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NEW ANALOGS OF LULIBERIN WHICH INHIBIT OVULATION IN THE RAT

K. Channabasavaiah and John M. Stewart

Department of Biochemistry, University of Colorado Medical School, Denver, Colorado 80262

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SUMMARY

Fifteen analogs of luliberin (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, LRH) were synthesized by the solid phase method and examined for their ability to block ovulation in the rat. Two analogs, [Ac-DAla^1,DPhe^2,DTrp^{3,6}]-LRH and [Ac-DPhe^1,DPhe^2,DTrp^{3,6}]-LRH, each blocked ovulation at a single injection dose of 250 µg administered at noon on the day of proestrus; three peptides, [Ac-DPro^1,DPhe^2,DTrp^{3,6}]-LRH, [Ac-DThi^1,DPhe^2,DTrp^{3,6}]-LRH and [Ac-DTrp^1,DPhe^2,DTrp^{3,6}]-LRH, were effective at doses of 500 µg each; and four others, [Ac-DTrp^1,DPhe^2,DTrp^3,DTrp(Nps)^6]-LRH, [Chlorambucil-DPhe^1, DPhe^2, DTrp^{3,6}]-LRH, [<DGlu^1,DThi^2,DTrp^{3,6}]-LRH and [(<DGlu-DThi-DTrp-Ser-Tyr)$_2$-DLys^6]-LRH, gave partial inhibition at doses tested.

INTRODUCTION

The hypothalamic hormone luliberin, which releases the gonadotropins LH and FSH, is either directly or indirectly responsible for normal fertility in animals. Thus inhibitory analogs of this hormone could form a basis for a novel approach to contraception. Such inhibitors have been prepared in the past by making substitutions of D-amino acids for the residues at positions 2, 3 and 6 of the parent hormone. The structure-activity relationships of luliberin analogs have been reviewed recently (1,2). From these studies it is evident that the configuration of the amino acid at position 2 is very important in deciding agonist/antagonist activity of the analog. For example, [LPhe^2]-LRH is an agonist (3) whereas [DPhe^2]-LRH is an antagonist (4). In trying to

Abbreviations used: LRH, Luliberin, gonadotropin releasing hormone; MBHA, *p*-methyl benzhydrylamine; Boc, *t*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; TFA, trifluoroacetic acid; TEA, triethyl amine; DMF, dimethylformamide; Tos, *p*-toluenesulfonyl (Tosyl); Dns, 5-dimethylaminonaphthalenesulfonyl (Dansyl); Chl, 4-N,N-bis(2-chloroethyl)aminophenylbutyryl (Chlorambucil); Bzl, benzyl; Brz, 2-bromobenzyloxycarbonyl; Nps, 2-nitrophenylsulfenyl; Trp(Nps), 2-(2-nitrophenylsulfenyl)tryptophan; CCD, countercurrent distribution; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; Thi, β-(2-thienyl)-alanine; Boc-ON, 2-*t*-butyloxycarbonyloxyamino-2-phenylacetonitrile; Ac, acetyl; <Glu, pyroglutamyl; ACTH, adrenocorticotropic hormone.

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understand the antagonistic nature of DPhe^2 analogs, it can be assumed that the presence of the D-amino acid leads to a conformation that still retains enough structural features to be recognized by the receptor and bind to it, without, however, causing gonadotropin release.

In order to find out whether the presence of an additional D-amino acid at position 1 would be complementary to the antagonistic potency of analogs having D-amino acids at positions 2, 3 and 6, we have synthesized a new series of analogs and examined them for antiovulatory activity in the rat (see Table I).

MATERIALS AND METHODS

Peptide syntheses were carried out by the Merrifield solid phase method (5,6) on a Beckman 990 Automatic Peptide Synthesizer. The starting material, Boc-Gly-MBHA resin (0.4 mM of Gly/g of resin), was prepared by the DCC mediated coupling of Boc-Gly with p-methyl benzhydrylamine resin (prepared in our laboratory by the procedure of Matsueda and Stewart (unpublished); see Stewart et al (7)). The program used to incorporate one amino acid residue consisted of the following steps: (all washes are with DCM for 1.5 min) (a) wash 3 x; (b) wash with 25% TFA in DCM (v/v; containing 0.1% indole, w/v; the reagent was allowed to stand at least 16 hr before use); (c) deprotect with the same TFA reagent, 30 min; (d) wash 6 x; (e) wash with 10% TEA in DCM (v/v); (f) neutralize with 10% TEA in DCM, 5 min; (g) wash 6 x; (h) Boc-amino acid, 2.5 equivalents, in DCM (DMF added if necessary), 5 min; (i) DCC, 2.5 equivalents as a 0.25 M solution in DCM, couple 2 hr; (j) wash 3 x.

