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SYNTHESIS OF LH-RH USING A NEW PHENOLIC POLYMER AS SOLID SUPPORT AND "BOP" REAGENT FOR FRAGMENT COUPLING

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Abstract—*p*-Hydroxyphenyl propionic resin (Table I, compound 4) was used to prepare the 1–6 protected fragment of LH–RH which was then condensed with "BOP" (benzotriazolyl *N*-oxytrisdimethylamino-phosphonium hexafluorophosphate) as coupling reagent to the 7–10 residue synthesized on the same resin. Peptidylresin was divided into two aliquots in order to obtain: LH–RH after aminolysis and treatment with liquid hydrogen fluoride; LH–RH–COOH after saponification followed by hydrogenation, or treatment with liquid hydrogen fluoride. The resulting hormones were rapidly purified by the sole means of two gel filtrations.

The stepwise solid phase method for peptide synthesis is fast but tedious purification of the final product is generally required involving the successive use of different methods such as gel filtration, ion exchange chromatography and solvent partition on a gel support. Solid phase fragment condensation has been proposed to minimize these difficulties. Its major advantage over the stepwise method is that the final product becomes easier to purify by gel filtration since the molecular weights of undesired or deleted sequences are sufficiently different from those of the expected product. Moreover each fragment can be carefully purified before coupling. This technique is becoming widely used[1–7] but the necessary preparation of fragments in solution limits its speed and many approaches have been proposed to accelerate the process by synthesizing the protected fragments on a solid support.[8–10] The use of such methods is often limited either by lack of generality or by slowness or an unsatisfactory yield.

The solid support

Phenolic resins of various structures have been proposed since 1968[20] but have not found a wide range of applications. The studies of Kenner[11–14] on the splitting of phenyl esters may give new impetus to the use of phenolic resins and a novel preparation of these esters has been described by our group.[15] Very recently, after our preliminary presentation of a phenolic resin[16] and during the preparation of this full paper, the utilisation of a phenolic resin has been reported by Kenner's group[34] for the synthesis of enkephalin analogs, supporting the renewed interest in these resins. In this polymer, phenolic groups are directly supported by the polystyrene backbone, being introduced as acetoxy styrene during polymerization.

We have chosen to prepare phenolic resins by chemical modification of commercial resins, chloromethyl polystyrene[35] or α-amino benzyl polystyrene,[20] in order to preserve a wide flexibility in the structural environment of the phenolic group.

Several different phenolic resins were prepared (Table 1) and Boc-Glycin was attached by the DCCI method. In order to check their utility for peptide synthesis, each glycyl-resin was exposed to a mixture of trifluoroacetic acid-methylene chloride (3/7, v/v) 4 hours, followed by diisopropylethylamine methylene chloride (12.5/87.5, v/v) 2 hours. This is the equivalent exposure time to these reagents during the preparation of a decapeptide. Only the hydroxy-phenyl-propionyl resin **4**, from which glycin was almost quantitatively retained after the model treatment, gave satisfactory results and this was used for further syntheses.

The advantage of resin **4** over the three others results from the location of the phenolic ester linkage remote from any electron withdrawing group. The position of phenolic groups remote from the polystyrene backbone may provide more ready access of reagents to the reactive sites.

