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Orthogonal Protecting Groups for N^{α}-Amino and *C*-Terminal Carboxyl Functions in Solid-Phase Peptide Synthesis

Abstract: *For the controlled synthesis of even the simplest dipeptide, the* N^{α}*-amino group of one of the amino acids and the* C*-terminal carboxyl group of the other should both be blocked with suitable protecting groups. Formation of the desired amide bond can now occur upon activation of the free carboxyl group. After coupling, peptide synthesis can be continued by removal of either of the two protecting groups and coupling with the free* C*-terminus or* N^{α}*-amino group of another protected amino acid. When three functional amino acids are present in the sequence, the side chain of these residues also has to be protected. It is important that there is a high degree of compatibility between the different types of protecting groups such that one type may be removed selectively in the presence of the others. At the end of the synthesis, the protecting groups must be removed to give the desired peptide. Thus, it is clear that the protection scheme adopted is of the utmost importance and makes the difference between success and failure in a given synthesis. Since R. B. Merrifield introduced the solid-phase strategy for the synthesis of peptides, this prerequisite has been readily accepted. This strategy is usually carried out using two main protection schemes: the* tert*-butoxycarbonyl/benzyl and the 9-flourenylmethoxycarbonyl/*tert*-butyl methods. However, for the solid-phase preparation of complex or fragile peptides, as well as for the construction of libraries of peptides or small molecules using a combinatorial approach, a range of other protecting groups is also needed. This review summarizes other protecting groups for both the* N^{α}*-amino and* C*-terminal carboxyl functions.* © 2000 John Wiley & Sons, Inc. Biopoly 55: 123–139, 2000

Keywords: *chemical libraries; combinatorial chemistry; orthogonal strategy; peptide synthesis; protecting group*

INTRODUCTION

Peptide synthesis is based on the appropriate combination of protecting groups together with an efficient method for the activation of the carboxyl group prior to reaction with the amino component.[1] In the solid-phase strategy developed by Merrifield, the *C*-terminal protecting group is in fact a polymeric carrier and, consequently, the synthesis is carried out on an insoluble support.[2] The solid-phase method now dominates synthetic peptide chemistry and the majority of peptides are made using this technique.

Since Merrifield described the solid-phase approach, only two *protection schemes* have been widely adopted. The *tert*-butoxycarbonyl (Boc)/benzyl (Bzl) strategy depends on graduated acid lability.[2] Thus, while the Boc group is removed by trifluoroacetic acid (TFA), Bzl, and related protecting groups are removed at the end of the synthetic strategy with strong acids such as HF or trifluoromethanesulfonic.

Biopolymers (Peptide Science), Vol. 55, 123–139 (2000)

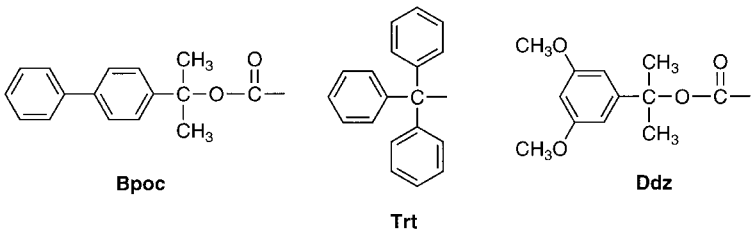

FIGURE 1 Structure of acid-labile protecting groups.

The main drawback associated with this strategy is a lack of flexibility. Long exposure of the peptide chain to TFA in the removal the Boc group can also cause premature removal of the benzyl protecting group. Furthermore, conditions for the removal of Bzl groups will always remove the Boc group. Finally, some peptides containing fragile sequences will not survive the strong acid conditions used to remove the Bzl groups. The alternative 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*-Bu) strategy[3,4] is based on the *orthogonal* concept[5] in the sense that the two protecting groups belong to independent classes and are removed by different mechanisms. The two groups can be removed therefore in any order in the presence of the group. Orthogonal protection schemes are milder because the selective deprotection is governed by alternative cleavage mechanisms rather than by reaction rates. The Fmoc group[6] is removed by piperidine through a β-elimination reaction and *t*Bu is removed by acidolysis with TFA. Although this strategy has several advantages with respect to the Boc/Bzl approach, the conditions are still, in some cases, too harsh and can be incompatible with certain sequences. Furthermore, the preparation of complex molecules such as cyclic or branched systems could require the use of other protecting groups.[7] In this report, other protecting groups for the amino function as well as for the carboxyl one are discussed.

