Structural Requirements in Positions 1, 2, 3, and 6 of the Luteinizing Hormone-Releasing Hormone (LH-RH) for Antiovulatory Activity[1]

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Sixteen analogues of the luteinizing hormone-releasing hormone (LH-RH) were synthesized by the solid-phase method. In new and surprising relationships, it was found that the substitution of D-Trp into position 3 of [D-<Glu[1],D-Phe[2],amino acid[3],D-Phe[6]]-LH-RH significantly enhanced the antiovulatory potency, but substitution by Pro, *N*-Me-Phe, *N*-Me-Leu, or L-Trp reduced antiovulatory activity. The substitution of L-<Glu in position 1 of [D-Phe[2],Pro[3],D-Phe[6]]-LH-RH by cyclohexylcarbonyl (Chc), benzoyl (Bz), Ac, Hyp, Ac-Met, hydrogen, Pro, and D-<Glu residues, and the substitution of D-Phe in position 2 by D-Trp, D-His, D-Phg, and L-Phe residues resulted in analogues with no antiovulatory activity at 750 μg/rat. Structural requirements for the design of inhibitors of higher potency have been discussed.

Our discovery of the LH-RH inhibitor [Leu[2],Leu[3]]-LH-RH[3] and the more potent inhibitor [Leu[2],Leu[3],D-Ala[6]]-LH-RH[4] led us and other investigators to synthesize more analogues based on a 2,3,6-trisubstituted LH-RH sequence. The search resulted in the further discovery that two Pro[3]-substituted peptides and one D-Trp[3]-substituted peptide, [D-Phe[2],Pro[3],D-Trp[6]]-LH-RH,[5] [D-Phe[3],Pro[3],D-Phe[6]]-LH-RH,[6] and [D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH,[7] were approximately equipotent and completely inhibited ovulation at 750 μg/rat sc. [D-Phe[2],Pro[3],D-Phe[6]]-LH-RH also inhibited the release of LH and FSH, in vitro, by [Phe[2]]-Met-enkephalinamide.[8] Although certain other 2,3,6-trisubstituted LH-RH sequences, for example, [D-Phe[2],*N*-Me-Leu[3],D-Phe[6]]-LH-RH,[9] were of comparable potency in the rat antiovulation assay, none was 100% effective at lower dosages.

In attempts to achieve more potent inhibitors, additional positions in the basic 2,3,6-trisubstituted LH-RH sequence have been modified. The replacement of the C-terminal segment, -Pro-Gly-NH_2, by the less polar Pro-NHEt residue has led to analogues with significant activity in vitro but with reduced antiovulatory activity.[10-12]

We have examined sequences having further modifications at position 1, a position also emphasized by Momany from theoretical conformational considerations.[13] The analogue [chlorambucil[1],Leu[2],Leu[3],D-Ala[6]]-LH-RH was the first reported irreversible inhibitor of LH-RH in vitro and was more potent than [Leu[2],Leu[3],D-Ala[6]]-LH-RH.[14] The inhibitors [cyclopentylcarbonyl(Cpc)[1],D-Phe[2],Pro[3],D-Phe[6]]-,[11] [Cpc[1],D-Phe[2],Pro[3],D-Trp[6]]-,[9] and [Cpc[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH[15] were of comparable potency in vitro to their corresponding <Glu[1] analogues but were inactive in the antiovulation assay at 750 μg/rat. The analogue [D-<Glu[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH was later found to be more potent in the rat antiovulatory assay than the corresponding L-<Glu analogue.[16]

Recently, we reported that Ac-[Pro[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH completely inhibited ovulation at 200 μg/rat and was as potent as the corresponding D-<Glu[1] analogue.[15] The related analogue Ac-[D-Pro[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH was less active, and 750 μg/rat was required to inhibit ovulation completely.[15]

Furthermore, we have also recently described a new category of ovulation inhibitors based on linear LH-RH analogues having more than ten residues.[17] The undecapeptide [(<Glu-Pro)[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH was found to completely inhibit ovulation at 200 μg/rat. This was the first report that inhibitors could be designed having sequences longer than a decapeptide.

