was used to obtain the best fit curve to the data. In all cases, the correlation coefficient was greater than 0.99.

Acknowledgment. We are grateful to the National Institutes of Health (Grant NS 15703) and the Shell Development Co. for financial support of this work.

Registry No. 1, 74936-17-7; 2, 98212-71-6; 3, 98212-80-7; 4, 98212-94-3; 5, 98212-95-4; 6, 103500-21-6; 6 (free base), 42303-42-4; 7, 85452-41-1; 8, 103500-22-7; 9, 103500-23-8; 10, 103500-24-9; 11, 103500-25-0; 12, 103500-26-1; 13, 103500-27-2; 13 (free base), 98212-93-2; MeOH, 67-56-1; $PhCH_2OCOCl$, 501-53-1; PhSeCl, 5707-04-0; EtOH, 64-17-5; 1-aminocyclopropanecarboxylic acid, 22059-21-8; triethyl phosphonoacetate, 867-13-0; γ-aminobutyric acid aminotransferase, 9037-67-6.

New Effective Gonadotropin Releasing Hormone Antagonists with Minimal Potency for Histamine Release in Vitro

Jean E. Rivier,* John Porter, Catherine L. Rivier, Marilyn Perrin, Anne Corrigan, William A. Hook,† Reuben P. Siraganian,† and Wylie W. Vale

Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, California 92037, and Clinical Immunology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892. *Received December 12, 1985*

In order to minimize the adverse effect of histamine release in the rat of some gonadotropin releasing hormone (GnRH) antagonists, such as [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH, new structures with modifications at positions 1, 2, 3, 5, 6, 7, and 10 were synthesized and tested in several biological systems. In vitro: the affinity for the pituitary GnRH receptor was measured as was the ability of the analogues to inhibit GnRH-stimulated release of luteinizing hormone (LH) by dispersed anterior pituitary cells in culture and to release histamine from rat mast cells. In vivo: inhibition of ovulation in the cycling rat was determined after subcutaneous (sc) injection of the peptides at noon on the day of proestrus; the duration of action of the peptides was evaluated by measuring LH levels in the castrated male rat after sc injection of some selected analogues. [Ac-D2Nal[1],D4ClPhe[2],D3Pal[3],Arg[5],D-4-*p*-methoxybenzoyl-2-aminobutyric acid[6],DAla[10]]-GnRH was found to be one of the most potent analogues of this series, causing a 100% inhibition of ovulation at 5 μg/kg or less. Release of histamine was observed at doses 10–25 times that required for [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH. Thus, introduction of arginine in position 5 with a hydrophobic amino acid in position 6 is compatible with high potency in several biological systems and results in compounds with lowered potency to release histamine compared to homologous peptides with tyrosine in position 5 and D-arginine in position 6.

It is now well-recognized that the decapeptide gonadotropin-releasing hormone (GnRH) plays a fundamental stimulatory role in the control of reproductive functions. Furthermore, the inhibitory effects of chronic administration of GnRH agonists or of acute administration of GnRH antagonists on reproductive functions have been demonstrated and recently summarized.[1] Because of the fact that these effects are desired for the management of several pathological, steroid-dependent conditions, or as a new means of contraception, potent analogues were developed.[2,3] Whereas superagonists seem to act by desensitization of the pituitary, a process that may take several weeks of treatment, antagonists inhibit LH within 10 min after the first injection of the peptides. In order to be effective, however, the antagonists have to occupy the GnRH receptor at all times, thus emphasizing the need for analogues with high affinity for the receptor and resistance to both degradation and elimination. New antagonists of GnRH have been reported recently that had modifications at positions 1, 2, 3, 6, 7, and 10,[4,5] the most potent of which, in an antiovulatory assay (AOA), had a basic D-amino acid in position 6.[6-8] Subcutaneous (sc) injection of one such analogue, [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH ($ED_{100} \leq 5$ μg/kg in the AOA), however, induced transient edema of the face and extremities in the rat;[9] this effect, which was maximal 3–5 h after peptide administration and which had subsided by 24 h, was not observed in mice, rabbits, and rhesus monkeys. Later studies indicated that this peptide and others with similar structural features such as a basic amino acid in position 6 and a hydrophobic N-terminus did release histamine from rat mast cells.[10] Interestingly, analogues such as the superagonists characterized by a hydrophobic D-amino acid in position 6 and the des-Gly[10],Pro[9]-NHEt substitution, or [Ac-Δ^3Pro[1],D4FPhe[2],DTrp[3,6]]-GnRH (ED_{100} = 25 μg/kg in the AOA), another potent GnRH antagonist, did not produce the in vivo edematous effects and were considerably less

† National Institute of Dental Research, NIH.

