Active Reduced-Size Hexapeptide Analogues of Luteinizing Hormone-Releasing Hormone[†,‡]

Fortuna Haviv,* Christopher A. Palabrica, Eugene N. Bush, Gilbert Diaz, Edwin S. Johnson, Stephen Love, and Jonathan Greer

Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064. *Received November 3, 1988*

A series of reduced-size hexapeptide analogues of LH-RH were synthesized that contain the residues corresponding to amino acid positions 4–9 and are linked to various carboxylic acids in place of residue 3. These compounds were tested in vitro in the rat pituitary receptor binding and LH release assays. A wide range of binding affinities was obtained up to and exceeding that of LH-RH. Both agonists and antagonists were obtained. From the SAR studies, it appears that a very precise size, length, and shape of the substituent at position 3 is required to achieve agonist activity, whereas the structural requirements for antagonist activity appear to be much less stringent. Depending on the nature of the substituent at positions 6 and 4, the biological response switches from antagonist to agonist or vice versa. The results suggest that conformational changes at position 6 or 4 feed back to the substituent at position 3, which induces the change from agonist to antagonist. The most potent compounds in the series were tested in vivo and found to be active.

The isolation, structure elucidation, and synthesis of luteinizing hormone-releasing hormone (LH-RH), p-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, by Schally and co-workers[1-5] and Guillemin and co-workers[6,7] in 1971, prompted an intensive search for more potent agonists and for active antagonists.[8-14] LH-RH agonists have proven efficacious in the treatment of infertility[15] and prostate cancer[16-19] and are being studied intensively in a wide variety of other endocrine diseases including endometriosis,[20,21] breast cancer,[22] precocious puberty,[23] and uterine fibroids[24] as well as a new mode of contraception.[25,26]

Since the replacement of the $Gly^{10}NH_2$ residue with *N*-ethylamide in the LH-RH peptide by Fujino and co-workers[27] in 1972, a large number of analogues have been synthesized.[10,11] Nevertheless, very few reports have appeared on additional reduction in the size of the molecule or active fragments of LH-RH. Sandow and co-workers[28] examined fragments of buserelin[29] where amino acids were removed sequentially from the N-terminus. Using the ovulation induction assay in the rat, they found that (3–9)buserelin[29] was the smallest fragment that retained significant ovulatory activity, about 23% of LH-RH activity. Although this activity was quite low, representing only 0.2% of buserelin[29] potency, we chose the (3–9)peptide as a departure point for the design of reduced-size LH-RH analogues.

Peptide Synthesis. Peptides were synthesized by the solid-phase method on a Merrifield resin according to techniques similar to those reported previously.[30] The peptides were cleaved from the resin upon treatment with anhydrous ethylamine at room temperature for 2 days. Afterward they were deprotected with anhydrous HF at 0 °C for 1 h in the presence of anisole and dimethyl phosphite.[30] The crude peptides were purified by HPLC using a C-18 reversed-phase column. The purity of the final product was greater than 95% on the basis of HPLC, FAB mass spectrometry (Tables I and II), and amino acid analyses.

[†] Part of this work has been presented at the International Symposium on GnRH Analogues in Cancer and Human Reproduction, Geneva, Switzerland, Feb 18–21, 1988. For abstract, see *Gynecological Endocrinology*; Parthenon Publishing Group Ltd., Park Ridge, NJ, 1988; Vol. 2, Suppl. 1, p 20.

[‡] Abbrevations: The abbreviations for the amino acids are in accordance with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise. Additional abbreviations: 1-Nal, 3-(1-naphthyl)alanine; Cha, 3-cyclohexylalanine; D-2-Nal, D-3-(2-naphthyl)alanine; Boc, *tert*-butoxycarbonyl; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; iv, intraveneous; RIA, radioimmunoassay; FAB, fast atom bombardment.

