Analogs of Luteinizing Hormone-Releasing Hormone Containing Derivatives of Phenylalanine in Place of Tyrosine

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The protected decapeptides pyroglutamyl-N^{im}-benzylhistidyltryptophyl-*O*-benzylseryl-*O*-methyltyrosylglycylleucyl-N^{G}-tosylarginylprolylglycine amide and pyroglutamylhistidyltryptophyl-*O*-benzylseryl-*p*-nitrophenylalanylglycylleucyl-N^{G}-tosylarginylprolylglycine amide were synthesized by solid-phase methods. They were converted to [5-(*p*-methoxyphenylalanine)]-LH-RH and [5-(*p*-nitrophenylalanine)]-LH-RH by reaction with sodium in liquid ammonia and anhydrous hydrogen fluoride, respectively. Treatment of the *p*-nitrophenylalanine-protected peptide with sodium in liquid ammonia resulted in both the reduction of the nitro group to an amino group and the removal of the protecting groups to give [5-(*p*-aminophenylalanine)]-LH-RH in good yield. These analogs were assayed *in vivo* against pure natural LH-RH and found to possess the following LH-releasing activities: [5-(*p*-aminophenylalanine)]-LH-RH, 37%; [5-(*p*-methoxyphenylalanine)]-LH-RH, 24%; [5-(*p*-nitrophenylalanine)]-LH-RH, 5%.

Inactivation studies[1] on LH-RH (pGlu-His-Trp-Ser-Gly-Leu-Arg-Pro-Gly-NH_2) suggested that the hydroxyl group of the tyrosine residue contributed little toward the biological activity of the molecule. This was later confirmed[2] when the 5-phenylalanine analogs was synthesized and found to have 64% of the LH-releasing activity of the natural hormone, in spite of the missing hydroxyl group. In one proposed[3] conformation of the LH-RH molecule the aromatic nuclei of the tryptophan and tyrosine residues lie parallel to each other in such a way that π-π electron overlap might be at a maximum. Substituents in either of these rings could influence this interaction and thus the preferred conformation of the whole molecule and its interaction with pituitary receptor sites. In addition, dipole moments created by substituents, their size, and hydrophobicity might be expected to influence biological activity. We have, therefore, synthesized three peptides containing new groups in the para position of the benzene ring in position five and have examined their biological activity.

Synthesis. An outline of the routes used in the synthesis of the peptides is shown in Figure 1. The *O*-methyltyrosine protected peptide I was prepared by an adaptation of the Merrifield method[4] which has been described previously,[2,5,6] amino acids with functional side chains being protected as follows: histidine, N^{im}-benzyl; serine, *O*-benzyl; tyrosine, *O*-Me; arginine, N^{G}-tosyl. Although it has been reported[7,8] that some racemization takes place during couplings with Boc-His(Bzl), the presence of D-histidine has not been detected by us in peptides prepared using this derivative. Thus, for instance, enzyme digests of the LH-RH C-terminal nonapeptide prepared[5] in this manner exhibit theoretically correct ratios for histidine. Presumably if racemization does occur traces of peptide containing D-His are removed during the purification of protected and free peptides. Ammonolysis of the peptide-resin released the protected peptide which was purified by reprecipitation and subjected to reaction[9] with sodium in liquid NH_3, whereupon protecting groups other than the *O*-Me group were removed. The crude peptide III was desalted on a column of Sephadex G-15 in 50% AcOH and purified by continuous gradient elution on CM-cellulose using NH_4Ac buffers.

The *p*-nitrophenylalanine protected peptide II was prepared in a similar fashion. However, tosyl group protection of the imidazole nitrogen of histidine enabled a peptide to be synthesized in which all protecting groups were removable with either Na in liquid NH_3 or with anhydrous, liquid HF. We have found in general that the synthesis of LH-RH analogs is facilitated by the use of the two deprotection methods interchangeably for if one fails to yield a readily purifiable product the alternate process is available in reserve. *p*-Nitrophenylalanine was introduced into the peptide in the normal manner as its Boc derivative and Boc groups were removed after the addition of each amino acid by treatment with 25% TFA in CH_2Cl_2. Peptide II was cleaved from the resin by ammonolysis and purified by reprecipitation.

