Application of the Trityl Group in Peptide Chemistry

IVO FRANCI EGGEN
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op het gebied van de Natuurwetenschappen

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IVO FRANCI EGGEN
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Promotores
Prof. Dr. G.I. Tesser
Prof. Dr. H. Höcker (RWTH Aachen)

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Prof. Dr. H. Zahn
Dr. P.B.W. ten Kortenaar

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Eggen, Ivo Franci

Application of the trityl group in peptide chemistry
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Ma salgo ancora nuove scale
e vedo ancora più in là
la luce chiara di domani
precipitando esplode già.

(Francesco De Gregori e Zucchero, “Pane e sale”)

I fiori son facili da dipingere
il mistero è nelle foglie.

(Francesco Messina e Alice, “In viaggio sul tuo viso”)

Za opa in omo
ki bosta vedno v mojem srcu
En voor Piš en Niš
Dankwoord

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List of Abbreviations


Additional abbreviations used are:

AAA: amino acid analysis; Acm: acetamidomethyl; AcOH: acetic acid; Al: allyl; b: resin loading; Aloc: allyloxy carbonyl; Boc: *tert*-butyloxy carbonyl; BOP: benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; Bpoc: 2-(4-biphenyl)isoproxy carbonyl; ’Bu: *tert*-butyl; Bum: *tert*-butyloxymethyl; BzI: benzyl; Clt: 2-chlorotriyl; CSpSS: convergent solid-phase peptide synthesis; DCA: dicyclohexylamine; DCC: *N,N*-dicyclohexylcarbodiimide; DCM: dichloromethane; DCU: *N,N*-dicyclohexylurea; Dde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl; DIPCDI: *N,N*-dipropionylcarbodiimide; DIPEA: *N,N*-dioxopropylethylamine; DMA: *N,N*-dimethylacetamide; Dmab: 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl; DMAP: 4-dimethylaminopyridine; DMF: *N,N*-dimethylformamide; DMSO: dimethyl sulfoxide; Dmt: 4,4’-dimethoxy-4’-dimethyltrityl; DTE: 1,4-dithioerythritol; DTT: 1,4-dithiothreitol; DVB-PS: divinylbenzene-poly styrene; El-MS: electron impact mass spectrometry; ES-MS: electrospray mass spectrometry; Et: ethyl; EtOAc: ethyl acetate; FAB-MS: fast atom bombardment mass spectrometry; Fmoc: 9-fluorenylmethoxy carbonyl; FT-IR: Fourier transform infrared spectroscopy; GC-MS: gas chromatography-mass spectrometry; HFIP: hexafluoroisopropanol; HOBt: 1-hydroxybenzotriazole; HONs: *N*-hydroxy succinimide; MALDI-TOF-MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Me: methyl; MeOH: methanol; meq: molar equivalent(s); Mmt: 4-methoxytrityl; Mtr: 4-methoxy-2,6,6-trimethylbenzenesulfonfyl; Mt: 4-methyltrityl; NBA: nitrobenzyl alcohol; NMM: *N*-methylmorpholine; NMP: *N*-methylpyrrolidone; NMR: nuclear magnetic resonance; Np: 4-nitrophenyl; PAS: photoacoustic spectroscopy; PE: petroleum ether; Ph: phenyl; Pmc: 2,2,5,7,8-pentamethylchroman-6-sulfonfyl; RP-HPLC: reversed-phase high-performance liquid chromatography; RT: room temperature; SARSIN: super acid sensitive resin; Scm-Cl: 5-carboxyethyl sulfenyl chloride; SIMS: secondary ionization mass spectrometry; TBTU: 2-[(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TDM: 4,4’-tetrathieldiamidodiphenyl methane; TES: triethylsilane; TFA: trifluorocetic acid; TFE: trifluoroethanol; TFMSA: trifluoromethanesulfonic acid; THF: tetrahydrofuran; TIS: triisopropylsilane; TLC: thin layer chromatography; TMEDA: tetramethylethylenediamine; TMS: trimethylsilyl; Tmt, TMT: 4,4’,4”-trimethyltrityl; *t*<sub>r</sub>: retention time; Tris: tris(hydroxymethyl)-aminomethane; Trt: trityl, triphenylmethyl; TTMG: tetramethyl-trityl-guanidine; UV: ultraviolet; VIS: visible; Z: benzyl oxycarbonyl.
Chapter 1

General Introduction

1.1. Solid-phase peptide chemistry

1.1.1. Principles of the solid-phase technique

The introduction of the solid-phase technique by Merrifield in 1963 revolutionized peptide chemistry. In classical peptide chemistry, laborious syntheses of larger peptides like 39-residue adrenocorticotropic hormone, 27-residue secretin and 51-residue insulin (two chains mutually connected by disulfide bridges) have been accomplished using stepwise acylation methods and fragment condensations [1-3]. The solid-phase method, developed in the course of about 25 years, did not only enable the synthesis of longer peptides [4], but would also pave the way to the design of peptide libraries, giving rise to exponential production of new peptides in a simple straightforward way [5]. The desirability of a fast method for the synthesis of peptides was posed by modern immunochemistry, drug design and investigations into structure-activity relationships. In order to obtain an analogue of a peptide hormone with the desired activity, many sequences with only minimal variations should be compared. The application of synthetic peptides as such is a big gain in important medical fields, such as cancer and AIDS research [6, 7].

Simultaneously with the development of applications of synthetic peptides, the method itself is also constantly subjected to a lively evolutionary process. Solid-phase peptide synthesis (SPPS) consists of the stepwise acylation of the amino terminus of a nascent peptide chain, which is attached to an insoluble polymeric support, with activated Nα-protected amino acids, the protecting group being of the urethane type [8]. The stepwise strategy for the synthesis of peptides, as depicted in Scheme 1, has been introduced by Bodanszky and du Vigneaud, who observed in their solution synthesis of oxytocin a complete absence of racemization, which is typical for this method [9]. The great advantage of synthesis on a solid support is the elimination of intermediate purifications, which depend on crystallization, on lengthy chromatographic operations or on counter-current distribution. Moreover, SPPS is readily amenable to automation.
\[
\text{X-Leu-ONp} + \text{H-Gly-O-Y} \rightarrow \text{X-Leu-Gly-O-Y} + \text{HONp}
\]

\[
\text{H-Leu-Gly-O-Y} \xrightarrow{\text{base}} \text{H}_2\text{Leu-Gly-O-Y Br}^-
\]

\[
\text{X-Pro-ONp} \rightarrow \text{X-Pro-Leu-Gly-O-Y} \rightarrow \text{etcetera}
\]

**Scheme 1:** The stepwise strategy of peptide synthesis according to Bodanszky and Du Vigneaud [9], where X denotes benzylloxycarbonyl, Y an acid-stable carboxyl protecting group and activations are by the p-nitrophenyl esters. In Merrifield’s solid-phase method (Section 1.1.2), X denotes tert-butyloxycarbonyl, Y methylpolystyrene and activations are with carbodiimide.

The strategy of an actual solid-phase synthesis is determined by the proper combination of support, protection scheme and reagents used. The protection scheme involves three levels: (a) the anchoring linkage, (b) the side chains of trifunctional aminoacyl groups and (c) the α-amino function, which is to be acylated. The protecting groups used for (a) and (b) have a semi-permanent character; they are not removed before the protected peptide is fully assembled. Protecting groups suitable for (c) have a temporary character, which means that they should be selectively removable in each cycle of the SPPS, to allow a new α-amino acylation to take place (Scheme 2 [10]).

**1.1.2. Boc chemistry**

The original SPPS system, as conceived by Merrifield [11], makes no distinction between the functions (a) and (b). It is based on the difference in sensitivity towards acidolysis of the benzyl-type adducts of (a) and (b) on the one hand and the tert-butyl-type adduct of (c) on the other hand. One cycle in this protocol includes:

- removal of the $N^\alpha$-tert-butyloxycarbonyl (Boc) protecting group with 50% TFA in DCM [12] or 4 M HCl in dioxane [13], leaving the α-amino function as the corresponding ammonium salt;
- deprotonation of the ammonium group with a tertiary base;
- acylation of the free α-amino function with the new $N^\alpha$-Boc-amino acid.
Scheme 2: General scheme for stepwise solid-phase peptide synthesis [10], where (a) denotes the anchoring linkage, (b) the semi-permanent side-chain protecting groups and (c) the temporary \( N^\alpha \)-protecting group. \( R \) denotes the solid support, \( AA^1 \) the N-terminal amino acyl residue and \( AA^n \) the C-terminal.

Merrifield originally chose chloromethylpolystyrene as the supporting resin, and cleaved both the anchoring linkage and the side-chain protecting groups (benzyl esters and benzyl urethanes (Bzl/Z)) by treatment with a strong acid, namely anhydrous HBr in TFA. Since the repetitive treatments with TFA or HCl to remove the temporary Boc groups proved incompatible with benzyl functions in
longer chains, the acid stability of adducts of both (a) and (b) had to be enhanced by the introduction of substituents, which hamper the formation of benzyl-type cations. Consequently resins of the PAM type* (Fig. 1) [14] and halogenated or nitrated benzyl-type protecting groups [15] were designed, respectively, necessitating the application of stronger acids, i.e. anhydrous HF or TFMSA, for final deprotection and cleavage of the peptide-resin link. Harsh acidolytic conditions in turn generate reactive carbonium ions, provoking side-chain modification of sensitive amino acid residues like methionine, tyrosine and tryptophan [16-18]. In order to reduce this risk, scavengers must be added.

![Chemical structure](image)

**Figure 1:** The phenylacetamidomethyl (PAM) resin, whose esters are cleavable by HF.

### 1.1.3. Orthogonality

The principle of Merrifield’s method was improved in 1977 with the introduction of orthogonal protection [19]. Barany and Merrifield exemplified their new concept with the application of the 2,3-dithiasuccinoyl (Dts) function as a new amino protecting group, which is stable to all kinds of acids and which can be removed by thiolysis. They defined an orthogonal system as “a set of completely independent classes of protecting groups”, each of which “can be removed in any order and in the presence of all other classes.”

The concept was subsequently applied by Meienhofer and Sheppard, when they combined the acid-resistant but base-labile 9-fluorenylmethylxyloxy carbonyl (Fmoc) group for the temporary α-amino protection with the Wang resin (Section 1.1.4.) and side-chain protecting groups of the tert-butyl type [20].

Three-dimensional orthogonality has been achieved with the introduction of the acid and base-stable protecting groups of the allyl type; these functions, which are ultimately designed for the synthesis of branched and cyclic peptides, are selectively removed by allyl transfer onto a nucleophile, using a palladium(0)-complex as the catalyst [21]. Application of extremely acid-labile adducts like the triphenylmethyl (trityl) derivatives may be reckoned to this methodology, but in the strict sense only conform with the second half of the definition of orthogonality.

* A more detailed discussion on PAM-type resins and the consequences of aromatic substitutions in linker systems is given in Chapter 3.
1.1.4. Linkers allowing milder cleavage conditions

The original supports in SPPS usually consisted of resins, which were directly modified with an atom or a functional group to enable anchoring of the peptide chain. Wang and Merrifield [22] realized that insertion of a bifunctional linker between peptide chain and support (Fig. 2) would exert a beneficial effect on yield and purity of the product. This concerns especially the final step of the synthesis: detachment from the support and acidolysis of the protecting groups would be effected under milder conditions, eliminating the generation of excessively aggressive carbonium ions.

![Figure 2: The Wang resin. Insertion of 4-(hydroxymethyl)phenol between the support and the peptidyl moiety leads to a more acid-sensitive benzyl ester.](image)

Handles contain on one end a reactive group (e.g. a carboxyl group) for coupling to a functionality on the support (an activated methylene group or an amino group), and on the other end a group allowing condensation with the C-terminus of the target peptide. The linkage with the peptide may be selectively cleavable once peptide assembly has been completed. Whilst the Wang linker exhibits the same acid-sensitivity as tert-butyl-type protecting groups and detachment thus affords the peptide in its unprotected form [22b], this is not an essential feature. Inasmuch as the handle molecule is a separate unit and usually contains a benzyl group, its sensitivity for acidolysis can be "fine-tuned" by introduction of substituents with a +I effect (electron-releasing). Linkers introduced later, such as in the "super acid sensitive resin" (SASRIN) and the Rink linker, use this possibility for synthesis of protected peptide acids and peptide amides (Fig. 3) [23,24]. Synthesis of peptides in the protected form does not only facilitate their further processing, but also allows the final deprotection to take place in diluted solution.

Three-dimensional orthogonality is encountered in systems using allyl-type linkers [25], silicon-containing handles, which are cleaved by fluorolysis [26] or ortho-nitrobenzyl-derived linkers, which are sensitive to photolysis or nucleophilic cleavage [27].

1.1.5. Physical nature of the solid support

SPPS can be performed in two modes. In the conventional batchwise synthesis the support is a low-crosslinked polystyrene (1-2%) [28]. The support does not dissolve in any solvent, but actively
absorbs some solvents and their solutes by swelling. This ensures transportation of reagents to the sites where the peptide chains are immobilized. The synthesis is performed with a suspension in a shaken reactor. The growing chains are evenly distributed over the interior of each grain of resin and not only at the surface [29]; this is the cause of the relatively high loading of the resins, which can amount to 1.0 mmol/g. Excess reagents and by-products are readily removed after each acylation by washing and filtration. Completeness of an acylation can be estimated in the Kaiser test with a few grains of resin [30]. Batchwise syntheses can also be performed with polyacrylamide gels [31] or polyethylene glycol grafted on polystyrene [32].

In the continuous-flow mode, the support is a bed of rigid, macroporous grains, packed in a short column [33]. Reagents are introduced by pumping solutions through the reactor, which is not agitated. The grains consist of kieselguhr, onto which mobile polyamide chains are grafted (commercially known as Pepsyn K) [34]. Non-swelling, highly cross-linked “macroceticular” polystyrene grafted with polyethylene glycol is also applied [32]. In this mode the reactions occur only at the surface of the support and the loading per gram of polymer is consequently lower. Linkers can be introduced in the same way as for swelling resins. The course of the synthesis can be followed by continuous spectrometric monitoring [35].

![SASRIN](image1)
![Rink](image2)

**Figure 3:** Linkers developed for the synthesis of protected peptide derivatives by increasing the stability of intermediate carbonium ions that arise upon mild acidolysis. The SASRIN linker affords protected peptide acids; the Rink linker also affords protected peptide acids ($X = OH$) or deprotected peptide amides ($X = NH_2$).
1.2. The trityl group as a protecting function

1.2.1. Chemistry of the trityl group

The triphenylmethyl (trityl) cation derives its stability completely from resonance, due to extended stabilization of the benzyl moiety by introduction of two extra phenyl groups; charge delocalization is even more enhanced by its propeller-shaped geometry, with the aromatic rings inclined approximately 30° out of plane [36]. The cationic character of the tertiary carbon is so strong, that trityl chloride, a highly polar compound that was discovered by Gomberg in 1900 [37], reacts rapidly with nucleophiles like amines, primary alcohols and water, affording N-trityl amines, trityl ethers or triphenylmethanol (trityl alcohol, tritanol), respectively. The propensity of the trityl moiety to alkylate is exploited in the preparation of adducts with nitrogen, oxygen and sulfur compounds, a category encompassing the functional groups that occur in amino acids. These reactions are driven to completion by neutralization of the proton ensuing from this attack. The trityl cation can also be generated in situ with acid, if the eventual adduct is less basic, i.e. with carboxamides and thiols (Scheme 3).

![Scheme 3: Typical synthetic routes affording trityl-type adducts. The last shown route is applicable to all pertinent functional groups except carboxamide. B denotes a base.]

The reverse route, cleavage of a trityl adduct with a nitrogen compound, an acid, an alcohol or a thiol, proceeds by acidolysis and is followed by the conversion of the trityl cation into a trityl ether or to tritanol, according to:
R—X—Trt $\xrightarrow{\text{H}^+}$ R$^+$—X—Trt $\xrightarrow{-\text{H}^+}$ R—XH $+$ Trt$^+$ $\xrightarrow{\text{ROH}}$ R'O—Trt

with X = NH (amino, amido, hydrazido, imidazolyl, guanidino), O (alkyloxy, acyloxy) or S (alkylthio). Detachment is preceded by protonation; the formed species then cleaves to release the trityl cation. The relative stabilities of the different trityl adducts are determined by the concentration of the protonated species on the one hand, and by the leaving ability of the eliminated group R-XH on the other hand. For amino acid side chains, the following order in trityl adduct stability towards TFA is observed [38]: Asp < Glu < Tyr < Thr < Ser < Lys < His < Cys < Gln < Asn < Arg. Lysine holds an intermediate position with complete removal of $N^\alpha$-trityl by 15% TFA. $N^\alpha$-Trityl protection is more labile, whereas acid lability of the tritylhydrazide bond may be expected to lie between those of the e-amine (Lys) and carboxamide (Gln) functions. Field effects are very conspicuous in the deprotection of Asn(Trt) and Gln(Trt) in N-terminal position, of monosubstituted guanidino groups in Arg(Trt), and of the imidazolyl moiety in His(Trt).

For historical reasons, the protection of the $\alpha$-amino function with the trityl group is described next, followed by an overview of specific features that apply to adducts with other functional groups occurring in amino acids.

1.2.2. The trityl group for $\alpha$-amino protection

Protection of $\alpha$-amino functions with the trityl group, as introduced by Helferich in 1925 [39], comprised an altogether new strategy, since for the first time alkylation was used for protection instead of acylation. The approach initially failed to have an impact on peptide chemistry, but was eventually reinvestigated in 1953 by Hillmann and co-workers [40]. Alkylation was then performed by the reaction of trityl chloride with amino acid esters in the presence of triethylamine. Due to electronic effects within the trityl moiety, saponification of the esters had to be performed at elevated temperatures, which was expected to cause racemization. Subsequently, Zervas and Theodoropoulos described the direct tritylation of free amino acids or peptides by trityl chloride in aqueous 2-propanol, in the presence of diethylamine. These reactions, however, also proceeded in rather low yields because of hydrolysis of the trityl chloride [41]. The standard procedure for $N^\alpha$-tritylation was developed by Barlos and colleagues and makes use of intermediate trimethylsilyl esters, that react with trityl chloride in the presence of triethylamine [42]. Alternatively, due to its higher reactivity and its reduced hygroscopic character with regard to the chloride, trityl bromide may be used in combination with the free amino acids to yield the $N^\alpha$-tritylamino acid trityl esters; subsequent methanolation, catalyzed by free protons, results in formation of the corresponding acids in high yields [43].
The tritylamine bond is completely stable towards bases, but labile towards catalytic hydrogenation [41]. It is extremely labile towards acidolysis. Removal of the trityl group may be effected by heating in 50% AcOH for 2 minutes [41], with 0.5 M HOBT in TFE [44], 1% TFA in DCM [42b] or HCl in 90% TFE at an apparent pH of 4, thereby permitting its use as a selectively cleavable moiety in the presence of Boc and Bpoc (2-(4-biphenyl)isopropylxycarbonyl) protecting groups [45]. Due to its high electronic density, the presence of the Nα-trityl group, ensures the chiral stability of the Cα-centre [46] and strongly reduces the reactivity of the Cα-carboxyl function. As mentioned above, Nα-tritylamino acid esters are difficult to hydrolyze, which led to a synthetic method for selective α-saponification and transesterification of glutamic acid diesters [47]. Reactions of trityl-aspartic anhydride with Grignard and Wittig reagents, bulky hydrides, and amines or alcohols of the benzhydryl type, led regioselectively to products arising from attack at the β-carbonyl function [48]. Only Nα-tritylglycine methyl ester is attacked by hydrazine [41] and acylation of an amino acid ester can only be performed with Nα-tritylglycine according to the mixed anhydride or p-nitrophenyl ester methods [49,50]. Few cases have been reported, in which other Nα-tritylamino acids have been successfully used as acylating agents. These include carbodiimide-mediated couplings in solution [51] as well as solid-phase syntheses employing aza derivatives of aminium or phosphonium salts as activating reagents [52] or preformed benzotriazolyl esters [53]. Nα-Trityl protection has been mainly used at the peptide rather than the amino acid level, to allow selective removal under very mild acidolytic conditions [51a,54].

1.2.3. The trityl group for protection of other functions

Since trityl cations, formed during mild acidolytic cleavage, display a low electrophilicity in contrast to tert-butyl cations, SPPS using Fmoc/Trt amino acids affords peptides of higher purity, compared to those prepared by the application of Fmoc/Bu amino acids [55]. In recent years, a complete set of Fmoc/Trt amino acids has been developed for use in this novel approach (Table 1).

Cysteine - thiol

Due to its high nucleophilicity, the thiol group of cysteine can be tritylated by several methods, using either trityl chloride [56] or trityl alcohol under acidic conditions [57]. Tritylthiol cleavage is a reversible reaction in TFA: evaporation of the solvent TFA drives the equilibrium towards tritylthiol bond formation [58], addition of a silane scavenger results in cleavage by reduction of the trityl cation [59]. Cleavage may furthermore be accomplished by silver [56] or mercury(II) ions [60], also yielding a free thiol function, by thiocyanogen [61], thallium(III) [62] or iodine oxidation [63], affording a disulfide, or by S-methoxycarbonylsulfonyl chloride (Scm-Cl) [64], producing an activated thiol derivative.
Table 1: Applications of the trityl moiety as amino acid side-chain protecting group.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Derivative</th>
<th>Tritylation conditions</th>
<th>Acidolytic cleavage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys</td>
<td>Trt*</td>
<td>Trt-Cl</td>
<td>95% TFA / TES</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-OH / BF₃ / AcOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trt-OH / TFA</td>
<td></td>
</tr>
<tr>
<td>Mmt*</td>
<td>Mmt-Cl</td>
<td></td>
<td>0.5% TFA / 5% TES / DCM</td>
</tr>
<tr>
<td>His</td>
<td>Trt*</td>
<td>Me₂SiCl₂, Trt-Cl / Et₃N</td>
<td>90% TFA</td>
</tr>
<tr>
<td>Mtt*</td>
<td>Me₂SiCl₂, Mtt-Cl / Et₃N</td>
<td>15% TFA</td>
<td></td>
</tr>
<tr>
<td>Mmt</td>
<td>Me₂SiCl₂, Mmt-Cl / Et₃N</td>
<td>5% TFA</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>Trt</td>
<td>Me₂SiCl₁, Trt-Cl / Et₃N</td>
<td>10-20% TFA</td>
</tr>
<tr>
<td>Mtt*</td>
<td>Me₂SiCl₁, Mtt-Cl / Et₃N</td>
<td>1% TFA</td>
<td></td>
</tr>
<tr>
<td>Orn</td>
<td>Trt</td>
<td>Me₂SiCl₁, Trt-Cl / Et₃N</td>
<td></td>
</tr>
<tr>
<td>Mtt*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn / Gln</td>
<td>Trt*</td>
<td>Trt-OH / Ac₂O / H₂SO₄</td>
<td>95% TFA</td>
</tr>
<tr>
<td>Mtt*</td>
<td>Mtt-OH / Ac₂O / H₂SO₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>Trt</td>
<td>Trt-Cl / NaOH</td>
<td>100% TFA for several hours</td>
</tr>
<tr>
<td>Tmt</td>
<td>Tmt-Cl / TTMG</td>
<td></td>
<td>5% TFA</td>
</tr>
<tr>
<td>Ser</td>
<td>Trt*</td>
<td>Trt-Cl / Et₃N</td>
<td>1% TFA / 5% TIS / DCM</td>
</tr>
<tr>
<td>Hse</td>
<td>Trt*</td>
<td>Trt-Cl / Et₃N</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>Trt*</td>
<td>Trt-Cl / Et₃N / DMAP</td>
<td>1% TFA / 5% TIS / DCM</td>
</tr>
<tr>
<td>Tyr</td>
<td>Trt*</td>
<td>Trt-Cl / Et₃N / DMAP</td>
<td>AcOH / TFE / DCM (2:2:6)</td>
</tr>
<tr>
<td>2-Chl⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Commercially available as N'-Fmoc derivative.

Incorporation of a 4-methoxy substituent into the S-trityl group (Mmt) allows complete deprotection by 0.5% TFA in the presence of triethylsilane, within 40 minutes at room temperature [65]. Several other modified trityl-type protecting groups for the cysteine side chain have been reported, including 2-chlorotrityl [55a], 4,4',4''-trimethoxytrityl [66], tritylsulfenyl [67] and the "safety-catch" 4,4''-bis(methylsulfinyl)trityl [68] protecting groups.
Chapter 6 of this thesis describes the role of Cys(Trt) in the synthesis of peptide disulfides, while Chapter 7 gives an extensive overview of protecting groups for the cysteine side chain.

**Histidine - imidazole**

The strongly nucleophilic and basic character of the imidazole ring in histidine has been the cause of many a problem in peptide synthesis. During coupling reactions it may be partially acylated, followed by transfer of the acyl residue to other nucleophilic sites of the peptide [69]. The major problem, however, consists of the strong tendency of the histidine Cα-centre to racemize, a process which is catalyzed by the proximity of the basic π-nitrogen in the imidazole ring [70]. This problem may be overcome by directly blocking the π-site or else by a substitution at the τ-nitrogen, thereby inactivating the π-site. Tritylation of the imidazole ring occurs selectively at the τ-site [42a]. The bulkiness of the trityl group prevents imidazole acylation, whereas its strong electron-withdrawing character reduces the basicity of the π-nitrogen (the pKₐ of the imidazole ring decreases from 6.2 to 4.7 [71]) and thereby the extent of racemization.

His(Trt) is stable towards 1 M HCl in acetic acid. Complete deprotection is effected by 90% TFA in one hour [71,72], but substantial loss of the trityl group is observed at TFA concentrations as low as 1%. In order to allow the synthesis of fully protected peptide fragments, several more stable trityl-type protecting groups for histidine were tested [55a,73], of which the trichlorotrityl (Tct) group proved stable towards diluted TFA during a reasonable period of time. 4-Methyl (Mtt) and 4-methoxy (Mmt)-substituted trityl derivatives could be cleaved by 15 and 5% TFA, respectively [74].

**Lysine and other ω-amines**

Reaction of lysine with trityl chloride yields the Nω,Nω-ditritylated product [42b,c]. Selective Nω-deprotection can be accomplished by treatment with excess HCl [75] or 1% TFA at 0°C [42b]. The resulting Nω-trityl-lysine may be used for the synthesis of α as well as α,ε-peptides of lysine [76].

Selective removal of lysine side chain protecting groups is required in the solid-phase synthesis of side chain-to-side chain cyclic peptides, branched peptides and oligolysine cores suitable for the assembly of MAPs (multiple antigenic peptides) [77]. Their synthesis is possible through Boc chemistry on HF-labile resins. Alternatively, Fmoc chemistry may be applied if the lysine side chain is blocked by orthogonal protecting groups [78] or by the 4-methyltrityl (Mtt) group, which can be selectively cleaved by AcOH/TFE/DCM (1:2:7) in the presence of tert-butyl-type protecting groups [79].

The 4-methoxytrityl (Mmt) group was used in the solid-phase synthesis of polyamide nucleic acids (PNAs) as the amino protecting group for its monomers, nucleobase-derivatized N-(2-amino-ethyl)glycines. Deprotections took place by 3-minute treatments with 3% trichloroacetic acid in DCM [80].
Asparagine and glutamine - carboxamide

The carboxamide side chains of asparagine and glutamine are often unprotected during SPPS because of their low nucleophilic character. However, they are known to undergo several side reactions, the most important of which is nitrile formation during the activation step, via dehydration of the carboxamide side chain [81]. In addition, peptides containing unprotected asparagine and glutamine residues tend to be insoluble due to formation of β-sheet aggregates, which may lead to inefficient deprotection and slow coupling rates during SPPS. Carboxamide protection by the 2,4,6-trimethoxybenzyl (Tmob) and 4,4'-dimethoxybenzhydryl (Mbh) groups suppresses side reactions and β-sheet formation, but must be avoided in combination with tryptophan, since the cations arising from acidolytic deprotection attack the indole moiety in position 2 [81b]. Trityl protection of asparagine and glutamine side chains by acid-catalyzed reaction with trityl alcohol effects the same advantages. As, in addition, trityl cations do not alkylate the indole moiety, the trityl group is the preferred protecting group in the presence of tryptophan [82].

Deprotection of Asn(Trt) and Gln(Trt) proceeds slowly. The tritylamide function can indeed be applied in Bpoc-SPPS, since it provides complete stability towards 0.5% TFA [83]. Selective removal of the Nα-Boc group of peptides in the presence of Asn(Trt) or Gln(Trt) may be accomplished by treatment with a solution of 1 M HCl in 50% 2-propanol during 30 minutes at 50°C [84]. Due to a field effect, deprotection of N-terminal Asn(Trt) cannot be brought to completion within a reasonable period of time [85]; therefore, the 4-methyltrityl (Mtt) group was introduced for carboxamide protection, allowing deprotection to proceed 2 to 3 times faster than in the unsubstituted trityl derivatives [86]. A more detailed discussion of the protection of the α-carboxamide function is presented in Chapter 7.

Protected amino acid Cα-amides could be tritylated under similar conditions as asparagine and glutamine side chains. Detritylation proceeded even more slowly as exemplified by Z-Phe-NHTrt, which was 20 times more stable towards TFA-dichloroethane (1:1) than Fmoc-Gln(Trt)-OH [82a].

Arginine - guanidine

The trifunctional guanidine side chain of arginine is a strong base and should therefore be protected during SPPS. Due to its high pKa value (~12.5), it can be selectively protected by protonation [87]. However, this type of protection is, because of its polarity, mainly restricted to solution-phase synthesis. In SPPS, protecting groups of the arylsulfonyl type like 4-methoxy-2,6,6-trimethylbenzenesulfonyl (Mtr) [88], 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) [89] and 2,2,4,6,7-pentamethylhydrobenzofuran-5-sulfonyl (Pbf) [90] are most commonly applied. Removal of these functions usually requires stringent conditions, necessitating the protection of sensitive residues like tryptophan and tyrosine against aggressive cations by addition of scavengers [91].
The known acid lability of the trityl group and the low electrophilicity of its cation was exploited with the application of α-Fmoc-ω-tritylarginine. Tritylation of arginine in the presence of a strong base led to α,ω-ditryptyl derivatives, which could be selectively detritylated at the Nα-position by treatment with a methanolic solution of HCl [50,92]. Although Fmoc-Arg(Trt) was successfully applied in the synthesis of thymopentin and atrial natriuretic peptide [92], it is seldom used in SPPS because it is still too stable towards acid and sparingly soluble in DCM/DMF. More labile side-chain tritylated derivatives were synthesized by Barlos and co-workers by substitution of the trityl moiety with electron-releasing groups. The tris(4-methoxy)-substituted derivative Fmoc-Arg(Tmt)-OH could be completely deprotected by 5% TFA within 10 minutes at room temperature [93].

Serine, threonine and tyrosine - hydroxyl

In synthesis schemes with minimal protection, the hydroxylic side chains of serine, threonine and tyrosine may remain unprotected as long as no strong activating agents such as DCC are applied [87a,94]. They are, however, normally protected as the tert-butyl ethers, whose cleavage by 95% TFA often proceeds very slowly, especially in the case of serine [91a]. Both strategies can present additional drawbacks. The use of unprotected serine and threonine may be accompanied by the formation of dehydration products, whereas the use of tert-butyl protection may lead to substantial deletion sequences in the final product, due to secondary structural effects during synthesis [35,95]. To overcome these problems and, moreover, to allow the selective deprotection of certain residues needed for site-specific post-synthetic modifications like phosphorylations, sulfations or glycosylations, trityl ether protection was introduced.

Trityl ether cleavage is an equilibrium reaction that is only driven to completion by addition of silanes to scavenge the formed trityl cations [96]. Acidolytic lability increases when going from serine over threonine to tyrosine [97]. Side-chain tritylated tyrosine proved so labile that its total deprotection could be performed simultaneously with peptide cleavage from 2-chlorotrityl chloride resin, with AcOH/TFE/DCM (2:2:6) as the cleavage mixture [98]. To ensure complete stability during coupling reactions in the presence of the weak acid HOBt, the more stable 2-chlorotritylated (2-Clt) tyrosine derivative has been recently introduced [99].

Carboxyl

2-Chlorotrityl chloride was applied by Barlos and co-workers for temporary C-terminal carboxyl protection of peptide fragments in a convergent synthesis scheme, i.e. the sequential condensation of protected fragments [100]. Selective cleavage of the resulting esters was accomplished within 30-60 minutes using an AcOH/TFE/DCM mixture.
Hydrazide

Weygand and Steglich used the trityl group as protection for the hydrazide function, by reacting N\textsuperscript{α}-trifluoroacetamino acids with tritylhydrazin after the DCC method. Peptides could be synthesized in C-terminal direction by alternating azide couplings and trityl deprotections in alcoholic HCl [101]. Alternatively, deprotection could be achieved with boiling acetic acid [102].

1.3. The trityl group as anchoring linkage

Applications of several trityl derivatives as anchoring linkages for solid-phase synthesis are summarized in Table 2.

Table 2: Applications of the trityl moiety as an anchoring linkage in solid-phase chemistry.

<table>
<thead>
<tr>
<th>Trityl substituents (^a)</th>
<th>Synthesis conditions</th>
<th>Attached functionalities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-alkyl- 4'-methoxy</td>
<td>iodinated DVB-PS + (^1) n-BuLi (^2) 4-methoxybenzophenone (^3) acetyl chloride</td>
<td>hydroxyl</td>
<td>Melby, 1967 [103]</td>
</tr>
<tr>
<td>4-alkyl- 4',4''-dimethoxy</td>
<td>DVB-PS + (^1) 4-anisoyl chloride (^2) 4-anisylimagnesium bromide (^3) acetyl chloride</td>
<td>hydroxyl</td>
<td>Cramer, 1968 [104]</td>
</tr>
<tr>
<td>4-alkyl</td>
<td>DVB-PS + (^1) benzoxy chloride (^2) phenylmagnesium bromide (^3) acetyl chloride</td>
<td>hydroxyl</td>
<td>Fréchet, 1975 [105]</td>
</tr>
<tr>
<td>4-alkyl</td>
<td>DVB-PS + (^1) n-BuLi/TMEDA (^2) benzophenone (^3) acetyl chloride</td>
<td>hydroxyl</td>
<td>Leznoff, 1976 [106]</td>
</tr>
<tr>
<td>4-alkyl-2'-chloro</td>
<td>DVB-PS + (^1) 2-chlorobenzoyl chloride (^2) phenylmagnesium bromide (^3) acetyl chloride</td>
<td>carboxyl, hydroxyl, amine, all amino acid side chain functions</td>
<td>Barlos, 1991 [109] (^b)</td>
</tr>
<tr>
<td>4-carboxamide</td>
<td>aminomethylated PEG-PS + (^1) 4-carboxytritol/DIC/CDI/HOBt (^2) acetyl chloride</td>
<td>carboxyl</td>
<td>Bayer, 1993 [114]</td>
</tr>
</tbody>
</table>

\(^a\) Some derivatives can have additional functionalities.

\(^b\) Additional functionalities may be present in some derivatives.
Table 2 (cont.)

<table>
<thead>
<tr>
<th>Trityl substituents&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Synthesis conditions</th>
<th>Attached functionalities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-fluoro(chloro)-4'-carboxamide</td>
<td>aminomethylated DVB-PS + 1. 2-fluoro(chloro)-4'-carboxytritantol/DIPCDI/HOBt&lt;sup&gt;b&lt;/sup&gt; 2. acetyl bromide</td>
<td>carboxyl</td>
<td>Zikos, 1994 [115]</td>
</tr>
<tr>
<td>4-alkoxy</td>
<td>aminomethylated DVB-PS + 1. Tesser’s handle (&lt;i&gt;cf.&lt;/i&gt; Figure 4)/TBTU/HOBt/NMM&lt;sup&gt;2&lt;/sup&gt; 2. thionyl chloride</td>
<td>thiol, hydroxyl, amine, hydrazide</td>
<td>Tesser, 1992 [116]</td>
</tr>
<tr>
<td>4-benzoxy-4',4''-dimethoxy</td>
<td>chloromethylated DVB-PS + 1. methyl 4-hydroxybenzoate 2. 4-anisylmagnesium bromide 3. acetyl chloride&lt;sup&gt;4&lt;/sup&gt; ammonia</td>
<td>amide</td>
<td>Voelter, 1994 [118]</td>
</tr>
<tr>
<td>4-benzoxy-2',4''-dimethoxy</td>
<td>chloromethylated DVB-PS + 1. 2,4-dimethoxy-4''-hydroxybenzophenone 2. phenylmagnesium bromide 3. acetyl chloride&lt;sup&gt;4&lt;/sup&gt; ammonia/hydrazine</td>
<td>amide, hydrazide</td>
<td>White, 1994 [119]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Italics denote the link to the polymer backbone.  
<sup>b</sup> An alternative method for the preparation of 2-chlorotrityl chloride resin has been recently presented by Orosz and Kiss, using phenyllithium instead of phenylmagnesium bromide [110b].

1.3.1. Trityl-type resins for oligonucleotide and organic syntheses

The trityl moiety was first used as an anchoring linkage in oligonucleotide solid-phase synthesis. Melby and Stroback started with a copolymer of styrene and 4-iodostyrene in a molar ratio of 4:1, crosslinked with 1% divinylbenzene. Lithium-iodine exchange, followed by reactions with 4-methoxybenzophenone and acetyl chloride, yielded a methoxy-substituted trityl chloride resin [103]. Cramer and Köster proceeded by Friedel-Crafts reactions with benzoyl or anisoyl chloride on divinylbenzene-styrene copolymers and subsequent Grignard reaction with 4-anisylmagnesium bromide, to obtain mono- and dimethoxy-substituted trityl-type resins [104]. Semi-protected thymidine derivatives were attached to the chloride form of the resins in the presence of pyridine. Assembled oligonucleotides could be cleaved from the resins by treatment with acetic acid.
In the mid-seventies, two Canadian research teams headed by Fréchet and Leznoff applied trityl-type resins in organic solid-phase synthesis. Trityl alcohol resins were prepared from 1-2% crosslinked divinylbenzene-styrene copolymers. These were either converted by Friedel-Crafts benzoylation and subsequent Grignard reaction with phenylmagnesium bromide [105], or by direct lithiation and subsequent reaction with benzophenone or 4,4'-dimethoxybenzophenone [106]. They found lithiation of polystyrene to lead to ambiguous products arising from both the desired para as well as meta substitution; more selectivity could be achieved by prior bromination.

The polymers were applied in the selective functionalization of polyhydroxy alcohols. Acetylation of resin-bound symmetrical diols resulted in considerable recovery of unreacted diol, due to “double-binding” of the diol to vicinal trityl groups. In order to prevent “double-binding”, conformationally adjacent lithiated sites could be quenched by methyl benzoate or phosgene, to yield trityl-type anchoring groups that were doubly or triply bound to the resin backbone. As a typical result, 1,10-decanediol could be monoacetylated in 90% overall yield [106a].

1.3.2. Trityl-type resins for carboxyl attachment

2-Chlorotrityl chloride resin

Barlos introduced the trityl-type resin in peptide chemistry. Initially, an unmodified trityl resin, as described by Fréchet, was used to couple amino acid trimethylsilyl esters through their α-amino group and subsequently modify their C-terminus by reaction with amino acid esters or amides, by esterification or by reduction [107]. For the synthesis of peptides, however, the stepwise methodology in N-terminal direction had to be employed. Therefore, amino acid esters of various trityl-type resins were tested on their stability in this respect; both the unsubstituted and the para-chloro-substituted resins had to be excluded, since they proved to be too labile in DCC/HOBt-mediated couplings [108]. The 2-chlorotrityl chloride resin* turned out to be the ideal resin in the preparation of protected peptide acids, offering numerous advantages over dialkoxybenzyl-type resins [109]. Esterification of the first amino acid proceeds in DCM in the presence of an excess of DIPEA, resulting in initial loadings up to 0.9 mmol Fmoc-amino acid/g resin (which is too high for practical use). Since electrophilic activation is avoided, no racemization occurs during loading, making this method especially advantageous in the synthesis of peptides with C-terminal cysteine and histidine residues. Loading proceeds without by-product formation even in the case of Fmoc-asparagine and Fmoc-glutamine. The trityl ester group prevents the formation of diketopiperazines and enables the synthesis of (protected) peptides with C-terminal glycine and proline residues; these are required in fragment condensations [110].

* Starting from Chapter 2, this resin will be referred to as trityl-type resin I.
Inasmuch as the link between the tertiary trityl carbon and a nitrogen or oxygen atom cannot be attacked by a nucleophile, SPPS according to the Fmoc protocol – which does not require treatments with acid, but base – is excellently suitable for the synthesis of longer peptides. Quantitative detachment of protected peptides from the resin is performed under very mild conditions, utilizing mixtures of AcOH/TFE/DCM in typical compositions of 1:1:8, 1:2:7 or 2:2:6 by volume, depending on the peptide, during short periods of time. This treatment guarantees complete stability of all tert-butyl-type and most trityl-type side-chain protecting groups. Applying such cleavage mixtures selective side-chain deprotection of incorporated Tyr(Trt) [98] and Lys(Mtt) [79] could be accomplished simultaneously to detachment from the resin. Even milder cleavage conditions are provided by a mixture of HFIP/DCM (1:4) [111]. The extreme stability of the immobilized trityl cations formed during the cleavage reaction excludes the attack of sensitive side chains of Trp, Met and Tyr, thus eliminating secondary fixation of a detached peptide to its former support. Addition of iodine to the cleavage mixture effects simultaneous oxidation of incorporated Cys(Trt) residues, to yield protected peptides containing disulfide bonds [112].

The growing peptide may also be attached to the resin via a side-chain carboxyl group, as was demonstrated in the synthesis of protected [Leu^{15}]gastrin I and cholecystokinin octapeptide from immobilized Fmoc-Asp(OH)-Phe-NH₂ [98]. Alternatively, the resin may be loaded with a (longer) protected peptide [113]. Section 1.3.3. describes the anchoring of other functional groups to this resin.

*Variations on the 2-chlorotrityl chloride resin approach*

In contrast to direct modification of the polymer backbone to yield trityl-type resins, as described above, these resins can also be prepared by applying preformed bifunctional trityl-type handles. The handle can be purified and characterized prior to attachment, leading to an unambiguous and fully characterized resin. However, solution-phase synthesis and purification of the handle is more laborious in comparison with direct resin modification to afford the anchoring unit.

Bayer et al. attached 4-carboxytrityl alcohol to an aminomethylated polyethylene glycol-poly styrene graft support, which after reaction with acetyl chloride resulted in a resin with properties similar to Barlos's 2-chlorotrityl chloride resin, but designed for use in continuous-flow SPPS [114]. Eleven fully protected fragments of the HIV-1 protease were synthesized and cleaved from the resin by treatment with 50% acetic acid in DCM. No diketopiperazine formation was observed in the synthesis of fragments with C-terminal glycine or proline residues.

More complex variations of this handle were reported by Zikos and Ferderigos [115]. 4-Bromobenzoic acid was lithiated with n-butyllithium and subsequently reacted with 2-fluoro- or 2-chlorobenzophenone, yielding 2-fluoro or 2-chloro-4'-carboxytrityl alcohol, respectively. Attachment to aminomethylated divinylbenzene-styrene copolymer and treatment with acetyl bromide resulted in
resins, that allowed cleavage of protected peptide acids with either AcOH/TFE/DCM (1:1:8) or 0.1% TFA in DCM, the fluoro-derivative being slightly more stable.

1.3.3. Trityl-type resins for attachment through other functions

Introduction of more labile trityl-type resins in SPPS

It was Tesser who first recognized, that the application of more labile trityl-type resins – the trityloxygen link being extremely labile and requiring a fairly stabilized system as opposed to nitrogen and sulfur adducts – would enable the anchoring through other functional groups to be eventually followed by detachment under mild conditions, thereby greatly extending the number of possible pathways leading to protected peptide fragments [116].

In our laboratories a trityl-type handle was prepared, consisting of a 4-hydroxytrityl moiety and a pentanoic acid spacer, with the intention to increase the distance between the polymer backbone and the anchoring linkage, resulting in a better accessibility of the latter to reagent molecules (Fig. 4). The handle was condensed to aminomethylated divinylbenzene-styrene copolymer, yielding Tesser’s trityl-type resin*.

The alcohol form of the resin was treated with cysteamine in TFA. Evaporation of the solvent TFA led to attachment of the thiol function to the resin [58]. Subsequent SPPS of a 10-residue sequence, corresponding to the DNA binding wing of the viral gene-V protein of the M13 phage, was concluded by the N-terminal incorporation of S-acetamidomethyl-cysteine (Cys(Acm)). Oxidative detachment by iodine in DMF led to the formation of the protected monomeric disulfide in high yield and purity. Oxidation in other solvent systems like dioxane and chloroform/methanol gave not only the monomeric product, but also substantial amounts of the parallel symmetrical dimer retaining Cys(Acm) at its N-termini [117]. A more detailed account of the mechanisms is given in Chapter 6 (Section 6.1.3).

Preliminary studies showed that chlorination of the trityl alcohol resin by thionyl chloride enables the attachment of nucleophilic nitrogen and oxygen compounds, like amino acid hydrazides, diaminohexane and 2-butene-1,4-diol. The diol can be used in the synthesis of peptide allylic esters, which are deprotected by palladium-catalyzed allyl transfer onto a nucleophile to afford the peptide acid.

* This resin will be further referred to as trityl-type resin V.
Other labile trityl-type resins

To enable the preparation of protected peptide amides, Voelter and co-workers designed three different alkoxy-substituted trityl resins [118]. In a four-step reaction sequence, chloromethylated divinylbenzene-styrene copolymer was transformed into the 4-benzyoxtritylamine derivative. The first reaction involved nucleophilic substitution of the chloride by methyl 4-hydroxybenzoate in the presence of sodium methoxide in DMA (1). Grignard reaction with phenylmagnesium bromide led to the alcohol form of the resin (2), which was converted into the amine resin by subsequent treatments with acetyl chloride (3) and ammonia in DCM (4). Analogous to this scheme, 4-benzyoxo-4’-methoxytritylamine resin was prepared by using 4-hydroxybenzophenone and 4-anisylmagnesium bromide in step (1) and (2), respectively, and 4-benzyoxo-4’,4”-dimethoxytritylamine resin by using methyl 4-methoxybenzoate and 4-anisylmagnesium bromide. Attachment of the first Fmoc-amino acid was accomplished by the DCC method and followed by standard Fmoc SPPS. Only the trisalkoxytrityl resin proved to be labile enough towards 1% TFA to allow quantitative cleavage of protected peptide amides. Addition of MeOH or TFE to the cleavage mixture resulted in increasing cleavage times and the number of by-products.

White developed various alkoxy-substituted trityl-type linkers [119] according to the same synthetic strategy as applied by Voelter. Chloromethylated divinylbenzene-styrene copolymer was converted with 2,4-dimethoxy-4’-hydroxy-benzophenone, a commercially available intermediate in the synthesis of the Rink resin [24]. The resulting adduct yielded, upon treatment with several different Grignard reagents, trityl-type resins with varying substitutions and hence varying acid sensitivities. Phenyl-, tolyl- and anisylmagnesium bromide were used. Subsequent treatments with acetyl chloride and ammonia or hydrazine afforded resins for use in the synthesis of peptide amides and hydrazides. The trisalkoxy-monomethylated resin gave the most satisfactory results in the synthesis of protected peptide amides, offering a good compromise between available loading and acid sensitivity. Peptide hydrazides were obtained in very low yields, even in the case of the least substituted resin, indicating instability of the tritylhydrazide bond towards the coupling conditions of the TBTU protocol.

Miscellaneous modes of attachment to 2-chlorotrityl chloride resin

Attachment to 2-chlorotrityl chloride resin is not restricted to carboxyl groups either, although at the cost of impairment of the original elegance, since in all reported cases listed below detachment from the resin was effected with a minimal TFA concentration of 50%, thus at the same time also removing side-chain protecting groups.

Wenschuh and co-workers loaded Fmoc-amino alcohols like Fmoc-phenylalaninol, valinol and leucinol onto the resin, using a binary mixture of DCM and DMF with pyridine as the base. Loadings of about 0.24 mmol/g were obtained after a 6-hour reaction period. Subsequent solid-phase assembly via Fmoc-amino acid fluorides resulted in the synthesis of naturally occurring peptaibols with
extremely hindered sequences [120]. Egner et al. treated 2-chlorotrityl chloride resin with putrescine (1,4-diaminobutane), whose unreacted amino functionality was subsequently converted [121]. Recently, Bernhardt and colleagues undertook the solid-phase synthesis of a set of tetrapeptide-4-nitroanilides with the general structure Suc-Ala-Phe-Pro-Xaa-NH-Np (Xaa = Arg, Asp, Cit, Cys, Gln, Glu, His, Lys, Orn, Ser, Thr, Tyr, Trp). Preformed Fmoc-Xaa-HN-Np were attached to 2-chlorotrityl chloride resin through their side-chain functionalities. After the standard Fmoc SPPS the products could be cleaved from the resin by treatment with 95% TFA; the arginine analogue caused difficulties in this preparation. Attachment of Fmoc-Xaa-HN-Np to the resin, with Xaa = Asp, Cys, Glu, Lys, Orn, was performed with 1 meq of the actual component and 2.5 meq DIPEA in DCM during 1 hour, resulting in loadings of 0.7, 0.2, 0.7, 0.3 and 0.4 mmol/g, respectively. Attachment for Xaa = Ser, Thr, Tyr was performed by treatment with 1 meq Fmoc-Xaa-HN-Np, 0.1 meq DMAP and 2.5 meq DIPEA in DCM, containing a small amount of DMF, during 2 hours, resulting in loadings of 0.2, 0.2 and 0.3 mmol/g, respectively. Finally, attachment for Xaa = Arg, Cit, Gln, His, Trp was performed using 1 meq Fmoc-Xaa-HN-Np and 5 meq Et$_3$N in DCM, containing a small amount of DMF, during 2 hours, resulting in loadings of 0.25, 0.1, 0.1, 0.25 and 0.1 mmol/g, respectively [122].

The 2-chlorotrityl chloride resin is commercially available and widely applied in the preparation of protected peptide acids. Also available are the unsubstituted as well as the more labile 4-methyl and 4-methoxy-substituted analogues, unloaded or loaded with a variety of functional moieties such as 5-nitroanthranilic acid (for the synthesis of 4-nitroanilides), amino alcohols, ethylene glycol, amino acid active esters, diaminoalkanes, piperazine, hydrazine, amino thiols and carboxy thiols for use in solid-phase organic combinatorial chemistry (SPOCC) [123]. So far few applications have been reported in literature.

1.4. Outline of the thesis

The trityl moiety proves to be widely applicable both on the anchorage and on the protection level due to three inherent properties. First of all, the trityl moiety can act as an anchoring or protecting group for a wide range of functional groups, including all functional amino acid side chains as well as several possible C-termini like carboxyl, alcohol, hydrazide and amide. Second, the lability of the bond between trityl and functional group, which depends on the nature of the latter, may be minutely adjusted by adding electron-releasing substituents to the trityl moiety to increase lability or by adding electron-withdrawing substituents to lower it. Finally, the trityl cation formed upon acidolytic cleavage shows little electrophilic character, thereby eliminating the danger of unwanted side reactions, like alkylation of sensitive side chains by the cation or reattachment to a trityl-type resin.
This thesis deals with the synthesis of modified trityl-type resins and their application in the solid-phase synthesis of different types of peptide derivatives. While peptides may be attached to the resins as in standard SPPS strategies via their C-terminus, attachment through a side-chain function opens many more hitherto unaccessible perspectives in SPPS. A simple synthetic pathway leading to C-terminal peptide esters and (substituted) amides is opened, as an alternative to classical routes. C-Terminal peptide allylic esters may be deprotected on the resin under mild conditions, to enable the on-resin head-to-tail cyclization of peptides, thus avoiding dimerization by pseudo-dilution, a kinetic phenomenon supposed to favour intramolecular processes [124]. The same phenomenon also ensures, under well-defined conditions, the iodolytic cleavage of peptides attached through a thiol function to a trityl-type resin, to yield monomeric peptides containing an intramolecular disulfide bond.

Two-directional SPPS is a term, coined by Barlos, to describe the process of fragment condensation at either terminus of a peptide, that was reattached to a methyltrityl chloride resin via a selectively deprotected N*-lysine functionality [125]. Carboxyl activation of an N*-Fmoc-amino acid, attached through its side chain to a trityl resin, proceeds without racemization [8], authorizing two-directional SPPS with absolute retention of chirality.

Three special applications of the 2-chlorotrityl chloride resin as carboxyl anchor are discussed in Chapter 2 of this thesis, namely the convergent solid-phase synthesis of “difficult sequences”, Scm-Cl activation of a thiol and subsequent disulfide bond formation on the resin and synthesis of an amphiphilic peptide by side-chain attachment of aminodicarboxylic acid derivatives.

Chapter 3 of this thesis describes the preparation of new trityl-type resins, either by direct well-defined modification of a polymeric support or via a preformed trityl-type handle. The resins are classified on the basis of the reactivity of their thioethers in acidic (TFA) solutions of various concentrations. The cleavage rate is determined by the degree of stabilization of the liberated trityl cation. Trityl-type resins prepared by direct modification of the polymeric support are preferred to resins prepared by introduction of a preformed handle. A colorimetric evaluation of the trityl group and its derivatives is also presented in this chapter.

Chapter 4 deals with the synthesis of protected peptide hydrazides on trityl-type resins. The synthesis commences with the attachment of preformed Fmoc-amino acid hydrazides, whose preparation is reported in Chapter 8.

In Chapter 5 trityl-type resins are used to attach amino functions. The synthesis of both C-terminally substituted N' and N*-lysine peptide amides as well as an aminoalkylamide is described. Anchoring Fmoc-Lys(H)-OAll may be applied in two-directional SPPS or in standard SPPS followed by on-resin head-to-tail cyclization.
Various applications of a trityl-type resin as a thiol-anchoring unit are presented in Chapter 6. In case of Fmoc-cysteine two-directional SPPS may be applied; alternatively, any Fmoc-cysteine amide may be attached to the resin. Release from the resin proceeds by iodosylation as a peptide disulfide, by TFA treatment as a free thiol or by Scm-Cl-mediated activation of the thiol function. Iodolytic cleavage follows the rules for solvent-dependency observed by Kamber [126].

In Chapter 7 a new trityl-type protecting group is introduced. 4,4'-Dimethoxy-4''-methyltrityl alcohol is an intermediate product in the synthesis of one of the new trityl-type handles. It is applied as a very acid-sensitive protecting group for the asparagine, glutamine and cysteine side chains. The first two complete the set of Fmoc/Irt-type amino acid derivatives, that are cleaved by low-concentration TFA. Synthesis protocols and analytical data of amino acid derivatives, including amino acid hydrazides, amides and allylic esters, applicable in the scope of this thesis, are given in Chapter 8.

Because of its tempting complexity, relaxin from rhesus monkey (Macaca mulatta) was chosen as a central model protein to study the possible applications of trityl-type resins in the synthesis of protected peptide derivatives. The choice of the actual fragments is elucidated in the next section.

1.5. Relaxin

1.5.1. Physiology and morphology

Relaxin is a hormone of reproduction, produced principally during pregnancy in the corpus luteum of the ovary and in the placenta [127]. It inhibits uterine contraction during gestation and causes widening of the birth canal in preparation for parturition. In males it is believed to play a part in sperm motility [128]. Findings of relaxin receptors in heart [129] and brain tissue [130] of rodents suggest a possible role in blood pressure regulation [131]. Two relaxin genes have been found in humans, only one of which, designated gene-2, is expressed in the ovary [132].

\[ QLYMTLSNKCCIGCTKKSLAKFC \]
\[ KWMDVIAKACGRELVRAQIAICGKSTLGKRS \]

**Figure 5:** Primary structure of rhesus monkey relaxin.

Relaxin is closely related to insulin and insulin-like growth factors [133]. Like insulin, relaxin is produced from a single prohormone precursor, prorelaxin, by enzymatic scission [134] and comprises two dissimilar peptide chains designated A and B. The two chains are linked by two disulfide bonds; the A chain contains a third intrachain disulfide (Fig. 5).
As shown in Figure 6, relaxin and insulin are closely related by their overall structure, whereas sequence homology is only about 25%. Unlike insulin, there is a considerable variation in primary structure of about 50% among relaxins from different species [135]. Residues that are conserved in all sequenced relaxins are, besides the three cystine bridges, Gly$_{114}$, Gly$_{111}$, Arg$_{112}$, Arg$_{116}$ and Gly$_{231}$, with numbering relative to rhesus monkey relaxin. The two conserved arginine residues are located on the surface of an α-helix and are believed to be essential for the function of the hormone [136].

**A-chain**

\[ h_{insulin} \quad G I V E Q C C T S I C S L Y Q L E N Y C N \]

\[ h_{2 \ relaxin} \quad Q L Y S A L N K C C H V G C T K R S L A R F C \]

\[ r m \ relaxin \quad Q L Y M T L S N K C C H I G C T K K S L A K F C \]

**B-chain**

\[ h_{insulin} \quad F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K T \]

\[ h_{2 \ relaxin} \quad D S W M E E V I K L C G R E L V R A Q I A I C G M S T W S K R S L \]

\[ r m \ relaxin \quad K W M D D V I K A C G R E L V R A Q I A I C G K S T L G K R S L \]

**Figure 6:** Sequence comparison of human insulin, human relaxin II and rhesus monkey (rm) relaxin. The numbering system is relative to rhesus monkey relaxin.

