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PEPTIDES—XXXV

SYNTHESIS OF THE 17–26 FRAGMENT OF A LYSOZYME ANALOGUE

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Abstract—The fully protected 17–26 fragment of a lysozyme analogue has been synthesised. A fragment condensation approach has been employed using the protected subfragments 17–19, 20–22 and 23–26. In the synthesis of the subfragments the use of the diphenylphosphinyl mixed anhydride method has been demonstrated.

As described in a previous paper[1] our aim is to synthesise an analogue of Hen Egg White Lysozyme (HEL). The general tactics and strategy employed in this synthesis have been described earlier[1] and here we describe our synthesis of the (17–26) subfragment. The sequence of the decapeptide (17–26) is shown below:

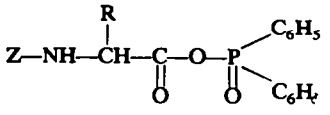
Leu. Ala. Gly. Tyr. Orn. Gly. Tyr. Ser. Leu. Gly‡
17 20 23 26

In the synthetic analogue the aspartylasparagine (18–19) sequence of HEL (aspartylglycine in Human Leukaemic Lysozyme[2]) has been replaced by alanylglycine in order to eliminate the possibility of α- to β- peptide rearrangement.[3] As these residues are found on the surface of the natural enzyme,[4] we believe that this and other similar changes are less likely to cause significant changes in tertiary structure. Also we have replaced the arginine 21 by ornithine, this change was initially made in order to facilitate purification, as fragments carrying a guanidinium group often do not chromatograph well on Sephadex LH20. On the other hand, recent preliminary studies using bis-adamantyloxycarbonyl arginine have shown that retention of the protected arginine residue would not cause any major synthetic upheaval.

Initially the synthesis of Z(17–26) OPh was explored with a view to coupling at the amino terminus. This would allow a (1–26) plus (27–37) condensation forming the (1–37) portion of the molecule. In the event it proved better to follow the alternative route of phenyl ester cleavage of Bpoc (17–26) OPh followed by coupling to the (27–37) fragment, providing Bpoc (17–37) OPh which could be combined with the (1–16) portion to form the (1–37) sequence. Here we describe the route to the Bpoc (17–26) OPh fragment (**32**).

The protected tripeptide Bpoc (17–19) OPh was synthesised by the route outlined in Scheme 1 which commenced with the protected dipeptide (**24**) which was obtained in excellent yield by active-ester coupling. A 12 h. hydrogenolysis carried out in the manner described previously[5] gave the *p*-toluenesulphonate as a white solid which was then coupled to *p*-biphenylylisopropoxycarbonyl-leucine[6] using the isobutyloxycarbonyl mixed anhydride to give a good yield of protected tripeptide (**25**).

The heptapeptide portion of the (17–26) fragment was synthesised by the route shown in Scheme 2. Synthesis of the protected dipeptide (**14**) by the active ester method has been described in the previous paper in the this series.[5] A rather extended hydrogenolysis (20 hr) under the normal conditions gave the *p*-toluenesulphonate as a gum. Activation of N-benzyloxycarbonyl-O-t-butylserine to give (**26**) was best achieved by reaction with diphenylphosphinyl chloride[7] giving the protected tripeptide in 73% yield, in contrast to the 43% yield obtained by activation with isobutyloxycarbonyl chloride. Intermediate mixed anhydrides of the type shown below have never in our experience given products associated with nucleophilic



attack at phosphorus. Attack at the CO is exclusive[7] giving only the required acylated amino-component. The relative yields and purity of the products are notably high when the R group is large, as demonstrated by this and further examples in this paper. A simple steric effect might apply whereby the large R group and the two aromatic rings interact in such a way as to expose the CO function. However, it is more likely that the nature

