounds showed only weak attractivity [245], however, they obviously act as oviposition deterrents [248, 249].

The interesting structures of the *Lasioderma* compounds have been the subject of many syntheses, serving as models for stereocontrolled approaches. More recent syntheses of serricornin form two groups: those using chiral auxiliaries (oxazolidinone [250], boronic esters [251], and SAMP/RAMP [252]) and those involving chemoenzymatic steps ([253–255]).



Scheme 14

The pheromone produced by females of the drugstore beetle was the first to be identified in an anobiid beetle: 2,3-dihydro-2,3,5-trimethyl-6-(1′-methyl-2′-oxobutyl)-4*H*-pyran-4-one (stegobinone) [256] which by independent synthesis [257] was shown to keep (2*S*,3*R*,1′*R*)-configuration **123.** A second compound

which, however, seems to be of minor importance in the communication system of the drugstore beetle was found to be (2*S*,3*R*,1′*S*,2′*S*)-2,3-dihydro-2,3,5-trimethyl-6-(2′-hydroxy-1′-methylbutyl)-4*H*-pyran-4-one **124** (stegobiol) [258, 259]. A non-natural stereoisomer, 1′-*epi*-stegobinone showing (2*S*,3*R*,1′*S*)-configuration strongly inhibits response [260]. The compound is easily formed from stegobinone upon enolization. Crystalline (2*S*,3*R*,1′*R*)-stegobinone was synthesized by careful oxidation of crystalline stegobiol, and its absolute configuration was confirmed by X-ray analysis [261, 262].

The furniture beetle *Anobium punctatum*, a death-watch beetle, seems to use the same communication system as the drugstore beetle [263, 264].

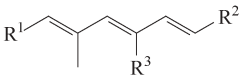
Comparison of the structures of the *Lasioderma* compounds **121** and **122** with the *Stegobium* compounds **123** or **124** reveals strong similarities even with respect to the stereochemistry. The biosyntheses may be very similar involving a C3-unit as the stereotypic building block. As already mentioned above (see introduction and Fig. 2) the skeletons of **123** and **124** would be formed when the methylmalonate (or propanoate) unit terminating the chain elongation of **121** and **122** would be replaced by malonate (or acetate), respectively.

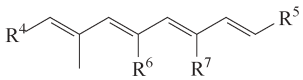
21
Cleridae (Checkered Beetles)

Defensive Compounds. Clerid beetles such as *Trichodes apiarius* were found to contain considerable amounts of cantharidin **48**, accompanied by small to minute amounts of palasonin **49** [122, 265]. Previously, the latter has been known only from seeds and fruits of the Indian shrub, *Butea frondosa* (Leguminaceae). It is suggested that these predatory beetles feed on cantharidin producing oedemerid and meloid beetles, see below. Several clerid species are canthariphilous [266, 267].

22
Nitidulidae (Sap Beetles)

Attractive Compounds. The male-produced pheromones of sap beetles, known so far, show the rather stereotypic structures **125–147** (Scheme 15): methyl- and ethyl-branched hydrocarbons with three or four (*E*)-configured conjugated double bonds [4]. Up to now, 23 compounds could be identified, forming species specific mixtures. Major components in the bouquets are (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene, **128**, in *Carpophilus davidsoni* [268] as well as in *C. freemani* [269], (2*E*,4*E*,6*E*)-4,6-dimethyl-2,4,6-nonatriene, **129**, in *C. truncatus* [270], (3*E*,5*E*,7*E*)-5-ethyl-methyl-3,5,7-undecatetraene, **132**, in *C. mutillatus* [271], (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene, **134**, in *C. hemipterus* [272] as well as *C. brachypterus* [273], (2*E*,4*E*,6*E*,8*E*)-3,5,7-tri-





	R^1	R^2	R^3
125	Me	Me	Me
126	Me	Me	Et
127	Me	Et	Me
128	Me	Et	Et
129	Et	Me	Me
130	Et	Et	Me
131	Et	Et	Et
132	Pr	Et	Et

	R^4	R^5	R^6	R^7
133	Me	Me	Me	Me
134	Me	Et	Me	Me
135	Me	Me	Et	Me
136	Me	Me	Me	Et
137	Et	Me	Me	Me
138	Me	Et	Et	Me
139	Me	Et	Me	Et
140	Me	Me	Et	Et
141	Et	Me	Et	Me
142	Et	Me	Me	Et
143	Et	Et	Me	Me
144	Me	Et	Et	Et
145	Et	Et	Me	Et
146	Et	Et	Et	Et
147	Pr	Et	Et	Et

Scheme 15

methyl-2,4,6,8-undecatetraene, **134**, in *C. obsoletus* [274], (*E*2,*E*4,*E*6,*E*8)-7-ethyl-3,5-dimehtylundecatetraene, **139**, in *C. lugubris* [275], and (*E*3,*E*5,*E*7,*E*9)-6,8-diethyl-4-methyldodeca-3,5,7,9-tetraene **146** in *C. antiquus* [276] as well as in *C. dimidiatus* [277]. The major components are accompanied by several homologues as minor components, and cross-attraction between species has been frequently observed [278]. Response of sap beetles to their natural pheromones is strongly inhibited by the (*Z*)-configured analogues [279, 280]. In contrast, pheromones are synergized by food and host volatiles [281, 282].

The biosyntheses of the sap beetle pheromones has been carefully investigated by Bartelt and his co-workers [47, 48]. The typical methyl-branching of the compounds originates from propanoate (or methylmalonate) units that form the principal structures (see Fig. 2). Replacement of propanoate by butyrate during chain elongation yields ethyl-branching. In about half of the compounds (**125–128**, **133–136**, **138–140**, and **144**) the structures suggest acetate to act as a starter while in **133** and **147** the starter should be butyrate. The chains

are built up by sequences of Claisen-type condensations of subunits, while in the final step decarboxylation provides the hydrocarbon structure.

Syntheses follow a kind of bio-mimetic approach [283, 284] in building up the chain during a sequence of Wittig-type reactions or Horner-Wadsworth-Emmons olefination, adding two carbons to the chain at a time with either methyl- or ethyl-branches. As the final products need to be highly pure (*E*)-stereoisomers, reaction steps and purification need to be carefully controlled.

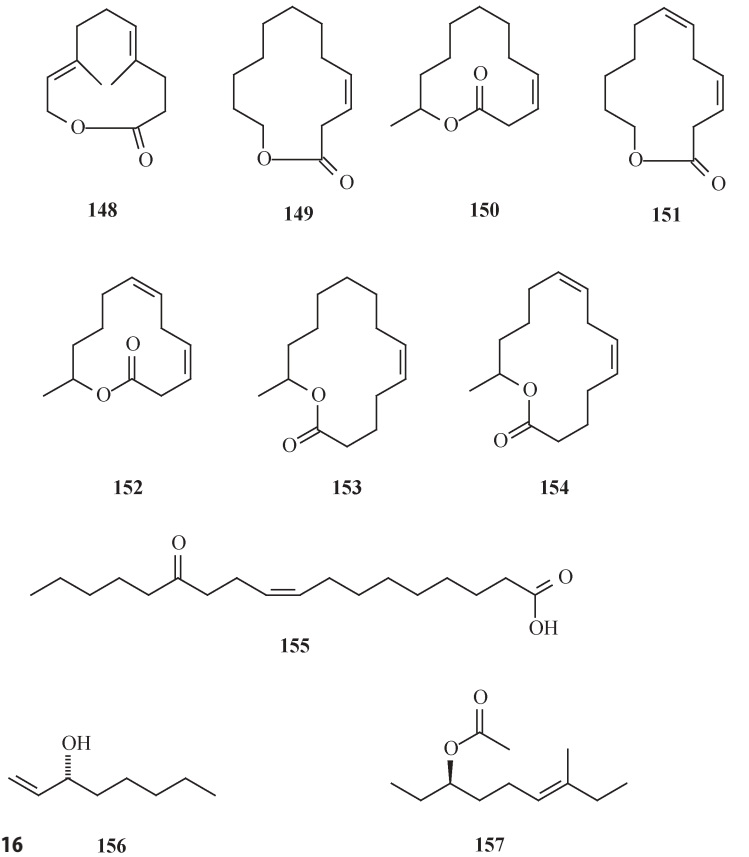
23
Cucujidae, Silvanidae/Laemophloeidae (Flat Bark Beetles, Grain Beetles)

Attractive Compounds. Macrocyclic lactones are typical components of the male produced aggregation pheromones of cucujid beetles [285]. Males and females are attracted to species-specific mixtures of these compounds, which have been given the trivial name cucujolides [286]. The following compounds have been identified: (4*E*,8*E*)-4,8-dimethyl-4,8-decadien-10-olide, cucujolide I **148** (formerly termed ferrulactone I), (*Z*)-3-dodecen-12-olide, cucujolide VIII **149** (Scheme 16), (*Z*)-3-dodecen-11-olide, cucujolide II **150** (formerly termed ferrulactone II), (3*Z*,6*Z*)-3,6-dodecadien-12-olide, cucujolide IX **151**, (3Z,6Z)-3,6-dodecadien-11-olide, cucujolide IV **152**, (*Z*)-5-tetradecen-13-olide, cucujolide III **153**, and (5*Z*,8*Z*)-5,8-tetradecadien-13-olide, cucujolide V **154**.

The biosynthesis of cucujolides has been investigated by Vanderwel et al. [287, 288]. With the exception of **148**, which shows a branched carbon skeleton, the compounds are biosynthesised from unsaturated fatty acids like oleic acid or linoleic acid. Chain-shortening and oxidation at the ω- or ω-1 position will furnish monounsaturated or doubly unsaturated lactones after ring closure. In the case of ω-1 oxidation, ring closure proceeds with high enantioselectivity. As shown by isotope labelling, **148** is of isoprenoid origin: oxidative cleavage of the last double bond of (*E*,*E*)-farnesol, followed by ring closure, yields cucujolide I.

The structures of (*Z*)-13-oxooctadec-9-enoic aid **155** and its bis-homologue (*Z*)-15-oxoicos-11-enoic acid (and their – doubly unsaturated? – precursors) are certainly related to the cucujolides, as corresponding sequences of chain shortening will provide unsaturated C12- or C14-acids. The two oxygenated fatty acids were identified in wheat flour infested by *Oryzaephilus surinamensis* but found to be absent in non-infested material. They seem to act as arrestants [289]. Similarly, 3-ketosteroids, cholestan-3-one, ergostan-3-one, and stigmastan-3-one were identified in wheat flour infested by *O. surinamensis* and described to be arrestant [290].

As already mentioned, the cucujolides form species specific mixtures of at least two compounds per species. Depending on the species, some of the compounds are active per se while others act as synergists. Species specificity also includes enantiomeric composition. While cucujolides II and III show (*S*)-configuration in *C. ferrugineus* and *C. pusillus*, respectively, cucujolies II, IV, and V



Scheme 16

show (*R*)-configuration in *O. mercator* and *O. surinamensis*. The pheromone of *C. turcicus* keeps a position in between, as cucujolide V shows an enantiomeric ratio of (*R*):(*S*)=85:15 synergized by cucujolide III of (*R*):(*S*)=35:65. Pure enantiomers of cucujolides V and III proved to be inactive in this species [286, 291]. For details see [7, 285].

The key step in Fürstner's elegant synthesis of racemic **153** furnishing a *Z*:*E*=7:3 mixture, used an intramolecular metathesis reaction of the ester **A** [292]. Employing optically active 9-decene-2-ol will certainly produce the desired enantiomer (Fig. 7).

A synthesis of **149**, cucujolide VIII, proceeded via the *tert*-butyldimethylsilyl-(TBS)-ether of methyl (*E*)-12-hydroxydodec-4-enoate **B** [293] (Fig. 7). Deprotonation in α-position and reaction with di(4-methoxyphenyl)diselenide furnished **C**. This was transformed to the macrolide **E** after saponification of the ester moiety, deprotection of the hydroxy group, and Mitsunobu lactonization. Alternatively, the unsaturated lactone **F** was synthesized from **B** following a sequence similar to that from **C** to **D**. Oxidative elimination of the arylseleno group

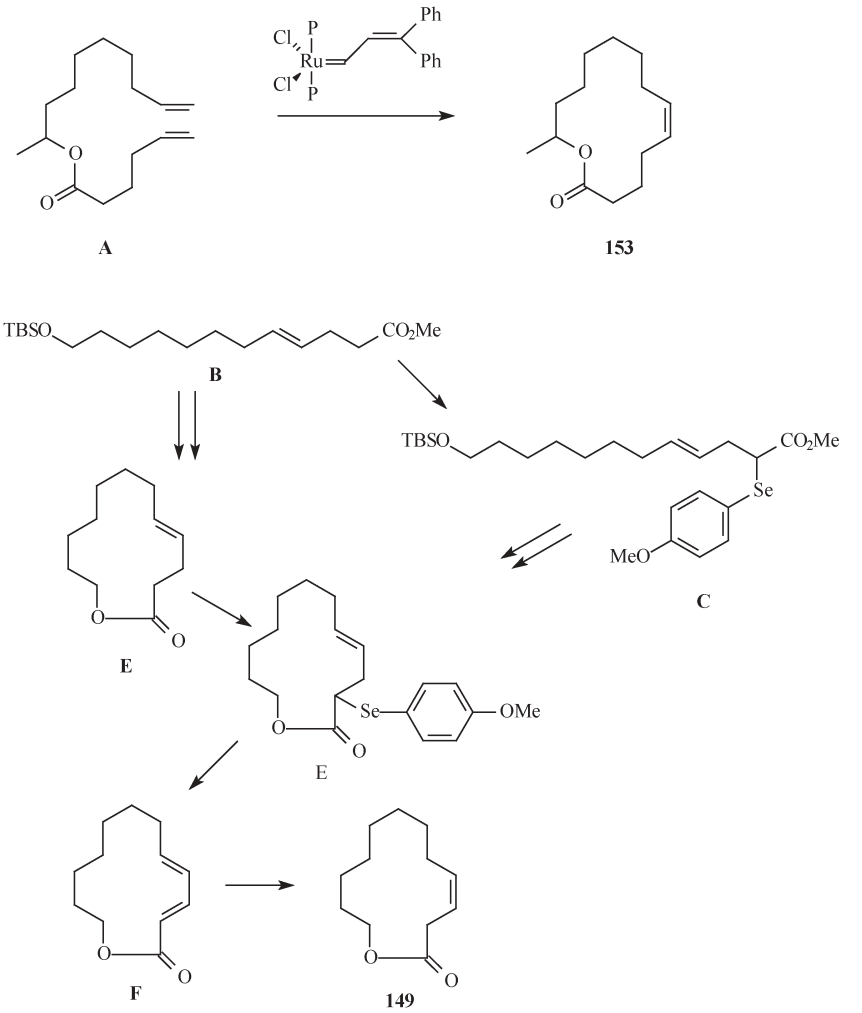


Fig. 7 Reaction scheme for the syntheses of cucujolide III **153** and cucujolide VIII **149**

in **D** gave (2,4)-dodecadiene-12-olide **G.** Subsequently, 1,4-*cis*-hydrogenation over (η 6-naphthalene) tricarbonylchromium afforded the target compound, **149.** Similarly, organoselenium chemistry and Mitsunobu lactonization have been applied in the synthesis of racemic **149** from commercially available methyl 10-undecenoate [294].

In addition to the cucujolides, (*R*)-1-octen-3-ol **156** has been described as a pheromone compound in *O. mercator* and *O. surinamensis* [295]. The alcohol is produced by both sexes at low population densities, and during a later stage of adulthood. It is reported to be attractive at low concentrations (supporting the attractivity of the cucujolies) but strongly repellent at high dosages. The

same alcohol has been reported as an aggregation pheromone, produced by both sexes of the foreign grain beetle *Ahasverus advenes* [296]. At this stage it should be noted that 1-octen-3-ol is a particularly wide-spread natural volatile, mostly associated with fungal activities.

The male-produced aggregation pheromone of the square-necked grain beetle, *Cathartus quadricollis* has been identified to be (3*R*,6*E*)-3-acetoxy-7-methylnon-6-ene **157** [297]. The compound, termed quadrilure, is attractive to both sexes, however, females are more sensitive at low concentrations. The (*S*)-enantiomer is biologically inactive.

Syntheses of both enantiomers of **157** are depicted in Fig. 8. Both approaches involve enzymatically controlled reactions during asymmetric syntheses.

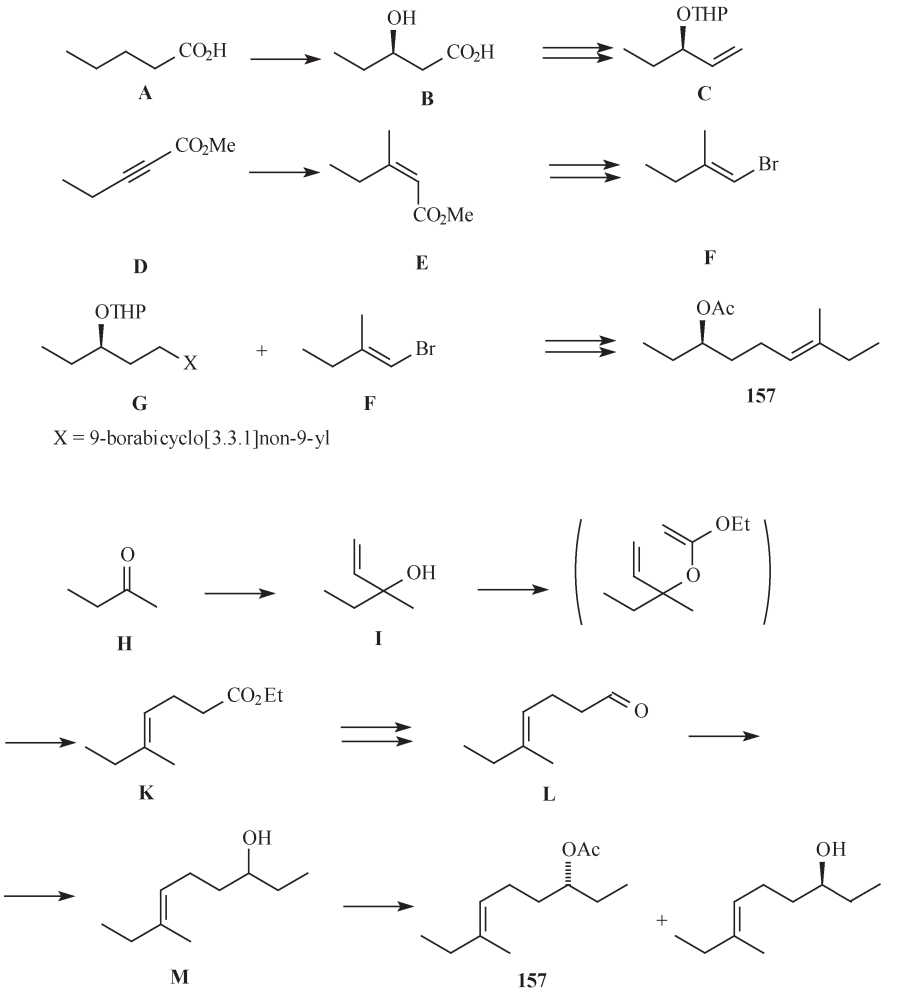


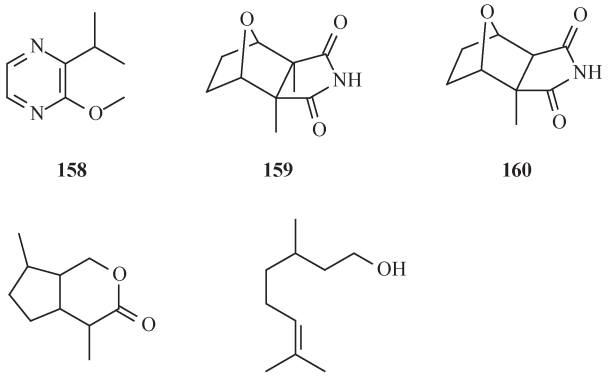
Fig. 8 Reaction scheme for the syntheses of optically active quadrilure **157**

Mori started with the early introduction of the chiral centre [298] in using β-oxidation of pentanoic acid **A** by the yeast, *Candida rugosa*, IFO 0750 [299]. The obtained (*R*)-3-hydroxypentanoic acid **B** was transformed into **C** in a few conventional steps. The second building block was prepared from methyl 2-pentynoate **D**: conjugate addition of lithium dimethyl cuprate yielded **E**, which was further converted into the *trans*-configured vinyl bromide **F**. Hydroboration of **C** yielded **G** which upon Suzuki's palladium catalysed cross-coupling with **F** furnished **157** after treatment of the reaction product with hydrochloric acid followed by chromatographic purification. The synthesis of *ent*-**157** used (*S*)-3-hydroxypentanoic acid.

In contrast to Mori's synthesis, Pawar and Chattapadhyay used enzymatically controlled enantiomeric separation as the final step [300]. Butanone **H** was converted into 3-methylpent-1-en-3-ol **I**. Reaction with trimethyl orthoacetate and subsequent Claisen-orthoester rearrangement yielded ethyl (*E*)-5-methylhept-4-enoate **K**. Transformation of **K** into the aldehyde **L**, followed by reaction with ethylmagnesium bromide furnished racemic (*E*)-7-methylnon-6-ene-3-ol **M**. Its enzyme-catalysed enantioselective transesterification using vinylacetate and lipase from *Penicillium* or *Pseudomonas* directly afforded **157**, while its enantiomer was obtained from the separated alcohol by standard acetylation.

24
Coccinellidae (Ladybird Beetles)

Attractive Compounds. While the defence chemistry of ladybird beetles has been extensively investigated, little is known about intraspecific communication. The role of chemical and behavioural cues has been described in mate recognition in *Adalia bipunctata*. Cuticular hydrocarbons, especially 7- and 9-methyltricosane seem to play an important role [301]. In *Coccinella septempunctata*, 2-isopropyl-3-methoxypyrazine **158** (see Scheme 17) accounting for the dis-



158 159 160

161 162

Scheme 17

tinctive odour of the secretion that these beetles release after molestation, was found to act as an aggregation pheromone of adult males and females [302]. The compound had previously been identified from several butterfly species as well as from coccinellids and has been described as alerting odour or warning signal to carnivores, announcing potent defence chemistry [303]. In some coccidophagous species, larvae produce chemical signals that prohibit ovipositon by adult conspecifics [304, 305]. In *Adalia bipunctata* this pheromone seems to consist of a mixture of hydrocarbons with *n*-pentacosane as the major component [305].

Defensive Compounds. The defensive chemistry of ladybird beetles was treated in the chapter by Laurent et al. in this volume.

25
Oedemeridae (False Blister Beetles)

Defensive Compounds. All developmental stages of oedemerid beetles contain and produce cantharidin as a defensive substance. The total amount of the terpenoid anhydride increases in successive instars [306]. Moreover, by using deuterium-labelled cantharidin it was found that males of *Oedemera femorata* transfer no or only very small amounts of cantharidin **48** to females during copulation. False blister beetles cause a severe dermatitis, i.e. blisters with burning and itching sensation a few hours after contact with oedemerid haemolymph [307].

26
Pyrochroidae (Fire-coloured Beetles)

Almost 30% of the world's pyrochroid genera are canthariphilous which indicates that most of these species may gain considerable amounts of cantharidin **48** from exogenous sources [121]. In the European genus *Schizotus* [306] and the North American genus *Neopyrochroa* [308, 309] an intersexual transfer of cantharidin during copulation from male to females was shown. In *Schizotus* the transfer was followed up by isotope techniques. Analyses of eggs and first instar larvae showed that a paternal allocation of cantharidin to developmental stages exists. In addition, males possess special head glands where cantharidin is excreted. During courtship, females test cantharidin titres of individual males and accept only those which contain elevated amounts of this nuptial gift [306, 308, 309].

27
Meloidae (Blister Beetles)

Defensive Compounds. Apart from cantharidin **48** and palasonin **49** the corresponding non-toxic imides cantharidinimide **159** and palasoninimide **160** could be identified in various bodyparts of the meloid beetle, *Hycleus lunata* [310, 311].

While the Indian shrub *Butea frondosa*, contains (*S*)-(–)-palasonin of high enantiomeric purity, palasonin from *Hycleus lunata* shows *a* low ee with the (*R*)-(+)-enantiomer (20–50 *ee*) prevailing. Despite this difference between the insect-derived and the plant-produced product, an uptake of palasonin from hitherto unknown plant sources in the environment of *Hycleus* appears to be highly unlikely, however, palasonin may be produced by oxidative demethylation of cantharidin [122].

The cantharidin titres of male and female specimens of *Epicauta occidentalis*, dead and live beetles as well as specimens stored under different conditions, were measured in detail [312].

Several predation tests especially with spiders and blister beetles [121, 313, 314], show that spiders exhibit a wide range of sensitivities to meloid beetles as prey. In the racoon *Procyon lotor* it was shown that they quickly form an aversion to blister beetle prey, which is induced by cantharidin [314].

28
Anthicidae (Antlike Flower Beetles)

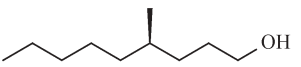
Defensive Compounds. Just as many male meloid beetles, both sexes of many anthicids possess paired mesothoracic gland reservoirs which open ventrally through an unpaired mesothoracic pore [315]. The reservoir surface is covered by secretory glands. The secretion has been shown to deter ants of the genera *Lasius* and *Myrmica*, and in addition, it shows a topical irritancy. Chemical constituents of the secretion were identified in the genera *Formicomus* and *Microhoria* and are represented by iridoids such as iridodial **17**, dolidodial **65**, iridomyrmecin **161**, dihydronepetalactone **67**, and actinidine **60**. Apart from citronellol **162**, citronellal, and isopropyl hexadecanoate the mesothoracic secretions contain alkanes ranging from tridecane to nonadecane (main constituents: pentadecane and heptadecane), 1-alkanols from 1-undecanol to 1-pentadecanol and 1-alkenes from 1-tridecene to 1-heptadecene.

Many anthicid species are known to be cantharipholous [121]. After take up, males store the toxin in the accessory glands and transfer it as a kind of nuptial gift to the females. Many male anthicid species are characterized by elytral exocrine glands which serve for excretion of cantharidin depending on the cantharidin titre. Similar to Pyrochroidae (see there) females test the cantharidin load of males before copulation and select those males which previously were able to incorporate this precious defensive compound from exogenous sources.

It has been stated that the biologically active gland secretion protects the adults whereas the haemolymph toxin which is transferred to females may serve for protection of both larvae and eggs.

29
Tenebrionidae (Darkling Beetles, Flower Beetles)

Attractive Compounds. The female-produced sex pheromone of the yellow mealworm beetle, *Tenebrio molitor*, is (*R*)-4-methyl-1-nonanol [316] **163** (Scheme 18). Careful investigations on the biosynthesis of this compound [317] revealed that it is produced through a modification of normal fatty acid biosynthesis (Fig. 1, Fig. 2): propanoate serves as the starter, while formal chain elongation with acetate, propanoate, and acetate (accompanied by removal of the oxygens) produces 4-methylnonanoate which yields the pheromone alcohol after reduction. The structures and role of proteins that are present in the hemolymph or secreted by the tubular accessory glands of *T. molitor*, and that may carry lipophilic chemical messengers (like pheromones) are under investigation [318, 319].



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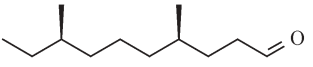

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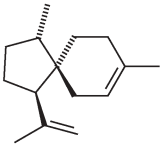

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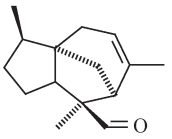

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Scheme 18

The male-produced sex pheromone of the red flour beetle, *Tribolium castaneum*, has been identified to be (4*R*,8*R*)-4,8-dimethyldecanal **164** (tribolure) [320, 321]. During bioassays, a mixture of the (4*R*,8*R*)- and (4*R*,8*S*)-stereoisomers proved to be more active than the pure (4*R*,8*R*)-enantiomer [322]. The exact enantiomeric composition of the natural product remains as yet unknown. 4,8-Dimethyldecanal was found in other *Tribolium* species, too [323]. Factors affecting the pheromone production in *T. castaneum* have been described by Hussain et al. [324].

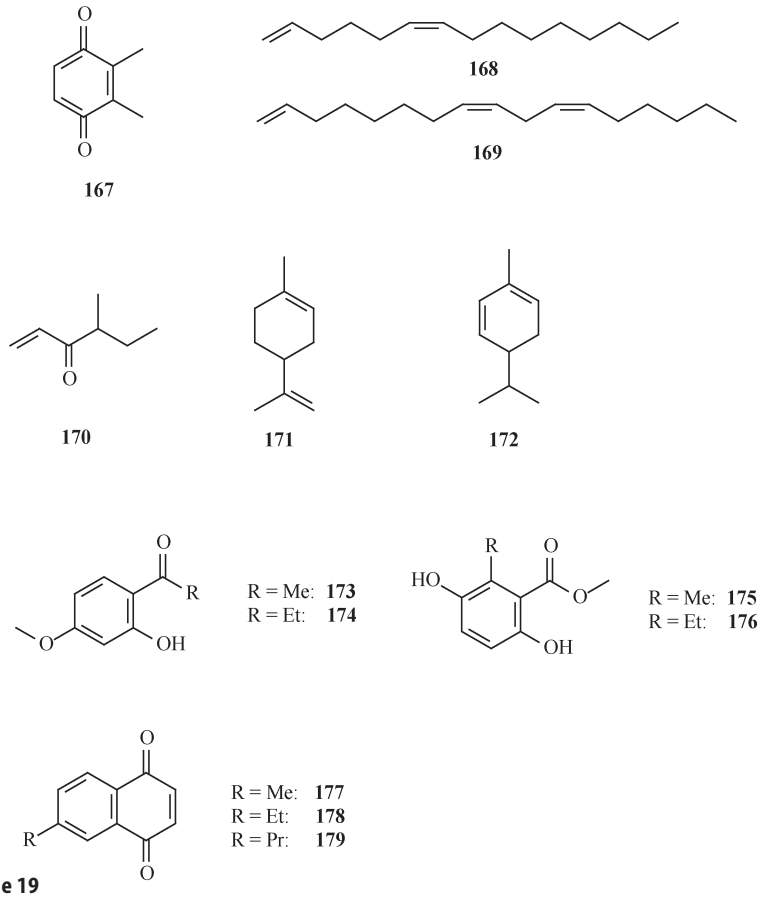
While the structure of 4,8-dimethyldecanal may suggest a tris-norsesquiterpene, produced upon degradation of a farnesol-precursor, it appears more likely that its biosynthesis follows a way similar to that of 4-methylnonanol: starting

with acetate followed by chain elongation with a sequence of propanoate-acetate-propanoate-acetate would yield 4,8-dimethyldecanoate which could be easily transformed to the corresponding aldehyde (see also Fig. 2).

The biological significance of 1-pentadecene and 1,6-pentadecadiene which have been shown to be common volatiles among flour beetles [323] remains to be investigated.

While the pheromones of *Tenebrio* and *Tribolium* originate from a mixed biosynthesis, those produced by males of the broad horned flour beetle, *Gnathocerus cornutus,* represent true terpenes. Initially, the configuration of this new pheromone had been erroneously proposed to be (1*R*,4*R*,5*S*) α-acoradiene [325]; however, independent syntheses of pure stereoisomers [326, 327] proved the correct structure to show (1*S*,4*R*,5*R*)-configuration **165**. The scope of the synthesis is shown by Mori (see chapter by Mori in volume 1 and [15]). A minor component of the *G. cornutus* was reported to be α-cedren-14-al **166** [328].

Defensive Compounds. Since the last review [8], secretions of another 88 species from 63 Australian tenebrionid genera and 23 tribes [329] as well as 10 species of Triboliini [330] have been analysed. They usually contain constituents previously identified from American and European species [329]. Most species produce toluquinone **7**, as well as the ethyl- and propyl-homologue. In addition, Australian species may contain 2,3-dimethyl-1,4-benzoquinone **167** (Scheme 19) or 2-methoxy-3-methyl-1,4-benzoquinone **54** admixed with a row of straight chain uneven numbered 1-alkenes from C_9-C_{19} as well as pentadecadiene, heptadecadiene and nonadecadiene. In *Palorus ratzeburgi* and various *Tribolium*-species, structures of polyenes were determined to be (1,6*Z*)-1,6-pentadecadiene **168**, (1,7*Z*)-1,7-hexadecadiene, (1,8*Z*)-1,8-heptadecadiene, and (1,8*Z*,11*Z*)-1,8,11-heptadecatriene **169**. The biosynthesis of the uneven numbered 1-alkenes starts with fatty acids. The process involves an enantiospecific cleavage of the C-H bond of the pro-(*S*) hydrogen at C3 and simultaneous decarboxylation of the acid form an 1-alkene and carbon dioxide via an *anti*-periplanar transition state geometry (*anti*-elimination). The stereochemistry of this biotransformation was shown to be identical in all respects with the same reaction in higher plants [331]. Further defensive compounds of Australian species are ethylbenzene, *m*-cresol **89**, 4-methyl-3-hexanone, 4-methylhex-1-en-3-one **170**, limonene **171**, α-pinene **45**, α-phellandrene **172**, hexadecyl acetate and tetradecyl acetate. The defensive glands of *Tribolium* additionally contained 2-hydroxy-4-methoxyacetophenone **173**, 2-hydroxy-4-methoxypropiophenone **174**, methyl 2,5-dihydroxy-6-methylbenzoate **175** and methyl 2,5-dihydroxy-6-ethylbenzoate **176**. The defensive secretion of *Blaps mucronata* was analysed in detail [332]. Most compounds correspond to substances of Australian tenebrionids; unusual components are tridecanone, pentadecanone and octanoic acid. As in staphylinid beetles [265] irritancies caused by tenebrionid secretion were determined by using bioassays with ants and cockroaches. Hydrocarbons of *Blaps*-secretions may serve as surfactants that promote spreading of the secretion over the beetles' body [332].



Scheme 19

In Australian tenebrionid beetles, defensive compounds and their patterns seem to be of only low chemotaxonomic value. However, the aforementioned aromatic compounds are restricted to the genus *Tribolium*. Abdominal defensive compounds were used as chemosystematic characters in order to construct a phylogenetic tree for the genus *Tribolium* [330]. The defensive secretion of adults of *Tenebrio molitor* was shown to contain toluquinone **7** and *m*-cresol **89** [333]. The quantification of benzoquinones in single individuals of *Tribolium castaneum* at different days after adult eclosion indicates that the amount of toxic quinone only shows a maximum subsequent to cuticle sclerotization. Obviously, there is a need for an adequate cuticular barrier for self-protection from these defensive compounds [334].

In order to determine whether the defensive compounds of hybrids of the two *Tribolium*- species *T. freemani* and *T. castaneum* represent simple mixtures of the parental phenotypes, different glandular samples were compared by GC-MS [335]. Concerning the qualitative and quantitative data of the quinones,

hydroquinones, propiophenone and alkenes/alkadienes (main compounds) only small differences could be observed. However, the pattern of saturated branched and straight chain hydrocarbons showed significant quantitative differences.

Acidic methanolic extracts of larvae of *Tenebrio molitor* contain toxic substances, so-called paralysins, which exhibit immediate paralytic effects on other insects upon injection [336].

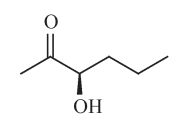
Larvae of the tenebrionid beetle *Hypophloeus versipellis* were shown to possess an unpaired defensive gland reservoir with an opening situated at the anterior border of the ninth tergite [126]. The secretion contains methyl-1,4-benzoquinone **7**, ethyl-1,4-benzoquinone **8**, ethylhydroquinone, and acetophenone as well as 6-methyl-1,4-naphthoquinone **177**, 6-ethyl-1,4-naphthoquinone **178**, and 6-propyl-1,4-naphthoquinones **179**. Several alkenes (probably 1-alkenes) like 1-tridecene, 1-tetradecene, 1-pentadecene (main constituent), 1-hexadecene, and 1-heptadecene may function as solvents for the solid biologically active compounds.

The secretion of *Hypophloeus* caused considerable amounts of mortalities when tested with co-occurring sciarid larvae and probably acts as a bactericide, fungicide, and as a fumigant [337]. 6-Alkyl-1,4-naphthoquinones are erratically distributed among arthropods but also occur in non-homologous paired defensive glands of adult darkling beetles of the genus *Argoporis* (see [8]).

30
Cerambycidae (Longhorn Beetles)

Attractive Compounds. Structures of pheromone components of longhorn beetles are surprisingly diverse (Scheme 20).

Various unbranched α-hydroxyketones were found in several species: (*R*)-3-hydroxy-2-hexanone **180** is the most important compound in the male-specific pheromone blend of the old house borer *Hylotrupes bajulus* and in *Pyrrhidium sanguineum* [338]. Additional compounds are 2-hydroxy-3-hexanone **181** (possibly an artefact produced from **180** upon hydrogen shift) and the reduction products (2*R*,3*R*)-hexanediol, (2*S*,3*R*)-2,3-hexanediol, the corresponding diketone, and 2-butanol. The latter compounds have consequently not been tested with respect to their biological activity; however, the diols appear to be important. The hydroxyketone **181** and its *bis*-homologue (*R*)-3-hydroxy-2-octanone **183** are male released pheromone constituents of *Anaglyptus subfasciatus* [339, 340]. The attractivity of a 25:1 blend of **180** and **182** is significantly enhanced by the addition of the floral attractant methyl phenylacetate [341]. The sex pheromones of *Xylotrechus* spp. consist of (2*S*,3*S*)-2,3-octanediol **183** and (*S*)-2-hydroxy-3-octanone [342, 343]. Structures of pheromone components in the coffee white stem borer, *Xylotrechus quadripes*, seem to follow the scheme of other *Xylotrechus* spp: (*S*)-2-hydroxy-3-decanone **184** (accompanied by the corresponding dione) was found to be weakly attractive



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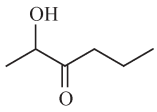

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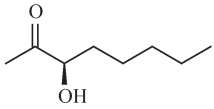

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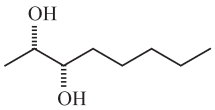

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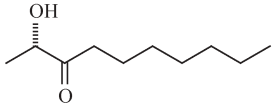

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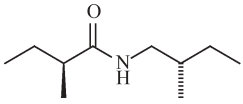

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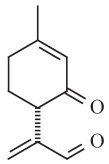

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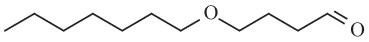

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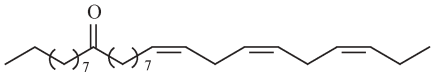

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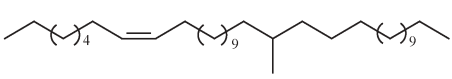


Scheme 20 **189**

[344]. All these compounds represent a row of bishomologues of acetoin **77**, the pheromone of the chafer *Amphimallon solstitiale* [167].

The female produced long range sex pheromone of *Migdolus fryans* is *N*-[(2′*S*)-2-methylbutyl]-(2*S*)-2-methylbutyramide **185** [345]. The acyl part as well as the alkyl part may be derived from isoleucine. Interestingly, this amide is accompanied by the ethyl ester of *N*-formyl isoleucine, which is also known from the scarab beetle, *Phyllophaga elenans* [181]. This amino acid derivative proved to be not attractive for both species; its biological significance remains to be clarified.

In contrast to the doubly oxygenated acetogenins **180–184** and the branched amide **185**, female specific semiochemicals of *Vesperus xytarti* are monoter-

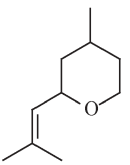
penes. The oxygenated isopiperitenone derivatives **186** and the corresponding primary alcohol were called vesperal and vesperol [346]. Independent syntheses proved the natural compounds to show (*S*)-configuration [346, 347] while bioassays established the role of **186** as the decisive pheromone component.

Two male specific volatiles of *Anoplophora glabripennis* were found to elicit strong electrophysiological responses in the antenna of both males and females. The very unusual 4-(*n*-heptyloxy)butanal **187** and the corresponding alcohol form a 1:1 mixture [348]. A synthetic blend proved to be attractive in laboratory bioassays.

While these functionalized ethers may be long range signals, long chain unsaturated ketones, isolated from the elytra of females of the related species *Anoplophora malasiaca*, act as contact pheromones. The mixture of 10-heptacosanone, (*Z*)-18-heptacosen-10-one, (18*Z*,21*Z*)-18,21-heptacosadien-10-one and (18*Z*,21*Z*,24*Z*)-18,21,24-heptacosatrien-10-one **188** proved to show pronounced biological activity [349].

Another contact sex pheromone was identified as a component of the cuticular lipids of females of *Psacothea hilaris* [350, 351]. Extracts of the elytra contained (*Z*)-21-methyl-8-pentatriacontene **189**. The synthetic compounds (both enantiomers were synthesized [352, 353]) induced precopulatory behaviour in males, however, its biological activity was considerably lower than that of the natural extract.

Defensive Compounds. In Cerambycinae, paired metasternal glands are situated in the thorax, while associated reservoirs open near the hind coxae [8]. Rose oxide **190** (Scheme 21) and iridodial **17** were identified from *Aromia moschata*



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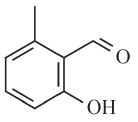

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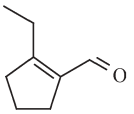

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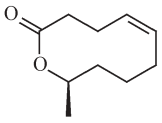

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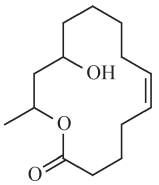

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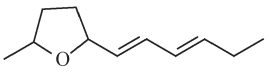


195

Scheme 21

[354]. *Phoracantha* species contained 6-methylsalicylic aldehyde **191**, the disubstituted cyclopentene phoracanthal **192**, the corresponding alcohol, phoracanthol, and the (*E*)- and (*Z*)-stereoisomers of the saturated system. In addition, methyl and ethyl esters of 2-methylbutyric acid and isovaleric acid as well as the macrocyclic lactones decan-9-olide (=phoracantholide I), (*Z*)-dec-4-en-9-olide (=phoracantholide J) **193**, and 11-hydroxytetradec-5-en-13-olide **194** [8, 355, 356]. As shown by independent syntheses of both enantiomers, the natural phoracantholides show (*R*)-configuration [357].

From the metasternal gland secretion of the locust tree borer *Megacyllene robiniae* Wheeler et al. [358] identified 2-(1,3-hexadienyl)-5-methyltetrahydrofuran **195** (no stereochemistry provided), hexadecyl acetate, octadecyl acetate, and 1-phenylethanol.

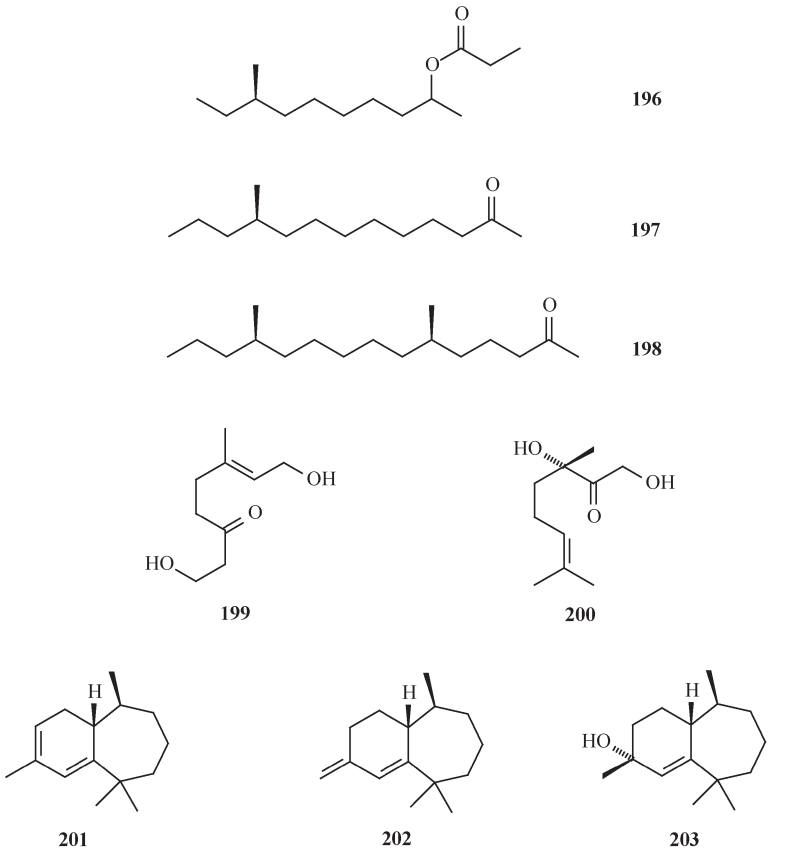
31
Chrysomelidae (Leaf Beetles)

Attractive Compounds. Despite the fact that defence chemistry and insect-plant interactions have been extensively investigated in many leaf beetle species, not too much is known about the chemical background of intraspecific communication.

1,7-Dimethylnonyl propanoate **196** (Scheme 22), the female produced sex pheromone of several corn root worm species, *Diabrotica* spp. keeps (*R*)-configuration at the methyl branching, whereas the stereochemistry at the oxygen function may vary with species (including the formation of mixtures) [359, 360]. The structure of the pheromone of the southern corn root worm *D. undecimpunctata*, (*R*)-10-methyltridecan-2-one **197**, is closely related to **196** [361, 362]. Compared with **196** and **197**, (6*R*,12*R*)-6,10-dimethylpentadecan-2-one **198**, the sex pheromone of *D. balteata* shows similar structural features [363, 364].

A more recent synthesis of **197** [365] is shown in Fig. 9. Enders introduced the stereogenic centre of (*S*)-lactic acid into the crucial position 10 in **197**. The vinylsulfone **B**, readily available from lactic acid, was transformed into the planar chiral phenylsulfonyl-substituted (η^3-allyl)tetracarbonyliron(+1) tetrafluoroborate **C** showing (1*R*,2*S*,3*R*)-configuration. Addition of allyltrimethyl silane yielded the vinyl sulfone **D** which was hydrogenated to **E**. Alkylation with the dioxolane-derivative of 1-bromoheptan-6-one (readily available from 6-bromohexanoic acid) afforded **F**. Finally, reductive removal of the sulfonyl group and deprotection of the carbonyl group furnished **197**. A similar approach was used for the synthesis of **198** [366].

The biosyntheses of these compounds may follow similar principles involving propanonate (methylmalonate) and acetate (malonate) units; however, the sequence seems to be less clear than in other branched chain structures. According to Fig. 2, incorporation of propanoate followed by chain elongation with acetate (including termination by either propanoate or acetate) would lead to an even number of methylene groups between the methyl branching



Scheme 22

and the oxygen function – which is not the case in the *Diabrotica* pheromones. However, a sequence of acetate-propanoate-acetate-acetatepropanoate followed by oxidative decarboxylation of the acyl-intermediate (see also remarks concerning the biosynthesis of lardolure [45]) and esterification would definitely yield **196**. Similarly, the introduction of oxygen into **197** and **198** may be introduced upon oxidative decarboxylation of an α-methyl acyl-precursor.

Another unusual structure was identified from cereal leaf beetles, *Oulema melanopus*: (*E*)-8-hydroxy-6-methyl-6-octen-3-one **199** was found to be a male-specific volatile. Electrophysiological investigations showed a sensitive detection of **199** by both sexes which is consistent with a male-produced aggregation pheromone [367]. The behaviour mediating capacity of the compound needs to be proven.

While the existence of a female produced sex pheromone in the Colorado potato beetle *Leptinotarsa decemlineata* has been the subject of controversy for many years (for a discussion see [368]) a male produced pheromone has

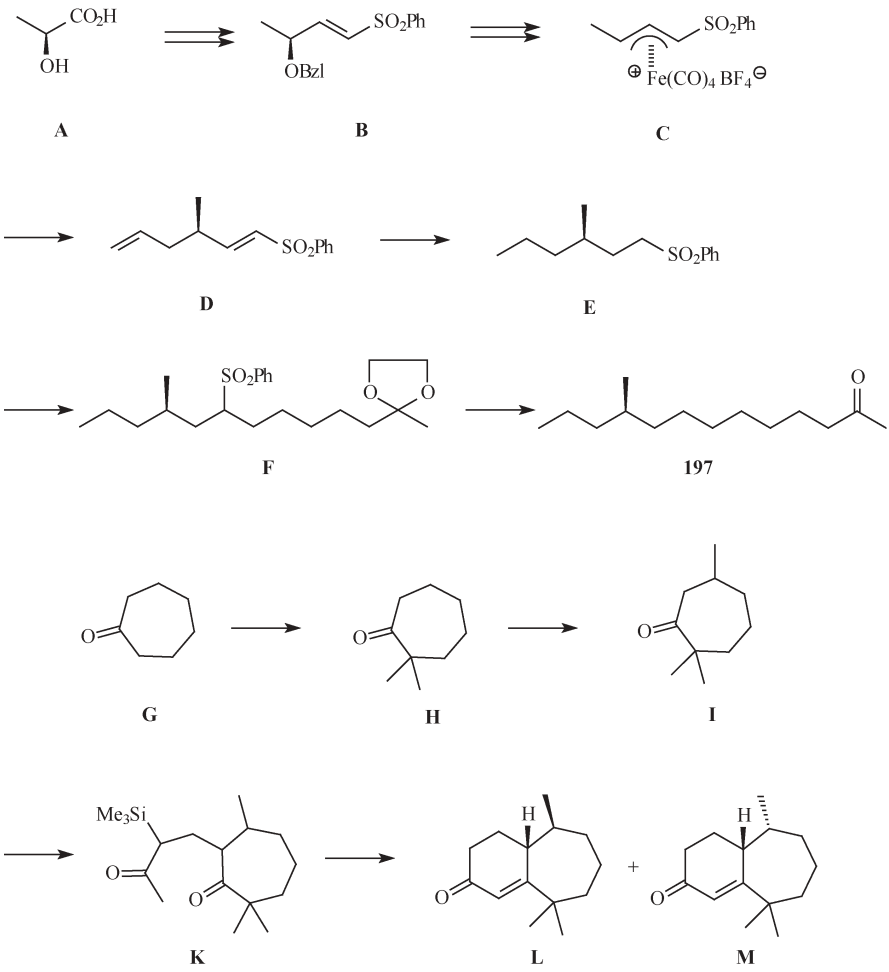


Fig. 9 Reaction schemes for the syntheses of (*R*)-10-methyltridecan-2-one and the sesquiterpenes of *Phyllotreta* and *Aphtona* spp.

recently been identified: (*S*)-3,7-dimethyl-2-oxo-6-octene-1,3-diol **200**. The structure suggests a highly oxygenated monoterpene [369]. Only the natural (S)-enantiomer proved to be active. Corresponding syntheses have been shown by Mori [15, 16]. It is obvious that **199** and **200** may share an isoprene subunit.

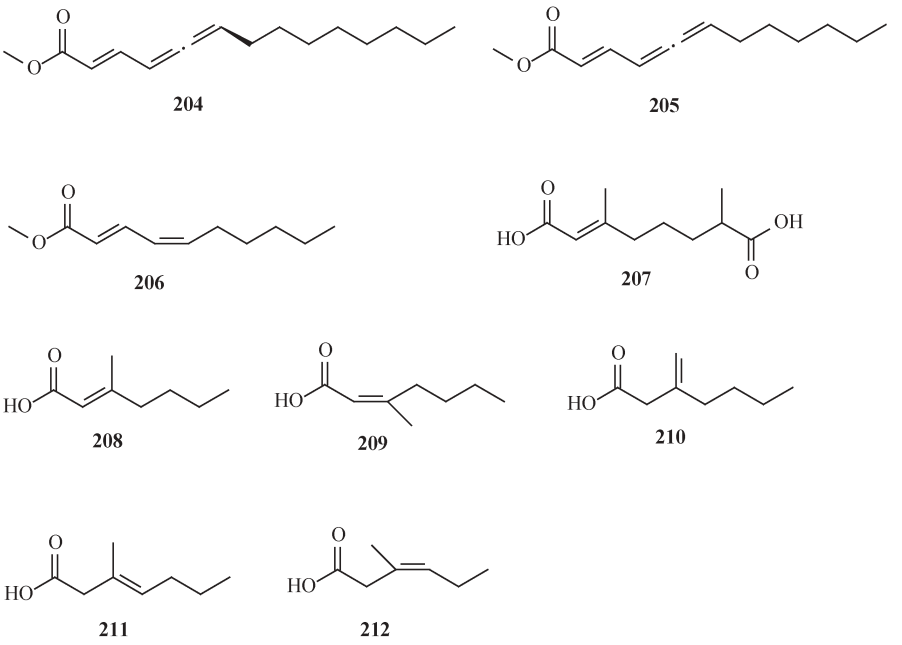
In some flea beetles, *Phyllotreta* and *Aphthona* spp., species specific, male produced blends of himachalene derivatives like **201, 202,** and **203** were identified. Structure elucidation was carefully carried out on the basis of spectroscopic methods, micro reactions, and independent syntheses [370, 371]. Compounds **201, 202, 203** are perceived by both male and female antennae, as would be expected for an aggregation pheromone. Investigations on the behaviour mediating capacity of the compounds are ongoing.

Syntheses of diastereomerically pure racemates of himachalene derivatives started from cycloheptanone **G** (Fig. 9). The sequence to **I** involved dimethylation to yield **H** followed by bromination/dehydrobromination and conjugate methylation using cuprate chemistry. The sequence furnishing **L** and **M** follows a Robinson-annelation type: Reaction of **I** with 3-(trimethylsilyl)but-3-en-2-one yielded **K**. Refluxing **K** with potassium hydroxide in ethanol removed the silyl group and cyclized the diketone to form a 97:3 mixture of racemic **L** and **M**. Occurring as a volatile in *A. flava*, **L** served as a versatile intermediate in the syntheses of other *Aphthona* compounds.

