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PEPTIDES—XXXXII

SYNTHESIS OF THE 76–93 FRAGMENT OF A LYSOZYME ANALOGUE

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Abstract—The synthesis of the (76–93) fragment of a lysozyme analogue was achieved using a fragment condensation approach employing the protected subfragments (76–79), (80–86), and (87–93). The utility of Bates' reagent in conjunction with *N*-hydroxysuccinimide was examined for the linking of fragments. The resulting protected peptide (76–93) was found to be one of the most insoluble encountered in this whole programme directed towards the synthesis of a lysozyme analogue.

Several preceding papers have described the preparation of two of the major fragments (1–37)[1] and (38–75)[2] of a lysozyme analogue. The series of papers now presented relate to the preparation of the fragments constituting the remaining (76–129) portion of the analogue employing tactics which were outlined at the onset of the researches.[3] We now describe the preparation of the (76–93) sub-fragment having the sequence shown below.

Cys. Asn. Ile. Pro. Cys. Ala. Ala. Leu. Nva. Ser.
76 79 80 82 83

Gly. Asp. Ile. Thr. Ala. Ser. Val. Gly.
86 87 93

Proline-79 and glycine-86 were chosen as the main fragmentation points, as coupling at these residues would not be subject to the risk of racemisation. Although initially it might appear that the (80–86) (87–93) heptapeptides would both require preparation by the fragment coupling method, in the event only the former peptide was so prepared; the latter heptapeptide being prepared by stepwise elongation from the C-terminus.

Initial attempts at the synthesis of the (76–79) tetrapeptide were made using the phenyl ester group[4] for protection of the proline-79 carboxyl function. Coupling with *Z*-isoleucine using the hydroxysuccinimide ester,[5] the isobutylchloroformate mixed anhydride method[6] or the Bates reagent[7] were not entirely successful as proline phenyl ester tends to cyclise to the dixopiperazine quite rapidly when it is liberated from the salt. Similarly, when coupling of *Z*-asparagine to isoleucylproline phenyl ester was attempted, cyclisation of the dipeptide phenyl ester was a major side reaction.

The use of proline benzyl ester[8] was then investigated as an alternative. Although better results were obtained, problems again were found due to the propensity towards dioxopiperazine formation. In addition, during mixed anhydride coupling, non regiospecific opening of the isobutylchloroformate mixed anhydride was observed. These findings, which are in agreement with those of other workers,[9] led us to consider a salt coupling approach as coupling of Boc-isoleucine hydroxysuccinimide ester with a sodium salt of proline had been found to give a high yield of the protected dipeptide acid.[9] The successful synthetic route to the protected (76–79) tetrapeptide acid is shown in Scheme 1.

Z-Isoleucine-*N*-hydroxysuccinimide ester was coupled with proline (20% excess) employing DMF as the solvent and triethylamine as the base. The resulting crystalline acid (**96**)† being obtained in 82% yield. After hydrogenolysis of compound (**96**) in the presence of *p*-toluenesulphonic acid, coupling was attempted with

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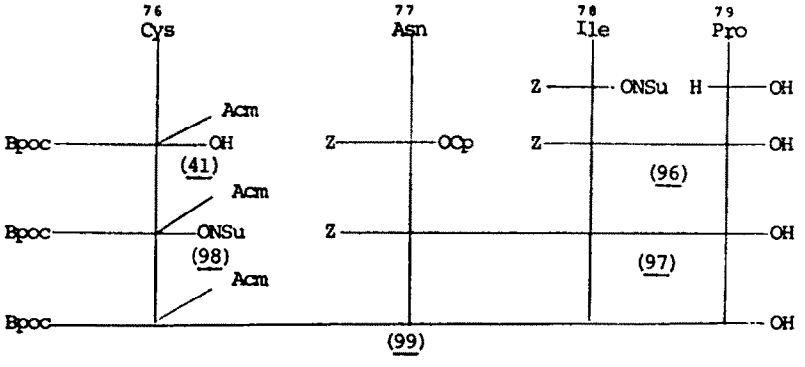
†The compound numbering sequence follows that established in earlier papers in this series.



Scheme 1. Synthesis of the protected (76–79) tetrapeptide (**99**).

Z-asparagine using the 2,4,5-trichlorophenyl ester[10] or the hydroxysuccinimide ester.[11] The yields using these two active esters were 65% and 72% respectively, however, the latter ester required much more careful handling due to its ease of decomposition to the corresponding imide. The trichlorophenyl ester method was therefore preferred as the ester was easier to prepare and the product (**97**) was easier to isolate even though the yield was slightly lower.

Hydrogenolysis gave the free tripeptide which was then coupled to the cysteine active ester (**98**) which had in turn been prepared from Bpoc-acetamidomethylcysteine (**41**)[12] using DCCI and hydroxysuccinimide. Coupling proceeded smoothly using triethylamine as the base giving a 79% yield of the protected tetrapeptide (**99**), after gel filtration on Sephadex LH-20 with DMF as eluant.

