Antiovulatory Potency and Conformation of an Antagonist of the Luteinizing Hormone-Releasing Hormone Having Six D-Amino Acids

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[N-Ac-Thr[1],D-Phe[2],D-Trp[3,6]]-LHRH was the model antagonist of LHRH, which was the basis for the design, synthesis and bioassay of seven peptides having four, five and six D-amino acids, which resulted from three single, three double, and one triple introductions of D-amino acids in positions 4, 5 and 8 of the model. Only the analog with six D-amino acids, [N-Ac-Thr[1],D-Phe[2],D-Trp[3],D-Ser[4],D-Tyr[5],D-Trp[6],D-Arg[8]]-LHRH, had antiovulatory activity which was higher than that of the model antagonist, *i.e.*, 70% antiovulatory activity at 25 μg/rat compared with 50% activity at 50 μg/rat, respectively. Empirical energy calculations gave a conformational structure for [N-Ac-Thr[1],D-Phe[2],D-Trp[3], D-Ser[4],D-Tyr[5],D-Arg[6],D-Arg[8]]-LHRH which is similar to that calculated for previous potent antagonists. These results are a basis of new designs of antagonists having D-substituents in up to ten positions toward effective inhibitors of ovulation by the parenteral and oral routes of administration.

Introduction

Since the elucidation of the chemistry and endocrinology of the luteinizing hormone-releasing hormone (LHRH), [1, 2], <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, several research groups sought to achieve an extraordinarily effective antagonist of this hormone which might function as a contraceptive for humans. Since 1973, these research groups have made stepwise progress in the design and synthesis of antagonists, and it was found that exemplary antagonists do show antiovulatory activity in a primate [3], as well as in the rat models.

Present objectives of these research groups are the design of antagonists which are more potent, have prolonged action, and oral activity.

We have studied an approach, by increasing the number of D-amino acids in an analog, not only toward increased potency, but toward a peptide of greater stability to proteolytic enzymes. Initially, we chose the antagonist [N-Ac-Thr[1], D-Phe[2], D-Trp[3,6]]-LHRH as the model for new analogs with additional D-amino acids.

Previously, our studies [4] and those of others had emphasized position 1, and it had been learned that reasonably potent antagonists may have either the L- or the D-configuration in position 1 as well as more than one amino acid moiety [5, 6] *i.e.*, undecapeptides, *etc.*

Many investigators found that single substitutions can be detrimental in one analog, but beneficial in an analog having a different sequence. For example, replacing D-Phe by D-pClPhe in [D-Phe[2], D-Ala[6]]-LHRH decreased antiovulatory activity [7], but the same change in the sequence [N-Ac-Thr[1], D-Phe[2], D-Trp[3,6]]-LHRH increased inhibitory activity *in vitro* as well as antiovulatory activity [6, 8]. An example of detrimental and beneficial effects on activity by an exchange of configuration in position 1 are the two peptides, [D-Phe[2], D-Trp[3], D-Phe[6]]-LHRH, [9], and [D-Phe[2], Pro[3], D-Phe[6]]-LHRH, [10], which differ by D-Trp[3] and Pro[3], respectively. When <Glu was changed to D-<Glu in position 1 for the first of these two analogs, the antiovulatory potency was increased but when <Glu in position 1 of the second of these two analogs was changed to D-<Glu, the antiovulatory activity decreased [11].

[N-Ac-Thr[1], D-Phe[2], D-Trp[3,6]]-LHRH has a well-tested antiovulatory activity [8]. For this antagonist, as a model analog, we now report the effects on antiovulatory activity of introducing one.

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two and three changes to the D-configuration in positions 4, 5 and 8 by the synthesis and bioassay of the 7 possible new analogs having 4, 5 and 6 D-amino acids.