Defensive Compounds. The defensive chemistry of leaf beetles was treated in the chapter by Laurent et al., this volume.

32
Bruchidae (Bean Weevils, Seed Beetles)

Attractive Compounds. The male produced sex pheromone of the dried bean beetle, *Acanthoscelides obtectus*, is an unusual methyl ester, methyl (*R*,2*E*)-2,4,5-tetradecatrienoate **204** [372] (Scheme 23). The compound was among the first pheromones identified from male beetles, and only very recently other insect volatiles showing allenic structures have been described [184]. Careful head



Scheme 23

space analyses of volatiles released by males of *A. obtectus* confirmed the presence of **204** as a major component but showed also other compounds like methyl 2,4,5-dodecatrienoate **205** and methyl (2*E*,4*Z*)-decadienoate **206** to be present [373]. No bioassays have been carried out with **205** or **206**.

While the unbranched **204–207** clearly originate from the acetate pool, the structure of (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid, callosobruchusic acid **207**, a female produced copulation releasing pheromone of the azuki bean weevil, *Callosobruchus chinensis* [374] points to a terpenoid structure. The synthetic enantiomers [375] proved to be equally effective in releasing copulation behaviour in males.

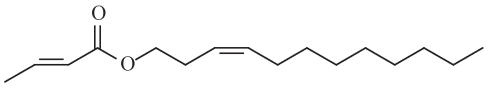
Females of the cowpea weevil, *Callosobruchus maculatus*, release a male attracting pheromone from the tip of their abdomen. The volatile signal contains five unsaturated, branched C8-acids **208–212** [376, 377]. Individual compounds proved to be active while mixtures showed additive effects. Similarly, compounds **208** and **209** have been identified as the female produced sex pheromone of *C. subinnotatus* [378], while **209** had been described as the sex pheromone of *C. analis* [379]. However, GC-MS analyses of female produced volatiles of *C. analis* failed to detect any of the *C. maculatus* compounds, but did find an unidentified C8-acid with a retention time different from any of the *C. maculatus* acids [377].

It is interesting to note that the *C. maculatus* compounds show an "isoprene sub-unit" which bears the unsaturation and the carboxylic moiety just like callosobruchusic acid **207**. A propanoate (or methylmalonate) starter would formally complete the biogenesis of the structures.

33
Curculionidae (Snout Beetles, Weevils)

Attractive Compounds. With the exception of (*Z*)-3-dodecenyl (*E*)-2-butenoate **213** (Scheme 24), the female produced sex pheromone of the sweetpotato weevil *Cylas formicarius* [389], the structures of weevil pheromones are represented by oxygenated monoterpenes, polyketides produced from propanoate units, and branched alcohols and ketones, probably originating from a mixed acetate-propanoate biosynthesis [5].

The male produced sex pheromone of the boll weevil, *Anthonomus grandis*, was the first weevil pheromone identified [381]. The bouquet is made up by four compounds, the tri-substituted cyclobutane **214**, grandisol (main component), and the cyclohexane derivatives (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexanethanol, (*Z*)-ochtoden-1-ol **215** (main component), **216**, and **217** (minor components). Upon comparison with synthetic samples, natural grandisol proved to show (1*R*,2*S*)-configuration [382, 383], its enantiomer is behaviourally inactive [384]. A close relative of the boll weevil, the pepper weevil, *Anthonomus eugenii*, does not produce grandisol but the three cyclohexane derivatives **215–217** and **218** as well as geraniol and geranic acid **219** [385].



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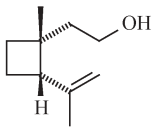

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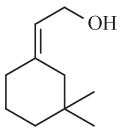

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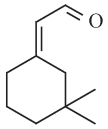

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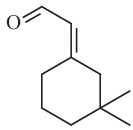

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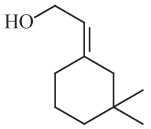

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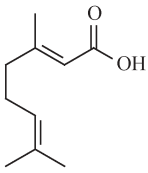

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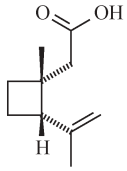

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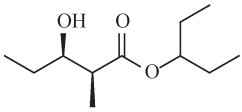

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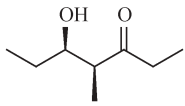

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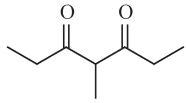

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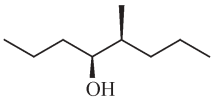

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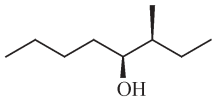

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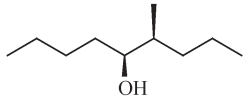

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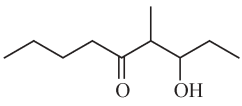

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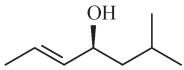

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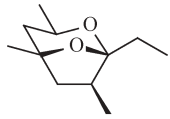

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Scheme 24

The pheromone bouquet of the pecan weevil, *Curculio caryae* is similar to that of the boll weevil; however, it also contains (1*S*,2*R*)-grandisol. The quantitative composition of the blend determines whether it is more attractive to the pecan weevil or to the boll weevil [386]. In some pine weevils, *Pissodes* species, grandisol and the corresponding aldehyde, grandisal, are components of a male produced pheromone. In *Pissodes nemorensis* and *Pissodes strobi* grandisol

shows almost 100% (1*R*,2*S*)-configuration. In contrast, *Pissodes nemorensis* releases nearly 100% pure (1*S*,2*R*)-grandisal, while in *Pissodes strobi* this enantiomer dominates only with 20% enantiomeric excess [387]. Finally, (1*R*,2*S*)-grandisoic acid **220** was identified as a component of the male produced aggregation pheromone of the plum curculio *Conotrachelus nenuphar* [388]. The male produced pheromone of the strawberry blossom weevil, *Athonomus rubi* consists of (1*R*,2*S*)-grandisol **214**, (*Z*)-ochtoden-1-ol **215**, and lavandulol **37** [389].

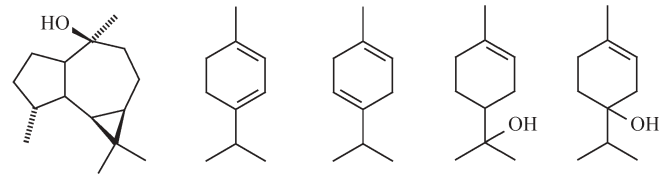
Two propanoate units (see Fig. 2) seem to be coupled in 1-ethylpropyl (2*S*,3*R*)-2-methyl-3-hydroxypropanoate **221**, the male produced aggregation pheromone of the granary weevil, *Sitophilus granarius* [390–393]. Even the ester-moiety may origin from two propanoate units after decarboxylation and reduction Three propanoate units (and decarboxylation, see Fig. 2) may produce (4*S*,5*R*)-5-hydroxy-4-methyl-3-heptanone, sitophilure **222**, the aggregation pheromone of the rice weevil, *Sitophilus oryzae* [394, 395]. The same carbon skeleton is present in the achiral 4-methylheptan-3,5-dione **223**, the pheromone of the pea weevil, *Sitona lineatus* [396–398].

Simple branched secondary alcohols have been identified as male-produced aggregation pheromones of *Rhynchophorus* and related species. With only few exceptions the compounds are not species specific. The pheromone of the palmetto weevil, *Rhynchophorus cruentatus* is (4*S*,5*S*)-5-methyl-4-octanol, cruentol, **224** [399, 400] whereas its isomer, (3*S*,4*S*)-3-methyl-4-octanol, phoenicol, **225**, is the pheromone of the African palm weevil, *Rhynchophorus phoenicis* [399–402]. The homologue of **225**, (4*S*,5*S*)-4-methyl-5-nonanol, ferruginol, **226**, was identified in the African palm weevil, *Rhynchophorus ferrugineus*, and several related species including *Dynamis borassi* [403–405]. Ferruginol is also the most important pheromone component of the sugar cane weevil, *Metamasius hemipterus*, where it is accompanied by 2-methyl-4-heptanol, 2-methyl-4-octanol, the corresponding ketones, 5-nonanol, and 3-hydroxy-4-methyl-5-nonanone **227** [406–408]. No bioassays with these minor components have been reported. The pheromone of the American palm weevil, *Rhynchophorus palmarum* is (2E,4*S*)-methyl-2-hepten-4-ol, rhynchophorol, **228** [409–411]. The corresponding epoxide was also found to be present [412], but no bioassays have been reported.

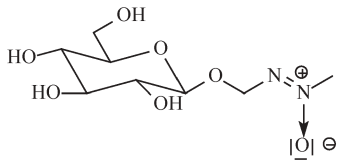
Weevils do not seem to be very sensitive to the presence of non-natural stereoisomers of their pheromones, since racemic mixtures proved to be active in the field. This greatly facilitates their use in large-scale integrated pest management. Some species also contain ketones, corresponding to the pheromone alcohols; however, they do not show behavioural activity.

A higher degree of oxygenation along the chain is represented in (1*S*,3*R*,5*R*,7*S*)-1-ethyl-3,5,7-trimethyl-2,8-dioxabicyclol[3.2.1]octane, sordidin, **229**, the aggregation pheromone of the banana weevil, *Cosmopolites sordidus* [413–415]. It is interesting to note, that the (1*R*,3*S*,5*S*,7*S*)-stereoisomer of sordidin is a biologically active compound in cadddisfly species (Trichoptera) [416].

The biological activity of the banana weevil pheromone and those of related palm weevil species is strongly enhanced by host plant volatiles [399, 417–419].



230 **231** **232** **233** **234**



Scheme 25 **235**

Defensive Compounds. Larvae of the weevil *Oxyops vitiosa* produce a shiny orange secretion that covers their integument and probably acts as deterrent against ants [420]. The composition of the secretion resembles the terpenoid pattern of the host foliage (*Melaleuca quinquenervia*) from where it is sequestered (concentration about twice that of the host foliage). It contains the sesquiterpene (+)-viridoflorol **230** (Scheme 25), the monoterpene hydrocarbons α-pinene **45**, β-pinene **46**, limonene **171**, α-terpinene **231**, and γ-terpinene **232** as well as the oxygenated monoterpenes1,8-cineole **58**, α-terpineol **233**, and terpinen-4-ol **234**.

In males and females of the weevil *Rhopalotria mollis* the sequestration of cycasin **235** known from the Mexican cycad *Zamia furfuracea* was reported [421].

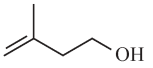
34
Scolytidae (Bark Beetles)

Attractive Compounds. Among the Coleoptera, bark beetles are the most intensively investigated family. Guided by chemical signals they typically colonize their host trees in large amounts (mass attack) to overcome jointly its resistance which is prerequisite for a successful breeding. In this context, typical sex pheromones produced by one sex to attract exclusively the other play a minor role. In contrast, intraspecific communication is mainly based on aggregation pheromones that attract both sexes. These aggregation pheromones are released by so-called pioneer beetles after landing on a host tree while mate-finding of the attracted conspecifics on the host tree surface seems to be a matter of close range orientation and statistics. In general, females of monogamous species select individual host trees while in polygamous species it is the males.

Host selection is largely influenced by the physical condition of the tree as well as by its inventarium of volatile and non-volatile compounds. In coniferous species, the composition of monoterpene hydrocarbons seems to be a major olfactory clue in bark beetle orientation (primary attraction); however, details are still not understood. The chemical signal is specified by pheromones (secondary attraction) which may serve as intraspecific attractants and interspecific repellents for species that compete for the same breeding place. At the same time predators may locate their prey by using the corresponding pheromones as kairomones. With a few exceptions, bark beetle species that have been investigated with respect to their communication systems attack coniferous trees. The intriguing mechanisms of host colonization as well as intra- and interspecific communication in bark beetles have been extensively reviewed [422–425]. This paragraph is focussed on chemical structures of compounds that are used in bark beetle communication rather than on pheromone biology, i.e. the intruding mechanisms of host selection, mate finding, and interspecific competition etc.

The hemiterpene 3-methyl-3-buten-1-ol **236** (Scheme 26), one of the two C5-building blocks of monoterpenes, is a pheromone of the larch bark beetle *Ips cembrae* [426]. While the other principal building block of monoterpenes, 3-methyl-2-buten-1-ol does not play a decisive role as a bark beetle pheromone, 2-methyl-3-buten-2-ol, **237**, the product of its allylic rearrangement is the main aggregation pheromone of several *Ips* and related species [427, 428]. In *Ips typographus* it proved to be synthesized de novo [429]. A higher oxygenated isoprenoid 3-hydroxy-3-methylbutan-2-one **238**, is a volatile constituent of ambrosia beetles [430] and induces an extremely high electrophysiological response in the antenna of *Xyloterus lineatus* [431].

The almost ubiquitous terpenoid 6-methyl-5-hepten-2-one may be produced either by degradation of a geranial precursor (oxidative cleavage of the allylic-double bond or a retroaldol type reaction) or by chain elongation of β,β-dimethylalkyl pyrophosphate with acetoacetate followed by decarboxylation. Reduction of 6-methyl-5-hepten-2-one yields the corresponding alcohol, sulcatol **239**, which is the aggregation pheromone of *Gnathotrichus* spp [432–434]. These ambrosia beetles produce species specific mixtures of the sulcatol enantiomers, and the natural proportions are essential for maximum response. Oxidation at the double bond of sulcatol (e.g. epoxidation) followed by ring closure will yield either 2-(1-hydroxy-1-methylethyl)-5-methyltetrahydrofuran, pityol **240**, or 2,2,6-trimethyl-3-hydroxytetrahydropyran, vittatol **241**. The elm bark beetle *Pteleobius vittatus* uses *cis*-pityol and *cis*-vittatol of as yet unknown absolute configuration as part of its aggregation pheromone [435]. Males of *Pityophthorus pityographus* release trans-pityol showing (2*R*,5*S*)-configuration [436]. The same stereoisomer is part of the aggregation pheromones of other *Pityophthorus* species: in *Pityophthorus carmeli* it is produced by the males and in *Pityophthorus nitidulus* as well as in *Pityophthorus setosus* by the females [437]. Moreover, females of cone beetles, *Conophthorus* spp, also produce (2*R*,5*S*)-pityol as an aggregation pheromone [438, 439]. The structural relations



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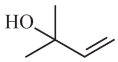

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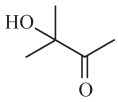

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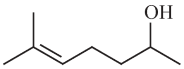

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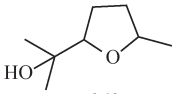

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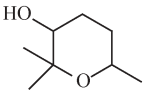

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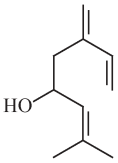

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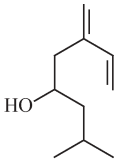

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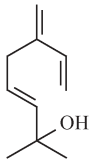

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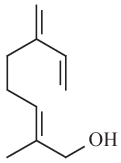

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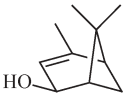

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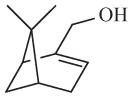

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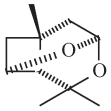

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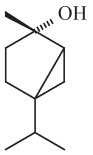

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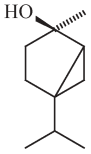

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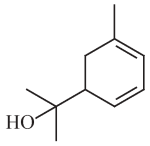

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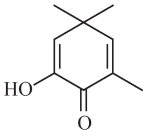

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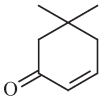

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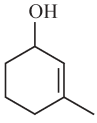

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Scheme 26

between **239**, **240**, and **241** are essentially the same as those between linalool and the furanoid or pyranoid forms of linalool oxide.

Monoterpenes play a particularly important role in host selection and mass aggregation of bark beetles. Insects attacking conifers have to overcome both physical and chemical obstacles as sticky and toxic oleoresin is involved in the defence mechanisms of trees. Bark beetles developed a series of strategies to survive which include detoxification through oxygenation. These oxygenation products may in turn be used as chemical signals indicating the attempts of an individual insect to attack a tree. Allylic oxygenation or hydration of unsaturated monoterpene hydrocarbons followed by secondary reactions such as further oxidation, hydrogenation, or rearrangement seem to be important mechanisms in the generation of bark beetle pheromones [440, 441] (Scheme 26).

Whereas some species oxidize host terpenes more randomly, producing an array of rather unspecific volatiles with little information, others use highly selective enzyme systems for the production of unique olfactory signals. However, apart from transformations of monoterpene hydrocarbons of host trees, oxygenated monoterpenes may well be biosynthesized de novo by the beetles (see below).

None of the monoterpene pheromones of bark beetles is represented by a specific compound per se; however, species specificity of the signal is accomplished by qualitatively and quantitatively fine-tuned mixtures including enantiomeric proportions.

The myrcene derivatives ipsdienol **242**, and ipsenol **243**, the first pheromone components identified from bark beetles [442], are typical male-specific aggregation pheromones of many Ips species, but they also play a role in host colonization by other species such as *Pityokteines* [443, 444] or *Xylocleptes bispinus* [445] (which attacks *Clematis vitalba* and is, thus, no truly conifer-breeder). Enantiomeric composition of these monoterpene alcohols is instrumental with respect to the behaviour mediating capacity of the signal [446], even in different populations of the same species [447]. The corresponding ketones, ipsdienone and ipsenone, were found in several *Ips* species, however, their biological significance is not yet clear. They may well be involved in transformation reactions leading from ipsdienol to ipsenol [448, 449]. The tertiary alcohol amitinol **244**, represents a product of an allylic rearrangement of ipsdienol [450, 451]. Another product of a formally allylic oxidation of myrcene, *trans*-myrcenol **245**, was also identified as a pheromone of *Ips* species [452].

While earlier it was generally thought that the acyclic monoterpene alcohols **242–245** are derived from the host tree's oleoresin component, myrcene [453], more recent results clearly show that at least in some species they are produced de novo [454–456].

Oxygenated monoterpenes which are found in almost every bark beetle species attacking coniferous trees, include *cis*-verbenol **246**, *trans*-verbenol **247**, and myrtenol **248**, representing primary products of allylic oxidation of the host terpene α-pinene **45**. Further oxidation of **247** or **248** leads to the

corresponding carbonyl compounds verbenone and myrtenal, which, too, are common bark beetle volatiles. 1,4-Elimination of water from verbenol yields verbenene **249**, which was found as a behaviour-mediating volatile emitted by females of *Dendroctonus rufipennis* [457]. Among the bicyclic terpenes, *cis*-verbenol is a particularly important component in the aggregation pheromones of *Ips* spp., whereas *trans*-verbenol is used by *Dendroctonus* spp. Both sexes of *Ips* species oxidise α-pinene enantioselectively [458, 459]: (4*S*)-*cis*-verbenol **246** is produced from (–)-α-pinene, whereas (+)-α-pinene yields (4*S*)-*trans*-verbenol **247**. Verbenone, which in bark beetles appears to be largely formed from verbenols due to the action of associated microorganisms [460, 461] seems to act as a general inhibiting signal which the beetles use to avoid overpopulation and which induces shifting of the attack to another tree [442, 462].

The close relationships between weevils and bark beetles becomes evident in the fact that (*E*)-ochtodenol **218**, and grandisol **214**, are components of the pheromone bouquet of *Pityogenes quadridens* [463]. In related *Pityogenes* species as well as in *Pityophthorus pityographus* grandisol shows (1*R*,2*S*)-configuration. The tricyclic acetal, lineatin **250**, a higher oxygenated derivative of grandisol (showing an additional oxygen at the position complementory to carbon 4 in ipsdienol) is an aggregation pheromone of several ambrosia beetles, *Xyloterus* (*Trypodendron*) spp. The natural product was shown to be the (1*S*,4*R*,5*S*,8*S*)-enantiomer [465, 466].

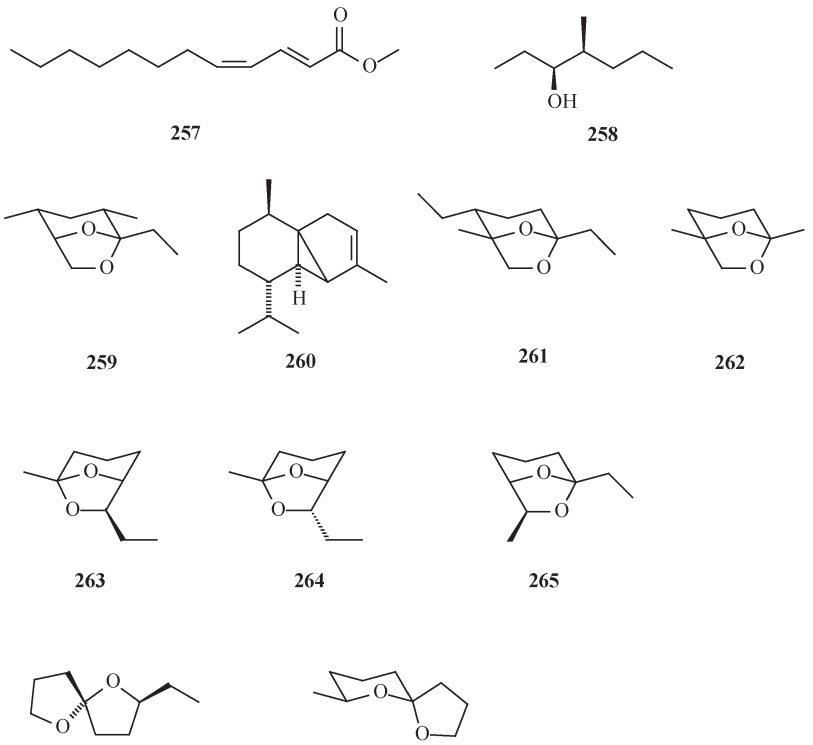
In *Polygraphus poligraphus* the (4*R*)-enantiomer of terpinen-4-ol **234**, acts as an aggregation pheromone [467]. The compound is accompanied by the thujanols **251** and **252** which may be biogenetically close to terpinen-4-ol and keep the same configuration at C4 [440].

The interesting *m*-menthadienol (3*S*)-1-methyl-5-(1-hydroxy-1-methylethyl)-1,3-cyclohexadiene **253**, is produced by *Ips sexdentatus*, boring under stress in 3-carene-rich, highly resinous pine trees and possibly released as a kind of warning signal to conspecifics to keep off [463].

2-Hydroxy-4,4,6-trimethyl-2,5-hexadien-1-one, lanierone, **254**, is a component of the complex aggregation signal of male *Ips pini* [468]. The carbon skeleton of **254** is the same as in isophorone **255**, which has been identified as a volatile constituents of females of *Ips typographus*. Whether these compounds are degradation products of higher terpenes awaits further investigations.

Another cyclohexane derivative is represented by 3-methyl-2-cyclohexen-1-ol seudenol **256**, a component of the female produced aggregation pheromone of Douglas-fir beetles, *Dendroctonus pseudotsugae* [470]. Enantiomeric compositions of the natural compound are reported to range between (*R*):(*S*)=2:1 and almost racemic [471]. Again, the product of an allylic rearrangement, 1-methyl-2-cyclohexen-1-ol, has been identified as an accompanying attractive compound [472]. The biosynthesis of **256**, and that of the corresponding ketone which acts as an intraspecific repellent (similar to verbenone in other species) is unknown, however, a simple acetogenin like 2,6-heptandione (derived from a fatty acid?) would easily produce 3-methyl-2-cyclohexen-1-one upon intramolecular aldol condensation.

An open chain fatty acid derivative is methyl (2*E*,4*Z*)-dodecadienoate **257**, an important component of the male produced aggregation pheromone of *Pityogenes chalcographus* [473]. A behaviour releasing capacity of ethyl dodecanoate, which has been found in several other *Pityogenes* spp [463], needs to be tested. Compounds with a chain length similar to the ester **257** are undecanal and decanal as well as 2-undecanone, 2-decanone, and 2-nonanone which apart from α- and β-pinene **45**, **46** were identified as attractive components of the olive bark beetle *Phloeotribus scarabaeoides* [474]. Nonanal fits to this row; it was found to be present in many bark beetle species; however, no significant behaviour mediating capacity of this compound has been reported. Somewhat shorter is 2-heptanol a principal pheromone constituent produced by female *Dendroctonous jeffreyi* [475]. The structure of (3*S*,4*S*)-4-methyl-3-heptanol **258**, strongly suggests a biosynthesis involving three propanoate units according to Fig. 2. The compound is an important component in the aggregation pheromones of several *Scolytus* species [476], whereas its (3*R*,4*S*)-diastereomer acts as a trail pheromone of the ant *Leptogenys diminuta* [477]. The corresponding ketone was also identified in *Scolytus* spp.; however, it did not decisively contribute to the biological activity of the pheromone bouquet [478].



257 258 259 260 261 262 263 264 265 266 267

Scheme 27

A group of bark beetle pheromones is represented by alkylated 6,8-dioxabicyclo[3.2.1]octanes. The biological significance, mass spectrometric fragmentation, and syntheses of these bicyclic acetals have been extensively reviewed [479]. An important pheromone component of several *Scolytus* species is (1*S*,2*R*,4*S*,5*R*)-multistriatus **259** [480, 481]. In the smaller European elm bark beetle, *Scolytus multistriatus*, it forms the aggregation signal along with (3*S*,4*S*)-4-methyl-3-heptanol **258** and the host tree sesquiterpene (–)-a-cubebene **260**. Similar to the alcohol **258**, the biosynthesis of the acetal **259** may involve propanoate units.

The male produced aggregation pheromone of the beech bark beetle, *Taphrorychus bicolor* is (1*S*,2*R*,5*R*)-bicolorin **261** [482, 483]. Its carbon skeleton may represent a rearranged terpene.

Frontalin **262** is a widespread pheromone of *Dendroctonus* species [285]. In those cases where the enantiomeric composition of naturally occurring frontalin is known, the (1*S*,5*R*)-enantiomer is always dominating. In females of *Dendroctonus frontalis*, it shows an enantiomeric excess of 70% [481] while males of *Dendroctonus simplex* produce it in high enantiomeric purity [483]. The biosynthesis of frontalin may involve 6-methyl-6-hepten-2-ol as precursor which upon epoxidation and ring closure would yield **262** [483]. The beetles produce the compound de novo along a mevalonate pathway [486, 487]. Interestingly, frontalin (unknown configuration) has been identified in the temporal gland secretion of male Asian elephant [488] and in the *Alnus* spp. red alder and Sitka alder [489].

Other important *Dendroctonus* pheromones are *exo*-brevicomin **263** [481, 490] as well as endo-brevicomin **264** [490]. In the monogamous *Dendroctonus* species, frontalin and brevicomin are part of intriguing dialogues between the sexes: attracted to the resin components of host trees, females of the southern pine beetle, *Dendroctonus frontalis*, release frontalin and the oxygenated monoterpene *trans*-verbenol **247** to attract both males and females [491]. The males joining the females strongly increase the attractivity of the system by contributing (1*R*,5*S*,7*S*)-*endo*-brevicomin [492, 493]. In contrast, females of the western pine beetle, *Dendroctonus brevicomis*, produce (1*R*,5*S*,7*R*)-*exo*-brevicomin which also attracts both sexes with a preponderance of males; after arrival, these release (1*S*,5*R*)-fontalin which is predominantly attractive to females [494]. As may be seen, brevicomin and frontalin are not always produced by the same sex. Brevicomin frequently occurs as a mixture of diastereomers with a large excess of the *exo*-isomer. In the mountain pine beetle, *Dendroctonus ponderosae*, males produce highly pure (1*R*,5*S*,7*R*)-*exo*-brevicomin [481, 495, 496]; however, the enantiomeric excess in the accompanying (1*R*,5*S*,7*S*)-*endo*-brevicomin ranged only between 65–70% depending on the population. The brevicomins were also identified in *Dryocoetes* species. Males of the European *Dryocoetes autographus* release the attractive compounds upon feeding [497]. Again, *exo*-brevicomin proved to be the very pure (1*R*,5*Z*,7*R*)-isomer, while *endo*-brevicomin showed an enantiomeric excess of only 63%. The American *Dryocoetes confusus* uses *exo*-brevicomin [498], and the same is true for

Dryocoetes affaber [499]. In the case of brevicomin, bark beetles seem to make use of all degrees of freedom which are opened by a compound showing two chiral centres: species specific mixtures may be generated by differences in relative proportions of enantiomers and diastereomers as well as by release of different absolute amounts. It is interesting to note that the parent carbon skeleton of the brevicomins is represented by (1*R*,5*S*)-6,8-dioxabicyclo[3.2.1]octane, while the other bicyclic acetal pheromones are alkylated enantiomers thereof. The biological significance of oxygenated brevicomins that have been identified in volatiles of the mountain pine beetle, *Dendroctonus ponderosae* [500], needs to be clarified. The same is true for an "*iso*-*exo*-brevicomin" **265** which compared to natural *exo*-brevicomin shows a kind of reverse substitution pattern and keeps (1*S*,5*R*,7*S*)-configuration, basically the opposite configuration of **263** [500]. The biosynthesis of *exo*-brevicomin has been thoroughly investigated in *Dendroctous ponderosae* [501, 502]. There is strong evidence that the compound is derived from the fatty acid pool and produced via an unsaturated ketone, 6-nonen-2-one, through epoxidation and ring closure. Similar to frontalin, the occurrence of brevicomin is not restricted to beetles as (1*R*,5*S*,7*S*)-*endo*-brevicomin (only 30% enantiomeric excess) was found among the volatiles of the orchid, *Ophrys speculum* [503].

Apart from those reviewed in the chapter written by Mori and in reference 479 of this chapter, only a few syntheses of the bicyclic acetals mentioned above have been published [504–506].

The spiroacetal **266**, chalcogran, is an important component in the male produced aggregation pheromone of spruce beetle, *Pityogenes* spp. [22, 507]. In *Pityographus chalcographus* it occurs as a pair of diastereomers showing (2S,5R)- and (2S,5S)-configuration [508, 509]. The weak biological activity of chalcogran is dramatically enhanced by the ester **257** [510, 511]. Field tests with pure stereoisomers of chalcogran showed that the biological activity rests with the (2*S*,5*R*)-enantiomer while its (2*S*,5*S*)-diastereomer is inactive. Racemic chalcogran proved to be strongly attractive to a predator, the Ostomid beetle, *Nemosoma elangatum* [512]. Similar to some bicyclic acetals, **266** was found to be a component of flower volatiles [513]. The spiroacetal conophthorin **267**, an isomer of chalcogran, plays a dual role in bark beetle-communication. The biological significance, mass spectrometric fragmentation and syntheses of volatile spiroacetals, has been extensively reviewed [514]. Apart from bark beetles, the relatively widespread **267** was found in the poison glands of wasps, in fruit flies, orchids, and in a couple of tree species [512]. In general, naturally occurring conophthorin shows (2*S*,5*S*)-configuration of rather high enantiomeric purity. In several bark beetle species, the male produced **267** shows a repellent effect as in *Leperisinus varius* and *Cryphalus piceae* [514] as well as in pine cone borers, *Conophthorus* spp. [438, 439], and in *Pityophthorus* spp. [437]. This may be interpreted as a "spacer" signal used during male-male competition and to ensure enough space for a successful establishment of the new brood system. In *Pityophthorus carmeli* conophtorin **267** and pityol **240** make up the male produced aggregation pheromone [437].

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Topics in Current Chemistry (2005) 240: 167–229
DOI 10.1007/b98317

Insect Chemical Defense

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Abstract Research on the defensive chemistry of insects during the last decade is reviewed, with special emphasis on non-volatile compounds. The isolation and structure determination of defensive chemicals, of glandular and non-glandular origins, are first discussed, followed by an overview of the synthesis and biological/pharmacological activities of some of them. Biosynthesis has been largely omitted since this topic has been addressed in a recent review. During the period covered, beetles (e.g., coccinellids and chrysomelids) and ants have undoubtedly been the most prolific producers of repellent and/or toxic compounds. This survey also shows that alkaloids are the most frequently encountered defensive compounds in insects.

Keywords Defensive compounds · Structure determination · Synthesis · Biological activity · Alkaloids

List of Abbreviations

CIMS	Chemical ionization mass spectrometry
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DIPE	Diisopropyl ether
DMA	Dimethylacetamide
DMDS	Dimethyldisulfide
Dppb	3,4-Di(bisphenylphosphino)butane
DQ-COSY	Double quantum correlation spectroscopy
EDCI	1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride
EIMS	Electron ionization mass spectrometry
ESI-MS	Electrospray mass spectrometry
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-CIMS	Gas chromatography coupled to chemical ionization mass spectrometry
GC-EIMS	Gas chromatography coupled to electron ionization mass spectrometry
GC-MS	Gas chromatography coupled to mass spectrometry
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear double quantum coherence
HPLC	High pressure liquid chromatography
HSQC	Heteronuclear single quantum coherence
IR	Infrared spectroscopy
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTPA	α-Methoxy-α-trifluromethylphenylacetamide
NMO	*N*-Methylmorpholine *N*-oxide
NMR	Nuclear magnetic resonance
Ns	2-Nitrophenysulfonyl (nosyl)
PAs	Pyrrolizidine alkaloids
PPL	Porcine pancreatic lipase
SAMP	(*S*)-(–)-1-Amino-2-methoxymethylpyrrolidine
TFEB	Trifluoroethyl butanoate
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
TPAP	Tetra *n*-propylammonium perruthenate
UV	Ultraviolet spectroscopy

1 Introduction

Insects are fascinating organisms. They constitute about 75% of all animal species and have colonized nearly every terrestrial habitat. Several reasons may be put forward to explain their ecological success, e.g., a high fecundity rate, a remarkable adaptation to different environments and climatic conditions, the evolution of specialized structures (e.g., carapace, wings, mandibles...) and, for some groups, the existence of highly organized societies. During evolution, insects have also acquired extremely diversified and sophisticated

communication systems that play a prominent role in their ecological success. Intraspecific communication systems based on pheromones are the major topic of this two volumes book. However, interspecific communication systems are also highly developed in insects. To quote Eisner: "Among terrestrial animals, none is perhaps more diversely endowed with chemical weaponry than the arthropods" [1]. With the exception of a few isolated studies, however, the field of insect defenses has not been investigated from a chemical point of view until the late 1950s. The pioneering work of Eisner, Meinwald, and Blum in the USA, of Schildknecht in Germany and Pavan in Italy, paved the way for the future developments in this field. It was not until 1970 that three important reviews devoted to insect chemical defenses were published [1–3]. This was followed in 1981 by the first book entirely devoted to arthropod chemical defenses [4]. Since then, numerous reviews covering particular aspects of this area of research have appeared. They will be mentioned at appropriate places in this chapter so that the reader can easily go back to earlier literature.

In this chapter, simple, volatile defensive compounds that can be identified by GC-MS are not discussed. This is also the case for alarm pheromones that may play a role in defense, particularly in social insects, since they are covered in the chapters of Millar, Francke and Keeling in this two volumes book. Protein venoms that are administered by stinging or biting are not included. Likewise, we have not surveyed the antibacterial peptides that insects synthesize in their hemolymph in reaction to bacterial aggression [5], nor the recently described "paralysins" found in some insect larvae and pupae [6]. We have also omitted the numerous examples of phytophagous insects taking up compounds from their host plant and using them for their own defense. However, some cases where the insect subsequently transforms the plant metabolites will be discussed. Literature coverage (not comprehensive) is from 1993 till mid 2003, except for topics covered in recent reviews. Earlier works are briefly mentioned when needed.

As in other areas of natural products chemistry, studies on insect chemical defenses comprise several different aspects: first come the isolation and structure determination of the compound(s) responsible for the defensive activity. The next step is the total synthesis of the identified compounds, in order to confirm the proposed structure, usually deduced from spectroscopic data only, and to get enough material for biological testing. Biosynthetic studies to determine the origin of the active compound(s) (biosynthesis by the insect itself or sequestration from the diet with or without metabolization) are sometimes performed. In some cases, the biological activities of the isolated compounds (repellency, toxicity...) and their possible pharmacological activities are also evaluated. This chapter is divided into four sections treating those different topics.

2
Isolation and Structure Determination of Insect Defensive Compounds

Obtaining a pure sample of the compound(s) responsible for the chemical defense of an insect (or of any other organism) is generally the most challenging task in this area of research. The purification and isolation of insect defensive chemicals are usually performed using the whole array of modern chromatographic techniques. When no clues are available concerning the type of molecules involved, the preliminary purification steps can be conveniently performed by size exclusion chromatography. This technique fractionates the sample molecules on the basis of their size, but other subtle interactions between solute and stationary phase are also at play [7]. Reversed phase chromatography that utilizes a non-polar stationary phase (e.g., C_8 or C_{18} bonded silica gel) and a polar mobile phase is a popular alternative to size exclusion chromatography. These two techniques limit the risk of degradation or irreversible adsorption of solutes that may happen when polar adsorbants such as silica gel and alumina are used. During all purification steps, one should be always careful to prevent the degradation of unstable compounds such as polyunsaturated long chains, enamines, some aldehydes etc., that are sensitive not only to chromatographic materials but also to oxygen. It is also important to recognize that the strategies followed to trace the compound(s) responsible for the chemical defense differ considerably depending on the location of the latter in the insect body. Generally, the compound(s) are either located in defensive glands or distributed in the whole insect body (in most cases, in the hemolymph). In the former case, it is usually possible to collect some material from the glands by "milking" the insects (as is the case for ants and leaf beetles, for example [8]), and to analyze it by using thin layer chromatography (TLC), gas chromatography (GC) or high-performance liquid chromatography (HPLC), depending on the type of compounds present and on the complexity of the mixture. In many cases, obtaining an ^{1}H NMR spectrum of the crude secretion may provide key information on the nature of the secretion components. This, in turn, should allow the researchers to devise an adequate isolation procedure. If the amount of material obtained by "milking" is not sufficient for complete identification, the partially characterized compounds can be isolated from whole insect extracts using the gland material as reference.

The problem is much more difficult when the defensive compounds are distributed throughout the insect body and no clues are available as to which type of compounds are present. In this case, a ^{1}H NMR spectrum on the insect total extract will usually not be helpful, and a reliable bioassay is needed to follow the biological activity through the fractionation process. Repellency bioassays using ants [9] or spiders [10] have been successfully used for this purpose. Chemotaxonomy can also be very helpful, as taxonomically related insects tend to produce the same kind of defensive chemicals. Thus, once the latter have been identified for a few species, the study of other species belonging to the same group is usually much simplified. A good example is provided by coc-

cinellid beetles which produce repellent and bitter alkaloids in their hemolymph (see below).

2.1 Coleoptera

2.1.1 Coccinellidae

2.1.1.1 Introduction

Amongst Coleoptera, the family Coccinellidae comprises over 5200 species worldwide and is divided into seven subfamilies (Sticholotidinae, Chilocorinae, Scymninae, Coccidulinae, Ortaliinae, Coccinellinae, and Epilachninae) which are further subdivided into tribes [11].

When disturbed or molested, these insects release small droplets of hemolymph from the tibio-femoral joints of their legs, and it is now well established that the deterrency exhibited by many species of coccinellids towards potential predators results from the presence of repellent and bitter alkaloids in that fluid [12, 13]. In ladybirds, this unpalatability is associated with a bright aposematic coloration and a characteristic smell due to 3-alkyl-2-methoxypyrazines [14, 15]. The beetles use these molecules not only to reinforce the visual alerting signal on an olfactory level, but also as aggregation pheromones [16].

While earlier reviews have covered both the chemistry and biology of coccinellids [12, 13], our knowledge of the defensive chemistry of these beetles has grown significantly during the past decade.

2.1.1.2 Tribe Coccinellini (Subfamily Coccinellinae)

The very first ladybird studied from a chemical point of view was the European *Coccinella septempunctata*, and the use of a bioassay-mediated fractionation of the methanolic extract [17] led to the isolation of the tricyclic *N*-oxide coccinelline (**1**), which was accompanied by the corresponding free base, precoccinelline (**2**) (Fig. 1) [12, 13]. Afterwards, most of the other alkaloids isolated from ladybirds were simply screened and located on TLC by spraying the plates with Dragendorff's reagent. Compounds **1** and **2** were the first representatives of a new family of alkaloids based on the 2-methylperhydro-9*b*-azaphenalene ring system. In this family, there are only three possible ring junction stereoisomers of this skeleton, and examples of each of these isomers have been isolated from coccinellids [12, 13]. From a biological point of view, Al Abassi et al. have shown that the wasp *Dinocampus coccinellae*, which parasitises the seven-spot ladybird, exploits some of these alkaloids [namely precoccinelline (**2**), myrrhine (**3**), and hippodamine (**4**)] as kairomones for locating its host [18].

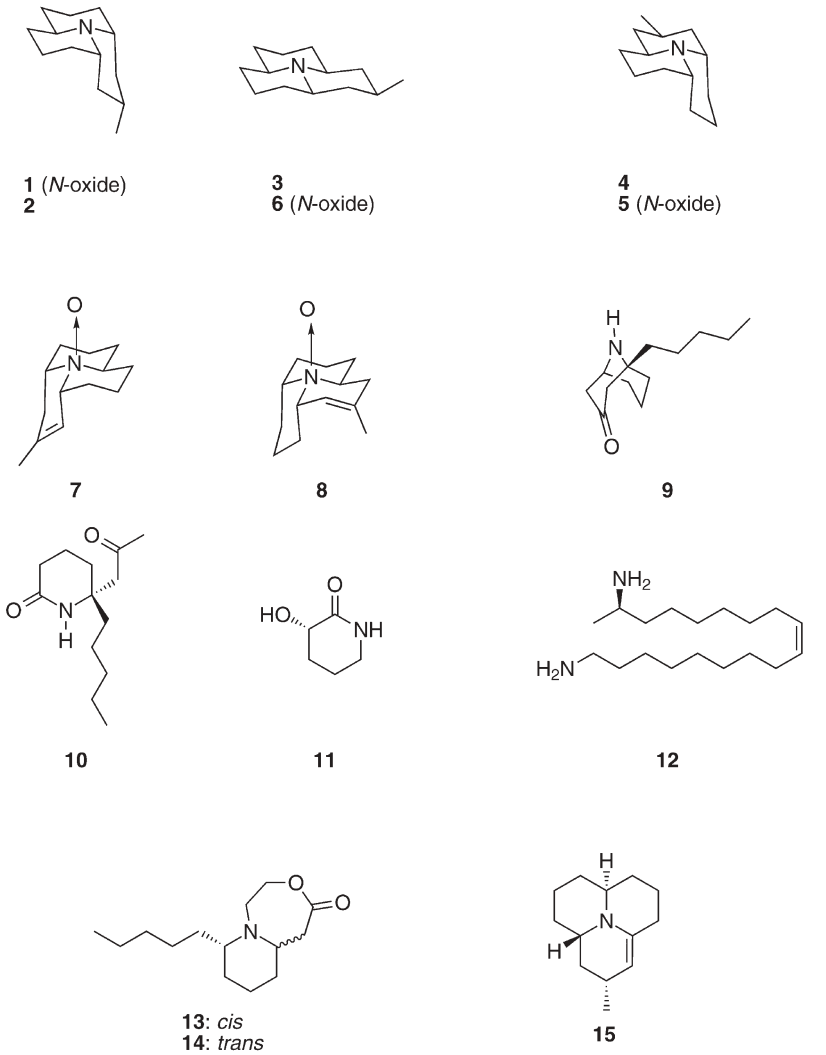


Fig. 1 Alkaloids found in the Coccinellini (except for **6**)

The unambiguous identification of these alkaloids needs the use of high field ^{1}H and ^{13}C NMR. However, although these molecules have been synthesized several times [13], the ^{1}H and ^{13}C NMR data available were either incomplete or tentative, as the assignments were made before two-dimensional methods were available [19, 20]. This situation prompted Lebrun et al. to report the complete assignments of all the signals of the ^{1}H and ^{13}C NMR spectra of **1**–**5** and of myrrhine *N*-oxide (**6**), which is not a natural compound (Fig. 1). These assignments also allowed the authors to analyze the influence of stereochemical parameters on the chemical shift of carbon and hydrogen

atoms, and to discuss the differences observed between the spectra of the free-bases, the *N*-oxides derived therefrom and the corresponding hydrochlorides [21].

The importance of ^{1}H and ^{13}C NMR assignments was once again proved in the isolation and structure determination of 2-dehydrococcinelline (**7**) from the European ladybird *Anatis ocellata* [22]. This alkaloid has indeed the same connectivity as hippocasine *N*-oxide (**8**), a defensive compound isolated nearly 30 years ago by Ayer et al. from *Hippodamia caseyi*, a ladybird indigenous to western Canada [12, 13]. At the time, neither ^{13}C nor high field ^{1}H spectrum of hippocasine *N*-oxide (**8**) had been reported, thus precluding an accurate comparison of the two molecules. However, NMR arguments unambiguously proved the structure and the relative configuration of **7**. Furthermore, a catalytic hydrogenation of 2-dehydrococcinelline (**7**) into precoccinelline (**2**) fully confirmed the proposed structure (Fig. 1) [22].

Although the alkaloid content of the European two-spot ladybird *Adalia bipunctata* had already been studied [12, 13], the analysis of a $CHCl_3$-soluble extract of *A. bipunctata* and *A. decempunctata* adults by GC-EIMS showed the presence, in addition to adaline (**9**), of a minor nitrogen-containing compound for which the name adalinine (**10**) was coined, and amounting to about 10% of the concentration level of **9** (Fig. 1) [23]. The structure of adalinine (**10**) was established by 2D NMR studies and unambiguously proved by comparison of the spectral properties of the natural compound with those of a synthetic sample [24]. The absolute configuration of natural (–)-adalinine was assigned as (*R*) by comparison of its optical rotation with that of optically pure adalinine obtained by an asymmetric total synthesis [25].

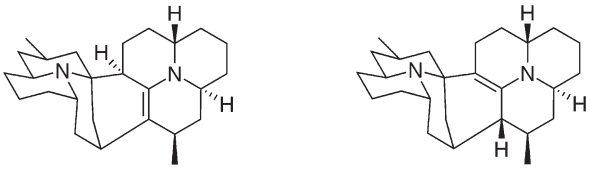
Another piperidone, (*S*)-3-hydroxypiperidin-2-one (**11**), was isolated from two coccinellid beetles *Harmonia axyridis* and *Aiolocaria hexaspilota* [26], together with harmonine (**12**), a compound previously reported from *Harmonia leis conformis*, *Harmonia 4-punctata*, *Adonia variegata*, *Semiadalia 11-notata* and *Hippodamia convergens* (Fig. 1) [12, 13].

The alkaloids of two ladybirds belonging to the genus *Calvia* have also been recently investigated. In *Calvia 14-guttata*, the major alkaloid is the piperidinic *cis* lactone **13**, which was named calvine. The corresponding *trans* lactone (2-epicalvine) **14** is also present as a minor constituent (about 10%) (Fig. 1). The structure of these alkaloids has been determined on the basis of their spectral properties and confirmed by a total synthesis of **13** and **14** [27]. Two different enantioselective syntheses of (+)-calvine and of (+)-2-epicalvine allowed the authors to assign the absolute configuration (2*S*,6*S*) to natural (+)-calvine and (2*R*,6*S*) to natural (+)-2-epicalvine [28]. Furthermore, a GC-EIMS injection of a fresh secretion of one *C. 14-guttata* beetle and of an authentic sample of adaline (**9**) showed also the presence of traces of the latter in the hemolymph of the ladybird [29]. Specimens of the closely related species *C. 10-guttata* were also collected and analyzed. Three alkaloids were recognized by GC-EIMS [27]: calvine (**13**), 2-epicalvine (**14**) and propyleine (**15**), an alkaloid already isolated from *Propylaea 14-punctata* [12, 13].

2.1.1.3 Tribe Psylloborini (Subfamily Coccinellinae)

Beside the Coccinellini, the only other tribe in the subfamily Coccinellinae studied from a chemical point of view, is the Psylloborini. *Psyllobora 22-punctata* [30], *Halyzia 26-guttata*, *Vibidia 12-guttata* [31] and *Cycloneda sanguinea* (unpublished results) were the four species investigated for their alkaloid content. This has led to the isolation and structure determination of psylloborine A (**16**) and isopsylloborine A (**17**), two closely related azaphenalene dimers (Fig. 2) [30, 31]. The structural assignments of those molecules were once again essentially based on 2D-NMR methods.

It is probably not by chance that the only true dimeric alkaloids to have been found till now in the Coccinellidae originate from Psylloborini species, but it is too early to draw any chemotaxonomic conclusions from these data.



16 17

Fig. 2 Alkaloids found in the Psylloborini

2.1.1.4 Tribe Chilocorini (Subfamily Chilocorinae)

In the subfamily Chilocorinae, only three species of one tribe (the Chilocorini) have been chemically studied. This has led to the isolation and structure determination of several alkaloids made up of the familiar 2-methylperhydro-9*b*-azaphenalene skeleton linked to an azaacenaphthylene ring system, which probably has a biogenetic origin similar to that of the former, but a different cyclization pattern (Fig. 3).

The first alkaloid based on these ring systems was discovered in 1992 in the European species *Exochomus quadripustulatus*. After recrystallization of its hydrochloride salt, a single-crystal X-ray diffraction study of exochomine (**18**) established the structure and absolute configuration of this alkaloid [32].

The second Chilocorini species studied, *Chilocorus cacti*, contained several alkaloids closely related to the former. The structure of the heptacyclic chilocorine A (**19**) was deduced from a series of NMR experiment (DEPT, HMQC, DQ-COSY, TOCSY) and from a comparison of its spectral properties with those of exochomine (**18**) from which it differ by having two C-C linkages between the two tricyclic partners. However, the configuration at the asymmetric center in the azaacenaphthylene ring was not established [33].

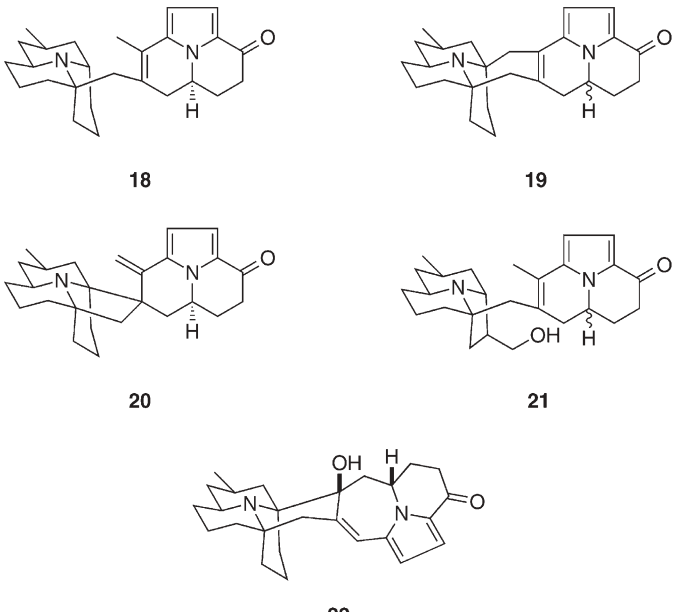


Fig. 3 Alkaloids found in the Chilocorini

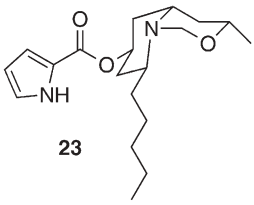
A third alkaloid of this type, chilocorine B (**20**), was isolated from *C. cacti*. In this molecule, NMR experiments showed that the 2-methylperhydro-9*b*-azaphenalene and the 3,4-dimethyloctahydro-8*b*-azaacenaphtylene subunits are linked in a spirocyclic fashion. An X-ray diffraction analysis fully determined the structure and relative stereochemistry of this alkaloid [34].

Furthermore, HPLC analyses of the crude extract of *C. cacti* revealed the presence of another minor compound, which showed a UV spectrum similar to those of chilocorines A and B. By several cycles of preparative TLC, chilocorine C (**21**) was isolated. Its hexacyclic structure was proposed on the basis of UV, IR, NMR and mass spectral evidence. Interestingly, the structure of chilocorine C incorporates a 1-(hydroxymethyl)-7-methylperhydro-8*b*-azaacenaphtylene skeleton in place of the classical 2-methylperhydro-9*b*-azaphenalene moiety [35].

Finally, the examination of *Chilocorus renipustulatus*, a European member of the *Chilocorus* genus, led to the isolation and structure determination of chilocorine D (**22**), a new heptacyclic alkaloid constituted of a hippodamine moiety linked to a modified octahydro-azaacenaphthylene skeleton encompassing a seven-membered ring [36].

2.1.1.5 Tribe Hyperaspini (Subfamily Scymninae)

In the subfamily Scymninae, only one species of the tribe Hyperaspini has been reported to contain alkaloids, that is the European *Hyperaspis campestris*.



23

Fig. 4 Structure of hyperaspine

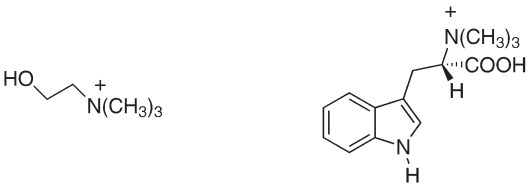
This ladybird is protected by hyperaspine (**23**) (Fig. 4), the structure of which was deduced from its 1D and 2D NMR data. These spectra disclosed the presence in the molecule of a 3-oxaquinolizidine skeleton substituted by a 2-pyrrolecarboxylate moiety, by a secondary methyl group, and by a *n*-pentyl side chain. The *cis*-fused ring conformation and the relative configuration of **23** were based on IR and 2D NMR methods [37].

Hyperaspine (**23**) is the first 3-oxaquinolizidine alkaloid reported so far from ladybird beetles. Its skeleton, however, which is based on a chain of 13 carbon atoms, is biosynthetically related to those of the homotropane and perhydroazaphenalene alkaloid already isolated from ladybirds [38].

