Synthesis of (−)-1-Cyclohexyl-4-(1,2-diphenylethyl)piperazine [(−)-5] Dihydrochloride from (*R*)-(−)-1,2-Diphenylethylamine. In EtOH (30 ml) were dissolved (*R*)-(−)-1,2-diphenylethylamine[12] (1.1 g, 5.6 mmol) and *N*-cyclohexyl-2,2′-dichlorodiethylamine hydrochloride (1.3 g, 5 mmol) and $NaHCO_3$ (1.4 g, 17 mmol) was added to the solution. The mixture was refluxed for 24 hr with stirring and the solvent was removed in vacuo. To the residue was added aqueous 10% Na_2CO_3 and the mixture was extracted with AcOEt. The organic layer was washed with H_2O and dried and the solvent was removed. The residue was treated with methanolic HCl, and the resulting crystals were recrystallized from MeOH to give 1.2 g (57%) of colorless needles: mp 275–277° dec; $[\alpha]^{28}D$ −54.6° (*c* 1.0, MeOH). By mixture melting point measurement and ir spectrum, this compound was identified with (−)-5·2HCl, previously described.

Synthesis of (+)-1-Cyclohexyl-4-(1,2-diphenylethyl)piperazine [(+)-5] Dihydrochloride from (*S*)-(+)-1,2-Diphenylethylamine. To a solution of *N*-cyclohexyl-2,2′-dichlorodiethylamine hydrochloride (0.99 g, 3.8 mmol) in $CHCl_3$ was added (*S*)-(+)-1,2-diphenylethylamine[13] (1.5 g, 7.6 mmol). After the solvent was removed, the mixture was heated at 110–120° for 2.5 hr and then at 120–130° for 0.5 hr. After being cooled, to the mixture was added aqueous 10% HCl and the mixture was cooled and the resulting crystals were collected, washed with a small amount of cold H_2O and acetone, and dried. The crystals were recrystallized from MeOH to give 0.64 g of colorless needles: mp 275.5–277° dec; $[\alpha]^{19}D$ +54.5° (*c* 2.0, MeOH). By mixture melting point measurement and ir spectrum, this compound was identified with (+)-5·2HCl previously described.

(*R*)-(−)-*N,N*-Dimethyl-1,2-diphenylethylamine hydrochloride:[3] ORD $[\phi]^{22}$ (nm) −258° (610), −273° (589), −3560° (275).

Analgesic Assay. The compounds listed in Table I were tested for analgesic activity by the following methods. D'Amour–Smith method.[14] Thermal pain was induced by radiating heat light on the tail of male mice (9–12 g) of ddN strain using the modified apparatus of D'Amour–Smith according to the procedure of Nakamura et al.[14b] Phenylquinone writhing method.[15] Chemical pain was induced by an intraperitoneal injection of phenylquinone in female mice (18–22 g) of ddN strain. Haffner method.[16] Mechanical pain was induced by pressing the tail of male rats (90–110 g) of Wister strain using the modified apparatus of Haffner.

Six to twelve animals were used for a dose, and the values of ED_{50} were calculated according to the Litchfield–Wilcoxon method.[22]

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Antagonism of Luteinizing Hormone Release and of Ovulation by an Analog of the Luteinizing Hormone-Releasing Hormone

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Two varients of LH-RH, <Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2 (I) and <Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-$NHCH_2CH_3$ (II), have been synthesized by solid-phase methods. Both peptides strongly inhibit the LH-RH induced secretion of LH in an in vitro assay; however, only I proved effective in preventing ovulation in the 4-day cycling rat.

Antagonists of the luteinizing hormone releasing factor (LH-RH) offer a basis for the design of a contraceptive agent.[1] A successful candidate would be expected to inhibit pituitary secretion of the gonadotropins FSH and LH which normally induce ovarian functions such as follicular development, ovulation, and gonadal steroid production.

