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SOLID PHASE SYNTHESIS ON SASRIN

SASRIN™ RESIN

A REVIEW OF ITS MANIFOLD APPLICATIONS INCLUDING MANY USEFUL PROCEDURES

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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
tBu	tert. Butyl
Bzl	Benzyl
Dde	1-(4,4-Dimethyl-2,6-dioxo-cyclohexylidene)-3-methylbutyl
Dmb	2,4-Dimethoxybenzyl
Dpm	Diphenylmethyl
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
Mtt	4-Methyltrityl
OAll	Allyl ester
OtBu	tert. Butyl ester
ODmab	4-{-[1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3-methylbutyl]amino}benzyl ester
OMpe	3-Methylpent-3-yl ester
OPp	2-Phenylisopropyl ester
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofurane-5-sulfonyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulfonyl
StBu	tert. Butylthio
Tnm	1,5-dioxaspiro[5.5]undecane-3-nitro-3-methoxycarbonyl
Trt	Trityl
Tos	p-Toluenesulfonyl
Xan	9-Xanthydryl
Z	Benzyloxycarbonyl

Reagents

BOP	Benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate
DBU	Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DEBPT	3-(Diethoxy-phosphoryloxy)-3H-benzo [d][1,2,3] triazin-4-one
DIBAH	Diisobutylaluminum hydride

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

DIPEA	Diisopropylethylamine
DMAP	N,N-Dimethylaminopyridine
EDT	Ethanedithiol
HATU	O-(7-Azabenzotriazolyl)-tetramethyluronium hexafluorophosphate*
HOAt	1-Hydroxy-7-aza-benzotriazole
HOBt	1-Hydroxybenzotriazole
TATU	(7-Azabenzotriazolyl) tetramethyluronium tetrafluoroborate*
TBTU	(Benzotriazolyl) tetramethyluronium tetrafluoroborate*
TFA	Trifluoroacetic acid
TNBS	2,4,6-Trinitrobenzenesulfonic acid
UNCA	Urethane N-carboxyanhydride

Solvents

AcOH	Acetic acid
DCM	Dichloromethane
DMA	N,N-Dimethylacetamide
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
EtOAc	Ethyl acetate
HFIP	Hexafluoroisopropanol
IPA	Isopropanol
NMP	N-Methylpyrrolidone
TCM	Chloroform
TFE	Trifluoroethanol
THF	Tetrahydrofuran

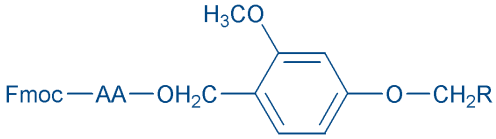
Miscellaneous

AA	Amino Acid
bp	Boiling Point
HMPB	4-Hydroxymethyl-3-methoxyphenyloxybutyric acid
Ⓟ	Polystyrene crosslinked with 1% divinylbenzene
RT	Room Temperature
SPOS	Solid-Phase Organic Synthesis
SPPS	Solid-Phase Peptide Synthesis
TASP	Template-Assembled Synthetic Protein
TLC	Thin Layer Chromatography

1. Introduction

As fully protected peptide fragments have become readily available by solid-phase peptide synthesis (SPPS), the synthesis of large peptides via fragment coupling (Giralt's «Convergent SPPS»[1]) has become a viable alternative to stepwise SPPS. These fragments may be assembled either in solution [2] or «on resin» [3].
This approach may seem more cumbersome than the classical stepwise method, but it usually leads to crude peptides of better purity, as deletion sequences accumulated in stepwise synthesis are much more difficult to remove compared to those obtained from incomplete fragment coupling. Difficult sequences which impede stepwise SPPS may be circumvented by adequate choice of segments and, especially, condensation sites [3b]. Furthermore, before being coupled, the fragments can be thoroughly analyzed, characterized, and purified, whereas in stepwise SPPS these operations are restricted to the final product.
Orthogonal N^{α}/side chain protection is the strategy of choice when synthesizing fully protected fragments. The combination of N^{α}-Fmoc and tert-butyl based side-chain protection has become most popular. This approach requires a peptide resin bond that can be cleaved either by acids weak enough (or sufficiently diluted) to leave the tert-butyl groups intact [4,5] or by a method employing neither acid nor base, such as catalysis by a Pd(0) complex [6] or photolysis [7].
The very acid-labile linkers themselves should withstand repeated contact with Fmoc amino acids and HOBt.
A few of such resin linker combinations have been made commercially available. Based on earlier work of Sheppard et al. [4a] on 2-methoxy-4-alkoxybenzyl alcohol type anchors, Bachem has introduced its Super Acid-Sensitive ResIN (SASRIN) in 1987 [5]. SASRIN is available in two different mesh sizes: 200 - 400 mesh (product number 4012712) and 100-200 mesh (4027899). Cleavage of the peptide from the resin requires 0.5 to 1% TFA in DCM [4b,5].
Peptide fragments containing Tyr(tBu), Lys(Boc), or His(Trt) can be safely obtained only under these conditions when the cleavage proceeds rapidly (see Table 1). Thus, SASRIN (2-methoxy-4-alkoxybenzyl alcohol resin) is sufficiently acid-labile so that Tyr(tBu), Lys(Boc), and His(Trt) are left intact during cleavage. On the other hand, it is stable against Fmoc amino acids and HOBt under normal coupling conditions.
Many fully protected peptide fragments have been obtained, also on large scale, in good yield and purity. But SASRIN can as well be applied for the classical stepwise SPPS of peptides with an additional advantage. Due to the acid-lability of the resin, samples can be easily and rapidly cleaved at any stage of the synthesis and the intermediates can be isolated and characterized. Thus, an unambiguous monitoring and batch documentation system is obtained. Thorough monitoring helps in solving synthetic problems or optimizing a large scale synthesis. After being cleaved from SASRIN, the (fully) protected peptide can be modified in solution, e.g. backbone cy-

**Table 1. Cleavage kinetics of very acid-labile resins in 1% TFA/DCM [4b].
Half-lives (as determined photometrically at 301 nm) in seconds.**



R =	-Ph-Ⓟ SASRIN [5]	-CH_2-CH_2-CO-NH-Ⓟ HMPB-linker [4b]	-CO-NH-Ⓟ Sheppard's linker [4a]
Fmoc-Gly	20	24	600
Fmoc-Ile	48	33	1500

clized (head-to-tail or suitable side chains). Some examples taken from the literature may illustrate various applications of SASRIN:

- synthesis of derivatives of the glycopeptide antibiotic vancomycin (even a molecule as complex as vancomycin could be coupled to SASRIN bound peptides in reasonable yields) [8].
- synthesis of peptide-containing templates to modify the surfaces of implanted biomaterials as to create specific interactions between implant and surrounding tissue. The aim is to develop materials for implants of improved biocompatibility [9].
- synthesis of linear and head-to-tail cyclized analogs of bradykinin and other kinins [10].
- synthesis of pseudoproline-containing peptides. Pseudoprolines (2,2-dimethyl-4-carboxythiazolidine and 4-carboxy(5-methyl)oxazolidine) are acid-labile derivatives of Cys, Ser and Thr, respectively. Pseudoproline formation serves as a solubilizing technique in convergent peptide synthesis [11a,b]. Keller et al. could obtain a nonapeptide by repetitively coupling of Fmoc-Pro-pseudoproline-OH building blocks to H-Pro-SASRIN [11c].
- synthesis of vasoactive intestinal peptide (VIP) (4011611) by a combination of solid-phase and solution methods [2b].
- the incorporation of Fmoc-N(2-hydroxy-4-methoxybenzyl)amino acids impedes the aggregation of peptide chains during SPPS («difficult sequences»). These compounds have also been used as building blocks for synthesis on SASRIN. Protected fragments containing such moieties are ideally suited for convergent synthesis [12].
- synthesis of a fluorescent analog of a peptide enhancing HIV infectivity [13a]. Fluorescein isothiocyanate adds (in solution) to a free N^{ε} of Lys of an otherwise protected peptide, which was obtained by on-resin Lys(Dde) deprotection [13b] with 2% hydrazine hydrate in NMP followed by cleavage with 1% TFA/DCM.
- synthesis of chemoselectively addressable templates (for the synthesis of TASP's) containing homocanaline ((S)-2-amino-5-aminooxy-pentanoic acid, H-Homocan-OH) employing Fmoc-Homocan(Aloc)-OH and Fmoc-Lys(Boc)-OH (4003151). Thus, peptides are linked to the template either via amide or via oxime bonds, an aldehyde group has to be generated beforehand in the latter case [14a]. SASRIN has already been used to synthesize other types of templates [14b], and, more recently, for synthesizing the building blocks of template-bound protein mimetics [14c]
- convergent synthesis of a complex glycopeptide antigen [15]
- synthesis of S-farnesylated peptide methyl esters [16]
- synthesis of thioxopeptides. The thioamide moiety is sensitive towards acids and bases. Nevertheless, a protection scheme compatible with this type of backbone modification could be developed by Wildemann et al. [17]. Nα-Bpoc protection was chosen, which could be selectively removed by treatment with $Mg(ClO_4)_2$ in acetonitrile. The peptide was cleaved from SASRIN with $ZnCl_2$ in ether which concomitantly removed the tBu-type side-chain protecting groups.

Last but not least the fully protected peptides obtained by synthesis on SASRIN may be purified before cleavage of the protecting groups. As final cleavage is subsequently performed in a homogeneous system, it is easier to optimize the conditions (composition of the cleavage cocktail, cleavage time). Peptides containing a C-terminal Lys or Orn may be obtained more smoothly by two-stage cleavage (but, even when applying 95% aq TFA or similar systems, they may be split off more readily from SASRIN than from Wang resin).

Further applications of SASRIN include on-resin modifications and cleavages with nucleophiles. These will be dealt with in detail in the following chapters. Another chapter will discuss properties and use of SASRIN derivatives.

Also available at Bachem but not further described in this review is the 2-chlorotrityl chloride resin (available in two bead sizes 4025425 (200-400 mesh) and 4040104 (100-200 mesh)) developed by Barlos [4c] for the synthesis of short fully protected peptide fragments.

Its use is especially recommended for the

synthesis of fragments with a C-terminal Pro since diketopiperazine formation (leading to substancial loss of peptide) is inhibited [18, 19].
Peptide fragments are cleaved from chlorotrityl resin, e.g. with AcOH/DCM/TFE (1:3:1) [4c], or, avoiding the use of a carboxylic acid, with HFIP/DCM (1:4) [19].

2. Preparation of SASRIN and Attachment of Amino Acid Derivatives

The «SASRIN linker» 3-methoxy-4-hydroxymethyl phenol can be synthesized from commercially available 3-methoxyphenol [20]. The Vilsmeier formylation of this compound leads to two isomers which are separated by extraction. As to be expected, reduction with sodium borohydride yields a very acid-sensitive alcohol, which is linked to chloromethyl polystyrene (known as Merrifield resin) employing the conditions described by Lu et al. [21].
The alkylation proceeds very smoothly, the resulting resin derivative contains only traces of chlorine (below 0.1%) [20,22].
Fig. 1 shows the synthesis of SASRIN.

Fmoc/tert-butyl amino acids were esterified to the resin employing DCC and catalytic amounts of 4-dimethylaminopyridine at low temperature. The conditions had to be optimized to achieve minimal racemization, maintaining a high load. Following this pathway good results were obtained for most amino acid derivatives. Only a few of them (e.g. Cys and His derivatives) showed a pronounced tendency to racemize upon activation. Hence an alternative way of synthesis had to be looked for. As it was known from the literature, protected amino acids can be esterified to Merrifield resin via nucleophilic substitution, employing, e.g. the cesium salts [23], with virtually no concomitant racemization. Thus, the polymer-bound benzyl alcohol had to be converted into a benzyl halide taking into account its acid-lability. The best results have been obtained with triphenylphosphine dichloride [24,25,26a] which was prepared *in situ* from triphenylphosphine and tetrachloromethane [26b] (SASRIN bromide could be obtained as well, by treating the resin with the dimethyl sulfide/N-bromosuccinimide complex [27]. Our own attempts to convert SASRIN into its bromide had failed). The resulting polymer is treated with the dry cesium salt of the desired Fmoc amino acid in DMA in the presence of sodium iodide to enhance nucleophilic displacement. As expected, minimal levels of racemization, but nevertheless high loads could be obtained [28]. Both strategies for attachment are summarized in Fig. 2.

The loading of the resin is determined photometrically. The Fmoc group is cleaved with piperidine/DMF (1:4), the absorption of the supernatant is measured at 301 nm (ε = 8100 in 20% piperidine in DMF [29], a value of ε = 7800 has also been published [30]).
The extent of racemization is determined as described by H. Frank et al. [31]. The optimization of both methods of esterification enables Bachem to offer a vast range of Fmoc amino acids coupled to SASRIN with guaranteed high loadings and excellent optical purity, please see pp. 37-38.
Fmoc-Asp and Fmoc-Glu derivatives have also been coupled via their side-chain to SASRIN for special applications (see Fig 3 and, as an example for an SPPS relying on side chain anchoring, reference [32]):

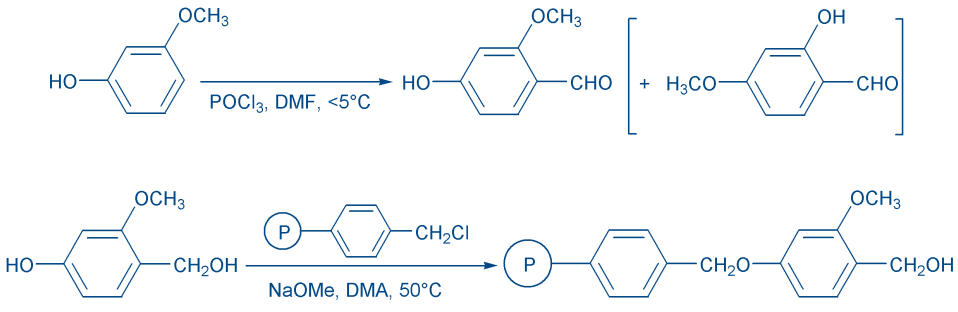


Fig. 1.
Synthesis of SASRIN.