We now describe a series of analogues which were designed to emphasize the structural requirements of positions 1 and 2 for antiovulatory activity. We will also examine the effect of structural variation at position 3 in sequences which have a D-<Glu residue in position 1.

Experimental Section

Amino acid derivatives were purchased from Peninsula Laboratories. α-Amino functions were protected by the Boc group, except for Arg in which case the Aoc group was used. Side-chain functionalities were protected by benzyl for Ser and Hyp, tosyl for Arg, and *o*-Br-Z for Tyr. L-Pyroglutamic acid was incorporated using Z-<Glu-OH. The other N-terminal residues were incorporated using their respective acids, except for acetyl for which acetic anhydride was used. Benzhydrylamine (BHA) resin hydrochloride was obtained from Beckman Bioproducts. The abbreviations Chc and Phg have been used to designate cyclohexylcarbonyl and phenylglycyl, respectively.

Attachment of the First Amino Acid. The peptides were synthesized by solid-phase methodologies in a Beckman Model 990 peptide synthesizer. The BHA resin hydrochloride was neutralized with 25% NEt_3 in CH_2Cl_2 (v/v) for 10 min, washed four times with CH_2Cl_2, and coupled with a threefold excess of Boc-Gly and DCC in CH_2Cl_2 for 4 h. The resin was given three washes with CH_2Cl_2, three washes with 10% NEt_3 in CH_2Cl_2 (v/v), four washes with CH_2Cl_2, and then submitted to a second coupling with Boc-Gly and DCC (threefold excess) for ca. 12 h. After successive washes with CH_2Cl_2, 2-propanol, and CH_2Cl_2, the resulting Boc-Gly-BHA resin gave a negative ninhydrin test.[18]

Peptide Elongation. The coupling program involved the following successive operations (number of times each step performed, mixing time): 1, CH_2Cl_2 (three washes, 2 min); 2, 50% TFA in CH_2Cl_2, w/v (one prewash, 2 min); 3, 50% TFA in CH_2Cl_2, w/v (deprotection, 30 min); 4, CH_2Cl_2 (three washes, 2 min); 5, 2-propanol (two washes, 2 min); 6, CH_2Cl_2 (four washes, 2 min); 7, 10% NEt_3 in CH_2Cl_2, v/v (two prewashes, 2 min); 8, 10% NEt_3 in CH_2Cl_2, v/v (neutralization, 10 min); 9, CH_2Cl_2 (four washes, 2 min); 10, amino acid derivative (addition of threefold excess, 2 min); 11, DCC in CH_2Cl_2 (addition of ca. threefold excess, coupling for 3 to 4 h); 12, CH_2Cl_2 (three washes, 2 min); 13, 2-propanol (three washes, 2 min); 14, CH_2Cl_2 (three washes, 2 min).

In order to ensure complete coupling of amino functions, as indicated by the ninhydrin test,[18] a double-coupling procedure was performed consisting of repeating operations 6 to 14.

Cleavage and Deblocking Procedure. The completed peptide-BHA resin was treated with anhydrous liquid HF containing ca. 20% anisole for 1 h at 0 °C as described.[4]

Purification Procedures. The chromatographic systems were as follows (v/v): Sephadex G-25 (96 × 2.75 cm) with 10% AcOH (A); 1.3% AcOH (B); by partition chromatography with 1-BuOH–AcOH–H_2O (4:1:5) (C); CM-Sephadex (26 × 1.4 cm) with a gradient of NH_4OAc buffers [1 mM (pH 4.5) to 250 mM] (D); Sephadex LH-20 (96 × 2.75 cm) with 1-BuOH–H_2O (6:100) (E);

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or with mixtures of 1-BuOH–AcOH–H_2O in the ratios of 9:10:90 (F), 6:10:90 (G), or 1:10:90 (H), or with pure MeOH (I); silica gel (15 × 1 cm) with 1-BuOH–AcOH–H_2O (4:1:5, upper phase) (J).

