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PEPTIDES—XXXIX

SYNTHESIS OF THE 50–67 FRAGMENT OF A LYSOZYME ANALOGUE

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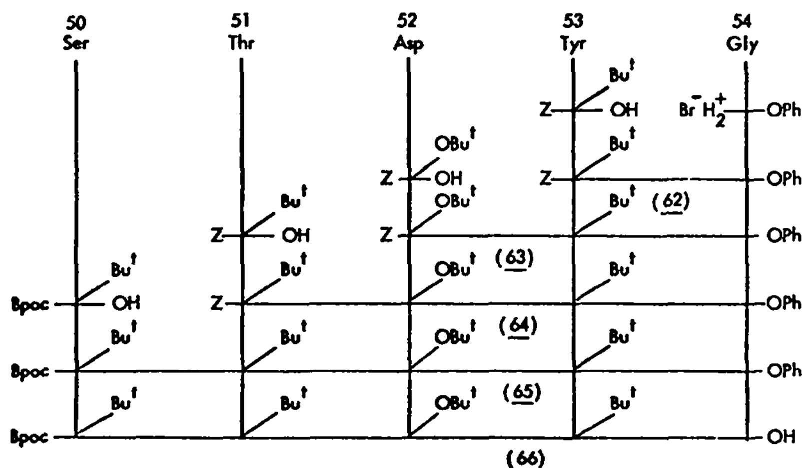
Abstract—The synthesis of the fully protected octadecapeptide fragment (50–67) was achieved by combination of the subfragments 50–54, 55–60 and 61–67. Single routes to two of these fragments are described but the fragment (55–60) was prepared by two different approaches. In this case salt-coupling techniques alleviated serious solubility problems. Fragment couplings were achieved using DCCI/HONSu.

In continuance of our general aim of synthesis of a Lysozyme analogue we wish to describe the synthesis of the (50–67) portion of the (38–75) fragment of the target molecule employing the general tactics and strategy developed in earlier papers of this series..[1–5] Two independent routes have been used for the preparation of the (50–75) fragment, and one of these uses the (50–67) subfragment as a major component whilst the second route utilises coupling of the (50–60) and (61–75) fragments which will be described in a subsequent paper.

The amino acid sequence of the (50–67) subfragment is

Ser. 50	Thr.	Asp.	Tyr.	Gly. 54	Leu.	Leu.	Gln.	Ile
Asn.	Ser. 60	Orn.	Trp.	Trp.	Cys.	Ala.	Asp.	Gly. 67

This subfragment may be dissected into 3 major portions in order to facilitate synthesis by maximising the use of stepwise procedures and also making use of Gly-54 in a subsequent fragment condensation step. The (50–54) pentapeptide was synthesised using the route shown in Scheme 1. All the coupling reactions were achieved using the pivalic mixed anhydride method; benzyloxycarbonyl groups being removed prior to amide formation by catalytic hydrogenolysis in the presence of *p*-toluene-sulphonic acid. It was found however, that prolonged hydrogenolysis of the protected tripeptide **63** or tetrapeptide **64** led to the formation of troublesome by-products although purification of both of these compounds could be achieved readily by gel filtration on LH20 Sephadex eluting with DMF.[6] The compound numbering sequence follows that established in certain papers in this series.[1–5] During optimisation of the synthesis of **66** Bates reagent[7] was evaluated as the coupling reagent but was found to be less convenient. The products obtained from both synthetic routes were shown to be identical by a variety of techniques including gel filtration, NMR at 220 MHz and by electrophoresis of the fully deprotected pentapeptide. Hydrolysis of the



Scheme 1. Synthesis of the protected (50–54) pentapeptide (**66**).

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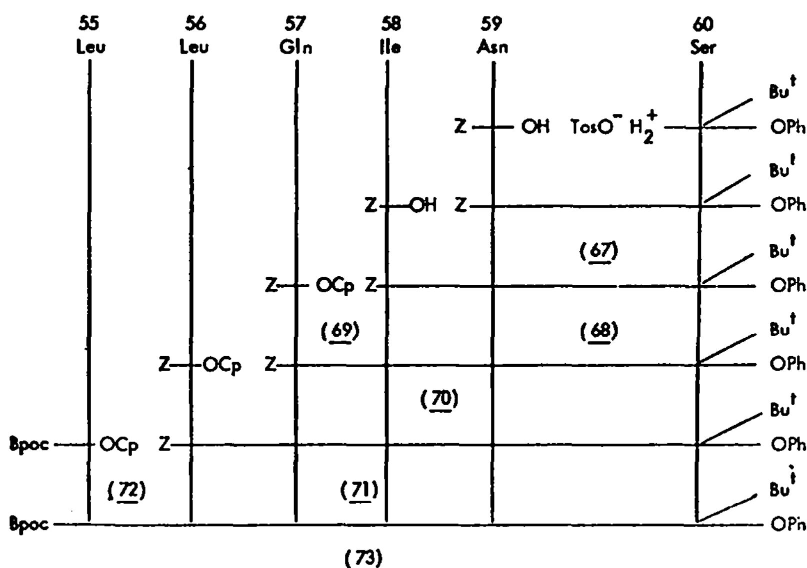
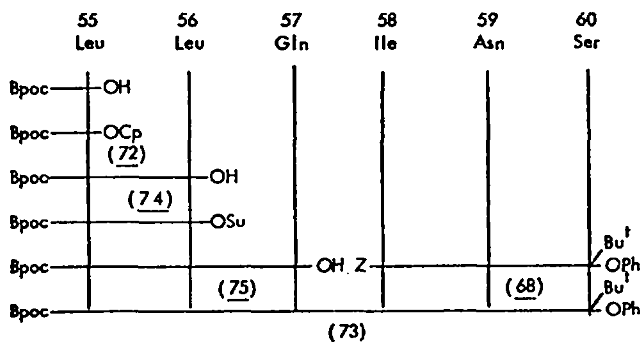
fully protected pentapeptide phenyl ester **65** was readily achieved under our standard conditions[8] employing hydrogen peroxide at pH 10.5, the cleavage being rapidly achieved using acetone as a solvent to give the protected pentapeptide acid **66** which was then subsequently used in the preparation of the larger fragment.

