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PEPTIDES—XXXVII[1]

SYNTHESIS OF THE 1-37 FRAGMENT OF A LYSOZYME ANALOGUE

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Abstract—Combination of the protected peptide fragments 1-16, 17-26 and 27-37 to yield the 1-37 portion of a lysozyme analogue is described. The fragments were combined using DCCI with the addition of HONSu, and the products purified mainly by gel filtration.

The first major subfragment of our lysozyme analogue[2] spans the residues 1-37, the sequence of which is shown below:

Lys.Val.Phe.Gly.Orn.Cys.Glu.Leu.
1

Ala.Ala.Ala.Nle.Lys.Ala.Leu.Gly.
10 16

Leu.Ala.Gly.Tyr.Orn.Gly.Tyr.Ser.
17 20

Leu.Gly.Asn.Trp.Nva.Cys.Ala:Ala.
26 27 30

Lys.Phe.Glu.Ser.Gly.
37

In the early stages of planning the 37 residue peptide was broken into three subfragments as determined by the strategy and tactics outlined in an earlier paper[2] in which the constituent fragments 1-16[3], 17-26[4] and 27-37[1] each has glycine at the C-terminus eliminating any concern over racemisation during the fragment coupling steps. Thus as these constituent fragments have been shown to be optically pure by enzyme digestion[3,4,1] no optical inhomogeneity should be introduced during combination of these fragments.

The first preliminary synthesis was carried out using Z(1-16)OH which was combined with $Cl^-H_2^+$(17-26)OPh containing free arginine at position 21. The Z(1-16)OH obtained by phenyl ester cleavage[5] was subjected to purification by counter current distribution ($CHCl_3$:CCl_4:MeOH:pyridinium acetate 0.1 M, pH7 3:1:3:1) with the arginine present as its *p*-toluenesulphonate, however, it was found that the carboxyl component was difficult to remove completely from the product. Coupling to $Cl^-H_2^+$(27-37)OPh was carried out on Z(1-16)OH in the hope that the Z(1-37)OPh would be easier to purify. These hopes were not realised as this compound also proved difficult to purify using the same counter current system. Purification by gel filtration on Sephadex LH20 eluting with DMF initially appeared to be useful but the 37 residue peptide eluted virtually at the void volume[6] thus preventing significant purification. Some broadening of the peaks was also observed, due to the presence of the arginine *p*-toluenesulphonate. The amino-acid analysis of the final material ($Lys/Orn_{3.85}Arg_{0.75}Asp_{1.02}Ser_{1.46}Glu_{2.00}Gly_{6.10}Ala_{7.03}Val_{1.12}Nva_{0.98}Leu_{4.46}Nle_{1.22}Tyr_{1.73}Phe_{1.74}$), although not totally acceptable, does indicate that a considerable degree of caution must be exercised when interpreting amino-acid analyses of relatively large synthetic peptides. When this product was deprotected and examined by electrophoresis at pH 2.2 and 6.5 it was found to be rather more heterogeneous than the above amino-acid analysis might suggest.

The problems of purification and the knowledge that the ultimate deprotection would have to employ anhydrous HF (to remove the N-Z group) prompted several changes to be made. These included changing the N-terminal protecting group to adamantyloxycarbonyl and replacing Arg-21 by N^{δ}-adamantyloxycarbonylornithine. The synthesis of the constituent subfragments Adoc(1-16)OPh (**23**), Bpoc(17-26)OPh (**32**) and Bpoc(27-37)OPh (**43**) has been described in the preceding papers.[3,4,1] Initially we considered the same approach as had been used for our earlier trial experiments. This would give Adoc(1-26)OH which would be coupled to a suitably deprotected 27-37 fragment. In the light of our general experience at this time we changed our method of attack, preferring to synthesise the 17-37 fragment first, and to finally couple this to the 1-16 portion. This change was made in an attempt to obtain products which would be more amenable to purification by gel filtration methods.

The final, most satisfactory route to Adoc(1-37)OPh (**48**) is shown in Scheme 1. Phenyl ester

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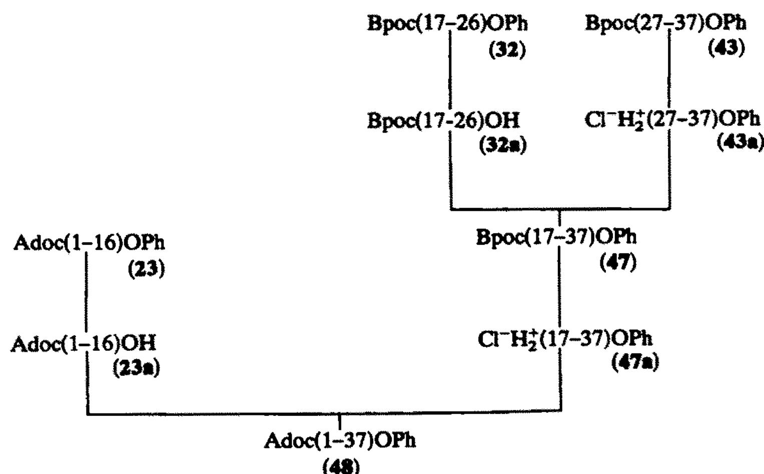

