Peptide Synthesis. Part 6.† Protection of the Sulphydryl Group of Cysteine in Solid-phase Synthesis using N_α-Fluorenylmethoxycarbonylamino Acids. Linear Oxytocin Derivatives

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The N_α-fluorenylmethoxycarbonyl derivatives of *S*-acetamidomethyl-, *S*-t-butyl-, and *S*–*S*-t-butylcysteine have been prepared and used in solid-phase peptide synthesis on polar, poly(dimethylacrylamide) supports. All three derivatives proved suitable as judged by synthesis of the linear oxytocin nonapeptide amide sequence.

In solid-phase peptide synthesis, protection of the side chains of polyfunctional amino acids presents major problems. Protecting groups have to be chosen so as (i) to suppress intrinsic reactivity of the amino acid side-chain; (ii) not to introduce any new and undesirable reactivities; (iii) to be completely stable to the reaction conditions of both acylation and deprotection steps, and yet (iv) to be ultimately removable under mild conditions which do not damage the newly assembled polypeptide chain. Frequently some compromise has been necessary in attempting to meet all these requirements. Thus in conventional solid-phase synthesis,[1] N_α-t-butoxycarbonyl (Boc) amino acids have most frequently been side-chain-protected as variously substituted benzyl ether, ester, or urethane derivatives. Both t-butyl- and benzyl-based protecting groups are labile to acids, and absolute specificity in cleavage cannot be expected or obtained. Thus requirement (iii) above cannot be met. Attempts to increase selectivity by use, for example, of substituted benzyl derivatives which are more stable to acid might be expected to worsen the situation regarding objective (iv).

The introduction of N_α-fluorenylmethoxycarbonyl (Fmoc) amino acids[2] into solid-phase synthesis[3,4] has reduced these conflicts. These derivatives are cleaved by anhydrous bases rather than acids, and can therefore be used in conjuction with acid-labile side-chain-protecting groups with the expectation of nearly absolute specific cleavage. The more labile t-butyl-based protecting groups are then to be preferred to benzyl derivatives for side-chain protection, permitting substantially milder reaction conditions. This 'Fmoc–t-butyl' combination forms the basis of our current protecting-group strategy in solid-phase peptide synthesis using polyamide supports.[3,5] Side-chains of serine, threonine, and tyrosine are protected as t-butyl ethers, and those of aspartic and glutamic acids as t-butyl esters. Repetitive cleavage of Fmoc groups by base [20% piperidine in dimethylformamide (DMF)] is achieved with seemingly complete selectivity in the presence of these protecting groups.

Other polyfunctional amino acids present individual problems. The symmetrical anhydride of N_α-Fmoc-N_ε-Boc-lysine does not always couple efficiently,[3,6] and the N_ε-trifluoroacetyl derivative may sometimes be used with advantage (see below.)‡ The indole side-chain of tryptophan does not require protection under the largely basic reaction conditions of the Fmoc-polyamide method.[7,8] On the other hand, difficult problems are presented by the basic amino acids, histidine and arginine. A number of possibilities are presented for the thiol amino acid cysteine. This paper reports exploratory experiments on the suitability of several side-chain-protecting groups for N_α-Fmoc-cysteine. It is anticipated that future parts in this series will deal similarly with arginine[9,10] and histidine.[10]

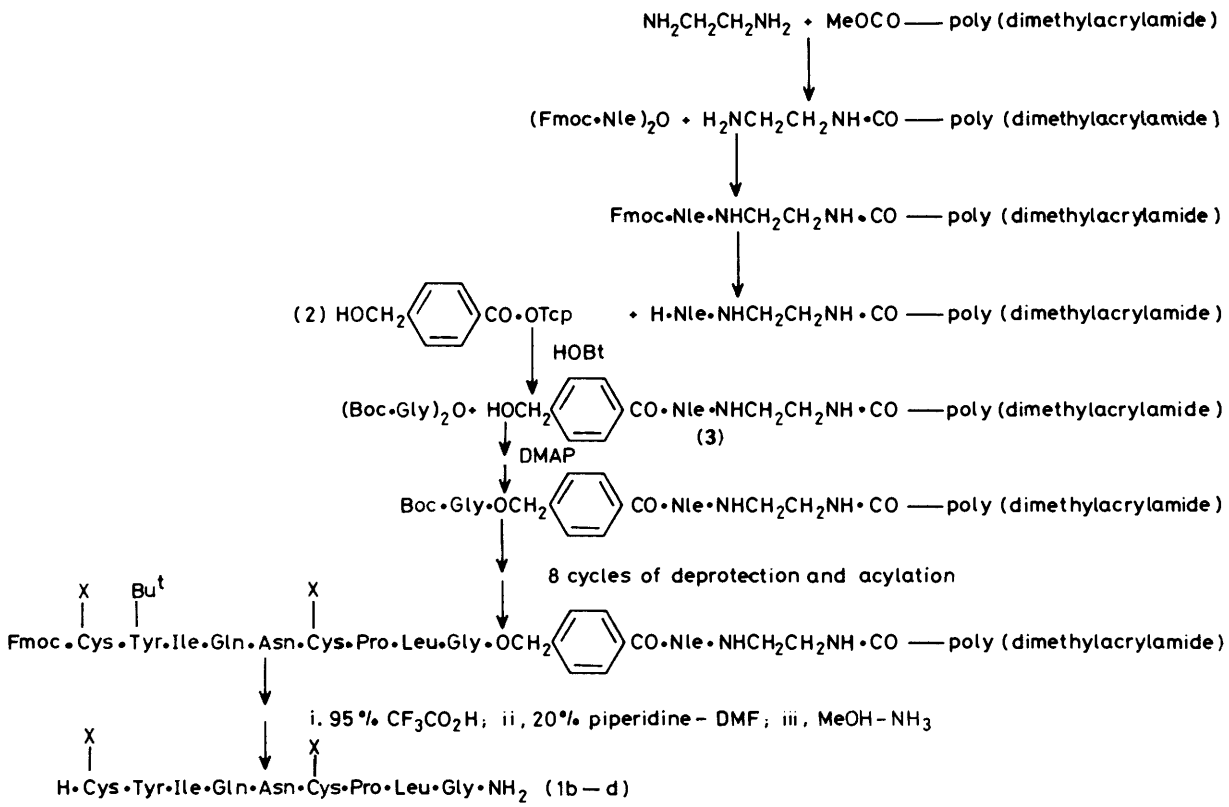
A wide range of *S*-protecting groups for cysteine have been recorded in the literature.[11] Those which require very strongly acidic reaction conditions (commonly liquid hydrogen fluoride) or other sometimes damaging reagents (*e.g.*, sodium in liquid ammonia) for their cleavage were excluded from consideration. Foremost amongst these is the classical *S*-benzyl derivative and a number of substituted variants. Use of such derivatives would vitiate the particularly advantageous mild reaction conditions associated with the Fmoc–t-butyl combination. Groups susceptible to nucleophilic displacement were similarly excluded because of their probable incompatibility with the Fmoc-cleavage conditions. Highly lipophilic sulphur-protecting groups, notably *S*-trityl and its relatives, were also not investigated because of the generally polar character of the polyamide solid-phase procedure. Excessive accumulation of apolar side-chains may lead to hydrophobic association of the resin-bound peptides in the polar environment. This effect was probably observed in an early synthesis of a cytochrome c part sequence,[12] and the use of polar N_ε-trifluoroacetyl-lysine was one factor in solving this problem. Of the remaining possibilities, the *S*-t-butyl,[13] *S*-acetamidomethyl,[14] and *S*-t-butylsulphenyl[15] groups were considered as promising candidates for the side-chain protection of Fmoc-cysteine. All were expected to be largely stable to the reaction conditions of the Fmoc-polyamide solid-phase method including the mildly acidic side-chain deprotection and resin-cleavage steps. Thus completely assembled peptides might be purified without the complication of free thiol groups, and the latter liberated or converted directly into disulphide at the very end of the synthesis. Selective cleavage of differentially protected cysteine residues was also conceivable, offering the prospect of selective disulphide bridge formation in polycysteine compounds.§