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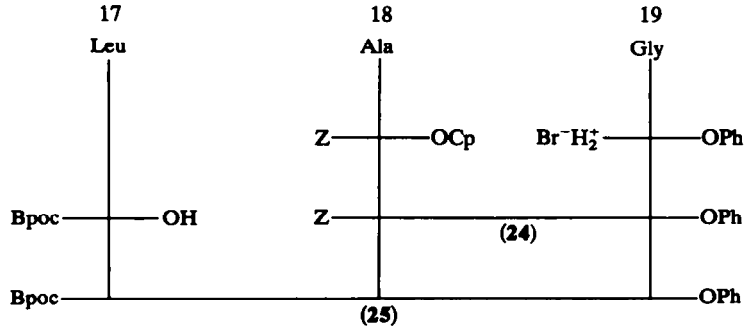
‡ All amino-acids are of the L-configuration, and nomenclature follows Specialist Periodical Reports *Amino-acids, peptides and proteins* (Edited by G. T. Young) Vol. 4, Chapter 5, Chemical Society, London (1972).

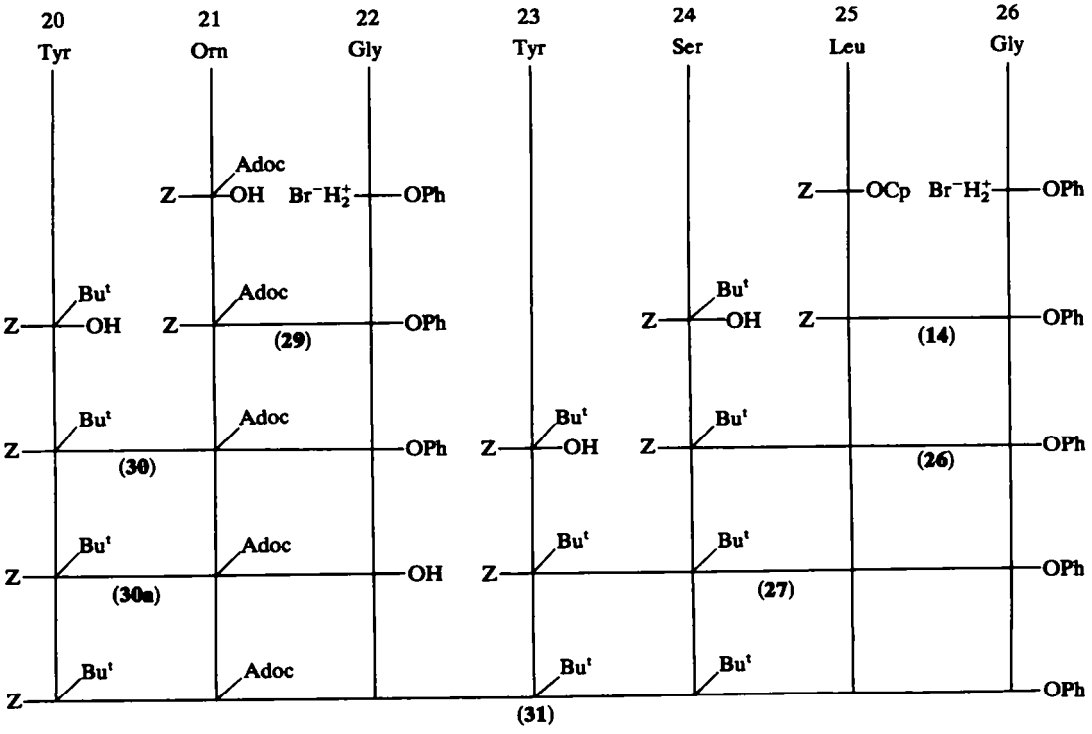


Scheme 1. Synthesis of the protected (17–19) tripeptide (25).

of the transition state is the dominant feature in determining the site of attack. The CO group will of course give a tetrahedral intermediate on nucleophilic attack whereas the phosphinic acid grouping will be in a five co-ordinate trigonal bipyramidal environment. Thus it would be expected that attack at the CO function would provide the easier course of reaction. In the earlier work within our group the Izumiya test[8] showed (5.7%) racemisation for activation by diphenylphosphinyl chloride in contrast to (2.6%) when using pivaloyl chloride. As expected, however, there was no sign of racemisation when the diphenylphosphinyl anhydride of a benzyloxycarbonyl amino-acid was used.

