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Convergent Solid-Phase Peptide Synthesis. X. Synthesis and Purification of Protected Peptide Fragments 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.

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Abstract: Protected peptide fragments corresponding to the 12-18, 31-38 and 59-67 segments of the Uteroglobin monomer have been synthesised on a solid support using the photolabile *ortho*-nitrobenzyl unit as a handle. Attachment of the first amino acid of the sequence has been carried out in three different ways and a new procedure for avoiding the formation of DKPs in the coupling of the third amino acid with this resin-handle is described. Photolytic detachment of the peptides from the solid support occurs in good yields. In spite of their low solubility in the normal solvents used in reverse-phase MPLC and HPLC techniques, protected peptide fragments can be purified by MPLC utilising solvents containing a high proportion of DMF.

INTRODUCTION

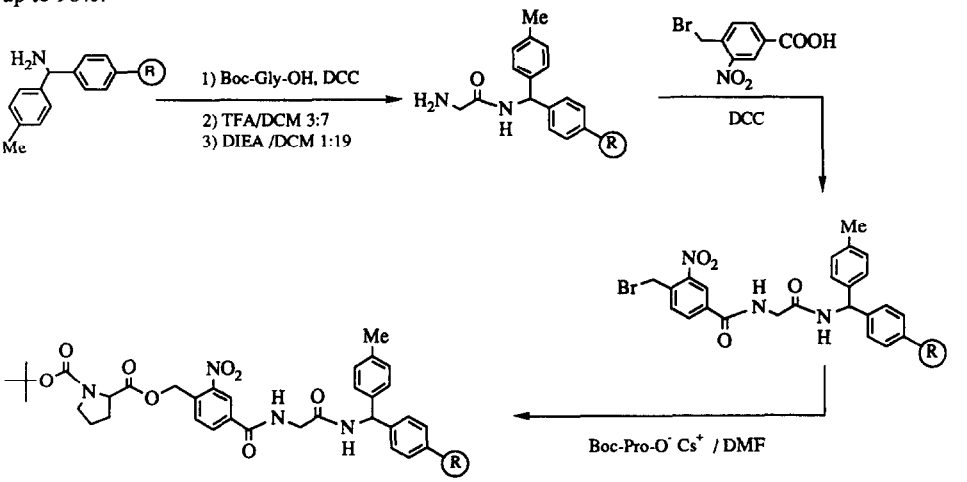
Convergent solid-phase synthesis is one of the most promising methodologies for the synthesis of peptides of high molecular weight or small proteins.[3] The method is based upon the synthesis of fully protected peptide fragments, which are then detached from the solid support and purified. Several purified protected peptides are then coupled together on a new solid support and the fully deprotected peptide is cleaved from the resin and purified. This method should avoid some of the drawbacks of the classical solid-phase approach, such as the formation of deletion and/or terminated sequences, and in principle should facilitate the final purification.

Of paramount importance in this strategy is the method of detaching the protected peptide from the resin. The protected peptide must be detached from the resin with all the protecting groups of the side-chains and also that of the N-terminal α-amino group intact. We have investigated the use of the photolabile Nbb-resin,[3b] for the synthesis of protected peptide fragments and our study has concentrated on the incorporation of the first amino acid, a new strategy for coupling the third residue in order to avoid the formation of diketopiperazines (DKPs), and also on the purification of protected peptide fragments by medium pressure liquid chromatography using solvent systems containing a high (up to 60%) proportion of DMF.

As a source of model peptides for our studies on the viability of this approach to peptide synthesis we have used the sequence of the Uteroglobin molecule,[4] a small protein consisting of two chains of 70 amino acids linked in an antiparallel sense, and have synthesised and purified several fragments of the monomer of this molecule. In this paper we describe the synthesis and purification of the peptides Boc-Leu-Thr-(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH, Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH and Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH which correspond to the fragments 59 to 67, 12 to 18 and 31 to 38 respectively of the Uteroglobin monomer.

