Peptide synthesis. Part 2.[1] Procedures for Solid-phase Synthesis using N^{α}-Fluorenylmethoxycarbonylamino-acids on Polyamide Supports. Synthesis of Substance P and of Acyl Carrier Protein 65–74 Decapeptide

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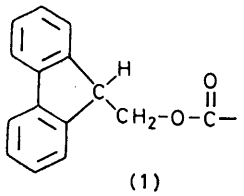
Use of base-labile N^{α}-9-fluorenylmethoxycarbonylamino-acids in combination with acid-labile t-butyl or *p*-alkoxybenzyl side-chain or carboxy-terminal (resin-linkage) protecting groups enables solid-phase peptide synthesis to be carried out under exceptionally mild reaction conditions. The repetitive and vigorous acidic treatments required in conventional synthesis are avoided. High-yield syntheses of a decapeptide and of an undecapeptide amide (Substance P) are described using this new combination of protecting groups on polyamide supports.

In the preceding paper [1] we described a procedure for solid-phase peptide synthesis utilising novel polar support materials. For the evaluation of these polyamide supports *vis à vis* polystyrene, other changes in the basic solid-phase system [2] were kept to a minimum. In particular a rather conventional combination of protecting groups was employed. Thus terminal amino-groups were protected as t-butoxycarbonyl (Boc) derivatives, and side-chain and carboxy-terminal (resin-linkage) groups as benzyl or substituted-benzyl ether or ester derivatives. This conventional combination is not necessarily optimum, and we now describe in detail [3,4] experiments and reasoning which have led us to adopt N^{α}-fluorenylmethoxycarbonyl (Fmoc) derivatives (1) [5] for routine use in polyamide-based solid-phase synthesis.



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From its beginnings [6] the Merrifield solid-phase technique has been firmly rooted in the near-exclusive use of acid-labile protecting groups. Differential cleavage has been achieved by use of groups of graded acid lability. This approach was typified in the original method [6] using *N*-terminal benzyloxycarbonyl derivatives together with a more stable nitrobenzyl ester resin linkage. Subsequently this combination was replaced by use of the Boc group for α-amino-protection with the permanent protection of trifunctional amino-acid side-chains and the resin linkage provided by benzyloxycarbonyl or benzyl ether or ester derivatives.[7] A number of other acid-labile protecting groups have been suggested and used from time to time,[2] but the Boc–benzyl combination has formed the basis for the vast majority of solid-phase syntheses reported.[2]

As solid-phase peptide synthesis has developed and the synthetic targets have become larger and more sensitive, the limitations of this system have become more apparent. Both the repetitive cleavage of Boc groups, and especially the final cleavage of benzyl derivatives involve treatment with strong acids which are clearly destructive to some peptide sequences. Typically acidolysis of Boc groups requires *ca.* 30 min treatment with *e.g.* trifluoroacetic acid per cycle, giving a total contact time of many hours in the synthesis of a substantial peptide. Likewise, final deprotection and detachment of the completed peptide from the resin requires treatment with even stronger acid, commonly liquid hydrogen fluoride, the damaging effects of which are well recognised.[2b] Selectivity in the cleavage of Boc groups in the presence of multiple benzyl functions may be inadequate, resulting in premature loss of side-chain protection [8] and detachment of peptide from the resin.[9] Several acid-induced side reactions of benzyl-protected amino-acid derivatives have been recognised, notably reaction of benzyl cations with the nucleophilic sulphur atom in methionine residues,[10] *O* ⟶ *C* migration in *O*-benzyl-tyrosine derivatives,[11] cyclic imide formation from β-benzyl aspartyl esters,[12] and formation of pyroglutamyl residues and other side-products derived from acylium ion intermediates in γ-benzyl-glutamate cleavage.[13] Some of these last problems together with the lack of absolute selectivity in repetitive end group cleavage have been minimised by the introduction of more stable, nuclear-substituted benzyl derivatives for particular amino-acids, *e.g.* ε-2,4-dichlorobenzyloxycarbonyl-lysine,[8b] *O*-2-bromobenzyloxycarbonyl-[14] and 2,6-dichlorobenzyl-tyrosine,[11] and *p*-chlorobenzyl aspartate and glutamate.[15] A more stable *p*-carbamoylbenzyl ester resin linkage has also been employed.[16] All these negatively substituted benzyl derivatives are of course cleaved more slowly than their unsubstituted parents, and may therefore require even more vigorously acidic final deprotection conditions, exacerbating the first-mentioned problem.

RESULTS AND DISCUSSION

It seemed to us that the difficulties described above warranted reconsideration of the protecting group and resin-linkage system as a whole rather than piecemeal. The foremost requirement is to reduce the overall severity of reaction conditions in solid-phase peptide

synthesis. This is a prerequisite to application to the synthesis of sensitive proteins. Any new system should, if possible, convey additional flexibility enabling, for example, synthesis and detachment of *protected* peptides useful in fragment condensation strategies. This also may ultimately prove to be essential for useful protein synthesis. It strongly suggests abandonment of the concept of graded lability to the same reagent type as a feature of protecting group design.

Our preferred system now uses *base-labile* groups for amino-protection in combination with *acid-labile* t-butyl-based derivatives for side-chain protection. Thus the multiple treatments with *e.g.* trifluoroacetic acid required for Boc-group cleavage are replaced by a single final treatment, and use of liquid hydrogen fluoride or other very strongly acidic reagents is dispensed with. Several possibilities were considered for the base-labile amino-protecting group, *viz.*, methylsulphonylethoxycarbonyl,[17] α-cyano-t-butoxycarbonyl,[18] 5-benzisoxazolylmethoxycarbonyl,[19] and fluorenylmethoxycarbonyl (1).[5] Only the last of these appeared to combine the required degree of base-lability, complete acid stability, and ease of preparation of crystalline, readily purified amino-acid derivatives. Fmoc-amino-acids were introduced into peptide chemistry by Carpino and Han [5] in 1970, but found little practical use until the present work [3] and a contemporary, independently conceived application to polystyrene-based solid-phase synthesis.[20] During the past year interest has quickened and detailed papers have now appeared on the preparation of a range of amino-acid derivatives [21] and of their activated esters.[22] Utility of Fmoc-derivatives in solution-phase peptide synthesis is being re-examined.[22,23]