The route taken by leading groups in this field involves the design of more potent agonists of LH-RH which could themselves form the basis for antagonist design, e.g., by deletion of histidine at position 2.[2-4] The potentiating effects of D-alanine at position 6 and of the N-terminal ethylamide modifications on agonist activity have been reported[3,4] and

Table I. Activities of LH-RH and Analogs in Monolayer Cultures of Pituitary Cells

Expt no.	LH-RH concn, ng/ml	Analog	Analog concn, ng/ml	LH released, ng/ml
1		None		79 ± 5
	50	None		244 ± 11
	5	None		153 ± 19
	5	I	1000	36 ± 2[a]
	5	I	100	81 ± 6[a]
	5	I	10	136 ± 20
	5	II	1000	52 ± 5[a]
	5	II	100	50 ± 4[a]
	5	II	10	55 ± 5[a]
2		None		137 ± 11
	50	None		467 ± 21
	5	None		388 ± 33
		II	1000	94 ± 15
	5	II	100	138 ± 16[a]
	5	II	10	184 ± 6[a]
	5	II	1	286 ± 14[a]
	5	II	0.1	400 ± 23
3		None		75 ± 10
	50	None		526 ± 10
	5	None		234 ± 21
		I	10000	77 ± 10
		I	1000	134 ± 33
		I	100	85 ± 7

[a]Dunnett's multiple comparison test vs. LH-RH alone, $p < 0.01$.

led to the synthesis of des-Gly[10]-[D-Ala[6]]-LH-RH-ethylamide, a very potent agonist analog of LH-RH with prolonged action in vivo.[5,6]

The separate introduction of the above modifications into des-His[2]-LH-RH has been shown to potentiate in vitro antagonist activity.[3,7] Vilchez-Martinez et al. have reported that chronic administration of des-His[2]-des-Gly[10]-LH-RH-ethylamide can delay spontaneous ovulation in the rat for at least 48 hr.[7]

Recently, Rees et al.[8] reported in vitro data for [D-Phe[2]]-LH-RH. Its inherent agonist activity was less than 0.001% that of LH-RH, yet was shown to antagonize the LH release induced by LH-RH at a ratio of 200:1, a fivefold improvement on the 1000:1 ratio for des-His[2]-LH-RH. In the hope of obtaining superior antagonists of LH-RH, the above information was combined in the synthesis of analogs I and II (vide infra) using conventional solid-phase methodology. Peptides were assayed for LH secretion by monolayer cultures of dispersed rat pituitary cells.[9,10] Both peptides were also tested for their ability to inhibit LH-RH stimulated secretion.[11]

<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10
LH-RH
<Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2
I, [D-Phe[2],D-Ala[6]]-LH-RH
<Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt
II, des-Gly[10]-[D-Phe[2],D-Ala[6]]-LH-RH ethylamide

Table I summarizes relevant cell culture data. Table II illustrates the degree of ovulation inhibition in the 4-day cycling rat. Details of the method are given in the Experimental Section.[12]

Table II. Ovulation Inhibition in the Unanesthetized Proestrous Rat

Peptide	Dose, μg per rat sc, × no. of injections	No. of ovulating rats/no. of rats	No. of ova/ovulating rat
Oil control		6/6	11.5
I	125 × 6	7/10	13.3
	500 × 6	1/20	12.5
	1000 × 6	0/5	0
II	1000 × 6	5/5	12.8
	1000 × 6	5/5	14.0
	4500 × 6	3/3	10.3

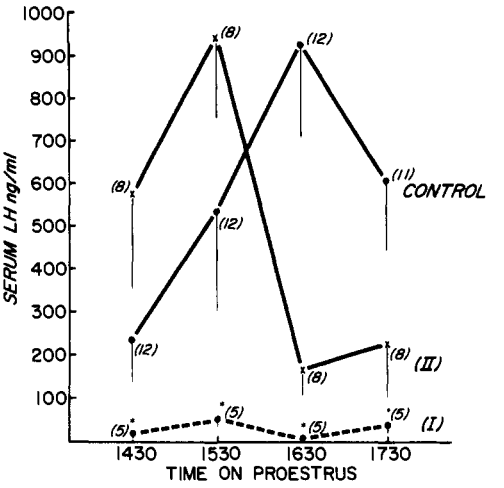

Figure 1. The effect of LH-RH analogs on the proestrous serum LH surge: dose, 6×10^3 μg sc; *, $p < 0.05$ from control; number of rats per point in parentheses, each point $\bar{x} \pm$ SE.

