Tetrahedron Vol. 49, No 44, pp. 10069-10078, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00

Convergent Solid-Phase Peptide Synthesis. XI. Synthesis and Purification of Protected Peptide Segments Spanning the Entire Sequence of the Uteroglobin Monomer Using the Photolabile Nbb-Resin.[1,2]

Paul Lloyd-Williams, Margarida Gairí, Fernando Albericio and Ernest Giralt*

Departament de Química Orgànica, Universitat de Barcelona,
Martí i Franquès 1-11, E-08028 Barcelona, Spain.

(*Received in UK* 28 *July* 1993)

Key Words: convergent solid-phase synthesis, HPLC, Nbb-resin, NPE-resin, photolysis, protected peptide

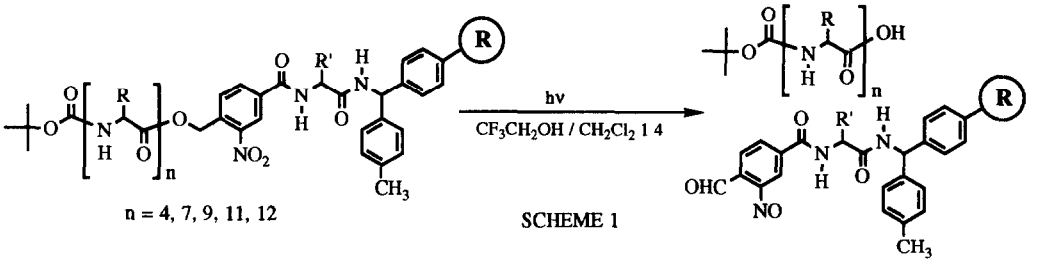
Abstract: Protected peptide segments corresponding to the 1-4, 5-11, 19-30, 39-49 and 50-58 segments of the Uteroglobin monomer have been synthesised on a solid support using the photolabile *ortho*-nitrobenzyl unit as a handle. Photolytic detachment of the peptides from the solid support occurred in good yields. The 50-58 protected peptide segment has also been synthesised using the base-labile NPE handle, and detached from the solid support in excellent yield by treatment with a 20% solution of piperidine in DMF. The protected peptide segments were purified by MPLC and/or preparative HPLC, using solvents containing DMF.

INTRODUCTION

Convergent solid-phase peptide synthesis[3] is one of the most promising approaches to the synthesis of large peptides or of small proteins. The method involves the solid-phase synthesis of fully protected peptide segments which are then purified in solution. The purified protected peptides are coupled together on a new solid support and once the desired amino acid sequence has been built up, the fully deprotected peptide is cleaved from the resin and purified. In this way some of the drawbacks of linear solid-phase synthesis, such as the formation of deletion and/or terminated sequences, should be avoided and in principle the final purification should be facilitated.

The protected peptides must be detached from the resin with all the protecting groups of the side-chain functionalities and also that of the N^{α}-amino group in place. We have investigated the use of the photolabile Nbb-resin[4] for the synthesis of such protected segments using the Boc/Bzl- peptide synthesis strategy. Irradiation of a suspension of this resin in a mixture of TFE/CH_2Cl_2 gives rise to the protected peptide carboxylic acids as shown in Scheme 1. As a source of model peptides for studies on the viability of a

convergent solid-phase approach to large peptide synthesis, we have used the sequence of Uteroglobin,[5] a small protein consisting of two chains of 70 amino acids linked in an antiparallel fashion.



SCHEME 1

In this paper we describe the synthesis and purification of the peptides Boc-Gly-Ile-Cys(Acm)-Pro-OH, Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OH, Boc-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Glu(OcHex)-Thr(Bzl)-Ser(Bzl)-Leu-Lys(ClZ)-Glu(OcHex)-Phe-Glu(OcHex)-Pro-OH, Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OH, and Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-OH. These protected peptides together with those which we have described previously, comprise the entire sequence of the Uteroglobin monomer.[1,6]

RESULTS AND DISCUSSION

The Uteroglobin monomer contains five Met residues. Met can undergo alkylation or oxidation during peptide synthesis but there appears to be no clear consensus on whether or not this residue should be incorporated in a protected form.[7] Attempted synthesis of the protected peptide Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-OH, corresponding to the 50-58 sequence of the molecule, on the Nbb-resin was not successful because of essentially quantitative oxidation of the initially unprotected Met residue to the corresponding sulfoxide Met(O), in the photolytic cleavage reaction.[6,8] The synthesis of the peptide on the Nbb-resin proceeded without problems. The first amino acid of the sequence, in this case Boc-Lys(ClZ)-OH was attached to the solid support incorporating Phe as an internal standard, using the cesium salt procedure.[9] This method was used for the attachment of the first amino acid to the Nbb-resin for all peptides described here. The third amino acid of the sequence, in this case Boc-Ile-OH was incorporated using the method described by ourselves for minimising the formation of DKPs.[10] This procedure or the one described by Suzuki,[11] was used for the incorporation of the third amino acid of all peptides synthesised on the Nbb-resin, both methods giving similar results. All other amino acids were incoporated using a single coupling protocol. Amino acid analysis of the peptide-resin after the synthesis gave satisfactory values. Photolysis of the peptide from the resin proceeded in 82% yield as judged by amino acid analysis of the peptide-resin after the cleavage, and all peptidic material obtained was found to contain Met(O), as demonstrated by FABMS and high-field ^1H NMR. Since we were unable to avoid this oxidation in the photolysis step, we synthesised this peptide using the base-labile NPE handle [12] Synthesis of the peptide using this support was carried out using normal protocols and cleavage of the protected peptide from the resin using 20% piperidine in DMF gave rise to a very high (>90%) yield of the crude protected segment. Comparison of the HPLC retention times of the protected peptide segments produced in both of these syntheses indicated that the peptide incorporating the Met(O) residue was somewhat more polar as judged by the HPLC retention times of the two protected peptides