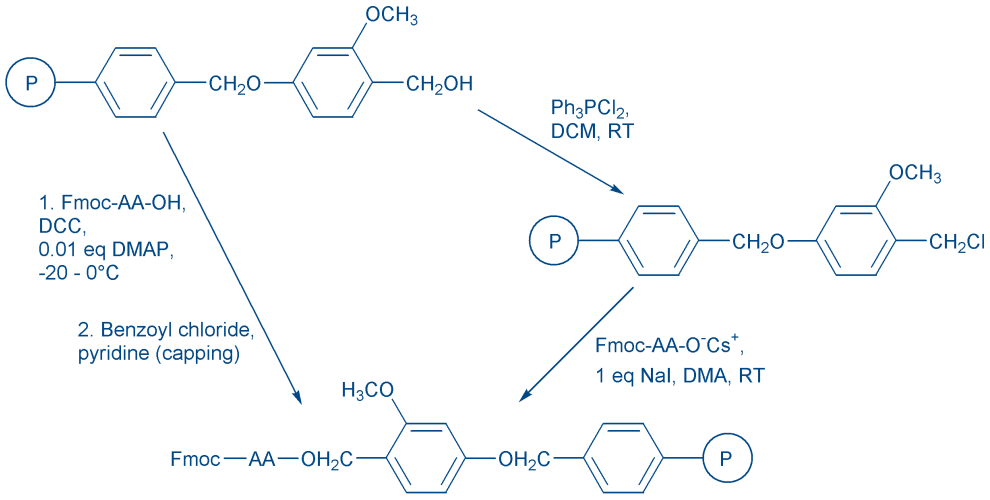


Fig. 2.
Loading of SASRIN.

3. Synthesis of Peptides Using SASRIN

3.1. General Remarks

A short introduction to Fmoc/tBu-based SPPS with emphasis on the characteristics of SASRIN will be presented in the following chapters.

SPPS on SASRIN can be performed either manually or automatically, any commercially available synthesizer suitable for polystyrene-based resins, especially for Wang resin, can be applied. Continuous-flow reactors for this type of resin have also been described [33]. SASRIN has already been applied in the fully automated simultaneous multiple peptide synthesis of 31 heptapeptide segments of endothelin [34].

A shaker with a vessel equipped with a fritted glass bottom will normally suffice for synthesis and cleavage. It can be operated manually or automatically. Except for the cleavage procedure, all synthetic protocols developed for Wang resin can be applied to SASRIN.

«Maximum protection» is recommended (exceptions see chapter 4), a choice of compatible protecting groups is listed in Table 2. The N-terminus of fragments is usually protected by Fmoc, the very last fragment to couple may be protected by Boc.

Trp(Boc) derivatives [35] have gained much importance in Fmoc-SPPS, as N^{in}-protection has been shown to be the most efficient method to suppress irreversible binding of the Trp-containing peptide to the resin during cleavage [2a,36].

Furthermore, the lipophilic protecting groups influence the solubility of the fragments [2].

Partially protected peptides may be obtained by choosing side-chain protecting groups labile towards 1%TFA/DCM (cf. Table 2), though unambiguous deprotection can only be achieved by employing orthogonal side-chain protection (e.g. OAll/Aloc [6b] or Dde/Dmab [13b]).

If possible, Gly or Pro are chosen as C-terminal amino acids of the fragments to avoid

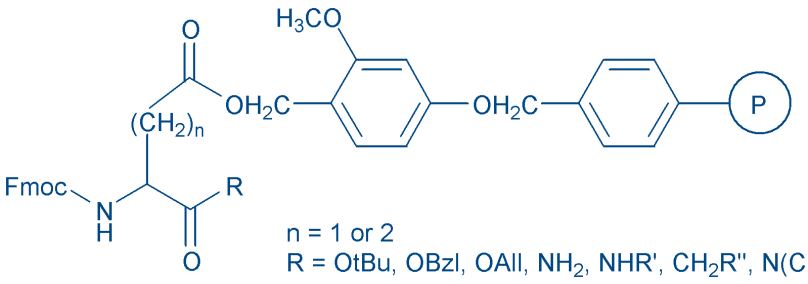


Fig. 3.
Anchoring via side-chain carboxyl moiety.

Table 2. Selection of commonly used side-chain protecting groups categorized according to their acid-lability. All derivatives are available from Bachem.

Amino Acid	Side-chain protecting group cleaved by:		
	95% aq TFA	1% TFA/DCM	HF or orthogonal
Arg	Pbf, Pmc	-	Nitro, Tos
Asn/Gln	Mtt, Trt, (Xan)[a]	-	-
Asp/Glu	OtBu, OMpe	OPp, ODmb	OAll, OBzl, ODmab
Cys	Trt, Dpm[b]	Mmt	Acm, StBu, Mbzl, Mob
His	Trt, Mtt	(Mtt)	Bom, Dnp
Met	-	-	sulfoxide
Lys	Boc	Adpoc, Mtt, Mmt	Aloc, Dde, ivDde, Tnm, Z
Ser/Thr/ Hyp/Tyr	tBu	Trt	Bzl
Trp	Boc	-	For

[a] Protecting groups in brackets - partial cleavage
[b] Use of Dpm in place of Trt [149] additionally reduces oxidation of Cys to cysteic acid.

racemization during coupling later on. When synthesizing fragments with a C-terminal Pro, as with Fmoc-Pro-O-Wang resin (product number 4003166 and 4028591), the formation of diketopiperazines has to be circumvented by coupling Fmoc-dipeptides instead of the penultimate Fmoc amino acid at the risk of concomitant racemization (diketopiperazine formation on chlorotrityl resin is impeded due to steric hindrance [4c,19]. In these critical cases we now recommend the use of chlorotrityl resin.

3.2. Cleavage of Fmoc

SPPS on SASRIN can be performed in DMF, NMP, or other polar aprotic solvents (as long as they swell the resin properly). Cleavage of the Fmoc group is usually performed with 20% piperidine in DMF (or NMP). After a few prewashes with the solvent, the resin is treated twice (e.g., 5 and 10 min) with this mixture. Diluted solutions of diazabicycloundecene (DBU, 2-5%) have also been recommended [37]. The mixture of piperidine (20%) and DBU (2%) in DMF is even more efficient for difficult Fmoc cleavages, but it should not be applied routinely (DBU, being a strong base, may cause side-reactions, namely aspartimide formation [41a,b]).
Cleavage with tetrabutylammonium fluoride [38a,38c] and piperidine-mediated cleavage at elevated temperatures (45°C [38b]) have (to our knowledge) not yet been applied to SASRIN.
A weaker base such as morpholine was recommended for the SPPS of base-sensitive glycopeptides [39]. In our hands, the combination N-methylpyrrolidine/hexamethyleneimine/HOBt/NMP/DMSO (50:4:4:71:71, v/v/w/v/v) [40] turned out to be a mild and reliable reagent for removing Fmoc from base-sensitive peptides [41a].
The completeness of Fmoc cleavage can be confirmed, e.g., by taking a sample to perform the «quantitative Kaiser test» (determining the amount of liberated amino groups [42]) or by UV-monitoring. The UV-spectrometric determination of the amount of dibenzofulvene-piperidine adduct formed requires special instrumentation [43]. Another method is cleaving a sample with 1% TFA/DCM and analyzing the peptide, e.g. by TLC or HPLC. Due to the high UV-absorption of the Fmoc group very small amounts of Fmoc peptide resulting from incomplete Fmoc cleavage can be detected. Alternatively, the peptide may be analyzed by HPLC after complete removal of acid-labile side-chain protection.

Recommended Standard Procedure:
Cleavage of Fmoc
20% piperidine in DMF
1 x 5 min
1x 10 min
wash until neutral, e.g. > 10 x DMF or 5 x DMF, 2 x IPA, 3 x DMF

3.3. Coupling

Piperidine has to be washed out completely before proceeding further, the last washings have to react neutral with a wet pH-indicator paper. Treatments with isopropanol or tert-butyl methyl ether will shrink the resin and accelerate piperidine removal, but the resin has to be swollen again with DMF or NMP. Washing protocols employing just a single solvent such as DMF may take longer, but the peptide resin is permanently swollen (shrinking may enhance aggregation).
Several color tests indicating free amino groups have been developed for qualitative monitoring, e.g. the Kaiser ninhydrin test [44], the 2,4,6,-trinitrobenzenesulfonic acid (TNBS) test [45] (not suitable for Pro), the acetaldehyde/chloranil test (especially recommended for Pro and secondary amino groups) [46], and the bromophenol blue test (giving ambiguous results in the presence of His(Trt)) [47], which should be positive by now.
As mentioned before, all coupling procedures and reagents applicable to Wang resin can be employed with SASRIN, coupling solutions containing acids (e.g., Fmoc amino acids, HOBt in DMF) need not be buffered (compare [2a,4b]). Couplings have been performed with DCC/HOBt in DMF either «one-pot» or, preferably, after preactivation and removal of the precipitated dicyclohexylurea (in automated synthesis, activation with diisopropylcarbodiimide (DIC or DIPCDI) /HOBt is preferred, as the corresponding urea is readily dissolved by the solvents used for washing). This method is useful especially in large scale synthesis, as these reagents are usually reliable and still the cheapest ones available. Adjustment of the pH (7-7.5), i.e. neutralisation of the liberated HOBt using bases such as DIPEA, N-methylmorpholine, or collidine (2,4,6-trimethylpyridine) can accelerate the coupling, but an excess of base has to be avoided.
The situation in small scale (automated) synthesis is somewhat different. There, the necessity to increase coupling rates and to avoid insoluble by-products such as dicyclohexylurea led to the application of a vast range of coupling reagents such as BOP, TBTU, HATU [48] in combination with a base such as DIPEA, N-methylmorpholine, or, especially when coupling Fmoc-Cys derivatives [49] or Fmoc-Ser derivatives [50], collidine. The coupling reagent DEPBT (4029249) [51] used in combination with DIPEA proved to be the superior reagent for coupling Fmoc-His(Trt)-OH (4011444), a derivative with a distinct tendency to racemize during activation [52a,b]. These compounds are more convenient to handle than carbodiimides. HATU/base should be chosen in case of steric hindrance, e.g., coupling to N-alkylamino acids. Stable activated amino acid derivatives such as UNCAs and Pfp-esters [53] have also been employed successfully with SASRIN. But, whichever coupling reagent is chosen, a significant excess of (activated) amino acid (e.g. threefold as a standard) has to be applied to drive the reaction to completion. The «consumption» of amino groups is monitored by the color tests mentioned above. The sensitivity of the TNBS and acetaldehyde/chloranil tests can be increased by using a microscope to inspect the beads (see Fig. 5) If the tests indicate complete acylation, the resin is washed carefully (see above). If, after 60 -120 minutes, the tests are still (slightly) positive, the coupling should be repeated. Capping, i.e. acylation of amino groups which might have remained undetected by the color tests, is optional (its efficacy has never unambiguously been proven to our knowledge), usually a large excess of acetic anhydride and pyridine, in the solvent system used for coupling is employed. After a few minutes reaction time the reagents have to be washed out carefully. The next coupling cycle starts again with the piperidine treatment. As the peptide chain grows longer, the swelling of the resin should increase slowly. A sudden decrease can mean aggregation of the peptide impeding further coupling and Fmoc cleavage steps (it can be

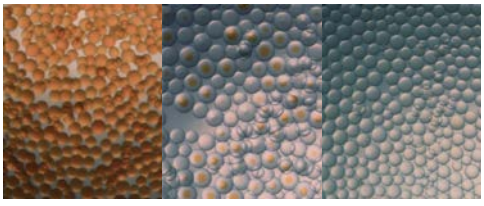


Fig. 4.
TNBS-test.
Beads inspected under the microscope.
left: positive
middle: incomplete coupling
right: negative.

assumed that syntheses on Wang resin and SASRIN take a similar course). Then monitoring by cleaving samples with 1% TFA/DCM turns out to be a valuable aid, as these samples reflect the actual state of the synthesis. Changes in the coupling protocol such as the change of solvent, application of additives [54], coupling at elevated temperatures [55], or a more effective coupling reagent may help to overcome such difficulties, their effect can be judged by cleaving samples for comparison.

Recommended Standard Procedure:
Coupling
3 eq. of Fmoc amino acid derivative
3 eq. TBTU/DIPEA (or collidine) or DCC/HOBt
Solvent: DMF
Monitoring: Kaiser and/or TNBS test after 30 - 60 min, acetaldehyde/chloranil test in case of coupling to secondary amino groups

4. On-resin Modification

The modification of resin-bound peptides is far more convenient than converting the protected peptide in solution, as a large excess of reagent may be added and, later on, simply be washed out. The progress of the reaction is monitored by cleaving and analyzing samples. On-resin reactions such as sulfation may be repeated, if the conversion was incomplete. As the modified peptide can be cleaved rapidly under mild conditions, SASRIN lends itself especially to the synthesis of acid-sensitive peptide derivatives such as sulfated peptides and O-glycosylated peptides. These labile moieties withstand the mild conditions of cleavage from SASRIN.
If even repeated treatments only yield unsatisfactory amounts of modified peptide, or a considerable amount of by-products is formed, if the reagent is too expensive to be used in large excess, or more than one amino acid (especially adjacent moieties) is modified, better yields may be obtained by coupling the corresponding amino acid derivatives (e.g. Fmoc- Tyr(SO_3Na)-OH, product number 4029106). This approach will equally benefit from the acid lability of SASRIN.