Results

LH-RH *releasing* activity could not be demonstrated for either peptide at levels indicated in Table I. Both peptides are, however, potent inhibitors of LH-RH release in cell cultures; II was active at an antagonist to agonist ratio of as low as 1 to 5, the corresponding value for I being only 20:1. When assayed for antiovulatory activity only I proved effective. A 95% antiovulatory block was obtained at a dose of 6×500 μg per rat while II was inactive at ten times the dose of I. This nonparallelism shows that potency for antagonism of LH-RH stimulated LH secretion in cell cultures, by itself, is an inadequate basis for predicting relative antiovulatory activity.

The apparent lack of interassay correspondence may be rationalized when the ability of I and II to suppress the proestrous preovulatory surge of LH is determined. The rat and human ovulatory cycles, when charted in terms of fluctuating blood levels of LH, are characterized by a sharp mid-cycle peak in LH levels which precede and result in ovulation.[13,14] Peptide I completely suppressed this surge in the rat at a 6×1000 μg dose level used for full antiovulatory block (Figure 1). Peptide II was totally ineffective.

Experimental Section

All protected amino acids were purchased from Bachem Laboratories, Marina Del Rey, Calif. Amino acid analyses were performed on a Durrum D-500 high-speed amino acid analyzer. Peptides were

hydrolyzed in evacuated sealed tubes for 18 hr at 125° in 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. R_f data are given for the following chromatography systems: (1) 1-butanol–acetic acid–water (4:1:5, top phase); (2) 1-butanol–acetic acid–water–ethyl acetate (1:1:1:1); (3) ethyl acetate–pyridine–acetic acid–water (5:5:1:3).

Boc-Gly-benzhydrylamine-resin. Benzhydrylamine resin (20 g) was washed thoroughly with ca. 200 ml of CH_2Cl_2, TFA–CH_2Cl_2 (1:1, twice), CH_2Cl_2, MeOH, $(Et)_3N$ [12.5% in DMF (twice)], MeOH (twice), and CH_2Cl_2 (twice). The resin was then suspended in 75 ml of CH_2Cl_2–DMF (1:1) and 3.65 g (21 mequiv) of Boc-Gly was added, followed by 25 ml of 1 *M* DCC–CH_2Cl_2 (25 mequiv) in two portions, 30 min apart, with gentle rocking. Rocking was continued overnight, the solvents were removed, and the resin was washed with MeOH and CH_2Cl_2. A sample was withdrawn and found to be negative in the ninhydrin test.[15] Acid hydrolysis of a sample in 6 *N* HCl for 4 hr at reflux and subsequent quantitative amino acid analysis showed it to be substituted by glycine to the extent of 0.30 mequiv/g of resin.

Boc-Tyr(Bzl)-D-Ala-Leu-Arg(NO_2)-Pro-Gly-benzhydrylamine-resin. The above resin was deblocked by treatment with TFA–CH_2Cl_2 (1:1) three times, each time for 15 min, and was then washed with CH_2Cl_2, MeOH, DMF [12.5% in $(Et)_3N$ (twice, each time for 10 min)], MeOH (twice), and CH_2Cl_2 (twice). A sample of the resin showed a very strong ninhydrin response. The resin was suspended as previously, and 4.48 g (21 mequiv) of Boc-Pro was added followed by 25 ml of 1 *M* DCC in CH_2Cl_2 (25 mequiv) in three portions over a period of 30 min. Rocking was then continued overnight. In essentially the same fashion, Boc-Arg(NO_2) (2.63 g, 21 mequiv), Boc-Leu (4.82 g, 21 mequiv), Boc-D-Ala (3.94 g, 21 mequiv), and Boc-Tyr(Bzl) (7.73 g, 21 mequiv) were added. After the appropriate washings the resin was removed from the reaction vessel and dried in vacuo for 24 hr: yield 36.0 g.