(Figure 1). The unavoidable oxidation of Met residues during the photolysis reaction together with the apparent greater polarity of the Met(O)-containing peptide led us to consider the protection of other Met residues in the sequence as their sulfoxides.[6] The greater polarity conferred upon these molecules by the presence of the sulfoxide might lead to increased solubility and hence to easier manipulation. A drawback of the use of Met(O) in peptide synthesis is that it leads to the formation of diastereomeric peptides, which in our experience are usually, but not always, resolvable by HPLC. The protected peptide Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met(O)-Lys(ClZ)-OH showed only a single peak upon HPLC analysis under a variety of conditions.

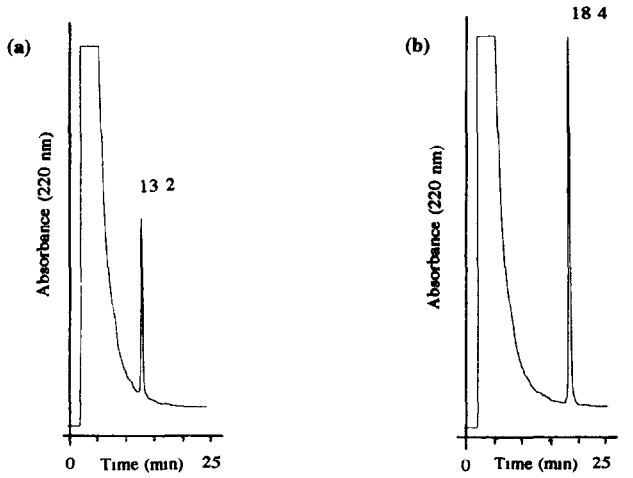


FIGURE 1. Analytical HPLC traces using a Vydac C_{18} reversed-phase column and eluting with a linear gradient from 50% to 60% of B in A over 25 min, where A is H_2O/0.045% TFA and B is MeCN/0.036% TFA at a flow rate of 1 mLmin^{-1} Detection by UV absorbance The large peak at the beginning of each chromatogram is due to DMF. **(a)** Purified Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met(O)-Lys(ClZ)-OH. **(b)** Purified Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-OH

Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OH corresponds to the 39-49 segment of the monomer and contains two Met residues protected as the sulfoxides. Amino acid analysis of the peptide resin after the synthesis gave satisfactory values. The yield for the photolytic cleavage reaction was 67% as judged by amino acid analysis of the peptide-resin afterwards. Careful HPLC analysis of the crude peptide showed the presence of the diastereomeric peptides corresponding to the desired amino acid sequence. Gratifyingly, in spite of its length, this peptide was found to be relatively soluble in DMF and it proved to be possible to purify it in a straightforward manner by preparative reversed-phase HPLC using eluents containing 30% DMF. It may be that the presence of the sequence -Met(O)-Gln-Met(O)- has a favourable effect with regard to increasing the polarity and solubility of this peptide in solvent mixtures of DMF, MeCN and H_2O.

We initially considered a synthetic strategy in which the 5-30 hexaeicosapeptide was incorporated as a single protected peptide segment. The synthesis of this segment was carried out on the Nbb-resin using the standard synthesis protocols required for the use of this resin. Amino acid analysis of the peptide-resin after the synthesis indicated the expected values. Photolytic cleavage of the peptide from the solid support gave rise to insoluble material and all our attempts to purify this peptide or indeed to analyse its purity by reversed-phase

HLPC were unsuccessful. Other authors[13] have also reported that the very limited solubility of longer protected peptides has necessitated a change in synthetic planning and a switch to the use of shorter protected peptides spanning the desired sequence. The poor solubility of longer protected peptides is a recurring theme in the literature and a satisfactory solution is as yet unavailable. We subsequently divided this sequence of the molecule into smaller and potentially more soluble units, namely the 19-30, 12-18 and 5-11 protected peptide segments.