Peptide peaks were located at 260 or 280 nM and were examined for purity by TLC with baths from the following systems (v/v): R_f^1, 1-BuOH–AcOH–EtOAc–H_2O (1:1:1:1); R_f^2, EtOAc–pyridine–AcOH–H_2O (5:5:1:3), R_f^3, 2-propanol–1 N AcOH (2:1); R_f^4, 1-BuOH–pyridine–AcOH–H_2O (30:20:6:24); R_f^5, $CHCl_3$–MeOH–concentrated NH_4OH (60:45:20); R_f^6, 1-BuOH–AcOH–H_2O (4:1:5, upper phase); R_f^7, EtOH–H_2O (7:3). Fraction cuts were made for purity at the expense of product yield. The latter was based on starting amino acid resin.

Amino acid analyses on ca. 0.5-mg samples hydrolyzed in 6 N HCl, with or without the addition of 4% thioglycolic acid, were performed as described.[6] Optical rotations were measured in a Perkin-Elmer 141 digital readout polarimeter.

[Chc^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification A, D; yield 55.8%; amino acid analysis gave Phe 2 × 0.97, Pro 2 × 1.07, Ser 0.97, Tyr 0.94, Leu 1.05, Arg 0.87, Gly 1.1; R_f^1 0.83, R_f^3 0.92, R_f^4 0.74; $[\alpha]^{24}_D$ −57.50° (*c* 0.9634, MeOH).

Bz-des-<Glu^1-[D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification G; yield 13%; amino acid analysis Phe 2 × 1.08, Pro 2 × 1.00, Ser 0.88, Tyr 1.05, Leu 0.91, Arg 0.88, Gly 0.91; R_f^1 0.58, R_f^2 0.79, R_f^3 0.49, R_f^5 0.69.

Ac-des-<Glu^1-[D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification A, D; yield 54.7% amino acid analysis gave Phe 2 × 1.05, Pro 2 × 0.98, Ser 0.95, Tyr 1.03, Leu 0.96, Arg 0.97, Gly 1.03; R_f^1 0.78, R_f^3 0.87, R_f^4 0.64.

[Hyp^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification D; yield 75.3%; amino acid analysis gave Phe 0.99, Pro 2 × 1.08, Ser 0.96, Tyr 0.96, Leu 1.03, Arg 0.86, Gly 1.05; R_f^1 0.76, R_f^3 0.87, R_f^4 0.43; $[\alpha]^{24}_D$ −57.63° (*c* 0.9952, MeOH).

Ac-[Met^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification H; yield 39.1%; amino acid analysis gave Met 1.00, Phe 2 × 1.05, Pro 2 × 0.8, Ser 1.1, Tyr 1.04, Leu 1.08, Arg 0.93, Gly 1.1; R_f^1 0.80, R_f^2 0.96, R_f^3 0.92, R_f^4 0.69; $[\alpha]^{24}_D$ −50.96° (*c* 0.9978, MeOH).

Des-<Glu^1-[D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification A, D, C; yield 35.8%; amino acid analysis gave Phe 2 × 0.99, Pro 2 × 1.05, Ser 0.90, Tyr 0.96, Leu 1.04, Arg 0.93, Gly 1.09; R_f^1 0.80, R_f^3 0.89, R_f^4 0.42; $[\alpha]^{24}_D$ −81.19° (*c* 0.9576, MeOH).

[Pro^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification F; yield 67%; amino acid analysis gave Pro 3 × 0.96, Phe 2 × 1.01, Ser 1.01, Tyr 0.98, Leu 1.05, Arg 1.02, Gly 1.04; R_f^1 0.65, R_f^2 0.92, R_f^3 0.8; $[\alpha]^{24}_D$ −88.14° (*c* 1.06, MeOH).

