BACHEM

SOLID PHASE PEPTIDE SYNTHESIS

TIPS AND TRICKS FOR SOLID PHASE PEPTIDE SYNTHESIS FROM THE EXPERTS AT BACHEM

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List of Abbreviations

Protecting Groups and Active Esters

Acm	Acetamidomethyl
Adpoc	2-(1'-Adamantyl)-2-propyloxycarbonyl
Aloc	Allyloxycarbonyl
Boc	tert. Butyloxycarbonyl
Bom	Benzyloxymethyl
2-BrZ	2-Bromobenzyloxycarbonyl
tBu	tert. Butyl
Bzl	Benzyl
2-ClZ	2-Chlorobenzyloxycarbonyl
Dde	1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl
2,6-diClBzl	2,6-Dichlorobenzyl
Dmb	2,4-Dimethoxybenzyl
Dnp	2,4-Dinitrophenyl
Fm	9-Fluorenylmethyl
Fmoc	9-Fluorenylmethyloxycarbonyl
For	Formyl
Hmb	2-Hydroxy-4-methoxybenzyl
MBzl	4-Methylbenzyl
Mmt	4-Methoxytrityl
Mob	4-Methoxybenzyl
Mtr	4-Methoxy-2,3,6-trimethylphenylsulfonyl
Mtt	4-Methyltrityl
Npys	3-Nitro-2-pyridylsulfenyl
OAll	Allyl ester
OtBu	tert. Butyl ester
OBt	3-Hydroxy-1,2,3-benzotriazole ester
OcHex	Cyclohexyl ester
OcPen	Cyclopentyl ester
ODhbt	3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester
ODmab	4-{-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl] amino}benzyl ester
OFm	9-Fluorenylmethyl ester
OMpe	3-Methylpent-3-yl ester
OPfp	Pentafluorophenyl ester
OPp	2-Phenylisopropyl ester
OSu	Hydroxysuccinimide ester
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulfonyl
StBu	tert. Butylthio
Tfa	Trifluoroacetyl
Tmob	2,4,6-Trimethoxybenzyl
Trt	Trityl
Tos	p-Toluenesulfonyl
Xan	9-Xanthydryl
Z	Benzyloxycarbonyl

Reagents

BTFFH	Bis(tetramethylene)fluoroformamidinium hexafluorophosphate
BOP	Benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate
DBU	Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DEPBT	3-(Diethoxy-phosphoryloxy)-3H-benzo [d][1,2,3] triazin-4-one
DIC	Diisopropylcarbodiimide
DTE	Dithioerythritol
DIPEA	Diisopropylethylamine
DMAP	N,N-Dimethylaminopyridine
EDT	Ethanedithiol
HATU	O-(7-Azabenzotriazolyl)-tetramethyluronium hexafluorophosphate*
HBTU	(Benzotriazole-1-yl) tetramethyluronium hexafluorophosphate*
HOAt	1-Hydroxy-7-aza-benzotriazole
HOBt	1-Hydroxybenzotriazole
PyBOP	(Benzotriazol-1-yl)oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
TATU	(7-Azabenzotriazolyl) tetramethyluronium tetrafluoroborate*
TBTU	(Benzotriazolyl) tetramethyluronium tetrafluoroborate*
TEA	Triethylamine
TFA	Trfluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
TES	Triethylsilane
TFFH	Tetramethylfluoroformamidinium hexafluorophosphate
TIS	Triisopropylsilane
TMSBr	Trimethylsilyl bromide
TMSCl	Trimethylsilyl chloride
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TNBS	2,4,6-Trinitrobenzenesulfonic acid

Resins

BHA	Benzhydrylamine
DHPP	4-(1',1'-Dimethyl-1'-hydroxypropyl)phenoxyacetyl alanyl aminomethylpolystyrene
MBHA	4-Methylbenzhydrylamine
PAM	Phenylacetamidomethyl
PDDM	Polymeric diphenyldiazomethane

* cf. I. Abdelmoty, F. Albericio, L.A. Carpino, B.M. Foxman, and S.A. Kates, Lett. Pept. Sci. 1 (1994) 57.

Solvents

AcOH	Acetic acid
DCM	Dichloromethane
DMA	N,N-Dimethylacetamide
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
HFIP	Hexafluoroisopropanol
IPA	Isopropanol
MTBE	Methyl tert. butyl ether
NMP	N-Methylpyrrolidone
TFE	Trifluoroethanol

Miscellaneous

AA	Amino Acid
DKP	Diketopiperazine
FTIR	Fourier Transformed Infra Red
HPLC	High Performance Liquid Chromatography
MALDIMS	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
MASNMR	Magic Angle Spinning Nuclear Magnetic Resonance
MS	Mass Spectrometry
SPOS	Solid Phase Organic Synthesis
SPPS	Solid Phase Peptide Synthesis
TLC	Thin Layer Chromatography

FOREWORD

This publication is a practical vademecum in which Bachem's chemists involved in solid phase synthesis for many years have gathered their knowledge and experience in SPPS.
The idea is to discuss the variables of solid phase synthesis and to present the choices, advantages and drawbacks of each one enabling an optimal selection for an «easy» and successful synthesis.
The procedures described in this brochure are routinely used but we can't guarantee that they can be applied in all cases. When in doubt it is strongly recommended to perform feasibility experiments before using the bulk of the material.
During the last years, several books have been published in which SPPS is a major topic. We want to cite them apart from the literature references.

- Methods in Enzymology 289, Solid Phase Peptide Synthesis, (G.B. Fields Ed) Academic Press 1997.
- Chemical Approaches to the Synthesis of Peptides and Proteins, (P. Lloyd-Williams, F. Albericio, E. Giralt Eds), CRC Press 1997.
- Fmoc Solid Phase Peptide Synthesis, A Practical Approach, (W.C. Chan, P.D. White Eds), Oxford University Press 2000.
- Solid Phase Synthesis, A Practical Guide, (S.F. Kates, F. Albericio Eds), Marcel Dekker 2000.
- Houben-Weyl E22a, Synthesis of Peptides and Peptidomimetics (M. Goodman, Editor-in- chief; A. Felix, L. Moroder, C. Toniolo, Eds), Thieme 2002, p.665ff.

I INTRODUCTION

1. Historical Background

Solid Phase Peptide Synthesis (SPPS) can be defined as a process in which a peptide anchored by its C-terminus to an insoluble polymer is assembled by the successive addition of the protected amino acids constituting its sequence.
Each amino acid addition is referred to as a cycle consisting of:

a) cleavage of the N^{α}-protecting group
b) washing steps
c) coupling of a protected amino acid
d) washing steps

As the growing chain is bound to an insoluble support the excess of reagents and soluble by-products can be removed by simple filtration. Washing steps with appropriate solvents ensure the complete removal of cleavage agents after the deprotection step as well as the elimination of excesses of reagents and by-products resulting from the coupling step.
For a general scheme of SPPS see Fig. 1 on p. 10. Table 1 gives an overview of important developments during the history of SPPS.

Table 1. 50 Years of history - A choice of key dates.

Year	Authors	Development
1963	Merrifield	Development of SPPS [1], insoluble carrier: crosslinked polystyrene; N^{α}-protecting group: Boc
1967	Sakakibara	HF-cleavage [2]
1970	Pietta & Marshall	Introduction of BHA-resin for the synthesis of peptide amides [3], MBHA-resin: Matsueda & Stewart 1981 [4]
1970	Carpino & Han	Fmoc, a base labile N^{α}-protecting group [5]
1973	Wang	Development of p-alkoxybenzyl alcohol resin (Wang resin) [6], cleavage: TFA; N^{α}-protection: Bpoc
1976	Burgus & Rivier	Application of preparative reversed phase HPLC for the purification of peptides prepared by Boc SPPS [7]
1977	Barany et al.	The concept of «orthogonal» protection schemes [8]
1978	Meienhofer et al.	Fmoc/tButyl strategy. Carrier: p-alkoxybenzyl alcohol resin; N^{α}-protection: Fmoc; side-chain protection: TFA- labile, e.g. Boc, tBu; final cleavage: TFA [9]
1985	Houghten and others	Simultaneous parallel peptide synthesis, synthesis of peptide libraries (T-bags, pins, etc.) [10,11]
1985	Rapp and others	Polystyrene-polyethylene glycol grafts e.g. TentaGel [12]
1987	Rink and others	Introduction of various TFA-labile linkers for the Fmoc/tBu SPPS of peptide amides [13–15]
1987	Sieber	«Xanthenyl linker» for the Fmoc/tBu SPPS of fully protected peptide amides, cleavage: 1% TFA/DCM [16]
1987	Mergler et al.	Development of 2-methoxy-4-alkoxybenzyl alcohol resin SASRIN (Super Acid Sensitive ResIN) for the Fmoc/tBu SPPS of fully protected peptide fragments, cleavage: 1% TFA/DCM [17]
1988	Barlos et al.	2-Chlorotritylchloride resin for the Fmoc/tBu SPPS of fully protected peptide fragments, cleavage: AcOH/TFE/ DCM (1:1:3) or HFIP/DCM (1:4) [18]
1993	Hobbs de Witt, Ellman and others	Combinatorial Chemistry; Solid Phase Organic Synthesis (for rapid synthesis of libraries of small molecules [19-22])
1995	Mutter et al.	Pseudoproline dipeptides [23]
2002	Gogoll and others	Microwave-accelerated SPPS [24]
2003	White and others	Fmoc SPPS of long peptides (100 AA) [25]

Although in general acidolytic cleavage from the resin is the method of choice to release the peptide at the end of the synthesis, a broad range of resins susceptible to be cleaved by nucleophiles such as the «Kaiser oxime resin» [26] and the p-carboxybenzyl alcohol linker [27] or by photolysis [28] has gained popularity.
Quite often, these moieties are not compatible with the conditions of Fmoc SPPS, whereas allyl-based anchors [29] are resistant towards the cleavage conditions of Boc as well as Fmoc protecting groups. The so-called «safety-catch linkers» are perfectly compatible with both Boc and Fmoc chemistries. Only after an activation step they are highly sensitive towards nucleophiles e.g. the sulfonamide linker [30] or 4-hydrazinobenzoic acid [31].

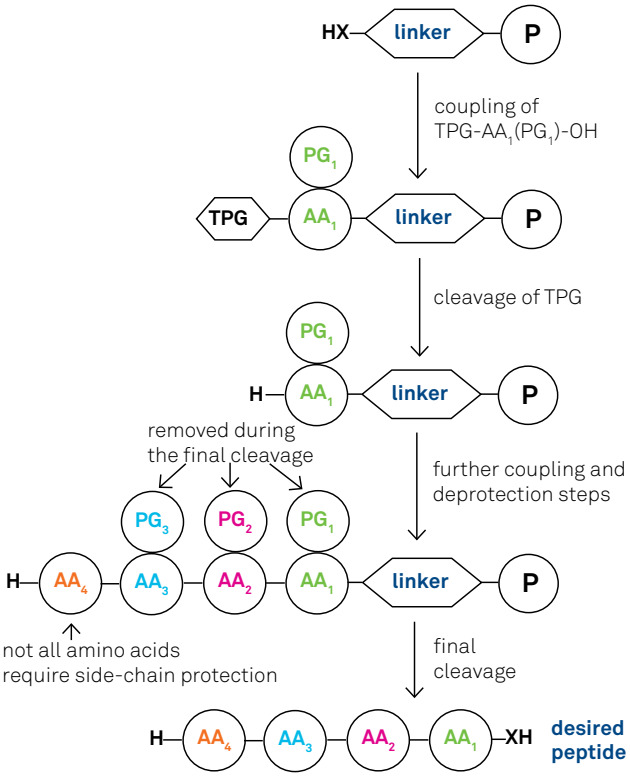


Fig. 1.
General scheme of SPPS.
X = O, NH
AA = Amino Acid
PG = Protecting Group
P = Polymer Support
TPG = Temporary Protecting Group

2. Boc or Fmoc?

The choice of an adequate combination of protecting groups/solid support is the first step on the way to achieve a successful synthesis. For standard SPPS this choice is generally limited to a Boc/benzyl or a Fmoc/tBu based scheme. During the first 15 years of SPPS, the Boc group has been used almost exclusively.

Even if this technique permitted remarkable synthetic achievements [32,33] the introduction of a new type of protecting group has offered more flexibility for the modification of the peptide chain and/or more specificity in the cleavage of the N^{α}- versus the side-chain protecting groups. The combination Fmoc/tBu has met these requirements and broadened the scope of SPPS. Moreover, the development of new resin derivatives has allowed the cleavage of fully protected sequences which can be further coupled in SPPS or in a classical solution process.

In addition, a variety of selectively cleavable protecting groups offers new perspectives for «on-resin» modification (cyclization, formation of disulfide bridges, derivatization of side chains, etc).

The main characteristics of the two general approaches are outlined in Table 2.

3. Equipment

3.1. Manual Synthesis

The «classical» reactor for SPPS merely consists of a cylindrical vessel with a fritted disc and a removable lid equipped with a mechanical stirrer. Shakers have already been used by Merrifield, for a popular model see the photograph on p. 74 in [34]. The resin may also be stirred by bubbling nitrogen through, however more elaborate equipment is required. For rapid small scale synthesis a small fritted glass funnel is sufficient. Oxygen and moisture need not be strictly excluded, but the cleavage of the N^{α} protecting group should be performed under a hood as to avoid exposure to piperidine (Fmoc cleavage) or TFA (Boc cleavage). The swelling of the resin has to be taken into consideration in the choice of the reactor size. Normally, the volume of the

Table 2. Fmoc/tBu or Boc/Bzl?

Topic	Fmoc/tBu	Boc/Bzl
Use	Routine synthesis	Requires special equipment
N^{α}/side chain protection	orthogonal[1)]	both acid labile
TFA treatment	final cleavage	repetitive cleavage
HF treatment	none	final cleavage
Automation	yes	yes
Scale	any scale, including final cleavage	HF cleavage: limited scale
Monitoring: N^{α}-deblocking, completion of coupling	UV-absorption, chromophores: Fmoc, dibenzofulvene-piperidine adduct	quantitative ninhydrin test: cumbersome
Synthetic steps	deblock, wash, couple, wash	additional neutralization step
Avoidance of DKP formation	circumvention tedious; synthesis on 2-chlorotrityl resin: suppression of DKP formation	change of coupling protocol: concomitant coupling/neutralization
Final cleavage	in SPPS vessel	special equipment required
Especially recommended for	acid sensitive peptides & derivates, e.g. O-glycosylated or sulfated peptides	base labile peptides; «difficult sequences», aggregation impeded by repetitive TFA treatment

[1)] For a definition see II.3.1.

swollen peptide resin will slowly increase during chain elongation. When synthesizing a medium-sized peptide (20–30 AA) using Fmoc SPPS, a 100–150 ml reactor will suffice for ca. 10 g of resin. The swelling will be more important in Boc SPPS mostly during the TFA deprotection step; a 250 ml reactor would be recommended for the above-mentioned synthesis. Vessels for small-scale SPPS are depicted in Fig 2a (p.12), Fig. 2b (p. 17) shows a large-scale reactor.

At the beginning of each coupling cycle, deblocking or washing step the resin and the solution have to be mixed thoroughly, followed by slow stirring or shaking for the remaining process. All the beads have to be suspended in the liquid for thorough washing, efficient coupling, and complete deblocking. It is important to watch for beads sticking to the wall of the vessel especially during the coupling and rinse them from the wall with a small amount of solvent if necessary. «Sticking beads» may become a problem when stirring too vigorously. Silylation of the glassware improves the surface hydrophobicity and prevents the beads from sticking to the wall of the vessel.

Solvents are filtered off by slight suction, or, more gently, by applying inert gas pressure. In Fmoc/tBu based SPPS the vessel may also be used for the final cleavage or for the cleavage of fully protected peptides from very acid-labile resins such as SASRIN. Using a manual synthesizer may be more cumbersome than employing a fully automated one, but any parameter can be changed at any time. A more thorough monitoring is possible as samples for analysis can be removed at each stage of the synthesis.

3.2. «Quasi Continuous Flow»

In this approach the solid support is packed into a column and the reagents and solvents are delivered by a pump. The resins used in this technique must be able to withstand considerable pressure and, at the same time, keep a constant volume while changing solvents. The standard polystyrene-based resin is not suitable for that purpose as the volume of the beads markedly depends on the solvent. Nevertheless, continuous-flow synthesizers taking into account the shortcomings of swollen polystyrene have been developed [35,36].

This type of synthesizer is best used for Fmoc-based protocols. The Boc protocols generate ionic species during the Boc cleavage, which cause considerable changes in swelling due to electrostatic forces.
A synthesizer has been developed in which swelling is monitored, considering that during Fmoc-SPPS, volume changes in a given solvent can only be caused by the growing peptide chain [37].
Composite material made from a rigid support such as Kieselguhr particles [38] or large pore crosslinked polystyrene [39] in which dimethylacrylamide [40] has been polymerized are used for continuous flow synthesis.
Poly(ethylene glycol)-based supports such as TentaGel or PEGA have been introduced for batch as well as continuous flow synthesis [41–43]. For a review of recent developments in this field see [12].

3.3. Fully Automated SPPS

A variety of fully automated synthesizers for batchwise and continuous flow SPPS is commercially available [44]. Fig. 2a shows the reaction vessels of such a machine allowing parallel small-scale syntheses. In the meantime, fully automated synthesizers employing microwave irradiation for accelerating the synthetic steps were successfully introduced to the market [24].
Fmoc/tBu SPPS permits automatic monitoring and adequate adjustment of deprotection and coupling times in order to achieve complete conversions. The monitoring relies on strong chromophores which are either released during deprotection [45] or «consumed» [46] (the Fmoc amino acid derivative) and concomitantly released (HOBt or HOAt) during coupling. Monitoring via changes of conductivity [46] allows the monitoring on a real-time basis and end point value can be given to determine the completion of the coupling reaction.