(1) Schally, A. V.; Arimura, A.; Baba, Y.; Nair, R. M. G.; Matsuo, H.; Redding, T. W.; Debeljuk, L.; White, W. F. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 393.
(2) Schally, A. V.; Nair, R. M. G.; Redding, T. W.; Arimura, A. *J. Biol. Chem.* **1971**, *246*, 7230.
(3) Matsuo, H.; Baba, Y.; Nair, R. M. G.; Arimura, A.; Schally, A. V. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1334.
(4) Baba, Y.; Matsuo, H.; Schally, A. V. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 459.
(5) Matsuo, H.; Arimura, A.; Nair, R. M. G.; Schally, A. V. *Biochem. Biophys. Res. Commun.* **1971**, *45*, 822.
(6) Amoss, M.; Burgus, R.; Blackwell, R.; Vale, W.; Fellows, R.; Guillemin, R. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 205.
(7) Burgus, R.; Butcher, M.; Amoss, M.; Ling, N.; Monahan, M.; Rivier, J.; Fellows, R.; Blackwell, R.; Vale, W.; Guillemin, R. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 278.
(8) Sandow, J.; Rechenberg, W. V.; König, W.; Hahn, M.; Jerzabek, G.; Fraser, H. *Hypothalamic Hormones. Chemistry, Physiology, and Clinical Applications*; Gupta, D., Voelters, W., Eds.; VCH Pubs, Inc.: New York, 1978, p 307.
(9) Rivier, C.; Rivier, J.; Perrin, M.; Vale, W. *Biol. Reprod.* **1983**, *29*, 374.
(10) Dutta, A. S. *Drugs Future*, **1988**, *13*, 43.
(11) Karten, M. J.; Rivier, J. E. *Endocr. Rev.* **1986**, *7*, 44.
(12) Hocart, S. J.; Nekola, M. V.; Coy, D. H. *J. Med. Chem.* **1987**, *30*, 735.
(13) Nestor, J. J.; Tahilramani, R.; Ho, T. L.; McRae, G. I.; Vickery, B. H. *J. Med. Chem.* **1988**, *31*, 65.
(14) Rivier, J.; Kupryszewski, G.; Varga, J.; Porter, J.; Rivier, C.; Perrin, M.; Hagler, A.; Struthers, S.; Corrigan, A.; Vale, W. *J. Med. Chem.* **1988**, *31*, 677.
(15) Hurley, D. M.; Brian, R.; Outch, K.; Stockdale, J.; Fry, A.; Hackman, C.; Clarke, I.; Burger, H. G. *N. Engl. J. Med.* **1984**, *310*, 1069.
(16) Warner, B.; Worgul, T. J.; Drago, J.; Demers, L.; Dufau, M.; Max, D.; Santen, R. J. *J. Clin. Invest.* **1983**, *71*, 1842.
(17) Trachtenberg, J. *J. Urol.* **1983**, *129*, 1149.
(18) Santen, R. J.; Dermers, L. M.; Max, D. T.; Smith, J.; Stein, B. S.; Globe, L. M. *J. Clin. Endocrinol. Metab.* **1984**, *58*, 397.
(19) Garnick, M. B.; Glode, M. L. *N. Engl. J. Med.* **1984**, *311*, 1281.
(20) Lemay, S.; MaLeux, R.; Faure, N.; Jean, C.; Fazekas, A. T. A. *J. Steroid Biochem.* **1984**, *20*, 1379.
(21) Okada, H.; Heya, T.; Ogawa, Y.; Shimamoto, T. *J. Pharmacol. Exp. Ther.* **1988**, *244*, 744.
(22) Klijn, J. G. M.; DeLong, F. H. *Lancet* **1982**, *1*, 1213.
(23) Boepple, P. A.; Mansfield, M. J.; Wierman, M. W.; Rudlin, C. R.; Bode, H. H.; Crigler, J. F., Jr.; Crawford, J. D.; Crowley, W. F. *Endocr. Rev.* **1986**, *7*, 24.
(24) Coddington, C. C.; Collins, R. L.; Shawker, T. H.; Anderson, R.; Loriaux, D. L.; Winkel, C. A. *Fertil. Steril.* **1986**, *45*, 624.
(25) Bergquist, C.; Nillius, S. J.; Wide, L. *Contraception* **1985**, *31*, 111.

0022-2623/89/1832-2340$01.50/0

Table I. Binding Affinities to Rat Pituitary Receptor and Potencies in LH Release or Inhibition of (4–9)LH-RH Analogues