RESULTS AND DISCUSSION

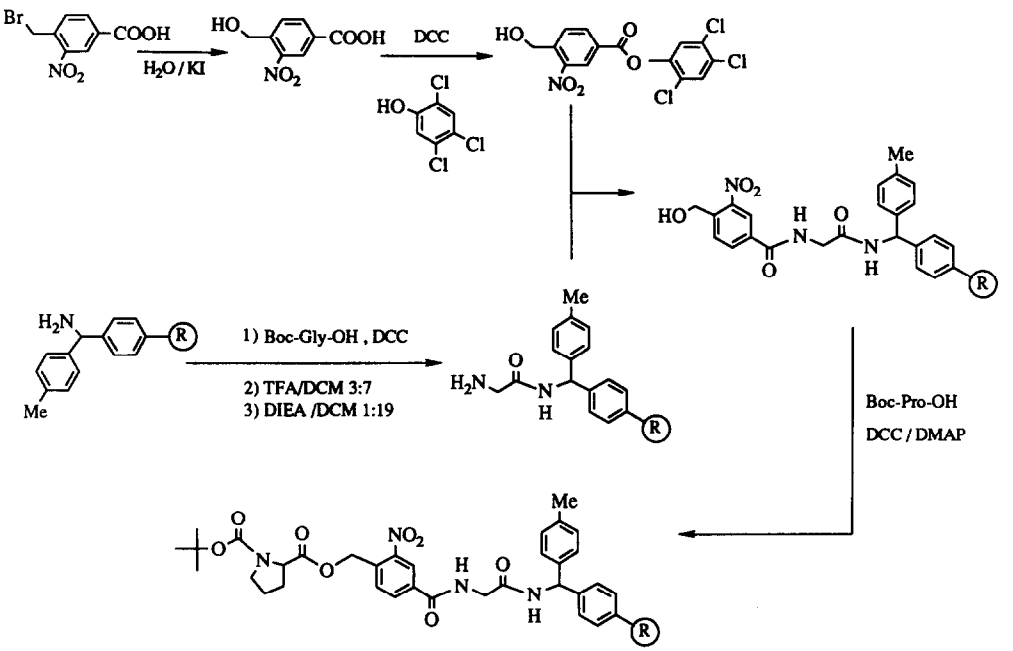
Traditionally when the photolabile Nbb handle is used the first amino acid is incorporated onto the resin using the cesium salt method first described by Gisin.[5] In most of the protected fragments we have synthesised to date, the C-terminal amino acid has been proline. Generally speaking, in our hands for Boc-proline the cesium salt procedure has worked well. Thus for example in Scheme 1, reaction of the cesium salt of N-Boc-proline with Nbb-resin to which glycine had been coupled in order to serve as an internal standard,[6] gives incorporation yields of up to 90%.



Scheme 1. Incorporation of Boc-proline using the cesium salt method.

However we have also found that in some cases yields for both Boc-proline and other amino acids can be low and poorly reproducible.[7] We have investigated two alternative approaches for the anchoring of the first amino acid. Firstly, by modifying the bromomethyl nitrobenzyl handle and converting it to the corresponding hydroxy derivative the first amino acid can be attached by a simple esterification. Thus 4-bromomethyl 3-nitrobenzoic acid was treated with water in the presence of potassium iodide and after work up gave the desired 4-hydroxymethyl 3-nitrobenzoic acid. This after purification was converted into its 2,4,5-trichlorophenyl ester and then coupled to a benzhydrylamine resin, containing glycine as an internal standard,[6] affording the hydroxymethyl Nbb-resin. Esterification of Boc proline onto this resin-handle using DCC and DMAP gave incorporation yields of 79%. See Scheme 2.

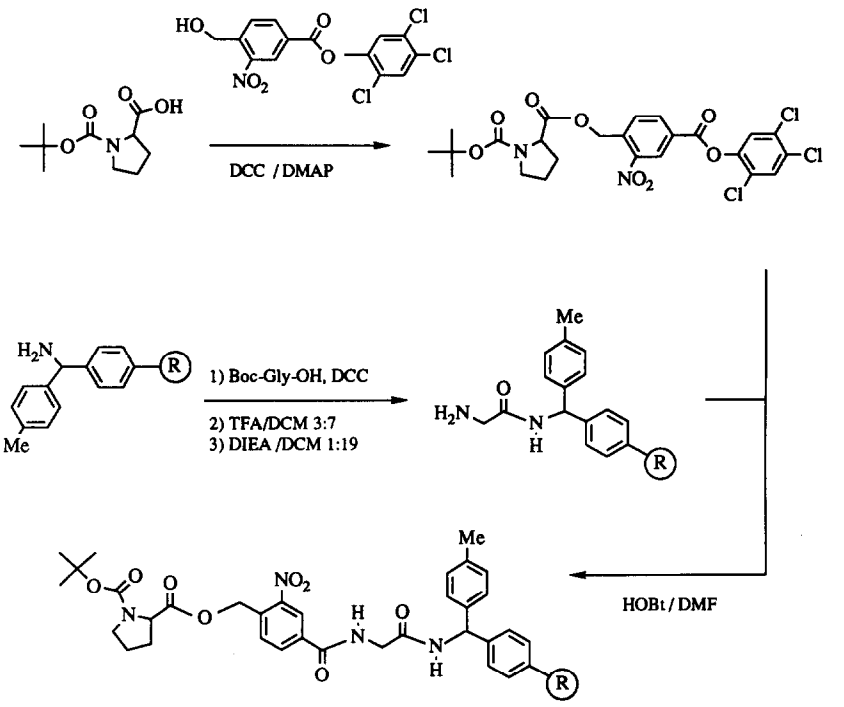
Our second alternative for the incorporation of the first amino acid was to first couple Boc-proline to the handle in solution and then after isolation and purification to attach this preformed handle[8] to the resin. Thus, Boc-Pro-OH was coupled to the 2,4,5-trichlorophenol ester of 4-hydroxymethyl 3-nitrobenzoic acid giving 2,4,5-trichlorophenyl 4-(Boc-prolyloxymethyl)-3-nitrobenzoate. See Scheme 3.