2.1.1.6 Tribe Epilachnini (Subfamily Epilachninae)

In the subfamily Epilachninae, only the predominantly phytophagous tribe Epilachnini has been studied till now.

Purifications of the methanolic extract of adults and larvae of the New Guinean species *Epilachna signatipennis* led to the isolation and structure determination of three nitrogen-containing secondary metabolites: choline (**24**), L-hypaphorine (**25**), and signatipennine (**26**) (Fig. 5). From a biosynthetic



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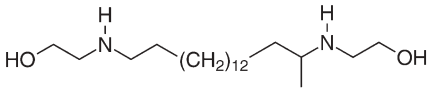

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Fig. 5 Nitrogen-containing secondary metabolites found in *Epilachna signatipennis*

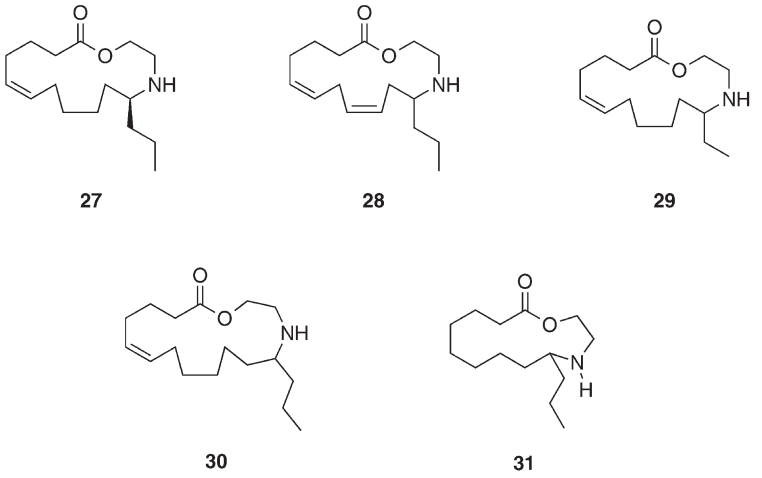


Fig. 6 Azamacrolides from the pupal hairs of *Epilachna varivestis*

point of view, it can be assumed that the latter might be derived from L-serine and stearic acid [39].

Microscopic examination showed the pupae of the Mexican bean beetle *Epilachna varivestis* to be covered with glandular hairs, each having a droplet of oil at its tip. Attygalle and co-workers undertook a chemical analysis of this secretion, which resulted in the identification of a novel family of alkaloids, the azamacrolides [40]. Five compounds (**27**–**31**) (Fig. 6) were identified by GC-EIMS from the secretion, with epilachnene (**27**) comprising over 90% of the volatile material.

The structure of this molecule was established by a combination of NMR, mass spectral and chemical studies [40], and was further confirmed by several total syntheses [13]. One of those syntheses, starting from (*R*)- or (*S*)-norvaline, furnished samples of enantiomerically pure (*R*)- and (*S*)-epilachnene [41]. The diastereoisomeric α-methoxy-α-trifluoromethylphenylacetyl amides of these synthetic samples were well resolved by gas chromatography. Analogous derivatization and gas chromatographic analyses of a sample of epilachnene from the pupal secretion of the beetle established that the natural product is (*S*)-epilachnene [41]. Moreover, both (*R*) and (*S*) enantiomers proved to be deterrent in a feeding bioassay with a predaceous coccinellid beetle (*Harmonia axyridis*) and active in a topical irritancy test with the cockroach *Periplaneta americana* [42].

On the other hand, adults of *E. varivestis* turned out to be the most versatile alkaloid producer of all ladybird species so far examined. Indeed, not less than 12 alkaloids have been isolated from this beetle, including euphococcinine (**32**), a complex mixture of simple piperidines (**33**–**36**), 2-phenylethylamine (**37**) and long chain pyrrolidines (**38**–**41**) (Fig. 7) [43–45]. The syntheses of optically pure (2*S*,12′*R*)-2-(12′-aminotridecyl)-pyrrolidine (**38**) and (2*S*,12′*R*)-1-(2″-hydroxy-

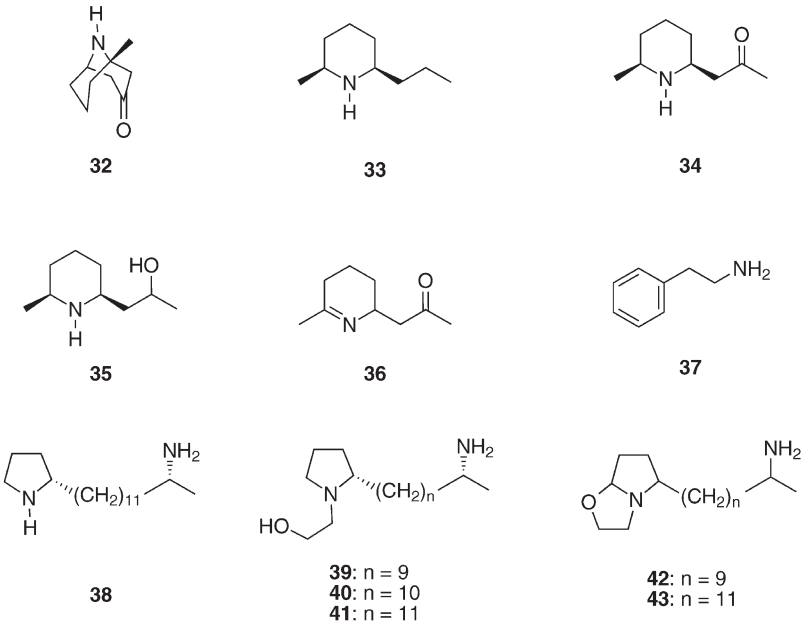


Fig. 7 Alkaloids found in adults of *Epilachna varivestis* and *E. borealis*

ethyl)-2-(12′-aminotridecyl)-pyrrolidine (**41**) allowed Shi et al. to assign the (2*S*,12′*R*) absolute configuration to these alkaloids by a comparison of the ^{1}H NMR spectra of MTPA derivatives of natural alkaloids with those of the synthetic standards [46, 47]. Other pyrrolidine alkaloids (**42** and **43**), structurally related to monocyclic **39** and **41**, were also isolated from adults of two species of ladybird beetles, *Epilachna varivestis* and *E. borealis*. Those bicyclic pyrrolidinooxazolidines were characterized on the basis of spectrometric and synthetic investigations [48].

GC analyses of the pupal secretion of *E. borealis* have indicated the presence of vitamin E acetate and other tocopherol derivatives [49, 50]. However, in tests with ants, these compounds proved to be essentially inactive, whereas the secretion itself was potently deterrent. To find and identify the active components in the pupal *Epilachna borealis* secretion, NMR spectroscopic studies on the fresh secretion were carried out. One and two-dimensional ^{1}H NMR experiments revealed that the tocopheryl acetates account for only a relatively small percentage of the beetles' total secretion (20%), whereas the major components represented a group of previously undetected compounds. By analysis of the COSY, HSQC and HMBC spectra of the mixture, these components were shown to be esters and amides derived from three (ω-1)-(2-hydroxyethylamino)alkanoic acids **44**–**46**. HPLC analyses coupled to a mass spectrometric detector revealed that the secretion contain a highly diverse mixture of macrocyclic polyamines, the polyazamacrolides (PAMLs) **47**–**52** (Fig. 8).

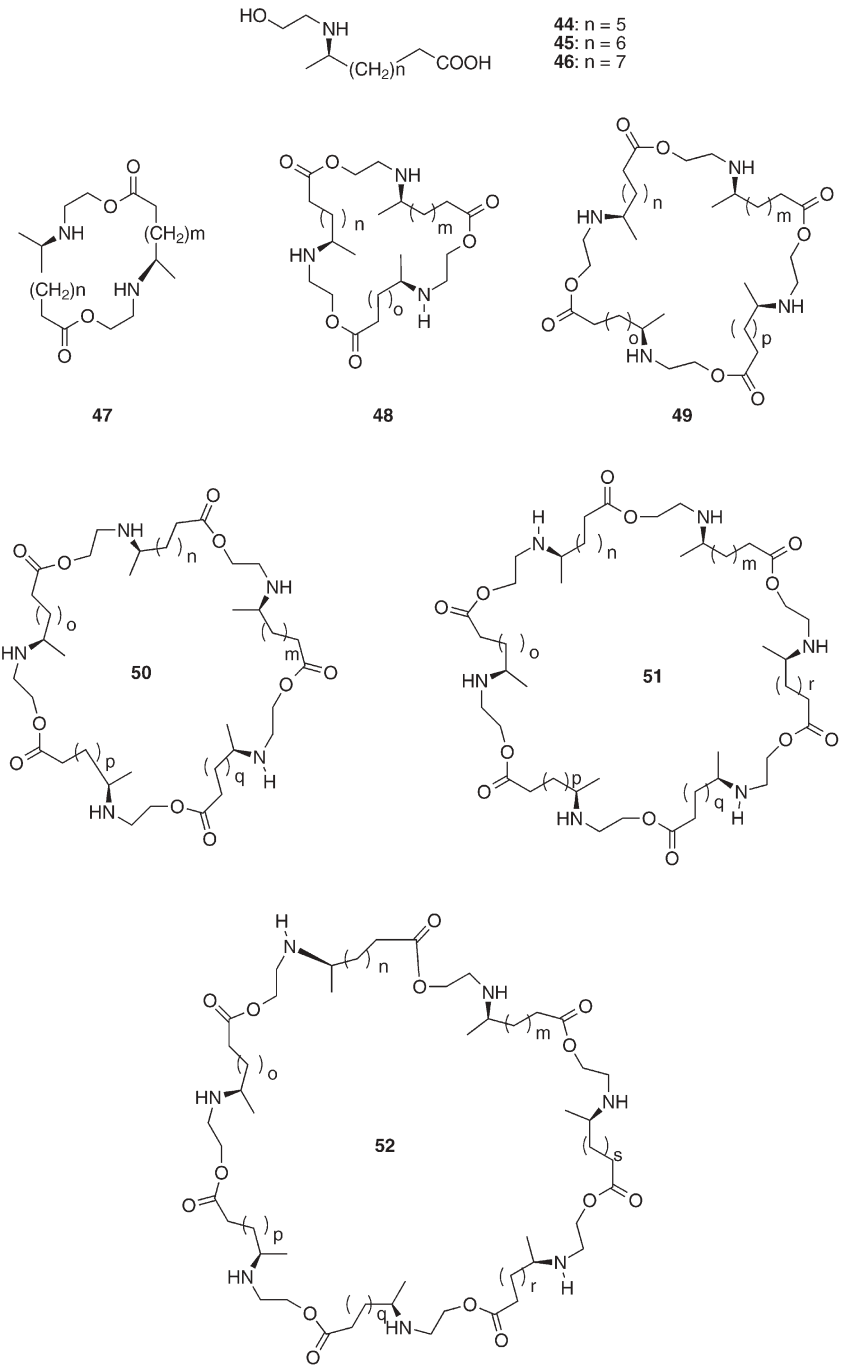


Fig. 8 Building blocks and PAMLs found in pupae of *Epilachna borealis*. In these formulas, each of the variables m to s can have the values 5, 6 or 7

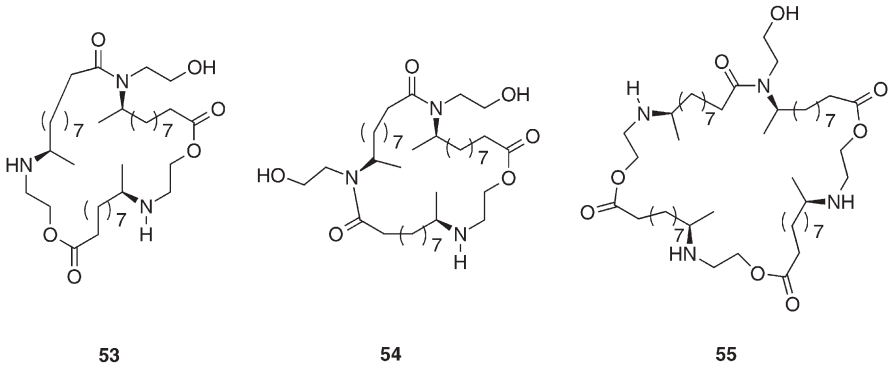


Fig. 9 Amide isomers isolated from pupae of *Epilachna borealis*

The major components are series of homologous trimers, tetramers, and pentamers of the three acids **44–46**, along with smaller quantities of dimers, hexamers, and heptamers. Furthermore, the secretion contains several isomers of each oligomer, furnishing a combinatorial library of several hundred macrocyclic polyamines [51, 52]. Using repeated preparative HPLC fractionation, the most abundant trimeric, tetrameric and pentameric earliest-eluting compounds were isolated. One and two-dimensional ^{1}H NMR spectroscopic analyses showed that these molecules were the symmetric macrocyclic lactones **48**, **49**, and **50** (m, n, o, p, q=7) derived from three, four or five units, respectively, of acid **46**. Moreover, using preparative HPLC and NMR methods, various amide isomers, such as **53**, **54**, and **55** (Fig. 9) were also isolated and characterized [51, 52].

The structural assignments were confirmed by several total syntheses of some PAMLs [51, 53], or analogues [54]. Gas chromatographic comparison of MTPA derivatives of synthetic enantiomerically pure aza-lactones with the MTPA derivatives of aza-lactones prepared from the natural material established that the polyazamacrolides have the (*R*) configuration at all stereogenic centers [55]. Despite the structural diversity of the natural material, the fresh secretion of *Epilachna borealis* does not contain detectable amounts of the monomeric azamacrolides, and only very low concentrations of open chain oligomers. Consequently, whereas the three building blocks **44–46** appear to be randomly incorporated into the oligomers, the oligomerization itself seems to be a well controlled process [51, 52].

In *Subcoccinella 24-punctata*, pupal surface also bears glandular hairs that produce a secretion principally made of the three PAMLs **58–60**, which correspond to the three possible dimers of the two unsaturated (ω-3)-(2-hydroxyethylamino)acids **56** and **57** (Fig. 10) [56]. The structure of the three PAMLs were based on NMR investigations and HPLC analyses, and were fully confirmed by total synthesis [57]. Smaller amounts of isomeric amides **61–64** and of the four possible cyclic trimers **65–68** were also detected (Figs. 10 and 11).

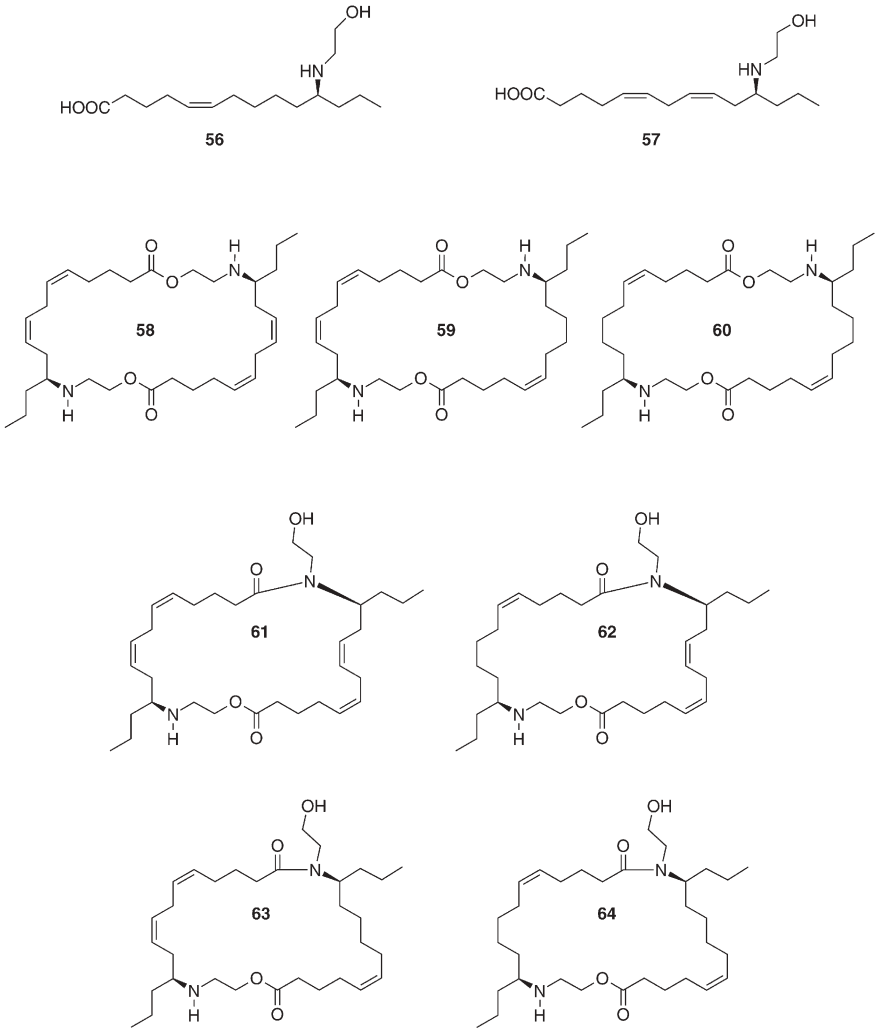


Fig. 10 Components identified in pupae of *Subcoccinella 24-punctata*, and their building blocks

In addition, the all-(*S*) absolute configuration of the *Subcoccinella 24-punctata* macrocycles was determined by chiral GC-MS comparison of derivatives of the natural material with optically pure synthetic samples [56]. Furthermore, it was demonstrated that this secretion serves as a potent antipredator defense: contact with it elicited pronounced cleaning activity by the predatory ant *Crematogaster lineolata*. Additionally, application of the secretion to palatable food items rendered them unacceptable to the ant [58].

As in *Epilachna varivestis*, pupae and adults of *Subcoccinella 24-punctata* produces totally different alkaloids. Thus, N_{α}-quinaldyl-L-arginine (**69**) (Fig. 11) was

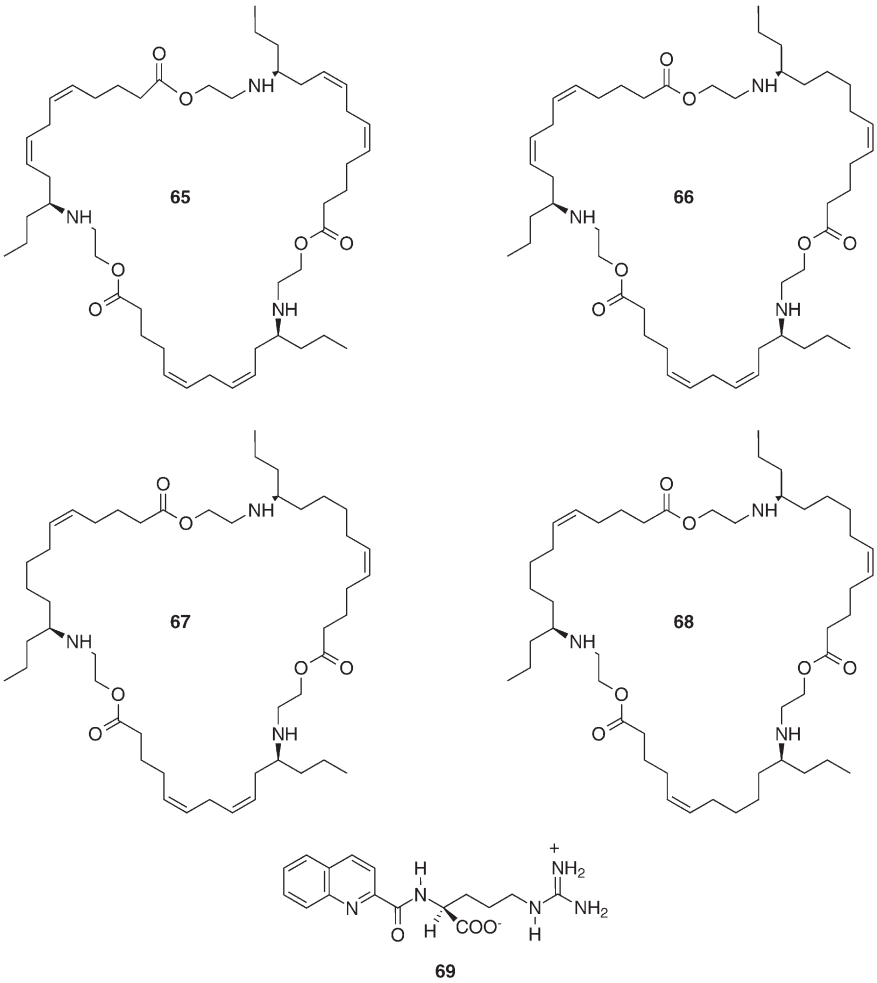


Fig. 11 Components identified in pupae and adults of *Subcoccinella 24-punctata*

isolated from adults of the latter beetle and was shown to be repulsive against *Myrmica rubra*. From a biogenetic aspect, this compound was not from dietary origin and must therefore be synthesized by the insect itself [59].

2.1.2 Chrysomelidae

In the family Chrysomelidae (leaf beetles), the presence of defensive glands located on the elytra and on the pronotum has been reported for adults of 4 of the 19 subfamilies. As these beetles are phytophagous, it is not surprising that their host plant chemistry frequently plays a prominent role in their defensive

strategy. However, the diversity of defensive chemicals produced by the insects themselves or through hemi-biosynthesis is amazing. This is illustrated by the identification in the defensive secretions of adult leaf beetles of cardenolide glycosides, polyoxygenated steroid glycosides, isoxazolinone glucosides esterified by one or two 3-nitropropanoate moieties, β,γ-unsaturated amino acid derivatives, and pyrrolizidine alkaloids. This topic has been reviewed several times between 1988 and 1994 [60–63] and will not be discussed here. Since then, the major breakthrough in this area was the discovery of oleanane glycosides in the defensive glands of New World adult chrysomelids belonging to the genera *Platyphora*, *Desmogramma*, *Leptinotarsa*, and *Labidomera* [64–67]. Till now, six oleanane glycosides (**70–75**) (Fig. 12) have been fully characterized from four species of leaf beetles. Recent HPLC-MS analyses of the secretions of about 30 other neotropical chrysomelids have shown that oleanane glycosides occur widely in these beetles [67]. This discovery was intriguing since it is well known that insects are unable to biosynthesize pentacyclic triterpenes. Moreover, the

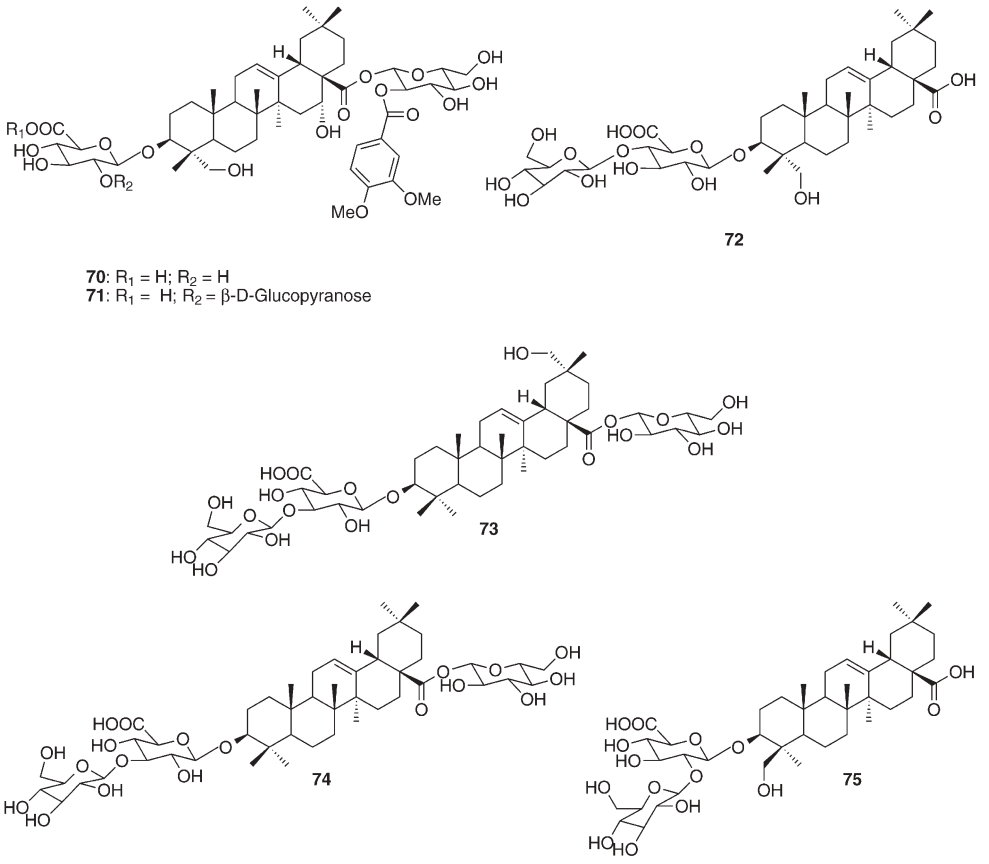


Fig. 12 Structures of oleanane glycosides from chrysomelid beetles

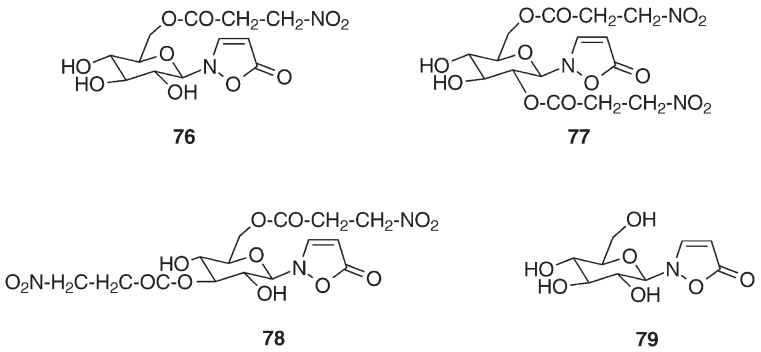


Fig. 13 Structures of isoxazolinone glycosides from chrysomelid beetles

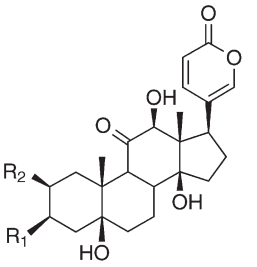
host plants of these beetles were found to be devoid of these glycosides [64]. It has been recently shown that these compounds are biosynthesized by the beetles from a dietary ubiquitous precursor, β-amyrin [68]. Besides oleanane glycosides, the defensive secretion of several *Platyphora* species also contains pyrrolizidine alkaloids (PAs) that are known to be efficient chemical defenses [69, 70]. It should be recalled that the sequestration of PAs by Old World *Oreina* spp., which are primarily cardenolide producers, had already been documented [71, 72]. However, the mechanisms of PAs sequestration and, in some cases, of partial metabolization differ considerably between the two genera [69]. This topic was surveyed in detail in a recent review [73] and will not be discussed further.

Adult secretions of four Japanese Chrysomelinae, *Gastrophysa atrocyanea, Plagiodera versicolora distincta, Chrysomela vigintipunctata costella*, and *Gastrolina depressa* were recently shown [74] to contain the same 3-nitropropanoyl-isoxazolinone glucosides (**76**–**78**) (Fig. 13) we had previously reported from several *Chrysomela* spp. and *Gastrophysa viridula* from Europe [60, 62, 75]. Free 3-nitropropanoic acid is a well-known vertebrate toxin, and recent experiments have shown that upon emission of the defensive secretions, the 3-nitropropanoyl-glucosides come into contact with an esterase which releases the free acid, thus enhancing its toxicity [76]. This explains why the concentration of dinitropropanoic acid glucosides **77** and **78**, which are the major compounds of the freshly emitted secretion, drops with time whereas the concentrations of the mononitropropanoyl glucoside **76** and of the non-esterified glucoside **79** increase. The distribution of defensive compounds in leaf beetles has been recently used to propose a classification of Holarctic and Neotropical leaf beetles genera in three groups that were recognized as natural supra-generic taxa [67, 77].

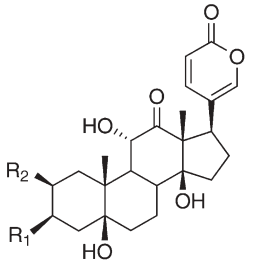
2.1.3 Other Beetles

In recent years, few studies have been devoted to families of beetles other than the two discussed above. The Cornell group, however, has pursued the investi-

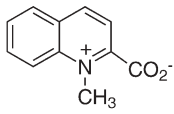
gation of the remarkable defensive strategies of fireflies (family Lampyridae, genus *Photinus*), which are known to be distasteful to a number of predators. This property is attributable, at least in part, to the presence of toxic steroidal pyrones (lucibufagins) in the beetles' tissues (references in [78]). Fireflies of another genus (*Photuris*) are also protected by lucibufagins, but it was recently shown by Eisner et al. that it is the female *Photuris* that acquires these compounds by feeding on male *Photinus* [78]. Moreover, the lucibufagins found in female *Photuris versicolor* are somewhat different from those of their male *Photinus ignitus* prey. Analyses of whole body extracts of the latter showed the presence of eight non-glycosylated lucibufagins (**80–87**) (Fig. 14), most of them being oxidized at C-3 and C-5 of the steroid skeleton. In contrast, the female *Photuris versicolor* that had preyed upon male *Photinus ignitus* contained six major lucibufagins (**80**, **81**, **83**, **88–90**). Three of these compounds were not present in their prey, and comprised the new xyloside **89**, and two other lucibufagins with a trioxygenated A ring (**88** and **90**). These results indicate that *Photuris* females transform the acquired lucibufagins by glycosidation and oxidation, thus increasing the polarity of the compounds, which could affect their transportability [79]. It was also discovered that females of *Photuris versicolor* contained another defensive compound in their blood, namely *N*-methylquinolinium 2-carboxylate (**91**), which was present whether they fed on *Photinus* or not. According to these authors, this compound strikingly reinforces the



80: R_1 = OH; R_2 = OH
83: R_1 = OH; R_2 = H
85: R_1 = OAc; R_2 = H
88: R_1 = OH; R_2 = OAc



81: R_1 = OH; R_2 = OH
82: R_1 = OAc; R_2 = H
84: R_1 = OH; R_2 = H
86: R_1 = OAc; R_2 = OAc
87: R_1 = OAc; R_2 = H
89: R_1 = O-β-D-Xyl; R_2 = H
90: R_1 = OH; R_2 = OAc



91

Fig. 14 Lucibufagins and *N*-methylquinolinium 2-carboxylate from fireflies

protective role of the lucibufagins [80]. It is worth noting that a compound having a quinoline 2-carboxylate moiety has also been isolated from a coccinellid beetle, *Subcoccinella 24-punctata* [59]. The same authors have also studied the chemical egg defense in *Photuris* spp. They found that *Photuris* females that fed on *Photinus* males endowed their eggs with both lucibufagins and the betaine **91**. In contrast, those females that did not feed on *Photinus* laid eggs that contained the betaine but were almost devoid of lucibufagins. Apparently, the use of both endogenous and exogenous chemicals allows the females to maximize the protection they offer to their offspring [81].

In addition to aromatic compounds secreted by their pygidial glands, dytiscid beetles possess a pair of exocrine prothoracic glands that discharge a milky fluid upon mechanical disturbance or predation [3, 82]. This fluid has been shown to contain steroid derivatives, e.g., cybisterone (**92**) (Fig. 15) which display anesthetic and toxic effects against a variety of mammals and fishes [3, 82, 83]. Recently, three further species of dytiscids have been studied. *Thermonectus marmoratus* produces as major steroid an 18-oxygenated pregnane, mirasorvone (**93**) (Fig. 15) [84]. Analysis of the prothoracic gland secretion of *Graphoderus cinereus* (subfamily Dytiscinae) by GC/MS after trimethylsilylation showed the presence of one major steroid (about 90% of the total peak area), identified as 3α,11α-dihydroxy-5β-pregnan-20-one (**94**); the same analysis performed on *Laccophilus minutus* (subfamily Laccophilinae) showed the presence of two major steroids, 3α,12α-dihydroxy-5β-pregnan-20-one (**95**) and 3α-hydroxy-5β-pregnan-20-one (**96**) (Fig. 15) [85]. It is the first time that saturated pregnanes are reported in the defensive glands of dytiscid beetles.

Palasonin (**97**), a cantharidin-related toxin, has recently been detected in the hemolymph and tissues of several families of beetles (Meloidae, Cleridae, Staphylinidae) that produce cantharidin or feed on cantharidin containing an-

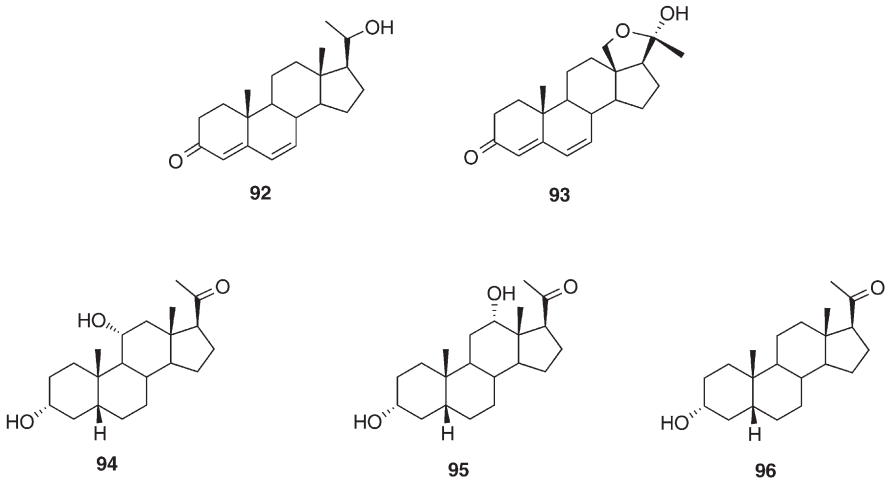


Fig. 15 Pregnane derivatives from dytiscid beetles

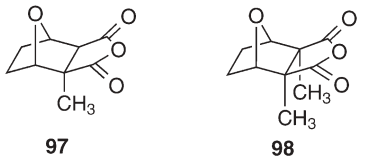


Fig. 16 Palasonin and cantharidin from Meloidae

imals (**98**) (Fig. 16). In contrast to plants which produce (*S*)-palasonin with a high ee (~99%), the insects contained palasonin of low ee (0–50%), with the (*R*)-(+)-enantiomer prevailing [86]. Finally, we should mention a review discussing cantharidin biological activity, mode of action, occurrence, and attractivity to cantharophilous insects. The transfer of cantharidin through developmental stages and the interspecific transfer of cantharidin were also covered [87].

Rove beetles of the sub-tribes Staphylinina and Philonthina are carnivorous insects containing paired abdominal defensive glands that secrete a complex mixture of volatile substances among which iridoids are prominent [88–90]. In particular, rove beetles of the genus *Philonthus* produce a defensive secretion dominated by the alkaloid actinidine (**99**) with minor amounts of chrysomelidial (**100**), plagiodial (**101**) and 8-hydroxygeraniol (**102**) (Fig. 17) [88]. With the exception of the large abundance of actinidine, such a pattern of iridoids is similar to that found in phytophagous leaf beetles [75]. To compare the monoterpene biosynthesis in these two groups of insects, Weibel et al. carried out in vitro incubation experiments of chiral deuterium-labeled substrates on tergal glands of *Philonthus* spp. specimens [91]. They demonstrated that these insects utilize the same monoterpenoid precursors and follow a comparable overall biosynthetic pathway as the leaf beetles [92, 93]. Interestingly, it was observed that the iridoid biosynthetic route exhibits distinct stereochemical

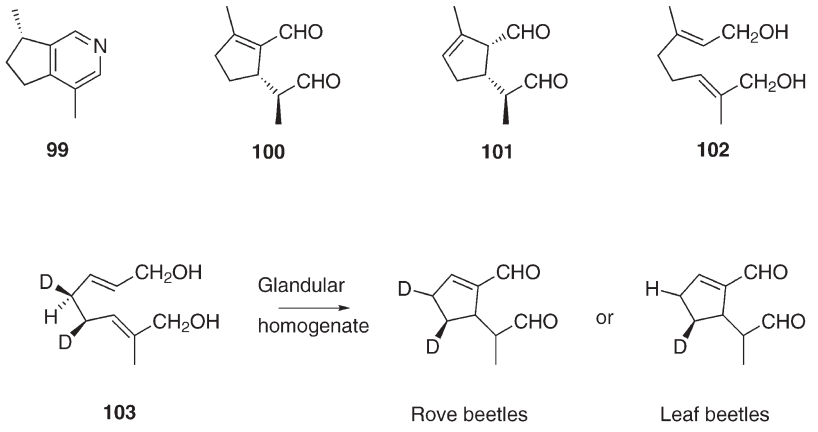
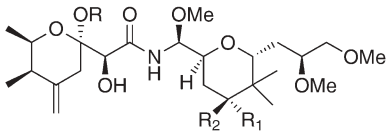


Fig. 17 Iridoid derivatives from rove beetles and divergent biosynthesis in chrysomelids and in rove beetles



104: R = Me, R_1 = H, R_2 = OH

105: R = Me, R_1 = R_2 = O

106: R = H, R_1 = H, R_2 = OH

Fig. 18 Pederin and related compounds from *Paederus* beetles

differences in carnivorous and in phytophagous beetles. Indeed, the ring closure of the deuterated precursor **103** proceeded with loss of the C(5)-H_R proton in rove beetles and with loss of the C(5)-H_S proton in leaf beetles (Fig. 17) [91].

The toxic effect of various species of rove beetles pertaining to the genus *Paederus* on the skin and eyes of mammals, including man, are due to the presence in their hemolymph of three vesicant amides: pederin (**104**), pederone (**105**) and pseudopederin (**106**) (Fig. 18) [94, 95], pederin being the major and most active of the three compounds. Their structure determination [96, 97] revealed rather unique substances until similar natural products with comparable biological activities were isolated from sponges of the genera *Mycale* [98, 99], *Stylinos* [100] and *Theonella* [101–104].

Only *Paederus* females are able to accumulate pederin. Moreover, they are polymorphic with regard to their ability to produce the toxin. (+)-Females endow their eggs with pederin whereas (–)-females lay eggs devoid of the substance [105, 106]. This biosynthetic capability is horizontally transmitted by ingestion of (+)-eggs and is markedly reduced through antibiotic treatment, heating or freezing [107, 108]. All these facts led Kellner to suggest that microorganisms are involved in the production of this unusual group of compounds. The endosymbiotic hypothesis has been supported by analysis of endobacterial 16S rDNA from (+)- and (–)-females of *P. sabaeus* that indicated a clear correlation between pederin biosynthesis ability and presence of ɣ-protobacteria belonging to the *Pseudomonas aeruginosa* group [109, 110]. Pederin appears to be the first insect defensive substance of endosymbiotic origin.

2.2 Hymenoptera

2.2.1 Formicidae

The vast majority of ant species use chemical secretions for defensive and offensive purposes [111–113]. Stinging is the most notorious, although not the only defensive mechanism in ants. Indeed, many ants do not sting, the sting

being reduced or completely absent as in the Formicinae which eject a secretion containing as much as 65% of formic acid [111]. Other ants possess a modified sting (e.g., spatula or drawing pen) which facilitates smearing or spilling the venom. In these ants either the poison gland secrete alkaloidal venoms or chemical defense is taken over by the Dufour gland as in the *Crematogaster* that secrete lipidic contact venoms [114].

In stinging ants, two major glands are associated with the sting, the poison and the Dufour glands. The poison gland secretes the venom that accumulates in the poison sac before being injected into the prey or the enemy. The constituents of this gland are usually proteinous. However in some groups (e.g., Myrmicinae) these protein venoms have been superseded by low-molecular weight alkaloids. In a few cases non-alkaloidal defensive compounds produced by the Dufour gland have also been isolated [114].

In this chapter, we will limit ourselves to non-proteinous poisons produced by either the venom or Dufour glands. Information on proteinous venoms can be found in the review of Schmidt [111] and the book of Blum [4].

2.2.1.1 Alkaloids

Alkaloids from ants have been comprehensively reviewed in 1987 by Numata and Ibuka [115], in 1990 by Braekman and Daloze [116] and more recently by Leclercq et al. [114]. We will thus report only the literature that has been published since and until October 2003.

Several species pertaining to the sub-family Myrmicinae (e.g., *Solenopsis* spp., *Monomorium* spp.) are characterized by a venom rich in dialkylated saturated nitrogen heterocycles (e.g., piperidine, pyrrolidine, indolizidine, pyrrolizidine). Exhaustive lists of these alkaloids have already been published [114–116]. Since then, only a few more of these alkaloids have been reported from a few further species.

Examination of the venom content of the workers of *Solenopsis maboya* revealed the presence of (5*Z*,9*Z*)-3-butyl-5-methylindolizidine (**107**), (5*E*,9*E*)-3-butyl-5-methylindolizidine (**108**) and *trans*-2-methyl-6-nonylpiperidine (**109**), while analysis of the extract of the queens revealed the presence of a single alkaloid, *cis*-2-heptyl-5-methylpiperidine (**110**) (Fig. 19) [117]. Such caste-specific alkaloidic contents has been already been found in other *Solenopsis* ants [118, 119] and suggest different roles for these compounds in each caste.

It had been already reported, that the venom of the fire ant *Solenopsis invicta* is composed of 2-methyl-6-alkyl- or 2-methyl-6-alkenylpiperidines, the carbon chain of which has an odd number of carbon atoms in the range of 11 to 17 [115, 116]. Recently, Deslippe and Guo [120] reported that in workers of *S. invicta* from Texas the relative abundance of each alkaloid was highly correlated with worker size as well as with the ratios of saturated to unsaturated alkaloids. Moreover, young and old workers produced less venom than ants of intermediate age

and ratios of saturated to unsaturated alkaloids increased significantly with worker age.

Until now, a total of over 500 lipophilic alkaloids pertaining to 22 structural classes have been detected in skin extracts of certain Neotropical frogs and toads [121, 122]. Such alkaloids were called "dendrobatid alkaloids" after the family Dendrobatidae, from which they were first isolated. Initially, they were thought to be synthesized by the frogs. However, it now seems that all dendrobatid alkaloids originate from their diet [119, 122–125], which for such frogs consists of small arthropods. Among these, myrmicine ants are likely sources of several of the "dendrobatid alkaloids" as many of them have been isolated from such type of ants. A list of ant alkaloids detected in skin extracts of alkaloid-containing frogs and toads has been published by Jones et al. [123]. In the same paper the isolation of 3 further alkaloids [4-methyl-6-propylquinolizidine (**111**), *cis*-5-methyl-2-propyldecahydroquinoline (**112**), *cis*-2-methyl-5-propyldecahydroquinoline (**113**)] from a Brazilian myrmicine ant of the *Solenopsis* (*Diplorhoptrum*) sp. *picea* group was also reported (Fig. 19). The same mixture of **111**, **112**, and **113** was found in frogs of the species *Mantella betsileo* [123].

Since then, the dietary hypothesis for the origin of several classes of lipophilic alkaloids in frog skins has been strengthened several times. Thus,

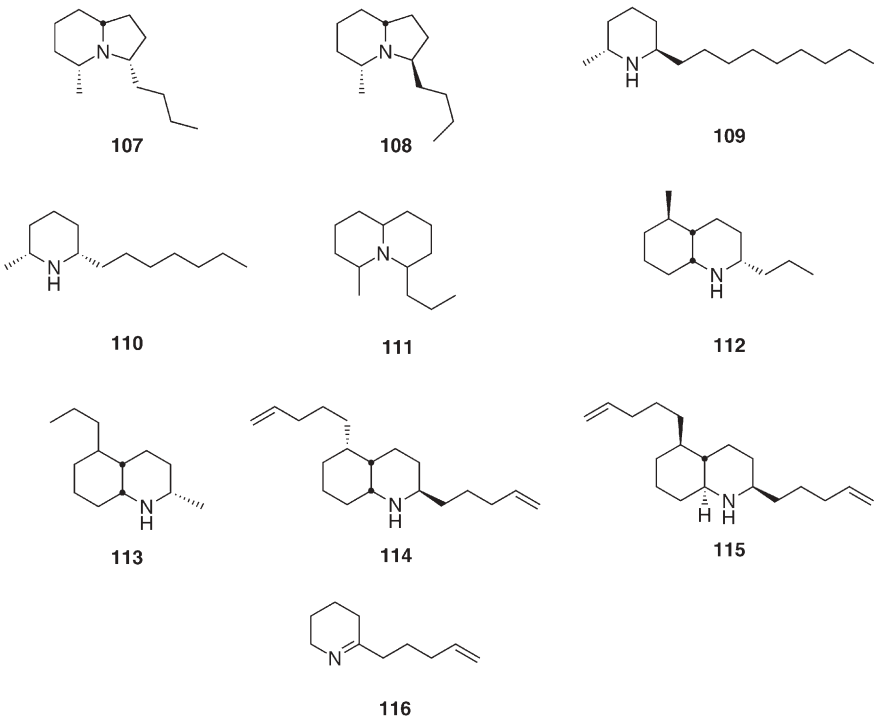


Fig. 19 Defensive alkaloids from myrmicine ants (structures **107** to **116**)

extracts of virgin queens of *Solenopsis* (*Diplorhoptrum*) *azteca* collected in Puerto Rico were found to contain the decahydroquinolines 5-epi-*cis*-275B′ (**114**) and 5-epi-*trans*-275B (**115**) while both workers and queens contained the piperideine **116** (Fig. 19) [119]. This is the first isolation from an ant of 2,5-disubstituted decahydroquinolines, a group of compounds that are frequently found in frog skins.

In an effort to identify possible sources of the 16 alkaloids found in the skin of the Panamanian poison frog *Dendrobates auratus*, ants from a total of 61 terrestrial nests were analyzed [124]. The alate queens of one species of myrmicine ants (*Solenopsis* (*Diplorhoptrum*) sp.) collected at Cerro Ancon were found to contain the decahydroquinoline (–)-*cis*-195A (**112**) which was also present as a minor alkaloid in the skin of the microsympatric population of *D. auratus*. Moreover, from wingless ants of two nests collected at Isla Taboga and identified as *Megalomyrmex silvestri*, the same workers isolated the stereoisomeric 3,5-disubstituted pyrrolizidines *cis*-251 K (**117**) and *trans*-251 K (**118**) in the same ratio 3:1 that was present in the skin of a microsympatric population of *D. auratus* (Fig. 20) [124].

Since the isolation of anabaseine (**119**) in 1981 by Wheeler et al. [126] in the venom gland of *Aphaenogaster fulva* and *A. tennesseensis*, this alkaloid together with anabasine (**120**) has been found in the poison gland of a few other myrmicine ants (Fig. 20) [115]. Recently, the absolute configuration of anabasine from further *Aphaenogaster* and *Messor* ants has been determined. In *M. sanctus* only (2′*S*)-anabasine was present whereas in *A. subterranea* and *A. miamiana*, (2′*S*)-anabasine was determined to have an *ee* of 78 and 24% respectively. Anabaseine was also detected in *A. subterranea* and *A. senilis* [127].

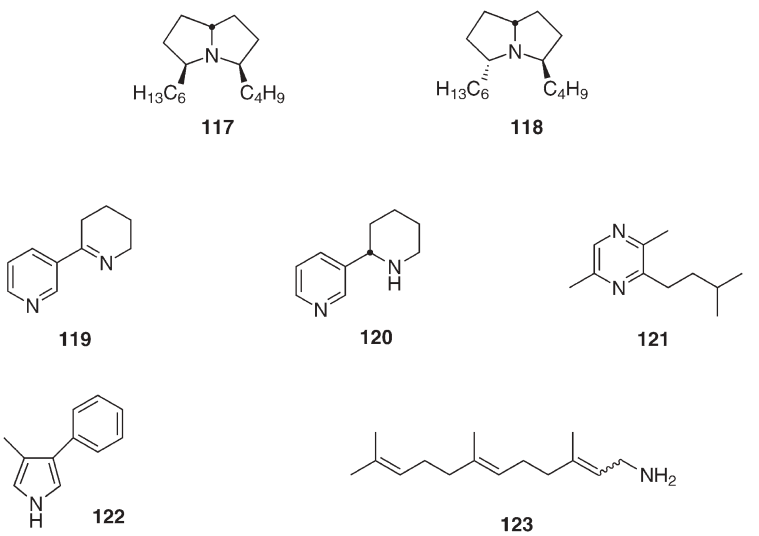


Fig. 20 Defensive alkaloids from myrmicine ants (structures **117** to **123**)

The cephalic extracts of the ants *Anochetus kempfi* and *A. mayri* were found to contain 2,5-dimethyl-3-isoamylpyrazine (**121**) and 3-methyl-4-phenylpyrrole (**122**) (Fig. 20). This is the first report of a phenylpyrrole in an insect [128]. These compounds are most likely mandibular gland products and have a pheromonal role.

(2*E*)- and (2*Z*)-Farnesylamine (**123**) (Fig. 20) were detected in the extracts of the myrmicine ant *Monomorium fieldi* from Australia. Their structures were established by comparison with synthetic material. This is the first report of a farnesylamine derivative from a natural source [129].

Finally, Garraffo et al. have demonstrated that ammonia chemical ionization tandem mass spectrometry (CIMS/MS) and collision activated dissociation may provide unique structural information for certain "dendrobatid alkaloids" found in frog skin and ants [130]. These techniques together with GC/FTIR and EIMS/MS were applied to analyze six ants of the genus *Tetraponera* [131]. Tetraponerines, the tricyclic alkaloids originally detected in the venom of *Tetraponera* sp. from Papua New Guinea [114, 132], were found to be present in two of the six species analyzed. An Indian ant (*T. allaborans*) had tetraponerine-2 (T-2) (**124**), tetraponerine-4 (T-4) (**125**) and tetraponerine-8 (T-8) (**126**), while a Chinese ant (*T. binghami*) had tetraponerine-5 (T-5) (**127**), tetraponerine-6 (T-6) (**128**), tetraponerine-7 (T-7) (**129**) and T-8 (**126**) (Fig. 21). The EIMS/MS fragmentations proved diagnostic for the ring system and the CIMS/MS patterns for the C-8 or C-9 substitution, while the FTIR spectra were diagnostic for the C-8 or C-9 configurations.

T. rufonigra from India and *T. penzigi*, *T. clypeata* and *T.* sp. cf. *emeryi*, all three from Africa, had no tetraponerines. Surprisingly, the extract from another collection of *T. allaborans* was also devoid of alkaloids. Thus, the presence or absence of tetraponerine alkaloids cannot be considered a taxonomic marker for the genus until the genetic or environmental factors responsible for their production are uncovered and understood [131].

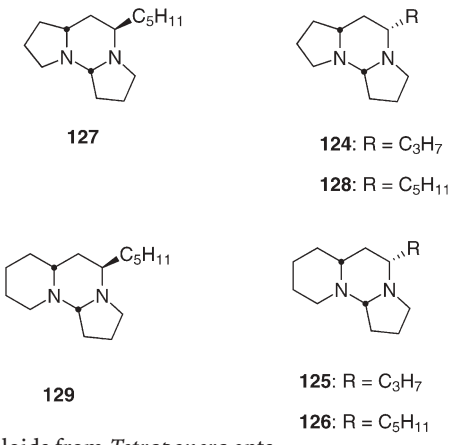
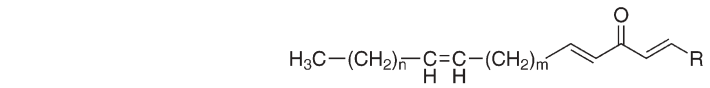


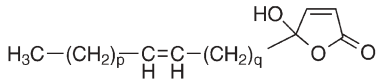
Fig. 21 Defensive alkaloids from *Tetraponera* ants

2.2.1.2 Defense Chemistry of Crematogaster Ants

Ants of the genus *Crematogaster* are characterized by a unique defense mechanism. These ants are able to raise their abdomen forward and over the thorax and head, which allows them to point their abdominal tip in nearly all directions. The sting is also modified, when compared to a normal ant sting: it has a spatulate tip that is not a suitable injection device. In many *Crematogaster* species, the venom is emitted as a froth that accumulates on the spatulate portion and at the basis of the sting, from where it can easily be applied to the integument of other insects. In this genus, the defense chemistry is also peculiar. Whereas most ants produce proteinaceous or alkaloidic venoms in their poison gland, *Crematogaster* ants produce lipidic defensive compounds (or the precursors thereof) in their Dufour gland. In the three European species of *Crematogaster* ants, the cooperation of the Dufour and poison glands in the production of both topical poison and alarm pheromone was demonstrated [133–135]. In these three species (*C. scutellaris*, *C. auberti*, and *C. sordidula*), the venoms consist of long chain unsaturated compounds, bearing an (*E,E*)-cross-conjugated dienone linked to a primary acetate function. In *C. scutellaris* the major compounds derive from an acetylated C_{21} long chain (e.g., **130d–f**), but minor homologs deriving from C_{19} or C_{23} chains are also present (**130a–c** and **130g–i**) (Fig. 22) [133–135].



	n	m	R=CH_2OAc	R=CHO	R=COOH
C_{19}	3	7	**130a**	**131a**	**132a**
	5	5	**130b**	**131b**	**132b**
	7	3	**130c**	**131c**	**132c**
C_{21}	3	9	**130d**	**131d**	**132d**
	5	7	**130e**	**131e**	**132e**
	7	5	**130f**	**131f**	**132f**
C_{23}	3	11	**130g**	**131g**	**132g**
	5	9	**130h**	**131h**	**132h**
	7	7	**130i**	**131i**	**132i**



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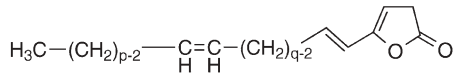
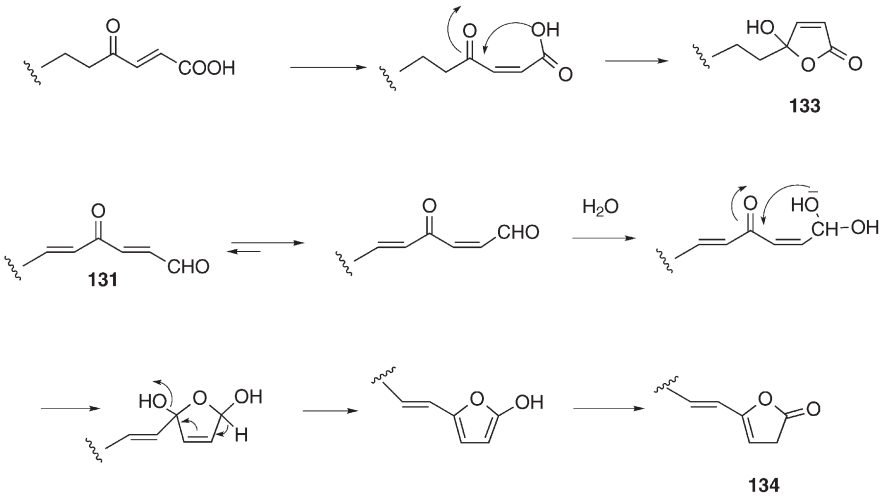


134

Fig. 22 Structures of electrophilic poisons from the Dufour gland of European *Crematogaster* ants

When the venom is emitted, these Dufour gland compounds are transformed into highly electrophilic 4-oxo-2,5-dienals (e.g., **131a–i**), by the action of two enzymes, an esterase and an oxygen-dependent alcohol oxidase, which are both stored in the poison gland. Thus, this elegant mechanism allows the ants to store venom precursors of relatively low toxicity, the production of the true toxins being triggered during the simultaneous emission of both the Dufour and the poison glands constituents. The presence of an esterase and of an oxygen-dependent oxidase in the poison gland was substantiated by in vitro experiments in phosphate buffer, using the poison gland as enzyme source [134]. Acetic acid released during this process, was identified in venom samples by GC analysis of its *tert*-butyldimethylsilyl derivative. It reinforces the efficiency of the defensive mechanism by acting as an alarm pheromone. Biological tests have demonstrated that the toxicity of the native Dufour gland constituents is markedly lower than that of the 4-oxo-2,5-dienals-enriched secretion [134]. It should also be mentioned that the 4-oxo-2,5-dienal derivatives are exceedingly unstable and that, once produced, they are quickly oxidized or rearranged into an array of compounds, among which the corresponding carboxylic acids **132**, lactols **133**, and α-angelica lactones **134** have been identified (Fig. 22) [133]. The formation of the lactols **133** and of the α-angelica lactones **134** may be rationalized as shown in Scheme 1 [133, 134].



