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Synthetic Peptides

A User's Guide

Edited by

Gregory A. Grant

Washington University School of Medicine

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For
Roger and Clarice
I hope you know.

I would like to thank Carolyn Stock; late of UWBC, and the people at W. H. Freeman, especially Ingrid Krohn and Penny Hull, who helped make the job easier and enjoyable. I would especially like to thank Ronald Niece for his efforts and support from the very beginning. Finally, I would like to thank the contributors for their excellent work; all the credit goes to them.

Gregory A. Grant

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3

Principles and Practice of Solid-Phase Peptide Synthesis

Gregg B. Fields, Zhenping Tian, and George Barany

Peptides play key structural and functional roles in biochemistry, pharmacology, and neurobiology. Naturally occurring and designed peptides are also important probes for research in enzymology, immunology, and molecular biology. The amino acid building blocks can be among the 20 genetically encoded L residues or they may be unnatural. Further, the sequences can be linear, cyclic, or branched. It follows that rapid, efficient, and reliable methodology for the chemical synthesis of these molecules is of utmost interest. A number of synthetic peptides are significant commercial or pharmaceutical products, ranging from the sweet dipeptide L-Asp-L-Phe-OMe (aspartame) to clinically used hormones such as oxytocin, adrenocorticotropic hormone, and calcitonin. Synthesis can lead to potent and selective new drugs by judicious substitutions that change functional groups, conformations, or both. These include introduction of *N*- or *C*-alkyl substituents, unnatural or D-amino acids, side-chain modifications, including sulfate groups, phosphate groups, or carbohydrate moieties, and constraints such as disulfide bridges between half-cystines or side-chain lactams between Lys and Asp or Glu (see Chapter 2). Most of the biologically or medicinally important peptides that are the targets for useful structure-function studies by chemical synthesis comprise fewer than 50 amino acid residues, but occasionally a synthetic approach can lead to important conclusions about small proteins in the 100-residue size range.

Methods for synthesizing peptides are divided conveniently into two categories: solution (classical) and solid phase (SPPS). The classical methods have evolved since the beginning of the twentieth century, and they are described amply in several reviews and books (Wünsch, 1974; Finn and Hofmann, 1976; Bodanszky and Bodanszky, 1984). The solid-phase alternative was conceived and elaborated by R.B. Merrifield beginning in 1959, and it has also been covered comprehensively (Erickson and Merrifield, 1976; Birr, 1978; Barany and Merrifield, 1979; Stewart and Young, 1984; Merrifield, 1986; Barany et al., 1987; Barany et al., 1988; Kent, 1988; Clark-Lewis and Kent, 1989; Atherton and Sheppard, 1989; Fields and Noble, 1990; Barany and Albericio, 1991b). Solution synthesis retains value in large-scale manufacturing and for specialized laboratory applications. However, the need to optimize reaction conditions, yields, and purification procedures for essentially every intermediate (each of which has unpredictable solubility and crystallization characteristics) renders classical methods time-consuming and labor-intensive. Consequently, most workers now requiring peptides for their research opt for the more accessible solid-phase approach.

In this chapter, we discuss critically the scope and limitations of the best available procedures for solid-phase synthesis of peptides. At the same time, we mention briefly important new developments and trends in this field. Literature citations are weighted toward detailed reports with full experimental descriptions, with some bias toward those describing procedures with which we and our collaborators have laboratory experience.

OVERVIEW OF SOLID-PHASE STRATEGY

The concept of SPPS (Figure 1) is to retain chemistry proved in solution (protection scheme, reagents), but adding a covalent attachment step (anchoring) that links the nascent peptide chain to an insoluble *polymeric support*. Subsequently, the anchored peptide is extended by a series of addition (deprotection/coupling) cycles, which are required to proceed with exquisitely high yields and fidelities. It is the essence of the solid-

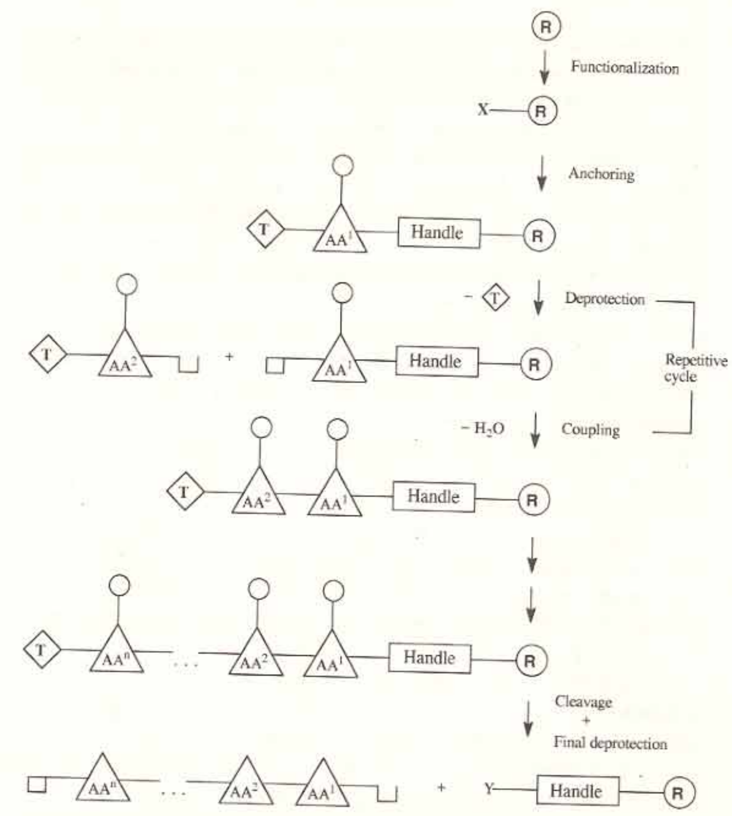


FIGURE 1 Stepwise solid-phase synthesis of linear peptides. **R**, insoluble polymeric support; AA^1, AA^2, . . . , AA^n, amino acid residues numbered starting from *C*-terminus; **T**, "temporary protection; O, "permanent" protection; ——┘, free carboxyl; ┌——, free amino group. See text for further details. Figure adapted from Barany et al., 1988.

phase approach that reactions are driven to completion by the use of *excess* soluble reagents, which can be removed by simple *filtration* and *washing* without manipulative losses. Because of the speed and simplicity of the repetitive steps, which are carried out in a single reaction vessel at ambient temperature, the major portion of the solid-phase procedure is readily amenable to *automation*. Once chain elaboration has been accomplished, it is necessary to *release* protected residues and to release (cleave) the crude peptide from the support under conditions that are minimally destructive toward sensitive residues in the sequence. Finally, there must follow prudent *purification* and appropriate *characterization* of the synthetic peptide product to verify that the desired structure is indeed the one obtained.

An appropriate polymeric support (resin) must be chosen that has adequate mechanical stability as well as desirable physicochemical properties that facilitate solid-phase synthesis (see Polymeric Support). In practice, such supports include those that exhibit significant levels of swelling in useful reaction/wash solvents. Swollen resin beads are reacted and washed batchwise with agitation and filtered either with suction or under positive nitrogen pressure. Alternatively, solid-phase synthesis may be carried out in a continuous-flow mode, by pumping reagents and solvents through resins that are packed into columns. The usual batchwise resins often lack the rigidity and strength necessary for column procedures. More appropriate supports, which are usually, but not always, lower in terms of functional capacity, are obtained when mobile polymer chains are chemically grafted onto, or physically embedded within, an inert matrix.

Regardless of the structure and nature of the polymeric support chosen, it must contain appropriate functional groups onto which the first amino acid can be anchored. In early schemes that still have considerable popularity, chloromethyl groups are introduced onto a polystyrene resin by a direct Friedel-Crafts reaction, following which an N^{α}-protected amino acid, as its triethylammonium or cesium salt, is added to provide a polymer-bound benzyl ester. More recently, it has been recognized that greater control and generality is possible by use of "handles," which are defined as bifunctional spacers that, on one end, incorporate features of a smoothly cleavable protecting group. The other end of the handle contains a functional group, often a carboxyl, that can be activated to allow coupling to functionalized supports, for example ones containing aminomethyl groups. Particularly advantageous, though more involved to prepare, are "preformed" handles, which serve to link the first amino acid to the resin in two discrete steps, and thereby provide maximal control over this essential step of the synthesis (see Attachment to Support).

The next stage of solid-phase synthesis is the systematic elaboration of the growing peptide chain. In the vast majority of solid-phase syntheses, suitably N^{α}- and side-chain protected amino acids are added stepwise in the $C \rightarrow N$ direction. A particular merit of this strategy is that the best practical realizations have been shown experimentally to proceed with only negligible levels of racemization. A "temporary" protecting group is removed quantitatively at each step to liberate the N^{α}-amine of the peptide resin, following which the next incoming protected amino acid is introduced with its carboxyl group suitably activated (see Formation of Peptide Bond). It is frequently worthwhile to verify that the coupling has gone to completion by some monitoring technique (see Monitoring).

Once the desired linear sequence has been assembled satisfactorily on the polymeric support, the anchoring linkage must be cleaved. Depending on the chemistry of the original handle and on the cleavage reagent selected, the product from this step can be a C-terminal peptide acid, amide, or other functionality. The cleavage can be conducted so as to retain "permanent" side-chain protecting groups and thus yield protected segments that, once purified, are suitable for further condensation. Alternatively, selected "permanent" groups can be retained on sensitive residues for later deblocking in solution. However, the approach that is most widely used involves final deprotection which is carried out essentially concurrent with cleavage; in this way, the released product is directly the free peptide.

PROTECTION SCHEMES

The preceding section outlined the key steps of the solid-phase procedure but dealt only tangentially with combinations of "temporary" and "permanent" protecting groups and the corresponding methods for their removal. The choice and optimization of protection chemistry is perhaps the key factor in the success of any synthetic endeavor. Even when a residue has been incorporated safely into the growing resin-bound polypeptide chain, it may still undergo irreversible structural modification or rearrangement during subsequent synthetic steps. The vulnerability to damage is particularly pronounced at the final deprotection/cleavage step, since these are usually the harshest conditions. At least two levels of protecting-group stability are required, insofar as the "permanent" groups used to prevent branching or other problems on the side chains must withstand repeated applications of the conditions for quantitative removal of the "temporary" N^{α}-amino protecting group. On the other hand, structures of "permanent" groups must be such that conditions can be found to remove them with minimal levels of side reactions that affect the integrity of the desired product. The necessary stability is often approached by kinetic "fine tuning," which is a reliance on quantitative rate differences whenever the same chemical mechanism (usually acidolysis) serves to remove both classes of protecting groups. An often limiting consequence of such schemes, based on graduated lability, is that they force adoption of relatively severe final deprotection conditions. Alternatively, *orthogonal* protection schemes can be used. These schemes involve two or more classes of groups that are removed by differing chemical mechanisms, and therefore can be removed in any order and in the presence of the other classes. Orthogonal schemes offer the possibility of substantially milder overall conditions, because selectivity can be attained on the basis of differences in chemistry rather than in reaction rates.

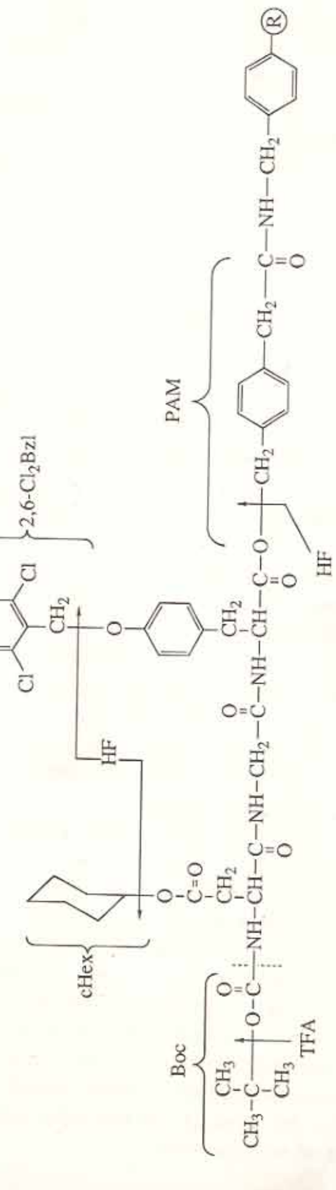


FIGURE 2 "Merrifield" protection scheme for solid-phase synthesis, based on graduated acidolysis. Temporary N^{α}-amino protection is provided by the Boc group, removed at each step by the moderately strong acid TFA. Permanent Bzl-based and cHex side-chain protecting groups, and the PAM linkage, are then cleaved simultaneously by HF or other strong acids, with a free peptide acid being formed in high yield.

"Temporary" Protection of N^{α}-Amino Groups

Boc Chemistry

The so-called "standard Merrifield" system is based on graduated acid lability (Figure 2, in a modern, improved version). The acidolyzable "temporary" N^{α}-tert-butyloxycarbonyl (Boc) group is introduced onto amino acids with either di-*tert*-butyl dicarbonate or 2-*tert*-butyloxycarbonyloximino-2-phenylacetonitrile (Boc-ON) in aqueous 1,4-dioxane containing NaOH or triethylamine (Et_3N) (Bodanszky and Bodanszky, 1984). The Boc group is stable to alkali and nucleophiles and removed rapidly by inorganic and organic acids (Barany and Merrifield, 1979). Boc removal is usually carried out with trifluoroacetic acid (TFA) (20 to 50%) in dichloromethane (DCM) for 20 to 30 minutes, and, for special situations, HCl (4 N) in 1,4-dioxane for 35 minutes. Deprotection with neat (100%) TFA, which offers enhanced peptide resin solvation compared to TFA-DCM mixtures, proceeds in as little as 4 minutes (Kent and Parker, 1988; Wallace et al., 1989). Following acidolysis, a rapid diffusion-controlled neutralization step with a tertiary amine, usually 5 to 10% Et_3N or *N,N*-diisopropylethylamine (DIEA) in DCM for 3 to 5 minutes, is interpolated to release the free N^{α}-amine. Alternatively, Boc amino acids may be coupled without prior neutralization by using "in situ" neutralization, i.e., coupling in the presence of DIEA or NMM (Suzuki et al., 1975; Schnolzer et al., 1992). "Permanent" side-chain protecting groups are ether, ester, and urethane derivatives based on benzyl alcohol, suitably "fine tuned" with electron-donating methoxy or methyl groups or electron-withdrawing halogens for the proper level of acid stability/lability. Alternatively, ether and ester derivatives based on cyclopentyl or cyclohexyl alcohol are sometimes applied, because their use moderates certain side reactions. These "permanent" groups are sufficiently stable to repeated cycles of Boc removal, yet they are cleaved cleanly in the presence of appropriate scavengers by the use of liquid anhydrous hydrogen fluoride (HF) at 0 °C or trifluoromethanesulfonic acid (TFMSA) at 25 °C (see Cleavage). The 4-(hydroxymethyl)phenylacetic acid (PAM) or 4-methylbenzhydrylamine (MBHA) anchoring linkages are similarly "fine tuned" to be cleaved at the same time (see Attachment to Support).

Fmoc Chemistry

A mild orthogonal alternative is constructed using Carpino's base-labile "temporary" N^{α}-9-fluorenylmethyloxycarbonyl (Fmoc) group (Figure 3). The optimal reagent for preparation of Fmoc amino acids is fluorenylmethyl succinimidyl carbonate (Fmoc-OSu), applied in a partially aqueous/organic mixture in the presence of base; the alternative procedure involving derivatization by Fmoc chloride is accompanied by unaccep-

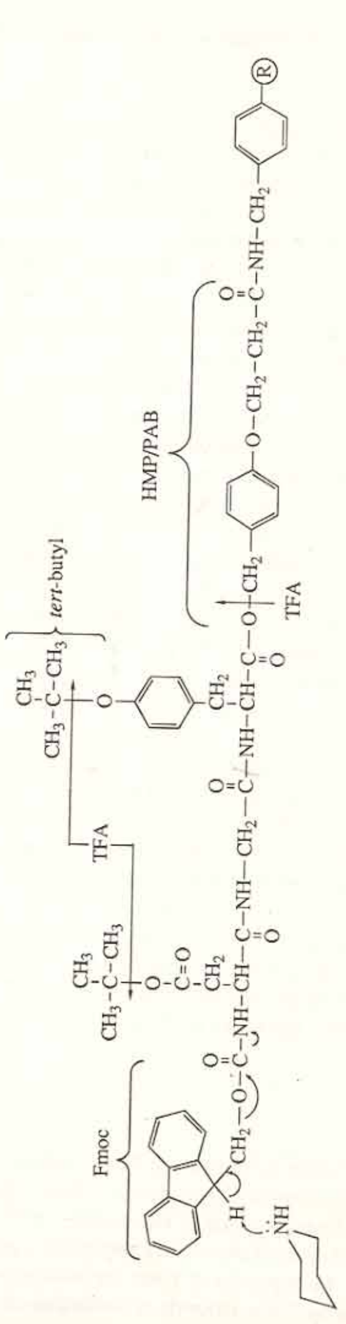


FIGURE 3 A mild two-dimensionsl orthogonal protection scheme for solid-phase synthesis. Temporary N^{α}-amino protection is provided by the Fmoc group, removed by the indicated base-catalyzed β-elimination mechanism. Permanent *t*Bu-based side-chain protecting groups and the HMP/PAB ester linkage are both cleaved by treatment with TFA to yield the free peptide acid. A third dimension of orthogonality may be added with an acid-stable, photolabile anchoring linkage (details in text).

A problem that occurs during preparation of Fmoc amino acids is the precipitation of either the Fmoc-OSu reagent or the base ($NaHCO_3$ or Na_2CO_3) upon mixing of the organic (1,4-dioxane, acetone, DMF, and acetonitrile have been proposed) and aqueous cosolvents. This problem is best overcome by use of aqueous dimethoxyethane with Na_2CO_3 (Fields et al., 1989; Netzel-Arnett et al., 1991). A solution of Fmoc-OSu (3.0 mmol) in dimethoxyethane (10 mL) is added slowly to the amino acid (2.0 mmol) dissolved in 10% aqueous Na_2CO_3 (10 mL); final yields of Fmoc amino acids after workup are in a range of 75 to 95%.

The Fmoc group has been shown to be completely stable to treatment with TFA, HBr in acetic acid (HOAc), or HBr in nitromethane for 1 to 2 days (Carpino and Han, 1972). Somewhat less stability was found in dipolar aprotic solvents (Atherton et al., 1979). Fmoc-Gly was deprotected after 7 days in dimethylacetamide (DMA), *N,N*-dimethylformamide (DMF), and NMP to the extent of 1, 5, and 14%, respectively. Although these low levels of decomposition are considered to be relatively insignificant, it is nevertheless prudent to purify the aforementioned solvents just before use. For NMP, Fmoc group removal is attributed directly to the presence of methylamine as an impurity (Otteson et al., 1989). The addition of HOBt (0.01 to 0.1 M) greatly reduces the detrimental effect of methylamine (Albericio and Barany, 1987a); Fmoc-Gly-HMP resin was less than 0.05% deprotected after 12 hours in NMP containing 0.01 M HOBt (Otteson et al., 1989). Fmoc amino acids can be stored in purified or synthesis-quality NMP with little decomposition for 6 to 8 weeks, in the dark at 25 °C (Kent et. al., 1991).

table levels (2 to 20%) of Fmoc dipeptide formation (Pacquet, 1982; Sigler et al., 1983; Lapatsanis et al., 1983; Tesser et al., 1983; Ten Kortenaar et al., 1986; Milton et al., 1987; Fields et al., 1989). The Fmoc group may also be added via [4-(9-fluorenylmethyloxycarbonyloxy)-phenyl]dimethylsulfonium methyl sulfate (Fmoc-ODSP) in H_2O with Na_2CO_3 or Et_3N (Azuse et al., 1989). Removal of the Fmoc group is achieved usually with 20 to 55% piperidine in DMF or N-methylpyrrolidone (NMP) for 10 to 18 minutes (Atherton et al., 1978a; Atherton et al., 1978b; Chang et al., 1980a; Albericio et al., 1990a; Fields and Fields, 1991); piperidine in DCM is not recommended, because an amine salt precipitates after relatively brief standing. The base abstracts the acidic proton at the 9 position of the fluorene ring system; β-elimination follows to give a highly reactive dibenzofulvene intermediate, which is trapped by excess secondary amine to form a stable, harmless adduct

(Carpino and Han, 1972). The Fmoc group may also be removed by 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF; however, this reagent is recommended for continuous-flow syntheses only, because the dibenzofulvene intermediate does not form an adduct with DBU and thus must be washed rapidly from the peptide resin (Wade et al., 1991). After Fmoc removal, the liberated N^{α}-amine of the peptide resin is free and ready for immediate acylation without an intervening neutralization step (compare to the previous paragraph on Boc chemistry). "Permanent" protection compatible with N^{α}-Fmoc protection is provided primarily by ether, ester, and urethane derivatives based on tert-butanol. These derivatives are cleaved at the same time as appropriate anchoring linkages by use of TFA at 25 °C. Scavengers must be added to the TFA to trap the reactive carbocations that form under the acidolytic cleavage conditions.

"Permanent" Protecting Groups for Reactive Amino Acid Side Chains

Once the means for N^{α}-amino protection has been selected, compatible protection for the side chains of trifunctional amino acids must be specified. These choices are made in the context of potential side reactions, which should be minimized. Problems may be anticipated either during the coupling steps or at the final cleavage/deprotection step. For certain residues (e.g., Cys, Asp, Glu, and Lys), side-chain protection is absolutely essential, whereas for others, an informed decision should be made depending upon the length of the synthetic target and other considerations. Most solid-phase syntheses follow maximal rather than minimal protection strategies. Almost all of the useful N^{α}-Boc and N^{α}-Fmoc protected derivatives can be manufactured in bulk, and they are found in the catalogues of the major suppliers of peptide synthesis chemicals. The most widely used "permanent" protecting groups for the trifunctional amino acids have been listed (Table 1), together with information on how derivatives are prepared, conditions for their intentional deblocking, and conditions under which the indicated side-chain protection is either entirely stable or cleaved prematurely by reagents used for peptide synthesis.

