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PEPTIDES—XXXXVI

STUDIES IN THE SYNTHESIS OF AN ANALOGUE OF HEN EGG WHITE LYSOZYME

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Abstract—The previously synthesised (1–37), (38–75), (76–93), (94–104), (105–117) and (118–129) fragments of the analogue were combined making extensive use of the DCCI/HONSu method. The final coupling involved the (1–75) and (76–129) sub-fragments. Aggregation of the latter fragment caused problems in purification by routine gel filtration methods employing Enzacryl K2 or Sephadex LH60. The fully protected (1–129) product was partially purified by washing, then deprotected and purified by gel filtration and ion exchange chromatography. Satisfactory removal of the acetamidomethyl group used for cysteine protection could not be achieved.

Throughout the preceding series of papers we have attempted to show that large polypeptide fragments may be assembled by total synthesis to the standards of purity acceptable to organic chemists. Certainly much effort was directed towards obtaining full sets of analytical data for the peptide fragments which frequently have molecular weights up to about 5000. The six assembled fragments were those which had been originally envisaged for the synthesis of the hen egg-white lysozyme analogue[1,2] shown below complete with protecting groups.

The first step in the sequence of events shown in the first paper of this series[2] leading to the synthesis of the 1–75 fragment, requires combination of the protected (1–37)[3] and (38–75)[4] fragments. The intermediate stages in this assembly are shown in Scheme 1.

The Adoc. (1–37)·OPh (**48**) was subjected to phenyl ester cleavage under our standard conditions employing trifluoroethanol as the solvent.[5] In the presence of dimethylsulphide at pH of 10.5 the addition of hydrogen peroxide brought about cleavage in 1 h, acidification yielding the required Adoc. (1–37)·OH (**48a**). In this case thin layer chromatography adequately showed the difference between the starting phenyl ester and the corresponding acid.

The *N*-α-protecting group was removed from Bpoc. (38–75)·OPh (**93**) under the standard conditions[6] which employ 0.05 M HCl in 90% trifluoroethanol to bring about the cleavage of the Bpoc protecting group, using

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Table 1.

Adoc-Lys(1)-Val-Phe-Gly-Orn(5)-Cys(Acm)-Glu(OBu^t)-Leu-Ala-Ala(10)-Ala-Nle-Lys(Adoc)-Ala-Leu(15)-Gly-Leu-Ala-Gly-Tyr(Bu^t)-20

Orn(Adoc)-Gly-Tyr(Bu^t)-Ser(Bu^t)-Leu(25)-Gly-Asn-Trp-Nva-Lys(Acm)(30)-Ala-Ala-Lys(Adoc)-Phe-Glu(OBu^t)(35)-Ser(Bu^t)-Gly-Phe-Asn-Thr(Bu^t)-40

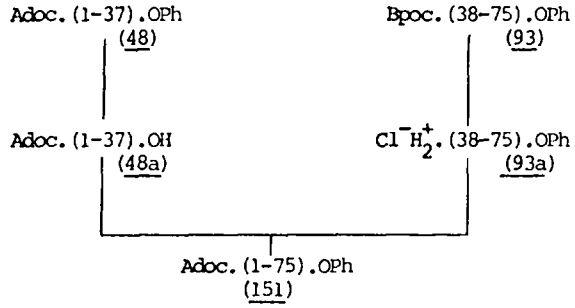
Gln-Ala-Thr(Bu^t)-Asn-Orn(Adoc)(45)-Asn-Thr-Glu(OBu^t)-Gly-Ser(Bu^t)(50)-Thr(Bu^t)-Asp-Tyr(OBu^t)-Gly-Leu(55)-Leu-Gln-Ile-Asn-Ser(Bu^t)-60

Orn(Adoc)-Trp-Trp-Cys(Acm)-Ala(65)-Asp-Gly-Orn(Adoc)-Thr(Bu^t)-Pro(70)-Gly-Ser(Bu^t)-Ala-Asn-Gly(75)-Cys(Acm)-Asn-Ile-Pro-Cys(Acm)-80

Ala-Ala-Leu-Nva-Ser(Bu^t)(85)-Gly-Asp(OBu^t)-Ile-Thr(Bu^t)(90)-Ala-Ser(Bu^t)-Val-Gly-Cys(Acm)(95)-Ala-Lys(Adoc)-Lys(Adoc)-Ile-Val-Ser(Bu^t)-100

Asp(OBu^t)-Gly-Asn-Gly-Nle(105)-Asn-Ala-Trp-Val-Ala(110)-Trp-Orn(Adoc)-Asn-Arg-(Adoc)$_2$-Cys(Acm)(115)-Lys(Adoc)-Gly-Ser(Bu^t)-Asp(OBu^t)-120

Ser(Bu^t)-Ala-Trp-Val-Orn(Adoc)(125)-Gly-Cys(Acm)-Gly-Leu(129)-OBu^t



Scheme 1. Synthesis of the fully protected Adoc·(1–75)·OPh fragment (**151**)‡.

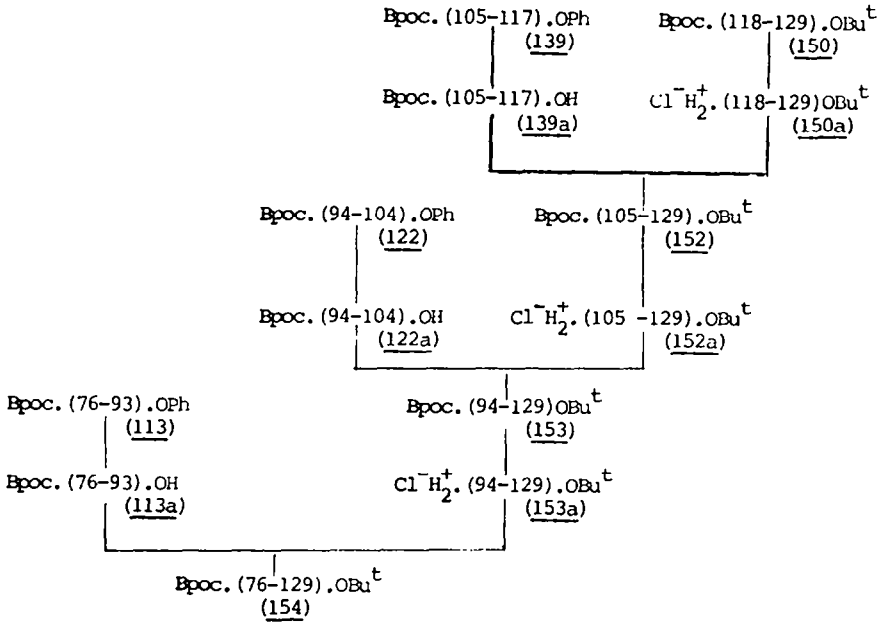
dimethylsulphide as a scavenger to prevent side reactions. Purity of the product could again be assessed from thin layer chromatography and an approximate estimation of the extent of cleavage was made by checking the UV absorbance of the ether washings at 258 nm.