The 19-30 dodecapeptide Boc-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Glu(OcHex)-Thr(Bzl)-Ser(Bzl)-Leu-Lys(ClZ)-Glu(OcHex)-Phe-Glu(OcHex)-Pro-OH was synthesised on the Nbb-resin in a straightforward manner and amino acid analysis of the peptide-resin after the synthesis gave the expected values. Photolytic cleavage from the resin occurred in a yield of 68%. This protected peptide was only sparingly soluble in DMF which made its purification difficult. Attempts to improve the purification procedure by carrying out HPLC using isopropanol in the eluants led to no significant improvement in the purity of the peptide. A partial purification was achieved by MPLC using a C_4 column.

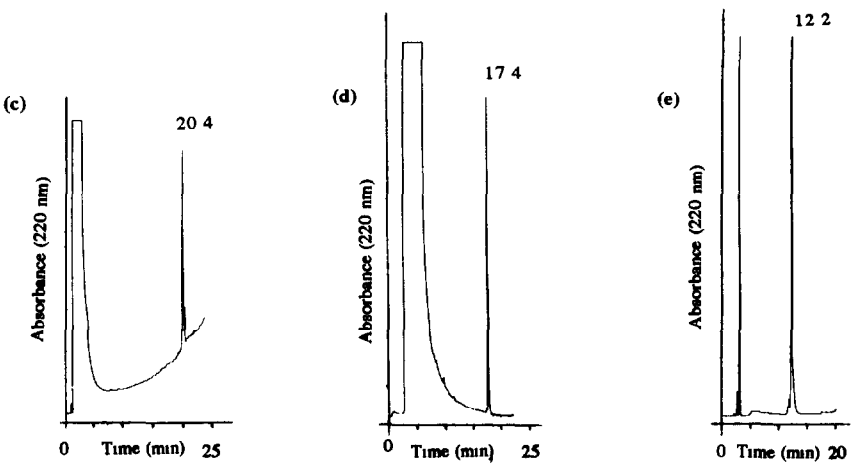


FIGURE 2. Analytical HPLC traces using a Nucleosil C_{18} reversed-phase column and eluting with a linear gradient from 10% to 100% of B in A over 25 min. For chromatogram (c), A is H_2O/0.1% TFA and B is MeCN/0.1% TFA. For chromatograms (d) and (e) A and B are as for FIGURE 1. Flow rate and detection as for FIGURE 1. The large peaks at the beginning of the chromatograms are due to DMF. (c) Purified Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OH. The complexity of the peak at 20 4 min. is due the presence of different sulfoxide diastereomers. (d) Purified Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OH. (e) Purified Boc-Gly-Ile-Cys(Acm)-Pro-OH.

The synthesis and purification of the Uteroglobin 12-18 protected peptide, Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH has previously been described by ourselves.[1] The peptide comprising the 5-11 sequence, Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OH contains the only His residue present in the Uteroglobin chain and we used the Bom[14] group for protection of the imidazole. The yield for the photolytic cleavage reaction was 61% as judged by amino acid analysis of the peptide-resin after the cleavage. This peptide was purified in a straightforward manner by semi-preparative HPLC.

The 1-4 tetrapeptide Boc-Gly-Ile-Cys(Acm)-Pro-OH was also synthesised on the Nbb-resin. Cleavage of the peptide from the resin was brought about by photolysis in a yield of 63% as judged by amino acid

analysis of the peptide-resin after cleavage. This peptide was purified by semi-preparative reversed-phase HPLC.

The peptides described here, in conjunction with those we have previously described, span the complete sequence of the Uteroglobin monomer. The protected peptides and the sequence of the monomer to which they correspond are shown below

(1-4) Boc-Gly-Ile-Cys(Acm)-Pro-OH
(5-11) Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OH
(12-18) Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH
(19-30) Boc-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Glu(OcHex)-Thr(Bzl)-Ser(Bzl)-Leu-Lys(ClZ)-Glu(OcHex)-Phe-Glu(OcHex)-Pro-OH
(31-38) Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH
(39-49) Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OH
(50-58) Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met(O)-Lys(ClZ)-OH
(59-67) Boc-Leu-Thr(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH

We have synthesised the *C*-terminal tripeptide (68-70) of the Uteroglobin monomer on a Pam-resin,[15] giving rise to Boc-Leu-Cys(Acm)-Met(O)-OCH_2-Pam-resin. As a first approximation to the total synthesis of Uteroglobin we propose to investigate a non-directed disulfide bond formation strategy and therefore have elected to protect both of the Cys residues of the monomer with the Acm group. We are at present investigating the coupling of the fully protected peptide segments described here onto the *C*-terminal tripeptide resin. These results will be reported in due course.

EXPERIMENTAL

Boc-amino acids were supplied by NovaBiochem AG (Läufelfingen, Switzerland), Bachem Feinchemikalien AG, (Bubendorf, Switzerland), Propeptide, (Vert-le-Petit, France) or Advanced ChemTech, (Maidenhead, England) and were used as received. BOP reagent was supplied by Richelieu Biotechnologies, (St. Hyacinth, Canada) and was used without further purification. DMF was supplied by Rathburn Chemicals, (Walkerburn, Scotland) and was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4Å molecular sieves. MeCN was HPLC grade, DCM and TFA were peptide synthesis grade and were used directly. Dioxane was distilled over KOH and then stored over sodium wire. All other solvents were distilled prior to use. Other reagents were used without further purification.

