Blackwell, R. Burgus, and R. Guillemin, *Science*, **176**, 933 (1972).
(2) J. A. Vilchez-Martinez, D. H. Coy, A. Arimura, E. J. Coy, Y. Hirotsu, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **59**, 1226 (1974).
(3) M. Monahan, M. Amoss, H. Anderson, and W. Vale, *Biochemistry*, **12**, 4616 (1973).
(4) M. Fujino, S. Kobayashi, M. Obayashi, S. Shinagawa, T. Fukuda, C. Kitada, R. Nakayama, I. Yamakazi, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Commun.*, **49**, 863 (1972).
(5) D. H. Coy, E. J. Coy, A. V. Schally, J. A. Vilchez-Martinez, L. Debeljuk, W. H. Carter, and A. Arimura, *Biochemistry*, **13**, 323 (1974).
(6) M. Fujino, I. Yamazaki, S. Kobayashi, T. Fukuda, S. Shinagawa, R. Nakayama, W. F. White, and R. H. Rippel, *Biochem. Biophys. Res. Commun.*, **57**, 1248 (1974).
(7) J. A. Vilchez-Martinez, A. V. Schally, D. H. Coy, E. J. Coy, L. Debeljuk, and A. Arimura, *Endocrinology*, **95**, 213 (1974).
(8) R. W. A. Rees, T. J. Foell, S.-Y. Chai, and N. Grant, *J. Med. Chem.*, **17**, 1016 (1974).
(9) W. Vale, G. Grant, M. Amoss, R. Blackwell, and R. Guillemin, *Endocrinology*, **91**, 562 (1972).
(10) N. H. Grant, D. E. Clark, and E. I. Rosanoff, *Biochem. Biophys. Res. Commun.*, **51**, 100 (1973).
(11) R. Guillemin, M. Amoss, R. Blackwell, R. Burgus, G. Grant, N. Ling, M. Monahan, J. Rivier, and W. Vale, *J. Reprod. Fertil., Suppl.*, **18**, 23 (1973).
(12) A. Corbin and C. W. Beattie, *Endocr. Res. Commun.*, 1, 23 (1975).
(13) M. Fukushima, V. C. Stevens, C. L. Gantt, and N. Vorys, *J. Clin. Endocrinol.*, **24**, 205 (1964).
(14) (a) S. M. McCann and V. D. Ramirez, *Recent Prog. Horm. Res.*, **20**, 131 (1964); (b) N. B. Schwartz, *Am. J. Physiol.*, **207**, 1251 (1964).
(15) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(16) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(17) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).

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

Table I. Chemical and Physical Properties of Synthetic LH-RH Analogs

No.	Peptide	Amino acid analysis: Glu	Phe	Trp	Ser	Tyr	Gly		Leu	Arg	Pro	$[\alpha]_D$[a]	R_f on TLC
1	LH-RH[b]	1.06		0.89	0.84	1.02	1.96	0.97[c]	1.00	1.02	1.11	−52.9	0.05[d]
2	[D-Phe^2]-LH-RH	1.00	1.00	0.84	0.70	0.98	2.00		1.00	1.03	1.04	−42.2	0.34[d]
3	[D-Phe^2-D-Ala^6]-LH-RH	0.92	1.03	0.92	0.85	0.95	1.00	0.97[e]	0.93	0.97	1.05	−38.1	0.25,[d] 0.69[f]
4	[D-Phe^2-D-Leu^6]-LH-RH		1.00	0.73	0.97	1.00	1.00		2.00	1.00	0.70	−26.0	0.49[d]
5	[D-Phe^2-D-Ser^6]-LH-RH	1.00	1.00	0.77	1.70	1.00	1.00		0.98	1.00	0.99	−41.3	0.29,[d] 0.65[f]
6	[D-Phe^2-D-Arg^6]-LH-RH	1.00	0.90	0.70	1.10	0.80	1.10		1.00	2.00	0.80	−24.3	0.28,[d] 0.65[f]
7	[D-Phe^2-D-(Ph)-Gly^6]-LH-RH	0.99	1.01	0.64	0.93	1.03	1.10	1.06[g]	1.00	1.04	0.95	−33.0	0.70[h]
8	[D-Phe^2-D-Phe^6]-LH-RH	0.94	1.95	~1.00	0.84	0.92	1.00		0.98	~1.00	0.90		0.24[d]
9	[D-Phe^2-D-Lys^6]-LH-RH	0.98	1.01	0.79	0.87	1.00	1.05	0.99[i]	1.00	1.01	0.94	−25.8	0.45[h]
10	[D-*p*-F-Phe^2-D-Ala^6]-LH-RH	1.00	1.01[j]	~1.00	0.79	0.96	1.01	1.00[e]	0.93	0.97	1.00	−36.6	0.18[d]
11	[D-*p*-Cl-Phe^2-D-Ala^6]-LH-RH	0.95	0.89[k]	0.97	0.74	0.99	1.03	1.00[e]	0.98	1.03	0.84		0.19[d]
12	[D-Phe^2-2-Me-Ala^6]-LH-RH	0.94	1.05	0.83	0.64	0.99	1.00	0.98[l]	0.93	0.98	1.01	−25.0	0.45[m]

