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PEPTIDES-XXXXI

SYNTHESIS OF THE 38-75 FRAGMENT OF A LYSOZYME ANALOGUE

I. J. GALPIN, B. K. HANDA, A. G. JACKSON, G. W. KENNER,† P. MCDOWELL, S. R. OHLSEN and R. RAMAGE‡
The Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, England

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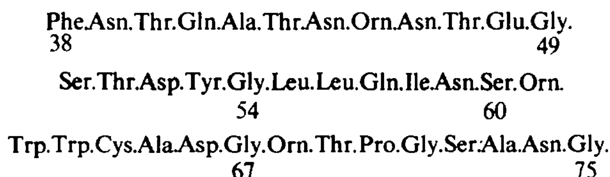
Abstract—Two routes to the 38-75 fragment of a lysozyme analogue are reported. The two syntheses use the protected peptide fragments 38-49, 50-54, 55-60, 61-67 and 68-75; the routes differ in the order of fragment combination with the DCCI/HONSu method being the preferred means of linking the fragments. Sephadex LH 60 proved to be a particularly useful matrix for the gel filtration of the large fragments involved in the synthesis.

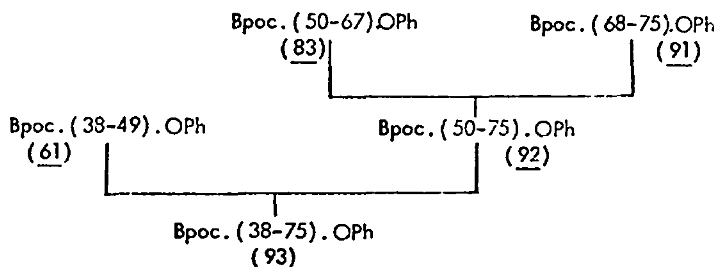
The second major subfragment of the lysozyme analogue[1] under investigation as a target for synthesis spans the region from residue -38 to residue -75 and has the sequence shown below:



In accordance with our initial strategy,[2] it may be seen that of the four glycine residues, three would provide adequate fragmentation points to enable reasonable size fragments to be assembled and the fourth at residue -75 would form the carboxyl-terminus of the whole (38-75) fragment ready for racemisation-free incorporation into the (1-129) major fragment. The position of the glycine residues thus dictated that the fragments synthesised should be (38-49), (50-54), (55-67) and (68-75). However, in the event the (56-67) fragment was severed at serine -60 in order to enable the (55-67) fragment to be assembled more readily. It is of interest to reflect that at the time this synthetic route was devised, few methods were available for racemisation-free coupling, although during the early stages of this work it rapidly became apparent that the DCCI/HONSu method[3] and the DCCI/HOBt method[4] were suitable alternatives to the azide procedure for achieving racemisation free coupling. Notwithstanding these important developments in peptide methodology we would still favour C-terminal glycine in the coupling of large fragments.

Preceding papers in this series have described the synthesis of the (38-49),[5] (50-54),[6] (55-60),[6] (61-67)[6] and (68-75)[7] fragments. The second paper in this group described the synthetic route to Bpoc.(50-67).OPh **83**. The compound numbering sequence follows that established in earlier parts of this series. This intermediate fragment was constructed by combination of the (50-54) portion with the (55-67) fragment which had itself been obtained by coupling at residue -60.

Our first synthesis (see Scheme 1) of Bpoc.(38-75).OPh **93** used the fragment **83** as one of



Scheme 1. The first route to the protected 38-75 fragment (**93**).

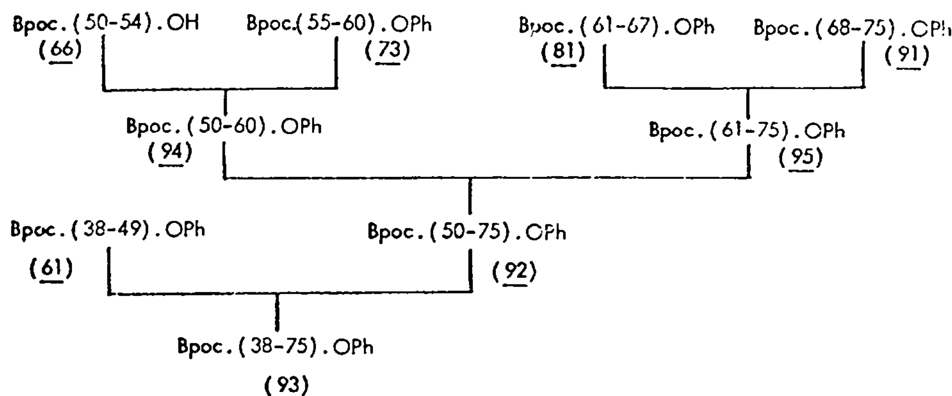
†Deceased, 25th June 1978.
‡Present address: Chemistry Department, UMIST, Sackville Street, Manchester.

