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PEPTIDES–XXXIV

SYNTHESIS OF THE 1–16 FRAGMENT OF A LYSOZYME ANALOGUE

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Abstract—The synthesis of the 1–16 fragment of a lysozyme analogue is described. Three protected subfragments 1–4, 5–10 and 11–16 were combined using the N, N′-dicyclohexylcarbodiimide/N-hydroxysuccinimide method. The fully protected hexadecapeptide was purified by gel filtration on Sephadex LH–60 eluting with N-methylpyrrolidone.

The aims and objectives of the synthesis of a lysozyme analogue have been described in the preceding paper.[1] Here we describe the present 'best route' to the 1–16 fragment of the analogue. The initial scheme proposed for the synthesis of the 1–16 fragment was found to be unsuitable and was considerably modified in the light of

Lys.Val.Phe.Gly.Orn.Cys.Glu.Leu.
1 5
Ala.Ala.Ala.Nle.Lys.Ala.Leu.Gly[2]
10 16

experience together with the advent of improved purification methods. It should be emphasised that in a project of this complexity it is not possible ever to say that the ultimate method of synthesis has been arrived at, because developments in methodology may rapidly outdate a previously acceptable synthesis.

When the synthesis was originally designed, it was unfortunately true that fragment coupling had to be carried out at glycine or proline in order to eliminate the possibility of racemisation. It would have been possible to use the azide fragment coupling method, but that brings its own problems. Subsequent publications, on racemisation whilst using the azide method, have shown that the method is not as free of racemisation as had earlier been believed.[3,4] In the early stages of the programme it became clear that the use of N, N′-dicyclohexylcarbodiimide (DCCI) with the addition of N-hydroxysuccinimide (HONSu)[5] or 1-H-N-hydroxybenzotriazole (HOBt)[6] provided a route whereby fragments could be joined at many residues with an acceptable risk of racemisation. These new methods allowed us to modify the original strategy to a considerable extent.

Initially, we set out to prepare the enzyme analogue with lysine-1 protected as its bis-benzyloxycarbonyl derivative[7]. This had the disadvantage that final deprotection would have to be carried out by treatment with anhydrous HF and studies within our group[8] had shown that reoxidation of HF-treated reduced Hen Egg White Lysozyme gave very low yields of active enzyme. In spite of this potential problem we decided to pursue the synthesis using bis-benzyloxycarbonyl lysine at residue-1.

The first syntheses of the Z(1–16)OPh fragment was achieved by using a (1–4)+(5–16) or (1–6)+(7–16) approach. However, it soon became clear that this tactic had its drawbacks in that, although fragments (1–4) and (1–6) were reasonably soluble in dimethylformamide (DMF), the fragments (5–16) and (7–16) were rather insoluble. A consequence of the use of either of these routes was the hydrogenolysis of the benzyloxycarbonyl group on residue-7. This required passing hydrogen through a suspension of 10% palladium on carbon catalyst in a hexamethylphosphoramide (HMPA)/DMF solution of the decapeptide for one week. An alternative method of synthesis rapidly evolved, utilizing a (1–10)+(11–16) approach since both of these fragments have good solubility in DMF. The (1–10) sequence was constructed by either a (1–4)+(5–10) or (1–6)+(7–10) strategy.

These methods enabled us to synthesise the Z(1–16)OPh fragment. Unfortunately, it was immediately clear that, in its current form, the fragment was unacceptably insoluble, as it could be dissolved only in hot DMF or warm HMPA. At this stage we replaced the two benzyloxylcarbonyl groups on lysine-1 by two adamantyloxycarbonyl groups, in the belief that the increasing lipophilic character of the protecting group would enhance solubility in the solvents commonly employed in peptide synthesis, and that the final stage of deprotection would be significantly improved.

Following the experience gained with the Z(1–16)OPh fragment, we chose the (1–10)+(11–16) method of fragment combination for the Adoc(1–16)OPh, constructing the decapeptide fragment by a (1–5)+(6–10) approach. The resulting Adoc (1–16)OPh (**23**) fragment was indeed found to have

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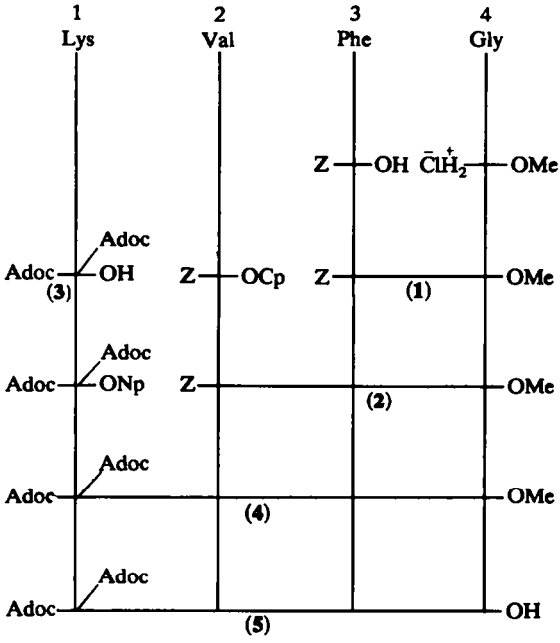
improved solubility over the corresponding benzyloxycarbonyl compound; however, it was clear that this part of the lysozyme sequence possessed inherent insolubility, at least in its fully protected form.

The (1–4) tetrapeptide was constructed by stepwise addition of protected amino-acids as shown in Scheme 1.

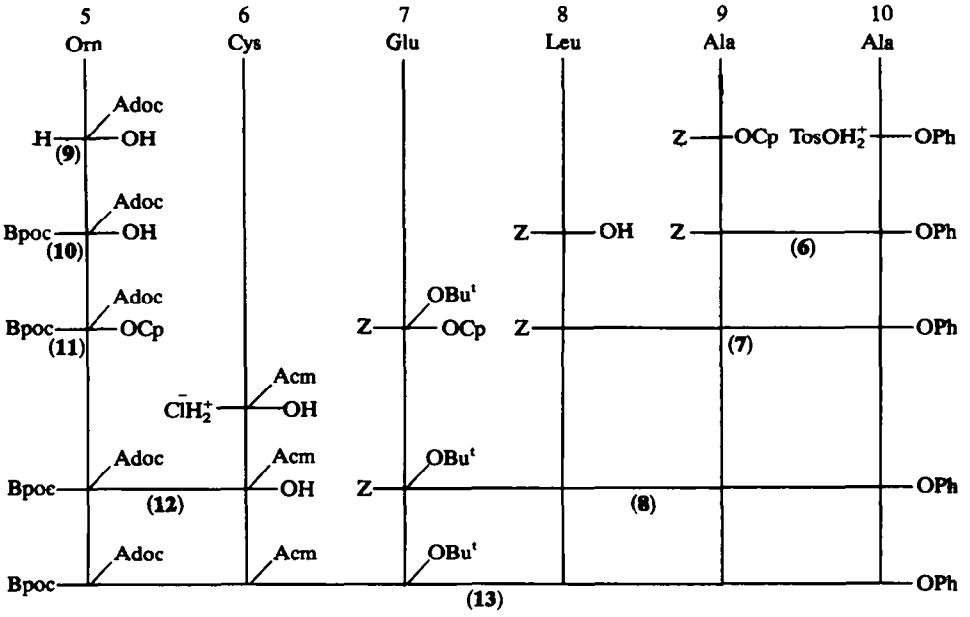


Scheme 1. Synthesis of the protected (1–4) tetrapeptide (5).