Two alternative schemes were tested for the preparation of the (55–60) hexapeptide. In the first of these, being shown in Scheme 2, the required asparagine and isoleucine derivatives were added to *O-tert*-butyl-serine phenyl ester-*p*-toluene-sulphonate giving the dipeptide **67** and tripeptide **68** respectively. After hydrogenolysis of the tripeptide **68** in the usual way Bpoc.Gln.OCp was coupled to yield the sparingly soluble tetrapeptide **70**. This lack of solubility was manifested during the coupling reaction by precipitation of the product from the DMF solution and led to problems during hydrogenolysis of **70** which had to be performed in a mixture of HMPA and DMF (1.6:1) using a Vibromixer for 3 days in order to achieve complete removal of the *N*-protecting group. Acylation of the *p*-toluene-sulphonate derived from the tetrapeptide **70** gave the even more insoluble pentapeptide **71** which required seven days for complete hydrogenolysis using an HMPA/DMF mixture, again using the Vibromixer. Acylation of this pentapeptide derivative with Bpoc.Leu.OCp **72** gave the slightly more soluble product **73** which could be purified by gel filtration on Sephadex LH20 eluting with DMF. Due to the difficulties encountered using this approach, the alternative synthetic procedure shown in Scheme 3 was explored in which the protected tripeptide fragment **75** was then coupled to the *p*-toluene-sulphonate derived from the tripeptide **68** which was available by the route shown in Scheme 2. In this approach both tripeptide fragments and the resulting hexapeptide **73** could be purified by gel filtration on Sephadex LH20 in the usual way. The identity of the product **73** obtained from both routes was demonstrated by TLC in a variety of systems and by electrophoretic examination of the free peptide at pH 6.5.



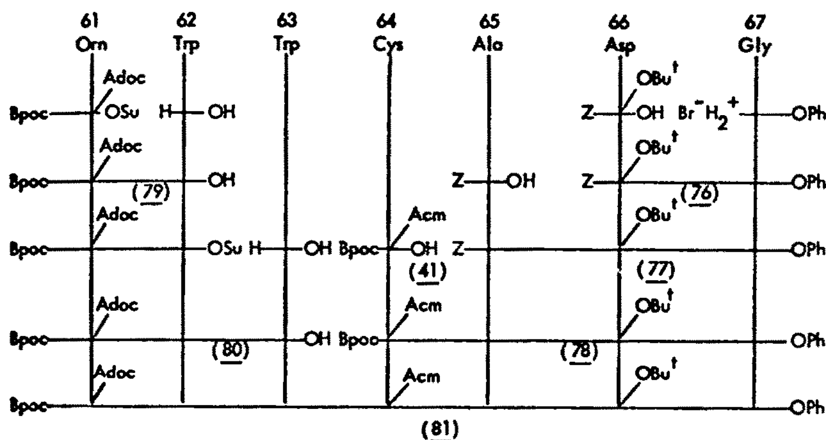
Scheme 2. Synthesis of the protected (55–60) hexapeptide (73).



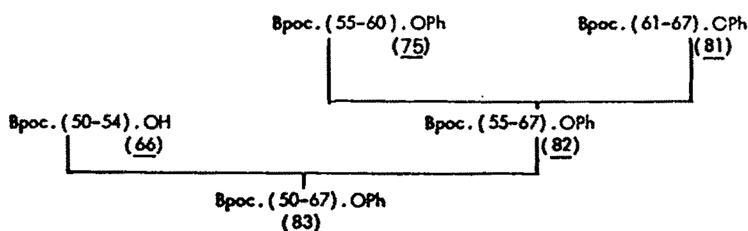
Scheme 3. Salt coupling route to the protected (55–60) hexapeptide (73).

The remaining (61–67) heptapeptide fragment was assembled by the 3 + 4 fragment coupling approach which is shown in Scheme 4. The dipeptide **76** was synthesised using the isobutyl chloroformate mixed anhydride method and then hydrogenolysed in the presence of *p*-toluene-sulphonic acid to give the corresponding *p*-toluene-sulphonate which was subsequently coupled with Z.Ala.OH using the same mixed anhydride method to afford the tripeptide **77** as a crystalline solid. After hydrogenolysis, Bpoc.Cys-(Acm).OH **41** was condensed using the DCCI/HONSu method to give the tetrapeptide **78** which was purified by chromatography on Sephadex LH20. The tetrapeptide **78** has also been prepared using Bates reagent employing a step-wise approach. Again, the product isolated from both synthetic routes was identical. When the addition of the cysteine derivative **41** was attempted using *N*-hydroxybenzotriazole (HOBt) as the additive in place of HONSu it was noted that substantially more impurities were produced suggesting that the HOBt was responsible for the partial removal of the acid-labile *N*-protecting group in accordance with the observation by Rudinger[9] who observed partial cleavage of the acid labile ONps group in the presence of HOBt employed as an additive in coupling reactions. The tripeptide acid **80** corresponding to the (61–63) sequence was most effectively synthesised using successive salt couplings employing hydroxysuccinimide active esters for activation. This approach was employed as it gave rise to a purer product than was obtained when *C*-terminal protection was used. The Bpoc protecting group was initially removed from the amino-terminus of the tetrapeptide **78** using 0.05 molar HCl in dichloromethane. However, the method developed by Ciba-Geigy[10] employing 0.05 molar HCl in 90% trifluoroethanol was subsequently found to be more satisfactory. Coupling of the resulting tetrapeptide hydrochloride to the tripeptide acid **80** by the DCCI/HONSu method gave the crystalline heptapeptide **81** which was isolated by gel filtration on Sephadex LH20.