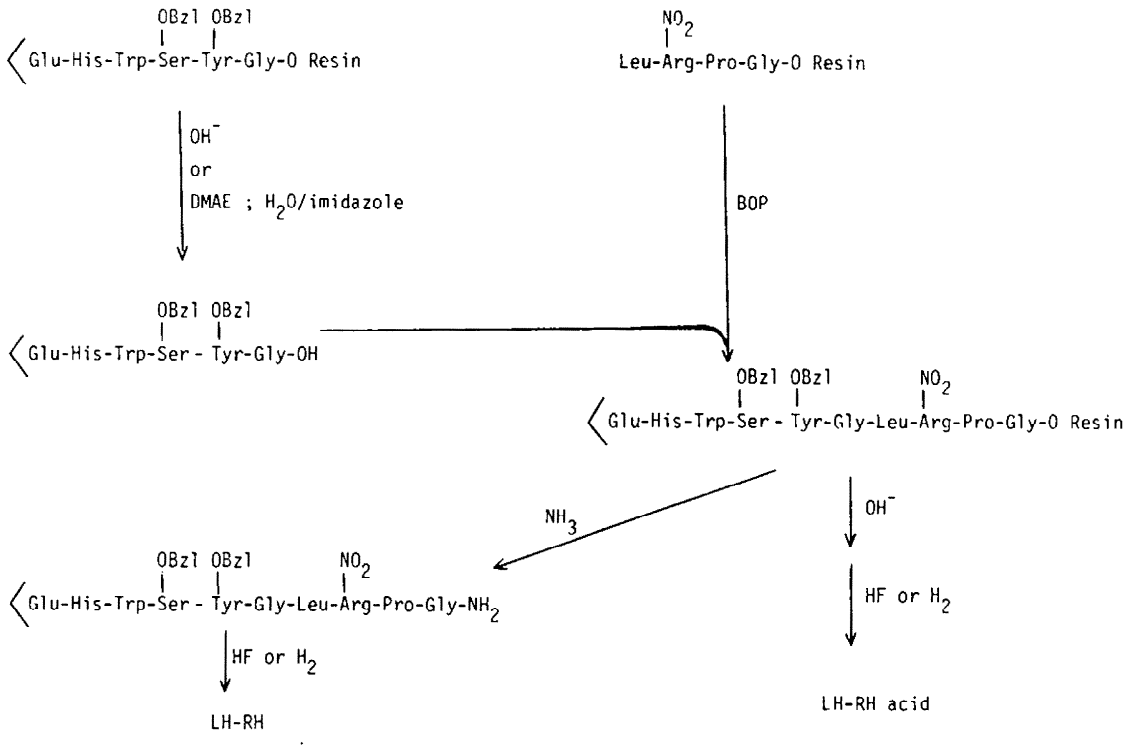
A model synthesis of LH–RH

In order to demonstrate the different possibilities offered by this new resin in peptide synthesis, we chose as example the synthesis of LH–RH.[16]

<Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2
1 2 3 4 5 6 7 8 9 10

This important releasing factor, synthesized many time by classical or solid phase method,[17] and also by another method of solid phase fragment condensation,[18] remains an excellent example to test the efficiency of a new procedure or reagent proposed for peptide synthesis:

—Nine amino-acids out of ten are different; the presence of tryptophan, histidine and arginine makes



its identification on thin layer chromatography or paper electrophoresis by specific color reaction (19) easier.

—Bio and radioimmunoassays are now routinely used to establish its biopotency.

The synthetic pathway is outlined in the diagram. It has been designed in order to illustrate the possibility of splitting a protected fragment from the resin and the efficiency of our BOP reagent[2–5] (benzotriazolyl oxy-N-tris-dimethylaminophosphonium hexafluorophosphate) for high yield fragment coupling in solid phase synthesis.[36]

Stepwise elongation of each fragment was then performed by the dicyclohexylcarbodiimide/hydroxybenzotriazole method according to the traditional solid phase procedure, except that to prevent any risk of transesterification, methanol was replaced by dry acetone or *t*-butanol in methylene chloride to shrink the resin. During the preparation of 1–6 fragment the tosyl protecting group of the imidazole of histidine was displaced by hydroxybenzotriazole.[21] Indeed the ninhydrine test used to control the coupling was found slightly positive in spite of repeated incorporations of pyroglutamic acid. Free imidazole did not affect the continuity of the synthesis, since imidazole substituents do not require protection when histidine is incorporated into a peptide chain.[23]

—1–6 fragment was split from the resin according to two procedures:

(1) Saponification in dimethylformamide with sodium hydroxide. We avoided Kenner's hydroperoxide procedure both because it could be damaging to the tryptophan residue and because of the presence of glycine at the C terminus. The Kenner procedure was designed especially to prevent racemization of phenylalanine phenyl esters.

(2) Transesterification in dimethylformamide with dimethylamino-ethanol followed by hydrolysis, as described by Barton has been shown to be a mild procedure for this reaction.[24] We respected strictly the reaction time given by these authors for the benzyl esters on the Merrefield resin but believe that in the case of phenolic resin the reaction is completed within a much shorter time.

The completion of each reaction, saponification or transesterification was estimated by amino-acid analysis,[27] which indicated that in neither case did any amino-acid remain on the polymer.

After purification on Sephadex LH 20 with dimethylformamide as eluant, 1-6 fragment was coupled to 7-10 peptidyl-resin with "BOP" reagent,[25] much easier to use than the dicyclohexylcarbodiimide/hydroxybenzotriazole method on account of:

—fast and quantitative coupling using a small (1.5 equiv.) excess of the acylating moiety
—avoidance of dicyclohexylurea formation
—solubility of each by-product
—the possibility of re-using the reaction mixture until the completion of coupling.

At the end of the reaction the peptidyl-resin was divided into two aliquots in order to prepare LH–RH and LH–RH acid simultaneously.

1. *LH–RH*

The peptide was cleaved from the resin by aminolysis in dimethylformamide, the reaction being complete within 6 hours as determined by amino-acid analyses made on the residual resin.

Table 1.