Scheme 2. Incorporation of Boc-proline onto hydroxymethyl-Nbb-resin.

This was then purified and attached to a benzhydrylamine resin, to which again glycine had been coupled in order to serve as an internal standard, using HOBt in DMF. In this way quantitative incorporation of the proline onto the resin was achieved. Although this procedure is somewhat lengthy due to the number of steps involved it is the only one which assures a well-defined starting point for the synthesis. It also avoids leaving unreacted bromomethyl- or hydroxymethyl-groups on the resin which can give rise to troublesome side-reactions and to undesired peptide sequences.

The formation of diketopiperazines[9] can be a problem when benzyl-type peptide-resin linkages are used for the synthesis of peptide fragments and even more so in the case of nitrobenzyl-type linkages. The formation of DKPs leads to the formation of undesired hydroxymethyl groups on the resin which can again be a starting point for the synthesis of undesired peptide sequences. We have investigated the use of BOP reagent[10] as a possible alternative to the well-known Suzuki[11] procedure for avoiding DKP formation. This new method also suppresses the formation of DKPs and has the advantages of requiring a shorter (approx. 60 min.) reaction time, of not requiring freshly-prepared HCl in dioxane and of being amenable to use in automatic peptide synthesis protocols. For our study on the use of the BOP reagent for this purpose we chose the tripeptide Lys-Ala-Pro, corresponding to the 4-6 fragment of the neurotoxin apamin as a model since we have had previous experience of the problem with this sequence.[3b] Boc-Phe-OH was attached directly to a *p*-MBHA resin and served as an internal standard for the estimation of DKP formation.



Scheme 3. Incorporation of pre-formed Boc-proline-handle onto the resin.

The incorporation of Lys was carried out by deprotecting the amino group of the dipeptide-Nbb-resin with TFA, and then without prior neutralisation, coupling Boc-Lys(ClZ)-OH using BOP reagent both in the presence and in the absence of additional HOBt[12] using either DIEA or NMM as base. This BOP-mediated coupling was then compared with both the Suzuki procedure and a standard protocol for the same amino acid coupling step. Our results are summarised in Table 1, entries 1-7.

DKP formation of the order of 59% was observed with a standard protocol whereas the Suzuki procedure completely suppressed DKPs. Using BOP, the amount of DKP formed was dependent upon the base employed. With NMM 20% DKP formation was observed, but use of DIEA led to almost complete suppression of DKPs and a considerably shorter reaction time. Addition of extra HOBt had little effect upon the amount of DKP formed.

In order to prove the effectiveness of this procedure more stringently we used BOP reagent with additional HOBt and DIEA as base in the coupling of Boc-Lys(ClZ)-OH to the dipeptide D-Val-L-Pro, since this sequence is known to be particularly susceptible to DKP formation. Thus the dipeptide-resin D-Val-L-Pro-Nbb-resin was deprotected with TFA and then subjected to a standard coupling procedure which gave rise to 91% formation of DKP whereas the Suzuki method led to less than 10% DKP. Deprotection of the dipeptide-resin with TFA followed by coupling of Boc-Lys(ClZ)-OH in the presence of additional HOBt and DIEA as base gave comparable results to the Suzuki procedure for this sequence. See Table 1.

Table 1

Entry	Coupling Procedure for the third amino acid[a]	Amino acid ratio[b]			Formation of DKPs (%)[c]
		Phe	Ala	Lys	
(1)	Standard protocol	1.00	0.36	0.26	59
(2)	Suzuki	1.00	0.88	0.92	0
(3)	BocaaOH/ BOP/ NMM[d] 3eq 3eq 6eq	1.00	0.70	0.68	20
(4)	BocaaOH/HOBt/BOP/ NMM 3eq 3eq 3eq 6eq	1.00	0.70	0.69	20
(5)	BocaaOH/ BOP/ NMM 3eq 1.5eq 6eq	1.00	0.76	0.77	12
(6)	BocaaOH/ BOP/ DIEA 3eq 3eq 6eq	1.00	0.82	0.94	5
(7)	BocaaOH/HOBt/BOP/ DIEA 3eq 3eq 3eq 6eq	1.00	0.85	0.83	2
		Phe	D-Val	Lys	
(8)	Standard protocol	1.00	0.07	0.05	91
(9)	Suzuki	1.00	0.72	0.68	8
(10)	BocaaOH/ BOP/ DIEA 3eq 3eq 6eq	1.00	0.71	0.72	9
(11)	BocaaOH/HOBt/ BOP/ DIEA 3eq 3eq 3eq 6eq	1.00	0.71	0.73	9

[a]Qualitative ninhydrin test[13] was used to determine completion of coupling.
[b]Determined by amino acid analysis.
[c]Calculated from the ratio of the second amino acid (Ala or D-Val respectively) to Phe after incorporation of Lys, divided by the same ratio before incorporation (0.87 or 0.78 respectively) and expressed as a percentage.
[d]Equivalents are quoted relative to the initial amino substitution of the resin.