The synthesis of the (50–54), (55–60) and (61–67) fragments thus provided the components of the (50–67) fragment which were to be assembled according to the sequence shown in Scheme 5. In our earlier synthesis a mixture of acetic acid, formic acid and water (7:1:2), in the presence of dimethylsulphide was used to remove the amino-protecting group from the fully protected fragment **81**. However, this was later superceded by 0.05 molar HCl in trifluoroethanol (90%) to remove the protecting group. Attempts at the cleavage using 0.05 molar HCl in DMF were unsuccessful as the cleavage was incomplete even after 4 hours. The phenyl ester group was removed from the Bpoc.(55–60).OPh fragment **73** by hydrolysis under the standard conditions using 20% aqueous HMPA as solvent leading to complete hydrolysis after 15 minutes. Interestingly the protected hexapeptide acid was found to have much better solubility properties than the corresponding phenyl ester. The (55–60) and (61–67) fragments were then combined using the DCCI/HONSu method and the resulting tridecapeptide **82** was isolated by gel filtration on Sephadex LH20. Cleavage of the amino



Scheme 4. Synthesis of the protected (61–67) heptapeptide (**81**).



Scheme 5. Synthesis of the protected (50–67) octadecapeptide (**83**).

protecting group **82** was effected by treatment with 0.05 molar HCl in trifluoroethanol (90%). The resulting hydrochloride was then coupled with the Bpoc.(50–54).OH **66** using the DCCI/HONSu method to give the Bpoc. (50–67). OPh fragment **83** which was purified by gel filtration on Sephadex LH20 eluting with DMF. In contrast to the component subfragments the peptide **83** had much better solubility properties, which may be due to the additional four *tert*-butyl side chain protecting groups which had been added in the fragment **66**. Electrophoresis and isoelectric focusing of the fully deprotected material obtained from the protected fragment **83** by treatment with 90% trifluoroacetic acid in the presence of mercaptoethanol showed the fragment to be homogeneous.

EXPERIMENTAL

The abbreviations, TLC systems and general experimental methods are those detailed in an earlier paper[2] in this series except for the TLC system (34) $CHCl_3$/MeOH/TFE (45:5:10).

Z.Tyr(Bu^t).Gly.OPh **62**. A solution of Z-Tyr(Bu^t)-OH (12.25 g, 33 mM) in THF (100 ml) was cooled to −18° and NMM (3.34 g, 33 mM) in THF (5 ml) added. Pivaloyl chloride (3.62 g, 30 mM) in THF (8 ml) was added dropwise over 5 min and a further 5 minutes allowed for completion of activation. A pre-cooled solution of $Br^- H_2^+$-Gly-OPh (6.96 g, 30 mM) in DMF (10 ml) was added followed by NMM (3.04 g, 30 mM), and the reaction mixture gradually allowed to warm to room temperature overnight. The solvents were evaporated and the resulting oil dissolved in EtOAc; this solution being washed with 1 M $NaHCO_3$ solution and 1 M HOAc solution. Drying and evaporation yielded a gel which was crystallised from EtOAc/hexane giving **62** (14.14 g, 93%), m.p. 82–84°; $[\alpha]_D^{24}$ + 9.7° (c = 2, $CHCl_3$), R_f(8) − 0.6, R_f(20) − 0.6, $Gly_{1.00}Tyr_{1.01}$. (Found: C, 68.89; H, 6.33; N, 5.56. $C_{29}H_{32}N_2O_6$ requires: C, 69.03; H, 6.39; N, 5.55%.)

Z-Asp(OBu^t)-Tyr(Bu^t)-Gly-OPh **63**. Compound **62** (14.13 g, 28 mM) and Tos.OH.H_2O (5.33 g, 28 mM) were dissolved in DMF (60 ml) and the solution subjected to hydrogenolysis for 6 h in the presence of 10% Pd/C (1.41 g). Filtration through Celite and evaporation yielded the salt as an oil which was used directly in the coupling. Z-Asp-(OBu^t)-OH (10.02 g, 31 mM) and NMM (3.14 g, 31 mM) were dissolved in THF (75 ml) and cooled to −18°. Pivaloyl chloride (3.38 g, 28 mM) was then added dropwise and a total of 10 min allowed for formation of the mixed anhydride. A pre-cooled solution of the salt of the amino component in DMF (15 ml) was added along with NMM (2.83 g, 28 mM) and the reaction mixture warmed to room temperature overnight. Evaporation gave an oil which was dissolved in EtOAc then washed with acid and base. Evaporation of the dried solution gave a gel which was crystallised from EtOAc/hexane giving **63**, (9.2 g, 49%), m.p. 126–127°, $[\alpha]_D^{24}$ − 11.6° (c = 2, $CHCl_3$), R_f(8) − 0.7, R_f(20) − 0.6, $Asp_{1.01}Gly_{1.00}Tyr_{1.00}$. (Found: C, 65.73; H, 6.78; N, 6.13. $C_{37}H_{45}N_3O_9$ requires: C, 65.76; H, 6.71; N, 6.22%.)

Z-Thr(Bu^t)-Asp(OBu^t)-Tyr-(Bu^t)-Gly-OPh **64**. The protected tripeptide **62** (9.13 g, 13.5 mM) and Tos.OH.H_2O (2.55 g, 13.5 mM) were dissolved in DMF (50 ml) and hydrogenolysed for 4 h in the presence of 10% Pd/C (1.0 g) in the usual way. This reaction required careful checking by TLC as a second unidentified product was formed on prolonged reaction. The reaction mixture was filtered through Celite and used directly in the coupling. Z-Thr(Bu^t)-OH (4.49 g, 14.5 mM) and NMM (1.50 g, 14.5 mM) were dissolved in THF (50 ml) and the solution cooled to −18°. Pivaloyl chloride (1.62 g, 13.5 mM) was slowly added and a total of 10 min. allowed for activation. The *p*-toluene-sulphonate of the amino-component was then added followed by NMM (1.49 g, 13.5 mM) and the reaction warmed gradually to room temperature overnight. The solution was evaporated and purified in the usual way to give an oil which was crystallised from EtOAc/hexane giving **64** (8.33 g, 74%), m.p. 106–108°, $[\alpha]_D^{24}$ − 6.3° (c = 2, $CHCl_3$), R_f(2) − 0.5, R_f(20) − 0.6, $Asp_{1.01}$ $Thr_{0.99}$* $Gly_{1.00}$ $Tyr_{1.00}$. (Found: C, 64.68; H, 7.54; N, 6.85. $C_{45}H_{61}N_4O_{12}$ requires: C, 64.89; H, 7.26; N, 6.73%) (*amino acid analysis corrected for degradation).