### 1.5.2. Synthesis

Relaxin can be directly isolated from ovaries, and subsequently be used in semi-synthetic procedures, that include enzymatic or chemical scission of the semi-protected form and assembly with synthetic peptide fragments, to yield analogues for the determination of structure-activity relationships [137]. Alternatively, the separate relaxin chains may be prepared by SPPS. Rhesus monkey, gorilla and rat relaxin were obtained by oxidative combination of the synthetic chains in solution, in overall yields between 0.2 and 0.6%, based on starting resin-bound B chain [138]. In most cases, A chains were prepared by Boc SPPS, since a C-terminally esterified cysteine residue is prone to significant racemization during basic $N^\alpha$-Fmoc removal [139]. Higher yields of synthetic relaxin were obtained by Büllesbach and Schwabe in their preparation of human relaxin II and its analogues and mouse relaxin by site-directed chain combination, using three sets of selectively removable cysteine protecting groups (Scheme 4) [136,140]. The A chain was
produced by Fmoc SPPS. The cysteine side chains were protected by the trityl group in positions A10 and A15, by the Acm group in A11 and 4-methylbenzyl (MeBzl) in A24. Cleavage from the resin with TFA, using thiophenol as a scavenger, yielded the A chain with two free thiol groups (A10 and A15) and two protected cysteines. Formation of the intrachain disulfide loop was accomplished by titration with iodine in 50% acetic acid. Prior to combination with the B chain, the MeBzl protecting group was removed by the action of HF. The B chain was produced by Boc SPPS, the cysteine side chains being protected with the Acm group in position B11 and the activating 3-nitro-2-pyridinesulfenyl (Npys) group in B23. Reaction of the two chains led to formation of the interchain cystine bridge A24/B23. The third disulfide link A11/B11 was formed by iodine oxidation in 70% acetic acid.

**Formation of the A chain loop**

![Diagram of the A chain loop formation](image)

**Chain combination**

![Diagram of chain combination](image)

**Scheme 4:** Schematic representation of the synthesis of human relaxin II after Bülliesbach and Schwabe.

1.5.3. Choice of fragments

In view of the aim of this thesis, several protected rhesus monkey relaxin fragments were selected for synthesis on trityl-type resins. The choice of these fragments was based on the unambiguous strategy of Kamber and co-workers, as applied in their synthesis of human insulin [64,141]. Starting point in their synthesis scheme was the unsymmetrical disulfide containing the A-chain residues A(20-21) and the B-chain residues B(17-20), formed after Scm-Cl-mediated activation of the latter fragment.
A second central fragment was the A(6-13) fragment, that contained S-trityl-protected cysteines in positions A6 and A11, and an S-Acm-protected cysteine in A7. The difference in reactivity towards iodine oxidation of the S-trityl and S-Acm moieties in trifluoroethanol led to selective formation of the intrachain disulfide loop A6/A11, without affecting the S-Acm moiety. Fragment assembly resulted in a protected insulin precursor, containing the two preformed cystine bridges A6/A11 and A20/B19 and two S-Acm-protected cysteines in positions A7 and B7 (Scheme 5). Synthetic human insulin with full biological activity was obtained by subsequent iodine oxidation and removal of the protecting groups with TFA.

Scheme 5: Schematic representation of key steps in Kamber's insulin synthesis.

During convergent syntheses, peptide fragments should be coupled either with C-terminal glycine or proline residues or otherwise by applying the azide method, to ensure maximum retention of chirality in the final product [142]. The chosen rhesus monkey relaxin fragments as well as the chapters in which their respective syntheses are described, are given in Table 3. In an eventual assembly of relaxin, analogous to Kamber's insulin synthesis, additional care would have to be taken to avoid racemization of the C-terminally esterified cysteine residue in position A24 during N'-Fmoc deprotections [143]. N'-Protection of the actual cysteine residue by the trityl group should be preventive in this respect [46] and was indeed applied in the synthesis of the A24-B(17-23) fragment reported in Chapter 6.
Table 3: Rhesus monkey relaxin fragments and chapters in which their respective syntheses are described.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(1-8)</td>
<td>Boc-Gln(Trt)-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-N2H3</td>
<td>4</td>
</tr>
<tr>
<td>A(9-16)</td>
<td>Fmoc-Lys(Boc)-Cys-Cys(Acm)-His(Burn)-Ile-Gly-Cys-Thr(H)-OAll</td>
<td>6</td>
</tr>
<tr>
<td>A24-B(17-23)</td>
<td>H-Cys-O'Bu</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys-Gly-OH</td>
<td></td>
</tr>
<tr>
<td>A24-B(17-23)</td>
<td>Trt-Cys-O'Bu</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys-Gly-OMe</td>
<td></td>
</tr>
<tr>
<td>B(1-11)</td>
<td>Boc-Lys(Boc)-Trp(Boc)-Met-Asp(O'Bu)-Asp(O'Bu)-Val-Ile-Lys(Boc)-Ala-Cys(Trt)-Gly-OH</td>
<td>2</td>
</tr>
<tr>
<td>B(1-16)</td>
<td>Boc-Lys(Boc)-Trp(Boc)-Met-Asp(O'Bu)-Asp(O'Bu)-Val-Ile-Lys(Boc)-Ala-Cys(Trt)-Gly-Arg(Pmc)-Glu(O'Bu)-Leu-Val-Arg(Pmc)-OH</td>
<td>2</td>
</tr>
<tr>
<td>B(12-16)</td>
<td>Fmoc-Arg(Pmc)-Glu(O'Bu)-Leu-Val-Arg(Pmc)-N2H3</td>
<td>4</td>
</tr>
<tr>
<td>B(24-32)</td>
<td>H-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-Leu-O'Bu</td>
<td>4</td>
</tr>
</tbody>
</table>

1.6. References

General Introduction


8 $N^\alpha$-Acyl amino acids tend to racemize during carboxyl activations via formation of azlactones, which facilitate the abstraction of the acidic proton from the chiral centre by resonance stabilization of the carbanion generated in this process. $N^\alpha$-Protecting groups of the urethane type, like Z, Boc and FMoc, destabilize the anion by electron release and hence prevent racemization during electrophilic activation. See also Section 4.1.1.


See Section 1.2.3. and references cited therein.


General Introduction


99 Novabiochem Letters 1/97, Calbiochem-Novabiochem GmbH, Switzerland.


See Section 4.1.1.

A problem not encountered in the convergent synthesis of human insulin, since in that case the corresponding cysteine residue is amidated with an additional asparagine residue.
Chapter 2

Special Applications of Trityl-Type Resin I
in the Synthesis of Peptide Acids

2.1. Introduction

This chapter deals with three special applications of trityl-type resin I (2-chlorotrttyl chloride resin, depicted in Figure 1), when used for anchoring of peptides through a carboxyl function. They comprise the use of solid-phase fragment condensation in the synthesis of peptides with multiple “difficult sequences”, cysteine activation and subsequent disulfide bond formation on the peptidyl resin and the synthesis of amphiphilic peptides via side-chain attachment of α-aminodicarboxylic acid derivatives.

2.1.1. Convergent solid-phase peptide synthesis

Convergent solid-phase peptide synthesis (CSPPS), which is also called solid-phase fragment condensation (SPFC), is applied in the synthesis of large peptides or proteins [1]. Fully protected peptide fragments are condensed sequentially on a suitable resin, preferably by activation of C-terminal glycine or proline residues to avoid racemization [2]. The approach offers a number of advantages over stepwise solid-phase synthesis. The protected fragments may be purified before being coupled; purification of the final product is simplified inasmuch as impurities arising from incomplete couplings differ from the target molecule by a considerable sequence rather than one amino acid. Moreover, sequence variation within a large peptide is easily accessible through the convergent approach.

The synthesis of fragments containing C-terminal glycine or proline residues is complicated by the formation of diketopiperazines (DKP). They originate from base or acid-catalyzed intramolecular attack of the free α-amino group of a dipeptide on the C-terminal carbonyl. When the dipeptide is C-terminally linked to a benzyl-type resin, base deprotection (in Fmoc SPPS) or neutralization (in Boc SPPS) can release the DKP, leaving the support as an alcohol. DKP formation is predominant with residues that can form cis peptide bonds like proline, glycine and sarcosine in either the first or the
second position [3]. One way to prevent DKP formation is to couple the preformed dipeptide to the amino acyl resin [4]. DKP formation is, however, totally suppressed when the C-terminal carboxyl function is esterified to a trityl group [5], since the trityloxy moiety cannot function as a leaving group in aminolysis [6]. Application of trityl-type resin I ensures total stability of the anchoring ester bond during SPPS, but at the same time allows the cleavage of fully protected fragments to proceed under very mild acidolytic conditions. Through CSPPS, 33-residue [Ser2,Val8,Gly35]calcitonin, 64-residue type III anti-freeze protein, 109-residue prothymosin α and 157-residue tumor necrosis factor α could be successfully assembled on this resin [7-10].

![Figure 1: Trityl-type resin I.](image)

As an extension of its applicability, we set out to synthesize the B(1-16) rhesus monkey relaxin fragment; this was accomplished by coupling the protected B(1-11) intermediate to the B(12-16) peptidyl resin. Both of these fragments contain a “difficult sequence”, i.e. a series of consecutive couplings that tend to remain incomplete [11]. Through the applied fragmentation we aimed to circumvent serious problems which were bound to arise in a total stepwise synthesis.

2.1.2. Cysteine activation and disulfide bond formation on a solid support

The regioselective synthesis of disulfide bonds within a peptide poses a continuing challenge to peptide chemists, and will be elaborately discussed in Chapter 6 of this thesis. At present, one specific aspect is treated, namely the incorporation of a cystine residue into a peptide. Brois and co-workers first reported on the reaction of a carboalkoxysulfenyl chloride with a thiol compound to give a sulfenylthiocarbonate, an activated species, which can be cleaved by a second thiol with concomitant formation of a mixed disulfide, carbonylsulfide and an alcohol [12]:

\[
R^1\text{-SH} + R^3\text{O-CO-S-Cl} \xrightarrow{-\text{HCl}} R^3\text{O-CO-S-S-R}^1 \xrightarrow{+ R^2\text{-SH}} R^1\text{-S-S-R}^2 + \text{COS} + R^3\text{OH}
\]
In peptide chemistry, the activation can also be achieved by treatment of Trt or Acm-protected cysteine residues with Scm-Cl ($R^1 = $ methyl) or Sce-Cl ($R^1 = $ ethyl) in alcoholic media [13,14]. To prevent the HCl formed from attacking acid-labile protecting groups within the activated peptide, the activation step is usually carried out at low temperature. Another drawback of this activation method is the possible (trans)esterification of (protected) carboxyl groups within the peptide, which is counteracted by the presence of diethylamine and low reaction temperatures [15]. A more secure way to attain the same goal consists of performing the activation and disulfide bond formation after solid-phase assembly, while the peptide is still attached to the solid support. Transesterification at the anchoring linkage now leads to detachment from the resin, thus simplifying purification. In addition, large excesses of reagents may be applied with no negative consequences for product purification.

Ten Kortenaar and van Nispen treated a Cys(Acm)-containing peptide, that was bound to Wang’s 4-alkoxybenzyl alcohol resin, with subsequently Scm-Cl and cysteine, to obtain the open-chain unsymmetrical cystine peptide upon simultaneous cleavage and deprotection [16]. In a similar approach, we assembled the A24-B(17-23) relaxin fragment on trityl-type resin I; in this case, however, the protecting groups may be preserved during the mild acidolytic detachment.

2.1.3. Amphiphilic peptides and proteins

Lipidation of proteins directs their translocation to the plasma membrane and may be essential for biological activity [17]. In vivo three types of lipidation are encountered, i.e. fatty acid acylation ($N$-myristoyl or $S$-palmitoyl), isoprenylation at a cysteine side chain near the C-terminus and covalent linkage to a specific modified phospholipid (GPI) [18]. Additionally, synthetic lipopeptides may incorporate non-natural $\alpha$-amino acids with long alkyl side chains (lipidic amino acids) [19]. Besides improving oral absorption and enhancing the activity of peptide drugs that act at membranes, lipidation may be applied as a means for increasing immunogenicity of peptide antigens [20,21].

In the present study, an attempt was made to increase the receptor affinity of human insulin through C-terminal amidation of the B-chain with stearyl amine. The translocation of the molecule to the membrane surface is thought to facilitate its interaction with the specific binding site on the insulin receptor by a change in dimensionality of movement. The ligand, which was originally in the large volume of the cytoplasm, may be constrained to move in the small two-dimensional water/membrane interface that also contains the binding site, and the collision frequency between the two components is consequently higher than in case of three-dimensional movement [22].

The stearyl insulin derivative is built up from desoctapeptide-insulin [23] and [Glu$^{30}$]B(23-30)-insulin $\alpha$-stearylamide. Preparation of the latter commenced with the attachment of Fmoc-Glu(OH)-NHC$_{18}$H$_{37}$ to trityl-type resin I via its side chain.
2.2. Synthesis of a multiple “difficult sequence” relaxin fragment through CSPPS

In 1991, van Woerkom and van Nispen published an evaluation of 696 couplings performed over the years on Wang’s 4-alkoxybenzyl alcohol resin [24]. Acylations proceeded in the presence of DCC/HOBt and completeness was monitored by the ninhydrin test developed by Kaiser [25]. The results of these tests were collected and led to a table predicting the occurrence of “difficult sequences”. They found the residues with aliphatic side chains (Ala, Ile, Leu and Val) and arginine derivatives to be most frequently involved in these sequences. Aggregation of peptide chains in the form of β-sheet secondary structures is generally assumed to be responsible for their occurrence [26]. From other studies, it is apparent that an accumulation of residues bearing tert-butyl-type protecting groups may exert a similar effect, whereas trityl-type protecting groups tend to moderate aggregations [27].

Upon comparison of the rhesus monkey relaxin B(1-16) sequence:

\[ \text{Lys}^1\text{-Trp-Met-Asp}^5\text{-Val-Ile-Lys-Ala-Cys}^{10}\text{-Gly-Arg-Glu-Leu-Val-Arg}^{16} \]

with the data collected by van Woerkom and van Nispen, “difficult sequences” can be expected to occur within the first five couplings and around the tenth coupling (~Ala-Lys(Boc)-Ile-Val~) in a stepwise solid-phase synthesis [N.B. SPPS proceeds from C to N-terminus]. Table 1 summarizes the data for completeness of a specific acylation as a function of the acylated N-terminal residue as well as the acylating residue, for the synthesis of the actual relaxin fragment. It should be noted that the data for Arg(Pmc) are not very reliable since they are based on only four couplings; furthermore, no data are available for Cys(Trt), Trp(Boc) and Boc-Lys(Boc)-OH (the last two are to be used in the two final couplings).

<table>
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<tr>
<th>AA</th>
<th>Arg(Pmc)</th>
<th>Val</th>
<th>Leu</th>
<th>Glu(O'Bu)</th>
<th>Arg(Pmc)</th>
<th>Gly</th>
<th>Cys(Trt)</th>
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<td>39</td>
<td>23</td>
<td>0</td>
<td>21</td>
<td>n.d.</td>
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<tr>
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<td>46</td>
<td>43</td>
<td>34</td>
<td>100</td>
<td>15</td>
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<table>
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<th>Val</th>
<th>Asp(O'Bu)</th>
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<tbody>
<tr>
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<td>22</td>
<td>18</td>
<td>35</td>
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<td>6</td>
</tr>
<tr>
<td>acylating Fmoc-AA-OH</td>
<td>45</td>
<td>27</td>
<td>43</td>
<td>46</td>
<td>7</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

* According to van Woerkom and van Nispen [24]; for further explanation, see text.
An additional complication for a stepwise synthesis of B(1-16) is indicated by the observation that acylation problems most frequently arise at a chain length between nine and fifteen residues [24,28]. Unfavourable folding of the growing peptide chain is thought to diminish the accessibility of the N-terminus to deprotecting, acylating and monitoring agents, thus resulting in deletion sequences [29]. In the pertinent fragment, couplings at this chain length are all the more hampered due to the constituent residues. We chose to circumvent this problem by dividing the fragment into two parts (synthesized by standard SPPS on trityl-type resin I) and subsequently performing a solid-phase fragment condensation at glycine residue 11 (Scheme 1).

\[
\text{Boc-Lys(Boc)-Trp(Boc)-Met-Asp(O' Bu)-Asp(O' Bu)-Val-Ile-Lys(Boc)-Ala-Cys(Trt)-Gly-OH} \quad 1
\]

\[+ \quad \text{H-Arg(Pmc)-Glu(O' Bu)-Leu-Val-Arg(Pmc)-O-resin I} \quad 2a \quad \rightarrow_{i,ii} \rightarrow
\]

\[
\text{Boc-Lys(Boc)-Trp(Boc)-Met-Asp(O' Bu)-Asp(O' Bu)-Val-Ile-Lys(Boc)-Ala-Cys(Trt)-Gly-Arg(Pmc)-Glu(O' Bu)-Leu-Val-Arg(Pmc)-OH} \quad 2b
\]

**Scheme 1:** Synthesis of protected rhesus monkey relaxin fragment B(1-16) (2b): i) DCC/HOBt; ii) AcOH/TFE/DCM (2:2:6).

As expected, in the SPPS of fragment 1, Fmoc-Lys(Boc)-OH, Fmoc-Ile-OH and Fmoc-Val-OH (residues 8 to 6) failed to give negative Kaiser tests after standard DCC/HOBt-mediated couplings and double acylations were therefore necessary. From residue 5 (Fmoc-Asp(O' Bu)-OH) on, acylations after the TBTU/HOBt/NMM protocol in NMP were complete within the usual 45-minute period. In the synthesis of peptidyl resin 2a, acylation times were doubled and no irregularities were found with the Kaiser tests. Both fragments gave the correct amino acid composition upon hydrolysis and the identity of compound 1 was further established by FAB-MS.

The actual fragment condensation to peptidyl resin 2a was performed in DMF with 2 molar equivalents of compound 1 in the presence of 2.2 and 2.4 equivalents of DCC and HOBt, respectively [30]. This combination has proved to be very suitable in fragment condensations for catalyzing the reaction and suppressing racemization (if glycine or proline is not in C-terminal position) [1]. The course of the reaction was monitored by Kaiser tests. Acylation was complete after 5 days, with in between addition of another half portion of DCC and HOBt, affording upon cleavage the desired fragment 2b with the correct amino acid composition in 83% overall yield, based on Fmoc-Arg(Pmc)-loaded trityl-type resin I.
2.3. Synthesis of a relaxin fragment containing an unsymmetrical cystine

Synthesis of the protected relaxin fragment containing the A24-B22 disulfide bridge was started from Fmoc-Gly-loaded trityl-type resin I. The resin loading, as in most other cases reported in this thesis, was assessed by cleaving the Fmoc group from a sample of the resin with piperidine/DCM (1:1) and subsequently measuring the absorbance of the arising dibenzofulvene-piperidine adduct in DCM [31]. In this case a rather high initial loading of 0.49 mmol/g could be used, since the opted selective method for disulfide bond formation precludes the formation of intermolecular links.

Standard SPPS was performed with incorporation of S-tritylcysteine in position B22 to yield the peptidyl resin 3a. The further transformation into resin 3c (Scheme 2) could be chromatographically monitored by acidolysis of a resin sample. The reaction of 3a with two equivalents of Scn-Cl was complete within 30 minutes at -10°C; at this temperature less than 5% of the peptide material was cleaved by the formed HCl. The resulting resin 3b, containing the activated cysteine, was converted into the cystine compound 3c by overnight reaction with 4 equivalents of cysteine tert-butyl ester (4b). Cleavage with an AcOH/TFE/DCM mixture yielded the protected title compound 3d in 83% overall yield, based on Fmoc-Gly-loaded trityl-type resin I.

\[
\text{Fmoc-X-Cys(Trt)-Gly-O-resin I } 3a \quad \overset{i}{\rightarrow} \quad \text{Fmoc-X-Cys(Scm)-Gly-O-resin I } 3b \quad \overset{ii}{\rightarrow} \\
\text{Fmoc-X-Cys(H-Cys-O}^\text{Bu})\text{-Gly-O-resin I } 3c \quad \overset{iii}{\rightarrow} \quad \text{Fmoc-X-Cys(H-Cys-O}^\text{Bu})\text{-Gly-OH } 3d
\]

**Scheme 2:** Synthesis of protected rhesus monkey relaxin fragment A24-B(17-23): i) Scn-Cl/Et₂NH; ii) AcOH; H-Cys(H)-O}^\text{Bu} (4b)/Et₃N; iii) AcOH/TFE/DCM (2:2:6). X = Ala-Gln(Trt)-Ile-Ala-Ile.

The purity of the crude product 3d, as estimated from the ES mass spectrum (Fig. 2), was significantly higher than the one achieved by ten Kortenaar and van Nispen using Wang's resin (35-40%) [16]; this might be partly due to the milder cleavage conditions applied in the present case. The minor peaks that were detected might stem from artefacts formed during the ES measurement, since none of them were found in the corresponding SIMS spectrum.

Cysteine tert-butyl ester was prepared in two steps from cystine (Scheme 3). Cystine was esterified through alkyl transfer from tert-butyl acetate and the product 4a was isolated as its readily crystallizable and stable acetate [32]. Reduction with tributylphosphine [33] yielded the free thiol 4b, whose acetate was used in the synthesis of 3c without further purification.

\[
[H-Cys-OH] \quad \overset{i,ii}{\rightarrow} \quad [\text{AcOH.H-Cys-O}^\text{Bu}] \quad 4a \quad \overset{iii,iv}{\rightarrow} \quad \text{AcOH.H-Cys(H)-O}^\text{Bu} \quad 4b
\]

**Scheme 3:** Preparation of H-Cys(H)-O}^\text{Bu} (4b): i) tert-butyl acetate/HClO₄; ii) 1 M AcOH/ether; iii) tributylphosphine; iv) 1 M AcOH/ether/petroleum ether.
Figure 2: ES-MS of crude product 3d ($C_{64}H_{82}N_{15}O_{12}S_{2}$ : $MW_{theor}$ 1314.6).

2.4. Synthesis of an amphiphilic insulin fragment

Side-chain carboxyl attachment to trityl-type resin I has been reported for Fmoc-Asp(OH)-Phe-NH$_2$ in the synthesis of protected [Leu$^{15}$]gastrin I and cholecystokinin octapeptide [34]. In an analogous way, we attached the lipidic amino acid derivatives Fmoc-Asp(OH)-NHC$_{18}$H$_{37}$ and Fmoc-Glu(OH)-NHC$_{18}$H$_{37}$, whose synthesis will be described in Chapter 8, to this resin, resulting in initial loadings of 0.50 and 0.56 mmol/g, respectively. These and similarly loaded resins may be utilized as a basis for the preparation of many amphiphilic peptides [35].

Fmoc-Glu(O-resin I)-NHC$_{18}$H$_{37}$  5a  $\rightarrow$ $i, ii$→

H-Gly-Phe-Phe-Tyr(Bu)-Thr(Bu)-Pro-Lys(Boc)-Glu(OH)-NHC$_{18}$H$_{37}$  5b  $\rightarrow$ $iii$→

H-Gly-Phe-Phe-Tyr(H)-Thr(H)-Pro-Lys(H)-Glu(OH)-NHC$_{18}$H$_{37}$  5c

Scheme 4: Synthesis of [Glu$^{10}$]B(23-30)-insulin stearylamide (5c): $i$ 7 cycles of Fmoc SPPS; $ii$ AcOH/TFE/DCM (2:2:6); $iii$ TFA/TEA/H$_2$O (95:2.5:2.5).

The glutamic acid derivative 5a was applied in the synthesis of the partially protected peptide 5b, which was obtained in 89% yield based on the starting resin; the product was identified by means of
MALDI-TOF-MS (Fig. 3a). No negative influence from the aliphatic end group, i.e. steric hindrance at the N-terminus, was observed during SPPS, inasmuch as all acylations were complete within the usual coupling time. Deprotection with a TFA/TES/H₂O mixture yielded the amphiphilic peptide 5c, which was more than 96% pure according to RP-HPLC (Fig. 3b) and could be used without further purification for the trypsin-catalyzed coupling to desoctapeptide-insulin [36]. Synthesis procedure and evaluation of the final product will be described elsewhere [37].

Figure 3: MALDI-TOF-MS of partially protected 5b (C₁₉H₁₇O₉N₁₀O₁₂ : MW₉₉₉ 1451.9) (a) and RP-HPLC of deprotected 5c (b). The two major peaks in (a) correspond to artefacts containing Na and K, respectively. The peak at [M+56] corresponds to an unidentified artefact present in most MALDI-TOF mass spectra presented in this dissertation.
2.5. Conclusions

Besides the straightforward application of trityl-type resin 1 for \(\alpha\)-carboxyl anchoring in the solid-phase synthesis of protected peptide fragments, several other pathways are intrinsically connected with trityl-type resins. This chapter focussed on three in particular, which are associated with carboxyl anchoring to trityl-type resin 1, but may also be combined with other modes of attachment to various other trityl-type resins, as will be reported in following chapters of this thesis.

Since diketopiperazine formation is hampered by the esterification with a trityl group, trityl-type resin I is very suitable for the synthesis of protected fragments with a C-terminal glycine or proline residue for convergent solid-phase synthesis. The usefulness of this method was elegantly demonstrated by the preparation of a 16-residue relaxin fragment containing two “difficult sequences”, whose synthesis by a total stepwise solid-phase approach would have been troublesome. A separation of the sequences through fragmentation at an internal glycine residue enabled the assembly of the total fragment via convergent SPPS in high yield and purity without major complications.

A second special application of trityl-type resin 1 consists of cysteine activation with Scn-Cl and subsequent unsymmetrical disulfide bond formation on the peptidyl resin. At \(-10^\circ\text{C}\), the peptide-resin linkage proved stable enough towards the hydrochloric acid, which is formed during the activation, to ensure high-yield product formation. A possible by-product, the methyl ester arising from trans-esterification, is easily washed from the resin. Following this procedure, an unsymmetrical cystine-containing relaxin fragment was prepared in its protected form for eventual use in a convergent relaxin synthesis. A completely different approach, which combines cysteine side-chain attachment to a trityl-type resin with simultaneous cleavage and activation by Scn-Cl, led to an analogous product and will be described in Chapter 6.

Finally, an amphiphilic insulin fragment was synthesized after the anchoring of Fmoc-glutamic acid, whose \(\alpha\)-carboxyl group was condensed to stearyl amine, via its side chain to trityl-type resin 1. Many lipopeptides thus become accessible by combining two approaches in peptide synthesis: (1) side-chain attachment of an \(\alpha\)-aminodicarboxylic acid or in fact any other functional amino acid to a trityl-type resin and (2) C-terminal condensation to a lipidic amine. Moreover, insertion of tris(hydroxymethyl)aminomethane allows the preparation of peptides derivatized with three fatty acid chains [38,39], which should increase the affinity for a membrane even more [22]. The full scope of side-chain attachment to resins will be discussed in Chapter 5.
2.6. Experimental section

Materials and methods
The FAB mass spectrum of compound 1 was measured on a Varian MAT 711 A mass spectrometer at a source temperature of 30°C, using an argon gun and NBA as the matrix. The ES mass spectrum of compound 3d was measured on an API III triple quadrupole mass spectrometer equipped with an IonSpray™ interface (Sciex, Thornhill, Canada). For further specifications, see Appendix A.

Coupling of the first Fmoc-amino acid to trityl-type resin 1
Trityl-type resin 1 (b = 1.41 mmol/g) was shaken in DCM (1g resin/5 ml DCM) during 4 h in the presence of 1 and 2 meq of the actual Fmoc-amino acid and DIPEA, respectively. The resin was filtered and, upon addition of a DCM/MeOH (1:1) mixture containing 250 µl DIPEA, shaken for an additional 15 min to destroy the active chloride that eventually remained unreacted. The resin was then washed five times with DMF, twice with 2-propanol and ether each, and dried. N.B. The same procedure was applied for the attachment of Fmoc-amino acid derivatives through a carboxyl side chain.

SPPS protocols
Syntheses were performed on trityl-type resin 1 according to the general Fmoc SPPS procedure described in Appendix A. The first five couplings in the synthesis of compound 1 were accomplished with DCC/HOBt in DMF (double couplings in case of Lys(Boc), Ile and Val), the last five with TBTU/HOBt/NMM in NMP. In the preparation of peptidyl resin 2a double coupling times (90 min) were standardly used in combination with the DCC/HOBt protocol. The synthesis of compound 3 was performed after the standard DCC/HOBt protocol, the synthesis of 5 after the TBTU/HOBt/NMM protocol. Synthesis of 1 was concluded with the incorporation of Boc-Lys(Boc)-OH.

Cleavage of protected peptide acids from trityl-type resin 1
The peptidyl resin was suspended in AcOH/TFE/DCM (2:2:6) (1 g resin/10 ml solution). After standing for 45 min, the resin was filtered and extracted twice with the cleavage mixture. The combined filtrates were evaporated in vacuo and the residue was triturated with ether.

Boc-Lys(Boc)-Trp(Boc)-Met-Asp(OBu)-Asp(OBu)-Val-Ile-Lys(Boc)-Ala-Cys(Tri)-Gly-OH (1)
Based on 1.5 g of Fmoc-Gly-O-resin 1 (b = 0.49 mmol/g). Yield: 1.20 g (81%). TLC: Rf (A) 0.28, Rf (B) 0.15. [α]D 25 -11.1 (c=1, DMF). FAB-MS: m/z 2041.9 [M+Na]+. Amino acid analysis: Asp 2.00 (2), Gly 1.09 (1), Ala 1.02 (1), Val 0.84 (1), Met 0.96 (1), Ile 0.82 (1), Lys 2.00 (2), Trp 0.48 (1). Glycine was slightly elevated because of cysteine decomposition; valine and isoleucine gave diminished values due to the poor hydrolyzability of the hydrophobic Val-Ile sequence [40].

H-Arg(Pmc)-Glu(OBu)-Leu-Val-Arg(Pmc)-O-resin 1 (2a)
Based on 0.4 g of Fmoc-Arg(Pmc)-O-resin 1 (b = 0.41 mmol/g), four cycles of SPPS afforded peptidyl resin 2a (b = 0.27 mmol/g, 86%, determined before the last Fmoc removal). A sample of the peptidyl resin was subjected
to standard cleavage and the resulting peptide hydrolyzed. Amino acid analysis: Glu 1.02 (1), Val 1.00 (1), Leu 1.00 (1), Arg 2.00 (2).

**Boc-Lys(Boc)-Trp(Boc)-Met-Asp(OBu)-Asp(OBu)-Val-Ile-Lys(Boc)-Ala-Cys(Trt)-Gly-Arg(Pmc)-Glu(OBu)-Leu-Val-Arg(Pmc)-OH (2b)**

To peptidyl resin 2a a solution of compound 1 (575 mg, 2.0 meq), DCC (64 mg, 2.2 meq) and HOBT (52 mg, 2.4 meq) in 5 ml DMF was added and the suspension was shaken for three days. After adding another half portion of DCC and HOBT, the suspension was shaken for two more days. The Kaiser test was then negative. The resin was washed and peptide 2b cleaved from the resin as described above. Yield: 445 mg (97% based on 2a). TLC: Rf (A) 0.62, Rf (B) 0.57. [α]D 25 -9.4 (c=1, NMP). Amino acid analysis: Asp 1.96 (2), Glu 0.98 (1), Gly 1.00 (1), Ala 1.00 (1), Val 1.69 (2), Met 0.45 (1), Ile 0.75 (1), Leu 1.00 (1), Lys 1.99 (2), Trp 0.51 (1), Arg 1.97 (2). Valine and isoleucine gave diminished values due to the poor hydrolyzability of the hydrophobic Val-Ile sequence [40].

**Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(H-Cys-OBu)-Gly-OH,AcOH (3d)**

Based on 0.3 g of Fmoc-Gly-O-resin 1 (b = 0.49 mmol/g). Peptidyl resin 3a, which was obtained after 6 cycles of SPPS and contained S-trityl cysteine, was suspended in 5 ml CHCl3/MeOH (2:1). Upon addition of 27 μl ScmCl (ca. 2 meq) at -10°C, the suspension was rotated for 30 min at this temperature. Then the resin was filtered off and washed with cold CHCl3, DMF, 2-propanol and ether, three times each, affording peptidyl resin 3b, which contained the Scm-activated cysteine. The resin was again suspended in 5 ml CHCl3/MeOH and AcOH-H-Cys-OBu (4b) (140 mg, 0.59 mmol) was added. The suspension was shaken for 20 h at room temperature; then the resin was washed as before. The resulting resin 3e was submitted to standard cleavage, eventually affording product 3d in 83% overall yield (170 mg). TLC: Rf (C) 0.69. ES-MS: m/z 1314.5 [M+H]+, minor peaks: m/z 1337.0 [M+Na]+, 1258.5 [M-1Bu]+, 1179.5, 1157.5, 1057.5. SIMS (+), DTE/DTT/sulfolane: m/z 1315.4 [M+H]+, minor peak: m/z 920.0. Amino acid analysis: Glu 1.03 (1), Gly 1.03 (1), Ala 2.00 (2), Ile/allo-Ile 1.98 (2). Isoleucine adjacent to cysteine leads to partial racemization during hydrolysis [41].

**[AcOH.H-Cys(0Bu)2]2 (4a)**

Cystine (1.75 g, 7.2 mmol) and 70% HClO4 (2.3 ml, 15.8 mmol) were dissolved in 220 ml tert-butyl acetate. The solution was stirred at room temperature for 5 days, during which a white product precipitated; this was filtered off and dissolved in 50 ml ether and 25 ml 1 M NaHCO3. The organic layer was washed with 1 M NaHCO3 (1×20 ml) and saturated NaCl (4×15 ml), dried over Na2SO4 and evaporated in vacuo. Recrystallization from 1 M acetic acid/ether afforded white crystals in 68% yield (2.3 g). Mp 82-84°C. TLC: Rf (B) 0.09, Rf (C) 0.65. [6]D 25 -34.5 (c=1, MeOH). 1H-NMR (CDCl3), δ (ppm): 1.49 (s, 18H, OBu); 2.04 (s, 8H, CH2COOH); 3.00/3.18 (dm, 4H, β-CH2); 3.84 (m, 2H, α-CH); 6.44 (s, 7H, α-NH). According to 1H-NMR, the product contained some residual acetic acid.

**AcOH.H-Cys(H)-OBU (4b)**

Tributylphosphate (315 μl, ca. 1.26 mmol) was added to a solution of 300 mg (0.63 mmol) of 4a in 10 ml 2-propanol. After stirring for 2.5 h at room temperature, the solution was evaporated in vacuo. Quick
crystallization from 1 M acetic acid/ether/petroleum ether afforded a white solid, which was thoroughly washed with petroleum ether and then dried under nitrogen. Yield: 240 mg (80%). TLC: Rf (C) 0.85, produced a pink spot upon spraying with nitroprusside solution [42].

**H-Gly-Phe-Phe-Tyr(Bu)-Thr(Bu)-Pro-Lys(Boc)-Glu(OH)-NHC\textsubscript{3}H\textsubscript{7}AcOH (5b)**

Based on 1 g of Fmoc-Glu(O-resin I)-NHC\textsubscript{3}H\textsubscript{7}, (5a, b = 0.56 mmol/g). Yield: 750 mg (89%). TLC: Rf (A) 0.63, Rf (B) 0.23. [α]\textsubscript{D}\textsuperscript{25} -14.6 (c=1, DMF). MALDI-TOF-MS: m/z 1507.7 [M+56]\textsuperscript{+}, 1489.7 [M+K]\textsuperscript{+}, 1473.8 [M+Na]\textsuperscript{+}.

**H-Gly-Phe-Phe-Tyr(H)-Thr(H)-Pro-Lys(H)-Glu(OH)-NHC\textsubscript{3}H\textsubscript{7}2TFA (5e)**

Compound (5b) (600 mg, 0.4 mmol) was dissolved in 10 ml of a TFA/TES/H\textsubscript{2}O (95:2.5:2.5) mixture. After standing for 90 min, the solution was evaporated in vacuo; the residue was suspended in 25 ml water and lyophilized. Yield: 580 mg (100%). RP-HPLC (30-90%/60 min): t\textsubscript{R} 50.1 min (96.4%). [α]\textsubscript{D}\textsuperscript{25} -21.3 (c=1, DMF). Amino acid analysis: Thr 0.87 (1), Glu 1.00 (1), Pro 1.00 (1), Gly 0.97 (1), Tyr 0.95 (1), Phe 1.94 (2), Lys 1.00 (1).

### 2.7. References

2. For a detailed account on the racemization mechanism occurring during the activation of acyl amino acids, see Section 4.1.1.
6. On the contrary, the tritylthio moiety may indeed act as a leaving group, due to the higher polarizability of the sulfur atom, and as such be regarded as a carboxyl activating group. The synthesis of peptide thiocarboxylic acids on a trityl-type resin consequently proved impossible, nearly all of the peptide material being lost from the resin within five cycles of SPPS [Personal communication by K. Barlos].
Special Applications of Trityl-Type Resin I in the Synthesis of Peptide Acids


Chapter 6 describes the attachment of Fmoc-Cys(H)-NHC₆H₄Cl₂ via its side chain to a trityl-type resin.


Forthcoming Ph.D. thesis of Dipl.-Chem. S. Lauter, member of the Insulin Department at the German Wool Research Institute in Aachen.

Chapter 8 describes the preparation of Fmoc-Phe-NHC(CH₂OH)₂, which can likewise be applied to other amino acids.


In: Aufbaureagenzien für die Dünnschicht- und Papier-Chromatographie (1970) E. Merck, Darmstadt, p. 79.
Chapter 3

Synthesis and Evaluation of Trityl-Type Resins

3.1. Introduction

In the introductory chapter the desirability was expressed of introducing more subtlety in the handling of compounds as delicate as peptides and proteins. In pursuit of this goal, the current chapter describes the development of sophisticated, mildly cleavable linkers of the trityl type for use in peptide synthesis.

3.1.1. The effect of substitutions on linker lability

The influence of substituents on the aromatic system is not only apparent in governing aromatic substitution reactions, but also in the (de)stabilization of non-intermediate charges of the system. The latter property is extensively applied in protection and anchoring strategies in peptide chemistry. One of the best examples is encountered in the group of solid-phase handles, that have been derived from the 4-hydroxymethylphenylacetic acid (PAM) handle, for coupling to amino-functionalized supports [1-8]. They can be comprised by the general formula:

\[
\text{HOCH}_2 - \quad \text{Y - X - CO}_2 \text{H}
\]

The PAM handle (X=CH\(_2\); Y=Z=H), applied in the synthesis of peptide acids after the Boc protocol, is cleavable with anhydrous HF. Derivatives that are completely stable to HF, but cleavable by nucleophiles like hydroxide and methoxide ion, ammonia and hydrazine, lack the CH\(_2\)-spacer (no X: Y=Z=H); additional ortho nitration enables cleavage by photolysis (no X: Y=NO\(_2\); Z=H).

Lengthening of the spacer X and addition of electron-releasing alkoxy substituents to the system leads to stabilization of the benzylic cation, arising from acidolytic cleavage, and thereby allows cleavage to proceed under milder conditions. Various PAM-derived linkers, applied in the SPPS of C-terminal
peptide acids, and their cleavage conditions are listed in Table 1. The trisalkoxybenzyl derivative is so labile, that its amidated form (PAL: X=O(CH$_2$)$_4$; Y=Z=OCH$_3$) allows the cleavage of C-terminal peptide amides by treatment with 70% TFA [9].

**Table 1**: *PAM-derived linkers and their cleavage conditions.*

<table>
<thead>
<tr>
<th>Handle</th>
<th>$X$</th>
<th>$Y$</th>
<th>$Z$</th>
<th>Cleavage conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONb</td>
<td>—</td>
<td>NO$_2$</td>
<td>H</td>
<td>nucleophile, hv (350 nm)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>H</td>
<td>H</td>
<td>nucleophile</td>
<td>2</td>
</tr>
<tr>
<td>PAM</td>
<td>CH$_2$</td>
<td>H</td>
<td>H</td>
<td>HF</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(CH$_2$)$_2$</td>
<td>H</td>
<td>H</td>
<td>HBr</td>
<td>4</td>
</tr>
<tr>
<td>HMPA</td>
<td>OCH$_2$</td>
<td>H</td>
<td>H</td>
<td>neat TFA</td>
<td>2</td>
</tr>
<tr>
<td>PAB</td>
<td>O(CH$_2$)$_2$</td>
<td>H</td>
<td>H</td>
<td>20-50% TFA</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>OCH$_3$</td>
<td>OCH$_3$</td>
<td>H</td>
<td>1% TFA ($t_h &gt; 600$ s)</td>
<td>6</td>
</tr>
<tr>
<td>HMPB</td>
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<td>OCH$_3$</td>
<td>H</td>
<td>1% TFA ($t_h = 30$ s)</td>
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</tr>
<tr>
<td>HAL</td>
<td>O(CH$_2$)$_4$</td>
<td>OCH$_3$</td>
<td>OCH$_3$</td>
<td>0.1% TFA</td>
<td>8</td>
</tr>
</tbody>
</table>

Similar stabilizing effects were observed in studies comparing the acidolytic cleavage conditions of peptide amides from various different benzhydrylamine [10] and xanthenylamine-type handles [11]. Besides increasing the number of electron-releasing substituents and the distance between the aromatic system and the electron-withdrawing carboxyl terminus of the handle, the substitution site also proved to exert a considerable influence on cation stabilization, *meta* substitution being much less effective, as expected.

### 3.1.2. Substitutions in trityl-type systems

The properties of the substituted trityl group have been extensively studied in the past, because of the vast application of the pertinent compounds as dyes and acid-base indicators and more generally because of the theoretical importance for carbonium ion chemistry [12]. Electronic “fine-tuning” of trityl-type systems adds an extra dimension to peptide chemistry in comparison with the above mentioned benzyl, benzhydryl and xanthenyl systems, since trityl-type adducts are, unlike these systems, not merely restricted to a limited number of functional groups, but may be applied with any functional group occurring in amino acids, each with its own adduct stability. Substituted trityl groups are hence widely being applied as side-chain protecting groups. Both derivatives of higher lability, 4-methyl- and 4-methoxytrityl, as well as some derivatives of decreased lability, in the case of histidine and tyrosine side-chain protection, are in use.
As comprehensive comparative studies concerning the acidolytic labilities (i.e., cation stabilizing potencies) of trityl-type resins have never been performed, we set out to synthesize a number of these resins according to three different strategies: direct well-defined modification of a polymeric support, synthesis through a preformed handle containing a spacer, and through a preformed handle and a separate spacer. The new resins were compared to earlier described trityl-type resins regarding the acidolytic labilities of their adducts with amino acid functionalities and the colorimetric properties of their cationic forms.

The relative acidolytic labilities had to be measured by means of a functional group, that exhibits comparable cleavage rates under equal acidic conditions when coupled to any of the resins. Most functional groups are unsuitable in this respect. The trityl ester, e.g., shows extreme lability with the most stabilized resin, Barlos’s trityl-type resin I, whereas the tritylamide bond is only readily cleavable in trisalkoxytrityl systems. The trityl thioether, however, proved to be the ideal system, showing comparable labilities towards low-concentration TFA solutions with all the studied trityl-type resins, which are listed in Figure 1. In this thesis the resins will be referred to by their numerical names (I to VIII).

3.2. Synthesis of trityl-type resins

A disadvantage in the preparation of Barlos’s trityl-type resin I is the rather heterogeneous nature of the initial Friedel-Crafts acylation, leading to a resin whose properties (swelling, reactivity, loading) are difficult to control. Greater reproducibility is achieved either by direct modification of the support in an unambiguous way or via a preformed handle.

3.2.1. Synthesis of trityl-type resins II, III and IV by direct modification of a polymeric support

Voelter presented a synthetic concept for the well-defined preparation of trityl-type resins starting from Merrifield’s chloromethylated divinyl benzene-styrene copolymer [13]. In this strategy, chlorine is substituted by a nucleophilic phenoxide, containing a carbonyl group. Subsequent treatment with a (substituted) phenyl Grignard reagent yields the desired trityl-type resin. We undertook the synthesis of trityl-type resins II, III and IV following this pathway. Resins II and IV have already been described in preliminary reports by Voelter [13] and White [14], respectively. The synthetic pathways, i.e., the applied carbonyl components and Grignard reagents, leading to the pertinent resins, are summarized in Table 2.
Figure 1: Trityl-type resins I to IV, prepared by direct modification of a polymeric support.
Figure 1: (cont.) Trityl-type resins V to VIII, prepared from a preformed handle.
Coupling of the carbonyl component to chloromethylpolystyrene was performed following a slightly adapted procedure of Wang’s synthesis of the 4-alkoxybenzyl alcohol resin [15], using 5 equivalents of the carbonyl component and sodium methoxide each, in DMA at 50°C for 96 hours. The IR spectrum of the resulting resin, in case of methyl 4-hydroxybenzoate, showed the characteristic C=O stretching band of esters at 1721 cm⁻¹, and with 2,4-dimethoxy-4’-hydroxybenzophenone the characteristic C=O stretching band of aryl ketones at 1657 cm⁻¹.

The intermediate carbonyl resins were suspended and refluxed for 24 hours in an in situ prepared solution of 10 (resin IV) or 20 equivalents (resins II and III) of the Grignard reagent in THF. Standard work-up yielded the desired trityl-type resins, showing the C=O stretching band, characteristic of aryl ethers, in the 1245-1252 cm⁻¹ region. The 1657 and 1721 cm⁻¹ C=O stretching bands had disappeared. Upon treatment with neat TFA, the resin beads showed a typical colouration, as will be discussed later in this chapter (Section 3.4.2).

**Table 2: Synthetic pathways leading to trityl-type resins II, III and IV.**

<table>
<thead>
<tr>
<th>Trityl resin</th>
<th>Carbonyl component</th>
<th>Grignard reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>methyl 4-hydroxybenzoate</td>
<td>phenylmagnesium bromide</td>
</tr>
<tr>
<td>III</td>
<td>methyl 4-hydroxybenzoate</td>
<td>4-tolylmagnesium bromide</td>
</tr>
<tr>
<td>IV</td>
<td>2,4-dimethoxy-4’-hydroxybenzophenone</td>
<td>phenylmagnesium bromide</td>
</tr>
</tbody>
</table>

**3.2.2. Synthesis of trityl-type resins V, VI and VII through a handle containing a spacer**

In order to enhance the accessibility of the anchoring trityl group as well as the acidolytic lability, we developed trityl-type resin V by insertion of an oxypentanoic acid spacer between polymer backbone and anchoring group [16]. Thespacer is part of the preformed handle 3a. (Scheme 1).

4-Hydroxytrityl alcohol 1a was prepared by the Grignard reaction of methyl (4-trimethylsilyloxy)benzoate and phenylmagnesium bromide. After quenching of the reaction mixture, by-products like 4-hydroxybenzophenone were removed by steam distillation. Crystallization from carbon tetrachloride afforded a yellow product in 84% yield.

Thin layer chromatography showed, that the yellow colour of the product was caused by a minor impurity. Beynon and Bowden reported earlier, that synthesis of 1a sometimes proceeds with concomitant formation of a quinoidal by-product, called fuchsone (Fig. 2: 4), depending on the crystallization conditions [17]. Crystallization from alcoholic ammonia gave a colourless product that decomposed at 159-160°C, whereas crystallization from 50% acetic acid led to formation of a yellow product that decomposed at 138-139°C. Treatment of our product 1a with a warm alcoholic ammonia
solution made the yellow chromatographic spot disappear, thus identifying the impurity as fuchsone (cf. Table 4, footnote d).

\[
\begin{align*}
\text{CH}_3\text{O} & \quad \text{OTMS} \\
\text{C} & \quad \text{ii} \\
\text{HO} & \quad \text{C} \\
\text{C} & \quad \text{OH} \\
\text{C} & \quad \text{OCH}_3 \\
\text{HO} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{HO} & \quad \text{C} \\
\text{O} & \quad \text{NHCH}_2 \\
\text{HO} & \quad \text{C} \\
\text{C} & \quad \text{NHCH}_2 \\
\end{align*}
\]

**Scheme 1:** Synthesis of trityl-type resin V: i) phenylmagnesium bromide; ii) aqueous H\textsubscript{2}SO\textsubscript{4}; iii) KH; iv) Br(CH\textsubscript{3})\textsubscript{2}COOCH\textsubscript{2}; v) dioxane/methanol/4 M NaOH (14:5:1); vi) TBTU/HOBt/NMM/aminomethylated DVB-PS; vii) capping of unreacted amino groups: Ac\textsubscript{2}O/DIPEA.

Compound 1a was treated with one equivalent of potassium hydride to give the phenolate, which reacted with methyl 5-bromopentanoate affording the methyl-protected handle 2a. Hydrolysis with a dioxane/methanol/4 M sodium hydroxide (14:5:1) mixture [18] yielded the handle 5-(hydroxytrityl-4-oxypentanoic acid 3a, which was condensed to aminomethylpolystyrene giving the trityl-type resin II. The N-H deformation band at 1688 cm\textsuperscript{-1} in the IR spectrum of the aminomethylated resin had
shifted towards 1664 cm$^{-1}$, confirming the amidation of the resin. The presence of the arylether function was identified by the C-O stretching band at 1246 cm$^{-1}$. From the decrease in nitrogen content of the resin upon derivatization, a capacity of 0.47 mmol/g could be calculated, corresponding to a coupling yield of 53%. Unreacted amino functions were acetylated.

As a next step, we wished to further increase the acidolytic lability of resin V by formal addition of methoxy substituents to the trityl anchor. Attempts to synthesize 4,4'-dimethoxy-4''-hydroxytrityl alcohol 1c by the same route as 1a failed, because of the sluggish transformation of anisyl bromide into its Grignard reagent. Therefore, compound 1c was prepared by lithium-bromine interconversion in 4-bromophenol, and subsequent condensation of the dianion with 4,4'-dimethoxybenzophenone (Scheme 2), according to Gilman and Arntzen [19]. The dialkoxy analogue 1b also became accessible by this approach. The orange colour of 1b and 1c preparations probably stems from the quinoidal by-products 4-methoxyfuchson and 4,4'-dimethoxyfuchsone, respectively.

Scheme 2: Synthesis of 4-hydroxy-4'-methoxytrityl alcohol (1b: R=H) and 4,4'-dimethoxy-4''-hydroxytrityl alcohol (1c: R=OCH$_3$), and trityl-type resins VI and VII: i) 2 meq n-BuLi, 0°C; ii) 4-methoxybenzophenone (1b) or 4,4'-dimethoxybenzophenone (1c); iii) aqueous NH$_4$Cl; iv) KH; v) Br(CH$_2$)$_3$COOCH$_3$; vi) dioxane/methanol/4 M NaOH (14:5:1); vii) TBTU/HOBt/NMM/aminomethylated DVB-PS; (viii) capping of unreacted amino groups: Ac$_2$O/DIPEA. Steps iv-viii proceed analogous to steps iii-vii in scheme 1.
Further transformation of 1b and 1c into the complete handles 3b and 3c proceeded analogous to the synthesis of handle 3a. Overall yields of these syntheses were rather low (48% for 3b and 31% for 3c, as opposed to 65% in case of the crystallized compound 3a), presumably due to low yields of the organolithium reactions. Both preparations showed the presence of one major product in their GC-MS spectra, their fragmentation patterns containing the mass peaks arising from both the spacer and the trityl moiety. Since only rather small quantities of 3b and 3c were available, the handles were coupled to the aminomethylated support without further purification. Purification should not be necessary, because coupling of the handle to the support and subsequent coupling of the first amino acid to the handle require the intended functionalities. Only the intact handle possesses both the carboxyl and the trityl moiety, so that each coupled amino acid can only be anchored to the resin through an intact handle. Coupling of unpurified handles might have a negative effect on the capacity of the resin, since the amino groups will to some extent be capped by impurities containing a carboxylic function. It does, however, not complicate cleavage experiments, the major aim at this point [20].

The IR spectra of trityl resins VI and VII were similar to the spectrum of resin V. In all three cases resin beads turned orange upon treatment with neat TFA.

3.2.3. Synthesis of trityl-type resin VIII through a handle and separate spacer

Coupling of carboxy-substituted trityl handles to amino-functionalized supports has been previously reported by Bayer [21] and by Zikos and Ferderigos [22]. Their goal was the preparation of resins with acidolytic labilities similar to trityl-type resin I, to be applied in the synthesis of protected peptide acids. We set off to synthesize a new trityl-type resin by the same approach. Our purpose was, however, to explore whether the stabilizing effect of the electron-withdrawing carboxyl substituent could be cancelled by methoxy substitutions, in order to obtain a resin with enhanced acidolytic lability similar to alkoxy-substituted trityl-type resins, for the anchoring through other functional groups. Carboxy-substituted handles might prove very useful for the derivatization of supports, which are not compatible with the harsher conditions used for direct modification. Trityl-type handle 5, 4-carboxy-4',4''-dimethoxytrityl alcohol, was synthesized in two ways.

In a first approach, trityl-type handle 5 was prepared by a lithium-bromine interconversion in 4-bromobenzoic acid, followed by condensation of the dianion with 4,4-dimethoxybenzophenone (Scheme 3). To avoid self-condensation, the reaction had to be performed at -100°C [23]. At this temperature, both the dianionic complex and 4,4-dimethoxybenzophenone displayed a diminished solubility in the solvent THF, which was eventually reflected by the rather poor yield of the reaction. Work-up by extractive methods proved to be difficult because of the amphiphilic nature of the product. Therefore the reaction mixture was quenched by the addition of solid NH₄Cl. The ammonia,
formed during quenching, was removed from the mixture by a nitrogen stream. Product 5 was collected as its DCA-salt in 37% yield, with a contamination of benzoic acid according to $^1$H NMR.

![Scheme 3](image)

**Scheme 3:** One-pot synthesis of the 4-carboxy-4',4''-dimethoxytrityl alcohol handle: i) 2 meq n-BuLi, -100 °C; ii) 4,4''-dimethoxybenzophenone; iii) NH$_4$Cl

Alternatively, trityl-type handle 5 was prepared by a Grignard reaction between 4-tolylmagnesium bromide and 4,4''-dimethoxybenzophenone, followed by oxidation of the methyl group to the carboxyl group (Scheme 4). The Grignard reaction proceeded in 94% yield. Residual 4,4''-dimethoxybenzophenone (less than 5%) could be removed by crystallization from ethanol, but this method was not applied because of concomitant etherification between ethanol and the trityl centre, as demonstrated by GC-MS and $^1$H-NMR spectroscopy. Instead, intermediate 6 was used for oxidation without further purification.

![Scheme 4](image)

**Scheme 4:** Two-step synthesis of the 4-carboxy-4',4''-dimethoxytrityl alcohol handle: i) tolylmagnesium bromide; ii) aqueous $\text{KHSO}_4$; iii) 2.1 meq KMnO$_4$/pyridine/water, 85 °C.
Selective oxidation yielding handle 5 was performed by treatment with 2.1 equivalents of potassium permanganate in a pyridine/water mixture, during 2 hours at 85°C and subsequently 20 hours at room temperature [24]. Treatment with more permanganate at higher temperatures led to formation of benzoic acid through oxidation of the trityl centre. The necessarily mild reaction conditions and difficult extractive work-up resulted in a rather low yield of 42%. The amorphous product 5 had a red colour, due to partial release of a water molecule, catalyzed by its acidic proton and leading to the dimethoxytritylcarboxylate zwitterion. This was confirmed by a second product peak in the GC-MS at [M-18]⁺, and by the fact, that the neutral DCA-salt of handle 5, which was collected in the one-step organolithium approach, appeared colourless. Moreover, ¹H-NMR spectroscopy clearly indicated a generally higher signal multiplicity for the red product, most apparent from the methyl ether signal (singlet in colourless 5, double singlet in red 5), due to quinoidal resonance forms of the zwitterion, *i.e.* oxonium rotamers (*cf.* structure 4 with a CH₃ substituent on the positively charged oxygen and para-methoxy and carboxylate substituents on the two remaining phenyl rings).

Direct coupling of handle 5 to aminomethylated divinylbenzene-styrene copolymer did not yield a stable adduct. Insertion of γ-aminobutyric acid as a spacer (*via* Fmoc-GABA) led to trityl-type resin VIII. The IR spectrum showed the characteristic C-O stretching band at 1251 cm⁻¹ and the amide I band at 1659 cm⁻¹. Resin beads turned red upon treatment with neat TFA.

The application of 4,4'-dimethoxy-4''methyltrityl alcohol (6: Dmt-OH) as a side-chain protecting group for asparagine, glutamine and cysteine will be described in Chapter 7 of this thesis.

### 3.3. Trityl-type resins: comparison of adduct stability towards acidolysis

#### 3.3.1. Coupling of Fmoc-cysteine to trityl-type resins

The tritylthiol adduct was chosen as a model system to measure the relative acidolytic labilities of the eight different trityl-type resins. For this purpose the initial loading of the resin has to be compared to the residual loading after treatment with the cleavage mixture. Cleavage yields are calculated from the following equation:

\[
\text{\%} = \frac{(b_i - b_n) \cdot 10^5}{b_i \cdot (1000 - \text{MW} \cdot b_n)}
\]

with \( b_i \) being the initial loading, \( b_n \) the (new) residual loading (both in mmol/g) and MW the net molar mass decrease upon cleavage (in g/mol). The equation is derived in Appendix B.