Experimental Section

Amino acid derivatives were purchased from Peninsula Laboratories (San Carlos, CA). The α-amino functions were protected by the BOC-group, except for Arg which had the AOC-group. Side-chain functions were protected by Z- for Ser and Thr, Tos- for Arg, and *o*-Br–Z for Tyr. Benzylhydrylamine (BHA) resin hydrochloride was obtained from Beckman Bioproducts (Palo Alto, CA). Dicyclohexylcarbodiimide and triethylamine were distilled prior to use. All other chemicals were reagent grade.

Synthesis

The peptides were synthesized by the solid-phase method using a Beckman Model 990 peptide synthesizer. The attachment of the first amino acid was achieved by using the double-coupling procedure.

The coupling program during the peptide chain elongation involved the following successive operations (mix-time): 1, CH_2Cl_2 (2×, 2 min); 2, 50% TFA in CH_2Cl_2, w/v (1×, 2 min); 3, 50% TFA in CH_2Cl_2, w/v (1×, 30 min); 4, CH_2Cl_2 (3×, 2 min); 5, 2-propanol (2×, 2 min); 6, CH_2Cl_2 (4×, 2 min); 7, 10% NEt_3 in CH_2Cl_2 v/v (2×, 2 min); 8, 10% NEt_3 in CH_2Cl_2 v/v (1×, 10 min); 9, CH_2Cl_2 (4×, 2 min); 10, amino acid derivative in CH_2Cl_2 (three-fold excess over peptide attached, 2 min); 11, DCC in CH_2Cl_2 (at least three-fold excess over peptide attached, 3 to 4 h); 12, CH_2Cl_2 (3×, 2 min); 13, 2-propanol (2×, 2 min); 14, CH_2Cl_2 (6×, 2 min).

In case the ninhydrin test [12] indicated an incomplete coupling, a double-coupling was performed consisting of operations 6 through 14.

The acetylation of the N-terminus was accomplished by adding 25% acetic anhydride in methylenchloride instead of steps 10 and 11 in the coupling program. The reaction time was 20 min.

The completed peptide – BHA resin was treated with anhydrous liquid HF containing *ca.* 20% anisole for 1 h at 0 °C as described [13].

Purification

The following chromatographic systems were employed: Gel filtration over Sephadex G-15 (100 × 2.75 cm) in 20% AcOH (A); Sephadex G-25 (100 × 2.75 cm) in 12% AcOH (B), and in 20% AcOH (C); partition chromatography over Sephadex G-25 in 1-BuOH, AcOH, water (4:1:5) (D); and chromatography over Sephadex LH-20 in 1-BuOH, AcOH, water (6:10:90) with 4–7% MeOH (E) or 1–10% MeOH (F).

Peptide peaks were located, and progress on purification was monitored on silica TLC plates using solvent system 1. Fraction cuts were made for purity at the expense of product yield. The purity was examined in the following TLC solvent systems: (1) 1-BuOH, AcOH, AcOEt, H_2O (1:1:3:1); (2) EtOH, H_2O (7:3); (3) 1-BuOH, AcOH, H_2O (4:1:5, upper phase); (4) 1-BuOH, pyridine, AcOH, H_2O (50:33:1:40); (5) 2-ProOH, 1 N AcOH (2:1); (6) 1-BuOH, pyridine, AcOH, H_2O (30:20:6:24).

Amino acid analyses on *ca.* 0.5 mg samples, hydrolyzed in 6 N HCl with or without the presence of traces of phenol, were performed on a Beckman Model 119 Amino Acid Analyzer.

Optical rotations were measured in a Perkin-Elmer 141 digital readout polarometer.

1. [N–Ac–Thr¹, D–Phe², D–Trp³, D–Ser⁴, D–Trp⁶]–LHRH

Purification C, 2× D. Amino acid analysis gave Thr 0.93 (1), Ser 0.98 (1), Pro 1.09 (1), Gly 1.00 (1), Leu 0.87 (1), Tyr 0.99 (1), Phe 0.96 (1), Arg 1.02 (1). R_f^1 0.69, R_f^2 0.79, R_f^4 0.71, R_f^5 0.91. $[\alpha]_D^{24} = -34.4°$ (c 0.407, MeOH).

