Analogs of Luteinizing Hormone-Releasing Hormone with Increased Biological Activity Produced by D-Amino Acid Substitutions in Position 6

David H. Coy,* Jesus A. Vilchez-Martinez, Esther J. Coy, and Andrew V. Schally

Department of Medicine, Tulane University School of Medicine, Veterans Administration Hospital, New Orleans, Louisiana 70112. *Received August 21, 1975*

The incorporation of simple D-amino acids in place of glycine in position 6 of the LH-RH decapeptide produces analogs which have far greater gonadotropin-releasing activities in vivo and in vitro than the natural hormone. An investigation of the structural features of the D-amino acids responsible for this phenomenon suggests that an increase in the lipophilic character and perhaps the size and aromaticity of the side chain coincides with an increase in biological activity. This is demonstrated by the LH-releasing activities of the following series of peptides which were assayed over a period of 6 h in immature male rats: [D-Glu^6]-, 1.8; [D-Ala^6]-, 7.0; [D-Leu^6]-, 9.0; [D-Phe^6]-, 10; [D-Trp^6]-LH-RH, 13 times more active than LH-RH itself. In contrast to previous results with [D-Ala^6]- and [D-Leu^6]-LH-RH, where the substitution of an ethylamide group for the glycine amide at the C-terminus produces large increases in LH/FSH releasing activity, the ethylamide derivatives of [D-Phe^6]- and [D-Trp^6]-LH-RH were actually less potent than their parent peptides. [(*N*-Me-D-Ala)^6]-LH-RH was found to be approximately 70 times less active than [D-Ala^6]-LH-RH which indicates that disruption of a preferred receptor-site binding conformation might be brought about by methylation of the amide linkage in this position.

One of the most important findings to come out of investigations on the relationships between the structure of luteinizing hormone-releasing hormone (LH-RH) and its biological activity was that [D-Ala^6]-LH-RH was considerably more active than LH-RH both in vitro[1] and in vivo.[2,3] The subsequent observation[4] that [D-Leu^6]-LH-RH was even more potent led us to the conclusion that the incorporation of other D-amino acids, particularly those with larger and more lipophilic side chains, would result in compounds with even higher gonadotropin-releasing properties. To test this hypothesis, [D-Phe^6]- and [D-Trp^6]-LH-RH, together with the more polar [D-Glu^6]-LH-RH, were synthesized and assayed against LH-RH and [D-Ala^6]-LH-RH. In addition, the effect which an *N*-methyl group might have when substituted on the amide linkage of this critical position was investigated by the synthesis and testing of [(*N*-Me-D-Ala)^6]-LH-RH.

Since the two most active LH-RH analogs to have been discovered so far are [D-Ala^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide[2,3,5,6] and [D-Leu^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide,[4,7] which are 4–5 times more active than the corresponding [D-Ala^6]- and [D-Leu^6]-LH-RH peptides, it was of interest to synthesize the ethylamide analogs of [D-Phe^6]- and [D-Trp^6]-LH-RH in the hope that still higher degrees of potency would result.

Synthesis. Peptides ending in glycine amide were assembled on a 1% cross-linked benzhydrylamine support[8] and cleaved and deprotected with HF–anisole directly as the amides. The two ethylamide analogs were assembled on a 1% cross-linked proline-Merrifield resin, cleaved by treatment with ethylamine,[9] and deprotected with HF–anisole. Free peptides were purified by gel filtration on Sephadex G-25 in 0.2 M AcOH, partition chromatography on Sephadex G-25, and, in the case of [D-Trp^6]-LH-RH, gradient elution on CM-cellulose.

Bioassays. LH and FSH releasing activities of all the analogs, except for [(*N*-Me-D-Ala)^6]-LH-RH, were measured over 6-h periods in 25-day-old male rats (six per group). Peptides were dissolved in 0.1% gelatin–0.9% saline in concentrations of 50 or 100 ng/0.2 ml and injected subcutaneously. Control groups were injected only with solvent. Serum gonadotropin levels were measured by radioimmunoassay[10,11] at intervals after injections. LH was expressed as NIH-LS-S17 and FSH as NIAMD-rat-FSH-RP-1. Mean serum gonadotropin concentrations in all groups at each time interval were calculated and plotted against time on an arithmetic graph (Figures 1–3). The gonadotropin releasing activity of a peptide was then arbitrarily considered to be proportional to the integral of the corresponding curve. Activities with respect to LH-RH are given in Table II.