The use of BOP reagent[14] under the appropriate conditions can be a practical and less time-consuming alternative to the Suzuki procedure for the coupling of the third amino acid in cases where DKP formation is a problem.

In the synthesis of the protected peptides Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH, Boc-Leu-Thr-(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH and Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH the incorporation of the first and third amino acids was accomplished as we have previously described. Methionine was incorporated as the sulphoxide in order to avoid the undesired oxidation of the unprotected residue during the photolysis and consequently two diastereomers were obseved for the Met-containing peptide. All other amino acids were protected with typical side-chain protecting groups: cHex for Glu and Asp, Bzl for Thr and Ser and ClZ for Lys. Once synthesised the protected peptides were detached from the resin by photolysis using a Rayonet apparatus and 4:1 toluene/TFE as solvent.

Purification of protected segments is a crucial step in our convergent solid-phase approach. Because of the poor solubility of protected peptides in most solvents the methods conventionally applied to the purification of unprotected peptides cannot be used. In our case, purification of each of the protected peptide fragments was achieved by dissolving the crude protected peptide (up to 100 mg) in DMF and subjecting it to MPLC with water and acetonitrile as solvents each containing DMF (up to 60% by volume) and 0.5-1% propionic acid. Monitoring was carried out by UV in the case of Tyr containing peptides or subjecting the column fractions to reverse-phase

analytical HPLC when a suitable chromophore was not present, using standard acetonitrile-water solvent systems and all peptide-containing fractions were combined, submitted to solvent removal and lyophilised. The pure products were characterised by amino acid analysis, high field ^{1}H-NMR and FAB-MS.

For the peptide Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH the photolytic cleavage from the resin gave the crude peptide in 70% yield. After purification by MPLC using DMF-containing solvents as described above a white powdery solid was obtained. The HPLC profile of the pure peptide is shown in Figure 1.

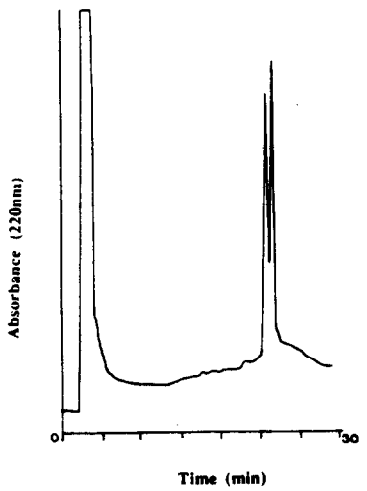


FIGURE 1. HPLC of pure Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH. Analytical HPLC was performed using a reversed-phase C_{18} column and eluting with a linear gradient from 10% to 100% of B in A over 20 min, followed by 10 min eluting with 100% B, where A is H_2O/0.045%TFA and B is MeCN/0.036% TFA, at a flow rate of 1.5 mLmin^{-1}. UV absorbance at 220 nm.

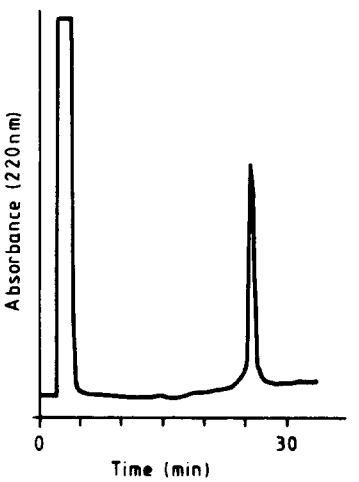


FIGURE 2. HPLC of pure Boc-Leu-Thr-(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH. Analytical HPLC was performed using a reversed-phase C_{18} column and eluting with a linear gradient from 10% to 100% of B in A over 25 min, followed by 10 min eluting with 100% B, where A is H_2O/0.045%TFA and B is MeCN/0.036% TFA, at a flow rate of 1 mLmin^{-1}. UV absorbance at 220 nm.

Photolysis of the peptide Boc-Leu-Thr-(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH gave the crude peptide in 74% yield and the peptide was purified by initially passing the crude mixture dissolved in DMF through a column of Sephadex LH 20 eluting with DMF. The product was collected and the solvent removed. The resulting yellow-white solid was then dissolved in DMF and subjected to reverse phase MPLC using solvents containing DMF. After work-up the pure peptide was obtained as a white solid whose HPLC profile is shown in Figure 2

Photolysis of the peptide Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH gave the crude peptide in 47% yield and the crude peptide dissolved in DMF was subjected to MPLC in a similar manner to that described for the previous peptide. The pure product was obtained after work-up as a white powdery solid whose HPLC profile is shown below (See Figure 3).

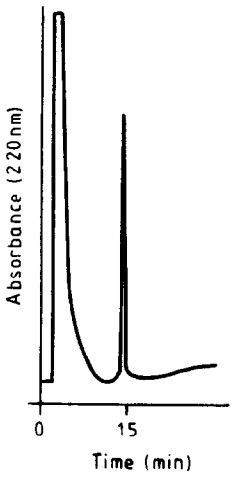


FIGURE 3. HPLC of pure Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH. Analytical HPLC was performed using a reversed-phase C_{18} column and eluting with a linear gradient from 10% to 100% of B in A over 20 min, where A is H_2O/0.045%TFA and B is MeCN/0.036% TFA, at a flow rate of 1.5 mLmin^{-1}. UV absorbance at 220 nm.