its major constituents. The phenyl ester function[8] was hydrolysed in 8 minutes by treatment with hydrogen peroxide at pH 10.5 using HMPA/water as solvent with dimethyl sulphide present to prevent untoward oxidation of the peptide. More recently, we have used trifluoroethanol/water (9:1) as a solvent for this cleavage as it aids the isolation of the product and it is readily removed by evaporation. A comparable yield was obtained in both cases. The hydrochloride of the (68–75) fragment was obtained by removing the Bpoc function from peptide **91** by treatment with 0.05 molar HCl in 90% trifluoroethanol[9]. Coupling of these two fragments was effected by DCCI/HONSu using HMPA and DMF as a mixed solvent system and required 4 days to reach completion. Although purification was achieved initially by gel filtration chromatography on Sephadex LH20, separation of the (50–67) fragment from the product was difficult since both compounds eluted close to the void volume of the column. More recently, the use of Sephadex LH 60 eluting with DMF[10] has allowed us to achieve a much better separation of these two peptides. The Bpoc.(50–75).OPh **92** obtained by this route was then subsequently used in the formation of the (38–75) fragment.

The Bpoc.(38–49).OPh **61** which had been obtained by the route outlined in an earlier paper[5] was subjected to phenyl ester cleavage using HMPA/water as solvent. The pH being adjusted to 10.5 by the addition of 1 M NaOH and the cleavage being carried out in the presence of 100 vol. hydrogen peroxide. After 3 min uptake of base ceased indicating rapid hydrolysis. The solution was then cooled and the pH adjusted to 3.5 with citric acid, thus causing the precipitation of the corresponding free acid. In this case 90% trifluoroethanol could not be used in the reaction medium as the solvent system was sufficiently acidic to cause slight cleavage of the Bpoc protecting function on residue -38. The hydrochloride of the (50–75) portion was obtained by acidolysis of the corresponding Bpoc compound **92**; the cleavage being achieved using HCl in trifluoroethanol. It should be noted that the rather insoluble peptide **92** required 5 hours for complete dissolution at 37° when using trifluoroethanol as the solvent, however, on addition of the acid only 20 min were required for complete cleavage of the Bpoc group. The two fragments were then linked using DCCI/HONSu as the coupling agent in HMPA/DMF as solvent mixture to achieve maximum concentration of reactants. This coupling required 5 days to reach completion as indicated by the absence of a fluorescamine positive spot on TLC. In our earlier preparations we used Sephadex G75 swollen in HMPA/water (95:5)[11] in order to achieve the purification of this fragment whereupon a 50% yield was obtained after rechromatography of the appropriate fractions on Sephadex G10. An improved separation was ultimately achieved using Sephadex LH 60 eluting with *N*-methylpyrrolidine (NMP).[10] In this way we achieved our first synthesis of the fully protected Bpoc.(38–75) fragment **93**. Clearly, however, many difficulties were encountered in the purification of intermediate peptides, and it was for this reason that we embarked on an alternative synthesis using the same fragments although combining them in a different manner.

The second approach, which is outlined in Scheme 2, again used the fragment (50–75), however, in this case it is constructed by combination of the approximately equal sized fragments (50–60) and the (61–75) which necessitated coupling with the racemisation-prone residue, serine-60.