4.1. Glycopeptides

Usually peptides are glycosylated either via nitrogen (N^{am} of Asn, N-terminus, N^{am} of peptide amides) or via oxygen (hydroxyl of Ser, Thr, C-terminus). Glycopeptides can be base-sensitive.
Therefore the weaker base morpholine should be used instead of piperidine to remove Fmoc [39], but see also [37b]. O-glycopeptides are known to be very acid-sensitive.
The synthesis of the O-glycosylated oncofetal fibronectin sequence Val-Thr(Gal-GalNAc)-His-Pro-Gly-Tyr on SASRIN has been published by Lüning et al. [56], employing the peracetylated diglycosyl derivative of Fmoc threonine. As Z-type side-chain protection had been chosen, a further acid treatment could be avoided (the glycosyl moiety was deprotected with sodium methylate).
Gobbo et al. [57,10b] applied the unprotected glycoside Fmoc-Thr(β-Gal)-OH in the synthesis of the bis-O-glycosylated vespulakinin I sequence Boc-Thr(tBu)-Ala-Thr(β-Gal)-Thr(β-Gal)-Arg(Pmc)-Arg(Pmc)-Arg(Pmc)-Gly-OH, which was then coupled to the free N^{ε}-(Lys) of a cyclopeptide.
The synthesis of a penta-O-glycosylated peptide, the penta-O-(N-acetyl-α-D-galactosaminyl)-MUC-1 eicosapeptide, H-Val-Thr(X)-Ser(X)-Ala-Pro-Asp(OtBu)-Thr(X)-Arg(Pmc)-Pro-Ala- Pro-Gly-Ser(X)-Thr(X)-Ala-Pro-Pro-Ala-His(Trt)-Gly-OH (X = α-D-GalNAc) has been described by Duprandeau et al. [58] employing the glycosylated Ser and Thr derivatives and recovering the excesses of these expensive compounds after coupling. N-glycosylated peptides have been obtained by on-resin glycosylation: Vetter et al. [59] synthesized a range of hexapeptides containing N-glycosylated Asn or Gln at varying positions. The corresponding glycosylamine was coupled to the ω-carboxyl of Asp or Glu generated from the ω-allyl ester by cleavage with $Pd(PPh_3)_4$ in TCM/AcOH/N-methylmorpholine and activated with DCC/pentafluorophenol. All these steps were performed «on the resin» and samples of all intermediates were cleaved and characterized. Even complex structures such as sulfated glycopeptides could be obtained.

4.2. O-Sulfated Peptides

O-sulfated peptides are notorious for their acid-lability, the corresponding salts (Ba or Na) are somewhat more stable. O-sulfated peptides can be obtained either by coupling O-sulfated Fmoc hydroxy amino acids (preferably, the Na sulfate salt, compare e.g. [60], or the tetrabutylammonium salt [61]), or by on-resin sulfation of hydroxyl groups left unprotected. If sulfation in solution is preferred, the suitably protected peptide can be cleaved from SASRIN before sulfation. All three approaches lead to selectively sulfated products.

Tyr and Thr can be coupled without side-chain protection, e.g. by activation with BOP [62].

But during the following coupling reactions care has to be taken: amino acids may not be «overactivated» during chain elongation to avoid esterification of the free hydroxy side-chain, i.e. branching. The fewer the synthetic cycles to follow the lower the risk of side reactions. The coupling of O-unprotected serine represents a risk of subsequent side reactions (Boc-Ser-OH (4001097) has been coupled employing BOP under certain precautions [62]), but orthogonal hydroxyl protection is feasible (e.g. the t-butyldimethylsilylether [63] cleavable - as Fmoc! - with 0.1 M tetrabutylammonium fluoride in DMF). On-resin sulfation can be achieved with pyridine - sulfur trioxide complex [64], the synthesis of a Tyr(SO_3Na)-containing peptide may illustrate the procedure:

As Tyr has to be incorporated unprotected into the otherwise fully protected peptide, Fmoc-Tyr-OH (4012011) has to be employed for coupling. Couplings are performed with e.g. BOP/DIPEA, an excess of base has to be avoided. The subsequent amino acids should be activated before being added to the peptide resin. Large excesses of all reagents should be avoided.

After coupling the N-terminal amino acid a sample should be cleaved to obtain the protected nonsulfated peptide. The C-terminus has to be blocked (e.g. by Fmoc). The peptide resin is prewashed with dry DMF/pyridine (2:1). It is suspended in the same solvent (6-8 ml/g resin) and a large excess (up to 40 eq) pyridine-SO_3 complex is added. Moisture has to be excluded.

After shaking (or stirring) overnight a sample is taken (but, before cleavage, the Na sulfate has to be formed (see below)). Normally, a short treatment with 1% TFA/DCM causes negligible desulfation. Then, the degree of conversion has to be assessed, e.g. by TLC. If only traces of peptides with a free phenolic hydroxyl are found (the use of a color test is strongly recommended), the resin has to be washed carefully with dry DMF/pyridine to remove excessive reagent. Only then it may be washed with DMF, aqueous DMF, and DMF containing diluted aqueous sodium hydrogen carbonate (or sodium carbonate) to form the less sensitive Na salt. Excess base and water have to be washed out carefully with DMF, which then has to be removed thoroughly with DCM (for cleavage see chapter 5). The salts of short sulfated peptides and salts of peptides containing more than one sulfate moiety may be scarcely soluble in 1% TFA/DCM, nevertheless, they are cleaved from the resin (which turns violet as usual) and washed out with a suitable solvent, e.g. DMF. The remaining protecting groups have to be removed in a separate step, then conditions should be optimized as to minimize the loss of sulfate groups [65].

Sulfation may be repeated with fresh reagent, if necessary. 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].

A range of sulfated hirudin fragments, cholecystokinin octapeptide (4033010), the bis-sulfated peptide cionine, and others have been obtained by this method. Sulfated resin-bound peptides may be modified even further, e.g. C-terminally acetylated. SPPS may also be continued risking a slight loss of sulfate groups (the fewer steps the better). Again, every additional step can be monitored by cleaving samples.

Recommended Standard Procedure:

On-Resin Sulfation of Tyr

Prewash resin with dry pyridine/DMF(1:2)
Use up to 40 eq. of pyridine-SO_3 complex
Solvent: dry pyridine/DMF(1:2)

Reaction time: overnight
Washes: pyridine/DMF, DMF
Na salt formation: aq. DMF, DMF/aq. Na_2CO_3 (or $NaHCO_3$), ca.2%
(a clear mixture should result)
Washes: aq. DMF, DMF, DCM or IPA to remove the DMF

4.3. Phosphopeptides

Phosphopeptides have gained growing interest as they have become readily accessible via Fmoc/tBu-SPPS: either by coupling the corresponding amino acid derivative or by «global phosphorylation», i.e. the phosphate moiety is incorporated after the assembly of the resin-bound peptide has been completed [66].

A range of O-phosphorylated amino acid derivatives has been developed for the former approach: phosphorylated Tyr can be introduced, e.g., as Fmoc-Tyr($PO_3(CH_2CH_2SiPh_2Me)_2$)-OH (4026422) [67] or Fmoc-Tyr(PO(OBzl)OH)-OH (4038299). Fmoc-Tyr(PO_3Me_2)-OH (4017013) and Fmoc-Tyr(PO_3H_2)-OH (4025426) may also be used for introduction of the phosphate moiety (the second one for incorporation at the N-terminus) but are not recommended. Phosphorylated derivatives of Fmoc-Ser-OH and Fmoc-Thr-OH (4015218) are difficult to handle, since phosphotriesters readily undergo β-elimination in the presence of bases (e.g. during Fmoc cleavage). Up to now, only Fmoc-Ser(P(O)(OX)OBzl)-OH (X=H,Na) turned out to be a sufficiently stable building block for assembling phosphoserine-containing peptides [68], the analogous Thr derivative Fmoc-Thr(P(O)(ONa)OBzl)-OH for the synthesis of phosphothreonine-containing peptides has also been described [68b].

Global Phosphorylation

To achieve post-assembly phosphorylation, an Fmoc hydroxy amino acid has to be coupled without or with orthogonal (e.g. t-butyldimethylsilyl [63b]) side-chain protection. Prior to the phosphorylation reaction described below the hydroxy function(s) to be phosphorylated must represent the only free functional moiety. Boc (or another protecting group requiring cleavage conditions other than bases) has to be chosen for N-terminal protection. The peptide resin has to be dried carefully, oxygen and moisture have to be strictly excluded during phosphitylation.
A sample should be taken before treatment with tetrazole and dibenzyl-N,N-diisopropylphosphoramidite (other phosphite protecting groups compatible with the synthetic strategy may be chosen). A range of reagents and protocols has been recommended for the subsequent oxidation step, two well-established oxidation procedures will be presented here.

Phosphite oxidation with tert-butylhydroperoxide

The resin is swollen in dry DMF or another suitable solvent (20-30 ml/g). 20 meq sublimed tetrazole are added followed by 10 meq dibenzyl-N,N-diisopropylphosphoramidite. After shaking (or stirring) for 1 to 3 hours the solution is sucked off and the resin is washed thoroughly with dry DMF. Oxidation is performed with 20 to 40 eq tert-butylhydroperoxide (the commercially available solution in toluene may be used) in DMF. After 30 minutes the solution is removed. The resin is carefully washed with DMF followed by IPA (or another solvent to remove the DMF), and dried. Met is oxidized to the sulfoxide, which has to be reduced later on, whereas Cys(Trt) is not attacked by this mild oxidant (as long as the oxidation medium is kept neutral or slightly basic). Cleavage with 1% TFA/DCM yields the fully protected phosphopeptide.

Phosphite oxidation with iodine/2,6-lutidine (especially recommended for Met and Trp containing peptides [69])

The resin is suspended in dry acetonitrile (DNA-synthesis grade, 20-30 ml/g). 15 meq sublimed tetrazole are added together with 10 meq dibenzyl-N,N-diisopropylphosphoramidite (both in acetonitrile). After stirring (or shaking) for up to 1 hour the solution is sucked off and the resin is washed thoroughly with dry acetonitrile. Oxidation is performed with a 1M solution of iodine in THF/2,6-lutidine (2,6-dimethylpyridine)/water 40/10/1 (v,v,v). After 30 minutes the solution is removed. The resin is carefully washed with THF followed by methanol

or ether, and dried. Met may be partially oxidized to the sulfoxide. Cleavage with 1% TFA/DCM yields the fully protected phosphopeptide. A different approach applying the less sensitive H-phosphonates has been described by Larsson & Lüning [70].

Recommended Standard Procedure:

On-Resin Phosphorylation

i. Oxidation with tert.-butylhydroperoxide
Swell resin in dry DMF (25 ml/g)
Add 20 meq tetrazole (sublimed) and 10 meq dibenzyl-N,N-diisopropylphosphoramidite
Reaction time: 1 to 3 hours
Washes: dry DMF
Add 20 to 40 meq tert.-butylhydroperoxide (in toluene)
Reaction time: 30 min
Washes: DMF, IPA
ii. Oxidation with iodine/2,6-lutidine
Suspend resin in dry acetonitrile (25 ml/meq)
Add 15 meq tetrazole (sublimed) and 10 meq dibenzyl-N,N-diisopropylphosphoramidite
Reaction time: 0.5 to 1 hr
Washes: dry acetonitrile
Add 1M I_2 in THF/2,6-dimethylpyridine/H_2O (40:10:1, v/v/v, 10ml/g resin)
Reaction time: 10 to 30 min
Washes: THF, MeOH

4.4. Cystine Peptides (S-S-Oxidation)

Cystine-containing peptides can be obtained from SASRIN by various synthetic strategies, which are summarized in Fig. 5. Either the (partially) protected peptide or the deprotected peptide may be oxidized in solution. To favor intramolecular disulfide formation, the oxidation is performed in very diluted solution. Thus work-up becomes rather tedious. Oxidation of a resin-bound peptide circumvents this problem. Quite a number of papers dealing with variations of this approach as employing different sulfhydryl protecting groups and oxidants has been published [71], though, to our knowledge, a systematic overview is still lacking. Cyclization yields depend on several factors, amongst them the peptide load (a low load should reduce intermolecular reaction and thus enhance cyclization, i.e. the pseudodilution effect), the size of the ring to be formed (probably best below 10 amino acids), and the solvent which should properly swell the peptide resin. Oxidations proceeding smoothly in solution may also work well if performed with the resin-bound peptide (for a noteworthy exception see [71b]). The N-terminus should be protected. The acidlability of SASRIN has to be taken into consideration, e.g. when oxidizing with iodine.