RESINS	Glycine retained
1- HO-⟨⟩-O-CH_2-●	0%
2-HO-⟨⟩-C(=O)-N(H)-C(H)(⟨⟩)-●	0%
3-HO-⟨⟩-CH_2-CH(AcO-NH)-C(=O)-N(H)-C(H)(⟨⟩)-●	0%
4-HO-⟨⟩-CH_2-CH_2-C(=O)-N(H)-C(H)(⟨⟩)-●	98±5%

●: Polystyrene 1% divinylbenzene.
Preparation of different resins by condensation of:
1-Hydroquinone under alkaline conditions to chloromethylated resin
2-p-Hydroxybenzoic acid on benzhydrylamine resin by the DCCI method
3-Tyrosine on benzhydrylamine resin, acetylation of the α NH_2 and saponification
4-p-Hydroxyphenylpropionic acid on benzhydrylamine resin by means of BOP reagent
Estimation of glycine retained on the resin

Comparative ninhydrin test were performed on an aliquot of Boc-glycyl-resin treated first with TFA/CH_2Cl_2 3:7 v/v, 20 min, then according to the conditions described in the text. Only resin 4 gave a persistently positive test. The percentage of glycine retained was estimated by performing Gisin's test[33] after treatment with TFA and checked by aminoacid analysis.

Removal of protecting groups from side-chain functions was performed with liquid hydrogen fluoride and gave crude LH-RH, which was analyzed by high pressure liquid chromatography (HPLC) and compared to crude LH-RH prepared stepwise on benzhydrylamine resin.[26] Comparison of elution profiles clearly shows the improvement brought about by these new tactic (Fig. 1). LH-RH was then purified by two successive gel filtrations, each chromatography being controlled by HPLC comparison against standard LH-RH (Roussel Uclaf, Ltd) in order to select the fractions containing this releasing factor. Hormone so obtained was as pure as the standard which had been successively purified by gel filtration, ion-exchange chromatography and finally by partition chromatography (Fig. 2).

2. LH RH acid

After saponification, protecting groups were removed by hydrogenolysis or treatment with hydrogen fluoride. Both products obtained were purified according to the procedure described above and were chromatographically and analytically identical.

Biological assays

In a radioimmunoassay system (Fig. 3), the synthetic decapeptide showed curves parallel to those of a hypothalamic acid extract (internal standard).

In a bioassay system (Fig. 3), the standard and the synthetic decapeptide gave similar release of LH. In summary the decapeptide synthesized on phenolic resin appeared identical with natural LH-RH and with another synthetic sample of high quality.

CONCLUSION

p-Hydroxyphenyl propionic resin **4** has been shown to be a convenient and rapid tool for preparing protected fragments as building units for peptide synthesis. It can be used also for their solid phase condensation. According to the method of cleavage, the peptide can be obtained in its amide form by aminolysis or its carboxylic form by saponification or transesterification with dimethylaminoethanol. In many cases, subsequent treatment with liquid hydrogen fluoride can be avoided. Since the preparation of this paper this polymer has been used to prepare other peptides, including the fragments 20–28,