S-Acetamidomethylcysteine is generally stable to both mild acids and bases,[14] but is rapidly cleaved by a range of electrophilic reagents, notably mercury(II) ion,[14] sulphenyl halides,[16] and iodine.[17] The last reagent yields disulphide derivatives directly. Of the three sulphur-protecting groups considered here *S*-acetamidomethyl has particular appeal for the polyamide method because of the further opportunity it provides to reduce the overall lipophilicity of the growing peptide chain. The acetamidomethyl group could be a valuable adjunct to N_ε-trifluoroacetyl-lysine mentioned above. This consideration may, however, be important only under the

† Part 5, E. Brown, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Perkin Trans 1*, 1983, 1161.

‡ The *p*-nitrophenyl ester of N_α-Fmoc-N_ε-Boc-lysine (B. E. B. Sandberg, personal communication) provides an alternative solution to this problem which we now favour.

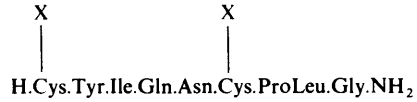
§ An example is provided by a recent synthesis of conotoxin (following paper).



Scheme. Solid-phase assembly of linear oxytocin derivatives.

special circumstances of particularly hydrophobic sequences. Similar stability towards acids and bases and reactivity towards mercury(II) ion is shown by the simple t-butyl thioether of cysteine,[13] and this easily prepared, less polar alternative was also included in our survey. Reactivity of a very different sort is shown by the t-butylsulphenyl derivative of cysteine [15] (a mixed disulphide). The same stability to mild acids and bases is present, but cleavage is by reduction of the disulphide bond, typically with phosphines [18] or thiols.[15] This protecting group may therefore be complementary to the previous two.

For our trial experiments, the linear nonapeptide amide sequence (**1a**) of oxytocin presented a realistic test case. This sequence is of significant complexity, containing as it does a range of amino acid types including two cysteine residues. One of these is located towards the carboxy terminus and would be exposed to six cycles of Fmoc-cleavage conditions. The other, the amino-terminal residue, would be affected more by the side-chain-protecting group and resin-cleavage conditions. Formation of the cyclic disulphide bond present in the natural hormone was an unnecessary complication which would not throw additional light upon the value of the thiol-protecting groups used. On the other hand, the well known instability of monomeric thiol peptides towards oxidation argued against isolation in the free deprotected form. We therefore envisaged characterisation of the synthetic products both in the initial *S*-protected forms (**1b**—**d**) as well as deprotected but derivatised by *S*-carboxymethylation, (**1e**).



(**1**) **a**; X = H
b; X = CH_2NHAc
c; X = Bu^t
d; X = SBu^t
e; X = CH_2CO_2H

Cross-linked poly(dimethylacrylamide) resin [5,*] functionalised with methoxycarbonyl groups was converted into the amino form by reaction with ethylene diamine (Scheme).[5] An internal reference amino acid (norleucine) was then added using the Fmoc-amino acid anhydride procedure.[3] The presence of this unique residue permanently bound to the resin and not appearing in the sequence to be assembled greatly facilitates calculation of yields and general analytical control through amino acid analysis. After cleavage of the Fmoc group, the reversible peptide-resin linkage agent was coupled as its 2,4,5-trichlorophenyl ester (**2**) in the presence of catalyst (hydroxybenzotriazole). This particular linkage agent [7] forms benzyl esters which are exceptionally acid-stable (even to liquid hydrogen fluoride) but are readily cleaved by nucleophiles. It is particularly appropriate for the preparation of peptide amides, as in substance P,[3] the gastrins,[7,8] and the linear oxytocin sequence (**1a**). The resulting functionalised resin (**3**) formed the basis for all three syntheses described below.

The following cycles of amino acid addition and deprotection (generalised in the Scheme) are detailed in the Experimental section and generally follow regular procedures [3,5] for coupling

* This support for both peptide and oligonucleotide synthesis is available from Cambridge Research Biochemicals, Ltd., Button End Industrial Estate, Harston, Cambridge CB2 5NX and Omnifit Ltd., 51 Norfolk Street, Cambridge.

Table 1. Assembly of (**1a**) using *S*-t-butylcysteine. Amino acid analysis of resin-bound peptides (columns 1—9; the column heading indicates the number of steps of amino acid addition for each analytical sample) and free peptides (columns A—C; A — total crude product; B — after chromatography on SP Sephadex C25; C — after carboxymethylation and desalting on Sephadex G15)

	1	2	3	6	9	A	B	C
Nle	1.09	1.10	1.22	1.23	1.30	—	—	—
Gly	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leu		0.99	0.98	0.97	1.02	1.00	1.05	1.01
Pro			0.97	1.18[a]	1.41[a]	1.32[a]	1.42	1.01
Cys				n.d.	n.d.	n.d.	n.d.	1.70[b]
Asp				0.94	0.97	0.93	0.97	1.01
Glu				0.99	1.02	0.98	1.05	1.01
Ile					0.99	0.95	1.01	0.95
Tyr					0.95	0.90	0.97	0.93

[a] Decomposition products of *S*-t-butylcysteine underlie the proline peak. [b] As *S*-carboxymethylcysteine using the ninhydrin colour constant for aspartic acid and without correction for destruction during hydrolysis.