The protected tripeptide (26) was then hydrogenolysed in the usual way and coupled to N-benzyloxycarbonyl-O-t-butyltyrosine by the diphenylphosphinyl mixed anhydride method. A 55% yield was obtained giving a cleaner product than the comparable trichlorophenyl active ester method, thus the synthesis of the protected tetrapeptide (27) was completed.

N^{α}-Benzyloxycarbonyl-N^{δ}-adamantyloxycarbonylornithine was prepared by treating N^{δ}-adamantyloxycarbonylornithine[5] with excess benzylchloroformate at 0° for 4 hr. The oily free acid obtained could be used directly or converted to its cystalline dicyclohexylammonium salt in 77% overall yield. The free acid was activated by reaction with diphenylphosphinyl chloride in the presence of N-methylmorpholine, 20 min at −20° being allowed for completion of activation. The mixed anhydride was coupled to glycine phenyl ester hydrobromide[9] overnight, work up and crystallisation give the pure dipeptide (29) in 60% yield. In an alternative synthesis using pivaloyl chloride for activation, a yield of 49% was obtained again demonstrating the advantage of using the phosphinic mixed anhydride method. The benzyloxycarbonyl group was removed by hydrogenolysis and N-benzyloxycarbonyl-O-t-butyltyrosine coupled by



Scheme 2. Synthesis of the protected (20–26) heptapeptide (31).

the diphenylphosphinic mixed anhydride method employing 20 min activation at −20° to give 81% yield of homogeneous crystalline protected tripeptide (**30**). The phenyl ester was then cleaved in 30 min at pH 10.5 by treatment with 1M NaOH in the presence of hydrogen peroxide. The phenol liberated in the cleavage was removed by chromatography on silica gel eluting first with diethylether and subsequently with methanol. Alternatively gel filtration on Sephadex LH20, eluting with DMF, may be used for purification, although this method is time consuming for large quantities of material.

The protected tripeptide acid (**30a**) was coupled by the DCCI/HONSu method to the *p*-toluenesulphonate obtained by hydrogenolysis of the protected tetrapeptide (**27**). After 3 days the mixture was filtered and evaporated to give a solid which was triturated with water and diisopropylether. Gel filtration on Sephadex LH20 eluting with DMF (Ve/Vt = 0.41) provided the final purification giving an overall yield of 67% of the protected heptapeptide (**31**).

The Bpoc (17–26) OPh fragment was then assembled from the tripeptide (**25**) and the heptapeptide (**31**). The protected tripeptide phenyl ester (**25**) was dissolved in 65% aqueous dioxan and the pH maintained at 10.5 by the addition of 1M sodium hydroxide solution for 30 min, hydrogen peroxide was added in the usual way to catalyse the hydrolysis. After removal of the liberated phenol acidification gave the acid (**25a**) in 83% yield. In this phenyl ester cleavage and many others it is useful to check the degree of cleavage by measuring the amount of liberated phenol by UV spectroscopy especially when dealing with the cleavage of phenyl ester groups from large peptides. The Z (20–26) OPh fragment (**31**) was readily hydrogenolysed in 2.5 hr by the standard method giving the *p*-toluenesulphonate (**31a**) in quantitative yield. The fragments (**25a**) and (**31a**) were combined by the DCCI/HONSu method employing a mixture of HMPA and DMF as solvent. After overnight reaction the mixture had become gelatinous and additional HMPA and DMF had to be added in order to facilitate further addition of DCCI and HONSu. A further 2 days reaction produced a highly viscous solution which was diluted with more HMPA and DMF then chromatographed directly on Sephadex LH20 eluting with DMF. The required product (**32**) eluted with (Ve/Vt) = 0.38 and was obtained by precipitation with water in an overall yield of 66%. The homogeneity of the product was confirmed by tlc in three solvent systems and by electrophoresis and isoelectric focussing of the totally deprotected fragment. Homogeneity was also demonstrated for a sample of the protected peptide phenyl ester (**32**) which had been treated with 90% TFA leaving the C-terminal protection intact; as expected, this phenyl ester and the totally deprotected peptide showed the same migration on electrophoresis at pH 2.2. The aminoacid analyses of the fragment confirm its structure and the enzyme digest results show clearly that no observable racemisation has taken place during the construction of the fragment. This was a particularly reassuring result as the diphenylphosphinyl mixed anhydride method was used extensively in this synthesis.