A direct way to measure the thiol loading of a trityl-type resin was applied in case of trityl-type resin II loaded with Fmoc-cysteine, making use of the procedure for thiol determination, developed by Ellman [25]. In our adaptation, the thiol was cleaved from the resin by a continous flow of TFA in
order to drive the cleavage equilibrium to completion. The thiol concentration of the filtrate was indirectly measured upon reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [26]. Since this procedure is rather laborious, we proceeded further by an indirect route, which consisted of coupling Fmoc-cysteine to the resin and determining the Fmoc loading of the resulting resin. An additional advantage of the tritylthiol adduct as a model system is, that thiols can be coupled to the alcohol form of trityl-type resins simply by evaporation of the two components in TFA [27], thus abandoning the extra step of prior chlorination of the resin. Fmoc-cysteine was prepared according to ten Kortenaar et al. [28] and directly coupled to the eight trityl-type resins. About ten equivalents of initial cysteine were used with respect to the maximum (theoretical) resin capacity [29]. The outcome of the couplings was qualitatively verified by suspending a sample of each loaded resin in a few drops of DCM containing iodine. The resulting solutions were spotted on a TLC chromatogram with Fmoc-cystine as a reference. Rs-values in two different solvent systems were equal in all cases. The IR spectra of the derivatized resins showed the characteristic C=O stretching band of carboxylic acids between 1725 and 1728 cm⁻¹.

Initial loadings of the resins, listed in Table 3, were all satisfactory, with somewhat lower yields in the case of resins VI and VII, as was expected because of the coupling of unpurified handles. Moreover, elemental analyses of the original aminomethylated resins showed, that the capacity of the batch, used in the preparation of resins VI, VII and VIII, was 20% lower than the capacity of the one that was used to prepare resin V. Loading of resin V proceeded quantitatively, based on the capacity of 0.47 mmol/g. Loading of resins II, III and IV proceeded in 71 to 74% yield, based on the capacity of the initial chloromethylpolystyrene, which is indicative of the high overall yield of direct modification of the polymeric supports. The validity of the Fmoc procedure was established by comparing the thus obtained loading of resin II (0.40 mmol/g) with loadings calculated from the sulfur content of the resin (0.40 mmol/g) and according to the Elliman method (0.38 mmol/g).

3.3.2. Cleavage of Fmoc-cysteine from trityl-type resins

The actual cleavage experiments were conducted by treating resin samples with solutions of TFA in DCM/TES (95:5) at various concentrations. Triethylsilane, a hydride donor, has to be added to scavenge trityl cations arising from cleavage [30]. Readdition of the thiol to the resin, due to the reversible nature of the tritylthiol bond-breaking reaction, is thus prevented. Upon addition of the cleavage mixture, resin beads show the typical cationic colouration, which disappears within a few seconds only if triethylsilane is present in the mixture, indicative of the scavenging process during which colourless triphenylmethane derivatives are formed.
**Table 3:** Cleavage yields of Fmoc-cysteine from trityl-type resins. The resins are listed in order of increasing acidolytic lability of the adducts.

<table>
<thead>
<tr>
<th>Trityl-type resin</th>
<th>Substituents a</th>
<th>Initial loading (nmol/g)</th>
<th>% Cleavage in 1.0 % TFA c</th>
<th>% Cleavage in 0.5 % TFA c</th>
<th>% Cleavage in 0.1 % TFA c</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cl / R</td>
<td>0.52</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>OR</td>
<td>0.41</td>
<td>46</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>2xOR / CA</td>
<td>0.35</td>
<td>56</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>2xOR</td>
<td>0.29</td>
<td>76</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>OB</td>
<td>0.40</td>
<td>82</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2xR / OB</td>
<td>0.39</td>
<td>93</td>
<td>62</td>
<td>15</td>
</tr>
<tr>
<td>VII</td>
<td>3xOR</td>
<td>0.31</td>
<td>78</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>2xOR b / OB</td>
<td>0.38</td>
<td>95</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

a R = alkyl; OR = alkoxy; CA = carboxamide; OB = benzoxy. b One alkoxy substituent is in ortho position. c 15-minute treatment with TFA solutions in DCM/ TES (95:5), MW = 325.4 except for resin I.

As evidenced by the cleavage data assembled in Table 3, the acidolytic lability of trityl-type resins is minutely determined by the ability of substituents on the trityl moiety to stabilize the liberated cation. Electron-withdrawing halogen (resin I) and carbonyl-type (resin VIII vs. VI) substituents lower the lability, whereas it is enhanced by electron-releasing alkyl, alkoxy and benzoxy substituents. Very conspicuous are the strong cation-stabilizing effect exerted by alkyl substituents (resin III vs. II) due to hyperconjugation, and the large increase in stabilization when replacing an alkoxy substituent by a benzoxy substituent (resin II vs. V and IV vs. VII), which is a consequence of the extension of the conjugated system with one phenyl ring. Ortho as opposed to para substitution may also have a slightly positive effect on stabilization; the increased stability in this respect is assumedly due to the relatively greater relief of steric strain for the ortho-substituted compound in going from the tetrahedral central carbon in the carbinol to the trigonal carbon in the carbonium ion (resin IV vs. VII) [31].

All studied trityl-type resins, except Barlos's resin I, would allow the cleavage of protected peptides with a free thiol function by one or more 15-minute treatments with 1% TFA. In case of very acid-sensitive peptides, such as those containing an Asp-Pro bond [32], use of resins IV and VII would be recommended. The applicability of the latter is, however, limited because of its instability, which will be discussed in Section 3.4.3.
3.4. Colorimetric properties of substituted trityl carbonium ions

3.4.1. Intermezzo: Principles of colour chemistry applied to the trityl group

Colorimetry deals with the absorption of light in the visible part of the electromagnetic spectrum, at wavelengths between 400 and 700 nm [33]. The absorption by a so-called chromophore is strongly correlated to its chemical structure of conjugated double bonds, expanding from an electron-deficient to an electron-donating site in the system. The absorbed energy is used for electron excitation. In molecular-orbital terms, electrons are promoted from a bonding (occupied) MO to an anti-bonding (unoccupied) MO. Absorption takes place if the energy of the light is equal to the energy difference between these levels. Inasmuch as the introduction of conjugation leads to a degeneration of MOs, the energy difference between the occupied π-MOs and the unoccupied π*-MOs becomes small enough to allow excitation by absorption of light of relatively low energy in the UV-VIS range. In general, the following law applies: the longer the conjugated system, the longer the wavelength of the absorption maximum.

An extension of the conjugated system by the addition of auxochromic groups results in a further reduction in the separation of energy levels, thus causing a bathochromic or red shift, i.e. a shift towards a longer wavelength, in the absorption spectrum. The opposite, a hypsochromic or blue shift, is caused by removal of conjugation. Auxochromic groups are substituents carrying a lone pair of electrons (e.g. OH, OR and NR₂); these electrons occupy p-orbitals which, because of their geometry, can overlap with the aromatic π-orbitals. Alternatively a group with π-orbitals itself (e.g. NO₂ and COOH) may function as an auxochrome, since overlapping of the two π-electron systems leads to new bonding and anti-bonding electronic states.

In trityl alcohol, the three conjugated subsystems, the phenyl rings, are isolated from each other and absorption consequently occurs in the UV region around 259 nm. In the presence of a strong acid, however, the trityl cation is formed: an electron deficiency is introduced at the trityl centre and by resonance delocalized over all three phenyl rings, thereby extending the conjugation over the entire system. A bathochromic shift is observed, affording a double absorption maximum at around 406 (γ band) and 432 nm (x band) [34]. The x band corresponds to a transition from the highest occupied MO (HOMO) to the lowest unoccupied MO (LUMO), whereas the γ band arises from excitation of an electron from the second highest occupied MO. In unsymmetrically substituted trityl-type compounds, the contribution from charge migration from an unsubstituted phenyl ring predominates in the γ band; the x band is essentially localized in the substituted part of the molecule [35].

Trityl-type compounds carrying auxochromes, like Malachite Green, Crystal Violet, fuchsine and benzaurine (7a-d), proved very efficient in their application as dyes due to high colour strength [36];
the absorption maxima may reach as far into the visible region as 640 nm in case of certain Malachite Green analogues. The acid-base indicator phenolphthalein (7e) also belongs to the extensive class of compounds, whose members are commonly depicted as their quinoidal resonance structures (Fig. 3).

![Chemical structures](image)

**Figure 3**: Examples of trityl-type compounds used as dyes or acid-base indicators: Malachite Green, Crystal Violet, fuchsine, benzaurine, and phenolphthalein (7a-e). The last two are depicted in the coloured anionic form present in alkaline solution. Notice the structural similarity between phenolphthalein and compound 5.

### 3.4.2. Colorimetric analysis of synthesized trityl-type compounds

Reference data of substituted trityl cations being abundantly available, we wished to characterize the compounds, that were isolated as precursors in the syntheses of trityl-type resins V, VI, VII and VIII, by means of their colorimetric properties in an acidic medium. For this purpose, the absorption spectra of commercially available trityl alcohol and compounds 1, 3a-c, 5 and 6 were measured in 1% TFA/DCM at similar concentrations. The results and literature data of related compounds [17,37-41] are collected in Table 4. The perceived colours range from bright yellow for trityl alcohol to red for compound 5.
Given that the absorption maxima of commercially available trityl alcohol appeared at somewhat longer wavelengths than reported in the literature, the maxima found for compounds 1a, 3a-c and 6 are in very good agreement with those of the corresponding reported hydroxy and methoxy-substituted analogues. In some cases, no second absorption maximum was reported in the cited literature.

Table 4: Colorimetric data of synthesized trityl-type compounds and related compounds from literature a.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituents b</th>
<th>λ_max (nm) y/x band</th>
<th>Relative intensities c</th>
<th>Literature compound</th>
<th>λ_max (nm) y/x band</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trt-OH</td>
<td></td>
<td>410/434</td>
<td>1.00:1</td>
<td>Trt-OH</td>
<td>404-408/431-433</td>
<td>34,37-39</td>
</tr>
<tr>
<td>1a</td>
<td>OH</td>
<td>405/465</td>
<td>0.85:1</td>
<td>1a = 4 d</td>
<td>401/464</td>
<td>17</td>
</tr>
<tr>
<td>3a</td>
<td>OR</td>
<td>399/480</td>
<td>0.31:1</td>
<td>Trt(OMe)-OH</td>
<td>n.d./470-476</td>
<td>37-40</td>
</tr>
<tr>
<td>3b</td>
<td>2xOR</td>
<td>417/505</td>
<td>0.47:1</td>
<td>Trt(OMe)2-OH</td>
<td>411/495-500</td>
<td>35a,38-40</td>
</tr>
<tr>
<td>3c</td>
<td>3xOR</td>
<td>489</td>
<td></td>
<td>Trt(OMe)3-OH</td>
<td>480-483</td>
<td>38-41</td>
</tr>
<tr>
<td>6</td>
<td>2xOR/R</td>
<td>435/496</td>
<td>0.67:1</td>
<td>Trt(Me)-OH</td>
<td>450</td>
<td>37,38</td>
</tr>
<tr>
<td>5</td>
<td>2xOR/CA</td>
<td>406/522</td>
<td>0.43:1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Para-substituted trityl-type compounds in their cationic forms. b OR = alkoxy; R = alkyl; CA = carboxamide. c Relative intensities of the two absorption maxima measured for the pertinent compound. d Beynon and Bowden used colorimetry as one means of proving the equivalence of fuchsone (4) and the yellow contamination present in 4-hydroxytrityl alcohol (1a), the absorption spectra in acidic medium being equal [17].

As expected, when going from a monoalkoxy to a dialkoxy-substituted compound (3a vs. 3b and 6, Trt(OMe)-OH vs. Trt(OMe)2-OH), a strong bathochromic shift of the spectrum is observed. Addition of a third substituent (3c, Trt(OMe)3-OH) is, however, deceptively accompanied by a small hypsochromic shift. Brand made an attempt to explain this phenomenon [40], which he also observed in case of dimethylamino and methylmercapto-substituted trityl derivatives, by assuming that the absorption maximum of the trisubstituted compound originates from a red shift of the y band of the disubstituted analogue. Its x band might have fused with the advancing y band. Indeed, the broad absorption peak of compound 3c is followed by a shoulder in the 520-570 nm region.

The red shift of the y band of compound 6 (435 nm) with regard to the corresponding maximum of compound 3b (417 nm) might be caused by the methyl substituent in the former; methyl substituents can exert this effect by hyperconjugation (cf. Trt-OH vs. Trt(Me)-OH). As mentioned earlier, the contribution of the unsubstituted phenyl ring predominates in the y band. The red colour of compound
As an extension of our studies, the eight trityl-type resins, upon treatment with neat TFA, were also subjected to colorimetric measurement by means of reflection spectra, using a device normally applied to evaluate textile dyeing. We obtained sigmoidal curves, whose starting points (\( \lambda \)) were taken as a qualitative measure for the bathochromic shifts caused by the different degrees of substitution on the trityl moiety of the resins (Table 5).

Table 5: Colorimetric data of cationic trityl-type resins.

<table>
<thead>
<tr>
<th>Trityl-type resin</th>
<th>Substituents (^a)</th>
<th>Colour</th>
<th>Reflection start ( \lambda ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cl / R</td>
<td>orange-brown</td>
<td>510</td>
</tr>
<tr>
<td>II</td>
<td>OB</td>
<td>orange</td>
<td>530</td>
</tr>
<tr>
<td>III</td>
<td>2xR / OB</td>
<td>orange</td>
<td>530</td>
</tr>
<tr>
<td>V</td>
<td>OR</td>
<td>orange</td>
<td>530</td>
</tr>
<tr>
<td>VI</td>
<td>2xOR</td>
<td>dark orange</td>
<td>545</td>
</tr>
<tr>
<td>VII</td>
<td>3xOR</td>
<td>dark orange</td>
<td>545</td>
</tr>
<tr>
<td>VIII</td>
<td>2xOR / CA</td>
<td>red</td>
<td>555</td>
</tr>
<tr>
<td>IV</td>
<td>2xOR (^b) / OB</td>
<td>dark red</td>
<td>570</td>
</tr>
</tbody>
</table>

\(^a\) R = alkyl; OR = alkoxy; CA = carboxamide; OB = benzoxy.

\(^b\) One alkoxy substituent is in ortho position.

The general tendencies are similar to those observed for the soluble trityl-type compounds. No differences were found between alkoxy and benzoxy-substituted systems (resins II, III and V), whereas the effect caused by the methyl substituents in resin III could not be detected with the presently used device. The red shift with regard to chloro-substituted resin I was not as big (20 nm) as the observed shift between unsubstituted trityl alcohol and its alkoxy-substituted analogues (ca. 45 nm for the \( \pi \) band); the chloro substituent displays an auxochromic character due to the interaction between its lone pair electrons and the aromatic \( \pi \)-system.

A difference between double and triple alkoxy substitution was not detected (resin VI vs. VII). Resin VIII showed the same red colour as its soluble counterpart 5. The exceptionally strong bathochromic shift measured with resin IV could not be anticipated on account of the number of the pertinent substituents. It may be attributed, though, to the larger out-of-plane angle of the phenyl rings imposed by the ortho substituent [42].
3.4.3. Solution colouration indicating resin instability

Apart from the resin beads, the solutions of resins II, III, IV, V, VI and VII when suspended in neat TFA, showed a pale colouration, which was (dark) yellow with the first five and red with the last mentioned resin. Addition of triethylsilane prevented the formation of a colour. We believe that this observation can be explained by two different processes, depending on the actual resin.

In case of the resins V, VI and VII, the colouration is probably caused by the presence of non-covalently bound handle molecules, that are captured within the polymeric matrix. During derivatization of the original aminomethylated resins, the anchoring sites might be difficult to access for the sterically bulky handles. Instead, loading by adsorption is enhanced because of the hydrophobicity of the handles (trityl moiety and pentanoic spacer) and the presence of a carboxylic function, enabling ample van der Waals interactions with the polymeric matrix and salt formation with the amino functions on the resin. Normal washing procedures were evidently insufficient for complete removal of non-covalently bound handle. Treatment with TFA of the derivatized resins leads to ionization of both covalently bound and adsorbed trityl handles. The presence of non-covalently bound handle is the cause of premature loss of peptide material from the resins during SPPS, as reported in the next chapter (Section 4.2.1).

Resins II, III and IV are linked to the polymer backbone as a benzyloxy ether. Penke and Rivier [43] and Voelter and co-workers [44] already noticed the exceptional lability of the ether linkage formed between chloromethylpolystyrene and the 4-hydroxybenzylamine handle towards 50% TFA, during the cleavage of peptide amides from the resin. The major product was always the 4-hydroxybenzyl-substituted amide analogue instead of the desired free amide. Riniker and Kamber found that cleavage of tryptophan-containing peptides from Wang’s 4-alkoxybenzyl alcohol resin led to partial alklylation of the indole moiety by the 4-hydroxybenzyl cation [45]. In case of the studied benzyloxy-substituted trityl-type resins, their cationic form, liberated upon treatment with TFA, might partly rearrange to a quinoidal fuchson analogue, as seen earlier in the synthesis of 1a-c (Fig. 2), with its concomitant release from the resin causing the colouration of the solution. A TFA-treated sample of resin II gave almost the same initial loading of Fmoc-cysteine as the untreated resin (0.39 vs. 0.40 mmol/g), indicating that rearrangement proceeds very slowly. The fission is inhibited by scavenging the trityl cations with hydride, through the addition of triethylsilane. In conclusion, we may say that the application of resins II, III and IV in the synthesis of deprotected peptides is not compromised by cleavage with undiluted TFA.
3.5. Conclusions

The synthesis of seven different trityl-type resins, applicable in SPPS, is reported, proceeding either by direct modification of a polymeric support or through condensation with a preformed handle. The first strategy starts with the nucleophilic substitution on chloromethylpolystyrene by a phenolate comprising a reactive carbonyl group, which is subsequently reduced with a Grignard reagent. This strategy is preferred because of its unambiguity, simple working method, low cost and high yield. Furthermore, variation of the carbonyl component or Grignard reagent allows the preparation of trityl-type resins with different levels of substitution. Derivatization of aminomethylated resins with a preformed bulky trityl-type handle is difficult to control, as the loading might be partially adsorptive. The acidolytic labilities of the synthesized trityl-type resins and Barlos’s trityl-type resin I have been compared by using the tritylthiol adduct as a model system. The lability of the anchoring bond is determined by the degree of stabilization of the incipient positive charge, arising upon cleavage, and can be minutely “fine-tuned” by varying the number and nature of the substituents on the trityl system. Electron-withdrawing substituents lower lability, whereas it is enhanced by electron-releasing substituents. The lability of resins prepared by the aforementioned direct-modification approach, containing a benzylox substituent (i.e. the linkage to the polymer backbone), is highly increased with respect to resins containing alkoxy substituents.

Easy “fine-tuning” promotes the general applicability of trityl-type resins in SPPS. The choice of the appropriate resin in a specific SPPS protocol depends on the attached functional group and the desired cleavage conditions, to yield either a protected or an unprotected peptide. The choice of trityl-type resins I and II for use in subsequent solid-phase syntheses is further elucidated in the next chapter.

The further exploration of compact carboxy-substituted trityl-type handles like compound 5 would be desirable for the derivatization of more labile types of (amino-functionalized) supports, that are not compatible with the relatively harsh reaction conditions encountered in the direct-modification approach. These handles might gain wide application in continuous-flow SPPS [21].

The last part of this chapter deals with the spectroscopic properties of trityl-type compounds. These, whether they are immobilized or present in solution, show bright colourations in their cationic forms. The observed absorption maxima, like the stabilities of the cations, depend on the degree of substitution: each auxochromic substituent (e.g. (ar)alkoxy or carboxamide) causes an increase in the number of possible energy levels of the system. The corresponding decrease in energy difference between the occupied and unoccupied MO levels results in a bathochromic shift in the absorption spectrum of the actual compound, eventually allowing its colorimetric characterization.
3.6. Experimental section

Materials and methods

Aminomethylated DVB-PS: %N 1.48 (batch used in the preparation of trityl-type resin V); 1.25 (batch used in the preparation of trityl-type resins VI, VII and VIII). IR (trityl-type resin I): 3063, 3026, 2925, 2851, 1602, 1568, 1493, 1447, 1340, 1272, 1187, 1161, 1126, 1054, 1019, 899, 818, 754, 701, 657, 626, 555 cm⁻¹; (chloromethylated DVB-PS): 3059, 3027, 2924, 2850, 1601, 1494, 1452, 1370, 1329, 1309, 1266, 1181, 1154, 1121, 1068, 1029, 906, 759, 701, 541 cm⁻¹; (aminomethylated DVB-PS): 3059, 3026, 2924, 2850, 1688, 1600, 1491, 1451, 1372, 1328, 1157, 1110, 1069, 1013, 907, 759, 701, 541 cm⁻¹. Aminomethylated and chloromethylated DVB-PS were purchased from Bachem. Trityl alcohol was purchased from Fluka. The GC-MS device consisted of a MFC 500 gas chromatograph (column: fused silica SE-54, 25 m x 0.25 mm, gas: helium, temperature program: 50°C stand-by, 1 min solvent delay 100°C, heating to 280°C at 5°C/min, 30 min isothermal) and a QMP 1000 Quadrupole EI mass spectrometer (70 eV), both from Carlo Erba Instruments; N-methyl-N-trimethylsilyl-heptafluorobutyramide was used for trimethylsilylation. The EI mass spectrum of compound 3a was measured on a VG 7070 mass spectrometer. Mentioned mass spectral data comprise only the molecular ion [M⁺] peak and/or the relevant fragmentation peaks. The reflection spectra of the cationic resins were recorded on a Datacolor 3890 colorimeter (Marl, Germany), using illuminant D65 and 10° observer. For further specifications, see Appendix A.

Trityl-type resin II

Finely cut sodium (800 mg, 35 mmol) was dissolved in 30 ml MeOH and the solution was evaporated in vacuo. To the residual NaOMe, 10 g Merrifield resin (0.7 mmol/g), 5.3 g methyl 4-hydroxybenzoate (35 mmol) and 60 ml DMA were added, and the suspension was refluxed at 50°C for 4 days. Then the resin was filtered and washed with subsequently DMF, dioxane, water, DCM and 2-propanol, each twice. After repetition of the same washing procedure, the resin was washed with ether. The dried resin was added to 70 ml of a 2 M phenylmagnesium bromide solution in THF at 0°C. The suspension was refluxed overnight at 60°C, after which a mixture of 3 ml concentrated sulfuric acid in 100 ml ice was added. The resin was then filtered off and washed as described above. IR (methyl 4-benzoxybenzoate resin): 3059, 3027, 2925, 2850, 1721, 1604, 1492, 1452, 1379, 1313, 1280, 1252, 1168, 1109, 1070, 1028, 906, 846, 762, 701, 542 cm⁻¹; (II): 3059, 3027, 2925, 2851, 1603, 1492, 1451, 1379, 1306, 1246, 1181, 1156, 1116, 1071, 1028, 906, 829, 759, 701, 576, 541 cm⁻¹.

Trityl-type resin III

According to the synthesis of resin II, using 4-tolylmagnesium bromide instead of phenylmagnesium bromide. IR: 3059, 3026, 2925, 2851, 1603, 1492, 1452, 1378, 1307, 1245, 1180, 1154, 1117, 1069, 1027, 907, 816, 759, 701, 577, 541 cm⁻¹.

Trityl-type resin IV

According to the synthesis of resin II, using 8.9 g 2,4-dimethoxy-4'-hydroxy-benzophenone (35 mmol) instead of methyl 4-hydroxybenzoate, and 70 ml of a 1 M phenylmagnesium bromide solution in THF instead of 2 M. IR (4-benzoxy-2',4'-dimethoxybenzophenone resin): 3059, 3026, 2925, 2850, 1657, 1602, 1493, 1452, 1377.
1310, 1250, 1210, 1160, 1121, 1029, 943, 908, 842, 762, 701, 542 cm\(^{-1}\); (IV): 3059, 3027, 2925, 2850, 1605, 1494, 1451, 1368, 1305, 1252, 1207, 1158, 1124, 1028, 906, 829, 759, 702, 541 cm\(^{-1}\).

4-Hydroxytrityl alcohol (1a)

A solution of 56 g (0.25 mol) methyl (4-trimethylsilyloxy)benzoate in 100 ml toluene was added to a stirred solution of phenylmagnesium bromide (285 ml, 0.57 mol, 2 M) in ether at 0°C. The reaction mixture was refluxed for 1 h, during which the colour of the solution turned from sepia to orange and a precipitate of the magnesium salt was formed. The suspension was subsequently poured into a mixture of 750 g ice and 25 ml of concentrated sulfuric acid. The organic layer was separated and the aqueous layer was extracted with ether (5x100 ml). The combined organic phases were washed with water (5x100 ml), dried over Na\(_2\)SO\(_4\) and evaporated in \textit{vacuo}. The resulting yellow oil was purified by steam distillation, affording a mixture of water and an orange oil. The oil was dissolved in 150 ml ether and the water layer was extracted with ether (3x50 ml). The combined ether extracts were washed with water (3x50 ml), dried over Na\(_2\)SO\(_4\) and evaporated in \textit{vacuo}. The orange residue was recrystallized from carbon tetrachloride affording a yellow solid in 84% yield (55.4 g). Mp 140-142°C. TLC: R\(_f\) (B) 0.77 (1a: UV\(^+\)) / 0.83 (4: yellow), R\(_f\) (D) 0.71 (1a: UV\(^+\)) / 0.45 (4: yellow). GC-MS (36.0 min, after trimethylsilylation, ca. 98%): m/z 348 [M+TMS]\(^+\). \(^1\)H NMR (CDCl\(_3\), δ (ppm)): 2.87 (sharp s, 1H, aliphatic OH); 5.52 (broad s, 1H, phenolic OH); 6.75 (d, 2H, J = 8.73 Hz, C(3,5)H); 7.10 (d, 2H, C(2,6)H); 7.28 (m, 10H, other aromatic protons).

5-(Hydroxytrityl-4-oxo)pentanoic acid (3a)

4-Hydroxytrityl alcohol 1a (10 g, 36 mmol) was added to a suspension of 1.44 g (36 mmol) KH in 70 ml DMF. After dropwise addition of 4.7 g (24 mmol) methyl 5-bromopentanoate, the reaction mixture was stirred for 3 h at 70°C. The resulting brown suspension was poured into 500 ml water, followed by extraction with ether (6x100 ml). The combined ether extracts were dried over Na\(_2\)SO\(_4\) and evaporated in \textit{vacuo}. The resulting yellow oil (2a) was taken up into 150 ml of a dioxane/MeOH/4 M NaOH (14:5:1) mixture and the solution was stirred for 20 h at room temperature. Addition of 250 ml water gave a milky suspension at pH 11. Residual phenolate was removed by washing with ether (6x100 ml). The pH of the aqueous layer was lowered to 1 with concentrated HCl at 0°C. The water layer was then extracted with ether (6x100 ml). The combined ether layers were extracted with 4 M NaOH (6x100 ml). The pH of the combined aqueous extracts was again lowered to 11 with HCl, followed by ether washings, pH lowering and ether extractions as described above. The combined ether extracts were dried over Na\(_2\)SO\(_4\) and evaporated in \textit{vacuo}. The residual orange residue crystallized spontaneously, yielding 7.0 g (77%) of a pale yellow product. Mp 94°C. TLC: R\(_f\) (B) 0.78, R\(_f\) (D) 0.37. EI-MS: m/z 376 [M]\(^+\). \(^1\)H NMR (CDCl\(_3\), δ (ppm)): 1.83 (m, 4H, C(β,γ)H); 2.44 (m, 2H, C(α)H); 3.96 (m, 2H, C(δ)H); 6.81 (d, 2H, C(3,5)H); 7.15 (d, 2H, C(2,6)H); 7.28 (s, 10H, other aromatic protons). The DCA-salt of 3a could be crystallized from ether. Recrystallization from ethyl acetate yielded a colourless solid in 55% yield. Mp 162°C.
4-Hydroxy-4'-methoxytrityl alcohol (1b)

4-Bromophenol (1.7 g, 10 mmol) and 40 ml ether were introduced, under nitrogen, into a three-neck flask equipped with a low-temperature thermometer, condenser and addition funnel connected to a nitrogen inlet tube. The reaction mixture was cooled to -20°C (solid carbon dioxide-acetone bath) and n-butyllithium (12.5 ml, 20 mmol, 1.6 M solution in hexane) was added slowly, keeping the temperature constant. After additional stirring for 1 h at room temperature, a solution of 1.1 g 4-methoxybenzophenone (5 mmol) in 40 ml THF was added, and the mixture was stirred for 15 h under reflux. The reaction was quenched with 40 ml 1 M aqueous NH₄Cl. The organic layer was subsequently washed with 1 M NaOH (2x25 ml), to remove the residual phenol, then with water (5x25 ml), dried over Na₂SO₄ and evaporated in vacuo, yielding 850 mg of an orange oil (56%).

4,4'-Dimethoxy-4'-hydroxytrityl alcohol (1c)

According to the synthesis of 1b, using 1.2 g (5 mmol) 4,4'-dimethoxybenzophenone instead of 4-methoxybenzophenone. Yield: 800 mg (48%).

5-(Hydroxy-4-methoxytrityl-4'-oxy)pentanoic acid (3b)

According to the synthesis of 3a, using 1b instead of 1a on a 2 mmol scale (610 mg 1b), and with 0.95 meq methyl 5-bromopentanoate instead of 0.67 meq. An orange oil was obtained in 85% yield (690 mg). TLC: R_f (B) 0.78, R_f (D) 0.27. GC-MS (38.9 min, after trimethylsilylation): m/z 289 [C(Ph)₃(PhOCH₂)O]⁻, 189 [O(CH₂)₃COOTMS]⁻.

5-(Hydroxy-4,4'-dimethoxytrityl-4'-oxy)pentanoic acid (3c)

According to the synthesis of 3a, using 1c instead of 1a on a 2 mmol scale (670 mg 1c), and with 0.95 meq methyl 5-bromopentanoate instead of 0.67 meq. An orange oil was obtained in 65% yield (570 mg). A sample of the oil had crystallized spontaneously upon standing for several months at room temperature. After washing with petroleum ether, orange crystals were collected with a melting point of 87-88°C. TLC: R_f (B) 0.79, R_f (D) 0.15. GC-MS (45.2 min, after trimethylsilylation): m/z 319 [C(Ph)(PhOCH₂)₃O]⁻, 189 [O(CH₂)₃COOTMS]⁻.

Trityl-type resins V, VI and VII

A suspension of aminomethylated DVB-PS copolymer in DMF (0.1 g/ml: 10.0 g (V) or 0.5 g (VI and VII) polymer) was shaken for 20 h in the presence of each 1.5 meq of the pertinent handle 3, TBTU and HOBt, and 2.25 meq NMM. After washing with DMF (four times) and 2-propanol (twice), the resin was capped with a solution of Ac₂O/DIPEA (2:1) in DMF, and again washed with DMF (four times), 2-propanol and ether (each twice). %N (V) = 1.23. IR (V): 3058, 3026, 2924, 2850, 1664, 1602, 1492, 1452, 1370, 1312, 1246, 1180, 1067, 1028, 905, 829, 759, 701, 627, 544 cm⁻¹; (VI): 3060, 3026, 2924, 2851, 1661, 1604, 1493, 1451, 1373, 1297, 1249, 1178, 1069, 1029, 906, 829, 759, 701, 621, 544 cm⁻¹; (VII): 3058, 3027, 2924, 2851, 1662, 1603, 1494, 1452, 1373, 1299, 1250, 1177, 1068, 1032, 908, 830, 759, 701, 583, 541 cm⁻¹.

4-Carboxy-4,4'-dimethoxytrityl alcohol (5) via organolithium reaction (Scheme 3)

4-Bromobenzoic acid (8.0 g, 40 mmol) and 300 ml THF were introduced, under nitrogen, into a three-neck flask equipped with a low-temperature thermometer, stirrer and addition funnel connected to a nitrogen inlet tube. The reaction mixture was cooled to -100°C (liquid nitrogen-ether bath) and n-butyllithium (50 ml, 80 mmol, 1.6 M
solution in hexane) was added slowly, so that the temperature did not exceed -95°C. After additional stirring for 30 min at -100°C, a solution of 9.7 g 4,4'-dimethoxybenzophenone (40 mmol) in 200 ml THF was added; the mixture was again stirred for 15 h, allowing the temperature to reach 0°C. The reaction was quenched by the addition of 6 g NH₄Cl and 2 g KHSO₄. The formed ammonia was removed by purging with nitrogen. Filtration and subsequent evaporation of the filtrate afforded a dark orange oil. The acid 5 was converted into its DCA-salt, which upon crystallization from ether afforded a colourless solid in 37% yield (8.0 g). Mp: 125-135°C (white crystals turning into a yellow oil). TLC: Rf (B) 0.74, Rf (D) 0.12. 1H NMR (CDCl₃), δ (> 3.5 ppm): 3.79 (s, 6H, Ph-OCH₃); 6.83 (d, 4H, J = 8.73 Hz, C(3',3'',5',5'')H); 7.17 (d, 4H, J = 8.73 Hz, C(2',2'',6',6'')H); signals at 7.26-7.50 and 7.87-8.05 are indiscernible because of a benzoic acid contamination.

4,4'-Dimethoxy-4''-methyltrityl alcohol (6)
A suspension of 24 g (99 mmol) 4,4'-dimethoxybenzophenone in 160 ml toluene was added dropwise to 300 ml of a 0.36 M phenylmagnesium bromide solution in ether at 0°C, during which the colour of the solution turned from yellow-green to orange. After refluxing overnight, the suspension was poured into 500 ml 10% KHSO₄ under ice cooling. The organic layer was separated and the water phase was washed with ether (6x100 ml). The combined organic layers were then washed with water (6x100 ml), dried over Na₂SO₄ and evaporated in vacuo, yielding 31 g (94%) of a yellow amorphous product. TLC: Rf (B) 0.93, Rf (D) 0.76. GC-MS (38.2 min, ca. 95%): m/z 334 [M]+. 1H NMR (CDCl₃), δ (ppm): 2.33 (s, 3H, Ph-CH₃); 2.76 (s, 1H, OH): 3.78 (s, 6H, Ph-OCH₃); 6.81 (d, 4H, J = 9.06 Hz, C(3,3',5,5')H); 7.12 (d, 4H, C(2,2',6,6')H); 7.17 (m, 4H, C(2''3'',5'',6'')H). After washing of residual dimethoxybenzophenone with ethanol, the major peak (ca. 80%) appeared at 37.6 min, m/z 363 [M+H][+]. 1H NMR (CDCl₃), δ (ppm): 1.21 (m, 2.3H, -OCH₂-CH₃); 2.30 (s, 3H, Ph-CH₃); 3.07 (q, 1.5H, J = 7.05 Hz, -OCH₂-CH₃); 3.77 (s, 6H, Ph-OCH₃); 6.81 (d, 4H, J = 9.05 Hz, C(3,3',5,5')H); 7.12 (m, 4H, C(2,2',6,6')H); 7.32 (m, 4H, C(2'',3'',5'',6'')H).

4-Carboxy-4',4''-dimethoxytrityl alcohol (5) via oxidation of 6 (Scheme 4)
To a solution of 1.0 g (3.0 mmol) 6 in 10 ml pyridine at 85°C, half of a solution of 1 g (6.3 mmol) KMnO₄ in 20 ml water was added. After stirring for 30 min at the mentioned temperature, the residual KMnO₄ solution was added. Stirring was continued for another 1.5 h at 85°C and overnight at room temperature. The solution was then filtered and concentrated in vacuo. The pH was lowered to 1 with concentrated HCl and the solution was extracted with DCM (5x10 ml). The combined organic layers were washed twice with water and subsequently extracted with 1.2 M NaOH solution (5x10 ml). The combined aqueous layers at pH 11 were washed twice with DCM, after which their pH was lowered to 4 with concentrated HCl. Finally, the water phase was extracted with DCM (5x10 ml). The combined organic layers were dried over Na₂SO₄ and evaporated in vacuo, yielding 0.43 g (42%) of a red amorphous product. TLC: Rf (B) 0.74, Rf (D) 0.12. GC-MS (43.3 min, ca. 30%): m/z 364 [M]+; (51.9 min, ca. 70%): m/z 346 [M-H₂O] [+]. 1H NMR (CDCl₃), δ (ppm): 3.76/3.81 (ds, 6H, Ph-OCH₃); 6.78/6.85 (dm, 4H, C(3,3',5,5')H); 7.17/7.28 (dm, 4H, C(2',2'',6',6'')H); 7.42-7.72 (m, 2H, C(2,6)H); 8.04/8.19 (dm, 2H, C(3,5)H).
Trityl-type resin VIII

Fmoc-γ-aminobutyric acid (0.44 g, 1.35 mmol) was coupled to aminomethylated DVB-PS copolymer (0.5 g, 0.9 mmol/g), followed by cleavage of the Fmoc group, after the general TBTU procedure for SPPS, described in Appendix A. Further derivatization proceeded according to the synthesis of resins V, VI and VII, using 5 instead of 3. IR: 3320, 3059, 3026, 2925, 2851, 1659, 1604, 1549, 1531, 1495, 1452, 1370, 1301, 1251, 1179, 1115, 1069, 1031, 908, 828, 760, 701, 584, 541 cm⁻¹.

Coupling of Fmoc-cysteine to trityl-type resins I to VIII

Cysteine hydrochloride (2.2 g, 14 mmol) was dissolved in 15 ml water in the presence of 3.9 ml triethylamine (14 mmol). To this mixture a solution of 4.4 g (12 mmol) Fmoc-ONsU in 15 ml dioxane was added in one portion. During the next 15 min the pH of the reaction mixture was maintained at 8.5-9.0 by the addition of triethylamine. After stirring for an additional 15 min, the mixture was concentrated in vacuo. The residue was poured into 50 ml of 1.5 M HCl, yielding an oily precipitate, which was extracted with ethyl acetate (3x30 ml). The combined organic phases were washed water and saturated NaCl, each twice, dried over Na₂SO₄ and evaporated in vacuo. The residual oil was dissolved in 40 ml TFA and divided into eight portions. To each portion 250 mg of the pertinent trityl-type resin was added. TFA was evaporated in vacuo. Residual TFA was removed by evaporation with ether (three times) until the resins attained a yellow colour, with the exception of resin VIII, which remained dark red. The loaded resins were subsequently washed with DMF, DCM, 2-propanol and ether, each twice. % S (Fmoc-Cys(II)-OH) = 1.29. IR (Fmoc-Cys(I)-OH): 3411, 3061, 3027, 2974, 2926, 2859, 1727, 1602, 1566, 1505, 1446, 1334, 1266, 1210, 1187, 1159, 1124, 1044, 1017, 905, 830, 759, 703, 634, 559 cm⁻¹; (Fmoc-Cys(II)-OH): 3059, 3027, 2924, 2852, 1728, 1601, 1493, 1451, 1379, 1316, 1246, 1181, 1153, 1116, 1070, 1030, 907, 822, 758, 701, 621, 541 cm⁻¹; (Fmoc-Cys(III)-OH): 3059, 3026, 2925, 2852, 1727, 1603, 1496, 1451, 1379, 1301, 1246, 1181, 1154, 1119, 1068, 1030, 906, 809, 760, 702, 540 cm⁻¹; (Fmoc-Cys(IV)-OH): 3060, 3026, 2925, 2851, 1727, 1605, 1495, 1452, 1381, 1307, 1250, 1211, 1181, 1160, 1118, 1031, 907, 825, 759, 701, 541 cm⁻¹; (Fmoc-Cys(V)-OH): 3059, 3026, 2924, 2851, 1726, 1666, 1603, 1493, 1451, 1316, 1247, 1183, 1066, 1030, 906, 822, 759, 701, 621, 540 cm⁻¹; (Fmoc-Cys(VIII)-OH): 3315, 3060, 3026, 2926, 2851, 1725, 1659, 1604, 1531, 1495, 1451, 1373, 1302, 1252, 1181, 1115, 1032, 906, 822, 759, 701, 540 cm⁻¹.

Determination of loading of Fmoc-Cys(resin II)-OH resin according to Ellman

TFA (10 ml) was continuously dropped on a glass filter holding 19.6 mg Fmoc-Cys(resin II)-OH resin. The filtrate was evaporated in vacuo and 50 ml of 40% DMF/water was added; 1 ml of the resulting solution was added to a glass tube containing 8 ml methanol and 1 ml 0.4 M Tris-HCl (pH 8.9). Then 0.1 ml of a 0.01 M DTNB solution in methanol was added. After 5 min, the absorbance at 412 nm appeared 0.190. A solution containing 1 ml of 40% DMF/water instead of 1 ml diluted filtrate was taken as a reference. The thiol loading was calculated from the following equation:

\[ b = \frac{(1.43 + 70.79 \cdot A_{412})}{2m} \]

with b being the loading (in mmol/g), \( A_{412} \) the measured absorbance (0.190) and m the original mass of the resin sample (19.6 mg). The calculated Fmoc-cysteine loading was 0.38 mmol/g.
Acidolytic cleavage of Fmoc-Cys from trityl-type resins I to VIII (Table 3)
To 40 mg of Fmoc-cysteine-loaded resin, 1 ml of the cleavage mixture was added. After standing for 14 min, the reaction tube was placed in an ultrasonic bath for one additional minute. Then the resin was filtered off and washed with DCM and ether, each twice.

Colorimetric measurement of cationic trityl-type resins I to VIII (Table 5)
The resin was loaded into a for this purpose prepared steel cylinder with a cylindrical hole of 5 mm diameter and 8 mm depth. After addition of a few drops TFA, enough to soak the entire resin sample, a glass slide was placed as a lid, and measurement was performed.

3.7. References


Resin V was developed in collaboration with Prof. Dr. G.I. Tesser, Miss A. van Vieth and Dr. R.H.P.H. Smulders.


Since trityl-type resins based on bulky preformed handles were found less suitable for SPPS (Section 4.2.1), no further attempts were made to purify compounds 3b and 3c.


Actually, the absorbance of the formed yellow 2-nitro-5-mercaptobenzoate is measured at 412 nm. The concentration of the initial thiol (in nmol/ml) is subsequently derived from a calibration line [concentration = 1.43 + 0.70.79 . A_{412}], which was obtained by measuring the absorbances upon reaction of DTNB with different concentrations of cysteine hydrochloride. For further details, see the Experimental section.


The maximum resin capacity b_{max} was calculated from the formula: b_{max} = (1000.b_1)/(1000-MW.b_2) with b_1 being the loading of the original resin (0.7 mmol/g for chloromethylpolystyrene resin and 0.9 mmol/g for aminomethylated resin) and MW the net molar mass increase upon derivatization (in g/mol). Maximum capacities varied from 0.58 mmol/g for trityl-type resin IV to 0.68 mmol/g for trityl-type resin V. For the couplings of Fmoc-cysteine, we took the mean value of 0.65 mmol/g as the maximum capacity.
Synthesis and Evaluation of Trityl-Type Resins

Chapter 4

Synthesis of Protected Peptide Hydrazides on Trityl-Type Resins

4.1. Introduction

4.1.1. Azide method for peptide bond formation

The standard strategy for peptide synthesis proceeding through stepwise acylation of the nascent peptide chain with (ar)alkoxy carbonylamino acids, is a direct result from the necessity of maintaining the chiral stability in the final product [1]. Upon activation of an acyl amino acid, formation of an intermediate 5(4H)-oxazolone or azlactone (Scheme 1) proceeds in competition with the acylation of the amino component. The oxazolone racemizes via its stabilized enolic mesomer, which is achiral. Aminolysis of the oxazolone consequently leads to significant levels of racemization of the introduced residue [2]. In amino acids, whose α-amino function is protected as a urethane (ar)alkoxy carbonyl protection), the oxazolone intermediate is very unlikely to form, due to either the decreased nucleophilicity of the urethane carbonyl, or the lowered acidity of the urethane proton, with regard to the carbonyl or proton of carboxamides. In this case carboxyl activation is permitted [3].

Scheme 1: Mechanism of racemization via intermediate oxazolone formation. L denotes the activating group.
Racemization becomes a serious problem during fragment condensations and cyclizations, which involve activation of the C-terminal (acyl) amino acid. The problem is circumvented when fragments are used with a C-terminal glycine residue, lacking a chiral centre, or a proline residue, which is an amino acid that is not prone to oxazolone formation.

The only other method that has, ever since its discovery by Curtius in 1902 [4], stood out for its absence of racemization was the coupling of peptide azides, whose electrostatic nature prevents attack of the carboxamide carbonyl upon the activated carbonyl and hence oxazolone formation (Fig. 1) [5].

The azide method involves conversion of a peptide hydrazide into the intermediate azide, which is directly applied in the acylation. The classical Curtius procedure for this conversion used sodium nitrite in aqueous acetic acid or hydrogen chloride-acetic acid mixtures. A reinvestigation by Honzl and Rudinger into the suppression of a competing side reaction (i.e. amide formation) resulted in the procedure that is commonly used today [6]. In this approach, conversion of the hydrazide is effected by treatment with anhydrous hydrogen chloride and a nitrite ester (tert-butyl or isoamyl nitrite) in an organic solvent at low temperature, and followed by neutralization with a tertiary amine. Racemization has been observed in some cases and was found to depend on the amount and the nature of the base. The use of sterically hindered amines like diisopropylethylamine (DIPEA) in equimolar amounts has been recommended as such [7]. The azide method thus performed is generally considered the least racemization-prone method of peptide bond formation.

4.1.2. Synthesis of peptide hydrazides

Ester hydrazinolysis

Hydrazides are commonly prepared by hydrazinolysis of the corresponding alkyl esters utilizing an alcohol or dimethylformamide as the solvent (Scheme 2) [8]. C-Terminal peptide esters are accessible through classical solution-phase methods. Alternatively, the C-terminal free acid may be prepared by SPPS and subsequently esterified with diazomethane or by reaction of its cesium salt with an alkyl halide [9,10]. A total SPPS procedure involves the hydrazinolysis of the assembled peptide from a benzyl-type resin. For this purpose chloromethylpolystyrene, its hydroxymethylated analogue, the SASRIN resin, and resins derivatized with a 2-nitro-4-carboxybenzyl-type handle have been employed [11-15]. Hydrazinolysis cleavage procedures from resins containing a phenyl or α-methylphenacyl ester anchoring linkage have also been reported [16,14a].

The use of a large excess of hydrazine in ester hydrazinolysis, necessary to prevent diacylation of the hydrazine and accelerate the reaction, poses limitations to the applicability of the method.
Hydrazinolysis of the arginine side chain to yield ornithine can be prevented by brief treatment with a very large excess of hydrazine [8d]. Incorporation of aspartic acid within a peptide gives rise to multiple problems. Its β-carboxy tert-butyl ester may not only, like the benzyl ester, be readily saponified by base treatment through the N'-succinimide intermediate, but also converted to the hydrazide by hydrazine [17,18]. Moreover, ester hydrazinolysis is not compatible with the Fmoc protecting group or in the presence of disulfides, because of the risk of base-catalyzed β-elimination [19,20].

ester hydrazinolysis: \( R\text{-COO}X + N_2H_4 \)

protected hydrazide: \( R\text{-CONHNH} \cdot Y + H^+ \text{ or } H_2/Pd \) → \( R\text{-CON}_2H_3 \)

carboxyl activation: \( R\text{-COOH} + \text{DCC/HOBt/N}_2H_4 \)

Scheme 2: Approaches applied in the synthesis of peptide hydrazides. \( X = \) alkyl group such as Me or Et, or benzyl-type resin; \( Y = \) protecting group such as Z, Boc or Trt, or immobilized analogue.

Protected-hydrazide approach

Hofmann circumvented the complications associated with hydrazine treatment by developing a totally different approach, in which (solution-phase) peptide synthesis was started with the C-terminal amino acid hydrazide in the \( N' \)-protected form [21]. Numerous applications have been reported employing \( Z, \) Boc or Trt groups as semi-permanent hydrazide protection [22-24]. Since an azide is less reactive with side-chain functional groups such as hydroxyl groups, only the ε-amino groups of incorporated lysines and the thiol groups of cysteines have to be orthogonally protected [25], to permit selective deprotection of the hydrazide before the intended azide coupling [26]. The protected-hydrazide approach allows the racemization-free stepwise synthesis of peptides in C-terminal direction [24a,27]. The solid-phase counterpart of the protected-hydrazide approach employs resins that contain linkers, which are derived from the \( Z, \) Boc, Trt or dibenzocyclohepta-1,4-dienyl moieties (Fig. 2). Wang developed the alkoxy-substituted \( Z \)-type resin as well as two different Boc-type resins, all three of which were applied in Bpoc/Bzl SPPS, eventually yielding the protected peptide hydrazides after treatment of the peptidyl resin with 50% TFA [28-30]. The trisalkoxytrityl and dibenzocyclohepta-1,4-dienyl hydrazide resins were used in Fmoc/\(^4\)Bu SPPS and allowed cleavage conditions of 1% TFA, although cleavage proceeded at a considerably slower rate in the latter case. Both of these systems revealed major drawbacks and require refinement. The trityl-type resin (IV, cf. Section 3.2.1) gave a rather low initial loading and showed a steady decline in loading during SPPS due to the
instability of the tritylhydrazide bond to TBTU coupling conditions [31]. The initial acylation in the dibenzocyclohepta-1,4-dienyl system also proceeded sluggishly, due to the steric hindrance of the adjacent NHBoc substituent, and higher TFA concentrations were recommended to improve cleavage yields [32].

\[ \text{Z(OAlkyl) type} \]
\[ \text{Boc type I} \]
\[ \text{Boc type II} \]
\[ \text{Trt type} \]

\[ \text{dibenzocyclohepta-1,4-dienyl type} \]

**Figure 2:** "Hydrazide resins" designed for the solid-phase synthesis of protected peptide hydrazides.
Carboxyl activation

A third method for the formation of peptide hydrazides consists of the direct carbodiimide-mediated coupling of the free C-terminus with hydrazine in the presence of 1-hydroxybenzotriazole [33]. Wang and co-workers converted several protected peptides in this way in 65 to 77% yield without detectable racemization [34].

4.1.3. Outline of the investigation

The most elegant route for the solid-phase synthesis of protected peptide hydrazides is beyond any doubt the direct synthesis on “hydrazide resins”, avoiding the harsh conditions of hydrazinolysis or the extra DCC/HOBt activation step with concomitant risk of racemization. However, no satisfactory Fmoc SPPS protocol involving a “hydrazide resin” was available hitherto. We started an investigation into peptide hydrazide SPPS on trityl-type resins, in which the stability of the tritylhydrazide bond was enhanced with respect to the aforementioned trisalkoxytrityl system due to a lower degree of substitution. Preliminary accounts on the use of trityl-type resin V for this purpose have been presented at various symposia over the past few years [35]. However, since yields proved to be rather disappointing, several of the other trityl-type resins, listed in Chapter 3, were also tested and both their stabilities during the actual SPPS as well as their cleavage behaviour were compared.

A general protocol was developed for the synthesis of protected peptide hydrazides on the two elected trityl-type resins I and II, in which an Fmoc-amino acid hydrazide is coupled to the resin as a first step in synthesis. In this manner, the sluggish acylation and decomposition of the tritylhydrazine adduct, which are the major causes of impure products and low initial loadings, are circumvented.

Three relaxin fragments, i.e. A(1-8), B(12-16) and B(24-31), were synthesized as their protected hydrazides according to the presented method. The validity of our approach was ascertained by resynthesizing the first of these fragments as a reference after the conventional hydrazinolytic method, and by actually applying the B(24-31) fragment in an azide coupling.

4.2. Development of a general protocol for hydrazide SPPS

4.2.1. Synthesis of peptide hydrazides on trityl-type resin V

Our initial studies regarding peptide hydrazide SPPS concentrated on trityl-type resin V. A number of model peptides were synthesized with qualitatively satisfactory results [36]. A thorough investigation
focussing on the quantitative aspects of such syntheses had, however, remained untouched and was now started with the syntheses of the following three relaxin fragments on the topical resin:

\[
\begin{align*}
A(2-8) & \quad \text{Fmoc-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-N}_2\text{H}_2\text{-resin V} \\
A(1-8) & \quad \text{Boc-Gln(Trt)-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-N}_2\text{H}_2\text{-resin V} \\
B(24-31) & \quad \text{Fmoc-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(Trt)-N}_2\text{H}_2\text{-resin V}
\end{align*}
\]

(1) (2) (3)

During the syntheses, two shortcomings in the actual protocol were identified, i.e. the instability of the tritylhydrazine adduct on the one hand and the instability of trityl-type resin V on the other hand.

Conversion of the trityl-type resin into the active chloride form by treatment with thionyl chloride is the first step in the preparation of peptide hydrazides. The most logical and generally applicable sequel at this point consists of the coupling of Fmoc-hydrazide to the resin, as was performed in the synthesis of fragment 1 in quantitative yield, based on the capacity of 0.47 mmol/g. After deprotection of the Fmoc group and coupling of the first Fmoc-amino acid (Fmoc-Asn(Trt)-OH) the loading had dropped by approximately 50% (Table 1). The cause for this decrease, decomposition of the tritylhydrazine adduct, will be discussed in Section 4.2.3. Quantitative amino acid loading of the resin was achieved by the attachment of preformed Fmoc-amino acid hydrazides, whose preparation is described in Chapter 8 of this thesis. This approach was applied in the synthesis of fragments 2 and 3. Coupling proceeded quantitatively in pyridine or a pyridine/DMF (3:1) mixture, with two molar equivalents of the Fmoc-amino acid hydrazide in the presence of one equivalent of DIPEA, within four hours. Addition of DMF was sometimes necessary to increase the solubility of the hydrazide. The active chloride that remained unreacted was destroyed by the addition of methanol and an excess of DIPEA, thus transforming the trityl chloride into the corresponding trityl methyl ether.

\begin{table}[h]
\centering
\begin{tabular}{lrrrr}
\hline
Peptidyl resin & Initial loading & Coupling yield (%) \\
 & (mmol/g) & (%) & overall & per cycle \\
\hline
1 & 0.21 & 51 & 57 & 91.1 \\
2 & 0.38 & quant. & 64 & 92.8 \\
3 & 0.39 & quant. & 34 & 85.7 \\
\hline
\end{tabular}
\caption{Results of hydrazide SPPS on trityl-type resin V.}
\end{table}

\textsuperscript{a} Fmoc-Asn(Trt) loading. \textsuperscript{b} Based on penultimate coupling.

SPPS proceeded after the standard TBTU/HOBt/NMM protocol. The overall coupling yields were determined by comparison of the Fmoc loading in the assembled resin-bound peptides with the initial
loading. Fmoc determinations in each cycle of the performed syntheses showed, in all three cases, a steady decline in Fmoc loading on the resin of about 10% per cycle. Analyses of the final products discarded premature capping as a possible reason. This observation is therefore in full agreement with the theory proposed in Chapter 3, about the partially adsorptive nature of the resin-linker adduct in trityl-type resins V, VI and VII, causing loss of peptide material from the resin during SPPS. A similar decrease in peptide loading was noted during the syntheses of peptides anchored through a thiol function to resin V (Section 6.2.3).

4.2.2. Evaluation of various trityl-type resins for hydrazide SPPS

The instability of trityl-type resin V prompted us to search for more suitable resins for hydrazide SPPS [37]. The model peptide Fmoc-Phe-Ala-Asn(Trt)-N₂H₃ was synthesized on several trityl-type resins, starting with the attachment of Fmoc-Asn(Trt)-N₂H₃. A bulky residue was chosen for anchoring to preclude speculations about non-compatibility of trityl-type resins due to steric hindrance [38].

| Resin | Substituents | Initial loading (mmol/g) | SPPS yield 2 cycles (%) | % Cleavage
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<tbody>
<tr>
<td>I</td>
<td>Cl / R</td>
<td>0.39</td>
<td>96</td>
<td>18 34 71 33</td>
</tr>
<tr>
<td>V</td>
<td>OR</td>
<td>0.38</td>
<td>83</td>
<td>36 49 74 36</td>
</tr>
<tr>
<td>VIII</td>
<td>2xOR / CA</td>
<td>0.18</td>
<td>quant.</td>
<td>65 76 80 54</td>
</tr>
<tr>
<td>VI</td>
<td>2xOR</td>
<td>0.20</td>
<td>73</td>
<td>– – – –</td>
</tr>
<tr>
<td>II</td>
<td>OB</td>
<td>0.33</td>
<td>quant.</td>
<td>74 88 95 20</td>
</tr>
<tr>
<td>VII</td>
<td>3xOR</td>
<td>0.16</td>
<td>44</td>
<td>– – – –</td>
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<tr>
<th>A</th>
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\(^a\) R = alkyl; OR = alkoxy; CA = carboxamide; OB = benzoxy. \(^b\) A: 1x45 min 0.5% TFA 5% MeOH/DCM; B: 1x45 min 1% TFA/5% MeOH/DCM; C: 4x15 min 1% TFA/5% MeOH/DCM; D: 1x45 min AcOH/TFE/DCM (2:2:6).

The initial loadings of Fmoc-Asn(Trt)-N₂H₃ were satisfactory for resins I (36%), II (88% [39]) and V; resins VI, VII and VIII showed rather low values (Table 2). The solid-phase coupling yields were (nearly) quantitative for resins I, II and VIII, as opposed to those determined for resins V, VI and VII. This finding presents the final confirmation for the partially adsorptive loading of the linker in case of the last mentioned group of resins. Instability of the tritylhydrazide adduct towards TBTU coupling
conditions, as in the case of the trisalkoxytrityl system (Fig. 2), can now be ruled out for resin V, since no loss of peptide material was observed with the far more labile adducts of resin II. Both factors contribute to the exceedingly great losses of peptide material in case of resin VII.

Cleavage experiments on the peptidyl resins yielded the same series of increasing acid labilities as observed for the Fmoc-cysteine-resin adducts (Chapter 3), ignoring the remarkable stability of the hydrazide-resin II bond towards acetic acid, for which we have no explanation at present. Repeated TFA treatments are preferred to a continuous cleavage. This procedure has been recommended for cleavage of protected fragments from SASRIN resin, and is especially advantageous, since each portion of cleavage mixture may be neutralized after a short period of time, thus reducing the time of exposure of the product to the acidic medium [40]. Side-chain deprotection of very acid-sensitive residues like Lys(Boc) and Tyr(Bu) is minimized by this approach.

