Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1014-1023

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF PSEUDOPEPTIDE ANALOGUES OF LH-RH: AGONISTS AND ANTAGONISTS

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Received September 22,1980

Summary

Using solid phase methods, seven agonist and antagonist analogues of LH-RH have been prepared containing enzyme-resistant CH_2S linkages as selected amide bond replacements. Agonists modified at the 5-6, 6-7 and 9-10 position had 2, < 0.1, and 10% of the in vitro activity of LH-RH, respectively. Among potential antagonists, 6-7 position analogues showed only minimal inhibitory activity but N- and C-terminal modified analogues retained substantial LH-RH-LH and FSH inhibitory activity. In addition, a 1-2 position methylene thioether analogue of the parent [Ac-Pro^1, D-Phe^2, D-Trp^{3,6}]LH-RH antagonist was completely inhibitory at 30 ng in vitro and represents the first such structure-modification that may be at least as active as its corresponding amide linked congener. However, neither 1-2 nor 9-10 methylene thioether position antagonists showed in vivo antiovulatory activity at the 250 µg level.

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** Abbreviations used: LH-RH, luteinizing hormone-releasing hormone; FSH, follicle stimulating hormone, LH, luteinizing hormone, Boc, t-butyloxycarbonyl, Cbz, benzyloxycarbonyl; DCC, dicyclohexylcarbodimide; DIEA, diisopropylethyl amine; TFA, trifluoroacetic acid; DMF, dimethylformamide; Tos, p-toluene sulfonyl; Bzl, benzyl; pGlu, pyroglutamyl; tlc, thin layer chromatography; HPLC, high pressure liquid chromatography; TEAP (Triethylammonium phosphate); ψ refers to the replacement of the normal amide linkage, [CONH] by the thiomethylene ether moiety, [CH_2S].

0006-291X/80/231014-10$01.00/0

INTRODUCTION

Both LH-RH superagonists and antagonists have potential as antifertility agents by virtue of their inhibition of LH and FSH release (1). Prolonged bioactivity would appear to be a necessary corequisite for useful candidates possessing antiovulatory activity. In an effort to prepare peptide analogues that would prove resistant to enzymatic degradation, we have examined the use of the thiomethylene ether moiety [CH_2S] as a replacement for the amide link [CONH] in critical positions within the LH-RH structure (I). Our major focus was predicated by the known lability of the 6-7 and 9-10 bonds of LH-RH as established by Marks and Stern (2) and by Koch et al., (3) but other position analogues have also been synthesized. Antagonists to LH-RH based on the potent inhibitor [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]LH-RH (4) have also been subjected to this selective modification with the objective of synthesizing pseudopeptide analogues that have long-lasting inhibitory activity.

LH-RH pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2 I

LH-RH Antagonist D-pGlu-D-Phe-D-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-GlyNH_2

The strategy chosen for the synthesis of these analogues has been first to prepare the suitable dipeptide which contains the amide bond replacement (amide bond surrogate) and then to incorporate it into the peptide. The Boc-protected dipeptides, GlyψX, were first prepared by Yankeelov et al., (5) followed by dipeptides of the Boc-XψGly type prepared by Spatola et al., (6). Also, since then we have prepared pseudodipeptides of the XψY type (where both X and Y represent chiral amino acids). (7) The procedure for incorporation of each of these three varieties of the pseudodipeptides into the peptides by standard solid phase methods has been described in the present study.

