SYNTHESIS AND BIOLOGICAL PROPERTIES OF [D-ALA-6, DES-GLY-NH_2-10]-LH-RH ETHYLAMIDE, A PEPTIDE WITH GREATLY ENHANCED LH- AND FSH-RELEASING ACTIVITY

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Summary: A nonapeptide analog of luteinizing hormone-releasing hormone (LH-RH), [D-Ala^6, des-Gly-$NH_2{}^{10}$]-LH-RH ethylamide, was prepared by solid-phase methodology. The peptide was assayed against LH-RH in two *in vivo* systems and was found to be many times more potent than the naturally occurring hormone. In one of the tests, based on elevation of LH and FSH levels after infusion into immature male rats, the analog showed LH-releasing activity of 1600% and FSH-releasing activity of 1200% compared to LH-RH.

The search for competitive inhibitors of LH-RH has resulted in considerable interest in analogs of this peptide which are more potent than the natural hormone because they might offer promising structures on which to base effective synthetic antagonists. The discovery (1,2) that the LH-releasing activity of [des-Gly-$NH_2{}^{10}$]-LH-RH ethylamide was approximately 300% greater than that of LH-RH itself quickly led to the synthesis of the [des-His^2, des-Gly-$NH_2{}^{10}$]-LH-RH ethylamide (3,4), the first inhibitor of LH-RH to be active *in vivo*. More recently, [D-Ala^6]-LH-RH was also shown (5) to have higher activity than LH-RH, a phenomenon which was attributed to the imposition of a preferred receptor-site-binding conformation on the peptide backbone.

It occurred to us that a combination of both these modifications within the same molecule might result in a spectacular increase in hormonal activity, and therefore [D-Ala^6, des-Gly-$NH_2{}^{10}$]-LH-RH ethylamide was prepared.

SYNTHESIS, PURIFICATION, AND CHARACTERIZATION

A protected peptide, pGlu-His(DNP)-Trp-Ser(Bzl)-Tyr(Bzl)-D-Ala-Leu-Arg(Tos)-Pro-resin, was assembled on its solid support in a Beckman Model 990 Peptide Synthesizer by an automated method which has been extensively described previously (4,6). The protected peptide was cleaved from the resin as its ethylamide by treatment (4) with ethylamine and benzyl and tosyl side-chain protecting groups were then readily removed by reaction with hydrogen fluoride in the presence of anisole (20%)at 0°. The free nonapeptide, pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NH-CH_2-CH_3, was purified in two stages. The first stage consisted of gel filtration on a column (2.7 x 91 cm) of Sephadex G-25 in 0.2M acetic acid and gave a major peak (R_f, 0.53). The second stage utilized partition chromatography on a column (1.8 x 115 cm) of Sephadex G-25 previously equilibrated with the lower phase, followed by the upper phase of a system of n-butanol:acetic acid: water (4:1:5); elution with upper phase gave a major peak (R_f, 0.22).

[D-Ala^6, des-Gly-NH_2^{10}]-LH-RH ethylamide was obtained in 32% yield, $[\alpha]_D^{25^{\circ}}$ -44°. The following R_f's were obtained in three different solvent systems by thin layer chromatography on either silica or cellulose plates (Brinkmann): n-butanol: acetic acid: water (4:1:5, upper phase), 0.66 (cellulose); ethyl acetate:pyridine:acetic acid: water (5:5:1:3), 0.71 (silica); n-butanol:ethyl acetate:acetic acid: water (1:1:1:1), 0.51 (silica). Amino acid analyses were carried out on a Beckman Model 119 amino acid analyser equipped with a System AA computing integrator on samples which were hydrolyzed at 110° (18 hr) in methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (7). The following ratios were obtained: Ser, 0.92; Glu, 1.00; Pro, 1.00; Ala, 0.95; Leu, 1.00; Tyr, 0.98; His, 0.97; Trp, 1.06; $EtNH_2$, 1.00; Arg, 0.97.

Abbreviations: DNP, dinitrophenyl; Bzl, benzyl; Tos, tosyl

TABLE 1: LH-RH Activity of [D-Ala^6, desGly-NH_2^{10}]-LH-RH Ethylamide Compared with that of LH-RH in Ovariectomized, Estrogen-Progesterone Treated Rats at Various Times after Injection.