These results indicate that protected peptide fragments can be synthesised and detached from the solid support in good yields. Attention must be paid to the method of anchoring the first amino acid to the resin. Among the three different alternatives proposed in this paper attachment of a preformed handle is the method of choice. Formation of diketopiperazines can be avoided in most sequences by using either the Suzuki method or BOP reagent. In spite of their low solubility in the normal solvents used in reverse-phase MPLC and HPLC techniques, protected peptide fragments can be purified by MPLC utilising solvents containing DMF. This enhances the viability of the fragment coupling approach to the chemical synthesis of large peptides.

Use of the photolabile Nbb-resin followed by purification of protected peptides by MPLC using solvents containing DMF is an efficient method for the preparation of fully protected peptides that can be further used either in solid-phase convergent synthesis or for large scale protected segment coupling in solution. Further work is in progress directed towards improving and refining these techniques.

MATERIALS AND METHODS

Chemicals

Boc-amino acids were supplied by Nova Biochem AG (Läufelfingen, Switzerland) and were used as supplied. 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 4A molecular sieves. MeCN was HPLC grade and used directly. Dioxane was distilled over KOH and then stored over sodium. All other solvents were distilled prior to use. Other reagents were used without further purification.

Methods

Peptide resins were hydrolysed using 12M HCl/propionic acid (1:1) at 110°C for 48 hours and peptides were hydrolysed using 12M HCl/propionic acid (1:1) at 110°C for 24 hours. Amino acid analyses were performed on a Beckmann System 6300 analyser. HPLC was carried out on a Shimadzu apparatus comprising two solvent delivery pumps model LC-6A, automatic injector model SIL-6B69A, 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) with 5 μm particle packing or Nucleosil (25 x 0.5 cm) with 10 μm particle packing.

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. A Merck LiChroprep RP-C8 column (1.2 x 20 cm) 40-63 μm was employed. This system was also used for Sephadex LH-20 columns supplied by Pharmacia.

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 carried out by rinsing the reaction vessel 3 or 4 times with a 10% solution of Me_3SiCl in toluene, followed by washing with absolute EtOH and drying. UV spectra were recorded on a Perkin-Elmer Lamda 5 spectrophotometer. ^1H NMR spectra were recorded at 60 MHz on a Perkin-Elmer R-24 instrument, at 200 MHz on a Varian XL-200 instrument and at 500MHz on a Varian XL-500 instrument, using TMS as an internal standard. Chemical shifts are quoted in ppm downfield from TMS.

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, 4x0.5 min; 2) 33% TFA/CH_2Cl_2, 1x1 min, 1x30 min; 3) CH_2Cl_2, 3x0.5 min; 4) 5% DIEA/CH_2Cl_2, 3x 0.5 min; 5) CH_2Cl_2, 4x0.5 min; 6) Boc-amino acid (3eq) in CH_2Cl_2, after 2 min add the equivalent amount of DCC in CH_2Cl_2, stand 90 min at r.t. with occasional agitation; 7) CH_2Cl_2, 4x0.5 min; 8) DMF, 4x0.5 min; 9) CH_2Cl_2, 4x0.5 min. The qualitative ninhydrin test was used to monitor the synthesis, 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 (max. 300mg) were suspended in 4:1 toluene/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 9-14 hours maintaining vigorous magnetic stirring during this time. The reaction crude was filtered and the resin washed well with 4:1 toluene/TFE, followed by CH_2Cl_2 and finally with MeOH and dried. The combined filtrates were then evaporated to dryness.

Incorporation of the First Amino Acid.

Method (a)

Bromomethyl-Nbb-Gly-resin. 4-Bromomethyl-3-nitrobenzoic acid (0.33g, 1.2mmol) was dissolved in CH_2Cl_2 and cooled to 0°C. A 1M solution of DCC in CH_2Cl_2 (1.2mL, 1.2mmol) was added and this mixture allowed to stand at 0°C for 15 min. The DCU formed was removed by filtration and the filtrate was added to pMBHA resin containing Gly as an internal standard (1.0g) and allowed to stand 120 min at r.t with occasional agitation. The resin was then washed well with CH_2Cl_2 and MeOH. The qualitative ninhydrin test was negative.

Cesium Boc-prolinate. Boc-Pro-OH (0.3g, 1.5mmol) was dissolved in 4:1 EtOH/H_2O (10mL) and a 1M solution of Cs_2CO_3 was added dropwise until the pH was 7.0. The solvent was removed, azeotroping with benzene and the resulting white solid was kept overnight *in vacuo* over P_2O_5.