Scheme 1. Synthesis of Adoc(1–37)OPh (**48**).

cleavage of the fully protected decapeptide Bpoc(17–26)OPh (**32**) was best effected by treatment of a solution of the peptide in TFE/H_2O (9:1) with hydrogen peroxide at pH 10.5[5]. After 15 min the mixture was adjusted to pH 3 by the addition of 10% citric acid. It should be noted that the use of stronger acids or acidification to a lower pH must be avoided as the Bpoc group is cleaved particularly rapidly when this solvent system is used.[7] The free acid (**32a**) was obtained in 97% yield after washing with $^{i}Pr_2O$ and Et_2O to remove phenolic impurities. Our original method of phenyl ester cleavage for this peptide employed 50% aqueous N,N[1]-dimethylacetamide as solvent but, although the method was satisfactory, the cleavage took 90 min. to reach completion and gave a slightly lower yield (91%).

Removal of the α-amino protecting group from Bpoc (27–37)OPh (**43**) by treatment of the compound with 0.05 M HCl in TFE/H_2O (9:1) afforded the hydrochloride (**43a**). The Bpoc(17–26)OH (**32a**) was then coupled to the free amine derived from hydrochloride (**43a**) by the DCCI/HOBt method using DMF/HMPA (1:1) as solvent. After allowing 2 days for complete reaction the crude product was precipitated with water, washed with Et_2O and dried to give the crude product (**47**) which was dissolved in NMP/HMPA (1:1) and purified by gel filtration on Sephadex LH60 eluting with NMP.[8] Although the major proportion of the product eluted at (Ve/Vt) = 0.55 giving an isolated yield of 35% it was found that some of the material, *ca* 5%, was present as an aggregate and eluted close to the void volume. This material showed a strong positive optical rotation suggesting some form of ordered secondary structure which could be disaggregated by treatment with urea in DMF. Furthermore when deprotected both the aggregate and normal material (eluting at (Ve/Vt) = 0.55) behaved in a similar manner when subjected to electrophoresis. The homogeneity of the product (**47**) was demonstrated by tlc and by electrophoresis and isoelectric focusing of the deprotected peptide.

In this coupling the diagnostic residues are Leu and Tyr (found exclusively in the carboxyl component) and Asp, Glu, Nva and Phe (found exclusively in the amino component). The ratio of these residues given in the Table 1 below are correct to within experimental error thus providing substantiation of the identity of the product.

Table 1. Diagnostic ratios for amino-acids contained in Bpoc(17–37)OPh (**47**)

		amino component				
Carboxyl component		Asp 0.96	Glu 1.05	Nva 0.98	Phe 1.02	Σamino 4.01
Tyr	1.96	2.04 (2)	1.87 (2)	2.00 (2)	1.92 (2)	
Leu	2.03	2.11 (2)	1.93 (2)	2.07 (2)	1.99 (2)	
Σ carboxyl	3.99					1.00 (1)

In the earlier preparations of (**47**) we carried out purification by gel filtration Enzacryl K2[9], the product eluted at (Ve/Vt) = 0.43 as a rather broad peak giving a yield of 60%. The isolated material appeared to be a single spot on tlc in several solvent systems and gave a good amino-acid analysis ($Lys/Orn_{1.99}Asp_{1.04}Ser_{1.60}Glu_{1.05}Gly_{4.01}$-$Ala_{3.00}Nva_{1.04}Leu_{2.00}Tyr_{1.96}Phe_{0.99}$) however electrophoresis, after deprotection, showed the presence of several minor impurities. Material obtained by this route was used initially but the advent of Sephadex LH60 allowed pure material to be obtained, albeit in lower yield. The hydrochloride (**47a**) was obtained from Bpoc(17–37)OPh (**47**) by acidolysis with 0.1 M HCl in TFE/H_2O (9/1) at pH 0.5. This cleavage, carried out in the presence of DMS, was complete in 1 h. giving 90% yield.