EXPERIMENTAL

The abbreviations, tlc systems and general experimental methods are detailed in the preceding paper.[5]

Scheme 1.

Z-Ala-Gly-OPh (**24**). Z-Ala-OCp (29.0 g, 72.8 mM) $Br^-H_2^+$-Gly-OPh (16.9 g, 72.8 mM) and TEA (10.2 ml, 72.8 mM) were dissolved in DMF (200 ml) and stirred for 3 days. The solvent was evaporated and the residue taken up in EtOAc (200 ml), this soln was filtered, washed with water, dried and evaporated. The resulting solid was crystallised from EtOAc-petroleum ether yielding the protected dipeptide (**24**) (24.2 g, 93%), m.p. 118–119°, $[\alpha]_D^{22} - 12.6°$ ($c = 4$, DMF), R_f(19)–0.45, R_f(26)–0.4, (Found: C, 63.78; H, 5.75; N, 7.71. $C_{19}H_{20}N_2O_5$ requires: C, 64.03; H, 5.66; N, 7.86.

Bpoc-Leu-Ala-Gly-OPh (**25**). A soln of (**24**) (12.5 g, 35 mM) in DMF (200 ml) was hydrogenolysed for 12 hr in the presence of Tos.OH.H_2O (7.3 g, 38 mM) and 10% Pd/C (2.0 g). Filtration and evaporation yielded a residue which on trituration with Et_2O gave the corresponding *p*-toluenesulphonate as a white solid (14.2 g, 100%). Bpoc-Leu-OH[6] was dissolved in CH_2Cl_2 (100 ml) and cooled to −20°, TEA (4.9 ml, 35 ml) and IBC (4.4 ml, 34 mM) were added and 15 min allowed for activation. The *p*-toluene-sulphonate and TEA (5.0 ml, 35 mM) were dissolved in CH_2Cl_2 (50 ml), cooled and added to the soln of the mixed anhydride. The mixture was stirred at −20° for 1 hr and then allowed to warm to room temp overnight. The solvent was evaporated and the residue dissolved in EtOAc (250 ml). This soln was washed and dried in the usual way, evaporation gave a gum which was crystallised from EtOAc-petroleum ether giving the required (**25**) (13.3 g, 68%), m.p. 127–129°, $[\alpha]_D^{22} - 30.4°$ ($c = 2$, DMF), R_f(2)–0.5, R_f(26)–0.3, $Gly_{1.00}$ $Ala_{0.97}Leu_{1.03}$, (Found: C, 68.84; H, 6.72; N, 7.13. $C_{33}H_{39}N_3O_6$ requires: C, 69.09; H, 6.85; N, 7.32.

Scheme 2

The synthesis of Z-Leu-Gly-OPh (**14**) was described in the previous paper of this series.[5]

Z-Ser(Bu^t)-Leu-Gly-OPh (**26**). A soln of the protected dipeptide (**14**) (16.7 g, 42 mM) and Tos.OH.H_2O (8.0 g, 42 mM) in DMF (100 ml) was hydrogenolysed over 10% Pd/C (2.5 g) for 20 hr. Work up in the usual way gave a gum which was dissolved in DMF (120 ml). Z-Ser(Bu^t)-OH (15.7 g, 55 mM) obtained from the DCHA salt by the usual procedure was dissolved in CH_2Cl_2 (120 ml) and cooled to −20°. NMM (5.36 g, 53 mM) and DPP.Cl (12.53 g, 53 mM) were added and the mixture stirred for 15 min. The DMF soln from above was added followed by NMM (5.55 g, 55 mM) and the soln allowed to reach room temp overnight. The mixture was dissolved in EtOAc and washed with acid and base in the usual way. This soln was washed with water, dried and evaporated to yield the protected tripeptide (**26**) which was crystallised from EtOAc-petroleum ether (19.8 g, 73%), m.p. 99°, $[\alpha]_D^{25} - 12.6°$ ($c = 1$, DMF), R_f(19)–0.6, R_f(20)–0.5, $Ser_{0.91}$ $Gly_{1.02}Leu_{0.98}$, (Found: C, 64.21; H, 7.22; N, 7.86. C_{29} $H_{39}N_3O_7$ requires: C, 64.31; H, 7.26; N, 7.76. IBC may be used for activation but the yield obtained was 43%.