2. [N–Ac–Thr¹, D–Phe², D–Trp³, D–Tyr⁵, D–Trp⁶]–LHRH

Purification C, D. Amino acid analysis gave Thr 0.92 (1), Ser 1.03 (1), Pro 1.17 (1), Gly 1.01, Leu 0.89 (1), Tyr 1.01, Phe 0.95 (1), Arg 1.03 (1). R_f^1 0.7, R_f^2 0.8, R_f^4 0.72, R_f^5 0.92. $[\alpha]_D^{24} = -30.77°$ (c 0.221, MeOH).

3. [N–Ac–Thr¹, D–Phe², D–Trp³, D-Trp⁶, D–Arg⁸]–LHRH

Purification C, D. Amino acid analysis gave Thr 0.94 (1), Ser 0.95 (1), Pro 1.09 (1), Gly 1.01 (1), Leu 0.99 (1), Tyr 0.98 (1), Phe 0.98 (1), Arg 1.03 (1). R_f^1 0.73, R_f^2 0.83, R_f^3 0.55, R_f^4 0.77.

4. [N–Ac–Thr¹, D–Phe², D–Trp³, D–Ser⁴, D–Tyr⁵, D–Trp⁶]–LHRH

Purification B, E. Amino acid analysis gave Thr 0.98, Ser 0.90 (1), Pro 1.08 (1), Gly 0.95 (1), Leu 0.95 (1), Tyr 1.02 (1), Phe 1.01, Arg 1.02 (1). R_f^1 0.57, R_f^2 0.73, R_f^3 0.45, R_f^4 0.69.

5. [N–Ac–Thr¹, D–Phe², D–Trp³, D–Ser⁴, D–Trp⁶, D–Arg⁸]–LHRH

Purification C, 2× D. Amino acid analysis gave Thr 0.80 (1), Ser 1.1 (1), Pro 1.12 (1), Gly 1.13 (1), Leu 0.93 (1), Tyr 0.94 (1), Phe 0.92 (1), Arg 1.06 (1). R_f^1 0.76, R_f^2 0.87, R_f^3 0.74, R_f^4 0.76. $[\alpha]_D^{24} = -19.78°$ (c 0.809, MeOH).

6. [N–Ac–Thr¹, D–Phe², D–Trp³, D–Tyr⁵, D–Trp⁶, D–Arg⁸]–LHRH

Purification A, D. Amino acid analysis gave Thr 0.96 (1), Ser 0.96 (1), Pro 1.05 (1), Gly 0.95 (1), Leu 0.91 (1), Tyr 0.99 (1), Phe 0.98 (1), Arg 0.91 (1). R_f^1 0.73, R_f^2 0.89, R_f^3 0.46, R_f^6 0.71.

7. [N-Ac-Thr¹, D-Phe², D-Trp³, D-Ser⁴, D-Tyr⁵, D-Trp⁶, D-Arg⁸]-LHRH

Purification C, D, F. Amino acid analysis gave Thr 0.96 (1), Ser 1.00 (1), Pro 1.00 (1), Gly 1.07 (1), Leu 0.99 (1), Tyr 1.00 (1), Phe 1.00 (1), Arg 0.99 (1). R_f^1 0.71, R_f^2 0.88, R_f^3 0.44, R_f^4 0.65.

Biological assays

The peptides were assayed for their LHRH agonist and antagonist activities, *in vitro*, using rat pituitaries, and for activity to inhibit ovulation in rats, as described [10].

Results and Discussion

The results of the *in vitro* and the antiovulatory assays in rats are shown in Table I and II, respectively. All of these analogs were essentially devoid of agonist activity at the highest dosages tested; for brevity, these data were omitted. The inhibitory effect on the FSH release parallels that on the LH release.