The peptide acid (**48a**) and the hydrochloride (**93a**) were coupled by the DCCI/HONSu method. On this occasion a mixture of HMPA,DMF and DMA were used as solvent with *N*-methylmorpholine being used as the base. During the reaction the physical consistency of the reaction changed dramatically by changing from a mobile solution at the beginning of the reaction to a rather gelatinous consistency after 24 h. After a further 4 days reaction the product was purified by gel filtration chromatography using Enzacryl K2 eluting with NMP.[7] Although the crude product had a reasonably acceptable amino acid analysis, the gel filtration purification could clearly be seen to improve some of the amino-acid ratios. A portion of the Adoc. (1–75)·OPh (**151**) was then deprotected using 90% TFA containing mercaptoethanol and anisole. The resulting product was chromatographed on Sephadex G50 eluting with 50% acetic acid. Ion exchange chromatography of the material isolated from the Sephadex G50 column showed a single component with a highly satisfactory amino acid analysis.

The second portion of the lysozyme analogue spanned the sequence (76–129), and the method of assembly is shown in Scheme 2.

Bpoc·(105–117)·OH (**139a**) was prepared from the corresponding phenyl ester (**139**)[8] by phenyl ester cleavage under the standard conditions[5] using DMF as solvent. At pH 10.5 the cleavage was complete after 1 h and acidification gave the required product in 93% yield. The hydrochloride of the protected (118–129) fragment (**150a**) was prepared from the corresponding Bpoc compound[9] by cleavage of the *N*-α-protecting group under the standard conditions[6] at pH0.5. In this case the hydrochloride was isolated in a yield of 81%.

The peptide acid (**139a**) and the hydrochloride salt (**150a**) were then coupled by the DCCI/HONSu method using HMPA as solvent and *N*-methylmorpholine as base, a second addition of DCCI and HONSu being made after 24 h. After a total reaction period of 6 days the reaction mixture was diluted with DMF and applied to an Enzacryl K2 column eluting with DMF. The major peak obtained centred around a (Ve/Vt) value of 0.48 was rather broad and hence the peak was divided into four parts and the fractions evaporated separately. Amino acid analyses of these fractions and TLC indicated that a satisfactory purification had not been



Scheme 2. Synthesis of the fully protected Bpoc·(76–129)·OBu^t fragment (**154**).

achieved. The fractions were therefore recombined and applied to Sephadex LH60 eluting with NMP.[10] The major peak in this case eluted with a (Ve/Vt) ratio of 0.53 giving an isolated yield of 34%, however, a little material appeared to elute at the void volume. When the material from the peak at (Ve/Vt) 0.53 was reapplied a major peak eluted again at (Ve/Vt) 0.53 along with a small peak again at the void volume. This observation was the first indication that some aggregation of the protected peptides was occurring. The homogeneity and identity of the two products was demonstrated by TLC and by paper electrophoresis of the deprotected product. Amino acid analysis after acid hydrolysis or enzymic digestion indicated a good degree of optical purity. The integrity of the tryptophan residues was also indicated from the enzyme digest result.

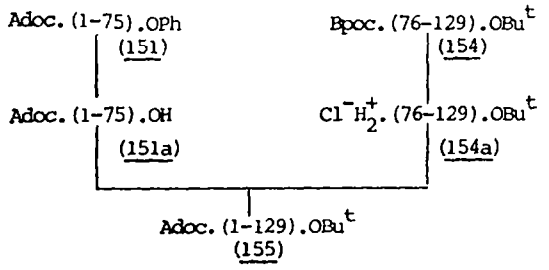
The hydrochloride of the protected (105–129) fragment (**152a**) was then obtained by removal of the Bpoc protecting group from the fully protected fragment (**152**). This was achieved by acidolysis with 0.1 M HCl in 90% trifluoroethanol at pH 0.5 in the presence of dimethylsulphide.[6] Cleavage was complete in 45 min giving a 95% yield of the required hydrochloride (**152a**). The carboxyl component Bpoc·(94–104)·OH (**122a**) was prepared from the corresponding phenyl ester (**122**)[11] by a standard phenyl ester cleavage using 98% aqueous trifluoroethanol as the solvent,[5] hydrogen peroxide and dimethylsulphide being added at pH 10.5. The cleavage was complete in 35 min giving the acid (**122a**) in 90% yield. This acid was identical with a sample of the decapeptide acid which had been prepared by an abortive salt fragment coupling method earlier in the work. It should be noted that a small but perceptable cleavage of the Bpoc-*N*-α-protecting group from this fragment occurred at this stage. However, this problem seems only to be appreciable when the amino-terminal residue is cysteine. Nevertheless, the problem could be minimised by adjusting the pH of the trifluoroethanol to 9 whilst dissolving the protected peptide.

Coupling of the peptide acid (**122a**) and the hydrochloride (**152a**) was again effected by the DCCI/HONSu method using a mixture of HMPA and DMF as solvent. A second addition of DCCI and HONSu was carried out after 2 days and a total reaction period of 4 days was allowed. At the end of this period the reaction mixture was diluted with a mixture of DMF and HMPA and was then chromatographed on Sephadex LH60 eluting with DMF.[10] As with the protected (105–129) fragment, aggregation was again observed but, on this occasion, in much greater amounts. Two widely separated peaks were observed at (Ve/Vt) = 0.25 and 0.47, the latter peak gave an isolated yield of the required fragment of 22% whereas the faster running peak gave a recovery of 76%. Both materials when rechromatographed showed both peaks indicating a re-equilibration of the aggregates. Also both fractions when dissolved in HMPA and chromatographed on Sephadex LH60 this time eluting with 2 M urea in DMF gave a single peak at (Ve/Vt) = 0.52, indicating that the aggregate had been disrupted. The amino acid analysis of the product was satisfactory and enzyme digest again confirmed the integrity of the tryptophan residue and indicate a reasonable degree of optical purity. As with the (105–129) fragment the combustion analysis was satisfactory after an adjustment for the level of hydration of the peptide.