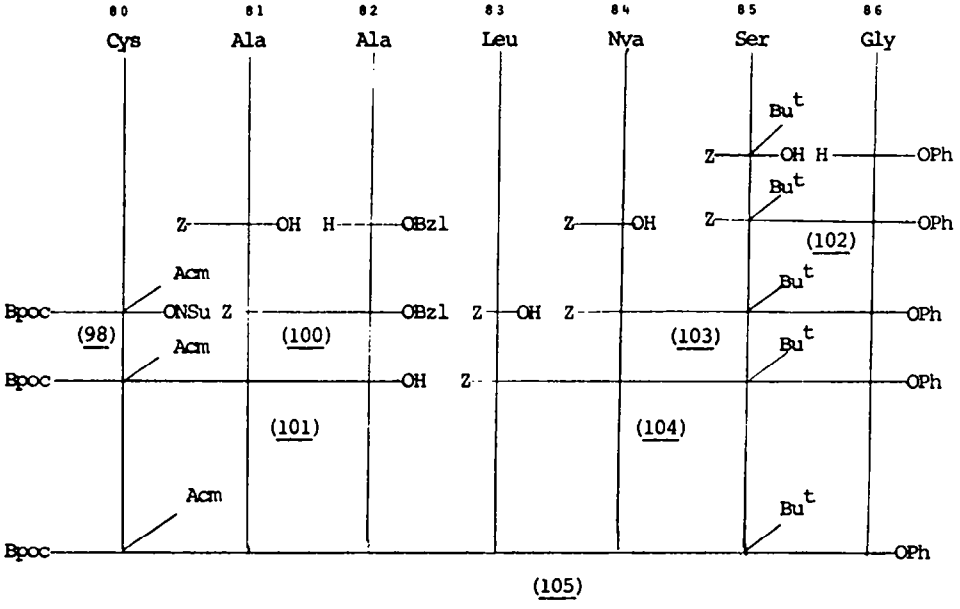
The synthesis of the (80–86) fragment was first investigated using a stepwise approach, however solubility problems were encountered at the tetrapeptide stage. It was for this reason that the alternative fragment condensation approach was examined. In this case either a 2 + 5 or 3 + 4 route was feasible using the materials which had already accumulated in the stepwise approach. In the event the 3 + 4 route was preferred, and Scheme 2 below illustrates the synthesis using this route.

Initially the preparation of Z-alanylalanine was envisaged as the first stage in a salt coupling approach to the (80–82) tripeptide. However, it was found that, when Z-alanine hydroxysuccinimide ester was coupled with alanine in the presence of triethylamine, some tripeptide was also generated. This was proven using mass spectroscopy which showed the presence of the tripeptide in the reaction product. Such a side reaction is probably the result of a reaction treatment similar to that shown in Scheme 3.

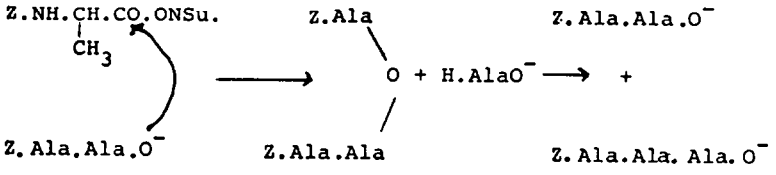
As a result of this finding an alternative approach was chosen in which Z-alanine was coupled to alanine benzyl ester by the pivalic mixed anhydride method. The resulting fully protected dipeptide (**100**) was then hydrogenolysed and coupled with the cysteine active ester (**98**) to give the protected tripeptide acid (**101**).

An alternative synthesis employing phenyl ester protection was abandoned as it was found that the peroxide catalysed cleavage of the protected (80–82) tripeptide phenyl ester gave two components. The impurity in this case derived from oxidation of the acetamidomethylcysteine residue. This is the only occasion of which this type of side reaction has been encountered and model building suggests that in this case it may be due to the proximity of the carboxylate group to the sulphur atom providing some catalytic effect. Certainly, at later stages in the synthesis of the (76–93) fragment no further evidence for this type of oxidation was found.

The synthesis of the dipeptide (**102**) could be achieved in two ways; either by active ester coupling using the 2,4,5-trichlorophenyl active ester or by using the pivalic mixed anhydride method. The latter method proved to be superior in giving higher yields of the product and not requiring the isolation of an intermediate active ester.



Scheme 2. Synthesis of the protected (80–86) heptapeptide (**105**).



Scheme 3. Formation of tripeptide during salt coupling.

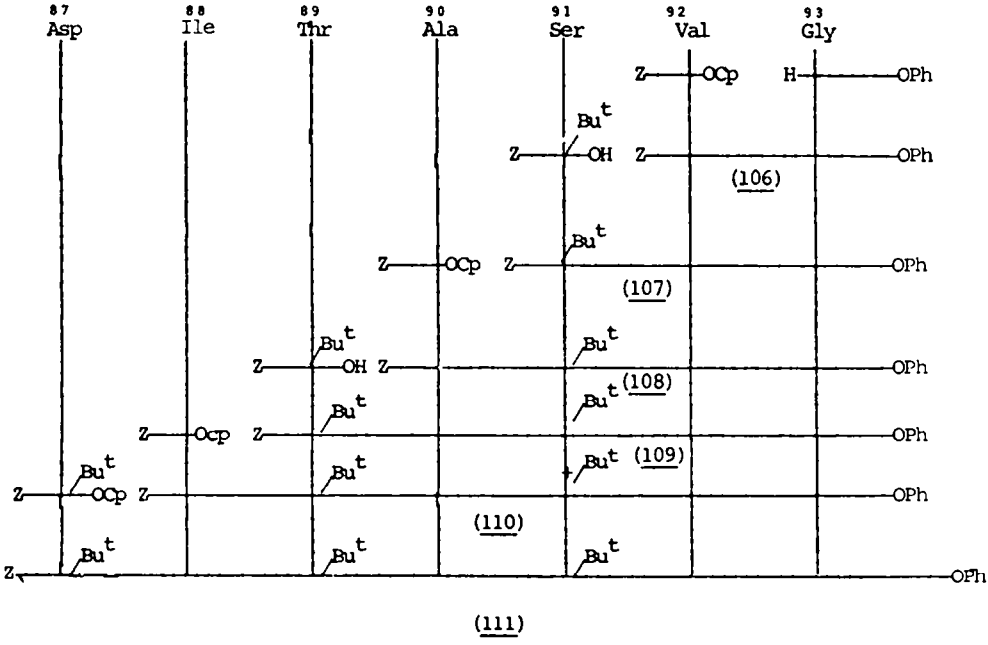
Hydrogenolysis of the protected dipeptide (**102**) followed by coupling to *Z*-norvaline was again most satisfactorily achieved using the pivalic mixed anhydride method. Extension to the tetrapeptide was carried out using the pivalic mixed anhydride of *Z*-leucine which gave a comparable yield to that obtained via the active ester method but did not require isolation of intermediates. The required (80–86) heptapeptide (**105**) was then obtained by DCCI/hydroxysuccinimide coupling using gel filtration on Sephadex LH-20 eluting with DMF as a means of purification. The product which was obtained in 70% yield gave only one product when subjected to phenyl ester cleavage conditions using hydrogen peroxide as catalyst. This acted to confirm that the (80–82) tripeptide was especially susceptible to oxidation as clearly no analogous oxidation was observed for the whole (80–86) fragment.