In our experience, Fmoc-amino-acids may be simply prepared by reaction of the appropriate amino-acid with a 10% excess of (crystalline) 9-fluoroenylmethyl chloroformate in 10% aqueous sodium carbonate–dioxan, essentially Carpino and Han's conditions.[5] Reaction is continued until a negative ninhydrin reaction (t.l.c.) is obtained; usually 1—1.5 h at room temperature is adequate. Unduly prolonged reaction periods may be deleterious (*cf.* ref. 21). Chemical characteristics of a wide range of Fmoc-amino-acids have been tabulated.[21] Although general procedures have been described [22] for *p*-nitrophenyl ester preparation, the derivatives of asparagine and glutamine which present special solubility problems are described in the Experimental section.

Fmoc-amino-acids and peptides show apparently quite exceptional lability towards secondary amines. In our hands, Fmoc-valine was cleaved within seconds by piperidine in dimethylformamide solution (Table 1). The rate of cleavage is evidently strongly influenced by both base strength and steric factors. The weaker but sterically similar bases piperazine and morpholine cleave Fmoc-valine at distinctly slower rates, although the former is still a practically useful reagent in solid-phase synthesis even at the lower concentration necessitated by its limited solubility. The more hindered secondary amine dicyclohexylamine is relatively ineffective, as are tertiary amines in general. The appreciable lability towards *p*-dimethylaminopyridine is of significance in connection with its use as a catalyst for ester-bond (resin-linkage) formation, and is considered further below. Approximate rates of cleavage of the Fmoc-group by other basic reagents have been tabulated by Chang *et al.*[21]

TABLE 1

Approximate half lives for cleavage of Fmoc-Val-OH by various amines in dimethylformamide

20% Piperidine	6 s
5% Piperazine	20 s
50% Morpholine	1 min
50% Dicyclohexylamine	35 min
10% *p*-Dimethylaminopyridine	85 min
50% Ethyldi-isopropylamine	10.1 h

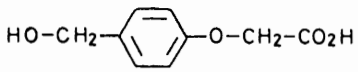
Of special importance in peptide synthesis is the possibility of Fmoc-group cleavage by the relatively weakly basic amino-component during the actual acylation reaction. If it occurred to a detectable extent, it would result in multiple addition of individual amino-acid residues. This possibility has recently been discussed elsewhere, both for the solution- [23] and solid-phase situations.[21] In solution-phase experiments, rates have been determined for cleavage of Fmoc-glycine and alanine by a range of amino-components at 0.2M concentration; half-lives were 10—70 h.[23] Our own experiments [24] on the cleavage of neutral Fmoc-derivatives by poly(dimethylacrylamide)-bound secondary amine at relatively high concentration (2—4 milliequiv. g^{-1}) again gave extended times (8—12 h) for complete reaction. Both the concentration and base strength of these resin-bound amines are very much higher than are encountered at the start of a typical polyamide solid-phase acylation reaction (*ca.* 0.3 milliequiv. g^{-1} amino-component, resin swollen volume *ca.* 20 ml, corresponding to *ca.* 0.015M), and this declines rapidly to near zero, usually within a few minutes as judged by the sensitive ninhydrin test.[25] Thus significant loss of Fmoc-groups during the acylation reaction would not be expected, and we have not yet detected side-products in which this has occurred. On the other hand it should be noted that Fmoc-derivatives are not completely stable to prolonged storage in polar aprotic solvents. Thus in dimethylacetamide, dimethylformamide, and *N*-methylpyrrolidone, Fmoc-glycine decomposed to the extent of 1, 5, and 14%, respectively during 7 days.[26] For this and other reasons [27] we now favour dimethylacetamide as a general solvent for peptide synthesis using Fmoc-derivatives, although much of the work described here utilises dimethylformamide. One cycle of solid-phase synthesis as described here occupying *ca.* 2 h would correspond to *ca.* 0.01% decomposition in dimethylacetamide. Both dimethylacetamide and dimethylformamide need to be completely freed from traces of dimethylamine and from acetic or formic acids by careful

fractional distillation *in vacuo* immediately before use in solid-phase synthesis, especially in view of the relatively large volumes of solvents involved.

The complete stability of Fmoc-derivatives to acidolysis was convincingly demonstrated by Carpino and Han,[5] and is in line with our own experience. In contrast, we were unable to confirm the reported resistance to hydrogenolysis.[5] In our hands, Fmoc-aniline and Fmoc-glycine were cleaved to 9-methylfluorene by hydrogenation over palladium–charcoal on palladium block catalysts under a variety of conditions with half-lives of 2—30 h.[26] Confirmatory results have since been obtained by others.[28] This surprising result indicates caution in applications involving hydrogenation of Fmoc-derivatives (*cf.* ref. 21).

The very high repetitive yields in deprotection and acylation reactions demanded by solid-phase synthesis require a large margin of safety in assessing minimum reaction conditions. At least 13 half-lives are required to ensure 99.99% reaction. An additional rather arbitrary factor of 6 has been included to allow for end group and possible sequence variations from the data in Table 1, as well as for the transfer from free solution to solid-phase reaction conditions. On this basis we have adopted a standard 10-min contact time with 20% piperidine–dimethylformamide for Fmoc-cleavage of resin-bound peptide. This treatment would seem to be without detectable effect on t-butyl-based side-chain protecting groups. The most base-labile t-butyl derivatives likely to be encountered are those protecting the side-chains of aspartyl residues, particularly when adjacent to glycine, serine, and threonine.[29] Boc-Asp(OBu^t)-Gly-OBu^t and an analogous resin-bound dipeptide gave no β-aspartylglycine * or other ninhydrin-reacting products after 24 h treatment with piperidine–DMF (equivalent to 144 reaction cycles) and acidic deprotection. The similarly base-labile asparaginyl-glycine sequence is present in the test decapeptide sequence described below; we have recently successfully synthesised a tridecapeptide containing the –Asp–Ser– sequence without undue difficulty.[30]