Peptide-resins were hydrolysed using 12M HCl/propionic acid (1:1) at 110°C for 48 hours and peptides were hydrolysed in a 6M aqueous HCl solution at 110°C for 24 hours. Amino acid analyses were performed on a Beckman System 6300 analyser. HPLC was carried out on a Shimadzu apparatus comprising two solvent delivery pumps model LC-6A, automatic injector model SIL-6B, variable wavelength detector model SPD-6A, system controller model SCL-6B and plotter model C-R6A. C_{18} Reverse-phase columns were used, either Vydac (25 x 0.5 cm, 5 μm) or Nucleosil (25 x 0.5 cm, 10 μm).

Reverse-phase MPLC was carried out using a LDC/MiltonRoy pump, an LKB 2158 Uvicord SD variable wavelength detector, an automatic fraction collector model LKB Ultrorac II 2070 and a Servoscribe 1s plotter. Preparative HPLC was carried out using a Labomatic apparatus comprising a Labomat VS-200 system

controller, a Labomatic HD-200 high pressure metering pump, a Labocord 700 uv/vis detector, a Labocol Roto 100 automatic fraction collector and a Servoscribe 1s plotter.

Photolyses were carried out on a Rayonet RPR-100 photochemical reactor supplied by the Southern New England Ultraviolet Company. The reaction vessel was silylated before photolysis of the peptide resin in order to prevent resin adhering to the walls of the vessel. This was done by rinsing the reaction vessel 3 or 4 times with a 10% solution of Me_3SiCl in CH_2Cl_2, followed by washing with absolute EtOH and drying. ^1H NMR spectra were recorded at 500 MHz on a Varian XL-500 instrument, using TMS as an internal standard. Chemical shifts are quoted in ppm downfield from TMS.

FAB mass spectra were recorded on a VG Quattro machine using 3-nitrobenzyl alcohol as matrix.

General Procedure for the Solid-Phase Assembly of Peptides

Peptide syntheses were performed manually in a 50 mL polypropylene syringe fitted with a polyethylene disc. Boc-amino acids (except the first and the third) were assembled using the following protocol: 1) CH_2Cl_2, 4 x 0.5 min; 2) 33% TFA/CH_2Cl_2, 1 x 1 min, 1 x 30 min; 3) CH_2Cl_2, 3 x 0.5 min; 4) 5% DIEA/CH_2Cl_2, 3 x 0.5 min; 5) CH_2Cl_2, 4 x 0.5 min; 6) Boc-amino acid (3 eq) in CH_2Cl_2 or DMF if necessary, after 2 min the equivalent amount of DCC in CH_2Cl_2 was added and the resin allowed to stand 60 min at r.t. with occasional agitation. For Asn, Gln, and Arg(Tos), the coupling was carried out using the HOBt active ester, formed by treating a solution of the amino acid (3eq) and HOBt (3eq) in DMF with a solution of DCC in CH_2Cl_2. After 30 min the mixture was filtered to remove the DCU formed, and the solution added to the resin; 7) CH_2Cl_2, 4 x 0.5 min; 8) DMF, 4 x 0.5 min; 9) CH_2Cl_2, 4 x 0.5 min. The qualitative ninhydrin test was used to monitor the synthesis.[16] If the test was positive the protocol was repeated from step 4.

General Procedure for the Photolytic Cleavage of o-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-Resins

Peptide-Nbb-resins were suspended in 4:1 CH_2Cl_2/TFE in a cylindrical reaction vessel. Prior to photolysis the peptide-resin suspension was degassed by evacuation at water-pump pressure and purging with argon three times in succession. The resin was photolysed for 7-15 hours maintaining vigorous magnetic stirring during this time. The reaction crude was filtered and the resin washed well with 4:1 CH_2Cl_2/TFE, CH_2Cl_2, DMF and then with MeOH. The combined filtrates were then evaporated to dryness.

Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-OH

Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-ONPE-resin (250 mg) was treated with a solution of 20% piperidine in DMF for 2 hours at room temperature. The mixture was filtered and the resin washed with 20% piperidine in DMF (3 x 1 min) and DMF (3 x 1 min). The combined filtrates were evaporated to dryness. The cleavage yield as judged by amino acid analysis was 91%.

The crude peptide (31 mg, 17.0 μmol) in DMF (2 mL) was loaded onto a Merck LiChroprep RP-C_8 column (440 mm by 37 mm, 40-63 μm), eluting with a convex gradient starting from DMF/MeCN/H_2O/propionic acid (30:30:39.5:0.5) (1L) to DMF/MeCN/H_2O/propionic acid (30:64.5:5:0.5) (1L) at a flow rate of 6 $mLmin^{-1}$. The volume of each individual fraction collected was 6 mL. Monitoring was carried out by subjecting the column fractions to analytical HPLC (using a Vydac C_{18} column and eluting with a linear gradient starting from 50% to 60% of B in A over 25 min, where A is H_2O/0.045% TFA and B is