Scheme 1 Hypothetical formation of lactols and α-angelica lactones in *Crematogaster* ants

More recently, three as yet unidentified species originating from Papua New Guinea have also been studied. *Crematogaster* sp. 1 was shown to contain, besides C_{25} homologs of the 4-oxo-2,5-dienals (**131**), a series of derivatives, whose structures were determined to be **135a–f**, on the basis of a complete 1D and 2D NMR study at 600 MHz (Fig. 23) [136].

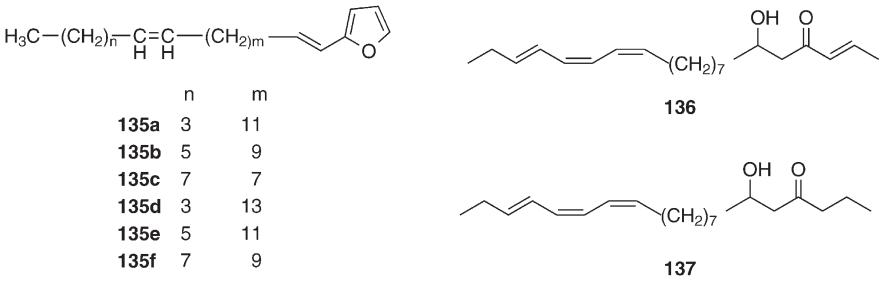
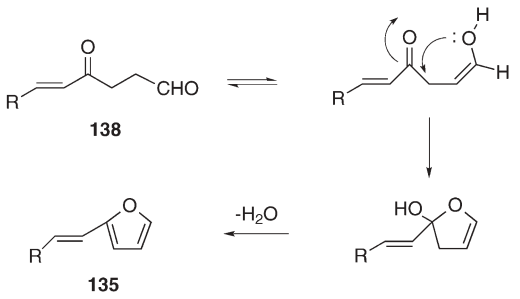


Fig. 23 Structures of Dufour gland components of New Guinean *Crematogaster* ants

In the latter compounds, the cross-conjugated dienone is replaced by a furan ring conjugated to an *E* double bond. Biosynthetically, it is not known if these compounds arise from 1,4-dione precursors such as **138** by a Paal-Knorr type cyclization (Scheme 2) or from the α-angelica lactones **134** by reduction of the lactone carbonyl followed by loss of water.

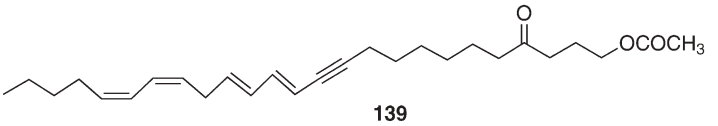
In this latter study, the position of the isolated double bond in the chains was established by DMDS treatment, followed by linked scan MS/MS analysis of the resulting mixture of adducts [136]. As in the case of the European species, there are always three position isomers of the isolated double bond for each chain length. Moreover, the positions of this double bond are always the same with



Scheme 2 Hypothetical formation of furans from 1,4-dione precursors

respect to the terminal methyl group, namely ω-5, ω-7, and ω-9. This observation could have interesting biosynthetic implications.

In contrast with all the other species of *Crematogaster* studied till now, the venom of *C.* sp. 2 from Papua New Guinea did not contain mixtures of homologous compounds. Two derivatives, **136** and **137**, characterized by the presence of a conjugated triene on one end of the chain, and by a 1,3-hydroxyketone at the other end, were isolated from this species (Fig. 23). These structures could constitute biosynthetic intermediates en route to the cross-conjugated dienone system. The venom of *C.* sp. 3 contained 4-oxo-2,5-dienyl acetates similar to



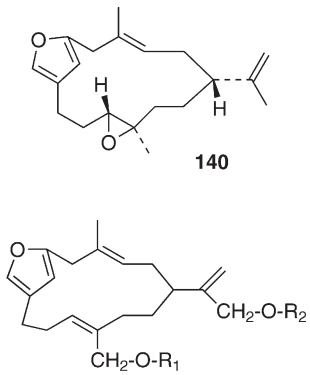
139

Fig. 24 Defensive compound from a Brazilian *Crematogaster* sp

those already reported from other species. It should be pointed out that in *C.* sp. 1 and 3 from Papua New Guinea, the major derivatives are based on a C_{25} chain, instead of C_{19}, C_{21} or C_{23} in the European species. This could constitute a useful taxonomic marker for these species.

A comparative study of *Crematogaster* species from the New World and from Africa has been also undertaken by the same authors to see whether there are variants to the peculiar defensive mechanism evidenced in European and New Guinean species. The Dufour gland secretion of an unidentified species from Brazil yielded as major component (13*E*,15*E*,18*Z*,20*Z*)-1-hydroxypentacosa-13,15,18,20-tetraen-11-yn-4-one 1-acetate (**139**), the first compound of this type to be isolated from ants (Fig. 24) [137]. The methylene group at C-17 is allylic both to a conjugated diene and to a conjugated dienyne, a characteristic that renders **139** highly sensitive to autoxidation, but we do not know yet if this has something to do with its toxicity.

Surprisingly, further investigations of two other Brazilian *Crematogaster* ants showed that they produce furanocembrene derivatives. The Dufour gland of *C. brevispinosa rochai* contained two epoxy-furanocembrenes, crematofuran (**140**) and its regioisomer **141** (Fig. 25). The structure, relative configuration and preferred conformations of these new compounds were determined by a combination of NMR and molecular mechanics (MM3) methods [138]. The related species, *C. brevispinosa ampla* produced as major compounds the dibutanoate furanocembrene **142**, accompanied by the corresponding monoacetate mono-



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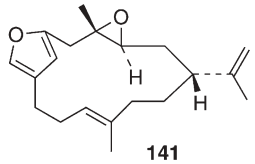
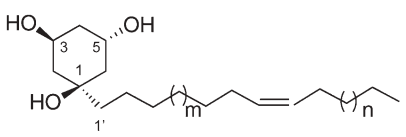


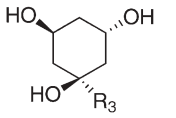
141

142: R_1=R_2=CO-*n*-C_3H_7
143: R_1= CO-*n*-C_3H_7, R_2= $COCH_3$
144: R_1=$COCH_3$, R_2= CO-*n*-C_3H_7
145: R_1=CO-*n*-C_3H_7, R_2=H
146: R_1=H, R_2=CO-*n*-C_3H_7

Fig. 25 Cembrane derivatives from New World *Crematogaster* ants



147a: m = 6; n = 1
147b: m = 4; n = 3
147c: m = 2; n = 5
147d: m = 8; n = 1
147e: m = 6; n = 3
147f: m = 4; n = 5



147g: $R_3 = C_{17}H_{35}$
147h: $R_3 = C_{19}H_{39}$

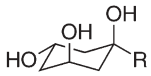


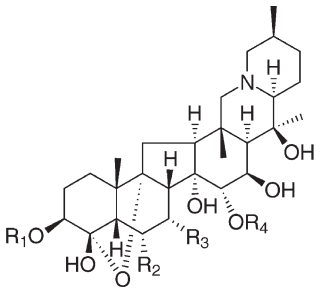
Fig. 26 Structures of trihydroxycyclohexane derivatives from an African *Crematogaster* ant

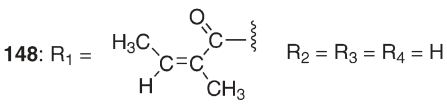
butanoates **143** and **144** and two monohydroxy monobutanoates **145** and **146** (Fig. 25) [139]. Indirect evidence points to a biosynthesis of these compounds by the ants themselves [138]. As is the case for other furan derivatives [140], compounds **140** and **141** proved to be toxic towards other insects, but their mechanism of action is not known [138]. It is interesting to note that, even if *C. brevispinosa ampla* produces ester derivatives, it is devoid of the esterase and oxidase enzymes that trigger the production of 4-oxo-2,5-dienals in European species [139].

Quite recently, the study of the *Crematogaster* genus was extended to an African species, *C. nigriceps* [141]. The Dufour gland of this species contains a complex mixture of at least eight trihydroxycyclohexane derivatives substituted at C-1 by an alkyl, an alkenyl or an alkadienyl chain. The major derivatives (about 57%) are the 1-heptadecenyl- (**147a–c**) and 1-nonadecenyl-1,3,5-trihydroxycyclohexane (**147d–f**) derivatives (Fig. 26). They are accompanied by the corresponding 1-heptadecyl- (**147g**) and 1-nonadecyl-1,3,5-trihydroxycyclohexane (**147h**) derivatives, and by small amounts of (*Z*,*Z*)-dienic derivatives (<10%). The structures, the relative and absolute configurations, and the preferred conformation of the cyclohexane ring of these new compounds have been established by spectroscopic and chemical methods, as shown in Fig. 26. The positions of the double bond in the mono-unsaturated compounds **147a–f** were again established by DMDS derivatization followed by linked scan EIMS [141].

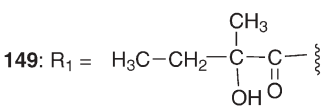
2.2.2 Tenthredinidae

Many sawfly larvae protect themselves against predation by sequestering toxic metabolites from their host plant. One strategy is to store these compounds in

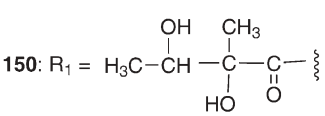




148: R_1 = $R_2 = R_3 = R_4 = H$



149: R_1 = $R_2 = R_3 =$ Ac R_4 =



150: R_1 = $R_2 = R_3 =$ Ac R_4 =

151: $R_1 = R_2 = R_3 = R_4 = H$

152: R_1 = Ac; $R_2 = R_3 = R_4 = H$

Fig. 27 *Veratrum* alkaloids from Tenthredinidae

their hemolymph. This is the case for iridoid glycosides [142], for glucosinolates, [143], and for *Veratrum* alkaloids [144, 145]. In the latter case, some metabolization of the alkaloids has been demonstrated. Indeed, whereas the major alkaloids present in the plant leaves are zygadenine angelic acid ester (**148**), protoveratrine A (**149**) and protoveratrine B (**150**), the larvae do not contain any of the latter compounds, but instead zygadenine (**151**) and zygacine (**152**), formed by hydrolysis of the ester functions of **148**, **149**, and **150**, and by partial re-esterification in the case of **152** (Fig. 27) [144, 145].

In the pine sawfly, *Neodiprion sertifer*, the larvae sequester the resin from pine needles in pouches in the foregut and use it as defense against predators. The resin contains mono- and sesquiterpenes, as well as resin acids, the major sesquiterpene being (5*S*,8*S*)-germacra-1,6-dien-5-ol (**153**) (Fig. 28) [146]. Sequestration of plant compounds is not the sole strategy in this group of insects, as was already demonstrated in 1984 by the detection of volatile compounds (mostly aldehydes) in the ventral gland secretions of eight nematine larvae [147]. More recently, the same strategy was found in two *Hoplocampa* larvae that produce a defensive secretion mainly composed of acetogenins. Major compounds are (*E*)-2-octenal (**154**), (*E,Z*)- and (*E,E*)-2,4-decadienal (**155** and

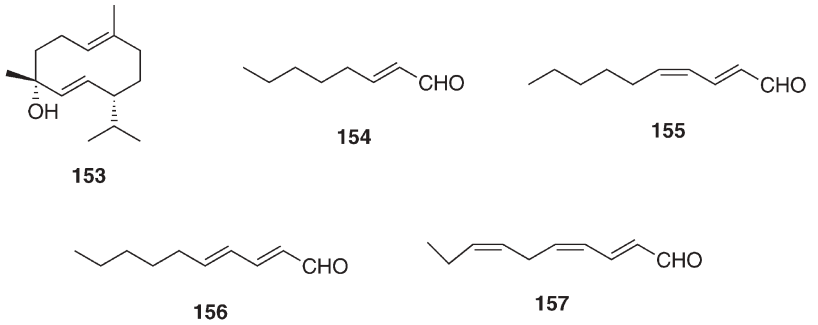


Fig. 28 (5*S*,8*S*)-Germacra-1,6-dien-5-ol from *Neodiprion sertifer* larvae and unsaturated aldehydes from *Hoplocampa* larvae

156), and (*E,Z,Z*)-2,4,7-decatrienal (**157**) (Fig. 28). Benzaldehyde was present in one of the species [148]. Boevé and Schaffner have put forwards the "harmful hemolymph hypothesis" [149]. This hypothesis is based on the observation that the integument of many sawfly larvae has such a low resistance that slight mechanical damage is enough to provoke the release of hemolymph. Moreover, they observed a negative correlation between integument resistance and hemolymph deterrence on *Myrmica rubra* workers. Thus, this hypothesis assumes a functional link between integument resistance and hemolymph deterrence, jointly acting as a chemical defense strategy [149].

2.3 Lepidoptera

The chemical defense of butterflies against predation has been studied since the nineteenth century and some detailed reviews have covered this area of research in the last ten years [150–152].

Many aposematic lepidopteran insects are associated with poisonous plants and sequester the toxins from their host instead of, or in some cases in addition to, biosynthesizing their own defensive compounds.

A classical example of sequestration is the monarch butterfly *Danaus plexippus* (Danainae) which feeds on leaves of *Asclepias curassavica* (Asclepiadaceae) and sequesters cardenolides such as calotropin (**158**) (Fig. 29). This molecule was shown to afford the butterfly an efficient protection against birds [150–152]. It was also demonstrated that its resistance to cardiac glycosides is due to a single amino acid mutation in the ouabain-binding site of the Na^+/K^+ ATPase [153, 154].

Pyrrolizidine alkaloids (PAs) such as senecionine (**159**) (Fig. 29) are also taken up from plants by various butterflies and moths belonging to the Danainae, Ithomiinae, and Arctiidae. The chemical ecology of PAs and their role in the interaction between plants and adapted Lepidoptera were reviewed several times in details during the last decade [155–159]. These insects use them for defense and for the production of male pheromones.

From a defensive point of view, it was shown that sequestered PAs constitute an efficient protection against the orb-weaving spider *Nephila clavipes*, which liberates butterflies unharmed from its web. In this study, *N*-oxides were shown to be more active than the corresponding free bases. This could be correlated with physicochemical properties of these molecules in interaction with the *Nephila* receptors. Moreover, there was a significant correlation between dosage and antipredator activity of PAs [160].

In larvae of *Tyria jacobaeae* (Arctiidae), the conversion of the free base to the *N*-oxide is made in the hemolymph by senecionine *N*-oxygenase, a flavin dependent monooxygenase with a high specificity for PAs [161]. Interestingly, *T. jacobaeae* from an alpine population, and living mostly on *Petasites* species, did not sequester PAs like its congener living on *Senecio* species, but terpenes such as petasol (**160**) and isopetasol (**161**) (Fig. 29). Nucleotide sequences of the mitochondrial 16S rDNA gene showed 1% sequence divergence, indicating that a genetical difference exist between the PA exploiting and the terpene-sequestering races of *T. jacobaeae* [162].

In addition to their defensive role, the plant-acquired PAs have also an ecological importance in the evolutionary biology of *Utetheisa ornatrix* (Arctiidae). An alkaloid deficiency seems to be a main cause of cannibalism: losers in the larval sequestration of alkaloids, which would result in a lack of chemical protection, cannibalize conspecific winners with the aim to accumulate PAs. This may contribute to a balanced regulation of the acquired alkaloid content in these arctiid populations [163]. Moreover, during mating, adult males of this moth transmit some of their alkaloid content to the female, thus protecting her against spiders from the moment she uncouples from the male [164]. The alkaloidal gift is then transmitted by the female to the eggs, which are protected as a result [165]. It is interesting to note that eggs may receive PAs from more than one male source, multiple mating resulting in an increased transmis-

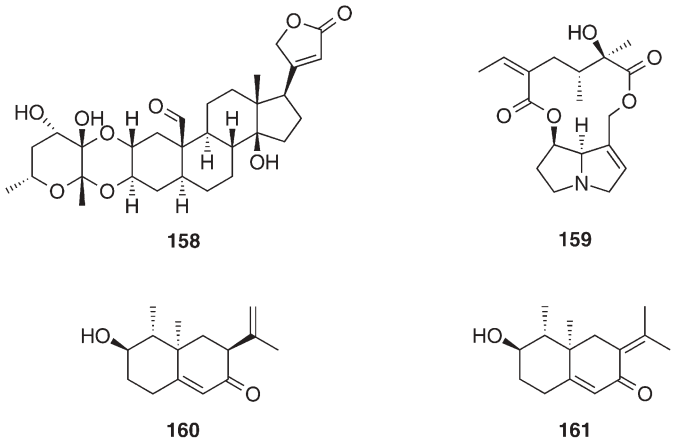


Fig. 29 Examples of sequestered defensive compounds from Lepidoptera

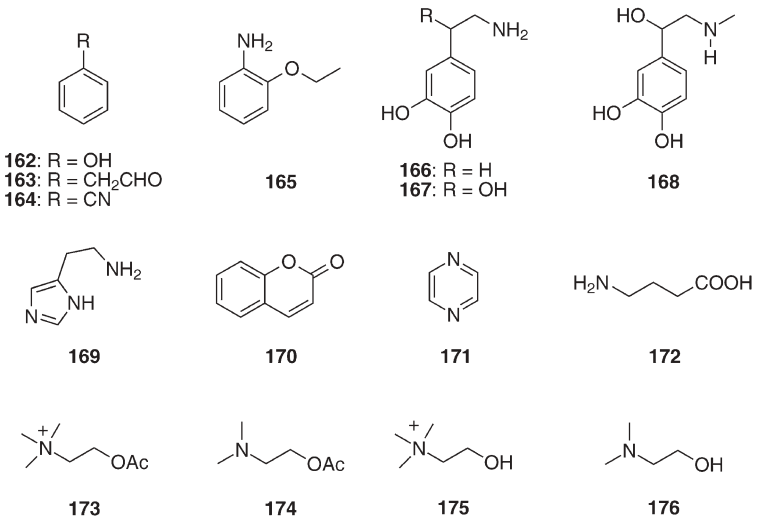


Fig. 30 Biogenic amines and aromatic compounds characterized from saturniid caterpillars

sion of alkaloidal gift to the eggs, and not in segregated allocation of these gifts [166].

If many unpalatable Lepidoptera obtain their defensive quality by sequestering plant-derived substances, various caterpillars fortify their bodies with spines and hairs containing various toxins [167]. Defensive secretions in specialized exocrine glands were also reported [150–152].

For example, *Attacus atlas*, a caterpillar belonging to the family Saturniidae, has the ability to eject an irritating spray when disturbed. By increasing hemolymph pressure, the lids closing the spraying gland are blasted off and the secretion spouts out. Several aromatic compounds, biogenic amines, and glycerol were detected in the emitted fluid and in the hemolymph (**162–176**) (Fig. 30). The results of an ant feeding deterrence test with *Lasius niger* indicated a highly significant deterrent effect of *Attacus atlas* secretion three minutes after the beginning of the test [168].

Biogenic amines and phenolic compounds were also characterized from the defensive secretion of other saturniid caterpillars such as *Saturnia pavonia*, *S. pyri*, and *Eupackardia calleta* [169] and the chemical ecology of Saturniidae and Lymantriidae was recently reviewed by Deml and Dettner [170].

In the latter family, exclusively first stage larvae of *Lymantria dispar* show two types of striking hairs (long acuminate hairs and balloon hairs), the chemical investigation of which indicates the presence of nicotine (**177**) and isopropylmyristate (only in acuminate hairs). Nicotine (**177**), nicotinamide (**178**), nicotinic acid (**179**), 2-pyrrolidone (**180**), *N*-methyl-2-pyrrolidone (**181**), and glycerol (Fig. 31) are found in larval hemolymph or larval gland secretions. These compounds were tested for their defensive efficiency against ants and could serve for defense against other predatory arthropods [171]. This cater-

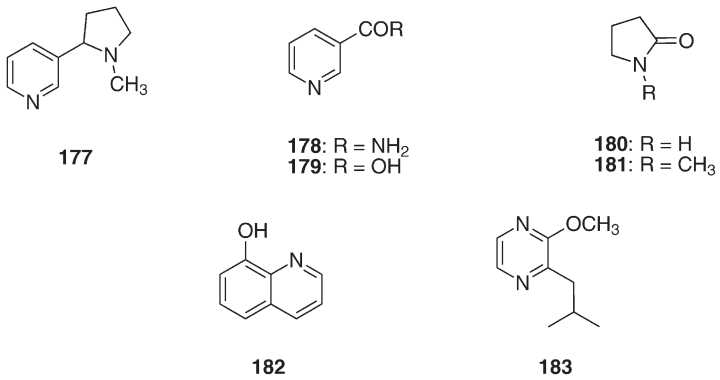
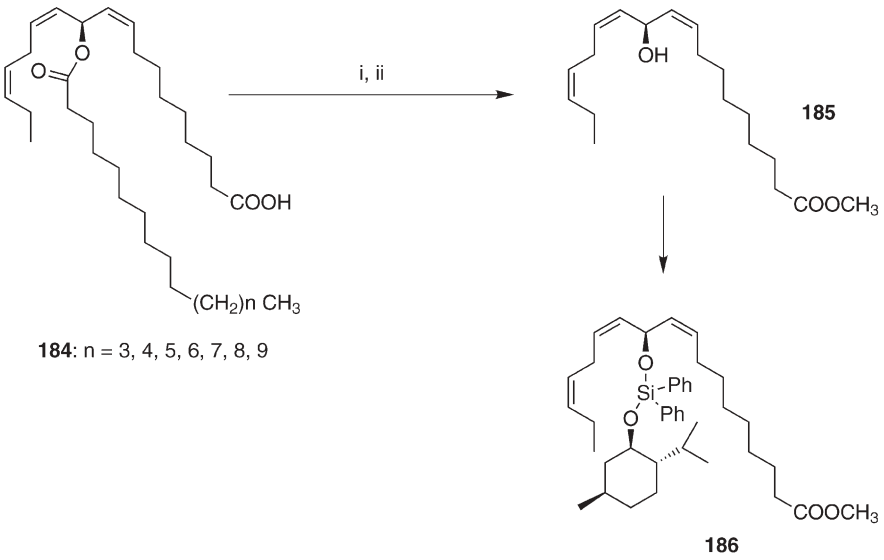


Fig. 31 Defensive compounds from *Lymantria dispar* larvae

pillar possess also unpaired dorsal abdominal glands on the sixth and seventh segments, and pairs of smaller glands on the first to fourth abdominal segments. The exudate emitted from these glands is an aqueous mixture of low molecular weight carboxylic acids. Moreover, 8-hydroxyquinoline (**182**) and 2-isobutyl-3-methoxypyrazine (**183**), a molecule considered to be a constituent of the warning odor in many aposematic insects, also occur in the secretion (Fig. 31). This mixture was a feeding deterrent to the fire ant *Solenopsis geminata* in a laboratory bioassay [172].



Scheme 3 Determination of the absolute configuration of the C-11 stereocenter in the mayolenes, defensive compounds from *Pieris rapae*

Finally, larvae of *Pieris rapae* (Pieridae), a widely distributed butterfly native from Eurasia and North Africa, are protected with glandular hairs, bearing droplets of a clear oily secretion at their tip. This fluid consist of a series of chemically labile, unsaturated lipids, the mayolenes (**184**) (Scheme 3), which are derived from 11-hydroxylinolenic acid. Their structures were established by a combination of NMR and ESI-MS analyses [173] and fully confirmed by a total synthesis [174]. To determine the absolute configuration of the C-11 stereocenter in the mayolenes, an elegant method using a new chiral silylation reagent was developed. Therefore, hydrolysis of the ester group in **184** with methanolic potassium hydroxide followed by methylation of the resulting carboxylic acid yielded **185**, containing a *bis*-allylic hydroxyl group. Derivatization of **185** with (–)-chloromenthoxydiphenylsilane provided the corresponding (–)-menthoxydiphenylsilyl ether **186** (Scheme 3). Comparison of the ^{1}H NMR spectrum of **186** prepared from the natural material with spectra of synthetic samples of both enantiomers of **186** demonstrate that the natural mayolenes exist as single enantiomers having the (11*R*) absolute configuration [173].

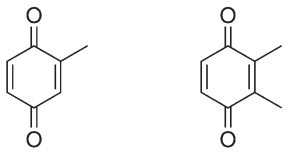
2.4 Other Insects

Many other insects are known for their use of repellent chemicals to defend themselves against predators [4].

Stink bugs (Heteroptera, Pentatomidae) for example are well known for the odorous volatile secretion they emit from their metathoracic glands when molested [175].

In the Dermaptera, the earwig *Doru taeniatum* (Forficulidae) has a pair of defensive glands, opening on the fourth abdominal tergite, from which it discharges a spray when disturbed. It aims the discharges by revolving the abdomen, a maneuver that enables it to use simultaneously its pincers in defense. The secretion contains methyl-1,4-benzoquinone (**187**) and 2,3-dimethyl-1,4-benzoquinone (**188**), present in the glands as a crystalline mass, together with pentadecene, and a (presumably) aqueous phase (Fig. 32) [176].

With maximal densities of 53,000 individuals/m^2, springtails (Collembola) are exceedingly abundant and are among the most important consumers in many soil ecosystems. Springtails also represent important and readily avail-



187 **188**

Fig. 32 Methyl-1,4-benzoquinone and 2,3-dimethyl-1,4-benzoquinone found in *Doru taeniatum*

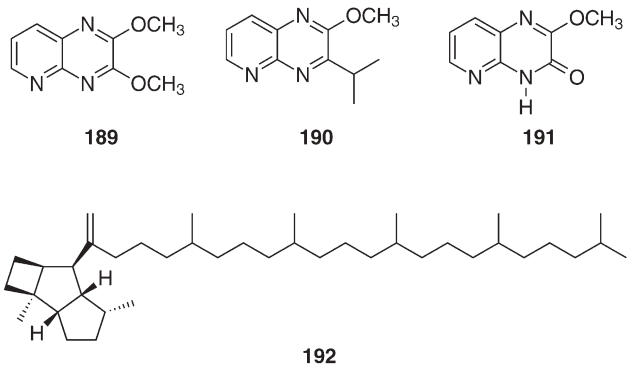


Fig. 33 Defensive compounds isolated from springtails

able prey for many predatory mites, spiders, beetles, bugs, or hymenopteran species. Their defensive chemistry was recently examined. The giant springtail, *Tetrodontophora bielanensis*, is characterized by integumental openings from which small droplets of a sticky fluid are secreted after molestation. Bioassays with topically treated ground beetle *Nebria brevicollis* showed that this fluid evokes a total disorientation and cleansing behavior of the beetle. The main constituents were identified as 2,3-dimethoxypyrido[2,3-*b*]pyrazine (**189**), 3-isopropyl-2-methoxypyrido[2,3-*b*]pyrazine (**190**), and 2-methoxy-4*H*-pyrido-[2,3-*b*]pyrazin-3-one (**191**) (Fig. 33) [177]. An unusual hydrocarbon tetraterpene, poduran (**192**) (Fig. 33), was also isolated from the springtail *Podura aquatica*. This molecule possesses a tricyclic head and a tail of five isoprene units. The structure was elucidated by one- and two-dimensional NMR experiments, mass spectrometric investigations as well as chemical transformations [178].

Insects of the order Phasmatodea, the so-called stick insects, comprise some 2500 species, mostly from the warmer parts of the world. Phasmids are generally slow and clumsy and, for these reasons, are vulnerable. Many, however are cryptically colored, protected by resemblance to twigs and leaves or armed with spines. Others, comprising only a few known species, are protected by defensive glands. When molested, the Peruvian fire stick *Oreophoetes peruana* ejects a white malodorous fluid from two glands in the prothorax. This secretion contains quinoline (**193**) (Fig. 34), an alkaloid proved to be repellent or topically irritant in assays with ants, spiders, cockroaches, and frogs [179].

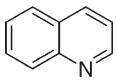


Fig. 34 Structure of the defensive alkaloid from *Oreophoetes peruana*

On the other hand, the defensive secretions of *Sipyloidea sipylus* and of *Megacrania tsudai*, two phasmid insects originating from Asia, contain volatile compounds, and, in both cases, the whole secretion has a repellent activity [180, 181].

Termites are living in rigid societies in which morphologically specialized individuals execute specific tasks: king and queen reproduce, the workers forage and feed. Finally, soldiers of many termite species are morphologically and physiologically specialized for defense [182].

In addition to their mandibles, which are used to bite, pierce, shear, and cut intruders, soldiers of *Coptotermes formosanus*, a Formosan subterranean termite, are also armed with chemical weaponry. When disturbed, the soldiers secrete a gluelike white fluid from the frontal gland, which often form a drop between the open mandibles. Lignoceric and hexacosanoic acids were the two major components identified in this secretion [183].

In European *Reticulitermes* termites however, 16 known terpene compounds were isolated from the soldier frontal gland secretion, including monoterpenes, sesquiterpenes, diterpenes, and one sesterterpene [184].

On the other hand, *Pseudacanthotermes spiniger*, an African fungus-growing termite, possesses, in addition to powerful mandibles, a small frontal gland and a pair of salivary glands, the reservoir of which fill more than half of their abdomen. When in close contact with enemies, the large soldiers emit a drop of saliva that paralyze and sometimes kills the aggressor. This defensive secretion contains three macrolactones, hexacosanolide (**194**), 22-keto-hexacosanolide (**195**), and 2-hydroxy-24-keto-octacosanolide (**196**) (Fig. 35), together with important amounts of a polysaccharide based mainly upon β-glucopyranose, β-glucosamine, and *N*-acetyl-β-glucosamine in equal amounts [185].

In the family Termitidae, one quarter of the species belong to the subfamily Nasutitermitinae. In many species of this subfamily, in particular those of the genera *Nasutitermes* and *Trinervitermes*, the soldiers caste have evolved large

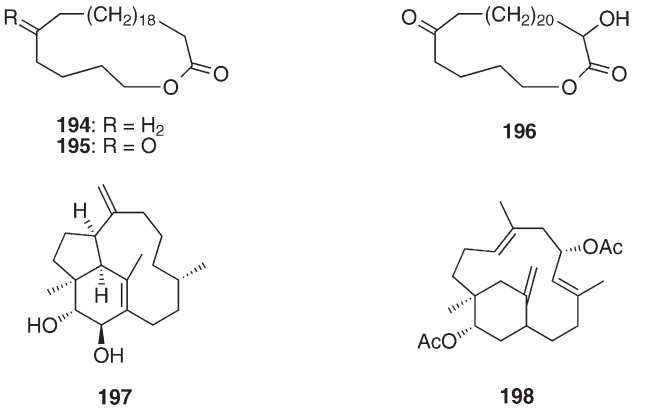


Fig. 35 Defensive compounds from termites

frontal glands and nozzle-like cephalic structures from which they eject irritating, entangling secretions. These defensive secretions contain monoterpenic hydrocarbons together with polycyclic diterpenes such as **197** (Fig. 35) [186].

Analysis of the CH_2Cl_2 extract of the Madagascan termite *Nasutitermes canaliculatus* yielded a major compound identified to 3α,10α-diacetoxy-7,16-secotrinervita-7,11,15(17)-triene (**198**) (Fig. 35) by X-ray crystallographic analyses combined with ^1H, ^{13}C NMR and MS experiments [187].

3 Synthesis

In this section, selected examples of syntheses of insect defensive compounds will be presented. The impact of asymmetric synthesis and the increasing use of organometallic reagents, notably transition metal catalysts, will be highlighted.

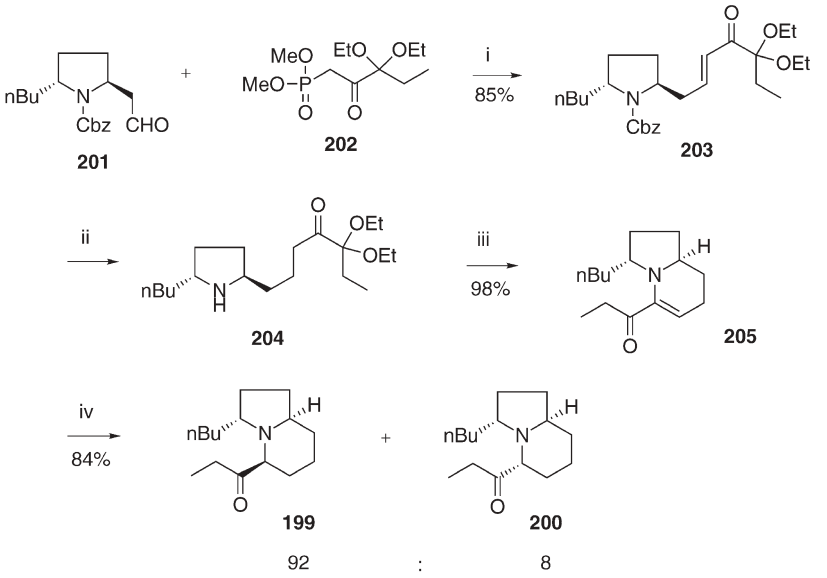
3.1 Alkaloids

Many insect defensive alkaloids have already been synthesized and several of these syntheses have already been reviewed (e.g., [13, 114, 116]). Here we will limit ourselves to the presentation of recent syntheses of *Myrmicaria* and *Tetraponera* ant alkaloids.

The venom of ants of the genus *Myrmicaria* is made up of indolizidine or pyrrole-indolizidine alkaloids [188, 189]. The synthesis of some of these alkaloids has already been reported in the review of Leclercq et al. [114]. Thus, we will report here only on the syntheses published since 1999.

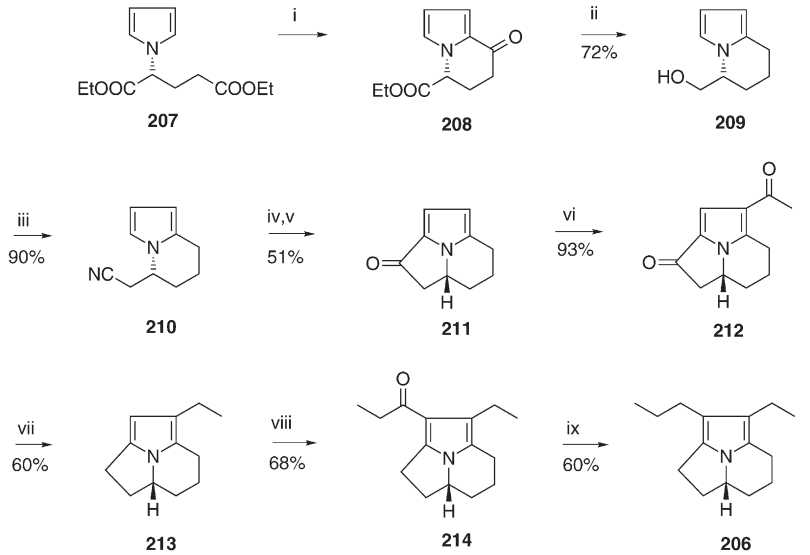
(–)-Myrmicarin 237A (**199**) and its epimer (+)-myrmicarin 237B (**200**) have been enantioselectively synthesized by Thanh et al. [190] from the chiral synthon **201** readily available from (*S*)-pyroglutamic acid [191] (Scheme 4). Thus, a Wittig-Horner condensation between the aldehyde **201** and phosphonate **202** gave in 85% yield the enone **203** that was then catalytically reduced to the keto pyrrolidine **204**. Acid deprotection of the keto group induced cyclization into enamine **205** that after reduction of the carbon-carbon double bond by sodium cyanoborohydride, led with a good diastereoselectivity to a mixture (ratio 92:8) of myrmicarins 237A (**199**) and 237B (**200**).

The first synthesis of non-racemic (*R*)-(+)-myrmicarin 217 (**206**) has been described by Sayah et al. starting from D-glutamic acid to introduce the stereogenic center [192]. The diethyl ester of D-glutamic acid was condensed with tetrahydro-2,5-dimethoxyfuran to give the pyrrole **207** which when treated by BBr_3 gave the bicyclic compound **208** (Scheme 5). A two-step strategy was followed using $NaBH_3CN$ in the presence of ZnI_2 to reduce the ketone group and $LiAlH_4$ to reduce the ester function. The alcohol **209** was transformed into a mesylate which upon treatment with cyanide anions gave nitrile **210**. Hy-



i: KHMDS, THF; ii: H_2, Pd/C, MeOH; iii: TFA/H_2O (1:1) then K_2CO_3;
iv: $NaBH_3CN$, HCl (1 eq).

Scheme 4 Total synthesis of (–)-myrmicarins 237A and 237B

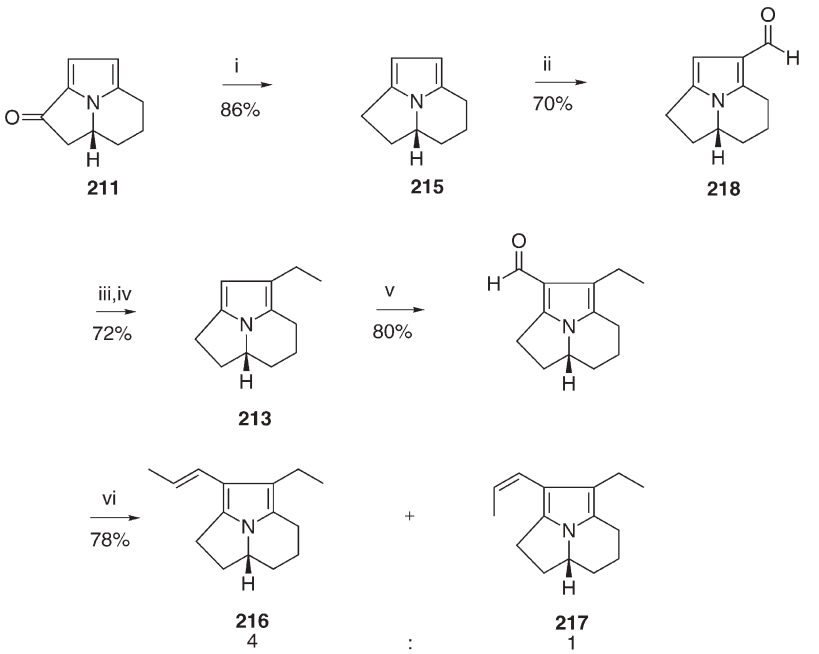


i: BBr_3; ii: $NaBH_3CN$,ZnI_2 then $LiAlH_4$, THF; iii: CH_3SO_2Cl, pyridine then NaCN, DMF; iv: NaOH, H_2O, MeOH;
v: EtOCOCl, Et_3N then $BF_3.OEt_2$; vi: AcCl, $AlCl_3$; vii $LiAlH_4$, dioxane; viii: $POCl_3$, $EtCON(Me)_2$; ix: $LiAlH_4$, dioxane.

Scheme 5 Synthesis of non-racemic (*R*)-(+)-myrmicarin 217

drolysis of the nitrile group followed by cyclization of the corresponding acid activated as an anhydride led to the tricyclic compound **211**. A Friedel-Crafts acylation was carried out to regioselectively introduce an acetyl group at position C-4 of the pyrrole ring. The two ketone groups of compound **212** were efficiently reduced with $LiAlH_4$ into methylene groups. A Vilsmeier-type reagent prepared by reaction of *N,N*-dimethylpropionamide with $POCl_3$ was reacted with compound **213** to provide ketone **214**. The last step of the synthesis was the reduction of the ketone function by $LiAlH_4$. The overall yield of the synthesis from pyrrole **207** was 7.5%.

After having performed the above synthesis, Sayah et al. studied the regioselectivity of the acylation of 1,2,5,6,7,7*a*-hexahydropyrrolo[2,1,5-*c,d*]indolizidine (**215**) using the Vilsmeier-Haack conditions [193]. They found that in toluene the major product of the reaction was the compound monoformylated at C-4. Only traces of the C-3 regioisomer and of the diformylated derivative were observed. Thus, this method could be regarded as useful for the synthesis of (*R*)-myrmicarins 215A (**216**) and 215B (**217**). Compound **218** was treated with methyllithium and in the same pot the resulting alcoholate was reduced by $LiAlH_4$ leading to the ethyl derivative **213** (Scheme 6). Then, compound **213** was formylated under Vilsmeier conditions and the obtained aldehyde

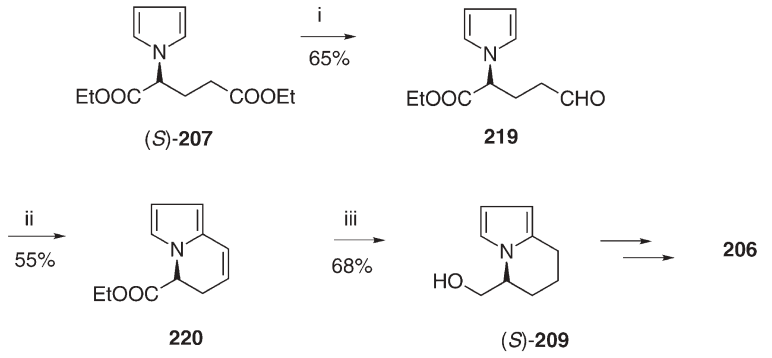


i: $LiAlH_4$, dioxane; ii: $POCl_3$, $(Me)_2NCHO$, toluene; iii: MeLi, THF; iv: $LiAlH_4$, THF then H_3O^+; v : $POCl_3$, DMF,CH_2Cl_2; vi: Ph_3PEtBr, NaH, THF.

Scheme 6 Synthesis of (*R*)-myrmicarins 215A and 215B

treated with the Wittig reagent obtained from ethyltriphenylphosphonium bromide. In this way a mixture of (*R*)-myrmicarins 215A (**216**) and 215B (**217**) was obtained (ratio A/B : 4/1) in 45% yield from **218**.

Recently, Settambolo et al. [194] reported a formal synthesis of (*S*)-myrmicarin 217 (**206**) using a sequence of reactions slightly modified from the one described in Scheme 4. Indeed, rather than to cyclize directly the diester (*S*)-**207**, the latter was first transformed into the aldehyde **219** (Scheme 7) that was further intramolecularly cyclodehydrated into ester **220**. The latter can be converted into (*S*)-myrmicarin 217 (**206**) via the alcohol (*S*)-**209** as described previously by Sayah et al. [192] (see Scheme 5).

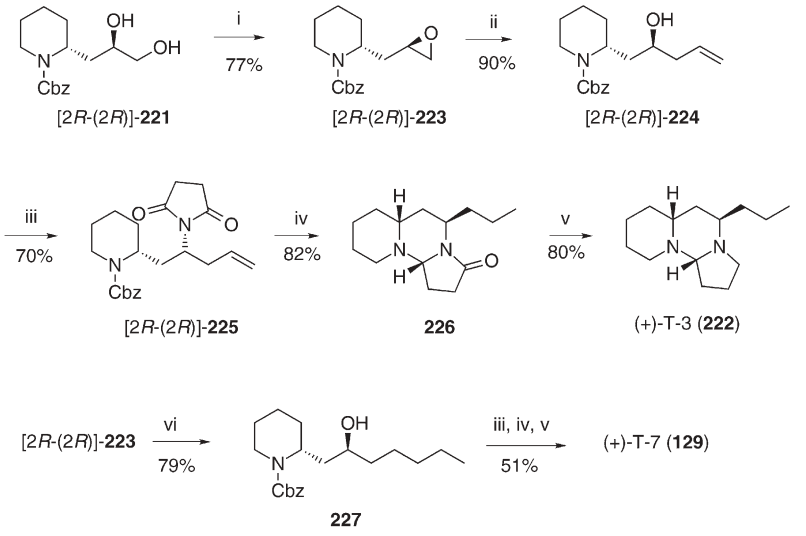


i: DIBALH, hexane; ii: DMSO, 100°C; iii: $LiAlH_4$, THF then H_2, Rh, Et_2O.

Scheme 7 Formal synthesis of (*S*)-myrmicarin 217

Since the discovery of tetraponerine-8 in 1987 by Braekman et al. [195] the tetraponerines, the defensive alkaloids of ants of the genus *Tetraponera*, have been the target of considerable synthetic efforts and have served to demonstrate the utility of various synthetic methodologies [114]. Recently a few further syntheses of these unusual tricyclic alkaloids have been reported.

Takahata et al. [196] have developed an efficient way to prepare with high *ee* (>98%) the four stereoisomers of 1-benzyloxycarbonyl-2-(2,3-dihydroxypropyl)piperidine (**221**) starting from 5-hexenylazide and using iterative asymmetric dihydroxylation. They demonstrated the utility of these stereoisomers for the synthesis of (+)-T-3 (**222**), T-4 (**125**), T-7 (**129**) and T-8 (**126**) after their conversion into the corresponding epoxides **223** by the Sharpless one-pot procedure [197]. The synthesis of (+)-T-3 (Scheme 8) began with the opening of the epoxide of [2*R*-(2*R*)]-**223** with vinylmagnesium bromide in combination with a cuprous bromide-dimethylsulfide complex. This afforded alcohol [2*R*-(2*R*)]-**224** in 90% yield. This alcohol was then submitted to a Mitsunobu reaction with succinimide to provide imide **225**. Catalytic reduction of **225** resulted in conversion into the tricyclic lactam **226** as a single isomer in 82% yield in a single step. Finally reduction of the lactam **226** with $LiAlH_4$ gave (+)-T-3 (**222**).



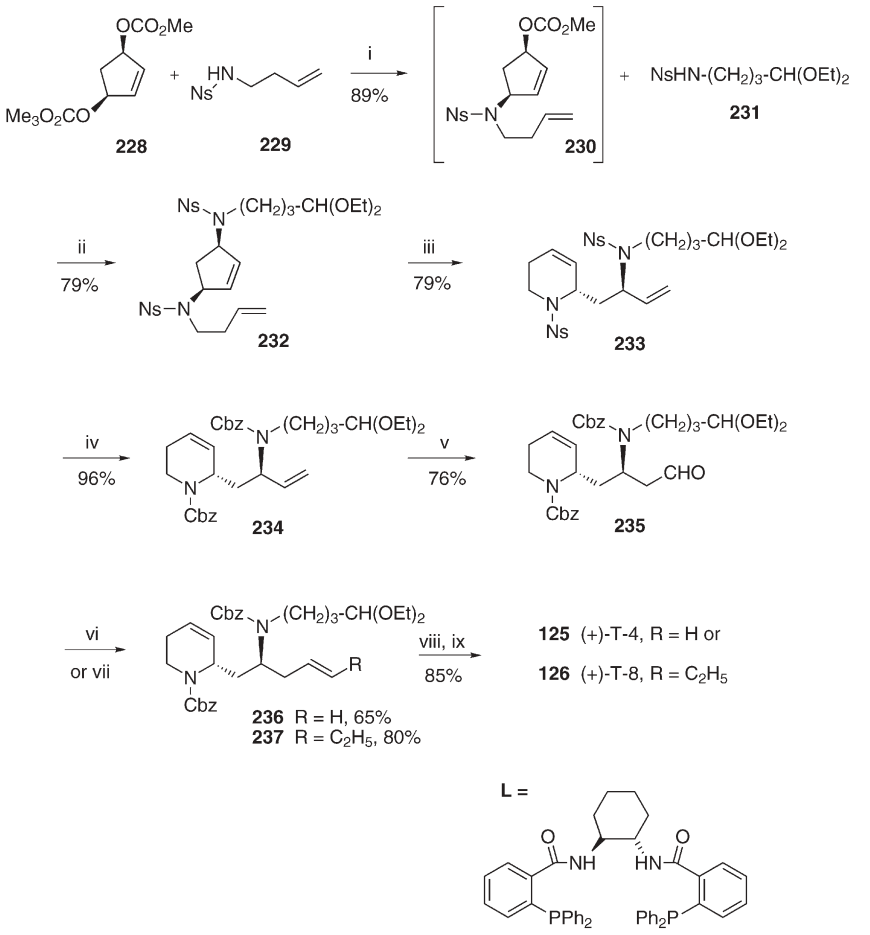
i: $(MeO)_3CMe$, PPTS then MeCOBr then K_2CO_3, MeOH; ii: $BrMgCH_2CHCH_2$, Me_2S-CuBr; iii: succinimide, Ph_3P, DEAD; iv: H_2, Pd/C; v: $LiAlH_4$; vi: Bu_2CuLi.

Scheme 8 Total synthesis of (+)-T-3 and (+)-T-7 according to Takahata et al

Similarly, the synthesis of (+)-T-7 (**129**) was achieved in a five-step sequence from [2*R*-(2*R*)]-**221** in 32% overall yield (Scheme 8). The regioselective opening of the epoxide of [2*R*-(2*R*)]-**223** with lithium dibutylcuprate afforded alcohol [2*R*-(2*R*)]-**227** that was transformed in a three-step sequence into (+)-T-7 (**129**) in 51% yield. In an analogous way (+)-T-8 (**126**) was obtained from [2*R*-(2*S*)]-**223** in four steps and 35% yield.

A further enantioselective synthesis of (+)-T-4 (**125**), T-6 (**128**), T-7 (**129**) and T-8 (**126**) has been reported by Stragies and Blechert [198]. Key steps are a Pd-catalyzed domino allylation and a Ru-catalyzed metathesis ring rearrangement. Their strategy represents a general approach towards all naturally occurring tetraponerines and will be illustrated here by the description of the syntheses of (+)-T-4 (**125**) and (+)-T-8 (**126**) (Scheme 9).

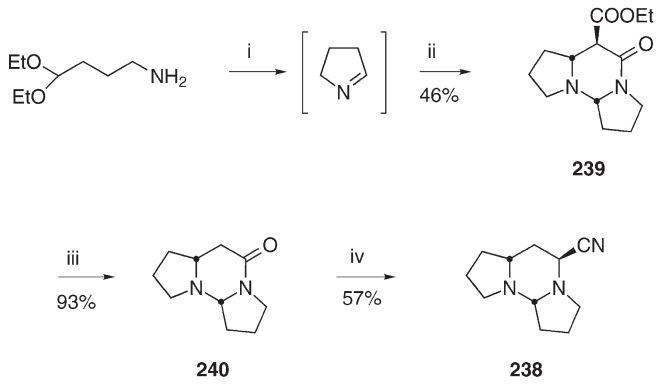
The selective allylic alkylation of dicarbonate **228** with *N*-nosyl-3-butenylamine (**229**) in the presence of a palladium catalyst (PdL_2) gave compound **230** in 89% yield. The addition of three equivalents of Et_3N was essential to obtain good yields and enantioselectivities. Exchange of the palladium ligand **L** against 3,4-di(bisphenylphosphino)butane (dppb) and addition of the second nucleophile **231** in a one-pot procedure led to the diaminated product **232.** The synthesis was continued using a metathesis ring rearrangement utilising the Grubbs' catalyst ([Ru]=$Cl_2(PCy_3)_2Ru$=CHPh) at 35 °C. Under these conditions, conversion of **232** into the tetrahydropyridine derivative **233** was achieved in 79% yield after two days. The ratio of **232/233** was 1:5.5 as determined by ^1H NMR. At this stage of the synthesis, the nosyl protecting groups of **233** were



i: PdL_2, -60°C, Et_3N, THF; ii: dppb, rt; iii: CH_2Cl_2, C_2H_4, [Ru], 35°; iv: K_2CO_3,DMF, PhSH then $PhCH_2OCOCl$; v: O_2, $PdCl_2$, CuCl; vi: $CHEtI_2$, $CrCl_2$; vii: CH_2I_2, $CrCl_2$, viii: H_2,Pd/C, EtOH then 5% HCl.