Bpoc-Ser(Bu^t)-Thr(Bu^t)-Asp(OBu^t)-Tyr(Bu^t)-Gly-OPh **65**. The tetrapeptide *p*-toluene-sulphonate was prepared by hydrogenolysis for 6 h in the usual way using **64** (8.33 g, 10 mM) and Tos.OH.H_2O (1.90 g, 10 mM) in DMF (45 ml) in the presence of 10% Pd/C (850 mg). A solution of Bpoc-Ser(Bu^t)-OH (4.41 g, 11 mM) and NMM (1.11 g, 11 mM) in THF (50 ml) was cooled to −20° and activated over 10 minutes with pivaloyl chloride (1.21 g, 10 mM). The pre-cooled DMF solution from hydrogenolysis was added followed by NMM (1.02 g, 10 mM) the reaction mixture being left to warm to room temperature overnight. Work up in the manner used in preparations of **62–64** gave a yellow foam (9.73 g) which was impure by TLC. Final purification was achieved by gel filtration on Sephadex LH20 eluting with DMF. The product **65** eluted with (Ve/Vt) = 0.46 giving 5.23 g, 48%), m.p. 108–109°, $[\alpha]_D^{24}$ + 6.8° (c = 1, $CHCl_3$), $[\alpha]_D^{24}$ + 8.4 (c = 1, DMF), R_f(2) − 0.6, R_f(20) − 0.6, $Asp_{1.00}Ser^*_{0.99}$ $Thr^*_{1.00}$ $Gly_{1.00}$ $Tyr_{1.00}$, (*corrected for degradation), material deprotected with 90% TFA, $E^{2.1}_{Dnp.Lys}$ 1.3 single spot/ninhydrin. (Found: C, 65.89, H, 7.57; N, 6.67. $C_{60}H_{81}N_5O_{13}$.H_2O requires: C, 65.61; H, 7.62; N, 6.38%.)

Bpoc-(50–54)-OH **66**. The protected phenyl ester **65** (4.3 g, 4 mM) was dissolved in acetone (48 ml) and water (12 ml) added dropwise to the stirred solution. The pH was then brought to 10.5 with NaOH 1 M and 100 vol. H_2O_2 (0.4 ml) added. The hydrolysis was complete in 5 min, with the uptake of NaOH 1 M being 4.3 ml and the solution was cooled to 0° and brought to pH 4 by the addition of 10% citric acid solution. The solid product was extracted into EtOAc and this organic solution washed four times with water. Drying and evaporation yielded a white foam which was crystallised from $CHCl_3$/petroleum ether giving the protected pentapeptide acid **66** (3.88 g, 97%), m.p. 150–155°, $[\alpha]_D^{24}$ − 27.1° (c = 1, DMF), R_f(7) − 0.4, R_f(30) − 0.7. (Found: C, 63.28; H, 7.61; N, 7.25. $C_{54}H_{77}N_5O_{13}$. H_2O requires: C, 63.45; H, 7.59; N, 6.85%.)

Z-Asn-Ser(Bu^t)-OPh **67**. A solution of Z-Asn-OH (3.67 g, 13.8 mM), pyridine (1.09 g, 13.8 mM) and NMM (1.40 g, 13.8 mM) in DMF was cooled to −20° and pivaloyl chloride (1.58 g, 13.1 mM) added. After 10 minutes activation a solution of $TosO^- H_2^+$-Ser(Bu^t)-OPh[8] (5.38 g, 13.1 mM) in DMF (10 ml) was added followed by NMM (1.33 g, 13.1 mM). The reaction was allowed to attain room temperature and stirred overnight, work up in the usual manner and crystallisation from EtOAc/hexane gave **67** (5.21 g, 82%), m.p. 135°, $[\alpha]_D^{24}$ − 10.6° (c = 2, DMF), R_f(2) − 0.4, R_f(20) − 0.2, $Asp_{1.00}Ser_{1.00}$. (Found: C, 62.00; H, 6.51; N, 8.65. $C_{25}H_{31}N_3O_7$ requires: C, 61.84; H, 6.44; N, 8.66%.)

Z-Ile-Asn-Ser(Bu^t)-OPh **68**. Compound **67** (17.4 g, 40 mM) and Tos.OH.H_2O (7.61 g, 40 mM) were dissolved in DMF (50 ml) and hydrogenolysed for 6 h in the usual way in the presence of 10% Pd/C (1.74 g). Z-Ile-OH (11.13 g, 42 mM) and NMM (4.25 g, 42 mM) were dissolved in DMF (60 ml) and cooled to −20°. A solution of pivaloyl chloride (4.82 g, 40 mM) in DMF (10 ml) was then added. After 10 minutes the cooled solution of the *p*-toluene-sulphonate of the amino-component obtained from the hydrogenolysis was added along with NMM (4.00 g, 40 mM), the reaction mixture then being allowed to warm to room temperature overnight. The volume of the reaction mixture was reduced and water added to precipitate the product which was washed with 1 M $NaHCO_3$ and 1 M HOAc solutions, then crystallised from DMF/H_2O yielding **68** (14.39 g, 60%), m.p. 212–215°, $[\alpha]_D^{24}$ − 11.4° (c = 2, DMF), R_f(7) − 0.6, R_f(20) − 0.2, $Asp_{1.01}$-$Ser_{1.00}Ile_{0.97}$. (Found: C, 62.15; H, 6.93; N, 9.16. $C_{31}H_{42}N_4O_8$ requires: C, 62.19; H, 7.07; N, 9.36%.)