The phenyl ester group was cleaved from Adoc(1–16)OPh (**23**) using TFE/H_2O (9:1) as solvent.[5] Hydrogen peroxide was added to the solution maintained at pH 10.5 in the presence of DMS which acted as a scavenger. The cleavage was complete in 30 min. giving the acid (**23a**) in 91% yield. Coupling of the two fragments (**23a**) and (**47a**) by the

DCCI/HONSu method was effected using a mixture of HMPA and DMF as solvent. A second addition of DCCI and HONSu was carried out after 1 day, and the components allowed to react 1 day more before precipitating with water to give the crude product which was best purified by gel filtration on Sephadex LH60 eluting with NMP.[8] As with Bpoc(17–37) OPh (**47**) aggregation was again observed with some material being eluted close to the void volume, however the major portion of the required product was eluted at (Ve/Vt) = 0.30 in a yield of 43%. In earlier work we had used the gel Enzacryl K2[9] giving (Ve/Vt) = 0.45 and obtained 55% yield, but the quality of the compound (**48**) was again inferior to that obtained using Sephadex LH60.

Adoc.Lys(Adoc).Val.Phe.Gly.Orn(Adoc).
Cys(Acm).Glu(OBu^t).Leu.Ala.Ala.Ala.Nle.
Lys(Adoc).Ala.Leu.Gly.Leu.Ala.Gly.Tyr(Bu^t).
Orn(Adoc).Gly.Tyr(Bu^t).Ser(Bu^t).Leu.Gly.Asn.Trp.
Nva.Cys(Acm).Ala.Ala.Lys(Adoc).Phe.Phe.
Glu(OBu^t).Ser(Bu^t).Gly.OPh

(**48**)

The diagnostic ratios for the material isolated from the Sephadex LH60 purification are given in Table 2.

The values appearing in the Table support the proposed structure; the value obtained for tyrosine 1.78 (2) must be due to loss on acid hydrolysis of the large fragment since the Bpoc(17–37)OPh (**47**) which had been used in the preparation had given a satisfactory value for tyrosine.

As a check on the purity of the fully protected Adoc(1–37)OPh we have removed the phenyl ester by the usual method[5] using TFE/H_2O (9:1) and then totally deprotected the peptide using aqueous TFA. By monitoring the cleavage using isoelectric focusing (using a 5% polyacrylamide gel with 3% crosslinking in the presence of pH 9–11 ampholine), it became clear that a 3 hr deprotection with 90% TFA was required to give a homogeneous product (isoelectric point-10.5). Less concentrated TFA or shorter cleavage time resulted in several partially cleaved products being observed. The purity of the deprotected 1–37 peptide was also checked by paper electrophoresis at pH 2.2 and 6.5 and by chromatography on Sephadex CM25 eluting on a gradient from NH_4OAc (0.1 M) pH 6.5 to "$(NH_4)_2CO_3$" (0.4 M pH) 8.5 which showed one major peak. The majority of the synthetic material (**48**) was conserved for use in further synthesis.

Table 2. Diagnostic ratios for amino-acids contained in Adoc(1–37)OPh (**48**).

		amino components			
Carboxyl component		Asp 0.99	Nva 1.05	Tyr 1.78	Σamino 3.82
Val	1.02	1.03 (1)	0.97 (1)	0.57 (0.5)	
Nle	0.96	0.97 (1)	0.92 (1)	0.54 (0.5)	
Σcarboxyl	1.98				0.52 (0.5)

EXPERIMENTAL

The general experimental methods, chromatographic systems and abbreviations are described in an earlier paper in this series.[3]

Scheme 1

Bpoc(17–37)OPh (**47**). Bpoc(17–26)OPh (**32**) (1.0 g, 0.6 mM) as dissolved in a mixture of HMPA (5 ml) and DMA (5 ml). A 50% aqueous soln of DMA (1 ml) was added and the pH brought to 10.5 with 1 M NaOH. 100 vol H_2O_2 (0.06 ml) was added and the pH maintained at the above value for 90 min [consumed 1 M NaOH (0.58 ml)]. The pH was adjusted to 3 with 10% citric acid causing the precipitation of the product, this was completed by the addition of sat NaCl. The ppt was washed with water, $^iPr_2O$ and Et_2O and dried over P_2O_5 giving (**32a**) (0.85 g, 91%), R_f(7)–0.45. The cleavage may also be carried out using 90% aqueous TFE as solvent, in this case no further addition of 1 M NaOH was required after reaching pH 10.5 and the reaction was complete in 15 min. Acidification must be carried out with caution as the buffering action of the TFE makes it easy to overshoot the pH 3 endpoint. The yield using the latter method was (0.93 g, 97%); the product being identical to that obtained above.

Bpoc(27–37)OPh (**43**) (1.0 g, 0.5 mM) was dissolved in 90% aqueous TFE (10 ml) containing DMS (0.5 ml). The pH was brought to 0.5 and maintained at this value for 45 min by the addition of 0.05 M HCl in 90% TFE. The soln was evaporated when acid consumption had ceased and the residue triturated with Et_2O, filtered, washed with Et_2O and dried giving (**43a**) (0.85 g, 100%), R_f(7)–0.4.

