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The Use of the Nbb-Resin for the Solid-Phase Synthesis of Peptide Alkylesters and Alkylamides. Synthesis of Leuprolide[1]

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Abstract : The use of nucleophiles for the cleavage of an *o*-nitrobenzylester peptide-resin bond in order to afford peptide alkylesters and alkylamides has been studied. With this purpose, three short peptide sequences anchored to a Nbb-resin were used as models. Peptides were cleaved from the polymeric supports by reaction with primary and secondary amines and by a transesterification process with yields that depended on the nucleophile and the *C*-terminal amino acid of the sequence. This methodology was applied to the synthesis of leuprolide, an ethylamide peptide of relevant pharmacological interest, which was obtained in a 70% overall yield.

The design and synthesis of biological active peptides has become one of the main goals in Bioorganic Chemistry. Tremendous efforts are being devoted to the development of this area in order to provide analogues with improved biological properties. A number of approaches to the problem have been described so far, which include the use of unnatural amino acids (D-amino acids, for example), peptide bond mimics, conformationally restricted peptides (homo and heterodetic cyclic peptides) or *C*-terminal and/or *N*-terminal substituted peptides.[2] Among them, peptide alkylesters[3] and peptide alkylamides[4] have proved to be interesting from the biological and pharmacological points of view. Thus, increased biological activity and proteolytic stability can be achieved by attaching a hydrophobic group at the *C*-terminus. Moreover, the lipophylicity of the peptide can be substantially changed in this way, which may improve the ability of the product to be distributed in tissues or to bind to receptors. Several methods have been described in the literature for the preparation of these derivatives. The synthesis of peptide *N*-alkyl amides can be performed by nucleophilic displacement of the peptide anchored to a Merrifield type resin[5], an oxime resin[6] or a polyacrylic resin.[7] Alternatively, the use of *N,N*-peptidylalkylaminomethyl type resins that can afford these peptide analogues by HF,[8] TFA[9] or photolytic cleavage[10] have also been reported. Peptide alkylesters have been obtained by transesterification processes using Merrifield-like resins[11] and SASRIN™.[12]

Attack of a suitable nucleophile at an activated peptide-resin bond seems to be one of the most general methods to synthesize these peptide analogues. This methodology has the advantage of providing different *C*-terminal modified peptides from a single peptide-resin depeding on the nucleophile that is used. In this sense, an optimal general strategy for the synthesis of a peptide *N*-alkylamide or a peptide alkylester would involve: (i) elongation of the peptidic chain using either Boc or Fmoc chemistry, (ii) cleavage of the peptide from the resin with a nucleophile and (iii) side chain deprotection with HF or TFA depending on the strategy that has been used in step (i). Fmoc chemistry would be preferred because the use of TFA is compatible with a broader range of peptides and is easier to handle than HF.

The use of handles containing electron-withdrawing groups can facilitate the nucleophilic attack at the peptide-resin bond. We considered that 4-bromomethyl-3-nitrobenzamido-benzylpolystyrene(4-bromomethyl-Nbb resin) had a potential interest from this point of view.[13,14] The 4-bromomethyl-Nbb resin has been widely used in our laboratory for the synthesis of protected peptides by both the Boc and the Fmoc strategies.[14] The cleavage of the peptide from the resin is performed usually under mild conditions by a photochemically induced redox process, in which the nitro group is involved. The fact that this electron-withdrawing group can also enhance the lability of the *o*-nitrobenzylester bond against nucleophiles, moved us to explore the possibility of performing the nucleophilic cleavage of the peptide-resin bond for the obtention of peptides with alkyl groups attached to the *C*-terminal position.[15] Considering that the lability of the ester linkage might depend not only on the nature of the nucleophile but also on the amino acid at this position of the peptide sequence, we decided to use the peptide-resins Ac-Tyr(tBu)-Gly-Gly-O-CH_2-Nbb (**1**), Ac-Lys(Boc)-Lys(Boc)-Ala-Ala-O-CH_2-Nbb (**2**) and Ac-Tyr(tBu)-Gly-Phe-O-CH_2-Nbb (**3**) as models to carry out this study. Once the potential usefulness of this approach was demonstrated, it was applied to the synthesis of the LHRH analogue leuprolide, an ethylamide peptide of pharmacological interest.[16]

The 4-bromomethyl-Nbb resin was prepared from a MBHA resin, incorporating an amino acid as internal standard and 4-bromomethyl-3-nitrobenzoic acid as handle. The first amino acid of the three peptide sequences was assembled using classical methodology, by reaction with the cesium salt of the corresponding Boc-amino acid.[17] The rest of the couplings were carried out using Boc or Fmoc chemistries[18] depending on the position of the residue in the sequence. The fact that the two first amino acids of the peptidyl resins are difunctional allows the synthesis to be started using Boc chemistry and to be continued with the Fmoc strategy in order to obtain a tBu based protected peptide after nucleophilic cleavage and prior to the final acid treatment. This strategy is prefered when a Nbb type resin is used since DKP formation[19] can be minimized by starting the synthesis with Boc chemistry, which allows the assembly of the third amino acid using a phosphonium salt as the coupling agent and with *in situ* neutralisation[20,21]. On the other hand, Fmoc

chemistry has the advantage that the amino acid side chain protecting groups that are used with this strategy can be removed with TFA-based cocktails, avoiding the concourse of HF. Amino acids were coupled using the standard protocols that are used in Boc and Fmoc strategies (DIPCDI/HOBt and BOP reagent for the assembly of the third amino acid).