[D-Trp^2,Pro^3,D-Phe^6]-LH-RH: purification A, D; yield 49.9%; amino acid analysis gave Glu 1.08, Pro 2 × 0.91, Ser 1.10, Tyr 1.04, Phe 0.99, Leu 0.88, Arg 1.02, Gly 0.92; R_f^1 0.66, R_f^2 0.78, R_f^3 0.77; $[\alpha]^{24}_D$ −61.36° (*c* 1.162, MeOH).

[D-His^2,Pro^3,D-Phe^6]-LH-RH: purification E, G, J; yield 6.2%; amino acid analysis gave Glu 0.96, His 0.78, Pro 2 × 1.05, Ser 0.92, Tyr 1.06, Phe 1.15, Leu 0.88, Arg 1.15, Gly 1.08; R_f^1 0.72, R_f^2 0.97, R_f^3 0.71, R_f^4 0.72, R_f^5 0.69.

[D-Phg^2,Pro^3,D-Phe^6]-LH-RH: purification E; yield 50.9%; amino acid analysis gave Glu 1.00, Phe 1.10, Pro 2 × 1.06, Ser 0.96, Tyr 1.03, Leu 1.07, Arg 0.92, Gly 1.00; R_f^1 0.54, R_f^2 0.84, R_f^4 0.76.

[L-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification A, D; yield 53.3%; amino acid analysis gave Glu 1.03, Phe 2 × 1.08, Pro 2 × 1.04, Ser 0.91, Tyr 0.97, Leu 0.93, Arg 0.96, Gly 0.97; R_f^1 0.75, R_f^2 0.87, R_f^3 0.77; $[\alpha]^{24}_D$ −78.17° (*c* 0.9722, MeOH).

[D-<Glu^1,D-Phe^2,D-Trp^3,D-Phe^6]-LH-RH: purification G; yield 36%; amino acid analysis gave Glu 1.07, Phe 2 × 0.9, Ser 1.04, Tyr 1.00, Leu 0.85, Arg 1.1, Pro 0.96, Gly 1.09; R_f^1 0.70, R_f^2 0.96, R_f^3 0.64, R_f^4 0.66, R_f^5 0.80, R_f^7 0.64.

[D-<Glu^1,D-Phe^2,Pro^3,D-Phe^6]-LH-RH: purification B, G; yield 19%; amino acid analysis gave Glu 1.07, Phe 2 × 0.95, Pro 2 × 0.97, Ser 1.06, Tyr 0.99, Leu 0.91, Arg 1.04, Gly 1.09; R_f^1 0.66, R_f^2 0.95, R_f^3 0.57, R_f^4 0.64, R_f^5 0.80, R_f^7 0.57; $[\alpha]^{24}_D$ −61.11° (*c* 1.106, MeOH).

[D-<Glu^1,D-Phe^2,MePhe^3,D-Phe^6]-LH-RH: purification B, D; yield 31%; amino acid analysis gave Glu 0.91, Phe 2 × 0,87, Ser 1.1, Tyr 0.94, Leu 1.08, Arg 1.03, Pro 1.0, Gly 1.1; R_f^1 0.69, R_f^2 0.96, R_f^3 0.63, R_f^4 0.67, R_f^5 0.85, R_f^7 0.62; $[\alpha]^{24}_D$ −64.56° (*c* 1.046, MeOH).

[D-<Glu^1,D-Phe^2,MeLeu^3,D-Phe^6]-LH-RH: purification B, E, G; yield 21%; amino acid analysis gave Glu 1.02, Phe 2 × 0.95, Ser 1.01, Tyr 1.07, Leu 0.92, Arg 0.99, Pro 1.08, Gly 1.02; R_f^1 0.69, R_f^2 0.97, R_f^3 0.63, R_f^4 0.67, R_f^5 0.84, R_f^7 0.63; $[\alpha]^{24}_D$ −81.83° (*c* 1.018, MeOH).

[D-<Glu^1,D-Phe^2,D-Phe^6]-LH-RH: purification G, D, I; yield 30%; amino acid analysis gave Glu 0.99, Phe 2 × 0.99, Ser 1.02, Tyr 1.06, Leu 0.99, Arg 0.95, Pro 0.99, Gly 1.03; R_f^1 0.63, R_f^2 0.98, R_f^3 0.79, R_f^5 0.47; $[\alpha]^{24}_D$ −55.23° (*c* 1.071, MeOH).

