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CONVERGENT SOLID PHASE PEPTIDE SYNTHESIS. I. SYNTHESIS OF PROTECTED SEGMENTS ON A HYDROXYMETHYLPHENYLOXYMETHYL RESIN USING THE BASE LABILE FMOC α -AMINE PROTECTION. MODEL SYNTHESIS OF LHRH.

E. PEDROSO, A. GRANDAS, M.A. SARALEGUI, E. GIRALT, C. GRANIER* and J. VAN RIETSCHOTEN*

Departament de Quimica Organica, Facultat de Quimica, Universitat de Barcelona Diagonal 645, Barcelona 28, Spain

*INSERM SC 10, Laboratoire de Biochimie, Faculté de Médecine, Secteur Nord, Bd. P. Dramard, 13326 Marseille Cedex 15, France

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ABSTRACT - Convergent solid phase peptide synthesis has been applied to yield LHRH. The segments 1-6 and 7-10 of LHRH were synthesized on a hydroxymethylphenyloxymethyl resin using the base labile Fmoc protecting group on the α -amines. The side chains were protected by HF labile groups. Purification of the segments was performed on Sephadex LH-20 columns and by HPLC on Silica Gel 60 columns. The two segments were then assembled on an α-aminobenzyl resin to yield entire sequence of LHRH. After HF treatment and standard purification on Sephadex G-15 and carboxymethylcellulose CM-52 the desired LHRH was obtained. Synthesis of the segments by the same strategy on carbazoyloxymethylphenyloxymethyl resin showed up unexpected difficulties.

Applying the common knowledge of peptide synthesis, it is clear that, at the moment, no general strategy is yet available to permit a secure synthesis of a peptide larger in size than thirty to forty amino-acids. At first sight, for such an undertaking, one might consider the stepwise solid phase method of Merrifield that has been employed with considerable success for obtaining hundreds if not thousands of biologically active peptides and analogues. Over the recent years improvements of this methodology (see the excellent review of Barany and Merrifield (1)) have been such (control of side reactions, yields of incorporation) that it is now the most reliable and wise way to undertake the first synthesis of any peptide smaller than twenty to thirty residues long. Still, for a larger peptide, if one excludes the problem of the unpredicted side reactions (which seems to happen in a similar way, whatever strategy is used), the main limitation of this method remains the potential accumulation on the resin of undesired deletion peptides. To circumvent this criticism, one will automatically consider that the most satisfying strategy to start with is that of fragment condensation with the idea that each protected fragment can be purified extensively and characterized as a very homogeneous product. Still, the few examples of possible comparison between fragment condensation technique and stepwise synthesis have shown no clear cut advantage (yield or purity of the final product) for one or the other technique in obtaining peptides ranging in size between 24 and 48 amino-acids (2). The group of Kenner, for the undertaking of the synthesis of a 129-peptide, decided to use the strategy of fragment condensation (3). In the subsequent studies, all the syntheses were done by the solution methods and two main drawbacks appeared while using this approach, the same ones as already mentioned in the review by Finn and Hofmann (4), i.e. the low yields for coupling of large segments and the unpredictable sparing solubility characteristics of some

protected intermediates.

One possible way to avoid some of these difficulties for the synthesis of large peptides may be the "convergent" (5) solid phase strategy which involves both solid phase fragment condensation and also solid phase synthesis of protected peptides. On one hand, use of the solid phase approach for the coupling of soluble segments has given encouraging results over the last years with reasonably high yields (6,7,8,9, see also 10 for coupling of segments on a polymer gel). On the other hand, solid phase synthesis of these protected segments may overcome the challenging manipulations for the purification of every intermediates which sometimes are insoluble and for which there exists no routine methods : only the final fragment here has to be soluble in a feasible solvent to permit its purification. Employing mostly the current methodology, we were eager to try the convergent solid phase synthesis approach on some small peptides.

The type of linkage between the growing fragment and the resin must be chosen in function of the combination of α-amine protection and side chain protection. Although the drastic HF treatment is probably at the origin of multiple side reactions, cleavages and also polymerisations, it has given many satisfying results and the experience with it is such that it remains one of the most convenient approaches in the protection strategy. So, with the HF labile side chain protection, five types of functions and reagents can be used and have been tested by different groups on resins to perform solid phase segment synthesis :

a) hydrazinolysis on an ester linkage (11-13) enabling further segments coupling by the azide method. There are two drawbacks to this approach : the rather strong basic conditions and the fact that it is not feasible when either a β-benzyl protected aspartic acid or a γ-benzyl glutamic acid is present in the segment.

b) catalytic hydrogenolysis of the ester linkage either with an O-nitrobenzoyl polyethylene glycol support (14) or with the standard oxymethyl polystyrene resin (15, 16) : side reactions are expected if the peptide chain contains cysteine or methionine residues.

c) mild acidolytic cleavage (50 % trifluoroacetic acid (TFA)/CH_2Cl_2) of a *p*-alkoxybenzyl ester linkage (12, 17 and 18) after synthesis of protected segment with combination of 2-(*p*-biphenylyl)propyl(2)-oxycarbonyl (Bpoc) group for α-amino protection and 0.5 % TFA/CH_2Cl_2 as the deprotection agent. Protected peptides have been obtained that way but a scheme with a better chemical selectivity is preferable.

d) base or nucleophile cleavage of a phenyl ester anchoring linkage (19,20). Severe peptide losses during base wash and coupling steps have been reported especially with proline residue as C-terminal amino acid.

e) photolysis of o-nitrophenyl ester linkage. This method has enabled synthesis of protected segments (21) with relatively good yields.

