A Reversible Protecting Group for the Amide Bond in Peptides. Use in the Synthesis of 'Difficult Sequences'

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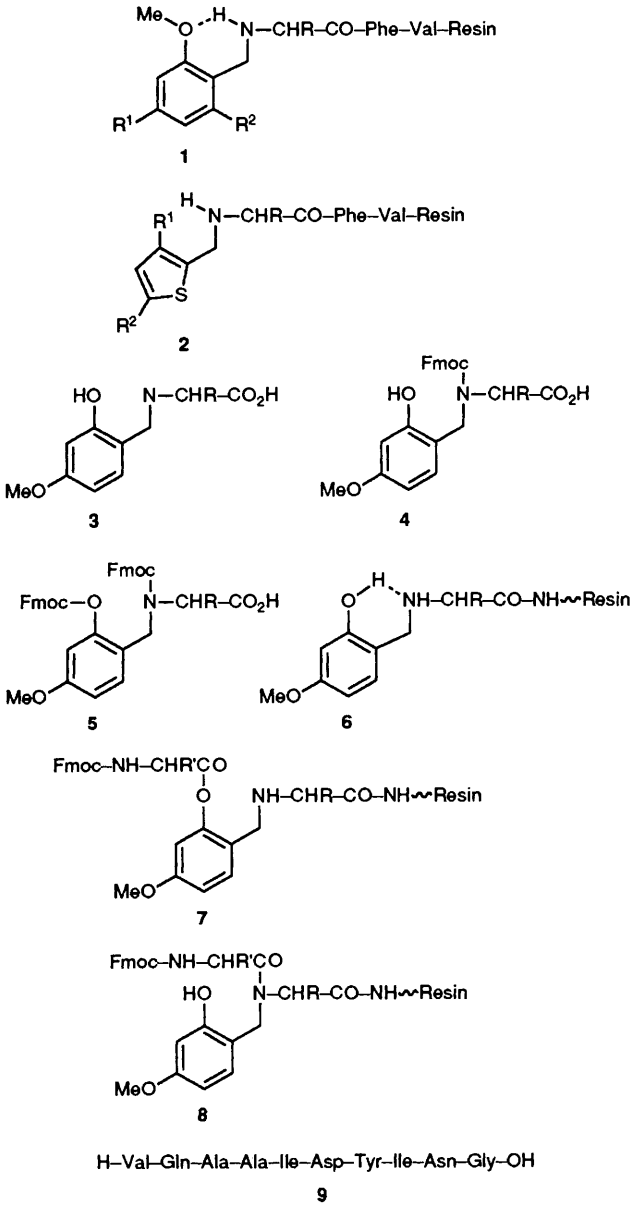
N,O-Bis-Fmoc (fluoren-9-ylmethoxycarbonyl) derivatives of N^{α}-(2-hydroxy-4-methoxybenzyl)amino acids **5** are useful intermediates for the preparation of peptides with reversibly protected (tertiary) peptide bonds; their value in inhibiting interchain association during solid phase peptide synthesis is demonstrated.

The secondary amide bond in peptides has hydrogen-bonding potential and other properties which cause frequent problems in peptide synthesis. A notable example is the occurrence of so-called 'difficult sequences' in solid phase synthesis in which intermediate resin-bound peptide chains are believed to associate in β-sheet or other structures.[1] Massive steric hindrance commonly results. This hydrogen bonded association could be prevented by replacement of secondary by tertiary amide bonds within the peptide chain.[2] A recent study[3] suggested that replacement at only about every sixth



residue could be sufficient to inhibit association. Introduction of tertiary amide bonds in peptides is most logically achieved through reversible *N*-substitution of precursor amino acid derivatives, but for amino acids other than glycine, this substitution itself usually causes serious hindrance in subsequent peptide bond formation.[4] We now describe a reversibly modifying group in which hindrance introduced at the substituted amino acid stage is substantially reduced by acyl capture and intramolecular transfer. Such a modifying group has a number of applications in peptide chemistry. Here we exemplify its value in solid phase synthesis of 'difficult sequences.'

