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Luteinizing Hormone Releasing Hormone. Solid-Phase Synthesis of a 5-Phenylalanine Analog Possessing High Biological Activity

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Received July 14, 1972

Since the initial isolation and characterization[1-3] of luteinizing hormone relasing hormone (LH-RH) of the porcine hypothalamus, the decapeptide pGlu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 has been synthesized by both classical[4-6] and solid-phase[5,7-10] methods. In order to establish structure-activity relationships for this peptide, with the ultimate goal of creating an inhibitor of LH release, it is essential that analogs of LH-RH be synthesized and their biological properties investigated.

Preliminary inactivation studies[11] on LH-RH indicated that the hydroxyl group of the tyrosine residue was not essential for LH release. We have, therefore, made a peptide in which the hydroxyl group on the aromatic nucleus is deleted.

Synthesis. Manning and coworkers[12] have recently developed a synthesis of LH-RH in which a peptide intermediate made by solid-phase reactions is deprotected by the sodium in liquid ammonia reduction method devised by Sifferd and du Vigneaud.[13] Readily purifiable products are obtained and this route has been employed by us in the preparation of [5-Phe]-LH-RH.

The synthetic procedure is outlined in Scheme I. The protected peptide **1** was prepared by the Merrifield method[14] with modifications.[15] Functional groups were protected as follows: histidine, N^{im}-benzyl; serine, *O*-benzyl; arginine, N^G-tosyl. The N^G-nitro group has commonly been used for arginine protection in previously published solid-phase syntheses of LH-RH peptides where it is finally removed by cleavage in liquid HF. Recent reports[16,17] indicate that this reaction is accompanied by the formation of considerable amounts of hard-to-separate ornithine-containing contaminants resulting from deamidation of the arginine side chain.

Scheme I. Outline of the Solid-Phase Synthesis of the Protected Decapeptide **1** and Its Reduction to [5-Phe]-LH-RH

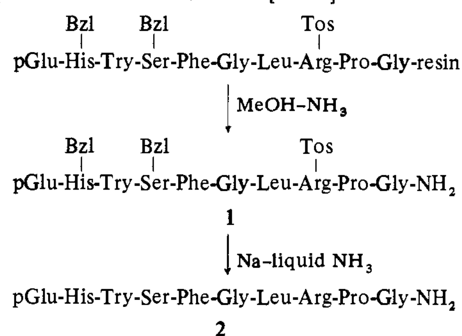

Table I. LH-RH Activity of Natural and [5-Phe]-LH-RH in Ovariectomized, Estrogen-Progesterone Treated Rats

Sample	Dose, ng/rat	Mean serum LH,[a] ng/ml ± S.E.
Saline		2.3 ± 0.9 (3)[b]
Natural LH-RH	0.5	13.6 ± 0.4 (3)
	2.5	70.0 ± 9.4 (3)
[5-Phe]-LH-RH	0.8	22.3 ± 1.1 (3)
	4.0	62.9 ± 8.7 (3)

[a]As NIH-LH-S-17. [b]Number of rats per group.

Peptide **1** was cleaved from the resin by ammonolysis and purified by recrystallization from MeOH. The three protecting groups were removed simultaneously by treatment with small amounts of sodium in refluxing NH_3. The crude peptide **2** was desalted by gel filtration on Sephadex G-15 in 50% AcOH and purified by ion-exchange chromatography on CM-cellulose using continuous gradient elution with ammonium acetate buffers.

Biological Results. LH-RH activities (Table I) were determined *in vivo* by stimulation of LH release at two dose levels in ovariectomized rats pretreated with estrogen and progesterone[2,3,18] followed by radioimmunoassay for LH.[19] Serum LH levels after injection of samples are compared with those obtained after administration of saline and two doses of natural LH-RH. Using a four-point factorial assay,[20] the LH-RH activity of the [5-Phe]-peptide was calculated to be 64% of the natural hormone with 95% confidence limits of 38-108%. In a separate, but similar assay, LH-RH prepared by a similar synthetic procedure[12] possessed 120% of the activity of the natural material with 95% confidence limits of 57-305%.

