SYNTHESIS OF LUTEINIZING HORMONE-RELEASING HORMONE CONTAINING TRITIUM-LABELLED PYROGLUTAMIC ACID

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Summary. A method of preparing luteinizing hormone-releasing hormone (LH-RH) pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, by a combination of solid-phase and classical reactions was employed to conveniently synthesize a tritium-labelled hormone by incorporation of 4-[^{3}H]-pyroglutamic acid into position 1 of the peptide chain. The tritiated LH-RH possessed a specific radioactivity of 18.3 Ci/mmole and a maximal biological potency.

The isolation and determination of the structure of porcine (1-3) and ovine (4) LH-RH of the hypothalamus and its synthesis (5-7) made possible some key physiological and clinical studies (8). Isotopically labelled, synthetic LH-RH of sufficient specific activity for detection in a number of biological systems is necessary for other studies on its half-life, distribution in the body and metabolism. We have, therefore, developed a relatively simple method for synthesizing such a material.

In view of the high cost of [^{3}H]-labelled amino acid starting material and thus the necessarily small scale of the synthesis it was essential that losses due to poor reaction yields and excessive manipulation be kept to a minimum. A synthesis of LH-RH was devised in which the last stage of the process involves the coupling in solution of pGlu to the C-terminal nonapeptide (des-pGlu-LH-RH) giving LH-RH directly in good yield. The labelled hormone was thus prepared in a one-step operation by substituting 4-[^{3}H]-pGlu in this reaction.

[Des-pGlu]-LH-RH was prepared by a modified version (9) of the Merrifield solid-phase method (10). The intermediate protected peptide was cleaved from the resin support by ammonolysis, purified by re-precipitation, and deprotected by sodium in liquid ammonia reduction (11). The free peptide was first desalted by gel filtration on Sephadex G-15 and purified by contin-

uous gradient elution on CM-cellulose with NH_4Ac buffers. Steric and chemical homogeneity of the product was demonstrated by enzymatic digestion and t.l.c. in a number of solvent systems. Pyroglutamic acid could readily be joined to this peptide by coupling in DMF* in the presence of DCI and N-hydroxysuccinimide (12). Upon purification, the LH-RH produced was homogeneous by t.l.c. and t.l.e. and was found to have a biological activity of 116% when assayed against natural LH-RH. When optimum conditions had been established, 4-[^{3}H]-pGlu (specific activity 20.7 Ci/mmole) was attached to the nonapeptide in an analogous fashion. The reaction mixture was purified on CM-cellulose and yielded isotopically labelled LH-RH with a specific activity of 18.3 Ci/mmole and a biological activity of 121%. Higher activity of these two synthetic LH-RH preparations can be readily explained by their higher peptide content (78%) as compared with 67.5% for natural LH-RH (2).

EXPERIMENTAL SECTION

With the exception of glycine, amino acids were of the L variety. Amino acid derivatives were checked for purity by t.l.c. and were purchased, unless otherwise stated, from Bachem, Inc., Marina del Rey, Calif. Amino acids with functional side chains were protected as follows: histidine, N^{im}-Bzl; serine and tyrosine, O-Bzl; arginine, N^G-Tos. Amino acid analyses were carried out on samples which were hydrolysed (18 h) in 6 M HCl containing 4% thioglycolic acid in sealed, evacuated ampoules at 110°. The following t.l.c. systems were used: R_f^1, n-BuOH-AcOH-H_2O (4:1:5); R_f^2, n-BuOH-Pyr-AcOH-H_2O (15:10:3:12); R_f^3, n-BuOH-AcOH-H_2O-EtOAc (1:1:1:1).

N^{im}-Bzl-His-Trp-O-Bzl-Ser-O-Bzl-Tyr-Gly-Leu-N^G-Tos-Arg-Pro-Gly-NH_2. A 2% cross-linked t-BOC-glycine resin (3.0 g, 1.02 mmoles) (Schwarz Bio Research, Inc.) was de-protected and neutralized. Amino acids (3.06 mmoles) were coupled in the presence of equivalent amounts of DCI. t-BOC-N^G-Tos-Arg and t-BOC-N^{im}-Bzl-His were coupled in DMF/CH_2Cl_2 (1:3), the remaining amino acids in CH_2Cl_2. Hydrogen chloride (1 M) in glacial acetic acid was used for removal of BOC groups and 1% EtSH was included in this

*Abbreviations used: DMF, dimethylformamide; DCI, dicyclohexylcarbodiimide; EtSH, mercaptoeth

reagent for de-protecting the Trp and His residues. Triethylamine (10%) in $CHCl_3$ was used for neutralization.

Protected nonapeptide resin (4.27 g) was suspended in dry MeOH (130 ml) and the mixture saturated with NH_3 at -2°. After stirring at room temperature (40 h) the NH_3 was partially removed at the water pump, and after filtration, the resin cake was extracted with DMF (3 x 20 ml). The combined filtrates were evaporated to dryness in vacuo and the cream-colored, amorphous solid re-precipitated from hot MeOH/H_2O. Yield, 1.15 g, 91% based on weight gain of resin. R_f^1 (silica), 0.50, single spot to Ehrlich, Pauly and I_2 reagents. Amino acid analysis of acid hydrolysate: Trp, 0.84; NH_3, 1.15; Arg, 1.00; Ser, 0.81; Pro, 0.88; Gly, 2.10; Leu, 1.14; Tyr, 0.98.