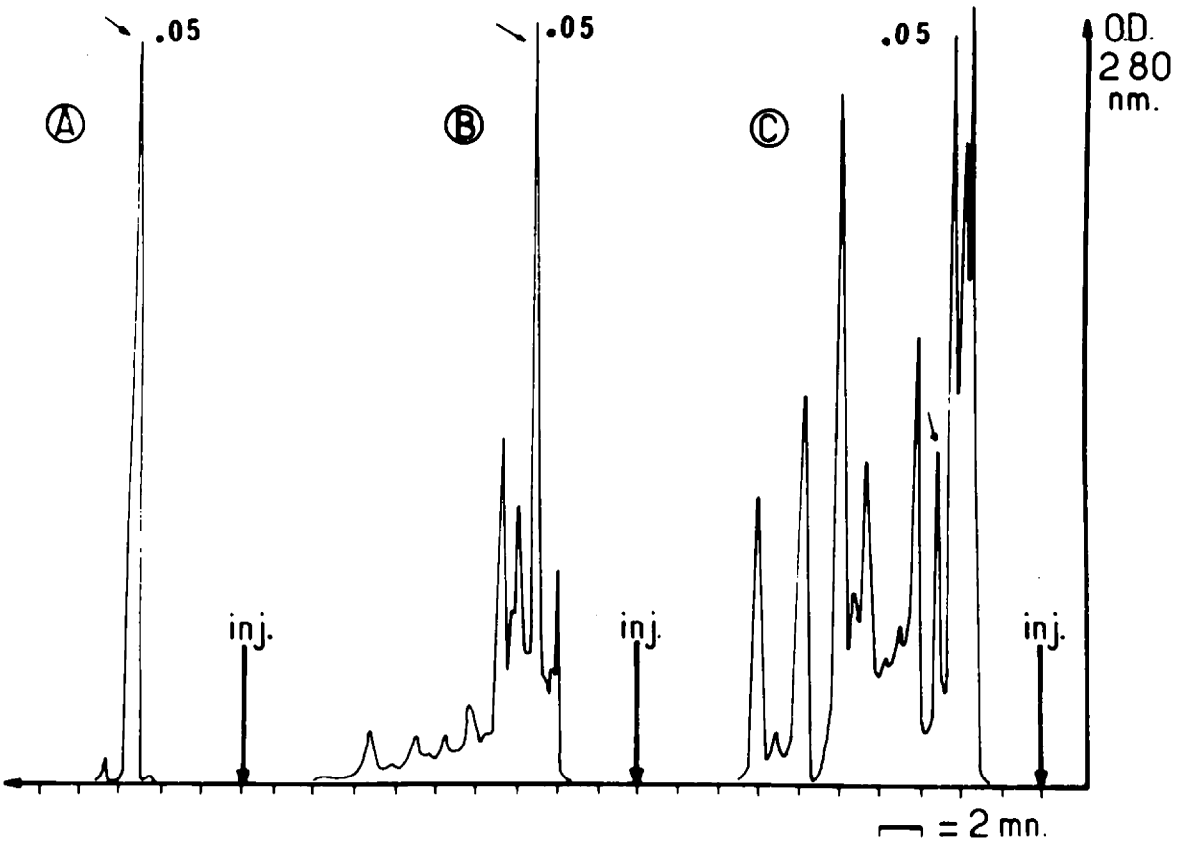


Fig. 1. A—LH–RH standard (Roussel Uclaf 7E/0950); B—crude LH–RH prepared with phenolic resin by fragment condensation; C–crude LH–RH prepared stepwise on benzhydrylamine resin. Solvent HCOOH 0.25 N + Et_3N → pH 3, CH_3CN 70:30 v/v. Numbers near the peaks indicate the O.D. for full scale.

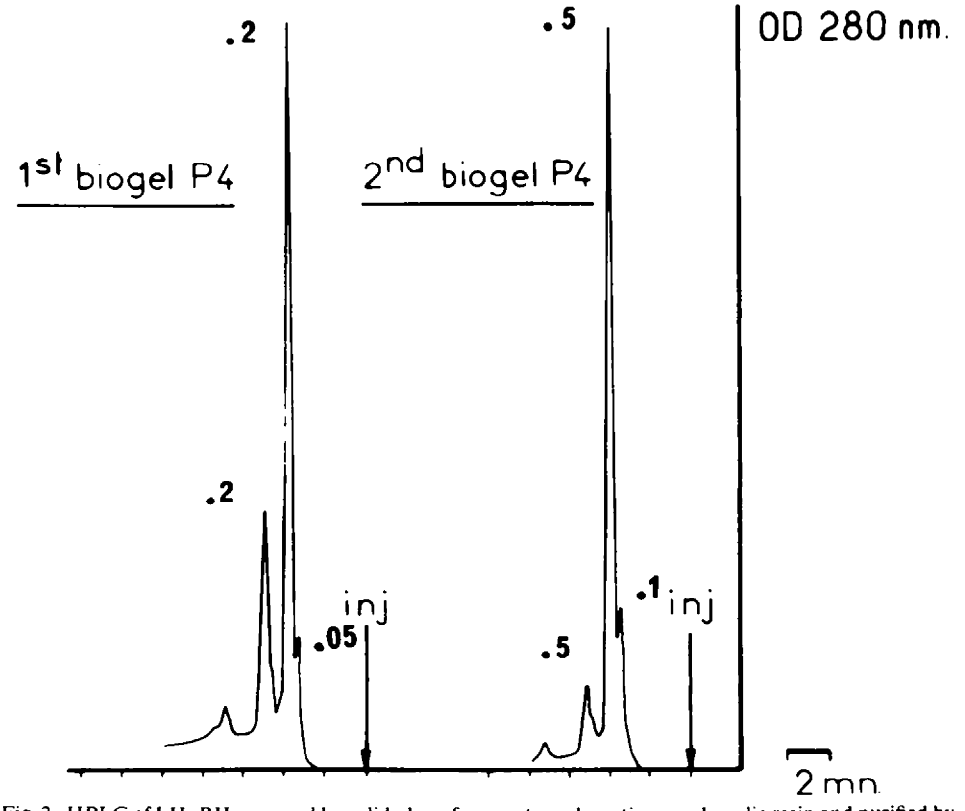


Fig. 2. HPLC of LH–RH prepared by solid phase fragment condensation on phenolic resin and purified by gel filtration on Biogel P_4.