II FMOC-BASED SPPS

1. Resins

1.1. General Remarks

All resins marketed by Bachem are obtained from beaded polystyrene crosslinked with 1% divinylbenzene (a mixture of the meta and the para isomer). This degree of crosslinking is optimal for SPPS. A higher level of crosslinking would reduce the swelling whereas a decrease would cause a considerable loss of mechanical stability in the swollen state.
The carrier resins for SPPS are obtained from this polymer or from the chloromethylated material. In the second case, the available load is restricted by the degree of chloromethylation. The average bead size is adjusted by the conditions of polymerization. Bachem offers the most popular size distribution 200–400 mesh (average diameter 38–75 µm). A variety of resin derivatives is also available as large beads: 100–200 mesh (average diameter 75–150 µm). With such resins, reaction times may have to be prolonged due to limited diffusion towards the interior of the beads.

Fig. 2a. Fully automated reactor for parallel small-scale SPPS, reaction vessels.

The load of the resins is adapted to the needs of routine SPPS: 0.7–1 meq/g before the loading of the first Fmoc amino acid. Loads may be deliberately reduced, e.g., for side-chain cyclization, for the synthesis of long peptide chains (above 30–40 residues), or for the preparation of sequences presenting intrinsic difficulties. Resins having a particularly high load can be prepared by Bachem on request.

1.2. General Handling of the Resin

The term «Solid Phase» peptide synthesis is actually misleading, gel-phase synthesis would be more appropriate [47]. The swelling, i.e. the solvation of the polystyrene chains and the functionalized moieties including the growing peptide, remains essential for successful SPPS and even more so in SPOS. The swelling volumes of polystyrene-based resins in the most important solvents have been determined by Santini [48]. The complete swelling of the dry resin may take up to 1 hour [48]. Unmodified crosslinked polystyrene and chloromethylated polystyrene swell very well in apolar solvents such as toluene, dioxane and DCM, moderately in DMF and poorly, if at all in alcohols and water. The swelling behaviour of derivatized polystyrene depends on the load and the polarity of the functional groups. These moieties are usually rather polar: amides, alcohols, amines, esters, ethers, etc., and improve the interactions with polar solvents whereas no «additional polarity» is gained when working with the «purely aromatic» 2-chlorotrityl chloride resin. So, after the loading with an Fmoc amino acid, the Fmoc group is split off with piperidine/DMF (1:4) considerably slower from the 2-chlorotrityl resin than from the Wang resin derivative.

SPPS relies on proper swelling in polar solvents as polar aprotic solvents facilitate coupling (see II. 4); good swelling means good accessibility of coupling sites and thus, a smooth reaction (even though a few exceptions to this rule have been observed [49]). Concurrently with the peptide elongation, swelling in DMF normally increases. But it should be kept in mind especially when synthesizing long peptides that swelling also means dilution of coupling sites and reagents. A slow and steady increase of their excess will compensate for this effect.

As mentioned above, the coupling rate is controlled by the diffusion of the activated species into the swollen bead, i.e. the larger the bead the slower the coupling. Thus, the coupling can't be accelerated by vigorous stirring. Slight stirring or shaking is sufficient to support the diffusion of reagents into the beads.

Sudden shrinkages should alarm the operator. This phenomenon is caused by the aggregation of the peptide chain which will impede the continuation of the synthesis. Complete coupling reaction and deblocking will be difficult to attain due to the steric hindrance created by the aggregation. A range of methods to improve the efficiency of these key steps will be dealt with later on.

As the peptide resin may be deliberately swollen or shrinked, washes with shrinking solvents such as IPA accelerate the removal of excesses of reagents, and time must then be allowed for proper swelling, e.g. in DMF. The peptide resin should not be shrinked when the peptide may aggregate, which is rather difficult to predict [50] and during the first cycle of a synthesis. On the other hand, when shrinking the resin with MTBE before coupling the maximum concentration of coupling reagents is attained. For fragment coupling it has even been recommended to treat the dried resin with a solution of the activated fragment [51].

A WASH represents a short treatment (1–5 min, depending on the amount) of the peptide resin with a solvent under gentle stirring. The swollen resin may be inspected under a microscope. Regular round spheres should be observed, but not neccesarily smooth surfaces. Torn beads and fines result from inappropriate treatment of the resin and will clog frits. Mechanical stress has to be minimized as swollen beads are rather susceptible to abrasion. So they should not be stirred with a magnetic bar (except for cleavage, as the carrier is normally not recovered). Reaction vessels and stirrer blades have to be designed to minimize shearing forces. As already mentioned, a slight stirring will suffice, and the system has to be thoroughly mixed only when starting a washing step or a reaction. Vigorous suction and suction to dryness will unnecessarily stress the peptide-resin. Applying inert gas pressure to remove the solvent is a gentle alternative. The inert atmosphere

may be beneficial, though inertization is not an esential requirement of SPPS.
The load of the carrier resin is determined by elemental analysis (N, Cl) and/or by coupling an Fmoc amino acid and determining the resulting conversion (see below). Resins may also be characterized, e.g., by FTIR-spectroscopy. The growing interest in SPOS led to renewed interest in the method and refined instrumentation for the characterization of solid samples [52]. MAS-NMR can also been carried out if the resin is properly swollen [53].
Requirements of storage depend on the nature of the resin: PDDM-resin and photolabile resins have to be protected from light, 2-chlorotritylchloride resin is sensitive to humidity; in most cases the resins have to be stored in the deep-freezer.

1.3. Resins Available from Bachem

1.3.1. Resins for the synthesis of peptide acids

Wang resin and preloaded Wang resins
(4003214 (200–400 mesh) and 4027344 (100–200 mesh))
Wang resin, i.e. p-alkoxybenzyl alcohol resin, may be termed the standard resin for Fmoc/tBu SPPS of «peptide acids». The tert. butyl type side-chain protection is concomitantly removed during acidolytic cleavage from this resin.
The esterification of Wang resin as well as of other resins bearing hydroxyl groups with Fmoc amino acids is a crucial step in SPPS. It is more difficult than it may seem considering that high conversion and, especially, minimal racemization are desired. We therefore recommend the use of preloaded resins. Bachem offers a broad range of Fmoc L- and D-amino acids coupled to Wang resin. If the resin derivative you need is not yet available please ask for a quotation. In any case, Bachem guarantees high loading and minimal racemization.

DHPP resin and Fmoc-Pro-DHPP resin
DHPP-resin, i.e. 4-(1',1'-dimethyl-1'-hydroxypropyl) phenoxyacetyl alanyl aminomethyl polystyrene, has been developed especially for the synthesis of peptides containing a C-terminal proline [54-56].
The bulkiness of the linker prevents diketopiperazine formation, but the esterification of Fmoc-Pro-OH (yielding a modified tert. butyl ester) is impeded as well. Bachem offers the preloaded Fmoc-Pro-DHPP resin (4019306).

Diphenyldiazomethane resin (PDDM-resin)
(4027172)
PDDM-resin, i.e. diphenyldiazomethane resin 4027172, readily reacts with carboxylic acids in DCM [57,58]. The rate correlates with the acidity of the substrate. Nitrogen, the only by-product, is evolved concomitantly. As the incoming amino acid is not activated racemization is suppressed; as a result, PDDM-resin lends itself especially for the synthesis of peptides containing a C-terminal Cys or His.
Due to the simple and reliable linkage protocol PDDM-resin is especially recommended for the anchoring of expensive amino acids. The excess of derivative can also be easily recovered as no coupling reagent is used. Bulky amino acids such as Fmoc-Aib and peptide fragments react readily with PDDM-resin. The acids don't have to be pure but they must not be contaminated by other acids of comparable strength or stronger; on the other hand, selective alkylation of the more acidic carboxyl group may be attained. PDDM-resin reacts preferentially with the α-carboxyl group of Fmoc-Glu-OH in DCM/DMF (3:1), the γ-carboxyl can be modified otherwise (M. Mergler, unpublished results). Loading of PDDM-resin is best performed in DCM but small amounts of other solvents such as DMF, THF, or dioxane may be added to improve solubility. In contrast to standard esterification procedures where conversion can't be easily followed, alkylation with PDDM-resin can by monitored visually due to the color change of the resin. The deeply violet resin turns yellowish while nitrogen evolves. After discoloration, shaking or stirring is continued for 4 to 6 hours and the resin is carefully washed with DCM.
Moreover, PDDM-resin is the resin of choice for the side-chain anchoring of Fmoc-Asp and Fmoc-Glu derivatives, especially when the standard esterification of Wang resin or SASRIN (e.g., with DCC/DMAP) has proven difficult. When anchoring Fmoc-Asp-NHR via the β-carboxyl functionality to PDDM-resin, losses due to aspartimide formation can't occur.

Peptides can be cleaved from PDDM-resin under the same conditions as from Wang resin, even though 2–5% TFA/DCM is sufficiently strong to promote cleavage. Fully protected peptide fragments may be obtained if Tyr(tBu), Lys(Boc), or His(Trt) are not present. Thus, for safe synthesis of fully protected peptide fragments, SASRIN or 2-chlorotritylchloride resin should be preferred.

1.3.2. Resins for the synthesis of peptide amides

A special functionality must be introduced on the resin to allow the release of the peptide as an amide.
These linkers possess an amino function to which the C-terminal amino acid is coupled and present an electronic structure such that the final acid treatment splits off the peptide as an amide.

Tricyclic amide linker resin
(4027095)
The 5-Fmoc-amino-10,11-dihydro-5H-dibenzo[a,d]cycloheptenyl-2-oxyacetyl linker based on the dibenzosuberyl protecting group of Pless [59] has been developed by Ramage [60] to enable smooth cleavage of peptide amides with concomitant side-chain deprotection. The linker is coupled to MBHA-resin modified with DL-norleucine. The final cleavage is performed using standard cocktails (see II. 5.)

«Rink amide» resins
(4014571 and 4026912)
Rink amide AM resin (or Knorr resin) 4014571 is obtained by the attachment of the linker Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine to aminomethyl resin [61].
The ether derivative 4-(2',4'-dimethoxy-phenyl-Fmoc-aminomethyl) phenoxymethyl polystyrene as originally described by Rink [13] is also available from Bachem (4026912).
Peptide amides are split off from these resins by 95% aqueous TFA; scavengers being added if necessary.

4,4'-Dialkoxybenzhydrylamine resin
(4014344)
N-Fmoc-4-Methoxy-4'-(-carbonylpropyloxy) benzhydrylamine is linked to H-Ala-aminomethyl resin [15]. The cleavage of peptide amides from this resin requires harsher conditions, e.g. a treatment with 95% TFA and scavengers at 35°C for 2 hours. Peptide amides containing a C-terminal Gly may be split off under milder conditions.

Other TFA-labile amide resins
Bachem also offers the well-established «PAL» resin 4-alkoxy-2,6-dimethoxybenzyl-amine resin [14] (4031247). Cleavage from PAL resin requires a lower concentration of TFA than cleavage from the resins described above. Additionally, peptides containing C-terminal Asn or Gln derivatives may be obtained by side-chain linkage of the corresponding Fmoc-Asp or Fmoc-Glu derivative followed by SPPS, acidolytic cleavage yields the C-terminal Asn or Gln [63].
The aldehyde resins 4029143 and 4029157 may be used for backbone (-CO-NH-) anchoring of peptides or SPPS of peptide N-alkylamides [64]. The appropriate educt resins are obtained via reductive amination of 4029143/4029157. 4029143 should be preferred for Fmoc-SPPS, as the final cleavage can be performed with TFA under standard conditions.

1.3.3. Resins for the synthesis of fully protected peptide fragments

SASRIN and preloaded SASRIN resins
(4012712 (200–400 mesh) and 4027899 (100–200 mesh))
SASRIN (*Super Acid-Sensitive ResIN)* corresponds to 2-methoxy-4-alkoxy-benzyl alcohol resin [17].
As already discussed before, we recommend the use of SASRIN preloaded with the desired Fmoc amino acid. Fully protected peptide fragments are obtained by cleavage with 0.5 to 1% TFA in DCM or by treatment with HFIP/DCM (1:4) [65,79]. Fully protected peptide hydrazides can be obtained conveniently by Fmoc SPPS employing the SASRIN-derivative 4027439.

Detailed cleavage protocols and comprehensive information concerning the use of SASRIN are contained in our brochure *SASRIN – a review of its manifold applications* which is available free of charge upon request. It can also be downloaded from our homepage at www.bachem.com.

2-Chlorotrityl chloride resin and preloaded 2-chlorotrityl resins
(4025425 (200–400 mesh) and 4040104 (100–200 mesh))
2-Chlorotrityl resin [18] is somewhat more acid-labile than SASRIN.
Loading of 2-chlorotritylchloride resin is achieved by treatment with the triethylammonium salt of the desired Fmoc amino acid, thus, concomitant racemization is minimized. To proceed with SPPS, Fmoc has to be split off, but the first deprotection with piperidine/DMF takes longer than usual (2 x 30 minutes).
On the other hand, Fmoc-AA-2-chlorotrityl resins are not stable, the Fmoc amino acid is slowly cleaved upon storage, whereas the H-AA-2-chlorotrityl resins can be stored.
Bachem offers a broad range of preloaded H-AA-2-chlorotrityl resins, resins with standard substitution as well as the corresponding low-load (LL) derivatives. The resin is especially suitable for the synthesis of fully protected peptides containing a C-terminal Cys or Pro. In case of C-terminal Pro, diketopiperazines can't be formed when proceeding with the SPPS due to the steric hindrance of the trityl moiety.
Moreover, 2-chlorotrityl resin is the optimal carrier for the synthesis of peptides containing a C-terminal tryptophan as the bulkiness of the chlorotrityl group prevents the alkylation of the indole moiety, and thus, irreversible binding of peptide during final cleavage.
Rapid cleavage of fully protected peptide fragments is attained by treatment with 0.5–1% TFA in DCM or HFIP/DCM (1:4 or 3:7) [66]; further cleavage mixtures have been described by Barlos et al. [18].

Xanthenyl linker resin
(for the synthesis of fully protected peptide amides)
(4026167)
Xanthenyl resin (or Sieber resin) has been developed for the synthesis of peptide amides allowing very mild cleavage conditions [16]. The 4-(9-Fmoc-aminoxanthen-3-yloxy) butyryl linker coupled to MBHA resin [68] yields fully protected peptide amides after repetitive short treatments of the peptide resin with 1% TFA/DCM. The resin will turn yellow during acidolytic cleavage. The cleavage protocol and work-up procedures described in the SASRIN brochure can also be applied to this product. The N-terminus may be deprotected before cleavage. Traces of carboxylic acids have to be carefully removed if the product is to be subjected to fragment coupling.
Fully protected fragments obtained from SASRIN or 2-chlorotrityl resin can be coupled in solution to amidated fragments cleaved from the title resin, providing a way for the synthesis of peptide amides by a convergent approach [69, 70].

PDDM-resin
For the synthesis of protected peptide fragments on PDDM-resin see p. 14.

1.3.4. Resins for the synthesis of peptide alcohols
Bachem offers a range of resins which can also be used for the synthesis of (fully protected) peptide alcohols and thiols.

SASRIN
The alcohol is generated by reductive cleavage. A detailed procedure is described in the SASRIN-brochure.

PDDM-resin
This resin readily alkylates Fmoc amino alcohols in the presence of a catalytic amount of $BF_3 \cdot Et_2O$ in DCM [71]. High loads can be obtained rapidly under these mild conditions provided that the alcohol is sufficiently soluble in DCM. Satisfactory loads have been obtained as well with secondary alcohols. The resulting benzhydryl ether can be cleaved by repetitive short treatments with 1–2% TFA/DCM. This simple and efficient approach is our preferred method for the preparation of protected peptide alcohols.
Thiols are alkylated by PDDM under the same conditions with equally good results. The resulting thioethers can be cleaved with TFA/phenol (9:1) [72] or 95% TFA.

2-Chlorotrityl chloride resin
This resin reacts with Fmoc amino alcohols in the presence of pyridine (or DIPEA/DMAP) in DCM/DMF. The reaction is slower than the alkylation with the PDDM-resin [73,74]. The resulting ethers can be cleaved by mild acid. Thiols react more readily with the resin.

3,4-Dihydro-2H-pyran-2-ylmethoxymethyl resin
(Ellman's dihydropyrane resin)
(4028707)
This resin has been conceived especially for the anchoring of alcohols [75].
It reacts with Fmoc amino alcohols in dichloroethane to form acetals. Acetalation and mild cleavage via transacetalation are catalyzed by strong acids such as benzene-sulfonic acid.

Further resins
For the preparation of C-terminally modified peptides, the side-chain hydroxyl groups of Ser and Thr can be alkylated with PDDM-resin or 2-chlorotrityl chloride resin [74] or acetalated by Ellman's resin.
The reaction is less favorable with Thr due to the steric hindrance of the amino acid side-chain.
Cys and cysteamine derivatives have been obtained from SASRIN (4026902 and 4026901, respectively), PDDM [72] and chlorotrityl resin.

Table 3 gives an overview of the resins available from Bachem.

Fig. 2b.
Large-scale reactor for SPPS allowing to produce kilograms of peptide.

