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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 at springerlink.com

Library of Congress Control Number: 2007927309

ISSN 0340-1022
ISBN 978-3-540-72509-1 Springer Berlin Heidelberg New York
DOI 10.1007/978-3-540-72510-7

Springer is a part of Springer Science+Business Media

springer.com

Cover design: WMXDesign GmbH, Heidelberg
Typesetting and Production: LE-TEX Jelonek, Schmidt & Vöckler GbR, Leipzig

Printed on acid-free paper 02/3180 YL – 5 4 3 2 1 0

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Preface

The modern billion-dollar drug-discovery process strongly relies on both high-throughput synthesis and screening methods. Whereas the latter is based on molecular biological methods, the efficient and reliable generation of compound collections often makes use of combinatorial chemistry. Discovered in the 1980s, this methodology was explored extensively in the 1990s by groups in academia and in industry. Without any doubt, combinatorial chemistry changed the whole drug-discovery process and found many applications in crop science and the material sciences.

However, since its implementation, solution- and solid-phase techniques have been competing with each other, and although many companies started their combinatorial chemistry program with solid-phase techniques, solution-phase combinatorial methods have taken over and now account for approximately 25% of all combinatorial efforts.

The syntheses of complex, non-polymeric structures, discovered in the 1960s by the late Bruce Merrifield, was largely ignored in the context of solid supports, mainly due to the fact that appropriate synthesis techniques were not available.

Since solid-phase chemical methodology strongly differs from traditional solution-phase chemistry, two chapters deal with this topic. The Bräse group (Jung, Wiehn, Bräse) gives an overview of multifunctional linkers, which can be used for the generation of diversity-oriented collections, simply by cleavage from resins.

Still in its infancy, solid-phase reactions employ "simple" amide chemistry in most cases due to their high-yielding, reliable protocols. Ljungdahl, Bromfield, and Kann address solid-phase organometallic chemistry, which is now one of the great challenges in reliable solid-phase organic synthesis.

The next four chapters address the construction of designed and native complex structures, such as polyamines (Hahn and Schepers), natural products (Mentel and Breinbauer) and peptides, with a focus on identification of bioactive hormone structures (Haack and Beck-Sickinger). Furthermore, the automated synthesis of carbohydrates is addressed in detail by Castagner and Seeberger.

Finally, Winssinger, Pianowski, Debaen give an overview of array techniques that are suitable for solid-phase chemistry.

In this volume, state-of-the-art solid-phase synthesis is presented from different angles. Ranging from methodology development to application in the synthesis of complex native and designed structures, a complete overview is presented.

We are confident that addressing the fascinating interface between chemistry and biology is only possible by innovative methods in both disciplines. Combinatorial chemistry is surely one of these.

The editor thanks the editorial staff of *Topics in Current Chemistry*, in particular Mrs. Kollmar-Thoni and Dr. Marion Hertel for their professional support.

Karlsruhe, April 2007 Stefan Bräse

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Volume Editor: Fumio Toda
ISBN: 978-3-540-22982-7

Top Curr Chem (2007) 278: 1–88
DOI 10.1007/128_2007_118

Published online: 11 April 2007

Multifunctional Linkers for Combinatorial Solid Phase Synthesis

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Abstract This review covers recent results in the area of multifunctional linkers for solid phase synthesis during the period 2000–2006.

Keywords Diversity-oriented synthesis · Linkers · Solid phase synthesis

Abbreviations

AA	amino acid
Ac	acetyl
acac	acetylacetonate
AIBN	azobisisobutyronitril
AM	aminomethyl
AMB	α-methyl benzyl
BAL	backbone amide linker
9-BBN	9-borabicyclo[3.3.1]nonane
BHA	benzhydrylamine
BME	β-mercapto ethanol
Bn	benzyl
Boc	*t*-butyloxycarbonyl
BOP	benzotriazole-1-(yloxy) tris-(dimethylamino) phosphonium hexafluorophosphate
BPO	benzoylperoxide
BSA	bovine serum albumin
BTC	*bis*-trichloromethyl carbonate
CAN	cerium ammonium nitrate
Cbz	carbobenzyloxy
CDI	carbonyl diimidazole
CSA	camphor sulfonic acid
DBU	diaza(1,3)bicyclo[5.4.0]undecane
DCC	dicyclohexyl carbodiimide
DCH	1,3-dichloro-5,5-dimethylhydantoin
DDQ	dichlorodicyanobenzoquinone
DEAD	diethylazodicarboxylate

DEAM	diethanolaminomethyl
DEPEC	diethyl phosphorocyanidate
DIBAL	diisobutylaluminumhydride
DIC	diisopropyl carbodiimide
DIEA	diisopropylethylamine
DMAP	diemthylaminopyridine
DMF	dimethylformamide
DMPU	*N*,*N*′-dimethylpropylene urea
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	desoxyribonucleic acid
DOS	diversity-oriented synthesis
dppe	1,3-bis(diphenylphosphino)ethane
dppf	1,3-bis(diphenylphosphino)ferrocene
dppp	1,3-bis(diphenylphosphino)propane
DSC	*N*,*N*-disuccinimidyl carbonate
DVB	divinylbenzene
EDCI	*N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimid
Fmoc	9-fluorenylmethyloxycarbonyl
FMP	4-formyl-3-(methoxyphenoxy)methyl-PS
Gly	glycin
HASC	heteroatom-substituted carbonyl linker
HAL	hypersensitive acid-labile
HFIP	hexafluoroisopropanol
HMDS	hexamethyldisiloxane
HMPA	hexamethylphosphoramide
HMPB	4-hydroxymethyl-3-methoxyphenoxy-butyric acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBT	1-hydroxybenzotriazol
LDA	lithiumdiisopropylamide
MAMP	Merrifield α-methoxyphenyl
MBHA	methylbenzhydrylamine
*m*CPBA	*m*-chlorperbenzoic acid
NBS	*N*-bromosuccinimide
NCS	*N*-chlorosuccinimide
NMM	*N*-methyl morpholine
NMP	*N*-methyl pyrrolidone
NPCF	4-nitrophenylchloroformate
NpSSMpact	2-methoxy-5-[2-((2-nitrophenyl)dithio]-1-oxopropyl)phenylacetic acid
Nu	nucleophile
PAC	peptide acid linker
PAM	phenylacetamidomethyl
PEG	polyethylene glycol
PEGA	polyethylene glycolpoly-(*N*,*N*-dimethyl-acrylamide)
PFS	perfluoroalkylsulfonyl
PPF	1,1′-bis(diphenylphosphino)ferrocene
PPTS	*p*-pyridinumtoluene sulfonic acid
PNA	peptide nucleic acid
PS	polystyrene
PTMSEL	(2-phenyl-2-trimethylsilyl)ethyl
Py	pyridine

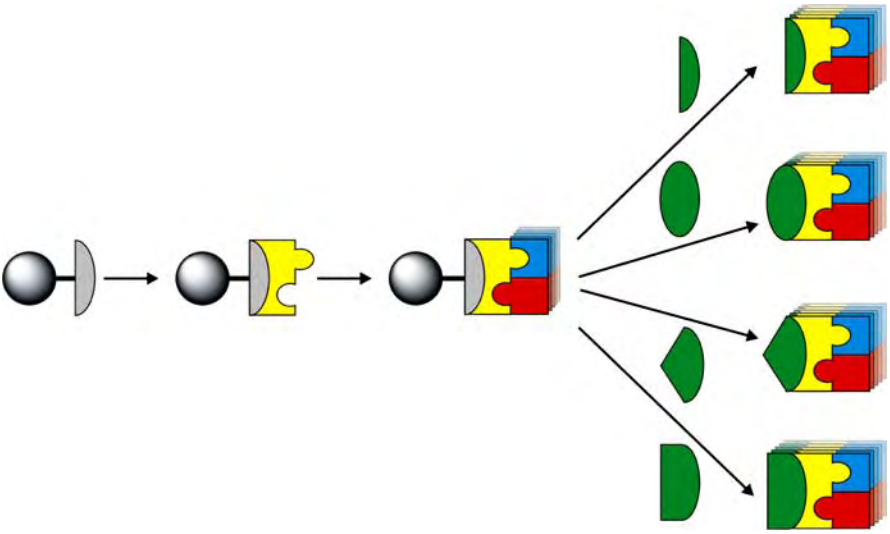
PyBrOP	bromo-tris-pyrrolidino phosphoniumhexafluorophosphate
RAM	Rink amide
RCM	ring-closing metathesis
RRTR	resin-to-resin transfer reaction
SAC	silyl acid
SASRIN	super acid sensitive resin
SCAL	safety catch acid labile
SEC	2-alkylsulfonylethyl carbamate
SPPS	solid phase peptide synthesis
TBAF	tetrabutylammoniumfluoride
TBDPS	*t*-butyldiphenylsilyl
TBTU	*O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
TEA	triethylamine
THF	tetrahydrofuran
Tf	trifluormethylsulfonyl
TFA	trifluoro acetic acid
TFAA	trifluoro acetic acid anhydride
THP	tetrahydropyran
TMEDA	tetramethylethylenediamine
TMG	2-*t*-butyl-1,1,3,3-tetramethylguanidine
TMS	trimethylsilyl
Trt	trityl
XAL	xanthenylamide linker
XAN	9-xanthenyl linker
XPHOS	2-dicyclohexylphosphino-2′,4′,6′-triisopropyl-biphenyl

1 Introduction

The advent of combinatorial chemistry being implemented in the modern drug discovery process in the 1990s [1] has reinitiated the use of solid phase synthesis originally developed by the late Bruce Merrifield [2]. While in the early stages of solid phase synthesis, first peptides and later nucleic acids were favorably synthesized using this technique due to the ease of automation [3], small molecular entities obeying the Lipinski rules have been prepared in the last decades with the notable exception by Frechet and others [4, 5]. In particular, the invention of the split-and-mix-technique by Furka [6] and later the technological platforms derived from this, e.g. the IRORI techniques [7], triggered the design and preparation of large compound libraries with more than 2 000 000 compounds [8]. Diversity-oriented synthesis (DOS), originally proposed by S. L. Schreiber [9, 10], is today used by many laboratories both in academia and industry. In particular solid phase synthesis has served as a technology platform and allows the rapid assembly of building blocks to generate quite complex structures in few synthetic steps. A crucial point in the design of compound libraries is the careful choice of the appropriate

linker attaching the molecule to the solid support [11, 12]. Linkers do not only serve as the point of attachment, they also control the chemistry being allowed during the assembly stage and importantly are directing the functional group being generated upon cleavage. While peptide synthesis requires more or less the detachment of carboxylic acids and amides, diversity-oriented synthesis strongly relies on the cleavage of various functional groups in order to avoid constraints. Thus, a high number of various linkers have been prepared and discussed in a number of reviews.

Linkers allowing the cleavage of one certain functional group have been named mono-functional linkers [13]. However, an attachment being cleavable to generate more than one functional group is named a multifunctional linker [14–16] (Scheme 1).



Scheme 1 Solid phase synthesis and multifunctional cleavage

We will define *multifunctional linkers as attachments which allow the generation of more than one functional group upon cleavage from a solid support either with or without implementation of building blocks.*

Linkers which allow cleavage of reactive functional groups that in turn can be reacted with added building blocks in a one-pot method are also called multifunctional.

In this review we will discuss the multifunctional linkers in terms of assembly on solid supports, stability towards reaction conditions, and finally the issue of introduction of multifunctionality.

2
Ester-Type Linkers

One of the oldest and up to now most important multifunctional linker classes is the ester-type linker. Ester linkers are of the general structure A or B (Fig. 1). Cleavage methods are similar for both types of linkage but the resulting molecules end up with different functional groups.

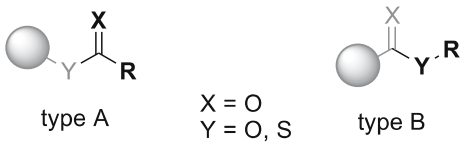


Fig. 1 General structures of esters type A and esters type B

2.1
Esters Type A

Esters of general structure A are the most common in solid phase synthesis due to the commercial availability of many building blocks such as carboxylic acids, acid chlorides and anhydrides. The synthetic route to an ester linkage is quite simple, fast and can often be performed quantitatively.

Figure 2 presents several ester linkers differing in linker length, aromatic substitution and sterical hindrance. Modifications of the crude linker core allow the modulation of the linker nature to the desired cleavage conditions. The influence of even smallest variations is given for different trityl linkers which require ester cleavage conditions from 1% TFA for a methoxy trityl linker to 100% TFA for the corresponding unsubstituted trityl linker. Linker modification can influence directly the multifunctionality of the system if the modification causes sterical hindrance and prevents nucleophilic attack on the carbonyl group (e.g. hindered handle) [17]. The result of linker modification is therefore a change of reactivity that may limit the theoretically possible cleavage protocols.

Some commercially available linker types which are important for ester linkage are presented in Fig. 2, others recently synthesized to perform solid phase synthesis for special purposes are, for example, the SAC linker [18, 19], PAC and methoxy-PAC linker [20–22] as well as various trityl linkers [23–29] (trityl-, 2-chlorotrityl, 4-methyltrityl, 4-methoxytrityl, 4-fluoro(chloro)-tritylcarboxyl, 4-cyanotrityl).

Scheme 2 presents the most applied cleavage strategies for esters of type A (carboxylic and sulfur esters) with an alkyl or an aryl substrate directly bound to the ester linkage. The bandwidth of products obtainable from carboxylic esters includes esters **14**, acids **13**, primary and tertiary alcohols

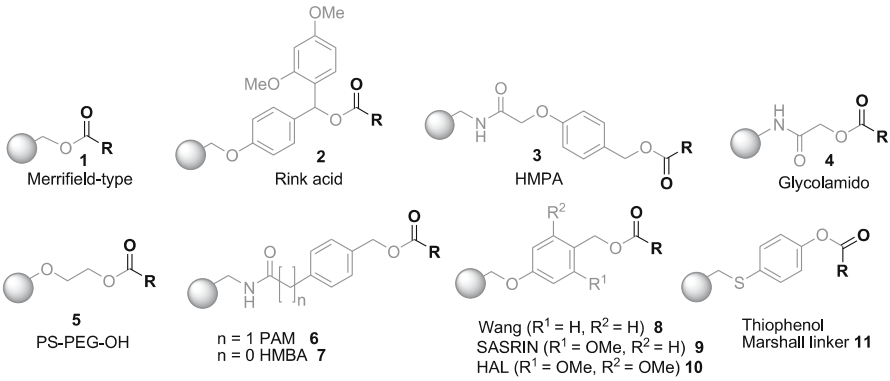
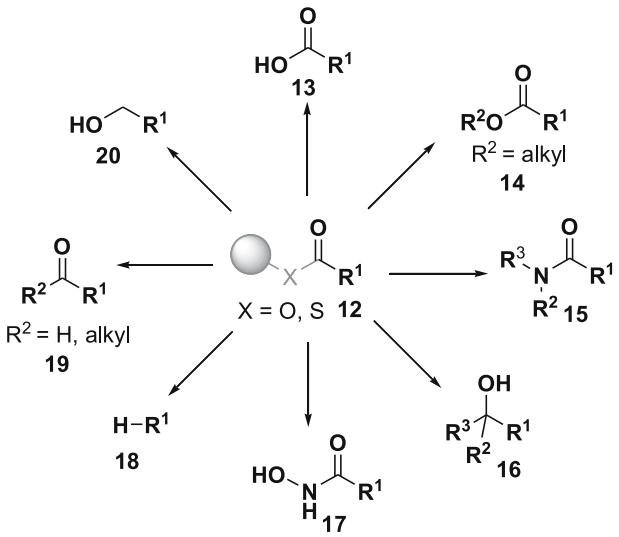


Fig. 2 Commercially available oxygen ester linkers (substrate added)

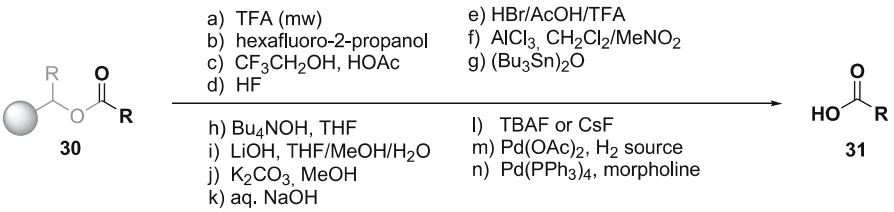


Scheme 2 Cleavage possibilities of ester and thioester linkers

20/16, aldehydes or ketones **19**, amides **15**, hydroxamic acids **17** and alkanes **18** as a result of the lability of esters towards acids, bases, nucleophiles and reducing agents [30–58].

In general, thioester linkers can be called multifunctional as carboxylic ester linkers. They are discussed within this work because cleavage strategies and products differ only slightly from those of esters. In fact, thioester linkers disclose possibilities where ester linkers traditionally fail.

In 1995, the group of Ley showed the utility of a simple ester linker **25** for multifunctional cleavage [59]. While reductive cleavage has always been performed with DIBAL-H and acidic cleavage with TFA, nucleophilic cleavage

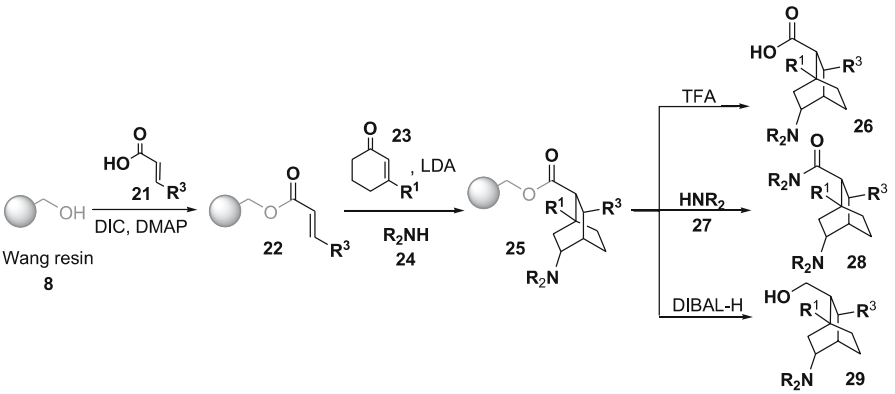


Scheme 3 Acidic, N-nucleophilic and reductive cleavage of ester linkers

with primary and secondary amines **27** has been proven by the synthesis of several examples (**28,** Scheme 3).

2.1.1 Cleavage Yielding Carboxylic Acids

Cleavage of esters **30** to give carboxylic acids **31** can be achieved under acidic conditions [30–41], basic conditions [42–50] and—for special linker types—by irradiation and/or activation of safety-catch linkers and other methods [51–58] (Scheme 4). The best-known method is the saponification with alkalimetal hydroxides or with strong acids like TFA or HBr.



Scheme 4 Cleavage of esters (**a**–**g**) acidic cleavage, (**h**–**k**) basic cleavage, (**l**) fluorous cleavage, (**m**) hydrogenation, (**n**) Pd-catalyzed cleavage

Especially in the last few years variations of the acidic method have been developed. Kappe et al. cleaved ester linkers with TFA/CH_2Cl_2 supported by microwave irradiation [33] and Gavriluk et al. used a solution of 20% hexafluoro-2-propanol in CH_2Cl_2 to release peptidic structures from 2-chlorotrityl ester resins [32]. Other types of ester linkers like the fluorine-derived linkers of Albericio et al. can be cleaved with morpholine/DMF or piperidine/DMF mixtures [60, 61] and there exist also silyl-derived linker

types that can be cleaved to give acids by the addition of fluoride nucleophiles like TBAF or CsF. This cleavage strategy is described by Turner et al. [19] as well as by Ramage et al. [62] and Chao et al. [18] [SAC (silyl acid linker)-like linkers] and was recently picked up by Kunz and coworkers [63]. The latter ones introduced the TBAF-labile PTMSEL linker that can be cleaved using nearly neutral conditions (see also Horst et al. [64] and Waldmann et al. [65]).

The safety-catch linker **34** developed by Balasubramanian and Lee in 1999 combines preactivation of the linker and cleavage through irradiation (photocleavable safety-catch linker) [66–69]. Preactivation of the linker means removal of the dithiane protection can be performed oxidatively by addition of H_5IO_6 or by addition of MeOTf (Scheme 5) while subsequent cleavage of the linker occurs with irradiation of 350 nm. The chemical stability of the linker has been tested under reductive/oxidative, basic/acidic and nucleophilic conditions as well as in ester coupling and carbon–carbon bond-forming reactions. The Balasubramanian linker is only one example of an ester linker class called photocleavable linkers. Some of them are summarized in Fig. 3 [70–77].

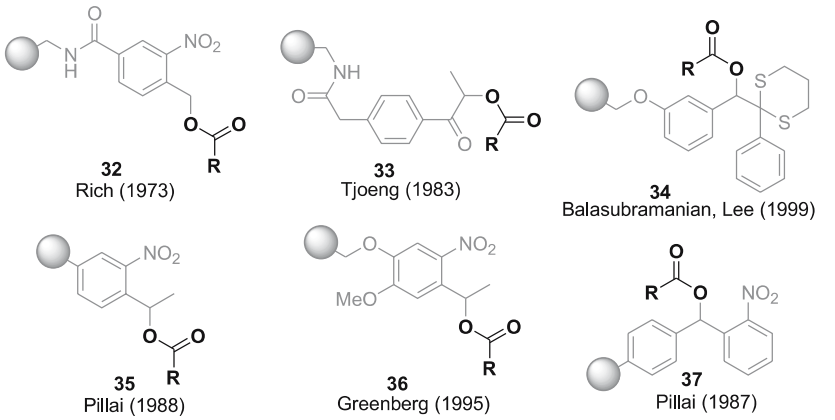
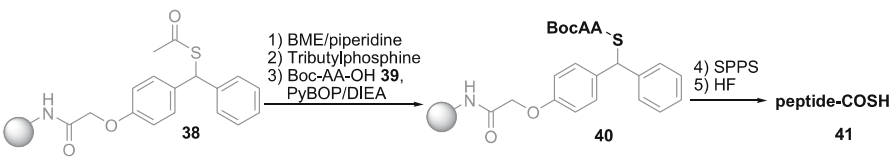


Fig. 3 Photolabile carboxylic acid linkers



Scheme 5 Synthesis of thioacids via acidic cleavage from thioester resins

Beyond those often-used linkage strategies, some more seldom carboxylic acid-forming variations should be mentioned. A quinoline linker system cleavable via two mild reactions was developed for peptide synthesis in 1999.

The approach of Wang et al. [78] uses the redox-sensitive nature of quinoline structures that form dihydroquinolines in the presence of mild reducing agents and cyclize after treatment with TBAF. Another special linker type is the class of allylic linkers because they can be cleaved by Pd-catalysis via allyl transfer to give carboxylic acids [79].

Thioesters are known to be cleaved by addition of 20% 1 M aqueous NaOH in dioxane to give carboxylic acids and thio-Wang resins [80] but they have also been cleaved under acidic conditions. An approach of Villain et al. describes the synthesis of thioacids by acidic cleavage of thioesters on solid support [81]. This procedure depends on a special type of linker (**38**) which is synthesized via addition of a mercapto-benzophenone derivative to aminomethyl-polystyrene. Cleavage of the resulting C-terminal thioacids **41** is induced by addition of HF (Scheme 5, for another application of thioester linkers for thioacid synthesis see Canne et al. [82]).

2.1.2 Cleavage Yielding Ketones, Aldehydes and Alcohols

Aldehydes and alcohols are obtainable from ester linkers by a few reducing methods (for a summary of linkers see Fig. 4). Martinez et al. [83] presented a methodology to cleave peptidic structures in form of their aldehydes by treating resin **49** according to a method of Zlatoidsky [84] with $LiAl(OtBu)_3H$ in THF (Scheme 6). The resulting aldehydes **50** could be isolated in 75% yield whereas over-reduction could not be fully prevented. The corresponding alcohols were formed in 25% yield.

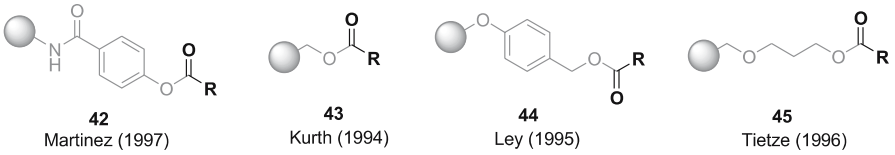
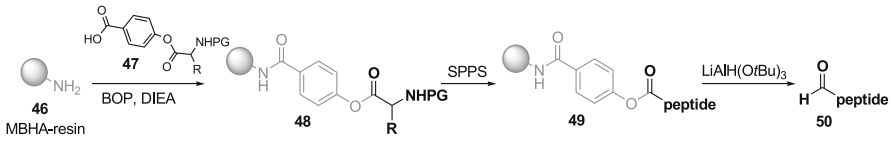


Fig. 4 Linkers for reductive cleavage to aldehydes and alcohols

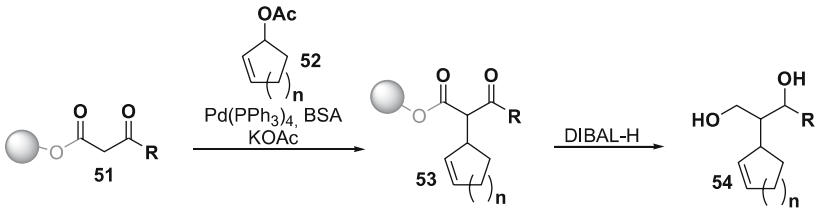


Scheme 6 Reductive cleavage yielding aldehydes

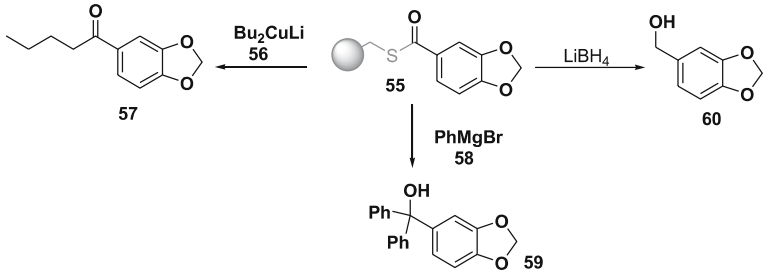
To our knowledge the synthetic route described in Scheme 6 is the only one successfully applied for the cleavage of esters forming aldehydes on solid

supports but there are other methods to synthesize aldehydes from thioesters (Scheme 9).

The reduction of esters to alcohols has been performed by Ley et al. [85], Kurth et al. [86] and Tietze et al. [87] using DIBAL-H as a hydride source (Scheme 7). There are no demands regarding the linker unit, i.e., esters directly bound to Merrifield resin can be cleaved.



Scheme 7 Reductive cleavage of esters yielding alcohols



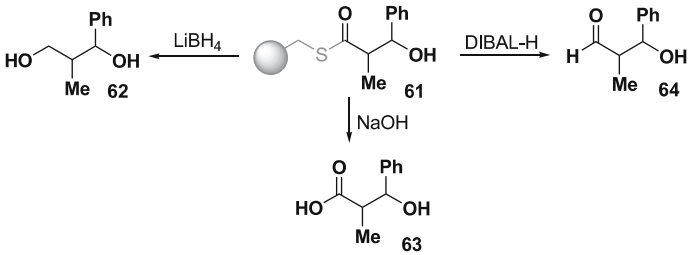
Scheme 8 Cleavage of thioester linker yielding ketones, primary and tertiary alcohols

Another possibility to release alcohols from ester resins is cleavage by carbon nucleophiles. Chandrasekhar et al. [88] proved the utility of diverse Grignard reagents for the cleavage of aliphatic as well as aromatic esters directly bound to Merrifield resin. Reactions were performed in THF/Et_2O with three equivalents of Grignard reagent and furnished products in 65–88% yield.

To our knowledge no possibility exists to cleave ester linkers with carbon nucleophiles to give ketones. In contrast, thioester linkers can be applied for these purposes as shown by Bradley et al. [89]. The thioester **55** in Scheme 8 was prepared easily by addition of an arylthioamide and sodium iodide to Merrifield resin in aqueous DMF. It was shown that thioesters without the need of special linker demands can be transformed into ketones **57** as well as into primary (**60**) and tertiary alcohols (**59**). The addition of Grignard reagents to thioesters gives tertiary alcohols whereas the addition of C-nucleophiles via organocuprates provides ketones in 53% yield. These results differ from those found by Vlattas et al. in 1997 who observed the

formation of ketones by treatment of an alkyl thioester bound to BHA resin with Grignard reagents. Remarkably, the synthesis of ketones on BHA-resin derivatives gave no formation of tertiary alcohols [90].

Earlier works on thioester reduction were performed by Kobayashi et al. on aliphatic thioesters [80, 91–93]. Thioesters **61** have been synthesized by addition of potassium thioacetate to Merrifield resin in DMF and following $LiBH_4$-reduction to the thiol. The group could selectively reduce alkyl thioesters **61** to give alcohols **62** by the addition of $LiBH_4$ and was successful in the reduction to aldehydes **64** via DIBAL-H (Scheme 9). To prove diversity potential of the linker, also hydrolysis to the corresponding carboxylic acid **63** has been performed.



Scheme 9 Synthesis of aldehydes from thioester resins

2.1.3 Traceless Cleavage

Traceless cleavage is a possibility to completely remove ester linkages from the substrate. For these purposes special linkers are needed. Traceless cleavage resulting in the formation of an aliphatic C – H bond includes a decarboxylation step initiated via an acid-catalyzed mechanism. Since Patchornik and Kraus described the decarboxylative cleavage from an ester for the first time [94], many other groups, among them Ganesan et al. [95, 96], Hoeg-Jensen et al. [97], Zaragoza [98], Abell et al. [99] and Kappe et al. [100] (Fig. 5), used this concept to perform cleavage to non-functionalized compounds.

Fig. 5 Linkers for traceless ester cleavage

Ganesan et al. presented in 1998 a traceless approach on solid phase synthesis of 4-hydroxyquinoline-2-(1*H*)-ones using the β-ketonitrile linker **66** that was generated within one step from Wang resin by addition of cyanoacetic acid and peptide coupling reagents [95]. Alkylation of the cyanoacetic ester can be performed using either carboxylic acids—which have to be activated in situ by DEPEC (diethyl phosphorocyanidate) and base—or using acyl chlorides or anhydrides. Cleavage of the target compounds is achieved by treatment with 70% TFA/triethylsilane/CH_2Cl_2. The same linker system has been used before by Zaragoza to synthesize cyanoacetamidines via ketene aminals on solid phase [98].

2.1.4 Cleavage Yielding Primary and Secondary Amides

The formation of amides from resin-bound esters is besides cleavage to give carboxylic acids/esters the most used cleavage procedure.

The usefulness of amines for cleavage of ester linkers depends strongly on the nature of the linker. Esters directly connected to Merrifield resin for example are rather unreactive towards aminolysis and even Wang linkers as well as SASRIN linkers afford special conditions like reactions under pressure. It can be stated in general that electron-withdrawing groups at aryl-containing linkers (e.g. HMBA) enhance cleavage from the resin and electron-donating groups decrease the cleavage rate. Besides, the nucleophilic cleavage depends on the nature of the used amine. Sterical aspects (bulky residues vs. linear aliphatic residues) are important as well as the grade of substitution. Ammonia mostly gives good yields whereas primary amines show lower reactivity and secondary amines require harsh conditions or show no conversion.

Because of the relation between linker structure and aminolysis stability of the ester linkers, a lot of—more or less similar—ester linkers have been developed to reach easy release and compatibility with all reaction steps (Fig. 6, [101–109]). Some stability data for selected nucleophiles are available: stabilities towards morpholine for example reach from resins with half-life times of 0.17 h to resins with half-life times of 50 h and more [110].

To overcome these problems some safety-catch linkers were developed by the groups of, for example, Merrifield [111], Cowell and Jones [112] as well as Main [110]. All studied *o*-hydroxy-phenyl esters because of their moderate stability concerning reactions with nucleophiles in their protected form and because of their low stability towards nucleophiles in the unprotected form (phenol-derivatives). These researches confirm former results describing on the one hand low reactivity concerning sterically hindered ester linkers and on the other hand high reactivity for arylesters bearing an *o*-hydroxy-substituent that supports nucleophilic cleavage via anchimeric as-

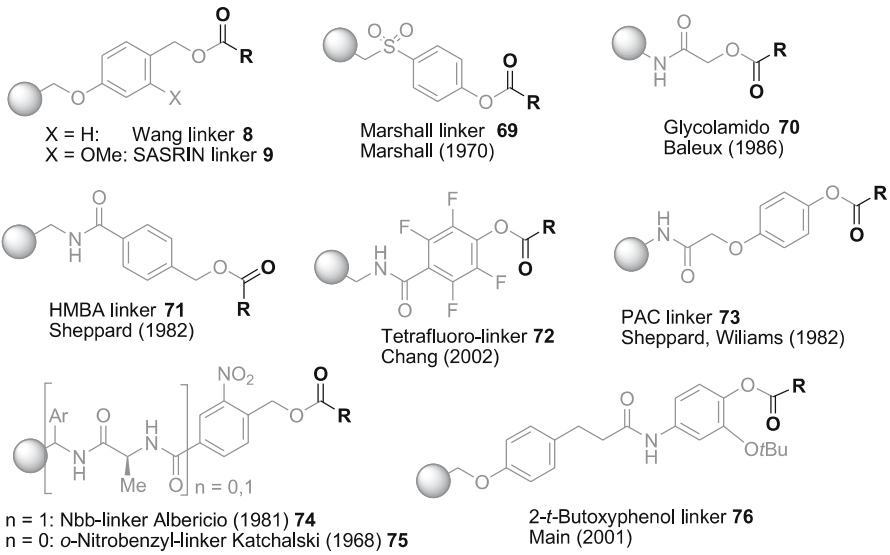
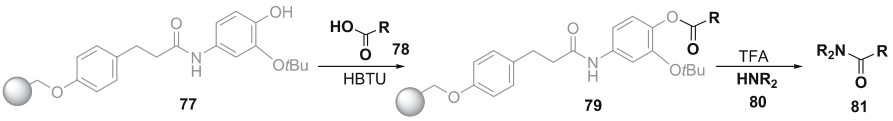


Fig. 6 Ester resins cleavable by aminolysis



Scheme 10 Safety-catch ester linker for amide synthesis

sistance. Activation of the safety-catch linker **77** is achieved by cleavage of the *t*-butylether with TFA (Scheme 10) [110].

There are some thioesters that can be used for release of amides via aminolysis developed by Vlattas et al. in 1997 (Fig. 7) [90].

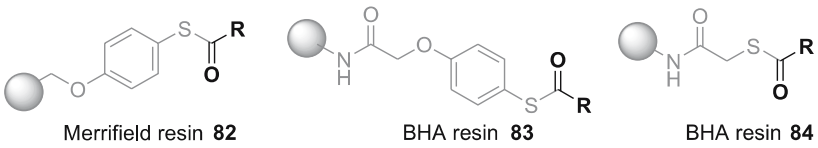
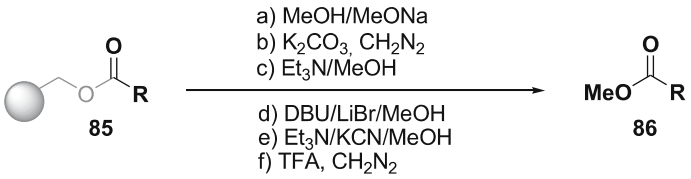


Fig. 7 Thioester linkers utilizable for aminolysis

Thioester **84** has been used for aminolysis by several primary amines (beyond that it was used for Grignard addition) to give the corresponding amides in non-hydroxylic solvents such as dioxane with 70–80% yield. For reaction of secondary amines with thioesters, more reactive thioesters like **82** and **83** were needed.

2.1.5
Cleavage Yielding Alkyl-O- and S-Esters

Transesterification of ester linkers can be conducted by many protocols. Scheme 11 gives a short overview illustrating the most popular procedures for a transesterification into methylesters [113, 114].

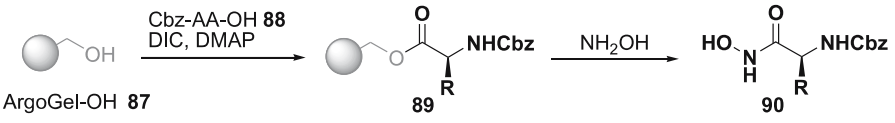


Scheme 11 Methods for transesterification of ester linkers

Transesterification of resin-bound esters to give thioesters is also a literature known procedure. Swinnen et al. released thioesters from solid-supported esters by addition of ethanethiol and Me_2AlCl (in situ formation of alkylaluminum thiolate) [115]. This strategy does not afford special linkers and can be performed for example on Wang or PAM resins. The reaction sequence favors side reaction of the ester species to thio-orthoesters and ketene thioacetals that could be transformed into the desired thioesters via treatment with TFA.

2.1.6
Cleavage Yielding Hydroxamates

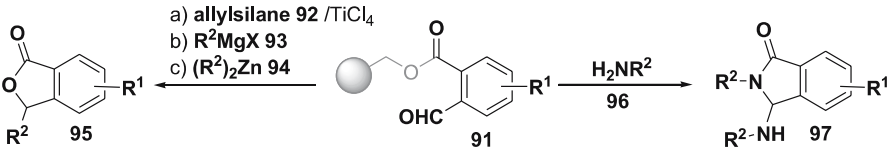
In 1998, Dankwardt used for the first time ester linkers for the synthesis of hydroxamates [116]. The synthesis of hydroxamates and hydroxamic acids is a known synthesis on solid supports but former approaches used special hydroxamate linkers that had to be synthesized in previous reactions. The methodology published by Dankwardt is very simple because ArgoGel-OH resins can be used without further derivatization to bind protected amino acids **88** in the presence of coupling reagents. Hydroxamic acids **90** were cleaved by the addition of hydroxylamine in 21% (sterically hindered amino acids) to quantitative yields (glycin, Scheme 12).



Scheme 12 Synthesis of hydroxamates via ester linker

2.1.7 Ring-Forming Strategies

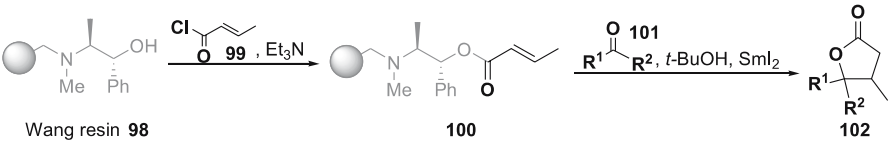
Ester linkers are generally suitable for cyclative cleavage with respect to the considerations of alkylester and amide formation. Cleavage of substrates from the resin via ring closing procedures provided a widely used tool for the synthesis of heterocycles on solid supports during the last few years. Especially lactam and lactone syntheses could be developed successfully in solid phase organic chemistry and could often be applied to automated synthesis because of their potency for diversity and the high purities of released compounds that do not require purification methods. Depending on the loaded residues on the ester linker unit several compound classes like tetramic acids [117–119], hydantoins [120–122], thiohydantoins [123, 124], sulfahydantoins [125], isoindoles **95** and phthalides **97** [126] (Scheme 13), alkaloid analogues [123, 127], pyrrolidines [128], diketopiperazines [122, 129], butyrolactones [130], diketomorpholines [131], 2*H*-pyranones [132] and ketopiperazines [133] have been synthesized.



Scheme 13 Synthesis of isoindoles and phthalides

For lactone synthesis there is no need for special ester linker requirements. It can be performed using an ester activating reagent like TFA that enhances intramolecular nucleophilic attack by an alkoxy functionality. The distance between the ester group and the alkoxy functionality determines the resulting ring-size and therefore whether γ- or δ-lactone-derivatives are released [134].

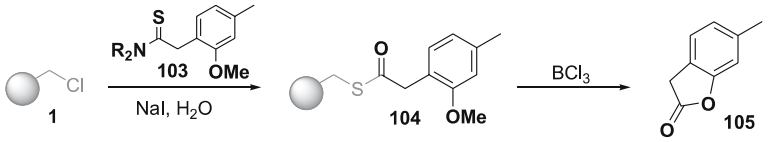
Beyond the usual cleavage strategies using catalytic amounts of acids to start cyclization processes, there are other approaches to initiate cleavage. Procter et al. synthesized a crotonate resin by addition of crotonyl chloride to ephedrin resin **98**. In the presence of SmI_2, aldehydes and ketones form ketyl radicals which give γ-butyrolactones **102** via conjugate addition to **100** and concomitant cyclative cleavage (Scheme 14) [135].



Scheme 14 Synthesis of γ-butyrolactones via cyclative cleavage

Matthews et al. described a synthetic route to hydantoins as well as thiohydantoins on solid-supported esters in 1997 [136, 137]. The hydantoin or thiohydantoin skeleton is synthesized on Wang resin by addition of an amino acid and following reductive alkylation. For the release of hydantoins, acylation with an isocyanate to urea derivatives with following base promoted cleavage takes place. For the preparation of thiohydantoins, isothiocyanates have to be reacted with the corresponding precursors; these intermediates cyclize without additional treatment with bases to the corresponding thiohydantoins.

Intramolecular cyclization mechanisms are also known for the related thioesters. Activation of solid-supported thioesters affords lactones in the case of O-nucleophiles and lactams in the case of N-nucleophiles. For these cyclizations no special linker structures are required and lactam as well as lactone formation have been published on the Vlattas-linker systems **82–84** (see Fig. 7). The lactone formation by Bradley et al. (Scheme 15) shows the utility of thioesters for intramolecular ester formation [138].



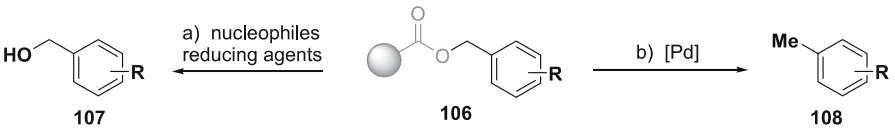
Scheme 15 Lactone-formation via thioester linkers

Applications for this lactone formation chemistry are manifold. Kobayashi et al. for example found a method to synthesize monosaccharide derivatives via cleavage of thioester linkers [139]. The resulting lactones had to be reduced with DIBAL-H to get glucose derivatives.

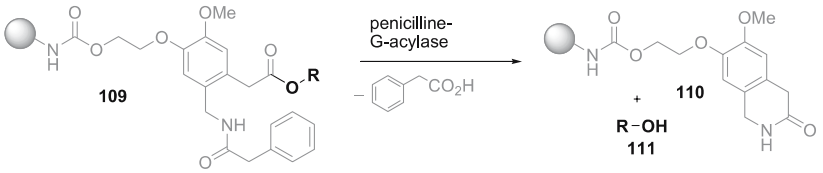
Thioesters in their function as activated derivatives of carboxylic esters are also important for lactam formation as shown in a synthesis published by Vlattas et al. in 1997 [90].

2.2 Esters Type B

Esters of general structure B can be cleaved with the same reagents described for the esters of general structure A. The multifunctionality of the resulting compounds is limited because the carbonyl part of the functionality remains on the resin. Most type-B esters are released as alcohols from the resin (path a) but there are a few examples which obtain other derivatives of the resin-bound structures (Schemes 16, 18 and 19). Path b in Scheme 16 illustrates the cleavage of methylarenes **108** on polymeric support published by Sucholeiki in 1997 [140]. Release from the resin affords addition of a palladium catalyst ($Pd(OAc)_2$) and formate reduction.



Scheme 16 Ester type B cleavage strategies



Scheme 17 Safety-catch approach to alcohol functionalities

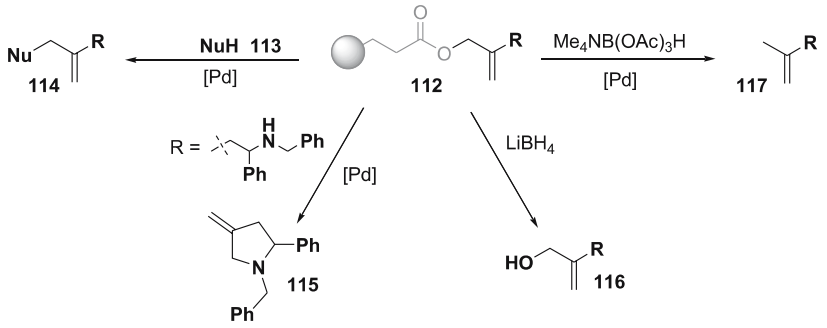
Release of alcohols **107** from ester linkers can be performed using various nucleophiles or reducing agents (path a). Nucleophilic cleavage is a very often used tool and is known to be intermolecular (e.g. by addition of NaOMe, NH_3 or N_2H_4) as well as intramolecular in the form of safety-catch linkers **109** [141]. An intramolecular approach was published by Waldmann et al. in 2000 [142]. The synthesis described in Scheme 17 has several advantages referring to the traditional nucleophilic cleavage. First of all the nucleophile is present but masked during the whole synthesis. The cleavage reagent does not need to be added in excess which would lead to the need for purifying methods. A further advantage is that additional ester groups or even less stable functionalities are tolerated under the cleavage conditions. The desired ester group can be cleaved selectively what is only hardly possible with intermolecular nucleophiles.

Other groups worked on similar approaches and whereas Waldmann reached activation of his linker by addition of enzymes, Subra et al. used conventional basic deprotection methodologies for the removal of Fmoc protecting groups resulting in the same activating effect [143].

2.2.1 Allylic Esters

The allylic esters represent a special group within the esters of type B and their cleavage possibilities are manifold (Scheme 18) [128, 144, 145]. The allylic component offers possibilities for palladium-catalyzed release of target structures (inter- as well as intramolecular cleavage and ring closure) whereas the ester bond can be reduced or attacked by nucleophiles.

The allylic ester linker has been extensively studied by Brown et al. [145]. The cleavage concept relies on the formation of an electrophilic π-allyl palladium complex that could react with nucleophiles in order to give cyclic

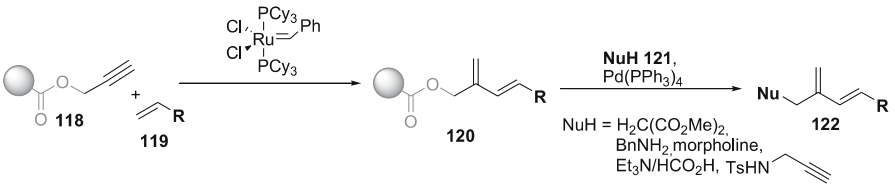


Scheme 18 Multifunctional cleavage of allylic esters type B

or non-cyclic substitution products. C-Nucleophiles as well as heteroatom-nucleophiles **113** work in this strategy and give product yields of about 80% for five steps starting from the carboxylic acid resin.

The allylic linker core **112** is formed via a two step synthetic route including addition of diethylmalonate to Merrifield resin and decarboxylation. Substrates for allylic substitution can be attached to the carboxylic acid linker via DIC/DMAP mediated coupling reaction. One cleavage strategy that uses nucleophiles results in the synthesis of pyrrolidines **115** or linear amine-containing compounds. Additionally, alternative reaction to the release of resin-bound substrates as alcohols **116** by addition of reducing agents like $LiBH_4$ occurs. The reductive removal of allylic esters as alcohols can be modified to give propene derivatives **117** upon cleavage. This reaction is always observed when traces of palladium catalyst are still remaining on the resin and a hydride source is added. Best results (69% of **117**) could be achieved by the addition of $Me_4NB(OAc)_3H$ in the presence of $Pd(acac)_2$ and dppe.

Scheme 19 presents the synthesis and cleavage methodologies published by Blechert et al. in 1998 [144]. This approach to a linker core **120** implies some disadvantages because metal catalysis is essential for the development of the allylic core. Therefore, the possibility of traces of remaining catalyst on the resin have to be considered while planning the following reaction sequences.



Scheme 19 Allylic ester linker synthesis and cleavage

3
Amide Linkers

Because of the origins of solid phase chemistry in peptide synthesis, amide formation is historically one of the most important reactions on solid supports. The most public resins are the Rink amide linker **123** [146, 147] and its derivatives that are formed by addition of the Knorr linker [148] to polystyrene-derived resins.

Solid phase amide synthesis is successful with all mentioned linker types (Fig. 8, [149–165]) and several cleavage protocols (Scheme 20, [166–175]) have been developed to release primary amides **138** from these solid supports always depending on the cleavage conditions required for special linker types **137**. Rink amide (**123**) or PAL (**124**) resins are for example cleaved with TFA mixtures from 50–95% whereas MBHA resins (**126**) require HF/anisole mix-

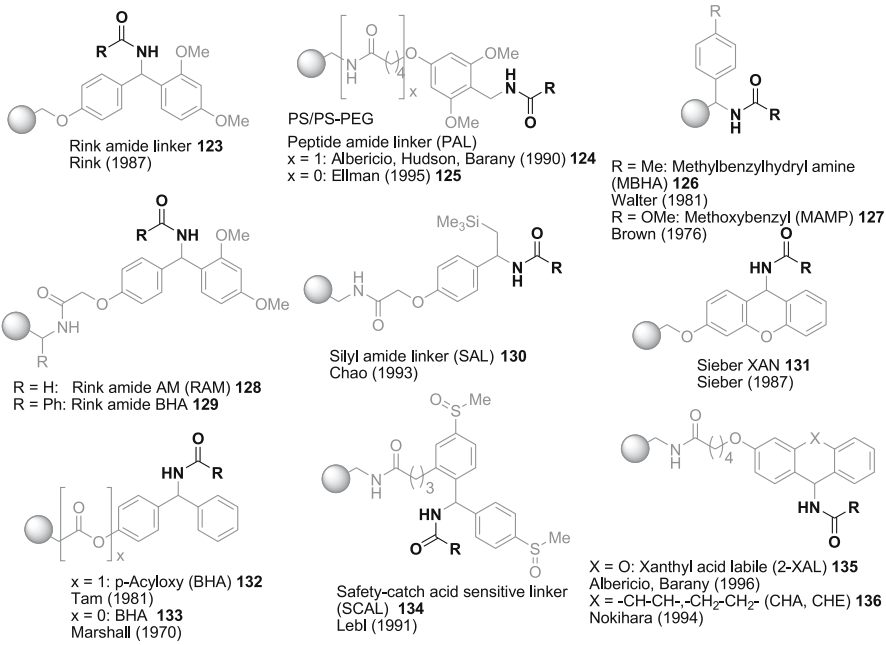
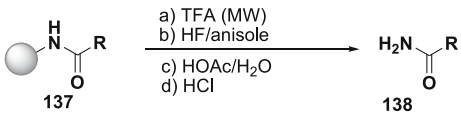


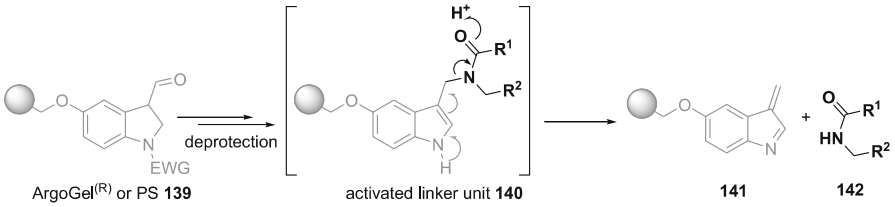
Fig. 8 Linkers for amide bond forming on solid phase



Scheme 20 Methods for the release of amides from amide resins

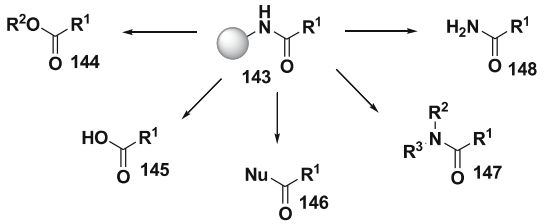
tures. The addition of scavengers is common to restrict the reversibility of cleavage procedures.

A more recent approach to safety-catch cleavage of resin-bound amides (PS or ArgoGel® resin) to their secondary amides **142** was published by Scicinski et al. in 2004 (Scheme 21) using an indole linker [176].



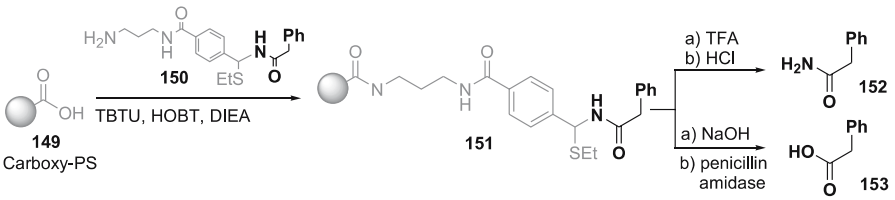
Scheme 21 Indole-based safety-catch linker by Scicinski et al.

Beyond the release of solid-supported amides as amides (Schemes 20 and 21), there are some possibilities to cleave resins via nucleophilic attack at the carbon center. An overview over the potential of amides as multifunctional linkers is given in Scheme 22.



Scheme 22 Possible products from resin-bound amides

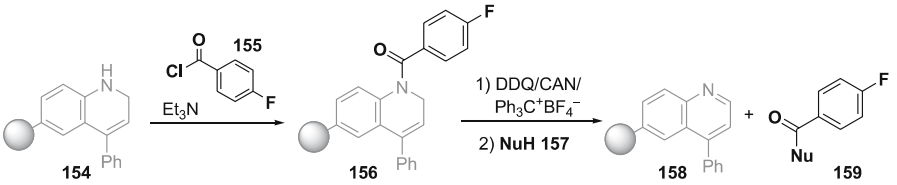
Starting from amides on solid supports one could cleave to get functionalities like carboxylic acids **145**, esters **144**, primary, secondary, tertiary amides (**147**, **148**) and other targets **146** obtainable via nucleophilic substitution. Carboxylic acids can be obtained by treatment of the resin with strong bases like aq. NaOH or alternatively by addition of enzymes under mild, neutral and aqueous conditions (Scheme 23) [177].



Scheme 23 Formation of amides and carboxylic acids via cleavage of amide linker

Cleavage yields for the latter enzyme-cleavage procedure depend strongly on the used resins; PEGA resins give only 25% yield whereas TentaGel seems to be more suitable showing up to 50% cleaved product. The use of other resins is restricted to those that are able to work in aqueous media.

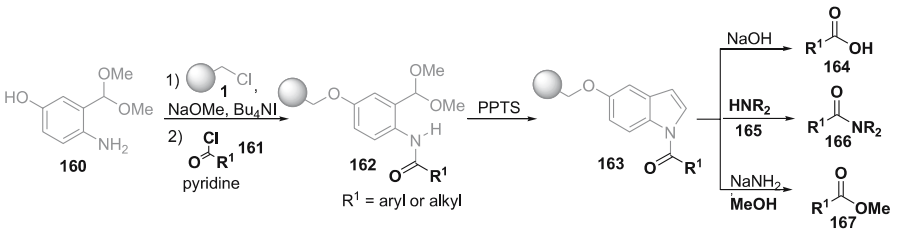
Scheme 24 describes an example for amide resins easily cleavable by various nucleophiles after activation. Without former activation, the linker is stable under acidic as well as basic hydrolytic conditions. Synthesis of the linker core **154** is performed starting from Merrifield resin within a three step sequence via sodium azide addition and subsequent acid-promoted Schmidt rearrangement to a phenyl-iminium intermediate. The desired 4-phenyl-1,2-dihydroquinoline resin **154** is obtained after addition of phenylacetylene [178].



Scheme 24 Nucleophilic replacement on amide resins

Wagner and Mioskowski developed that kind of linker type for the safety-catch synthesis of amides and carboxylic acids using oxidizing reagents like DDQ, ceric ammonium nitrate (CAN) and triphenylcarbenium tetrafluoroborate ($Ph_3C^+BF_4^-$) as activating reagents. These activating reagents induce the formation of a *N*-acyl quinolinium species that is washed and treated with excess of the nucleophilic component **157**. Products could be isolated in up to 59% yield.

Two other safety-catch linkers should be mentioned because of their options in multifunctional cleavage. The first one published by Abell et al. in 1999 offers the possibility to release carboxylic acids, carboxylic esters and amides (Scheme 25) [179].

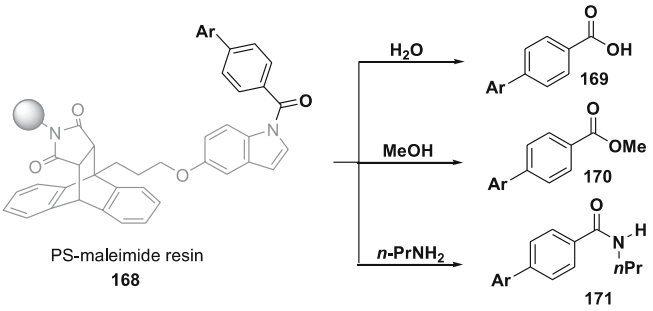


Scheme 25 Applications of safety-catch linker from Abell et al.

Abell et al. synthesized the linker within a four-step synthetic route in overall 47% yield. Starting with 2-nitro-5-hydroxytoluene, benzylation of the

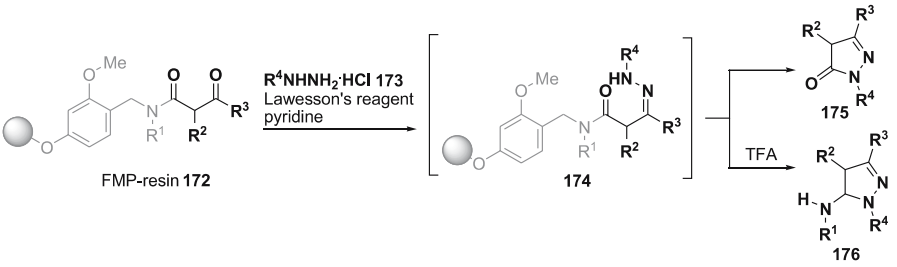
phenol functionality and subsequent reaction with *N*,*N*-dimethylformamide dimethyl acetal gives the enamine derivative of compound **160**. The linker unit is finished by quantitative conversion of the enamine to dimethylacetal and reduction of the nitro functionality to the amine.

Another multifunctional amide-containing linker has also been published recently by Abell et al. [180]. The synthesis of the new anthracenyl-based linker **168** involves again four synthetic steps in solution phase that can be conducted in 53% overall yield. Different to the other linker strategies is the attachment to the resin because the linker core and the substrate are connected in solution phase and are afterwards linked to a maleimide-polystyrene resin. To prove the utility of the new resin, N- and O-nucleophiles were added to give amides **171**, esters **170** and carboxylic acids **169** (Scheme 26).



Scheme 26 Anthracenyl-based amide linker

Cyclative cleavage of polymer-bound substrates is also a known procedure for amide resins. Recently, Dodd et al. released 5-aminopyrazoles **176** from resins linked to β-ketoamides (Scheme 27) [181]. As a side reaction, they found the formation of 5-pyrazolones **175** which are formed via cyclative cleavage from the resins prior to product isolation. The whole synthesis has been performed on 4-formyl-3-(methoxyphenoxy)methylpolystyrene (FMP) resin with immobilized amines that react with β-ketoamides to give **172**.

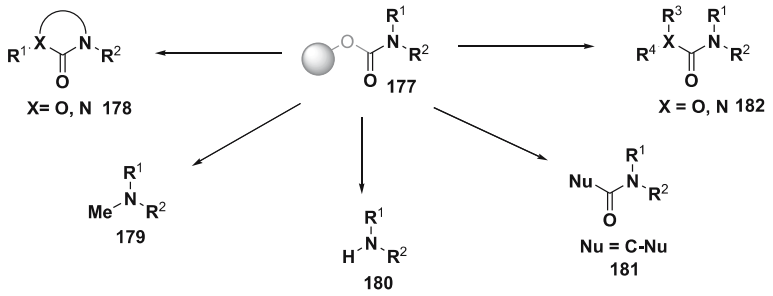


Scheme 27 Cyclization strategies on resin-bound amides

4
Carbamate and Carbonate Linkers

4.1
Carbamate Type A

Carbamates can be divided into type A and type B according to the approach for esters (Fig. 1). The positions of the N- and O-atoms define the linker type (O next to the resin, N next to R^1 = type A). Scheme 28 shows possible cleavage reactions on carbamate linkers which include the release of cyclic and acyclic urea derivatives **178/182** by the addition of amines, the formation of amides **181** and the reduction of the linker to end up with methylamines **179.**



Scheme 28 Cleavage reactions producing diversity on solid-supported carbamate linkers

Carbamate linker systems (Fig. 9, [19, 182–191]) are often produced by the transformation of an alcohol to a carbonate functionality. Afterwards, the carbonate can be reacted with amines and amide coupling reagents to give carbamates. This strategy is for example used by Undén et al. [192] by the addition of 4-nitrophenylchloroformate (4-NPCF) to a 4-methylbenzhydrylamine polystyrene (MBHA-PS)-based resin and further addition of an alkylamine. A similar sequence is conducted with a solid-supported hydroxy-functionality that is converted via phosgene addition into the chloroformate. Addition of amines then gives resin-bound carbamates [193].

Another possibility to synthesize carbamates on a solid support is the addition of activating reagents that transform alcohols directly into (activated) carbamates like *N*,*N*-disuccinimidyl carbonate (DSC) or carbonyl diimidazole (CDI). These activated carbamates then are easily reacted to the target linker via addition of amines [193, 194].

Good results are also achieved by a recently developed approach that is successful through the coupling of isocyanates on hydroxyl-containing resins [195].

The group of Hiemstra developed a reduction-cleavage protocol for carbamates on solid supports [184]. In order to get methyl amines, the activated

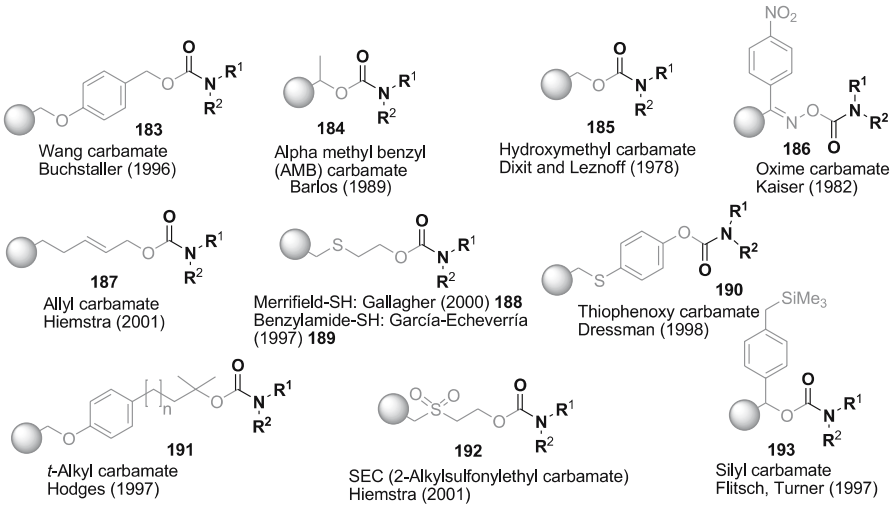
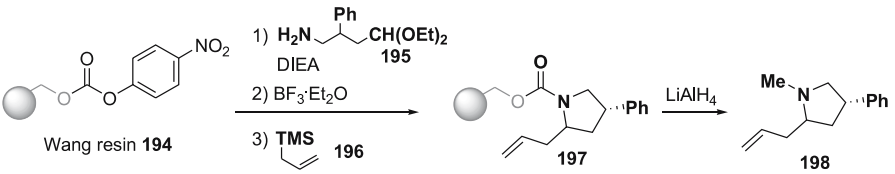


Fig. 9 Summary of linker systems containing carbamates

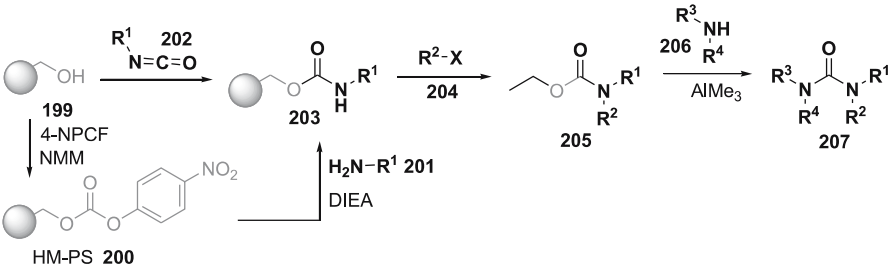
carbonate resin **194** has been treated with an amine in the presence of base to obtain polymer-bound carbamate **197** that could be further derived. Reduction of these carbamates with $LiAlH_4$ gave methyl amines **198** in 70% yield (Scheme 29).



Scheme 29 Reductive cleavage from carbonate resins

More common than reductive methods on carbamate resins are substitution protocols which result in formation of urea-type compounds **207** that can be acyclic or even cyclic if the attacking amine is part of the solid-supported carbamate.

Scheme 30 shows two alternative pathways to end up with the same monosubstituted carbamate resin **203**. The first possible strategy is to form an activated carbonate derivative and to substitute it by a primary amine. The second approach uses isocyanates **202** which are added to the free alcohol functionality on the solid support. Further reaction of the resulting resin with alkyl halogenides **204** leads to twofold substituted carbamates that are released in the presence of primary or secondary amines **206** and



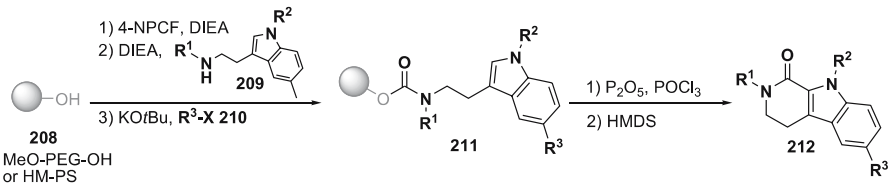
Scheme 30 Synthesis of resin-bound carbamates and cleavage of urea-type compounds thereof

trimethylaluminum (formation of aluminum amide complex) to give ureas **207** [196].

Similar concepts have been proven to give urea-type compounds by several groups including Dressman et al. [186] and Fitzpatrick et al. [197]. The group of Dressman performed the cleavage/urea-forming process by the addition of base that causes an equilibrium of the resin-bound carbamate and the corresponding isocyanate. This intermediate is then converted upon reaction with primary or secondary amines into the desired target ureas.

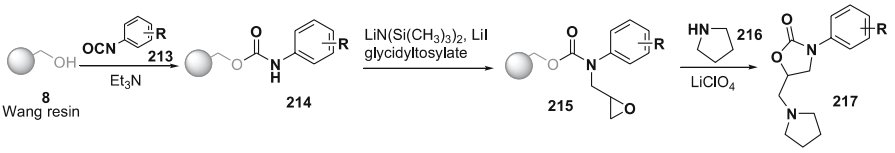
The common problem of all these cleavage protocols is the release of a more or less large amount of amine that is formed as a side product via solvolysis reaction. Sometimes, these amine contaminations can be removed in a separate step by the addition of scavenger resins but often the crude products have to be purified by chromatography [196, 197].

The cleavage via Bischler–Napieralski reaction is an example for the nucleophilic cleavage of carbamates by the addition of carbon nucleophiles (Scheme 31). This methodology has been developed by Li et al. who recently synthesized carbolinones in an at least three-step procedure on solid phase [198]. Starting from MeO-PEG-OH or hydroxymethyl-PS resin (**208**), the hydroxy functionality has been activated as carbonate and further coupled with a secondary amine **209**. Various transformations can be performed on the target core before elimination of the linker residue gives carbolinones **212** in overall 50–72% yield. Former problems to release the desired compounds could be overcome by the addition of hexamethyldisiloxane (HMDS).



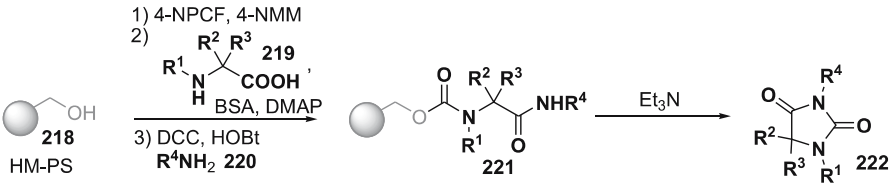
Scheme 31 Cleavage from carbamate resins via Bischler–Napieralski reaction

The release of carbamates from carbamate linkers has been shown by Buchstaller [195] who published a synthesis for oxazolidinones **217** on solid supports (Scheme 32). Starting from Wang resin which is reacted with isocyanates **213**, Buchstaller formed the reactive hydroxyl group via addition of pyrrolidine to an epoxide **215** former introduced via N-alkylation of the core structure **214** on bead. Nucleophilic ring opening results in the formation of an aminoalcohol intermediate that cyclizes spontaneously and gives free oxazolidinones **217** in an overall 71% to quantitative yield in three steps.



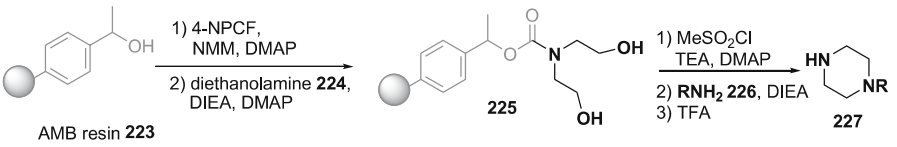
Scheme 32 Release from carbamate-resins via O-nucleophilic attack

Cyclization can also be performed via nucleophilic attack of nitrogen nucleophiles as shown for the release of cyclic urea derivatives. Dressman et al. examined a methodology to cleave hydantoins **222** via treatment of carbamate linkers with an intramolecular amide functionality and base [188]. In the case of resin-bound substrate **221** (Scheme 33), the resulting heterocycle is a five-membered ring system but other heterocycles containing a urea-type unit can also be synthesized as published by diverse other groups (c.f. benzimidazolone-synthesis of Li et al. [199]).



Scheme 33 Cyclization-cleavage strategy for the release of hydantoins

Release of compounds from polymer-bound carbamates has not to be performed via nucleophilic attack but can also be induced by the addition of acids like, for example, TFA in various concentrations. The released target compounds are either primary or secondary amines. The synthesis described in Scheme 34 has been recently published by Dolle et al. [183]. Synthesis of piperazines **227** has been conducted on a α-methylbenzyl resin **223** that has been activated with 4-NPCF and that has been further reacted with diethanolamine **224**. After mesylation of the two resulting terminal hydroxy groups, reaction with a primary amine **226** and base gave immobilized piper-



Scheme 34 Release of amines from resin-bound carbamates

azines. These could be released with yields from 82% to quantitative yield in the presence of TFA/CH_2Cl_2 (1 : 1).

4.2 Carbamates Type B

In comparison to carbamates type A, carbamates of general structure B are not very often used linkers (Fig. 10, [200, 202]). There are only a few cleavage reactions that are known for producing functional diversity.

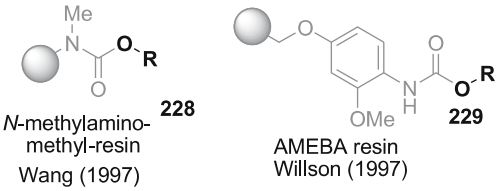
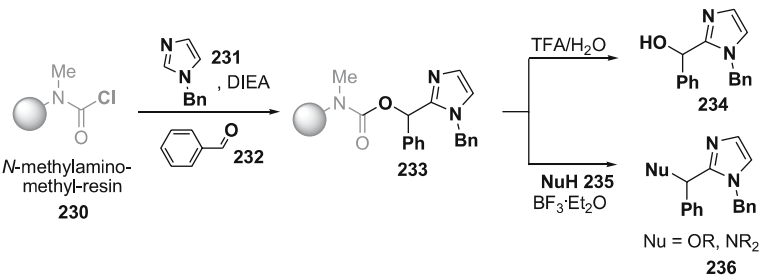


Fig. 10 Known linker structures of carbamate type B

Linker **228** (Fig. 10 and Scheme 35) has been synthesized following a procedure of Wang et al. [201] starting from *N*-methylaminomethyl polystyrene which is converted in the presence of DIEA and phosgene (triphosgene) into the carbamylchloride resin **230**. Addition of benzaldehyde (**232**), 1-benzylimidazole (**231**) and base gave the solid-supported imidazole derivative **233** (Scheme 35). Cleavage can be performed by acidic hydrolysis or by nucleo-



Scheme 35 Cleavage of carbamate resins type B

philic displacement of the linker unit via attack of various amines, alcohols and aldehydes **235** [202].

The yield of target compounds depends strongly on the pK_a value of the nucleophilic part. For amines that show high pK_a values (pK_a of 1-methylpiperazine = 9.7; traces of product) yields are generally worse than yields for amines with lower pK_a (pK_a of aniline = 4.7; 85% yield). These problems could be overcome by the addition of strong Lewis acids like boron trifluoride and the use of microwave-assisted heating procedures.

4.3 Carbonate Linkers

There are many strategies for the synthesis of carbonates [19, 203] (Fig. 11) on polymeric supports. Some of the most common ones are the activation of an alcohol via addition of *bis*-trichloromethyl carbonate (BTC) and base [204], the activation of alcohols via for example carbonyl diimidazole and subsequent transformation with alcohols [19] or the direct conversion of resin-bound alcohols into carbonates via reaction with chloroformates.

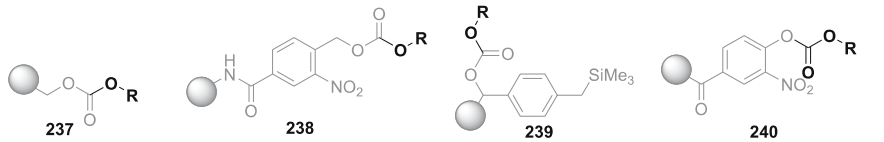
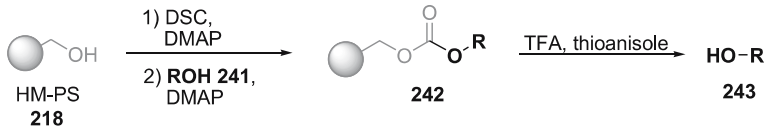


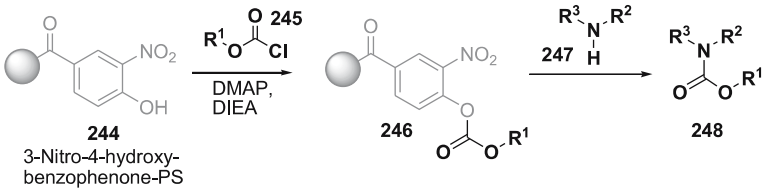
Fig. 11 Carbonate-containing linker systems on solid phase

Carbonate linkers have been transformed into carbamates and alcohols via cleavage from the solid support. Alcohols **243** can be produced by the addition of TFA/thioanisole to the resin-bound substrates **242** (Scheme 36). They can also be obtained by photolytic cleavage from resins with general linker structure **238** or via cleavage with fluorine reagents from linkers like **239** in Fig. 11 [205, 206].



Scheme 36 Release of alcohols from carbonate resins

The nitrophenyl-linker [207] part in the synthetic route described in Scheme 37 is due to its activated nature a good leaving group and cleavage



Scheme 37 Cleavage of carbamates from carbonate linkers

of this linker to give released carbamates **248** has been conducted successfully [204].

5 Weinreb Derivatives and Hydroxamates

Weinreb amides (Fig. 12) [208–212] can be cleaved to give hydroxamates on the one hand and to give aldehydes or ketones on the other hand (Scheme 38) while regenerating the starting polymer-bound alkoxyamine.

Conversion of solid-supported hydroxamates to aldehydes is a useful tool for the generation of C-terminal peptide-aldehydes. Martinez et al. firstly used $LiAlH_4$-reduction for the cleavage of hydroxamates from solid supports and succeeded in synthesizing tripeptide aldehydes that could be obtained in about 40% yield after purification [83]. Reduction of Weinreb amides on

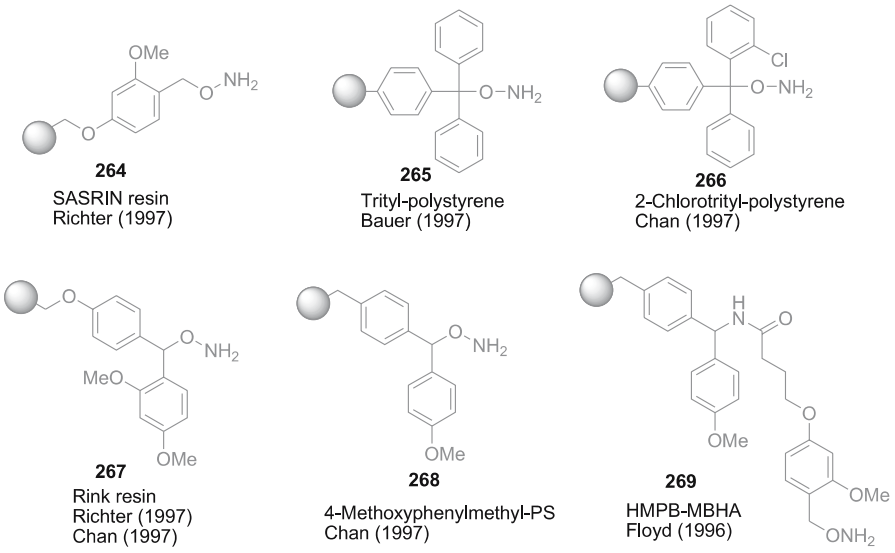
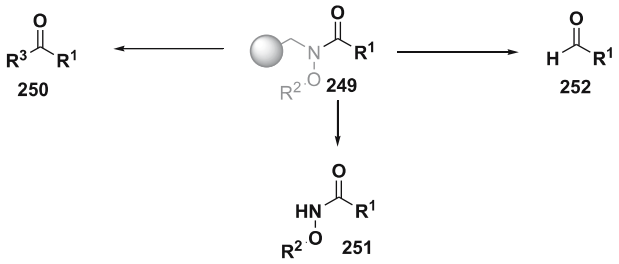


Fig. 12 Hydroxylamine linker systems for solid phase synthesis of hydroxamate-linkers

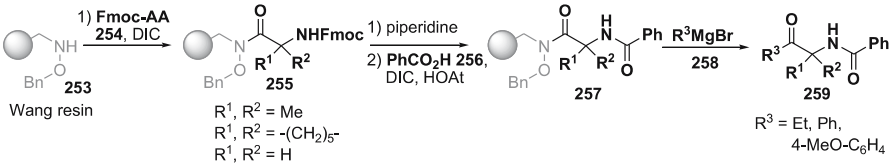


Scheme 38 Cleavage of Weinreb derivatives on solid phase

solid supports became a commonly used tool during the last years and is performed on slightly different resin types (e.g. BAL- and Rink-linker).

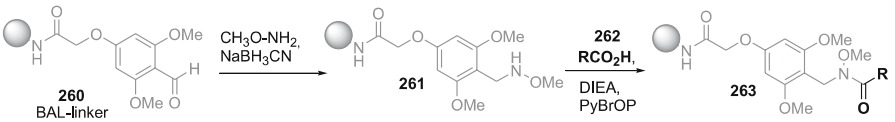
The reaction of Weinreb amides to ketones can be performed cleanly using Grignard reagents in large excess. Yields of that transformation range from 16% up to 78% and no overaddition was observed as described by Armstrong et al. on Rink resin [213].

Tice and Albericio et al. renewed the synthetic idea of Armstrong et al. using a Weinreb *O*-benzyl derivative on Wang resin [214]. Their concept included the synthesis of Weinreb amide linker **255** starting from benzyloxyamino resin **253** (Wang derivative [215]) and following addition of N-protected amino acids **254** with peptide coupling reagents. After deprotection, the terminal nitrogen was converted with benzoic acid into resin **257** and the target substances were cleaved as ketones **259** via Grignard reaction with ethyl-, phenyl- and 4-methoxyphenyl magnesiumbromides **258** (Scheme 39). Yields up to 51% based on the initial loading of Wang resin could be achieved.



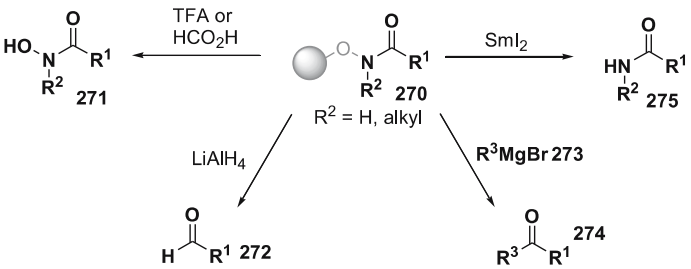
Scheme 39 Synthesis of ketones via Weinreb amides on solid phase

Scheme 40 presents a synthetic route to polymer-supported Weinreb amides starting from commercially available BAL-linker [216]. Whilst most approaches to Weinreb linkers start with the conversion of alcohol functionalities (and following Mitsunobu approach), the BAL linker **260** has to be reacted via reductive amination into the desired methoxyamines **261**. Afterwards resin **261** is treated with carboxylic acids **262** in the presence of coupling reagents to give amides **263** containing the target structure.



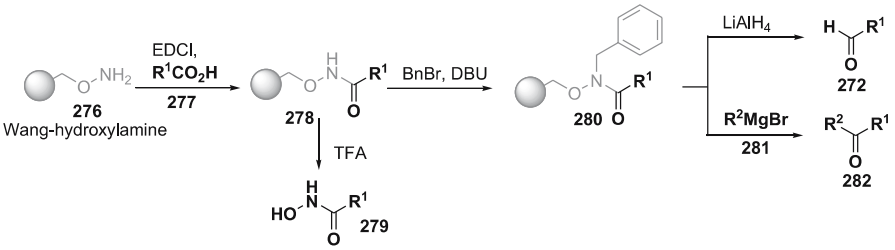
Scheme 40 Weinreb amide synthesis from backbone amide linker (BAL)

Hydroxamates can be cleaved to give four different functionalities (Scheme 41). Treatment of immobilized hydroxamates **270** with TFA/CH_2Cl_2 (chlorotrityl resin **266**) or HCOOH/THF (1 : 3 for trityl resin **265**) gives hydroxamic acids **271** as shown for peptidyl hydroxamic acids by Chan et al. [211], for alkyl amides by Bauer et al. [210] and for sulfonylated diamino acid hydroxamate derivatives by Delaet et al. [217]. Addition of SmI_2 initiates the release of amides **275** [218, 219] and reduction with $LiAlH_4$ produces aldehydes **272** [220]. Similar to the reaction of Weinreb amides, ketones **274** can be cleaved from resins containing a secondary nitrogen with Grignard reagents [215].



Scheme 41 Cleavage of hydroxamates on solid phase

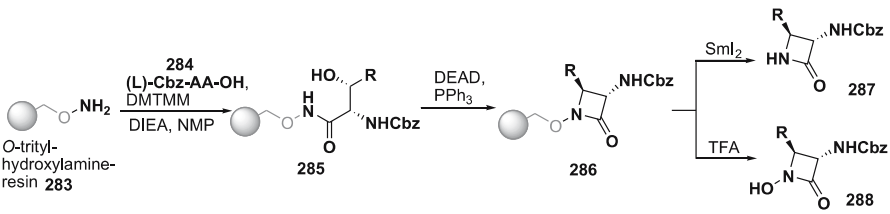
Salvino et al. showed the synthesis of a simple linker to generate aldehydes and ketones from the corresponding acids via immobilization as hydroxamate resin. The starting resins **276** were obtained by synthesis of hydroxylamines on solid support (from Wang resin, Scheme 42) that were coupled to carboxylic acids and N-protected amino acids **277**. Depending on the substi-



Scheme 42 Synthesis of aldehydes and ketones from resin-bound hydroxamic acids

tution of the linker nitrogen, either hydroxamic acids (NH, **279**) or aldehydes and ketones (NBn, **272/282**) are released. Yields up to 54% for reductive cleavage to aldehydes and 68% for alkylation to ketones were obtained.

The strategy of releasing amides (Scheme 43) can be applied to the synthesis of lactams by immobilization of acids containing a β-hydroxy functionality **284**. Ring closure can be carried out under Mitsunobu conditions as shown in a publication of Taddei et al. in 2001 [219]. The authors chose an *O*-trityl-hydroxylamine resin for the reaction sequence because other linker types as for example a Wang-type resin carrying a hydroxylamine functionality failed.



Scheme 43 β-Lactams and 1-hydroxy-β-lactams via resin-bound hydroxamic acids

Taddei et al. used SmI_2 to cleave N – O bonds in hydroxamic acids and adapted a method for the first time used in solid phase synthesis by Abell et al. [220]. Former applications in solution phase [221] showed that SmI_2 is a useful reagent for many transformations which avoids acidic conditions for cleavage of amides. Another example for the release of cyclic substrates from hydroxamate linkers was also given by Taddei et al. [218].

In solution phase, hydroxamic acids can be synthesized by addition of hydroxylamine to esters. This attempt is not successful for the solid phase synthesis of similar substrates thus the following method developed by Floyd et al. is of special importance [212]. In the approach of Floyd et al. a linker core for resin-bound hydroxamic acids is achievable within a three-step procedure in supposed quantitative conversion. To Wang resin as staring material is added *N*-hydroxyphthalimide that can be attached via Mitsunobu reaction. Subsequent cleavage of the phthalimide moiety is conducted with hydrazine in THF. An alternative strategy applicable for Rink acid, SASRIN and Wang resins has been published by Richter et al. using *N*-hydroxyphthalimide/Cs_2CO_3/NMP instead of the Mitsunobu approach [208].

6
Triazene Linkers

The chemistry of diazonium salts provides tremendous opportunities for the construction of various compounds, with particular emphasis on heterocyclic

structures [222]. Triazenes, which correspond to protected diazonium ions, do not only offer interesting possibilities for activation of the *ortho*-position of arenes by coordination of metal ions and/or by lowering the electron density of the arene ring, they are also quite robust precursors for diazonium salts. Inspired by the flexible use of triazenes in the total synthesis of the complex glycopeptide natural product vancomycin by the Nicolaou group [223, 224] and the pioneering work of Moore et al. [225] and Tour et al. [226] in the synthesis of triazenes on a solid support to produce iodoarenes, two sets of triazene linker systems shown in Fig. 13 were proposed by Bräse et al. [227].

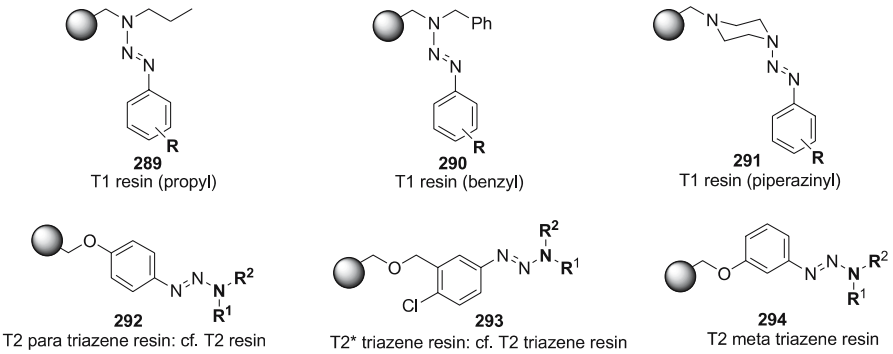


Fig. 13 The triazene linkers

The T1 linker system consists of 3,3-dialkyl-1-aryl triazenes bound to the support via the alkyl chain (either via a dibenzyl type or a piperazinyl type anchoring). The T2 linker family is based on immobilized arene diazonium salts [228–231].

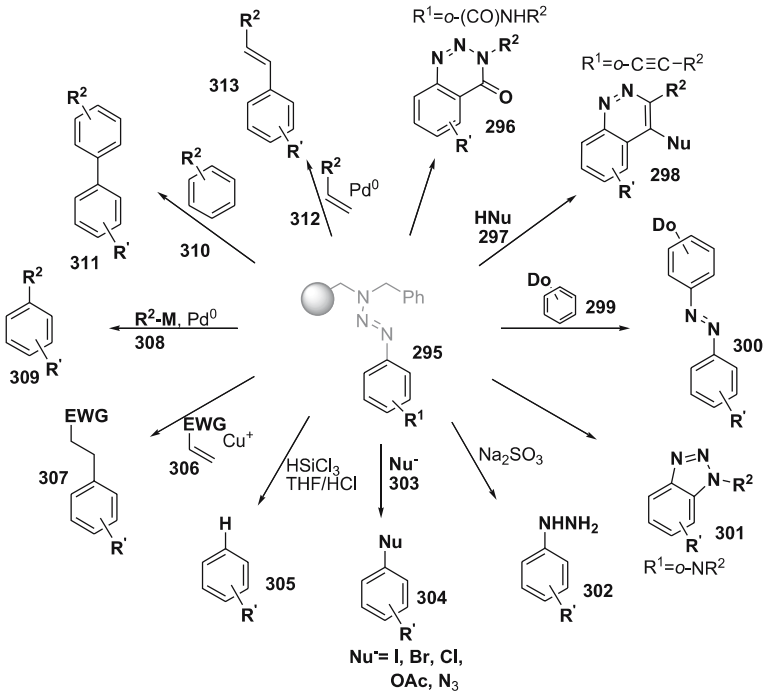
Triazenes are in general stable towards daylight, oxygen (air), moisture, reducing agents, oxidizing reagents and transition-metal complexes. Alkyl lithium reagents can be used for the metalation of simple alkyl-substituted triazenes, whereas benzylic-substituted triazenes undergo a metalation/fragmentation reaction due to their enhanced acidity [232, 233]. However, triazenes are generally labile towards Brønsted acids and certain Lewis acids producing diazonium salts and amines.

6.1 The Triazene T1 Linker

The triazene T1 linker has been successfully used as a linker for arenes. Up to now, more than 100 different anilines have been immobilized by the Bräse group and others [234, 235]. In general, the synthesis starts with diazotation of an aniline derivative in an organic solvent using alkyl nitrite reagents. The immobilization on solid supports has been successfully carried out using

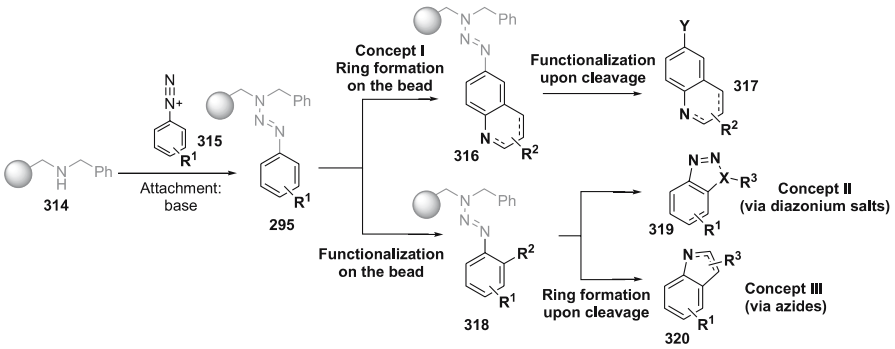
benzylaminomethylpolystyrene or piperazinylmethylpolystyrene resin, each one accessible from Merrifield resin in only one step. Although both linkers are equally suitable, the benzylamino resin is more sensitive toward strong bases (e.g. BuLi) [232].

Functionalization on the polymer bead has been demonstrated extensively. Acidic cleavage of the triazene resin yields the recyclable amine resin and the modified aryl diazonium salts, which can be further transformed directly during the cleavage step in high yields (> 90%) and purities (> 90%) (Scheme 44).



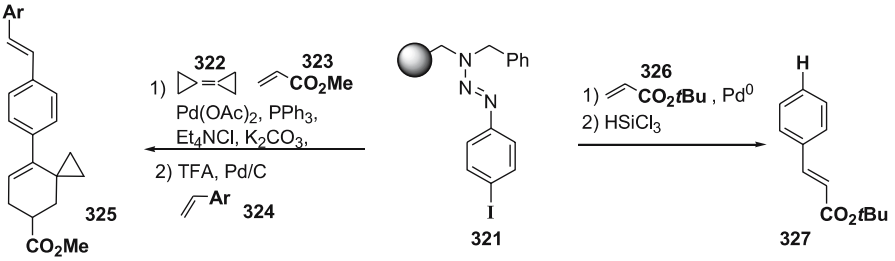
Scheme 44 Some possibilities of the T1 triazene linker (Do = electron-donating group, EWG = electron-withdrawing group, Nu = Hal, N_3, OAc …)

Bräse et al. therefore anticipated using the triazene T1 linker for the synthesis of diverse heterocycle libraries. While in concept II (Scheme 45) the diazonium ion was directly incorporated into the heterocyclic core to add two extra nitrogen atoms, the flexibility of the azide functionality has been advantageously used in concept III (Scheme 45) to yield heterocycles **320** with an odd number of nitrogen atoms. In concept I, the heterocyclic core **316** is generated on the bead using (classical) ring-formation reactions followed by a subsequent multifunctional cleavage hence leading to the library members **317**.



Scheme 45 Concept for cleavage/heterocyclization with the T1 System

As pointed out above, acidic media cleave the triazenes to yield the diazonium salts. The diazonium salts can be further functionalized as demonstrated in the case of the reduction to the hydrocarbon **327** in THF with the aid of ultrasound via a radical pathway [227]. A recently found reagent for this reduction was trichlorosilane [232] which does not only serve as a source of trace-quantity hydrochloric acid to cleave the triazene moiety, but also as a hydride donor cleanly reducing the diazonium ions (Scheme 46).



Scheme 46 The T1 linker for traceless cleavage

As already shown by Moore et al. and Tour et al., addition of methyl iodide to a triazene resin at elevated temperature (110 °C) gives rise to aryliodides **304** (Nu = I) in excellent yields [225, 226]. It was shown that aryl halides are readily available using lithium halides in the presence of an acidic ion exchange resin or with the corresponding trimethylsilyl halide at room temperature. The introduction of oxygen functionalities is viable using a mixture of acetic anhydride and acetic acid to produce the corresponding phenol acetates **304** (Nu = OAc) in good yields (Scheme 44).

While the range of electrophiles that could be employed is quite broad, the most versatile benefit was the development of a cleavage cross-coupling strategy [236–238]. Starting from modified triazene resins, a one-pot cleavage

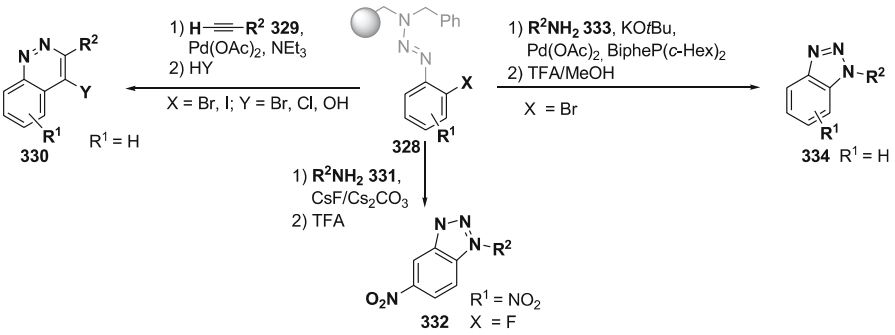
cross-coupling reaction was conducted with two equivalents of trifluoroacetic acid in MeOH at 0 °C to yield a diazonium intermediate **315**. In situ coupling with both electron-deficient or electron-rich alkenes **312** in the presence of catalytic amounts (5 mol %) of palladium(II) acetate provides the corresponding products **313** in excellent yields and purities. This one-pot cleavage cross-coupling reaction affords salt-free products since the present resin **314** also participates as a "scavenger-resin" trapping the excess of trifluoroacetic acid. Using palladium on charcoal as the catalyst in the cross-coupling reaction has the advantage of decreasing palladium-contamination as well as providing the conditions for a subsequent hydrogenation reaction. This salt-free cleavage cross-coupling strategy allows the clean synthesis of substituted (cyclo)alkenyl and (cyclo)alkyl (hetero)arene derivatives and it is especially suitable for automated synthesis since there is no need for purification of the final compounds. This modulated protocol is amenable to virtually any amino arene as well as alkenes and alkynes tolerating most functional groups providing a synthetic pathway for the formation of highly lipophilic molecules. Multi-component Heck reactions (i.e. domino Heck Diels–Alder reaction shown in Scheme 46, compound **325**) are possible in this context leading to further diversification [236, 237].

Concept II (Scheme 45) represents a straightforward synthesis of benzoannelated heterocycles (e.g. cinnolines, benzotriazoles, etc.) via a diazonium intermediate and a nucleophilic *ortho*-substituent. While the diazonium group is lost as dinitrogen upon cleavage from the resin, a suitable nucleophilic *ortho*-substituent favors cyclization to give heterocyclic structures **319**. Benzotriazoles, for example, are accessible from *o*-aminoaryl-substituted triazenes after a two-step reaction sequence: a nucleophilic displacement followed by cleavage/heterocyclization [239].

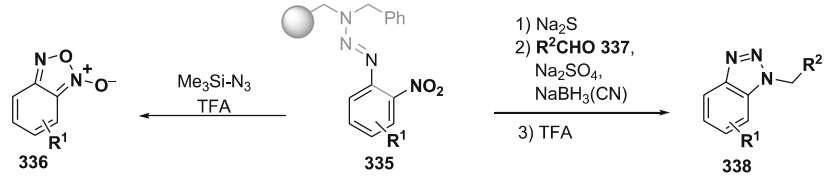
Starting from commercially available 2-fluoro-5-nitroaniline, this aniline was diazotized and coupled to benzylaminomethylpolystyrene to give the immobilized triazene **328** (R = NO_2, X = F, equivalent to Sanger reagent). After nucleophilic displacement with primary amines to furnish an aniline resin, the cleavage with trifluoroacetic acid in dichloromethane proceeds smoothly at room temperature within minutes resulting in the desired 1-alkyl-5-nitro-1*H*-benzotriazoles **332** in excellent yield and purities (Scheme 47). This route was successfully adopted for the synthesis of a 200-member library by means of automated synthesis (Lormann et al., 2007, personal communication).