Z-Gln-OCp **69**. Z-Gln-OH (7.06 g, 25 mM) and 2,4,5-trichlorophenol (6.17 g, 32 mM) were dissolved in DMF

(30 ml) and cooled to −18°. A solution of DCCI (5.76 g, 27.5 mM) in DMF (25 ml) was added dropwise and the reaction mixture stirred at room temperature overnight. The reaction mixture was cooled to 0° and 2 drops of glacial HOAc added. Filtration and evaporation yielded an oil which was dissolved in EtOAc, after washing with acid and base the solution was concentrated. Spontaneous crystallisation occurred giving the required active ester **69** (8.80 g, 77 %), m.p. 171.5–172°, $[\alpha]_D^{24}$ − 17.5° (c = 1, DMF) $R_f(2)$ − 0.3. (Found: C, 49.86; H, 3.76; N, 6.03. $C_{19}H_{17}N_2Cl_3O_5$ requires: C, 49.64, H, 3.73, N, 6.09 %.)

Z-Gln-Ile-Asn-Ser(Bu^t)-OPh **70**. The protected tripeptide **69** (11.49 g, 19 mM) and Tos.OH.H_2O (3.65 g, 19 mM) were dissolved in DMF (160 ml) and hydrogenolysed for 12 h in the presence of 10 % Pd/C (1.15 g). The solution was filtered and the active ester **69** (12.20 g, 19.2 mM) and di-isopropylethylamine (2.48 g, 19.2 mM) was added. The reaction mixture was stirred for 4 h at 37° and for 2 days at room temperature, during which time the peptide began to precipitate from solution. The resulting suspension was poured into a mixture of Et_2O/H_2O (1:1) and the precipitated white solid collected and washed thoroughly with IPA and Et_2O. The product was recrystallised from DMF/H_2O giving (12.51 g, 89 %), m.p. 270° (dec), $[\alpha]_D^{24}$ − 25.3° (c = 1, HMPA), $R_f(34)$ − 0.6, $Asp_{1.01}Glu_{1.02}Gly_{1.00}Ile_{0.96}$. (Found: C, 59.13; H, 6.97; N, 11.36. $C_{36}H_{50}N_6O_{10}$ requires: C, 59.49; H, 6.93; N, 11.56 %.)

Z-Leu-Gln-Ile-Asn-Ser(Bu^t)-OPh **71**. The protected peptide **70** (12.50 g, 17.2 mM) and Tos.OH.H_2O (3.28 g, 17.2 mM) were dissolved in HMPA (160 ml) and 10 % Pd/C (1.5 g) added. Hydrogenolysis was carried out over 3 days using a Vibromixer. Addition of an equal volume of MeOH and filtration through Celite gave a clear solution of the *p*-toluene-sulphonate. A mixture of water and ether was added to precipitate the flocculent product which was filtered and dried. A microanalysis for sulphur confirmed the completion of the hydrogenolysis. (Found: 4.25 %, calculated for the *p*-toluene-sulphonate 4.18 %.) This salt (13.01 g, 17.01 mM) was dissolved in DMF (75 ml) and Z-Leu-OCp (9.15 g, 20.6 mM) and di-isopropylethylamine (2.21 g, 17.01 mM) added. The reaction mixture was stirred at room temperature and after about 30 min the formation of a solid was observed. After 4 days the reaction mixture was poured into a mixture of Et_2O/H_2O to complete precipitation. The solid was washed with IPA and Et_2O then crystallised from DMF/H_2O giving the product **71** (12.94 g, 91 %), m.p. 280–285° (dec), $[\alpha]_D^{24}$ − 25.3° (c = 1, HMPA), $R_f(34)$ − 0.6, $Asp_{1.00}Ser_{0.99}{}^*Glu_{1.03}Leu_{1.01}Ile_{0.97}$ (*corrected for degradation). (Found: C, 58.95; H, 7.20; N, 11.42. $C_{42}H_{61}N_7O_{11}.H_2O$ requires: C, 58.79; H, 7.40; N, 11.43 %.)

Bpoc-Leu-OCp[12] **72**. Bpoc-Leu-OH (7.70 g, 21 mM) and 2,4,5-trichlorophenol (4.34 g, 22 mM) were dissolved in EtOAc (40 ml). The solution was cooled to −5° and a solution of DCCI (4.54 g, 22 mM) in EtOAc (20 ml) added over 10 min. After stirring overnight the reaction mixture was filtered and the filtrate evaporated to give an oil which was triturated with petroleum ether then crystallised from Et_2O/petroleum ether to give the active ester **72** (11.0 g, 82 %) m.p. 109–110°, $[\alpha]_D^{23}$ − 30.0° (c = 2, $CHCl_3$), $R_f(2)$ − 0.8. (Found: C, 61.39; H, 5.37; N, 2.56. $C_{28}H_{28}NCl_3O_4$ requires: C, 61.29; H, 5.14; N, 2.55 %.)