The protected dipeptide (1) was obtained in high yield by (DCCI) coupling. (The ^{1}H proton NMR spectra of the vast majority of the compounds prepared have been recorded at 220, 100 or 60 MHz although the details of the assignments are not included in the experimental section. Although the NMR spectra of such compounds are complex and frequently difficult to interpret fully, it is important to realise that the information obtained often furnishes the best check of the integrity of the protecting groups which give simple, clear signals which are easy to integrate). The hydrogenolysis of (1) was effected by the general method, using hydrogen passed through a solution of the appropriate peptide in the presence of Pd/C catalyst and *p*-toluene sulphonic acid monohydrate. The use of this acid is preferred because it can be accurately weighed into the reaction vessel and does not suffer from the disadvantages often encountered with other acids. In addition, when required, highly crystalline salts may readily be isolated. The active ester coupling to yield the tripeptide (2) was straightforward, using a slight excess (1.1 equivs) of active ester to decrease the possibility of diketopiperzine formation. The excess active ester was removed by using N, N-dimethylaminopropylamine.[9] Bisadamantyloxycarbonyl lysine (3) was prepared by treating lysine monohydrate with 2.2 equivalents of adamantylchloroformate[10] at pH 11.0 the product could not be crystallised, and it was obtained in an amorphous form. Frequently we found that although the presence of the adamantyloxycarbonyl group considerably enhanced solubility in organic solvents, it had an adverse effect on crystallinity. Conversion of the lysine derivative (3) to the corresponding *p*-nitrophenylester did not improve the crystallinity. Hence the active ester was coupled directly with the *p*-toluenesulphonate of the tripeptide (2) to yield the fully protected tetrapeptide (4). The methyl ester was hydrolysed in dioxan/water (4/1) at pH 12.0, 4 h being required to complete the cleavage.

In the synthesis of the (1–4) fragment the methyl ester function has been used in place of the phenyl ester for carboxyl protection. This change in our normal strategy[11] was brought about after initial experiments indicated that the hydrogenolysis of the protected dipeptide (Z.Phe.Gly.OPh) did not produce a single product. The reason for the formation of byproducts in this reaction is not immediately clear, although it is possible that cyclisation may be enhanced in this particular case. This would agree with the observed lack of side-reactions in the case of the methyl ester as phenoxide is a better leaving group than methoxide and this would facilitate cyclisation in the phenyl ester case. It should be added at this point that this is the only occasion on which we have encountered such a side-reaction whilst using phenyl esters.

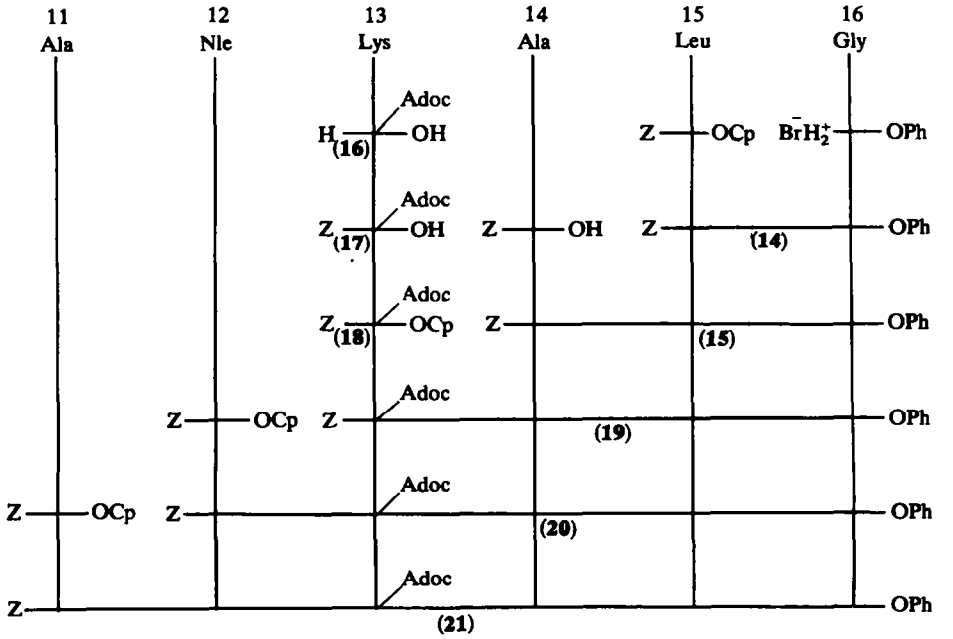
The (5–10) fragment (Scheme 2) was prepared by coupling the (5–6) dipeptide to the (7–10) tetrapeptide (8) which was assembled in high yield by a stepwise procedure using an isobutyloxycarbonyl mixed anhydride for the introduction of Leucine-8. Hydrogenolysis in the presence of 10% Pd/C was used for the removal of benzyloxycarbonyl groups. When fragments with phenyl ester for C-terminal protection are hydrogenolysed it is usually preferable to use DMF as solvent since alcoholic solvents, e.g., methanol, involve the risk of transesterification. Glacial acetic acid may be used if required, as the phenyl ester is known to be stable to this reagent. N-Adamantyloxycarbonyl ornithine hemihydrate (9) was obtained by treating the copper(II) complex of ornithine with adamantylchloroformate[10] at pH 10, the free zwitterion being liberated from its copper(II) complex by the action of hydrogen sulphide. As the overall reaction sequence consisted of three distinct steps the relatively low yield was considered to be acceptable. The N-*p*-biphenylylisopropoxycarbonyl derivative (10) was prepared by reacting the zwitterion (9) with the *p*-bi-phenylylisopropoxycarbonyl azide[12] in the presence of N,N,N,N′-tetramethylguanidine (TMG). During the work up, 40% Triton B in methanol was added, as this prevented the protected amino-acid anion from moving into the organic phase. If this precaution was omitted, appreciable amounts of the TMG salt were to be found in the ether washings; this is believed to be due to the rather lipophilic nature of the compound (10). The crystalline 2,4,5-trichlorophenyl ester (11) was subsequently prepared in high yield by treatment with the corresponding phenol and DCCI. This active ester was coupled for two days with S-acetoamidomethylcysteine hydrochloride[13] in the presence of



Scheme 2. Synthesis of the protected (5–10) hexapeptide (13).

