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Synthesis and Biological Activity of LH-RH Analogs Modified at the Carboxyl Terminus

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Des(Pro^9,Gly^{10})-LH-RH ethylamide, des(Pro^9,Gly^{10})-LH-RH butylamide, desGly^{10}-LH-RH 2-aminoethylamide, and desGly^{10}-LH-RH hydrazide were synthesized by a solid-phase method involving cleavage of protected peptide intermediates from their resin support by reaction with ethylamine, butylamine, ethylenediamine, and hydrazine, respectively. In the assay utilizing steroid pretreated, ovariectomized rats, the peptides were found to have the following LH-releasing activities when compared with natural LH-RH: ethylamide, 0.2%; butylamide, 0.1%; 2-aminoethylamide, 2.4%; hydrazide, 12%. DesGly^{10}-LH-RH hydrazide was used as a precursor in the synthesis of desGly^{10}-LH-RH allylamide and desGly^{10}-LH-RH propargylamide by conversion to the azide and reaction with allylamine and propargylamine, respectively. LH and FSH levels were measured over a 4-hr period after subcutaneous injection of these two peptides into immature male rats in order to detect any prolongation of activity. The allylamide analog was quite active, releasing 1.7 times more LH and 1.3 times more FSH than the same dose of LH-RH. The propargylamide analog was considerably less active, exhibiting 50% LH-releasing activity and 64% FSH-releasing activity. Neither peptide appeared to be longer acting than LH-RH.

Since Fujino and coworkers found[1,2] that desGly^{10}-LH-RH ethylamide (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$NHCH_2CH_3$) and propylamide, nonapeptides in which the glycinamide moiety of LH-RH is replaced by ethylamide and propylamide groups, exhibited greatly enhanced gonadotropin-releasing and ovulation-inducing activities, much attention has been focused on the synthesis and biological evaluation of C terminally modified analogs. This has now led to the preparation of even more potent, related peptides such as desGly^{10}-LH-RH 2,2,2-trifluoroethylamide,[3] analogs containing the ethylamide modification in conjunction with D-alanine[3,4] and D-leucine[5] in position 6 of the chain which induce massive and prolonged release of gonadotropins, as well as peptides with improved antagonism toward LH-RH.[3,6]

In a continuing study of those structural requirements at the C terminus necessary for maintaining very high biological activity, we have synthesized four peptides containing new replacements, similar in size to the original ethyl and propyl groups, for the glycinamide residue in LH-RH. Two octapeptides were also prepared in which Pro-Gly-NH_2 was replaced by an ethylamide and a butylamide group in order to examine the effects that removal of proline would have on biological potency.

Synthesis. The automated procedure used for assembling the protected peptide intermediates, beginning with either an arginine or proline 1% divinylbenzene–cross-linked polystyrene resin, has been described in detail elsewhere.[7] The protected peptide intermediates for the ethylamide, butylamide, 2-aminoethylamide, and hydrazide analogs were removed from the resin by direct treatment (0°, 6 hr) with ethylamine, butylamine, ethylenediamine, and hydrazine, respectively. All these reactions were accompanied by simultaneous removal[8] of the dinitrophenyl group protecting the imidazole ring of histidine. The remaining protecting groups were then eliminated in the usual fashion with anhydrous hydrogen fluoride in anisole (20%). The free peptides were purified by gel filtration on Sephadex G-25 followed by partition chromatography on Sephadex G-25.

DesGly^{10}-LH-RH allylamide and propargylamide were made by a novel route from desGly^{10}-LH-RH hydrazide by its conversion to the azide followed by reaction with allylamine and propargylamine in good yield. In this manner, undesirable side reactions between HF and the olefinic and acetylenic groups were avoided. The two peptides were readily purified by partition chromatography alone.

Homogeneity of all peptides was demonstrated in several tlc solvent systems and by amino acid analysis.