Table 3. Resins for Fmoc-SPPS

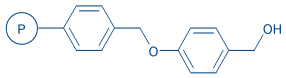
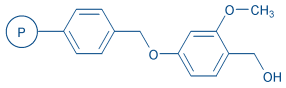
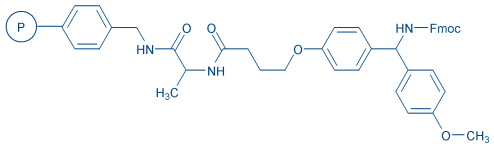
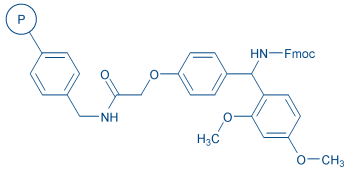
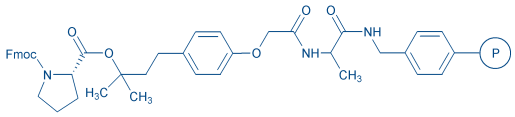
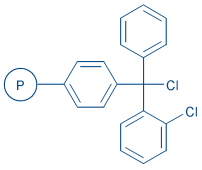
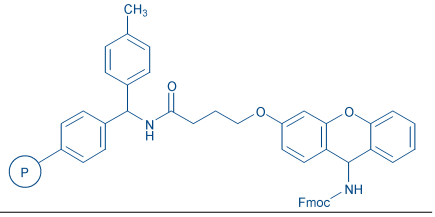
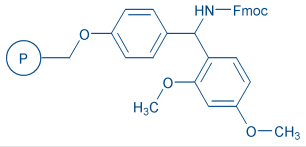
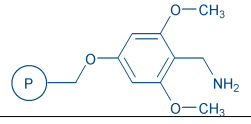
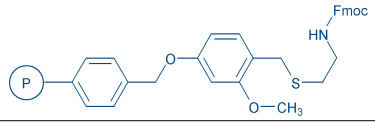
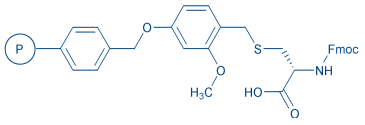
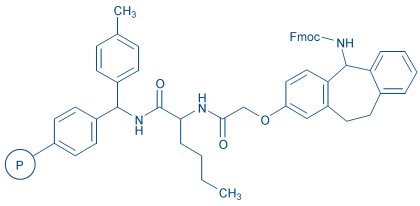
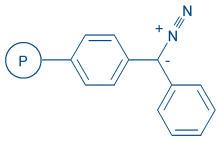
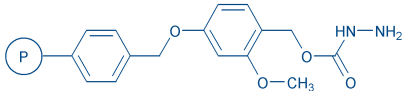
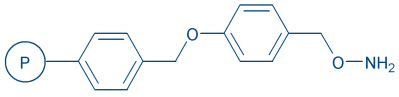
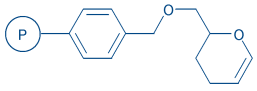
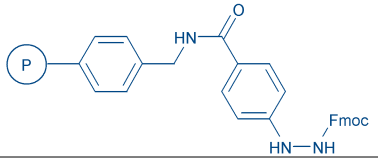
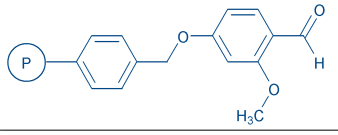
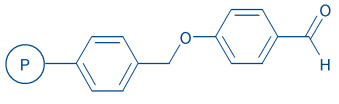
Product No.	Name	Structure
4003214 (200-400 mesh) 4027344 (100–200 mesh)	Wang resin (4-Alkoxybenzyl alcohol resin)	
4012712 (200-400 mesh) 4027899 (100–200 mesh)	SASRIN™ resin (2-Methoxy-4-alkoxybenzyl alcohol resin)	
4014344 (200–400 mesh)	Fmoc-4-methoxy-4'-(γ-carboxypropyloxy)-benzhydrylamine linked to Alanyl- aminomethyl resin	
4014571 (200–400 mesh)	Rink amide AM resin (Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine linked to Aminomethyl resin)	
4019306 (200–400 mesh)	Fmoc-Pro-DHPP-resin	
4025425 (200–400 mesh) 4040104 (100-200 mesh)	2-Chlorotrityl chloride resin	
4026167 (200-400 mesh)	Xanthenyl linker resin (Sieber resin, 4-[9-Fmoc-amino-xanthen-3-yloxy]-butyryl)-4-methyl-benzhydrylamide resin)	
4026912 (200-400 mesh)	4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-polystyrene resin (Rink resin)	
4031247 (200–400 mesh)	PAL resin (4-Alkoxy-2,6-dimethoxybenzylamine resin)	
4026901 (200–400 mesh)	Fmoc-cysteamine-SASRIN™ (Fmoc-2-aminoethanethiol-SASRIN™)	

Table 3. Resins for Fmoc-SPPS (continued)

Product No.	Name	Structure
4026902 (200–400 mesh)	Fmoc-Cys(SASRIN™)-OH	
4027095	Tricyclic amide linker resin (200–400 mesh) (Ramage resin, 5-Fmoc-amino-10,11-dihydro-5H-dibenzo [a,d]cycloheptenyl-2-oxyacetyl-DL-Nle- 4-methyl-benzhydrylamide resin)	
4027172 (200–400 mesh)	PDDM-resin	
4027439 (200-400 mesh)	SASRIN™-carbazate	
4027663 (200-400 mesh)	Hydroxylamine-Wang-resin	
4028707 (200–400 mesh)	3,4-Dihydro-2H-pyran-2-ylmethoxymethyl resin (Ellman resin)	
4029093 (200-400 mesh)	4-(Fmoc-hydrazino)-benzoyl aminomethyl resin	
4029143 (200–400 mesh)	4-Formyl-3-methoxy-phenyloxymethyl polystyrene resin	
4029157	4-Formyl-phenyloxymethyl polystyrene resin (200-400 mesh) (4-Alkoxybenzaldehyde resin)	

1.4. Linkers

Linkers are bifunctional molecules anchoring the growing peptide to the insoluble carrier. Linkers may be coupled to any carrier suitable for SPPS, an important option if alternatives to polystyrene-based resins have to be considered.
The C-terminal Fmoc amino acid may be coupled to the linker yielding the so-called handle which can be purified before loading the polymer. High loads regardless of the bulkiness of the amino acid are obtained by coupling these handles.
The linkers available from Bachem are presented in Table 4. Some of them are better suited for use in solid phase organic synthesis.

4015248
Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine
(Rink amide linker),
for the synthesis of peptide amides [61].

4009593
4-Formyl-3-methoxy-phenoxyacetic acid,
may be reduced to the alcohol before or after coupling [64,76,77]; it can also be used as a formyl anchor e.g. for reductive amination [64].

4025325
2-Hydroxy-5-dibenzosuberone,
reacts with resins carrying chloromethyl groups.

4009594
4-Hydroxymethylbenzoic acid (HMBA),
an anchor especially suited for cleavage with nucleophiles, thus Boc should be preferred for N^{α}-protection [78].

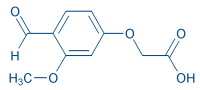
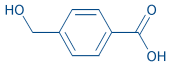
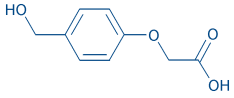
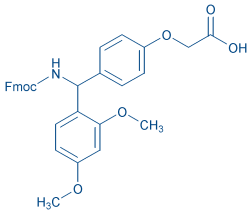
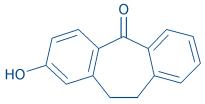
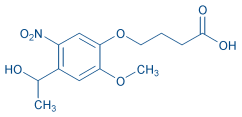
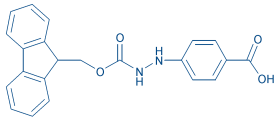
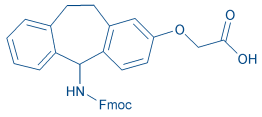
4009590
4-Hydroxymethyl-phenoxyacetic acid
(HMP linker),
a «Wang equivalent» [78].

4029124
4-(Fmoc-hydrazino)-benzoic acid,
acid-base stable linker which can yield various esters/amides upon the cleavage from the resin requiring a Cu(II) catalyst and a nucleophile [31].

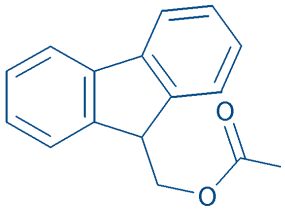
4027672
4(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)-butyric acid,
a photolabile linker releasing the peptides as carboxylic acids [80].

4025359
Fmoc-Suberol (5-Fmoc-amino-2-carboxymethoxy-10,11-dihydro-5H-dibenzo[a,d]cycloheptene),
«Ramage linker», for the synthesis of peptide amides [60].

Table 4. Linkers for Fmoc SPPS

Product No.	Name	Structure
4009593	4-Formyl-3-methoxy-phenoxyacetic acid	
4009594	4-Hydroxymethyl-benzoic acid (HMBA)	
4009590	4-Hydroxymethyl-phenoxyacetic acid (HMPA, HMP linker)	
4015248	Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)- benzhydrylamine (Rink linker)	
4025325	2-Hydroxy-5-dibenzosuberone	
4027672	4-(4'-[1-Hydroxyethyl]-2'-methoxy-5'-nitrophenoxy) butyric acid	
4029124	4-(Fmoc-hydrazino)-benzoic acid	
4025359	Fmoc-Suberol (5-Fmoc-amino-2-carboxymethoxy-10,11-dihydro-5H-dibenzo[a,d]cycloheptene) (Ramage linker)	

2. The Fmoc Group



2.1. General Remarks

Due to the development of strategies based on orthogonal protection, Fmoc has become the most important base-labile N-protecting group. The main stability features of the Fmoc group are summarized below:

Stability	Fmoc is acid-stable, withstands cleavage of Boc/tBu (TFA) and Z/Bzl (HF). Fmoc is stable under the cleavage conditions of Aloc/OAll (Pd°).
Limited stability	Limited stability towards tertiary amines such as DIPEA, pyridine [81]; the relative stability depends on base concentration, solvent and temperature. Stability towards hydrogenolysis is controversial [82] and should be evaluated for each individual case.
Lability	Lability towards bases, especially secondary amines [81] (piperidine > diethylamine). Fortunately the Fmoc group is less labile towards primary amines, including the amino group of the amino acid involved in the coupling reaction. Premature Fmoc cleavage may nevertheless occur during very slow couplings. N-silylation of the coupling site prevents this side reaction, it can accelerate the coupling [83].

The Fmoc group is removed via base-induced β-elimination (see Fig. 3). As a result dibenzofulvene and carbon dioxide are split off. Secondary bases such as piperidine add to the former molecule whereas bases such as DBU don't react with the dibenzofulvene. Hence, it has to be removed rapidly from the peptide resin or scavenged by a secondary amine such as piperidine to avoid irreversible attachment to the liberated amino group. Since both cleavage products are strong chromophores the deblocking can be monitored by UV spectroscopy.

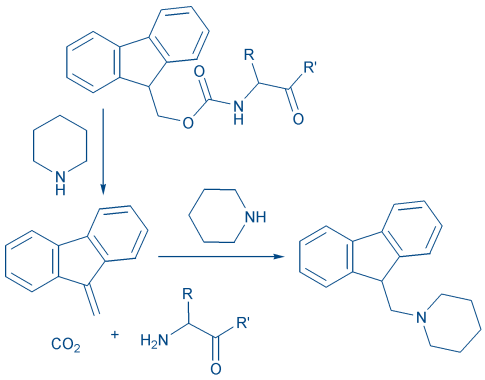


Fig. 3. Removal of the Fmoc group with piperidine

By-products generated by the repetitive treatment with base have been described. Aspartimide formation is the best documented side-reaction (see II.6.2). Epimerization and subsequent piperidide formation have been detected, even though the bulky tert. butyl group impedes reactions involving the β-carboxy group. Aggregation during chain elongation interferes with the couplings as well as with the Fmoc cleavages. If an incomplete deblocking occurs or is suspected, more active cleavage reagents should be tested (see below).

2.2. Cleavage Procedures

Usually, Fmoc is split off by a short treatment (3 to 5 minutes) with piperidine/DMF (1:4). In general this treatment is repeated and slightly prolonged (7 to 10 minutes). Under those conditions complete deblocking is attained in most cases. Thus, deviations are restricted to cases of sluggish cleavage (see difficult couplings, II.4.5.) or base-sensitive sequences.

Harsher alternatives to piperidine/DMF have been developed as well as milder cleavage reagents [84]. In case of sluggish deblocking, even slight variations of the

reagent may considerably accelerate the cleavage, e.g.:
• 1 to 5% DBU/DMF, more reactive than piperidine [85], for glycopeptides [86],
• 20% piperidine and 1–5% DBU in DMF, for difficult deblockings,
• morpholine/DMF (1:1), milder than piperidine for highly sensitive glycopeptides [87],
• piperidine/DMF (1:4) at 45°C, for «difficult sequences» [88],
• acceleration by microwave treatment [24],
• 0.1 M HOBt in piperidine/DMF (1:4), suppression of DKP and aspartimide formation [89],
• $Bu_4N^+F^-$ in DMF and other tetraalkylammonium fluorides [90] (not recommended),
• 2% HOBt, 2% hexamethyleneimine, 25% N-methylpyrrolidine in DMSO/NMP 1:1, mild cleavage conditions keeping thioesters intact [91] and reducing aspartimide formation [92].
Whichever cleavage reagent is preferred, it has to be washed out very carefully after Fmoc removal and the last washing must be neutral.
When synthesizing large peptides the duration of Fmoc cleavage should be gradually increased. For safe removal of the deblocking reagent the resin may have to be washed more often.

Recommended Standard Procedure

Fmoc Cleavage

Prewash with DMF (2x)
Treat with piperidine/DMF (1:4), 5 and 10 min, 10 mL of reagent/g peptide-resin.
Wash alternately with DMF and IPA until neutral pH.

As mentioned above, the generation and disappearance of Fmoc based chromophores allows the monitoring of the synthesis. Furthermore, samples may be taken to determine the load of Fmoc peptide. The completion of the deprotection reaction may be checked by cleaving samples and analyzing the obtained peptide.

Recommended Standard Procedure

Determination of Load

A sample of peptide-resin is washed 4x DMF, 5x IPA and 2x MeOH or ether, and dried to constant weight.
10 to 20 mg of dried resin are weighted exactly into a 100 ml measuring flask (the amount of resin depends on the expected load, for a load of ca. 0.5 meq/g 20 mg is sufficient).
Piperidine/DMF (1:4) is added to the mark, beads sticking to the neck have to be carefully rinsed off.
The mixture is shaken thoroughly and left to settle for 25 to 30 min.
The resin is filtered off and the absorbance of the filtrate is measured at 301 nm (ε=7800).

Recommended Standard Procedure

Determination of the Completion of Fmoc Cleavage

The sample is washed and dried as described above. The sample is cleaved with the appropriate reagent.
Super acid-sensitive resins: the sample is thoroughly washed with DCM and treated with 1% TFA/DCM (ca. 5 min) or HFIP/DCM (1:4) (15 min - 1 hr).
The resulting solution can be applied directly to a TLC plate, which should be dried in vacuo before development. Fmoc is readily detected at 254 nm, but Pmc, Pbf, Mtt, and Trt are strong chromophores as well.
For further analysis e.g. by HPLC, the peptide may be isolated and deprotected with TFA as described below.
Other resins (and large peptides, as TLC may show ambiguous results): treat with 95% aq TFA containing 5% EDT (or TIS) for at least 1 hr.
Filter off the resin and precipitate the peptide with MTBE.
Minute amounts of Fmoc peptide can be detected by HPLC, TLC or MS.

3. Fmoc-Amino Acid Derivatives

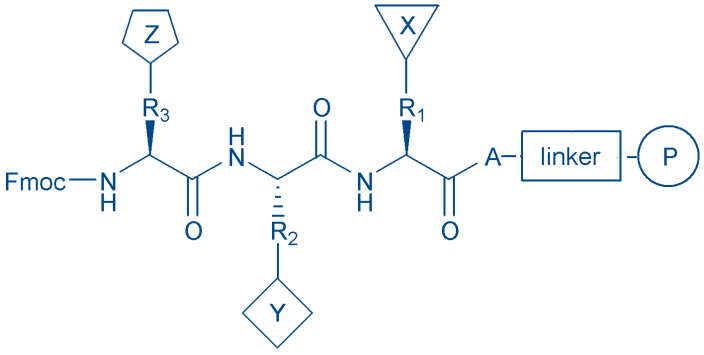
3.1. Side-Chain Protecting Groups

Fmoc/tBu probably represents the most popular «orthogonal» combination of protecting groups. The term «orthogonal» was coined by Barany and Merrifield in 1977 to designate «classes of protecting groups which are removed by differing chemical mechanisms. Therefore they can be removed in any order and in the presence of the other classes. Orthogonal protection schemes allow for milder overall reaction conditions as well as the synthesis of partially protected peptides» [8].

Table 5. Orthogonality of protecting groups.

Strategic choices	
A–Linker cleaved by strong acid (Wang, Ramage)	
Side-chain protecting groups	**Options**
X, Y, Z cleaved by strong acid	No cleavage specificity, standard synthesis. Final cleavage from the resin provides deprotected peptide.
X, Y cleaved by strong acid (tBu, Boc, ...) Z orthogonal (Allyl, ...) cleaved by weak acid (Trt, Mtt, ...)	Partial and specific on-resin deprotection and modification of the peptide chain.
A–Linker cleaved by weak acid (SASRIN, 2-Chlorotrityl, Xanthenyl)	
Side-chain protecting groups	**Options**
X, Y, Z cleaved by strong acid or orthogonal	Cleavage from the resin provides fully protected peptide for future chain elongation (convergent synthesis) or chain modification in solution.
X, Z cleaved by strong acid Y orthogonal	Partial and specific on-resin deprotection and modification of the peptide chain. Cleavage of the modified peptide from the resin with or without concomitant removal of side- chain protection.