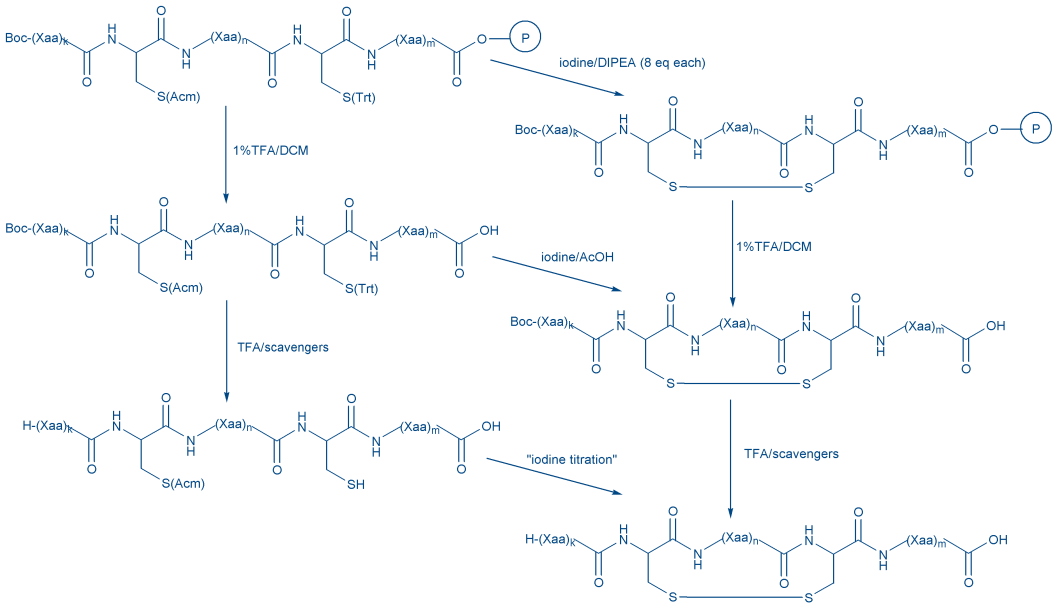


Fig. 5. Synthesis of peptides containing an intramolecular disulfide bridge (Ⓟ = SASRIN).

S-S-Cyclization has been performed successfully on SASRIN. The conditions described here have been evaluated for [Cys(Acm)[1],Cys(Trt)[7]]-calcitonin (1-10) (salmon) and [Cys(Acm or Trt)[2], Cys(Trt)[7]]-α-CGRP(1-14) (human) [72].
The resin (load of fully protected peptide approximately 0.1 meq/g or below) is left to swell in DCM/MeOH/H_2O (60:25:4,v/v/v, approx. 18 ml/g resin) for at least 30 minutes. A solution of iodine (8eq) in DCM (12 ml/g resin) is added rapidly, followed by DIPEA (max 8 eq) to neutralize the hydrogen iodide evolved during the reaction. Oxidation time should not exceed one hour, the rate has to be determined by taking samples and quenching them with ascorbic acid before cleavage and analysis. Eventually excess iodine is removed by filtering and washing the resin with DMF and DMF/ascorbic acid in aqueous buffer of pH7.
Nevertheless the resin may remain slightly yellow. As usual, all polar impurities have to be removed before cleaving with 1% TFA/DCM. The yield obtained may be lower than the cleavage yield of the linear fully protected peptide. Yields depend on the conditions of oxidation, especially on the solvent. Thus, preliminary experiments optimizing this parameter should be conducted if possible.
Other synthetic strategies may be chosen, e.g. generation of SH-groups and oxidation as separate steps. If S-tert-butylthio protection has been employed, the peptide resin is treated with tributylphosphine [73,74] (or β-mercaptoethanol), thoroughly washed and then treated with an oxidant.

Recommended Standard Procedure:
On-Resin Cys Oxidation with Iodine
Swell resin in DCM/MeOH/H_2O (60:25:4), 18 ml/g resin, for 0.5 to 1 hr
Add 8 eq I_2 in DCM, 12 ml/g resin
Add 8 eq DIPEA
Reaction time: 1 hr or below
Washes: DMF, aq. ascorbic acid/ DMF, aq. DMF, DMF, DCM or IPA

4.5. Further Applications

Reduced peptide bonds can be generated by reductive alkylation of peptides bound to SASRIN. Fmoc α-amino aldehydes are reacted with the free amino group of a resin-bound peptide yielding an aldimine which is reduced with sodium cyanoborohydride (cf.[75]) as shown in Fig. 6. The reductive alkylation has to be monitored by cleaving samples as the secondary amine formed leads to ambiguous color tests. Furthermore it represents, albeit a less reactive, coupling site (for introduction of a Ψ[CH_2NH] protecting group cf. [75d]). Thus, «overactivation», prolonged coupling, and capping have to be avoided.
On the other hand, the unprotected secondary amino group may be deliberately reacted before cleavage [75c].
Reagents for Aloc/OAll removal such as $Pd(PPh_3)_2Cl_2/Bu_3SnH$ or $Pd(PPh_3)_4/PhSiH_3$ [6b,c] have been successfully applied in our laboratories to SASRIN bound peptides. Samples were cleaved to check for completeness of the reaction. The Pd treatment can be repeated if necessary.

5. Cleavage with Acids

5.1. General Remarks

i. Dry DCM should be used throughout the procedure. Before cleavage, the resin has to be washed thoroughly with DCM to remove all polar impurities (especially remains of polar solvents). The importance of these washings cannot be overemphasized.
ii. Special care has to be taken when cleaving Lys, Tyr, and, above all, His containing peptides, as their side-chain protecting groups (N^{ε}-Boc, tBu, and especially N^{im}-Trt) are known to be considerably more acid-sensitive than the other protecting groups applied in the synthesis of fully protected peptides (cf. Table 2). Prolonged treatment with 1% TFA/DCM can cause cleavage of these groups [76]. Thus, acid treatment has to be kept as short as possible. Cleavage solutions containing the fully protected peptide fragment have to be neutralized immediately after removal of the resin.
iii. The cleavage, i.e. acid treatment, will also

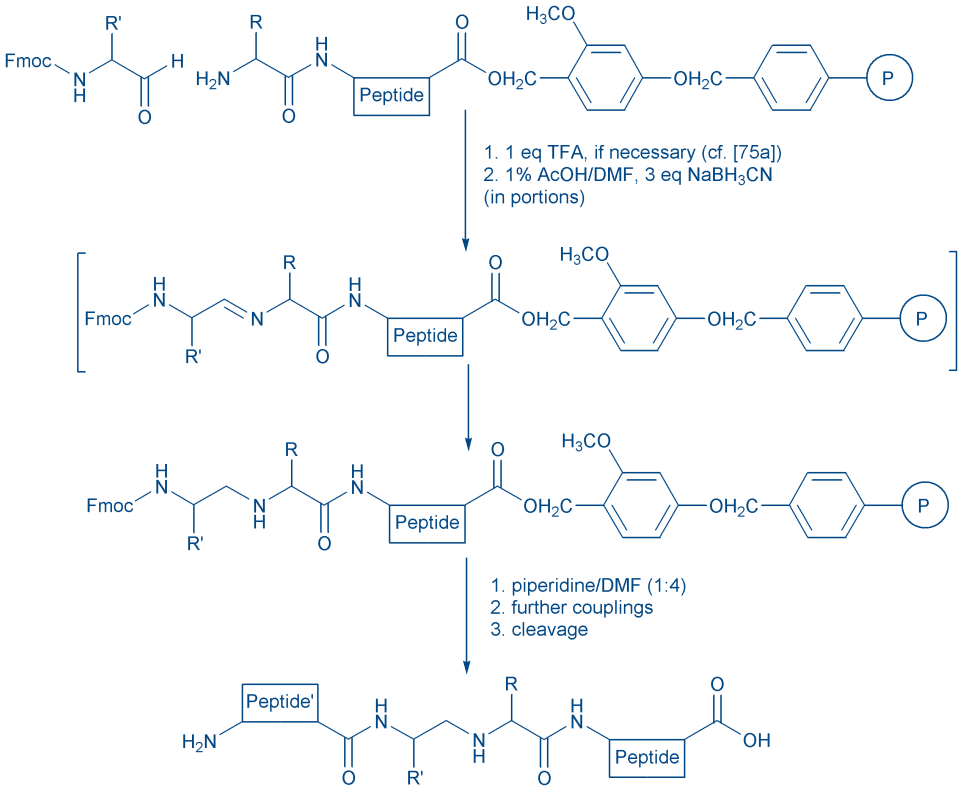


Fig. 6.
Synthesis of the Ψ[CH_2-NH] pseudopeptide bond on SASRIN (cf. [75a]).

yield a relatively stable deeply violet resin-bound cation which, by alkylating indoles (Trp) and thioethers (Met, Cys), can bind peptides irreversibly leading to a considerable loss of peptide fragment. The addition of scavengers (e.g. 5% EDT [2a,4b,10d,36] or 3% triisopropylsilane [77]) increases the yield, but cleavage may take about twice as long. The loss due to indole alkylation can be kept low by employing N^{in} protection (again, the use of Fmoc-Trp(Boc)-OH (product number 4017674) [35] is strongly recommended) and a scavenger (EDT [36]).

5.1. Cleavage with 1% TFA/DCM

5.1.1. The Cleavage Procedure

In the meantime, cleavage protocols superior to the one described originally [5a] have been developed [1b,2a] (cf. [4b,78]). Cleavage times have been rigorously shortened, so that even His(Trt)-containing peptides could be obtained without loss of imidazole protection. Upon treatment with 1% TFA/DCM the resin-bound peptide is protonated at first (donor groups: amide bonds etc) [2a]. Thus, a certain amount of TFA is consumed; rapid acidolytic cleavage will only take place with excess TFA, i.e. with further portions of 1% TFA/DCM.

The simple cleavage procedure described below is also applicable to large batches of resin.

The peptide resin (swollen or dry) is placed into a fritted-glass funnel and washed carefully (at least 5 times) with copious portions of DCM. Then it is treated with 1% TFA/DCM (10-15 ml/g, 5% EDT (if necessary)) for 2 to 5 minutes (depends on the amount of resin) with slight agitation.

The cleavage solution is sucked (or removed by applying inert gas pressure) into a vessel containing at least 2 eq of pyridine (per eq of TFA) and a little methanol (1ml/10ml solution), thus neutralizing the TFA immediately. The treatment is repeated with further portions of 1% TFA/DCM, lumps of resin should be dispersed. Collecting the filtrates in separate vessels and pooling the peptide-containing fractions later on simplifies the work-up when cleaving large amounts of peptide resin. The resin will change color gradually via pink to deeply violet (no scavenger used, Met, Cys, Trp not present, Trt, Mtt side-chain protecting groups not present).

As part of the TFA is «consumed» during the first treatment, the second and third portion will often contain most of the peptide. Completeness of cleavage, i.e. the peptide contents of the (last) fractions, can be determined by TLC.
Fully protected peptide fragments are usually soluble in 1% TFA/DCM, some will precipitate or form a gel upon neutralization (i.e. deprotonation). For a noteworthy exception see [8]: the desired product containing unprotected glycoside moieties had to be washed out with DMSO after treatment with 2% TFA/DCM (which, on the other hand, removed less polar impurities).
Generally, it has to be kept in mind that removal of highly acid-labile side-chain protecting groups such as Mtt(Lys), Trt(Ser), or OPp(Asp,Glu) may reduce the solubility of the fragment in 1% TFA/ DCM as do «salt-like» moieties such as Tyr(SO_3Na), even though cleavage from the resin is not impeded. In case of reduced solubility TLC monitoring is of utmost importance. Undissolved product can be detected by TLC (or HPLC) when triturating a sample of the «cleaved resin» with a highly polar solvent (DMF, DMSO...) which should dissolve the peptide. Accordingly, work-up has to include treatments with a suitable solvent.
Alternatively, TFE may be added to the 1%TFA/DCM to dissolve the insoluble cleavage product forming a visible layer or a gel on the otherwise violet resin beads. Mixtures of TFE and DCM (e.g., TFE/DCM (1:1)) are excellent solvents for fully protected peptide fragments [79].
Nevertheless, the TFE has to be washed out thoroughly with DCM, if a repetition of the acid treatment is intended.
A gentle and rapid removal of cleavage solutions can be achieved by applying inert gas pressure, when cleaving in a suitable vessel (see chapter 3). Samples for monitoring can be cleaved in a small frit, the (neutralized) filtrate is applied directly to a TLC plate, which is dried *in vacuo* and developed in a suitable system. Thus, this reliable method of monitoring is not as cumbersome as it may seem.
When cleaving with 0.5% TFA/DCM [80,81] the amount of solvent has to be increased to at least 20 ml/g (again, the acid is «consumed» by the peptide prior to cleavage, see above).
If desired, cleavage can also be performed with 95% aq TFA or TFA/DCM (1:1) containing scavengers (cf. Wang resin) leading to deprotected peptides.

Recommended Standard Procedure:
Cleavage with 1% TFA/DCM
Wash the resin thoroughly with DCM (5 to 10 times)
Treat with 1% TFA/DCM (10 ml/g resin) for 2 minutes, filter off
Neutralize filtrate immediately with at least 2 eq. pyridine (in 1ml MeOH/g resin)
Check filtrate for peptide, e.g., by TLC
Repeat steps 2 (treatment 2 to 5 minutes), 3, and 4, as long as peptide is split off the resin, usually 3 to 4 times
Pool peptide-containing fractions
Remove at least one half of the DCM
Further work-up depends on the solubility of the fragment; pyridinium trifluoroacetate has to be removed completely by all means[89]

5.2.2. Work-up

Utmost care has to be taken to remove pyridinium trifluoroacetate and remainders of trifluoroacetic acid completely before using the fragment for subsequent coupling reactions. Work-up procedures vary depending on the solubility of the fragment. At first a large part of the DCM (> 50%) should be removed. When insoluble, the fragment is filtered off. When soluble, it is precipitated by adding tert-butyl methyl ether (after checking its solubility in the latter solvent). Redissolution in a water-miscible solvent (e.g. DMF, DMA), and precipitation with water or, if the fragment is soluble in water-immiscible solvents (e.g. EtOAc, DCM, TCM), aqueous extraction will remove the hazardous contaminants mentioned above. The purity of the fragment has to be checked by TLC and HPLC, and its structure has to be confirmed by MS, amino acid analysis, NMR, and other methods, as the formation of by-products during SPPS can never be ruled out completely. As solid phase fragment condensation has gained much interest, further methods to characterize and purify fully protected peptide fragments have been developed accordingly [1,2a,82].

