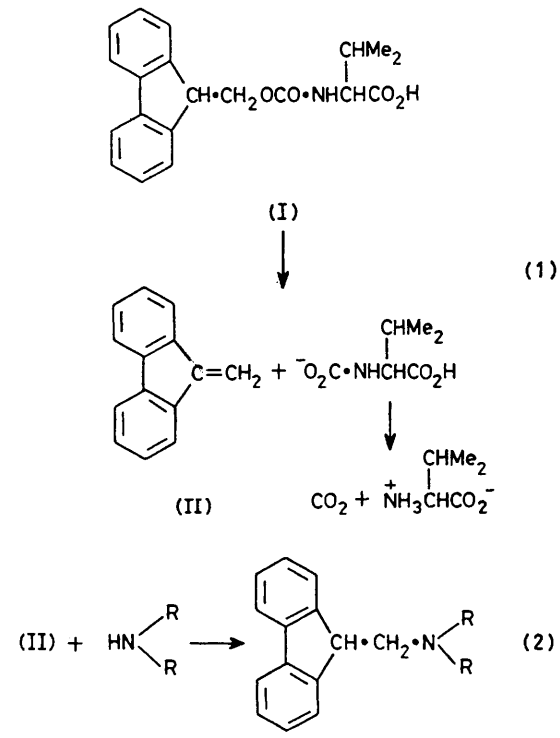
A Mild Procedure for Solid Phase Peptide Synthesis: Use of Fluorenylmethoxycarbonylamino-acids

By E. ATHERTON, HAZEL FOX, DIANA HARKISS, C. J. LOGAN, R. C. SHEPPARD, and B. J. WILLIAMS
(*Medical Research Council Laboratory for Molecular Biology, Hills Rd., Cambridge* CB2 2QH)

Summary Use of base-labile *N*-fluorenylmethoxycarbonyl-amino-acids, t-butyl based side chain protecting groups, and a *p*-alkoxybenzyl ester resin linkage provides a simple, rapid, and exceptionally mild strategy in solid phase peptide synthesis.

PRESENT day strategies of solid phase peptide synthesis[1] rely almost exclusively on *N*-t-butoxycarbonylamino-acids and on benzyl ether, ester, and urethane side chain protecting groups. Peptide resin linkages are also commonly benzyl ester or benzhydrylamine derivatives. All these benzyl-based groups require very strongly acidic conditions (anhydrous HF or HBr–CF_3CO_2H) for their final cleavage. Likewise the repetitive cleavage of Boc-groups by, *e.g.*, CF_3CO_2H or HCl–AcOH may be deleterious in the synthesis of long peptides or proteins when the total exposure to acid may exceed 1–2 days. We now describe a new combination of amino and side chain protecting groups and resin linkage which enables solid phase synthesis to proceed under much milder reaction conditions.

Current techniques for the removal of Boc-groups involve successive acidic (cleavage) and basic (neutralisation) treatments. We sought to eliminate the repetitive acid treatment and use instead mild basic conditions for the actual cleavage of the N^{α}-protecting group. Fluorenylmethoxycarbonyl[2] (Fmoc) derivatives appeared to possess reactivity appropriate to polyamide-based[3] solid phase synthesis.† Solution experiments using Fmoc-valine (I) showed a remarkable sensitivity to cleavage by secondary amines (equation 1) [$t_{\frac{1}{2}}$ *ca.* 1 min in 50% morpholine, *ca.* 30 s in 5% piperazine, and *ca.* 6 s in 20% piperidine, all in dimethylformamide (DMF) solution], and relative inertness to tertiary amines ($t_{\frac{1}{2}}$ *ca.* 22 h in 50% *N*-methylmorpholine, and *ca.* 10 h in 50% di-isopropylethylamine).‡ Selection of



† It is not to be assumed that the fluorenylmethoxycarbonyl group will be equally labile under the relatively non-polar conditions operating in conventional polystyrene-based synthesis.