The acid labile amino-protecting group was then removed from the fully protected (94–129) fragment (**153**) by a 45 min treatment with 0.1 M HCl in 90% trifluoroethanol at a pH of 0.5[6], affording the free peptide hydrochloride in 91% yield. Bpoc. (76–93)·OH (**113a**) was obtained from the corresponding phenyl ester (**113**) by cleavage under standard conditions as indicated in our earlier paper.[12] We found that it was important to monitor this reaction carefully as some cleavage of the Bpoc function from cysteine-76 was observed under the phenyl ester cleavage conditions in an analogous fashion to the (94–104) fragment. After a detailed examination we found that the loss of α-amino protection could not be brought below 2.5%, and this level although undesirable could be tolerated. Coupling of the two fragments (**113a**) and (**153a**) to give the second major fragment (**154**) was again brought about by the DCCI/HONSu method using a mixture of HMPA in DMF as solvent, a second edition of DCCI and HONSu being carried out after 2 days. During the complete reaction period of 5 days the reaction changed in consistency from a mobile liquid to a rather rigid gel. Precipitation of the product at the end of this period with brine gave a 91% yield of the crude product. As with the preceding large fragments, purification was attempted using Enzacryl K2 eluting with NMP.[7] A major peak which eluted at (Ve/Vt) = 0.38 gave a reasonable amino acid analysis but TLC of the protected material and electrophoresis and isoelectric focussing on the deprotected peptide showed that the material was slightly impure and therefore would require further purification. This was achieved by chromatography on Sephadex LH60 eluting with NMP.[10] Indeed some aggregation was again observed but the bulk of the material eluted at a (Ve/Vt) ratio of 0.42 giving a 46% yield of the fragment Bpoc. (76–129). OBu^t (**154**) which had a good amino acid analysis and was found to be homogeneous by TLC and isoelectric focussing of the deprotected material. Amino-acid analysis of material purified on Sephadex CM25 gave good ratios for the constituent amino acids.

The routes shown in Schemes 1 and 2 thus provided us with the (1–75)[13] and (76–129)[14] fragments of the lysozyme analogue. Having obtained both fragments in a reasonable state of purity and in good quantities we were able to contemplate the assembly of the fully protected target molecule Adoc.(1–129)·OBu^t (**155**). The final coupling has been carried out on three occasions and the results from each experiment were found to be internally consistent although, as might be anticipated for a molecule of this size, absolute reproducibility was impossible. The stages leading to the final coupling are indicated in Scheme 3.

The Adoc·(1–75)·OH (**151a**) was obtained by standard phenyl ester cleavage[5] on the corresponding phenyl ester (**151**). In this case the cleavage was carried out in



Scheme 3. The fragment coupling route to Adoc·(1–129)·OBu^t (**155**).

aqueous trifluoroethanol, the pH being adjusted to 10.5 with 0.1 M sodium hydroxide. Dimethylsulphide was added in the usual way as a scavenger prior to the addition of hydrogen peroxide. Thin layer chromatography indicated that the cleavage was complete within 1 h and work up gave a 91% recovery of the required acid (**151a**). On this occasion the phenol liberated was estimated by UV absorption and an 80% yield for the cleavage was indicated.

Cleavage of the Bpoc function from the fully protected (76–129) fragment (**154**) was readily achieved in 90% trifluoroethanol solution by treatment with 0.05 M HCl at pH 0.5 with dimethyl sulphide as scavenger. The cleavage conditions were maintained for 1.25 h at which time workup gave a 98% yield of the corresponding hydrochloride (**154a**). A UV estimation of this cleavage could only be estimated to ± 10% due to the scale of operations.

The peptide acid (**151a**) and the hydrochloride (**154a**) were then combined once again by the DCCI/HONSu method, a (2:1) mixture of HMPA and DMF was used as solvent and *N*-methylmorpholine as base. A second addition of DCCI and HONSu being made after 24 h and a total of 5 days was allowed for complete reaction. Once again a dramatic change in viscosity was observed during reaction period and after 5 days brine was added to precipitate the crude product (**155**) which was washed with water, ether and isopropanol before drying. TLC of the isolated material was surprisingly good indicating one major component with a trace of fluoroescamine positive material remaining at the origin in each system. The amino-acid analysis of the precipitated material was exceptionally encouraging and it gave satisfactory ratios for the majority of the amino-acids present even though little had been done to purify this product. In fact, due to its high insolubility in a range of solvents including trifluoroethanol, *N*-methylpyrrolidone and dimethylacetamide we concluded that our standard methods employing Enzacryl K2[7] would be unsatisfactory and that further purification would be best achieved after deprotection.

The precipitated protected protein (**155**) was deprotected by treatment with 90% trifluoroacetic acid under nitrogen using anisole and mercaptoethanol as scavengers. After a 3 h treatment which was shown to be optimal by polyacrylamide gel isoelectric focussing, the product was precipitated by the addition of ether. This product ((**156**) having the sequence set out in the first paper of this series, was then chromatographed on Sephadex G75 eluting with 0.4% ammonium bicarbonate whereupon peaks were observed at (Ve/Vt) values of 0.33, 0.70 and 0.91 (see Fig. 1a). The peak at (Ve/Vt) = 0.70 contained the majority of the product, the latter peak containing anisole and other small molecules. The UV spectrum of materials contained in peaks (a) and (b) was compared and only peak (b) was found to resemble native hen egg white lysozyme. It was also found that octa-carbamidomethylated reduced hen egg white lysozyme (CHEL)[15] was eluted at a (Ve/Vt) value of 0.66 on the Sephadex G75 column. The material from peak (b) was then applied to Sephadex CM25 eluting on a gradient running from 0.1 M ammonium acetate pH to 0.4 M ammonium carbonate pH 8.5. Two peaks were obtained as shown in Fig. 1(b). The weight destribution in the two peaks (c) and (d) being 1.2 and 4.6 mg respectively after dialysis. The samples were then run on polyacrylamide gel isoelectric focussing in comparison with HEL and