Bpoc cleavage from the available fragment Bpoc.(55–60).OPh **73** was achieved using 0.1 M HCl in 90% aqueous trifluoroethanol the cleavage being complete in 30 minutes. The hydrochloride thus obtained was coupled with the Bpoc.(50–54).OH **66** by the DCCI/HONSu method using HMPA/DMF as a solvent over a period of 3 days which was required to bring about complete coupling. In this case gel filtration on Sephadex LH20 afforded adequate separation of starting materials and products and led to the isolation of Bpoc.(50–60).OPh **94**. The Bpoc.(61–75).OPh **95**, was obtained by linking the already available fragments **81** and **91**. The free acid of the former compound was obtained by phenyl ester cleavage in 90% trifluoroethanol in the usual way, and the hydrochloride of **91** was obtained by Bpoc cleavage in 90% aqueous trifluoroethanol. The two fragments were linked using DCCI/HONSu as the coupling method employing only DMF as the solvent and the coupling required a total of 5 days to reach completion. Gel filtration on Sephadex LH 60 eluting with DMF permitted the isolation of the fully protected fragment **95** in reasonable yield, then the Bpoc protection was removed by acidolysis in 90%



Scheme 2. The second route to the protected 38–75 fragment (**93**).

aqueous TFE in the usual way to give the hydrochloride which was used directly in the preparation of **92**. The phenyl ester function at the Ser(Bu^t) terminus was cleaved from Bpoc.(50–60).OPh **94** in 1 hour using 90% aqueous trifluoroethanol as solvent at pH 10.3. The acid obtained was then coupled with the hydrochloride described above by the DCCI/HONSu method using HMPA/DMF (1:1) as the solvent. A 6 day coupling and a subsequent purification on Sephadex LH 60 eluting with DMF gave the required fully protected peptide Bpoc.(50–75).OPh **92**, again in reasonable yield. In this case the product **92** was well resolved from any of the starting materials; in contrast to the purification experienced in the route outlined in Scheme 1. Clearly then, the route shown in Scheme 2 is a superior method of preparing the fragment **92**.

Coupling between the (38–49) and (50–75) fragments was then carried out in a manner similar to that described in Scheme 1. In this case, however, the product was immediately chromatographed on Sephadex LH 60 and eluted with NMP; the required product being well resolved once again from any of the starting materials. We have thus synthesised the Bpoc.(38–75).OPh fragment **93** by two separate approaches and it was immediately reassuring to find that the material from either route had similar physical properties. The melting point, optical rotation and thin layer chromatographic behaviour was similar for compounds prepared by either method. In addition, total deprotection of the material prepared by the second route, followed by chromatography on Sephadex G25 eluting with 50% acetic acid gave a peptide which had a highly satisfactory amino-acid analysis. The Bpoc.(38–75).OPh fragment **93** was thus prepared ready for use in the final stages of our long-term plan for the synthesis of a lysozyme analogue.

EXPERIMENTAL

The abbreviations, TLC systems and general experimental techniques have been reported in an earlier paper in this series, except for TLC system (35) $CHCl_3$/MeOH/AcOH/Py (90:30:5:5).

Bpoc.(50–75).OPh **92** (*a*) *Phenyl ester cleavage from* **83**. (2.94 g, 1 mM) dissolved in HMPA (40 ml) to which water (20 ml) was then added, employed dimethylsulphide (DMS) (4 ml), at pH 10.5 (arrived at with 1 M NaOH solution) and 100 vol H_2O_2 (0.11 ml). The cleavage was allowed to run to completion (8 minutes) with 1 M NaOH being added from a pH stat. The solution was cooled to 0° and the pH adjusted to 3.5 with 10% citric acid to precipitate the peptide which was filtered and washed with IPA and Et_2O giving (2.86 g, 100%). When the cleavage was carried out in TFE/H_2O (9:1) a yield of 92% was obtained.

(b) *Bpoc cleavage from* **91**.*Bpoc.(68–75).OPh* **91** (3.97 g, 3 mM) was dissolved in 0.05 M HCl in DMF (120 ml). After 20 min TLC indicated that removal of the amino-protection was complete, and the solvent was evaporated. The resulting solid was triturated with Et_2O and EtOAc giving the corresponding hydrochloride (3.30 g, 99%). This cleavage has also been carried out in 92% yield using 0.05 M HCl In TFE/H_2O (9:1).