The side-chain carboxyls of Asp and Glu are protected as benzyl (OBzl) esters for Boc chemistry and as tert-butyl (O*t*Bu) esters for Fmoc chemistry. A sometimes serious side reaction with protected Asp residues involves an intramolecular elimination to form an aspartimide, which can then partition in water to the desired α-peptide and the undesired by-product with the chain growing from the β-carboxyl (Bodanszky and Kwei, 1978; Barany and Merrifield, 1979; Tam et al., 1988). Aspartimide formation is sequence dependent, with Asp(OBzl)-Gly, -Ser, -Thr, -Asn, and -Gln sequences showing the greatest tendency to cyclize under basic conditions (Bodanszky et al., 1978; Bodanszky and Kwei, 1978; Nicolás et al., 1989); the same sequences are also quite susceptible in strong acid

(Barany and Merrifield, 1979; Fujino et al., 1981; Tam et al., 1988). For models containing Asp(OBzl)-Gly, the rate and extent of aspartimide formation was substantial both in base (100% after 10 minutes treatment with 20% piperidine in DMF, 50% after 1 to 3 hours treatment with Et_3N or DIEA) and in strong acid (a typical value is 36% after 1 hour treatment with HF at 25°C). Sequences containing Asp(O*t*Bu)-Gly are somewhat susceptible to base-catalyzed aspartimide formation (11% after 4 hours treatment with 20% piperidine in DMF) (Nicolás et al., 1989), but they do not rearrange at all in acid (Kenner and Seely, 1972).

To minimize the imide/$\alpha \rightarrow \beta$ rearrangement side reaction, Fmoc-Asp may be protected with the 1-adamantyl (O-1-Ada) group (Okada and Iguchi, 1988) and Boc-Asp with either the 2-adamantyl (O-2-Ada) (Okada and Iguchi, 1988) or cyclohexyl (OcHex) (Tam et al., 1988) groups. The base-labile 9-fluorenylmethyl (OFm) group offers orthogonal side-chain protection for Boc-Asp/Glu (Bolin et al., 1989; Albericio et al., 1990c; Al-Obeidi et al., 1990), while the palladium-sensitive allyl (OAl) group (Belshaw et al., 1990; Lyttle and Hudson, 1992) offers orthogonal side-chain protection for both Boc- and Fmoc-Asp/Glu; neither of the esters mentioned in this sentence are as yet available commercially.

The side-chain hydroxyls of Ser, Thr, and Tyr are protected as Bzl and *t*Bu ethers for Boc and Fmoc SPPS, respectively. In strong acid, the benzyl (Bzl) protecting group blocking the Tyr phenol can migrate to the 3-position of the ring (Erickson and Merrifield, 1973a). This side reaction is decreased greatly when Tyr is protected by the 2,6-dichlorobenzyl (2,6-Cl_2Bzl) (Erickson and Merrifield, 1973a) or 2-bromobenzyloxycarbonyl (2-BrZ) (Yamashiro and Li, 1973) group; consequently, the latter two derivatives are much preferred for Boc SPPS. No corresponding *C*-alkylation occurs in Fmoc chemistry.

The ε-amino group of Lys is best protected by the 2-chlorobenzyloxycarbonyl (2-ClZ) or Fmoc group for Boc chemistry and, reciprocally, by the Boc group for Fmoc chemistry. The 2-ClZ group offers the desired acid stability for Boc chemistry, by comparison to the benzyloxycarbonyl (Z) and other ring-chlorinated Z groups. Branching due to premature side-chain deprotection by TFA is avoided, but the 2-ClZ group is still readily removable by strong acids (Erickson and Merrifield, 1973b). Orthogonal side-chain protection for both Boc- and Fmoc-Lys is provided by the palladium-sensitive allyloxycarbonyl (Aloc) group (Lyttle and Hudson, 1992).

The highly basic trifunctional guanidino side-chain group of Arg may be protected or unprotected (i.e., protonated). Appropriate benzenesulfonyl derivatives are the 4-toluenesulfonyl (Tos) or mesitylene-2-sulfonyl (Mts) groups in conjunction with Boc chemistry, and either 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) or 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) with Fmoc chemistry. These groups most likely block the

Table 1 Amino Acid Side-Chain Protection for Solid-Phase Peptide Synthesis[a]

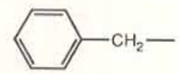
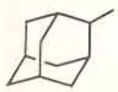
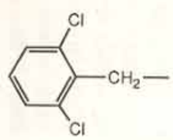
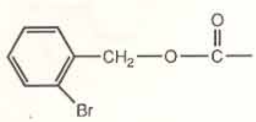
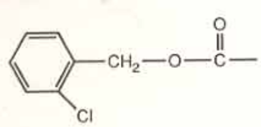
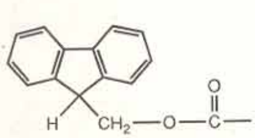
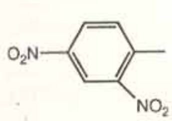
Side-Chain Protecting Group	*Protected Amino Acid Derivative*	*Stability*	*Removal[a]*	*Reagent(s) for Introduction*	*Reference(s)[a]*
Compatible with Boc chemistry					
Benzyl 	Asp/Glu(OBzl)	TFA	Strong Acid	Bzl-OH + H^+	Barany and Merrifield, 1979[#] Bodanszky and Bodanszky, 1984[*b] Tam and Merrifield, 1987[#]
	Ser/Thr/Tyr(Bzl)	TFA Base	Strong Acid	Bzl-Br + base	Yajima et al., 1988[#] Kiso et al., 1989[#]
2-Adamantyl 	Asp(O-2-Ada)	TFA Piperidine 1 M HCl	Strong Acid	Ada-2-OH + DCC	Okada and Iguchi, 1988[*#]
Cyclohexyl 	Asp(OcHex)	TFA	Strong Acid	cHex-OH + carbodiimide + DMAP *or* cHex-OH + H^+	Tam and Merrifield, 1987[#] Tam et al., 1988[*#] Yajima et al., 1988[#] Penke and Tóth, 1989[*] Kiso et al., 1989[#]
2,6-Dichlorobenzyl 	Tyr(2,6-Cl_2Bzl)	TFA	Strong Acid	2,6-Cl_2Bzl-Br + base	Erickson and Merrifield, 1973a[*#] Tam and Merrifield, 1987[#] Yajima et al., 1988[#] Kiso et al., 1989[#]
2-Bromobenzyloxycarbonyl 	Tyr(2-BrZ)	TFA	Strong Acid	2-BrZ-ONp + base	Yamashiro and Li, 1973[*#] Tam and Merrifield, 1987[#]
2-Chlorobenzyloxycarbonyl 	Lys(2-ClZ)	TFA	Strong Acid	2-ClZ-OSu + base	Erickson and Merrifield, 1973b[*#] Bodanszky and Bodanszky, 1984[*] Tam and Merrifield, 1987[#] Kiso et al., 1989[#]
9-Fluorenylmethyloxycarbonyl 	Lys(Fmoc)	TFA	Piperidine TBAF	Fmoc-N_3 + MgO	Albericio et al., 1990c[*#]
2,4-Dinitrophenyl 	His(Dnp)	Acids	Thiophenol	1-fluoro-2,4-dinitrobenzene + base	Chillemi and Merrifield, 1969[*#] Stewart et al., 1972[#] Tam and Merrifield, 1987[#] Applied Biosystems, Inc., 1989a[#]

Table 1 (continued)

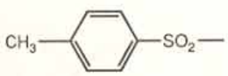
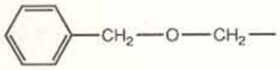
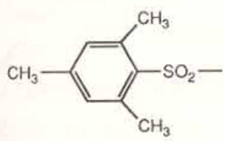
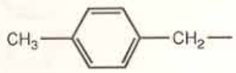
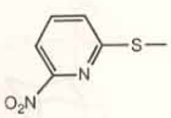
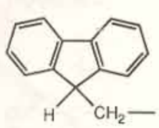
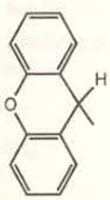
Side-Chain Protecting Group	*Protected Amino Acid Derivative*	*Stability*	*Removal*[a]	*Reagent(s) for Introduction*	*Reference(s)*[a]
4-Toluenesulfonyl 	His(Tos)	TFA	Strong acid Ac_2O-pyridine HOBt[@]	Tos-Cl + base	Stewart et al., 1972[#] Barany and Merrifield, 1979[#] van der Eijk et al., 1980[#] Bodanszky and Bodanszky, 1984* Tam and Merrifield, 1987[#]
	Arg(Tos)	HBr-TFA TFA	HF	Tos-Cl + base	
Benzyloxymethyl 	His(Bom)	TFA	Strong acid	$ClCH_2OBzl$	Brown et al., 1982*[#] Tam and Merrifield, 1987[#] Kiso et al., 1989[#]
Mesitylene-2-sulfonyl 	Arg(Mts)	TFA	Strong acid	Mts-Cl + base	Yajima et al., 1978*[#] Tam and Merrifield, 1987[#] Yajima et al., 1988[#]
4-Methylbenzyl 	Cys(Meb)	TFA	Strong acid $Tl(Tfa)_3$	Meb-Br + base	Erickson and Merrifield, 1973a*[#] Barany and Merrifield, 1979[#] Tam and Merrifield, 1987[#] Fujii et al., 1987[#]
3-Nitro-2-pyridinesulfenyl 	Cys(Npys)	TFA HF[c]	Thiols HF[c] HOBt[@] Piperidine[@]	Npys-Cl	Matsueda and Walter, 1980* Albericio et al., 1989b[#] Rosen et al., 1990[#]
9-Fluorenylmethyl 	Cys(Fm)	TFA HF	Piperidine[d]	Fm-OTos + base	Albericio et al., 1990c*[#]
	Asp/Glu(OFm)	TFA HF	Piperidine TBAF	Fm-OH + DCC/DMAP	Albericio *et al.*, 1990c*[#]
9-Xanthenyl 	Asn/Gln(Xan)	Base	Strong acid TFA[@]	Xan-OH + HOAc	Dorman et al., 1972*[#] Stewart and Young, 1984[#] Tam and Merrifield, 1987[#]
Formyl (on N^{in})	Trp(CHO)	TFA HF[c]	TFMSA piperidine HF[c]	HCOOH	Bodanszky and Bodanszky, 1984* Tam and Merrifield, 1987[#] Yajima et al., 1988[#]

Table 1 (continued)

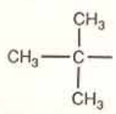
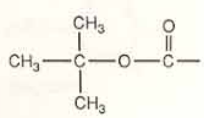
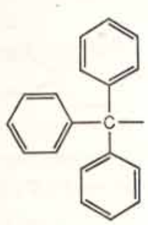
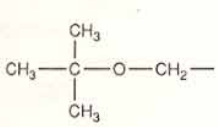
Side-Chain Protecting Group	*Protected Amino Acid Derivative*	*Stability*	*Removal[a]*	*Reagent(s) for Introduction*	*Reference(s)[a]*
Sulfoxide (on thioether)	Met(O)	TFA Base HF[c]	MMA DMF-SO_3 HF[c] NH_4I TMSBr + thioanisole	H_2O_2	Houghton and Li, 1979[#] Bodanszky and Bodanszky, 1984* Tam and Merrifield, 1987[#] Fujii et al., 1987[#] Futaki et al., 1990b[#]
Compatible with Fmoc chemistry					
tert-Butyl 	Asp/Glu(O*t*Bu)	Base	TFA	C_4H_8/H^+ *or* Boc-F	Chang et al., 1980b* Bodanszky and Bodanszky, 1984* Meienhofer, 1985# Loffet et al., 1989* Lajoie et al., 1990*[e] Greene, 1991[#]
	Ser/Thr/Tyr(tBu)	Base	TFA	C_4H_8/H^+	
1-Adamantyl 	Asp(O-1-Ada)	Base	TFA	Ada-1-OH + DCC	Okada and Iguchi, 1988*#
tert-Butyloxycarbonyl 	Lys(Boc)	Base	TFA	Boc_2O *or* Boc-ON	Bodanszky and Bodanszky, 1984* Meienhofer, 1985#
	His(Boc)	Piperidine	TFA	Boc_2O[f]	Atherton and Sheppard, 1989*[#]
Triphenylmethyl 	His(Trt)	Piperidine 1 N HCl	TFA	Trt-Cl + Me_2SiCl_2	Barlos et al., 1982* Sieber and Riniker, 1987[#]
	Cys(Trt)	Piperidine	HOAc TFA Hg(II) I_2	Trt-OH $BF_3 \cdot Et_2O$	Photaki et al., 1970[#] Bodanszky and Bodanszky, 1984* Meienhofer, 1985[#] Greene, 1991[#]
	Asn/Gln(Trt)	Piperidine 1 N HCl	TFA	Trt-OH Ac_2O/H^+	Sieber & Riniker, 1991*[#]
tert-Butoxymethyl 	His(Bum)	Piperidine	TFA	$ClCH_2OtBu$	Colombo et al., 1981*#

Table 1 (continued)

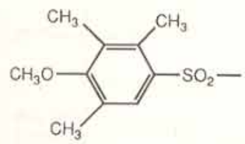
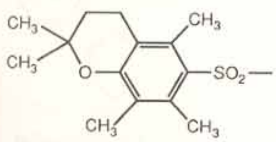
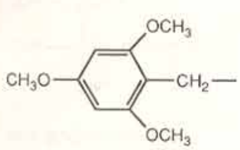
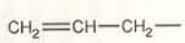
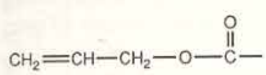
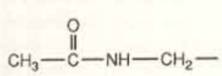
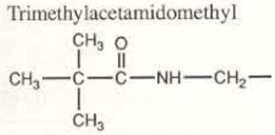
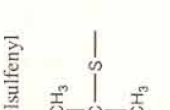
Side-Chain Protecting Group	*Protected Amino Acid Derivative*	*Stability*	*Removal*[a]	*Reagent(s) for Introduction*	*Reference(s)*[a]
4-Methoxy-2,3,6-trimethylbenzenesulfonyl 	Arg(Mtr)	Piperidine	TFA[g]	2,3,5-Trimethylanisole + $ClSO_3H$	Fujino et al., 1981*# Atherton and Sheppard, 1989#
2,2,5,7,8-Pentamethylchroman-6-sulfonyl 	Arg(Pmc)	Piperidine	TFA	2,2,5,7,8-Pentamethylchroman + $ClSO_3H$	Ramage and Green, 1987*#
2,4,6-Trimethoxybenzyl 	Asn/Gln(Tmob)	Piperidine	TFA	Tmob-NH_2[h] + DCC/HOSu	Weygand et al., 1968a* Hudson, 1988b#
	Cys(Tmob)	Piperidine	TFA	Tmob-OH + TFA	Munson et al., 1992*#
Compatible with both Boc and Fmoc chemistries					
Allyl 	Asp/Glu(OAl)	Acids Base	Pd(0)	Al-OH + TMS-Cl *or* H_2SO_4	Belshaw et al., 1990* Greene, 1991*# Lyttle and Hudson, 1992*#
Allyloxycarbonyl 	Lys(Aloc)	Acids Base	Pd(0)	Aloc-Cl	Lyttle and Hudson, 1992*#
Acetamidomethyl 	Cys(Acm)	Piperidine Acids	Hg(II) I_2 $Tl(Tfa)_3$	Acm-OH/TFA	Veber et al., 1972*# Bodanszky and Bodanszky, 1984* Atherton et al., 1985a# Albericio et al., 1987a*e Tam and Merrifield, 1987# Albericio et al., 1987*g Fujii et al., 1987# Brady et al., 1988# McCurdy, 1989#
Trimethylacetamidomethyl 	Cys(Tacm)	Piperidine Acids	Hg(II) I_2 $AgBF_4$	Tacm-OH/TFA	Yoshida et al., 1990# Kiso et al., 1990*#

Table 1 (concluded)

Side-Chain Protecting Group	*Protected Amino Acid Derivative*	*Stability*	*Removal*[a]	*Reagent(s) for Introduction*	*Reference(s)*[a]
tert-Butylsulfenyl 	Cys(S*t*Bu)	Piperidine Acids	Thiols Phosphines	*t*Bu-SH *t*Bu-SCN	Wünsch and Spangenberg, 1971* Atherton et al., 1985a# Romani et al., 1987#

[a] The protecting group structure drawn in the left column does not include the functional group that is being protected through a point of attachment at the far right of the structure. The order corresponds, by category, to first mention in the text. Abbreviations are provided in the second column. Conditions or reagents listed under Removal are intended for quantitative deprotection (see Cleavage), unless marked by @, in which case the removal is an unacceptable side reaction that indicates an incompatibility with the protecting group. Strong acid means HF, TFMSA, or equivalent reagents (see Cleavage). TFA cleavages are best carried out in the presence of appropriate scavengers (see Cleavage). References marked by * refer to preparation of the side-chain derivative; references marked by # refer to stability and lability of each derivative.

[b] Boc-Thr(Bzl)-OH is best prepared by the procedure of Chen et al., 1989.

[c] Stable to "high" (90%) HF, but removed by "low" HF-scavengers; see Cleavage.

[d] Complete deprotection requires 4-hour treatment by piperidine-DMF (1:1).

[e] One-pot synthesis of the Fmoc-amino acid side-chain protected derivative.

[f] Fmoc-His(Boc)-OH is prepared by reacting (Boc)2O with commercially available Fmoc-His(Fmoc)-OH.

[g] Arg(Mtr) deprotection by TFA can be slow, especially in multiple Arg(Mtr)-containing peptides. See Cleavage.

[h] Tmob-amine is reacted with a carboxyl protected Asp or Glu to produce Asn(Tmob) or Gln(Tmob), respectively.

ω-nitrogen of Arg, and their relative acid lability is Pmc > Mtr >> Mts > Tos (Fujino et al., 1981; Green et al., 1988). The Tos and Mts groups are removed by the same strong acids that cleave Bzl-type groups. The Mtr group may require extended TFA-thioanisole treatment (2 to 8 hours) for removal, while Arg(Pmc) is deprotected readily by 50% TFA (< 2 hours). A number of other Arg protecting groups have been proposed, particularly N^{ω}-mono or $N^{\delta,\omega}$-bis-urethane derivatives. However, based on current information, the aforementioned benzenesulfonyl derivatives seem to offer the best prospects of clean incorporation of Arg without contaminating ornithine (Orn) (Rink et al., 1984).

Due to the high pK_a of the guanidino group (~12.5), it can be protected selectively by protonation with HCl or HBr. Successful SPPS using protonated Arg requires a proton source (i.e., HOBt) for all subsequent coupling steps. This protocol is recommended to suppress intermolecular acylation of the guanidino group, which would lead to Orn formation (Atherton et al., 1984; Atherton and Sheppard, 1989).

A common side reaction of most Boc/Fmoc-Arg derivatives is δ-lactam formation (Barany and Merrifield, 1979; Rzeszotarska and Masiukiewicz, 1988). During carbodiimide activation, δ-lactam formation (intramolecular aminolysis) competes with peptide bond formation (intermolecular aminolysis). Acylations in the presence of HOBt are commonly used to inhibit δ-lactam formation. N^{α}-protected Arg derivatives may also be coupled as preformed esters, from which δ-lactam side products have been separated from the desired ester prior to use (Atherton et al., 1988b). However, preformed esters will undergo conversion to the δ-lactam relatively shortly after dissolving in DMF (D. Hudson, unpublished results).

Activated His derivatives are uniquely prone to racemization during stepwise SPPS due to an intramolecular abstraction of the proton on the optically active α-carbon by the imidazole π-nitrogen (Jones et al., 1980). Racemization could be suppressed either by reducing the basicity of the imidazole ring or by blocking the base directly (Riniker and Sieber, 1988). Consequently, His side-chain protecting groups can be categorized depending on whether the τ- or π-imidazole nitrogen is blocked. The Tos group blocks the N^{τ} of Boc-His and is removed by strong acids. However, the Tos group is also lost prematurely during SPPS steps involving HOBt; this allows acylation or acetylation (during capping) of the imidazole group, followed by chain termination due to $N^{im} \rightarrow N^{\alpha}$-amino transfer of the acyl or acetyl group (Ishiguro and Eguchi, 1989; Kusunoki et al., 1990). Therefore, HOBt should never be used during couplings of amino acids once a His(Tos) residue has been incorporated into the peptide resin. An HF-stable, orthogonally removable, N^{τ}-protecting group for Boc strategies is the 2,4-dinitrophenyl (Dnp) function. Final Dnp deblocking is best carried out at the peptide resin level prior to the HF cleavage step by use of thiophenol in DMF

Dnp removal from His(Dnp)-containing peptide resins is achieved by use of 20 mmol thiophenol per millimole His(Dnp) residue. Peptide resin is suspended in DMF (5 mL per gram of resin), thiophenol is added, and the reaction proceeds for 1 hour at 25 °C. After thorough washing of the Boc-peptide resin with DMF, H2O, ethanol, and DCM, the N^{α}-Boc group is removed and the peptide is cleaved with HF or TFMSA. If Dnp groups still remain, the peptide is dissolved in 6 M guanidine-HCl, 50 mM Tris acetate, pH 8.5 (10 to 20 mg peptide per mL solution), then deprotected by adding 2-mercaptoethanol to 20% (v/v) and treating for 2 hours at 37 °C. The peptide should then be purified immediately by gel filtration or HPLC (Applied Biosystems, Inc., 1989a).