(1) Rivier, C.; Vale, W.; Rivier, J. *J. Med. Chem.* **1983**, *26*, 1545–1550.
(2) Dutta, A. S.; Furr, B. J. A. *Annu. Rep. Med. Chem.* **1985**, *20*, 203–214.
(3) Karten, M.; Rivier, J. *Endocr. Rev.* **1986**, *7*, 44–66.
(4) Rivier, J.; Rivier, C.; Perrin, M.; Porter, J.; Vale, W. In *LHRH Peptides as Female and Male Contraceptives*; Zatuchni, G. I., Shelton, J. D., Sciarra, J. J., Eds.; Harper and Row: Philadelphia, 1981; pp 13–23.
(5) Coy, D. H.; Nekola, M. V.; Erchegyi, J.; Coy, E. J.; Schally, A. V. In *LHRH Peptides as Female and Male Contraceptives*; Zatuchni, G. I., Shelton, J. D., Sciarra, J. J., Eds.; Harper and Row: Philadelphia, 1981; pp 37–45.
(6) Rivier, J.; Rivier, C.; Perrin, M.; Porter, J.; Vale, W. In *LHRH and Its Analogs—Contraceptive and Therapeutic Applications*; Vickery, B. H., Nestor, J. J., Jr., Hafez, E. E. E., Eds.; MTP: Lancaster, UK, Advances in Repro., 1984; pp 11–22.
(7) Nestor, J. J., Jr.; Ho, T. L.; Tahilramani, R.; Horner, B. L.; Simpson, R. A.; Jones, G. H.; McRae, G. I.; Vickery, B. H. In *LHRH and Its Analogs—Contraceptive and Therapeutic Applications*; Vickery, B. H., Nestor, J. J., Jr., Hafez, E. E. E., Eds.; MTP: Lancaster, UK, Advances in Repro., 1984; pp 23–33.
(8) Nekola, M. V.; Coy, D. H. In *LHRH and Its Analogs—Contraceptive and Therapeutic Applications*; Vickery, B. H., Nestor, J. J., Jr., Hafez, E. E. E., Eds.; MTP: Lancaster, UK, Advances in Repro., 1984; pp 125–136.
(9) Schmidt, F.; Sundaram, K.; Thau, R. B.; Bardin, C. W. *Contraception* **1984**, *29*, 283–289.
(10) Hook, W. A.; Karten, M.; Siraganian, R. P. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1985**, *44*, 1323.

0022-2623/86/1829-1846$01.50/0

Table I. Chemical Characterization of GnRH Analogues

no.	compounds structure	$[\alpha]_D$[a]	retention time in HPLC[b] system A % CH_3CN	system A t_R, min	system B % CH_3CN	system B t_R, min
1	[Ac-D2Nal[1],D4ClPhe[2],D3Pal[3],Arg[5],D2Nal[6],DAla[10]]-GnRH	-34.3	33.0	7.19	36	14.50
2	,DTrp[6],DAla[10]]-GnRH	-30.3	29.4	8.40	36	5.70
3	,1FormylDTrp[6],DAla[10]]-GnRH	-24.3	30.0	8.01	36	6.16
4	,6NO_2DTrp[6],DAla[10]]-GnRH	-35.0	30.6	6.00	36	5.84
5	,DTyr[6],DAla[10]]-GnRH	-29.8	24.6	6.20	36	3.06
6	,D3Pal[6],DAla[10]]-GnRH	-29.8	22.8	5.47	36	3.20
7	,imBzlDHis[6],DAla[10]]-GnRH	-23.0	25.2	8.00	36	6.50
8	,DGlu[6],DAla[10]]-GnRH	-24.0	24.0	6.77	30	4.10
9	,DGlu[6](AA)*,DAla[10]]-GnRH[c]	-27.8	31.2	6.09	36	6.20
10	]-GnRH	-26.5	31.2	5.41	36	5.44
11	,*N*MeLeu[7],DAla[10]]-GnRH	-31.9	33.0	5.96	36	10.92
12	[Ac-Δ^3Pro[1], Tyr[5], ,DAla[10]]-GnRH	-58.5	27.6	7.09	33	4.57