(c) *Fragment coupling.* The peptide acid obtained from (a) above (2.86 g, 1 mM) and the hydrochloride from (b) (2.25 g, 2 mM) were dissolved in a mixture of HMPA (20 ml) and DMF (6 ml) and cooled to −8°. NMM (203 mg, 2 mM) in DMF (1 ml) and HONSu (230 mg, 2 mM) in DMF (1.5 ml) were added followed by a solution of DCCI (226 mg, 1.1 mM) in DMF (1.5 ml). After 2 h at room temperature the reaction was cooled and further portions of DCCI (115 mg) and HONSu (113 mg) were added. The reaction was stirred at room temperature for 4 days and then the reaction mixture applied directly to Sephadex LH 20 eluting with DMF. The required product eluted with (Ve/Vt) = 0.31 although separation from the (50–67) fragment was not ideal. Isolation of the product **92** gave (3.12 g, 79%), m.p. > 340°, $[\alpha]_D^{19}$ + 0.6° (c = 1.5, HMPA), R_f(3) – 0.7, R_f(35) – 0.9, $Orn_{2.01}Trp_{1.97}Asp_{3.93}Thr_{1.96}Ser_{2.94}Glu_{1.00}Pro_{0.99}Gly_{4.00}Ala_{1.99}Ile_{0.98}Leu_{2.00}Tyr_{0.98}$. (Found: C, 57.03; H, 7.61; N, 12.00. $C_{198}H_{290}N_{34}O_{47}S.12H_2O$ requires: C, 57.34; H, 7.63; N, 11.48%). An improvement in separation from the (50–67) fragment was achieved if the material from the LH 20 purification was rechromatographed on Sephadex LH60 eluting with DMF. The product **92** in this case eluted at (Ve/Vt) = 0.52 and has the amino acid analysis: $Orn_{1.92}$ $Asp_{4.05}$ $Thr_{1.81}$ $Ser_{2.57}$ $Glu_{1.09}$ $Pro_{1.05}$ $Gly_{3.91}$ $Ala_{2.06}$ $Ile_{0.94}$ $Leu_{1.99}$ $Tyr_{0.87}$.

Bpoc.(38–75).OPh **93**. (*a*) *Phenyl ester cleavage from* **61**. Bpoc.(38–49).OPh **61** (1.22 g, 0.6 mM) was dissolved in HMPA (24 ml) and water (6 ml) carefully added. The pH was brought to 10.5 with 1 M NaOH and 100 vol. H_2O_2 (0.1 ml) added, base uptake was rapid and after 3 min no more 1 M NaOH was consumed. The solution was cooled and the pH adjusted to 3.5 by the addition of ice-cold saturated citric acid solution. The precipitated free acid was washed with water, IPA and Et_2O giving (1.12 g, 95%).

(b) *Bpoc cleavage from* **92**. Bpoc.(50–75).OPh **92** (1.00 g, 0.25 mM) was dissolved in TFE (25 ml) over 5 h at 37°. The solution was cooled to 20° and DMS (1 ml) and 0.3 M HCl in TFE (5 ml) was added. The cleavage was complete after 20 min as indicated by TLC whereupon the solution was evaporated and the residue triturated with Et_2O giving the corresponding hydrochloride (919 mg, 96%).

(c) *Fragment coupling.* Bpoc.(38–49).OH from (a) (976 mg, 0.5 mM) and the hydrochloride from (b) (919 mg, 0.25 mM) were dissolved in HMPA (60 ml). Six hours at 35° was required to obtain a clear solution. This solution was cooled to 0° and NMM (25.5 mg, 0.25 mM) in DMF (10 ml), HONSu (90 mg, 0.78 mM) in DMF (1.5 ml) and DCCI (107 mg, 0.52 mM) in DMF (2 ml) added. After 2 h the reaction mixture was recooled and further portions of HONSu (45 mg) and DCCI (53 mg) added. After 5 days brine was added to precipitate the crude product, this was then washed with IPA and dried. Gel filtration on Sephadex G75 in HMPA/H_2O (95:5) gave the purified fragment eluting with (Ve/Vt) = 0.46 followed by isolation by chromatography on Sephadex G10 in DMF gave **93** (700 mg, 50%), m.p. > 340°, $[\alpha]_D^{19}$ – 58.4° (c = 1, HMPA), R_f(31) – 0.5, R_f(7) – 0,8, $Orn_{3.40}$Trp-$Asp_{7.02}Thr_{4.57}Ser_{2.26}Glu_{3.22}Pro_{0.96}Gly_{4.88}Ala_{2.97}Ile_{0.99}Leu_{1.98}Tyr_{0.84}Phe_{1.02}$. (Found: C, 57.64; H, 7.72; N, 12.23. $C_{278}H_{419}N_{51}O_{70}S.9H_2O$ requires: C, 57.67; H, 7.61; N, 12.34%.) An improved separation was achieved using Sephadex LH60 eluting with NMP, the product eluting with Ve/Vt) = 0.41.