Under conditions of ammonolysis the tosyl group on the imidazole ring of histidine is known† to be removed. The strongly positive reaction of the protected peptide to Pauly's reagent and its elemental analysis confirmed that the tosyl group was no longer present. Treatment of the compound with HF in the presence of anisole at 0° gave the peptide IV which was purified by chromatography on CM-cellulose.

[5-(*p*-Aminophenylalanine)]-LH-RH (V) was prepared in a one-step process by Na in liquid NH_3 reduction of protected peptide II. Removal of the protecting groups was accompanied by the simultaneous reduction of the NO_2 group and the free peptide was purified in an analogous manner to peptide III.

The presence of *p*-nitro- and *p*-aminophenylalanines in peptides IV and V was confirmed by the detection of the two amino acids on the amino acid analyzer after determining their retention times. Acid hydrolysates of peptides containing *p*-nitrophenylalanine were invariably contaminated with traces of *p*-aminophenylalanine, presumably originating from oxidation-reduction reactions occurring during the hydrolysis.

Biological Results. LH-RH activities (Table I) were determined *in vivo* by the stimulation of LH release at two dose levels in ovariectomized rats pretreated with estrogen and progesterone[10,11] followed by radioimmunoassay[12] for LH. Serum LH levels were compared with those obtained after administration of saline and two doses of natural LH-RH.

The *p*-aminophenylalanine analog, containing a group similar in size and electronic properties to a hydroxyl group, as expected, possessed the greatest LH-releasing activity (37%) of the three compounds. It is interesting to note that this analog, in addition to being highly active, also contains a chemically reactive group through which this molecule could be coupled to larger carrier proteins. Such a complex could be used for induction of antibody formation of LH-

†J. M. Stewart, personal communication.

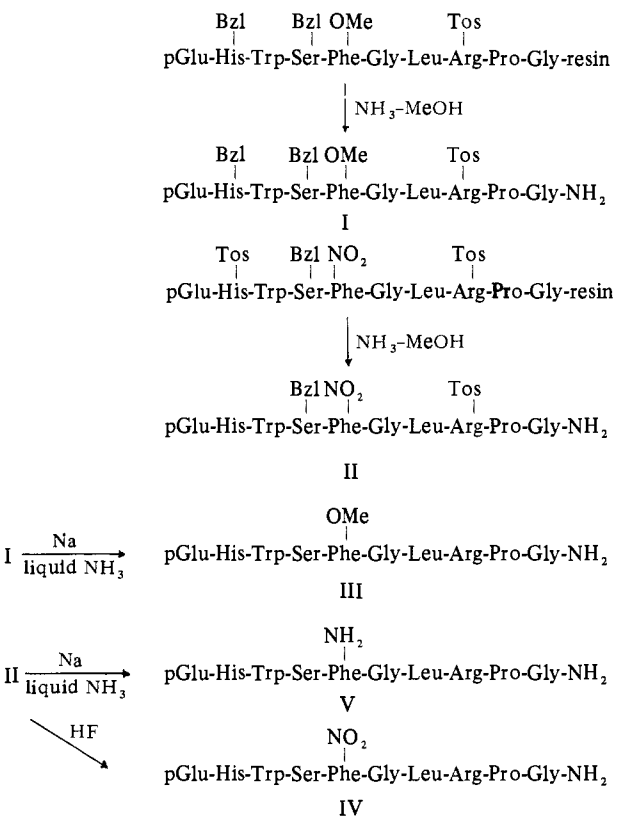

Figure 1. Outline of the solid-phase synthesis of the protected peptides I and II and their conversion to the LH-RH analogs III, IV, and V.

RH. The *p*-methoxyphenylalanine peptide was also quite active (24%); however, the presence of the bulky methyl group does cause a fall in activity. The sharpest drop in activity was exhibited by the *p*-nitrophenylalanine analog (5%). As well as being larger than the other groups, the NO_2 group is strongly electron withdrawing and is also capable of undergoing strong hydrogen bonding with suitable neighboring groups. All these properties could effect binding with receptor sites and/or mechanisms governing LH release.