The first three analogs (1, 2, 3 in Table I), are those with a single configurational change in position four, and five and eight of the model antagonist, [N-Ac-Thr¹, D-Phe², D-Trp³,⁶]-LHRH. The inhibitory activities, *in vitro*, of these three analogs did not parallel the antiovulatory activities. Of these three analogs, the D-Tyr⁵ (2) and the D-Arg⁸ (3) analogs showed decreased inhibitory activity in comparison to the D-Ser⁴ (1) analog. The D-Ser⁴-analog (1) inhibited ovulation in the rat by 25% at 200 μg/rat; the D-Tyr⁵ and D-Arg⁸-analogs (2, 3) showed 0% and 40% inhibition of ovulation, respectively, at the same level. It appears that the change of configuration in position 4 allows a conformation which still resembles that of the model antagonist, because both peptides are active, but differ by a

Table I. *In vitro* antagonist activity of the analog as compared to the model analog: [N-Ac-Thr¹,D-Phe²,D-Trp³,⁶]-LHRH.

Analog				Dose* analogue ng/ml medium	LH Δ ng/ml medium	SEM (±)	P	FSH Δ ng/ml medium	SEM (±)	P
	Pos. 4	Pos. 5	Pos. 8							
	Parent sequence			–	562	158		6530	1218	–
				30	60	12		956	303	<.01
				100	5	37		—168	244	<.001
				1000	33	24		459	42	<.001
1.	D-Ser			–	208	48	–	5486	817	
				30	54	24	<.02	2968	332	<.02
				100	27	15	<.01	923	389	<.01
2.		D-Tyr		–	208	48	–	5486	817	–
				30	86	15	~.05	3380	608	n. s.
				100	106	7	n. s.	3500	121	~.05
				1000	8	3	~.001	297	210	<.001
3.			D-Arg	–	208	48	–	5486	817	–
				30	112	31	~.01	3903	524	n. s.
				100	80	22	~.05	4131	1023	n. s.
				1000	5	31	~.01	1003	460	<.001
4.	D-Ser	D-Tyr		–	784	56	–	7746	430	–
				10	191	32	<.001	2415	366	<.001
				30	152	20	<.001	1980	364	<.001
				100	48	22	<.001	969	315	<.001
5.	D-Ser		D-Arg	–	344	19	–	8213	187	–
				30	247	39	.05	4909	644	<.001
				100	210	28	~.01	4095	406	<.001
				1000	183	47	~.01	3142	466	<.001
6.		D-Tyr	D-Arg	–	315	37	–	5540	570	–
				3	186	22	<.02	3548	461	.02
				30	263	39	n. s.	4302	355	n. s.
				300	276	32	n. s.	3801	258	.02
7.	D-Ser	D-Tyr	D-Arg	–	335	35	–	6028	460	–
				10	27	7	<.001	1277	248	<.001
				30	75	14	<.001	1744	306	<.001

* In presence of 0.6 ng/ml LHRH in medium.

Table II. Antivulatory activity of the analog as compared to the model analog: [N-Ac-Thr[1],D-Phe[2],D-Trp[3,6]]-LHRH.

	Pos. 4	Pos. 5	Pos. 8	Dosage μg/rat	Rats ovulating/treated	No. ova/ovulating rat ± SEM	% inhibition
	Model Analog			200	0/6	0 ± 0	100
	Control			–	5/5	10.6 ± .7	–
				100	2/9	1.8 ± 1.3	78
				50	4/9	5.2 ± 2.1	56
	Control			–	6/6	13.2 ± 1.1	–
1.	D-Ser			200	3/4	6.8 ± 3.1	25
	Control			–	8/8	11.9 ± .7	–
2.		D-Tyr		200	5/5	13.2 ± 1.0	0
	Control			–	8/8	11.9 ± .7	–
3.			D-Arg	200	3/5	9.4 ± 4.4	40
	Control			–	6/6	13.0 ± 1.1	–
4.	D-Ser	D-Tyr		200	5/5	9.6 ± 1.0	0
	Control			–	5/5	9.0 ± 1.3	–
5.	D-Ser		D-Arg	200	4/4	12.5 ± 0.75	0
	Control			–	6/6	13.0 ± 1.1	–
6.		D-Tyr	D-Arg	200	5/5	13.4 ± .5	0
	Control			–	8/8	11.9 ± .7	–
7.	D-Ser	D-Tyr	D-Arg	25	2/7	2.3 ± 1.6	71
				25	2/4	4.0 ± 2.3	50
				12.5	4/6	7.7 ± 2.5	33
	Control				5/5	12.4 ± 1.1	