The influence of scavengers during the cleavage reaction was also examined. Both the resin I and resin II peptidyl adducts were treated during 45 minutes with a 1% TFA mixture, containing either 5% triethylsilane, methanol or no scavenger. Residual loadings corresponded to less than 25% in all cases, except the cleavage of the resin I adduct in the presence of methanol, which gave a high value of 66%. Addition of a scavenger obviously has no influence in case of the very labile adducts of resin II. During the more sluggish cleavage from resin I, triethylsilane has a slightly accelerating effect, whereas methanol reduces acidity and thus the cleavage rate to a large extent.

All products originating from the cleavage experiments were chromatographically the same and pure in two solvent systems.

### 4.2.3. Problems associated with the tritylhydrazine adduct: attachment of Fmoc-N₂H₃

The model peptide Fmoc-Phe-Ala-Asn(Trt)-N₂H₃ was also synthesized on trityl-type resins I and II by initial attachment of Fmoc-N₂H₃ [41]. After removal of the Fmoc group and coupling of Fmoc-Asn(Trt)-OH, the Fmoc loading had decreased by 14 and 41%, respectively. In a similar synthesis of the relaxin B(12-16) fragment Fmoc-Arg(Pmc)-Glu(O'Bu)-Leu-Val-Arg(Pmc)-N₂H₃ on resin I, the Fmoc loading had dropped by 22%. A 49% decrease was measured in case of the above described synthesis of fragment A(2-8) on resin V (Section 4.2.1, compound 1).

A second irregularity was found when analyzing the amino acid composition of the two resin I products:

- Fmoc-Phe-Ala-Asn(Trt)-N₂H₃ : Asp 0.83. Ala 0.79, Phe 1.00 and
- Fmoc-Arg(Pmc)-Glu(O'Bu)-Leu-Val-Arg(Pmc)-N₂H₃ : Glu 1.00, Val 0.94, Leu 0.92, Arg 1.70.

The results indicate a sluggish acylation of the tritylhydrazine adduct. Following the course of this reaction by Kaiser tests is hampered by the orange instead of blue colour of the resin beads at the tritylhydrazine stage, which makes it very difficult to estimate completion of the reaction. i.e.
disappearance of the colour in a Kaiser test. Incomplete acylation by the first amino acid would allow acylation of the tritylhydrazine to occur in a later cycle during the SPPS, thus accounting for the increased content of the "later" amino acids phenylalanine and glutamic acid.

In addition, the steep decrease in Fmoc loading after coupling of the first Fmoc-amino acid to resins II and V suggests a decomposition of the tritylhydrazine adduct in systems bearing electron-donating substituents; formation of an intermediate positive charge on the trityl centre is obviously part of its reaction mechanism. Accordingly, no by-product formation was observed using Fmoc-N₂H₂-Trt as a non-substituted model compound in a similar reaction sequence (Fmoc deprotection/coupling of Fmoc-Gly-OH).

4.2.4. General discussion

The solid-phase synthesis of peptide hydrazides on trityl-type resins is evidently accompanied by a number of complications due to either instability of the trityl-type resin (1), the tritylhydrazide bond (2), or the tritylhydrazine adduct (3), sluggish acylation of the hydrazine adduct (4) or combinations of these. In developing a general procedure for the synthesis of peptide hydrazides, all these factors had to be taken into account. A brief summary of the problems and their solutions is now presented, leading to the protocol applied in the next section.

Ad 1.) The partially adsorptive nature of the resin-linker adduct in resins derived with a preformed bulky trityl-type handle (V, VI and VII) was first suggested by the colouration of the solution, when these resins were treated with trifluoroacetic acid. Further evidence came from the strong decrease in Fmoc loading after each cycle of the SPPS of peptide hydrazides and thioles, which was indicative of the loss of peptide material, since premature capping was excluded by the correct amino acid composition of the products. Moreover, loss of peptide material due to instability of the tritylhydrazide bond towards TBTU coupling conditions could be ruled out on the basis of quantitative couplings using a more labile trityl-type resin (II). The obviously inherent instability of the resins V, VI and VII makes this kind of resins less suitable for peptide hydrazide SPPS and indeed any SPPS.

Ad 2.) Trisalkoxytrityl-type resins (IV and VII) display a too high lability in their linkage to hydrazides to be compatible with TBTU/HOBt-mediated SPPS. These resins are ultimately designed for the synthesis of protected peptide amides [31,42].

Ad 3-4.) Decomposition of the tritylhydrazine adduct leads to low initial loadings and is encountered in systems, that stabilize the intermediate positive charge on the trityl centre through electron-releasing substituents (trityl-type resins II and V). Sluggish acylation of the tritylhydrazine adduct, resulting in impure products, is consequently more prominent in less labile systems (resin I). Both
complications are circumvented, when the synthesis of peptide hydrazides on trityl-type resins starts with the coupling of the first Fmoc-amino acid as a hydrazone rather than Fmoc-hydrazone. It is important to note that according to our experience the commercially available hydrazine 2-chlorotrityl resin, although kept strictly under argon, is very difficult to load [43].

4.3. Synthesis of three relaxin fragments as their hydrazides

4.3.1. Synthesis of the fragments on trityl-type resins

On the basis of the aforementioned considerations, trityl-type resins I and II were selected as the most suitable resins for the solid-phase synthesis of peptide hydrazides. Their applicability in SPPS will also be demonstrated with the anchoring through amino and thiol functions, as will be reported in the next two chapters. Three fragments of the rhesus monkey relaxin were synthesized as their protected hydrazides, A(1-8) on resin II and B(12-16) and B(24-31) on resin I:

\begin{align*}
A(1-8) & \quad \text{Boc-Gln(Trt)-Leu-Tyr(}^\text{Bu}\text{-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-N}_2\text{H}_3 \\
B(12-16) & \quad \text{Fmoc-Arg(Pmc)-Glu(}^\text{OBU}\text{-Leu-Val-Arg(Pmc)-N}_2\text{H}_3 \\
B(24-31) & \quad \text{Fmoc-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-N}_2\text{H}_3
\end{align*}

(4) (5) (6a)

Syntheses started with the coupling of the first Fmoc-amino acid hydrazide to the chloride form of the actual resin (1 g each) in the presence of DIPEA. In case of fragment 6a, side-chain protected Fmoc-Ser(Trt)-N₂H₃ was used to prevent attachment via the hydroxy function, although in retrospect this precaution appeared to be unnecessary due to the low tendency of this function to bind to the trityl moiety [44]. The trityl protecting group was for the greater part removed during the cleavage from the resin.

Fmoc-Arg(Pmc)-N₂H₃ was required in the synthesis of fragment 5. The preparation of hydrazides of Fmoc-arginine derivatives was accompanied by a complicating side reaction. DCC-mediated activation of both Fmoc-Arg(Pmc)-OH and Fmoc-Arg(Mtr)-OH in the presence of HOSt or HOBT led to formation of the corresponding δ-lactam (Chapter 8) [45]. The contamination could not be removed by recrystallization of the product. However, since the δ-lactam structure lacks the nucleophilic hydrazide function (Fig. 3), purification was quasi effected by

![Figure 3: δ-Lactam derived from Fmoc-Arg(Pmc).](image-url)
coupling the crude product to the resin, yielding a resin loaded with the pure Fmoc-Arg(Pmc)-N$_2$H$_3$ in this case, as judged by thin layer chromatography upon cleavage from a resin sample. Solid-phase syntheses were performed after the standard TBTU/HOBt/NMM protocol. Cleavage was effected by treatment of the peptidyl resins with a 1% TFA/5% TES/DCM mixture. After each 15-minute period, the cleavage mixture was neutralized with pyridine and fresh mixture was added to the resin. Completion of the cleavage was determined chromatographically. In case of fragment 4, no peptide material was detected in the cleavage mixture after two treatments, whereas resin I (fragments 5 and 6a) required six treatments.

**Table 3: Quantitative evaluation regarding the synthesis of three relaxin fragment hydrazides on trityl-type resins I and II.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Resin</th>
<th>Initial loading</th>
<th>SPPS yield (%)</th>
<th>Overall yield$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(mmol/g)</td>
<td>(%)</td>
<td>overall</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>0.33</td>
<td>65 / 88$^b$</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>0.35</td>
<td>32</td>
<td>96</td>
</tr>
<tr>
<td>6a</td>
<td>I</td>
<td>0.35$^a$</td>
<td>31</td>
<td>86</td>
</tr>
</tbody>
</table>

$^a$ Loading of Fmoc-Ser(Tri)-N$_2$H$_3$.  
$^b$ The first value is based on the capacity of the original chloromethyl-polystyrene, the second on the assumed derivatization yield of resin II (74%, concluded from the maximum Fmoc-cysteine loading (Section 6.2.1)).  
$^c$ Based on initial loading.

Initial loadings, solid-phase coupling yields and overall product yields, as listed in Table 3, were quite satisfactory in all cases. The somewhat lower overall yield of fragment 4 is attributable to the tedious work-up of the sparingly soluble product. Data obtained from amino acid analysis and mass spectrometry were in accordance with the desired products. The presence of the hydrazide functions was confirmed by the development of blue spots after spraying thin layer chromatograms of the products with modified Barton’s reagent [46].

### 4.3.2. Further evaluation of the products

The relaxin A(1-8) hydrazide fragment was chosen for synthesis via a conventional method, to serve as a reference for the product synthesized on a trityl-type resin (Scheme 4). The corresponding C-terminal peptide acid 7a was synthesized on trityl-type resin I according to the standard procedures described in Chapter 2 (TBTU protocol). After quantitative conversion into the methyl ester 7b by treatment with diazomethane, hydrazinolysis followed using DMF as a solvent. Methanol and dioxane were not applicable in this respect due to low solubility of the ester 7b. The N-terminal Boc group
and pertinent (protected) side chains are compatible with ester hydrazinolysis. The hydrazide 7c was obtained in 58% overall yield, based on the initial loading of Fmoc-Asn(Trt)-OH (0.43 mmol/g). This corresponds to roughly the same molar yield/g resin as achieved in the total solid-phase approach. Analytical data sustain the equality of the products 4 and 7c and hence both methods.

\[ \text{Boc-Gln(Trt)-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-OH} \quad 7a \quad \rightarrow \]

\[ \text{Boc-A(1-8)-OMe} \quad 7b \quad \rightarrow \quad \text{Boc-A(1-8)-N}_2\text{H}_3 \quad 7c \]

**Scheme 4:** Synthesis of relaxin A(1-8) hydrazide fragment by combined solid and solution-phase procedures. i) diazomethane; ii) \( \text{N}_2\text{H}_2\text{H}_2\text{O/DMF} \), A(1-8) = Gln(Trt)-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trt).

Product 6a was applied in an azide coupling to yield fragment B(24-32) as the C-terminal tert-butyl ester (Scheme 5). Hydrazide 6a was transformed into the activated azide 6b by treatment with tert-butyl nitrite in the presence of hydrogen chloride. The reaction was performed at -30°C to minimize side reactions and preserve side-chain protecting groups. After neutralization with DIPEA, leucine tert-butyl ester was added for the coupling reaction.

\[ \text{Fmoc-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-N}_2\text{H}_3 \quad 6a \quad \rightarrow \]

\[ \text{Fmoc-B(24-31)-N}_3 \quad 6b \quad \rightarrow \quad \text{Fmoc-B(24-31)-Leu-O}^1\text{Bu} \quad 6c \quad \rightarrow \quad \text{H-B(24-31)-Leu-O}^1\text{Bu} \quad 6d \]

**Scheme 5:** Synthesis of relaxin B(24-32) fragment via azide coupling: i) tert-butyl nitrite, HCl; ii) DIPEA, H-Leu-O(Bu),AcOH; iii) 1% TFA/5% TES/DCM; iv) piperidine/DMF (1:4). B(24-31) = Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H).

The resulting nonapeptide still contained some trityl contaminant originating from the Fmoc-Ser(Trt)-N\(_2\)H\(_3\), that was used for resin attachment. Normally, this would have been removed by repeated treatments with 1% TFA in the presence of triethylsilane [47]. During the cleavage from the resin, however, the first portion of cleavage mixture containing most of the product was immediately neutralized; some trityl residues evidently escaped acidolysis. Three subsequent treatments with the cleavage mixture at this stage, followed by Fmoc removal with piperidine, yielded the pure fragment 6d in 78% overall yield, based on starting hydrazide 6a. Amino acid analysis of the final product confirmed the presence of the second leucine residue. This model synthesis demonstrates that peptide hydrazides obtained by direct synthesis on a trityl-type resin are suitable for use in azide couplings without further purification.
4.4. Conclusions

Trityl-type resins I and II proved well-suited for the high-yield synthesis of protected peptide hydrazides by a total solid-phase approach. This way, hydrazinolysis of a peptide ester or activation of a peptide acid are avoided. Synthesis should start with the attachment of an Fmoc-amino acid hydrazide to the actual resin in its chloride form. The preparation of the hydrazides of the relevant amino acids is described in Chapter 8 of this thesis. The stable tritylhydrazide adduct can then be used for further assembly of the peptide according to conventional SPPS procedures. Due to instability of the resin or the tritylhydrazide bond, resins derivatized with a preformed bulky trityl linker (V, VI and VII) and trisalkoxytrityl-type resins (IV and VII) were found less suitable for peptide hydrazide synthesis. The completed peptide hydrazides are cleaved from resins I or II by repeated treatments with 1% TFA, yielding sufficiently pure products to be directly applied in azide couplings.

4.5. Experimental section

Materials and methods
Modified Barton's reagent was freshly prepared by mixing equal volumes of 15% FeCl₃ and 1% K₃Fe(CN)₆ solutions in water, and used for the detection of hydrazides on TLC. For further specifications, see Appendix A.

Conversion of trityl-type resins into the chloride form
The resin in its hydroxide form was shaken for 5 min with a 15% solution of thionyl chloride in DCM and subsequently washed with DCM containing 2% trimethylsilyl chloride. This procedure was repeated four times. The resin was additionally washed twice with the trimethylsilyl chloride solution and immediately used for coupling.

Coupling of Fmoc-hydrazide or Fmoc-amino acid hydrazide to trityl-type resins
The trityl-type resin in its chloride form was shaken in pyridine (1g resin/8 ml pyridine) during 4 h in the presence of 2 and 1 meq of the pertinent Fmoc-amino acid hydrazide and DIPEA, respectively. Half of these equivalent quantities were taken with trityl-type resin I; in case of Fmoc-N₂H₂ and Fmoc-Asn(Trt)-N₂H₂ a mixture of pyridine/DMF (3:1) was used. The resin was filtered and, upon addition of a DCM/MeOH (1:1) solution containing 250 μl DIPEA, shaken for an additional 15 min. The resin was then washed five times with DMF, twice with 2-propanol and ether each, and dried.

Fmoc-N₂H₂-Trt
Fmoc-N₂H₂ (0.50 g, 2 mmol, preparation described in Chapter 8), Trt-Cl (0.55 g, 2 mmol) and DIPEA (0.34 ml, 2 mmol) were dissolved in 20 ml DCM. After stirring for 1 h at room temperature, the solution was washed with saturated NaCl (4x15 ml), dried over Na₂SO₄ and evaporated in vacuo, yielding 0.88 g (89%) of a white amorphous product. Mp 188-189°C. TLC: Rₜ (D) 0.79.


Cleavage experiments (Table 2)  
To 40 mg of peptidyl resin, 1 ml of the cleavage mixture was added. The resin was either kept in the same solution for the given period of time, or the resin was filtered every 15 min and the solution renewed. In both cases, after each 14-min period, the reaction tube was placed in an ultrasonic bath for one minute. The resin was then filtered and washed with DCM and ether, each twice. Cleavage yields were calculated from comparison of the Fmoc loadings before and after cleavage (Appendix B).

Analytical data for Fmoc-Phe-Ala-Asn(Trrt)-N$_2$H$_3$ synthesized on trityl-type resin II, using Fmoc-Asn(Trrt)-N$_2$H$_3$:  
TLC: R$_f$ (A) 0.63, R$_f$ (B) 0.49. [α]$_D$ $^{25} -5.1$ (c=0.5, DMF). Amino acid analysis: Asp 1.00 (1), Ala 1.00 (1), Phe 0.97 (1).

Cleavage of protected peptide hydrazides from trityl-type resins  
Typically, 1 g of peptidyl resin was suspended in 20 ml of 1% TFA/5% TES/DCM. After 15 min, the resin was filtered and the filtrate collected in a vessel containing pyridine (1.1 ml in case of trityl-type resin II, 2 ml in case of resin I). The procedure was repeated once for resin II and five times for resin I, until no more peptide material was detected in the cleavage mixture (TLC). The resin was additionally extracted with the cleavage mixture (2×20 ml) and DCM (2×20 ml). The combined filtrates were washed with 10% KHSO$_4$ (2×40 ml) and saturated NaCl (4×40 ml), dried over Na$_2$SO$_4$ and evaporated in vacuo.

Peptidyl resins 2 and 3, cleavage products  
Analytical amounts of peptide material were cleaved from peptidyl resins 2 and 3 according to the above described procedure. TLC values for the products were in agreement with the ones found for 4 and 6a, respectively. [α]$_D$ $^{25} -6.2$ (c=1, DMF). Amino acid analyses:

2 Asp 1.04 (1), Thr 0.94 (1), Ser 0.83 (1), Glu 0.99 (1), Met 0.64 (1), Leu 2.00 (2), Tyr 0.86 (1);  
3 Thr 0.86 (1), Ser 1.70 (2), Gly 1.02 (1), Leu 1.00 (1), Lys 2.00 (2), Arg 1.00 (1).

Boc-Gln(Trrt)-Leu-Tyr(Bu)-Met-Thr(H)-Leu-Ser(H)-Asn(Trrt)-N$_2$H$_3$ (4)  
Yield: 0.64 g. TLC: R$_f$ (A) 0.59, R$_f$ (B) 0.25. [α]$_D$ $^{25} -10.8$ (c=1, DMF). SIMS (+), NBA: m/z 1780.1 [M+NBA]$^+$, 1646.1 [M+Na]$^+$; SIMS (-), NBA: m/z 1755.7 [M+Cs]$^+$. Amino acid analysis: Asp 1.00 (1), Thr 0.92 (1), Ser 0.78 (1), Glu 1.03 (1), Met 1.00 (1), Leu 1.96 (2), Tyr 1.00 (1).

Fmoc-Arg(Pmc)-Glu(OBu)-Leu-Val-Arg(Pmc)-N$_2$H$_3$ (5)  
Yield: 0.66 g. TLC: R$_f$ (A) 0.58, R$_f$ (B) 0.32. [α]$_D$ $^{25} -7.0$ (c=1, DMF). SIMS (+), DTE/DTT/sulfolane: m/z 1497.2 [M+H]$^+$, 1275.1 [M-Fmoc]$^+$, 1231.0 [M-Pmc]$^+$, 1008.8 [M-Fmoc-Pmc]$^-$. SIMS (-), DTE/DTT/sulfolane: m/z 1272.6 [M-Fmoc]. Amino acid analysis: Glu 1.00 (1), Val 1.00 (1), Leu 1.00 (1), Arg 1.96 (2).

Fmoc-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-N$_2$H$_3$ (6a)  
Yield: 0.61 g. TLC: R$_f$ (A) 0.30, R$_f$ (B) 0.11. [α]$_D$ $^{25} -6.1$ (c=1, DMF). SIMS (+), DTE/DTT/sulfolane: m/z 1601.2 [M+Na]$^+$, 1579.2 [M+H]$^+$, 1379.0 [M-2Boc]$^+$, 1312.9 [M-Pmc]$^+$. Amino acid analysis: Thr 0.87 (1), Ser 1.64 (2), Gly 1.03 (1), Leu 1.00 (1), Lys 1.92 (2), Arg 1.00 (1).
Fnoc-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-Leu-O\textsubscript{Bu} (6c)

A solution of compound 6a (0.40 g, 0.25 mmol) in 2.5 ml DMF was cooled to -30°C and 4 M HCl in dioxane (0.17 ml) was added with stirring, followed by terti-butyl nitrite (33 µl, 0.30 mmol). The solution was stirred for 45 min at this temperature and then neutralized with DIPEA (0.13 ml) to an apparent pH 7 (moist pH paper). To the azide 6b, leucine terti-butyl ester acetate (0.31 g, 1.25 mmol) was added and the reaction mixture stored at 0°C. After three days the pH was readjusted to 7 with NMM. After another three days 25 ml water was added and the precipitate collected by centrifugation. Three washings with water, using centrifugation, afforded a product that was slightly positive for trityl groups when spotted on a TLC plate. It was therefore dissolved in 5 ml 1% TFA/5% TES/DCM and after 5 min precipitated with petroleum ether. This procedure was repeated twice and the resulting product was additionally washed twice with petroleum ether. Yield: 0.36 g (82%). Since product 6c was quite insoluble, no chromatographic data are available; analysis took place at the next stage (6d).

H-Lys(Boc)-Ser(H)-Thr(H)-Leu-Gly-Lys(Boc)-Arg(Pmc)-Ser(H)-Leu-O\textsubscript{Bu} (6d)

Compound 6c (0.26 g, 0.15 mmol) was dissolved in 5 ml piperidine/DMF (1:1). After standing for 10 min, the product was precipitated by addition of 25 ml petroleum ether and washed three times with ether, affording 6d as a white solid in 95% yield (0.22 g). TLC: R\textsubscript{f} (A) 0.19, [\alpha]\textsubscript{D}\textsuperscript{25} -10.7 (c=1, DMF). Amino acid analysis: Thr 0.92 (1), Ser 1.64 (2), Gly 1.00 (1), Leu 2.00 (2), Lys 1.98 (2), Arg 1.01 (1).

Boc-Gln(Trt)-Leu-Tyr\textsubscript{Bu}-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-OH (7a)

Fragment 7a was prepared on trityl-type resin I (1.0 g) after standard methods described in Chapter 2. Yield: 0.80 g (0.5 mmol, 88% based on initial Fmoc-Asn(Trt)-loaded trityl-type resin I). TLC: R\textsubscript{f} (A) 0.31, R\textsubscript{f} (B) 0.17. [\alpha]\textsubscript{D}\textsuperscript{25} -11.4 (c=1, DMF). Amino acid analysis: Asp 1.00 (1), Thr 0.91 (1), Ser 0.73 (1), Glu 1.02 (1), Met 0.95 (1), Leu 2.00 (2), Tyr 0.97 (1).

Boc-Gln(Trt)-Leu-Tyr\textsubscript{Bu}-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-OMe (7b)

To a solution of 7a (0.70 g, 0.44 mmol) in 30 ml CHCl\textsubscript{3}/MeOH (1:1), a 0.3 M solution of diazomethane in ether was added dropwise until the yellow colour of the solution remained. After additional stirring for 10 min, the solution was evaporated in vacuo. Yield 0.74 g (100%). TLC: R\textsubscript{f} (A) 0.65, R\textsubscript{f} (B) 0.48. [\alpha]\textsubscript{D}\textsuperscript{25} -10.2 (c=1, DMF).

Boc-Gln(Trt)-Leu-Tyr\textsubscript{Bu}-Met-Thr(H)-Leu-Ser(H)-Asn(Trt)-N\textsubscript{2}H\textsubscript{2} (7c)

Peptide ester 7b (0.40 g, 0.25 mmol) was dissolved in 2 ml DMF and 7 µl hydrazine hydrate (1.5 mmol) was added. While stirred overnight a colourless gelatinous aggregate was formed, which dissolved after the addition of another 2 ml DMF. After stirring for another 24 h, the reaction mixture was poured into 25 ml DCM and the diluted solution was washed with saturated NaCl (4x10 ml). To enhance the solubility of the product in the organic phase, MeOH (3 ml) was added during the washings. The organic phase was subsequently dried over Na\textsubscript{2}SO\textsubscript{4} and evaporated in vacuo. The resulting oil crystallized from DCM/petroleum ether and was collected by centrifugation as a white solid. Yield: 0.26 g (66%). TLC: R\textsubscript{f} (A) 0.59, R\textsubscript{f} (B) 0.25. [\alpha]\textsubscript{D}\textsuperscript{25} -10.0 (c=1, DMF). MALDI-TOF-MS: major peak at m/z 1645.5 [M+Na]\textsuperscript{+}.
4.6. References


15. SASRIN resin is depicted in Figure 3/Chapter 1; a 2-nitro-4-carboxybenzyl-type linker (ONb) is listed in Table 1/Chapter 2.


25 Selectivity through graduated acid lability is also allowed.


35 See reference 116 in Chapter 1.


In fact, this finding initiated the synthesis of the other trityl-type resins described in the previous chapter. Chronologically, the current chapter should therefore be situated before Chapter 3.


See footnote b to Table 3.


The coupling of Fmoc-N₂H₂ to trityl-type resin II resulted in a quantitative loading of 0.42 mmol/g, which corresponds to 75% based on the original chloromethylpolystyrene.


See Section 5.3.1.

A comparative investigation into the extent of this side reaction using various activation methods has been reported recently [Cezari, M.H.S. and Juliano, L. (1996) Peptide Res. 9, 88-91].


Chapter 5

Synthesis of Amino Compounds on Trityl-Type Resins

On-resin head-to-tail cyclization

5.1. Introduction

In standard SPPS the growing peptide chain is anchored to the solid support via its C-terminus. Lengthening of the peptide proceeds in N-terminal direction through stepwise addition of protected amino acids, or protected fragments in case of the convergent variant (CSPPS). The anchoring of amino acids or peptides through a side chain allows modification of both backbone termini. Strategies thus come within reach for the direct solid-phase synthesis of peptides with a modified C-terminus, for two-directional SPPS as well as on-resin head-to-tail cyclizations. Applications of these strategies are reported in this and the next chapter.

5.1.1. Synthesis of peptides with modified C-termini

Synthesis of C-terminally modified peptide derivatives via classical methods

Several kinds of C-terminal modifications like esterification, amidation or reduction to an alcohol belong to the peptide chemist’s tool kit. Whereas C-terminal primary amides are often encountered in natural products such as hormones and neuropeptides [1], other modifications are primarily of methodical importance. The ester function is commonly applied as a protecting moiety, which is to be removed or transformed at a later stage during synthesis [2]. Introduction of an alcohol, alkylamide or ω-aminoalkylamide group at the C-terminal end of a biologically active peptide improved, in numerous cases, metabolic stability as well as pharmacokinetic properties [3-5]. Peptide-(ω-aminoalkyl)amides can also be prepared as epitopes for C-terminal conjugation. Methyl amide protection has been employed to prevent introduction of a negative charge at the C-terminus of a peptide, yet retain solubility in aqueous media [6].

Strategies leading to the C-terminal modifications are merely variants on the three global methods described in the previous chapter for the synthesis of peptide hydrazides, i.e. conversion of esters (a),
direct incorporation of the function in a protected form (b) and conversion of the carboxyl moiety (c), and they are consequently associated with the same problems.

a) Conversion of esters
The ammonolysis of peptide alkyl esters is the eldest method for the preparation of peptide amides [7]. As a synthetic method it is suffering from serious limitations, just like its solid-phase analogue, i.e. the nucleophilic cleavage* of peptides esterified to benzyl-type resins [8]. The strongly basic conditions, that are required to convert the unactivated ester function, may give rise to a number of deleterious side reactions including transesterification, if an alcohol is used as the solvent, partial racemization at the C-terminal residue, α→ω rearrangement at aspartyl and glutamyl residues and β-elimination of protected derivatives of serine, threonine and cysteine; in case of cystine, β-elimination leads to scrambling of disulfide bonds.

Several analogues of oxytocin and luteinizing hormone releasing hormone (LH-RH) have been cleaved from Merrifield’s resin by the action of primary and secondary amines [9,4]. Moreover, a series of publications by Mergler and Nyfeler describes the various modes of cleavage from SASRIN resin, which include, besides acidolysis (as the peptide acid) and hydrazinolysis (hydrazide), cleavage by aminolysis (alkylamine or (ω-aminoalkyl)amide), transesterification (ester) or reduction (alcohol) [10-14].

b) Direct incorporation
Alcohol, ester and amide functions require no additional protection to be directly incorporated in a stepwise synthesis scheme, since as such they display no nucleophilic character [3,15,16]. Unsymmetrically protected diaminoalkanes are, however, required in the synthesis of peptide-(ω-aminoalkyl)amides [5,17].

The solid-phase variant has been most successfully investigated for the synthesis of primary peptide amides on substituted benzyl-, benzhydryl-, trityl- and xanthylamine-type resins [18-21]. C-Terminal peptide alcohols have been prepared on Barlos’s trityl-type resin I, peptide alkenamides on the corresponding aminoalkylated methylpolystyrenes, and peptide-(ω-aminoalkyl)amides on a Z(ωAlkyl)-type resin [22-24]. In all cases, cleavage from the resin proceeded by acidolysis. In addition, protected peptide amides could be photolytically removed from ortho-nitrat ed benzylamine-type resins [25]. Esterification of N-protected amino alcohols to a succinic-acid linker, followed by SPPS, afforded peptide alcohols upon saponification [26].

* Ammonolysis, aminolysis, hydrazinolysis, base-catalyzed alcoholysis and alkaline hydrolysis.
c) Conversion of the carboxyl group

C-Terminal activation of a peptide acid should be circumvented whenever possible to avoid the risk of racemization. Nevertheless, DCC-mediated activation has been reported in the synthesis of primary amides; the activated benzotriazolyl or succinimidyl esters were converted to the corresponding amides with aqueous ammonia or dianinomethane (an ammonia-releasing agent) without detectable racemization [27,28]. Substituted peptide amides were obtained through reaction of protected peptide acids with alkylammonium HOBT salts in the presence of DIPCDI [29]. The preferred method for the esterification of peptide acids proceeds through reaction of their cesium salts with an alkyl halide [30]. Alternatively, methyl and tert-butyl esters may be formed under very mild conditions, using diazomethane and tert-butyl-2,2,2-trichloroacetimidate (TBTA), respectively [31,32]. An elegant synthesis of peptide 2-chlorotryptyl esters has been recently reported by Barlos and co-workers (cf. attachment of Fmoc-amino acids to trityl-type resin I, Chapter 2) [33].

Synthesis of C-terminally modified peptide derivatives via side-chain attachment to resins

The most convenient method for the synthesis of C-terminally modified peptides is the direct solid-phase synthesis approach. Yet no general protocol is available: each functional group requires another anchoring linkage and certain functions are even excluded from anchoring (ester and tertiary amide). All C-terminal modifications are, however, accessible if attachment of the growing peptide chain does not occur through this C-terminus, but via the side chain of the C-terminal amino acid or even any other side chain within the actual peptide. The flexibility of this approach was first recognized by Meienhofer and Trzeciak when they coupled Boc-Lys(H)-Gly-NH₂ via the lysine side chain to a Z-type resin [34]. Subsequently, Glass and co-workers reacted methylpolystyrene glycinate with 1,5-difluoro-2,4-dinitrobenzene, leading to a dinitrophenyl (Dnp)-type resin (Fig. 1). The remaining fluoride was substituted by the τ-nitrogen of the imidazole ring in Boc-histidine or by the thiol group in Boc-cysteine. C-Terminal coupling of an amino acid or peptide amide was followed by standard Boc SPPS in N-terminal direction. Thiolysis afforded protected peptide amides [35]. With these syntheses, two-directional SPPS was introduced into peptide chemistry.

![Figure 1: Dnp-type resin for imidazole and thiol attachment [35].](image-url)
Trityl-type resins should be particularly suitable for this approach, since they allow the attachment of any side-chain functional group so that the choice of an appropriate residue near the C-terminus is hardly limited [36]. In case of a functional C-terminal residue, it may be attached to the resin via the side chain as the $N^\alpha$-Fmoc-protected ester, amide or alcohol, followed by standard SPPS [37]. If a functional residue is present in the penultimate position, the protected dipeptide (with the desired C-terminal modification) may be coupled to the resin [38]. Alternatively, the attachment of an arbitrary functional $N^\alpha$-Fmoc-amino acid via its side chain allows C-terminal extension with any amino compound to proceed without risk of racemization owing to the $N^\alpha$-urethane protecting group. Further assembly of the peptide may then occur in N-terminal direction. The exact procedures of two-directional SPPS will be investigated for lysine side-chain attachment in this chapter and for cysteine side-chain attachment in the next.

5.1.2. Cyclic peptides

General aspects of cyclization

Cyclic peptides form a large group of naturally occurring and synthetic compounds, that have attracted considerable interest in recent years, owing to their explicit biological properties [39]. In general, cyclic peptides exhibit improved stabilities towards proteolysis in comparison with their linear counterparts, resulting in a prolonged duration of action of cyclic peptide hormones. A variety of biological studies further suggests more controlled bioavailabilities, increased potencies and better receptor selectivities as inherent properties of cyclic structures. Since cyclization reduces the flexibility of the peptide chain, it is an effective way to stabilize the bioactive conformation [40]. Potent cyclic analogues hence provide a powerful tool for studying structure-activity relationships, ultimately leading to the development of selective agonists and antagonists. More recently, cyclic peptides have been employed as synthetic immunogens, as restriction of the conformational freedom of a peptide is likely to result in a conformation, which more closely mimics that of the (linear or non-linear) epitope as presented on the surface of the native protein [41].

Cyclic peptides may be roughly divided into two groups. In homodetic cyclopeptides the constituent amino acids are only connected by peptide (lactam) linkages, whereas heterodetic cyclopeptides contain other structural moieties such as disulfide, ester (lactone), ether and thioether bridges that contribute to their ring(s). Cyclic peptides constituted of both amino as well as hydroxy acids, containing ester linkages within the main chain, are known as cyclodepsipeptides [42]. Cyclic disulfide peptides will be elaborately discussed in the next chapter of this thesis. The current treatise deals with the synthesis of homodetic cyclopeptides. A further classification can be made on the basis of their topological forms. Besides the obvious head-to-tail cyclizations, amino
well as in the synthesis of branched cyclopeptides (Fig. 2 [43]). Concretely, side chains of α,ω-diaminocarboxylic acids (lysine/ornithine and their lower homologues) and α-aminodicarboxylic acids (aspartic/glutamic acid and their higher homologues) are involved.

![Head-to-tail and side chain-to-side chain](image1)

**Figure 2:** Topological forms of homodetic cyclic peptides. Arrows point in the C-terminal direction of the peptide backbone [43].

Several difficulties are encountered during cyclization reactions. In cyclizations comprising the activation of the C-terminal carboxyl function as an active ester [44] or via standard in situ coupling reagents like DCC, BOP or TBTU [45-47], absolute retention of chirality can only be expected in the case of glycine or proline. Application of the azide method minimizes the risk of racemization [48]. The main problem associated with cyclization reactions, however, lies in the formation of di- and oligomeric products. To avoid intermolecular processes, cyclization has to take place at high dilution in the range of $10^{-3}$ to $10^{-4}$ M. As a consequence, the activation and cyclization steps must be separable, and since cyclization may be a slow process, the activated species should not undergo unimolecular or solvent-induced decomposition.

As an alternative to cyclization in highly diluted solutions, ring closure may take place while the peptide is still attached to the resin, taking advantage of pseudo-dilution to solely effect the intramolecular reaction [49].
**On-resin homodetic cyclization of C-terminally attached peptide derivatives**

Three-dimensional orthogonality is required in the on-resin cyclization of peptides. The amino and carboxyl group involved in the cyclization reaction must be selectively deprotected, while all other protected functional groups and the anchoring linkage remain intact. If the peptide is attached to the resin through its C-terminal carboxyl group, be it as an ester or amide, in principle, two modes of cyclization are possible, viz. side chain-to-side chain and side chain-to-N-terminal.

One way of achieving orthogonality during SPPS is to selectively apply Fmoc/tBu chemistry in a Boc/Bzl-type system. The anchoring linkage and all permanent side-chain protections are of the benzyl-type and cleavable by HF treatment, whereas the TFA-labile Boc group is used for α-amino protection. Only the side chains involved in cyclization are protected as a 9-fluorenylmethyl urethane (Lys(Fmoc) or its homologues) or ester (Asp(OFm) or its homologues) and selectively removable by treatment with a base [50]. Since Boc chemistry requires a neutralization step in each cycle of the SPPS, partial loss of the 9-fluorenyl-type protecting groups through the action of the tertiary amine cannot be totally excluded. Alternatively, Fmoc can be used for α-amino protection and tert-butyl, either as the urethane (Lys(Boc) or its homologues) or ester (Asp(OtBu) or its homologues), in semi-permanent side-chain protecting groups [50e,51].

![Scheme 1](image)

**Scheme 1:** Palladium(0)-catalyzed allyl transfer onto a nucleophile: the catalyst first reacts with the allyl group in the protected compound to give a palladium(II) π-allyl complex, causing concomitant release of the deprotected component Z. In the second step, the π-allyl moiety is trapped by the nucleophilic scavenger (Nu') and the catalyst regenerates [53b].

* Two-dimensional orthogonality with two of the three protection levels being differentiated kinetically within the same class of reagents (e.g. acids), is also included.
Allyl-based protecting groups brought truly three-dimensional orthogonality into Fmoc SPPS [52]. Selective removal of the side-chain protection in Lys(Aloc) or Asp/Glu(OAll) by palladium(0)-catalyzed allyl transfer onto a nucleophile [53], as depicted in Scheme 1, may be followed by on-resin cyclization [54]. A further dimension of orthogonality was added with the introduction of the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine)ethyl (Dde) group for lysine and 4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester (ODmab) for aspartic/glutamic acid side-chain protection, both removable by the action of 2% hydrazine in DMF (Fig. 3) [55,56]. The former has been used in combination with Glu(OAll) in the synthesis of a branched cyclic peptide [57]. The incorporation of Lys(Mtt) during SPPS offers yet another means of selective lysine side-chain deprotection, using 1% TFA [58].

![Chemical structures](image)

**Figure 3:** Amino-protecting Dde group and carboxyl-protecting Dmab ester, removable with 2% hydrazine.

When the C-terminal carboxyl group of a peptide is anchored to the resin via an activated ester linkage, head-to-tail cyclization will proceed with concomitant cleavage from the resin. Fridkin et al. described the esterification of protected peptides to poly-4-hydroxy-3-nitrostyrene, resulting in an immobilized activated ester derivative. Upon acidolytic removal of the N-terminal benzzyloxycarbonyl (Z) protecting group and subsequent neutralization, cyclic peptides were obtained in 60-80% yield [59]. Flanigan and Marshall applied the 4-(aralkylthio)phenyl group as an anchoring moiety in Boc SPPS. After assembly of the peptide on the support, the thioether linkage was oxidized to the corresponding sulfone, thus activating the anchoring C-terminal ester of the peptide. Detachment as the cyclic peptide occurred again after N-terminal deprotection and neutralization [60]. Both methods suffer from serious drawbacks. Fridkin's method excludes the solid-phase assembly on the actual resin and is only applicable with preformed peptides. The "safety-catch" method is limited in its application by the inherent oxidation step. A third comparable but more effective procedure employs a 4-nitrobenzophenone oxime-derivatized resin [61].
On-resin homodetic cyclization of peptide derivatives attached through a side chain

The more obvious method for on-resin head-to-tail cyclization starts with the attachment of a transiently C-terminal amino acid via its side-chain function. Most widely applied is the side-chain attachment of aspartic or glutamic acid derivatives, enabling the synthesis of peptides with C-terminal (or internal, if cyclization occurs) Asp or Glu residues on ester anchoring resins, as well as Asn or Gln residues on amide anchoring resins. In the synthesis of linear peptides, Boc-Asp/Glu(OH)-OBzl and Fmoc-Asp/Glu(OH)-O\textsuperscript{Bu} are applicable in Boc and Fmoc SPPS, respectively [62,63]. The difficult esterification of Fmoc-asparagine or glutamine to 4-alkoxybenzyl alcohol-type resins is thereby circumvented.

As mentioned above, on-resin cyclization requires a third degree of selectivity. Systems exploiting graduated acid lability in combination with one fully orthogonal protection class, employ Boc-Asp/Glu(OH)-OFm (Boc SPPS) or Fmoc-Asp/Glu(OH)-ODmb (Fmoc SPPS) [64,65]. The latter 2,4-dimethoxybenzyl ester is selectively cleavable on the peptidyl resin with 1% TFA [41]. C-Terminal protection as a 2-chlorotrityl ester enhances acid lability even more [66]. Similar to the case of side-chain protecting strategies, truly three-dimensional orthogonality is achieved with the incorporation of Fmoc-Asp/Glu(OH)-OAll or Fmoc-Asp/Glu(OH)-ODmab into a SPPS procedure [67,56].

Besides α-aminocarboxylic acids, any amino acid with a functional side chain might in principle be used for side-chain attachment to a resin, with the ultimate goal of performing on-resin head-to-tail cyclizations. To our knowledge, only two examples have been reported so far, employing histidine and ornithine. Isied and co-workers reacted a Dnp-type resin, similar to the one depicted in Figure 1, with Boc-histidine. C-Terminal condensation with glycine benzyl ester was succeeded by standard Boc SPPS in N-terminal direction. After assembly of the peptide, both terminal protecting groups were removed by acidolytic treatment. Following neutralization, the peptide was cyclized according to an in situ activation method and detached from the resin by the action of thiophenol [68]. Sklyarov and Shashikova employed a Z-type resin for side-chain attachment of ornithine, incorporated in an otherwise protected peptide. A similar procedure to the one applied by Isied eventually led to the desired cyclopeptide. In this case, however, C-terminal blocking was provided by the methyl ester, which had to be sapomified on the resin [69].

Side-chain attachment of other amino acid derivatives would greatly extend the scope of on-resin head-to-tail cyclizations, beyond cyclopeptides encompassing asparagine, glutamine, aspartic acid or glutamic acid. Trityl-type resins seem, as for two-directional SPPS, extremely suitable for this purpose. Combination with orthogonal protecting groups like allyl and Dmab esters, cleavable under very mild and highly selective conditions, should emphasize their general applicability.
5.1.3. Outline of the investigation

Synthesis scheme
This chapter deals with the attachment of peptides to trityl-type resins via an amino group, analogous to the Lys(Trt) and Lys(Mtt) adducts, and ultimately leads to on-resin head-to-tail cyclizations. In preliminary experiments, cleavage conditions for trityl-type resins I and II were compared, employing the model peptide-(6-aminohexyl)amide Fmoc-Leu-Val-Dah, that was anchored through its free amino function. To further establish the applicability of SPPS via amine anchoring, several types of peptide derivatives with free amino functions were synthesized on trityl-type resins I and II.

Application of trityl-type resins allows coupling of unprotected diaminoalkanes to precede solid-phase synthesis. Site isolation is evidently effective in preventing "double-binding" [20c,70,71]. We synthesized a hexapeptide-(6-aminohexyl)amide on trityl-type resin I. Next, Fmoc-Lys(H)-NHCH₃ and H-Lys(Fmoc)-NHCH₃ were attached to resins I and II, as a first step in the synthesis of α or ε-lysine peptide amides. The unusual ε-amide bond is found in some natural products such as bioeytin and bacitracin A [72,73]. Lysine also serves as a branching point in collagen [74]. At this stage, cleavage conditions for the detachment of selectively protected peptide fragments (containing only the anchoring amino function in its unprotected form) were studied.

As an introduction to two-directional SPPS, the ease of attachment of several functional groups, among them the carboxyl and α and ε-amino groups, was compared in a competitive experiment, to establish which groups can be selectively anchored in the presence of others under weakly basic conditions. As the difference in coupling rate between the carboxyl and the ε-amino group in Fmoc-lysine was found to be small, selectivity is endangered in the loading of a trityl-type resin. Conversion of the compound to its bis(trimethylsilyl) derivative prior to reaction with the resin, would have been effective in providing the desired selectivity [75]. A more stable carboxyl protection, however, would afford a general solution. These considerations led to the application of the orthogonally protected compound Fmoc-Lys(H)-OAll. After its attachment to trityl-type resin I, two-directional SPPS could only proceed upon removal of the allyl protecting group. A similar route was followed by Kaspari et al., who utilized Fmoc-Glu(OH)-OAll in the synthesis of peptides with a C-terminal glutamine-4-nitroanilide moiety [76].

Analogous to strategies involving Fmoc-Asp/Glu(OH)-OAll, the Fmoc-Lys(resin I)-OAll adduct was also applied in the synthesis of head-to-tail cyclic peptides. In this case, the allyl ester was cleaved after assembly of the peptide; cyclization was accomplished on the solid support. During this part of the study, two complicating side-reactions were encountered, namely diketopiperazine formation and allyl transfer onto an unprotected N-terminal proline residue.
Model peptide ODN-7

All peptides synthesized in the course of this study (except for Fmoc-Leu-Val-Dah), are derived from the ODN-7 sequence Pro-Gly-Leu-Leu-Asp-Leu-Lys. The sequence corresponds to amino acids 44-50 of rat DBI (diazepam binding inhibitor) [77]. DBI is a protein that displaces ligands bound to the β-carboline/benzodiazepine recognition site, an allosteric modulatory site of the type A γ-aminobutyric acid (GABA_A) receptor complex. The complex additionally contains a chloride channel subunit. Benzodiazepines such as diazepam (Valium) prolong chloride channel opening effected by GABA, and thereby play a role in reducing pathological anxiety. Both β-carboline and the endogenous DBI exert the opposite effect and induce anxiety, which under some circumstances can be an entirely appropriate response. The 18-amino acid peptide octadecaneuropeptide (ODN), a tryptic fragment of DBI, contains the functional domain and proved to be even more active than the parent compound [78]. ODN-7 is a reduced but still active form of ODN [79].

5.2. Application of amine anchoring in standard SPPS

5.2.1. Cleavage experiments on resin-bound Fmoc-Leu-Val-Dah

The title compound was synthesized on trityl-type resins I and II. Resin II was first converted into the chloride form by treatment of the original trityl alcohol resin with thionyl chloride. After reaction of the resins in the presence of a large excess of 1,6-diaminohexane, the active chloride that eventually remained unreacted was destroyed by the addition of methanol and excess of DIPEA. Standard SPPS, during which no major detachment of the product was observed, yielded the peptidyl resins with loadings of 0.44 mmol/g (resin I: 40% overall coupling yield) and 0.22 mmol/g (resin II: 41% coupling yield based on the original chloromethylpolystyrene).

Results of cleavage experiments on these resins are summarized in Table 1. Conditions normally used for the detachment of protected peptides, anchored through a carboxyl function to Barlos's trityl-type resin I (entry no.1 [80]), proved insufficient in case of the topical amino anchoring to resin I, but gave good results for resin II. Quantitative cleavage from resin II could also be effected with 0.5% TFA in DCM, whereas the resin I adduct required the tenfold concentration (entries 3-5 vs. 8-11). Addition of triethylsilane to the TFA-containing mixtures is not strictly necessary (entry 5 vs. 4 and 9 vs. 8): contrary to the tritylthiol adduct, cleavage of the tritylamino bond is an irreversible reaction, because the amino function, being more basic than the thiol function, becomes protonated. Addition of methanol, however, reduces the acidity of the solution by binding protons, and hence exerts a strongly
negative effect on the cleavage rate (entry 10 vs. 8). Acidity was virtually “eliminated” at low TFA concentration (entry 6 vs. 4).

### Table 1: Cleavage yields of Fmoc-Leu-Val-Dah from trityl-type resins.

<table>
<thead>
<tr>
<th>Entry no.</th>
<th>Cleavage conditions</th>
<th>% Cleavage resin I</th>
<th>% Cleavage resin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>45 min AcOH/TFE/DCM (2:2:6)</td>
<td>14</td>
<td>88</td>
</tr>
<tr>
<td>2.</td>
<td>45 min 0.1% TFA/DCM</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>45 min 0.5% TFA/DCM</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>4.</td>
<td>15 min 0.5% TFA/DCM</td>
<td>n.d.</td>
<td>85</td>
</tr>
<tr>
<td>5.</td>
<td>15 min 0.5% TFA/5% TES/DCM</td>
<td>n.d.</td>
<td>78</td>
</tr>
<tr>
<td>6.</td>
<td>15 min 0.5% TFA/5% MeOH/DCM</td>
<td>n.d.</td>
<td>0</td>
</tr>
<tr>
<td>7.</td>
<td>45 min 1% TFA/DCM</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td>8.</td>
<td>45 min 5% TFA/DCM</td>
<td>41</td>
<td>n.d.</td>
</tr>
<tr>
<td>9.</td>
<td>45 min 5% TFA/5% TES/DCM</td>
<td>57</td>
<td>n.d.</td>
</tr>
<tr>
<td>10.</td>
<td>45 min 5% TFA/5% MeOH/DCM</td>
<td>23</td>
<td>n.d.</td>
</tr>
<tr>
<td>11.</td>
<td>4x15 min 5% TFA/5% TES/DCM</td>
<td>76</td>
<td>n.d.</td>
</tr>
<tr>
<td>12.</td>
<td>15 min 10% TFA/5% TES/DCM</td>
<td>50</td>
<td>n.d.</td>
</tr>
<tr>
<td>13.</td>
<td>15 min 4 M HCl/dioxane</td>
<td>19</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

The reactivity in 4 M HCl/dioxane, a condition which normally affects the cleavage of the Boc-amino bond as an alternative to treatment with 50% TFA [81], proved to be remarkably slow, with a cleavage half-time of about forty minutes, comparable to that in 5% TFA (entry 13 vs. 9). This phenomenon can be explained by the poor accessibility of the hydrophobic polystyrene matrix to small inorganic particles with a high charge density. Tesser and co-workers encountered a similar problem, when exploring cleavage conditions for their 2-hydroxyethylsulfonylethoxycarbonyl (Msc) group for amino acid derivatives is readily cleaved by the action of aqueous sodium hydroxide [83]. Using a mixture with dioxane, detachment from the resin proceeded with very poor results, clearly because of the unfavourable penetration of hydroxide ions into the matrix core, which contained the anchoring sites in close proximity. Addition of methanol to the mixture, resulting in Tesser’s base cocktail, provided methoxide ions and led to instantaneous cleavage. The same principle would in the present case account for the decreased cleavage rate in HCl/dioxane with respect to TFA/DCM. The products, as evaluated from the crude cleavage mixtures, were chromatographically pure in two solvent systems.
5.2.2. Synthesis of a peptide aminoalkylamide, and α and ε-lysine peptide amides

Trityl-type resins I and II were applied in the SPPS of five derivatives of ODN-7 with varying C-terminal residues, according to Scheme 2. Synthesis of the (6-aminohexyl)amide compound 1b was initiated with the coupling of 1,6-diaminohexane to resin I, as described above. Attachment of Fmoc-Lys(H)-NHCH₃.HCl in the presence of DIPEA eventually yielded the α-lysine methylamide derivative 2b (resin I) and its semi-protected form 5c (resin II, chloride form), attachment of the isomeric H-Lys(Fmoc)-NHCH₃.HCl led to the ε-derivative 3b (resin I) and semi-protected 4b (resin I), which is equal to 6c prepared on resin II (chloride form). The preparation of all lysine derivatives, used in the current study, is described in Chapter 8.

Fmoc-Pro-X(O'Bu)-Dah(resin I) 1a $\rightarrow_{i, ii}$ H-Pro-X(OH)-Dah 1b

Fmoc-Pro-X(O'Bu)-Lys(resin I)-NHCH₃ 2a $\rightarrow_{i, ii}$ H-Pro-X(OH)-Lys(H)-NHCH₃ 2b

(resin I)-Lys(Fmoc-Pro-X(O'Bu)-)-NHCH₃ 3a $\rightarrow_{i, ii}$ H-Lys(H-Pro-X(OH)-)-NHCH₃ 3b

(resin I)-Lys(Boc-Pro-X(O'Bu)-)-NHCH₃ 4a $\rightarrow_{iii}$ H-Lys(Boc-Pro-X(O'Bu)-)-NHCH₃ 4b

Fmoc-X(O'Bu)-Lys(resin II)-NHCH₃ 5a $\rightarrow_{i, iv}$

Boc-Pro-X(O'Bu)-Lys(resin II)-NHCH₃ 5b $\rightarrow_{iii}$ Boc-Pro-X(O'Bu)-Lys(II)-NHCH₃ 5c

(resin II)-Lys(Fmoc-X(O'Bu)-)-NHCH₃ 6a $\rightarrow_{i, iv}$

(resin II)-Lys(Boc-Pro-X(O'Bu)-)-NHCH₃ 6b $\rightarrow_{iii}$ H-Lys(Boc-Pro-X(O'Bu)-)-NHCH₃ 6c

Scheme 2: Synthesis of an aminoalkylamide and several α and ε-acylated lysine methylamides, representing analogues of ODN-7: i) piperidine/DMF (1:4); ii) TFA/TES/H₂O (95:2.5:2.5); iii) AcOH/TFE/DCM (2:2:6); iv) Boc-Pro-OH, TBTU/HOBt/NMM. X(O'Bu) = Gly-Leu-Leu-Asp(O'Bu)-Leu; X(OH) = Gly-Leu-Leu-Asp(OH)-Leu. N.B. 4b = 6c.

The results of the syntheses are summarized in Table 2. Initial loadings and SPPS coupling yields were calculated from determinations of the Fmoc loading at various stages of the synthesis.
Attachment via the α-amino group proceeded in higher yields than attachment via the ε-amino group (roughly 1:0.64). Experiments to determine the minimal TFA concentration effecting quantitative cleavage of semi-protected “peptide amines” showed a similar difference in cleavage rate, with the α-aminotrityl adduct being far more labile (Table 3). These observations can be explained by considering the nature of the trityl adduct formation and cleavage reactions, as presented in Chapter 1 (Section 1.2.1). Both are completed with the breaking of a bond between the amino group and an electrophilic species, the proton or the trityl cation, respectively. Due to its lower pKₐ-value and hence greater leaving ability, these processes are favoured with the α-amino group, thus accounting for enhanced adduct formation and cleavage rates. The same principle rules the selective N²-detritylation of N²,N³-ditrityl lysine with 1% TFA [75a].

**Table 2:** Quantitative evaluation regarding the synthesis of the aminohexylamide and α- and ε-lysine methylamide derivatives of ODN-7.

<table>
<thead>
<tr>
<th>Peptidyl resin</th>
<th>Initial loading</th>
<th>SPPS yield (%)</th>
<th>Product</th>
<th>Overall yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmol/g)</td>
<td>(%)</td>
<td>overall</td>
<td>per cycle</td>
</tr>
<tr>
<td>1a</td>
<td>0.35</td>
<td>29</td>
<td>75</td>
<td>94.5</td>
</tr>
<tr>
<td>2a</td>
<td>0.42</td>
<td>35</td>
<td>97</td>
<td>99.5</td>
</tr>
<tr>
<td>3a</td>
<td>0.58</td>
<td>51</td>
<td>69</td>
<td>94.0</td>
</tr>
<tr>
<td>4a a</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5a</td>
<td>0.18</td>
<td>30 / 40 b</td>
<td>95</td>
<td>98.9</td>
</tr>
<tr>
<td>6a</td>
<td>0.30</td>
<td>53 / 72 b</td>
<td>86</td>
<td>96.9</td>
</tr>
</tbody>
</table>

*See SPPS of 3a, with Boc-Pro-OH instead of Fmoc-Pro-OH in the last cycle. b The first value is based on the capacity of the original chloromethylpolystyrene, the second on the assumed derivatization yield of resin II (74%, concluded from the maximum Fmoc-cysteine loading (Section 6.2.1)). c Based on initial loading.

The SPPS yields varied from 94% to nearly quantitative per cycle, with higher yields in case of ε-amino attachment. This might be partly due to the greater lability of the α-amino adduct, but cannot be entirely accounted for, as similarly lowered yields were also encountered with diaminohexane (peptidyl resin 1a) or Fmoc-Lys(II)-OAll (Section 5.3.2) as the anchoring residue. No significant difference between resins I and II was observed in this respect.

SPPS on resin II could not be monitored by the usual performance of Kaiser tests after each step (ninhydrin monitoring) [84]. The test results were always positive, probably due to instability of the benzoxytrityl-amino adduct towards thermal treatment as part of the protocol. Instead, the TNBS (2,4,6-trinitrobenzenesulfonic acid) method was applied [85].
In all cases, the overall synthesis yields were quite satisfactory and the products obtained were pure according to amino acid analysis and TLC. Since dianinohexane was not detected in amino acid analysis, product 1b was characterized by SIMS. RP-HPLC and mass spectrometry provided purities of at least 80% for all products. A minor contamination in the fully deprotected products 2b and 3b was identified by MALDI-TOF-MS as the desired compound still bearing the tert-butyl protecting group ([M+56]⁺). To exemplify the typical high purity of the products, RP-HPLC and MALDI-TOF-MS of crude 2b are presented in Figure 4.

**Figure 4:** RP-HPLC (a) and MALDI-TOF-MS (b) of crude product 2b (C₃₀H₂₆N₈O₂ : MW_{theor.} 768.0). For a complete assignment of the peaks in (b), see the experimental section.
The syntheses of semi-protected 4b and 6c on resin I and II, respectively, resulted in the same products of similar purity and in the same molar yield (0.22 mmol/g resin), although the final cleavage yield was significantly lower in case of 4b (55 vs. 89%). The data in Table 3 clearly evidence the enhanced lability of resin II in comparison to resin I under very mild acidolytic conditions. As an alternative to the synthesis of semi-protected 6c on resin II, synthesis of the same compound using resin I (4b) may therefore be optimized by utilizing a 1% TFA solution instead of AcOH/TFE/DCM as the cleavage mixture. The latter was used to avoid the extra washing step, inherent to cleavage procedures with dilute TFA solutions [86]. Instead, simple evaporation of the cleavage solution suffices for the recovery of the crude product.