Boc- or Fmoc-amino acid anhydrides or *p*-nitrophenyl esters.* The latter were used only for introduction of the side-chain amides, asparagine and glutamine. The choice of Boc-glycine (rather than the Fmoc derivative) for esterification of the first residue to the linkage agent in the presence of 4-(dimethylamino)pyridine follows early observations[3] on the slow but appreciable cleavage of Fmoc groups by this reagent. We would not now regard it as a universally necessary precaution, especially for the acylation of the hydroxy group of resin-bound linkage agent (**3**) which is particularly rapid.

(i) *Synthesis of* S,S'-*Di-t-butyl Nonapeptide Amide* (**1c**) *using Fmoc.Cys*(*Bu*^{t}).—This assembly was unexceptional apart from one acylation step. Coupling of Fmoc-proline anhydride to amino-terminal leucine resin was incomplete after 105 min as judged by both ninhydrin and trinitrobenzene-sulphonic acid colour tests. The resin was washed and the acylation step was repeated; it was then complete after 15 min. This difficulty was not encountered in the subsequent syntheses of (**1b** and **d**), even though all three resins were at this stage supposedly identical and must most likely be attributed to some unrecognised problem in initial formation of Fmoc-proline anhydride. Amino acid analysis of the intermediate resin-bound peptides was carried out at five stages and in every case indicated excellent amino acid incorporation (Table 1).

The t-butyl ether protecting the tyrosine residue was cleaved with aqueous trifluoroacetic acid (TFA) and the terminal Fmoc-group was removed with piperidine. The *S*-t-butyl protecting groups as well as the peptide-resin linkage were completely stable to these treatments. The nonapeptide amide (**1c**) was then detached from the resin support by ammonolysis with excess of methanolic ammonia. H.p.l.c. of the total crude peptide product (98% cleaved based on amino acid analysis of the residual resin) on μ-Bondapak C_{18} showed the presence of one very major peak with trailing impurities (Figure 1). Subsequent experiments in which the ammonolysis time was shortened indicated that products of incomplete reaction, *e.g.* the nonapeptide methyl ester (*cf.* ref. 7), might have contributed to the contaminants present, but not to a major extent.

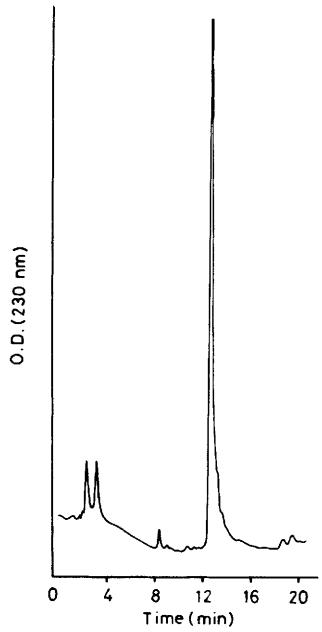


Figure 1. H.p.l.c. of crude bis(*S*-t-butyl) nonapeptide amide (**1c**) obtained directly from ammonolysis of the peptide resin. For h.p.l.c. conditions see Experimental section.

The bis-t-butyl nonapeptide amide (**1c**) was purified by ion-exchange chromatography on sulphopropyl Sephadex C25, from which it eluted as one very major and one earlier eluting, minor peak. The recovery in the main peak was 77%; the amino acid analysis is given in Table 1. H.p.l.c. gave a single peak (Figure 2) but with some indication of persistence of the earlier observed contaminants.

The above evidence indicated a satisfactory solid-phase assembly of the nonapeptide amide (**1c**) using Fmoc.Cys(Bu^t).

The *S*-t-butyl groups were easily removed using mercury(II) ion in aqueous ammonium trifluoroacetate, pH 4. A time-course study using h.p.l.c. showed that all starting material had been consumed after 5.5 h but not after 1 h. The rate was not greatly different in the absence of ammonium trifluoroacetate at the same pH. The h.p.l.c. profile of the reaction mixture after 18 h is shown in Figure 3. After reaction with excess of iodoacetic acid and desalting on Sephadex G15, the crude bis(carboxymethyl) derivative gave a single major peak on h.p.l.c. (Figure 4) with evidence of only minor impurities. Thus protection of the cysteine thiol group as its t-butyl thioether was satisfactory also in terms of ease of removal by mercury(II) ion. Although the cleavage reaction was relatively slow compared with that of the corresponding bis(acetamidomethyl) derivative (see below), it did not apparently cause substantial alterations elsewhere in the molecule.

(ii) *Synthesis of* S,S'-*Bis*(*acetamidomethyl*) *Nonapeptide Amide* (**1b**) *using Fmoc.Cys*(*Acm*).—The assembly of this sequence proceeded smoothly. All acylations were complete at the time of the first colour test (*ca.* 30 min) and no repeated couplings were necessary. The symmetrical anhydride of

* Our initial (E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Am. Chem. Soc.*, 1975, **97**, 6584) and current[3,5] procedures for solid-phase synthesis on polydimethylacrylamide resins utilise DMF as the sole solvent throughout. However, much of the work described here uses dimethylacetamide (DMA). Changes of solvent in the intermediate period were made when it was discovered that Fmoc-derivatives had better long-term stability in DMA. Later it was found that acylation reactions, especially esterification of the first residue to the support (N. L. Benoiton, unpublished observations), proceed appreciably faster in DMF. We therefore reverted to exclusive use of this solvent. Because of the slow release of dimethylamine when DMF is kept for any time, we consider it absolutely essential that all DMF be purified by fractional distillation under reduced pressure shortly before use.

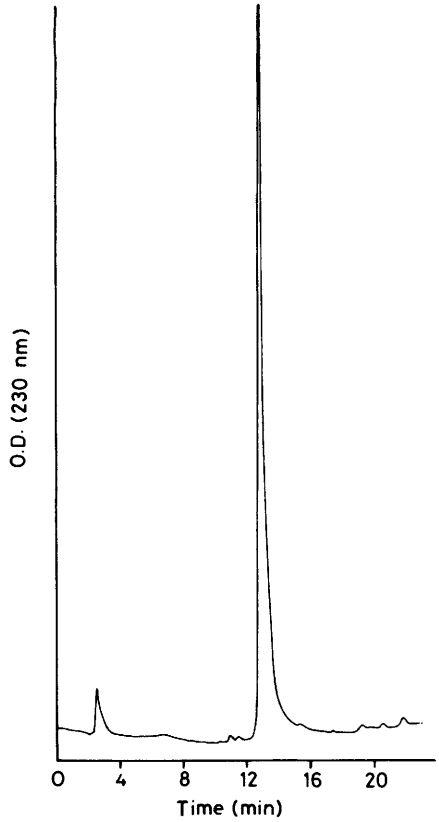


Figure 2. H.p.l.c. of bis(*S*-t-butyl) nonapeptide amide after purification on sulphopropyl Sephadex C25. For conditions see Experimental section.