Boc-Trp-Ser(Bzl)-Tyr(Bzl)-D-Ala-Leu-Arg(NO_2)-Pro-Gly-benzhydrylamine-resin. The synthesis was continued on half of the above resin (18.0 g) by the addition of Boc-Ser(Bzl) (3.07 g, 10 mequiv) followed by Boc-Trp (3.17 g, 10 mequiv). After washing and drying, the resin weighed 20.8 g.

<Glu-D-Phe-Trp-Ser(Bzl)-Tyr(Bzl)-D-Ala-Leu-Arg(NO_2)-Pro-Gly-benzhydrylamine-resin. After the incorporation of Boc-Trp, the deblocking step was carried out under the addition of 5% ethanedithiol in TFA–CH_2Cl_2 (1:1 mixture). A sample of 2.0 g of the above peptide was deblocked and then coupled with 0.360 g (1.4 mequiv) of Boc-D-Phe (2 ml, 1 *M* DCC) and, after the appropriate washings and deprotection steps, with 0.352 g (2.7 meqquiv) of <Glu (3.4 ml, 1 *M* DCC). The resin was again removed from the vessel and dried in vacuo: weight 2.18 g.

<Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH_2 (I). All of the above peptide resin was suspended in 25 ml of liquid HF and 10 ml of anisole and stirred for 60 min at 0°. The HF was then removed by aspirator and the anisole by washing with ether. The resin was subsequently extracted with 10% HOAc and filtered. The aqueous phase was lyophilized overnight and the crude peptide was recovered as a fluffy powder.

The crude peptide was dissolved in a minimum volume of 0.2 *N* acetic acid, applied to a Bio-Gel P-2 200–400 mesh gel-permeation column (2.5 × 90 cm), and eluted with the same solvent. Fractions of 9 ml each were collected and the effluent was monitored on a Beckman DU instrument at 280 mμ. The major fraction was eluted in fractions 32–38 (252 mg).

The above material was then dissolved in minimum volume of the upper phase of BAW (4:1:5) and applied to a partition column prepared on a bed of Sephadex G-25 fine (2.5 cm o.d. and 90 cm height) previously equilibrated with lower phase and eluted with upper phase, and 9-ml fractions were collected. In fractions 41–49, a single sharp peak was eluted (142.8 mg): $[\alpha]_D$ −36.5° (*c* 0.987, 1% HOAc). The peptide was homogeneous in three TLC (silica gel) systems: R_f (1) 0.25; R_f (2) 0.69; R_f (3) 0.89. Amino acid analysis: Ser (0.97), Glu (1.0), Pro (1.0), Gly (1.0), Ala (1.0), Leu (0.98), Tyr (1.0), Phe (0.99), NH_3 (1.2), Trp (0.93), Arg (0.97).

<Glu-D-Phe-Trp-Ser(Bzl)-Tyr(2,6-Cl_2-Bzl)-D-Ala-Leu-Arg(Tos)-Pro-resin. Boc-Pro-resin (20 g, substitution 0.5 mequiv/g), prepared by the method of Gisin[16] on a Bio-Beads SX1 resin (chloromethylated capacity 0.89 mequiv/g), was placed in a peptide synthesis vessel and subjected to the following wash cycle: (a) CH_2Cl_2; (b) TFA–CH_2Cl_2 (1:1) (three times for 5-, 15-, and 15-min periods); (c) CH_2Cl_2 (twice); (d) DMF; (e) 12.5% $N(Et)_3$ in DMF (twice, each time for 10 min); (f) DMF; (g) CH_2Cl_2 (twice); (h) MeOH (twice); (i) CH_2Cl_2 (three times), allowing a contact time of at least 2 min each, if not indicated otherwise.

The resin so prepared was gently stirred with Boc-Arg (Tos) (25 mequiv) in 1:1 CH_2Cl_2–DMF during 5 min followed by the addition of 1 *M* DCC (25 ml, 25 mequiv) in two portions 30 min apart. Stirring was continued during 18 hr. The peptide resin was washed successively with (j) MeOH, (k) CH_2Cl_2, (l) MeOH (twice), and (m) CH_2Cl_2 (twice). Usually to test for completeness of reaction, the peptide-resin was subjected to a ninhydrin test following the procedure of Kaiser et al.[15] Proline, however, is anomalous giving a weak color reaction in the above test so the resin was subject to a 4-hr recouple with Boc-Arg(Tos) (8.3 mequiv, 8.3 mequiv of DCC) to eliminate possible unreacted amine groups.