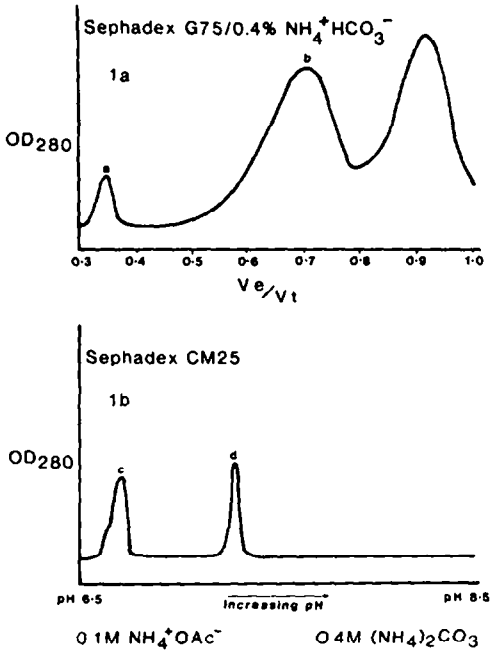


Fig. 1. Chromatography of octa-Acm (1–129) (**156**).

CHEL. Sample (c) had a pI of approx. 10 whereas (d), HEL and CHEL showed a pI of approx. 10.5. However, it should be borne in mind that this was rather close to the limit of the gel. Sample (d) was subjected to amino acid analysis and the majority of residues were found to give reasonably acceptable values; tyrosine, however was noticeably low. Interestingly, a comparative analysis on CHEL again showed a low figure for tyrosine. Comparison of the UV profile of the material from (d) with HEL and CHEL again showed a close similarity between the samples (see Fig. 2), thus supporting the fact that tryptophan was present in the correct ratio and that the tyrosine was also present in the correct ratio even though the amino acid analysis had indicated a low value.

Although we considered that at this point the characterisation of the octa Acm(1–129) protein (**156**) was rather inadequate, we decided to proceed further and to examine the removal of the Acm groups.[16]

A sample of the material (**156**) was dissolved in 50% acetic acid and mercuric acetate added. After stirring for 70 min under nitrogen at room temperature mercaptoethanol was added and the reaction mixture stirred for a further 19 h. The whole of the reaction mixture was then applied to Sephadex G15, on this occasion eluting with 0.1 M acetic acid. A sharp peak eluted at the void volume along with several other components at larger elution volumes. Pooling of the fractions at the void volume gave a yield of 53%, the UV profile of this material being the same as that before removal of the Acm groups and identical with HEL. The thiol content of this product was then estimated using Ellman reagent[17] giving a value of 4.01 SH groups per molecule using the OD_{280} value to estimate the protein concentration. A repeat of this experiment gave a value of 4.16 SH groups per molecule. As this value was only 50% of that anticipated, we then repeated these experiments in the presence of 8 M urea in the hope that this would bring about sufficient exposure of the Acm functions to the

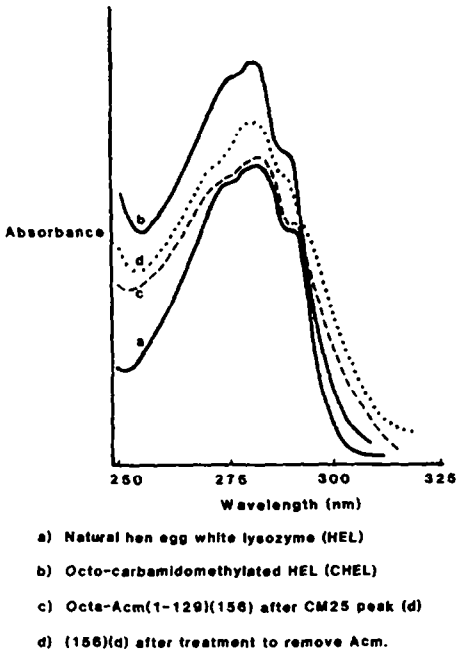


Fig. 2. Partial UV spectra for natural and synthetic materials at approximately the same concentrations.

reagents. In this experiment values of 6.1 and 6.0 were obtained after gel filtration indicating that indeed some of the Acm groups were probably not accessible to the reagent in the earlier experiments. However, even under these conditions only 75% of the thiol groups present were liberated. Even with this number of thiol groups present the chances of correct oxidation of the product were incredibly small, nevertheless trial oxidations were in fact carried out under conditions identical with those required to oxidixe reduced hen egg white lysozyme at the same concentration.[18] Although we could readily regenerate the activity of hen egg white lysozyme using natural reduced HEL we did not observe any lysozyme activity for the synthetic material, thus we concluded that there was just too small an amount of the octa. SH synthetic protein present in our reaction product and that it was most likely that a narrow distribution of thiol species containing approx. 6 SH groups per molecule was present in the final product.

In conclusion therefore, we would claim that large polypeptides of the (1–75) or (76–129) size can certainly be assembled, and that although the evidence is not fully convincing we believed that a peptide chain of 129 residues (the linear sequence of the hen egg white lysozyme analogue) had been assembled. If this is true this would satisfy the first requirement of our activities, as stated by one of us (G.W.K.).[1] That is, that we should demonstrate that a peptide of 129 residues can be assembled by the synthetic methods presently available. The major disappointment, however, must be that no material with lysozyme activity was obtained although this goal was considered to be subsidiary rather than one of the main objectives.[1]

It appears therefore that the majority of methods of protection are adequate for a peptide of this kind although the introduction of protecting groups which on cleavage do not give electrophilic species would be a considerable step forward. To this end protection based on phosphorus compounds has been investigated intensively by members of our group.[19] A very serious question, however, arises over the protection of cysteine residues since the low recovery of cysteine after removal of the acetamido-methyl function may well be the major factor in preventing us from obtaining a satisfactory product. However, the low thiol values we encountered may in fact only be a reflection of the highly convoluted nature of the octa-Acm protein (**155**) although reoxidation experiments with native hen egg white lysozyme would suggest that this is not the case. We would therefore conclude that some improvements in the protection of cysteine residues are urgently required.

During the course of this work coupling methods have improved considerably with the DCCI/HONSu[20] and DCCI/HOBt methods[21] becoming common place. Nevertheless, we have developed two new coupling methods during the course of this programme, these being the Bates reagent[22] and the triphenylphosphine–SO_3 reagent.[23]

One of the major problems encountered in this research was the purification of large fully protected, rather hydrophobic peptides. In the main, for smaller peptides, LH20 chromatography eluting with DMF served us well,[24] however, during the programme we have developed the use of Enzacryl K2[7] and Sephadex LH60[10] for the purification of large fully protected peptides.