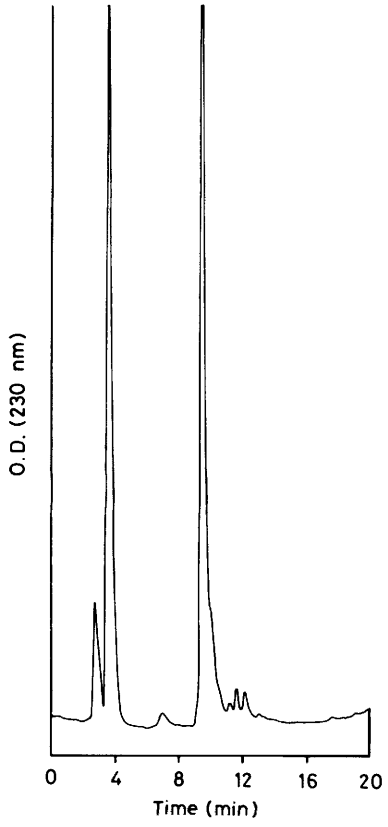


Figure 3. H.p.l.c. of the *S*-t-butyl cleavage reaction mixture after 18 h. For conditions see Experimental section. The early eluting peaks are derived from the reagents.

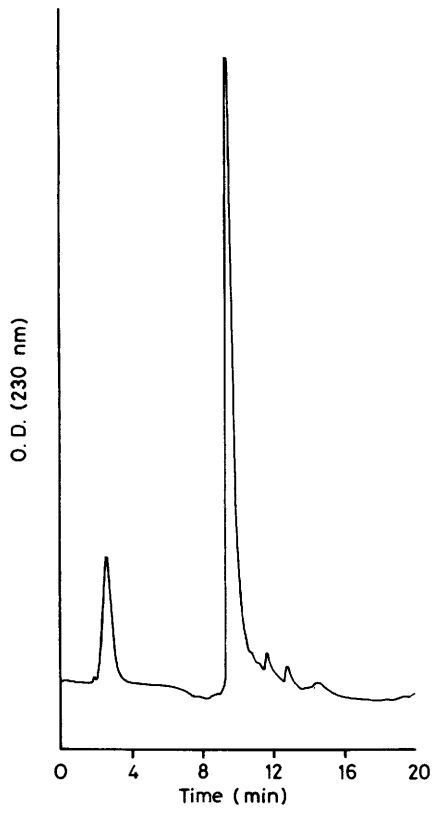


Figure 4. H.p.l.c. of the crude carboxymethylated nonapeptide amide (**1e**) after desalting on Sephadex G15. For conditions see Experimental section.

Table 2. Assembly of (**1a**) using *S*-acctamidomethylcysteine. The column headings and footnotes are as for Table 1

	2	3	6	9	A	B	C
Nle	1.15	1.22	1.22	1.22	—	—	—
Gly	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leu	0.94	0.99	0.99	1.00	1.01	1.02	1.02
Pro		0.91	1.17[a]	1.25[a]	1.40[a]	1.45[a]	0.92
Cys			n.d.	n.d.	n.d.	n.d.	1.78[b]
Asp				0.98	0.98	0.98	1.04
Glu				1.02	0.99	1.06	1.01
Ile				0.87	0.89	0.91	0.88
Tyr				0.82	0.90	0.93	0.93

Fmoc.Cys(Acm) proved to be rather insoluble in dichloromethane and precipitated along with dicyclohexylurea during its preparation. This commonly occurs with certain other Fmoc-amino acids (glycine, alanine, phenylalanine, ε-trifluoroacetyllysine). Our normal practice under these circumstances is to evaporate the whole reaction mixture to dryness, dissolve the anhydride in DMF or DMA, and to remove most of the urea by filtration at this stage. This procedure was satisfactory in the present case. Intermediate resin samples were removed for amino acid analysis as before. The results are collected in Table 2.

The nonapeptide amide was detached using methanolic ammonia (residual resin analysis indicated 97% cleavage), and the crude product was purified on sulphopropyl Sephadex as before. Seventy-one per cent of the material was recovered in the main peak which gave an h.p.l.c. profile (Figure 5) comparable with that of the bis(*S*-t-butyl) derivative (Figure 2), *i.e.*

Table 3. Assembly of peptide (**1a**) using *S*-t-butylsulphenylcysteine. The column headings and footnotes are as for Table 1.

	1	2	3	6	9	A	B	C
Nle	1.10	1.15	1.20	1.20	1.22	—	—	—
Gly	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leu		0.91	0.94	0.95	1.02	1.01	1.02	1.01
Pro			0.92	1.04[a]	1.19[a]	1.23[a]	1.19[a]	1.17
Cys				n.d.	n.d.	n.d.	n.d.	n.d.
Asp				0.92	0.91	0.93	1.04	1.01
Glu				0.94	0.93	0.95	1.03	1.01
Ile					0.81	0.80	0.89	0.89
Tyr					0.84	0.84	0.94	0.93

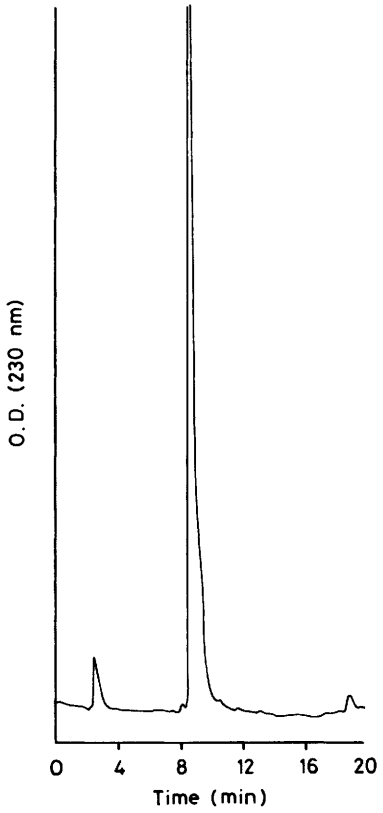


Figure 5. H.p.l.c. of bis(*S*-acetamidomethyl) nonapeptide amide (**1b**) after chromatography on sulphopropyl Sephadex C25. For conditions see Experimental section.

indicating a single very major component with traces of a less polar, trailing contaminant. This pattern was seen repeatedly throughout the whole of the present work. The contaminant is therefore unlikely to be associated with the particular sulphur-protecting group employed in each case.