X, Y, Z: protecting groups / A = O, yields peptide acid / A = NH, yields peptide amide:



The combination Fmoc/tBu is truly orthogonal whereas Boc/ Bzl is not, at least not under the conditions of SPPS as they both are cleaved by acids. As Boc can be selectively removed in the presence of Z/Bzl, the combination has been termed «quasi-orthogonal».
A brochure *Orthogonality of Protecting Groups* proposing combinations of selectively cleavable side-chain protecting groups for use in Fmoc-SPPS is also available from Bachem and can be downloaded from our homepage.

3.2. Side-Chain Protection Schemes

As mentioned earlier the choice of orthogonal protecting groups has broadened the scope of the Fmoc/tBu based SPPS. The solid support to which the C-terminal amino acid is linked is a protecting group as well. When conceiving the synthesis of complex or «modified» peptides (e.g. side-chain cyclized peptides) the tactics of synthesis, that is, the combination of side-chain protecting groups and type of resin has to be considered thoroughly. A few combinations are presented in Table 5.
For example, side-chain cyclization, usually via amide bond (e.g. for increasing the rigidity of a peptide and thus stabilizing desired conformations) has become an important structural element in designing peptide analogues. Appropriate synthetic strategies relying on (quasi) orthogonal side-chain protecting groups have been conceived, cyclization may be achieved either «on-resin» or following cleavage of the (partially) protected linear precursor from the resin.

Table 6. Side-chain protected Fmoc derivatives of proteinogenic amino acids.

Amino Acid	Protecting Group	Cleavage Conditions	Remarks
Arg	Pmc	95% aq TFA	standard
	Pbf	95% aq TFA	standard, slightly more acid labile than Pmc
	Mtr	95% aq TFA (35°C)	more acid stable than Pmc
Asn/Gln	Trt	95% aq TFA	standard, more stable to acidolysis than Mtt
	Mtt	95% aq TFA	standard
	Xan	95% aq TFA	standard
Asp/Glu	OtBu	95% aq TFA	standard
	OMpe	95% aq TFA	Asp: suppression of aspartimide formation
	OPp	1% TFA/DCM	on-resin modification
	OBzl	H_2/Pd or HF	acid sensitive peptides, SASRIN then H_2/Pd, rarely used
	OAll	$Pd(PPh_3)_4$	orthogonal to Fmoc/tBu/resin linkage
	ODmab	2% $N_2H_4 \cdot H_2O$/DMF	quasi orthogonal to Fmoc, acid stable
Cys			see II.3.3.
His	Trt	95% aq TFA	standard
	Mtt	95% aq TFA	standard
Met	O	NH_4I/Me_2S	rarely used (see II.6.7.)
Lys	Boc	95% aq TFA	standard
	Aloc	$Pd(PPh_3)_4$	orthogonal to Fmoc/tBu/resin linkage
	Adpoc	1% TFA/DCM	on-resin modification
	Mtt	1% TFA/DCM	on-resin modification
	Dde	2% $N_2H_4 \cdot H_2O$/DMF	cf. Dmab
	ivDde	2% $N_2H_4 \cdot H_2O$/DMF	improved stability towards piperidine
	Z	H_2/Pd or HF	rarely used
	Fmoc	20% piperidine/DMF	multiple antigenic peptides, dendrimers
Ser/Thr/ Tyr	tBu	95% aq TFA	standard
	Trt	1% TFA/DCM	on-resin modification
	Bzl	H_2/Pd or HF	rarely used
Trp	Boc	95% aq TFA then aq AcOH	standard

The combinations All/Aloc and Dmab/Dde have become very popular for side-chain cyclization (via an amide bond) of resin bound peptides.
If treatment with 1% TFA/DCM is kept sufficiently short, Mtt (or Adpoc) in combination with OPp may be employed in combination with a Wang type resin [93,94] as well.
For peptides synthesized on SASRIN, side-chain modifications of the partially protected peptide are performed in solution after cleavage from the resin.

A choice of side-chain protected Fmoc amino acids is presented in Table 6. For further information on these and other derivatives please go to our online shop at www.bachem.com

Arginine
Pmc and Pbf are mostly used for the protection of the guanidino function of Arg. The cleavage is accelerated in the presence of thiols in the cocktail (see II.5.1.). The cleavage of the Mtr group requires prolonged reaction time or reaction at elevated temperature which can lead to undesired side reactions [95].

Asparagine and Glutamine
The Trt and Mtt protected amino acids are perfectly suited for Fmoc based SPPS. The protecting groups are efficient in impeding the dehydration of the side chain carboxamide during the activation step. In addition these protecting groups increase the solubility of the poorly soluble Fmoc-Asn-OH

and Fmoc-Gln-OH. As Trt is removed rather sluggishly from an N-terminal Asn, Mtt carboxamide protection should be preferred in this position [96].

Aspartic Acid and Glutamic Acid
In routine Fmoc SPPS the side-chain is protected as the tert. butyl ester. This protecting group is stable under the conditions of SPPS and is readily removed during the final TFA cleavage. The OMpe derivative is less prone to base-catalyzed aspartimide formation [97]. OPp, OAll, or ODmab-esters are used when an additional level of orthogonality is required, for on-resin cyclization for example. These esters can be cleaved specifically in the presence of other protecting groups such as Boc, OtBu, Fmoc.

Cysteine
See also II.3.3.
Cys derivatives are notorious for base-catalyzed racemization during activation and coupling [98]. Considerable amounts of D-Cys epimer are obtained when coupling Cys(Trt) derivatives in the presence of bases. Cys(Acm) derivatives show a lower tendency to racemize. They tolerate weak bases as collidine. Attempted syntheses of peptides containing several disulfide bridges following standard Fmoc protocols may have failed for this reason. The extent of this side-reaction can be reduced by using weak bases as collidine in combination with uronium/aminium or phophonium reagents or, more effectively, by coupling in the absence of bases, e.g. with carbodiimides and HOBt (or HOAt). Racemization is further impeded by using less polar solvents for the coupling.

Histidine
Trt or Mtt are the most common protecting groups for the protection of the imidazole ring of His. Due to steric hindrance tritylation occurs exclusively at the N^{τ} (1-position). Trt and Mtt are stable under the conditions of SPPS but they don't prevent catalysis of racemization during activation by the imidazole moiety (free N^{π}) [99]. Fortunately, a coupling protocol minimizing this side reaction has been described. See 4.2.2. [100, 101].

Lysine
In routine Fmoc SPPS, Boc is used for the protection of the amino function. It is cleaved during the final TFA cleavage. Special orthogonal protecting groups exist for syntheses in which on-resin derivatization is desired. Among them the most commonly used are Aloc, cleaved by nucleophiles in the presence of Pd. Adpoc and Mtt are more acid labile than Boc and are cleaved by repeated treatment with 1–2% TFA/DCM. The combination of the various Asp/Glu and Lys/Orn protecting groups has enabled the synthesis of complex molecules benefitting from the «multidimensional orthogonality» [102].

Serine, Threonine and Tyrosine
The tert. butyl ethers possess the qualities of good protecting groups and are widely used. Trt can be used for the protection of Ser and Thr if on-resin derivatization is required. Thr and Tyr may also be coupled with unprotected side-chain functionalities .

Tryptophan
Trp has been used without protection, however the indole nucleus can then be alkylated in the final TFA cleavage. For that reason we strongly advise coupling indole-protected tryptophan. Fmoc-Trp(Boc) has become the standard derivative for incorporating the amino acid . During the final TFA cleavage the Boc group yields isobutylene and leaves the N(indole)-carboxy moiety which prevents alkylation of the indole nucleus. This intermediate is decarboxylated during a subsequent treatment with diluted AcOH.

3.3. Protection of Cys During Fmoc SPPS of Peptides Containing Disulfide Bonds

A brochure on cysteine and further mercapto amino acids, including a compilation of derivates offered by Bachem, can be downloaded from our web page.
Cys has always required particular attention in peptide chemistry. Protection of the highly reactive side chain thiol function during peptide synthesis is mandatory, and peptides containing free cysteines have to be protected from random oxidation.
See Table 7 for a choice of commonly used S-protecting groups. In most cases, the liberated sulfhydryl moieties are oxidized to

Table 7. Side-chain protected Fmoc-Cys-OH.

Cys (R) R =	Cleavage conditions	Remarks
Trt	95% TFA + scav[1]	standard
Dpm	95% TFA + scav	more stable than Trt[2]
Acm	Hg (II)	I_2/AcOH[3]
Mob	TFMSA	
Mmt	1% TFA/DCM + scav	on-resin cleavage in presence of Trt
Mbzl	HF	orthogonal
StBu	Bu_3P	orthogonal

[1] scavengers; [2] Use of Dpm in place of Trt reduces oxidation of Cys to cysteic acid.
[3] for oxidative deprotection

selectively generate intra-or intermolecular disulfide bonds. By choosing appropriate protecting groups, the disulfide bridges may be formed at various stages of the synthesis: on-resin as well as in solution. Furthermore, removal of S-protection and oxidation yielding the disulfide bridge may be performed either as separate steps or concomitantly, i.e. oxidative deprotection. If selective on-resin Cys deprotection is required, the Mmt derivative should be incorporated [103]. If two or more disulfide bonds have to be formed either pairwise orthogonal protection is used allowing selective, consecutive disulfide bridge formation or a single Cys protecting group is chosen [104]. Then all the sulfhydryls are liberated simultaneously followed by bridging in buffered solutions containing redox systems such as oxidized and reduced glutathione; the term «oxidative folding» as been created for this approach.

3.3.1. Peptides containing a single disulfide bridge

Standard strategies for synthesizing peptides containing a disulfide bond are assembled in Table. 8

Recommended Standard Procedure

Formation of Disulfide Bridges by Air Oxidation

The peptide is dissolved at a concentration of 10^{-3} to 10^{-4} M in dilute acetic acid or 0.05 M ammonium acetate; the pH is adjusted between 7.5 and 8 and the solution is stirred at room temperature in the presence of atmospheric oxygen. Additives such as 1–10 % DMSO or 3% H_2O_2 can be used to accelerate the reaction. H_2O_2 must not be used if peptides contain a Met residue.The progress of the reaction is followed by HPLC. After disulfide formation the solution is acidified with acetic acid and can be used directly for preparative HPLC purification.

3.3.2. Peptides containing two disulfide bridges

This procedure allows to avoid the mispairing which could occur if the simultaneous air oxidation of the 4 free sulfhydryls was performed. The adequate combination

Table 8. Strategies for obtaining peptides with a single disulfide bridge.

Cys protecting groups	Cleavage of Cys protecting groups	Cyclization conditions
Cys(Trt) + Cys(Trt)	95% aq TFA + 5% EDT	standard, separate steps, liberation of SH followed by oxidation (air, H_2O_2, iodine, ...)
Cys(Acm) + Cys(Acm)	iodine in 80% AcOH[1]	standard, oxidative cleavage, orthogonal to tBu/Wang
Cys(Trt) + Cys(Acm)	95% aqTFA + 5% EDT; 1 eq. iodine in 80% AcOH (iodine titration)	separate steps, Acm is cleaved concomitantly with bridge formation by titration with iodine [105]

[1] Precautions have to be taken in the presence of Tyr, His, Met and especially Trp. Trp can be protected by adding a large excess of Ac-Trp-OMe. Protected peptides are less susceptible to iodine-induced side-reactions.

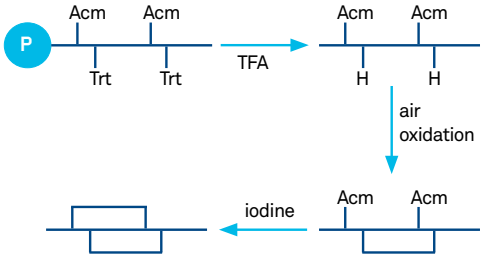


Fig. 4.
Synthesis of peptides containing two disulfide bridges via consecutive S-S-bond formation.

of the protecting groups allows specific cleavages and the use of optimal cyclization conditions. Numerous combinations of protecting groups are possible [106]. We will describe in our Recommended Standard Procedure merely the popular combination Trt/Acm as depicted in Fig. 4.
The first oxidation follows the protocol described in the preceeding paragraph.

Recommended Standard Procedure

Formation of Disulfide Bridges from Bis-Acm Peptides

The peptide is dissolved at a concentration of 10^{-3} to 10^{-4} M in 40% aqueous acetic acid. Iodine (25 to 50 fold excess) dissolved in 80% acetic acid (or in methanol) is added. The solution is stirred at room temperature and the progression of the reaction is monitored by analytical HPLC. At the end of the reaction the excess of iodine is destroyed with 1 M ascorbic acid in water (the ascorbic acid solution is added up to disappearance of the iodine color). The solution is diluted with water to an acetic acid concentration of approximately 10% and is used directly for preparative HPLC purification.

3.3.3. Peptides containing three disulfide bridges

The combination of protecting groups presented in Table 9 has been successfully used for the preparation of several peptides (relaxin, defensins, sapecin) [107-109] having three disulfide bridges, following an approach of selective bridging.
The experimental conditions for the formation of the first two bridges have been descibed earlier in this chapter.

Recommended Standard Procedure

Cleavage of Mob Group and Disulfide Formation

The cleavage solution (TFMSA/TFA/anisole 1:8:1) is prepared and cooled in an ice bath. The cooled solution is added to the peptide (3 mg/mL). The reaction is carried out at 0°C for 45 min in the ice bath. The reaction solution is diluted 50 times with iced water and extracted 3 times with ether. The water layer is recovered and DMSO (10% of total volume) is added. The cyclization is monitored by analytical HPLC and the reaction solution is used directly for preparative HPLC purification at the end of the reaction, after dilution with water. The cyclization can also be achieved by iodine titration: addition of a methanolic solution of iodine up to persistent yellow color and destruction of the excess of iodine with ascorbic acid.

It should be clear that the successive formation of the disulfide bridges is a long and tedious process. When the peptide to be prepared corresponds to a naturally occuring sequence, with the natural bridging pattern, the simultaneous disulfide bridges formation (random oxidation) can yield very good results [112]

3.3.4. Simultaneous disulfide bridge formation

Following this approach only a single kind of protecting group is chosen for the protection of all the sulfhydryl functions of a peptide. The most commonly used one is Trt which is cleaved during the final TFA deprotection; Mtt, Mmt and Tmob can also be chosen. The crude peptide can be prepurified or employed directly in the oxidation reaction.
The Acm group is stable to acidolysis, and the peptide obtained after the final TFA deprotection has its SH functions still protected, which facilitates the purification. The Acm group can then be cleaved by mercuric acetate followed by oxidative folding. This approach has been used in the preparation of muscarinic toxin 1 (MTX1) [113] a 66 residues peptide containing 5 disulfide bridges following a convergent Boc synthesis based on a combined solid phase-solution approach.

Table 9. Consecutive formation of three disulfide bonds

Cys protecting group pairing	Cleavage of Cys protecting groups	Cyclization conditions
Cycle 1 – Trt	TFA/H_2O/ scavengers	air oxidation
Cycle 2 – Acm	Concomitant cleavage and cyclization with iodine	
Cycle 3 – Mob[1)]	TFMSA/TFA/ anisole	air oxidation (DMSO) or iodine titration

[1)] can be replaced by Dpm [110].

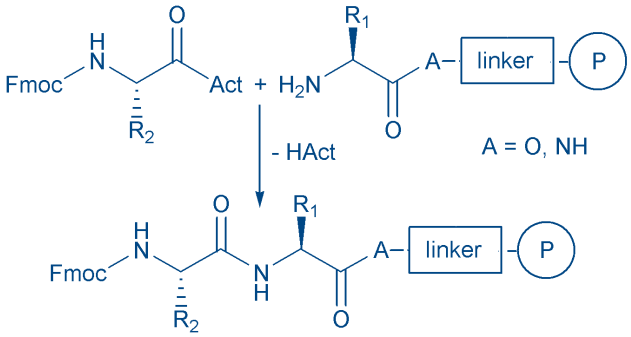


Fig. 5.
A coupling reaction.
Act: Activator, e.g. OBt, OAt, OSu.

Recommended Standard Procedure

Simultaneous Formation of Multiple Disulfide Bridges

The linear peptide is dissolved at a concentration of 10^{-3} to 10^{-4} M in 0.05 M ammonium acetate /0.1 M guanidinium hydrochloride (optional) and the pH is adjusted to 7.5. Redox additives are often used to accelerate the reaction; typically the couple glutathione reduced (GSH)/glutathione oxidized (GSSG) is added to the oxidation solution in the ratio peptide/GSH/GSSG 1:100:10. The reaction is monitored by analytical HPLC and at the end, after acidification with acetic acid, the solution is directly used for preparative HPLC purification.

However, it has been observed that in certain cases a product showing a non-natural folding pattern was isolated as main product [114]. Optimization of the cyclization conditions (reaction at 5°C in the presence of 2 M sodium sulfate) has resulted in the isolation of the naturally folded molecule as the main product.

4. Coupling Reagents and Methods

4.1. General Remarks

The coupling of an amino acid to the peptide resin, i.e. the elongation step, is the crucial part of the SPPS cycle (see Fig 2 and 5). Rate and yield of the reaction are influenced by various parameters such as

• The choice of solvent:
The solvent not only determines the swelling of the peptide-resin and thus influences the accessibility of the reactive sites; it also directly affects the kinetics of the coupling reaction.

• The steric hindrance:
It is determined by the nature of the side chains (R_1 and R_2) and their protecting groups.

• The reactivity of the activated carboxylic acid:
It determines the acylation rate, as well as the extent of side reactions, such as racemization.