factor of 4. The presumed increase of resistance against enzymatic degradation at position 4 may not compensate for a decreased binding strength of the peptide which can be the cause of the lower activity of the analog. The difference in antiovulatory activity between the D-Tyr[5] and the D-Arg[8] analogs 2 and 3 may be caused by the larger gain of stability against enzymatic attack for the D-Arg[8] analog 3.

The second group of three analogs (4–6) consists of sequences with configurational changes in two of the positions four, five, and eight of the model antagonist. None of these three peptides showed antiovulatory activity at the level of 200 μg/rat. The order of *in vitro* inhibitory potency was [D-Ser[4],D-Trp[5]] (4) > [D-Ser[4],D-Arg[8]] (5) > [D-Tyr[8],D-Arg[5]] (6). Of significance, analog 4 showed higher inhibitory activity *in vitro* than analog 1 which has only one configurational change. Both analog 5 and 6 were less active than any of the analogs with single changes. The order of *in vitro* inhibitory potencies indicates that the potency decreases if the configurational changes are shifted from the N-terminal toward the C-terminal region of the analog. This decrease in activity may be caused by the greater influence of configurational changes in the C-terminal region on the overall conformation of the molecule. This effect was previously indicated by the data on the peptides with the single changes.

The relationships between the D-configurations and the activity, *in vitro*, for these seven analogs, as based on the model antagonist, may be expressed by the following five observation.

(a) All three single substitutions of the D-configuration in positions 4, 5 and 8 resulted in a reduction of potency.

(b) All three two-fold substitutions of D-configurations in positions 4, 5 and 8 resulted in reduction of potency.

(c) Only the two-fold substitution of the D-configurations in positions 4 and 5 resulted in an increase in activity in comparison with the three single substitutions of the D-configurations in positions 4, 5 and 8.

(d) The two-fold substitutions of the D-configurations in positions 4 and 5 resulted in an increase in activity in comparison with the two-fold substitutions in positions 4 and 8 and in 5 and 8.

(e) The two-fold substitution in positions 4 and 8 resulted in an increase in activity over that of substitutions in positions 5 and 8.

[N-Ac-Thr^1, D-Phe^2, D-Trp^3, D-Ser^4, D-Tyr^5, D-Trp^6, D-Arg^8]-LHRH, analog 7, inhibited the release of LH *in vitro* at the same level as that of the model antagonist, but afforded the same degree of inhibition of ovulation at 1/2 the dosage for the model antagonist. This result indicates that analog 7 is bound at the receptor with an affinity comparable to that of the model antagonist. Analog 7 may be presumed to have an increased resistance against enzymatic degradation. Possibly, differences in effects of analog 7 on transport, *in vivo*, after administration can be excluded, because the polarities of analog 7 and the model are not expected to be different.

Empirical energy calculations on [N-Ac-Thr^1, D-Phe^2, D-Trp^3, D-Ser^4, D-Tyr^5, D-Trp^6, D-Arg^8]-LHRH gave a structure to be described separately [14], which is in favorable agreement with structures calculated for other potent antagonists of LHRH. These energy calculations were based upon those which had been made in 1978 for LHRH by Momany [15].

[N-Ac-Thr^1, D-Phe^2, D-Trp^3, D-Ser^4, D-Tyr^5, D-Trp^6, D-Arg^8]-LHRH, analog 7, contains the most D-substitutions of any known effective antagonist of LHRH, and is a basis for new designs of antagonists of up to ten D-substituents which might have even higher antiovulatory activity and enzymatic stability, perhaps toward effective orally active inhibitors of ovulation.

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