(see box). The τ-nitrogen of Fmoc-His can be protected by the Boc and triphenylmethyl (Trt) groups. The commercially available N^{τ}-protected Fmoc-His(Fmoc) derivative is not recommended because it is poorly soluble; furthermore, immediate side-chain deprotection at the first exposure to piperidine allows a variety of side reactions (Bodanszky et al., 1977; Barany and Merrifield, 1979; Riniker and Sieber, 1988). When His is N^{τ}-protected by the Boc group, the basicity of the imidazole ring is reduced sufficiently so that acylation by the preformed symmetrical anhydride (PSA) method proceeds with little racemization (Atherton and Sheppard, 1989). Contrary to earlier reports, His(Boc) now appears to be reasonably stable to repetitive base treatment (Atherton and Sheppard, 1989). His(Trt) is completely stable to piperidine, but it is removed with TFA (Sieber and Riniker, 1987). The Trt group reduces the basicity of the imidazole ring (the pK_a decreases from 6.2 to 4.7), although racemization by the PSA method is not eliminated completely (Sieber and Riniker, 1987). Since Dnp and Trt N^{τ}-protection do not allow PSA coupling with low racemization, it is recommended that the appropriate derivatives be coupled as preformed esters or in situ with carbodiimide in the presence of HOBt (Sieber and Riniker, 1987; Riniker and Sieber, 1988). Boc-His (Tos) is coupled efficiently using benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (3 equiv) in the presence of DIEA (3 equiv); these conditions minimize racemization and avoid premature side-chain deprotection by HOBt (Forest and Fournier, 1990).

Blocking of the π-nitrogen of the imidazole ring has been shown to be effective in reducing His racemization (Fletcher et al., 1979). The N^{π} of His is protected by the benzyloxymethyl (Bom) and *tert*-butoxymethyl (Bum) groups for Boc and Fmoc chemistry, respectively. Couplings using N^{π}-protected Boc-His(Bom) or Fmoc-His(Bum) PSAs result in racemization-free incorporation of His (Brown et al., 1982; Colombo et al., 1984). HF deprotection of His(Bom) and TFA deprotection of

Boc- and Fmoc-His are prepared by the general procedures described earlier for Boc and Fmoc amino acids. Crude Boc-His is dissolved in methanol (MeOH)-0.1 M pyridinium acetate buffer (pH 3.8) (1:1) and purified by ion-exchange chromatography over Dowex 50 (H^+ form) using a pH gradient from 3.8 to 5.8 (Kawasaki et al., 1989). Crude Fmoc-His is purified by washing with H2O and hot MeOH (Kawasaki et al., 1989). N^{τ}-protected Boc- and Fmoc-His derivatives are prepared by straightforward reactions of appropriate side-chain derivatizing reagents with either N^{α}-protected or free His (see references in Table 1). N^{π}-protected derivatives are prepared by protecting His-OMe at the N^{α} (by the Z or Boc group) and N^{τ} (by the Boc group), derivatizing N^{π} with the appropriate chloromethyl ether (simultaneously removing the N^{τ}–Boc group), and saponifying the methyl ester with NaOH (Brown et al., 1982; Colombo et al., 1984). The N^{α}-Z group is removed by hydrogenolysis and replaced by the Fmoc group (Colombo et al., 1984).

His(Bum) liberates formaldehyde, which can modify susceptible side chains (see Cleavage).

The carboxamide side chains of Asn and Gln are often left unprotected in SPPS, but this approach leaves open the danger of dehydration to form nitriles upon activation with in situ reagents. On the other hand, acylations by activated esters result in minimal side-chain dehydration (Barany and Merrifield, 1979; Mojsov et al., 1980; Gausepohl et al., 1989b) (see Formation of Peptide Bond). Nitrile formation is also inhibited during in situ carbodiimide acylations when HOBt is added (Mojsov et al., 1980; Gausepohl et al., 1989b) (see Formation of Peptide Bond). However, the presence of HOBt does not effectively inhibit N^{α}-protected Asn dehydration during BOP in situ acylations (Gausepohl et al., 1989b).

At the point where an N^{α}-amino protecting group is removed from Gln, the possibility exists for an acid-catalyzed intramolecular aminolysis, which displaces ammonia and leads to pyroglutamate formation (Barany and Merrifield, 1973; Barany and Merrifield, 1979; DiMarchi et al., 1982; Orlowska et al., 1987). Cyclization occurs primarily during couplings; N^{α}-protected amino acids and HOBt promote this side reaction (DiMarchi et al., 1982). Consequently, it is recommended that the incoming residue that is to be incorporated onto Gln be activated as a non-acidic species, e.g., PSA or a preformed ester (see Formation of Peptide Bond).

Although conditions are available for the safe incorporation of Asn and Gln with free side chains during SPPS, there are compelling reasons for their protection. Side-chain protecting groups, such as 9-xanthenyl (Xan), 2,4,6-trimethoxybenzyl (Tmob), and Trt minimize the occurrence

> In peptides where several Asn, Gln, His, and Cys residues are close in sequence, it may be worthwhile to limit the global use of Trt side-chain protection. Interspersing side-chain unprotected Asn and/or Gln residues in such congested sequences should limit difficult couplings due to steric hinderance. In addition, Asn or Gln adjacent to Trp should be left unprotected, since the Tmob, Trt, and Xan side-chain protecting groups can modify Trp during TFA deprotection/cleavage (Southard, 1971; see also Cleavage for additional references).

of dehydration (Mojsov et al., 1980; Hudson, 1988b; Gausepohl et al., 1989b; Sieber and Riniker, 1990) and pyroglutamate formation (Barany and Merrifield, 1979), and they may also inhibit hydrogen bonding that otherwise leads to secondary structures that substantially reduce coupling rates. Unprotected Fmoc-Asn and -Gln have poor solubility in DCM and DMF; solubility is improved considerably by Tmob or Trt side-chain protection. The Xan group has been used in Boc chemistry, but it does not entirely survive the TFA deprotection conditions (Dorman et al., 1972; Stewart and Young, 1984).

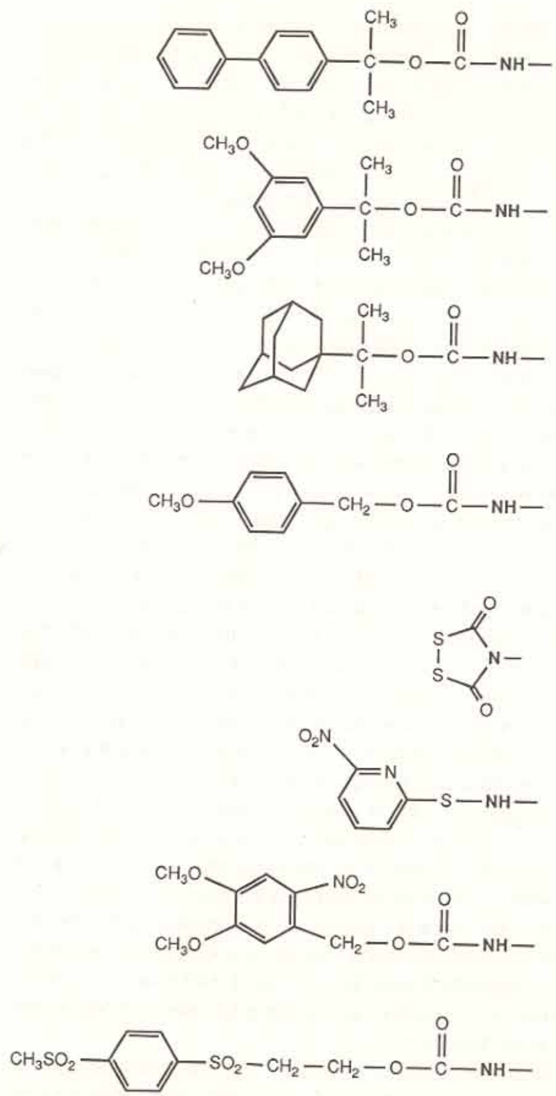
The highly sensitive side chains of Trp and Met generally survive cycles of Fmoc chemistry, but their protection during Boc chemistry is often advisable. For these purposes, the base-labile N^{in}-formyl (CHO) and reducible sulfoxide functions are applied respectively. Trp(CHO) is best deprotected at the peptide resin level by treatment with piperidine-DMF (9:91), 0 °C, 2 hours, *prior* to HF cleavage; the formyl group also is removed by 20 to 25% HF in the presence of dimethylsulfide and 4-thiocresol (see Cleavage). Smooth deblocking of Met(O) occurs in 20 to 25% HF in the presence of dimethylsulfide (see Cleavage), or by *N*-methylmercaptoacetamide (MMA) (10%) in 10% aqueous HOAc at 37 °C for 24 to 36 hours (Houghten and Li, 1979), NH_4I-dimethylsulfide (20 equiv each) in TFA at 0 °C for 1 hour (Fujii et al., 1987), or DMF·SO_3-EDT (5 equiv each) in 20% pyridine/DMF at 20 °C for 1 hour (Futaki et al., 1990b). DMF·SO_3-EDT treatment of Met(O) can be carried out only while hydroxyl residues are side-chain protected, because free hydroxyls will be sulfated (Futaki et al., 1990a). Unprotected Trp may be incorporated by Boc chemistry when 2.5% anisole plus either 2% dimethyl phosphite or indole are added to the TFA deprotection solution (Stewart and Young, 1984; Hudson et al., 1986).

The most challenging residue to manage in peptide synthesis is Cys, which is required for some applications in the free sulfhydryl form and, for others, as a contributor to a disulfide linkage. Another issue is the selective formation of multiple disulfides by the concurrent use of two or more classes of Cys protecting groups (see Post-Translational Modifications and Unnatural Structures). Compatible with Boc chemistry are the

4-methylbenzyl (Meb), acetamidomethyl (Acm), trimethylacetamidomethyl (Tacm), *tert*-butylsulfenyl (S*t*Bu), 3-nitro-2-pyridinesulfenyl (Npys), and Fm β-thiol protecting groups; compatible with Fmoc chemistry are the Acm, Tacm, S*t*Bu, Trt, and Tmob groups. The Trt and Tmob groups are labile in TFA; due to the tendency of the resultant stable carbonium ions to realkylate Cys (Photaki et al., 1970), effective scavengers are needed (see Cleavage). The Meb group is optimized for removal by strong acid (Erickson and Merrifield, 1973a); Cys(Meb) residues may also be directly converted to the oxidized (cystine) form by thallium (III) trifluoroacetate [$Tl(Tfa)_3$], although some cysteic acid forms at the same time. Cys(Npys) and Cys(Fm) are stable to acid and cleaved, respectively, by thiols and base. The Acm and Tacm groups are acid- and base-stable and removed by mercuric (II) acetate or silver tetrafluoroborate, followed by treatment with H_2S or excess mercaptans to free the β-thiol. In multiple Cys(Acm)-containing peptides, mercuric (II) acetate may not be a completely effective removal reagent (Kenner et al., 1979). Alternatively, Cys(Acm) and Cys(Tacm) residues are converted directly to disulfides by treatment with I_2, $Tl(Tfa)_3$, or methyltrichlorosilane in the presence of diphenylsulphoxide. Finally, the acid-stable S*t*Bu group is removed by reduction with thiols or phosphines.

C-terminal esterified (but not amidated) Cys residues are racemized by repeated piperidine deprotection treatments during Fmoc SPPS. Following 4 hours exposure to piperidine-DMF (1:4), the extent of racemization found was 36% D-Cys from Cys(S*t*Bu), 12% D-Cys from Cys(Trt), and 9% D-Cys from Cys(Acm) (Atherton et al., 1991). At least two examples have been provided where the use of Cys(S*t*Bu) as the *C*-terminal residue, esterified to an HMP/PAB-type resin, was entirely incompatible with formation of the desired peptide. Instead, TFA cleavage gave reduction-resistant by-products (structures not fully determined), retaining the *t*Bu group but evidently missing two molecules of water based on mass spectrometric evidence (Eritja et al., 1987). *N*-terminal Cys residues are modified covalently by formaldehyde, liberated during HF deprotection of His(Bom) residues (see Cleavage). Additional difficulties, often poorly understood, have arisen with a range of protected Cys derivatives in a variety of applications (Barany and Merrifield, 1979; Atherton et al., 1985b).

As is clear from the preceding discussion, the Boc and Fmoc groups have risen to the fore as the most widely used and commercially viable N^{α}-amino protecting groups for SPPS. A plethora of other N^{α}-amino protecting groups, some illustrating remarkably creative organic chemistry, have been proposed over the years (Figure 4). Among these, the 2-(4-biphenyl)propyl[2]oxycarbonyl (Bpoc) (Wang and Merrifield 1969; Kemp et al., 1988), 2-(3,5-dimethoxyphenyl)propyl[2]oxycarbonyl (Ddz) (Birr et al., 1972; Voss and Birr, 1981), 1-(1-adamantyl)-1-methylethoxycarbonyl (Adpoc) (Voelter et al., 1987; Shao et al., 1991),



Bpoc
Ddz
Adpoc
Moz
Dts
Npys
Nvoc
Mpc

FIGURE 4 Alternative N^{α}-amino protecting groups for SPPS. The protected nitrogen that is part of the amino acid is shown in boldface.

and 4-methoxybenzyloxycarbonyl (Moz) (Wang et al., 1987; Chen et al., 1987) groups are removed in dilute TFA, the dithiasuccinoyl (Dts) (Barany and Merrifield, 1977; Barany and Albericio, 1985; Albericio and Barany, 1987a; Zalipsky et al., 1987), and 3-nitro-2-pyridinesulfenyl (Npys) (Matsueda and Walter, 1980; Wang et al., 1982; Ikeda et al., 1986; Hahn et al., 1990) groups are removed by thiolysis, the 6-nitroveratryloxycarbonyl (Nvoc) group is removed by photolysis (Patchornik et al., 1970; Fodor et al., 1991), and the 2-[4-(methylsulfonyl)phenylsulfonyl]ethoxycarbonyl (Mpc) is base-labile (Schielen et al., 1991). Chemistries relying on these protecting groups are beyond the scope of this chapter.

POLYMERIC SUPPORT

The term *solid phase* often conjures misleading images among the uninitiated. Supports that lead to successful results for macromolecule synthesis are far from static, and because of the need for reasonable capacities, it is rare for solid-phase chemistry to take place exclusively on surfaces. The resin support is quite often a polystyrene suspension polymer cross-linked with 1% of 1,3-divinylbenzene; the level of functionalization is typically 0.2 to 1.0 mmol/g. Dry polystyrene beads have an average diameter of about 50 μm, but with the commonly used solvents for peptide synthesis, namely DCM and DMF, they swell 2.5- to 6.2-fold in volume (Sarin et al., 1980). Thus, the chemistry of solid-phase synthesis takes place within a well-solvated gel containing mobile and reagent-accessible chains (Sarin et al., 1980; Live and Kent, 1982). Polymer supports have also been developed based on the concept that the insoluble support and peptide backbone should have comparable polarities (Atherton and Sheppard, 1989). A resin of copolymerized dimethylacrylamide, *N,N′*-bisacryloylethylenediamine, and acryloylsarcosine methyl ester (typical loading 0.3 mmol/g), commercially known as polyamide or Pepsyn, has been synthesized to satisfy this criterion (Arshady et al., 1981). Under the best solvation conditions for both polystyrene and polyamide supports, reaction rates approach, but generally do not reach, those attainable in solution. It has been shown for a polystyrene carrier that macroscopic dimensions of both dry and solvated beads change dramatically once an appreciable level of peptide has been built up (Sarin et al., 1980). Thus, for this specific case, reactions continued to occur efficiently throughout the interior of a peptide resin that was fourfold the weight of the starting support.

A fertile area of inquiry has been the testing of supports with macroscopic physical properties and possibly other characteristics differing from 1% cross-linked polystyrene and polyamide gel beads. These include membranes (Bernatowicz et al., 1990), cotton and other appropriate carbohydrates (Frank and Döring, 1988; Lebl and Eichler, 1989;

Eichler et al., 1989), controlled-pore silica glass (Büttner et al., 1988), and linear polystyrene chains grafted covalently onto dense Kel-F particles (Tregear, 1972; Kent and Merrifield, 1978; Albericio et al., 1989a) or polyethylene sheets (Berg et al., 1989). Supports developed specifically to withstand the back pressures that arise during continuous-flow procedures have been low-density, highly permeable inorganic matrices with polyamide embedded within. These embedded matrices include polyamide-kieselguhr (known commercially as Pepsyn K) (Atherton et al., 1981b) and polyamide-Polyhipe (Small and Sherrington, 1989). Pepsyn K has a typical loading of 0.1 mmol/g, while Polyhipe loadings range from 0.3 to 1.8 mmol/g. An exciting recent development involves the use of polyethylene glycol-polystyrene graft supports (0.1 to 0.4 mmol/g), which swell in a range of solvents and have excellent physical and mechanical properties for both batchwise and continuous-flow SPPS (Hellermann et al., 1983; Zalipsky et al., 1985; Bayer and Rapp, 1986; Bayer et al., 1990; Barany et al., 1992). Poly *N*-[2-(4-hydroxyphenyl)ethyl]-acrylamide (core Q) is also suitable for both batchwise and continous-flow SPPS with high loading capacities (5 mmol/g) (Epton et al., 1987; Baker et al., 1990).

ATTACHMENT TO SUPPORT

Almost all syntheses by the solid-phase method are carried out in the C → N direction and, therefore, generally start with the intended *C*-terminal residue of the desired peptide being linked to the support either directly or by means of a suitable handle. Anchoring linkages have been designed so that eventual cleavage provides either a free acid or amide at the *C*-terminus, although, in specialized cases, other useful end groups can be obtained. The discussion that follows focuses on linkers that are either commercially available, readily prepared, and/or of special interest (Table 2); more complete listings are available (Barany et al., 1987; Fields and Noble, 1990).

Note that several handles (Table 2) have a free or activated carboxyl group that is intended to attach to the polymeric support. Such handles are most frequently coupled onto supports that have been functionalized with amino groups. Aminomethyl-polystyrene resin is optimally prepared essentially as described by Mitchell et al. (1978), except that methanesulfonic acid (0.75 g per 1 g polystyrene) is preferred as the catalyst instead of the originally described TFMSA (S.B.H. Kent and K.M. Otteson, unpublished results). Amino groups are introduced onto a variety of polyamide supports by treatment with ethylenediamine to displace carboxylate derivatives (Atherton and Sheppard, 1989). All else being equal, there are significant advantages to those anchoring methods in which the key step is amide bond formation by reaction of an activated handle carboxyl with an amino support, since such reactions can be made

to go readily to completion. This approach allows control of loading levels and obviates difficulties that may arise due to extraneous or unreacted functionalized groups. As indicated earlier, the best control is achieved by coupling "preformed handles," which are protected amino acid derivatives that have been synthesized and purified in solution prior to the solid-phase anchoring step.

Peptide Acids

For Boc chemistry, the most common approach to peptide acids uses substituted benzyl esters that are cleaved in strong acid at the same time that other benzyl-type protecting groups are removed (Figure 2). The classical procedures starting with chloromethyl resin are still favored by many (Gutte and Merrifield, 1971; Gisin, 1973; Stewart and Young, 1984), although preformed handle approaches with 4-(hydroxymethyl)phenylacetic acid (PAM) (Mitchell et al., 1978; Tam et al., 1979; Clark-Lewis and Kent, 1989) are preferable for a number of applications. Other resins and linkers proposed for Boc chemistry are cleaved by orthogonal modes, allowing their use for the preparation of partially protected peptide segments (see Auxiliary Issues). The 4-(2-hydroxyethyl)-3-nitrobenzoic acid (NPE) (Eritja et al., 1991; Albericio et al., 1991b) and 9-(hydroxymethyl)-2-fluoreneacetic acid (HMFA) (Liu et al., 1990) linkers are cleaved by bases (see Cleavage). The HMFA linker is also cleaved by free N^{α}-amino groups from the peptide resin; the addition of HOBt during SPPS inhibits premature cleavage from this source (Liu et al., 1990). A 4-nitrobenzophenone oxime resin (DeGrado and Kaiser, 1980; DeGrado and Kaiser, 1982; Findeis and Kaiser, 1989; Scarr and Findeis, 1990) yields a peptide acid upon cleavage by either *N*-hydroxypiperidine (HOPip) or amino acid tetra-*n*-butylammonium salts [AA^{-} $^{+}N(nBu)_4$] (Findeis and Kaiser, 1989; Lansbury et al., 1989; Sasaki and Kaiser, 1990). Note that in the latter mode of oxime resin cleavage, the penultimate residue of the desired peptide is the one initially attached to the support. Finally, the acid-stable 2-bromopropionyl (α-methylphenacyl ester) linker (Wang, 1976) is of interest because it can be cleaved by photolysis (350 nm).