Biological Assays. The LH-releasing activities of des-(Pro^9,Gly^{10})-LH-RH ethylamide and butylamide and desGly^{10}-LH-RH 2-aminoethylamide and hydrazide (Table I) were determined by stimulation of LH release at two appropriate dose levels after administration to ovariectomized, estrogen–progesterone pretreated rats. Serum LH levels were measured 30 min after injection by radioimmunoassay.[9] The LH levels were compared to those found after administration of two doses of natural LH-RH, allowing relative activities to be calculated with 95% confidence limits.

In view of the delayed peak gonadotropin responses[10] of desGly^{10}-LH-RH propylamide, it was considered advisable to test desGly^{10}-LH-RH allylamide and propargylamide in greater depth. An immature male rat system[10] was chosen

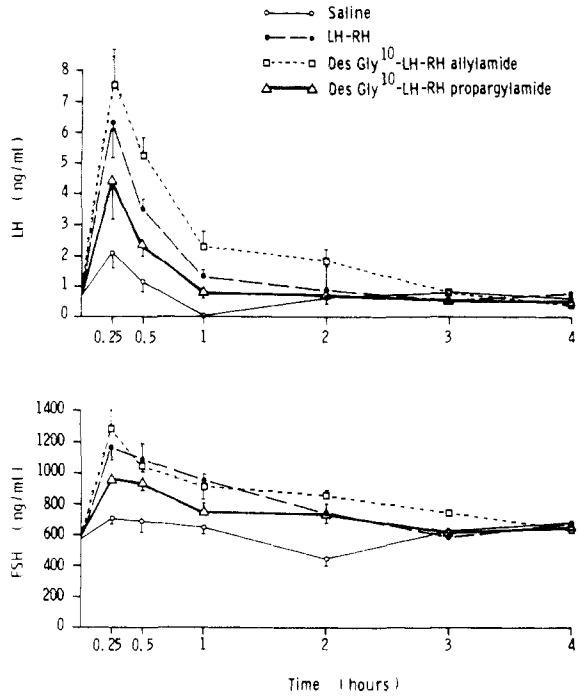

Figure 1. Serum LH and FSH concentrations at 0–4 hr after sc injection of immature male rats with 50-ng amounts of LH-RH, desGly[10]-LH-RH allylamide, and desGly[10]-LH-RH propargylamide.

which has been successful for assaying peptides with enhanced activity and a delayed peak of gonadotropin-releasing activity. The peptides and LH-RH were dissolved in 0.1% gelatin–0.9% saline solution in concentrations of 50 ng/0.2 ml and these amounts were injected subcutaneously into 25-day-old male rats (four per group) of the Sprague–Dawley strain. A control group was injected with the dissolving solution alone. Serum LH and FSH levels at 15 and 30 min and 1, 2, 3, and 4 hr after injection were then measured by radioimmunoassay.[9,11] Mean serum LH and FSH concentrations in all groups at each time interval were calculated, compared by Duncan's multiple range test, and plotted on an arithmetic graph against time (Figure 1). The gonadotropin-releasing activity of a peptide was then arbitrarily considered to be proportional to the integral of the corresponding curve.

The ratios of the integrated serum LH levels for desGly[10]-LH-RH allylamide and desGly[10]-LH-RH propargylamide gave activities of 170 and 47%, respectively, when compared to LH-RH itself (accepted as 100% activity). The two analogs were found to have FSH-releasing activities of 130 and 64%, respectively.

Discussion

In extensive studies on desGly[10]-LH-RH alkylamide analogs, Fujino, *et al.*,[1,12] discovered that the ethylamide and propylamide peptides were the most potent. Those analogs containing alkyl groups which were larger or smaller had much lower activities. With the exception of desGly[10]-LH-RH hydrazide, the desGly[10]-LH-RH alkylamides prepared here have C-terminal groups similar in size to a propyl group, but with lipophilicities which decrease in the order $-NHCH_2CH{=}CH_2$ > $-NHCH_2C{\equiv}CH$ > $-NHCH_2CH_2NH_2$. The gonadotropin-releasing activities of the peptides also decreased in that order, all the peptides being less potent than desGly[10]-LH-RH propylamide. It appears, therefore, that with end groups within a certain size range, there is a relationship between lipophilicity and biological activity, particularly when one considers that desGly[10]-LH-RH trifluoroethylamide,[3] containing a group which is even more hydrophobic than an ethyl group, is about twice as active as desGly[10]-LH-RH ethylamide. The hydrazide group of desGly[10]-LH-RH hydrazide is outside the size range for optimum activity and is no more active than desGly[10]-LH-RH amide.[13] However, it is considerably less active than desGly[10]-LH-RH methylamide[1] which is about equipotent with LH-RH itself. Unlike the propylamide analog,[10] neither the allylamide nor propargylamide peptides displayed patterns of gonadotropin release which were significantly different from those produced by LH-RH. There was no dissociation between the LH- and FSH-releasing activities.