Study of the Cleavage of the Peptide-Resin Bond with Nucleophiles

Table 1 summarises the results that were obtained with the use of some nucleophiles to perform the cleavage of the peptide-resin bond on resins **1**, **2** and **3**. In order to explore the scope of this methodology for the preparation of different peptide derivatives, several nucleophilic cleavage methods were tested. The hydrolysis of the *o*-nitrobenzylester bond, a transesterification reaction and the use of amines as nucleophiles were considered in this study as examples of the obtention of peptide carboxylic acids (table 1, entry a), peptide alkylesters (table 1, entries b and c) and peptide alkylamides (table 1, entries d-h), respectively. The percentages of cleavage that are shown in Table 1 were determined by amino acid analysis of the resins before and after nucleophilic treatment. All peptide derivatives were characterised by reverse phase HPLC and further amino acid analysis of aliquots of the collected peaks.[22] As it can be seen in the table, the steric hindrance around the peptide-resin bond seems to play a crucial role in the process. Thus, comparing the yields of cleavage that were obtained for each of the nucleophiles, resin **1** proved to be the most reactive (glycine at the C-terminal position) and resin **3** was less prone to nucleophilic attack (phenylalanine at the C-terminal position). A dependence of the yield of cleavage on the nature of the C-terminal amino acid of the sequence was already detected for the oxime[6] and SASRIN resins.[12]

Treatment of resins **1**, **2** and **3** with TBAF in DMF afforded the corresponding peptide carboxylic acids in excellent yields. This result indicates that the cleavage of the *o*-nitrobenzylester bond by an hydrolytic process could be an appropiate alternative to photolysis for the obtention of protected peptides in order to be used in convergent solid phase peptide synthesis.[23] However, special attention has to be paid if the peptide contains Asp residues because this reagent can provoke aspartimide formation.[24] Concerning the use of alcohols for carrying out the synthesis of peptide alkylesters, the KCN promoted methanolysis of the peptide-resin bond afforded percentages of cleavage for the three polymeric supports that were similar to those obtained with the hydrolytic process (table 1, entry b). Moreover, the same nucleophile proved to be less effective in the presence of DIEA (table 1, entry c), although the yields could be improved with longer reaction times and/or the use of DMF in order to achieve a better swelling of the resin.

Peptide alkylamides are among the most interesting peptide analogues from the biological point of view. In order to evaluate the usefulness of the Nbb resin for the obtention of such derivatives, three primary amines and two secondary amines were utilised in this study. Methylamine and dimethylamine, apart from being the simplest primary and secondary amines, were considered to be good examples for determining how steric hindrance of the nucleophile affects the cleavage of the peptide from the resin. Piperidine, a cyclic amine, was also included in this preliminary work. Hexylamine and stearylamine were chosen as examples of amines that could afford highly hydrophobic peptide analogues.

Table 1. Reaction of Peptidyl Resins **1**, **2** and **3** with the Nucleophiles Used in this Study[a].

		percentage of cleavage			
entry	reagent/catalyst	**1**	**2**	**3**	product
a	H_2O / TBAF	91	93	54	carboxilic acid
b	MeOH / KCN	89	84	49	methyl ester
c	MeOH / DIEA	77	66	42	methyl ester
d	CH_3NH_2	89	70	49	*N*-methylamide
e	$CH_3(CH_2)_5NH_2$	67	52	25	*N*-hexylamide
f	$CH_3(CH_2)_{17}NH_2$	82	50	47	*N*-estearylamide
g	$(CH_3)_2NH$	85	38	2	*N*,*N*-dimethylamide
h	piperidine	21	2	-	piperidide

a. See experimental part for reaction conditions.

As it was pointed out for the hydrolysis and the transesterification processes, high yields of cleavage were achieved when peptidyl-resin **1** was treated with the three primary amines (70-90% yield), but the reactions were less efficient with the other two peptidyl-resins (table 1, entries d-f). The fact that hexylamine proved to be less reactive than estearoylamine can be attributed to the conditions that were used to carry out the experiments (reaction time and/or a solvent effect). The reactivity of dimethylamine to peptidyl-resin **1** was similar to that of the primary amines (table 1, entry g); however, only 2% cleavage was observed when peptidyl-resin **3** was used. The steric effects play an important role in this case because of the structure of the amine (a secondary amine) and the fact that the C-terminal amino acid of the peptide sequence corresponding to the peptidyl-resin **3** is Phe. The percentage of cleavage fell dramatically when piperidine was used with peptidyl-resin **1** (table 1, entry h) and the other two peptidyl-resins showed no reactivity whatsoever to this amine. These results indicate that primary amines are suitable reagents for the obtention of

peptide alkylamides, but steric effects are determinant in order to use secondary amines for the cleavage of the peptide from the resin.