[a] All optical rotations were obtained with a Carl Zeiss LEP-A2 photoelectric precision polarimeter and all were determined in 1% acetic acid solution with the exception of that for compound 7, which was determined in 30% acetic acid solution. [b] All peptides for AAA were hydrolyzed in 6 *N* HCl, with the exception of compound 8 which was hydrolyzed in methanesulfonic acid. [c] His. [d] *n*-BuOH-AcOH-H_2O (4:1:5). [e] Ala. [f] *n*-BuOH-AcOH-H_2O-EtOAc (1:1:1:1). [g] (Ph)Gly. [h] *n*-BuOH-H_2O-AcOH-Pyr (30:24:6:20). [i] Lys. [j] *p*-F-Phe. [k] *p*-Cl-Phe. [l] 2-Me-Ala. [m] Isoamyl alcohol-Pyr-H_2O (7:7:6).

Table II. Antiovulatory Effect of LH-RH Analogs

Analog	No. of rats	Dose, μg/rat × 6	% inhibn of ovulation	No. of rats ovulating	$\bar{X}$ ova ovulated
[D-Phe^2]-LH-RH	5	1000	20	4	13.3
[D-Phe^2-D-Ala^6]-LH-RH	24	1000	96	1	14.0
	20	500	95	1	10.0
	10	125	30	7	13.3
[D-Phe^2-D-Leu^6]-LH-RH	10	1000	70	3	13.3
[D-Phe^2-D-Ser^6]-LH-RH	5	1000	0	5	13.5
[D-Phe^2-D-Arg^6]-LH-RH	5	750	40	3	10.3
[D-Phe^2-D-(Ph)Gly^6]-LH-RH	4	1000	100	0	0.0
	5	350	0	5	13.2
[D-Phe^2-D-Phe^2]-LH-RH	10	1000	80	2	4.0
[D-Phe^2-D-Lys^6]-LH-RH	5	1000	0	5	13.8
[D-*p*-F-Phe^2-D-Ala^6]-LH-RH	5	1000	100	0	0.0
	5	125	60	2	14.0
[D-*p*-Cl-Phe^2-D-Ala^6]-LH-RH	5	1000	0	5	13.5
[D-Phe^2-2-Me-Ala^6]-LH-RH	5	1000	100	0	0.0
	14	250	64	5	10.5
	10	125	80	2	11.5
Oil	68		1	67	13.7

[D-Phe^2-D-Ala^6]-LH-RH increased antiovulatory activity below the 6-mg dose level, although the MED_{100} was again 6 mg. Interestingly, the closely related [D-*p*-Cl-Phe^2-D-Ala^6]-LH-RH was completely inactive, indicating that the size of the para substitution in the phenyl moiety of phenylalanine may be crucial for activity.

Table III illustrates the in vivo gonadotrophin-releasing activity of several of the active antiovulatory analogs as compared with LH-RH. In this extremely sensitive model a minimal LH-releasing activity relative to LH-RH is common to all the antiovulatory peptides tested; however, as much as a 1000-fold difference between minimum LH-releasing doses exists between analogs exhibiting comparable antiovulatory activity, such as [D-Phe^2-D-Ala^6]-LH-RH or [D-Phe^2-2-Me-Ala^6]-LH-RH; [D-Phe^2]-LH-RH, which has little in vivo or in vitro releasing activity, is only minimally

Table III. In Vivo LH Agonist Activity of LH-RH Analogs[a]

Analog	Min releasing concn, ng/ml iv	Releasing act. rel to LH-RH, %	% increase in LH at min releasing dose
[D-Phe^2]-LH-RH	1000	0.0001	45
[D-Phe^2-D-Ala^6]-LH-RH	1	0.1	120
[D-Phe^2-Leu^6]-LH-RH	10	0.01	68
[D-Phe^2-D-Arg^6]-LH-RH	100	0.001	60
[D-Phe^2-2-Me-Ala^6]-LH-RH	1000	0.0001	42
LH-RH	0.1	100	65

[a]Ovariectomized, estrogen-progesterone treated rats.