[a] Concentration in 50% AcOH = 1. [b] Peptides (10 μg/10 μL) dissolved in A buffer were applied to a Vydac C_{18} end-capped (5 μm, 300 Å pore size; 4.5 × 250 mm) column under isocratic conditions at a flow rate of 2.0 mL/min. UV detection was 0.2 AUFS at 210 nm. Solvent system A was TEAP, pH 2.25, with the concentration of acetonitrile shown. Solvent system B was TEAP/H_2O (1/2), pH 7.2, with the concentration of acetonitrile shown. [c] (*) DGlu[6](AA) = DGlu[6](anisole adduct) = 4-(*p*-methoxybenzoyl)-D-2-aminobutyric acid[6].

Table II. Biological Characterization of GnRH Analogues

compd	relative potencies in vitro[a]	K_D, nM	AOA[b]	in vitro histamine release:[c] ED_{50} ± SE, μg/mL
1	0.88 (0.59–1.31)	5.0 (3.8–6.6)	1.0 (8/10)	1.9 ± 0.10
2	0.44 (0.28–0.63)	0.96 (0.57–1.5)	0.5 (2/10), 1.0 (0/6)	2.1 ± 0.23
3	0.95 (0.67–1.35)	0.48 (0.31–0.72)	0.5 (9/20)	3.4 ± 0.60
4	1.16 (0.85–1.57)	0.63 (0.42–0.95)	0.5 (7/20)	2.7 ± 0.55
5	1.35 (0.98–1.91)	0.18 (0.12–0.26)	0.5 (11/15)	3.7 ± 0.51
6	0.91 (0.76–1.10)	0.24 (0.17–0.34)	0.5 (5/10), 1.0 (0/10)	2.9 ± 0.33
7	0.50 (0.35–0.72)	0.54 (0.37–0.78)	0.5 (8/10)	4.4 ± 0.93
8	0.09 (0.07–0.12)	0.16 (0.11–0.23)	10 (9/10)	17 ± 3.7
9	1.01 (0.80–1.30)	0.67 (0.43–1.0)	0.5 (8/16), 1.5 (0/10)	1.6 ± 0.66
10		0.60 (0.38–0.93)	1.0 (1/10), 2.5 (0/10)	3.2 ± 0.52
11	0.75 (0.55–1.01)	0.77 (0.51–1.0)	1.0 (8/10)	4.6 ± 0.82
12	0.13 (0.10–0.17)	0.31 (0.25–0.38)	5.0 (10/10)	30.3 ± 4.95

[a] Pituitary cell culture assay[16] internal standard was [Ac-Δ^3Pro[1],D4FPhe[2],DTrp[3,6]]-GnRH = 1. ED_{50} ± SE in the in vitro histamine release test was 39 ± 5.6. [b] AOA = antiovulatory assay: dosage in micrograms (rats ovulating/total). [c] ED_{50} for [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH was 0.17 ± 0.01 μg/mL.

potent than [Ac-D2Nal[1],D4FPhe[2],DTrp[3],DArg[6]]-GnRH in releasing histamine from rat mast cells.[29]

We report here on the synthesis of several GnRH analogues whose structures were based on [Ac-D2Nal[1],D4ClαMePhe[2],DTrp[3],Arg[5],DTyr[6],DAla[10]]-GnRH, which was shown by Roeske et al.[11] to retain potent inhibitory activity in the antiovulatory assay. These new antagonists, which were tested in several assay systems in which some analogues are shown to be equipotent to the most potent analogues reported to date ($ED_{100} \leq 5$ μg/kg in the AOA), did not induce edema of the face and paws of the rat at the doses tested (500 μg/kg); additionally, they were significantly less potent in releasing histamine from rat mast cells than analogues with a D-arginine in position 6.