The ingenious undemanding Hartwig–Buchwald palladium-catalyzed carbon-nitrogen coupling can also be favorably used for the synthesis of benzotriazoles (**332**). This way, both the amine and the arylhalide can be immobilized. Crucial for the success is the careful choice of the ligand used (Scheme 47).

Other heterocyclic systems that can be prepared include cinnolines **338** which are available from *o*-alkynylaryl or *o*-alkynylaryl triazenes via a cleavage-cyclization strategy. Starting with the immobilization of diverse



Scheme 47 Arylhalides as precursors for Hartwig–Buchwald, Sonogashira and von Richter reaction



Scheme 48 Nitroarenes as precursors for five-membered heterocycles

o-haloaryl diazonium compounds, the palladium-catalyzed cross-couplings were performed under standard conditions [$Pd(OAc)_2$, NEt_3] with different alkynes affording *o*-alkynylarene resins. The von Richter cleavage reactions were conducted with aqueous hydrogen chloride or hydrogen bromide to generate the expected cinnoline **330** on a library format (146 members) in a 47–95% yield range and with 60–95% purity without any further purification (Scheme 47).

However, due to the fact that the strategy outlined in Scheme 47 is limited to the generation of 5-nitro-substituted benzotriazoles **332**, a different access to *o*-amino-substituted triazenes was sought. Starting from *o*-nitro triazenes **335**, reduction under newly developed conditions (Zimmermann et al., 2007, personal communication) suitable for acid sensitive linkers gave access to the *o*-amino-substituted resins. Consequently, a reductive-amination strategy was also viable. In this case, 3-alkylmethyl or 3-arylmethyl benzotriazoles **338** are accessible in good yields (Scheme 48).

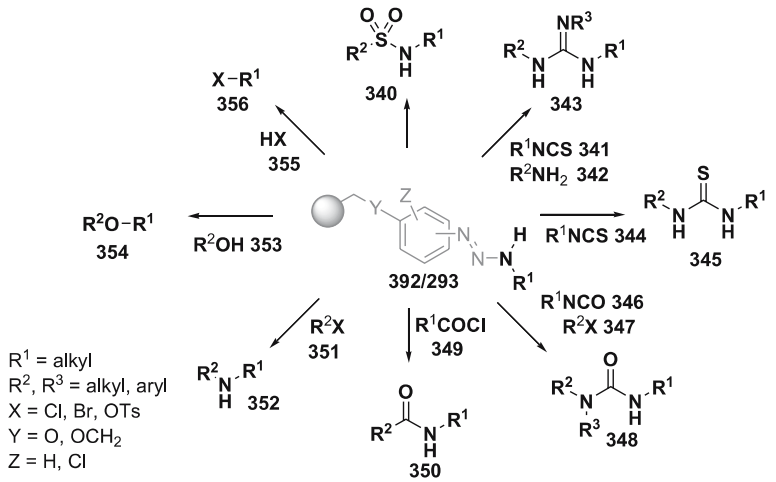
Since the attack of a neighboring nucleophile to the diazonium moiety proceeds in an intramolecular coupling step and the compounds produced are largely less acid sensitive than dialkyl-substituted triazenes, even the weak nucleophilicity of secondary amides or arenes can be used for the generation of heterocycles [240, 241]. For example benzo[a][1,2,3]triazinones **296** can therefore be formed upon cyclization of suitable substituted benzamides. The latter ones are accessible from carboxylate resins and amines by peptide coupling methods.

Another multifunctional cleavage of the T1 linker is based on concept III which uses the flexibility of the azide functionality. Upon thermolysis or photolysis, appropriately substituted aryl azides lose nitrogen gas to give intermediate nitrenes, which in turn cyclize with suitable *ortho*-substituents to give benzoannelated heterocycles. An unsaturated *ortho*-substituent consisting of double bonds including carbon, oxygen or nitrogen atoms then gives indoles/carbazoles (starting from C = C), indazoles (C = N), benzoisozazoles (C = O), benzooxadiazoles (benzofurazanes) (N = O), benzimidazoles (N = C) or benzotriazoles (N = N) (Scheme 48). Alternatively, the intra- or intermolecular *aza*-Wittig reaction might also be envisaged [242] in which case larger ring sizes (six- and seven-membered rings) are also obtainable.

The synthesis of azides (Nu = N_3) **304** was achieved via cleavage of the triazene resin with 10% TFA in dichloromethane at room temperature in the presence of trimethylsilyl azide in good yields (mostly > 90%) and high purities (> 95%) without any further purification [243, 244]. The required mild cleavage conditions allow the synthesis of various functionalized arenes.

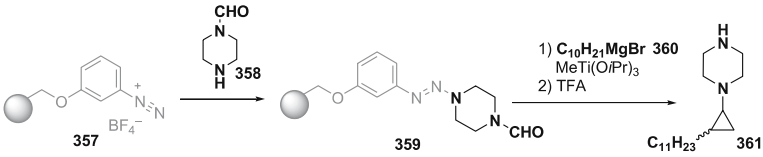
6.2
The Triazene T2 Linker

Coupling of the diazonium resins such as the T2 or T2* diazonium resins with various primary or secondary amines smoothly leads to the formation of a series of triazene resins **292** and **293** (Scheme 49), respectively [245, 246]. The Bräse group demonstrated the use of this anchoring group in the



Scheme 49 Possibilities with the T2 linker

synthesis of ABT 594 analogues and in the synthesis of cyclopropylpiperazines using the titanium-mediated Kulinkovich–de Meijere transformation of *N*-formyl amines (Scheme 50, for another application of T2 linker see Rich et al. [247]).



Scheme 50 Synthesis of cyclopropylpiperazines

In addition to secondary amines, attachment of primary amines, hydroxylamine, hydrazines, sulfoximines, or phenols proceeds equally well (Scheme 49). Secondary amines can be cleaved directly from the resin, while primary amines lead to a different reaction pathway.

Primary amines can be derivatized on the free NH-functionality and therefore be modified to an array of products. Thus, ureas **348**, thioureas **345** [248], guanidines **343** [248] and amides **350** were prepared in excellent yields (Scheme 49).

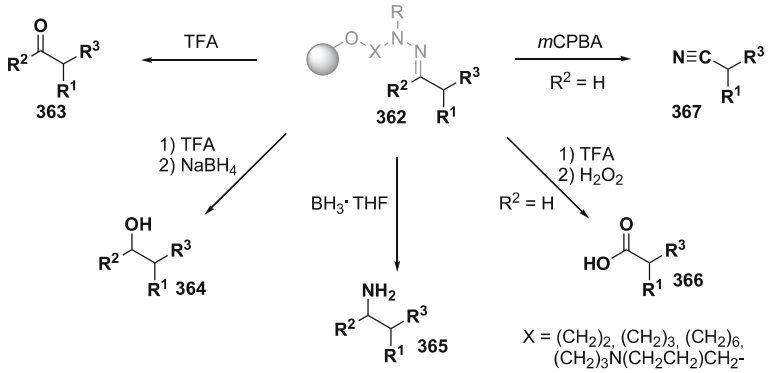
While the cleavage of trisubstituted triazenes gives rise to the formation of secondary amines in excellent yields, the cleavage of disubstituted triazene leads to aliphatic diazonium salts. The newly formed diazonium ion undergoes substitution with the nucleophile present in the reaction mixture. Therefore, alkyl halides [249], alcohols, ethers as well as alkyl carboxylic esters, sulfonic esters [250], phosphoric esters [251], and phosphinic esters can be formed by cleavage with trimethylsilyl halides (X = I, Br, Cl), aqueous trifluoroacetic acid, carboxylic acids, sulfonic acids, phosphoric acids, and phosphinic acids, respectively (compounds **355** Scheme 49) [252]. The regioselectivity of the cleavage can be explained by the presence of one tautomer of the triazene in which the hydrogen atom is on the triazene-nitrogen linked to the arene ring.

7
Hydrazone Linkers

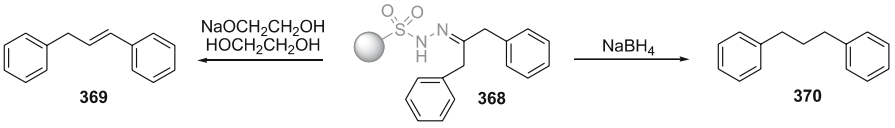
A recent and important example for a N-containing linker system is the hydrazone linker developed by the group of Lazny [253]. This system is useful for the immobilization of aldehydes and ketones via hydrazone bonding. The linker was synthesized starting from commercially available 2-(*N*-methylamino)ethanol (or corresponding aminoalcohols) which is nitrosated with *t*-butylnitrite and subsequently reduced with lithium aluminum hydride

to give the corresponding hydrazine. After conversion with aldehydes or ketones to hydrazones, the finished linker unit was attached to Merrifield resin using potassium *t*-butoxide as base.

The polymer-supported hydrazones were subjected to α-alkylation using LDA and alkylhalides followed by multifunctional cleavage (Scheme 51). Treatment of the immobilized hydrazones with TFA afforded the alkylated carbonyl compounds **363**. Thus, oxidative protocols provided carboxylic acids **366** (using hydrogen peroxide) or nitriles **367** (using *m*CPBA) while reductive cleavage with borane-THF or reductive postcleavage workup (e.g. sodium borohydride) gave primary amines **365** and alcohols **364**, respectively. Similar hydrazone linkers yielding amines (Enders et al. [254, 255]) or the corresponding carbonyl compounds (Ellman et al. [256]) have been reported before. The sulfonyl hydrazone linker **368** developed by Kamogawa et al. was the first example of a traceless hydrazone linker [257]. With this system ketones and aldehydes could be attached to the resin (Scheme 52) and—after being modified on the solid support—could be converted either into the corresponding alkanes **370** by reaction with sodium borohydride or into the corresponding alkenes **369** under basic conditions (Bamford–Stevens reaction).



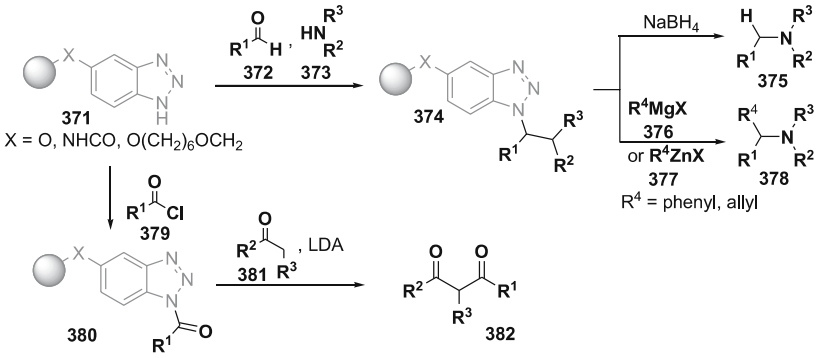
Scheme 51 Multifunctional cleavage from the hydrazone linker



Scheme 52 First traceless linker by Kamogawa et al.

8
Benzotriazole Linkers

Another example for a N-containing linker system is the benzotriazole linker that can be generated by several ways of attaching benzotriazole to solid supports [258, 259]. This linker is often used for the synthesis of amine libraries (Scheme 53). Therefore the benzotriazole linker **371** is reacted with aldehydes **372** and amines **373** to form Mannich-type adducts **374** which can be detached from the resin either by Grignard or zinc reagents or under reductive conditions using sodium borohydride [260].



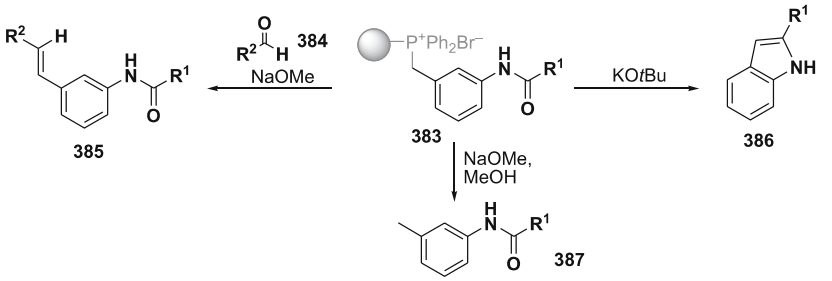
Scheme 53 Cleavage of benzotriazole linkers

A further application of the benzotriazole linker is the synthesis of β-diketones **382.** Polymer-supported benzotriazoles **371** were transformed into the corresponding azolides which were cleaved with various ketone lihio enolates to build diketones **382** [261]. Other cleavage reactions with nucleophiles should be possible as benzotriazole auxiliaries are often used as advantageous N, C-, S- and O-acylating reagents [262]. A well-known application is the solid phase synthesis of unsymmetric ureas ($R^1 = NR_2$) with secondary amines as cleaving nucleophiles [258].

9
Phosphonium Linkers

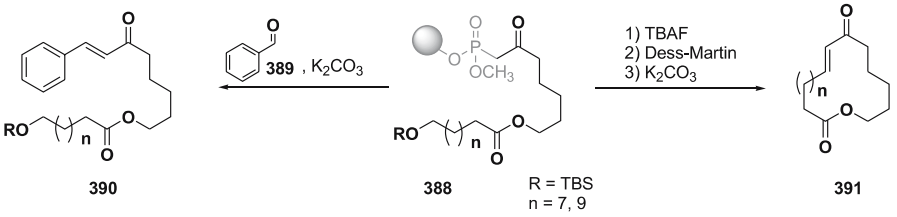
Hughes exploited a polymer-bound phosphonium-salt as a traceless linker for the synthesis of alkyl, alkenyl and heteroaryl products [263]. The linker system **383** was prepared from commercially available resin-bound triphenylphosphine and nitrobenzyl bromide with following reduction of the nitro group and acylation.

Products can be cleaved using three different pathways. Wittig cleavage with aldehydes **384** produces the corresponding olefins **385** and intramolecular Wittig reactions cleaving the molecules to give the indole derivatives **386** are possible. Cleavage of the carbon-phosphonium bond with NaOMe/MeOH affords the toluene derivatives **387** (Scheme 54). Another application of resin-bound triphenylphosphine was achieved by Slade et al. who used it to immobilize 4-fluoro-3-nitrobenzyl bromide for the synthesis of 2-alkylbenzimidazoles [264]. Cleavage with sodium hydroxide led to the formation of a methyl group at the attachment point.



Scheme 54 Multifunctional cleavage from the phosphonium linker by Hughes

A similar system was used by Johnson/Zhang [265] and by Nicolaou et al. [266]. Both groups cleaved molecules attached via a phosphonate linker from the resin **388** in a Horner–Wadsworth-Emmons reaction with benzaldehyde **389** in order to form the corresponding olefins **390**. This reaction can be performed in an intramolecular way as well to give cyclic olefins **391** (Scheme 55). Another possibility to cleave the linker system is the hydrolysis using trimethyl silyl iodide to give the corresponding phosphonic acids [267]. The release of those acid groups can lead to the formation of cyclic structures, e.g. to cyclic PNA and PNA-DNA chimeras as Di Fabio et al. showed [268].



Scheme 55 Cleavage from the phosphonate linker via Horner–Wadsworth-Emmons reaction

10
Sulfur Linkers

Sulfur linkers play an important role in solid phase synthesis and a set of modern linkers based on sulfur chemistry can be found in the literature (Figs. 14 and 15). The linkers can be synthesized following various synthetic routes. One possibility is the reaction of Merrifield resin with potassium thioacetate and subsequent reduction using $LiBH_4$ to give the free thiol **393**. Another way to get the desired thio resin starts from Merrifield resin and thiourea followed by treatment with *n*-butylamine [109, 269].

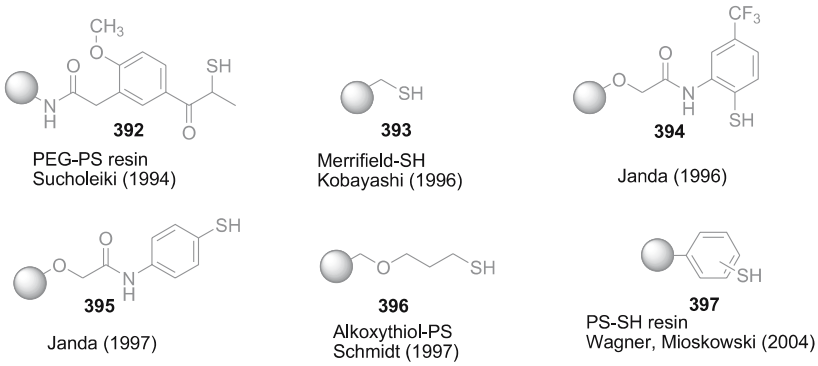


Fig. 14 Thioether-based linkers on solid support (see also Vlattas linkers in Fig. 7)

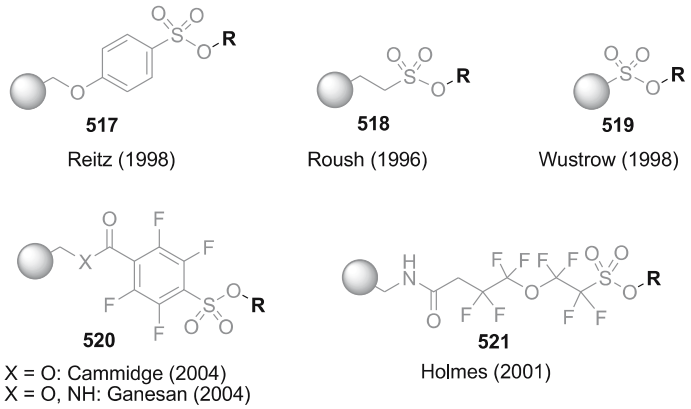
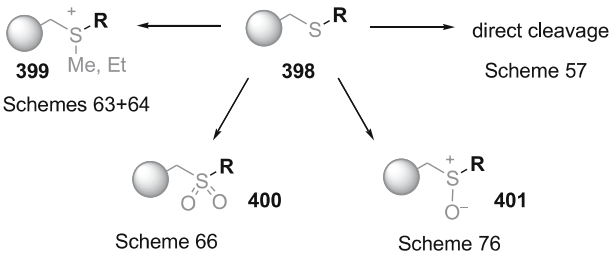


Fig. 15 Polymer-bound sulfonate ester linkers (substrates immobilized)

Sulfur-containing linkers (Fig. 14) [91, 270, 271] are a widely used class of linkers not at least because of the large amount of mild cleavage methods. Release of target compounds from sulfur linked resins depends strongly on

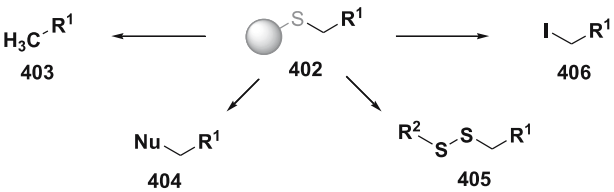


Scheme 56 Possible pathways for cleavage/derivatization of sulfides

the oxidation state of the sulfur atom. All types of sulfur linkers are generally synthesized starting from a simple sulfur(II) linkage **398**. After attachment to the resin, the heteroatom is often alkylated or oxidized to oxidation levels IV or VI but also direct cleavage without further derivation is very common (Scheme 56).

10.1 Cleavage of Non-Diversified Thioether Linkers

Direct cleavage of thioether derivatives **402** can be performed resulting in alkanes **403** or in the iodo-substituted derivatives **406**. Iodo-transformations have been known since a publication by Crosby et al. in 1977 [272]. Starting from a methyl sulfide resin and following lithiation with *n*-butyllithium, alkylarylthioethers have been synthesized that could be cleaved in 82% yield via the addition of a mixture containing MeI and NaI (Scheme 57).

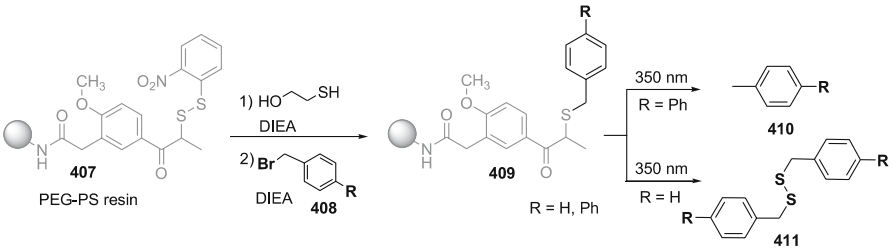


Scheme 57 Cleavage of sulfur linked aliphatic compounds

Cleavage protocols for the release of aliphatic molecules are very common [273, 274]. There exist, for example, procedures for photolabile linkers, linkers that are thermally cleavable and linkers that release target structures upon addition of hydrogenating reagents. Linkers for these methodologies are of a different nature as a result of their need to fulfil special demands.

Cleavage products from thioether linkers do not only depend on the nature of the linker and the cleavage procedure. Even slight differences concerning the loaded substrate itself can have an influence on the cleavage result as

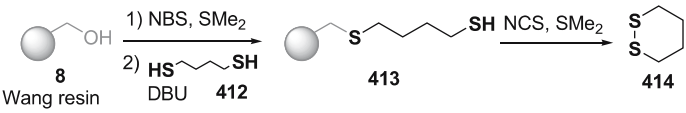
shown in Scheme 58. The example published by Sucholeiki points out that electronic effects of R can determine whether disulfide-bridged molecules **411** or the desired aliphatic derivatives **410** are formed [275, 276].



Scheme 58 Cleavage of aliphatic compounds from thioethers on solid supports

The Sucholeiki thioether linkage can be synthesized via a disulfide bond (NpSSMpact) and addition of benzylbromide **408** or alternatively via the chlorine derivative and following reaction with benzylmercaptan. Other syntheses start from thiols that are converted with electrophiles.

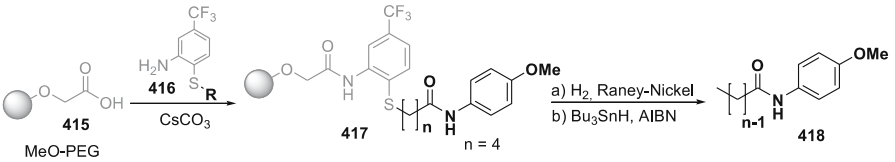
Zoller et al. used sulfide linker systems for the release of compounds containing a disulfide bridge [277]. The group investigated a methodology to obtain cyclic disulfides **414** using a complex of NCS and dimethylsulfide for the on-bead formation of an intermediate dimethyl(thio)sulfonium-ion which is attacked by an intramolecular nucleophile **413** and cyclizes spontaneously at room temperature (Scheme 59).



Scheme 59 Cleavage to give cyclic disulfides

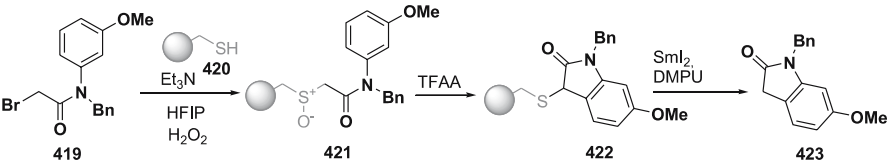
The arylthioether linker of Janda et al. has been synthesized using MeO-PEG **415** as a polymer support [273]. The sulfur-containing unit **416** has been previously prepared by adding a commercially available benzenethiol to an alkylbromine compound. The finished linker-substrate combination was then immobilized on the resin via amide coupling and examined concerning cleavage reactions. Comparison of cleavage conditions shows that the desulfurizative Raney–Nickel approach is successful with 94% yield of the product **418** within 3 h (path a). In analogy to that, conversion via a radical pathway with Bu_3SnH and AIBN gave only 40% yield within 18 h (path b, Scheme 60).

A new method of releasing compounds from solid supports is the application of SmI_2 that has been used before, for example, to cleave ether bonds on solid supports (HASC linker) [278]. The group of Procter applied this idea



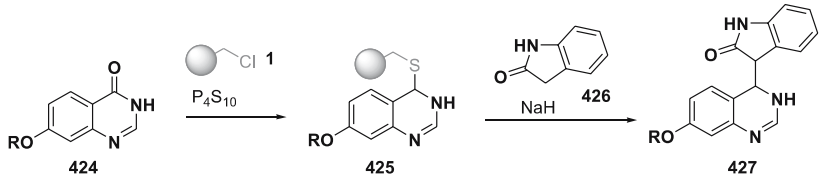
Scheme 60 Cleavage from thioether resins via hydrogenation

to the cleavage of resin-bound oxindoles **422** that are released in a traceless manner as shown in Scheme 61 [269]. Alternatively, the same oxindoles can be further manipulated and cleaved via oxidation of the sulfur atom (see SO_2-linker).



Scheme 61 Traceless cleavage from thioether resins with SmI_2

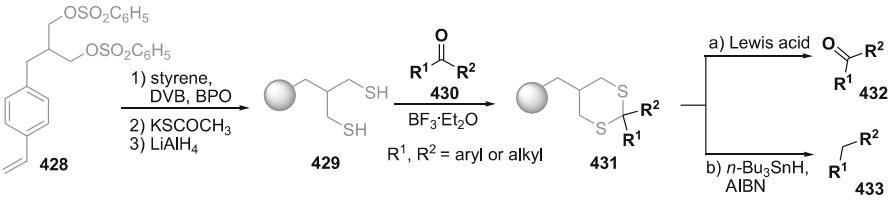
To our knowledge there is only one publication concerning cleavage of thioether linkers via attack of carbon nucleophiles. The approach developed by Hennequin et al. in 1999 describes the utility of deprotonated oxindol ring systems **426** for the nucleophilic displacement of the solid support **425** [279]. The substrate-resin complex has been synthesized very easily by coupling of a quinazoline core **424** to Merrifield resin (**1**) in the presence of P_4S_{10}. The resin-bound thioquinazolone **425** was found to be stable under Mitsunobu conditions and towards an acidic environment. Conversion with oxindole-anions has to be conducted with a great excess of the reagent due to its tendency towards polymerization. After purification, the target compounds **427** have been isolated in 35–68% yield (Scheme 62).



Scheme 62 Replacement of thioether linkers by carbon-nucleophiles

Dithianes are known as protecting groups for safety-catch linkers [66] but they can also be used as linker systems for the synthesis of either carbonyl-containing or aliphatic compounds. Bertini et al. [280] synthesized the first

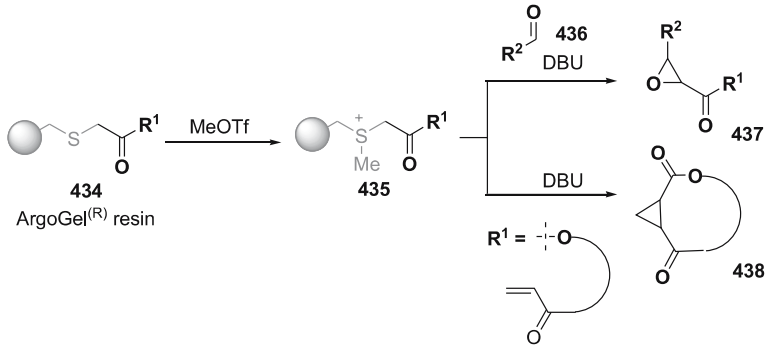
linker unit that can be attached to polymers and that is able to immobilize ketones and aldehydes in the form of their dithiane derivatives (Scheme 63). Starting from 4-chloromethylstyrene, the linker **428** could be prepared within a three-step synthetic route. The styrene derivative **428** was then added to styrene and radical starters to give the desired polymer support by copolymerization. Transformation to the dithiol derivative **429** has been performed via addition of potassium thioacetate and reduction of the resulting thioesters with $LiAlH_4$. The dithiol linker is able to catch ketones and aldehydes **430** if catalysts like $BF_3{\cdot}Et_2O$ are added. Aliphatic compounds **433** can be obtained by a cleaving strategy using *n*-Bu_3SnH and AIBN (path b), whereas the corresponding carbonyls **432** are obtained by the addition of different Lewis acids or other deprotecting methods known for dithianes (path a) [281, 282]. A similar dithian linker was developed by Huwe and Künzer in 1999 [283].



Scheme 63 Synthesis and cleavage possibilities of dithiane-linkage

10.2 Cleavage via Sulfonium-Ions

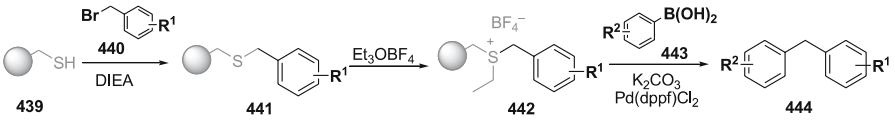
Thioether-containing substances can be transformed into the corresponding sulfonium ions via addition of Et_3OBF_4 or MeOTf. Alkylation leads to an activation of the sulfur atom and can be used for the release of epoxides or cyclopropanes from solid supports as shown by Gennari et al. [284] (Scheme 64).



Scheme 64 Cleavage of sulfonium-ions

Starting from ArgoGel®-SH resin (**434**) the linker core has been synthesized within four steps. After modification of the substrate on the solid support, the linker core is activated by the addition of an alkylating agent in combination with DBU to create the sulfonium ylide **435**. Reaction of these ylides with various aldehydes **436** results in the formation of epoxides **437**. The cleavage reaction is very selective and therefore gives products of high purity. A similar methodology has been applied in order to synthesize cyclopropane derivatives via 1,4-addition of ylides to Michael acceptors. As the Michael acceptor is part of the linker core, macrocyclic compounds **438** are released in a *trans*-selective manner upon activation with MeOTf and base-induced formation of ylides. This synthetic route has been proven on functionalized Merrifield resins and on ArgoGel resin.

Another carbon–carbon bond-forming methodology has been published by Wagner and Wioskowski who released biarylmethanes from benzylthio-resins in excellent yields [285]. The approach is successful by addition of benzylbromides **440** carrying electron-withdrawing, electron-donating or heterocyclic units to alkylthio-Merrifield resin **439** and following activation/alkylation of the resin. To achieve C – C bond formation to give biarylmethanes **444**, areneboronic acids **443** were added and cross-coupled in the presence of $Pd(dppf)Cl_2$ (Scheme 65).

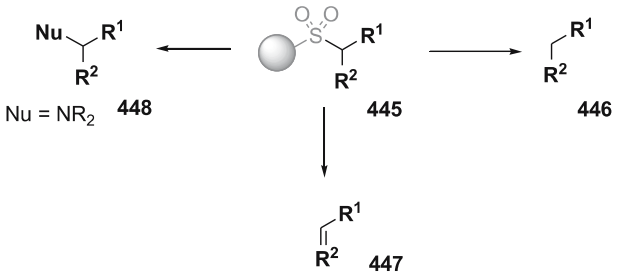


Scheme 65 Synthesis of biarylmethanes via sulfonium-ions

10.3
Cleavage via Oxidation to Sulfones/Attachment of Sulfones

There are a few routes to synthesize sulfone functionalities for solid phase chemistry. One possibility is the reaction of lithiated polystyrene beads with sulfur dioxide to deliver polymer-bound lithium phenyl sulfinate as a linker system to which allylic compounds can be easily attached [286]. Other syntheses start from the corresponding thioether linkage followed by oxidation to the sulfone. Mostly, sulfur-containing ether-linkers that are oxidized just before cleavage from the resin are presented separately from those which are directly bound to the resin in their oxidized form. This differentiation is not done in this review because it does not make any difference concerning the cleavage possibilities that are shown in Scheme 66.

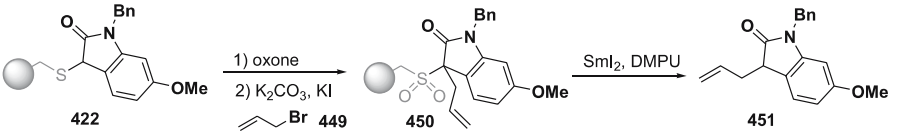
Depending on the nature of the resin-bound substrates, cleavage protocols can provide an (additional) alkene functionality **447** or compounds **448** that are substituted by nucleophiles on the sulfone anchoring side.



Scheme 66 Cleavage possibilities from sulfone linkers

It was proven earlier by Janda et al. that resin **394** can be alkylated by various alkylbromines to give either secondary or tertiary carbon centers. These compounds can be cleaved to give alkanes by the use of quite harsh reaction conditions like Raney–Nickel [273]. With the oxidation of the sulfide linker to the sulfone linker **445**, smoother and more selective reagents can be used for the release of hydrogenated compounds **446**. Oxidation was performed using oxone and the resulting linker could be cleaved via addition of 5% Na – Hg.

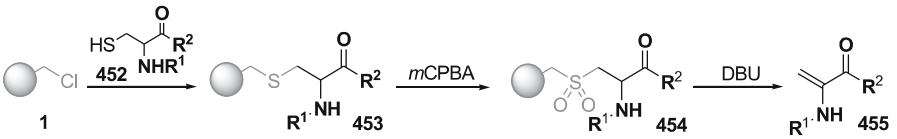
More recently published procedures describe the traceless release of aliphatic structures from simple benzylthiol resins **422** via oxidation with oxone and following treatment with SmI_2 (Scheme 67) [269].



Scheme 67 Cleavage of aliphatic compounds via SmI_2/DMPU procedure

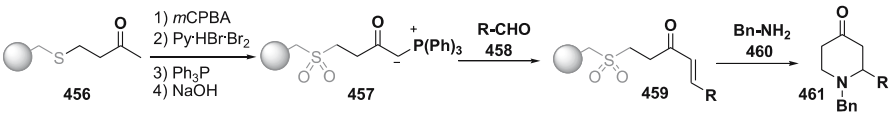
Sulfone linkers, especially those bearing carbonyl functionalities in the β-position can possess acidic protons. Via addition of base, these protons tend to be eliminated and therefore induce the release of olefinic compounds. Yamada et al. [287] as well as Barco et al. [288] used these properties to synthesize α,β-unsaturated carbonyl-containing compounds.

Yamada et al. released enamine-derived structures **455** from resin-bound cysteine derivatives **453** via β-elimination and simultaneous cleavage of the sulfone. DBU was found to be the right base for inducing the elimination process (Scheme 68).



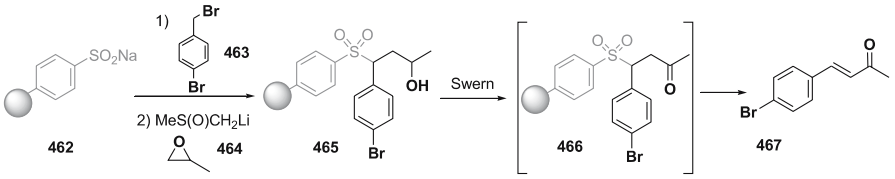
Scheme 68 Enamine formation via β-elimination on sulfone linkers

A similar strategy has been investigated by Barco et al. in their approach to the synthesis of piperidin-4-one derivatives **461** (Scheme 69). Starting from Merrifield-SH resin, Barco et al. prepared 4-benzylsulfonyl-1-triphenylphosphoranylidene-2-butanone **457** via addition of butanone/AcOH, oxidation of the sulfide to sulfone and selective bromination of the terminal methyl group. After reaction with aldehydes **458** to α,β-unsaturated ketones **459** a cyclization-elimination process occurs via addition of N-nucleophiles like benzylamine **460.**



Scheme 69 Elimination from sulfone linkers and incorporation of nucleophiles

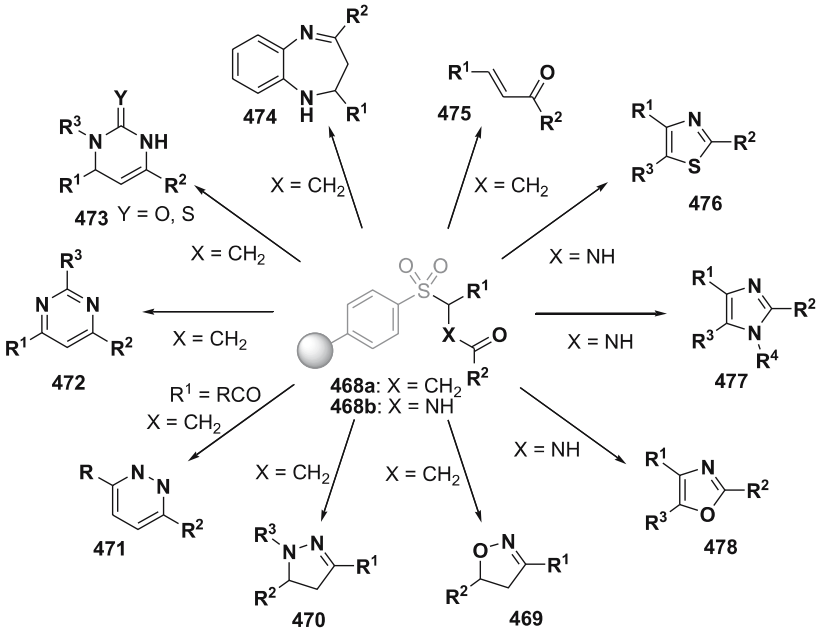
Kurth et al. developed a methodology to cleave resin-bound β-carbonyl-containing sulfones via deprotonation in the α-position and following β-elimination of the sulfinate. This concept is applicable for substances bearing alkyl or aryl residues in the α-position to the sulfone (Scheme 70) [289].



Scheme 70 Synthesis of α,β-unsaturated ketones

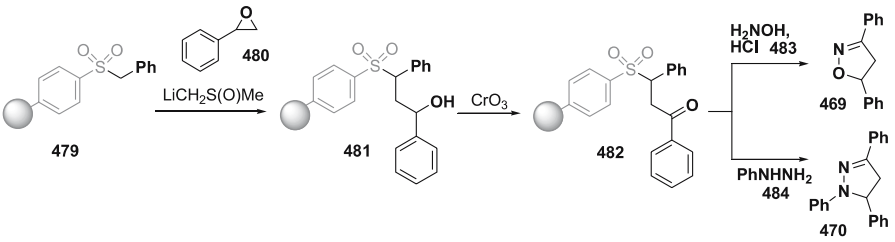
The synthesis of the linker starts with resin-bound sulfinate **462** that is alkylated firstly with bromobenzylbromide **463** and secondly with propylene oxide **464.** Selective monoalkylation has been achieved for both steps and the resulting alcohol **465** was oxidized via Swern oxidation yielding ketone **466.** The basic conditions of Swern oxidation are in this case sufficient for elimination and thus α,β-unsaturated ketones **467** are released from the resin. By slight variation of the aliphatic resin-bound compound, Kurth et al. proved the success of the oxidation/elimination model for the release of cyclic derivatives like cyclopentenones [290].

The protocol consisting of alkylation followed by oxidation and base-induced elimination of sulfones can also be found in the strategy of other solid phase syntheses. Lam et al. for example constructed α,β-unsaturated ketones **475,** [291] as well as various heterocyclic compounds **469–474** and **476–478** following this route. Scheme 71 gives an overview of possible structures obtainable from sulfone-linked β-carbonyl compounds **468a,b.**



Scheme 71 Cleavage of sulfone linkers containing β-carbonyl functionality

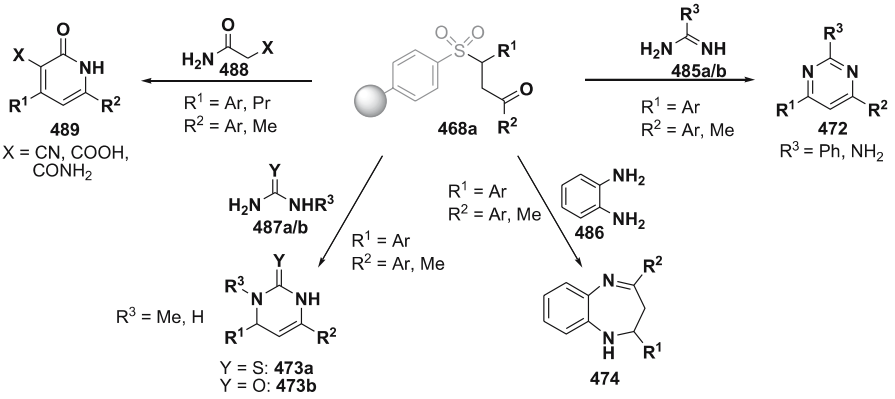
Lam et al. synthesized isoxazolines **469** and pyrazolines **470** via monoalkylation with dimsyl anion and styrene oxide **480** and following an oxidation/elimination strategy [292]. Oxidation of secondary alcohols **481** is performed via Jones oxidation and cyclization with cleavage from the resin follows in a separate step. Diverse elements can be introduced to the heterocycle using either hydroxylamine **483** or hydrazine derivatives **484** (Scheme 72). Corresponding six-membered heterocycles like pyridazine derivatives **471** can be obtained when the linked molecule **482** bears a benzoyl substituent instead of the phenyl substituent at the α-C-atom [293].



Scheme 72 Synthesis of isoxazolines and pyrazolines

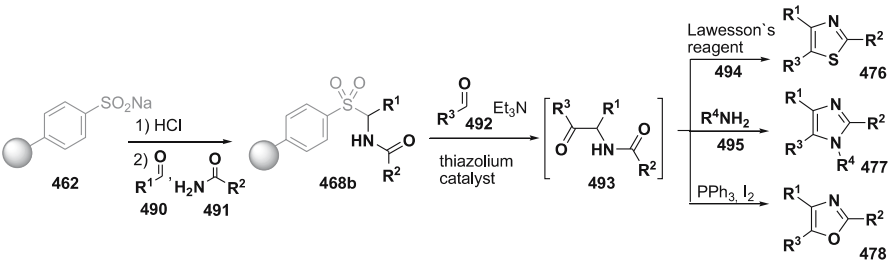
Pyrimidine-2-thiones **473a**, pyrimidine-2-ones **473b**, pyrimidines **472** and benzodiazepines **474** have been synthesized on solid phase via a similar re-

action pathway in order to find compounds with enhanced binding affinity to neuronal sodium channels. The synthesis of the core linker unit **468a** is performed by alkylation of sulfinate **462** and a second alkylation step in the presence of a dimsyl anion. As a second alkylation reagent epoxides were used which produce the desired γ-hydroxy functionality while adding to the resin. After Jones oxidation the addition of various thioureas **487a**, ureas **487b**, benzamidine hydrochlorides **485a**, guanidine hydrochlorides **485b** and phenylene diamines **486** afforded the target compounds by a one-pot elimination-cyclization process (Scheme 73) [294, 295]. Different 2-pyridones **489** have been synthesized via addition of cyanoacetamide, malonamide or methylmalonate monoamide **488** to the linker core unit.



Scheme 73 Synthesis of 2-pyridones, pyrimidine-2-thiones, pyrimidine-2-ones, pyrimidines and benzodiazepines

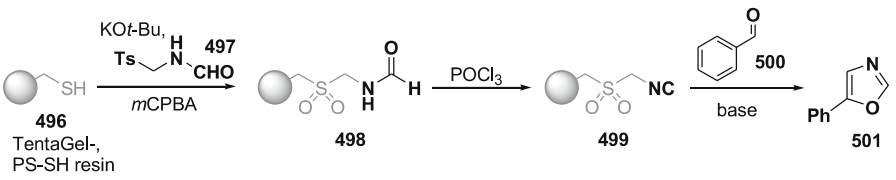
A further synthesis by Lam et al. dealing with β-ketosulfones is described in Scheme 74. In that approach, resin-bound arylsulfonylamides **468b** are synthesized instead of their ketone equivalents **468a** (Scheme 73). The synthesis of the linker includes the reaction of the sulfinate resin **462** to sulfinic acid with subsequent addition of aldehydes **490** and amides **491** to give amide-



Scheme 74 Synthesis of imidazoles, oxazoles and thiazoles

containing sulfone resin **468b**. Cleavage of the intermediates from the resin can be achieved by treatment with thiazolium catalyst and triethylamine to result in acylamines that are coupled with aldehydes **492** to give α-ketoamides **493**. Depending on the desired heterocyclic target structure, diversity containing reagents are added to induce cyclization procedures to give thiazoles **476** (addition of Lawesson's reagent **494**), imidazoles **477** (addition of amines **495**) or oxazoles **478** (addition of PPh_3, I_2) [296].

Another approach to oxazoles was published by Ganesan and Kulkami in 1999 [297]. The amide-containing linker was synthesized by addition of KO*t*-Bu and *N*-(p-tosylsulfonylmethyl)formamide **497** to TentaGel-SH resin **496**. The resulting thioether is oxidized by *m*CPBA to the corresponding sulfone formamide **498** and further derived to give resin-bound isonitriles **499** in the presence of $POCl_3$. Oxazoles **501** are built up using these isocyanide linkers by reaction with benzaldehyde **500** and an appropriate base. Reaction yields depend strongly on the choice of base as a comparison of several bases show. Yields rank from 0% for NaOEt up to 50% for tetrabutylammoniumhydroxide. It is also noticed that the purities of the released oxazoles are quite good if polystyrene-SH resins are used but that—due to a possible breakdown of the PEG linker—impurities increase as soon as TentaGel-SH is chosen (Scheme 75).

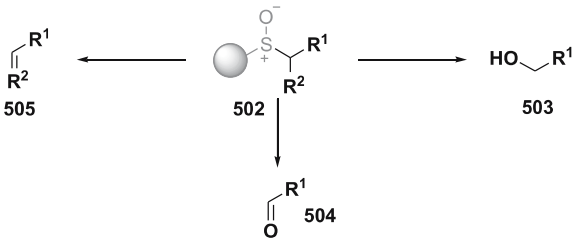


Scheme 75 Synthesis of oxazoles via benzaldehyde addition to sulfones

10.4 Cleavage via Sulfoxide-Linkers

Sulfoxide linkers can be synthesized via addition of a sulfoxide linker to the resin or via transformation of a sulfide linker to the corresponding oxo-derivative. Many oxidation reagents like ozone or *m*CPBA to convert thioethers are very unselective regarding sulfoxide/sulfone formation but methodologies like acid-catalyzed oxaziridine oxygen transfer [298], H_2O_2/HFIP oxidation [299] or reaction with a *t*BuOOH/CSA-mixture [300] can be applied successfully.

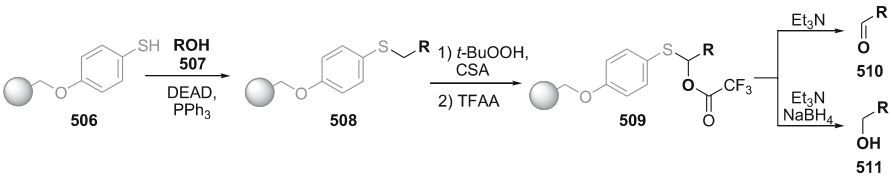
Sulfoxides in general are first of all multifunctional linkers because of their possible over reaction to the corresponding sulfones and the resulting possibilities of sulfone cleavage. Additionally, the sulfoxide linker **502** can release primary alcohols **503**, aldehydes **504** and alkene derivatives **505** without fur-



Scheme 76 Cleavage possibilities from sulfoxide linkers

ther over-oxidation. Scheme 76 demonstrates possible transformations via cleavage from the solid phase but it has to be considered that these cleaving procedures depend strongly on the nature of R^2.

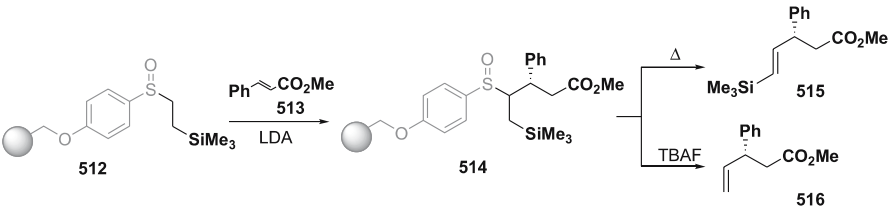
Scheme 77 presents a safety-catch linker for the preparation of alcohols or aldehydes developed by Li et al. [300]. The recently published method describes the attachment of various alcohols **507** to a modified Merrifield resin with hydroxythiophenol structure **506** under Mitsunobu conditions. The resin-bound substrates **508** can be further modified via, for example, alkylation and introduction of diversity containing reagents. The release from the resin includes three steps: firstly the oxidation of the sulfur atom with *t*-BuOOH/CSA, secondly the addition of trifluoroacetic acid anhydride to give resins that can perform Pummerer rearrangements. Structures like trifluoroacetoxythioacetals are formed in consequence and can be cleaved to give aldehydes **510** in a third step by treatment with base. The aldehydes can be reduced in situ to the corresponding alcohols **511**. Various electrophiles have been attached to the Merrifield-hydroxythiophenol resin and gave alcohols and aldehydes in 48–84% yield.



Scheme 77 Release of primary alcohols and aldehydes from sulfoxide resin

A similar approach was followed one year earlier by Solladié et al. in order to release alcohols from sulfoxide resins [298]. In contrast to the method of Li et al. a Wang-type linker system was used on which the sulfur-linker unit is attached. Beyond the Pummerer-arrangement induced cleavage methodology, the linker can also be cleaved giving phenol derivatives according to its Wang-type origin.

Toru et al. [301] developed a protocol for the release of alkenes from trimethylsilylethylsulfoxide resins (Scheme 78). The linker core **512** was syn-



Scheme 78 Cleavage protocol for alkenes from sulfoxide linkers

thesized via addition of previously formed hydroxyphenyl β-silylethyl sulfoxide to Merrifield resin. Conjugate addition to the sulfoxylinker with methyl cinnamate **513** in the presence of LDA was performed to construct the target structure **514** on a polymer support. Cleavage occurs via thermally induced elimination that is controlled by the trimethylsilyl functionality. The target structure (*R*)-**515** is obtained with 75% ee. The resulting yields and stereoselectivities of the conjugate addition depend on the nature of the spacer. If the spacer is not phenyl as shown in the example but biphenyl, yields as well as the ees increase. With a butanediol-linker unit between the Merrifield resin and target structure, yields are quite good at the expense of enantiomeric excess. The linker can also be cleaved to give vinyl-derivatives **516** while adding TBAF to the resin.

11 Sulfonyloxy Linkers

The importance of sulfonyloxy—or sulfonate ester—linkers results from their stability under several conditions including compatibility with Grignard additions, Wittig reactions, $NaBH_4$-reduction, reductive aminations, acylations, Suzuki couplings and treatment with various other electrophiles. Some linkers of the sulfonate ester type are shown in Fig. 15.

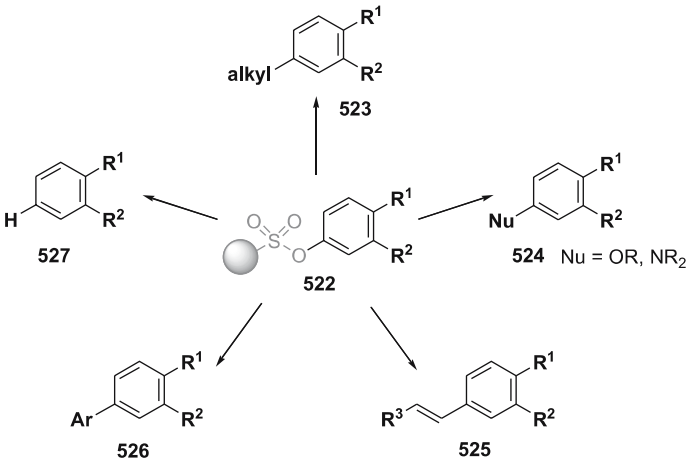
The cleavage from arylsulfonate ester linkers on solid phase goes back to the investigations of Wustrow et al. in which the palladium-catalyzed reductive cleavage of arylsulfonate esters to their hydrogenated equivalents via aryl – O bond break is examined [302].

Tsukamoto et al. recently reinvestigated the utility of sulfonate ester linkers for deoxygenation and multifunctional purposes [303]. The linker-substrate moiety was synthesized by addition of phenolic components to commercially available polystyrene sulfonyl chloride. As non-activated benzenesulfonyloxy linker units are quite unreactive towards palladium catalyzed reactions, the choice of adequate ligands can help to overcome this problem. Cleavage studies showed that benzene derivatives with various functional groups could be released with the optimized protocol including the use of PPF-*t*-Bu as catalyst in an amount of 20% at 120 °C. Although the benzenesulfonyloxy

linker is stable under conditions applicable for many cross coupling reactions, multifunctional cleavage is achieved by the use of $Ni(PCy_3)_2Cl_2/PCy_3$- or $Pd(OAc)_2$/XPHOS-based catalytic systems in combination with microwave irradiation for the latter one. Miyaura–Suzuki cross-coupling reactions could be used to cleave substrates by conversion of the resin-bound substrate with methylboronic acid, aminations could be performed using *N*-methylaniline and α-arylation of cycloheptanone has also been performed.

It has been shown recently by Park et al. that also Grignard reagents can be used to cleave sulfonate esters [304]. For this traceless biphenyl-forming reaction Ni(O)-catalysts ($NiCl_2$dppe) have to be added to permit *ipso* nucleophilic aromatic substitution of the alkyloxysulfonyl groups.

Scheme 79 [302, 305–307] shows possible cleavage strategies that can be conducted starting from aryl-residues bound to sulfonate ester linkers. Most of these transformations have been proven for the tetrafluoroarylsulfonate ester linker **520** synthesized by the groups of Cammidge et al. [306] and Ganesan et al. [307]. Both groups synthesized a tetrafluoroaryl linker with benzoic acid chloride and sulfonic acid chloride functionality. The linker core has been tethered to different resins via ester or amide bonding and allows the attachment of various alcohols using DIEA as the base.

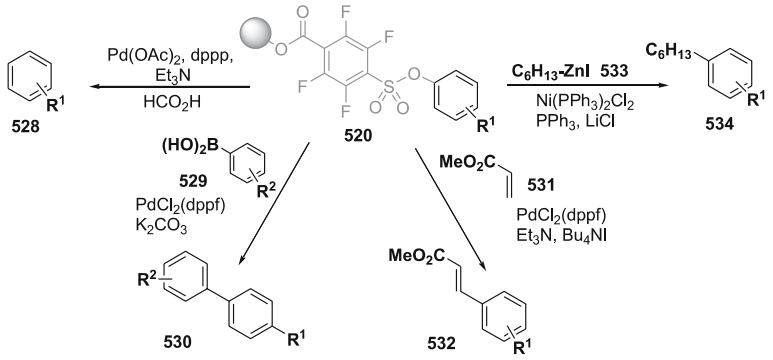


Scheme 79 Cleavage opportunities for aryl components attached on sulfonate ester linker

The tetrafluoroarylsulfonate ester linker **520** is sometimes regarded as a solid phase equivalent to the triflate group in solution phase organic chemistry. Although the benzenesulfonyloxy linker can be cleaved via aryl-O bond cleavage, its reactivity is, due to its modest activating ability, quite low. In contrast, conversion of alcohols with a fluorinated linker results in the formation of a good leaving group and conversion of phenols with this linker type gives esters with very weak electron-deficient O-aryl bonds. For this reason, the in-

sertion of transition metals is facilitated and many reactions comparable to those of arylbromides are possible. A similar effect can be achieved with only two fluorines on the aryl resin. This linker type has also been synthesized but the electron-withdrawing nature of that linker is weaker and harsher cleavage conditions have to be chosen.

The sulfonate ester **520** is able to release hydrogenated or deuterated compounds **528** by addition of HCO_2H or DCO_2D, triethylamine and $Pd(OAC)_2$-dppp as the catalyst-ligand system. Carbon–carbon bond-forming reactions have also been combined with the simultaneous cleavage of the linker. Thus, the Mizoroki–Heck reaction as well as Suzuki–Miyaura reaction have been performed on solid phase and gave styrene **532** and biaryl systems **530**, respectively (Scheme 80).



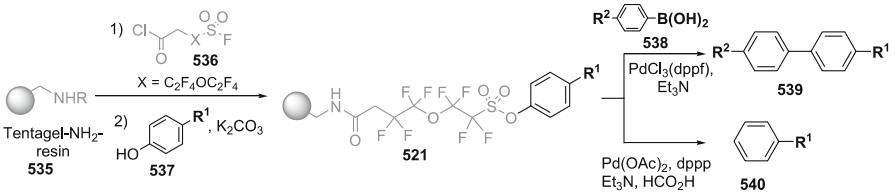
Scheme 80 Cleavage possibilities of fluorinated arylsulfonate esters

All cross-coupling approaches on the sulfonate ester linker have been tested for various substrates R^1 bound to the polymer, proving that the methodology is compatible with several aromatic systems containing ketones, pyridines, lactones, aldehydes and fluoro-substituents.

Beyond the C – C bond-forming approaches of Ganesan et al. who formed biarylic compounds and studied Heck reactions on sulfonate ester linkers, Cammidge et al. developed methods to create carbon–carbon bond-forming reactions for aryl as well as alkyl moieties. For the addition of aliphatic residues to aryl components the Negishi coupling has been successfully adapted to the solid phase to give target compounds **534** in about 75% yield.

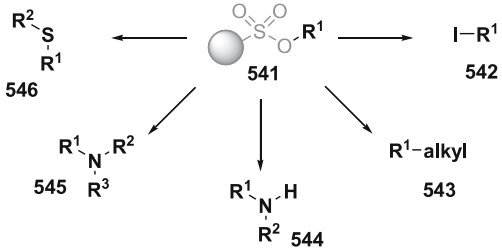
Other fluorine-containing linker systems have been developed to release target substances via cross-coupling reactions. Holmes et al. published an access to polyfluorous alkyl linker systems that are compatible with cross coupling reactions and reduction. The perfluoroalkylsulfonyl (PFS) linker **536** was synthesized via four steps from its commercially available iodide [308, 309]. Reaction of the terminal iodide with ethyl vinyl ether and following hydrolysis to an aldehyde prolonged the alkyl chain by two carbons. Oxida-

tion of the aldehyde with NaO_2Cl and chlorination using oxalylchloride gave acid chloride **536** (overall yield of about 60%) which was attached to resin **535**. Suzuki couplings were performed yielding compounds **539** and traceless detachment of the substrates to give the corresponding arenes **540** were successful as well (Scheme 81).



Scheme 81 Cleavage from perfluoroalkylsulfonyl (PFS) linker

Cleavage protocols for aliphatic compounds bound to arylsulfonate ester linkers are mostly concentrated on substitution via nucleophilic cleavage of the substrates via attack of heteroatom nucleophiles like amines, thiolates or imidazoles as shown in Scheme 82 [310–313].



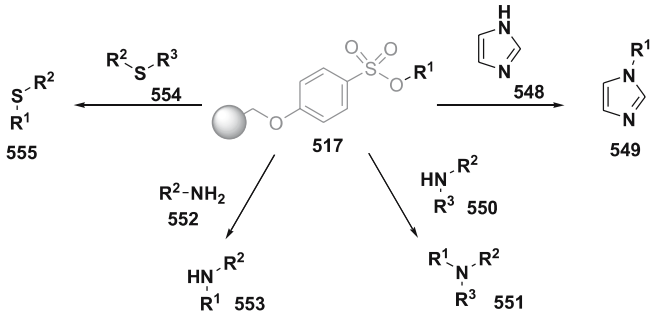
Scheme 82 Multifunctional cleavage of sulfonate esters

A simple route to sulfonate esters has been presented by Reitz et al. [310, 311] who converted Merrifield resin with *p*-hydroxy arenesulfonylchlorides to give the corresponding sulfonylchloride resin to which alkylhydroxides can be easily attached in the form of their sulfonate esters **517**.

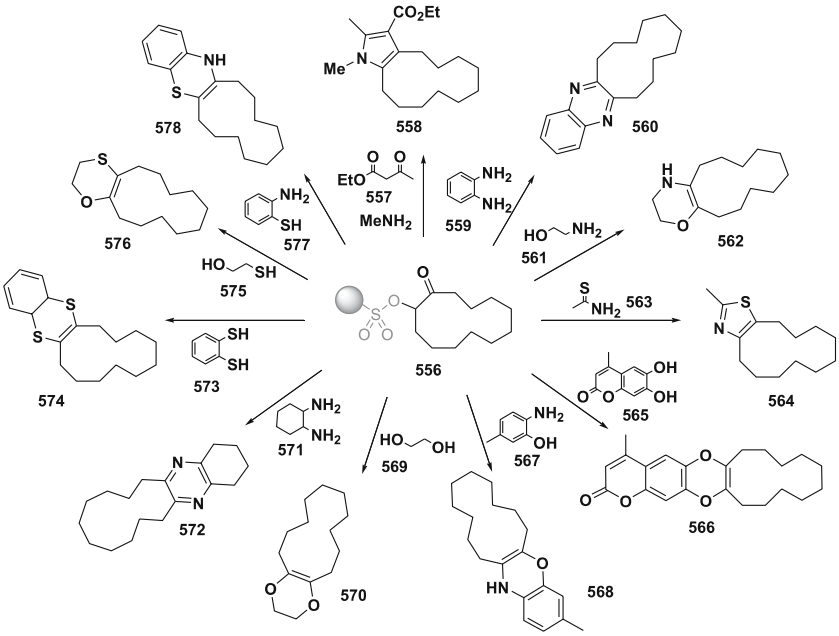
The developed sulfonated ester linker **517** has been employed for the synthesis of various amines **549**, **551**, **553** and thioethers **555** (Scheme 83). The nucleophilic cleavage of the alkyl-O bond is facilitated because of the transformation of hydroxy functionalities into good leaving groups.

Other nucleophiles can be chosen to perform substitutions on sulfonate ester linkers. Roush et al. synthesized alkyliodides **542** via cleavage of oligosaccharides from Merrifield-type resins **541** by addition of NaI to the resin-bound substrates [314].

Scheme 84 is just a selection of possible reaction pathways cleaving sulfonate esters **556** with keto-functionality. Nicolaou et al. developed a method-



Scheme 83 Nucleophilic substitution on polymer-supported sulfonate esters

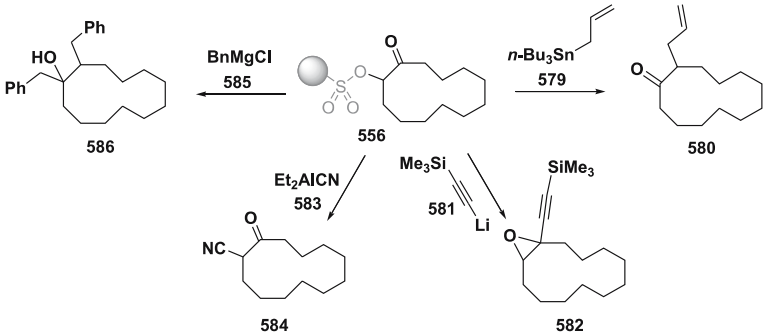


Scheme 84 Reaction of resin-bound α-ketone sulfonate esters with various nucleophiles

ology to introduce diversity during the release of α-sulfonated ketones from the resin giving heterocyclic structures [315]. The cycloalkanone has been synthesized on a polymer support via addition of the corresponding alkene or epoxide to polystyrene sulfonic acid. The resulting carbonyl group in the α-position to the sulfonate ester is able to activate the linker unit for nucleophilic substitution but other transformations can also be used to create diverse structures. Photolytic cleavage of the polymer-bound sulfonate ester as well as oxidation-cleavage strategies have been performed successfully and reactions with compounds containing at least two heteroatoms (e.g.

ethylene glycol **569**) result in the formation of heterocyclic structures. This heterocycle-release strategy has been applied to the synthesis of dioxines **566**, dihydrodioxines **570**, dihydrooxazines **562**, thiazoles **564**, thianthrenes **574**, oxathienes **576**, pyrazines (**560**,**572**), benzoxazines **568**, benzothiazines **578** and pyrroles **558**. Besides these examples, other nucleophiles like phenols, thiols and carboxylic acids have been shown to be able to cleave sulfonate esters **556** via nucleophilic attack.

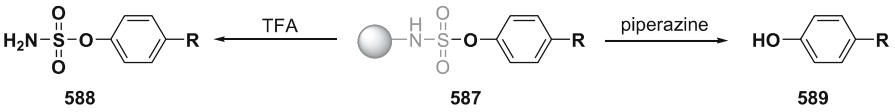
Nicolaou et al. used the same sulfonate ester system to prove the compatibility of sulfonate esters with carbon–carbon bond-forming reactions [305]. Functionalization and a simultaneous cleavage procedure have been applied successfully by the use of Grignard reagents **585**, the Nagata reagent **583** and stannane as well as lithium organic compounds (**579**, **581**). All compounds namely α-allyl ketone **580**, epoxide **582**, α-cyano ketone **584** and carbinol **586** could be isolated in 65–85% yield (Scheme 85).



Scheme 85 Carbon–carbon bond-forming reactions on resin-bound sulfonate esters

12
Sulfamate Linkers

Another example for a multidetachable linker system is the sulfamate linker **587** used by Ciobanu and Poirier to develop α-substituted estradiol sulfamate and phenol libraries [316]. The linker was synthesized via the attachment of a sulfamate group (bearing steroidic compounds) to trityl chloride resin. After modification of the steroids on solid supports, the target molecules



Scheme 86 Cleavage from sulfamate linker yielding sulfamates or phenols

could be cleaved by two pathways either releasing the sulfamates **588** or yielding the corresponding phenols **589** (Scheme 86).

13 Selenium Linkers

Selenium linkers show reactivities comparable to those of sulfur linkers but selenium has several advantages due to the weaker C-Se bond. In addition to that, selenium can often be oxidized faster than sulfur and selenium linkers are therefore easier to cleave.

The first synthetic examinations of selenium linkers were conducted on resins that were either synthesized via polymerization of *p*-vinylphenylselenol or by modification of bromopolystyrene with K_2Se [317].

Selenium-based linkers **590** can also be synthesized from polystyrene via lithiation and subsequent addition of dimethyldiselenide to the polymer support. Reaction of bromine gives selenenyl bromide that can be reduced to the lithium selenium resin in the presence of $LiBH_4$ that is alkylated subsequently [318]. Other approaches include the addition of former synthesized selenium-containing building blocks to the resin, for example, the linker systems generated by Elofsson et al. [319] and Nakamura et al. [320, 321].

In a very simple manner, starting from Merrifield resin, reaction with KSeCN forms a resin-bound methylselenocyanate that can be alkylated to give immobilized compound **591** [322].

Selenium linker systems tend to be oxidized in the presence of reagents like *m*CPBA or H_2O_2 and their cleavage properties are similar to those of sulfur linkers. Two general possible pathways to release compounds from the selenium linker systems **597** are responsible for a lot of functionalities that can be formed successfully from those linkers (Scheme 87). On the one hand,

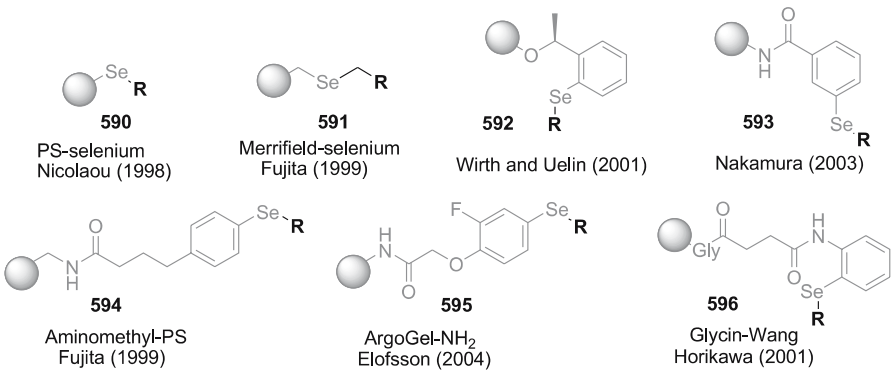
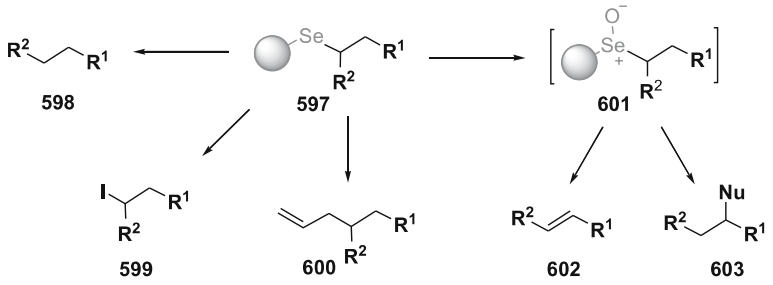


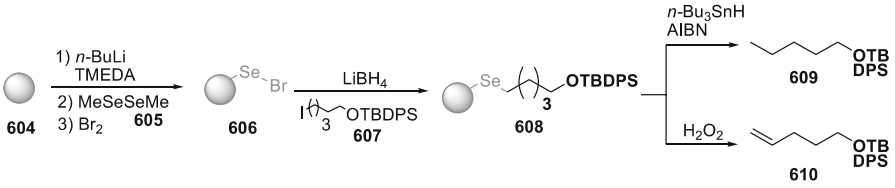
Fig. 16 Selenium-based linkers



Scheme 87 Possible cleavage reactions from selenium-based linkers

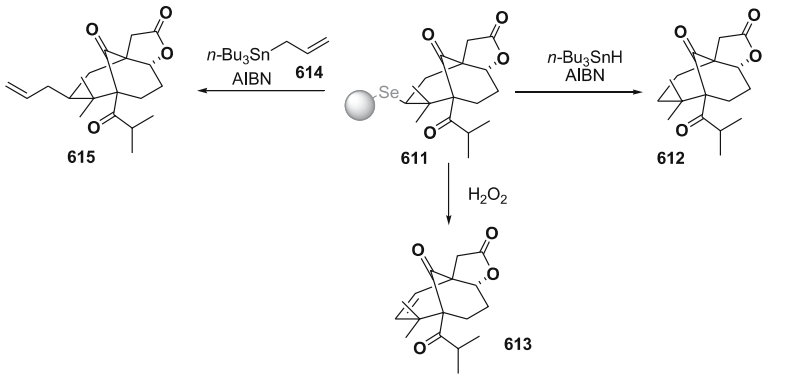
the selenium linkers can be directly cleaved radically by the addition of reducing agents like *n*-Bu_3SnH in the presence of AIBN to give the aliphatic derivatives **598**. Substitution via the attack of iodine or other nucleophiles is also performed on the non-oxidized resins. On the other hand, the oxidation of selenium linkers to their corresponding selenoxide resins **601** offers the possibility to firstly convert the immobilized structures via elimination into alkenes **602**. Secondly, this elimination reaction pathway can be used to add nucleophiles at the α-position of the selenoxide linker to give compounds **603**.

Scheme 88 demonstrates the compatibility of selenium linker systems with reductive, eliminative and nucleophilic conditions. The linker has been synthesized from polystyrene **604** via lithiation and quenching with dimethyldiselenide **605** as mentioned above. Reaction to terminal alkenes **610** is performed via an oxidation-elimination process, induced by the addition of H_2O_2. Alkyl compound **609** is released using radical chemistry with a combination of AIBN and *n*-Bu_3SnH [323].



Scheme 88 Reduction and elimination during cleavage reaction

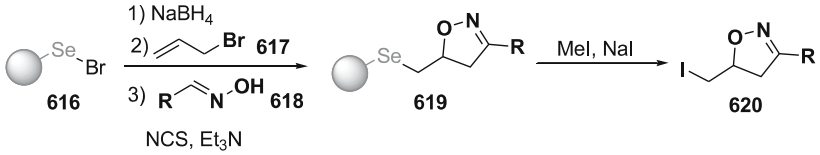
Scheme 89 presents the work of Nicolaou et al. concerning synthesis and release of substituted [3.3.1]bicycles on solid supports [324]. In order to investigate selenium-mediated cyclization reactions, Nicolaou et al. constructed a selenium-based linker system on solid phase and performed selenium-induced endocyclizations which gave immobilized polyfunctionalized bicycles **611**. These structures can be cleaved to give the alkyl and alkenyl derivatives **612** and **613** but Nicolaou proved also that carbon-carbon bond-forming reactions can be applied to that linker chemistry. Using a radical mechanism, the group succeeded in the coupling of an allyl group in the *ipso*-position



Scheme 89 Multifunctional cleavage from selenium linkers

to the former selenium linker via addition of AIBN and organo-stannane reagent **614.**

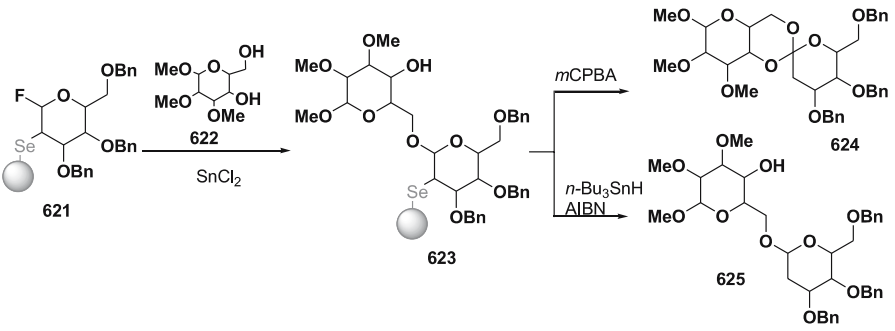
Scheme 90 shows a synthetic pathway to terminal iodines via cleavage from selenium linkers that was found by Huang et al. [325]. The transformation into the iodine derivatives **620** has the advantage that harsh cleavage conditions can be avoided and further transformations can be done after isolation of the product. Moreover, the same group developed a selenium linker which was applied to generate methylenecyclopentanes via an oxidation-elimination reaction using H_2O_2. Alternatively, cyclopentanylmethyl alcohols were obtained by employing a hydroboration-oxidation process using 9-BBN, H_2O_2 and NaOH [326].



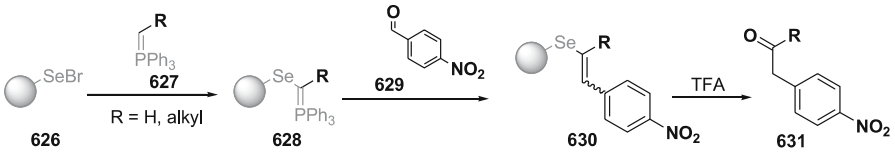
Scheme 90 Release of selenium-bound compounds as their iodides

The addition of nucleophiles to the α-center of the selenium linker has been described by several groups among them Nicolaou et al. who investigated the formation of several spiro compounds **624** upon cleavage from solid supports [327] (Scheme 91). Crucial for these transformations are heteroatom-nucleophiles which can attack the released compounds after selenium linker oxidation and elimination. Following the above-mentioned and similar routes, Nicolaou et al. synthesized diverse orthoesters and allylic orthoesters [328].

Beyond these general possible cleavage reactions, there are other possibilities to cleave from selenium-containing resins that should be mentioned



Scheme 91 Orthoformate cleavage from selenium linkers



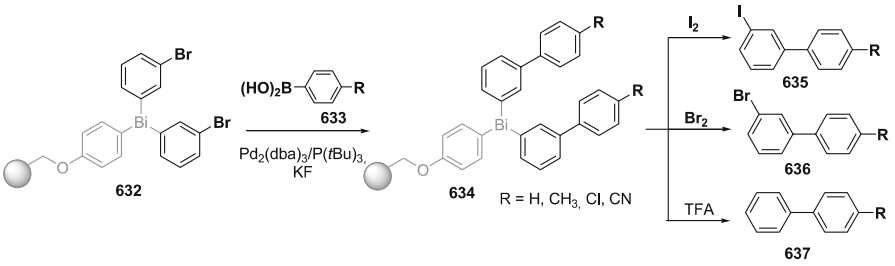
Scheme 92 Release of ketones from selenium linkers

here. Is for example the selenium linker attached to an unsaturated carbon atom, the addition of TFA leads to an additional cleavage opportunity [329]. Compounds of this vinylic linker **630** with hydrogen residue R are cleaved as aldehydes **631** (R = H) whereas compounds with R = alkyl are released as their ketone-derivatives (Scheme 92).

14
Bismuth Linkers

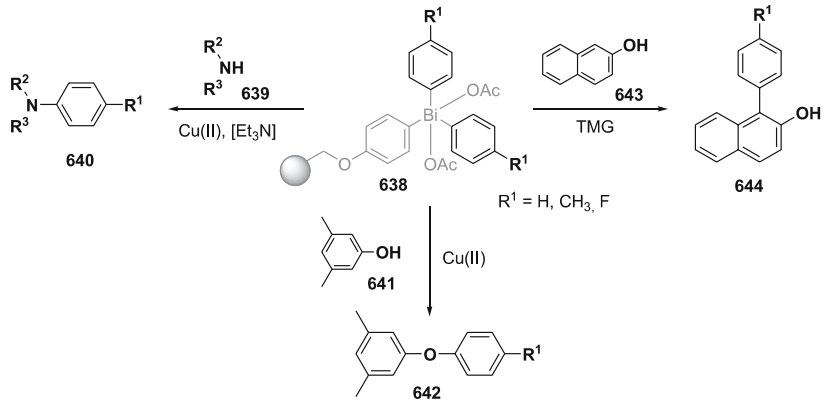
There are only two examples for multifunctional bismuth linker systems developed by Ruhland et al. The first one is the triarylbismuthane linker **632,** [330] that is generated starting from dibromobenzene, *n*-butyllithium and trichlorobismuthane to give tris(3-bromophenyl) bismuthane which is converted into the corresponding *bis*(3-bromophenyl)bismuth triflate·2 HMPA complex. This complex reacts with resin-bound Grignard reagent affording the resin-bound triarylbismuthane linker system **632.**

Different Suzuki coupling reactions were performed on solid-supported bismuth linkers. Afterwards the molecules were cleaved either by halodebismuthylation with a solution of iodine or bromine in THF or dichloroethane to give the corresponding halogenated products **635** and **636** in 48–83% yield. Traceless cleavage using TFA/dichloromethane affords the biphenyls **637** in 58–68% yield (Scheme 93).



Scheme 93 Multifunctional cleavage from the triarylbismuthane linker

The second bismuthane linker developed by Ruhland et al. is the triaryl bismuth diacetate linker **638** [331] that is generated by oxidation of the triaryl bismuthane linker **632** with diacetoxy iodobenzene. Performing the cleavage of the aryl groups from the resin, the triaryl bismuth diacetates can be treated with different nucleophiles, resulting in the formation of C – N, C – O and C – C bonds (Scheme 94). The N- and O-arylation reactions are carried out using 10% copper(II) acetate or pivaloate to give compounds **640** and **642**, respectively. In the absence of copper additives, *ortho*-arylation of phenolic substrates takes place, for example, the cleavage with β-naphtol yielding the C-arylated derivative **644** is performed using 2-*t*-butyl-1,1,3,3-tetramethylguanidine (TMG) as base.



Scheme 94 Multifunctional cleavage from the bismuth diacetate linker

15
Silyl Linkers

Silyl linkers are very common in solid phase chemistry in their function as alcohol-protecting groups [332–338]. Cleavage from those silyl ether resins

can be achieved by several fluorine sources to give the corresponding alcohols. Beyond these Si – O protecting group linkers, silicon-based solid supports became more and more relevant because of their ability to immobilize aryl moieties (Fig. 17) [339–348]. Cleavage of such aryl compounds can be achieved by addition of various electrophiles, by addition of acids or fluorides. The required cleavage conditions strongly depend firstly on the nature of the linker and secondly on the nature of the substituents on the aromatic moiety. While, for example, electron-poor systems require TFA, CsF or even HF treatment for cleavage, more electron-rich systems can be released with TBAF.

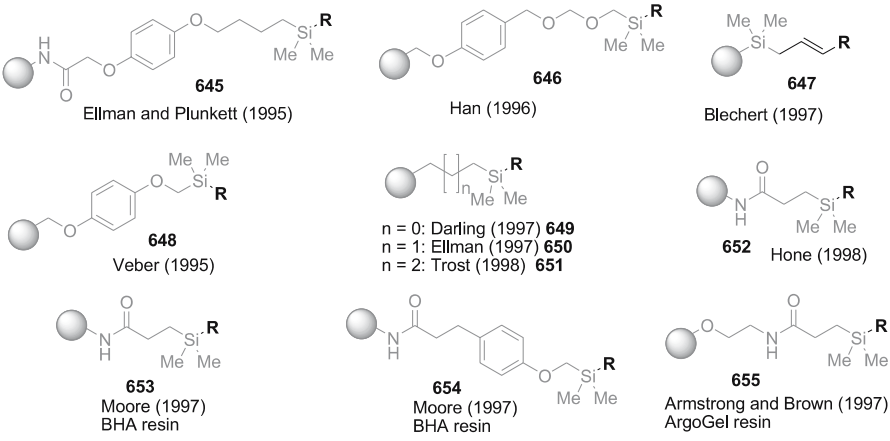


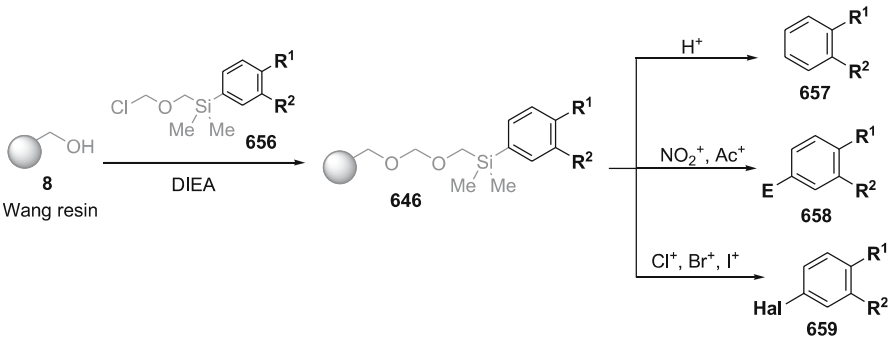
Fig. 17 Silyl linkers

Figure 17 presents resin-linker cores known to be compatible with C – Si bond formation and cleavage. The synthesis of those linkers is more difficult than that for the alcohol linkers because chloro-silyl resins, the often-used precursors of Si – C bond formation, are quite unstable on solid supports. Some groups, among them Hone et al. [341], Ellman et al. [344], Moore et al. [345], Showalter et al. [349] and Bäuerle et al. [350], avoided this problem and coupled the silicon-containing linker in a solution phase to the compounds that have to be immobilized.

A few silicon-containing linkers have been synthesized on solid phase and are used to tether diverse aryl moieties without former preloading of the linker. Ellman et al. attached aromatic and heteroaromatic compounds directly to a silicon-substituted support for the first time. The nature of the resin anchoring of those silyl linkers has to be chosen carefully because cleaving conditions for the release of non-alcoholic compounds may induce destruction (and therefore release) of the silicon linker as well. Ellman et al. synthesized a heteroatom free, masked and therefore stable silyl linker **650** starting from allylsilanes and bromo-substituted polystyrene that can be eas-

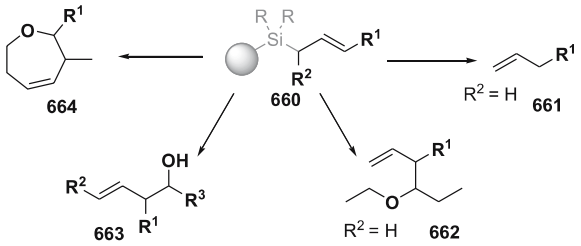
ily converted into the active silyl chloride derivative prior to use via conversion with HCl [346]. The linker has been successfully tested for its utility in solid phase organic synthesis of heterocycles.

Scheme 95 illustrates the possibilities for electrophilic cleavage of aryl compounds bound to silyl linkers. The linker developed by Han et al. is formed by addition of former synthesized chlorides **656** to Wang resin **8** [347]. Via cleavage with halogen cations, protons or other electrophiles, compounds **657–659** were released.



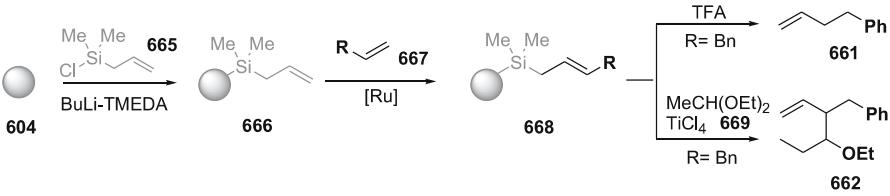
Scheme 95 Release of substituted aryl compounds by electrophilic cleavage

Allylic silyl linkers **660** can traditionally be cleaved via a β-elimination process (Scheme 96). Blechert et al. synthesized an allylic silyl linker system by immobilization of diverse alkenes to the resin via cross-metathesis [348]. Addition of electrophiles (Scheme 97) results in *ipso*-substitution via β-silicon-stabilized carbocations.



Scheme 96 Cleavage of allyl–silyl linkers

Appropriate allylic linker systems can be obtained with a three-step synthetic procedure starting from polystyrene. Selective lithiation of polystyrene **604** (1% DVB crosslinked) was achieved by addition of BuLi in combination with TMEDA and subsequent conversion with allyldimethylsilyl chloride gave resin **666** with an average silicon content of 1.3 mmol/g. Diversity could

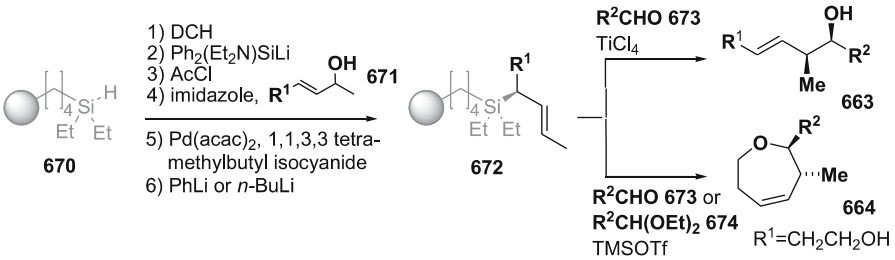


Scheme 97 β-Elimination from allylic silyl linkers

be introduced by ruthenium catalyzed cross-metathesis reaction with various terminal alkenes **667**. The compatibility of protodesilylation cleavage methodology (TFA addition) could be proven for metathesis products that contain esters and glycosides but was not successful in the case of hydroxyl allylic compounds or those with esters in an allylic position. The reaction of allyl silyl linkers **668** with carbon electrophiles is demonstrated by the synthesis of compound **662** resulting from addition of diethoxyethane **669** and $TiCl_4$ to the metathesis-derived polymer.

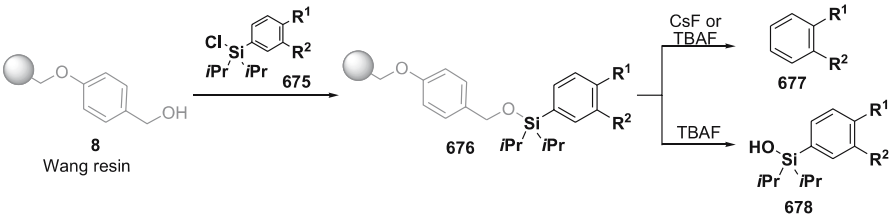
Another approach to allyl-containing silyl linkers has been investigated by Suginome and Ito [351]. It was found that polymer-supported allylsilanes can be synthesized enantiomerically enriched by conversion of commercially available hydrosilane resin **670** into the chlorosilane resin using DCH (1,3-dichloro-5,5-dimethylhydantoin) and addition of (diethylamino)diphenylsilyllithium. The resulting diethylamino functionality has been substituted to give the corresponding chloride. The following steps include reaction with allylic alcohols **671** in the presence of imidazole and treatment with a catalyst-system containing $Pd(acac)_2$/1,1,3,3-tetramethylbutyl isocyanide. Treatment with PhLi or *n*-BuLi gave resin **672** that was subjected to cleavage reactions. The release of target compounds via addition of carbon electrophiles is based on a former method published by Panek and Zhu [352] who transferred solution phase crotylation reactions on solid phase. Allylation has been performed using $TiCl_4$ as a Lewis acid and several aldehydes **673** as electrophilic compounds. For the use of acetaldehyde, yields of 54% over three steps could be achieved whereas for aldehydes bearing more bulky substituents the yield decreased to 34% for the allylation of isopropylaldehyde. Diastereoselectivities for the formation of the desired *syn*-homoallylic alcohols **663** are in any case better than the values achieved in solution phase. Suginome and Ito used a very similar methodology to synthesize oxacycloheptenes **664** as well. The reaction of resin **672** with aldehydes **673** or acetals **674** in the presence of TMSOTf proceeded in yields of 59–70% and with ees up to 99.2% (Scheme 98).

To the best of our knowledge only two groups have been working on the investigation of dialkylsilyloxy linkers up to now [349, 350, 353]. The synthesis of the linker core is performed in both cases in solution phase and is later on transferred to the resin.



Scheme 98 Crotylation via allylic silyl-containing linkers

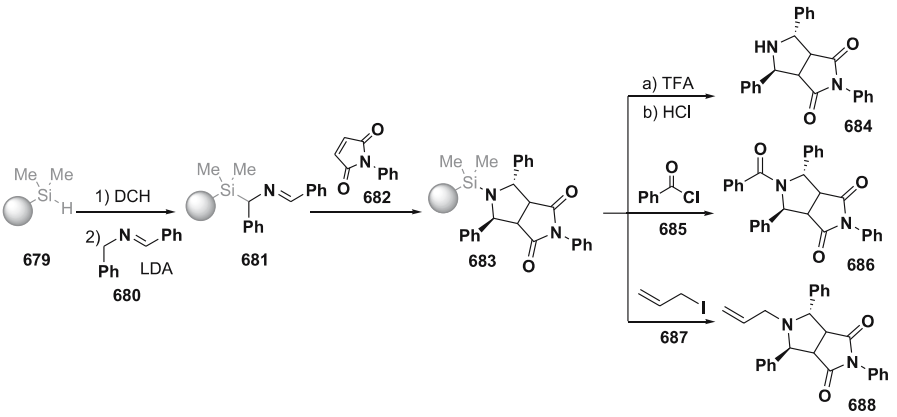
The linker created by Showalter et al. offers the possibility to cleave (depending on reaction temperatures) substrates by addition of TBAF or CsF to produce the desired silanolbenzopyranones **678** or to cleave in a traceless manner to isolate products **677** [349] (Scheme 99).



Scheme 99 Cleavage possibilities from dialkylsiloxy linkers

A similar approach has been published by Bäuerle et al. who synthesized a silyloxylinker by the addition of dialkylchlorosilanes to Wang resin in the presence of imidazole following the procedure of Showalter et al. [350]. The resin-bound intermediates were coupled to boronic acids and therefore show compatibility with the conditions of Suzuki reactions.

Beyond the above-mentioned importance of the attachment of alcohols to silyl linkers, there are also examples for the attachment of nitrogen-containing compounds. Komatsu et al. synthesized an allylic linker that is connected to the resin via a nitrogen-silicium bond [354]. The linker core was synthesized by addition of allylmagnesium chloride to Merrifield resin and conversion of the resulting terminal alkene with Me_2SiH_2 in combination with catalytic amounts of $RhCl(PPh_3)_3$ to resin **679**. That universally usable silyl linker unit can be chlorinated using DCH (1,3-dichloro-5,5-dimethylhydantoin) and is then able to immobilize several nucleophiles like *N*-benzylidene-*N*-benzylamine **680** (in situ formation of an aza-allyl anion in the presence of LDA). The resulting α-silylimines **681** are converted with *N*-phenylmaleimide **682** into pyrrolidines **683** within one step. Some other transformations starting with resin **679** have been tested for further



Scheme 100 Cleavage of pyrrolidines immobilized via silyl linkers

derivatization of the resin. Olefinic or acetylenic dipolarophiles can be added to the silyl-linker containing resin **681** in order to perform 1,3-dipolar cycloadditions. Polymer Si-N-bound compounds were successfully released by the addition of acids (HCl, TFA), benzoyl chloride **685** or allyl iodide **687** (Scheme 100).

16
Germanium Linkers

There are only a few examples for germanium-based linkers in solid phase synthesis. In general, the linker systems and the cleavage conditions are very similar to the silicon-based linkers that were introduced before. Two important germanium linkers are shown in Fig. 18.

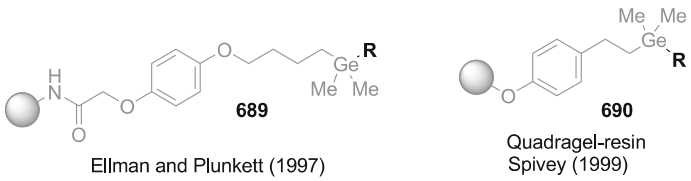
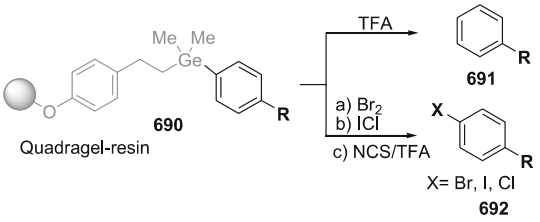


Fig. 18 Germanium-based linkers

As a consequence of the stronger β-effect of germanium, arylgermanes undergo more easily cleavage via electrophilic aromatic substitution than the corresponding arylsilanes. In 1997 Ellman and Plunkett [355] generated the first multifunctional germanium linker **689** that could be cleaved either in a traceless manner using trifluoracetic acid to give the arenes or using

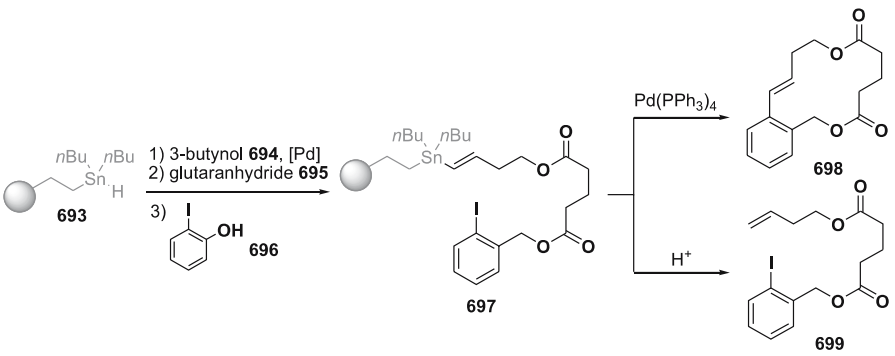
bromine as the electrophile to yield the brominated aryl compounds. Spivey et al. [356] refined and extended this germanium-based diversity linker strategy when they described the preparation and application of the linker system shown in Fig. 18. The linker **690** was synthesized starting from Quadragel-OH resin and different *ortho*-germylethyl phenols to give the corresponding ethers. After modification of the solid support, for example to give a library of pyrazole structures [357], the arenes **691**, aryl bromides, aryl iodides or aryl chlorides **692** were obtained by treatment with TFA, bromine, iodine monochloride or *N*-chloro-succinimide, respectively (Scheme 101).



Scheme 101 Germanium-based linkers for traceless release and formation of haloarenes

17
Stannane Linkers

Stannanes have become prominent members in the field of multifunctional anchoring groups. Nicolaou et al. [358] used polymer-bound tin hydride **693** to hydrostannylate alkynes **694** under palladium-catalysis to give polymer-bound alkenyl stannanes. Alternatively, the latter ones can be prepared from a polymer-bound tin chloride and an alkenyl-lithium or -magnesium halide reagent. These alkenyl stannanes were employed in intermolecular as well

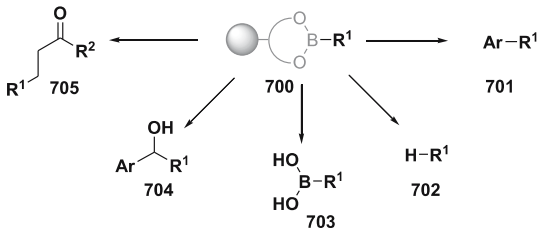


Scheme 102 Stannane linker according to Nicolaou et al.

intramolecular Stille reactions to give compounds **698**. Alkenyl stannanes can also undergo protonation to give alkenes **699** in a traceless fashion (Scheme 102).

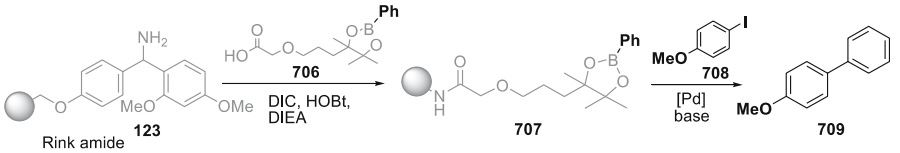
18 Boron Linkers

Boronic esters can be applied for several metal-catalyzed conversions in the same fashion as boronic acids. Scheme 103 gives an overview of the reactions of immobilized boronic acids **700** (in form of their esters) that include multifunctional cleavage.



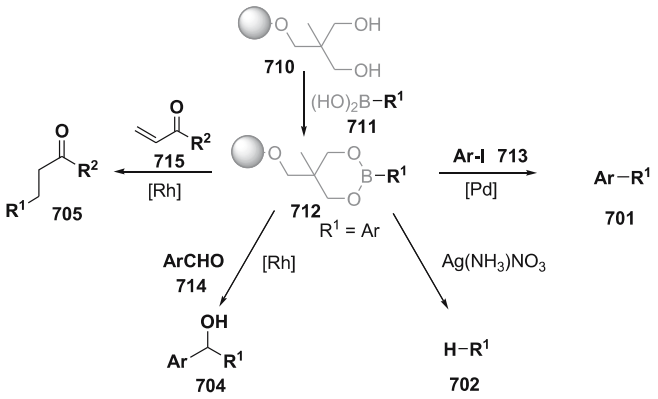
Scheme 103 Cleavage from polymer-bound boronic ester

The first carbon–carbon bond-forming cleavage reaction via boronic esters on solid supports was described by Burgess et al. who synthesized a β-turn mimetic by a simultaneous solid phase Suzuki coupling and release strategy [359]. The synthesis of the linker core bases on the coupling of a former prepared boronic ester-spacer unit **706** to Rink amide resin **123** via peptide coupling methods (Scheme 104). The boronic ester resin **707** was then treated with 4-iodomethoxy benzene **708** under various conditions for Suzuki couplings. Several catalysts as well as bases have been tested and $PdCl_2$binap in combination with K_3PO_4 gave the best results with 85% yield and purities over 95%. Similar couplings have been performed on MBHA as well as TentaGel resins where macrolactonization to the desired β-turn mimetic could be achieved in about 30% yield.