The polyamide solid-phase method allows complete flexibility in the choice of peptide-resin linkage groupings.[31] For use in combination with N^{α}-Fmoc and t-butyl-based side-chain protection, the acid-labile *p*-alkoxybenzyl ester resin linkage [32] is generally applicable. This is easily achieved through the linkage agent (2), analogous to (3) previously employed in combination with benzyl-based side chain protection.[1] Esters of the benzylic hydroxy-group in (2) are cleaved under the same acidic conditions (*e.g.* trifluoroacetic acid, 30 min) as are t-butyl derivatives, enabling simultaneous side-chain deprotection and detachment of completed peptides. Esters of (3) require the more drastic hydrogen fluoride treatment associated with benzyl ester derivatives of normal reactivity. Essentially complete acid stability (unaffected by liquid hydrogen fluoride) and enhanced nucleophilic lability is obtained with esters derives from the *p*-carboxy-substituted benzyl alcohol (4). Application of this linkage agent to the preparation of a peptide amide (Substance P) is illustrated below. Other potentially useful linkage agents have been tabulated elsewhere.[31] An additional reagent (5) offers special promise for the photolytic detachment [33] of



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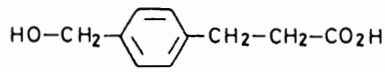

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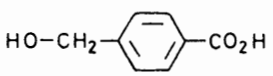

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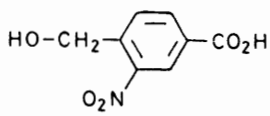

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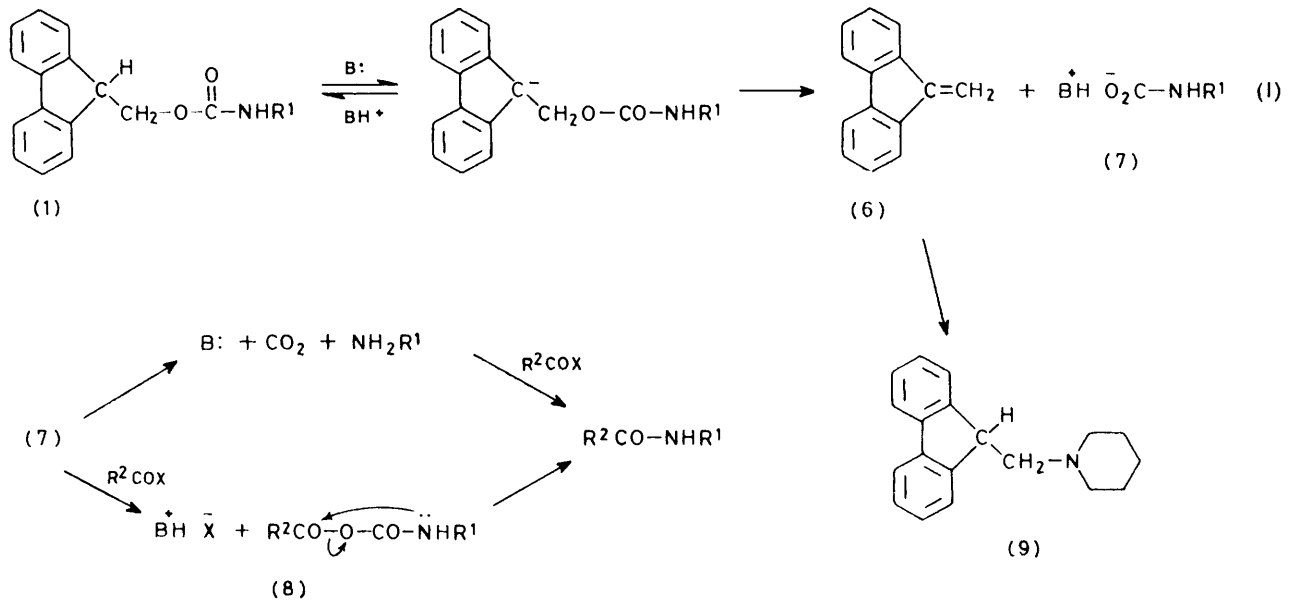
protected peptides for use in solid-phase fragment condensation strategies.[34]

The general protocol for use of Fmoc-amino-acid anhydrides and activated esters in polyamide solid-phase synthesis is shown in Table 2, column A, together with the comparable procedure (column B) for Boc-amino-acid derivatives.[1] In procedure (A) all the reactions are carried out in a single polar reaction medium (dimethylformamide or dimethylacetamide), resulting in considerable advantage in terms of speed and simplicity over (B). No special steps are taken, or appear to be necessary, to ensure decarboxylation of the presumed intermediate carbamic acid salt (7) [Scheme, equation (1)] which could be stable under the basic conditions of its formation. It is possible that peptide bond formation occurs by acylation of this carboxylate and rearrangement of the resulting *N*-carboxy-anhydride (8) (arrows in Scheme). This reaction path may be compared to the formation of amide bonds through reaction of isocyanates with carboxylic acids.[35] No mechanistic studies have apparently been carried out on the base-catalysed decomposition of fluorenylcarbamates, but fluoroenylmethanol itself follows an *E*lcb mechanism.[36] A similar path of initial conjugate base formation [equation (1)] would be favoured in the polar resin–polar solvent environment provided in the polyamide solid-phase method, and this may be a significant factor in the relative applicability of fluorenylmethoxy-carbonylamino-acids in polyamide solid-phase synthesis compared with more apolar polystyrene-based systems. No firm evidence has been obtained for significant (irreversible) back addition of dibenzofulvene (6) to

* Authentic samples of α- and β-aspartyl-glycine were kindly provided by the late Professor G. W. Kenner.

newly liberated terminal amino-groups, although trace impurities observed during purification procedures may show relatively high u.v. absorption compared with their peptide content (see below). Qualitative experiments with alanine t-butyl ester showed that this addition is a slow process, and rapid reaction [5] with the very large excess of deprotecting secondary base (piperidine) to form (9) would be expected to predominate.



