Synthesis and Bioassay of Antagonists of the Luteinizing Hormone Releasing Hormone Having the Azagly^{10} Moiety

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Seven new analogs of the luteinizing hormone releasing hormone (LHRH), having an Azagly^{10} moiety, and three corresponding Gly^{10}-analogs were synthesized for bioassay and comparison of inhibitory potencies. This study was toward a possible advantage of the Azagly^{10} moiety to minimize C-terminal degradation, *in vivo*. Of the three procedures which were studied to achieve Azagly^{10}-peptides, the reaction of cyanate ion with hydrazides was the most favorable. Variations of substitution in position 1 were also studied. The data from the antiovulatory assay showed that an Azagly^{10} moiety may not depress activity, and may allow equal or even higher activity than the Gly^{10} moiety, depending on the analog. [N-Ac-D-Thr^1,D-*p*-Cl-Phe^2,D-Trp^{3,6},Azagly^{10}]-LHRH was more inhibitory than the corresponding Gly^{10}-analog. Based on pairs of analogs, the following relationships appeared: (1) N-Ac-D-Thr^1 was more effective than N-Ac-*p*-Cl-Phe^1; (2) The L-configuration of Ala as N-Ac-Ala^1– was more effective than the D–; (3) N-Ac-Ala^1 appeared more effective than the N-Ac-D-Thr^1; (4) D-Trp^6 appeared more effective than D-Phe^6. In an ultimate clinical use of an antagonist of LHRH to block ovulation, the Azagly^{10} moiety may be advantageous for limitation of enzymatic degradation.

Introduction

The luteinizing hormone releasing hormone (LHRH) functions in the mechanisms of evolution and conception. Over a thousand analogs of LHRH have apparently been synthesized, some of which were significant agonists, super-agonists or antagonists.

We have synthesized and bioassayed a series of analogs (Table I), most of which have the azaglycineamide (Azagly) moiety, I, in position 10. For three of these analogs, the corresponding peptides having the glycinamide (Gly) moiety, II, in position 10 were synthesized for comparison of inhibitory activities.

I, $-NH-NH-CO-NH_2$ II, $-NH-CH_2-CO-NH_2$

The rationale for synthesizing and bioassaying these analogs with Azagly was that the C-terminal might be protected, *in vivo*, against enzymic degradation in contrast to the corresponding Gly^{10} analogs. Such protection might not be observed by routine *in vivo* assays for activity, but might be important for subsequent studies toward the ultimate clinical use of an antagonist of LHRH to prevent ovulation.

Tab. I. Analogs of LHRH.

1. [N-Ac-D-*p*-Cl-Phe^{1,2},D-Trp^{3,6},Azagly^{10}]-LHRH
2. [(N-Ac-Pro-Pro)^1,D-*p*-Cl-Phe^2,D-Trp^{3,6}, Azagly^{10}]-LHRH
3. [(N-Ac-Pro-Pro)^1,D-*p*-Cl-Phe^2,D-Trp^{3,6}]-LHRH
4. [N-Ac-D-Ala^1,D-*p*-Cl-Phe^2,D-Trp^{3,6},Azagly^{10}]-LHRH
5. [N-Ac-Ala^1,D-*p*-Cl-Phe^2,D-Trp^{3,6},Azagly^{10}]-LHRH
6. [N-Ac-D-Thr^1,D-*p*-Cl-Phe^2,D-Trp^{3,6},Azagly^{10}]-LHRH
7. [N-Ac-D-Thr^1,D-*p*-Cl-Phe^2,D-Trp^{3,6}]-LHRH
8. [N-Ac-Thr^1,D-Phe^2,D-Trp^{3,6},Azagly^{10}]-LHRH
9. [N-Ac-Thr^1,D-Phe^2,D-Trp^{3,6}]-LHRH
10. [N-Ac-Thr^1,D-Phe^{2,6},D-Trp^3,Azagly^{10}]-LHRH

In all of the analogs, the amino acid residues in positions 4, 5, 7, 8, and 9 were identical to those in the same positions of LHRH, *i.e.*, Ser, Tyr, Leu, Arg and Pro. Variations were introduced at positions 1, 2, 3, 6, and 10. N-terminal residues (D-Ala, L-Ala, D-Thr, D-*p*-Cl-Phe, L-Thr and the dipeptide L-Pro-L-Pro) were N-acetylated.