Tam and coworkers (22,23) have developped some interesting solid supports called multidetachable resins which can be associated with several of the above mentioned types of reactions to yield solid phase synthesized segments. The versatility of these resins should find soon applications in this direction.

We felt that the methodology of solid phase segment synthesis should rely on a minimum of orthogonality as defined by Barany and Merrifield (24), . at least either α-amine deprotection or resin linkage cleavage ought to be performed unrelated to acidolytic treatment. In order to gain some experience with the convergent solid phase peptide synthesis strategy, two of the five possible ways to perform solid phase segment synthesis have been tested with modifications. In this paper is presented use of the *p*-alkoxybenzyl ester type resin in combination with 9-fluorenylmethyloxycarbonyl (Fmoc) group as α-amino protection, and benzyl type or tosyl as side chain protection for the preparation of the two segments 1-6 and 7-10 of luliberin (LHRH) (25). Purification of these fragments was performed,

followed by assemblage on an α-aminobenzylcopoly-(styrene-1%-divinylbenzene) (benzhydrylamine resin) to yield entire LHRH. In the paper just next to this one, use of photolysis is presented for synthesis of the segment 1-6 of apamin on an α-(4-bromomethyl-3-nitrobenzamido)benzylcopoly-(styrene-1%-divinylbenzene) (bromomethyl-Nbb-resin) (26), followed by the assemblage on an α-aminobenzyl resin with the 7-18 portion to yield entire apamin.

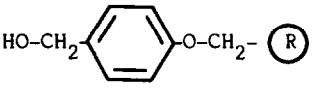
The *p*-alkoxybenzyl ester type resin has been used with success for either direct solid phase synthesis of peptides with tertiobutyl protecting groups on the side chains and Bpoc or Fmoc on the α-amines (2, 27-31), or for solid phase synthesis of segments with benzyl blocking groups on the side chains and Bpoc on the α-amines (11, 17 and 18). In both cases, behaviour of this solid support looked fine with high yields in the final cleavage by trifluoroacetic acid of the peptides or segments. In order to improve somewhat the orthogonality and also to avoid use of the too acid sensitive Bpoc groups, we felt inclined to try on this resin the combination of the base labile Fmoc group for α-protection with the HF labile side chain protections. Sequence of LHRH (Fig. 1) is particularly well suited for such a test as it can be divided

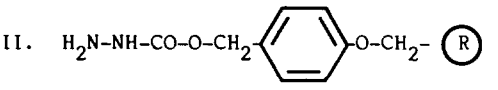
*p*Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Fig. 1. Amino acid sequence of LHRH

in two segments bearing glycine on their C-terminus and most of the amino acids are trifunctional. LHRH is also well known and characterized and any side reaction would be easily detected.

Using Fmoc as α-protection and the following HF labile groups for side chain protection : tosyl (Tos) for histidine and arginine, benzyl (Bzl) for serine and 2,6-dichlorobenzyl (Dcb) for tyrosine, we undertook building of the two segments LHRH 1-6 and LHRH 7-10 on both the p-alkoxybenzyl resin (I) and on its derived carbazoyloxybenzyl resin (II)

I. 

II. 

Ⓡ = copoly(styrene-1%-divinylbenzene)

to yield the segments with their C-terminal carboxyl either free or as a hydrazide.

RESULTS

Synthesis of Fmoc-Leu-Arg(Tos)-Pro-Gly-OH (III) and of pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-OH (IV)

Fmoc-glycine was attached to resin I with dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) following the same procedure as described by Meienhofer and coworkers (29). Substitution varied from 0.36 to 0.41 mmol NH_2 /g of H-Gly-resin I depending on the synthesis. Remaining hydroxyl groups were benzoylated. Next amino acid derivatives were incorporated via their preformed symmetrical anhydrides (1.5 fold excess) and completeness of couplings was controlled by a fluorescamine test (32).

For synthesis of segment III a 48 % drop in the substitution level was observed after incorporation of Fmoc-arginine. It has been mainly attributed to diketopiperazine formation. No such drop was observed during synthesis of segment IV probably because of the less favourable amino acid sequence. In both cases, after incorporation of the third amino acid derivative, acetylation was performed to block the eventual hydroxyl groups. Trifluoroacetic acid treatment (in presence of mercaptoethanol in the case of segment IV to protect its tryptophane) yielded the crude segments which were chromatographed on LH-20 columns in methanol (MeOH) and on Silica Gel 60 columns by HPLC. Purified protected segments could thus be obtained with yields varying from 23 % to 37 % of

the crude mixture. The peaks were cut off shortly and no trial was made to optimize the yields by recycling the interfaces of the peaks. Homogeneity of the purified segments was assessed by a correct amino acid analysis and by detection of a single homogeneous spot by TLC.

Synthesis of Fmoc-Leu-Arg(Tos)-Pro-Gly-NH-NH_2 (V) and of pGlu-His(Tos)-Trp-Ser(Bzl)--Tyr(Dcb)-Gly-NH-NH_2 (VI)

Wang (17) introduced the use of resin II to yield solid phase synthesized segment hydrazides but in literature no further experiments have been described with this solid support. We wanted to try this resin for synthesis of the segments V and VI, compare the results to those of the synthesis of the segments III and IV, and eventually determine their usefulness in the couplings via their azides.