Generally speaking, the first priority is to find the optimal combination of high

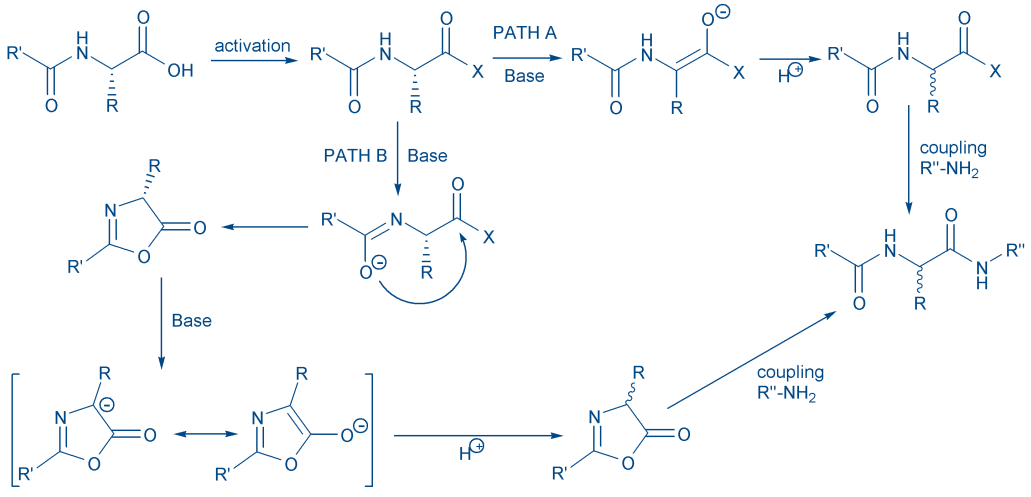


Fig. 6.
Mechanism of base-catalyzed racemization during activation.

coupling rate and minimal racemization. Contrary to widespread assumptions N^{α}-urethane protected amino acids can racemize considerably under inadequate coupling conditions [98, 99,114,115]. The mechanism of this reaction is presented in Fig. 6.
«Standard» coupling conditions may result in incomplete coupling and formation of by-products such as epimers, truncated peptides and deletion sequences, products of guanylation [116], or products generated by premature Fmoc cleavage. More potent coupling reagents such as HATU [117] or very active Fmoc amino acid derivatives such as the acid fluorides [118] may drive the coupling to completion, N-silylation with bis(trimethylsilyl)acetamide (the use of TMS-Cl is restricted to Boc-SPPS) [83,119] may be an additional means to promote the reaction.
Better results will be obtained by repeating a coupling with fresh reagents (and changing coupling parameters if a low conversion was obtained) rather than by prolonging the reaction. Generally, coupling protocols may be changed in the course of a synthesis, especially when optimizing an SPPS.
A special paragraph will be devoted to the problems caused by peptide aggregation in the course of the synthesis.
This phenomenon is a major cause of trouble as it is difficult to predict. Aggregation is sequence-dependent and no universal solution for avoiding it has been found up to now.
Numerous methods for activating the carboxylic function can be found in the literature. Here we want to present in detail the activation protocols most often used in SPPS.

4.2. Activation Methods

4.2.1. Carbodiimide – Carbodiimide/HOBt

DCC activation has been used from the first days of the solid phase technique [120] and is still popular today. DIC [121] is also frequently used especially in automated SPPS, because the corresponding urea is more soluble than the one obtained from DCC. However the activation by carbodiimides presents several drawbacks:
• The coupling (acylation of the amino function) rate is not particularly high, and the activated species can be deactivated by rearrangement into an N-acyl urea (Fig. 7).
• Racemization can occur following cyclization of the activated species to a 5(4H)-oxazolone via enolization. The subsequent reopening of the ring by the amino component yields epimers [122].
These problems can usually be avoided by the introduction of additives to the reaction mixture. The most popular additive in SPPS is HOBt [123].
In a standard coupling procedure the HOBt ester is generated by the reaction between the protected amino acid and HOBt. The reaction is mediated by DCC or DIC. HOAt, an aza analogue of HOBt has been reported to markedly accelerate the acylation and also to reduce racemization [124]. This additive is widely expected to overcome problems related to «difficult» coupling.
Whereas DCM proves to be the optimal solvent for carbodiimide activation, it is less suitable for the subsequent coupling step which proceeds more rapidly in more polar solvents such as DMF. Therefore a mixture of DCM and DMF is often used for both the activation and the coupling steps.

Recommended Standard Procedure

Activation and Coupling with DCC/HOBt

After deprotection of the amino function the resin is thoroughly washed. DMF has to be used for the last wash. The appropriate color tests must be positive. The reagents are used in the following molar ratio: Free amino function/Fmoc-amino acid/DCC/HOBt 1:3:3:3. The protected amino acid is dissolved in DMF or DCM/DMF. 1 equivalent of HOBt dissolved in DMF or DCM/DMF is added followed by 1 equivalent of DCC dis-

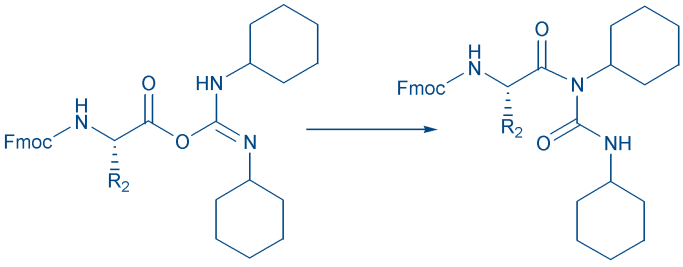


Fig. 7.
N-Acylurea formation from the Fmoc-amino acid isoacyl urea.

solved in DMF or DCM/DMF. The reaction mixture is stirred at room temperature for 15–20 min. The urea is filtered off and the filtrate is added to the resin. The coupling is monitored by the appropriate color tests.

CAUTION: DCC is an aggressive allergen. Inhalation and contact must be avoided. Adequate protective clothing and goggles must be worn when working with DCC, an efficient ventilation is mandatory!

The preactivation time must be kept at a minimum when generating the Fmoc-Arg(Pmc/Pbf)-OBt derivative as the activated Arg derivatives may cyclize yielding an unreactive lactam.
This also applies for the activation of Fmoc-His(Trt)-OH since racemization catalyzed by the nitrogen of the imidazole ring may occur.
For the coupling of especially bulky amino acids such as Aib, Tic, ... or in the case of recognized difficult coupling we recommend the replacement of HOBt by HOAt, or the use of other activating agents (see below).
On the other hand, carbodiimide/HOBt (or HOAt) activation in DCM/DMF turned out to be superior protocol for coupling Fmoc Cys derivatives with minimal concomitant racemization [125].

4.2.2. Activation by phosphonium and uronium/aminium salts

In recent years acylphosphonium (BOP, PyBOP) [126,127], DEPBT [128] and acyluronium/aminium salts (HBTU, TBTU) [129] have become extremely popular coupling agents in SPPS.
TBTU converts the Fmoc amino acid into the active OBt ester in the presence of one equivalent of base. DIPEA is routinely used though it has been demonstrated that it can induce racemization in the coupling of Fmoc-Cys(Trt)-OH [98] and of Fmoc-Ser(tBu)-OH [115]. The use of collidine has been recommended as a substitute for DIPEA [130]. Nevertheless large excess of base is to be avoided even though coupling is accelerated at a slightly basic pH.
For the coupling of very bulky amino acids such as N-alkylamino acids or α-dialkylamino acids, the moderately reactive TBTU should be replaced by more potent reagents such as HATU [117], TATU, or PyBOP [127].

Recommended Standard Procedure

TBTU/DIPEA (collidine) Activation

The resin is prepared as described in the previous procedure. Equimolar amounts of protected amino acid and TBTU are dissolved in DMF (usually a 3-fold excess). An equimolar amount of base (DIPEA or collidine) is added. The solution is stirred for 3 to 8 min and added to the resin. When working with an excess of base, 20 to 25% of the original molar amount of base are added 20 min after the start of the coupling reaction.

The comments concerning the preactivation of Fmoc-Arg (Pmc, Pbf)-OH and Fmoc-His(Trt)-OH are also valid in this case. Racemization of His can be considerably reduced by activating Fmoc-His(Trt)-OH with DEPBT/DIPEA [100] which generates the ODhbt ester. Additionally, DEPBT/DIPEA in DCM/DMF (1:1) is an excellent choice for coupling Cys derivatives [131].
The preactivation step has to be carried out especially when working with uronium derivatives such as TBTU or TATU, as they can react with the free amino group of the peptide-resin yielding a substituted guanidine [116].

4.2.3. Fmoc-amino acid active esters

Active esters have been used in peptide synthesis long before the introduction of the solid phase technique.
Several classes of active esters are sufficiently stable for isolation, prolonged storage and still react rapidly in peptide coupling. Among them Fmoc-AA-OPfp esters [132] and Fmoc-AA-ODhbt esters [133] have become popular in automated SPPS, they are merely dissolved and added to the resin (in case of Pfp esters the addition of HOBt to increase their reactivity has been recommended [134]).
Small scale manual SPPS as well as multiple parallel peptide synthesis for obtaining peptide libraries can be performed very rapidly and conveniently with preformed active esters. Fmoc-AA-ONp and Fmoc-AA-OSu have found only restricted application in SPPS.

4.2.4. Fmoc-amino acid fluorides and chlorides

The crystalline, rather stable Fmoc amino acid fluorides have gained much interest [118]. They may also be obtained *in situ* by treating the Fmoc amino acids with TFFH [135].
Even Fmoc amino acids bearing acid-labile side-chain protection can be converted into stable acid fluorides (except for Arg and His derivatives for which the corresponding acid fluorides should only be generated *in situ*). TFFH [135] or BTFFH [136] can be used for the *in situ* preparation of Fmoc amino acid fluorides.
Fmoc amino acid chlorides can also be used. Their applicability is more limited [137] and acid-sensitive groups may not be present when treating Fmoc amino acids with oxalyl chloride or thionyl chloride to obtain them [139,140]. Falb et al.have described the *in situ* preparation of Fmoc amino acid chlorides by reaction with bis (trichloromethyl) carbonate and their use in difficult couplings [140]. The conditions developed by the authors are even compatible with tBu side chain protection, see also [141].

4.3. Monitoring of Coupling and Deblocking

In Fmoc SPPS the monitoring of the completion of the Fmoc cleavage and of the coupling reaction is essential.
Reliable methods detecting minute amounts of unreacted amino groups are mandatory for the monitoring of the coupling reaction. On the other hand the ability of detecting small quantities of Fmoc peptide allows the control of the completion of the Fmoc cleavage.
A range of color tests for the qualitative monitoring of the coupling reaction has been developed and we describe the most commonly used ones here below. Each one of these tests having its limitations, we recommend to perform two independent tests in the monitoring of the completion of the coupling.
It must also be noted that during the synthesis of very long peptides the detection of the remaining free amino group will usually become difficult with increasing peptide length. Thus, for the SPPS of long peptides we recommend a protocol including systematic double coupling followed by acapping step. This also applies when the growing peptide chains aggregate which limits the accessibility of the free amino groups. For protocols for disrupting aggregated sequences see II.4.5.
The tests described in this brochure should then be performed with small samples of washed resin. It is recommended to perform control tests with protected amino function (negative control) and deprotected amino function (positive control).
For a review on qualitative colorimetric tests see [142].

4.3.1. Kaiser test

The Kaiser test [143] is based on the reaction of ninhydrin with amines. It is a very sensitive test for primary amines, visualized by an intense blue color, and somewhat less suitable for secondary amines, being detected by development of a brownish red color.
Usually the color is developed mainly in the beads and partly in the supernatant. For spectrometric quantitative determination of the amount of unreacted amino groups the color has to be transferred completely to the solution [144]. The intensity of the color depends on the nature of the amino terminus to be detected, rather unspecific shades are obtained with N-terminal (side-chain protected!) Asp, Asn, Cys, Ser, and Thr. Brownish red beads result with N-terminal Pro. As the resin sample has to be heated, «hidden» NH_2-groups may become more accessible and thus detectable. However, prolonged heating as well as overheating should be avoided as it may cause cleavage of Lys(Boc) or Fmoc removal (by pyridine).

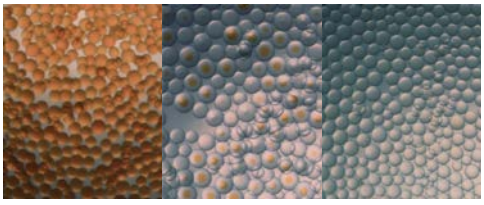


Fig. 8.
TNBS-test.
Beads inspected under the microscope.
left: positive
middle: incomplete coupling
right: negative.

Recommended Standard Procedure

Kaiser Test

Solution 1: 5 g ninhydrin in 100 ml ethanol-
Solution 2: 80 g phenol in 20 ml ethanol
Solution 3: 2 ml 0.001 M aqueous KCN in 98 ml pyridine
A few resin beads are placed in a small test tube and 2–5 drops of each solution are added. The tube is placed in an oven and the reaction left to develop for 5 min at 100°C.
Results: resin and solution blue (variable intensity – from light to dark blue): positive, resin and solution colorless to yellowish: negative.

A modified Kaiser test has been developed for the quantitative monitoring of the coupling [143].

4.3.2. TNBS test

The TNBS (2,4,6-trinitrobenzenesulfonic acid) test [145] is easier to perform and of a nearly equivalent sensitivity as the Kaiser test. It can only be used for detecting primary amino groups. Only the beads will turn orange-red and the intensity of the color does not depend on the nature of the N-terminal amino acid. In the monitoring of the completion of the coupling, it is recommended to inspect the beads under a microscope (see Fig. 8), as a slightly orange discoloration in the core of the beads can't be detected by visual inspection.
We recommend the use of the combination Kaiser/TNBS tests in the routine detection of primary amino function.

Recommended Standard Procedure

TNBS test

Solution 1: 10% DIPEA in DMF
Solution 2: 1 M aqueous TNBS
A few resin beads are placed in a small test tube and 1–3 drops of each solution are added. After a short mixing the mixture is left at room temperature for 10 min and the beads inspected. Except for distinctly positive tests, all tests should be inspected under a microscope.
Results: intensely orange or red beads: positive, yellow or slightly orange beads or cores: slightly positive, colorless beads: negative.

4.3.3. Acetaldehyde/Chloranil test

This sensitive test has been developed for reliable detection of secondary amino groups [146] but it will also detect primary amines. As for the TNBS test, only the beads will be colored in case of a positive test, they should be inspected under a microscope.

Recommended Standard Procedure

Acetaldehyde/Chloranil Test

Solution 1: 2% acetaldehyde in DMF
Solution 2: 2% chloranil in DMF
The stock solutions should be kept in the refrigerator and for one month maximum.
A few beads of resin are placed in a small test tube and 2–5 drops of each solution are added. After a short mixing the mixture is left at room temperature for 5 min and the beads inspected.
Results: dark blue to green beads: positive, colorless to yellowish beads: negative

4.3.4. Bromophenol Blue test

As all types of amines can be detected applying this test [147] traces of DIPEA or collidine have to be removed very carefully. Traces of acid lead to false negative results. False positives are obtained in the presence of His(Trt). The beads may be inspected under a microscope.
Minute amounts of bromophenol blue have been added to couplings in progress to monitor the conversion. The end-point is indicated by discoloration [148].

Recommended Standard Procedure

Bromophenol Blue Test

Reagent solution: 0.05% bromophenol blue in DMA
A few resin beads are placed in a small test tube and 10–15 drops of reagent solution are added. The beads are immediately inspected.
Results: blue beads: positive ,colorless beads: negative.

Further color tests can be found in the literature.

4.3.5. Cleavage of samples

Cleavage of samples may seem a rather elaborate way of monitoring an SPPS, but it is the most comprehensive method. It is especially useful when the synthesis has to be optimized or documented or when negative color tests results are obtained because of scarcely accessible coupling sites.
The cleavage of a sample is also a reliable

method to monitor the completion of the Fmoc cleavage.
After the cleavage the sample is analyzed by HPLC as Fmoc protected and free amino sequences are usually well separated in a standard HPLC gradient.
If necessary additional information can be gained from mass spectrometry or micro-sequencing. This approach can even be further simplified and accelerated, as MALDI-MS turned out to be a simple and very effective method for monitoring an SPPS. Only a few beads of peptide resin have to be cleaved to obtain sufficient material for a mass spectrum [149–151].

Recommended Standard Procedure

TFA Cleavage of In Process Samples

We recommend the use of a standard cleavage cocktail, e.g. TFA/H2O/EDT 90:5:5. Approx. 50 mg of peptide-resin are cleaved for 2 hrs with 500 µl of cocktail.The reaction mixture is filtered into cold MTBE. The precipitated peptide is isolated by filtration or centrifugation. The solid is washed with MTBE. After drying, the crude product is analyzed by HPLC and MS.

4.4. Capping

If the color tests are still positive after the predetermined coupling time the resin is filtered off, washed with DMF and IPA, and a second coupling is usually performed.
If the test is slightly positive the recoupling is usually performed with a lower amount of reagents, typically 50% of the quantities used in the first coupling.
One or two parameters may be changed in the second coupling. However changes of coupling conditions are certainly due if positive tests are obtained after the second coupling!
Some possible changes:
• Change solvent, DMF/DCM 1:1 instead of DMF,
• Change coupling reagent, TATU instead of TBTU,
• Change additive, TBTU+HOAt instead of TBTU+HOBt.
In case the color tests keep revealing the presence of unreacted amino functions after recoupling, they have to be blocked («capped») as to avoid the formation of deletion sequences.
Capping will yield a truncated sequence (shortened peptide). Truncated sequences generally differ considerably from the final peptide and can be readily separated.
Capping is achieved by a short treatment of the peptide resin with a large excess of a highly reactive unhindered acid derivative and a base, usually acetic anhydride or benzoyl chloride and pyridine. At the end of the capping step the reagents are filtered off and the resin is carefully washed before proceeding to the next deprotection step.
For the synthesis of long peptides we recommend to use a protocol in which systematic double coupling is followed by capping.