In orienting studies, a number of methyl- and methoxy-substituted *N*-benzylamino acids and analogous furan and thiophene derivatives were examined. Acid lability of the substituent compatible with Fmoc-*tert*-butyl peptide synthesis strategies (completely cleaved from *N*-acyl derivatives† by 95% aq. trifluoroacetic acid in less than 15 min) was shown by 2,4-dimethoxy and 2,4,6-trimethoxybenzyl derivatives, and by

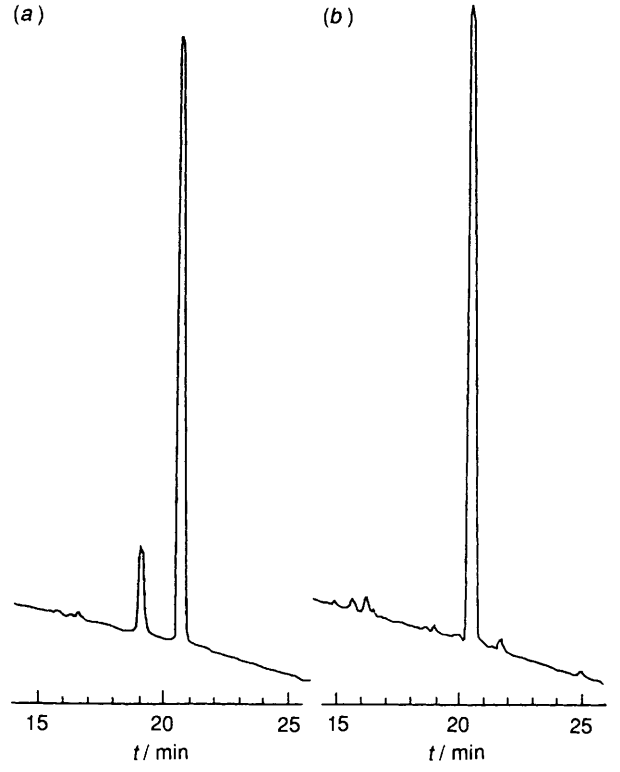


Fig. 1 HPLC profiles of crude acyl carrier protein decapeptide **9** synthesised (*a*) using standard 45 min pentafluorophenyl ester–HOBt couplings throughout; and (*b*) with replacement of residue 7 (from the C-terminus) by **5** (R = Me). The impurity peak eluting at 19 min in (*a*) corresponds to the des-valine nonapeptide. In both syntheses the peptide was cleaved from the resin using 95% aq. trifluoroacetic acid for 15 min.

3- and 5-methoxythienylmethylamino acids.‡ All, however, also caused massive steric hindrance of the substituted amino group for residues other than glycine.

Several observations suggested that hydrogen-bonded complexes with *ortho*-substituents (as in **1**) were important in determining the reactivity of the secondary amino group towards acylating agents. *N*-2,4,6-Trimethoxybenzylpeptide resin§ **1** (R^1 = R^2 = MeO) was more easily acylated than the less hindered 2,4-dimethoxybenzyl peptide **1** (R^1 = MeO, R^2 = H), and 3-methoxythienylmethyl peptide **2** (R^1 = MeO, R^2 = H) more easily than 5-methoxythienylmethyl isomer **2** (R^1 = H, R^2 = MeO). *ortho*-Methoxy-substituents thus facilitated rather than hindered acylation. Similar *ortho*-hydroxy groups could therefore be particularly well placed for internally base-catalysed *O*-acylation and intramolecular acyl transfer reactions **6** → **7** → **8**, processes which would not be subject to the steric hindrance of direct *N*-acylation. This was confirmed by comparison of 2-methoxy (**1**, R = Me, R^1 = R^2 = H) and 2-hydroxybenzylalanine derivatives. The latter was acylated completely under maximum concentration conditions by Fmoc-alanine anhydride (10 equiv.) within 1 h and by Fmoc-alanine pentafluorophenyl ester (Fmoc-Ala-OPfp, 10 equiv.) in the presence of hydroxybenzotriazole catalyst (HOBt, 10 equiv.) in 11 h. Under these last conditions, *N*-2-methoxybenzylalanine peptide **1** (R = Me, R^1 = R^2 = H) was acylated to the extent of only 2%.