Fmoc-glycine was incorporated on resin II by a standard coupling procedure and remaining amine groups were acetylated. Substitution was then of 0.55 mmol NH_2 /g of H-Gly-resin II. Coupling of the second amino acid derivative, Fmoc-proline or Fmoc-tyrosine(O-2,6-dichlorobenzyl) yielded a considerable drop of the functionalization on the resin : 36 % drop for segment V and 73 % drop for segment VI. Investigation by amino acid analyses of the 6 N HCl hydrolyzates of the filtrates from the Fmoc-glycine-resin II deprotection step showed large amounts of glycine, but the nature of its derivative was not elucidated. After acetylation at this step, assembly was carried out and proceeded smoothly. The segments obtained after trifluoroacetic cleavage were purified on LH-20 columns and on Silica Gel 60 HPLC. Due to the early losses of functionalization too low amounts of segments V and VI were recovered to perform the segment coupling procedure. Furthermore segment VI showed special difficulties in purification giving multiple peaks at each step and also a slurry spot on TLC, which was not the case for segment IV.

Segment assembly on an α-aminobenzyl resin and purification of LHRH

Segment III was coupled onto α-aminobenzyl resin (0.84 mmol NH_2 /g) by the dicyclohexylcarbodiimide and hydroxybenzotriazole (HOBt) procedure in dimethylformamide (DMF). Although the amines on the solid support were in a three fold excess over the peptide, only 21 % of the segments could be incorporated. Remaining amines were acetylated and after Fmoc deprotection, segment IV (2.5 excess) was then coupled by the same procedure. A negative ninhydrin test (33) indicated completeness of incorporation.

Crude LHRH peptide obtained after HF treatment (59 % cleavage yield) showed by amino acid analysis a ratio of N-terminal amino acids over C-terminal residues close to one. After Sephadex G-15 and carboxymethylcellulose CM-52 chromatographies followed by a last desalting on Bio-Gel P2, the purified LHRH (56 % yield over crude peptide) showed a correct amino acid analysis and an excellent homogeneity on HPLC.

DISCUSSION

Combination of Fmoc α-protection, HF labile side protection and *p*-alkoxybenzyl type resin can provide useful means for preparing peptide segments. Still a few difficulties appeared in the course of the work.

The first C-terminal Fmoc-amino acid was incorporated to resin I with DCC and DMAP (29). Once our synthesis work was completed, Atherton and coworkers (34) mentioned a slight instability of the Fmoc-group to DMAP. As a matter of fact, repeating the experiment of incorporation of Fmoc-Gly onto resin I, we could detect presence of about 9% of the dipeptide Gly-Gly by ion exchange chromatography on the amino acid analyzer. Purification of segments III and IV probably removed the segments bearing the Gly-Gly sequence at the carboxyl end because the amino acid ratio of glycine improved markedly

from the crude to the purified segments. Still for later syntheses, one will have to improve the methodology for incorporating the first amino acid derivative. The drop of functionalization found after incorporation of the arginine derivative of segment III (48 % drop) can be explained by diketopiperazine formation which is sequence dependant : no loss was detected during synthesis of segment IV, although in that case protocole was slightly modified (see experimental). The method of Suzuki (35) is not applicable when dealing with Fmoc-amino acid derivatives and the best alternative would be to couple directly the dipeptide onto the C-terminal glycine on the resin. One could also introduce the second amino acid as its Bpoc derivative and then apply the Suzuki's procedure.

The main advantage in making segments is the possibility to purify them before further incorporation. Use of preparative HPLC on Silica gel has not yet solved this problem in a simple way. Purified segments have been obtained but the yields are too low to considere this method appropriate.

Use of resin I seemed finally more convenient than that of resin II which was disappointing by unpredictible loss of functionalization after deprotection of C-terminal Fmoc-glycine. This embarrassing loss might be due to an intramolecular cyclization by aminolysis.

Despite the few difficulties that still need improvements, especially the methods for purifying the segments, the combination of Fmoc α-protection and use of *p*-alkoxybenzyl type resin for preparing segments with HF labile side chain protections may prove useful in the strategy of convergent solid phase peptide synthesis.

EXPERIMENTAL PART

Amino acid symbols follow mostly IUPAC-IUC recommendations (Biochem. J. 126, 773 (1972)). Abreviations not previously defined are as follows : DIEA, diisopropylethylamine ; DMA, dimethylacetamide ; *i*PrOH, 2-propanol ; AcOH, acetic acid ; Ac_2O, acetic anhydride ; $(Fmoc\text{-}AA)_2O$, Fmoc-amino acid anhydride.

Chloromethylcopoly-(styrene-1%-divinylbenzene) was from Bio-Rad Laboratories; α-aminobenzyl resin was synthesized from Bio-Beads copoly-(styrene-1%-divinylbenzene) by the Leuckart reaction (36) ; Fmoc-L-amino acids have been fashion made and purchased at Bachem. HF reaction was performed in a Toho-Kasei installation (Japan). Hydrolysates for amino acid analyses from peptide samples were prepared by treatment with 6 N HCl for 20 hr in vacuum degazed sealed tubes at 110°, and amino acid analyses were run on a Beckman 120 C autoanalyzer. The ratios were calculated on the basis of the data obtained for the stable amino acids and no correction was made for destruction of the unstable ones. Thin-layer chromatography was carried out on precoated Silica Gel 60 (F-254) plates (Merck), with the solvent system indicated at every case. Analytical HPLC was performed in a Waters Associates ALC/GPC 205 U apparatus. CH_2Cl_2 was dried over anhydrous K_2CO_3 and distilled over it immediately before use. DMF was dried over 4 Å molecular sieves and freed of amines by nitrogen bubbling until negative 1-fluoro-2,4-dinitrobenzene test (37). Piperidine was distilled under N_2 from NaOH pellets and redistilled under N_2 . Peroxide-free dioxane was used. 2-propanol was dried with calcium chloride and barium oxide prior to distillation. All other solvents and chemicals were reagent grade.

