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PEPTIDES—XXXX

SYNTHESIS OF THE 68–75 FRAGMENT OF A LYSOZYME ANALOGUE

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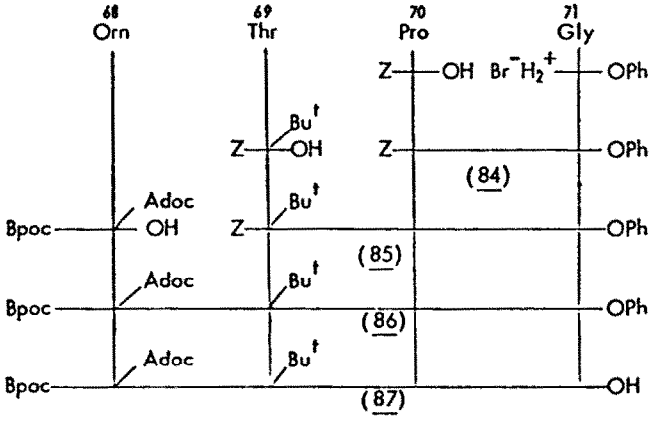
Abstract —The 68–75 subfragment has been prepared by a 4 + 4 fragment condensation using DCCI/HOBt. The constituent 68–71 and 72–75 fragments being prepared stepwise by the pivalic mixed anhydride method. The octapeptide prepared by this approach was shown to be identical with that synthesised by a solid phase method using a phenolic polymer as the support.

The work described in this paper relates to the synthesis of the 68–75 fragment of our lysozyme analogue[1] by application of the general methodology described in an earlier paper in this series.[2] The amino acid sequence of this fragment is

Orn.	Thr.	Pro.	Gly.	Ser.	Ala.	Asn.	Gly.
68			71				75

Synthesis of the fragment has been accomplished by two totally independent methods. The first, involving solid phase synthesis utilised a phenolic polymer with the Bpoc group for *N*-terminal protection, has been described previously.[3] In the solid phase synthesis[3] the Bpoc-glycine phenolic resin ester was prepared by reacting the caesium salt of Bpoc–glycine with deacylated resin. After acetylation Boc–Asn–OH, Boc–Ala–OH and Bpoc–Ser (Bu^t)–OH were added using DCCI to achieve the successive coupling reactions. The remaining amino acids were added as their Bpoc derivatives because of the presence of the acid-labile side chain protection on Ser-72. When the octapeptide had been assembled, the peptide resin was carefully washed and dried and the phenyl ester cleavage was then effected in the presence of hydrogen peroxide using a dioxan/water (9:1) mixture, whilst maintaining the pH at 10.5 by the addition of 1 molar tetramethylguanidine. After the cleavage was complete (50 minutes) as evidenced by cessation of base uptake,[4] acidification to pH 3.5 with potassium hydrogen sulphate gave the crude protected octapeptide **91**. The numbering of compounds follows from the sequence used in earlier papers. The trace impurities were removed by gel filtration on Sephadex LH20 in DMF, the required product eluting with a (Ve/Vt) ratio of 0.39. Isolation of the purified peptide by precipitation from DMF/water gave the product as a white powdery material.

For large scale synthesis we have also prepared this fragment by a 4 + 4 fragment coupling involving union at the Gly-71 residue to avoid racemisation. The (68–71) and (72–75) tetrapeptides required for the fragment condensation synthesis of the (68–75) fragment were prepared in a stepwise manner. Z–Pro–OH was coupled to glycine phenyl ester hydrobromide by the pivalic mixed anhydride method as shown in Scheme 1, giving the crystalline dipeptide **84** in 68% yield. This peptide was then deprotected by catalytic hydrogenolysis in the presence of *p*-toluenesulphonic acid to



Scheme 1. Synthesis of the protected (68–71) fragment (**87**)

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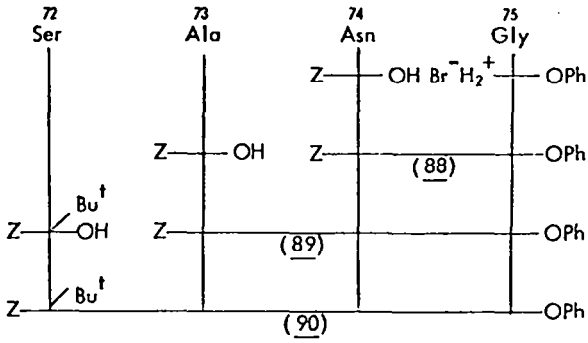