Finally, therefore, we would say that the assembly of polypeptide chains of this size (in the region of 129 residues) is certainly possible but that clearly enormous resources must be brought to bear in order to complete such a project.

EXPERIMENTAL

The general experimental techniques used, abbreviations and TLC systems are those which have been described in earlier papers in the series. In this paper theoretical amino-acid ratios are shown as a subscript following the amino-acid in question.

Adoc·(1–37)·OH (**48a**). Adoc·(1–37)·OPh (**48**) (100 mg, 0.018 mM) was dissolved in TFE and DMS (0.15 ml) added. The pH of the solution was adjusted to 10.5 with 2 M NaOH (1.14 ml), H_2O (1 ml) and H_2O_2 (20 μl) added, and the solution stirred at that pH for 1 h. The pH was then brought to 3 by the addition of 1 M HCl before evaporation of the solvent. The residue was triturated with water and Et_2O then dried over P_2O_5 giving (55 mg, 56%), R_f(11) −0.4, R_f(28) −0.1, cf. R_f(28) −0.6 for compound (**48**).

$Cl^-H_2^+$·(38–75)·OPh (**93a**). Bpoc. (38–75)·OPh (**93**) (94.0 mg, 0.017 mM) was dissolved in TFE (2.2 ml) and water (0.2 ml) added. DMS (60 μl) was added and the pH brought to 0.5 with 0.5 M HCl in 90% TFE (aq). After standing for 1 h the solvent was evaporated and the residue triturated with Et_2O giving the required hydrochloride (85 m g, 92%), R_f(23) −0.2 cf. 0.9 for (**93**). An approximate UV estimation of cleavage coproducts absorbing a 258 nm gave the yield as 77%.

Adoc·(1–75)·OPh (**151**). Adoc·(1–37)·OH (**48a**) (86 mg, 16 μM) and $Cl^-H_2^+$·(38–75)·OPh (**93a**) (85 mg, 16 μM) were dissolved in a solvent mixture containing HMPA (1 ml), DMF (0.5 ml) and DMA (0.2 ml). HONSu (5 mg, 48 mM) and a solution of NMM in DMF (0.16 ml) (solution contained 0.1 mM/ml NMM) were added and the solution cooled to −5°. DCCI (5 mg, 24 μM) was then added and the reaction mixture stirred overnight. After 24 h the reaction mixture had become gelatinous and thus a further portion of HMPA (0.2 ml) added. The solution was cooled and further portions of DCCI (5 mg) and HONSu (5 mg) added before stirring for a further 4 days. Brine was then added to precipitate the product. The white precipitate being washed several times with water, IPA and Et_2O finally drying *in vacuo* over P_2O_5. The product obtained (130 mg, 79%), R_f(23) −0.9 (streak extending 0.7–0.9), R_f(7) −0.8 had amino acid analysis: $Lys/Orn_8$7.63

$Asp_8$7.53 $Thr_5$3.42 $Ser_5$3.08 $Glu_5$5.10 Pro_1 0.95 Gly_{11} 11.8 Ala_{10} 10.50 Val_1 1.02, Nva_1 1.23 Ile_1 1.07 Leu_6 6.50 Nle_1 1.07 Tyr_3 2.89 Phe_3 2.79. This material was then further purified by gel filtration on Enzacryl K2 eluting with NMP. The major peak eluted at K_{av} −0.18 and thus could be separated from (**48a**) [K_{av} −0.29 for the phenyl ester (**48**)] and (**93a**) [K_{av} −0.10) for the Bpoc compound (**93**)] although from the TLC data it was clear that no fluorescamine positive material was present in the crude product isolated. The yield of purified (**151**) was (79 mg, 48%), R_f(23) −0.9, R_f(7) 0.8, Lys/Orn_8 7.78 Asp_8 7.79 Thr_5 4.35 Ser_5 4.63 Glu_5 5.29 Pro_1 0.80 Gly_{11} 11.00 Ala_{10} 10.10 Val_1 0.89 Nva_1 1.20 Ile_1 1.12 Leu_6 6.37 Nle_1 0.96 Tyr_3 3.04 Phe_3 2.92. A portion of this product was totally deprotected by treatment with 90% TFA (3 ml) containing mercaptoethanol (0.5 ml) and anisole (0.1 ml) and was chromatographed on Sephadex G50 eluting with 50% HOAc. The single peak (Ve/Vt) = 0.56 from this chromatography was rechromatographed on Sephadex CM25 using a gradient elution from 0.1 M ammonium acetate (pH 6.5) to 0.4 M ammonium carbonate (pH 8.5). The material contained in the single peak had amino acid analysis: Lys/Orn_8 8.53 Asp_8 7.73, Thr_3 3.77 Ser_5 4.40 Glu_5 5.37 Pro_1 0.95 Gly_{11} 10.40 Ala_{10} 9.94 Val_1 1.14 Nva_1 0.84 Ile_1 1.18 Leu_6 5.98 Nle_1 1.10 Tyr_3 2.78 Phe_3 3.05.

Bpoc·(105–117)·OH (**139a**). Bpoc·(105–117)·OPh (**139**) (877 mg, 0.33 mM) was dissolved in DMF (8 ml) and a mixture of DMF (2 ml) and H_2O (4 ml) added dropwise followed by DMS (1.25 ml, 1.7 mM). The pH was adjusted to 10.5 with 0.5 M NaOH and 100 vol. H_2O_2 (0.033 ml, 0.33 mM) added. After 1 h base uptake had ceased and the pH was adjusted to 4 with ice cold 5% citric acid. Water was added and the precipitated product filtered, washed with more water, Et_2O then dried giving the required peptide acid (**139a**) (0.78 g, 93%) R_f(17) −0.2, R_f(2) −0.6.

$Cl^-H_2^+$·(118–129)·OBu^t (**150a**). Bpoc·(118–129)·OBu^t (**150**) (0.64 g, 0.34 mM) was dissolved in TFE (9 ml) and water (1 ml) added. DMS (1 ml) was then added and the pH brought to 0.5 with 0.1 M HCl in 90% TFE (aq). After maintaining that pH for 45 min the solvent was evaporated and the residue triturated with Et_2O. The resulting solid was washed with water and Et_2O then dried, giving the required hydrochloride (0.48 g, 81%), m.p. >230°, $[\alpha]_D^{20}$ −29.2° (c = DMF/HMPA 1:1). R_f(11) −0.6, R_f(25) −0.1, (Found: C, 54.83; H, 7.75; N, 11.93; $C_{82}H_{134}N_{15}O_{20}SCl·4H_2O$ requires: C, 55.03; H, 7.97; N, 11.74%).