Scheme 9 Synthesis of (+)-T-4 and (+)-T-8 reported by Stragies and Blechert

replaced by benzyloxycarbonyl groups owing to their better compatibility with the subsequent reaction steps. The terminal double bond of the metathesis product **234** was cleanly transformed into the corresponding aldehyde **235** using the conditions of the Wacker oxidation. Product **236** was obtained in 80% yield when performing the olefination reaction with $CrCl_2$ and $CHEtI_2$ in THF. The yield of **237** in this reaction with CH_2I_2 was 65%. The synthesis was achieved with the cleavage of the protecting groups with concomitant hydrogenation of the double bonds followed by an acidic treatment that led to deprotection of the ketal group and cyclization into (+)-T-4 (**125**) from **236** (overall yield 24%) and (+)-T-8 (**126**) from **237** (overall yield 31%).

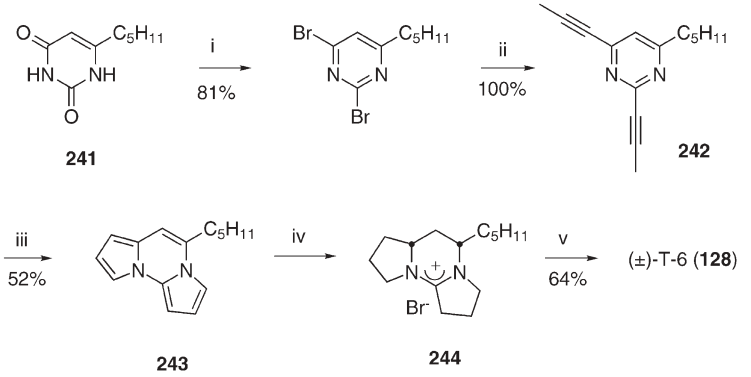


i: 10% HCl, 20°C, 30 min; ii: 1M KOH --> pH 12 then diethyl malonate; iii: 5% KOH/MeOH then 10% HCl --> pH 4.5; iv: DIBALH, THF then $HClO_4$/EtOH --> pH 3 then KCN.

Scheme 10 Formal synthesis of racemic T-1, T-2, T-5, and T-6

In addition to the two asymmetric syntheses above described, two racemic syntheses of tetraponerines based on the 5=6-5 tricyclic skeleton have been published. Thus, Plehiers et al. [199] have reported a short and practical synthesis of (±)-decahydro-5*H*-dipyrrolo[1,2-*a*:1′,2′-*c*]pyrimidine-5-carbonitrile (**238**), a pivotal intermediate in the synthesis of racemic tetraponerines-1, -2, -5 and -6, in three steps and 24% overall yield from simple and inexpensive starting materials. The key reaction of the synthesis was a one-pot stereoselective multistep process, whereupon two molecules of Δ^1-pyrroline react with diethylmalonate to afford the tricyclic lactam ester **239**, possessing the 5-6-5 skeleton (Scheme 10). Hydrolysis of the carboethoxy group of **239** followed by decarboxylation yielded lactam **240**, that was converted into α-aminonitrile **238** identical in all respects with the pivotal intermediate described by Yue et al. [200] in their tetraponerine synthesis.

The procedure used by Kim and Gevorgyan [201] to synthesize (±)-tetraponerine-6 (**128**) is based on the double pyrrolization of pyrimidine derivatives into bis-pyrrolopyrimidines via the Cu-assisted cycloisomerization of alkynylimines. The synthesis began with pyrimidinedione **241** (Scheme 11). Treatment of **241** with $POBr_3$ followed by Sonogashira coupling with propyne gave bispropynylpyrimidine **242**. The next step, a sequential double pyrrolization of **242**, led to the tricyclic bis-pyrrolopyrimidine **243**. The complete reduction of the heteroaromatic ring proved not to be simple and was performed in two steps. First, catalytic hydrogenation of **243** under acidic conditions gave amidinium salt **244** that was further reduced with $LiAlH_4$ to give (±)-T-6 (**128**) as the sole diastereoisomer in 64% yield.

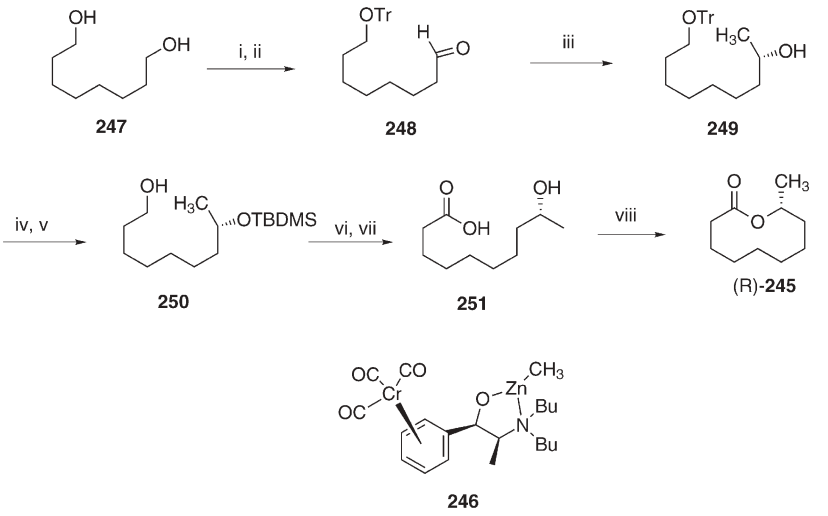


i: $PhN(Me)_2$, $POBr_3$, C_6H_6; ii: CuI, $Pd(PPh_3)_2Cl_2$, propyne, Et_3N; iii: CuBr, Et_3N/DMA; iv: H_2, PtO_2, HBr, MeOH; v: $LiAlH_4$, THF.

Scheme 11 Total synthesis of (±)-T-6

3.2 Non-Alkaloid Compounds

In the latter years, few non-alkaloid insect defensive compounds have been synthesized. In this section, only enantioselective syntheses will be considered.



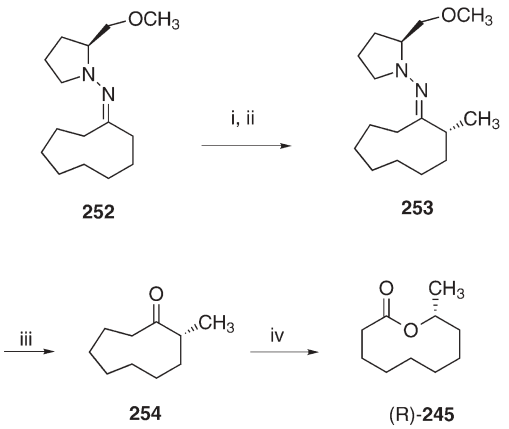
i: TrCl, Et_3N, DMF (98%); ii: Dess-Martin periodinane, CH_2Cl_2 (99%); iii: $(CH_3)_2Zn$, toluene, -5°C, 0.05 eq. **246** (84%, >88% ee); iv: TBDMSOTf, 2,6-lutidine (97%); v: BCl_3, CH_2Cl_2 (96%); vi: PCC/DMF (95%); vii: HF-py (100%); viii: Ph_3P, $(pyS)_2$, $AgClO_4$ (75%).

Scheme 12 Synthesis of (*R*)-phoracantholide using a chiral arene chromium tricarbonyl catalyst

(*R*)-(–)-Phoracantholide I (**245**), a component of the defensive secretion of the eucalyptus longicorn *Phoracantha synonyma* [202], proved to be a popular synthetic target. The most interesting approaches towards this compound reported during the last ten years are summarized hereunder.

In one approach, the asymmetric center was introduced by using the chiral arene chromium tricarbonyl based catalyst **246** to mediate the addition of dimethylzinc to the functionalized aldehyde **248** (Scheme 12) [203, 204]. The choice of the catalyst and of a suitable protecting group for the primary alcohol function proved to be critical. Optimum conditions were found using 5 mol% of the *N,N*-dibutyl catalyst **246** and the trityloxy protecting group. Thus, commercially available 1,9-nonanediol (**247**) was first monoprotected, thence oxidized with Dess-Martin reagent to afford the protected hydroxyaldehyde **248**. The latter was subjected to enantioselective methylation to give **249**. After protection of the secondary hydroxyl group with TBDMSOTf, and selective trityloxy deprotection with boron trichloride, compound **250** was obtained in good yields. Oxidation of the primary alcohol to the carboxylic acid with PDC in DMF, deprotection of the secondary alcohol and macrolactonisation of the resulting hydroxyacid **251** produced (*R*)-(–)-phoracantholide I (**245**) in high optical purity.

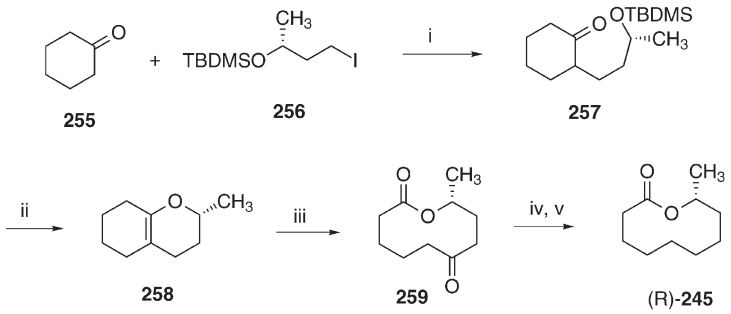
Enders and co-workers based their synthesis of (*R*)-(–)-phoracantholide I (**245**) on their well-known SAMP methodology (Scheme 13) [205]. Thus, the SAMP derivative of cyclononanone (**252**) was deprotonated with LDA at 0 °C, and the resulting enolate was treated with CH_3I at –100 °C to afford the α-methylated product **253** with >93% de. The latter was ozonized at –78 °C, and the resulting ketone (**254**) subjected to a Bayer-Villiger reaction to afford (*R*)-(–)-**245** with 91% ee.



i: LDA, THF, 0°C; ii: MeI, -100°C (85%, >93% de);
iii: O_3, CH_2Cl_2, -78°C (70%); iv: *m*-CPBA (74%, 91% ee).

Scheme 13 Synthesis of (*R*)-phoracantholide using a ring enlargement of cyclohexanone

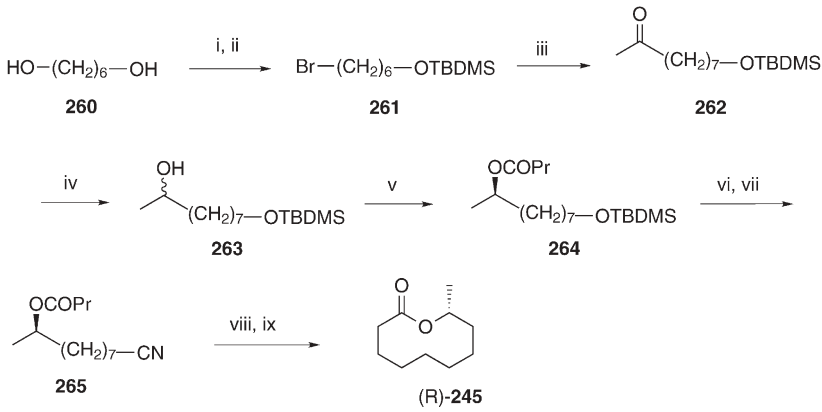
Another approach to (*R*)-(–)-phoracantholide I (**245**) used a ring enlargement of cyclohexanone (**255**) which had been alkylated with chiral synthon **256** (Scheme 14) [206]. Thus, compound **257** was prepared in 35% yield on a 7-g scale by alkylation of cyclohexanone with chiral **256.** Cyclization with Amberlyst A-15 provided enol ether **258** that was directly submitted to ruthenium tetroxide oxidation to give oxolactone **259** in a 47% yield. Reduction of the latter with catecholborane via its tosylhydrazone afforded (*R*)-(–)-phoracantholide I (**245**) in 31% yield.



i: (35%); ii: Amberlyst A-15; iii: RuO_4 (47%); iv: TsNH-NH_2; v: catecholborane (31% in two steps).

Scheme 14 Synthesis of (*R*)-phoracantholide using the Enders methodology

A rather lengthy chemoenzymatic synthesis of (*R*)-(**245**) was also reported (Scheme 15) [207]. Carbinol **263,** obtained in four steps from 1,6-hexanediol (**260**), was submitted to enantioselective acylation catalyzed by porcine pan-

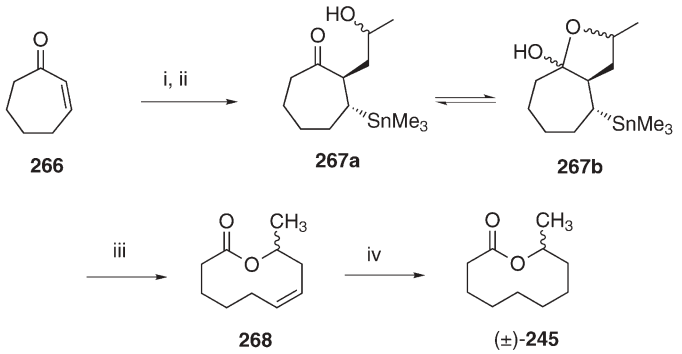


i: 48% HBr, benzene, Δ (60%); ii: TBDMSCl, Et_3N, DMAP, CH_2Cl_2 (88%); iii: $CH_3COCH_2CO_2Me$, NaOH, MeOH; Aqueous NaOH, H^+ (48%); iv: $NaBH_4$, MeOH (78%); v: PPL, TFEB, DIPE (26%, 93% ee); vi: Bu_4NF, THF (84%); vii: TsCl, py, CH_2Cl_2 ; KCN, DMSO, Δ (68%); viii: Conc. HCl, Δ; Alcoholic KOH, H^+ (61%).

Scheme 15 Synthesis of (*R*)-phoracantholide using a PPL-mediated resolution step

creatic lipase using trifluoroethyl butanoate as acyl donor. This procedure afforded the (*R*)-butanoate **264** with 93% ee at 30% conversion. After desilylation of the primary alcohol, tosylation followed by reaction with KCN afforded nitrile **265**. This, on acid hydrolysis, alkaline treatment and subsequent acidification gave (*R*)-**245** with 93% ee.

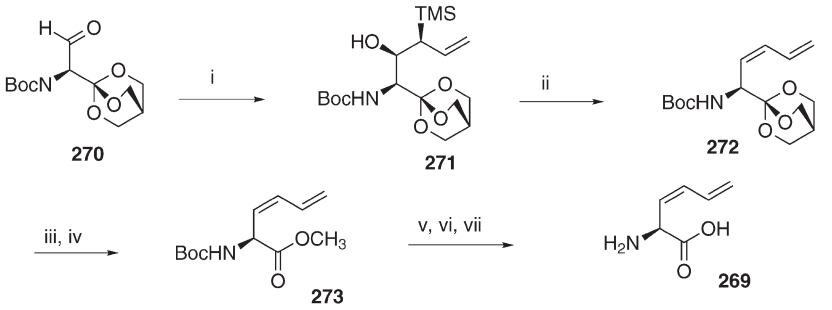
Recently, the shortest, and probably most elegant synthesis of phoracantholide, was reported by Posner et al. (Scheme 16) [208]. It is based on the discovery that $BF_3.Et_2O$ activates 1-substituted and 1,2-disubstituted epoxides towards nucleophilic opening by ketone enolates under mild conditions. This affords useful γ-lactols that can undergo n+3 ring expansion reactions. Thus, treatment of 2-cycloheptenone (**266**) with $LiSnMe_3$, followed by addition of (±)-propylene oxide in the presence of $BF_3.Et_2O$ at –78 °C gave the γ-hydroxyketone **267a** which is in equilibrium with hemiketal **267b**. The latter, upon treatment with $Pb(OAc)_4$ and $CaCO_3$, produced a ten-membered lactone bearing a Δ^6 double bond (**268**). Catalytic hydrogenation of the latter gave (±)-**245** in four steps and 45% overall yield. Since both enantiomers of propylene oxide are commercially available, enantiomerically pure (+)- and (–)-phoracantholide can be prepared via this methodology.



i: $LiSnMe_3$; ii: propylene oxide, BF_3-Et_2O, -78°C, 1h; iii: $Pb(Oac)_4$, $CaCO_3$;
iv: H_2, $(Ph_3P)_3RhCl$ (45% overall yield).

Scheme 16 Synthesis of (±)-phoracantholide based on nucleophilic opening of epoxides by ketone enolates

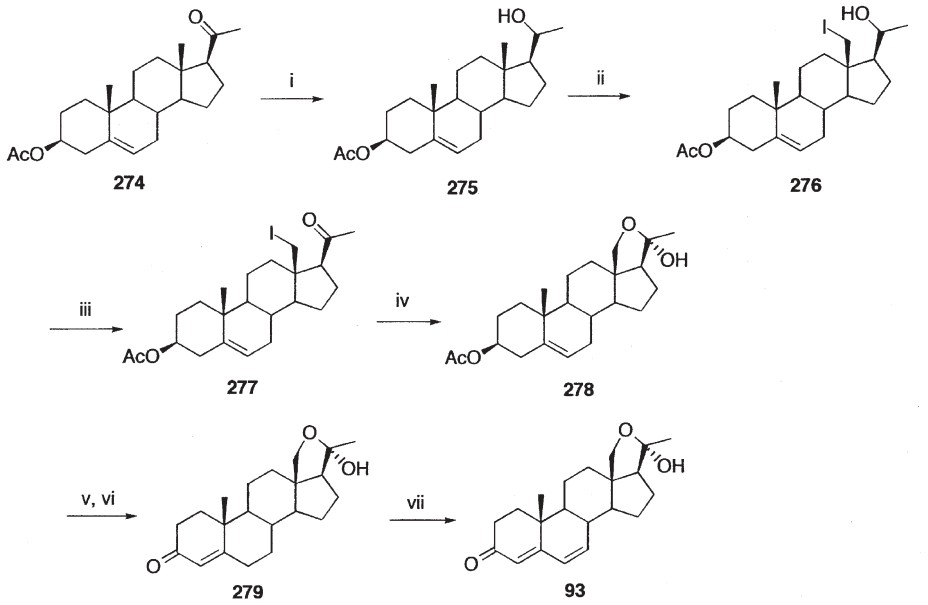
The unusual amino acid (*S*)-2-amino-(*Z*)-3,5-hexadienoic acid (**269**), which is a component of the toxic γ-glutamyl dipeptide isolated from the defensive glands of the Colorado beetle [209], has been synthesized along Scheme 17, after two initial attempts had proved unsuccessful due to the instability of **269** towards various oxidation conditions [210]. Scheme 17 relies on the hydrolysis of an ortho ester to generate the required carboxylic acid. Thus, the L-serine aldehyde equivalent **270** was treated with (*E*)-1-trimethylsilyl-1-propene-3-boronate to give the addition product **271**. Reaction of **271** with KH gave the stereochemically pure (*Z*)-diene **272**. Mild acid treatment of **272** followed by



i: Pinacol (*E*)-1-trimethylsilyl-1-propene-3-boronate, CH_2Cl_2, 4 days, then triethanolamine;
ii: KH, THF, -10°C; iii: TsA, MeOH; iv: K_2CO_3, MeOH (aq.) (55% for the 4 steps);
v: 10% aq. $CsCO_3$, MeOH (); vi: HBr/AcOH; vii: DOWEX 50WX2-200 eluted with H_2O,
then 10% NH_4OH solution.

Scheme 17 Synthesis of (*S*)-2-amino-(*Z*)-3,5-hexadienoic acid

transesterification with potassium carbonate in aqueous methanol afforded the protected amino dienoic acid **273** in 55% yield from **272.** Deprotection of **273** was accomplished by treatment with 10% aqueous cesium carbonate in methanol followed by acidolysis of the Boc group to afford **269,** but the yield was not reported.

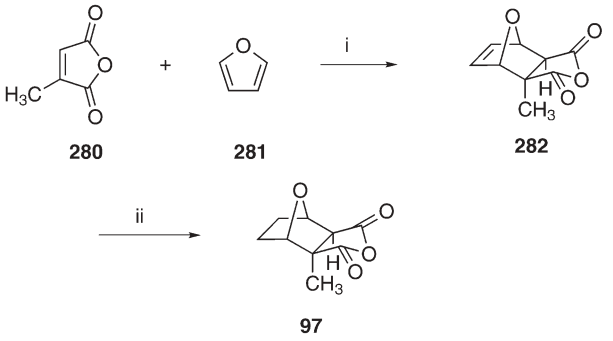


i: $NaBH_4$, THF:MeOH (1:1) (85%); ii: $Pb(OAc)_4$, I_2, $CaCO_3$, cyclohexane, hν, reflux; iii: CrO_3, H^+, acetone, 0°C, 10 min., (50% for two steps); iv: AgOAc, 1,4-dioxan, H_2O (9:1), 65°C, 12h (92%); v: 10% NaOH, MeOH, r.t., 30 min. (95%); vi: $Al(OPr^i)_3$, *N*-methyl-4-piperidone, toluene, reflux, 5h (72%); vii: chloranil, *tert*-butanol, reflux, 30 min. (54%).

Scheme 18 Synthesis of mirasorvone from dytiscid beetles

The pregnane steroid mirasorvone (**93**) discussed above has been synthesized from pregnenolone acetate (**274**) as outlined in Scheme 18 [211]. Treatment of pregnenolone acetate (**274**) with sodium borohydride yielded 3β-acetoxypregn-5-en-20β-ol (**275**) in 85% yield. Irradiation of **275** in the presence of iodine, lead tetraacetate and dry calcium carbonate, using a 500-W tungsten lamp, produced the iodide **276**, which was oxidized with Jones reagent without purification to give the unstable iodoketone **277** in 50% yield. Reaction of **277** with silver acetate in aqueous dioxane provided the 18,20 hemiketal **278**. Saponification of the latter in 95% yield followed by oxidation using a modified Oppenauer method, yielded enone **279**. Dehydrogenation with chloranil in *tert*-butanol afforded the target **93**. The 20α-hydroxy configuration of **93** was determined by ^{1}H NMR spectroscopy and confirmed by X-ray diffraction analysis on intermediates **278** and **279**.

In view of the biological activity of cantharidin (**98**) and its analogues (see below), Dauben and coworkers devised a total synthesis of (+)- and (–)-palasonin (**97**) [212]. As is also the case for cantharidin, palasonin (**97**) is not an easy target despite its structural simplicity: the only way to assemble its ring system (Scheme 19) was through a Diels-Alder reaction at 8 kbar between citraconic anhydride (**280**) and excess furan (**281**), yielding (±)-dehydropalasonin (**282**). The latter was then subjected to a catalytic hydrogenation reaction to afford (±)-palasonin (**97**). All attempts to circumvent the use of high pressures (normal pressure Diels-Alder reaction under hydrogenation conditions, use of $LiClO_4$) failed. (±)-Palasonin was resolved by reaction with two equivalents of (S)-(–)-α-methylbenzylamine to yield a pair of diastereomeric diamides, which were separated and subsequently saponified to the diacids. The indi-



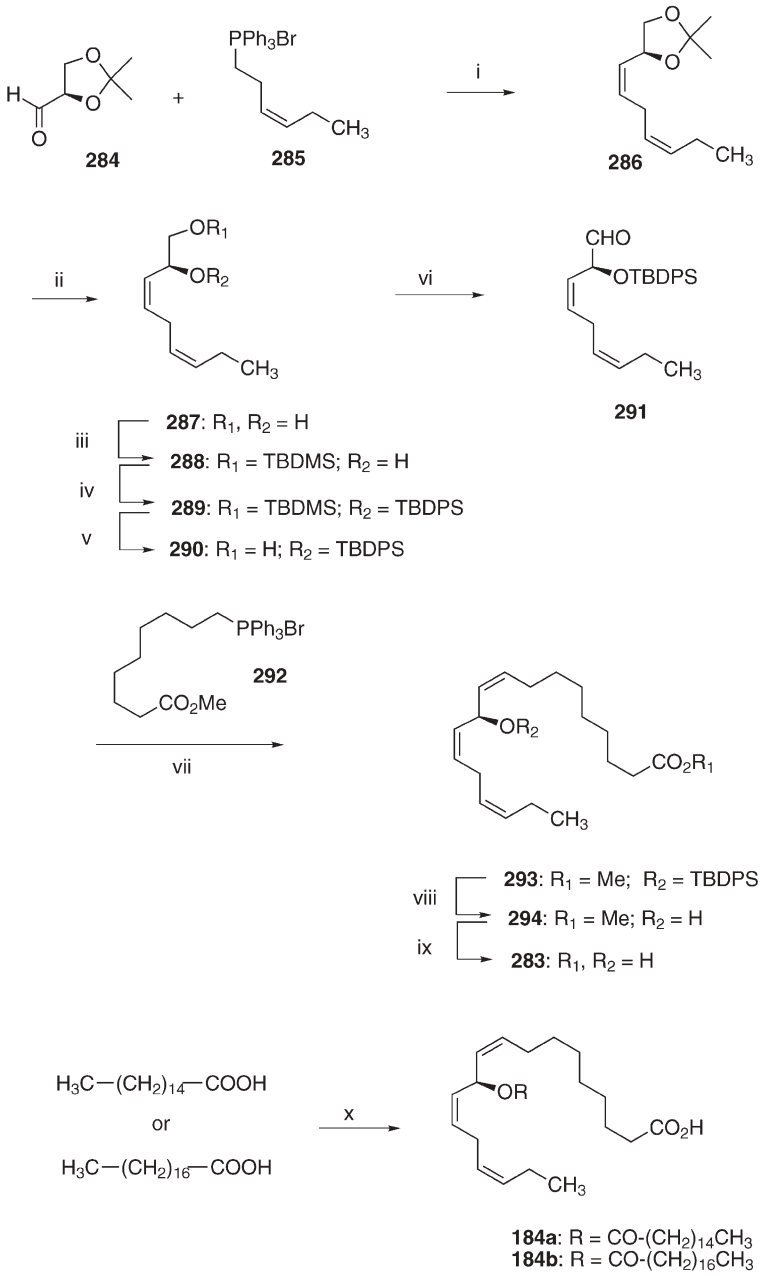
280 **281** **282** **97**

i: 8 kbar, 4 days (82%); ii: H_2, Pd/C, THF, 20h (90%).

Scheme 19 Synthesis of (+)- and (–)-palasonin

vidual diacids upon reaction with thionyl chloride yielded (+)-palasonin and (–)-palasonin.

A synthesis of the mayolenes, defensive lipids from the glandular hairs of the cabbage butterfly *Pieris rapae* (see above), has recently been described [174].



i: LiHMDS, -78°C, THF (69%); ii: HCl, MeOH (90%); iii: TBDMSCl, Et_3N, DBU, CH_2Cl_2 (85%); iv: TBDPSCl, DMAP, CH_2Cl_2 (89%); v: PPTS, EtOH (92%); vi: TPAP, NMO, CH_2Cl_2 (81%); vii: LiHMDS, -78°C, THF (55%); viii: TBAF, THF (99%); ix: LiOH, MeOH (95%); x: EDCI, DMAP, CH_2Cl_2, then 96 (59%).

Scheme 20 Synthesis of the mayolenes

These compounds, e.g., mayolene-16 (**184a**) and mayolene-18 (**184b**) [173], are based on (11*R*)-hydroxylinolenic acid (**283**) [(11*R*)-HLA] esterified with a series of homologous saturated fatty acids. The synthetic challenge resides in the bis-allylic hydroxyl group of 11-HLA, which is particularly prone to 1,4-elimination. The route depicted in Scheme 20 is based on two consecutive Wittig reactions to construct the C_9-C_{10} and C_{12}-C_{13} *Z* double bonds. Both enantiomers of 11-HLA could be prepared from (*R*)-glyceraldehyde acetonide (**284**) by simply inverting the order of the two Wittig reactions. Thus, the ylide derived from (3*Z*)-3-hexenyltriphenylphosphonium bromide (**285**) was treated with (*R*)-**284**, providing diene **286** with a *Z*:*E* ratio >99:1. Deprotection of **286** afforded diol **287**. The latter was selectively protected at the primary alcohol group with TBDMSCl and triethylamine in the presence of DBU to afford **288**. Next, protection of the secondary alcohol of **288** as the *tert*-butyldiphenylsilyl ether **289** followed by selective deprotection of the primary alcohol with PPTS afforded alcohol **290**. Oxidation of this alcohol to aldehyde **291** followed by Wittig reaction of the latter with ylide **292** gave trienoate **293**. Treatment of **293** with TBAF afforded alcohol **294**, which on lithium hydroxide treatment and careful neutralization afforded (11*R*)-HLA (**283**). The synthesis of the labile mayolene-16 and -18 could be achieved by preactivating palmitic or stearic acid with EDCI in the presence of DMAP, followed by the addition of **283**. Careful neutralization of the solution with a pH 5.75 buffer followed by extraction and chromatography afforded pure **184a** and **184b** (Scheme 19) [174].

4 Biosynthesis

Despite the large number of defensive compounds already isolated from insects, few studies have addressed their biosynthetic origin. This situation undoubtedly reflects the many problems that hamper this area of research. Finding the right method to administer the labeled precursor(s) (feeding, injection...) is one of the most important problems. Insects being complex organisms, how to be sure that the precursor(s) will reach the site of biosynthesis intact? Also, the administration of relatively large doses of labeled material may lead to channel overload and disturb physiological processes. On the other hand, with insects, low incorporation rates are generally observed with little advanced precursors such as acetate. Thus, in most cases radioactive precursors must be used, which necessitate both special laboratory facilities and the set up of degradation experiments in order to locate the label in the molecule.

Several biosynthetic studies of defensive compounds produced by insects have been reported, but they will not be dealt with here, since we have published quite recently a review on this topic [73], and the earlier literature has been adequately covered [3, 4, 157, 213].

5
Biological and Pharmacological Activities

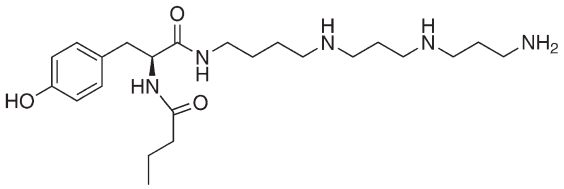
Although many insect defensive compounds are endowed with diverse biological activities, few of them have been studied in depth to evaluate their potential pharmacological activities. Cantharidin (**98**) (Fig. 16), pederin (**104**) (Fig. 18), and some of the alkaloids isolated from ants (e.g., the solenopsins **109** and **110**, Fig. 19) have been the subject of most of the investigations. These topics have been reviewed several times [111, 214, 215], and we will only summarize here the most recent data.

Cantharidin (**98**), the toxic, irritating principle from blister beetles (*Mylabris* spp.) and the Spanish fly (*Cantharis vesicatoria*), has been (in)famous for centuries due to its alleged aphrodisiac properties [87]. Recent work has shown that this compound and its analogs belong to the okadaic acid class. They bind with high affinity to a specific protein isolated from mouse liver, identified as protein phosphatase 2A (PP2A) [216]. The syntheses of several cantharidin analogs and the evaluation of their effects on PP2A have shown that cantharidin was the most active of all compounds tested. It was also demonstrated that both the 7-oxa group and the anhydride system are important for the activity. Removal of the bridgehead methyl groups also reduced the activity [217]. Some cantharidin analogs like palasonin (**97**) (Fig. 16) are anthelmintic. This has been ascribed to inhibition of energy metabolism and/or alteration in the motor activity of the parasite [218].

Pederin (**104**), the powerful cytotoxin of staphylinid beetles of the genus *Paederus*, has been the object of renewed interest due to the unexpected discovery of a series of closely related compounds in marine sponges that display antitumor activities. The latter, as well as pederin, were prepared by total synthesis and their biological activities were reported [219, 220]. A recent review summarizing present knowledge on this family of compounds has been published [221].

Farnesylamine (**123**) (Fig. 20), a sesquiterpene alkaloid, was recently detected in whole extracts of *Monomorium fieldi* [129]. This compound had already be prepared by synthesis and found to display a whole range of biological activities. Among others, it inhibits arthropod molting, squalene synthesis, and the growth of malignant tumor cells, modulates human T cells and has anti-osteoporosis activity [129].

Before closing this section, we should also briefly mention the work performed by Nakanishi's group on philanthotoxin (PhTX-433) (**295**) (Fig. 36), a toxin present in the venom of the wasp *Philanthus triangulum* F. [222]. PhTX-433 is a non-competitive, reversible antagonist of the quisqualate-sensitive glutamate receptor (qGlu-R) of locust skeletal muscle and of some vertebrate glutamate receptors, which acts by open channel blocking. It is closely related to the numerous polyamines isolated from spider venoms [223]. Extensive structure-activity studies performed with approximately hundred PhTX-433 analogs have provided several analogs with higher activity than the natural



295

Fig. 36 Structure of philanthotoxin (PhTX-433) from *Philanthus triangulum*

product. Photolabile or radioactive groups were incorporated at various sites of the molecule, thus allowing the authors to perform studies of a nicotinic acetylcholine receptor. In particular, photoaffinity labeling studies have led to a model showing the mode of binding of PhTX to this receptor [224].

Finally, the antifungal properties of α-pinene and limonene, two monoterpenes frequently found in the defensive secretion of *Nasutitermes* termites, was studied. In vitro assays showed that these molecules reduce spore germination of the fungus *Metarhizium anisopliae* through direct and indirect (vapor) contact [225]. Moreover, some diterpenes isolated from these insects have also an antibiotic activity on *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis* [226].

6 Conclusions and Perspectives

In the last ten years, research in the field of insect defensive chemistry has made remarkable breakthroughs which would not have been possible without the advances in separation techniques, structure determination methods, and synthetic methodology. The structures of structurally complex compounds can now be determined on less than 1 mg of material, as exemplified by the hexa- and heptacyclic coccinellid alkaloids. Moreover, in-depth investigations on the biosynthetic origins of some of the defensive compounds are now made possible and will surely bring interesting data in the future.

However, it should be pointed out that success in this area cannot be achieved without a close association, and mutual understanding, between entomologists and chemists, a condition that, so far, does not seem easy to achieve.

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Topics in Current Chemistry (2005) 240: 231–278
DOI 10.1007/b98318

Mammalian Semiochemicals

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Abstract Progress that has been made in research on the chemical aspects of mammalian semiochemistry over the past decade is discussed on the basis of examples from the most topical problem areas. The chemical characterization of the volatile organic constituents of the urine, anal gland secretions and exocrine gland secretions of rodents, carnivores, proboscids, artiodactyls and primates, and their possible role in the semiochemical communication of these mammals are discussed, with particular emphasis on the advances made in the elaboration of the function of proteins as controlled release carrier materials for the semiochemicals of some of these animals.

Keywords Mammalian semiochemicals · Mammalian pheromones · Mammalian exocrine secretions · Chemical communication · Territorial marking

List of Abbreviations

CI	Chemical ionization
FID	Flame ionization detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GCXGC	Comprehensive two-dimensional gas chromatography
MS	Mass spectrometry
MUPs	Major urinary proteins
SBSE	Stir bar sorptive extraction
SPME	Solid phase microextraction
TOF	Time-of-flight mass spectrometry

1 Introduction

Mammalian semiochemical communication has enjoyed continued interest from behavioral scientists, zoologists and molecular biologists during the past decade. In chemical circles, however, there seems to be more interest in insect semiochemicals than in mammalian chemical communication. There are several possible reasons for this. First, mammalian semiochemical secretions are almost invariably very complex and, in most cases, it is not possible to simplify the analytical problems resulting from this complexity by applying the so-called response-guided strategy [1]. In some cases, scientists are not even sure whether mammalian exocrine secretions are used exclusively for chemical communication or whether they also play a part in maintaining the health of the animal. Second, mammals are generally not considered to be as serious pests as many insect species, and it is therefore often difficult to obtain funding for chemical work on mammals. A further, not altogether unimportant, reason may be that, with some notable exceptions, the constituents of some mammalian semiochemical secretions are often rather boring compounds, which do not attract the same amount of attention from synthetic chemists as do many insect pheromones.

Only a few compounds or mixtures of compounds have been shown beyond doubt to be mammalian pheromones. This is the main reason why the subject matter of this chapter is not restricted to pheromones and why exocrine secretions and other mammalian excretions in general will be discussed as possible sources of pheromones, even though their role in the chemical communication of the species under discussion has not yet been established. Feeding deterrents are not discussed. In general defensive secretions are also not discussed, but the anal sac secretions of the mustelids are included, because it is possible that these secretions could also fulfill a semiochemical role, in addition to being used for defense.

2
Isolation and Identification

Techniques developed for the identification of insect semiochemicals and the determination of environmental contaminants have been used equally effectively in chemical work on mammals. Some of these methods will therefore be discussed only as far as their application is of particular significance in mammalian semiochemistry. Examples can be found in the literature of cases in which conclusions were drawn from results that were obtained by using inappropriate or, at least, doubtful analytical techniques. A few of the problem areas will be highlighted without giving the relevant literature references.

2.1
Sample Collection

In semiochemistry the selection of a suitable secretion collection method depends on the chemical nature, consistency and quantity of the material that has to be collected and on how easy it is to restrain the animal during the collection process. Glass vials with Teflon-faced (PTFE-faced) screw caps are preferred for the storage of collected material at the lowest possible temperature for as short a time as possible before the samples are analyzed. Plastic bottles have to be avoided at all costs because organic compounds and oxygen can diffuse through plastic materials and even through Teflon. Rubber stoppers are totally unacceptable because the volatile organic constituents of secretions will gradually be extracted from samples by sorption into the rubber, while antioxidants, residual polymerization monomers and other contaminants in the rubber will diffuse into the sample. The diffusion of impurities into samples is probably the source of *tert*-butylphenols and other artifacts that have been reported as constituents of some mammalian secretions.

After the vials have been cleaned in the conventional manner, they can be heated to high temperatures to get rid of residual volatiles [2]. It is of course not necessary to sterilize vials cleaned in this manner by rinsing them with ethanol. An example is known of the practically complete conversion of carboxylic acids

into ethyl esters in a secretion collected in a vial unnecessarily sterilized with ethanol. Secretions can be scooped from certain glandular structures with a vial, the top of the vial cleaned with pre-cleaned gauze or tissue paper, and the vial closed with its Teflon-faced screw cap. Heavier, waxy material has to be collected by wiping it off or out of glandular structures with gauze from which any volatile organic material has been extracted with a suitable solvent (see below) in a Soxhlet apparatus [2]. The collection of material in methanol or ethanol is extremely risky because these solvents can react with some of the constituents of the collected material. In one case, for example, the collection of material in ethanol resulted in the complete alcoholysis (transesterification) of a long-chain alkenyl pentanoate.

2.2 Sample Preparation

2.2.1 Extraction

There is no universal or ideal solvent for the extraction of volatile organic material from urine or exocrine secretions. Although the commercially available solvents for pesticide analysis are quite expensive, it is not prudent to try saving money by using cheaper products. Hexane is a very popular solvent for the extraction of organic compounds from collected secretions, especially amongst biologists, but it is the worst solvent to use if short-chain, polar compounds have to be extracted from a secretion that also contains polar material and especially water. One example of the inappropriate use of this solvent is the attempted extraction of organic material from 1 g of secretion with 0.2 ml of hexane. Needless to say, only a few long-chain compounds were isolated. Dichloromethane is a moderately polar solvent with a relatively low boiling point that is available in very high purity. If it is of residue analysis grade, it can be used without further purification. Unfortunately, it is not an ideal solvent for the extraction of polar compounds. Old dichloromethane has to be discarded because it will contain phosgene. Diethyl ether is a better solvent than dichloromethane for the extraction of polar compounds, and it also has a conveniently low boiling point. However, it is sold with various stabilizers, which have to be removed before it can be used. This can best be done by passing the solvent through a column of aluminum oxide which has been activated at 800 °C to remove organic impurities. At the same time residual moisture and other polar impurities are also removed from the solvent. Care has to be taken to prevent the formation of ether peroxide in this purified ether in the absence of a stabilizer. Ethyl acetate, although an excellent solvent for many organic compounds, has a relatively high boiling point, which can lead to the loss of the volatile constituents of the extracted material when the extract is concentrated, and also to overlapping of the solvent peak with some of the analytes in GC and GC-MS analyses.

Soxhlet extraction is used to extract organic compounds from larger quantities of gauze, cotton wool or other material on which a secretion has been collected. This technique normally requires the use of relatively large volumes of solvent, even when a specially made small Soxhlet apparatus is used. Some of the extracted volatile material will be lost when such large volumes are evaporated from extracts obtained in this manner. The evaporation of the solvent is best done very slowly in an inert gas atmosphere without blowing the gas directly into the sample vial [2]. Extracting very small quantities of organic volatiles from gauze, hair or particulate matter is best done without using a Soxhlet apparatus. Wetting the carrier material with the minimum volume of solvent and filtration in a centrifuge was found to produce good results in difficult cases [2]. This method has been used very effectively to extract volatile organic material from the coarse hair of antelope.

2.2.2 Sample Enrichment

Various sample enrichment techniques are used to isolate volatile organic compounds from mammalian secretions and excretions. The dynamic headspace stripping of volatiles from collected material with purified inert gas and trapping of the volatile compounds on a porous polymer as described by Novotny [3], have been adapted by other workers to concentrate volatiles from various mammalian secretions [4–6]. It is risky to use activated charcoal as an adsorbent in the traps that are used in these methods because of the selective adsorption of compounds with different polarities and molecular sizes on different types of activated charcoal. Due to the high catalytic activity of activated charcoal, thermal conversion can occur if thermal desorption is used to recover the trapped material from such a trap.

It is surprising that solid phase micro extraction (SPME) [7] has so far not been used much for the headspace sampling of volatiles in mammalian semiochemistry. This technique, although very convenient and powerful in qualitative analysis, has to be used with discretion in the comparison of the quantitative composition of the volatile organic fraction of samples with large variations in their water and/or heavy lipid content. In water-containing samples, for example, the water competes with the SPME fiber for the polar constituents and the quantitative results will thus be distorted if the secretions that are compared contain widely varying amounts of water. This method can, however, be used with confidence to follow changes in the rate at which volatiles are released from a territorial mark, for example [8]. If the target compounds are present in very low concentrations in aqueous media or in waxy or proteinaceous matrices, the enrichment factor obtained with SPME could be very low. The recently introduced stir bar sorptive extraction (SBSE) technique [9] will give far better results in such difficult cases, because a larger volume of sorptive material is used in this technique than in SPME. Enrichment factors of more than two orders of magnitude higher than those obtained with SPME can be achieved.

An excellent review of modern sorptive sampling techniques that could be considered for the enrichment of volatiles from mammalian secretions appeared recently [10]. To be on the safe side, more than one sample preparation and sample enrichment method should be used to analyze mammalian secretions. If GC and GC-MS analyses are employed, the results obtained with split/splitless, on-column, SPME and solventless (solid) sample introduction methods [11, 12] should be compared.

2.3 Identification

GC-MS analysis has become standard practice in semiochemical research. There is, however, a real danger that information based exclusively on the results of computerized library searches without mass spectral and retention-time comparison with authentic synthetic material can be introduced into the literature. This could be problem especially in mammalian semiochemistry, because researchers often are faced with the problem of having to identify large numbers of compounds of which many may have very uninformative mass spectra. A critical reader of the original publication could still be aware of the unverified nature of some of the information, but this may not be pointed out in later references to the work.

High resolution MS and chemical ionization mass spectrometry (CI-MS) with methane, isobutane or ammonia as reactant gases are used less frequently in semiochemical analysis (e.g. [13, 14]). Nitric oxide (NO) is a most versatile reactant gas for chemical ionization mass spectrometry. CI(NO)-MS can be used to determine the positions of double bonds in unsaturated compounds without having to derivatize the unsaturated compounds [15] and it also allows distinction between primary, secondary and tertiary alcohols. The reaction of NO^+ with primary alcohols affords abundant $(M-3)^+$, $(M-1)^+$ and $(M-2+NO)^+$ ions, whereas $(M-OH)^+$, $(M-1)^+$ and $(M-2+NO)^+$ ions are generated from secondary alcohols and a single $(M-OH)^+$ ion from tertiary alcohols [16]. CI(NO)-MS has been utilized to determine the position of double bonds in some of the constituents of mammalian secretions [17, 18]. Unfortunately, NO is an aggressive gas which requires the use of an iridium filament (cathode) coated with thorium oxide. Otherwise, a glow discharge ion source, which obviates the use of a filament, can be used [19]. The derivatization with dimethyl disulfide in conjunction with electron impact mass spectrometry of the resulting derivatives therefore still remains the most used method for the determination of double bond positions [20, 21].

Due to its relatively low sensitivity, the combination of gas chromatography with Fourier transform infrared spectroscopy (GC-FTIR) is not a standard technique in semiochemical research. Nevertheless, it could come in handy for the identification of some compounds with utterly uninformative mass spectra [22].

Enormous progress has recently been made in the development of a powerful new technique known as comprehensive two-dimensional gas chro-

matography (GCXGC). The maximum number of peaks that can theoretically be accommodated in a two-dimensional chromatogram is in this technique increased by more than one order of magnitude, so that the chances of having overlapping peaks are reduced. It could thus be possible to detect trace compounds that would remain undetected in conventional capillary gas chromatography, especially when minor constituents are overlapped by the broad peaks of the major constituents of a sample. So far results have been very promising [23–25] and this technique is expected to become the method of choice for the gas chromatographic separation of complex mixtures of the type that is often encountered in mammalian semiochemical studies. A tabletop GCXGC-TOF-MS is expected to become commercially available in 2004. The separation of the complex preorbital secretion of the steenbok, *Raphicerus campestris*, probably the first example of a GCXGC separation of a mammalian exocrine secretion, is shown in Fig. 1 [26]. The apolar first column that was used in this analysis gave very broad, pre-tailing peaks for the fatty acids in the sample in the first dimension, resulting in this, not yet quite optimal, two-dimensional separation. Better results can be expected if OV-1701, a phase that elutes fatty acids as sharp peaks, is used in the first dimension and a highly polar phase is used in the second column. This chromatogram, nevertheless, demonstrates the power of this technique.

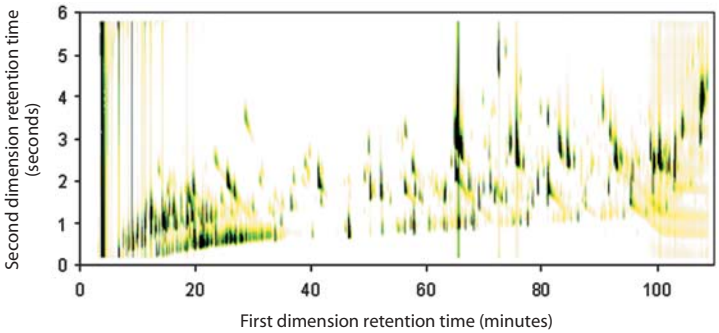


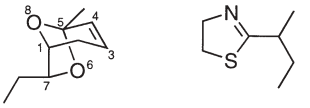
Fig. 1 A contour plot presentation of a two-dimensional gas chromatogram (GCXGC) of an extract of the preorbital secretion of the steenbok, *Raphicerus campestris* [26]. The x- and y-axes reflect a volatility-based separation on an apolar column in the first dimension, and a polarity-based separation on a more polar column in the second dimension, respectively. The compounds eluting at low retention times in the second dimension are therefore apolar compounds or compounds with a lower polarity than the compounds eluting at higher second-dimension retention times. This technique has the advantage that compounds belonging to the same compound class, appear as groups or bands in the contour plot, as can be seen in the lower left hand corner of the chromatogram. Gas chromatographic conditions: first column, 30 m×0.25 mm ID coated with 0.5 µm PS-255 (apolar phase); second column, 1 m×0.20 mm ID coated with 0.14 µm OV-1701; mean H_2 flow at 40 °C, 64 cm/s; ten-stage thermal modulator array pulsed at 300 ms/segment, pause time, 3 s [27]. In the GCXGC technique a modulator is used to focus fractions of the eluent from the first column and to release the focused material into the second column as sharp pulses

3
Rodents

Nocturnal habits and dark living environments have led to the evolution of olfaction as a major method of communication in many rodents. Although the dark and cramped habitats of many rodents complicate the study of their behavior, they can mostly be bred in large numbers under controlled conditions at modest cost and they are therefore ideal animals to use in the study of certain semiochemical phenomena. A considerable body of information on the chemical cues regulating the social and reproductive systems of these animals has thus been gathered over the last two to three decades.

3.1
Mice

Probably the most progress in this field has been made in the study of the role of constituents of the urine of the house mouse, *Mus domesticus*. 3,4-Dehydro-*exo*-brevicomin **1** and 2-*sec*-butyl-4,5-dihydrothiazole **2** (Fig. 2), identified in male urine, were found to elicit inter-male aggression in the house mouse. Whereas the urine of castrated males did not elicit this response, spiking castrated male urine with these two compounds restored the activity of the urine. The compounds were effective only when perceived in the context of the general odor of mouse urine and males did not show a significant increase in aggression towards castrated males painted with pure water spiked with the compounds [28]. Comparison of the urine of dominant and subordinate males showed that the levels of (*E,E*)-α-farnesene and (*E*)-β-farnesene were elevated in the urine of dominant males. These compounds are absent from bladder urine and originate from the animals' preputial glands, and were found to be responsible for the male aversion signal produced by dominant males. In this case the compounds were effective whether present in urine matrix or plain water [29]. These two compounds are also effective in estrus induction [30]. 2-Heptanone, (*E*)-5-hepten-2-one, (*E*)-4-hepten-2-one, pentyl acetate, (*Z*)-2-pentenyl acetate, and 2,5-dimethylpyrazine were identified as the constituents of a pheromone in the urine of adult females that delays puberty in juvenile females. These compounds were found to be active when added in their natural concentrations to previously inactive urine or to plain water [31]. The androgen-dependent compounds 6-hydroxy-6-methyl-3-heptanone, 3,4-dehydro-*exo*-brevicomin, 2-*sec*-butyl-4,5-dihydrothiazole, α-farnesene, and β-farnesene were found to be independently capable of accelerating the onset of puberty [30]. In synergism, 3,4-dehydro-*exo*-brevicomin **1** and 2-*sec*-butyl-4,5-dihydrothiazole **2** are responsible for the attraction of females to intact male urine, whereas if either of the compounds are added to castrate urine, its attractiveness remains the same [32]. Apparently they also play a role in estrus synchronization [33]. It was suggested that (*E,E*)-α-farnesene and (*E*)-β-farnesene also play a role in the recognition of sexually mature and socially dominant males [34].



1 2

Fig. 2 Two of the semiochemicals identified in the urine of the house mouse

These two compounds were as effective as intact preputial homogenate in eliciting this effect, while an extract of castrate preputial tissue did not show any pronounced effect [35].

An investigation of the stereochemistry of the urinary substances eliciting intermale aggression in the house mouse established that (1*R*,7*R*)-3,4-dehydro-*exo*-brevicomin is present in the urine. Due to extremely facile racemization under very mild conditions, it was concluded that 2-*sec*-butyl-4,5-dihydrothiazole **2** it is present in the urine as the racemate. It was suggested that the acidity of mouse urine probably promotes racemization of the optically active compound derived biosynthetically from an amino acid [36]. The observation that female house mice prefer the urinary odors of males uninfected by the intestinal nematode *Heligmosomoides polygyrus* suggests that urine may also be important in conveying information on the infection status of males [37].

The urinary profiles of adult male and female California mice, *Peromyscus californicus*, were examined to determine the volatile compounds that may affect reproduction. Several ketones, pyrazines, alkanes, as well as benzonitrile and benzaldehyde were identified as the constituents that are not specific to either one of the sexes. The urine of both sexes contains a remarkably large number of pyrazine derivatives that are present in unusually high concentrations [38]. To provide a broad characterization of the urinary volatiles as potential semiochemicals in the deermouse, *Peromyscus maniculatus*, the urine from adult and young animals of both sexes, as well as from castrated animals, was examined by Ma et al. [39]. The major constituents were identified as the various aldehydes, ketones, pyrazines, pyridines and quinolines, a thiazole, an amide and a nitrile, which are listed in Table 1. While none of these compounds were found to be unique to gender or age, quantitative analyses revealed that numerous compounds exhibited significant variations among different groups. It was concluded that the endocrine dependency of these compounds could suggest a potential chemosignaling function.

In common with other rodents, mice exhibit an obligate proteinuria in the form of major urinary proteins MUPs, a heterogeneous group of 19-kDa proteins with isoelectric points in the region of pH 4.2–4.7, of which males excrete 5 to 20 times as much as females [40]. The role of these proteins in chemical communication in mice has been studied in great detail during the past decade (e.g. [40–46]). When isolated from urine of the house mouse, these proteins are odorless, but after denaturation and extraction with a solvent, the organic phase had a strong mousy odor [41]. The proteins were found to bind 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyl-4,5-dihydrothiazole [41, 47, 48].