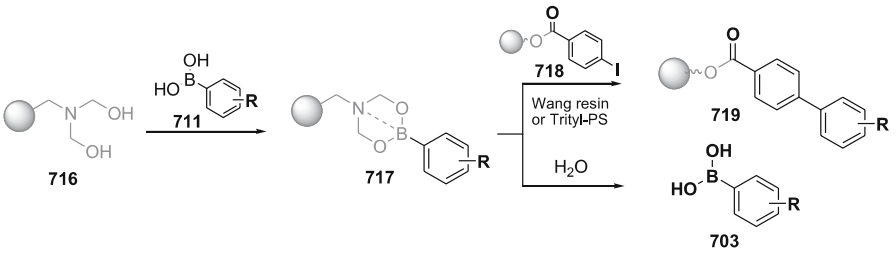
Scheme 104 Synthesis and application of boronic ester derived linker

Another boronic acid linker has been investigated by Carboni et al. who proved the compatibility of such linkers with multifunctional cleavage [360, 361]. The linker **712** was synthesized by the addition of boronic acid **711** to 1,3-diol functionalized resin **710** generated earlier [362]. The addition of aryliodides **713** under Suzuki conditions gave biaryls **701** in up to 75% yield. Rhodium-catalyzed addition of aldehydes **714** has been conducted to release secondary alcohols **704** in one step and reaction of the resin with enones **715** gave β-functionalized ketones **705** via 1,4-addition. A fourth possibility to release compounds from boronic acid linkers is the traceless cleavage to give arenes **702.** The detachment is achieved by treating the solid-supported substrates with $Ag(NH_3)NO_3$-solution in THF. The exact mechanism of this silver-ion catalyzed protodeboration has not been cleared up in detail, but the hypothesis of an arylmetal intermediate is strongly favored. Yields for these cleavage steps range from 43% to 81% referring to the synthesis of secondary alcohols and from 47% to 60% for the release of ketone derivatives. Purities are in all cases higher than 90% (Scheme 105).



Scheme 105 Multifunctional cleavage of boronic ester linkers

In 2002, Hall et al. employed *N*,*N*-diethanolaminomethyl polystyrene **716** to immobilize various boronic acids [363, 364]. The DEAM-PS resin could be generated in two different ways: either via reaction of aminomethyl polystyrene with ethylene oxide or via attachment of diethanolamine to Merrifield resin. The boronic acids **711** were attached and several transformations modifying the solid-supported compounds were performed, for example nucleophilic substitutions, reductions or amide coupling reactions. The resulting products were either released as free boronic acids **703** via hydrolysis or transferred to other, iodide-functionalized resins **718** via Suzuki coupling reactions and iminium-functionalized resins via borono-Mannich reactions. These resin-to-resin transfer reactions (RRTR) are performed using phase-transfer reagents which detach the compounds from one bead and couple

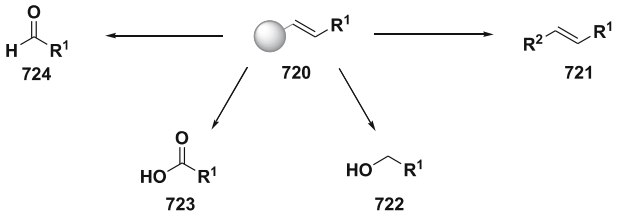


Scheme 106 Cleavage or resin-to-resin transfer reaction from boronic ester linker

them in situ to another. This concept is particularly attractive in combinatorial chemistry for the use in automated library synthesis and allows furthermore coupling of fragments for which a linear SPOS strategy would involve incompatible reaction conditions (Scheme 106).

19
Olefinic Linkers

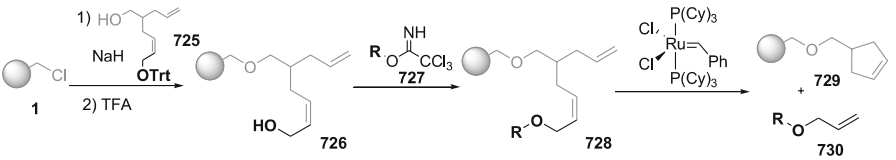
In general, there are three main methodologies to cleave the presented double bond containing linker systems **720** (Scheme 107). First of all, oxidative methods that can be used to convert alkenes into glycol-type structures and furthermore to cleave the C – C single bond in solution phase can be applied in solid phase chemistry. This methodology is suitable to release compounds which are immobilized via a double bond in form of their aldehydes **724**. The release of the corresponding alcohols **722** or carboxylic acids **723** should be possible adding reducing or oxidizing reagents, respectively. Secondly, Diels–Alder reactions can be performed on some solid phase systems including either a dienophile or a diene element and moreover, metathesis reactions using metallorganic reagents can be performed to give olefinic compounds **721**. The latter reactions are often used to produce target compounds that should contain terminal or internal double bonds after cleavage from the resin.



Scheme 107 Possible transformations of alkene containing linkers

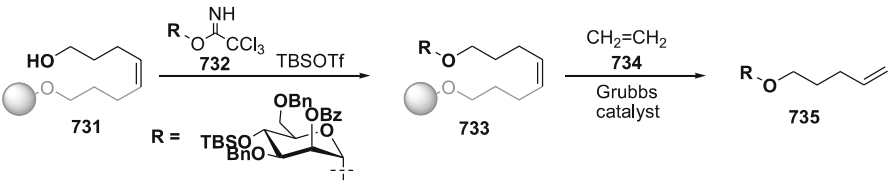
Metathesis reactions are very common on solid phases and syntheses of small molecules as well as cyclizations of macrocycles have been performed. The cyclative cleavage via ring-closing metathesis reaction (RCM) was published for the first time by Maarseveen et al. in 1996 [365]. The products obtained by ring-closing metathesis are either cycloolefins that are generated from immobilized alkenes containing another intramolecular double bond or acyclic olefins that include an additional substituent on the double bond introduced by RCM. Diolefinic linkers are able to release products containing a terminal double bond as shown by Knerr and Schmidt [366, 367].

The same linker type has also been used by other groups for example Waldmann et al. [368, 369] and Blechert et al. [370] to release terminal alkenes. The synthesis of the linker affords the addition of diolefinic structures **725** that have to be prepared via a three-step synthetic route to Merrifield resin **1**. After removal of the alcoholic protection group on the bead, substrates **727** like, for example, saccharides can be anchored on the resin, modified via diverse steps and cleaved to give compounds **730** (Scheme 108).



Scheme 108 Release of terminal olefins from olefinic linker systems

Another method to cleave olefinic structures as their non-cyclic derivatives is the conversion with an excess of olefin in solution. Scheme 109 shows the application of olefinic linker systems for the synthesis of terminal alkenes via reaction of immobilized alkenes with ethylene [371, 372]. The linker unit containing one double bond has been synthesized by addition of monoprotected octenediol to Merrifield resin in the presence of sodium hydride. Capping of the unreacted functionalities with methanol as well as removal of the protecting group followed before the substrate **732** could be anchored to the resin **731** via glycosylation. Cleavage of the olefin-containing product was conducted by the use of Grubbs catalyst and ethylene **734** giving a yield of 53% over six steps.

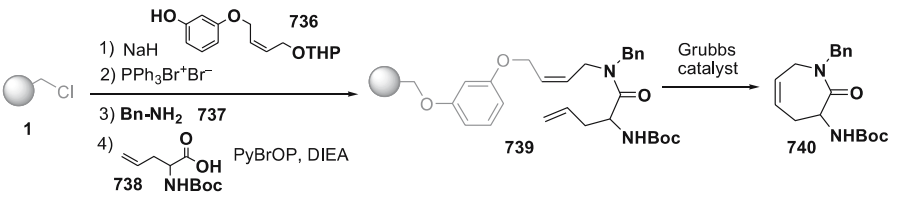


Scheme 109 Cleavage with ethylene via formation of terminal alkenes

A similar method has been developed by Seeberger et al. for metathesis reaction with pentene to give octene-substituted monosaccharide derivatives [373]. In previous solution phase experiments three catalysts were screened at different temperatures and in different solvents which suggest that metathesis proceeds best in dichloromethane at 0 °C and with $[(H_2Imes)(3-Br-py)_2-(Cl)_2Ru = CHPh]$ as the catalyst. All solid phase experiments have been performed on Merrifield octenediol linker **731** and have been checked for the cleavage reaction with ethylene as described in Scheme 109 for the synthesis of terminal olefins.

Several cyclic compound classes have been synthesized on solid phase using cyclative cleavage methodology based on RCM including dihydropyrans [374], seven-membered cycloolefins as Feidinger lactams or sulfonamides [365, 375–377], tetrapeptidic structures [378], amino esters [379], mannitol-derived oxazycles [380], sugar-derivatives [373], epothilone A precursors [381] and macrolactams [382].

The metathesis-concept developed by Maarseveen et al. offers the possibility to synthesize small ring sizes as well as macrocycles by adding Grubbs catalyst to a diene attached to the solid support (Scheme 110). After the release of the target cycloolefin **740**, the Grubbs catalyst remains immobilized via a double bond on the resin and can be released by addition of ethylene. Yields of seven-membered ring systems were only moderate (54%) even with a stoichiometric amount of the "catalyst" probably because of dimerization reactions on bead which gave macrocyclic structures. The yields of such metathesis ring-closure reactions could be increased by Blechert et al. [378] who used a spacer unit to overcome the low macrocycle yields for RCM on a solid support. By these improvements, yields of macrocycles could be increased from 30% up to 70%. The group of Brown used the investigations of Blechert et al. and synthesized seven-membered sulfonamide cycles via metathesis [375]. The spacer-resin part consisted of 2-carboxyethylpolystyrene to which an alcohol moiety with two double bonds had to be attached. Ring-closing metathesis with Grubbs catalyst gave the desired sulfonamides in up to 66% yield. Further experiments showed that a change from the ester spacer to alcohol spacers can improve the yield to give nearly quantitative conversions in the presence of only 5% of the Grubbs catalyst.



Scheme 110 Synthesis of seven-membered ring systems according to Maarseveen et al.

Ozonolysis has been very often used to convert double bonds into carbonyl-containing compounds on solid phase but there are only a few examples for the release of olefinic structures from polymer support using this methodology. While transformations including the use of ruthenium catalysts (see metathesis cleavage) tolerate many functionalities including carboxylic acids and their anhydrides, amides, aldehydes, ketones, alcohols and sulfonamides [370], ozonolysis stands for harsh conditions and compatibility with only a few functional groups. Nevertheless, there are examples for the release of diverse compounds from solid phase as demonstrated by Hall and Sutherland [383], Frechet and Schuerch [384] and Martinez et al. [385, 386].

Linker systems outlined in Fig. 19 can be synthesized by several pathways. Starting from hydroxymethyl-polystyrene **208**, Hall and Sutherland prepared linker **744** and connected it to the desired substrate within three steps via bromination of the benzylic position and following conversion into a Wittig-type salt **745** anchored to the resin. Treatment with base and addition of an aldehyde **746** affords the olefinic structure **748** (Scheme 111). After modification on solid supports, the olefin is cleaved via ozonolysis to give the corresponding carbonyl compound **749**. Martinez et al. used a very similar approach performing Wittig reactions as well. Another possible synthetic route to olefinic linkers shown by Frechet and Schuerch is the transformation of Merrifield resin into the corresponding aldehyde and reaction with malonic acid in the presence of pyridine. The following reduction gives the corresponding allylic alcohol to which various substrates might be attached.

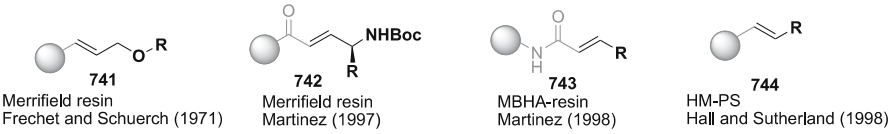
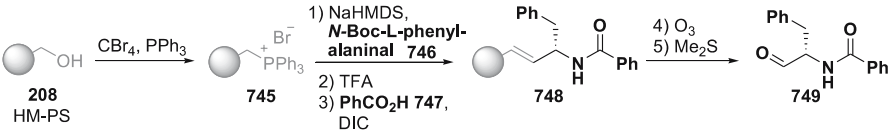


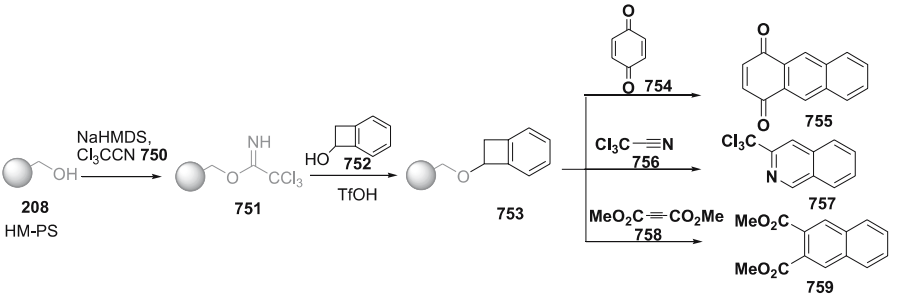
Fig. 19 Linkers developed for release of target compounds via ozonolysis



Scheme 111 Ozonolysis of alkene linkers to give aldehydes

Diels–Alder reactions as well as hetero-Diels–Alder reactions are known procedures on solid phase [387, 388]. The application of that methodology to the cleavage of solid-supported compounds has been investigated by two groups namely by Quadrelli et al. [389] and Craig et al. [390].

The synthetic route developed by Craig et al. examines a traceless cleavage model for resin-bound *o*-quinodimethane (**753**) and the transformation into naphthalene-derivative **759**, 2,3-naphthoquinone (**755**) and 3-(trichloromethyl)-isoquinoline (**757**) (Scheme 112). The linker unit synthesized via a three-step procedure contains no visible dien structure to undergo Diels–Alder reactions but can be seen as a precursor for a solid-supported dien.



Scheme 112 Cleavage via Diels–Alder reaction

Other Diels–Alder approaches for the release of compounds from olefin-containing resins have been published by the group of Quadrelli. Their methodology implicates a retro-Diels–Alder step and capture of the resulting nitroso-carbonyl benzene structures by the addition of dienes or enes.

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Top Curr Chem (2007) 278: 89–134
DOI 10.1007/128_2007_125

Published online: 11 April 2007

Solid Phase Organometallic Chemistry

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Abstract This review covers recent results in the area of solid phase organometallic chemistry during the period 2000–2006. The focus is on carbon–carbon bond formation, but other aspects of organometallic reactions are also discussed. This includes the use of metal complexes as linkers, synthesis of biologically active compounds and complex natural product analogues, development of diversifying cleavage strategies, and testing the stability of new linkers and polymers under different reaction conditions. In many cases a direct comparison has been made between solid phase and solution phase chemistry. Thus, the advantages of performing reactions on polymer-supported substrates in terms of avoiding side reactions and facilitating product/metal separation are highlighted.

Keywords Cross-coupling · Metal carbonyl complexes · Metathesis · Organometallic chemistry · Solid phase synthesis

Abbreviations

BINAP	2,2′-Bis(diphenylphosphino)-1,1′-binaphthalene
Cbz	Carbobenzyoxy
Cy	Cyclohexyl
dba	Dibenzylideneacetone
DIC	*N*,*N*′-Diisopropylcarbodiimide
dppf	Bis(diphenylphosphino)ferrocene
dppp	1,3-Bis(diphenylphosphino)propane
Fmoc	Fluorenyl-methoxy-carbonyl
L	Ligand
MW	Microwave
NMP	*N*-Methyl-2-pyrrolidone
RCM	Ring closing metathesis
TFP	Tetrafluorophenyl
TPPDS	Di(*m*-sulfonylphenyl)phenylphosphine

1 Introduction

Although the use of solid phase synthesis was pioneered in the 1960s and 1970s through the work of Merrifield [1], Fréchet [2], Leznoff [3] and others, the application of organometallic reactions in solid phase chemistry has evolved more recently. Early studies focused on the use of palladium-catalyzed cross-coupling reactions but the field has now expanded to encom-

pass a wide variety of transition metals. This review covers the literature dealing with organometallic chemistry on solid phase from January 2000 until September 2006. For earlier reports in this area, we refer to reviews by Kurth and coworkers, describing solid phase carbon–carbon bond formation and covering the years 1990–1999 [4, 5], and a review by Kingsbury et al. [6] as well as one by Andres and coworkers [7] on transition-metal catalyzed reactions on solid phase, encompassing the same period. An extensive review by Bräse et al. covers more specifically palladium-catalyzed reactions on solid phase and includes literature up to the end of 2002 [8]. Another review by Gradén et al. includes other types of organometallic reactions but excludes palladium-catalyzed cross-coupling [9]. Brill describes the more practical aspects of carbon–carbon bond-forming reactions on solid phase in a review that also contains experimental procedures [10]. There are also several books available on the subject of organic synthesis on solid phase that cover organometallic methods and can be useful complementary reading [11–13].

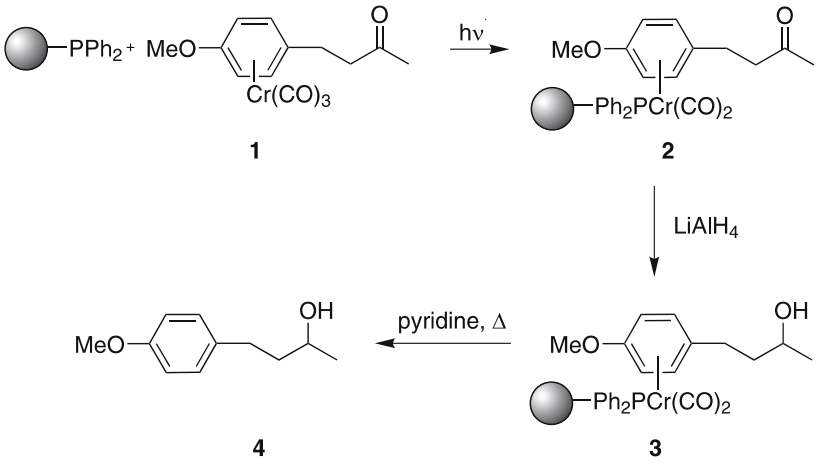
The vast amount of reports in the area of solid phase organometallic chemistry has made it impossible for us to include all work in this area over the time frame stated. Therefore, a certain selection has been made, both in terms of subjects covered and the research cited. Polymer-supported reagents are not included in this report and we here refer the readers to comprehensive reviews in this area by Ley [14], Bradley [15], Leadbeater [16, 17], and Bräse [18]. Grignard reactions have been included, while copper-catalyzed conjugate addition as well as transformations involving zinc and lithium reagents have not. A review by Reginato and Taddei covers these excluded areas [19]. Likewise, publications concerning radical mediated reactions are numerous enough to merit a review of their own. For cross-coupling reactions, focus has been on the more commonly used palladium reactions, i.e., the Suzuki, Stille, Sonogashira, and Heck reactions and concentrates on the more recent reports in this area.

2
Metal Carbonyl Complexes

2.1
Chromium

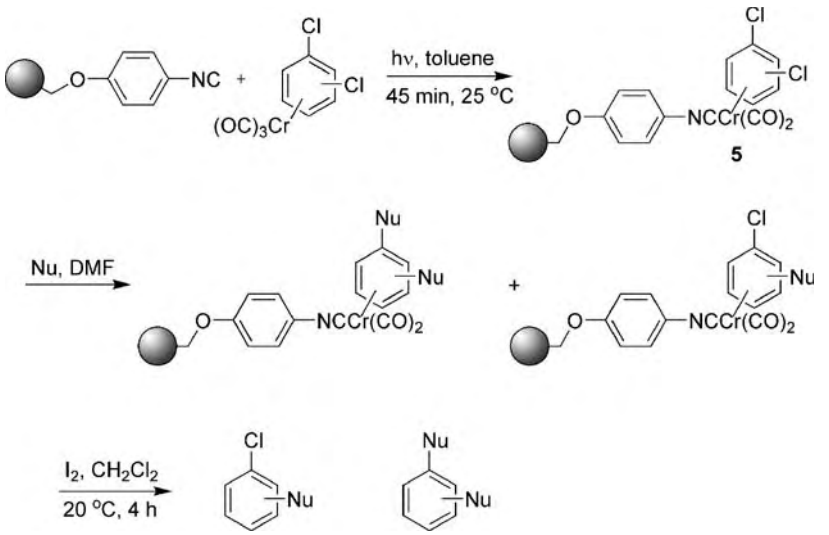
Using transition metal complexes as linkers for solid phase synthesis has some advantages compared to the substrate being attached via an organic linker. The organometallic linker complexes are stable to many of the chemical operations performed, and may be cleaved by a known set of conditions that are unlikely to affect the carbon scaffold. Comely et al. [20] used commercially available and relatively inexpensive polymer-bound triphenylphos-

phine to attach a chromium carbonyl linker in order to demonstrate the usefulness of π-bound ligands in linker chemistry (Scheme 1). The resin was irradiated with chromium complex **1** in THF, using a 125 W Hg vapor lamp, producing complex **2** in 40% yield according to ^{31}P NMR. The ketone moiety was then reduced by $LiAlH_4$, with the IR spectrum of **3** indicating the complete absence of the ketone functionality. However, the ^{31}P NMR revealed that the loading was only 25%, giving a yield of 62% for this step. The reason for this loss of material may be due to nucleophilic addition to the carbonyl ligands. The final product **4** was cleaved from the polymer through decomplexation, by heating in pyridine, in 92% yield.



Scheme 1 Use of a chromium carbonyl arene complex as a linker

The above strategy was applied to biologically important phenylalanine in order to investigate whether the chromium–phosphine linkers were stable to operations usually performed in peptide chemistry. As such, Fmoc-PheO^{t}Bu was attached to polymer-bound triphenylphosphine through a chromium carbonyl complex of the phenyl ring. Subsequent Fmoc deprotection followed by coupling of Fmoc-Val-OH was successful. Aerial oxidative cleavage resulted in the corresponding dipeptide in 90% yield. Although the above strategy worked well, one may wonder if the phosphine, being a donor ligand, might decrease the activation of the aromatic ring towards nucleophilic substitution. Baldoli et al. [21] used isonitrile to anchor the complex to the polymer and maintain the activation of the aromatic ring (Scheme 2). The authors carried out extensive studies on complexes of the type **5**, anchored to a hydroxymethyl polystyrene resin, varying the reaction conditions, substitution pattern, nucleophile, and solvent. It was found that the reactivity of polymer-bound complexes, including dihalogenated aromatics, was comparable with the corresponding complexes in solution, in-

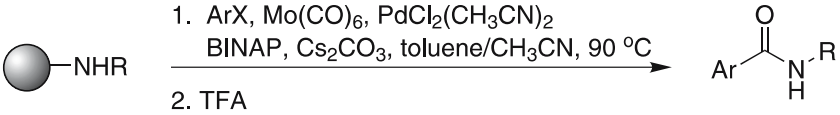


Scheme 2 Nucleophilic aromatic substitution on solid phase

dicating that the isonitrile anchor maintained the activity of the parent $Cr(CO)_3$ complexes. The polymer-bound complexes were then subjected to several different transformations and were found to be stable under the reaction conditions.

2.2 Molybdenum

$Mo(CO)_6$ can be used as a source of carbon monoxide in palladium-catalyzed carbonylations via a ligand-exchange reaction, as reported by Yamazaki and Kondo [22]. Two different routes to amides were described, with either the aryl halide or the nucleophile attached to the solid phase (Scheme 3). These methods were subsequently applied in the synthesis of heterocycles.

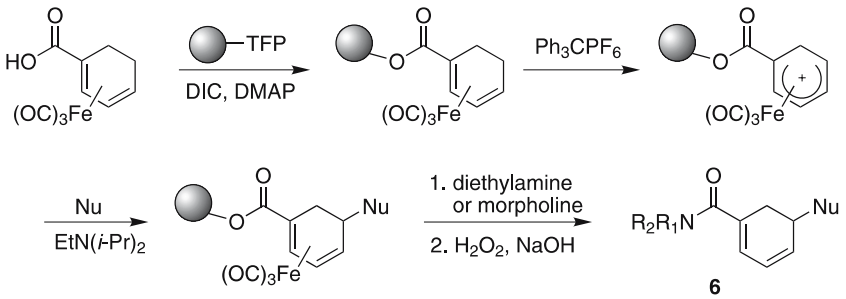


Scheme 3 Palladium-catalyzed carbonylation employing $Mo(CO)_6$ as a carbonyl source

A similar application of $Mo(CO)_6$ was described by Larhed and coworkers where carbonylation of immobilized reactants was assisted by microwave heating [23, 24]. Different carbon monoxide sources were also investigated. The authors found that $Cr(CO)_6$ and $W(CO)_6$ worked almost as well as the molybdenum analogue, while iron and cobalt carbonyl were not as efficient.

2.3 Iron

Although the development of carbon–carbon bond forming reactions on solid phase has been very rapid, examples of reactions involving sp^3-hybridized carbon atoms are limited. Gradén et al. studied cationic iron dienyl complexes and their reaction with nucleophiles [25]. The complexes were attached to commercially available tetrafluorophenol resin, and treated with carbon, oxygen, nitrogen, or phosphorous nucleophiles (Scheme 4). Cleavage with a secondary amine and subsequent decomplexation with hydrogen peroxide gave the desired products **6**. Noteworthy is the fact that the same conditions were used in all the reactions despite the different nature of the nucleophiles. In another study by the same group, the use of solid-supported active esters as linkers was studied [26]. Iron cyclohexadienyl carboxylate complex was attached via three different activated ester linkers to the solid phase, and the effect on the rate of aminolysis in each case was investigated.

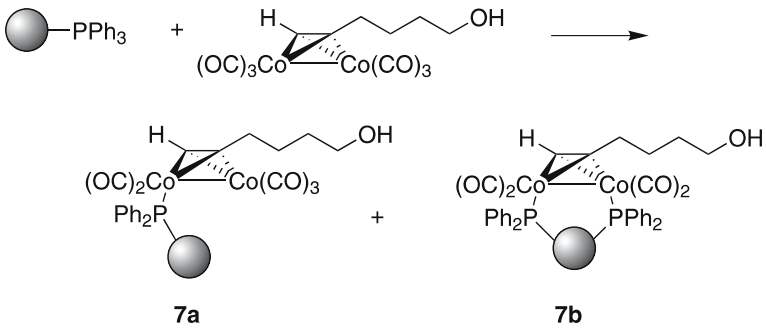


Scheme 4 Nucleophilic addition to polymer-bound iron carbonyl cations on solid phase

2.4 Cobalt

Gibson et al. have used polymer-bound cobalt complexes for immobilizing alkynes [27]. Scheme 5 demonstrates the indirect loading approach, meaning that the alkyne–cobalt complex is formed prior to the attachment onto solid phase. The alcohol moiety of **7a/7b**, formed as a mixture, can then be subjected to a number of transformations, and the alkyne is subsequently released from the polymer by aerial oxidation. The direct loading approach is also described, where the cobalt complex is formed on solid phase before the alkyne complexation step. This approach gives a somewhat lower substrate loading, but the subsequent steps proceed with almost the same efficiency in both cases.

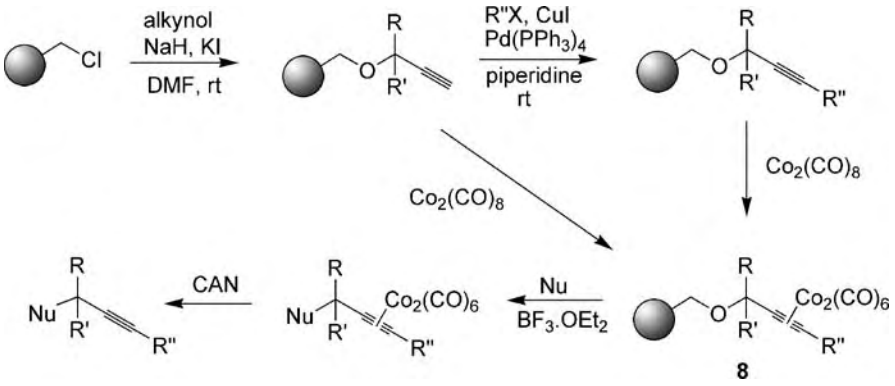
Another important application of cobalt carbonyl complexes is the Nicholas reaction, i.e., creating stable carbocations from propargylic alcohols



Scheme 5 Cobalt carbonyl complexes as linkers for alkynes

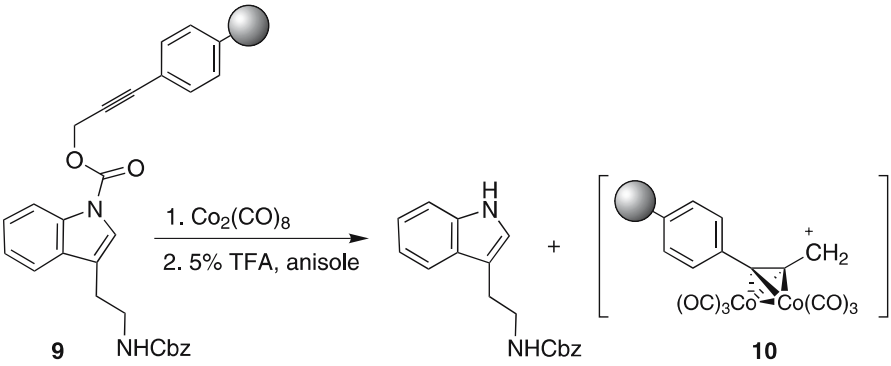
and subsequently exposing them to different nucleophiles. The first example of performing this reaction on solid phase was reported by Cassel et al. [28]. The strategy involved the attachment of propargylic alcohols to a solid phase, followed by formation of cobalt complex **8** (Scheme 6). The Nicholas reaction was then used as a diversifying cleavage step, allowing the introduction of different substituents simultaneously. Incorporation of the Sonogashira reaction into the sequence increased the diversity of the product compounds. In a later publication by the same group, the reaction sequence was optimized and two combinatorial alkyne libraries were synthesized [29]. By using microwave heating, the reaction time for the whole sequence was decreased dramatically, and the final purification step could be eliminated. The Nicholas reaction was also applied in the preparation of potential galectin inhibitors, using different carbon and oxygen nucleophiles to introduce diversity [30].

Cobalt carbonyl complexation can be used in conjunction with a propargylic linker as a safety-catch cleavage method, as described by Fürst et al. [31]. The propargylic functionality itself is stable under acidic conditions. How-



Scheme 6 The Nicholas reaction on solid phase

ever, formation of an alkyne–cobalt complex alters the reactivity, and subsequent treatment with acid allows cleavage. To visualize the potential of the linker, Cbz-tryptamine was attached to a propargylic linker (Scheme 7). Resin **9** was dried and the linker was activated by addition of dicobalt octacarbonyl. After treatment with TFA, Cbz-tryptamine was liberated, albeit in a low yield, together with the stabilized cation **10**. This confirms that the strategy of using propargylic linkers for solid-phase synthesis is feasible, although the reaction efficiency could be improved.



9 **10**

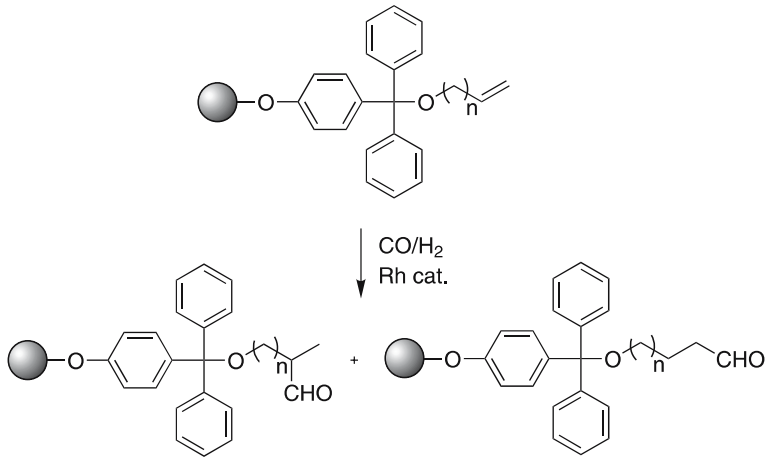
Scheme 7 Safety-catch linker activation by cobalt carbonyl complexation

2.5 Rhodium

Hydroformylation of alkenes in the presence of carbon monoxide, hydrogen and a rhodium catalyst is one of the most important processes for the industrial production of aldehydes. However, the major drawback of the so-called oxo-process is the very expensive catalyst. Several methods have been developed in order to facilitate separation of the catalyst from the product aldehyde, one example being the use of polymer-supported alkenes. Taddei and coworkers [32, 33] used immobilized alkenes of different length to obtain aldehydes in quantitative yields (Scheme 8). Varying the catalytic precursor changed the ratio between the linear and the branched aldehydes, resulting in excellent chemoselectivity. The method was also efficient for alkenes supported through an ester linkage as well as in tandem hydroformylation–reductive amination sequences.

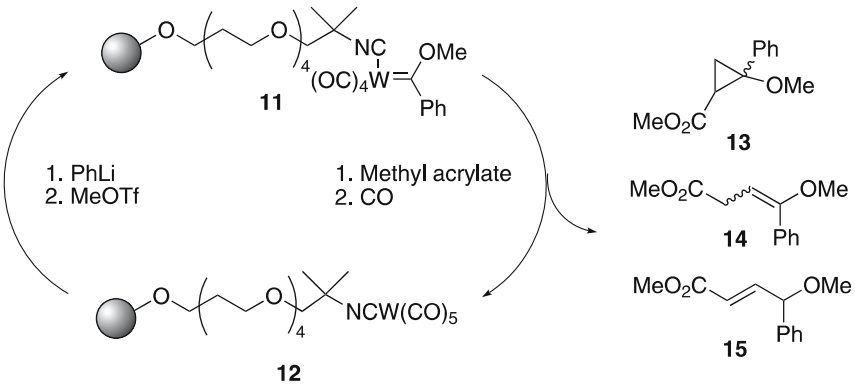
2.6 Metal Carbenes

The development of transition metal carbene complexes has been quite slow compared to other areas of organometallic chemistry on solid phase. Group 6



Scheme 8 Hydroformylation on solid phase

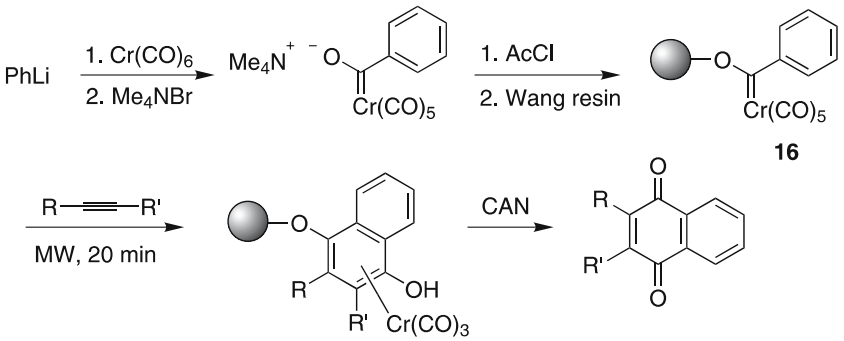
Fischer carbene complexes have proven to be useful in a number of selective syntheses, although the major drawback of these systems is the need for a stoichiometric amount of metal. One way to circumvent this problem is to employ polymer-bound complexes that can be recycled. Barluenga et al. [34] prepared polymer-bound Fischer tungsten carbene complexes from the corresponding pentacarbonyl metal compounds The reactivity and reusability of the polymer-supported complexes was exemplified by a cyclopropanation reaction (Scheme 9). Carbene **11** was heated with methyl acrylate in dichloroethane, and after cooling, saturation with CO, filtration and washing, the polymer **12** could be re-converted to a carbene and recycled. The product of the reaction was a mixture of cyclopropane **13** and C – H inser-



Scheme 9 Cyclopropanation on solid phase using polymer-bound Fischer carbene complexes

tion products **14** and **15**, in a total yield of 40%. The recovered complex **11** was used in a new reaction cycle, affording the same product mixture in 20% yield.

One of the major applications of Fischer carbene complexes is the formation of substituted phenols, known as Dötz benzannulation. The first example of this reaction on solid phase was reported by the group of Martinez [35]. The Fischer carbene complex **16** was obtained with a loading of 95% (Scheme 10), and the reaction could be monitored by colorimetric analysis. Microwave-assisted Dötz benzannulation allowed a sixfold decrease in the reaction time. Investigations into various reaction conditions showed that the reaction was almost independent of solvent, and that elevated temperature and prolonged reaction time did not have a major influence on the yield.



Scheme 10 Dötz benzannulation on solid phase

3
Metathesis

Recently, olefin metathesis has attracted much attention as a versatile carbon–carbon bond-forming reaction. In addition to methods using polymer-bound substrates, which are covered in this section, there are numerous examples of metathesis where the catalyst is immobilized on a solid support with a recent review published on this subject [37].

3.1
Ring-Closing Metathesis

Ring-closing metathesis (RCM) is a powerful method for synthesizing ring systems. One application presented by Arya and coworkers [38, 39] used the Grubbs 2nd generation catalyst **17** (Fig. 1) in a ring-closing metathesis to create tetrahydroquinoline-based polycycles having different ring skeletons (Scheme 11), where the overall yields for the sequence were typically around

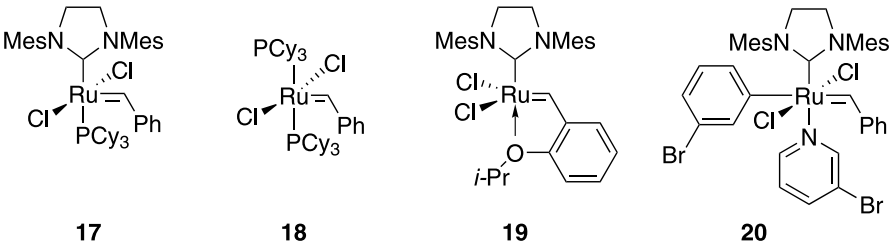
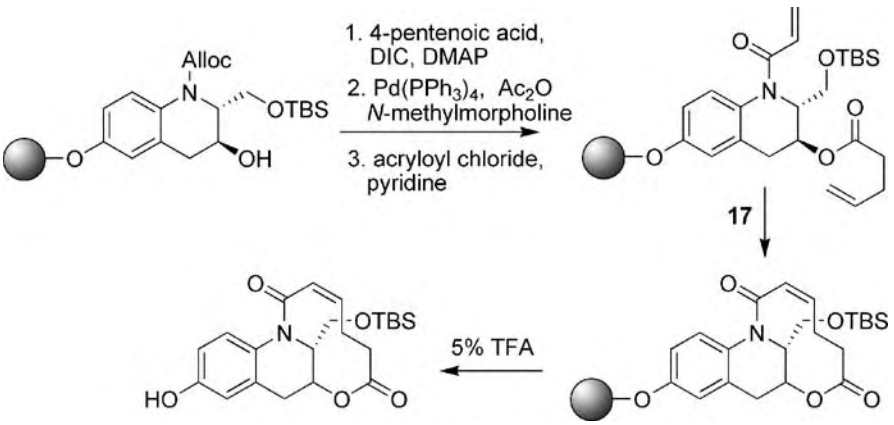


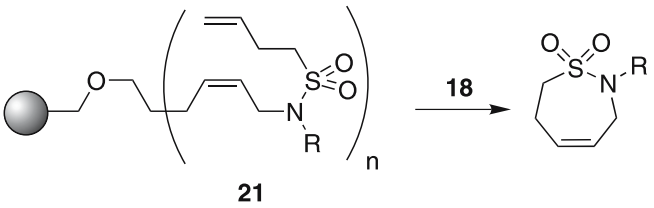
Fig. 1 Grubbs catalysts used for ring-closing metathesis



Scheme 11 Tetrahydroquinoline synthesis employing ring-closing metathesis

45%. Such polycyclic systems are capable of acting as modulators of protein–protein interactions.

Despite all the benefits of using solid phase synthesis, the major disadvantage is the introduction of two additional steps to the reaction sequence, i.e. attachment and cleavage, that may result in a less efficient synthetic route in terms of time and yield. In some of the examples of RCM on solid phase the cleavage is achieved in the same step as the cyclization. In a report by Moriggi et al. [40], this strategy was implemented in the synthesis of novel seven-membered cyclic sulfonamides (Scheme 12). To evaluate the

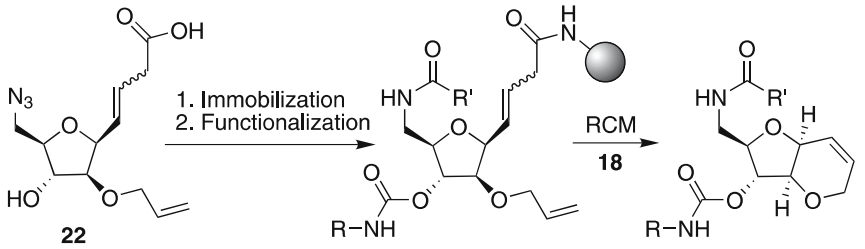


21

Scheme 12 Synthesis of cyclic sulfonamides via metathesis cyclization-cleavage

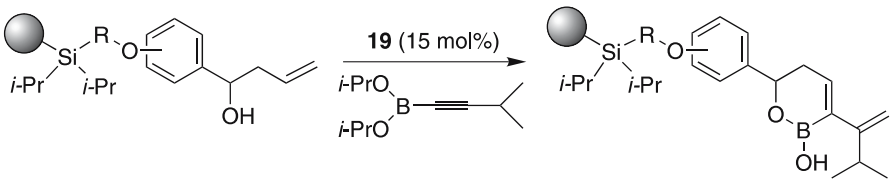
cyclization-cleavage strategy, resin **21** was refluxed with the Grubbs 1st generation catalyst (**18**, Fig. 1) forming a cyclic sulfonamide in good yield. A series of *N*-alkylated analogues were prepared with excellent results.

Another example of a single-step cyclization-cleavage is demonstrated in the synthesis of a nine-membered library, where the scaffold was derived from naturally occurring D-(+)-mannitol [41]. Compound **22** was attached to a polymeric support, and after functionalization of the resin-bound products, the subsequent treatment with catalyst **18** produced a library of pyranofurans in excellent yields (Scheme 13).



Scheme 13 Preparation of a pyranofuran library

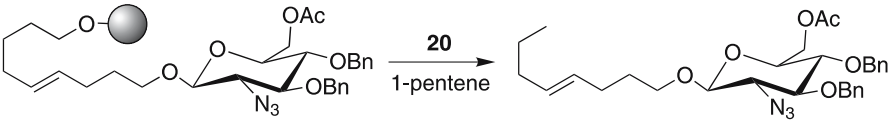
In a comparative study carried out by Brittain et al. [42], the Hoveyda–Grubbs catalyst **19** (Fig. 1) was found to give higher conversion in the solid phase synthesis of dialkenylboronates (Scheme 14) than the Grubbs 2nd generation catalyst **17**, while the reverse results were obtained for the corresponding reactions in solution phase. To investigate this further, a detailed mechanistic study was performed. It was established that the trend was the same for aliphatic and aromatic substrates, and was not affected by variation of the boronic ester. Also, phosphine-free catalysts were more effective for metathesis on solid phase. Using the proposed catalytic cycle for metathesis, and considering the fact that the transition from solution to solid phase can be troublesome, it was proposed that the conversion of the metal alkylidene to the diene was the limiting step.



Scheme 14 Solid phase synthesis of dialkenylboronates

3.2
Cross-Metathesis

In the solid phase synthesis of oligosaccharides, several criteria should be carefully considered, including the choice of protective groups and linkers. In some cases the linker can be used as a polymer-bound protective group. One such linker was developed by Kanemitsu et al. [43] and incorporated a double bond, enabling the use of cross-metathesis to release the product from the solid support (Scheme 15). The conditions for the cleavage step were compatible with azides, which are often used as masked amine functionalities. An extensive solution phase study, performed in order to determine the optimal reaction conditions, showed that catalysts containing phosphine ligands were not compatible with the azide groups. However, the use of a phosphine-free catalyst gave the desired products in high yields and the sequence could be applied to polymer-supported substrates with good results.



Scheme 15 Linker cleavage via metathesis

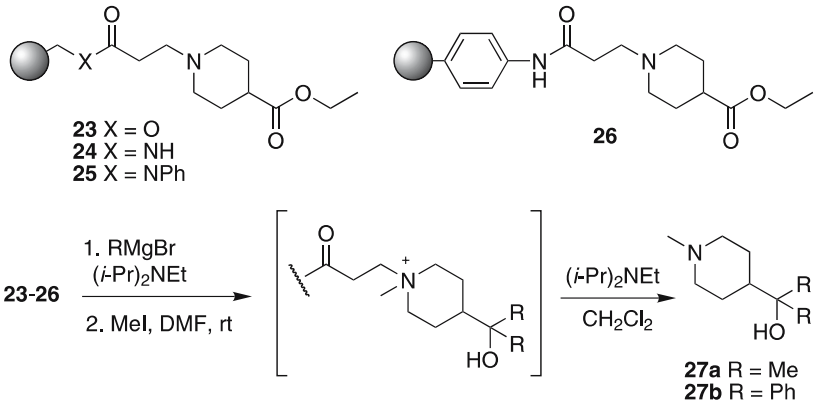
Dimeric carbohydrate scaffolds are important natural product mimics due to their ability to activate cellular processes and to increase the binding affinity. One strategy for the dimerization of carbohydrates on solid support used cross-metathesis of two identical olefins. Liao and coworkers [44] used a split-pool method to create polymer-bound benzo[*b*]furans, which were subsequently treated with Grubbs catalyst, thus undergoing metathesis and forming dimeric molecules.

4
Grignard Reactions

4.1
Addition of Grignard Reagents to Polymer-Bound Carbonyl Compounds and Imines

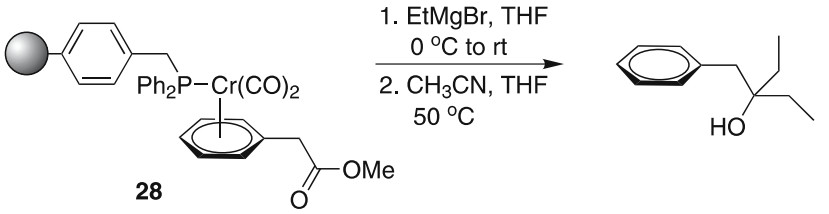
The addition of an organomagnesium reagent to a polymer-bound aldehyde, ketone, or ester to form an alcohol, is the most common application of Grignard reagents on solid phase and with many examples in the literature. A review by Franzén covers the earlier reports up to 1999 [45]. In many cases, the Grignard reaction has been used to test the compatibility of new linkers towards various reaction conditions. One such example concerns a modified

REM resin for the preparation of tertiary amines on solid phase. The original REM-resin contains an ester linkage that limits its utility in terms of reactive nucleophiles. Morphy and coworkers replaced the ester with an amide linkage to form new resins termed AM REM resins, and subsequently compared the stability of the original and the new resins towards Grignard reagents, along with other strongly basic reagents [46]. Resins **23**–**26**, derivatized with ethyl isonipecotate, were treated with either methyl or phenyl magnesium bromide (Scheme 16). The original REM resin **23** was cleaved under these conditions, while the newer resins were more robust and showed selective reaction of the pendant ester moiety in the presence of the amide linkage. The best stability was obtained with resin **26**, probably due to deprotonation of the amide hydrogen, deterring further reaction at this site, and affording products **27** in 39–45% yield for the three-step sequence.



Scheme 16 Modified REM resin suitable for reactions involving Grignard reagents

Another modification of an existing linker in order to improve its stability was described by Lazny and Nodzewska [47], who incorporated 3- or 6-carbon spacers into the T2 triazene-type linkers developed by Bräse [48], and subsequently carried out a Grignard reaction on anchored nortropinone. Waldmann's traceless phenylhydrazide linker, has been applied in the reaction of allylmagnesium bromide and phenyl lithium with polymer-bound carbonyl compounds (ester, aldehyde, ketone) with good results [49]. Another type of traceless linkage was employed by Rigby and Kondratenko, where aromatic ketones and esters were attached to a polymer-bound phosphine via a π-arene chromium carbonyl complex and subsequently treated with different Grignard reagents, affording tertiary alcohols in high yields (Scheme 17) [50]. For ester **28** the corresponding reaction of the unbound substrate was also carried out, affording only unreacted starting material after workup. Quenching of the solution phase reaction with D_2O instead of NH_4Cl, indicated a substantial amount of enolization of the substrate by the



Scheme 17 Grignard reactions of π-arene chromium carbonyl linked ketones and esters

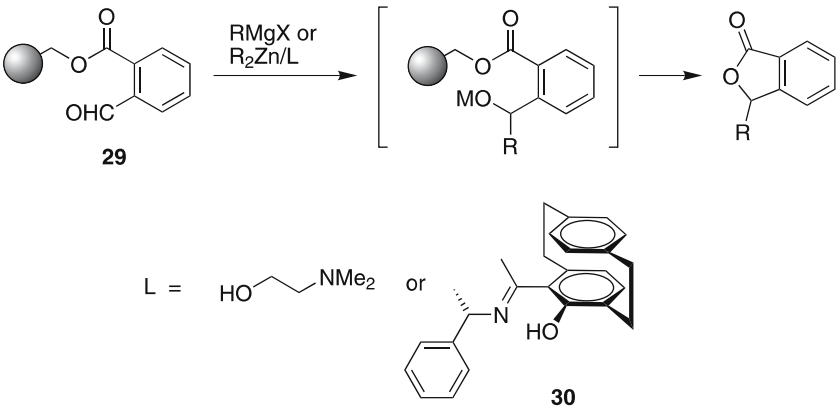
Grignard reagent, thus demonstrating the advantages of performing this reaction on solid support.

Other examples of linker compatability in solid phase Grignard reactions include that of Cossy et al. [51] who prepared optically active amino alcohol derivatives in excellent yields by ring-opening of *N*-tosyl lactams attached to polystyrene via the Ellman DHP linker [52]. Harikrishnan and Showalter have employed Grignard additions to polymer-bound aldehydes as a means of accessing benzopyran-4-ones [53], while Chandrasekhar and coworkers used the Grignard reaction as a cleavage method for an ester linker, forming a library of 20 tertiary alcohols [54].

Imines are also versatile electrophiles for reaction with Grignard reagents. Wendeborn has introduced diversity onto a 4-hydroxyquinoline scaffold, attached to Merrifield resin via a 1,6-hexanediol spacer, by reaction with a wide range of organomagnesium halides [55]. Substituents on the Grignard reagents included alkyl-, cycloalkyl-, alkenyl-, aryl, allyl-, and alkynyl-groups, and additional steps subsequently gave access to a 2,3-dihydroquinolin-4-one library. A similar approach was used by Bazin and Kuhn in the preparation of 4-amino-2-alkyl-1,2,3,4-tetrahydroquinolines on solid phase [56], and by Munoz and coworkers in the synthesis of substituted dihydropyridones on solid support [57]. Grigg and et al. have used Grignard reactions in conjunction with a hydroxylamine linker to prepare tertiary methylamines, α-methylpargyline and also (±)-Tramadol [58]. Furthermore, Sher and coworkers added different benzyl and allyl Grignard reagents, as well as organozinc reagents, to polymer-bound imines in a modular synthesis of α-branched secondary amines intended as β-3 adrenergic receptor agonists [59].

4.2
Cyclative Cleavage Involving Grignard Reagents

A cyclative cleavage approach employing Grignard reagents has been applied in the synthesis of benzobutyrolactones (phtalides). Many biologically active natural products incorporate phtalimidine and phtalide moieties, and Bräse and coworkers have developed a solid phase approach to such compounds starting from polymer-bound 2-formyl benzoic acids [60]. To access the phtalides, **29** was treated with alkylmagnesium halides or organozinc reagents

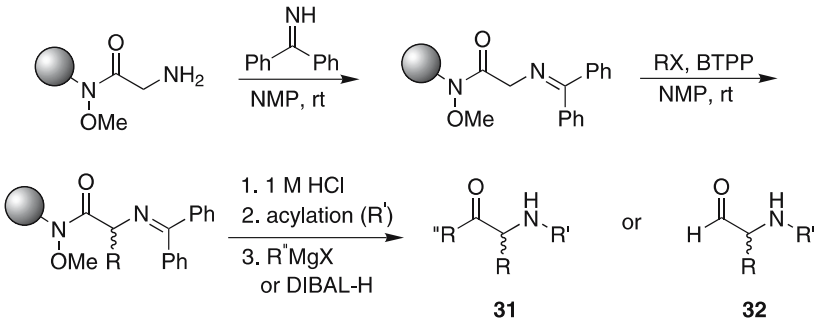


Scheme 18 Phtalide synthesis employing a cyclative cleavage approach

to induce cyclative cleavage via an intermediate alkoxide (Scheme 18). A chiral ligand (**30**) was used in conjunction with the zinc reagent, effecting an asymmetric addition to the polymer-bound aldehyde. However, the resulting enantiomeric excess was rather low.

4.3
Formation of Ketones from Weinreb Amides

Ketones can be prepared from Weinreb-type amides upon reaction with an organomagnesium reagent, and there are several reports of reactions of this type on solid support. O'Donnell et al. have prepared libraries of unnatural amino acids and peptides by attaching glycine to commercially available Fmoc-Weinreb AM resin [61]. The glycine moiety was alkylated in the α-position after activation of the amino group as a Schiff base, followed by additional steps (Scheme 19). The final products were cleaved from the resin by reaction with Grignard reagents, forming ketone products **31**, or alter-



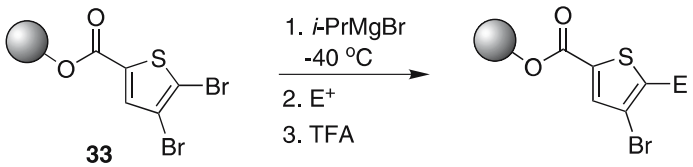
Scheme 19 Synthesis of unnatural amino acids via supported Weinreb amides

natively with DIBAL-H, to produce the corresponding aldehydes **32**. Sixteen unnatural amino acids and eight peptide derivatives were prepared in parallel using this strategy.

Albericio and coworkers investigated a similar strategy in the synthesis of α-acylaminoketones as possible ecdysone agonists [62]. However, due to problems with sterical hindrance in the cleavage step, they opted for a combined solution/solid phase approach with the Weinreb amide positioned in the exterior part of the scaffold rather than used as a linker [63, 64].

4.4 Polymer-Bound Grignard Reagents

One of the more interesting reports on the use of Grignard reagents in solid phase synthesis is the extension of Knochel's magnesium–halogen exchange on polymer-bound functionalized heterocycles [65, 66] to encompass dibrominated compounds [67]. Dibromothiophene **33**, attached to Wang resin, underwent selective reaction of one bromide at low temperature (Scheme 20). The method was extended to four different brominated thiophenes (including two mono-brominated compounds), resulting in a total of 13 highly functionalized thiophene products upon quenching with different electrophiles, with yields ranging from 83% to 99%.



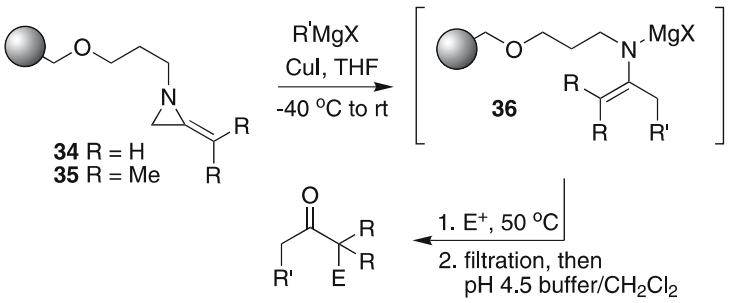
Scheme 20 Selective magnesium–halogen exchange of dibromohalogens on Wang resin

A library of substituted imidazoles, prepared via a magnesium–halogen exchange, was reported by Timmerman and coworkers [68]. Polymer-bound 4-iodoimidazole was treated with ethyl magnesium bromide, and the formed polymer-bound Grignard reagent was subsequently quenched with different electrophiles (aldehydes, ketones, acid chlorides, nitriles, and isothiocyanates). In those cases where a polymer-bound keto-imidazole was formed, the intermediate product was subjected to a second Grignard step, affording imidazoles with a tertiary alcohol in the 4-position after cleavage.

4.5 Miscellaneous Reactions Involving Grignard Reagents

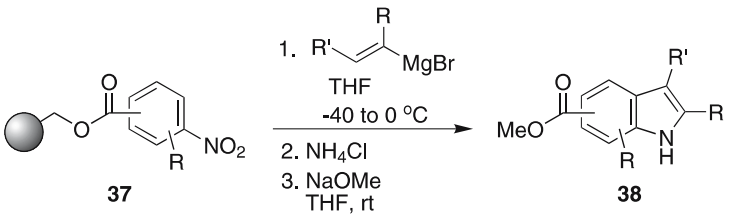
Substituted ketones can be prepared on solid support employing a copper-catalyzed multicomponent reaction involving Grignard reagents. Shipman

and coworkers prepared a library of 15 different ketones starting from aziridines **34** and **35** attached to Merrifield resin (Scheme 21) [69]. Treatment with five different Grignard reagents effected ring-opening of the aziridines affording intermediates **36**, which were subsequently quenched with various electrophiles. The imines thus formed were then cleaved hydrolytically from the resin, affording the desired ketones in high yields and excellent purities after a short gel filtration.



Scheme 21 Solid phase synthesis of substituted ketones using aziridine multicomponent reaction

Cheng and Kurth have effected ring-opening of epoxides by Grignard reagents and organocuprates on solid phase as a means of accessing substituted cyclopent-2-enones, using a sulfone functionality to link the cyclopentenyl scaffold to a polystyrene resin [70]. Schobert et al. have prepared a polymer-bound cumulated ylide, Ph_3PCCO, that was subsequently treated with butylmagnesium bromide to afford an acid ylide that could be applied directly in Wittig reactions with aldehydes [71]. Knepper and Bräse have employed alkenyl Grignard reagents in a solid phase version of the Bartoli indole synthesis [72]. Various polymer-bound nitro benzoic acids **37** were attached to Merrifield resin and treated with different Grignard reagents (Scheme 22). Reduction of the nitro group followed by attack of the Grignard reagent on the aromatic ring effected what is presumed to be an electrocyclic reaction to form indoles. Subsequent cleavage from the resin with sodium methoxide, afforded a library of 16 indoles (**38**) of high purity.



Scheme 22 Bartoli indole synthesis on solid support

Takahashi and coworkers have utilized a copper-catalyzed Grignard reaction in an elegant synthesis of vitamin D_3 analogues on solid phase [73]. A 72-membered library was prepared using radiofrequency encoded combinatorial chemistry, with the final Grignard cleavage step being performed in a Quest 210 synthesizer. The same group also used difunctionalized Grignard reagents as a means of attaching a silyl linker to different chloromethylated resins under copper-catalyzed conditions. Both polystyrene and Argogel were investigated [74]. Conjugate addition of Grignard reagents to a polymer-bound bismethylene cyclic malonic acid ester derivative has been effected by Tang and Huang as a means of preparing quinolone derivatives [75]. Finally, Solladié and coworkers have shown that menthyl sulfinates bound to a Wang resin can be efficiently converted to the corresponding sulfoxides by treatment with a variety of Grignard reagents [76].

5
Palladium-Catalyzed Cross-Coupling Reactions

Metal-catalyzed cross-coupling reactions have been used extensively in solid phase organic synthesis for the introduction of new carbon–carbon bonds. The first major review in this area was undertaken by Franzén in 2000 and summarized the development of the palladium-catalyzed Suzuki, Heck, and Stille reactions on solid supports since 1980 [77]. At this time, there was only one example where the Stille reaction had been applied to a large library synthesis (hundreds of compounds) and there were no examples of the Suzuki or Heck reactions being used in the generation of libraries containing more than 20 compounds. A more extensive review of palladium-catalyzed reactions in solid phase organic synthesis was published by Bräse in 2003 [8]. This included the literature up to November 2002 and highlighted the dominance of this chemistry for the introduction of C – C bonds which is of importance for the efficient synthesis of pharmaceutically significant molecules.

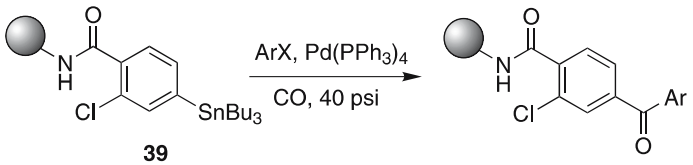
Although Sect. 5 has been subdivided into different parts according to the type of reaction employed, there are a few reports that describe several types of cross-coupling reactions used in the same context. Grether and Waldmann investigated the stability of a safety catch linker to Heck, Suzuki, Stille, and Sonogashira reactions, where the enzyme labile linker was found to be stable under the reaction conditions. Activation by hydrolysis of a pendant phenylacetamide by penicillin G acylase, triggered a cyclative cleavage reaction and released the target molecule [78]. A phenylhydrazide linker developed by the same group was also employed in various cross-coupling reactions, as well as in Wittig and Grignard methodology [49]. A new silylated linker was applied in Heck, Sonogashira, and Suzuki reactions to prove its utility, as reported by Duboc, Savignac, and Genêt [79]. Bräse and coworkers have investigated the effect of nitrogen functionalities

in palladium-catalyzed reactions by subjecting different triazene-linked aromatic bromides, with and without amino substituents, to Heck, Suzuki, and Sonogashira conditions [80]. A free amino group on the aromatic scaffold was found to inhibit the Heck and Suzuki reactions, while a copper-free Sonogashira reaction could be performed in the presence of a free amine in the *ortho* position. Berteina-Raboin and colleagues have compared conventional and microwave heating in the preparation of 2-substituted melatonin derivatives using different palladium-catalyzed cross-coupling methods to diversify the 2-position of a polymer-bound indole moiety, attached to polystyrene via a Rink amide linker [81]. Microwave heating was found to give better results in Stille, Sonogashira, and Heck reactions, while Suzuki reactions worked better with conventional heating. Agrofoglio and coworkers employed the same four reactions to prepare uridine derivatives on solid support, with Sonogashira and Stille coupling giving the best results [82]. Louërat, Gros, and Fort elaborated a pyridylpiperazine scaffold using Stille, Negishi, Suzuki, and Sonogashira reactions [83], while Burgess derivatized macrocyclic β-turn mimetics with alkynes and arenes via palladium-catalyzed reactions [84].

5.1 The Stille Reaction

5.1.1 Polymer-Bound Stannanes

Diaryl ketones are important scaffolds in biologically active compounds, and although several synthetic pathways are available in solution, their application in solid phase synthesis is rare. Yun et al. [85] have developed a high-throughput synthesis of diaryl ketones via a Stille carbonylation on solid phase. Immobilized arylstannanes **39** were subjected to Stille coupling with aryl halides in presence of carbon monoxide (Scheme 23). Not only did the reaction afford products of high yield and purity, but it was also tolerant towards most functional groups.



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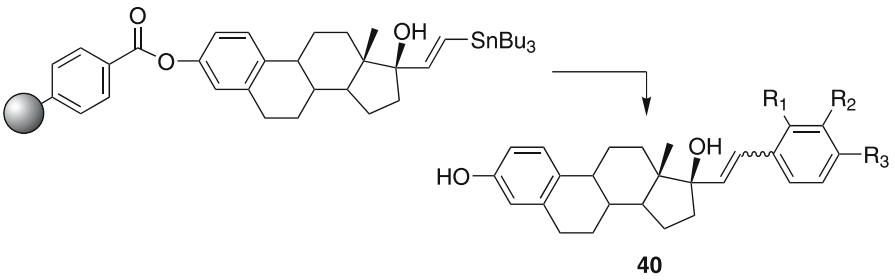
Scheme 23 Solid phase Stille carbonylation for the synthesis of diaryl ketones

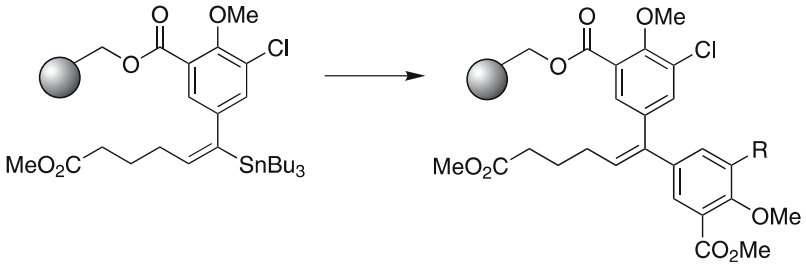
Another application of the Stille coupling on solid phase was described by Lee et al. [86], who designed and created a series of 17 α-*E*/*Z*-(X-phenyl)-



Scheme 24 Solid phase synthesis of estradiols involving a Stille reaction

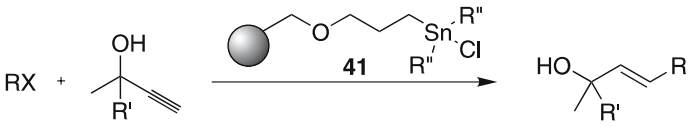
vinyl estradiols as potential agents against breast cancer (Scheme 24). One of the challenges in this synthetic strategy was the choice of the linker, due to the necessity for mild conditions for the attachment and cleavage steps. The resin of choice was carboxylated polystyrene, which resulted in a quantitative attachment. The ratio of *E* and *Z* isomers is dependent on the reaction temperature, time and stoichiometric ratio of $HSnBu_3$ to alkyne, and in this case **40**-*E* was formed almost exclusively.

Alkenyldiarylmethanes (ADAMs) have shown activity as HIV-1 reverse transcriptase inhibitors. Xu et al. [87] reported the first synthesis of such compounds with non-identical aromatic moieties, both in solution and on solid phase (Scheme 25). The yields of the Stille coupling were low in the initial attempts, and the reaction conditions had to be carefully optimized. It was found that $Pd_2(dba)_3/AsPh_3/CsF/DMF$ afforded the coupling product in high yield and retained the stereochemistry at the double bond.



Scheme 25 Stille coupling in the synthesis of alkenyldiarylmethanes

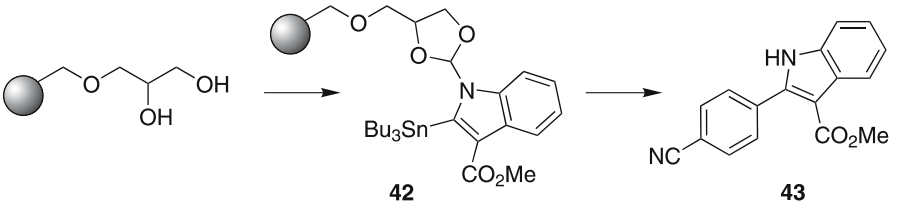
One major drawback of the Stille coupling in general is the presence of tin impurities in the final product. To solve this problem, polymer-bound tin reagents can be used. Hernan et al. [88] synthesized both dimethyl- and dibutyltin chloride resins, and used them in a model reaction shown in Scheme 26. Substoichiometric rather than catalytic amounts of resin **41** were needed in order to obtain acceptable yields, and the usual Stille conditions had to be optimized to fit this specific reaction. The catalytic system consisted



Scheme 26 Stille coupling using polymer-bound tin reagents

of a mixture of $PdCl_2(PPh_3)_2$, $Pd_2(dba)_3$ and tri-2-furylphosphine, with polymethyl hydrosiloxane added to regenerate the immobilized $R''Me_2SnH$. The same group was also able to obtain polymer-bound distannanes by reduction of **41** and subsequent cyclization. Immobilized distannanes were used in a two-atom transfer reaction of iodides [89], and for radiolabeling of aromatic substrates [90]. However, the radiolabeling attempts failed due to coordination of tin to the oxygen atom in the linker.

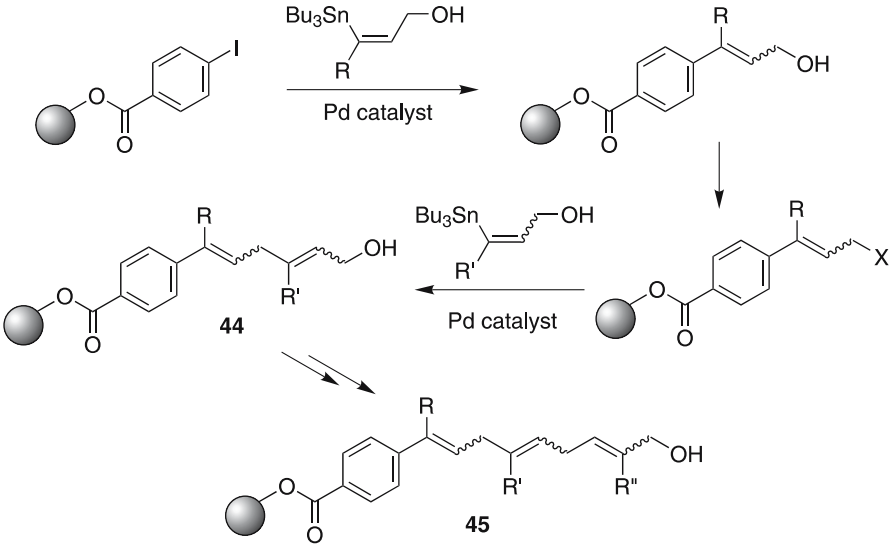
The use of traceless linkers is becoming more popular as it avoids the need for functional groups such as carboxylic acids or amides to anchor the substrate to the polymer. Kraxner et al. used a traceless diethoxymethyl linker to anchor the nitrogen atom of indoles [91]. Compound **42** was then subjected to a Stille coupling using $Pd_2(dba)_3$/*t*-Bu_3P/CsF as the catalytic system (Scheme 27). After cleavage, compound **43** was obtained in 66% yield, along with recovered starting material.



Scheme 27 Stille coupling on polymer-bound indoles

5.1.2 Stannanes in Solution

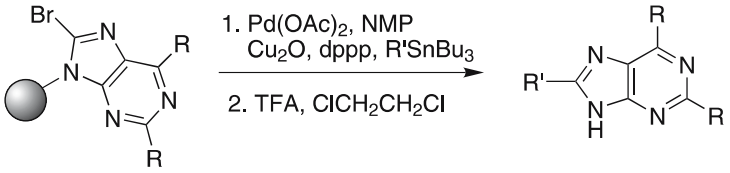
Havranek et al. [92] used repeated Stille coupling of immobilized 3-(butylstannyl)allyl alcohols to create libraries of dienes (**44**) and trienes (**45**, Scheme 28). When the catalytic system $Pd_2(dba)_3 \cdot CHCl_3/AsPh_3$ was used, the yields were much lower compared to the same cross-coupling reaction in solution. After some optimization, the best conditions were found using $Pd_2(dba)_3 \cdot CHCl_3/P(o-tolyl)_3$, affording complete conversion of the starting iodobenzoate. When the reaction sequence was applied towards library syntheses, the amount of catalyst and the temperature were increased in order to ensure quantitative consumption of the starting material, producing the



Scheme 28 Synthesis of dienes and trienes via Stille coupling

library members in high yield and purity. The diversity of the products could be increased by using substituted iodobenzoic acids instead.

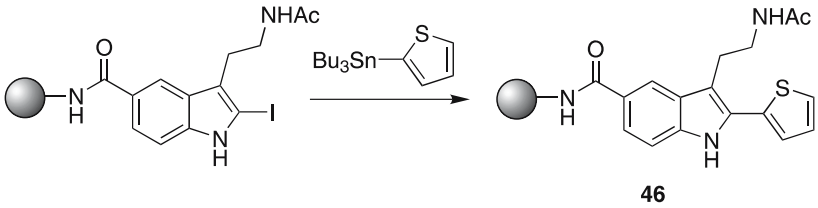
Purines, targeting nucleotide-binding proteins, are usually substituted at the 2-, 6-, and 9-positions. The work published by Brill et al. [93] describes a synthetic strategy that includes bromination at the 8-position, followed by a Stille coupling (Scheme 29). The reaction turned out to be problematic, affording products in low yields accompanied by side products arising from dehalogenation. After extensive investigation, including variation of the catalysts, co-catalysts and solvents, the optimal reaction conditions were determined, allowing the introduction of aromatic, acetylenic and vinylic functionalities at the 8-position.



Scheme 29 Purine derivatization via Stille coupling

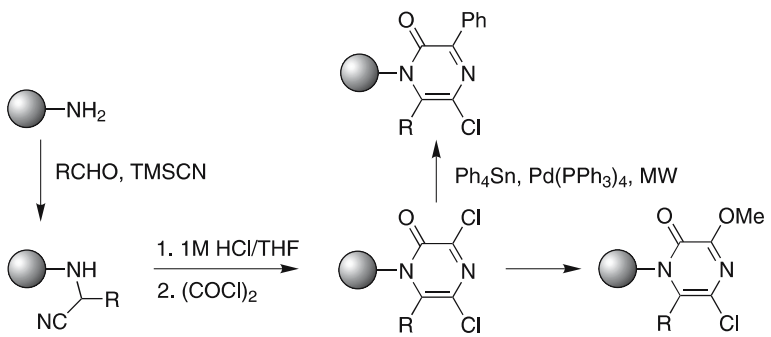
Melatonin analogues are known for their biological activity in humans, with their indole core modified in a number of ways. Berthault et al. [81] carried out a comparative study using conventional and microwave heating on solid support to generate melatonin analogues modified at the 2-position.

Compound **46** was obtained in good yield, and the reaction time was reduced from 24 h to 40 min using microwave heating (Scheme 30). However, the resin had to be washed after the first 20 min and then subjected to an identical reaction for another 20 min in order to achieve full conversion, probably due to catalyst poisoning.



Scheme 30 Synthesis of melatonin analogues via Stille coupling on solid support

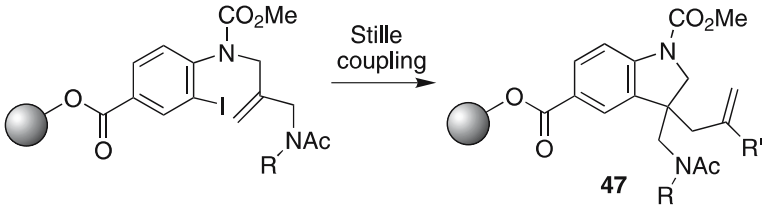
Pyrazinones and their derivatives have interesting biological activities, and have been used as thrombin and HIV inhibitors. They are also important building blocks for further derivatization. Kaval et al. [94] synthesized 2(1H)-pyrazinones with the possibility of introducing diversity at the C6 position (Scheme 31). The group also used different microwave-assisted cross-coupling reactions to increase the number of possible synthetic products by variation of the substitution pattern at position C3.



Scheme 31 Diversification of pyrazinones

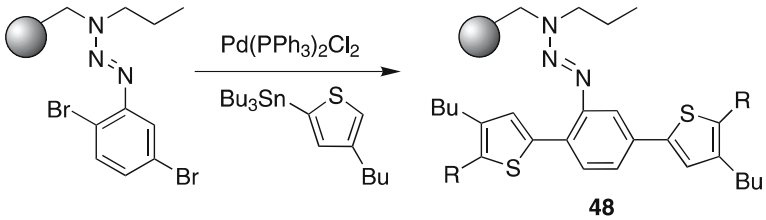
Palladium-catalyzed cyclization was used by Grigg et al. [95] to construct complex heterocycles **47** (Scheme 32). Due to the excellent regioselectivity of the hydrostannylation of terminal alkynes, only one major isomer was formed in most cases. The strategy was used for preparation of combinatorial libraries.

The preparation of conjugated molecules of precise length has evolved rapidly due to their applications as molecular wires and in molecular elec-



Scheme 32 Construction of heterocycles on solid phase

tronics. However, the synthesis is often troublesome due to solubility and purification issues. Zhang et al. [96] described a solid-phase approach for the preparation of oligothiophenes, which allowed chain growth in both directions and utilized butyl side-chains to enhance the solubility (Scheme 33). Bromination of the R-positions in **48** and subsequent Stille coupling added another unit in the chain. The sequence was repeated until the desired length was achieved, with the final product readily cleaved by MeI.



Scheme 33 Synthesis of oligothiophenes

5.2
The Sonogashira Reaction

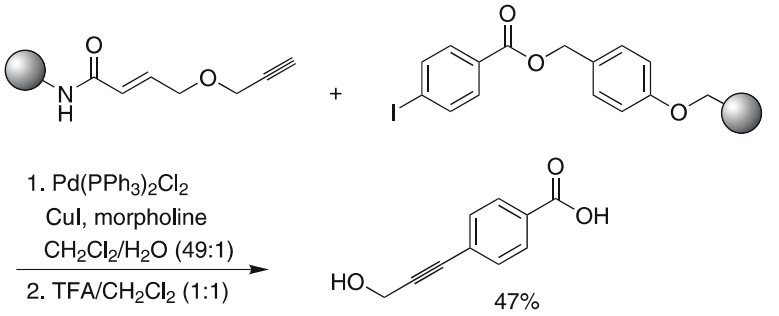
Like the Suzuki reaction, the Sonogashira reaction is a robust transformation that has found wide application in solid phase methodology, from the synthesis of heterocycles to construction and derivatization of scaffolds of biological interest. Cyclization reactions, joining two segments both attached to a polymer via a Sonogashira coupling, have been reported, as well as the assembly of alkyne oligomers, while microwave heating has been used to enhance reaction performance.

5.2.1
Methodology and Linker Testing

Erdélyi and Gogoll have previously reported the use of microwave heating for the Sonogashira reaction in solution, to shorten the often lengthy reac-

tion times needed for complete conversion [97]. This methodology has now been extended to encompass the corresponding reactions of polymer-bound aryl halides with trimethylsilyl acetylene [98]. Reactions were carried out in a microwave process vial, modified by the introduction of a polypropylene frit and a screw cap to facilitate the workup procedure. Optimal conditions for the cross-coupling of trimethylsilyl acetylene with aryl iodides, attached to polystyrene via a Rink amide linker, were found to involve the use of 5% $Pd(PPh_3)_2Cl_2$ together with 10% CuI at 120 °C in a 3 : 1 mixture of diethylamine and DMF. This afforded complete conversion in 15 min with excellent yields. Aryl bromides reacted somewhat more sluggishly and required a larger amount of catalyst along with the addition of 20% PPh_3, while aryl chlorides as well as amino-substituted aryl halides did not react at all.

Tulla-Puche and Barany employed Sonogashira chemistry in a resin-to-resin transfer reaction (RRTR) where an alkyne and an aryl halide are linked to different solid supports via Wang or allyl linkers [99]. In situ cleavage of the allyl-linked fragment with a palladium catalyst afforded a free alkyne moiety. This was then transferred to the polymer-bound aryl halide via a Sonogashira reaction under the same reaction conditions used for the initial cleavage (Scheme 34). The alternative strategy, with the aryl halide attached to the allyl linker resin, was also performed. However, a third reaction protocol, where both components were attached to allyl resins with the subsequent reaction taking place in solution, was found to give the best results. For the more successful RRTR reactions, equimolar amounts of palladium, in comparison to the resin-bound substrate, were needed.

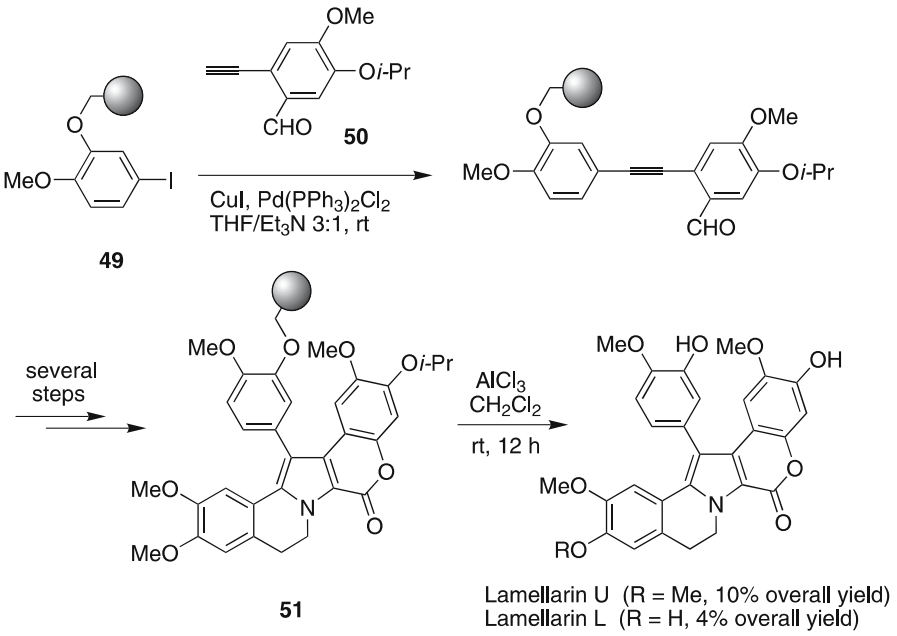


Scheme 34 Resin-to-resin transfer reactions involving a Sonogashira reaction

Finally, Fukase and coworkers have used the Sonogashira reaction to anchor glycoside moieties with a pendant acetylenic side chain to polymer-bound aryl iodides, forming a novel alkyne linker system [100].

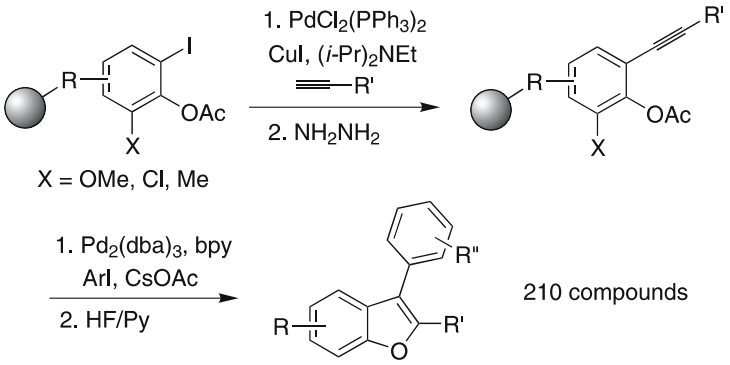
5.2.2
Heterocycles

Alkynes are versatile functionalities for the construction of benzannulated heterocyclic systems. The Sonogashira reaction provides a way of introducing substituents onto the newly formed ring by derivatization of the terminal acetylenic carbon prior to annulation. Albericio and Álvarez have used this concept in the synthesis of lamellarins U and L on solid phase [101]. The lamellarins are a class of marine natural products belonging to the alkaloid family, with interesting biological properties that include antitumor and anti-HIV-1 activity. Polymer-bound aryl iodide **49** was reacted with the aromatic acetylenic aldehyde **50** under Sonogashira conditions, using $Pd(PPh_3)_2Cl_2$ and CuI in a 3 : 1 mixture of THF and triethylamine (Scheme 35). Further transformations on solid phase, involving a Baeyer–Villiger oxidation and a [3+2] cycloaddition afforded **51**, which upon cleavage with $AlCl_3$ in CH_2Cl_2 produced a mixture of lamellarins U and L, separable by semipreparative HPLC.



Scheme 35 Total synthesis of lamellarins U and L on solid support

Likewise, Hu, Fathi and coworkers have employed the Sonogashira reaction as a key step in the preparation of a 2,3-diarylbenzo[*b*]furan library [102]. Five different polymer-bound iodophenols were coupled with seven acetylenic compounds under Sonogashira conditions employing IRORI



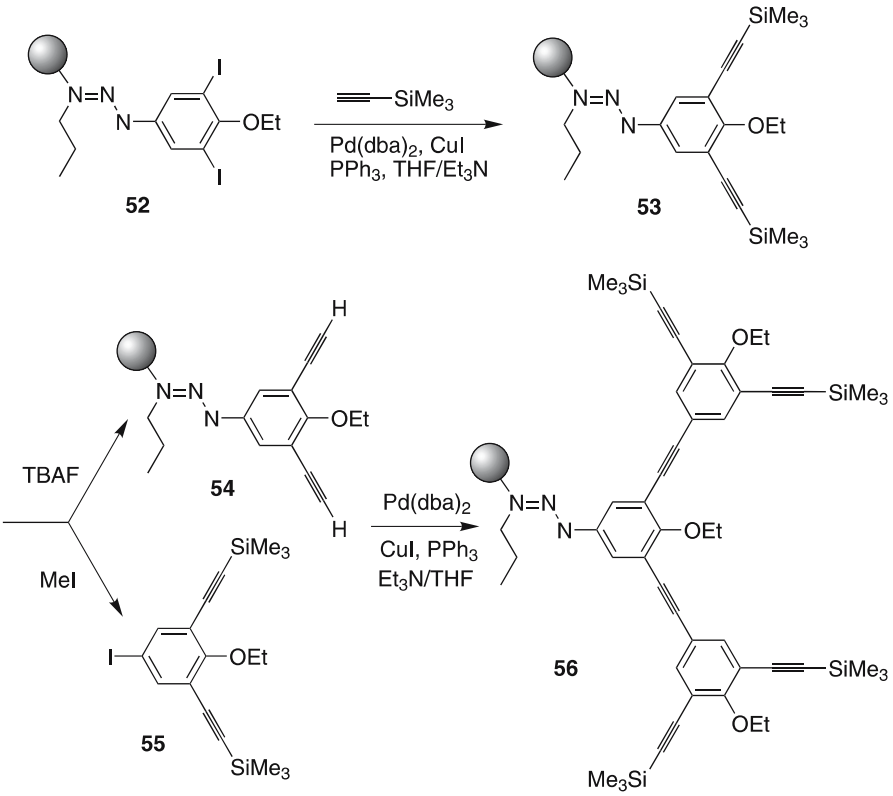
Scheme 36 Synthesis of 2,3-diarylbenzo[*b*]furans via Sonogashira reaction and palladium-catalyzed annulation

Micro-Kans and radiofrequency labeling (Scheme 36). After deacetylation, the formed *o*-alkynylphenols were treated with different aryl iodides in the presence of a $Pd_2(dba)_3$, effecting a diversifying annulation reactions. Cleavage with HF generated a library of 210 substituted benzofurans.

5.2.3 Oligomer Synthesis and Cyclization Reactions

The robustness of the Sonogashira reaction makes it well suited for the formation of acetylenic oligomers and polymers. Wang and coworkers have employed the Sonogashira reaction in the preparation of phenylacetylene dendrimers [103]. 3,5-Diiodo-4-ethoxyaniline was converted to the corresponding diazonium ion and attached to *n*-propylaminomethyl polystyrene to form **52** (Scheme 37). Sonogashira coupling with trimethylacetylene using $Pd(dba)_2$ and CuI gave **53**, which was split into two portions for the ensuing reaction. The first portion was treated with TBAF to remove the trimethylsilyl group, affording **54**, while the second portion was cleaved with methyl iodide, giving **55**. These two products were then combined under Sonogashira conditions to afford second generation monodendron **56**. Repeating these steps gave a fourth generation monodendron that could be functionalized with a ferrocenyl group on the original aromatic ring after cleavage.

Oligomerization on solid phase via the Sonogashira reaction has also been carried out by Hwang and Tour in the preparation of 24 tetrameric oligo(phenylene ethynylene)s [104], as well as by Diederich and coworkers [105, 106], who prepared poly(triacetylene)-derived oligomers displaying high fluorescence intensities. Triazene linkers, to attach the scaffold to a polystyrene resin, were also employed in these two cases. On-bead homo-



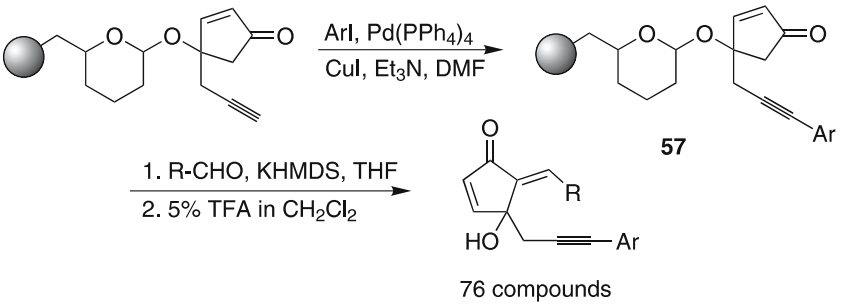
Scheme 37 Solid phase synthesis of dendrimers employing a Sonogashira coupling protocol

coupling of acetylenes under Sonogashira conditions has been effected by Liao et al. to form symmetric dimeric diacetylenes [107], while Spivey et al. performed macrocyclization of a linear polymer-bound peptide chain with an ethynylbenzamide moiety in one terminal position and an iodobenzene functionality in the other [108].

5.2.4
Applications in the Synthesis of Biologically Active Molecules

There are numerous applications of the Sonogashira reaction in the solid phase synthesis of molecules of biological interest, in some cases as part of a synthetic sequence but in the majority of examples for scaffold diversification purposes, and only a few can be mentioned here. Takahashi and coworkers prepared a library of 96 cross-conjugated dienones by subjecting a pendant acetylenic moiety on a cyclopentenone scaffold, attached to polystyrene via the Ellman THP-linker, to a Sonogashira coupling reaction

(Scheme 38) [109]. Derivatization of the α-position of the cyclopentenone **57** via an aldol reaction followed by cleavage with TFA afforded 76 of the desired compounds according to mass analysis, of which 11 showed strong cytotoxic activity in HeLaS3 cells.



Scheme 38 Preparation of a cross-conjugated dienone library with cytotoxic activity

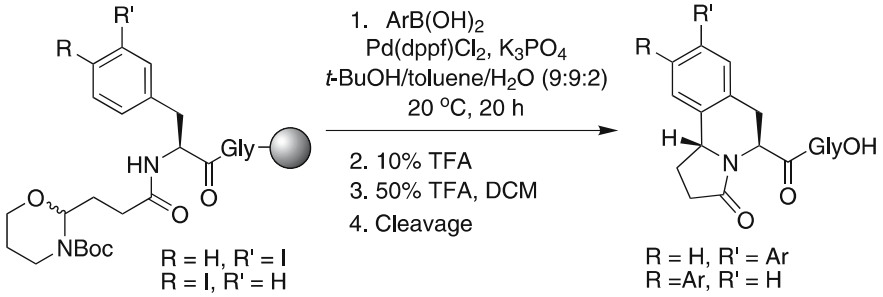
Schreiber and coworkers have employed the Sonogashira reaction for elaboration of a spirooxindole core structure [110]. A thiourea-based scavenger, i.e., glyoxal bis(thiosemicarbazone), was employed to reduce the metal content (Pd, Cu) after the Sonogashira step, thus avoiding contamination in the cellular screening. 2,6,9-Trisubstiuted purines, attached to Merrifield resin via a valeric acid linker, have been alkynylated in the C-2 position via a Sonogashira reaction in the preparation of a library of cyclin-dependent kinase (CDK) inhibitors, as reported by Legraverend and coworkers [111, 112]. Use of $PdCl_2(PPh_3)_2$ caused precipitation of Pd(0), indicating that the active catalyst underwent irreversible loss of its phosphine ligands, probably due to complexation of Pd(0) to the heteroaromatic purine system. However, this problem was solved upon switching to the more stable $Pd(dppe)Cl_2$. Additional examples include the diversification of pyrazinones via the Sonogashira reaction under microwave conditions as described by Van der Eycken [94], and the introduction of alkynyl substituents on the 5-position of pyrimidines in oligonucleotides as investigated by Richert and coworkers [113].

5.3 The Suzuki Reaction

Due to the substantial amount of literature, even when excluding supported catalysts, only selected articles on the solid phase Suzuki reaction are mentioned here, concentrating on the more recent publications. Apart from the general palladium reviews cited in Sect. 5, some examples of solid phase synthesis can be found in a review of applications of the Suzuki–Miyaura cross-coupling reactions in organic synthesis by Kotha in 2002 [114].

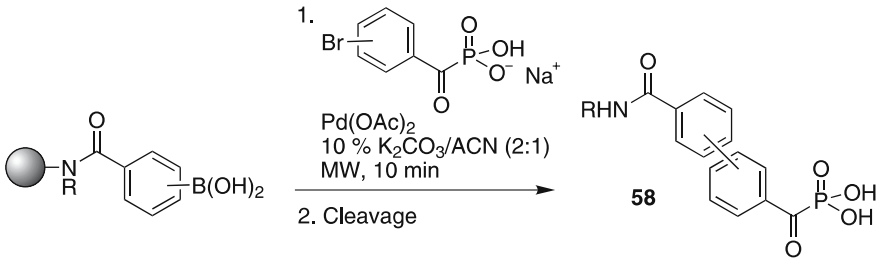
5.3.1
Biarylic Compounds

A recent example of the application of solid phase Suzuki reactions to the synthesis of pharmaceutically important biaryl compounds was shown by Nielsen et al. [115]. A range of aryl-substituted pyrroloisoquinolines were synthesized from biarylalanine precursors in high purity (Scheme 39). This involved a Suzuki coupling of solid-supported iodophenylalanine derivatives containing a masked aldehyde to various boronic acids, followed by liberation of the aldehyde, TFA-mediated intramolecular Pictet–Spengler reaction, and cleavage.



Scheme 39 Sequential Suzuki and Pictet–Spengler reaction on solid phase

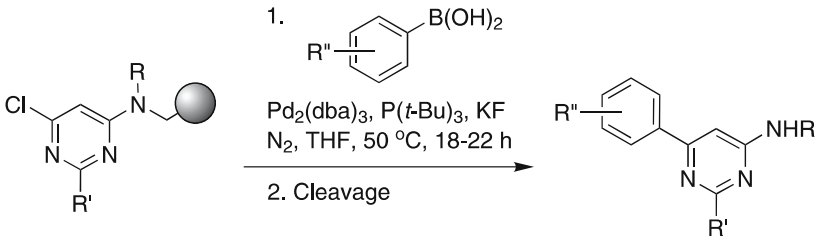
Another example is the synthesis of functionalized biaryl α-ketophosphonic acids, a class of compound with a wide range of biological activity [116]. Initial solution phase Suzuki reactions showed that the α-ketophosphonic acid moiety was unaffected by the reaction conditions, which were then transferred to the solid phase synthesis. This involved the synthesis of several polymer-supported amide-arylboronic acids and their coupling to three different bromobenzyl α-ketophosphonates (Scheme 40). Microwave heating was utilized to produce functionalized biaryl α-ketophosphonic acids **58** in good yield.



Scheme 40 Microwave Suzuki reactions to form biarylic compounds

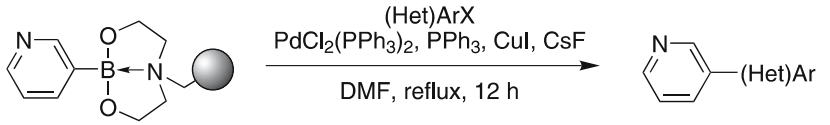
5.3.2
Aryl-Heteroarylic and Biheteroarylic Compounds

An example of the solid phase synthesis of aryl-heteroaryl compounds is shown by the work of Wade and Krueger [117]. Their strategy was one of the first to investigate Suzuki couplings with resin-supported dichloropyrimidines, due to the unavailability of the more reactive iodide or bromide derivatives. Under inert atmosphere, several catalysts, ligands, bases, and solvents were investigated. The optimized conditions were then applied to the synthesis of a 96-compound library with a double coupling, as shown in Scheme 41.



Scheme 41 Aryl-heteroaryl compounds via Suzuki coupling

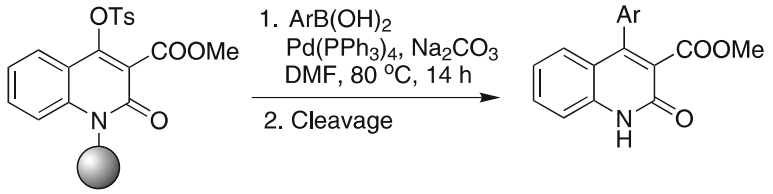
A novel route for the synthesis of aryl-heteroaryl and diheteroaryls by Gros et al. involved the immobilization of 2-pyridylboronate on a solid support (Scheme 42) [36]. Heteroaryl-boronic pyridines are particularly unstable and their use is very limited. However, via solid phase attachment, Suzuki couplings to a range of heteroaryl halides were possible in good yield (50–85%).



Scheme 42 Derivatization of pyridines

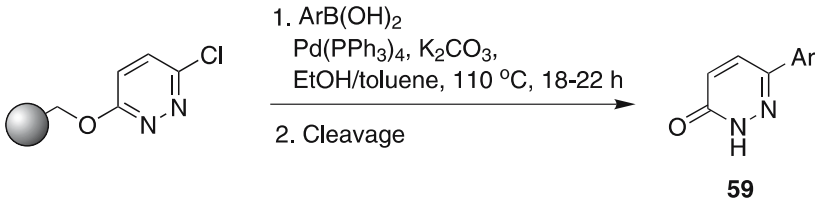
A recent example of the application of Suzuki reactions to introduce diversity is shown by the synthesis of 4-carbon-based substituted quinolinones [118]. There are limited methods for the synthesis of such derivatives and the outlined solid phase approach involved the synthesis of a resin-bound 3-methoxycarbonyl 4-tosyl quinolinone and subsequent Suzuki coupling with several aryl boronic acids and cleavage (Scheme 43). One drawback was the formation of hydrolysis byproducts, lowering the yield.