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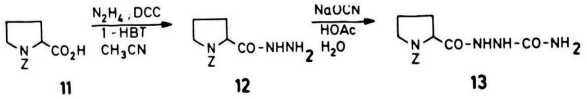
Variations of the solid phase method of Merrifield were used to synthesize the peptides. The glycinamide analogs (compounds **3**, **7**, and **9**) were prepared from benzhydrylamine resins. The azaglycinamide analogs (compounds **1**, **2**, **4**, **5**, **6**, **8** and **10**) were prepared from chloromethyl Merrifield resins by attaching the penultimate amino acid (Pro) to the resin and completing of the synthesis to the N-terminus. The C-terminal Azagly moiety was then introduced after the peptide was cleaved from the resin.

Two methods for introducing the Azagly residue were studied. Analogs **4**, **6**, **8**, and **10** were synthesized by cleaving the peptide-Merrifield resin with hydrogen fluoride to give the unprotected N-acetylated peptide carboxylic acids. Condensation of the latter with semicarbazide (azaglycinamide) by means of N–N′-dicyclohexylcarbodiimide (DCC) afforded the peptides for purification.

Analogs **1**, **2**, and **5** were synthesized by a variation of the azide procedure of Dutta *et al.* [1] which gave better yields and fewer difficulties with purification than the method which coupled semicarbazide by DCC. In this azide procedure, the peptide-Merrifield resins were subjected to hydrazinolysis to afford protected peptides as hydrazides. Treatment of these hydrazides with butyl nitrite in acidic dioxan gave azides which were then allowed to react with semicarbazide. The protecting groups were then removed with liquid HF. The conditions of hydrazinolysis may have possibly resulted in minor loss of the N-terminal acetyl group. Phillips [2], and Schmer and Kreil [3], have shown that complete removal of N-acetyl groups by hydrazinolysis is possible at 100 °C for about 17 h. All of the Azagly-containing peptides gave strong positive tests [4] for hydrazine following complete acid hydrolysis.

A new method of introducing the azaglycine moiety was extended. Dutta and Morley [5] reported the use of cyanate ion to convert hydrazides to aza-amino acid derivatives. In this method, a peptide hydrazide was treated with cyanate ion in acidic solution. We used N–Z–L-proline (**11**) as a model. Condensation of **11** with hydrazine by means of DCC in the presence of 1-hydroxybenzotriazole (1-HBT) afforded the N–Z–L-prolylhydrazide (**12**), which was isolated as the *p*-toluene sulfonate salt. When **12** was allowed to react with sodium cyanate in dilute aqueous acetic acid, N–Z–L-prolylazaglycinamide (**13**) was formed in high yield. This method appears to offer advantages for continuation of the synthesis of new types of Azagly[10]-analogs of LHRH.



All of the peptides were purified by combinations of gel-filtration counter-current distribution (CCD) partition chromatography, and high-performance liquid chromatography (HPLC).

Experimental

Amino acid derivatives were purchased from Peninsula Laboratories, San Carlos, California except for D-*p*-chlorophenylalanine which was provided by the Southwest Foundation for Research and Education, San Antonio, Texas. Alpha amino functions were protected by the Boc group except that the Aoc group was used for arginine. Side chain functions were protected by Z(Ser,Thr), Tos(Arg) and *o*-Br–Z(Tyr). Benzhydrylamine (BHA) resin was purchased from Beckman Bioproducts, Palo Alto, California. DCC, Et_3N, CH_2Cl_2, and DMF were distilled prior to use. All other chemicals were reagent grade.

General method of synthesis

The peptides were synthesized by the solid phase method using a Beckman Model 990 peptide synthesizer. Attachment of the first amino acid to a BHA resin was by double DCC-mediated coupling. Attachment of the first amino acid to a chloromethyl resin was by single coupling of the Boc amino acid cesium salt. Two coupling programs were employed for peptide chain elongation. Program B was identical to Program A except that step 14 was replaced by two steps, 14 and 15; step 14: DMF (3×2 min) and step 15: CH_2Cl_2 (3×2 min). If a ninhydrin test [7] indicated an incomplete acylation of amino groups, a second coupling was performed by a repetition of steps 6 through 14. Sequences resulting from incomplete coupling, even after double coupling were terminated by acetylation. Acetylation of the N-terminal amino acid moiety was accomplished by adding 25% acetic anhydride in CH_2Cl_2-pyridine in place of steps 10 and 11 of the coupling program. Peptides were removed from BHA resins by treatment with anhydrous liquid HF containing *ca.* 20% anisole for 1 h at 0 °C, as described [8].