Table 1 Compounds[a] identified in the urine of the deermouse, *Peromyscus maniculatus* [39]

Butanone	Octanal
3-Methyl-2-butanone	2,4,6-Trimethylpyridine
2-Pentanone	6-Methyl-2-octanone
3-Pentanone	Acetophenone
4-Methyl-2-pentanone	Unidentified ethyldimethylpyrazine
2-Methyl-3-pentanone	Unidentified ethyldimethylpyrazine
3-Hexanone	Propiophenone
4-Methyl-3-penten-2-one	Unidentified constituent
trans-2-Hexenal	Unidentified methylacetophenone
5-Methyl-2-hexanone	2-Methylacetophenone
2-Heptanone	Phenylacetonitrile
2,6-Dimethylpyridine	6-Methyl-2-nonanone
3-Heptanone	Naphthalene[b]
2-Heptanone	Formanilide
Heptanal	Benzothiazole
Ethylpyrazine	2-Methylquinoline
Benzaldehyde	Unidentified dimethylnaphthalene[b]
4-Cycloheptenone	Unidentified methylethylquinoline
6-Methyl-5-heptenal	Geranylacetone
2,3-Ethylmethylpyrazine	

[a] Listed in order of gas chromatographic elution from an apolar column. Nomenclature as in [39].
[b] Most likely contaminants.

Apparently there is some specificity to the binding of these two ligands, with the brevicomin being associated with certain proteins, whereas the thiazole binds to all the major urinary protein subclasses [41]. The results of an X-ray crystallographic study of mouse major urinary protein corroborated the role of MUPs in pheromone transport in the house mouse [48]. Surprisingly, the major urinary proteins recovered from territorial marks deposited by wild house mice (*M. domesticus*) did not contain brevicomin and the dihydrothiazole as ligands. Instead, less volatile components, in particular menadione (vitamin K_3), were found to be present [42]. The displacement of natural ligands by lipophilic competitors such as menadione offers possibilities to use it as a tool for the exploration of the role of MUPs as slow release carriers for their associated volatiles [49]. However, MUPs could be more than simple delivery agents for pheromone molecules [50–52]. For example, recent evidence showed that the MUPs themselves are an integral part of the male mouse pheromonal complex and that the vomeronasal system is involved in the estrus-stimulating effect of the MUPs [46]. It is possible that the pattern of MUPs present in male urine can act as a type of individuality bar code that signals the identity of the owner of a scent mark [53, 54]. Because it has been found that female mice that had no previous experience with adult male-derived chemical signals are not attracted to male-soiled bedding, it has also been suggested that the primary sexual

attractant could be nonvolatile compound(s), e.g. MUPs [55]. On the other hand, it has been found that the high molecular fraction of male urine containing MUPs is ineffective in eliciting pregnancy block [56]. As far as the semiochemical role of MUPs is concerned, the overall picture is apparently still not quite clear.

3.2 Hamsters

The golden hamster, *Mesocricetus auratus,* utilizes the secretions of a variety of scent sources such as urine, vaginal secretions, and secretions of the Harderian, preputial and androgen-dependent sebaceous flank glands [57, 58]. Hamsters engage in two types of scent-marking behavior: flank marking, which is associated with competition and aggression, and vaginal marking, which is used for sexual solicitation.

A component of the vaginal secretion, dimethyl disulfide, was found to be the major sex attractant of the golden hamster [59]. Volatile alcohols, fatty acids and, interestingly, dimethyl trisulfide in the secretion do not appear to enhance the attractancy of the secretion [60]. However, proteins in the mass range of 15–16 kDa that are present in the vaginal secretion act as a mounting pheromone [61]. No comprehensive chemical characterization of the semiochemical secretions of golden hamsters has yet been undertaken.

The dwarf hamster, *Phodopus sungorus*, also known as the Djungarian, striped or hairy-footed hamster, is a small rodent that was first described in the 1960s. It is unusually resistant to the low temperatures occurring in its habitat on the dry steppes of Mongolia and Western Siberia (e.g. [62]). Olfactory marking in this hamster includes marking with feces, urine and secretions from the ventral gland [63]. Feoktistova [64] investigated the behavioral responses of adult, sexually experienced males toward different olfactory cues, such as the urine, bedding material and integumentary skin gland secretions of conspecific males and diestrous females. The animals have well-developed glands, of which the ventral sebaceous gland, which is located along the axis bodyline before the genitals, is one of the most important. The hamsters use the secretion of this gland to mark their home ranges.

In addition to the ventral gland, both male and female dwarf hamsters produce buccal secretions from the supplementary sacculi situated at the opening of their cheek pouches. Very little information is available on this subject. Functions attributed to this secretion in the subspecies *P. s. campbelli* include the communication of information about sex, identity, female breeding condition, and even the regulation of certain physiological functions [65]. It was found that the surgical removal of the sacculi did not influence the development of the pups when they could consume secretion from parents and littermates, but that restriction of, or the inability to consume secretion, led to a significant increase in pup mortality, accompanied by conditions such as nonspecific enterocolitis and disbacteriosis and a significant delay in growth and reproductive devel-

Table 2 Constituents of secretions of the ventral gland and supplementary sacculi (buccal secretion) of the dwarf hamster, *Phodophus sungorus sungorus*

Ventral secretion[a]		Buccal secretion[b]	
Alcohols	**Carboxylic acids**	Carbon dioxide	Octa-9,12-dien-1-yl acetate
Hexadecan-1-ol	C_{16}, C_{18}, C_{20}, C_{22}, C_{24} unbranched acids[c]	Hydrogen sulfide	Icosanoic acid
Heptadecan-2-ol	13-Methyltetradecanoic acid[d]	Trimethylamine	2-Hydroxyoctadec-1-yl acetate
Nonadecan-2-ol	14-Methylpentadecanoic acid[d]	Acetic acid	2-Hydroxyicos-1-yl acetate
Henicosan-2-ol	15-Methylhexadecanoic acid[d]	Propanoic acid	Tetracosadien-4-olide[f]
Hexadecane-1,2-diol	17-Methyloctadecanoic acid[d]	2-Methylpropanoic acid	Cholesterol
Heptadecane-1,2-diol	18-Methylnonadecanoic acid[d]	Hexanal	Unidentified steroid
Saturated ketones	19-Methylicosanoic acid[d]	Butanoic acid	Pentacosadien-4-olide[f]
Tridecan-2-one	11-Methyltridecanoic acid[e]	3-Methylbutanoic acid	Desmosterol
Pentadecan-2-one	14-Methylhexadecanoic acid[e]	2-Methylbutanoic acid	Lanosterol
Hexadecan-2-one	(*Z*)-Octadec-9-enoic acid	Pentanoic acid	
Heptadecan-2-one	3-Phenylpropanoic acid	4-Methylpentanoic acid	
Octadecan-2-one	**Hydroxyesters**	Phenol	
Nonadecan-2-one	1-Hydroxyhexadec-2-yl pentanoate	Hexanoic acid	
Icosan-2-one	2-Hydroxyhexadec-1-yl pentanoate	1-Acetylimidazole	
Henicosan-2-one	1-Hydroxyheptadec-2-yl pentanoate	2-Piperidone	

Table 2 (continued)

Ventral secretion[a]		Buccal secretion[b]
Tricosan-2-one	2-Hydroxyheptadec-1-yl pentanoate	Oct-1-yl acetate
7 C_{16}-C_{21} branched alkan-2-ones	2-Hydroxyicos-1-yl acetate	Indole
Unsaturated ketones	**Butanolides (γ-lactones)**	3-Phenylpropanoic acid
(*Z*)-Hexadec-8-en-2-one	Icosadien-4-olide[f]	3-Hydroxyacetophenone
(*Z*)-Heptadec-8-en-2-one	Henicosadien-4-olide[f]	Oxindole
(*Z*)-Heptadec-10-en-2-one	**Other**	Hexadecane
(*Z*)-Octadec-8-en-2-one	2-Piperidone	Tetradecanoic acid
(*Z*)-Octadec-11-en-2-one	Cholesterol	Octadecane
(*Z*)-Nonadec-8-en-2-one	Desmosterol	Hexadecanoic acid
(*Z*)-Nonadec-12-en-2-one	Lanosterol	(*Z*)-Octadec-9-enoic acid
(*Z*)-Henicos-8-en-2-one	7 Unidentified compounds	(*E*)-Octadec-9-enoic acid
(*Z*)-Henicos-14-en-2-one		Octadecanoic acid

[a] Listed as compound classes; data from Burger et al. [66].
[b] Listed in order of elution from an apolar column; data from Burger et al. [67].
[c] Unbranched chains.
[d] Iso-branched.
[e] Anteiso-branched.
[f] Positions of double bonds unknown.

opment [65]. The buccal secretion thus appears to play an important role in the survival and development of juvenile dwarf hamsters. Although polar involatile compounds could be responsible for at least some of the functions attributed to the ventral and buccal secretions, the chemical characterization of their volatile organic constituents was undertaken as a first step in the investigation of the function of these secretions. In captivity the ventral gland of females produced so little material that it could not be collected for analysis [66]. Most of the constituents of these two glands, which are listed in Table 2, or closely related compounds, have already been identified in other mammalian secretions. Large variations were found in the relative concentrations of the short-chain carboxylic acids present in the buccal secretions of individual animals [67]. Although this aspect was not investigated in sufficient detail, these differences could play a role in individual recognition in this species.

It was recently found that females exposed to bedding from mature intact males showed a significantly higher incidence of proestrus than females exposed to the bedding of mature gonadectomized males [68]. It has, however, not yet been established whether this effect arises from the ventral secretion of the males, from constituents of male urine, or from some other source.

3.3 Beavers

Beavers have two pairs of secretory organs, anal glands and castor sacs. The latter contain castoreum, a brownish paste with a strong animal smell. Castoreum, which is believed to be composed of secondary metabolites from urine, is applied by beavers to mud piles on the banks of their ponds [69, 70]. It is generally believed that this territorial marking behavior serves to signal that the inhabitants will defend their habitat against conspecific intruders. The castor sac is a pocket lined with a layer of nonsecretory epithelium, whereas the anal gland of the animals is a holocrine secretory gland. Both of these structures open into the urogenital pouch [71].

Until recently, most of the chemical research on the contents of these structures was directed at the identification of the constituents of castoreum. In the late 1940s Lederer [72, 73] identified 36 compounds and some other incompletely characterized constituents in castoreum of uncertain origin. Other constituents were subsequently identified in the material [74–77]. In a reinvestigation aimed specifically at the phenol content of the material, Tang et al. [69] identified 10 previously unreported phenols in the castoreum from the North American beaver, *Castor canadensis.* Of the 15 phenols reported elsewhere, only five were confirmed in this analysis, in addition to 10 phenolic compounds that were not reported elsewhere. It was concluded that the 10 previously identified phenols that were not found in the study by Tang et al. were either absent or were not volatile enough to be detected by the methods employed. This was most probably because a relatively low maximum column temperature of only 210 °C was employed in the GC-MS analyses. The compounds identified by Lederer,

and subsequently by other workers, are listed in Table 3 and the structures of some of these compounds are given in Fig. 3.

Recently, Rosell and Sundsdal [78] tentatively identified 21 of the 43 constituents present in 96 scent marks deposited directly on snow or ice mounds by the Eurasian beaver, *C. fiber*. In this study, a higher final oven temperature was employed and several steroids were eluted from the capillary column. Because the main focus of this study was to determine whether the beaver uses castoreum and/or anal gland secretion for scent marking and not the full chem-

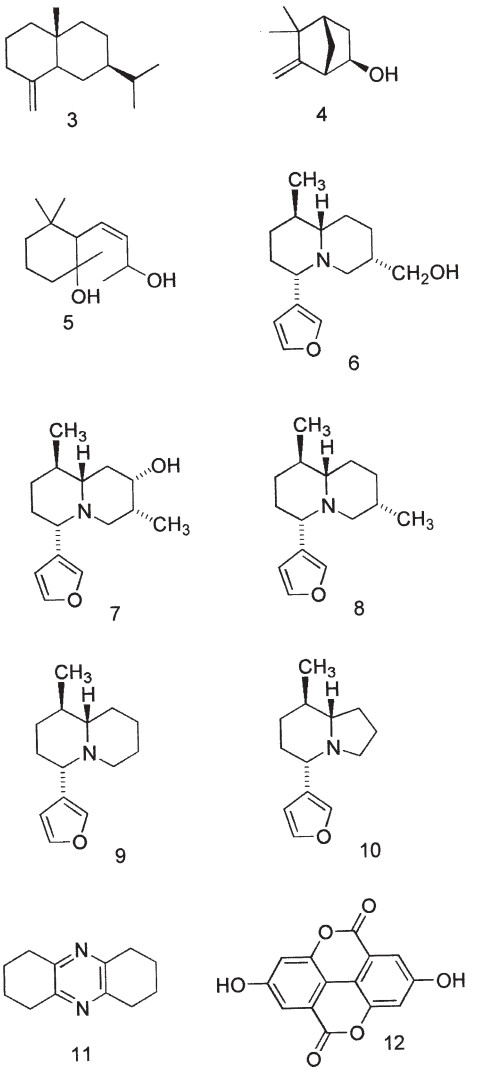


Fig. 3 Examples of the compounds identified in castoreum

Table 3 Constituents of castoreum

Phenols	**Alcohols**	**Aldehydes, Ketones**
Phenol[f]	6-Methyl-1-heptanol[f]	Benzaldehyde[f]
o-Cresol[f]	4,6-Dimethyl-1-heptanol[f]	Salicylaldehyde[b,f]
m-Cresol[f]	Junenol[f]**3**	Acetophenone[b,f]
p-Ethylphenol[b,f]	*trans*-Pinocarveol[f]	*p*-Hydroxyacetophenone[b]
p-Propylphenol[b,f]	Nojigiko alcohol[f]**4**	*p*-Methoxyacetophenone[b]
Pyrocatechol[b,f]	(1*R*)-Myrtenol[f]	3,4-Dimethoxyacetophenone[f]
Quinol[b]	Borneol[b,f]	Pinocamphone[f]
Quinol monomethylether (4-Methoxyphenol)[b]	*cis*-5-Hydroxytetrahydroionol[b]**5**	Isopinocamphone[f]
p-Methylguaiacol[f]	*cis*-Cyclohexane-1,2-diol[d]	Verbenone[f]
p-Ethylguaiacol[f]	Benzyl alcohol[b,f]	Unidentified aromatic ketone[b]
p-Propylguaiacol[f]	Cholesterol[b]	Two unident. isomeric hydroxyketones[b]
Chavicol (4-Allylphenol)[b]	β-Cholestanol[b]	**Carboxylic acids**
4-Methylpyrocatechol[f]	Mannitol[b]	Benzoic acid[b]
4-Ethylpyrocatechol[f]	**Amines, nitrogen bases**	2-Phenylpropanoic acid[b]
2-Hydroxy-5-ethylanisole[b,f]	Castoramine[b,c], (–)-castoramine[e]**6**	Cinnamic acid[b]

Table 3 (continued)

3,5-Dimethoxyphenol[f]
2,6-Dimethoxy-4-methylphenol[f]
Betuligenol [4-(4′-Hydroxyphenyl)-2-butanol][b]
2,4′-Dihydroxydiphenylmethane[b]
2',3″-Dihydroxydibenz-2-pyrone[b]
Unidentified phenolic ether[b]
Acetovanillone[f]
5-Indanol[f]
4-(4′-Hydroxyphenyl)-2-butanone[f]
Cyclic ethers
cis-Linalool oxide[f]
trans-Linalool oxide[f]

(–)-Isocastoramine[e] **7**
(–)-Deoxynupharidine[e] **8**
(–)-7-*epi*-Deoxynupharidine[e]
(–)-1-*epi*-Deoxynupharidine[e]
(–)-1-*epi*-7-*epi*-Deoxynupharidine[e]
(–)-7-Demethyldeoxynupharidine[e] **9**
5-(3-Furyl)-8-methyloctahydroindolizine[e] **10**
Trimethylpyrazine[e]
Tetramethylpyrazine[e]
5,6,7,8-Tetrahydroquinoxaline[e]
1,3,6,6-Tetramethyl-5,6,7,8-tetrahydroisoquinolin-8-one[e]
2-Methyl-5,6,7,8-tetrahydroquinoxaline[e]
2,3-Dimethyl-5,6,7,8-tetrahydroquinoxaline[e]
1,2,3,4,6,7,8,9-Octahydrophenazine[e] **11**

Salycilic acid[b]
m-Hydroxybenzoic acid[b]
p-Hydroxybenzoic acid[b]
Gentisic acid[b]
p-Anisic acid[b]
5-Methoxysalicylic acid[b]
Stearic acid[b]
Esters
Cholesterol oleate[b]
Esters of benzyl alcohols[b]
Esters of gentisic acid[b]
Esters of phenols[b]
Esters of ceryl alcohol[b]
Lactone
4,4′-Dihydroxydiphenic acid dilactone[b] **12**

Arranged according to compound class, although some may fall into more than one class. Nomenclature as in the original articles. Data were abstracted from: [a] Lederer [72, 73], [b] Valenta, Khaleque [74], [c] Valenta et al. [75], [d] Maurer, Ohloff [76], [e] Tang et al. [69, 77].

ical characterization of the secretion, the identification of the constituents of the scent marks was, unfortunately, not verified by comparison with authentic synthetic compounds.

Using chemical ionization mass spectrometry, Grønneberg [79] studied the chemical constitution of the anal gland secretion of the Eurasian beaver, *C. fiber*. A large number of unbranched, iso-branched, saturated and unsaturated wax esters were found in the male secretion, with the acid and alcohol moieties of the esters having carbon chain lengths of C_5–C_{22} and C_{14}–C_{19}, respectively. These compounds were not found in females. In the study by Rosell and Sundsdal [78] on the possible role of anal gland secretion in the territorial behavior of *C. fiber,* some of the 160 constituents present in the secretion collected from 20 dead male beavers were tentatively identified. The compounds found in the castoreum and anal gland secretions of the dead beavers were compared with the compounds found in the 96 scent marks on the snow. All of these marks contained compounds from castoreum, whereas compounds from anal gland secretion were found in only four scent marks. It was concluded that beavers do not specifically deposit anal gland secretion on scent mounds. Recent research indicates that *C. fiber* beavers do not mark their own pelage with castoreum or anal gland secretion, and that castoreum is the main scent signal used in the defense of beaver territories during winter [80].

The differences between castoreum from different species and sources still need to be investigated. As suggested by Tang et al. [69] it is quite likely that these differences could be diet related because the phenols are most likely derived from the diet of these animals. Indeed, already in 1949 Lederer [73] recognized this possibility and pointed out that 2′,3″-dihydroxydibenz-2-pyrone and 4,4′-dihydroxydiphenic acid dilactone **12** (Fig. 3) are closely related to ellagic acid, which is abundant in the bark of trees. A comprehensive chemical analysis of the polyphenols present in the preferred diet of the beavers and a comparison of these compounds with the phenols present in castoreum from animals fed exclusively on this diet could shed some light on this unresolved problem.

4
Insectivores

4.1
European Mole

The European mole, *Talpa europaea,* lives a solitary life in its own subterranean tunnel system for most of the year and actively avoids contact with conspecifics. During the mating season, male moles migrate into female territories to mate [81, 82]. Khazanehdari et al. [83] have found that the territorial behavior of this animal coincides with profound changes in the carboxylic acid content of the glands in the inguinal region of both sexes, shown to be anal glands [84]. The compounds in the secretions of adult males appear to vary little throughout the

year and are similar to those from the adult female outside the breeding season. A large number of alcohols, aliphatic carboxylic acids, esters, aldehydes, ketones and dimethyl disulfide were identified in the anal secretions of this species. Outside the breeding season the secretions are dominated by large concentrations of C_5–C_{10} carboxylic acids. Female glands regress as they enter proestrus, accompanied by early disappearance of the carboxylic acids. In juvenile moles, the composition of the secretion changes as the animal matures, with carboxylic acids becoming dominant only as the animal reaches maturity. The carboxylic acids apparently serve as a pheromone that helps to protect the individual's tunnel system. In female moles, this signal is interrupted only in the breeding season with the regression of the anal glands.

Bacterial activity has been shown to be responsible for volatile organic compounds in, among many others, the exocrine secretions of beavers (see above), the anal sacs of the red fox, *Vulpes vulpes,* and lion, *Panthera leo* [85], and the inguinal pouch of the rabbit, *Oryctolagus cuniculus* [86]. No bacteria were, however, found in the anal gland of the European mole, *T. europaea* [87].

5 Carnivores

Albone [88] has reviewed the literature on anal sac secretions up to the early 1980s. Organosulfur compounds are particularly plentiful in many of these secretions and are responsible for their offensive odors. In general, predator odors seem to be repulsive to potential prey. Epple et al. [89] have speculated that the reason for the repellent properties of the feces and urine of carnivores could be diet related. It would be logical to argue that organosulfur compounds derived from a protein-rich diet could be a cue by which prey can distinguish a potential predator. The results so far are consistent with this hypothesis [90].

It is surprising that, with the exception of the wolf, *Canis lupus*, and the red fox, *Vulpes vulpes* [88], relatively little information is available on the chemical composition of the urine of carnivores. Cat species have two distinct behavioral patterns to deposit fluid from the bladder via the urinary tract: normal urination and spray marking, in which a spray of urine is ejected by the animal while it stands with its hindquarters towards a target object with its tail lifted vertically [91]. The fluid deposited in both these behaviors comprises normal urine mixed with a whitish lipid precipitate in some species. In the tiger, for example, the white material contains lipids such as cholesteryl and wax esters, triglycerides, free fatty acids, sterols, and phospholipids [92]. This material originates from somewhere inside the urinary system and not from the anal glands, the products of which only mark the feces [93]. On the other hand, Asa [94] has found that the bladder urine of the leopard, *Panthera pardus*, puma, *Felis concolor*, and cheetah, *Acinonyx jubatus*, does not contain any lipids.

Very little reliable data on the chemical composition of the volatile fraction of urine of the large cats are available. The urine of the lion has a strongly

offensive odor to the human nose and is used in some parts of Africa to repel dogs from rural gardens. It is surprising that the possible role of this animal's urine in the chemical communication of the species has not attracted much more attention from chemists working in this field. Analyses of the urine of captive male and female lions revealed the presence of a large number of compounds [91]. Unfortunately bladder urine from only one juvenile female could be obtained in this study and sampling methods, such as squeezing the urine from sawdust bedding material, probably introduced artifacts into the samples that were used for analysis. The results of this study can therefore perhaps best be summarized as indicating that lion urine appears to contain many aldehydes, ketones, furan derivatives, and a few amines and organosulfur compounds.

5.1 Cheetah

In contrast to the other large cats, the urine of the cheetah, *A. jubatus,* is practically odorless to the human nose. An analysis of the organic material from cheetah urine showed that diglycerides, triglycerides, and free sterols are possibly present in the urine and that it contains some of the C_2–C_8 fatty acids [95], while aldehydes and ketones that are prominent in tiger and leopard urine [96] are absent from cheetah urine. A recent study [97] of the chemical composition of the urine of cheetah in their natural habitat and in captivity has shown that volatile hydrocarbons, aldehydes, saturated and unsaturated cyclic and acyclic ketones, carboxylic acids and short-chain ethers are compound classes represented in minute quantities by more than one member in the urine of this animal. Traces of 2-acetylfuran, acetaldehyde diethyl acetal, ethyl acetate, dimethyl sulfone, formanilide, and larger quantities of urea and elemental sulfur were also present in the urine of this animal. Sulfur was found in all the urine samples collected from male cheetah in captivity in South Africa and from wild cheetah in Namibia. Only one organosulfur compound, dimethyl disulfide, is present in the urine at such a low concentration that it is not detectable by humans [97].

The presence of elemental sulfur in the urine is quite surprising. Until more information has been obtained, it is speculated that sulfur could possibly be a cheetah pheromone. There could, however, also be another reason for the presence of sulfur in the animal's urine and the almost total absence of organosulfur compounds. Although the cheetah can reach speeds of more than 110 km per hour in short bursts, it is not very powerful and cannot defend itself against the lion and hyena. The conversion of organosulfur compounds to elemental sulfur could therefore be a mechanism to avoid detection by stronger predators.

The secretion of elemental sulfur by a carabid beetle [98] is the only other known example of the production of elemental sulfur by an animal. However, it might have been overlooked in the urine and/or feces of other species, because sulfur is not detected by FID. Furthermore, due to the interconversion of S_2–S_8 species in the gas chromatographic column, it is eluted as a very broad

band that could easily be mistaken for a rising base-line hump in GC and GC-MS analyses.

Although too little information on the composition of the urine of the large cats is at present available to reach any final conclusion, there seem to be some similarities between the urine of the cheetah and that of the spotted hyena, *Crocuta crocuta* [99].

5.2 Mustelids

The interest of chemists and biologists in the anal sac secretions of a variety of mustelid species most probably arose from the aggressively malodorous nature of these secretions and led to many attempts, over more than 140 years [100], to elucidate the structures of the organosulfur compounds responsible for the offensive smell of the secretions. From the late 1960s to the 1980s, secretions of the mink, *Mustela vison*, stoat, *M. erminea*, polecat, *M. putorius*, and ferret, *M. putorius furo*, were investigated and were found to contain mixtures of substituted thietanes and dithiolanes [101–106], as well as diisopentyl disulfide, indole and quinoline in the ferret [101, 105], indole and 2-aminoacetophenone, ethanol, *S*-methylthioacetate, isopentenyl methyl sulfide, dimethyl disulfide, dibutyl disulfide, butyl isopentyl disulfide and di-(3-methylbutyl) disulfide in the mink [102, 106]. This research was recently extended to include the anal sac secretions of the Siberian weasel, *M. sibirica*, and the steppe polecat, *M. eversmanni* [5], using headspace sampling and GC-MS identification. The organosulfur compounds identified in other mustelids were also found in the secretions of these two animals. No age-specific compounds were found. In *M. sibirica*, 2-ethylthietane was found only in the female. The relative abundance of several of the compounds was significantly different between males and females. In *M. eversmanni*, no sex-specific volatile compounds were found. It was concluded that the organosulfur compounds could be used to communicate information about species, sex, age and territoriality, as has already been suggested by Erlinge in 1982 [107].

In contrast to the relatively limited number of organosulfur compounds in these species, the anal sac secretions of skunks contain large numbers of malodorous compounds, which are very effectively utilized in the defensive behavior of these species. During the early years of capillary gas chromatography, Andersen et al. [108] found that 150 of the 160 components detected in the anal gland secretion of the striped skunk, *Mephitis mephitis,* contained sulfur. The results of recent chemical studies on North American skunks by Wood et al. [109] are summarized in Table 4. Only three of the compounds reported in this table are common to the secretions of all four skunk species and, in two of these three secretions, the common compound is present in concentrations of about 1% or less. Although it is clear that skunks use their anal gland secretions primarily for defensive purposes, it does not rule out the possibility that the difference in the composition of these secretions could also be utilized for

Table 4 Quantitative composition of the volatile fraction of anal sac secretion from four species of North American skunks [109][a]

Compound	Amount (%)			
	Hooded skunk	Striped skunk	Spotted skunk	Hog-nosed skunk
(*E*)-2-Butene-1-thiol	32	38–40	30–36	71
3-Methyl-1-butanethiol	39	18–26	48–66	
S-(*E*)-2-Butenyl thioacetate	16	12–18		17
S-3-Methylbutanyl thioacetate	7	2–3		
Phenylmethanethiol	0.3		0.2–0.8	1
2-Phenylethanethiol	1.4		2–5	
2-Methylquinoline	2.3	4–11	0.3–0.9	2
Bis[(*E*)-2-butenyl] disulfide	Trace		Trace	3
S-Phenylmethyl thioacetate	Trace			
(*E*)-2-Butenyl 3-methylbutyl disulfide	0.5	0.2–1.6	0.2–0.6	
Bis(3-methylbutyl) disulfide	Trace		0.1–0.2	
S-2-Phenylethyl thioacetate	0.2			
2-Quinolinemethanethiol	1.3	4–12	0.2–0.3	0.5
S-2-Quinolinemethyl thioacetate	Trace	1–4		

[a] In order of elution from an apolar column. Nomenclature as in the original article. Compounds less than 1% not included.

territorial marking or to transmit other semiochemical information. Be that as it may, there is still a lot of scope for analytical work in this field, albeit only to demonstrate the complexity of these secretions and the power of modern analytical methods.

Albone [88] has discussed the role of bacteria in the production of anal s ecretions of various types in great detail. However, not all the constituents of anal secretions are produced by bacteria. As mentioned earlier, no bacteria could, for example, be found in the anal gland of the European mole, *Talpa europaea* [87].

6 Proboscids

6.1 Elephants

Elephants have conspicuous temporal glands that are unique to these animals. They are modified apocrine structures imbedded in the subcutaneous tissue on each side of the head, midway between the eye and the ear. Male elephants experience an annual rut-like period of heightened aggressiveness and elevated

testosterone levels, known as musth. Elephant bulls in musth secrete copious amounts of temporal gland secretion. Females can distinguish males in musth using cues such as cyclohexanone, a component of the male temporal secretion of the Asian elephant, *Elephas maximus* [110], and the urine of males in musth [111]. Older males, young males and females also respond to different degrees to frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane), a bark beetle pheromone (see elsewhere in this volume), which has been detected in older musth males [112, 113].

Other secretions and excretions have been suggested as possible sources of chemical signals in the elephant, notably the feces, genital tract mucus, saliva and secretion from the interdigital glands [111]. The unsaturated ester, (*Z*)-7-dodecen-1-yl acetate, which is a constituent of many insect pheromones, has been identified in the urine of the female elephant. In behavioral studies conducted with groups of male elephants, the synthetic *Z* isomer elicited multiple flehmen responses, erections and pre-mating behavior from test animals. The *E* isomer was found to be inactive. (*Z*)-7-Dodecen-1-yl acetate is not detectable during the luteal phase, but increases in concentration during the follicular phase to about 33 μg per ml just before ovulation [114, 115]. It was furthermore found that only dominant males responded to the ester, whereas younger subordinate males ignored or backed away from test samples. The odorant-binding proteins in the mucus of the elephant's trunk apparently only moderately facilitate transport of the compound through the mucus of the olfactory sensory epithelium [116]. It was suggested that this result could potentially have implications as far as the interaction of (*Z*)-7-dodecen-1-yl acetate with insect antennal proteins is concerned.

Recent results indicate that the air-borne steroids 5α-androst-2-en-17β-ol and 5α-androst-2-en-17-one, which are present in the urine of female *E. maximus* during the luteal phase at levels that are increased 10- to 20-fold, could be responsible for the synchronization of estrus in females living in close social relationships [117]. The ketone has previously been identified as a product of the incubation of androsterone sulfate with human axillary bacterial isolates [118].

Because the African elephant, *Loxodonta africana*, is a very large, unpredictable and dangerous animal, it is difficult to study its chemical ecology under natural conditions. Although both sexes of the African elephant produce temporal secretions more often than their Asian counterparts, they have not enjoyed the same attention from researchers. Earlier work revealed the presence of two phenols and the sesquiterpenoids (*E,E*)-farnesol **13**, (*E*)-3,7,11-trimethyl-2,10-dodecadien-1,7-diol **14** and (*E*)-3,7,11-trimethyl-2-dodecen-1,7,11-triol **15** in the temporal secretion of the African elephant [119]. Two further sesquiterpenes, drimane-8α,11-diol **16**, a component of Greek tobacco [120] and (*E*)-2,3-dihydrofarnesol **17**, a bumblebee pheromone [121] and a component of the Dufour gland secretion of an army ant [122], were subsequently reported in this secretion [123] and recently (*E*)-nerolidol **18**, albicanol **19**, 3,7,11-trimethyl-10-dodecen-1,7-diol **20**, (*E*)-3,7,11-trimethyl-6-dodecen-1,11-diol **21**, and 3,7,11-

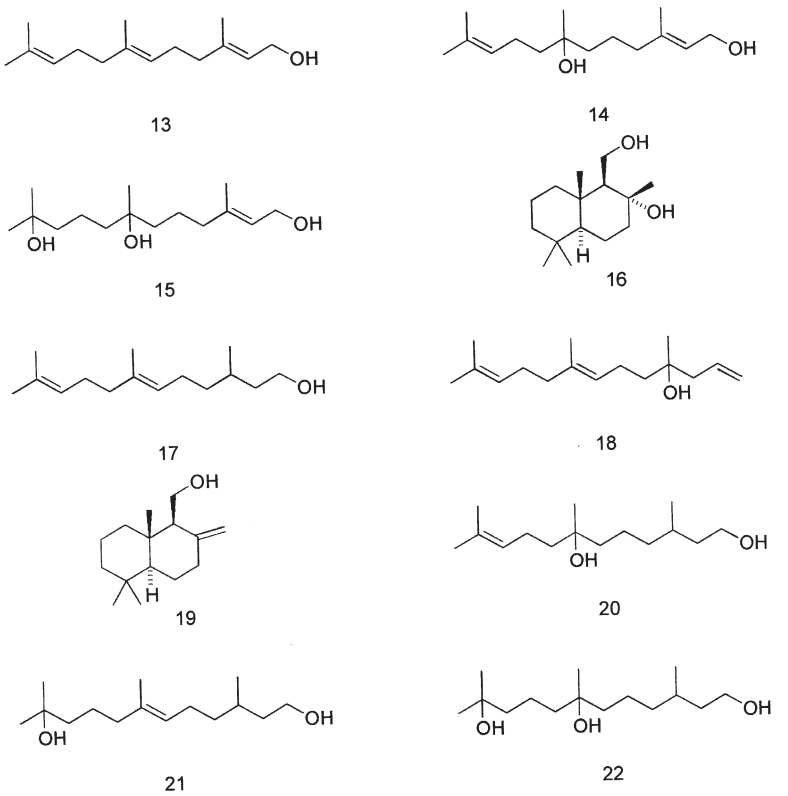


Fig. 4 Terpenoids identified in the temporal secretion of the African elephant

trimethyl-1,7,11-dodecanetriol **22** were added to the growing list of terpenoids (Fig. 4) in the temporal secretion of this animal [124].

7 Artiodactyls

The hoofed mammals or ungulates of the order Artiodactyla are richly endowed with specialized skin glands. Caudal, preorbital, dorsal, ventral, tarsal, metatarsal, interdigital and inguinal glands are examples of the glands found in these animals. Observations and the results of many investigations indicate that the secretions of these glands are involved in semiochemical communication. If it is taken into consideration that some artiodactyls have several exocrine glands, surprisingly little chemical research has so far been done on these secretions. One reason for this situation could be that many of these secretions are extremely complex, with more than 200 constituents that can be detected by currently available instrumentation. Another is that, for several reasons, it is

just about impossible to apply the so-called response-guided strategy [1] in semiochemical research on these animals under natural conditions in the wild. The best approach is probably to obtain the best possible qualitative and quantitative information on the secretions from as many individual animals as possible of both sexes, different age groups and social strata from different habitats. As envisaged in a chemical image approach to research of this type [1], this information and chemical data from related species with similar behavioral patterns could then be used to obtain an idea of how a secretion or some of its constituents could possibly be utilized for the transmission of semiochemical information.

7.1 Deer

Tarsal, metatarsal, caudal, interdigital and preorbital glandular structures have been described in the black-tailed deer, *Odocoileus hemionus columbianus*. The tarsal organ received considerable attention from chemists and behavioral scientists during the early years of chemical research on mammalian semiochemicals. The major constituent of the complex mixture of volatile compounds associated with the tarsal hair tuft of this mule deer, (*Z*)-6-dodecen-4-olide [125], was subsequently found to be a mixture of the *R* and *S* enantiomers in a ratio of 89:11 respectively [126]. It was later found that this compound does not originate in the tarsal structure itself, but that it is extracted from the animal's urine by the tarsal hair tuft, which is specially adapted to extract lipids from urine [127].

Mule deer have interdigital glands that can be described as pockets or pouches between the primary digits of both the forefeet and hind feet. Three ketones, 2-tridecanone, (*E*)-3-tridecen-2-one and (*E*)-4-tridecen-2-one, were identified in extracts from the interdigital secretion of the black-tailed deer. (*E*)-3-Tridecen-2-one, the major constituent of this secretion, inhibits the growth of several species of fungi and gram-positive bacteria. The fungus *Trichophyton mentagrophytes,* which is responsible for athlete's foot in humans and infections in other animals, is also strongly inhibited by this unsaturated ketone. 2-Tridecanone was found to be inactive against all of the 14 organisms tested [128].

In a more comprehensive study, 46 constituents were identified in the interdigital secretion of the white-tailed deer, *O. virginianus* [129]. Only relatively volatile compounds up to methyl salicylate were identified in the secretion, because samples for GC-MS analysis were enriched from the headspace gas of the secretion collected on cotton swabs. Some variations in the relative concentrations of the compounds between the secretions from dominant and subordinate animals were observed, but it was not possible to conclude definitely whether these differences were related to age or dominance.

Both male and female black-tailed deer, *O. h. columbianus*, rub their foreheads on twigs and branches. Several studies have indicated that this behavior functions as visual and olfactory signposts [130]. A qualitative and quantitative

Table 5 Volatile compounds recovered from forehead hairs of male white-tailed deer, *Odocoileus virginianus* [6][a]

Alkanes	**Arenes**	**Ketones**
4-Methylnonane	Toluene	Fenchone
β-Ocimene	Styrene	Pinocamphone
Decane	*p*-Cymene	Acetophenone
β-Pinene	Naphthalene	Carvone
Allo-ocimene	1,3-Dimethylbenzene	Verbenone
Myrcene	**Aldehydes**	**Alcohols**
Undecane	Hexanal	1-Octen-3-ol
Limonene	Heptanal	Linalool
4-Carene	Octanal	Sabinol
Dodecane	Benzaldehyde	Carveol
Tridecane	Nonanal	Borneol
Tetradecane	Decanal	α-Terpineol
	Myrtenal	**Unidentified**
		18 Unidentified constituents

[a] Nomenclature as in the original article.

comparison of the volatile organic compounds extracted from hair samples from the forehead and back of male white-tailed deer, *O. virginianus*, revealed that 17 of the 57 extracted constituents were terpenoids (Table 5) [6]. It was concluded that, although some of the terpenoids could be plant-derived, most of them were probably produced by the apocrine glands and that the variation in hair volatiles among individuals might be indicative of an individual-specific odor that could aid in identification.

Behavioral observations of male white-tailed deer indicate that urine could play a role in olfactory communication in this animal [131]. To extend the knowledge of the urinary volatiles of the white-tailed deer and to investigate the possibility that vaginal mucus could also carry semiochemical information, Jemiolo et al. [132] studied the qualitative and concentration changes in the profiles of the volatiles present in these excretions. Forty-four volatiles were found in the mucus and 63 in female urine. The volatiles common to both vaginal mucus and urine included alcohols, aldehydes, furans, ketones, alkanes, and alkenes. Aromatic hydrocarbons were found only in the mucus, whereas pyrans, amines, esters and phenols were found only in the urine. Both estrous mucus and estrous urine could be identified by the presence of specific compounds that were not present in mid-cycle samples. Numerous compounds exhibited dependency on ovarian hormones.

Qualitative and quantitative comparisons of the constituents of the urine of male and female white-tailed deer indicated that the presence and concentration of the urinary compounds depend on the season, reproductive status and social rank of the animals [132, 133]. Of the 63 and 55 compounds characterized in female and male urine, respectively, 27 were common to both sexes, 36

Table 6 Compounds identified in the urine of male white-tailed deer, *Odocoileus virginianus*, during the breeding season [122][a]

Common to urine of dominant and subordinate males	Exclusively in urine of subordinate males	Exclusively in urine of dominant males
2,2,4,6,6-Pentamethylheptane[b]	Octane	Hexane
3-Methyl-3-buten-1-ol	Decane	2-Methylhexane
2-Methylenepentanol	α-Phellandrene	1,3-Pentadiene
4-Methyl-1,4-butanediol	Limonene	3-Methyl-2-buten-2-ol
Benzaldehyde	β-Phellandrene	1-Hexanol
Dihydrocarvone	1-Penten-3-ol	3-Methyl-1,3-butanediol
Phenylacetonitrile	Ethyl thiolacetate	Unidentified ketone (mol mass 138)
3,4,5,6-Tetrahydropyridine	6-Methyl-2-heptanone	2,5-Dimethylfuran
	3-Ethylcyclopentanone	Isobutyronitrile
	3-Pentylcyclopentanone	
	6-Methyl-3,5-heptadien-2-one	
	6-Methyl-2-cyclohexen-1-one	
	2,3-Dihydrocarvone	
	Unidentified dimethylacetophenone	
	2-Butyrylfuran	
	Dimethyl disulfide	
	2,6-Dimethylpyridine	
	Isobutylbenzene	
	Unidentified acetal (mol mass 154)	

[a] Nomenclature as in the original article.
[b] Probably an artifact.

occurred in females only, and 28 were present exclusively in males. Alcohols, aldehydes, alkanes, alkenes, amines, ethers, furans and ketones occurred in the urine of both sexes. Thiol esters, benzene, ketals, disulfides and nitriles were found in male urine, but not in female urine. Phenols were found in female urine, but not in male urine. The relevant data are summarized in Table 6 [133]. As far as the urine from dominant and subordinate male deer during the breeding and non-breeding seasons is concerned, nine compounds were common to both dominants and subordinates during the breeding season. Of these nine compounds, three were present in higher concentrations in dominants and six were higher in subordinates. A further nine compounds were found exclusively in the urine of dominants and 19 exclusively in the urine of subordinates during the breeding season. The concentrations of several compounds were dependent on the time of the year. It was suggested that differing concentrations of these suites of compounds might be more important for the identification of social status than the presence of individual compounds. Since mature male white-tailed deer frequently urinate on their tarsal glands during the breeding season, this behavior may allow a deer simultaneously to scent-mark its environment and carry intraspecific cues indicative of social status [133].

7.2 Antelope

Chemical work on the exocrine secretions of African antelope has concentrated on the interdigital secretions of members of the subfamily Alcelaphinae and the preorbital secretions of members of the subfamilies Antilopinae and Cephalophinae.

7.2.1 Interdigital Secretions

African alcelaphine bovids have interdigital glands only on their forefeet. So far chemical work has been done on the interdigital secretions of the bontebok, *Damaliscus dorcas dorcas,* and another subspecies, the blesbok, *D. d. phillipsi,* the gnu or black wildebeest, *Connochaetes gnou,* and the red hartebeest, *Alcelaphus buselaphus caama.*

The bontebok is a strongly territorial antelope found in the southernmost parts of South Africa, while the blesbok inhabits the arid plains of the central parts of the country. It is practically impossible to translocate these animals even over small distances of a few hundred meters by driving them into unfamiliar areas. In game catching operations, the majority of the driven animals mostly run with their heads down, as though they are more interested in information on the ground than in the source of the threat. This is possibly also the reason why this animal was hunted almost to extinction by the early settlers in South Africa. The territorial behavior of the animal is attributed to territorial mark-

ing with secretions of its interdigital glands. The general character of the secretion suggests that it very likely could be used for long-term territorial marking. The secretion's odor is so persistent that the human nose can still detect it on objects in the laboratory after many years.

Following the identification of nine of the constituents of the bontebok's interdigital secretion during the 1970s, another 76 compounds belonging to widely differing compound classes were characterized in a reinvestigation of the secretion [2]. These 85 compounds are listed in Table 7. No qualitative differences were found between the secretions of free-ranging bontebok and blesbok from different habitats, or between the secretions of the two sexes of both the subspecies. The available information indicates that the small quantitative differences between the secretions of individual animals probably do not have major semiochemical implications. The compounds that have strong odors and are present in the secretion in relatively high concentrations, such as *m*-cresol, 2-heptylpyridine, (*Z*)-6-dodecen-4-olide and, to a lesser extent, α-terpineol and hexanal, are responsible for the pleasant herbal smell of the secretion as sensed by the human nose. The chiral, unsaturated γ-lactone was recently found to be present in the secretion as the pure *S* enantiomer [134], in contrast to the mixture of *R* and *S* enantiomers present in the tarsal tuft of the black-tailed deer [126].

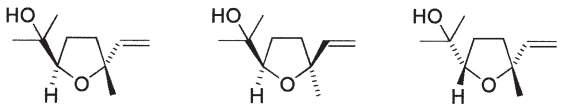
The interdigital secretions of the two subspecies contain a series of long-chain hydroxyalkyl esters of the type first identified in the dorsal secretion of the springbok, *Antidorcas marsupialis* [135], and subsequently also in, amongst others, the preorbital secretions of the grysbok, *R. melanotis* [18], and the ventral gland of the dwarf hamster, *P. s. sungorus* [66]. It has been speculated that these compounds could be formed by nucleophilic substitution of long-chain epoxides by carboxylic acids. A comparison of the structures of these compounds reveals that each species has its own profile of carboxylic acid residues present in its hydroxyalkyl esters. The interdigital secretions of the bontebok and blesbok, for example, contain one hydroxyalkyl ester with an acetate moiety and others with methylpropanoate, butanoate and pentanoate moieties, whereas the grysbok's preorbital secretion contains only two hydroxyalkyl acetates [18], and that of the steenbok, *R. campestris*, contains several acetates and butanoates [136]. The stereochemistry of these hydroxyalkyl esters has so far not been studied and no explanation has been found for their presence in these secretions.

Only two species of bacteria, *Bacillus brevis* and *Planococus citreus*, were found in the interdigital pouches of male and female members of the two *D. dorcas* subspecies, regardless of the habitat of the animals [2]. Although many of the products could not be identified in microbiological experiments, using acetate as carbon source, *B. brevis* was found to synthesize, among other unidentified products, (*Z*)-3-penten-2-ol, 2-hexanone, 2-octanone, 2-nonanone, the C_{14}, C_{15}, C_{17} and C_{18} carboxylic acids, (*Z*)-9-hexadecenoic acid and isopropyl hexadecanoate in vitro, while *P. citreus* produced, among others, the γ-lactones dodecan-5-olide and (*Z*)-6-dodecen-4-olide. The latter is one of the major

Table 7 Compounds identified in the interdigital secretions of the bontebok, *Damaliscus dorcas dorcas* and blesbok, *D. d. phillipsi* [2]

Alkanes	**Aldehydes**	**Ketones**	**Hydroxyesters**
Octane	Pentanal	Pentan-2-one	2-Hydroxyoctadec-1-yl acetate
Nonane	Hexanal	Methylbutan-2-one	2-Hydroxyoctadec-1-yl 2-methylpropanoate
Tetradecane	(*E*)-Hex-2-enal	Hexan-2-one	1-Hydroxyoctadec-2-yl butanoate
Hexadecane	Heptanal	Heptan-2-one	2-Hydroxyoctadec-1-yl butanoate
Terpenoids	(*E*)-Hept-2-enal	Octan-4-one	1-Hydroxyoctadec-2-yl pentanoate
γ-Terpinene	(*Z*,*Z*)-Hepta-2,4-dienal	Oct-7-en-2-one	2-Hydroxyoctadec-1-yl pentanoate
Linalool	Octanal	Octan-3-one	2-Hydroxynonadec-1-yl butanoate
p-Cymen-α-ol	(*E*)-Oct-2-enal	Octan-2-one	2-Hydroxyicos-1-yl 2-methylpropanoate
α-Terpineol	(*E*)-Non-2-enal	Nonan-4-one	2-Hydroxyicos-1-yl butanoate
Squalene	(*Z*,*Z*)-Deca-2,4-dienal	Nonan-2-one	2-Hydroxyicos-1-yl pentanoate
Alcohols	**Carboxylic acids**	Decan-2-one	2-Hydroxyhenicos-1-yl butanoate
(*Z*)-Pent-3-en-2-ol	Hexanoic acid	(*Z*)-Undec-5-en-2-one	2-Hydroxydocos-1-yl butanoate
Hexan-2-ol	Heptanoic acid	(*Z*)-Undec-7-en-2-one	**Phenols**
Heptan-1-ol	Octanoic acid	Undecan-2-one	Phenol
Nonan-2-ol	Decanoic acid	Decane-2,5-dione	3-Methylphenol
Hexadecan-1-ol	Dodecanoic acid	Undecane-2,5-dione	3-Ethylphenol
Octadecan-1-ol	Tetradecanoic acid	(*Z*)-Tridec-7-en-2-one	3-Propylphenol
Icosan-1-ol	Pentadecanoic acid	**Lactones**	**Pyridines**
Henicosan-1-ol	(*Z*)-Hexadec-9-enoic acid	(*Z*)-Dodec-6-en-4-olide[a]	2-[(*E*)-Hept-4-en-1-yl] pyridine
Docosan-1-ol	Hexadecanoic acid	Dodecan-5-olide	2-Heptylpyridine
Tetracosan-1-ol	Heptadecanoic acid	**Ester**	2-Heptanoylpyridine
Hexacosan-1-ol	(*Z*,*Z*)-Octadeca-9,12-dienoic acid	Isopropyl hexadecanoate	**Steroids**
	(*Z*)-Octadec-9-enoic acid		Cholesterol
	Octadecanoic acid		Desmosterol
	Icosanoic acid		**Other**
			Dimethylsulfone

[a] Subsequently identified as (*S*,*Z*)-Dodec-6-en-4-olide [134].



23 24 25

Fig. 5 Cyclic terpenoid ethers identified in the interdigital secretion of the red hartebeest

constituents of the interdigital secretions of the two subspecies. An analysis of the volatile organic constituents of the glandular tissue gave a chemical image totally different from that of the secretion itself and, although more work needs to be done on this aspect, it was concluded that microbiological activity in the interdigital pouch most likely makes a large contribution to the complexity of the secretion. It is, nevertheless, clear that the unsaturated γ-lactone is not extracted from the urine of the animal as in the case of the black-tailed deer [127].

Facilitating the translocation of the two subspecies by using a trail-marking pheromone composed of the major constituents of the secretion is being considered. Because the cost of synthesizing these compounds in relatively large quantities would make this procedure prohibitively expensive, the possibility of using a culture broth for this purpose is being investigated. The choice of the correct medium seems to be extraordinarily important, because the unsaturated γ-lactone, for example, was not produced at all in the absence of acetate as carbon source [137].

The interdigital secretion of the red hartebeest, *A. b. caama*, consists of fewer compound classes. It contains a few alkanes and short-chain, branched alcohols, fatty acids, including a few of the higher fatty acids up to octadecanoic acid, an epoxide and the cyclic ethers, *trans*-(2*R*,5*R*)-furanoid linalool oxide **23**, *cis*-(2*R*,5*S*)-furanoid linalool oxide **24** and *cis*-(2*S*,5*R*)-furanoid linalool oxide **25** (Fig. 5) in a ratio of 2.5:1:1.5 respectively [138]. From the point of view that many of the constituents of the interdigital secretion of this animal are probably of microbial origin, it is interesting that *cis*- and *trans*- furanoid linalool oxides have also been found in castoreum [77].

The secretion of the red hartebeest is characterized by its high aldehyde content. Of an estimated 100 detectable constituents, 25 are saturated and unsaturated aliphatic aldehydes [138]. Because the aldehydes are highly susceptible to autoxidation, the secretion could therefore only be used for short-term territorial marking. On the other hand, the conversion of the aldehydes to carboxylic acids could also be transmitting information with a "date stamp". In this regard, it is debatable whether fatty acids, which are almost ubiquitous in the animal world, really are such major carriers of semiochemical information in all of the many species in which they are purported to fulfill this role.

No significant qualitative and quantitative differences were found between male and female secretions in any one of these alcelaphine bovids.

7.2.2 Preorbital Secretions

7.2.2.1 Alcelaphinae

Alcelaphine bovids also possess preorbital glands. The 42 compounds identified in the preorbital secretions of the bontebok and blesbok include pentane, heptane, acetic acid, 2-methylbutanoic acid, (*E*)-2-methyl-2-butenoic acid, benzoic acid, 4-hydroxybenzoic acid, saturated and unsaturated long-chain carboxylic acids, long-chain alcohols and aldehydes, 2-heptanone, piperitone, dimethyl sulfone, isopropyl tetradecanoate, isopropyl hexadecanoate, hexadecyl acetate, alkan-5-olides (δ-lactones), cholesterol, α-tocopherol and squalene. Although 19 compounds are common to both the interdigital and preorbital glands, no exchange of constituents takes place between the two glands in these animals [139].

Because 2-heptanone, 1-octen-3-ol, and 1-henicosanol are completely absent from the male secretions, and piperitone, hexadecyl acetate and hexadecan-5-olide are completely absent from the female secretions of both subspecies, the difference in the quantitative composition of the male and female preorbital secretions of these animals could be construed as evidence that their preorbital secretions play a role in sexual recognition. The varying concentrations in which these and the other compounds are present in the secretions could also be interpreted in terms of the preorbital secretion being employed for individual recognition. Even the human nose can detect the differences between mixtures of chemicals formulated according to the available quantitative data. However, in this regard, more quantitative data from a larger sample of unrelated animals from different social levels will have to be obtained to find out whether the preorbital secretion is indeed employed for individual recognition

7.2.2.2 Antilopinae

Members of the subfamily Antilopinae, known as dwarf antelope, have well-developed preorbital glands. In both sexes the preorbital gland is a thin-walled pocket anterior to the forward corner of the eye, the secretions of which are used for territorial marking. It would be impossible to present and discuss in detail the enormous volume of chemical information on the preorbital secretions of these animals that has been accumulated over more than 30 years (e.g. [8, 17, 18, 136, 140–143]). The compounds identified in the preorbital secretion of the Cape grysbok, *Raphicerus melanotis*, are, however, listed in Table 8 [18] to exemplify the compounds that are typically found in these secretions, with one notable exception (see below).