4-hydroxymethylphenyloxymethyl resin (I)

This resin was obtained from chloromethylcopoly(styrene-1%-divinylbenzene) (0.89 mmol/g ; Bio Rad Laboratories), by reaction with methyl 4-hydroxybenzoate/CH_3ONa and further reduction with $LiAlH_4$ according to the procedure described by Wang (17). The resin showed no detectable amount of chlorine and had an identical IR spectrum to that previously reported.

Fmoc-Gly-O-CH_2-C_6H_4-O-CH_2-resin

Fmoc-Gly-OH was attached to hydroxymethylphenyloxymethyl resin with dicyclohexylcarbodiimide and 4-dimethylaminopyridine following the same procedure as Meienhofer et al. (29). After several washings (CH_2Cl_2, DMF and *i*PrOH) the Fmoc-Gly-O-CH_2-C_6H_4-O-CH_2- resin was treated with an excess of benzoyl chloride in the presence of pyridine and a catalytic amount of 4-dimethylaminopyridine to block the unreacted hydroxymethyl sites.

General synthetic procedure

The following protocol was performed at every α-amino deprotection step with ca 15 ml of solvents :

1) CH_2Cl_2 (3x2 min), 2) 50 % piperidine/CH_2Cl_2 (1x5 min and 1x25 min), 3) CH_2Cl_2 (4x2 min), 4) DMF (1x2min). The collected filtrates from treatment and washings 2,3 and 4 were combined and, after the adequate dilution with CH_2Cl_2 , used directly for the spectrophotometric determination of N-(9fluorenylmethyl)piperidine at 301 nm (ε 7.800). Whenever a second deprotection was performed over the same amino acid, virtually no amount of N-(9-fluorenylmethyl)piperidine could be detected.

Amino acids were assembled via preformed

symmetrical anhydrides synthesized by mixing two cold solutions of DCC and Fmoc-amino acid (double molar amount) in CH_2Cl_2 /DMF 2:1. After 30 min at 0°C, N,N'-dicyclohexylurea was filtered off and washed with the same solvent. Coupling was performed with the combined filtrate and washings in the minimal total amount of solvent, ca 10 ml. The program used consisted of :
1) DMF (2x2 min), 2) dioxane/H_2O 2:1 (2x5 min), 3) DMF (2x2 min), 4) CH_2Cl_2 (3x2 min), 5) $(Fmoc\text{-}AA)_2O$ (1x15 min), 6) addition of 1 eq DIEA in CH_2Cl_2 (1x45 min), 7) CH_2Cl_2 (3x2 min), 8) DMF (3x2 min), 9) iPrOH (3x2 min), 10) steps 7, 8 and 9 repeated.

Couplings were performed with 1.5 fold excess of Fmoc-amino acid anhydride unless otherwise indicated. The completeness of coupling was controlled by means of a fluorescamine test, and if positive, steps 4 to 10 of the general coupling program were repeated, this time with only one equivalent of Fmoc-amino acid anhydride. If the fluorescamine test was still positive, the remaining amino groups were blocked by acetylation, following this program : 1) CH_2Cl_2 (3x2 min), 2) DMF (2x2 min), 3) five fold excess of Ac_2O in DMF (1x10 min), 4) addition of an equimolar amount of DIEA (1x10 min), 5) DMF (3x2 min), 6) CH_2Cl_2 (3x2 min), 7) steps 5 and 6 repeated.
Cleavage of the peptide resin bond was performed by a one hour treatment with 55 % TFA in CH_2Cl_2 preceeded and followed by CH_2Cl_2 washings (4x5 min). The combined filtrates of the treatment and later washings were evaporated to dryness affording the crude peptide.

Fmoc-Leu-Arg(Tos)-Pro-Gly-O-CH_2-C_6H_4-O-CH_2-resin

The synthesis was started with 1.93 g of Fmoc-Gly-O-CH_2-C_6H_4-O-CH_2-resin with a substitution level determined spectrophotometrically (UV absorption of N-(9-fluorenylmethyl)piperidine after deprotection) and by nitrogen analysis of 0.41 mmol/g H-Gly-resin 1. Two couplings of $(Fmoc\text{-}Pro)_2O$ (1.5 equivalents each) were needed to obtain a negative fluorescamine test. After coupling of Fmoc-Arg(Tos)-OH (2 fold excess of anhydride) deprotection gave a substitution level of 0.20 mmol/g H-Gly-resin I showing a 48 % loss of peptide probably due mainly to diketopiperazine formation. After acetylation Fmoc-Leu-OH was incorporated through two couplings with 2 and 1.5 equivalents of amino acid anhydride respectively.

pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-O-CH_2-C_6H_4-O-CH_2-resin