Biological Assays. The peptides were assayed for their agonist and LH-RH antagonist activities in vitro using whole rat pituitaries and for their ability to inhibit ovulation in rats as described.[6]

Results and Discussion

The results of the assays in vitro are in Table I. The analogues were essentially devoid of agonist activity at the highest dosages tested.

The first group of analogues was synthesized to evaluate the importance of position 1 (<Glu) for the activity of [D-Phe^2,Pro^3,D-Phe^6]-LH-RH, which completely inhibited ovulation at 750 μg/rat and suppressed the release of LH and FSH by 0.6 ng of LH-RH in vitro at 0.1 μg. The analogues which contained Chc (**1**), Bz (**2**), Ac (**3**), Hyp (**4**), Ac-Met (**5**), hydrogen (**6**), Pro (**7**), and D-<Glu (**13**) residues in position 1 were all inactive at 750 μg/rat as ovulation inhibitors. Analogues **1**, **3**, **4**, **6**, and **13** did significantly inhibit at 0.1 μg in vitro, especially analogue **3** and the shortened chain analogue **6**, but, in general, reduced inhibition potencies were observed.

The second group of analogues consisted of sequences based on the formula [aromatic amino acid^2,Pro^3,D-Phe^6]-LH-RH in which D-Trp (**8**), D-His (**9**), D-Phg (**10**), and L-Phe (**11**) were in position 2. These analogues did not inhibit ovulation at 750 μg/rat, and the in vitro inhibition potency decreased in the order D-Phe > D-Trp > D-Phg and L-Phe > D-His.

Therefore, the effect on in vitro inhibition potency of the large side-chain aromatic moiety in analogue **8** was less than that resulting from omission of the side-chain $-CH_2-$ as in analogue **10**, which, in turn, was less than that resulting from the substitution of the more polar imidazole group of analogue **9**. The retention of low in vitro inhibition activity by analogue **11** was anticipated, since des-Gly^{10}-[Phe^2,Leu^3,D-Ala^6]-LH-RH ethylamide had previously been found to inhibit the in vitro response of 0.3 ng of LH-RH at dosages of 0.1 and 1 μg.[10]

The third group of analogues was synthesized to evaluate the effect of substitution of amino acid residues in position 3 when a D-<Glu residue was in position 1. The D-Trp^3 analogue **12** inhibited ovulation in nine out of ten rats (90%) at 350 μg/rat (no. of ova/ovulating rat equals 1.5 ± 1.5) and in three out of four rats (75%) at 200 μg/rat (no. of ova/ovulating rat equals 2.25 ± 2.25). Analogue **12** was, therefore, more active than [D-Phe^2,Pro^3,D-Phe^6]-LH-RH. In contrast, the analogues with Pro (**13**), *N*-Me-Phe (**14**), *N*-Me-Leu (**15**), and L-Trp (**16**) residues in position 3 did not inhibit ovulation at 750 μg/rat. The observation that analogue **12** had enhanced antiovulatory activity with respect to [D-Phe^2,Pro^3,D-Phe^6]-LH-RH but that analogues **13** and **15** had greatly reduced activity was unexpected, because [D-Phe^2,Pro^3,D-Phe^6]-LH-RH, [D-Phe^2,Pro^3,D-Trp^6]-LH-RH, [D-Phe^2,*N*-Me-Leu^3,D-Phe^6]-LH-RH, and [D-Phe^2,D-Trp^3,D-Trp^6]-LH-RH were essentially equipotent as ovulation inhibitors.

In the in vitro assays, analogue **12** gave complete inhibition of LH and FSH release at 0.1 and 0.03 μg. Analogue **15** also strongly inhibited at 0.1 μg, but the Pro^3 analogue **13** and analogues **14** and **16** were less active.