No quantitative and only small, probably insignificant, differences were found between male and female secretions in the grysbok, and marking activ-

Table 8 Compounds identified in the preorbital secretion of the grysbok, *Raphicerus melanotis* [18]

Alkanols
Undecan-1-ol
Dodecan-1-ol
Tridecan-1-ol
Tetradecan-1-ol
Pentadecan-1-ol
Alkenols
(*Z*)-Dodec-6-en-1-ol
(*Z*)-Tridec-6-en-1-ol
(*Z*)-Tridec-7-en-1-ol
(*Z*)-Tetradec-8-en-1-ol
(*Z*)-Tetradec-7-en-1-ol
(*Z*)-Pentadec-8-en-1-ol
(*Z*)-Pentadec-6-en-1-ol
(*Z*)-Hexadec-8-en-1-ol
(*Z*)-Hexadec-7-en-1-ol
(*Z*)-Heptadec-8-en-1-ol
(*Z*)-Nonadec-10-en-1-ol
(*Z*)-Henicos-12-en-1-ol
(*Z*)-Tricos-14-en-1-ol
Alkadienols
(*Z*,*Z*)-Trideca-4,7-dien-1-ol
(*Z*,*Z*)-Tetradeca-5,8-dien-1-ol
(*Z*,*Z*)-Pentadeca-6,9-dien-1-ol
(*Z*, *Z*)-Heptadeca-8,10-dien-1-ol
Alkyl formates
Undec-1-yl formate
Dodec-1-yl formate
Tridec-1-yl formate
Tetradec-1-yl formate
Pentadec-1-yl formate
Hexadec-1-yl formate
Heptadec-1-yl formate
Octadec-1-yl formate
Nonadec-1-yl formate
Icos-1-yl formate
Henicos-1-yl formate
Docos-1-yl formate
Tricos-1-yl formate
Tetracos-1-yl formate
Pentacos-1-yl formate
Alkenyl formates
(*Z*)-Tridec-7-en-1-yl formate
(*Z*)-Tetradec-7-en-1-yl formate
(*Z*)-Tetradec-8-en-1-yl formate
(*Z*)-Pentadec-6-en-1-yl formate
(*Z*)-Pentadec-8-en-1-yl formate
(*Z*)-Hexadec-7-en-1-yl formate
(*Z*)-Hexadec-8-en-1-yl formate
(*Z*)-Heptadec-8-en-1-yl formate
(*Z*)-Octadec-9-en-1-yl formate
(*Z*)-Octadec-10-en-1-yl formate
(*Z*)-Nonadec-10-en-1-yl formate
(*Z*)-Icos-10-en-1-yl formate
(*Z*)-Icos-11-en-1-yl formate
(*Z*)-Henicos-12-en-1-yl formate
(*Z*)-Docos-13-en-1-yl formate
(*Z*)-Tricos-14-en-1-yl formate
(*Z*)-Tetracos-15-en-1-yl formate
(*Z*)-Pentacos-16-en-1-yl formate
Alkadienyl formates
(*Z*,*Z*)-Tetradeca-5,8-dien-1-yl formate
(*Z*,*Z*)-Pentadeca-6,9-dien-1-yl formate
(*Z*,*Z*)-Heptadeca-8,11-dien-1-yl formate
(*Z*,*Z*)-Nonadeca-10,13-dien-1-yl formate
(*Z*,*Z*)-Henicosa-12,15-dien-1-yl formate
Alkanals
Dodecanal
Tridecanal
Alkenal
(*Z*)-Tridec-3-enal
Alkanoic acids
Tetradecanoic acid
Pentadecanoic acid
Hexadecanoic acid
Heptadecanoic acid
Octadecanoic acid
Icosanoic acid
Alkanolides
Pentadecan-4-olide
Hexadecan-4-olide
Heptadecan-4-olide
Octadecan-4-olide
Icosan-4-olide
Hexadecan-5-olide
Sulfides
Heptadec-1-yl methyl sulfide
Methyl nonadec-1-yl sulfide
Hydroxyesters
2-Hydroxyoctadecan-1-yl acetate
2-Hydroxyicosan-1-yl acetate
Other
19 Unidentified constituents

ity is only rarely observed in females. The secretion is remarkably rich in saturated and unsaturated long-chain formates, a compound type that is present in the preorbital secretions of many dwarf antelope.

The steenbok, *R. campestris*, is another member of the Antelopinae. Its habitat overlaps that of the grysbok. However, the external structures of the orbital glands of these two species are totally different. In the grysbok, the secretion accumulates in a shallow hollow, the size of a small pea, from which up to a few hundred milligrams of black material can be collected twice a day, whereas the secretion of the steenbok accumulates in a more prominent, slit-like invagination of the skin. Very small quantities of the secretion can be collected from the steenbok's preorbital glands only about once a week. This secretion is much more complex than that of the grysbok and is now estimated to contain more than 300 volatile organic constituents, such as saturated and unsaturated, long-chain alcohols, carboxylic acids and formates [136], similar to those in the secretion of the grysbok [18]. However, there is not such a preponderance of formates and, in addition, the secretion also contains quite a number of acetates, as well as hydroxyalkyl esters of the type that has been found, amongst others, in the interdigital secretion of the bontebok [2] (see Table 7). The secretions of male and female animals were found to be qualitatively identical, regardless of the reproductive state of the animals, and it is therefore unlikely that this secretion is used for sex or individual recognition.

The oribi, *Ourebia ourebi*, and the suni, *Neotragus moschatus*, which are both found in the eastern tropical and subtropical parts of Africa, are two further members of the Antilopinae. Of these antelope, male oribi have the highest marking rate of up to 45 per hour [144]. Female oribi do not seem to produce any preorbital secretion, although they have an externally visible glandular structure. Only one male oribi has so far been available for chemical work and it would therefore be premature to make generalizations as far as the nature of the male secretion is concerned. However, the secretion of this male was found to differ from the previously analyzed secretions in that it had a finite volatility range and contained a limited number of compounds, which were almost completely resolved on a capillary column coated with an apolar phase. Once again, a large number of long-chain alcohols, formates and acetates, but only a few aldehydes and carboxylic acids, were present in this secretion [17].

Male and female suni both produce preorbital secretions. However, these secretions have totally different chemical images. The male secretion consists of a rather complex mixture of straight-chain and methyl-branched alkanes, a large number of alkenes, a few aldehydes, carboxylic acids, including benzoic acid, and long-chain saturated and unsaturated formates [140]. With the exception of benzoic acid, none of these compounds were found in the female secretion, which contains small quantities of straight-chain and branched C_{14}–C_{24} carboxylic acids, including oleic and linoleic acids, and long-chain hydroxyalkyl butanoates and -pentanoates with C_{21}–C_{24} alcohol moieties [145]. However, the most significant difference is that more than 90% of the female secretion consists of a mixture of only six compounds, each with a base peak

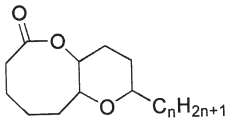


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Fig. 6 Bicyclic lactones tentatively identified in the preorbital secretion of the female suni

at *m*/*z* 153 in their mass spectra. These compounds have so far not been reported in the literature. Using the whole repertoire of 600 MHz ^{1}H and ^{13}C NMR techniques, high resolution MS and GC-IR analysis, it was concluded that these compounds probably have the bicyclic structures **26** with fused lactone and ether rings depicted in Fig. 6 [145]. These compounds could conceivably be synthesized in the animal through the hydroxylation and cyclization of alkadienoic acids, but this has not yet been confirmed.

Marking with preorbital secretion appears to play an extraordinarily important role in the territorial behavior of the klipspringer, *Oreotragus oreotragus*, another member of the Antilopini. Either partner carries out scent marking but, if the female scent marks a carefully selected twig, the male usually follows and overmarks. The male almost invariably licks a territorial mark before overmarking. The secretion is produced at such a high rate that it is unlikely that bacterial action could contribute to the production of its volatile constituents. In one experiment, the secretion was found to be produced at a rate of about 100 mg in 22 min [8]. Territorial marks have a shiny black appearance and can accumulate to form rod-like structures, which can reach the size of a man's little finger. The secretion of this animal is totally different from those of the other members of the subfamily, in that it contains only eight quite volatile compounds, the ketones 3-pentanone, 4-methyl-2-pentanone, 5-methyl-3-hexanone and 4-methyl-3-hexanone, and the esters ethyl propanoate, isobutyl acetate, ethyl 3-methylbutanoate and isobutyl propanoate [8]. In addition to trace quantities of these compounds and water, the secretion consists entirely of a simple mixture of a few proteins, the major one of which has a molecular mass of about 18 kDa and an amino acid composition characteristic of a small globular albumin-like protein. Another protein appears to be a glycoprotein or mucoprotein. In a simple experiment, it was found that the affinity of the proteins for volatiles of the secretion is similar to that of albumin. Trypsin has a very low affinity for these volatiles and lysozyme has an extremely high affinity for ethyl 3-methylbutanoate. Convincing evidence was found in another experiment that the proteinaceous material of the secretion serves as a controlled-release agent for the volatiles [8].

The results of the chemical characterization of the preorbital secretions of these dwarf antelope are summarized in Table 9 [140]. Due to the large number of double-bond positional isomers in the secretions of some of the members of the tribe, it is impossible to include all the relevant information in the table.

Table 9 Compound types[a] identified in the preorbital secretions from antelope of the subfamily Antilopini [140]

Compound types	Male & female grysbok, *Raphicerus melanotis*	Male & female steenbok, *R. campestris*	Male oribi, *Ourebia ourebi*	Male suni, *Neotragus moschatus*	Male klipspringer, *Oreotragus oreotragus*
Unbranched alkanes	–	8–10, 12	–	15, 17, 19, 21, 23	–
Isoalkanes	–	–	–	15, 17, 18, 19, 21–25	–
Alkenes	–	–	–	17, 19–23 (30)[b]	–
Alkan-1-ols	11–15	8–16, 20, 23–27	9, 10	–	–
Alken-1-ols	12–17, 19, 21, 23 (13)[b]	7, 10–15 (9)[b]	10, 12–14 (4)[b]	–	–
Alkadien-1-ols	13–15, 17 (4)[b]	17 (1)[b]	13–16 (4)[b]	–	–
Alkan-1-yl formates	11–25	11–13, 20–28	8–16	11–24	–
Alken-1-yl formates	13–25 (18)[b]	11–15 (6)[b]	9–15 (8)[b]	13–15, 17, 19–25 (19)[b]	–
Alkadien-1-yl formates	14, 15, 17, 19, 21 (5)[b]	–	12–18 (7)[b]	–	–
Alkan-1-yl acetates	–	13, 15, 16	8–17	–	–
Alken-1-yl acetates	–	13, 15, 19, 21, 23, 24 (6)[b]	10–17 (10)[b]	–	–
Alkadien-1-yl acetates	–	–	13–18 (6)[b]	–	–

[a] Numbers denote the number of carbon atoms in unbranched chains.
[b] Total number of double-bond positional and configurational isomers.

Table 9 (continued)

Compound types	Male & female grysbok, *Raphicerus melanotis*	Male & female steenbok, *R. campestris*	Male oribi, *Ourebia ourebi*	Male suni, *Neotragus moschatus*	Male klipspringer, *Oreotragus oreotragus*
Alkanals	12, 13	6, 7, 9	9–13	6–9	–
Alkenals	13 (1)[b]	–	11–14 (4)[b]	–	–
Alkadienals	–	10 (2)[b]	13–16 (4)[b]	–	–
Cycloalkanones	–	16–21	–	–	–
Alkanoic acids	14–18, 20	4, 8, 10, 12, 14–16, 18, 20	16–18	5–8, 16, 18	–
Alkenoic acids	–	12–14, 16, 18 (5)[b]	–	–	–
Alkadienoic acids	–	18 (1)[b]	–	–	–
Alkan-4-olides (γ-lactones)	15–18, 20	–	–	–	–
Alkan-5-olides (δ-lactones)	16	–	–	–	–
1-Hydroxyalk-2-yl acetates	–	18, 20–22	–	–	–
2-Hydroxyalk-1-yl acetates	–	17, 18, 20–23	–	–	–
1-Hydroxyalk-2-yl butanoates	18, 20	18, 20, 22	–	–	–
2-Hydroxyalk-1-yl butanoates	–	14, 16, 18, 20, 22	–	–	–

Table 9 (continued)

Compound types	Male & female grysbok, *Raphicerus melanotis*	Male & female steenbok, *R. campestris*	Male oribi, *Ourebia ourebi*	Male suni, *Neotragus moschatus*	Male klipspringer, *Oreotragus oreotragus*
Other	Heptadec-1-yl methyl sulfide	Limonene	13-Me-(*Z*)-8-C_{15} formate	Cholesterol	3-Pentanone
	Methyl nonadec-1-yl sulfide	Squalene	–	Benzoic acid	4-Methyl-2-pentanone
	–	5-Methyl-3-hexanol	–	–	5-Methyl-3-hexanone
	–	Cholesterol	–	–	4-Methyl-3-hexanone
	–	2-Methylbutanoic acid	–	–	Ethyl propanoate
	–	3-Methylbutanoic acid	–	–	2-Methylpropyl acetate
	–	Isopropyl tetradecanoate	–	–	Ethyl 3-methyl-butanoate
	–	Isopropyl hexadecanoate	–	–	2-Methylpropyl propanoate
	–	α-Tocopherol	–	–	–

There is also no simple rule as to where double bonds are found in different members of the tribe. For example, the double bonds occur in positions 2 and 6 in the steenbok, whereas, in other species, there seems to be a tendency for the double bonds to be at specific distances from the C-terminal end of the unsaturated compounds.

The overall picture of the secretions of the dwarf antelope seems to suggest that secretions that are produced slowly are more complex. This could be explained in terms of microbiological action, which has more time to contribute to the complexity of a secretion, the slower it is produced. If this is indeed the reason for the complexity of secretions that are produced very slowly, it is possible that, in these animals, with exception of the klipspringer, the long-chain lipid constituents of the secretions could be controlled-release carrier materials rather than semiochemicals. If these heavy compounds were semiochemicals, it could be asked why is it necessary for an animal to spend so much energy to regularly renew its territorial marks. In retrospect, it is possible that up to now too much attention could have been devoted to the heavy constituents of the secretions, while the semiochemically active constituents could have been overlooked because they could be present in such low concentrations that they were not detected by the methods that were employed.

7.2.2.3 Cephalophinae

The red duiker, *Cephalophus natalensis*, is threatened with extinction and normally does not survive in captivity. Red duiker are strongly territorial and both males and females almost constantly mark their home ranges with urine, dung and preorbital secretion. In this antelope, the preorbital secretion is an inhomogeneous mixture of a clear, colorless liquid and a somewhat more viscous, opaque liquid containing varying quantities of heavy black, involatile material. It has been noticed that the proportion in which these substances are released from the gland depends on the pressure applied to the glandular surface, almost as though the animal could choose to leave one or both of the liquids on objects marked with the secretion. Centrifuging the freshly collected secretion at 3000 rpm for about 15 min, during which time the temperature is allowed to rise to about 30 °C, results in a sharp separation of a layer of a clear liquid and a supernatant layer of waxy material. On cooling down to room temperature, the solidified wax can be lifted and removed from the underlying liquid. Chemical characterization of the volatile organic constituents of these two layers revealed quite a clean separation of the two groups of compounds listed in Table 10 [22, 141]. The mucous phase contains mainly alcohols, ketones, spiroacetals, and the two thiazoles, whereas the wax phase contains aldehydes and fatty acids, although there is some cross contamination between the two phases.

All 12 thermodynamically stable enantiomers and traces of a few of the less stable enantiomers of the spiroacetals **27–30** depicted in Fig. 7 are present in the secretion. This is the first identification in a mammal of spiroacetals previously

Table 10 Compoundsidentified in the mucous and wax phases of the preorbital secretion of the redduiker, *Cephalophus natalensis*[22,141]

Constituents of the mucous phase		Constituents of the wax phase
Alkan-1-ols (C_5, C_9, C_{10})	1-Methoxy-2-methylpropane	Undec-1-ene
Alkan-2-ols (C_7, $C_{8)}$	1-Methoxy-3-methylbutane	Alkanals (C_{10}, C_{11}, C_{12}, C_{13}, C_{15}, C_{17}, C_{19})
Alkan-3-ols (C_6, C_7, C_8)	2-Methylpropan-1-ol	Alkanoic acids (C_{12}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18})
Alkan-4-ols (C_7, C_8, C_{14})	3-Methylbutan-1-ol	Alkan-4-olides (γ-lactones) (C_{14}, C_{15}, C_{16})
Alkan-6-ols (C_{14}, C_{15})	Methylbutanone	1-Methoxy-3-methylbutane
Alkan-4-ones (C_7, C_8, C_9, C_{13}, C_{14}, C_{15})	3-Methylbutyronitrile	3-Methylbutan-1-ol
Alkan-6-ones (C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17})	1-Nitropentane	1-Nitropentane
2-Isobutyl-1,3-thiazole	2-Nitropentane	2-Isobutyl-4,5-dihydro-1,3-thiazole
2-Isobutyl-4,5-dihydro-1,3-thiazole	Nonan-4-olide	Phenylacetonitrile
Enantiomers of 7-methyl-1,6-dioxaspiro[4.5]decane **27**	Decan-4-olide	(*Z*,*Z*)-Octadeca-9,12-dienoic acid
Enantiomers of 2,7-dimethyl-1,6-dioxaspiro[4.5]decane **28**	Benzaldehyde	(*Z*)-Octadec-9-enoic acid
Enantiomers of 2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane **29**	2-Phenylethanol	
Enantiomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane **30**	Phenylacetonitrile	
5-Methylheptan-2-ol	Isobutyl benzoate	
(*Z*)-Non-3-en-1-ol	Isopentyl benzoate	
3,7-Dimethyloctan-1-ol	Hexadecanoic acid	
	17-Hydroxyandrost-4-en-3-one	

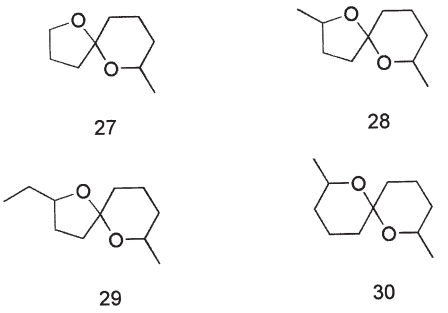

Fig. 7 Basic structures of the spiroacetals of which all the stable enantiomers were identified in the preorbital secretion of the red duiker

identified as pheromones in several insects of the orders Coleoptera, Diptera, and Hymenoptera [146] (see elsewhere in this volume). The identification of 1-nitropentane in the urine of the female rat, *Rattus rattus* [147], is the only other example of the production of nitro compounds in mammalian metabolites.

8 Primates

Possible sources of semiochemicals in primates include the scalp, hair, axillary region, genitals, chest and/or breast, feet and skin. As possible starting points for studies on human semiochemicals, the constituents present in the effluvia, excretions and secretions of humans have been characterized. For example, a large number of constituents of normal human urine have been identified since modern gas chromatographic techniques became available for this type of analysis. The results of these earlier studies on human effluvia and urine have been reviewed by Albone [148].

The observed synchronization of the menstrual cycles of women living together in an all-female institution is ascribed to the effect of a primer pheromone and led to several earlier studies (e.g. [149, 150]). Later results supported the pheromonal explanation of synchrony [151]. In an evaluation of the research on menstrual synchrony between mothers and daughters against the background of the results of other investigations, Weller and Weller [152] concluded that there are indications that menstrual synchrony could be affected by both pheromonal and environmental influences, and the interaction between them.

Most of the research on human exocrine secretions and excretions has probably been devoted to the chemistry of human skin lipids. To a certain extent, this could be due to the interest of the cosmetic industry in the chemical compounds found on the human skin and the chemical processes they are subjected to by the environment. It is possible that some of the proprietary in-

formation resulting from these studies has not been reported in the literature. The literature on the chemistry and function of the sebaceous lipids of mammals in general was reviewed by Steward and Downing in 1991 [153].

In addition to sebaceous glands, humans, monkeys and apes have apocrine glands and eccrine sweat glands on the general body surface, with higher concentrations in places such as on the chest and in friction areas. The sebaceous glands do not seem to have any vital function in humans. In addition, the axillary armpit scent gland complex is unique to man, the gorilla and the chimpanzee. Several studies have suggested that extracts made from human axillary secretions can alter the length and timing of the human menstrual cycle [154, 155]. Initially, the steroid biochemistry occurring in the underarm region was emphasized [156]. However, more recent work has shown that a large number of C_6–C_{11} saturated, branched and unsaturated acids, with (*E*)-3-methyl-2-hexenoic acid as the major component of this fraction, and not the odoriferous steroids, such as androstenol and androstenone, are responsible for the typical odor of axillary secretions. (*Z*)-3-Methyl-2-hexenoic is present in these secretions at a concentration of about 10% of that of the *E* isomer [157]. The secretions of males and females with the same micro flora are similar, with only minor qualitative differences [158]. The two 3-methyl-2-hexenoic acid isomers and several other axillary odor compounds appear to be carried to the axillary skin surface bound to water-soluble proteins present in the secretions [159, 160].

The existence of releaser pheromones in humans has been investigated since the mid-1970s. Perhaps the most convincing evidence that releaser pheromones are found in humans is that humans are capable of discriminating between kin and non-kin by olfactory cues alone [161]. Neonates are, for example, able to distinguish the odor of their mothers from that of other women [162] and mothers can already identify their infants' garments within the first few days after delivery [163]. Although indications have been found that the odors eliciting responses in the neonates originate from the mother's breast region, breast-feeding infants also orientate preferentially to the odor of their own mother's underarm pad [161]. It has recently been found that neonates are attracted to the odor of amniotic fluid, which suggests that they may have become familiar with that substance prior to birth [164, 165].

Not only the sensitivity of the human olfactory system to androstenol (5α-androst-16-en-3α-ol) and androstenone (5α-androst-16-en-3-one), but also the difference in the sensitivity with which individuals can detect these compounds and the fact that they are implicated in the semiochemical communication of the pig, *Sus scrofa* [166, 167], have led to them being considered human pheromones. Final confirmation that they are human pheromones is still outstanding. However, it was recently found that passive inhalation of another related steroid, androsta-4,16-dien-3-one, can influence the physiological state of humans by increasing a positive mood in test persons [168]. It has yet to be determined whether humans exude concentrations of this chemical information that are adequate for communication within social contexts.

9
Concluding Remarks

Reading the literature on mammalian semiochemistry over the past decade, a chemist is impressed by the enormous volume of biological information that has been gathered in well planned and meticulously executed studies of the modulation of the behavior of mammals by the chemicals released by conspecifics. One cannot, however, escape the impression that the chemical basis of many of these studies is lacking. Some of the problem areas were pointed out in the foregoing sections. To a certain extent there seems to be lack of appreciation of the diffusion rates of compounds with different volatilities and of the extent to which these differences can influence the outcome of behavioral tests. It is difficult to make an estimate of the persistence of semiochemicals that are released into the laboratory atmosphere or that are left on objects or surfaces in arenas in which tests are conducted. From what is known about the evaporation rate of some heavy compounds that are considered to be semiochemicals, it could take several weeks or even months for these compounds to be depleted to levels that cannot be detected by currently available instrumentation; levels at which meaningful information could still be available to experimental animals. This then leaves the question unanswered as to when it would be safe to conduct behavioral experiments in a laboratory or arena that had been occupied by conspecifics.

In some research fields the full benefits of well-planned research and much hard work could not be reaped, because the full potential of modern analytical instrumentation was not fully exploited. More and better information could have been obtained if the expertise of an experienced analytical chemist had been available.

Although quite a detailed picture of the compound types that are generally found in the exocrine secretions of the artiodactyls is slowly emerging, attempts to demonstrate the semiochemical activity of these compounds in field tests were not met with unqualified success, probably because it is mostly not feasible to apply the so-called response-guided strategy in work on the artiodactyls. Nevertheless, an enormous volume of chemical information has been made available to zoologists that are interested in studying these animals.

On a more positive note, the concerted attempts of scientists over the past few decades to unravel the semiochemical communication of mice and especially the work on the MUPs of these animals, could well serve as an excellent example of how this type of research should be approached and of the benefits that can be reaped from interdisciplinary collaboration.

Understandably, there is a lot of general and also commercial interest in research aimed at the identification and evaluation of human pheromones. In as much as humans can motivate their responses to test compounds, it should be simpler to work with humans than with other mammals. However, although much has already been accomplished in this field, pheromone communication in humans, by and large, still remains an enigma.

Acknowledgement The invaluable assistance of my wife, Wina, in writing this chapter is gratefully acknowledged.

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Topics in Current Chemistry (2005) 240: 279–315
DOI 10.1007/b98319

Extracellular Communication in Bacteria

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Abstract Populations of bacterial cells often coordinate their responses to changes in their local environmental conditions through "quorum sensing", a cell-to-cell communication system employing small diffusible signal molecules. While there is considerable diversity in the chemistry of such signal molecules, in different Gram-positive and Gram-negative bacteria they control pathogenicity, secondary metabolite production, biofilm differentiation, DNA transfer and bioluminescence. The development of biosensors for the detection of these signal molecules has greatly facilitated their subsequent chemical analysis which in turn has resulted in significant progress in understanding the molecular basis of quorum sensing-dependent gene expression. Consequently, the discovery and characterisation of natural molecules which antagonize quorum sensing-mediated responses has created new opportunities for the design of novel anti-infective agents which control infection through the attenuation of bacterial virulence.

Keywords Quorum sensing · Cell-cell signalling · *N*-Acylhomoserine lactones · Antibacterial agents · Bacterial pheromones

List of Abbreviations

AHLs	*N*-Acyl-L-homoserine lactones
DCCI	*N*,*N*′-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	*N*,*N*′-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
Fmoc	9-Fluorenylmethoxycarbonyl
HOBt	1-Hydroxybenzotriazole
HSL	L-Homoserine lactone
QS	Quorum sensing
RP-HPLC	Reversed-phase high performance liquid chromatography
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TsOH	Toluene-*p*-sulfonic acid

1 Introduction

Unicellular bacteria, in common with higher, multicellular organisms, possess the ability to communicate using small diffusible chemical signal molecules. Bacterial cell-to-cell communication facilitates the control of gene expression in a cell population density dependent manner and is exploited for the control of traits likely to benefit the population as a whole. These include the acquisition of new genes through conjugal DNA transfer, swarming and swimming motility (population migration) and the elaboration of mechanisms for niche protection and for combating human, animal and plant host defence mechanisms during infection [1]. Indeed, the threat posed to medicine and public health by the rise of antimicrobial resistance and the emergence of strains resistant to multiple therapeutic agents has stimulated interest in novel strategies which attenuate virulence (i.e. the capacity of bacteria to cause disease) rather than kill the infecting bacteria such that the infection is cleared by the innate immune defences [2, 3]. In this context, the extracellular communication systems employed by pathogenic bacteria to control virulence are attractive targets for the design of new anti-infective agents.

Bacterial cell-to-cell communication is often referred to as "quorum sensing" (QS) and relies on the activation of a sensor kinase or response regulator protein by a diffusible, low molecular weight signal molecule (sometimes referred to as a "pheromone" or "autoinducer") [1, 4]. Consequently, in QS, the concentration of the signal molecule is a reflection of the number of bacterial cells in a particular niche and the perception of a threshold concentration of that signal molecule indicates that the population is "quorate", i.e. ready to make a behavioural decision. QS is viewed as a primitive mechanism for multicellular behaviour in prokaryotes and has been described as "the most consequential molecular microbiology story of the last decade" [5]. This chapter will present the structure, mode of action and functions of bacterial quorum sensing signal molecules, their analysis and synthesis, their ecological role and their potential as novel antibacterial targets.

1.1 Bacterial Cell-Cell Communication Mechanisms

In any quorum sensing regulatory cascade there are several key elements to be considered: (i) the gene(s) involved in signal synthesis, (ii) the gene(s) involved in signal transduction and (iii) the QS signal molecule(s) (Fig. 1). During the growth of a bacterial population, the concentration of signal molecules increases. These either diffuse or are exported out of the cell into the surrounding environment before acting at the surface or inside neighbouring bacterial cells. The accumulation of QS signal molecules to a critical threshold concentration results in the activation of a sensor/response regulator, responsible for signal transduction, which in turns triggers the expression of multiple genes

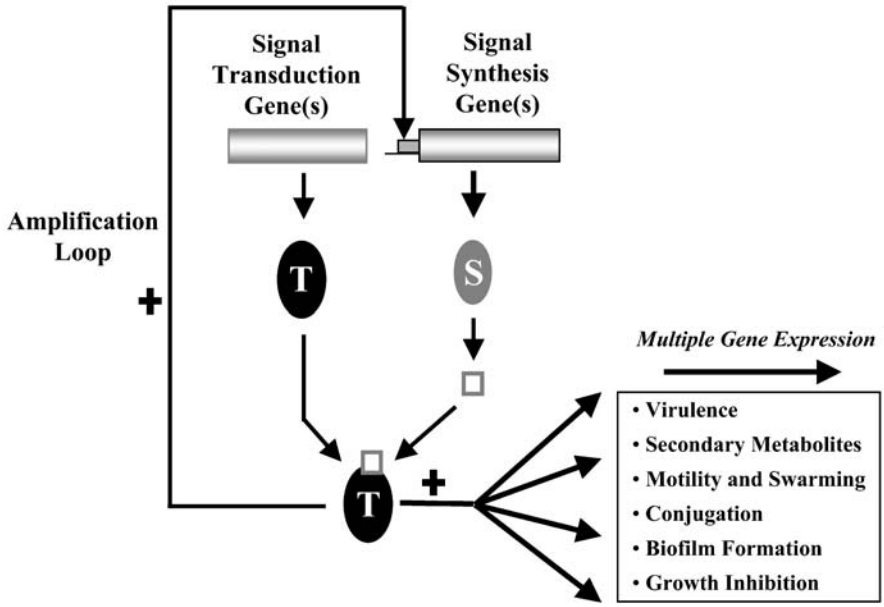


Fig. 1 QS-dependent activation of multiple gene expression

and often also incorporates a positive, autoinductive feedback loop to amplify QS signal molecule generation. Hence the term "autoinducer" is sometimes used to describe the QS signal molecule. Although the principle behind QS-mediated gene expression in both Gram-positive and Gram-negative bacteria is shared, the molecular mechanisms and signal molecules involved are different [2, 3]. In Gram-negative bacteria, the most intensively investigated QS signalling systems are those employing *N*-acylhomoserine lactones (AHLs) as QS signal molecules. AHLs were originally discovered to control light emission in bioluminescent marine vibrios such as *Vibrio fischeri* and to be synthesized via the LuxI protein and sensed by the LuxR protein. The latter acts as both AHL sensor and response regulator by directly binding to target gene sequences once activated. Members of the LuxI and LuxR protein families are widely distributed amongst many different Gram-negative genera. No AHL-producing Gram-positive bacteria have been identified although the *Streptomyces* produce structurally related ɣ-butyrolactones [1]. Gram-positive bacteria such as the staphylococci employ post-translationally modified peptides as QS signals which are sensed via two component phosphorelay systems consisting of a cytoplasmic membrane bound sensor kinase protein and a cytoplasmic response regulator protein. The QS signalling peptides are produced in the cytoplasm and then modified during their secretion. Accumulation of the signal molecule at the cell surface results in activation of the phosphorelay system through direct binding to a sensor kinase protein resulting the activation/inhibition of multiple gene expression [2, 3].

1.2 Extracellular Signal Molecule or Metabolite?

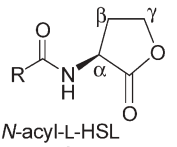
A vast number of chemically diverse extracellular metabolites are present in culture media after bacterial growth and, theoretically, any of these has the potential to serve as a QS signal. It is therefore important to define the features that distinguish QS signal molecules from other metabolites [6]. The classification of a molecule as a true QS signal molecule requires that: (i) the production of the QS signal takes place during specific stages of growth, under certain physiological conditions, or in response to environmental changes; (ii) the QS signal accumulates in the extracellular milieu and is recognized by a specific bacterial receptor; (iii) the accumulation of a critical threshold concentration of the QS signal generates a concerted response; (iv) this cellular response extends beyond physiological changes required to metabolise or detoxify the molecule [6]. Unless these four criteria are met, a molecule cannot be classified as a true QS signal molecule as there are many other extracellular metabolites that meet the first three. Examples of these are toxic bacterial metabolites that accumulate and trigger a coordinated stress responses in bacterial populations once they reach a critical concentration. These metabolites cannot be considered as intercellular communication signals, as the cells are only responding to the toxicity of the molecule itself. Similarly, there are metabolites which can induce, during their temporary release, the expression of their own uptake systems and the production of enzymes required for their turnover. This could indirectly influence the expression of genes from other linked metabolic pathways and emphasizes the importance of criterion (iv) when defining QS.

An important concept in the understanding QS and the size of the "quorum" is the idea of 'compartment sensing' [6]. As noted above, the concentration of a given QS signal molecule may be a reflection of bacterial cell number, or at least the minimal number of cells (quorum) in a particular physiological state. To achieve the accumulation of a QS signal there is a need for a diffusion barrier, which ensures that more molecules are produced than lost from a given microhabitat. This type of 'compartment sensing' enables the QS signal molecule to be both a measure of the degree of compartmentalization and the means to distribute this information through the entire population. Likewise, the diffusion of QS signal molecules between detached subpopulations may convey information about their numbers, physiological state and the specific environmental conditions encountered.

2 Structural Diversity in QS Signal Molecules

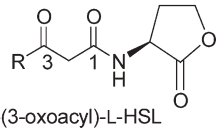
To date, a diverse range of bacterial QS signal molecules has been isolated and identified. By far the most studied is the AHL family produced by various Gram-negative bacterial genera. All AHLs reported to-date are characterized by

a homoserine lactone ring unsubstituted in the β- and γ-positions which is N-acylated with a fatty acyl group at the α-position **1**. The acyl chain has various lengths, saturation levels and oxidation states. In most cases the chain has even number of carbons (4–18 C). Examples of different AHLs identified in Gram-negative are shown in Table 1. They belong to either the *N*-acyl **1**, *N*-(3-oxo-acyl) **2** or *N*-(3-hydroxyacyl) **3** class of compounds [7–13]. In addition, some AHLs also have unsaturation with *Z* stereochemistry in the 7 position in a chain of 14 carbons **4** [11, 12].



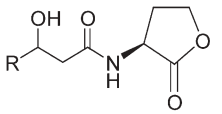
N-acyl-L-HSL

1



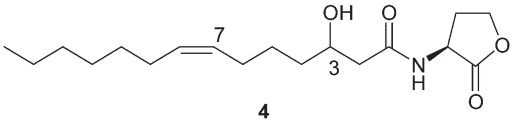
N-(3-oxoacyl)-L-HSL

2



N-(3-hydroxyacyl)-L-HSL

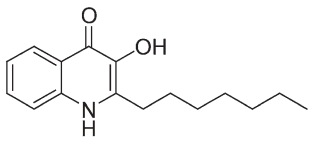
3



4

Stereochemistry at the α-centre of the homoserine lactone (HSL) ring has been unequivocally established to be L for the *V. fischeri* autoinducer, *N*-(3-oxo-hexanoyl)homoserine lactone and by analogy it is extrapolated that all other natural AHLs have the same configuration. In some cases D-isomers have been synthesised and shown to lack activity [14–17].

Besides AHLs (Table 1), the opportunistic human pathogen *Pseudomonas aeruginosa* also produces another chemically distinct QS signal molecule, 2-*n*-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS **5**) [18]. PQS is an integral component of the QS hierarchy and plays an important role in regulating virulence gene expression [19].

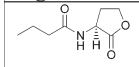
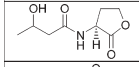
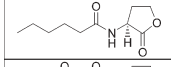
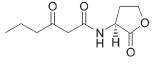
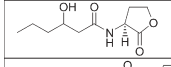
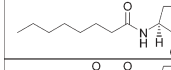
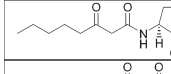
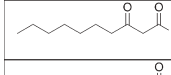
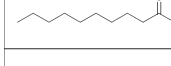
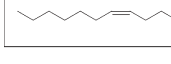


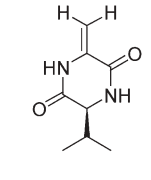
PQS

5

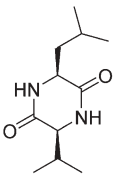
The cyclic dipeptides or diketopiperazines (DKPs) **6–10** are yet another class of small diffusible signal molecules that has been isolated from the culture supernatants of *P. aeruginosa, Pseudomonas putida WCS* 358 and other Gram-negative bacteria. Their role in cross-talk with AHL-dependent QS has been demonstrated but their physiological function is not known [20, 21].

Table 1 Bacterial *N*-acylhomoserine lactones and their associated phenotypes

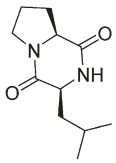
Signal Molecule	Name and Abbreviation	Organism	Phenotype(s)
	*N*-butanoyl-L-homoserine lactone, BHL or C_4-HSL	*Aeromomas hydrophila* *Aeromonas salmonicida* *Pseudomonas aeruginosa,* *Serratia liquefaciens*	Extracellular protease, biofilm formation. Extracellular protease. Virulence factors:- alkaline protease, cyanide, elastase, haemolysin, lectins, pyocyanin, rhaminolipid, RpoS Swarming, protease.
	*N*-(3-hydroxybutyryl)-L-homoserine lactone, HBHL or 3-OH, C_4-HSL	*Vibrio harveyi* *Xenorhabdus nematophilus*	Bioluminescence, polyhydroxybutyrate metabolism. Virulence, extracellular lipase.
	*N*-hexanoyl-L-homoserine lactone, HHL or C_6-HSL	*Chromobacterium violaceum* *Pseudomonas aureofaciens* *Yersinia enterocolitica*	Antibiotics, cyanide, exoenzymes, violaien. Phenazine antibiotic Swimming and swarming motility
	*N*-(3-oxohexanoyl)-L-homoserine lactone, OHHL or 3O, C_6-HSL	*Enterobacter agglomerans* *Erwinia carotovora* *Pantoea stewartii* *Nitrosomonas europea* *Obesumbacterium proteus* *Vibrio fischeri* *Yersinia enterocolitica* *Yersinia pseudotuberculosis*	? 5(*R*)-Carbapen-2-em-3-carboxylic acid antibiotic, virulence factors:- extracellular protease, cellulases, pectinases. Virulence factors:- protease, cellulases, exopolysaccharide. Emergence from lag phase. Bioluminescence. Swimming and Swarming Motility Swimming motility, cell aggregation, biofilm maturation
	*N*-(3-hydroxyhexanoyl)-L-homoserine lactone, HHHL or 3-OH, C_6-HSL	*Vibrio anguillarum*	C_{10}-HSL production
	*N*-octanoyl-L-homoserine lactone, OHL or C_8-HSL	*Vibrio fischeri* *Ralstonia solanacearum* *Yersinia pseudotuberculosis*	Bioluminescence. ? Swimming motiliy, cell aggregation, biofilm maturation
	*N*-(3-oxooctanoyl)-L-homoserine lactone, OOHL or 3O, C_8-HSL	*Agrobacterium tumefaciens* *Vibrio fischeri*	Conjugal transfer of Ti plasmid. Bioluminescence
	*N*-(3-oxodecanoyl)-L-homoserine lactone, ODHL or 3O, C_{10}-HSL	*Vibrio anguillarum*	?
	*N*-(3-oxododecanoyl)-L-homoserine lactone, OdDHL or 3O, C_{12}-HSL	*Pseudomonas aeruginosa*	Virulence factors:- alkaline protease, elatase, exotoxin A, haemolysin, neuraminidase, exoenzyme S, Xcp secretion, RhlR, biofilm formation.
	*N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone, 7Δ HtDHL or 7Δ, 3-OH, C_4-HSL	*Rhizobium leguminosarum* *Rhodobacter sphaeroides*	Nodulation, growth inhibition, expression of rhizosphere genes, plasmid transfer Community escape



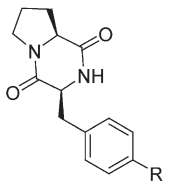
cyclo (ΔAla-L-Val)
6



cyclo (L-Leu-L-Val)
7

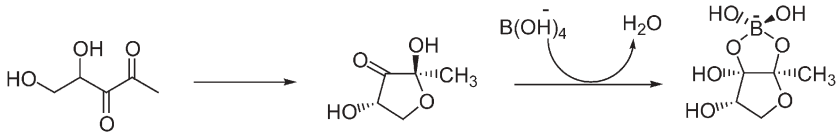


cyclo (L-Leu-L-Pro)
8



9 = H cyclo (L-Phe-L-Pro)
10 = OH cyclo (L-Pro-L-Tyr)

A QS signal molecule termed autoinducer-2 (AI-2) is produced by *Vibrio harveyi*, where it exerts its activity in conjunction with an AHL via a complex phospho-relay system to control bioluminescence. AI-2 production has been demonstrated for many other bacterial species, both Gram-negative and Gram-positive, implying the widespread existence of AI-2-based QS. The chemical identity of AI-2 as a furanosyl borate diester **11** follows from the crystal structure of an AI-2 sensor protein, LuxP, in a complex with autoinducer (Scheme 1) [22]. However, the relative stability of the AI-2 intermediates in vivo seems uncertain, as is the availability of boric acid [6]. In addition, the function of AI-2 as a QS signal molecule in bacteria other than vibrios has been questioned and this molecule has been suggested, for most bacteria to be a metabolic side product in the activated methyl pathway [2, 23].



4,5-dihydroxy-2,3-pentanedione

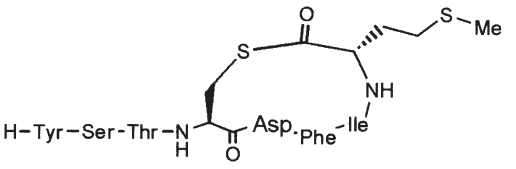
Pro-AI-2

AI-2
11

Scheme 1 Production of AI-2 from DPD and borate

In contrast to Gram-negatives, many Gram-positive bacteria employ post-translationally modified peptides processed from larger precursors as QS signal molecules. In *Staphylococcus aureus*, for example, a family of peptide (7–9 amino acid residues) thiolactones which vary in the primary amino acid sequence but contain a conserved cysteine at position 5 control the expression of cell wall colonization factors and exotoxins [24–26].

Interestingly, the peptide thiolactones activate virulence gene expression in the producer strain but inhibit virulence gene expression in staphylococcal strains belonging to other peptide thiolactone groups [24, 25]. For example, a group I *S. aureus* peptide thiolactone **12** is an activator of group I *S. aureus* strains but an antagonist of strains producing peptide thiolactones belonging to groups II, III and IV [24, 25]. Due to the variety and complexity of bacterial QS systems, this review will focus on the synthesis, analysis, role and exploitation of AHLs in Gram-negative bacteria.



12

3 Nomenclature and Abbreviations of AHLs

The names of AHLs listed in Table 1 are the non-IUPAC description of compounds as *N*-acyl derivatives of L-homoserine lactone. Alternatively, IUPAC chemical designations for the compounds can be used. These are based on the amide unit as the principal function. Thus *N*-(3-oxohexanoyl)-L-homoserine lactone is named as: 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)hexanamide.

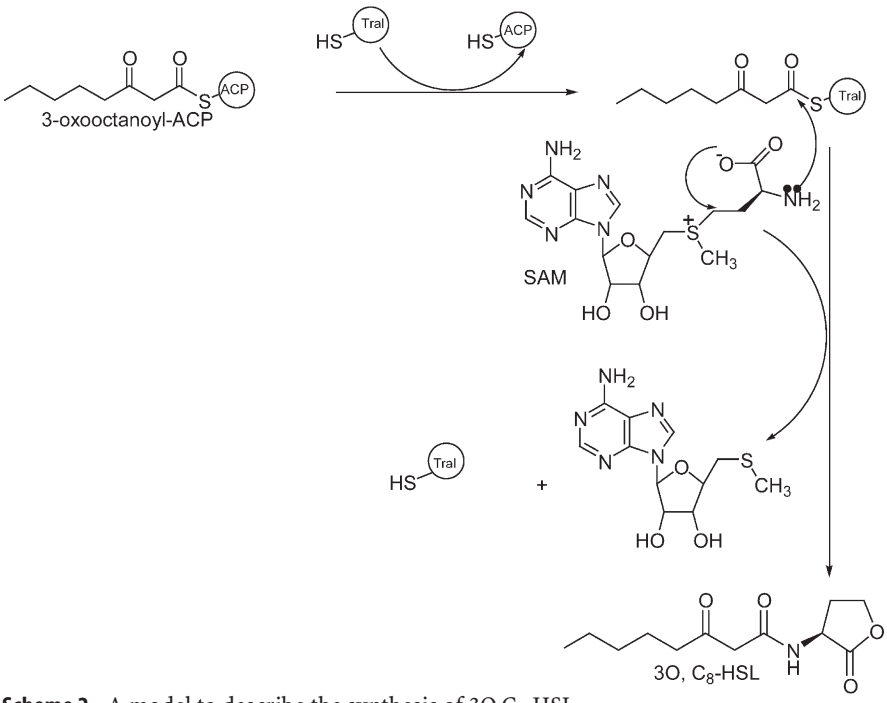
In addition, other systems of nomenclature for AHLs have also appeared in the literature describing them as derivatives of furanone, γ-butyrolactone or 4-butanolide. For example, the above *V. fischeri* autoinducer can also be named as (*S*)-*N*-(3-oxohexanoyl)-3-aminodihydro-2(3*H*)-furanone, (*S*)-α-(3-oxohexanoyl)amino-γ-butyrolactone or (*S*)-2-(3-oxohexanoyl)amino-4-butanolide (another IUPAC name) [27].

Although the most frequently used nomenclature for AHLs was initially based on a 3–5 letter codes based on names e.g. BHL for *N*-butyryl-L-homoserine lactone, OHHL for *N*-(3-oxohexanoyl)-L-homoserine lactone or OdDHL for *N*-(3-oxododecanoyl)-L-homoserine lactone, the rapid expansion in the range of AHL molecules discovered has led to changes in this nomenclature. Currently, the accepted abbreviations are a structure-based short-hand notations e.g. C_4-HSL for *N*-butyryl-L-homoserine lactone, 3-oxo-C_6-HSL (or 3O,C_6-HSL) for *N*-(3-oxohexanoyl)-L-homoserine lactone or 3-hydroxy-C_{12}-HSL (or 3OH,C_{12}-HSL) for *N*-(3-hydroxydodecanoyl)-L-homoserine lactone.

4 Biosynthesis of AHLs

In vitro studies using purified AHL synthases have revealed that AHLs are synthesised from precursors derived from amino acid and fatty acid metabolism, with the LuxI proteins being the predominant family of AHL synthases [28] although at least one other family of unrelated synthases, the LuxM family has been identified [13]. Since LuxI proteins are not particularly closely related it has been impossible to predict the nature of the AHL produced by a given LuxI protein. Nevertheless, in vitro studies using recombinant proteins have shown that LuxI-type proteins catalyse the synthesis of AHLs using the appropriately charged acyl carrier protein (acyl-ACP), as the main acyl chain donor, and *S*-adenosyl methionine (SAM) as the source for the homoserine lactone moiety

[29–32]. Scheme 2 shows the biosynthesis of *N*-(3-oxooctanoyl)-L-homoserine lactone by TraI protein from *Agrobacterium* using 3-oxooctanoyl-ACP, derived from fatty acid metabolism, as a substrate [29, 33]. Recently, the first crystal structure of a LuxI protein homologue [34] has provided new insights into the function of AHL synthases which will aid the design of novel inhibitors of AHL biosynthesis.



Scheme 2 A model to describe the synthesis of 3O,C_8-HSL

5 Detection of AHLs

Since the discovery of AHLs as QS signal molecules, a variety of methods have been developed to detect the presence of these molecules as well as their structure. This section provides some examples.

5.1 AHL Biosensors

One of the first cues for the presence of QS-mediated gene regulation in bacteria is the appearance of certain phenotypes (e.g. the production of antibiotics or exoproteases) in a cell population density dependent fashion and the increased expression of these phenotypes upon addition of spent culture supernatants as potential AHL sources. To detect the presence of AHLs, a number of biosensor

strains have been developed. These biosensors manifest an obvious phenotypic change such as light emission (bioluminescence), fluorescence or pigment production in the presence of AHLs. There are several bioluminescence-based AHL biosensors available which couple an AHL-activated promoter (e.g. the *luxI* promoter) to the bioluminescence encoding *lux* operon from *V. fischeri* or *Photorhabdus luminescens* together with the corresponding *luxR* homologue activator gene [35–37]. Plasmids carrying the engineered reporter gene fusions are introduced in *E. coli* such that the recombinant strain is dark and will only emit light in the presence of exogenously supplied AHL(s). In other biosensors the *lux* operon has been replaced by the gene encoding the green fluorescence protein *gfp* resulting in the generation of fluorescence, rather than bioluminescence [38]. In addition, one of the most frequently employed AHL biosensors reported in the literature is that based on the AHL-dependent regulation of the purple pigment violacein by a mutant of *Chromobacterium violaceum* (CV026). This mutant is unable to make AHLs but responds to their presence synthesising violacein [37, 39]. Alternative biosensors have been developed by Fuqua and Winans [40], Passador et al., [41] Wood and Pierson [42] and Wood et al., [43].

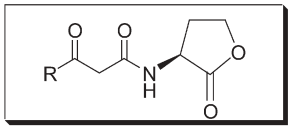
AHLs can be tentatively identified by comparison of the unknown with synthetic AHL standards after Thin Layer Chromatography (TLC) in which the plates are overlaid with agar containing one of the AHL biosensors described above [37, 39, 44, 45]. However, for the unequivocal identification of AHLs the use of more powerful methods such as LC-mass spectrometry, nuclear magnetic resonance and infrared spectroscopy as described below are required.

5.2 HPLC

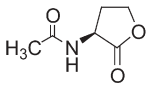
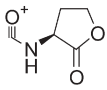
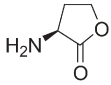
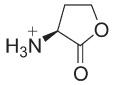
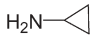
HPLC has been shown as an effective method in the fractionation and preparation of AHLs for structural analysis. Preparation of AHL-containing samples for HPLC analysis requires their extraction with organic solvents such as dichloromethane or ethyl acetate [37]. Usually, C8 reverse-phase columns are employed and samples eluted with either gradient or isocratic mobile phases, e.g. acetonitrile-water. Fractions are analysed for the presence of AHLs using the biosensors described in the previous section. AHLs from active fractions can then be identified using more powerful techniques (see following sections).

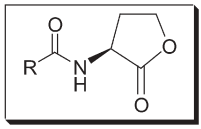
5.3 Mass Spectrometry

Once a single HPLC peak able to activate the AHLs biosensors has been obtained, it is important to unequivocally assign a structure to the QS signal molecule on the basis of its spectroscopic properties. The final structural confirmation needs to be obtained by chemical synthesis to demonstrate that the properties of both natural and synthetic materials are identical.



Principal fragment ions

1. M^+
2. R^+
3. $R.CO^+$
4. $[R\text{-}CH_2]^+$
5. $M\text{-}[R\text{-}CH_2]^+ = 185$
6. $[R.CO.CH_2.CO]^+$
7.  = 143
8.  = 128
9.  = 101
10.  = 102
11.  = 99 and/or 98
12.  = 84 and/or 85
13.  = 57 and/or 58
14. $H_2N.CH{=}CH_2 = 43$



As above except fragments 5 and 6

Fig. 2 Mass spectrometry data of AHLs

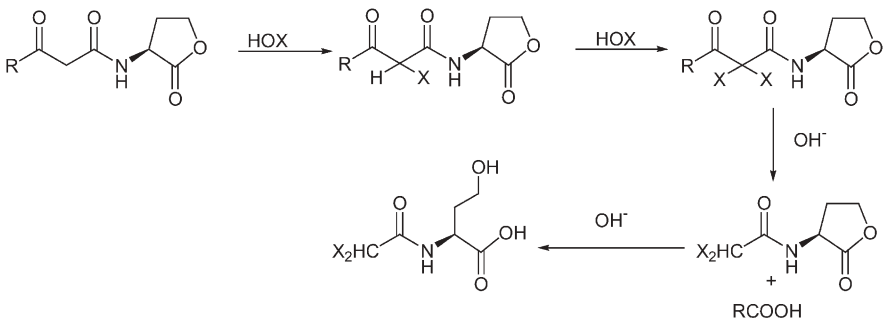
Mass spectrometry (MS) has been shown as the most valuable tool for the identification and characterisation of AHLs with detection levels in the picomole range. The major fragmentation ions in the electron impact ionisation-MS (EI-MS) of the *N*-acyl and *N*-(3-oxoacyl) classes of AHLs are summarised in Fig. 2 [37].

Furthermore it may be noted that the series of *N*-(3-hydroxyacyl)-L-HSLs can be readily characterized by MS as these show initial loss of a molecule of water during fragmentation, leading to a characteristic ion at M-18 [12, 44].

Lately, electrospray ionisation technique (ESI-MS) which is compatible with RP-HPLC has been routinely used. This allows labile molecules to be studied intact. Sample molecules are simultaneously nebulised and ionised at atmospheric pressure in the presence of several thousand volts. The resulting ions can be multi-protonated (multiply charged) and relatively stable. This mode of ionisation has recently been used in the development of RP-HPLC coupled with positive ion ESI-MS and ion-trap MS protocols for the identification and

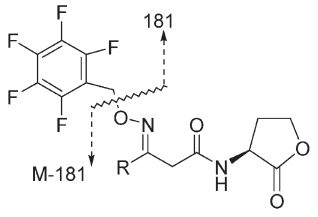
quantification of AHLs in crude cell-free supernatants of bacterial cultures. This method circumvents the multi-step procedures previously described and employs an online LC-MS-MS technique which is fast and can detect eleven different AHLs. The selectivity is based on the MS-MS fragment ions of the molecular $[M+H]^+$ ions and on their relative intensities. For quantification, the m/z 102 ion, specific for the lactone ring and detected with a good signal to noise ratio, allows low detection limits even in complex matrix samples (0.28 up to 9.3 pmol) [46].