The surprisingly high activity of [5-Phe]-LH-RH demonstrates conclusively that the hydroxyl group of the tyrosine residue of LH-RH is not essential for either binding to the receptor site or in the mechanism governing the release of LH.†

Experimental Section

Melting points are uncorrected. Amino acid derivatives used as starting materials were the pure L isomers and were purchased from Bachem, Inc., Marina del Rey, Calif. Microchemical analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Amino acid analyses were carried out with a Beckman Amino Acid Analyser Model 120C on samples which were hydrolyzed (18 hr) in 6 *M* HCl containing 4% thioglycolic acid[22] in sealed, evacuated ampoules at 110°. The following tlc systems were used: R_f^1,

†The FSH releasing activity of the [5-Phe]-peptide when assayed *in vitro* as described by Schally, *et al.*, was found to be 98% (50-265%) that of the natural hormone.

n-BuOH:AcOH:H_2O (4:1:5 upper phase); $R_f{}^2$, *n*-BuOH:AcOH:H_2O:EtOAc (1:1:1:1); $R_f{}^3$, EtOH:H_2O (7:3).

pGlu-N^{im}-Bzl-His-Try-*O*-Bzl-Ser-Phe-Gly-Leu-N^G-Tos-Arg-Pro-Gly-NH_2 (1). *tert*-Butyloxycarbonylglycine resin (2.26 g, 0.63 mmol of Gly) (purchased from Schwarz Bio Research, Inc.) was added to the reaction vessel and, after deprotection and neutralization, each new amino acid (*tert*-Boc derivatives with the exception of pGlu) was coupled on successive days by a nine-cycle procedure described previously.[15] Amino acids (1.89 mmol) were coupled in the presence of equivalent amounts of DCI. *tert*-Boc-N^G-Tos-Arg, *tert*-Boc-N^{im}-Bzl-His, and pGlu were coupled in DMF, the remaining amino acids in CH_2Cl_2. Hydrogen chloride (1 *M*) in glacial AcOH was used for the removal of *tert*-Boc groups and, following the incorporation of *tert*-Boc-Try, 1% EtSH was included in this reagent.

After completion of the synthesis, drying *in vacuo* gave 2.98 g of protected decapeptide resin (86% incorporation based on initial butyloxycarbonylglycine content). Peptide resin (1.40 g) was suspended in dry MeOH (90 ml) which was saturated at $-2°$ with dry NH_3. The mixture was stirred at room temperature in a stoppered flask (40 hr). NH_3 was partially removed *in vacuo* and, after filtration and extraction of the resin with DMF (three 15-ml portions), the combined filtrates were evaporated to dryness *in vacuo*.

Recrystallization of the residue (290 mg) from MeOH gave a white powder (176 mg, 40% based on initial Gly attached to resin): mp 162–164°; $[\alpha]^{26.5}D$ −25.3° (*c* 1.10, DMF); $R_f{}^1$ (silica) 0.46; single spot to Ehrlich, Pauly, and I_2-starch reagents. Amino acid analysis of acid hydrolysate: Try, 0.9; NH_3, 1.2; Arg, 1.0; Ser, 0.7; Glu, 1.0; Pro, 1.1; Gly, 2.0; Leu, 0.9; Phe, 0.9. *Anal.* ($C_{76}H_{93}N_{17}O_{14}S \cdot 3H_2O$) C, H, N.