Some conclusions are evident for the design of ovulation inhibitors based on the LH-RH sequence. The minimum structural requirements are: (1) substitution in positions

Table I. In Vitro Agonist and Antagonist Activity of the Analogues[a]

		dose		LH			FSH		
no.	analogues of LH-RH	peptide, μg/mL of medium	LH-RH ng/mL of medium	Δ, ng/mL of medium	SEM (±)	*p*	Δ, ng/mL of medium	SEM (±)	*p*
1	[Chc[1],D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	457	26		5824	706	
		0.1	0.6	172	24	<0.001	2418	167	<0.001
		1	0.6	22	19	<0.001	160	93	<0.001
				51	22		268	204	
		10		23	35	ns	214	83	ns
2	Bz-des-<Glu[1]-[D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	408	87		2330	275	
		0.1	0.6	292	31	ns	2584	637	ns
		1	0.6	106	40	~0.01	783	240	<0.01
		10	0.6	56	15	<0.01	322	56	<0.001
				−9	6		60	43	
		100		28	6	<0.01	0.25	59	ns
3	Ac-des-<Glu[1]-[D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	442	6		5705	669	
		0.1	0.6	56	20	<0.001	427	87	<0.001
		1	0.6	16	12	<0.001	31	158	<0.001
				4	5		151	54	
		100		8	4	ns	−106	139	ns
4	[Hyp[1],D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	269	20		5726	663	
		0.1	0.6	127	18	<0.001	1828	216	<0.001
		1	0.6	112	27	<0.01	1567	251	<0.001
		10	0.6	32	9	<0.001	123	193	<0.001
		100	0.6	−11	14	<0.001	−110	198	<0.001
				14	3		156	75	
		100		0	6	ns	−7	88	ns
5	Ac-[Met[1],D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	151	32		1359	32	
		0.1	0.6	437	157	ns	2292	356	<0.05
		1	0.6	190	17	ns	754	198	<0.02
		10	0.6	43	20	<0.05	316	146	<0.001
		100	0.6	10	10	<0.01	215	171	<0.001
				−8	11		−57	58	
		100		25	14	ns	214	171	ns
6	des-<Glu[1]-[D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	321	56		2933	233	
		0.1	0.6	84	12	<0.01	1087	198	<0.001
		1	0.6	14	3	<0.001	112	124	<0.001
				−2	3		−63	84	
		100		13	2	<0.01	77	45	ns
7	[Pro[1],D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	645	12		3350	492	
		1	0.6	628	20	ns	1317	301	<0.01
		100	0.6	67	27	<0.001	404	41	<0.001
		100		29	10	ns	35	26	ns
				7	15		48	71	
8	[D-Trp[2],Pro[3],D-Phe[6]]-LH-RH		0.6	145	12		3698	634	
		0.1	0.6	109	15	ns	3943	413	ns
		1	0.6	56	10	<0.001	1984	286	<0.05
		10	0.6	25	5	<0.001	1417	308	<0.01
				12	4		119	152	
		10		8	1	ns	454	66	ns
9	[D-His[2],Pro[3],D-Phe[6]]-LH-RH		0.6	227	46		2243	224	
		1	0.6	346	90	ns	3113	290	0.05
		10	0.6	115	10	<0.05	1697	158	ns
		100	0.6	6	2	~0.001	748	68	<0.001
				−42	25		−53	110	
		100		−2	1	ns	49	59	ns
10	[D-Phg[2],Pro[3],D-Phe[6]]-LH-RH		0.6	243	30		9523	869	
		1	0.6	181	42	ns	4953	1028	<0.01
		10	0.6	28	19	<0.001	890	544	<0.001
				12	4		650	182	
		100		2	4	ns	19	93	<0.02
11	[L-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.6	295	53		3673	463	
		0.1	0.6	313	38	ns	4128	401	ns
		1	0.6	100	33	~0.01	1582	290	<0.01
		10	0.6	11	25	<0.001	293	169	<0.001
				−25	14		−61	69	
		100		5	21	ns	−298	212	ns
12	[D-<Glu[1],D-Phe[2],D-Trp[3],D-Phe[6]]-LH-RH		0.47	451	15		5173	506	
		0.03	0.47	88	17	<0.001	2330	570	<0.02
		0.1	0.47	63	17	<0.001	1120	135	<0.001
		1	0.47	13	6	<0.001	871	207	<0.001
		100		8	9	ns	255	99	ns
				−15	29		445	362	
13	[D-<Glu[1],D-Phe[2],Pro[3],D-Phe[6]]-LH-RH		0.47	451	15		5173	506	
		0.03	0.47	197	42	<0.001	3406	190	<0.01
		0.1	0.47	144	10	<0.001	3282	242	<0.01
		1	0.47	42	6	<0.001	1300	112	<0.001
		100		31	14	ns	262	110	ns
				−15	29		445	362	