Bpoc.(50–60)OPh **94**. (a) *Bpoc cleavage from Bpoc.(55–60).OPh.* Bpoc.(55–60).OPh **73** (1.50 g, 1.4 mM) was dissolved in TFE/H_2O (9:1) (150 ml). The pH of the hazy solution was adjusted to 0.5 with 0.1 M HCl in 90% TFE and TLC showed that after 30 minutes the cleavage was complete. The solvent was evaporated and the residue triturated with Et_2O and dried yielding the corresponding homogeneous hydrochloride (1.08 g, 94%).

(b) *Fragment coupling.* Bpoc.(50–54).OH **66** (1.52 g, 1.5 mM) and the hydrochloride from (a) (1.08 g, 1.35 mM) were dissolved in HMPA/DMF (3:2) (25 ml), HONSu (305 mg, 2.6 mM) was added and the solution cooled to −20°. DCCI (322 mg, 1.56 mM) and NMM (0.115 ml, 1.35 mM) were added and the reaction mixture stirred for 24 h at room temperature. Further portions of DCCI (1.53 mg, 0.75 mM) and HONSu (153 mg, 1.3 mM) were added at −20°. After 3 days at room temperature the reaction was applied directly to Sephadex LH 20 and eluted with DMF. The product **94**

eluted with (Ve/Vt) = 0.38, isolation yielding (1.19 g, 66%), m.p. 260–265° (dec), $[\alpha]_D^{24}$ – 43.0° (c = 1, HMPA), $R_f(12)$ – 0.6, $R_f(7)$ – 0.7, $Asp_{1.96}Thr_{0.80}Ser_{1.43}Glu_{0.97}Gly_{0.94}Ile_{1.03}Leu_{2.10}Tyr_{0.95}$. (Found: C, 61.58; H, 7.88; N, 10.27. $C_{94}H_{141}N_{13}O_{22}.1\frac{1}{2}H_2O$ requires C, 61.62; H, 7.92; N, 9.94%.)

Bpoc.(61–75).OPh **95**. (a) *Phenyl ester cleavage from Bpoc.(61–67).OPh.* Bpoc.(61–67).OPh **81** (2.43 g, 1.6 mM) was dissolved in TFE/H_2O (9:1) (50 ml) together with DMS (8 ml) and 100 vol. H_2O_2 (0.5 ml). The pH was brought to and maintained at 10.5 for 30 min by the addition of 1 M NaOH, at this stage TLC indicated the cleavage to be complete. The pH was then carefully adjusted to 4 with 10% citric acid and the solvent evaporated. The residue was triturated with water and ether, then dried giving the free acid (1.90 g, 82%).

(b) *Bpoc cleavage from Bpoc.(68–75).OPh.* The protected fragment **91** (2.0 g, 1.5 mM) was dissolved in TFE/H_2O (9:1) (30 ml), DMS (1 ml) added and the pH brought to 0.5 by the addition of 0.1 M HCl in 90% aqueous TFE. After 30 min TLC indicated the cleavage to be complete, and the solvent was evaporated to give a residue which was triturated with Et_2O and dried to give the hydrochloride (1.62 g, 99%).

(c) *Fragment coupling.* Bpoc.(61–67).OH (1.76 g, 1.25 mM), $Cl^-H_2^+$.(67–75).OPh (1.29 g, 1.15 mM) and HONSu (278 mg, 2.4 mM) were dissolved in DMF (10 ml) and cooled to –20°. DCCI (308 mg, 1.5 mM) and NMM (0.12 ml, 1.2 mM) were added and the reaction mixture stirred at room temperature for 24 h. Further portions of DCCI (150 mg) and HONSu (150 mg) were added to the recooled solution and the reaction allowed to continue for 4 days at room temperature. The reaction mixture was filtered and concentrated to (5 ml) then applied directly to a Sephadex LH 60 column eluting with DMF and gave the product **95** (1.61 g, 57%), m.p. 166–170°, $[\alpha]_D^{24}$ – 20.0° (c = 1.9, DMF), $R_f(23)$ – 0.9, $R_f(7)$ – 0.7, $Orn_{1.86}Asp_{2.04}Thr_{0.95}Ser_{0.97}Pro_{0.99}Gly_{2.97}Ala_{1.99}$. (Found: C, 59.68; H, 6.85; N, 11.84. $C_{126}H_{171}N_{21}O_{28}S.4H_2O$ requires C, 59.77; H, 7.12; N, 11.62%.)