Recommended Standard Procedure

Capping

After removal of the coupling solution the peptide-resin is washed several times with DMF, IPA; the last washing being DMF. A 50 fold molar excess of acetic anhydride and DIPEA (or pyridine) is dissolved in DMF and added to the peptide resin. The reaction is left for 30 min at room temperature. The solution is filtered and the peptide resin is washed alternately with DMF and IPA.
A color test is performed before proceeding to the next step.

Capping can also be used to reduce the loading of a resin.
For that purpose a deficient amount of the C-terminal or penultimate Fmoc amino acid is coupled to the unloaded or preloaded resin. The resulting load is determined and when the desired level of subsitution has been reached the remaining free amino groups are blocked by acetylation.

4.5. Aggregation/Difficult Sequences

Repeated incomplete deprotection of the α-amino group as well as difficulties in obtaining complete conversion during coupling are some of the problems caused by the on-resin aggregation of the peptide chain.
This phenomenon has been attributed to the self-association of the peptide chain by formation of hydrogen bonds [152]. Aggregationis sequence-dependent. Up to now, no «Golden Rule» allowing to predict its occurence during the synthesis of a new sequence exists!
A few indications can be drawn from the

sequence: Aggregation has a tendency to occur in clusters of hydrophobic residues; it has been described as starting from the 5/6th residue from the C-terminus but not occurring after the 21st one [153].
Two approaches can be used to disrupt the aggregation; the first one consists in modifying the environment in which the synthesis is performed and to introduce elements known to disrupt hydrogen bonds; among them we list:
• Addition of solvents such as DMSO [154]
• Addition of chaotropic salts [155]
• Addition of nonionic detergents and of ethylene carbonate
(«Magic Mixture») [156]
• Coupling at elevated temperature [157]
• Sonication of the coupling reaction [158]
• Reduction of the peptide load on the resin
• Microwave [24]
The other approach is to introduce backbone protecting groups which will prevent the formation of hydrogen bonds. Such protection is achieved by modifying the amide bond with the Hmb group [159]. It has been shown that the presence of a Hmb unit every 6–7 residues is sufficient to disrupt peptide aggregation [160]. Hmb-protected amino acids were incorporated as N,O-bis-Fmoc-N-(2-hydroxy-4-methoxybenzyl) derivatives, the O-Fmoc protection being cleaved during the following piperidine treatment. Fortunately, O-protection can be omitted [161], the corresponding derivatives are more readily available. At the end of the synthesis the Hmb group is removed during the final TFA cleavage. In the meantime, 2,4-dimethoxybenzyl (Dmb) was introduced as backbone protecting group and turned out to be a superior alternative to Hmb [162].
Another highly efficient method to achieve backbone protection is to couple Fmoc-dipeptides containing a pseudoproline residue derived from Ser, Thr, or Cys, since Pro is known to disrupt aggregation [23, 163]. Suitably protected pseudoproline dipeptides for incorporating Xaa-Ser and Xaa-Thr motifs during Fmoc-SPPS are available from Bachem for most proteinogenic amino acids. Please see the brochure *Pseudoproline Dipeptides* which can be downloaded from our homepage.
At the end of the synthesis the Ser or Thr will be regenerated during the final TFA cleavage step. Sampson et al. showed that introduction of pseudoprolines is superior to Hmb backbone protection in disrupting aggregates [164]. The introduction of pseudoprolines moiety allows the stepwise SPPS of long peptides [25] and facilitates cyclization.
Esterification to Ser or Thr will efficiently disrupt aggregation as well. After cleavage from the resin, the resulting depsipeptide is rearranged in slightly basic aqueous solution yielding the desired peptide (see II.6.8. and III.6.4.) [165,166].

5. Cleavage from the Resin

5.1. Simultaneous Cleavage from the Resin and of the Side-Chain Protecting Groups

Concentrated TFA (95% aqueous TFA) is the standard reagent to perform the final cleavage of the peptide from the resin. The side-chain protecting groups are removed concomitantly.
During the reaction highly reactive carbocations are generated. They have to be trapped as to avoid undesired reactions with sensitive amino acids such as Cys, Met, Ser, Thr, Trp, Tyr.
For this reason so-called scavengers are added to the cleavage solution.
Water is a moderately efficient scavenger and can be used as single scavenger for the cleavage of peptides devoid of Cys, Met and Trp.
EDT and DTE are often-used and efficient scavengers for peptides containing sensitive amino acids.

Starting Cocktail: TFA/water 95:5
Add:

Amino acid present	Recommended scavengers
Cys	DTE, EDT, TES
Met	DTE, EDT, 2-mercaptoethanol, ethyl methyl sulfide, TES
Ser, Thr, Tyr	m- or p-cresol, DTE, EDT, TES
Trp (unprotected)	DTE, EDT, 2-Me-indole, Ac-Trp-OMe, tryptamine, TIS

Several papers have been published analyzing the advantages of different cleavage cocktails [167,168] and recommending
Reagent K:
TFA/thioanisole/water/phenol/EDT (82.5 : 5 : 5 : 5 : 2.5)
The use of sulfur-containing scavengers is recommended in the cleavage of sequences containing Met, Cys and Arg. Care must be taken when using thioanisole as it has been suggested that the thioether participates in the premature cleavage of Acm, StBu, or tBu from Cys residues [169].
Silane derivatives (TES, TIS) can successfully replace the malodorous EDT [170-172] as well. They show good efficacy in quenching carbocations in sequences containing Arg and Trp residues.
It is strongly advised to perform sample cleavages using different cocktail compositions and to compare yield and quality of the crude product before choosing the final one. These sample cleavages should be monitored by analytical HPLC.
During the development of the cleavage conditions it is also advisable to perform a simultaneous time course evaluation of the cleavage. It should be complete in 2–3 hours at room temperature in order to minimize side reactions arising from long exposure to strong acids.
The table below shows the recommended scavengers in relation to the amino acids present in the sequence.

5.2. Mix Your Own Cocktail

Sample and bulk cleavages should be performed in a well-ventilated hood wearing appropriate protective equipment (gloves, goggles; mask if necessary) during the operation!

A growing proportion of scavengers means reduction of the amount of TFA, thus, cleavage with cocktails containing a considerable amount of scavenger or many different scavengers may take longer.
The Recommended Standard Procedure for a sample cleavage is described on p. 33.
Having optimized the composition of the cocktail and the reaction time it is possible to perform the cleavage of the bulk of peptide-resin.
One of the principal advantages of Fmoc SPPS is the possibility of scaling up the cleavage batch size without having to change to special equipment.

Recommended Standard Procedure

TFA Cleavage

The cleavage cocktail (10–15 mL/g peptide-resin) is prepared in a reactor or a round-bottom flask equipped with a stirrer and cooled in an ice-bath during the preparation. The cold mixture is flushed with argon or nitrogen. The peptide resin is slowly added under inert atmosphere and the temperature is controlled. When the addition is completed the cooling bath is removed and the reaction is allowed to proceed at ambient temperature for the predetermined time. The cleavage mixture is filtered and the resin rinsed with neat TFA (or 95% TFA/water). The filtrate is slowly added to cooled MTBE (8–10 mL ether/mL cleavage cocktail). The precipitate is left to settle and filtered off. The solid is resuspended in the ether, stirred and left to settle in the cold and filtered off. The operation is repeated up to the elimination of TFA and scavengers (pH control). After the last filtration, the solid is dried under vacuum. The crude peptide can be stored as a dry powder or dissolved in an appropriate solvent for purification purposes.

Filtrates containing sulfur scavengers have to be oxidized to eliminate the pervasive obnoxious smell. For that purpose the TFA filtrate is neutralized (NaOH/ice) before addition of an oxidant (e.g. bleach or a saturated solution of iodine in ethanol).

5.3. Cleavage of Protected Peptide Fragments

Protected peptide fragments can be obtained from SASRIN or 2-chlorotrityl type resins (see II.1.3.3.). Detailed descriptions of cleavages from SASRIN are found in our SASRIN brochure [173].
Cleavages from xanthenyl resin (cf. p. 16) follow the same protocol.
A range of cleavage reagents for peptides synthesized on 2-chlorotrityl resin has been described. TFE/AcOH/DCM (1:1:3) has been developed by Barlos [18]. Cleavage is also rapidly attained with 0.5% TFA/DCM as well

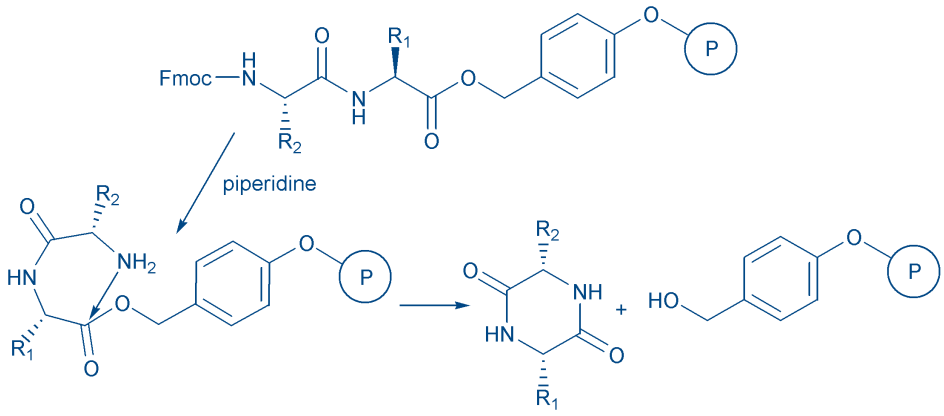


Fig. 9.
Diketopiperazine formation after deprotection of the penultimate amino acid.

as with HFIP/DCM (1:4 or 3:7) [66].
The latter system is especially suitable for the cleavage of fully protected fragments to be coupled to another fragment in solution, as it eliminates the use of a carboxylic acid in the cleavage step.

Recommended Standard Procedure

Cleavage from 2-Chlorotrityl Resin

The peptide-resin is stirred about 15 min in HFIP/DCM 1:4 (10 mL/g peptide-resin). The reaction mixture is filtered and the resin rinsed with HFIP/DCM. The filtrates are pooled and the solvents evaporated under vacuum. At the end the peptide can be precipitated with MTBE or with water depending on its hydrophobicity and recovered after filtration and drying.

6. Side Reactions in Fmoc SPPS

6.1. Diketopiperazine Formation

Diketopiperazine formation is a notorious side-reaction during peptide synthesis at the dipeptide stage. It is particularly prone to occur in Fmoc based SPPS due to its protocol, see Fig. 9.
During the base-induced deprotection of the penultimate amino acid, the liberated amino function may cleave the resin ester linkage whilst forming a piperazinedione, i.e. the dipeptide is split off.
Good accessibility to the ester bond facilitates this side-reaction as does a cis-amide bond making sequences containing a C-terminal Pro susceptible to this side-reaction. Nearly quantitative loss may occur.
The extent of diketopiperazine formation depends on the nature of the C-terminus as well as on the penultimate amino acid, especially on the bulk of the side-chains.
Diketopiperazine formation may be circumvented in Fmoc/tBu SPPS:

- by using 2-chlorotrityl chloride resin or other bulky resins such as DHPP-Resin (II.1.3.1.2.)
- by coupling the appropriate Fmoc-dipeptide in lieu of the penultimate amino acid. In that case a risk of epimerization exists if the C-terminal amino acid of this dipeptide is not Gly nor Pro.
- by coupling the appropiate Trt-amino acid [174]. Deblocking with dilute TFA yields the protonated dipeptide cf III.6.1. The amino group is liberated during the proceeding coupling by an additional equivalent of base. Albeit they couple sluggishly, Trt-amino acids are optically rather stable.

6.2. Aspartimide Formation

This sequence-dependent side reaction is catalyzed by acids as well as by bases.
In Fmoc based SPPS aspartimide can thus be generated during the final TFA cleavage as well as during the chain elongation; this cyclization is accompanied by epimerization. The tert. butyl side-chain protecting group normally provides sufficient protection.
Several examples of base-catalyzed aspartimide formation during the peptide elongation have been reported, together with the reopening of the aspartimide cycle during the subsequent Fmoc deprotection step, yielding piperidides [175-177] and diketopiperazine formation with the subsequent amino acid. [178]. Fig. 10 gives an overview of the numerous potential by-products.
The addition of HOBt to the piperidine solution helps to reduce the formation of aspartimide. Bulkier β-carboxy protecting groups as OMpe [97] or ODie [179] prevent

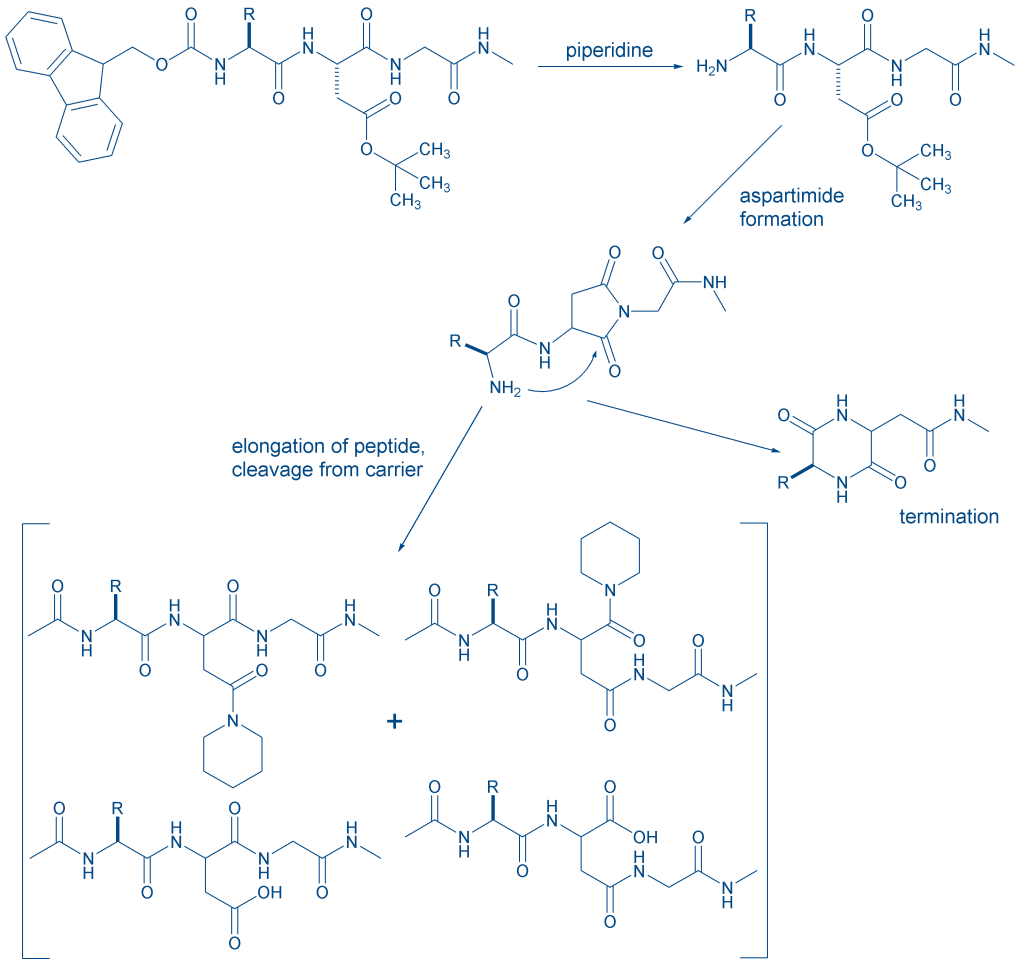


Fig. 10.
Base-catalyzed aspartimide formation and subsequent reactions.

cyclization more efficiently than OtBu. Another approach is to introduce the residue preceding Asp as the Fmoc(Hmb or Dmb)-protected amino acid [162, 180]. The most sensitive Asp-Gly motif is reliably stabilized by coupling Fmoc-Asp(OtBu)-HmbGly-OH (4035481) [181] or Fmoc-Asp(OtBu)-DmbGly-OH (4062763) [162]. For an Fmoc cleavage protocol reducing aspartimide formation [92] see 2.2.

Base-catalyzed aspartimide formation will be facilitated considerably when replacing OtBu by less hindered groups such as OAll, OBzl and ODmab.

Glu may be involved in base-catalyzed side reactions [182] as well, cyclization can yield acylated pyroglutamic acid or glutarimide.

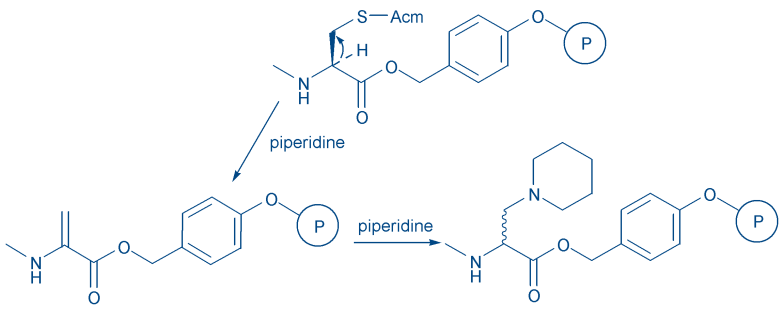


Fig. 11.
Base-induced β-elimination of C-terminal cysteine.