For Fmoc chemistry, peptide acids have been generated traditionally using the 4-alkoxybenzyl alcohol resin/4-hydroxymethylphenoxy (HMP/PAB) linker (Wang, 1973; Lu et al., 1981; Sheppard and Williams, 1982; Colombo et al., 1983; Albericio and Barany, 1985; Bernatowicz et al., 1990), which is cleaved in 1 to 2 hours at 25 °C with 50 to 100% TFA. The precise lability of the resultant 4-alkoxybenzyl esters depends on the spacer between the phenoxy group and the support. The HMP/PAB moiety can be established directly on the resin, or it can be introduced as a handle; preformed handles are best coupled to amino-functionalized resins as their 2,4,5-trichlorophenyl- or 2,4-dichlorophenyl-activated

Table 2 Resin Linkers and Handles[a]

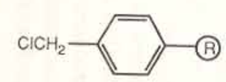
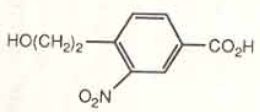
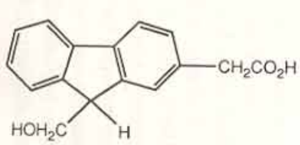
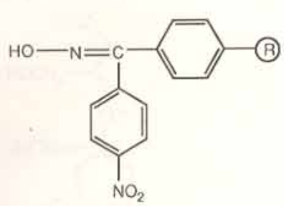
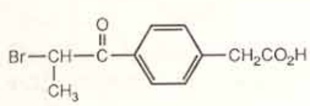
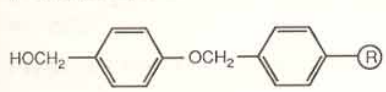
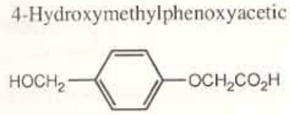
Linker/Handle/Resin	*Cleavage Conditions*	*Resulting C-Terminus*	*Reference(s)*
4-Chloromethyl resin 	Strong acid	Acid	Gutte and Merrifield, 1971 Stewart and Young, 1984
4-Hydroxymethylphenylacetic acid (PAM) 	Strong acid	Acid	Mitchell et al., 1978 Tam et al., 1979
3-Nitro-4-(2-hydroxyethyl)benzoic acid (NPE) 	Piperidine DBU	Acid	Eritja et al., 1991 Albericio et al., 1991b
9-(Hydroxymethyl)-2-fluoreneacetic acid (HMFA) 	Piperidine	Acid	Mutter and Bellof, 1984[c] Liu et al., 1990
4-Nitrobenzophenone oxime resin 	HOPip $AA^{-}\ {}^{+}N(nBu)_4$ $AA\text{-}NH_2$	Acid[b] Amide	DeGrado and Kaiser, 1982[c] Findeis and Kaiser, 1989 Scarr and Findeis, 1990
α-Bromophenacyl 	hν (350 nm)	Acid	Wang, 1976
4-Alkoxybenzyl alcohol resin 	TFA	Acid	Wang, 1973[c] Lu et al., 1981
4-Hydroxymethylphenoxyacetic acid (HMPA/PAB) 	TFA	Acid	Sheppard and Williams, 1982

Table 2 (continued)

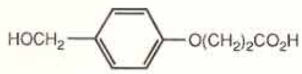
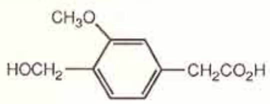
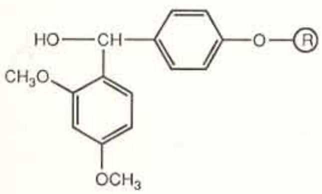
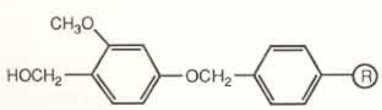
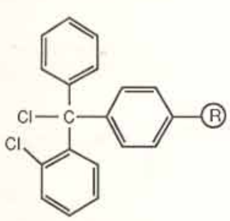
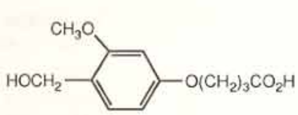
Linker/Handle/Resin	*Cleavage Conditions*	*Resulting C-Terminus*	*Reference(s)*
3-(4-Hydroxymethylphenoxy)propionic acid (PAB) 	TFA	Acid	Albericio and Barany, 1985
3-Methoxy-4-hydroxymethylphenoxyacetic acid 	Dilute TFA	Acid	Sheppard and Williams, 1982
4-(2′,4′-Dimethoxyphenylhydroxymethyl) phenoxymethyl resin 	Dilute TFA	Acid	Rink, 1987
2-Methoxy-4-alkoxybenzyl alcohol resin (SASRIN™) 	Dilute TFA	Acid	Mergler et al., 1988a
2-Chlorotrityl chloride resin 	TFA, HOAc	Acid	Barlos et al., 1989 Barlos et al., 1991a
4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) 	Dilute TFA	Acid	Flörsheimer and Riniker, 1991

Table 2 (continued)

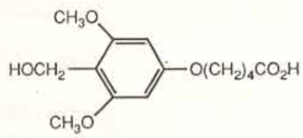
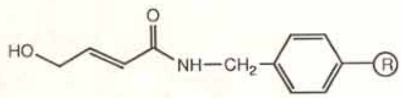
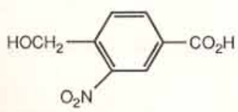
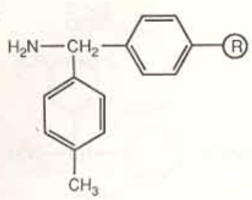
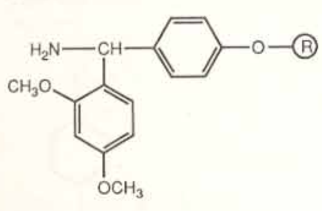
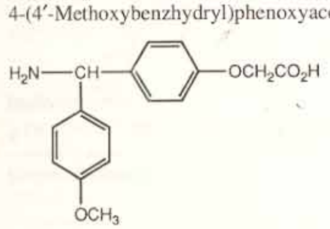
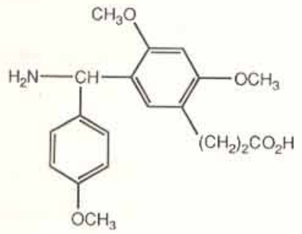
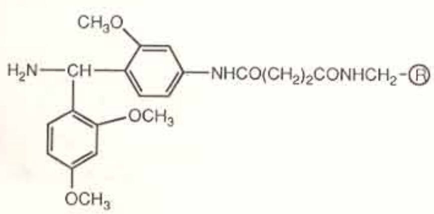
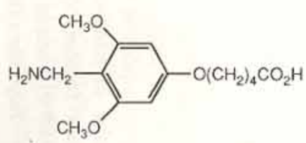
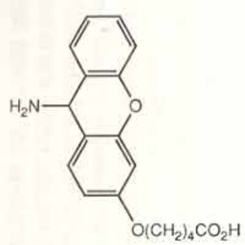
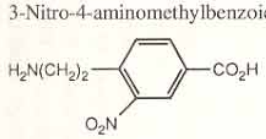
Linker/Handle/Resin	*Cleavage Conditions*	*Resulting C-Terminus*	*Reference(s)*
5-(4-Hydroxymethyl-3,5-dimethoxyphenoxy)valeric acid (HAL) 	Dilute TFA	Acid	Albericio and Barany, 1991
Hydroxy-crotonyl-aminomethyl resin (HYCRAM™) 	Pd(0) + NMM *or* dimedone	Acid	Kunz and Dombo, 1988 Guibé et al., 1989 Lloyd-Williams et al., 1991b
3-Nitro-4-hydroxymethylbenzoic acid (ONb) 	hv (350 nm)	Acid	Rich and Gurwara, 1975[c] Giralt et al., 1982 Barany and Albericio, 1985 Kneib-Cordonier et al., 1990
4-Methylbenzhydrylamine resin (MBHA) 	Strong acid	Amide	Matsueda and Stewart, 1981 Gaehde and Matsueda, 1981
4-(2′,4′-Dimethoxyphenylaminomethyl)phenoxymethyl resin 	Dilute TFA	Amide	Rink, 1987
4-(4′-Methoxybenzhydryl)phenoxyacetic acid (Dod) 	TFA	Amide	Stüber et al., 1989

Table 2 (concluded)

Linker/Handle/Resin	*Cleavage Conditions*	*Resulting C-Terminus*	*Reference(s)*
3-(Amino-4-methoxybenzyl)-4,6-dimethoxyphenyl-propionic acid 	Dilute TFA	Amide	Breipohl et al., 1989
4-Succinylamino-2,2′,4′-trimethoxybenzhydrylamine resin (SAMBHA) 	TFA	Amide	Penke et al., 1988
5-(4-Aminomethyl-3,5-dimethoxyphenoxy)valeric acid (PAL) 	TFA	Amide	Albericio and Barany, 1987b Albericio et al., 1990a
5-(9-Aminoxanthen-2-oxy)valeric acid (XAL) 	Dilute TFA	Amide	Sieber, 1987c[c] Barany and Albericio,1991a Bontems et al., 1992
3-Nitro-4-aminomethylbenzoic acid (Nonb) 	hν (350 nm)	Amide	Hammer et al., 1990

[a] Structural diagrams are oriented so that the resin or point of attachment to support is on the far right, and the site for anchoring the *C*-terminal amino acid residue is on the far left. Benzyl ester type linkages may also be cleaved by a range of nucleophiles to give acids, esters, and other derivatives. See text discussion under Cleavage, and consult Barany and Merrifield (1979) and Barany et al. (1987) for further examples.

[b] Cleavage by aminolysis results in 0.6 to 2% racemization (DeGrado and Kaiser, 1980).

[c] Reference for historical reasons; preparation of linker/resin has been improved in later references.

esters, sometimes in the presence of HOBt (Albericio and Barany, 1985; Bernatowicz et al., 1990; Albericio and Barany, 1991). A number of supports and linkers are available that can be cleaved in dilute acid; under optimal circumstances, these can be used to prepare protected peptide segments retaining side-chain *tert*-butyl protection. These include 3-methoxy-4-hydroxymethylphenoxyacetic acid (Sheppard and Williams, 1982), 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)phenoxy resin (Rink acid) (Rink, 1987; Rink and Ernst, 1991), 2-methoxy-4-alkoxybenzyl alcohol (SASRIN™) (Mergler et al., 1988a), 2-chlorotrityl-chloride resin (Barlos et al., 1989; Barlos et al., 1991a), 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) (Flörsheimer and Riniker, 1991), and 5-(4-hydroxymethyl-3,5-dimethoxyphenoxy)-valeric acid (HAL) (Albericio and Barany, 1991). Because of its acute acid lability, and in order to prevent premature loss of peptide chains, the Rink acid linker is used in conjunction with N^{α}-protected amino acid preformed HOBt esters in the presence of excess DIEA (3 equiv) (Rink and Ernst, 1991). The HMP/PAB and SASRIN™ linkers are available as the corresponding chlorides or bromides (Colombo et al., 1983; Mergler et al., 1989a; Bernatowicz et al., 1990).

Linkers for preparing peptide acids that are compatible with both Boc and Fmoc chemistries include hydroxy-crotonyl (HYCRAM™) (Kunz and Dombo, 1988; Kunz, 1990; Lloyd-Williams et al., 1991b), which is cleaved by Pd(0) catalyzed transfer of the allyl linker to a weak nucleophile, and 3-nitro-4-hydroxymethylbenzoic acid (ONb) (Rich and Gurwara, 1975; Giralt et al., 1982; Barany and Albericio, 1985; Kneib-Cordonier et al., 1990), which is cleaved photolytically at 350 nm.

All of the anchoring linkages that ultimately provide peptide acids are esters; rates and yields of reactions for ester bond formation (Table 3) are lower than those for corresponding methods for amide bond formation (see Formation of Peptide Bond). Consequently, compromises are needed to achieve reasonable loading reaction times and substitution levels, while ensuring that the extent of racemization remains acceptably low. As a point of departure, esterification of N^{α}-protected amino acid PSA catalyzed by 1 equiv of 4-dimethylaminopyridine (DMAP) in DMA results in significant (1.5 to 20%) racemization (Atherton et al., 1981a). In general, racemization levels can be reduced to acceptable levels (0.2 to 1.2%) when catalytic (0.06 equiv) amounts of DMAP are used and loadings are performed with carbodiimides in situ (Mergler et al., 1988a), sometimes in the presence of *N*-methylmorpholine (NMM) (0.9 equiv) (D. Hudson, personal communication). Alternatively, in situ carbodiimide loading with HOBt (2 equiv) and DMAP (1 equiv) at low temperature (0 to 3 °C) provides a good compromise of minimized racemization (0.1 to 0.3%) and reasonable loading times (16 hours) (van Nispen et al., 1985). No racemization was detected (0.05%) when in situ loadings were carried out at 25 °C with only *N,N′*-dicyclohexylcarbodiimide (DCC) (4 equiv) and HOBt (3 equiv) and no DMAP (Grandas et al., 1989a). Esterifications of Fmoc amino acids mediated by *N,N*-dimethylformamide dineopentyl acetal (Albericio and Barany, 1984; Albericio and Barany, 1985), 2,6-dichlorobenzoyl chloride (DCBC) (Sieber, 1987a), diethyl azodicarboxylate (DEAD) (Sieber, 1987a; Stanley et al., 1991), or 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazolide (MSNT) (Blankemeyer-Menge et al., 1990) have all been reported to suppress racemization, as is the case with preformed Fmoc amino acid 2,5-diphenyl-2,3-dihydro-3-oxo-4-hydroxythiophene dioxide (OTDO) esters (Kirstgen et al., 1987; Kirstgen et al., 1988; Kirstgen and Steglich, 1989), Fmoc amino acid chlorides (Akaji et al., 1990a), or Fmoc amino acid *N*-carboxyanhydrides (NCA) (Fuller et al., 1990). The well-established cesium salt method (Gisin, 1973) also allows loading of N^{α}-protected amino acids to chloromethyl linkers and resins with low levels of racemization (Colombo et al., 1983; Mergler et al., 1989b) while effectively preventing alkylation of susceptible residues (Cys, His, Met) (Gisin, 1973). Bromomethylated linkers may be loaded directly by Boc amino acids in the presence of KF (Tam et al., 1979) or by Fmoc amino acids in the presence of DIEA (Bernatowicz et al., 1990), in each case with little racemization. While premature removal of the Fmoc group during loading can result in dipeptide formation (Pedroso et al., 1983), the efficient ester bond formation methods described in this paragraph minimize this side reaction. However, care should be taken when preparing cesium salts of Fmoc amino acids, because Cs_2CO_3 may promote partial removal of the Fmoc group. Of the ester bond formation methods discussed here, the most generally applicable are esterification of N^{α}-protected amino acids in situ by carbodiimide (DCC or DIPCDI) in the presence of catalytic amounts of DMAP (0.06 to 0.1 equiv), or by *N,N*-dimethylformamide dineopentyl acetal. For esterifications in the presence of DMAP, time and temperature should be carefully mediated, and HOBt (1 to 2 equiv) may be included.

Fmoc-His and Fmoc-Cys derivatives are particularly difficult to load efficiently while suppressing racemization. Low racemization loadings have been documented using the cesium salts of Fmoc-His(Trt) (0.4% D-His) and Fmoc-Cys(Acm) (0.5% D-Cys) (Mergler et al., 1989b) and for Fmoc-His(Bum) (0.3% D-His) esterification by MSNT (Blankemeyer-Menge, 1990). Since the last-mentioned result is undoubtedly due to the fact that the Bum group blocks N^{π} of His, it may be noted that a more efficient loading procedure involves Fmoc-His(Bum) (2 equiv) esterified in situ by *N,N′*-diisopropylcarbodiimide (DIPCDI) (2 equiv) and DMAP (0.16 equiv) in DCM-DMF (1:3) for 1 hour (Fields and Fields, 1990). The best reported results for loading Fmoc-Cys(Trt) and Fmoc-Cys(S*t*Bu) are 2.1% D-Cys during Fmoc-Cys(S*t*Bu) esterification by MSNT (Blankemeyer-Menge, 1990) and 2.0 to 2.5% D-Cys during Fmoc-Cys(Trt) cesium salt loading (Mergler et al., 1989b) or in situ

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Table 3 Formation of Ester Bonds to Attach N^{α}-Protected Amino Acids to Linkers[a]

Reagent(s)	*Linker*[a]	*Stoichiometries*[b]	*Conditions*[c,d,e]	*Racemization (%)*[e]	*Loading (%)*[e]	*Reference*
Boc-AA-OH: DCHA	Br-PAM*	2 : 2 : 1	DMF (4 h, 50 °C + 14 h, 25 °C)	<0.1	86	Mitchell et al., 1978
Boc-AA-OH : KF	Br-PAM*	2.2 : 1.1 : 1	CH_3CN (18-48 h)	N.R.	~100	Tam et al., 1979
Fmoc-AA-OH : DMF-dineopentyl acetal	HO-HMP*,#	1.7 : 1.7 : 1	DMF (72 h)	<0.05	67 - 75	Albericio and Barany, 1984 Albericio and Barany, 1985
Fmoc-AA-OH : DMAP : HOBt : DCC	HO-HMP#	2 : 2 : 4 : 2 : 1	DMF (18 h, 0 °C)	0.1 - 0.3	47	van Nispen et al., 1985
Fmoc-AA-OH : DCBC : pyridine	HO-HMP#	2 : 2 : 3.3 : 1	DMF (15-20 h)	0.1 - 0.7 Arg(Mtr) <1.0 His(Trt) 27.0 His(Bum) 2.2 Cys(Acm) 1.2 Cys(Trt) 4.0	55 - 66 His(Bum) 16	Sieber, 1987a
Fmoc-AA-OH : DEAD : Ph_3P	HO-HMP#	3 : 3 : 3 : 1	THF (16 h, 0 °C)	0.3 Cys(Acm) 1.6	53 - 61	Sieber, 1987a Stanley et al., 1991
Fmoc-AA-OH : DCC : DMAP	HO-SASRIN#	1.5 : 1.2 : 0.01-0.1	DMF-DCM (1:3) (20 h, 0 °C)	0.2 - 1.0 Ile 1.2 Cys(Acm) 4.0 Cys(Trt) 18.3 His(Trt) 26.0	~80	Mergler et al., 1988a Mergler et al., 1989b
Fmoc-AA-OH : DCC : HOBt	HO-HMP#	4 : 4 : 3 : 1	DMA (17 h)	<0.05	60 - 94 Arg 6, Asn 31 Gln 29, Pro 54 .	Grandas et al., 1989a
Fmoc-AA-OH : MSNT : MeIm	HO-HMP#	2 : 2 : 1.5 : 1	DCM[f] (0.5 h, twice) His(Bum) $CHCl_3$	0 - 0.6 Cys(S*t*Bu) 2.1 Asp(O*t*Bu) 1.4	72 - 100	Blankemeyer-Menge et al., 1990
Fmoc-AA-OH : DIEA	Br-HMP*	1.1 : 1 : 1	DMF (2-3 h)	<0.1	61 - 99	Bernatowicz et al., 1990
Fmoc-AA-OH : DIEA	Cl-Trt#	0.3-1.0 : 2.5 : 1.6	DCE (0.5 h)	<0.05	61 - 99, Trp 51	Barlos et al., 1991a
Fmoc-AA-O^- Cs^+	Cl-CH_2-®#	1 : 1	DMF (16 h, 50 °C)	N.R.	72 - 95	Gisin, 1973
Fmoc-AA-O^- Cs^+	Cl-HMP#	2 : 1	DMA (15-24 h, 50 °C)	0.01 - 0.07	89 - 98	Colombo et al., 1983
Fmoc-AA-O^- Cs^+ : NaI	Cl-SASRIN#	1.5-3.0 : 1 : 1	DMA (24 h)	0.1 - 0.7 Cys(Trt) 2.5	N.R.	Mergler et al., 1989b
Fmoc-AA-OTDO : DIEA	HO-HMP#	40 mM : 1 : 1	DCM[f] (1-2 h), Ile 10 h	<0.2 Cys(Acm) 1.0	81 - 97	Kirstgen et al., 1987 Kirstgen and Steglich, 1989
Fmoc-AA-Cl	HO-HMP#	5 : 1	pyridine-DCM (2:3), (1 h)	< 0.5 Met 1.7, Ala 0.7	~100	Akaji et al., 1990a
Fmoc-NCA : NMM	Rink acid#	3 : 0.02 : 1	toluene[f] (0.5-1 h)	<0.1	N.R.	Fuller et al., 1990

[a] The column entitled Linker distinguishes between ester bond formation *first* to provide a preformed handle (*) (often the preferred route, as discussed further in the text) and ester bond formation *directly* to the linker resin (#).

[b] Stoichiometries (equivalents) are stated in the same order as the reagents are listed, followed last by the linker or resin.

[c] When publications state overnight reactions, this table indicates 18 h.

[d] Reactions are at room temperature (25 °C) unless otherwise stated.

[e] Values are representative for incorporation of most amino acids. Individual amino acids falling outside of the general range are also listed.

[f] The solvent must be dry, or yields decrease dramatically.

Fmoc-Cys(Trt) loading with DCC and HOBt (Atherton et al., 1991). Loading of Fmoc-Cys(Acm), Fmoc-Cys(Trt), and Fmoc-His(Boc) to bromomethylated-linkers in the presence of DIEA has been reported to result in less than 0.1% D-isomers (Bernatowicz et al., 1990).

Peptide Amides

Most anchoring linkages that ultimately provide *C*-terminal peptide amides in a useful and general manner are benzhydrylamide derivatives. The attachment step is a direct coupling of an N^{α}-protected amino acid by means of its carboxyl to an appropriate benzhydrylamine resin, with eventual cleavage at a different locus providing the desired carboxamide. The 4-methylbenzhydrylamine (MBHA) linkage has been fine tuned with an electron-donating 4-methyl group (Matsueda and Stewart, 1981; Gaehde and Matsueda, 1981) to cleave in strong acid with good yields, yet it is completely stable to the conditions of Boc chemistry. The benzhydrylamide system also has been fine tuned with electron-donating methoxy groups to create the TFA-sensitive 4-(2′,4′-dimethoxyphenyl-aminomethyl)phenoxy (Rink amide) (Rink, 1987), 4-(4′-methoxy-benzhydryl)phenoxyacetic acid (Dod) (Stüber et al., 1989), 3-(amino-4-methoxybenzyl)-4,6-dimethoxyphenylpropionic acid (Breipohl amide) (Breipohl et al., 1989), and 4-succinylamino-2,2′,4′-trimethoxy-benzhydrylamine (SAMBHA) (Penke et al., 1988) linkers for use in Fmoc chemistry. Other structural themes are compatible with Fmoc chemistry and provide anchoring linkages that cleave in TFA to give peptides amides. These include the 5-(4-aminomethyl-3,5-dimethoxy-phenoxy)valeric acid (PAL) (Albericio and Barany, 1987b; Albericio et al., 1990a) and 5-(9-aminoxanthen-2-oxy)valeric acid (XAL) (Sieber, 1987c; Barany and Albericio, 1991a; Bontems et al., 1992) handles, both of which have highly desirable features by direct comparison to alternative procedures. Also, the photolabile 3-nitro-4-aminomethylbenzoic acid (Nonb) handle is an option with both Boc and Fmoc chemistries (Hammer et al., 1990). Dod, Breipohl amide, XAL, PAL, and Nonb handles in their *N*-protected (usually Fmoc) forms are attached to the appropriate amino-functionalized supports in situ with DIPCDI or BOP/DIEA in the presence of Dhbt-OH or HOBt (Stüber et al., 1989; Breipohl et al., 1989; Albericio et al., 1990a; Hammer et al., 1990).