Bpoc-Leu-Leu-Gln-Ile-Asn-Ser(Bu^t)-OPh, Bpoc.(55–60).OPh **73**. The protected pentapeptide **71** (13.40 g, 16 mM) and Tos.OH.H_2O (3.04 g, 16 mM) were dissolved in a mixture of HMPA (200 ml) and DMF (100 ml). 10 % Pd/C (1.5 g) was added and the compound hydrogenolysed for 7 days with a Vibromixer providing efficient mixing, additional catalyst (0.8 g) was added after 2 days. Filtration through Celite and partial evaporation of solvent gave an HMPA/DMF solution which was used directly in the subsequent coupling. Bpoc-Leu-OCp **72** (10.12 g, 18.5 mM) was added to the above solution followed by a solution of di-isoproylethylamine (1.94 g, 15 mM) in DMF (5 ml). The reaction mixture was stirred for 4 days at room temperature and at 37° for 12 h. The reaction mixture was poured into a mixture of Et_2O/H_2O (1:1) giving the crude product (13.21 g). This material was chromatographed on Sephadex LH20 eluting with DMF giving the purified **73** with (Ve/Vt) = 0.44 (9.61 g, 61 %), m.p. 230° (dec), $[\alpha]_D^{24}$ − 16.4° (c = 0.5, HMPA), $R_f(34)$ − 0.5, $Asp_{1.00}Ser_{0.99}{}^*Glu_{1.01}Leu_{2.00}Ile_{0.99}$ (*corrected for degradation), the fully deprotected material showed a single spot on electrophoresis $E^{6.5}_{Dnp.Lys}$ 1.1. (Found: C, 61.95; H, 7.80; N, 10.21. $C_{56}H_{80}N_8O_{12}$. $2H_2O$ requires: C, 61.52; H, 7.74; N, 10.25 %.)

Bpoc-Leu-Leu-OH **74**. Leucine (8.99 g, 68.5 mM) and TEA (6.94 g, 68.5 mM) were dissolved in DMF (250 ml) and Bpoc-Leu-OCp **72** (30.0 g, 54.5 mM) and HONSu (6.29 g, 55 mM) added. The reaction was stirred overnight at room temperature then excess leucine removed by filtration. Evaporation of the solvent yielded a gum which was dissolved in EtOAc and this solution was washed with 10 % citric acid and four times with water, then dried and evaporated. The resulting oil was crystallised from E OAc/petroleum ether giving **74** (22.0 g, 84 %), m.p. 98–100°, $[\alpha]_D^{22}$ − 35.5° (c = 1.1, $CHCl_3$), $R_f(30)$ − 0.6. (Found: C, 69.68; H, 7.81; N, 5.91. $C_{28}H_{38}N_2O_5$ requires: C, 69.68; H, 7.94; N, 5.81 %.)

Bpoc-Leu-Leu-Gln-OH **75**. Compound **74** (13.0 g, 27 mM) was dissolved in dimethoxyethane (500 ml) and cooled to −20°. HONSu (6.25 g, 54 mM) and DCCI (5.63 g, 27 mM) were added and the reaction stirred at 0° for 2 h then at room temperature overnight. The DCU was removed by filtration and the solvent evaporated to give an oil. This oil was dissolved in DMF (500 ml) and the solution cooled to 0°. A suspension of glutamine (3.9 g, 27 mM) in DMF (250 ml) was then added along with 1 M NaOH (27 ml), the reaction mixture was then stirred for 2 days at room temperature. The reaction mixture was filtered and 3-dimethylaminopropylamine (2 ml) added, the resulting solution being stirred for 2 h. The DMF was evaporated and the resulting solid washed with water and dissolved in EtOAc. This solution was washed with 10 % citric acid, water, and brine, and was then dried and evaporated. The resulting white foam (16.25 g) was shown to contain a minor impurity by TLC, thus the whole of the product was chromatographed on Sephadex LH20, the pure product **75** eluted (DMF) with (Ve/Vt) = 0.51, isolation gave the product as a dry foam (11.6 g, 70.5 %), $R_f(2)$ − 0.6, m.p. of DCHA salt) 122–124° $[\alpha]_D^{22}$ (of DCHA salt) − 27.0° (c = 3.4, $CHCl_3$). (Found: C, 66.54; H, 8.54; N, 8.37. $C_{45}H_{69}N_5O_7.H_2O$ (DCHA salt) requires: C, 66.71; H, 8.83; N, 8.65 %.)

Bpoc.(55–60).OPh **73**; (*second route*). The protected tripeptide **68** (10.11 g, 17 mM) and Tos.OH.H_2O (3.22 g, 17 mM) were dissolved in DMF (200 ml). 10 % Pd/C (2 g) was added and the mixture hydrogenolysed for 12 h, the reaction mixture then being filtered to give a solution which was evaporated and the resulting oil used directly in the subsequent coupling. The carboxyl component, **75** (10.14 g, 17 mM) and HONSu (3.78 g, 33 mM) were dissolved in DMF/HMPA (1:1) (80 ml) and the solution cooled to −20°. DCCI (3.41 g, 17 mM) and di-isopropylethylamine were then added and the reaction mixture stirred for 2 days at room temperature. The resulting viscous solution was added to water and the precipitate which formed was washed with warm IPA. The material was chromatographed on Sephadex LH20 eluting with DMF giving the product (Ve/Vt) = 0.45 (10.36 g, 60 %), m.p. 229° (dec), $[\alpha]_D^{23}$ − 18.0° (c = 0.5, HMPA), $R_f(34)$ − 0.5, $Asp_{1.05}Ser^*_{0.96}Glu_{1.02}Leu_{1.98}Ile_{0.96}$ (*corrected for degradation). (Found: C, 62.36; H, 7.49; N, 10.70. $C_{56}H_{80}N_8O_{12}.H_2O$ requires: C, 62.55; H, 7.67; N, 10.42 %.)

Z-Asp(OBu^t)-Gly-OPh **76**. This protected dipeptide was prepared by the isobutoxycarbonyl mixed anhydride method by the procedure described in the earlier paper in this series.