Sample	Dose (ng/rat)	Mean LH (ng/ml ± S.E.) 30 min.	60 min.	90 min.	120 min.
Saline	---	2.7 ± 0.7	4.2 ± 0.4	5.6 ± 0.8	5.9 ± 1.1
LH-RH*	0.5	5.7 ± 1.4	4.6 ± 0.4	5.2 ± 0.4	4.0 ± 0.4
	2.5	18.7 ± 2.7	8.8 ± 1.5	7.2 ± 1.1	6.8 ± 2.1
[D-Ala^6, desGly-NH_2^{10}]	0.25	5.7 ± 0.4	5.8 ± 0.5	6.4 ± 0.3	6.2 ± 0.5
LH-RH ethylamide	1.25	31.7 ± 4.2	24.1 ± 3.5	21.2 ± 5.0	11.8 ± 2.7

* Natural LH-RH, AVS 77-33, #215-269.

TABLE 2: FSH-RH and LH-RH Activity of [D-Ala^6]-LH-RH and [D-Ala^6, des-Gly-NH_2^{10}]-LH-RH Ethylamide when infused into Immature Male Rats.

Sample	Dose (ng/rat)	LH (ng/ml)	FSH (ng/ml)
Saline	---	1.29 ± 0.25	455.52 ± 70.4
LH-RH	50	12.58 ± 1.52	1988.33 ± 97.95
	150	30.46 ± 2.52	4278.33 ± 285.74
[D-Ala^6]-LH-RH	10	20.20 ± 2.17	2315.00 ± 135.24
	30	28.27 ± 3.92	5976.66 ± 756.06
[D-Ala^6, desGly-NH_2^{10}]	5	17.76 ± 0.93	2066.66 ± 353.13
LH-RH ethylamide	15	43.35 ± 4.76	5286.00 ± 375.20

	FSH Potency with 95% Confidence Limits (%)
[D-Ala^6]-LH-RH	727 (529-1064)
[D-Ala^6, desGly-NH_2^{10}] LH-RH ethylamide	1243 (978-1611)

	LH-RH Potency with 95% Confidence Limits (%)
[D-Ala^6]-LH-RH	801 (568-1252)
[D-Ala^6, desGly-NH_2^{10}] LH-RH ethylamide	1579 (1194-2221)

BIOLOGICAL ASSAYS - DISCUSSION

The LH-RH activity of the analog (Table I) was first determined *in vivo* at two dose levels by the stimulation of LH release in ovariectomized rats (4 per group), pretreated (8,9) with estrogen and progesterone, followed by radioimmunoassay (10) for serum LH. Serum LH levels at 30, 60, 90, and 120 minute intervals were compared with amounts present after administration of saline and LH-RH.

The extremely high potency of the peptide is only fully manifested when the assay is continued over a considerable period of time because of the prolonged activity of the material. A similar situation was found (4) to exist with [des-Gly-NH_2^{10}]-LH-RH ethylamide in this and other (11,12) assay systems. Thus, the activity of the analog relative to LH-RH was at a minimum of 343 (210-620)% at 30 minutes and at a maximum of 862 (257-1055)% at 90 minutes. Levels of LH produced by the 1.25 ng dose of the analog were still significantly higher than in the controls even at 120 minutes after injection.

In another experiment, the FSH-releasing activity and LH-releasing activity of [D-Ala^6,des-Gly-NH_2^{10}]-LH-RH were determined by four hour infusion (13) into 25-day-old male rats (6 per group). For comparison, [D-Ala^6]-LH-RH (5), prepared by us, was also assayed simultaneously. The results are shown in Table II. Since levels of LH and FSH released over a four hour period are measured, this assay gives more realistic relative activities when analogs having a more prolonged effect than LH-RH are tested. The LH/FSH-releasing potencies of [D-Ala^6,desGly^{10}]-LH-RH ethylamide were 1500-1200% or roughly twice those of [D-Ala^6]-LH-RH. In an identical assay (4), [des-Gly-NH_2^{10}]-LH-RH possessed LH/FSH activities of about 250%. Thus, the ethylamide modification has similar reinforcing effects on the potencies of both LH-RH and [D-Ala^6]-LH-RH.

Preliminary results from a time study (14) comparing total amounts of LH and FSH released by identical doses of LH-RH and the analog reveals that the latter is ca. 30 times more active than LH-RH. Consequently, we expect that it will have important clinical and veterinary applications when prolonged release of LH and FSH is desirable, for example, in ovulation induction. In addition, this structure appears to offer an excellent starting point for the synthesis of competitive LH-RH inhibitors.

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