The importance of the aryl pyridazinone moiety in biologically active compounds has lead to the development of solid phase routes to novel derivatives for general screening. Salives et al. were the first to investigate such



Scheme 43 Diversification of quinolinones via Suzuki coupling

a solid phase reaction and utilized Suzuki couplings for introduction of the aryl moiety (Scheme 44) [119]. Several aryl or heteroaryl boronates were coupled to resin-bound chloropyridazine in high yield (as determined from elemental analysis). Cleavage gave aryl pyridazinones (**59**), with the phenyl pyridazinone obtained in 59% yield from Wang resin.



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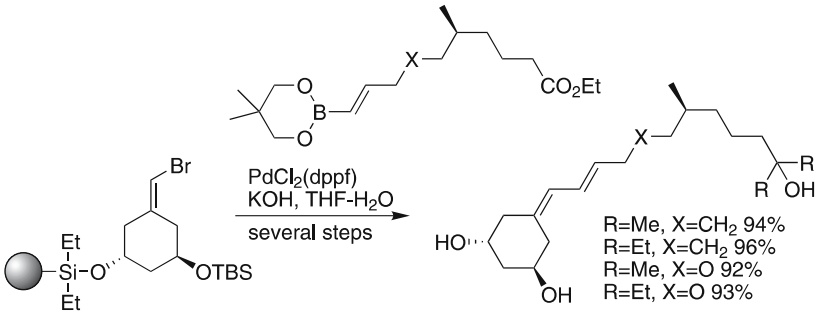
Scheme 44 Solid phase synthesis of aryl pyridazinones

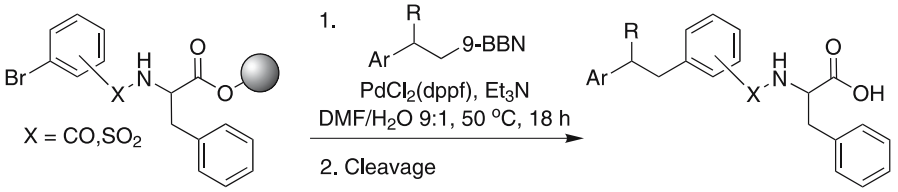
5.3.3
Miscellaneous Compounds Prepared via the Suzuki Reaction

The Suzuki coupling has also been undertaken utilizing alkenyls instead of aryls. One example of this on the solid phase is the synthesis of the hormonal, biological form of vitamin D_3 and several derivatives [120]. This involved the coupling of a solid-supported alkenylbromide to two different alkenylboronates (Scheme 45). Further derivatization and cleavage gave 19-*nor*-1α,25-dihydroxyvitamin D_3 and three new derivatives.

The dihydrostilbene (bibenzyl) fragment is an important substructure of many drugs and protein ligands, which has been accessible through solution phase Sonogashira couplings followed by reduction. An alternate method is needed for the solid phase synthesis and one such example utilized 9-BBN-mediated Suzuki coupling [121]. This involved an initial investigation of a wide variety of reaction conditions, including catalyst, base, and solvent for the Suzuki coupling of a resin-supported arylbromide with the adduct of 9-BBN and styrene, followed by the application of the preferred reaction conditions to the synthesis of a small combinatorial library (Scheme 46). The reactions generally gave high conversions and showed little difference



Scheme 45 Synthesis of vitamin D_3 derivatives



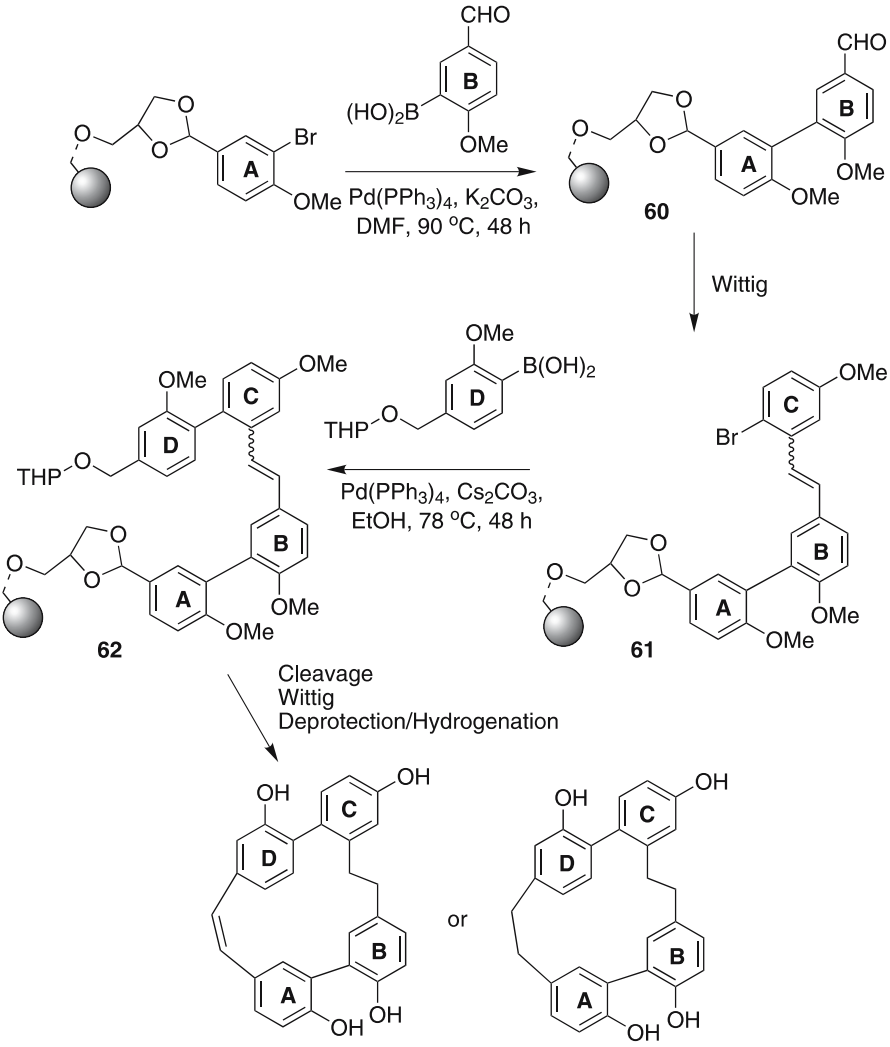
Scheme 46 Synthesis of dihydrostilbenes on solid support

between *para*- and *meta*-substituted arylbromides, or sulfonyl or carbonyl amide groups. With regard to the styrene, high yields were observed with both electron-withdrawing and electron-donating substituents on the phenyl ring. However, *ortho*-methoxy styrene failed, and heteroaryl olefins also gave only fair to poor yields. An example of an alkyl olefin was shown to give high conversion.

Another recent example of the synthesis of bibenzyls is illustrated by the work of Speicher et al. in their synthesis of cyclic bisbibenzyls of the isoplagiochin type found in liverworts [122]. Suzuki reactions played a key role in the strategy which involved the coupling of fragments A and B to form **60**, followed by a Wittig reaction to introduce fragment C in **61** and another Suzuki coupling to attach fragment D, affording **62** (Scheme 47). Hydrogenation, cleavage from the solid support, Wittig cyclization, and further deprotection/hydrogenation led to the synthesis of two cyclic bisbibenzyls in 17–20% overall yield.

5.3.4 Methodology Studies

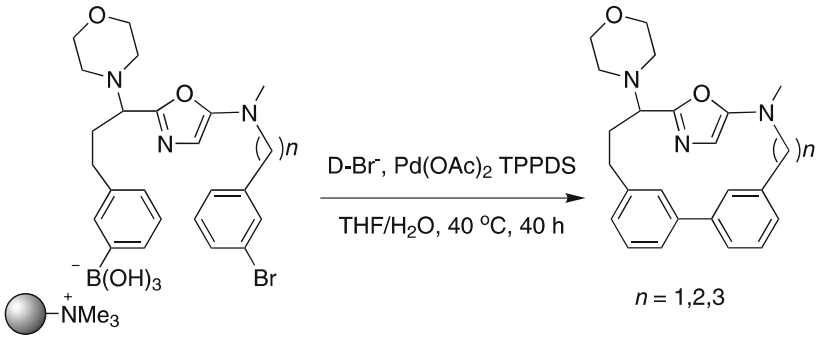
The Suzuki reaction has been applied to the synthesis of a biarylcyclopeptide through a "resin-capture-release" methodology [123]. This involved the capture of aryl boronic acids, containing a remote aryl halide, on ammonium hydroxide-form Dowex ion exchange resin, followed by a releasing



Scheme 47 Combined Suzuki–Wittig approach to bisbibenzyls

Suzuki cyclization (Scheme 48). Interestingly, this reaction was promoted by the attachment, as the free boronic acid did not undergo cyclization when subjected to the same Suzuki conditions. This approach allowed the synthesis of three different macroheterocycles.

Another methodology application utilizing the Suzuki reaction is chemical tagging for encoding during combinatorial synthesis. Todd and Abell have demonstrated that *p*-substituted alkyl phenol tags could be introduced via Suzuki couplings of aryl diboronic acids or aryl iodides under mild conditions that are tolerant of a variety of functional groups [124]. The tag itself is



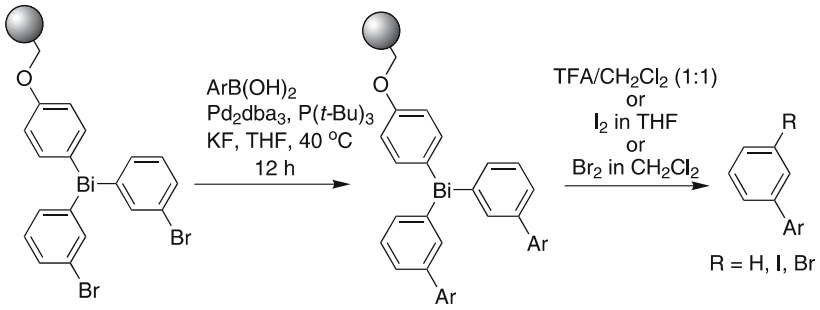
Scheme 48 Catch-and-release Suzuki coupling

inert and hydrophobic (well suited to chemistry on polystyrene beads), while it is possible to introduce polar groups on cleavage to aid flight and identification by accurate mass spectrometry. This method allowed the decoding of a tripeptide attached to a single resin bead and should be applicable to formal split-and-mix libraries.

The use of electron-rich phosphine ligands in palladium couplings has facilitated couplings of even the least reactive species. An application of this in solid phase synthesis was shown by Ohnmacht et al. [125], in which addition of 1,3,5,7-tetramethyl-2,4,8-trioxa-6-phenyl-6-phospha-adamantane as a ligand allowed the coupling of solid-supported aryl halides with an array of boronic acids at room temperature and low palladium loading. The enhancement was particularly evident for couplings of solid-supported *para*-bromobenzoic acid, while the corresponding aryl iodide showed little difference as it is already activated.

It should be noted that the success of solid phase Suzuki reactions can be dependant on the linker. In a study by Fernandez et al., optimal conditions for the Suzuki coupling of solid-supported 5-bromonicotinic acid with various boronic acids were found to vary for three different resins [126]. For amide resins, i.e., Rink and BAL, aqueous base in a toluene/EtOH solvent gave high yields and purities for a range of boronic acids. However, for ester-bound Wang resin, anhydrous conditions were required to achieve good conversion over a wide variation in the aryl boronic acid.

The development of multidirectional and/or traceless linkers for solid phase organic synthesis is of importance and as such, the use of a resin-bound bismuth complex has been investigated with Suzuki reactions to give functionalized biphenyl structures of pharmaceutical interest [127]. It should be noted that immobilization of the bromophenyl bismuthane on the solid phase prevents the bismuthane from coupling with the arylbromide that would otherwise be a problem in solution chemistry (Scheme 49). Also, mild conditions are needed to prevent side reactions of the bismuthane, and several solvents, palladium sources, ligands, and promoters were investigated. Once



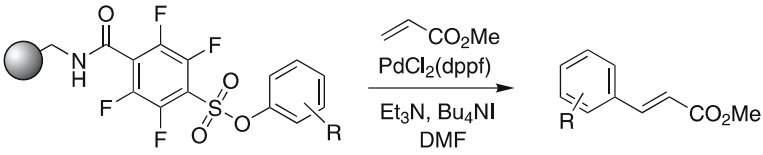
Scheme 49 Use of a bismuth linker in conjunction with the Suzuki reaction

coupled, it was shown that cleavage could be affected with either TFA, to give biphenyls (isolated yields of 58–68%), or using halo-debismutylation, to give iodo-substituted (isolated yields of 48–83%) and bromo-substituted (isolated yields of 60–69%) biphenyls.

5.4
The Heck Reaction

5.4.1
Method Development, Including the Use of New Linkers and Polymers

One of the more interesting applications of the Heck reaction on a polymer-bound substrate was reported by Revell and Ganesan [128]. Cross-coupling on solid phase generally involves attaching either the organometallic component to a solid phase or sequestering the aryl halide. In the latter case, this is generally done by linking the aryl moiety, leaving the halide free for reaction. If the halide component could be used as the linking point, a concomitant cross-coupling and cleavage could take place, but due to the fact that halides in general bond to one atom only, this is not possible. By replacing the halide by a pseudo-halide, in this case a triflate, attachment to the polymer via the carbon undergoing reaction is possible. Polymer-bound tetrafluorobenzenesulfonate **63** was reacted with methyl acrylate in the presence of $PdCl_2(dppf)$, affording different vinyl arene derivatives in 64–81% yield (Scheme 50). Suzuki reactions could also be performed using this linker system.



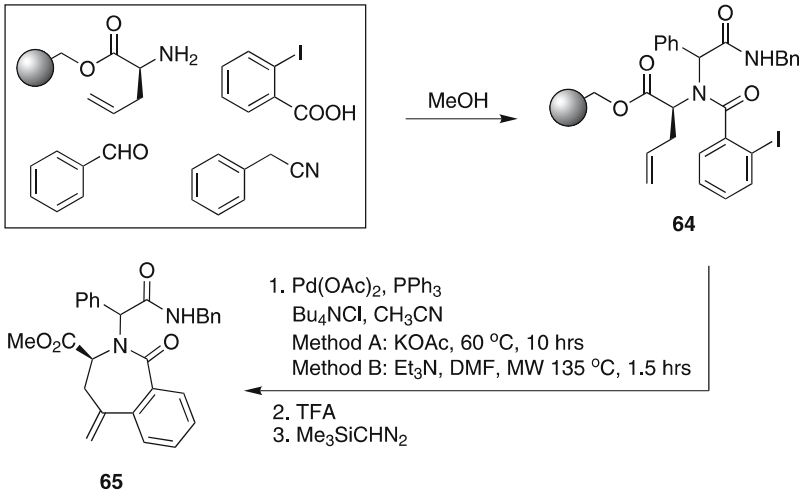
Scheme 50 Heck reaction employing a polymer-bound aryl sulfonate as a pseudohalide

Carbonneau et al. have carried out Heck reactions on a styrene-derived xerogel (a hybrid organic–inorganic solid phase) with moderate to good results depending on the proportions of the organic/inorganic monomers used when preparing the xerogel [129]. Transesterification was found to be the main side reaction. Dendrimers attached to a Wang resin core have also been used as supports for Heck reactions, as reported by Dahan and Portnoy [130]. Concerning variations of the reaction conditions, supercritical carbon dioxide has been employed by Holmes and coworkers as a solvent in polymer-supported Heck reactions with excellent results, REM resin being used as the alkene component in this case [131], while Morphy et al. have augmented the yields of solid phase Heck reactions by using extremely small solvent volumes even in the presence of a solid catalyst system [132]. Macrocyclization of peptidic derivatives, analogous to those described in the Sonogashira section (Sect. 5.2), have also been performed using the Heck reaction as reported by Akaji et al. [133]. Chlorotrityl resin gave better results than Wang resin in this case, and the cyclization reaction was found to perform better on solid phase than the corresponding reaction in solution. Takahashi and coworkers have reported Heck reactions on polymer-supported dehydroalanines, followed by asymmetric hydrogenation using a Rh(I)-DuPHOS catalyst system, in the preparation of a library of 36 dipeptides containing unnatural amino acids [134]. Finally, Dupont and colleagues have utilized solid phase Heck reactions as a probe to investigate the homogeneous/heterogeneous nature of a palladacycle catalyst precursor derived from the chloropalladation of *N*,*N*-dimethyl-1-phenylpropargylamine [135].

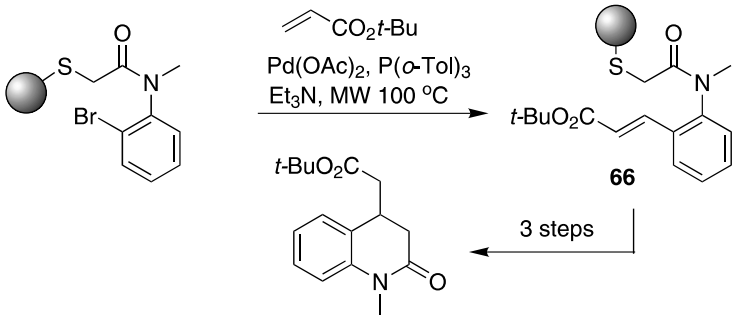
5.4.2
Heterocyclic Compounds Prepared via the Heck Reaction

In comparison with the other common cross-coupling methods, there are fewer examples of the use of the Heck reaction for the construction of heterocyclic systems on solid phase, although some interesting reports have appeared. Gracias et al. have prepared a seven-membered ring on Wang resin, employing a Ugi four-component reaction, forming **64**, followed by a cyclative Heck reaction (Scheme 51) [136]. Conventional heating gave a somewhat higher overall yield of **65** (44%) as compared to microwave heating (33%).

Procter and coworkers have applied a microwave-assisted Heck reaction as an intermediate step in the construction of tetrahydroquinolones on solid phase (Scheme 52) [137]. Optimization of the reaction conditions showed DMF to be the optimal solvent for the solid phase Heck reaction, and a small library of tetrahydroquinolones was prepared using this approach. Additional studies showed that intermediates of the type **66** could be cyclatively cleaved with SmI_2 in one step if the sulfur linker was replaced by a sulfoxide group (i.e., the HASC linker).



Scheme 51 Preparation of a seven-membered heterocycle on solid phase employing a sequential Ugi/Heck reaction

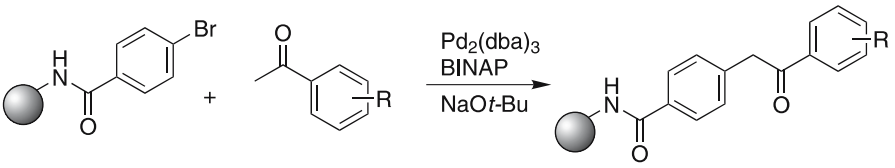


Scheme 52 Tetrahydroquinolone synthesis on solid phase involving a Heck coupling

5.5
α-Arylation and Aryl Amination

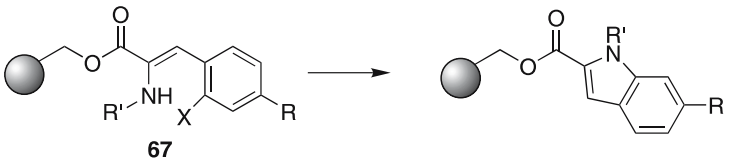
α-Arylation of ketones is a powerful tool for creation of drug-like compounds. The first example of this reaction on solid phase is described by Limbeck et al. [138], who performed α-arylation of aromatic, aliphatic, heteroaromatic, and cyclic ketones as well as 1,3-diketo compounds (Scheme 53). It was discovered that neither cyclic ketones nor 1,3-diketo compounds were suitable for α-arylation, while the other compounds mentioned above gave the desired products in satisfactory yields and excellent purities.

Indoles are known to posses various biological activities, and there is a need for diversely substituted indoles as potential drug candidates. One solution to this problem is the use of palladium-catalyzed cyclization of



Scheme 53 α-Arylation of ketones on solid phase

polymer-supported enaminoesters, presented by Yamazaki et al. [139, 140]. The resin **67** was heated in the presence of 15 mol % $Pd(OAc)_2$ or $Pd_2(dba)_3$ and NEt_3 (Scheme 54). The addition of $P(o-Tol)_3$ improved the reaction outcome, and the desired products were obtained in good to moderate yields. The group also investigated intramolecular amination of immobilized *N*-acetyl- or *N*-Cbz-dehydrobromophenylalanine. The reaction conditions were similar to those in solution, i.e., heating in the presence of $Pd_2(dba)_3$ and a ligand along with $MeNCy_2$. The desired indoles were obtained in good yields, but for some of the substrates the reaction conditions needed optimization. The one-pot synthesis of indoles by tandem *C*,*N*-arylation was also investigated. The desired compounds were obtained in satisfactory yields, but the reaction was not selective, and the method is thus limited to symmetric substrates.

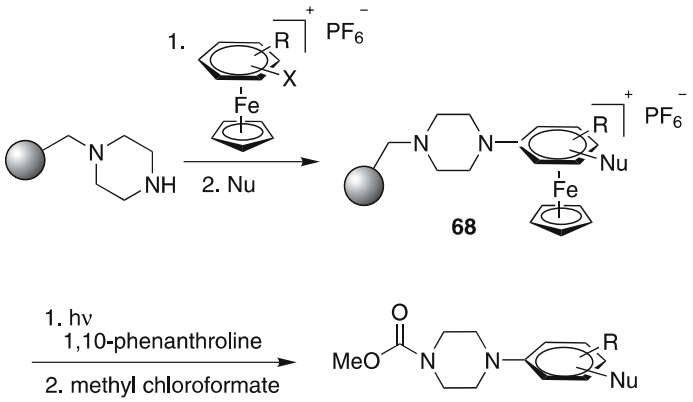


Scheme 54 Indole synthesis via palladium-catalyzed aryl amination

6
Miscellaneous Reactions

6.1
Nucleophilic Aromatic Substitution Involving Iron Carbonyl Complexes

The first example of iron-activated nucleophilic aromatic substitution on solid phase has been presented by Ruhland et al. [141], who attached [(cyclopentadienyl)-benzene $Fe(II)]^+PF_6^-$ to polymer-bound piperazine (Scheme 55). The complex **68** was subjected to a variety of nucleophiles using different protocols. The decomplexation was achieved by irradiation in the presence of phenanthroline, and in the final step the resin-bound products were cleaved with methyl chloroformate to give corresponding carbamates in good yields.

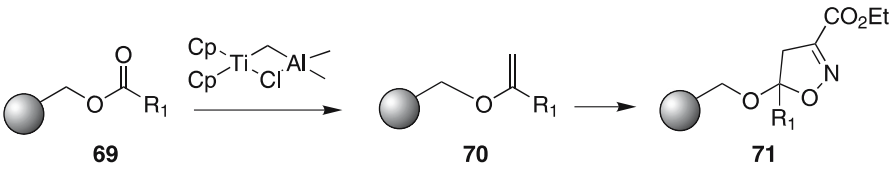


Scheme 55 Iron-assisted nucleophilic aromatic substitution on solid phase

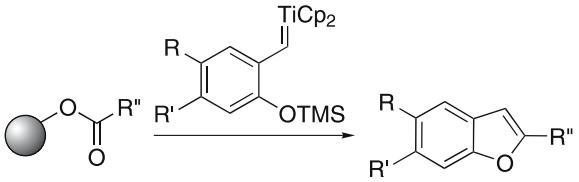
6.2 The Tebbe Reaction

As has been shown previously, the choice of the linker is of crucial importance in solid phase synthesis. Barrett et al. [142] showed that immobilized carboxylic esters **69** undergo Tebbe olefination resulting in polymer-bound vinyl ethers **70**, that can be used in different chemical transformations such as [3+2] cycloadditions of nitrile oxides (Scheme 56). This reaction is known to be troublesome in solution; however, when performed on solid phase, the resulting products **71** are obtained in satisfactory yields and excellent purities.

Titanium(IV) benzylidenes bearing a masked oxygen or nitrogen nucleophile can be used in the synthesis of benzofurans and indoles. Macleod



Scheme 56 Tebbe olefination followed by dipolar cycloaddition



Scheme 57 Synthesis of benzofurans using Tebbe reagents

et al. [143] used titanium benzylidenes derived from thioacetals to introduce the masked nucleophile in the *ortho*-position (Scheme 57). Treatment of the immobilized enol ethers with TFA gave the products in excellent purity with no further purification required.

7 Concluding Remarks

Due to the large number of publications on this topic, this review summarizes only a fraction of the latest developments in solid phase organometallic chemistry. Of special interest, however, is that the scope of metal complexes has expanded to encompass much more than cross-coupling. Also, the advantages of performing organometallic reactions on a solid phase in terms of avoiding side reactions like homocoupling and dimerization, have been capitalized upon. The number of examples of the application of organometallic chemistry on polymer-bound substrates for library generation or for the preparation of natural product analogues has increased, indicating that these methods are robust and synthetically useful. Oligomer synthesis on support, mimicking the long-established solid phase peptide synthesis, is a new developing area, while reactive intermediates like carbenes are becoming more frequent in solid phase chemistry. However, some topics still remain for development, such as palladium-catalyzed α-arylation, the wide scope of rhodium-catalyzed reactions, and the application of metal-mediated asymmetric chemistry on solid phase, and provide a challenge for future researchers in this area.

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Top Curr Chem (2007) 278: 135–208
DOI 10.1007/128_2007_135

Published online: 7 June 2007

Solid Phase Chemistry for the Directed Synthesis of Biologically Active Polyamine Analogs, Derivatives, and Conjugates

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Abstract Polyamines in naturally occurring compounds are biologically active molecules that do not display a great diversity but are very abundant in almost all organisms. Their functions comprise antibiotics, immunosuppressants, toxins, and many others. In most

biologically active compounds the polyamine chain is coupled to other functionalities making the polyamine an excellent template for solid phase chemistry and combinatorial approaches. This review comprises common techniques for the synthesis of polyamines and polyamine derivatives on solid support. It includes the description of basic synthesis requirements such as of suitable resins, new linkers, reaction conditions for generation of biologically active polyamines and polyamines in natural products as well as the description of protection groups. Moreover, it will focus on the modular coupling of the amine building blocks using different synthesis strategies. Thus, the advantages of performing reactions on polymer-supported substrates in terms of avoiding side reactions and facilitating product/metal separation are highlighted.

Keywords Alkylation · Philanthotoxin · Polyamines · Polycationic compounds · Solid phase synthesis

Abbreviations

ADDP	1,1′(Azodicarbonyl)dipiperidine
Ac	Acetyl/acyl
Alloc/Aloc/	Allyloxycarbonyl
ADP	Adenosine diphosphate
AMD	Age-related macular degeneration
AMP	Adenosine monophosphate
AMPA	2-Amino-3-(3-hydroxy-5-methyl-4-isooxazolyl)propionic acid
ATP	Adenosine triphosphate
BAL	Backbone amide linker
Bn	Benzyl
Boc	*tert*-Butyloxycarbonyl
Bu	Butyl
t-Bu/^{t}Bu	*tert*-Butyl
Cy	Cyclohexyl
Cys	Cysteine
DBU	1,8-Diazabicyclo-[5.4.0]-undec-7-ene
DCC	*N*,*N*′-Dicyclohexylcarbodiimide
DIC	*N*,*N*′-Diisopropylcarbodiimide
Dde	1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl
DEAD	Diethylazodicarboxylate
DiPEA	Diisopropylethylamine
DMAP	Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMTr/DMT	4,4′-Dimethoxytrityl
DNA	Desoxy-ribonucleic acid
Dod	4,4′-Dimethoxydityl
Et	Ethyl
Fmoc	9-Fluorenylmethyl-oxycarbonyl
FMOP	4-Formyl-3-methoxy-phenoxymethyl-polystyrol
Glu	Glutamate
Gly	Glycine
HBTU	2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphat
HOBt	1-Hydroxybenzotriazol

HODhbt	3,4-Dihydro-5-hydroxy-4-oxo-1,2,3-benzotriazine
LPS	Lipopolysaccharide
MBHA	*p*-Methylbenzhydrylamine
Me	Methyl
Mesyl	Methylsulfonyl
Mmd	4-Monomethoxydityl
MMTr/MMT	4-Monomethoxytrityl
NMDA	*N*-Methyl-D-aspartate
NMP	*N*-Methyl-2-pyrrolidone
Nosyl/Ns	Nitrophenylsulfonyl
NPTX	Nephilatoxin
o	Ortho
ODC	L-Ornithine decarboxylase
p	Para
PAO	Flavin-dependent polyamine oxidase
PEG	Polyethylenglycol
Pfp	Pentafluorophenyl
Ph	Phenyl
Pht	Phthaloyl
PhTX	Philanthotoxin
PS	Polystyrene
RNA	Ribonucleic acid
rt	Room temperature
SAMDC	*S*-Adenosyl methionine decarboxylase
siRNA	Small interfering RNA
SMO	Spermine oxidase
SPS	Solid phase synthesis
SSAT	Spermine/spermidine acetyltransferase
TBAF	Tetrabutylammonium fluoride
Teoc	2-(Trimethylsilyl)-ethoxycarbonyl
Tfa/TFA	Trifluoracetic acid/trifluoroacetyl
TFMSA	Trifluormethanesulfonic
THF	Tetrahydrofuran
TMOF	Trimethylorthoformiate
Tosyl	*p*-Toluenesulfonyl
Tr/Trityl	Triphenylmethyl
Z	Benzyloxycarbonyl

1 Naturally Occurring Polyamines and Their Function in Vivo

Most of the naturally occurring polyamine structures that are ubiquitously present in all prokaryotic and eukaryotic cells are based on simple aliphatic structures such as in putrescine (**1**), spermidine (**2**), spermine (**3**), and cadaverine (**4**) (Fig. 1)

The diversity is low but the cellular concentration within a millimolar range is rather high giving rise to a variety of important biological

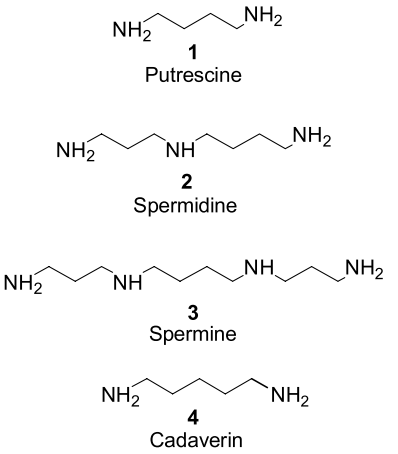


Fig. 1 Naturally abundant polyamines

functions. Their biosynthesis in bacteria is based on the decarboxylation of L-arginine to putrescine while mammals use L-ornithine as a precursor (Fig. 2). L-Ornithine, an amino acid that is not used for protein biosynthesis, is synthesized during the urea cycle and is expressed mainly in the liver but is present in almost all tissues. It provides the 1,4-diaminobutane unit, whereas the aminopropyl unit comes from L-methionine [1]. Interestingly, the main natural and biogenic polyamines are made almost exclusively with 1,3-diaminopropyl and 1,4-diaminobutyl units and display little diversity in their sequence and length as compared to other natural compounds.

Apart from biosynthesis, a large amount of polyamines are obtained from the diet and from bacteria residing in the gut [5, 6]. These polyamines are transported through the mucosa, presumably by passive diffusion through the paracellular pathway [7] and reach systemic circulation. Eventually, they are distributed throughout the body and are widely implicated in cell growth, development of tissues, and tissue repair [2]. They have been involved not only in the growth of normal tissue but also in cell proliferation of malignant tumors. Further, it has been implicated that they play an important role in the development of the gut and the repair of gastric and duodenal injuries as well as in other wound healing processes. Increase in polyamine levels in several other tissues has been associated with the normal growth and hypertrophy (increase in tissue size or cell size) of several tissues such as skin, breast, kidney, and heart. Since polyamines are protonated under physiological conditions, they can interact with many negatively charged or zwitterionic biomolecules such as DNA, RNA, phosphate groups of phospholipids, and negatively charged amino acids of proteins such as aspartate and glutamate and many more. Spermine, in particular, (**3**) has been shown to condense DNA [8–10] and chromatin [11] and to stabilize DNA conformation as well as

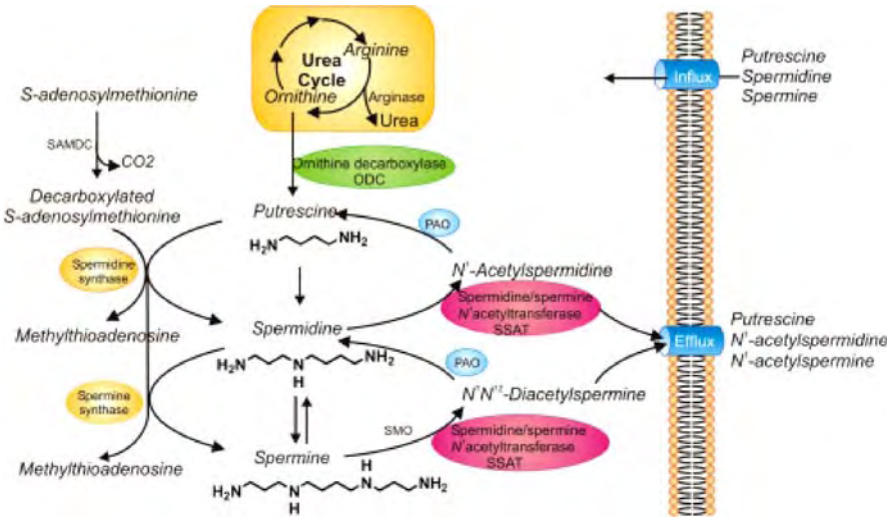


Fig. 2 Schematic overview of polyamine metabolism in mammalian organisms (modified from Gerner and Meyskens [2]). Besides their cellular import from nutrition, polyamines are synthesized intracellularly. L-Ornithine, the precursor of natural polyamines, is derived from L-arginine, which is produced in the urea cycle through the action of arginase. In the first step of polyamine synthesis, ornithine is decarboxylated by L-ornithine decarboxylase (*ODC*) yielding putrescine. In the second and third reactions spermidine and spermine are produced by the spermidine and spermine synthases, which transfer a propyl amine moiety derived from the decarboxylation of *S*-adenosylmethionine, by *S*-adenosylmethionine decarboxylase (*SAMDC*), respectively. Eventually, spermine and spermidine can be mono- or bis-acetylated by the spermidine/spermine N^1-acetyltransferase (*SSAT*) for either cellular export and excretion into the urine or conversion into putrescine by flavin-dependent polyamine oxidase (*PAO*). In addition, there are first reports on a spermine oxidase (*SMO*) that can oxidize non-acetylated spermine to produce spermidine [3, 4]

DNA triplexes to either modulate protein–DNA interactions or to protect the DNA from degradation [12, 13]. The hydrophilicity/hydrophobicity level of most polyamines is balanced, allowing them to perform physiological functions by interacting with some of these anionic structures, without impairing the functionality of others.

Besides the genuine aliphatic polyamines, numerous naturally occurring polyamine conjugates have been isolated and characterized displaying a broad range of biological functions and activities. They comprise compounds that range from potent immunosuppressive agents such as deoxyspergualin (**5**) [14, 15], over antibacterial substances and broad-spectrum antibiotics such as squalamine (**6**) [16] edeine A (**7**) [17] guanidospermidines **8**, and glycocinnamoylspermidines **9**, to alkaloids such as kukoamine A (**10**), wasp toxins, and spider venoms [18, 19] (Fig. 3 and Sect. 2.2).

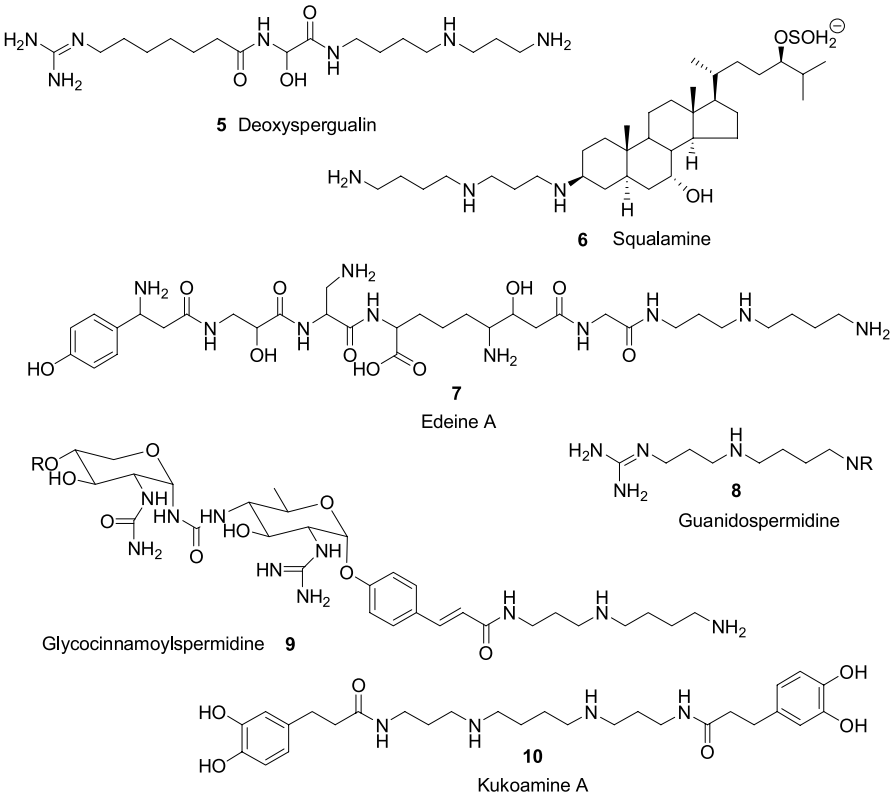


Fig. 3 Selection of naturally occurring polyamines, which display a variety of biological functions

Likewise, due to their cationic properties polyamine moieties were found to enhance water solubility and cellular uptake of many therapeutic molecules, making them potential drug delivery agents. For most of the properties of polyamines the biological mechanisms are not well understood or are still in debate. Therefore, current research on natural and synthetic polyamines requires the synthesis of those compounds and many derivatives to investigate their function and to gain novel mimics for therapeutic applications.

Up to the year 2000 many reviews emerged that described the classes of naturally occurring polyamines and the research on their synthetic polyamine conjugates [1]. Recently, polyamine alkaloids, toxins, and polyamines in cancer therapy have been reviewed in detail [2, 20–22]. In this review, we will group the polyamines considering their properties, and focus exclusively on the therapeutic applications of polyamine compounds that gained interest from 1996 until the present, as well as on the new advances in polyamine synthesis and modification using solid phase

chemistry and combinatorial approaches [23–31] (for further references see Sect. 3).

2
Polyamines with Therapeutic Properties

To date, many properties of polyamines are known to be interesting for therapeutic application. These applications can be classified into five groups: DNA recognition and binding; development of antibiotics, bactericides, and antiparasitics; natural product synthesis of toxins; cancer therapy; and drug delivery.

2.1
DNA Recognition and Binding

A number of studies have suggested that DNA is a major target for the function of polyamines. As already described above (for review see [1]), aliphatic polyamines have been shown to either condense or bend DNA by binding to the nucleic acid via strong salt bridges, or to disturb the DNA structure by introducing interchalating conjugates. This interaction has a great influence on DNA conformational transitions, condensation/decondensation processes and DNA stabilization [32, 33]. Thereby, stabilization of specific DNA conformations may be important for the nucleosome formation, chromatin condensation, and gene expression. Binding of the polyamines alters the DNA structure from the common B-helix to the A and Z forms or leads to aggregation at higher concentrations [34]. Such an effect might be important for DNA physiology, as a tight connection occurs between transcriptional activity on DNA and acquisition of the Z form [35, 36]. The stability of the double helix increases with the ratio of the positively charged amino groups to the negatively charged phosphate groups [37], meaning that longer polyamines can lead to a better protection of the DNA. Not only the binding but also the bending and the conformational change of the DNA by polyamines lead to a protection from DNA damaging agents such as reactive oxygen species and from endonucleases like DNAse I [36, 38–42]. The condensation effect prevents the DNA fragmentation thus inhibiting apoptosis. Reactive oxygen species and singlet oxygen induce oxidative damage to DNA by single-strand breaks [38]. Further, polyamines also have the ability to promote specific modifications in assorted RNA molecules [10, 43–46] or to stabilize and stimulate the action of ribonucleases [10, 47] and ribozymes [48–55].

Functional interaction between DNA and polyamines extends to DNA–protein binding, resulting in regulatory complexes to regulate gene expression [56]. As a result of the structural similarity, polyamine analogs

compete with natural polyamines for the DNA binding and interaction with proteins. The ability of polyamines to alter DNA–protein and protein–protein interactions might be revealed in disturbance of cellular functions, when defects in catabolism or export lead to an accumulation of polyamines [57].

One of the main functions of polyamines is their ability to interact with DNA for the regulation of gene expression and apoptosis. Besides their function in promoting cell growth, polyamines also play an important role in facilitating cell death [57], which seems at first to be contradictory. This potential makes the polyamines and especially their analogs good candidates for anticancer therapeutics. Interactions of polyamines with DNA have therefore been thoroughly studied in order to allow for the selective design of anticancer drugs by the synthesis of a series of analogs.

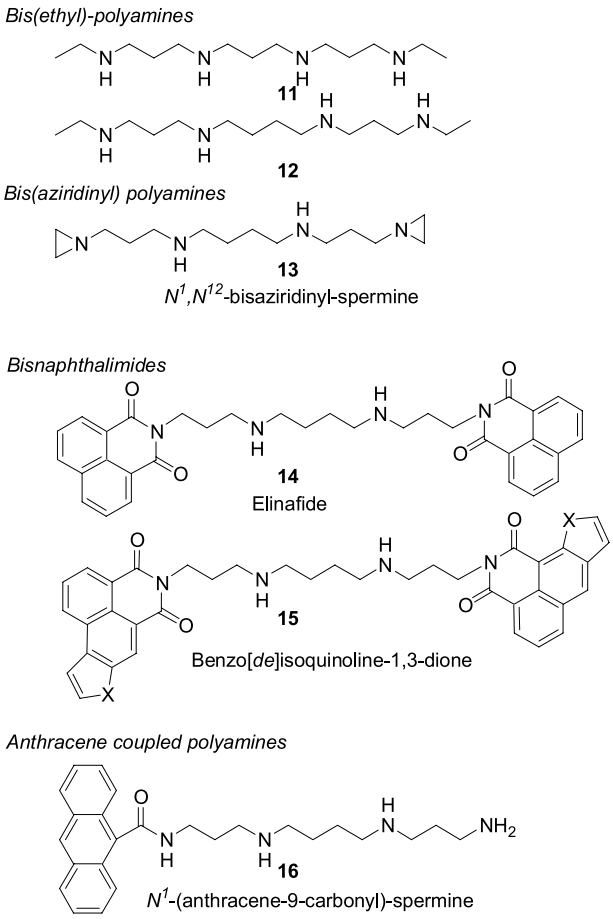


Fig. 4 Polyamines with DNA binding and interchalating activities

To date, most of the polyamine analogs that induce apoptosis in a variety of tumor cell lines are bis(ethyl)polyamine analogs (**11**, **12**) [58–60] (Fig. 4 and Sect. 2.4). However, the mechanism by which the apoptosis occurs differs between polyamines and their analogs. Interesting DNA-interacting polyamine analogs are naphthalimides and bis-naphthalimides (**14**, **15**) (Fig. 4), which have been shown to interchalate into the DNA backbone [61]. Other polyamine analogs using the same mechanism are, for example, N^1-(anthracene-9-carbonyl)-spermine (**16**) [62–64] and N^1,N^{12}-bisaziridinyl-spermine (**13**) (Fig. 4). The polyamine skeleton of these compounds enhances the cellular uptake by cells and facilitates the interchalating effect of the anthracene moiety by bringing the compound closer to the DNA [65].

2.2
Polyamine Conjugates from Spiders and Wasps

In the last couple of years many groups focused their research on the synthesis of polyamines present in spider and wasp toxins such as nephilatoxin-643 (NPTX-643) (**24**), philanthotoxin-433 (PhTX-433) (**26**) (Fig. 5) and many analogs (Fig. 6). There are several reviews [20, 21] that describe these toxins in more detail, while we will mainly focus on the synthesis of those polyamine conjugates already performed on solid support and the first combinatorial libraries (see Sect. 3). These natural toxins (Fig. 5) are mainly present in the venom glands of the spider or wasp and serve as a paralyzing toxin for their prey.

Most of the polyamine toxins show a high degree of structural similarity implying that the polyamine part of the toxin has an important function. The interest in these acylpolyamines arises from their ability to act as potent antagonists of the mammalian neuroexcitatory or ionotropic glutamic acid receptors (iGluR) or as inhibitors of the nicotinic acetylcholine receptors. The family of iGlu receptors comprises the *N*-methyl-D-aspartate (NMDA), the 2-amino-3-(3-hydroxy-5-methyl-4-isooxazolyl)propionic acid (AMPA), and the kainate receptor, which are blocked by the polyamine toxins in an open cationic channel conformation [21]. Attempts have been made to exploit this interaction for the therapy or prevention of neurotoxicity, epilepsy, and neurodegenerative diseases [66, 67].

As described for many polyamine toxins, the polyamine moiety is coupled via an amide bond to an aromatic moiety, which mostly consist of 2,4-dihydroxyphenylacetyl or indol-3-acetyl moieties. Nakanishi and coworkers as well as Blagbrough and colleagues conducted structure and function studies of polyamine toxin analogs to improve their potential as inhibitors of the nicotinic acetylcholine receptor (for review see [21]). To date, many groups have focused on the development of novel polyamine toxin analogs as therapeutically active agents. Most of the syntheses in this field are already performed on solid phase and will be discussed in Sect. 3. Some of the analogs (**27**–**35**) are depicted in Fig. 6.

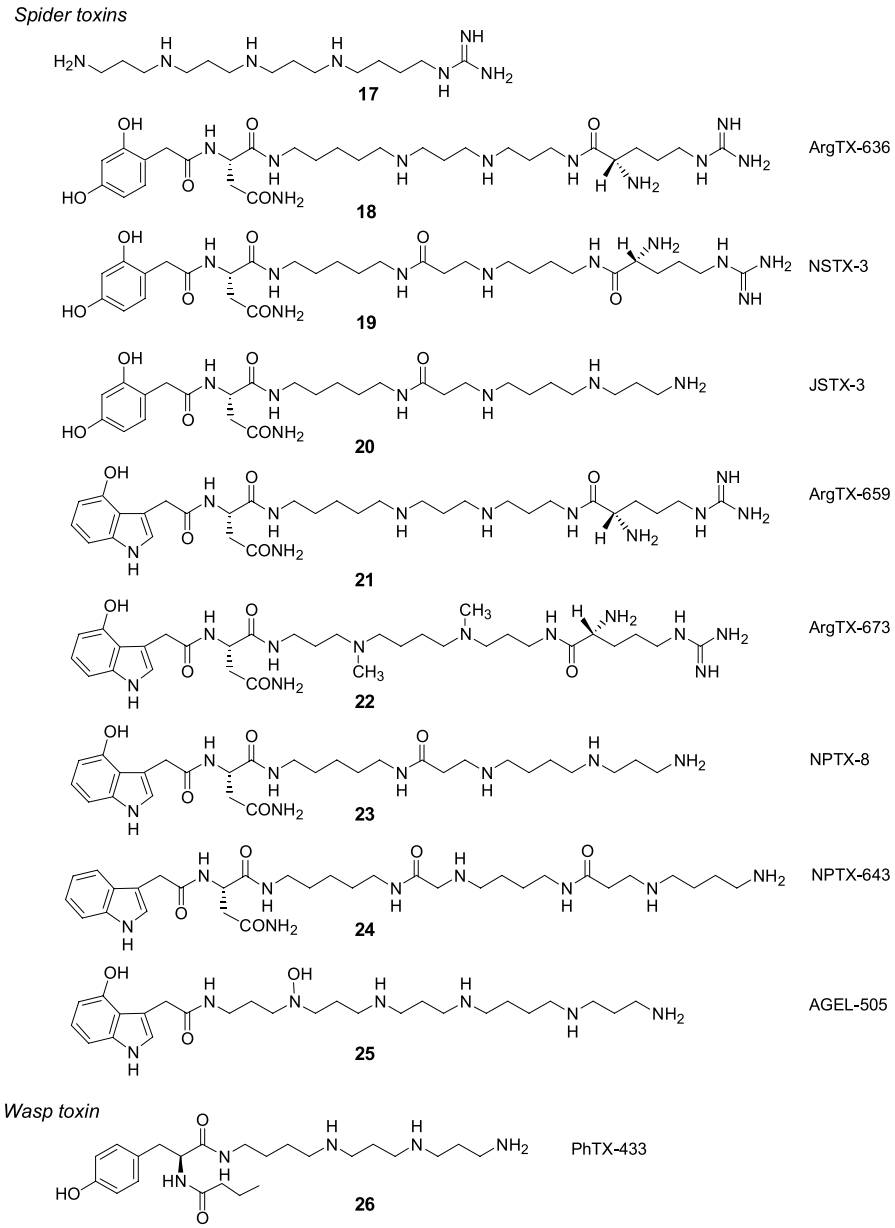
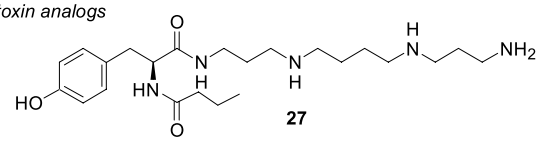


Fig. 5 Naturally occurring spider (**17**–**25**) and wasp (**26**) toxins containing polyamine backbones. Many analogs of nephilatoxin-643 (NPTX-643) (**24**), philanthotoxin-433 (PhTX-433) (**26**) have been generated using solid phase chemistry (see Sect. 3)

Fig. 6 Some examples of polyamine toxin analogs. They are divided into different subgroups such as the analogs with bulky headgroups or analog-conjugates ►

Philantotoxin analogs



PhTX-343

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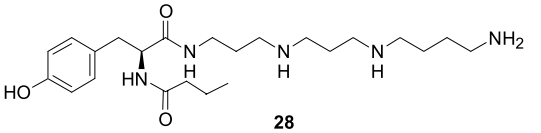

PhTX-334

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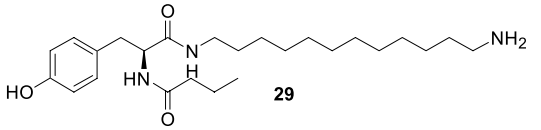

PhTX-12

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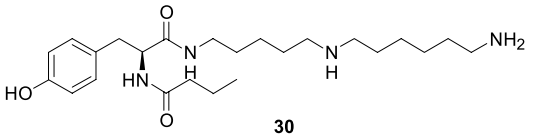

PhTX-56

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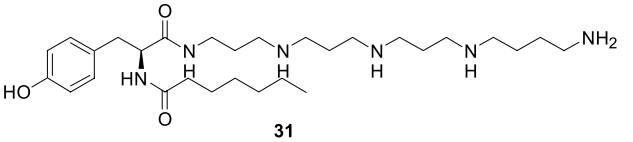

C_7-PhTX-3334

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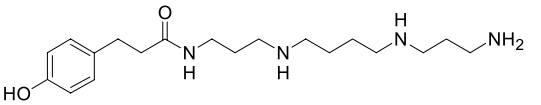

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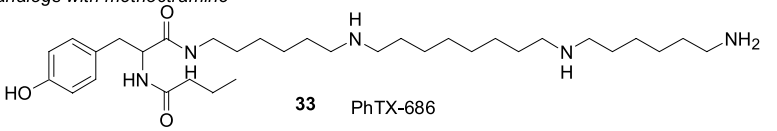
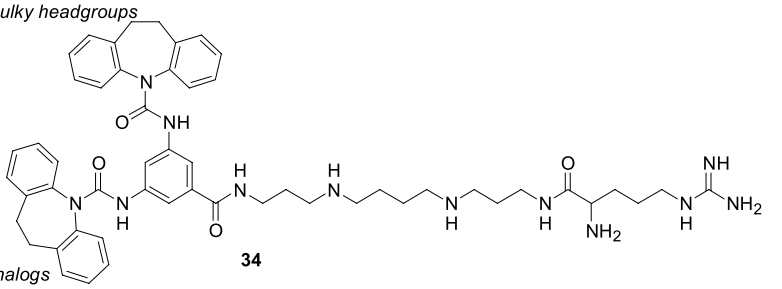
Hybrid analogs with methoctramine



33 PhTX-686

Analogs with bulky headgroups



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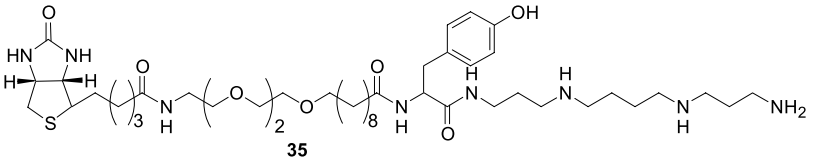
Biotinylated analogs



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2.3 Polyamines and Polyamine Conjugates with Antibiotic, Bactericidal, or Antiparasitic Properties

The emergence of multidrug-resistant microorganisms, such as the methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and drug-resistant *Mycobacterium tuberculosis*, constantly require the development of novel antibiotics [68–70]. As the majority of the resistant bacteria are gram-negative, the outer membrane of those bacteria is protected with a barrier of crosslinked lipopolysaccharides (LPS) that not only can be penetrated by metal-chelating agents but also by polycations such as basic peptides or polyamines. Thereby, the polyamines are supposed to interact with the lipid A moiety of the LPS, leading to a perturbation of the bacterial membrane. While there are already reports on the antibiotic activity of polycationic peptides, there are no comprehensive studies on the antibiotic action of polyamine analogs. This might be due to first therapeutic studies with the polycationic peptides requiring large dose applications while showing lack of oral bioavailability, strong immunogenicity, and non-specific cytotoxicity [71].

Research on polycationic mimics and analogs revealed that the cationic amphipathity is not only required for the uptake of such antibiotics but also for the antibiotic action. Mimics that are currently under research are polyamines substituted with long alkyl side chains, the so-called lipopolyamines. To date, it is known that *N*-acylated spermine derivatives **36**–

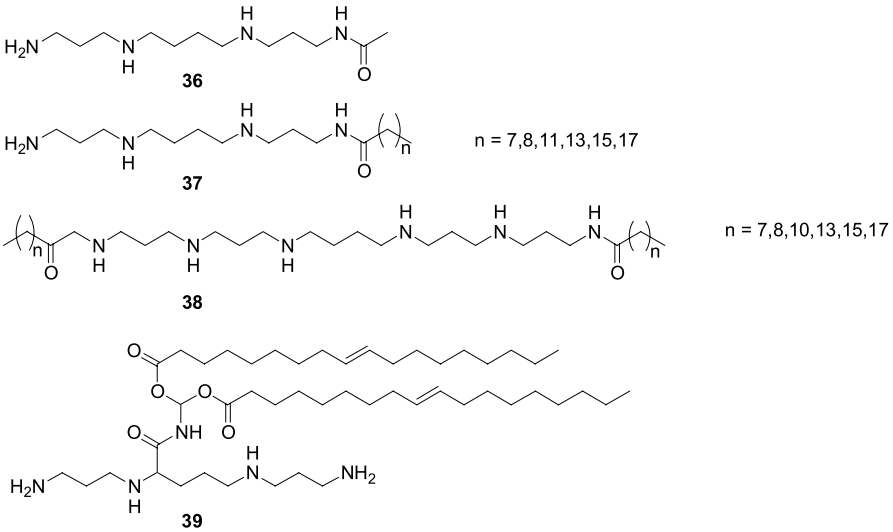


Fig. 7 Mono- and bis-acyl homospermines as recently synthesized and tested for their use in sepsis treatment by David and coworkers [73, 74]

39 form stable complexes with LPS and are effective in preventing endotoxic shock while being non-toxic due to their metabolization to physiological components such as polyamines and fatty acids [72–74] (Fig. 7). Antibacterial activity of 1,3-di-oleoyloxy-2-(6-carboxyspermyl)-propylamide (**39**) (DOSPER) has been determined at concentrations up to 75 $\mu g\,mL^{-1}$ [73] due to its extremely labile ester-linked acyl moieties. It has been shown that in lipopolyamines and other polycationic species a correlation exists between the antiendotoxic and the antibiotic activities [75–77]. Combination of both properties can regulate the antibiotic-induced endotoxin release [78–80].

2.3.1 Antibiotic Polyamine Conjugates with Steroids

In recent years an interesting broadband antibiotic has been isolated from the dogfish shark *Squalus acanthias*, which was designated as squalamine (**6**) (3-β-*N*-1-(*N*-[3-(4-aminobutyl)]-1,3-diaminopropane)-7-α-24-ζ-dihydroxy-5-α-cholestane-24-sulfate) (Fig. 8). Squalamine is a cationic steroid characterized by a condensation of an anionic bile salt intermediate with spermidine and exhibits potent antibiotic and microbiotic activity against both gram-negative and gram-positive bacteria as well as against fungi [16, 70, 81].

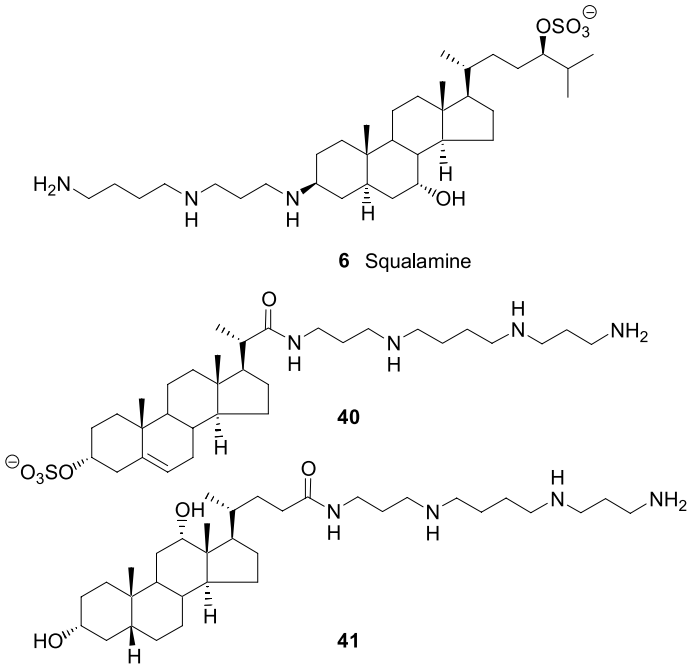
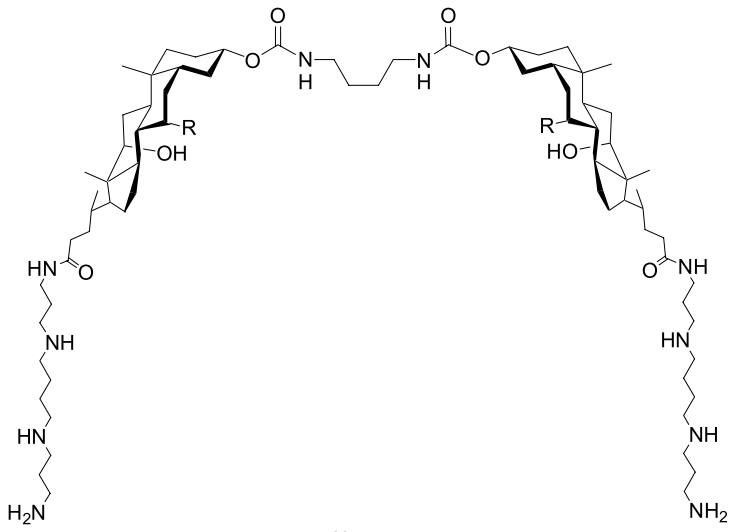


Fig. 8 Squalamine (**6**) and its analogs (**40** and **41**)

In addition, squalamine induces osmotic lysis of protozoa [16] and shows antiangiogenic activity, making it useful for the treatment of different diseases such as lung, ovarian, brain, and other cancers as well as age-related macular degeneration (AMD) and the control of body weight in mammalian organisms [82]. Its amphiphilic structure derived from the hydrophilic spermidine chain and the hydrophobic sterol scaffold enables the squalamine to interfere with membrane lipids and to enhance the cellular uptake. Moreover, some of the squalamine mimics (**40**, **41**) (Fig. 8) and related compounds based on the squalamine structure effectively permeabilize the outer membranes of gram-negative bacteria sensitizing these organisms to hydrophobic antibiotics [83].

The cellular recognition and selectivity of those steroid–polyamine based antibiotics differs between eukaryotic and prokaryotic cells but share mechanistic aspects with cationic peptide antibiotics [83]. It has been reported that squalamine mimics (i.e., **41**) lacking the sulfate residue retain their antibiotic activity, indicating that the amphiphilic or cationic structure is important for the mechanism of action [81]. As already mentioned, the structure is membrane-active. A kinetic analysis of the membrane activity of squalamine analogs supported a model in which monomer and a noncovalently linked dimer coexist in equilibrium at the membrane [81]. From this notion, molecular umbrella molecules **42** (Fig. 9) were generated, in which two molecules of squalamine or its derivatives are covalently linked to form dimers. Those dimers can be coupled with a variety of cargo and drug molecules promoting their delivery into cells and tissue (Fig. 10). For



42

Fig. 9 Squalamine-based umbrella transporter

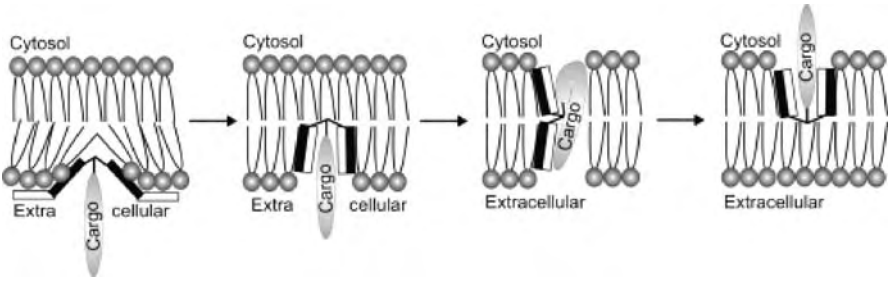


Fig. 10 Proposed mechanism of squalamine dimer-supported drug delivery through cellular membranes. The mechanism is also proposed for the bactericidal mode of action with non-covalently coupled squalamine residues. Modified from [81, 87]

the last couple of years Regen and coworkers have focused on different coupling strategies and cargos, which ranged from small molecules such as one amino acid up to ATP, AMP, glutathione, anti-HIV drugs, and even oligonucleotides [81, 84–87].

2.3.2 Antimicrobial Alkaloid Polyamines

Other polyamines with antimicrobial activity are secondary metabolites from marine sponges, such as pseudokeratidine (**43**) [88–90], which is used in antifouling paints. The majority of those alkaloid polyamines contain a spermidine moiety such as ptilomycalin A (**44**) [91], a marine alkaloid possessing antibiotic activity (Fig. 11).

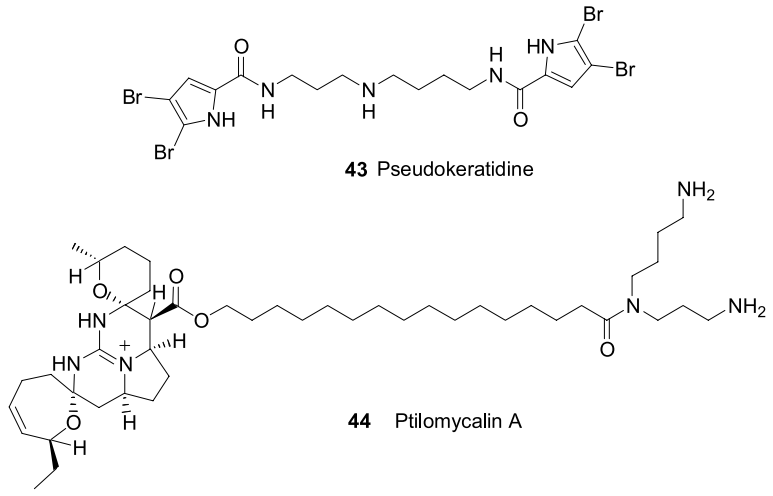


Fig. 11 Selection of antimicrobial and antibiotic polyamine alkaloids

2.3.3
Antiparasitic Alkaloid Polyamines

Besides their antibiotic properties, some polyamines display activity against parasites such as *Trypanosoma cruzi* and *Leishmania*. These parasites are transmitted to humans by blood-sucking triatomine bugs, by blood transfusion, and transplacentally and show diverse manifestations affecting the heart, intestines, and nervous systems. There are many metabolic pathways such as the phosphoinositol pathway and key enzymes such as the trypanosome-specific trypanothione reductase are involved in the survival of the parasites. Trypanothione (**45**) (Fig. 12) plays a key role in the antioxidant metabolism of the parasite and serves as an antioxidant in the defence of *Trypanosoma* and *Leishmania* against oxidative stress. The uniqueness of trypanothione makes the metabolism of this molecule an attractive target in antitrypanosomal and antileishmanial drug design. Trypanothione is a conjugate of two glutathiones with spermidine, which derives from L-ornithine. Its free thiol groups are oxidized by reactive oxygen species to reveal a cyclized disulfide-bridged compound **46**, which is eventually reduced by the trypanothione reductase to restart the cycle (Fig. 12).

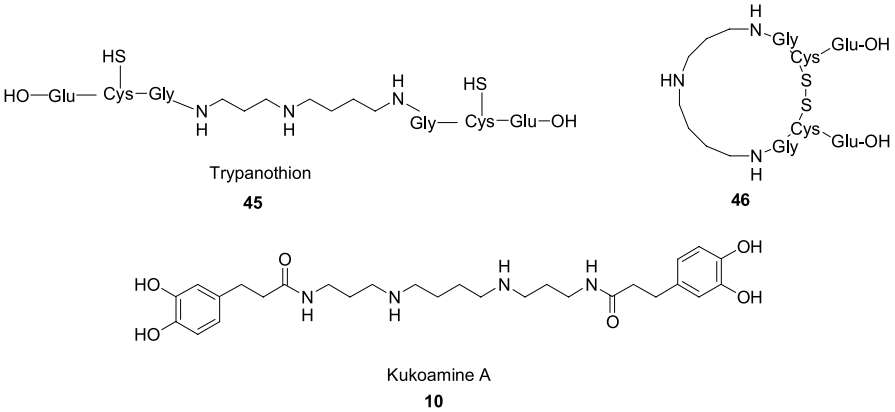


Fig. 12 Natural polyamine trypanthione (**45**) and its oxidized form (**46**), which are involved in the antioxidant metabolism of *T. cruzi* and the inhibiting polyamine analog kukoamine A (**10**)

A common inhibitor of the latter enzyme is the polyamine alkaloid kukoamine A (**10**). The synthesis of trypanothione and kukoamine A and their analogs was recently performed on solid support, as described in Sect. 3.2.

2.3.4 Cytotoxic Polyamines

Most of the polyamines and their conjugates exhibit several features. Besides their DNA or nucleic acid binding capacity, they often display anticancer activity, drug delivery properties, or cytotoxicity. It has long been known that cytotoxic properties of many polyamine analogs depend on the number of nitrogen atoms in the chain and therefore on the charge distribution of these molecules at physiological pH. They further rely on the distance between the nitrogen atoms and the nature of the terminal alkyl substituents. Examples for cytotoxic polyamines are anticancer derivatives of oxa-polyamines and bisoxyanaphtalimides **47**–**49** (Fig. 13), which reveal strong cytotoxic effects.

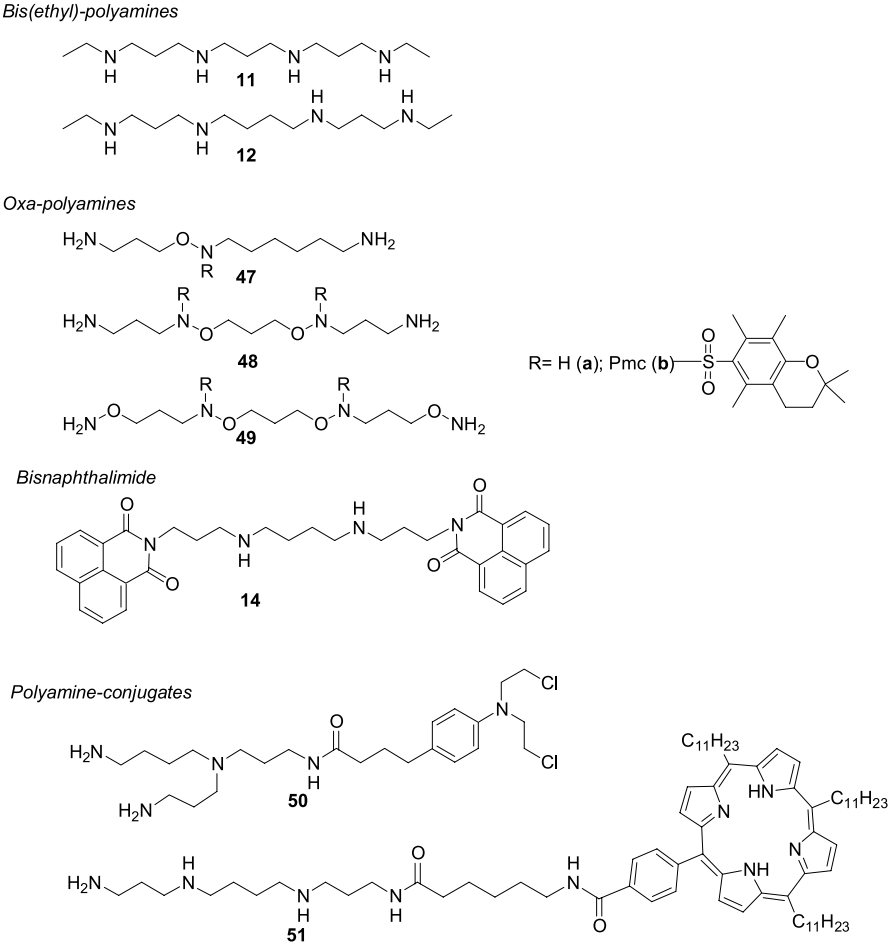


Fig. 13 Selection of polyamines displaying cytotoxic activities

While the parent oxa-polyamines **47a–49a** do not show biological activity, their sulfoamino derivatives **47b–49b** exhibit cytotoxicity against a broad panel of malignant and proliferating cells [92–96].

As described in Sect. 2.1, bisnaphthalimido compounds **14** and **15** (Fig. 4, Fig. 13) show cytotoxic effects but are usually insoluble in polar solvents. After improving the solubility by introduction of a polyamine chain of at least four nitrogen atoms, bisnaphthalimido derivatives **14** and **15** exert strong dose- and time-dependent cytotoxic effects by inducing apoptosis [92]. Other polyamines that cause cytotoxic effects are based on spermidine metabolites, isolated from a soft coral [97, 98].

In recent years many synthetic polyamine conjugates have been synthesized where the polyamine moiety is covalently coupled to cytotoxic compounds such as chlorambucil **50** [99, 100] or porphyrins **51** (Fig. 13) (Hahn et al. submitted for publication). The function of the polyamine is to enhance the solubility and the cellular uptake of the cytotoxic compounds. In high concentration the polyamine itself massively disturbs the membrane and increases the influx of the conjugated polyamines. Within the cell the porphyrin–polyamine conjugate **51** generates reactive oxygen species upon illumination with light of 650 nm, not depending on the polyamine moiety, while the chorambucil crosslinks the B-helix of the DNA after polyamine–DNA interaction.

Polyamine analogs for cancer therapy often induce apoptosis, but so far the mechanisms have not been satisfactorily defined. Elevated levels of H_2O_2 as a byproduct of the catabolism of polyamines by spermine/spermidine acetyltransferase (SSAT) are assumed to induce apoptosis by oxidative stress. In a recent study, Chen et al. demonstrated that a suppression of SSAT prevented the depletion of polyamine pools by polyamine analog-induced apoptosis in human melanoma cells [101]. The observation that this phenomenon is mainly related to the polyamine analogs opens a new route for the development of novel analogs inhibiting the SSAT induction that could lead to the disruption of the tumor, but not generally of normal tissues.

2.4
Polyamine Functions in Cancer and Cancer Treatment

A large data set indicates that the polyamine pathway can be a molecular target for therapeutic interference in several types of cancers [57]. There are numerous studies that have correlated elevated polyamine levels not only with diseases such as cystic fibrosis, muscular dystrophy, psoriasis, and diabetes [102–105] but also with abnormal or rapid cell growth as shown for malignant tumors (for review see [2, 22]). Over the last few years, research on the importance of polyamines in cancer progression has revealed many conflicting results. While increased polyamine levels are associated with increased cell proliferation, decreased apoptosis and increased expression of

genes affecting tumor progression and metastasis, suppression of polyamine levels has also been shown to be responsible for decreased cell growth, increased apoptosis, and decreased expression of genes affecting tumor progression [2, 106]. The cellular pools of polyamines from biosynthesis and influx from nutrition are strongly regulated by a number of processes, which are disturbed in several cancer types exhibiting an increase in biosynthesis, decreased catabolism, and elevated uptake of exogenous polyamines. Therefore, not only polyamine biosynthesis inhibitors but also polyamine analogs as well as polyamines covalently linked to chemotherapeutic drugs are promising drug candidates for the treatment of cancer [57]. Effective strategies, however, will certainly require a combination of agents acting at multiple sites of the polyamine biosynthesis and influx and efflux pathways [107].

To date, polyamine analogs are known to inhibit tumor progression at least partially by mimicking some of the regulatory functions of natural polyamines described above. To treat diseases with altered cell proliferation anticancer compounds have to be developed that are able to reduce the cellular polyamine levels. This can be reached by different mechanisms, which include protein–antizymes either for the downregulation of polyamine biosynthesis and import or for the upregulation of polyamine export. Further, polyamine mimics or analogs can be designed that directly diminish the natural polyamine synthesis [108].

Recently, a high throughput assay was established to allow the rapid screening of a 750-member polyamine analog library for compounds that induce antizyme frameshifting but fail to substitute for the natural polyamines in cell proliferation. From this library, some xylene (1,4-dimethyl benzene)-containing analogs were found to be equally or more active than spermidine at stimulating antizyme frameshifting. However, they turned out to be inefficient at rescuing cell proliferation after natural polyamine depletion [109]. Besides, polyamines are necessary for blood vessel development (angiogenesis) occurring in response to damage to normal tissues or in tumor growth. Inhibition of polyamine synthesis blocks angiogenesis in tumor models [110, 111].

Over the last two decades, interest has increased in compounds that target the regulation of polyamine metabolism rather than directly inhibiting the enzymes involved in polyamine biosynthesis or import and export [22]. The research on these compounds resulted in the generation of a variety of polyamine analogs **11**, **12**, **52–82** and conjugates that effectively display cytotoxicity (Figs. 14 and 15). Having similar structure to natural polyamines, these analogs efficiently enter the cells via the polyamine transport and uptake machinery. At the same time they have better specificity for cancer than normal cells exhibiting lower toxicity to the latter and are metabolically stable.

The cytotoxicity of these analogs for cancer cells has been mainly ascribed to the induction of the catabolic enzymes or to the reduction in activity of the

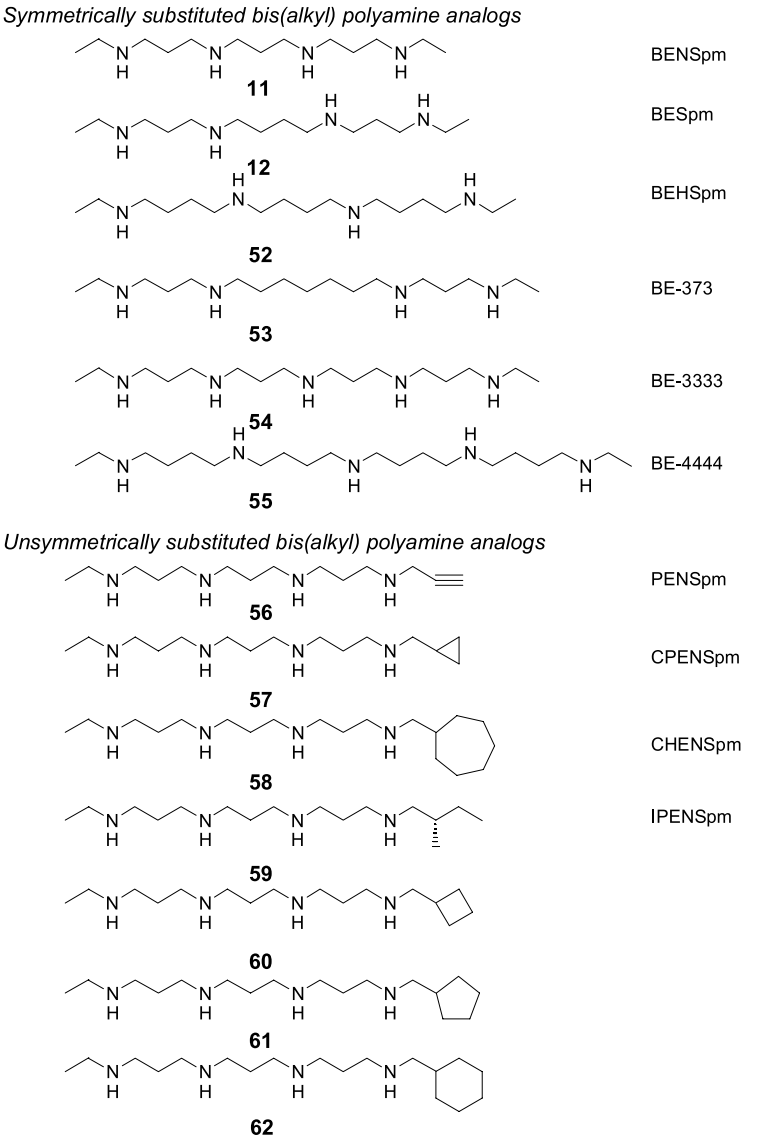
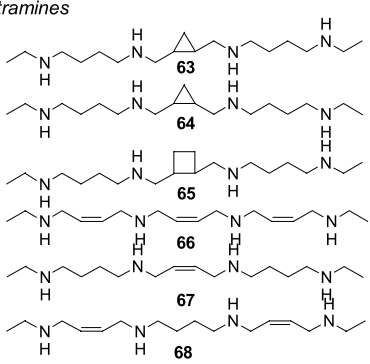


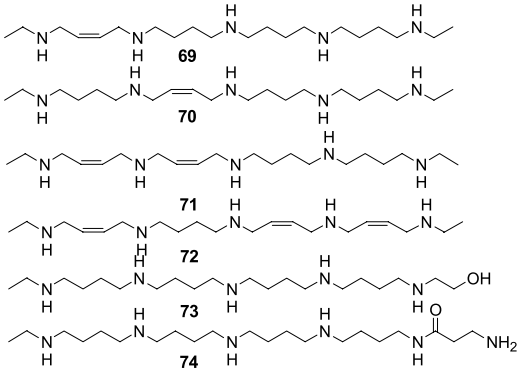
Fig. 14 Selection of symmetrically (**11**, **12**, **52–55**) and unsymmetrically substituted bis(alkyl)polyamine analogs (**56–62**)

Fig. 15 A selection of conformationally restricted bis(alkyl)polyamine analogs. These analogs were designed on the basis of the structure of natural polyamines and their interactions with DNA [1, 22] ►

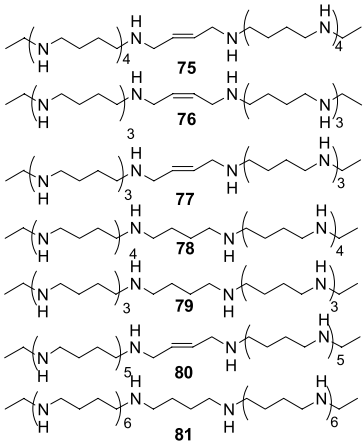
Tertramines



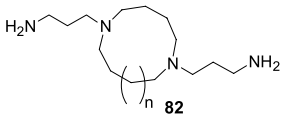
Pentamines



Oligoamines



Cyclopolyamines



anabolic enzymes. Both mechanisms serve to reduce the polyamine content of the cell.

Once in the cell, they can mimic intracellular polyamines, depleting them from their binding sites rather than substituting their growth-promoting properties [22]. They lower the polyamine levels below the concentration needed for cell survival. Some of these polyamine analogs are also able to interfere with the polyamine uptake from nutrition and to accelerate the degradation and the export of the natural polyamines [112–115]. The structure of many of the first generation analogs were based on symmetrically substituted bis(alkyl)polyamines **11**, **12**, **52–55** (Fig. 14, Sect. 2.4.1)

Later, Woster and colleagues generated unsymmetrically substituted bis(alkyl)polyamine analogs **56–62** (Fig. 14, Sect. 2.4.2) using solution phase chemistry. These show more defined functions in regulating polyamine metabolism and tissue specificity [116]. To date, conformationally restricted **63–74**, cyclic **82**, and long-chain oligoamine analogs **75–81** are gaining importance since they provide a tool for analyzing the mechanisms by which those analogs target polyamine metabolism and prevent tumors from growing (Fig. 15, Sect. 2.4.3) [22, 117–120].

2.4.1
Symmetrically Alkylated Polyamine Analogs

Many of the symmetrically alkylated polyamine analogs (Fig. 14) such as N^1,N^{11}-bis(ethyl)norspermine (**11**) (BENSpm or BE-333), N^1,N^{12}-bis(ethyl) spermine (**12**) (BESpm, or BE-343), N^1,N^{14}-bis(ethyl) homospermine (**52**) (BEHSpm or BE-444), N^1,N^{15}-bis-[3-(ethylamino)-propyl]-1-17-heptanediamine (**53**) (BE-373), N^1,N^{15}-bis(ethylamino)-4,8,12-tria-zapentadecane (**54**) (BE-3333), and N^1,N^{19}-bis(ethylamino)-5,10,15-triazanonadecane (**55**) (BE-4444) [121] utilize the polyamine transport system to get into tumor cells, where they lead to a depletion of the naturally occurring polyamines and exert a cytotoxic response [22, 115, 122]. One of the most successful symmetrically alkylated polyamines is N^1,N^{11}-bis(ethyl)norspermine (**11**) (BENSpm, BE-3333) in in vitro and in vivo applications, although a recent clinical phase II study on breast cancer patients revealed that it cannot be administered as a single drug against cancer [123, 124].

2.4.2
Unsymmetrically Substituted Polyamine Analogs

Besides the symmetrically alkylated polyamines, unsymmetrically substituted bis(alkyl)polyamines such as N^1-propargyl-N^{11}-ethylnorspermine (**56**) (PENSpm), N^1-(cyclopropylmethyl)-N^{11}-ethyl-4,8-diazaundecane (**57**) (CPENSpm) [116], N^1-(cycloheptylmethyl)-N^{11}-ethyl-4,8-diazaundecane (**58**) (CHENSpm), and (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8-diazaundecane

(**59**) (IPENSpm) [125, 126] were developed (Fig. 14). These compounds show an increase in cytotoxicity compared to their symmetrical analogs. The cytotoxicity of N^1-propargyl-N^{11}-ethylnorspermine (**56**) (PENSpm) and N^1-(cyclopropylmethyl)-N^{11}-ethyl-4,8-diazaundecane (**57**) (CPENSpm) is based on the upregulation of the spermine/spermidine N^1-acetyltransferase, which converts natural spermine and spermidine to their N^1-acetylspermine and N^1-acetylspermidine derivatives. The following cleavage into 3-acetamidopropanal, spermidine, and putrescine through the action of FAD-dependent polyamine oxidase (PAO) generates H_2O_2, which is accompanied by oxidative stress induced cell death [57]. This function differentiates the bis(alkyl)polyamines **56** and **57** from **58** and **59**, which solely suppress tumor growth without affecting SSAT activity. These analogs, even though structurally similar and related, behave very differently from spermine and produce considerably different cellular effects. Some of the analogs, such as **58**, initiate cell death by a G2/M cell cycle arrest, whereas others interfere with normal tubulin polymerization [126]. Thus, decreasing levels of cellular polyamines appears not to be the only essential step for the initiation of apoptotis [127]. Nevertheless, polyamine analogs that can alter tubulin polymerization provide lead structures that display the same function as paclitaxel-conjugates but are much less elaborate to synthesize [126]. Comparison with known cytotoxic drugs, such as etoposide, revealed significant similarity in terms of apoptotic phenotype, altered cell morphology, DNA fragmentation, and IC_{50} value [127].

2.4.3
Conformationally Restricted Polyamine Analogs

It has been shown that conformationally restricted polyamines **63–74**, **82** (Fig. 15) can introduce a bending in the structure of polyamine-binding partners that differs from natural polyamine binding [117–120].

Free rotation of the single bonds is inhibited by introduction of one or two *cis* double bonds or *trans* double bonds into the hydrocarbon backbone [108, 119].

Most of the compounds that show markedly increased cytotoxicity against tumor cells are synthesized as oligoamines **75–81** (Fig. 15), since they induce DNA aggregation and collapse at much lower concentrations (2–4 μM) than spermine (50–100 μM) [22, 118, 120, 128]. Although, the exact mechanisms implicated in this cytotoxicity have not yet been fully elucidated. There are some studies proposing that the cytotoxicity of the polyamines derives from their ability to change the DNA conformation, since the tumor cells treated with cytotoxic polyamines are more sensitive to nuclease digestion than those treated with the non-toxic analogs [22, 129]. Cytotoxicity can even be enhanced by modification of the charge distribution along the surface of the aliphatic polyamine backbone [129].

Comparing the concentrations of oligoamines required to induce DNA aggregation with those necessary to kill tumor cells, it is obvious that there is a correlation between the aggregation ability of the oligoamines and their cytotoxicity [115, 130–133]. These studies provide evidence to suggest that modification of polyamine analog structures may affect their DNA binding abilities and make them cytotoxic by inducing DNA breakdown and apoptosis.

However, the mechanism by which apoptosis is induced has not been defined. As described in Sect. 2.3.4, the catalysis of polyamines produces H_2O_2 as a byproduct, suggesting that apoptosis may be, in part, due to oxidative stress [134]. Treatment with other polyamine analogs revealed that multiple apoptotic mechanisms were involved. Typical features of apoptosis including cytochrome c release, activation of caspase-3, and cleavage of poly(ADP-ribose) polymerase (PARP) occur after treatment of tumor cells.

2.5 Polyamines in Drug Delivery

Since polyamines are highly positively charged at physiological pH, they can interact with biological membranes that mainly contain zwitterionically and anionically charged components. These components are phospholipids and negatively charged proteins. Electrostatic interaction with membranes often leads to internalization of the bound molecules via endocytotic mechanisms. Additionally, many organisms have an active polyamine uptake system, although not much is known about the uptake mechanism. Polyamines and polyamine conjugates with lipophilic anchors can be therefore exploited as drug delivery moieties when covalently bound to a therapeutically active drug. The polyamine moiety thereby often serves as solubilizing agent for very hydrophobic drugs in the aqueous environment, additionally increasing the electrostatic interaction with the biological membrane. Further, the polyamine moiety can serve as a complexing agent to neutralize negatively charged drugs that never would interact with a mainly negatively charged membrane and would therefore be excluded from endocytotic uptake.

To date, polycationic moieties have been used for a variety of drugs to enhance their cellular uptake. Most of them are either based on naturally occurring polycationic peptides **83a–c**, polycationic peptides such as poly- or oligo- arginines or lysines **84**, **85** or their analogous β-peptides **86**, on oligocarbamates **88**, on other polycationic peptide scaffolds **87** (Fig. 16) or on polyamine-modified liposomes comprising the polyamine moiety covalently bound to lipids or steroids **41**, **88**, **90** as well as on polyamine-modified dendrimers (Fig. 17), as discussed in the next section.

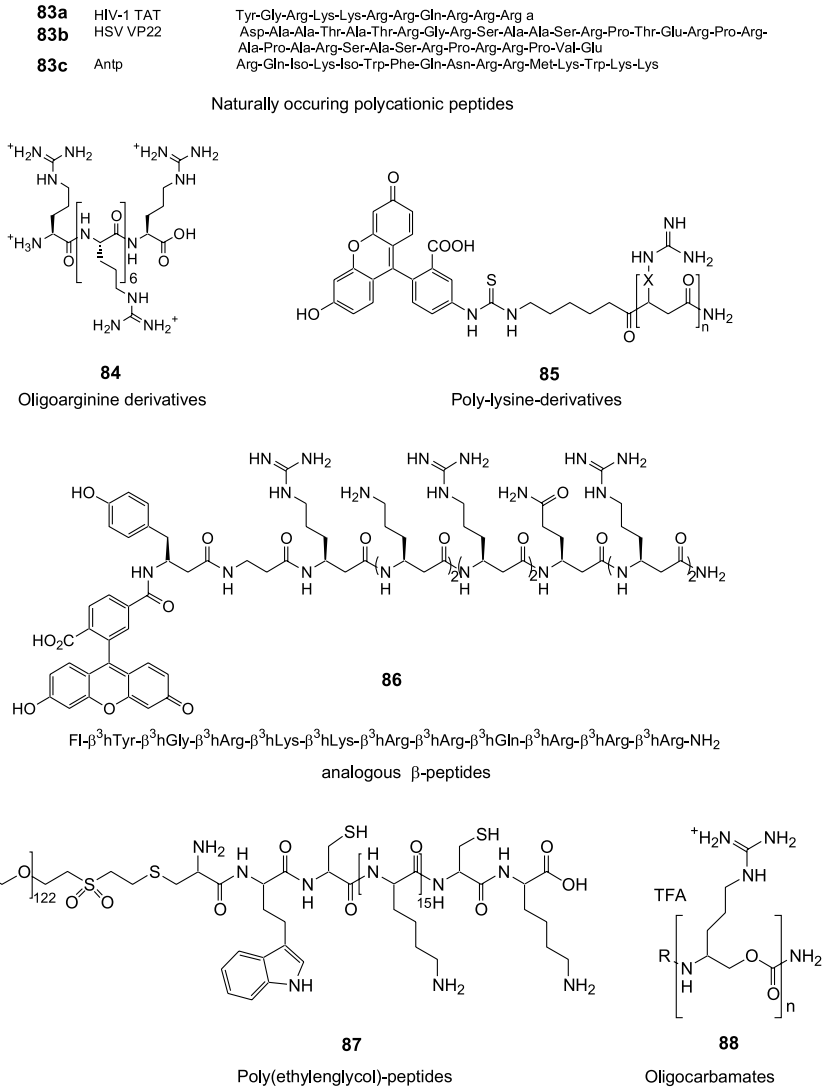
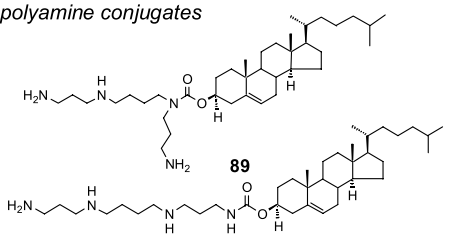


Fig. 16 Known naturally and synthetic polycationic molecular transporters

2.5.1 Synthetic Polyamines as Non-viral Vectors for Gene Delivery

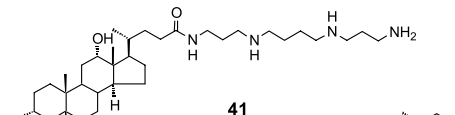
Increasing attention has been paid to technologies used for the delivery of genetic materials into cells for gene therapy and the generation of genetically engineered cells. To date, viral vectors have often been used since they display an overall high transfection efficiency of genes. However, there are of-

Steroid-polyamine conjugates

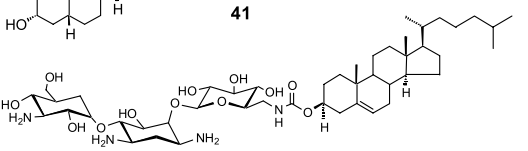


89

90



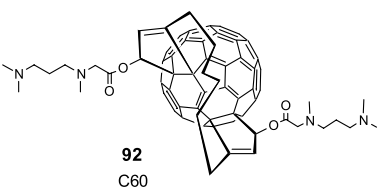
41



91

KanaChol

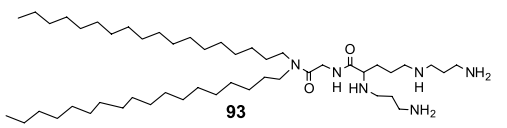
Fullerene-polyamine conjugates



92

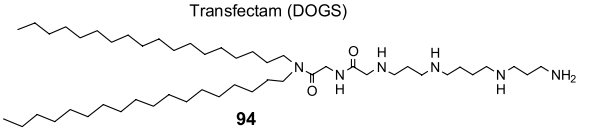
C60

Polyamine conjugates with long aliphatic chains



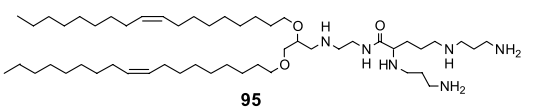
93

Transfectam (DOGS)



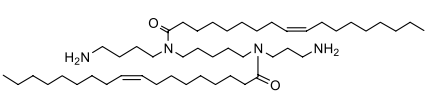
94

RPR 120535



95

DOSPA



96

LipoGen (N^2,N^3 dioleoylspermine)

◀ **Fig. 17** Selection of polyamines for transfection of DNA and nucleotides

ten problems due to the pathogenicity or immunogenicity of the viral vector. These problems have been resolved for clinical applications by using so-called non-viral gene delivery vectors [135]. So far, many groups have focused on the development of non-viral systems, which allow the introduction of nucleic acids as efficiently. Polyamine scaffolds have been chosen as precursors for DNA delivering agents since they display some important features.

Prerequisites for delivery of DNA across the cellular membrane are certainly the condensation of the DNA and the masking of the negative charges of the nucleotide backbone [136]. Due to their positive charges polyamines have the ability to condensate the DNA and to neutralize the charges [137, 138]. Spermine and spermidine were first discovered in the nuclei of sperm where they help the histones in the packaging and condensation of the DNA. Thus, polyamines have the ability to reduce the volume of naked DNA by non-covalent electrostatic interaction to particles of 50–150 nm, while neutralizing its negative charge. Conjugates of polyamines with long aliphatic chains **93–96** [139], bile acids **41** [140] cholesterol and steroids **41**, **89**, **90** [141–144] as well as other lipid moieties [72, 145] and hydrophobic compounds such as Buckminster fullerenes **92** and dendrimers have been recently developed as gene delivery agents (Fig. 17).

The attachment of hydrophobic residues such as a lipid moiety further enhances the condensation and the permeability of the hydrophobic membrane part [146, 147]. The mechanism by which these compounds causes introduction into cells is still poorly understood. It was proposed that the polyamines still interact with the phospholipid headgroups of the membrane lipids leading to a close neighborhood of the DNA and the membrane, which eventually become endocytosed, delivering the bound DNA into the endosomes. Finally, the DNA is proposed to leave the endosomes by the so-called endosomal escape [148]. The mechanism, by which the endosomal escape functions, is so far not fully understood. There are many proposed mechanisms, such as the proton sponge theory or the retrograde transport of DNA via the Golgi apparatus. The first hypothesis is based on the buffering capability of polyamines. Since primary amines contain a pKa of around 10.5 [34], polyamines can buffer the endosome while the V-ATPase proton pump is constantly pumping protons into its lumen. This increases the proton and water flux, finally leading to a decrease of the pH from 7.4 to 5.5. Swelling and osmotic disruption of the endosome leads to the escape of the DNA into the cytosol from where it will be translocated into the nucleus [148, 149].

First combinatorial approaches on solid phase have been made to synthesize amphiphilic polyamine conjugates as non-viral gene delivery agents. Bradley and coworkers reported the synthesis of three different libraries **x.1–x.31** (**x=97–99**) based on the polyamine moieties **97–99** as depicted in

Fig. 18 [150, 151]. The compounds were tested in DNA transfection assays in comparison to the commercially available DNA transfection agent Effectene™ (Qiagen). The highest activity was detected with library 2 compounds, which contain two guanidinium headgroups and, in comparison to

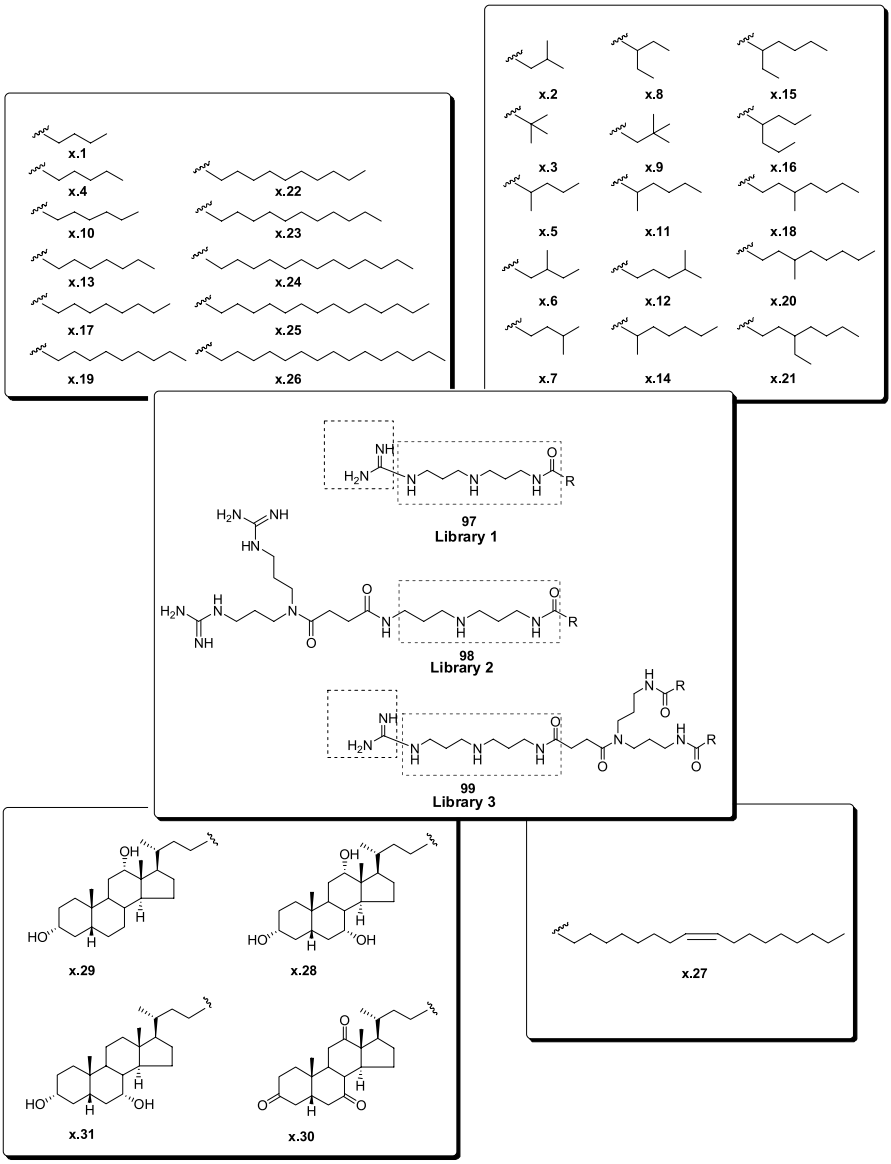


Fig. 18 Combinatorial approach for non-viral gene delivery vectors by Bradley and coworkers [150, 151]

the library 3 compounds, only one aliphatic or hydrophobic chain. While in this assay nearly all steroid conjugates showed poor DNA binding and transfection efficiency, some of the straight aliphatic chain conjugates such as those containing **x.23**–**x.27** display good transfection activities.