Synthesis of Leuprolide

With the object of demonstrating the usefulness of this methodology, it was applied to the synthesis of leuprolide, an analogue of the luteinizing hormone releasing factor (LHRH, figure 1). We considered this peptide ethylamide as an interesting target molecule because it contains Pro at the C-terminal position. LHRH is a decapeptide carboxamide that is secreted by the human body. It stimulates the synthesis of the luteinizing hormone, which regulates the production of sexual hormones. Its crucial biological role has provoked the development of numerous analogues of higher biological activity in order to treat infertility problems and, in general, illnesses related to secretion of sexual hormones in men and women. Leuprolide (figure 1) is one of the most interesting peptide analogues, with a biological activity fifteen times higher than that of the natural peptide. The structural differences that this compound has with respect to LHRH is a D-Leu residue in substitution to Leu at position 6, the absence of Gly at position 10 and the C-terminal extreme as ethylamide.

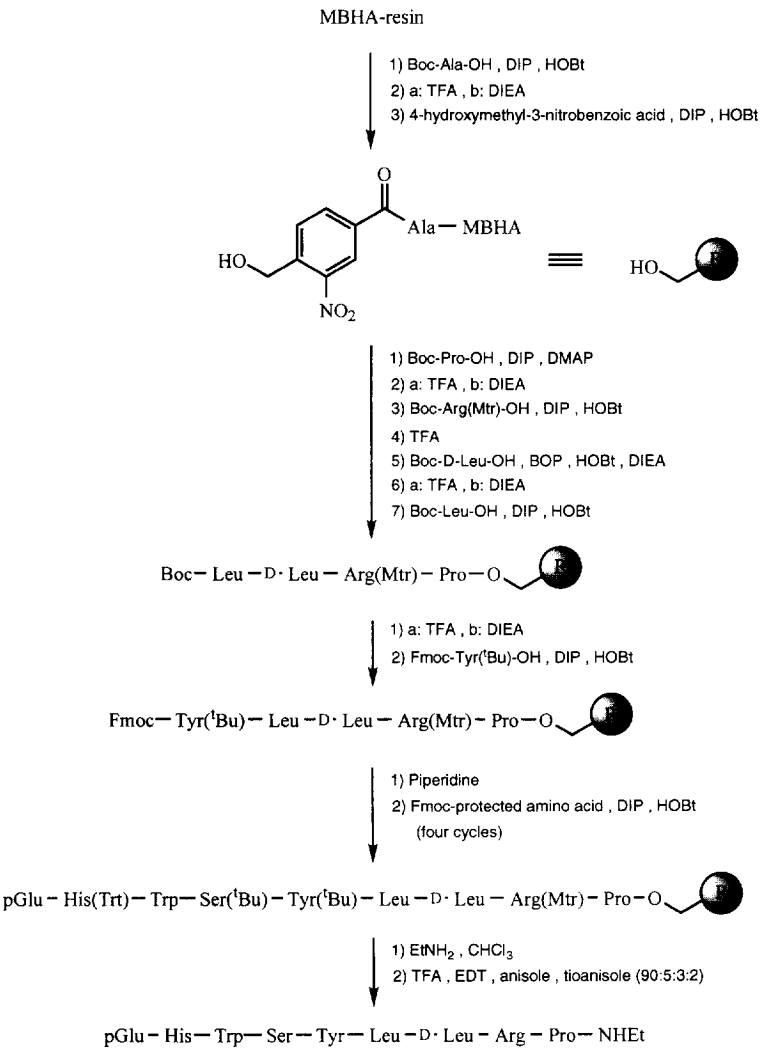
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 LHRH

pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NH-Et Leuprolide

figure 1

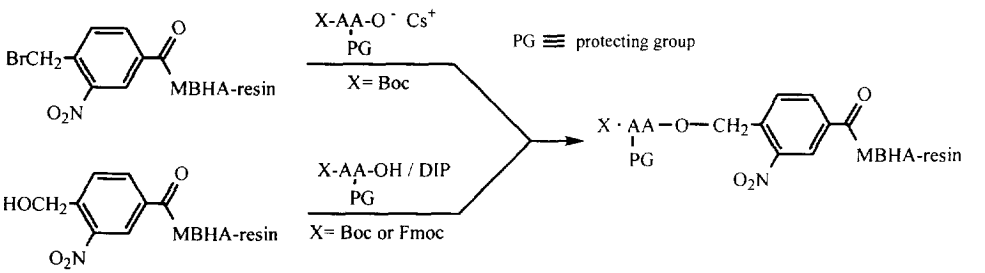
The synthesis of this peptide has been described using a Merrifield-like resin and the Boc strategy. The protected peptide was cleaved from the polymeric support using either MeOH/TEA followed by treatment of the ester with ethylamine,[25] or by cleavage of the peptide from the resin with the amine followed by removal of side chain protecting groups with HF[26] (25% and 40% yields respectively). We focused our attention on the use of Fmoc chemistry in order to perform the final deprotection of the peptide with TFA. The fact that the *o*-nitrobenzylester bond is stable to this acid allows an alternative method that consists of side chain deprotection of the peptide anchored to the polymeric support prior to the nucleophilic displacement of the peptide from the resin to be considered.[27]

Scheme 1 shows the total synthesis of leuprolide. The synthesis of leuprolide was carried out on a 4-hydroxymethyl-Nbb resin in this particular case. The 4-bromomethyl-Nbb resin has been generally used for the synthesis of peptides on a polymeric support through a *o*-benzylester linkage. The coupling of the first amino acid on this resin has usually been performed using the cesium salt of the Boc-amino acid with moderate heating. However, the yields (40-90%) are very often unreproducible and the method has proved to be uncompatible with Fmoc-amino acids.