N^{α}-AMINO PROTECTING GROUPS

Acid-Labile Protecting Groups

Groups that can be removed through a milder acid in comparison to the Boc group are 2-(4-biphenyl)isopropoxycarbonyl (Bpoc),[8] triphenylmethyl (trityl, Trt),[9] and α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz)[10] (Figure 1).

The Bpoc group can be removed by treatment with 0.2–0.5% TFA* in CH_2Cl_2 and has been used in combination with *t*-Bu side-chain protecting groups in the early stage of solid-phase peptide synthesis (SPPS).[11,12]

The main disadvantage of these derivatives is that most are obtained as oils that undergo an autocatalytic decomposition to the amino acid, CO_2, and the Bpoc olefin and its dimer over a half-life of weeks.[13] Thus, they have to be isolated and stored as cyclohexyl ammonium (CHA) or dicyclohexyl ammonium (DCHA) salts. Liberation of the free carboxyl compounds is not a straightforward process due to the extremely high acid lability of the protecting group. Recently, a convenient method for the preparation of the *O*-pentafluorophenyl (*O*-Pfp) esters has been reported.[13] Most of these compounds are obtained as crystalline solids. Furthermore, the Pfp ester is intended both to protect the highly acid-labile urethane from autocatalytic cleavage by the free acid during storage and to activate the derivative for acylation during peptide coupling reactions. The preparation of the Pfp esters of Bpoc-amino acids was performed by first reacting the starting amino acid derivative with either Bpoc-phenyl carbonate, Bpoc-azide, or Bpoc-*p*-methoxycarbonylphenyl carbonate to form the free Bpoc-amino acids, which were immediately esterified with dicyclohexylcarbodiimide (DCC).

SPS of several model peptides has been performed using Pfp esters of Bpoc-amino acids (4 equiv) in the presence of 1-hydroxybenzotriazole (HOBt; 4 equiv), an additive to form in situ the OBt esters and therefore to increase the reactivity, and diisopropylethylamine (DIEA, 8 equiv) to neutralize Pfp-OH and HOBt, both of which are sufficiently acidic to present a threat to the stability of the Bpoc group, in CH_2Cl_2/*N*,*N*-

* When oxymethyl-containing resins such as poly(ethylene glycol)-based resins are used, higher contents of TFA for this and other acid-labile protecting groups could be necessary because some of the TFA will be employed for the protonation of the oxymethyl moieties.

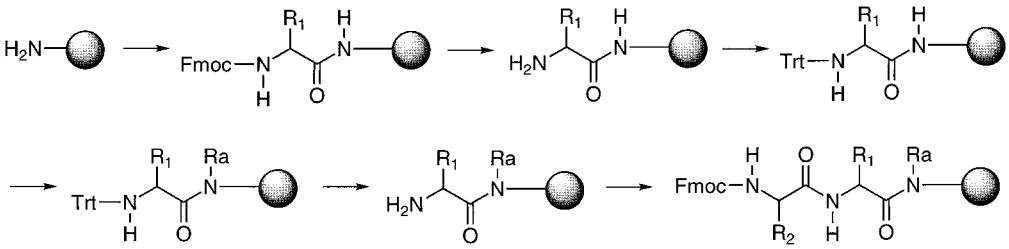

FIGURE 2 Scheme for the alkylation of amide bonds. Adapted from Ref. 22.

dimethylformamide (DMF) (1:1). Removal of the protecting group was achieved with 0.5% TFA in CH_2Cl_2 (1×3 min + 1×15 min). In this regard, the use of scavengers such as benzyl mercaptan and thiophenol has been also reported.[14]