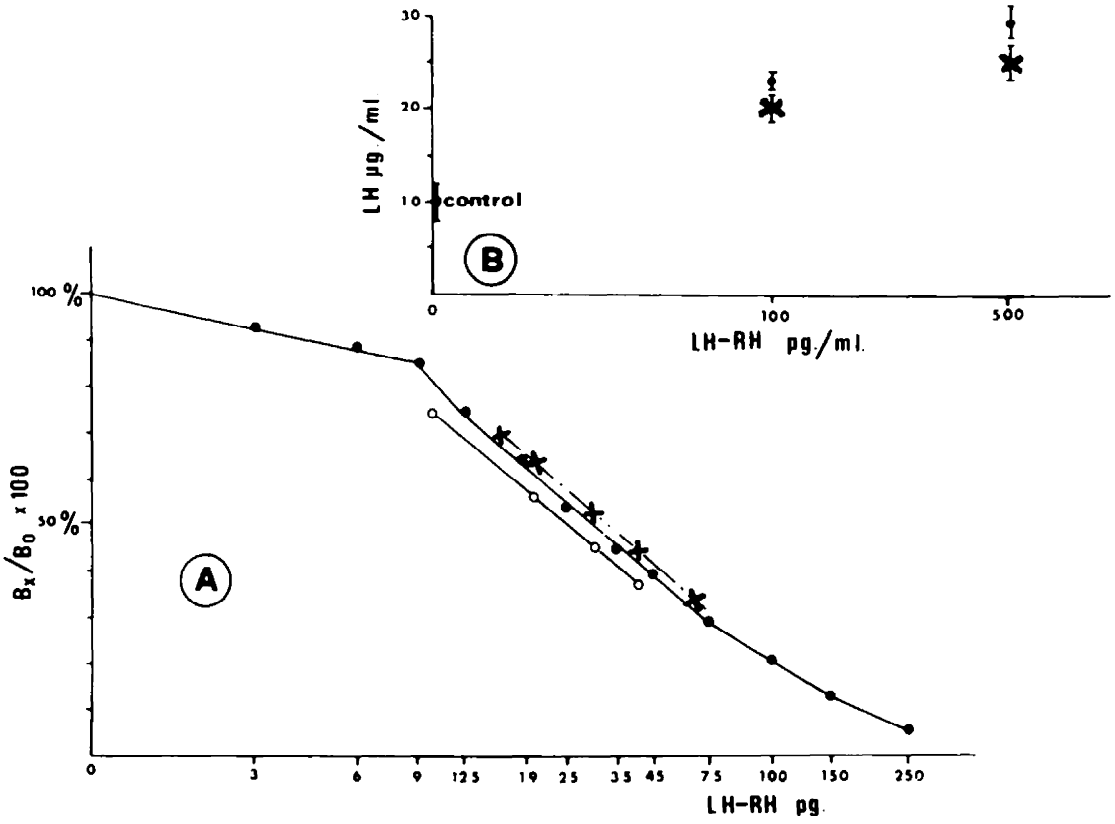


Fig. 3. A—LH–RH radioimmunoassay. ·--· standard LH–RH, x——x decapeptide synthesized on phenolic resin O——O hypothalamic acid extract. B_0: total binding of the radiolabeled hormone. B_x: residual binding of the radiolabeled hormone in the presence of the standard, the decapeptide or a hypothalamic acid extract. B—LH–RH bioassay. LH amounts (means ± SEM for 10 values) released by standard LH–RH (●) and by the decapeptide synthesized on phenolic resin (×).

11–19 and 1–10 of human calcitonin. It has been found satisfactory even with cysteine, lysine and alanine as the carboxyl terminal residue. The only failure encountered was in the attempted preparation of the tetrapeptide valyl-glycyl-alanyl-proline, presumably because under the conditions for regeneration of the amino function of alanine, the formation of diketopiperazine facilitated by the pyrole group of proline led to rupture of the phenolic linkage. The resin may be re-used indefinitely since elimination of the peptide chain by saponification is complete. "BOP" is a highly efficient reagent for fragment coupling in solid phase. Now that it is more readily available it is also used in our laboratory for stepwise peptide synthesis as in preparation of the fragments described above.

EXPERIMENTAL

All amino-acids, except glycine were of the L-configuration. Standard abbreviations for amino-acids, protecting groups and peptides are as recommended by the IUPAC-IUB commission on Biochemical Nomenclature. Other abbreviations used include: BOP, benzotriazolyl-N-oxytrisdimethylaminophosphonium hexafluorophosphate; DCCI, dicyclohexylcarbodiimide; DIEA, di-isopropylethylamine; DMAE, dimethylamino-ethanol; DMF, dimethylformamide; HOBT, 1-hydroxybenzotriazole; HF, hydrogen fluoride; TFA, trifluoroacetic acid; Tos, tosyl.