TMG to give the rather amorphous protected dipeptide acid (12). The *p*-toluenesulphonate obtained from a 15 hr hydrogenolysis of the protected tetrapeptide (8) was coupled with (12) by the DCCI/HONSu method. A high yield could be obtained by a washing procedure followed by recrystallisation from ethyl acetate. Often, however, trace impurities remained which were best removed by gel filtration on Sephadex LH20 eluting with DMF[14] (Ve/Vt) = 0.39).

The remaining (11–16) hexapeptide fragment was assembled in a stepwise manner by the route shown in Scheme 3 by the addition of protected amino-acid 2,4,5-trichlorophenyl active esters with the exception of alanine-14 which was coupled as its isobutyloxycarbonyl mixed anhydride in order to avoid diketopiperazine formation. The ε-amino group of lysine was protected by an adamantyloxycarbonyl group. This was introduced by treatment of the copper complex with adamantylchloroformate[10] under conditions similar to those described earlier for the corresponding ornithine derivative (9). In this case however the disodium salt of ethylene diamine tetra-acetic acid was used to break up the copper complex. The free zwitterion was converted to the N-benzyloxycarbonyl active



Scheme 3. Synthesis of the protected (11–16) fragment (21).

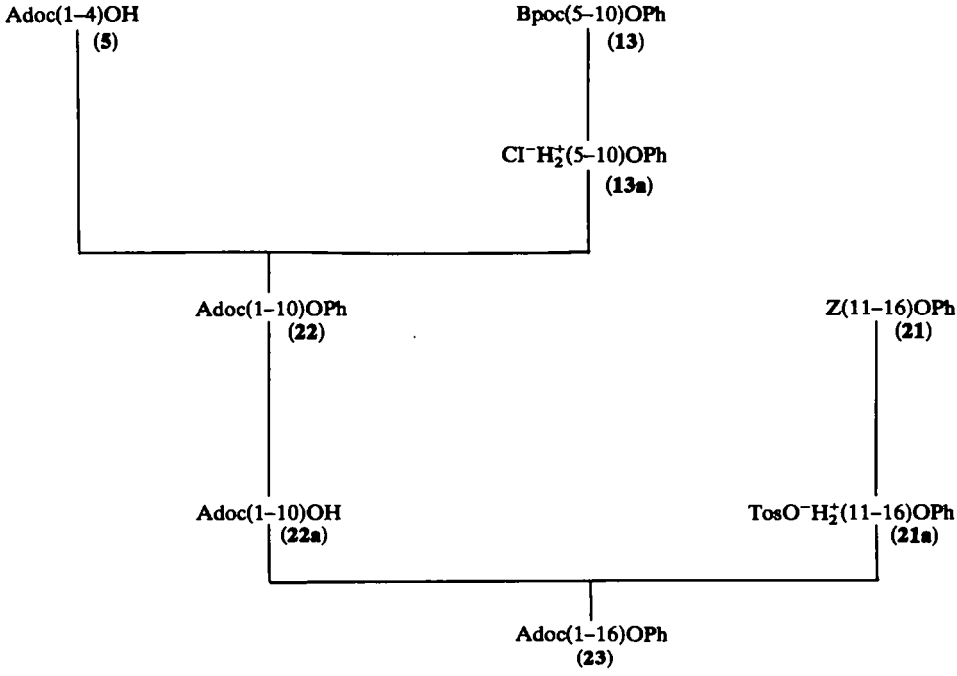
ester (**18**) by routine methods. Although the hexapeptide could be recrystallised from ethyl acetate often it was simpler to purify the coupling mixture directly on Sephadex LH20 eluting with DMF.[14] Assembly of the 1–16 fragment was then accomplished by the route shown in Scheme 4.

The N-protecting group was cleaved from the hexapeptide (**13**) by a 2 hr treatment with a mixture of acetic acid, formic acid and water (7:1:2).[12,15] Dimethyl sulphide was added to act as a scavenger for carbonium ions thus preventing side-reactions with the S-acetamidomethylcysteine residue. The free peptide was isolated as its hydrochloride (**13a**) after a double treatment with 2.5 equivalents of 0.05 M HCl in DMF. Coupling to the protected peptide acid (**5**) was effected by the DCCI/HONSu method, with a second addition of reagents at 24 hr. After a total of 3 days reaction time the Adoc (1–10) OPh fragment (**22**) was isolated by gel filtration on Sephadex LH20, eluting with DMF ((Ve/Vt) = 0.40). The phenyl ester function was then removed by treatment with 2 M NaOH in the presence of one equivalent of 100 volume hydrogen peroxide.[11] Dimethylsulphide was again used as a scavenger, this time preventing oxidative side-reactions. The solvent of choice for this cleavage was ultimately found to be 2,2,2,trifluoroethanol (TFE) which superseded the use of HMPA as it gave a higher yield, was easier to remove and as far as is known does not have toxic properties; it is, however, expensive. The resulting acid (**22a**) was coupled with the *p*-toluenesulphonate (**21a**) obtained by hydrogenolysis of peptide (**21**). The DCCI/HONSu method was used employing a mixture of HMPA and DMF (5:3) solvent. The crude product was obtained by precipitation after 4 days reaction. Originally purification was carried out by gel filtration on Sephadex G50 eluting with HMPA/H_2O (19:1) the HMPA being removed by a further passage through Sephadex G10 eluting with DMF.[14] A considerable improvement was then brought about by using N-methylpyrrolidone (NMP) as eluant, initially Enzacryl K2[16] was used as the support but the best purification at present may be achieved using Sephadex LH60.[17]

The product (**23**) from this coupling was isolated in reasonably high yield (75%). Homogeneity has been demonstrated by tlc, electrophoresis, isoelectric focussing, amino-acid analysis and combustion analysis. The amino-acid analysis was carried out by acid hydrolysis and by digestion of the deprotected peptide with pronase and aminopeptidase. The latter method indicated that within the constraints of the analytical technique the peptide was optically pure. Thus no racemisation had occurred whilst fragment couplings at positions 6 and 10 were being carried out.

EXPERIMENTAL

Abbreviations not in common use or not previously defined are as follows: TEA-triethylamine, DCU-N,N'-dicyclohexylurea, DCHA-dicyclohexylamine, IBC-isobutylchloroformate, Triton-B benzyltrimethylammonium methoxide, HOCp-2,3,5-trichlorophenol, DMS-dimethylsulphide, Z.Cl-benzylchloroformate, DPP.Cl-diphenylphosphinyl chloride, DMA-N,N-dimethylacetamide, NMM-N-methylmorpholine. Tlc was carried out on silicagel GF_{254} using Merck prepared plates in the following systems: (1) $CHCl_3/Me_2CO$ 2/1, (2) $CHCl_3$/MeOH 9/1, (3) $^nBuOH/Py/AcOH/H_2O$ 60/20/6/24, (4) $CHCl_3/^iPrOH/AcOH$ 90/10/1, (5) EtOAc, (6) EtOAc/benzene 3/7, (7) $CHCl_3$/MeOH 3/1, (8) $CHCl_3$/MeOH 19/1, (9) $CHCl_3/^iPrOH$ 9/1, (10) EtOAc/benzene 2/3, (11) $CHCl_3$/MeOH 4/1, (12)



Scheme 4. Synthesis of the fully protected (1–16) fragment (**23**). The protecting groups are as shown in Schemes 1–3.