X³-Ser-Tyr-Z⁶-Leu-Arg-ProNHEt

compd	X³	Z⁶	MH⁺[a]	t_R[b]	pK_I[c]	pD_2[c]	pA_2[c]
1	Trp	D-Leu	961[d]	13.6	7.07		6.06
2	Trp	D-Trp	1034[e]	13.8	8.33		7.24
3	1-Nal	D-Leu	972	13.8	7.30		
4	3-indolylpropionyl	D-Leu	946	17.6	6.71	6.93	
5	3-indolylpropionyl	D-Trp	1019	24.8	8.34	7.35	
6	3-indolylpropionyl	D-2-Nal	1030	24.4	8.77		7.60
7	3-indolylpropionyl	D-Phe	980	30.3	7.78		6.07
8	3-indolylpropionyl	D-Cha	986	22.4	7.38		6.44
9	3-indolylcarbonyl	D-Leu	918	15.5	6.69		6.72
10	3-indolylacetyl	D-Leu	932	13.67	5.87		5.56
11	3-indolylbutyryl	D-Leu	960	19.3	6.91		6.72
12	3-indolylbutyryl	D-2-Nal	1044	22.4	8.86		8.07
13	1-naphthylpropionyl	D-Leu	957[f]	22.5	7.60		6.60
14	1-naphthylpropionyl	D-Trp	1030	36.3	9.28		8.74
15	1-naphthylpropionyl	D-2-Nal	1041	24.2	9.55		9.25
16	1-naphthylcarbonyl	D-Leu	929	18.9	6.12	6.00	
17	1-naphthylacetyl	D-Leu	943	19.0	7.17	6.85	
18	1-naphthylacetyl	D-Trp	1016	17.2	8.65		8.18
19	1-naphthylacetyl	D-2-Nal	1027	25.8	8.58		8.20
20	1-naphthylacetyl	D-Phe	977	20.27	8.01		7.83
21	1-naphthylacetyl	D-Cha	983	24.09	7.44		6.08
22	2-naphthylacetyl	D-Trp	1016	26.18	8.43		7.80
23	1-naphthylacrylyl	D-Leu	955	31.90	7.30		6.69
24	1-naphthoxyacetyl	D-Trp	1032[g]	20.6	8.72		7.89
25	*trans*-4-MeO-cinnamoyl	D-Trp	1008[h]	26.75	6.80		6.46
26	*cis*-4-MeO-cinnamoyl	D-Trp	1008[i]	28.00	8.85		7.22
27	3-pyridylpropionyl	D-Leu	908[i]	15.24	5.79	4.96	
28	3-pyridylacrylyl	D-Leu	906[k]	15.66	5.83		5.72
29	1-adamantylacetyl	D-Trp	1024	23.6	9.34		8.50
LHRH					8.90	9.27	
[*N*Ac-D-4-Cl-Phe¹·², D-Trp³, D-Arg⁶, D-Ala¹⁰]LH-RH					10.74		9.44

[a] Values determined by FAB mass spectrometry. [b] HPLC retention time in minutes. For details see Experimental Section. [c] For definitions see Bioassays. [d] Trp 0.90. [e] Trp 1.92. [f] Tyr 0.92. [g] Tyr 0.90; Trp 0.92. [h] Tyr 0.92; Trp 0.88; Leu 0.92. [i] Tyr 0.90; Trp 0.85; Pro 0.85. [j] Tyr 0.89; Leu 1.80; Prp 0.85. [k] Tyr 0.85.

Table II. Binding Affinities to Rat Pituitary Receptor and Potencies in LH Release or Inhibition of [Ser⁴(*O*-Bzl)]LH-RH(4–9) Analogues

X³-Ser(*O*-Bzl)-Tyr-Z⁶-Leu-Arg-ProNHEt

compd	X³	Z⁶	MH⁺[a]	t_R[b]	pK_I	pD_2[c]	pA_2[c]
30	1-naphthylpropionyl	D-Leu	1047	27.40	7.64	7.93	
31	1-naphthylpropionyl	D-Trp	1120[d]	40.20	8.20		7.10
32	1-naphthylacetyl	D-Leu	1032	26.28	7.70		7.49
33	1-naphthylacetyl	D-Trp	1106[e]	31.48	8.91		8.52
34	1-naphthylacetyl	D-2-Nal	1117	30.61	7.43		8.44
35	1-naphthylacetyl	D-Phe	1067	24.6	8.46		7.98

[a] Values determined by FAB mass spectrometry. [b] HPLC retention time in minutes. For details see Experimental Section. [c] For definitions see Bioassays. [d] Phe 0.92. [e] Trp 1.10.

Bioassays. Peptides were tested in vitro in the rat pituitary receptor binding assay[31] and in the LH release from cultured rat pituitary cells assay.[32,33] The binding affinities are reported as pK_I, the negative logarithm of the equilibrium dissociation constant. The LH release potencies for agonists are reported as a pD_2, which is defined as the negative logarithm of the concentration of agonist that produces 50% of the maximum release of LH in response to the test compound. Antagonist potencies are reported as a pA_2, the negative logarithm of the concentration of antagonist that shifts the LH release dose–response curve produced by the agonist leuprolide[34] to 2-fold higher concentration. The most potent peptides were also tested in vivo as an iv infusion for 120 min in mature castrate male rats. LH plasma levels were determined by using RIA (see Experimental Section).