Table I (Continued)

no.	analogues of LH-RH	dose: peptide, μg/mL of medium	dose: LH-RH, ng/mL of medium	LH: Δ, ng/mL of medium	LH: SEM (±)	LH: p	FSH: Δ, ng/mL of medium	FSH: SEM (±)	FSH: p
14	[D-<Glu[1],D-Phe[2],MePhe[3],D-Phe[6]]-LH-RH		0.6	599	14		3350	492	
		0.1	0.6	433	60	<0.05	3020	269	ns
		1	0.6	91	24	<0.001	897	134	<0.001
		10	0.6	47	29	<0.001	266	83	<0.001
		10		42	14	ns	71	129	ns
				16	27		48	71	
15	[D-<Glu[1],D-Phe[2],MeLeu[3],D-Phe[6]]-LH-RH		0.6	537	87		2642	423	
		0.03	0.6	228	27	<0.01	1006	177	<0.01
		0.1	0.6	15	40	<0.001	481	222	~0.001
		100		85	63	ns	84	243	ns
				−50	47		276	133	
16	[D-<Glu,D-Phe[2],D-Phe[6]]-LH-RH		0.6	622	59		2945	387	
		0.03	0.6	507	52	0.05	3232	187	ns
		0.1	0.6	273	14	<0.001	1725	175	<0.02
		1	0.6	31	18	<0.001	232	71	<0.001
		100		63	17	<0.01	597	101	<0.001
				−9	6		−19	47	

[a] For brevity, not all dosages have been reported.

2 and 3, which generates inhibitory activity in vivo; (2) substitution by D-Phe or D-Trp in position 6, which enhances potency and stability in vivo; (3) a D-Phe residue is apparently very important in position 2.

For 2,3,6-trisubstituted sequences, position 3 can accomodate such residues as Pro, *N*-Me-Leu, and D-Trp and retain full activity at 750 μg/rat. Other substitutions have also been reported[6] for position 3 that resulted in partial activity at this dosage.

In the case of 1,2,3,6-tetrasubstituted analogues, the structural requirements for position 3 are more critical. The reduced activity of the Pro[3] and *N*-Me-Leu[3] analogues, as opposed to the high activity of the D-Trp[3] analogues, may be a consequence of a detrimental shift in the equilibrium of trans and cis conformers caused by substitution in position 1.[19]

To date, the most potent ovulation inhibitors, which are completely effective at 200 μg/rat, are based on the formula [residue[1],D-Phe[2],D-Trp[3],D-Trp[6]]-LH-RH. Position 1 can equally well accomodate residues of the L configuration, such as Ac-Pro and Ac-Hyp[9], and the D configuration, such as D-<Glu. The observation that the fragment <Glu-Pro- is also completely acceptable further illustrates the lack of specificity of position 1 in this sequence and now allows a more detailed examination to be made of the effect of peptide elongation from the N terminus.

It is interesting to note that the presence of such nonpolar residues as Cpc and Chc in both Pro[3] and D-Trp[3] sequences results in the elimination of antiovulatory activity at 750 μg/rat. Presumably, this indicates a requirement for polar character at position 1 and may be a consequence of an unfavorable change in transport properties in vivo.

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