Synthesis, Purification, and Characterization (See Table I). Analogues were synthesized automatically on a Beckman 990 peptide synthesizer with use of previously described protocols.[12] Briefly, *tert*-butyloxycarbonyl (Boc) was used for N-terminal protection with N-terminal deblocking achieved with 60% TFA in CH_2Cl_2 and 1–2% ethanedithiol. Washes included 2-propanol, CH_2Cl_2, and MeOH. Triethylamine (10% in CH_2Cl_2) was used for neutralization. Couplings were mediated by dicyclohexylcarbodiimide (DCCI) in CH_2Cl_2 or 50% DMF/CH_2Cl_2, depending on the solubility of the Boc-amino acids. Unnatural amino acids 3-(2-naphthyl)-D-alanine (D2Nal), 4-chloro-D-phenylalanine (D4ClPhe), and 3-(3-pyridyl)-D-alanine (D3Pal) were synthesized de novo, resolved, and appropriately protected for SPPS (see Experimental Section). 6-Nitro-D-tryptophan (6NO_2DTrp) was obtained by nitration of D-tryptophan (DTrp) by use of the method of Moriya et al.[13] HF cleavages were carried out under different conditions depending on the desired products (**8** vs. **9**). In order to optimize the conditions leading to the formation of **9**, HF cleavages were performed at room temperature for an extended period of time.[14] With the exception of 4-(*p*-methoxybenzoyl)-D-2-aminobutyric acid in position 6, referred to as DGlu[6] (anisole adduct), which is obtained as the result of the reaction of the carboxyl acylium ion with anisole in the presence of HF,[14] all other D-amino acids in position 6 had been previously used in that position with varying degrees of success in agonist as well as antagonist series.[3]

Peptides were purified by preparative HPLC as previously reported[15] and characterized as shown in Table I. Amino acid analyses including quantitation of the un-

(11) Roeske, R.; Chaturvedi, N. C.; Rivier, J.; Vale, W.; Porter, J.; Perrin, M. In *Peptides Structure and Function*, Proceedings of the Ninth American Peptide Symposium; Deber, C. M., Hruby, V. J., Kopple, K. D. Eds.; Pierce Chemical Co., Rockford, IL, 1986; pp 561–564.
(12) Marki, W.; Spiess, J.; Tache, Y.; Brown, M.; Rivier, J. E. *J. Am. Chem. Soc.* **1981**, *103*, 3178–3185.
(13) Moriya, T.; Hagio, K.; Yoneda, N. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 2217–2218.
(14) Feinberg, R. S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1975**, *97*, 3485–3496.
(15) Rivier, J.; McClintock, R.; Galyean, R.; Anderson, H. *J. Chromatogr.* **1984**, *288*, 303–328.

Table III. Time Course of Action of [Ac-Δ^3Pro^1,D4FPhe^2,DTrp^3,D2Nal^6]-GnRH (**13**) and **9** Administered Sc on LH Secretion in the Castrated Male Rat

	LH levels, ng/mL						
compound	T = 0	T = 1 h	T = 19 h	T = 43 h	T = 49 h	T = 67 h	T = 73 h
control	12.60 ± 0.95	9.20 ± 0.67	10.79 ± 0.64	9.92 ± 0.21	9.17 ± 0.77	8.92 ± 0.87	10.28 ± 0.96
13 (25 μg)	10.25 ± 0.94	5.68 ± 0.35[a]	2.78 ± 0.04[a]	4.38 ± 0.61[a]	5.46 ± 0.60[a]	8.23 ± 0.60	9.15 ± 1.30
13 (125 μg)	12.47 ± 0.78	6.17 ± 0.54	3.00 ± 0.30[a]	2.63 ± 0.30[a]	2.50 ± 0.23[a]	2.74 ± 0.45[a]	4.58 ± 1.13[a]
9 (125 μg)	13.38 ± 1.48	6.31 ± 0.55	3.68 ± 0.35[a]	3.27 ± 0.35[a]	2.94 ± 0.28[a]	3.67 ± 0.43[a]	5.06 ± 0.62[a]

[a] $P \leq 0.01$. Between group comparisons were performed with the multiple range comparison test of Duncan.

natural amino acids gave the expected ratios (details are to be reported elsewhere). Purity of the isolated peptides was calculated to be greater than 93% on the basis of absorbance data obtained on HPLC in two systems.