MATERIALS AND METHODS

Synthetic routes for the pseudodipeptides used in this study have been described elsewhere (7). The incorporation of the Boc-protected derivatives

Table I Methodology For Solid Phase Peptide Synthesis

Step	Reagent	Time (Min)
1	CH_2Cl_2 (3x)	1
2	TFA/anisole/CH_2Cl_2 (40:2:58)	5 and 25
3	CH_2Cl_2 (5x)	1
4	DIEA/CH_2Cl_2 (10:90) (2x)	2-10
5	CH_2Cl_2 (3x)	1
6	Boc-amino acid/CH_2Cl_2 (2.5 equiv.)[a]	-
7	DCC/CH_2Cl_2 (2.5 equiv.)[a]	90[b]
8	CH_2Cl_2 (3x)	1
9	EtOH (3x)	1

a. Substituted by two couplings of 1.5 thru 3.0 equivalents for Boc-pseudodipeptides.

b. Substituted by two couplings of 2-12 hr. each for Boc-pseudodipeptides.

into peptides related to LH-RH follows standard methods used in our laboratory. Table I describes the methodology employed for solid phase peptide synthesis; chiral amino acids are of the L-configuration except where otherwise specified. Side chain protecting groups used were tosyl (Arg and His) and benzyl (Ser and Tyr). Common protected amino acids were purchased from Vega Biochemicals, Bachem, or Beckman Biochemicals. Benzhydrylamine resins used were 1% cross-linked polystyrene polymers (0.4-0.5 meq. nitrogen/g) from Beckman Biochemicals, condensed (DCC mediated) with Boc-Glycine or the Boc-Pro ψ Gly pseudodipeptide to the extent of 0.15-0.35 mmol/g. No unusual precautions were necessary in the coupling reactions involving Boc-pseudodipeptides, although scavengers and/or antioxidants were employed during acid-catalyzed deprotections, hydrogen fluoride cleavage and amino acid hydrolyses.

Peptides were cleaved from the resins by treatment with anhydrous hydrogen fluoride containing 10% anisole and 10% ethyl methyl sulfide for 30 min. at 0° C. The extracted products were lyophilized and desalted over Sephadex G-15 using 25% or 30% HOAc. The major peak was collected, lyophilized

and purified by gel filtration. The final purification was by semi-preparative reversed phase liquid chromatography (Dupont Model 850) using Zorbax C-18 columns, employing methanol/ammonium acetate buffer gradient solvent systems and monitoring at 254 nm. The major peak was collected, lyophilized repeatedly to remove ammonium acetate salt, and analyzed by standard techniques including tlc on solvents x, y, and z (Table II), amino acid analysis (Table III), and by analytical HPLC employing a triethylammonium phosphate/acetonitrile gradient system and monitoring at 210 nm to detect any possible weakly absorbing impurities. The analytical purity by this procedure showed all products free of HPLC-separable impurities to $\geq$ 95% as monitored on a Hewlett-Packard Model 3837 integrating recorder. In several cases, the amino acid hydrolysis conditions caused loss (through apparent rearrangement) of the pseudodipeptide units to ninhydrin-negative products. The presence and position of the pseudodipeptide units in compounds II and III were confirmed via partial enzymatic degradation, derivatization, and analysis of the resulting fragments via chemical ionization mass spectrometric analyses. Details of the latter procedure will be reported elsewhere.

The in vitro biological tests were performed in two distinct systems. The first involved the use of hemipituitaries of 20-day old female Sprague-Dawley rats. The LH and FSH agonist and antagonist activities were determined by incubating the hemipituitaries in Ringers solution and adding LH-RH and test peptides over 6 h as described elsewhere (8). Values were calculated in terms of nanograms of the standards LH-LER-1240-2 (0.60 NIH-LH-S1 (units/mg) and NIAMDD rat FSH RP-1 (2.1 x NIH-FSH-S1 units/mg.).

The second in vitro system utilized primary cell cultures of enzymatically dissociated rat anterior pituitary cells (9). Multiple dose levels of pseudopeptides and an LH-RH standard were tested for their abilities to increase the luteinizing hormone secretion rates by these cells. The ability of analogues to inhibit the release of luteinizing hormone stimulated by a constant concentration of LH-RH was the basis of the antagonist assay, where [D-pGlu^1,

Table II. Properties of Synthetic LH-RH Analogues.