Table IV. Effect of LH-RH Analogs on the Proestrus–Estrus Gonadotropin Surge[a]

	LH[c]							FSH[c]								
	Proestrus					Estrus		Proestrus							Estrus	
Analog	1330[a]	1430	1530	1630	1730	800	900	1330	1430	1530	1630	1730	1830	1930	800	900
Oil	110.0[b] ± 20.5 (25)	242.9 ± 102.5 (25)	531.3 ± 238.9 (22)	1193.0 ± 121.9 (22)	812.4 ± 131.3 (22)	82.7 ± 25.2 (5)	45.8 ± 35.8 (5)	210.0 ± 15.0 (22)	197.1 ± 15.0 (22)	262.0 ± 47.2 (22)	414.4 ± 45.0 (22)	360.4 ± 48.9 (2)	354.6 ± 42.1 (6)	591.9 ± 42.9 (6)	653.7 ± 58.3 (6)	728.1 ± 69.0 (5)
[D-Phe^2-D-Ala^6]-LH-RH	85.0 ± 10.0 (6)	74.5 ± 15.1 (6)	70.5* ± 20.5 (6)	339.6* ± 79.8 (6)	287.2* ± 62.8 (6)	188.9 ± 80.3 (6)	186.8 ± 89.3 (3)	110.2* ± 7.5 (6)	107.3* ± 20.1 (6)	137.5* ± 10.5 (6)	466.2 ± 59.7 (6)	503.2 ± 53.3 (6)	478.8 ± 63.3 (6)	362.8* ± 28.6 (6)	394.3* ± 47.8 (6)	385.8 ± 55.1 (6)
[D-Phe^2-2-Me-Ala^6]-LH-RH		221.4 ± 93.0 (4)	437.9 ± 169.1 (4)	273.5* ± 125.8 (4)	192.1* ± 97.1 (4)		880.4 ± 451.8 (4)		594.1 ± 151.1 (4)	649.5 ± 112.7 (4)	533.1 ± 159.3 (4)	640.6 ± 174.2 (4)				463.4 ± 134.7 (4)

[a]Hours sampled. [b]Mean ± SE, ng/ml of serum. Numbers in parentheses refer to numbers of animals per group. [c]An asterisk indicates significantly different from oil control ($p < 0.05$).

effective as an antiovulatory agent. Also, this latter analog has been reported to antagonize LH-RH-induced LH release in vitro,[4] suggesting that minimal in vivo/in vitro LH-releasing ability or antagonism of an LH-RH induced LH release in vitro is only marginally predictive of antiovulatory activity.

Conversely, potent antiovulatory analogs of LH-RH suppress the proestrous, preovulatory serum LH surge and may also reduce the estrous morning surge of FSH (Table IV), clearly suggesting that this is the basis for their antiovulatory activity.

In support of this mechanism is the significant decrease in proestrous gonadotrophin levels and ovulation following peptide administration earlier in the estrous cycle,[8] coupled with negligible LH stimulation after antiovulatory doses in cycling rats (C. W. Beattie, unpublished observations) and inability to induce ovulation (100 μg iv) in the nembutalized rat.[7]

Experimental Section

All protected amino acids with the exception of Boc-D-*p*-F-Phe and Boc-D-*p*-Cl-Phe 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 either hydrolyzed in evacuated sealed tubes for 18 hr at 125° in 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole or hydrolyzed in 6 *N* HCl containing 0.59% thioglycolic acid for 4 hr at 145° in a closed system under nitrogen. Purity of the individual samples was determined by TLC and the shape of their elution curves from the respective partition columns. TLC was performed on Brinkman silica gel plates, irrigated with the respective solvents listed in Table I. The samples were applied at the 0.03-mg level and the spots were visualized with iodine vapor, Ehrlich spray, and chloramin T spray. By these criteria all samples were judged to be essentially pure.

[*p*-Cl-Phe] and [*p*-F-Phe] were resolved by selective α-chymotrypsin hydrolysis of their respective ethyl esters by a procedure described earlier by Tong et al.[12] The respective D-amino acids were first isolated and then converted to their Boc derivatives by conventional methods.[13]

[D-*p*-Cl-Phe]: $[\alpha]^{25}D$ +3.03° (*c* 1.98, 1 *N* HCl) (lit.[12] $[\alpha]D$ +3.3°). [Boc-D-*p*-Cl-Phe]: mp 100–103°. Anal. Calcd for $C_{14}H_{18}O_4NCl$: C, 56.10; H, 6.05; N, 4.67; Cl, 11.93. Found: C, 56.36; H, 6.16; N, 4.66; Cl, 11.60.