3
Polyamine Synthesis on Solid Support

Polyamine chemistry often encounters problems on the selective protection or the directed reaction of the various secondary and primary amino functionalities [152]. In particular, the asymmetric derivatization of the symmetric spermine usually turns out to be difficult. Moreover, the coupling of polyamines to many hydrophilic or hydrophobic moieties often requires the use of polar solvents and odd purification protocols. To overcome these problems solid phase chemistry has recently been established for the polyamine synthesis.

There are basically two strategies for this. The first one is the attachment of whole and commercially available polyamines like spermine or their appropriate derivatives to solid phase. This mostly requires protection of the polyamine and at least one tedious chromatographic step before linking it to the solid support. All earlier examples in the literature go back on this approach and they mostly adopt protection group strategies for tri- and tetraaza-polyamines, which have already been established in solution phase. The differentiation between primary and secondary amines is a crucial factor. Depending on the type of resin (see Table 1) and the chosen substrate, the crosslinking reactions of symmetrical molecules must also be taken into account as these can decrease the overall yield and purity of the final products.

Anyway, it is possible to generate large amounts of useful starting blocks, while excess can be recovered after the attachment step. If synthesis is focused on a particular polyamine this is probably the best solution.

In cases where either the polyamine moiety differs distinctly from the commercially available building blocks or polyamines with more diversity are required, the backbone is preferentially generated by stepwise and modular elongation. Diversity can be achieved by the introduction of different aliphatic chains between the nitrogens, branching of the skeleton in alkyl- or amino position, and the introduction of other functional groups into the backbone. This finally opens access to numerous compounds that are different in detail but structurally closely related.

Methods for the modular generation of polyamine backbones are alkylation by S_N2-displacement, Fukuyama alkylation, reduction of amides, and reductive amination. As for the non-modular approach mentioned above the methods are all adopted from solution phase chemistry, but some effort has been made in optimizing them for solid phase chemistry. Although they all

Table 1 Common resins currently in use for solid phase synthesis of polyamines

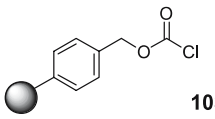
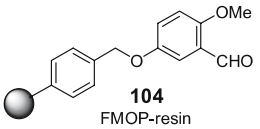
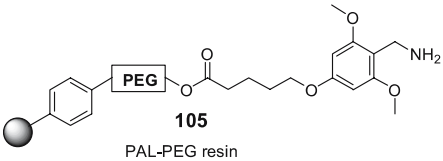
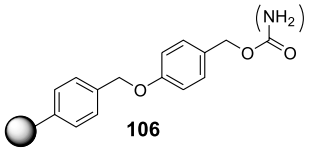
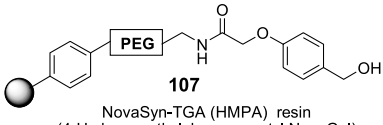
Resins	Remarks	Refs.
 **103** Methylchloroformylated Merrifield resin		[156] [158] [159]
 **104** FMOP-resin (4-Formyl-3-methoxy-phenoxymethyl-)	This resin is cleaved with high concentrations of TFA 50–95% and is acid-stable	[166]
 **105** PAL-PEG resin		[19]
 **106** Wang carbamate resin		[167]
 **107** NovaSyn-TGA (HMPA) resin (4-Hydroxymethylphenoxyacetyl NovaGel) HMPA linker	The resin was prepared by derivatization of aminomethyl Novagel resin with the TFA-labile 4-hydroxymethyl-phenoxyacetic acid linker and combines excellent swelling properties in polar solvents such as PEG-resins. More robust than Wang resin **101** under acidic condition due to the lack of benzylic ether linkage	[168]

Table 1 (continued)

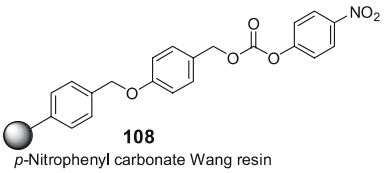
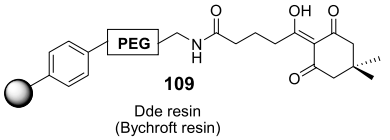
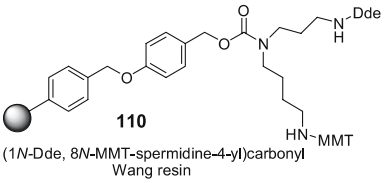
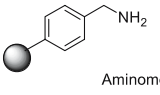
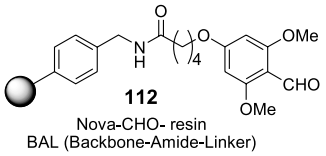
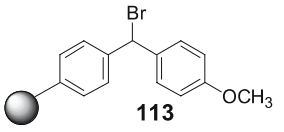
Resins	Remarks	Refs.
	p-Nitrophenyl carbonate esters can react with amines to provide resin-bound carbamates. The resin can be cleaved with TFA to liberate the amine, or with lithium aluminium hydride to produce the N-methylated amines	[167] [169–173]
		[174] [175]
		[176]
	Urethane linkages are cleavable under strong acidic conditions with 10 equiv TMSA/TFA	[150–152] [162] [163]
		[177]
	For secondary amines, which do not couple well to trityl-type resins. Derivatization of the resin is achieved by treating the resin with the nucleophile in the presence of a base. Amines produced in this manner can be readily converted to acid-sensitive sulfonamides and amides that can be cleaved with TFA/DCM/water 23:75:2	

Table 1 (continued)

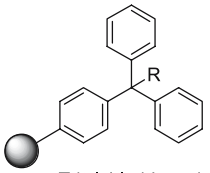
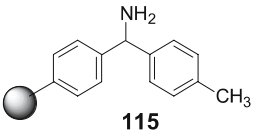
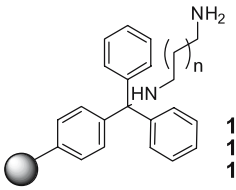
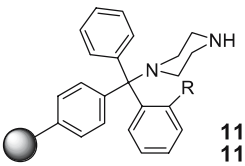
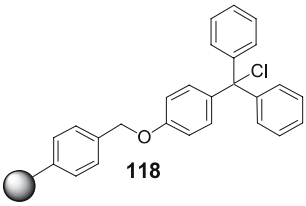
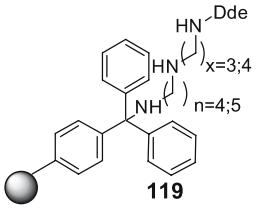
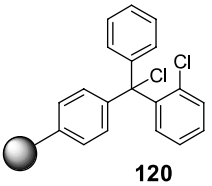
Resins	Remarks	Refs.
 **114a** R=Cl **114b** R=Br Tritylchloride resin	An acid-labile resin for the immobilization of amines. Cleavage of the product is achieved using 1–5% TFA containing 1% TIS. Amines can also be cleaved with 30% HFIP in dichloromethane. The recycling is performed under mild reaction conditions utilizing thionyl chloride as chlorinating agent	[30] [178–185]
 **115** 4-Methylbenzylhydrylamine resin (MBHA)	Carboxamides can be cleaved from the MBHA resin by strong acids such as HF or TFMSA	[23] [24] [186–191]
 **116a** n=0 **116b** n=1 **116c-f** n=2-5 1,3-Diaminopropane trityl resin (Manku resin)	1,5-diaminopentane resin is also available that can be used in the preparation of nephilatoxin analogs	[30] [192]
 **117a** R=H **117b** R=Cl Piperazine trityl resin	This preloaded resin has been employed for the synthesis of a polyamine toxin library	[179] [193]
 **118** *p*-Alkoxytrityl chloride Merrifield resin	One of the most acid-labile resins. Release of amines can be achieved using 0.1–0.5% TFA in dichloromethane containing 5% TIS. Careful handling is necessary	[23] [24]

Table 1 (continued)

Resins	Remarks	Refs.
 **119** Dde-triamine trityl resins (Merck)	Commercially available at Merck	
 **120** 2-Chlorotrityl chloride resin (Barlos resin)	Extremely versatile acid-labile resin for the attachment of amines, especially where racemization is a concern. Cleavage can be achieved with AcOH/TFE/dichloromethane, 0.5% TFA, or HFIP Acid-free cleavage: trifluoroethanol/dichloromethane	[194–201]

display advantages and restrictions, finally they are helpful tools and, depending on the target molecule, one or the other will be favorable. They both require a well-planned protection group strategy as there are several amino groups present in the molecules, which should be distinguishable.

3.1 Resins and Linkers

Most of the polyamine syntheses on solid support have been carried out on resins that are modified from Merrifield (**100**), Wang (**101**), and Rink-Amide resins (**102**) (Fig. 19) depending on the synthesis strategies, the coupling procedures, and the branching. In this review, we illustrate the polystyrene (PS) scaffold of the resins as depicted in Fig. 19. Table 1 presents an overview of the most common resins used in polyamine synthesis (**103–120**), which will be described in the remaining sections.

During the past decade the development of novel protection groups for the directed protection of primary and secondary amines led to a large improvement of the linkers and resins to serve the different needs. The first solid phase syntheses of polyamines were reported by Sergheraert and coworkers [156]. The synthesis of reduced trypanothione disulfide (**45**) and its oxidized form (**46**) involved the selective protection of the primary amines of spermidine with a *tert*-butyldiphenylsilyl (TBDPS)

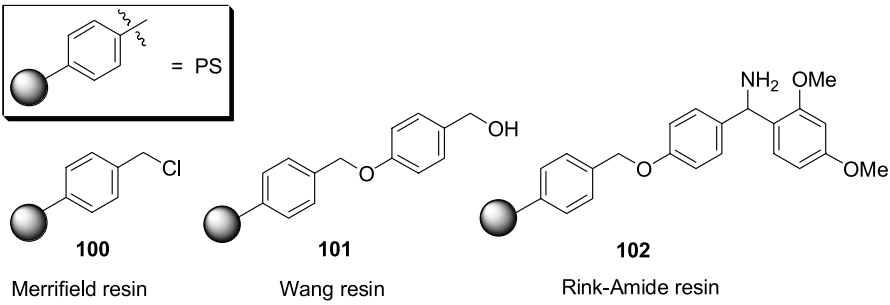


Fig. 19 Standard resins for solid phase chemistry. After attachment, amines can be cleaved from the resin with strong acids such as HF or TFMSA from **100** [153], with 95% TFA in CH_2Cl_2 from **101**, or with TFA/H_2O, CH_2CL_2 (5:5:90) from **102** [154, 155]

group (**149**) [157] (Table 2) followed by coupling the disilyl derivative on a methylchloroformylated Merrifield polystyrene resin **103** (Table 1) [158, 159]. After deprotection and assembly of the peptidic chain the resin was treated with HF, followed by oxidation and purification. To avoid these harsh cleaving conditions using HF, Bychroft and colleagues performed the first synthesis on an Fmoc (**131**)-protected PAL-PEG-polystyrene resin (**105**) (peptide amide linker [5-((4-Fmoc-aminomethyl)-3,5- dimethoxyphenoxy)valeric acid] poly(ethyleneglycol)-polystyrene resin) (Table 1). Since then, a variety of different resins and linkers have been developed to allow the synthesis of diverse polyamine analogs and coupling products. In particular, the improvement of orthogonal protection strategies for the different amino groups within a polyamine chain, allowing mild deprotection reactions, led to the development of novel linkers that also can be cleaved under mild conditions. As described in more detail in Sect. 3.2, Bycroft et al. developed Dde (**141**) as a protecting group for peptides, which is fully orthogonal to the deprotection conditions of Boc (**128**) and Fmoc (**131**) (Table 2) [19]. Since then it has frequently been used to discriminate between primary and secondary amines [19, 160, 161]. It was used to protect a NovaSynTGA resin with an HMPA linker (**107**) (Table 1), which permits the coupling under more hydrophilic conditions due to the PEGylation.

Cleavage of the final polyamine conjugates was achieved with TFA at higher concentrations [19].

A variety of polyamine linkers were developed by Bradley and colleagues [152, 162, 163]. They mainly use an aminomethylated polystyrene resin **111** (Table 1). An urethane linkage was found to be cleavable using strong acidic conditions (10 equiv trifluoromethanesulfonic acid–trifluoroacetic acid, TFMSA/TFA) and hence is potentially useful for resin screening purposes. Other linkers such as the trityl linkers (**114**, **116**–**120**) (Table 1) are readily cleavable with TFA and are ideal for solution screening applications.

Furthermore, modified Wang resins (**101**, **106**, **108**, **110**) have been used to synthesize libraries of polyamines [164] (Table 1). After reaction with phosgene or activated carbonates such as bis(*p*-nitrophenyl)carbonate (to yield **108**) Wang resins can serve as urethane-based protecting groups during immobilization of polyamines or amines.

However, the cleavage of many polyamine-coupled biomolecules require even milder cleavage conditions from the resin, such as DNA conjugates that are hydrolyzed when treated with high concentrations of TFA.

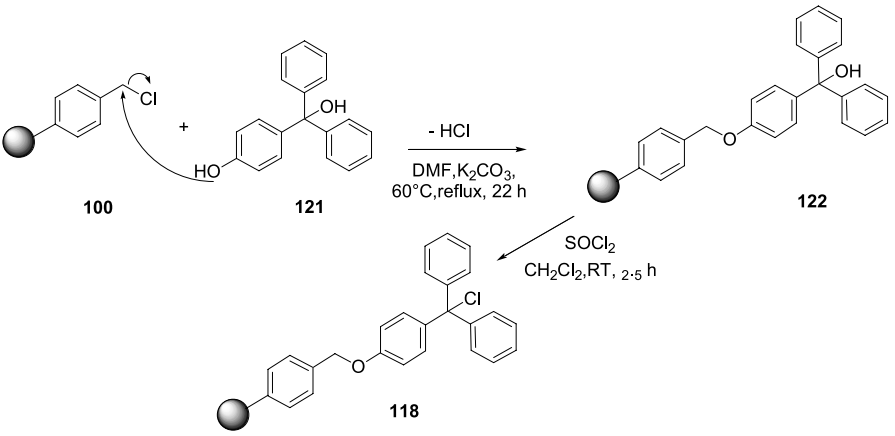
Firstly, Byk et al. developed a solid phase methodology that allows quick and easy access to a high number of monofunctionalized geometrically varied polyamines using mild cleavage methods [165]. They used a trityl-tethered resin **120**, which can be cleaved using low concentrations of TFA (Table 1).

The trityl resins **114** and **116–120** (Table 1) are extremely powerful as they are gradually sensitive to acids such as TFA in CH_2Cl_2 (for amines), AcOH in CH_2Cl_2, or HFIP in CH_2Cl_2 (for carboxylic acids) at various concentrations. They are already commercially available with a hydrophobic PS scaffold or with a more hydrophilic PS-PEG version. Recently, Fukuyama et al. developed a novel alkoxytrityl chloride linker tethered Merrifield resin **118** [24] for extremely mild cleavage of polyamine conjugates with 0.1–0.5% TFA [23, 24] (Scheme 1), which permits the coupling of highly acid-labile biomolecules such as DNA and RNA to the polyamine moiety.

Additionally, Fukuyama and coworkers also created a highly efficient and versatile synthetic route to polyamines using nitrobenzenesulfonamides (Ns-amides) **144a–c** (Table 2) both as a protecting and activating group [23]. The alkylation of N-monosubstituted Ns-amides either proceeded conventionally or under Mitsunobu conditions to provide the N,N-disubstituted sulfonamides. With this strategy the Ns group (**144a–c**) can be removed easily with soft nucleophiles to yield the corresponding secondary amines. The major advantage of this protocol is that both alkylation and deprotection proceed under mild conditions. Thus, with this methodology, the total synthesis of linear and/or macrocyclic natural polyamines can be accomplished more efficiently.

To date, most of the solid phase syntheses for polyamines take advantage of resins with derivatized trityl linkers such as the Barlos linker **120**, the Fukuyama linker (**118**), or the 1,3-diaminopropane-modified trityl linker (**116b**) reported by Manku and Hall [30].

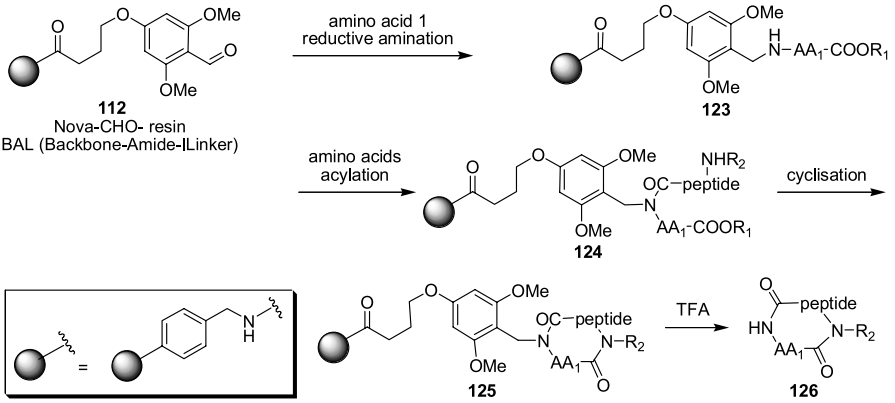
Besides the alkylation reactions in polyamine synthesis, the reductive amination approach found broad entry in solid phase chemistry in the backbone amide linker (BAL) strategy [202] (Scheme 2). Thereby, amino acids are bound to a linker via imine reduction bearing a secondary amine, which can be further acylated to obtain peptides with a variety of C-terminal functionalities. Besides the effective synthesis of C-modified peptides, this also opens access to amide formation in peptides if C- and N-terminal residues are deprotected, commonly leading to cyclic peptides [177]. Cleavage from the resin



Scheme 1 Synthesis of a *p*-alkoxytrityl chloride Merrifield resin (**118**) as described by Fukuyama et. al [23, 24]

112 can be performed by treatment with diluted TFA, somewhat milder conditions than those for the methylchloroformylated Merrifield resin **103.**

Various other linker systems such as FMOP (4-formyl-3-methoxy-phenoxymethyl-polystyrol) linker (**104**) are based on reductive amination [166, 203–206].



Scheme 2 Schematic synthesis of a cyclic peptide using the BAL strategy

The externally controlled cleavage of covalently linked prodrugs, proteins, or solid-phase formulation vehicles offers potential advantages for controlled drug or gene delivery. Diamond and coworkers tested a series of *o*-nitrobenzyl ester compounds that allowed a systematic study of resins with linkers for polyamine synthesis expressing a photolability for mild cleavage of the polyamine conjugate. They observed that an *o*-nitrobenzyl ester was

Table 2 Amine protection groups used in solid phase synthesis of polyamines and polyamine conjugates

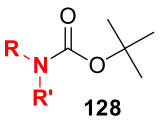
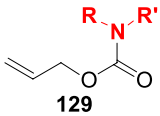
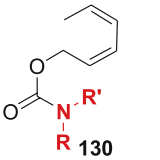
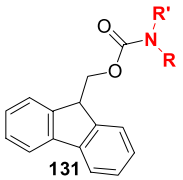
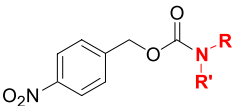
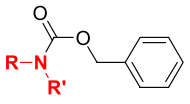
Protecting group	Deprotection	Refs.
 **128** t-Butyl carbamate (Boc-NRR')	50–80% TFA, H_2O TFA, PhSH AcCl, MeOH 3 M HCl, EtOAc, 25 °C	[157, 162] [174, 192] [197] [209–219]
 **129** N -Allyl carbamate (Aloc-NRR')	$Pd(Ph_3P)_4$, Bu_3SnH, AcOH $Pd(Ph_3P)_4$, Me_2NTMS	[24, 220]
 **130** Hexadienyloxycarbamate (Hdoc-NRR')	Mild cleavage 1% TFA, CH_2Cl_2 stable to base and Pd(0) (alternative to trityl groups)	[221]
 **131** 9-Fluorenylmethyl carbama (Fmoc-NRR')	5–20% piperidine or 50% morpholine or amine base	[162] [174, 175] [213–215] [222]
 **132** p-Nitrobenzyloxycarbamate (PNZ-NRR')	Deprotection by quantitative Kaiser test conditions	
 **133** Benzyl carbamate (Z-NRR', Cbz-NRR')	Hydrogenation, catechol borane or H_2, Pd/C, MeOH, NH_3 or NH_4Ac	[223–228]

Table 2 (continued)

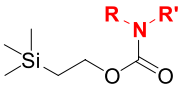
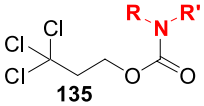
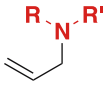
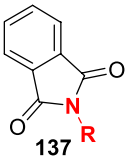
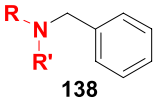
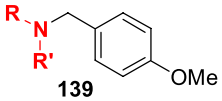
Protecting group	Deprotection	Refs.
 **134** 2-(Trimethyl-silyl)ethyl carbamate (Teoc-NRR')	TFA, TBAF, KF-H_2O, CH_3CN TASF	[179, 213]
 **135** 2,2,2-Trichloroethyl carbamate (Troc-NRR')	Zn, AcOH, THF Cd, AcOH	
 **136** Allyl group (Allyl-NRR')	Pd(Ph_3P), RSO_2Na, CH_2Cl_2	[209–211]
 **137** Phthalimide (Pht-NRR')	Hydrazine, MeOH	[28, 152] [209–211] [229, 230]
 **138** Benzyl group (Bn-NRR')	Hydrogenetion or dissolved metal reduction	[229–231]
 **139** *p*-Methoxybenzyl group (PMB-NRR')	DDQ, CH_2Cl_2	[226]

Table 2 (continued)

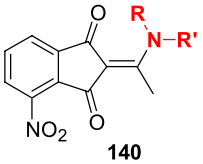
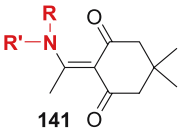
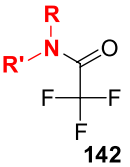
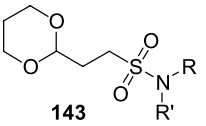
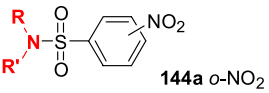
Protecting group	Deprotection	Refs.
 **140** N-1-(4-nitro-1,3-dioxoinda 2-ylidene)ethyl group (Nde-NRR')	Mild conditions 2% hydrazine, DMF	[233, 234]
 **141** 1-(4,4-dimethyl-2,6-dioxocyc-lohexylidene)ethyl group (Dde-NRR')	Mild conditions 2–10% hydrazine DMF. Exclusively reacts with primary amines. Disadvantage: transamination onto other primary amines present in the molecule	[160, 167] [174, 175] [197, 220] [234–236]
 **142** Trifluoroacetamide (Tfa-NRR')	Mild hydrolysis K_2CO_3, MeOH H_2O	[216, 218] [219, 237] [238]
 **143** 2-(1,3-Dioxan-2yl)ethylsul nyl group (Dios-NRR')	TFA/H_2O 4:1 65 °C TFA/H_2O, 0 °C allows selective deprotection of Boc groups	[239]
 **144a** *o*-NO_2 **144b** *p*-NO_2 **144c** *d*-NO_2 p -Nitrobenzenesulfonyl group (Nosyl; Ns-NRR')	PhSH, Hünig's base, DMF	[23, 24] [167, 175] [179] [240–243]

Table 2 (continued)

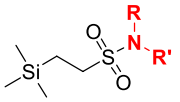
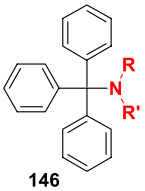
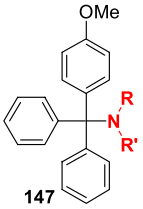
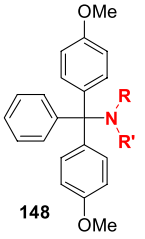
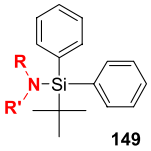
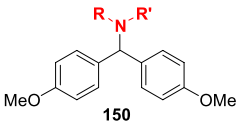
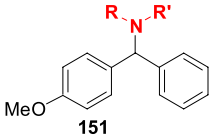
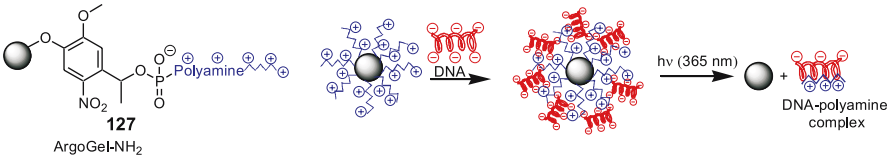
Protecting group	Deprotection	Refs.
 **145** 2-Trimethylsilylethane sulfonyl group (SES-NRR')	CsF, DMF, 90 °C, 37 h Bu_4NF, THF Can be used together with Boc, Pht, allyl, pyridine-2-sulfonyl groups	[209–211] [224, 244]
 **146** Triphenylmethylamine (Tr-NRR')	Mild cleavage 0.2%TFA, 1% H_2O CH_2Cl_2	[231, 245] [246]
 **147** 4-Monomethoxytrityl group (MMT-NRR')		[247]
 **148** 4,4'-Dimethoxytrityl group (DMT-NRR')	Deprotection with 0.1 M chloroacetic acid is faster than cleavage of trityl linkers	[193]
 **149** t-Butyldiphenylsilyl group (TBDPS-NRR')		[156, 157]

Table 2 (continued)

Protecting group	Deprotection	Refs.
 150 4,4'-Dimethoxydityl group (Dod-NRR')		[222, 248]
 151 4-Monomethoxydityl group (Mmd-NRR')	TFA/CH_2Cl_2	[222, 248]

strictly required for photolability, while imido esters were not photolabile. The degradation kinetics of 1-*o*-phenylethyl ester was an order of magnitude faster than that of *o*-nitrobenzyl ester. Tosylate, phosphate, and benzoate derivatives of 1-*o*-nitrophenylethyl displayed > 80% decomposition within 10 min at 3.5 mW cm^{-2} at 365 nm. However, *O*-2-nitrobenzyl *O*′,*O*″-diethyl phosphate displayed the fastest decomposition at photoirradiation conditions suitable for most of the biological systems [207, 208].

They reported a solid phase synthesis with spermine or polyethyleneimines (PEI, 600 MW) tethered via *o*-nitrobenzyl linkages to polyethylene oxide beads (Argo*Gel*-NH_2; Scheme 3). The photolysis of *O*-2-nitrophenylethyl *O*′,*O*″-diethyl phosphate or solid phase with *o*-nitrobenzyl group as synthetic linker was completely degradable after photoirradiation at 365 nm for 10–18 min at 3.5 mW cm^{-2}. With this linker system DNA-loaded solid phase is assumed to be exploited for spatially, temporally, or dose-controlled release of DNA–polyamine complexes, at extracellular or intracellular sites [207].



Scheme 3 DNA conjugation to polyamines on Argo*Gel*-NH_2, as reported by Kim and Diamond [207]

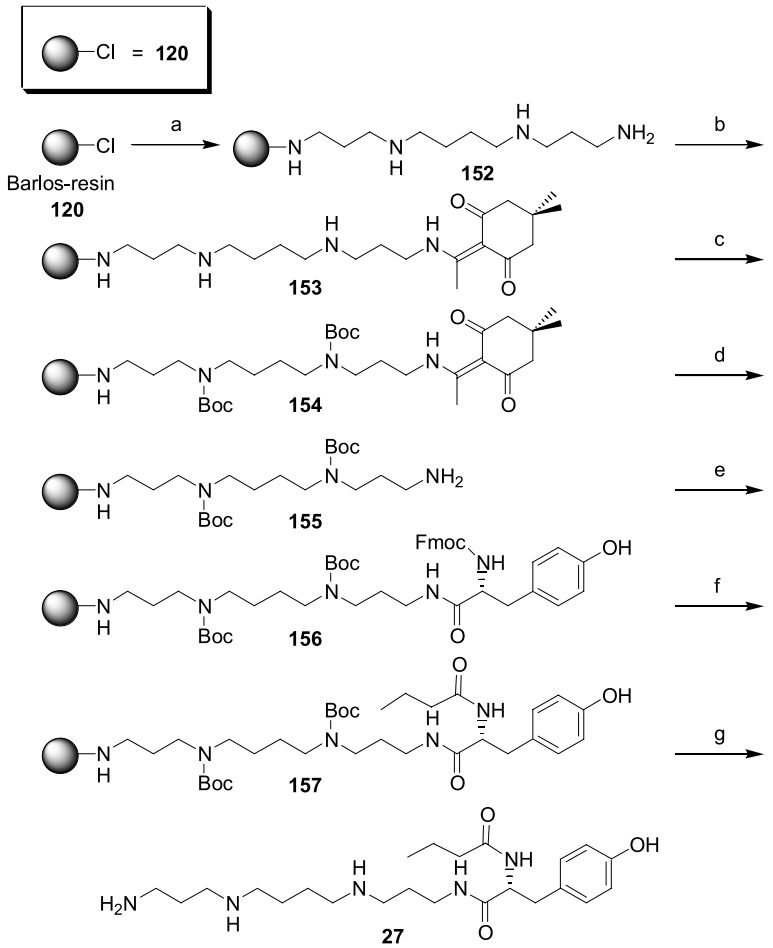
3.2
Direct Attachment of Long Aliphatic Polyamines

Besides the stepwise alkylation of amines, a polyamine backbone can also be generated by the direct attachment of larger building blocks to the solid phase. Usually, the building blocks have to be prepared by solution phase chemistry protocols before attachment to the solid phase using appropriate protection group strategies. The adaptation of the solution phase chemistry to solid phase chemistry often facilitates the protection of the functional groups due to the binding to the resin. However, the protection and the deprotection strategies have to be compatible with the linker chemistry to avoid premature cleavage from the resin. Table 2 gives an overview on the common amine protection groups **128–151** that are so far used in solid phase chemistry.

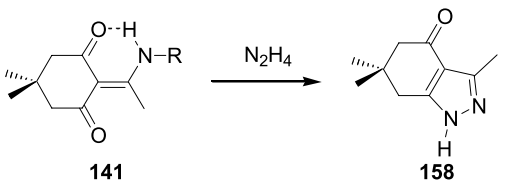
An adaptation of the protection strategies for polyamines from solution phase chemistry was first mentioned for solid phase synthesis of PhTX-343 (**27**) published by Bycroft et al. in 1996 [197] (Scheme 4). Therein, they attached unmodified spermine on a Barlos resin (**120**) and used the enamine of acetyldimedone-(Dde) (**141**) and the Boc-group (**128**) to differentiate between primary and secondary amines (Table 2). It is known that Dde-OH exclusively reacts with primary amines in the presence of secondary amines even if large excess of Dde-OH is used. Investigation of the crude product after Dde-protection showed no bis-Dde-protected spermine, indicating that only the primary amines reacted with the 2-chlorotrityl resin (**120**) in the initial step.

Bycroft et al. developed Dde (**141**) in 1993 as a protecting group for peptides that is fully orthogonal with the deprotection conditions of Boc (**128**) and Fmoc (**131**) [160, 161]. It has since then frequently been used to discriminate between primary and secondary amines [19, 160, 161]. It can be completely removed with 2% hydrazine in polar solvent within 3 min. The driving force of this quick and exhaustive reaction is the formation of 3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol, whose formation can be monitored by UV absorption at 270 or 290 nm (Scheme 5). A drawback of this protecting group is the transamination onto other primary amines present in the molecule [19]. This reduced the half-life time of monoprotected polyamine and requires either its fast subsequent processing or a different protection group strategy ab initio. Nevertheless, its selectivity for primary amino functions makes it a helpful tool for the synthesis of appropriately protected polyamine building blocks.

Another example from the early years of polyamine solid phase chemistry comes from Byk et al. [165] (Scheme 6). After immobilization of 2-bromoacetic acid on a Barlos resin (**120**) they coupled spermine by S_N2-displacement of the bromine. The spermine is either completely protected with Boc-groups (**128**) or, according to Bycroft et al., the primary and secondary amines are differentiated by the use of Dde-/Boc-strategy [197].



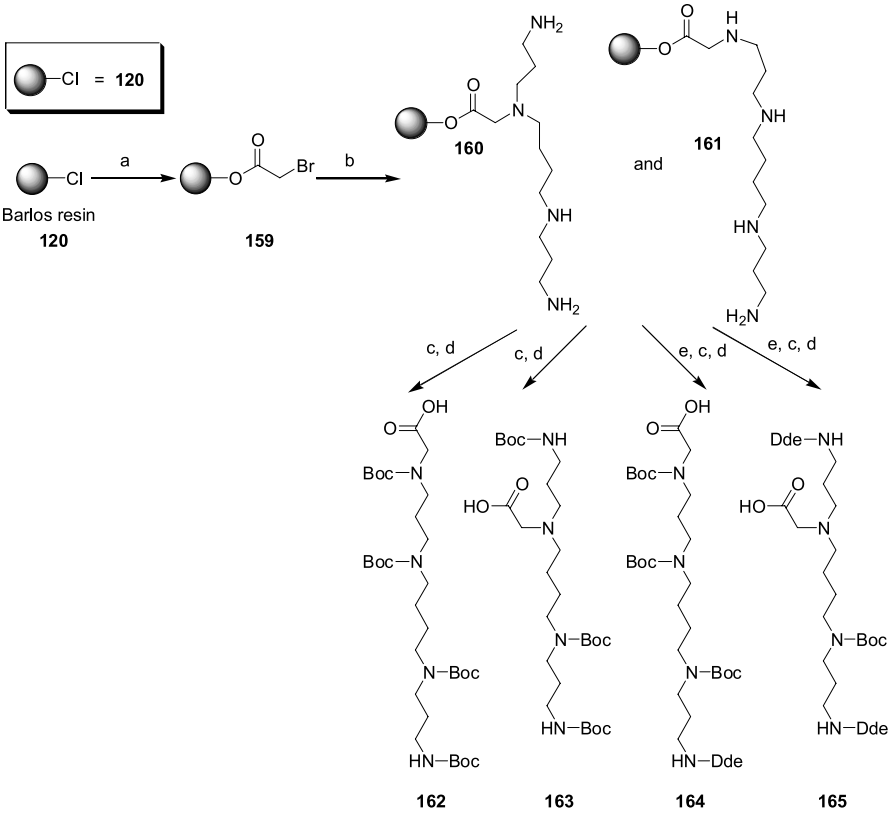
Scheme 4 Synthesis of PhTX-343 (**27**) using Dde-(**141**) and Boc-(**128**) protecting groups. Reagents and conditions: *a* spermine, CH_2Cl_2; *b* 2-acetyldimedon, DMF; *c* Boc_2O, DiPEA; *d* 2% N_2H_4 in DMF; *e* Fmoc-L-Tyr-(*O*-*t*Bu)-OH, HBTU, HOBt, DiPEA, DMF; *f* 20% piperidine in DMF; *g* Fmoc-L-Tyr-(*O*-*t*Bu)-OH, HBTU, HOBt, DiPEA, DMF [197]



Scheme 5 Deprotection of Dde group (**141**). 3,6,6-Trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol is formed

After acid-free cleavage from the resin **120** with 1,1,1-trifluoroethanol in CH_2Cl_2 and chromatographic purification, building blocks **162–165** are obtained in yields of 8–40%. Although the yields of the products **162** and **164** are enhanced compared to **163** and **165** (40%:8% and 17%:12%, respectively), a completely selective synthesis does not seem to be possible by this approach. An interesting feature here was the publication of an acid-free procedure for the cleavage of amines from trityl resins and a refinement of the Kaiser test.

The Kaiser test is an adaption of the ninhydrin test to solid phase conditions and allows the visual detection of free amines in resin-bound substrates. The possibility of qualitatively or quantitatively tracking the reaction progress of polyamine modification makes it a very helpful tool for solid phase chemistry. The general solution phase methods (e.g., thin layer chromatography) are not suitable. Here, the Kaiser test was used to additionally



Scheme 6 Monofunctionalization of spermine by Byk et al. [165]. Reagents and conditions: *a* bromoacetic acid, DiPEA, CH_2Cl_2, rt; *b* spermine, CH_2Cl_2; *c* Boc_2O, CH_2Cl_2; *d* trifluoroethanol/CH_2Cl_2; *e* Dde-OH, DMF

discriminate between primary and secondary amines. It should be noted that the cold Kaiser test is positive within 1 min only, when primary amines are present, whereas the conventional method at 110 °C shows positive results after 15 s for both primary and secondary amines.

Other protecting groups that were also shown to enable the primary–secondary amine differentiation are trifluoroacetyl (Tfa) (**142**) [216, 218, 219, 237, 238], phthaloyl (Pht) (**137**), benzyloxycarbonyl (Z) (**133**) [227, 228], *t*-butyloxycarbonyl (Boc) (**128**) [212], trityl (Tr) (**146**) [245], and monomethoxytrityl (MMT) (**147**) [247] (Table 2). Some valuable, protected polyamine derivates **166–169** are shown in Fig. 20.

	R^1	R^2	R^3	R^4	R^5
166a	Z	Z			
166b	Boc	Boc			
166c	Tfa	Tfa			
166d	Dde	Dde			
166e	Tfa	$(CH_2)_3NHTfa$			
166f	Dde	$(CH_2)_3NHDde$			
166g	Tr	$(CH_2)_3NHTr$			
167a	Bn				
167b	Boc				
167c	Tr				
168a	Teoc	Boc			
168b	Z	Z			
168c	Boc	Boc			
169a	Boc	SES	Allyl	Tfa	N_3
169b	Boc	SES	Allyl	Tcboc	Tfa
168c	Boc	SES	Allyl	Tcboc	ArCO
168d	Boc	SES	PyrS	Allyl	Pht

ArCO = *p*-$CH_3OC_6H_4CH$=CHCO
PyrS = pyridine-2-sulfonyl-

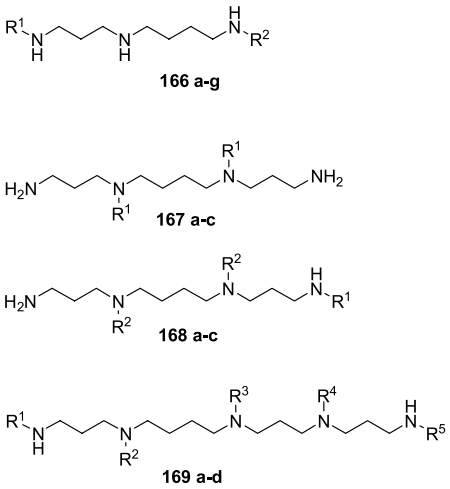
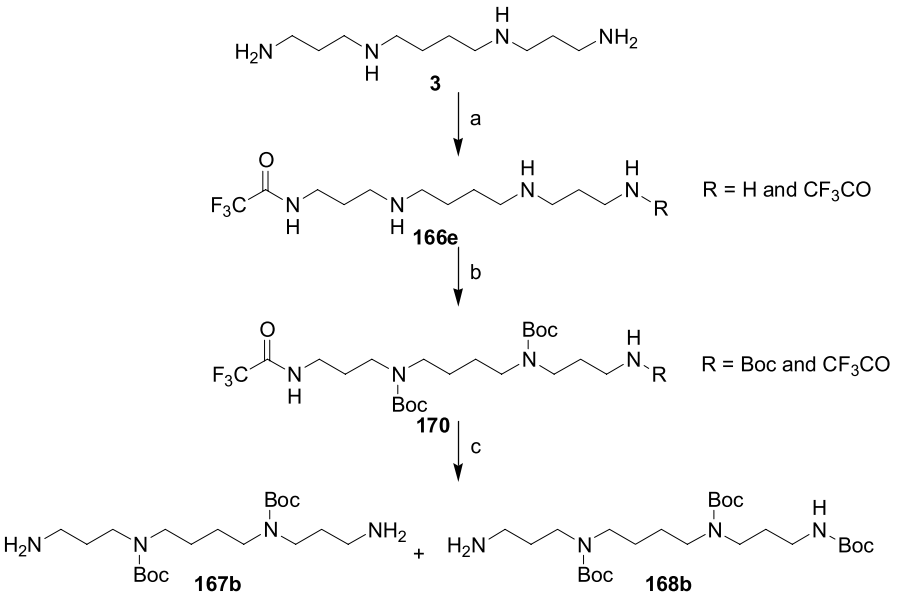


Fig. 20 Selection of useful protected polyamine building blocks for solid phase synthesis [249]. Although **169a–d** derived from solution phase studies [209–211] of asymmetric protection of pentamines, similar protection strategies can be used for the solid phase synthesis

The selective protection of secondary amines is accomplished by indirect methods after blocking of the primary amines. A typical procedure is shown in Scheme 7. Therein, Tfa (**142**) partially or fully protects spermine at its primary amines, while the remaining amines can eventually be saturated with Boc-groups (**128**). After cleavage of the Tfa group with aqueous NaOH, three different Boc-spermines are obtained. By choosing the excesses of ethyltrifluoroacetate the amounts of di- and tri-Boc-spermine can be controlled. Both are helpful synthetic building blocks for solution and solid phase chemistry.

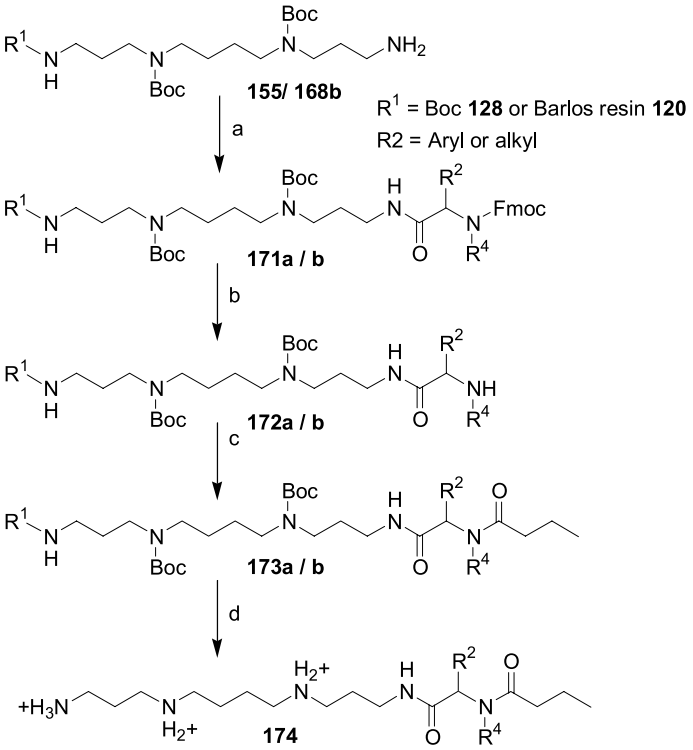


Scheme 7 Representative synthesis of di- and tri-Boc protected spermine (**167b**, **168b**). The Tfa group (**142**) is used for temporary blocking the primary amines. Di- and tri-Boc-spermine are obtained in 18% and 43% yield after chromatographic purification by vacuum liquid chromatography. Reagents and conditions: *a* 1.2 eq Tfa-OEt, MeOH, –50 °C; *b* Boc_2O, MeOH, 0 °C; *c* $NaOH_{conc}/H_2O$ [216].

This reaction has been used for several applications [216, 217, 219, 250]. Jaroszewski et al. used di- and tri-Boc spermine in the synthesis of philanthotoxin analogs [219] (Scheme 8). Firstly, they acylated building blocks **155** and **168b** with a variety of hydrophobic amino acids and those subsequently with butanoic acid using solid and solution phase synthesis. In general, the solution phase experiments showed higher yields for the synthesis of philanthotoxins with comparable residues R^2 (70–90% vs. 42–54% overall yield). One possible reason for decreasing yields of the solid phase synthesis could be the crosslinking of di-Boc-spermine.

The vice-versa strategy was also reported by the same group [213] (Scheme 9). Therein, the Fmoc-protected and Pfp-activated tyrosine moiety is anchored to a trityl bromide resin **114b** by ether formation of the side-chain hydroxyl function. Amide generation with an asymmetrically protected spermine building block **176** and cleavage gave PhTX-343 (**27**) in 43% overall yield.

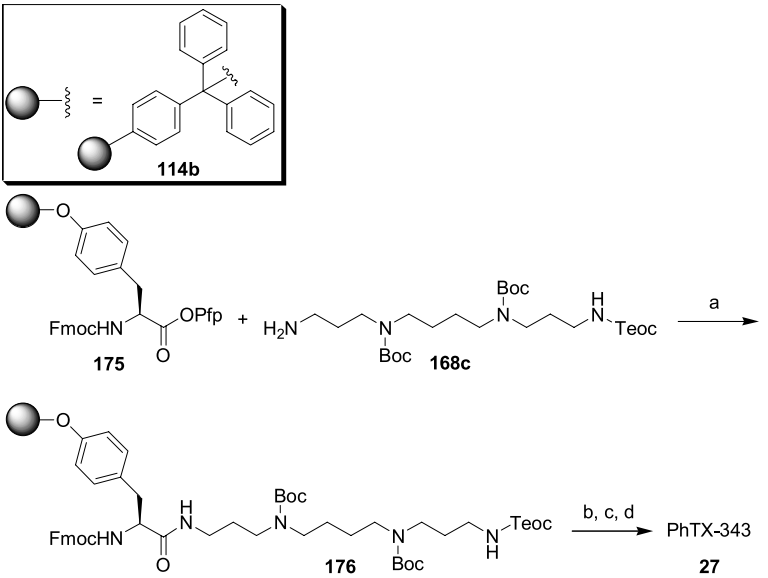
In 1996 and 1997 Bradley et al. published several papers, in which they described using protected spermidine building blocks to start divergent solid phase synthesis of polyamine–peptides, e.g., oxidized trypanothione (**46**) [162, 163, 215]. The spermidine was synthesized by condensa-



Scheme 8 Solid and solution phase synthesis of PhTX-343 (**27**) analogs. Reagents and conditions: Solution phase *a* FmocNR^4-(CHR^2)-COOPfp, Et_3N, CH_2Cl_2, rt; *b* 1-octanethiol, DBU, THF, rt; *c* $C_3H_7COOPfp$, CH_2Cl_2, rt; *d* 20% TFA in CH_2Cl_2, rt. Solid phase *a* FmocNR^4-(CHR^2)-COOPfp, DiPEA, HODhbt, DMF, rt; *b* 20% piperidine in DMF, rt; *c* $C_3H_7COOPfp$, DiPEA, HODhbt, DMF, rt; *d* CH_2Cl_2/TFA/triisopropylsilane (78:20:2), rt [219]

tion of N^1-Boc-N^4-benzylputrescine and *N*-protected aminopropyltosylates or bromides. The secondary amine is protected by *O*-(*p*-nitrophenyl)-*O*′-(methoxycarbonyl)benzylcarbonate that also acts as a handle, by which the whole construct can be attached to an aminomethyl resin **111** (Table 1). Acylation with different amino acids opens access to a broad number of symmetrical and asymmetrical polyamino peptides. This approach has been recently used for a library generation [215] (Scheme 10).

The same group extended the concept of this “handle linker” to spermine derivatives. Symmetrical spermine conjugates like kukoamine A (**10**) were synthesized to prove the versatility of this method [214] (Scheme 11).



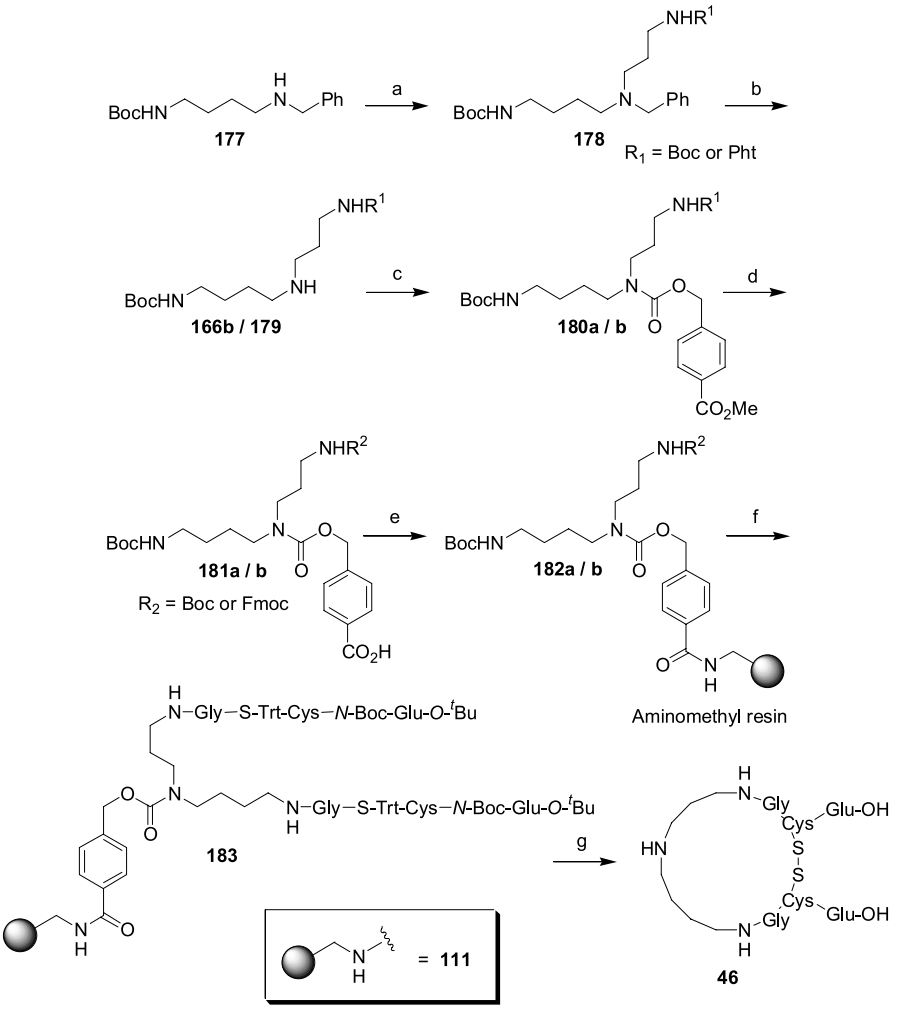
Scheme 9 Synthesis of PhTX-343 (**27**). Reagents and conditions: *a* DiPEA, HODhbt; *b* 20% piperidine in DMF; *c* $C_3H_7COOPfp$, DiPEA, HODhbt; *d* TFA in CH_2Cl_2

3.3
Modular Synthesis of Polyamine Backbones

3.3.1
S_N2-alkylation

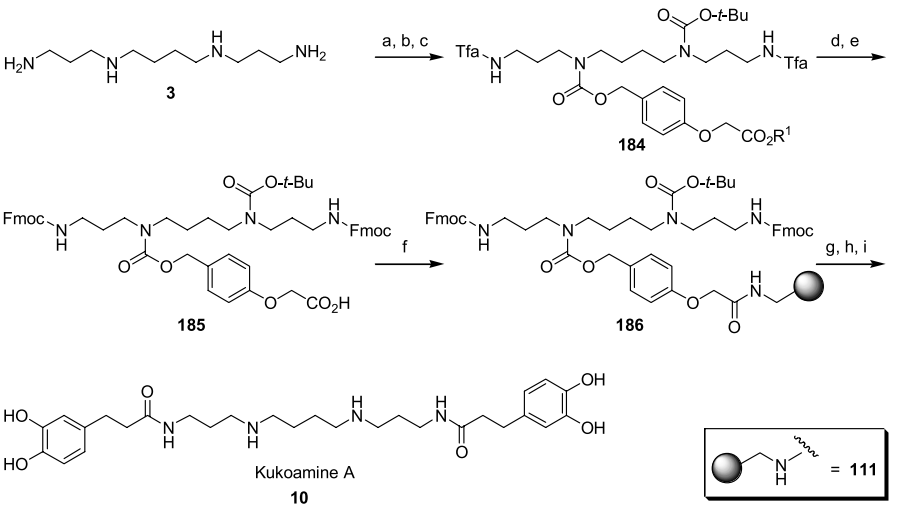
SPS approaches for both amine and electrophile bound to the resin have been reported. The latter is superior and most examples found in the recent literature refer to it, because efforts for the direct generation of secondary amines often led to overalkylation. However, this strategy is not suitable in all cases. Crosslinkage between solid phase bound electrophiles and primary amines must be taken care of [185, 251]. Reaction of a primary amine with two solid phase bound electrophiles can sometimes result in the formation of tertiary amines as side products, which may significantly lower yield and purity.

Thereby, common S_N2-alkylation is mostly limited to the homo-dialkylation of primary amines and monoalkylation of secondary amines. Solid phase bound electrophiles can be halides, sulfonates (mesyl-, tosyl-, and nosylates) and in the broader sense epoxides and Michael systems. The last two are obviously limited in their applicability and therefore references in the context of solid phase polyamine synthesis are rare or non-existent. For examples of those reactions in solution phase polyamine synthesis one should refer to the corresponding literature.



Scheme 10 Synthesis of oxiized trypanothione (**46**). Either the di-Boc- or the Boc-Fmoc-spermidine is coupled to the resin **111**. After deprotection, the primary amines are further derivatized [162, 163, 215]. Reagents and conditions: *a* Boc-HN-$(CH_2)_3$OTs or 3-bromopropylphthalimide, Na_2CO_3, KI, *n*-BuOH; *b* Pd/C, H_2, EtOH, AcOH; *c* *O*-(*p*-nitrophenyl)-*O′*-(methoxycarbonyl)benzylcarbonate, NEt_3, DMF, 97%; *d* aq. NaOH, dioxane; in the case of R_1 = Pht: $N_2H_4 \cdot H_2O$, EtOH then Fmoc-succinimide; *e* DIC, HOBt, DMAP, aminomethyl resin, DMF or CH_2Cl_2; *f* deprotection, Fmoc-AS-peptide coupling; *g* TFA/TFMSA/ethanedithiol/PhSMe; I_2/MeOH [162]

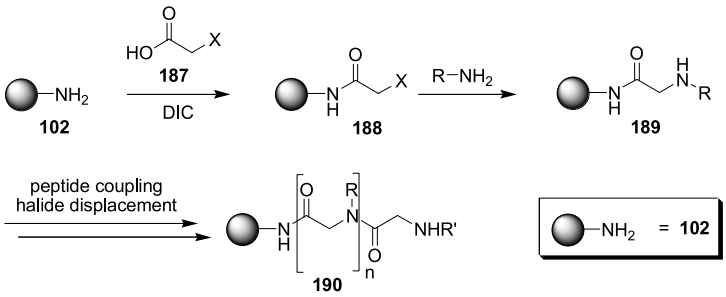
Zuckermann et al. published the first examples of SPS-halide displacement in the synthesis of peptoids in 1992 [252] (Scheme 12). The utilized strategy is called sub-monomer synthesis and the halide was introduced by amide formation of halogenated carboxylic acids. To elongate the peptoid back-



Scheme 11 Synthesis of kukoamine A (**10**) using the handle linker approach. Reagents and conditions: *a* CF_3CO_2Et, MeCN, H_2O; *b* 1 eq Boc_2O, NEt_3, DMAP, DMF; *c* $EtO_2CH_2OphCH_2OCO_2Ph\text{-}4\text{-}NO_2$, NEt_3, DMF; *d* NaOH, dioxane; *e* pH 8.5, Fmoc-OSu, H_2O, dioxane; *f* Aminomethyl resin **111**, DIC, HOBt, CH_2Cl_2; *g* 20% piperidine in DMF; *h* $HO_2CH_2CH_2Ph\text{-}3,4\text{-}(OH)_2$, DIC, HOBt, CH_2Cl_2; *i* TFA/TIS/thioanisole/H_2O [214]

bone, acylation and halogen-displacement steps are alternately repeated. In the halogen-displacement step, iodo- and bromoacetic acids were superior to chloroacetic acid when weakly nucleophilic amines like aniline (> 79% vs. < 5% yields) but not aliphatic amines (> 75% yield) were used.

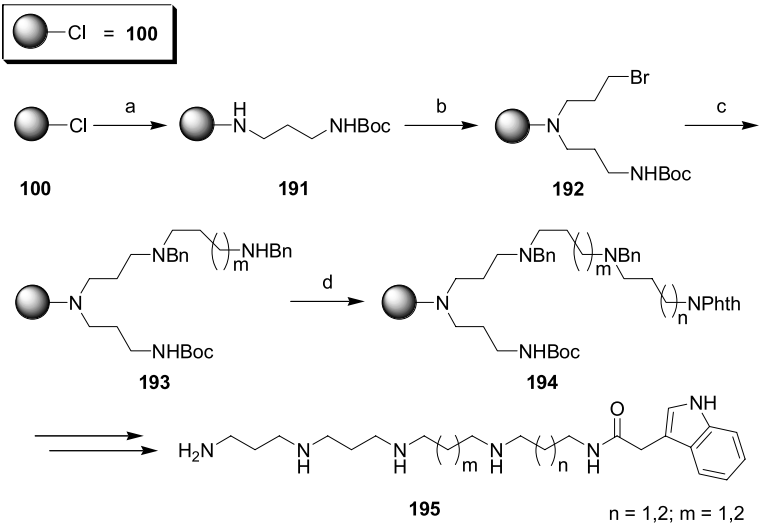
Nevertheless, the use of chloroacetic acid is superior to the other acids, when using unprotected side-chain heterocycles [253]. This is due to the irreversible alkylation reaction that can occur with nucleophiles in the side chains. The lowered reactivity of the chloride can be enhanced by previous addition of, e.g., KI, which enables the generation of 15-mers in > 80% aver-



Scheme 12 Peptoid synthesis by alternate peptide coupling–halide displacement [252]

age purity. Use of bromoacetic acid monomers gave only undetectable traces of the desired product.

An example that starts the synthesis of polyamine toxins from a central amino group is shown in Scheme 13 [229, 230]. The backbone is elongated by alternate coupling of benzyl-protected amines and bromides. Due to the protection group strategy consisting of orthogonal Boc- (**128**), benzyl- (**138**), and phthalimides (**137**) it is possible to synthesize polyamines from center to tail. Unfortunately, after three alkylation steps the overall yield is only 30%, which drops the overall yield to 10–30%.

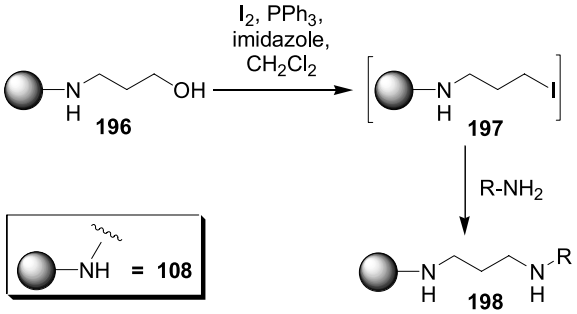


Scheme 13 Asymmetric synthesis of polyamine **195** starting from an internal amino functionality. Reagents and conditions: *a* *N*-Boc-diaminopropane, DiPEA; *b* dibromopropane, DiPEA; *c* N^1,N^5-dibenzyldiaminopropane, DiPEA; *d* N^1-benzyl-N^5-phthaloyldiaminopropane [229, 230]

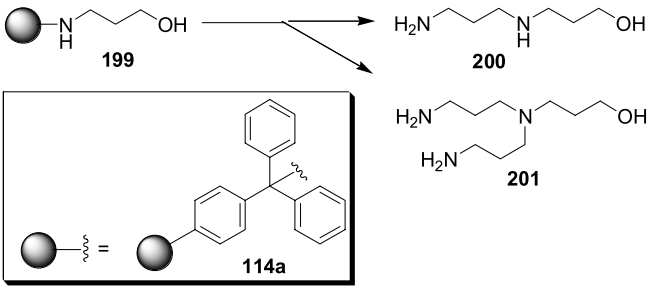
Another example shows similar results for the yields of SP-alkylation of bromides and iodides generated from alcohols if the reaction is carried out on a trityl resin **114a** [185]. Interestingly, the change to a *p*-nitrophenylcarbonate-activated Wang resin **108** afforded significantly higher overall yields for both steps, ranging up to quantitative yields (Scheme 14).

A common problem in the last-mentioned examples is the formation of side products by crosslinking [185, 251] (Scheme 15). This obstacle cannot be solved by the use of large excess of amine or electrophile but can be largely suppressed by the use of partially loaded resins, with loadings up to 0.5 mmol g^{-1}.

The use of the more reactive *p*-nosyl (*p*-Ns) group (**144b**) was also reported [241, 242]. Overall yields of 45–64% were obtained for a 12-step



Scheme 14 Conversion of some simple alcohols (**196**) into iodides (**197**) and subsequent substitution with primary or secondary amines [185]

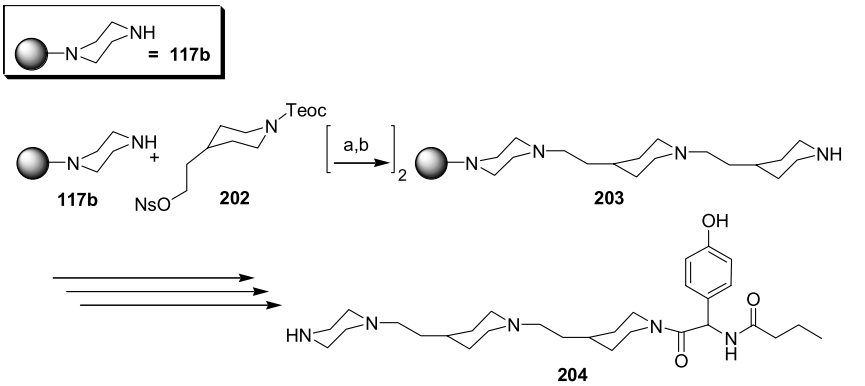


Scheme 15 Cross-linking produces at least one side product [185]

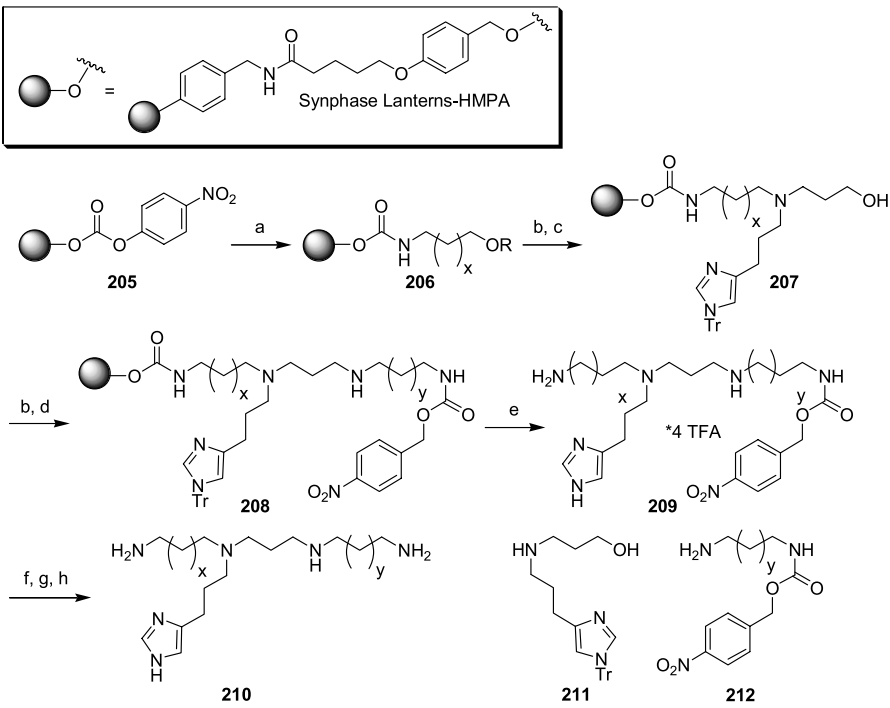
reaction sequence, in which the displacement of the nosylate leads to the formation of a secondary amine.

Olsen et al. used 2-nitrophenylsulfonate (**144a**) for the alkylation of cyclic secondary amines [179] (Scheme 16). The overall yield (26%) of this synthesis containing two alkylation steps is usually lower, because the cyclic amine is sterically more demanding than the primary amine. If only one piperidine moiety is introduced before acylation the overall yield increases up to 60% for the shorter polyamine analogs, confirming that the alkylation step is the crucial one. Nevertheless, the *o*-nosyl-group (**144a**) seems to be the leaving group of choice. If the appropriate mesylate is used no product **204** but side products with only one or no piperidine blocks are obtained.

Mesylates found an application in the solid phase synthesis of polyamine–imidazole conjugates and usnic acid, both described by Uriac et al. [164, 246] (Scheme 17). They showed that the use of longer reaction times (32 h) and higher temperatures (70 °C) gave good yields for the alkylation of secondary amines. Primary amines are sufficiently alkylated in much shorter time (6 h) and at lower temperatures (50 °C).



Scheme 16 Synthesis of a conformationally rigid polyamine **204** by nosylate displacement. Reagents and conditions: *a* DiPEA, THF/PhMe, 50 °C, 16 h; *b* TBAF, DMF, 50 °C, 0.5 h [179]



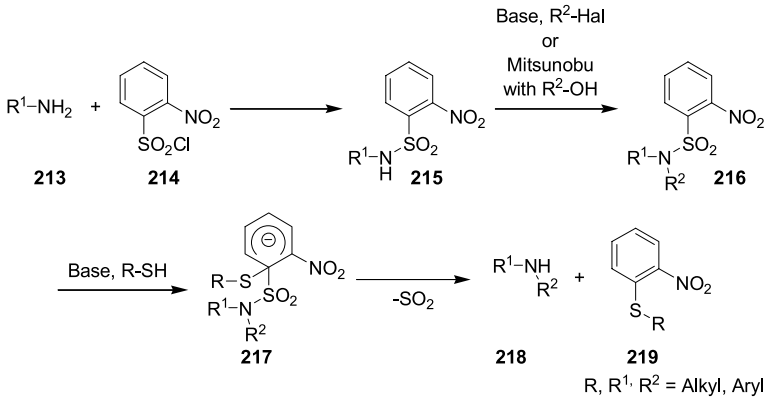
Scheme 17 Synthesis of polyamine–imidazole conjugates. Reagents and conditions: *a* aminoalcohol, CH_2Cl_2, 2 h, rt; *b* methanesulfonyl chloride, pyridine, 30 min, rt; *c* **211**, DMSO, 32 h, 70 °C; *d* **212**, DMSO, 5 h, 50 °C; *e* 1:1 TFA:CH_2Cl_2, 1 h, rt; *f* NaOH then CH_2Cl_2, chromatography; *g* 20% $Pd(OH)_2$/C, H_2, MeOH, 4 h; *h* H_2O, HCl/EtOH, 3 h, 0 °C then rt [246]

3.3.2 Fukuyama Alkylation

The Fukuyama alkylation (Scheme 18) is a special case of S_N2-reaction with activated amines. It was developed by Fukuyama et al. in 1995 [240] for solution phase synthesis and it was first mentioned for solid phase chemistry in 1997 by Nguyen et al. [254]. It is particularly useful for the selective monoalkylation of primary amines under mild conditions. This is a major advantage over the other methods for the modular generation of polyamine backbones described above.

S_N2-displacement produces low yields and easily leads to over-alkylation if the secondary amine is not sterically hindered. This problem can be largely suppressed for the reductive alkylation of ketones and aldehydes [222, 232, 248] and is not existent for the reduction of *N*-monoalkyl amides, which lead very selectively to the corresponding secondary amines. The latter two methods, however, suffer more from the necessity to utilize strong reducing agents like $Na(CN)BH_3$, $LiAlH_4$, or diborane. In comparison, the conditions of the Fukuyama alkylation (Scheme 18) seem very mild and generally applicable.

The reaction proceeds as shown in Scheme 18. Sulfonylbenzyl-type protecting groups can either be 2-nitro-(*o*-Ns) (**144a**), 4-nitro-(*p*-Ns) (**144b**) or

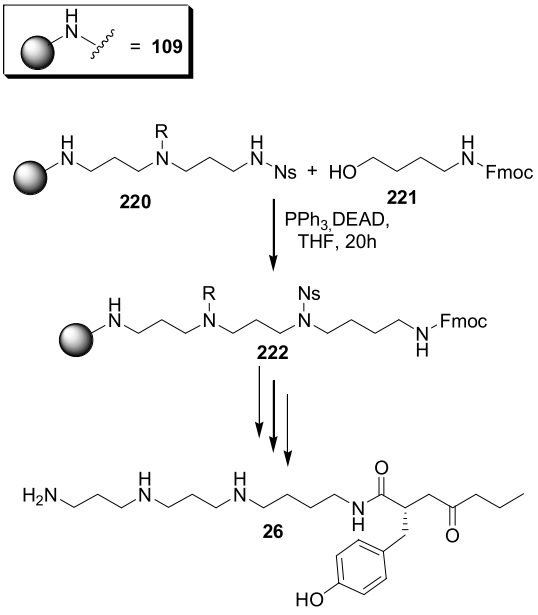


Scheme 18 General mechanism of Fukuyama alkylation with *o*-Ns (**144a**) as activating group. After introduction of the *o*-nitrobenzenesulfonyl-protecting group **144a** a *N*-alkylsulfonamide (**215**) is obtained, in which the acidity of the NH-proton is significantly enhanced. After deprotonation of the sulfonamide it can displace a halide or an alcohol in a Mitsunobu-type reaction. The former activating group now changes its function to a protecting group until it is removed by reaction with thiolates. Those form a Meisenheimer complex (**217**) with the electron-deficient ring of the Ns-group that decomposes under loss of SO_2 and nitrophenyl thioether (**219**) to release the secondary amine **218** [240]

2,4-dinitrobenzenesulfonates (d-Ns) (**144c**) (Table 2). One advantage of d-Ns over mono-Ns (**144a** and **b**) is an increased rate in sulfonamide formation. Due to the enhanced electron-withdrawing effect of the second nitro group, the amide proton of the d-Ns amide additionally possesses a lower pK_a leading to higher rates in alkylation reactions and making it even more susceptible to the basic deprotection conditions used for *o*-Ns. On one hand, this permits the use of *o*-Ns and d-Ns as fully orthogonal protecting groups [243], but on the other hand also limits the applicability of the d-Ns group. Weak bases like *n*-propylamine and higher temperatures partially deprotect the amine, which makes the use of d-Ns problematic and unsuitable for multiple alkylation steps on the resin [167, 184].

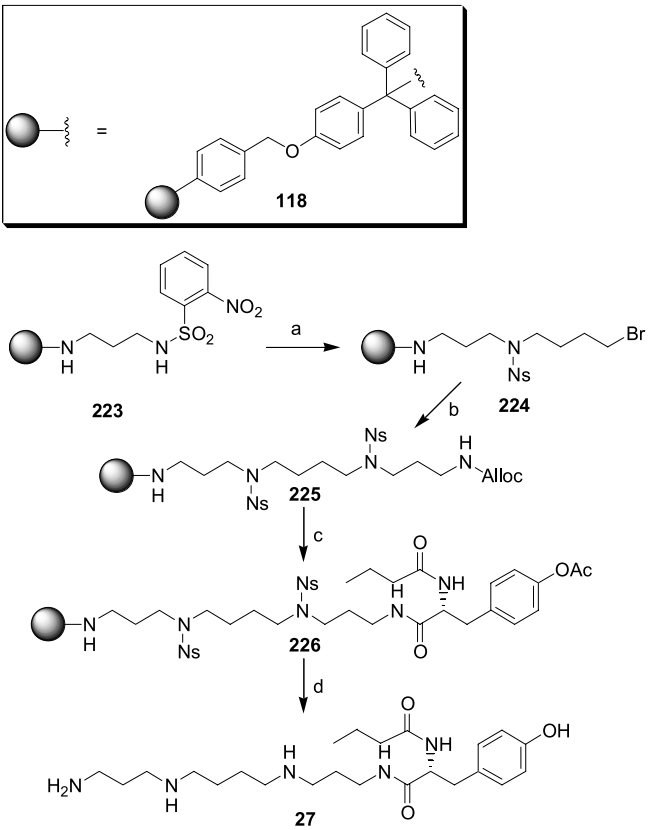
Alkylation with tosyl- and trifluoracetamides has also been reported but their harsh deprotection conditions prevent their suitability for solid phase chemistry of polyamines [254–256].

The first application of Ns-amides in the synthesis of polyamines was reported in 2000 [175] (Scheme 19). Bycroft et al. used it to integrate a C_4-unit into the polyamine backbone of PhTX-433 (**26**) under Fukuyama–Mitsunobu conditions [175].



Scheme 19 Fukuyama–Mitsunobu alkylation for introduction of a C_4-moiety [175]

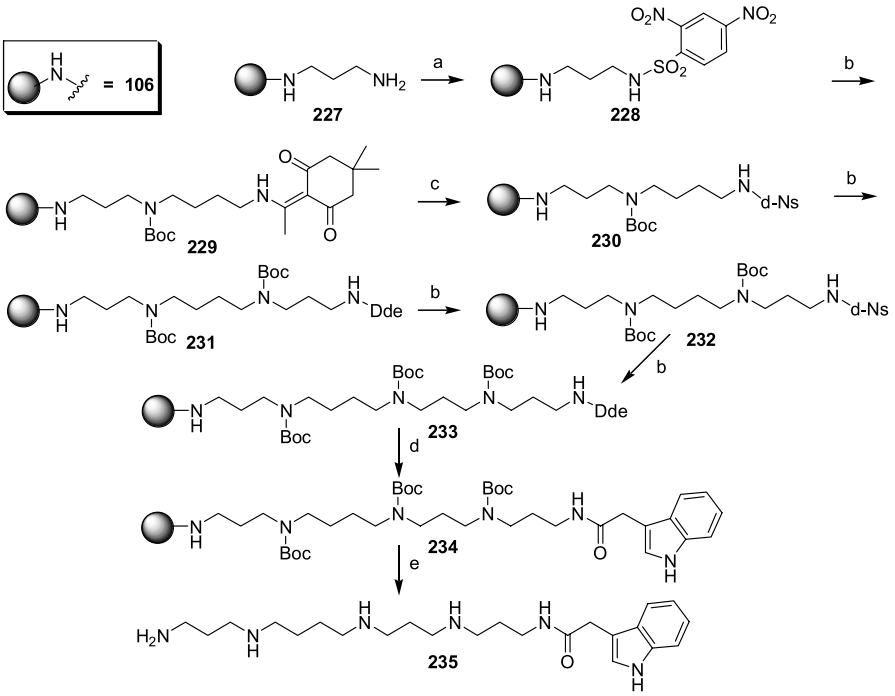
After publishing a mixed solution–solid phase synthesis of polyamine toxin HO-416b (**274**) in 1999 [257], Fukuyama et al. demonstrated the versatility of the Ns-strategy for the complete synthesis of longer polyamines on solid phase by the synthesis of PhTX-343 (**27**) [24, 257] (Scheme 20). The



Scheme 20 Synthesis of PhTX-343 (**27**) using an *o*-Ns group (**144a**) and primary bromides for establishing the polyamine backbone. Reagents and conditions: *a* 1,4-dibromobutane, K_2CO_3, DMF, 60 °C; *b* *N*-Ns, *N*′-alloc-diaminopropane, K_2CO_3, DMF, 60 °C; *c* $Pd(PPh_3)_4$, pyrrolidine, DMF, rt; *N*-butyryl-*O*-acetyl-L-Tyr-*p*-nitrophenol ester, CH_2Cl_2, rt; *d* K_2CO_3, MeOH; mercaptoethanol, DBU, DMF, rt; TFA, CH_2Cl_2, rt [24]

product was isolated in 75% yield. A possible source of yield loss during this synthesis is the expected crosslinking in the attachment of unprotected diaminopropane to the resin and step a.

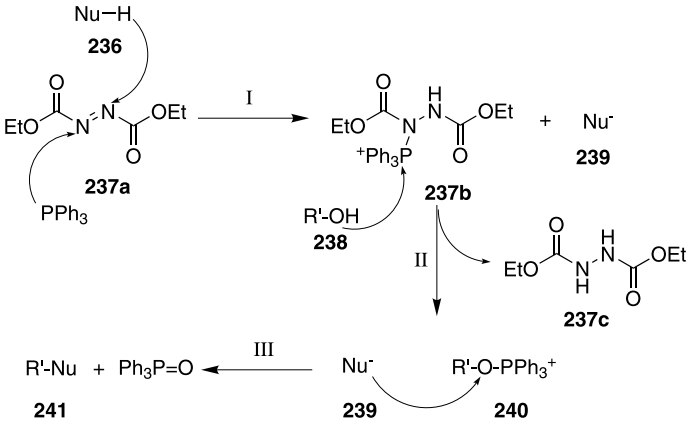
This problem is circumvented by the use of monoprotected diamine building blocks or amino alcohols that can be alkylated under Mitsunobu conditions. Hone et al. showed the utility of this method in the synthesis of spider toxin HO-416a (Agel 416) (**235**) in 2000 [167] (Scheme 21). The whole 16-step reaction sequence gave HO-416a (**235**) in 88% yield and 94% purity. Herein, the d-Ns group (**144c**) was used, which was probably responsible for the high overall yield compared to the example in Scheme 20 by Fukuyama et al. However, its above-mentioned restrictions made it necessary to use a laborious protecting group strategy.



Scheme 21 Synthesis of HO-416a (Agel 416) (**235**) using d-Ns-amide (**144c**) alkylation as central tool. Reagents and conditions: *a* 2,4-dinitrobenzenesulfonyl chloride, 2,6-lutidine, CH_2Cl_2, 3 h; *b* 1. *N*-Dde butanolamine or *N*-Dde propanolamine, PPh_3, DEAD,THF, 3 h; 2. Mercaptoacetic acid, DiPEA, CH_2Cl_2, 3 h; 3. Boc_2O, DiPEA, CH_2Cl_2, 3 h; *c* hydrazine-hydrate (2% in DMF), 30 min; 2,4-dinitrobenzenesulfonyl chloride, 2,6-lutidine, CH_2Cl_2, 3 h; *d* hydrazine-hydrate (2% in DMF), 30 min; *N*-Boc indole-3-acetic acid, HOBt, DIC, CH_2Cl_2, 1 h; *e* 5% TFA in CH_2Cl_2, 5 min; 4 M HCl in dioxane, 3 h [167]

Since then, the alkylation of amines using Mitsunobu–Fukuyama conditions has been extensively studied and applied in the synthesis of polyamines and polyamine analogs. The mechanism is shown in Scheme 22.

According to Hone et al., Stromgaard et al. showed its applicability in the small library synthesis of philanthotoxin-433 analogs [184]. Reagent concentration, excess ratios, reaction time, number of reaction cycles, and order of reagent addition were investigated. It appeared that with a reagent concentration of 200 mM, reagents added in the order tributyl phosphine (TBP) and then 1,1′-(azodicarbonyl)dipiperidine (ADDP), and reaction times of 3×3 h, the first alkylation step yielded 100% conversion of the educt. After one further alkylation and the acylation procedures mentioned above PhTX-433 analogs are obtained in high purities of 98–99% and overall yields of 23–40%. This suggests a distinct loss of conversion in either the second alkylation or the acylation and deprotection steps.



Scheme 22 Mechanism of the Mitsunobu reaction. The essential compounds are a phosphane and a diazocompound that is able to generate the oxophosphonium intermediate in the presence of alcohols. The S_N2-reaction in *step III* is the rate-determining step. The outcome of the reaction strongly depends on the steric environment of the alcohol and the acidity of the nucleophile. In this case the traditional Mitsunobu reagents diethylazodicarboxylate and triphenylphosphane are shown as participants of the reaction [258]

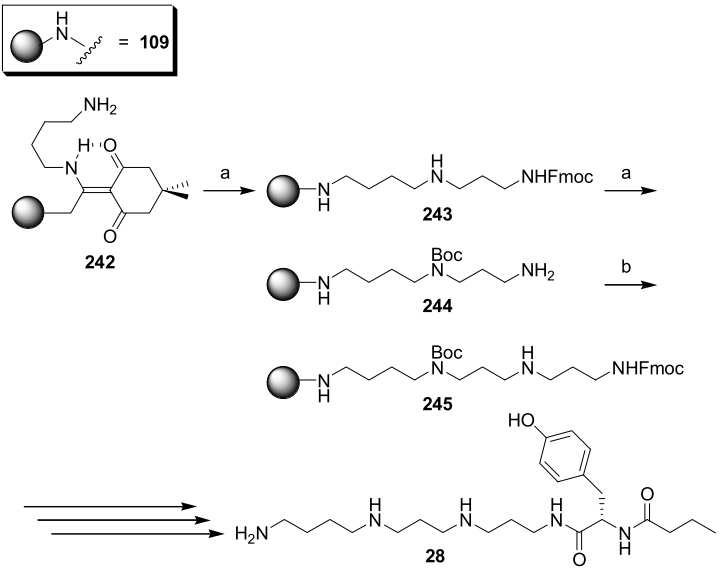
Further refinements were made by Olsen et al., who tested the influence of base addition, different solvent systems, and Mitsunobu reagent pairs [259]. The use of DEAD in combination with PPh_3 or PEt_3 seems favorable, although obviously dependent on the substrate type. The effort to synthesize curtatoxins with more than five amino groups did not show any of the desired products. The results for the alkylation with secondary alcohols were even more distinct. In agreement with the work of Stromgaard, this shows that the scope of *o*-Ns Fukuyama–Mitsunobu alkylation with protected amino alcohols is limited to fewer steps, if acceptable yields are demanded.

3.3.3 Reductive Amination

Reductive amination was the first method described for amine production on solid phase. It was first mentioned in 1987 by Coy et al. [260, 261]. They used a combination of $Na(CN)BH_3$ and HOAc for the subsequent reduction of intermediately formed imides in the synthesis of somatostatin octapeptide analogs. Due to its early development, this alkylation method has found entry to the synthesis of diverse substance classes like N-terminal-modified peptides, carbohydrate mimetics [262], glycopeptides [263, 264], oligonucleotides [265], and of course polyamines [174, 175, 222, 232, 248, 266, 267]. For the same reasons as the amide reduction approach, it is powerful enough to be used in the production of several compound libraries [177, 262–264, 268].

As already mentioned in Sect. 3.1, the reductive amination approach plays an important role for many linker systems [202].

In 2000, Bycroft and coworkers used this approach for the first time for the generation of a polyamine backbone (Scheme 23). They synthesized PhTX-334 (**28**) [174]. After attachment of 1,4-butanediamine to the resin the asymmetric backbone of the polyamine moiety was built up stepwise. Fmoc-protected 3-aminopropanal building blocks were used to generate an imine, which was eventually reduced with $Na(CN)BH_3$/HOAc. Protection group conversion and repetitive chain elongation yielded tetraamine **245**. Tyrosine and butanoic acid were coupled by previously described methods to yield PhTX-334 (**28**) in 65% overall yield and 90% purity (Scheme 23).



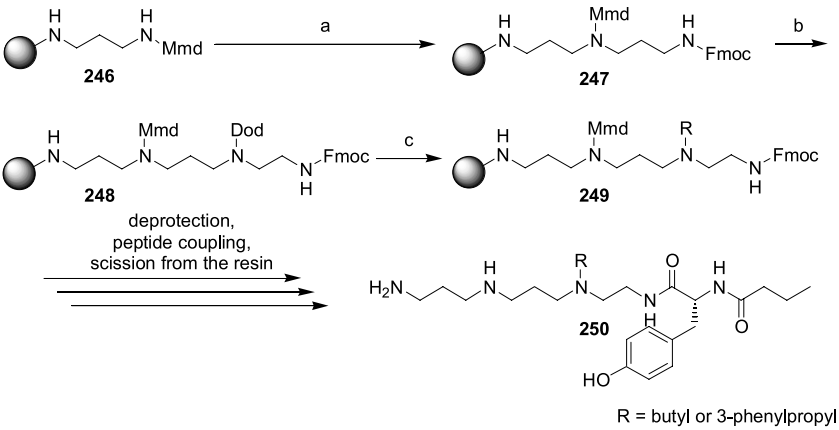
Scheme 23 First use of reductive amination in the solid phase preparation of a PhTX-334 (**28**) by Chhabra. Reagents and conditions: *a*(i) $FmocNHC_2H_4CHO$, 1% AcOH/DMF, 1.5 h; *a*(ii) $Na(CN)BH_3$, 1% AcOH/DMF, 10 min; *b*(i) Boc_2O, DIPEA, DMF, 20 h; *b*(ii) 20% piperidine in DMF; *c*(i) $FmocNHC_2H_4CHO$, 1% AcOH/DMF, 1.5 h; *c*(ii) $Na(CN)BH_3$, 1% AcOH/DMF, 10 min [174]

Although the reductive amination is very common in the synthesis of polyamines, it is not possible to generate protected C_4- and C_5-aminoaldehydes. This limits the procedure to the synthesis of philanthotoxins, in which the terminal alkyl spacer is not a C_3 building block. For synthesis of the corresponding PhTX-433 (**26**), Bychroft and coworkers used the above described Fukuyama–Mitsunobu alkylation conditions [175].

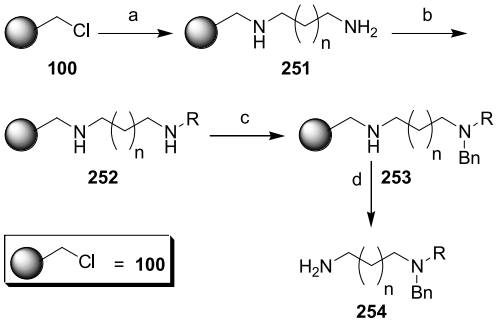
Jönsson accomplished the synthesis of branched polyamines using reductive amination together with a complex protecting strategy compris-

ing 4-methoxy dityl- (Mmd) (**151**) and 4,4′-dimethoxy dityl (Dod) groups (**150**) [222, 248] (Scheme 24). Mmd and Dod differ in the number of their methoxy groups making the Dod group more sensitive against acids than the Mmd group. This differential acid sensitivity facilitates the selective removal of Dod in the presence of the Mmd group. The products were obtained in > 80% purity and 75–80% overall yield.

One possible problem of the reductive amination approach, analogous to the S_N2-reaction, is the risk of over-alkylation. This can either be circumvented by an introduction of a temporary protecting group as described in



Scheme 24 Synthesis of PhTX-233 (**250**) by Jönsson. Reagents and conditions: *a*(i) *N*-Fmoc-aminopropanal; *a*(ii) Na(CN)BH3, 3% AcOH, NMP, 40 °C; *b*(i) piperidine in DMF; *b*(ii) Dod-Cl, DiPEA, CH_2Cl_2; *b*(iii) 5% TFA; *b*(iv) *N*-Fmoc-2-aminoacetaldehyde; *b*(v) $Na(CN)BH_3$, 3% AcOH, NMP; *c*(i) 5% TFA in CH_2Cl_2; *c*(ii) butyraldehyde or 3-phenylpropionaldehyde; *c*(iii) $Na(CN)BH_3$, 3%AcOH, NMP, 40 °C [222]



Scheme 25 Excess of 2.5 equiv of aldehyde for the reductive amination selectively leads to the formation of secondary amines **254**. Reagents and conditions: *a* diamine, DiPEA, DMF; *b* 1–2.5 equiv R-CHO, CH_2Cl_2:TMOF, 2-$NaBH(AcO)_3$, AcOH, DMF; *c* BnBr, DBU, DMF; *d* 10% TFA in CH_2Cl_2

Scheme 24 [222, 248] or by the use of an appropriate excess of aldehyde. According to the current literature 2.5 equiv of aldehyde lead to the formation of primary amines whereas 5 equiv completely alkylate to the tertiary amine **254** [232] (Scheme 25).

3.3.4 Reduction of Amides

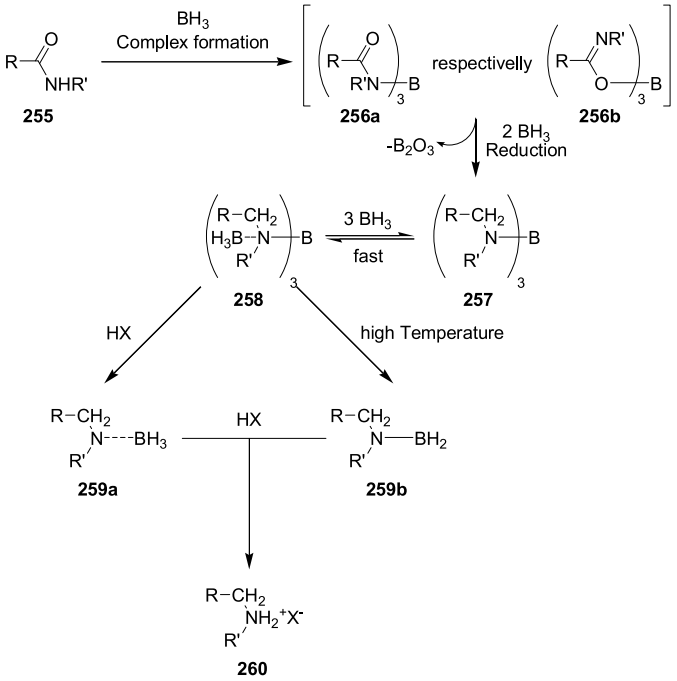
The first reports of amide reduction on solid phase were published by Schultz et al. for the synthesis of *N*,*N*-dialkylated oligocarbamates. They used diborane as a relatively mild reducing agent. BH_3 is favored in the recent literature as the stronger reducing agent. $LiAlH_4$, which has been used in solution phase, is too harsh against several reducible groups and linker systems [231, 269, 270].

The method itself reveals high yields and, in combination with the large number of commercially available acylation reagents, it opens access to a broad range of structurally diverse polyamines.

Scheme 26 shows the supposed mechanism for the reduction of amides by BH_3 [30]. The reaction proceeds well up to the formation of aminoborane **257**. Its complexation with three additional equivalents of borane occurs faster than the reduction of further amides. **258** is stable and can only be transformed to **259b** at high temperatures (110 °C in toluene). The aminoborane is converted to the secondary amine **260** by acid treatment. These conditions are not suitable for solid phase chemistry and make a different workup procedure necessary. If reduction is the terminal step of a synthesis, complex **258** can easily be destroyed during the cleavage from the resin with strong acids.

Alternative decomplexation conditions are the use of DBU in NMP:MeOH (9:1) [271], piperidine [193, 272], and conditions known from solution phase, i.e., exposure to refluxing methanol [273] and basic hydrolysis with hydrochloric acid or saturated ammonium chloride [274]. However, those conditions often do not fit most of the resin requirements, commonly used linker systems, and amino-protection groups. Therefore, Hall et al. developed a milder method employing iodine in a *i*-Pr_2NH/HOAc buffered solution for polyamine synthesis on a trityl linker [275]. They suggest that the acetate anion is able to rapidly cleave the covalently bound aminoborane unit in **258** (Scheme 26). The hydrides in **259a** can be successively removed by iodine, which itself can be displaced by another acetate ion in a simple S_N2-reaction. Back bonding of the oxygen weakens the boron–nitrogen bond and the complex decomposes irreversible after protonation of the resulting amine.

Karigiannis et al. used amide reduction in the synthesis of kukoamine isomers **266** [231] (Scheme 27). The spermine backbone is generated by amide formation of diaminobutane and trityl-protected and resin-bound β-alanine (**261**). The amide groups are reduced with BH_3, and kukoamine C (**266**) is ob-

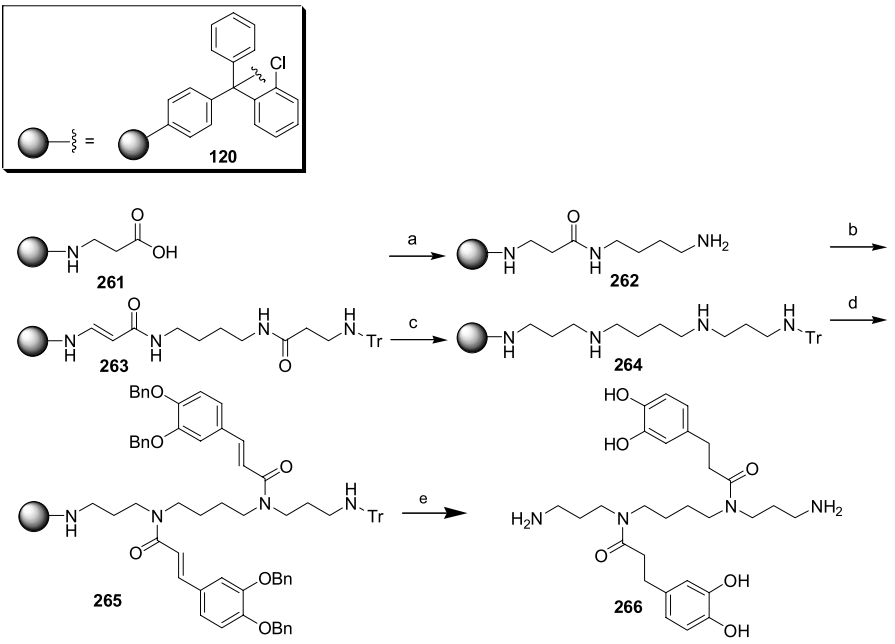


Scheme 26 Supposed mechanism of amide reduction by BH_3 and subsequent liberation of the resulting amino–borane complex [30]

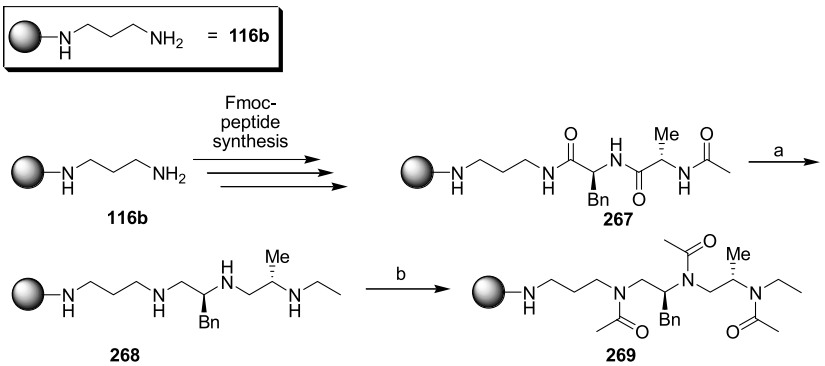
tained in 65% overall yield after further acylation, cleavage from the resin, and reduction of the side chain with concurrent cleavage of the benzyl groups with Pearlmans catalyst. There was no mention of the destruction of the amine–borane complex.

The synthesis of chiral polyamines by reduction of the precursor amides was also shown to work without loss of optical activity [30] (Scheme 28). Stepwise coupling of L-amino acids and successive reduction of the tripeptide leads to the formation of the polyamine backbone. The aminoborane complex is destroyed by the iodine procedure mentioned above and the resulting secondary amines were acylated. The products were obtained in almost quantitative yield and high purity. A comparable study with a piperidine treatment showed that the iodine treatment is preferred. Analysis of the products showed no racemization at the stereogenic centers.

Yields dropped notably, when this procedure was applied to synthesize branched polyamines. This comes from the slow reduction rate of the tertiary amide that leads mainly to only partially reduced side products, even after 5 days at 65 °C. The problem was satisfactorily solved for aliphatic substituents by alkylating the secondary amine by reductive amination with aldehydes using $NaBHAc_3$ as an additional hydride source.