† Determined for *N*-phenylacetyl derivatives. Acid cleavage is inhibited if the substituted amino group is free, and slowed if the substituted residue is adjacent to the free *N*-terminal residue of the peptide. Substituents are cleaved from *N*-Fmoc-amino acids exceptionally rapidly.

‡ Acylated *N*-5-methoxyfurfurylamino acids were also very acid labile but did not regenerate the parent acylamino acid.

§ In the following, 'resin' refers to functionalised and cross-linked polydimethylacrylamide supported in macroporous Kieselguhr (Pepsyn KA). Internal association during solid phase synthesis occurs with both polystyrene and polyamide resins.

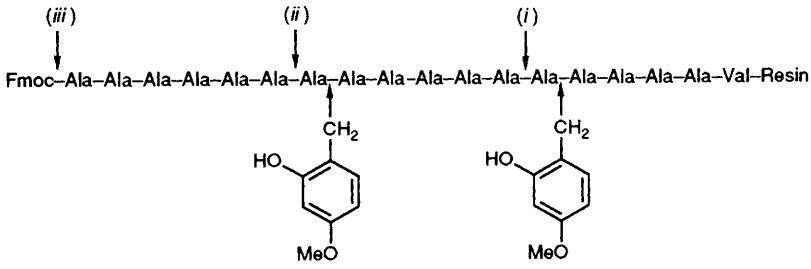


Fig. 2 Assembly of Ala[17]-Val-resin and intermediate peptides: (*i*) interchain association detected with no *N*-substituents; (*ii*) with *N*-substitution at residue 5; and (*iii*) with *N*-substitution at residues 5 and 11

The practical system required an additional methoxy substituent to generate appropriate acid lability of the benzyl group. *N*-2-Hydroxy-4-methoxybenzylamino acids **3** [R = H, Me, $CHMe_2$, CH_2CHMe_2, CH(Me)Et, and CH_2Ph] were readily prepared from 2-hydroxy-4-methoxybenzaldehyde by *in situ* reduction of Schiff bases using sodium borohydride.[5] Reaction with fluoren-9-ylmethyl chloroformate (Fmoc-Cl) in aqueous sodium carbonate–dioxane gave first the optically pure¶ *N*-Fmoc derivatives **4** and then the fully protected *N,O*-bis-Fmoc-substituted amino acids **5**.‖ Activated pentafluorophenyl (Pfp) ester derivatives[7] were readily obtained. The substituted bis(Fmoc)amino-acids **5** coupled normally to resin-bound peptides using diisopropylcarbodiimide or through the Pfp esters. (The Pfp method would now be preferred.) Deprotection with piperidine–dimethylformamide (DMF) in the usual way yielded *N*-substituted peptide derivatives **6**.

The value of the new amide protecting group in inhibiting interchain association was shown by synthesis of the well known 'difficult sequence' acyl carrier protein 65–74 decapeptide **9**. This decapeptide undergoes strong inter-chain association after addition of the penultimate glutamine residue.[3] Addition of the final valine is very strongly hindered and is invariably 10–15% incomplete [Fig. 1(*a*)] under normal Fmoc-polyamide continuous flow conditions (Pfp ester–HOBt couplings, 45 min in DMF) which are completely adequate for the earlier residues. Insertion of the *N*-substituted alanine derivative **3** (R = Me) at residue 7 enabled the final valine (residue 10) to be coupled completely under standard conditions [Fig. 1(*b*)]. This 'long range' effect of introduced *N*-substituted residues was anticipated from earlier studies[3] and was further demonstrated in syntheses of resin-bound polyalanine sequences. H-Valyl resin was extended by a series of standard continuous flow Fmoc-Ala-OPfp–HOBt couplings until strong internal association was indicated by the spectrometric deprotection profiles.[3]** The assembly was then repeated with replacement of the preceding alanine by the *N*-substituted derivative **3** (R = Me). The following alanine was added as its anhydride, and then chain extension continued under normal Pfp ester conditions. The process was repeated for incorporation of a second *N*-substituted residue. Although strong association begins in this series at the fifth alanine added, only two substitutions were required for assembly to the octadecapeptide level (Fig. 2).