Experimental Section

Amino acid derivatives used as starting materials were, with the exception of glycine, the pure L isomers and were purchased from Bachem, Inc., Marina del Rey, Calif. Microchemical analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., on samples which were dried *in vacuo* over P_2O_5 at 80°. Amino acid analyses were carried out on samples hydrolyzed (18 hr) in 6 *M* HCl containing 4% thioglycolic acid[13] to inhibit the oxidation of tryptophan, using a Beckman Model 120 C analyzer equipped for the standard 4-hr run. All operations on the resin took place under nitrogen in a Beckman Model 990 automatic peptide synthesizer. The following tlc solvent systems were used: R_f^1, *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f^2, *n*-BuOH–AcOH–Pyr–H_2O (15:3:10:12); R_f^3, *n*-BuOH–AcOH–EtOAc–H_2O (1:1:1:1); R_f^4, EtOH–H_2O (7:3). Sample sizes of *ca.* 50 μg were spotted and solvent fronts allowed to travel *ca.* 15 cm.

***tert*-Butyloxycarbonyl-*p*-nitrophenylalanine.** For incorporation into the peptide chain *p*-nitrophenylalanine was converted to its Boc derivative by the method of Schwyzer, *et al.*[14] The derivative was obtained in 66% yield after recrystallization from ethyl acetate–hexane: mp 106.5°; $[\alpha]^{27}D$ +7.91° (*c* 1.05, MeOH) [lit.[15] mp 107.1°; $[\alpha]^{25}D$ +7.94° (*c* 1.55, MeOH)]. *Anal.* ($C_{14}H_{18}N_2O_6$) C, H, N.

To aid in the identification of synthetic peptides containing *p*-nitrophenylalanine retention times were determined for the amino acid on the ion-exchange columns of the analyzer. On the short column a sharp peak was observed at 2.4 min before Trp. On the long column a broad peak was eluted at 37 min after Phe.

Table I. LH-RH Activity of the Three LH-RH Analogs Compared with That of the Natural Hormone in Ovariectomized, Estrogen–Progesterone Treated Rats

Sample	Dose, ng/rat	Mean LH,[a] ng/ml + S.E.	Potency % with 95% confidence limits
Saline		9.1 ± 1.2	
Natural LH-RH	0.5	21.8 ± 2.7	
	2.5	74.1 ± 6.1	
[5-(*p*-MeO-Phe)]-LH-RH	1	5.5 ± 1.2	24 (17–32)
	5	47.1 ± 2.9	
Saline		7.2 ± 1.4	
Natural LH-RH	0.5	18.4 ± 1.5	
	2.5	52.4 ± 6.5	5 (3–9)
[5-(*p*-NO_2-Phe)]-LH-RH	20	31.9 ± 3.1	
	100	67.0 ± 4.0	
Saline		8.8 ± 0.4	
Natural LH-RH	0.5	33.0 ± 2.0	
	2.5	97.0 ± 0.0	
[5-(*p*-NH_2-Phe)]-LH-RH	2	41.3 ± 8.5	37 (27–53)
	10	125.0 ± 4.2	

[a] As NIH-LH-S-17.

***p*-Aminophenylalanine.** A sample of this amino acid for standardization purposes was prepared by the reduction of *p*-nitrophenylalanine with tin in HCl.[16] It was eluted at 4.8 min before Trp on the short column.

pGlu-His(Bzl)-Trp-Ser(Bzl)-Tyr(Me)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (I). Boc-protected amino acids were coupled (two treatments of 1.50 mmol each) successively in the presence of DCI (1.50 mmol) to a 2% cross-linked, polystyrene–divinylbenzene, glycine resin (1.80 g, 0.50 mmol of Gly) (Schwarz Bio-research, Inc.) by a procedure which has been described before.[2,5,6] Boc-protecting groups were removed at each stage by treatment with 1.0 *M* HCl in glacial acetic acid, 1% mercaptoethanol being included in this reagent after the incorporation of Trp. Boc-Arg(Tos), Boc-His(Bzl), and pGlu were coupled in a 1:3 mixture of DMF–CH_2Cl_2 for reasons of solubility, the remaining amino acids in CH_2Cl_2.

The dried peptide-resin weighed 2.52 g (100% incorporation based on initial glycine content of resin). This material (1.50 g) was suspended in dry MeOH (100 ml) which was saturated with redistilled anhydrous NH_3 at 0°. After stirring at room temperature (18 hr) in a tightly stoppered flask, the NH_3 was partially removed at the water pump and the mixture filtered. Extraction of the resin with DMF (three 15-ml portions), combination of the filtrates, and evaporation to dryness *in vacuo* resulted in a viscous, semisolid residue. This was dissolved in MeOH (10 ml) and ether (100 ml) was added to produce a copious precipitate which, upon drying, gave peptide I as a white powder (408 mg). This was purified by reprecipitation from refluxing MeOH (175 mg, 45% based on initial Gly attached to resin): single spot to Ehrlich reagent and I_2 vapor; R_f^1 (silica), 0.41. Amino acid analysis of acid hydrolysate gave Trp, 0.96; NH_3, 1.09; Arg, 0.95; Ser, 0.86; Glu, 1.04; Pro, 0.91; Gly, 2.20; Leu, 1.08; Tyr, 0.90. *Anal.* ($C_{77}H_{95}N_{17}O_{15}S \cdot 4H_2O$) C, H, N.