Scheme 1

Our interest on the search for a polymeric support of general application prompted us to use the 4-hydroxymethyl-Nbb resin[14c,14e] instead of the traditional 4-bromomethyl-Nbb resin because the former would allow the anchoring of both Fmoc and Boc amino acids using, for example, the carbodiimide method (scheme 2). This resin was prepared from 4-hydroxymethyl-3-nitrobenzoic acid and an MBHA resin incorporating alanine as internal standard. The handle was obtained in good yields by treatment of 4-bromomethyl-3-nitrobenzoic acid with saturated aqueous Na_2CO_3 solution and was attached to the MBHA resin using DIPCDI. The first amino acid of leuprolide was efficiently incorporated using the same coupling agent in the presence of catalytic quantities of DMAP.[28]

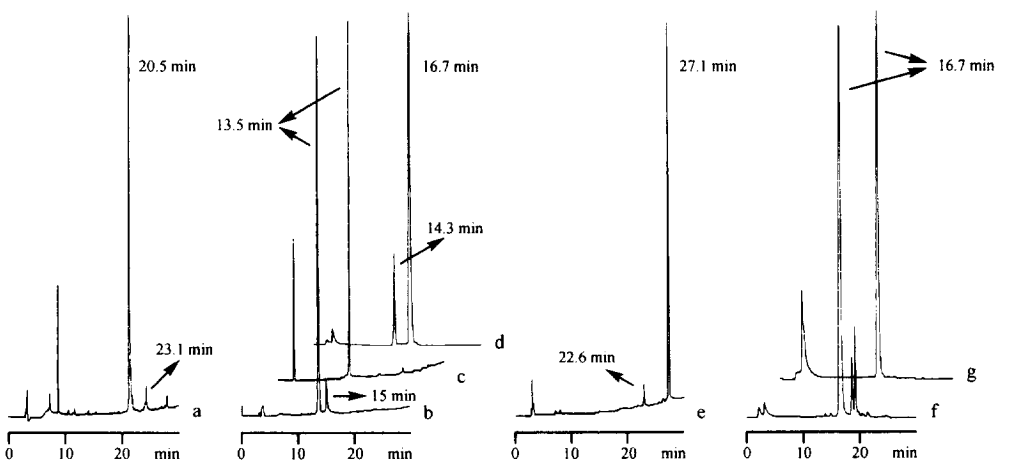


Scheme 2

The first four amino acids from the *C*-terminus of leuprolide were coupled as Boc derivatives while the Fmoc strategy was employed for the assembly of the rest of the amino acids of the sequence (scheme 1). The ^{t}Bu group was used for Ser and Tyr side chain protection, the Trt group for His, and Trp was utilised unprotected. However, unprotected and Mtr protected Arg were considered to be suitable alternatives to carry out the synthesis. Amino acid couplings were performed with DIPCDI/HOBt, except for the third amino acid which was coupled with BOP/DIEA in order to avoid diketopiperazine formation.[19] The synthetic protocols were similar to those already mentioned for peptidyl-resins **1**, **2** and **3**. Special attention had to be paid to the use of piperidine to deprotect the N^{α}-position of Fmoc-amino acids because, as a nucleophile, this reagent could provoke premature loss of peptide chains from the polymeric support. The results shown in table 1 (entry h) indicate that piperidine can be a problem when a long peptide with Gly at its C-terminal position has to be synthesized. Two 1 min treatments per cycle should be carried out to remove the Fmoc group in order to minimize this drawback.[29] However, according to the results that were obtained with peptidyl-resins **2** and **3**, the *o*-nitrobenzylester peptide-resin bond is practically stable when the C-terminal amino acid of the sequence has a substituted side chain. The fact that leuprolide has Pro at the C-terminal

position should prevent the peptide-resin bond from the attack of piperidine. That was confirmed by amino acid analysis of the solution that resulted from joining all the filtrates corresponding to the treatments with piperidine and the following washings that were carried out during the assembly of the peptide chain.

The synthesis of the peptide was initially carried out without protection for Arg, which was coupled using a large excess of amino acid with the object of overcoming competition with δ-lactam formation.[30] The peptide was cleaved from the resin using a mixture of 70% aqueous ethylamine solution and DCM (1:1) (16 h, 90% of cleavage) and side chain protecting groups were removed with TFA/EDT (9:1). Figure 2 shows the HPLC analyses of the crude resulting from the treatment of the peptidyl resin with ethylamine (chromatogram a) and the material after prepurification by size exclusion chromatography (chromatogram b). As indicated in the figure, 2% of peptide without Arg was detected (r.t. 23.1 min and 15 min, respectively).[31] The final product was obtained after purification by reverse phase chromatography and was characterised by amino acid analysis, reverse phase HPLC (figure 2, chromatogram c) and FAB-MS spectrometry (49% overall yield).