Biological Activities and Relative Potencies (See Table II). In vitro, the peptides were tested for their ability to inhibit GnRH-mediated LH secretion by cultured pituitary cells.[16] Potencies of GnRH antagonists were expressed relative to a standard ([Ac-Δ^3Pro^1,D4FPhe^2,DTrp^{3,6}]-GnRH). In binding studies, the K_D for the potent agonist [DAla^6,*N*MeLeu^7,Pro^9-NEt]-GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_D values were calculated from the potencies of the analogues (relative to the standard) determined from displacement data.[17] The in vivo antiovulatory assay was carried out as described by Corbin and Beattie.[18] Results are expressed in terms of the ratio of GnRH-antagonist-treated rats ovulating over vehicle-administered controls. The peptides were dissolved in saline unless it is stated otherwise and injected subcutaneously in a volume of 200 μL at noon on the day of proestrus.

Results and Discussion

Earlier results indicating that the Arg^5 substitution could be introduced with retention of considerable potency[11] in the AOA led us to synthesize and test in several biological systems a series of analogues having the hydrophobic Ac-D2Nal^1-D4ClPhe^2 substitutions at the N-terminus including the D3Pal^3 substitution first reported by Folkers et al.[19] We systematically varied the amino acid at the 6-position while also introducing the DAla^{10} substitution first suggested by Coy et al.[5] We had hoped that this homologous series would permit a semiquantitative analysis of structure-activity relationships.

We had suggested in a prior publication[4] that HPLC retention times under isocratic conditions with use of a buffer at pH 7.2–7.4 could be a measure of a peptide's overall relative hydrophobicity within an homologous family. However, no good direct correlation between hydrophobicity and biological potency in vitro could be found, suggesting that other parameters such as the aromatic or aliphatic character of the side chain or even the electronic properties and location of substituents on an aromatic side chain may play an important role. Compounds **1**–**9** are part of an homologous family and were analyzed with HPLC with use of TEAP buffers at pH 2.25 and 7.2. The order of the hydrophobicity when analyzed at pH 7.2 was **8** < **5** < **6** < **2** < **4** < **3** < **9** < **7** < **1**. A good correlation of these data with relative in vitro potencies or with the affinity of these peptides for the GnRH receptor (Table II) may be difficult to obtain because of the inability of the GnRH receptor assay to discriminate the effects of drastic changes in the nature of the side chain of the amino acid at the 6-position,[20] or because relative potencies are too similar. Since the conditions in the three assays (in vitro cell culture, binding, and in vivo) are very different and potencies may reflect differential rates of absorption, or degradation in vivo, or of association and dissociation from the receptors in vitro, it is not surprising that a good correlation cannot be obtained. An attempt at correlating the order of elution with in vivo data is also hampered by the cost of obtaining reliable ED_{50} values in the antiovulatory assay.

Independent of these considerations, peptides **1**–**7**, **9**, and **10** belong to a new class of GnRH analogues with high potencies in inhibiting ovulation in the rat. Of interest are **5** and **8**, which are more hydrophilic at physiological pH than their homologues. Of the series, **5** and **8** exhibit the highest affinity for the GnRH receptor under the test conditions. The fact that **8** is not very potent in the in vitro and in vivo assays was expected in view of earlier results indicating the deleterious effects on bioactivity of the DGlu^6 substitution in an agonist.[20] *N*MeLeu^7, a substitution known to improve potency of certain antagonists,[21] was introduced in **11** since it was proposed that this modification may render the bond between residues 6–7 more resistant to enzymatic degradation;[20] however, the low potency of **11** in vivo may suggest that the extra methyl group interferes with transport to, recognition by, or activation of the receptor. Introduction of the Ac-Δ^3Pro^1 substitution, recognized to be more hydrophilic than the Ac-D2Nal^1 substitution, and keeping tyrosine in position 5 yielded **12**, which has high affinity for the GnRH receptor as measured in the cell culture and binding assays; **12**, however, is more than 10 times less potent in vivo than **9**, suggesting that both Ac-DNal^1 and Arg^5 substitutions confer desirable characteristics on this class of analogues.