The (87–93) heptapeptide was prepared by stepwise approach without complications according to the plan outlined in Scheme 4.*Z*-Valine was condensed with glycine phenyl ester to give a 68% yield of the protected dipeptide (**106**) which was coupled after hydrogenolysis to *Z-O*-tert-butyl serine using the mixed anhydride derived from isobutyl chloroformate. Using this approach a 69% yield was obtained and no diketopiperazine was observed in the reaction mixture as the amino terminus was rapidly acylated. Hydrogenolysis of the tripeptide (**107**) followed by active ester coupling gave the tetrapeptide (**108**). Extension to the pentapeptide was again best achieved using the isobutyl chloroformate mixed anhydride which gave an 87% yield of the pentapeptide (**109**). Addition of isoleucine-88 and tert-butyl aspartic acid-87 were achieved by the combination of hydrogenolysis and active ester couplings to produce the fully protected heptapeptide (**111**) in 92% yield after crystallisation.

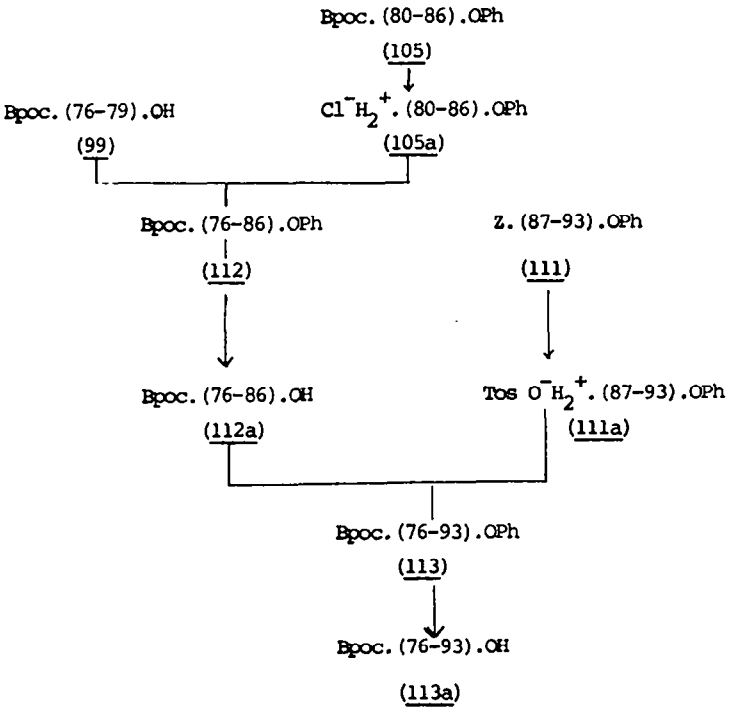
Having assembled the three constituent fragments the method of fragment condensation must be considered. It would be possible to combine the (76–79) fragment with the (80–86) fragment and then to couple the (87–93) portion or to form the 86/87 bond and then to subsequently couple the (76–79) fragment to the resulting (80–93) portion. Trial experiments indicated that the former method would be most satisfactory. DCCI/hydroxysuccinimide coupling of the (80–86) fragment with the (87–93) portion gave a 45% yield of a product which was totally insoluble in DMF. However, the alternative successful approach outlined in Scheme 5 gave an intermediate fragment which could more easily be purified.

A variety of coupling methods were studied using the tetrapeptide free acid (**99**) and the hydrochloride of the (80–86) fragment (**105a**). This hydrochloride was obtained by Bpoc cleavage using 0.5 M hydrogen chloride in DMF or by treatment with acetic acid/formic acid/water (7:1:2), although the latter method gave a purer product after the anion had been exchanged to hydrochloride prior to coupling. The coupling conditions explored are shown in Table 1, showing that coupling proceeds more efficiently when a large excess of the carboxyl component is present. Although the table indicates that the diphenylphosphinic mixed anhydride method gives the highest yield, the method was not used in the final preparation as a trace of impurity was present in the final product (**112**) and at this time the use of this reagent had not been firmly established. It was found that the most successful method of combination was to use the Bates reagent[7] in the presence of *N*-hydroxysuccinimide as an additive, using a 50% excess of the carboxyl component. This gave the protected undecapeptide (**112**) in a yield of 62%.