The long range effect of *N*-substitution gives useful flexibility in the choice of residue to be replaced. *O*-Acylation followed by O → N migration will inevitably be slower than direct, unhindered *N*-acylation, and it would be unwise to choose unnecessarily an intrinsically hindered site containing β-branched residues. The steric constraints were explored in a series of model couplings of **6** [R = H, Me, $CHMe_2$, CH_2CHMe_2, CH(Me)Et, and CH_2Ph, peptide resin as in **1**] with a range of Fmoc-amino acid anhydrides,†† from which the following general conclusions were drawn. No special difficulties are to be expected by replacement of glycine by the derivative **6** (R = H) which was fully acylated within 1 h by a variety of residues including valine, isoleucine and *O-tert*-butylthreonine. Conversely, **6** [R = CH(Me)Et, *N*-substituted isoleucine] required 20 h for full reaction with unhindered residues (glycine, alanine, *tert*-butyl glutamate), and was incompletely acylated by leucine (96%), N^{ε}-Boc-lysine (90%) and *O-tert*-butyl serine (65%) after this time.†† β-Branched *O-tert*-butylthreonine failed to react appreciably. The corresponding valine derivative **6** (R = $CHMe_2$) behaved similarly. Valine, isoleucine and probably *O-tert*-butylthreonine should therefore be avoided as sites for *N*-substitution except when the next residue to be coupled is particularly unhindered. Intermediate *N*-substituted residues (*e.g.* *N*-substituted leucine, **6**; R = CH_2CHMe_2) may require extended reaction periods for addition of residues other than glycine; *e.g.* alanine, leucine, and *O-tert*-butyl serine coupled to the extent of 85, 50, and 25% after 15 min.†† Intermediate residues should not be selected if the following residue has a β-branched structure.

Caution is indicated in applications to peptides containing *in*-unprotected tryptophan. The cleavage product of peptides containing **3** behaves differently from simple alkoxylated benzyl cations which are completely scavenged by triisopropylsilane,[8] and *ca.* 10% of modified peptide is obtained on synthesis of H-Leu-Ala-Gly-Trp-Val-OH using **5** (R = H). This problem is solved by the use of N^{α}-Fmoc-N^{in}-Boc-tryptophan.[9]

We conclude that the *N,O*-bis(Fmoc) derivatives of N^{α}-2-hydroxy-4-methoxybenzylamino acids **5** are likely to be useful intermediates in the synthesis of sequences made difficult by inter-chain association. The dissociating effect of *N*-substitution extends for some residues towards the amino terminus, enabling unhindered regions to be chosen for modification. In solution as opposed to solid phase synthesis, a correspond-

¶ The *N*-substituents were cleaved with trifluoroacetic acid and then piperidine, and the optical purity of the resulting free amino acid established with Marfey's reagent.[6]

‖ Good analytical data and FAB-MS molecular weights were obtained in all cases.

** The deprotection profiles in these and in the previous acyl carrier protein decapeptide synthesis also showed that no significant further acylation of the phenolic hydroxy group occurred in the absence of the basic (unacylated) *N*-substituted amino group.

†† These experiments were carried out with 10 equiv. of symmetrical anhydride in dichloromethane solution at maximum concentration. For structures of type **6** but not for normal anhydride acylations (*e.g.* of isoleucine peptides by Fmoc-valine anhydride) this solvent gives faster reaction rates than DMF, supporting the idea that formation of H-bonded complexes is important in reactions of **6**.

ingly useful increase in solubility is anticipated.[4a] Other applications are under study.

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