Z-Tyr(Bu^t)-Ser(Bu^t)-Leu-Gly-OPh (**27**). Compound (**26**) (6.4 g, 11.8 mM) in DMF (25 ml) was hydrogenolysed overnight in the presence of Tos.OH.H_2O (2.2 g, 11.8 mM) and 10% Pd/C (0.65 g). Work up in the usual way gave a gum which was dissolved in the minimum volume of CH_2Cl_2. Z-Tyr(Bu^t)-OH obtained from the corresponding DCHA salt (7.75 g, 14 mM) was dissolved

in the minimum volume of CH_2Cl_2 and cooled to −20°, NMM (1.42 g, 14 mM) and DPP.Cl (3.3 g, 14 mM) were added and 15 min allowed for activation. The CH_2Cl_2 soln of the salt of the amino-component was added followed by NMM (1.2 g, 11.8 mM). After warming to room temp. overnight the usual work up procedure gave the required (**27**) on crystallisation from hot EtOAc (4.2 g, 55%), m.p. 194–197°, $[\alpha]_D^{25}$ − 13.2° (*c* = 1, DMF), R_f(19)–0.6, R_f(20)–0.4, $Ser_{0.86}Gly_{1.03}Leu_{1.01}Tyr_{0.95}$, (Found: C, 66.09; H, 7.35; N, 7.29. $C_{42}H_{56}N_4O_9$ requires: C, 66.30; H, 7.42; N, 7.35. An active ester coupling with Z-Tyr(Bu^t)-OCp gave 50% yield.

Z-Orn(Adoc)-OH.DCHA (**28**). A suspension of (**9**)[5] (31.0 g, 0.1 M) in water (150 ml) was treated with 1 M NaOH (160 ml, 0.16 M), and cooled to 0°. 2 M NaOH soln (100 ml) and Z-Cl (21.2 ml, 0.15 M) were simultaneously added dropwise with constant stirring maintaining the soln at pH 10. After stirring for 4 hr at 0°, water (1.5 l) was added and the soln extracted with Et_2O. Acidification of the aqueous phase with 10% aqueous citric acid to pH 2.5–3.0 gave an oil which was extracted into EtOAc (3 × 150 ml). The organic phase was backwashed with water and brine then dried and evaporated. The free acid thus obtained (39.2 g, 88%) was dissolved in anhyd Et_2O and DCHA (16.0 g, 88 mM) added. The pure crystalline salt (**28**) was then filtered (48.8, 77%), m.p. 136–138°, $[\alpha]_D^{26}$ + 7.6° (*c* = 1, DMF), R_f(17)–0.4, R_f(30)–0.8, (Found: C, 69.00; H, 8.54; N, 6.81. $C_{36}H_{55}N_3O_6$ requires: C, 69.09; H, 8.86; N, 6.71.

Z-Orn(Adoc)-Gly-OPh (**29**). Z-Orn(Adoc)-OH (11.1 g, 25 mM) obtained from salt (**28**) in the usual way was dissolved in CH_2Cl_2 (150 ml) and cooled to −20°. NMM (2.5 g, 25 mM) and DPP.Cl (5.4 g, 23 mM) were added and 20 min. allowed for activation. A soln of $Br^-H_2^+$-Gly-OPh[9] (6.4 g, 27.5 mM) and NMM (2.78 g, 27.5 mM) in CH_2Cl_2 (50 ml) was added and the mixture allowed to warm to room temp. overnight. The solvent was evaporated and the resulting gum dissolved in EtOAc. This soln was washed with 5% $NaHCO_3$, 10% citric acid and water then dried and evaporated. Crystallisation of the gum obtained from EtOAc-petroleum ether gave the protected (**29**) (7.8 g, 60%), m.p. 109–110°, $[\alpha]_D^{24}$ −8.4°, (*c* = 1, DMF), R_f(21)–0.6, R_f(22)–0.8, $Orn_{0.99}$ $Gly_{1.01}$, (Found: C, 66.56; H, 6.84; N, 7.39. $C_{32}H_{39}N_3O_7$ requires: C, 66.53; H, 6.80; N, 7.27%). Using a mixed anhydride formed with PivCl a 49% yield was obtained.