pGlu-His-Try-Ser-Phe-Gly-Leu-Arg-Pro-Gly-NH_2 (2). The protected peptide **1** (145 mg) was dissolved in 250 ml of anhydrous, liquid ammonia. Sodium was added to the gently boiling, stirred solution from a small-bore glass tube until a faint, persistent blue color was observed. This was discharged immediately with 2 drops of dry AcOH and the NH_3 removed under anhydrous conditions. The residue was applied to a column (1.7 × 110 cm) of Sephadex G-15 and eluted in 50% AcOH. The peptide emerging close to the void volume of the column was recovered by lyophilization. This material was dissolved in water (6 ml) and loaded on a column (0.9 × 91 cm) of CM-cellulose equilibrated with 0.002 *M* NH_4Ac buffer (pH 4.6). After 40 ml had been collected, a pH and concentration gradient was begun by introducing 0.1 *M* NH_4Ac buffer (pH 7.0) through a 250-ml mixing flask containing starting buffer. [5-Phe]-LH-RH (**2**) was located between elution volumes of 575 and 650 ml by measurement of the optical density at 280 nm. The corresponding fractions were pooled and lyophilized. Relyophilization from 0.1 *M* AcOH to constant weight gave peptide **2** (41 mg, 35%): $[\alpha]^{26.5}D$ −66.0° (*c* 1.16, 10% AcOH); single spot to Ehrlich, Pauly, and I_2-starch reagents; $R_f{}^1$ (cellulose) 0.70, $R_f{}^2$ (silica) 0.56, $R_f{}^3$ (silica) 0.34; single component moving in direction of cathode after TLE in pyridine acetate buffers at pH 4.5 and 6.4. Amino acid analysis: Try, 1.2; His, 1.0; NH_3, 1.2; Arg, 0.9; Ser, 0.7; Glu, 1.0; Pro, 1.0; Gly, 2.2; Leu, 1.0; Phe, 0.8. *Anal.* ($C_{55}H_{75}N_{17}O_{12} \cdot 6CH_3COOH \cdot 3H_2O$) C, H, N.

Acknowledgment. This work was supported by grants from the Veterans Administration, U. S. Public Health Service Grant AM-07467, NIH Contract No. NICHD 72 2741, and the Population Council, N. Y. We would particularly like to thank Mr. Weldon H. Carter for bioassays and the statistical evaluation of results.

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Novel Analgetics and Molecular Rearrangements in the Morphine–Thebaine Group. 29.[1] Aryl and Arylalkyl Tertiary Alcohols in the 6,14-*endo*-Ethenotetrahydrothebaine Series

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Received May 3, 1972

In the homologous series of C-19 tertiary alcohols 1 derived from the thebaine–methyl vinyl ketone adduct, analgetic activity is maximal when *n* is 2 or 3 and thereafter becomes smaller.[2] The effect of increasing chain length is more pronounced in the analogous series 2 (also C-19) in which a phenyl group is placed at the end of the alkyl chain (Table II).[3] Related series of C-7 tertiary alcohols **3** and **4** have now been prepared in which the hydrocarbon side chain is directly attached to C-7.

6,14-*endo*-Etheno-7-oxotetrahydrothebaine (**5**)[1] reacted with methylmagnesium iodide to give a mixture of epimeric alcohols from which the 7α-methyl epimer **3** (*n* = 0) was isolated by repeated crystallization. The specificity of the reaction increased with the size of the Grignard reagent (Table I); 7α-benzyl-6,14-*endo*-etheno-7β-hydroxytetrahydrothebaine (**4**, *n* = 1) was obtained in best yield (64%). Assignment of structure to the isolated products was made from the position of the C-5β proton signal in the nmr spectrum. This appeared at δ 4.5[4] and is attributed to 1,3 deshielding by the 7β-hydroxyl group.[5] Attack of the Grignard reagents from the α face of ring C is preferred on steric grounds; approach from the β face is hindered by the C-5 and C-15 β-hydrogen atoms.

Structure–Activity Relationships. The analgetic potencies of the new tertiary alcohols in the rat tail pressure test,[6] when administered intraperitoneally, are shown in Table II. The α-alkyl alcohols **3** (*n* = 0, 2) are somewhat less potent than morphine; these levels are very similar to those of their isomers **6** and **7** in which the hydroxyl group is at C-19 instead of C-7.[2] In the homologous series **4** there are tenfold increases in potency between phenyl