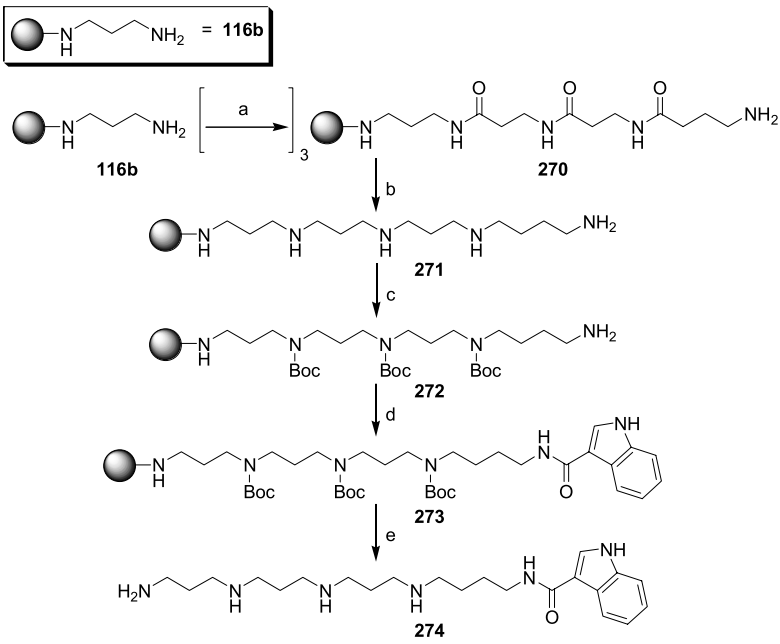


Scheme 27 Synthesis of kukoamine C (**266**). Reagents and conditions: *a* DIC/HOBt, diaminobutane; *b* TrHN-β-Ala-COOSucc, triethylamine; *c* BH_3; *d* *O*,*O*′-benzylcaffeyl chloride, triethylamine; *e*(i) TFA, Et_3SiH; *e*(ii) H_2/Pd-C [231]



Scheme 28 Synthesis of chiral polyamine **269** by solid phase coupling of amino acids and subsequent reduction. Reagents and conditions: *a*(i) 1 M BH_3, THF, 65 °C, 24 h; *a*(ii) I_2, 1:2:7 *i*-Pr_2EtN/AcOH/THF, rt, 4 h; *a*(iii) neutralization; *b*(i) Ac_2O, Et_3N, DMF; *b*(ii) 5% TFA in CH_2Cl_2 [30]

Hall and coworkers also demonstrated the versatility of amide reduction for the synthesis of philanthotoxins HO-416b (**274**) and PhTX-433 (**26**) [192] (Scheme 29). For HO-416b the first solid phase synthesis was obtained in 57%

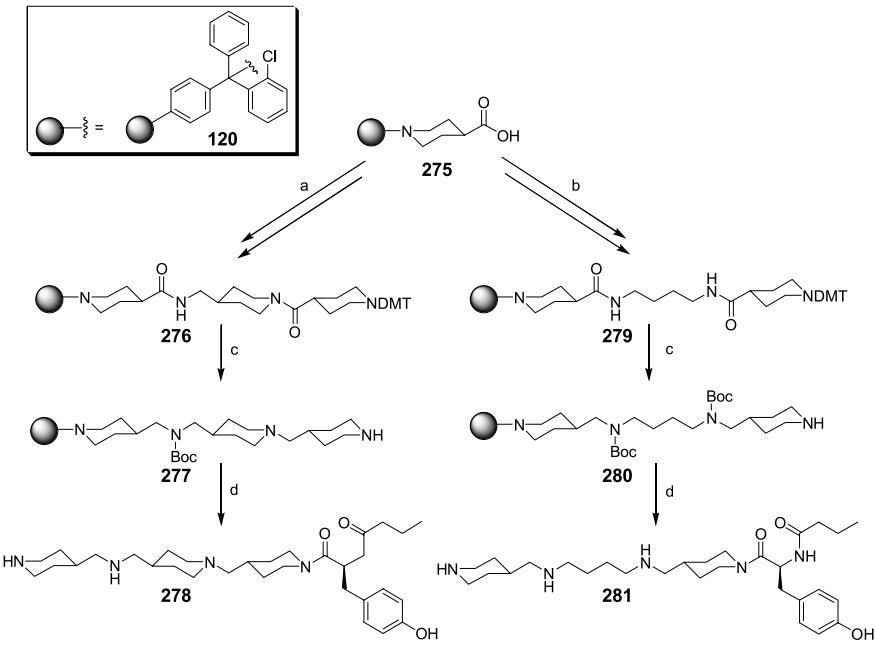


Scheme 29 Synthesis of HO-416b (**274**) by amide formation/reduction. Reagents and conditions: *a*(i) Fmoc-HN-$(CH_2)_2$ – COOH or FmocHN-$(CH_2)_3$-COOH, HBTU, HOBt, DiPEA, DMF; *a*(ii) 20% piperidine in DMF; *b*(i) 1 M BH_3/THF, 65 °C, 48 h; *b*(ii) I_2, THF/DiPEA/AcOH (7:1:2), rt, 4 h; *c*(i) Dde-OH, DMF, rt, 2 h; *c*(ii) Boc_2O, DiPEA, CH_2Cl_2, rt, 12 h; *c*(iii) 2% N_2H_4 in DMF; *d* *tert*-butyloxy-2-indoleacidanhydride, Et_3N, DMAP, CH_2Cl_2, rt; *e* TFA/H_2O/*i*-Pr_3SiH (95:2.5:2.5) [192]

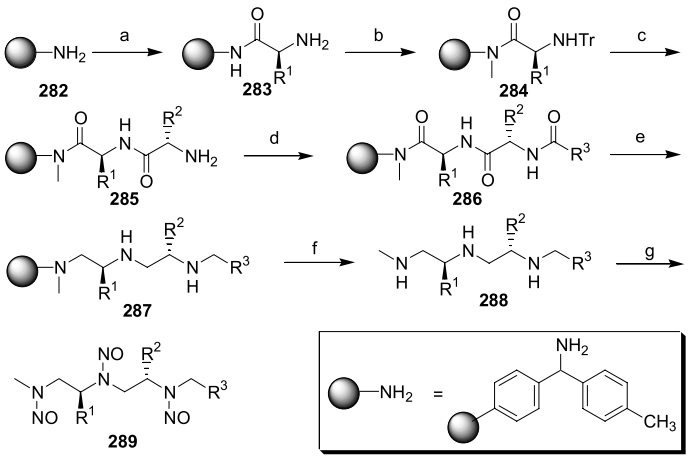
yield and 81% purity after cleavage from the resin. The synthesis of PhTX-433 gave 77% overall yield and 80% purity.

Franzyk et al. reported another variant of amide reduction in the synthesis of polyamine-based philanthotoxin analogs using piperidyl moieties in order to increase conformational rigidity, proteolytic properties, and lipophilicity of the polyamine [193] (Scheme 30). The generation of the backbone comprises amide formation of secondary and primary amines with different carboxyl group activating agents. Investigation of some of the commonly used activating agents showed that DIC/HOBt and PyBOP/DiPEA are preferred. An interesting feature of this approach is the use of DMT (**148**) as an amino protecting group on a chlorotrityl resin **120**. It has been shown that the deprotection from DMT already occurs with 0.1 M chloroacetic acid compared to chlorotrityl (**120**) or trityl (**114a**) linkers, which are not cleavable under those acidic conditions. The workup of the amine–borane complex is accomplished with piperidine at 60 °C.

There are numerous advantages of the amide-formation/BH_3-reduction strategy. Due to the well-investigated methods for amide formation known



Scheme 30 Synthesis of two conformationally rigid philanthotoxin analogs (**278**, **281**). Reactions: *a* peptide coupling: 2× DIC/HOBt; *b* peptide coupling: (i) PyPOB, (ii) DIC/HOBt; *c*(i) BH_3-reduction/workup; *c*(ii) Boc-protection; *c*(iii) DMT-deprotection; *d*(i) peptide coupling; *d*(ii) cleavage from resin **120** [193]



Scheme 31 Amide reduction in "libraries from libraries". Polyamines are intermediates in the conversion to nitrosamines **289**. Reagents and conditions: *a*(i) Boc-NH-CHR^1-COOH, peptide coupling; *a*(ii) 5% TFA in CH_2Cl_2; *b*(i) TrCl, DiPEA, CH_2Cl_2; *b*(ii) CH_3I, *t*BuOLi, DMSO; *c*(i) 2% TFA in CH_2Cl_2; *c*(ii) 5% DiPEA in CH_2Cl_2; *c*(iii) Fmoc-NH-CHR^2-COOH, peptide coupling; *c*(iv) 20% piperidine in DMF; *d* R_3COOH, DIC, HOBt, DMF; *e* BH_3·THF, 65 °C; *f* HF/anisole, 0 °C; *g* EtONO, CH_2Cl_2 [286]

from peptide chemistry and the high yields of the reducing step it opens access to polyamine synthesis with high overall yields. The selective synthesis of branched polyamines is possible and the large amount of commercially available acyl building blocks supports the generation of large compound libraries.

Recently, Hall et al. and Houghten et al. used this method for the generation of their "libraries from libraries" concept [29, 31, 186–189, 197, 272, 276–286]. Therein, the polyamines are only intermediates en route to other functional groups. The number of their published examples would go beyond the scope of this chapter so that only one example will be mentioned as a representative for the whole concept (Scheme 31). *N*-Alkylated tripeptides were synthesized on solid support, reduced, and after cleavage from the support converted to nitrosamines **289** [286].

4 Concluding Remarks

As described in this review, polyamines have been shown to possess many interesting biological features such as their interaction with DNA and nucleic acids and their potential in drug delivery and cancer treatment, while being non-immunogenic. However, naturally occurring polyamines are difficult to modify since they comprise several primary and secondary amines and are often symmetric molecules. During the last 10 years the research on solid phase synthesis of polyamines and their respective conjugates emerged rapidly and opened up the possibility of synthesizing asymmetrically substituted polyamine compounds, which can also be easily purified. Several combinatorial approaches have been reported that allow synthesis of an enormous number of natural polyamine analogs and eventually screening for therapeutic applications. These techniques will certainly be very helpful in the near future to exploit the polyamines for the treatment of many severe diseases and for the understanding of biological mechanisms that use polyamines at high concentrations.

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Top Curr Chem (2007) 278: 209–241
DOI 10.1007/128_2006_107

Published online: 2 February 2007

Combinatorial Solid-Phase Natural Product Chemistry

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Abstract One major challenge in combinatorial chemistry is to find biologically relevant starting points in the chemical universe around which compound libraries should be produced. Natural products represent such biologically validated starting points. Progress in Solid-Phase Organic Synthesis (SPOS) has enabled now the combination of natural product synthesis with combinatorial methods. Two strategies can be distinguished: (1) Attachment of a natural product core structure onto solid phase and subsequent modification of functional groups, or (2) total synthesis of the complete natural product scaffold on solid phase. A complementary approach identifies privileged structures among natural product scaffolds, which serve as inspiration for library synthesis. In this review the current status and the challenges of combinatorial natural product synthesis on solid phase are presented.

Keywords Chemical biology · Combinatorial chemistry · Natural products · Organic synthesis · Solid phase

Abbreviations

Ac	acetyl
AcOH	acetic acid

Bn	benzyl
BOP	(1,2,3-benzotriazol-1-yloxy)-tris(dimethylamino)phosphoniumhexaflurophosphate
BuLi	butyllithium
Bz	benzoyl
CSA	camphersulfonic acid
dba	1,2-dibenzylidenacetone
DDQ	2,3-dichloro-5,6-dicyano-quinone
DCE	1,2-dichloroethane
DCM	dichloromethane
DEAD	diethyldiazodicarboxylate
DIAD	diisopropyldiazodicarboxylate
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
dppf	1,1′-bis(diphenylphosphino)ferrocene
Eq	equivalent
Et	ethyl
Fmoc	fluorenylmethoxycarbonyl
HATU	7-aza-3-[(dimethyliminium)(dimethylamino)methyl]-1,2,3-benzotriazol-1-ium-1-olate hexafluorophosphate
HBTU	3-[(dimethyliminium)(dimethylamino)methyl]-1,2,3-benzotriazol-1-ium-1-olate hexafluorophosphate
HOAt	3-hydroxy-3*H*-[1,2,3]triazolo[4,5-*b*]pyridine
HOBt	1-hydroxybenzotriazol
iPr	isopropyl
LDA	lithiumdiisopropylamide
mCPBA	meta-chloroperbenzoic acids
Me	methyl
MEM	methoxymethyl
OTf	trifluormethanesulfonate
PPTS	pyridinium p-toluenesulfonate
PTSA	p-toluenesulfonic acid
rt	room temperature
SAR	structure activity relationship
TBAF	tetrabutylammonium fluoride
TBS	tetrabutyldimethysilyl
tBu	tert.-butyl
THF	tetrahydrofurane
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
Thr	threonin
TIPS	triisopropylsilyl
TMOF	trimethylorthoformiate
TMS	trimethylsilyl
Trt	trityl

1
Introduction

The continued quest for new medicines to cure discomfort and diseases coupled with man's intrinsic curiosity to reveal nature's hidden secrets provide a powerful motivation for research in the life sciences. Although the last decades have produced a tremendous gain in understanding the cell at its molecular level, we still are overwhelmed by the intrinsic complexity of this system and struggle with revealing its inner secrets. At the moment most scientists agree on the notion that cellular mechanisms have to be studied by using several complementary research techniques at the same time taking advantage of their complementary strengths and cancelling out their individual weaknesses. Recently, the use of small molecule probes that selectively modulate the function of a specific protein have been rediscovered as a powerful tool in cell biology [1–3]. This strategy of "Chemical Genetics" complements genetic approaches for the study of cellular processes, allowing the identification of potential biological targets for the treatment of our burning medical needs curing numerous diseases, including cancer, neurodegenerative diseases, malaria, AIDS, or hepatitis. In order to cure these diseases new drugs, preferentially in the form of small organic molecules, are needed which inhibit or promote the target's biological function and, in the ideal case, avoid side effects with the required level of selectivity. For both goals, the study of protein function by small molecule modulators using "Chemical Genetics" in basic research, as well as in the search for new drugs addressing specific prevalidated biological targets, small molecules which selectively bind to proteins are needed. Although Organic Chemistry has in its 180-year-old history achieved a level of maturity, in which it prides itself to be able to synthesize any stable organic molecule [4], there is of course a continuing need for improvements in efficiency and speed. Organic chemists have invented two tools—solid-phase synthesis and combinatorial chemistry—which when combined might in principle be able to address these challenges.

1.1
Combinatorial Libraries and Hit Rates

Solid-phase synthesis techniques applied either in a parallel fashion in automated synthesis or in split-pool-synthesis enable the rapid production of compound libraries containing thousands or even millions of members. The original expectation that the synthesis of million compound libraries will produce as many or even more drug candidates as historical libraries of pharmaceutical companies and that it will thereby overcome the problem of efficient hit and lead finding was not fulfilled [5]. The design of these libraries was dominated by the principle of synthetic accessibility and the availability

of building blocks. As a result libraries were created which were largely composed of oligopeptides assembled via amide bond formation between amino acid building blocks. But many of these libraries contained hardly any hit. Obviously the underlying structure of the individual library members was biologically not relevant.

It was soon recognized that not the number game will determine the quality of a library, but its "diversity" [6–8] and its "drug-likeness" [9, 10]—qualitative parameters which are considered in current library design [11, 12]. As a consequence the hit rate of modern combinatorial libraries has strongly improved, and at the moment the following paradigms are followed in library design:

Screening libraries are compound collections which are used in the initial High-Throughput Screening campaign against a new drug target. In most cases, the complete historical compound collection of a pharmaceutical company is screened to identify hit compounds which are active against this target. An ideal screening library represents a huge portion of the chemical universe [13], and therefore should be as structurally and functional diverse as possible [14–16]. Nevertheless, already at this stage the important question of bioavailability for development of a hit to a drug demands that screening compounds should obey Lipinski's and Veber's rules of bioavailability and should not contain functional groups which lead to promiscuous binding, such as strong electrophiles like epoxides or α,β-unsaturated carbonyl groups, or strong nucleophiles [17]. For this endeavor the objective of combinatorial chemistry is to provide pure and structurally defined compounds which can be added to these depositories to increase the overall size and diversity of the screening collection.

Once a hit has been found, medicinal chemists prepare analogs around this structure to investigate the SAR of this compound. Support through combinatorial chemistry at this stage requires a focused library of medium size, which explores a narrow part of the chemical universe around the hit compound.

A few years ago accounts were published which celebrated compounds originating from combinatorial chemistry entering clinical trials [18]. Such an assignment is not possible anymore as combinatorial chemistry has become an integral part of drug discovery and the origin of individual compounds is no longer discussed. Anecdotal evidence, such as the fascinating story of the discovery of BAY 59-7939, an oral factor Xa inhibitor, gives testimony of the impact combinatorial chemistry has made in drug discovery [19].

As we return to the important goal of increasing the quality of screening libraries, two challenges have to be addressed: The first is related to the incredible size of the chemical universe, which some authors have suggested to contain up to 10^{62} thinkable compounds with MW< 500 and being built up by only nine chemical elements [13]. But realistically, only a tiny part of this universe will be translated into synthesized compounds and here one should

ask: Where in the almost indefinite space of thinkable chemical compounds are the structures which are of biological relevance? The second challenge is related to the problem of the druggable proteome [10, 20]. Big pharmaceutical companies have recognized that for some biological targets a HTS campaign against their compound collection with 10^6 compounds did not deliver any hit. This happens quite often with new, potentially very interesting biological targets, but which are obviously difficult to address with the available screening collection of small molecules. It seems that for addressing protein-protein interaction sites with small molecules or GPCRs which have peptides as their natural ligand, new types of organic molecules have to be designed and synthesized.

In developing new concepts and logics for meeting this demand the central and crucial tasks are to identify compound classes that represent already biologically validated starting points in structural space, to find a synthetic access to them that is amenable to combinatorial variation and to design and synthesize combinatorial libraries centered on the identified underlying structural framework of these compound classes.

1.2 Natural Products

Natural products have been identified as the active principle of herbs and extracts used in folk medicine [21]. The importance of natural products in the pharmaceutical industry has continued to the present day and is reflected by the fact that close to half of the best selling pharmaceuticals are either natural products (e.g. cyclosporine, Taxol, FK 506) or derivatives (e.g. statins, many antibiotics) thereof [22–25]. In high-throughput screening processes performed by the pharmaceutical industry natural product extracts exhibit a hit rate which is estimated to be substantially higher than the hit rate of random libraries from combinatorial chemistry [5]. Natural products such as epothilones, lactacystin, or ecteinascidin are promising clinical candidates for future cancer treatment. Chemoinformatic studies of compound collections in pharmaceutical companies showed that natural products differ from synthetic compounds in size, molecular weight, number of oxygen and nitrogen atoms, number of stereocenters, and several other descriptors [26, 27]. Recently, several excellent review articles have highlighted the importance of natural products as tool compounds in Chemical Biology and drug leads [28–32].

Despite this proven record of biological significance there had been some doubts if natural products are suitable and accessible lead structures for combinatorial libraries by solid-phase synthesis. In contrast to the diversity-oriented approach of library design, which is driven by the underlying chemistry of reliable reactions with broad substrate scope [14], natural product library synthesis involves multi-step sequences requiring careful opti-

mization, which ultimately leads to the chosen target structure (focused library).

This extra-mile in synthetic effort should be rewarded by a higher hit rate in biological screens and what would be even more significant: biologically validated hits. The unique role of natural products can be understood in the light of recent results from protein biochemistry and bioinformatics. Because of the evolutionary relationship between proteins of different function within an organism and the phylogenetic relationship between different species, it becomes less surprising that a small molecule, like discodermolide, synthesized by a marine sponge to defend itself against being eaten by a fish, at the same time represents a promising lead compound for the cure of breast cancers in mammals. As a small molecule natural product has been biosynthesized by enzymes and exerts its biological function by addressing a specific target protein, most natural products have interactions with at least two different proteins. Only a limited number of protein folds exists and there is considerable similarity between the binding pockets of protein ligands. Consequently, the probability that natural products bind to other proteins is significantly higher than for other random chemical structures. On the basis of these assumptions one can reason why one should not only synthesize the natural product itself but a library thereof: the fine-tuning of substituents and variation of the molecular framework will allow for the requirements of different binding pockets and the desired selectivity to distinguish between similar protein domains. Additionally, it should be taken into account that natural products which are descended from marine or tropical sources are not optimized by nature for human cells, i.e. the fine work for getting better selectivity and less side reactions is due to pharmacological and combinatorial chemistry [5].

1.3 Synthetic Challenges

Concluding from the arguments outlined above, it would be desirable to combine the demonstrated synthetic efficiency of solid-phase organic synthesis using combinatorial methods with the proven biological relevance of natural product synthesis. Paramount to the success of this approach is that efficient and reliable methods and multi-step sequences for the total synthesis of natural products and analogs thereof on polymeric supports are available. The corresponding transformations must proceed with a degree of selectivity and robustness typical of related classical solution phase transformations, irrespective of the stringencies and differing demands imposed by the anchoring to the polymeric support.

At present the current state of SPOS has not yet reached the level requested above [33]. We see its current status by classifying the tool box of organic reactions implemented in SPOS by distinguishing three types of maturity:

Type 1 reactions are reactions which work for a broad range of substrates on solid phase, for which robust reaction protocols exist. These reactions have typically a long history in solid phase synthesis and through optimization very reliable reaction protocols have been established (e.g. oligopeptide synthesis, oligonucleotide synthesis). Another characteristic element of these reactions is that the reaction products represent thermodynamic sinks, which form easily and are less prone to further reactions leading to side products. The most useful C – C-bond forming reaction on solid phase is Pd-catalyzed cross-coupling reactions.

Type 1 reactions:

1. amide bond formation;
2. nucleotide oligomerization;
3. esterification/saponification;
4. protection/deprotection;
5. reductive amination;
6. Pd-catalyzed cross-coupling;
7. Knoevenagel condensation;
8. heterocycle condensations;
9. reactions with isocyanates/thioisocyanates;
10. oxidation of alcohols;
11. reductions of amides;
12. ozonolysis.

Type 2 reactions are reactions which have already been implemented on solid phase but with unexplored scope. As a rule, the reaction protocols are limited to the few reported substrates in the few reported applications. Interestingly, the most important C – C-bond forming reactions in solution organic synthesis show limitations on solid phase: Stereoselective aldol reactions have been reported so far only by a few groups [34–37]. Cycloadditions have been described more often, but still limitations are imposed by the resin, which makes both low temperature as well as high reaction temperature conditions problematic. In general, the use of stereoselective and especially enantioselective methods has largely been neglected so far [38].

Type 2 reactions:

1. cycloaddition;
2. aldol reaction;
3. allylation;
4. reaction of solid-phase bound electrophiles with organometallic reagents;
5. many functional group transformations;
6. stereoselective (especially enantioselective) reactions;
7. photochemical reactions.

Type 3 reactions are reactions which have not been applied on solid phase yet or if they have been, then only in proof-of-concept experiments. For a more general application several intrinsic problems have to be solved. Catalytic hy-

drogenations or electro-organic reactions use heterogeneous reagents which cannot interact with the substrates bound within the gel matrix of a polymer bead, a limitation which can only be overcome using either soluble mediators or soluble polymeric supports [39, 40]. Other reactions suffer from the fact that they require very well-defined reaction conditions, but diligent control of reaction stoichiometry, reaction time, exclusion of water, and low temperature conditions are difficult to achieve due to the intrinsic heterogeneous reaction environment of an insoluble support.

Type 3 reactions:

1. catalytic hydrogenation;
2. electro-organic synthesis;
3. organometallic reagents on solid phase (Grignard, Li-organyls, ...);
4. reactions involving carbocations (skeletal rearrangements, etc.);
5. reactions involving radicals, radical cations, radical anions, and other high energy intermediates.

1.4 Scope of the Review

Only recently has the progress in solid-phase synthesis begun to meet the demands of the intrinsic complexity of natural product library synthesis on a solid support. The aim of this work is to describe the different approaches followed in this science and to highlight these with notable examples demonstrating the current state-of-the-art.

This review is complemented by a series of excellent reviews of natural products in combinatorial chemistry [41–60]. Because of space limitations we will not be able to discuss the synthesis of oligomeric natural products, such as oligopeptides or polysaccharides despite their paramount biological importance. Recently, an excellent review article has been published covering the latter subject [61]. An amazing source of information are the annual comprehensive surveys of combinatorial library synthesis, which have been published by Dolle since 1998, covering essentially all efforts in small molecule library synthesis starting from the beginnings of this field in 1992 [62–71]. The journal "QSAR Comb Sci" presents in each issue literature highlights in library synthesis on solid phase.

2 Solid-Phase Organic Synthesis of Natural Products Libraries

Two strategies have emerged for the synthesis of natural-product libraries on solid phase. In the first approach, a natural product skeleton is immobilized onto a solid support which is then subjected to diversification by decorating the scaffold. While the core structure remains unchanged, building blocks are

attached to already existing functional groups. This approach requires a precursor molecule which should be easily accessible either from natural sources, degradation chemistry, or solution phase total synthesis. Furthermore, an appropriate site on the scaffold for attachment onto a solid support must be identified to facilitate reliable loading and release, as well as installation of the highest possible structural and functional diversity. The second approach involves building the entire core structure on a solid support, a very challenging proposition, since it often requires a multi-step synthesis applying a large range of organic reactions and often leads to the demand for the development of new methods. However, it allows maximum diversity in the core structure (e.g. ring size, chain length), by both variation of stereochemistry as well as the introduction and derivatization of functional groups. While the first strategy provides the advantage that nearly each step on the solid support leads to diversification, the second one often requires considerable effort just to build up the scaffold, but in principle should lead to a skeletally more diverse library [16].

2.1 Modification of Core Structures

2.1.1 Overview

Historically, building up a library around a given core structure has been the first strategy to access natural product libraries. This approach is still the preferred way to prepare either focused libraries to study the SAR of certain natural products or to build libraries of very complex natural product scaffolds which cannot be constructed on solid phase yet. In Fig. 1 two notable structures of natural product libraries built by core structure modification are highlighted, such as an Indolactam library by Waldmann [72], and a Taxoid library by Xiao [73].

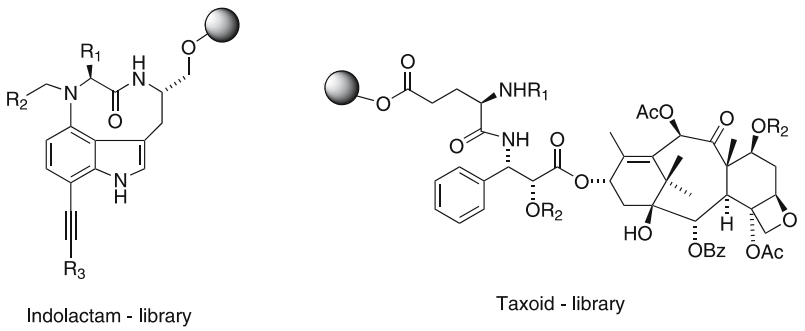
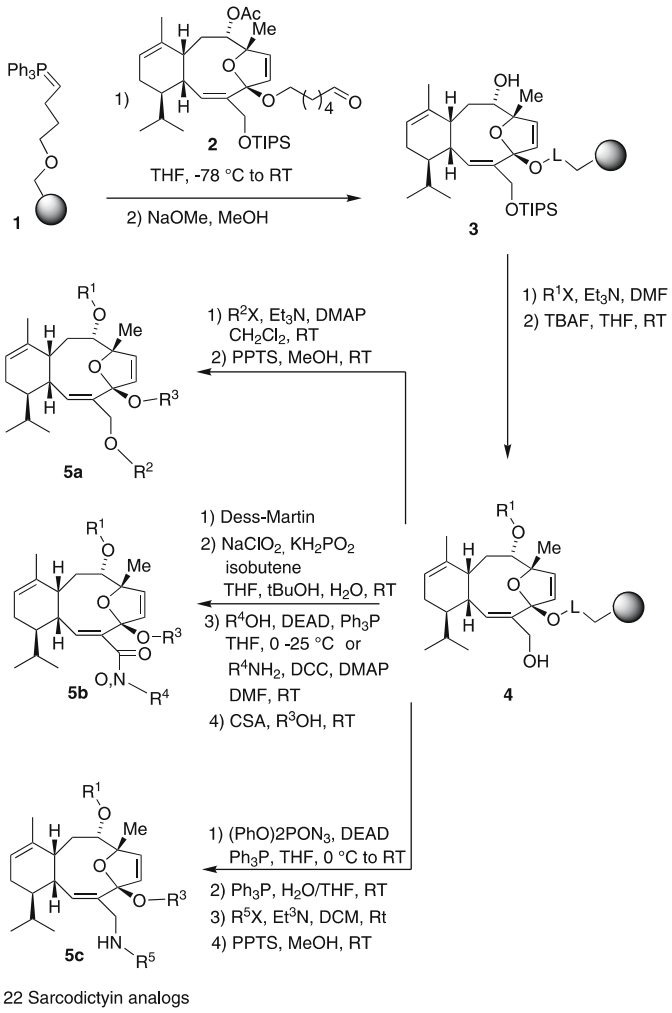


Fig. 1 Examples of natural product libraries by modification of core structures

2.1.2
Representative Examples

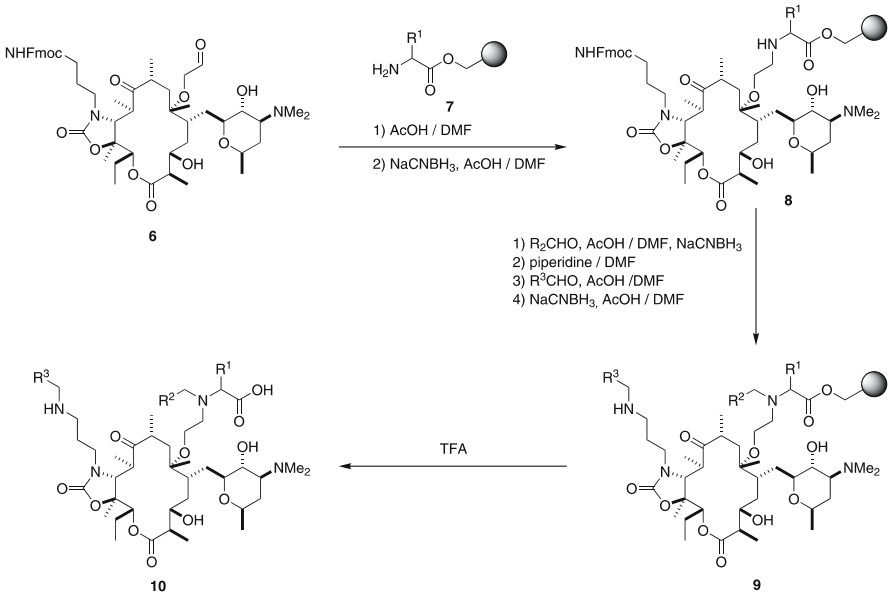
Nicolaou et al. have prepared a library of 22 Sarcodictyin-analogs by attaching core structure **2** onto solid phase using polymer-bound Wittig-ylide **1** (Scheme 1) [74]. The OH-groups of the core scaffold were decorated by alkylation or converted to carboxylic acid or amino functionalities which could be derivatized as esters, carbamates, or secondary amines. Cleavage off the solid phase was performed via trans-ketalization, a reaction step which was exploited to increase the diversity by using different kind of alcohols.



Scheme 1 Synthesis of a Sarcodictyin library

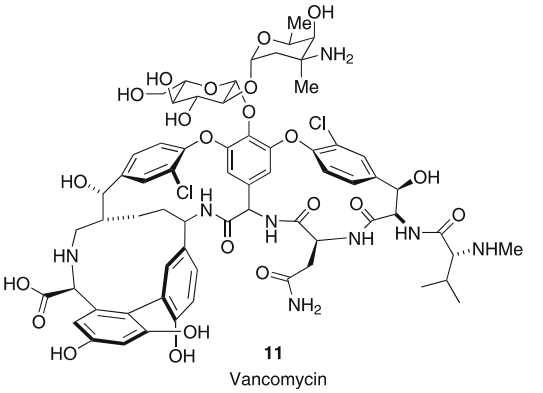
Akritopoulou-Zanze et al. have described the combinatorial synthesis of macrolide analogs based on Erythromycin A, which is an important antibiotic used for the treatment against Gram-positive bacteria. One disadvantage of Erythromycin A is that it is partially deactivated in the stomach due to the strongly acidic conditions at this site. As a result, efforts have been made to improve the acid stability and hence increase the bioavailability by means of combinatorial chemistry. The synthesis by Akritopoulou-Zanze et al. is the first synthesis of a macrolide library on a solid support (Scheme 2) [75]. The precursor molecule **6** was made in solution employing a six-step synthetic sequence starting from 6-*O*-Allyl-Erythromycin. Macrolide **6** was attached to a solid support via reductive amination to a pre-bound amino acid (R^1) (**7**). The resulting amine was then reacted with a second aldehyde (R^2) to afford an intermediate tertiary amine. Deprotection of the primary amine in the side chain of the oxazolidinone moiety and reductive amination with another aldehyde (R^3) afforded compound **9**. Acidic cleavage off the solid support delivered the macrolide analogues **10** in high purity and reasonable to good yields. This synthesis is distinguished by applying the very reliable reductive amination protocol three times for introducing essentially all diversity elements. Thus, high yields and high purity are achieved through the use of this highly optimized reaction (Type 1, according to the classification in Sect. 1.3). Another advantage of this reaction is the great variety of accessible building blocks.

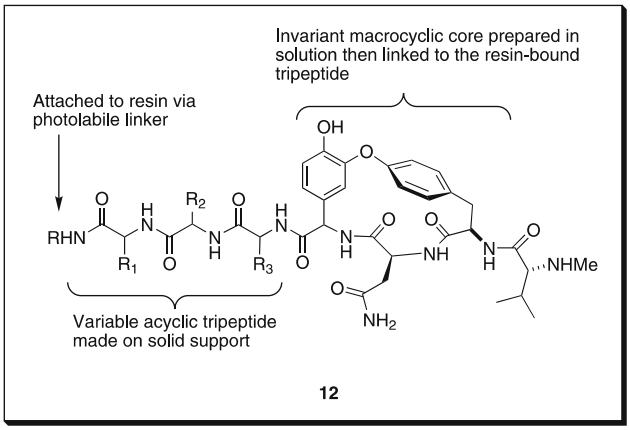
The glycopeptide Vancomycin exhibits a structural complexity which is still beyond reach by current SPOS, but has been successfully used in semi-

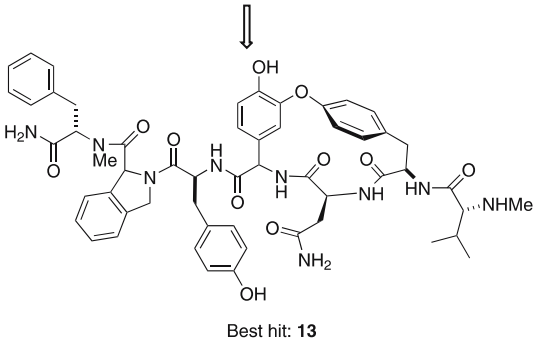


Scheme 2 Synthesis of an Erythromycin library

synthetic library synthesis [76, 77]. Vancomycin (**11**) is the drug of last resort for treating multidrug-resistant Gram-positive bacterial infections; consequently the emergence of vancomycin resistance presents a serious threat to







Scheme 3 Identification of a Vancomycin-type receptor overcoming antibiotic resistance

public health. Vancomycin inhibits the maturation of the peptidoglycan layer surrounding bacterial cells by binding to the terminal D-Ala-D-Ala-L-Lys fragment found in peptidoglycan precursors. Resistance to vancomycin arises when microorganisms acquire genes that lead to the substitution of D-Ala-D-Ala-L-Lys by D-Lac-D-Ala-L-Lys, which decreases vancomycin's binding affinity 1000-fold, and, as a consequence, renders the antibiotic ineffective against such mutants. A combinatorial approach by Ellman et al. to synthetic receptor molecules (**12**) targeting vancomycin-resistant bacteria was therefore designed to bind to the mutant sequence D-Lac-D-Ala-L-Lys (Scheme 3) [76]. The right-hand binding pocket of vancomycin was preserved to retain key hydrogen bonding sites and hydrophobic interactions, while the left-hand side was replaced with a variable tripeptide unit. The tripeptide was left acyclic to allow free rotation which eliminates the unfavorable electrostatic interaction observed between the phenylglycine carbonyl of vancomycin and the oxygen of the lactate group. The vancomycin E-ring chlorine and sugar residues were not incorporated into **12** in order to expedite receptor library synthesis and because each of these groups contribute less than 2- to 3-fold to the binding of vancomycin to *N*-Ac_2-L-Lys-D-Ala-D-Ala. A library of 39304 members (**12**) from 34 amino acid building blocks was prepared on a solid support by split synthesis, which was followed by attachment of the macrocyclic template. The amino acids were selected on the basis of the side chain display found in the proteinogenic amino acids and on the degree of rigidity which they can give to the tripeptide. The resin bound library was then screened against both *N*-Ac_2-L-Lys-D-Ala-D-Lac and *N*-Ac_2-L-Lys-D-Ala-D-Ala labelled with the fluorophore nitrobenzodioxazole. To determine the structures of the most active receptors, the stained beads were decoded using a matrix-assisted laser desorption ionization mass spectrometry sequencing method. Consensus sequences arising from the screening led to **13** as the most active inhibitor. By microcalorimetry **13** exhibited slightly lower binding affinity to *N*-Ac_2-L-Lys-D-Ala-D-Ala than vancomycin, but, more significantly, showed five times greater binding affinity to *N*-Ac_2-L-Lys-D-Ala-D-Lac. The significance of these results is that receptors less structurally complex than vancomycin can exhibit comparable and even enhanced binding affinity toward the same target. It also represents the first successful example on the identification of synthetic receptors that bind to a small molecule target in aqueous solution.

2.2
Synthesis of Natural Products on Solid Phase

2.2.1
Overview

The considerable progress in method development in SPOS has allowed synthesizing more and more complex molecular skeletons on solid phase, mak-

ing it possible to synthesize the complete structural framework of natural products on solid phase. This usually requires a multi-step synthesis applying a large range of organic reactions and often leads to the demand for the development of new methods. However, it allows maximum diversity in the core structure (e.g. ring size, chain length), by both variation of stereochemistry as well as the introduction and derivatization of functional groups. In Fig. 2 a selection of notable examples of natural product syntheses is given, such as the Muscone [78] and Zearalenone [79] syntheses by Nicolaou, the Tentoxin synthesis by Albericio [80], the synthesis of a FR225659 derivative

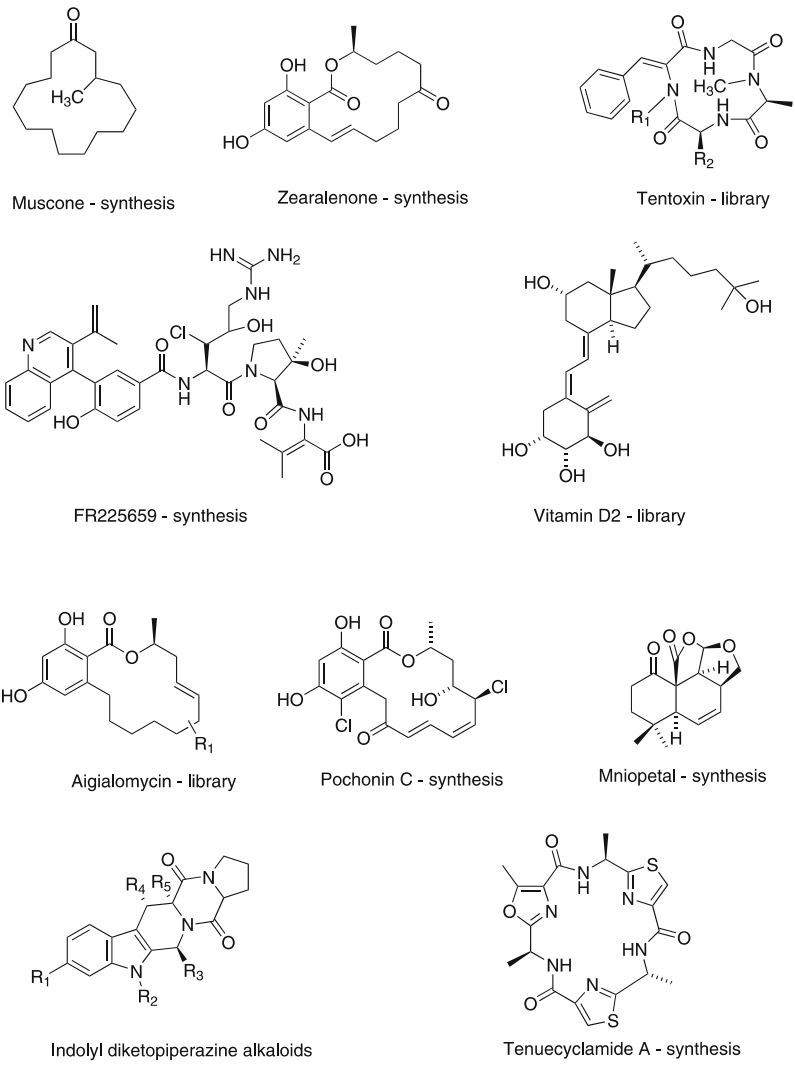
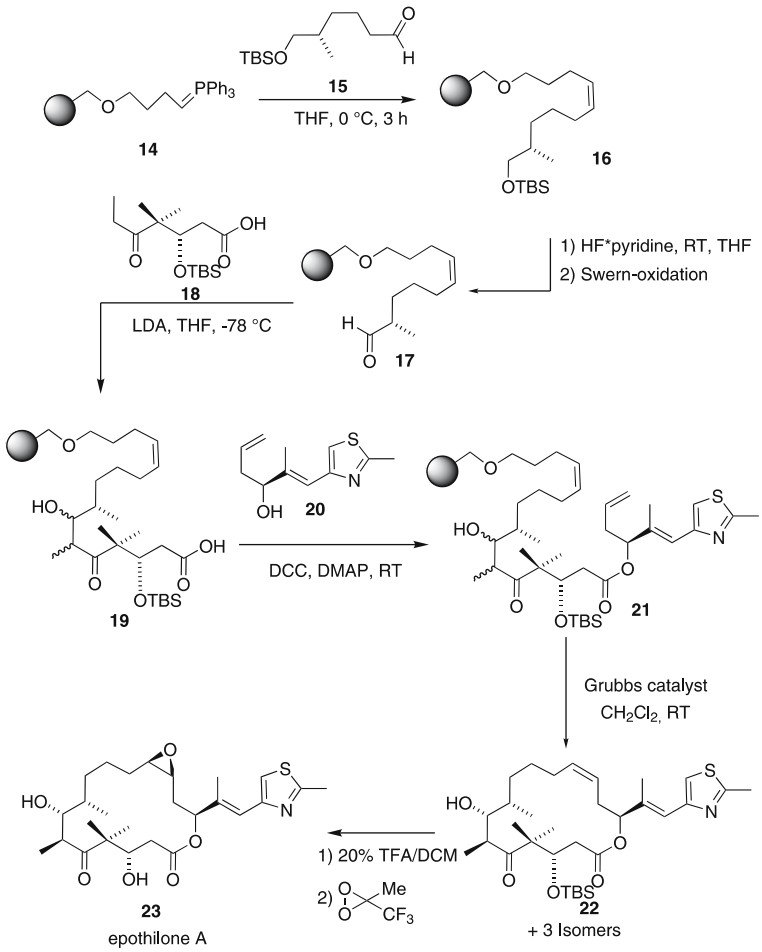


Fig. 2 Examples of total syntheses of natural products on solid phase

for receptor screening by Handa and Takahashi [81], the synthesis of Vitamin D by Takahashi [82, 83], a synthesis of Pochonin C [84] and a library of Aigialomycin-analogs [85] by Winssinger, the synthesis of Mniopetal scaffold by Jauch [86], the synthesis of indolyldiketopiperazine natural products and analogues by Ganesan [87, 88] and Koomen [89], and the synthesis of Tenuecyclamides by Kelly [90].

2.2.2 Representative Examples

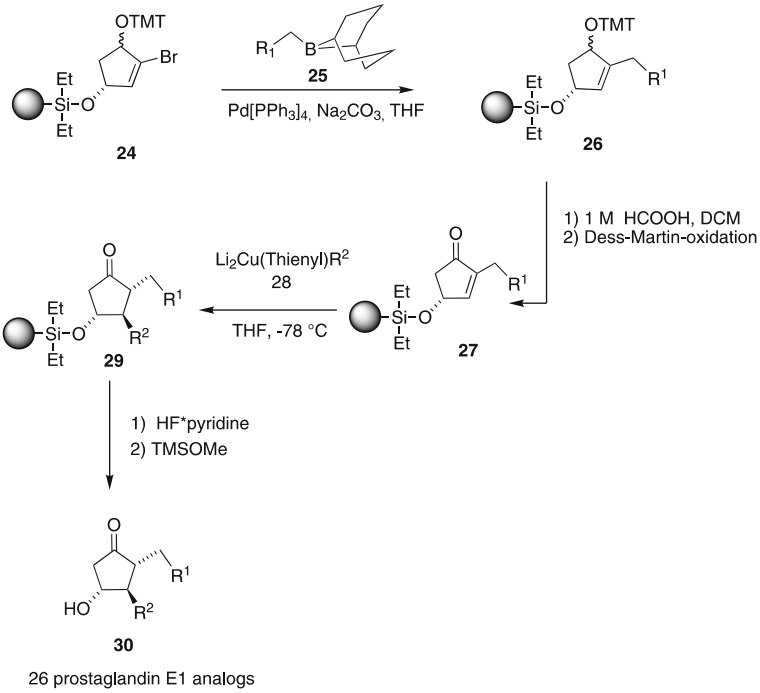
Epothilones are natural products isolated from myxobacteria, which have been found to exhibit cytotoxic activity against Paclitaxel-resistent tumor



Scheme 4 Total synthesis of Epothilon A on solid phase by Nicolaou et al.

cell lines, by inducing tubulin polymerization. The successful synthesis of epothilone A (**23**) by the Nicolaou group, represents a landmark in the solid-phase synthesis of complex molecules on a solid support [91]. A polymer-bound Wittig ylide **14** was further elaborated by reaction with building blocks **15**,**18**,**20** via an olefination reaction, an aldol reaction, and an esterification (Scheme 4). In the next step the macrocycle **22** was formed from the acyclic precursor through a ring-closing olefin metathesis reaction mediated by Grubbs' catalyst liberating the substrate from the solid support. This cyclorelease strategy pioneered by Rapoport offers the additional advantage that only molecules which undergo the desired transformation will be found in the cleavage solution [92–94]. After deprotection and epoxidation of the less substituted double bond in solution epothilone A (**23**) was isolated. It deserves special attention because this example demonstrated for the first time the principal feasibility of multi-step natural product synthesis on a solid support. In a subsequent paper Nicolaou et al. prepared a library of further analogs, which helped to establish structure-activity relationships within this compound class [95].

Prostaglandines play a prominent role in a wide variety of physiological processes and exhibit a very subtle structure-activity relationship, which

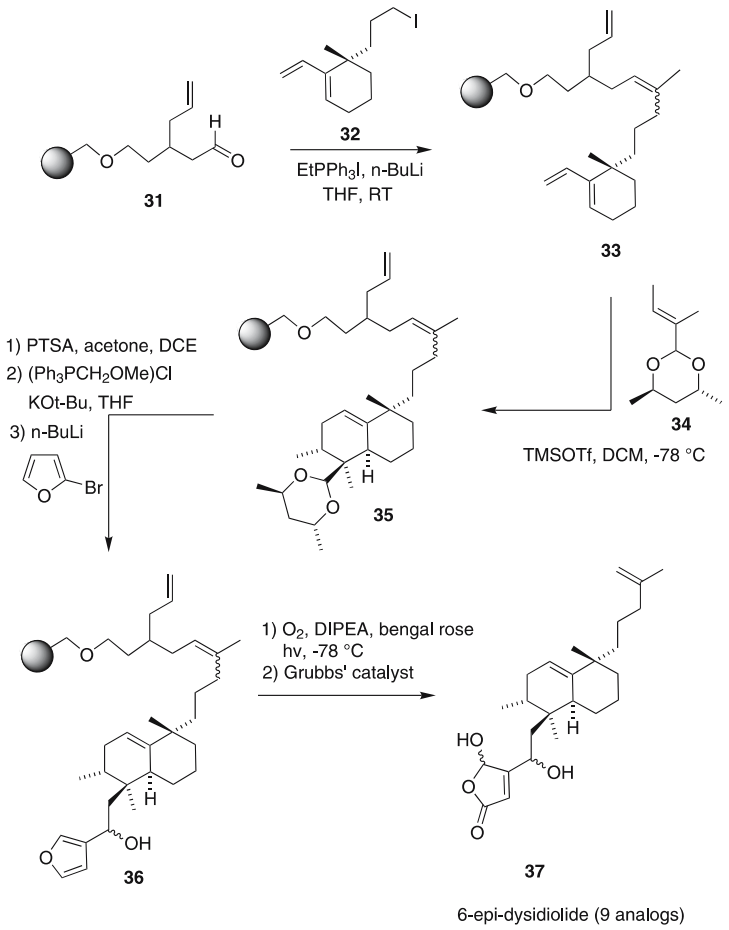


Scheme 5 Synthesis of a prostaglandine library by Ellman et al.

make them a target for combinatorial chemistry of highest interest and significance. The group of J.A. Ellman has disclosed the solid phase synthesis of a 26-member library of prostaglandin E_1 analogues (**30**). After modification of the core structure **24** via Suzuki-coupling with building block **25**, the relative stereochemistry of the two carbons bearing the side chains was set by a diastereoselective Michael addition of a higher order cuprate **28** across the enone **27** (Scheme 5) [96, 97].

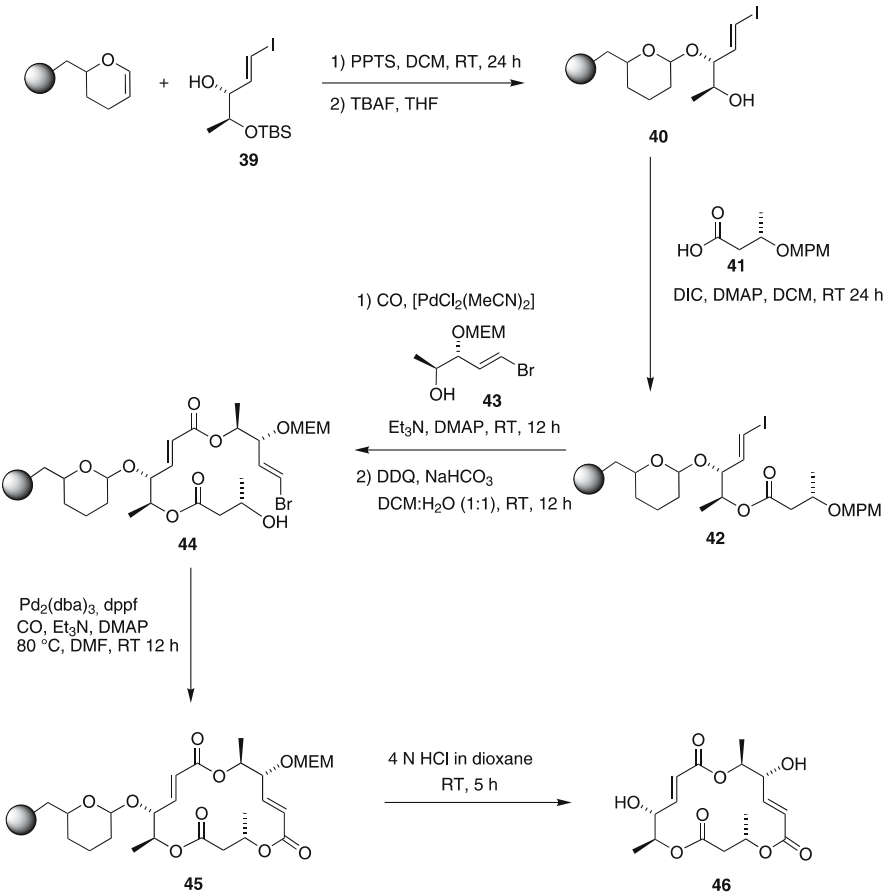
Notable features of this synthesis are the Suzuki-reaction between sp^3-sp^2-carbons and the use of a cuprate reagent on solid phase. Janda et al. have published a prostaglandin synthesis using a soluble polymeric support [98].

Waldmann et al. have synthesized a library of analogs of the anti-tumor active phosphatase inhibitor 6-*epi*-dysidiolide (**37**) [99, 100]. A notable feature



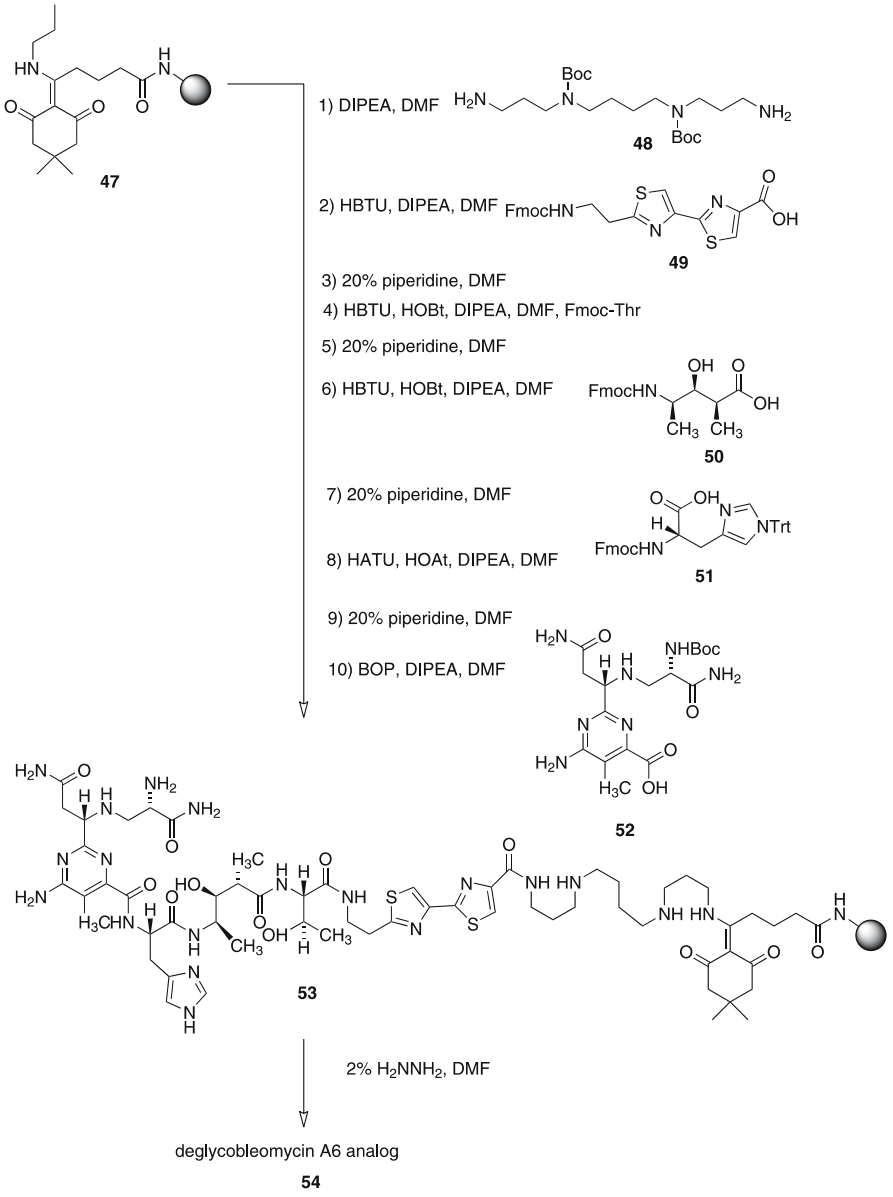
Scheme 6 Synthesis of a library of analogs of 6-epi-Dysidiolide by Waldmann et al.

of this 11-step reaction sequence on solid phase is that a wide range of transformations with vastly differing requirements could successfully be developed. Key transformations of the synthesis include an asymmetric Diels–Alder reaction with chiral dienophile **34**, and an oxidative elaboration of furan **36** with singlet oxygen on solid phase, as well as the traceless cleavage of the products via olefin-metathesis from the support (Scheme 6). The sequence rapidly yielded access to eight analogs of the natural product and led to the identification of a potent inhibitor of the cell-cycle-controlling phosphatase cdc25c which displays a very promising selectivity pattern. Interestingly, the different stereochemical position of the residues at the 6-position does not obscure the overall biological activity in comparison to the natural product, indicating that in many cases not an exact match of the underlying natural product lead has to be synthesized. In addition, this synthesis is a rare example of a solid phase synthesis of a terpene natural product on solid phase.



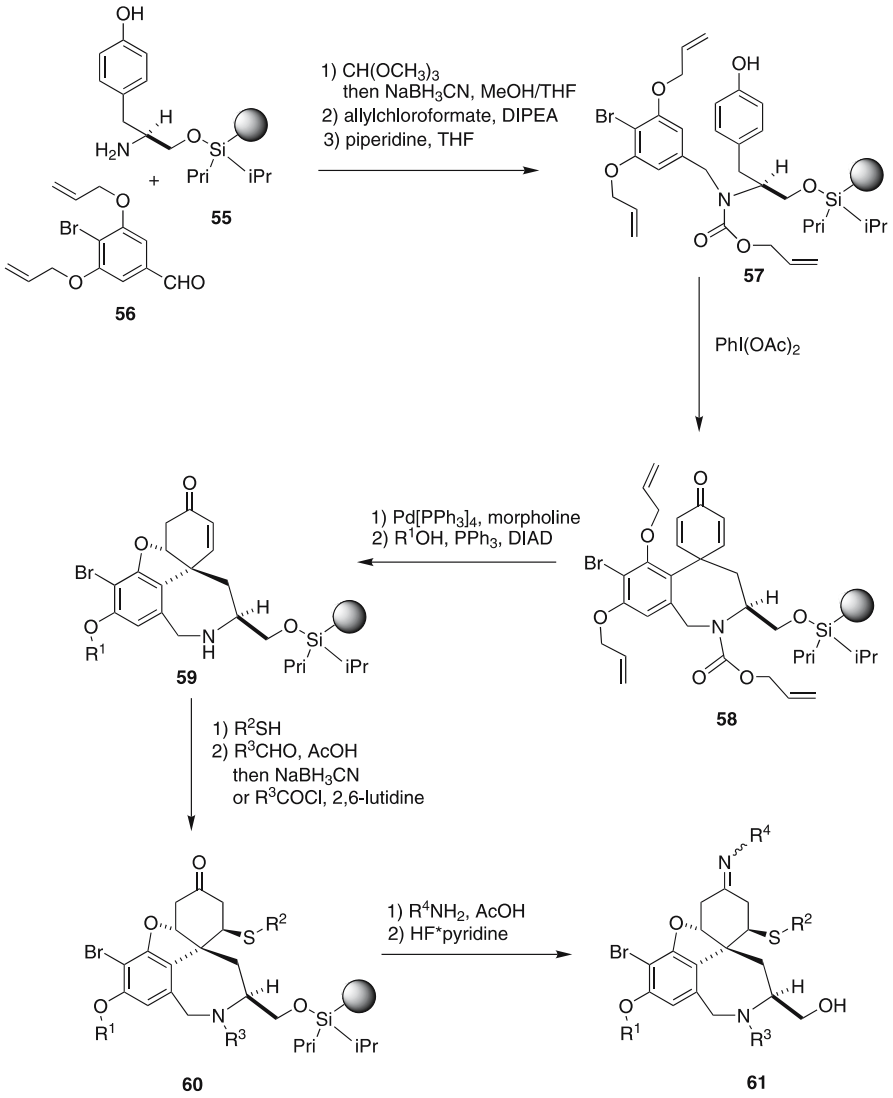
Scheme 7 Synthesis of Macrosphelide A on solid phase using Pd-catalyzed carbonylations

Takahashi et al. have reported on a combinatorial synthesis of a library of 122 macrosphelides, which uses a three-component coupling in which not only the building blocks are connected by a Pd-catalyzed carbonylation, but also this reaction is used for macrocyclic ring closure on a solid support (Scheme 7) [101].



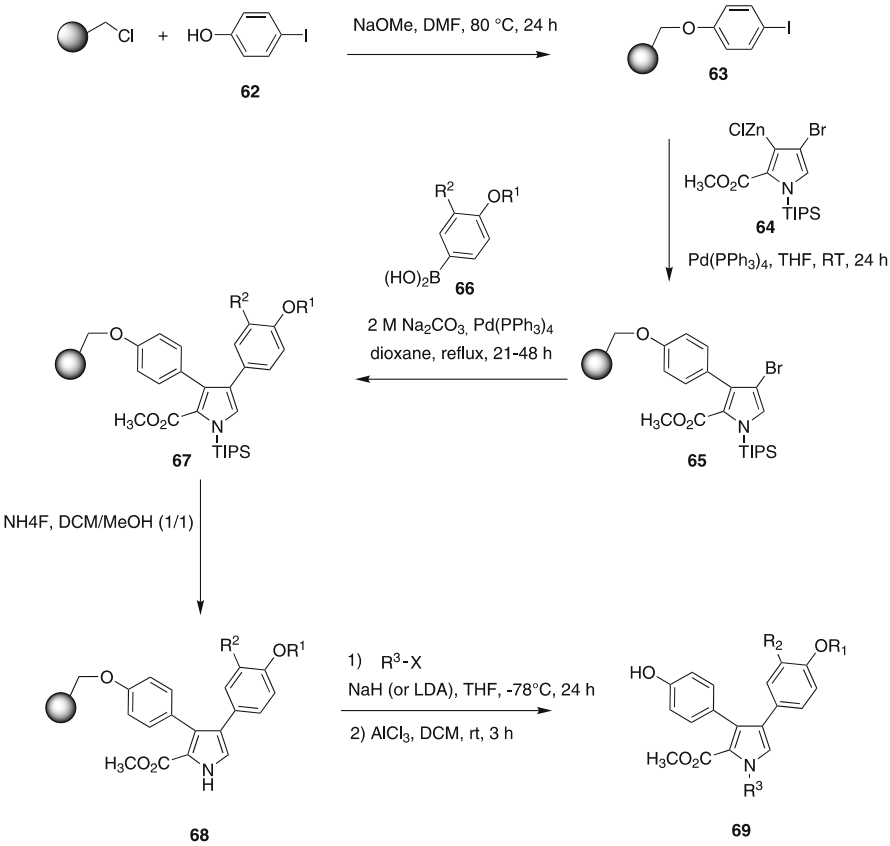
Scheme 8 Synthesis of Deglycobleomycin library by Hecht et al.

Bleomycin and deglycobleomycin are antibiotics which mediate oxidative damage to DNA and RNA. Hecht et al. have prepared 108 analogs of deglycobleomycin using a modular approach in which building blocks, such as **49–52**, are connected via amide bond formation, furnishing deglycobleomycin analog **54** (Scheme 8) [102]. Two members of the library exhibited higher activity in supercoiled plasmid DNA relaxation than the parent deglycobleomycin molecule.



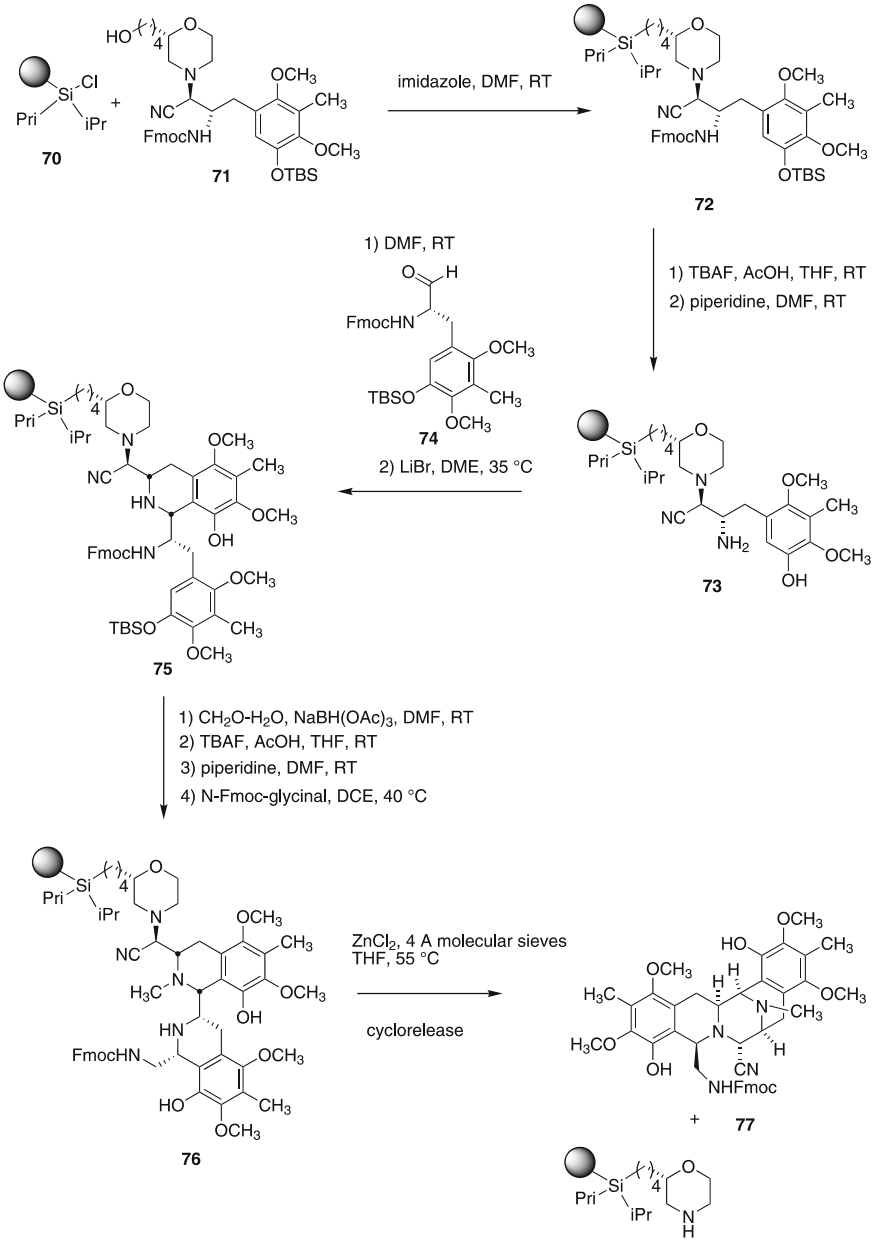
Scheme 9 Synthesis of a Galanthamine-based library by Shair et al.

Shair et al. have produced a 2527-membered library based on the alkaloid natural product galanthamine using an elegant biomimetic oxidative cyclization reaction (Scheme 9) [103]. Starting from a tyrosine-derivative (**55**), attached onto a solid support via an acid labile silyl-ether linkage, adduct **57** was synthesized after coupling of building block **56** via reductive amination. In the following key step a $PhI(OAc)_2$-mediated oxidative cyclization produced the spiroazepine ring. The newly generated dienone **58** was further elaborated via two Michael-additions involving an internal phenolate- and external *S*-nucleophile. Further *O*- and *N*-alkylation, and imine formation of the keto-group enabled the introduction of four different diversity elements. After cleavage from the solid support and biological screening of library **61** using a phenotypic assay, a library member was identified that perturbs the secretory pathway in mammalian cells—a process unrelated to the acetylcholine esterase inhibitory activity of the lead structure galanthamine. This compound, named Secramine, has recently been shown to inhibit ac-



Scheme 10 Synthesis of Lamellarins by Albericio and Alvarez

tivation of the Rho GTPase Cdc42 [104]. Using a similar oxidative addition strategy the Shair group has also prepared a 10 000 member large library of carpanones, which they also screened for phenotypic changes in vesicular



Scheme 11 Total Synthesis of Saframycin A analogs on solid phase by Myers et al.

trafficking [105, 106]. By its clever combination of scaffold building and subsequent modification, in which each synthetic step contributes to the overall diversity of the library, the galanthamine and carpanone-libraries by the Shair group have set a benchmark for future library design.

The lamellarins are an important group of marine natural compounds having a pyrrole ring as a core component of their skeleton. Albericio and Alvarez have found a modular approach to these natural products **69** on solid phase by assembling appropriate building blocks via Pd-catalyzed coupling reactions introducing three points of diversity (Scheme 10) [107].

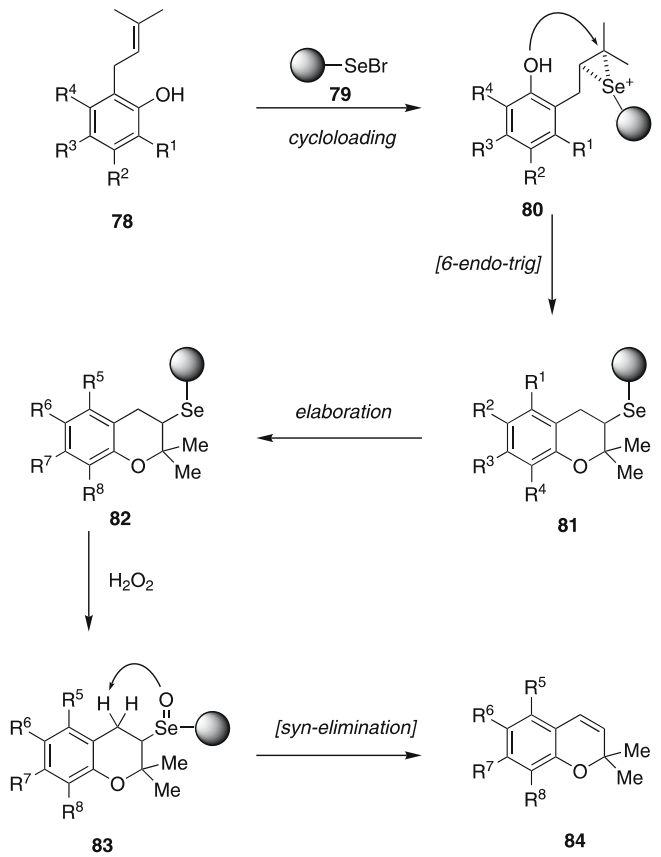
The group of Myers has published a very impressive solid-phase synthesis of 23 analogs of the structurally complex alkaloid Saframycin A by two Pictet–Spengler-reactions (Scheme 11) [108]. After attachment of starting material **71** to the solid support via silyl linkage, the amino functionality of **72** was deprotected to deliver **73** which could react with aldehyde **74** to tetrahydroisoquinoline **75** via Pictet–Spengler cyclization. Reductive amination and deprotection reactions set the stage for a second Pictet–Spengler cyclization delivering **76**, which upon warming in the presence of $ZnCl_2$ underwent cyclization-autorelease producing **77** in high diastereoselectivity. This sequence produces the Saframycin analogs in 9–26% overall yield (ten steps) in excellent purity and highlights the structural complexity which can now be accessed in natural product solid-phase synthesis.

2.3 Privileged Structures

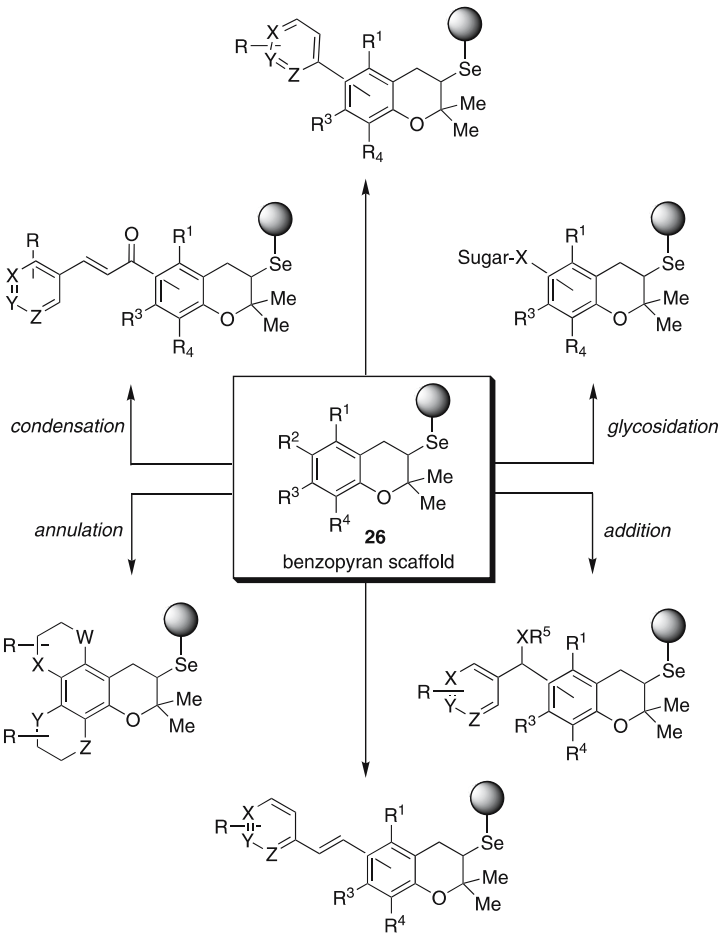
The concept of privileged structures is based on common structural motifs that are capable of interacting with a variety of seemingly unrelated biomolecular targets. This term was coined by Evans et al. who were the first to recognize that the benzodiazepine-scaffold is represented in several marketed drugs addressing different protein targets [109]. Since then many privileged structures have been identified [110, 111], such as benzoazepines, benzamidines, biphenyltetrazoles, spiropiperidines, indoles, or benzylpiperidines, and have been advantageously used in library synthesis. As has been outlined in Sect. 1.2 natural products can also be regarded as privileged structures, exhibiting biological activity against different protein targets. Therefore, libraries built around a core molecule, which represents a frequently occurring natural product scaffold, can be expected to deliver higher hit rates than other libraries built around randomly chosen scaffolds. That this assumption holds true in real life has been recently demonstrated for several cases.

Nicolaou et al. constructed a 10 000-membered natural-product-like library based on the 2,2-dimethylbenzopyran scaffold [112–114]. The benzopyran motif can be found in many bioactive natural products and pharmaceutically designed compounds, and it is therefore an excellent choice for

combinatorial derivatization. For the synthesis of the 2,2-dimethylbenzopyran loading and cleavage steps were chosen in a way that they already contribute to the complexity of the target structure, i.e. operations which do not serve the complexity built up of the structure are reduced (usually loading and cleavage) and the efficiency of the combinatorial synthesis is increased (Scheme 12). Nicolaou used a polystyrene-based selenyl bromide resin (**79**), which can be used to load substrates by electrophilic cyclization reactions. In this case *ortho*-prenylated phenol **78** reacted with the selenyl bromide **79** to form the benzopyrane scaffold **81** via a 6-*endo-trig* cyclization. The high chemical stability of the pyran linked via the selenyl ether bridge allowed further elaborations on all four possible positions on the aromatic ring such as annulations, condensations, aryl/vinyl couplings, glycosidations, and organometallic additions (Scheme 13). Finally, the benzopyran analogs were released by oxidation of the selenide followed by *syn*-elimination fur-



Scheme 12 Synthesis of Benzopyrans on solid phase

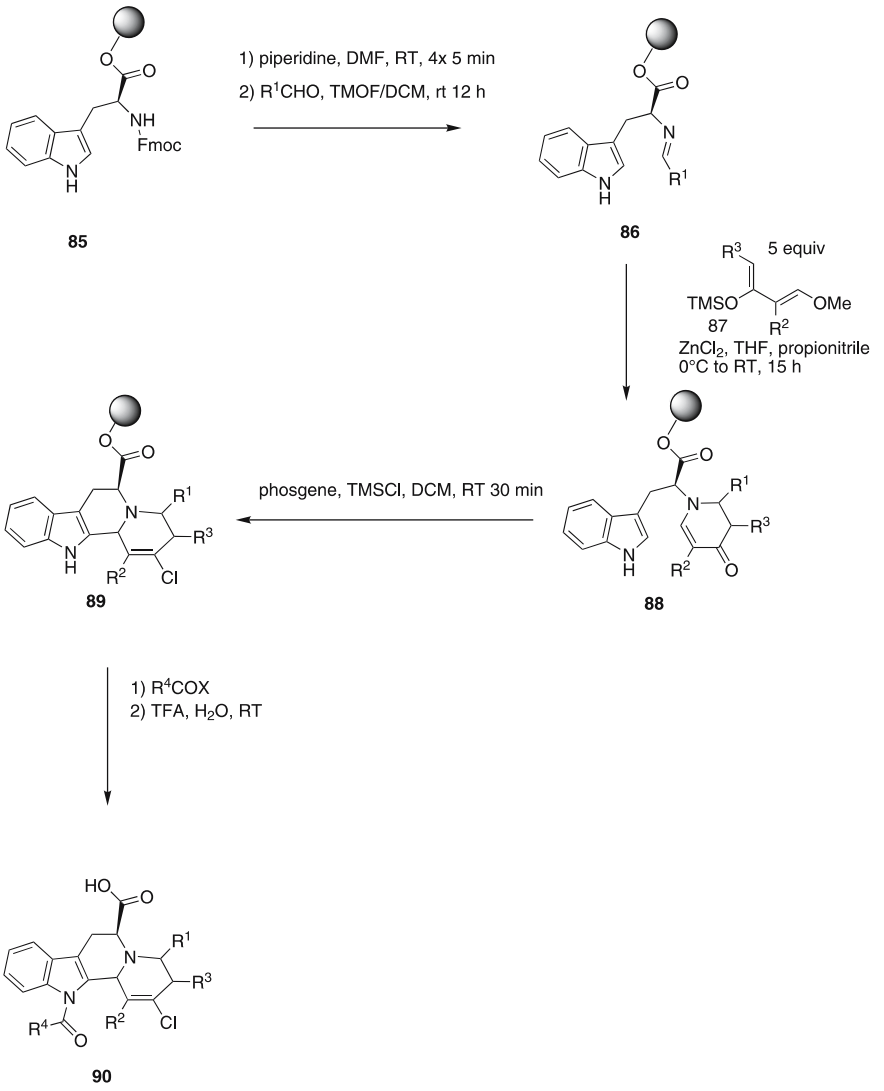


Scheme 13 Accessible Benzopyran Scaffolds on solid phase

nishing the benzopyrans **84**. A 10 000-membered natural product-like library was constructed by directed split-pool techniques employing the NanoKan optical encoding platform. An automated cleavage protocol employing hydrogen peroxide furnished 2–3 mg quantities of each library member. Biological testings in 96-well microtiter plates led to the identification of a novel structural class of antibacterial agents and a series of potent inhibitors of the NADH:ubiquinone oxidoreductase enzyme.

Waldmann et al. have performed a chemoinformatic analysis of all available natural product structures and classified the most frequent occurring scaffolds in a hierarchical tree (Structural Classification of Natural Products, SCONP) [115]. This analysis represents for the first time a quantitative description of privileged structures found among natural products. The Wald-

mann group has since initiated a program (Biology Oriented Synthesis, BIOS) in which library scaffolds are selected based on relevance to and prevalidation by nature [116, 117]. One beautiful example of such a library is the synthesis of 450 indoloquinolizidine derivatives (**90**) on solid phase, using a Lewis acid-mediated Mannich–Michael reaction between immobilized tryptophan imines **86** and electron-rich silyloxy dienes **87** followed by on resin functionalization (Scheme 14). This library exhibited a hit rate of 2.4% for the protein phosphatase MptpB [117].

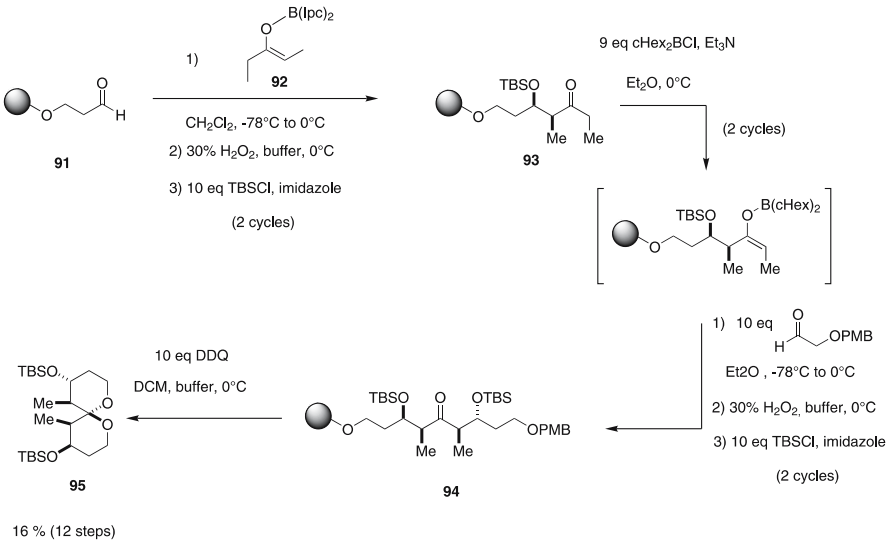


Scheme 14 BIOS of Indoloquinolizidines by Waldmann et al.

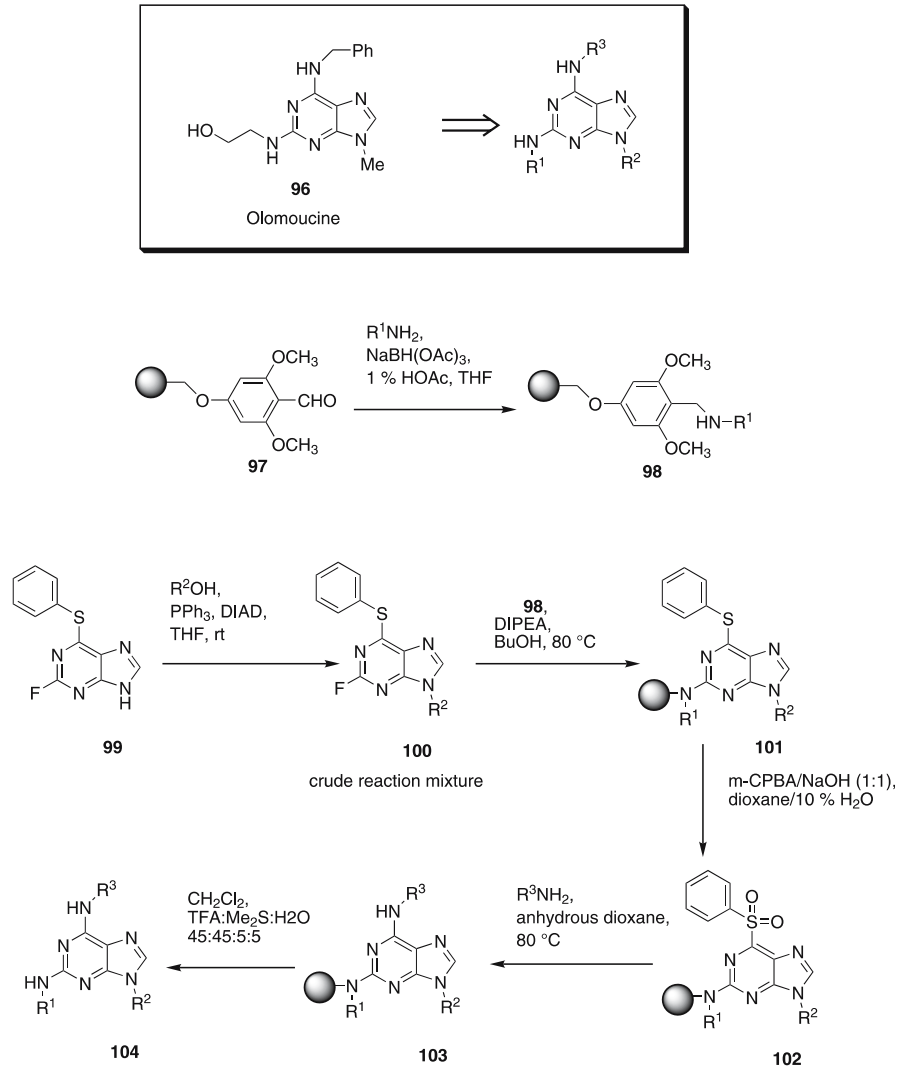
The spiroacetal moiety is another frequently occurring structural element occurring in potent natural anticancer substances, such as spongistatin. The groups of Paterson [35, 36] and Waldmann [37] have been the first to successfully synthesize libraries of this privileged structure using enantioselective aldol reactions—a reaction type which turned out to be difficult for implementation on solid phase (Scheme 15).

The adenine derivative olomoucine (**96**) was found to inhibit several cyclin dependent kinases up to the micromolar range. It is a selective competitive inhibitor of CDK2/cyclin A with an IC_{50} of 7 μM. Cyclin-dependent kinases, such as CDK2, play a significant role as regulators of cell cycle events including DNA replication and cellular division. From the crystal structure of the olomoucine-CDK2 complex it is known that the purine-based molecule occupies the adenine binding pocket of ATP although the ring structure lies in a drastically different orientation. Upon examination of olomoucine's structure, it was reasoned that diversification of the substituents appended at C6, C2, and N9 of the purine scaffold may allow a significant improvement in the binding affinity and selectivity. Schultz and co-workers have synthesized a series of libraries, following a strategy in which a properly substituted purine building block is reacted with different types of nucleophiles (Scheme 16), leading to the identification of purvalanol B, a 6 nM inhibitor [118–120].

A selection of other libraries synthesized around privileged structures is depicted in Fig. 3 [121–123].



Scheme 15 Synthesis of spiroketals by Waldmann et al.



Scheme 16 Purine-based library of kinase inhibitors by Schultz et al.

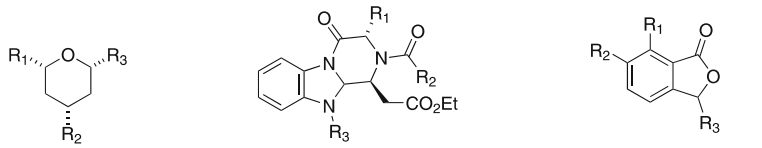
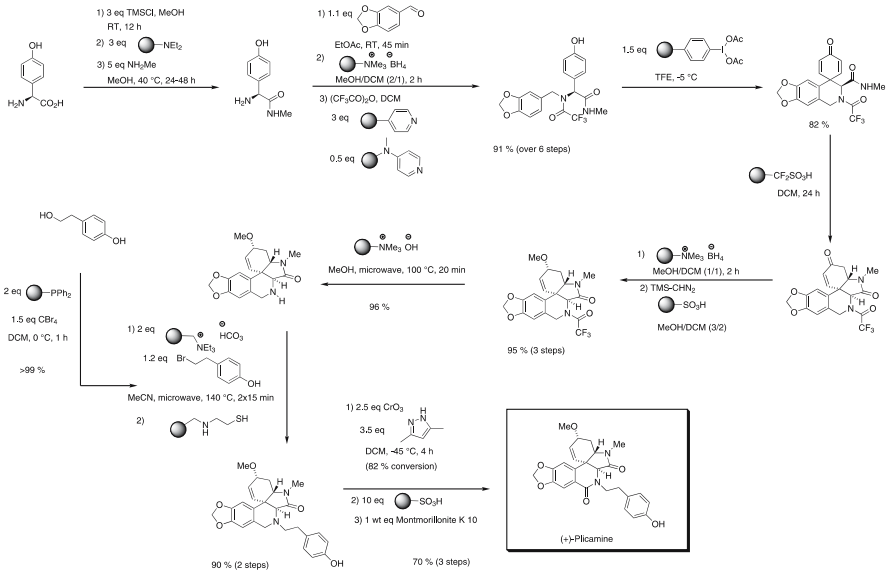


Fig. 3 Examples of privileged natural product scaffolds

3
Solid-Supported Reagents

Recently a different strategy, in which not the substrate but the reagents are bound to insoluble supports, has received considerable attention [124]. Although still relying on filtration as the preferred workup, this approach offers significant advantages complementary to SPOS: (1) no additional steps are required for attaching and cleaving the substrate, (2) reaction monitoring can be carried out by using conventional techniques, such as TLC, NMR, GC, and HPLC, (3) reaction protocols resembling those developed for solution phase organic synthesis can be introduced, (4) through compartmentalization mutually reactive reagents, such as acid and bases, or electrophiles and nucleophiles can be used in the same pot, and (5) by definition it allows the use of heterogeneous reagents, such as the important reaction class of catalytic hydrogenations using supported noble metal catalysts. Solid-supported reagents have been used for a long time for the modification or decoration of molecules, but just recently especially the group of S.V. Ley has demonstrated that such an approach is applicable for the total synthesis of structurally diverse natural products, highlighted by their syntheses of complex molecules such as epibatidine, plicamine [125] (Scheme 17), and epothilone using a wide selection of supported reagents of different type. Although such an approach will not become routine in the near future, the use of supported reagents will definitely influence the way how molecular scaffolds or natural products will be decorated with diversifying elements.



Scheme 17 Total synthesis of Plicamine using solid-supported reagents by Ley et al.

4
Conclusions and Outlook

The ongoing progress in the development of solid-phase techniques, including reaction design and automation, has enabled the multi-step synthesis of complex synthetic targets on solid phase. The examples above illustrate the structural complexity of natural product molecules which is already accessible by current methods. It can be anticipated that in the future even more challenging and demanding synthetic goals will be successfully accomplished. Considering the proven biological relevance of natural products these structures deserve more attention for the design of future combinatorial libraries. New genome and proteome sequence data combined with the tool of bioinformatics will assist the chemist in the selection of both the synthetic and biological target. Future libraries will also be measured by their design, in which as many steps as possible in the multi-step-sequence should contribute to the diversity of the library. The expansion and refinement of the toolbox of organic reactions applicable to solid-phase synthesis will remain an important task in order to exploit the potential of SPOS as a drug discovery tool further.

Acknowledgements Our research has been generously supported by the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, and the Universities of Dortmund and Leipzig. R.B. is indebted to Herbert Waldmann (Max-Planck-Institute and University of Dortmund) for many inspiring discussions about the subject of natural product inspired combinatorial chemistry.

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Top Curr Chem (2007) 278: 243–288
DOI 10.1007/128_2006_106

Published online: 2 February 2007

Multiple Peptide Synthesis to Identify Bioactive Hormone Structures

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Abstract Being unable to penetrate the cell membrane, peptide hormones and neuropeptides achieve their effects by interaction with transmembrane receptors which transmit the signal into the cell. G protein-coupled receptors occur ubiquitously in eukaryotes and mediate a multitude of biological functions. The development of drugs targeting these receptors to treat diseases rising from a dysfunction requires the knowledge of ligand-receptor interaction sites as well as of the bioactive conformation of the peptide hormone. In this review article we present concepts and methods in multiple peptide synthesis to determine structural requirements of the ligand for binding to and activation of the corresponding receptor. These include the investigation by alanine and D-amino acid scans to probe the importance of distinct side chains and conformations as well as approaches to obtain conformationally restricted and stabilized distinct structures within the peptide hormone, such as cyclization and introduction of turn mimetics. Examples are taken from several multi-ligand/multi-receptor systems including that of the neu-

ropeptide Y family. Moreover, binding and functional assays that are used to investigate the interaction of ligand and receptor are briefly reviewed.

Keywords Peptide synthesis · Peptide hormone · Structure-activity relationship · Neuropeptide Y · Bioactive conformation

Abbreviations

AC	adenylate cyclase
ACTH	adrenocorticotropin hormone
AgRP	agouti-related peptide
Ahx	ε-aminohexanoic acid
ARC	arcuate nucleus
ATP	adenosine triphosphate
AVP	arginine vasopressin
βACC	β-aminocyclopropane carboxylic acid
BK	bradykinin
BRET	bioluminescence resonance energy transfer
CaMKII	Ca^{2+}/calmodulin-activated protein kinase II
cAMP	cyclic adenosine monophosphate
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRF	corticotrophin-releasing factor
CRH	corticotrophin-releasing hormone
DAG	diacylglycerol
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fluorescence correlation spectroscopy
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
GAL	galanin
GDP	guanosine diphosphate
GHS-R	growth-hormone secretagogue receptor
GIP	glucose-dependent insulinotropic polypeptide
GIP	GPCR-interacting protein
GLP	glucagon-like peptide
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
hNPS	human neuropeptide S
HPLC	high performance liquid chromatography
I	inhibitor
IP	inositol phosphate
IP_3	inositol-1,4,5-triphosphate
L	ligand
MCH	melanin-concentrating hormone
MSH	melanocyte-stimulating hormone
NKA	neurokinin A

NKB	neurokinin B
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NPγ	neuropeptide γ
NPK	neuropeptide K
NPY	neuropeptide Y
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC_β	phospholipase C_β
PNS	peripheral nervous system
POMC	pro-opiomelanocortin
PP	pancreatic polypeptide
PYY	peptide YY
R	receptor
SAR	structure-activity relationship
SP	substance P
SPA	scintillation proximity assay
SPR	surface plasmon resonance
SPS	solid-phase synthesis
TR	time-resolved
U-II	urotensin II
URP	urotensin II-related peptide
VIP	vasoactive intestinal peptide

Amino acid and peptide nomenclature conforms to IUPAC-IUB guidelines [1].

1 Introduction

The regulation of metabolism, growth and differentiation as well as the response to external signals is one of the fundamental principles of life. Only a few hormones are able to penetrate biological membranes and act more directly on gene expression by binding to nuclear hormone receptors. The majority of such regulatory processes rather involves interactions of membrane-spanning receptors responsible for the recognition and transduction of a specific endogenous signal into an intracellular response. The heptahelix and predominantly G protein-coupled receptors (GPCRs) comprise by far the largest family of proteins encoded by more than 850 genes [2–6]. They form an extremely diverse and versatile family of receptors [7, 8] and are activated by a variety of extracellular messengers, including peptides and proteins [9–11]. Because of the limited availability of structural data on GPCRs and the much more laborious investigation of the receptor itself by means of site-directed mutagenesis, the design of ligands mainly relies on ligand-based drug design techniques. Therefore, structure-activity relationship (SAR) studies, in which the structure of a chemically modified ligand is related to receptor affinity and/or biological activity, are a powerful tool

for the identification of the bioactive conformation of a ligand or at least the understanding of its interactions at a distinct receptor subtype. Herein, the native ligand often provides a good starting point and is modified in order to determine structural elements important for binding and activation.

Owing to their involvement in physiological key functions, GPCRs are the target of a majority of drugs on the market and in development stages [12]. More than 30 to 40% of all marketed therapeutics act on about 30 members of the GPCR family, mainly biogenic amine receptors [13–16]. Changes in the structure of either the receptor or the ligand by mutation may result in the disruption or dysfunction of signal transmission and subsequently in a related disease pattern. Loss- and gain-of-function mutations in GPCR-encoding genes have been identified as the cause of an increasing number of retinal, endocrine, metabolic, and developmental disorders [17]. It is therefore an important aim of modern drug development to identify the specific interactions of peptide hormones and neuropeptides with their receptors or even distinct receptor subtypes.

This review summarizes approaches based on multiple peptide synthesis for the determination of structural and conformational requirements of peptide hormones for binding at and activation of their specific receptors. These include various systematic scans, several methods to stabilize distinct local conformations as well as secondary or even tertiary structures. Examples of their application are mainly taken from various multi-ligand/multi-receptor systems including that of the neuropeptide Y (NPY) family. Moreover, basic ligand-receptor binding assays and functional cell-based in vitro assays, which are used to investigate the interaction of native and modified peptide ligands with their receptors, are briefly reviewed.

1.1 Peptide Hormones and Neuropeptides

Today, more than 35 different receptor families that bind peptide ligands have been identified, with multiple receptor subtypes in most of these families [18]. With only a few exceptions, they all belong to the superfamily of GPCRs, most of them assigned to family A; the rest can be classified as family B. Activation of these receptors is achieved by more than 50 known peptide hormones and neuropeptides. The ligand-binding domains of these peptidergic G protein-coupled receptors involve the amino terminus and extracellular domains and are of interest due to the discovery of many small-molecule non-peptide ligands that can act with high potency at peptide receptors as antagonists and agonists [19]. However, small-molecule ligands of peptide receptors may not necessarily share an overlapping binding site with the endogenous peptide agonist [20].

Endogenous peptide ligands are generated by enzymatic cleavage of a prepro-precursor to give fragments ranging from 4–90 amino acid residues

in length. Intriguingly, different biologically active peptides specific for one or more GPCRs within a family can be generated from a single precursor [21–23]. Thereby, the regulation of which peptide is to be produced from the prohormone is carried out by various processing enzymes [24–26]. In addition, biologically active peptides can be further processed in different ways. Truncation may result in altered receptor specificity, as is the case for dipeptidyl peptidase-IV processing of neuropeptide Y (NPY) and peptide YY (PYY) to give truncated NPY_{3-36} and PYY_{3-36}. Both truncated peptides bind preferentially to the NPY Y_2 receptor subtype [27]. In contrast, the full length peptides display equal affinity for both NPY Y_1 and Y_2 receptor subtypes [28, 29]. Cleavage of already biologically active peptides and proteins also serves as an additional mechanism to generate novel peptide ligands. Aspartic proteinases such as pepsin and cathepsin E cleave the 25-amino acid xenin belonging to the neurotensin/xenin family from its precursor, the α-cytosolic coat protein [30]. Alternative splicing has been shown to generate the primary translation products prepro-ghrelin, prepro-des-Gln^{14}-ghrelin, and exon-4-deleted prepro-ghrelin which are further processed to give the bioactive isoforms of ghrelin and des-Gln^{14}-ghrelin, as well as obestatin and terminal peptides [31]. Post-translational modifications of single amino acid residues may regulate the potency or specificity of the peptide ligand or may even be absolutely necessary for signaling. C-terminal α-amidation is required for function of several peptides, including neuropeptide Y [32], gastrin and cholecystokinin (CCK) [33] as well as orexins A and B [34]. Full potency at the CCK1 receptor requires sulfation of Tyr^7 in CCK but not of Ala^7 in gastrin. Contrary to this, the CCK2 receptor has high affinity for both sulfated and nonsulfated forms of CCK and gastrin, respectively [33]. Similarly, biological activity of ghrelin requires octanoylation at Ser^3 [31]. A relatively uncommon event and generally restricted to intracellular proteins is the N_α-acetylation. However, among others melanocortins and endorphins are secreted peptides that undergo acetylation, which results in increased resistance to peptide degradation and increased biological activity [35].