Results and Discussion

To evaluate the in vitro potencies and to study the structure–activity relationships (SAR) of the synthesized analogues, we used the in vitro receptor binding affinities and the LH release data. LH-RH was used as a standard agonist, which had a pK_I of 8.9 and a pD_2 of 9.27 (Table I) and [*N*-Ac-D-4-Cl-Phe¹·²,D-Trp³,D-Arg⁶,D-Ala¹⁰]LH-RH[35]

(26) Dewart, P. J.; McNeilly, S.; Smith, S. K.; Sandow, J.; Hillier, S. G.; Fraser, H. M. *Acta Endocrinol.* **1987**, *114*, 185.
(27) Fujino, M.; Kobayashi, S.; Obayashi, M.; Sinagawa, S.; Fukuda, T.; Kitada, C.; Nakayama, R.; Yamazaki, I.; White, W. F.; Rippel, R. H. *Biochem. Biophys. Res. Commun.* **1972**, *49*, 863.
(28) Sandow, J.; Konig, W. *Endocrinology* **1979**, *81*, 175.
(29) Buserelin = [D-Ser⁶(O-*t*-Bu),Pro⁹NHEt]LH-RH.
(30) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Merrifield, R. B., Ed.; Pierce Chemical Co., Rockford, IL, 1984; p 85.
(31) Marian, J.; Cooper, R. L.; Conn, P. M. *Mol. Pharmacol.* **1981**, *19*, 399.
(32) Jinnah, H. A.; Conn, P. M. *Am. J. Physiol.* **1985**, *249*, E619.
(33) Vale, W.; Grant, G.; Amoss, M.; Blackwell, R.; Guillemin, R. *Endocrinology* **1974**, *91*, 562.
(34) Leuprolide = [D-Leu⁶,Pro⁹NHEt]LH-RH.
(35) Nekola, M. V.; Horvath, A.; Ge, L. J.; Coy, D. H.; Schally, A. V. *Science* **1982**, *218*, 160.

as a standard antagonist, which had a pK_I of 10.74 and pA_2 of 9.44 (Table I).

The first compound synthesized, [D-Leu^6,Pro^{9}NHEt]-LH-RH(3-9) (1), had a pK_I of 7.07. It did not release LH but rather antagonized its release with a pA_2 of 6.06 (Table I). Similarly, [D-Trp^6,Pro^{9}NHEt]LH-RH(3–9) (2) was also a potent antagonist with pA_2 of 7.24. These results were surprising, since (3–9)buserelin[29] had been reported to be an agonist and was active in the ovulation induction test in the rat.[28] To further explore this site, we substituted 1-Nal for Trp^3 to produce [1-Nal^3,D-Leu^6,Pro^{9}NHEt]-LHRH(3–9) (3), which had a pK_I of 7.30. However, neither pD_2 nor pA_2 could be determined for this compound, suggesting that this peptide either is a very weak antagonist or is perhaps degraded either chemically or by proteolytic enzymes in the pituitary cell culture medium during the course of the LH release assay incubation.

Our design strategy involved modifying the moiety at position 3 of the (3–9)peptide and following the affinity and biological potency as the substituent at position 6 was varied. The first structural change introduced was replacement of the Trp^3 of 1 with desamino-Trp or 3-indolepropionic acid to give compound 4 with virtually the same receptor binding affinity (Table I). This indicates that the α-amino group and asymmetric center of position 3 are not essential for receptor binding. Interestingly, though, removal of this α-amino group converts the molecule from antagonist to agonist. Even more remarkable is that one observes the typical, expected increases in affinity and potency as the residue at position 6 is changed from D-Leu to D-Trp and D-2-Nal (Table I); however, while the D-Leu^6 (4) and D-Trp^6 (5) forms are agonists, the D-2-Nal^6 (6), D-Phe^6 (7), and D-Cha^6 (8) analogues are antagonists. These results demonstrate that in this series the delicate balance between agonist versus antagonist activity can be influenced by the substituent at position 6 as well as by that at position 3. The highest affinity molecules of this series (Table I) have about one-fifth (for agonist 5) to equal (for antagonist 6) the affinity of LH-RH. The affinity of antagonist 6 was one-hundredth the affinity of [*N*-Ac-D-4-Cl-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH, a potent, commercially available, decapeptide antagonist.[36]

Having achieved affinity levels around those of LH-RH in a molecule that is composed of only six amino acids, we decided to synthesize several additional compounds in order to further explore the structure–activity relationships. The length of the aliphatic chain connecting the indole group at position 3 to the molecule was varied (Table I). Shortening the chain in 4 by one methylene converted the compound from agonist to antagonist (10), however with a large loss in binding affinity. Further shortening of the chain by an additional methylene (9) restored receptor binding affinity, and the molecule was still an antagonist. Lengthening the chain in 4 by one methylene also converted the compound from agonist to antagonist (11), in this case maintaining binding affinity. In this series, [*N*-(3-indolylbutyryl)Ser^4,D-2-Nal^6,Pro^{9}NHEt]LH-RH(4–9) (12) showed the highest binding affinity, pK_I of 8.86, which is equal to that of LH-RH. Thus, once again the molecule could be shifted between agonist and antagonist, this time solely by changing the number of methylene groups in the side chain at position 3. The results in this series suggest that there is an optimal size, length, and shape for substituents at position 3, for affinity, for biological potency, and for achieving agonist versus antagonist activity. These are unprecedented results, since antagonists were originally discovered by deleting the His^2 residue in the LH-RH peptide[37,38] or by replacing residues 2, 3, and sometimes 1 and 10 with D-amino acids.[10]