Amino-acid analysis

(1) Free peptides. Samples (50 µg) were hydrolyzed with constant boiling 6N HCl (200 µl) to which 5% of thioglycolic acid was added for protection of tryptophan. Ampoules were evacuated several times and refilled with nitrogen before sealing under vacuum and placed in an oven at 110° for 24 hrs.

(2) Peptidyl-polymer. Resin (400 µg) was hydrolyzed under similar conditions by sealing with a 1/1 v/v HCl 12 N, propionic acid mixture, at 130° for 2 hrs.[27] Analyses were performed on a Jeol model 6 HA amino-acid analyses. Optical rotations were measured in 2 ml cell with a Jobin et Yvon model R 3 micropolarimeter.

High performance liquid chromatography was performed on a Waters Associates apparatus provided with a 6000 A pump, a 6 U6K injector and a 440 U.V. detector at 280 nm coupled to a GA 12 Mettler chart recorder.

Stainless steel columns of µ Bondapak C 18 (2 × 30 cm × 4 mm Ø) were purchased prepacked from Waters Associates. Solvents were glass distilled, filtered through millipore (0.4 µ) and degassed with ultrasound just before use.

General procedure

(1) *HF treatment.* The peptide was placed in a Kel F reaction vessel and 1 ml anisole and 150 mg indole per/g were added. 10 ml/g of liquid HF was distilled in and the reaction was continued for 1 hr at 0°, after which the HF was quickly evaporated *in vacuo*. The dried residue was washed several times with peroxide free ether, then the peptide was dissolved in acetic acid 0.1 M (10–20 ml). The precipitate was removed by centrifugation and the solution was lyophilized.

(2) *Gel filtration.* Biogel P4 was packed in a Pharmacia column (2.5 × 100 cm). 5 ml fraction were collected with LKB model Ultrorak fraction collector. The eluant (0.1 M ACOH, flow rate 25 ml/h) was monitored at 254 and 280 nm with a LKB model Uvicord III spectrophotometer.

Fractions corresponding to the peaks of elution were reanalyzed with HPLC (0.25 N H-COOH + Et_3N to pH 3 70‰ CH_3CN 30% as solvent at 1.5 ml/mn, 2500 psi) (28). Fractions corresponding to the standard were collected and freeze-dried.

(3) *Amino-acids of L configuration* (Bachem, Bubendorf, Switzerland) were protected as follow: t-butoxycarbonyl for α amino functions and for the side chain functions: 0-benzyl ether for the hydroxyl of serine, 0-2,6-dichlorbenzyl for the hydroxyl of tyrosine, tosyl for the imidazole of histidine and nitro for the guanidine of arginine.

(4) *Solvents and reagents.* Dichloromethane (Merck, Darmstadt, Germany) was redistilled from phosphorus pentoxide immediately before use. Dimethylformamide was azeotropically distilled with anhydrous benzene, under vacuum (N_2) and stored over molecular sieve "4 Å" (Prolabo). Di-isopropylethylamine (Merck) and trifluoro-acetic acid (Merck) solutions in methylene chloride were freshly made up every day.

(5) *Thin layer chromatography.* Solvent A: n-ButOH, Pyr, AcOH, H_2O: 15, 10, 3, 12, v/v; Solvent B: n-ButOH, AcOH, H_2O: 4, 1, 1, v/v; Solvent C: $CHCl_3$, MeOH, 32% AcOH: 12, 9, 4, v/v; Solvent D: $CHCl_3$, MeOH, 24% NH_4OH: 12, 9, 4, v/v.

Precoated plates were purchased from Schleicher and Schüll (G 1500 Silicagel, G 1440 cellulose).

(6) *Amino-acid couplings.* Operations were made manually in a reaction vessel previously described.[26]

(a) Boc cleavage. For 1 g of the resin 15 ml of each solvent or reagent was used. Wash dichloromethane 3 × 1 mn: Prewash 30% TFA/CH_2Cl_2 1 × 1 mn; Treatment 30% TFA/CH_2Cl_2 1 × 20 mn; Wash dichloromethane 3 × 1 mn; Neutralization 12.5% DIEA/CH_2Cl_2 1 mn; Neutralization 12.5% DIEA/CH_2Cl_2 10 mn; Wash dichloromethane/acetone alternately 4 × 1 mn; Wash acetone 3 × 1 mn; Neutralization 12.5% DIEA/CH_2Cl_2 1 mn; Wash dichloromethane/acetone alternately 4 × 1 mn; Wash dichloromethane 1 mn.