‡ In contrast, methylsulphonylethoxycarbonyl-leucine (G. I. Tesser in 'Peptides 1974,' Proceedings of the Thirteenth European Peptide Symposium, Wiley, New York, 1975, p. 53) was largely unaffected by 5% piperazine or 50% triethylamine during 4 and 2 h respectively.

the Fmoc group for N^{α}-protection permitted use of acid labile t-butyl based side chain protecting groups and a *p*-alkoxybenzyl ester resin linkage.[4] The latter was conveniently introduced by way of the reagent (III).§

p-$HOCH_2 \cdot C_6H_4 \cdot OCH_2CO \cdot OC_6H_2Cl_3$-2,4,5

(III)

Val·Gln·Ala·Ala·Ile·Asp·Tyr·Ile·Asn·Gly

(IV)

In a preliminary experiment, a satisfactory synthesis of the Merrifield–Dorman test tetrapeptide[5,6] was obtained. A much more stringent test is the decapeptide sequence corresponding to residues 65—74 of acyl carrier protein (IV).[3a,7] Assembly of this decapeptide was initiated by addition of the linkage agent (III) to the leucyl-β-alanyl-polydimethylacrylamide resin using the activated ester programme already described.[3a] Because of potential dangers in the base-catalysed formation of ester bonds in the presence of base-labile protecting groups, the first amino-acid of the sequence (glycine) was introduced as the anhydride of its biphenylisopropoxycarbonyl (Bpoc) derivative[8]

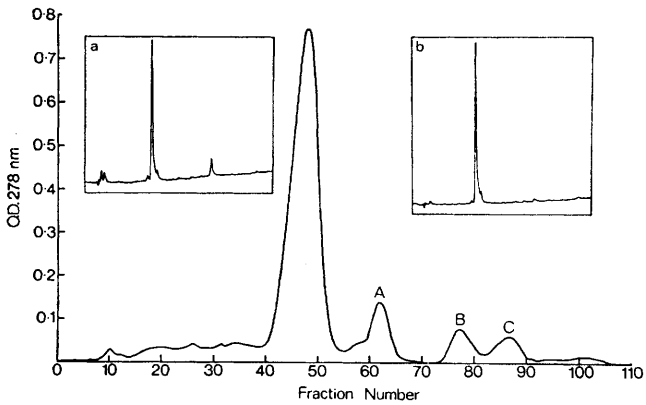


FIGURE. Ion exchange chromatography of synthetic decapeptide (IV) on diethylaminoethyl cellulose DE52. Eluant: linear gradient of 0·01—0·5 M ammonium hydrogen carbonate, pH 8·1. Insets: Analytical h.p.l.c. on Partisil ODS. Eluant: 5—60% MeCN in 0·01 M NH_4OAc, pH 4·5. (a) Total reaction product before fractionation; (b) main peak from ion-exchange chromatogram.

in the presence of *p*-dimethylaminopyridine.[9] The loading was 0·30 mequiv. g^{-1}. The Bpoc group was cleaved with 0·09N HCl–AcOH and the resin neutralised with 10% diisopropylethylamine in DMF. The following eight amino-acid residues were introduced as Fmoc-derivatives and the synthesis was conveniently completed with Boc-valine. All coupling reactions utilised immediately preformed symmetrical anhydrides except for asparagine and glutamine (*p*-nitrophenyl esters).¶ One synthetic cycle comprised the following steps: (1) DMF, 5 × 1 min; (2) 20% piperidine in DMF, 3 and 7 min; (3) DMF, 10 × 1 min; (4) coupling, six-fold excess of Fmoc-amino-acid anhydride in DMF, 60–120 min; (5) DMF, 5 × 1 min. For the active ester reactions, step (4) was replaced by: (4a) 1-hydroxybenzotriazole (6 equiv.) and Fmoc-amino-acid *p*-nitrophenyl ester (6 equiv.) in DMF. Negative ninhydrin tests for residual amine[10] were usually obtained within a few minutes of the start of the acylation reactions. Amino-acid incorporation was comparable to that previously obtained[3a] using Boc-amino-acids with the same polydimethylacrylamide resin. The leucine: glycine ratio in the final resin indicated retention of approximately 92% of the initial peptide chains.