$CHCl_3$/MeOH/AcOH 90/9/1, (13) $CHCl_3$/MeOH/33% NH_3 90/17/3, (14) ^nBuOH/3% NH_3 3/1, (15) $CHCl_3$/^iPrOH 6/1, (16) $CHCl_3$/^iPrOH/AcOH 19/10/1, (17) CH_3CN/H_2O 9/1, (18) $CHCl_3$MeOH 5/1, (19) $CHCl_3$/^iPrOH 19/1, (20) EtOAc/benzene 2/1, (21) $CHCl_3$/^iPrOH 5/1, (22) $CHCl_3$/^iPrOH 3/1, (23) ^nBuOH/AcOH/H_2O 3/1/1, (24) benzene/MeOH/AcOH 10/2/1, (25), $CHCl_3$/MeOH 6/1, (26) benzene/EtOAc 1/1, (27) CHCl/^iPrOH 7/1, (28) EtOAc/Py/AcOH/H_2O 60/20/6/1, (29) $CHCl_3$/MeOH/TFE 90/5/10, (30) $CHCl_3$/MeOH/AcOH/H_2O 60/18/2/3, (31) ^nBuOH/Py/AcOH/H_2O 30/24/6/20.

Compounds were visualised by one or more of the following methods: Iodine vapour, chlorine/starch/KI[18], ninhydrin or fluorescamine[19] for peptides with free N amino group and UV absorption at 254 nm. Deprotections were carried out using 90% trifluoroacetic acid (TFA) with mercaptoethanol and anisole as scavengers. Free peptides were subject to high voltage paper electrophoresis using a refrigerated Pherograph instrument[20] and to flat-bed isoelectric focussing on polyacrylamide gels using an LKB 2117 multiphore. Amino-acid analyses were determined on a Jeol 5 AH or 6 AH with integrator after hydrolysis (a) by sealed tube hydrolysis with 6 M HCl at 110° for 24 hr or (b) by digestion with pronase (24 hr) followed by amino-peptidase M (24 hr) in 0.1 phosphate buffer at pH 7.5 at 37°. All evaporations were *in vacuo* at the minimum possible temp, DMF and other high boiling solvents being removed at 20° under an oil pump vacuum. A radiometer autotitrator was used for the maintenance of constant pH. M.ps are uncorrected. The preparations given are typical rather than those giving maximum yield and are not necessarily on the largest scale studied.

Scheme 1

Z-Phe-Gly-OMe (**1**). A soln of Z-Phe-OH (12.4 g, 40 mM) and glycine methyl ester hydrochloride (5.0 g, 40 mM) in CH_2Cl_2 (60 ml) was cooled to −10°. TEA (5.5 ml, 40 mM) and DCCI (9.0 g, 44 mM) were added to the stirred mixture which was maintained at −10° for 2 hr. After warming to room temp overnight a few drops of glacial AcOH were added and after a further 0.5 hr the DCU ppt was removed by filtration. The organic phase was washed with water dried and evaporated to yield a gum which crystallised from EtOAc-petroleum ether (13.0 g, 88%), m.p. 120–121°, $[\alpha]_D^{25}$ −16.5° ($c = 1$, meOH), $R_f(1)$ −0.6, (Found: C, 64.57; H, 5.93; N, 7.73. $C_{20}H_{22}N_2O_5$ requires: C, 64.85; H, 5.99; N, 7.56).

Z-Val-Phe-Gly-OMe (**2**). The protected dipeptide (**1**) (11.1 g, 30 mM) and Tos.OH.H_2O (5.7 g, 30 mM) were dissolved in DMF (20 ml), Pd/C (10%) catalyst (1.5 g) was added and hydrogen passed through the soln overnight. The catalyst was removed and evaporation yielded a gum which was dissolved in DMF (75 ml). Z-Val-OCp (14.4 g, 33 mM) and TEA (4.2 ml, 30 mM) were added and the mixture stirred for 48 hr at room temp. The solvent was removed and the residue dissolved in MeOH (40 ml), N,N-dimethylaminopropylamine[9] (0.6 ml) was added and the soln stirred for 0.5 hr. The mixture was poured into water, the ppt filtered off, washed with 5% HCl, and water until neutral. Recrystallisation of the dried solid from MeOH gave (**2**) (12.0 g, 82%), m.p. 182–3°, $[\alpha]_D^{20}$ −20.9° ($c = 1$, DMF), $R_f(1)$ −0.7, $R_f(2)$ −0.75, $Val_{1.00}Phe_{1.00}Gly_{1.00}$, (Found: C, 63.74; H, 6.63; N, 8.72. $C_{25}H_{31}O_6N_3$ requires: C, 63.95; H, 6.65; N, 8.95.

Adoc-Lys(Adoc)-OH (**3**). A suspension of lysine monohydrochloride (7.3 g, 40 mM) at 0° was brought to pH 11.0 with 1 M NaOH. A soln of adamantyl chloroformate[10] (18.2 g, 88 mM) was dissolved in dioxan (50 ml) and added to the above vigorously stirred suspension in five portions over 1 hr the pH being maintained at 11.0 by the addition of 1 M NaOH. Et_2O (100 ml) was added and stirring continued at 0° for a further 2 hr. After warming to room temp overnight the soln was washed with Et_2O and acidified to pH 3 with solid citric acid. The soln was extracted with EtOAc (3 × 100 ml) and the combined extracts washed with water, dried and evaporated to give a gum. This gum could not be crystallised but was solidified under n-pentane at 0° giving (13.4 g, 67%), m.p. 65°, $[\alpha]_D^{25}$ −0.9° ($c = 1$, DMF), $R_f(3)$0.65, $R_f(4)$0.65, (Found: C, 66.94; H, 8.62; N, 5.50. $C_{28}H_{42}O_6N_2$ requires: C 66.90; H, 8.42; N, 5.57%).