(b) DCCI/HOBT coupling procedure: 1.5 fold excess of Bocamino-acid. DCCI and HOBT were shaken for an average of 4 hours in dichloromethane or DMF for the coupling of Boc-NO_2-Arg.

Preparation of the p-Hydroxyphenylpropionic resin. To 5 g of benzhydrylamine resin (substitution 4 meq NH_2/g) swelled in 20 ml DMF (3 fold excess) of the following reagents were added; 997 mg of 4-hydroxyphenylpropionic acid, 758 μl of N-methylmorpholine and finally 2.65 g of BOP. The reaction mixture was shaken overnight. The resin was filtered on sintered glass and washed alternately with DMF (20 ml), MeOH (20 ml), dichloromethane (20 ml), MeOH (20 ml) in order to remove all trace of impurities. The resin was treated overnight with 20 ml of 50% piperidine in 50% of DMF (v/v) and was washed as previously described. Kaiser's test performed on an aliquot of resin revealed that all the NH_2 groups were substituted by the 4-hydroxyphenylpropionic acid. Substitution of the first amino-acid: 4 mM/g.

Preparation of fragment 1-6.

Attachment of Gly as first amino-acid. To 2 g of the phenolic resin swelled in 20 ml CH_2Cl_2 was added 1.2 mM (1.5 excess) of Boc-Gly (210 mg) and DCCI (246 mg). The reaction vessel was shaken overnight and this procedure was repeated 3 times. In order to block the potentially free hydroxyl groups, AcOH (1 mM, 60 μl) and DCCI (1 mM, 206 mg) in dichloromethane (20 ml) were allowed to react for 4 hrs with the resin which was then washed and treated as described above. Gisin's test[33] or amino-acid analysis revealed the substitution of 0.3 mM/g of Gly.

Peptidyl-resin was divided into two aliquots, one for the synthesis of fragment 7-10, the other for fragment 1-6.

Sequence elongation 1-6 *and* 7-10 *polymer respectively.* To 1 g of glycyl-resin in 15 ml of methylene chloride, 6 mM, 1.5 excess of each amino-acid, 6 mM (123 mg) of DCCI and 6 mM (92 mg) of HOBT were added and the reaction vessel was shaken for 4 hours. The resin was then washed; the completeness coupling was estimated by the ninhydrin test[22] and the procedure repeated if necessary.

Cleavage of fragment 1-6. (1) *Saponification.* 700 mg of the 1-6 peptidyl-polymer was treated for 45 mn in DMF 5 ml + NaOH 2N 5 ml. The filtered solution was acidified to pH 4 with HCl N/10; water was added in order to precipitate the protected fragment amino-acid analysis of crude product: His 0.90 (1), Ser 0.73 (1), Glu 0.75 (1), Gly 1.19 (1), Tyr 1.33 (1).

The precipitate (≃200 mg) was applied to a column (2.5 × 45 cm) of LH 20 and eluted with DMF. Fractions of 3 ml were collected and every third fraction was checked by TLC on silicagel (solvent A). Fractions positive for Pauly's and Erlich's tests were selected and collected. DMF was evaporated under vacuum (35–40°) and the peptide precipitated with water was centrifuged and dried. Yield of the peptide based on the substitution of the first amino-acid 127 mg (48%) $(\alpha)_D^{20} = -12.35$ (c = 1, DMF), Rf Solvent A (Silicagel) = 0.50. Amino-acid analysis: His 1.04 (1), Ser 0.87 (1), Glu 0.95 (1), Gly 1.22 (1), Tyr 1.15 (1).

(2) *Transesterification.* 700 mg of 1-6 peptidyl-resin was mechanically stirred for 24 hrs in 15 ml of 50% DMAE, 50% dry DMF (v/v). Resin was filtered off and washed with a few ml of DMF (4-5 ml). Solutions were combined, water (15 ml) and a trace of imidazole were added and the mixture was allowed to stand for 24 hrs at room temperature. Solvents were evaporated under vacuum (35–40°) and the residue was applied to the column of LH 20 for the purification previously described. Yield 137 mg (52%) $(\alpha)_D^{20} = 12.35$ (c = 1, DMF) Rf Solvent A (Silicagel) = 0.50, Amino-acid analysis: His 0.90 (1), Ser 1.01 (1), Glu 1.15 (1), Gly 1.25 (1), Tyr 0.97 (1). Found: C, 60.48; H, 5.14; N, 12.70. $C_{50}H_{51}N_9O_9Cl_2$ requires C, 61.03; H, 5.19; N, 12.81%.