Table I. LH Releasing Activity of [(*N*-Me-D-Ala)^6]-LH-RH in Ovariectomized, Estrogen-Progesterone-Treated Rats

Sample	Dose, ng/rat[a]	Mean LH, ng/ml ± SE 30 min	60 min
Saline		5.3 ± 1.5	6.0 ± 0.4
LH-RH	1.0	24.5 ± 2.4	9.6 ± 0.7
	5.0	47.0 ± 4.6	15.4 ± 1.8
[(*N*-Me-D-Ala)^6]-LH-RH	10.0	22.9 ± 3.0	8.9 ± 0.8
	50.0	47.8 ± 4.1	18.6 ± 2.5

[a] Six per group.

The LH releasing activity of [(*N*-Me-D-Ala)^6]-LH-RH (Table I) was determined by stimulation of LH release at two dose levels in ovariectomized rats pretreated with steroid.[12] Serum LH concentrations at 30 and 60 min were compared with those obtained after injection of saline and two doses of LH-RH. Its activity with respect to LH-RH (Table II) was calculated for the 30-min interval.

Results and Discussion

[D-Phe^6]-LH-RH (I) and [D-Trp^6]-LH-RH (II) exhibit patterns of LH release which are similar, but more intense, than [D-Ala^6]-LH-RH with peak activities 2 h after injection as compared to 15 min for LH-RH (Figure 1). [D-Glu^6]-LH-RH (III) was comparatively far less potent and showed a peak response 15–60 min after administration (Figure 2). The order of LH releasing activities (Table II) increased as follows: [D-Glu^6]-, 1.8; [D-Ala^6]-, 7.0; [D-Phe^6]-, 10; [D-Trp^6]-LH-RH, 13 times the activity of LH-RH. FSH releasing activities (Table II) followed

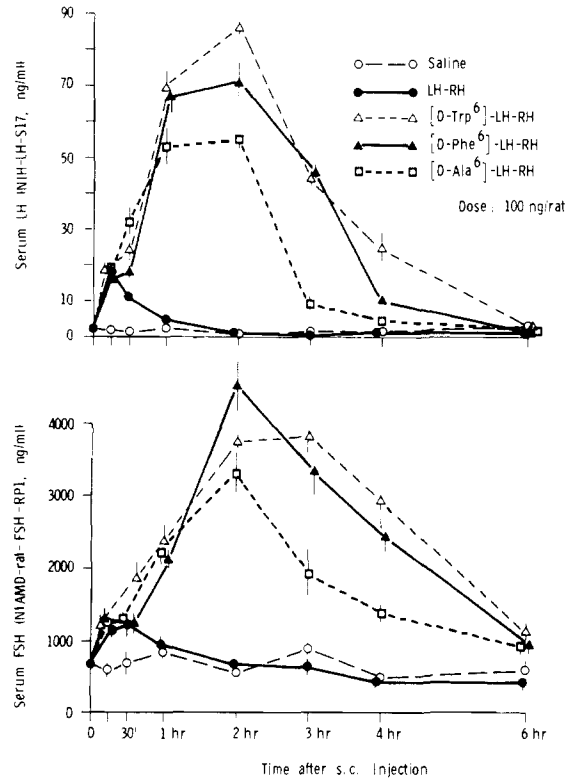

Figure 1. Serum LH and FSH concentrations at 15 min to 6 h after sc injection of immature male rats with LH-RH, [D-Ala^6]-LH-RH, [D-Phe^6]-LH-RH (I), [D-Trp^6]-LH-RH (II), and saline.