Z-Tyr(Bu^t)-Orn(Adoc)-Gly-OPh (**30**). The protected dipeptide (**29**) (7.8 g, 13.6 mM) in DMF (50 ml) was hydrogenolysed for 4 hr in the presence of Tos.OH.H_2O (2.6 g, 13.6 mM) and 10% Pd/C (0.8 g). Work up in the usual way gave a gum R_f(21)–0.7, which was dissolved in CH_2Cl_2 (20 ml). Z-Tyr(Bu^t)-OH obtained from the corresponding DCHA salt (9.0 g, 16.3 mM) was dissolved in CH_2Cl_2 (150 ml) and cooled to −20°. DPP.Cl (3.8 g, 16.2 mM) and NMM (1.63 g, 16.2 mM) were added consecutively. After 20 min the soln of the product from the hydrogenolysis was added followed by NMM (1.37 g, 13.6 mM). The soln was allowed to attain room temp and stirred overnight, the solvent was evaporated and the residue dissolved in EtOAc. This soln was washed with 5% $NaHCO_3$, 10% citric acid and water, then dried and evaporated. The resulting (**30**) was crystallised from EtOAc-petroleum ether giving (8.8 g, 81%), m.p. 101°, $[\alpha]_D^{25}$ − 11.8°, (*c* = 1, DMF), R_f(9)–0.6, R_f(20)–0.5, $Orn_{0.99}$ $Gly_{1.02}Tyr_{0.99}$, (Found: C, 67.50; H, 7.26; N, 7.23. $C_{45}H_{56}N_4O_9$ requires: C, 67.82; H, 7.08; N, 7.03.

Z-Tyr(Bu^t)-Orn(Adoc)-Gly-Tyr(Bu^t)-Ser(Bu^t)-Leu-Gly-OPh (**31**). The protected peptide phenyl ester (**30**) (6.0 g, 7.5 mM) was dissolved in dioxan (90 ml) and water (40 ml). The pH was brought to 10.5 with 1 M NaOH and 100 vol H_2O_2 (0.75 ml, 7.5 mM) added. After maintaining the pH at this value for 1 hr with 1 M NaOH, it was adjusted to 7 and the solvent evaporated. The residue was dissolved in EtOAc and washed with 10% citric acid and water. Evaporation gave a residue which was dissolved in benzene and chromatographed on silica gel, the column was prepared in $CH Cl_3$, initially eluting with Et_2O and finally with MeOH. The pure gum, R_f(23)–0.7, was solidified under petroleum ether in the fridge giving (**30a**) (4.5 g, 75%). Alternatively the crude hydrolysate may be purified by chromatography on LH20 eluting with DMF (Ve/Vt) = 0.47.

Compound (**27**) (5.0 g, 6.6 mM) and Tos.OH.H_2O (1.24 g, 6.6 mM) were dissolved in DMF (25 ml) and hydrogenolysed over 10% Pd/C (0.5 g) 5 hr. Filtration and evaporation gave a residue which was solidified by trituration with water; after drying over P_2O_5 the white amorphous product was obtained (5.2 g, 100%) R_f(9)–0.5).