Bpoc.(50–75).OPh **92**. (a) *Phenyl ester cleavage from Bpoc.(50–60).OPh.* Bpoc.(50–60).OPh (1.88 g, 1 mM) was dissolved in TFE/H_2O (9:1) (20 ml), 100 vol. H_2O_2 (0.16 ml) and DMS (1.0 ml) were added and the pH taken to 10.3 with 1 M NaOH. After 1 h TLC indicated the reaction to be complete and the pH was brought to 7 with 10% citric acid. The solvent was evaporated and the residue triturated with 10% citric acid, washing with water and Et_2O gave the free acid (1.74 g, 81%).

(b) *Bpoc cleavage from Bpoc.(61–75).OPh.* The fully protected peptide **95** (1.4 g, 0.6 mM) was dissolved in TFE/H_2O (9:1) (15 ml). DMS (1.8 ml) was added and the solution taken to pH 0.5 with 0.1 M HCl in 90% aqueous TFE. After 1 hour the solvent was evaporated and the residue triturated with Et_2O, filtration gave the required hydrochloride (1.26 g, 96%).

(c) *Fragment coupling.* Bpoc.(50–60).OH (1.05 g, 0.6 mM) and $Cl^-H_2^+$.(61–75).OPh (1.14 g, 0.5 mM) were dissolved in HMPA/DMF (1:1) (16 ml) and cooled to –15°. HONSu (141 mg, 1.22 mM), DCCI (125 mg, 0.6 mM) and NMM (0.056 ml, 0.5 mM) were added and the reaction mixture stirred at room temperature for 24 h. Additional portions of DCCI (81 mg) and HONSu (81 mg) were added to the recooled solution and the reaction continued for 6 days at room temperature. The product was precipitated with water, washed with Et_2O and dried. This material was purified on Sephadex LH 60 eluting with DMF, the required product **92** had (Ve/Vt) = 0.52; isolation gave (804 mg, 41%) m.p. > 340°, $[\alpha]_D^{15}$ – 18°. (c = 1, TFE), $R_f(3)$ – 0.7, $R_f(35)$ – 0.9, $Orn_{1.94}Asp_{3.89}Thr_{1.89}Ser_{2.20}Glu_{1.09}Pro_{1.04}Gly_{4.05}Ala_{1.91}Ile_{1.00}Leu_{2.09}Tyr_{0.98}$. (Found: C, 58.57; H, 7.55; N, 11.93. $C_{198}H_{290}N_{34}O_{47}S.7H_2O$ requires: C, 58.59, H, 7.60; N, 11.73%.)

Bpoc.(38–75).OPh **93**. Bpoc.(38–49).OH **61** (143 mg, 0.07 mM) and $Cl^-H_2^+$.(50–75).OPh (220 mg, 0.06 mM) were dissolved in HMPA/DMF (3:1) (2 ml) and HONSu (15 mg, 0.13 mM) added. The solution was cooled to – 15° and DCCI (15 mg, 0.07 mM) added along with NMM (6 μl, 0.06 mM). The reaction mixture was stirred for 24 h then recooled to – 15° and further portions of DCCI (10 mg) and HONSu (10 mg) added. After 10 days the product was precipitated by the addition of water. The solid was washed with Et_2O and chromatographed on Sephadex LH 60 eluting with NMP, the required product had (Ve/Vt) = 0.41. Isolation by evaporation of the appropriate fractions gave **93** (206 mg, 61%) m.p. > 340°, $[\alpha]_D^{24}$ – 56.4° (c = 1, HMPA), $R_f(31)$ – 0.5, $R_f(7)$ – 0.8, $Orn_{3.41}$Trp–$Asp_{7.01}Thr_{4.40}Ser_{1.99}Glu_{3.18}Pro_{0.95}Gly_{5.02}Ala_{3.03}Ile_{1.01}Leu_{2.25}Tyr_{0.78}Phe_{1.01}$. De-protection of a sample of this material by phenyl ester cleavage and treatment with TFA gave a product which eluted as a single peak on Sephadex G25 when eluted with 50% HOAc (Ve/Vt) = 0.46 and gave the following amino-acid analysis $Orn_{2.94}Asp_{6.65}Thr_{3.50}Ser_{1.31}Glu_{3.13}Pro_{1.10}Gly_{5.15}Ala_{2.82}Ile_{1.03}Leu_{2.23}Tyr_{1.05}Phe_{0.92}$ (30 hour hydrolysis).

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