Esterification of N^{α}-protected Asn and Gln can be sluggish (Barany and Merrifield, 1979; Wu et al., 1988; Fields and Fields, 1990). As an alternative, Boc-Glu(OH)-OBzl has been coupled (by means of an unprotected γ-carboxyl side chain) to benzhydrylamine resin, with HF cleavage yielding a peptide containing C-terminal Gln (Li et al., 1976). In parallel fashion, Fmoc-Asp(OH)-O*t*Bu or Fmoc-Glu(OH)-O*t*Bu have been coupled (by means of an unprotected β- or γ-carboxyl side-chain) to PAL, Rink amide, or Breipohl amide, with TFA cleavage yielding peptides containing *C*-terminal Asn or Gln (Albericio et al., 1990b; Breipohl et al., 1990; Fields and Fields, 1990).

The substitution level of Fmoc amino acid resins is determined by quantitative spectrophotometric monitoring following piperidine deblocking. Fmoc amino acyl resins (4 to 8 mg) are shaken or stirred in piperidine-DMF (3:7) (0.5 mL) for 30 minutes, following which MeOH (6.5 mL) is added, and the resin is allowed to settle. The resultant fulvene-piperidine adduct has UV absorption maxima at 267 nm (ε = 17,500 $M^{-1}cm^{-1}$), 290 nm (ε = 5800 $M^{-1}cm^{-1}$), and 301 nm (ε = 7800 $M^{-1}cm^{-1}$). For reference, a piperidine-DMF-MeOH solution (0.3:0.7:39) is prepared. Spectrophotometric analysis is typically carried out at 301 nm, with comparison to a free Fmoc amino acid (i.e., Fmoc-Ala) of known concentration treated under identical conditions. The substitution level (mmol/g) = (A_{301} × 106 μmol/mol × 0.007 L/7800 $M^{-1}cm^{-1}$ × 1 cm × mg of resin) (Meienhofer et al., 1979; D. Hudson, unpublished results).

FORMATION OF PEPTIDE BOND

There are currently four major kinds of coupling techniques that serve well for the *stepwise* introduction of N^{α}-protected amino acids for solid-phase synthesis. In the solid-phase mode, coupling reagents are used in excess to ensure that reactions reach completion. The ensuing discussion will skirt some of the rather complicated mechanistic issues and focus on practical details. Recommendations for coupling methods are included in Tables 4 and 5.

In situ Reagents

The classical example of an in situ coupling reagent is *N,N′*-dicyclohexylcarbodiimide (DCC) (Rich and Singh, 1979; Merrifield et al., 1988) (see Figure 5). The related *N,N*-diisopropylcarbodiimide (DIPCDI) is more convenient to use under some circumstances, because the resultant urea coproduct is more soluble in DCM. The generality of carbodiimide-mediated couplings is extended significantly by the use of 1-hydroxybenzotriazole (HOBt) as an additive, which accelerates carbodiimide-mediated couplings, suppresses racemization, and inhibits dehydration of the carboxamide side chains of Asn and Gln to the corresponding nitriles (König and Geiger, 1970a; König and Geiger, 1973; Mojsov et al., 1980). Recently, protocols involving benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), and 2-[2-oxo-1(2H)-pyridyl]-1,1,3,3-bispenta-

Table 4 Typical Protocols for Automated Boc Chemistry SPPS[a]

Cycle	*Function*	*Time*
1	DCM wash	3 × 1 min
2	TFA-DCM (1:1) deprotection	2 + 20 min
3	DCM wash	3 × 1 min
4	DIEA-DCM (1:9) neutralization	2 × 2 min
5	DCM wash	3 × 1 min
6	DMF or NMP wash	3 × 1 min
7a	Boc-amino acid (3 equiv) in DMF	5 min
7b	DIPCDI (3 equiv) in DMF[b]	60 min
or		
7a	Boc-amino acid (3 equiv) in DMF	5 min
7b	BOP (3 equiv):DIEA (5.3 equiv) in DMF[c]	45 min
or		
7	Boc-amino acid PSA (2 equiv) in DMF or NMP[c]	60 min
or		
7	Boc-amino acid preformed ester (3 equiv) in DMF or NMP	60 min
8	DMF or NMP wash	3 × 1 min

[a] Refer to original research papers for additional specifications and variations.
[b] HOBt (3 equiv) is added for Boc-Asn, -Gln, -Arg(Tos), -Arg(Mts), and -His(Dnp).
[c] Boc-Asn and -Gln require side-chain protection in this variation.

methyleneuronium tetrafluoroborate (TOPPipU) have deservedly achieved popularity. BOP, HBTU, TBTU, and TOPPipU require a tertiary amine, such as NMM or DIEA, for optimal efficiency (Dourtoglou et al., 1984; Fournier et al., 1988; Ambrosius et al., 1989; Gausepohl et al., 1989a; Seyer et al., 1990; Fields et al., 1991; Knorr et al., 1991). HOBt has been reported to accelerate further the rates of BOP- and HBTU-mediated couplings (Hudson, 1988a; Fields et al., 1991). In situ activations by excess HBTU or TBTU can cap free amino groups (Gausepohl et al., 1992); it is not known whether HOBt can suppress this side reaction. Acylations using BOP result in the liberation of the carcinogen hexamethylphosphoramide, which might limit its use in large-scale work. The modified BOP reagent benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) liberates potentially less toxic by-products (Coste et al., 1990). Protocols have been reported for the use of BOP to incorporate side-chain unprotected Thr and Tyr (Fournier et al., 1988; Fournier et al., 1989).

Table 5 Typical Protocols for Automated Fmoc Chemistry SPPS[a]

Cycle	*Function*	*Time*
1	DMF or NMP wash	3 × 1 min
2	Piperidine-DMF or NMP (1:4) deprotection	3 + 17 min
3	DMF or NMP wash	3 × 1 min
4a	Fmoc-amino acid (4 equiv) in DMF or NMP	5 min
4b	DIPCDI (4 equiv):HOBt (4 equiv) in DMF	60 min
or		
4a	Fmoc amino acid (4 equiv) in DMF or NMP	5 min
4b	BOP (3 equiv):NMM (4.5 equiv):HOBt (3 equiv) in DMF[b]	45 min
or		
4a	Fmoc amino acid (4 equiv): HBTU (3.8 equiv): HOBt (4 equiv) in DMF[b]	5 min
4b	DIEA (7.8 equiv)	45 min
or		
4	Fmoc-amino acid preformed ester (4 equiv) in DMF or NMP	60 min
5	DMF or NMP wash	3 × 1 min

[a] Refer to original research papers for additional specifications and variations.
[b] Fmoc-Asn and -Gln require side-chain protection in this variation.

Active Esters

A long-known but steadfast coupling method involves the use of active esters. The classical 2- and 4-nitrophenyl esters (ONo and ONp, respectively), used in DMF, allow relatively slow but dehydration-free introduction of Asn and Gln (Mojsov et al., 1980) (see Figure 6). ONo and ONp esters of Boc- and Fmoc amino acids are prepared from DCC and either 2- or 4-nitrophenol, and the undesired nitrile contaminant is separated easily (Bodanszky et al., 1973; Bodanszky et al., 1980). *N*-hydroxysuccinimide (OSu) esters of Fmoc amino acids have been used successfully in SPPS (Fields et al., 1988), but they are not recommended for general use due to the formation of succinimidoxycarbonyl-β-alanine-*N*-hydroxysuccinimide ester (Gross and Bilk, 1968; Weygand et al., 1968b).

More recently, workers have concentrated on pentafluorophenyl (OPfp), HOBt, 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine (ODhbt), and substituted 1-phenylpyrazolinone enol esters. Boc and Fmoc amino acid OPfp esters are prepared from DCC and pentafluorophenol (Kisfaludy et al., 1973; Penke et al., 1974; Kisfaludy and Schön, 1983) or pentafluorophenyl trifluoroacetate (Green and Berman, 1990). Although

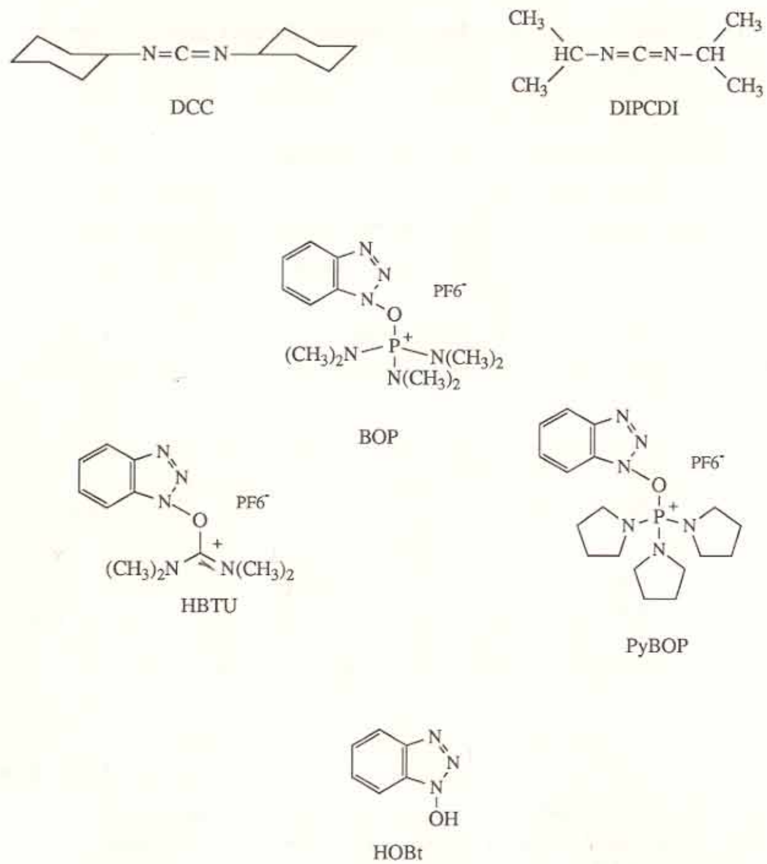

FIGURE 5 In situ coupling reagents and additives for SPPS.

OPfp esters alone couple slowly, the addition of HOBt (1 to 2 equiv) increases the reaction rate (Atherton et al., 1988a; Hudson, 1990b). Fmoc-Asn-OPfp allows for efficient incorporation of Asn with little side-chain dehydration (Gausepohl et al., 1989b). HOBt esters of Fmoc amino acids are formed rapidly (with DIPCDI) and highly reactive (Harrison et al., 1989; Fields et al., 1989), as are Boc amino acid HOBt esters (Geiser et al., 1988). N^{α}-protected amino acid ODhbt esters suppress racemization and are highly reactive, in similar fashion to HOBt esters (König and Geiger, 1970b). Preparation of ODhbt esters (from Dhbt-OH and DCC) is accompanied by the formation of the by-product 3-(2-azidobenzoyloxy)-4-oxo-3,4-dihydro-1,2,3-benzotriazine (König and Geiger,

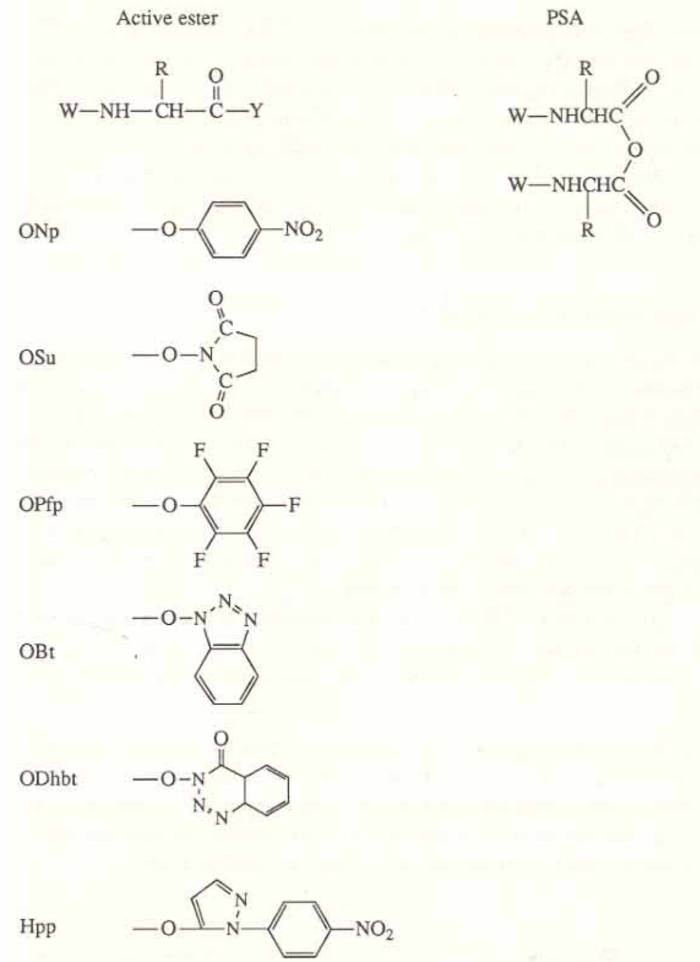

FIGURE 6 Activated N^{α}-protected amino acids. W is either Boc or Fmoc, Y is the structure specified next to the abbreviation of the active ester derivative.

1970c). Fmoc amino acid ODhbt esters are far more stable than HOBt esters and, therefore, can be isolated from the side product before use (Atherton et al., 1988b). Aminolysis by Fmoc amino acid esters of 1-(4-nitrophenyl)-2-pyrazolin-5-one (Hpp), 3-phenyl-1-(4-nitrophenyl)-2-pyrazolin-5-one (Pnp), and 3-methyl-1-(4-nitrophenyl)-2-pyrazolin-5-

one (Npp) also proceeds rapidly (Hudson, 1990a; Johnson et al., 1992). Competition experiments have shown ester reactivity usually to be Pnp > Hpp ~ Npp > ODhbt > OPfp > OSu > ONp > ONo (Hudson, 1990a; Hudson, 1990b; Johnson et al., 1991), although Hpp esters were found to be superior to Pnp and Npp esters for "difficult" couplings (Johnson et al., 1991). Both Fmoc-Tyr and Fmoc-Ser have been incorporated successfully as preformed active esters without side-chain protection (Fields et al., 1989; Otvös et al., 1989a).

Preformed Symmetrical Anhydrides

Preformed symmetrical anhydrides (PSAs) are favored by some workers because of their high reactivity (see Figure 6). They are generated in situ from the corresponding N^{α}-protected amino acid (2 or 4 equiv) plus DCC (1 or 2 equiv) in DCM; following removal of the urea by filtration, the solvent is exchanged to DMF for optimal couplings. Detailed synthetic protocols based on PSAs have been described for Boc (Merrifield et al., 1982; Yamashiro, 1987; Geiser et al., 1988; Kent and Parker, 1988; Wallace et al., 1989) and Fmoc (Chang et al., 1980a; Heimer et al., 1981; Atherton and Sheppard, 1989) chemistries.

The use of the PSA procedure to introduce Boc/Fmoc-Gly or -Ala occasionally results in inadvertent coupling of a diglycyl or dialanyl unit (Merrifield et al., 1974; Benoiton and Chen, 1987; Merrifield et al., 1988). Also, side-chain unprotected Asn and Gln, all Arg derivatives, and N^{τ}-protected His should not be used as PSAs due to the potential side reactions discussed previously (see Protection Schemes). The solubilities of some Fmoc amino acids make PSAs a less-than-optimum activated species. Not all Fmoc amino acids are readily soluble in DCM, thus requiring significant DMF for solubilization. Optimum activation conditions, which require neat DCM (Rich and Singh, 1979), cannot be obtained. In addition, the resulting Fmoc amino acid PSAs are even less soluble than the parent Fmoc amino acid (Harrison et al., 1989).

> Efficient couplings using Boc amino acid PSAs are critically dependent on the concentration of the activated species in solution. It has been recommended that Boc amino acid PSA couplings proceed at a concentration of 0.15 M and that double couplings be standard practice for syntheses of >50 residues (Kent and Parker, 1988).

> Fmoc amino acids have variable solubility properties in relatively nonpolar solvents, such as DCM. Fmoc-Asp(O*t*Bu), -Glu(O*t*Bu), -Ile, -Leu, -Lys(Boc), -Ser(*t*Bu), -Thr(*t*Bu), and -Val are soluble in DCM, while Fmoc-Ala, -Gly, -Met, -Trp, and -Tyr(*t*Bu) require the presence of a more polar solvent (i.e., DMF) for solubilization. Fmoc-Asn, -Gln, -His(Bum), and -Phe require at least 60% DMF to remain in solution. Conversion of Fmoc amino acids to the corresponding PSAs results in poorer solubilities in nonpolar solvents. Thus, whether Fmoc amino acids are coupled in situ or as preactivated species, relatively polar solvents (DMF or NMP) should be used to ensure that all reactants are in solution.

Acid Halides

N^{α}-protected amino acid chlorides have a long history of use in solution synthesis. Their use in solid-phase synthesis has been limited, because the Boc group is not completely stable to reagents used in the preparation of acid chlorides. The Fmoc group, on the other hand, survives acid chloride preparation with thionyl chloride (Carpino et al., 1986), while both the Fmoc and Boc groups are stable to acid fluoride preparation with cyanuric fluoride (Carpino et al., 1990; Bertho et al., 1991; Carpino et al., 1991a). For derivatives with tBu side-chain protection, only the acid fluoride procedure can be used (Carpino et al., 1990). Fmoc amino acid chlorides and fluorides react rapidly under SPPS conditions in the presence of HOBt/DIEA and DIEA, respectively, with very low levels of racemization (Carpino et al., 1990; Carpino et al., 1991b).

MONITORING

A crucial issue for stepwise solid-phase peptide synthesis is the repetitive yield per deprotection/coupling cycle. There are a number of ways of monitoring these steps, including some with a possibility for "real-time" feedback based on the kinetics of appearance or disappearance of appropriate soluble chromophores measured in a flow-through system. Most accurate and meaningful are qualitative and quantitative tests for the presence of unreacted amines after an acylation step. Such tests should ideally be negative before proceeding further in the chain assembly. For certain active ester methods, the leaving group has "self-indicating" properties insofar as a colored complex is noted for as long as unreacted amino groups remain on the support. These various techniques reveal that high efficiencies can, indeed, be achieved in stepwise synthesis.

The best known qualitative monitoring methods are the ninhydrin (Kaiser et al., 1970) and isatin (Kaiser et al., 1980) tests for free N^{α}-amino and -imino groups, respectively, where a positive colorimetric response to an aliquot of peptide resin indicates the presence of unreacted N^{α}-amino/imino groups. These tests are easy, reliable, and require only a

Free amino groups are quantitated based on their reaction with ninhydrin to produce Ruhemann's purple. Three reagent solutions are required: solution 1 is phenol-ethanol (7:3), solution 2 is 0.2 mM KCN in pyridine, and solution 3 is 0.28 M ninhydrin in ethanol. A sample of Boc-peptide resin (2 to 5 mg) is incubated with 75 μL of solution 1, 100 μL of solution 2, and 75 μL of solution 3 for 7 minutes at 100 °C (Sarin et al., 1981; Applied Biosystems, Inc., 1989c). For Fmoc-peptide resins, premature removal of the Fmoc group (by pyridine) is minimized by adding 2 to 3 drops (20 to 40 μL) of glacial HOAc to each resin sample and heating the reaction mixture for 5 minutes instead of 7 minutes (Applied Biosystems, Inc., 1989b). Immediately following the designated incubation time, 60% aqueous ethanol (4.8 mL) is added to each sample with vigorous mixing. Once the peptide resin has settled, the absorbance of each sample solution is read at 570 nm; 60% ethanol is used as a reference. The concentration of free amino groups (mmol/g) = ($A_{570} \times 10^6$ μmol/mol × 0.005 L)/(15000 $M^{-1}cm^{-1} \times 1$ cm × mg of resin).

few minutes to perform, allowing the chemist to make a quick decision concerning how to proceed. A highly accurate quantitative modification of the ninhydrin procedure has been developed (see Box).

Other monitoring techniques exist that are generally nondestructive (noninvasive) and, therefore, can be carried out on the total batch. Resin-bound N^{α}-amino groups can be titrated with picric acid, 4,4′-dimethoxytrityl chloride, bromphenol blue dye, and quinoline yellow dye. Picric acid is removed from resin-bound amines with 5% DIEA in DCM, and the resulting chromophore is quantitated at 362 nm (Hodges and Merrifield, 1975; Arad and Houghten, 1990). For trityl monitoring, 4,4′-dimethoxytritylchloride and tetra-n-butylammonium perchlorate are reacted with the resin, released with 2% dichloroacetic acid in DCM, and quantitated at 498 nm (Horn and Novak, 1987; Reddy and Voelker, 1988). The effect of the dilute acid on Fmoc amino acid side-chain protecting groups and linkers has not been reported. For bromphenol blue and quinoline yellow monitoring, the dye is bound to free amino groups following deprotection, then displaced as acylation proceeds. Quantitative monitoring can be carried out at 600 and 495 nm for bromphenol blue and quinoline yellow, respectively (Krchňák et al., 1988; Flegel and Sheppard, 1990; Young et al., 1990). Gel-phase nuclear magnetic resonance (NMR) spectroscopy has been proposed for direct examination of resin-bound reactants (Epton et al., 1980; Giralt et al., 1984; Butwell et al., 1988), but that procedure would appear to suffer from the problems of sensitivity, expense, and time needed to accumulate data.