<table>
<thead>
<tr>
<th>Peptidyl resin (trityl resin / attachment)</th>
<th>% TFA</th>
<th>% Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a (resin I / ε-amino)</td>
<td>1.0</td>
<td>22</td>
</tr>
<tr>
<td>3a (resin I / α-amino)</td>
<td>1.0</td>
<td>96</td>
</tr>
<tr>
<td>3a (resin I / α-amino)</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>5a (resin II / ε-amino)</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>6a (resin II / α-amino)</td>
<td>0.1</td>
<td>97</td>
</tr>
</tbody>
</table>

* TFA concentration in 5% TES/DCM, 45-min treatment.

The experiments, described in this section, demonstrate the general applicability of trityl-type resins I and II in the SPPS of amino compounds. We prefer the use of resin I in the synthesis of unprotected "peptide amines", because of higher initial loadings. Resin II is recommended for the synthesis of semi-protected "peptide amines", because milder conditions may be used for detachment, ensuring an easier work-up procedure.

5.3. Application of amine anchoring in two-directional SPPS

5.3.1. Competition experiment concerning ease of attachment

To consolidate or reject the possible use of Fmoc-lysine in two-directional SPPS, the preferential binding of the ε-amino group to trityl-type resins in the presence of the carboxyl function had to be established. Therefore, we developed a competition experiment, in which trityl-type resin I was allowed to react in the presence of several amino acid derivatives, one molar equivalent each, and
each containing a different unprotected functional group, under weakly basic conditions. Various combinations were used to preclude, as much as possible, effects due to steric hindrance of the side chains. Upon hydrolysis of the resulting loaded resins, amino acid analysis provided the relative coupling rates for the various functional groups. The derivatization scheme as well as analysis results are given in Table 4.

**Table 4:** Competition experiment on trityl-type resin I. Compounds used and relative molar quantities recovered after hydrolysis of the resulting adducts, according to amino acid analysis (AAA).

<table>
<thead>
<tr>
<th>Carboxyl compound</th>
<th>AAA</th>
<th>Hydrazide compound</th>
<th>AAA</th>
<th>Amino compound</th>
<th>AAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Pro-OH</td>
<td>1.00</td>
<td>Fmoc-Ile-N₂H₃</td>
<td>0.50</td>
<td>Fmoc-Lys(H)-NHCH₃</td>
<td>0.87</td>
</tr>
<tr>
<td>Fmoc-Glu(OH)-OAll</td>
<td>1.00</td>
<td>Fmoc-Asp(O'Bu)-N₂H₃</td>
<td>1.57</td>
<td>Fmoc-Lys(H)-OAll</td>
<td>0.61</td>
</tr>
<tr>
<td>Fmoc-Asp(OH)-OAll</td>
<td>1.00</td>
<td>Fmoc-Glu(O'Bu)-N₂H₃</td>
<td>1.04</td>
<td>Fmoc-Lys(H)-OAll</td>
<td>0.37</td>
</tr>
<tr>
<td>Fmoc-Val-OH</td>
<td>1.00</td>
<td>Fmoc-Ala-N₂H₃</td>
<td>1.28</td>
<td>H-Lys(Z)-OMe</td>
<td>1.22</td>
</tr>
<tr>
<td>Fmoc-Gln(Trt)-OH</td>
<td>1.00</td>
<td>Fmoc-Ala-N₂H₃</td>
<td>1.30</td>
<td>H-Lys(Z)-OBzl</td>
<td>1.17</td>
</tr>
<tr>
<td>Fmoc-Glu(O'Bu)-OH</td>
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<td>Fmoc-Lys(Boc)-N₂H₃</td>
<td>0.75</td>
<td>H-Leu-OMe</td>
<td>0.88</td>
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<tr>
<td>Fmoc-Phe-OH</td>
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<td>Fmoc-Ile-N₂H₃</td>
<td>0.52</td>
<td>H-Leu-OMe</td>
<td>1.08</td>
</tr>
</tbody>
</table>

Coupling reactions with various carboxyl, hydrazide and amino compounds revealed the following relation for case of attachment to trityl resin I:

\[
\text{carboxyl} : \text{hydrazide} : \epsilon\text{-amino} : \alpha\text{-amino} = 1.00 : 0.99 : 0.62 : 1.09.
\]

No preference was observed for either carboxyl or hydrazide compounds. The \(\alpha\)-amino group couples slightly faster, whereas coupling of the \(\epsilon\)-amino group is significantly more sluggish; their relative coupling rates are in good accordance with the ones observed in the aforementioned syntheses of the ODN-7 derivatives (Section 5.2.2). The difference in coupling rate between the carboxyl and \(\epsilon\)-amino group is so small that no preferential mode of binding can be expected to prevail in case of Fmoc-lysine. Consequently, temporary blocking of its carboxyl group cannot be circumvented when aiming at two-directional SPPS.

A similar competition experiment was carried out using Fmoc-Glu(O'Bu)-N₂H₃, Fmoc-Leu-NH₂, Z-Ser(H)-OBzl and Trt-Arg(H)-OMe. The only amino acid recovered after hydrolysis of the resulting loaded resin was glutamic acid, indicating that carboxyl, hydrazide and amino groups can be selectively coupled to trityl-type resins under weakly basic conditions, in the presence of unprotected amide, alcohol [87] or guanidino groups [88].
5.3.2. Application of Fmoc-Lys(H)-OAll: two-directional SPPS

The allyl ester was chosen as a temporary (orthogonal) protecting group for the carboxyl function in Fmoc-lysine. The preparation of Fmoc-Lys(H)-OAll.HBr is reported in Chapter 8. Coupling to trityl-type resin I in the presence of DIPEA yielded the derivatized resin 7a with an initial loading of 0.59 mmol/g (52%). The further course of the topical two-directional synthesis is depicted in Scheme 3.

Fmoc-Lys(resin I)-OAll  7a  \( \rightarrow \)
Fmoc-Lys(resin I)-OH  7b  \( \rightarrow \) Fmoc-Lys(resin I)-Leu-O\(^{\text{tBu}}\)  7c  \( \rightarrow \)
Fmoc-Pro-Gly-Leu-Leu-Asc(O\(^{\text{tBu}}\))-Lys(resin I)-Leu-O\(^{\text{tBu}}\)  7d  \( \rightarrow \)
H-Pro-Gly-Leu-Leu-Asc(OH)-Lys(H)-Leu-OH  7e

Scheme 3: Synthesis of [Lys\(^{\text{6}},\text{Leu}^{\text{7}}\)ODN-7 (7e): i) [(C\(_{5}\)H\(_{5}\)]\(_{2}\)Pd/N-methylaniline; ii) H-Leu-O\(^{\text{tBu}}\), TBTU/HOBt/NMM; iii) 5 cycles of Fmoc SPPS. iv) piperidine/DMF (1:4); v) TFA/TES/H\(_{2}\)O (95:2.5:2.5).

To remove the allyl protecting group, resin 7a was suspended in dichloromethane under nitrogen, followed by the addition of ca. 0.25 molar equivalents of tetrakis(triphenylphosphine)palladium(0) and 5 equivalents of N-methylaniline. Use of this not too basic allyl group scavenger ensures stability of the Fmoc moiety [67a]. The course of the reaction could be monitored chromatographically by treating an aliquot of the resin with a 10% TFA solution. After five hours, the reaction was complete and the resin was extensively washed to remove the catalyst.

The actual two-directional SPPS commenced with the acylation of leucine tert-butyl ester by the in situ activated carboxyl group in resin adduct 7b. Acylation proceeded quantitatively with 5 molar equivalents of the ester in DMF, in the presence of 1.3 equivalents of TBTU and HOBt each. During 20 hours of coupling, the pH of the suspension was maintained at 7 by the regular addition of NMM. The course of the reaction was again chromatographically monitored. Standard SPPS on 7e eventually afforded peptidyl resin 7d with a loading of 0.33 mmol/g. The overall coupling yield was 72% (94.7% per coupling). No exceptional loss of material from the support was observed during the allyl deprotection step.

After Fmoc removal and treatment with concentrated TFA, the fully deprotected [Lys\(^{\text{6}},\text{Leu}^{\text{7}}\)ODN-7 peptide 7e was obtained in 70% overall yield, based on initial loading. Purity of the crude product was 76% according to RP-HPLC.
5.4. On-resin head-to-tail cyclization via amine anchoring

5.4.1. Application of Fmoc-Lys(H)-Pro-OAll: diketopiperazine formation

C-Terminal carboxyl activation of peptides usually involves some degree of racemization at the C-terminal residue, unless this is glycine or proline, or activation proceeds via the azide method. Since in cyclic ODN-7, proline is C-terminally adjacent to the (anchoring) lysine residue, the most obvious synthetic strategy towards the topical on-resin head-to-tail cyclization would start with the attachment of Fmoc-Lys(H)-Pro-OAll to trityl-type resin I. After further assembly of the peptide by standard Fmoc SPPS, Fmoc-glycine being the last residue to be coupled, deprotection of both peptide termini would allow the cyclization to take place.

Reaction of Fmoc-Lys(H)-Pro-OAll.HCl with resin I resulted in an initial loading of 0.53 mmol/g (50%). After one cycle of SPPS (Fmoc-leucine) the loading had dropped by 90% to 0.05 mmol/g. TLC following cleavage showed one and the same ninhydrin positive spot, even after attempted continuation of the SPPS, indicating high-yield on-resin diketopiperazine formation (Fig. 5) [89]. We did not observe DKP formation during the assembly of the sequence Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys-Gly-OMe, anchored to trityl-type resin II via the cysteine side chain, as described in the next chapter of this thesis (Section 6.2.3). Apparently, with lysine side-chain attachment the distance between the anchoring trityl moiety and the dipeptide backbone is so large (5 atoms instead of 2 with cysteine attachment), that the resulting higher motility of the anchored dipeptide favours the carbonyl attack that precedes DKP formation. Moreover, the tendency for DKP formation is generally higher with a C-terminal proline residue than with glycine [90].

Geyseren and co-workers performed Fmoc SPPS on a Boc-Lys(Fmoc)-Pro-support. Subsequently, tert-butyl-type protecting groups were cleaved by TFA treatment. Addition of a phosphate buffer of pH 7 resulted in detachment of peptides with the C-terminal DKP moiety in high yields, indicative of the strong propensity of the Lys-Pro sequence to form DKPs [91].

5.4.2. Application of Fmoc-Lys(H)-OAll: on-resin head-to-tail cyclization

Attachment of the preformed tripeptide Fmoc-Lys(H)-Pro-Gly-OAll would eliminate the precarious dipeptide stage, though it would at the same time, because of its complexity, reduce the practical value of the method. As a compromise, we started the synthesis of cyclic ODN-7 with the attachment
of Fmoc-Lys(H)-OAl to trityl-type resin 1, fully aware of the risk of racemization during the cyclization reaction.

Standard SPPS on 7a led to peptidyl resin 8a (80% overall coupling yield, 96.3% per cycle), which was protected at its N-terminus by the Fmoc group and at its C-terminus as an allyl ester (Scheme 4). One can now proceed in two ways: (a) removal of the allyl function from the completely protected peptidyl resin, or (b) removal of the allyl function after deprotection of the N-terminus. Both routes were investigated.

a) The recommended route to cyclic ODN-7

Allyl ester cleavage on 8a proceeded analogous to the aforementioned protocol leading to 7e (Section 5.3.2), without loss of peptide material from the resin. Following deprotection of the N-terminus, cyclization was effected on the resin using 1.3 molar equivalents of TBTU and HOBr each (Scheme 4). Another half portion of both reagents was added after 24 hours. The pH of the suspension was maintained at 7 by the regular addition of NMM. A negative Kaiser test after 48 hours indicated completion of the reaction. BOP-mediated cyclization is likely to proceed faster [50a,b]. Treatment with concentrated TFA afforded cyclic ODN-7 (8e) in 69% overall yield, with a purity of 72% according to RP-HPLC. No second diastereomeric peak was detected. SIMS confirmed the identity of the product. Transfer of the tetramethyluronium group from TBTU to the free amino group, as previously reported for HBTU during on-resin side chain-to-side chain cyclization [51e], had not occurred.

Linear ODN-7 (8f) was obtained from peptidyl resin 8e in 76% overall yield and 90% purity.

\[
\text{Fmoc-Lys(resin 1)-OAl} \quad 7a \quad \rightarrow \quad \text{Fmoc-Pro-X(OBu)-Lys(resin 1)-OAl} \quad 8a \quad \rightarrow
\]

\[
\text{Fmoc-Pro-X(OBu)-Lys(resin 1)-OH} \quad 8b \quad \rightarrow \quad \text{H-Pro-X(OBu)-Lys(resin 1)-OH} \quad 8c \quad \rightarrow
\]

\[
\text{cyclo(Pro-X(OBu)-Lys(resin 1))} \quad 8d \quad \rightarrow \quad \text{cyclo(Pro-(OH)-Lys(H))} \quad 8e
\]

\[
\text{H-Pro-X(OBu)-Lys(resin 1)-OH} \quad 8c \quad \rightarrow \quad \text{H-Pro-(OH)-Lys(H)-OH} \quad 8f
\]

**Scheme 4: Synthesis of cyclic (8e) and linear (8f) ODN-7:** i) 6 cycles of Fmoc SPPS; ii) \[\{\text{C,H}_{2},\text{Pr}\},\text{Pd/N-methylaniline}\]; iii) piperidine/DMF (1:4); iv) TBTU/HOBt/NMM; v) TFA/TES/H_{2}O (95:2.5:2.5). \(X(OBu) = \text{Gly-Leu-Leu-Asp(OBu)-Leu} \), \(X(OH) = \text{Gly-Leu-Leu-Asp(OH)-Leu}\).

The order of removal of the terminal orthogonal protecting groups is not strictly determined by any rules [92], and Fmoc removal prior to allyl ester cleavage may be contemplated as an alternative route towards cyclic ODN-7 [93].
b) The dissuaded route, accompanied by allyl transfer onto proline

Following the route depicted in Scheme 5, a mixture of ODN-7 (8f) and a second unexpected compound was obtained after the final deprotection with TFA (Fig. 6a). The identity of both components of the product mixture was established with mass spectrometry. The new compound 9c still contained an allyl group (Fig. 6b). By \(^1\)H-NMR its position could be assigned to the amino function of the N-terminal proline residue: the three allyl proton signals at 5.9, 5.2 and 4.1 ppm could be clearly discerned, the last of which, derived from the ~N-CH\(_2\)-CH=CH\(_2\) protons, lies in between the values for ~C(O)NH-CH\(_2\)-CH=CH\(_2\) (3.7 ppm) and ~C(O)O-CH\(_2\)-CH=CH\(_2\) (4.6 ppm) [94]. This conclusion was further supported by amino acid analysis, which indicated a diminished proline content (74%). The presence of the unprotected N-terminal proline thus complicates the synthesis and impedes the amenability to cyclization. Besides the desired peptidyl resin 8c, with both termini deprotected, intramolecular allyl transfer via the palladium(0) complex resulted in the irreversibly modified peptidyl resin 9b.

Fmoc-Pro-X(O'Bu)-Lys(resin I)-OAll 8a \(\rightarrow\) H-Pro-X(O'Bu)-Lys(resin I)-OAll 9a \(\rightarrow\)

H-Pro-X(O'Bu)-Lys(resin I)-OH 8e \(\rightarrow\) All-Pro-X(O'Bu)-Lys(resin I)-OH 9b \(\rightarrow\)

H-Pro-X(OH)-Lys(H)-OH 8f \(\rightarrow\) All-Pro-X(OH)-Lys(H)-OH 9c

H-Pro-X(O'Bu)-Lys(resin I)-OAll 9a \(\rightarrow\) H-Pro-X(OH)-Lys(H)-OAll 9d

Scheme 5: Synthetic strategy leading to allyl transfer onto the C-terminal proline residue, including the synthesis of ODN-7 C-terminal allyl ester (9d): i) piperidine/DMF (1:4); ii) \([C_\text{6}H_\text{4}P]_\text{4}\text{Pd/N-methylaniline}; iii) TFA/TES/H\(_2\)O (95:2.5:2.5). X(O'Bu) = Gly-Leu-Leu-Asp(O'Bu)-Leu; X(OH) = Gly-Leu-Leu-Asp(OH)-Leu.

Palladium(0)-catalyzed allyl transfer requires the presence of a nucleophilic species to scavenge the liberated allyl cation. Most commonly used for this purpose are carbonic acids of low pK such as dimedone or N,N'-dimethylbarbiturate [95,96] or weakly basic amines such as morpholine, N-methylaniline, pyrrolidine or N-methylmorpholine [97-99,54b]. An N-terminal proline might be considered as the (1-carboxamide)-substituted derivative of pyrrolidine and hence is a likely allyl acceptor.

The C-terminal allyl ester of ODN-7 (9d) was obtained from peptidyl resin 9a in 82% overall yield and 88% purity.
Figure 6: RP-HPLC (a) and MALDI-TOF-MS (b) of product mixture $8f/9e$ ($8f$ $C_{36}H_{60}N_8O_{10}$: $MW_{theor.}$ 754.9, $9e$ $C_{38}H_{54}N_8O_{10}$: $MW_{theor.}$ 793.0). (a) $8f$: $t_r$ 14.7 min (48.2%), $9e$: $t_r$ 16.2 min (42.7%). (b) For a complete assignment of the peaks, see the experimental section.

5.5. Scope and limitations

This chapter deals with the synthesis of nine analogues of the ODN-7 peptide. All compounds were synthesized on trityl-type resins I or II after attachment of the first residue via an amino group. The first residue may be a diamine or a diaminocarboxylic acid (lysine and its homologues). The carboxyl function of the latter must be blocked as an ester or an amide, and one of its amino functions must be protected by the Fmoc group, in order to allow selective attachment to precede Fmoc SPPS. In this way various peptide derivatives become accessible such as C-terminal peptide aminoalkylamides and
α or ε-lysine peptide amides or esters. Trityl-type resin I is particularly suited for the synthesis of fully deprotected peptides, whereas the use of trityl-type resin II is recommended in the preparation of their semi-protected counterparts.

The scope of the presented method can be widely extended when the carboxyl group of the first residue is protected as an orthogonally cleavable ester. Coupling of Fmoc-lysine allyl ester may be directly succeeded by palladium(0)-catalyzed allyl cleavage, thereby allowing two-directional SPPS to take place. The general procedure for two-directional SPPS should consist of four distinct reaction series: (1) anchoring of an Fmoc-amino acid derivative with a free side chain and a protected Cα-function, (2) selective deprotection of the Cα-function, (3) coupling at the Cα-function of one amino acid or peptide, which is itself C-terminally protected, and (4) standard Fmoc SPPS in N-terminal direction.

Alternatively, the C-terminal allyl ester may be cleaved after the peptidyl resin has been fully assembled by standard SPPS. In the course of allyl ester cleavage, allyl transfer onto an unprotected N-terminal proline residue was observed as an important side reaction; removal of the N-terminal Fmoc group should therefore not precede allyl ester cleavage. Activation of the liberated carboxyl function may ultimately lead to on-resin head-to-tail cyclization or fragment condensation. Fmoc-lysine allyl and Dmab esters, in combination with trityl-type resins, will provide a suitable alternative to the corresponding aspartic and glutamic acid derivatives as key compounds in the easy high-yield synthesis of homocyclic cyclic peptides, whose application range in the chemical and biomedical field is growing at a steady pace.

5.6. Experimental section

Materials and methods
All SPPS were performed according to the TBTU/HOBt/NMM protocol, described in Appendix A, starting with 1 g of the actual resin (trityl-type resin I or II, as indicated). For further specifications, see also Appendix A.

TNBS test
A small sample of the resin was suspended in a few drops of DMF containing 1% of 2,4,6-trinitrobenzene sulfonic acid trihydrate. Addition of one drop of NMM resulted in a dark orange colour of the resin beads if unblocked amino groups were present.

Conversion of trityl-type resin II into the chloride form
Resin II was shaken for 5 min with a 15% solution of thionylchloride in DCM and subsequently washed with DCM containing 2% trimethylsilyl chloride. This procedure was repeated four times. The resin was additionally washed twice with the trimethylsilyl chloride solution and immediately used for coupling.
Coupling of 1,6-diaminohexane to trityl-type resins
The trityl-type resin in its chloride form was shaken for 4 h in the presence of 2.5 (resin I) or 5 meq (resin II) of the diamine in DCM (1 g resin/5 ml DCM). The resin was filtered and, upon addition of a DCM/McOH (1:1) solution containing 250 μl DIPEA, shaken for an additional 15 min. The resin was then washed five times with DMF, twice with 2-propanol and ether each, and dried.

Coupling of lysine derivatives to trityl-type resins
According to the coupling of 1,6-diaminohexane, with 1 and 2 (resin I) or 2 and 4 meq (resin II) of the lysine derivative and DIPEA, respectively, instead of the diamine.

Cleavage experiments (Tables 1 and 3)
To 40 mg of peptidyl resin, 1 ml of the cleavage mixture was added. The resin was either kept in the same solution for the given period of time, or the resin was filtered every 15 min and the solution renewed (Table 1: entry 11). In both cases, after each 14-min period, the reaction tube was placed in an ultrasonic bath for one minute. The resin was then filtered and washed with DCM and ether, each twice. Cleavage yields were calculated from comparison of the Fmoc loadings before and after cleavage (Appendix B).

Cleavage from the resin affording the deprotected peptide (1b, 2b, 3b, 7c, 8e, 8f, 8f9c, 9d)
The peptidyl resin was suspended in TFA/TES/H₂O (95:2:5:2.5) (1 g resin/10 ml solution). After standing for 2 h, the resin was filtered and washed twice with the cleavage mixture. The filtrate was evaporated in vacuo. The oily residue was triturated and washed twice with tert-butyl methyl ether by centrifugation. The remaining solid was then dissolved in water and lyophilized.

Cleavage from the resin affording the semi-protected peptide (4b, 5c, 6c)
The peptidyl resin was treated with AcOH/TFE/DMC (2:2:6) (1 g resin/10 ml solution) for 45 min. The work-up proceeded analogous to that in the aforementioned cleavage with concentrated TFA.

Competition experiment (Table 4)
Trityl-type resin I (70 mg, 1.41 mmol/g) was suspended in 1.5 ml of a DCM/DMF (1:1) solution, containing 110 μmol of each of the three compounds mentioned and 70 μl DIPEA (400 μmol), and shaken for 4 h. The resin was then washed three times with DMF, twice with 2-propanol and ether each, and dried. The reaction was performed for all seven compound combinations. The amino compounds were used as their hydrochlorides, except for Fmoc-Lys(H)-OAll.HBr. The resulting derivatized resins (5 mg each) were used for hydrolysis, followed by amino acid analysis.

Allyl ester cleavage on the resin (7a, 8a, 9a)
During 30 min, a stream of nitrogen was bubbled through a suspension of the peptidyl resin in DCM (1 g resin/10 ml DCM), which contained 5 meq of N-methylaniline. About 0.25 meq of [(C₅H₅)Pd]₄Pd were then added and the suspension was shaken for 5 h in a stoppered vessel. The resin was successively washed with DCM, DMF, 0.5% DIPEA/DMF, DMF, 2-propanol and ether, each twice, and dried.
C-Terminal coupling of leucine tert-butyl ester (7b → 7c)

Leucine tert-butyl ester (340 mg, 1.8 mmol), TBTU (150 mg, 0.47 mmol) and HOBr (72 mg, 0.47 mmol) were dissolved in 3 ml DMF and the resulting solution was added to resin adduct 7b (600 mg, 0.36 mmol). The apparent pH was adjusted to 7 (moist pH paper) with NMM, and kept constant during 20-h shaking. The resin was then washed three times with DMF, twice with 2-propanol and ether each, and dried.

On-resin head-to-tail cyclization (8c → 8d)

TBTU (42 mg, 130 μmol), HOBr (20 mg, 130μmol) and NMM (22 μl, 200 μmol) were dissolved in 3 ml DMF and the resulting solution was added to peptidyl resin 8c (285 mg, 100 μmol). After shaking the suspension for 24 h, TBTU (21 mg) and HOBr (10 mg) were added, and shaking continued for another 24 h. The apparent pH of the suspension was maintained at 7 (moist pH paper) by the regular addition of NMM. The resin was then washed three times with DMF, twice with 2-propanol and ether each, and dried.

**Analytical data**

<table>
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<tr>
<th>Peptidyl resin</th>
<th>Loading (mmol/g)</th>
<th>Peptide</th>
<th>TLC Rf (E)</th>
<th>RP-HPLC tR (min)</th>
<th>[α]D 25°C (c=1, DMF)</th>
<th>Amino acid analysis</th>
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<td>1a</td>
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<td>1b</td>
<td>0.71</td>
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<td>7e</td>
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<td>9d</td>
<td>0.76</td>
<td>22.8</td>
<td>-31.4</td>
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a Preceding peptidyl resin, which was used for determination of the Fmoc loading and hence SPPS coupling yield; peptidyl resins 4a, 8c and 8c/9b are derived from 3a, 8b and 8a, respectively. b Product mixture (cf. Scheme 5).
Mass spectrometry

<table>
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<tr>
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<tr>
<td>8e</td>
<td>SIMS, NBA</td>
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</tr>
<tr>
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<td>SIMS</td>
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<tr>
<td></td>
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<tr>
<td>8f/9c</td>
<td>MALDI-TOF-MS</td>
<td>755.2 [M_{sr}+H]^+, 777.2 [M_{sr}+Na]^+, 793.1 [M_{sr}+K]^+, 795.2 [M_{sr}+H]^+, 811.1 [(M_{sr}+Bu)+H]^+, 817.2 [M_{sr}+Na]^+, 833.1 [M_{sr}+K]^+, 851.1 [(M_{sr}+Bu)+H]^+</td>
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</tbody>
</table>

* Depicted in Figure 4b.  
* Product mixture (cf. Scheme 5).  
* Depicted in Figure 6b.

5.7. References


Synthesis of Amino Compounds on Trityl-Type Resins


In Chapter 2, Fmoc-Glu(OH)-NHCl, H2, has been applied as the first residue in the solid-phase synthesis of the amphiphilic insulin B(23-30) analogue on trityl-type resin I.


See Chapter 4.


Also apparent in the aforesaid SPPS of model compound Fmoc-Leu-Val-Dah.


Synthesis of Amino Compounds on Trityl-Type Resins


Repeated treatments with 1% TFA require intermittent neutralization with pyridine to avoid loss of tert-butyl-type protecting groups, especially from Tyr(Bu) and Lys(Boc). Pyridinium trifluoroacetate needs to be subsequently washed from the reaction mixture [Product Information Sheet 'Peptide Fragment Synthesis using SASRIN®-Resin', Bachem].


We also attempted SPPS through side-chain attachment of arginine to trityl-type resin I. Trt-Arg(H)-OMe was coupled to the resin in the presence of tetramethyl-trityl-guanidine [Barlos, K., Gatos, D. and Eleftheriou, S. (1995) in: 'Peptides 1994' Proc. 23rd Eur. Peptide Sympos. (Maia, H.L.S., ed.) ESCOM, Leiden, pp. 149-150]. After removal of the N'-trityl group with 1% TFA and neutralization, Fmoc-Ala-OH was coupled, resulting in a loading of 0.27 mmol/g (22%). Subsequent SPPS was performed in reasonable yields (91.2% per cycle). Cleavage, however, proceeded very slowly, with only 20% yield after 5-h treatment with TFA/TES/DCM (65:3:32) at 40°C, refreshing the cleavage mixture every hour. Higher TFA concentrations resulted in even lower yields. TLC of the product showed one Sakaguchi-positive spot [(a) Acher, R. and Crocker, C. (1952) *Biochim. Biophys. Acta* 9, 704-705; (b) Alexeenko, L.P. and Orekhovich, V.N. (1970) *Int. J. Protein Res.* II, 241-246).

At present, the attachment of peptides via an amidino group to trityl-type resins is being explored in collaboration with the Institute of Biochemistry and Biophysics at the Friedrich-Schiller University in Jena, Germany.

The mechanism of diketopiperazine formation was treated in Section 2.1.1.


The definition of an orthogonal system states that they “can be removed in any order and in the presence of each other.


Reported in Chapter 8 for Fmoc-Phe-NH-Allyl and various allyl esters.


Chapter 6

Application of Trityl-Type Resin II in the Synthesis of Protected Peptide Disulfides

6.1. Introduction

The regioselective formation of disulfide bonds has always been one of the great challenges in peptide chemistry [1]. Disulfide bonds are important in stabilizing conformational features within proteins like insulin and ribonuclease. The importance of disulfide-stabilized conformations is also evident in studies regarding structure-activity relationships of small synthetic peptides. The optimization of the active conformation by cyclic constraints has been most significant in the synthesis of superactive analogues of somatostatin [2]. This chapter further explores the scope of side-chain attachment to a trityl-type resin, concentrating on the versatile class of cysteine-containing peptides.

6.1.1. Disulfide bond formation in solution

Non-specific thiol oxidation

Disulfide bond formation may proceed along the three different pathways depicted in Scheme 1. Non-specific air oxidation of unprotected thiols in dilute solutions is the most commonly used disulfide forming reaction [3]. Because air oxidation is a slow process, it allows equilibration of different conformers to yield thermodynamically controlled products. Satisfactory results are obtained with the reoxidation of natural proteins like ribonuclease, whose folding is governed by their primary structure [4]. In case of synthetic peptide analogues, the thermodynamically favoured isomer may not coincide with the synthetic target molecule. Moreover, products are often contaminated with by-products arising from oligomerization.

An alternative to air oxidation, proceeding by another mechanism, is the treatment of polythiols with a mixture of reduced and oxidized glutathione [5] or cysteine [6], a so-called oxidoreduction buffer, which catalyzes the net oxidation by thiol-disulfide exchange reactions. Other faster methods, whose outcome is governed by kinetics, make use of potassium ferricyanide, 1,2-dibromoethane, dimethyl-sulfoxide or hydrogen peroxide as oxidizing agents [7-10].
non-specific thiol oxidation: \( \text{R}^1\text{-SH} + \text{R}^2\text{-SH} \)

oxidative deprotection: \( \text{R}^1\text{-S(X)} + \text{R}^2\text{-S(Y)} \rightarrow \text{R}^1\text{-S-S-R}^2 \)

unsymmetrical thiol activation: \( \text{R}^1\text{-S(A)} + \text{R}^3\text{-SH} \)

**Scheme 1:** Approaches applied in disulfide bond formation

\( X, Y = \) protecting group; \( A = \) activating protecting group; \( \text{ox.} = \) oxidant.

**Oxidative deprotection**

A more sophisticated approach, which is especially advantageous in the regioselective synthesis of peptides with more than one intramolecular disulfide bridge, applies orthogonal pairs of protecting groups. An outstanding example is the synthesis of human relaxin II by Büellesbach and Schwabe, described in the opening chapter of this thesis (Section 1.5.2) [11].

Kamber and co-workers have elaborated the prototype oxidative deprotection strategy, by treating Cys(Trt) and Cys(Acm) residues with iodine [12]. The reaction mechanism proceeds through an intermediate sulfenyl iodide, that may either react with another sulfenyl iodide or attack an unchanged protected thiol; both pathways lead to the formation of a disulfide. The course of the reaction in the presence of both Cys(Trt) and Cys(Acm) strongly depends on the nature of the solvent used. Kamber distinguished three groups of solvents, summarized in Table 1.

**Table 1:** Half-times \( (t_h) \) for the iodine oxidation of Boc-Cys(Trt)-Gly-Glu(OBu)_2 and Boc-Cys(Acm)-Gly-Glu(OBu)_2 in various solvents *, according to Kamber [12].

<table>
<thead>
<tr>
<th>Group</th>
<th>Solvent</th>
<th>( t_h ) for S-.Tr</th>
<th>( t_h ) for S-Acm</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>MeOH</td>
<td>3-5 s</td>
<td>1 min</td>
</tr>
<tr>
<td>( t_h ) (Trt) &lt; ( t_h ) (Acm)</td>
<td>AcOH</td>
<td>70-80 s</td>
<td>40-45 min</td>
</tr>
<tr>
<td></td>
<td>dioxane</td>
<td>1 min</td>
<td>1.5-2 h</td>
</tr>
<tr>
<td></td>
<td>MeOH/CHCl(_3) 1:1</td>
<td>2-4 s</td>
<td>15 min</td>
</tr>
<tr>
<td>II</td>
<td>DCM, CHCl(_3)</td>
<td>1-2 s</td>
<td>1.5-2 h</td>
</tr>
<tr>
<td>( t_h ) (Trt) &lt;&lt; ( t_h ) (Acm)</td>
<td>HFIP/CHCl(_3) 1:1</td>
<td>1-2 s</td>
<td>&gt; 2 h</td>
</tr>
<tr>
<td></td>
<td>TFE/CHCl(_3) 1:1</td>
<td>5-6 s</td>
<td>&gt; 2 h</td>
</tr>
<tr>
<td>( t_h ) (Trt) &gt; ( t_h ) (Acm)</td>
<td>DMF</td>
<td>25-35 s</td>
<td>2-3 s</td>
</tr>
</tbody>
</table>

* peptide concentration = 5 \( \times \) 10\(^{-3}\) M; iodine concentration = 15 \( \times \) 10\(^{-3}\) M; temperature 20-25°C.
In solvents of group II, including chloroform and dichloromethane, the conversion of S-Trt proceeds much faster, so that the sole product is the symmetrical dimer arising from two S-Trt functions, while S-Acm remains unaffected [13]. The difference in reaction rate is less pronounced in solvents of group I, the major reaction product being the unsymmetrical dimer arising from one S-Trt and one S-Acm function. DMF is the only solvent, in which reactivities are reversed, leading to the symmetrical dimer from two S-Acm functions as the major product recovered. During iodine oxidation of Cys(Trt) and Cys(Acm), no exchange of existing disulfides has been observed, making this method particularly suitable for selective disulfide formation in peptides with more than one disulfide bridge.

Oxidative deprotection may also proceed by treatment of Cys(Trt) with thiocyanogen [14], Cys(Fm) (9-fluorenylmethyl) or Cys(Dnpe) (2-(2,4-dinitrophenyl)ethyl) with piperidine/DMF (1:1) [15,16], or treatment with N-iodosuccinimide or methyltrichlorosilane/diphenylsulfoxide, both applicable in combination with several S-protecting groups like Acm, MeOBzI (4-methoxybenzyl) and MeBzI (4-methylbenzyl) [17,18]. The most versatile agent applied in this approach, affording disulfides in high yield and purity, is thallium(III) trifluoroacetate, which can be used with a wide range of S-protecting groups including Acm, Trt, Bu, MeOBzI and MeBzI [19]. An overview of the various classes of thiol protecting groups will be presented in the next chapter (Section 7.1.1).

Unsymmetrical thiol activation

A third approach for disulfide bond formation under very mild conditions is preceded by the unsymmetrical activation of one thiol of a pair, which is to react with the other thiol in its unprotected form. In Chapter 2, the application of Scm-Cl and See-Cl as an important means of activating S-Trt and S-Acm-protected cysteines was already discussed [20]. Treatment of a thiol with azodicarboxylic acid di-tert-butyl ester (Boc-Ne-N-Boc) leads to an activated sulfinylhydrazide derivative, whereas treatment with Ellman's reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) yields an activated mixed disulfide [21,22]. Alternatively, the activated cysteine residue can be directly incorporated in the course of the peptide-backbone assembly as its 3-nitro-2-pyridinesulfenyl (Npys) protected derivative [23]. Disulfide bond formation takes place upon side-chain deprotection of a second cysteine residue present.

6.1.2. Disulfide bond formation on a solid support

The majority of syntheses involving disulfide bond formation has been carried out in solutions of high dilution, thereby minimizing the extent of oligomerization. The same result may be achieved by performing the deprotection/oxidation reaction with the peptide chain still anchored to the support, taking advantage of pseudo-dilution to minimize the formation of oligomeric by-products [24]. The effective site isolation is in the first place determined by the density of active sites on the resin.
(loading or capacity). It is further influenced by the motility of its polymer chains, which depends on the degree of crosslinking, temperature and solvent used. Site isolation should not be taken for granted as evidenced by dimerization studies performed by Lunkenheimer and Zahn [25]. Iron(II)-catalyzed oxidation of resin-bound Boc-Cys(H)Gly by oxygen in DMSO gave the symmetrical peptide disulfide in 52.2% yield. Addition of dichloromethane, which promotes the swelling of the resin and thereby chain motility, led to an even higher degree of dimerization (74.8%).

Intramolecular cyclizations have been performed on resin-bound peptides, with equal or better results than in the corresponding solution-phase oxidations [26], applying established strategies like oxidation of dithiols by air or oxygen [27], ferricyanide [28], 1,2-diodoethane [29] or DTNB [26a], oxidative deprotection of Cys(Acm) and Cys(Trt) using iodine [26,30] or thallium(III) [26a,31], oxidative deprotection of Cys(Acm) or Cys(MeOBzl) using N-iodosuccinimide [17], oxidative deprotection of Cys(Fm) or Cys(Dnpe) with piperidine [26a,32] and unsymmetrical cysteine activation by Scn-Cl [33] or by the incorporation of Cys(Npys) [34]. Ten Kortenaar and van Nispen treated a resin-bound peptide, containing Cys(Acm), with subsequently Scn-Cl and cysteine, to obtain the open-chain unsymmetrical cysteine peptide [35].

6.1.3. Disulfide bond formation using trityl-type resins

We set out to investigate a new method for the iodolytic detachment of monomeric peptide disulfides from solid supports, thereby exploiting the solvent dependency of iodolysis and the pseudo-dilution caused by immobilization. Barlos had previously reported a similar cleavage of a peptide, anchored through its C-terminal carboxyl function and containing two Cys(Trt) residues, from trityl-type resin I. Simultaneous formation of a disulfide bond was effected by the addition of iodine to the acidic cleavage mixture [36]. In this case, however, the pseudo-dilution principle was not a priori applicable, since cyclization might have occurred in solution after cleavage from the resin.

In our approach, the peptide is anchored to a trityl-type resin through its thiol function. Initial studies concentrated on the use of trityl-type resin V in the synthesis of an 11-residue cyclic analogue of the ssDNA binding loop of gene-V protein encoded by the M13 phage [37]. Cysteamine was coupled to the resin by evaporation of a suspension of both components in TFA. Subsequent SPPS was concluded with the incorporation of a Cys(Acm) residue. The outcome of the iodolytic detachment appeared to be strongly dependent on the solvent used and in agreement with the findings of Kamber (Table 1). Treatment of Cys(Acm) or Cys(Trt) with iodine leads to formation of intermediate sulfenyl iodides (Scheme 2). In DMF, the higher reactivity of Cys(Acm) results in formation of a sulfenyl iodide at the N-terminus of the peptide. Due to site isolation, the only possible continuation should be the attack on the anchoring thiol function (N.B. a Cys(Trt) derivative), thus effecting the cyclization
and concomitant release from the resin (pathway a). Indeed, the sole product of the reaction was the monomeric disulfide 1.

![Diagram of reaction pathways](image)

**Scheme 2**: Possible reaction pathways during iodo-lytic detachment from trityl-type resin V [37].

With other solvent systems, like dioxane or chloroform/methanol (1:1), the outcome of the reaction was not quite as unambiguous. In these cases, the anchoring trityl thioether is first attacked by iodine, effecting the release of the C-terminal sulfenyl iodide from the resin. The pseudo-dilution principle no longer applies now. Attack of the sulfenyl iodide on the N-terminal Cys(Acm) residue leads to the formation of the monomeric disulfide 1 (pathway b). Alternative pathways consist of the attack on another detached sulfenyl iodide (c) or on an anchoring trityl thioether (d), both yielding the parallel symmetrical dimer 2 with unaffected Cys(Acm) at the two identical N-termini. The product composition upon cleavage in chloroform/methanol was further influenced by the degree of mechanical agitation applied during the process. With gentle agitation compound 2 was the major product formed, whereas compound 1 was formed preferentially (ca. 70%) with vigorous agitation.
This result can be explained by considering, that dimerization via pathway d requires only half the amount of iodine compared to cyclization; cyclization is therefore not a favoured process. Vigorous agitation, however, promotes the diffusion of iodine into the polymer matrix and to the "active" sites, thus enhancing the formation of cyclic product 1.

6.1.4. Outline of the investigation

In the current study, the application of trityl-type resin II in the synthesis of peptide disulfides was explored. Several different cysteine derivatives were coupled to the resin. Fmoc-cysteine was anchored for application in two-directional SPPS, the urethane-type protecting group preventing racemization during activation of the carboxyl function. In the two-directional synthesis strategy, the intermediate protection of the carboxyl function, as applied with Fmoc-lysine in the previous chapter, can be omitted now, because the conditions used for the anchoring of Fmoc-cysteine preclude formation of the trityl ester.

Anchoring of Fmoc-cysteine amides, followed by Fmoc SPPS, is also allowed, since no racemization of cysteine amides has been observed under Fmoc-deprotection conditions [39]. This was confirmed in the current study by establishing the chemical and chiral stability of the model peptide Boc-Gly-Cys(Trt)-NH₂ in piperidine/DMF (1:3). C-Terminal cysteine esters are prone to significant racemization under these conditions.

Three different methods of tritylthiol cleavage were examined with peptides anchored to trityl-type resin II through a cysteine side chain, namely iodine oxidation, activation by Scn-Cl and treatment with TFA to yield the free thiol.

First of all, half-times for the iodolytic detachment in DMF and DCM were determined, to confirm the validity of the solvent dependency, observed by Kamber. Two different monomeric disulfides were synthesized analogous to the aforementioned synthesis of the ssDNA binding loop, by oxidation in DMF (Scheme 2; pathway a). One peptide contained a C-terminal cysteine amide, the other was C-terminally elongated according to the two-directional synthesis strategy. A third peptide was synthesized, containing in its sequence both a Cys(Acm) residue as well as an additional Cys(Trt) residue. Iodolytic detachment in DCM should provide the monomeric disulfide, derived from the Cys(Trt) residue and the anchoring trityl thioether, with intact Cys(Acm) residue.

Detachment with Scn-Cl was studied during the synthesis of the open-chain unsymmetrical cystine peptide, derived from the rhesus monkey relaxin sequence A24-B(17-23). Several resin-bound peptides were used to examine the optimal conditions for TFA-effected thiol cleavage. Peptides that contain a free thiol function may be used for non-specific oxidation reactions or in the unsymmetrical thiol activation approach leading to specific disulfide bond formation. Their most promising

application lies, however, in the synthesis of artificial antigens [40]. A cysteamide group at the C-terminus of a peptide has been used as an anchor for fluorescent labels [41].

6.2. Chain assembly on trityl-type resin II

6.2.1. Anchoring of thiol compounds

Free thiols were coupled to trityl-type resin II by evaporation of a suspension containing both components in TFA. The lability of thiols towards air oxidation had to be considered in this reaction. Fmoc-cysteine was coupled directly after its preparation, as described in Chapter 3 (Section 3.3.1). Side-chain deprotection of Fmoc-Cys(Trt)-NH\(_2\) by TFA in the presence of triethylsilane yielded the corresponding free thiol, which was used without further purification. Fmoc-cysteine methyl amide and the corresponding octadecyl amide were obtained upon reduction of the disulfides with tributylphosphine [42] and immediately used for attachment. The synthesis of the protected amides will be described in Chapter 8.

Table 2: Coupling of thiols to trityl-type resin II.

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Equivalents (^a)</th>
<th>Loading (mmol/g)</th>
<th>Coupling yield (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Cys(H)-OH</td>
<td>2</td>
<td>0.20</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>0.30</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.40</td>
<td>74</td>
</tr>
<tr>
<td>Fmoc-Cys(H)-NH(_2)</td>
<td>3</td>
<td>0.20</td>
<td>32</td>
</tr>
<tr>
<td>Fmoc-Cys(H)-NHCH(_3)</td>
<td>5</td>
<td>0.38</td>
<td>70</td>
</tr>
<tr>
<td>Fmoc-Cys(H)-NHC(<em>{18}H</em>{37})</td>
<td>5</td>
<td>0.18</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) Molar equivalence of initial cysteine hydrochloride, initial Fmoc-Cys(Trt)-NH\(_2\), initial [Fmoc-Cys-NHCH\(_3\)], and initial [Fmoc-Cys-NHC\(_{18}H_{37}\)], respectively, with respect to maximum capacity of 0.60 mmol/g. \(^b\) Based on the capacity of the initial chloromethylpolystyrene (0.70 mmol/g).

The outcome of the couplings was qualitatively controlled by suspending a sample of each loaded resin in a few drops of DCM containing iodine. The resulting solutions were spotted on a thin layer chromatogram with the corresponding disulfides as reference. In all cases, \(R_f\)-values in two different solvent systems were in agreement. The IR spectra of the derivatized resins showed characteristic C=O stretching bands between 1667 and 1728 cm\(^{-1}\).
A quantitative evaluation of the couplings is summarized in Table 2. Coupling yields were determined by measuring the Fmoc loading of the derivatized resin. They were satisfactory in all cases. The lower yield for the octadecyl amide might be a consequence of its poor solubility, although at some point during the evaporation of the suspending TFA, all amide appeared to have gone into solution. A more probable reason may be the low accessibility of the aliphatic amide to the anchoring sites within the resin matrix.

Coupling yields varied with the molar equivalence of thiol used, as exemplified by the various couplings of Fmoc-cysteine. Initial loading can thus be controlled, depending on the length of the actual peptide to be synthesized. An increase in molar equivalence from 5 to 10 (cf. Section 3.3.1) did not improve the coupling yield, indicating that the maximum loading was achieved at 0.40 mmol/g and that the conversion of chloromethylpolystyrene into trityl-type resin II must have proceeded in an overall yield of approximately 74%.

### 6.2.2. Choice of model peptides

The resins, derivatized with Fmoc-cysteine and Fmoc-cysteine amide, were applied in the SPPS of the following peptides:

**E. Coli glutaredoxin (10-15)**

Fmoc-Gly-Cys(Acm)-Pro-Tyr(Bu)-Cys(resin II)-Val-OMe

(3a)

**Crustacean cardioactive peptide (CCAP)**

Fmoc-Pro-Phe-Cys(Acm)-Asn(Trt)-Ala-Phe-Thr(Bu)-Gly-Cys(resin II)-NH₂

(4a)

**Rhesus monkey relaxin A (9-16)**

Fmoc-Lys(Boc)-Cys(Trt)-Cys(Acm)-His(Bum)-Ile-Gly-Cys(resin II)-Thr(H)-OAll

(5a)

**Rhesus monkey relaxin B (17-23)**

Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(resin II)-Gly-OMe

(6a)

**Rabbit bone marrow glutaredoxin (77-83)**

Fmoc-Asp(O'Bu)-Cys(Acm)-Ile-Gly-Gly-Cys(resin II)-Ser(H)-OMe

(7)

Glutaredoxins, like thioredoxins, belong to a family of small redox-active proteins. The catalytic centres of these so-called thiol-protein oxidoreductases are located within thiol/disulfide segments, which exhibit the characteristic bis(cysteinyl) sequence motif Cys-X-X-Cys [43]. The main function of glutaredoxins in cells is probably their part in a hydrogen donor system, known to catalyze the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase for DNA synthesis.
The other components of this system are glutathione, NADPH and glutathione reductase [44]. In contrast to the E. Coli protein [45], glutaredoxins isolated from rabbit bone marrow and calf thymus contain two additional half-cystines in positions 78 and 82, which may be of regulatory significance [46]. Both half-cystine pairs are represented in the chosen fragments: peptide 3a contains the active site sequence, whereas the additional pair is included in peptide 7.

Crustacean cardioactive peptide is a highly conserved neuropeptide among arthropoda [30a,47]. Stanger and co-workers reported the synthesis of CCAP by on-resin iodine oxidation of the bis(Acm) analogue [30a]. Both native and synthetic CCAP displayed high accelerating activity on heart preparations.

6.2.3. Further assembly of model peptides: two-directional SPPS

The first step in the synthesis of peptides 3a, 5a, 6a and 7 was the acylation of the pertinent amino acid ester hydrochloride by the in situ activated carboxyl group of Fmoc-cysteine-loaded trityl-type resin II. In case of peptide 6a, a resin with an initial loading of 0.30 mmol/g was used, whereas the others were based on a loading of 0.40 mmol/g. Acylation proceeded analogous to the protocol applied with the Fmoc-lysine-loaded resin (Section 5.3.2), with 5 molar equivalents of the ester in DMF, in the presence of 1.3 equivalents of TBTU and HOBT each. During 20 hours of coupling, the pH of the suspension was maintained at 7 by the regular addition of NMM. The course of the reaction was followed by chromatographic comparison of an iodine-containing suspension of a resin sample with Fmoc-cysteine.

Peptide 4a was synthesized on resin II loaded with Fmoc-cysteine amide (b= 0.30 mmol/g). To test the chiral stability of the system towards Fmoc deprotection conditions, the model peptide Boc-Gly-Cys(Trt)-NH₂ (8) was synthesized in solution. The optical rotation of this compound remained constant during 6-hour treatment with piperidine/DMF (1:3), corresponding to twenty deprotection cycles. Its chemical stability under these conditions was established by TLC and ¹H-NMR.

Table 3: Coupling yields during the synthesis of peptides 3a to 7.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Overall yield (%)</th>
<th>Yield per cycle (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>quant.</td>
<td>quant.</td>
</tr>
<tr>
<td>4a</td>
<td>92</td>
<td>99.0</td>
</tr>
<tr>
<td>5a</td>
<td>quant.</td>
<td>quant.</td>
</tr>
<tr>
<td>6a</td>
<td>84</td>
<td>97.2</td>
</tr>
<tr>
<td>7</td>
<td>86</td>
<td>97.6</td>
</tr>
</tbody>
</table>
Further assembly of the peptides proceeded according to the standard TBTU/HOBt/NMM protocol. As shown in Table 3, in all cases, synthesis could be accomplished in high overall yield. Yields for all separate coupling steps were also determined through the Fmoc loading and showed no major irregularities. Most remarkably, no diketopiperazine formation was observed during the synthesis of peptide 6a.

Similar syntheses on trityl-type resin V proceeded in somewhat lower yields between 91 and 97% per cycle [38], probably due to the partially adsorptive loading of the linker in this resin, as discussed in Chapters 3 and 4 (cf. Section 4.2.4).

6.3. Oxidative detachment with iodine

6.3.1. Preliminary study regarding solvent dependency

A quantitative study to determine the half-time for iodolytic detachment and its solvent dependency was performed with the resin-bound model peptide Fmoc-Ile-Cys(resin II)-Gly-OMe (b = 0.26 mmol/g) (6b), an intermediate in the synthesis of peptide 6a. A resin sample was treated with 4 molar equivalents of iodine in DCM or DMF, the resulting concentration being 3.75 mM for the resin-bound peptide and 15 mM for iodine. The reaction was performed in an ultrasonic bath, to preclude diffusion-governed effects. Quenching occurred at different time intervals by addition of ascorbic acid. Triethylamine was added to neutralize the hydrogen iodide formed. Ascorbic acid was chosen in preference to aqueous thiosulfate, since the latter caused a decolouration of the resin, that was too slow on the used time scale. Cleavage yields could be calculated by comparison of the Fmoc loading before and after iodolytic detachment. The results are graphically represented in Figure 1. Best fitting curve techniques gave the following equations for the loading after detachment (in %):

**DCM:** \[ % = 7.8 + 92.2 \exp (-0.367 t) \]

**DMF:** \[ % = 6.5 + 93.5 \exp (-0.051 t) \]

with \( t \) being the elapsed reaction time in seconds. Small levels of residual loading were observed even after prolonged reaction times. The half-times for iodolytic detachment of thiols from trityl-type resin II, calculated from the two equations, were 1.9 s in case of DCM as the solvent and 13.6 s in case of DMF. The value for DCM is in good accordance with the one for Cys(Trt) measured by Kamber (1-2 s). The cleavage rate in DMF is somewhat enhanced compared to Kamber’s findings (\( t_h = 25-35 \) s), as a consequence of the greater lability towards an electrophilic agent of the anchoring tritylthiol adduct, bearing a benzoxy substituent, with regard to Cys(Trt). The smaller difference in reactivity between Cys(Acm) (\( t_h = 2-3 \) s) and the anchoring tritylthiol adduct in DMF should not lead, however, to a
lower selectivity during cyclization reactions, since this effect is counteracted by the pseudo-dilution phenomenon.

![Graph showing residual loading over reaction time](image)

**Figure 1:** The course of iodolytic detachment of model peptide Fmoc-Ile-Cys-Gly-OMe from trityl-type resin II, in DCM and DMF.

### 6.3.2. Cleavage of model peptides using DMF as the solvent

Peptidyl resins 3a and 4a were treated with iodine in DMF for ten minutes, as described above, leading to the protected monomeric disulfides 3b and 4b, according to pathway a in Scheme 2:

\[
\text{Fmoc-Gly-Cys-Pro-Tyr(Bu)-Cys-Val-OMe} \quad (3b)
\]

\[
\text{Fmoc-Pro-Phe-Cys-Asn(Trt)-Ala-Phe-Thr(Bu)-Gly-Cys-NH}_2 \quad (4b)
\]

Detachment proceeded in 90 and 94% yield, respectively. Although the products were chromatographically pure, the SIMS spectrum of compound 3b showed a minor peak corresponding to twice the expected molecular weight. This phenomenon, which is encountered more often in the MS of cystine peptides, is probably caused by non-covalent dimerization occurring during spectral measurements [37]. MALDI-TOF-MS afforded unequivocal results (Fig. 2).

Amino acid analysis of compound 4b gave somewhat decreased values for Pro and Phe, also after prior removal of the hydrophobic Fmoc protecting group. Similar results were obtained earlier by Stangier et al. with isolated and synthetic CCAP [30a]. Since no irregularities were observed during
Fmoc determinations in each step of the current synthesis, and RP-HPLC of the fully deprotected peptide showed only one major product peak, the incorrect values from the amino acid analysis may probably be neglected in this case.

Figure 2: MALDI-TOF-MS of crude products 3b (C_{43}H_{70}N_8O_{15}S_2; MW_{theor.} 931.1) (a) and 4b (C_{36}H_{60}N_{10}O_{14}S_2; MW_{theor.} 1476.8) (b). The two major peaks in both spectra correspond to artefacts containing Na and K. respectively; the peaks at [M+56] correspond to unidentified artefacts present in most MALDI-TOF mass spectra presented in this dissertation.
6.3.3. Cleavage of a model peptide using DCM as the solvent

Peptidyl resin 5a was treated with iodine in DCM during two minutes, leading to the protected monomeric disulfide 5b with intact Cys(Acm), in 62% yield:

\[
\text{Fmoc-Lys(Boc)-Cys-Cys(Acm)-His(Bum)-Ile-Gly-Cys-Thr(H)-OAll} \quad (5b)
\]

Mass spectrometry and chromatography identified a product carrying a trityl group. Incomplete cyclization is a highly improbable explanation for its occurrence, because of the high reactivity of the Cys(Trt) moiety in DCM \((t_{1/2} = 1-2 \text{ s})\). Moreover, iodine treatment of a product sample for an additional five minutes did not lead to a decrease of the by-product. Based on these findings, we suggest a trityl migration from Cys(Trt) onto the unprotected threonine side chain in position A16. At the moment of the actual disulfide bond formation, threonine is in close proximity to the arising trityl cation in one of several possible pathways leading to compound 5b (Scheme 3: pathway b). Similar S-O shifts have been reported for the benzylic carbonium ion originating from Cys(McBzl) in an acidic medium [48], and for the acetamidomethyl carbonium ion during oxidations by thallium(III) [49]. No addition of iodine to the allyl group was observed.

**Scheme 3:** Possible reaction pathways during iodolytic treatment of peptidyl resin 5a in DCM.
A higher yield might have been achieved by treatment over a somewhat longer period in the presence of TFE. This solvent not only lowers the reactivity of the S-Acm moiety, which allows selectivity of disulfide bond formation to be maintained during a longer reaction time. It also acts as a trityl cation scavenger, thereby impeding the formation of the assumed by-product.

During iodine oxidations on peptidyl resins in DCM, the pseudo-dilution principle no longer applies when detachment from the resin occurs before disulfide bond formation [50]. Detached peptide sulfenyl iodides might be involved in intermolecular disulfide bond formations, leading to oligomeric by-products. In the present study, no such compounds were detected, because a mass spectrum could only be successfully measured on a reducing matrix. The product was chromatographically pure.

### 6.4. Other modes of detachment

#### 6.4.1. Detachment with Scm-Cl

Treatment with Scm-Cl of a peptide containing Cys(Trt) leads to removal of the trityl group and simultaneous activation of the thiol as its sulfenylthiocarbonate. Likewise, peptidyl resin 6a was treated with 5 molar equivalents of Scm-Cl, leading to the activated compound 6e in 86% yield:

\[
\text{Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Scm)-Gly-OMe} \quad (6c)
\]

The activated thiol was coupled to Trt-Cys(H)-O'Bu (11), to give the protected unsymmetrical cystine peptide 6d in 90% yield:

\[
\text{Trt-Cys-O'Bu} \\
\text{Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys-Gly-OMe} \quad (6d)
\]

Although product 6d was chromatographically pure, its mass spectra (SIMS, positive and negative ion mode, and MALDI-TOF-MS) showed rather complicated fragmentation patterns. In all spectra, a peak was found at a mass corresponding to compound 6d missing the Trt-Cys-O'Bu moiety. Its occurrence is probably a consequence of the strong ionizing power inherent to the applied techniques, the relative abundancies of the major peaks differing in all three spectra.

\[
[H\text{-Cys-OH}]_2 \quad \text{\textit{i,ii}→} \quad \text{[AcOH.H-Cys-O'Bu]}_2 \quad \text{\textit{iii,iv}→} \quad \text{[Trt-Cys-O'Bu]}_2 \quad \text{\textit{v}→} \quad \text{Trt-Cys(H)-O'Bu}
\]

**Scheme 4: Synthesis of Trt-Cys(H)-O'Bu (11):** i) tert-butyl acetate/HClO₄; ii) 1 M AcOH/ether; iii) Me₃SiCl/ Et₃N; iv) Trt-Cl/Et₃N; v) tributylphosphine.
Trt-Cys(H)-O\textsuperscript{t}Bu (11) was prepared in three steps from cystine (Scheme 4). As already reported in Chapter 2 (Section 2.3), cystine was esterified through alkyl transfer from tert-butyl acetate and the product 9 was isolated as its acetate [51]. Tritylation and subsequent reduction with tributylphosphine [42] yielded compound 11, which was used in the synthesis of 6d without further purification.