To continue the structure–activity study of the substituents at position 3, 3-(1-naphthyl)propionic acid, corresponding to desamino-1-Nal, was placed at this position (compounds 13–15 in Table I). Compounds with very potent receptor binding affinity were obtained including the antagonist [*N*-[3-(1-naphthyl)propionyl]-Ser^4,D-2-Nal^6,Pro^{9}NHEt]LH-RH(4–9) (15), which displayed a binding affinity 4 times higher than that of LH-RH and virtually the same antagonist potency (pA_2) as the potent standard antagonist [*N*-Ac-D-4-Cl-Phe^{1,2},D-Trp^3,D-Arg^6,D-Ala^{10}]LH-RH. These results are remarkable considering that 15 contains only six amino acids (versus ten in the reference antagonist) and only one of the D configuration (versus five in the reference antagonist). Once again, the length of the aliphatic chain connecting the naphthyl group to the molecule was varied. When it was shortened by one or two methylenes (17 and 16, respectively), the biological response switched, this time from antagonist to agonist (Table I). Since it had reverted to agonist, we studied the influence of changes at position 6 on the biological activity of the *N*-(1-naphthylacetyl)-Ser^4 (compounds 18–21 in Table I). Again, as the size of the residue at position 6 was increased from D-Leu to D-Cha, D-Phe, D-Trp, or D-2-Nal, the molecule changed from an agonist to an antagonist all the while displaying the expected enhancement in receptor binding.

To further probe the properties of the side chain at position 3, we decided to synthesize several more radical substitutions for position 3 in this (4–9)LH-RH series. The data for compounds 22–29 (Table I) show that a wide range of affinities were obtained, from very weak for the *N*-[3-(3-pyridyl)propionyl]-Ser^4 (27) to quite high affinity for the *N*-(adamantylacetyl)-Ser^4 (29) with binding affinity 3-fold higher than that of LH-RH.

As a final series, among the compounds described above, several byproducts of the solid-phase synthesis were obtained with an *O*-benzyl group on the Ser^4 side chain (Table II). In general, these Ser^4(*O*-Bzl) peptides had only slightly different binding affinity from their respective parent, sometimes greater, sometimes lower, indicating that the free hydroxyl of the Ser^4 is not essential for binding. However, as in all these series, the biological activity results were more anomalous. For example, in the case of [*N*-[3-(1-naphthyl)propionyl]-Ser^4,D-Leu^6,Pro^{9}NHEt]LH-RH(4–9) (30), the molecule was transformed from an antagonist (13) to an agonist by the *O*-benzyl substitution. In another case, [*N*-(1-naphthylacetyl)-Ser^4,D-Leu^6,Pro^{9}NHEt]LH-RH(4–9) (32), the reverse effect was observed; the *O*-benzyl group changed agonist 17 into antagonist 32.

In order to test the efficacy of these compounds in vivo, the most active antagonist, 15, was administered to male castrate rats as an iv infusion at 10 μg/kg/min over 120 min. In total, 1.2 mg/kg of compound was administered to each animal. The LH levels were monitored over the infusion period by RIA. The results show that, at this dose, the level of LH was greatly suppressed, indicating clearly that this antagonist is both active and a potent LH

(36) Purchased from Sigma Chemical Co., St. Louis, MO.

(37) Guillemin, R.; Amoss, M.; Blackwell, R.; Burgus, R.; Grant, G.; Ling, N.; Monahan, M.; Rivier, J.; Vale, W. *Gynecol. Invest.* **1972**, *72*, 2.

(38) Vale, W.; Grant, G.; Rivier, J.; Monahan, M.; Amoss, M.; Blackwell, R.; Burgus, R.; Guillemin, R. *Science* **1972**, *176*, 933.

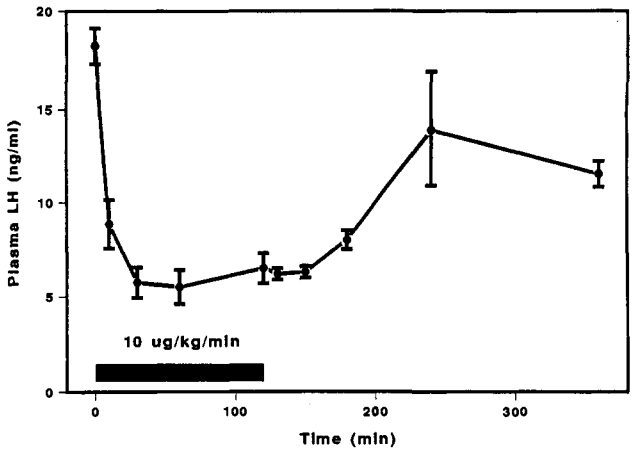

Figure 1. Plot of the level of LH in mature castrate male rats during and after iv infusion of the hexapeptide antagonist **15** at 10 μg/kg/min for 120 min (shown by solid bar). The infusion began at time 0. The level of LH was suppressed 70% at this rate of compound administration, showing that this compound is effective in vivo. Data are presented as mean ± SEM for two rats.