TLC was performed on E. Merck silica gel G plates with detection by fluorescence, chlorine-tolidine spray, and ferric ferricyanide. Values for R_f^1, R_f^2, R_f^3 and R_f^4 refer to the systems: 1-butanol-acetic acid-ethyl acetate-water (1:1:3:1), ethanol-water

(7:3); 2-propanol-1M acetic acid (2:1); and 1-butanol-pyridine-acetic acid-water (50:33:1:40), respectively. Optical rotations were measured on a Perkin-Elmer Model 141 digital readout polarimeter. Amino acid analyses were performed on a Beckman Amino Acid Analyzer Model 119. Samples were hydrolyzed with constant boiling hydrochloric acid containing about 1 mg of phenol for 24 h at 110 °C in sealed evacuated tubes.

Biological assays for *in vitro* LH releasing and *in vivo* antiovulatory activity were carried out, as described [9], with one exception. The *in vitro* assay of [N-Ac-Ala[1],D-*p*-Cl-Phe[2],D-Trp[3,6],Azagly[10]]-LHRH was conducted using a culture of enzymatically dispersed anterior pituitary cells according to Vale *et al.* [10].

DCC couplings

Synthesis of [N-Ac-D-Thr[1], D-p-Cl-Phe[2], D-Trp[3,6], Azagly[10]]LHRH: To a mixture of 90 mg of [N-Ac-D-Thr[1],D-*p*-Cl-Phe[2],D-Trp[3,6],desGly[10]]-LHRH, 450 mg of semicarbazide, and 5 ml of DMF, was added 490 mg af DCC. The mixture was stirred for 28 h at room temperature, treated with 15 g of ice, and then the mixture was extracted 4 × with 20 ml portions of ethyl acetate. The organic extract was washed with 30 ml of water. Lyophilization of the combined aqueous extract afforded the crude peptide which was purified by gel filtration in 2 M acetic acid on a column of Sephadex G-25 and by semipreparative HPLC on a column of Bondapak C_{18} with 50% CH_3CN–0.01 M ammonium acetate, pH 5, as the eluting solvent; 15.2 mg of peptide was obtained. Analogs **4**, **6**, **8** and **10** were synthesized similarly.

Protected peptide hydrazides

To about 1 g of the protected peptide-Merrifield resin suspended in DMF, was added 0.77 ml of anhydrous hydrazine, and the mixture stirred 4 d at room temperature. The cleaved resin was filtered and washed with DMF. The combined filtrate and washings were evaporated, *in vacuo* (oil pump), and the residue was dried overnight over concd. H_2SO_4. After an ether wash, the residue was dissolved in an appropriate solvent. The following hydrazides were obtained by using the indicated dissolution and precipitation solvents [11, 12].

14 N-Ac-D-*p*-Cl-Phe-D-*p*-Cl-Phe-D-Trp-Ser(Bzl)-Tyr(BrZ)-D-Trp-Leu-Arg(Tos)-Pro-$NHNH_2$ (precursor of 1); methanol/ether.