This peptide fragment was synthesized starting with 1.87 g of Fmoc-Gly-OCH2-C6H4-OCH2-resin with a substitution level of 0.36 mmol/g H-Gly-resin I as determined spectrophotometrically. Fmoc-Tyr(Dcb)-OH, Fmoc-Trp-OH, Fmoc-His(Tos)-OH and pGlu-OH were incorporated by double coupling of the symmetric anhydrides following the general procedure as described above. Acetylation was necessary after tyrosine and histidine couplings. In the case of pyroglutamic acid a third direct DCC mediated coupling with a three fold excess of amino acid, was needed to afford a negative fluorescamine test.
In order to minimize diketopiperazine formation Fmoc-Ser(Bzl)-OH was incorporated in a five fold excess and DIEA was added simultaneously to the symmetric anhydride. After that, a negative fluorescamine test was obtained and blockade of the possible hydroxymethyl sites liberated after diketopiperazine formation was performed by acetylation with Ac_2O and pyridine for 30 min. Deprotection of Fmoc-peptidyl resin at this stage showed no loss of functionalization. By weight increase, yield of incorporation was about 90 % of expected. Deprotection after incorporation of the histidine showed an abnormal increase of functionalization but deprotection of the Fmoc-His(Tos)-OH as amino acid derivative showed already the same kind of excess N-(9-fluorenylmethyl)piperidine.

Fmoc-Leu-Arg(Tos)-Pro-Gly-OH (III)

TFA treatment of Fmoc-Leu-Arg(Tos)-Pro-Gly-OCH_2-C_6H_4-OCH_2-resin afforded 198 µmol (59% cleavage yield) of the crude protected peptide with the following amino acid analysis : Pro 0.75, Gly 1.10, Leu 1.30 and Arg 0.86. Two more treatments were performed, one in the previous conditions and the other with 100 % TFA for one hour, but only 0.6 % and 0.2 % yields were respectively obtained. Two different chromatographic methods of purification were used. All chromatographic runs were monitored by UV absorption at 301 nm.
a) 20 µ mol of the crude protected fragment were chromatographed on a Sephadex LH-20 column (100 x 1 cm) with MeOH. The flow rate was 6 ml/h. The desired fractions were pooled, evaporated to dryness, and applied to a pre-packed Silica Gel 60 column (Merck size A, 24 x 1 cm). The product was eluted with $CHCl_3$/*i* PrOH/AcOH 88:5:2 with a flow rate of 120 ml/h collecting fractions of 2 ml. Finally the peptide was rechromatographed under the same conditions affording 4.6 µmol (23 % yield) of the purified protected peptide.
b) 79.2 µ mol of the product were first cleaned on a 12 g Silica Gel, 60 open column (40-63 µm) eluted with $CHCl_3$/MeOH/-AcOH 70:20:5. All the peptide containing fractions were pooled together and applied to a Silica Gel 60 cartridge (28 x 5 cm) of a Prep LC/System 500 Waters Instrument. Elution at 200 ml/min flow rate was performed with a three step gradient of CH_2Cl_2 /*i* PrOH/AcOH 88:1.5:0.5 (3400 ml), CH_2Cl_2 /*i* PrOH/AcOH 88:10:5 (2600 ml) and CH_2Cl_2 /MeOH/AcOH 88:10:5 (2000 ml) (Fig. 2). The desired peptide, eluting with the last solvent system, was rechromatographed on the same column with CH_2Cl_2 /MeOH/AcOH 88:8:4. The product was obtained in a 35 % total purification yield. The purified protected peptide was homogeneous by TLC ; Rf = 0.55 in CH_2Cl_2 /MeOH/ AcOH 70:20:5, and had the following amino acid analysis after Fmoc removal : Pro 1.04, Gly 0.99, Leu 0.97, Arg 0.96. Its specific rotation was $[\alpha]_D$ = - 44.1° (c 0.4, DMF).

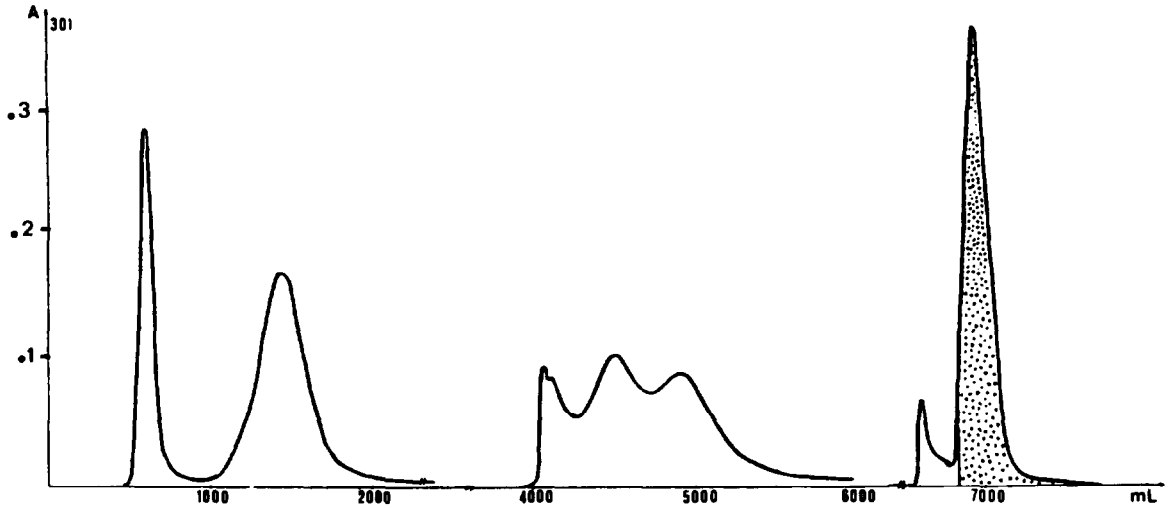


Fig.2. Preparative HPLC on Silica Gel of crude segment III (see text).

pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-OH (IV)

Treatment of the peptide-resin with 55 % TFA/CH_2Cl_2 in the presence of 2 % mercaptoethanol afforded 325 µmol (56 % cleavage yield) of the crude protected peptide with the following amino acid analysis : Ser 0.92, Glu 2.38, Gly 1.16, Tyr 0.98, His 0.94. A second treatment with 30 % TFA/CH_2Cl_2 and 1 % mercaptoethanol gave only a 1.3 % yield.

The chromatographic purifications of this peptide were monitored by UV absorption at 280 nm. First, the protected fragment was subjected to a gel filtration on a Sephadex LH-20 column (100 x 1 cm) with MeOH (flow rate 6 ml/h, 2 ml per fraction). All the crude product was purified in five chromatographic runs. The desired fractions (Fig. 3) were assembled, applied to a Silica Gel cartridge of Prep LC/System 500 Waters instrument and eluted with CH_2Cl_2 /MeOH/H_2O 57:40:3 (flow rate 200 ml/min). Finally the product was rechromatographed on a Sephadex LH-20 in the same previous conditions.

The purified peptide fragment obtained in a 37 % total purification yield had the following amino acid analysis : Ser 0.85, Glu 1.05, Gly 0.98, Tyr 0.97, His 1.04. The product was homogeneous by TLC : Rf=0.43 $CHCl_3$ /MeOH/NH_3 10:10:1 (Ehrlich identification spray (37)) and had a specific rotation $[\alpha]_D$ = -13.7° (c 1.2, DMF).

H-Leu-Arg(Tos)-Pro-Gly-NH-CHØ-resin

Fmoc-Leu-Arg(Tos)-Pro-Gly-OH, 47 µ mol, dissolved in 4 ml of DMF and 47 µmol of HOBt in 1 ml DMF were added to 177 mg

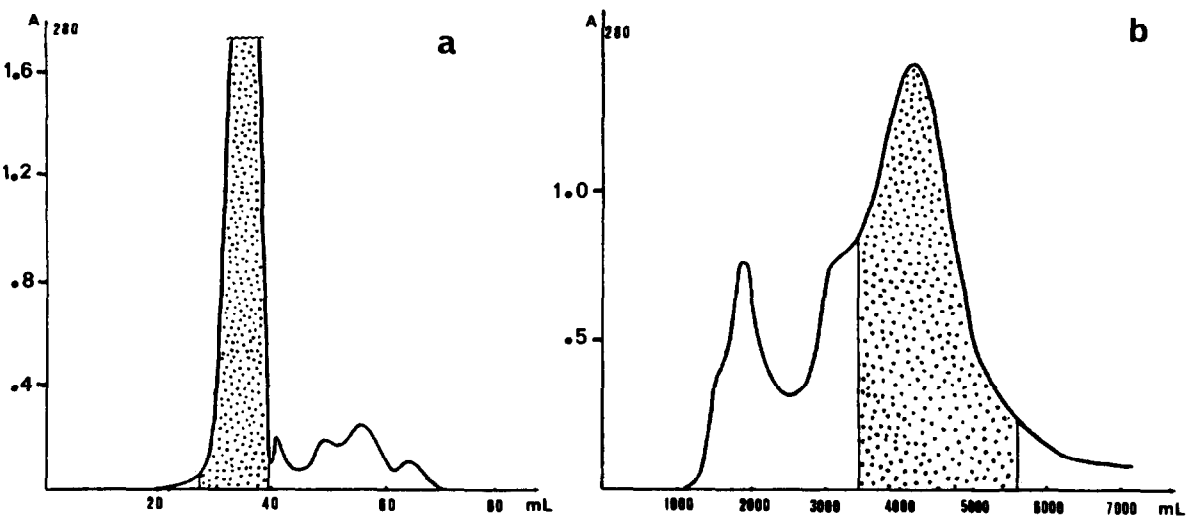


Fig.3. Purification of segment IV.
a) Sephadex LH-20 chromatography ;
b) Preparative Silica Gel HPLC of the peptide containing fractions obtained after Sephadex LH-20 chromatographies.