H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. The protected peptide (200 mg) was reduced by treatment with Na in refluxing, dry NH_3 under conditions described previously (9). The residue which was obtained by removal of NH_3 in vacuo was de-salted on Sephadex G-15 by elution in 50% AcOH and applied to a column (0.9 x 91 cm) of CM-cellulose equilibrated with 0.002 M NH_4Ac buffer. A gradient was started immediately by introducing 0.1 M NH_4Ac buffer (pH 7.0) through a 250 ml mixing flask containing starting buffer. The nonapeptide was eluted between 910 and 1060 ml and after lyophilization yielded 94 mg (60%) of pure material. R_f^1 (cellulose) 0.46; R_f^2 (cellulose) 0.71; R_f^3 (silica) 0.49. $[\alpha]_D^{26°}$ - 35.5° (c 1.02, 1% AcOH) (lit. (13). $[\alpha]_D^{28°}$ -30.6° (c 1.0, 1 M AcOH)). Amino acid analysis of acid hydrolysate: Trp, 1.05; His, 0.89; NH_3, 0.97; Arg, 0.87; Ser, 0.75; Pro, 1.03; Gly, 2.10; Leu, 1.07; Tyr, 1.02. Amino acid ratios in a leucine aminopeptidase digest: Trp, 0.94; His, 0.97; Arg, not found; Ser, 1.00; Pro, not found; Gly, 1.34; Leu, 1.03; Tyr, 0.93.

LH-RH. Nonapeptide (20.0 mg, 18.7 μmoles) and pGlu (4.8 mg, 37.6 μmoles) were dissolved in re-distilled DMF (600 μl). N-Hydroxysuccinimide (4.3 mg, 37.6 μmoles) and DCI (8.4 mg, 41.4 μmoles) were added consecutively to this solution. The mixture was gently agitated (18 h)

and then dried in vacuo. The gummy residue was dissolved in water (10 ml), applied on the CM-cellulose column and purified under conditions identical to those employed for the chromatography of the nonapeptide. Lyophilized fractions from elution volumes 530 to 570 ml gave pure LH-RH (11.4 mg, 50% based on nonapeptide). R_f's of this material in solvent systems 1,2, and 3 were identical to an authentic sample of the hormone. $[\alpha]_D^{25^\circ}$ - 50.0° (c 1.0, 1% AcOH) (lit. (6)$[\alpha]_D$- 50° (c 1.0, 1% AcOH)). Amino acid analysis of acid hydrolysate : Trp, 1.00; His, 0.94; NH_3, 1.01; Arg, 1.08; Ser, 0.83; Glu, 1.00; Pro, 0.96; Gly, 2.10; Leu, 0.92; Tyr, 0.87. (Peptide content 78%).

1-(4-[^{3}H]-pGlu)-LH-RH. Nonapeptide (13.3 mg, 12.5 µmoles) and 4-[^{3}H]-pGlu (3.27 mg, 25.0 µmoles, 510 mCi) (Purchased from New England Nuclear, Boston, Mass.) were dissolved in DMF (500 µl). N-Hydroxysuccinimide (2.9 mg,25.0 µmoles) followed by DCI (5.6 mg, 27.6 µmoles) were added to the solution which was agitated (18 h) and dried. Purification on CM-cellulose as described above yielded two fractions: (1) elution volume 550 to 570 ml gave 5.0 mg of labelled LH-RH which was homogeneous in solvent systems 1 and 3; (2) elution volume 571 to 600 ml yielded a mixture (6.7 mg) containing LH-RH contaminated with a faster moving (system 1) component. The mixture upon re-chromatography on the same column afforded a further 0.8 mg of labelled hormone. Total yield = 5.8 mg, 40%.

Appropriate aliquots of an aqueous solution of the tritiated LH-RH in 10 ml of Dimlume scintillation solution (Packard Instrument Co., Downers Grove, Ill.) were counted in a liquid scintillation spectrometer to less than 2% probable error. The samples were corrected to 100% efficiency by the addition of an internal standard and the results expressed in dpm. The specific activity of the tritiated LH-RH was calculated as 12.2 mCi/mg or 18.3 Ci/mmole when based on a peptide content of 78%. Thin layer radiochromatograms of tritiated LH-RH which had been stored in 10% ethanol/0.1 M acetic acid for three weeks at -30° C did not show any decomposition products. Loss of tritium by exchange with the solvent was less than 0.1% as determined by distillation of water from the mixture.

TABLE I

LH-RH ACTIVITY OF SYNTHETIC LH-RH AND

1-(4-[^{3}H]-PYROGLUTAMIC ACID)LH-RH

Sample	Dose ng/rat	Mean serum LH ng/ml ± S.E.	Relative potency* with 95% limits
Saline	---	8.8 ± 0.4	
Natural LH-RH	0.5	33.0 ± 2.0	
	2.5	97.0 ± 0.0	
Synthetic LH-RH	0.5	44.3 ± 12.3	
	2.5	96.6 ± 15.9	116 (43 - 358) %
Saline	---	10.8 ± 0.8	
Natural LH-RH	0.5	15.6 ± 0.8	
	2.5	36.8 ± 1.6	
[^{3}H]-LH-RH	0.5	13.6 ± 1.3	
	2.5	45.0 ± 4.8	121 (76 - 198) %

* Accepting natural LH-RH as 100%

Biological Assays. LH-RH activity of synthetic material was measured at two dose levels in vivo by stimulation of LH release in ovariectomized rats (3 per group) pre-treated with estrogen and progesterone (2,14) followed by radioimmunoassay (15) for serum LH. Serum LH levels after injection of samples were compared with those obtained after administration of saline and two doses of natural LH-RH. The results obtained are shown in Table I.

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