15 N-Ac-Pro-Pro-D-*p*-Cl-Phe-D-Trp-Ser(Bzl)-Tyr(BrZ)-D-Trp-Leu-Arg(Tos)-Pro-$NHNH_2$ (precursor of 2); ethanol/ether.

16 N-Ac-Ala-D-*p*-Cl-Phe-D-Trp-Ser(Bzl)-Tyr(BrZ)-D-Trp-Leu-Arg(Tos)-Pro-$NHNH_2$ (precursor of **5**); DMF/H_2O.

Azide couplings

Synthesis of N-Ac-D-p-Cl-Phe-D-p-Cl-Phe-D-Trp-Ser(Bzl)-Tyr(BrZ)-D-Trp-Leu-Arg(Tos)-Pro-NHNH-CO-NH₂: The general procedure of Klausner and Bodanszky [13] was followed. To a solution of 0.467 g (0.266 mM) of hydrazide 14 in 2.5 ml of DMF at −30 °C, was added 0.22 ml (1.33 mM) of 6 M HCl in dry dioxan, followed by 0.08 ml of 50% isoamyl nitrite in isoamyl alcohol (0.298 mM, 12% excess). After 30 min at −25 to −30 °C, the solution was cooled to −60 °C. Triethylamine (0.185 ml, 1.33 mM) was added, followed by semicarbazide hydrochloride (35.6 mg, 0.319 mM, 20% excess), and additional triethylamine (0.044 ml, 0.319 mM). The suspension was stirred at 4 °C for 24 h then at room temperature for two days. A further 35.6 mg-portion of semicarbazide and 0.044 ml of triethylamine were added, and the mixture was stirred for three days. The product was precipitated with water and filtered. The same procedure was used for all three hydrazides.

N-Z-L-Prolylhydrazide p-toluenesulfonate 12: To 598 mg (2 mM) of N-Z-L-proline and 280 mg (2 mM) of 1-HBT in 6 ml of CH_2Cl_2, was added 458 mg (2.4 mM) of DCC. The mixture was swirled for *ca.* 5 min at room temperature, and filtered into a solution of 1.30 g (39.2 mM) of anhydrous N_2H_4 in 8 ml of CH_3CN with vigorous magnetic stirring. After 5 min, the mixture was evaporated to a small volume on a rotating evaporator. To remove residual N_2H_4, 3 ml of 1-butanol was added and evaporated, and this step was repeated. Ethyl acetate (5 ml) was added, and some insoluble material was removed by filtration. The filtrate was treated with 380 mg of *p*-toluenesulfonic acid monohydrate which resulted in a clear solution, from which a crystalline solid rapidly separated. Filtration afforded 773 mg (89%) of a solid, m.p. 154–166 °C. Two recrystallizations from methanol/ethyl acetate raised the m.p. to 177.5–180 °C; $[\alpha]_D^{23}$ −31.4 °C (c 1.04 MeOH) TLC R_f^1 0.71, R_f^2 0.63, R_f^3 0.72, R_f(0.64. The analytical data were in agreement with $C_{20}H_{25}N_3O_6S$.

N-Z-L-Prolylazaglycinamide (**13**): To 179 mg (0.41 mM) of **12** in 2 ml of warm water containing 0.05 ml of acetic acid, was added 31 mg (0.48 mM) of sodium cyanate. The solution was kept at room temperature for 45 min and at 4 °C for 18 h. Filtration afforded 101 mg (80%) of **13**, 187–189 °C. Recrystallization of the product from 2-propanol raised the m.p. to 187–189.5 °C (reported [1] m.p. 189–190 °C); $[\alpha]_D^{25}$ −56.6 °C (c 1.44 MeOH), $[\alpha]_D^{23}$ −90.6 °C (c 1.42 DMF) (reported [1] $[\alpha]_D^{24}$ −43.6 °C (c 1.4 DMF)); TLC R_f^1 0.75, R_f^2 0.73, R_f^3 0.73, R_f^4 0.69. The analytical data were in agreement with $C_{14}H_{18}N_4O_4$.

Results and Discussion

Dutta *et al.* [1] reported the synthesis of some analogs of LHRH which have Azagly[10] moieties.

They found that some of their analogs had agonist and others had antagonist activities, in certain assays. Simpson *et al.* [14] found [D-Nal(2)^6, Azagly^{10}]-LHRH (Nal(2) is 3(2-naphthalene)-alanine) to be one of the most potent known agonists. Since the Azagly residue in position 10 of antagonists might protect the C-terminal from enzymatic degradation, *in vivo*, in contrast to enzymic attack of the naturally occurring Gly^{10} in LHRH, it was considered important to extend the chemical and biological data on Azagly^{10} analogs of LHRH, and particularly for analogs which have multiple substitutions for the amino acids in LHRH. These substitutions have been based upon the results of investigations by several groups of investigators, including Ling and Vale [15]; Prasad *et al.* [16]; Coy *et al.* [17]; Rivier *et al.* [18]; Channabasavaiah and Stewart [19].