5.3. Cleavage with HFIP/DCM

Fully protected peptide fragments have been obtained from 2-chlorotrityl chloride resin by cleavage with HFIP/DCM (1:4 or 3:7) [19]. These mixtures are excellent solvents for such compounds [79a] and, due to the absence of other carboxylic acids, work-up is reduced to evaporation of the solvents and, when sufficiently pure, the fragments obtained can be directly activated and coupled. This straightforward method has been applied to SASRIN with excellent results, as fully protected peptide fragments could be obtained in good yields by treatment with HFIP/DCM (1:4), for 3 to 5 hours (or with HFIP/DCM (3:7), for 1 hour). Tyr(tBu) and Lys(Boc) were left intact, whereas His(Trt) is partially cleaved (this lability has been noted even with the more labile chlorotrityl resin [19]). The peptide resin which should be free from polar impurities and solvents will turn violet during the cleavage. The side-chain alkylation of Cys, Met, and, especially, Trp as known from the cleavage with TFA/DCM may occur as well. The influence of scavengers has not been studied yet, whereas a slight influence of the nature of the C-terminal amino acid on rate and yield was observed (see also Table 1) [83]. A range of examples is compiled in Table 3a/b. This simple procedure is particularly convenient when cleaving large batches.

The dry peptide resin is subjected to cleavage (as the DCM content of the swollen resin can only be roughly estimated) with HFIP/DCM. It has to be freed from remains of polar non-volatile solvents (DMF, NMP,...) beforehand, e.g. by thorough washing with IPA followed by ether or methanol, or by washes with DCM, and drying *in vacuo*. Dry DCM (16ml/g resin) is added and the resin is left to swell. The cleavage is started by adding HFIP (4ml/g resin). After being shaken or stirred for ca. 15 minutes the resin will turn pink (except for Trp, Met, Cys being present).
This color will intensify subsequently, whereas in the presence of Trt or Mtt groups a yellow color (or even none) is observed. Hence, color formation is not a prerequisite of cleavage. After being agitated for 3 to 5 hours, the resin is filtered off and washed with HFIP/DCM and DCM, it should be kept as it may be necessary to repeat the cleavage. The filtrate is evaporated *in vacuo*, the residue is taken up in a suitable volatile solvent and reevaporated several times. Further work-up depends on the properties of the fragment though the crude residue obtained as above can be used for fragment couplings or other modifications. Cleavage with HFIP/DCM (3:7) is performed analogously, though it proceeds more rapidly. Prolonged treatment of peptides containing Tyr(tBu) or, in particular, Lys(Boc) should be avoided.
In order to avoid the use of halocarbons, other acids such as p-toluenesulfonic acid and solvents, e.g., toluene, have been evaluated as cleavage reagents, but none of the systems tested so far could compete with the yields and purities obtained by 1% TFA/DCM or HFIP/DCM treatment.

Recommended Standard Procedure:

Cleavage with HFIP/DCM (1:4)

Wash thoroughly with IPA (or DCM) to remove remains of coupling solvents, then with ether or methanol
Dry in vacuo
Add dry DCM (16ml/g resin) and allow to swell for 30 minutes Add HFIP (4ml/g resin) and agitate for 3 to 5 hours Filter off resin and wash with HFIP/DCM (1:4) and DCM
Remove solvent in vacuo, do not heat As to remove traces of HFIP, redissolve in a suitable volatile solvent (e.g. DCM) and evaporate in vacuo

Recommended Standard Peocedure

Cleavage with HFIP/DCM (3:7)

as above, except for:
Add dry DCM (14ml/g resin) and allow to swell
Add HFIP (6ml/g resin) and agitate for not more than 1 hour

Table 3. Cleavage with HFIP/DCM (1:4).

a) Fully protected peptide fragments

Fmoc-peptide-OH	Cleavage (hrs)	Yield[b] (%)
Gly-Tyr(tBu)-Lys(Boc)-Ile-Gly[a]	1	62
do.	2	74
do.	3	80
do.	4	82
do.	5	84
do.	1[c]	78
Ala-His(Trt)-Gly-Tyr(tBu)-Lys(Boc)-Ile-Gly	1.5[c]	80[e]
Ala-Asp(OtBu)	3	83
Boc-Cys(Acm)-Asn(Mtt)-Ser(tBu)-Phe-Arg(Pmc)-Tyr(tBu)	2	58[d]
(ANF(23-28))	3	75[d]
Boc-Cys(Acm)-Lys(Boc)-Val-Leu-Arg(Pmc)-Arg(Pmc)-His(Trt)	2	48[d,e]
(BNP(26-32))	3	65[d,e]

b) Fmoc Amino Acids

Fmoc-AA-OH[f]	Yield (%)[b]
Ala	85
Phe	80
Leu	66
Met	68
Trp	20
Trp(Boc)	27g

[a] A model peptide for studying the behavior of Lys(Boc) and Tyr(tBu).
The cleavage products containing deprotected Lys and/or Tyr can easily be detected by TLC, at most, traces were found. Lability: Lys(Boc) > Tyr(tBu), HFIP/DCM 3:7 > 1:4.
[b] Fmoc determination before and after treatment with HFIP/DCM.
[c] HFIP/DCM (3:7)
[d] Determined by weight loss ≈ amount of isolated material
[e] Partial cleavage of His(Trt).
[f] Duration of cleavage: 3 hours.
[g] Better yields were obtained with non-C-terminal Trp(Boc).

6. Cleavage with Nucleophiles

6.1. General Remarks

i. Usually, cleavage rates and yields are distinctly influenced by steric hindrance, i.e. by the type of α-substituent of the C-terminal amino acid as well as by the bulk of the attacking nucleophile. The ease of cleavage with any nucleophile decreases approximately in the order:

Gly >	Asp(OtBu) ≥	Ser(tBu) ≥	Phe >	Leu >	Pro >	Val >	Thr(tBu) >	Ile
	Met	Glu(OtBu)	Tyr(tBu)					
	Arg(Pmc)	Lys(Boc)	Trp					
	Cys(Acm)	Ala						

ii. Base-catalyzed C-terminal racemization may become a problem if the cleavage is sluggish. The amount of undesired epimer formed further depends on the nature of the C-terminus, e.g. Phe is especially prone to racemization.
iii. If treated with nucleophiles, Asp(OtBu)-containing peptides are susceptible to side reactions, especially Asp(OtBu)-Gly and similar sequences (then the repetitive piperidine treatment may also generate by-products, see, e.g. [41a]). Glu(OtBu) tends to be less sensitive. Side reactions involving Asn are suppressed by employing side chain protection (Trt, Mtt).
iv. Good swelling facilitates the attack of the nucleophile. Thus, if a solvent has to be used, its ability to swell the resin should be tested. If the solution of the nucleophile dissolves the fully protected fragment (obtained by cleaving a sample with 1% TFA/DCM), the desired product will probably be dissolved as well.
v. The cleavage time has to be optimized individually (only average values can be taken from the following chapters), and it always should be kept as short as possible. Repetitive short treatments may be more effective than a single prolonged cleavage. The resin should not be discarded before a satisfactory yield has been obtained. Sonification or increase of temperature can accelerate the cleavage.
vi. An unambiguous correlation between the length of the fragment and cleavage yield has not been noticed. A decreasing cleavage rate has been observed in some cases with increasing length (up to 10 amino acids).
vii. The N-terminus need not be protected. Fmoc is removed under most of the conditions applied (for exceptions see chapter 6.5.) Fig. 7 summarizes the options of this approach.

6.2. Hydrazinolysis

Azide coupling is one of the oldest methods in peptide synthesis but it has retained its popularity because of the minimal concomitant racemization (in most cases). Peptide azides are usually generated from the corresponding peptide hydrazides and coupled at low temperatures [84].
Surprisingly little use has been made of the option that fully Bzl-type protected peptide hydrazides can be cleaved from Merrifield resin with hydrazine (hydrate) in a polar solvent [85]. Equally well fully tert-butyl protected peptide hydrazides are obtained from SASRIN by treatment with 10-20% hydrazine hydrate in DMA [86]. Only peptides with β-branched amino acids at the C-terminus require harsher conditions such as anhydrous hydrazine in DMA:
C-terminal Gly, Lys(Boc), Phe and others - 10% hydrazine hydrate, 4-6 hours
C-terminal Leu, Pro - 10-20% hydrazine hydrate, 24 hours
C-terminal Val, Thr(tBu), Ile - 20% hydrazine hydrate, 48 hours (or hydrazine)
The dry peptide resin is merely suspended in DMA (18ml/g resin) and left to swell. Then hydrazine hydrate is added (2ml/g resin) to obtain a 10% solution (else 16 ml DMA and 4 ml hydrazine hydrate/g resin).
The suspension is agitated for 4 to 24 hours, then the resin is filtered off and washed with DMA. The filtrate is evaporated *in vacuo* (no heating!) to dryness. Further work-up, e.g. via precipitation, depends on the solubility of the hydrazide. The remaining resin is washed and dried. DMA can be replaced by NMP or N,N'-dimethylpropylene urea. Better yields have been obtained using these solvents but they slowly react with

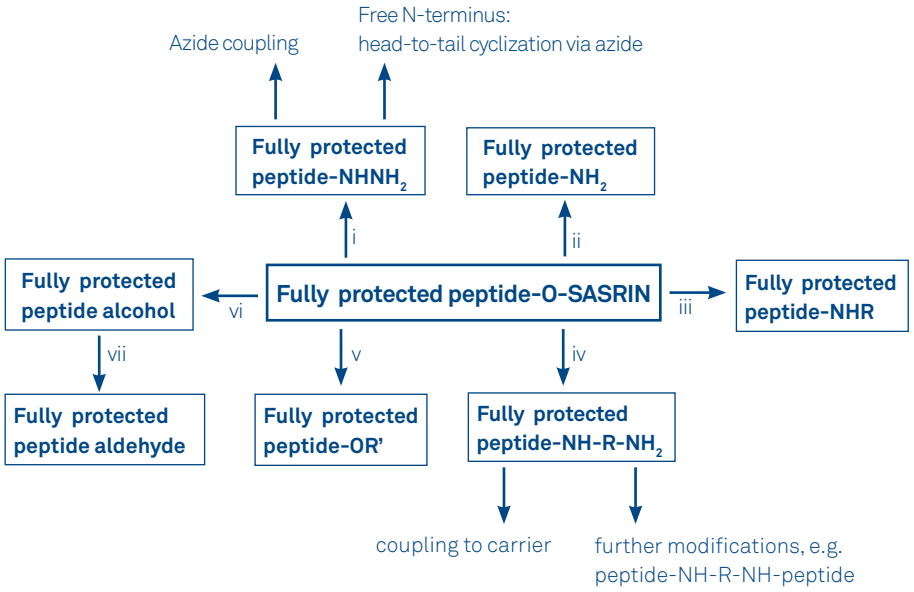


Fig. 7.
Fully protected peptide derivatives obtained from SASRIN via cleavage with nucleophiles).

i) hydrazine hydrate/DMA (1:9) or (1:4)
ii) NH_3, NH_4Cl (or LiCl), under pressure
iii) RNH_2 (neat if possible)
iv) $NH_2R'NH_2$ (neat if possible)
v) R"OH, DMA, KCN (or LiBr/DBU (5:2) (molar ratio))
vi)$NaBH_4$, LiCl, THF (or THF/EtOH)
vii) pyridine · SO_3, Et_3N, DMSO

hydrazine hydrate.
Baur and Pennington obtained an N-terminally labeled peptide hydrazide by this method, transformed it into the azide, which was coupled to a fluorescent label [87].
As Fmoc is concomitantly cleaved, hydrazinolysis yields an excellent precursor for head-to-tail cyclization via azide [85b,88a] (for synthesis of peptide C-terminal derivatives from protected hyrazides cf. [88b]).

Recommended Standard Procedure:
Hydrazinolysis
Swell peptide resin in DMA, 18 ml/g resin
Add hydrazine hydrate, 2 ml/g resin
Reaction time: 4 to 24 hours
Filter off resin and wash with DMA
Remove hydrazine hydrate and (part of the) DMA rapidly in vacuo
Triturate residue with water (or add water to precipitate the peptide hydrazide)
Further work-up depends on the solubility of the product

6.3. Ammonolysis and Aminolysis

6.3.1. Gaseous Amines: NH_3, $MeNH_2$, $EtNH_2$

Better results, i.e. purer products, have been obtained by cleaving with neat amines under pressure rather than by employing solutions (such as methanolic ammonia, 30% aqueous methylamine, or 70% aqueous ethylamine), the resulting products being contaminated by the corresponding methyl ester, or the free acid [89]. The three gases are readily condensed, pressures at RT are comparably low (see Table 5):
LHRH analogs have already been obtained in good yield by cleaving the peptide resin (Merrifield) with condensed ethylamine [90]. Treatment of a SASRIN-bound (des-Gly^{10})-LHRH analog with ethylamine at r.t. under a slight overpressure resulted in a better yield than cleavage with liquid ethylamine at 0°C. Ammonolysis [91] is known to be catalyzed by ammonium salts, but by adding solid ammonium chloride yields were only moderately increased, the addition of lithium chloride led to about the same increase [92].
A simple steel autoclave equipped with a manometer will suffice for ammonolysis.