MeCN/0.036% TFA, at a flow rate of 1 mLmin^{-1}; retention time of the peptide under these conditions is 18.4 min). All tubes containing pure peptide were combined and subjected to solvent removal by high-vacuum rotatory evaporation, maintaining the water-bath as cool as possible, until the volume of the peptide solution was 1-2 mL, water was then added to precipitate the protected peptide and this suspension was lyophilised to afford a white solid (17 mg, 9.2 μmol, 54% recovery) whose HPLC profile is shown in Figure 1(b) : composition Asp : 1.04; Thr : 1.68; Glu : 2.09; Met : 0.47; Ile : 1.00; Lys : 1.09; Arg : 0.81 ; FABMS *m/z* 1828.49 [(M+Na)$^+$, 100%], 1806.71 [(M+1)$^+$, 55%], $C_{86}H_{122}ClN_{15}O_{22}S_2$ requires M$^+$ 1805.55; ^{1}H NMR (500 MHz, d_6-DMSO) 8.20-6.60 [m, amide-H Asn, Thr, Glu, Gln, Met, Ile, Lys, Arg, NH Arg, NH_2 Asn, Gln, aromatics Thr(Bzl), Lys(ClZ), Arg(Tos)], 5.10 [s, benzylics Lys(ClZ)], 4.60-4.10 [m, α-H Asn, Thr, Glu, Gln, Met, Ile, Lys, Arg, benzylics Thr(Bzl)], 4.00-3.90 (m β-H Thr), 3.17-2.95 (m, δ-H Arg, ε-H Lys), 2.50-2.00 (m, β-H Asn, γ-H Gln, Glu, Met), 2.31 (s, Me Tos), 1.99 (s, Me Met), 1.90-1.40 [m, β-H Gln, Glu, Met, Ile, Lys, Arg, γ-H Lys, Arg, Glu(OcHex)], 1.34 (s, Bu^t), 1.30-1.20 (m, γ-H Ile, δ-H Lys), 1.08 (d, *J* 6 Hz, γ-H Thr), 1.04 (d, *J* 6 Hz, γ-H Thr), 0.80-0.70 (m, γ-H Ile, δ-H Ile).

Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met(O)-Lys(ClZ)-OH

Boc-Gln-Thr(Bzl)-Thr(Bzl)-Arg(Tos)-Glu(OcHex)-Asn-Ile-Met-Lys(ClZ)-OCH_2-Nbb-resin (550 mg) was photolysed (see General Procedure for the Photolytic Cleavage of *o*-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-Resins). Amino acid analysis of the peptide-resin before and after the photolysis reaction indicated a yield of 82% for the cleavage.

The crude peptide (46 mg, 25 μmol) in DMF (4 mL) was loaded onto a Merck LiChroprep RP-C_8 column (440 mm by 37 mm, 40-63 μm), eluting isocratically with DMF/MeCN/H_2O (5:50:45) (1.5 L) at a flow rate of 6 mLmin^{-1}. The volume of each individual fraction collected was 12 mL. Monitoring was carried out by subjecting the column fractions to analytical HPLC (using a Vydac C_{18} column and eluting with a linear gradient starting from 50% to 60% of B in A over 25 min, where A is H_2O/0.045% TFA and B is MeCN/0.036% TFA, at a flow rate of 1 mLmin^{-1}; retention time of the peptide under these conditions is 13.2 min). All tubes containing pure peptide were combined and subjected to solvent removal by high-vacuum rotatory evaporation, maintaining the water-bath as cool as possible for the elimination of DMF, until the volume of the peptide solution was 1-2mL, water was then added to precipitate the protected peptide and this suspension was lyophilised to afford a white solid (19 mg, 10.6 μmol, 43% recovery) whose HPLC profile is shown in Figure 1(a) : composition Asp : 1.05; Thr : 1.49; Glu : 2.05; Met : 0.64; Ile : 0.94; Lys : 1.00; Arg : 0.93 ; FABMS *m/z* 1844.26 [(M+Na)$^+$, 100%], 1822.35 [(M+1)$^+$, 11%], $C_{86}H_{122}ClN_{15}O_{23}S_2$ requires M$^+$ 1821.45; ^{1}H NMR (500 MHz, d_6-DMSO) 8.20-6 60 [m, amide H Asn, Thr, Gln, Glu, Met, Ile, Lys, Arg, NH Arg, NH_2 Asn, Gln, aromatics Thr(Bzl), Lys(ClZ), Arg(Tos)], 5.10 [s, benzylics Lys(ClZ)], 4.60-4.10 [m, α-H, Asn, Thr, Gln, Glu, Met, Ile, Lys, Arg, benzylics Thr(Bzl)], 4.00-3.90 (m, β-H Thr), 3.19-2.95 (m, δ-H Arg, ε-H Lys), 2.51 [s, Me Met(O)], 2.50-2.00 (m, β-H Asn, γ-H Gln, Glu, Met), 2.31 (s, Me Tos), 1.90-1.40 [m, β-H Gln, Glu, Met, Ile, Lys, Arg, γ-H Lys, Arg, Glu(OcHex)], 1.34 (s, Bu^t), 1.30-1.20 (m, γ-H Ile, δ-H Lys), 1.08 (d, *J* 6 Hz, γ-H Thr), 1.04, (d, *J* 6 Hz, γ-H Thr), 0.80-0.70 (m, γ-H Ile, δ-H Ile).

Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OH

Boc-Met(O)-Gln-Met(O)-Lys(ClZ)-Lys(ClZ)-Val-Leu-Asp(OcHex)-Ser(Bzl)-Leu-Pro-OCH_2-Nbb-resin (315 mg) was photolysed (see General Procedure for the Photolytic Cleavage of *o*-Nitrobenzyl Ester

Anchoring Linkages of Peptide-Nbb-Resins). Amino acid analysis of the peptide-resin before and after the photolysis reaction indicated a yield of 67% for the cleavage.

The crude peptide (54 mg, 26 μmol) in DMF (8 mL) was loaded onto a Vydac C_8 column (250 mm by 50 mm, 15-20 μm), eluting with a linear gradient starting from 10% B taken to 80% B over 25 min, followed by 10 min at 80% B, then from 80% B to 90% B over 10 min followed by 10 min at 90% B. Where A is DMF/H_2O/propionic acid (30:69.5:0.5) and B is DMF/MeCN/propionic acid (30:69.5:0.5) at a flow rate of 55 $mLmin^{-1}$. The volume of each individual fraction collected was 23 mL. Monitoring was carried out by subjecting the column fractions to analytical HPLC (using a Nucleosil C_{18} column and eluting with a linear gradient starting from 10% to 100% of B in A over 25 min, where A is H_2O/0.1% TFA and B is MeCN/0.1% TFA, at a flow rate of 1 $mLmin^{-1}$; retention time of the peptide under these conditions is 20.4 min. All tubes containing the pure desired peptide were combined and subjected to solvent removal by high-vacuum rotatory evaporation, maintaining the water-bath as cool as possible for the elimination of DMF, until the volume of the peptide solution was 1-2mL, water was then added to precipitate the protected peptide and this suspension was then lyophilised to afford a white solid (22 mg, 11.0 μmol, 42% recovery) whose HPLC profile is shown in Figure 2(c) : composition Asp : 1.04; Ser : 0.16; Glu : 1.00; Pro 1.16: Val : 1.01; Met : 1.08; Leu : 2.24; Lys : 2.08 ; FABMS *m/z* 1970.03 [$(M+K)^+$, 19%], 1954.02 [$(M+Na)^+$, 100%], $C_{90}H_{134}Cl_2N_{14}O_{24}S_2$ requires M^+ 1931.15; ^1H NMR (500 MHz, d_6-DMSO) 8.20-7.00 (m, amide-H Asp, Ser, Gln, Val, Met, Leu, Lys, NH_2, Gln), 7.5-7.2 [m, aromatics Ser(Bzl), Lys(ClZ)], 5.05 [s, benzylics Lys(ClZ)], 4.70-4.00 [m, α-H Asp, Ser, Gln, Val, Met, Leu, Lys, Pro, benzylics Ser(Bzl), β-H Thr], 3.00-2.40 (m, ε-H Lys, β-H Asp, Pro, γ-H Thr, Met), 2.50-2.495 (s, Me Met), 2.20-1.10 [m, β-H Gln, Val, Met, Leu, Lys, γ-H Gln, Lys, Pro, δ-H Lys, Asp(OcHex)], 1.36 (s, Boc), 0.84 (d, *J* 6.5 Hz, γ-H Val), 0.80-0.74 (m, δ-H Leu).

Boc-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Glu(OcHex)-Thr(Bzl)-Ser(Bzl)-Leu-Lys(ClZ)-Glu(OcHex)-Phe-Glu(OcHex)-Pro-OH

Boc-Ser(Bzl)-Ser(Bzl)-Tyr(BrZ)-Glu(OcHex)-Thr(Bzl)-Ser(Bzl)-Leu-Lys(ClZ)-Glu(OcHex)-Phe-Glu(OcHex)-Pro-OCH_2-Nbb-resin (315 mg) was photolysed (see General Procedure for the Photolytic Cleavage of *o*-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-Resins). Amino acid analysis of the peptide-resin before and after the photolysis reaction indicated a yield of 68% for the cleavage.