pGlu-His-Trp-Ser-Tyr(Me)-Gly-Leu-Arg-Pro-Gly-NH_2 (III). The protected peptide I (100 mg) was dissolved in 200 ml of anhydrous liquid NH_3 which had been freshly distilled from Na. 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 1 drop of dry AcOH and the NH_3 was allowed to evaporate. The residue was applied to a column (1.7 × 110 cm) of Sephadex G-15 and eluted with 50% AcOH. Peptide emerging close to the void volume was recovered by lyophilization, dissolved in distilled water (6 ml), and loaded onto a column (0.9 × 91 cm) of CM-cellulose equilibrated with 0.002 *M* NH_4Ac buffer (pH 4.6). After 30 ml had been collected, a pH and concentration gradient was started by introducing 0.1 *M* NH_4Ac buffer (pH 7.0) through a 250-ml mixing flask containing starting buffer. The required peptide III was located as a discrete peak between elution volumes of 550 and 650 ml by measurement of optical density at 280 nm. The corresponding fractions were pooled and lyophilized to constant weight from water to yield 28 mg (35%) of white powder: $[\alpha]^{25}D$ −42.3° (*c* 1.03, 0.1 *M* AcOH); single spot to Ehrlich, Pauly, and Cl-tolidine reagents; R_f^1 (cellulose), 0.54; R_f^3 (silica), 0.60; R_f^4 (silica), 0.30; single component moving in the direction of the cathode after tlc at pH 4.6 and 6.4 in pyridine acetate buffers. Amino acid analysis gave Trp, 1.00; His, 0.96; NH_3,

1.04; Arg, 1.00; Ser, 0.80; Glu, 1.01; Pro, 0.95; Gly, 2.08; Leu, 1.00; Tyr, 0.85. *Anal.* ($C_{56}H_{77}N_{17}O_{13}\cdot 3CH_3COOH\cdot 4H_2O$) C, H, N.

pGlu-His-Trp-Ser(Bzl)-Phe(4-NO_2)-Gly-Leu-Arg(Tos)-Pro-Gly-NH_2 (II). The protected peptide was synthesized beginning with Boc-glycine resin (2.94 g, 1.0 mmol of Gly) in an analogous fashion to peptide I. However, tosyl group protection was used for His and Boc groups were removed by treatments (5 and 25 min) with 25% TFA in CH_2Cl_2. Acetic acid washes before and after deprotection were replaced by washes with CH_2Cl_2.

The dried peptide-resin weighed 4.55 g (118% incorporation) and 2.0 g of this material were ammonolyzed and extracted as described. The crude powder (700 mg) was reprecipitated from refluxing MeOH–EtOAc (3:1) to yield 285 mg (40%) of protected peptide II: single spot to Ehrlich and Pauly reagents and I_2 vapor; R_f[1] (silica), 0.41. Amino acid analysis gave Phe(4-NO_2), 0.93; Trp, 0.87; His, 0.99; NH_3, 1.10; Arg, 1.00; Ser, 0.83; Glu, 1.10; Pro, 0.97; Gly, 2.10; Leu, 1.00. *Anal.* ($C_{69}H_{86}O_{16}S\cdot 3H_2O$) C, H, N, S.