Chromatograms a-d correspond to the synthesis of leuprolide using unprotected arginine (a, protected peptide; b-d, unprotected peptide); chromatograms e-g correspond to the synthesis of leuprolide using Mtr protected arginine (e, protected peptide; f and g, unprotected peptide). Chromatographic conditions: Vydac C-18 (25 x 0.4 cm, 5 μm) (a-c) or Nucleosil C-18 (25 x 0.5 cm, 10 μm) (d-g). A: H_2O/0.045% of TFA (a, b, c and e) or 0.05 M aqueous NH_4OAc buffer (pH 7)(d, f and g); B: CH_3CN/0.036% of TFA (a, b, c and e) or CH_3CN (d, f and g). Linear gradient from 10% to 100% of B in 30 min, 1 mL/min, 220 nm. See the text for explanations.

figure 2.

The mass spectrum of the final product showed the peak corresponding to Leuprolide (1209.9, M+1) and some typical fragmentations of the peptide chain. A peak with a m/z of 1182.9 was also detected, which

may indicate the presence of the peptide carboxylic acid. The fact that one peak was only observed by chromatographic analysis moved us to change the HPLC conditions in order to confirm the formation of the by-product. As shown in figure 2, the use of a 0.05 M aqueous NH_4OAc buffer (pH 7) instead of water/0.045% of TFA resolved the peak of chromatogram c at 13.5 min into two new peaks, the one with r.t. 14.3 min corresponding to the peptide carboxylic acid (25% by the integration of the areas). The two products were separated by ionic exchange chromatography, using a Sephadex-CMC column and a gradient of ionic strength from 0.05 M to 0.5 M of aqueous NH_4OAc. This result indicated that water competed in nucleophilic attack at the peptide bond under the conditions that were used. This moved us to explore other conditions for peptide cleavage from the resin in order to improve the yields. A new synthesis was carried out using Mtr protection for Arg with the object of avoiding the formation of the des-Arg peptide. The protected leuprolide was obtained with anhydrous ethylamine in this case. A concentrated solution of the amine in $CHCl_3$ at room temperature proved to be efficient (95% yield of cleavage).[32] The chromatographic analysis of the crude showed the presence of the desired product as the majoritary component (figure 2. chromatogram e; r.t. 27.1 min).[33] The use of reagent R (TFA/EDT/anisole/thioanisole, 9:0.5:0.3:0.2) for removing side chain protecting groups proved to be suitable in this particular case (figure 2. chromatogram f; r.t. 16.7 min). The final product was obtained in a 70% yield after a simple work-up and purification of the crude by ion exchange chromatography (figure 2, chromatogram g).

CONCLUSIONS

The potential usefulness of the *o*-nitrobenzyl ester linkage for the obtention of C-terminal modified peptides by the solid phase methodology has been demonstrated. Peptide alkylesters and peptide alkylamides can be easily prepared using the appropriate nucleophile to carry out the clevage of the peptide from the resin. 4-Bromomethyl-3-nitrobenzamido-benzylpolystyrene is a suitable polymeric support for this purpose, but 4-hydroxymethyl-3-nitrobenzamido-benzylpolystyrene can be a good alternative because it avoids problems related to the coupling of the first amino acid and allows the use of both Boc and Fmoc chemistries. When Fmoc strategy is used, precautions have to be taken in the case of sequences with glycine at the C-terminal position in order to minimize the loss of peptide chains with the use of piperidine. As an example of the usefulness of this methodology, leuprolide, a nonapeptide *C*-ethylamide of pharmacological interest, has been prepared in good yield. We believe that this methodology can be useful for the preparation of peptide libraries based on modifications at the *C*-terminal end. The extension of this work to the synthesis of non-peptide libraries is in progress.

EXPERIMENTAL

Protected Boc-amino acid and Fmoc-amino acid derivatives were either from Novabiochem (Läufelfingen, Switzerland) or from Propeptide (Vert-le Petit, France). MBHA resins were from Bachem Feinchemikalien (Bubendorf, Switzerland). DMF was peptide synthesis grade from Scharlau (Barcelona, Spain) and was flushed with nitrogen and kept stored over molecular sieves (4 Å). TFA was purchased from Kali-Chem, Piperidine from Aldrich, DIEA from Acros (Geel, Belgium), ethylamine, $CsCO_3$, HOBt and DIPCDI from Fluka Chimie (Buchs, Switzerland), BOP reagent from Richelieu Biotechnologies (St. Hyacinth, Canada), PyBOP from Novabiochem, DCM and $CHCl_3$ from Scharlau and DMAP from Jansen (Geel, belgium). Hexylamine and methylamine were supplied by Aldrich, dimethylamine by Scharlau, stearoylamine by Sigma, KCN by Merk and TBAF by Fluka. 4-Bromomethyl-3-nitrobenzoic acid was synthesized as described before. CH_3CN (Scharlau) and THF (Merk) were HPLC grade.