The Trt group can be removed in solution with 1% TFA in CH_2Cl_2[15] and 0.1*M* HOBt in trifluoroethanol (TFE).[16] To extend the application of Trt-amino acids in solid-phase synthesis, as well as their compatibility with hyperacid-labile resins and acid-sensitive biomolecules, the use of mild acid conditions was investigated.[17] The results indicate that 0.1*M* 7-aza-1-hydroxybenzotriazole (HOAt)/0.12*M* Me_3SiCl in TFE, 0.25*M* Me_3SiCl in TFE, 0.2% TFA/1% H_2O in CH_2Cl_2, and 3% trichloroacetic acid (TCA) in CH_2Cl_2 all quantitatively remove the Trt group and are compatible with the 3-(4-hydroxymethylphenoxy)propionic acid (AB) linker. Furthermore, solutions of 0.2% TFA/1% H_2O in CH_2Cl_2, and 3% TCA in CH_2Cl_2 are compatible with the most labile Riniker handle and the synthesis of oligonucleotide–peptide conjugates, respectively.

The synthesis of Trt-amino acids can be carried out starting either from the amino acid or the methyl ester.[9,18] The former method involves in situ trimethylsilyl ester formation to protect the carboxylic acid followed by reaction with Trt-Cl. The latter method requires reaction with Trt-Cl followed by hydrolysis of the methyl ester with $LiOH/H_2O/CH_3OH$ at 25–40°C. In our laboratories the second strategy is preferred since the overall yields are better (>75% as opposed to 40−60%) and column chromatography is not required to obtain pure derivatives.[19]

The main drawback associated with Trt-amino acids is that these compounds couple with other amino acid derivatives in lower yields when compared with carbamate-based protected amino acids.[9] SPPS with Trt-amino acids have proceeded in good yields when *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5]*b*]-pyridino-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) in the presence of DIEA was used as the coupling reagent. Thus, several model peptides have been synthesized using single couplings with HATU/DIEA (4 equiv:8 equiv) for 20 min and with 3% TCA in CH_2Cl_2 (2×3 min) for removal of the Trt group.[19]

The Trt group could be used in combination with an Fmoc strategy in order to avoid diketopiperazine (DKP) formation in a similar manner to that described earlier for a Boc strategy.[20] This procedure avoids the presence of the free amino function of the penultimate residue and incorporates the third residue with neutralization in situ. Thus, in those sequences that are prone to DKP formation, the following experimental protocol should be used: (i) incorporation of the penultimate residue as its Trt derivative, (ii′) selective detritylation with $TFA/H_2O/CH_2Cl_2$ (2:1:97) (5×1 min) for AB-type resins or (ii″) $TFA/H_2O/CH_2Cl_2$ (0.2:1:99; 5×1 min) for Riniker-based resin, and (iii) incorporation of the third residue as its Fmoc derivative under in situ neutralization/coupling conditions mediated by 7-azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/DIEA (5 equiv each) in DMF for 1 h.[21]

The Trt protecting group has also been used in a strategy developed to successively alkylate each amide bond following its formation.[22] Thus, after the coupling of an Fmoc-amino acid, the Fmoc group is removed and the *N*-terminal amino function is reprotected with Trt by reaction with Trt-Cl in the presence of DIEA. Treatment of the Trt-peptide resin with lithium *t*-butoxide in tetrahydrofuran (THF) leads to the formation of the amide anion. Following removal of excess base, the alkylating reagent in DMSO is reacted with the Trt-peptide resin. The alkylation reaction mixture is then removed and the base and alkylation treatments repeated to drive the alkylation to completion. Removal of the Trt group, followed by coupling of the next Fmoc-amino acid allows the formation of the next amide bond, which can again be alkylated by following the same protocol involving the concourse of Trt group (Figure 2).