Table 5. Boiling points and pressure at room temperature of ammonia and gaseous alkylamines.

	ammonia	methylamine	ethylamine
bp (°C)	-33	-6	+16.6
pressure at RT (bar)	1 - 15	5	<2

Equal amounts of dry peptide resin and dry ammonium chloride (or lithium chloride) are weighed into the tube, then ammonia is condensed (10ml/g resin). After tightly closing the lid, the mixture is agitated for 24 hours (C-terminal Gly) or up to 96 hours (C-terminal Leu). After evaporating the ammonia the resin is extracted with a suitable solvent. The crude peptide amide has to be freed from adherent ammonium chloride (or lithium chloride).
Better yields have been obtained with condensed methylamine or ethylamine. Thus, the LHRH analog mentioned above has been obtained in good yield despite the C-terminal Pro. This method lends itself especially to the synthesis of Trp-containing peptide (N-alkyl)-amides, whereas it should not be applied for the derivatization of Asp(OtBu)-containing peptides. An alternative method for obtaining peptide N-alkyl-amides employing SASRIN is described in chapter 7.1.

Recommended Standard Procedure:
Ammonolysis
Weigh equal amounts of peptide resin and ammonium chloride (or lithium chloride) into the tube of a steel autoclave
Condense ammonia in a separate vessel and add it rapidly (10ml/g resin)
Close the autoclave tightly and agitate for 24 to 96 hours at room temperature
Evaporate the ammonia and extract the resin with DMF or another suitable solvent
Further work-up which has to include the removal of the salt depends on the solubility of the peptide amide

6.3.2. Liquid and Solid Amines

Only slight differences in the bulk of the amine decrease the cleavage rate markedly. Thus, the best yields have been obtained with undiluted linear aliphatic amines and diamines [89].
When adding a solvent, only exceptional swelling will compensate for the dilution of the amine and the concomitant decrease of the reaction rate (catalysis by the corresponding ammonium salts has not been evaluated). Solid amines such as hexamethylenediamine have to be applied as saturated solutions in a solvent with optimal swelling capacity.
Various amines R-NH_2 have been employed neat except those marked * [89]; in the order of reactivity:

R = propyl ≈butyl > octyl > allyl
R = benzyl > phenethyl > cyclohexyl >>> piperidine
R = ω-aminoalkyl* [93] >> ω-hydroxyalkyl ≈ ω-carboxyalkyl*

Sluggish aminolyses can be accompanied by substantial racemization, especially at C-terminal Phe. Fortunately, unwanted peptide cleavage with piperidine in DMF is rather slow, even with the base-labile Fmoc-Asp(OtBu)-Gly terminus [94]. The total contact time with 20% piperidine/DMF is short enough for safe synthesis of medium-sized peptides.
Fully protected peptide ω-aminoalkyl amides [93] and similar derivatives can be further derivatized or coupled to insoluble carriers.
The peptide resin is simply suspended in the dry amine or in a saturated solution of the solid amine in a dry solvent (this solution should properly swell the resin); in both cases 10 to 20 ml/g resin. Agitation is continued for 24 hours. Moisture and oxygen have to be excluded.
The cleavage of unhindered C-termini with linear amines or diamines may take less time. In any case, the resin is filtered off and washed with a little amine and a suitable solvent. Excess amine has to be removed rapidly *in vacuo* (if possible). Further work-up (e.g. precipitation, chromatography) depends on the solubility of the product.
The resin should be washed (to remove the excess amine) and dried for storage.

6.4. Transesterification

Fully protected peptide esters can be obtained by base-catalyzed transesterification [95].
Reasonable yields have been obtained only with primary alcohols (e.g. methanol, benzyl alcohol).
Low cleavage rates due to steric hindrance can be dramatically increased by adding a cosolvent properly swelling the resin, e.g. DMA. Even transesterifications with a solid alcohol are feasible in such a solvent. The best results were obtained employing either KCN [96] or LiBr/DBU [97] as a basic catalyst. Moisture has to be excluded rigorously, as saponification will also be promoted.
tert-Butyl esters (especially Asp(OtBu)) may also be transesterified, but much slower, under these conditions. Unfortunately, racemization is also enhanced by adding a solvent, the amount of undesired epimer formed depends on the C-terminus (Phe >>Ala > Val > Leu > Pro) and the reaction rate. Thus, a compromise, e.g. short repetitive treatments, between yield and racemization has to be worked out.

6.4.1 KCN as Catalyst

The peptide resin and the solvents employed have to be dried carefully before use, all components have to withstand prolonged KCN-treatment. Transesterification will occur, even if the solubility of KCN is low, residual salt did not interfere (crown ethers were never added [96b,98]). The peptide resin is suspended in a mixture of the desired alcohol and the cosolvent (usually 1:1, (10 to 20 ml/g resin), methanol and benzyl alcohol may be employed without cosolvent in case of unhindered C-termini). After 30 minutes, sufficient solid KCN is added, so that a 0.08 M solution is obtained (or, at least, saturation as only methanol and methanol/DMA readily dissolve the salt). After stirring for 24 hours, the resin (which may contain solid KCN!) is filtered off and washed with the cosolvent. The catalyst must be destroyed immediately, e.g. by vigorously shaking the filtrate with sufficient solid anhydrous $FeCl_2$. Iron blue will flock out, it is left to settle for approx. 30 minutes and filtered off. The filtrate may remain greenish. Further work-up depends on the solubility of the product. As the catalyst is removed, the ester may (and should!) be treated with water, e.g. after removing alcohol and DMA *in vacuo*, the residue is taken up in EtOAc or TCM for subsequent aqueous extraction (to remove salts).

Recommended Standard Procedure:
Transesterification with KCN
Swell the dry resin in alcohol/DMA (1:1, both carefully dried), 10-20ml/g resin
Add sufficient solid KCN to obtain a 0.08M solution (or at least, saturation)
Stir for 24 hours, filter off, and wash with dry DMA
Add an excess of solid anhydrous $FeCl_2$ to the filtrate, shake vigorously
The precipitate is left to settle and filtered off
Further work-up depends on the solubility of the product

6.4.2. LiBr/DBU as Catalyst

LiBr or DBU alone do not catalyze transesterification, but the mixture (5M LiBr : 2M DBU represents the optimal molar composition) exhibits a far better catalytic activity than KCN [97].
Moisture has to be excluded even more rigorously, a stock solution of LiBr in dry DMA (approx. 0.5M) may be employed (LiBr is rather hygroscopic). Again, a mixture of DMA stock solution and the desired alcohol (1:1, 15 to 20 ml/g resin) is used and the resin is left to swell for 30
minutes. Only when DBU (approx. 0.1mol/l mixture) is added the reaction will start.
The reaction mixture is stirred for 4 hours. Work-up again depends on the solubility of the peptide ester.
The catalyst has to be quenched instantaneously with acid, it also promotes saponification upon contact with water. The resin is filtered off, washed with small portions of dry DMA and then copiously with dry EtOAc (DCM, TCM ... depending on the solubility of the fragment).
The filtrate is further diluted if necessary and extracted with 0.5 to 1N aqueous HCl to remove the catalysts.

Recommended Standard Procedure:
Transesterification with LiBr/DBU (5:2)
Special care has to be taken to exclude moisture rigorously!

Dissolve LiBr in DMA (0.5mol/l)
Add an equal volume of the desired alcohol
Add the peptide resin (10-15ml DMA/alcohol(1:1)/g resin) and leave it to swell for 30 minutes
Add DBU (0.1mol/l), which starts the reaction
Stir for 4 hours
The catalyst is quenched by adding acid
Filter off the resin, wash with a minimum of DMA
Further work-up depends on the solubility of the peptide. If possible, it should be taken up in a water-immiscible solvent

6.5. Reductive Cleavage

6.5.1. Peptide Alcohols

Like any «ordinary» ester, peptide-resin esters can be reduced and concomitantly cleaved to the corresponding fully protected peptide alcohols with complex hydrides such as lithium borohydride in THF [99]. Fewer by-products are formed when this reducing agent is generated *in situ* from $NaBH_4$ (cheaper and more convenient to handle) and LiBr in THF [100,101]. Peptides prone to side reactions should be cleaved reductively by employing only a slight molar excess of $NaBH_4$ and LiCl (1.2 eq each) and adding a small amount of ethanol to the solvent [100].
The temperature may be lowered to 0°C. The yield does not depend on the C-terminus, but it can decrease with increasing peptide length (in such cases the reduction should be repeated). Very mild conditions have to be chosen when cleaving peptide alcohols containing Pro or N-alkylamino acids. Especially the amide bond between Gly and Pro is prone to reductive cleavage [102]. Any Gly-Xaa bond [103] and urethane-type protecting groups (especially Z [104]) may be attacked by $LiBH_4$ under harsher conditions. When applying THF alone, Fmoc is rather stable, but it is cleaved in THF/EtOH. Asp(OtBu) may be partially reduced to homoserine, whereas Glu(OtBu) is more resistant to reduction.
$NaBH_4$ (1.5 to 2 molar eq) is suspended in dry, peroxide-free THF (16ml/g resin) under argon.
After cooling to approx. 0°C, dry ethanol (4ml/g resin) is added (if desired) yielding a clear solution. After adding the equivalent amount of LiBr (or LiCl), the suspension is stirred vigorously for 15 minutes. Then the dry resin is added. The suspension is stirred for 24 hours at RT (sensitive peptides: 0°C), then the resin is filtered off and washed thoroughly with THF and EtOH, final washes are performed with EtOAc. A small amount of acetic acid – just enough to decompose excessive borohydride – is added, thus a neutral mixture should result. The mixture is evaporated *in vacuo* (no heating), but not to complete dryness. The residue is taken up in equal amounts of water and EtOAc (or another water-immiscible solvent dissolving the desired product). The organic phase is washed with water to remove the salts, and eventually with brine followed by evaporation. Otherwise, further work-up depends on the solubility of the peptide alcohol. The resin must be carefully dried, if the reductive cleavage has to be repeated.

6.5.2. Peptide Aldehydes

Our attempts to stop the reduction at the aldehyde stage (employing DIBAH in toluene at –70°C [105]) failed, only small amounts of mixtures containing the desired aldehyde were obtained. Otherwise, the fully protected peptide alcohols which are rapidly available as described above can be oxidized to yield aldehydes, e.g. with pyridine·SO_3 complex, DMSO and triethylamine (Parikh-Doering method) [106].
To meet the increasing demand for peptide aldehydes, especially those containing aspartic aldehyde such as the ICE-inhibitor Ac-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO, product number 4018830), an alternative method to obtain Asp-containing peptide alcohols had to be developed, since reductive cleavage of Asp(OtBu) containing peptides from SASRIN (as described in chapter 6.5.1.) will yield rather impure products. Additionally, we have developed a more generally applicable method to obtain fully protected peptide alcohols: Fmoc amino alcohols are etherified by reaction with diphenyldiazomethane resin [107,108] followed by standard Fmoc/tBu-SPPS. Cleavage with 1 to 2% TFA/DCM yields the protected peptide alcohol which may contain Asp(OtBu) or a C-terminal Pro

[109] in high purity to be directly oxidized as mentioned above. Diphenyldiazomethane resin is available from Bachem (4027172). Nα-protected amino aldehydes have been obtained by reducing the corresponding methyl esters with DIBAH [106b, 110], so peptide aldehydes may also be obtained when reducing a peptide methyl ester (obtained by transesterification, see chapter 6.4.) in a homogeneous system.

Recommended Standard Procedure:
Reductive Cleavage
Suspend 1.5 eq of $NaBH_4$ in peroxide-free THF, 16 ml/g resin, under argon
Cool the suspension to 0°C and add dry ethanol, 4 ml/g resin
Add 1.5 eq of LiBr (or LiCl)
Stir vigorously for 15 min
Add the resin and stir for 24 hours at RT
Filter off and wash thoroughly with THF, EtOH, EtOAc
Adjust pH of filtrate to about pH7 with AcOH (decomposition of remaining borohydride)
Remove solvents and take up in water/ water-immiscible solvent,if soluble
Wash organic phase with water and brine, remove solvent in vacuo
Further work-up depends on the solubility of the product

7. SASRIN Derivatives

7.1. «SASRIN Chloride» and Nucleophilic Substitution Thereof

As described in chapter 2, «SASRIN chloride» (cf. Fig. 2) was originally conceived for anchoring Fmoc amino acids by nucleophilic substitution in order to suppress concomitant racemization [24,28]. Due to the increasing demand for polymeric carriers suitable for solid phase organic synthesis the halides of Wang resin and SASRIN have regained considerable interest, as evidenced by the number of papers dealing with the synthesis and use of these derivatives [25,26a,27,111].
The resins obtained by reacting SASRIN chloride with nucleophiles may also find applications in peptide synthesis as can be seen in Fig. 6. It should be emphasized that the acid-lability of these derivatives can differ considerably from that of SASRIN esters.