Adoc-Lys(Adoc)-Val-Phe-Gly-OMe (**4**). The tripeptide derivative (**2**) (2.2 g, 4.9 mM) was hydrogenolysed in the usual way overnight in the presence of Tos.OH.H_2O (0.93 g, 4.9 mM) and 10% Pd/C (0.6 g). Filtration and evaporation yielded the corresponding *p*-toluenesulphonate (1.9 g, 3.8 mM). Adoc-Lys(Adoc)-OH (**3**) 2.0 g, 4.0 mM) was dissolved in EtOAc (5 ml) and *p*-nitrophenol (0.68 g, 4.8 mM) and DCCI (0.85 g, 4.0 mM) added whilst stirring at 0°. After stirring for 2 hr at 0° and overnight at room temp a few drops of glacial AcOH were added and the reaction stirred for a further 3 hr. The mixture was then cooled to 0°, filtered and the soln evaporated to give a non-crystallisable oil (2.4 g, 100%), homogeneous $R_f(2)$ − 0.8. The *p*-toluenesulphonate and *p*-nitrophenyl ester were dissolved in DMF (5 ml), TEA (0.39 g, 3.8 mM) was added and the mixture stirred at room temp for 3 days. Evaporation gave a residue which was dissolved in EtOAc (250 ml), washed with 5% citric acid (100 ml), 0.5 M NH_4OH until free of *p*-nitrophenol, 5% citric acid (3 × 100 ml) and water. The soln was dried and evaporation gave a gum which was crystallised from EtOAc-petroleum ether giving the protected tetrapeptide (**4**) (1.9 g, 65%), m.p. 118°, $[\alpha]_D^{25}$ −16.9° ($c = 1$, DMF), $R_f(5)$ − 0.8, $R_f(6)$ − 0.3, $Lys_{0.96}Gly_{1.05}Val_{1.04}Phe_{0.95}$, (Found: C, 66.10; H, 8.06; N, 8.84. $C_{45}H_{65}N_5O_9$ requires: C, 65.91; H, 7.99; N, 8.54%).

Adoc-Lys(Adoc)-Val-Phe-Gly-OH (**5**). The protected tetrapeptide ester (**4**) (0.82 g, 1 mM) was dissolved in dioxan/water (4 : 1, 30 ml) and the pH brought to 12.0 by the addition of 1 M NaOH. The pH was maintained for 4 hr by the addition of 1 M NaOH, the soln was then poured into 5% citric acid (500 ml). The ppt was washed with water, dried, redissolved in MeOH (1 ml) and precipitated with ether, filtration gave the protected peptide acid (**5**) (0.6 g, 76%), m.p. 120°, $[\alpha]_D^{25}$ −18.1° ($c = 1$, DMF), $R_f(7)$ − 0.5, (Found: C, 62.53; H, 8.01; N, 8.40. $C_{44}H_{63}N_5O_9.2H_2O$ requires: C, 62.76; H, 8.02; N, 8.32%).

Scheme 2

Z-Ala-Ala-OPh (**6**). Z-Ala-OPh[11] (18.0 g, 60 mM) in DMF (100 ml) was hydrogenolysed overnight in the presence of Tos.OH.H_2O (11.4 g, 60 mM) and 10% Pd/C (3.0 g). Removal of the catalyst and evaporation gave a residue which was redissolved DMF (75 ml). Z-Ala-OCp (26.2 g, 65 mM) and NMM (6.6 ml, 60 mM) were added and the reaction mixture stirred for 2 days at room temp. The solvent was evaporated and the residue dissolved in EtOAc, washed with 5% $NaHCO_3$, 5% citric acid and water. The dried soln was evaporated to give a white solid which was crystallised from EtOAc-petroleum ether giving the required product (16.7 g, 75%), m.p. 126–7°, $[\alpha]_D^{22}$ −44.6° ($c = 2$, DMF), $R_f(8)$ − 0.45, (Found: C, 64.62; H, 5.75; N, 7.37. $C_{20}H_{22}N_2O_5$ requires: C, 64.85; H, 5.99; N, 7.56%).

Z-Leu-Ala-Ala-OPh (**7**). Compound (**6**) (16.7 g, 45 mM) in DMF (75 ml) was hydrogenolysed overnight in the presence of Tos.OH.H_2O (8.6 g, 45 mM) and 10% Pd/C (2.7 g). Filtration and evaporation produced a crystalline material which was dissolved in DMF (20 ml). Z-Leu-Oh [obtained from Z-Leu-OH.DCHA (22.3 g, 50 mM) by liberation with 10% citric acid and extracting into EtOAc] was dissolved in CH_2Cl_2 (75 ml) and cooled

to −20°, NMM (5.5 ml, 50 mM) and IBC (6.5 mM) were added allowing 15 min. for activation at −20°. The DMF soln and NMM (4.95 ml, 45 mM) were then added and the mixture allowed to attain room temp over 24 hr. Evaporation of the solvents gave a residue which was dissolved in warm EtOAc; washing with water, drying and evaporation crystallised from EtOAc (16.8 g, 78%), m.p. 179°, $[\alpha]_D^{30}-50.2°$ ($c=0.5$, DMF), $R_f(9)-0.73$, $R_f(10)-0.22$, $Leu_{1.00}Ala_{2.04}$, (Found: C, 64.62; H, 6.89; N, 8.95. $C_{26}H_{33}N_3O_6$ requires: C, 64.58; H, 6.88; N, 8.69%).

Z-Glu(OBu^t)-Leu-Ala-Ala-POh (**8**). The protected tripeptide **7** (9.7 g, 20 mM) was hydrogenolysed overnight in the presence of Tos.OH.H_2O (3.8 g, 20 mM) and 10% Pd/C (1.0 g). Work up in the usual way gave a residue which was dissolved in DMF (50 ml) along with Z-Glu(OBu^t)OCp (11.3 g, 22 mM) and NMM (2.2 ml, 20 mM). The sol was stirred for 3 days and the solvent evaporated to give a residue which was dissolved in warm EtOAc (750 ml), washed with water and dried. Evaporation gave the solid product which was crystallised from EtOAc (14.8 g, 80%), m.p. 160°, $[\alpha]_D^{30}-53.8°$ ($c=0.5$, DMF), $R_f(1)-0.5$, $R_f(2)-0.6$, $Glu_{1.00}Ala_{1.94}Leu_{1.00}$, (Found: C, 61.94; H, 7.34; N, 8.05. $C_{35}H_{48}N_4O_9.0.5H_2O$ requires: C, 62.02; H, 7.29; N, 8.26%).

H-Orn(Adoc)-OH (**9**). Ornithine hydrochloride (84.3 g, 0.5 M) was dissolved in 1 M NaOH (1 l) and a soln of $CuSO_4.5H_2O$ (62.5 g, 0.25 M) in water (750 ml) added. After stirring for 15 min, the soln was cooled to 0° and a soln of Adoc.Cl in dioxan added (1 l. containing 0.55 M). Solid Na_2CO_3 (159.0 g, 1.5 M) was added as required to maintain the pH at approximately 10. The sol was maintained at 0° for 2 hr then allowed to warm to room temp overnight. The resulting blue suspension was diluted with water (2 l.) and filtered. The solid was washed with water until the washings were colourless, then washed with ice cold EtOH and Et_2O to remove unreacted Adoc.Cl. The finely powered copper complex (94.0 g, 138 mM) was suspended in a mixture of water (3 l.), AcOH (60 ml) and MeOH (1 l.) and H_2S passed through the vigorously stirred suspension for 4 hr. Air was passed through the suspension to remove excess H_2S, which was then warmed to near boiling and filtered using a double fluted filter paper. The volume of the filtrate was then reduced by evaporation *in vacuo* until the crystalline product was obtained, this was then washed with cold water yielding (**9**) (59.7 g, 39%); recrystallisation may be carried out using MeOH–Et_2O, m.p. 214–216°, $[\alpha]_D^{24}-7.8°$ ($c=1$, MeOH), $R_f(23)-0.4$, $R_f(31)-0.6$, (Found: C, 59.85; H, 8.40; N, 8.53 $C_{16}H_{26}N_2O_4.0.5H_2O$ requires: C, 60.15; H. 8.52: N, 8.77%).