### 6.4.2. Acidolytic detachment

Peptidyl resin 7 was used to optimize the conditions for cleavage of protected peptides with a free thiol group from trityl-type resin II. Continuous cleavage was compared to repeated TFA treatments during shorter periods of time and cleavage in the presence of triethylsilane as a scavenger was compared to cleavage without a scavenger. Cleavage yields were determined by measurement of the Fmoc loading before and after cleavage. The results, combined with results from cleavage experiments on two other peptidyl resins (5a and a precursor of 3a), were used to calculate the half-times for TFA cleavage, as summarized in Table 4.

<table>
<thead>
<tr>
<th>Cleavage condition</th>
<th>Peptidyl resin</th>
<th>% Cleavage</th>
<th>( t_h (\text{min}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x45 min 1% TFA/DCM</td>
<td>5a</td>
<td>38</td>
<td>73 ± 8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>4x15 min 1% TFA/DCM</td>
<td>3a\textsuperscript{a}</td>
<td>59</td>
<td>44 ± 3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>1x45 min 1% TFA/5% TES/DCM</td>
<td>5a</td>
<td>55</td>
<td>38 ± 1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4x15 min 1% TFA/5% TES/DCM</td>
<td>7</td>
<td>89</td>
<td>19</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Peptidyl resin used contained \textit{E. Coli} glutaredoxin (12-15) sequence (b= 0.35 mmol/g).

Cleavage yields for different peptidyl resins under equal conditions were in very good agreement, although cleavage of Fmoc-cysteine from resin II proceeded much faster (Section 3.3.2: \( t_h = 6 \text{ min} \) in 1% TFA/5% TES/DCM). This result indicates that cleavage might be primarily influenced by chain length – due to basicity of the backbone amides [52] – and solubility, rather than the actual amino acid sequence. Repeated TFA treatments gave rise to a significant reduction of the cleavage time by approximately 50% with respect to continuous cleavage; this approach guarantees the preservation of all tert-butyl-type side-chain protecting groups [53].

The tritylthiol cleavage in Cys(Trt) is a reversible reaction in TFA, which can be driven to completion by reducing the liberated trityl cation through the addition of a silane [54]. Likewise, in case of thiol
cleavage from trityl-type resin II, readdition of the liberated thiol was impeded in the presence of triethylsilane, leading to an additional reduction of the cleavage time by 50%.
Optimal cleavage of protected peptide thiols is hence effected by treatment of the peptidyl resin with 1% TFA/DCM in the presence of triethylsilane. After 15 minutes, the mixture is filtered and the filtrate neutralized with pyridine. The cleavage operation is repeated several times. The course of the reaction can be monitored by thin layer chromatography. The combined filtrates are subjected to a standard work-up to yield the protected peptides containing a free thiol function.

6.5. Scope and limitations

New methods for the synthesis of protected cyclic and unsymmetrical open-chain peptide disulfides and peptide thiols were developed, by varying the cleavage conditions for peptides immobilized through a cysteine side chain to trityl-type resin II.

The first method involves oxidative detachment from the resin by iodine treatment. When Cys(Acm) and/or Cys(Trt) residues are incorporated into the peptide sequence, product formation is governed by the pseudo-dilution principle as well as the rules for solvent dependency, observed by Kamber [12]. Incorporation of Cys(Acm) eventually gives the monomeric cyclic disulfides in high yield and purity, if iodolytic detachment is performed in DMF. If detachment takes place in less polar solvents like DCM, the S-Acm moiety remains unaffected, ultimately leading to monomeric disulfides (if Cys(Trt) was incorporated) or symmetrical dimers (no Cys(Trt) incorporated), with additional Acm-protected cysteines.

Activating detachment by Sem-CI and subsequent thiolysis is a most promising strategy in the synthesis of open-chain unsymmetrical peptide disulfides. The third method applies acidolytic cleavage by dilute TFA in the presence of a scavenger, affording protected peptide fragments with a free thiol function, which can be used for conjugation.

Variation of the initial thiol, coupled to the resin, allows further differentiation with respect to the C-terminal function of the product. Coupling of Fmoc-cysteine amide and its substituted analogues eventually leads to peptides with chirally unchanged C-terminal cysteine amides. Amphiphilic peptides become accessible through lipophilically substituted amides, like the octadecyl analogue. Alternatively, C-terminal peptide esters may be synthesized through initial coupling of Fmoc-cysteine, followed by two-directional SPPS. The tendency of C-terminal cysteine esters to racemize during the performance of the Fmoc SPPS protocol puts a limit to the synthetic possibilities, although the chiral stability of the 2-chlorotrityl ester [55], whose formation may occur on the resin, deserves a thorough examination; its application may prove a “remedy” for this shortcoming in the presented strategies.
The ultimate challenge lies in the combined application of selectively cleavable C-terminal esters with the adducts arising from side-chain immobilization of Fmoc-cysteine to a trityl-type resin. It paves the way to regioselective synthesis of complex peptides containing multiple disulfide bonds. Allyl esters are stable towards acid treatment, but may be deprotected under very mild conditions by allyl transfer onto a nucleophile [56]. The reaction is catalyzed by tetrakis(triphenylphosphine)-palladium(0), which unlike many other transition metal catalysts is not poisoned by the presence of sulfur-containing amino acids [57]. Contrary to general belief, the allyl group proved to be stable during short treatment with iodine in DCM, necessary for oxidative detachment from a trityl-type resin. Likewise, 2-chlorotrityl and Dmab esters may prove valuable synthetic building blocks [55,58]. These esters allow the selective liberation of the C-terminal carboxyl function, while the actual peptide is still attached to the resin, enabling continuation by two possible reaction series. The first consists of either two-directional solid-phase fragment condensation or on-resin head-to-tail cyclization, followed by cleavage from the resin according to any of the three methods presented. Head-to-tail cyclization in combination with oxidative detachment leads to bicyclic products. In the second approach, the peptide with an unprotected C and/or N-terminus may be cleaved from the resin by treatment with iodine or dilute TFA. The resulting protected fragment, containing a disulfide bridge or a free thiol, may be further used in either solution or solid-phase convergent synthesis of complex peptides containing more than one disulfide link. Activating detachment by Scn-Cl is less appropriate in this approach, as cleavage might proceed with concomitant C-terminal (trans)esterification.

We believe that the concept of peptide immobilization via an amino acid side chain opens ample new perspectives in the solid-phase assembly of complex peptides and small proteins. More specifically, this chapter has demonstrated the application of thiol immobilization as a powerful tool for regioselective disulfide bond formation in high yield and purity.

6.6. Experimental section

Materials and methods

Nitroprusside solution was used for the detection of free thiols on TLC, producing pink spots [59]. For further specifications, see Appendix A.

\textbf{Fmoc-Cys(\textit{H})-NH}_2

Fmoc-Cys(Trt)-NH\_2 (2.9 g, 5 mmol) was dissolved in 10 ml TFA/TES (95:5). After standing for 1h, the solution was evaporated \textit{in vacuo}. Petroleum ether was added and evaporated. The residual oil was triturated with ether. The precipitate was collected by centrifugation and subsequently washed twice with ether, yielding 1.5 g (88\%) of a white amorphous powder. TLC: \textit{R}_f (A) 0.53, \textit{R}_f (B) 0.75.
Fmoc-Cys(H)-NHCH₃

Tributylphosphine (150 μl, approximately 0.51 mmol) was added to a suspension of 120 mg (0.17 mmol) of [Fmoc-Cys-NHCH₃]₂ in 12 ml DCM. After stirring for 2.5 h at room temperature, the suspension was evaporated in vacuo. The white residue was washed twice with tert-butyl methyl ether using centrifugation. TLC: Rₜ (A) 0.78, Rₜ (B) 0.76.

Fmoc-Cys(H)-NHC₃H₇₇

According to the synthesis of Fmoc-Cys(H)-NHCH₃ₓ, using 200 mg (0.17 mmol) [Fmoc-Cys-NHC₃H₇₇]₂ instead of [Fmoc-Cys-NHCH₃]₂. TLC: Rₜ (A) 0.93, Rₜ (B) 0.98.

Coupling of thiols to trityl-type resin II

The thiol component (meq listed in Table 2) was dissolved in TFA and trityl-type resin II (bₘₐₓ = 0.60 mmol/g) was added. The suspension was evaporated in vacuo. Residual TFA was removed by evaporation with ether (three times) until the residue attained its yellow colour. The loaded resins were washed with DMF, DCM, 2-propanol and ether, each twice. IR (Fmoc-Cys(resin II)-OH): see Chapter 3; (Fmoc-Cys(resin II)-NH₂): 3059, 3027, 2925, 2852, 1691, 1602, 1492, 1451, 1380, 1297, 1247, 1180, 1153, 1117, 1072, 1030, 907, 826, 759, 701, 620, 541 cm⁻¹; (Fmoc-Cys(resin II)-NHC₃H₇₇): 3060, 3027, 2924, 2852, 1667, 1603, 1492, 1451, 1364, 1309, 1241, 1204, 1179, 1156, 1083, 1027, 905, 849, 828, 759, 701, 542 cm⁻¹.

C-Terminal coupling of amino acid esters to Fmoc-Cys(resin II)-OH

DMF containing 5 meq of amino acid ester (3a: H-Val-OMe, 5a: H-Thr(H)-OAll, 6a: H-Gly-OMe, 7: H-Set(H)-OMe) hydrochloride, and 1.3 meq of TBTU and HOBT each, was added to Fmoc-Cys(II)-OH resin, so that the resulting concentration amounted to 1 g resin/5 ml DMF. The apparent pH was adjusted to 7 (most pH paper) with NMM, and kept constant during 20-h shaking. The resin was then three times washed with DMF, twice with 2-propanol and ether each, and dried.

Boc-Gly-Cys(Trt)-NH₂ (8)

Boc-Gly-ONBu (0.90 g, 2.5 mmol) and H-Cys(Trt)-NH₂ (0.76 g, 2.8 mmol) were dissolved in 25 ml dioxane, and the apparent pH of the resulting solution was adjusted to 7.5 by the addition of 0.34 ml triethylamine (2.5 mmol). After stirring for 1 hr at 10°C, the pH was readjusted with 0.17 ml triethylamine, and the solution was stirred for an additional 20 h at room temperature. The solution was then concentrated and the residue dissolved in 50 ml DCM. After washing with 10% KHSO₄, 1 M NaHCO₃ and saturated NaCl (3x25 ml each), the organic layer was dried over Na₂SO₄ and evaporated in vacuo. Crystallization from DCM/petroleum ether gave 1.15 g of compound 8 as white needles (89%). Mp 185-187°C. TLC: Rₜ (A) = 0.64, Rₜ (C) 0.88, Rₜ (F) 0.77. [α]D [25 -6.2 (c=5, DMF), [α]D [25 -9.3 (c=3, piperidine/DMF (1:3)). ¹H-NMR (CDCl₃), δ (ppm): 1.40 (s, 9H, Boc); 2.51/2.80 (dm, 2H, β-CH₂-Cys); 3.65 (d, 2H, J = 5.70 Hz, α-CH₂-Gly); 4.21 (m, 1H, α-CH-Cys); 5.35 (m, 1H, α-NH-Gly); 5.77/6.39 (d, 2H, -CO-NH₂); 6.71 (d, 1H, J = 7.72 Hz, α-NH-Cys); 7.17-7.31 (m, 9H, arom Trt); 7.37-7.43 (m, 6H, arom Trt).

The optical rotation was stable during 6 h in piperidine/DMF (1:3). A sample of the product (200 mg) was treated with the same solvent mixture during 1.5 h. Thin layer chromatography in all three solvent systems
showed no change after this period. Evaporation and crystallization from DCM/petroleum ether gave 180 mg (90%) of a product with the same melting point and optical rotations.

**Iodosyl cleavage experiments in DCM on peptidyl resin 6b (Fig. 1)**

Peptidyl resin 6b (30 mg, 0.26 mmol/g) was suspended in 2.1 ml DCM containing 7.9 mg iodine (31 μmol, 15 mM). After a cleavage period of 3, 5, 8, 10 or 20 s, a solution of 41 mg ascorbic acid (233 μmol) and 22 μl triethylamine (158 μmol) in 5 ml DCM was added, which caused an immediate decolouration. All operations were carried out in an ultrasonic bath. The resin was then filtered off and washed with DMF, 2-propanol and ether, each twice.

* The solution was prepared by dissolving the ascorbic acid and triethylamine in 375 μl DMF, and adding DCM up to 5 ml.

**Iodosyl cleavage experiments in DMF on peptidyl resin 6b (Fig. 1)**

Peptidyl resin 6b (30 mg, 0.26 mmol/g) was suspended in 2.1 ml DMF containing 7.9 mg iodine (31 μmol, 15 mM). After a cleavage period of 8, 15, 30, 45, 60, 75, 120 or 180 s, a solution of 41 mg ascorbic acid (233 μmol) and 22 μl triethylamine (158 μmol) in 375 μl DMF was added, which caused an immediate decolouration. All operations were carried out in an ultrasonic bath. The resin was then filtered off and washed with DMF, 2-propanol and ether, each twice.

**Fmoc-Gly-Cys-Pro-Tyr(Bu)-Cys-Val-OMe (3b)**

Peptidyl resin 3a (540 mg, 0.32 mmol/g) was suspended in 50 ml DMF containing 175 mg iodine (0.7 mmol, 15 mM). After 10-min treatment in an ultrasonic bath, a solution of 900 mg ascorbic acid (5.1 mmol) and 0.48 ml triethylamine (3.4 mmol) in 10 ml DMF was added, which caused an immediate decolouration. The resin was filtered off and washed twice with DMF. The combined filtrates were evaporated *in vacuo* below 40°C. The residual oil was precipitated with water, upon which a few drops of pyridine were added to maintain neutrality. The precipitate was collected by centrifugation and washed twice with water. Drying over KOH yielded 145 mg (90%) 3b as a white amorphous powder. TLC: Rf (A) 0.91, Rf (B) 0.68. [α]D 25°-23.1 (c=1, DMF). SIMS (+), NBA: m/z 1863.2 [2(M+H)]+, 953.5 [M+Na]+, 931.6 [M+H]+; SIMS (-), NBA: m/z 1083.2 [M+NBA]-, 929.3 [M-H]-, 707.4 [M-Fmoc]. MALDI-TOF-MS: m/z 987.1 [M+56]+, 969.1 [M+K]+, 953.1 [M+Na]+. Amino acid analysis: Pro 0.99 (1), Gly 1.00 (1), Val 1.02 (1), Tyr 0.84 (1).

**Fmoc-Pro-Phe-Cys-AsnTru-Ala-Phe-Thr(Bu)-Gly-Cys-NH2 (4b)**

According to the synthesis of 3b, using 500 mg peptidyl resin 4a (0.15 mmol/g) instead of 3a, All mentioned quantities were adjusted (to 45%). Yield: 104 mg (94%). TLC: Rf (A) 0.69, Rf (B) 0.45. [α]D 25°-21.4 (c=0.5, DMF). SIMS (+), NBA: m/z 1498.9 [M+Na]+, 1476.9 [M+H]+, 1254.9 [M-Fmoc]; SIMS (-), NBA: m/z 1628.6 [M+NBA]-, 1607.5 [M+Cs]+, 1474.6 [M-H], 1253.2 [M-Fmoc]. MALDI-TOF-MS: m/z 1532.5 [M+56]+, 1514.5 [M+K]+, 1498.6 [M+Na]+. Amino acid analysis: Asp 1.00 (1), Thr 0.92 (1), Pro 0.90 (1), Gly 1.02 (1), Ala 1.02 (1), Phe 1.94 (2). Threonine was determined separately after hydrolysis in the absence of thioglycolic acid.
A 3-mg sample of the product 4b was subjected to complete deprotection. Cleavage of the N-terminal Fmoc group was effected by treatment with 100 μl piperidine/DMF (1:3) during 20 min. After precipitation and three washings with tert-butyl methyl ether by centrifugation, Asn(Trt) and Thr(Bu) were deprotected by dissolving the pellet in TFA/TES/water (95:2.5:2.5). After standing for 2.5 h, precipitation and washings followed as aforementioned. RP-HPLC (20-60%/40 min): t_R 20.8 min (71.7%).

\[ \text{Fmoc-Lys(Boc)-Cys(Acm)-His(Bum)-Ile-Gly-Cys-Thr(H)-OAll (5b)} \]

Peptidyl resin 5a (400 mg, 0.27 mmol/g) was suspended in 30 ml DCM containing 110 mg iodine (0.43 mmol, 15mM). After 2-min treatment in an ultrasonic bath, a solution of 275 mg Na_2S_2O_3·5H_2O (1.1 mmol) in 30 ml water was added, which caused immediate decolouration of the solution. The aqueous phase was extracted with DCM (3×30 ml). The resin, residing at the interface of the aqueous and the organic layer, was removed during this process. The combined organic layers were washed with water (3×30 ml), dried over Na_2SO_4 and evaporated in vacuo. The yellow product was recovered in 62% yield (95 mg). TLC: R_f (A) 0.94, R_f (B) 0.89 (Trt+). SIMS (+), DTE/DTT/sulfolane: m/z 1624.1 [M+Trt]^+, 1381.6 [M+H]^+. Amino acid analysis: Thr 0.93 (1), Gly 1.06 (1), Ile 1.00 (1), Lys 1.00 (1), His 0.90 (1). Glycine was slightly elevated because of cysteine decomposition; threonine was determined separately after hydrolysis in the absence of thioglycolic acid.

\[ \text{Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Scm)-Gly-OME (6c)} \]

Peptidyl resin 6a (900 mg, 0.21 mmol/g) was suspended in 15 ml chloroform/methanol (2:1), containing 85 μl Scm-Cl (0.95 mmol) and 40 μl diethylamine (0.38 mmol), at 0°C. After shaking for 1 h at 0°C and for another 75 min at room temperature, the resin was filtered off and washed three times with DCM and once with DMF. The combined filtrates were washed with water (3×20 ml), dried over Na_2SO_4 and evaporated in vacuo. The residue was triturated with ether to yield 200 mg (86%) of a pale yellow powder. TLC: R_f (A) 0.81, R_f (B) 0.59. [α]_D^25 -23.9 (c=0.5, DMF).

\[ \text{Fmoc-Ala-Gln(Trt)-Ile-Ala-Ile-Cys(Trt-Cys-O\text{'}Bu)-Gly-OME (6d)} \]

Peptide 6c (100 mg, 0.08 mmol), Trt-Cys(H)-O\text{'}Bu (200 mg, 0.48 mmol) and triethylamine (2 μl, 0.02 mmol) were suspended in 10 ml DCM. After stirring for 20 h, additional Trt-Cys(H)-O\text{'}Bu (200 mg) was added, followed by stirring for another 24 h. The suspension was then evaporated in vacuo and the residue washed with DCM/petroleum ether and ether. Yield: 115 mg (90%). TLC: R_f (A) 0.86, R_f (B) 0.73. [α]_D^25 -15.2 (c=0.5, DMF). SIMS (+), NBA: m/z 1594.0 [M+Na]^+, 1328.6 [M-Trt]^+, 1175.6 [M-(Trt-Cys-O\text{'}Bu)+Na]^+. SIMS (-), NBA: m/z 1346.5 [M-Fmoc]^-, 929.2 [M-(Trt-Cys-O\text{'}Bu)-Fmoc]^-. MALDI-TOF-MS: m/z 1592.7 [M+Na]^+, 1350.7 [M-Trt+Na]^-, 1175.7 [M-(Trt-Cys-O\text{'}Bu)+Na]^+. Amino acid analysis: Glu 1.01 (1), Gly 1.60 (1), Ala 2.02 (2), lle/allo-Ile 2.00 (2). Isoleucine adjacent to cysteine leads to partial racemization during hydrolysis [60].

\[ \text{[AcOH.H-Cys-O\text{'}Bu]_2 (9)} \]

See Chapter 2, Experimental Section, compound 4a.
[Trt-Cys-\(\text{O}^\text{Bu}\)] \(\text{II}\). (10)

A solution of 1.6 g of compound 9 (3.4 mmol), 0.86 ml trimethylsilyl chloride (6.8 mmol) and 1.9 ml triethylamine (13.6 mmol) in 30 ml DCM was refluxed for 2 h. Trityl chloride (2.1 g, 7.5 mmol) and triethylamine (1.05 ml, 7.5 mmol) were then added and reflux was continued for another 20 h. After addition of 1.2 ml MeOH (30 mmol), the solution was evaporated \text{in vacuo}. The oily residue was dissolved in 25 ml ether. The ether phase was washed with 10% KHSO\(_4\) (2x15 ml), 1 M NaHCO\(_3\) (2x15 ml) and water (4x15 ml), dried over Na\(_2\)SO\(_4\) and evaporated, to yield 2.85 g of a yellow oil, which was used for reduction to 11 without further purification. TLC: \(R_f\) (B) 0.88.

\text{Trt-Cys(\text{H})-\text{O}^\text{Bu}}\) \(\text{II}\). (11)

Tributylphosphine (150 \(\mu\)l, ca. 0.6 mmol) was added to a solution of 500 mg (0.6 mmol) of 10 in 5 ml 2-propanol. After stirring for 2.5 h at room temperature, 135 mg (0.6 mmol) cysteaminium dihydrochloride was added to quench any residual tributylphosphine and the solution was stirred for additional 10 min. The suspension was taken up in 30 ml ether. The ether phase was washed with 10% KHSO\(_4\) (2x15 ml), 1 M NaHCO\(_3\) (2x15 ml) and water (4x15 ml), dried over Na\(_2\)SO\(_4\) and evaporated \text{in vacuo}. The resulting yellow solid (460 mg, 92%) was immediately used in the synthesis of 6d. TLC: \(R_f\) (B) 0.95.

\text{Fmoc-Asp(\text{O}^\text{Bu})-Cys(\text{Acm})-\text{Ile-Gly-Gly-Cys(resin II)-Ser(\text{II})-OMe}}\) (7)

Loading: b = 0.28 mmol/g. Amino acid analysis: Asp 1.00 (1), Ser 0.84 (1), Gly 2.17 (2), Ile/allo-Ile 1.00 (1). Glycine was elevated because of cysteine and serine decomposition.

Acidolytic cleavage experiments (Table 4)

To 40 mg of peptide resin, 1 ml of the cleavage mixture was added. The resin was either kept in the same solution for 45 min, or the resin was filtered every 15 min and the solution renewed. In both cases, after each 14-min period, the reaction tube was placed in an ultrasonic bath for one minute. The resin was then filtered and washed with DCM and ether, each twice. Cleavage yields were calculated from comparison of the Fmoc loadings before and after cleavage (Appendix B).

6.7. References


20 See Section 2.1.2.


Application of Trityl-Type Resin II in the Synthesis of Protected Peptide Disulfides


38 Reference 37 and unpublished results by I.F. Eggen.


The pseudo-dilution principle only applies in pathway a (Scheme 3), when detachment proceeds with concomitant disulfide bond formation.


Chapter 7

Evaluation of a Novel Trityl-Type Protecting Group for the Asparagine, Glutamine and Cysteine Side Chains

7.1. Introduction

The preparation of 4,4'-dimethoxy-4''-methyltrityl alcohol as a precursor of trityl-type resin VIII has been reported in Chapter 3 (Section 3.2.3). In the current chapter, this moiety is applied as a protecting group for the carboxamide functions in asparagine and glutamine, thereby aiming at a mildly cleavable adduct for Fmoc SPPS, which was not available hitherto. The compatibility as thiol protecting group in cysteine is also investigated.

7.1.1. Cysteine side-chain protection

Protecting groups for the strongly nucleophilic thiol side chain in cysteine may be roughly classified into three categories, i.e. thioethers, thioether aminals and sulfenyl derivatives [1]. A brief overview regarding the synthesis and cleavage of the most important ones is given below and their structures are depicted in Figure 1. It should be noted that, unless indicated otherwise, deprotection conditions only include those that lead to the free thiol. Oxidative cleavage schemes, directly leading to disulfides, have been discussed in the previous chapter.

Thioethers

Thioether derivatives are generally prepared by nucleophilic substitution reactions in which the thiol function acts as the nucleophile. The reactions are carried out either under basic conditions in the presence of an (ar)alkylhalide, or under acidic conditions promoting the formation of a cation which alkylates the thiol function [2]. Historically, the first cysteine protecting group was the benzyl thioether [3]. For a long time, the only available method for its removal was sodium in liquid ammonia [4], but HF treatment is now preferred [5]. As both methods are quite harsh towards complex peptides, a set of acidolytically more labile protecting groups has been developed by
electronic “fine-tuning”. Acid lability increases with the stability of the liberated carbonium ion [6] in the approximate order: benzyl (BzI) ~ tert-butyl ("Bu) [7] < 4-methylbenzyl (McBzl or Mb) [8] < 4-methoxybenzyl (McOBzl or Mob) [9] ~ benzhydryl (Dpm) [10] < trityl (Trt) [11] ~ 4,4'-dimethoxy- benzhydryl (Ddm) [12] < 2,4,6-trimethoxybenzyl (Tmob) [13] < 4-methoxytrityl (Mmt) [14] < 4,4',4''-trimethoxytrityl (TMTtr) [15]. The first of these may be used in combination with Boc chemistry; the limit is reached with the McOBzl group, whose application in Boc SPPS is restricted to the synthesis of smaller peptide sequences due to diminished stability towards 50% TFA [8,16]. The others are suitable for use in Fmoc SPPS.

The S-trityl group requires 2-hour treatment with 90% TFA and the presence of a scavenger for its complete removal. Tritylthiol cleavage is a reversible reaction in TFA: evaporation of the solvent TFA or addition of trityl alcohol drives the equilibrium towards tritylthiol bond formation [12b], addition of a silane scavenger towards cleavage by reduction of the trityl cation [17]. The latter condition enhances the reduction of the indole ring in tryptophan to the indoline, and therefore a more labile protection is preferred in a synthetic scheme, in which acidolysis leads to the free thiol. Application of the more labile Tmob group also leads to modification of tryptophan by the cation produced upon cleavage. However, addition of one methoxy substituent to the trityl moiety (Mmt) allows complete deprotection by 0.5% TFA in the presence of triethyilsilane to proceed within 40 minutes at room temperature, not affecting tryptophan [14].

The unmodified S-trityl protecting group is perfectly suitable in an oxidative deprotection or activation strategy towards regioselective disulfide bond formation [18]. Cleavage may furthermore be accomplished by mercury(II) [19] or silver ions [10], also affording a free thiol function. The base-labile S-9-fluorenylmethyl (Fm) and S-2-(2,4-dinitrophenyl)ethyl (Dnpe) derivatives provide disulfides by β-elimination upon treatment with piperidine/DMF (1:1) [20,21].

**Thioether amininals**

The mostly applied thioether amininal-type protecting function for cysteine is the S-acetamidomethyl (Acm) group, introduced by acid-catalyzed reaction of cysteine with acetamidomethanol [22]. It is stable towards acid and base treatment and may be removed by mercury(II) or silver ions [23]. Acm blocking is very often used in conjunction with Trt blocking in oxidative deprotection and activation schemes [18]. Protecting groups that have been derived from the Acm moiety include trimethyl- (Tacm) and phenylacetamidomethyl (Pacm) [24,25]; they exhibit similar stability and deprotection behaviour, but in addition the Pacm group may be enzymatically cleaved by penicillin amidohydrolase.
Figure 1: Protecting groups for the thiol moiety in cysteine.
Sulfenyl derivatives

Of the sulfenyl-type protecting groups, the tert-butylsulfenyl (S'Bu) group has found ample application in the synthesis of cysteine-containing peptides [26]. A general method for the facile preparation of S-(alkylsulfenyl)cysteines, including SEt, S'Bu and STrt derivatives, has been described by Rietman et al., and involves Scm-activation of the parent cysteine, followed by reaction with the pertinent thiol [27]. Removal of sulfenyl-type protecting groups is accomplished by reduction with thiols or trialkylphosphines [26b,28].

Besides unsymmetrical disulfides, the vast group of sulfenyl derivatives includes cystine peptides and S-sulfonates (SO₃H) [29,30]. S-Carboalkoxysulfenyl (Scm, Sce) and S-(3-nitro-2-pyridinesulfenyl) (Npys) derivatives of cysteine directly lead to unsymmetrical disulfides upon thiolysis [31,32]. Recently, the orthogonal coupling between a peptide thiocarboxylic acid and a peptide, containing an S-(2-pyridinesulfenyl)-protected (Pyr) cysteine at its N-terminus, has been reported [33].

7.1.2. Carboxamide protection

Side reactions associated with the unprotected carboxamide function

Although asparagine and glutamine may be incorporated in peptide synthesis without side-chain protection, their use as such is accompanied by several side reactions [34].

During the activation of N°-protected asparagine, and to a lesser extent glutamine, with a condensing agent such as DCC or BOP, dehydration of the carboxamide function results in the formation of the corresponding nitrile. The intermediate in this reaction is believed to be an isocyanide (Scheme 1). Its occurrence is somewhat suppressed by the addition of HOBr; alternatively, preformed activated esters may be used. Dehydration is mainly a problem associated with Fmoc chemistry, since in Boc chemistry, the nitrile is rehydrated to a large extent in the final HF cleavage.

Scheme 1: Intramolecular dehydration of N°-protected asparagine upon activation with DCC.

A second side reaction, the intramolecular cyclization of asparagine and glutamine peptides and esters to give the succinimide and glutarimide derivatives, is catalyzed by base. Two possible pathways are depicted for asparagine in Scheme 2.
Scheme 2: Intramolecular cyclic imide formation of asparagine.

Pathway a involves the acylation of an internal peptide bond with concomitant release of ammonia. Hydrolytic ring opening affords a mixture of the α and β-peptide in case of asparagine. Imide formation is largely sequence dependent and occurs most frequently within the asparaginyl-glycyl sequence as well as in aspartyl containing peptides. Cyclization along route b starts with the nucleophilic attack of the side-chain carboxamide nitrogen on the α-carbonyl group, which is accompanied by cleavage of a peptide or ester bond. This mechanism is only of importance in case of a C-terminal asparagine residue.

Finally, the intramolecular aminolytic cyclization of an N-terminal glutamine residue to its pyroglutamyl analogue gives rise to irreversible premature chain termination during peptide synthesis (Scheme 3). The reaction is promoted by weakly acidic conditions and observed during deprotection of the $N^\alpha$-Boc protecting group as well as in couplings using HOBT.

Scheme 3: Intramolecular pyroglutamate formation.
In addition to these side reactions, the presence of an unprotected carboxamide group tends to lower the solubility of both the amino acid derivative and the resultant peptide, and enhance the formation of gelatinous β-sheet aggregates due to extensive hydrogen bridging. As a consequence, chemical reactions are strongly retarded. Protection of the carboxamide side chain of asparagine and glutamine has therefore become standard nowadays.

*Protecting groups for the carboxamide function*

The carboxamide group gives rise to adducts of similar stability compared to the thiol group. Consequently, a range of acidolytically cleavable side-chain protecting groups, analogous to the above listed set of thioethers, has been developed for asparagine and glutamine (Fig. 2). Acid lability of the carboxamide adducts [35] increases in the approximate order: 4-methoxybenzyl (Mob) [36] < benzhydryl (Dpm) [5] < 2,4-dimethoxybenzyl (Dmob) [37] < 4,4'-dimethoxybenzhydryl (Mbh or Ddm or Dod) [38] < 2,4,6-trimethoxybenzyl (Tmob) [39] ~ 9-xanthenyl (Xan) [40] < trityl (Trt) [41] < cyclopropyl(dimethylcarbinyl (Cpd) [42] ~ 4-methyltrityl (Mtt) [43] < 2,2',4,4'-tetramethoxybenzhydryl (Tbh) [44]. Most groups are introduced by alkylation of the amide function by the pertinent carbonium ion under acidic conditions, whereas DCC-mediated condensation of selectively blocked aspartic or glutamic acid with substituted benzylamines or 2,2',4,4'-tetramethoxybenzhydrylamine afforded the benzyl-type and Tbh derivatives, respectively.

![Diagram](image-url)

*Figure 2: Protecting groups for the carboxamide moiety in asparagine and glutamine.*
Widely applied are Xan, Mbh, Tmob, Trt and Mtt protection. Xan has been utilized for carboxamide blocking in Boc chemistry. Although it is prematurely lost after several cycles of repetitive deprotection, no significant side reactions are to be expected once it has been incorporated into the peptide chain [45]. Its applicability in Fmoc chemistry is hampered due to difficult preparation and unfavourable solubility. The use of Xan, Mbh (benzhydryl-type, also Tbh [44]) and Tmob (benzyl-type) protection is moreover limited to peptides that do not contain tryptophan, since the cations liberated upon cleavage alkylate the indole ring in position 2 [46].

Trityl-type cations do not attack the indole ring and the corresponding protecting groups are therefore most suitable for general application. As TFA deprotection of an N-terminal Asn(Trt) residue tends to remain incomplete due to a repulsive field effect exerted by the positive charge on the proximate amino group – this one, being the most nucleophile, is the first to be protonated and subsequently counteracts the protonation of the carboxamide moiety leading to deprotection –, the more acid-labile Mtt group is preferred, but still requires high-concentration TFA treatment for its complete removal [47].

In general, glutamine is more easily deprotected than asparagine, because the aforementioned field effect between main-chain amine/amide and side-chain amide is exerted over a longer distance in glutamine (one extra methylene group) and hence of somewhat less importance.

7.1.3. 4,4'-Dimethoxy-4''-methyltrityl (Dmt) protection in the Fmoc/Trt strategy

Mild deprotection conditions and low electrophilicity of the formed cations favour the application of trityl-type protection rather than tert-butyli-type protection for amino acid side chains in high-purity peptide synthesis. Barlos and co-workers first established the superiority of an Fmoc/Trt protection scheme as opposed to the conventional Fmoc/4Bu variant in SPPS by synthesizing several tryptophan and methionine-containing peptides, using a set of amino acid derivatives with only trityl-type side-chain protecting groups, which could be cleaved by treatment with 3% TFA or less. Purity of all products was greater than 90%, while the conventional Fmoc/4Bu method afforded products of typically 40-80% purity [14,48].

In the Fmoc/Trt strategy, side-chain protection of maximum acidolytic lability is provided by the 2-chlorotrityl (Clt) group for Asp, Glu and Tyr [49,50], the unmodified trityl (Trt) group for Ser and Thr [51], 4-methyltrityl (Mtt) for Lys [52], 4-methoxytrityl (Mmt) for Cys and His [14,53], and 4,4',4''-trimethoxytrityl (Tmt) for Arg [54]. Utilizing these, treatment with 5% TFA in DCM/TESE (95:5) would allow the quick complete removal of all, Arg(Tmt) being the threshold residue. No side-chain protection for Trp would be required under these conditions.
A shortcoming of the method is the lack of very acid-labile trityl-type protecting groups for the carboxamide functions in asparagine and glutamine. In an attempt to complete the set of highly labile Fmoc/Trt-type amino acid derivatives, we used the 4,4'-dimethoxy-4''-methyltrityl (Dmt) group [55] for protection of the carboxamide function and evaluated its applicability by establishing the minimum conditions for complete deblocking. Likewise, the Dmt group was tested for cysteine thiol protection as a (more labile) alternative to the Mmt group.

7.2. Synthesis and evaluation of Dmt-protected amino acid derivatives

7.2.1. Dmt-protected asparagine and glutamine derivatives

For derivatization of amino acid side chains, Dmt alcohol 1 was converted into the chloride 2 with acetyl chloride [56]. At this stage, a contamination of residual 4,4'-dimethoxybenzophenone (cf. Chapter 3, Section 3.2.3) was precipitated by addition of petroleum ether to a solution of crude 2 in dichloromethane and subsequently removed by filtration. Pure compound 2 was then used directly for thiol protection (Section 7.2.2) or further converted into the corresponding amine 3 by treatment with ammonia (Scheme 4), for application in carboxamide protection (Scheme 5) [57]. The route to tritylamide adducts, proceeding via acid-catalyzed alkylation of the carboxamide function by a trityl-type cation, was successfully applied in the preparation of the Trt and Mtt derivatives [41,43], but failed for Dmt presumably due to the enhanced acid lability of the resulting adduct.

Scheme 4: Preparation of Dmt-Cl (2) and Dmt-NH₂ (3) from Dmt-OH (1): i) acetyl chloride; ii) NH₂/DCM
In one of our preliminary communications, we addressed the difficulties of synthesizing immobilized tritylamide adducts [58]. Although couplings of the β or γ-carboxyl group to an (ar)alkylamide proceed inherently more difficult than couplings between an α-carboxyl and an α-amino group [59], the best route, originating from the preparation of benzyl-substituted amides [36,37], proved to be the DCC-mediated condensation of selectively blocked aspartic and glutamic acid derivatives to tritylamine linkers [57]. Unexpectedly, addition of the standard catalysts HOBt or HONSu appeared to slow down the reaction and was consequently abandoned, which is permissible since there is no risk of racemization during the activation of the β or γ-carboxyl function [60].

Based on these considerations, the synthesis of Fmoc-Asn/Gln(Dmt)-OH (6a/b) was started with the condensation of Fmoc-Asp/Glu(OH)-OAll (4a/b) [61] to Dmt-NH₂ (1.8 molar equivalents), mediated by DCC (1.1 equivalents) in dichloromethane, during 24 hours at room temperature. The resulting crude Dmt-amides 5a/b were subjected to allyl ester cleavage, catalyzed by tetrakis(triphenylphosphine)palladium(0) and with N-methylaniline as the allyl-accepting nucleophile [62]. Since the excess of 3 could not be removed by crystallization, the products 6a/b were subjected to treatment with a strong basic anion exchanger. In this protocol, solutions of the crude products in methanol were rotated overnight with Amberlite resin in the acetate form, exchanging the acetate ion for the Fmoc-Asn/Gln(Dmt)-OH anion. Washing of the resulting loaded resins with an acetic acid-containing solution effected the release of the pure amorphous compounds in 24 and 28% overall yield, respectively. Loss of material occurred during the final purification, which demands optimization for routine use.

Fmoc-AA(OH)-OAll 4a/b →i→ Fmoc-AB(Dmt)-OAll 5a/b →ii→ Fmoc-AB(Dmt)-OH 6a/b

Scheme 5: Synthesis of Fmoc-Asn(Dmt)-OH (6a) and Fmoc-Gln(Dmt)-OH (6b): i) DCC/Dmt-NH₂ (3); ii) [(C₅H₅)₂Pd(N-methylaniline). AA = Asp (a) or Glu (b); AB = Asn (a) or Gln (b).

The Dmt-amide adducts 6a/b proved to be completely stable towards weak acid, i.e. 3-hour treatment with AcOH/TFE/DCM (2:2:6) at room temperature. The conditions for acidolysis of the carboxamide protections in Fmoc-Asn(Trt)-OH, Fmoc-Asn(Dmt)-OH, Fmoc-Gln(Trt)-OH and Fmoc-Gln(Dmt)-OH were assessed by thin layer chromatography, following treatment with TFA solutions in DCM/TES (95:5) of different concentrations. The results are summarized in Table 1.

The cleavage experiments clearly indicate a big difference in stability between Asn(Xyz) and Gln(Xyz) on the one hand (field effect) and between Trt and Dmt on the other hand. Fmoc-Gln(Dmt)-OH is, due to the presence of three cation-stabilizing substituents in the trityl system, a valuable addition to the set of very acid-sensitive Fmoc/Trt-type derivatives, being completely deprotected by 5% TFA within 5 minutes. Fmoc-Asn(Dmt)-OH is somewhat more stable in acidic medium: complete
deprotection is accomplished by 10% TFA within 30 minutes. Although this compound should be a good alternative to the much less labile Asn(Trt) analogue, whose complete deprotection could not be effected with 10% TFA even after prolonged treatment, enhanced lability towards 5% TFA, or indeed 1% TFA in case of Gln, might only be achieved after adding a third alkoxy substituent to the trityl moiety (4,4',4''-trimethoxytrityl). A similar conclusion has been drawn regarding the synthesis of protected peptide amides on trityl-type resins [57,60].

Table 1: Results from acidolytic deprotections of side-chain carboxamides in DCM/TES (95:5) by TLC.

<table>
<thead>
<tr>
<th>Cleavage condition</th>
<th>Fmoc-AB(Xyz)-OH</th>
<th>% Cleavage after 5 min</th>
<th>% Cleavage after 30 min</th>
<th>% Cleavage after 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% TFA</td>
<td>Asn(Trt)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asn(Dmt)</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln(Trt)</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln(Dmt)</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% TFA</td>
<td>Asn(Trt)</td>
<td>4</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asn(Dmt)</td>
<td>40</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln(Trt)</td>
<td>40</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln(Dmt)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10% TFA</td>
<td>Asn(Trt)</td>
<td>2</td>
<td>30</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Asn(Dmt)</td>
<td>40</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Gln(Trt)</td>
<td>10</td>
<td>60</td>
<td>100</td>
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<tr>
<td></td>
<td>Gln(Dmt)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

7.2.2. Dmt-protected cysteine derivatives

S-Dmt-Cysteine 7 was synthesized analogous to the Mmt derivative [14] by S-alkylation of cysteine hydrochloride with Dmt-Cl 2 (0.5 molar equivalents) in DMF, during 24 hours at room temperature (Scheme 6). The nucleophilicity of the thiol function is high enough to allow reaction with the small portion of the trityl component, that is present in the cationic form; the protonation of the amino group prevents alkylation of the latter. Without further purification the Fmoc group was introduced using Fmoc-ONSu after the procedure by ten Kortenaar et al. [63]. The crude product 8 could not be purified by crystallization and was therefore also treated with Amberlite resin (cf. Section 7.2.1).
affording the pure amorphous compound 8 in 38% overall yield. A higher yield may be achieved by optimizing the purification procedure.

\[
\text{HCl·H-Cys(H)-OH} \quad \rightarrow \quad \text{H-Cys(Dmt)-OH} \quad 7 \quad \rightarrow \quad \text{Fmoc-Cys(Dmt)-OH} \quad 8
\]

**Scheme 6: Synthesis of Fmoc-Cys(Dmt)-OH (8):** i) Dmt-Cl (2); ii) Fmoc-OSu/Et$_3$N.

Like S-Trt and S-Mmt, the S-Dmt adduct is stable towards bases and very weak acids. Treatment of 7 with 30% piperidine in DMF for 24 hours at room temperature left the Dmt function completely unaffected, thus supporting the applicability of Fmoc-Cys(Dmt)-OH 8 in Fmoc SPPS [64]. Likewise, compound 8 remained intact during 45-minute treatment with a 2:2:6 mixture of AcOH/TFE/DCM, which is used for the cleavage of protected peptide fragments from trityl-type resin I. Compound 8 was completely deprotected within 25 minutes by treatment with 0.5% TFA in DCM/TES (95:5). While under similar circumstances after 30 minutes, 2-3% of the S-Trt and 98% of the S-Mmt groups had been removed, the increase in acid lability with S-Dmt will promote the elaboration of synthesis strategies involving the highly selective acidolytic deprotection of specific cysteines (preferably protected by Dmt) in the presence of other cysteines, protected by Trt, and other amino acids, whose side chains are blocked by protecting groups of the tert-butyl-type [14].

Iodolytic oxidation and activation with Scm-Cl yielded the same products for both Fmoc-Cys(Dmt)-OH and Fmoc-Cys(Trt)-OH.

### 7.3. Conclusions

The 4,4'-dimethoxy-4''-methyltrityl (Dmt) derivatives of asparagine, glutamine and cysteine, presented in this chapter, are new members in the class of protected amino acids of the Fmoc/Trt type, which are required in the SPPS of sensitive peptides. Although their preparation needs optimization and the actual applicability in SPPS remains to be tested, these new derivatives are very promising. Their acid labilities are highly enhanced with regard to the residues that are most commonly used nowadays (Trt or Mmt protection for Cys, and Trt or Mtt for Asn/Gln). The Dmt cation has a specific absorption maximum at 496 nm; this allows the monitoring of its cleavage in the presence of, yet without interference of the Trt cation, which has its maximum absorption at a lower wave length [65]. Incorporation of Dmt-protected cysteines during peptide assembly would allow the subsequent selective deblocking of these cysteines by short treatment with 0.5% TFA in DCM/TES (95:5). Under these conditions the indole ring of tryptophan is not reduced. Removal of the topical protection while
the peptide is still attached to the solid support, may be followed by on-resin disulfide bond formation or modification of the thiol group (e.g. fatty acid acylation [66]).

The increased aciolytic lability of the new asparagine and glutamine derivatives ensures complete deprotection of the side chains by treatment with dilute TFA (5-10%) during a short period of time, yielding stable cations that would not, like the Xan, Tmob and Mbh cations, attack the indole ring. Substitution on the trityl-type protecting group by a third alkoxy group should however be pursued, especially in the case of asparagine, to further enhance lability, also in this last “stronghold”.

### 7.4. Experimental section

**Materials and methods**

The Amberlite resin in its hydroxide form was purchased from Sigma (A-4269). For further specifications, see Appendix A.

**Dmt-Cl (2)**

Dmt-OH (1) (2 g, 6 mmol) was dissolved in 10 ml acetyl chloride. The solution was stirred overnight at room temperature and then evaporated in vacuo. Three evaporations from PE afforded a dry solid, which was dissolved in 20 ml DCM. Contaminating 4,4’-dimethoxybenzophenone was precipitated by addition of PE and filtered off. The filtrate was evaporated in vacuo, suspended in PE and once more evaporated, yielding 1.60 g (76%) of a yellow-green amorphous powder. Mp 84-86°C. TLC: Rf (D) 0.74, produced a dark orange spot upon spraying with H2SO4/MeOH (1:1).

**Dmt-NH2 (3)**

Dmt-Cl (2) (1 g, 2.8 mmol) was dissolved in 25 ml NH3-saturated DCM and the solution was stirred overnight at room temperature. The formed NH3Cl was then filtered off and washed with DCM. The combined filtrates were evaporated in vacuo, and triturated with PE, yielding 0.84 g (89%) of a yellow amorphous powder. TLC: Rf (D) 0.37, produced a yellow spot upon spraying with H2SO4/MeOH (1:1).

**Fmoc-Asn(Dmt)-OH (6a)**

A solution of 0.60 g Fmoc-Asp(OH)-OAll (4a, 1.5 mmol), 0.34 g DCC (1.65 mmol) and 0.90 g Dmt-NH2 (3, 2.7 mmol) in 30 ml DCM was stirred for 24 h at RT. The formed DCC was then filtered off and washed with 15 ml DCM. The combined filtrates were washed with 1% KHSO4, water and saturated NaCl (each 2x15 ml), dried over Na2SO4 and evaporated in vacuo, affording compound 5a as a yellow oil, with Rf (G) 0.82. To cleave the allyl ester, the oil was taken up in 80 ml of nitrogen-flushed CHCl3, containing 0.40 g [(C6H5)2P]2Pd (0.35 mmol) and 0.83 ml N-methylaniline (7.5 mmol). After 4-h stirring, work-up proceeded as above. The remaining oil was dissolved in 60 ml MeOH and, upon addition of 7 g Amberlite resin in its acetate form, rotated overnight. The resin was then filtered off, washed with MeOH, resuspended in 60 ml of a 95:5:3 mixture of CHCl3/MeOH/AcOH, rotated for 3 h, again filtered off and washed with 40 ml CHCl3. The combined filtrates
were washed with water (6x20 ml), dried over Na₂SO₄ and evaporated in vacuo. The oily residue was suspended in PE, affording dark orange amorphous 6a (0.24 g, 24%) upon evaporation. Mp 112-114°C. TLC: R₅ (C) 0.80, R₅ (H) 0.85. [α]D²⁵ -11.3 (c=1, MeOH). ¹H NMR (CDCl₃), δ (ppm): 2.25 (s, 3H, CH₃-Dmt); 2.65/2.87 (dm, 2H, β-CH₂); 3.70 (s, 6H, OCH₃-Dmt); 4.12 (t, 1H, CH-Fmoc); 4.36 (m, 2H, CH₂-Fmoc); 4.63 (m, 1H, α-CH); 6.06 (d, 1H, J = 8.73 Hz, α-NH); 6.75 (d, 4H, J = 8.73 Hz, arom Dmt); 7.02 (m, 8H, arom Dmt); 7.21-7.41 (m, 4H, arom Fmoc); 7.54 (m, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

Reference Fmoc-Asn(Trt)-OH, TLC: R₅ (C) 0.77, R₅ (H) 0.84.

**Fmoc-Gln(OMe)-OH** (6b)

According to the synthesis of 6a, using 0.62 g of Fmoc-Glu(OH)-OAll (4b) instead of Fmoc-Asp(OH)-OAll (4a). Yield: 0.29 g (28%). Mp 106-108°C. TLC: R₅ (C) 0.82, R₅ (H) 0.81. [α]D²⁵ -2.5 (c=1, MeOH). ¹H NMR (CDCl₃), δ (ppm): 2.09 (m, 2H, β-CH₂); 2.29 (s, 3H, CH₃-Dmt); 2.53 (m, 2H, γ-CH₂); 3.73 (s, 6H, OCH₃-Dmt); 3.98 (m, 1H, α-CH); 4.12-4.46 (m, 3H, CH-Fmoc/CH₂-Fmoc); 5.91/6.05 (dd, 1H, J = 7.39 Hz, α-NH); 6.70 (m, 1H, γ-NH); 6.79 (d, 4H, J = 8.73 Hz, arom Dmt); 7.05 (m, 8H, arom Dmt); 7.23-7.42 (m, 4H, arom Fmoc); 7.57 (m, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

Compound 5b, TLC: R₅ (G) 0.77. Reference Fmoc-Gln(Trt)-OH, TLC: R₅ (C) 0.81, R₅ (H) 0.86.

**H-Cys(Dmt)-OH** (7)

Cysteine hydrochloride (0.95 g, 6 mmol) and Dmt-Cl (2) (1.06 g, 3 mmol) were dissolved in 6 ml DMF. After stirring for 24 h at RT, the solution was concentrated in vacuo, 25 ml water was added and the pH of the suspension was adjusted to 6 by the addition of 1 M NaOH. The white precipitate was collected by centrifugation and washed with water and ether, three times each. Yield: 1.15 g (88%). Mp 155-166°C (dec.). TLC: R₅ (C) 0.42, R₅ (H) 0.86.

Reference H-Cys(Trt)-OH, TLC: R₅ (C) 0.46, R₅ (H) 0.82.

**Fmoc-Cys(Dmt)-OH** (8)

Compound 7 (1.1 g, 2.5 mmol) was dissolved in 4 ml water in the presence of 0.35 ml triethylamine (2.5 mmol). To this mixture a solution of 0.8 g (2.4 mmol) Fmoc-ONSu in 4 ml dioxane was added in one portion. The pH of the reaction mixture was maintained at 8.5-9.0 for 15 min by the addition of triethylamine. After stirring for an additional 15 min, the mixture was taken up in 20 ml ethyl acetate. The resulting solution was washed with 10% KHSO₄, water and saturated NaCl (each 2x10 ml), dried over Na₂SO₄ and evaporated in vacuo. Further purification by treatment with Amberlite anion exchanger (12 g) was performed as described in the synthesis of 6a, yielding product 8 (0.7 g, 43%) as a pale orange amorphous solid. Mp 70-85°C (dec.). TLC: R₅ (C) 0.87, R₅ (H) 0.89. [α]D²⁵ +7.0 (c=1, CHCl₃). ¹H NMR (CDCl₃), δ (ppm): 2.23 (s, 3H, CH₃-Dmt); 2.68 (m, 2H, β-CH₂); 3.66 (s, 6H, OCH₃-Dmt); 4.12 (m, 2H, α-CH/CH-Fmoc); 4.24 (m, 2H, CH₂-Fmoc); 5.34 (d, 1H, α-NH); 6.70 (d, 4H, arom Dmt); 7.03-7.39 (m, 12H, arom Fmoc(4H)/arom Dmt(8H)); 7.52 (m, 2H, arom Fmoc); 7.69 (d, 2H, arom Fmoc).

Reference Fmoc-Cys(Trt)-OH, TLC: R₅ (C) 0.88, R₅ (H) 0.91.
7.5. References


2. The same principles basically apply to the tritylation of any functional group (Chapter 1, Scheme 3).


29 The relaxin fragment A24-B(17-23) was designed for use in a convergent synthesis scheme, Cys^{A24} and Cys^{B22} being "each other's protection" (Chapters 2 and 6).


31 See Chapter 2 (Section 2.1.2) on the use of Scm-Cl and Scm-Cl.


Preparation and use of Fmoc-Asp(Glu(OCH)3)-OH are yet to be reported, but the Clt group has proved efficient for α-carboxyl protection in convergent peptide synthesis [Athanassopoulos, P., Barlos, K., Gatos, D., Hatzi, O. and Tzavara, C. (1995) Tetrahedron Lett. 36, 5645-5648].

Novabiochem Letters L/97, Calbiochem-Novabiochem GmbH, Switzerland.


The abbreviation “Dmt” has been used once before in 1995 by Barlos et al. [Reference 54] to denote the 4,4’-dimethoxytrityl moiety for arginine side-chain protection; no mention has appeared since.


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Birmingham, pp. 701-704.


61 These two derivatives are commercially available, but a new synthesis route is presented in Chapter 8 of this thesis (Section 8.3).

62 See Chapter 5.


64 We realize that the application of (neutral) Ac-Cys(Dmt)-NHR in this experiment would lead to a more conclusive result; H-Cys(Dmt)-OH was however used in analogy to H-Cys(Mnt)-OH in Reference 14.

65 See Chapter 3, Section 3.4.2. A typical procedure for testing the completeness of Dmt removal involves short treatment of a sample of the pertinent peptide or peptidyl resin with TFA in DCM, followed by spectroscopic measurement.

Chapter 8
Preparation of Amino Acid Derivatives
Hydrazides, amides and allyl esters

8.1. Introduction

Several protected amino acid derivatives were encountered in the course of our studies regarding trityl-type resins. Fmoc-amino acid hydrazides were used as the first residues to be coupled to these resins in the synthesis of peptide hydrazides, since yields starting from universally applicable Fmoc-hydrazide were unsatisfactory (Chapter 4). The recommended approach presupposes the easy conversion of all Fmoc-amino acids into the corresponding hydrazides. A general procedure is described in the current chapter and the accompanying analytical data for the new derivatives are listed. The same procedure was used for the preparation of Fmoc-amino acid amides.

In the second part of this chapter, a route leading to some useful Fmoc-amino acid allyl ester derivatives is reported. Both these and the amides are valuable building blocks in the synthesis of peptides with modified C-termini via side-chain attachment to solid supports; they may be either directly coupled to the resin or, in the N'-deprotected form, used for C-terminal elongation in two-directional SPPS. Allyl ester deprotection at a later stage of the synthesis may be followed by on-resin head-to-tail cyclization (Chapter 5).