Removal of the phenyl ester protecting group at glycine-86 was readily achieved in 30 min using the standard hydrogen peroxide catalysed base cleavage, which gave the free acid (**112a**). Hydrogenolysis of the benzyloxycarbonyl function of the protected heptapeptide (**111**) gave the *p*-toluenesulphonate (**111a**) whereupon the coupling between these two fragments was then examined using the DCCI/hydroxysuccinimide method



Scheme 4. Synthesis of the protected (87–93) heptapeptide (**111**).



Scheme 5. Fragment combination route to Bpoc.(76-93).OH (**113a**).

and the Bates/hydroxysuccinimide method. The latter method was preferred as the by-products appeared to be more easily removed and the resulting phenyl ester (**113**) was found to be highly insoluble even in warm DMF. Purification could only be achieved by gel filtration using Sephadex G-25 eluting with hexamethylphosphoramide containing 5% water.[13] However, it appeared that washing the product on a sinter with a variety of solvents gave a comparable product in 83% yield. The highly insoluble Bpoc. (76–93). OPh (**113**) was then subjected to phenyl ester cleavage. In this case trifluoroethanol containing a little water had to be used in order to maintain solubility during hydrolysis. It was found that the resulting protected peptide acid (**113a**) was slightly more soluble than the parent phenyl ester and could be purified by gel filtration on Sephadex LH-60 providing that *N*-methyl pyrrolidone was used as eluant.[14] The homogeneity of the resulting Bpoc. (76–93). OH (**113a**) was demonstrated by thin layer chromatography and by polyacrylamide gel electrophoresis of the totally deprotected peptide.

The preparation of this highly insoluble fragment demonstrates the way in which the order of combination of fragments may be chosen to circumvent solubility problems which would prejudice the purification of products.

EXPERIMENTAL

The general experimental techniques, abbreviations, and TLC systems are those which have been described in earlier papers in this series except for the TLC system (36) EtOAc/Py/HOAc/H_2O (60:20:6:11).

Z-Ile-Pro-OH (**96**). A solution of Z-Ile-ONSu (24.6 g, 68 mM), proline (9.4 g, 81 mM) and TEA (11.4 ml, 81 mM) in DMF (80 ml) was stirred for 3 days at room temperature. The solvent was removed and the resulting oil dissolved in water (200 ml) containing $NaHCO_3$ (8 g). The solution was extracted with EtOAc then acidified to pH 1 with 3 M hydrochloric acid. Extraction with Et_2O and backwashing with water and saturated sodium

Table 1.

Coupling Reagent	Excess carboxyl %	Yield %
DCCI/HONSu	10	43
DCCI/HOBt	20	43
pivaloyl chloride	50	54
Bates	20	40
Bates	200	59
DPPCl	200	74
Bates/HONSu	20	50
Bates/HONSu	50	62

chloride solution followed by evaporation of the ethereal solution gave an oil which crystallised on standing for 1 week giving (**96**) (20.3 g, 82%), m.p. 96–97°, $[\alpha]_D^{22}$ −61.8° (c = 2, DMF), R_f(14) −0.4 (Found: C, 62.70; H, 7.36; N, 7.93; $C_{19}H_{26}N_2O_5$ requires: C, 62.97; H, 7.23; N, 7.73%).

Z-Asn-Ile-Pro-OH (**97**). Compound (**96**) (14.5 g, 40 mM) and Tos.OH.H_2O (7.6 g, 40 mM) were dissolved in DMF (150 ml) and hydrogenolysed in the presence of 10% Pd/C (1.3 g) for 4 hr. Work up in the usual way gave a solution which was evaporated to 100 ml. Z-Asn-OCp (18.7 g, 42 mM) and TEA (11.2 ml, 80 mM) were added and the solution stirred for 3 days. Evaporation gave an oil which was dissolved in $CHCl_3$, the resulting solution being shaken with 10% citric acid then dried. The aqueous phase was further extracted with butan-1-ol, and the organic phases combined and evaporated. The resulting oil was dissolved in warm EtOAc and dicyclohexylamine (9 ml, 45 mM) added. The salt precipitated and was recrystallised from EtOAc giving (**97** as DCHA salt) (16.3 g, 62%), m.p. 107–108°, $[\alpha]_D^{25}$ −59.2° (c = 2, MeOH), R_f(36) −0.7, R_f(23) −0.4, $Asp_{0.98}Pro_{1.02}Ile_{1.00}$ (Found: C, 61.67; H, 8.47; N, 10.48; $C_{35}H_{55}N_5O_7$. 1.5 H_2O requires: C, 61.38; H, 8.54; N, 10.23%).

Bpoc-Cys(Acm)-ONSu (**98**). Bpoc-Cys-(Acm)-OH (**41**) (12.6 g, 29 mM) and HONSu (5.1 g, 44.5 mM) were dissolved in 1,2-dimethoxyethane (50 ml). After cooling to 0° a precooled solution of DCCI (6.7 g, 32 mM) in 1,2-dimethoxyethane (25 ml) was added and the solution stirred overnight at room temperature. Filtration and evaporation gave an oil which was dissolved in EtOAc, after further cooling a small amount of DCU was removed by filtration and the resulting solution was washed with 5% citric acid water, 5% $NaHCO_3$ solution, water and brine. Drying and evaporation gave (**98**) as a dry foam under high vacuum. (15.4 g, 98%), m.p. 50–51°, $[\alpha]_D^{23}$ −40.1° (c = 2, DMF), (Found: C, 58.16; H, 5.94; N, 7.73. $C_{26}H_{29}N_3O_7S$. 0.5 H_2O requires: C, 58.20; H, 5.64; N, 7.83%).