For the preparation of peptides bearing acetyl, benzoyl, p-toluenesulfonyl and 5-dimethylaminonaphthalenesulfonyl groups on the α-amino group, the peptide resins after coupling of the last Boc-amino acid were taken through deprotection and neutralization (Steps (a) through (g)) and then acylated with acetic anhydride, benzoyl chloride, tosyl chloride and dansyl chloride, respectively, in the presence of one equivalent of TEA. Chlorambucil was coupled with DCC in the normal manner, as described previously (8). For the synthesis of peptides containing the Trp(Nps) residue, the peptide resins, Boc-Trp-Ser(Bzl)-Tyr(BrZ)-DPhe-Leu-Arg(Tos)-Pro-Gly-MBHA and Boc-DTrp-Leu-Arg(Tos)-Pro-Gly-MBHA, were treated with Nps-Cl (3 equivalents) in 10% HOAc in DCM for 2 hr and then taken through the rest of the synthesis in the standard way. The DLys^6 branched chain peptide was prepared using Boc-DLys(Boc). The poly-lysine conjugate was prepared as follows: [<DGlu^1,DPhe^2,DTrp^3,DLys^6]-LRH (XVI) was treated with an excess of succinic anhydride in 0.01 M sodium phosphate (pH 8.0) and the resulting hemi-acid was coupled to poly-lysine (MW 70,000; Miles Biochemicals) using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (Ott Chemicals) in the same buffer.

Peptides were cleaved from the resins by treatment with anhydrous liquid HF containing 10% anisole for 30 min at 0°. The crude products were purified by CCD using a Post automatic instrument. The purity of the finished peptides was checked by TLC, HPLC and amino acid analysis. TLC was carried out on Silica Gel F-254 (E. Merck) plates. The peptide spots were detected by scanning under a UV lamp and by spraying with ninhydrin, Sakaguchi, Pauly and Ehrlich sprays as appropriate.

Table I: Antivoulatory Activity of Synthetic LRH Analogs.

Peptide No.	Structure	Actvity[a]	Dose µg/rat
I	Ac-DAla-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	0/10 2/10	250 100
II	Ac-DPro-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	0/10 4/10 5/10	500 250 100
III	Ac-DPhe-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	0/10 3/10	250 100
IV	Ac-DThi-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	0/10 1/10 4/10	500 250 100
V	Ac-DTrp-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	0/10 1/10 4/10	500 250 100
VI	Ac-DTrp-DPhe-Trp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	10/10	1000
VII	Ac-DTrp-DPhe-Trp(Nps)-Ser-Tyr-DPhe-Leu-Arg-Pro-Gly-NH_2	10/10	1000
VIII	Ac-DTrp-DPhe-DTrp-Ser-Tyr-DTrp(Nps)-Leu-Arg-Pro-Gly-NH_2	8/10	500
IX	Benzoyl-DTrp-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	9/10	500
X	Tos-DTrp-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	10/10	500
XI	Dns-DTrp-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	10/10	500
XII	Chl-DPhe-DPhe-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	7/10	1000
XIII	<DGlu-DThi-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2	3/10	500
XIV	[<DGlu-DThi-DTrp-Ser-Tyr]$_2$-DLys-Leu-Arg-Pro-Gly-NH_2	7/10	500
XV	[<DGlu-DPhe-DTrp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-NH_2]$_{13}$ / Succinyl-poly-Lysine	10/10	500

a Number of rats ovulating/number treated.

Analytical HPLC was carried out in a system consisting of an Altex Model 100 pump, a Valco Model CU-6 injection valve, two Lichrosorb RP-18 (10µ, 1x25 cm) columns connected in series, a Hitachi Model 155-40 spectrophotometer equipped with an Altex flow cell and a Linear Model 355 recorder. About 2 to 10 µg of peptides were injected, dissolved in methanol:0.025 M $HCOONH_4$ (9:1, pH 5.0), and eluted with the same solvent at a flow rate of 5 ml/min. The eluent was monitored at 215 nm. The physical characteristics of peptides as well as the solvent systems used for CCD and TLC are given in Table II.

For amino acid analysis, the peptides were hydrolyzed by heating in nitrogen flushed sealed tubes with 6 N HCl containing 1 mg/ml each of 2-mercaptoethanol and phenol for 22 hr at 110°. This procedure allows fair recovery of tryptophan if the hydrolysates are worked up and carried through analysis rapidly.

Table II: Properties of Synthetic Peptides.