Another application of LC-MS has been reported to study the degradation pathway of AHLs by halogen antimicrobials (hypochlorite and stabilized hypobromite) (Scheme 3). An RP-HPLC separation using a cyano column was developed to detect the parent lactones, lactonolysis products and halogenation products. This study demonstrated that only *N*-(3-oxoacyl)-HSL signal molecules are halogenated, where normal *N*-acyl-HSL are not. These results are of significance for the control of industrial biofilms [47].



Scheme 3 The complete pathway of the reaction between 3O,C_n-HSL and halogenated antimicrobials

Yet another sensitive procedure for the quantification of a diverse range of 3-oxo-AHLs (3O,C_n-HSL) using gas chromatography-mass spectrometry (GC-MS) has been developed by Charlton et al. [48]. The method entails the derivatisation of 3-oxo functionality with pentafluorohydroxylamine hydrochloride to furnish derivatives **13** where pentafluorobenzyloxime moiety serves



13

as the electron capturing group. These derivatives when analysed by GC-MS generate the M-181 (target) ions which were used in a quantitative assay employing [$^{13}C_{16}$]-3O,C_{12}-HSL as the internal standard. The assay was successfully applied to the quantification of 3O,C_n-HSL (n=8, 10, 12, 14) in biofilm and effluent of a green fluorescent protein (GFP)-expressing strain of *Pseudomonas aeruginosa* (6294) culture grown in flow cells.

5.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

Further confirmation of the structure of the AHLs often follows from the determination of their proton and carbon-13 NMR spectra. A large number of *N*-acyl, *N*-(3-oxoacyl) and *N*-(3-hydroxyacyl)-L-HSL derivatives have been prepared and their NMR data reported [14–16]. Also a detailed study by Lao et al. [49] on the complete assignments of the ^{13}C NMR resonances of *N*-acyl and *N*-(3-oxoacyl)-L-HSL derivatives has been published. These assignments were made by comparison with values for *N*-butanoyl-L-HSL (C_4-HSL), whose structure was comprehensively established by a combination of 1-D ^1H and ^{13}C spectra and 2-D COSY, NOESY, HSQC and HMBC experiments. The assignments

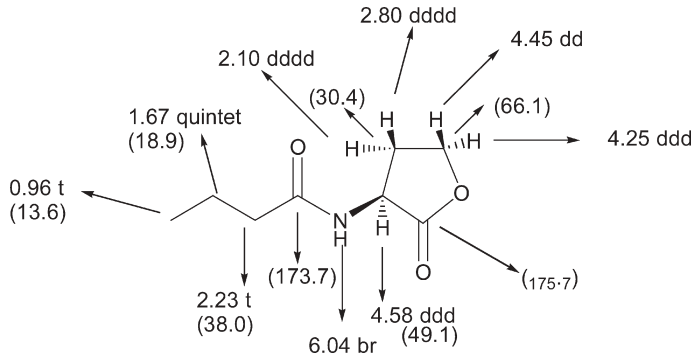


Fig. 3 ^1H and ^{13}C (*in parentheses*) assignments for C_4-HSL

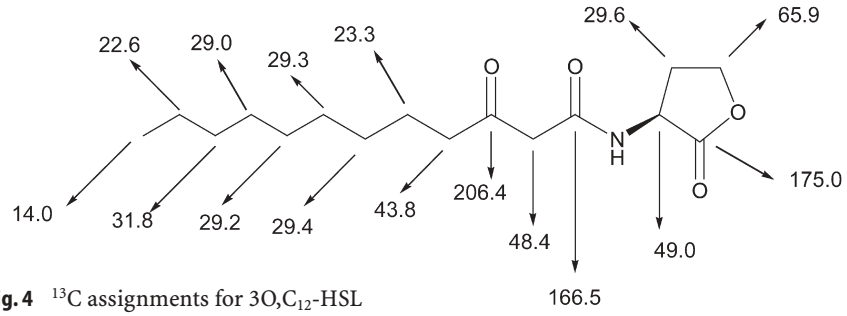
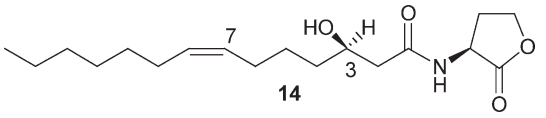


Fig. 4 ^{13}C assignments for 3O,C_{12}-HSL

for the ^{1}H and ^{13}C (values shown in parentheses) NMR of C_4-HSL and ^{13}C NMR of *N*-(3-oxododecanoyl)-L-HSL are summarized in Fig. 3 and Fig. 4 respectively.

The detailed structure of the *small* bacteriocin (isolated from the culture broth of the Gram-negative bacterium *Rhizobium leguminosarum*) as *N*-[(3*R*)-hydroxy-7-*cis*-tetradecenoyl]-L-HSL **14** was elucidated largely by the 1-D and 2-D ^{1}H and ^{13}C spectroscopy. The absolute configuration of both asymmetric carbon atoms in the molecule was determined by the use of chiral solvating agents (*S*)-(+)- and (*R*)-(–)-2,2,2-trifluoro-1-(9-anthryl)ethanol [11].



14

5.5 Infrared (IR) Spectroscopy

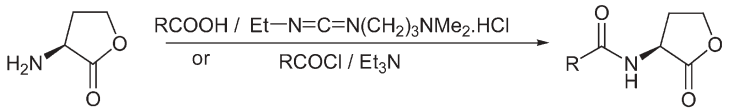
IR spectroscopy is useful for the identification of some of the functional groups in an organic molecule. The technique also provides a 'fingerprint' of the molecule and its comparison with authentic specimen often confirms the structure of that molecule. The IR spectra of AHLs show characteristic absorption peaks at 1780, 1710, 1650 cm^{-1} arising from the lactone ring, 3-oxo (when present), and amide carbonyl, respectively [15, 16].

6 Chemical Syntheses of AHLs

A number of synthetic methods to prepare all the three main classes of AHLs have appeared in the literature. Initially the methods were developed to prepare the authentic AHLs with defined stereochemistry to confirm the identity of the natural signal molecule. Subsequently, when some of these molecules, e.g. *N*-(3-oxododecanoyl)-L-homoserine lactone were found to impact on eukaryotic signalling systems [16, 50–52], detailed studies not only of their preparation but also of their structural analogues were undertaken by many laboratories.

6.1 Synthesis of *N*-Acyl-L-HSL

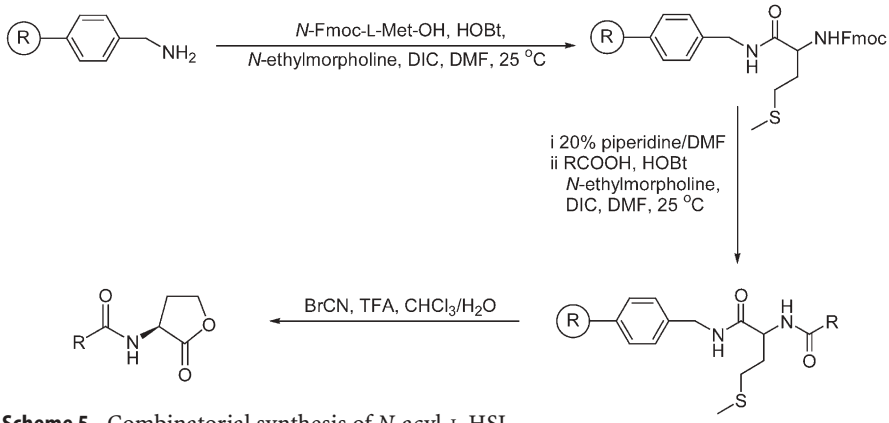
N-Acyl-L-HSL can be conveniently prepared in excellent yields by the acylation of L-HSL either with the corresponding carboxylic acids activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in a water/1,4-dioxane (1:1) solvent system or with the corresponding acid chlorides in dichloromethane (DCM) in the presence of triethylamine (Scheme 4) [15, 16, 37, 53].



Scheme 4 Synthesis of *N*-acyl-L-HSL

L-HSL is commercially available and in the laboratory can be easily prepared by the acid catalysed lactonisation of L-homoserine. The latter and its D-isomer can be prepared by the regioselective reduction of L- or D-aspartic acid either with BH_3-THF [31] or with $NaBH_4$ after activation with an alkyl chloroformate [54].

Solid-phase combinatorial synthesis of *N*-acyl-L-HSL has also been reported. The procedure entails the DIC/HOBt catalysed acylation of methionine functionalised resin with a carboxylic acid followed by BrCN-mediated cyclisation process to produce HSL libraries with retention of stereochemistry (Scheme 5) [55].

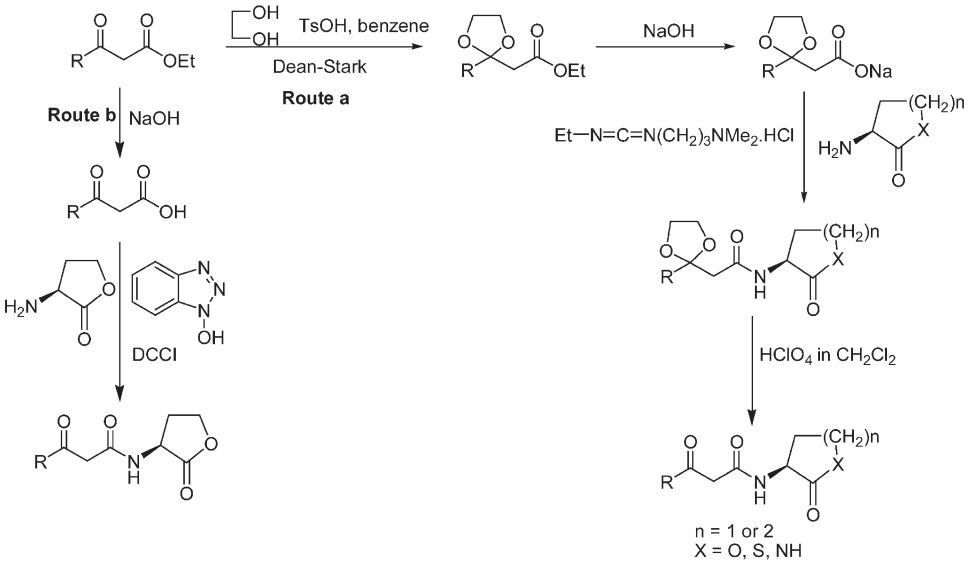


Scheme 5 Combinatorial synthesis of *N*-acyl-L-HSL

6.2 Synthesis of *N*-(3-Oxoacyl)-L-HSL

The signal molecule, 3O,C6-HSL and number of its analogues, with variations in the acyl chain and the hetero-ring, have been prepared [15, 56, 57] to investigate the mechanism of induction of carbapenem and luminescence in *Erwinia carotovora* and *V. fischeri* respectively. Essentially, the acylation of L-HSL with 3-oxoalkanoic acid by the same method as outlined for the preparation of *N*-acyl-L-HSL delivers the desired derivatives. However, as the β-keto acids are thermally labile, these were prepared from the corresponding β-keto ester after the initial protection of the β-keto function as ethylene glycol ketal (**route a**, Scheme 6).

The overall yield of the 3-oxo derivatives was 26% for the two steps from the ketal of 3-oxo acid. Dekhane et al. [58] found that by using 1-hydroxybenzo-

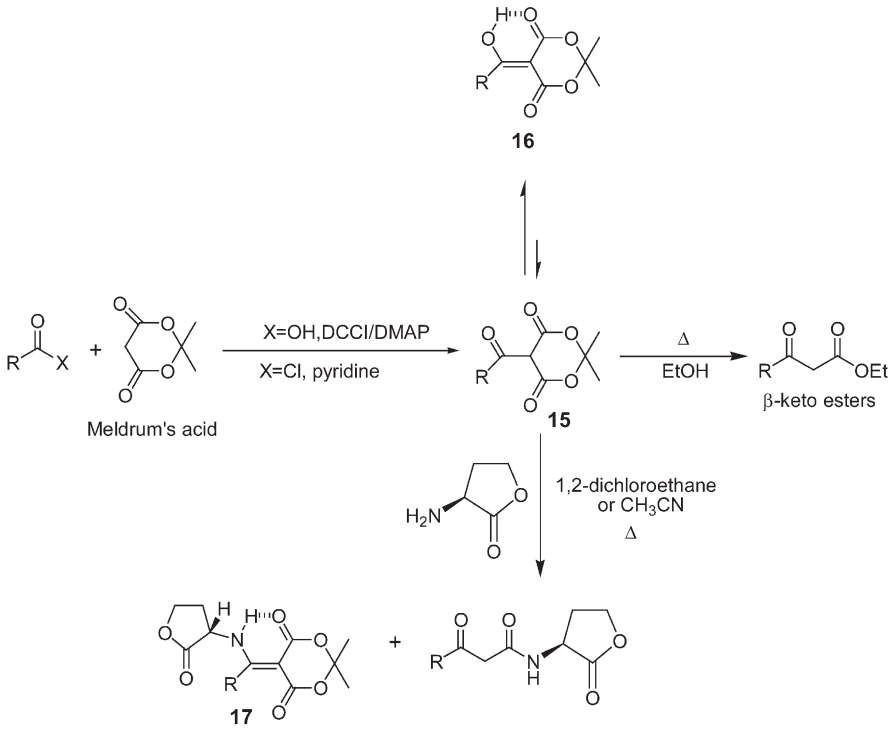


Scheme 6 Synthesis of *N*-(3-oxoacyl)-L-HSL and hetero-ring analogues

triazole and DCCI in non-aqueous media there was no need to protect the 3-oxo functionality (**route b,** Scheme 6). This led to an improved yield of 61% with fewer steps.

Ethyl 3-oxoalkanoates when not commercially available can be prepared by the acylation of *tert*-butyl ethyl malonate with an appropriate acid chloride by way of the magnesium enolate derivative. Hydrolysis and decarboxylation in acid solution yields the desired 3-oxo esters [59]. β-Keto esters can also be prepared in excellent yields either from 2-alkanone by condensation with ethyl chloroformate by means of lithium diisopropylamide (LDA) [60] or from ethyl hydrogen malonate and alkanoyl chloride using butyllithium [61]. Alternatively β-keto esters have also been prepared by the alcoholysis of 5-acylated Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione). The latter are prepared in almost quantitative yield by the condensation of Meldrum's acid either with an appropriate fatty acid in the presence of DCCI and DMAP [62] or with an acid chloride in the presence of pyridine [62] (Scheme 7).

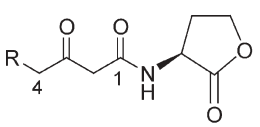
5-Acylated Meldrum's acids **15** exist as enolic structure **16** as evidenced from their NMR spectra which show a low field signal at 13–14 ppm assignable to enolic proton. These derivatives have been directly amidated with L-HSL for the preparation of *N*-(3-oxoacyl)-L-HSL (Scheme 7) [16, 57]. This route is quite concise and high yielding when R is C_6 or longer and has recently been exploited by Chhabra et al. (2003) [16] for the preparation of a large number of synthetic analogues of 3O,C_{12}-HSL to assess their potential as novel immune modulatory agents. When R is shorter than C_6, small amount of a side product **17** arising from the condensation of L-HSL with the exocyclic carbonyl in **15** is



Scheme 7 Synthesis of *N*-(oxoacyl)-L-HSL via 5-acylated Meldrum's acid

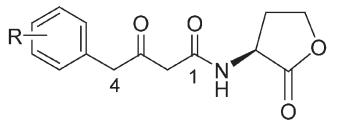
produced. Fortunately this can be easily removed by chromatographic separation on a silica column.

The acylated Meldrum's acid approach has also been used by Shaefer et al. [57] and recently by Reverchon and coworkers [53] to prepare novel synthetic *N*-(3-oxoacyl)-L-HSL derivatives with their acyl side-chain modified by introducing unsaturation, ramified alkyl, cycloalkyl **18** or aryl **19** substituents at the C-4 position.



R = iPr, *tert*-butyl, cycloalkyl

18



R = H, F, Cl, Br, CH_3, CF_3, OCH_3

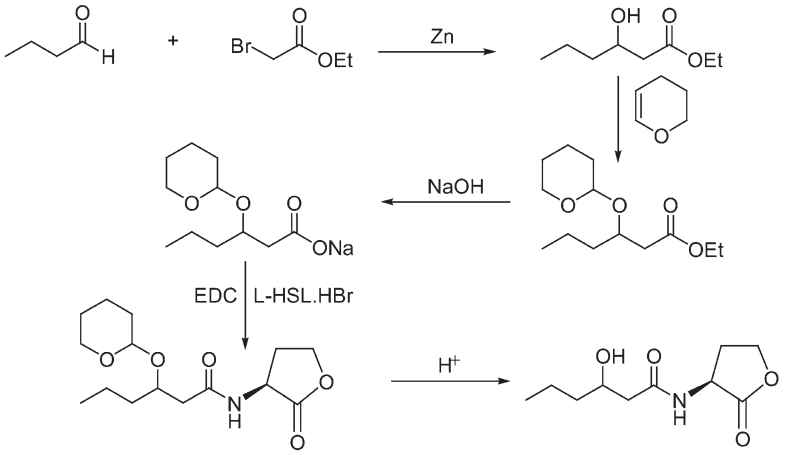
19

All the analogues were evaluated for both their activity using AHL biosensors and their ability to competitively inhibit the action of 3O,C6-HSL, the natural inducer of bioluminescence in *V. fischeri*. A similar protocol was also

employed by Zhang et al. [64] to prepare a selected number of *N*-(3-oxoacyl)-L-HSL derivatives, with variations in the length and nature of the acyl side-chain, to study the genetic regulation of conjugation in *Agrobacterium*.

6.3 Synthesis of *N*-(3-Hydroxyacyl)-L-HSL

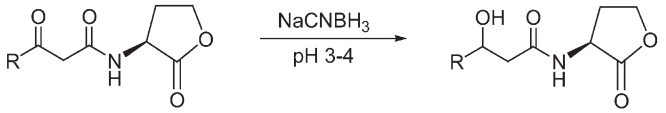
QS signal molecules possessing a 3-hydroxy substituent in the acyl chain have been isolated from *V. harveyi*, *V. anguillarum* and *R. leguminosarum*. The synthesis of *N*-(3-hydroxybutyryl)-L-HSL, the *V. harveyi* autoinducer, was achieved by the acylation of L-HSL.HBr with β-hydroxybutyric acid in the presence of water soluble carbodiimide, EDC, in water at room temperature overnight. The yield after purification by HPLC or silica-gel chromatography was only 7% [10]. To prepare the 3-hydroxy derivatives with a longer chain, Eberhard et al. prepared the requisite β-hydroxy acids by Reformatsky reactions [56, 65]. Thus the 3-hydroxyhexanoyl analogue was made by coupling butanal with ethyl bromoacetate using zinc, then protecting the resulting 3-hydroxy ester with dihydropyran before processing it for acylating the L-HSL (Scheme 8) [56].



Scheme 8 Synthesis of *N*-(hydroxyacyl)-L-HSL via Reformatsky reaction

This lengthy procedure requiring protection and deprotection of the hydroxy group can be circumvented by preparing the 3-hydroxy derivatives in 60–65% yield by the straightforward reduction of the corresponding 3-oxo derivatives with sodium cyanoborohydride (Scheme 9) [15, 16].

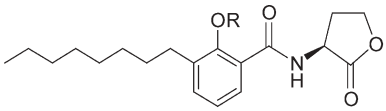
3-Hydroxyacyl analogues were prepared as a mixture of two diastereomers. In the case of 3-hydroxyhexanoyl (R=C_3H_7), the two diastereoisomers were successfully separated by HPLC. The absolute stereochemistry at the 3-hydroxy centre in either of the isomer was not established [15].

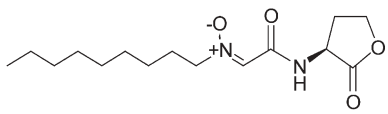


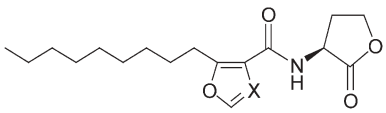
Scheme 9 Synthesis of *N*-(hydroxyacyl)-L-HSL via sodium cyanoborohydride reduction

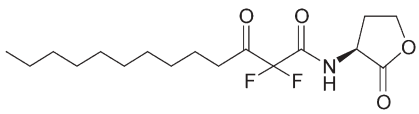
6.4 Synthesis of Conformationally Restricted Analogues of AHLs

Kline and co-workers describe the preparation and biological evaluation of constrained analogues of 3O,C_{12}-HSL, the longer chain AHL produced by *P. aeruginosa*. Their compounds **20**–**23** differ from the previous studies as these were designed to have a rigid functional group in order to probe the bioactive tautomer of the 3-oxo amide functionality [66].









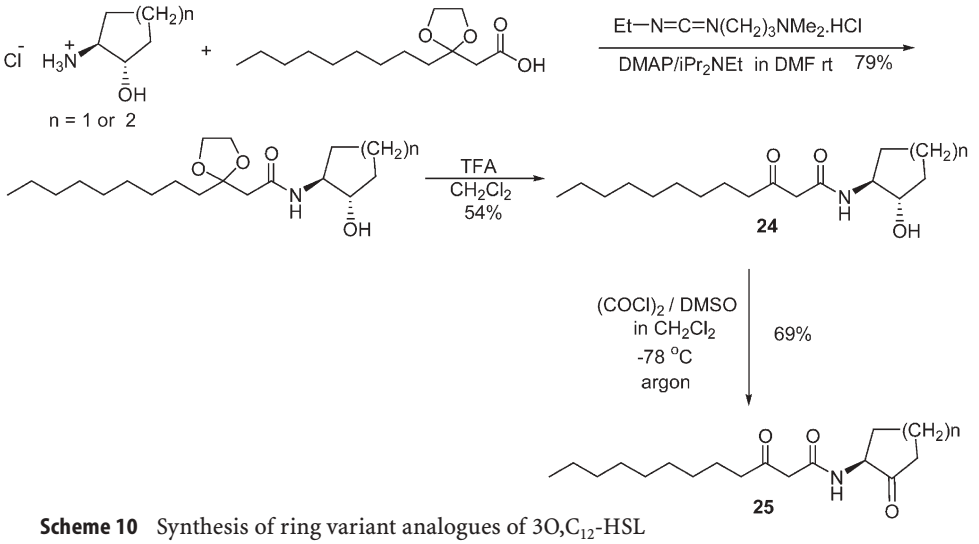
The salicylamides **20** and β-nitrone **21** structures were constructed to lock in the *Z*-enol conformer while the furanyl (**22**, X=CH) or oxazinyl (**22**, X=N) derivatives were expected to enforce the *E* relationship of the enolic oxygen with respect to the amide carbonyl. The gem difluoro substituted analogues **23** were likely to restrict tautomerisation while inducing minimal steric perturbation. Except **23** none of the constrained enolic analogues showed agonist or antagonist activity against LasR, the *P. aeruginosa* LuxR homologue activated by 3O,C_{12}-HSL.

6.5 Synthesis of Lactone Ring Variants of AHLs

Some of the lactone ring variants with variations in the hetero-atom and ring-size have already been described above. Smith et al. [67] recently reported the

synthesis of *P. aeruginosa* AHL analogues **24**–**27** in which the HSL ring has been replaced either with 2-aminocycloalkanone or 2-aminocycloalkanol rings.

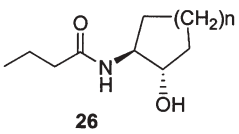
The derivatives **24** were synthesised by the acylation of *trans*-2-aminocyclopentanol (n=1) or *trans*-2-aminocyclohexanol (n=2) with 3,3-ethylenedioxydodecanoic acid in the presence of EDC, DMAP and DIPEA followed by acidolysis with THF in DCM. The cycloalkanone analogues **25** were obtained by Swern oxidation of the corresponding cycloalkanols **24** (Scheme 10).



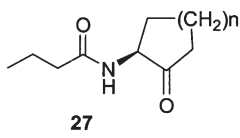
Scheme 10 Synthesis of ring variant analogues of 3O,C_{12}-HSL

The derivatives **26** and **27** were prepared by the same procedure using butyric acid.

These two sets of new analogues were screened for the activation of the QS transcription factors LasR and RhlR (for which C_4-HSL is the cognate AHL) respectively and discovered that 3-oxo-C_{12}-(2-aminocyclohexanol) (**24**, n=2) was an agonist capable of activating LasR.



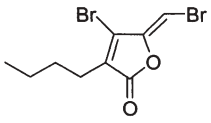
26



27

6.6 Synthesis of Lactone Ring Substituted Analogues of AHLs

Considering that the halogenated furanone type QS inhibitors (e.g. **28,** see below) isolated from the red marine alga *Delisea pulchra* [68, 69] have substituents in the 3- and 4-positions, Olsen et al. [70] reported on the parallel synthesis of new AHL analogues in which the substituents were introduced into the 3- and 4-position of the lactone ring.



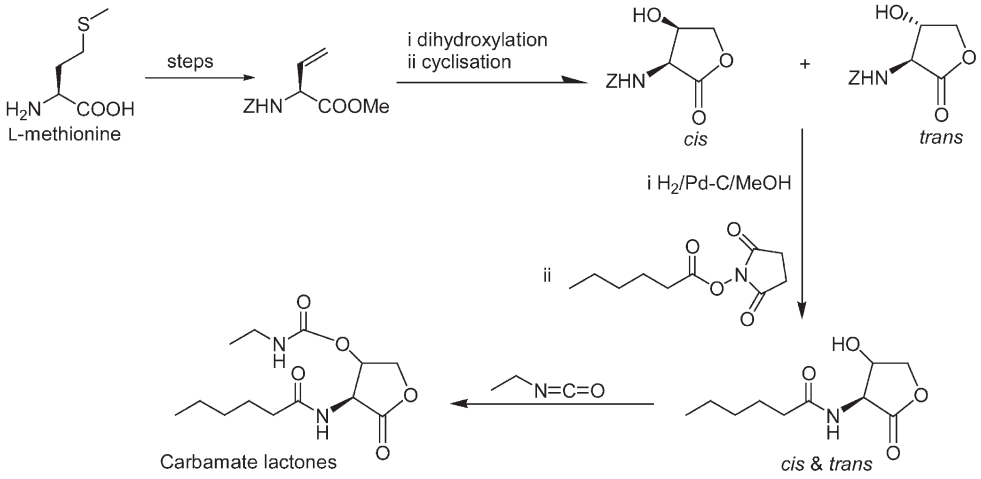
Inhibitor of AHL-dependent quorum sensing

28

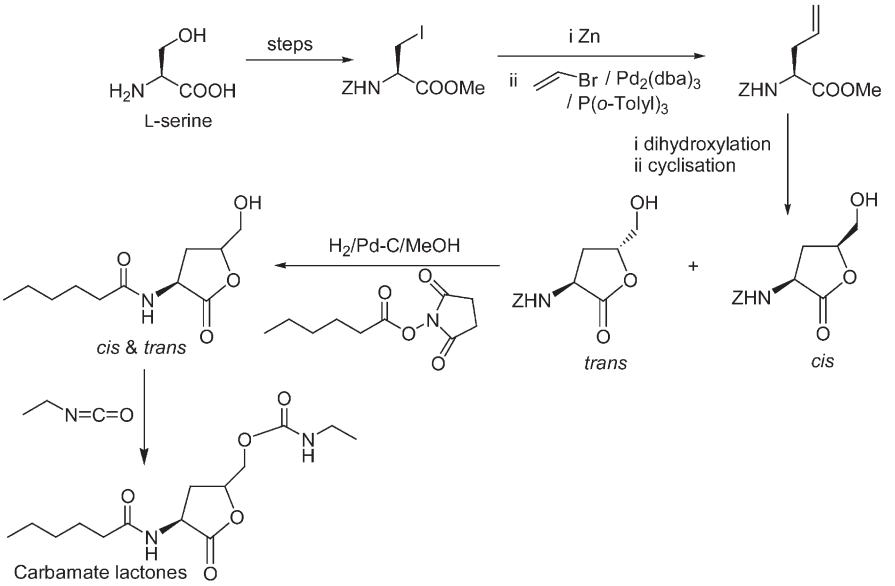
These new analogues were elaborated from enantiopure *cis*- and *trans*-3-hydroxy (Scheme 11) and 4-hydroxymethyl (Scheme 12) substituted L-HSL [70].

The hydroxy derivatives on reaction with ethyl isocyanate in the presence of CuCl in THF gave the corresponding carbamate lactones.

All the analogues including carbamate lactones were screened for their ability to activate and inhibit a *V. fischeri* AHL-dependent QS biosensor system. Z-protected lactones did not activate or inhibit the QS system, thereby underlining the importance of acyl side-chain. 4-Substituted-C_6-HSL analogues were only weak activators whereas the 3-substituted-C_6-HSL derivatives were significantly more potent as activators.



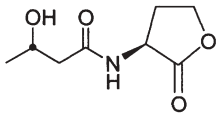
Scheme 11 Synthesis of *cis* and *trans* 3-hydroxy substituted C_6-HSL



Scheme 12 Synthesis of *cis* and *trans* 1-hydroxymethyl substituted C_6-HSL and their carbamate derivatives

6.7 Synthesis of Radio-labelled Derivatives of AHLs

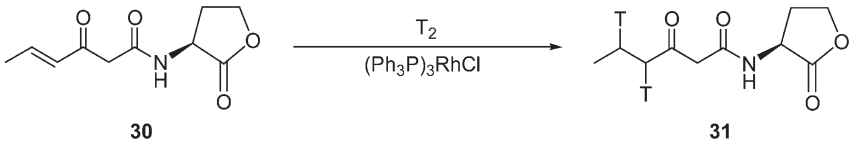
Only two reports appear in the literature describing the preparation of radio-labelled AHLs. ^{14}C-Labelled *V. harveyi* AHL **29** was synthesised using β-[3-^{14}C]hydroxybutyrate (43.4 mCi/mmol) [10].



N-([3-^{14}C][3-hydroxybutyryl)-L-HSL

29

The tritium labelled *V. fischeri* AHL **31** was prepared with a specific activity of 45–55 Ci/mmol by the tritiation of the corresponding unsaturated precursor, *N*-(3-oxo-4-hexenoyl)-L-HSL **30** in the presence of a homogeneous Wilkinson's catalyst, tris(triphenylphosphine)rhodium[I] chloride (Scheme 13) [71].



Scheme 13 Synthesis of *N*[3-oxo-(4,5-$^3H_2$]-hexanoyl]-L-HSL

7
Ecological Aspects of AHL-Mediated Bacterial Cell-Cell Signalling

7.1
Diversity and Habitats of AHL-Producing Bacteria

AHL-producing bacteria have so far only been found among the eubacteria and in particular within the alpha-, beta- and gamma-groups of proteobacteria [72]. Recently, it was reported that ethyl acetate extracts from the archaeon *Natronococcus occultus* activated the *Agrobacterium* AHL biosensor [73] implying the presence of AHLs in this organism. However, the compounds were not chemically characterized. Interestingly, all known AHL producers are not only Gram-negative but also mesophilic and aerobic or facultative anaerobic bacteria. So far, no AHL-producers have been found among strict anaerobes. AHL-producing bacteria have been isolated from humans, animals (including fish) and plants. With respect to the latter, these include pathogens such as *A. tumefaciens*, *E. carotovora* and *Pseudomonas syringae* and several symbiotic *Rhizobium* strains [74]. A study using *Pseudomonas* strains revealed that, in contrast to soil isolates, all those isolated from plants produced AHLs [75]. AHL producers can also be associated with animals such as the symbiosis of *V. fischeri* with the squid *Euprymna scolopes* and the insect pathogen *Xenorhabdus nematophilus* which lives in the intestines of the parasitic nematode *Steinernema carpocapsae* [76]. While the presence of AHLs in rumen fluid has been reported [77], no characteristic rumen bacteria have been shown to produce AHLs. Finally, AHL-producing enterobacteria have been isolated from the gut of wood-feeding termites (Philipp, unpublished).

Free-living AHL-producing bacteria have been isolated from various environments including freshwater, seawater and soil. In addition AHLs have been detected in natural habitats such as naturally occurring biofilms or bacterial aggregates on limestone rocks from a river bed [78], microbial mats from a Swiss alpine lake [79] or marine snow [80]. The presence of AHLs has also been shown in sections of biofilm-covered catheters freshly removed from patients [81] and in extracts of spoiled cold-smoked salmon [82]. AHLs have also been directly detected in the sputum of cystic fibrosis patients infected with *P. aeruginosa* and *Burkholderia cepacia* [83] and in the tissues of salmon infected with *Vibrio anguillarum* [84]. In many of these studies, the AHLs were detected using a range of different biosensor systems, mainly *Agrobacterium* and *Chro-*

mobacterium in combination with TLC. However, AHL biosensors may be activated by compounds other than AHLs, for example the diketopiperazines produced by various bacteria [20] or plant exudates, which can mimic AHL-activity [85]. The development of more advanced techniques for the detection and identification of AHLs in situ will certainly increase the knowledge of AHL-signalling in natural systems. Examples for this are AHL-detection at the single cell-level using *gfp* biosensors [38], which can be combined with flow cytometry [86].

7.2 Ecological Functions of AHLs

In laboratory systems, the function of AHLs in the producer bacteria is generally believed to mediate cellpopulation-density dependent gene regulation. In natural systems, environmental conditions are less constant and available nutrients have to be shared with a variety of other organisms. Therefore, AHL-producing bacteria will have to compete and co-operate with other species. It is not known whether QS as we understand it from laboratory studies operates in the same way under natural conditions [72]. However, in the last few years a number of intriguing observations have been made suggesting an ecological role for AHLs in natural bacterial habitats.

A major issue in microbial ecology is the development of strategies to increase the cultivation efficiency of bacteria since usually only up to 1% of bacteria in an environmental sample can be grown in culture [87]. One approach has been to incorporate QS signal molecules such as the AHLs into culture media. The addition of a mixture of different AHLs to growth media for lake water bacteria resulted in an increase in cultivation efficiency determined by the most-probable-number (MPN) method [88]; this increase was not significant compared to MPN counts without AHLs but was supported by the solvent control. The addition of C_4-HSL to growth media for MPN counts caused an increase of ca. 20% in cultivation efficiency of heterotrophic marine bacteria [89]. However, the molecular mechanisms by which AHLs exhibit general growth stimulating effects are not known. In *Nitrosomonas europaea* for example, AHLs have been shown to have a positive effect on starvation recovery [90]. Furthermore, in *Rhizobium leguminosarum*, addition of *N*-tetradecanoyl-L-homoserine lactone (C_{14}-HSL) was shown to cause increased survival upon entry into stationary phase [91]. In both examples, AHLs must induce a physiological status that results in higher cultivation efficiency upon starvation. Considering that starvation is the normal situation for bacteria in many of their habitats, this induction could be one mechanism by which AHLs stimulate growth.

Since different bacterial species can synthesise structurally similar or even identical AHLs, stimulation by AHLs produced by neighbouring species should be possible. In fact, such AHL-mediated cross-talk has been shown between *B. cepacia* and *P. aeruginosa* [92]. AHLs have also been shown to influence

bacteria that do not produce AHLs themselves. This is the case for *Salmonella* which can detect and respond to AHLs via the LuxR type regulator SdiA [93, 94]. Furthermore, the widespread distribution of diketopiperazines [20], which can also activate AHL-biosensors, supports the idea of bacterial interspecies communication as a common phenomenon.

Interestingly, AHLs can also be perceived by eukaryotes. A striking example is the attraction of zoospores from the green algae *Enteromorpha* by AHLs [95]. These zoospores exhibit chemotaxis for AHLs leading to their enhanced settlement on AHL-producing biofilms. In addition, the legume plant *Medicago trunculata* responds to AHLs as shown by proteomic analysis as well as activation of tissue-specific reporter gene fusions [96]. Furthermore, long chain AHLs such as those produced by the opportunistic pathogen *P. aeruginosa* have been shown to cause immunomodulatory and cardiovascular effects in mammalian hosts [16, 51].

7.3 AHLs as Nutrients; Metabolic and Chemical Conversions of AHLs

AHLs constitute attractive nutrients for bacteria as the homoserine lactone can be easily channelled into the amino acid pool and the acyl side chain is readily degradable via β-oxidation. The soil bacterium *Variovorax paradoxus* can grow using different AHLs as the sole source of carbon, energy and nitrogen [97]. In this organism, the degradation pathway starts with the cleavage of the acyl side chain releasing homoserine lactone and a carboxylic acid. This acid is used as carbon and energy source while the homoserine lactone can only be used as nitrogen source. Another soil isolate, *Arthrobacter*, can grow using the ring opened form of 3O,C_6-HSL as sole source of carbon and energy [98]. In addition, co-culture of *V. paradoxus* and the *Arthrobacter* isolate results in more effective degradation of AHLs and higher growth yields. The physiological basis behind the positive effect of this cooperation is still unknown.

Several strains of the *Bacillus cereus* group including various *B. thuringiensis* strains produce an enzyme which hydrolyses the homoserine lactone ring of AHLs. This lactonase, termed AiiA, is highly conserved and analysis of their deduced amino acid sequence revealed a characteristic zinc binding motif reminiscent of that present in metallo-β-lactamases [99, 100]. Expression of *aiiA* in *E. carotovora* [101] or in plants [102] significantly enhanced the resistance of the plants against *Erwinia* infection. Similarly, expression of *aiiA* in *P. aeruginosa* reduced the production of QS-regulated virulence factors such elastase, rhamnolipid, hydrogen cyanide and pyocyanin [103]. Open-ring forms of the AHLs, i.e. the corresponding *N*-acyl homoserines are generally considered biologically inactive. Consequently, the ecological function of AiiA may be the inactivation of AHL-mediated signalling systems, representing a strategy used by Gram-positive bacteria to compete against Gram-negatives. Interestingly, *A. tumefaciens* makes AHLs but also has its own growth phase-regulated AHL-lactonase AttM [104] the production of which is de-repressed in stationary

phase. Recently, an AHL-specific acylase (AiiD) has been cloned from *Ralstonia* sp. from a mixed species biofilm [105] and a related AHL acylase (PvdQ) with specificity for long chain AHLs has been reported in *P. aeruginosa* [106]. This enzyme may be involved in the turnover of endogenous AHLs. Consequently, the increasing number of metabolic conversions of AHLs found in different bacteria suggests that AHLs are common substrates found in natural environments.

AHLs are also subject to chemical decay as the lactone ring can be easily hydrolysed at alkaline pHs; C_4-HSL for example is 50% hydrolysed at pH 7 [107]. Since pH values above 7 are prevailing in many environments, the occurrence of hydrolysed AHLs should be rather common. Accordingly, the *Arthrobacter* isolate described above appears to have specialised in the degradation of hydrolysed AHLs [98]. With increasing length of the acyl side chain, the pH stability of the lactone ring also increases [107]. In addition, hydrolysed AHLs with acyl side chains of 10 or more carbon atoms show reformation of the lactone ring at neutral pH when interacting with hydrophobic surfaces.

Hypochlorite and hypobromite disinfectants have been shown to chemically degrade AHLs with 3-oxo substitutions (Scheme 3) [47]. Similarly, the brown algae *Laminaria digitata* produces a haloperoxidase which generates hypobromite and consequently can inactivate AHLs [108].

7.4 Role of QS in Biofilm Development

Biofilms are now considered to represent the natural mode of life of bacteria and are thought to be ubiquitous in nature [109]. In most cases biofilms form at the interface between a solid surface and an aqueous phase. According to the prevailing conceptual model, bacterial development proceeds through a temporal series of stages [110]. In the initial phase, bacteria attach to a surface, aggregate and then proliferate to form microcolonies. These microcolonies are hydrated structures in which bacterial cells are enmeshed in a matrix of self-produced slime, referred to as exopolymeric substances (EPS). With time, as substrate availability becomes limiting due to increased diffusion distances, growth will decrease and biofilm development will reach a steady-state. Such mature biofilms typically consist of 'towers' and 'mushrooms' of cells enmeshed in copious amounts of EPS separated by channels and interstitial voids to allow convective flow to transport nutrients to interior parts of the biofilm and remove waste products.

Biofilm formation is a major challenge for living organisms including humans, animals and marine eukaryotes [111, 112], as this sessile lifestyle of bacteria poses many problems in industrial settings, ranging from corrosion and biofouling to food contamination. In clinical microbiology, biofilms have attracted particular attention as many persistent and chronic infections, including pulmonary infections of cystic fibrosis patients, periodontitis, otitis media, biliary tract infection, endocarditis as well as the colonization of med-

ical implants are now believed to be intrinsically linked to the formation of bacterial biofilms [113]. A recent public announcement from the US National Institutes of Health stated that more than 60% of all microbial infections involve biofilms [114]. The capability of forming a biofilm within the human body is therefore considered to represent a pathogenic trait per se. Importantly, treatment of biofilm infections is particularly problematic as bacteria living in biofilms can withstand host immune responses and are markedly more tolerant to antibiotics, often exceeding the highest deliverable doses of antibiotics and thus making an efficient treatment impossible [114].

The contribution of QS to biofilm development has been extensively studied in *P. aeruginosa.* This human pathogen possesses two linked AHL-dependent QS systems: LasR/LasI and the RhlR/RhlI with their cognate QS signal molecules 3O,C_{12}-HSL and C_4-HSL respectively [115, 116]. The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two QS systems of *P. aeruginosa* are hierarchically arranged with the *las* system being at the top of this QS signalling cascade [115, 116]. The two QS circuits orchestrate the expression of a symphony of virulence factors including exoproteases, siderophores, exotoxins and several secondary metabolites and, as discussed below, participate in the development of biofilms [117–120].

The involvement of QS in the regulation of biofilm formation was originally reported for *P. aeruginosa* [120]. In this study the authors reported that a *lasI* mutant of *P. aeruginosa* only forms flat and undifferentiated biofilms when compared with the wild type, which formed characteristic microcolonies separated by water channels. This led the authors to suggest that the *las* system is required for development of the characteristic biofilm architecture. A role for AHL-mediated quorum sensing in biofilm formation has also been demonstrated for *B. cepacia* [121, 122], *Aeromonas hydrophila* [123], *Pseudomonas putida* [124]. AHL-negative mutants of *B. cepacia* and *A. hydrophila* showed defects in the late stages of biofilm development and thus were unable to form biofilms with a typical wild type structure that is comprised by 'mushroom-like' microcolonies separated by water-filled channels. The *P. putida* wild type forms very homogenous rather unstructured biofilms, while a QS mutant was shown to form structured biofilms similar to those of *B. cepacia* and *A. hydrophila*. Most interestingly, when AHL signal molecules were added to the medium the mutant biofilm lost its structure and converted into an unstructured biofilm that was similar to that formed by the wild type [124].

Several examples support the view that QS in fact plays a role in natural biofilms. By the use of AHL biosensors it has been shown that AHL molecules are present in natural biofilms growing on submerged rocks in a river as well as in biofilms formed on urethal catheters [39, 81]. Direct chemical evidence for the presence of high concentrations of AHL signal molecules in *P. aeruginosa* biofilms has been presented by Charlton et al. [48], who reported that the concentration of 3O,C_{12}-HSL is approximately 45-fold higher in biofilms relative to suspended cultures.

8
Inhibition of Quorum Sensing as an Anti-infective Strategy

Given that the QS systems of various bacterial pathogens are central players in the elaboration of virulence, they represent highly attractive targets for the development of novel therapeutic agents. Importantly, inhibition of QS specifically abolishes expression of pathogenic traits but does not affect bacterial growth. However, without production of virulence factors the bacteria can no longer adapt to the host environment and consequently are cleared by innate host defences.

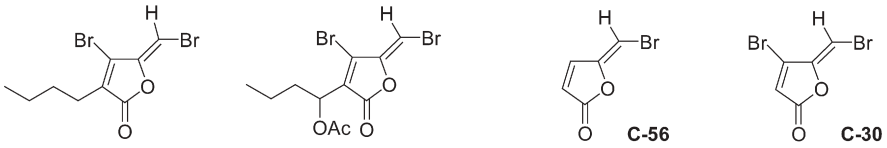
Disruption of the cell-cell signalling cascade could be accomplished either by blockade of AHL signal synthesis, AHL signal molecule degradation, or inhibition of AHL signal reception. At present the most promising strategy for successful disruption of QS appears to be the blocking of QS signal reception by an antagonist capable of competing or interfering with the cognate AHL signal for binding to the LuxR-type receptor. Competitive inhibitors are likely to be structurally related to the cognate AHL signal in order to bind to and occupy the AHL binding-site but without activating the LuxR-type protein receptor. Non-competitive inhibitors may show little or no structural similarity to AHL signals as these molecules are likely to bind to different sites on the relevant receptor protein.

Previous work undertaken to demonstrate the feasibility of AHL-analogues to activate or inhibit the QS circuits of various bacteria, suggested that a tight structure-function relationship exists and thus only AHL analogues with conservative changes were capable of antagonizing cognate AHL signals. From studies in which the acyl side chains of various analogues were modified it was concluded that chain length plays a critical role for biological activity. For *E. carotovora* increasing the acyl side chain of the cognate signal 3O,C_6-HSL by one methylene unit reduced activity by 50%, whereas a two unit's extension reduced activity by 90%. Decreasing the chain length by one methylene unit decreased activity to 10% [15]. A study of the *P. aeruginosa* LasR protein receptor revealed that the fully extended acyl chain geometry is necessary for activation as constrained analogues locked into different conformations showed no activity. This suggested that the minimum acyl side chain length determined by the cognate AHL signal is required for binding to LuxR-type proteins [125]. The flexibility of the acyl side chain also appears to be important for binding to LuxR-type proteins. For instance, reduction of the chain rotation by introduction of an unsaturated bond close to the amide linkage almost completely abolished binding [15, 41, 56, 64]. In accordance with this hypothesis, no natural AHL signal molecule with a 2,3 unsaturated bond has so far been identified.

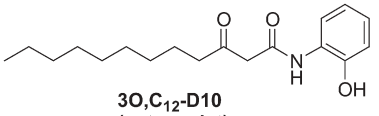
The substitution at the 3-position in the acyl side chains is important for the agonistic activity of AHLs, but so far no clear prediction on the antagonistic effect of a modification at this position can be made [57]. Recently however, the biological impact of chain length variation and hydroxylation of C_6-HSL was investigated [70]. Based on the halofuranone **26** from *Delisea pulchra*, a small

combinatorial library of ring 4-hydroxy and 5-hydroxymethyl derivatives of C_6-HSL and their corresponding ethyl carbamates were synthesized (Schemes 12 and 13). Interestingly, only the molecules carrying the free hydroxyl groups proved active. Thus, both the *cis* 4-hydroxy and the *trans* 4-hydroxy analogues of 3O,C_6-HSL were able to activate a LuxR-based QS system. Indeed, the *cis* 4-hydroxy homoserine lactone was a more potent activator than C_6-HSL. Among the C_6-HSL derivates tested, only the *cis* 5-hydroxymethyl homoserine lactones were able to antagonize LuxR.

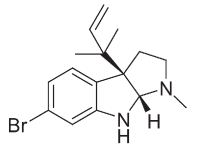
As noted above, the chirality of the homoserine lactone moiety is crucial to biological activity. Natural AHL signals are L-isomers whereas D-isomers generally are devoid of biological activity [15, 17, 39]. Importantly, L-isomer activities were not inhibited by D-isomers indicating that the D-isomers do not bind to the LuxR-type receptor [17]. Conversion of the homoserine lactone ring to a lactam ring results in a molecule without agonistic or antagonistic properties [15, 41]. Interestingly, switching to a homoserine thiolactone ring appears permissible for several quorum sensing systems [39, 41, 57]. The effects of changes in the composition and size of the homoserine lactone ring with either a 3O,C_{12} or a C_4 acyl side chain was recently investigated by Smith et al. [126]. From the screening and testing of combinatorial libraries it was concluded that ring size, the keto group adjacent to the amino group and the presence of saturated carbons in the ring strongly affected the inhibitory activity of the molecule on the *las* system of *P. aeruginosa*. Only slight variations in these key positions, such as a change from a saturated ring to an aromatic benzene ring (Fig. 5) transformed the molecule from an agonist into an antagonist. On the basis of these data it was suggested that the presence of an aromatic ring interferes with the ability of the antagonist to activate LasR. Interestingly, halogenated furanones such as **28**, which are natural QS inhibitors, have five-membered rings containing unsaturated bonds [68].



Furanones from *D. pulchra* and their synthetic derivatives C-56 and C-30



3O,C_{12}-D10 (antagonist)



Brominated alkaloids from *F. foliacea*

Fig. 5 Structures of some inhibitors of AHL-dependent quorum sensing

8.1 Naturally Occurring Quorum Sensing Blockers

Evidence has accumulated that inhibition of QS is a strategy commonly adopted by eukaryotic organisms to combat potentially pathogenic bacteria. The production of AHL antagonists has been demonstrated for the marine red alga *Delisea pulchra* [68], higher plants [85] as well as the animal *Flustra foliacea* [127]. Compounds with QS-inhibiting activity have been obtained from *D. pulchra* and *F. foliacea* (Fig. 5) while the structures of the plant-derived substances have yet to be elucidated.

The first reported example of a eukaryotic organism producing metabolites that specifically interfere with bacterial QS signalling was the Australian macroalgae *D. pulchra* [68]. It produces a range of halogenated furanone compounds [128], which display antifouling and antimicrobial properties [128–130]. This particular alga originally attracted the attention of marine biologists because it is devoid of extensive microbial surface colonization, i.e. biofouling, unlike other plants in the same environment. Biofouling is primarily caused by marine invertebrates and plants, but bacterial biofilms are believed to be essential for providing the initial conditioning biofilm to which other marine organisms attach [95, 131].

The *D. pulchra* furanone compounds generally consist of a furan ring structure with a substituted alkyl chain at the C-3 position and a bromine substitution at the C-4 position (Fig. 5). The substituent at the C-5 position may vary in terms of side chain structure. The natural furanones are halogenated at various positions by bromine, iodine, or chlorine [128]. *D. pulchra* produces at least 30 different halogenated furanones which are stored in specialized vesicles and are released at the surface of the thallus at concentrations ranging from 1 to 100 ng/cm^2 [132]. Field experiments have demonstrated that the surface concentration of furanones is inversely correlated with the degree of colonization by marine bacteria [133].

Givskov et al. [68] hypothesized that furanones of *D. pulchra* constitute a specific means of eukaryotic interference with bacterial QS. Extensive experimental evidence in support of this model includes the observations that furanones repress AHL-dependent expression of *V. fischeri* bioluminescence [69], displace AHL signal molecules from LuxR [69], inhibit AHL-controlled virulence factor production [117], surface motility, biofilm formation and colonization of *S. liquefaciens* [68], accelerate the degradation of the LuxR receptor [69] and finally inhibit the QS-controlled luminescence and in vivo virulence of the black tiger prawn pathogen *V. harveyi* [134] and the virulence of *E. carotovora* [135]. In addition, work in progress has identified a number of food relevant bacteria [136] which employ QS to control the process of food deterioration. Recent results suggest that these functions can also be controlled by furanone compounds (Givskov, unpublished).

The natural furanone compounds exhibited little or no effect on the QS systems of *P. aeruginosa* and so have been chemically modified and screened for

increased efficacy [70]. Two compounds, C-56 and C-30 (Fig. 5), were shown to repress QS and hence virulence factor production in *P. aeruginosa* [137]. Compared with growing cells, biofilm bacteria exhibit an increased tolerance to antibiotic treatment [113, 114, 138–140], and it has been proposed that diffusion barriers and the special physiological condition of the cells may contribute to this phenomenon. Hence, synthetic QS antagonists that function well on planktonic cells might be less efficient on biofilm cells. The construction of novel AHL biosensors which allowed for microscopic analysis of QS and its inhibition in biofilms made estimates of penetration efficacies and half-lives of the QS antagonists possible [137]. Surprisingly C-56 and C-30 were equally active on biofilm bacteria compared with planktonic cells and the compounds markedly affected biofilm development, resistance and persistence [117, 137]. It is well established that classical dose regimens for the treatment of *P. aeruginosa* infections requires 100- to 1000-fold higher concentrations of antibiotics to eradicate biofilm bacteria when compared to their growing, planktonic counterparts [138–140]. Most interestingly, the furanones exhibited synergistic effects with tobramycin, an aminoglycoside antibiotic routinely used in treatment of cystic fibrosis patients, as this antibiotic eradicated biofilm cells much more efficiently when the biofilm was pre-treated with furanones [117].

Although our studies of agonist and antagonist activities have generated substantial knowledge of AHL structure-function relationships, the value of these data for the rational design of potent QS inhibitors is not clear. Currently, furanone C-30 (which exhibits the least amount of structural homology with the cognate AHL signal molecules) shows the broadest spectrum of antagonistic activity. C-30 has proved to be highly specific for blocking the expression of QS-controlled genes in *P. aeruginosa* as determined using transcriptomics [117].

9 Concluding Remarks

QS enables bacterial populations to coordinate their behaviour and facilitate adaptation to changing environmental conditions such as those encountered during the infection of the tissues of higher organisms. Moreover, QS may confer on bacteria a mechanism for minimizing host responses by delaying the production of tissue-damaging virulence factors until sufficient bacterial cells have been amassed to overwhelm host defence mechanisms and to establish a successful infection. However, AHL production may disclose the presence of the bacterium to other organisms whether prokaryotic or eukaryotic. The central role of QS systems in controlling the expression of host-associated phenotypes including virulence factor production and biofilm development and the fact that they function by means of low molecular weight signal molecules substances makes QS an attractive therapeutic target for plant, animal and human infections.

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