In order to assess the duration of action on LH secretion of compound **9** as compared to another known antagonist, saline, **9**, and [Ac-Δ^3Pro^1,D4FPhe^2,DTrp^{3,6}]-GnRH were injected subcutaneously to castrated male rats and blood samples were drawn at several time points. Figure 1 shows that the effect of the antagonist is rapid in its onset and provides a significant ($P \leq 0.01$) inhibition of LH secretion for at least 26 h. No statistical difference between **9** administered at two doses and [Ac-Δ^3Pro^1, D4FPhe^2,DTrp^{3,6}]-GnRH can be seen. In another experiment, whereby [Ac-Δ^3Pro^1,D4FPhe^2,DTrp^3,D2Nal^6]-GnRH (**13**)[4] and **9** were administered sc at the same dose (125 μg) and LH secretion was followed over time, both peptides showed undistinguishable responses at times up to 73 h

(16) Vale, W.; Grant, G. In *Methods in Enzymology, Hormones and Cyclic Nucleotides*; O'Malley, B. W., Hardman, J. G., Eds.; Academic: New York, 1984; pp 213–219.
(17) Perrin, M. H.; Haas, Y.; Rivier, J. E.; Vale, W. W. *Mol. Pharmacol.* **1983**, *23*, 44–51.
(18) Corbin, A.; Beattie, C. W. *Endocr. Res. Commun.* **1975**, *2*, 1–23.
(19) Folkers, K.; Bowers, C. Y.; Kubiak, T.; Stepinski, J. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 1089–1095.
(20) Vale, W.; Rivier, C.; Brown, M.; Leppaluoto, Y.; Ling, N.; Monahan, M.; Rivier, J. *Clin. Endocrinol. (Oxford)* **1976**, *5*, 261s–273s.
(21) Ling, N.; Vale, W. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 801–806.
(22) Kornreich, W.; Anderson, H.; Porter, J.; Vale, W.; Rivier, J. *Int. J. Pept. Protein Res.* **1984**, *25*, 414–420.

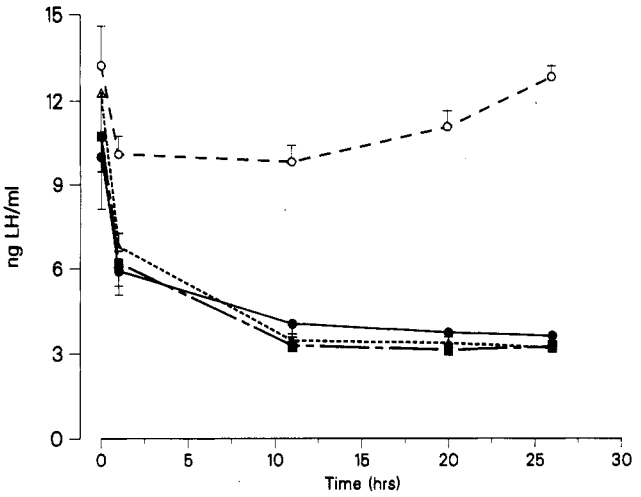


Figure 1. Duration of action on LH secretion in castrated male rats. Rats received saline (O) or antagonist subcutaneously (200 μL). Compounds were **9** (25 μg, Δ; 100 μg, ■) and [Ac-Δ^3Pro^1,D4FPhe^2,DTrp^{3,6}]-GnRH (100 μg, ●). LH levels were measured at times 1, 11, 20, and 26 h. Each point represents the mean ± SEM of six animals. The analogues significantly ($P \leq 0.01$) lowered plasma LH levels at all times tested. Between group comparisons were performed with the multiple range comparison test of Duncan.

when inhibition had returned to approximately half maximal (see Table III).