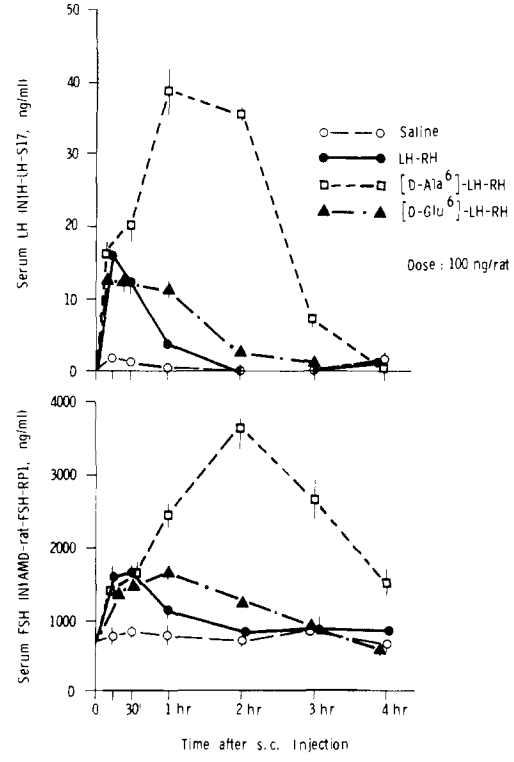

Figure 2. Serum LH and FSH concentrations at 15 min to 6 h after sc injection of immature male rats with LH-RH, [D-Ala^6]-LH-RH, [D-Glu^6]-LH-RH (III), and saline.

basically the same trend. These results confirm our original conclusions concerning those features which govern increased potency in analogs with changes in position 6; thus, in this limited series of peptides, the greater the hydrophobic character and size of the side chain of the

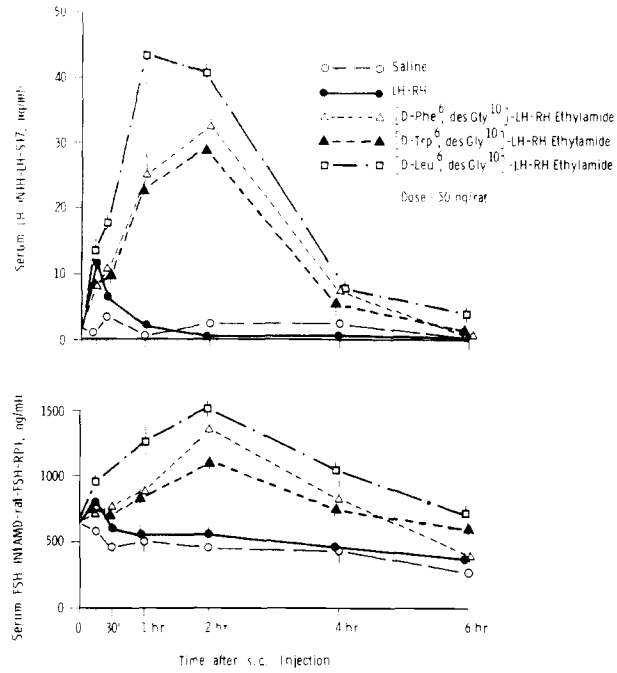

Figure 3. Serum LH and FSH concentrations at 15 min to 6 h after sc injection of immature male rats with LH-RH, [D-Leu^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide (VI), and [D-Trp^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide (VI), and saline.

Table II. Activities of Analogs and LH-RH Expressed as Ratios of Integrated Serum Gonadotropin Levels in Figures 1-3

Peptide	Activity LH	Activity FSH
LH-RH	1.0	1.0
[D-Ala^6]-LH-RH	7	13
[D-Phe^6]-LH-RH	10	20
[D-Trp^6]-LH-RH	13	21
[D-Glu^6]-LH-RH	1.8	1.5
[(*N*-Me-D-Ala)^6]-LH-RH[a]	0.01	
[D-Leu^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide	15	11
[D-Phe^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide	8	7
[D-Trp^6,des-Gly-NH$_2^{10}$]-LH-RH ethylamide	7	6

[a] LH releasing activity in ovariectomized rat assay (Table I) calculated for 30-min time interval.

D-amino acid, the higher the activity of the analog, with the added possibility that aromaticity is also a contributing factor.

[(*N*-Me-D-Ala)6]-LH-RH (IV) was only 10% as active as LH-RH and did not manifest the delayed peak response and prolonged activity of the superactive analogs.