Bpoc-Cys(Acm)-Asn-Ile-Pro-OH (**99**). The DCHA salt of (**97**) (10.9 g, 16.0 mM) and Tos.OH.H_2O (6.3 g, 33.2 mM) were dissolved in DMF (100 ml) and hydrogenolysed in the presence of 10% Pd/C (1 g) for 16 hr. The catalyst was removed and the solution concentrated to 60 ml, Bpoc-Cys(Acm)-ONSu (**98**) (9.6 g, 18.3 mM) and TEA (4.6 ml, 33.2 mM) were added and the reaction stirred for 3 days. The solvent was evaporated and the resulting oil dissolved in the minimum volume of acetone. This solution was poured into ice cold citrate buffer (pH 3.5) and the resulting solution extracted with $CHCl_3$ (×3) and butan-1-ol (×1). Combination of the organic phases and evaporation gave an oil which was purified by gel filtration on Sephadex LH-20 eluting with DMF. The fractions containing product (Ve/Vt) = 0.48 were pooled and concentrated, addition of ether yielding the free acid (**99**) (9.7 g, 79%), m.p. 77–78°, $[\alpha]_D^{25}$ −51.9° (c = 2, DMF), R_f(36) −0.3, R_f(14) −0.5, $Asp_{1.00}Pro_{0.98}Ile_{1.01}$.

Z-Ala-Ala-OBzl (**100**). Z-Ala-OH (7.61 g, 34.1 mM), and TEA (4.8 ml, 34.1 mM) were dissolved in CH_2Cl_2 (50 ml) and cooled to −20°. Pivaloyl chloride (4.1 ml, 33.8 mM) was added and 15 minutes allowed for activation, alanine benzyl ester hydrochloride (6.69 g, 31 mM) and TEA (3.34 ml, 31 mM) were added and the reaction mixture stirred overnight at room temperature. The solvent was evaporated and the resulting oil dissolved in EtOAc, this solution being washed with 5% citric acid, water, 5% $NaHCO_3$ solution, water and brine. After drying the solution was warmed to about 60° and hot petroleum ether added. Spontaneous crystallisation took place to give the required product (**100**) (9.92 g, 83%), m.p. 139–140°, $[\alpha]_D^{22}$ −20.9° (c = 2, $CHCl_3$), R_f(2) −0.7, (Found: C, 65.55; H, 6.27; N, 7.14; $C_{21}H_{24}N_2O_5$ requires: C, 65.61; H, 6.29; N, 7.29%). (lit. m.p.[15] 138°, m.p.[16] 141°; m.p.[17] 109°), $[\alpha]_D^{25}$ −1.7° (c = 1, $CHCl_3$); (m.p.[18] 113°), $[\alpha]_D^{20}$ −3.2° (c = 2, $CHCl_3$).

Bpoc-Cys(Acm)-Ala-Ala-OH (**101**). The protected dipeptide (**100**) (3.84 g, 10 mM) and Tos.OH.H_2O (1.90 g, 10 mM) were dissolved in DMF (25 ml) and hydrogenolysed for 6 hr in the presence of 10% Pd/C (0.5 g). After filtration and concentration to 20 ml TEA (1.8 ml, 20 mM) and Bpoc.Cys(Acm).ONSu (**98**) (5.80 g, 11 mM) were added and the solution stirred for 3 days. Evaporation gave an oil which was dissolved in acetone and shaken with ice cold citrate buffer (pH 3.5). The product was extracted into $CHCl_3$ (×3) and n-BuOH (×1) and the combined organic phases evaporated, the resulting oil being chromatographed on Sephadex LH-20 eluting with DMF. The product (**101**) eluted with (Ve/Vt) = 0.50, concentration of the combined fractions and precipitation with Et_2O gave (4.85 g, 83%), m.p. 55–57° $[\alpha]_D^{23}$ −24.0° (c = 2, DMF), R_f(14) −0.2, R_f(17) −0.3, (Found: C, 56.61; H, 7.18; N, 10.05; $C_{28}H_{36}N_4O_7S.H_2O$ requires: C, 56.93; H, 6.49; N, 9.48%).

Z-Ser(Bu^t)-Gly-OPh (**102**). Z-Ser(Bu^t)-OH (49.0 g, 157.5 mM) and NMM (17.3 ml, 157.5 mM) were dissolved in CH_2Cl_2 (300 ml) and cooled to −20°. Pivaloyl chloride (19.0 ml, 154.5 mM) was added and 15 min allowed for activation after which time a solution of $Br^-H_2^+$.Gly.OPh (34.8 g, 150 mM) and NMM (16.5 ml, 150 mM) in DMF (100 ml) was also added. After stirring overnight the solvents were evaporated and the product dissolved in EtOAc. This solution was washed with 5% citric acid, water, 5% $NaHCO_3$, water and brine then dried and concentrated to 150 ml. Addition of hot petroleum ether (750 ml) gave the product (**102**) as a white crystalline solid (53.1 g, 83%), m.p. 98–99°, $[\alpha]_D^{25}$ +0.7° (c = 2, DMF) R_f(2) −0.7, $Ser_{0.97}$ (corrected) $Gly_{1.00}$ (Found: C, 64.49; H, 6.79; N, 6.39; $C_{23}H_{28}N_2O_6$ requires: C, 64.47; H, 6.59; N, 6.54%).