Bpoc-Orn(Adoc)-OH (**10**). A soln of (**9**) (6.2 g, 20 mM), TMG (4.9 ml, 40 mM) and Bpoc.N_3[12])(6.8 g, 24 mM) in DMF (50 ml) was stirred for 2 days. The DMF was evaporated and the residue partitioned between water and Et_2O after the addition of 40% Triton B in MeOH (10 ml). A further extraction with Et_2O was carried out and the aqueous phase acidified with 10% citric acid. The resulting oil was extracted into EtOAc, washed with water, dried and the solvent evaporated to give a foam which was solidified by trituration with Et_2O-petroleum ether (1/3) giving (9.3 g, 75%), m.p. 110–112°, $[\alpha]_D^{30}+8.6°$ ($c=0.5$, DMF), $R_f(11)-0.44$, $R_f(12)-0.50$, (Found: C, 70.30; H, 7.22; N, 5.35. $C_{32}H_{40}N_2O_6$ requires: C, 70.04; H, 7.34; N, 5.10%).

Bpoc-Orn(Adoc)-OCp (**11**). The acid (**10**) (5.5 g, 10 mM), HOCp (2.0 g, 10 mM) and DCCI (2.3 g, 11 mM) were dissolved in EtOAc (100 ml) at 0°. The mixture was stirred for 2 hr at 0° and at room temp overnight. The resulting DCU was filtered and the solvent evaporated to give a residue which was crystallised from benzene-petroleum ether giving (**11**) (6.7 g, 90%), m.p. 95–98°, $[\alpha]_D^{30}-21.3°$ ($c=1$, DMF), $R_f(1)-0.45$, $R_f(2)-0.8$, (Found: C, 62.44; H, 5.51; N, 3.77. $C_{38}H_{41}N_2O_6Cl_3$ requires: C, 62.68; H, 5.67; N, 3.84%).

Bpoc-Orn(Adoc)-Cys(Acm)-OH (**12**). $\bar{C}l$ H_2^+Cys-(Acm)OH (2.3 g, 10 mM) was dissolved in warm DMF (15 ml), Bpoc-Orn(Adoc)-OCp (**11**) (6.7 g, 9 mM) and TMG (2.48 ml, 20 mM) were added and the mixture stirred for 2 days. The solvent was evaporated and water (250 ml) added, this soln was acidified to pH 3 with 10% icecold citric acid, extracted with EtOAc (3 × 150 ml) and the combined organic phases backwashed with water. Drying and evaporation produced a dry foam which was solidified by treatment with petroleum ether. This solid was washed with $^iPr_2O$ to remove unreacted active ester leaving the pure (**12**) (6.0 g, 92%), m.p. 105°, $[\alpha]_D^{30}+4.3°$ ($c=1$, DMF), $R_f(7)-0.65$, $R_f(12)-0.4$, $R_f(13)-0.3$, $R_f(14)-0.5$, (Found: C, 63.14; H, 7.18; N, 7.54; S, 4.37. $C_{38}H_{50}N_4O_8S$ requires: C, 63.14; H, 6.97; N, 7.70; S, 4.44%).

Bpoc-Orn(Adoc)-Cys(Acm)-Glu(OBu^t)-Leu-Ala-Ala-OPh (**13**). The protected tetrapeptide (**8**) (3.0 g, 4.5 mM) in DMF (25 ml) was hydrogenolysed for 15 hr in the presence of Tos.OH.H_2O (0.61 g, 3.2 mM) and 10% Pd/C (0.25 g). Filtration and evaporation gave a gum which was solidified by trituration with water. Filtration and drying over P_2O_5 gave the *p*-toluenesulphonate salt (3.0 g, 94%), $R_f(7)$–0.3. The protected dipeptide acid (**12**) (0.56 g, 0.78 mM) and the above *p*-toluenesulphonate (0.5 g, 0.71 mM) were dissolved in DMF (3 mM) and a 2% soln of NMM in DMF were added (3.75 ml, 0.71 mM) and the mixture was allowed to warm at room temp. After stirring for 48 hr the DCU was filtered and the solvent evaporated, precipitation with water gave a solid which was washed with 5% ice-cold citric acid, 5% $NaHCO_3$ water $^iPr_2O$. The resulting solid was recrystallised from EtOAc† giving the required product (**13**) (0.83 g, 93%), m.p. 155°, $[\alpha]_D^{25}-28.8°$ ($c=1$, DMF), $R_f(15)-0.5$, $R_f(16)-0.55$, $Orn_{1.00}Glu_{1.00}Ala_{2.18}Leu_{0.99}$ (trace of cystine appears as shoulder on Ala), (Found: C, 62.18; H, 7.27; N, 9.06. $C_{65}H_{90}N_8O_{14}S.H_2O$ requires: C, 62.08; H, 7.29; N, 8.91%).

Scheme 3

Z-Leu-Gly-OPh (**14**). Z-Leu-OCp (67.0 g, 150 mM), $Br^-H_2^+$-Gly-OPh (32.5 g, 140 mM) and TEA (19.0 ml, 140 mM) were dissolved in DMF (200 ml) and the resulting soln stirred for 2 days. Evaporation of the solvent gave a residue which was dissolved in EtOAc (750 ml), the soln was washed with 5% $NaHCO_3$, 5% citric acid and water, then dried and evaported. The resulting residue was crystallised from EtOAc-petroleum ether giving the required (**14**) (31.0 g, 71%), m.p. 124–125°, $[\alpha]_D^{30}-39.1°$ ($c=1$, DMF), $R_f(1)-0.8$, $R_f(2)-0.6$, (Found: C, 66.33; H, 6.61; N, 7.23. $C_{22}H_{26}N_2O_5$ requires: C, 66.31; H, 6.78; N, 7.03%).

Z-Ala-Leu-Gly-OPh (**15**). The protected dipeptide (**14**) (27.9 g, 70 mM) in DMF (100 ml) was hydrogenolysed overnight in the presence of Tos.OH.H_2O (13.3 g, 70 mM). After warming to room temp overnight the soln gave an oil. Z-Ala-OH (16.7 g, 75 mM) was dissolved in CH_2Cl_2 (50 ml) and cooled to −20°, TEA (10.5 ml, 75 mM) and IBC (9.8 ml, 75 mM) were added allowing 15 min for activation. The oil from the above was dissolved in DMF (30 ml) and added, followed by TEA (9.2 ml, 70 mM). After warming to room temp overnight the soln was evaporated and the residue dissolved in warm EtOAc. This soln was washed with water, dried and evaporated to give a residue which was crystallised from EtOAc

† On some occasions the mixture was applied directly to an LH-20/DMF column to effect purification (Ve/Vt) = 0.39.

yielding (27.0 g, 82%), m.p. 161–163°, $[\alpha]_D^{30}$–41.6° ($c = 1$, DMF), $R_f(1)-0.5$, $R_f(2)-0.55$, $Ala_{1.03}$-$Leu_{0.99}Gly_{0.97}$, (Found: C, 63.86; H, 6.78; N, 9.12. $C_{25}H_{31}N_3O_6$ requires: C, 63.94; H, 6.68; N, 8.95%).