In contrast to the three- to fourfold increase in activity[2-4,7] which results when an ethylamide group is substituted for the glycine amide portion of [D-Ala6]-LH-RH and [D-Leu6]-LH-RH, the ethylamide derivatives of [D-Phe6]- and [D-Trp6]-LH-RH (V and VI) were surprisingly less potent than the parent peptides and, hence, far less active than the ethylamide analogs of this type reported previously. There appears to be a limiting activity which is reached with [D-Leu6,des-Gly-NH$_2$10]-LH-RH ethylamide but can possibly not be exceeded by the combination of other position 6 and C-terminal modifications. An analogous situation was observed[13] previously with [D-Ala6,des-Gly-NH$_2$10]-LH-RH trifluoroethylamide which is only equipotent with [D-Ala6,des-Gly-NH$_2$10]-LH-RH ethylamide, even though [des-Gly-NH$_2$10]-LH-RH trifluoroethylamide is almost twice as active as the corresponding ethylamide analog.

Experimental Section

Amino acid derivatives were purchased from Bachem, Inc., Marina del Rey, Calif. Amino acid analyses were carried out on a Beckman Model 119 analyzer equipped with a System AA computing integrator on samples which were hydrolyzed (110°, 18 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[14] The TLC solvent systems described[15] in a previous paper in this series were employed and sample sizes of ca. 20 μg were spotted on Merck silica gel 60 plates unless otherwise indicated. Solvent fronts were allowed to travel 10–15 cm and spots were visualized by exposure to I_2 vapor, ninhydrin reagent (all compounds negative), and Ehrlich reagent in succession.

<Glu-His-Trp-Ser-Tyr-D-Phe-Leu-Arg-Pro-Gly-NH_2 (I). The following side-chain protecting groups were used while coupling *tert*-butyloxycarbonylamino acids to the growing peptide on its benzhydrylamine resin[8]: Arg, tosyl; Tyr, 2-bromocarbenzoxy; Ser, benzyl; His, tosyl. *tert*-Butyloxycarbonylamino acids (3 mmol) were coupled in a Beckman Model 990 automatic peptide synthesizer to the 1% cross-linked resin (2.22 g, 1.0 mequiv, of free amino groups) in the presence of dicyclohexylcarbodiimide (3 mmol). Boc-protecting groups were removed at each stage by treatment with 33% TFA in CH_2Cl_2. The events of the automatic cycle have been described previously.[16] The synthesis yielded 4.07 g of dry protected decapeptide-resin (98% of theoretical weight gain). Weight gains approaching theoretical values were obtained for all the solid-phase syntheses described here.

Part of this resin (1.5 g) was stirred at 0° (30 min) with HF (24 ml) and anisole (8 ml). HF was then removed as rapidly as possible (ca. 60 min) in vacuo and EtOAc was added to the residue. Solid material was filtered, washed with EtOAc, dried, and extracted with 2 M AcOH. Lyophilization gave a white powder which was purified by gel filtration on a column (2.5 × 95 cm) of Sephadex G-25 (fine) by elution with 2 M AcOH. Material from a major peak was then applied to a column (2.5 × 95 cm) of Sephadex G-25 (fine) previously equilibrated with the lower phase followed by the upper phase of the 1-BuOH–AcOH–H_2O (4:1:5) solvent system. Elution with upper phase gave a major peak (R_f 0.32) and peptide from this area was collected, concentrated to dryness, and lyophilized from dilute AcOH to give analog I (158 mg) as a white powder: $[\alpha]^{25}D$ −57° (*c* 0.55, 0.1 M AcOH); R_f^1 0.14, R_f^2 0.41, R_f^3 0.47, R_f^4 0.68. Amino acid analysis gave Glu, 1.01; His, 0.97; Trp, 0.90; Ser, 0.92; Tyr, 1.00; Phe, 0.96; Leu, 1.00; Arg, 1.02; Pro, 0.95; Gly, 1.00; NH_3, 1.00.

<Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2 (II). This peptide was prepared in an analogous fashion to peptide I. HF cleavage and deprotection of 1.5 g of resin gave material of lower homogeneity which was purified by gel filtration and partition chromatography (R_f 0.33) under the conditions described. In an additional purification step, the peptide was eluted on a column (1.4 × 90 cm) of CM-cellulose with an exponential gradient (500-ml mixing flask) of NH_4Ac buffers (0.002 M, pH 4.6, to 0.1 M, pH 7.0). Appropriate fractions (1050–1090 ml), after repeated lyophilization, gave peptide II (80 mg): $[\alpha]^{23}D$ −59° (*c* 0.33, 0.1 M AcOH); R_f^1 0.25, R_f^2 0.38, R_f^3 0.51, R_f^4 0.63. Amino acid analysis gave Glu, 1.08; His, 0.95; Trp, 2.00; Ser, 0.94; Tyr, 0.97; Leu, 0.93; Arg, 0.98; Pro, 1.00; Gly, 1.02; NH_3, 1.03.