pGlu-His-Trp-Ser-Phe(4-NO_2)-Gly-Leu-Arg-Pro-Gly-NH_2 (IV). The protected peptide II (100 mg) and anisole (3 ml) were placed in the reaction vessel of an inert vacuum system (Peninsula Laboratories, San Carlos, Calif.) and thoroughly degassed. Anhydrous HF was condensed on top and the mixture stirred at 0° (1 hr). Excess HF was removed *in vacuo* and the peptide-anisole mixture distributed between 0.1 *M* AcOH and EtOAc. The aqueous layer (50 ml) was extracted with EtOAc (10 ml) and ether (three 10-ml portions) and lyophilized to yield crude IV (89 mg). This was dissolved in water (8 ml) and purified on the CM-cellulose column under the described conditions. Fractions eluted between 620 and 700 ml were pooled and lyophilized to constant weight from water to give peptide IV (46 mg, 62%): $[\alpha]^{26}D$ −58° (*c* 1.10, 0.1 *M* AcOH); single spot to Ehrlich, Pauly, and Cl-tolidine reagents; R_f[1] (cellulose), 0.61; R_f[2] (silica), 0.65; R_f[3] (cellulose), 0.53; single component moving in the direction of the cathode after tlc at pH 4.6 and 6.4. Amino acid analysis gave Phe(4-NO_2), 1.03; Trp, 1.00; His, 0.99; NH_3, 1.13; Arg, 0.92; Ser, 0.93; Glu, 1.08; Pro, 0.89; Gly, 2.20; Leu, 0.98. *Anal.* ($C_{55}H_{74}N_{18}O_{14}\cdot 2CH_3COOH\cdot 2H_2O$) C, H, N.

pGlu-His-Trp-Ser-Phe(4-NH_2)-Gly-Leu-Arg-Pro-Gly-NH_2 (V). The protected peptide II (150 mg) was dissolved in NH_3 (250 ml) and treated with small amounts of Na under conditions described above. During the addition the color of the reaction mixture changed progressively from pink to colorless to brown to colorless to permanent blue, the latter color being discharged immediately with 1 drop of AcOH.

The residue, after removal of NH_3, was desalted and subjected to chromatography on CM-cellulose. Peptide eluted between 565 and 640 ml weighed 46 mg (44%) after lyophilization: $[\alpha]^{26}D$ −50.6° (*c* 1.06, 0.1 *M* AcOH); single spot to Ehrlich, Pauly, and Cl-tolidine reagents; R_f[1] (cellulose), 0.39; R_f[2] (cellulose), 0.56; R_f[4] (silica), 0.18; single component moving in the direction of the cathode after tlc at pH 4.6 and 6.4. Amino acid analysis gave Phe(4-NH_2), 0.97; Trp, 1.03; His, 0.93; NH_3, 1.00; Arg, 0.99; Ser, 0.92; Glu, 1.07; Pro, 0.94; Gly, 2.20; Leu, 0.92. *Anal.* ($C_{55}H_{76}N_{18}O_{12}\cdot 3CH_3COOH\cdot 5H_2O$) C, H, N.

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Factors That Influence the Antagonistic Properties of Angiotensin II Antagonists

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Synthesis of [Suc[1],Ala[8]]-, [Ala[4],Ile[8]]-, and [D-*a*Ile[8]]angiotensin II was carried out by solid-phase procedure. Angiotensin II was synthesized as a model compound for separation of D-histidine containing octapeptide, formed as a side product during the synthesis, from the L-histidine containing parent hormone by ion-exchange chromatography followed by partition chromatography on Sephadex G-25. [D-His[6]]angiotensin II had 4% pressor response of angiotensin II. Antagonism to myotropic activity of angiotensin II on rabbit aortic strips was reduced when position 1 (Asp) in [Ala[8]]angiotensin II was substituted with succinic acid residue (log K_2 7.04) or when position 4 (Tyr) in [Ile[8]]angiotensin II was replaced with Ala (log K_2 6.55). Similar results were obtained when position 8 in [Ile[8]]angiotensin II was replaced with D-*allo*isoleucine (log K_2 7.33). The antagonistic activities of all the analogs were reduced when position 6 (L-histidine) was substituted with D-histidine. As compared to angiotensin II, pressor activity (vagotomized, ganglion-blocked rats) of all the analogs was less than 0.72%. These results indicate that although position 8 in angiotensin II is responsible for determining the agonistic or antagonistic properties of the compound, the degree of activity and duration of action are very much influenced by the nature and optical orientation of substituents in other positions of the molecule. Changes in positions other than in position 8 may exert their influence by affecting position 8 side group orientation with respect to other necessary side groups.

Lack of parallelism between the myotropic activity and the ability to release catecholamines from adrenal medulla[1] led to the discovery of the antagonistic potential of 8-substituted analogs of angiotensin II.[2,3] Subsequent investigations in our laboratories revealed that substitution of the aromatic ring in position 8 (Phe) by an aliphatic residue