The acetamidomethyl groups were cleanly and rapidly removed by mercury(II) ion at pH 4, the reaction apparently being complete after 45 min. It was terminated after 3 h. The product was characterised as its bis(carboxymethyl) derivative which gave a single peak on h.p.l.c. (Figure 6), identical in position to that of the corresponding product from the *S*-t-butyl series run under the same conditions.

Thus the acetamidomethyl group was also satisfactory for use in combination with the Fmoc–t-butyl protection strategy. The products from the synthesis were, if anything, somewhat more pure than those of the earlier synthesis using *S*-t-butyl protection, and the yields were comparable.

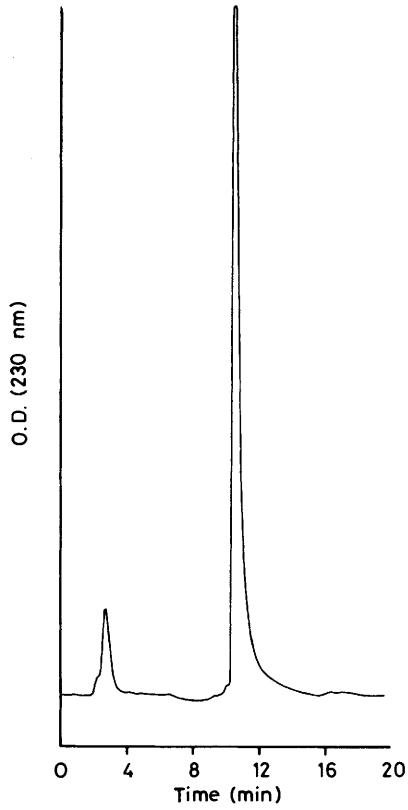


Figure 6. H.p.l.c. of carboxymethylated nonapeptide amide (**1e**) prepared from the bis(*S*-acetamidomethyl) derivative (**1b**). A linear gradient of 15—60% B in A (see Experimental section) was used; the same product from the bis(*S*-t-butyl) nonapeptide amide (**1c**) was shown to co-elute with the above-mentioned amide (**1e**) under these conditions.

(iii) *Synthesis of* S,S′-*Bis*(*t-butylsulphenyl*) *Nonapeptide Amide* (**1d**) *using Fmoc.Cys*(*SBu*^{t}).—This assembly was carried out as in the synthesis (ii) above, except that a punched-tape-controlled synthesiser (Beckman Model 990) was used in place of a simple manually controlled wrist-action shaker. The procedures were essentially the same as those in the manual syntheses. Symmetrical anhydrides were prepared manually outside the synthesiser immediately prior to completion of the deprotection step. Intermediate resin samples were removed for analysis as before. The results are collected in Table 3.

The peptide resin was treated just as in the foregoing section. The *S*,*S*′-protected nonapeptide amide (**1d**) was detached with methanolic ammonia (residual resin analysis indicated 97% cleavage), and the crude product was examined by h.p.l.c. The elution profile is shown in Figure 7. Chromatography of this material on sulphopropyl Sephadex did not proceed as smoothly as previously. On this occasion a long trailing peak was produced which was examined along its length by h.p.l.c. analysis. Contaminants X and Y (Figure 7) were concentrated in the earlier fractions but were not separated from the major product which appeared throughout the entire peak. The peak was collected in two halves (see Experimental section) with recoveries of 36.6 and 37.1%. Reductive cleavage of the second, most pure fraction with tributylphosphine proceeded smoothly. Reaction was nearly complete after 30 min and was terminated after 3 h. Carboxymethylation of the dithiol gave, after desalting, the h.p.l.c. profile shown in Figure 8a. In a previous experiment the earlier eluted, less pure fractions from the sulphopropyl Sephadex chromatography were also reduced

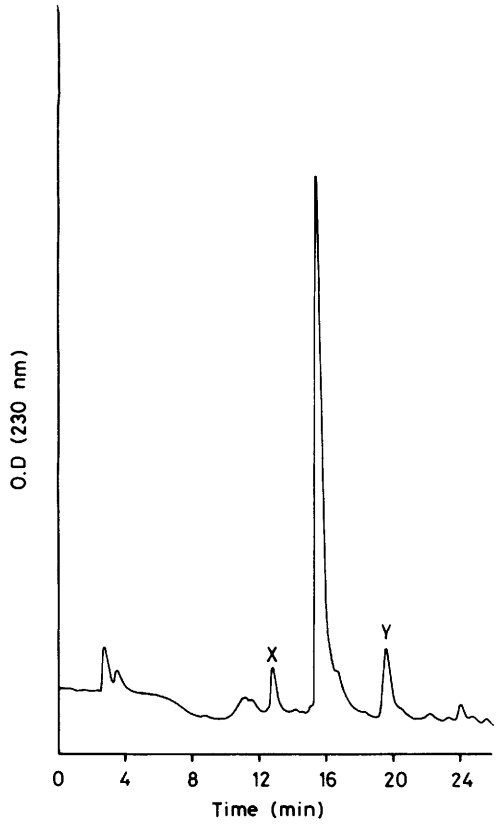


Figure 7. H.p.l.c. of crude *S,S'*-protected nonapeptide amide (**1d**). For conditions see Experimental section.

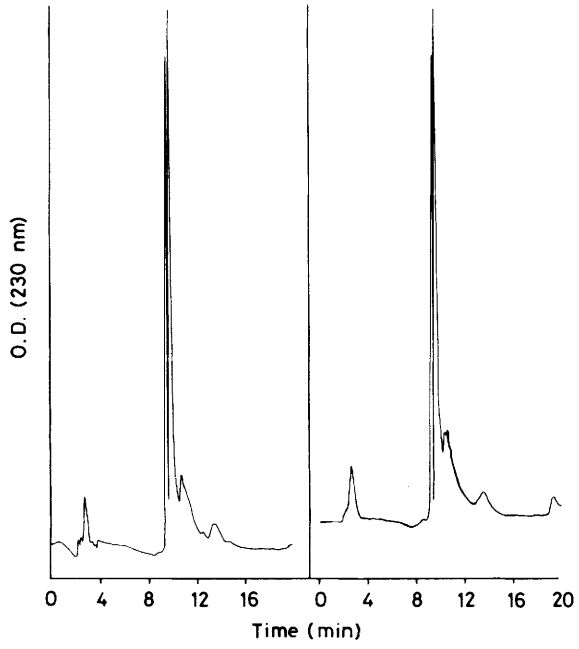


Figure 8. H.p.l.c. of two samples of carboxymethylated nonapeptide amide (**1e**) prepared from the bis(S-S-Bu^t) derivative (see text). For h.p.l.c. conditions see Experimental section.

with tributylphosphine. On this occasion the reaction appeared to be much slower. Multiple additions of reductant were made over a period of 18 h. Nevertheless, the final product gave a carboxymethylated derivative not greatly different (Figure 8b) from that (Figure 8a) described above. In both cases, however, the purity of the final derivatised nonapeptide amide (**1e**) was significantly lower than with the previously studied *S*-t-butyl and *S*-acetamidomethyl syntheses. The recoveries were comparable in all three cases.