Z-Nva-Ser(Bu^t)-Gly-OPh (**103**). The protected dipeptide (**102**) (53.1 g, 124 mM) and Tos.OH.H_2O (33.6 g, 124 mM) were dissolved in DMF (200 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (6 g), then filtered and concentrated to 100 ml. Z-Nva-OH (37.4 g, 149 mM) was dissolved in CH_2Cl_2 (170 ml) and NMM (16.4 ml, 149 mM) added. After cooling to −20° pivaloyl chloride (17.8 ml, 145 mM) was added and the solution stirred for 20 min before the precooled DMF solution from above was added. Following the addition of NMM (13.6 ml, 124 mM) the reaction was stirred at −20° for 3 hr then maintained at room temperature overnight. The solution was evaporated and the product dissolved in EtOAc; washing with acid and base in the usual way followed by crystallisation from EtOAc/petroleum ether yielded (**103**) (53.1 g, 81%), m.p. 122–122.5°, $[\alpha]_D^{23}$ −1.6° (c = 2, DMF), R_f(2) −0.7, $Ser_{1.02}$ (corrected) $Gly_{1.00}Nva_{0.98}$ (Found: C, 63.36; H, 7.09; N, 8.00. $C_{28}H_{37}N_3O_7$ requires: C, 63.74; H, 7.07; N, 7.96%).

Z-Leu-Nva-Ser(Bu^t)-Gly-OPh (**104**). Compound (**103**) (31.6 g, 60 mM) and Tos.OH.H_2O (11.4 g, 60 mM) were dissolved in DMF (100 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (3 g) then worked up in the usual way. Z-Leu-OH was liberated from its DCHA salt (35.7 g, 80 mM) by the standard method, the resulting oil was dissolved in CH_2Cl_2 (300 ml) then cooled to −20° and NMM (8.8 ml, 80 mM) and pivaloyl chloride (8.9 ml, 72 mM) added. After 20 minutes activation the amino component from above was added in DMF (100 ml) along with NMM (6.6 ml, 60 mM). Following evaporation the residual oil was dissolved in $CHCl_3$/EtOAc (3:1) (400 ml) and washed with acid and base in the usual way. Gradual evaporation gave the crystalline product (**104**) (33.4 g, 87%), m.p. 226–228°, $[\alpha]_D^{23}$ −10.7° (c = 2, DMF), R_f(2) −0.7, $Ser_{0.89}Gly_{0.99}Leu_{1.01}Nva_{0.94}$ (Found: C, 63.42; H, 7.58; N, 8.74. $C_{34}H_{48}N_4O_8$ requires: C, 63.73; H, 7.55; N, 8.74%).

Bpoc-Cys(Acm)-Ala-Ala-Leu-Nva-Ser(Bu^t)-Gly-OPh (**105**). The protected tetrapeptide (**104**) (2.44 g, 3.8 mM) and Tos.OH.H_2O (0.73 g, 3.8 mM) were dissolved in DMF (15 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (0.2 g). After filtration the solution volume was reduced to 7 ml and the tripeptide acid (**101**) (2.21 g, 4.2 mM) added. The solution was cooled to −10° and HONSu (0.88 g, 7.6 mM), DCCI (0.94 g, 4.6 mM) and NMM (0.42 ml, 3.8 mM) added. After stirring at room temperature for 24 hr the solution was recooled and additional portions of HONSu (0.44 g, 3.8 mM) and DCCI (0.47 g, 2.3 mM) added. A further 2 days reaction was allowed at room temperature before the reaction mixture was concentrated and applied to Sephadex LH-20 for elution with DMF. The fractions eluting at (Ve/Vt) = 0.45 were combined and concentrated, addition of Et_2O giving the required product (**105**) (2.8 g, 70%), m.p. chars at 250°, $[\alpha]_D^{23}$ −28.2° (c = 1, DMF), R_f(2) −0.6, R_f(9) −0.3, R_f(14) −0.75, $Ser_{1.00}$ (corrected) $Gly_{0.98}Ala_{2.10}Leu_{1.00}Nva_{0.95}$ (Found: C, 60.36; H, 7.36; N, 10.42; $C_{54}H_{76}N_8O_{12}S$.0.5 DMF requires: C, 60.72; H, 7.30; N, 10.84%).

Z-Val-Gly-OPh (**106**). Z-Val-OCp (21.5 g, 50 mM) and $Br^-H_2^+$.Gly.OPh (11.6 g, 50 mM) were dissolved in DMF (100 ml) along with TEA (7 ml, 50 mM). After stirring for 3 days the solution was evaporated and the residue taken up in $CHCl_3$. This solution was washed with acid and base then evaporated to give an oil which was crystallised from EtOAc/petroleum ether yielding the product (12.8 g, 68%), m.p. 164–167°, $[\alpha]_D^{21}$ −14.4° (c = 2, DMF), $R_f(2)$ −0.4, $Gly_{1.01}Val_{0.99}$ (Found: C, 65.80; H, 6.53; N, 7.23; $C_{21}H_{24}N_2O_5$ requires: C, 65.61; H, 6.29; N, 7.29%).

Z-Ser(Bu')-Val-Gly-OPh (**107**). The protected dipeptide (**106**) (9.6 g, 25 mM) was dissolved in DMF (125 ml) and 10% Pd/C (1 g) was added along with 8.3 M HCl in dioxan 3.9 ml, 32 mM). After hydrogenolysis overnight the reaction mixture was processed in the standard way. *Z*-Ser(Bu')-OH obtained from the DCHA salt (1.92 g, 25 mM) in the usual way was dissolved in CH_2Cl_2 (100 ml) and cooled to −20°. TEA (3.5 ml, 25 mM) and IBC (3.2 ml, 25 mM) were added and 10 min allowed for activation. The amino component from the above hydrogenolysis in DMF (100 ml) was added followed by TEA (3.5 ml, 25 mM). After stirring overnight the solution was evaporated and the resulting oil dissolved in EtOAc. Work up in the usual way followed by crystallisation from EtOAc/petroleum ether gave (7.8 g, 69%), m.p. 159–160°, $[\alpha]_D^{21}$ −4.8° (c = 2, DMF), $R_f(2)$ −0.7, $Ser_{0.94}$ (corrected) $Gly_{1.02}Val_{0.98}$. (Found: C, 63.96; H, 7.26; N, 7.95. $C_{28}H_{37}N_3O_7$ requires: C, 63.74; H, 7.07; N, 7.97%).