Our synthesis of seven new Azagly^{10} analogs resulted from our use and comparison of three procedures to introduce the Azagly^{10} moiety. The newest procedure involved the use of cyanate ion in an acidic medium to convert hydrazides into Azagly moieties, and appeared to provide a simpler and better procedure. For a preliminary model, this method was used to synthesize Z–L–prolylazaglycinamide (**13**); the analytical data and melting point of **13** were in agreement, but the optical rotation was considerably higher than that in data reported by Dutta *et al.* [1].

The general experience of investigators, who have synthesized analogs of LHRH toward more effective and potent inhibitors of the release of LH for the control of ovulation, has been that relationships between sequence and anti-ovulatory activity may have some generally, but the generality may be limited. The relationships may be applicable to restricted groups of sequence changes. On the basis of such generality and limitation, the following interpretations can be made of the antiovulatory activities of the ten new analogs according to the *in vivo* data of Table III.

Analog 1 with the N–Ac–D-*p*-Cl–Phe^1 moiety is compared with analog **6**, with the N–Ac–D–Thr^1 moiety; the rest of the sequence of both analogs is identical, [–D-*p*-Cl–Phe^2, D–Trp^{3,6}, Azagly^{10}]–LHRH. The N–Ac–D–Thr^1-peptide showed 100% inhibition at a dosage of 25 μg/rat, but the N–Ac-*p*-Cl–Phe^1-analog was inactive at this dosage, which shows the superiority of N–Ac–D–Thr^1 over N–Ac-*p*-Cl–Phe^1, for this pair.

Analog **2** has the Azagly^{10} moiety and analog **3** has the Gly^{10} moiety, with the rest of the sequence of both analogs being identical, [(N–Ac–Pro–Pro)^1, D-*p*-Cl–Phe^2, D–Trp^{3,6}–]–LHRH. The analog with Azagly^{10} showed 60% inhibition at a level of 50 μg/rat and the Gly^{10} analog showed 20% inhibition at 25 μg/rat. Analog **2** may have contained a little of the deacetylated peptide. Possibly, the

Tab. II. Amino acid analytical data[c].

Peptides	Arg	Tyr	Leu	Pro	Ser	Ala	Thr	Gly	Phe	NH_3	*p*ClPhe
1	1.05	0.98	0.99	1.10	1.00						2.3
2[a]	1.02	1.02	1.06	3 × 1.01	1.00					0.87	
3	0.97	0.96	0.87	3 × 0.99	0.99			0.98		1.27	
4[b]	1.09	0.99	1.01	1.04	0.91	0.96					1.08
5	1.00	1.07	1.03	1.01	0.98	1.06					
6	0.95	0.98	1.02	1.11	0.97		0.97			1.95	
7	1.03	0.97	0.99	1.13	0.93		0.96	0.99		1.77	
8	0.98	1.00	1.02	0.98	0.96		0.89		1.00	1.17	
9	0.93	0.95	0.98	1.07	1.04		0.99	1.07	0.95	1.16	
10	1.06	0.89	0.98	1.16	1.00		0.94		2 × 0.98	1.16	

[a] This product behaved as a mixture of two peptides, which were difficult to separate. The limitation of sample and lack of feasibility to resynthesize this peptide justified assay of the sample. This sample may have contained a little of the deacetylated peptide; [b] Purification of this analog was difficult. Eventually, a preparation was obtained which showed two approximately equal peaks by HPLC and two spots by TLC; R_f values of 0.73 and 0.92. The spot having R_f[3] 0.73 was ninhydrin positive, but the spot of R_f[3] 0.62 was ninhydrin negative. Since the analysis for amino acids was satisfactory, it was concluded that the peptide with R_f 0.73 may be deacetylated and the peptide with R_f 0.62 is the N–Ac–D–Ala^1-peptide; [c] Qualitative tests for hydrazine in the hydrolysate were positive. Tryptophan was generally detected, but not quantitated. Parachlorophenylalanine was detected, but not quantitated. Parachlorophenylalanine and tryptophan were quantitated as a pair using a separate standard containing these two amino acids.

Azagly[10]-analog might be more effective than the Gly[10] analog.