Peptide resins were hydrolyzed in 12 N HCl-propionic acid (1:1, v/v) at 115°C for 48 hours or at 155°C for 2 hours. Amino acid analyses were carried out in a Beckman System 6300 analyzer. Analytical HPLC was performed using Nucleosil C-18 (25 x 0.5 cm, 10 μm) or Vydac C-18 (25 x 0.4 cm, 5 μm) reverse-phase columns 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. Size exclusion chromatography and ion exchange chromatography were carried out on a Sephadex G-15 column (Pharmacia) and a CMC column (Whatman, England), respectively, using a LKB peristaltic pump (Microperpex 2132), an LKB 2158 Uvicord SD variable wavelength detector and a Servoscribe 1s plotter and a LKB Ultrorac II 2070 automatic fraction collector. Preparative MPLC chromatography was performed with a Vydac C-18 column, using a LDC/MiltonRoy pump and the system already described.

Positive-ion electrospray (ES-MS) and positive-ion fast atom bombardment mass spectrometry (FAB-MS) were carried out on a Fisons VG-Quattro instrument with matrices of thioglycerol and magic bullet (DTT / DTE, 3:1). ^{1}H-NMR spectra were recorded in a 200-MHz Varian XL-200 instrument.

General Procedure for the Synthesis of Peptidyl Resins Ac-Tyr(tBu)-Gly-Gly-O-CH_2-Nbb (1), Ac-Lys(Boc)-Lys(Boc)-Ala-Ala-O-CH_2-Nbb (2) and Ac-Tyr(tBu)-Gly-Phe-O-CH_2-Nbb (3)

The synthesis of the peptidyl resins **1**, **2** and **3** were carried out manually in polypropylene syringes fitted with a polyethylene disc. 4-Bromomethyl-3-nitrobenzoic acid was coupled to the polymeric support (1 g, 0.72 meq/g) using the following protocol : CH_2Cl_2 (4 x 0.5 min); 30% TFA/ CH_2Cl_2 (1 x 1 min and 1 x 30

min); CH_2Cl_2 (4 x 0.5 min); 5% DIEA/ CH_2Cl_2 (3 x 1 min); CH_2Cl_2 (4 x 0.5 min); handle (5 eq) and DIPCDI (6 eq), 3 h; CH_2Cl_2 (4 x 0.5 min); DMF (4 x 1 min). The first amino acid of the sequence (Boc-Gly-OH, Boc-Phe-OH or Boc-Ala-OH; 3 eq) was coupled through its cesium salt (10 mL of DMF, 40°C, overnight) and the second amino acid (Boc-Gly-OH or Boc-Ala-OH; 3 eq) was assembled using a protocol similar to the above mentioned (DIPCDI, 3 eq; 2 h). The third amino acid (Fmoc-Tyr(tBu)-OH or Fmoc-Lys(Boc)-OH; 3 eq) was coupled with BOP in DMF without prior neutralisation (HOBt, 3 eq; BOP, 3 eq; DIEA, 6 eq; 1 h) and the fourth amino acid (Fmoc-Lys(Boc)-OH) was assembled using the following protocol: DMF (4 x 0.5 min); 20% piperidine/DMF (2 x 1 min); DMF (4 x 0.5 min); amino acid (3 eq) and DIPCDI (3 eq), 3 h; DMF (4 x 0.5 min). Acetylations were carried out in DMF with Ac_2O in the presence of DIEA (3 eq of each, 2 h) after α-amino deprotection with 20% piperidine/DMF (2 x 1 min). The qualitative ninhydrin test was used to monitor the syntheses and final functionalisations were determined by amino acid analysis (85-90% yields).

Study of the Cleavage of the Peptide from the Resin

Samples of peptidyl resin (50 mg) were placed in 5 mL screw-cap tubes and were suspended in 1 mL of solvent. Reagents (see next paragraph) were added and the mixtures were left at 25°C with magnetic stirring. They were filtered in polypropylene syringes fitted with a polyethylene disc and the resins were washed with solvent (4 x 0.5 min) and MeOH (4 x 0.5 min). Cleavage yields were determined by amino acid analysis of the polymeric supports and products were identified by HPLC of filtrates and amino acid analysis of the collected peaks (Vydac C-18, 25 x 0.4 cm, 5 μm; A: H_2O with 0.045% of TFA, B: CH_3CN with 0.036% of TFA; from 10% to 100% of B in 30 min, 1 mL/min, 220 nm). Peptide esters were also identified by basic hydrolysis, affording the corresponding free carboxylic acid peptides wich were characterized by amino acid analysis as above mentioned.