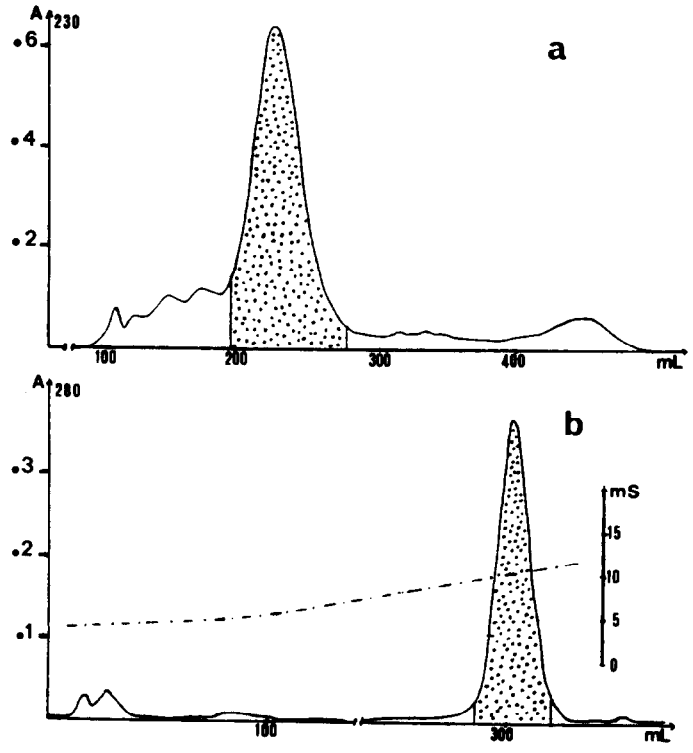


Fig.4. Purification of LHRH. a) Sephadex G-15 chromatography of the crude LHRH ; b) CM-52 chromatography of the main fraction obtained after Sephadex G-15 chromatography.

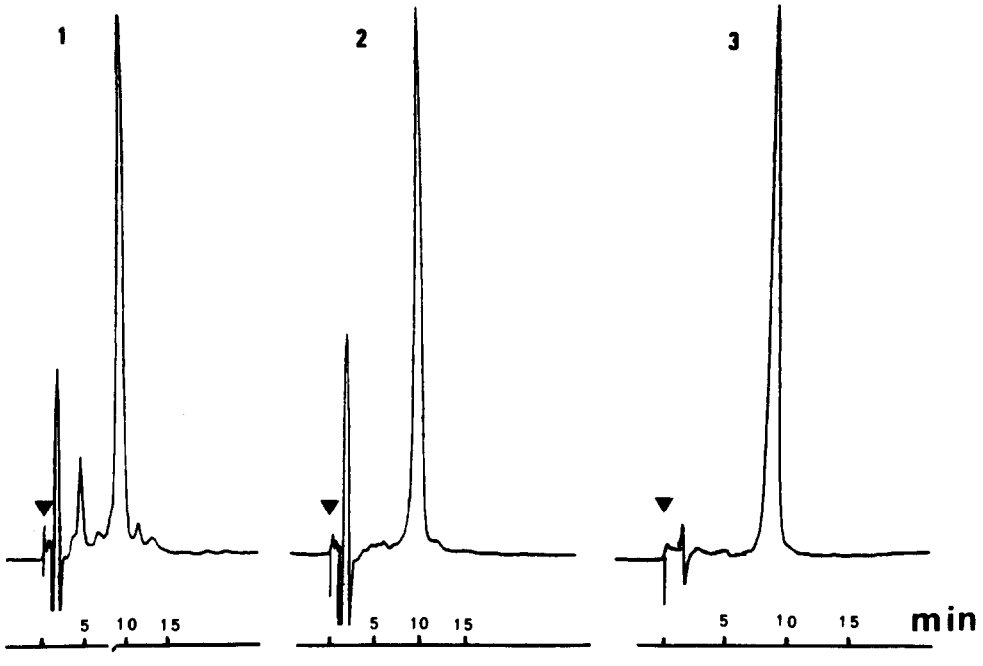


Fig.5. Analytical HPLC on reverse phase C18 of LHRH at different stages of purification : 1) crude peptide ; 2) main fraction obtained after Sephadex G-15 ; 3) purified LHRH.

of α-aminobenzyl resin (0.84 mmol NH_2 /g) and the mixture was shaken at 0°C for 10 min. After addition of 47 μmol of DCC in 1 ml DMF the reaction was continued for two hours at 0°C and 46 h at room temperature. The peptide-resin was filtered and washed with DMF, CH_2Cl_2 and MeOH (4 x 2 min each) and again with the same solvents (2 x 2 min, each).
Unreacted amino groups were blocked by two successive acetylations with Ac_2O (0.12 ml) and DIEA (0.20 ml) in DMF under the same conditions described for the synthesis of the fragments. Negative ninhydrine test was obtained.
Deprotection with 50 % piperidine/CH_2Cl_2 showed that only 9.8 μmoles of segment IV were incorporated which means a yield of 21 %.

pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-Leu-Arg(Tos)-Pro-Gly-NH-CHØ-resin
Segment IV (24.6 μmol) was coupled to H-Leu-Arg(Tos)-Pro-Gly-NH-CHØ-resin (9.8 μmol) with 24.6 μmol of both DCC and HOBt in the same conditions as described above. After this single coupling and washings a negative ninhydrin test was obtained.