H-Lys(Adoc)-OH (**16**). Lysine hydrochloride (91.3 g, 0.5 M) was reacted with Adoc.Cl[10] (0.55 M) under identical conditions to those used for the preparation of (**9**) yielding the copper complex (100.0 g, 141 mM). The finely powered complex was added to a boiling soln of EDTA.2Na (142.0 g) in water (3.6 l) and stirred until dissolved. The soln was allowed to cool to room temp and the product crystallised. After cooling to 0° for 2 hr to complete crystallisation the crystals were filtered and washed with ice-water yielding (**16**) (65.0 g, 40%), recrystallisation may be carried out from MeOH-Et_2O, m.p. 227–229°, $[\alpha]_D^{24}-1.3°$ ($c = 1$, MeOH), $R_f(23)-0.4$, $R_f(31)-0.65$, (Found: C, 61.28; H, 8.75; N, 8.58. $C_{17}H_{28}N_2O_4.0.5H_2P$ requires: C, 61.23; H, 8.76; N, 8.40%).

Z-Lys(Adoc)-OH (**17**). 1 M NaOH (90 ml) was added to an ice cold suspension of (**16**) (29.2 g, 90 mM) in water (50 ml). Z.Cl (15.0 ml, 110 mM) and 4 M NaOH (25.0 ml, 100 mM) were added dropwise over 1 hr and the mixture stirred for a further 1 hr at 0°. After stirring overnight the soln was extracted with Et_2O (2 × 150 ml) and the aqueous phase acidified to pH 3 with 20% citric acid. The resulting oil was extrated into EtOAc and washed with water, drying and evaporation of the organic phase gave the product as a gum which resisted all attempts at crystallisation (39.0 g, 95%), $R_f(7)-0.7$. The DCHA salt crystallised with difficulty from acetone –Et_2O m.p. 112°, $[\alpha]_D^{30}+0.9°$ (c = 1, DMF), (Found: C, 68.92; H, 8.91; N, 6.64. $C_{37}H_{57}N_3O_6$ requires: C, 69.45; H, 8.98; N, 6.57%).

Z-Lys(Adoc)-OCp (**18**). Z-Lys(Adoc)-OH (**17**) (35.0 g, 76.4 mM), HOCp (15.0 g, 76.4 mM) and DCCI (17.3 g, 84 mM) were dissolved in EtOAc (110 ml) at 0°. After stirring for 1 hr at 0° and overnight at room temp the resulting DCU was removed by filtration and the filtrate washed with 1 M $NaHCO_3$, 2 N citric acid and water. The dried organic phase was evaporated to give an oil which resisted all attempts at crystallisation, (45.8 g, 94%), $R_f(1)-0.6$, $R_f(2)-0.75$, $R_f(9)-0.7$, $R_f(26)-0.7$, NMR (100 MHz), δ($CDCl_3$: 7.47 and 7.09, (2H, 2xs, $C_6H_2Cl_3$); 7.28, (5H, s, C_6H_5); 5.78 (1H, d, xNH); 5.09, (2H, s, Ph.CH_2–); 4.74 (1H, t, NH); 4.3–4.7 (1H, complex, x CH); 2.9–3.2 (2H, broad, CH_2); 1.4–2.2 (2–H, complex, $C_{10}H_{15}+3\times CH_2$).

Z-Lys(Adoc)-Ala-Leu-Gly-OPh (**19**). A soln of (**15**) (0.94 g, 2 mM) in DMF (10 ml) was hydrogenolysed for 15 hr in the presence of TosOH.H_2O (0.38 g, 2 mM) and 10% Pd/C (0.1 g). Processing in the usual way gave a residue which was dissolved in DMF (25 ml). Z-Lys(Adoc)-OCp (**18**) (1.67 g, 2.0 mM) and NMM (0.28 ml, 2 mM) were added and the mixture stirred for 48 hr. Evaporation of the solvent gave a residue which was triturated with water and Et_2O, the resulting solid was crystallised from IPA to give (**19**) as a gelatinous solid (1.00 g, 65%), m.p. 175°, $[\alpha]_D^{30}-18.6°$ (c = 1, DMF), $R_f(2)-0.7$, $R_f(9)-0.6$, $R_f(17)-0.8$, $Lys_{0.94}Ala_{1.03}$-$Leu_{1.03}Gly_{1.00}$, (Found: C, 64.13; H, 7.25; N, 8.90. $C_{42}H_{57}N_5O_6.0.5H_2O$ requires: C, 64.26; H, 7.45; N, 8.92.

Z-Nle-Lys(Adoc)-Ala-Leu-Gly-OPh (**20**). A soln of (**19**) (15.5 g, 20 mM) in DMF (25 ml) was hydrogenolysed for 15 hr in the presence of TosOH.H_2O (3.8 g, 20 mM) and 10% Pd/C (1.0 g). Work up in the usual way gave a residue which was dissolved in DMF (100 ml). Z-Nle-OCp (9.9 g, 22.5 mM) and NMM (2.2 ml; 20 mM) were added to this soln and the mixture stirred for 48 hr. The soln was poured into a mixture of water and Et_2O and the resulting ppt. filtered off, washed with Et_2O and dried The solid was dissolved in $CHCl_3$ and excess IPA added, after stirring overnight at 0° the required (**20**) was filtered off giving (14.4 g, 81%), m.p. 224–227°, $[\alpha]_D^{30}-27.2°$ ($c = 1$, DMF), $R_f(2)-0.4$, $Nle_{1.01}Lys_{0.95}Ala_{1.03}Leu_{1.01}$-$Gly_{0.99}$, (Found: C, 64.04; H, 7.71; N, 9.35. $C_{48}H_{68}N_6$-$O_{10}0.5H_2O$ requires: C, 64.19; H, 7.74; N, 9.36.

Z-Ala-Nle-Lys(Adoc)-Ala-Leu-Gly-OPh (**21**). Hydrogenolysis of (**20**) (14.0 g. 16 mM) in DMF (20 ml) in the presence of TosOH.H_2O (3.0 g, 16 mM) and 10% Pd/C (1.0 g) for 16 hr and subsequent work up gave a residue which was dissolved in DMF (100 ml). Z-Ala-OCp (7.1 g, 17.6 mM) and NMM (1.76 ml, 16 mM) were added to the soln which was stirred for 48 hr. The mixture was poured into a mixture of water and Et_2O to give a white solid which was washed with Et_2O and dried. Recrystallisation from IPA† gave the protected hexapeptide (**21**) (14.5 g, 94%), m.p. 210–214°, $[\alpha]_D^{30}-25.0°$ ($c = 1$, DMF), $R_f(2)-0.55$, $Lys_{0.95}Leu_{1.02}Ala_{1.97}Nle_{1.05}Gly_{1.02}$, (Found: C, 62.32; H, 7.49; N, 10.17. $C_{51}H_{73}N_7O_{11}.H_2O$ requires: C, 62.62; H, 7.73; N, 10.02%).