The crude peptide (34 mg, 13.7 μmol) in DMF (1 mL) was loaded onto a Vydac C_4 column (270 mm by 25 mm, 15-20 μm), eluting with a linear gradient starting from 50%B taken to 100%B over 60 min, followed by 60 min at 100%B, where A is DMF/H_2O/propionic acid (60:39.5:0.5) and B is DMF/MeCN/propionic acid (60:39.5:0.5) at a flow rate of 6 $mLmin^{-1}$. The volume of each individual fraction collected was 12 mL. Monitoring was carried out by subjecting the column fractions to analytical HPLC (using a Nucleosil C_4 column and eluting with a linear gradient starting from 10% to 100% of B in A over 25 min, followed by 5 min eluting with 100% of B, where A is H_2O/0.1% TFA and B is MeCN/0.1% TFA, at a flow rate of 1 $mLmin^{-1}$; retention time of the peptide under these conditions is 25.5 min). All tubes containing the desired peptide were combined and subjected to solvent removal by high-vacuum rotatory evaporation, maintaining the water-bath as cool as possible for the elimination of DMF, until the volume of the peptide solution was 1-2mL, water was then added to precipitate the protected peptide and this suspension was then lyophilised to afford an off-white solid (10 mg, 4.9 μmol, 36% recovery) : composition Thr : 0.76; Ser : 1.02; Glu : 2.98; Leu : 1.00; Tyr : 0.47; Phe : 0.92; Lys : 0.94 ; FABMS *m/z* 2542.71 [$(M+K)^+$, 20%], 2526.82[$(M+Na)^+$, 100%], 2505. 82 [$(M+1)^+$, 15%],

$C_{130}H_{163}BrClN_{13}O_{30}$ requires M^+ 2503.86; ^1H NMR (500 MHz, d_6-DMSO) 8.25-6.80 [m, amide-H Thr, Ser, Glu, Leu, Tyr, Phe, Lys, aromatics Ser(Bzl), Thr(Bzl), Tyr, Tyr(BrZ), Phe, Lys(ClZ)], 5.28 [s, benzylics Tyr(BrZ)], 5.05 [s, benzylics Lys(ClZ)], 4.70-4.10 [m, α-H Thr, Ser, Glu, Phe, Leu, Tyr, Lys, Pro, benzylics, Thr(Bzl), Ser(Bzl)], 4.00-2.00 (m, β-H Thr, Ser, Leu, Tyr, Phe, Pro, ε-H Lys, γ-H Glu), 2.00-1.10 [m, β-H Glu, Lys, γ-H Lys, δ-H Lys, Glu(OcHex)], 1.34 (s, Boc), 1.06 (d, γ-H Thr), 0.74 (d, γ-H Leu), 0.71 (d, γ-H Leu).

Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OH

Boc-Arg(Tos)-Phe-Ala-His(Bom)-Val-Ile-Glu(OcHex)-OCH_2-Nbb-resin (340 mg) was photolysed (see General Procedure for the Photolytic Cleavage of *o*-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-Resins). Amino acid analysis of the peptide-resin before and after the photolysis reaction indicated a yield of 61% for the cleavage.

The crude peptide was purified by repetitive semi-preparative HPLC (using a Vydac C_{18} column, 250 mm by 10 mm, 5 μm) and eluting with a linear gradient starting from 10% to 100% of B in A over 25 min, followed by 10 min eluting with 100% of B, where A is H_2O/0.045% TFA and B is MeCN/0.036% TFA, at a flow rate of 2.5 $mLmin^{-1}$. The pure peptide fractions were combined and the MeCN removed by rotatory evaporation, leaving an aqueous solution which was then lyophilised to afford a white solid (3 mg) whose HPLC profile (Nucleosil C_{18} column, eluting with a linear gradient starting from 10% B taken over 25 min to 100% B, where A and B are as for the semi-preparative run, at a flow rate of 1 $minmL^{-1}$; retention time of the peptide under these conditions is 17.4 min) is shown in Figure 2 (d) : composition Glu : 1.00; Ala : 1.04; Val : 0.68; Ile 0.54; Phe : 1.03; His : 0.78; Arg : 1.06 ; FABMS *m/z* 1328.62 [$(M+1)^+$, 30%], 266(100%) $C_{66}H_{94}N_{12}O_{15}S$ requires M^+ 1327.61; ^1H NMR (500 MHz, d_6-DMSO) 8.30-6.80 [m, amide-H Glu, Ala, Val, Ile, Phe, His, Arg, aromatics Phe, His, His(Bom), Arg(Tos)], 4.80-4.10 (m, α-H Glu, Ala, Val, Ile, Phe, His, Arg), 3.00-2.50 (m, β-H Thr, Glu), 2.30 [s, Me Arg(Tos)], 2.25 (m, γ-H Glu), 2.00-0.80 [m, β-H Ala, Val, Ile, γ-H Val, Ile, Glu(OcHex)], 1.38 (s, Boc).

Boc-Gly-Ile-Cys(Acm)-Pro-OH

Boc-Gly-Ile-Cys(Acm)-Pro-OCH_2-Nbb-resin (100 mg) was photolysed (see General Procedure for the Photolytic Cleavage of *o*-Nitrobenzyl Ester Anchoring Linkages of Peptide-Nbb-Resins). Amino acid analysis of the peptide-resin before and after the photolysis reaction indicated a yield of 63% for the cleavage.