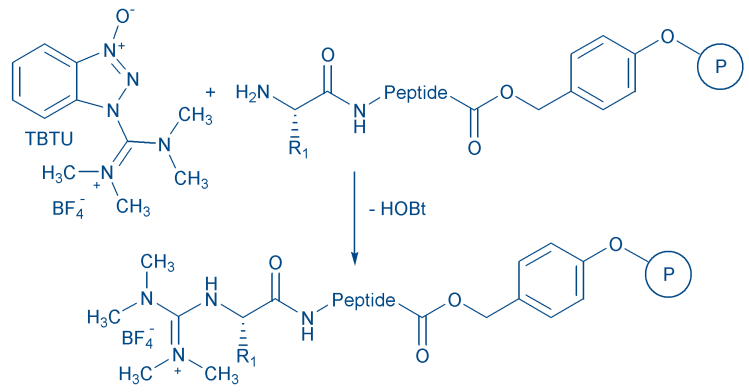


Fig. 12.
N-Terminal guanidinylation by the coupling reagent.

6.3. Transfer of Pmc from Arg to Trp During TFA Deprotection

This reaction is observed when Trp is inserted without indole protection. The addition of Pmc (or Pbf) to the indole ring will occur and the amount of by-product is related to the distance between the Trp and the Arg residues [183].

6.4. 3-(1-Piperidinyl)alanine Formation

This modified amino acid can be obtained when synthesizing peptides with C-terminal cysteine.

The base-catalyzed elimination of the sulfhydryl protecting group affords dehydroalanine and the subsequent addition of piperidine yields the C-terminally modified peptide, see Fig. 11 [184]. This side reaction is minimized (but not avoided!) when trityl is used for the protection of the C-terminal Cys.

6.5. Incomplete Fmoc Cleavage

It has been demonstrated [82] that the incomplete deprotection is associated with slow or incomplete coupling. In such cases, prolonged or repeated piperidine treatment, in combination with a change of solvent or the addition of 1-2% DBU can help to reduce the problem (cf. II.2.2).
Again this phenomenon is difficult to predict. Therefore, determination of the extent of cleavage is mandatory when doubts arise.

6.6. Guanidinylation of Free Amino Moieties During Coupling

As mentioned in II.4.2.2. this reaction occurs during couplings mediated by uronium/aminium reagents or carbodiimides. Fig. 12 shows an N-terminal guanidinylation with TBTU. This side reaction can be avoided by preactivation of the amino acid (i.e. the coupling reagent is consumed).
The side reaction can't occur when activating with phosphonium salts (Bop, PyBop).

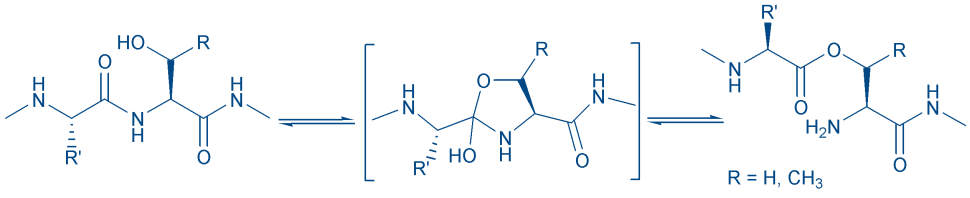


Fig. 13.
N-O shift involving serine or threonine.

6.7. Side Reactions of Methionine
The Met thioether is readily (and usually inadvertently) oxidized to the sulfoxide, whereas oxidation to the sulfone requires strong oxidants. The extent of sulfoxide formation usually can't be predicted. Fortunately it may be reversed by treatment with, e.g., NH_4I/Me_2S [185]. The sulfoxide may be deliberately introduced for increasing the polarity of a «difficult sequence» [183]. cf. II.4.5.
Side-chain tert. butylation of Met during the final cleavage usually does not pose a problem. It is reversed if the peptide is left standing in dilute aq. AcOH [99].

6.8. N-O Shift (cf. III.6.4)
Sampson et al observed that rearrangement of Ser/Thr-containing peptides may occur even during cleavage with TFA [164]. Reversal is induced by bases, e.g. aq. NH_3. The isoacyl peptide approach for synthesizing aggregation-prone peptides as β-amyloid peptides or IAPP is based on the rearrangement shown in Fig. 13 [166, 187].

7. Standard Fmoc Cycle

In Table 10 you can find a standard protocol used at Bachem for routine Fmoc SPPS.

Table 10 . Synthesis cycle during Fmoc-SPPS

Step No.	Solvent/reagent		Duration	Remarks
1	DMF	2x	1 min	
2	20% piperidine/DMF	1x	5 min	
3	20% piperidine/DMF	1x	10 min	can be extended to 20 min for peptides of 30 + residues
4	DMF	3x	1 min	alternate 4 and 5
5	IPA	3x	1 min	
6	DMF	3x	1 min	
7	color tests: Kaiser/TNBS			Chloranil replaces TNBS for Pro
8	Preactivation Fmoc-AA 3 fold excess TBTU 3 fold excess DIPEA 3 fold excess		3 to 8 min	DIPEA can be replaced by collidine
9	start coupling		1 to 18 hrs	
10	DIPEA 20% of excess in 8		after 20 min of coupling	optional
11	DMF	2x	1 min	alternate 11 and 12
12	IPA	3x	1 min	
13	color tests: Kaiser/TNBS			negative: go to 1 positive: go to 6 for recoupling

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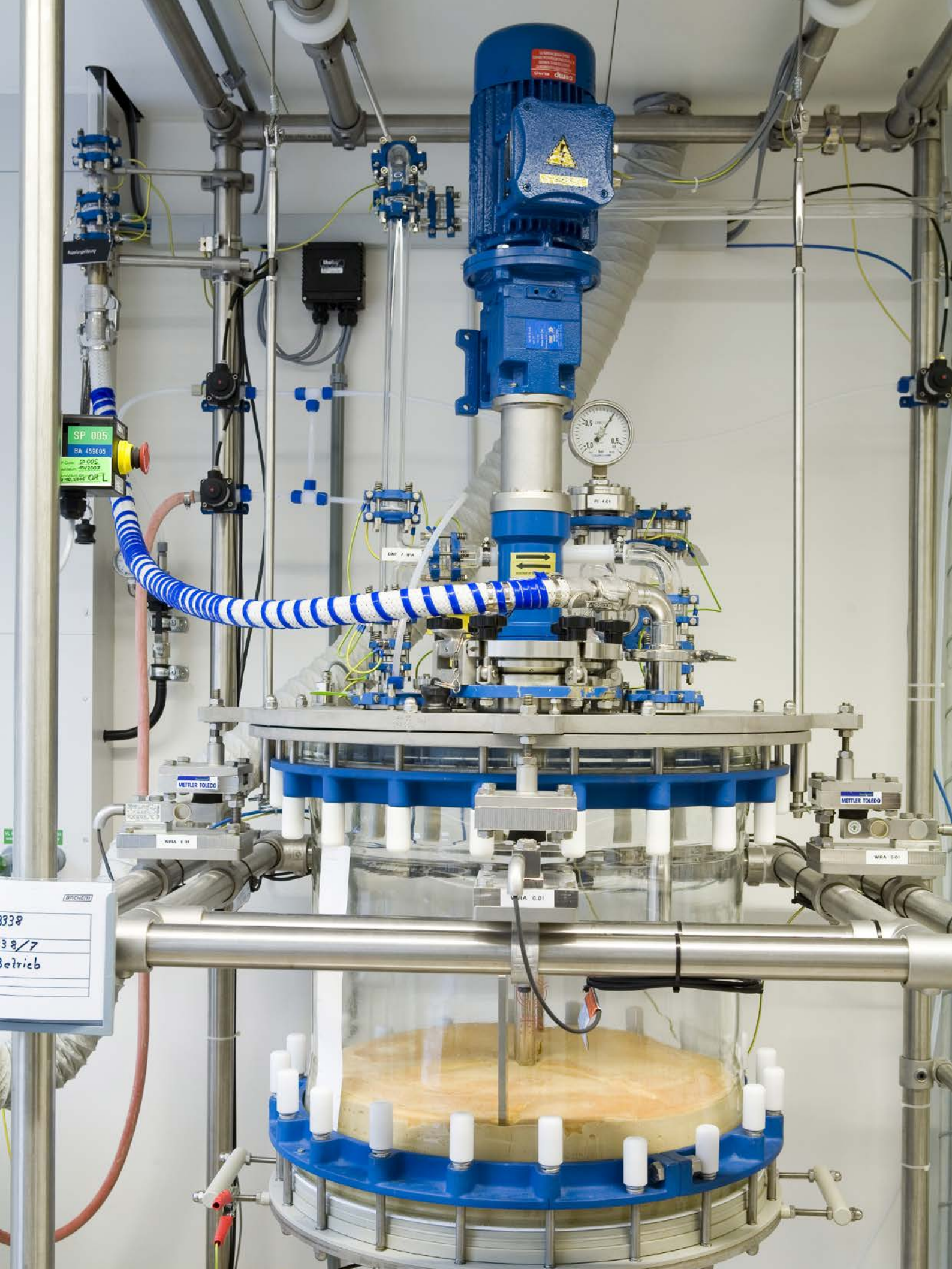
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III BOC-BASED SPPS

1. Resins

The general considerations formulated in the introduction to the Fmoc chapter are also valid for the Boc based SPPS approach. As for the Fmoc based SPPS, the resins can be divided into two main classes (v.i.), the resins available at Bachem are listed in Table 11.

1.1. Resins for the Synthesis of Peptide Acids

Chloromethyl polystyrene (Merrifield resin)
(4031224 (200–400 mesh) and 4031246 (100–200 mesh))
Chloromethylpolystyrene is the classical resin used in Boc based SPPS. The first amino acid is introduced as a salt which insures a racemization free esterification. In the original procedure [1] the triethylammonium salt of the Boc-amino acid was used but the formation of quaternary triethylammonium salt between the chloromethylated resin and the triethylamine limits the usefulness of the method. Presently the Boc-amino acids are esterified via their cesium salt, following the method of Gisin [2]. The deprotection (cleavage of the Boc group) is achieved using a 50% solution of TFA in DCM.
The benzyl ester linkage between the C-terminal amino acid and the polystyrene can be prematurely cleaved in the deprotection step and important losses can occur during the construction of a long peptide chain [3].

PAM-Resin
(only available as preloaded resin)
To avoid these losses the less acid-labile phenylacetamido linker has been inserted between the support and the C-terminal amino acid. The stabilization introduced by this linker reduces the loss of peptide at each deprotection step and the peptide acid is released from the resin by HF [4,5].
For the preparation of the Boc-amino acid-PAM resin, the preformed Boc-amino acid-PAM handle is coupled to aminomethyl resin. The esterification of the Boc-amino acid to the hydroxymethylphenylacetamidomethylpolystyrene using DCC/DMAP bears the risk of concomitant racemization.
In addition, the coupling of the anchor leaves unreacted hydroxyl groups on the resin and it is advisable to cap them by acetylation [6] or benzoylation [7].
For both types of support synthesis can be successfully performed on resins with a substitution in the range of 0.5 to 1.0 mmole/g. However certain sequences are more conveniently prepared with a resin of lower substitution.
As for Fmoc based SPPS, Bachem offers complete range of presubstituted Merrifield and PAM-resins.
The trifunctional amino acids are introduced with the most commonly used side-chain protecting groups and the substitution of the resin is generally in the range of 0.5 to 1.0 mmol/g.

1.2. Resins for the Synthesis of Peptide Amides

The standard Merrifield resin can be used for the construction of the peptide chain and the cleavage of the peptide from the resin can be achieved by ammonolysis [8,9]. This procedure presents several drawbacks and is rarely employed. Special resins have been developed which allow the recovery of the peptide amide following HF cleavage.
BHA-resin
(4013542)
The BHA-resin has been introduced by Pietta and Marshall [10] for the preparation of peptide amides. However it was rapidly recognized that the stability of the amide bond between the C-terminal amino acid and the resin depends on the amino acid. The enhanced stability can prevent an effective cleavage of the peptide from the resin. Orlowsky [11] has developed the more labile 4-methoxybenzhydrylamine resin but in this case undesirable losses of peptide have been encountered during the TFA cleavage of the Boc group.

MBHA-resin
(4013545 (200–400 mesh) and 4049216 (100–200 mesh))
This resin has been introduced by Stewart and Matsueda [12] and shows an optimal balance between stability towards TFA and lability towards HF. The MBHA resin has be-

come the support of choice for the preparation of peptide amides in the Boc SPPS.
The coupling of the first amino acid is achieved following a standard coupling protocol (DCC/HOBt, HBTU, TBTU, ...).
A substitution level between 0.5 and 1.0 mmole/g is routinely used as mentioned above. The optimal load for a specific sequence can only be determined after comparison of test syntheses.

1.3. Further Resins

4-Formyl-phenoxymethyl polystyrene (4029157)
This resin has been designed for the preparation of cyclic peptides, the cyclization is achieved on-resin and the cycle peptide is cleaved from it by HF [13].

2. The Boc Group

2.1. General Remarks

The Boc group has been described by Carpino in 1957 [14] and has directly been applied in peptide synthesis.

Merrifield rapidly realized that the relative labilities towards acid of the Boc and benzyl groups would be compatible with an effective temporary vs. permanent protecting scheme and very soon published the solid phase synthesis of Bradykinin using Boc protected amino acids [15].
Along the years numerous protecting groups for the side-chains of multifunctional amino acids have been developed. We will mention here only the protecting groups and their combinations which have been

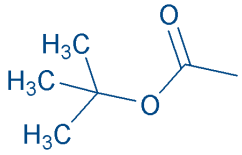
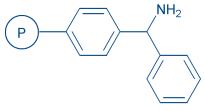
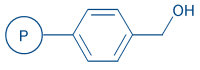
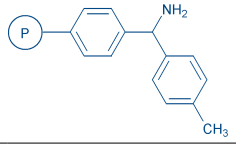
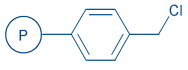
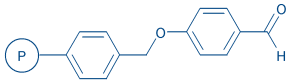
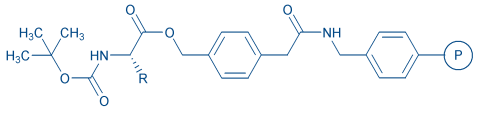


Table 11. Resins for Boc-based SPPS available from Bachem

Product No.	Name	Structure
4013542 (200–400 mesh)	Benzhydrylamine resin (BHA resin)	
4014323 (200–400 mesh) 4031238 (100–200 mesh)	Hydroxymethyl resin	
4013545 (200–400 mesh) 4049216 (100–200 mesh)	4-Methyl-benzhydrylamine resin (MBHA resin)	
4031224 (200–400 mesh) 4031246 (100–200 mesh)	Merrifield resin (Chloromethyl polystyrene-divinylbenzene)	
4029157 (200–400 mesh)	4-Formyl-phenyloxymethyl polystyrene resin (4-Alkoxybenzaldehyde resin)	
	Boc-amino acids linked to PAM resin	

applied successfully for the preparation of a vast number of different sequences (Table 12). The typical flow chart of a routine Boc based synthesis is presented in III.7 (Table 13) and we will just comment the steps which are typical for a Boc based protocol.

2.2. Deprotection

In most cases the cleavage of the Boc group is performed with a 50% TFA/DCM solution. A short prewash (5 minutes) is followed by the cleavage reaction (15 to 25 minutes). The use of a scavenger is recommended when Cys, Met or Trp are present in the sequence. In that case 0.5% DTE are added to the TFA/DCM solution.

Neat TFA has also been used for the cleavage of the Boc group [16], this protocol is rarely used.

After the cleavage, the peptide-resin is washed with DCM followed by shrinking with an alcohol to «extract» most of the remaining TFA.

IPA is preferred to MeOH because its use reduces the risk of transesterification with the Asp/Glu side chain protecting groups, and also because of a lower toxicity.

Table 12. Side-chain protected Boc-amino acids

Amino Acid	Protecting Group	Cleavage Conditions	Remarks
Arg	Tos	HF	standard
	NO_2	HF or hydrogenolysis	rarely used anymore
Asp/Glu	OcHex	HF	standard
	OcPen	HF	similar to OcHex
	OFm	secondary amine	orthogonal; can be used for on- resin cyclization
	OAll	$Pd(PPh_3)_4$	orthogonal to the linker and protecting groups
Asn/Gln	Xan	HF	standard
Cys	Mob	HF	standard
	MBzl	HF	standard
	Fm	secondary amine	orthogonal, S protected after HF cleavage
	Npys	thiolysis, reduction	orthogonal, S protected after HF cleavage
	StBu	S-S interchange reduction	orthogonal, S protected after HF cleavage
His	Tos	HF	standard
	Dnp	thiolysis (before HF)	standard
	Bom	HF	special caution during HF cleavage
Lys	2-ClZ	HF	standard
	Fmoc	secondary amine	orthogonal, can be used for on- resin cyclization
	Aloc	$Pd(PPh_3)_4$	orthogonal
	Tfa	piperidine	orthogonal, but rarely used
	Npys	phosphine reduction	orthogonal, but rarely used
Met	none		
Ser/Thr	Bzl	HF	standard
Trp	none		standard, risk of alkylation of indole ring
	For	piperidine or hydrazine (before HF)	standard
		HF + thiol	
Tyr	2-BrZ	HF	standard
	2,6-diClBzl	HF	standard

Recommended Standard Procedure

Boc Cleavage

The peptide-resin is swollen with DCM. The peptide-resin is stirred in 50% TFA/DCM for 5 min (10 mL/g peptide-resin). After filtration, a fresh solution of 50% TFA/DCM is introduced and the peptide resin is stirred for an additional 20 min. When Cys, Met and Trp are present, 0.5% DTE are added to the TFA solution. The peptide-resin is washed with DCM (2x) and IPA (2x) before neutralization.

2.3. Neutralization

The deprotection reaction leaves the amino group as a trifluoroacetate. It is necessary to neutralize that salt before the coupling. This is realized in two steps in which the peptide-resin is suspended and stirred in a 10% TEA/DCM solution. The resin is then carefully washed before proceeding to the coupling step.