The Ddz-amino acids, which are the most commercially available, are more stable in the presence of acids than both the Bpoc and the Trt analogues. The

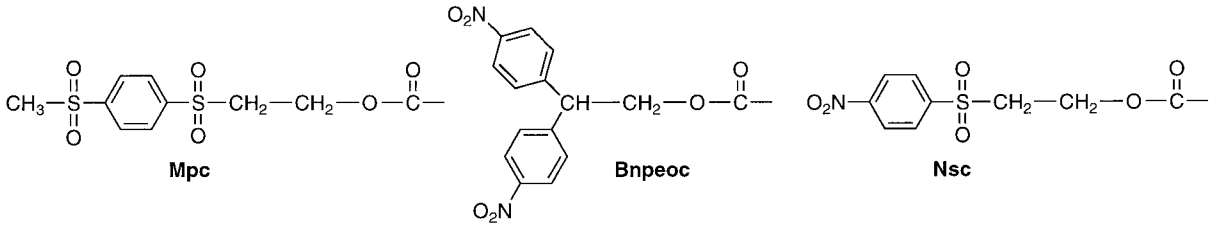

FIGURE 3 Structure of protecting groups that are base labile through a a β-elimination reaction.

Ddz group can be removed with 1–5% TFA in CH_2Cl_2 and the protected amino acids are described as being fully compatible with *t*-Bu side-chain protecting groups.[23] Furthermore, Ddz is also removed by photolysis at wavelengths above 280 nm. This property also makes the Ddz group potentially very useful in SPS library screening procedures. Ddz derivatives have a very characteristic uv absorption pattern with maxima at 230, 276, and 282 nm. Therefore, the Ddz fission product allows a very precise determination of the initial loading of the first amino acid on the resin as well as of the growing Ddz-peptide resin.

Ddz-amino acids have also been used to avoid DKP formation when a Backbone Amide Linker (BAL) strategy is used.[24] As mentioned above for AB- and Riniker-type resins, the incorporation of the penultimate residue is performed using the Ddz derivative. Removal of the protecting group is achieved with TFA–H_2O–CH_2Cl_2 (3:1:96) for 6 min before incorporation of the third residue as its Fmoc derivative under in situ neutralization/coupling conditions mediated by PyAOP. The advantage of the Ddz protecting group over the Trt group is that the former couples more efficiently, which is an especially important factor when the penultimate residue has to be incorporated to a second and/or hindered amine.[24]

Base-Labile Protecting Groups

Although the use of Fmoc-based SPPS has increased enormously in recent years to the point where it is now probably the method of choice for the chemical synthesis of peptides, there could also be a need to search for other, base-labile amino protecting groups. The main drawbacks associated with the use of Fmoc-amino acids are related to their hydrophobicity. For example, some derivatives possess low solubility in the solvents commonly used in SPPS, which can cause low coupling yields and/or problems associated with automation. Solubility issues are more critical during the manipulation of Fmoc-protected peptides in a convergent strategy.[25,26]

During the last few years, several N^{α}-amino protecting groups have been reported to overcome some of the problems described above. Examples of these are 2-[4-(methylsulphonyl)phenylsulphonyl]ethyloxycarbonyl (Mpc),[27] 2,2-bis-(4′-nitrophenyl)ethyloxycarbonyl (Bnpeoc),[28] and 2-(2,4-dinitrophenyl)ethyloxycarbonyl (Dnpeoc)[29] (See Figure 3).

Particularly appealing is the 2-(4-nitrophenylsulfonyl)ethoxycarbonyl (Nsc) group,[27,30,31] which can be considered from the point of chemical composition to be a combination of the Dnpeoc and the Mpc groups, since both nitro and sulfonyl groups are incorporated into the structure. These two functional groups should enhance the solubility of an amino acid and prevent hydrophobic interactions between the peptide chains, a situation that should subsequently reduce the degree of failure or truncated sequences during chain elongation.[32,33] Nsc-amino acids are synthesized easily from the corresponding succinimidyl carbonate and are crystalline compounds, a physical property strongly preferred for automated SPPS. Furthermore, the mechanism for the removal of the Nsc group is similar to that for Fmoc deprotection and is based upon a base-catalyzed β-elimination reaction. Therefore, the chemistry and instrumentation described for the Fmoc strategy can be readily adapted to this protecting group.