For the validation of the Fmoc-polyamide procedure in actual synthesis, we initially employed the same test decapeptide sequence (10) as before.[1] The original β-alanine-containing poly(dimethylacrylamide) support [1,27] was employed with initial addition of leucine as internal stable biphenylisopropoxycarbonyl (Bpoc) protecting group for esterification of the first residue (glycine) to

H–Val–Gln–Ala–Ala–Ile–Asp–Tyr–Ile–Asn–Gly–OH
(10)

H–Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2
(11)

the resin with subsequent very mild acidic treatment. It is not at all certain that this precautionary strategem will prove to be generally necessary (*cf.* ref. 20). However, on occasions when fluorenylmethoxycarbonyl-amino-acid derivatives are used in the presence of *p*-dimethylaminopyridine for attachment of the first reference amino-acid and the acid-labile *p*-alkoxybenzyl alcohol linkage agent (2). As mentioned previously, fluorenylmethoxycarbonyl groups are cleaved at an appreciable rate by *p*-dimethylaminopyridine, the usual

TABLE 2

Procedures for polyamide solid-phase peptide synthesis using (A) N^{α}-Fmoc protection: (B) N^{α}-Boc protection (HOBt = 1-hydroxybenzotriazole)

	(A) α-Fmoc-ω-butyl		(B) α-Boc-ω-benzyl	
			$EtCMe_2OH$	5 × 2 min
	DMF *	5 × 1 min	AcOH	5 × 2 min
Deprotection:	Piperidine–DMF	3 + 7 min	HCl–AcOH	5 + 25 min
	DMF	10 × 1 min	AcOH	5 × 2 min
			$EtCMe_2OH$	5 × 2 min
			DMF	5 × 2 min
Neutralisation:			Pri_2NEt–DMF	3 × 2 min
			DMF	5 × 2 min
Coupling:	Boc-AA-anhydride or active ester/ HOBt–DMF	60—120 min	Boc-AA-anhydride or active ester/ HOBt–DMF	60—120 min
	DMF	5 × 1 min	DMF	5 × 2 min
	Total operations:	23		41
	Cycle time:	2—3 h		5—6 h

* Dimethylacetamide may universally replace dimethylformamide (see text).

amino-acid, it will be appropriate to test by cleavage of a small resin sample that no dipeptide has been formed through premature loss of amino-protection and further acylation. This is our present practice.

The following eight amino-acid residues were introduced using exclusively Fmoc-amino-acids and the synthesis conveniently terminated using Boc-valine. Full details of the synthetic and purification procedures are given in the Experimental section. Two complete syntheses were carried out. In the first, 30-min exposure to 5% piperazine was used for repetitive deprotection; in the second, 10 min with 20% piperidine. Essentially identical results were obtained in both cases, the overall yield of decapeptide purified by a single ion-exchange chromatography being 75%. The elution profile for the second synthesis is shown in the Figure. On the rela-

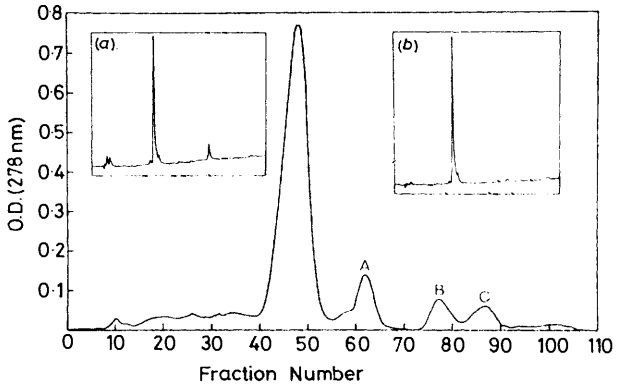


FIGURE Ion-exchange chromatography of the synthetic decapeptide (10) on diethylaminoethyl cellulose DE52: eluant, linear gradient of 0.01—0.5M ammonium hydrogencarbonate, pH 8.1. Insets: analytical h.p.l.c. on Partisil ODS; eluant 5—60% MeCN in 0.01M NH_4OAc, pH 4.5: (*a*) total reaction product before fractionation: (*b*) main peak from ion-exchange chromatogram

tively small scale employed for research experimentation, an indeterminate but significant part of the resin is removed for analytical control during the course of the synthesis. The yield quoted therefore reflects the efficiency of repetitive peptide-bond formation and protecting-group cleavage, retention of peptide chains on the resin, and the recoveries during the detachment, protecting-group cleavage, and all purification steps. Peptide-resin loss for analytical or other reasons is not included; previous experience [1] suggests that little if any polyamide resin is lost through physical attrition, providing its swollen gelatinous nature is retained throughout. The overall yield of decapeptide obtained is nearly two-fold better than that of the comparable synthesis utilising Boc and benzyl protecting groups and strongly acidic reagents.[1] In the latter case, though, significant losses were probably incurred through inefficiencies in the ion-exchange chromatographic procedure.[1]

A second test synthesis was provided by the undecapeptide amide Substance P (11) utilizing the newer sarcosine-containing polyamide support.[1,4] This resin is functionalised at the polymerisation stage with methoxycarbonyl-groups, and is converted to an amino-resin by aminolysis with an excess of ethylenediamine.[1] An internal reference amino-acid (norleucine) was added by the standard Fmoc-amino-acid anhydride procedure given in Table 2 (A), followed by active ester coupling of the acid-stable linkage agent (4). A second treatment with the active ester of (4) was necessary before a negative ninhydrin reaction was obtained, although this has not proved generally necessary. Thereafter the synthesis proceeded similarly to that described above. The t-butoxycarbonyl derivative of methionine was employed in the resin esterification step, avoiding the problem of lability of Fmoc-groups to dimethylaminopyridine. The following nine amino-acid residues were incorporated as Fmoc-anhydrides or *p*-nitrophenyl esters (glutamine) following the standard procedure. Although reaction with Fmoc-Lys(Boc) anhydride was complete as judged by ninhydrin reaction, amino-acid analysis revealed that this and all subsequent residues were incorporated to the extent of only 70% (see Experimental section). This problem is one which has recurred in the synthesis of other sequences utilising the ε-Boc-derivative of Fmoc-lysine (*e.g.* ref. 36), and is clearly associated with the particular derivative. It was not encountered in a later synthesis of substance P using the ε-trifluoroacetyl derivative of Fmoc-lysine,[36] nor in a very recent synthesis of a part sequence of cytochrome C.[37] The ε-trifluoroacetyl group is stable to prolonged treatment to piperidine,[36] and provides a useful solution to the problem. The present synthesis was completed using the hydrochloride salt of Boc-arginine for the final coupling. Insolubility of this derivative precluded activation to the symmetrical anhydride with dicyclohexylcarbodi-imide in dichloromethane in the usual way,[1] but a good incorporation was obtained by treatment with dicyclohexylcarbodi-imide in dimethylformamide and addition of the whole activation mixture to the resin after 5 min. Recent results indicated that the N^{α}-Fmoc derivative of ωω-bis(adamantyloxycarbonyl)arginine [38] may provide a general reagent for the introduction of this amino-acid.