Bpoc·(105–129)·OBu^t (**152**). The peptide acid (**139a**) (0.64 g, 0.25 mM), the salt (**150a**) (0.43 g, 0.25 mM) and NMM (2.5 ml of 0.1 mM/ml solution in DMF) in HMPA (10 ml) was cooled to 0° and treated with HONSu (58 mg, 0.5 mM) and DCCI (65 mg, 0.31 mM). After 2 h at 0°, the solution was allowed to attain room temperature for 24 h, then it was recooled and HONSu (29 mg, 0.25 mM) and DCCI (33 mg, 0.16 mM) added. The reaction was allowed to proceed for 5 days, then the reaction mixture was diluted with DMF and applied directly to Enzacryl K2 eluting with DMF. The slightly impure product (0.78 g, 74%) eluted with (Ve/Vt) = 0.48 and was subsequently further purified by chromatography on Sephadex LH60, again eluting with DMF. The product (**152**) eluted with (Ve/Vt) = 0.52 and was precipitated with water after pooling the appropriate fractions. After drying, the homogeneous product (**152**) was obtained in a yield of 0.37 g (34%), with m.p. >230°, $[\alpha]_D^{20}$ +1.6° (c = 0.5, DMF), R_f(11) −0.8, R_f(25) −0.4 R_f(12) −0.6, Lys/Orn_3 2.85 Arg_1 0.98 $Asp_3$2.99 Ser_2 1.76 Gly_3 3.08 Ala_3 2.98 Val_3 3.10 Leu_1 1.03 Nle_1 1.04; (Enzyme digest pronase/APM) Trp_3 3.02 Lys/Orn_3 3.19 Arg_1 0.90 Cys (ACM) + Cys_2 1.84 Asp_1 1.00 Asn/Ser_4 3.73 Gly_3 2.92 Ala_3 2.89 Val_2 Leu_1 1.13 Nle_1 1.08 (Found: C, 58.48; H, 7.35; N, 12.50; $C_{214}H_{312}N_{38}O_{46}S_2·9H_2O$ requires: C, 58.69; H, 7.60; N, 12.15%).

Bpoc·(94–104)·OH (**122a**). A solution containing the phenyl ester (**122**) (0.5 g, 0.26 mM) in TFE (8 ml) was adjusted to pH 9 with 0.5 M NaOH. Water (2 ml) and DMS (1.0 ml, 13.9 mM) were added and the pH readjusted to 10.5. Following the addition of 100 vol. H_2O_2 (0.026 ml, 0.2 mM) the cleavage was allowed to proceed for 35 min then the pH was brought to 3 with ice-cold 5% citric acid. This solution was then poured into brine to precipitate the peptide acid (**122a**). After washing with water and Et_2O the homogeneous product was obtained (0.44 g, 90%), $[\alpha]_D^{24}$ −6.5° (c = 1, DMF), R_f(11) −0.2, R_f(25) −0.1, R_f(23) −0.8. This material was identical in most respects with the same peptide acid prepared by a salt fragment coupling method. This product having m.p. >260°, $[\alpha]_D^{22}$ −5.8° (c = 1, DMF), R_f(11) −0.2, R_f(23) −0.8, (Found: C, 57.97; H, 7.47; N, 10.70; $C_{93}H_{141}N_{15}O_{23}S·4H_2O$ requires: C, 57.54; H, 7.74; N, 10.82%).

$Cl^-H_2^+$·(105–129)·OBu^t (**152a**). Bpoc·(105–129)·OBu^t (**152**) (0.342 g, 0.081 mM) was dissolved in TFE (5.4 ml) and water (0.6 ml) added along with DMS (0.5 ml, 6.85 mM). The pH was brought to 0.5 by the addition of 0.1 M HCl in 90% TFE (aq) and maintained at this value for 45 min until TLC indicated the cleavage to be complete. The solvent was evaporated and the residue triturated with Et_2O and washed with water and Et_2O giving the required hydrochloride (**152a**) (0.310 g, 95%), R_f(11) −0.3, R_f(22) −0.3.

Bpoc·(94–129)·OBu^t (**153**). The peptide acid (**122a**) (0.16 g, 0.086 mM) and the salt (**152a**) (0.20 g, 0.05 mM) were dissolved in HMPA (1 ml) and the solution cooled to 0° prior to the addition of HONSu (20 mg, 0.172 mM), DCCI (22 mg, 0.108 mM) and NMM (0.5 ml of a 0.1 mM/ml solution in DMF). The reaction mixture was stirred for 2 days then recooled to 0° and further portions of HONSu (10 mg) and DCCI (11 mg) added, stirring was then continued for 4 days. The reaction mixture was diluted with DMF (3 ml) and HMPA (3 ml) and the resulting solution applied directly to Sephadex LH60 being eluted with DMF. The product was found to be located in two widely separated peaks having (A) (Ve/Vt) = 0.25 and (B) 0.47, the isolated material being identical by TLC R_f(7) −0.8, R_f(30) −0.4 (see discussion of experimental above). Precipitation of the material contained in the major peak (Ve/Vt) = 0.25 gave (152 mg, 54%), Lys/Orn_5 4.98 Arg_1 1.02 Asp_5 5.08 Ser_3 3.16 Gly_5 5.14 Ala_4 4.06 Val_4 3.75 Ile_1 0.87 Leu_1 1.05 Nle_1 1.05. The peak at (Ve/Vt) = 0.47 gave (62 mg, 22%), Lys/Orn_5 5.21 Arg_1 1.05 Asp_5 5.00 Ser_3 2.88 Gly_5 4.97 Ala_4 4.02 Val_4 3.34 Ile_1 0.88 Leu_1 1.02 Nle_1 1.07. A sample of (A) (100 mg) was dissolved in HMPA (2 ml) and DMF (2 ml). This solution was then applied to Sephadex LH60 and eluted with 2 M urea in DMF giving a single peak at (Ve/Vt) = 0.52. [A sample of (B) similarly gave a single peak at (Ve/Vt) = 0.52]. After evaporation of the solvent and precipitation with brine the residue was washed with water. The hazy solution eventually yielded (**153**) (25 mg, 25%), m.p. >230°, $[\alpha]_D^{20}$ +3.1° (c = 1, HMPA/DMF, 4:1), $[\alpha]_D^{20}$ −2.8° (c = 0.5, TFE), R_f(7) −0.4, R_f(30) −0.4, R_f(11) −0.5, Lys/$Orn_5$4.67 Arg_1 1.02 Asp_5 4.96 Ser_3 2.59 Gly_5 5.04 Ala_4 4.01 Val_4 4.04 Ile_1 0.89 Leu_1 1.04 Nle_1 1.08, (promase/APM) Trp_3 3.00 Lys/Orn_5 5.15 Arg_1 0.97 Cys(Acm) + Cys_3 3.30 Asp_2 2.06 Asn/Ser_6^+ 4.50 Gly_5^+ 5.73 Ala_4 4.02 Val_4 4.18 Ile_1 1.18, Leu_1 0.98 Nle_1 0.95 (+error probably due to poor resolution), (Found: C, 57.82; H, 7.43; N, 12.25; $C_{291}H_{437}N_{53}O_{66}S_3·11H_2O$ requires: C, 57.98; H, 7.67; N, 12.31%).