The crude peptide was purified by repetitive semi-preparative HPLC (using a Vydac C_{18} column, 250 mm by 10 mm, 5 μm) and eluting with a linear gradient starting from 10% to 100% of B in A over 25 min, followed by 10 min eluting with 100% of B, where A is H_2O/0.045% TFA and B is MeCN/0.036% TFA, at a flow rate of 2.5 $mLmin^{-1}$. The pure peptide fractions were combined and the MeCN removed by rotatory evaporation, leaving an aqueous solution which was then lyophilised to afford a white solid (2 mg) whose HPLC profile (Vydac C_{18} column, eluting with a linear gradient starting from 10% B taken over 25 min to 100% B, where A and B are as for the semi-preparative run, at a flow rate of 1 $minmL^{-1}$; retention time of the peptide under these conditions is 12.2 min) is shown in Figure 2 (e) : composition Gly : 1.00; Ile : 0.87 ; Pro : 0.87 ; FABMS *m/z* 582.21 [$(M+Na)^+$, 66%], 427.11 (100%), $C_{24}H_{41}N_5O_8S$ requires 559.67; ^1H NMR (500MHz, d_6-DMSO) 8.49 (t, amide-H Acm), 8.36 (d, amide-H Cys or Ile), 7.56 (d, amide-H, Ile or Cys), 7.00 (t, amide-H Gly), 4.60-4.14 (m, α-H Cys, Gly, Ile, Pro, CH_2 Acm), 3.59-3.60 (m, β-H Gly), 3.58-3.40

(m, β-H Cys, δ-H Pro), 2.90 (m, β-H Pro), 2.66 (m, β-H Pro), 2.10 (m, β-H Ile), 1.88-1.81 (m, γ-H Pro), 1.83 (s, Me Acm), 1.63 (γ-H Pro), 1.36 (s, Bu^t), 1.00 (m, γ-H Ile), 0.80 (m, δ-H Ile).

Acknowledgements We thank Ministerio de Ciencia y Educación, Madrid for a research award to M.G. This work was supported by grant PB 92-0864 from Comisión de Investigación Cientifica y Técnica, Madrid and by grant SC1-CT91-0748 from the Commission of the European Communities.

REFERENCES

1 For Part X, see Lloyd-Williams, P.; Gairí. M.; Albericio, F.; Giralt, E. *Tetrahedron*, **1991**, *47*, 9867-9880.

2 Abbreviations used in this paper for amino acids and for the designations of peptides follow the rules of the IUPAC-IUB Comission of Biochemical Nomenclature in *European J. Biochem.*, **1984**, *138*, 9-37 and *J. Biol. Chem.*, **1989**, *264*, 633-673. The following additional abbreviations are used: Boc, *tert*-butyloxycarbonyl; Bom, benzyloxymethyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; FABMS, fast atom bombardment mass spectrometry; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; *p*-MBHA, *p*-methylbenzhydrylamine; MeCN, acetonitrile; MPLC, medium-pressure liquid chromatography; Nbb-, nitrobenzamidobenzyl; -resin, poly(styrene-*co*-1% divinylbenzene); NMR, nuclear magnetic resonance; NPE, nitrophenylethyl; Pam. phenylacetamidomethyl-; TMS, tetramethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

3 For a recent review see Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron*, in press.

4 a) Giralt, E.; Albericio, F.; Andreu, D.; Eritja, R.; Martin, P.; Pedroso, E. *An. Quím.*, **1981**, *77C*, 120-125, b) Giralt, E.; Albericio, F. A.; Pedroso, E.; Granier, C.; van Rietschoten, J. *Tetrahedron*, **1982**, *38*, 1193-1208.

5 Morize, I.; Surcouf, E.; Vaney, M.C.; Epelboin, Y.; Buchner, M.; Fridlansky, F.; Milgrom, E.; Mornon, J.P. *J. Mol. Biol.*, **1987**, *194*, 725-739.

6 Lloyd-Williams, P.; Albericio, F ; Giralt, E. *Int. J. Peptide Protein Res.*, **1991**, *37*, 58-60.

7 Barany, G.; Merrifield, R. B. *In* "The Peptides", (Gross, E.; Meienhofer, J., eds.), Volume 2, Academic Press, New York, 1979, pp 223-233.

8 Hammer, R.; Albericio, F.; Gera, L.; Barany, G. *Int. J. Peptide Protein Res.*, **1990**, *36*, 31-45.

9 Gisin, B. F. *Helv. Chim. Acta*, **1973**, *56*, 1476-1483.

10 Gairí, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron Lett.*, **1990**, *31*, 7363-7366.

11 Suzuki, K.; Nitta, K.; Endo, N. *Chem. Pharm. Bull.*, **1975**, *23*, 222-224.

12 Albericio, F.; Giralt, E.; Eritja, R. *Tetrahedron Lett.*, **1991**, *32*, 1515-1518.

13 Sasaki, T.; Findeis, M. A.; Kaiser, E. T. *J. Org. Chem.*, **1991**, *56*, 3159-3168.

14 Brown, T.; Jones, J. H. *J. Chem. Soc., Chem. Commun.*, **1981**, 648-649.

15 Mitchell, A. R.; Erickson, B. W.; Ryabtsev, M. N.; Hodges, R. S.; Merrifield, R. B. *J. Am Chem. Soc.*, **1976**, *98*, 7357-7362.

16 Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal Biochem.*, **1981**, *117*, 147-157.