The two peptides in which both the C-terminal proline and glycinamide residues are replaced by an ethylamide or a butylamide group have extremely low LH-RH activities. This indicates, as might be expected, that proline is very much involved in maintaining a preferred binding conformation. This was also concluded previously[8] when it was found that replacement of proline by a noncyclic amino such as leucine resulted in severe loss of activity.

Table I. LH-Releasing Activities of Several LH-RH Analogs Compared with LH-RH in Ovariectomized, Steroid-Blocked Rats

Sample	Dose, ng/rat	Mean plasma LH, ng/ml ± S.E.	Potency,[a] % (with 95% limits)
Saline		5.3 ± 1.5	
LH-RH	1.0	24.5 ± 2.4	
	5.0	47.0 ± 4.6	
DesGly[10]-LH-RH 2-aminoethylamide	50.0	30.5 ± 2.3	2.4 (1.2–4.9)
	250.0	45.4 ± 2.8	
DesGly[10]-LH-RH hydrazide	10.0	25.8 ± 4.6	12 (40–52)
	50.0	52.4 ± 11.2	
Saline		1.8 ± 0.2	
LH-RH	0.5	4.3 ± 1.0	
	2.5	34.3 ± 4.9	
Des(Pro[9],Gly[10])-LH-RH ethylamide	100.0	4.0 ± 1.1	0.2 (0.1–0.4)
	500.0	13.7 ± 1.8	
Saline		4.8 ± 0.8	
LH-RH	1.0	20.2 ± 5.8	
	5.0	57.4 ± 9.8	
Des(Pro[9],Gly[10])-LH-RH butylamide	100.0	6.0 ± 0.2	0.14 (0.003–0.52)
	500.0	15.3 ± 9.8	

[a] Pure natural LH-RH (AVS 77-33, no. 215-269) assumed 100%.

Experimental Section

Amino acid derivatives were of the L configuration and were purchased from Bachem Inc., Marina del Rey, Calif. Amino acid analyses were performed on a Beckman Model 119 amino acid analyzer equipped with a System AA computing integrator on samples which were hydrolyzed (110°, 18 hr) in 4 *M* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[14] A modified single-column elution system was used. Buffer 1 (pH 3.4) contained 1% 1-propanol, buffer 2 (pH 4.25) 0.15% 1-propanol, and buffer 3 (pH 6.4) 4% 1-propanol. Buffer change times were set at 73 and 132 min. Under these conditions, ethylamine was eluted 20 min after tryptophan, butylamine as a broad peak 170 min after arginine, allylamine 15 min after arginine, and propargylamine with tryptophan at 236 min. Hydrazine and ethylenediamine could not be eluted from the ion-exchange resin with the standard buffers.