LHRH
198 mg of peptide resin were treated with 5 ml of anhydrous HF in the presence of 0.5 ml of anisole for one hour at 0°C. 5.54μmol of crude LHRH were obtained (59% yield) with the following amino acid analysis : Ser 0.80, Glu 0.94, Pro 0.90, Gly 2.09, Leu 1.07, Tyr 0.65, His 0.71, Arg 1.08. 2.47 μmol of the crude peptide were chromatographed on a Sephadex G-15 column (150 x 1.2 cm) with 0.1 M ammonium acetate pH 8.5 at 24 ml/h flow rate (Fig. 4a). The desired fractions (analyzed by UV absorption at 230 and 280 nm and reverse phase HPLC) were lyophilized, applied to a carboxymethylcellulose (CM-52 Whatman) 25 x 1.2 column, and the peptide eluted by developing a linear gradient of ammonium acetate (buffer A : conductivity 4 mS, pH 5.10 ; buffer B : conductivity 12 mS, pH 6.30). The major peptide peak (Fig. 4b) was finally desalted on a Bio-Gel P2 column (100 x 1.2 cm) with 0.1 M acetic acid. LHRH was obtained in a 56 % total purification yield. Losses due to the samplings for amino acid analyses and HPLC analyses have not been taken into account and the low amount of starting material explains probably some losses on the columns.
The progress of the purification was followed by analytical HPLC : RP-18 Lichrosorb column 25 x 0.4 cm, elution with 20% CH_3-CN/0.05 M pH 3.0 triethylammonium phosphate at 2 ml/min (Fig. 5). The purified LHRH was homogeneous by HPLC and had the following amino acid analysis : Ser 0.93, Glu 0.99, Pro 0.97, Gly 2.11, Leu 0.97, Tyr 0.98, His 0.98, Arg 0.97, Trp 0.96. Tryptophane was determined after hydrolysis of the peptide by toluene sulfonic acid.

Fmoc-Gly-NH-NH-CO-O-CH_2-C_6H_4-O-CH_2-resin
4-carbazoyloxymethylphenyloxymethylcopoly-(styrene-1%-divinylbenzene) (resin II) was synthesized by reaction of 4-hydroxymethylphenyloxymethyl resin with phenyl chloroformate followed by hydrazinolysis according to the procedure described by Wang (17). The resin was obtained with a substitution level of 0.65 mmol/g as determined by nitrogen analysis.
Fmoc-Gly-OH was incorporated to the solid support as its symmetric anhydride (1.5 fold excess) in the same way as described above. After that the unreacted-NH-NH_2 groups were blocked by acetylation, cleavage of Fmoc group showed a substitution level of 0.55 mmol NH_2 /g of H-Gly-resin II.

Fmoc-Leu-Arg(Tos)-Pro-Gly-NH-NH-CO-O-CH_2-C_6H_4-O-CH_2-resin
The synthesis started with 2.19 g of Fmoc-Gly-resin II and was performed according to the general synthetic procedure.
Fmoc-Pro-OH was incorporated by a double coupling but for Fmoc-Arg(Tos)-OH and Fmoc-Leu-OH a single coupling was enough. After incorporation of proline, deprotection of an aliquot showed that there had been a 36 % loss of functionalization, so, before incorporation of the arginine, acetylation was performed to block possible reactive groups on the resin.

pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-NH-NH-CO-O-CH_2-C_6H_4-OCH_2-resin
This synthesis was also performed by the usual procedure, starting with 2,25 g of Fmoc-Gly-resin II.
Fmoc-amino acids were incorporated by single coupling of their symmetric anhydrides except for Fmoc-His(Tos)-OH (two couplings) and pGlu-OH (two couplings and a third directly DCC mediated coupling).
Although [Fmoc-Tyr(Dcb)]$_2$O was introduced in a two fold excess an even greater drop of functionalization (73 %) was observed after deprotection of an aliquot. Like in the precedent synthesis acetylation was performed before α-amine deprotection and coupling of serine.
Deprotection after histidine coupling showed the same abnormal increase of functionalization like in the synthesis of segment IV.

Fmoc-Leu-Arg(Tos)-Pro-Gly-NH-NH_2 (V)
Fmoc-Leu-Arg(Tos)-Pro-Gly-resin II was treated with 55 % TFA in CH_2Cl_2 to give 372 μmol (60 % cleavage yield) of crude protected peptide with the following amino acid analysis: Pro 1.01, Gly 1.25, Leu 0.98, Arg 0.77.
The product was purified by HPLC on Silica Gel 60 monitoring the UV absorption at 301 nm. Three runs were performed in the following conditions : 1) $CHCl_3$ /MeOH/-AcOH 80:10:2 with a flow rate of 240 ml/h on a pre-packed Merck column, size B ; 2) the same conditions ; 3) CH_2Cl_2 /MeOH/AcOH 87:10:3 eluting at 200 ml/min in a Prep LC/System 500 Waters Instrument. 25 μmol of the purified protected segment (7% purification yield) were obtained. The peptide was homogeneous by TLC (Rf= 0.44 $CHCl_3$ /EtOH 3:1) and had the following amino acid analysis after Fmoc removal : Pro 1.02, Gly 1.05, Leu 0.96, Arg 0.97.

pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Dcb)-Gly-NH-NH_2
55 % TFA/CH_2Cl_2 treatment in the presence of 2 % mercaptoethanol of pGlu-His(Tos)-

Trp-Ser(Bzl)-Tyr(Dcb)-Gly-resin II afforded 120 μmol (54 % cleavage yield) of crude protected peptide with the following amino acid analysis : Ser 0.99, Glu 2.38, Gly 1.71, Tyr 1.05, His 0.96.
Purification was started with gel filtration on a Sephadex LH-20 column (100 x 1 cm) eluting with 10 % DMA in methanol (flow rate 7.5 ml/h). After that, several trials of purification were made on silica gel 60 in a Prep LC/System 500 Waters Instrument eluting with different ratios of CH_2Cl_2 /MeOH/NH_3 giving several fractions of product with a rather good amino acid analysis but in low amounts and difficult to analyze on TLC due to slurry spots.

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