The removal of the Nsc group proceeds at a rate three to ten times slower than the removal of the Fmoc group, and is dependent on the nature of the base and solvent (Table I).[34] The high stability of Nsc in DMF is of particular importance when this group is to be left on for long period of time. Thus, the Nsc group is more suitable for use in automatic synthesizers where amino acid derivatives are stored in solution for relatively long periods or when protected peptides are left to react in a convergent strategy. In this sense, when traces of piperidine remain, the removal of Fmoc is still much faster.[34] Nsc is clearly a better protecting group as far as stability is concerned. Conditions recommended for the removal of Nsc are 20% piperidine in DMF, or preferably DMF–dioxane (1:1), for 15 min. The addition of the stronger base

Table I Comparison Between Fmoc and Nsc Protected Peptides[a]

	Fmoc	Nsc
Cleavage rate ($t_{1/2}$)		
20% Piperidine/DMF	10–15 s	90–110 s
1% DBU/20% piperidine/DMF	—	12–15 s
Decomposition in DMF solution		
1 week	10%	< 1%
3 weeks	40%	2%
Olefin-amine adduct formation	Fast and reversible	Very fast and irreversible
Polymerization during removal	Yes	No
uv monitoring range	302 nm	380 nm

[a] *Source:* Ref. 34.

1,8-diazabicyxlo[5.4.0]undec-7-ene (DBU) can compensate for the slower rate of N^{α}-deprotection.[30] In this case, caution is required because the use of DBU, even at a concentration of 1% (v/v), can cause aspartimide formation in sensitive sequences.[35] Other advantages that Nsc has over the Fmoc group are that the formation of the olefin-amine adduct is irreversible and takes place faster than with Fmoc.[34] The Nsc group absorbs at 380 nm, which allows selective online monitoring during SPPS.

Coupling kinetic studies have indicated that while Fmoc-amino acids at times coupled slightly faster than Nsc derivatives, both were globally within the same range of reactivity.[36]

Premature removal of the N^{α}-Fmoc protecting group has been observed during the synthesis of polyproline-containing peptides.[37] This phenomenon is presumably due to the secondary amine character of the α-amino function of the Pro. Electrospray mass spectroscopy analysis of the sequence H–PPPPPPA–NH_2 prepared with Nsc–Pro–OH showed no evidence of Pro insertion (detected as an [M + 97] peak), which would be indicative of premature deprotection of a Pro and the subsequent coupling of another protected Pro.[36]

The loss of configuration at the *C*-terminal carboxyl residue is perhaps the most important side reaction in peptide synthesis.[38] This is often enhanced by the use of highly potent coupling reagents, which usually convert the carboxyl function to a derivative bearing a good leaving group. Such leaving groups tend to increase the acidity of the α-proton and favor oxazolone formation, both of which lead to racemization.[39] This risk is more accentuated when residues such as His,[40] Cys,[41,42] and Ser[43] are incorporated into a sequence. To compare the racemization potential of Nsc- and Fmoc-amino acids, the model tripeptides H–Gly–Xxx–Phe–NH_2 (where Xxx = His, Cys, and Ser)[42,43] were manually synthesized by each method in the solid phase.[36] Preactivation of the carboxyl group was avoided to minimize racemization of the susceptible residues. High performance liquid chromatography (HPLC) analysis (Table II) revealed that for the Nsc-amino acids, racemization was undetected for Ser and reduced by more than a half for Cys and His. This illustrates the value of N^{α}-Nsc Cys and His residues since Fmoc-amino acids gave unacceptable results. Presumably, the higher stability of the Nsc-derivatives is correlated with the reduced acidity of the β-proton of the protecting group, which trans-

Table II Racemization Studies[a]

H–Gly–aa–L-Phe–NH_2

	aa: His		aa: Cys		aa: Ser	
	% L,L	% D,L	% L,L	% D,L	% L,L	% D,L
Fmoc–L-aa–OH	97.5	2.5	89.9	10.1	98.9	1.1
Fmoc–D-aa–OH	2.1	97.9	4.9	95.1	1.0	99.0
Nsc–L-aa–OH	98.9	1.1	95.7	4.3	99.9	0.1

[a] *Source:* Ref. 36.

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