The following tlc solvent systems were used to demonstrate purity of final peptides: R_f[1], *n*-BuOH–AcOH–H_2O (4:1:5, upper

phase); R_f^2, *i*-PrOH–1 *M* AcOH (2:1); R_f^3, *n*-BuOH–AcOH–H_2O–EtOAc (1:1:1:1); R_f^4, EtOAc–Pyr–AcOH–H_2O (5:5:1:3). Sample sizes of *ca.* 30 μg were spotted on Brinkmann Silplates and solvent fronts allowed to travel 10–15 cm. Spots were visualized by exposure to I_2 vapor, ninhydrin reagent (all compounds negative except for peptide III), and Ehrlich reagent in succession. The hydrazide (IV) gave spots which were positive to hydrazide reagent.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-NHCH$_2$CH$_3$ (I). During the solid-phase part of the synthesis, the following protecting groups were used for those amino acids with reactive side chains: histidine, dinitrophenyl; tyrosine, 2-bromocarbenzoxy; serine, benzyl; arginine, tosyl. Boc-protected amino acids (3.0 mmol) were coupled in a Beckman Model 990 automatic peptide synthesizer to a 1% cross-linked divinylbenzene–polystyrene-tosyl-arginine resin (4.6 g, 1.0 mmol of amino acid) in the presence of dicyclohexylcarbodiimide (3.0 mmol). α-Boc-protecting groups were removed at each stage by treatment with 25% TFA in methylene chloride. The events in the total automated cycle have been described previously.[7] No reducing agents were added to this reagent after the incorporation of tryptophan.[8]

The protected peptide resin (1.4 g) was allowed to react with ethylamine (15 ml) under anhydrous conditions at 0° for 6 hr, after which time excess amine was rapidly removed *in vacuo* at 0°. The residue was extracted with MeOH and the protected peptide intermediate (250 mg) precipitated by addition of EtOAc.

Part of this material (200 mg) in anisole (6 ml) was stirred (45 min) with anhydrous HF (24 ml) at 0°. Hydrogen fluoride was then removed as rapidly as possible (*ca.* 75 min) *in vacuo* and the peptide–anisole mixture distributed between 0.1 *M* AcOH and EtOAc. The aqueous layer (50 ml) was extracted three times with EtOAc and lyophilized to give a cream-colored powder which was first eluted on a column (2.5 × 95 cm) of Sephadex G-25 (fine) in 0.2 *M* AcOH to give a major peak (elution volume 330–380 ml). The partially purified peptide was then fractionated by partition chromatography on a column (1.4 × 95 cm) of Sephadex G-25 (fine) previously equilibrated with the lower phase followed by the upper phase of a system of *n*-BuOH–AcOH–H_2O (4:1:5). Elution with the upper phase yielded pure peptide I (96 mg, R_f 0.33–0.23): $[\alpha]^{27}D$ −20° (*c* 1.04, 0.1 *M* AcOH); R_f^1, 0.22; R_f^2, 0.50; R_f^3, 0.60; R_f^4, 0.54. Amino acid analysis gave Glu, 1.00; His, 1.00; Trp, 0.84; Ser, 0.90; Tyr, 0.97; Gly, 1.01; Leu, 1.04; Arg, 0.95; $EtNH_2$, 0.97.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-NHCH$_2$CH$_2$CH$_2$CH$_3$ (II). Protected peptide resin (1.4 g), prepared in the synthesis of peptide I, was allowed to react with *n*-butylamine (15 ml) at 0° for 6 hr, after which time the amine was rapidly removed *in vacuo* at room temperature. The residue was extracted with MeOH and the protected peptide (225 mg) precipitated by the addition of EtOAc.

Peptide obtained from the HF deprotection of part of this material (195 mg) was chromatographed under the same conditions described for peptide I. Gel filtration gave a major peak (320–370 ml) and partition chromatography yielded 74 mg of homogeneous peptide II (R_f 0.30–0.23): $[\alpha]^{27}D$ −32° (*c* 1.14, 0.1 *M* AcOH); R_f^1, 0.25; R_f^2, 0.55; R_f^3, 0.62; R_f^4, 0.59. Amino acid analysis gave Glu, 1.04; His, 0.98; Trp, 1.02; Ser, 0.97; Tyr, 1.00; Gly, 1.00; Leu, 1.01; Arg, 0.95, *n*-$BuNH_2$, 0.99.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH$_2$CH$_2$NH$_2$ (III). A protected nonapeptide resin was prepared using the reagents and conditions described in the synthesis of peptide I, beginning with a 1% cross-linked proline resin (2.78 g, 1.0 mmol of amino acid). Part of the completed peptide resin (1.0 g) was stirred with ethylenediamine (2 ml) in DMF (10 ml) at 0° for 6 hr and the volatile components were then removed *in vacuo* at 30°. The residue was extracted with MeOH and the cleaved peptide precipitated with EtOAc to yield 178 mg of powder.