We conclude that all three sulphur-protecting groups may be used for the incorporation of cysteine residues in Fmoc–t-butyl polyamide solid-phase syntheses with good prospect of success. In the present test series, purer products were obtained using the very stable t-butyl and acetamidomethyl protecting groups; the choice between them may involve consideration of their respective hydrophobic and hydrophilic characters and the influence they have on the efficiency of the whole synthesis. Direct disulphide formation by iodine oxidation of *S*-acetamidomethyl derivatives may also be an important factor, although this was not tested in the present work. On the other hand, the very different reductive reaction conditions for cleavage of the t-butylsulphenyl-protected cysteine residues introduces an element of flexibility and versatility into the synthesis of cysteine and especially of cystine peptides. Selective disulphide formation is easily possible using Cys(SBu^t) in combination, for example, with Cys(Acm). An example of this application in the conotoxin series is described in the following paper.

Experimental

General procedures using the Fmoc–t-butyl combination with poly(dimethylacrylamide) resins have previously been described.[3] Fmoc-amino acids were checked for purity and identity by m.p., optical rotation, and t.l.c. on silica [chloroform–methanol–acetic acid (85:10:5 v/v/v)]. Except where stated otherwise, h.p.l.c. was carried out on a μ-Bondapak C_{18} reverse-phase analytical column utilising a phosphoric acid system. Reservoir A contained 0.1% phosphoric acid and reservoir B 90% acetonitrile, 10% A. Elution was by a linear gradient 20—80% B during 20 min at a flow rate of 1.5 ml min^{-1}; the effluent was monitored at 230 mn using a Cecil spectrophotometer and 1 cm path-length cell.

S-*Acetamidomethyl*-N_α-*fluorenylmethoxycarbonyl*-L-*cysteine*.—*S*-Acetamidomethyl-L-cysteine monohydrate (3.15 g, 15.0 mmol) was dissolved in a mixture of 10% aqueous sodium carbonate (45 ml) and dioxane (20 ml). The solution was stirred and cooled to ice temperature and a solution of fluorenylmethoxycarbonyl chloride (4.41 g, 17.0 mmol) in dioxane (15 ml) was added dropwise during 30 min. The ice-bath was removed and the mixture was stirred for 1.5 h at room temperature, when t.l.c. indicated almost complete reaction. After a further 1 h the reaction mixture was poured into water (400 ml), extracted with ether (3 × 100 ml), and acidified with 10% citric acid to pH 3. The resultant white crystalline solid was collected and freed from citric acid by partitioning between ethyl acetate and water, and the organic phase was washed several times with water before being dried (Na_2SO_4) and evaporated. After several unsuccessful attempts to crystallise the product, it was dissolved in propan-2-ol and then water was added to cloud point. The mixture was cooled and the *title product* crystallised (4.5 g, 75%), m.p. 150—154 °C; $[\alpha]_D$ −27.5° (*c* 1 in ethyl acetate) (Found: C, 60.25; H, 5.4; N, 6.9. $C_{21}H_{22}N_2O_5S$ requires C, 60.86; H, 5.34; N, 6.76%).

In other preparations the compound failed to crystallise in a pure form and was purified on a Lobar C silica column using chloroform–methanol–acetic acid (370:20:10 v/v/v) as eluant prior to recrystallisation.

N_α-*Fluorenylmethoxycarbonyl*-S-(*t-butylsulphenyl*)-L-*cysteine*.—*S*-(t-Butylsulphenyl)-L-cysteine (3.14 g, 15.0 mmol)

was dissolved in water (27 ml) and the solution was treated dropwise with 1M sodium hydroxide (14 ml). Sodium carbonate (1.69 g, 16.0 mmol) was added and the resulting solution was cooled in an ice-bath, water (10 ml) and dioxane (21 ml) were added, followed by a solution of fluorenylmethoxycarbonyl chloride (3.9 g, 15.07 mmol) in dioxane (39 ml) dropwise during 15 min. The mixture was stirred in the ice-bath for 2 h and then for 15 min at room temperature after which time t.l.c. indicated the presence of only trace amounts of the starting cysteine derivative. The mixture was poured into ice–water (450 ml), washed with ether (2 × 100 ml), then acidified to pH 2 and the product was extracted into ether. The ether layer was washed with water (4 × 100 ml), dried (Na_2SO_4), and evaporated to give a foam which was crystallised from dichloromethane–n-hexane to give the title compound (5.45 g, 84%), m.p. 74—76 °C; $[\alpha]$ −87.0 (*c* 1 in ethyl acetate) [lit.,[19] m.p. 74—76 °C; $[\alpha]_D$ −84.6 (*c* 1 in ethyl acetate)].

N_α-*Fluorenylmethoxycarbonyl*-S-*t-butyl*-L-*cysteine*.—*S*-(t-Butyl)-L-cysteine hydrochloride (3.2 g, 15.0 mmol) was dissolved in a mixture of 10% aqueous sodium carbonate (60 ml) and dioxane (30 ml). The solution was cooled in an ice-bath and stirred, and a solution of fluorenylmethoxycarbonyl chloride (4.41 g, 17.0 mmol) in dioxane (15 ml) was added dropwise during 15 min. The ice-bath was removed and after the mixture had been stirred for 30 min, t.l.c. indicated very little starting material remaining. After a further 30 min the mixture was poured into water (400 ml), extracted with ether (3 × 100 ml), the solution was acidified with solid citric acid, and the resultant oil was extracted into ether (3 × 100 ml). The latter ether extracts were combined, washed thoroughly with water, dried (Na_2SO_4), and evaporated to give a foam which was crystallised from dichloromethane–light petroleum to give the title compound as white needles (4.7 g, 79%), m.p. 135—136 °C; $[\alpha]_D$ −24.0° (*c* 1 in DMF) [lit.,[19] m.p. 135—136 °C; $[\alpha]_D$ − 23.2° (*c* 1 in DMF)].