Z-Ala-Ser(Bu')-Val-Gly-OPh (**108**). Compound (**107**) (7.2 g, 13.7 mM) in DMF (75 ml) was hydrogenolysed overnight in the presence of 10% Pd/C (0.8 g) and 5 M HCl in dioxan (3.1 ml, 15 mM). The solution was filtered and evaporated in the usual way, the residual oil being dissolved in DMF (40 ml). *Z*-Ala-OCp (6.05 g, 15 mM) and TEA (1.82 ml, 13.5 mM) were added and the reaction stirred for 3 days at room temperature. Evaporation gave a gel which was dissolved in $CHCl_3$/EtOAc and processed in the usual way. Crystallisation from EtOAc gave (7.0 g, 85%), m.p. 197–202°, $[\alpha]_D^{22}$ −3.86° (c = 2, DMF), $R_f(2)$ −0.6, $Ser_{0.73}Gly_{0.99}Ala_{1.00}Val_{1.01}$, (Found: C, 61.93; H, 7.11: N, 9.40; $C_{31}H_{42}N_4O_8$ requires: C, 62.19; H, 7.07; N, 9.36%).

Z-Thr(Bu')-Ala-Ser(Bu')-Val-Gly-OPh (**109**). The compound (**108**) (6.6 g, 11 mM) was dissolved in DMF (100 ml), 10% Pd/C (0.6 g) and 5 M HCl in dioxan (2.4 ml, 12 mM) added and the mixture hydrogenolysed overnight. Work up in the usual way gave the salt as an oil *Z*-Thr(Bu')-OH prepared from the nitrobenzyl ester (6.7 g, 15 mM) was dissolved in CH_2Cl_2 (30 ml), TEA (1.7 ml, 12 mM) added and after cooling (1.6 ml, 12 mM) of IBC added. After 15 min activation a cooled solution of the amino component from above in DMF (80 ml) was added along with TEA (1.6 ml, 11.6 mM). After overnight reaction the solvents were evaporated and the solution worked up in the usual way. Trituration with Et_2O gave the product (**109**) (7.6 g, 87%), m.p. 203–206°, $[\alpha]_D^{22}$ +3.86° (c = 2, DMF), $R_f(2)$ −0.7, $Ser_{0.98}Thr_{0.80}Gly_{0.98}Ala_{1.00}Val_{1.01}$. (Found: C, 61.87; H, 7.79; N, 9.27; $C_{39}H_{57}N_5O_{10}$ requires: C, 61.96; H, 7.60; N, 9.27%).

Z-Ile-Thr(Bu')-Ala-Ser(Bu')-Val-Gly-OPh (**110**). The preceding protected peptide (**109**) (6.0 g, 8 mM) was dissolved in DMF (200 ml) and hydrogenolysed in the presence of 10% Pd/C (0.6 g) and 5 M HCl in dioxan (1.8 ml, 9 mM) for 16 hr. Work up by the standard procedure gave an oil which was dissolved in DMF (80 ml). *Z*-Ile-OCp (3.9 g, 8 mM) and TEA (1.11 ml, 7.95 mM) were added and the mixture stirred for 2 days at 40°. A further portion of the active ester (1.1 g, 2.4 mM) and TEA (0.22 ml, 1.6 mM) were added and stirring continued for 3 days at 40°. Evaporation gave a solid which was worked up in the usual way and crystallised from EtOAc/petroleum ether giving (**110**) (6.0 g, 86%), m.p. 235–240°, $[\alpha]_D^{21}$ +3.7° (c = 2, DMF), $R_f(2)$ −0.7, $Ser_{0.94}Thr_{0.87}Gly_{1.03}Ala_{1.05}Val_{0.98}Ile_{0.95}$, (Found: C, 61.03; H, 7.75; N, 9.40. $C_{45}H_{68}N_6O_{11}.H_2O$ requires: C, 60.93; H, 7.95; N, 9.48%).

Z-Asp(Bu')-Ile-Thr(Bu')-Ala-Ser(Bu')-Val-Gly-OPh (**111**). The above hexapeptide (**110**) (5.3 g, 6 mM) was dissolved in DMF (75 ml) and 10% Pd/C (0.5 g) and 5 M HCl in dioxan (1.4 ml, 7 mM) added. After hydrogenolysis overnight the mixture was worked up in the usual way. The salt was dissolved in DMF (75 ml) and Z-Asp(Bu')-OCp (4.0 g, 8 mM) and TEA (0.84 ml, 6 mM) added. After stirring for 2 days at 45° the reaction mixture had thickened to such an extent that an additional portion of DMF (25 ml) was added to facilitate stirring. After a further 2 days the reaction mixture was worked up using the usual acid/base washing procedure with the product being crystallised from $CHCl_3$ giving (5.8 g, 92%), m.p. 260–262°, $[\alpha]_D^{23}$ −6.8° (c = 2, DMF), $R_f(2)$ −0.8, $Asp_{1.03}Ser^{\dagger}_{1.02}Thr^{\dagger}_{1.02}Gly_{0.98}Ala_{1.01}Val_{0.98}Ile_{0.96}$(* corrected), (Found: C, 60.75; H, 71.83; N, 9.71. $C_{53}H_{81}N_7O_{14}$ requires: C, 61.19; H, 7.85; N, 9.43%).