Peptide obtained from the HF deprotection of part of this material (200 mg) was subjected to gel filtration (major peak at 300–380 ml) and partition chromatography (R_f 0.12–0.07) to yield 63 mg of homogeneous peptide III: $[\alpha]^{27}D$ −58° (*c* 1.27, 0.1 *M* AcOH); R_f^1, 0.044; R_f^2, 0.085; R_f^3, 0.14; R_f^4, 0.55. Amino acid analysis gave Gly, 1.05; His, 1.08; Trp, 0.92; Ser, 0.91; Tyr, 0.91; Gly, 1.03; Leu, 0.97; Arg, 0.97; Pro, 1.00.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHNH$_2$ (IV). The protected peptide resin (1.8 g), prepared in the synthesis of peptide III, was stirred with anhydrous hydrazine (1 ml) in DMF (10 ml) at 0° for 6 hr and the volatile components were then removed *in vacuo* at 30°. The residue was extracted and the protected nonapeptide precipitated twice from MeOH with EtOAc to give 309 mg of powder.

Peptide obtained from the HF deprotection of part of this material (287 mg) was subjected to gel filtration (major peak at 310–380 ml) followed by partition chromatography (R_f 0.23–0.15) and yielded 70 mg of homogeneous peptide IV: $[\alpha]^{26}D$ −45° (*c* 1.07, 0.1 *M* AcOH); R_f^1, 0.24; R_f^2, 0.55; R_f^3, 0.55; R_f^4, 0.51. Amino acid analysis gave Glu, 1.05; His, 0.95; Trp, 0.85; Ser, 0.98; Tyr, 1.01; Gly, 1.00; Leu, 1.01; Arg, 0.96; Pro, 0.98.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH$_2$CH=CH$_2$ (V). DesGly^{10}-LH-RH hydrazide (IV, 50 mg, 50 μmol) was dissolved in DMF (1.5 ml) and cooled to −20°. Hydrogen chloride (5.4 *M*) in dioxane (57 μl, 300 μmol) followed by *i*-$AmNO_2$ (14 μl, 300 μmol) was added and stirred at −20° (10 min). After addition of allylamine (50 μl, 600 μmol), the solution was maintained at −10° for 1 hr and 4° overnight. The DMF was removed *in vacuo* and the residue eluted directly on the partition column under the conditions already described. Homogeneous peptide V (26 mg) was recovered with an R_f of 0.35–0.27: $[\alpha]^{26}D$ −54° (*c* 0.52, 0.1 *M* AcOH); R_f^1, 0.22; R_f^2, 0.37; R_f^3, 0.50; R_f^4, 0.60. Amino acid analysis gave Glu, 1.04; His, 0.94; Trp, 0.90; Ser, 0.97; Tyr, 1.01; Gly, 1.00; Leu, 1.00; Arg, 0.95; Pro, 1.00; allylamine, 1.01.

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH$_2$C≡CH (VI). The hydrazide IV (50 mg, 30 μmol) was converted to the azide under the conditions described for the preparation of the allylamide analog V. The azide was allowed to react with propargylamine (23 μl, 600 μmol), also in a similar fashion. On the partition column, homogeneous peptide VI (26 mg) was obtained with an R_f of 0.35–0.26: $[\alpha]^{26}D$ −26° (*c* 0.63, 0.1 *M* AcOH); R_f^1, 0.22; R_f^2, 0.39; R_f^3, 0.51; R_f^4, 0.57. Amino acid analysis gave Glu, 1.04; His, 0.97; Trp, 1.10; Ser, 0.96; Tyr, 1.02; Gly, 1.00; Leu, 1.01; Arg, 0.98; Pro, 1.00; propargylamine, 1.10.

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