Solid-phase Assembly of the S,S′-*Di-t-butyl* (**1c**) *and* S,S′-*Bis*(*acetamidomethyl*) (**1b**) *Cysteine Nonapeptides*.—The acryloylsarcosine–poly(dimethylacrylamide) resin[5] (1 g, 0.30 mequiv.) was shaken overnight with ethylene diamine (32 ml) and then washed with DMA (2 min; 20 × 15 ml), 10% di-isopropylethylamine–DMA (3 × 15 ml), and DMA (5 × 15 ml). Acylation with the symmetrical anhydride of Fmoc.Nle (1.8 mmol) in DMA (15 ml) was carried out for 1 h, when negative ninhydrin[20] and trinitrobenzenesulphonic acid[21] tests were obtained. After the resin had been washed with DMA (1 min; 5 × 15 ml), it was subjected to the standard Fmoc-group cleavage and amino acid coupling cycle, *viz.*: DMA, 5 × 1 min; 20% piperidine–DMA, 3 + 7 min; DMA, 10 × 1 min; acylation: DMA, 5 × 1 min. In this cycle the resin was acylated with a mixture of 2,4,5-trichlorophenyl 4-hydroxymethylbenzoate (1.8 mmol) in the presence of 1-hydroxybenzotriazole (1.8 mmol) in DMA (15 ml) overnight (18 h), the resin then giving negative colour tests. The resin was washed with DMA (1 min; 5 × 15 ml); 10% di-isopropylamine–DMA (3 × 15 ml); and DMA (5 × 15 ml) and was then esterified with Boc-glycine anhydride (1.8 mmol) in the presence of 4-dimethylaminopyridine (1.8 mmol) for 75 min. Amino acid analysis of a small sample of washed and deprotected resin indicated 92% acylation [Found: Gly, 1.00; Nle, 1.09]. The washed resin was divided by volume into two equal parts which were used for separate assembly of the nonapeptide sequences (**1b**) and (**1c**).

Removal of the Boc group and acylation with Fmoc-leucine anhydride (0.9 mmol) in DMA (7.5 ml) was achieved using the sequence: 2-methylbutan-2-ol, 5 × 1 min; acetic acid, 5 × 1 min; 1.5M HCl–acetic acid, 5 + 25 min; acetic acid, 5 × 1 min; 2-methylbutan-2-ol, 5 × 1 min; DMA, 10 × 1 min; 10% di-isopropylethylamine–DMA, 3 × 1 min; DMA, 5 × 1 min; acylation, 60 min; DMA, 5 × 1 min. The volumes of the reagents–solvents used was *ca.* 7.5 ml in every case.

Both assemblies were then completed according to the standard cycle[3] for Fmoc-group removal, amino acid addition using Fmoc-amino acid anhydrides (0.9 mmol) or, in the case of asparagine and glutamine, *p*-nitrophenyl esters (0.9 mmol) in the presence of 1-hydroxybenzotriazole (0.9 mmol). With one exception, negative colour tests were obtained within the first 30 min of the acylation reaction period; reactions were allowed to proceed for a total of 60—75 min. In the coupling of Fmoc-proline anhydride during the assembly of nonapeptide (**1c**), positive colour tests indicated incomplete reaction after 105 min. The resin was washed with DMA, 5 × 1 min, and the coupling was repeated. Negative colour tests were then obtained within 15 min.

Fmoc-acetamidomethylcysteine anhydride precipitated during preparation. The dichloromethane was evaporated from the total mixture, DMA (7.5 ml) was added, and most of the dicyclohexylurea was filtered off prior to addition of the solution to the resin.

Resin samples for amino acid analysis were removed just prior to addition of the acylating component at various stages in the assemblies and are summarised in Tables 1 and 2.

Assembly of the S,S′-*Bis*(*t-butylsulphenylcysteine*)*Peptide* (**1d**).—The nonapeptide was assembled in the same manner as described above for acryloylsarcosine–poly(dimethylacrylamide) resin (0.5 g, 0.15 mequiv.) but utilising a modified Beckmann 990 synthesiser. No special problems were encountered. Samples for amino acid analysis were removed as before; the results are summarised in Table 3.

Cleavage and Isolation of the Bis(*carboxymethyl*) *Derivative of Oxytocin*.—(i) S,S′-*Bis*-(*t-butylcysteine*) *assembly*. Approximately one-quarter of the swollen resin (by volume) was deprotected at the amino-terminal and tyrosine residues by treatment with 2-methylbutan-2-ol, 5 × 1 min; acetic acid, 5 × 1 min; 90% aqueous trifluoroacetic acid, 5 + 30 min; acetic acid, 5 × 1 min; 2-methylbutan-2-ol, 5 × 1 min; DMA, 5 × 1 min; 20% piperidine–DMA, 3 + 7 min; DMA, 10 × 1 min. After removal of a small sample for amino acid analysis (Table 1), the DMA wet resin was treated in a tightly sealed round-bottomed flask with anhydrous ammonia in methanol (20 ml), saturated at 0 °C for 2 h at room temperature. The resin was filtered off and washed with methanol and the combined filtrate and washings were evaporated to yield the bis(*S*-t-butyl) peptide amide (22.9 μmol, 62% based on the amount of resin at the start of the synthesis and not taking into account resin removed for analytical purposes). Analysis of the residual resin after further thorough washing indicated 97.8% cleavage (Found: Gly, 0.018; Nle 1.00). H.p.l.c. of the cleaved peptide on C_{18} μ-Bondapak gave the profile shown in Figure 1.

A part (11.4 μmol) of the cysteine-protected derivative, dissolved in aqueous acetic acid (20 ml) pH 3.8, was applied to a sulphopropyl Sephadex C25 (1 × 10 cm) column which had been equilibrated with 0.02M ammonium acetate, pH 4.6. The column was eluted with the same buffer (61 ml), and then with a gradient of 0.02M ammonium acetate, pH 4.6, to 0.24M ammonium acetate, pH 5.4 (250 ml in each chamber). Effluent was continuously monitored at 278 nm with a flow rate of 0.6 ml min^{-1}. A major peak which eluted between 75 and 119 ml was freeze-dried and gave the bis(*S*-t-butyl)-protected cysteine derivative (8.3 μmol, 77%) (amino acid analysis, Table 1; h.p.l.c., Figure 2).

A part (4.4 μmol) was dissolved in water (1.5 ml), two drops of TFA was added, and the solution was adjusted to pH 4.0 with dilute ammonium hydroxide. Mercury(II) acetate (12.8 mg) was

added to this solution (total vol. 9 ml) and the mixture was stirred at room temperature. Samples for h.p.l.c. analyses were removed after 1, 5.5, and 18 h, saturated with hydrogen sulphide, centrifuged, and applied to μ-Bondapak C18. The 5.5 h sample indicated absence of starting material but the presence of some intermediate products which were still present in trace amounts in the 18 h sample (Figure 3). A sample deprotected in the absence of TFA gave very similar results. After overnight reaction,the bulk solution was treated with hydrogen sulphide and centrifuged, and the clear supernatant was purged with nitrogen, dithiothreitol (7.7 mg) was added, and the mixture was adjusted to pH 8 with dilute aqueous sodium hydroxide. A solution of 0.2M iodoacetic acid (1 ml) adjusted to pH 8 with sodium hydroxide was added (total volume *ca.* 10 ml), and the mixture was stirred at room temperature for 1 h and then desalted on Sephadex G15 in 2M acetic acid. The peptide peak was collected, freeze-dried, and dissolved in water (1 ml). No free thiol groups were detectable by the Ellman test and the product (1.8 μmol, 41.6%) gave the amino acid analysis indicated in Table 1 and the h.p.l.c. profile shown in Figure 4.