Free carboxylic acid peptides . TBAF (8 eq) in CH_3CN, 40 min or LiOH (15 eq) in water, 10 min. rt's : **1**, 11.0 min; **2**, 13.1 min; **3**, 14.5 min. *Peptide methyl esters* . KCN (8 eq) in MeOH, 6 h or DIEA (25 eq) in MeOH, 23 h. rt's : **1**, 12.2 min; **2**, 15.0 min; **3**, 16.3 min. *Peptides methylamide* . Ethylamine (> 200 eq) in CH_2Cl_2,[34] 15 h. rt's : **1**, 11.0 min;[35] **2**, 13.6 min; **3**, 15.0 min. *Peptides hexylamide* . Hexylamine (15 eq) in DMF, 16 h. rt's : **1**, 16.9 min; **2**, 18.2 min; **3**, 22.0 min. *Peptides dimethylamide* . Dimethylamine (>200 eq) in CH_2Cl_2,[34] 15 h. rt's : **1**, 11.7 min; **2**, 14.1 min; **3**, not determined. *Peptide stearoylamide* . Stearoylamine (10 eq) and DIEA (10 eq) in CH_2Cl_2, 5 days. **1**, rt 14.8 min (A: H_2O with 0.1% of TFA, B: THF; from 50% to 65% of B in 30 min). FABMS *m/z* 914.2 $[M+1+estearoylamine]^+$, 667.1 $[M + Na]^+$, 645.2 $[M + 1]^+$, 589.1 $[M + 2 - tBu]^+$; $C_{37}H_{64}N_4O_5$ requires M^+ 644.9.

Solid Phase Synthesis of Leuprolide

4-hydroxymethyl-3-nitrobenzoic acid. 4-Bromomethyl-3-nitrobenzoic acid (5 g, 19.2 mmol) was dissolved in an aqueous saturated solution of Na_2CO_3 (125 mL). The solution was heated to 80°C and left for 1 h at this temperature with magnetic stirring. The solution was cooled down and was acidified with 2 M aqueous HCl. The precipitate was filtered and crystallized in H_2O/EtOH, affording 3 g of pale brown crystals (79% yield). Mp 166-168°C; Rf ($CHCl_3$/MeOH/AcOH, 100:50:0.1), 0.30; NMR (DMSO-d_6) ppm (TMS as internal reference) : 4.9 (2H, s), 8.0 (1H, d, J_1= 9.8 Hz); 8.2 (1H, dd, J_1=9.8 Hz and J_2=1.1 Hz); 8.4 ppm (1H, d, J_2 = 1.1 Hz).

Syinthesis of leuprolide using unprotected arginine. Leuprolide was prepared manually in a polypropylene syringe fitted with a polyethylene disc. MBHA resin (1 g, 0.76 meq/g) was washed as above mentioned and Boc-Ala-OH was incorporated as internal standard (amino acid, 5 eq; DIPCI, 5 eq; DMF, 1 h). After the aminoacyl resin was deprotected, 4-hydroxymethyl-3-nitrobenzoic acid was assembled following a protocol similar to that described for 4-bromomethyl-3-nitrobenzoic acid (handle, 1.3 eq; DIPCI, 1.3 eq; HOBt, 1.3 eq; DMF, 60 min; two cycles). The first amino acid of the sequence (Boc-Pro-OH) was coupled in CH_2Cl_2 with DIPCI (5 eq of each) in the presence of DMAP (0.5 eq; 1 h; two cycles). The protocols for the assembly of the rest of the amino acid derivatives (Boc-Arg-OH, Boc-D-Leu-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Trp-OH, Fmoc-His(Trt)-OH and Pyr-OH) were similar to those used for the preparation of peptidyl resins **1**, **2** and **3**. Thus, couplings were carried out with DIPCI except in the case of Boc-D-Leu-OH, which was assembled with BOP reagent. The following modifications were introduced: couplings were left for 1.5 h, 10 eq of Boc-Arg-OH (with 10 eq of DIPCI) were used and, after the coupling of this amino acid, the polymeric support was washed with 0.1 M solution of HOBt in DMF (4 x 0.5 min) before each coupling step. The qualitative ninhydrin test was used to monitor the synthesis and the final functionalisation was determined by amino acid analysis (78% yield).

A 70% aqueous solution of ethylamine (1 mL) was shaken vigorously with DCM (1 mL). The resulting organic solution was placed in a screw-cap tube and 350 mg of the peptidyl resin were added. After the suspension was stirred during 16 h at 25°C, it was filtered in a polypropylene syringe fitted with a polyethylene disc and the resin was washed with DCM (4 x 0.5 min) and MeOH (4 x 0.5 min). The cleavage yield was 90% as determined by amino acid analysis of an aliquote of the polymeric support. Volatiles were removed under vacuum and the resulting material was treated with with 5 mL of TFA/EDT (9:1). The mixture was left for 2 h with magnetic stirring when volatiles were removed under vacuum and 5 mL of a 10% aqueous solution of AcOH were added. The crude material that was obtained after lyophilisation (93 mg),