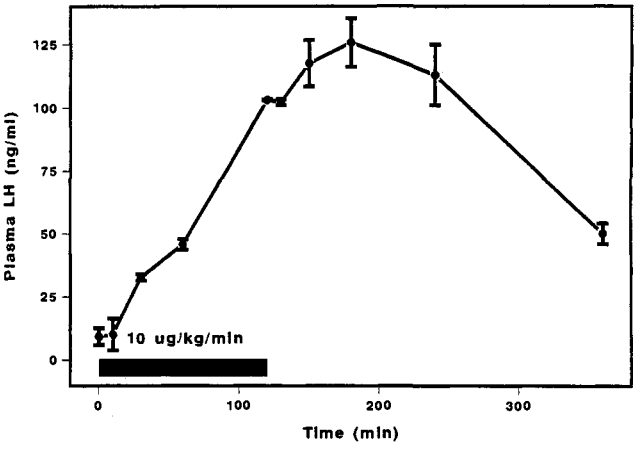

Figure 2. Plasma LH in mature castrate male rats during and after iv infusion of the hexapeptide agonist **5** at 10 μg/kg/min for 120 min (solid bar), beginning at time 0. The plasma level is increased 13.6-fold versus basal, and the increase is sustained for more than 4 h postinfusion.

suppressor in vivo (Figure 1). Compound **5**, the most active agonist in the series, when administered at 1 and 3 mg/kg doses by iv bolus, showed a significant dose-dependent rise in the circulating LH levels. Infusion iv of 10 μg/kg/min for 120 min produced a sustained increase in circulating LH levels (Figure 2).

Conclusions

Several novel conclusions have emerged from these studies that are very important for further development of reduced size and nonpeptidic LH-RH analogues. It is clear from the results reported here that a wide range of receptor binding affinities can be achieved with the hexapeptide LH-RH analogues up to and exceeding the activity of LH-RH itself. Even more interesting and important, by manipulating the nature of the group at position 3, the molecule can be easily transformed from agonist to antagonist. In examining the structure–activity relationships, it appears that a very precise size, length, and shape of side-chain substituent is required at position 3 to achieve agonist activity. If the group is either too large, too small, too long, or too short, the molecule becomes an antagonist. Nevertheless, the receptor binding site for position 3 seems to be formed in a way that permits the response to switch between agonist and antagonist quite readily depending upon changes in the size and shape of the substituent that occupies the site; yet these changes may have rather little effect upon the receptor binding affinity.

A novel result has been obtained upon modifying the nature of the side chain at position 6 in order to improve potency. While still performing its usual function of modulating receptor binding affinity in much the same way that it does in the nonapeptide or decapeptide LH-RH analogues, i.e., D-2-Nal > D-Trp > D-Leu, in the hexapeptide series, position 6 somehow feeds back to the residue at position 3 to change the compound from agonist to antagonist as the side chain gets larger. Even modification of the serine side chain at position 4 by O-substitution with a benzyl group has been observed to trigger the switch from agonist to antagonist in either direction depending upon the nature of the substituent at position 3. These results suggest that it is not a direct effect of the benzyl group, but once again the feeding back of a conformational change, probably small, to the 3-position, which influences the agonist to antagonist transition.

Finally, the high potencies which are observed in vitro can be translated into biological activity in vivo with almost complete suppression of LH by the antagonist and large LH release by the agonist, when the compounds were administered by infusion to rats. Taken together, these results suggest that compounds of the (4–9)LH-RH series described here represent a new departure in the design of a novel class of active LH-RH analogues which can be used to treat a wide range of endocrine disorders and conditions.