The undecapeptide was detached from the resin by ammonolysis, side-chain protecting groups were removed by a single trifluoroacetic acid treatment, and the amide purified by ion-exchange chromatography. Isolation was complicated by the presence in the crude product of a substantial proportion of methionine sulphoxide derivative, easily distinguished by reversed-phase h.p.l.c. The oxide was smoothly reduced by excess of dithiothreitol [39] and the purified substance P finally separated by Sephadex chromatography. The overall yield of material identical with authentic substance P by amino-acid analysis, h.p.l.c., and radio-immunoassay was 47%.

These results show that fluorenylmethoxycarbonyl derivatives of amino-acids may be usefully employed in solid-phase peptide synthesis. Their application is not limited to polyamide-based systems although the polar reaction media characteristic of the latter may favour

smooth base-catalysed β-elimination. Conditions for cleavage of fluorenylmethoxycarbonyl groups are exceptionally mild. Combination with t-butyl-based side-chain protecting groups and an appropriately labile peptide–resin linkage enables a substantial reduction in the overall severity of solid phase reaction conditions. Further applications embracing a wide range of amino-acid types have been reported.[35,36]

EXPERIMENTAL

p-Bromomethylbenzoic Acid.—α-Bromo-*p*-toluonitrile (20 g 0.102 mol) was heated with constant-boiling hydrobromic acid (200 ml) for 5 h under reflux. After cooling the solid was collected, washed with water, and dried *in vacuo*. Recrystallisation from ethyl acetate gave *p*-bromomethylbenzoic acid (12.5 g, 57%), m.p. 231—232 °C. A second crop (4.5 g, 20.5%) had m.p. 225—227 °C (lit.,[40] 223—224 °C).

p-Hydroxymethylbenzoic Acid.—*p*-Bromomethylbenzoic acid (12.5 g. 0.058 mol) was boiled in water (500 ml) for 1 h after dissolution. After cooling, the crystalline product was collected and recrystallised from water. *p*-Hydroxymethylbenzoic acid (7.8 g, 88%) had m.p. 181—183 °C (lit.,[40] m.p. 179—181 °C).

p-Hydroxymethylbenzoic Acid 2,4,5-Trichlorophenyl Ester.—*p*-Hydroxymethylbenzoic acid (4.5 g, 30 mmol) and 2,4,5-trichlorophenol (6.5 g, 33 mmol) were dissolved in dimethylformamide (40 ml), cooled to 0 °C, and a solution of dicyclohexylcarbodi-imide (6.8 g, 33 mmol) in dimethylformamide added dropwise with stirring. The reaction mixture was stirred and allowed to warm to room temperature overnight. The dicyclohexylurea was filtered off and the solution evaporated. The residual oil was dissolved in ethyl acetate (50 ml), insoluble urea filtered off, and petrol added. On cooling the *trichlorophenyl ester* (8.5 g, 86%) crystallised, m.p. 130—135 °C. An analytical sample melted at 139.5—141 °C (Found: C, 50.85; H, 3.05. $C_{14}H_9Cl_3O_3$ requires C, 50.71; H, 2.74%).

p-Chloromethylphenoxyacetic Acid.—A mixture of phenoxyacetic aid (24.5 g, 0.16 mol) and 40% w/v aqueous formaldehyde (38 ml) was stirred in an ice–salt bath until the temperature fell to −5 °C. A gentle stream of hydrogen chloride was passed through the stirred mixture for 40 min. Excess of water was then added and after cooling in ice briefly the product was collected by filtration, washed well with water, and dried. The combined yield from four such experiments (106.4 g) was recrystallised from chloroform to give a first crop consisting of 43.6 g (34%), m.p. 125—129 °C. A sample recrystallised for analysis had m.p. 128—129 °C (Found: C, 53.9; H, 4.55. $C_9H_9ClO_3$ requires C, 53.88; H, 4.52%).

p-Hydroxymethylphenoxyacetic Acid.—The chloromethyl derivative (25 g, 0.123 mol) was dissolved with effervescence in saturated aqueous sodium hydrogencarbonate (500 ml) and set aside overnight. The cloudy solution was filtered, washed with ethyl acetate (3×), acidified to pH 2 with concentrated hydrochloric acid (cooling), and extracted again with ethyl acetate (3×). The ethyl acetate extracts were washed with water, dried (Na_2SO_4), and evaporated to give 17.1 g of *product*. Recrystallisation from ethyl acetate–light petroleum (b.p. 60—80 °C) gave 12.14 g (53%), m.p. 111—112.5 °C. The analytical sample had m.p. 110.5—112 °C (Found: C, 59.25; H, 5.52. $C_9H_{10}O_4$ requires C, 59.34; H, 5.53%).

The *2,4,5-trichlorophenyl ester* was prepared from the foregoing acid (12.0 g, 65 mmol), 2,4,5-trichlorophenol (14.3 g. 72 mmol). and dicyclohexylcarbodi-imide (13.6 g, 65 mmol) in ethyl acetate (80 ml) initially at ice–salt temperature and then overnight at room temperature. Recrystallisation of the product from ethyl acetate–light petroleum (b.p. 60—80 °C) gave 11.7 g (49%) m.p. 100—102 °C. An analytical sample had m.p. 97—105 °C (Found: C, 49.85; H, 3.2. $C_{15}H_{11}Cl_3O_4$ requires C, 49.82; H, 3.07%).