Z-Ala-Asp(Bu^t)-Gly-OPh **77**. Compound **76** (11.59 g, 25.4 mM) and Tos.OH.H_2O (4.81 g, 25.4 mM) were dissolved in DMF (50 ml), 10 % Pd/C (1.16 g) was added and the mixture was hydrogenolysed for 4 h. Filtration and evaporation gave a yellow oil which was used directly in the coupling reaction. Z-Ala-OH (6.23 g, 28 mM) and NMM (2.7 g, 27 mM) were dissolved in DMF (30 ml) and cooled to −20°. IBC (3.64 g,

27 mM) was added dropwise and 10 min were allowed for activation. The above *p*-toluene-sulphonate (12.51 g, 25 mM) was dissolved in DMF (35 ml) and the cooled solution added to the solution of the mixed anhydride, followed by NMM (2.57 g, 25 mM). After warming to room temperature the solvent was removed *in vacuo* and the residue dissolved in EtOAc. Washing, drying and evaporation in the usual way gave a yellow oil which was crystallised twice from EtOAc/hexane giving the product **77** (8.81 g, 66%), m.p. 125.5–126°, $[\alpha]_D^{23}$ – 30.3° (c = 2, MeOH), $R_f(2)$ – 0.7, $R_f(22)$ – 0.3, $Asp_{1.00}Gly_{1.00}Ala_{1.00}$. (Found: C, 61.24; H, 6.38; N, 7.99. $C_{27}H_{33}N_3O_8$ requires: C, 61.47; H, 6.31; N, 7.97%.)

Bpoc-Cys(Acm)-Ala-Asp(OBu^t)-Gly-OPh **78**. The protected peptide **77** (9.25 g, 17.5 mM) and Tos.OH.H_2O (3.33 g, 17.5 mM) were dissolved in DMF (60 ml) and hydrogenolysed in the presence of 10% Pd/C (0.9 g) for 6 h. Work-up gave the salt as a crystalline solid (8.75 g, 94%), m.p. 160.5–161°. Bpoc-Cys-(Acm)-OH (6.33 g, 14.7 mM) and the *p*-toluene-sulphonate (8.32 g, 14.7 mM) were dissolved in DMF (50 ml) and cooled to –20°, NMM (1.49 g, 14.7 mM) and HONSu (1.86 g, 16.2 mM) were added along with a pre-cooled solution of DCCI (3.64 g, 17.6 mM) in DMF (25 ml) and the reaction was permitted to warm to room temperature overnight. The solvent was removed and the residue was dissolved in EtOAc and DCU was removed by filtration. Drying and evaporation yielded a yellow solid which was crystallised from EtOAc/hexane giving **78** (10.0 g, 84%), m.p. 132.5–133.5°, $R_f(2)$ – 0.5, $R_f(22)$ – 0.2. (Found: C, 60.37; H, 6.45; N, 8.52. $C_{41}H_{51}N_5O_{10}.\frac{1}{2}H_2O$ requires: C, 60.42; H, 6.43; N, 8.59%.)

Bpoc-Orn(Adoc)-Trp-OH **79**. Tryptophan (5.71 g, 28 mM) was dissolved in a mixture of 1 M NaOH solution (28 ml) and DMF (30 ml) and a solution of Bpoc-Orn(Adoc)-ONSu (18.12 g, 28 mM) in DMF (50 ml) added. The pH dropped from 9.5 to 6.5 during the 12 h reaction period. The pH was adjusted to 4 by the addition of 2 M HCl and the DMF evaporated to yield a white foam which was dissolved in EtOAc, washed with water, dried and evaporated to yield a white foam which was triturated with petroleum ether to produce **79** as a white powder (16.8 g, 82%), m.p. 98.5–100° (dec), $[\alpha]_D^{24}$ + 22.2° (c = 2, EtOAc), $R_f(7)$ – 0.6, $R_f(13)$ – 0.3, $Orn_{1.00}Trp_{0.99}$. (Found: C, 69.31, H, 7.01; N, 6.96. $C_{43}H_{50}N_4O_7.\frac{1}{2}H_2O$ requires: C, 69.43; H, 6.90; N, 7.53%.)

Bpoc-Orn(Adoc)-Trp—Trp-OH **80**. Compound **79** (16.8 g, 23 mM) was dissolved in dimethoxyethane (150 ml) and cooled to –18°. HONSu (5.3 g, 46 mM) in DMF (10 ml) and DCCI (5.6 g, 27 mM) were then added and the solution allowed to reach room temperature overnight. The reaction mixture was filtered and evaporated, the resulting oil being dissolved in EtOAc. This solution was washed rapidly with ice-cold 1% $NaHCO_3$ and water then dried and evaporated to yield a pale yellow foam (19.3 g, 97%). This active ester (18.6 g, 22 mM) in DMF (50 ml) was then added to a solution of tryptophan (4.1 g, 20 mM) in 1 M NaOH (20 ml) and DMF (20 ml) and the reaction mixture stirred for 24 h. The pH was reduced to 6.5 and the solvent evaporated to produce a residue which was suspended in EtOAc; this suspension (the sodium salt) was filtered and washed with EtOAc and Et_2O. The free acid was then obtained by partitioning the salt between ice-cold 10% mineral acid and EtOAc and the organic phase containing the product was washed with water and dried. Evaporation gave the protected tripeptide acid **80** as a white amorphous powder (15.6 g, 85%), m.p. 151–154°, $[\alpha]_D^{24}$ – 13.1° (c = 2, EtOAc), $R_f(7)$ – 0.6, $R_f(13)$ – 0.6, $Orn_{1.00}$ $Trp_{1.97}$. (Found: C, 68.69; H, 6.72; N, 9.23. $C_{54}H_{59}N_6O_8.H_2O$ requires: C, 69.13; H, 6.55; N, 8.95%.)