The following amino acid residues were introduced sequentially onto a washed (steps j–m), deprotected, and neutralized (steps a–c) peptide resin: Boc-Leu hydrate (25 mequiv), Boc-D-Ala (25 mequiv), Boc-Tyr(2,6-Cl_2-Bzl) (25 mequiv), Boc-Ser(Bzl) (25 mequiv), Boc-Trp (25 mequiv), and Boc-D-Phe (25 mequiv). All couplings were mediated using 1 *M* DCC in CH_2Cl_2 (25 mequiv) as described for the addition of Boc-Arg(Tos) except for the case of Boc-Leu, and DCC reagent being added first to reduce the possibility of peptide loss via diketopiperazine formation.[17] Dithioerythritol (5 g/1 l.) was added to the TFA–CH_2Cl_2 reagent after addition of Tyr. The resin was washed (steps j–m) and dried to give 30 g of peptide-resin. The final amino acid <Glu (10 mequiv, 10 mequiv of DCC) was attached using 10 g of peptide-resin. The resin was washed and dried in vacuo to give the title compound (ca. 10 g).

<Glu-D-Phe-Trp-Ser(Bzl)-Tyr(2,6-Cl_2-Bzl)-D-Ala-Leu-Arg(Tos)-Pro-$NHCH_2CH_3$. The above resin (10 g) in ethylamine (100 g) was stirred in a pressure bottle during 15 hr. The initial temperature of −5° was allowed to rise to room temperature over several hours. Ethylamine was removed under reduced pressure and the residue washed with DMF (four times). The combined filtrates were evaporated in vacuo below 35° and the residue in MeOH reprecipitated with ether to give the protected peptide (1.9 g).

<Glu-D-Phe-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-$NHCH_2$-CH_3. The protected peptide produce (1.9 g) from the previous experiment was treated with hydrogen fluoride (80 ml) and anisole (20 ml) at 0° for 1 hr. The hydrogen fluoride was removed by room temperature vacuum distillation and the anisole removed by trituration with ether. The crude peptide was dissolved in 10% acetic acid and lyphilized to give ca. 850 mg of a pale buff powder.

Purification was effected in two stages. The first stage consisted of gel permeation on a column of Sephadex G-15 fine (2.5 × 150 cm) in 33⅓% acetic acid. Peptide material was located by Ehrlich spot test and ultraviolet analysis at 280 mμ. Fractions (5.4 ml) eluted in tubes 48–63 were pooled and lyophilized. The second stage utilized partition chromatography on a column of Sephadex G-25 (100 × 2.6 cm) using the BAW (4:1:5) system described in I. A symmetrical peak eluted in fractions (8.4 ml) 37–51 was collected and lyophilized to afford 410 mg of product: $[\alpha]^{26}_D$ −36° (*c* 1.02, 1% HOAc). The peptide was homogeneous in three TLC (silica gel) systems: R_f (1) 0.54; R_f (2) 0.35; R_f (3) 0.81. Amino acid analysis: Ser (0.93), Glu (0.99), Pro (1.0), Ala (1.0), Leu (1.0), Tyr (0.94), Phe (0.99), NH_3 (0.34), Trp (0.68), Arg (1.0), $EtNH_2$ (1.0).

Ovulation-Inhibition Test. Sprague–Dawley (Charles River) rats, 200–250 g BW, were acclimated to laboratory conditions and maintained on a 14 light/10 dark lighting schedule with midnight the midpoint of the dark period. Only rats exhibiting at least two consecutive 4-day cycles were used.

On the afternoon of proestrus the animals received subcutaneous injections of I or II in corn oil at 12:00, 12:30, 1:00, 1:30, 2:00, and 2:30. The next morning (estrus), the rats were sacrificed and the number of animals ovulating, together with the number of ova identified under a dissecting microscope, were recorded. The absence of or a significant decrease in the number of animals ovulating was the criterion for an antiovulatory effect.

Blood samples for serum LH determination were taken by cardiac puncture (0.5–1.0 ml; volume replaced ip with saline) hourly from 2:30–5:30 proestrus.