Nα-Fluorenylmethoxycarbonyl-L-glutamine p-Nitrophenyl Ester.—Fluorenylmethoxycarbonyl-L-glutamine (4 g. 10.9 mmol) and *p*-nitrophenol (8.06 g. 57.9 mmol) were suspended in dimethylformamide (20 ml) and warmed gently until dissolution was complete. Dicyclohexylcarbodi-imide (2.45 g. 11.9 mmol) dissolved in dimethylformamide (10 ml) was added dropwise to the stirred and cooled (ice-bath) solution. The mixture was stirred and allowed to warm to room temperature overnight. The precipitated dicyclohexylurea was filtered off and washed with ethyl acetate, and the combined filtrate and washings evaporated to an oil which solidified on addition of ether. The product was collected, washed well with ether. and crystallised from ethanol–dimethylformamide containing a few drops of acetic acid. The yield was 2.7 g (51%), m.p. 192—193 °C, $[\alpha]_D^{18}$ −46.5° (*c* 1.01 in DMF) {lit.,[23] m.p. 182—185 °C, $[\alpha]_D^{22}$ −40.3° (*c* 1—2 in DMF–1% AcOH)} (Found: C, 63.95; H, 4.95; N, 8.55. Calc. for $C_{26}H_{23}N_3O_7$: C, 63.80; H, 4.73: H, 8.58%).

Nα-Fluorenylmethoxycarbonyl-L-asparagine p-Nitrophenyl Ester.—The asparagine derivative was prepared similarly to the foregoing ester and crystallised from nitromethane in 52% yield, m.p. 188—189 °C. $[\alpha]_D^{18}$ −39.1° (*c* 1.02 in DMF) {lit, [23] m.p. 170—172°, $[\alpha]_D^{22}$ −37.8° (*c* 1—2 in DMF–1% AcOH)} (Found: C, 62.85; H, 4.50; N. 8.95. Calc. for $C_{25}H_{21}N_3O_7$, C, 63.15; H. 4.45: N, 8.84%).

Rates of Cleavage of Fmoc-valine by Amines.—*E.g.* by 50% morpholine: Fmoc-valine (33.213 mg, 98 μmol) was dissolved in dimethylformamide (10 ml). Aliquots (50 μl) were withdrawn from this solution with a Hamilton syringe, treated with morpholine (50 μl), and quenched at time intervals of 0.5, 1, 2, 3, 4, 10, and 32 min by addition of 5 ml of 0.2M sodium citrate buffer, pH 2. Free valine was determined in the resulting solutions by amino-acid analysis The approximate half-life determined graphically was 1.0 min. Reaction rates with other amines were determined similarly (Table 1).

Stability of Boc-Asp(OBu^t)-Gly-OBu^t *to Piperidine.*—The *dipeptide derivative* (38%) was obtained by reaction between Boc-Asp(OBu^t)-OH (from the dicyclohexylammonium salt, 0.94 g, 2 mmol), glycine t-butyl ester dibenzosulphonimide salt (0.857 g, 2 mmol). triethylamine (0.28 ml. 2 mmol), and dicyclohexylcarbodi-imide (0.412 g, 2 mmol) in chloroform overnight, m.p. 81—83 °C (from ether–light petroleum) (Found: C, 56.75; H, 8.45; N, 7.15. $C_{19}H_{34}N_2O_7$ requires C, 56.72; H, 8.46; N, 6.96%). The dipeptide ester (6.3 mg, 15.65 μmol) was dissolved in DMF containing piperidine (5 ml, 20% v/v) and aliquots (0.1 ml) removed after 5 min, 1, 4, 24, and 96 h and quenched by addition to an excess of trifluoroacetic acid. After 20 min, individual samples were evaporated rapidly, dissolved in pH 2.2 citrate buffer (5 ml) and subjected to analytical ion-exchange chromatography (JEOL 5AH Amino-Acid Analyser). A single integratable ninhydrin-reacting peak eluting coincident to authentic α-aspartylglycine at *ca.* 140 min was found for reaction times of up to 24 h (equivalent to 144 deprotection

cycles). The 96 h reaction showed a second integrated peak (equivalent in ninhydrin colour yield to 7.5% of the main peak) eluting at 86 min. Authentic β-aspartyl-glycine eluted at 65 min under the same conditions.

A similar experiment was carried out using 5% piperidine–DMF with resin-bound Boc-Asp(OBu^t)-Gly-OCH$_2$-C$_6$H$_4$-O-CH$_2$-CO-Ala-Ala-polymer prepared by the general procedures described below. Aliquots (3—6 mg) of the peptide resin (Found: Ala 2.18, Gly 1.00, Asp. 1.03) were treated for 15 min, 2, 6, 18, and 42 h with a solution of 5% piperidine in DMF. The resin was washed thoroughly with DMF, t-pentyl alcohol, and acetic acid, and then treated with excess of trifluoroacetic acid for 1 h. The trifluoroacetic acid solution and washings were evaporated and the residue dissolved in pH 2.2 citrate buffer (10 ml) and analysed as above. A single ninhydrin-reacting peak eluting in the position of authentic α-aspartyl-glycine was found in each case. A similar experiment with 5% piperazine in DMF gave the same results. In a control experiment, 95% of the dipeptide was cleaved from the resin by trifluoroacetic acid in 1 h. as indicated by residual resin analysis.