The tripeptide acid (**30a**) (4.5 g, 5.6 mM) in DMF (25 ml) was cooled to −10°, DCCl (1.28 g, 6.2 mM), HONSu (1.43 g, 12.4 mM), the amorphous *p*-toluenesulphonate (4.55 g, 5.6 mM) and NMM (0.56 g, 5.5 mM) were added in the order indicated and the mixture allowed to reach room temp. overnight. After recooling HONSu, (0.28 g, 2.4 mM) and DCCl (0.25 g, 1.2 mM) were added, the reaction was then stirred for 2 days at room temp. The resulting DCU was removed by filtration and the solvent evaporated to give a residue which was triturated with water and $^iPr_2O$. After drying over P_2O_5 this material was purified by gel filtration on LH20 eluting with DMF(Ve/Vt) = 0.41, the product was precipitated with water and washed with Et_2O giving (**31**) (5.0 g, 67%), m.p. 225°, $[\alpha]_D^{25}$ − 5.3°, (*c* = 1, DMF), R_f(4)–0.5, R_f(21)–0.7, $Orn_{0.99}Ser_{0.85}Gly_{2.05}Leu_{1.00}Tyr_{2.09}$, (Found: C, 65.45; H, 7.73; N, 8.36. $C_{73}H_{100}N_8O_{15}.0.5H_2O$ requires: C, 65.50; H, 7.60; N, 8.37.

Bpoc(17–26)OPh

Bpoc(17–19)Oh (**25a**). The peptide phenyl ester (**25**) (2.87 g, 5 mM) was dissolved in dioxan (30 ml) and water (16 ml) added. The pH was brought to 10.5 with 1 M NaOH and maintained at this figure for 0.5 hr. after the addition of 100 vol H_2O_2 (0.5 ml, 5 mM). The soln was brought to pH 7 using 0.5 M HCl and the solvent evaporated, dissolving in water and washing with Et_2O removed phenolic material. After cooling in ice the pH was brought to 4 and the free acid extracted into EtOAc, this soln was washed with water, dried and evaporated. The resulting oil solidified under petroleum ether in the fridge giving the tripeptide acid (**25a**) (2.07 g, 83%), R_f(23)–0.78.

TosO$^-$$^+H_2$(20–26)OPh (**31a**). Z(20–26)OPh (**31**) (2.34 g, 1.76 mM) and Tos.OH.H_2O (0.335 g, 1.76 mM) were dissolved in DMF (75 ml). This soln was hydrogenolysed for 2.5 hr in the presence of 10% Pd/C (1.2 g) and then filtered and evaporated. The residue was triturated with water, washed with $^iPr_2O$ and dried for 24 hr over P_2O_5 giving (**31a**) (2.45 g, 100%), R_f(9)–0.4.

Bpoc(17–26)OPh (**32**). The tripeptide acid (**25a**) (1.1 g, 2.2 mM) was dissolved in a mixture of HMPA (5 ml) and DMF (5 ml) and cooled to 0°. HONSu (0.5 g, 4.4 mM), DCCl (0.45 g, 22 mM), (**31a**) (2.45 g, 1.76 mM) and a 10% soln of NMM in DMF (1.78 ml, 1.76 mM) were added, and the mixture allowed to attain room temp. overnight. During this period the mixture became a thick gel so additional HMPA (5 ml) and DMF (2.5 ml) were added. After cooling to 0° additional portions of HONSu (0.051 g, 0.44 mM) and DCCI (0.045 g, 0.22 mM) were added, the mixture was then allowed to reach room temp and stirred for a further 48 hr. The mixture was then diluted with HMPA (10 ml) and DMF (10 ml) and the resulting soln applied directly to an LH20 column which was eluted with DMF. The product with (Ve/Vt) = 0.38 was precipitated with water and washed with ether giving (**32**) (1.9 g, 66%), m.p. 250°, $[\alpha]_D^{24}$ − 0.9° (*c* = 1, HMPA),

R_f(4)–0.45, R_f(7)–0.8, R_f(29)–0.5, $Orn_{0.99}Ser_{0.84}Gly_{3.01}Ala_{1.00}Leu_{2.01}Tyr_{2.03}$, (pronase/APM) $Orn_{0.99}Ser_{1.00}Gly_{2.92}Ala_{1.00}Leu_{2.03}Tyr_{2.01}$, (Found: C, 65.88; H, 7.79; N, 9.18. $C_{92}H_{127}N_{11}O_{18}$ requires: C, 65.96; H, 7.64; N, 9.20.

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