Peptide No.	CCD[a] Solvent System	k	%Yield[b]	TLC[a] R_fG	R_fH	HPLC t_e/t_o
I	A	0.42	30	0.47	0.64	1.50
II	A	0.35	32	0.40	0.63	1.52
III	B	1.10	22	0.52	0.69	1.62
IV	C	0.24	34	0.51	0.68	2.30
V	A	0.81	35	0.50	0.71	1.96
VI	A	1.00	27	0.51	0.72	2.00
VII	D	0.50	22	0.59	0.78	1.59
VIII	C	0.85	20	0.54	0.69	1.64
IX	A	2.00	20	0.57	0.67	1.92
X	C	0.66	24	0.54	0.71	1.80
XI	A	1.10	39	0.55	0.67	1.90
XII	E	0.48	15	0.57	0.68	2.30
XIII	C	0.20	22	0.50	0.68	1.50
XIV	B	0.35	15	0.46	0.71	1.40
XVI	F	0.50	37	0.16	0.52	1.80

a. Solvent systems used: A, n-BuOH:HOAc:H_2O:EtOH:Hexane (3:1:5:1:2); B, n-BuOH:HOAc:H_2O:EtOH:EtOAc:Hexane (6:2:10:3:3:4); C, n-BuOH:HOAc:H_2O:EtOH:ETOAc:Hexane (6:2:10:3:3:6); D, n-BuOH:HOAc:H_2O:EtOH:Hexane (5:2:10:3:5); E, n-BuOH:HOAc:H_2O:EtOH:ETOAc:Hexane (2:1:4:1:13); F, n-BuOH:HOAc:H_2O (4:1:5); G, n-BuOH:HOAc:H_2O (4:1:1); H, n-BuOH:HOAc:H_2O:Pyridine (15:3:12:10).

b. Yields are overall, calculated from the glycine content of the starting Boc-Gly-MBHA Resin.

Table III: Amino Acid Compositions of Synthetic Peptides.

Peptide No.	Amino Acid Ratios											
	Trp	Lys	Arg	Ser	Glu	Pro	Gly	Leu	Thi	Tyr	Phe	Ala
I	1.65		0.98	0.95		1.02	1.03	1.00		1.00	0.98	0.99
II	1.64		0.95	0.96		2.04	1.06	0.96		1.01	1.01	
III	1.83		1.02	0.95		1.04	1.03	0.99		0.99	1.97	
IV	1.66		1.03	0.97		1.08	1.05	0.96	0.97	0.98	0.95	
V	2.65		1.01	0.97		1.03	1.03	1.00		1.02	0.91	
VI	2.62		1.00	0.95		1.05	1.00	0.97		0.98	0.98	
VII	1.85		1.07	0.99		0.99	1.07	0.96		1.05	1.93	
VIII	2.39		0.98	0.95		1.05	1.04	1.01		1.01	0.92	
IX	2.40		1.04	0.92		1.06	1.04	0.99		0.99	0.94	
X	1.54		0.99	0.91		1.01	1.03	0.96		1.00	0.95	
XI	1.86		1.07	0.89		1.05	0.94	0.97		0.99	0.93	
XII	1.82		0.99	0.96		1.05	0.97	0.98		0.98	1.97	
XIII	1.99		0.96	0.91	1.01	0.98	1.00	0.97	1.07	0.99		
XIV	1.80	0.97	1.03	1.83	1.93	1.01	1.04	1.03	1.93	1.96		
XVI	0.88	1.04	0.97	0.94	1.02	1.04	1.00	1.04		0.97	0.97	

Amino acid ratios in the hydrolysates were determined on a Beckman 120C analyzer When both lysine and tryptophan were present in hydrolysates, they were separted on the analyzer short column by first eluting it for 10 min with 0.175 M sodium citrate (pH 5.28) before passing through the standard 0.35 M buffer. Table III lists amino acid compositions of peptides.

The derivative Boc-DThi was prepared according to Itoh et al (9) starting from D-thienylalanine (kindly supplied by Dr. F.W. Dunn) and Boc-ON (Aldrich).

Antiovulatory Assays: These assays were carried out at the Mason Research Institute, Worcester, Mass., using female Sprague-Dawley rats exhibiting normal four-day cycles. Analogs suspended in corn oil were injected subcutaneously at 12 noon on the day of proestrus. On the following day (estrus), the rats were sacrificed, their fallopian tubes and uteri flushed with saline and the washes examined for ova. The results are summarized in Table I.

RESULTS AND DISCUSSION

The peptides described here were synthesized by the solid phase method on a modified benzhydrylamine (MBHA) resin in resonably good yields. The bond linking the peptide to the polymer is less stable in the MBHA resin than with the usual BHA resin and thus permits better yields of peptide amides upon HF cleavage. Some peptide amides cannot be cleaved from the BHA resin by the standard HF treatment; the bond is too stable (7). The purity of the synthetic LRH analogs was 95% or more as estimated by TLC, HPLC and amino acid analysis.