Solid-phase Synthesis of Acyl Carrier Protein Residues 65—74.—(a) *Using piperazine for cleavage of fluorenylmethoxycarbonyl protecting groups.* General instructions for solid phase synthesis using polydimethylacrylamide resins have been given previously.[1] The Boc-β-alanyl resin [1] (1 g) was swollen in dimethylformamide (DMF) and acylated with pre-formed [1] Boc-leucine anhydride (2 mmol) in DMF (15 ml) for 1 h using the coupling reaction, deprotection, and neutralisation cycle for Boc-amino-acids previously described.[1] The incorporation of leucine was 0.26 milliequiv. g^{-1}. *p*-Hydroxymethylphenoxyacetic acid 2,4,5-trichlorophenyl ester (2 mmol) was similarly coupled in the presence of hydroxybenzotriazole (2 mmol) in DMF (15 ml) for 20 min. To the washed resin, dimethylaminopyridine (2 mmol) in DMF (7.5 ml) was added followed after 5 min by a solution of Bpoc-glycine (from the dicyclohexylammonium salt, 4 mmol) and dicyclohexylcarbodiimide (2 mmol) in DMF (7.5 ml). After 17 h, the resin was thoroughly washed (DMF) and one-half stored under DMF–N$_2$ at 0 °C.

The remainder was deprotected using 0.09M HCl–AcOH and the hydrochloride salt neutralised.[1] The following *N*-protected amino-acid anhydrides (1 mmol),[1] or their active esters (1 mmol) in the presence of hydroxybenzotriazole (1 mmol) were coupled in DMF (7.5 ml) for the times indicated using a modified [1] Beckman 990 Peptide Synthesiser. The reaction cycle was as in Table 2(A) except that 5% piperazine in DMF (5 + 25 min.) was used as deprotecting reagent: Fmoc-Asn-ONp (17 h); Fmoc-Ile-OH (70 min); Fmoc-Tyr(Bu^t)-OH (66 min); Fmoc-Asp(OBu^t)-OH (66 min); Fmoc-Ile-OH (99 min); Fmoc-Ala-OH (65 min); Fmoc-Ala-OH (65 min); Fmoc-Gln-ONp (2 h); Boc-Val-OH (99 min). Samples of resin were removed for qualitative ninhydrin tests after every amino-acid addition and deprotection step, and for amino-acid analysis [1] after each complete cycle. Negative ninhydrin tests were obtained after each acylation. The amino-acid ratios in Table 3 (expressed relative to glycine = 1.00) were obtained. The final loading of glycine on the resin was 0.17 milliequiv. g^{-1}; 96% of the peptide chains were retained on the resin (Leu/Gly = 1.04). A sample of peptide–resin (53.8 mg) which had been washed with t-pentyl alcohol, acetic acid, t-pentyl alcohol, DMF, and dichloromethane, and dried *in vacuo* was treated with anisole (0.05 ml) and trifluoroacetic acid (10 ml). After 50 min stirring the resin was filtered off, washed with trifluoroacetic acid (3 × 10 ml) and then as above and dried (39.6 mg, Gly/Leu = 0.145. 15% residual peptide). The filtrate and trifluoroacetic acid washings were combined and evaporated to yield 7.4 μmol (81%) of peptide (Found: Gly, 1.00; Asp, 1.99; Ile, 1.93; Tyr, 0.96; Ala. 2.06; Glu, 0.97: Val, 0.89). This product (7.1 μmol) was applied to a column of diethylaminoethyl-cellulose (Whatman DE52) (34 cm × 1.5 cm diameter) and eluted with a linear gradient of 0.01M to 0.5M ammonium bicarbonate, pH 8.1. Flow rate 0.9 ml/min. The main peak emerged at 294—336 ml (6.81 μmol, 96% recovery, overall yield 74.6%) (Found: Gly, 1.00; Asp, 2.00; Ile, 1.96; Tyr, 1.03; Ala, 2.04; Glu. 1.03; Val, 1.01). This product was identical on t.l.c. and paper electrophoresis with that prepared previously.[1]

TABLE 3

Step	1	2	3	4	5	6	7	8	9	10
Leu	1.00	0.97	1.03	0.97	1.02	1.03	1.05	1.07	1.05	1.04
Gly	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Asp		1.00	1.01	1.03	2.02	2.03	2.01	2.06	1.95	2.04
Ile			0.94	0.87	0.91	1.87	1.88	1.91	1.87	1.88
Tyr				0.96	0.91	0.98	0.96	0.99	0.95	0.98
Ala							0.95	1.97	1.89	1.88
Glu									0.93	0.98
Val										0.90

(b) *Using piperidine as deprotecting agent.* The assembly was carried out essentially as in (a) above with the principal exception that 20% piperidine in DMF (3 + 7 min) was used for the deprotection step [Table 2(A)]. The coupling time for Fmoc-Asn-ONp was reduced to 70 min. Samples for qualitative ninhydrin tests were withdrawn at every coupling and deprotection step as before, but amino-acid analysis was performed only after addition and deprotection of residues 1 (Bpoc-Gly-OH), 4 [Fmoc-Tyr(Bu^t)-OH], 7 (Fmoc-Ala-OH), and 10 (Fmoc-Val-OH). The initial and final glycine loading were 0.305 and 0.195 milliequiv. g^{-1} respectively. 92% of the peptide chains were retained on the resin (Leu/Gly = 1.09). A sample (117 mg) of washed and dried peptide resin was treated with trifluoroacetic acid and anisole as in (a) above, yielding 21.15 μmol of peptide (93%) (Found: Gly, 1.00; Asp, 2.04; Ile, 1.88: Tyr, 1.00; Ala, 1.94; Glu, 0.97; Val, 0.84). Part (20.44 μmol) of this product was chromatographed on a column of DE52 anion-exchange resin as before, giving the elution profile of the Figure. The principle peak contained 18.03 μmol of peptide, 88% recovery, overall yield 74.5% (Found: Gly, 1.00, 1.00; Asp, 2.04, 2.00; Ile, 1.91, 2.06; Tyr, 1.00, 1.07; Ala, 1.98, 2.07; Glu, 1.02, 1.00; Val, 1.00, 1.04). The impurity peaks A, B, and C contained respectively 0.075, 0.21, and 0.13 μmol of peptide, total 0.415 μmol (2.0%). The decapeptide was chromatographically identical with that obtained in (a) above. H.p.l.c. profiles

Step	1	4	7	10
Leu	0.97	1.06	1.10	1.09
Gly	1.00	1.00	1.00	1.00
Asp		0.97	1.95	1.95
Ile		0.89	1.87	1.84
Tyr		0.91	0.97	0.96
Ala			0.95	1.96
Glu				0.97
Val				0.91

of the crude and single ion-exchange purified decapeptide are also shown in the Figure.