Analog **4**, having the N-Ac-D-Ala moiety and analog **5** having the N-Ac-Ala[1] moiety, have the same remaining sequence, [-D-*p*-Cl-Phe[2], D-Trp[3,6], Azagly[10]]-LHRH. The N-Ac-Ala[1] analog elicited 50% inhibition at a level of 6 μg/rat and the N-Ac-D-Ala[1] analog exhibited 14% inhibition at a level of 6.5 μg/rat. This comparison indicates that the L-configuration of Ala in position 1 is superior to the D-configuration. Analog **4** may have contained a little of the deacetylated peptide.

Analog **5**, with the N-Ac-Ala[1] moiety, and analog **6**, with the N-Ac-D-Thr[1] moiety, have the same remaining sequence, [-D-*p*-Cl-Phe[2], D-Trp[3,6], Azagly[10]]-LHRH. The N-Ac-D-Thr-analog caused 100% inhibition at 25 μg/rat and the M-Ac-Ala[1] analog caused 50% inhibition at a level of 6 μg/rat. Although there was a 4-fold difference in the test levels, the biological data indicate preference of N-Ac-Ala over N-Ac-D-Thr for position 1 in this pair of analogs.

Analog **6** has the Azagly[10] moiety and analog **7** has the Gly[10] moiety, but both analogs are otherwise identical, having [N-Ac-D-Thr[1], D-*p*-Cl-Phe[2], D-Trp[3,6]-]-LHRH. The Azagly[10] analog caused 50% inihibition at a level of 6.5 μg/rat and the corresponding Gly[10] analog at the same level caused

Table III. Antiovulatory activities.

Analog	Dose μg/rat	Rats ovulating/treated	No. of ova/SEM ovulating rat (±)	Inhibition %
1	25	6/6	12.3 ± 0.7	0
Control	–	5/5	12.4 ± 1.1	0
2	50	2/5	4.4 ± 2.8	60
Control	–	5/5	12.4 ± 1.1	0
3	25	4/5	9.6 ± 2.5	20
4	25	2/6	3.8 ± 2.5	67
	6.5	6/7	11.9 ± 2.0	14
Control	–	11/11	13.4 ± 0.6	0
5	6	4/8	5.6 ± 2.4	50
	–	6/6	11.5 ± 1.5	
6	25	0/6	0	100
	12	3/7	5.4 ± 2.6	57
	6.5	5/10	6.8 ± 2.3	50
Control	–	11/11	13.4 ± 0.6	0
7	25	1/5	0.8 ± 0.8	80
	6.25	4/5	8.4 ± 2.3	20
Control	–	8/8	11.9 ± 7	0
8	200	0/11	0	100
Control	–	15/15	12.5 ± 0.72	0
	50[a]	3/6	4.3 ± 2.3	50
	50[b]	1/6	0.17 ± 0.17	83.3
	50[c]	1/8	1.3 ± 1.2	87.5
Control	–	5/5	9.0 ± 1.3	0
9	200	1/7	1.28 ± 1.28	86
	100	2/9	1.8 ± 1.3	77.8
	50	4/9	5.2 ± 2.1	55.6
Control	–	6/6	13.16 ± 1.11	0
	200[d]	0/6	0	100
Control	–	5/5	10.6 ± 0.7	0
10	100	0/6	0	100
	50	7/10	7.1 ± 1.9	30
Control	–	5/5	9.0 ± 1.3	0

[a] Dispersed in medium 30–40 min before injection; [b] dispersed in medium 60–90 min before injection; [c] dispersed in medium 195 min before injection; [d] reassayed after 3 months.

a 20% inhibition, showing a superiority of Azagly[10] over Gly[10].

Analog 8 having the Azagly[10] moiety and analog **9** having the Gly[10] moiety were otherwise identical, having [N-Ac-Thr[1], D-Phe[2], D-Trp[3,6]-]-LHRH. The Azagly[10] analog caused 100% inhibition at a level of 200 μg/rat and the corresponding Gly[10] analog caused an 86–100% inhibition at 200 μg/rat, which possibly favors the Azagly[10] analog, although the error of the assay limits appraisal. Interestingly, when analog 8 having the Azagly[10] moiety was dispersed in the medium for assay, the effectiveness of inhibition was improved, since one-fourth (50 μg) of the test level increased inhibition from 50 to 83%. The dispersion of the sample led to more effective inhibition.