This allows us to conclude that the introduction of arginine in position 5 does not seem to render the molecule significantly more labile to enzymes or more amenable to elimination than a standard compound with significantly different substitutions. These data provide evidence that LH secretion could be consistently suppressed for three days in the rat by a single administration of at least one member of this new series of analogues. Furthermore, the rats treated sc with **9** (500 μg/kg) were observed to show no sign of swollen paws or snout. This is significant in light of the observation that these disturbing undesirable effects were obtained with compounds such as [Ac-D2Nal^1,D4ClPhe^2,DTrp^3,DArg^6]-GnRH[9] when injected at similar doses. At a higher dose of **9** (1000 μg/kg), no effect could be seen either (Sundaram, K., private communication). Since these effects may be related to local release of histamine and concomitantly of serotonin, relative potencies on histamine release in an in vitro mast cell assay were obtained (Table II). As can be seen, compounds **1–12** are at least 10 times less potent than the standard in releasing histamine (ED_{50} for [Ac-D2Nal^1,D4FPhe^2,DTrp^3,DArg^6]-GnRH is 0.17 ± 0.01 μg/mL), indicating that significant dissociation of activities (histamine release vs. inhibition of ovulation, for example) can be achieved. It is noteworthy that **8** and **12**, which are much less potent in the AOA, also release less histamine. In the case of compound **8**, one may suggest that a zwitterionic structure may be responsible for masking the positive charge of the Arg^5 side chain while that charge is altogether absent in **12**. Interpretations of these results in terms of the different receptor requirements for biological expression must await a better knowledge of the nature of the bioactive tertiary structures of the ligands. Progress in the development of less flexible GnRH antagonists[23] (cyclic analogues) should allow us to propose more accurate models of this interaction.

Experimental Section

Instruments. Amino acid analyses after 4 N methanesulfonic hydrolysis at 110 °C for 24 h were performed on a Perkin-Elmer high-pressure liquid chromatograph using orthophthalaldehyde postcolumn derivatization and fluorescence detection. HPLC were run on a Beckman system using two 100A pumps, an Altex Model 420 controller, a Kratos Model 757 UV detector, and an Omniscribe Series D500 dual-pen recorder from Houston Instruments, a Shimadzu Chromatopac EIA integrator, and a rheodyne Model 7125 injector. The peptide synthesizer used was Beckman Model 990. The HF cleavage line was designed in-house and allowed for HF elimination under high vacuum.

Starting Materials. The methyl benzhydrylamine-resin used (3 g per peptide) was obtained according to published procedures[24] with use of *p*-toluoyl chloride instead of benzoyl chloride during the Friedel–Crafts step. Resins with substitutions varying from 0.3 to 0.65 mequiv/g were used with no observable differences.

Boc-(1-formyl)DTrp, Boc-DTrp, Boc-(imBzl)DHis, Boc-(OBzl)Ser, Boc-(2,6-Cl_2Bzl)Tyr, Boc-(Tos)Arg, Boc-(*N*Me)Leu, Boc-DAla, Boc-Leu, Boc-Pro, DTrp, and Boc-carbonate were from Bachem (Torrance, CA). Δ^3Pro^1 was obtained from the Contraceptive Development Branch of NICHD at NIH and derivatized (Boc) by use of standard procedures. All solvents were reagent grade; DCCI was distilled under high vacuum.

3-(2-Naphthyl)-DL-alanine (DL-2-Nal) was synthesized by use of the method of Dittmer et al.[25]

***N*-(Trifluoroacetyl)-3-(2-naphthyl)-DL-alanine** was obtained by use of the method of Weygand and Geiger.[26] Dry racemic 2Nal (90 g, 0.42 mol) was dissolved in trifluoroacetic acid (200 mL) with stirring. After cooling to 5 °C, with use of an ice bath, trifluoroacetic anhydride (75 mL, 1.2 equiv) was added dropwise (15 min) to the stirred solution and allowed to react for 2 h at 5 °C and then overnight at room temperature. The solvent was removed by rotary evaporation under reduced pressure, leaving a white solid. The crude product was taken up in ethyl acetate (600 mL), washed with water (3 × 300 mL), and dried over $MgSO_4$. Solvent evaporation under reduced pressure gave 99.2 g (0.32 mol, 76%: mp 139–141 °C.