Recommended Standard Procedure

Neutralization

The peptide-resin is suspended and stirred in 10% TEA/DCM (10 mL/g peptide-resin) for 2x10 min. After filtration it is washed alternately with DCM and IPA (3x each) before being washed with the solvent used for the coupling.

3. Boc Amino Acid Derivates

As mentioned earlier, the basis of the development of the Boc/benzyl protecting scheme relies on the relative lability towards acid of the temporary protecting group (Boc) vs. that of the permanent (side chain) protecting groups.
The following table shows the Boc protected trifunctional amino acid derivatives used in standard protocols. All are available from Bachem.

Arginine

The NO_2 group offers a good protection but its removal by HF is not always complete after a standard cleavage procedure and may require «forced» treatment which can be detrimental for other sensitive amino acids present in the sequence.
The Tos group is almost exclusively used for the protection of the guanidino function of Arg. It presents an excellent stability towards TFA and can only be cleaved by HF or TFMSA/TFA.
This exceptional stability must be taken into account for the optimization of the final cleavage conditions. It is sometimes necessary to increase the reaction time to insure complete cleavage, mainly for sequences containing several Arg residues.

Aspartic Acid and Glutamic Acid

The β- and γ-benzyl esters have always been used for the side chain protection of these two amino acids.
The main side reaction encountered with aspartic acid is the cyclization to the aspartimide. It has been demonstrated that the use of the β-cyclohexyl ester could substantially decrease the amount of cyclized product [17].
Consequently Boc-Asp(OcHex)-OH has become the derivative of choice for Boc based SPPS. The use of the benzyl or cyclohexyl ester for the protection of the side chain of glutamic acid is less critical.
The OFm is stable towards the acidic treatment and thus represents a good orthogonal protecting group to be used for special syntheses (post synthetic modifications) [18]. However the cleavage of the OFm group with piperidine presents a risk of piperidide formation for other Asp or Glu present in the sequence.
The OAll also represents an orthogonal protecting group. The ester is stable to the synthesis conditions, stable to HF and can be specifically deprotected by palladium-catalyzed transfer [19].

Asparagine and Glutamine

The protection of the side chain of these amino acids has two functions. First the problem of dehydration of the side chain carboxamide during the activation of the α carboxylic function is eliminated. Furthermore the solubility is significantly increased in comparison to the unprotected derivatives.
Xan [20] has a limited stability towards TFA and is partially cleaved in the subsequent deprotection steps. This has no impact on the quality of the final product as the purpose of the Xan group is limited to the coupling reaction.

Cysteine
Mob [21] and MBzl [22] are the most commonly used protecting groups for the sulfhydryl function of Cys in Boc based SPPS. MBzl is more stable towards repetitive acidolysis of the deprotection step and it is the best choice for the preparation of Cys-containing long peptides.
Both groups are cleaved during the final HF treatment and yield free sulfhydryl functions.
Orthogonal protection of the sulfhydryl function can be achieved using Fm [23], Acm [24], StBu [25] or Npys [26].

Histidine
Tos is commonly used for the protection of the histidine side chain. Even if it is effective in suppressing racemization during coupling its use is not devoid of drawbacks. It is indeed cleaved by HOBt and the subsequent tosylation of the α amino function may cause chain termination.
To avoid this side reaction it is possible to completely remove the Tos group after coupling of the residue and to pursue the synthesis with a deprotected imidazole ring. The ring will be partially acylated in the following coupling step but TFA applied for the Boc deprotection cleavage will liberate the imidazole group again.
In addition, Gly insertion via $N^{im} - N^{\alpha}$ transfer following the loss of the Tos group has been reported [27].
Another possibility is to proceed without HOBt (or HOBt related substance) from the introduction of the His residue up to the end of the synthesis.
Dnp is also frequently used for the His side chain protection. It is stable under the conditions of the synthesis, and also stable towards HF. An additional thiolysis step before the HF treatment is required for its removal. It has however been observed that the cleavage of the Dnp group is not always complete and it is recommended to avoid the use of Dnp protected His in the preparation of a sequence containing several His residues.
Bom is also effective in preventing racemization during coupling and is readily cleaved by HF, however during this cleavage, formaldehyde is released. The reaction of formaldehyde with N-terminal Cys yields a thiazolidyl residue [28,29]. The use of a «formaldehyde trap» such as resorcinol, cysteine hydrochloride or EDT will greatly reduce the extent of this side reaction [30].

Lysine
2-ClZ is mostly used for the protection of the amino function of lysine. This group is indeed stable during the synthesis and is cleaved upon final HF treatment.
On the other hand orthogonal protecting groups can be used for special applications (specific protection of one Lys residue, cyclization ...). Fmoc is the most widely used one; it is stable to the conditions used in Boc based SPPS and can be easily and specifically cleaved by piperidine.
Aloc can also be applied for orthogonal protection. Aloc is selectively cleaved by palladium-catalyzed transfer [31]. Tfa and Npys are also available but less frequently used. [32,33].

Serine and Threonine
The Bzl group is almost exclusively used for the protection of the hydroxyl groups of these amino acids. The benzyl ether is stable under the conditions of the synthesis and is readily cleaved during the final HF treatment.

Tryptophan
It is not compulsory to protect the indole ring of the Trp residue. Nevertheless, due to its reactivity and susceptibility to oxidative degradation this protection is strongly recommended.
The indole ring can be protected by For to avoid tert. butylation during the TFA cleavages of the Boc group in the construction of the peptide chain [34]. This group is cleaved by treatment with piperidine prior to HF cleavage, or by thiolysis performed concomitantly with the HF cleavage.

Tyrosine
The protection of the phenolic function is usually achieved by 2-BrZ or by 2,6-diClBzl. Both are stable during the synthesis and cleaved without noticeable side-reactions with HF.

4. Coupling Reagents and Methods

No special reagent or protocol has been developed for Boc based SPPS and therefore the reader may refer to II.4.

5. Cleavage from the Resin

In this last step of the synthesis the peptide is cleaved from the resin together with the side chain protecting groups at the exception of the ones which are orthogonal to this acidolytic reaction.
Among these, two must be cleaved from the amino acid before the final step: Dnp from His and For from Trp.

Recommended Standard Procedure

Dnp Cleavage

The peptide-resin is swollen in DMF and a 20 fold excess of thiophenol in DMF is added. The peptide-resin is stirred overnight. After filtration the resin is washed with DMF, IPA and MTBE and dried before proceeding to the final cleavage.

Recommended Standard Procedure

For Cleavage

A solution of 10% piperidine in DMF is cooled in an ice bath. The peptide-resin is added to the solution and stirred for 2 hrs at 0–5°C. After filtration the peptide-resin is washed with DMF, IPA, MTBE and dried before proceeding to the final cleavage.

5.1. HF

Liquid hydrogen fluoride is without any doubt the most commonly used reagent for the cleavage of the peptide from the resin, with concomitant cleavage of the side-chain protecting groups [35].
The main drawback of the cleavage method is the handling of the acid:
HF is extremely toxic, corrosive and volatile.
A special all-fluorocarbon apparatus is required for reactions involving HF since it reacts with glass. Such equipment is commercially available and it is recommended to follow precisely the manufacturer's instructions in view of the risk represented by the use of HF.
The limited availability of this special equipment in large volume units prevents the scale-up of the procedure. The cleavage reaction is usually performed for 45 to 60 minutes at a temperature close to 0°C. It can be prolonged if several Arg(Tos) residues are present in the sequence.
Due to the high concentration of alkylating species in the reaction medium, it is mandatory to add scavengers to the cleavage mixture. Anisole has been widely used, the standard ratio is HF/anisole 9:1. Sulfur-containing scavengers such as thiocresol and dimethyl sulfide (DMS) are also recommended.
At the end of the reaction the HF is evaporated under vacuum and the peptide extracted from the resin and isolated.

Recommended Standard Procedure

HF Cleavage

The peptide-resin is placed in the reactor equipped with a teflon coated stirring bar. The scavenger is added, usually anisole in a ratio HF/anisole 9:1. The reactor is closed, connected to the apparatus and cooled in a dry ice/methanol bath for 10–15 min. HF is distilled under vacuum into the reaction vessel in a ratio of 10 mL HF/g peptide-resin. When the necessary quantity of HF has been distilled the dry ice/methanol bath is replaced with an ice bath and the reaction mixture is stirred for 45 to 60 min. At the end the HF is evaporated under vacuum. The cleaved peptide and the resin are suspended in ether and thouroughly washed with ether. After drying, the peptide is dissolved in 50% aqueous acetic acid, separated from the resin by filtration and the solution diluted with water. The peptide can be recovered by lyophilization or the solution can be used directly for preparative HPLC.

Besides the standard HF procedure, the low-high HF cleavage protocol of Tam and Merrifield [36] consists of a two-steps procedure. In the first step, a low concentration of HF in DMS (1:3) cleaves most of the protecting groups following an S_N2 mechanism (vs. S_N1 under the standard conditions). The formation of carbocations and most of the side reactions are prevented.
After evaporation of HF and DMS, a high HF cleavage is performed to cleave the functionalities resistant to the low HF conditions such as Arg(Tos).

The large volumes of DMS and the extra time needed to evaporate the first DMS/HF mixture represent the main drawbacks of the method.

Recommended Standard Procedure

Low-High HF Cleavage

The peptide-resin is placed in the reactor together with DMS/p-cresol (ratio HF/DMS/p-cresol 25:65:10 – p-thiocresol if Trp(For) is present). The required volume of HF is distilled into the reactor and the reaction mixture is stirred for 2 hrs at 0–5°C. HF and DMS are evaporated under vacuum. After evaporation the peptide-resin is removed from the reactor and washed with DCM. The peptide-resin is put back in the reactor with p-cresol. The high HF cleavage is performed for 1 hr at 0–5°C using HF/p-cresol 9:1 (10 mL/g peptide-resin). After evaporation of HF, the peptide is extracted as described in the standard HF procedure.

5.2. TFMSA

TFMSA can be used as an alternative for HF [37]. The main advantage of TFMSA is that glass is inert towards this acid and normal laboratory glassware can be used for the cleavage.

On the other hand, TFMSA will not cleave Arg (Tos) or Arg (NO_2). Also, Asp(OcHex) and Cys(MBzl) are not cleaved by TFMSA.

In addition TFMSA will not cleave the peptide from BHA-resin and requires prolonged reaction time for the cleavage from MBHA-resin.

In view of those potential problems it is recommended to optimize the reaction conditions performing small scale orientation cleavages.

Recommended Standard Procedure

TFMSA Cleavage

The peptide-resin is placed in a round-bottom flask with a stirring bar. Thioanisole/EDT (2:1) are added (3mL/g peptide-resin) and nitrogen or argon is used to generate an inert atmosphere. The flask is placed in an ice bath and 20 mL of TFA are added; the mixture is stirred for 10–15 min and 2 mL of TFMSA (2 mL/g peptide-resin) are SLOWLY added with vigourous stirring. The reaction mixture is stirred for 1–2 hrs at 0–5°C. The reaction mixture is filtered and the resin rinsed with neat TFA. The filtrate is added to a 10 fold volume of cold MTBE and the peptide extracted with 100 ml water (3 times). The water phases are combined and extracted twice with MTBE. The water phase is cooled in an ice bath and an ammonia solution is added up to pH 2–3. This solution is used as is for purification (ion exchange or RP HPLC).

5.3. TMSOTf

TMSOTf represents an alternative to HF [38]. The cleavage of Arg(Tos) requires a longer reaction time. Cys(Bzl), Cys(Acm) and Arg(NO_2) are stable to TMSOTf treatment.

A two step cleavage procedure has also been developed using TMSBr/thioanisole/TFA in the first step (cleavage of the protecting groups) and TMSOTf/thioanisole/TFA in the second step (cleavage from the resin) [39].

5.4. HBr/TFA

HBr/TFA has been used for the cleavage of the peptide resin linkage from the first days of the solid phase technique [40]; however the reagent has shown severe limitations and has been displaced by HF.

A special mixture (HBr/TFA/pentamethylbenzene/thioanisole) has been developed allowing the cleavage of a peptide amide from MBHA resin [41], the general benefits of this method have not been demonstrated.

6. Side Reactions in Boc SPPS

The repetitive TFA treatments together with the final HF cleavage represent sources of side-reactions for the most sensitive amino acids such as Cys, Met and Trp.

Alkylation by carbocations generated in the acidolytic cleavage is the most prominent side-reaction. The judicious choice of scavengers will help to minimize unwanted modifications.

6.1. Diketopiperazine Formation

(see II.6.1. and Fig. 9 on p. 37)

The extent of this side-reaction can be reduced, contrary to the situation in Fmoc based SPPS, by slight changes in the protocol. Indeed, after the deprotection reaction in Boc SPPS the α-amino function is protonated and less prone to attack the benzyl ester bond.

Protocols have been developed in which the neutralization step is performed concomitantly with the coupling [42,43] to suppress this side-reaction.

6.2. Aspartimide Formation

(see II.6.2.)
Aspartimide formation can be catalyzed by acids as well as by bases. During Boc-SPPS, the growing peptide chain repeatedly comes into contact with both types of reagent. Hence aspartimide formation can be a source of by-products, particularly in peptides containing the Asp-Gly, Asp-Ala or Asp-Ser motif.
As mentioned earlier (III. 3.) the OcHex protecting group reduces the extent of aspartimide formation.

6.3. Homoserine Lactone Formation

C-terminal Met can cyclize to homoserine lactone [44] during the HF cleavage if t-butyl type protecting groups have not been cleaved before the HF treatment (Fig. 14).

6.4. N-O Shift

(see II.6.8. and Fig. 13 on p. 39)
N → O shift can occur during the treatment of peptides containing Ser or Thr with strong acid [45]. The reaction can be reversed by base treatment.

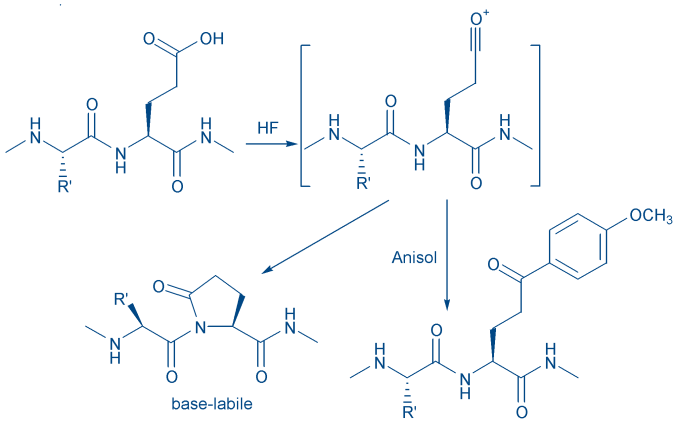


Fig. 15. Side reactions of glutamic acid during Boc-SPPS.

6.5. Side Reactions Involving Glu

The carboxyl function of Glu, protonated in strong acid, can lose water and form an acylium ion which will cyclize to a pyrrolidone or be trapped by a scavenger such as anisole to yield a ketone (Fig. 15) [46].

6.6. Asp-Pro Cleavage

The cleavage of the Asp-Pro bond has been observed during HF treatment [47].

7. Standard Boc Cycle

A standard protocol used at Bachem for routine Boc SPPS can be found in Table 13.

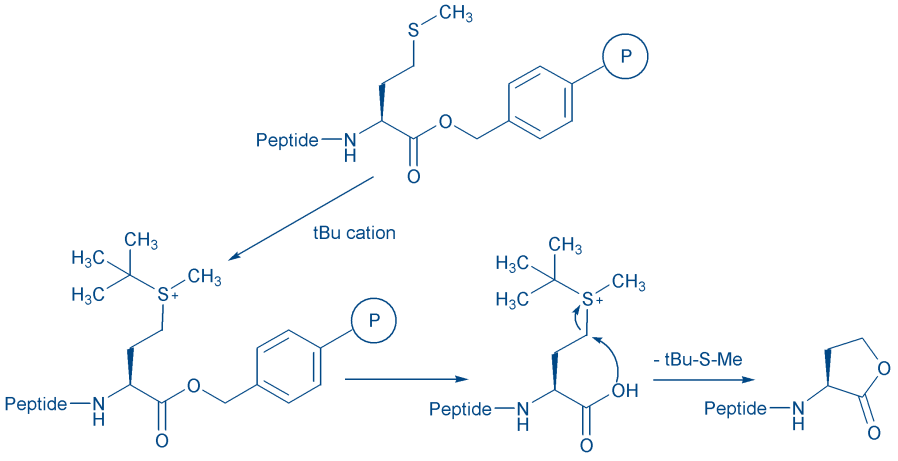


Fig. 14. Formation of homoserine lactone from C-terminal Met.

Table 13. Synthetic cycle during Boc-SPPS

Step No.	Solvent/reagent		Duration	Remarks
1	DCM	2x	1 min	
2	50% TFA/DCM	1x	5 min	+ 0.5% DTE if Cys, Met, Trp present
3	50% TFA/DCM	1x	15 min	
4	DCM 1 min	2x	1 min	alternate 4 and 5
5	IPA	1x	1 min	
6	DCM	1x	1 min	
7	10% TEA/DCM	2x	5 min	
8	DCM	1x	1 min	
9	IPA	2x	1 min	alternate 9 and 10
10	DCM	2x	1 min	
11	color tests: Kaiser/TNBS			Chloranil replaces TNBS for Pro
12	Preactivation Boc-AA 3 fold excess TBTU 3 fold excess DIPEA 3 fold excess		1 to 18 hrs	
13	coupling			
14	DIPEA 20% of excess in 12		after 20 min of coupling	optional
15	DMF	1x	1 min	
16	IPA	2x	1 min	alternate 16 and 17
17	DCM	2x	1 min	
18	color tests: Kaiser/TNBS			negative: go to 1 (2nd step) positive: go to 7

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