S-Alkylation predominates when treating SASRIN chloride with cysteamine hydrochloride and 2eq of base, i.e., the amino function need not be protected, but the resulting resin should be converted to the Fmoc derivative for increased shelf-stability. SPPS employing this derivative yields deprotected peptide N-(2-thioethyl) amides [112] which can be linked to carriers or proteins, e.g. via Michael addition to carriers functionalized with 2-maleinimidopropionic acid [113]. The analogous Cys derivative may serve the same purpose, but the Fmoc-Cys-OH linked to SASRIN may be activated and treated with amines, alcohols etc. to yield C-terminally modified peptides after SPPS and cleavage from the resin (cf. side-chain linked Asp and Glu, and [114a] for S-linked cysteine). Cleavage effected by oxidation (to the sulfone) and β-elimination yields a dehydroalanine-containing product [114b]. Both derivatives are available from Bachem: Fmoc-cysteamine-SASRIN (product number 4026901); Fmoc-Cys(SASRIN)-OH (4026902).
The SASRIN-N-alkylamines have also been obtained by reductive amination of polymer bound 2-methoxy-4-alkoxybenzaldehyde [115,116]. When generating the resin by nucleophilic substitution [117], a large excess of amine is needed to minimize bis-alkylation (which would probably cause - except for some crosslinking - no additional problems during Fmoc/tBu-SPPS).
The SASRIN-N-ethylamine resin allows the rapid synthesis of (des-Gly^{10}-$ProNHEt^{9}$)-LHRH analogs such as leuprolide (4033014) and histrelin (4035442). The C-terminal Pro does not lead to additional synthetic problems, since the diketopiperazine cannot be formed, at least not under standard SPPS conditions. Diketopiperazine formation leads to great losses of load and has to be prevented by coupling an Fmoc dipeptide instead of the penultimate amino acid when obtaining these analogs by SPPS on SASRIN followed by cleavage with ethylamine.
Furthermore, Asp-containing peptide N-alkylamides which cannot be obtained by aminolytic cleavage are readily available by synthesis on SASRIN-N-alkylamine. The N-alkyl group markedly increases the acid-lability of the SASRIN amines and amides, as shown by the cleavage behavior of peptides

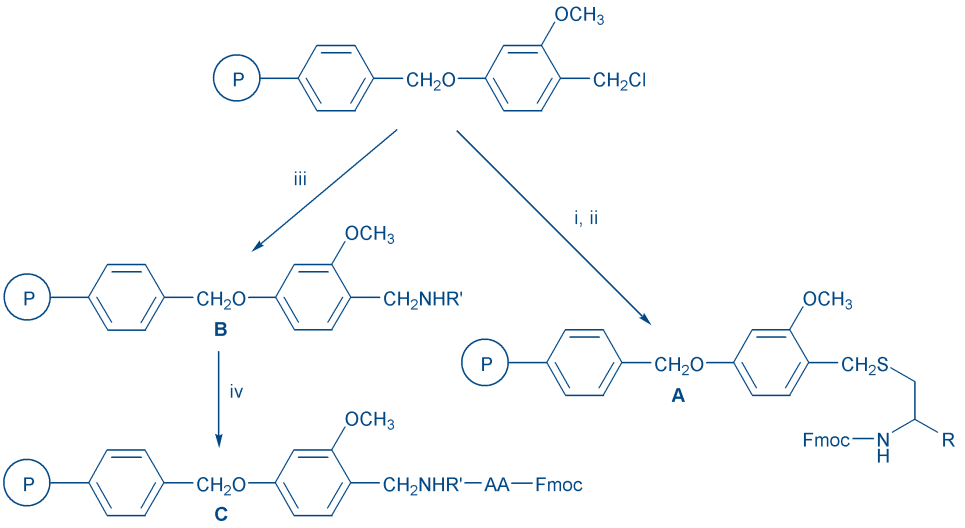


Fig. 8.
Treatment of SASRIN chloride with nucleophiles.

Acid lability:
SASRIN e.g. 1% TFA/DCM.
A considerably decreased, e.g. 95%aq TFA, 5% EDT.
B moderately decreased, e.g. 5% TFA/DCM.
C markedly decreased, e.g. 30% TFA/DCM [115,116].
i) NH_2CHRCH_2SH, DIPEA, DMF (R=H,COOH); ii) Fmoc-Cl, DIPEA; iii) $R'NH_2$/DMF 1:2 (R'=e.g. n-alkyl); iv) Fmoc-AA-OH, TATU, collidine.

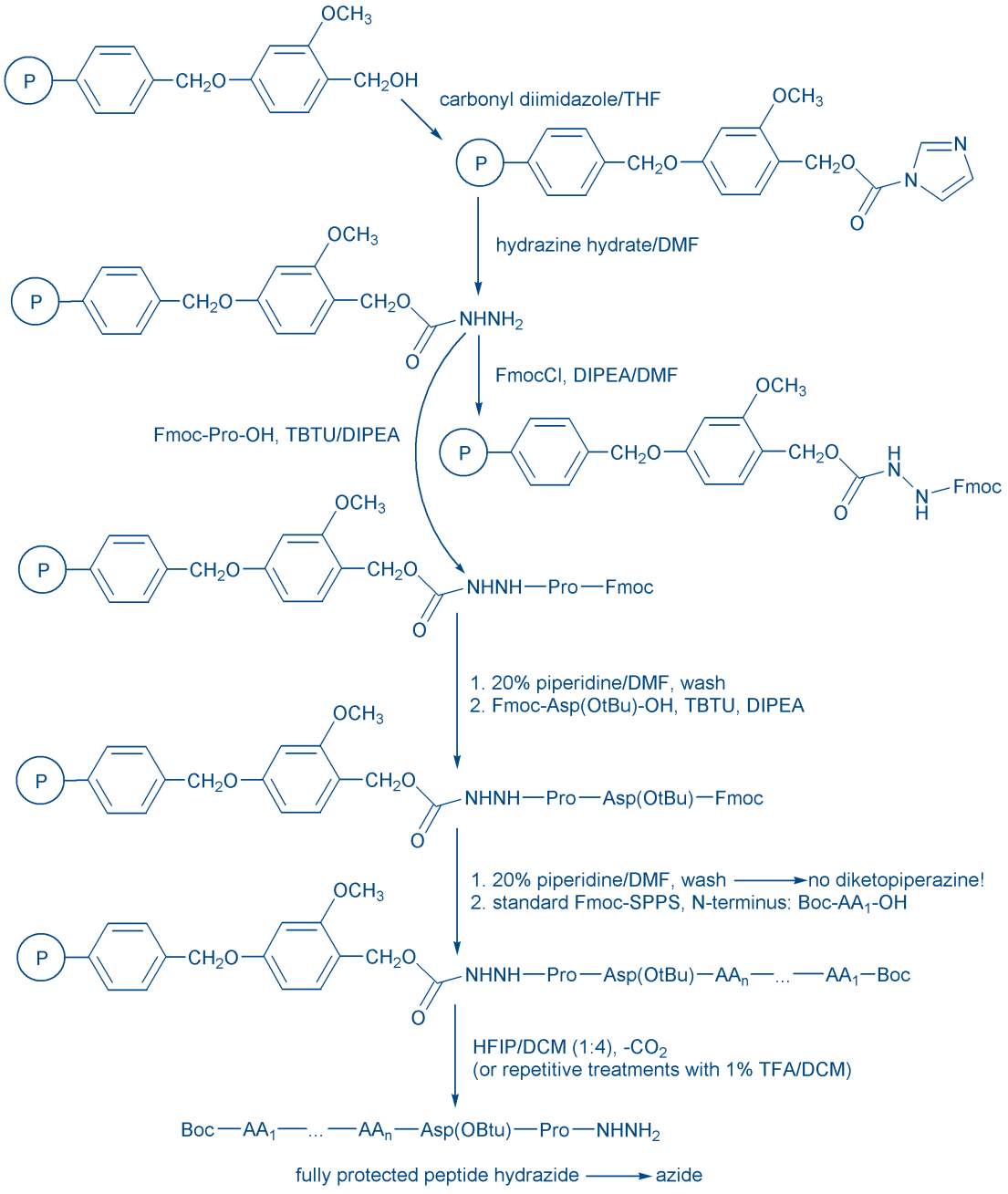
synthesized on N-unsubstituted SASRIN-amine: SASRIN-amine was obtained by mesylation of SASRIN followed by nucleophilic exchange with Na azide, treatment with triphenylphosphine, and hydrolysis of the iminophosphorane formed. Additionally, the synthesis of SASRIN-amine via reduction of the aldoxime was described. The resin turned out to be an excellent carrier for the traceless solid-phase synthesis of 1,2,4-triazoles [118].
Cleavage from this resin to yield peptide amides required rather harsh conditions such as treatment with TFA and thioanisol at elevated temperatures [119]. Thus, a third alkoxy function is needed to decrease acid-lability sufficiently to cleave peptide amides from the resin under more appropriate conditions [120]. It should be reemphasized that SASRIN-N-alkyl and N-arylamines tend to be rather acid-labile, even 5% TFA/DCM causes notable cleavage. Hence, SASRIN chloride may also find use for anchoring amines and modifying their substituents, cf.[121]. Moreover, SASRIN-N-alkyl-sulfonamides are considerably more acid-labile than the corresponding carboxamides, 5% TFA/DCM is sufficiently strong to promote cleavage [116].
Fig. 8 summarizes the conversions of SASRIN chloride described above.

The alkoxyamine derivative of SASRIN has been synthesized by Gordeev et al. [122] (see also [123]). N-Hydroxyphthalimide was linked to SASRIN via Mitsunobu coupling, the phthaloyl group was removed with methylhydrazine in THF. Protected peptide hydroxamic acids can be obtained by standard Fmoc/tBu-SPPS followed by cleavage with 1% TFA/DCM in the presence of triisopropylsilane. The resin may also be used as a carrier for carbonyl compounds, the acidolytic cleavage under anhydrous conditions may yield the oxime. Harsher cleavage conditions (20% TFA/DCM) are required, when hydroxylamine derivatives are linked to SASRIN via nitrogen [124].
SASRIN ethers should be about as acid-labile as SASRIN esters. The synthesis and application of SASRIN ethers, e.g. side-chain linkages of Ser and Tyr, have not been tackled yet, whereas ethers of Wang [125] and Merrifield [126] resins have found applications in solid phase organic chemistry.

7.2. SASRIN Carbonylimidazolide and SASRIN Carbazate

Hydroxymethylated polystyrene is smoothly converted into the chloroformate by treatment with a large excess of phosgene in toluene [127,128]. This highly reactive resin derivative will acylate amines, alcohols, and

Fig. 9.
Synthesis and use of SASRIN carbazate.



other nucleophiles yielding a vast range of useful resin derivatives.
Chloroformate formation failed with Wang resin [129], thus, SASRIN was not subjected to phosgene treatment. Nevertheless, reactive carbonate derivatives of Wang resin could be obtained by treatment with carbonylimidazole [129] or N,N'-disuccinimidylcarbonate [130] (both in THF) or related compounds.
By analogy, SASRIN could be converted using a large excess of carbonyldiimidazole in THF into the carbonylimidazolide (N,N'-disuccinimidylcarbonate reacted as well). Treatment with amines yields highly acid-labile carbamates.
SASRIN carbonylhydrazide (or carbazate) [131] will result when treating the carbonylimidazolide with an excess of hydrazine hydrate in DMF. Fmoc/tBu-SPPS followed by cleavage with 1% TFA/DCM or HFIP/DCM yields fully protected peptide hydrazides. Peptide hydrazides containing Asp(OtBu) or a C-terminal Pro are readily available by this

method. In addition, synthesis of hydrazides which could be obtained only in low yield by hydrazinolysis from SASRIN (cf. chapter 6.2.) becomes feasible. Peptide hydrazides containing base-labile moieties, e.g. an N-terminal Fmoc group, should not be cleaved from SASRIN carbazate with HFIP/DCM. For determination of the load, SASRIN carbazate is treated either with Fmoc-Cl/DIPEA or with an activated Fmoc amino acid. These reactions are monitored by color tests as described in chapter 3.2.
The resin is carefully washed and dried, its load is determined photometrically as explained in chapter 3.1. Fig. 9 summarizes the conversions of SASRIN described in this chapter.

7.3. SASRIN p-Aminophenylcarbamate and the Synthesis of Peptide p-Nitroanilides (Peptides pNAs)

SASRIN carbonyl(p-aminoanilide) [132] could not be obtained by reacting SASRIN carbonylimidazolide with a large excess of phenylenediamine, cf. [128], whereas SASRIN reacted smoothly with p-nitrophenylisocyanate; the resulting SASRIN-p-nitrophenylcarbamate could be reduced with surprising ease to the p-aminophenylcarbamate applying 2M $SnCl_2 \cdot 2\ H_2O$ in DMF [81,111e,133]. Minimal loss of the aniline moieties was observed, even though HCl is formed during the reduction. Unintended cleavage of the linker causes a decrease of the nitrogen content of the resin and thus can easily be detected by elemental analysis.
Nevertheless, the duration of the reduction should not exceed 8 hours. Thorough washing with DMF is carried out to remove unreacted $SnCl_2$ and by-products of the reduction. The «aniline resin» is acylated with Fmoc chloride and DIPEA to increase shelf stability, this reaction can be monitored by the color tests described in chapter 3.1 (development of color may take somewhat longer). Photometric determination of the load of Fmoc groups is the method of choice to assess the yield of reduction.