Scheme 4

Adoc(1–10)OPh (**22**). The protected hexapeptide (**13**) (2.44 g, 2.0 mM) was dissolved in AcOH:$H.CO_2H$:H_2O (7:1:2) (44 ml) in the presence of DMS (4.1 ml). The soln was stirred for 2 hr evaporated and the residue dissolved in DMF containing 2.5 equivs of 0.05 M HCl. The solvent was evaporated and the residue retreated with a 2.5 equivs of HCl in DMF, evaporation gave a product which solidified on trituration with Et_2O. Filtration and drying over P_2O_5 gave (**12a**) (1.75 g, 86%), $R_f(7)$–0.2. This hydrochloride and the protected tetrapeptide acid (**5**) (1.63 g, 2.0 mM) were dissolved in DMF:HMPA (1:1) (6 ml) and cooled to −20°. HONSu (0.47 g, 4.05 mM), DCCI (0.5 g, 2.43 mM) and a 1% soln of NMM in DMF (1.7 ml, 1.6 mM) were added and the mixture stirred for 24 hr at room temp. The mixture was again cooled, HONSu (0.12 g, 1.0 mM) and DCCI (0.13 g, 0.6 mM) added and then stirred for a further 48 hr at room temp. The mixture was diluted with DMF (15 ml) and applied directly to an LH20 column eluting with DMF. The protected decapeptide (**22**) had (Ve/Vt) = 0.40 and was isolated by precipitation with water and washing with ether giving (1.55 g, 55%), m.p. 250°, $[\alpha]_D^{25}-33.5°$ ($c = 0.5$, HMPA), $R_f(7)-0.4$, $R_f(29)-0.45$, $Lys/Orn_{2.01}$-$Glu_{1.00}Gly_{1.01}Ala_{2.02}Val_{1.00}Leu_{1.00}Phe_{1.00}$, (Pronase/APM) $Lys/Orn_{1.90}Cys(Acm)+Cys_{0.98}Glu_{0.95}Gly_{1.04}Ala_{2.14}$-$Val_{1.05}Leu_{0.93}Phe_{1.00}$, (Found: C, 61.51; H, 7.79; N, 9.94. $C_{93}H_{137}N_{13}O_{20}S.H_2O$ requires: C, 61.80; H, 7.75; N, 10.08%).

Adoc(1–16)OPh (**23**). The phenyl ester may be cleaved from (**22**) by two alternative methods. (a) Adoc(1–10)OPh (**22**) (0.8 g, 0.45 mM) and DMS (5 ml) were dissolved in HMPA (15 ml) and a mixture of HMPA: water (2:1) (15 ml) added. The pH was brought to 10.5 by the addition of 1 M NaOH, 100 vol H_2O_2 (0.05 ml) was added and the pH maintained at 10.5 by the addition of 1 M NaOH (0.45 ml) for 30 min by which time consumption of base had ceased. The pH was then brought to 3 by the addition of 1 M HCl, causing precipitation of the product. Sat NaCl was added and the product filtered, washed with water and Et_2O then dried over P_2O_5 giving **22a** (0.77 g, 86%), $R_f(7)-0.4$, $[\alpha]_D^{25}-10.4°$ ($c = 0.5$, HMPA).

(b) The phenyl ester (**22**) (0.6 g, 0.34 mM) and DMS (3 ml) were dissolved in TFE (10 ml), the pH was adjusted to 10.5 with 2 M NaOH (0.4 ml) and water (1.6 ml) added. 100 vol H_2O_2 (0.1 ml) was added and the pH maintained at 10.5 for 30 min. The pH was then brought to 3 and the solvent volume reduced to a minimum, working up as in (a) above gave (**22a**) (0.55 g, 96%), $F_f(7)$–0.4, $[\alpha]_D^{30}-10.9°$ ($c = 0.5$, DMF).

† The compound sometimes required further purification by gel filtration on LH20 in DMF (Ve/Vt) = 0.46.

The protected hexapeptide (**2**) (0.96 g, 1 mM) was hydrogenolysed in DMF (10 ml) over 10% Pd/C (0.2 g) for 15 hr in the presence of $TosOH.H_2O$ (0.19 g, 1 mM). Filtration and evaporation gave a residue which was triturated with water and Et_2O then dried over P_2O_5 yielding (**21a**) (0.88 g, 89%), $R_f(7)$–0.35.

The *p*-toluenesulphonate (**21a**) (0.38 g, 0.38 mM) and the protected peptide acid (**22a**) (0.50 g, 0.29 mM) were dissolved in HMPA:DMF (5 ml:3 ml). After cooling to 0° HONSu (68 mg, 0.6 mM), DCCl (74 mg, 0.36 mM) and a 20% soln of NMM in DMF (0.19 ml) were added and the soln stirred overnight at room temp. The mixture was recooled to 0°, HONSu (20 mg) and DCCl (19 mg) added and then allowed to warm to room temp. After 3 days the solvent volume was reduced to a minimum and brine added to precipitate the crude product, this was washed with water and Et_2O then dried over P_2O_5. The crude material was initially purified by gel filtration on Sephadex G50 eluting with $HMPA:H_2O$ (19:1), (Ve/Vt) = 0.6, the HMPA being removed by rapid gel filtration on Sephadex G10 eluting with DMF, (Ve/Vt) = 0.36. Some improvement was achieved by using Enzacryl K2 eluting with NMP, (Ve/Vt) = 0.54 although the best purification was ultimately brought about by the use of Sephadex LH60 eluting with NMP, (Ve/Vt) = 0.61. In all cases the product was precipitated with water after evaporation giving (0.56 g, 75%), m.p. 250°, $[\alpha]_D^{25} + 2.6°$ ($c = 0.5$, HMPA), $R_f(7) - 0.85$, $R_f(18) - 0.6$, $Lys/Orn_{2.89}$-$Glu_{1.06}Gly_{2.01}Ala_{3.91}Val_{0.99}Leu_{2.07}Nle_{0.94}Phe_{1.03}$, (pronase/APM) $Lys/Orn_{2.89}Cys(Acm)_{1.05}Glu_{1.05}Gly_{1.99}Ala_{3.80}$-$Val_{1.01}Leu_{2.12}Nle_{1.05}Phe_{1.13}$, (Found: C, 60.24; H, 7.86; N, 10.69. $C_{130}H_{198}N_{20}O_{28}S.4H_2O$ requires: C, 60.21; H, 8.00; N, 10.80.

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