8.2. Preparation of Fmoc-amino acid hydrazides and amides

The preparation of Fmoc-amino acid hydrazides and amides is hampered by the same problems as encountered for their peptidic analogues (Sections 4.1.2 and 5.1.1). Moreover, apart from those side reactions associated with specific amino acid side chains, the presence of the base-labile Fmoc group as such precludes the application of alkaline reaction media and hence hydrazinolysis and aminolysis of unactivated esters.
In the past, various approaches have been employed in the synthesis of amides and \( N' \)-protected hydrazides (\(-\text{CO-}N_2H_2-Z/\text{Boc}/\text{Trt}\)), including the use of preformed activated esters and \textit{in situ} activation as the mixed anhydride or with dicyclohexylcarbodiimide (DCC) [1-3]. Superior yields and suppression of racemization (during activation of \( N \)-acyl amino acids) were achieved by performing DCC-mediated couplings in the presence of \( N \)-hydroxysuccinimide (HONSu) or 1-hydroxybenzotriazole (HOBT) (Fig. 1); the intermediate \( O \)-acylisourea, which tend to yield by-products through rearrangement, are immediately converted into the reactive ONSu or OBt esters, eventually resulting in products of higher purity [4,5]. Protected amino acids and peptides could be transformed into the corresponding hydrazides and primary amides by DCC/HOBt-mediated couplings in the presence of hydrazine or aqueous ammonia, respectively [6,7]. Substituted peptide amides were obtained by DIPCIDI-mediated reaction of protected peptide acids with alkylammonium HOBT salts [8].

\[
\begin{align*}
\text{HONSu} & \quad \begin{array}{c}
\text{HOBT}
\end{array}
\end{align*}
\]

\textit{Figure 1: Additives in DCC-mediated couplings.}

Our approach towards the preparation of hydrazides and amides, as depicted in Scheme 1, is similar to the one used by Galavera and co-workers [9]. They preactivated protected amino acids and dipeptides with DCC in the presence of HONSu, in a solvent, in which DCU precipitated and could be removed prior to reaction of the activated species with dianimomethane dihydrochloride, an ammonia-releasing agent, affording primary amides. Likewise, we used THF as the solvent for preactivation; only in case of the very insoluble Fmoc-His(Bum)-OH, DMF had to be added to the medium. After a 5-hour reaction, precipitated DCU was removed by filtration; besides the obvious aim of higher-purity products, this method has an additional advantage for the purification of compounds that crystallize spontaneously during the subsequent formation. This conversion of the activated ONSu-ester was accomplished by treatment with the desired amino component upon its neutralization with acetic acid [10].

\[
\begin{align*}
\text{Fmoc-AA-OH} & \quad -i,ii \rightarrow \text{Fmoc-AA-ONSu} \quad -iii \rightarrow \text{Fmoc-AA-NH-X}
\end{align*}
\]

\textit{Scheme 1: Preparation of Fmoc-amino acid hydrazides and amides:} \( i \) DCC/HONSu, \( ii \) removal of DCU by filtration; \( iii \) \( X \)-NH\(_2\), at pH 6. \( X = \text{NH}_2, \text{Boc-NH}, \text{H or (ar)alkyl} \).
<table>
<thead>
<tr>
<th>Hydrazide</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>$[\alpha]_D$</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-$N_2$H$_3$</td>
<td>90</td>
<td>170 (lit. 171)</td>
<td>---</td>
<td>0.68</td>
</tr>
<tr>
<td>Fmoc-Ala-$N_2$H$_3$</td>
<td>85</td>
<td>193-195</td>
<td>-25.3$^{1a}$</td>
<td>0.44</td>
</tr>
<tr>
<td>Fmoc-Cys(Ala)-$N_2$H$_3$</td>
<td>90</td>
<td>132-134</td>
<td>-26.2$^{1a}$</td>
<td>0.28</td>
</tr>
<tr>
<td>Fmoc-Cys(Trt)-$N_2$H$_3$</td>
<td>80</td>
<td>208-210</td>
<td>-5.8$^{1a}$</td>
<td>0.74</td>
</tr>
<tr>
<td>Fmoc-Asp(O'Bu)-$N_2$H$_3$</td>
<td>89</td>
<td>92-94</td>
<td>-20.4$^{1a}$</td>
<td>0.58</td>
</tr>
<tr>
<td>Fmoc-Glu(O'Bu)-$N_2$H$_3$</td>
<td>84</td>
<td>154-156</td>
<td>-17.8$^{1a}$</td>
<td>0.52</td>
</tr>
<tr>
<td>Fmoc-Phe-$N_2$H$_3$</td>
<td>70</td>
<td>203-205</td>
<td>-17.3$^{1a}$</td>
<td>0.54</td>
</tr>
<tr>
<td>Fmoc-His(Boc)-$N_2$H$_3$</td>
<td>92</td>
<td>94-104</td>
<td>-2.9$^{1b}$</td>
<td>0.20</td>
</tr>
<tr>
<td>Fmoc-Ile-$N_2$H$_3$</td>
<td>80</td>
<td>230-233</td>
<td>-2.9$^{1b}$</td>
<td>0.54</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-$N_2$H$_3$</td>
<td>87</td>
<td>137-139</td>
<td>-18.1$^{1a}$</td>
<td>0.47</td>
</tr>
<tr>
<td>Fmoc-Leu-$N_2$H$_3$</td>
<td>92</td>
<td>172-174</td>
<td>-36.2$^{1a}$</td>
<td>0.52</td>
</tr>
<tr>
<td>Fmoc-Met-$N_2$H$_3$</td>
<td>82</td>
<td>184-186</td>
<td>-7.9$^{1c}$</td>
<td>0.49</td>
</tr>
<tr>
<td>Fmoc-Asn(Trt)-$N_2$H$_3$</td>
<td>89</td>
<td>195-197</td>
<td>-10.9$^{1a}$</td>
<td>0.58</td>
</tr>
<tr>
<td>Fmoc-Gln(Trt)-$N_2$H$_3$</td>
<td>87</td>
<td>180-183</td>
<td>-10.2$^{1a}$</td>
<td>0.55</td>
</tr>
<tr>
<td>Fmoc-Ser(O'Bu)-$N_2$H$_3$</td>
<td>81</td>
<td>160-162</td>
<td>+2.5$^{1b}$</td>
<td>0.64</td>
</tr>
<tr>
<td>Fmoc-Ser(Trt)-$N_2$H$_3$</td>
<td>97</td>
<td>178-180</td>
<td>+19.3$^{1a}$</td>
<td>0.62</td>
</tr>
<tr>
<td>Fmoc-Thr(O'Bu)-$N_2$H$_3$</td>
<td>92</td>
<td>139-141</td>
<td>+6.5$^{1a}$</td>
<td>0.69</td>
</tr>
<tr>
<td>Fmoc-Val-$N_2$H$_3$</td>
<td>85</td>
<td>236</td>
<td>-15.2$^{1d}$</td>
<td>0.49</td>
</tr>
<tr>
<td>Fmoc-Trp-$N_2$H$_3$</td>
<td>86</td>
<td>189-190</td>
<td>-16.1$^{1c}$</td>
<td>0.40</td>
</tr>
<tr>
<td>Fmoc-Tyr(O'Bu)-$N_2$H$_3$</td>
<td>69</td>
<td>106-108</td>
<td>-7.4$^{1a}$</td>
<td>0.62</td>
</tr>
<tr>
<td>Fmoc-Phe-$N_2$H$_3$-Boc</td>
<td>62</td>
<td>174-176</td>
<td>-29.8$^{1a}$</td>
<td>0.78</td>
</tr>
<tr>
<td>Fmoc-Pro-$N_2$H$_3$-Boc</td>
<td>72</td>
<td>144-145</td>
<td>-65.3$^{1c}$</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Universal applicability was demonstrated by the easy preparation of hydrazides (including Fmoc-$N_2$H$_3$ [11]), $N'$-Boc-hydrazides (Table 1), primary amides (Table 2) and several secondary amides (Table 3) in high yield and purity, using hydrazine acetate, tert-butylcarbazate, ammonium acetate and the corresponding (ar)lylammonium acetates for aminolysis, respectively. Due to insolubility of ammonium acetate and n-octadecylamine, the solvent THF was substituted by DMF in these cases.
The presented method for condensation of tris(hydroxymethyl)aminomethane to amino acid derivatives is superior to the original protocol by Whittaker and co-workers, which involved the alkaline aminolysis of unactivated esters during a period of several days [12]. Sluggish crystalization of primary amides was reflected in relatively low yields. The specific problem regarding the synthesis of arginine hydrazides was addressed in Chapter 4 (Section 4.3.1).

To allow direct anchoring to a solid support, several secondary amides derived from Asp, Glu, Lys, Ser, Thr and Tyr were selectively deprotected at their side chains by acidolysis (Table 4). Side-chain deprotection of Fmoc-Ser(Bu)-NH-Octadecyl was incomplete even after 4-hour treatment with neat TFA; the O-Trt analogue, whose complete deprotection required 30-minute treatment with 5% TFA in the presence of TES, was used instead. The conversion of Cys/Cyt amide derivatives into the free thiols and their applications have been described in Chapter 6.

All products were identified by $^{1}$H-NMR, with the exception of [Fmoc-Cys-NH-Octadecyl], owing to its insolubility in the available deuterated solvents; characterization of this compound took place by means of SIMS. The presence of rotamers due to partial $sp^2$ hybridization of the amide C-N bond in methyl and isopropyl amides is evidenced by a degeneracy of the methyl signal in the $^{1}$H-NMR-spectra of these compounds.

**Table 2:** Fmoc-amino acid primary amides (Footnotes: see page 179).

<table>
<thead>
<tr>
<th>Amide</th>
<th>Yield (%)</th>
<th>$mp$ (°C)</th>
<th>$[al_D]^{25}$</th>
<th>$R_f^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Ala-NH$_2$</td>
<td>63</td>
<td>189-190</td>
<td>-12.7$^{1a}$</td>
<td>0.52</td>
</tr>
<tr>
<td>Fmoc-Cys(Trt)-NH$_2$</td>
<td>70</td>
<td>94-97</td>
<td>-5.2$^{1b}$</td>
<td>0.76</td>
</tr>
<tr>
<td>Fmoc-Phe-NH$_2$</td>
<td>46</td>
<td>234</td>
<td>-21.4$^{1c}$</td>
<td>0.61</td>
</tr>
<tr>
<td>Fmoc-Gly-NH$_2$</td>
<td>58</td>
<td>168-169</td>
<td>---</td>
<td>0.37</td>
</tr>
<tr>
<td>Fmoc-Leu-NH$_2$</td>
<td>63</td>
<td>185-186</td>
<td>-27.5$^{1a}$</td>
<td>0.62</td>
</tr>
<tr>
<td>Fmoc-Thr(Bu)-NH$_2$</td>
<td>54</td>
<td>109-111</td>
<td>+19.5$^{1b}$</td>
<td>0.75</td>
</tr>
<tr>
<td>Fmoc-Val-NH$_2$</td>
<td>65</td>
<td>203-205</td>
<td>+2.2$^{1b}$</td>
<td>0.44</td>
</tr>
</tbody>
</table>
### Table 3: Fully protected Fmoc-amino acid secondary amides (Footnotes: see page 179)

<table>
<thead>
<tr>
<th>Amide</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>$[\alpha]_D^{25}$</th>
<th>$R_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Fmoc-Cys-NH-Methyl]$_2$</td>
<td>82</td>
<td>212-214</td>
<td>-86.9$^{1b}$</td>
<td>0.82</td>
</tr>
<tr>
<td>[Fmoc-Cys-NH-Octadecyl]$_2$</td>
<td>98</td>
<td>190-193</td>
<td>-30.3$^{1b}$</td>
<td>0.93</td>
</tr>
<tr>
<td>Fmoc-Asp(O'Bu)-NH-Octadecyl</td>
<td>62</td>
<td>73-76</td>
<td>$+2.5^{1r}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Glu(O'Bu)-NH-Octadecyl</td>
<td>80</td>
<td>102-104</td>
<td>-3.4$^{1g}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Allyl</td>
<td>80</td>
<td>179-180</td>
<td>-18.6$^{1a}$</td>
<td>0.86</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Benzyl</td>
<td>88</td>
<td>199-201</td>
<td>-20.4$^{1c}$</td>
<td>0.91</td>
</tr>
<tr>
<td>Fmoc-Phe-NH(CHOH)$_3$</td>
<td>55</td>
<td>150-153</td>
<td>-6.5$^{1a}$</td>
<td>0.49</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Cyclohexyl</td>
<td>67</td>
<td>218-219</td>
<td>-13.1$^{1c}$</td>
<td>0.90</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Decyl</td>
<td>74</td>
<td>125-127</td>
<td>-12.1$^{1a}$</td>
<td>0.90</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Isopropyl</td>
<td>74</td>
<td>193-194</td>
<td>-9.7$^{1c}$</td>
<td>0.86</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Methyl</td>
<td>75</td>
<td>192-193</td>
<td>-15.4$^{1a}$</td>
<td>0.84</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Phenyl</td>
<td>75</td>
<td>242-243</td>
<td>$+5.6^{1c}$</td>
<td>0.90</td>
</tr>
<tr>
<td>Fmoc-Phe-NH-Octadecyl</td>
<td>89</td>
<td>124-126</td>
<td>-13.0$^{1b}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-NH-Methyl</td>
<td>92</td>
<td>149-151</td>
<td>-11.0$^{1a}$</td>
<td>0.73</td>
</tr>
<tr>
<td>Boc-Lys(Fmoc)-NH-Methyl</td>
<td>91</td>
<td>143-145</td>
<td>-4.2$^{1a}$</td>
<td>0.72</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-NH-Octadecyl</td>
<td>30</td>
<td>126-127</td>
<td>$+5.2^{1g}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Ser(Boc)-NH-Octadecyl</td>
<td>68</td>
<td>99-101</td>
<td>$+17.7^{1g}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Ser(Trt)-NH-Methyl</td>
<td>71</td>
<td>199-200</td>
<td>$+17.5^{1a}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Ser(Trt)-NH-Octadecyl</td>
<td>83</td>
<td>oil</td>
<td>$+15.1^{1b}$</td>
<td>0.92</td>
</tr>
<tr>
<td>Fmoc-Thr(Boc)-NH-Cyclohexyl</td>
<td>61</td>
<td>141-143</td>
<td>$+13.0^{1a}$</td>
<td>0.93</td>
</tr>
<tr>
<td>Fmoc-Thr(Boc)-NH-Methyl</td>
<td>87</td>
<td>93-96</td>
<td>$+14.6^{1a}$</td>
<td>0.91</td>
</tr>
<tr>
<td>Fmoc-Tyr(Boc)-NH-Methyl</td>
<td>94</td>
<td>151-153</td>
<td>-4.9$^{1a}$</td>
<td>0.86</td>
</tr>
</tbody>
</table>
Table 4: Semi-protected Fmoc-amino acid secondary amides (Footnotes: see page 179).

<table>
<thead>
<tr>
<th>Amide</th>
<th>Yield (%)</th>
<th>mp (°C)</th>
<th>([\alpha_l]_D^{25})</th>
<th>(R_f^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Asp(OH)-NH-Octadecyl</td>
<td>82</td>
<td>144-147</td>
<td>-12.0(\text{a})</td>
<td>0.48</td>
</tr>
<tr>
<td>Fmoc-Glu(OH)-NH-Octadecyl</td>
<td>76</td>
<td>141-143</td>
<td>+3.7(\text{b})</td>
<td>0.55</td>
</tr>
<tr>
<td>HCl.Fmoc-Lys(H)-NH-Methyl</td>
<td>97</td>
<td>121-124</td>
<td>+7.2(\text{c})</td>
<td>0.05</td>
</tr>
<tr>
<td>HCl.H-Lys(Fmoc)-NH-Methyl</td>
<td>97</td>
<td>132-134</td>
<td>+16.6(\text{c})</td>
<td>0.12</td>
</tr>
<tr>
<td>Fmoc-Ser(H)-NH-Methyl</td>
<td>90</td>
<td>197-198</td>
<td>+6.2(\text{c})</td>
<td>0.52</td>
</tr>
<tr>
<td>Fmoc-Ser(H)-NH-Octadecyl</td>
<td>81</td>
<td>124-127</td>
<td>+3.8(\text{b})</td>
<td>0.68</td>
</tr>
<tr>
<td>Fmoc-Thr(H)-NH-Methyl</td>
<td>92</td>
<td>177-180</td>
<td>+3.0(\text{c})</td>
<td>0.54</td>
</tr>
<tr>
<td>Fmoc-Tyr(H)-NH-Methyl</td>
<td>94</td>
<td>232</td>
<td>-10.5(\text{c})</td>
<td>0.55</td>
</tr>
</tbody>
</table>

8.3. Preparation of allyl ester derivatives

The classical route towards allyl esters involves the acid-catalyzed reaction of the free carboxyl group with allyl alcohol [13]. Based on the protocol by Wang and co-workers, who reported the synthesis of various amino acid and peptide esters without detectable racemization by reaction of the cesium salts with alkyl halides [14], Kunz used allyl bromide, either in solution or immobilized to a solid support, as the alkylating reagent [15,16].

We started the synthesis of various allyl ester derivatives for Fmoc SPPS by treatment of the corresponding Boc-amino acid cesium salts with allyl chloride (Scheme 2). This approach was chosen to prevent cleavage of the Fmoc moiety during neutralization of the amino acid derivative with Cs₂CO₃-solution. The Boc-amino acid allyl esters were obtained as oils or low-melting solids in 77-87% yield (Table 5). The routes for further processing towards the synthetic targets Fmoc-Asp(OH)-OAll, Fmoc-Glu(OH)-OAll, Fmoc-Lys(H)-OAll and Fmoc-Lys(H)-Pro-OAll are depicted in Scheme 2; analytical data can be found in Table 6.

*Fmoc-Asp(OH)-OAll* and *Fmoc-Glu(OH)-OAll*

The title compounds were prepared from Boc-Asp(O'Bu)-OAll and Boc-Glu(O'Bu)-OAll. Their treatment with 95% TFA for 3 hours resulted in complete removal of the Boc protecting group, whereas cleavage of the side-chain 'Bu-ester remained incomplete due to a repulsive field effect exerted by the positive charge arising on the α-amino group [17]. After introduction of the \(N^\alpha\)-Fmoc group, according to ten Kortenaar et al. [18], the development of a charge is impeded; a subsequent 1-hour treatment with TFA resulted in complete removal of the 'Bu-group, affording Fmoc-Asp(OH)-
OAll and Fmoc-Glu(OH)-OAll in 55 and 71% overall yield, respectively. The products were identified by $^1$H-NMR and elemental analysis; values for the optical rotation were in close agreement with those measured for the commercially available compounds.

Several different routes towards Fmoc-Asp/Glu(OH)-OAll have been reported in the past, all starting from Fmoc-Asp/Glu(O'Bu)-OH. In the approach by Trzeciak and Bannwarth, the allyl esters were formed through reaction with allyl bromide, using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base [19]. Barany and Albericio either applied a combination of allyl bromide and DIPEA or allyl alcohol and DCC [20]. In all cases, side-chain deprotection with neat TFA led to the title compounds.

Melting points of the reported glutamic acid derivatives corresponded to the one found by us (116-117 [19], 120-121 [20] and 113-125 (Novabiochem) vs. 114-116°C), whereas the melting points measured for Fmoc-Asp(OH)-OAll varied significantly depending on the preparation: 92-93°C [19,20], 125-140°C (Novabiochem) and 146-148°C (our product). The disagreement may be explained by the different conditions used for crystallization.

\[
\begin{align*}
\text{Boc-} &\text{AA-OH} \quad \text{\(-i\rightarrow Boc-\text{AA-}O \quad \text{Cs}^+ \quad \text{\(-ii \rightarrow Boc-\text{AA-OAll}\)}
\end{align*}
\]

\[
\begin{align*}
\text{Boc-Asp/Glu(O'Bu)-OAll} \quad \text{\(-iii \rightarrow TFA.H-Asp/Glu(OH)-OAll + TFA.H-Asp/Glu(O'Bu)-OAll\)}
\end{align*}
\]

\[
\begin{align*}
\text{\(-iv \rightarrow Fmoc-Asp/Glu(OH)-OAll + Fmoc-Asp/Glu(O'Bu)-OAll \quad \text{\(-iii \rightarrow Fmoc-Asp/Glu(OH)-OAll}\)}
\end{align*}
\]

\[
\begin{align*}
\text{\(-iv \rightarrow TFA.H-Lys(Z)-OAll \quad \text{\(-iv \rightarrow Fmoc-Lys(Z)-OAll \quad \text{\(-v \rightarrow HBr.Fmoc-Lys(H)-OAll\)}\)}
\end{align*}
\]

\[
\begin{align*}
\text{Boc-Pro-OAll} \quad \text{\(-vi \rightarrow HCl.H-Pro-OAll \quad \text{\(-vi \rightarrow Fmoc-Lys(Boc)-Pro-OAll \quad \text{\(-vi \rightarrow HCl.Fmoc-Lys(H)-Pro-OAll\)}\)}
\end{align*}
\]

**Scheme 2:** Synthesis of allyl ester derivatives: i) 20% aqueous $\text{C}_2\text{CO}_2$; ii) allyl chloride; iii) $\text{TFA}/\text{H}_2\text{O (95:5)}$; iv) Fmoc-ON$_2$/Et$_3$N; v) 33% HBr/4cOH; vi) 4 M HCl/dioxane; vii) Fmoc-Lys(Boc)-OPfp/Et$_3$N

**Fmoc-Lys(H)-OAll and Fmoc-Lys(H)-Pro-OAll**

Preparation of the new compound Fmoc-Lys(H)-OAll proceeded in 44% overall yield based on starting Boc-Lys(Z)-OAll. The Boc group was selectively removed from the latter by short treatment with 95% TFA and substituted by the Fmoc group. Cleavage of the benzyloxy carbonyl (Z) side-chain protecting group with HBr in acetic acid afforded the desired product according to $^1$H-NMR.
The dipeptidic product was synthesized by an active ester coupling of Fmoc-Lys(Boc)-OPfp with H-Pro-OAll, the latter being obtained from 4 M HCl/dioxane treatment of Boc-Pro-OAll. The resulting Fmoc-Lys(Boc)-Pro-OAll led to the second title compound in 95% overall yield after a similar acid treatment. As with Fmoc-Lys(Boc)-NH-Methyl and Boc-Lys(Fmoc)-NH-Methyl, a prolonged reaction time needed for complete N'-Boc removal (3 vs. 0.5-2 hours) indicates the higher stability of N'-adducts in comparison with Nα-adducts; this has previously also been observed for resin-bound lysine derivatives (Section 5.2.2).

Table 5: Boc-amino acid allyl esters (Footnotes: see next page).

| Allyl ester   | Yield (%) | mp (°C) | $\left[\alpha\right]_D^{25}$ | $R_F$  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc-Asp(O'Bu)-OAll</td>
<td>87</td>
<td>oil</td>
<td>n.d.</td>
<td>0.92</td>
</tr>
<tr>
<td>Boc-Glu(O'Bu)-OAll</td>
<td>77</td>
<td>51-52</td>
<td>n.d.</td>
<td>0.95</td>
</tr>
<tr>
<td>Boc-Lys(Z)-OAll</td>
<td>83</td>
<td>oil</td>
<td>-17.5$^b$</td>
<td>0.85</td>
</tr>
<tr>
<td>Boc-Pro-OAll</td>
<td>82</td>
<td>oil</td>
<td>-49.3$^b$</td>
<td>0.92</td>
</tr>
<tr>
<td>Boc-Thr(H)-OAll</td>
<td>86</td>
<td>oil</td>
<td>-11.1$^b$</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Table 6: Miscellaneous allyl ester derivatives (Footnotes: see next page).

| Allyl ester   | Yield (%) | mp (°C) | $\left[\alpha\right]_D^{25}$ | $R_F$  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Asp(OH)-OAll</td>
<td>55</td>
<td>146-148</td>
<td>-15.4$^h$ (ref. -15.4)</td>
<td>0.80</td>
</tr>
<tr>
<td>Fmoc-Glu(OH)-OAll</td>
<td>71</td>
<td>114-116</td>
<td>-18.2$^h$ (ref. -19.0)</td>
<td>0.77</td>
</tr>
<tr>
<td>TFA.H-Lys(Z)-OAll</td>
<td>99</td>
<td>oil</td>
<td>n.d.</td>
<td>0.50</td>
</tr>
<tr>
<td>Fmoc-Lys(Z)-OAll</td>
<td>60</td>
<td>101-103</td>
<td>+3.3$^b$</td>
<td>0.94</td>
</tr>
<tr>
<td>HBr.Fmoc-Lys(H)-OAll</td>
<td>74</td>
<td>90-92</td>
<td>-11.1$^h$</td>
<td>0.14</td>
</tr>
<tr>
<td>HCl.H-Pro-OAll</td>
<td>100</td>
<td>oil</td>
<td>n.d.</td>
<td>0.23</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-Pro-OAll</td>
<td>95</td>
<td>foam</td>
<td>-37.4$^b$</td>
<td>0.86</td>
</tr>
<tr>
<td>HCl.Fmoc-Lys(H)-Pro-OAll</td>
<td>100</td>
<td>foam</td>
<td>-42.3$^b$</td>
<td>0.08</td>
</tr>
<tr>
<td>HCl.H-Thr(H)-OAll</td>
<td>100</td>
<td>oil</td>
<td>n.d.</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Footnotes to Tables 1-6:

1a (c=1, dioxane); 1b (c=1, NMP); 1c (c=1, DMF); 1d (c=1, AcOH); 1e (c=0.25, NMP); 1f (c=0.25, CHCl₃); 1g (c=1, CHCl₃); 1h (c=1, MeOH).

₂ CHCl₃/MeOH 9:1 (v/v); CHCl₃/MeOH/AcOH 95:5:3 (v/v/v); Fmoc-Phe-OH $R_f$ (A) 0.65, $R_f$ (B) 0.74.

₃ CHCl₃/MeOH/AcOH 95:5:3 (v/v/v); EtOAc/MeOH/H₂O 80:30:5 (v/v/v); EtOAc/PE 1:1 (v/v); Fmoc-Phe-OH $R_f$ (B) 0.74, $R_f$ (C) 0.81, $R_f$ (G) 0.41.

₄ Based on successively Boc-Asp(OrBu)-OAll, Boc-Glu(OrBu)-OAll, Boc-Lys(Z)-OAll, TFA-H-Lys(Z)-OAll, Fmoc-Lys(Z)-OAll, Boc-Pro-OAll, Fmoc-Lys(Boc)-OPfp, Fmoc-Lys(Boc)-Pro-OAll and Boc-Thr(H)-OAll.

8.4. Conclusions

Various amino acid derivatives were synthesized for use in combination with trityl-type resins in Fmoc SPPS. The easy preparation of Fmoc-amino acid hydrazides has been demonstrated for all common residues and favours the general procedure for the high-yield synthesis of protected peptide hydrazides on trityl-type resins as proposed in Chapter 4 of this thesis.

Next, the preparation of Fmoc-amino acid primary and secondary amides was explored. For economic reasons Fmoc-phenylalanine was used as a model starting compound, but as evident from many other examples the presented route can be transferred onto residues containing protected functional side chains. Upon deprotection of these side chains, a pathway leading to peptides with modified C-termini becomes available via side-chain attachment to trityl-type resins. Alternatively, deprotection of the $N^\alpha$-amino group yields compounds to be applied in two-directional synthesis schemes; the less expensive $N^\alpha$-Boc derivatives as such would not be compatible with selective side-chain deprotection.

The numerous synthetic goals originating from the presented amidation and SPPS strategies range from lipophilic peptides on the basis of octadecyl or Tris (–NHC(CH₃OH)₂) [21] amides (Chapter 2) to fully protected peptide primary amides (Chapter 6: CCAP).

Side-chain deprotected allyl ester derivatives are ideal starting compounds in on-resin head-to-tail cyclization procedures. Whereas Fmoc-Asp(OH)-OAll and Fmoc-Glu(OH)-OAll have been used before, the application of Fmoc-Lys(H)-OAll as such was first reported in Chapter 5; the use of Fmoc-Lys(H)-Pro-OAll is limited due to diketopiperazine formation. Preparation of other Fmoc-amino acid allyl esters as well as Dnab esters with functional side chains should further broaden the scope of on-resin head-to-tail cyclization as well as two-directional synthesis schemes, offered by trityl-type resins.
8.5. Experimental section

Materials and methods

Reference compounds Fmoc-Asp(OH)-OAll and Fmoc-Glu(OH)-OAll were purchased from Novabiochem. Methylamine was used as a 35% aqueous solution. The GC-MS device consisted of a MFC 500 gas chromatograph (column: fused silica SE-54, 25 m x 0.25 mm, gas: helium, temperature program: 50°C stand-by, 1 min solvent delay 100°C, heating to 280°C at 5°C/min, 30 min isothermal) and a QMP 1000 Quadrupole EI mass spectrometer (70 eV), both from Carlo Erba Instruments. For further specifications, see Appendix A.

General procedure for the preparation of Fmoc-amino acid hydrazides

To a solution of 1.0 g Fmoc-amino acid in 10 ml THF at 0°C, 1.1 meq of HONu and DCC each are added. After stirring for 1 h at 0°C and then 4 h at room temperature, the formed DCU is filtered off and washed with 10 ml THF. To the combined filtrates, hydrazine acetate (5 ml) is added at 0°C; this is prepared by adjusting the pH of hydrazine hydrate to 6 with acetic acid (moist pH paper). The suspension is stirred for 1 h at 0°C and overnight at room temperature and then concentrated in vacuo. Ethyl acetate (30 ml) is added and the resulting solution is washed with water and saturated NaCl (3x15 ml each). The organic phase is dried over Na2SO4 and evaporated; the residue is recrystallized from DCM/PE. N.B. In the preparation of Boc-hydrazides, tert-butylcarbazate (Boc-N3H3, 1.3 meq) is added instead of hydrazine acetate.

General procedure for the preparation of Fmoc-amino acid amides

Analogous to the preparation of Fmoc-amino acid hydrazides, using the actual amine (4 meq in the acetate form) instead of hydrazine acetate. N.B. In the preparation of primary and octadecyl amides, the THF solution containing the activated ONSu-ester is evaporated and the residue dissolved in 10 ml DMF; a suspension of ammonium acetate (4 meq) or n-octadecylamine (0.95 meq) in 10 ml DMF is then added, further following the general procedure. In the work-up of the octadecyl amides, ethyl acetate is replaced by DCM and recrystallization occurs from methanol.

* In case of a solid amine, it is dissolved in little THF prior to neutralization.

Variations in the preparation and/or work-up procedure (hydrazides, amides)

Fmoc-\(N\_2H\_3\) Starting from commercially available Fmoc-ONSu, using EtOAc instead of THF. The product, which crystallized within 5 min, was filtered off and extensively washed with cold EtOAc and water.

Fmoc-His(Bum)-\(N\_2H\_3\) To enhance solubility, the reactions were carried out in THF/DMF (1:1); DCU did not fully precipitate in this solvent mixture. Upon overnight treatment with hydrazine acetate, the solution was concentrated in vacuo and the residue taken up in 30 ml DCM, followed by standard work-up.

Fmoc-Ile/Asn(Tri)/Val-\(N\_2H\_3\) The products crystallized spontaneously during overnight stirring; they were collected by filtration and extensively washed with cold THF and water. A second crop was obtained after submitting the concentrated washing solutions to the standard work-up.

Fmoc-Phe-\(N\_2H\_3\)-Boc Recrystallization from 2-propanol.

[Fmoc-Cys-NH-Methyl]. During treatment with methylammonium acetate, a gelatinous precipitate was formed after 15 min, which was resuspended through the addition of 20 ml ethyl acetate. After stirring overnight, the
product was collected by filtration, extensively washed with cold ethyl acetate and water and recrystallized from DCM/MeOH.

[Fmoc-Cys-NH-Octadecyl]: The crude product was insoluble in DCM and was as such extensively washed with cold MeOH and water.

Fmoc-Phe-NH-Benzyl/Cyclohexyl/Phenyl: The crude products were insoluble in ethyl acetate and were as such extensively washed with cold ethyl acetate and water. N.B. The apparent pH of aniline solution is neutral, so that no addition of acetic acid was required.

Fmoc-Phe-NH-Decyl: The ethyl acetate used in the final work-up was slightly warmed to enhance the solubility of the product.

Fmoc-Thr(Bu)-NH-Cyclohexyl: Recrystallization from DCM.

Fmoc-Thr(Bu)-NH-Cyclohexyl: Recrystallization from MeOH.

General procedure for the preparation of Boc amino acid allyl esters

Water (5 ml) is added to a solution of Boc-amino acid (25 mmol) in methanol (50 ml). The mixture is neutralized with a 20% aqueous solution of Cs₂CO₃ and then evaporated *in vacuo*; the residue is reevaporated three times from 15 ml DMF at 40°C. The remaining solid cesium salt is stirred overnight with 4 ml (ca. 2 meq) allyl chloride in DMF (40 ml). Upon filtration of the formed cesium chloride and evaporation of the filtrate, the residue is dissolved in 50 ml CHCl₃, which is subsequently washed with 10% KHSO₄, 1 M NaHCO₃, water and saturated NaCl (2x25 ml each), dried over Na₂SO₄ and evaporated *in vacuo*, generally yielding a colourless oil. Only in case of Boc-Glu(O'Bu)-OAll the product crystallized spontaneously.

**Preparation of Fmoc-amino acid allyl esters (Asp, Glu, Lys(Z))**

The crude N°-deprotected compound (10 mmol, product from Boc-Asp/Glu(O'Bu)-OAll or Boc-Lys(Z)-OAll deprotection, see below: Protocols for acidolytic deprotection) was dissolved in 10 ml dioxane/water (1:1) in the presence of 2.8 ml triethylamine (20 mmol). To this mixture a solution of 3.2 g (9.6 mmol) Fmoc-ONSu in 10 ml dioxane was added in one portion. The pH of the reaction mixture was maintained at 8.5-9.0 for 15 min by the addition of triethylamine. After stirring for an additional 15 min, the mixture was concentrated *in vacuo* and 50 ml of 1.5 M HCl was added. The product was extracted with ethyl acetate (3x25 ml); the combined organic layers were washed with water and saturated NaCl (2x25 ml each), dried over Na₂SO₄ and evaporated *in vacuo*, leaving a yellow oil. The oil crystallized spontaneously in case of Fmoc-Lys(Z)-OAll and, upon washing with ether, afforded a white solid.

The Asp and Glu product mixtures (Fmoc-Asp/Glu(OH)-OAll and Fmoc-Asp/Glu(O'Bu)-OAll) were subjected to a second TFA treatment (see also below). After 1 h, the cleavage solutions were concentrated and the residues dissolved in 40 ml ethyl acetate; work-up proceeded as above, resulting in spontaneously crystallizing oils.

**Preparation of Fmoc-Lys(Boc)-Pro-OAll**

At 20°C, Fmoc-Lys(Boc)-OPfp (1 g, 1.6 mmol) and HCL-H-Pro-OAll (0.6 g, 3.2 mmol) were dissolved in 30 ml dioxane; the apparent pH of the resulting solution was adjusted to 7.5 through the addition of triethylamine. After stirring for 20 h, during which period the pH was kept constant, the formed precipitate was filtered off. The filtrate was concentrated *in vacuo* and the residue dissolved in 30 ml DCM, which was subsequently washed...
with 10% KHCO₃, 1 M NaHCO₃, water and saturated NaCl (2x15 ml each), dried over Na₂SO₄ and evaporated in vacuo. The remaining oil was triturated with DCM/PE, affording a yellow foam upon evaporation.

**Protocols for acidolytic deprotection (O'Bu, 'Bu, Boc, Trt, Z)**

The fully protected amino acid derivative was dissolved in diluted acid up to a concentration of 1 g/10 ml solvent, or in a minimal volume of concentrated acid. Specific reaction conditions are listed in the following table. After stirring during the indicated period of time and in vacuo concentration in case of dilute solutions, the product was precipitated, washed three times with the same solvent and collected by filtration.

<table>
<thead>
<tr>
<th>Protected compound</th>
<th>Cleavage mixture</th>
<th>Cleavage time</th>
<th>Precipitating solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Asp(O'Bu)-NH-Octadecyl</td>
<td>TFA/H₂O (95:5)</td>
<td>1.5 h</td>
<td>MeOH</td>
</tr>
<tr>
<td>Fmoc-Glu(O'Bu)-NH-Octadecyl</td>
<td>TFA/H₂O (95:5)</td>
<td>1.5 h</td>
<td>MeOH</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-NH-Methyl</td>
<td>4 M HCl/dioxane</td>
<td>3 h</td>
<td>DCM/PE</td>
</tr>
<tr>
<td>Boc-Lys(Fmoc)-NH-Methyl</td>
<td>4 M HCl/dioxane</td>
<td>1 h</td>
<td>Et₂O</td>
</tr>
<tr>
<td>Fmoc-Ser(Trt)-NH-Methyl</td>
<td>TFA/TES/DCM (5:5:90)</td>
<td>30 min</td>
<td>'BuOMe</td>
</tr>
<tr>
<td>Fmoc-Ser('Bu)-NH-Octadecyl</td>
<td>TFA/H₂O (95:5)</td>
<td>4 h (incomplete)⁹</td>
<td>MeOH</td>
</tr>
<tr>
<td>Fmoc-Ser(Trt)-NH-Octadecyl</td>
<td>TFA/TES/DCM (5:5:90)</td>
<td>30 min</td>
<td>MeOH</td>
</tr>
<tr>
<td>Fmoc-Thr('Bu)-NH-Methyl</td>
<td>TFA/H₂O (95:5)</td>
<td>2 h</td>
<td>'BuOMe</td>
</tr>
<tr>
<td>Fmoc-Trt('Bu)-NH-Methyl</td>
<td>TFA/H₂O (95:5)</td>
<td>1 h</td>
<td>'BuOMe</td>
</tr>
<tr>
<td>Boc-Asp(O'Bu)-OAll</td>
<td>TFA/H₂O (95:5)</td>
<td>3 h (O'Bu incomplete)⁸</td>
<td>'BuOMe (evaporation)</td>
</tr>
<tr>
<td>Boc-Glu(O'Bu)-OAll</td>
<td>TFA/H₂O (95:5)</td>
<td>3 h (O'Bu incomplete)⁸</td>
<td>'BuOMe (evaporation)</td>
</tr>
<tr>
<td>Boc-Lys(Z)-OAll</td>
<td>TFA/H₂O (95:5)</td>
<td>10 min (Z intact)</td>
<td>'BuOMe (evaporation)</td>
</tr>
<tr>
<td>Fmoc-Lys(Z)-OAll</td>
<td>33% HBr/AcOH</td>
<td>1 h</td>
<td>Et₂O</td>
</tr>
<tr>
<td>Boc-Pro-OAll</td>
<td>4 M HCl/dioxane</td>
<td>2 h</td>
<td>'BuOMe (evaporation)</td>
</tr>
<tr>
<td>Fmoc-Lys(Boc)-Pro-OAll</td>
<td>4 M HCl/dioxane</td>
<td>3 h</td>
<td>Et₂O (trituration)</td>
</tr>
<tr>
<td>Boc-Thr(H)-OAll</td>
<td>4 M HCl/dioxane</td>
<td>30 min</td>
<td>'BuOMe (evaporation)</td>
</tr>
</tbody>
</table>

⁸ The O-Trt derivative was used instead for the preparation of Fmoc-Ser(H)-NH-Octadecyl (next entry). ⁹ After introduction of an N⁰-Fmoc group, the deprotection was repeated (1 h), yielding pure Fmoc-Asp(Glu(OH))-OAll.

**Miscellaneous analytical data**

**Fmoc-Arg(Pmc)-N₂H₃** TLC: Rₚ (A) 0.21/0.37, Rₚ (B) 0.22/0.88 [hydrazide/δ-lactam].

[Fmoc-Cys-NH-Octadecyl]: SIMS (+), DTE/DTT/sulfolane: m/z 1187.4 [M+H]⁺, 595.3 [M₂H+H]⁺.

**Boc-Asp(O'Bu)-OAll** GC-MS (21.7 min, ca. 99%): m/z 330 [M+H]⁺ and fragmentation peaks.

**Boc-Glu(O'Bu)-OAll** GC-MS (23.9 min, ca. 99%): m/z 344 [M+H]⁺ and fragmentation peaks.

**Fmoc-Asp(OH)-OAll** Analysis: calc. C 66.83, H 5.35, N 3.54; found C 66.65, H 5.36, N 3.70.

**Fmoc-Glu(OH)-OAll** Analysis: calc. C 67.47, H 5.66, N 3.42; found C 67.68, H 5.93, N 3.61.
\textsuperscript{1}H NMR data / Fmoc-amino acid hydrazides

\textit{Fmoc-Ala-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.36 (d, 3H, \(J = 7.05\) Hz, \(\beta\)-CH\textsubscript{3}); 4.19 (m, 2H, \(\alpha\)-CH/CH-Fmoc); 4.39 (m, 2H, CH\textsubscript{2}-Fmoc); 7.28-7.45 (m, 4H, arom Fmoc); 7.61 (d, 2H, \(J = 7.38\) Hz, arom Fmoc); 7.77 (d, 2H, \(J = 7.39\) Hz, arom Fmoc).

\textit{Fmoc-Cys(Acm)-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 2.00 (s, 3H, CH\textsubscript{3}-Acm); 2.79/2.92 (dm, 2H, \(\beta\)-CH\textsubscript{2}); 4.19-4.44 (m, 6H, \(\alpha\)-CH/CH\textsubscript{2}-Acm/CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.27-7.44 (m, 4H, arom Fmoc); 7.61 (d, 2H, \(J = 7.38\) Hz, arom Fmoc); 7.76 (d, 2H, \(J = 7.38\) Hz, arom Fmoc).

\textit{Fmoc-Cys(Trt)-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 2.58 (d, 2H, \(J = 6.71\) Hz, \(\beta\)-CH\textsubscript{3}); 3.87 (t, 1H, \(\alpha\)-CH); 4.19 (t, 1H, \(J = 6.72\) Hz, CH-Fmoc); 4.29-4.45 (m, 2H, CH\textsubscript{2}-Fmoc); 7.17-7.31 (m, 11H, arom Fmoc(2H)/arom Trt(9H)); 7.35-7.42 (m, 8H, arom Fmoc(2H)/arom Trt(6H)); 7.58 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 7.76 (dd, 2H, \(J = 7.38\) Hz, arom Fmoc).

\textit{Fmoc-Asp(O\textsubscript{2}Bu)-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.43 (s, 9H, O\textsubscript{2}Bu); 2.70 (d, 2H, \(J = 5.71\) Hz, \(\beta\)-CH\textsubscript{2}); 4.21 (t, 1H, \(J = 6.38\) Hz, CH-Fmoc); 4.34-4.53 (m, 3H, \(\alpha\)-CH/CH\textsubscript{2}-Fmoc); 7.27-7.44 (m, 4H, arom Fmoc); 7.60 (d, 2H, arom Fmoc); 7.77 (d, 2H, \(J = 7.38\) Hz, arom Fmoc).

\textit{Fmoc-Glu(O\textsubscript{2}Bu)-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.43 (s, 9H, O\textsubscript{2}Bu); 2.01 (m, 2H, \(\beta\)-CH\textsubscript{3}); 2.34 (m, 2H, \(\gamma\)-CH\textsubscript{2}); 4.19 (m, 2H, \(\alpha\)-CH/CH-Fmoc); 4.38 (d, 2H, \(J = 6.87\) Hz, CH\textsubscript{2}-Fmoc); 7.25-7.42 (m, 4H, arom Fmoc); 7.57 (d, 2H, arom Fmoc); 7.75 (d, 2H, \(J = 7.41\) Hz, arom Fmoc); 7.99 (s, 1H, hydrazide).

\textit{Fmoc-Phe-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 2.95/3.06 (dm, 2H, \(\beta\)-CH\textsubscript{2}); 4.14 (t, 1H, \(J = 6.72\) Hz, CH-Fmoc); 4.23-4.44 (m, 3H, \(\alpha\)-CH/CH\textsubscript{2}-Fmoc); 7.14-7.44 (m, 9H, arom Fmoc(4H)/arom Ph(5H)); 7.53 (m, 2H, arom Fmoc); 7.76 (d, 2H, \(J = 7.39\) Hz, arom Fmoc).

\textit{Fmoc-His(Bum)-N\textsubscript{2}H\textsubscript{4}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 1.19 (s, 9H, O\textsubscript{2}Bu-Bum); 2.93 (m, 2H, \(\beta\)-CH\textsubscript{3}); 4.13-4.32 (m, 4H, \(\alpha\)-CH/CH-Fmoc/CH\textsubscript{2}-Fmoc); 5.33 (s, 2H, CH\textsubscript{2}-Bum); 7.29-7.46/7.69/7.84/7.89 (m, 10H, arom Fmoc(8H)/arom His(2H)); 9.18 (s, 1H, hydrazide).

\textit{Fmoc-Ile-N\textsubscript{2}H\textsubscript{4}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 0.82 (m, 6H, \(\gamma\)-CH\textsubscript{3}/\(\delta\)-CH\textsubscript{3}); 1.10 (m, 1H, \(\beta\)-CH); 1.49/1.70 (dm, 2H, \(\gamma\)-CH\textsubscript{3}); 3.79 (m, 1H, \(\alpha\)-CH); 4.24 (m, 3H, CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.29-7.50 (m, 4H, arom Fmoc); 7.87 (m, 4H, arom Fmoc); 9.09 (s, 1H, hydrazide).

\textit{Fmoc-Lys(Boc)-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.25-1.82 (m, 5H, \(\beta\)-CH\textsubscript{3}(1H)/\(\gamma\)-CH\textsubscript{2}/\(\delta\)-CH\textsubscript{2}); 1.43 (s, 9H, Boc); 1.89 (m, 1H, \(\beta\)-CH\textsubscript{3}); 3.06 (m, 2H, \(\varepsilon\)-CH\textsubscript{2}); 4.07 (t, 1H, \(\alpha\)-CH); 4.20 (t, 1H, CH-Fmoc); 4.40 (m, 2H, CH\textsubscript{2}-Fmoc); 7.27-7.44 (m, 4H, arom Fmoc); 7.60 (d, 2H, arom Fmoc); 7.76 (d, 2H, \(J = 7.39\) Hz, arom Fmoc).

\textit{Fmoc-Leu-N\textsubscript{2}H\textsubscript{4}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 0.91 (m, 6H, \(\delta\)-CH\textsubscript{3}); 1.26 (m, 1H, \(\gamma\)-CH); 1.59 (m, 2H, \(\beta\)-CH\textsubscript{3}); 4.18 (m, 2H, \(\alpha\)-CH/CH-Fmoc); 4.38 (m, 2H, CH\textsubscript{2}-Fmoc); 5.56 (d, 1H, \(J = 8.39\) Hz, \(\alpha\)-NH); 7.22-7.42 (m, 4H, arom Fmoc); 7.54 (d, 2H, \(J = 7.38\) Hz, arom Fmoc); 7.74 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 8.04 (s, 1H, hydrazide).

\textit{Fmoc-Met-N\textsubscript{2}H\textsubscript{4}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 1.85 (m, 2H, \(\beta\)-CH\textsubscript{3}); 2.04 (s, 3H, \(\sim\)-S-CH\textsubscript{3}); 2.44 (m, 2H, \(\gamma\)-CH\textsubscript{2}); 4.22 (m, 4H, \(\alpha\)-CH/CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.29-7.46 (m, 4H, arom Fmoc); 7.56 (d, 1H, \(J = 8.06\) Hz, \(\alpha\)-NH); 7.74 (dd, 2H, arom Fmoc); 7.90 (d, 2H, \(J = 7.72\) Hz, arom Fmoc); 9.13 (s, 1H, hydrazide).
\textbf{Fmoc-Asn(Trt)-N\textsubscript{2}H\textsubscript{3}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 3.01 (m, 2H, \(\beta\)-CH\textsubscript{2}); 4.12-4.47 (m, 4H, \(\alpha\)-CH/CH-Fmoc/CH\textsubscript{2}-Fmoc); 6.95-7.09/7.15-7.45/7.62-7.72 (m, 21H, arom Fmoc(6H)/arom Trt(15H)); 7.57 (d, 1H, \(J = 8.39\) Hz, \(\alpha\)-NH); 7.88 (d, 2H, \(J = 7.72\) Hz, arom Fmoc); 9.26 (s, 1H, hydrazide).

\textbf{Fmoc-Gln(Trt)-N\textsubscript{2}H\textsubscript{3}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.93 (m, 2H, \(\beta\)-CH\textsubscript{2}); 2.38 (m, 2H, \(\gamma\)-CH\textsubscript{2}); 4.05 (m, 1H, \(\alpha\)-CH); 4.17 (t, 1H, CH-Fmoc); 4.37 (d, 2H, \(J = 7.06\) Hz, CH\textsubscript{2}-Fmoc); 7.15-7.41 (m, 19H, arom Fmoc(4H)/arom Trt(15H)); 7.56 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 7.73 (d, 2H, \(J = 7.39\) Hz, arom Fmoc).

\textbf{Fmoc-Ser(Bu)-N\textsubscript{2}H\textsubscript{3}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 1.12 (s, 9H, \textsuperscript{1}Bu); 3.42 (m, 2H, \(\beta\)-CH\textsubscript{2}); 4.09 (m, 1H, \(\alpha\)-CH); 4.25 (m, 3H, CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.29-7.46 (m, 4H, arom Fmoc); 7.75 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 7.89 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 9.14 (s, 1H, hydrazide).

\textbf{Fmoc-Ser(Trt)-N\textsubscript{2}H\textsubscript{3}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 3.24/3.61 (dm, 2H, \(\beta\)-CH\textsubscript{2}); 4.15 (t, 1H, CH-Fmoc); 4.34 (m, 1H, \(\alpha\)-CH); 4.37 (d, 2H, CH\textsubscript{2}-Fmoc); 5.48 (d, 1H, \(\alpha\)-NH); 7.19-7.31/7.34-7.41 (m, 19H, arom Fmoc(4H)/arom Trt(15H)); 7.51 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 7.64 (s, 1H, hydrazide); 7.75 (d, 2H, \(J = 7.39\) Hz, arom Fmoc).

\textbf{Fmoc-Thr(Bu)-N\textsubscript{2}H\textsubscript{3}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.03 (d, 3H, \(J = 6.38\) Hz, \(\gamma\)-CH\textsubscript{3}); 1.26 (s, 9H, \textsuperscript{1}Bu); 4.14 (m, 2H, \(\alpha\)-CH/\(\beta\)-CH\textsubscript{2}); 4.23 (t, 1H, \(J = 7.05\) Hz, CH-Fmoc); 4.40 (d, 2H, \(J = 7.05\) Hz, CH\textsubscript{2}-Fmoc); 5.93 (d, 1H, \(\alpha\)-NH); 7.26-7.44 (m, 4H, arom Fmoc); 7.61 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 7.75 (d, 2H, \(J = 7.72\) Hz, arom Fmoc); 7.98 (s, 1H, hydrazide).

\textbf{Fmoc-Val-N\textsubscript{2}H\textsubscript{3}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 0.86 (dd, 6H, \(\gamma\)-CH\textsubscript{3}); 1.93 (m, 1H, \(\beta\)-CH); 3.77 (m, 1H, \(\alpha\)-CH); 4.24 (m, 3H, CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.29-7.49 (m, 4H, arom Fmoc); 7.76 (d, 2H, arom Fmoc); 7.89 (d, 2H, \(J = 7.39\) Hz, arom Fmoc); 9.15 (s, 1H, hydrazide).

\textbf{Fmoc-Trp-N\textsubscript{2}H\textsubscript{3}} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 3.02 (m, 2H, \(\beta\)-CH\textsubscript{2}); 4.16 (m, 2H, \(\alpha\)-CH/CH-Fmoc); 4.30 (m, 2H, CH\textsubscript{2}-Fmoc); 6.95-7.10/7.18-7.45 (m, 8H, arom Fmoc(4H)/arom Trp(4H)); 7.56-7.71 (m, 3H, arom Fmoc(2H)/arom Trp(1H)); 7.87 (d, 2H, \(J = 7.38\) Hz, arom Fmoc); 9.28 (s, 1H, hydrazide).

\textbf{Fmoc-Tyr(Bu)-N\textsubscript{2}H\textsubscript{3}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.28 (s, 9H, \textsuperscript{1}Bu); 2.99 (d, 2H, \(J = 6.40\) Hz, \(\beta\)-CH\textsubscript{2}); 4.14 (t, 1H, CH-Fmoc); 4.24-4.45 (m, 3H, \(\alpha\)-CH/CH\textsubscript{2}-Fmoc); 5.66 (d, 1H, \(\alpha\)-NH); 6.88 (d, 2H, \(J = 8.24\) Hz, arom Tyr); 7.05 (d, 2H, arom Tyr); 7.24-7.41 (m, 4H, arom Fmoc); 7.51 (d, 2H, \(J = 7.42\) Hz, arom Fmoc); 7.74 (d, 2H, \(J = 7.42\) Hz, arom Fmoc).

\textbf{Fmoc-Phe-N\textsubscript{2}H\textsubscript{2}-Boc} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.46 (s, 9H, Boc); 2.95/3.20 (dm, 2H, \(\beta\)-CH\textsubscript{2}); 4.11 (t, 1H, CH-Fmoc); 4.21/4.36 (dm, 2H, CH\textsubscript{2}-Fmoc); 4.47 (m, 1H, \(\alpha\)-CH); 7.14-7.43 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.52 (m, 2H, arom Fmoc); 7.75 (d, 2H, \(J = 7.72\) Hz, arom Fmoc).

\textbf{Fmoc-Pro-N\textsubscript{2}H\textsubscript{2}-Boc} (DMSO-d\textsubscript{6}), \(\delta\) (ppm): 1.38 (s, 9H, Boc); 1.55-2.33 (m, 4H, \(\beta\)-CH\textsubscript{2}/\(\gamma\)-CH\textsubscript{2}); 3.38 (m, 2H, \(\delta\)-CH\textsubscript{2}); 3.52 (m, 1H, \(\alpha\)-CH); 4.23 (CH-Fmoc/CH\textsubscript{2}-Fmoc); 7.31-7.46 (m, 4H, arom Fmoc); 7.67 (m, 2H, arom Fmoc); 7.90 (m, 2H, arom Fmoc); 8.78 (d, 1H, \(J = 14.4\) Hz, hydrazide).

\textsuperscript{1}H NMR data / Fmoc-amino acid primary amides

\textbf{Fmoc-Ala-NH\textsubscript{2}} (CDCl\textsubscript{3}), \(\delta\) (ppm): 1.38 (d, 3H, \(J = 7.05\) Hz, \(\beta\)-CH\textsubscript{3}); 4.21 (m, 2H, \(\alpha\)-CH/CH-Fmoc); 4.41 (d, 2H, \(J = 6.71\) Hz, CH\textsubscript{2}-Fmoc); 7.28-7.43 (m, 4H, arom Fmoc); 7.60 (d, 2H, \(J = 7.38\) Hz, arom Fmoc); 7.76 (d, 2H, \(J = 7.38\) Hz, arom Fmoc).
Preparation of Amino Acid Derivatives

Fmoc-Cys(Trrl)-NH₂ (CDCl₃), δ (ppm): 2.64 (m, 2H, β-CH₂); 3.83 (m, 1H, α-CH); 4.18 (t, 1H, CH-Fmoc); 4.41 (d, 2H, J = 6.87 Hz, CH₂-Fmoc); 5.02 (d, 1H, α-NH); 5.48/5.75 (d, 2H, −CO-NH₂); 7.15-7.31 (m, 11H, arom Fmoc(2H)/arom Trt(9H)); 7.34-7.43 (m, 8H, arom Fmoc(2H)/arom Trt(6H)); 7.56 (d, 2H, J = 7.41 Hz, arom Fmoc); 7.74 (dd, 2H, arom Fmoc).

Fmoc-Phe-NH₂ (DMSO-d₆), δ (ppm): 2.82/3.03 (dm, 2H, β-CH₂); 4.09-4.26 (m, 4H, α-CH/CH-Fmoc/CH₂-Fmoc); 7.05-7.75 (m, 11H, arom Fmoc(6H)/arom Phe(5H)); 7.87 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Gly-NH₂ (CDCl₃), δ (ppm): 3.81 (s, 2H, α-CH₃); 4.21 (t, 1H, J = 6.72 Hz, CH-Fmoc); 4.42 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 7.27-7.43 (m, 4H, arom Fmoc); 7.60 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Leu-NH₂ (CDCl₃), δ (ppm): 0.91 (m, 6H, δ-CH₃); 1.45-1.77 (m, 3H, β-CH₂CH₂-CH); 4.21 (m, 2H, α-CH/CH-Fmoc); 4.40 (d, 2H, CH₂-Fmoc); 5.54 (d, 1H, α-NH); 5.95/6.31 (d, 2H, −CO-NH₂); 7.24-7.42 (m, 4H, arom Fmoc); 7.56 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.74 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Thr(Bu)-NH₂ (DMSO-d₆), δ (ppm): 1.04 (d, 3H, J = 6.04 Hz, γ-CH₃); 1.14 (s, 9H, t-Bu); 3.92 (m, 2H, α-CH/β-CH); 4.20-4.39 (m, 3H, CH-Fmoc/CH₂-Fmoc); 6.76 (d, 1H, J = 8.73 Hz, α-NH); 7.16/7.22 (d, 2H, −CO-NH₂); 7.29-7.45 (m, 4H, arom Fmoc); 7.75 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.89 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Val-NH₂ (DMSO-d₆), δ (ppm): 0.88 (dd, 6H, γ-CH₃); 1.99 (m, 1H, β-CH); 3.83 (m, 1H, α-CH); 4.19-4.33 (m, 3H, CH-Fmoc/CH₂-Fmoc); 7.05 (m, 1H, α-NH); 7.24-7.46 (m, 6H, arom Fmoc(4H)/−CO-NH₂); 7.76 (d, 2H, arom Fmoc); 7.89 (d, 2H, J = 7.39 Hz, arom Fmoc).

¹H NMR data / Fully protected Fmoc-amino acid secondary amides

[Fmoc-Cys(NH-Methyl)] (DMSO-d₆), δ (ppm): 2.61 (d, 6H, J = 4.36 Hz, −NH₂CH₃); 2.90/3.13 (dm, 4H, β-CH₂CH₂); 4.18-4.37 (m, 8H, α-CH/CH-Fmoc/CH₂-Fmoc); 7.27-7.44 (m, 8H, arom Fmoc); 7.71 (m, 4H, arom Fmoc); 8.88 (d, 4H, arom Fmoc); 7.99 (m, 2H, −NH₂CH₃).

Fmoc-Asp(O'Bu)-NH-OctadecyL (CDCl₃), δ (ppm): 0.88 (t, 3H, J = 6.72 Hz, 18-CH₃); 1.25 (m, 32H, (2-17)-CH₃); 1.45 (s, 9H, O'Bu); 2.91 (m, 2H, β-CH₂); 3.22 (m, 2H, −NH₂CH₂); 4.22 (t, 1H, CH-Fmoc); 4.41 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 4.47 (m, 1H, α-CH); 5.98 (d, 1H, J = 8.39 Hz, α-NH); 6.48 (t, 1H, −NH₂CH₂); 7.26-7.44 (m, 4H, arom Fmoc); 7.58 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.77 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Glu(O'Bu)-NH-OctadecyL (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.25 (m, 32H, (2-17)-CH₃); 1.45 (s, 9H, O'Bu); 1.94/2.06 (dm, 2H, β-CH₂); 2.29/2.45 (dm, 2H, γ-CH₃); 3.23 (m, 2H, −NH₂CH₂); 4.19 (m, 2H, α-CH/CH-Fmoc); 4.37 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 5.82 (d, 1H, α-NH); 6.36 (t, 1H, −NH₂CH₂); 7.26-7.44 (m, 4H, arom Fmoc); 7.59 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Phe-NH-AllyL (CDCl₃), δ (ppm): 3.06 (m, 2H, β-CH₂); 3.74 (m, 2H, −NH₂CH₂); 4.15 (t, 1H, J = 6.72 Hz, CH-Fmoc); 4.22-4.48 (m, 3H, α-CH/CH₂-Fmoc); 4.98 (m, 2H, −CH₂); 5.61 (m, 1H, −CH=); 7.09-7.59 (m, 11H, arom Fmoc); 7.75 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Phe-NH-Benzyl (CDCl₃), δ (ppm): 3.04 (m, 2H, β-CH₂); 4.25 (m, 3H, CH₂-Benzyl/CH-Fmoc); 4.40 (m, 3H, α-CH/CH₂-Fmoc); 7.00-7.47 (m, 14H, arom Fmoc(4H)/arom Phe(5H)/arom Bzl(5H)); 7.55 (d, 2H, arom Fmoc); 7.75 (d, 2H, J = 7.41 Hz, arom Fmoc).
Fmoc-Phe-NHC(CH₂OH)$_₂$ (CDCl₃), δ (ppm): 3.03 (m, 2H, β-CH₂); 3.53 (m, 6H, ~C(CH₂OH)$_₂$); 4.17 (t, 1H, CH-Fmoc); 4.26-4.43 (m, 3H, α-CH(CH₂)₂-Fmoc); 7.16-7.44 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.55 (m, 2H, arom Fmoc); 7.76 (d, 2H, J = 7.72 Hz, arom Fmoc).