When coupling an Fmoc amino acid to the «aniline resin», a more reactive activating reagent such as TATU is required (coupling with TBTU proceeds rather slowly), but the following steps of the Fmoc/tBu-SPPS can be performed according to standard protocols (e.g. as described in chapter 3). The fully protected peptide p-aminoanilides can be cleaved from the resin either with 1% TFA/DCM (the cleavage solution should be neutralized with diluted aqueous $NaHCO_3$ to safely obtain the free base) or with HFIP/DCM. In the next step the peptide is oxidized with an excess of sodium perborate in glacial acetic acid [128,134] to yield the corresponding p-nitroanilide. The excess of oxidant has to be optimized when oxidizing sensitive substrates.
It should exceed 5 molar equivalents (3 molar equivalents would be the stoichiometric amount of perborate!). 16 mol eq [128] and 10 mol eq [135] have been recommended in the literature.
The course of oxidation is monitored by HPLC, usually complete conversion is attained after 8 to 16 hours. Work-up has to begin with the removal of unreacted perborate. The method is chosen according to the solubility of the product. Protected peptide pNAs are often soluble in water-immiscible solvents in which case they can be freed from salts by aqueous/organic extraction after thorough removal of AcOH *in vacuo*. Further purification is achieved by chromatography on silica gel, whereas unprotected peptide pNAs are desalted and purified by preparative RP-HPLC if sufficiently soluble in water-containing eluents needed for this method (unfortunately, purification is quite often impeded by low solubility). Thus, as to underline one of the advantages of obtaining peptide pNAs employing SASRIN, the products may be purified before or after deprotection, whichever method is more efficient. The application of SASRIN for the synthesis of peptide pNAs is illustrated in Fig. 10.
Peptide pNAs bearing acid-labile groups such as Boc-Ala-Ala-Pro-Ala-pNA (4015678) (an elastase substrate) or the subtilisin substrate Boc-Gly-Gly-Leu-pNA (4006106) are readily available by this method.
Moreover, undesired oxidation of sensitive moieties such as phenols and indoles may be prevented by side-chain protecting groups. Tyr(tBu) and Trp(Boc) showed increased stability towards sodium perbo-

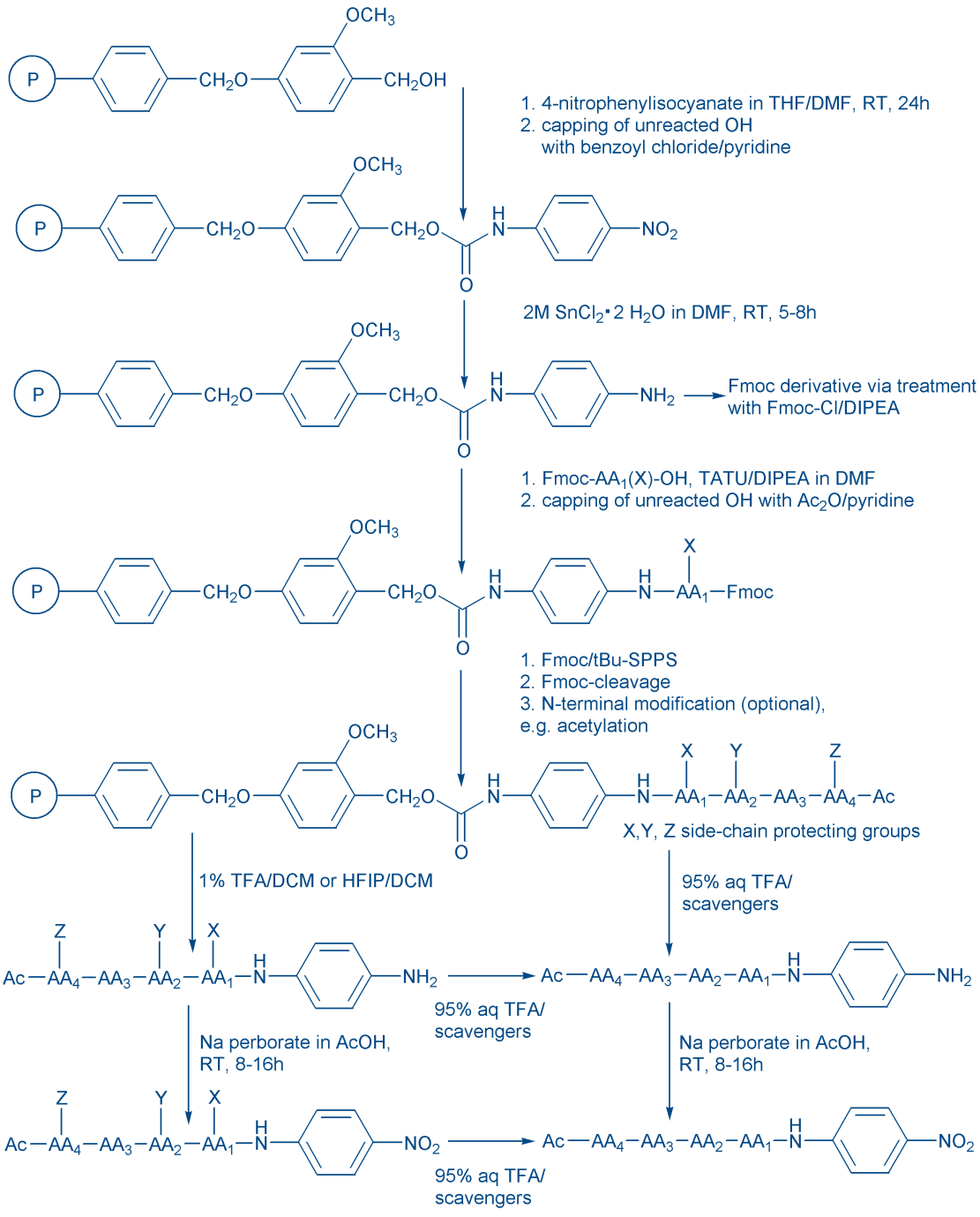


Fig. 10.
Synthesis of SASRIN carbonyl(p-aminoanilide) and its application for the synthesis of peptide p-nitroanilides.

rate (whereas Trp is rapidly decomposed by treatment with the oxidant).
Protecting groups can improve the solubility of the peptide p-aminoanilide in AcOH and thus facilitate its oxidation. Unfortunately, Met and Cys(Acm) are rapidly oxidized to the corresponding sulfones, whereas Cys(Trt) is cleaved to yield trityl alcohol. Thus, Cys and Met-containing peptide pNAs cannot be obtained by this method.

8. Application of SASRIN in Solid-phase Organic Synthesis

Due to the growing demand for suitable carriers for automated combinatorial chemistry the resins routinely used in SPPS have found a vast range of additional applications. Organic synthesis applying insoluble carriers has experienced a revival, and expectedly SASRIN was employed in the solid phase synthesis of acid-labile low molecular weight compounds.
Below you will find a choice of papers in this field:

- Synthesis of 4-aryl substituted β-lactams employing Suzuki and Heck couplings [136]
- Synthesis of β-sultams [137]
- Generation of secondary alkylamines by borane reduction [138]
- Synthesis of quinazoline-2,4-diones [122a]
- FT-IR and Raman spectroscopic monitor ing of reactions on a single SASRIN bead [139]
- Solid phase synthesis of pyridines and pyrido[2,3-d]pyrimidines [140]
- Synthesis of substituted benzoic acids by Suzuki coupling [141]
- Mass-spectrometric monitoring: evaluation of mass spectrometric methods applicable to the direct analysis of non-peptide bead-bound combinatorial libraries [142]
- Construction of a family of biphenyl combinatorial libraries (containing amino and carboxy substituted biphenyls) [143]
- Synthesis of 3-oxo-1,4-benzodiazepine derivatives via Heck reaction and regioselective alkylation at the N(4)-position [144]
- Solid-phase synthesis of 1,2,3,4-tetrahydropyridonecarboxylic acids [145]
- Synthesis of L- and D-cycloserine derivatives [124]
- Synthesis of tetrahydro-2H-1,3,5-thiadiazin-2-thione (TTT) derivatives [146]
- Solid-phase synthesis of peptidomimetics containing an epoxide moiety [147]
- Total synthesis of the quinazoline alcaloids verrucine A and B and anacine employing simultaneous cyclization and cleavage from the carrier [148]
- Synthesis of aminothiazole-based inhibitors of cyclin-dependent kinase-2 via the resin-bound heterocycle [26a]
- Generation of and 1,3-dipolar cycloaddition to SASRIN bound N-(4-carboxyphenyl)-maleinimide [81]

Apart from SASRIN derivatives, we offer a wide range of resin derivatives, educts, and reagents for use in combinatorial synthesis. For more information on our products please go to our online shop at **shop.bachem.com**

9. Final Remarks

In this review we have demonstrated the versatility of SASRIN and its derivatives. Beneath the standard applications for synthesis of peptides and peptide fragments a broad range indeed of useful peptide derivatives can be obtained, all from one single peptide synthesis using SASRIN. The scope of application could be extended by introducing new SASRIN derivatives and by employing SASRIN in solid phase organic synthesis. Further derivatives of SASRIN may be marketed in response to the needs of scientists working in the field of peptide synthesis or combinatorial chemistry.
Along with SASRIN's growing application the available range of Fmoc-L and D amino acids coupled to SASRIN (available in bulk quantities) has also steadily increased. For our complete offer, please visit us online at shop.bachem.com
If you do not find the product you need in our online shop or if you prefer another mesh size, please ask about our custom synthesis service: take advantage of our expertise and let our chemists work for you.
And, finally, if you have any questions or suggestions with regard to SASRIN, please contact us. We would like to do our best to help you.

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Proteinogenic Amino Acids

Product No.	Name
4012735	Fmoc-Ala-SASRIN™ resin (200-400 mesh)
4010296	Fmoc-D-Ala-SASRIN™ resin (200-400 mesh)
4019092	Fmoc-Arg(Pbf)-SASRIN™ resin (200-400 mesh)
4018539	Fmoc-D-Asn(Trt)-SASRIN™ resin (200-400 mesh)
4012736	Fmoc-Asp(OtBu)-SASRIN™ resin (200-400 mesh)
4013511	Fmoc-Cys(Trt)-SASRIN™ resin (200-400 mesh)
4026902	Fmoc-Cys(SASRIN™ resin)-OH (200-400 mesh)
4016126	Fmoc-Gln(Trt)-SASRIN™ resin (200-400 mesh)
4012721	Fmoc-Glu(OtBu)-SASRIN™ resin (200-400 mesh)
4017861	Fmoc-D-Glu(OtBu)-SASRIN™ resin (200-400 mesh)
4012730	Fmoc-Gly-SASRIN™ resin (200-400 mesh)
4025532	Fmoc-D-His(1-Trt)-SASRIN™ resin (200-400 mesh)
4012713	Fmoc-Leu-SASRIN™ resin (200-400 mesh)
4012718	Fmoc-Lys(Boc)-SASRIN™ resin (200-400 mesh)
4017862	Fmoc-D-Lys(Boc)-SASRIN™ resin (200-400 mesh)
4013502	Fmoc-Met-SASRIN™ resin (200-400 mesh)
4018503	Fmoc-D-Phe-SASRIN™ resin (200-400 mesh)
4013506	Fmoc-Pro-SASRIN™ resin (200-400 mesh)
4012722	Fmoc-Ser(tBu)-SASRIN™ resin (200-400 mesh)
4013509	Fmoc-Thr(tBu)-SASRIN™ resin (200-400 mesh)
4013504	Fmoc-Trp-SASRIN™ resin (200-400 mesh)
4025296	Fmoc-Trp(Boc)-SASRIN™ resin (200-400 mesh)
4013923	Fmoc-Tyr(Bzl)-SASRIN™ resin (200-400 mesh)
4012738	Fmoc-Val-SASRIN™ resin (200-400 mesh)

Unusual Amino Acids

Product No.	Name
4026901	Fmoc-cysteamine-SASRIN™ resin (200-400 mesh)
4029525	Fmoc-Dap(Dnp)-SASRIN™ resin (200-400 mesh) (Fmoc-Dpa-SASRIN™ resin)
4013926	Fmoc-Sar-SASRIN™ resin (200-400 mesh)

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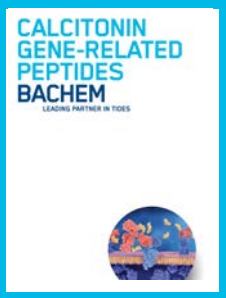
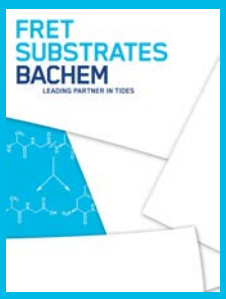
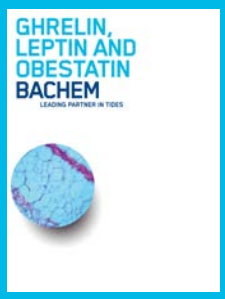
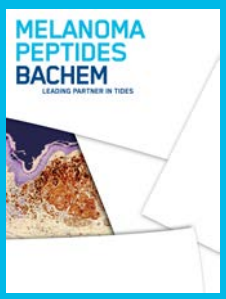
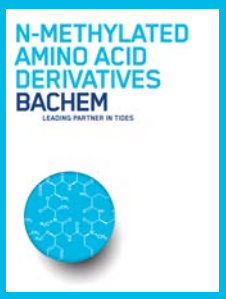
PRODUCT BROCHURES

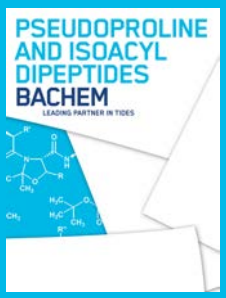












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2002628 published by Global Marketing, Bachem AG, May 2020