Bpoc.(61–67).OPh **81**. The protected tetrapeptide **78** (8.01 g, 10 mM) and DMS (20 ml) were dissolved in Ch_2Cl_2 (300 ml), 10 mM) and DMS (20 ml) were dissolved in CH_2Cl_2 (300 ml), stirred at room temperature for 20 min. Evaporation gave the corresponding hydrochloride salt of the tetrapeptide as a white solid (6.0 g, 98%). This hydrochloride (4.2 g, 7 mM) and the tripeptide acid (6.78 g, 8 mM) were dissolved in DMF (40 ml) and cooled to –20°. Dry nitrogen was bubbled through the stirred solution, when HONSu (1.8 g, 16 mM) and NMM (708 mg, 7 mM) were added followed by a solution of DCCI (1.98 g, 9.6 mM) in DMF (10 ml). The reaction was allowed to attain room temperature and was then stirred for a further 4 days. The solution was then cooled and the DCU removed by filtration; evaporation gave a residue which was purified initially by crystallisation from EtOAc/acetone/hexane (3:1:2) and subsequently by gel filtration on Sephadex LH20 eluting with DMF. The product **81** had (Ve/Vt) = 0.40 and isolation gave (6.15 g, 60%), m.p. 198–201°, $[\alpha]_D^{23}$ – 22.9° (c = 2, DMF), $R_f(2)$ – 0.40, $R_f(20)$ – 0.2, $Orn_{1.01}$ $Trp_{1.97}$ $Asp_{1.00}Gly_{1.00}Ala_{1.00}$, electrophoresis of the totally deprotected material gave a single spot $E_{Dnp.Lys}^{6.5}$ 1.1. (Found: C, 63.28; H, 6.67; N, 10.13. $C_{79}H_{93}N_{11}O_{15}S.2H_2O$ requires: C, 63.05; H, 6.50; N, 10.23%). Material obtained by an alternative route using intermediate phenyl ester protection in the synthesis of **80** had m.p. 197–200°, $[\alpha]_D^{23}$ – 22.8° (c = 1, DMF) and (Ve/Vt) (LH20/DMF) = 0.40.

Bpoc.(55–67).OPh **82**. (a) Bpoc cleavage from **81** (5.8 g, 4 mM) and DMS (8 ml), dissolved in TFE/H_2O (9:1) (50 ml) and the pH adjusted to 0.5 with 0.05 M HCl in 90% TFE, gave the hydrochloride (4.9 g, 97%) after 1 h (solvent was evaporated and the resulting solid washed with Et_2O).

(b) Phenyl ester cleavage from **73** (3.38 g, 3.2 mM), dissolved in HMPA (80 ml) at 37° and water (20 ml) added, the pH was then brought to 10.5 by the addition of 1 M NaOH, by H_2O_2 100 vol (0.35 ml) and addition of 1 M NaOH to maintain the pH at 10.5. After 15 min, base uptake ceased and the solution was cooled to 0° and the pH brought to 3.5 with 10% citric acid. The product precipitated as a fine solid and was isolated by centrifugation; washing with water and drying gave the peptide acid (2.92 g, 93%).

(c) Bpoc.(55–60).OH (3.92 g, 4 mM) and $Cl^-H_2^+$.(61–67).OPh (4.61 g, 3.6 mM) were dissolved in a mixture of HMPA (30 ml) and DMF (25 ml) and cooled to –18°. NMM (368 mg, 3.6 mM) and HONSu (920 mg, 8 mM) were added followed by a solution of DCCI (866 mg, 4.2 mM) in DMF (6 ml). The reaction warmed to room temperature overnight, and was cooled to –18° for a second addition of HONSu (460 mg, 4 mM) and DCCI (433 mg, 2.1 mM). After a further 5 days at room temperature the mixture was cooled to –10° and the DCU removed by filtration. The filtrate was then applied to Sephadex LH20 eluting with DMF, the product **82** had (Ve/Vt) = 0.39 and gave (5.01 g, 58%), m.p. > 340°, $[\alpha]_D^{23}$ – 50.8° (c = 2, DMF), $R_f(34)$ – 0.6, $R_f(7)$ – 0.7, electrophoresis of the fully deprotected peptide showed a single spot $E_{Dnp.Lys}^{2.1}$ 1.2, $Orn_{1.01}Trp_{1.97}Asp_{2.03}Ser^*_{0.99}Glu_{1.02}$-$Gly_{0.99}Ala_{1.00}Ile_{0.98}Leu_{2.02}$ (*corrected for degradation). (Found: C, 59.88; H, 7.13; N, 11.66. $C_{113}H_{156}N_{19}O_{24}S.4H_2O$ requires: C, 59.82; H, 7.28; N, 11.73%.)

Bpoc.(50–67).OPh **83**. (a) The protected peptide **82** (3.5 g, 1.6 mM) and DMS (3 ml) were dissolved in TFE/H_2O (9:1) (25 ml) and the pH was adjusted to, and maintained at, 0.5 by the addition of 0.05 M HCl in 90% TFE. After 1 h the solvent was evaporated to give a white solid. This was triturated with Et_2O, and dried giving (3.2 g, 99%) and used directly in the coupling reaction.

(b) The peptide acid **66** (3.2 g, 3.2 mM) and the hydrochloride from (a) (3.2 g, 1.6 mM) were dissolved in a mixture of HMPA (20 ml) and DMF (30 ml) and cooled to –10°. NMM (162 mg, 1.6 mM) and HONSu (368 mg, 3.2 mM) were added followed by a solution of DCCI (660 mg, 3.2 mM) in DMF (3 ml). After 2 h at room temperature the solution was cooled and further portions of HONSu (368 mg, 3.2 mM) and DCCI (330 mg, 1.6 mM) added, the reaction mixture being stirred for 4 days. The reaction mixture was then directly applied to Sephadex LH20 and eluted with DMF, the fractions corresponding to the peak at (Ve/Vt) = 0.35 were evaporated giving the product **83** (3.0 g, 65%), m.p. > 340°, $[\alpha]_D^{19}$ – 27.4° (c = 1, HMPA), $R_f(34)$ – 0.6, $R_f(3)$ – 0.7, $R_f(23)$ – 0.7, $Orn_{1.01}Trp_{1.96}Asp_{2.97}Thr_{0.75}Ser_{1.47}Glu_{1.02}$ $Gly_{1.94}Ala_{1.09}Ile_{0.97}Leu_{2.00}Tyr_{0.84}$. (Found: C, 60.02; H, 7.25; N, 11.18. $C_{151}H_{214}N_{24}O_{34}S.4H_2O$ requires: C, 60.18; H, 7.42; N, 11.15%.)

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