Scheme 2. Synthesis of the protected (72–75) fragment (**90**)

give the corresponding salt as a gum. Z–Thr (Bu^t)–OH (obtained from the DCHA salt) was again coupled by the pivalic mixed anhydride method giving the tripeptide **85** as an oil in 84% yield. Unfortunately all attempts at crystallization were unsuccessful, however TLC showed that this material was sufficiently pure for use in the next stage of the synthesis. The salt resulting from hydrogenolysis of the protected tripeptide **85** was coupled with Bpoc–Orn (Adoc)–OH[5] by the pivalic mixed anhydride method and the final fully protected peptide was subjected to gel filtration on Sephadex LH20 in DMF. The phenyl ester of the protected peptide **86** was rapidly cleaved by treatment with sodium hydroxide in the presence of hydrogen peroxide at pH 10.5 using acetone/water (4:1) as the solvent. The homogeneous tetrapeptide acid **87** was obtained as a white powder in 89% yield.

The (72–75) subfragment (see Scheme 2) was then assembled by stepwise addition of Z–Asn–OH, Z–Ala–OH, and Z–Ser (Bu^t)–OH employing in each case, the pivalic mixed anhydride procedure using NMM as base. The crude protected tetrapeptide **90** was sufficiently pure after reprecipitation from DMF with water to be utilized in the subsequent fragment coupling.

The benzyloxycarbonyl function was then removed from Z.(72–75).OPh by catalytic hydrogenolysis in the usual way to give the resulting *p*-toluenesulphonate salt which was then coupled with the tetrapeptide acid **87** by the DCCI/HOBt method.[6] In this particular example it was found that HOBt was superior to HONSu and that no cleavage of the Bpoc protecting group was observed. The resulting octapeptide **91** was purified by gel filtration on Sephadex LH20 eluting with DMF to give a yield of 69%. The product **91** was shown to be homogeneous by the usual criteria and by electrophoresis at pH 2.1 of the fully deprotected octapeptide.

The identity of the peptide **91** produced by the solid phase method and the solution method was readily ascertained and clearly demonstrates the potential of the phenolic polymer in peptide synthesis; the advantage over many of the obvious alternatives being that the protected peptide may be removed from the resin and purified without loss of the main-chain and side-chain amino-protecting groups. The resulting acid (or hydrazide which would result from hydrazinolysis of the peptide phenyl ester linkage) could then be used to build up larger fragments either in solution or on the polymeric support.

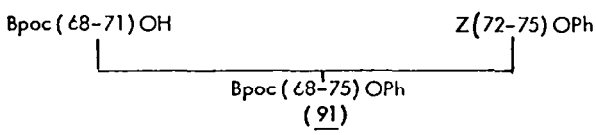
EXPERIMENTAL

The abbreviations, TLC systems and general experimental methods are those described in earlier papers in this series.

Z–Pro–Gly–OPh **84**. Z–Pro–OH (24.9 g, 100 mM) and NMM (10.1 g, 100 mM) were dissolved in CH_2Cl_2 (100 ml) and the solution cooled to −10°. Pivaloyl chloride (12.0 g, 100 mM) was added and after activation (20 min) a precooled solution of $Br^-H_2^-$—Gly–OPh (23.2 g, 100 mM) and NMM (10.1 g, 100 mM) were added. The reaction mixture was stirred overnight at room temperature then evaporated and the residue dissolved in EtOAc. This solution was washed with acid and base then dried and evaporated. The product **84** was crystallised from EtOAc/petroleum ether giving (25.8 g, 68%), m.p. 74–75°, $[\alpha]_D^{24}$ − 63.6° (c = 1, DMF), $R_f(24)$ − 0.5, $R_f(18)$ − 0.6, $Pro_{0.97}Gly_{1.03}$. (Found: C, 66.22; H, 5.94; N, 7.63. $C_{21}H_{22}N_2O_5$ requires: C, 65.96; H, 5.80; N, 7.33%.)

Z–Thr(Bu^t)–Pro–Gly–OPh **85**. The dipeptide **84** (24.9 g, 65 mM) and Tos.OH.H_2O (12.4 g, 65 mM) were dissolved in DMF (50 ml) and hydrogenolysed over 10% Pd/C (3.25 g) for 4 h, then worked up in the usual way to give a gum. Z–Thr (Bu^t)–OH.DCHA (33.1 g, 65 mM) was converted in the usual way to the free acid which was dissolved in EtOAc (100 ml) and cooled to −10°. NMM (6.58 g, 65 mM) and pivaloyl chloride (7.8 g, 65 mM) were added and the solution stirred for 20 minutes. A precooled solution of the *p*-toluene-sulphonate of the amino-component in DMF (50 ml) was added followed by NMM (6.58 g, 65 mM) and the reaction stirred overnight at room temperature. Removal of the solvent and work up in the usual manner gave a homogeneous oil which defied all attempts at crystallisation (29.4 g, 84%) $R_f(2)$ − 0.5, $R_f(26)$ − 0.3, $Thr_{0.87}Pro_{1.04}Gly_{1.00}$.