was purified by size exclusion chromatography (Sephadex G-15, 2.5 x 90 cm; 0.1 M aqueous solution of AcOH; 42 mL/h, 220 nm) and reverse phase MPLC (Vydac C-18, 2.5 x 30 cm, 15-20 μm; A: 400 mL of CH_3CN/H_2O, 1:9, 0.1% TFA and B: 400 mL of CH_3CN/H_2O, 3:7, 0.1% TFA; 127 mL/h, 220 nm). Leuprolide was separated from the corresponding free C-terminus peptide by ionic exchange chromatography (CMC, 2.7 x 10 cm; A: 200 mL of 0.05 M aqueous NH_4OAc and B: 200 mL of 0.5 M aqueous NH_4Ac; 25 mL/h, 280 nm), yielding 53 mg (36.6%) of the final product as a homogeneous material by HPLC (rt, 17.8 min; nucleosil C-18, 25 x 0.5 cm, 10 μm; A: 0.01 M aqueous NH_4Ac, B: CH_3CN; from 10% to 100% of B in 30 min, 1 mL/min, 220 nm). Leuprolide: ESMS *m/z* 1209, $C_{59}H_{84}N_{16}O_{12}$ requires 1209; acid ESMS *m/z* 1182, $C_{57}H_{79}N_{15}O_{13}$ requires 1182.

Synthesis of leuprolide using Mtr protection for arginine . A MBHA resin of 0.56 meq/g (0.5 g) was used in this case. The protocols were similar to those reported in the first synthesis. The following modifications were introduced: the couplings were performed with 5 eq of reagents (1 h), Boc-D-Leu-OH was assembled with PyBOP (3 eq of each, 1h) and washings with HOBt/DMF solution were removed. The final functionalisation was 0.25 meq/g (90% yield).

A solution of 1 g of ethylamine in 3 mL of $CHCl_3$ was prepared in a 10 mL screw-cap tube and 230 mg of peptide-resin were added. The tube was stopped and the mixture was left for 15 h with mechanical stirring. The organic solution was filtered under vacuum and a second treatment with $EtNH_2$ was carried out. The two organic solutions were joined in a 100 mL round bottom flask and volatiles were removed under vacuum. 114 mg of a yellowish solid were obtained (94.5% yield of cleavage by amino acid analysis of the final resin). To the residue thus obtained were added 10 mL of reagent R (TFA, 9; EDT, 0.5; anisol, 0.3; tioanisol, 0.2) and the resulting orange solution was left for 4 h with magnetic stirring. Volatiles were removed under vacuum by trapping with Et_2O (6x30 mL, the reaction mixture was cooled on an ice bath the first time to avoid warming during the addition of Et_2O) and 25 mL of H_2O were added. The aqueous mixture was washed with 3x25 mL of AcOEt and was liophilized. The resulting yellow-white solid was purified by ionic exchange (two batches), resulting in 48 mg of the final product as a white solid (70% yield).

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REFERENCES AND NOTES

1. 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: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazolyl-*N*-oxytrisdimethylaminophosphonium hexafluorophosphate; ^{t}Bu, *tert*-butyl; CMC, carboxymethylcellulose; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine, DIPCDI, *N,N'*-diisopropylcarbodiimide; DMAP, 4-*N,N*-dimethylaminopiridine; DMF, *N,N'*-dimethylformamide; EDT, ethanditiol; ES-MS, electrospray mass spectrometry; DTE, dithioerythritol; DTT, dithiothreitol; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MBHA, *p*-methylbenzhydrylamine; MPLC, medium-pressure liquid chromatography; Mtr, 4-methoxy-2,3,6-trimethyl-benzenesulfonyl; Nbb-, nitrobenzamidobenzyl; -resin, poly(styrene-*co*-1% divinylbenzene); NMR, nuclear magnetic resonance; PyBOP, (benzotriazolyl)-*N*-oxypyrrolidinephosphonium hexafluorophosphate; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; THF, tetrahydrofurane; TMS, tetramethylsilane; Trt, trityl. Amino acid symbols denote L-configuration unless indicate otherwise.
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28. Precautions have to be taken when histidine or cysteine are C-terminal amino acids in order to avoid racemization of these residues. In such cases, alternative methodologies are recomended; see for example (a) Akaji, K.; Kuriyama, N.; Kimura, T.; Fujiwara, Y.; Kiso, Y., *Tetrahedron Lett.* **1992**, *33*, 3177-3180. (b) Green, J.; Bradley, K., *Tetrahedron* **1993**, *49*, 4141-4146. (c) Zhu, Y.-F.; Blair, R.K.; Fuller, W.D., *Tetrahedron Lett* **1994**, *35*, 4673-4676.
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31. Confirmed by amino acid analysis and FAB-MS spectrometry (retention time of the desired peptide, 13.5 min; retention time for the desarginine peptide, 15 min (see figure 2 for chromatographic conditions).
32. Dichloromethane was also used, but better yields were achieved with chloroform.
33. Peptide with unprotected arginine was also detected in the HPLC crude (peak at 22.6 min in chromatogram e of figure 2). Premature deprotection of Arg resulted from TFA treatments for removing N^{α}-Boc groups during the synthesis. Nevertheless, unstability of Mtr protecting group under these conditions did not supose a drawback to achieve the synthesis of leuprolide in good yields.
34. The amine was extracted from 1 mL of a 70% aqueous solution of ethylamine.
35. The free carboxylic acid peptide and the peptide methylamide of **1** had the same chromatographic behaviour; however, the amino acid analysis of the latter showed a peak at 51 min corresponding to methylamine

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