As an alternative to quantitating resin-bound species, soluble reactants or coproducts can be analyzed. Continuous measurement of electrical conductivity can be used to evaluate coupling and Fmoc deprotection efficiencies (Nielson et al., 1989; Fox et al., 1990; McFerran et al., 1991). The progress of Fmoc chemistry can be evaluated by observing at 300 to 312 nm, the decrease of absorbance when Fmoc amino acids are taken up during coupling and by the increase in absorbance when the Fmoc group is released with piperidine (Chang et al., 1980a; Atherton and Sheppard, 1985; Frank and Gausepohl, 1988; Atherton and Sheppard, 1989). Monitoring a decrease in Fmoc amino acid concentration at 300 nm can be complicated when OPfp esters are utilized (Atherton et al., 1988b). More straightforward acylation monitoring is possible when Fmoc amino acid ODhbt, Hpp, Pnp, and Npp esters are used. During the coupling of Fmoc amino acid ODhbt esters, the liberated HO-Dhbt component binds to free N^{α}-amino groups, producing a bright yellow color, which diminishes as acylation proceeds (Cameron et al., 1988). Real-time spectrophotometric monitoring proceeds at 440 nm (Cameron et al., 1988). In a similar way, ionization of the leaving group from Fmoc amino acid Npp esters by free N^{α}-amino groups results in a blood-red color (Hudson, 1990a). As coupling proceeds, the color change (to gold) can be monitored at 488 nm.

Unfortunately, continuous-flow monitoring is inherently insensitive for direct judgment of reaction endpoints. Assuming an initial twofold excess of activated incoming amino acid, absorbance will drop from 2.00 units to 1.05 units or 1.01 units, respectively, with 95 percent or 99 percent coupling. It is difficult to distinguish accurately between 1.05 and 1.01. In contrast, if unreacted resin-bound components are titrated for the same two cases, the fivefold difference between 0.05 and 0.01 is easily noted. The sensitivities of techniques monitoring resin-bound components is limited by nonspecific binding or irreversible reactions of the titrant with the protected peptide or resin, which contribute to background readings despite complete reactions.

Invasive monitoring of both synthetic efficiency and amino acid composition of peptide resins can be achieved by a powerful quantitative variation of the Edman sequential degradation, called preview analysis (Tregear et al., 1977; Matsueda et al., 1981; Kent et al., 1982). Crude peptide resins are sequenced directly; each Edman degradation cycle serves to identify a primary amino acid residue and preview the next amino acid in the sequence. Because preview is cumulative, quantitation of peaks after a number of cycles indicates the average level of deletion peptides and thus the overall synthetic efficiency. Since the linkers as well as most side-chain protecting groups used in Boc chemistry are stable to the conditions of Edman degradation, sequencing is a true solid-phase process; moreover, identification of amino acid phenylthiohydantoins requires a set of protected standards (Simmons and Schlesinger, 1980; Steiman et al., 1985). Preview sequence analysis of peptide resins made by Fmoc chemistry requires initial TFA cleavage, followed by immobilization (covalent or noncovalent) of the crude peptide on a suitable support (Kochersperger et al., 1989). Most side-chain protecting groups used in conjunction with Fmoc chemistry are not stable to the conditions of Edman degradation; hence, the usual free side-chain phenylthiohydantoin standards can be used.

The technique of "internal reference amino acids" (IRAA) is often very useful to accurately measure yields of chain assembly and retention of chains on the support during synthesis and after cleavage (Matsueda and Haber, 1980; Atherton and Sheppard, 1989; Albericio et al., 1990a). In addition, amino acid analysis of peptide resins may be used to monitor synthetic efficiency; the advent of microwave hydrolysis technology may permit rapid analysis (Yu et al., 1988).

AUTOMATION OF SOLID-PHASE SYNTHESIS

A significant advantage of solid-phase methods lies in the ready automation of the repetitive steps (see Tables 4 and 5). The first instrument for synthesis of peptides was built by Merrifield, Stewart, and Jernberg (1966) and is currently on display at the Smithsonian Institution. Numerous models for both batchwise and continuous-flow operation at various scales of operation are now commercially available. Some of these instruments include features to facilitate monitoring (compare to previous section). Supported procedures have also been introduced for the generation of large numbers of (usually related) peptide sequences in a reasonably short time, although with some sacrifices in the usual standards for purity and characterization.

Automated Synthesizers, 1-3 Simultaneous Syntheses

The Applied Biosystems, Inc., Models 430A (Kent et al., 1984) and 431A utilize either Boc or Fmoc chemistries, with reaction mixing by vigorous vortex or gas bubbling. The automated 430A and 431A cycles for Boc amino acids (PSA in DMF, HOBt ester in NMP) and Fmoc amino acids (HOBt ester in either DMF or NMP) have been described in detail (Geiser et al., 1988; Fields et al., 1989; Fields et al., 1990). The Boc amino acid PSA cycles feature solvent exchange, so that activation occurs in DCM and coupling in DMF. The 430A and 431A also use fully automated HBTU + HOBt in situ cycles (Fmoc chemistry in NMP), called *FastMoc*™ (Fields et al., 1991). The synthesis scale is from 0.1 to 0.25 mmol, with microprocessor control by means of an internal computer. The MilliGen/Biosearch 9600 also utilizes either Boc or Fmoc chemistries, with reaction mixing by nitrogen bubbling. Coupling for both Boc and Fmoc amino acids can be in situ (In-Reservoir Activation™) with BOP/HOBt, or by solution sampling and preactivation using DIPCDI or DIPCDI/HOBt. Cycle control is by Sequence Driven™ "Expert System" software utilized with an IBM PC/AT Synthesis Workstation. Advanced ChemTech Models 100, 200, and 400 use either Boc or Fmoc chemistries, with reaction mixing by nitrogen bubbling or oscillation. Coupling for the Models 200 and 400 is by preformed mixed or symmetrical anhydrides (with solvent exchange) or by preformed HOBt esters. The Model 100 does not preactivate, and thus it must use preformed or in situ species. Cycles for all Advanced ChemTech instruments are controlled by means of an IBM PS/2. Pharmaceutical considerations are fulfilled by the Model 400 (Birr, 1990a; Birr, 1990b), because it can utilize 100 g or more of resin. The Eppendorf Synostat™ P is compatible with either Boc or Fmoc chemistries, utilizing in situ HBTU or BOP activation at scales from 0.1 to 5.0 mmol with adjustable vortex mixing. The Rainin/Protein Technologies PS3 features coupling by in situ BOP with Fmoc amino acids only (all prepackaged) at scales from 0.1 to 0.5 mmol with nitrogen bubbling for reaction mixing. The MilliGen/Biosearch EXCELL is also an Fmoc only instrument, using in situ DIPCDI (in DMF-DCM) or BOP/HOBt for coupling on a 0.1 mmol scale. The Biotech Instruments BT 7600 is designed for Fmoc chemistry and operates at scales from 0.05 to 1.0 mmol, using preformed OPfp esters with continuous conductivity monitoring.

The just-mentioned instruments are designed for batchwise syntheses. Continuous-flow instruments (Fmoc chemistry only) include the MilliGen/Biosearch 9050, the NovaSyn™ Crystal, and the NovaSyn™ Gem. The MilliGen/Biosearch 9050 (Kearney and Giles, 1989) is a 3-column automated instrument with a synthesis scale of 0.1 to 1.0 mmol and flow rates typically from 5 to 15 mL/min. Coupling proceeds by means of in situ BOP/HOBt or DIPCDI, or preformed OPfp/HOBt esters with continuous spectrophotometric monitoring of both coupling and deprotection at 365 nm. An NEC APC IV computer controls the MilliGen Express-Peptide™ software. The NovaSyn™ Crystal (AminoTech, 1991) is, in similar fashion to the MilliGen/Biosearch 9050, a 3-column automated instrument with a synthesis scale of 0.05 to 0.7 mmol. Acylation methods include pre–formed OPfp and ODhbt esters, in situ PyBOP/HOBt, or PSA with continuous spectrophotometric analysis of both coupling (by counterion distribution monitoring) and deprotection. The software is controlled by an IBM compatible 1120/S. The NovaSyn™ Gem is a semiautomated, 2-column instrument with similar acylation and monitoring features as the NovaSyn™ Crystal (AminoTech, 1991).

Automated and Semiautomated Multiple Peptide Synthesizers

The Zinsser Analytic SMPS 350/Advanced ChemTech 350 utilizes two independent robotic arms, controlled through an IBM PS/2, to synthesize up to 96 peptides simultaneously. Only Fmoc chemistry is used, with coupling by PSAs or HOBt esters or in situ DIPCDI or TBTU, on a 0.5 mmol scale (Schnorrenberg and Gerhardt, 1989; Groginsky, 1990). The ABIMED Model AMS 422 (Gausepohl et al., 1990; Gausepohl et al., 1991) uses Fmoc chemistry to synthesize up to 48 peptides on a scale from 5 to 50 μmol with activation by in situ PyBOP. A single robotic arm dispenses reagents, while resins are contained in fritted polypropylene tubes. The manual Multiple Peptide Synthesis Tea Bag method can synthesize up to 120 peptides simultaneously, using either Boc or Fmoc chemistry and coupling by PSAs or in situ DCC/DIPCDI (Houghten et al., 1986; Beck-Sickinger et al., 1991). The Tea Bag method has been semiautomated (Beck-Sickinger et al., 1991) and commercialized by Labostec and Biotech Instruments (BT 7500). Biotech Instruments also supplies a PepSeal heat sealer for Tea Bag preparation. Cambridge Research Biochemicals markets the Pepscan/PIN method (Geysen et al., 1984; Hoeprich, et al., 1989; Arendt and Hargrave, 1991), which uses either Boc or Fmoc chemistry and coupling in situ with DCC or BOP/HOBt to synthesize up to 96 peptides (10 to 100 nmol) simultaneously. The semiautomated Dupont RaMPs™ (Wolfe and Wilk, 1989) synthesizes up to 25 peptides simultaneously by either Boc or Fmoc chemistry using PSAs or OPfp/HOBt esters. Finally, the just-described Affymax Parallel Chemical Synthesis system (VLSIPS, for "very large-scale immobilized polymer synthesis"), using the photolabile Nvoc N^{α}-protecting group and preformed HOBt esters, can be used for simultaneous synthesis of an extraordinarily high number of related peptides (e.g., $1024 = 2^{10}$ by 10 stages requiring 2.5 hours each) (Fodor et al., 1991).

CLEAVAGE

Boc SPPS is designed primarily for simultaneous cleavage of the peptide anchoring linkage and side-chain protecting groups with strong acid (HF or equivalent), while Fmoc SPPS is designed primarily to accomplish the same cleavages with moderate strength acid (TFA or equivalent). In each case, careful attention to cleavage conditions (reagents, scavengers, temperature, and times) is necessary in order to minimize a variety of side reactions. Considerations for separate removal of acid-stable side-chain protecting groups have been covered earlier (see Protection Schemes). Nonacidolytic methods for cleavage of the anchoring linkage, each with certain advantages as well as limitations, may also be used in conjunction with either Boc or Fmoc chemistries.

Hydrogen Fluoride (HF)

Treatment with HF simultaneously cleaves PAM and MBHA linkages and removes the side-chain protecting groups commonly applied in Boc

> HF cleavage procedures require a special all-fluorocarbon apparatus. The standard method uses HF-anisole (9:1) (1 mL per 20 μmol peptide) at 0 °C for 1 hour, with the addition of 2-mercaptopyridine (10 equiv) for Met-containing peptides. For Cys- and Trp-containing peptides, HF-anisole-dimethylsulfide-4-thiocresol (10:1:1:0.2) is recommended. Following cleavage, HF is evaporated carefully under aspirator suction with ice-bath cooling, and most of the anisole is removed by vacuum from an oil pump. The vessel is triturated with ether to remove benzylated scavenger adducts; at the same time, the ether facilitates transfer of the cleaved resin (with trapped peptide) to a fritted glass funnel. Next, 30% aqueous HOAc (twice, 1.5 mL per 20 μmol peptide) is used to rinse the cleavage vessel and extract the resin on the fritted glass filter. The combined filtrates are diluted with H_2O to bring the HOAc concentration to <10%, and the peptide is usually recovered by lyophilization (Stewart and Young, 1984; Tam and Merrifield, 1987; Applied Biosystems, Inc., 1989c). "Low-high" HF cleavages are carried out following the detailed description of the original and later publications (Tam et al., 1983; Tam and Merrifield, 1985; Tam and Merrifield, 1987).

chemistry (Figure 2), i.e., Bzl (for Asp, Glu, Ser, Thr, and Tyr), 2-BrZ or 2,6-Cl_2Bzl (for Tyr), cHex (for Asp), 2-ClZ (for Lys), Bom or Tos (for His), Tos or Mts (for Arg), Xan (for Asn and Gln), and Meb (for Cys) (Tam and Merrifield, 1987). HF cleavages are always carried out in the presence of a carbonium ion scavenger, usually 10% anisole. For cleavages of Cys-containing peptides, further addition of 1.8% 4-thiocresol is recommended. Additional scavengers, such as dimethylsulfide, 4-cresol, and 4-thiocresol, are used in conjunction with a two-stage "low-high HF" cleavage method that provides extra control and thereby better product purities (Tam and Merrifield, 1987). Both Trp(CHO) and Met(O) can be deprotected under "low HF" conditions [20 to 25% HF–0 to 5% 4-thiocresol–70 to 80% dimethylsulfide, 0 °C for 1 hour; 4-thiocresol is necessary only for Trp(CHO)] (Tam and Merrifield, 1987). In the presence of the large levels of dimethylsulfide used in "low HF" conditions, Tyr(Bzl) undergoes little C-alkylation (Tam and Merrifield, 1987).

In strong acid, the γ-carboxyl group of Glu can become protonated and lose water. The resulting acylium ion is then trapped either intramolecularly by the N^{α}-amide nitrogen to give a pyrrolidone or (more likely) intermolecularly with the commonly used scavenger anisole (Feinberg and Merrifield, 1975). This serious problem can be controlled by attenuation of the acid strength (i.e., "low HF" conditions) and caution with regard to cleavage temperature (Tam and Merrifield, 1987). Strong acid can also cause an N → *O* acyl shift in Ser- and Thr-containing peptides, resulting in the thermodynamically less stable *O*-acyl species (Fujino et al., 1978). This process can be reversed for simple cases by treating the cleaved, deprotected peptide with 5% aqueous NH_4HCO_3, pH 7.5, at 25 °C for several hours or in 2% aqueous NH_4OH at 0 °C for 0.5 hour (Barany and Merrifield, 1979); reversal of the $N \rightarrow O$ acyl shift in multiple Ser- and Thr-containing peptides may be more problematic. HF-liberated Boc groups can modify Met residues (Noble et al., 1976); therefore, the N^{α}-Boc group should be removed prior to HF cleavage. HF deprotection of His(Bom) liberates formaldehyde, resulting in methylation of susceptible side chains and cyclization of *N*-terminal Cys residues to a thiazolidine (Mitchell et al., 1990; Gesquière et al., 1990; Kumagaye et al., 1991). These side reactions may be inhibited by including in the HF cleavage mixture a formaldehyde scavenger, e.g., resorcinol (0.27 M), or Cys or $CysNH_2$ (30 to 90 equiv), and purifying the peptide immediately after cleavage (Mitchell et al., 1990; Kumagaye et al., 1991). Serious Trp alkylation side reactions have been observed during workup after HF cleavage of peptides containing Cys or Met adjacent to Trp; the problem may be mitigated by adding free Trp (10 equiv) as a scavenger during cleavage (D. Hudson, unpublished results) or during the initial lyophilization (Ponsati et al., 1990a).

Other Strong Acids

The alternative strong acids listed here, and with further examples elsewhere (Barany and Merrifield, 1979), are very likely to promote the same side reactions just described for HF. TFMSA (1 M)-thioanisole (1 M) in TFA cleaves PAM and MBHA linkers (Tam and Merrifield, 1987; Bergot et al., 1987), and removes many side-chain protecting groups used in Boc chemistry, e.g., Mts (for Arg), Bzl (for Asp, Glu, Ser, Thr), cHex (for Asp), Meb (for Cys), 2-ClZ (for Lys), 2-BrZ or 2,6-Cl_2Bzl (for Tyr), and Bom or Tos (for His) (Tam and Merrifield, 1987), without requiring a special apparatus. A "low-high" method can be used with TFMSA, in similar fashion to HF (Tam and Merrifield, 1987). Tetrafluoroboric acid (HBF_4) (1 M)-thioanisole (1 M) in TFA offers a similar range of side-chain deprotection as TFMSA (Kiso et al., 1989; Akaji et al., 1990b).

Trimethylsilyl bromide (TMSBr) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) have also been used for strong acid cleavage and deprotection reactions, which are accelerated by the presence of thioanisole as a "soft" nucleophile (Yajima et al., 1988; Nomizu et al., 1991). TMSBr (1 M)-thioanisole (1 M) removes Mts (for Arg), Bzl (for Asp, Glu, Ser, Thr, and Tyr), 2,6-Cl_2Bzl (for Tyr), and 2-ClZ (for Lys) as well as reducing Met(O) to Met (Yajima et al., 1988). Although not specifically stated, TMSBr-thioanisole probably deprotects His(Tos), Cys(Meb), and Tyr(2-BrZ). TMSOTf (1 M)-thioanisole (1 M) additionally removes Bom from His (Yajima et al., 1988) and efficiently

For TFMSA cleavages, peptide resin (100 mg) is stirred in thioanisole (187 μL) and 1,2-ethanedithiol (EDT) (64 μL) in an ice bath for 10 minutes. TFA (1.21 mL) is added, and after equilibration for 10 minutes, TFMSA (142 μL) is added *slowly*. Cleavage and deprotection proceeds for 1 hour (unless the MBHA linker is being cleaved, in which case cleavage proceeds for 1.5 to 2.5 hours). During cleavage and deprotection, the ice bath is removed, but precautions are taken to ensure that the temperature of the reaction does not increase rapidly. Subsequently, the cleavage mixture is filtered through a fritted glass funnel directly into methyl *t*Bu ether, to rapidly precipitate the peptide and remove the acid and scavengers. The precipitated peptide should be washed with methyl *t*Bu ether and dried under vacuum overnight (Bergot et al., 1987; Fields and Fields, 1991).

cleaves PAM and MBHA linkages (Akaji et al., 1989; Nomizu et al., 1991). Stable Cys side-chain protection should be used during TMSBr-thioanisole cleavages.

Trifluoroacetic Acid (TFA)

The combination of side-chain protecting groups, e.g., *t*Bu (for Asp, Glu, Ser, Thr, and Tyr), Boc (for His and Lys), Bum (for His), Tmob (for Asn, Cys, and Gln), and Trt (for Asn, Cys, Gln, and His), and anchoring linkages, e.g., HMP/PAB or PAL, commonly used in Fmoc chemistry (see Figure 3), are simultaneously deprotected and cleaved by TFA. Such cleavage of *t*Bu and Boc groups results in *tert*-butyl cations and *tert*-butyl trifluoroacetate formation (Jaeger et al., 1978a; Jaeger et al., 1978b; Löw et al., 1978a; Löw et al., 1978b; Lundt et al., 1978; Masui et al., 1980). These species are responsible for *tert*-butylation of the indole ring of Trp, the thioether group of Met, and, to a very low degree (0.5 to 1.0%), the 3′-position of Tyr. Modifications can be minimized during TFA cleavage by utilizing effective *tert*-butyl scavengers. An early comprehensive study showed the advantages of 1,2-ethanedithiol (EDT) (Lundt et al., 1978); this thiol has the additional virtue of protecting Trp from oxidation that occurs due to acid-catalyzed ozonolysis (Scoffone et al., 1966). To avoid acid-catalyzed oxidation of Met to its sulfoxide, a thioether scavenger, such as dimethylsulfide, ethylmethylsulfide, or thioanisole, should be added (Guttmann and Boissonnas, 1959; King et al., 1990). TFA deprotection of Cys(Trt) is reversible in the absence of a scavenger, and it can occur readily following TFA cleavage if solutions of crude peptide in TFA are concentrated by rotary evaporation or

Cleavage and side-chain deprotection of peptide resins assembled by Fmoc chemistry is carried out in TFA in the presence of carefully chosen scavengers. The text discussion should be consulted with respect to peptide sequence and potential side reactions. It is recommended that small-scale cleavages (<10 mg peptide resin) be performed and analyzed before proceeding to large-scale cleavages. Standard cleavages of HMP/PAB, Dod, and PAL linkers and simultaneous side-chain deprotection proceed by stirring peptide resin (50 to 200 mg) in 2 mL of the appropriate, *freshly* prepared cleavage cocktail for 1.5 to 2.5 hours at 25 °C. The resin is filtered over a medium fritted glass filter and rinsed with 1 mL of TFA. The combined filtrate and TFA rinse are either (a) precipitated with methyl *t*Bu ether (~50 mL) or (b) dissolved in ~40 mL H_2O and extracted six times with ~40 mL methyl *t*Bu ether. Following (a), the mixture is centrifuged at 3000 rpm for 2 minutes and decanted. The peptide pellet is dispersed with a rubber policeman, washed thoroughly with methyl *t*Bu ether, and dried overnight in a lyophilizer (King et al., 1990; Albericio et al., 1990a). Following (b), the H_2O layer is loaded directly to a semipreparative HPLC column and the peptide is purified.

lyophilization (Photaki et al., 1970; D. Hudson, unpublished results). EDT, triethylsilane, or triisopropylsilane are recommended as efficient scavengers to prevent Trt reattachment to Cys (Pearson et al., 1989); this recommendation extends to prevent reattachment of Tmob as well (Munson et al., 1992). TFA deprotection of His(Bum) liberates formaldehyde, in a similar fashion to HF deprotection of His(Bom) (Gesquière et al., 1992). Cyclization of *N*-terminal Cys residues to a thiazolidine is only partially (60%) inhibited by even complex TFA/scavenger mixtures, such as reagent K (see following discussion).