<Glu-His-Trp-Ser-Tyr-D-Glu-Leu-Arg-Pro-Gly-NH_2 (III). This peptide was prepared in an analogous fashion to peptide I. HF cleavage and deprotection of 1.5 g of resin gave material which was purified by gel filtration followed by partition chromatography in the system 1-BuOH–2-PrOH–AcOH–H_2O (7:1:2:10) (R_f 0.36) to yield 200 mg of peptide III: $[\alpha]^{26}D$ −35° (*c* 0.78, 0.1 M AcOH); R_f^1 0.05, R_f^2 0.32, R_f^3 0.47, R_f^4 0.65. Amino acid analysis gave Glu, 2.10; His, 0.93; Trp, 1.05; Ser, 0.96; Tyr, 1.00; Leu, 1.00; Arg, 1.00; Pro, 1.02; Gly, 1.00; NH_3, 1.13.

<Glu-His-Trp-Ser-Tyr-*N*-Me-D-Ala-Leu-Arg-Pro-Gly-NH_2 (IV). This peptide was prepared in an analogous fashion to peptide I. HF cleavage and deprotection of 0.5 g of resin gave material which was purified by gel filtration followed by partition chromatography (R_f 0.39) to yield 51 mg of peptide IV: $[\alpha]^{26}D$ −33° (*c* 0.73, 0.1 M AcOH); R_f^1 0.19, R_f^2 0.38, R_f^3 0.42; R_f^4 0.71. Amino acid analysis gave Glu, 0.93; His, 0.96; Trp, 0.91; Ser, 0.87; Tyr, 0.93; Leu, 0.96; Arg, 0.97; Pro, 1.00; Gly, 1.02; NH_3, 1.06. The presence of *N*-Me-Ala was determined by TLC of the hydrolysate on SiO_2 using $CHCl_3$–MeOH–17% NH_3 (2:2:1) as the developing medium.

<Glu-His-Trp-Ser-Tyr-D-Phe-Leu-Arg-Pro-NHCH$_2$CH$_3$ (V). The peptide was assembled on a 1% cross-linked Pro-Merrifield resin (2.0 g, 1.0 mmol of Pro) using the same conditions and protecting groups employed for analog I, with the exception that dinitrophenol group protection was used for the imidazole group of histidine. The peptide-resin (3.45 g) was stirred with ethylamine (20 ml, 0°) for 6 h and excess amine removed in vacuo. Protected peptide was extracted with MeOH and precipitated by the addition of a large excess of EtOAc to give 1.16 g of material. Part of this (250 mg) was deprotected with HF–anisole under the usual conditions and crude peptide was purified by gel filtration followed by partition chromatography (R_f 0.34) to yield homogeneous peptide V (87 mg): $[\alpha]^{22}D$ −54° (*c* 0.40, 0.1 M AcOH); R_f^1 0.19, R_f^2 0.47, R_f^3 0.56, R_f^4 0.71. Amino acid analysis gave Glu, 1.05; His, 0.98; Trp, 0.93; Ser, 0.94; Tyr, 1.00; Phe, 0.97; Leu, 0.99; Arg, 1.02; Pro, 1.00; $EtNH_2$, 0.98.

<Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NHCH$_2$CH$_3$ (VI). This peptide was prepared in an analogous fashion to peptide V. The protected peptide (250 mg) was treated with HF–anisole and the free peptide purified by gel filtration followed by partition chromatography (R_f 0.39) to yield homogeneous analog VI (50 mg): $[\alpha]^{24}D$ −61° (*c* 0.37, 0.1 M AcOH); R_f^1 0.28, R_f^2 0.47, R_f^3 0.59, R_f^4 0.70. Amino acid analysis gave Glu, 1.13; His, 0.99; Trp, 2.02; Ser, 0.90; Tyr, 1.00; Leu, 0.99; Arg, 1.01; Pro, 0.99; $EtNH_2$, 0.98.

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