Boc-Pro-OCH$_2$-Nbb-Gly-resin. Boc-Pro-O$^-$Cs$^+$ was dissolved in DMF (5 mL) in a previously silylated flask (see Methods) and the bromomethyl-Nbb-Gly-resin was added. The mixture was stirred mechanically for 22 hours at 35°C. The resin was washed as follows: DMF (3x1min), DMF/H_2O 9:1 (5x2min), DMF/H_2O 4:6 (5x2min), DMF/H_2O 9:1 (5x2min), DMF (3x1min) and finally with MeOH. Amino acid analysis of the resin hydrolysate after coupling gave a yield of 93% for the incorporation of proline.

Method (b)

4-Hydroxymethyl-3-nitrobenzoic acid. 4-Bromomethyl-3-nitrobenzoic acid (4.6g, 17.7mmol) was added to a suspension of KI (0.46g, 2.8mmol) in H_2O (40mL) with vigorous stirring and the mixture was refluxed for 18 hours. The mixture was allowed to cool and filtered washing well with H_2O. The off-white crude product was recrystallised from H_2O affording 4-hydroxymethyl-3-nitrobenzoic acid as pale yellow needles, (1.1g, 29%), m.p. 162-166°C (lit.[3g] 174-175°C). It had δ (60 MHz, $CDCl_3$) 8.62 (d, J =1.7 Hz, 1H), 8.25 (dd, J_1 =8.7 Hz, J_2 = 1.7 Hz, 1H), 7.85 (d, J = 8.7 Hz, 1H), 5.00 (s, 2H).

2,4,5-Trichlorophenyl 4-hydroxymethyl-3-nitrobenzoate. A solution of 2,4,5-trichlorophenol (0.61g, 3.0mmol) and DCC (0.64g, 3.01mmol) in CH_2Cl_2 (4mL) was added to a solution of 4-hydroxymethyl-3-nitrobenzoic acid and the mixture was stirred for 18 hours. The mixture was cooled to 4°C and maintained at this temperature for 2 hours in order to precipitate the DCU formed. This was removed by filtration. The filtrate was washed with a pH=9.5 buffer and then with saturated NaCl solution and then dried (Na_2SO_4). Solvent removal gave the product as a yellow solid (0.68g, 58%),m.p.143-147°C (lit.[3g] 158-161°C). It had δ (200 MHz,$CDCl_3$) 8.89 (d, J = 1.7Hz, 1H), 8.46 (dd, J_1 = 8.7Hz, J_2 =1.7Hz, 1H), 8.04 (d, J =8.7 Hz, 1H), 7.61 (s, 1H), 7.44 (s, 1H), 5.13 (s, 2H).

4-Hydroxymethyl-Nbb-Gly-resin. Boc-Gly-resin (2g) was deprotected with 33% TFA in CH_2Cl_2. 2,4,5-Trichlorophenyl 4-hydroxymethyl-3-nitrobenzoate (0.58g, 1.5mmol) in DMF (2mL) was added to the resin followed by HOBt (0.28g, 2mmol) and the mixture was allowed to stand at room temperature for 18 hours with

occasional agitation. The resin was then washed well with DMF followed by CH_2Cl_2. The qualitative ninhydrin test was negative.

Boc-Pro-OCH$_2$-Nbb-Gly-resin. Boc-Pro-OH (0.66g, 3mmol) in CH_2Cl_2 (1 mL) was added to the 4-hydroxymethyl-Nbb-resin above, followed by a 0.5 M solution of DCC in CH_2Cl_2 (6 mL, 3mmol) and DMAP (38 mg, 0.3mmol) and the mixture was allowed to stand at r.t. for 180 min with occasional agitation. Amino acid analysis of the resin hydrolysate after coupling gave a yield of 79% for the incorporation of proline.

Method (c)

2,4,5-Trichlorophenyl 4-(Boc-prolyloxymethyl)-3-nitrobenzoate. A solution of 2,4,5-trichlorophenyl 4-hydroxymethyl-3-nitrobenzoate (1.1g, 2.92mmol) in CH_2Cl_2 (42mL) was added to a solution of Boc-Pro-OH (0.68g, 3.16mmol) in CH_2Cl_2 (32mL). The mixture was cooled to 4°C and stirred magnetically. A 1M solution of DCC (3.1mL, 3.1mmol) was added followed by DMAP (0.04g, 0.32 mmol). The reaction was stirred 30min at 4°C and the 90 min at r.t. After this time t.l.c. indicated that the reaction was complete and the mixture was cooled to 4°C and filtered to remove the DCU precipitated. The filtrate was diluted with EtOAc and washed with pH=9.5 buffer and then with saturated NaCl solution. Drying (Na_2SO_4) followed by solvent removal gave a yellow oil (1.32g). Purification of the crude product by "flash chromatography" [15] (silica, hexane/EtOAc 2:1) gave a clear oil (0.75g, 57%). It had δ (200 MHz, $CDCl_3$) 8.89 (d,1H), 8.42 (dm, 1H), 7.97 (d, 1H),7.61 (s, 1H),7.44 (s, 1H), 5.7-5.6 (m, 2H), 4.6-4.2 (m, 1H), 3.6-3.2 (m, 2H), 2.0-2.2 (4H), 1.4 (s, 9H).