The indole ring of Trp can be alkylated irreversibly by Mtr and Pmc groups from Arg (Sieber 1987b; Harrison et al., 1989; Riniker and Hartmann, 1990; King et al., 1990), Tmob groups from Asn, Gln, or Cys (Gausepohl et al., 1989b; Sieber and Riniker, 1990), and even by some TFA-labile ester and amide linkers (Atherton et al., 1988a; Riniker and Kamber, 1989; Albericio et al., 1990a; Gesellchen et al., 1990). Cleavage of the Pmc group may also result in *O*-sulfation of Ser, Thr, and Tyr (Riniker and Hartmann, 1990; Jaeger et al., 1992). Two efficient cleavage "cocktails" for Mtr/Pmc/Tmob quenching and preservation of Trp, Tyr, Ser, Thr, and Met integrity are TFA-phenol-thioanisole-EDT-H_2O (82.5:5:5:2.5:5) (reagent K) and TFA-thioanisole-EDT-anisole (90:5:3:2) (reagent R) (Albericio et al., 1990a). Recent studies on Trp preservation during amino acid analysis (Bozzini et al., 1991) has led to the development of reagent K′, where EDT is replaced by 1-dodecanethiol (Fields

and Fields, unpublished results). H_2O is an essential component of reagents K and K′, but phenol is necessary only with multiple Trp-containing peptides (King et al., 1990). Thioanisole, a soft nucleophile, accelerates TFA deprotection of both Arg(Mtr) and Arg(Pmc). Triethylsilane (4 equiv) in MeOH-TFA (1:9) has been reported to efficiently cleave and scavenge Pmc groups (Chan and Bycroft, 1992). Given a choice for Arg protection, Pmc is preferred because it is more labile and it gives less Trp alkylation during unscavenged TFA cleavage; the recommendation for Pmc is particularly appropriate for sequences containing multiple Arg residues (Green et al., 1988; Harrison et al., 1989; King et al., 1990). The Trt group, instead of Tmob, is suggested for Asn/Gln side-chain protection in Trp-containing peptides, because Trt cations are easier to scavenge (Sieber and Riniker, 1990).

Nonacidolytic Cleavage Methods

Benzyl ester anchoring linkages can be cleaved usefully under nonacidic conditions. An interesting alternative to standard HF cleavages for Boc chemistry is catalytic transfer hydrogenolysis (CTH), which removes Bzl side-chain protecting groups (from Asp, Glu, Ser, Thr, and Tyr) and cleaves benzyl ester anchors to provide a *C*-terminal carboxyl group (Anwer and Spatola, 1980; Anwer and Spatola, 1983). Peptide resin (1 g) is treated initially with palladium (II) acetate (1 g) in DMF (13 mL) for 2 hours; ammonium formate (1.3 g) is then added, and the reaction proceeds for an additional 2 hours (Anwer and Spatola, 1983). CTH can reduce Trp to octahydrotryptophan (Méry and Calas, 1988). Benzyl ester linkages also may be cleaved by 2-dimethylaminoethanol (DMAE)-DMF (1:1) for 70 hours, with subsequent treatment of the peptide-DMAE ester by DMF-H_2O (1:5) for 2 hours, yielding the peptide acid (Barton et al., 1973). For some applications when peptide amides are required, benzyl ester-type linkages are treated with NH_3 in anhydrous MeOH, 2-propanol, 2,2,2-trifluoroethanol (TFE), or MeOH-DMF for 2 to 4 days at 25 °C (Atherton et al., 1981c; Stewart and Young, 1984; Story and Aldrich, 1992), although these conditions will also convert Asp(OBzl) and Glu(OBzl) residues to Asn and Gln, respectively. Additionally, ethanolamine in DMF or MeOH cleaves benzyl ester linkages (8 to 40 hours, 45 to 60 °C) to provide an ethanolamidated peptide *C*-terminus (Prasad et al., 1982; Fields et al., 1988; Fields et al., 1989; Prosser et al., 1991). Nucleophilic cleavages of benzyl esters can be accompanied by side reactions, including racemization of the *C*-terminal residue (Barany and Merrifield, 1979). On the other hand, base cleavages of the NPE and HMFA linkers appear to be quite safe and general. Peptide acids are obtained upon treatment with either piperidine (15 to 20%)-DMF or DBU (0.1 M)-1,4-dioxane, after 5 minutes (for HMFA) to 2 hours (for NPE) at 25 °C (Liu et al., 1990; Albericio et al., 1991b).

Following synthesis, peptide resins should be well dried, and then stored in a desiccator at 4 °C with the N^{α}-terminus protected. As a general practice, peptides should never be stored in the solid state after being lyophilized from moderate or strong acid; deamidation, among other side reactions, may proceed rapidly (see Auxiliary Issues). If stored in solution following purification, peptides should be used for biological or chemical studies as soon as possible. Met-containing peptides oxidize rapidly upon storage in solution, especially when repeated freeze and thawing occurs (Stewart and Young, 1984), while Asn-containing peptides can hydrolyze spontaneously in solution (see Auxiliary Issues).

Palladium-catalyzed peptide resin cleavage is used for the HYCRAM™ linker (Kunz and Dombo, 1988; Guibé et al., 1989). The fully protected peptide resin (0.1 mmol of peptide) is shaken for 6 to 18 hours under N_2 or argon atmosphere in 8 mL of DMSO-THF-0.5 M aqueous HCl (2:2:1) in the presence of 50 to 190 equiv of either NMM (for Boc-peptides) or dimedone (for Fmoc-peptides) as well as 0.015 equiv of the tetrakis(triphenylphosphino)palladium(0) catalyst. The reaction mixture is filtered and washed with DMF, DMF-0.5 M aqueous HCl (1:1), and DMF. The filtrate and washings are combined and evaporated to minimal volume; the peptide is then precipitated with methyl *t*Bu ether (Orpegen, 1990; Lloyd-Williams et al., 1991b). The crude peptide acid (0.7 mmol scale) can be converted to an amide by dissolving in dry DMF (50 mL) at 25 °C, adding NMM (80 μL), cooling to −20 °C, adding isobutylchloroformate (90 μL) and, after 8 minutes, 25% aqueous NH_4OH (0.3 mL), and stirring for 2 to 12 hours. The solution is evaporated and the peptide amide dried over P_2O_5 in vacuo (Orpegen, 1990).

Photolytic cleavage at 350 nm under inert (N_2, Ar) atmosphere is used for the ONb, 2-bromopropionyl, and Nonb linkers. The most efficient photolysis of the ONb and Nonb linkers is achieved when peptide resins are swollen with 20 to 25% 2,2,2-trifluoroethanol in either DCM or toluene (Giralt et al., 1986; Kneib-Cordonier et al., 1990; Hammer et al., 1990). Photolytic cleavage yields after 9 to 16 hours range from 45 to 70 percent for relatively small peptides (5 to 9 residues). The 2-bromopropionyl linker is cleaved in DMF with a yield of 70 percent after 72 hours for a tetrapeptide (Wang, 1976).

POST-TRANSLATIONAL MODIFICATIONS AND UNNATURAL STRUCTURES

Peptides of biological interest often include structural elements beyond the 20 genetically encoded amino acids. This section summarizes the best current methods to duplicate by chemical synthesis the post-translational modifications achieved in nature, including the alignment of half-cystine residues in disulfide bonds. This section also covers a set of unnatural structures that are of considerable interest for peptide drug design, namely side-chain to side-chain lactams, and lastly describes the steps needed to elicit good antibody production from synthetic peptides.

Hydroxylated Residues

Hydroxyproline (Hyp) has been incorporated successfully without side-chain protection in both Boc (Felix et al., 1973; Stewart et al., 1974) and Fmoc (Fields et al., 1987; Netzel-Arnett et al., 1991) SPPS. Alternatively, the usual hydroxyl protecting groups Bzl (Cruz et al., 1989) for Boc and *t*Bu (Becker et al., 1989) for Fmoc can be used. Fmoc SPPS of unprotected Hyp-containing peptides can be carried out without affecting the homogeneity of the product (Fields and Noble, 1990).

Hydroxylysine (Hyl) has been incorporated in SPPS as FmocHyl-(Boc,*O*-Tbdms). This derivative was prepared by protecting the N^{ε}-amino group of acetyl-Hyl by Boc-N_3, removing the N^{α}-acetyl group enzymatically with acylase I, adding the Fmoc group, and, finally, blocking the side-chain hydroxyl group with tert-butyldimethylsilyl chloride (Penke et al., 1989).

γ-Carboxyglutamate

Acid-sensitive γ-carboxyglutamate (Gla) residues have been identified in a number of diverse biomolecules, such as prothrombin and the "sleeper" peptide from the venomous fish-hunting cone snail (*Conus geographus*). Fmoc chemistry has been utilized for the efficient SPPS of the 17-residue sleeper peptide, with the five Gla residues incorporated as Fmoc-Gla(O*t*Bu)$_2$ (Rivier et al., 1987). Cleavage and side-chain deprotection of the peptide resin by TFA-DCM (2:3) for 6 hours resulted in no apparent conversion of Gla to Glu.

Phosphorylation

Incorporation of side-chain phosphorylated Ser and Thr by SPPS is especially challenging, because the phosphate group is decomposed by strong acid and lost with base in a β-elimination process (Perich, 1990). Boc-Ser(PO_3Ph_2) and Boc-Thr(PO_3Ph_2) have been used, where HF or hydrogenolysis cleaves the peptide resin, and hydrogenolysis removes the phenyl groups from the phosphate (Perich et al., 1986; Arendt et al., 1989). Alternatively, peptide resins that were built up by Fmoc chemistry to include unprotected Ser or Thr side chains may be treated with a suitable phosphorylating reagent, e.g., *N,N*-diisopropyl-bis(4-chlorobenzyl)phosphoramidite or dibenzylphosphochloridate. The desired phosphorylated peptide is then obtained in solution following simultaneous deprotection and cleavage with TFA in the presence of scavengers (Otvös et al., 1989a; de Bont et al., 1990).

Side-chain phosphorylated Tyr is less susceptible to strong acid decomposition, and it is not at all base-labile. Thus, SPPS has been used to incorporate directly Fmoc-Tyr(PO_3Me_2) (Kitas et al., 1989), Fmoc-Tyr(PO_3Bzl_2) (Kitas et al., 1991), Fmoc-Tyr(PO_3tBu_2) (Perich and Reynolds, 1991), and Boc-Tyr(PO_3^{2-}) (Zardeneta et al., 1990). Syntheses incorporating Fmoc-Tyr(PO_3Bzl_2) use 2% DBU in DMF for N^{α}-amino deprotection, because piperidine was found to remove the benzyl protecting groups from phosphate (Kitas et al., 1991). TFMSA or TMSBr can be used for peptide resin cleavage and removal of the methyl phosphate groups without O-dephosphorylation (Kitas et al., 1989; Zardeneta et al., 1990), while TFA is used for removal of the benzyl and *tert*-butyl phosphate groups (Kitas et al., 1991).

Sulfation

Gastrin, cholecystokinin, and related hormones contain sulfated Tyr; thus, incorporation of this residue into synthetic peptides is of great interest. Synthesis of Tyr sulfate-containing peptides is difficult, as a result of the substantial acid lability of the sulfate ester; also, most sulfating agents are more reactive toward the hydroxyls of Ser or Thr with respect to the phenol of Tyr. While there is an elegant history of success in solution chemistry (Beacham et al., 1967; Ondetti et al., 1970; Pluscec et al., 1970; Wünsch et al., 1981), this brief discussion focuses on the best SPPS approaches. Side-chain unprotected Tyr can be incorporated by Boc or Fmoc chemistry, and sulfation is carried out while the otherwise fully protected peptide remains anchored to the support, achieved by use of pyridinium acetyl sulfate (Fournier et al., 1989). Base- or acid-promoted deprotection/cleavage follows under conditions that are carefully optimized to avoid or minimize desulfation. Alternatively, sulfated Tyr can be incorporated directly by use of Fmoc-Tyr($SO_3^-Na^+$)-OPfp, Fmoc-Tyr($SO_3^-Na^+$), or Fmoc-Tyr($SO_3^-Ba_{1/2}^{2+}$) in situ with BOP/HOBt (Penke and Rivier, 1987; Penke and Nyerges, 1989; Penke and Nyerges, 1991; Bontems et al., 1992). A brief and carefully optimized acidolytic cleavage/deprotection is then used to minimize desulfation.

Glycosylation

Methodology for site-specific incorporation of carbohydrates during chemical synthesis of peptides has developed rapidly. The mild conditions of Fmoc chemistry are more suited for glycopeptide syntheses than Boc chemistry, because repetitive acid treatments can be detrimental to sugar linkages (Kunz, 1987). Fmoc-Ser, -Thr, -Hyp, and -Asn have all been incorporated successfully with glycosylated side chains. Side-chain glycosylation is performed with glycosyl bromides or glycose-$BF_3{\cdot}Et_2O$ for Ser, Thr, and Hyp, and glycosylamines for Asp (to produce a glycosylated Asn). The side-chain glycosyl is usually hydroxyl protected by either the Bzl or acetyl group (Paulsen et al., 1988; Torres et al., 1989; Paulsen et al., 1990; Jansson et al., 1990; de la Torre et al., 1990; Bardají et al., 1991; Biondi et al., 1991), although some SPPS have been successful with no protection of glycosyl hydroxyl groups (Otvös et al., 1989b; Otvös et al., 1990; Filira et al., 1990). Glycosylated residues are incorporated as preformed Pfp esters or in situ with DCC/HOBt (Paulsen et al., 1988; Paulsen et al., 1990; Bardají et al., 1991; Meldal and Jensen, 1990; Filira et al., 1990; Jansson et al., 1990; Otvös et al., 1990; Biondi et al., 1991). These sugars are relatively stable to Fmoc deprotection by piperidine or morpholine (Paulsen et al., 1988; Paulsen et al., 1990; Meldal and Jensen, 1990; Jansson et al., 1990; Filira et al., 1990; Otvös et al., 1990; Bardají et al., 1991; Biondi et al., 1991), brief treatments with TFA for side-chain deprotection and peptide resin cleavage (Paulsen et al., 1988; Filira et al., 1990; Paulsen et al., 1990; Meldal and Jensen, 1990; Jansson et al., 1990; Otvös et al., 1990; Bardají et al., 1991; Biondi et al., 1991), and palladium treatment for peptide resin cleavage from HYCRAM™ (Kunz and Dombo, 1988). Deacetylation and debenzylation are performed with hydrazine-MeOH (4:1) prior to glycopeptide resin cleavage (Kunz, 1987; Bardají et al., 1991).

Disulfide Bond Formation

In the majority of cases, intramolecular disulfides or simple intermolecular homodimers have been formed from purified linear precursors by nonspecific oxidations in dilute solutions. An even number of Cys residues are brought to the free thiol form by removal of the same *S*-protecting group, following which disulfide formation is mediated by molecular O_2 (from air), potassium ferricyanide [$K_3Fe(CN)_6$], DMSO, or others from a lengthy catalogue of reagents (Hiskey, 1981; Stewart and Young, 1984; Gariépy et al., 1987; McCurdy, 1989; Tam et al., 1991b). Accomplishing the same end, but proceeding by a different mechanism, the polythiol can be treated with a mixture of reduced and oxidized glutathione, which catalyzes the net oxidation by thiol-disulfide exchange reactions (Ahmed et al., 1975; Lin et al., 1988; Pennington et al., 1991). These procedures, which require scrupulous attention to experimental details, have often given the desired disulfide-containing peptide products in acceptable yields. However, even under the best conditions, significant levels of dimeric, oligomeric, or polymeric materials are observed. The nonmonomeric material has usually proved to be difficult to "recycle" by alternating reduction and reoxidation steps.

A more sophisticated approach, which also requires dilute solutions, involves selective pairwise co-oxidations of two designated free or protected sulfhydryl groups. Such reactions are best carried out in intramolecular fashion, because if the paired groups are on separate chains, there is the problem that homodimers form along with the desired heterodimer. If the thiols have already been deblocked, oxidation follows using procedures mentioned earlier. The prototype oxidative deprotections involve I_2 treatments on Cys(Trt) or Cys(Acm) residues (Kamber et al., 1980). These reactions are carried out in neat or mixtures of the solvents TFE, MeOH, 1,1,1,3,3,3-hexafluoroisopropanol, HOAc, DCM, and chloroform and often proceed in modest to high yield; however, side reactions have been observed at Trp residues, resulting in Trp-2′-thioethers (Sieber et al., 1980) and β-3-oxindolylalanine (Casaretto and Nyfeler, 1991). Pairwise oxidative removal of appropriate Acm or Tacm Cys protecting groups with $Tl(Tfa)_3$ or methyltrichlorosilane (in the presence of diphenylsulfoxide) also furnishes the disulfide directly (Fujii et al., 1987; Kiso et al., 1990; Akaji et al., 1991). However, Trp and Met must be side-chain protected during such treatments. As a final example in this category, Cys(Fm) residues form disulfides directly upon treatment with piperidine (Ruiz-Gayo et al., 1988; Ponsati et al., 1990b).

Most general, but also most demanding in terms of the range of selectively removable sulfhydryl protecting groups required, are unsymmetrical directed methods of disulfide bridge formation (Barany and Merrifield, 1979; Hiskey, 1981). For example, Cys(Acm) or Cys(Trt) residues in peptides can be reacted with methoxy- or ethoxycarbonylsulfenyl chloride to form Cys(Scm) or Cys(Sce) residues, respectively, which are attacked by the free thiol of a deprotected Cys residue to form a disulfide bond (Kullmann and Gutte, 1978; Mott et al., 1986; Ten Kortenaar and van Nispen, 1988). Disulfide bonds may also be formed by a free thiol attack of Cys(NBoc-NHBoc) residues, which are prepared by treatment of Cys with azodicarboxylic acid di-*t*Bu ester (Romani et al., 1987). Cys(Npys) residues form disulfides upon reaction with deprotected Cys residues (Bernatowicz et al., 1986). Directed methods are particularly suited for linking two separate chains.

As already alluded to, directed methods require at least two classes of selectively removable Cys protecting groups; the same holds true for experiments aimed at controlled formation of multiple disulfide bridges by sequential pairwise deprotection/co-oxidations. An overriding concern with all such chemical approaches is to develop conditions that avoid scrambling (disulfide exchange). Oxidation by I_2 in TFE allows for selective disulfide bond formation between Cys(Trt) residues in the presence of Cys(Acm) residues; in DMF, I_2 oxidation is preferred between Cys(Acm) residues in the presence of Cys(Trt) residues (Kamber et al., 1980). Direct I_2 oxidation of Cys(Acm) or Cys(Trt) residues is particularly advantageous in that existing disulfides are not exchanged (Barany and Merrifield, 1979; Kamber et al., 1980; Hiskey, 1981; Gray et al., 1984; Atherton et al., 1985b; Ponsati et al., 1990b). Since the Acm group is essentially stable to HF, an Acm/Meb combination of protecting

groups facilitates selective disulfide formation in Boc chemistry (Gray et al., 1984; Tam et al., 1991a).

The alternative of carrying out deprotection/oxidation of the Cys residues while the peptide chain remains anchored to a polymeric support is of obvious interest and has received some recent attention. Such an approach takes advantage of *pseudo-dilution*, which is a kinetic phenomenon expected to favor facile intramolecular processes and thereby minimize dimeric and oligomeric by-products (Barany and Merrifield, 1979). Disulfide bond formation on peptide resins has been demonstrated by $K_3Fe(CN)_6$, air, dithiobis(2-nitrobenzoic acid), or diiodoethane oxidation of free sulfhydryls, direct deprotection/oxidation of Cys(Acm) residues by $Tl(Tfa)_3$ or I_2, direct conversion of Cys(Fm) residues by piperidine, and nucleophilic attack by a free sulfhydryl on either Cys(Npys) or Cys(Scm) (Gray et al., 1984; Mott et al., 1986; Buchta et al., 1986; Eritja et al., 1987; Ploux et al., 1987; Ten Kortenaar and van Nispen, 1988; García-Echeverría et al., 1989; Albericio et al., 1991a, and references to earlier work cited in these papers). The most generally applicable and efficient of these methods is direct conversion of Cys(Acm) or Cys(Trt) residues by I_2 (10 equiv in DMF), Cys(Acm) residues by $Tl(Tfa)_3$ (1.5 equiv in DMF) (Albericio et al., 1991a), and Cys(Fm) residues by (a) piperidine-DMF (1:1) for 3 hours at 25 °C (Ponsati et al., 1990b; Albericio et al., 1991a) or (b) piperidine-DMF-2-mercaptoethanol (10:10:0.7) treatment for 1 hour at 25 °C, followed by air oxidation in pH 8.0 DMF for 1 hour at 25 °C (Albericio et al., 1991a). The best solid-phase yields were at least as good and, in some cases, better than the results from corresponding solution oxidations.

Side-Chain Lactams

Intrachain lactams are formed between the side chains of Lys or Orn and Asp or Glu to conformationally restrain synthetic peptides, with the goal of increasing biological potency and/or specificity. The residues used to form intrachain lactams must be selectively side-chain deprotected, while all side-chain protecting groups of other residues remain intact. Selective deprotection is best achieved by using orthogonal side-chain protection, such as Fmoc and Fm for Lys/Orn and Asp/Glu, respectively, in combination with a Boc/Bzl strategy (Felix et al., 1988a; Felix et al., 1988b; Hruby et al., 1990). A more complicated but equally efficient approach is to use side-chain protection based on graduated acid-lability (Schiller et al., 1985; Sugg et al., 1988; Hruby et al., 1990). Cyclization is carried out most efficiently with BOP (3 to 6 equiv, 2 hours, 20 °C) in the presence of DIEA (6 to 7.5 equiv) while the peptide is still attached to the resin (Felix et al., 1988a; Plaué, 1990), taking advantage of the pseudo-dilution phenomenon discussed in the previous section.

Peptide Antigens

For the production of antipeptide antibodies, the peptide must be attached to a carrier. The simplest, although not necessarily most effective, way to accomplish this goal is to make direct use of peptide resins in which the side chains have been freed but where the anchoring linkage is stable to the appropriate deprotection conditions. Polyamide-type and polyethylene glycol-polystyrene resins have been applied according to this approach (Chanh et al., 1986; Kennedy et al., 1987; Goddard et al., 1988; Fischer et al., 1989; Bayer, 1991). Peptides may also be synthesized on a scaffold that, following cleavage and deprotection, is used directly for immunization. This scaffold, consisting of branched Lys residues, is referred to as a "multiple antigen peptide" system (MAP) (Tam, 1988; Tam and Lu, 1990). MAPs may be prepared by either Boc (Tam, 1988) or Fmoc (Pessi et al., 1990; Drijfhout and Bloemhoff, 1991; Biancalana et al., 1991) chemistry.