Bpoc.(76–86).OPh (**112**). Bpoc.(80–86). OPh (**105**) (2.12 g, 2 mM) was dissolved in $AcOH/H.CO_2H/H_2O$ (7:1:2) (50 ml) containing DMS (6 ml, 80 mM) and stirred for 2 hr. The solvent was evaporated and the residue treated with 0.05 M HCl in dioxan (80 ml, 4 mM) containing DMS (6 ml, 80 mM). After evaporation of the solvent the anion exchange was repeated. The resulting solid was washed with Et_2O and dried, then dissolved in HMPA (15 ml) containing NMM (0.22 ml, 2 mM) over 30 min. Bpoc.(76–79).OH (**99**) (2.22 g, 3 mM), HONSu (0.69 ml, 6 mM), Bates reagent (2.32 g, 4.5 mM) and NMM (0.99 ml, 9 mM) were added and the reaction stirred overnight. The solution was applied directly to the column of Sephadex LH-20 and eluted with DMF. The product (**112**) eluted with (Ve/Vt) = 0.41, and after combination of the appropriate fractions evaporation and precipitation with Et_2O gave (1.94 g, 62%), m.p. 178° (amorph.), $[\alpha]_D^{23}$ −28.8° (c = 2, DMF) $R_f(23)$ −0.3, $R_f(17)$ −0.5, $R_f(7)$ −0.6, $Asp_{0.95}Ser_{0.89}Pro_{0.97}Gly_{1.00}Ala_{2.12}Nva_{0.93}Ile_{0.98}Leu_{1.04}$. (Found: C, 56.35; H, 7.21; N, 12.34. $C_{75}H_{110}N_{14}O_{18}S_2$; $2H_2O$ requires: C, 56.45; H, 7.20; N, 12.29%).

Bpoc.(76–93).OPh (**113**). (a) *Phenyl ester cleavage.* Bpoc. (76–86).OPh (**112**) (265 mg, 0.17 mM) was dissolved in DMF (7 ml) and water (1.2 ml). DMS (0.62 ml, 8.5 mM) was added and the pH adjusted to 10.5 with 0.1 M NaOH solution. One equivalent of 100 vol.H_2O_2 was added and the solution maintained at pH 10.5 by the addition of 0.1 M NaOH solution from a pH stat. After 30 minutes base uptake was complete, the solution was cooled to −5° and acidified with 5% citric acid to pH 3.5. On pouring into brine the free acid (**112a**) was precipitated, the resulting solid was washed with ether and water then dried giving (233 mg, 93%), $R_f(2)$ −0.2, $R_f(23)$ −0.1, $R_f(14)$ −0.3.

(b) *Benzyloxycarbonyl cleavage.* Z. (87–93).OPh (**111**) (208 mg, 0.2 mM) and Tos.OH.H_2O (38 mg, 0.2 mM) were dissolved in DMF (7 ml) and hydrogenolysed overnight in the presence of 10% Pd/C (50 mg). The reaction mixture was then filtered and the solvent evaporated giving a solid which was triturated with Et_2O to yield (**111a**) 196 mg, (91%), $R_f(7)$ −0.6, $R_f(17)$ −0.7.

(c) *Coupling.* Bpoc.(76–86).OH (**112a**) (110 mg, 0.075 mM), $TosO^-H_2^+$. (87–93). OPh (**111a**) (54 mg, 0.05 mM), HONSu (17 mg, 0.15 mM) and Bates reagent (58 mg, 112 mM) were dissolved in HMPA (2 ml). 1 M NMM in DMF (0.28 ml, 0.28 mM) was added and the reaction stirred at room temperature overnight. The solution was poured into brine and the precipitate filtered, washed with water, propan-2-ol and Et_2O then dried giving (98 mg, 83%), m.p. 239–245°, $[\alpha]_D^{22}$ −24.8° (c = 1, TFE), R_f (TFE on alumina) −0.7, compound would not run in any other solvent system, $Asp_{2.04}Thr_{0.96}Ser_{1.84}Pro_{0.97}Gly_{2.03}Ala_{3.23}Val_{1.13}Nva_{0.92}Ile_{1.95}Leu_{1.00}$. Found: C, 55.92; H, 7.84; N, 11.63; $C_{114}H_{179}N_{21}O_{29}S_2.4H_2O$ requires: C, 56.03; H, 7.71; N, 12.04%).

Bpoc.(76–93).OH (**113a**). A solution of Bpoc. (76–93).OPh (98 mg, 0.042 mM) in TFE (1.7 ml) was adjusted to pH 9 with 0.5 M NaOH solution; water (0.3 ml) and DMS (0.2 ml, 2.74 mM) were added and the pH carefully raised to 10.5 with 0.5 mM NaOH solution. H_2O_2 (0.42 ml of a 1% solution in TFE) was added and the solution stirred for 35 minutes by which time the reaction was shown to be complete by tlc on alumina with TFE as eluant. The reaction mixture was acidified to pH 3.5 with ice cold 5% citric acid then poured into water to precipitate the product (**113a**). Filtration, washing with water and Et_2O gave (80 mg, 83%), m.p. 230°, $[\alpha]_D^{20}$ −48.4° (c = 0.59, TFE), R_f (all systems silica 0.0), R_f (TFE on alumina) 0.0 *cf.* 0.7 for the phenyl ester, (Found: C, 54.21; H, 7.76; N, 12.61. $C_{108}H_{175}N_{21}O_{29}S_2.5H_2O$ requires: C, 54.37; H, 7.82; N, 12.33%).

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