The protected peptide acid (**32a**) (1.28 g, 0.8 mM) and the hydrochloride (**43a**) (1.56 g, 0.92 mM) were dissolved in a mixture of HMPA (8 ml) and DMF (8 ml). After cooling to 0° HONSu (221 mg, 1.92 mM) and DCCI (198 mg, 0.96 mM) were added along with a 10% soln of NMM in DMF (0.95 ml, 0.94 mM). The reaction was allowed to reach room temp over 24 hr, then cooled again to 0° and further portions of HONSu (110 mg, 0.96 mM) and DCCI (94 mg, 0.48 mM) added. After stirring had been continued for a further 3 days at room temp the solvent volume was reduced to a minimum and water added. The resulting solid was filtered washed with Et_2O and dried. The crude solid was purified by gel filtration on Sephadex LH60 eluting with NMP, the product (**47**) having (Ve/Vt) = 0.55 was precipitated with water and washed with Et_2O producing the purified product (1.05 g, 35%), m.p. 250° charred, $[\alpha]_D^{25} - 2.1°$ ($c = 1$, HMPA), R_f(12) – 0.30, R_f(18) – 0.7, R_f(23) – 0.9, $Lys/Orn_{2.03}$-$Asp_{0.96}Ser_{1.69}Glu_{1.05}Gly_{4.02}Ala_{3.12}Nva_{0.98}Leu_{2.03}Tyr_{1.96}$-$Phe_{1.02}$, (Found: C, 61.51; H, 7.46; N, 11.20. $C_{168}H_{238}O_{36}N_{26}S.2H_2O$ requires: C, 61.78; H, 7.47; N, 11.15%). A small amount of aggregated material was eluted at the void volume (150 mg, 5%).

Adoc(1–37)OPh (**48**). Adoc(1–16)OPh (**23**) (0.5 g, 0.2 mM) was dissolved in TFE (16 ml) in the presence of DMS (2.5 ml) and the pH adjusted to 10.5 by the addition of 2 M NaOH (2.5 ml). Water (2.0 ml) was added to bring the total water content to 25%, and 100 volume H_2O_2 (0.05 ml) added. After 30 min at pH 10.5 the pH was lowered to 3 by the addition of 10% citric acid and the solvent evaporated. The residue was triturated with water, washed with water and Et_2O then dried giving (**23a**) (0.46 g, 95%), R_f(7) – 0.4, R_f(18) – 0.15. No trace of unhydrolysed ester was found. However if the hydrolysis

was carried out in an aqueous solution of HMPA and DMA in a similar manner the yield was (87%) and the trace of uncleaved phenyl ester remains, even after 1.5 hr.

Bpoc(17–37)OPh (**47**) (0.33 g, 0.1 mM) was dissolved in 90% TFE (25 ml) in the presence of DMS (1 ml). The pH was brought to 0.5 by the addition of 0.1 M HCl in 90% TFE, and maintained at this value until the consumption of acid had ceased (1 hr). The solvent was evaporated and the residue triturated with Et_2O, filtered and dried to give (**47a**) (0.31 g, 90%), $R_f(18)-0.3$.

The protected peptide acid (**23a**) (243 mg, 0.099 mM) and the hydrochloride (**47a**) (275 mg, 0.091 mM) were dissolved in a mixture of HMPA (3 ml) and DMF (3 ml) and cooled to 0°. HONSu (28 mg, 0.24 mM) and DCCI (24 mg, 0.12 mM) were added followed by a 2% soln of NMM in DMF (0.46 ml, 0.091 mM). The mixture was stirred for 24 hr at room temp then cooled again to 0° when HONSu (15 mg) and DCCI (12 mg) were added. After stirring for a further 3 days at room temp. the solvent volume was reduced to a minimum by evaporation and the product precipitated with water. This material was washed with Et_2O and dried, then purified by gel filtration on Sephadex LH60 eluting with NMP. A very small quantity of product was isolated as an aggregate eluting at the void volume. The product (**48**) with (Ve/Vt) = 0.30 was obtained by precipitation with water and washing with Et_2O yielded (212 mg, 43%), m.p. 250° (charred), $[\alpha]_D^{24} + 9.0°$ ($c = 1$, TFE), $R_f(12)-0.4$, $R_f(18)-0.7$, $R_f(23)-0.9$, Lys/$Orn_{5.08}Asp_{0.99}Ser_{1.57}$-$Glu_{2.16}Gly_{5.92}Ala_{7.02}Val_{1.02}Nva_{1.05}Nle_{0.96}Tyr_{1.78}Phe_{2.04}$, (Found: C, 59.99; H, 7.64; N, 11.91. $C_{276}H_{416}O_{61}N_{46}S_2 \cdot 5H_2O$ requires: C, 60.18; H, 7.79; N, 11.70%).

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