Solid-phase Synthesis of Substance P.—The dimethylacrylamide–ethylenebisacrylamide–acryloylsarcosine methyl ester copolymer [1] (0.5 g) was shaken with redistilled ethylenediamine (16 ml) overnight at room temperature. The excess of ethylenediamine was removed and the resin washed thoroughly with 1.5% aqueous KCl, water, dioxan, and DMF. (More recent procedures have successfully employed a simplified washing procedure using only DMF, 10% ethyldi-isopropylamine in DMF, and DMF). The amino resin was acylated with symmetrical anhydride prepared [1] from Fmoc-Nle-OH (2 mmol, 120 min) and the Fmoc-group cleaved with 20% piperidine–DMF [Table 2, column (A)]. The incorporation of norleucine was 0.24 milliequiv. g^{-1}. The amino-resin was acylated with the 2,4,5-trichlorophenyl ester of *p*-hydroxymethylbenzoic acid (4), (1 mmol) in DMF (7.5 ml) containing hydroxybenzotriazole (1 mmol). After 80 min a resin sample gave a strongly positive ninhydrin reaction. The resin was washed and the acylation repeated as above for 17 h, when reaction was complete (negative ninhydrin test). Boc-Methionine anhydride was prepared from the dicyclohexylammonium salt (2 mmol) and coupled with the hydroxymethyl resin in DMF containing *p*-dimethylaminopyridine (1 mmol). The Boc-group was cleaved with HCl–AcOH and neutralised [Table 1, procedure (B)] and the synthesis continued by procedure (A) using pre-formed symmetrical anhydrides (1 mmol) of the following protected amino-acids or their *p*-nitrophenyl esters (1 mmol) in the presence of hydroxybenzotriazole (1 mmol) for the times indicated: Fmoc-Leu-OH (90 min): Fmoc-Gly-OH (60 min); Fmoc-Phe-OH (60 min); Fmoc-Phe-OH (60 min); Fmoc-Gln-ONp (60 min); Fmoc-Gln-ONp (60 min); Fmoc-Pro-OH (60 min); Fmoc-Lys(Boc)-OH (60 min); Fmoc-Pro-OH (99 min). Synthesis was completed with Boc-Arg(HCl)-OH (2 mmol) activated by addition of dicyclohexylcarbodi-imide (1 mmol) to the protected amino-acid hydrochloride salt in DMF (7.5 ml) 5 min prior to addition of the whole reaction mixture to the resin. Acylation was continued for 140 min. Samples were removed at each acylation and deprotection step for qualitative ninhydrin tests. Negative tests were obtained after each acylation. Samples for amino-acid analysis were removed after addition and deprotection of residues 1 (methionine), 4 (phenylalanine), 6 (glutamine), 9 (lysine), and 11 (arginine). The ratios in Table 4 were obtained expressed relative to norleucine (step 1) and leucine thereafter.

TABLE 4

Step	1	4	6	9	11
Nle	1.00	1.06	1.07	1.07	1.07
Met *	0.80	0.94	0.85	0.99	0.97
Leu		1.00	1.00	1.00	1.00
Gly		1.01	0.97	1.00	0.99
Phe		1.01	2.03	2.05	2.02
Glu			0.97	2.02	2.02
Pro				1.00	1.69
Lys				0.70	0.66
Arg					0.64

* Not including methionine sulphoxide.

Approximately one-third of the residual resin (dry weight 99 mg Leu = 0.16 mmol g^{-1}) was swollen in DMF for 2 h, filtered off, and suspended in ice-cold methanolic ammonia (saturated, 20 ml) in a stoppered flask. After 160 min stirring at room temperature the resin was filtered off and washed well with DMF (Leu/Nle = 0.029, 3% residual peptide content). The combined filtrate and washings were evaporated, and the residue washed with ether, dried, and treated with 90% aqueous trifluoroacetic acid (10 ml) for 30 min before evaporation and re-evaporation several times with ether (Found: Met, 0.97; Leu, 1.00; Gly, 1.06: Phe, 2.03; Glu, 2.16; Pro, 1.70; Lys, 0.71; Arg, 0.70), total peptide content 14.9 μmol, 94.3% recovery from the resin. The product (14.6 μmol) was applied to a column (10 cm × 1 cm diameter) of carboxymethylcellulose CM52 and eluted with a linear gradient of 0.03—0.3M ammonium acetate, pH 6.6, flow rate 0.6 ml min^{-1}. The column effluant was monitored continuously at 234 nm. The main peak eluted at 312—384 ml and contained 8.2 μmol (56% recovery) (Found: Met, 0.88; Leu, 1.00; Gly, 1.03; Phe, 2.08; Glu, 2.09; Pro, 2.07: Lys, 1.00; Arg, 1.00). H.p.l.c. on μ-Bondapak C18 (linear gradient of 25—50% MeCN in 0.01M NH_4OAc, pH 4.5 over 20 min, flow rate 1.5 ml min^{-1}) showed the product to be a mixture of Substance P (co-eluting with authentic undecapeptide amide, $t = 11$ min, and its sulphoxide (co-eluting with hydrogen peroxide oxidised substance P. $t = 6$ min). The peptide mixture (7.8 μmol) was dissolved in water (2 ml), purged with nitrogen, and dithiothreitol (0.2 g) added.[39] The mixture was kept for 18 h at 38 °C and 6 h at 45 °C, and then applied to a column (84 cm × 2.5 cm) of Sephadex G-25 equilibrated with 0.2M AcOH. Elution with the same solvent afforded Substance P (7.55 μmol, 96% recovery) (Found: Met, 1.02; Leu, 1.00; Gly, 1.01; Phe, 1.92; Glu, 2.07; Pro. 2.15; Lys, 0.97; Arg, 0.98). The product co-eluted with authentic Substance P on h.p.l.c. and was equipotent in radio-immunoassay.

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