Analog **9** having a D-Trp[6] moiety and analog **10** having a D-Phe[6] moiety were otherwise identical with the sequence, [N-Ac-Thr[1], D-Phe[2], Azagly[10]]-LHRH. The D-Trp[6]-analog elicited 100% inhibition at a level of 200 μg/rat and the corresponding D-Phe[6]-analog elicited 100% inhibition at 100 μg/rat indicating superiority of D-Trp[6] over D-Phe[6]; a level of 50 μg of the D-Trp[6]-analog inhibited 50% in comparison with 50 μg of the D-Phe[6]-analog which inhibited 30%.

The data on the *in vitro* assay of these analogs for antagonist activity are in Table IV. All of the analogs except **5** were assayed as described by Bowers *et al.* [20], which was based upon using pituitaries from 20-day old female rats of the CDl strain. The analog and LHRH were added at appropriate periods of incubation, and the release of LH and FSH were recorded as ΔLH and ΔFSH. The LH and FSH values are in nanograms, based on standards. Analog **5** was assayed *in vitro*, by the

Table IV. *In vitro* antagonist activity.

Analog	dose* ng/ml	LH Δng/ml	SEM (±)	P	FSH Δng/ml	SEM (±)	P
1	–	724	24	–	6816	224	–
	1	585	36	< 0.05	7546	797	n.s.
	3	419	28	< 0.001	6942	266	n.s.
	10	477	118	n.s.	6729	594	n.s.
	30	233	40	< 0.001	5146	466	< 0.01
2	–	336	21	–	4505	266	–
	3	228	7	< 0.001	4710	498	n.s.
	10	151	43	< 0.01	1834	713	< 0.01
	30	59	24	< 0.001	1411	217	< 0.001
	100	69	17	< 0.001	1639	308	< 0.001
3	–	336	21	–	4505	266	–
	1	237	35	< 0.05	4070	489	n.s.
	3	185	15	< 0.001	3308	325	< 0.02
	10	204	9	< 0.001	3233	363	< 0.002
	30	36	46	< 0.001	1784	311	< 0.001
4	–	465	20	–	9052	885	–
	3	164	18	< 0.001	5097	295	< 0.01
	10	64	17	< 0.001	1347	214	< 0.001
	30	4	13	< 0.001	555	242	< 0.001
5	–	123	20	–			
	0.02	702	47	< 0.001			
	0.67	410	22	< 0.001			
	2.0	389	10	< 0.001			

Analog	dose* ng/ml	LH Δng/ml	SEM (±)	P	FSH Δng/ml	SEM (±)	P
6	–	487	45	–	9811	980	–
	3	58	17	< 0.001	1526	289	< 0.0
	10	45	13	< 0.001	1111	109	< 0.0
	30	25	10	< 0.001	555	112	< 0.0
	–	465	20	–	9052	885	–
	1	125	11	< 0.001	4409	276	< 0.0
	3	14	31	< 0.001	1179	859	< 0.0
	10	14	61	< 0.001	928	201	< 0.0
7	–	343	47	–	5428	745	–
	1	139	25	< 0.001	3345	663	n.
	3	118	30	< 0.001	2771	503	< 0.0
	10	25	12	< 0.001	138	244	< 0.0
	30	37	39	< 0.001	—319	240	< 0.0
8	–	560	86	–	5769	482	–
	10	113	13	< 0.001	1721	94	< 0.0
	30	28	6	< 0.001	261	198	< 0.0
	100	14	10	< 0.001	—54	219	< 0.0
	1000	9	4	< 0.001	—152	141	< 0.0
	–	487	45	–	9811	980	–
	3	284	40	< 0.01	8154	1048	n.
	10	188	39	< 0.001	3743	629	< 0.0
	30	32	10	< 0.001	780	115	< 0.0
9	–	562	158	–	6530	1218	–
	30	60	12	< 0.01	956	303	< 0.0
	100	5	37	< 0.01	—168	244	< 0.0
	1000	33	24	< 0.01	459	42	< 0.0
10	–	784	56	–	7746	430	–
	10	188	38	< 0.001	2412	570	< 0.0
	30	72	46	< 0.001	1586	323	< 0.0
	100	49	37	< 0.001	359	328	< 0.0
	1000	52	16	< 0.001	416	100	< 0.0

* In presence of 0.6 ng of LHRH per ml of medium.

procedure of Vale *et al.* [10], which uses cultures of dispersed pituitary cells. Analogs **1**, **3**, and **7** at levels of 1 μg significantly ($p < 0.05$–0.001) inhibited the release of LH. Analogs **3**, **4**, and **6** significantly ($p < 0.001$) inhibited at levels of 3 μg. Analogs **8**, **9**, and **10** showed significant ($p < 0.01$–0.001) inhibition at levels of 10, 30 and 10 μg, respectively.