Bpoc–Orn(Adoc)–Thr(Bu^t)–Pro–Gly–OPh **86**. The protected tripeptide **85** (29.4 g, 54.6 mM) and Tos.OH.H_2O



Scheme 3. Synthesis of Bpoc (68–75) OPh (**91**).

(10.4 g, 54.6 mM) were dissolved in DMF (100 ml) and hydrogenolysed for 4 h in the presence of 10% Pd/C (2.75 g). Work up in the usual way gave the salt as an oil. Bpoc–Orn (Adoc)–OH (34.0 g, 62 mM) and NMM (6.2 g, 61 mM) were dissolved in EtOAc (100 ml) and cooled to −10°. Pivaloyl chloride (7.3 g, 61 mM) was added and 20 min allowed for activation. A precooled solution of the *p*-toluene-sulphonate in DMF (50 ml) was added followed by NMM (5.5 g, 54 mM) and the reaction mixture stirred overnight at room temperature. The solvent was evaporated and the residue applied directly to a column of Sephadex LH20 and eluted with DMF. The product was isolated by combination of fractions with (Ve/Vt) = 0.44 to afford **86** (38.5 g, 77%), m.p. 105–107°, $[\alpha]_D^{24}$ − 25.5° (c = 1, DMF), $R_f(22)$ − 0.4, $R_f(9)$ − 0.7, $Orn_{0.94}Thr_{1.05}Pro_{0.94}Gly_{1.06}$. (Found: C, 66.73; H, 7.27; N, 7.66. $C_{53}H_{69}N_5O_{10}.H_2O$ requires: C, 66.72; H, 7.50; N, 7.34%.)

Bpoc.(68–71)*.OH* **87**. The peptide phenyl ester **86** (8.9 g, 9.5 mM) was dissolved in acetone/H_2O (4:1) (50 ml) and the pH brought up to 10.5 by the addition of 1 M NaOH. 100 vol H_2O_2 (1.0 ml, 10 mM) was added and the pH maintained at 10.5 by the addition of 1 M NaOH (a total of 10.0 ml was required), after 15 min base uptake ceased indicating completion of the hydrolysis. The acetone was evaporated and the solution diluted with water and washed with Et_2O. The aqueous phase was cooled to 10° and acidified to pH 3.5 with 10% citric acid to give a solid which was extracted into EtOAc and the solution was washed with water and dried. Evaporation gave the pure tetrapeptide acid **87** after trituration with water and drying (8.3 g, 89%), m.p. 118–121°, $R_f(23)$ − 0.9, $R_f(31)$ − 0.8, (Found: C, 63.62; H, 7.53; N, 8.08. $C_{47}H_{65}N_5O_{10}.1\frac{1}{2}H_2O$ requires: C, 63.64; H, 7.56; N, 7.90%.)

Z–Asn–Gly–OPh **88**. Z–Asn–OH (26.6, 100 mM), pyridine (8 ml, 100 mM) and NMM (10.1 g, 100 mM) were dissolved in DMF (75 ml) and cooled to −10°. Pivaloyl chloride was added and 20 min allowed for activation. A precooled solution of $Br^-H_2^+$—Gly–OPh (23.2 g, 100 mM) in DMF (75 ml) was added followed by NMM (10.1 g, 100 mM) and the reaction permitted to reach room temperature overnight. Evaporation gave an oily residue which was solidified by addition of 1 M $NaHCO_3$ solution. The solid was washed on the sinter alternately with 1 M AcOH and 1 M $NaHCO_3$ then finally with Et_2O. Drying *in vacuo* gave the required product **88** (33.0 g, 83%), m.p. 171–173°, $[\alpha]_D^{24}$ − 10.5° (c = 1, DMF), $R_f(17)$ − 0.7, $R_f(23)$ − 0.8. (Found: C, 59.84; H, 5.31; N, 10.60. $C_{20}H_{21}N_3O_6$ requires: C, 60.14; H, 5.30; N, 10.52%.)