Many peptides are synthesized and released from the gastrointestinal tract in response to changes in the nutritional state. These hormones influence central mechanisms involved in the regulation of energy balance. Since there is a growing worldwide epidemic in obesity, more potent pharmacological targets need to be identified. The discovery of new hormones such as ghrelin and leptin along with deeper understanding of the functions of previously described gut hormones, such as PYY, pancreatic polypeptide (PP), CCK, and glucagon-like peptide 1 (GLP-1), has led to a rapid increase in our knowledge of the regulation of energy homeostasis [36]. Gut hormones may inhibit or stimulate appetite through interactions with appropriate receptors that can be found on two distinct neuronal populations within the arcuate nucleus (ARC) of the hypothalamus [37–39]. One group contains neurons express-

ing anorexigenic peptides derived from pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). The second population of neurons is characterized by the expression of the orexigenic peptides NPY and agouti-related peptide (AgRP) [40, 41]. Hence, the ARC, that is believed to have an incomplete blood-brain barrier which allows access for various hormones and peptides from the bloodstream [42], plays an integrative role in appetite regulation.

In addition to gut hormones that are expressed from cells in the gastrointestinal tract, neuropeptides are contained in and released from a wide range of neuronal and even glial cells (e.g. NPY and substance P). Chemically distinct, they exhibit characteristic patterns of localization within the peripheral (PNS) and central nervous system (CNS) and possess the ability to stimulate a range of diverse biological activities [10]. To a large extent neuropeptides are part of a complex neuroendocrine system and involved in the hypothalamic regulation of energy homeostasis through exhibiting orexigenic (such as NPY, AgRP and melanin-concentrating hormone, MCH) and anorexigenic actions (e.g. POMC, CART and corticotrophin-releasing hormone, CRH), respectively. Intriguingly, structurally identical (such as calcitonin-gene related peptide, CGRP) or similar peptides (PYY and NPY) can also be released from non-neuronal cells and act via the same receptors as their neuronal counterparts making their overall functional significance more complex.

1.2 Multi-Ligand/Multi-Receptor Systems

Several multi-ligand/multi-receptor systems are known in which multiple structurally related peptide ligands bind to various GPCRs that belong to a common receptor family. The neuropeptide Y family, the tachykinin system and the melanocortin system, just to mention a few, are known to exhibit important functions in the organism and therefore have been intensively investigated for years by SAR studies based on synthetically derived analogs.

Sharing several common features, neuropeptide Y (NPY) as well as its structurally related analogs peptide YY (PYY) and pancreatic polypeptide (PP) belong to a peptide ligand family of neuroendocrine hormones and have been of increasing interest in the last two decades. All of these so-called NPY family peptides consist of 36 amino acid residues, of which several are tyrosines, exhibit a C-terminal amidation that is necessary for biological activity, and are believed to have a common tertiary structure called the PP-fold. The latter is based on X-ray crystallography using crystals of symmetric dimers of avian PP [43]. Herein the monomers have been characterized by an extended polyproline type II helix (residues 1–8) that is followed by a β-turn (residues 9–13). Residues 14–31 form an amphiphilic α-helix ending in the flexible loop conformation of the five most carboxy-terminal residues. Be-

cause of the high sequence homology among the members of the NPY family, the hairpin-like fold, which is stabilized by hydrophobic interactions of the N-terminal part and the α-helix, was assumed to be adopted by all the peptides [44]. Accordingly, although NPY and PYY are believed to be monomeric at physiologically relevant concentrations, NMR studies revealed a PP-like fold for monomeric PYY [45, 46]. The solution structure of human NPY in contrast has been described both by Darbon et al. [47] and Monks et al. [48] which found that only the C-terminal part of NPY forms a stable α-helix, that extends to residue 35 and 36, respectively. The conformation of this segment of NPY could be further confirmed by ^{1}H-NMR investigations in various solvents, including water, dimethylsulfoxide and trifluoroethanol [49, 50]. Owing to the small number of sequential NOEs, a secondary structure of the segment 1–10 could not be determined by Darbon et al. However, a series of long-range NOEs allowed establishing the position and orientation of the N-terminal amino acids relative to the C-terminal helix and led to the proposal of a polyproline type II helix that bends back onto the C-terminal helix. In contrast, Monks et al. postulates that the N-terminus is fully flexible which is supported by ^{15}N-relaxation [51] and FRET data [52]. Because of this controversial discussion in the literature, the structure of the N-terminus of NPY in solution and bound to the receptor as well as its importance for receptor recognition is not entirely clear yet; and further investigations are performed to disclose this secret. All three peptides can be found in the circulation and exert their hormonal and neuronal functions that are mainly related to the regulation of food intake in an orexigenic (NPY) and anorexigenic (PYY and PP) manner, respectively, through binding at several NPY Y receptor subtypes that belong to the class A of the GPCR superfamily. Four human NPY Y receptor subtypes are known, namely Y_1, Y_2, Y_4 and Y_5, which mediate either inhibition of adenylate cyclase or increases in intracellular calcium. NPY and PYY bind with high affinity to the Y_1, Y_2 and Y_5 receptor subtypes, which in turn show only moderate (Y_5) or low affinity for PP (Y_1 and Y_2). In contrast, the Y_4 receptor subtype is preferentially activated by PP but only less by NPY and PYY. As mentioned above, further enzymatic processing of NPY and PYY by dipeptidyl peptidase-IV results in the truncated peptides NPY_{3-36} and PYY_{3-36} and subsequently in an altered binding pattern.

The melanocortin receptor system is unique among GPCRs as it possesses both endogenous agonists and antagonists binding to five known melanocortin receptor isoforms MC1R-MC5R. They play a vital role in physiological functions and activate adenylate cyclase upon agonist binding as well as phospholipase C-β [53, 54]. The four naturally occurring peptide agonists, namely α-, β- and γ-melanocyte stimulating hormones (α-, β-, γ-MSH) as well as adrenocorticotropin hormone (ACTH), are derived from the precursor molecule pro-opiomelanocortin (POMC) via tissue-specific post-translational cleavage and are characterized by a conserved His-Phe-

Arg-Trp sequence that has been attributed to receptor selectivity and stimulation [55–58]. Moreover, agouti and agouti-related peptide (AgRP) are the endogenous peptidic antagonists of the MC3 and MC4 receptors [59]. Besides other physiological functions, particularly the participation of melanocortin agonists in energy and weight homeostasis, which is achieved by activation of MC3R and MC4R, as well as the involvement of MC1R in melanoma has made them subject to intensive academic and industrial research. Thus, the melanocortin ligands, both endogenous and synthetic, have been lead compounds in many SAR studies that identified amino acid residues to be important for receptor binding and stimulation [60, 61].

On the basis of their common carboxy-terminal amino acid sequence Phe-Xaa-Gly-Leu-Met-NH_2 (in which Xaa is always a hydrophobic residue that is either aromatic or β-branched aliphatic), substance P (SP), neurokinins A and B (NKA and NKB) as well as neuropeptides γ (NPγ) and K (NPK) belong to the tachykinin family [62]. Their biological activities are mediated by G protein-linked neurokinin receptors, which are divided into at least three subtypes, named NK_1, NK_2 and NK_3 receptor. Recently, Page et al. could show that there are additional non-neuronal members of this peptide family, the endokinins and hemokinins [63]. Whereas the C-terminal region is essential and central for activation of the NK receptors, the N-terminal segments of the tachykinins possess divergent and hydrophilic regions and are believed to be responsible for receptor selectivity [64, 65]. Although they do not act as exclusive agonists for a distinct receptor, SP shows the highest affinity for the NK_1 receptor subtype, whereas NKA and NKB preferentially bind to NK_2 and NK_3 receptors, respectively. The activation of the neurokinin receptors has been implicated in a wide variety of physiological and pathological systems [66].

The above ensembles of various receptor subtypes and peptide ligands make multi-ligand/multi-receptor systems an excellent example for the complexity of the regulation of important basic physiological functions within the organism. Knowledge of the specific interactions of one receptor subtype with the appropriate ligand is of basic necessity for the treatment of metabolic disorders and even cancer. Structure-affinity/activity relationship studies based on modifications of the ligand allow the determination of its structural requirements for binding at the specific receptor and, as a consequence, the development of agonists and antagonists. Several of the multiple peptide synthesis approaches that will be described in detail in the following section have been, among others, applied to the peptides of the NPY and melanocortin systems, and have been very beneficial in the search of the bioactive conformation at the various receptors; and finally, they have led to a set of peptide analogs specifically binding to distinct receptor subtypes. The application of multiple solid phase peptide synthesis which has experienced a multitude of efforts and improvements since its development more than 30 years ago is therefore an essential tool in the search of a hormone's bioactive

conformation and the subsequent development of potent and specific agonists and antagonists.

2 Peptide Ligand Design

Specific interactions are postulated to occur between a ligand and its receptor, which include the molecular recognition, the binding of the ligand and, subsequently, the activation of the receptor. Mostly due to the lack of adequate quantities of GPCRs for direct characterization of the receptor or the specific ligand-receptor complex by means of X-ray crystallography and NMR spectroscopy, indirect approaches that probe the structure of the complexes can be based on structure-affinity and structure-activity relationship studies, respectively. Many attempts have been made to infer biologically relevant conformations of peptides. Since most of the endogenously occurring neuropeptides and peptide hormones are relatively small and have a lower volume-to-surface-area ratio than most proteins, the intramolecular stabilization of any particular conformer of the peptide is limited, making peptides inherently more flexible than proteins. Thus, correlating a so-called bioactive conformation with functional activity is challenging. However, structure-affinity/activity-relationship studies can be used to characterize the interaction between hormone and receptor. Essential segments of the peptide hormone for receptor recognition can be identified and distinguished from nonessential ones by using truncated analogs. And the significance of each sequence position can be assessed by the substitution of amino acid

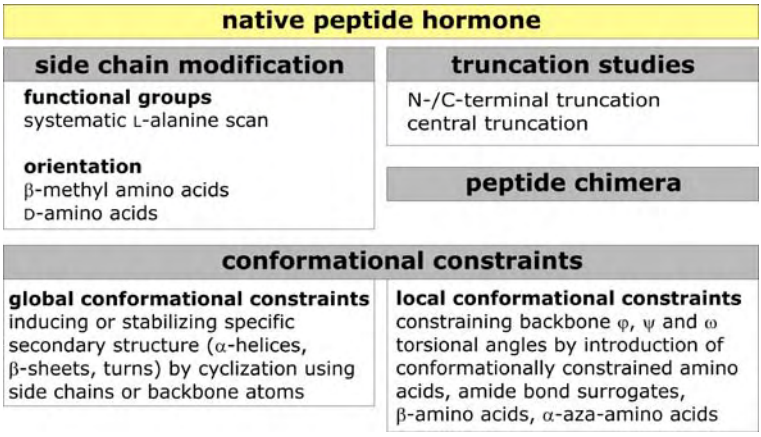


Fig. 1 Overview of peptide design approaches to investigate ligand-receptor interactions by structure-affinity and structure-activity relationship studies

residues to alter the side chain functionality, its spatial orientation or even the backbone conformation by conformationally restricted peptidomimetics or non-peptidomimetics, respectively. Various peptide design approaches are shown in Fig. 1.

2.1 Multiple Peptide Synthesis

To develop "A new approach to the continuous, stepwise synthesis of peptides" (Merrifield's notebook entry of May 26, 1959), Merrifield started the search for the best resin, linker, amino protecting group and cleavage procedure in 1959, which led to the synthesis of a tetrapeptide by the end of 1962 [67]. Peptide chemistry, up to this time rather inefficient, was revolutionized, and, having had a great impact on scientific capabilities, solid-phase synthesis (SPS) has proven a paradigm shift for the chemistry community as well as its dominance in combinatorial chemistry [68]. Together with improvements in both purification and characterization techniques such as HPLC, NMR and mass spectrometry solid-phase chemistry emerged as an extremely feasible method. Since only addition of solvent and reagent, shaking and filtration are involved, the simplicity of these steps together with the practical advantages in handling allowed for automation of SPS. The discovery of novel biologically active peptides, the systematic and detailed exploration of ligand-receptor interactions as well as the search for peptides that inhibit these interactions require large numbers of chemically diverse peptides. The development of various multiple synthesis techniques that enable parallel and simultaneous synthesis of hundreds of individual peptides has taken this need into account [69]. Synthetic peptide arrays and peptide combinatorial libraries as versatile tools for the characterization and mapping of ligand-receptor interactions as well as for the de novo identification of biologically active peptides have been reviewed in detail by Beck-Sickinger and Weber [70] and, more recently, by Eichler [71], Falciani et al. [72] and Shin et al. [73]. Peptide libraries on solid phases can be obtained by the application of different approaches. In parallel synthesis, peptides are synthesized in individual reaction vessels leading to pure, separated and well-determined products. In contrast, split-and-mix synthesis approaches apply the three processes: splitting, coupling in mixtures and recombination. Repetitive cycles can provide mixtures of random and large peptide libraries. The resulting combinatorial library, in which one bead often displays only one peptide (one bead-one compound, OBOC), is then screened for specific biological properties and potent peptides are identified. Moreover, besides parallelization, the use of polymeric materials, either as supports for SPS or as solid-supported reagents and scavengers, has had an additional impact on automation which, in turn, can make a significant improvement at each stage in the drug discovery process [74].

2.2
Side Chain Modifications

2.2.1
Alanine Scan Studies and Single Amino Acid Replacements

Alanine scan studies are a classical method to evaluate amino acid residues involved in ligand–receptor interactions. Therefore, a set of analogs is synthesized in which each amino acid residue in the native peptide is systematically replaced by L-alanine. L-alanine itself may be replaced by glycine. Since alanine consists of a methyl side chain that does not alter the secondary structure, thus maintaining the backbone conformation, only functional groups are deleted. Alanine scans allow the identification of residues responsible for or contributing to the biological properties of the ligand. Only with the emergence of simultaneous multiple peptide synthesis, the realization of scan studies that require a high number of hormone analogs has become easier [69]. Once a critical position has been identified, the side chain can be further characterized by the replacement by amino acids that have an influence on hydrophobicity, charge, dipole–dipole interaction, steric and electronic effects in order to recover affinity. In addition to naturally occurring amino acids, noncoded amino acids with unique side chains can be applied to modify distinct positions. Today, a multitude of Fmoc- and Boc-protected building blocks is commercially available.

Beck-Sickinger et al. performed a complete alanine scan of NPY, in which the binding was investigated by displacement of ^{125}I-labeled NPY from human neuroblastoma cell lines SK-N-MC and SMS-KAN [75]. Additionally, 16 selected alanine-substituted NPY analogs have been investigated for their binding affinity to human Y_4 and Y_5 receptor subtypes by Eckard et al. [76]. All NPY receptor subtypes have been shown to be sensitive to the positions of Pro and Tyr. Pro^2, Pro^5, Tyr^{27} and Tyr^{36} proved to be the most important positions for the Y_1 receptor subtype, Pro^5 and Tyr^{36} for the Y_2 receptor, Pro^8 and Tyr^{27} for the Y_4 and Y_5 receptor subtypes. Moreover, substitution of arginine residues also led to different receptor affinities. Whereas Arg^{19} is of importance for the Y_1 and Y_2 receptor subtypes, Arg^{25} replacement resulted in the decrease of affinity at Y_1 and Y_5 receptors. In contrast, arginine residues at position 33 and 35 are essential for the binding at all four subtypes. Hence, the C-terminal pentapeptide does not only represent the binding site of NPY for the Y_1 and Y_2 receptors as stated before [77], this is also the case for the subtypes Y_4 and Y_5. The importance of single amino acid replacements by alanine, other naturally occurring amino acids and *p*-benzoyl-L-phenylalanine for binding at the four receptor subtypes has been reviewed by Cabrele et al. [78]. Moreover, the replacement of Ile^{31} and Thr^{32} by Ala^{31} and α-amino-isobutyric acid in position 32 (Aib^{32}), respectively, resulted in the first Y_5 receptor-selective analog [79]. Further studies showed that this selec-

tivity of the Ala^{31}–Aib^{32} motif derives from a specific conformation that must be correlated with the bioactive conformation of NPY at this receptor subtype [80]. In addition, Söll et al. presented the first NPY-based analogs with Y_1 receptor preference and agonistic properties [81]. Amongst the analogs tested in this study, [Phe^7,Pro^{34}]-NPY showed the most significant Y_1 receptor preference with sub-nanomolar affinity and a receptor selectivity of 1:3000 with respect to Y_2 and Y_5 receptor subtypes.

The first characterization of the functional properties of the widespread neuropeptide vasoactive intestinal peptide (VIP) for interaction with the two human VIP receptor subtypes $VPAC_1$ and $VPAC_2$ has been reported by Nicole et al. [82]. They combined data of a set of alanine-substituted VIP analogs obtained from a ligand–receptor binding assay using ^{125}I-labeled VIP and adenylyl cyclase assay with ab initio molecular modeling. Modeling of the three-dimensional structure of native VIP and analogs showed that substitutions of His^1, Val^5, Arg^{14}, Lys^{15}, Lys^{21}, Leu^{23} and Ile^{26} decreased biological activity without altering the predicted structure. It was therefore suggested that these residues are directly involved in the interaction with the $VPAC_1$ receptor subtype. Since the interaction with the $VPAC_2$ has been very similar, with three remarkable exceptions, they were able to develop the first highly selective $VPAC_1$ receptor agonist, [Ala^{11,22,28}]-VIP.

Studies have investigated truncated ghrelin peptides and have revealed the importance of the first 14 amino-terminal residues [83–86]. Ghrelin, a 28-amino acid peptide with an acyl group esterifying the hydroxyl function of Ser^3 and being necessary for binding and activation, is the natural ligand of the growth-hormone secretagogue receptor (GHS-R). Van Craenenbroeck et al. determined the importance of each of the first 14 residues by alanine replacement on the recombinant GHS-R expressed in HEK293 or CHO cells by binding, IP and Ca^{2+} assays [87]. This scan revealed the importance of the N-terminal positive charge that is essential for efficient binding and activation. Moreover, Phe^4 has been found to be crucial for receptor recognition. All other positions between 1 and 14 of ghrelin could be modified with little or no effect.

Structure-activity relationship studies on the vasoactive cyclic undecapeptide urotensin II (U-II) by Guerrini et al. using 31 peptide analogs with single replacements by alanine, the corresponding D-amino acid or even noncoded amino acids, such as norvaline, norleucine, ornithine, citrulline, (4-pyridyl)-alanine and various substituted phenylalanine derivatives, indicated that the sequence Phe^6–Trp^7–Lys^8–Tyr^9 is essential for biological activity [88]. Moreover, the positive charge of the primary aliphatic amine at position 8 and its relative spatial orientation has been found to be crucial for both receptor binding and activation, while the only chemical requirement at position 9 is the presence of an aromatic moiety.

Lang et al. used the shortest active analogue of the neuropeptide orexin B, orexin B(6–28), to screen for important amino residues by L-alanine and L-proline replacement scans [34]. Herein, [Ala^{27}]-orexin B(6–28) and [Pro^{11}]-

orexin B(6–28) have been identified as being highly potent orexin 2 receptor-selective peptides. The C-terminal half region of orexin A exhibits both hydrophobic and hydrophilic residues on separate surfaces to provide an amphiphilic character in helices I and II. Since the nine hydrophobic residues are also well conserved in orexin B and their substitution with alanine resulted in a significant drop in the functional potency at the receptors, it is now suggested that they form the surface responsible for the main hydrophobic interaction with the receptors [89].

Recently, alanine scan studies of the seven most amino-terminal residues in glucose-dependent insulinotropic polypeptide (GIP) were performed to evaluate specific residues that play a role in insulin secretion [90]. Alaña et al. could demonstrate the importance of the N-terminus in glucose-dependent insulin release since all analogs, with exception of [Ala^6]-GIP, showed reduced or no insulinotropic activity in cultured pancreatic BRIN-BD11 cells.

Substitution of each amino acid residue by L-alanine within the sequence of human neuropeptide S (hNPS), the endogenous ligand for the hNPS receptor and involved in biological functions such as sleeping/wakening, locomotion anxiety and food intake, revealed that Phe^2, Arg^3 and Asn^4 are crucial for biological activity as determined by their ability to stimulate calcium release in HEK293 cells expressing the human recombinant NPS receptor. Moreover, Roth et al. could show that the sequence Thr^8–Gly^9–Met^{10} is important for receptor activation [91].

2.2.2 D-Amino Acid Scan Studies and Single Replacements

Whereas an L-alanine scan allows the investigation of direct interactions of side chains at the receptor, the utilization of D-amino acid analogs provides information on the orientation and steric prerequisites [92]. In D-enantiomers the side chains remain unchanged and the overall hydrophobicity and dipole moment are only slightly changed.

Kirby et al. reported a complete D-scan of NPY in which the amino acids in all 36 positions have been systematically replaced by the corresponding D-enantiomer (achiral Gly^8 has been replaced by Ala and D-Ala) and in which the analogs have been tested for their Y_1 and Y_2 receptor binding affinities by using human neuroblastoma cell lines SK-N-MC and SK-N-BE2, respectively [93]. Chiral inversion of residues in position 2–5, 20 and 27 showed the strongest reduction of Y_1 affinity in addition to substitutions within the C-terminal octapeptide. Exchange of the configuration of any residue of the segment NPY_{29-36} was not tolerated and led to more than 100-fold loss of affinity. With the exception of residues 30–35, binding affinities of all analogs for the Y_2 receptor subtype have been in the low or sub-nanomolar range. Only [D-Arg^{33}]-NPY and [D-Gln^{34}]-NPY displayed no measurable binding affinity. In spite of the fact that incorporation of a single D-amino acid may

confer a conformational perturbation, it was concluded that interaction with the receptor was only affected when certain critical residues were modified. Because of the previous finding that D-Trp imparts antagonistic properties to other peptides, this unnatural amino acid has been chosen for substitutions within the C-terminal segment of NPY. One of these analogs, [D-Trp^{32}]-NPY, was originally reported to antagonize NPY-induced food intake [94], but was identified as a selective Y_5 receptor agonist later on [95]. Parker et al. identified [D-Trp^{34}]-NPY as a potent and selective Y_5 receptor agonist with greater in vivo orexigenic activity than [D-Trp^{32}]-NPY [96].

Recent structure-activity studies on hNPS by Roth et al. also included a systematic D-amino acid replacement of each residue of the peptide primary sequence. Whereas inversion of the most N-terminal residue Ser^1 did not affect potency and efficacy of the peptide, the configuration of Phe^2, Arg^3 and Asn^4 seemed to be of high importance since these analogs led to a significant loss of potency (10- to 30-fold) on Ca^{2+} mobilization. These findings are in agreement with the results obtained from the alanine scan as mentioned above. Furthermore, D-amino acid substitutions from residue 8 to 20 did not modify either potency or efficacy of the peptides, producing hNPS analogs that behaved as full agonists [91].

In addition to U-II, the existence of a paralog named U-II-related peptide (URP) has been demonstrated in mouse, rat and human [97]. A series of URP analogs has been studied for their affinity to the urotensin receptor (previously hGPR14) in transfected cells, for their potency to increase Ca^{2+} mobilization as well as in an aortic ring contraction assay. The alanine scan revealed significantly reduced binding affinities for all analogs of this cyclic octapeptide with exception of the Ala^8-substituted one. Whereas the replacement of Phe^3, Lys^5 and Tyr^6 by the corresponding D-enantiomer decreased binding affinity, the [D-Trp^4]-URP analog substantially retained binding affinity. In agreement with this finding, it has been reported that Trp^7 in human U-II is the only position within the cyclic region, in which the D-isomer substitution is sufficiently tolerated [88, 98]. Moreover, the [D-Trp^4]-URP analog turned out to be a partial agonist with moderate potency and a full antagonist with low potency.

2.3 Truncation Studies

The amino acids of a bioactive peptide hormone exhibit different functions. Whereas some are directly involved in the ligand–receptor interaction, others are important for the overall conformation or only necessary to provide the required space. Thus, amino acids that do not contribute to the binding are responsible for orientating them in the required tertiary structure. Besides the above-mentioned approaches to investigate the significance of functionality and spatial orientation of the side chain in each sequence position,

truncation studies are routinely performed to determine the minimal sequence to illicit a pharmacological response, thereby reducing the synthetic effort that is required to produce subsequent analogs. Such studies involve the selective removal of N- and/or C-terminal residues, followed by evaluation of the truncated analogs for binding and/or functional activity at each receptor subtype. They are therefore an important approach for the development of selective and pharmacologically active hormones and neuropeptides. Owing to the spatial relation of the remaining amino acid side chains, central truncation frequently leads to an almost complete loss of affinity. However, it has also been reported that removal of the central peptide segment can lead to discontinuous analogs that are still active [78].

By progressive deletion of ten residues from both sides, Roth et al. recently investigated the contribution of the N- and C-terminal segments of hNPS to the biological effects of the peptide [91]. Whereas the deletion of Ser^{1} produced a significant reduction in the potency without modifying the efficacy and further N-terminal truncations led to inactive peptides, deletion of all of the ten C-terminal amino acid residues did not affect either peptide potency or efficacy. As hNPS(1–10) maintained the same biological activity as the native peptide, further truncations have been performed in order to identify the minimum sequence required for hNPS receptor activation. As a result, the hNPS sequence 1–10 has been identified to be the smallest fragment able to activate the hNPS receptor with similar potencies and efficacies as full-length hNPS. However, hNPS(1–10) did fully mimic the in vitro effects of the natural ligand but failed to exhibit the same in vivo biological activity, which may be due to a different susceptibility to peptidases.

Corticotropin releasing factor (CRF) is a linear 41-amino acid peptide amide that is believed to be involved in endocrine illnesses such as feeding disorders as well as in neurological and psychiatric illnesses such as major depression and anxiety-related disorders. CRF antagonists are therefore considered promising for treatment of these disorders. Previous SAR studies led to the discovery of astressin, cyclo(30–33)[D-Phe^{12},$Nle^{21,38}$,Glu^{30},Lys^{33}]-CRF(12–41), the most potent peptide-based antagonist described so far [99, 100]. Since truncated peptides may serve as valuable lead structures for the development of small, non-peptidic CRF antagonists, Rijkers et al. reported the design, synthesis and biological evaluation of 91 truncated astressin analogs in order to deduce the pharmacophoric amino acid residues [101]. Finally, this study showed that the first 15 N-terminal residues of astressin can be deleted without a significant loss of biological activity. However, further alanine scan studies with the truncated analog revealed that all residual amino acids of the smallest peptide with CRF antagonistic activity, astressin (27–41), are required for biological activity. Accordingly, scaffolding of the pharmacophoric amino acid residues to mimic the bioactive conformation would be difficult.

Earlier binding studies in the late 1980s using synthetic segments of NPY revealed the importance of the C-terminal part for its biological activity, and

N-terminal segments, such as NPY(1–12) and NPY(1–24) have been found to be completely inactive [102–105]. Moreover, the Y_1 receptor subtype can be distinguished from the Y_2 receptor, since the former is unable to recognize the C-terminal segment alone, thus only the full-length NPY possess high Y_1 receptor affinity [106]. Deletion of the two N-terminal residues does not affect the binding to the Y_2 receptor subtype and the C-terminal segments NPY(13–36), NPY(16–36), NPY(18–36) and even NPY(25–36) have been identified as agonists in different assays [107–113]. Thus, the Y_2 receptor can be considered as resistant to N-terminal deletions. However, the minor binding affinity of the acetylated dodecapeptide NPY(25–36) could be enhanced by introduction of special amino acids with hydrophobic and conformationally restricted side-chains up to 20-fold [114]. As already suggested by the small effect of Tyr^1 substitution by L-Ala, the Y_5 receptor tolerates the deletion of the N-terminal amino acid in NPY to yield NPY(2–36) [115, 116]. The above findings demonstrate that terminal truncation studies may be a simple approach that results in analogs which are able to distinguish between various receptor subtypes based on different minimal structural requirements. Assuming the necessity of the juxtaposition of N- and C-terminus of the peptide hormone, as for NPY at the Y_1 receptor subtype, central truncation studies can be applied to further characterize ligand–receptor interactions for receptor recognition and activation. On the basis of earlier studies which revealed [Ahx^{5-24}]-NPY, in which the first four N-terminal amino acid residues are linked via ε-aminohexanoic acid (Ahx) to the C-terminal partially α-helical peptide segment 25–36, to be the first potent NPY agonist, which is considerably reduced in size compared to the native hormone [117, 118], as well as to be one of the most active analogs in receptor binding and in vivo assays [113], Rist et al. synthesized four sets of centrally truncated analogs and tested their affinity for the Y_1 receptor subtype on human SK-N-MC cells [119]. Keeping the N-terminal part in each set constant while varying the length of the C-terminal part, only one optimal length of the C-terminal segment could be found in each set which suggests that the three-dimensional arrangement and orientation of the amino acids are rather more important than the residues themselves.

2.4 Global Conformational Constraints

Biologically active peptides are cyclized to achieve metabolic stability, to increase agonist or antagonist potency, to confer or improve receptor selectivity and to enhance bioavailability, thus controlling pharmacological characteristics to convert natural bioactive peptides into peptidomimetic drugs. Relating structural rigidification and activity, in combination with conformational analysis, gives insight into the biologically active conformation of linear peptides. Moreover, cyclization is also used to stabilize distinct secondary and tertiary structure motifs of proteins. Cyclization can be distinguished in

Table 1 Secondary structure elements resulting from the introduction of distinct structural constraints

Constraints	Secondary structure elements
Lactam bridge (side chain-to-side chain, side chain-to-end)	α-helices, β-turns
Disulfide bridge	β/γ-turns, α-helices, β-sheets
backbone-to-backbone cyclization	β/γ-turns, β-sheets

short-range cyclizations, which means a cyclization from residue i to residue i or $i + 1$, and in medium- and long-range cyclizations between residues i and $i + n$ (with $n > 1$). Moreover, cyclization can be achieved by (i) a disulfide formation between two Cys residues, (ii) lactamization of N- and/or C-terminus or by the amino- and carboxy group-containing side chains of naturally and non-naturally occurring amino acids (side chain-to-side chain, end-to-end, and side chain-to-end cyclization), (iii) backbone cyclization with backbone, side chain or N-/C-terminal end [120]. Cyclization of a peptide chain can dramatically change the overall conformation of a ligand favoring certain secondary structures; some types of structural constraint are listed in Table 1.

2.4.1 Cyclization Involving Side Chains and Termini

Small, linear peptides exist in a fast equilibrium of interchanging conformations with different conformers being able to bind and activate different receptor subtypes. Structural modifications that conformationally constrain linear peptides in a global (cyclization) or local manner (specialized amino acids) are made to overcome this lack of receptor selectivity and play a crucial role in designing functional ligands [121]. Medium- and long-range cyclization can be achieved by linking N- and/or C-terminal as well as side chain functional groups. If not already present in the native sequence, functional groups will be introduced by substitution of amino acids by either other natural (Asp, Glu, Lys and Cys) or even unnatural amino acids such as α,β-diaminopropionic acid (Dpr) or α,γ-diaminobutyric acid (Dab). Primarily applied methods are summarized in Fig. 2. The availability of a variety of selectively cleavable protecting groups for amines and carboxylic acids allows for several approaches to the synthesis of monocyclic, bicyclic lactam-bridged peptides by solid-phase methods. Lactam bridges linking (i and $i + 3$), (i and $i + 4$) and (i and $i + 7$) spaced residues have proven to be useful for the stabilization of α-helices, and (i and $i + 3$)-linked residues have additionally been found to stabilize β-turns. Synthetic approaches for the introduction of one or more lactam bridges have been recently reviewed by Taylor et al. [122] and John Davies [123]. In general, cross-linking side chains by amide bond forma-

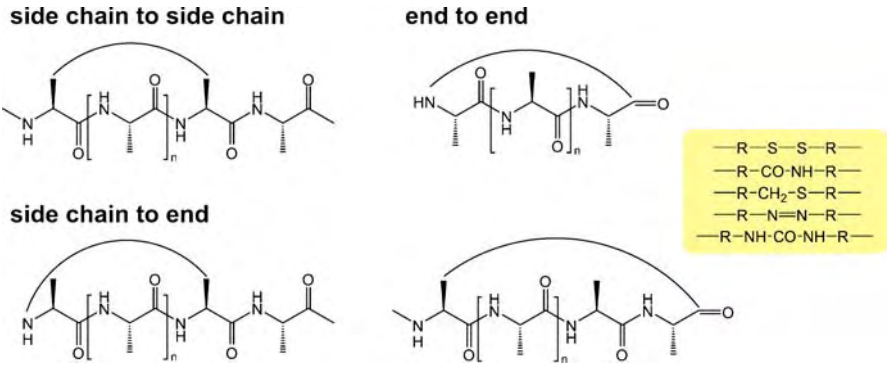


Fig. 2 Modes of cyclization involving amino acid side chains and/or N- or C-terminus

tion offers two significant advantages. Whereas disulfide bonds may exchange at neutral pH to relieve a strain or otherwise more stable folded conformation or may be reduced to their thiol form in intracellular environments, amide bonds are chemically inert to most of the conditions peptides are subjected to. Moreover, synthesis of peptides is easier to perform by using trifunctional amino acids with amino or carboxylic acid groups in their side chain since a variety of protecting groups is available that can be cleaved under orthogonal or highly selective conditions. Recently, additional approaches to join side chains have been reported, including carbonyl bridges and azo-cyclization.

The importance of cyclization has been so well established that modern day schemes for ligand-based drug design often start with cyclization of linear peptides to rigidify peptide structure, to limit its conformational possibilities, and to find key pharmacophore elements in three-dimensional space. Analogues of the α-melanocyte stimulating hormone (α-MSH) have been subjected to cyclization studies for years to develop ligands that show improved binding affinity, receptor selectivity, efficacy and biostability. Cyclic α-MSH analogs such as MT-II and SHU-9119 have been found to be potent, and SAR studies and molecular modeling therefore have been shown to be more useful for creating new pharmacophore templates [124]. As a result and based on the MT-II and SHU-9119 templates, several highly potent and more selective analogs have been reported [125–128].

The synthesis and investigation of six novel cyclic enkephalin analogs by Pawlak et al., in which two basic amino acids have been linked by a carbonyl bridge achieved by treatment with bis(4-nitrophenyl)carbonate led to a model of the bioactive conformation of this class of opioid peptides [129].

Recently, Fridkin et al. reported on the synthesis of gonadotropin-releasing hormone (GnRH) analogs containing a *p*-aminophenylalanine residue in their sequence [130]. Azo-cyclization has been performed in solution by diazotization of the *p*-aminophenylalanine residue followed by intramolecular coupling of the formed diazo salt with either tyrosine or histidine side chains

present in the sequence. However, the enhanced molecular rigidity of this novel family of cyclic GnRH analogs did not result in an increase of receptor recognition or higher biological potency.

By cyclization of the centrally truncated analog [Ahx$^{5-24}$]-NPY, peptides have been obtained that showed strong affinity at the Y_2 receptor subtype but no affinity to the Y_1 receptor. This cyclic compound [Ahx$^{5-24}$,γ-Glu^{2}-ε-Lys^{30}]-NPY displayed a Y_2 selectivity that is 40-fold higher compared to the linear NPY(13–36), which has been used to determine Y_2 receptor selectivity so far [131]. Molecular dynamic simulations showed that due to the cyclization a large hydrophobic site becomes accessible to the receptor [132]. Using 2D-NMR and molecular modeling techniques, Rist et al. suggested a model of the bioactive conformation of NPY at the human Y_2 receptor based on the structure of the C-terminal dodecapeptide NPY(25–36) cyclized by a lactam bridge between position 28 and 32, which showed a 100-fold increase in affinity compared to the linear peptide [133]. Additional analogs of the C-terminal dodecapeptide have been synthesized, in which size, position, orientation, configuration and location of the cycle have been varied, and found to play an important role in receptor recognition [134]. It has been further shown that a turn-like structure and the juxtaposition of the N- and C-termini of the cyclic dodecapeptide play a major role for high affinity binding.

2.4.2 Backbone Cyclization

Whereas the above-mentioned cyclization modes are restricted to cyclization through the side chains of a few amino acids and/or the amino or carboxy terminal groups, Gilon et al. developed a method of backbone cyclization wherein the ring closure is achieved by a bond formation between two functional groups linked by alkyl spacers to the amide nitrogen and/or the α-carbon in the backbone [120]. This allows cyclization without substitutions in the native sequence. Thus, side chains that are mostly important for ligand–receptor interaction remain unchanged. The concepts of N-backbone and C-backbone cyclization are summarized in Fig. 3. Although it may be advantageous to constrict the peptide backbone by cyclization, the bridge can prevent the formation of hydrogen bonds and thus sterically disturb the interaction with the receptor. Nevertheless, backbone cyclization has emerged as an important method in the search for the bioactive conformation and biologically potent peptide analogs.

The method of backbone cyclization was first exemplified on substance P analogs [135], and later applied to other peptide hormones, including bradykinin and somatostatin. Gazal et al. reported on the synthesis and SAR studies of compounds related to a previously described cyclic somatostatin analog, whose lactam bridge has been substituted by a backbone disulfide bridge using novel sulfur-containing building units for on-resin backbone

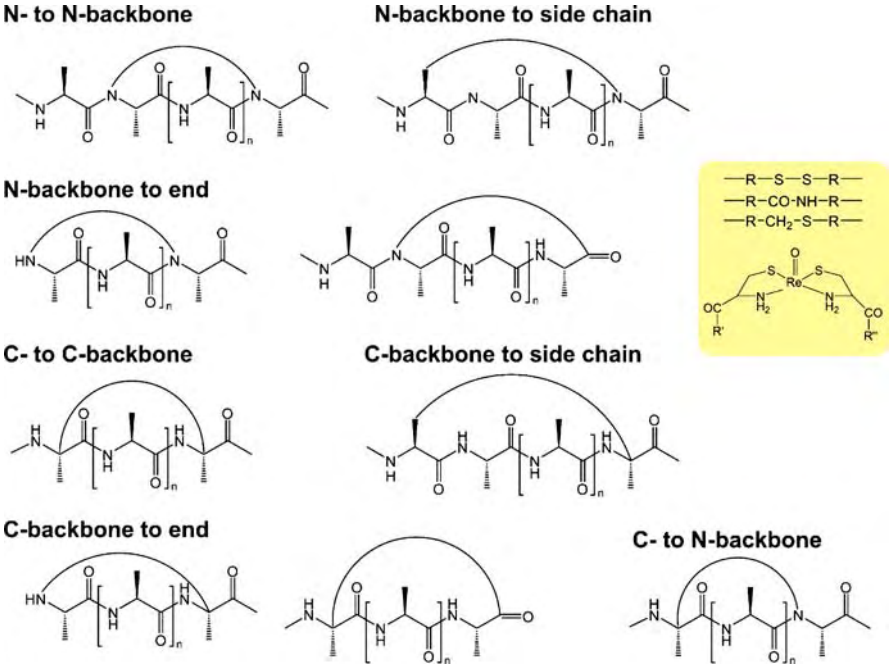


Fig. 3 Modes of cyclization involving backbone nitrogen and α-carbon atoms as well as amino acid side chains and/or N- or C-terminus

cyclization [136]. Besides a significant metabolic stability, these disulfide backbone cyclic analogs revealed different receptor selectivity profiles in comparison to their prototypes, which implies that ring chemistry, ring size and ring position may affect the receptor binding selectivity.

Schumann et al. synthesized a series of conformationally constrained cyclic analogs of the peptide hormone bradykinin (BK) to investigate different turn structures that have been proposed for the bioactive conformation of BK agonists and antagonists [137]. Besides lactam-bridged analogs with cycles of different ring size and direction of the amide bond in both the C- and N-terminal segments, mainly *N*-amino alkylated and *N*-carboxy alkylated amino acids have been introduced into the peptide chain allowing backbone cyclization to preserve side chains for receptor interaction. Thereby, dipeptide building units with more bulky amino acids as well as an alternative building unit with an acylated reduced peptide bond for backbone cyclization at the C-terminus have been pre-built in solution before coupling on the solid phase. The results could support the hypothesis of turn structures in both parts of the molecule as a requirement for BK antagonism.

The group of Gilon, who developed the method of backbone cyclization, has recently reported on a novel approach for simultaneous peptide cyclization and radiolabeling of GnRH and somatostatin analogs. Therein, the

metallo-cyclic peptides have been prepared from their linear precursors through complexation of a metal atom via two hemi-chelating arms located on the peptide backbone. Fridkin et al. found five rhenium-cyclic somatostatin analogs to be most potent with IC_{50} values in the low-nanomolar range making them promising leads for further development of tumor diagnostic and therapeutic radiolabeled agents. Further, they successfully prepared one ^{99m}Tc somatostatin analog by the same method [138]. Application of this approach resulted in the synthesis of rhenium-and technetium-labeled cyclic analogs of GnRH [139].

2.5 Local Conformational Constraints

Local conformational constraints that are able to restrict the backbone φ, ψ and ω torsional angles (for definition see Fig. 4) can often provide insights into the structural basis of agonist activity. Binding of a conformationally flexible peptide to its receptor is accompanied with the adoption of distinct secondary structures such as α-helix, β-sheet or extended β-turn conformations [140, 141]. Thus, a major aim of the application of conformational constraints is to determine which of these structures will be adopted by the receptor-bound ligand, in particular with respect to various receptor subtypes, which may recognize different conformations of the same ligand. Two major approaches may be distinguished, both directly affecting the peptide backbone and reducing the flexibility of the peptide chain: (i) the introduction of nonproteinogenic, conformationally constrained amino acids and (ii) the incorporation of amide bond surrogates.

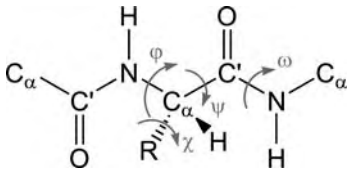


Fig. 4 Conventional notations of the torsion angles in a peptide chain

2.5.1 Amide Bond Replacements

Amide bond surrogates are bioisosteric groups that resemble an amide and range from simple olefinic groups to more sophisticated heterocycles. They have the additional advantage of not being cleaved by proteases which may possibly lead to orally active drugs with longer duration of action. Quite a number of amide bond replacements have been reported in the literature

in the last 15 years, but only a few of them are routinely used in the search for the bioactive conformation of peptide hormones and neuropeptides today. Among these amide bond replacements the most widely used methods result in pseudopeptides, depsipeptides, azapeptides and β-peptides and are sum-

Table 2 Structure, characteristics and recent applications of amide bond surrogates

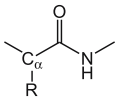
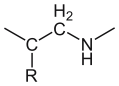
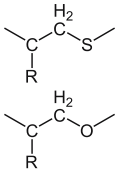
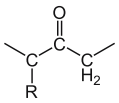
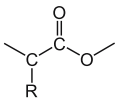
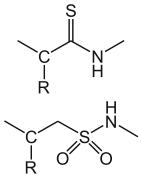
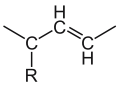
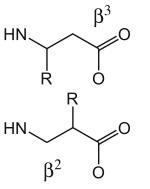
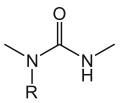
Type	Characteristics and application
	**Amide bond**
	**Aminomethylene, Ψ[CH_2NH]** • introduction of a new basic center due to reduction of the peptide bond and an ionizable site that can restore some hydrophilic properties often required for receptor recognition and binding • enhanced flexibility of pseudopeptides resulting in a more rapid synthesis of cyclic analogs • allows to investigate the importance of the carbonyl function Synthesis of pseudopeptide analogs of the C-terminal tridecapeptide of gastrin [237]. Synthesis and investigation of opioid activity of dynorphin analogs [238]. Development of gastrin-releasing peptide antagonists [239]. Synthesis of stabilized neurotensin analogs as potential radiopharmaceuticals [240]. SAR studies on nociceptin/orphanin FQ analogs [241]
	**Thiomethylene, Ψ[CH_2S]; oxomethylene, Ψ[CH_2O]** • polar, flexible and proteolytically inert surrogates; • preferred orientation of oxomethylene similar to the *trans*-amide bond; • thiomethylene less polar and acting as hydrogen bond acceptor only Synthesis of bombesin-like pseudopeptide agonists and antagonists [242, 243]. Synthesis of substance P analogs containing reduced peptide bonds in the C-terminal region may result in antagonists at NK receptors [244].
	**Ketomethylene, Ψ[$COCH_2$]** Synthesis of a series of Ψ[CH_2NH] and Ψ[$COCH_2$] pseudopeptide analogues of the C-terminal hexapeptide of neurotensin (NT8-13) revealed the importance of the CO group in the amide or surrogate linkage [245]
	**Ester, Ψ[COO]** • ester bonds of resulting depsipeptides exhibit many structural similarities to amide bonds since they are planar, strongly favor *trans*-conformation and possess similar bond angles and length • lack of hydrogen bond donating properties and descreased ability of the carbonyl oxygen to act as hydrogen bond acceptor allows to evaluate the role of hydrogen bonding in α-helices [246]

Table 2 (continued)

Type	Characteristics and application
	**Thioamide, Ψ[CSNH]; sulfonamide, Ψ[CH_2SO_2NH]** • Thioamide replacement restricts the allowable φ and ψ angles in the vicinity of the thioamide linkages Application of sulfonamide surrogates in a positional scanning of Leu-enkephalin revealed an increased stability toward proteolytic degradation [247, 248]. Synthesis and evaluation of beta-sulfonamido gonadotropin-releasing hormone analogs [249].
	**(*E*)-alkene, Ψ[(*E*)–CH=CH]** • *trans*-carbon–carbon double bond isosteres best mimic the transoid nature of the amide bond with respect to rigidity, planarity and bond length, thus providing valuable information of each amide bond in the peptide chain Synthesis and investigation of CCK-4 analogs [250].
	**β/γ-amino acids** • Oligomers thereof can fold into well-defined bioactive conformations containing secondary structures such as helices, sheets and turns that are analogous to those of proteins [251] Synthesis of human parathyroid hormone hPTH(1-34) analogs containing β-amino acid residues to investigate the importance of two helical segments that are located at the N- and C-terminal sequences for biological activity and binding affinity [252]. Substitution of the C-terminal α-helix of human interleukin-8 (hIL-8) by a modelled helical β-peptide undecamer resulted in the first active hIL-8 analog with slightly reduced affinity for CXC receptor 1 (CXCR1) while maintaining the selectivity for CXCR1 versus CXCR2, (personal communication with Ralf David, 2006)
	**α-aza-amino acids** • Isoelectronical replacement of the α-carbon of common amino acids with a trivalent nitrogen atom results in loss of chirality and in a structure that can be considered intermediate in configuration between D- and L-amino acids • Reduction of flexibility of the linear peptide has been predicted to occur in aza-peptides due to the more rigid urea $N_\alpha - C(O)$ structure • unproblematic synthesis allowing retention of amino acid side chain Partial aza-amino acid scans on potent tetrapeptide melanocortin receptor agonist, growth hormone secretagogue hexapeptide and human calcitonin-gene related peptide antagonist for probing the existence and importance of b-turn conformations in bioactive peptides [253]. Further applications have been recently reviewed [254].

marized in Table 2. Their characteristics and examples of recent application will also be mentioned.

2.5.2 Conformationally Constrained Amino Acids

The restriction of the conformational flexibility of the peptide backbone can also be achieved by the use of constrained amino acids. Thereby, introduction of a single amino acid as structural constraint, if carefully chosen, may have a dramatic effect on the structure of the whole molecule. Thus, various constrained amino acids can have direct influences on the shape of the peptide [142]. Methylation or even larger alkylation in the N- or C_α-position causes steric hindrance in the main chain but also in the side chain, resulting in conformational rigidity and also affects the hydrophobicity [143].

One of the most frequently used amino acids is α-aminoisobutyric acid (Aib). This achiral C_α-dimethylated amino acid exhibits interesting structural properties due to the steric hindrance of the extra methyl group. It is known to be a very strong β-bend and helix promoter, preferentially adopting 3_{10}-helical or α-helical structures. In recent years, Aib-containing N-terminal analogs of parathyroid hormone have been intensively studied with respect to conformation and biological activity [144–146]. The conformational preferences of other C_α-methylated amino acids have been reviewed by Toniolo et al. [147]. Different C_α-substituents forming quaternary amino acids lead to increased occurrence of β-turn structures as the steric hindrance increases [148].

N-Methylation of the amide nitrogen eliminates the potential formation of hydrogen bonds with the NH group in the peptide backbone. Moreover, the conformational space of amino acids is restricted to the β-region in a linear peptide; preventing interstrand hydrogen bonding at the same time, N-methylation can also act as a β-sheet breaker. The presence of the methyl group does not affect the modified amino acid thereby; steric interactions are exerted with the side chain of the adjacent residue. Furthermore, methylation of the amide nitrogen may prevent degradation of the peptide bond by endopeptidases and is therefore used to improve peptide stability. N-methylation has recently been applied to perform SAR studies on analogs of arginine vasopressin (AVP) [149], shortened insulin analogs [150], glucose-independent insulinotropic polypepeptide (GIP) [151] and in the search for potent somatostatin antagonists [152, 153].

An additional group of constrained amino acids is formed by the so-called cyclic amino acids, which are characterized by both amino and carboxylic acid groups being exocyclic to the ring. The introduction of β-aminocyclopropane carboxylic acids (βACC) into the C-terminal dodecapeptide of NPY, NPY(25-36), to rigidify the peptide backbone and to induce or stabilize distinct secondary structure motifs has been reported by Koglin et al. [154]. Strongly dependent on the position of substitution and configuration, βACC-containing analogs could be obtained, which bind with high affinity and selectivity to the Y_1 receptor subtype.

Depending on their structure, N_α-C_α-cyclic heterocyclic amino acids are constraining the peptide backbone dihedral torsion angle φ (similar to proline), restrain the side chain torsion angle χ and may provide an additional aromatic and hydrophobic residue in the peptide. Their incorporation into the peptide backbone has been therefore an increasingly popular way to elicit conformational constraints. The structures of several conformationally constrained cyclic and heterocyclic α-amino acids are shown in Fig. 5. Beck-Sickinger et al. have introduced various nonproteinogenic amino acids, among these cyclic and heterocyclic ones, to bridge N- and C-terminal segments of NPY to investigate the importance and requirements of the spacer [155]. The introduction of various nonproteinogenic amino acids instead of tryptophane in a melanocortin tetrapeptide revealed the importance of the tryptophane indole moiety. Whereas β-(2-naphthyl)-alanine has been tolerated, 1,2,3,4-tetrahydronorhaman-3-carboxylic acid and 4-phenylphenylalanine lead to an decrease of affinity at melanocortin receptor subtypes 3 and 4, but not at subtypes 1 and 5 [156].

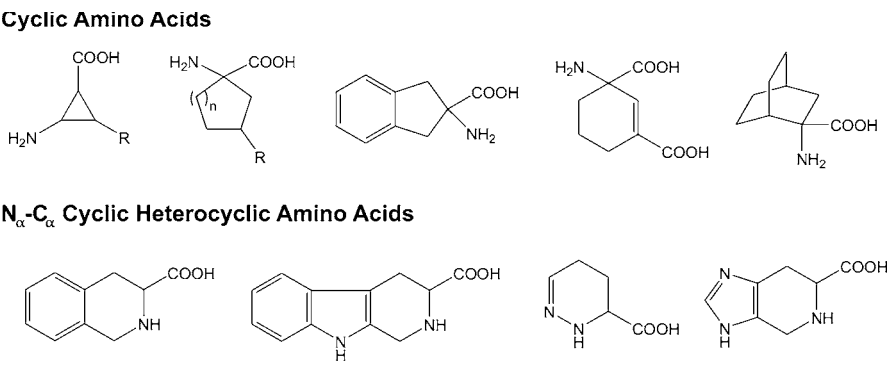


Fig. 5 Structures of conformationally constrained cyclic and N_α-C_α-cyclic heterocyclic α-amino acids

Elicitation of β-turn structures cannot only be achieved by lactamization or backbone-to-backbone cyclization as mentioned above; various bicyclic and spirocyclic compounds have been designed to mimic β-turns with decreased numbers of amide bonds. These originate from Freidinger lactams those synthesis and application have been recently reviewed by Freidinger [157] and Perdih et al. [158]. Some heterocyclic dipeptides which have been proposed to induce or stabilize β-turns in peptides are shown in Fig. 6. Martín-Martínez et al. reported on the comparative substitution of Met-Asp in CCK4 analogs by recognized β-turn inductor frameworks and a β-turn dipeptide to test whether a turnlike arrangement is involved in the bioactive conformation of CCK4 [159]. Whereas the dipeptide introduction led to inactive compounds, incorporation of the β-turn mimetics resulted in selec-

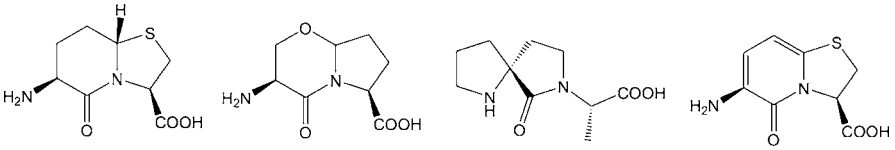


Fig. 6 Selected heterocyclic dipeptides that have been proposed to induce β-turns

tive and active CCK_1 receptor compounds. Seger and Geyer recently reported on the synthesis of a novel pyridone dipeptide chromophore [160], which is characterized by an N-terminal dehydroalanine moiety twofold linked to a C-terminal thiaproline. This N-terminally completely planar ($\varphi = 180°$, $\psi = 180°$) bicycle has been introduced into the N-terminus of NPY to further investigate the importance of this segment for the overall peptide structure in solution as well as for binding to the receptor [161].

2.6 Peptide Chimeras

The creation of chimeras, in which one segment of the molecule originates from one peptide, whereas the other part originates from a second peptide of the same ligand/receptor family, can be a useful method to determine structural characteristics that are the precondition for subtype specificity. Moreover, even the combination of segments from different peptide families has been reported in the literature.

Chimeric peptides have been used to study the interaction between galanin (GAL) and its three receptor subtypes. In recent years several chimeric peptides consisting of the N-terminal fragment 1–13 of galanin and C-terminal fragments of other bioactive peptides, such as substance P, neuropeptide Y, mastoparan and bradykinin, have been synthesized and investigated for their biochemical actions [162–166]. However, it is still unclear why some of these peptides act as antagonists whereas others act as agonists. Since different GAL fragments might be recognized as ligands by different receptors in a species and locus-specific manner, some authors suggest that the effects of some of the chimeric GAL ligands are mediated through other receptors that are still not characterized [162, 167, 168].

The peptides of the neuropeptide Y family have also been subjected to chimeric studies. Cabrele et al. reported on the synthesis of 38 full-length PP/NPY chimeras with binding properties that have been completely different from those of the two native ligands [169]. Structure-affinity relationship studies using transfected cell lines selectively expressing the human Y_1, Y_2, Y_4 and Y_5 receptor subtypes revealed the crucial role of the central NPY segment 19-23 for induction of the helical peptide conformation, as shown by circular dichroism, as well as for high affinity at all four receptor subtypes. Moreover, its presence in human PP (hPP) analogs increased their affinity at Y_1, Y_2 and

Y_5 receptor subtypes. Furthermore, position 34 has been found to play a role in inducing the bioactive conformation of the C-terminus of NPY, since substitution by Pro led to a complete loss of affinity at the Y_2 receptor, was tolerated at the Y_1 and Y_5 receptor subtypes, and could even enhance the affinity for the Y_4 receptor. Finally, the combination of the chicken PP(1–7) sequence with the NPY(19–23) sequence and the chicken PP residue His^{34} led to a human PP analog with sub-nanomolar affinity at the Y_5 receptor subtype. This has been the most potent Y_5 receptor ligand so far, as it shows 15-fold higher affinity than NPY. However, since this analog did not show selectivity, binding also to the Y_4 receptor subtype, Cabrele et al. combined the primary sequence of the highly potent chimera with the key motif Ala^{31}-Aib^{32} to obtain peptides with high affinity as well as high selectivity at the Y_5 receptor subtype. As a result, the porcine NPY analog combining the human PP(1-17) sequence and the turn-inducing Ala-Aib motif, [hPP^{1-17},Ala^{31},Aib^{32}]-pNPY, remained selective for the Y_5 receptor while showing sub-nanomolar affinity [80]. On the basis of the above findings, Dumont et al. developed and characterized a highly selective Y_5 receptor agonist radioligand, [^{125}I][hPP^{1-17},Ala^{31},Aib^{32}]-NPY [170]. However, the nonspecific binding obtained with this radioligand was too high to allow for detailed receptor autoradiography studies. The development of a new Y_5 receptor agonist radioligand, [^{125}I][cPP^{1-7},NPY^{19-23},Ala^{31},Aib^{32},Gln^{34}]-hPP, based on a peptide chimera that has been shown to be most potent at the Y_5 receptor subtype [80], turned out to be a useful tool to study the unique feature of the Y_5 receptor subtype [171].

2.7 Fluorescence Labels

The fluorescence labeling of peptides has been well established in order to study their uptake by cells [172–175], to investigate the internalization of ligand–receptor complexes upon ligand binding [176, 177], to probe the binding domain of the ligand at its specific receptor [178–180] or to develop FRET-based assays for the investigation of enzymes, such as proteases or deacetylases [181–183]. But nearly nothing has been reported on the application of fluorescence labeling techniques in order to determine the bioactive conformation of neuropeptides and peptide hormones. However, Bettio et al. reported on the synthesis of fluorescently labeled NPY analogs to study the solution conformation of NPY by fluorescence resonance energy transfer (FRET) [52]. A series of analogs with a Trp residue as fluorescence donor in the C-terminal segment and the dansyl fluorophor as fluorescence acceptor at various positions in the helical and N-terminal segments has been synthesized. They could show that FRET is a useful and convenient technique to investigate the structure of small proteins in highly diluted concentrations (μM range) where NMR cannot be applied. They could further show that the dissociation of the NPY dimer is not accompanied by a folding back of the

N-terminal segment on the C-terminal amphiphatic α-helix. They concluded that the PP-fold structure is not adopted by the NPY monomer in solution. Further studies using other FRET pairs investigating the conformation of the ligand on the surfaces of membranes, whole cells or solubilized receptors are currently in progress.

3
Investigation of the Ligand-Receptor Interaction

Specific interactions of ligands and receptors play a crucial role within biological systems. Approaches to understand and measure these constitute the basis for the identification of the bioactive conformation of the ligand and are an important part of modern drug development. A wide range of methods is available to investigate the binding of ligands at their receptors and to probe the intracellular effects on the signal cascade following the GPCR activation, including the determination of second messengers or nuclear activation. For a better understanding, the basic signaling pathways are shown and described in Fig. 7.

Functional assays in which cellular levels of second messengers, such as Ca^{2+} and IP_3, can be determined, as well as basic ligand binding assays based on radioactive and nonradioactive methods are shown in Fig. 8 and briefly reviewed in the following. Methods that can be applied to cell-based in vitro assays using eukaryotic cell-lines are exclusively discussed since there is a trend towards the development of such assays, for example, to replace animal studies. Therefore, receptor–ligand pairs with high specificity and/or activity are required, in which the given receptor is characterized by its ability to distinguish between structurally similar compounds and the ligand by its ability to activate intracellular signal pathways [187]. According to their signal transduction activities, ligands can be classified into agonists, antagonists and inverse agonists.

3.1
Theories of Receptor Binding

Traditionally and based on a two-state model of receptor function, ligands acting on GPCRs have been classified as agonists, partial agonists and antagonists. This model has been included in the ternary complex model, which was first published by De Lean and colleagues and describes a receptor that, when activated by an agonist, moves laterally within the cell membrane to physically couple to a heterotrimeric G protein [188]. The finding of constitutive activity (elevated agonist-independent activity) by Costa and Herz revealed a receptor behavior that could not be described by the original ternary complex model [189]. The extension of this model describes a receptor that exists in a spontaneous equilibrium between two conformations, active (R_a) and in-

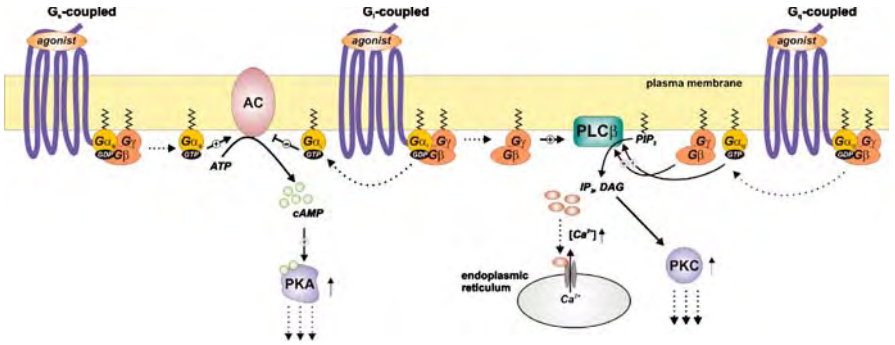


Fig. 7 Schematic illustration of classical GPCR signaling. Binding of an agonist causes a conformational change which allows the binding of an inactive heterotrimeric G protein [184, 185]. The exchange of G protein-bound GDP to GTP is accompanied by the dissociation of the ternary GPCR-$G_{\alpha\beta\gamma}$-GTP complex into free receptor, GTP-bound α-subunit and the $\beta\gamma$-dimer. Both GTP-bound α-subunit and $\beta\gamma$-dimer have the capacity to forward the signal by activation/stimulation or inhibition of effector proteins. At least 17 mammalian G protein α-subunits are known and classified into four classes based on their primary structure and the dependent signaling cascade. The $\beta\gamma$-dimers are formed by combination of at least five β-subunits and 12 γ-subunits [186]. The signal transduction of G_s protein-coupled receptors is characterized by the stimulation of adenylate cyclases (AC) by GTP-bound members of the $G_{\alpha s}$ family leading to an increased level of cyclic adenosine monophosphate (cAMP). This second messenger subsequently activates protein kinase A (PKA), which in turn can phosphorylate nearby substrates through its catalytic subunits. In contrast, receptors coupled to members of the $G_{\alpha i/o}$ family inhibit adenylate cyclase whereas the $\beta\gamma$-dimer acts stimulating on phospholipase C_β (PLC_β). PLC_β, which is also activated by the four members of the $G_{\alpha q/11}$ family, generates the water-soluble second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2). Cytosolic IP_3 triggers the release of calcium ions (Ca^{2+}) into the cytosol from intracellular stores (endoplasmic reticulum, ER) which subsequently regulate the activation of Ca^{2+}/calmodulin-activated protein kinase II (CaMKII). DAG remains at the membrane and increases the activity of membrane-bound protein kinase C (PKC). Both PKC and CaMKII are able to phosphorylate various substrates. Each isoform of $G_{\beta\gamma}$ dimers can act on a large set of effectors and regulators, including phospholipases, ion channels, phosphoinositide kinases and the ras/raf/ERK pathways

active (R_i), that are named for their ability to activate G proteins [190]. In this so-called extended ternary complex model, which is routinely used to simulate quantitatively GPCR behavior, *full agonists* stabilize the R_a conformation shifting the equilibrium toward the active state to generate full receptor activity and a maximal response. Ligands that have a lower intrinsic efficacy produce a sub-maximal system response and a potential attenuation of full agonist activation and are therefore called *partial agonists*. Agonists that are able to shift the equilibrium toward the inactive conformation, thus acting as antagonists in nonconstitutively acting systems, are named *inverse agonists*. They are able to actively reduce receptor-mediated constitutive activity

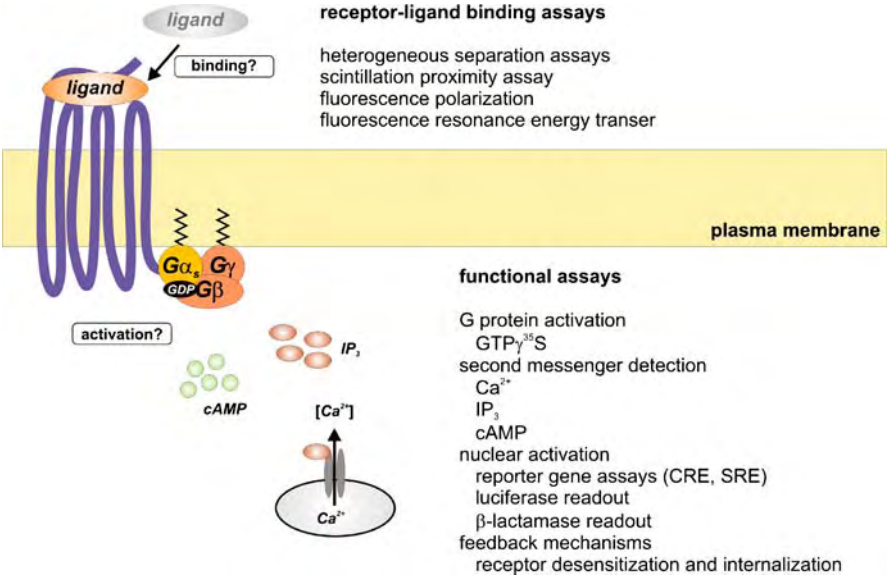


Fig. 8 Overview of widely used methods to investigate ligand–receptor interactions. Today, several radioactivity- and fluorescence-based methods are available to investigate the binding of a ligand to the receptor and to determine binding parameters such as K_d and IC_{50} as well as dissociation (k_{-1}) and association rates (k_{+1}). However, they do not allow drawing conclusions on the intrinsic efficacy of the ligand. Therefore, various so-called functional assays have been developed to detect G protein activation and measure changes in the intracellular level of second messengers. Moreover, since changes in the level of second messengers produce alterations in the gene transcription or protein activity, also these events can be measured. Finally, as a consequence of prolonged activation, receptors can be subject to feedback mechanisms including desensitization and internalization, which in turn can be measured by sub-cellular imaging techniques

in GPCR systems by binding preferentially to R_i. In contrast, *antagonists* bind indiscriminately to both R_a and R_i and produce no physiological response but block the response of agonists.

Since it is known that different areas of the cytosolic loops of receptors activate different G proteins [191], it is evident that agonists can stabilize multiple active receptor conformations, thus changing not only the degree, but also the "quality" of receptor activation [192]. Consequently, three-state to multistate models have been developed. Furthermore, in thermodynamic terms must be a provision for the inactive receptor to also interact with G proteins, which lead to a more complex model for GPCRs, named the cubic ternary complex model [193]. Taking into account the concomitant binding of an orthosteric ligand led to a thermodynamically complete, extended model, named the quaternary complex model [194]. The above mentioned and additional models have been recently reviewed by Maudsley et al. [192], Kenakin et al. [195] and Christopoulos et al. [196]. Therein, the potential for allosteric

effects arising from receptor oligomerization and formation of complexes with accessory cellular proteins are discussed, which consequently led to allosteric receptor models. Furthermore, the importance of pharmacological receptor theory for drug design in the past 25 years has been reviewed.

3.2 Receptor-Ligand Binding Assays

The application of receptor–ligand binding assays in basic research and analytical chemistry is versatile. Numerous assay formats are available that can be used to screen and quantify receptor ligands as well as for the investigation of the receptor itself by determining receptor distribution and identifying receptor subtypes. On the one hand, assay technologies can be classified into radioactive and nonradioactive ones. While radio binding assays are fast, easy to use and reproducible, their major disadvantage lies in their risk for human health and in the production of radioactive waste. This has led to the development and enhancement of assays based on optical methods, such as surface plasmon resonance (SPR), fluorescence correlation spectroscopy (FCS), fluorescence polarization (FP) and fluorescence resonance energy transfer (FRET). On the other hand, receptor binding assays may be classified according to the need for separation of bound from free ligand into heterogeneous, homogeneous and nonseparating homogeneous assays. In heterogeneous assays the free ligand has to be separated from the bound fraction by centrifugation, filtration or dialysis before measurement. In contrast, homogeneous assays do not require any washing or separation step before measurement and are therefore called mix-and-measure assays. If the signal is centered on or around a solid phase which contains the immobilized receptor or ligand, one will speak of a nonseparating homogeneous assay format in which a physical separation of free and bound fractions is not needed [197]. Most of the assays require labeling of either the ligand or the receptor, but there are also label-free screening methods available [198]. Despite the advantages of radio-isotopic labels, such as ^{3}H, ^{125}I and ^{32}P, which have only a negligible less effect on the affinity of the ligand towards the receptor, efforts have increased to develop new technologies based on either colorimetric, fluorescence or luminescence detection systems. Today, a multitude of small-molecular fluorophores exhibiting improved physical properties (e.g. water solubility, pH stability) as well as a high quantum yield, a high extinction coefficient to enable sensitive detection in aqueous media, photostability and a high excitation wavelength to reduce autofluorescence are available. Preferably, they can be readily attached to regions of the ligand that have been found to be not involved in binding [199, 200]. However, fluorescence-based detection methods exhibit also some disadvantages since fluorescence signals may be quenched by other assay compounds, the biological matrix or even plastic materials. Furthermore, fluorescence from proteins or other cellular

components can give rise to high background signals and, much more important, introduction of the fluorescence label may affect the affinity. Some of the problems can be overcome by the utilization of long-lifetime lanthanides that allow time-resolved fluorescence measurements with higher sensitivity and precision [201–203]. The generation of chemoluminescence or bioluminescence upon oxidation of luminol by peroxidase and luciferin by luciferase, respectively, offers an alternative detection technique [204]. Furthermore, luminescence can be generated nonenzymatically from acridinium esters upon addition of hydrogen peroxide in an alkaline environment [205]. Advantages of luminescence-based methods are the lack of background of matrix or scattering and an outstanding sensitivity.

The basic principle of receptor binding assays is the competition between an unlabeled ligand I (analyte, inhibitor) and a labeled ligand L for binding to a certain receptor R. The relationship between the labeled ligand, the receptor and the resulting ligand–receptor complex is given in Eq. 1 and follows the law of mass action assuming reversible binding.

$$[\mathrm{R}] + [\mathrm{L}] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [\mathrm{RL}] \,. \tag{1}$$

At equilibrium, the dissociation constant K_d, given as ratio k_{-1}/k_{+1}, can be determined as shown in Eq. 2. It represents the amount of ligand that saturates 50% of the binding sites.

$$K_\mathrm{d} = \frac{k_{-1}}{k_{+1}} = \frac{[\mathrm{L}] \cdot [\mathrm{R}]}{[\mathrm{RL}]} \,. \tag{2}$$

Kinetic experiments, in which different incubation times are studied, investigate association and dissociation of the ligand–receptor complex, respectively. The association rate k_{+1} characterizes the time to reach equilibrium at certain concentrations of ligand and receptor. In turn, the dissociation rate k_{-1} describes the time of the ligand bound to the receptor. The determination of the binding constant K_d is performed by saturation of the receptor binding sites at a high concentration of labeled ligand ($> 10\ K_\mathrm{d}$). Such assays measure the amount of receptor-bound ligand as a function of the ligand concentration. Further, the total number of specific binding sites B_max can be derived from Eq. 3 and allows the calculation of the average number of receptors per cell. Today, calculation of K_d and B_max is achieved by nonlinear regression using curve fitting/biostatistic programs such as GraphPad Prism or Systat SigmaPlot.

$$[\mathrm{RL}] = \frac{[\mathrm{L}] \cdot B_\mathrm{max}}{[\mathrm{L}] + K_\mathrm{d}} \,. \tag{3}$$

Introduction of a competing inhibitor results in the formation of two different receptor complexes. The inhibitor will displace a certain amount of the labeled ligand, depending on its concentration and own affinity to the

receptor. Utilization of a well-characterized labeled ligand and receptor at constant concentrations while simultaneously varying the concentration of the unlabeled ligand allows the construction of inhibition curves. From these curves, the IC_{50} value, which represents the inhibitor concentration needed to displace 50% of the bound labeled ligand, can be determined. This IC_{50} value is related to the affinity constant K_i of the inhibitor as expressed by the Cheng–Prusoff equation (Eq. 4) [206]. K_i allows the comparison of data from different experiments and systems since it is independent of the labeled ligand concentration and even the method.

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}} . \tag{4}$$

In case of high receptor concentration or high affinity of the labeled ligand for the receptor resulting in ligand depletion, the equation loses its validity since it is based on the assumption that the free and total labeled ligand concentrations are approximately equal [207].

Figure 9 summarizes and briefly explains the basic principles of heterogeneous receptor–ligand binding assays as well as of fluorescence polarization, fluorescence resonance energy transfer and the scintillation proximity assay (SPA). Additional fluorescence-based methods that are used for the investigation of receptor–ligand interactions have been discussed in detail in the literature and recently reviewed by de Jong et al. [197]. These methods include fluorescence correlation spectroscopy [208–210], chip-based assays such as total internal reflection fluorescence [211–213] and surface plasmon resonance [198, 214], flow cytometry [215, 216], AlphaScreen™ [217] and the fluorometric microvolume assay technology [218]. Since for most of them, immobilization of the receptor (or ligand) is required, they are not applicable for the investigation of all ligand–receptor complexes.

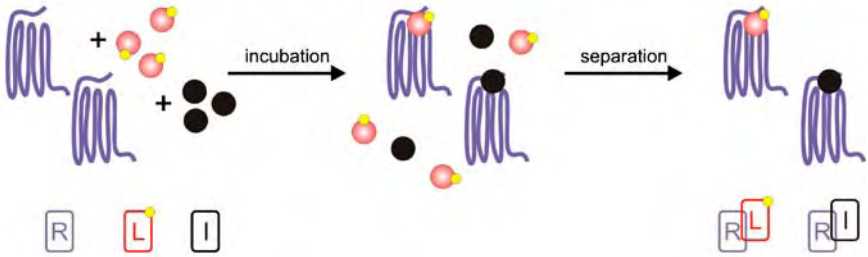
However, receptor–ligand binding assays do not allow the determination of the intrinsic efficacy (agonistic or antagonistic) of these compounds, and functional studies have to be conducted [219].

3.3 Functional Assays

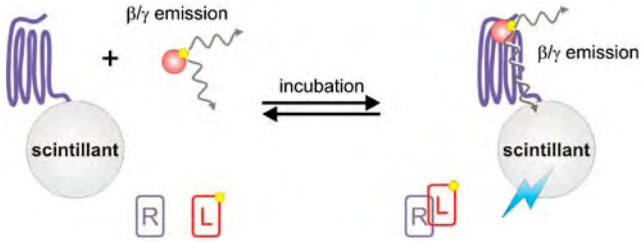
Both affinity and efficacy are two fundamental and distinct characteristics of the ligand–receptor pair pharmacology and, in cell-based GPCR assays, the question arises to measure affinity or efficacy [228, 229]. Functional cellular assays based on G protein activation, determination of second messengers or transcription activation can yield more information than ligand–receptor binding assays. Key advantages of employing these assays are their ability to distinguish antagonists from agonist or inverse agonists, their improved sensitivity to agonism and, finally, they allow the search for allosteric modulators

Fig. 9 **A** Heterogeneous receptor–ligand binding assays are based on the competitive interaction between a labeled ligand (L) and a non-labeled inhibitor (I) for the same receptor binding site (R). They have been developed using radioactively labeled ligands for binding to a membrane bound receptor first [220]. Advantages of radioligand binding assays are their sensitivity, specificity and ease of use. These assays usually require only one labeling step leading to a ligand with only low or even not reduced affinity for the receptor. However, a drawback of these assays is that after incubation and before measurement free ligand has to be removed from the bound fraction by filtration, centrifugation or dialysis, what makes them relatively slow. And, along with that, the dissociation of the ligand has to be much slower than the time needed to perform the separation of bound and free ligand. In principle, fluorescently labeled ligands can be used instead of radioactively labeled ones. Herein, the major disadvantage of significant background fluorescence can be overcome by the application of lanthanide-chelated ligands in combination with time-resolved fluorescence measurements [201, 202]. **B** Scintillation proximity assays (SPAs) have been developed to overcome the necessity to separate the free from the bound fraction [221, 222]. SPAs require the immobilization of the receptor on a solid surface (bead) and the ligand labeled with a radioactive isotope. The bead contains a scintillant emitting light as a result of energy transfer and upon binding of the ligand to the receptor, thus attaining close proximity to the bead. Despite the mix-and-read format of this assay that makes it easy to automate and enhances reliability, major drawbacks are the necessity to immobilize the receptor on a solid surface, where it should remain stable and maintain its affinity, the sensitivity that is limited by the receptor binding capacity on the beads and the long incubation time (18 h) in comparison to a filtration assay (90 min) [197]. **C** Fluorescence resonance energy transfer (FRET) is based on the transfer of energy upon excitation from a donor molecule via dipole–dipole interactions to the acceptor molecule, without emission of a photon [223]. The acceptor molecule has to be in close proximity to the donor molecule (distance smaller than 10 nm, e.g. after binding to the receptor which bears a donor molecule). Moreover, the excitation spectrum of the acceptor and the emission spectrum of the donor should interfere with each other. The principle of FRET is used for most of the mix-and-measure assays. Disadvantages of this method are the strict distance constraints and the need to label both receptor and ligand. If an antibody is used to label the receptor, one has to ensure that the antibody will not block the ligand-binding site. To overcome the drawback of background fluorescence, again lanthanide chelates with a long excited state lifetime can be used as donor molecules in time-resolved FRET studies (TR-FRET) [224]. The utilization of a bioluminescent donor in combination with a fluorescent acceptor is called bioluminescence resonance energy transfer (BRET) and is more sensitive than FRET since the enzyme reaction leading to bioluminescence does not produce background fluorescence [225]. **D** In the fluorescence polarization or fluorescence anisotropy assay the change in polarization of light emitted from a fluorescently labeled ligand as a consequence of the change of its mobility is measured [226, 227]. Because of the rapid tumbling of a free low-molecular weight ligand, excitation by polarized light results in the emission of depolarized light. In contrast, binding of the ligand to the receptor results in the decrease of the rotational speed of the high-molecular weight receptor–ligand complex. Consequently, the emitted light remains partially polarized. The advantage of this very simple technique over FRET is the requirement of only one labeling step. However, disadvantages will be a lack of precision at low nanomolar concentrations and the restriction to low-molecular weight ligands (< 5 kDa), if fluorophores with short excited lifetimes are used ►

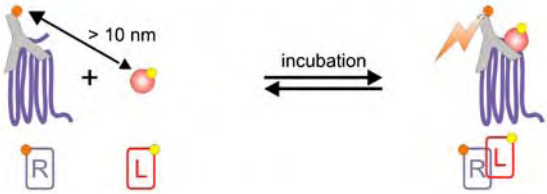
A Heterogeneous Receptor-Ligand Binding Assay



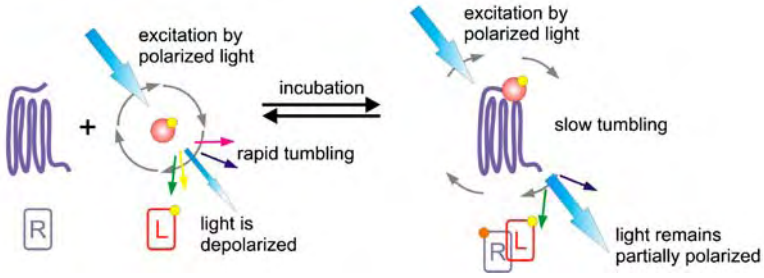
B Scintillation Proximity Assay



C Fluorescence Resonance Energy Transfer



D Fluorescence Polarization



that act at receptor sites other than the binding site of the endogenous agonist. Before applying or developing functional assays, various considerations have to be made regarding the functional protein equipment of the cell line to choose for recombinant receptor expression and thus for the assay. Not only is it that most GPCRs undergo post-translational modifications [230] or that their expression, pharmacology and signaling profile may be influenced by GPCR-interacting proteins (GIPs) [231], they also may undergo homodimerization and/or heterodimerization, which can also influence receptor expression and pharmacology [232]. In addition, several GPCRs have been shown to possess multiplicity of coupling, thus activating multiple G proteins [233, 234], or even showing a G protein-independent pathway (David R, personal communication). In contrast, most functional assays will capture only one signaling pathway resulting in the missing of a potentially valuable compound if this compound does display functional selectivity.

Common functional assays for screening GPCRs have been reviewed recently in detail by Thomsen et al. [236]. The basic principles, advantages and drawbacks of selected assays are summarized in Table 3.

Table 3 Widely used functional assays for screening GPCRs

Biological measurement	Basic principle	Advantages, disadvantages
G protein activation		
membrane-based GPCR-mediated guanine nucleotide exchange	irreversible [^{35}S]-GTPγS binding to receptor-activated G proteins, radiometric endpoint measurement	early event in signal transduction cascade, thus less subject to amplification or regulation by other cellular processes; generally limited to $G_{\alpha i/o}$-coupled receptors; assay usually requires separation step [255], which can be avoided using SPA beads [256, 257]
Determination of second messengers		
cell-based cAMP accumulation	detection of changes in intracellular cAMP by the competition between cellular cAMP and a labeled form of cAMP for binding to an anti-cAMP-antibody	screening of $G_{\alpha s}$-coupled receptors generally straightforward; need of forskolin stimulation of the adenylate cyclase to maximize the inhibition signal [258, 259]
	[^{125}I]-cAMP: radiometric measurement using SPA beads or scintillant coated plates	homogeneous assays, which do not require washing steps, amenable to automation

Table 3 (continued)

Biological measurement	Basic principle	Advantages, disadvantages
	biotinyl-cAMP with biotin-moiety binding with high affinity to streptavidyl-"donor" beads: decrease of luminescence upon competition of biotinyl-cAMP by cellular cAMP for binding to antibody immobilized on "acceptor" beads	homogeneous assay, high sensitivity, temperature- and light-sensitive
	fluorescently labeled cAMP: decrease of fluorescence polarization upon binding to the antibody or decrease of FRET upon binding to a fluorescently labeled antibody	homogeneous assay, lower signal-to-noise ratio
cell-based inositol phosphate (IP) accumulation	quantification of PLC stimulation by measuring the amount of either total IP or individual phospho-inositides (IP, IP_2, IP_3) by radioactive phospho-inositol turnover assay	screening of $G_{\alpha q}$-coupled receptors, non-homogeneous, low throughput [260, 261]
cell-based increase in intracellular Ca^{2+}	increased fluorescence upon binding of intracellular calcium chelating dye	screening of $G_{\alpha i}$- and $G_{\alpha q}$-coupled receptors, homogeneous assay, sensitive [262, 263]
	NMR frequency shift of Ca^{2+} chelators with magnetic properties of ^{19}F upon binding of calcium	application especially for opaque cell suspensions and tissues that cannot be measured by fluorescence techniques [264]
Nuclear activation		
cell-based, increases in reporter gene expression due to increase in second messenger	changes in intracellular second messenger levels alter expression of a selected reporter gene, for which several promoters are commercially available. Detection via fluorescence, luminescence or absorbance	screening of $G_{\alpha i/o}$-, $G_{\alpha s}$- and $G_{\alpha q}$-coupled receptors, homogeneous assay, sensitive, long incubation time, distal to receptor activation, thus subject to regulation or amplification by other cellular processes [265]

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Top Curr Chem (2007) 278: 289–309
DOI 10.1007/128_2007_124

Published online: 11 April 2007

Automated Solid Phase Oligosaccharide Synthesis

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Abstract Of the three classes of biopolymers—nucleic acids, proteins and glycoconjugates, nucleic acids and proteins have seen the most breakthroughs in understanding their biological role, in part due to their ready availability. The automation of oligonucleotide and peptide synthesis has been fruitful in providing biologists and biochemists with pure, well-defined structures. This work reviews the recent developments in the automated synthesis of oligosaccharides, the third class of biopolymers. Both glycosyl phosphates and glycosyl trichloroacetimidates have been used successfully in the automated assembly of oligosaccharides employing an octenediol-functionalized polystyrene resin. The product was cleaved either by methanolysis of an ester bond or by olefin cross metathesis. Several biologically important carbohydrates have been synthesized by automation, in a fraction of the time needed to synthesize them by traditional methods. For example, the tumor associated antigens Lewis *Y*, Le^y-Le^x, were synthesized by automation. A *Leishmania* cap tetrasaccharide and a malaria toxin vaccine candidate were also assembled.

Keywords Automation · Carbohydrate synthesis · Oligosaccharides · Solid-phase

Abbreviations

CM	Cross-metathesis
DIPC	Diisopropylcarbodiimide
DMDO	Dimethyldioxirane
DMT	4,4′-Dimethoxytrityl
Fmoc	9-Fluorenylmethoxycarbonyl
Lev	Levulinoate
Phth	Phtalimido
Pip	Piperidine
TCA	Trichloroacetate
TMSOTf	Trimethylsilyl trifluoromethanesulfonate

1 Introduction

Three repeating biopolymers dominate the signal transduction processes in living organisms—nucleic acids, proteins and glycoconjugates. The role of nucleic acids and proteins has been the subject of intense research and development. The synthetic advances have fueled the emergence of several powerful tools for the identification of carbohydrate structure, function and interactions with other biomolecules. In turn, these tools, have allowed more discoveries. The pharmaceutical and biotechnology areas have seen several fundamental breakthroughs in genomics and proteomics. Detailed pictures of interactions of nucleic acids and proteins with each other or with themselves are starting to emerge (Fig. 1). These insights have led to the identification of many therapeutic approaches and opportunities. Glycomics, however, has been less understood, which leaves important areas of research and many therapeutic opportunities untapped.

Carbohydrates are mostly present in nature in the form of glycoconjugates (glycoproteins and glycolipids) [1]. Their role is unambiguously important but remains often vague. If the understanding of the biological role of carbohydrates is to approach that of nucleic acids and proteins, access to well-defined pure oligosaccharide structures will have to be improved. Compared to the other two biopolymers, the availability of a wide range of pure and defined oligosaccharides has been lagging behind. Several facts are responsible. First, there is no amplification system analogous to the polymerase chain reaction (PCR) of nucleic acids. Second, carbohydrates have been difficult to isolate from natural sources because of their often-heterogeneous nature. There is no recombinant DNA technique to produce them like for proteins. Third, the chemical synthesis of carbohydrates has been more challenging than that of peptides and oligonucleotides. Unlike amino acids and

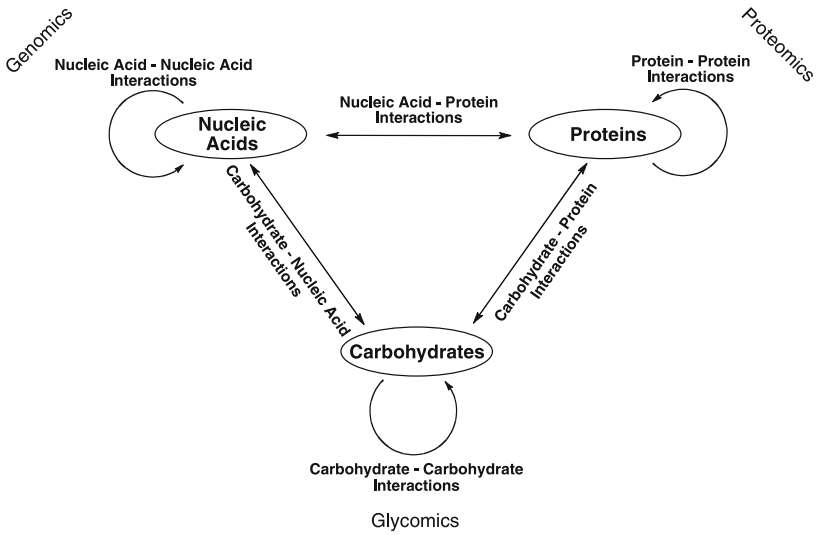


Fig. 1 Biopolymer interactions

nucleotides, monosaccharides have several possible points of attachment and the stereochemistry of the glycosidic bonds has to be defined. The lack of information regarding the structure of oligosaccharides has aggravated this situation. While the sequencing of samples of oligonucleotides and proteins is routine and has been automated, carbohydrate sequencing has been particularly challenging. More breakthroughs will be needed in this field even though progress is being made [2].

Both oligonucleotides [3] and peptides [4] can now be synthesized in an automated fashion, making them widely available. Non-experts can perform the synthesis of oligonucleotides and peptide on a routine basis. That development has allowed much progress in understanding the precise role of proteins and nucleic acids in biological systems.

Synthetic carbohydrate chemists have developed powerful methods to assemble complex carbohydrates [5]. Still, oligosaccharide synthesis remains technically challenging, and is limited to some expert labs. The number of different approaches that are available are not necessarily compatible with each other. A more unified and simplified approach for the synthesis of carbohydrates could tremendously benefit the field of glycomics by making a large variety of compounds available.

Automated oligosaccharide synthesis constitutes a useful tool. The basic idea of automation has now attracted the attention of several groups and drastically different approaches are being pursued in this direction. The group of Takahashi focused on automated parallel solution-phase synthesis of oligosaccharides based on a one-pot procedure [6]. A reactivity-based one-pot reaction has been pursued by Wong [7, 8]. Several groups are working on

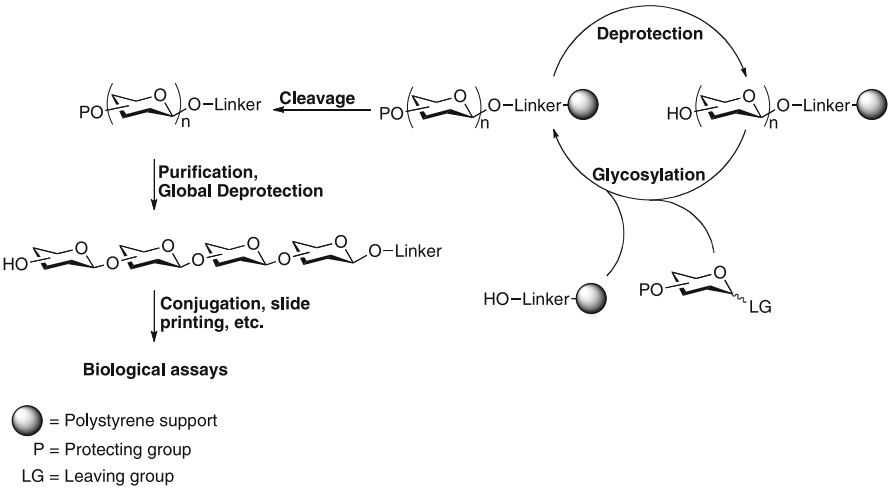


Fig. 2 Automated solid phase oligosaccharide synthesis

a fluorous linker-based strategy for carbohydrate synthesis that is potentially amenable to automation [9–11]. Nishimura's group developed the "Golgi" apparatus [12] based on enzymatic glycosylations. The basis of automation of oligonucleotides and peptides was the use of solid phase synthesis, where excess of reagents can be used to drive the coupling reactions to completion and no purification is necessary between the different steps. Solid phase oligosaccharide synthesis [13] has been performed since the early 1970s [14], but was not automated until 2001 [15]. The general scheme is depicted in Fig. 2. A linker is glycosylated followed by removal of excess reagent by washing. Then, a deprotection step reveals a new acceptor hydroxyl group. This cycle is repeated as many times as needed, after which, the resin-bound carbohydrate is cleaved from the solid support. Purification and deprotection yields an oligosaccharide that can be used in several different experiments. Several aspects of automated solid phase synthesis of oligosaccharides will be discussed here.

2
Automation Platform

Different instruments are now available for automated synthesis on solid phase. The Applied Biosystems peptide synthesizer ABI 433 (Fig. 3) was chosen as an initial platform due to its wide availability and the few modifications necessary for its application to oligosaccharide synthesis. The custom-made reaction vessel is constructed of jacketed glass to allow cooling. The building blocks are placed in cartridges to be delivered sequentially. The synthesizer allows for nine different solvents and reagents. A new, more versatile and less

Fig. 3 Modified peptide synthesizer

expensive platform, based on syringe pumps is currently being explored in our laboratory.

The programming of the instrument can be modified easily to accommodate different cycles of glycosylations, deprotections as well as different washing procedures.

3 Synthetic Strategy

Two general strategies are available for solid phase oligosaccharide synthesis, the acceptor-bound and the donor-bound strategy (Fig. 4). Both can also be combined in a bi-directional approach.

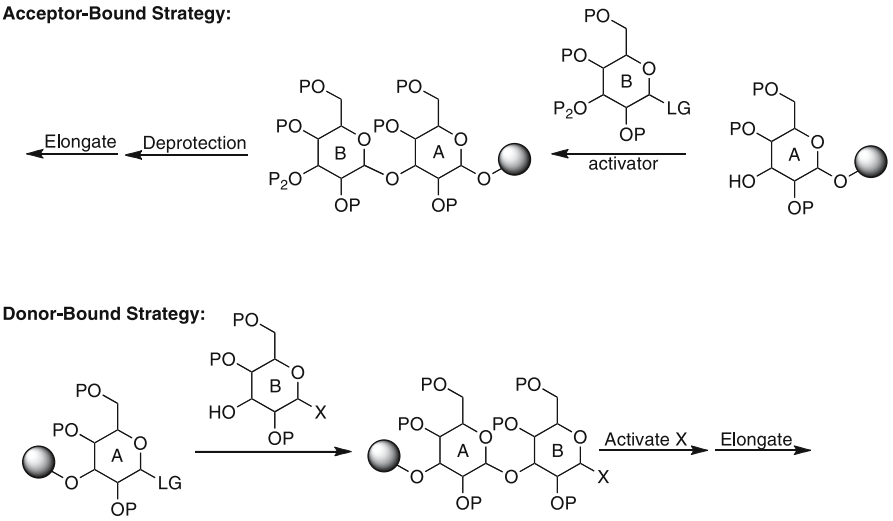


Fig. 4 Synthetic strategies

The acceptor-bound strategy has the "reducing end" of the oligosaccharide bound to the resin exhibiting a free alcohol. A building block and activator are added, commonly used in excess, to glycosylate the hydroxyl group. One or more hydroxyl groups on the newly incorporated sugar can be deprotected for elongation. The donor-bound strategy anchors the "non-reducing end" of the oligosaccharide to the solid support. An acceptor building block with a free alcohol and a stable anomeric leaving group precursor is added to the activated resin-bound donor. The newly added building block is then transformed into a donor and activated for elongation.

The acceptor-bound strategy permits more reactive building blocks, because decomposition of the donor results in soluble by-products that can be washed away from the resin-bound oligosaccharide. This strategy was ultimately used for automation.

4
Support and Linker

4.1
Solid Support

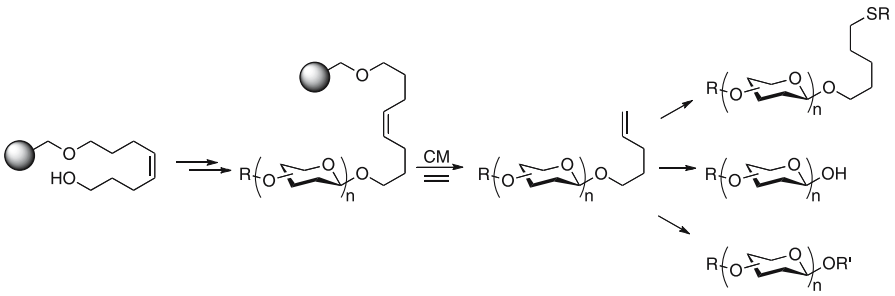
Non-soluble polymers were chosen as support for automation rather than soluble supports due to the ease of filtration through regular filters. A variety of polymer supports are commercially available including polystyrene-based

polymer, such as Merrifield [4] and TentaGel [16], and non-swelling controlled pore glass (CPG) support used in DNA synthesis.

Polystyrene resins have been successfully used in peptide synthesis and in organic solid-phase synthesis [17]. Polystyrenes exhibit good swelling properties in organic solvents that are commonly used in glycosylation reactions, such as dichloromethane and toluene. Swelling in more polar solvents is more difficult, but some polar solvents like DMF and THF exhibit good swelling properties. The overall stability of polystyrene towards acids and bases is an attractive feature for automated solid-phase carbohydrate synthesis.

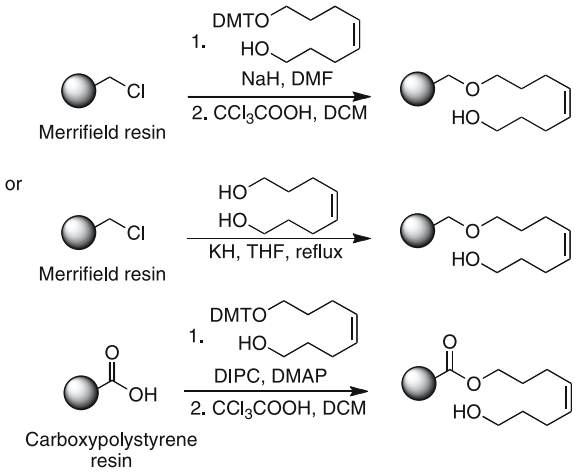
4.2 Linker

The polystyrene resin can be functionalized in various ways. The linker is of crucial importance for successful solid-phase synthesis because it has to remain intact during the synthesis cycles but should be easily cleaved in high yield with reagents that are not going to affect the synthesized carbohydrate. The two linkers used successfully for automated synthesis to date were both based on an octenediol linker (Scheme 1) attached to the resin via an ether [18] or an ester bond [19]. The octenediol linker has several advantages. First, the double bond is inert to many glycosylation strategies with the notable exception of thioglycosides. Second, the linker can be cleaved selectively by olefin cross-metathesis (CM). Finally, the resulting pentenyl group on the anomeric position can be activated [20] for glycosylation or hydrolysis or functionalized by radical-initiated addition of a thiol [21].