Experimental Section

All the peptides were synthesized by using a Biosearch Model 9500 automated peptide synthesizer. Amino acid derivatives Boc-Arg(Tos), Boc-L- and -D-Leu, Boc-L- and D-Trp(*N*-indolylformyl), Boc-D-2-Nal, Boc-Tyr(*O*-2-Br-Cbz), and Boc-Ser(*O*-Bzl) were obtained from Bachem Inc. (Torrance, CA). Boc-Pro-Merrifield resin with a capacity of 0.7–0.5 mmol/g was obtained from the same company. 1-Nal and TFA were obtained from Chemical Dynamics Corp. (South Plainfield, NJ) and Kali-Chemie Co. Inc. (Greenwich, CT), respectively. All the solvents were obtained from Fisher Scientific Co. (Fair Lawn, NJ). All other chemicals were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). Analytical and preparative HPLC were run on a Waters Associates (Milford, MA) Delta Prep 3000. FAB mass spectra were run on a Kratos MS 50. Amino acid analyses were performed on a Beckman Model 6300 amino acid analyzer. The peptides were hydrolyzed with 6 N HCl containing 0.3% phenol at 150 °C for 2 h. If the peptides contained Trp, 0.3% phenol was replaced with 5% thioglycolic acid. Amino acid analyses for compounds **1–35** were obtained. The content of Tyr, Trp, Leu, Pro, and Phe were within ±5% (otherwise when so indicated in the table footnotes). The content of Ser was generally low, within ±20%, and that for Arg was slightly high, within ±20%, due to interference of the ethylamide residue.

General Synthetic Method for the Solid-Phase Synthesis and Purification of Peptides 1–29. Merrifield resin was used. Double couplings in (1:1) DMF/CH_2Cl_2 were performed with 1,3-diisopropylcarbodiimide (0.4 M in CH_2Cl_2) for 1 h each. A 3-fold excess of 0.4 M protected amino acid (on the basis of the original substitution of the BOC-Pro-O-resin) in DMF was used. Deblocking was performed in 45% TFA, 50% CH_2Cl_2, 2.5% anisole, and 2.5% dimethyl phosphite for 20 min followed by neutralization with 10% *N,N'*-diisopropylethylamine in CH_2Cl_2. The peptide was cleaved from the resin (1 g) upon treatment with anhydrous ethylamine (10 mL) at room temperature for 48 h. The ethylamine was evaporated, and the resin was stirred in methanol for 10 min and filtered. The filtrate was concentrated in vacuo, and the residue was triturated with water. The solid product was filtered and dried over P_2O_5 overnight. The dry protected peptide was treated with anhydrous HF (15 mL) at 0 °C for 1 h in the presence of anisole (1.0 mL) and dimethyl phosphite (0.5 mL).

The excess of HF was removed in vacuo. The residue was triturated with anhydrous ether and then was dissolved in (1:1:0.01) water–acetonitrile–acetic acid and lyophilized to give the crude peptide. The product was purified with HPLC using a Dynamax C-18 300A 12-μm (21.4 × 250 mm) column equilibrated with 10% acetonitrile/90% water containing 0.1% TFA at a flow rate of 20 mL/min. The peptide was eluted with a linear gradient from 10% to 51% acetonitrile over a period of 50 min. UV detection was run at 260 nM. The product was collected as a single peak. Selected fractions were lyophilized to give over 95% pure product, on the basis of analytical HPLC, FAB mass spectrometry, and amino acid analysis.

Biological Assays. The receptor binding assay was modified from a literature procedure.[31] A rat pituitary plasma membrane fraction was prepared as a source of LH-RH receptors, and ^{125}I-[Tyr^5]leuprolide[34] was made via chloramine-T oxidation and purified by using ion-exchange chromatography. Several concentrations of test compounds were coincubated for 120 min at 4 °C with ^{125}I-[Tyr^5]leuprolide[34] and receptors to achieve equilibrium binding; the bound tracer was separated from free by centrifugation. The ability of compounds to release luteinizing hormone (LH) from cultured pituitary cells[32,33] was utilized to establish their biological activities. Pituitaries were dispersed with collagenase and hyaluronidase, and the cells were plated for 72 h. The cells were incubated with test compounds for 3 h at 37 °C, and then the medium was harvested for determination of released LH via RIA. The RIA utilized ^{125}I-LH (ovine) as a tracer, immunoaffinity-purified anti-rat LH rabbit IgG, and rat LH (NIH-RP2) standard. Anti-LH rabbit IgG was obtained from Dr. P. M. Conn of the University of Iowa. Bound tracer and free tracer were separated with immobilized protein A.

In vivo testing of compounds was conducted in urethane anesthetized (800 mg/kg, ip) mature castrate (30-day) male rats which have 10–20-fold elevated basal plasma LH levels.[39] Compounds were administered by iv infusion for 120 min through the tail vein in 20% propylene glycol in normal saline, at a rate of 1 mL/h. Blood samples of 0.4-mL volume were drawn by jugular venipuncture periodically over the 2-h infusion period and for 4 h after the infusion was discontinued. Plasma samples were assayed for LH by using the same RIA for rat LH described above, except that goat anti-rabbit antibody was used to precipitate bound tracer.

Acknowledgment. We are indebted to Dr. P. Michael Conn of the University of Iowa for providing the anti-rat LH antibody and for his many helpful suggestions regarding biological methodology.