	Peptide	HPLC Solvent System[a]	HPLC Ret'n Time (MIN)	TLC[b] R_f^x	TLC[b] R_f^y	TLC[b] R_f
I	LH-RH	A	9.05	0.24	0.35	
II	[Gly ψ Leu^{6-7}]LH-RH	A	12.7	0.43	0.39	-
III	[Tyr ψ Gly^{5-6}]LH-RH	A	11.9	0.52	0.50	-
IV	[Pro ψ $GlyNH_2^{9-10}$]LH-RH	A	12.0	0.42	0.44	-
V	[$DpGlu^1$, $D\text{-}Phe^2$, $D\text{-}Trp^3$, Gly ψ Leu^{6-7}]LH-RH	B	12.9	0.57	0.56	0.61
VI	[$D\text{-}pGlu^1$, $D\text{-}Phe^2$, $D\text{-}Trp^3$, D-Ser ψ Leu^{6-7}]LH-RH	B	10.7	0.56	0.53	0.60
VII	[$D\text{-}pGlu^1$, $D\text{-}Phe^2$, $D\text{-}Trp^{3,6}$ Pro ψ $GlyNH_2^{9-10}$]LH-RH	C	12.0	0.60	0.66	0.68
VIII	[Ac-Pro ψ $D\text{-}Phe^{1-2}$, $D\text{-}Trp^{3,6}$] LH-RH	C	14.7	0.64	0.64	0.67

a. HPLC Solvent System: A. Acetonitrile/0.25M Triethylammonium phosphate; pH 3.5; 12-25% gradient (15 min)
B. Acetonitrile/0.25M Triethylammonium phosphate; pH 3.5; 20-30% gradient (15 min)
C. Acetonitrile/0.25M Triethylammonium phosphate; pH 3.5; 25-50% gradient (25 min)

b. Tlc Solvent Systems: x, n-BuOH:HOAc:H_2O:pyridine (15:3:12:10); y, n-BuOH:HOAc:EtOAc:H_2O (1:1:1:1); z, EtOH:H_2O (7:3).

Table III. Amino-acid Analysis Ratios of LH-RH Analogs

Structure No.	Amino Acid Ratios										
	Glu	Phe	Trp	His	Ser	Tyr	Gly	Leu	Arg	Pro	Other[a,b]
II	1.00		0.84	0.81	0.91	0.97	1.04		0.87	1.13	0.92 Gly ψ Leu
III	0.98		0.81	0.91	0.78		1.00	1.03	1.02	1.02	N.D. Tyr ψ Gly
IV	0.99		0.83	1.02	1.00	1.08	1.00	1.03	0.90		N.D. Pro ψ Gly
V	0.96	1.04	0.90		0.93	0.92	1.01		0.94	1.05	1.06 Gly ψ Leu
VI	0.99	c	0.76		0.89	1.02	1.00		0.96	1.02	c
VII	1.05	1.10	1.64		0.92	1.03		0.96	0.90		N.D. Pro ψ Gly
VIII			1.76		0.82	0.98	1.00	0.99	0.94	1.10	N.D. Pro ψ D-Phe

a. Incorporated Pseudodipeptide

b. N.D. = not determined due to low ninhydrin response

c. D-Ser ψ Leu + Phe = 1.89, amino-acids peaks are virtually superimposable.

D-Phe^2, D-Trp^{3,6}]LH-RH was used as a standard and arbitrarily assigned a potency of 1.

Antiovulatory assays were carried out at the Mason Research Institute, Worcester, Mass., using female Sprague-Dawley rats. The peptides were dissolved in corn oil and injected subcutaneously on noon of proestrus and the ova were counted the next morning.