Treatment of part (0·117 g, final loading 0·195 mmol g^{-1}) of the peptide resin with trifluoroacetic acid (15 ml) in the presence of anisole (0·13 ml) for 50 min gave 21·15 μmol (93%) of crude decapeptide (found: Asp, 2·04; Glu, 0·97; Gly, 1·00; Ala, 1·94; Val, 0·84; Ile, 1·88; Tyr, 1·00), of which 20·44 μmol was chromatographed on diethylaminoethyl cellulose DE52 using a linear gradient of 0·01–0·5 M ammonium hydrogen carbonate, pH 8·1. The elution profile is shown in the Figure. The main peak eluted in the same position as previously found[3a] for the decapeptide (IV), and yielded 18·03 μmol (88%) (found: Asp, 2·04; Glu, 1·02; Gly, 1·00; Ala, 1·98; Val, 1·00; Ile, 1·91; Tyr, 1·00). A single ninhydrin and fluorescamine-reacting spot, R_{Asp} 0·21, was obtained on paper electrophoresis at pH 6·5 and 3 kV. The overall yield from starting glycyl resin was 74·5%. Analysis of the minor peaks on the ion-exchange chromatogram gave peptide contents of A, 0·075; B, 0·21; and C, 0·13 μmol. The high u.v. absorption of peak A is associated with the presence of anisole in the cleavage reaction.

Almost identical results were obtained in a second similar experiment in which 5% piperazine (5 and 25 min) was used for the cleavage of Fmoc-derivatives.

In this synthesis, the acidic treatments previously employed using Boc-amino-acids[3a] (9 × 30 min in 2N HCl–AcOH and 60 min in anhydrous HF) were reduced to 30 min in 0·09 N HCl–AcOH and 50 min in anhydrous trifluoroacetic acid, with substantial improvement in yield. The present treatments with secondary amine appear to be without effect on the amino-acid side chains involved. Boc–Asp(OBu^t)·Gly·OBu^t or the corresponding resin bound dipeptide derivative was unchanged after treatment with 5% piperazine, 5—20% piperidine, or 50% triethylamine in DMF over 15 h, as judged by analytical ion-exchange chromatography of the deprotected products.** The similarly labile asparaginyl–glycine sequence is present in (IV). The mildness of the acidic treatments and the avoidance of benzyl-based side chain groups reduces or eliminates other potential side reactions, *e.g.* rearrangement of protected tyrosine derivatives.[11] A significant additional advantage is the use of a single solvent medium throughout, permitting a much simplified and shortened reaction cycle. Six amino-acid residues may be added per day without difficulty. The efficiency of coupling reactions using Fmoc

§ The 2,4,5-trichlorophenyl ester (III), m.p. 103–104·5°, was prepared in the usual manner (J. Pless and R. A. Boissonas, *Helv. Chim Acta*, 1963, **46**, 1609) from *p*-hydroxymethylphenoxyacetic acid, m.p. 111·5–113°, itself obtained by chloromethylation and alkaline hydrolysis of phenoxyacetic acid.

¶ Use of Fmoc·Asn(Mbh) and Fmoc·Gln(Mbh) anhydrides gave inferior results.

** We thank Professor G. W. Kenner for authentic samples of α- and β-aspartylglycine derivatives.

derivatives appears to be equal to that using Boc amino-acids. No evidence was obtained for significant back addition to the peptide chain of dibenzofulvene (II) liberated in the base catalysed cleavage reaction. Cyclic secondary amines are efficient trapping agents for this reactive product (equation 2).[2] On the evidence presented, we believe that Fmoc-amino-acids will prove valuable reagents in solid phase peptide synthesis, particularly using polar resins and reaction media.

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