(ii) S,S'-*Bis*(*acetamidomethylcysteine*) *assembly*. Approximately one-quarter of the DMA swollen resin (by volume) was treated in exactly the same manner as the bis(*S*-t-butylcysteine) peptide resin. After ammonolysis the residual resin (Found: Gly, 0.024; Nle, 1.00) indicated 97% cleavage and 17.7 μmol [*ca.* 48.7%, based on starting resin, see (i) above] of the bis(acetamidomethyl) derivative was isolated; amino acid analysis, Table 2. A part (8.8 μmol) of this derivative was purified on sulphopropyl Sephadex C25 as above, to give 6.3 μmol (71%); amino acid analysis, Table 2; h.p.l.c., Figure 5. A portion (3.1 μmol) dissolved in dilute acetic acid (8.5 ml) at pH 4.0 was treated with mercury(II) acetate (13.7 mg), and the mixture was readjusted to pH 4 and stirred gently at room temperature. After 45 min a sample was removed, saturated with hydrogen sulphide, and centrifuged. H.p.l.c. indicated nearly complete reaction. After 3 h hydrogen sulphide was passed through the total reaction mixture for 15 min, the precipitate was removed by centrifugation, and then the supernatant was filtered through a Millipore membrane. The clear solution was flushed with nitrogen (15 min), adjusted to pH 8.0 with dilute ammonium hydroxide, and dithiothreitol (7.7 mg) was added followed by 0.2M iodoacetic acid, pH 8.0 (1 ml), to give a total volume of 10 ml. After 1 h at room temperature the product was desalted on G15 Sephadex with 2M acetic acid as eluant and the peptide peak was collected and freeze-dried to give 1.4 μmol (45%); amino acid analysis, Table 2; h.p.l.c., Figure 6.

(iii) S,S'-*Bis*(*t-butylsulphenylcysteine*) *assembly*. Approximately one-quarter (by volume) of the swollen peptide resin was treated as in (i) above. After ammonolysis, residual resin analysis (Found: Gly, 0.025; Nle, 1.00) indicated 97% cleavage, and 20.6 μmol [*ca.* 56.6%, based on starting resin, see (i) above] of the bis(S–S Bu^t-cysteine) peptide amide was isolated; amino acid analysis, Table 3; h.p.l.c., Figure 7. A part (10.3 μmol) was purified on sulphopropyl Sephadex C25 as previously described. The peak tailed between 68.4 ml and 216 ml with contaminants X and Y (h.p.l.c., Figure 7) concentrated in the earlier eluting part of the peak. Fractions comprising 68.4—108 ml and 108—216 ml were combined separately and freeze-dried to yield 3.77 μmol (36.6%) and 3.82 μmol (37.1%) respectively. The purer, later eluting material (3.8 μmol) was dissolved in a mixture of water (1 ml), propan-1-ol (2.5 ml), 1.0M aqueous sodium carbonate (1 ml), and 0.5M sodium carbonate (0.5 ml) and the solution was purged with nitrogen. To this solution, under nitrogen, was added 0.3M tributylphosphine (0.5 ml) in propan-1-ol and the course of the reductive deprotection was monitored by h.p.l.c. After 30 min a trace of starting material remained and after 60 min the reaction was complete. Iodoacetic acid (18.5 mg) dissolved in 0.1M aqueous sodium hydroxide was then added and the pH was adjusted to 8.5 by the addition of solid iodoacetic acid. After 45 min at room temperature, h.p.l.c. indicated complete conversion; the mixture was desalted, as above, to yield 2.94 μmol (77.3%); amino acid analysis, Table 3; h.p.l.c., Figure 8.

References

1 G. Barany and R. B. Merrifield, in 'The Peptides,' eds. E. Gross and J. Meienhofer, Academic Press, New York, 1980, vol. 2, p. 3.
2 L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, 1970, **92**, 5748.
3 E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1978, 537; E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
4 J. Meienhofer, M. Waki, E. P. Heimer, T. J. Lambros, R. C. Makofski, and C.-D. Chang, *Int. J. Pept. Protein Res.*, 1979, **13**, 35.
5 R. Arshady. E. Atherton, D. L. J. Clive, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 529.
6 E. Atherton, M. Caviezel, H. Fox, D. Harkiss, H. Over, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1983, 65.
7 E. Brown, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1983, 75.
8 E. Brown, R. C. Sheppard, and B. J. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1983, 1161.
9 Preliminary communication, E. Atherton, R. C. Sheppard, and J. Wade, *J. Chem. Soc., Chem. Commun.*, 1983, 1060.
10 E. Atherton, L. E. Cammish, P. Goddard, J. D. Richards, and R. C. Sheppard, Proceedings of 18th European Peptide Symposium, Djuronassett, Sweden, 1984, p. 153.
11 For a recent review, see R. G. Hiskey, in 'The Peptides, Analysis, Synthesis, Biology,' eds. E. Gross and J. Meienhofer, Academic Press, New York, 1981, vol. 3, p. 137, and references cited therein.
12 E. Atherton, V. Wooley, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1980, 970.
13 A. Chimiak, in 'Peptides 1962,' ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 37; H. C. Beyerman, *ibid.*, p. 53.
14 D. F. Veber, J. D. Milkowski, S. L. Vargas, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, 1972, **94**, 5456.
15 U. Weber and P. Hartter, *Z. Physiol. Chem.*, 1970, **351**, 1384.
16 A. Fontana, *J. Chem. Soc., Chem. Commun.*, 1975, 976.
17 B. Kamber, *Helv. Chim. Acta*, 1971, **54**, 927.
18 U. T. Ruegg and H. G. Gattner, *Z. Physiol. Chem.*, 1975, **356**, 1527.
19 C.-D. Chang, M. Waki, M. Ahmad, J. Meienhofer, E. O. Lundell, and J. D. Haug, *Int. J. Pept. Protein Res.*, 1980, **15**, 59.
20 E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.
21 W. S. Hancock and J. E. Battersby, *Anal. Biochem.*, 1976, **71**, 260.

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