$Cl^-H_2^+$·(94–129)·OBu^t (**153a**). Bpoc·(94–129)·OBu^t (**153**) (182 mg, 0.032 mM) was dissolved in TFE (3.6 ml), water (0.4 ml) and DMS (0.2 ml, 2.74 mM) added and the pH brought to 0.5 by the addition of 0.1 M HCl in 90% TFE (aq). After 45 min TLC indicated the cleavage to be complete and the solvent was evaporated leaving a residue which was triturated with Et_2O. This solid was washed with water and Et_2O then dried giving the required salt (**153a**) (165 mg, 91%), R_f(7) −0.6.

Bpoc·(76–129)·OBu^t (**154**). Bpoc·(76–93)·OH (**113a**)[12] (53 mg, 0.023 mM) and the hydrochloride (**153a**) (120 mg, 0.023 mM) were dissolved in HMPA (1 ml) and DMF (0.5 ml). NMM [(0.23 ml) of a 0.1 mM/ml solution in DMF] was added and the solution cooled to 0° before treating with HONSu (5 mg, 0.046 mM) and DCCI (7 mg, 0.46 mM). After stirring for 2 days at room temperature the reaction mixture was recooled to 0° and further portions of HONSu (3 mg) and DCCI (4 mg) added. The reaction mixture had set to a thick gel after a further 3 days and was precipitated with water, being washed with DMF, MeOH and Et_2O to give the crude product (**154**) (165 mg, 91%), Lys/Orn_5 4.60 Arg_1 1.00 Asp_7 7.10 Thr_1 1.12 Ser_5 4.19 Pro_1 1.00 Gly_7 6.94 Ala_7 7.06 Val_5 4.92 Nva_1 1.27 Ile_3 2.88 Leu_2 2.18 Nle_1 0.98.

Purification was initially carried out on Enzacryl K2 eluting with NMP the required product being contained in the peak eluting with (Ve/Vt) = 0.38 giving Lys/Orn_5 4.78 Arg_1 1.03 Asp_7 7.26 Thr_1 0.94 Ser_5 4.01 Pro_1 1.03 Gly_7 6.94 Ala_7 7.21 Val_5 4.61 Nva_1 1.10 Ile_3 2.71 Leu_2 2.00 Nle_1 1.29 TLC electrophoresis and isoelectric focussing showed that this material was slightly impure

and therefore further purification was carried out by dissolving in HMPA and rechromatography on Sephadex LH60 eluting with NMP. Some aggregation was observed, but the bulk of the material eluted with (Ve/Vt) = 0.42, work up giving (**154**) (84 mg, 46%), $R_f(7)$ −0.8, $R_f(23)$ −0.6, $R_f(31)$ −0.6, Lys/Orn_5 4.46 Arg_1 1.01 Asp_7 7.12 Thr_1 1.12 Ser_5 4.57 Pro_1 1.01 Gly_7 7.12 Ala_7 7.31 Val_5 4.71 Nva_1 1.14 Ile_3 3.01 Leu_2 2.01 Nle_1 1.01; deprotected material run on Sephadex CM25 eluting with 0.1 M pyridinium acetate pH 6.5 (0.1 M wrt NaCl) running a gradient to 0.1 M pyridinium acetate pH 8.5 (1 M wrt NaCl) gave Lys/Orn_5 5.05 Arg_1 0.98 Asp_7 7.00 Thr_1 0.90 Ser_5 4.67 Pro_1 0.98 Gly_7 7.15 Ala_7 6.93 Val_5 4.97 Nva_1 1.05 Ile_3 2.64 Leu_2 2.18 Nle_1 1.05.

Adoc·(1–75)·OH (**151a**). Adoc·(1–75)·OPh (151) (120 mg, 11.6 μM) was dissolved in TFE (5.8 ml), H_2O (0.6 ml) and DMS (0.6 ml) added and the pH adjusted to 10.5 with 0.1 M NaOH. A solution of H_2O_2 [0.1 ml of a solution containing 100 vol. H_2O_2 (0.1 ml) in H_2O (10 ml)] was added and the cleavage allowed to proceed for 1 h. The pH was then brought to 3.5 with ice cold 10% citric acid and concentrated prior to the addition of a saturated solution of NaCl. The resulting precipitate was filtered, washed with Et_2O and dried giving the required peptide acid (**151a**) (107 mg, 91%), $R_f(23)$ −0.8, $R_f(7)$ −0.7 (cf. 0.9 and 0.8 respectively for the corresponding phenyl ester). A UV estimation of the phenol liberated indicated an 80% yield of phenol.

$Cl^-H_2^+$·(76–129)·OBu^t (**154a**). Bpoc·(76–129)·OBu^t (**154**) (111 mg, 13.8 μM) was dissolved in TFE (9 ml), H_2O (0.9 ml) and DMS (0.9 ml). At this stage the reaction mixture was very hazy but adjusting the pH to 0.5 with 0.05 M HCl in 90% TFE (aq) rapidly brought about a clear solution. The reaction mixture was maintained at pH 0.5 for 1.25 h then evaporated to give a dry foam which was triturated with Et_2O giving (**154a**) (105 mg, 96%). $R_f(7)$ −0.2, $R_f(23)$ −0.4 [cf. 0.8 and 0.6 for the corresponding Bpoc derivative (**154**)]. A UV estimation indicated complete cleavage.