As a group, these nine analogs were more inhibitory for the release of LH than for FSH, since analogs **1**, **2**, **3**, and **7** did not inhibit release of FSH at levels effective for inhibition of the release of LH, but analogs **4**, **6**, **8**, **9**, and **10** did inhibit release of FSH at the same level which inhibited LH. By the culture of dispersed pituitary cells, analog **5**, at a level of 0.02 nanograms, significantly ($p < 0.001$) inhibited the release of LH.

It is evident that the Azagly^{10} moiety in comparison with the Gly^{10} moiety in analogs which are otherwise identical does not cause a significant reduction in activity for inhibition of ovulation. The Azagly^{10} analogs are apparently equal to the Gly^{10} analogs in activity, and perhaps may be desirable to study the metabolic stability of Azagly^{10} analogs under conditions which more closely simulate potential clinical use.

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[1] A. S. Dutta, B. J. A. Furr, and M. B. Giles, Chem. Soc. Perkin I **1979,** 379.
[2] D. M. P. Phillips, Biochem. J. **86,** 397 (1963).
[3] G. Schmer, and G. Kreil, Analytical Biochem. **29,** 186 (1969).
[4] F. Feigl and V. Anger in: Spot Tests in Inorganic Analysis, Elsevier Biomedical Press, New York 1975, p. 340.
[5] A. S. Dutta and J. S. Morley, Chem. Soc. Perkin I **1975,** 1712.
[6] J. Humphries, Y.-P. Wan, T. Wasiak, K. Folkers, and C. Y. Bowers, J. Med. Chem. **22,** 774 (1979).
[7] E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. T. Cook, Anal. Biochem. **34,** 595 (1970).
[8] J. Humphries, G. Fisher, Y.-P. Wan, K. Folkers, and C. Y. Bowers, J. Med. Chem. **17,** 569 (1974).
[9] J. Humphries, Y.-P. Wan, K. Folkers, and C. Y. Bowers, J. Med. Chem. **20,** 1674 (1977).
[10] W. Vale, G. Grant, M. Amoss, R. Blackwell, and R. Guillemin, Endocrinol. **91,** 562 (1972).
[11] M. Ohno and C. B. Anfinsen, J. Am. Chem. Soc. **89,** 5994 (1967).
[12] J. K. Chang, M. Shimizu, and S. Wang, J. Org. Chem. **41,** 3255 (1976).
[13] Y. S. Klausner and M. Bodanszky, Bioorg. Chem. **2,** 354 (1973).
[14] J. J. Nestor (Jr.), T. L. Ho, R. A. Simpson, B. L. Horner, and G. H. Jones, Seventh American Peptide Symposium, Abstracts, June 14–19, 1981, Madison, Wisconsin, p. 138.
[15] N. Ling and W. Vale, Biochem. Biophys. Res. Commun. **63,** 801 (1975).
[16] K. U. Prasad, R. W. Roeske, R. L. Weitl, J. A. Vilchez-Martinez, and A. V. Schally, J. Med. Chem. **19,** 492 (1976).
[17] D. H. Coy, J. A. Vilchez-Martinez, E. J. Coy, and A. V. Schally, J. Med. Chem. **39,** 423 (1976).
[18] J. Rivier, M. Brown, C. Rivier, and W. Vale in: Peptides 1976, A. Loffet, Ed., Editions del-Universite de Bruxelles, Brussells 1977, p. 427.
[19] K. Channabasavaiah and J. M. Stewart, Biochem. Biophys. Res. Commun. **86,** 1266 (1979).
[20] C. Y. Bowers, J. Humphries, T. Wasiak, K. Folkers, G. A. Reynolds, and L. E. Reichert (Jr.), Endocrinol. **106,** 674 (1980).