Registry No. 1, 121962-61-6; 2, 121962-62-7; 3, 121962-63-8; 4, 121962-64-9; 5, 121962-65-0; 6, 121962-66-1; 7, 121962-67-2; 8, 121962-68-3; 9, 121962-69-4; 10, 121962-70-7; 11, 121962-71-8; 12, 121962-72-9; 13, 121962-73-0; 14, 121962-74-1; 15, 121962-75-2; 16, 121962-76-3; 17, 121962-77-4; 18, 121962-28-5; 19, 121962-79-6; 20, 121987-55-1; 21, 121962-80-9; 22, 121962-81-0; 23, 121987-56-2; 24, 121962-82-1; 25, 121962-83-2; 26, 122045-80-1; 27, 121962-84-3; 28, 121962-85-4; 29, 121962-86-5; 30, 121962-87-6; 31, 121962-88-7; 32, 121962-89-8; 33, 121962-90-1; 34, 121962-91-2; 35, 121987-57-3; LH, 9002-67-9.

(39) Romano, M. I.; Machiavelli, G. A.; Alonso, G. E.; Burdman, J. A. *Horm. Metab. Res.* **1986**, *18*, 31.

Tyrphostins I: Synthesis and Biological Activity of Protein Tyrosine Kinase Inhibitors

Aviv Gazit,[†] Pnina Yaish,[‡] Chaim Gilon,[†] and Alexander Levitzki*[,‡]

Departments of Organic Chemistry and Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.
Received January 18, 1989

A novel class of low molecular weight protein tyrosine kinase inhibitors is described. These compounds constitute a systematic series of molecules with a progressive increase in affinity toward the substrate site of the EGF receptor kinase domain. These competitive inhibitors also effectively block the EGF-dependent autophosphorylation of the receptor. The potent EGF receptor kinase blockers examined were found to competitively inhibit the homologous insulin receptor kinase at 10^2–10^3 higher inhibitor concentrations in spite of the significant homology between these protein tyrosine kinases. These results demonstrate the ability to synthesize selective tyrosine kinase inhibitors. The most potent EGF receptor kinase inhibitors also inhibit the EGF-dependent proliferation of A431/clone 15 cells with little or no effect on EGF independent cell growth. These results demonstrate the potential use of *protein* tyrosine kinase inhibitors as selective antiproliferative agents for proliferative diseases caused by the hyperactivity of protein tyrosine kinases. We have suggested the name "tyrphostins" for this class of antiproliferative compounds which act as protein tyrosine kinase blockers.

Among the numerous oncogene products, many exhibit protein tyrosine kinase (PTK) activity, which is essential for their biological activity (see ref 1 and 2, for review). Similarly, the initial event in mitogenesis induction by plasma growth factors is the ligand-induced autophosphorylation of the cell surface receptor and the phosphorylation of a host of intracellular substrates.[2-9] Similarly, the hormone insulin induces autophosphorylation of its receptor on specific tyrosine residues as well as hormone-induced tyrosine phosphorylation of intracellular target proteins. (See ref 10, for review.) Site-directed mutagenesis within the insulin receptor[10] and the epidermal growth factor (EGF) receptor[11,12] molecules which eliminate their PTK activities nullify their biological activity. These findings, which demonstrate that PTK

* Author to whom correspondence should be addressed.
[†] Department of Organic Chemistry.
[‡] Department of Biological Chemistry.

(1) Carpenter, G. *Annu. Rev. Biochem.* **1987**, *56*, 881.
(2) Yarden, Y.; Ulrich, A. *Annu. Rev. Biochem.* **1988** *57*, 443.
(3) Erneaux, C.; Cohen, S.; Garbers, G. L. *J. Biol. Chem.* **1983**, *258*, 4137.
(4) Sawyer, S. T.; Cohen, S. *J. Biol. Chem.* **1985**, *260*, 8233.
(5) Gilmore, T.; Martin, G. S. *Nature* **1983**, *306*, 487.
(6) Bertics, P. J.; Gill, G. N. *J. Biol. Chem.* **1985**, *260*, 14642.
(7) Downward, J.; Waterfield, M. D.; Parker, P. *J. Biol. Chem.* **1985**, *260*, 14538.
(8) Gullick, W. J.; Downward J.; Waterfield, M. D. *EMBO J.* **1985**, *4*, 2869.
(9) Cohen, S.; Fara, R. A. *J. Biol. Chem.* **1985**, *260*, 12351.
(10) Rosen, O. M. *Science* **1987**, *237*, 1452.
(11) Honegger, A. M.; Szopary, D.; Schmidt, A.; Lyall, R.; Van Oberghen, E.; Dull, T. J.; Ulrich, A.; Schlessinger, M. *J. Mol. Cell. Biol.* **1987**, *7*, 4568.

0022-2623/89/1832-2344$01.50/0