Scheme 1 Octenediol linker cleavage and modifications

The linker was synthesized from Merrifield resin or carboxypolystyrene and a DMT-protected diol (Scheme 2). The cleavage of the resulting DMT-protected linker allowed for UV quantitation of the resin loading [22]. The linker can also be installed directly from the diol [23]. The loading can be determined by Fmoc quantitation [24].



Scheme 2 Linker syntheses

5 Building Blocks

Carbohydrates contain a large variety of possible attachment and branching points. If one considers the ten mammalian monosaccharides [glucose (Glc), galactose (Gal), mannose (Man), sialic acid (Sia), glucosamine (GlcNAc), galactosamine (GalNAc), fucose (Fuc), xylose (Xyl), glucuronic acid (GlcA) and iduronic acid (IdoA)] 224 different building blocks would be

Table 1 The most abundant monosaccharide units found in mammalian glycans

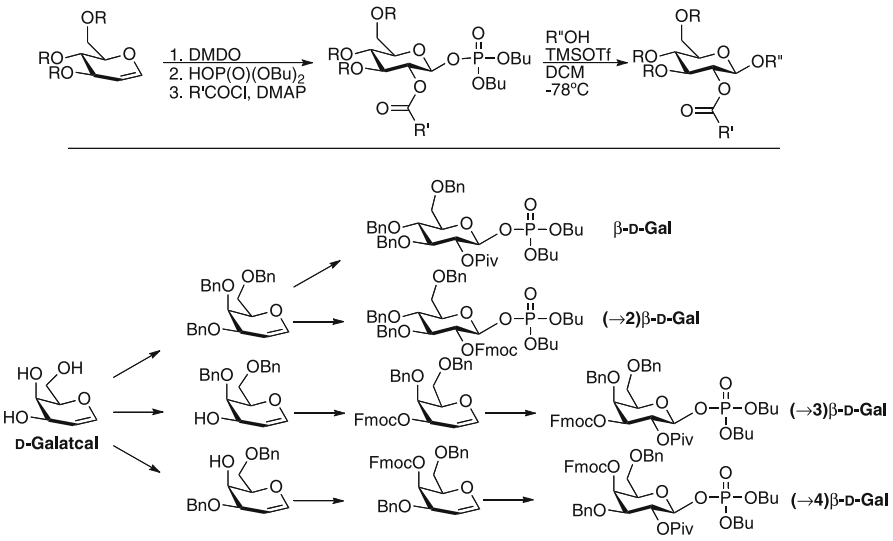
Monosaccharide unit	Abundance (%)
(→4)β-D-GlcNAc	21.7
(→3)β-D-Gal	9.5
α-D-Sia	8.8
α-L-Fuc	8.0
β-D-Gal	7.8
(→2)α-D-Man	7.1
(→3)(→6)β-D-Man	4.7
α-D-Man	2.8
β-D-GlcNAc	2.7
(→6)β-D-Gal	2.4
(→3)(→4)β-D-GlcNAc	2.3
(→2)(→4)α-D-Man	2.1
(→4)β-D-Glc	1.6
(→2)β-D-Gal	1.5

required theoretically to assemble any given oligosaccharide. A detailed statistical analysis (Seeberger, unpublished results) of the online database *glycosciences.de* [25] revealed that a far smaller number of building blocks would be necessary to construct a large fraction of the mammalian *N*- and *O*-linked oligosaccharides. In fact, a set of 25 building blocks would suffice to assemble 60% of the 3266 mammalian oligosaccharides contained in the database, and 65 building blocks would enable access to 90% of these oligosaccharides. A ranking of the most important monosaccharide units by abundance is depicted in Table 1.

Several building blocks giving access to these linkages have already been successfully used on solid support. A building block for automated solid phase synthesis needs to meet several criteria: it must contain a stable anomeric leaving group that can easily be activated. The temporary protecting group should easily be removed for the next glycosylation but needs to be stable to the glycosylation conditions. The glycosylation should be stereoselective, which usually implies an ester participating group on the C2 hydroxyl. The two types of anomeric leaving groups used for automated synthesis are glycosyl phosphates and glycosyl trichloroacetimidates.

5.1 Glycosyl Phosphate Building Blocks

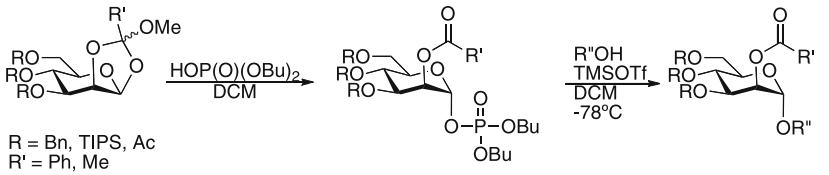
Glycosyl phosphates are efficient glycosylating agents for oligosaccharide synthesis [26–32] and can be readily obtained from glycals [33] via a one-pot



Scheme 3 One-pot formation of different glycosol phosphates

procedure [30, 31] (Scheme 3). The glycal precursors simplify the protection scheme as only three hydroxyls have to be differentiated. For example, galactal can yield four different building blocks - terminal, 2-linked, 3-linked, or 4 linked in only two or three steps.

The glycosyl phosphates can also be obtained from orthoesters in a single, high-yielding reaction (Scheme 4) [34].

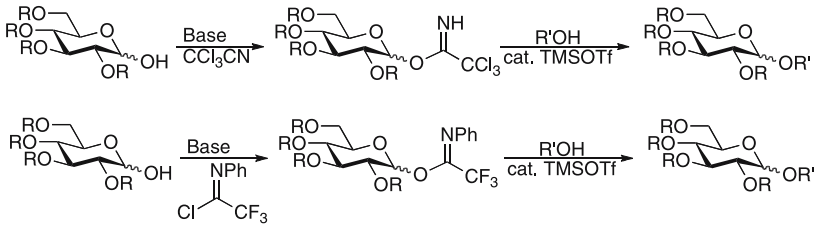


Scheme 4 Glycosyl phosphate synthesis from orthoesters

The activation of a phosphate group requires a stoichiometric amount of Lewis acid. Glycosyl phosphates can generally be stored for several months in the freezer without significant degradation.

5.2 Glycosyl Trichloroacetimidate Building Blocks

Glycosyl trichloroacetimidates [35] have been used most extensively in solution phase chemistry. These building blocks are easily obtained from the free anomeric lactol and can be activated under mild conditions with a catalytic amount of Lewis acid (Scheme 5). More recently, a variation of this important class of glycosylating agent gained some attention. The *N*-phenyl trifluoroacetimidate is as easily prepared and is activated under similar conditions [36]. This type of anomeric leaving group has shown advantageous reactivities when compared to the trichloroacetimidate [37]. This glycosylating agent can also be used on solid support [23].

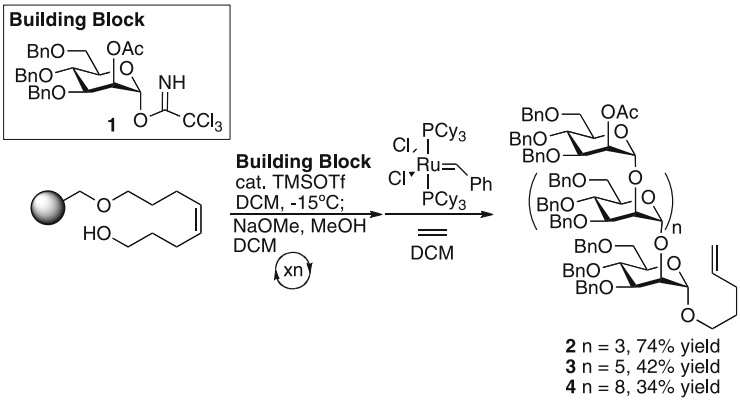


Scheme 5 Glycosyl trichloroacetimidate and *N*-phenyl trifluoroacetimidate building blocks

6
Automated Synthesis

6.1
α-Mannan and β-Glucan

The Seeberger group reported the first automated synthesis of oligosaccharides in 2001 [15] using the synthesis of poly α-(1→2) mannosides (Scheme 6) and a phytoalexin elicitor β-glucan (Scheme 7) as examples. The polymannoside was assembled using mannosyl trichloroacetimidate building block **1**. The building block was used in a ten-fold excess to react with the octenediol functionalized resin in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate for 30 minutes. The glycosylation was repeated once and the resin was washed with several solvents. The C2 hydroxyl was deprotected by treatment of the resin with an excess of sodium methoxide in a methanol/dichloromethane mixture. This sequence was repeated a number of times to obtain penta-, hepta- and deca-saccharide **2**, **3** and **4** in good overall yield after cross-metathesis with ethylene.



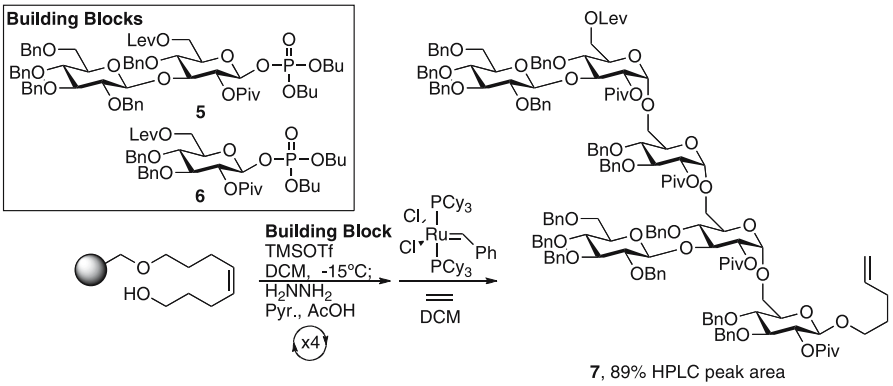
Scheme 6 Automated polymannose synthesis

The coupling cycle used for this synthesis is shown in Table 2. One cycle is completed in 2.6 h. The cycle is repeated for each building block incorporation such that the pentasaccharide was assembled in only 14 h.

Soybean plants can produce the antibiotic phytoalexin when exposed to a fungal β-glucan. A phytoalexin elicitor hexasaccharide was synthesized by automation using glycosyl phosphate building blocks (Scheme 7). The functionalized resin was submitted to building blocks in a five-fold excess with stoichiometric amounts of trimethylsilyl trifluoromethanesulfonate for 15 minutes at – 15 °C. The resin was washed and treated with hydrazine to affect the deprotection of the C6 hydroxyl. The sequence was repeated three times,

Table 2 Coupling cycle for automated synthesis using trichloroacetimidate building blocks and acetate protecting group

Step	Function	Reagent	Time (min.)
1	Couple	10 eq. Building block and 0.5 eq. TMSOTf	30
2	Wash	CH_2Cl_2	6
3	Couple	10 eq. Building block and 0.5 eq. TMSOTf	30
4	Wash	CH_2Cl_2	6
5	Wash	1:9 Methanol:CH_2Cl_2	6
6	Deprotection	2 x 10 eq. NaOMe (1:9 Methanol:CH_2Cl_2)	60
7	Wash	1:9 Methanol:CH_2Cl_2	4
8	Wash	0.2M Acetic acid in THF	4
9	Wash	THF	4
10	Wash	CH_2Cl_2	6

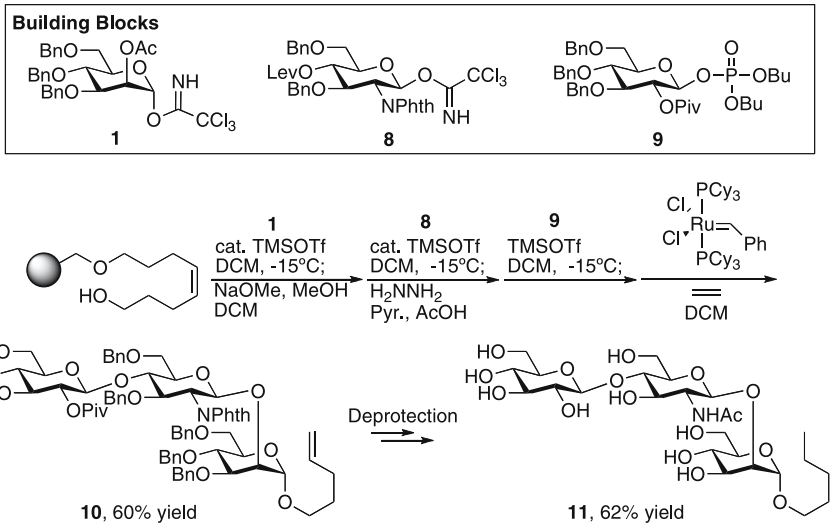


Scheme 7 Automated phytoalexin elicitor hexasaccharide synthesis

alternating the building blocks. A crude HPLC after cross-metathesis revealed a product peak accounting for 89% of the mixture.

These syntheses established the automated synthesis as an efficient and rapid way of assembling oligosaccharides. Both trichloroacetimidates and phosphates were competent donors and acetate and levulinoate could be used as temporary protecting groups for the synthesis. These two approaches were combined for an automated synthesis of a trisaccharide on a 100 μmol scale (Scheme 8).

Product **10**, obtained in 60% yield, was then fully deprotected to afford trisaccharide **11** in 62% yield or 37% overall yield from the functionalized resin. This example demonstrates the validity of the automated solid phase method to produce oligosaccharides in their deprotected form, ready for biological assays.



Scheme 8 Automated synthesis of a deprotected trisaccharide

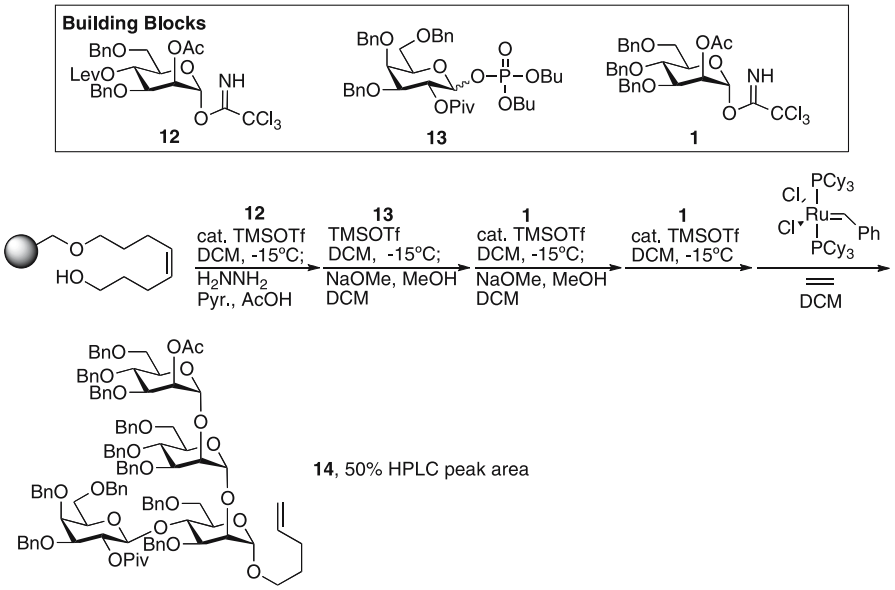
6.2
Leishmania Cap Tetrasaccharide

Leishmaniasis is a disease that affects over 12 million people worldwide. One of the carbohydrates that is exposed on the *Leishmania* parasite surface is a structure consisting of a glycosylphosphatidylinositol (GPI) anchor, a repeating phosphorylated disaccharide and tetrasaccharide cap **14**. The tetrasaccharide cap was synthesized by automation [38] using three building blocks (Scheme 9).

Levulinoate and acetate groups were used as temporary protecting groups during this assembly. Branching was achieved by having both ester protecting groups on the same building block and deprotecting the levulinoate in the presence of the acetate using hydrazine. The tetrasaccharide was the major product in the mixture as determined by HPLC. This synthesis demonstrates the possibility of producing branched structures by automation, with the use of orthogonal ester protecting groups.

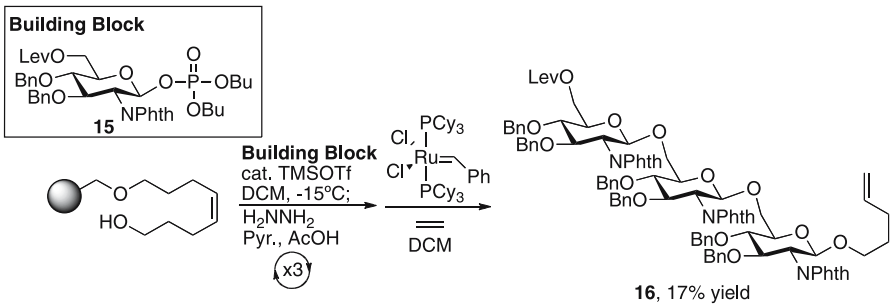
6.3
Oligoglucosamine

Glucosamines are involved in several important biological processes including cell-cell adhesion and immune response. Poly $\beta(1\rightarrow6)$glucosamines in particular, is an in vivo-expressed surface polysaccharide in human *Staphylococcus aureus* infections [39] and is a vaccine candidate [40]. A methodology



Scheme 9 Automated *Leishmania* cap tetrasaccharide synthesis

to assemble polyglucosamine by automation was developed [32]. A solution phase study was first conducted to identify a suitable building block for this purpose. A participating phtalimido group as a masked nitrogen and a phosphate as the leaving group in building block **15** were identified as optimal (Scheme 10).

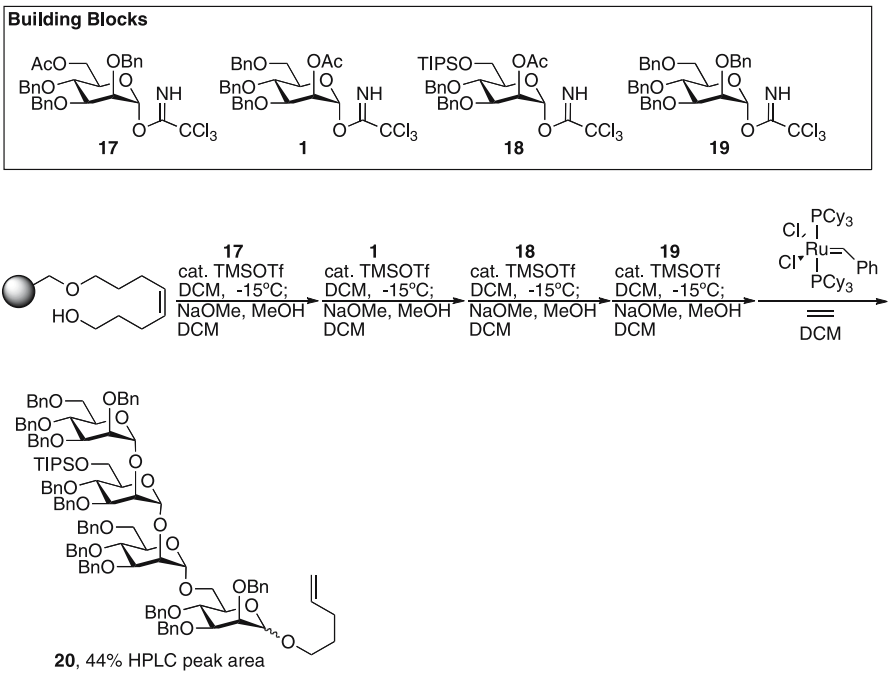


Scheme 10 Automated triglucosamine synthesis

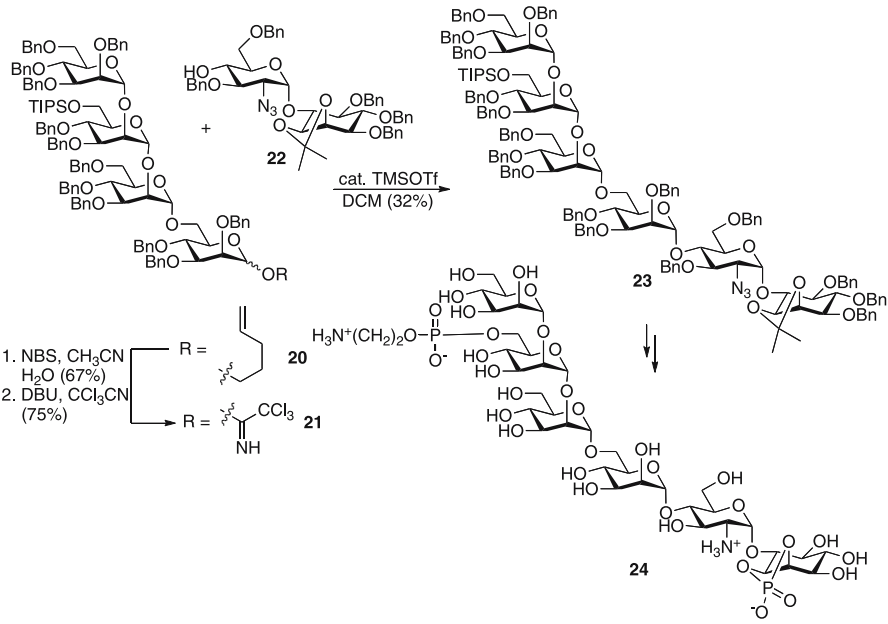
The synthesis was performed by treating the resin with five equivalents of building block and five equivalents of trimethylsilyl trifluoromethanesulfonate as activator. Deprotection of the levulinoate between glycosylations was performed with hydrazine. Cross-metathesis afforded the trisaccharide in 17% overall yield, along with 9% of the disaccharide deletion sequence.

6.4
Malaria Vaccine Tetrasaccharide

A GPI toxin [41] is responsible for the pathogenesis of malaria that induces cytokine and adhesin expression in macrophages and the vascular endothelium. The glycan portion of this GPI was synthesized in solution and conjugated to a protein to form a vaccine construct. The vaccine candidate protected mice against malaria pathogenesis. An automated synthesis of this carbohydrate and analogues would allow for rapid screening of various vaccine candidates. To this end, the synthesis of the tetramannose portion of the glycan was automated (Scheme 11) [42]. The building blocks were chosen considering the overall protecting group strategy of the complete malaria vaccine glycan. Benzyl and triisopropylsilyl ethers were used as orthogonal permanent protecting groups, whereas acetyl groups were used as the temporary protecting group to be removed prior to elongation. Four building blocks were needed. The glycosylation of building block **1** with the octenediol resin was not selective, as no participating group was used. However, further functionalization of the anomeric position renders this selectivity inconsequential. The automated synthesis afforded the product as the major



Scheme 11 Automated tetramannose synthesis



Scheme 12 Elaboration to the unprotected vaccine candidate **24**

component of the cleaved mixture. The mixture of anomers was purified by HPLC and further elaborated in solution (Scheme 12).

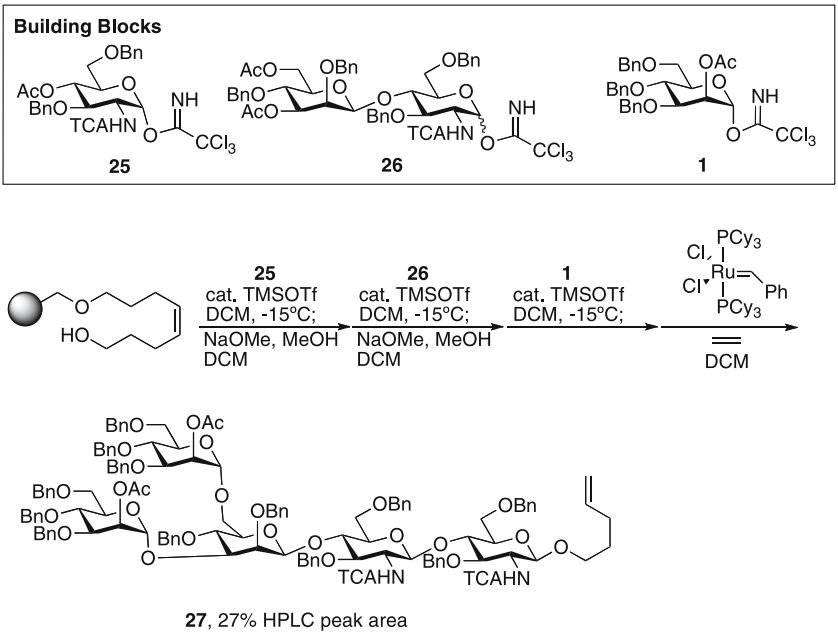
Although direct couplings of pentenyl glycosides are reported [20], a model donor failed to glycosylate the pseudo disaccharide **22.** The pentenyl glycoside **20** was converted to trichloroacetimidate donor **21** that was then used to glycosylate the disaccharide in moderate yield. Hexasaccharide **23** was further functionalized and deprotected to afford the antigenic glycan portion of the malaria toxin (compound **24**).

This synthesis opens the possibility of assembling various constructs for evaluation as vaccines. The anomeric pentenyl was successfully converted to a trichloroacetimidate donor, opening the way to building large carbohydrates from smaller fragments.

6.5 *N*-Linked Glycoprotein Core Pentasaccharide

There are three classes of asparagine-linked, or *N*-linked glycans: high-mannose, hybrid and complex-type mannans. All possess an identical core pentasaccharide **27**, comprised of a chitobiose ($\beta(1\rightarrow4)$diglucosamine) followed by a β-mannose branching two α-mannose residues. These glycans are not only found in mammals, but also on the glycoproteins of pathogens, including HIV [43], Ebola [44] and some coronaviruses [45].

The core pentasaccharide has been the subject of several solid-phase syntheses [46, 47] prior to our automated synthesis (Scheme 13) [48]. The automated assembly was performed using three different glycosyl trichloroacetimidate building blocks. The challenging β-mannosyl linkage was installed in solution and the disaccharide was transformed into building block **26.** Acetyl groups were used as temporary protecting groups. The resin was glycosylated twice with 3.5 equivalents of building block except for building block **26,** where the glycosylation was repeated three times. After cross-metathesis, the product peak area was 27% and could be isolated by preparative HPLC.

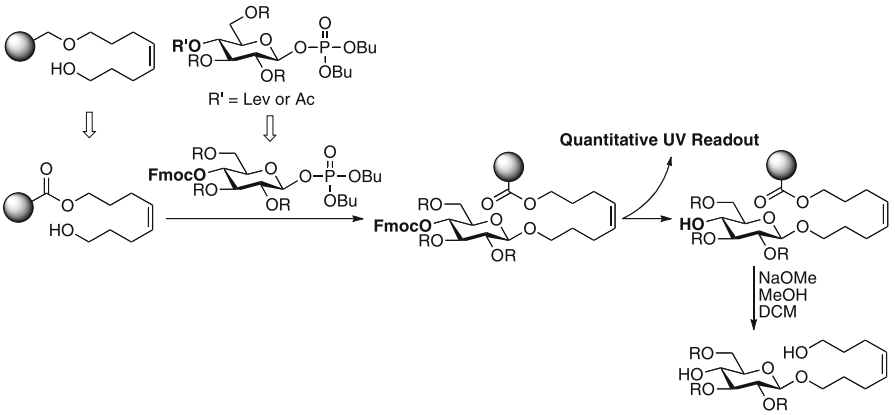


Scheme 13 Automated core pentasaccharide synthesis

6.6 Type II Lewis Blood Group Oligosaccharides

Lewis^y hexasaccharide and Le^y-Le^x nonasaccharide are tumor markers that are currently being evaluated as cancer vaccines [49]. Their complex structures constitute a challenge for synthesis both in solution and on solid phase [50, 51]. The successful automation [19] of this synthesis came after a solution phase [52] study and some modification to the previous automated syntheses (Scheme 14).

First, the resin was modified to include an ester functionality between the linker and the resin. This allowed for faster and more reliable cleavage

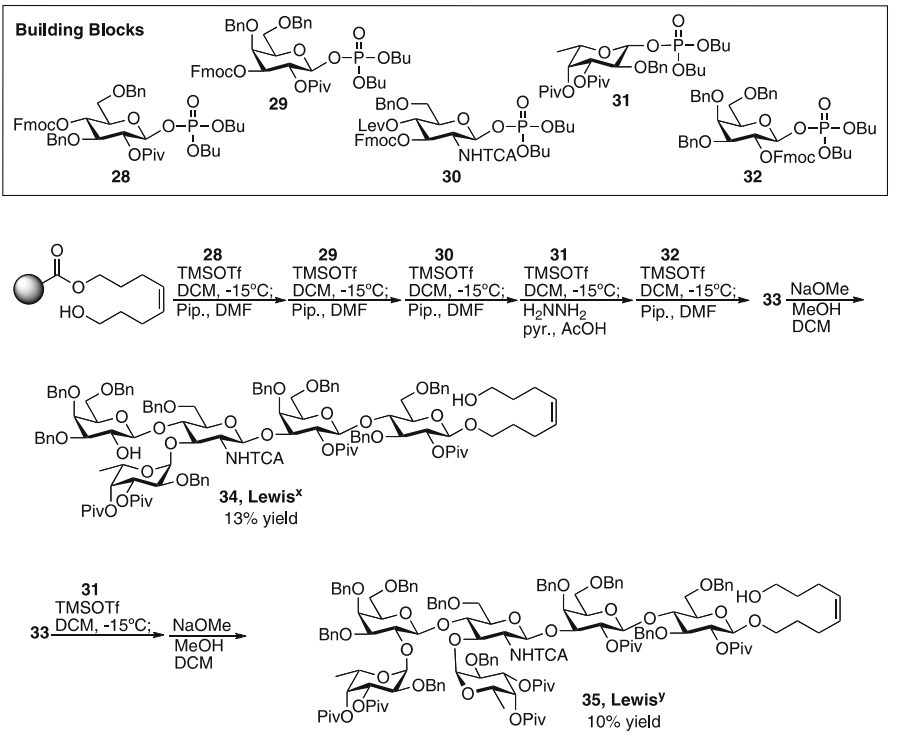


Scheme 14 Modifications from previous syntheses

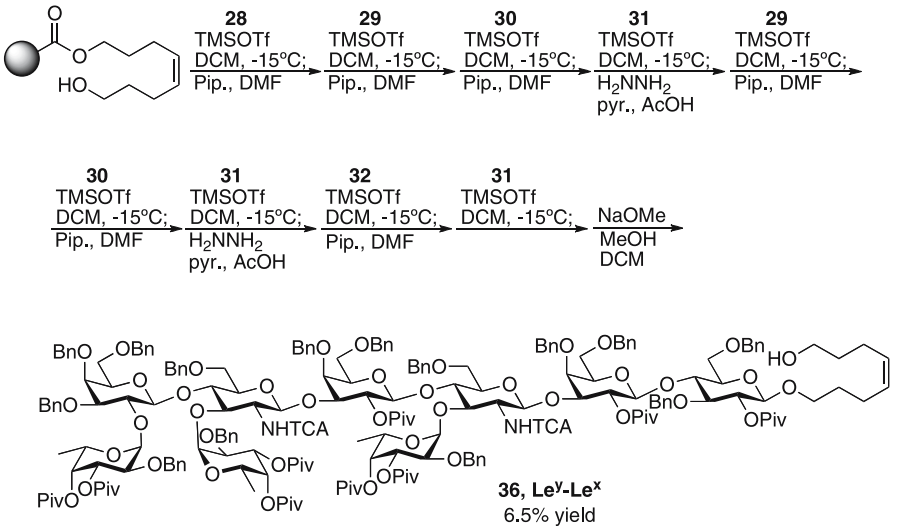
of the carbohydrate after the synthesis. The presence of this ester precluded the use of acetate or benzoate as a temporary protecting group since the conditions to remove them would also cleave the oligosaccharide from the support. Two orthogonal protecting groups had to be used for the branching glucosamine building block. The Fmoc group [46, 51, 53] can be removed by treatment with weak amine bases such as piperidine or triethylamine, and is orthogonal to levulinoate esters that require stronger basic conditions. The Fmoc deprotection is very rapid and can be quantitated by UV analysis. This procedure has the advantage of providing immediate feed-back regarding the efficiency of each glycosylation, information that is particularly important at the development stage of the synthesis.

The synthesis of Lewis *X* pentasaccharide **34** and Lewis *Y* hexasaccharide **35** required five different building blocks (Scheme 15). The glucose and galactose building blocks **28**, **29** and **32** were assembled efficiently using the previously described one-pot method based on the use of glycals. The glycosylations were performed with the standard five equivalents at – 15 °C repeated once, except for the glucosamine building block **30**, that was incorporated using three times 3.5 equivalents. This protocol was devised to ensure complete glycosylation despite competing decomposition of the building block under glycosylation conditions. The resin-bound pentasaccharide **33** could be released from the resin to afford pentasaccharide **34** in 13% overall isolated yield or fucosylated with building block **31** to give hexasaccharide **35** in 10% overall isolated yield.

The $Le^{y}Le^{x}$ dimer **36** was assembled in a similar fashion (Scheme 16) to give the nonasaccharide in 6.5% overall isolated yield after nine glycosylations, eight deprotection steps and one cleavage and purification step. The size and complexity of this oligosaccharide underscores the power of this methodology.



Scheme 15 Automated Lewis^x and Lewis^y synthesis



Scheme 16 Automated Le^y-Le^x synthesis

7 Conclusion

Automated solid phase synthesis has provided access to a broad range of biologically relevant oligosaccharides including cancer antigens, vaccine candidates and the *N*-linked core pentasaccharide, in a fraction of the time it would take to produce them by conventional means. Rapid access to a vast number of oligosaccharides has become possible and is a powerful tool for glycomics research that is currently being further improved.

Acknowledgements Funding from the ETH Zürich, the Swiss National Science Foundation (SNF Grant 200121-101593) are gratefully acknowledged.

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Top Curr Chem (2007) 278: 311–342
DOI 10.1007/128_2007_109

Published online: 10 March 2007

Probing Biology with Small Molecule Microarrays (SMM)

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Abstract In the continuous drive to increase screening throughput and reduce sample requirement, microarray-based technologies have risen to the occasion. In the past 7 years, a number of new methodologies have been developed for preparing small molecule microarrays from combinatorial and natural product libraries with the goal of identifying new interactions or enzymatic activities. Recent advances and applications of small molecule microarrays are reviewed.

Keywords Activity profiling · Combinatorial libraries · Diagnostic · Screening · Small molecule microarray (SMM)

Abbreviations

Ab	Antibody
AGT	Alkyl guanine transferase
Boc	*tert*-Butoxycarbonyl
Cy3, Cy5	Cyanine-3, cyanine-5
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocarbamate
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GST	Glutathione-*S*-transferase
MALDI	Matrix-assisted laser desorption ionization
NHS	*N*-Hydroxysuccinimide
NVOC	Nitroveratryloxycarbonyl
PNA	Peptide nucleic acid
SMM	Small molecule microarray
SPR	Surface plasmon resonance

1 Introduction

Astute observation of unanticipated results has often contributed to scientific breakthroughs. As famously said by Louis Pasteur, "chance favors the prepared mind". To explore biology, screening for small molecules that perturb processes has been a fruitful approach by providing the means to dissect the role of individual actors in complex biological networks. With the advent of automation, much effort has been devoted to increase our capability for discovery-driven research and to investigate the millions of interactions that make up a biological organism. Combinatorial chemistry has enabled small teams of chemists, as well as academic laboratories, to prepare large compound libraries. As for screening, the 96-well plate has gradually been substituted by the 386- or 1544-well plate, reducing the sample requirement from 50 μL/well to 5 μL/well. This means that 10 000 compounds now require a total volume of 50 mL using 1544-well plates (5 μL/well). Using microarrays, 10 000 compounds can be screened in less than 100 μL without sophisticated automation. More importantly, ligands can be identified for

proteins obtained from crude cell extract without further purification or labeling, thus enabling discoveries of unanticipated interactions. This level of miniaturization is not only important for screening but also to measure multiple enzymatic activities in parallel from complex proteomic mixtures. While the first small molecule microarrays (SMM) were reported in the early 1990s using photolithography for their preparation, it was the success of DNA microarrays that inspired the widespread exploration of SMM as a tool to probe biological events. This chapter is divided into two main sections: (i) the preparation of SMM with a discussion of microarray surfaces, in situ microarray synthesis or immobilization of existing libraries, and (ii) the screening of SMM with a discussion of ligand discovery, enzymatic activity measurement, and carbohydrate recognition as well as non-immobilized SMM for multicomponent screening and cellular assays.

2 SMM Preparation

Several techniques have been reported for preparing microarrays (including photolithography, contact printing, and inkjet), yielding arrays with densities ranging from 1000 to 500 000 features per square centimeter [1, 2]. While photolithography is used on an industrial scale (Affymetrix) to pro-

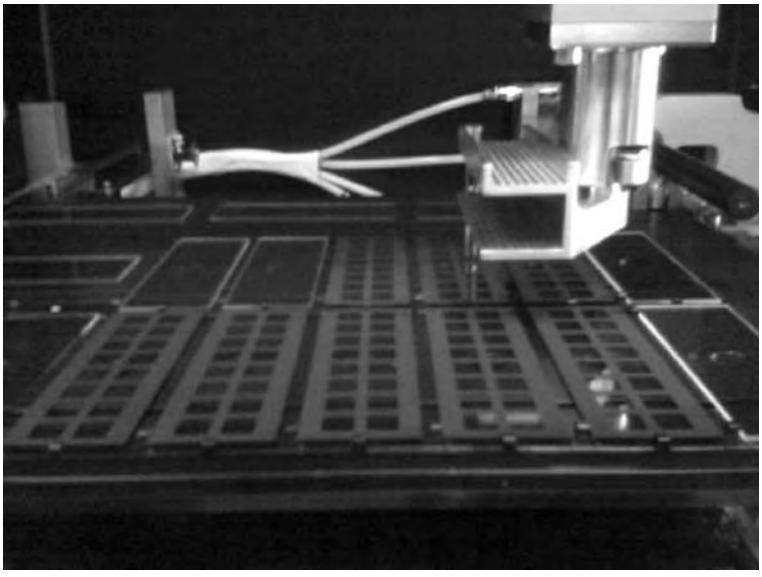

Fig. 1 Contact printing robot: expanded view of the needles with capillaries for solution pick up and delivery

duce oligonucleotide microarrays with feature size from 14 to 50 μm, most SMM have been prepared using a contact printing robot. In its simplest format, the robot picks up a solution from a microtiter plate by capillary action and delivers nanodroplets of solution (ca. 1 nL) on the array simply by getting into contact with the surface in a similar fashion as does an ink pen (Fig. 1). Depending on the conditions, features ranging from 100 to 300 μm are obtained by this method. The first such robot was assembled according to a protocol published by the Brown laboratory in the mid 1990s [3]. By the end of the 1990s, such robots had become commercially available; current versions have the ability to print 10–50 microarrays containing 10 000 features in a matter of hours.

2.1 Microarray Surface

Most contact printing is carried out on standard 25 mm × 75 mm glass microscope slides, which can be functionalized but are otherwise chemically inert and have low intrinsic fluorescence. As shown in Fig. 2, the silanol glass surface can be treated with (3-aminopropyl)triethoxysilane, which reacts covalently with the glass via hydrolysis/condensation resulting in aminosilanized slides (careful preparation is crucial to ensure an even surface) [4]. The amino groups can be subsequently coupled with a bifunctional linker (e.g., Fmoc-8-amino-3,6-dioxaoctanoic acid [5], poly(ethylene glycol) diglicydyl ether [6], or 1,8-diamine-3,6-dioxaoctane via carbonate formation [7]) and termi-

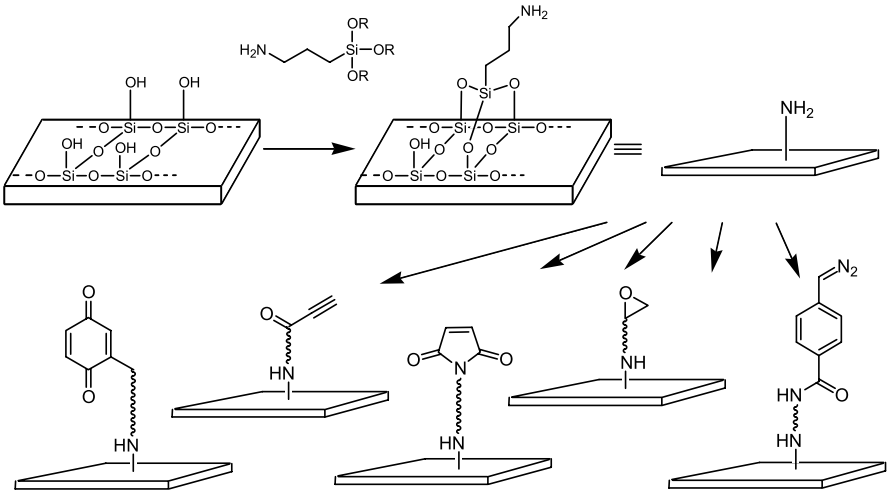

Fig. 2 Functionalization of glass slides. The silanol surface is reacted with aminopropylsilane, which is further reacted with a bifunctional linker and adequate spacers to obtain the desired functionalized surface

nated with a functional group for covalent attachment of small molecules (maleimide, diazobenzylidene, *N*-hydroxysuccinimide, epoxide, alkyne, isocyanate, etc.). While it has been demonstrated that a small molecule–protein interaction is possible with small molecules attached directly to the glass surface [8], it has generally been noted that higher sensitivity and better signal-to-noise ratios are obtained if a PEG spacer is used [7, 9, 10]. To improve loading and homogeneity of the glass surfaces, aminosilylation followed by coupling with dendritic molecules (PAMAM) and subsequent crosslinking was explored. Optimized procedures led to a chemically activated polymer film with a tenfold increase in loading (150 fmol/mm^2 vs. 5 fmol/mm^2 for the aminosilane slides) and high resistance [11]. The polarity of the surface formed was modulated by changing crosslinking reagents, which allowed modifications of the spot size and sharpness. Alternatively, uncoated glass slides were functionalized with methacryloxypropyl-trimethoxysilane and incubated with tetraethylenepentamine, thereby introducing multiple reactive groups per attachment point to the surface. This surface was further functionalized with acryloyl chloride, epichlorohydrin, or 1,4-butanediol diglicydyl ether to obtain acrylic, epoxy hydrophobic, and epoxy hydrophilic surfaces, respectively [12]. Aside from glass, arrays were also prepared on a gold surface, which can be used directly for surface plasmon resonance (SPR) or mass spectroscopies. Generally, a self-assembled alkanethiolate monolayer was partially functionalized with a handle for subsequent derivatization such as a hydroquinone [13], or carboxylic [14] or NHS groups [15].

2.2 In Situ Synthesis

2.2.1 Light-Directed Synthesis

The first microarrays reported in the literature were prepared by synthesizing the small molecule directly on the array surface in a combinatorial fashion using monomers blocked with a photolabile protecting group (nitroveratryloxycarbonyl, NVOC) and photolithography masks to achieve selective deprotection at a given coordinates (Fig. 3). This light-directed in situ synthesis was demonstrated to be effective for the preparation of oligopeptide and oligonucleotide microarrays [16] and was later used for the preparation of unnatural oligomers (carbamates) [17]. Larger oligonucleotide arrays were subsequently reported using a new photolabile protecting group – α-methyl-6-nitropiperonyloxycarbonyl (MeNPoc) – allowing the synthesis to be carried out on features of 5–10 μm (10^6 sequences/cm^2) [18]. The spatial resolution achieved using that method is close to the physical limitations of diffraction for high contrast. Using an effect of the non-linear response of semiconducting photoresistant films to light, thus lowering the contrast requirements,

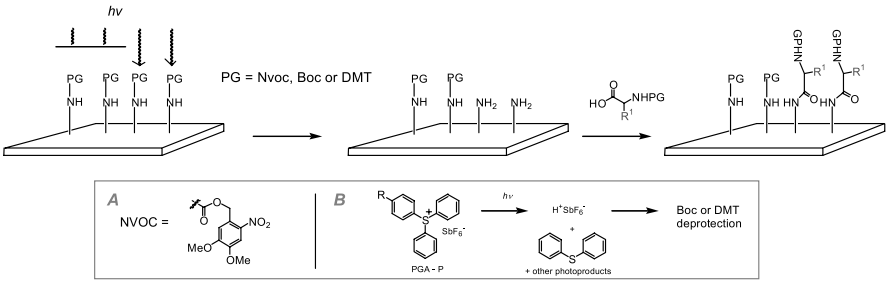

Fig. 3 Light-directed synthesis. An area of the array is selectively irradiated with UV light using photolithographic masks to trigger the deprotection of photolabile groups such as Nvoc (**a**) or the generation of an acid for deprotection of acid labile groups (**b**)

it is possible to further improve the density. The polymeric film is used to construct a pattern, which masks selected regions of the substrate from exposure to standard chemical reagents during synthesis, whereas the unprotected areas are exposed to coupling of new monomers. A microarray of 8 μm feature size was prepared with this method, raising the possibility of further optimization [19]. Alternatively, as the cost of preparing photolithographic masks is considerable, a maskless method using a digital micromirror device (digital light processor, DLP) was developed [20]. Using the DLP technique and a single photolabile reagent (MeNPoc-protected glycolic acid) a peptoid array was synthesized [21]. Shortly after, a strategy for the synthesis of spatially addressable arrays of cyclic peptides was also reported [22]. These experiments expanded the scope of light-directed synthesis of microarrays beyond specialized environments. DLP has also been applied to the preparation of oligonucleotide and peptide microarrays using building blocks with standard acid-labile protecting groups (DMT and Boc, respectively). Instead of directly removing the protecting group, light is used to generate an acid with spatial resolution resulting in deprotection of the standard Boc and DMT groups (Fig. 3b) [23–25]. Although the microarray density is lower than in the masking methods (ca. 3000 features/cm^2), the technique is more flexible than classical mask photolithography.

2.2.2 SPOT-Synthesis

In the late 1980s, Frank and coworkers developed a method for parallel solid phase synthesis where the molecules were linked to a membrane rather than to conventional polystyrene beads [26, 27]. Libraries were prepared by adding the different reagents at each of the spots on the membrane. This SPOT-synthesis method can be carried out on cellulose and polypropylene sheets (loading: 100–10 000 nM/cm^2 or 10–100 nM/cm^2, respectively) and requires no specialized infrastructure to obtain libraries. The mean spot size

is 6 mm, which makes these arrays several orders of magnitude less dense than microarrays. However, the spots are sufficiently large that they can be excised and the molecules recovered from the membrane after cleavage [28]. For preparation of small molecule arrays, the cellulose has to be activated (epibromohydrin or tosyl chloride) to introduce a flexible diamino spacer allowing for further functionalization. In the case of polypropylene membranes, photoinduced coupling with acrylic acid or methyl acrylate prior to the spacer introduction proved to be effective. Such surfaces can be further modified with linker such as Rink or Wang linkers. Macroarrays of 1,3,5-triazines [29], cyclic peptidomimetics [30], 1,3,5-hydantoins [31], natural product fragments (from sorangicin and epothilone) [32], chalcones [33], and α-acyl amino amides [34] have been reported, testifying to the flexibility of this method. The synthesized libraries can be screened against the biological targets directly on the support (e.g., protein binding, antibody binding, or metal binding assays) or reformatted in a microtiter plate or microarray after cleavage.

2.3
Chemoselective Immobilization

As an alternative to in situ synthesis, molecules can be immobilized to the surface after their synthesis. To this end, it is critical to have a method that is chemoselective and operates under mild conditions to avoid degradation of the molecule or to preclude it from interacting with a protein. In the case of combinatorial libraries, the design of the synthesis can include a specific functionality, which can be leveraged to ligate the small molecule to an appropriately functionalized surface with controlled orientation. Existing methodologies are listed below (Fig. 4).

2.3.1
Immobilization of Thiols on a Maleimide Surface

The first SMM microarray prepared by contact printing using a chemoselective immobilization was reported by Schreiber and coworkers in 1999 [35]. Amine-functionalized slides were treated with a bifunctional linker (NHS/maleimide) to obtain a maleimide-functionalized surface (Fig. 4a). Three molecules (biotin, digoxigenin, and a pipecolyl α-ketonamide – an FKPB ligand) conjugated to a cysteine residue were spotted and the array was then probed simultaneously with FITC-conjugated streptavidin, Cy3-conjugated DI-22 (an anti-digoxigenin Ab), and Cy5-conjugated FKPB. After a brief washing, the three differently labeled proteins were detected at the respective wavelength of their fluorophore only in the locations corresponding to their cognate ligands, thereby demonstrating that SMM could be used to probe small molecule–protein interaction and be multiplexed.

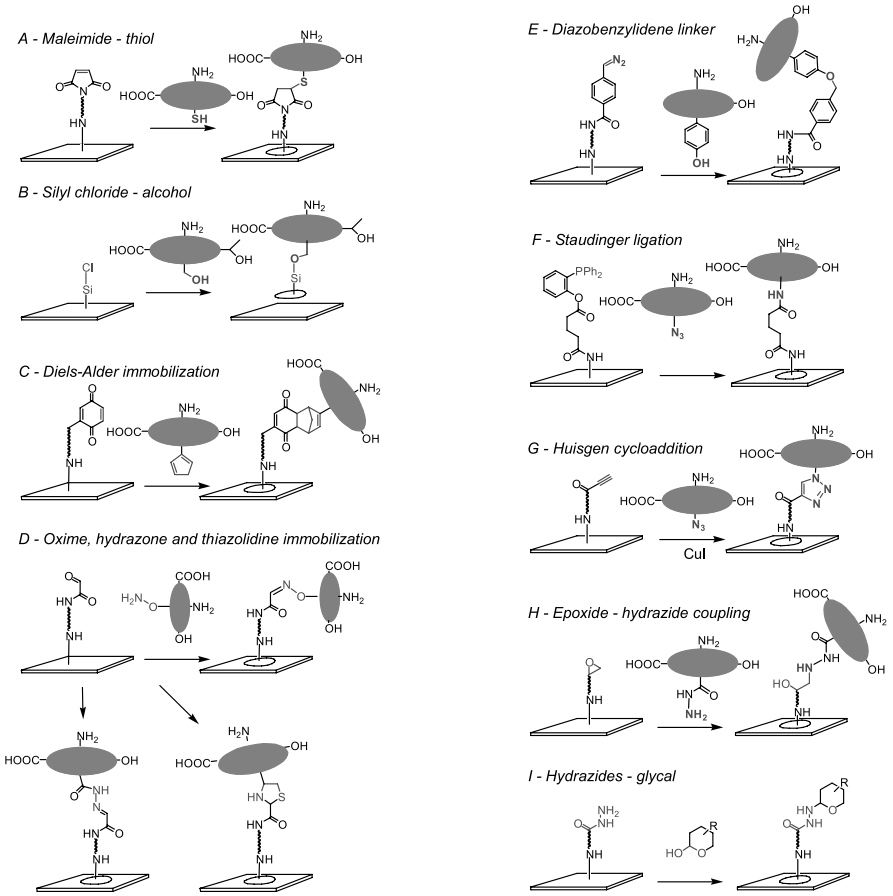

Fig. 4 Chemoselective ligation methods: **a** maleimide linker, **b** silyl linker, **c** Diels–Alder ligation, **d** glyoxylyl linker, **e** diazobenzylidene linker, **f** Staudinger ligation, **g** Huisgen [3 + 2] cycloaddition, **h** hydrazide linker, **i** glycal immobilization

2.3.2 Immobilization of Alcohol on a Silyl Chloride Surface

While most SMM have some form of a linker that distances the small molecule from the surface so as to minimize steric interactions between the protein and the surface, direct immobilization on the glass surface has also been reported to be effective. Treatment of the glass surface with $SOCl_2$ in THF using catalytic amounts of DMF transformed silanols on the surface into chlorosilanes [8]. This chlorosilane was found to react readily with primary alcohols but rather slowly with secondary alcohols or phenols (Fig. 4b). To validate the detection of small molecule–protein interactions, the interactions of three known ligand–protein pairs (α-ketoamide, digoxigenin, and biotin)

were investigated and led to the observation that, despite the lack of linker, binding was detected. An important motivation for the development of this immobilization strategy was that it is compatible with libraries synthesized by the same group using a silyl linker. The viability of this immobilization method beyond the proof of concept has been validated with the discovery of new ligands from large libraries (> 1000 microarrayed compounds) [8, 36]. Furthermore, it could also be used to immobilize natural products, which often bear a primary hydroxyl group.

2.3.3 Immobilization Through Formation of Oximes, Hydrazones, and Thiazolidines

Hydroxylamines or hydrazines are known to react selectively with aldehydes and even faster with glyoxylyl groups. Glyoxylyl-functionalized slides were obtained by derivatizing aminosilanized glass slides with serine which, after deprotection, was oxidized with $NaIO_4$ to obtain the glyoxyl functionality [9]. Alternatively, direct coupling of protected glyoxalic acid to aminosilanized glass slides and its subsequent deprotection also afforded glyoxylyl-functionalized slides. This surface was shown to react selectively with peptides labeled with hydroxylamine or a terminal cysteine residue, which yielded a thiazolidine ring (Fig. 4c). The authors noted that improved results were obtained in the spotting when the surface was prepared with a mixture containing protected glyoxalic acid and stearic acid to increase the hydrophobicity of the surface. The immobilization chemistry was validated using Cy3-labeled streptavidin to detect immobilized biotin. The possibility of using this array for functional assays was also demonstrated by treating an immobilized kinase substrate (octapeptide) with Src kinase in the presence of [γ-^{33}P]-ATP followed by autoradiography imaging of the phosphorylated peptide. It was further shown that arrayed peptides could be used for cell adhesion assay by means of a known ligand/cell surface receptor pair. For the purpose of cell adhesion screens, non-specific binding could be eliminated by derivatizing the glass surface with $PEG_{5000}NHNH_2$ after spotting of the small molecules.

Conversely, peptide microarrays prepared from glyoxylyl-labeled peptides and a semicarbazone-derivatized surface were also reported [37]. Glass slides covered by a semicarbazide sol-gel layer were found to reduce non-specific protein absorption and improve signal-to-noise ratio for antibody detection. Using three immobilized peptide epitopes from hepatitis C and Epstein-Barr virus, the concentration of specific antibodies in patients' sera could be measured. The microarray format was compared to traditional ELISA using 130 samples of sera from HCV-infected individuals, resulting in superior sensitivity and selectivity for the microarray format (there were 13 false positives by ELISA). This ligation method was also found to be effective for antibody immobilization. Periodate oxidation of the antibodies' carbohydrates yielded aldehydes that were conjugated to the semicarbazone.

2.3.4
Immobilization via Diels–Alder Cycloaddition

The orthogonality of the Diels–Alder reaction and its compatibility with most functional groups defined it as a suitable reaction for chemoselective immobilization (Fig. 4d) [13]. A self-assembled monolayer of alkanethiolates on a gold surface terminated with hydroquinone was activated after a mild reduction that converted the hydroquinone into benzoquinone, an excellent dienophile. Three known peptide substrates to kinases (*c-Src*, PKA, and c-Abl) were labeled with cyclopentadiene and then arrayed. Using [γ-^{33}P] ATP, it was shown that only the matched substrate was phosphorylated with Src kinase. It has also been demonstrated that a Diels–Alder reaction can be used to immobilize proteins as well [38]. In this case, a maleimide surface was used as the dienophile and spotted with hexadienoic acid-labeled proteins obtained by ligation reaction. As thiol groups from cysteine residues also reacted with the maleimide, the cysteine residues were protected prior to spotting using Ellman's reagent.

2.3.5
Immobilization to a Diazobenzylidene Surface

To broaden the range of functional groups compatible with an immobilization reaction, a diazobenzylidene-functionalized surface (Fig. 4e) was developed as this functional group is known to selectively react with heteroatoms bearing acidic protons (such as phenols, carboxylic acids, or sulfonamides) [39]. An aminosilane surface was derivatized with the toluenesulfonylhydrazone of 4-carboxybenzaldehyde, which was converted to the desired diazobenzylidene functionality following base-induced elimination. The functional group compatibility of this immobilization method was evaluated with a series of FKPB12 and biotin derivatives, demonstrating that the diazobenzylidene functionality reacts only with heteroatoms having a proton with a $pK_a < 11$. The effectiveness of this strategy for ligand discovery was confirmed with the discovery of new calmodulin ligands from a library of > 6000 immobilized phenols.

2.3.6
Immobilization via Staudinger Ligation

The mildness and orthogonality of the Staudinger ligation with respect to most functional groups [40, 41] makes this reaction ideal for immobilization of small molecules on an array with controlled orientation (Fig. 4f). To this end, an amine-functionalized slide with PAMAM dendrimer was derivatized with glutaric anhydride to introduce terminal COOH groups, which were subsequently esterified with 2-(diphenylphosphanyl)phenol.

Small molecules labeled with an azide were conveniently obtained directly from solid phase cleavage by using a Kenner-type linker followed by cleavage using 6-azidohexylamine. The immobilization chemistry was validated with a series of biotinylated peptides and a biphenyl-antibiotic, as well as manose, which were recognized selectively either by Cy5-labeled anti-biotin Ab or fluorescently labeled concanavalin A, which binds to manose. It was shown that the array could be washed and reused with nearly the same sensitivity of detection, which attests to the stability of the crosslinked dendritic surface. Concurrently, an alternative Staudinger ligation with a thioester was also used to immobilize peptides [42]. The authors prepared a 15-mer peptide which binds to RNase S and showed that the immobilized RNase S had nearly full activity. More recently, the Staudinger ligation was applied to site-selective covalent immobilization of proteins [43]. The azide-modified C-terminus of a protein was prepared by expressed protein ligation (EPL) in vitro. This chemoselective immobilization was validated with the microarraying of Ras, which was detected using a Cy5-labeled anti-Ras Ab.

2.3.7
Immobilization via Huisgen Cycloaddition

The Cu-catalyzed [3 + 2] Huisgen cycloaddition of azides [44] to alkynes has proven itself to be a very mild and reliable reaction, proceeding in aqueous environments and orthogonal to most other functional groups, thus being highly suitable for conjugation of highly functionalized molecules (Fig. 4g). This reaction was exploited for the preparation of carbohydrate arrays [45–48]. Commercially available amine-coated and NHS-functionalized glass slides were coupled with a linker terminated with an electron-poor alkyne and having an internal disulfide bond. The latter can be reductively cleaved from the support to analyze the degree and quality of carbohydrate immobilization. The reliability and efficiency of this method were not only demonstrated with several lectin-binding assays but also by its use to map epitopes of therapeutically important antibodies (vide infra).

2.3.8
Immobilization Using Hydrazides

Epoxides react faster with hydrazides than other nucleophilic functionalities such as hydroxyl, amine, carboxylic, and even thiol, which should allow for selective ligation of small molecules having hydrazide functionality (Fig. 4h). The epoxide surface was prepared from amine-coated slides immersed in a solution of poly(ethylene glycol) diglicydyl ether. Careful analysis of functional group compatibility using carbohydrate ligands showed that the immobilization reaction was selective for hydrazides in the presence of thiols at pH > 5 [10]. Carbohydrate arrays were also prepared from unlabeled saccha-

rides using a hydrazide surface (Fig. 4i). The unfunctionalized carbohydrates containing a hemiacetal at the reducing end were immobilized and probed with lectins. Notably, it was found that a hydrazide surface was superior to a hydroxylamine surface due to the predominance of the acyclic product oxime generated in the latter reaction, whereas the hydrazide coupling yielded mostly the β-anomeric cyclic product. Using this technique, glycan microarrays were prepared and their ability to detect pathogens was demonstrated [49].

2.3.9 Self-Sorting Supramolecular Immobilization

Instead of screening biological samples directly on the surface, as in the case of covalent attachment of small molecules or proteins to the glass slide, an alternative method has been developed where the small molecules are covalently tethered to a peptide nucleic acid (PNA) tag such that libraries can be screened in solution prior to self-assembly by sequence-specific hybridization to an oligonucleotide microarray [50, 51]. Aside from minimizing potential problems associated with the display of ligands or substrates on a surface (non-specific interactions with the surface, high local concentration), it also allows separation of ligands that are bound to a protein from unbound ones prior to hybridization and as such offers a detection method that is not possible with covalently immobilized compounds (vide infra) [52]. A second asset of the PNA tag is that it can be used to encode libraries prepared by mix-and-split combinatorial synthesis by using a unique PNA codon for every building block in the library [53]. Upon cleavage from the solid phase, the library is obtained as a mixture in solution; however, it sorts itself into an addressable microarray upon hybridization (Fig. 5). This PNA-encoded strategy has been validated with the discovery of inhibitors from libraries of > 1000 compounds and the profiles of substrate specificity and enzymatic activity from complex proteomic mixtures [54–58].

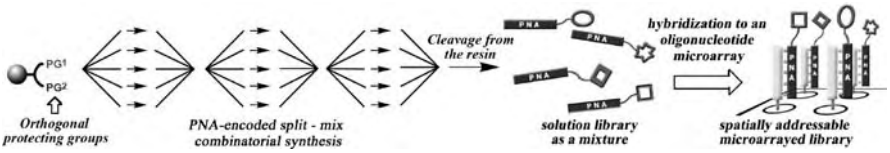

Fig. 5 Immobilization through self-sorting of PNA-encoded libraries. Starting from a bifunctional linker with orthogonal protecting groups, a library is prepared by split and mix combinatorial syntheses where a specific PNA codon is used to encode every building block. The libraries are then cleaved from the solid phase to obtain a mixture in solution, which is converted to a SMM by hybridization to an oligonucleotide microarray [51]

2.3.10
Physisorption Using Lipophilic Tags or Fluorous Tags

Rather than using a covalent reaction to immobilize the small molecule to a surface, a small molecule tagged with a tail having special physical properties could adhere to a surface by physiosorption. This was demonstrated for the preparation of carbohydrate arrays in microtiter plates where carbohydrate labeled at the anomeric position with saturated carbon chains between 13 and 15 carbons were retained on a hydrophobic surface and were resistant to aqueous washing. The viability of this immobilization was demonstrated with the detection of several carbohydrate–lectin interactions [59]. Conversely, it was also shown that such lipophilic tails could be introduced via Huisgen cycloaddition [60].

The unique properties of fluorinated alkanes (immiscible with water or organic solvent) have prompted the development of perfluorocarbon chains linked to small molecules as a handle for purification [61]. Recently, this principle has been applied to the preparation of carbohydrate microarrays [62]. Four sugar derivatives with a prefluorooctane tag linked to the anomeric position were prepared and spotted by a standard DNA arraying robot onto a commercially available glass microscope slide coated with a Teflon/epoxy mixture, or onto a surface prepared by the reaction of a fluoroalkylsilane with uncoated glass slides. The array was then successfully screened against respective fluorescently labeled lectins and the result was reproducible after rinsing with water, buffers, and detergent (Tween-20).

2.4
Non-specific Immobilization

All the specific immobilization methods listed above require a certain functional group to be present in the immobilized compounds. These methods are desirable for combinatorial libraries where the required functionality may be introduced but lend themselves poorly to immobilize natural products or known drugs that do not have a single common functionality that can be targeted. Two methods have been reported to address immobilization of natural products or libraries lacking a common functionality: photocrosslinking, and reaction with an isocyanate surface (Fig. 6).

2.4.1
Photocrosslinking

Amine-coated glass slides were derivatized with a linker terminated with the known photoaffinity reagent trifluoromethyldiazirinbenzoyl (Fig. 6), which upon UV irradiation releases a nitrogen molecule and is converted into a highly reactive carbene that reacts indiscriminately with small molecules.

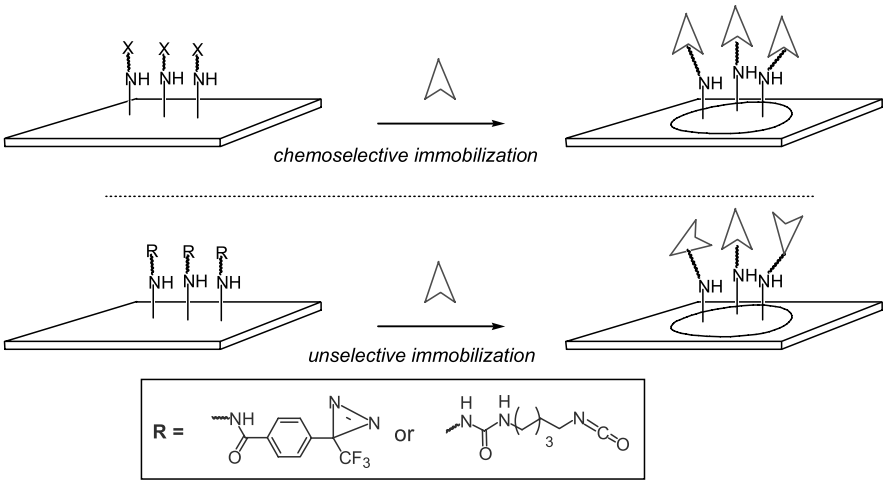

Fig. 6 Non-specific immobilization methods with covalent bond formation. Chemoselective immobilization will yield a SMM with a defined orientation (*top*) whereas unselective immobilization will allow different orientations of the small molecule on the array (*bottom*)

The lack of selectivity in the photocrosslinking was seen as advantageous because it means that small molecules will be presented into different orientations, which reduces the chance that the attachment point to the surface interferes with binding to its target. The method was validated with the immobilization of a variety of natural products such as the steroidal glycosides digoxin, digitoxin and digoxigenin, FK506, rapamycin and cyclosporine A. All the immobilized products were successfully recognized in a specific manner by their protein targets or antibodies [7].

2.4.2 Immobilization to an Isocyanate Surface

Aminosilanized glass slides were coupled with an ω-aminoacid linker functionalized with α,ω-diisocyanatohexane yielding an isocyanate-functionalized surface (Fig. 6). Isocyanates react with many nucleophilic functional groups and should also produce attachment of product in multiple orientations. It was demonstrated that alcohols, phenols, carboxylic acids, amines, and anilines all reacted with comparable efficiency on this surface. A "diversity microarray" containing nearly 10 000 bioactive small molecules, natural products, and compounds originated from several diversity-oriented syntheses were prepared and detected with antibodies against compounds of interest. It was also shown that by using an appropriate PEG spacer to avoid non-specific protein interactions, a crude proteomic mixture could be used directly in the screening without prior purification [5].

2.5 Other Microarray Preparation

2.5.1 Dip-Pen Nanolithography

Protein nanoarrays were prepared using a scanning probe microscopy (SPM)-based lithography technique by deposition of lines and dots of 16-mercaptohexadecanoic acid (MHA) [14] or 16-mercaptoundecanoyl-NHS [15] on a gold thin-film substrate. The spot size ranged from 100 to 600 nm, which was significantly lower than the standard 5–20 μm features achievable by photolithography or the 100–300 μm achievable by contact printing. The surrounding area was passivated with 11-mercaptoundecyl-tri(ethylene glycol). Proteins were then immobilized by simple adsorption (MHA) or reaction with the NHS. In a proof of principle, it was shown that protein–protein interaction could be detected by atomic force microscopy or fluorescence.

2.5.2 Microarrays with Non-immobilized Small Molecules

While screening immobilized compounds in a microarray has already enabled the discovery of important inhibitors, the fact that the small molecules are covalently attached to the surface limits the types of screens that can be used, most notably in cell-based assays. Two approaches have been developed to address these limitations: (i) microarrays of nanodroplets, which have been used for multicomponent enzymatic assays, and (ii) microarrays of small molecules embedded in a biodegradable polymer for cell-based screens. For nanodroplet microarrays, it was found that small molecules could be deposited in a microarray format as glycerol nanodroplets (1.6 nL) by standard contact printing at a density of 400 spots/cm (similar density to contact printing) and that reagents or enzymes could be metered into each droplet using aerosol deposition without any cross-contamination amongst nanodroplets (Fig. 7). Because of water's fast evaporation in these small volumes, multiple additions by aerosol are possible. The utility of this method was demonstrated by screening a library of 352 compounds against thrombin, chymotrypsin, and three caspases [63]. More recently, this method has been used to identify inhibitors of SARS. A potential limitation of this approach is that the assays need to be carried out in a high concentration of glycerol to reduce the evaporation rate, but it was shown that at least several therapeutically important enzymes (proteases and kinases) are functional under these conditions.

To extend the utility of SMM towards cell-based screens, small molecules impregnated in a biodegradable polymer solution were microarrayed by contact printing on standard glass slides. Cells were cultured on top of the

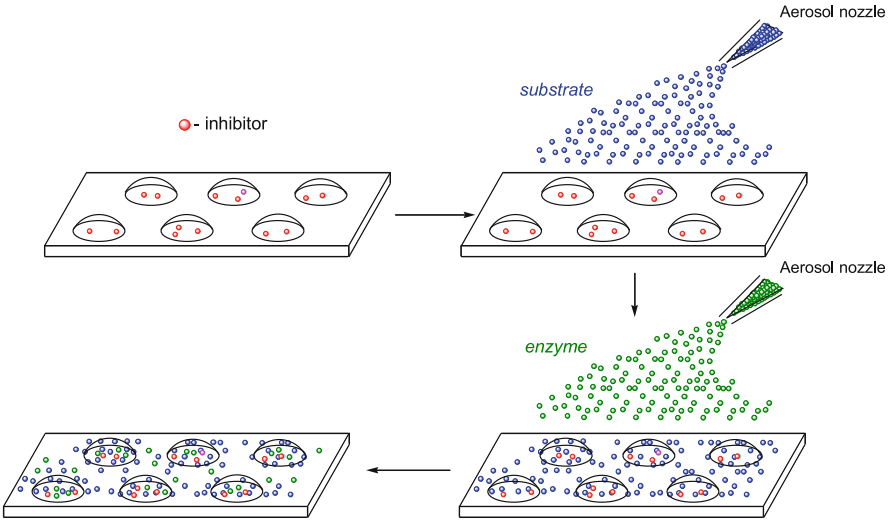

Fig. 7 Microarray of nanodroplets as individual reaction vessels. Small molecules are arrayed in a glycerol solution and the subsequent reagents are added as an aerosol

array allowing each compound to slowly diffuse out of the polymer and affect neighboring cells. After screening several polymers, polylactide/glycolide copolymer (PLGA) was identified as the best candidate [64]. As a proof of principle, it was shown that cytotoxic compounds indeed killed cells in the proximity of the compound spot but not beyond. It was also shown that this format was compatible with other phenotypes than cell death, using rapamycin for the inhibition of ribosomal phosphorylation. The readout was achieved by staining the cells with a specific anti-phosphoribosome Ab after they were fixed and permeabilized. This method was then used to evaluate the synergistic effect of 70 small molecule inhibitors in combination with seven different siRNA. These results demonstrated that high content cell-based assays can be performed in a highly miniaturized microarray format that does not require sophisticated automation. Using the standard microarray density with a 200 μm spot, less than 100 cells are necessary to screen each compound.

2.6 Commercially Available Functionalized Surfaces

Slides for microarrays have become commercially available with a variety of functionalized surfaces for oligonucleotide, protein, and small molecule immobilization. Aminopropylsilane with different surface properties are available from Asper, Corning, and Schott; amine-reacting NHS slides are available from GE Healthcare and Schott; epoxide-functionalized slides are available from Corning and Schott; aldehyde-functionalized surface for im-

mobilization through reductive amination are available from Schott; and isothiocyanate-functionalized surface are available from Asper. Industrialized and standardized preparation conditions ensure high and reproducible quality of the surfaces, which are often superior to those prepared from the underivatized slides directly before microarray spotting.

3 SMM Screening

As seen in the previous section, a number of methods have been developed for displaying small molecules in a microarray format. This section describes various methods for SMM screening [65]. For screening of protein–ligand interactions, the simplest method is to obtain a fluorescently labeled protein; however, such labeling procedure can be cumbersome and may potentially inactivate the protein. Alternatively, specific antibodies can be used if available or the protein can be genetically tagged with a His-6 tag, GST tag, with fluorescent proteins such as GFP [66], or with AGT, which specifically reacts with 6-*O*-alkylguanine-fluorophore conjugates (Fig. 8) [67]. Fluorescently tagged proteins have also been obtained from crude lysates by labeling the C-terminus using a fluorophore–puromycin conjugate [68].

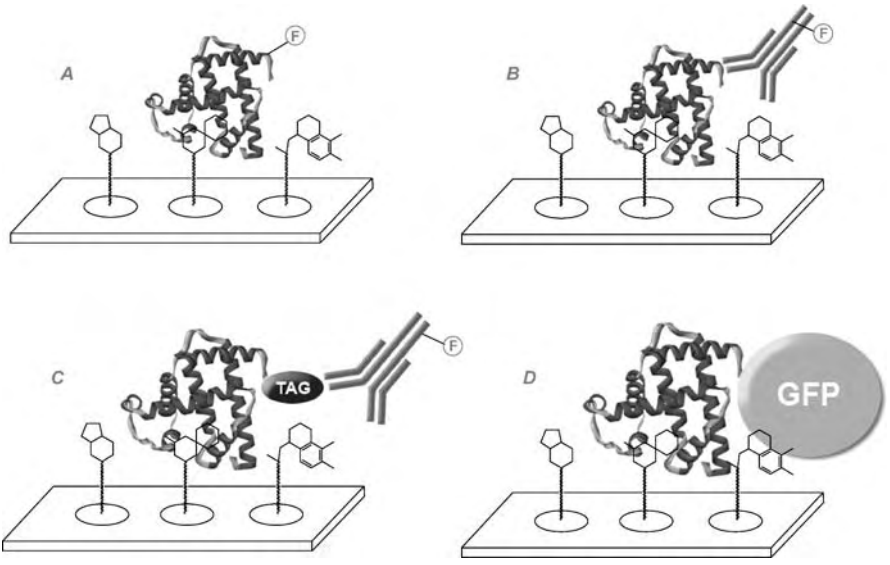

Fig. 8 Detection of a small molecule ligand interaction. **a** The protein of interest is itself labeled with a fluorophore F. **b** A specific labeled antibody is used for detection. **c** The protein of interest is expressed with a tag such as His-6 or GST and a labeled anti-tag Ab is used. **d** The protein of interest is expressed fused to green fluorescent protein (GFP)

3.1
Screening Ligand–Protein Interactions

Most methods that have been reported in the literature to immobilize small molecules on microarrays validate the immobilization strategy using well-known interactions such as biotin-streptavidin, digoxigenin-Ab or a known carbohydrate–lectin interaction. Beyond these proofs of principle, the first report of a new small molecule–ligand interaction was reported by Schreiber and coworkers with the discovery of a ligand for Ure2p [36], a central repressor of genes involved in yeast nitrogen metabolism. While Ure2p has been widely studied, there was no known small molecule inhibitor of this protein. Screening a library of 3780 small molecules microarrayed on the silyl chloride slides with fluorescently labeled Ure2p, the first ligand for this protein was discovered and named uretupamine (Fig. 9). Interestingly, uretupamine was found to inhibit only a subset of Ure2p's functions and as such provided the means to deconvolute Ure2p's different roles. It should be noted that the screen only required 4 μg of protein! Whole-genome transcription profiling and *URE2* gene depletion experiments showed that this inhibitor modulated the glucose-sensitive genes controlled by Ure2p. This discovery not only shed new light on the multiple function of Ure2p but also demonstrated the power of SMM in providing rapid screens for the small molecule probes necessary to dissect complex biological networks. Using the same approach, but an extended microarray containing 12 396 small molecules from several different

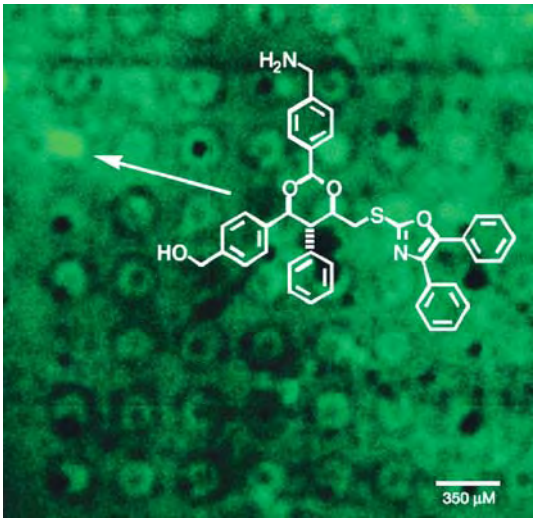

Fig. 9 Expanded view of the region of the 3780 compound SMM showing the signal from Cy5-labeled Ure2p interaction with uretupamine (reprinted with permission from reference [36])

combinatorial libraries, Hap3p, a subunit of a yeast transcription factor complex involved in yeast aerobic respiration, was screened. For this purpose, Hap3p was expressed with a GST tag and visualized with a Cy5-labeled Ab against GST. Two hits were identified from the microarray and one was reconfirmed to be a good Hap3p ligand by SPR (K_d 5 μM). Interestingly, the other hit was a GST ligand. Importantly, the identified molecule was found to function in vivo using a reporter assay [69]. In a third example from the same group, a microarray containing 6336 phenols immobilized using the diazobenzylidene was screened with Cy5-labeled calmodulin. Sixteen hits were identified of which 13 were reconfirmed using SPR (K_d 0.12 μM for the best compound) [39]. Together these results clearly show the power of SMM screen to discover new ligands for proteins of interest.

The first peptide microarrays were used to map the epitope of antibodies [16]. More recently, researchers have used an array of peptides to find an inhibitor of angiotensin II, one of the strongest vasopressors that regulates the cardiovascular system and blood pressure. An array of 8mer peptides spanning the sequence of the endogenous receptor of angiotensin II was prepared by SPOT-synthesis. Screening of this macroarray containing 352 unique peptides with fluorescently labeled angiotensin II afforded four concurring hits [70]. It was further shown that the best hit inhibited the contractile response of angiotensin II in a phenotypic assay.

All those reports required pre-purification or modification of the target protein with a label in order to visualize the signal on the array. Such labeling has limitations, including the need for additional steps in or prior to the assay in order to chemically or genetically encode the tag. Furthermore, proteins may need to be in a complex to be functional or properly folded. Perhaps the most important limitation of labeling is the inability to identify unanticipated (and thus unlabeled) proteins in crude cell lysates. Furthermore, it would be difficult for labeling techniques to be useful for diagnostic purposes because they are not applicable to complex mixture of proteins such as crude cell lysates. Nevertheless, they remain essential for screening SMM against target enzymes. In an alternative strategy, it was shown that PNA-encoded small molecules could be screened against crude cell lysates by using a size-exclusion separation to remove small molecules that do not interact with a macromolecule (Fig. 10) [52]. Since all the PNA tags also bear a fluorophore, hybridization of the selected compounds to a DNA microarray reveals the hits. Following speculations that acute respiratory allergy may be accentuated by residual proteolytic activity in allergens such as dust mite feces, a 4000 compound library targeting cysteine proteases was screened against crude fecal extracts, resulting in the discovery of a potent inhibitor of Derp1 [56]. Using a phenotypic assay, this inhibitors was used to correlate the function of Derp1 and T-cell replication, the phenotype of allergy. In a more targeted approach, it was also shown that the inhibitors bound to an enzyme could also be isolated using a gel-based separation. This approach was used to iden-

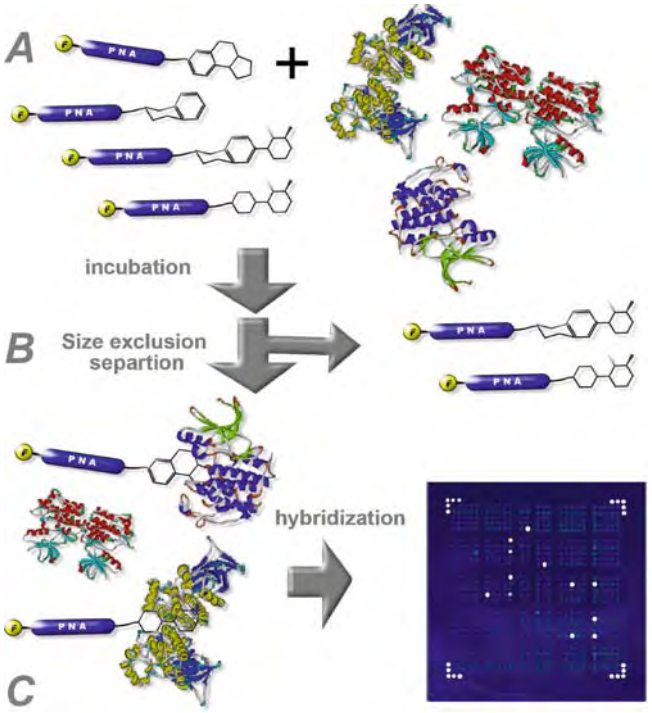

Fig. 10 Screening PNA-encoded libraries. **a** PNA-encoded library is incubated with unlabeled proteomic mixture. **b** The mixture is passed through a size exclusion filter and PNA-encoded molecules that are not bound to a protein are removed. **c** The hits are hybridized to the microarray for readout (all PNA are labeled with a fluorophore)

tify orthogonal inhibitors to the closely related cathepsins K and F. While several compounds inhibited both cathepsins, two compounds were found to be nearly ten times more potent for their respective targets [54]. Recently, it has been shown that an optimized microarray surface containing short PEG spacer could also be used to screen proteins directly from crude cell lysates using either genetically tagged proteins (GST) or specific antibodies towards untagged proteins. The ability to screen directly from crude lysates is significant as it saves substantial time and effort and may be more relevant as many proteins require a partner to remain active [5].

As protein rarely binds a single compound in a combinatorial library, the pattern of binding can be used as a specific fingerprint of a given protein [71]. To demonstrate that principle, a microarray of peptoids (7680) was probed with three proteins: maltose-binding protein, glutathione *S*-transferase, and ubiquitin. Each of these proteins gave a unique pattern for interaction. Knowing the fingerprint of a protein, such array could be used to detect specific proteins from proteomic mixtures. In a related effort to find a general ligand for IgG as an alternative method to protein A or G for antibody purifica-

tion, a SMM of 2688 triazines was screened, yielding a ligand with a K_d of 2 μM [72].

3.2
Profiling Substrate Specificity of an Enzyme or Enzymatic Activity from Complex Mixtures

A second important application of SMM has been to profile the substrate specificity of given enzymes or to measure the activity of enzymes from crude cell lysates. This has been demonstrated for kinases, proteases, and glycosidases.

3.2.1
Kinases

Phosphorylation of proteins by kinases provides an essential posttranslational mechanism to regulate the activity of targeted proteins. Kinases are involved in most signal transduction pathways and have been implicated in cell proliferation, differentiation, metabolism, and apoptosis. Information about cellular substrates of kinases and a method to measure kinase activity in relation to a particular cellular state are essential for dissection of kinase networks. Researchers in the kinase area recognized the power of peptide libraries toward this goal. It was demonstrated in 1995 that macroarrays on cellulose paper could be used to define optimal substrates for PKA and PKG [73] or to define peptide sequence with high affinity to cGPK [74]. As previously discussed, these macroarrays are two to three orders of magnitude larger than microarrays and require significantly more protein for screens.

More recently, a number of reports have appeared demonstrating that phosphorylation could be measured in a microarray format using a known kinase–substrate pair. Two different detection approaches have generally been used, the first makes use of [γ-$^{32/33}$P]-ATP to label the immobilized substrate with a radioactive phosphate, which can be detected by autoradiography, phosphor imager, or silver staining [9, 13, 75–79]. The second detection method relies on phosphospecific antibodies, which are fluorescently labeled [13, 51, 58, 76, 80]. While the fluorescent detection may be preferable as it avoids working with radioactive ATP, it has been shown that only monoclonal anti-phosphotyrosine antibodies showed reliable results [76]. Alternatively, fluorescently labeled phosphor-chelators have been used to detect the phosphorylated peptide in an array [81]. Preliminary results have also been reported for mass spectrometry detection and surface plasmon detection (vide infra).

In an impressive step from a proof of principle to a useful tool, Schutkowski and coworkers reported the preparation of peptide microarrays (13mers) containing 700 to 1300 kinase substrates identified bioinformatically from se-

quence analysis of the human genome [78]. Using [γ-^{33}P]-ATP, the authors showed that these arrays could be used to identify the preferred substrate of a given kinase, as exemplified by profiling two kinases – protein kinase A (PKA) (Fig. 11) and 3-phosphoinositide-dependent protein kinase (PDK1). The same strategy was used to identify the preferred substrates of CK2, a serine/threonine kinase [76]. Rather than using specific peptide sequences of predicted phosphorylation sites from database analysis, it was also shown that the preferred substrate of Abl could be inferred from the phosphorylation of a random array of 1433 peptides using a weight matrix-nearest neighbor algorithm [77]. In an other example, this technique was used to define the substrate specificity of Dbf2 a yeast kinase which, together with its binding partner Mob1, is an important component of the mitotic exit signaling network. The Dbf2–Mob1 complex was found to preferentially phosphorylate substrates that contain an RXXS motif and it was shown that proteins containing this motif were phosphorylated in vivo. However, the relatively low degree of sequence restriction suggested that Dbf2 achieves specificity by docking its substrates at a site that is distinct from the phosphorylation site [75]. Together, these studies clearly demonstrate that peptide microarrays represent a useful tool in identifying the preferred substrate of a kinase, which can be used to predict the cellular target of a kinase or for screening and diagnostic applications.

It was also demonstrated in a model system that the microarray format could be used to determine the K_i [13] and selectivity [82] of inhibitors. In

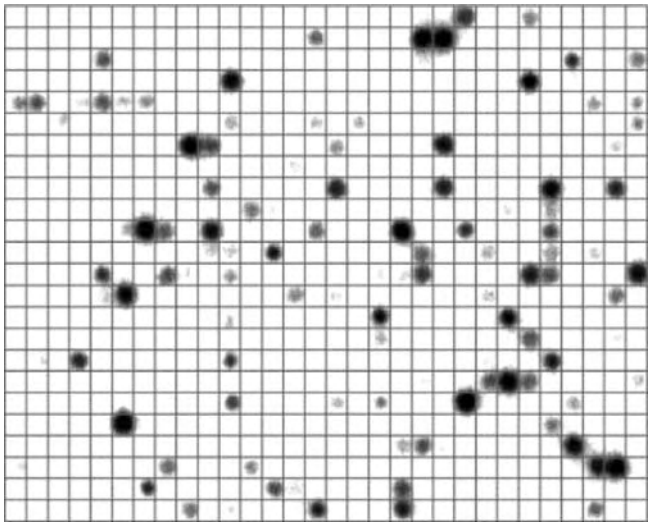

Fig. 11 Profiling substrate selectivity of PKA. A peptide microarray displaying 710 peptides derived from annotated human phosphorylation sites after incubation with PKA and ^{32}P-ATP and phosphoimaging (reprinted with permission from reference [78])

the later case, microfluidics was used to deliver separate kinase/inhibitor mixtures over a set of kinase substrates.

3.2.2 Proteases and Hydrolases

Proteases hydrolyse the amide bonds of proteins. While some proteases have a purely metabolic function, a number of proteases are involved in the post-translational regulation of protein activity and are essential for cellular function. Many pathways such as hormone activation, apoptosis, coagulation, or viral infection are dependent on the action of specific proteases. As for kinases, there is widespread interest in methods that define the preferred substrate of a protease and in being able to correlate their activity to the cellular state. Three detection methods have been developed based on irreversible inhibitors that selectively label active proteases [52], fluorogenic substrates [55, 83], and substrates flanked by two FRETing fluorophores [58].

The first proof of concept that protease activity could be measured from crude cell lysates using microarray-based technologies was reported in 2002. Using a set of PNA-encoded irreversible inhibitors to label active proteases in complex mixtures, the authors showed that they could measure the difference in activity of caspase-3 between apoptotic and healthy cells [52]. Concurrently, it was shown that immobilized coumarin-based fluorogenic substrates (Fig. 12) could be used to define substrate specificity of proteases, as exemplified with thrombin. Importantly, it was shown that the relative K_{cat}/K_m was comparable for solution substrates and immobilized substrates [83]. It was later shown that PNA-encoding could also be used to prepare fluorogenic substrate libraries. Aside from defining the substrate specificity of a given protease, the method was shown to be robust enough to be used with more complex mixtures such as crude cell lysates or clinical blood samples. It was

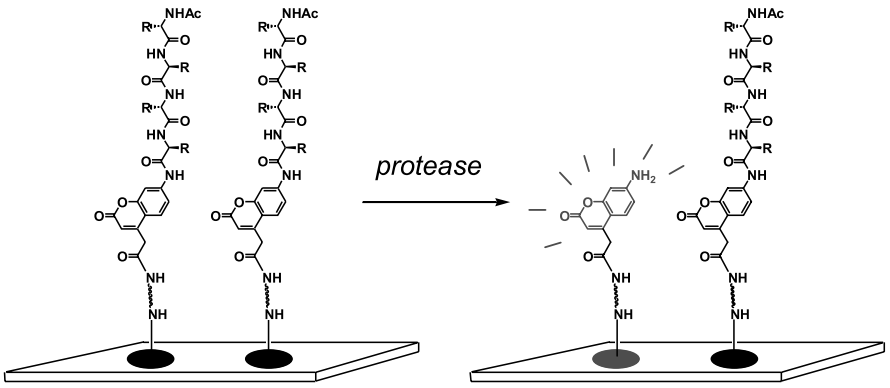

Fig. 12 Profiling substrate selectivity of protease using fluorogenic substrates

also sufficiently sensitive to measure differences in proteolytic activity between apoptotic and healthy cells as well as between sera from patient on anticoagulation therapy and healthy patients [55]. Conversely, it was also shown in a proof of principle that the activity of pepsin could be detected from a PNA-encoded library of substrates using dabcyl as an internal quencher and fluorescein [58]. More recently, PNA-encoded substrate containing fluorescent probes was used to perform β-secretase assay, a protease involved in Alzheimer's disease. In addition to the gain in miniaturization, the authors reported that this assay format was ten times more sensitive than assays in solution [57].

Alternatively, it was shown that fluorogenic substrates could be deposited as a glycerol/DMSO nanodroplet on a surface of a glass slide with the same density as covalent microarrays and that an enzyme of interest could be introduced by aerosol [84, 85].

3.2.3 Glycosidase

Shin and coworkers demonstrated that enzymatic glycosylations were possible on microarrays by converting GlcNAc to Sialyl-Lex using three separate glycosidase (β-1,4-galactosyltransferase, α-2,3-sialyl transferase and α-1,3-fucosyl transferase), followed by the detection of the product using a fluorescently labeled anti-Sialyl-LeX Ab [86]. An interesting application of this principle would be to use a microarray of substrates to profile the substrate specificity or activity of carbohydrate-processing enzymes. As a model study for this, a microarray containing GlcNAc and fucose was treated with β-1,4-galactosyltransferase and UDP-Gal. Revealing the product of the reaction with specific lectins showed that only GlcNAc was converted suggesting that this method should be more broadly applicable.

3.3 Carbohydrate Arrays

The glycobiology community was quick to recognize the potential of microarray technologies to screen glycan interactions. Microarrays are particularly well suited for this purpose as complex glycans are difficult to obtain in large quantities and the presentation of glycans on a surface with controlled density mimics the natural display of carbohydrates on the surface of proteins or phospholipid bilayers. This "cluster" effect may also be important to yield strong multivalent interactions, which have been shown to be important for affinity and selectivity in natural systems. Oligosaccharides have been immobilized using maleimide–thiol chemistry [86–88], Diels–Alder reaction [89], the reaction of *p*-aminophenyl glycosides with cyanuric chloride-coated slides [90], Huisgen cycloaddition [45, 46, 48, 59, 60], Staudinger lig-

ation [91], amine-NHS chemistry [92], and hydroxylamine-aldehyde [93]. Several reviews have already appeared highlighting the first proofs of principle in preparing and screening carbohydrate microarrays [10, 47, 94]. Notable applications of carbohydrate arrays have been to profile the specificity of therapeutically relevant antibodies against a panel of oligosaccharide. Wong and coworkers used a panel of epitopes to dissect the specificity of 2G12, a human antibody that neutralizes a broad range of HIV-1 strains and is known to recognize mannose-rich carbohydrate ($Man_9GlcNAc_2$) present on gp120. The profile revealed that the carbohydrate specificity of 2G12 is less restrictive than originally believed and that in a multivalent display, a single Manα1-2Man is sufficient for strong binding [46]. In a similar study, Wong and coworkers profiled the specificity of monoclonal antibodies to Globo H, a hexasaccharide antigen found on the surface of several cancer cell lines [48]. For this purpose they prepared a microarray with seven oligosaccharides (the Globo H hexamer and truncated forms) at 15 different concentrations. It was found that in a multivalent format, the truncated tetrasaccharide had similar activity to the Globo H hexasaccharide, suggesting that the more accessible tetrasaccharide should also be effective for vaccine preparation. Carbohydrate and glycoprotein microarrays have also proven useful to study carbohydrate–protein interactions beyond the known model systems. Seeberger and coworkers used an array of seven carbohydrates and five glycoproteins to define the interactions of gp120 and its glycans with five different binding proteins (CD4, CVN, Scytovirin, 2G12, and SC-SIGN) [88]. Aside from CD4–gp120, all other interactions required the glycan. However, the promiscuity of binding varied greatly amongst the four proteins, with Scytovirin being the most specific for the exact glycan structure present on gp120. More recently, Seeberger and colleagues used an array of six carbohydrates of varying length and sulfation pattern spotted at 12 different concentrations to probe interactions between these heparin-like glycans and fibroblast growth factors (FG1 and 2) [92]. This analysis led to the identification of a monosaccharide ligand bearing a sulfation pattern not found in nature that had similar affinity to tetra- and hexasaccharides. In another study on the sulfation pattern of chondroitin, a glycosamine glycan, Hsieh-Wilson and coworkers discovered a novel and specific interaction between chondroitin sulfate and TNF-α that can inhibit its activity [93]. In the most extensive study of lectin interactions, an array of 69 carbohydrates and glycoproteins were probed with 24 lectins at varying concentrations. This extensive study led to the identification of several unexpected lectin interactions and in a larger context clearly demonstrated the potential throughput of carbohydrate microarrays [95].

3.4
Solution-Based and Cell-Based Screening in a Microarray Format

Beyond proof of concept, nanodroplet technology was used to screen a library of 352 small molecules against three caspases leading to the identification of a selective inhibitor (high μM) [63]. This method was later used to screen 1000 pharmaceutically active compounds against human cathepsin L, a cysteine protease, based on the speculation that cathepsin L may be important in the activation of severe acute respiratory syndrome coronavirus (SARS). The screen led to the identification of a dipeptide aldehyde with an IC_{50} of 2.5 nM for cathepsin L. This compound was then shown to block SARS entry into host cells, thereby validating the role of cathepsin L in the pathology of SARS. This compound could potentially be used to develop a new therapy against the SARS virus [96].

The SMM developed for cell-based screens [64] using biodegradable polymer to imbed the small molecule was used to evaluate the synergistic effect of 70 small molecule inhibitors at three different concentrations in combination with seven different siRNA in two different cell lines. This small pilot experiment required the collection of 50 000 data points, which attests to the necessity for miniaturization. The screen led to the identification of four hits where a compound had reduced effect in the presence of an siRNA. These compounds were retested in conventional cytotoxic assays, which confirmed that macbecin II had reduced cytotoxicity when TSC2 was knocked down, suggesting that enhanced cellular growth resulting from decreased TSC2 activity can reduce a cell's sensitivity to DNA damage. Together, these results demonstrated that high content cell-based assays can be performed in a highly miniaturized microarray format, which greatly reduces the need for complex automation.

3.5
Beyond Fluorescent Readout

Fluorescence has thus far been the method of choice for detecting interactions in most microarray applications. As discussed earlier, detection of phosphorylated substrates has been successfully achieved using [γ-$^{33-32}$P] ATP for the phosphorylation followed by autoradiography as there are a number of limitations with fluorescent detection of phosphorylated residues [9, 13, 77, 78]. Alternatively, Mrksich and coworkers have also shown that mass spectrometry (MALDI) can be used to quantify phosphorylation of substrates microarrayed on gold surfaces [82] as well as small molecule–protein and protein–protein interactions [97]. Becker and coworkers have shown that MALDI is also suitable to detect protein–protein interactions for protein microarrays prepared by self-assembly of DNA-tagged proteins onto a DNA microarray [98]. Although the throughput of MALDI is much lower than fluorescence,

it provides a means to identify the protein and as such may lead to discovery of unanticipated interactions. Alternatively, surface plasmon resonance (SPR) has been used to detect phosphorylated peptides. As the difference between a peptide and the phosphopeptide is not sufficient to produce a change in SPR, an antiphosphotyrosine [13] and a novel zinc-based phosphochelator group coupled to biotin/streptavidin [99] were used to produce a shift in SPR. In a proof of concept, it was also shown that SPR could be used to detect interaction between small molecules immobilized via photocrosslinking (vide supra) and proteins or antibodies [100].

4 Conclusion

Over the past 7 years a number of new technologies have been reported for the preparation of small molecule microarrays and there is now a broad repertoire of chemistries to immobilize small molecules with controlled or random orientation. Several groups have reported the preparation of SMM with libraries ranging from 1000 to over 10 000 compounds. The efficiency of SMM to screen and discover new ligands has been demonstrated with several landmark studies providing new ligands to explore important biological problems. The applicability of SMM to profile or to measure enzymatic activity from complex proteomic mixtures has also been demonstrated beyond simple proof of principle for kinases and proteases. In the carbohydrate field, the cluster effect of microarrays appears to be beneficial and carbohydrate arrays have also led to important new discoveries regarding therapeutically important antibodies, lectin specificity, and carbohydrate sulfation pattern. While the use of SMM is more challenging to implement than oligonucleotide microarrays, based on early success, there is little doubt that this format will become more widespread and will find applications in diagnostics.

Acknowledgements We thank our collaborators who have contributed to our work in the area of SMM (J. Harris, U. Urbina, B. Jost, C. Bole-Feysot, D. Mason) and the funding agencies Human Frontier Science Program, Agence National de Recherche, and Marie Curie. This review was not intended to be comprehensive but rather to highlight some recent work and we apologize for unenviable arbitrary omissions.

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