The assay results shown in Table I indicate that the analogs with the general structure [Ac-D-amino acid^{1},DPhe^{2},DTrp^{3,6}]-LRH are potent inhibitors of ovulation in the rat. [Ac-DAla^{1},DPhe^{2},DTrp^{3,6}]-LRH (I) and [Ac-DPhe^{1},DPhe^{2},DTrp^{3,6}]-LRH (III) completely blocked ovulation (100% inhibition) at a dose of 250 µg, and gave 80% and 70% inhibition, respectively, at a dose of 100 µg. Recently, Rivier and Vale (10) reported that [<DGlu^{1},DPhe^{2},DTrp^{3,6}]-LRH caused complete inhibition of ovulation in the rat at a dose of 250 µg, and 60% inhibition at a dose of 100 µg. Thus, peptides I and III are either equipotent or better than the best known LRH inhibitors. Other peptides in this series were somewhat less potent in inhibiting ovulation.

The analog VI, which is similar to the potent inhibitor V except for having L-Trp instead of D-Trp at position 3, was inactive at a dose as high as 1000 µg. This result agrees with an earlier observation that substituting D-Trp for L-Trp at position 3 leads to better antagonists (11).

We have observed a close structural similarity between LRH and the N-terminal decapeptide of ACTH (see Figure 1). This and other sequence homologies have been described in detail elsewhere (12). The presence of Trp(Nps) in ACTH has been

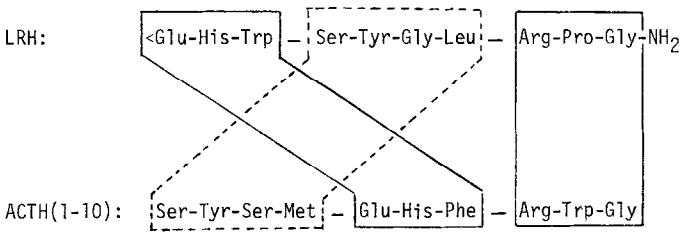

Figure 1. Structural similarity between LRH and ACTH(1-10): In derivation of the LRH sequence from the presumed more primitive ACTH, Glu-His-Phe has evidently been transposed by one turn of DNA helix from its original position matching <Glu-His-Trp. The amino acids, Trp and Phe, Gly and Ser, and Leu and Met, differ by a single point mutation of the DNA.

reported to cause dissociation of the activities of this hormone (13). Therefore, we decided to incorporate Trp(Nps) into LRH antagonists. Treatment of tryptophan in acid solution with Nps-Cl yields 2-(2-nitrophenylsulfenyl)tryptophan (14). In a similar fashion tryptophan present in peptide resins was alkylated with Nps-Cl to yield, after completion of the synthesis and purification, analogs VII and VIII. The peptide VII, having a Trp(Nps) residue at position 3, was inactive at a dose of 1000 µg. Similar derivatization of D-Trp at position 6 (analog VIII) caused loss of potency compared to peptide V, a somewhat surprising observation since hydrophobic residues at this position are expected to give better antagonists of LRH.

Analogs which have bulky aromatic acylating moities like benzoyl (IX), tosyl (X), dansyl (XI) and chlorambucil (XII) showed decreased potency as compared to the corresponding acetyl peptides (V and III)

Analogs of bradykinin in which the two phenylalanine residues at positions 5 and 8 are replaced by thienylalanine (isosteric with phenylalanine) exhibit enhanced biological activity (15). Two LRH analogs having a similar substitution of D-Thi for D-Phe, [<DGlu^1,DThi^2,DTrp^{3,6}]-LRH (XIII) and [(<DGlu-DThi-DTrp-Ser-Tyr)$_2$-DLys^6]-LRH (XIV) gave 70% and 30% inhibition, respectively, at a dose of 500 µg. Thus there is a reduction in activity in going from D-Phe to D-Thi at position 2 and from the D-Trp^6 single chain analog (XIII) to the D-Lys^6 branched-chain peptide (XIV). Seprodi et al (16) reported that [DPhe^2,DTrp^3,

DPhe^6]-LRH and [(<Glu^1,DPhe^2,DTrp^3,DPhe^6]-LRH inhibited ovulation in the rat by 83% and 73%, respectively, at a dose of 1000 μg. These authors suggested, however, that on a molar basis the branched-chain analog was the more potent of the two.

The polymer conjugate (XV), which was expected to lead to better binding to the receptor membrane due to ionic interactions, was inactive at a dose of 500 μg.

Thus, in conclusion, the presence of an acylated D-amino acid at position 1 is not only acceptable , but when it is combined with other suitable modifications results in highly potent inhibitors of ovulation. The enhanced antiovulatory activity exhibited by these analogs may be due to a molecular geometry which elicits better receptor recognition and binding or to incresed resistance to enzymatic degradation. A recent study suggests that enzymes which degrade LRH by opening the pyroglutamyl ring are present in the brain (17).

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