Condensation of fragment 1-6 *on* 7-10 *resin.* In a 5 ml reaction vessel were placed 487 mg of 7-10 polymer, 274 mg of fragment 1-6, 129 mg of BOP, 74 μl of 4-methylmorpholine and 2 ml of DMF. After 36 hrs shaking the resin was washed with DMF and acetone as previously described and dried (weight increase: 199 mg th. with 4 mM/g of substitution 182 mg). Amino-acid analysis: His 1.29 (1), Arg 0.66 (1), Ser 0.84 (1), Glu 1.02 (1), Pro 0.99 (1), Gly 2.4 (2), Leu 0.92 (1), Tyr 0.75 (1). A part of Arg is transformed into ornithine.

Aminolysis. 686 mg of the peptide-resin conjugate was stirred in 2-3 ml of dried DMF, and NH_3 (dried over KOH) was gently bubbled for 5 hours. Resin was filtered off and carefully washed with DMF (3 × 2 ml). The solution was evaporated to small volume (200 μl), water was added and the precipitate obtained was centrifuged and dried. Yield: 160 mg, 58%; Rf solvent A: 0.67.

The precipitate was treated by HF (Cf above) and the crude product purified by two gel filtrations on biogel P 4. Fractions containing exclusively LH–RH as determined by HPLC were collected and freeze dried (yield: 50 mg, 37%). Amino-acid analysis: His 0.76 (1), Trp 1.03 (1), Arg 1.19 (1), Ser 1.00 (1), Glu 0.92 (1), Gly 2.21 (2), Leu 1.04 (1), Tyr 0.81 (1), Pro 1.04 (1) $(\alpha)_D^{20} = -49$ (c = 1, AcOH 14%)

Rf Solvent A (Silicagel) = 0.54 identical to standard LH–RH; Solvent B (Silicagel) = 0.37 identical to standard LH–RH; Solvent C (Cellulose) = 0.72 identical to standard LH–RH; Solvent D (Cellulose) = 0.74 identical to standard LH–RH.

Found: C, 51.66; H, 6.50; N, 16.85. $C_{55}H_{77}N_{17}O_{13}$, $2CH_3CO_2H$, $4H_2O$ requires: C, 51.56; H, 6.62; N, 17.33%.

Preparation of LH–RH COOH. 168 mg of peptide-resin were treated in DMF 500 μl + NaOH N/10 500 μl for 30 mn. The resin filtered off was washed with DMF (2 × 1 ml) and

the solution brought to pH 4 by HCl N/10 was evaporated under vacuum. The precipitate obtained after addition of water was centrifuged and dried yield 56 mg.

Removal of the protecting groups. (1) *By HF.* 28 mg of the preceding product were treated with liquid HF and the product obtained was directly chromatographied (2x) on biogel P4 as described before for LH-RH. Yield, 10 mg 42%, $(\alpha)_D^{20} = -30$ (c = 1, AcOH 1%).

Rf Solvent A (Silicagel) = 0.45; Solvent B (Silicagel) = 0.34; Solvent C (Cellulose) = 0.74; Solvent D (Cellulose) = 0.71.

Amino-acid analysis: His 1.09 (1), Trp 1.01 (1), Arg 0.83 (1), Ser 1.15 (1), Glu 1.05 (1), Gly 2.14 (2), Leu 1.12 (1), Tyr 0.98 (1), Pro 0.82 (1).

(2) *By hydrogenolysis.* The remaining 28 mg of the protected peptide in solution in 5 ml of DMF plus 50 μl of acetic acid and 10 mg of Pd 5% on $BaSO_4$ were hydrogenated for 24 hours. The catalyst was eliminated by filtration and the crude product obtained was treated as previously described. Yield 8 mg, 37%, $(\alpha)_D^{20} = -30$ (c = 1, AcOH 1%).

Rf Solvent A (Silicagel) = 0.45; Solvent B (Silicagel) = 0.34; Solvent C (Cellulose) = 0.74; Solvent D (Cellulose) = 0.71. Amino-acid analysis: His 1.10 (1), Trp 1.03 (1), Arg 0.77 (1), Ser 1.20 (1), Glu 0.86 (1), Gly 2.09 (2), Leu 1.12 (1), Tyr 1.03 (1), Pro 0.80 (1).

Biological assays. LH-RH radioimmuno-assay was carried out with the antibody[29] according to the method of Nett.[30] Synthetic LH-RH (Roussel Uclaf, 7E/0950) was used as a standard and for radioiodination.

LH release by the hypophysis during incubation with different doses of synthetic or standard peptides was used as an LH-RH bioassay according to the method of Kordon.[31] The amounts of LH released were measured by radioimmuno-assay according to the method of Niswender[32] and expressed as μg/ml of NIAMDD rat LH-RH.

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