Fmoc-Phe-NH-Cyclohexyl (CDCl₃), δ (ppm): 0.92 (m, 2H, 4-CH₂-cHex); 1.08/1.29 (ddm, 4H, 3.5-CH₂-cHex); 1.70 (m, 4H, 2,6-CH₂-cHex); 2.98 (d, 2H, J = 7.05 Hz, β-CH₂); 3.60 (m, 1H, 1-CH-cHex); 4.17 (t, 1H, CH-Fmoc); 4.23-4.42 (m, 3H, ω-CH(CH₂)₂-Fmoc); 7.14-7.45 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.56 (m, 2H, arom Fmoc); 7.76 (d, 2H, J = 7.72 Hz, arom Fmoc).

Fmoc-Phe-NH-Decyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 10-CH₃); 1.20 (m, 16H, (2-9)-CH₃); 2.95-3.25 (m, 4H, β-CH₂~NH-CH₂~); 4.17 (t, 1H, CH-Fmoc); 4.25-4.44 (m, 3H, α-CH(CH₂)₂-Fmoc); 5.58 (d, 1H, α-NH); 5.73 (m, 1H, ~NH-CH₂~); 7.05-7.44 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.53 (m, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Phe-NH-Isopropyl (CDCl₃), δ (ppm): 0.91/1.02 (dd, 6H, ~CH(CH₂)₂~CH(CH₃)₂); 2.96/3.11 (ddm, 2H, β-CH₂); 3.97 (m, 1H, ~CH(CH₂)₂); 4.17 (t, 1H, J = 7.05 Hz, CH-Fmoc); 4.23-4.44 (m, 3H, α-CH(CH₂)₂-Fmoc); 5.51 (d, 1H, NH); 5.64 (d, 1H, NH); 7.11-7.43 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.54 (m, 2H, arom Fmoc); 7.75 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Phe-NH-Methyl (CDCl₃), δ (ppm): 2.68 (s, 3H, ~NH-CH₃); 3.01 (m, 2H, β-CH₂); 4.15 (t, 1H, CH-Fmoc); 4.23-4.43 (m, 3H, α-CH(CH₂)₂-Fmoc); 7.09-7.45 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.53 (m, 2H, arom Fmoc); 7.75 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Phe-NH-Phenyl (DMSO-d$_6$), δ (ppm): 2.97 (m, 2H, β-CH₂); 4.19 (m, 3H, CH-Fmoc/CH₂-Fmoc); 4.46 (m, 1H, α-CH); 7.02-7.47/7.65/7.87 (m, 11H/4H/3H, arom Fmoc(8H)/arom Phe(5H)/~NH-Phenyl(5H)).

Fmoc-Phe-NH-Octadearyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.26 (m, 32H, (2-17)-CH₂); 2.88-3.23 (m, 4H, β-CH₂~NH-CH₂~); 4.17 (t, 1H, J = 6.87 Hz, CH-Fmoc); 4.24-4.45 (m, 3H, α-CH(CH₂)₂-Fmoc); 5.53 (m, 1H, NH); 5.67 (m, 1H, NH); 7.09-7.44 (m, 9H, arom Fmoc(4H)/arom Phe(5H)); 7.54 (m, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.42 Hz, arom Fmoc).

Fmoc-Lys(Boc)-NH-Methyl (CDCl₃), δ (ppm): 1.24-1.98 (m, 6H, β-CH₂γ-CH₂δ-CH₂); 1.43 (s, 9H, Boc); 2.77 (d, 3H, ~NH-CH₃); 3.10 (m, 2H, ε-CH₂); 4.08-4.22 (m, 2H, α-CH(CH₂)₂-Fmoc); 4.39 (d, 2H, J = 6.71 Hz, CH₂-Fmoc); 4.69 (t, 1H, ε-NH); 5.69 (d, 1H, α-NH); 6.38 (m, 1H, ~NH-CH₂); 7.26-7.42 (m, 4H, arom Fmoc); 7.57 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.75 (d, 2H, J = 7.39 Hz, arom Fmoc).

Boc-Lys(Fmoc)-NH-Methyl (CDCl₃), δ (ppm): 1.22-1.98 (m, 6H, β-CH₂γ-CH₂δ-CH₂); 1.42 (s, 9H, Boc); 2.78 (d, 3H, J = 5.03 Hz, ~NH-CH₃); 3.16 (m, 2H, ε-CH₂); 4.08 (m, 1H, α-CH); 4.19 (t, 1H, CH-Fmoc); 4.38 (m, 2H, CH₂-Fmoc); 5.05 (t, 1H, ε-NH); 5.27 (d, 1H, J = 8.39 Hz, α-NH); 6.46 (m, 1H, ~NH-CH₂); 7.26-7.42 (m, 4H, arom Fmoc); 7.57 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Lys(Boc)-NH-Octadearyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.26 (m, 32H, (2-17)-CH₂); 1.35-1.94 (m, 6H, β-CH₂γ-CH₂δ-CH₂); 1.43 (s, 9H, Boc); 3.10 (m, 2H, ε-CH₂); 3.21 (m, 2H, ~NH-CH₂~); 4.11 (m, 1H, α-CH); 4.19 (t, 1H, J = 7.05 Hz, CH-Fmoc); 4.38 (d, 2H, J = 6.72 Hz, CH₂-Fmoc); 4.63 (t, 1H, ε-NH); 5.60 (d, 1H, α-NH); 6.23 (m, 1H, ~NH-CH₂~); 7.25-7.42 (m, 4H, arom Fmoc); 7.56 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).
Fmoc-Ser(Bu)-NH-Octadecyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.20 (s, 9H, tBu); 1.25 (m, 32H, (2-17)-CH₂); 3.27 (m, 2H, ~NH-CH₂-); 3.81 (dd, 2H, β-CH₂); 4.12-4.27 (m, 2H, α-CH(CH₂-Fmoc); 4.41 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 7.31-7.43 (m, 4H, arom Fmoc); 7.60 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Ser(Trt)-NH-Methyl (CDCl₃), δ (ppm): 2.83 (d, 3H, J = 4.70 Hz, ~NH-CH₃); 3.21/3.65 (dm, 2H, β-CH₂); 4.16 (t, 1H, CH-Fmoc); 4.29 (m, 1H, α-CH); 4.57 (d, 2H, CH₂-Fmoc); 5.48 (m, 1H, NH); 6.32 (m, 1H, NH); 7.10-7.45 (m, 19H, arom Fmoc(4H)/arom Trt(15H)); 7.53 (d, 2H, arom Fmoc); 7.75 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Ser(Trt)-NH-Octadecyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.26 (s, 32H, (2-17)-CH₂); 3.15-3.32 (m, 3H, β-CH₂(1H)~NH-CH₂(2H)); 3.62 (m, 1H, β'-CH₂); 4.16/4.33 (m, 4H, α-CH(CH₂-Fmoc/CH₂-Fmoc); 7.17-7.44 (m, 19H, arom Fmoc(4H)/arom Trt(15H)); 7.53 (m, 2H, arom Fmoc); 7.72 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Thr(Bu)-NH-Cyclohexyl (CDCl₃), δ (ppm): 1.04 (t, 3H, J = 6.05 Hz, γ-CH₃); 1.29 (s, 9H, tBu); 1.06-2.00 (m, 10H, (2-6)-CH₂-cHex); 3.82 (m, 1H, 1-CH-cHex); 4.13 (m, 2H, α-CH/β-CH); 4.23 (t, 1H, J = 7.39 Hz, CH-Fmoc); 4.38 (m, 2H, CH₂-Fmoc); 6.07 (d, 1H, J = 4.70 Hz, ~NH-cHex); 7.04 (d, 1H, J = 7.72 Hz, α-NH); 7.25-7.43 (m, 4H, arom Fmoc); 7.61 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.32 Hz, arom Fmoc).

Fmoc-Thr(Bu)-NH-Methyl (CDCl₃), δ (ppm): 1.04 (d, 3H, J = 6.04 Hz, γ-CH₃); 1.27 (s, 9H, tBu); 2.86 (d, 3H, J = 4.70 Hz, ~NH-CH₃); 4.15 (m, 2H, α-CH/β-CH); 4.23 (t, 1H, J = 7.05 Hz, CH-Fmoc); 4.40 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 6.05 (m, 1H, ~NH-CH₃); 6.88 (d, 1H, α-NH); 7.25-7.44 (m, 4H, arom Fmoc); 7.61 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.38 Hz, arom Fmoc).

Fmoc-Tyr(Bu)-NH-Methyl (CDCl₃), δ (ppm): 1.30 (s, 9H, tBu); 2.66 (d, 3H, ~NH-CH₃); 3.00 (m, 2H, β-CH₂); 4.16 (t, 1H, CH-Fmoc); 4.24-4.50 (m, 3H, α-CH/CH₂-Fmoc); 5.58 (d, 1H, α-NH); 5.83 (m, 1H, ~NH-CH₃); 6.90 (d, 2H, J = 8.39 Hz, arom Tyr); 7.07 (d, 2H, arom Tyr); 7.25-7.43 (m, 4H, arom Fmoc); 7.54 (d, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

1H NMR data / Semi-protected Fmoc-amino acid secondary amides

Fmoc-Asp(OH)-NH-Octadecyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.25 (m, 32H, (2-17)-CH₂); 2.74/2.98 (dd, 2H, β-CH₂); 3.22 (m, 2H, ~NH-CH₂-); 4.22 (t, 1H, CH-Fmoc); 4.42 (d, 2H, CH₂-Fmoc); 4.55 (m, 1H, α-CH); 6.09 (d, 1H, α-NH); 6.58 (m, 1H, ~NH-CH₂-); 7.24-7.43 (m, 4H, arom Fmoc); 7.56 (d, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

Fmoc-Glu(OH)-NH-Octadecyl (CDCl₃), δ (ppm): 0.88 (t, 3H, 18-CH₃); 1.25 (m, 32H, (2-17)-CH₂); 1.98/2.09 (dm, 2H, β-CH₂); 2.40-2.51 (dm, 2H, γ-CH₂); 3.22 (m, 2H, ~NH-CH₂-); 4.19 (t, 1H, CH-Fmoc); 4.25-4.42 (m, 2H, α-CH(CH₂-Fmoc); 5.84 (d, 1H, α-NH); 6.57 (m, 1H, ~NH-CH₂-); 7.24-7.43 (m, 4H, arom Fmoc); 7.57 (d, 2H, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

HCl/Fmoc-Lys(H)-NH-Methyl (DMSO-d₆), δ (ppm): 1.32 (m, 2H, γ-CH₂); 1.58 (m, 4H, β-CH₂/β-CH₃); 2.60 (d, 3H, J = 4.70 Hz, ~NH-CH₃); 2.75 (m, 2H, ε-CH₂); 3.93 (m, 1H, α-CH); 4.18-4.32 (m, 3H, CH-Fmoc/CH₂-Fmoc); 7.30-7.46 (m, 4H, arom Fmoc); 7.52 (d, 1H, J = 8.06 Hz, α-NH); 7.75 (m, 2H, arom Fmoc); 7.90 (d, 2H, J = 7.39 Hz, arom Fmoc); 8.09 (s, 3H, ε-NH₃⁺).
**HCl-Lys(Fmoc)-NH-Methyl** (DMSO-d$_6$), δ (ppm): 1.29/1.40 (dm, 4H, γ-CH$_2$/δ-CH$_2$); 1.71 (m, 2H, β-CH$_2$); 2.65 (d, 3H, J = 4.36 Hz, ~NH-CH$_3$); 2.96 (m, 2H, ε-CH$_2$); 3.72 (m, 1H, α-CH); 4.21 (t, 1H, CH-Fmoc); 4.30 (d, 2H, J = 6.71 Hz, CH$_2$-Fmoc); 7.30-7.41 (m, 5H, arom Fmoc(4H)/ε-NH(1H)); 7.70 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.88 (d, 2H, J = 7.39 Hz, arom Fmoc); 8.34 (s, 3H, α-NH$_3$); 8.64 (q, 1H, J = 4.70 Hz, ~NH-CH$_3$).

**Fmoc-Ser(H)-NH-Methyl** (CDCl$_3$), δ (ppm): 2.79 (s, 3H, ~NH-CH$_3$); 3.70/3.87 (dm, 2H, β-CH$_2$); 4.08-4.25 (m, 2H, α-CH/CH-Fmoc); 4.42 (d, 2H, CH$_2$-Fmoc); 7.27-7.46 (m, 4H, arom Fmoc); 7.62 (m, 2H, arom Fmoc); 7.78 (d, 2H, arom Fmoc).

**Fmoc-Ser(H)-NH-Octadecyl** (CDCl$_3$), δ (ppm): 0.88 (t, 3H, 18-CH$_3$); 1.15 (m, 32H, (2-17)-CH$_2$); 3.20 (m, 2H, ~NH-CH$_2$-); 3.48/3.65 (dm, 2H, β-CH$_2$); 4.06-4.25 (m, 2H, α-CH/CH-Fmoc); 4.42 (d, 2H, CH$_2$-Fmoc); 5.87 (d, 1H, α-NH); 6.57 (m, 1H, ~NH-CH$_2$-); 7.24-7.46 (m, 4H, arom Fmoc); 7.58 (d, 2H, J = 7.42 Hz, arom Fmoc); 7.76 (d, 2H, arom Fmoc).

**Fmoc-Thr(H)-NH-Methyl** (CDCl$_3$), δ (ppm): 1.04 (d, 3H, J = 6.38 Hz, γ-CH$_3$); 2.61 (d, 3H, J = 4.36 Hz, ~NH-CH$_3$); 3.88 (dd, 1H, α-CH); 3.97 (m, 1H, β-CH); 4.20-4.40 (m, 3H, CH-Fmoc/CH$_2$-Fmoc); 5.07 (m, 1H, ~NH-CH$_3$); 6.99 (d, 1H, J = 8.72 Hz, α-NH); 7.29-7.46 (m, 4H, arom Fmoc); 7.76 (d, 2H, arom Fmoc); 7.90 (d, 2H, J = 7.39 Hz, arom Fmoc).

**Fmoc-Tyr(H)-NH-Methyl** (DMSO-d$_6$), δ (ppm): 2.60 (d, 3H, J = 4.36 Hz, ~NH-CH$_3$); 2.69 (dd, 1H, β-CH$_2$); 2.85 (dd, 1H, β'-CH$_2$); 4.05-4.29 (m, 4H, α-CH/CH-Fmoc/CH$_2$-Fmoc); 6.67 (d, 2H, J = 8.39 Hz, arom Tyr); 7.06 (d, 2H, J = 8.40 Hz, arom Tyr); 7.26-7.45 (m, 4H, arom Fmoc); 7.55 (d, 1H, J = 8.40 Hz, α-NH); 7.66 (m, 2H, arom Fmoc); 7.89 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.89 (m, 1H, ~NH-CH$_3$).

### 1H NMR data / Boc-amino acid allyl esters

**Boc-Lys(Z)-OAll** (CDCl$_3$), δ (ppm): 1.30-1.72 (m, 4H, γ-CH$_2$/δ-CH$_2$); 1.43 (s, 9H, Boc); 1.81 (m, 2H, β-CH$_2$); 3.17 (m, 2H, ε-CH$_2$); 4.30 (m, 1H, α-CH); 4.62 (m, 2H, ~O-CH$_2$-); 4.87 (m, 1H, ε-NH); 5.09 (s, 2H, CH$_2$-Bz); 5.29 (m, 2H, ~CH$_2$-); 5.90 (m, 1H, ~CHJ-); 7.34 (m, 5H, arom Bz).

**Boc-Pro-OAll** (CDCl$_3$), δ (ppm): 1.44 (d, 9H, J = 15.8 Hz, Boc); 1.80-2.05 (m, 3H, β-CH$_3$(1H)/γ-CH$_2$(2H)); 2.23 (m, 1H, β'-CH$_2$); 3.48 (m, 2H, δ-CH$_2$); 4.25/4.35 (dm, 1H, α-CH); 4.61 (m, 2H, ~O-CH$_2$-); 5.29 (m, 2H, =CH$_2$); 5.91 (m, 1H, ~CHJ-).

**Boc-Thr(H)-OAll** (CDCl$_3$), δ (ppm): 1.26 (d, 3H, J = 6.38 Hz, γ-CH$_3$); 1.46 (s, 9H, Boc); 4.29 (α-CH/β-CH); 4.68 (2H, J = 5.70 Hz, ~O-CH$_2$-); 5.31 (m, 2H, =CH$_2$); 5.43 (m, 1H, α-NH); 5.92 (m, 1H, ~CHJ-).

### 1H NMR data / Miscellaneous amino ester derivatives

**Fmoc-Asp(OD)-OAll** (CDCl$_3$), δ (ppm): 2.94/3.11 (dm, 2H, β-CH$_2$); 4.22 (t, 1H, CH-Fmoc); 4.31-4.55 (m, 3H, α-CH/CH$_2$-Fmoc); 4.66 (d, 2H, J = 5.71 Hz, ~O-CH$_2$-); 5.28 (m, 2H, =CH$_2$); 5.88 (m, 2H, ~CHJ/α-NH); 7.24-7.42 (m, 4H, arom Fmoc); 7.59 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.74 (d, 2H, J = 7.39 Hz, arom Fmoc).

**Fmoc-Glu(OD)-OAll** (CDCl$_3$), δ (ppm): 1.98/2.24 (dm, 2H, β-CH$_2$); 2.44 (m, 2H, γ-CH$_2$); 4.20 (t, 1H, CH-Fmoc); 4.34-4.52 (m, 3H, α-CH/CH$_2$-Fmoc); 4.63 (d, 2H, J = 5.71 Hz, ~O-CH$_2$-); 5.29 (m, 2H, =CH$_2$); 5.51 (d, 1H, J = 8.39 Hz, α-NH); 5.89 (m, 1H, ~CHJ-); 7.24-7.42 (m, 4H, arom Fmoc); 7.58 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.74 (d, 2H, J = 7.38 Hz, arom Fmoc).
Fmoc-Lys(Z)-OAll (CDCl₃), δ (ppm): 1.38/1.52 (dm, 4H, γ-CH₂δ-CH₃); 1.70/1.85 (dm, 2H, β-CH₂); 3.17 (m, 2H, ε-CH₂); 4.21 (t, 1H, CH-Fmoc); 4.32-4.45 (m, 3H, α-CH/CH₂-Fmoc); 4.64 (d, 2H, J = 5.71 Hz, −O-CH₂−); 4.85 (m, 1H, ε-NH); 5.08 (s, 2H, CH₂-Bzl); 5.29 (m, 2H, −CH₂); 5.44 (d, 1H, J = 8.39 Hz, α-NH); 5.89 (m, 1H, −CH=); 7.29-7.43 (m, 9H, arom Fmoc(4H)/arom Bzl(5H)); 7.59 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.75 (d, 2H, J = 7.38 Hz, arom Fmoc).

HBr.Fmoc-Lys(H)-OAll (CDCl₃), δ (ppm): 1.31-1.99 (m, 6H, β-CH₂γ-CH₂δ-CH₃); 3.07 (m, 2H, ε-CH₂); 4.13 (t, 1H, CH-Fmoc); 4.20-4.40 (m, 3H, α-CH/CH₂-Fmoc); 4.55 (d, 2H, J = 8.39 Hz, −O-CH₂−); 5.20 (m, 2H, −CH₂); 5.98 (m, 1H, −CH=); 7.19-7.36 (m, 4H, arom Fmoc); 7.48 (d, 1H, α-NH); 7.57 (d, 2H, arom Fmoc); 7.68 (d, 2H, J = 7.38 Hz, arom Fmoc); 7.75 (s, 3H, ε-NH₃⁺).

Fmoc-Lys(Boc)-Pro-OAll (CDCl₃), δ (ppm): 1.20-1.60 (m, 4H, γ-CH₂-Lys/δ-CH₂-Lys); 1.43 (s, 9H, Boc); 1.69/1.81 (dm, 2H, β-CH₂-Lys); 2.01 (m, 3H, β-CH₂-Pro(1H)/γ-CH₂-Pro(2H)); 2.24 (m, 1H, β-CH₂-Pro); 3.13 (m, 2H, ε-CH₂-Lys); 3.58 (m, δ-CH₂-Pro); 4.20 (t, 1H, CH-Fmoc); 4.35 (d, 2H, J = 7.05 Hz, CH₂-Fmoc); 4.46-4.63 (m, 2H, α-CH-Lys/α-CH-Pro); 4.64 (d, 2H, −O-CH₂−); 4.85 (t, 1H, ε-NH-Lys); 5.30 (m, 2H, −CH₂); 5.67 (d, 1H, J = 8.40 Hz, α-NH-Lys); 5.91 (m, 1H, −CH=); 7.26-7.43 (m, 4H, arom Fmoc); 7.59 (d, 2H, J = 7.39 Hz, arom Fmoc); 7.76 (d, 2H, J = 7.39 Hz, arom Fmoc).

HCl.Fmoc-Lys(H)-Pro-OAll (CDCl₃), δ (ppm): 1.40-2.27 (m, 10H, β-CH₂-Lys/γ-CH₂-Lys/δ-CH₂-Lys/β-CH₂-Pro/γ-CH₂-Pro); 3.07 (m, 2H, ε-CH₂-Lys); 3.70 (m, 2H, δ-CH₂-Pro); 4.17 (m, 1H, CH-Fmoc); 4.28 (m, 2H, CH₂-Fmoc); 4.50 (m, 2H, α-CH-Lys/α-CH-Pro); 4.60 (m, 2H, −O-CH₂−); 5.29 (m, 2H, −CH₂); 5.89 (m, 1H, −CH=); 6.35 (m, 1H, α-NH-Lys); 7.19-7.42 (m, 4H, arom Fmoc); 7.61 (d, 2H, arom Fmoc); 7.72 (d, 2H, arom Fmoc); 8.22 (m, 3H, ε-NH₃⁺).

8.6. References


Tert-butylcarbazate, n-octadecylamine and aniline are neutral as such and do not require the addition of acetic acid.


Whittaker, R.G., Hayes, P.J. and Bender, V.J. (1993) Peptide Res. 6, 125-128.


This phenomenon was previously encountered in the difficult removal of the trityl side-chain protecting group from an asparagine residue in N-terminal position [Friede, M., Denery, S., Neimark, J., Kieffer, S., Gausepohl, H. and Briand, J.P. (1992) Peptide Res. 5, 145-147].


Summary

Application of the trityl group in peptide chemistry

The triphenylmethyl (trityl) group allows the application of mild reaction conditions in solid-phase peptide chemistry. In recent years, there has been a tendency towards the synthesis of protected fragments on the solid support. Not only does this facilitate further processing of the peptide, but it also allows the final deprotection to take place in diluted solution, thus minimizing the risk of modification of sensitive amino acid side chains by the liberated cations, which eventually results in products of higher yield and purity.

Applications of the trityl group as a protecting function are numerous and a summary is given in Chapter 1. The versatility may be attributed to three inherent properties. Most typically, the trityl moiety may form adducts with oxygen, nitrogen and sulfur compounds and as such act as a protecting or anchoring group for a wide range of functional groups, including all functional amino acid side chains as well as several possible C-termini (carboxyl, alcohol, amide and hydrazide). Second, the lability of the bond between trityl and functional group may be minutely adjusted by adding electron-releasing substituents to the trityl moiety to increase lability or by adding electron-withdrawing substituents to lower it. Finally, the trityl cation formed upon acidolytic cleavage shows little electrophilic character, thereby circumventing undesired side reactions, like alkylation of the sensitive side chains in tryptophan and methionine by the cation or reattachment to a trityl-type resin.

Until recently, the use of trityl-type resins in Fmoc SPPS had been mainly limited to the synthesis of protected peptide acids on 2-chlorotrityl chloride resin (trityl-type resin I). In Chapter 2, three special applications of this procedure are reported, namely the condensation of protected fragments onto the solid support in the synthesis of "difficult sequences", the activation of thiols and subsequent disulfide bond formation on the resin, and the synthesis of lipophilic peptides through side-chain attachment of aminodicarboxylic acid derivatives to the resin.

In SPPS, a fine balance has to be found between stability of the peptide-resin link during assembly of the peptide on the one hand and its lability during cleavage of the peptide from the resin on the other hand. Synthesis of protected fragments in an Fmoc/Bu protocol limits cleavage conditions, which are determined by the actual linking functional group, to a maximum trifluoroacetic acid (TFA) concentration of 1%. A chloro-substituted trityl system is suitable for carboxyl anchoring, whereas the very stable carboxamidite adducts require trisalkoxycarbonyl systems to allow cleavage in the protected form; other functional groups are intermediate. With the general aim of synthesizing protected fragments through several modes of attachment, solid supports were modified with trityl-type linkers bearing
various degrees of substitution. Modifications took place either in a direct way or by coupling preformed trityl-type handles. This resulted in seven different trityl-type resins, which are listed in Chapter 3. Their relative stabilities towards acidolysis were investigated by means of the tritylthiol model and appeared to obey the general electronic rules regarding aromatic substitutions. A large increase in lability was observed for benzoxy-substituted systems; this finding in combination with its easy preparation prompted us to use the 4-benzoxytrityl alcohol resin (trityl-type resin II) in further syntheses. A colorimetric evaluation of the trityl moiety and its derivatives is also presented in this chapter.

Chapter 4 describes the synthesis of protected peptide hydrazides on trityl-type resins I and II. Synthesis in each case was started with the base-catalyzed attachment of an Fmoc-amino acid hydrazide to the resin in its chloride form. During the course of preliminary experiments, resins based on a preformed bulky trityl-type handle were found less suitable for use in SPPS, due to the partially adsorptive nature of the link between polymeric matrix and the handle and its subsequent instability during the actual peptide synthesis.

Having examined several aspects of the applicability of trityl-type resins for C-terminal attachment, Chapter 5 introduces a general concept involving the attachment of peptides via a functional side chain and the synthetic implications originating from this approach. First, peptides with various modified C-termini become easily accessible by the use of merely one class of resins. Fmoc-amino acids or peptides containing a free side chain may be attached as their esters, alcohols or primary as well as substituted amides, the latter also encompassing lipidic amides.

Second, side-chain attachment offers the possibility of two-directional SPPS. A racemization-free strategy should generally consist of three distinct reaction series: (1) attachment of the first Fmoc-amino acid via a free side chain to the resin, (2) elongation at the C-terminus with one amino acid or peptide, which is C-terminally protected, and (3) standard Fmoc SPPS in N-terminal direction. As we found that base-catalyzed side-chain attachment to a trityl-type resin cannot proceed selectively in the presence of an unprotected carboxyl function, we suggest the application of orthogonal allyl or Dmb esters for temporary C-terminal protection, which is to be removed after initial attachment. In case of acid-catalyzed Fmoc-cysteine attachment, this precaution is not necessary.

A third synthetic pathway stems from the application of these same orthogonal esters, but in this approach they are deprotected after complete assembly of the peptide. Taking advantage of the pseudo-dilution principle, which is likely to apply to syntheses on a solid support, head-to-tail cyclization of the peptide may occur while it is still attached to the resin.

All three strategies were investigated for the case of amine attachment to trityl-type resins I and II. Besides 1,6-diaminohexane, several lysine derivatives, including the new compound Fmoc-lysine allyl ester, were applied in the synthesis of both linear and cyclic ODN-7 analogues. While hitherto strategies employing side-chain attachment were merely based on aspartic and glutamic acid
derivatives, it is our strong belief that the application of trityl-type resins, which allow the attachment of a vast variety of functional groups, in combination with new orthogonal protecting groups, will greatly enlarge the scope of SPPS.

Another fine example of the numerous possibilities offered by side-chain attachment is presented in Chapter 6. Cysteine immobilization onto trityl-type resin II eventually led to the synthesis of protected disulfide-containing peptides by iodolytic detachment. Upon incorporation of an S-Acm protected cysteine into the peptide sequence, product formation could be influenced by the choice of the solvent, following the general rules concerning the reactivity of Cys(Acm) and Cys(Trt) observed by Kamber. In DMF, cyclic monomers of high purity were obtained, thereby supporting the validity of the pseudo-dilution principle. Peptides could also be cleaved from the solid support by S-carbomethoxysulfenyl chloride yielding the thiol in its activated form, or by 1% TFA in the presence of a silane scavenger affording the free thiol.

Returning to the trityl moiety as a protecting group, Chapter 7 describes the application of the 4,4'-dimethoxy-4''-methyltrityl (Dmt) group, an intermediate in the synthesis of one of the trityl-type resins, for asparagine, glutamine and cysteine side-chain blocking. Very recently, the superiority of an Fmoc/Trt protection scheme towards the conventional Fmoc/Bu scheme has been proved. The new derivatives are very suitable in the Fmoc/Trt approach. While Cys(Dmt) was quickly deprotected by 0.5% TFA, Asn(Dmt) and Gln(Dmt) required 5-10% TFA-treatment. These conditions are much milder than the ones commonly applied in Fmoc SPPS and hence provoke less side reactions.

Chapter 8 deals with the preparation of various Fmoc-amino acid derivatives, including hydrazides, amides and allyl esters, which are useful in the scope of trityl-type resin application. An extensive analytical evaluation of these new compounds is also presented.
Samenvatting

Toepassing van de tritylgroep in de peptidechemie

De triphenylmethyl- of tritylgroep maakt de toepassing van milde reactiecondities in de “solid-phase” peptidechemie (SPPS) mogelijk. In de afgelopen jaren is er een sterke tendens waarnembaar met betrekking tot de synthese van beschermde fragmenten aan de vaste drager. Dit vergemakkelijkt niet alleen de verdere verwerking van het peptide, maar maakt het ook mogelijk de definitive ont- scherming plaats te laten vinden in een verdunde oplossing. Hierdoor wordt de kans op modificatie van gevoelige aminozuurzijkgetens aanzienlijk verkleind, hetgeen uiteindelijk leidt tot schoonere producten in een hogere opbrengst.

Vele toepassingen van de trityleenheid als beschermgroep zijn bekend en deze zijn samengevat in Hoofdstuk 1. De veelzijdigheid kan worden toegeschreven aan drie inherente eigenschappen. De meest kenmerkende is de mogelijkheid tot het vormen van adducten met zowel zuurstof-, stikstof- als zwavelverbindingen, hierdoor kan de trityleenheid worden gebruikt als bescherm- of verankeringsgroep voor een brede selectie van functionele groepen, waaronder alle zijketens van aminozuren als ook verschillende mogelijke C-terminale eindgroepen (carboxyl, alcohol, amide en hydrazide).

Verder kan de labiliteit van de binding tussen tritylgroep en functionele groep nauwkeurig worden bijgesteld: de toevoeging van elektronenstuwende substituenten aan het tritylskelet verhoogt de labiliteit, terwijl de toevoeging van elektronenzuigende substituenten die verlaagt. Tenslotte vertoont het tritylkation, dat ontstaat na zure afsplitting, een gering elektrofiel karakter, waardoor nevenreacties, zoals de alkylieering van de gevoelige zijketens van tryptofaan en methionine door het kation of de terugadditie aan een hars van het trityltype, worden omzeild.

Lange tijd was het gebruik van tritylharsen in de Fmoc-SPPS grotendeels beperkt tot de synthese van beschermde peptidexuren aan de 2-chloortritylchloride hars (tritylhars I). In Hoofdstuk 2 worden drie speciale toepassingen van deze procedure beschreven, namelijk de condensatie van beschermde fragmenten aan de vaste drager in de synthese van “moeilijke sequenties”, de activering van een thiol en aansluitende disulfidevorming aan de hars, en de synthese van lipoïefiepeptiden door zijketenaanhechting van aminodicarbonzuurderivaten aan de hars.

In de “solid-phase” synthese dient een goede balans gevonden te worden tussen de stabiliteit van de hars-peptide binding gedurende de opbouw van het peptide enerzijds, en de labiliteit van deze tijdens de afsplitting van het peptide van de hars anderzijds. De synthese van beschermde fragmenten volgens het Fmoc/Bu protocol staat als afsluitingsconditie, die wordt bepaald door de functionele groep in kwestie, een maximale trifluorazijnzuur (TFA) concentratie van 1% toe. Een chloor-
gesubsuteerd tritylsysteem is geschikt voor carboxylverankering, terwijl de zeer stabiele amide-adducten een trisalkoxytrityl systeem vereisen, wil afsluiting in beschermde vorm mogelijk zijn; andere functionele groepen liggen qua stabilitiet van de gevormde adducten er tussenin. In Hoofdstuk 3 zijn vaste dragers gomodificeerd met trityllinkers van een verschillende substitutiegraad met als algemeen doel: de synthese van beschermde fragmenten via diverse manieren van aanhechting. De modificaties vonden ofwel direct aan de hars plaats, of door koppeling van een voorgevormde "handle" van het trityltype; deze aanpak leidde tot zeven verschillende tritylharsen. Hun relatieve stabiliteiten ten opzichte van zure afsluiting werden onderzocht met behulp van het tritylthiol-model en bleken geheel te voldoen aan de algemene regels betreffende het elektronisch gedrag van aromatische substituenten. Een grote labiliteit werd gemeten voor benzyoxy-gesubsuteerde systemen; dit gegeven in combinatie met zijn eenvoudige bereiding maakt de 4-benzyoxytritylalcohol hars (tritylhars II) uitmate geschikt voor de verder beschreven syntheses. Een colorimetrische evaluatie van de trityeenheid en zijn derivaten komt in dit hoofdstuk tevens aan bod.

Hoofdstuk 4 behandelt de synthese van beschermde peptidehydraziden aan de tritylharsen I en II. De eerste stap in de synthese was steeds de base-gekatalyseerde aanhechting van een Fmoc-aminozuur hydrazide aan de chlorendvorm van de hars. In de loop van voorbereidende experimenten bleken harsen, die afgeleid waren van voorgevormde sterisch grote "handles" van het trityltype, niet geschikt voor gebruik in de SPPS, vanwege het gedeeltelijk adsorptieve karakter van de binding tussen de polymere matrix en de "handle" zelf en de daaruit volgende instabiliteit tijdens de eigenlijke peptide synthese.

Na het onderzoek naar enkele aspecten van de aanhechting over de C-terminale eindgroep aan tritylharsen, wordt in Hoofdstuk 5 een algemeen concept uitgewerkt betreffende de aanhechting van peptiden via een functionele zijketen en de synthetische gevolgen die uit deze benaderingswijze voortvloeien. Allereerst kunnen peptiden met verschillende gomodificeerde C-terminale functies gesynthetiseerd worden aan één type hars. Fmoc-aminozuuren of peptiden met een vrije zijketen kunnen worden opgehangen als ester, alcohol of primair dan wel gesubstitueerd amide; tot de laatste behoren tevens vetzure amiden.

Ten tweede biedt zijketenaanhechting de mogelijkheid tot bidirectionele SPPS. Een racemiserings-vrije strategie bestaat uit drie afzonderlijke reactiestappen: (1) aanhechting van het eerste Fmoc-aminozuur via een vrije zijketen aan de hars, (2) verlenging aan het C-terminale uiteinde met één aminozuur of peptide, dat C-terminaal beschermd is, en (3) standaard Fmoc SPPS in N-terminale richting. Omdat uit onderzoek van ons bleek, dat de base-gekatalyseerde aanhechting over een zijketen aan een tritylhars niet selectief kan optreden in aanwezigheid van een onbeschermd carboxyl functie, is het nodig deze tijdelijk te beschermen. Wij adviseren hiervoor de orthogonale allyl of Dmab esters, die verwijderd kunnen worden na aanhechting aan de hars. In het geval van de zuur-gekatalyseerde aanhechting van Fmoc-cysteïne is deze extra maatregel overbodig.
Een derde strategie komt voort uit het gebruik van deze zelfde orthogonale esters, die echter nu pas ontschermd worden na de opbouw van het peptide. Gebruik makend van het pseudooverdunnings-principe, dat aan de vaste drager wellicht van toepassing is, kan een cyclisatie van het peptide “van kop tot staart” plaatsvinden, terwijl dit nog aan de hars vastzit.

De drie genoemde strategieën werden onderzocht met betrekking tot de aanhechting over een aminogroep aan de tritylharzen I en II. Behalve 1,6-diaminohexaan werden verschillende lysinederivaten, waaronder de nieuwe verbinding Fmoc-lysine allyl ester, toegepast in de synthese van zowel lineaire als cyclische ODN-7 analoga. Terwijl tot nog toe strategieën, die gebruik maakten van de aanhechting over een zijketen, louter uitgaven van asparagine- en glutaminezuurderivaten, zal de toepassing van tritylharzen, die aanhechting over vele verschillende functionele groepen mogelijk maken, in combinatie met nieuwe orthogonale beschermgroepen, de reikwijdte van de SPPS sterk doen toenemen.

Een ander fraai voorbeeld van de vele mogelijkheden, die zijketenaanhechting biedt, wordt gepresenteerd in Hoofdstuk 6. De immobilisering van cysteïne aan tritylhars II heeft uiteindelijk na jodolytische afspilting geleid tot beschermde peptiden met een inwendig disulfide. Na inbouw van een met S-Acm beschermd cysteïne in de sequentie van het peptide, kon de productvorming gestuurd worden door de keuze van het oplosmiddel; deze was geheel in overeenstemming met de algemene regels, die Kamber constateerde met betrekking tot de reactiviteit van Cys(Acm) en Cys(Trt). In DMF werden cyclische monomeren van een hoge zuiverheid gevormd, waarmee een bewijs werd geleverd voor de geldigheid van het pseudooverdunningsprincipe. De peptiden konden tevens van de hars worden gesplitst met S-carbomethoxysulfonylchlooride, waarbij een geactiveerd thiol ontstond, of met 1% TFA in het bijzijn van een silaan “scavenger”, wat leidde tot het vrije thiol.

Terugkerend naar de toepassing van de trityleenheid als beschermgroepe, beschrijft Hoofdstuk 7 het gebruik van de 4,4'-dimethoxy-4''-methyltrityl (Dmt) groep, een tussenproduct in de synthese van één van de tritylharsen, als zijketenbeschermgroep voor asparagine, glutamine en cysteïne. Onlangs werd eenduidig aangetoond, dat een Fmoc/Trt beschermingschema betere resultaten oplevert dan het conventionele Fmoc/Bu schema. De nieuwe derivaten zijn zeer geschikt voor toepassing in de Fmoc/Trt strategie. Terwijl Cys(Dmt) snel ontschermd werd door 0,5% TFA, was in het geval van Asn(Dmt) en Gin(Dmt) een behandeling met 5 tot 10% TFA vereist. Deze afspiltingscondities zijn veel milder dan die normaal in de Fmoc-SPPS toegepast worden en gaan hierdoor met minder nevenreacties gepaard.

Hoofdstuk 8 behandelt de synthese van verschillende Fmoc-aminozuurderivaten, waaronder hydraziden, amiden en allyl esters, welke binnen het bestek vallen van de veelzijdige toepassingen van tritylharzen. De nieuwe verbindingen zijn uitgebreid geanalyseerd.
Zusammenfassung

Anwendung der Tritylgruppe in der Peptidchemie

Die Triphenylmethyl- oder Tritylgruppe gestattet die Anwendung milder Reaktionsbedingungen in der Festphasenpeptidchemie. In den vergangenen Jahren gab es eine starke Tendenz zur Synthese geschützter Fragmente am festen Träger. Das erleichtert nicht nur die weitere Verarbeitung des Peptids, sondern ermöglicht auch die Abspaltung der Schutzgruppen in verdünnter Lösung. Dadurch nimmt das Risiko der Modifizierung sensitiver Seitenketten durch die entstehenden Kationen erheblich ab, was letztendlich zu reinen Produkten in höherer Ausbeute führt.


Bis vor kurzem war die Anwendung von Harzen des Trityltyps hauptsächlich auf die Synthese geschützter Peptidsäuren am 2-Chlortritylchlorid-Harz (Tritylharz 1) beschränkt. In Kapitel 2 werden drei spezielle Anwendungen dieses Verfahrens beschrieben, nämlich die Kondensation geschützter Fragmente an die feste Phase bei der Synthese "schwieriger Sequenzen", die Aktivierung eines Thiols und nachfolgende Disulfidbrückenschließung am Harz, sowie die Synthese lipophiler Peptide durch Kupplung von Aminodicarbonsäurederivaten über die Seitenkette an das Harz.

In der Festphasensynthese muß die richtige Balance zwischen der Stabilität der Peptid-Harzbinding während des Aufbaus des Peptids einerseits und deren Labilität bei der Abspaltung des Peptids vom Harz andererseits gefunden werden. Die Synthese geschützter Fragmente nach der Fmoc/ButStrategie schränkt die Abspaltungsbedingungen, die durch die jeweilige funktionelle Gruppe bestimmt werden, auf eine maximale Trifluoressigsäure (TFA) Konzentration von 1% ein. Das Chlor-substituierte Tritylsystem wird für die Carboxylankupplung benutzt, während die sehr stabilen Amidaddukte für die Abspaltung in der geschützten Form ein Trisalkoxytritylsystem benötigen;

Kapitel 4 zeigt die Synthese geschützter Peptidhydrazide an den Tritylharzen I und II. Die Synthese begann immer mit der Basen-katalysierten Ankupp lung eines Fmoc-Aminosäurehydrazids an das Harz in der Chloridform. In Vorversuchen wurde deutlich, daß Harze, die entstanden nach Modifizierung mit einem sterisch anspruchsvollen "Handle" vom Trityltyp, für die Festphasensynthese weniger geeignet sind, weil die Anbindung des "Handles" an die polymere Matrix teilweise nur adsorptiv erfolgte und deshalb während des nachfolgenden Peptidaufbaus nicht stabil war.


Eine dritte Synthesesstrategie beruht auf dem Pseudoverdünnungsprinzip, das am festen Träger Gültigkeit haben sollte. Sie geht von der Verwendung der gleichen orthogonalen Ester aus, die aber
jetzt erst nach vollständiger Herstellung des Peptids gespalten werden. Das Peptid kann daraufhin "Kopf/Schwanz"-cyclisiert werden, während es noch am Harz verankert ist.
Die drei vorgestellten Strategien wurden alle erprobt für die Ankopplung von Aminen an die Tritylharze I und II. Außer 1,6-Diaminohekan wurden verschiedene Lysinderivate, darunter der neue Fmoc-Lysin-Allylester, zur Synthese linearer und cyclischer ODN-7 Analoga eingesetzt. Während bisherige Strategien zur Ankopplung über die Seitenkette fast ausschließlich auf Asparagin- und Glutaminsäurederivaten beruhten, ermöglicht die Anwendung von Tritylharzen die Verankerung über viele verschiedene funktionelle Gruppen. In Kombination mit neuen orthogonalen Schutzgruppen, eröfnen sich für die Festphasensynthese von Peptiden viele interessante Variationsmöglichkeiten.
Ein weiteres Beispiel wird in Kapitel 6 vorgestellt. Die Immobilisierung über die Cystein-Seitenkette an das Tritylharz II führte letztendlich zur Synthese geschützter disulfidhaltiger Peptide durch jodolytische Abspaltung. Nach Einbau eines S-Acm geschützten Cysteins in die Peptidsequenz konnte die Produktbildung durch die Wahl des Lösungsmittels gesteuert werden; sie folgte den Regeln, die Kamber in bezug auf die Reaktivität von Cys(Acm) und Cys(Trt) beobachtete. In DMF wurden cyclische Monomere von hoher Reinheit erhalten; gleichzeitig wurde damit die Theorie der Pseudo verdünnung bestätigt. Peptide konnten auch mit S-Carbomethoxysulfenylchlorid vom Harz abgespalten werden unter Bildung des aktivierten Thiols oder mit 1% TFA in Gegenwart eines Silanscavegers, wobei das freie Thiol entstand.
Zurückkehrend zur Anwendung der Trityleinheit als Schutzgruppe, wurde in Kapitel 7 die 4,4'-Dimethoxy-4'-methyltrityl (Dmt) Gruppe, ein Zwischenprodukt bei der Herstellung eines der Tritylharze, als Seitenkettenschutz von Asparagin, Glutamin und Cystein verwendet. Vor kurzem wurde eindeutig gezeigt, daß Peptidsynthese nach der Fmoc/Trt-Schutzgruppen-Taktik bessere Resultate als nach der konventionellen Fmoc/Bu-Taktik liefert. Die neuen Derivate eignen sich gut für die Anwendung in der Fmoc/Trt-Strategie. Während Cys(Dmt) schnell mit 0,5% TFA gespalten wurde, war für die vollständige Spaltung von Asn(Dmt) und Gln(Dmt) 5 bis 10% TFA erforderlich. Diese Deblockierungsbedingungen sind erheblich milder als die üblichen Verfahren und verursachen daher weniger Nebenreaktionen.
Appendix A

Materials and Methods

Materials and devices

Chemicals

All amino acid derivatives were purchased from Bachem or Novabiochem, unless otherwise stated. 2-Chlorotrityl chloride resin (b = 1.41 mmol/g) was purchased from Alexis Corporation. Tetrakis(triphenylphosphine)palladium(0) and S-carboxymethoxysulfenyl chloride were purchased from Aldrich. Solvents were freshly distilled and dried according to standard procedures.

Analytical devices

Melting points were determined with a Monoskop IV apparatus from H. Bock, Frankfurt, and are uncorrected. Optical rotations were measured with a Perkin Elmer 241 polarimeter. Elemental analyses were performed on a Carlo Erba EA 1108. ¹H-NMR spectra were recorded on a Varian VXR 300 MHz spectrometer, FT-IR spectra on a Nicolet 60 SXR spectrometer (PAS technique) and UV-VIS spectra on a Shimadzu UV-160A spectrometer.

Analytical RP-HPLC chromatography was performed on a LC 41 CD apparatus from Bruker-Franzen Analytik GmbH in Bremen (column: Nucleosil 100-5C₁₈, 250 x 4 mm, eluents: A 0.1% TFA/water, B 84% CH₃CN/0.085% TFA/water, detection: 220 nm). SIMS spectra were measured on a MAT 95 from Finnigan MAT GmbH (cesium bombardment) and MALDI-TOF spectra on a BRUKER BIFLEX™ MALDI-TOF mass spectrometer from Bruker-Franzen Analytik GmbH (ion source: MultiPROBE, laser system: pulsed nitrogen laser at 337 nm and 3 ns pulse width, matrix: sinapinic acid). Solid-phase peptide syntheses were performed either manually on a shaking-and-suction device developed at the German Wool Research Institute, or on the fully automatic ACT 200 Peptide Synthesizer from Advanced Chemtech.

Amino acid analyses

Peptides were hydrolyzed in 6 M HCl for 24 hours at 110°C. Evaluation took place on Biotronik LC 2000 and LC 5000 amino acid analyzers using the ninhydrin protocol [1]. With peptides containing cysteine, methionine, tryptophan and tyrosine, a small amount of thioglycolic acid was added [2]. Values for histidine, methionine, tryptophan and tyrosine are usually somewhat low; values for threonine and serine typically amount to 0.92 and 0.84, respectively [3]. Cysteine was not determined.
Thin layer chromatography

Thin layer chromatography was performed on pre-coated silica gel plates Kieselgel 60F254 (Merck). Chromatographical purity was assessed using 2 µl of a 1% solution of the actual sample.

Solvent systems (v/v/v)

A chloroform / methanol (9:1)
B chloroform / methanol / acetic acid (95:5:3)
C ethyl acetate / methanol / water (80:30:5)
D diisopropyl ether
E n-butanol / pyridine / acetic acid / water (15:10:3:12)
F chloroform / methanol / acetic acid (95:20:3)
G ethyl acetate / petroleum ether (1:1)
H chloroform / methanol / water (80:30:5)

Detection methods (reagent, application)

UV

chlorine / TDM reagent [4] aromatic groups
ninhydrin (5% in n-butanol / 2 M acetic acid (95:5)), 110°C [5] NH moiety
sulfuric acid / methanol (1:1) free amino groups
sodium nitroprusside (10% in water) [6] trityl-type groups
modified Barton’s reagent (15% FeCl₃ and 1% K₃Fe(CN)₆ in water) [7] free thiols
hydrazides, free thiols

Standard procedures in Fmoc solid-phase peptide synthesis

Determination of resin loading by Fmoc cleavage

A known quantity (m in mg) of dry resin, between 10 and 15 mg, is rotated for 30 min in 1 ml piperidine/DCM (1:1). Then the resin is filtered and washed twice with alternatingly DCM and 2-propanol. The filtrate is evaporated in vacuo, and the residue dissolved in 50 ml DCM (V). The absorbance ($A_{280}$) of this solution is measured at 267 nm against DCM as a reference [8]. The resin loading (in mmol/g) can be calculated from the formula given in Appendix B.

Protocol for Fmoc SPPS

The standard protocol for Fmoc SPPS is schematically depicted on the following page. SPPS was always performed after the TBTU procedure when Fmoc-serine and/or Fmoc-threonine were used without side-chain protection, to avoid undesired side reactions at the hydroxyl group [9]. In all syntheses described in this thesis, the actual procedure is specified, as well as the method for attachment of the first amino acid derivative.
## STANDARD PROTOCOL FOR FMOC SPPS

<table>
<thead>
<tr>
<th>Step</th>
<th>Operation</th>
<th>Solvent / Reagent</th>
<th>Time</th>
<th>Multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Swelling of the resin</td>
<td>DMF</td>
<td>1 min</td>
<td>once</td>
</tr>
<tr>
<td>2)</td>
<td>Removal of Fmoc group</td>
<td>20% piperidine/DMF</td>
<td>6 min</td>
<td>3 times</td>
</tr>
<tr>
<td>3)</td>
<td>Wash</td>
<td>DMF</td>
<td>0.5 min</td>
<td>3 times</td>
</tr>
<tr>
<td>4)</td>
<td>Wash</td>
<td>2-propanol</td>
<td>0.5 min</td>
<td>twice</td>
</tr>
<tr>
<td>5)</td>
<td>Kaiser test [10] *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6)</td>
<td>Swelling of the resin</td>
<td>DMF</td>
<td>1 min</td>
<td>once</td>
</tr>
<tr>
<td>7a)</td>
<td>Acylation / DCC procedure</td>
<td>Fmoc-amino acid (3 meq), DCC (3.3 meq) and HOBT (3.6 meq) are dissolved in 5 ml DMF and added to the resin</td>
<td>45 min</td>
<td>once</td>
</tr>
<tr>
<td>7b)</td>
<td>Acylation / TBTU procedure</td>
<td>Fmoc-amino acid, TBTU, HOBT (3 meq each) and NMM (4.5 meq) are dissolved in 5 ml DMF and added to the resin</td>
<td>45 min</td>
<td>once</td>
</tr>
<tr>
<td>8)</td>
<td>Wash</td>
<td>DMF</td>
<td>0.5 min</td>
<td>3 times</td>
</tr>
<tr>
<td>9)</td>
<td>Wash</td>
<td>2-propanol</td>
<td>0.5 min</td>
<td>twice</td>
</tr>
<tr>
<td>10)</td>
<td>Kaiser test [10] **</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11)</td>
<td>Swelling of the resin</td>
<td>DMF</td>
<td>1 min</td>
<td>once</td>
</tr>
<tr>
<td>12a)</td>
<td>Capping / DCC procedure</td>
<td>400 μl Ac₂O and 200 μl DIPEA are dissolved in 5 ml DMF and added to the resin</td>
<td>10 min</td>
<td>once</td>
</tr>
<tr>
<td>12b)</td>
<td>Capping / TBTU procedure</td>
<td>0.83 g AcONSi and 250 μl DIPEA are dissolved in 5 ml DMF and added to the resin</td>
<td>30 min</td>
<td>once</td>
</tr>
<tr>
<td>13)</td>
<td>Wash</td>
<td>DMF</td>
<td>0.5 min</td>
<td>3 times</td>
</tr>
<tr>
<td>14)</td>
<td>Wash</td>
<td>2-propanol</td>
<td>0.5 min</td>
<td>twice</td>
</tr>
</tbody>
</table>

* Proceed if positive (Pro is negative).
** If negative proceed; if positive resume from step 6, with half of the quantities in step 7.
References

6 In: Anfärberreagenzien für die Dünnschicht- und Papier-Chromatographie (1970) E. Merck, Darmstadt, p. 79.
Appendix B

Mathematical Formulas in SPPS

Calculation of coupling yield (%) and mean coupling yield per step (%s)

\[
\begin{align*}
\frac{b_i}{m_i} &= \frac{q}{b_i} & \Rightarrow & & m_i = \frac{q}{b_i} \\
\frac{b_n}{m_n} &= \frac{x \cdot q}{b_n} & \Rightarrow & & m_n = \frac{x \cdot q}{b_n} \\
\Rightarrow & & \% = 100 \cdot x = \frac{b_n \cdot 10^3}{b_i \cdot (1000 - MW \cdot b_n)} \\
& & \Rightarrow & & \%_s = \left( \frac{b_n \cdot 10^3}{b_i \cdot (1000 - MW \cdot b_n)} \right)^{1/n}
\end{align*}
\]

b\(_i\) initial resin loading [mmol/g]; b\(_n\) new resin loading [mmol/g]; m\(_i\) initial mass; m\(_n\) new mass; q arbitrary molar quantity; x fraction of q that is coupled to; MW net molar mass difference upon coupling [g/mol]; % (overall) coupling yield; %\(_s\) mean coupling yield per step; n number of steps.

Calculation of cleavage yield (%)

\[
\begin{align*}
\frac{b_i}{m_i} &= \frac{q}{b_i} & \Rightarrow & & m_i = \frac{q}{b_i} \\
\frac{b_n}{m_n} &= \frac{(1 - x) \cdot q}{b_n} & \Rightarrow & & m_n = \frac{(1 - x) \cdot q}{b_n} \\
\Rightarrow & & \% = 100 \cdot x = \frac{(b_i - b_n) \cdot 10^3}{b_i \cdot (1000 - MW \cdot b_n)} \\
& & \Rightarrow & & \% = \left( \frac{b_i - b_n}{b_i \cdot (1000 - MW \cdot b_n)} \right)^{1/n}
\end{align*}
\]

b\(_i\) initial resin loading [mmol/g]; b\(_n\) new resin loading [mmol/g]; m\(_i\) initial mass; m\(_n\) new mass; q arbitrary molar quantity; x fraction of q that is cleaved; MW net molar mass difference upon cleavage (MW≥0) [g/mol]; % cleavage yield.
Calculation of resin loading (b) through Fmoc determination

\[ A = \varepsilon \cdot c \cdot d \quad \Rightarrow \quad c = \frac{A}{\varepsilon \cdot d} \]

\[ b = \frac{1000 \cdot c \cdot V}{m \cdot 10^{-3}} \quad \Rightarrow \quad b = \frac{500 \cdot A_{267}}{175 \cdot m} \]

\[ \varepsilon = 17500, \quad d = 1, \quad V = 0.05 \]

A absorbance; \( \varepsilon \) molar extinction coefficient [1000 cm\(^2\)/mol]; c concentration of measured solution [mol/L]; d cuvette length [cm]; V volume [L] (see Appendix A); b resin loading [mmol/g]; m mass of resin sample used for determination [mg]; \( A_{267} \) measured absorbance at 267 nm.

Calculation of resin loading (b) through other modes of determination

**gravimetric determination**

\[ b = \frac{1000 \cdot (m_n - m_i)}{MW \cdot m_n} \]

**sulfur determination after Ellman\(^a\)**

\[ b_i = \frac{(1.43 + 70.79 \cdot A_{412})}{2 \cdot m} \]

**sulfur elemental analysis\(^b\)**

\[ b_i = \frac{\%S}{3.2} \]

**nitrogen elemental analysis\(^c\)**

\[ b_i = \frac{1000 \cdot (%N_i - %N_n)}{MW \cdot %N_i} \]

b resin loading [mmol/g]; \( b_i \) initial resin loading [mmol/g]; \( m_n \) new resin mass [mg]; \( m_i \) initial resin mass [mg]; MW net molar mass difference upon coupling [g/mol]; \( A_{412} \) absorbance at 412 nm; m mass of resin sample used for determination [mg]; \( \%S \) measured sulfur content; \( \%N_n \) measured nitrogen content upon coupling; \( \%N_i \) measured nitrogen content before coupling.

Applied in Chapter 3 to determine the initial loading of: \(^a\) Fmoc-Cys(resin II)-OH (see also Experimental section and Footnote 26); \(^b\) Fmoc-Cys(resin II)-OH; \(^c\) trityl-type resin V.

Example of \textbf{MW calculation} for coupling of Fmoc-Gly-OH to 2-chlorotrityl chloride resin:

\[ MW = 223.251 \text{(Fmoc)} + 57.052 \text{(Gly)} + 15.999 \text{(O)} - 35.453 \text{(Cl)} = 260.849 \]
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