[D-*p*-F-Phe]: $[\alpha]^{25}D$ +6.31° (*c* 2.04, 1 *N* HCl) (lit.[12] $[\alpha]D$ +5.6°). [Boc-D-*p*-F-Phe]:[14] mp 70–72°. Anal. Calcd for $C_{14}H_{18}O_4NF$: C, 59.35; H, 6.40; N, 4.94. Found: C, 58.99; H, 6.29; N, 4.86.

All peptides were assayed for antiovulatory activity in adult (200–250 g) female Sprague–Dawley rats acclimated to laboratory conditions and maintained on a 14-hr light/10-hr dark lighting schedule with midnight the midpoint of the dark cycle. Only those rats exhibiting a least two consecutive 4-day cycles were used. All peptides were administered sc in corn oil (1 mg/0.2 ml) in varying doses on a half-hourly basis from 1200 to 1430 hr on the day of proestrus (D_3). All rats were autopsied on the morning of estrus (D_4) and the presence or absence of ova was ascertained under a dissecting microscope. The absence of or a significant decrease in the number of rats ovulating was the criterion for an antiovulatory effect.

Active antiovulatory analogs were assayed for LH release in vivo using ovariectomized, estrogen–progesterone-primed rats[15] and compared with LH-RH. Briefly, 50 μg of 17β-estradiol and 25 mg of progesterone were administered sc to rats 3 weeks post ovariectomy. Later (72 hr) analogs (1.0–1000 ng/ml/rat) in saline were administered iv via the jugular vein under light ether anesthesia, immediately after withdrawing a 1-ml blood sample. Twenty minutes post-treatment, rats were again sampled (1 ml) via the jugular vein for determination of serum LH–FSH.

In addition, the ability to suppress the proestrous, preovulatory surge of gonadotrophin in rats was assessed for two of the most active antiovulatory analogs ([D-Phe^2-D-Ala^6]-LH-RH and [D-Phe^2-2-Me-Ala^6]-LH-RH). Peptides were administered at 6 half-hourly (1 mg) sc injections from 1200 to 1430 hr on the day of proestrus. Blood samples for serum LH–FSH determination were taken by cardiac puncture (0.5–1.0 ml, volume replaced ip with saline). All serum hormone levels were determined by double-antibody radioimmunoassay using reagents and standards supplied by the NIAMDD Rat Pituitary Hormone Distribution Program through Dr. A. Parlow.

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References and Notes

(1) W. Vale, G. Grant, J. Rivier, M. Monahan, M. Amoss, R. Blackwell, R. Burgus, and R. Guillemin, *Science*, **176**, 933 (1972).
(2) H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, **43**, 1334 (1971).
(3) R. Burgus, M. Butcher, N. Ling, M. Monahan, J. Rivier, R. Fellows, M. Amoss, R. Blackwell, W. Vale, and R. Guillemin, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **273**, 1611 (1971).
(4) R. W. A. Rees, T. J. Foell, S. Y. Chai, and N. Grant, *J. Med. Chem.*, **17**, 1016 (1974).
(5) M. Monahan, W. Vale, J. Rivier, G. Grant, and R. Guillemin, *Proc. Meet. Endocr. Soc., 55th*, 194 (1973).
(6) A. Corbin and C. W. Beattie, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **34**, 240 (1975).
(7) A. Corbin and C. W. Beattie, *Endocr. Res. Commun.*, **2**, 1 (1975).
(8) C. W. Beattie and A. Corbin, *Proc. Meet. Endocr. Soc., 57th*, 194 (1975).
(9) J. P. Yardley, T. J. Foell, C. W. Beattie, and N. H. Grant, *J. Med. Chem.*, in press.
(10) T. J. Foell and J. P. Yardley, U.S. Patent 3855199 (Dec 17, 1974).
(11) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(12) J. H. Tong, C. Petitclerc, A. D'Iorio, and N. L. Benoiton, *Can. J. Biochem.*, **49**, 877 (1971).
(13) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969, p 29.
(14) The interaction of Boc-*p*-F-Phe salt has been reported by P. Marbach and J. Rudinger, *Helv. Chim. Acta*, **57**, 403 (1974).
(15) V. D. Ramierez and S. M. McCann, *Endocrinology*, **73**, 193 (1963).