The more traditional (and still most common) methods for preparing peptide antigens start with free synthetic peptides that have been cleaved from the support and deprotected. In one variation, the peptide is conjugated to a protein carrier, e.g., bovine serum albumin, by means of a water soluble carbodiimide (see Deen et al., 1990, for a recent example). Alternatively, the peptide can be extended at the *C*- or *N*-terminus with a Cys residue, which is subsequently used to form a disulfide bridge with a free sulfhydryl on the carrier or is attached to the carrier using a heterobifunctional cross-linking reagent. Cystine formation is best achieved by the direct attack of a carrier protein thiol onto a peptide Cys(Npys) residue. Thiol groups are introduced on the carrier protein either by reduction to form free Cys (Albericio et al., 1989b; Ponsati et al., 1989) or by functionalization of Lys using *S*-acetylmercaptosuccinic anhydride (Drijfhout et al., 1988). Peptide-carrier conjugation is quantitated by monitoring the liberation of the Npys group at 329 nm (Drijfhout et al., 1988). For peptides synthesized by Fmoc chemistry, Boc-Cys(Npys) is incorporated as the *N*-terminal residue, thus avoiding additional piperidine treatments that would remove the Npys group (Albericio et al., 1989b). A heterobifunctional reagent, such as *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Lerner et al., 1981), can also be used to link sulfhydryl-containing synthetic peptides to lysine-containing carrier proteins such as keyhole limpet hemocyanin and bovine serum albumin. Maleimide activated carrier protein is available commercially (Pierce Chemical Co.).

AUXILIARY ISSUES

This final section of the chapter covers a variety of practical considerations that researchers in SPPS should be aware of. Included are some potential side reactions that do not fit neatly with categories covered earlier, and ways to mitigate the extent of their occurrence. A logical culmination of expertise in SPPS is the successful preparation of long sequences, and we outline current achievements and possible ways to improve on them in the future.

Diketopiperazine Formation

The free N^{α}-amino group of an anchored dipeptide is poised for a base-catalyzed intramolecular attack on the *C*-terminal carbonyl (Gisin and Merrifield, 1972; Barany and Merrifield, 1979; Pedroso et al., 1986). Base deprotection (Fmoc) or neutralization (Boc) can thus release a cyclic diketopiperazine while a hydroxymethyl-handle leaving group remains on the resin. With residues that can form *cis* peptide bonds, e.g., Gly, Pro, *N*-methylamino acids, or D-amino acids, in either the first or second position of the (C $\rightarrow$ N) synthesis, diketopiperazine formation can be substantial (Albericio and Barany, 1985; Pedroso et al., 1986; Gairi et al., 1990). For most other sequences, the problem can be adequately controlled. In Boc SPPS, the level of diketopiperazine formation can be suppressed either by removing the Boc group with HCl and coupling the NMM salt of the third Boc amino acid without neutralization (Suzuki et al., 1975) or else by deprotecting the Boc group with TFA and coupling the third Boc amino acid in situ using BOP, DIEA, and HOBt without neutralization (Gairi et al., 1990). For susceptible sequences being addressed by Fmoc chemistry, the use of piperidine-DMF (1:1) deprotection for 5 minutes (Pedroso et al., 1986) or deprotection for 2 minutes with a 0.1 M solution in DMF of tetrabutylammonium fluoride ("quenched" by MeOH) (Ueki and Amemiya, 1987) has been recommended to minimize cyclization. Alternatively, the second and third amino acids may be coupled as a preformed N^{α}-protected-dipeptide, avoiding the diketopiperazine-inducing deprotection/neutralization at the second amino acid.

> For continuous-flow Fmoc SPPS, diketopiperazine formation is suppressed by deprotecting for 1.5 minutes with piperidine-DMF (1:4) at an increased flow rate (15 mL/min), washing for 3 minutes with DMF at the same flow rate, and coupling the third Fmoc amino acid in situ with BOP, NMM, and HOBt in DMF (MilliGen/Biosearch, 1990). For batchwise SPPS, rapid (a maximum of 5 minutes) treatments by piperidine-DMF (1:1) should be used, followed by DMF washes and then in situ acylations mediated by BOP or HBTU (Pedroso et al., 1986).

Capping

Some workers choose to "cap" unreacted chains, thereby substituting a family of terminated peptides for a family of deletion peptides. In either case, these by-products must ultimately be separated from the desired product. Intentional termination of chains may be carried out when there is an indication of unreacted sites. In the simplest case, capping is carried out with reactive acetylating agents, such as acetic anhydride (Ac_2O) or *N*-acetylimidazole in the presence of tertiary base (Bayer et al., 1970; Stewart and Young, 1984). An alternative capping reagent is 2-sulfobenzoic acid cyclic anhydride (OSBA). Application of OSBA/tribenzylamine results in a negatively charged amino terminus; the desired product with the positively charged amino terminus may then be isolated after purification by ion-exchange chromatography (Drijfhout and Bloemhoff, 1988). In a reciprocal strategy, chains capped by acetylation are separated by modifying the *N*-terminus of the desired peptide with Fmoc-derivatives. These derivatives include the 9-(2-sulfo)fluorenylmethyloxycarbonyl (Sulfmoc) (Merrifield and Bach, 1978) or 9-(hydroxymethyl)fluorene-4-carboxylate group, where the carboxylate at the 4-position is in turn derivatized with Lys, Glu, or 2-aminodecanoic acid (Ball et al., 1990; Ball et al., 1991). After ion-exchange or reverse-phase purification, the modified Fmoc group is removed by base.

Deletions

The standard explanation for deletion peptides relates to incomplete couplings, which can usually be diagnosed by qualitative or quantitative monitoring tests (see Monitoring). In contrast, a chemically plausible side reaction that suggests a different reason for deletion peptides has been elucidated (Kent, 1983). Resin-bound aldehyde groups can form a Schiff's base with deprotected amino groups of the peptide chain. Those amino groups that are involved in a Schiff's base are prevented from acylation by the next incoming protected amino acid. The blockage is not permanent, i.e., terminated peptides are not formed. Rather, ready amine exchange of the Schiff's base renders a *different* set of amino groups temporarily inaccessible at a subsequent coupling, and thus deletion peptides result. The side reaction is *not* minimized by capping steps; in fact, amines blocked as Schiff's bases contribute to negative ninhydrin tests. The formation of aldehyde sites on polystyrene resins can be minimized by using a strong acid Friedel-Crafts catalyst during the original functionalization step. In any case, it is crucial to quantitate aldehyde concentrations of prepared or purchased resins (Kent, 1983).

Acid-Sensitive Side Chains and Bonds

Trp is quite sensitive to acid conditions, undergoing reactions with carbonium ions and molecular oxygen (see Cleavage). Synthesis of Trp-containing peptides is thus best approached by means of Fmoc chemistry, where acidolysis is kept at a minimum. Gramicidins A, B, and C (where either 3 or 4 of the 15 residues are Trp) have been synthesized efficiently by Fmoc chemistry; acid was avoided entirely throughout the synthesis and final nucleophilic cleavage was achieved by ethanolamine (Fields et al., 1988; Fields et al., 1989). Efficient Fmoc SPPS of indolicidin (which contains 5 Trp out of 13 residues) used an optimized TFA-scavenger mixture (reagent K) to prevent modification of Trp during acidolytic cleavage and side-chain deprotection (King et al., 1990; Selsted et al., 1992). Fmoc chemistry also has been suggested for incorporation of ^{2}H-labeled amino acids, because the repetitive acidolyses of Boc chemistry can exchange out the ^{2}H label (Fields and Noble, 1990; Prosser et al., 1991). Finally, Fmoc chemistry may be the better choice for the synthesis of peptides containing the acid-labile Asp-Pro bond. SPPS of baboon β-chorionic gonadotropin 109-145, which contains 2 Asp-Pro bonds, was reported to be successful by Fmoc chemistry only, because Boc chemistry resulted in acid-promoted cleavage of the Asp-Pro bonds (Wu et al., 1988).

Imide Formation

Asn residues can cyclize to form a succinimide, which can yield both α-and β-Asp peptides. Imide formation is largely sequence-dependent, with Asn-Gly showing the greatest tendency to rearrange (Stephenson and Clarke, 1989). Succinimide formation can be significant for peptides stored in solution (Stephenson and Clarke, 1989; Patel and Borchardt, 1990a; Patel and Borchardt, 1990b). In addition, peptides containing Asn (and even Gln) stored in the solid state with residual acid can undergo deamidation (Ten Kortenaar et al., 1990). Therefore, Asn- and Gln-containing peptides should never be stored in a solid form with residual acid present, and samples stored in solution should be monitored carefully for deamidation and decomposition.

Solvent Preferences

Effective solvation of the peptide resin is perhaps the most crucial condition for efficient chain assembly. Under proper solvent conditions, there is no decrease in synthetic efficiency up to 60 amino acid residues in Boc SPPS (Sarin et al., 1984). The ability of the peptide resin to swell increases with increasing peptide length due to a net decrease in free energy from solvation of the linear peptide chains (Sarin et al., 1980). Therefore, there is no theoretical upper limit to efficient amino acid couplings, provided that proper solvation conditions exist (Pickup et al., 1990). In practice, obtaining these conditions is not always straightforward. "Difficult couplings" during SPPS have been attributed to poor solvation of the growing chain by DCM. Infrared and NMR spectroscopies have shown that intermolecular β-sheet aggregates are responsible for lowering coupling efficiencies (Live and Kent, 1983; Mutter et al., 1985; Ludwick et al., 1986). A scale of β-sheet structure-stabilizing potential has been developed for Boc amino acid derivatives (Narita and Kojima, 1989). Enhanced coupling efficiencies are seen upon the addition of polar solvents, such as DMF, TFE, and NMP (Yamashiro et al., 1976; Live and Kent, 1983; Geiser et al., 1988; Narita et al., 1989; Fields et al., 1990; Fields and Fields, 1991). It has been suggested that chaotropic salts may be added to organic solvents in order to disrupt β-sheet aggregates (Stewart and Klis, 1990; Thaler et al., 1991).

Aggregation also occurs in regions of apolar side-chain protecting groups, sometimes resulting in a collapsed gel structure (Atherton et al., 1980; Atherton and Sheppard, 1985). In cases where aggregation occurs due to apolar side-chain protecting groups, increased solvent polarity may not be sufficient to disrupt the aggregate. A relatively unstudied problem of Fmoc chemistry is that the lack of polar side-chain protecting groups could, during the course of an extended peptide synthesis, inhibit proper solvation of the peptide resin (Atherton et al., 1980; Fields and Fields, 1991). To alleviate this problem, the use of solvent mixtures containing both a polar and nonpolar component, such as THF-NMP (7:13) or TFE-DCM (1:4), is recommended (Fields and Fields, 1991). The partial substitution or complete replacement of *t*Bu-based side-chain protecting groups for carboxyl, hydroxyl, and amino side chains by more polar groups would also aid peptide resin solvation (Atherton et al., 1980; Fields and Fields, 1991).

Long Syntheses (>50 Residues)

Many impressive long-chain syntheses (>50 residues), including ribonuclease A (124 residues) (Gutte and Merrifield, 1971), human parathyroid hormone (84 residues) (Fairwell et al., 1983), interleukin-3 (140 residues) (Clark-Lewis et al., 1986), HIV-1 aspartyl protease (99 residues) (Schneider and Kent, 1988; Nutt et al., 1988), HIV-1 vpr protein (95 residues) (Gras-Masse et al., 1990), and insulin-like growth factor (70 residues) (Bagley et al., 1990), have been carried out using Boc methodology. There have also been recent successful long-chain syntheses by Fmoc chemistry, including HIV-1 Tat protein (86 residues) (Cook et al., 1989; Chun et al., 1990), preprocecropin A (64 residues) (Pipkorn and Bernath, 1990), ubiquitin (76 residues) (Ramage et al., 1989; Ogunjobi and Ramage, 1990), yeast actin-binding protein 539-588 (50 residues) (King et al., 1990), pancreastatin (52 residues) (Funakoshi et al., 1988), and human β-chorionic gonadotropin 1-74 (Wu et al., 1989). Both chemistries appear susceptible to the same difficult couplings (Meister and Kent, 1983; J. Young et al., 1990; van Woerkom and van Nispen, 1991), and side-by-side syntheses for moderate-length chains (~30 residues) are comparable (Atherton et al., 1983; Wade et al., 1986). However, there are two additional considerations when using Fmoc, rather than Boc, chemistry for long-chain syntheses. First, the efficient solvation of hydrophobic side-chain protecting groups used in conjunction with Fmoc chemistry, which was discussed previously, can become more critical for extended syntheses (Fields and Fields, 1991). Second, deprotection of the Fmoc group can proceed slowly in certain sequences (Atherton and Sheppard, 1985; Larsen et al., 1991). By monitoring deprotection as the synthesis proceeds, one can extend base deprotection times and/or alter solvation conditions as necessary (Ogunjobi and Ramage, 1990).

Segment Condensation

The advantages of segment condensation procedures for the synthesis of large peptides have been well described (Barany and Merrifield, 1979; Kaiser et al., 1989; Kneib-Cordonier et al., 1990), but to date there are relatively few examples for polymer-supported procedures. A significant aspect of the problem involves ready access to pure partially protected peptide segments, which are needed as building blocks. The application of solid-phase synthesis to prepare the requisite intermediates depends on several levels of selectively cleavable protecting groups and anchoring linkages. Combination of the Boc/Bzl strategy with the 4-nitrobenzophenone oxime resin (DeGrado and Kaiser, 1982; Kaiser et al., 1989; Landsbury et al., 1989; Sasaki and Kaiser, 1990), base-labile linkers (Liu et al., 1990; Albericio et al., 1991b), palladium-labile linkers (Kunz and Dombo, 1988; Guibé et al., 1989; Lloyd-Williams et al., 1991b) or photolabile linkers (Rich and Gurwara, 1975; Albericio et al., 1987b; Lloyd-Williams et al., 1991a), and of the Fmoc/*t*Bu strategy with dilute acid-labile linkers (Mergler et al., 1988b; Barlos et al., 1989; Atherton et al., 1990; Albericio and Barany, 1991; Barlos et al., 1991b) or photolabile linkers (Kneib-Cordonier et al., 1990) has proved successful for the generation of N^{α}-amino and side-chain protected segments with free C^{α}-carboxyl groups. Methods for subsequent solubilization and purification of the protected segments are nontrivial (Atherton et al., 1990; Lloyd-Williams et al., 1991a) and beyond the scope of this review.

In recent years, solid-phase assembly of protected segments has proved successful for a 44-residue model of apolipoprotein A-1 (Nakagawa et al., 1985), human cardiodilatin 99-126 (Nokihara et al., 1989), human gastrin-I (17 residues) (Kneib-Cordonier et al., 1990), *Androctonus australis Hector* toxin II (64 residues) (Grandas et al., 1989b), λ-Cro DNA binding protein (66 residues) (Atherton et al., 1990), and prothymosin α (109 residues) (Barlos et al., 1991b). One-percent 1,3-divinylbenzene cross-linked polystyrene and polyamide resins have been shown to be suitable supports for solid-phase segment condensations

(Albericio et al., 1989c). Individual rates for coupling segments are much lower than for activated amino acid species by stepwise synthesis, and there is always a risk of racemization at the *C*-terminus of each segment. Careful attention to synthetic design and execution may minimize these problems.

SUMMARY

The solid-phase method has made the synthesis of peptides widely accessible. With the increased sophistication of commercial automated instrumentation, the appeal is ever broadening. The majority of solid-phase peptide syntheses of less than 50 residues can be performed with high efficiencies by either Boc or Fmoc chemistry. The Bzl-based side-chain protecting group strategy is used routinely for Boc chemistry and the *t*Bu-based side-chain protection strategy is usually used for Fmoc chemistry. Manufacturers generally suggest specific chemistry packages with their instruments, which represent some variation of either of these two strategies.

It is important not to lose sight of the fact that each synthetic procedure has limitations and that even in the hands of highly experienced workers, certain sequences defy facile preparation. Common residue-specific side reactions that may lead to failed syntheses include (a) dehydration of Asn and Gln to the respective nitriles (see Protection Schemes, In Situ Reagents, and Active Esters), (b) racemization of His (see Protection Schemes), (c) aspartimide formation from Asp (see Protection Schemes), (d) alkylation of Trp and Glu (see Cleavage), and (e) acidolysis of Asp-Pro bonds (see Auxiliary Issues). Additional sources of synthetic problems are sterically hindered couplings and diketopiperazine-forming sequences (see Auxiliary Issues). In all of the aforementioned examples, deleterious side reactions or other difficulties can be minimized somewhat by careful examination of a peptide sequence prior to synthesis. Appropriate precautions as outlined in this chapter (alternative side-chain protecting groups, use of additional reagents during coupling or cleavage, etc.) can be taken.

The maturation of high-performance liquid chromatography (HPLC) has been a major boon to modern peptide synthesis, because the resolving power of this technique facilitates removal of many of the systematic low-level by-products that accrue during chain assembly and upon cleavage. Nowadays, the homogeneity of synthetic materials should be checked by at least two chromatographic or electrophoretic techniques, e.g., reverse-phase and ion-exchange HPLC and capillary zone electrophoresis. Also, determination of a molecular ion by fast atom bombardment mass spectrometry (FABMS) or a related mild ionization method is almost *de rigueur* for proof of structure. Synthetic peptides must be checked routinely for the proper amino acid composition, and, in

some cases, sequencing data are helpful. Spectroscopic measurements, particularly through the use of one-and two-dimensional nuclear magnetic resonance (NMR), at the least provide insights on structure and purity, and they can often give conformational information as well.

Improvements in the chemistry of SPPS continue apace. This chapter has touched on most of the key issues and discussed the recent status for each of them. There is every reason to be optimistic that peptide synthesis will continued to play an important role in the elucidation of biological processes.

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ABBREVIATIONS

Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. 247: 977-983 (1972). The following additional abbreviations are used: AA, amino acid; $Ac_4\beta$Gal, 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl; Acm, acetamidomethyl; Ada, adamantyl; Al, allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Boc-ON, 2-*tert*-butyloxycarbonyloximino-2-phenylacetonitrile; Bom, benzyloxymethyl; BOP, benzotriazolyl *N*-oxytris(dimethylamino)phosphonium hexafluorophosphate; 2-BrZ, 2-bromobenzyloxycarbonyl; Bum, *tert*-butoxymethyl; Bzl, benzyl; cHex, cyclohexyl; Cs, cesium salt; 2,6-Cl2Bzl, 2,6-dichlorobenzyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCBC, 2,6-dichlorobenzoyl chloride; DCC, *N,N'*-dicyclohexylcarbodiimide; DCE, 1,2-dichloroethane; DCM, dichloromethane (methylene chloride); DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMA, *N,N*-dimethylacetamide; DMAP, 4-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; Dnp, 2,4-dinitrophenyl; Dod, 4-(4'-methoxybenzhydryl)phenoxyacetic acid; EDT, 1,2-ethanedithiol; Et_3N, triethylamine; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-OSu, fluorenylmethyl succinimidyl carbonate; HAL, 5-(4-hydroxymethyl-3,5-dimethoxyphenoxy)valeric acid; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HF, hydrogen fluoride; HMFA, 9-(hydroxymethyl)-2-fluoreneacetic acid; HMP, 4-hydroxymethylphenoxy; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HOPip, *N*-hydroxypiperidine; HPLC, high performance liquid chromatography; Hpp, 1-(4-nitrophenyl)-2-pyrazolin-5-one; HYCRAM™, hydroxycrotonylaminomethyl; MBHA, 4-methylbenzhydrylamine (resin); Meb, 4-methylbenzyl; MeIm, 1-methylimidazole; MeOH, methanol; MMA, *N*-methylmercaptoacetamide; Mob, 4-methoxybenzyl; MSNT, 2,4,6-mesitylene-sulfonyl-3-nitro-1,2,4-triazolide; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; Mts, mesitylene-2-sulfonyl; NCA, *N*-carboxyanhydride; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; Nonb, 3-nitro-4-aminomethylbenzoic acid; NPE, 4-(2-hydroxyethyl)-3-nitrobenzoic acid; Npp, 3-methyl-1-(4-nitrophenyl)-2-pyrazolin-5-one; N.R., not reported; Nvoc, 6-nitroveratryloxycarbonyl (4,5-dimethoxy-2-nitrobenzyloxycarbonyl); ODhbt, 1-oxo-2-hydroxydihydrobenzotriazine; ONb, 2-nitrobenzyl ester; ONo, 2-nitrophenyl; ONp, 4-nitrophenyl; OPfp, pentafluorophenyl; Orn, ornithine; OSu, *N*-hydroxysuccinimidyl; OTDO, 2,5-diphenyl-2,3-dihydro-3-oxo-4-hydroxythiophene dioxide; PAB, 4-alkoxybenzyl; PAL, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid; PAM, 4-hydroxymethylphenylacetic acid; PEG, polyethylene glycol; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Pnp, 3-phenyl-1-(4-nitrophenyl)-2-pyrazolin-5-one; PSA, preformed symmetrical anhydride; PyBOP, benzotriazole-yl *N*-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; SASRIN™, 2-methoxy- 4-alkoxybenzyl alcohol; Scm, *S*-carboxymethylsulfenyl; SPPS, solid-phase peptide synthesis; S*t*Bu, *tert*-butylsulfenyl; Tacm, trimethylacetamidomethyl; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TFMSA, trifluoromethanesulfonic acid; $Tl(Tfa)_3$, thallium (III) trifluoroacetate; Tmob, 2,4,6-trimethoxybenzyl; TMSBr, trimethylsilyl bromide; TMSOTf, trimethylsilyl trifluoromethanesulfonate; Tos, 4-toluenesulfonyl; Trt, triphenylmethyl; XAL, 5-(9-aminoxanthen-2-oxy)valeric acid; Xan, 9-xanthenyl; Z, benzyloxycarbonyl. Amino acid symbols denote the L-configuration where applicable, unless indicated otherwise.

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