Boc-Pro-OCH$_2$-Nbb-Gly-resin. 2,4,5-Trichlorophenyl 4-(Boc-prolyloxymethyl)-3-nitrobenzoate (0.75g, 1.3mmol) in DMF (2mL) was added to the resin followed by HOBt (0.31g, 2.3mmol) in DMF (1mL) and the reaction was allowed to stand 18 hours at r.t. with occasional agitation. The resin was washed well with DMF followed by CH_2Cl_2 and allowed to dry. Amino acid analysis of a portion of the resin indicated quantitative incorporation of the Pro.

Coupling of the Third Amino acid

Standard Method (Table 1, entries 1 and 8). The Boc-dipeptide-resin was deprotected with 33% TFA in CH_2Cl_2 and then neutralised with 5% DIEA in CH_2Cl_2 (see General Procedure for Solid-phase Assembly of Peptides). A solution of Boc-Lys(ClZ)-OH (3 eq) in CH_2Cl_2 was added to the resin. A 1M solution of DCC in CH_2Cl_2 (3 eq) was then added and the resin allowed to stand at r.t. 60min with occasional agitation. The resin was washed well with DMF followed by CH_2Cl_2 and allowed to dry.

Suzuki method (Table 1, entries 2 and 9). The Boc-dipeptide-resin was deprotected with 4M HCl in dioxane and a solution of Boc-Lys(ClZ)-OH (3 eq) in CH_2Cl_2 to which NMM (3 eq) had previously been added, was added to the resin followed by a 1M solution of DCC in CH_2Cl_2 (3 eq). This mixture was then allowed to stand at r.t. for 300min with occasional agitation. The resin was washed well with DMF followed by CH_2Cl_2 and allowed to dry.

BOP Method (Table 1, entries 3, 4, 5, 6, 7, 10 and 11). The Boc-dipeptide-resin was deprotected with 33% TFA in CH_2Cl_2 and after washing with CH_2Cl_2 the following were added to the resin in this order. Boc-Lys(ClZ)-OH (3 eq) in DMF, HOBt (3 eq entries 4, 7 and 11) in DMF, BOP (1.5 eq, entry 5; 3 eq, entries 3, 4, 6, 7, 10 and 11) in DMF and finally NMM (6 eq, entries 3, 4 and 5) or DIEA (6 eq, entries 6, 7, 10 and 11) and the mixture was allowed to stand at r.t. for 60min with occasional agitation. The resin was washed well with DMF and CH_2Cl_2 and allowed to dry.

Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OH.[1b] Boc-Asp(OcHex)-Asp(OcHex)-Thr(Bzl)-Met(O)-Lys(ClZ)-Asp(OcHex)-Ala-Gly-OCH_2-Nbb-resin (100mg) 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 70% for the cleavage.

The crude peptide (30mg, 20μmol) in DMF (1mL) was loaded onto a Merck LiChroprep RP-C-8 column (440mm by 37mm), eluting with a convex gradient starting from DMF/H_2O/propionic acid (60:39:1) (500mL) to DMF/MeCN/propionic acid (60:39:1) (500mL) at a flow rate of 3mLmin^{-1}. The volume of each individual fraction collected was 10mL. 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 10% to 100% of B in A over 20 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 1.5mLmin^{-1}; retention time of the peptide under these conditions is 23 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 (11mg, 7.6μmol, 37% recovery) whose HPLC profile showed two peaks (Figure 1) : composition Asp : 2.96; Thr : 1.11; Gly : 1.09 ; Ala 1.12; Met : 1.00; Lys : 0.83 ; FABMS *m/z* 1494 [(M+23), 100%], 1472 [(M+1), 50%]; 1H N.M.R.(500MHz, d_6 DMSO) 8.3-7.0 (m, aromatics Thr, Lys, amide H Lys, Thr, Met, Asp, Ala, Gly), 5.1 (s, benzylics Lys), 4.75-3.5 (m, α H, Asp, Thr, Met, Lys, Ala, Gly, β H Thr), 3.40 (ε-H, Lys), 3.00-2.5 (m, β-H, Asp, Thr), 2.459 (s, Me, Met, one diastereomer), 2.46 (s, Me, Met, second diastereomer), 1.8-1.04 (m, β-H Lys, Ala, γ-H, Lys, Thr, δ-H Lys).

Boc-Leu-Thr(OBzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(OBzl)-Pro-OH. Boc-Leu-Thr(OBzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(OBzl)-Pro-OCH_2-Nbb-Gly-resin (100mg) 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 74% for the cleavage.