LH in serum (as well as in cell culture filtrates) was determined by radioimmunoassay using reagents supplied by the NIAMDD rat pituitary hormone program. Values are expressed in terms of LH-RP-1.

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Notes

Luteinizing Hormone-Releasing Hormone. Antiovulatory Activity of Analogs Substituted in Positions 2 and 6

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Ten analogs of luteinizing hormone-releasing hormone (LH-RH) substituted in position 2 with D-amino acids and at 6 with either a D-amino acid or a nonasymmetric amino acid were synthesized by solid-phase methodology and assayed for antiovulatory activity. [D-Phe^2]-LH-RH substituted in the 6 position with D-Ala, D-Leu, D-Arg, D-(Ph)Gly, D-Phe, or 2-Me-Ala possessed varying degrees of antiovulatory activity. [D-*p*-F-Phe^2-D-Ala^6]-LH-RH was one of the most active antiovulatory compounds, while the [D-*p*-Cl-Phe^2-D-Ala^6]-LH-RH analog was devoid of activity at a comparable dose.

Following the first report[1] on luteinizing hormone-releasing hormone (LH-RH) antagonists, considerable effort was directed toward elucidating novel compounds of this class possessing antiovulatory activity. This study reports on several analogs of LH-RH which may prove to be effective precoital contraceptives by virtue of possessing such activity.

Replacement of histidine [His^2] in LH-RH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)[2,3] with D-phenylalanine [D-Phe^2] gives an analog with minimal agonist activity and considerable antagonism to LH-RH-induced luteinizing hormone (LH) release in vitro.[4] Replacement of glycine [Gly^6] with D-alanine [D-Ala^6] gives an analog with a high degree of LH-releasing activity in vitro and in vivo.[5] A recent report from our laboratories demonstrated that incorporating [D-Phe^2] and [D-Ala^6] into the LH-RH molecule instead of [His^2] and [Gly^6] results in analogs with marked antiovulatory and contraceptive activity.[6-10] This observation formed the basis for further examination of possible structure–activity relationships among other LH-RH analogs with different D-amino acid or nonasymmetric amino acid substitutions for histidine and glycine.[6] The synthesis and/or biological activities of the following LH-RH derivatives are described: [D-Phe^2]-LH-RH, [D-Phe^2-D-Ala^6]-LH-RH, [D-Phe^2-D-Leu^6]-LH-RH, [D-Phe^2-D-Ser^6]-LH-RH, [D-Phe^2-D-Arg^6]-LH-RH, [D-Phe^2-D-(Ph)Gly^6]-LH-RH, [D-Phe^2-D-Phe^6]-LH-RH, [D-Phe^2-D-Lys^6]-LH-RH, [D-Phe^2-2-Me-Ala^6]-LH-RH, [D-*p*-F-Phe^2-D-Ala^6]-LH-RH, and [D-*p*-Cl-Phe^2-D-Ala^6]-LH-RH.

Synthesis. All peptides were prepared by the Merrifield method[11] on benzhydrylamine resin by a previously described procedure.[4,9] The analytical data are given in Table I.

Biological Activities. Table II illustrates the antiovulatory activity of [D-Phe^2]-LH-RH analogs substituted in the 6 position by representative lipophilic (D-Ala, D-Leu, 2-Me-Ala), hydrophilic (D-Ser), basic (D-Lys, D-Arg), or aromatic [D-Phe, D-(Ph)Gly] amino acids.

The data show that no D-amino acid or nonasymmetric amino acid substitution in positions 6 of [D-Phe^2]-LH-RH was more effective than D-alanine in potentiating antiovulatory activity. The minimum effective dose (MED_{100}) for full ovulatory blockade was 6 mg for that analog; partial inhibition could be achieved at the 1.25-mg dose level. [D-Phe^2-2-Me-Ala^6]-LH-RH was equipotent with [D-Phe^2-D-Ala^6]-LH-RH (100% inhibition) at the 6-mg level, but lower doses gave only partial inhibition, with an erratic dose–response curve. A [D-*p*-F-Phe^2] substitution at position 2 in