Adoc·(1–129)·OBu^t (**155**). Adoc·(1–75)·OH (**151a**) (105 mg, 9.89 μM), $Cl^-H_2^+$·(76–129)·OBu^t (**154a**) (77 mg, 9.89 μM) and HONSu (4.6 mg, 22 μM) were dissolved in a mixture of HMPA (2 ml) and DMF (1 ml). After cooling to 0° a solution of NMM in DMF (0.1 ml) was added (0.1 m/ml NMM) along with DCCI (4.6 mg, 22 μM) and the reaction mixture stirred overnight at room temperature. The solution was recooled and further portions of DCCI (2.3 mg) and HONSu (2.3 mg) added before stirring for an additional 4 days. The solution, which had increased in viscosity during the reaction period, was then poured into brine to precipitate the product. The resulting solid was washed with water, Et_2O and IPA before drying over P_2O_5 which gave the crude product (170 mg, 91%), $R_f(3)$ −0.8, $R_f(15)$—streak to 0.6, $R_f(7)$ −0.8, $R_f(17)$ −0.6, a trace of fluorescamine positive material remained at the origin in all systems. AAA: Lys/Orn_{13} 12.08 Arg_1 1.00 Asp_{15} 14.81 Thr_6 5.50 Ser_{10} 8.27 Glu_5 5.27 Pro_2 2.08 Gly_{18} 17.93 Ala_{17} 16.98 Cys_8 8.06 Val_6 5.93 Nva_2 2.51$^+$ Ile_4 4.29 Leu_8 8.27 Nle_2 2.04 Tyr_3 2.94 Phe_3 2.82 (carried out in the presence of Nps·Cl; +occurred at the buffer change).

The isolated material was only slightly soluble in a wide variety of solvents and trial gel filtration chromatography in TFE, NMP or DMA with Enzacryl K2 or Sephadex LH60 indicated that deprotection of this material followed by further purification would be the best course of action.

Deprotection studies on Adoc·(1–129)·OBu^t (**155**). The deprotection experiments were run many times and therefore the experimental details which follow may be taken as representative of the whole series of experiments. The precipitated protein (**155**) (24 mg, 1.7 μM) was treated with anisole (0.3 ml) and mercaptoethanol (0.3 ml) then dissolved in 90% TFA (aq) (3 ml) under N_2 in the dark. After 3 h Et_2O was added to precipitate the product which was washed with Et_2O then dried and dissolved in 0.4% $NH_4^+HCO_3^-$ prior to being applied directly to a Sephadex G75 column. Peaks were observed at (Ve/Vt) (a) 0.33, (b) 0.70 and (c) 0.91; peak (a) contained 1.4 mg (b) 7.1 mg and (c) anisole and other small molecules present. Material from (a) did not have the required UV profile whereas the material in peak (b) resembled native HEL. Also a marker, octa-*S*-carbamidomethylated-reduced HEL (CHEL) eluted at (Ve/Vt) = 0.66 on the G75 column.

The material from peak (b) was then applied to Sephadex CM25 eluting on a gradient running from 0.1 M NH_4OAc (pH 6.5) to 0.4 M "$(NH_4)_2CO_3$" (pH 8.5). The two peaks were obtained as indicated in the discussion section, the peak at lowest pH giving (c) (1.2 mg) and that at higher pH (d) (4.6 mg) after dialysis against distilled water and lyophilisation (see Fig. 1).

Samples were run on polyacrylamide gel isoelectric focussing being compared with HEL and CHEL being stained with Coomasie Blue. Sample (c) had a pI of 10 whereas (d), HEL and CHEL all showed a pI of 10.5, however, this was rather close to the limit of the gel. AAA (6 M HCl, 24 h, 110°) for (d) gave: Lys/Orn_{13} 13.33 Arg_1 1.00 Asp_{15} 15.71 Thr_6 6.19 Ser_{10} 9.76 Glu_5 5.26 Pro_2 2.62 Gly_{18} 18.27 Ala_{17} 16.96 Val_6 6.45 Nva_2 1.88 Ile_4 4.91 Leu_8 8.07 Nle_2 2.19 Tyr_3 1.69 Phe_3 2.32. A parallel analysis on CHEL gave: Lys_6 5.12 His_1 1.12 Arg_{11} 11.22 $Cys(CH_2CO_2H)_8$ 7.18 Asp_{21} 19.69 Thr_7 5.68 Ser_{10} 7.08 Glu_5 5.61 Pro_2 2.40 Gly_{12} 12.00 Ala_{12} 12.00 Val_6 6.02 Met_2 2.49 Ile_6 5.54 Leu_8 7.26 Tyr_3 2.53 Phe_3 3.04. The UV profiles of material from (d), HEL and CHEL were virtually identical (see Fig. 2) supporting the integrity of the tryptophan side chain.

Study of Acm removal from octa-Acm(1–129) (**155**). Similar material to that obtained above by gel filtration and ion exchange chromatography was used in the two following experiments.

(a) The material (**155**) (10 mg) was dissolved in 50% HOAc and $Hg(OAc)_2$ (15.1 mg) in 50% HOAc (0.17 ml) added. After stirring under N_2 for 70 min at room temperature mercaptoethanol (0.7 ml) was added and the reaction mixture stirred for 19 hours, during this time the reaction mixture became hazy. The whole reaction mixture was then applied directly to Sephadex G15 eluting with 0.1 M HOAc. A sharp peak eluted at the void volume along with several other components with larger elution volumes. Pooling of the fractions at the void volume and UV estimation of protein at 280 nm indicated a yield of (53%). The UV profile of the material being the same as that before removal of the Acm groups and identical with HEL (see Fig. 2).

The thiol content of the product was then estimated using the Ellman reagent giving a value of 4.01 SH groups per molecule (using OD_{280} to estimate protein concentration using ϵ_{280} = 37017 calculated for the lysozyme analogue, HEL ϵ_{280} = 35712). A repeat of this experiment gave a value of 4.16 SH groups per molecule.

(b) The experiment was then repeated in duplicate after adding urea to a concentration of 8 M. This brought the SH content to 6.15 and 6.00 after gel filtration on Sephadex G15 eluting with 0.1 M HOAc.

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