The crude peptide 12.7μmol in DMF (1mL) was loaded onto a Sephadex LH-20 column and was eluted with DMF at a flow rate of 0.5mLmin^{-1}, detecting by U/V absorption at 280 nm. All fractions of the column which contained peptide material as evidenced by analytical HPLC (Nucleosil C-18, linear gradient from 10% to 100% of B in A over 25 min, followed by 10 min eluting with 100% B, where A is 0.045% TFA in H_2O and B is 0.036% TFA in MeCN at a flow rate of 1mLmin^{-1}; retention time of peptide under these conditions is 26 min) 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. The peptide was then redissolved in DMF (1mL) and subjected to reverse-phase MPLC using a Merck LiChroprep RP-8 (C-8) column (440mm by 37mm), eluting

with a convex gradient starting from DMF/MeCN/H_2O/propionic acid (50:10:39.5:0.5) (1000mL) to DMF/MeCN/propionic acid (50:49.5:0.5) (1000mL) and then eluting at isocratic conditions with DMF/MeCN/propionic acid (50:49.5:0.5) (1000mL) at a flow rate of 5mLmin^{-1}. Detection was carried out by U/V absorption at 280nm and the volume of each individual fraction collected was 10mL. The column fractions were subjected to analytical HPLC under the above mentioned conditions and all tubes containing the pure desired peptide were combined and subjected to solvent removal by high-vacuum rotatory evaporation, 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 (8.38μmol, 66% recovery) whose HPLC profile showed a single peak (Figure 2) : composition Ser: 0.76; Lys: 1.94; Val: 0.77; Ile: 0.78; Glu: 0.84; Thr: 0.88; Leu: 1.00 ; FABMS, *m/z* 1737 [(*M*+23), 85%], 1715 [(M+1), 20%], 1615 (75%), 1135 (100%) ; ^{1}H N.M.R. (200MHz, d_6, DMSO) 7.4-7.2 (m, aromatics and amide H, Lys, Ser, Thr), 7.18 (d, amide, Glu), 5.07 (s, benzylics Lys), 4.4-3.8 (m, α-H Pro, Ser, Lys, Leu, Glu, Val, Ile, β-H Ser, Thr, benzylics Thr, Ser), 2.3 (t, γ-H, Glu), 1.95-1.45 (m, β-H Val, Ile, Lys,Pro, γ-H, Pro, Leu, δ-H, Lys), 1.35 (s, Boc), 1.07 (d, γ-H, Thr), 0.95-0.85 (m, γ-H, Val, Ile , δ-H, Ile, Leu).

Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OH. Boc-Asn-Leu-Leu-Leu-Gly-Thr(Bzl)-Pro-OCH_2-Nbb-Gly-resin (100mg) 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 47% for the cleavage.

The crude peptide (14.3μmol) in DMF (1mL) was subjected to MPLC in a similar manner to that described for the peptide Boc-Leu-Thr-(Bzl)-Glu(OcHex)-Lys(ClZ)-Ile-Val-Lys(ClZ)-Ser(Bzl)-Pro-OH, using a Merck LiChroprep RP-C8 column (440mm by 37mm), eluting with a convex gradient starting from DMF/H_2O/propionic acid (60:39:1) (500mL) to DMF/MeCN/propionic acid (60:39:1) (500mL) at a flow rate of 3 mLmin^{-1}. Finally the column was eluted under isocratic conditions with 60% DMF in MeCN/ 1% propionic acid (1000mL). Monitoring was carried out by subjecting the column fractions to analytical HPLC using a reverse-phase C_{18} column and eluting with a linear gradient from 10% to 100% of B in A over 20 min, where A is H_2O/0.045% TFA and B is MeCN/0.036% TFA, at a flow rate of 1.5 mL min^{-1}. The relevant fractions from the column were combined and the solvent was removed by high vacuum rotatory evaporation. After addition of H_2O, the resulting oil was lyophilised affording a white powdery solid (10mg, 10.9μmol, 76% recovery) whose HPLC profile showed one single peak (see Figure 3): composition Asp : 0.74 ; Thr : 0.87 ; Gly : 0.99 ; Leu : 3.27 ; FABMS *m/z* 940 [(M+23), 100%] ; ^{1}H N.M.R. (200MHz, d_6 DMSO) 8.2-7.1 (m, aromatics, amide H, Asn, Leu, Gly, Thr, Pro), 4.92 (s, benzylics, Thr), 4.8-3.25 (m, α-H, Pro, Leu, Thr, Asn, Gly, β-H, Thr), 2.35-0.48 (m, β-H, Asn, Leu, Pro, γ-H, Pro, Leu, Thr, δ-H, Leu), 1.3 (s, Boc).

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2. Abbreviations used in this paper: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, *N,N'*-dicyclohexylurea; DIEA, diisopropylethylamine; DMAP, 4-(N,N-dimethyl)-aminopyridine; DMF, *N,N*-dimethylformamide; EtOAc, ethyl acetate; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; p-MBHA, p-methylbenzhydrylamine; MeCN, acetonitrile; MeOH, methanol; Nbb-, nitrobenzamidobenzyl; -resin, poly(styrene-*co*-1% divinylbenzene); NMM, *N*-methylmorpholine; TMS, tetramethylsilane; TFE, trifluoroethanol; TFA, trifluoroacetic acid; t.l.c., thin-layer chromatography.
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