Z–Ala–Asn–Gly–OPh **89**. Compound **88** (32.0 g, 80 mM) and Tos.OH.H_2O (15.2 g, 80 mM) were dissolved in DMF (100 ml) and hydrogenolysed overnight over 10% Pd/C (4.0 g). Work up in the usual way gave the salt as a gum. Z–Ala–OH (17.9 g, 80 mM) and NMM (8.09 g, 80 mM) were dissolved in CH_2Cl_2 (100 ml) and cooled to −10°. Pivaloyl chloride (9.6 g, 80 mM) was added and 20 min allowed for activation. A solution of the *p*-toluene-sulphonate of the amino-component from above was dissolved in DMF (75 ml) and added to the solution of the mixed anhydride with NMM (8.1 g, 80 mM) and the reaction mixture stirred at room temperature overnight. The solvent was evaporated and the residue solidified on addition of brine. Washing with acid and base on the sinter gave the protected tripeptide **89** (18.7 g, 50%), m.p. > 250°, $[\alpha]_D^{24}$ − 5.8° (c = 1, DMF), $R_f(17)$ − 0.6, $R_f(28)$ − 0.9, $Asp_{1.01}Gly_{1.01}Ala_{0.98}$. (Found: C, 58.42; H, 5.50; N, 11.95. $C_{23}H_{26}N_4O_7$ requires: C, 58.72; H, 5.57; N, 11.91%.)

Z–Ser(Bu^t)–Ala–Asn–Gly–OPh **90**. The tripeptide **89** (15.1 g) 32.1 mM) and Tos.OH.H_2O (6.1 g, 32.1 mM) were dissolved in DMF (250 ml) and hydrogenolysed for 4 h in the presence of 10% Pd/C (1.6 g). Filtration and removal of solvent gave a gum which was used in the coupling reaction. Z–Ser (Bu^t)–OH (10.2 g, 34.6 mM) and NMM (3.5 g, 34.6 mM) were dissolved in EtOAc (70 ml) and cooled to −10°. Pivaloyl chloride (4.2 g, 34.6 mM) was added and the solution stirred for 20 min. A precooled solution of the *p*-toluene-sulphonate of the amino-component in DMF (150 ml) was added along with NMM (3.2 g, 31.5 mM) and the reaction allowed to reach room temperature overnight. Removal of solvent gave a solid which was triturated with 10% citric acid, 1 M $NaHCO_3$ solution and water, then reprecipitated from DMF by the addition of water giving **90** (12.0 g, 64%), m.p. 198–201°, $[\alpha]_D^{24}$ −4.30° (c = 1, DMF), $R_f(22)$ − 0.6, $R_f(28)$ − 0.9, $Asp_{1.01}Ser_{0.81}Gly_{0.97}Ala_{1.05}$. (Found: C, 58.11; H, 6.29; N, 11.48. $C_{30}H_{39}N_5O_9.\frac{1}{2}H_2O$ requires: C, 57.87; H, 6.48; N, 11.25%.)

Bpoc.(68–75)*.OPh* **91**. The protected tetrapeptide **90** (11.2 g, 18.3 mM) and Tos.OH.H_2O (3.5 g, 18.3 mM) were dissolved in DMF (200 ml) and hydrogenolysed over 10% Pd/C (1.0 g) for 5 h. Filtration and evaporation gave the corresponding *p*-toluene-sulphonate as a gum. This gum was dissolved in DMF (100 ml) and the peptide acid **87** (3.3 g, 3.8 mM) added. After cooling to −5° HOBt (1.0 g, 7.7 mM), DCCI (790 mg, 3.8 mM) and NMM (388 mg, 3.8 mM) were added and the reaction mixture stirred overnight. The reaction mixture was then cooled to −5° and additional portions of HOBt (207 mg, 1.5 mM) and DCCI (158 mg, 0.77 mM) added. After stirring for a further 2 days the solution was purified by gel filtration on Sephadex LH20 eluting with DMF. The required product **91** eluted with (Ve/Vt) = 0.41, isolation gave (3.51 g, 69%), m.p. 128–131°, $[\alpha]_D^{24}$ − 12.7° (c = 1, DMF, $R_f(22)$ − 0.7, $R_f(23)$ − 0.8, $Orn_{0.95}Asp_{1.03}Thr_{0.96}Ser_{0.93}Pro_{1.04}Gly_{2.06}Ala_{1.04}$. (Found: C, 59.53; H, 7.15; N, 10.22. $C_{69}H_{96}N_{10}O_{16}.2\frac{1}{2}H_2O$ requires: C, 59.61; H, 7.45; N, 10.25%.)

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