RESULTS AND DISCUSSION

As shown in Table IV, LH-RH analogues whose only structural variations involved amide bond replacements at 5-6, 6-7 and 9-10 displayed widely divergent biological activities. Though none were as active as LH-RH, LH-RH pseudopeptides III and IV with amide bond replacements at 5-6 or 9-10 were 1-2 orders of magnitude more active with respect to LH and FSH release than the analogue containing a 6-7 modified linkage. Since the 6-7 linkage has been shown to be the most susceptible site of degradation of the LH-RH molecule[2,3],

TABLE IV. IN VITRO AGONIST ACTIVITY OF PSEUDOPEPTIDES RELATED TO LH-RH

Pseudopeptide Analogue	Dose		LH[a]			FSH[a]			Relative Potency[b] LH-RH = 1
	Peptide ng/ml of medium	LH-RH ng/ml of medium	Δ, ng/ml of medium	SEM (±)	p	Δ,ng/ml of medium	SEM (±)	p	
[Gly ψ Leu$^{6-7}$]LH-RH	-	-	3	5	-	470	133	-	
		0.6	450	23	-	7856	1462	-	< 0.001
	100		5	3	ns	304	87	ns	
	1000		187	44	.01	5562	648	.001	
	10000		849	198	.001	9332	981	.001	
[Tyr ψ Gly$^{5-6}$] LH-RH	-	-	29	15	-	179	33	-	
		0.6	1057	148	-	5840	607	-	
	30		595	74	.001	4487	702	.001	0.02
	100		1256	105	.001	8364	829	.001	
	1000		>1607	-		12846	1106	.001	
[Pro ψ GlyNH$_2$$^{9-10}$]LH-RH	-	0.6	407	49	-	5627	281	-	
	0.6		-6	11	.001	-155	112	.001	0.10
	6		25	14	.001	-26	240	.001	
	60		140	8	.001	1966	282	.001	

[a]As determined by hemipituitary incubations - See experimental section.

[b]As determined in pituitary cell culture vs. standard - See experimental section.

the above results suggest that the 6-7 pseudolinkage induces an unfavorable conformational change. Perhaps pertinent is that the proposed Momany model(s) for LH-RH contains a bend or hairpin turn at the 6-7 junction (10), and, thus, insertion of the pseudo linkage at this site may have reduced the content of a preferred low energy conformer since the CH_2S substitution is more flexible than the amide bond. While a β-turn per se at this position seems to have been ruled out by the equipotent activity of an N^{α}-Me-Leu^7 LH-RH analogue (11), some type of bend in this region is likely, especially in view of residual activity found for cyclic analogues that link amino acids 1 and 10 (12). An additional correlation among these analogues is the greater overall lipophilicity observed in the 6-7 substituted analogue II , as ascertained by HPLC retention times (Table II); the interpretation that folding more readily exposes the now-modified bend region to the lipophilic reversed phase column substrate with which it may more readily interact seems possible although still speculative.

A substantially greater structure activity dependence of the pseudopeptides was noted in the in vitro results of the antagonist analogues (Table V). While the 6-7 amide bond proved intolerant to substitution even with a 6-D-residue

TABLE V. IN VITRO ANTAGONIST ACTIVITY OF PSEUDOPEPTIDES RELATED TO LH-RH.

Pseudopeptide Analogue	Dose		LH[a]			FSH[a]			Relative Potency[b]
	Peptide ng/ml of medium	LH-RH ng/ml of medium	Δ, ng/ml of medium	SEM (±)	p	Δ, ng/ml of medium	SEM (±)	p	[DpGlu^1, D-Phe^2, D-Trp^{3,6}]-LH-RH = 1
[D-pGlu^1, D-Phe^2, D-Trp^3, Gly ψLeu$^{6-7}$]LH-RH	-	-	6	6	<.001	467	124	<.001	< 0.001
	-	.6	450	23	-	7856	1462	-	
	10	.6	366	74	ns	4400	563	.05	
	100	.6	415	20	ns	7091	672	ns	
	1000	.6	380	53	ns	7061	803	ns	
	10,000	.6	268	15	.001	5807	373	ns	
[D-pGlu^1, D-Phe^2, D-Trp^3 D-Ser ψLeu$^{6-7}$]LH-RH	-	-	7	23	-	127	185	-	
	-	.6	532	144	-	5339	1282	-	
	100	.6	594	82	ns	4265	250	ns	< 0.001
	10000	.6	553	108	ns	4600	542	ns	
	10,000	.6	563	97	ns	4988	871	ns	
	50,000	.6	226	16	.05	2081	371	.05	
[D-pGlu^1, D-Phe^2, D-Trp^{3,6} Pro ψ GlyNH$_2$$^{9-10}$]LH-RH	-	-	-2	10		375	207		0.47 (.30-.71)
	-	.6	636	72	-	7241	953	-	
	10	.6	251	31	.001	3852	1251	.05	
	30	.6	164	27	.001	2420	305	.001	
	100	.6	19	25	.001	360	287	.001	
	1000	.6	-20	13	.001	-283	165	.001	
[Ac-Pro ψD-Phe$^{1-2}$, D-Trp^{3,6}]LH-RH	-	-	-16	2	<.001	-15	153	<.001	0.60 (0.40-1.0)
	-	.6	526	41	-	7871	231	-	
	30	.6	26	10	.001	773	225	.001	
	100	.6	25	24	.001	386	156	.001	
	1000	.6	9	5	.001	-23	75	.001	
	10,000	.6	5	11	.001	-117	96	.001	

[a] As determined by hemipituitary incubations - see experimental section.

[b] As determined in pituitary cell culture vs. standard - see experimental section.

(peptide VI), the 1-2 and 9-10 amide bonds were considerably more expendable. Peptide VII with the 9-10 pseudopeptide substitution retained more than 50% of its parents in vitro biological activity; moreover, compared to the corresponding amide linked structure, the [Ac-Pro ψ D-Phe$^{1-2}$, D-Trp^{3,6}]LH-RH antagonist (VIII) proved to be at least as active in the hemipituitary assay. These results and those of Pro ψ GlyNH$_2$$^{9-10}$ LH-RH suggest the possible importance of the carbonyl function of the peptide bond at 1-2 and 9-10 for agonist activity. Whether increased lipophilicity at the 1-2 position after substitution of the pseudopeptide linkage plays a role in these structure activity relationships is unknown at this time.

Both antagonists displaying substantial in vitro activity were next assayed for their in vivo antiovulatory activity in female adult rats by administering 250 μg peptide/animal on the day of proestrus.

Surprisingly both of the pseudopeptide analogues were inactive at dosages at which the corresponding analogues with amide linkages have been fully active in the rat antiovulatory assay (11). Work is in progress to determine to what extent this may be due to a transport problem, preferred degradation of the amide surrogates by unexpected mechanisms, or non-reversible binding to extracellular components, and to establish whether such findings are unique to LH-RH pseudopeptides. Other highly lipophilic analogues have shown a similar lack of correlation between in vitro and in vivo results of LH-RH analogue antagonists (14). By the use of suitably modified peptide analogues, it may be possible to distinguish between the problems of local and global lipophilicity of the analogues which perhaps are involved in the tight bending at the LH-RH receptor (in vitro) and the possible poor transportation of the analogue after subcutaneous injection (in vivo).

Our results suggest that the replacement of amide bonds in LH-RH analogue antagonists is not incompatible with retention of full biological potency, even when the replacement involves substantial steric and polarity changes. The site of the replacement does appear to be critical and this once again raises the question as to whether backbone elements in peptides may have a functional role. The observation that amide replacements at N and C terminal positions can yield highly active analogues tends to support the use of such substitutions as a mode of retarding or blocking peptide degradation by endo- and exopeptidases in the search for orally active analogues.

ACKNOWLEDGEMENTS

Appreciation is expressed to the NIH Contraceptive Development Branch for support of this work through Contract NO 1-HD-8-2882 and to Dr. Marvin Karten for helpful discussions. We are indebted to Mr. Scott Mack, Mr. Benjamin Van Osdol, Ms. Georgeann Reynolds and Ms. Karen Von Dessonneck for expert technical assistance.

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