***N*-(Trifluoroacetyl)-3-(2-naphthyl)-D-alanine.** The trifluoroacetylated amino acid (99 g, 0.32 mol) was dissolved in water (3 L) by addition of 2 N sodium hydroxide by use of an automatic titrator set at pH 7.2 until base uptake ceased (8 h). The solution was filtered to remove undissolved solids and warmed to 37 °C with stirring, and the pH was adjusted to 7.2 with the titrator delivering 0.2 N sodium hydroxide. Carboxypeptidase A type II (1000 units from Sigma) was added, via a syringe, to effect asymmetric hydrolysis. As the concentration of hydrolyzed L-2Nal increased (by 24 h), the reaction rate slowed down. Removal of L-2Nal by filtration and addition of enzyme to the filtrate drove the resolution to completion. This last step may have to be repeated depending upon the quality of the particular batch of enzyme. When base uptake ceased, the solution was filtered and acidified with 6 N HCl to pH 2.5. Ethyl acetate extraction (3 × 300 mL) and drying over $MgSO_4$ and solvent removal under reduced pressure gave the unhydrolyzed *N*-(trifluoroacetyl)-3-(2-naphthyl)-D-alanine (49.9 g, 0.15 mol): $[\alpha]_D$ −34.8° (*c* 1, EtOAc); mp 144 °C.

3-(2-Naphthyl)-D-alanine. The resolved N-trifluoracetylated amino acid (49.9 g, 0.15 mol) was dissolved in ethanol (250 mL) and treated with 2 N sodium hydroxide (185 mL, 0.37 mol, 2.5 equiv). The solution was stirred overnight at 37 °C, filtered to remove any solid material, and then acidified with 6 N HCl to pH 5.5. Collection by filtration and drying overnight under vacuum gave 31.1 g (0.145 mol, 95%): mp 247 °C; $[\alpha]_D$ +26.4° (*c* 1, HOAc).

Boc-3-(2-naphthyl)-D-alanine. Treatment of D-2Nal (31 g, 0.145 mol) with Boc-carbonate (35 g, 1.2 equiv) in the usual

(23) Rivier, J.; Varga, J.; Porter, J.; Perrin, M.; Hass, Y.; Corrigan, A.; Rivier, C.; Vale, W. In *Peptides Structure and Function*, Proceedings of the Ninth American Peptide Symposium; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co., Rockford, IL, 1986, pp 541–544.

(24) Rivier, J.; Vale, W.; Burgus, R.; Ling, N.; Amoss, M.; Blackwell, R.; Guillemin, R. *J. Med. Chem.* **1973**, *16*, 545–549.

(25) Dittmer, K.; Herz, W.; Cristol, S. J. *J. Biol. Chem.* **1948**, *173*, 323–326.

(26) Weygand, F.; Geiger, R. *Chem. Ber.* **1955**, *84*, 647.

manner gave after workup 41.4 g of Boc-D2Nal (0.131 mol, 90%); mp 94–95 °C; $[\alpha]_D$ −42.6° (c 1, EtOH).

Boc-6-nitro-D-tryptophan. $6NO_2$DTrp was obtained by nitration of DTrp by use of the method of Moriya et al.[13] $6NO_2$DTrp (5 g, 0.02 mol) was dissolved in H_2O (40 mL) and *tert*-butyl alcohol (40 mL); pH was adjusted to 9 with 1 N NaOH. Boc-carbonate (1.2 equiv, 4.8 g) was added over 25 min, while the pH was maintained at 9 by use of an automatic titrator delivering 1 N NaOH. After 2 h of stirring, water (200 mL) was added and the resulting mixture extracted with hexane (2 × 100 mL). The aqueous phase was then acidified to pH 2.0 by use of sodium hydrogen sulfate. Extraction with ethyl acetate (2 × 100 mL) and drying ($MgSO_4$) followed by rotary evaporation gave an oil (6.4 g, 0.018 mol, 90%), which solidified upon standing. Recrystallization from ethyl acetate/hexane gave 5.7 g: mp 134 °C; $[\alpha]^{22}_D$ +7.1° (c 1, MeOH).

Diethyl Acetamido(3-pyridylmethyl)malonate. Sodium metal (28 g, 1.21 mol) was dissolved by stirring in ethanol (0.6 L), previously rendered dry by distillation from magnesium turnings, and then treated with diethyl acetamidomalonate (132 g, 0.61 mol). Solid 3-picolyl chloride hydrochloride from Aldrich (100 g, 0.61 mol) was suspended in benzene, rotary evaporated to eliminate residual water, and further dried overnight under high vacuum. It was added to the resulting clear yellow sodiomalonate solution and stirred overnight at 25 °C. The precipitated salts were removed by filtration, and the filtrate was rotary evaporated to dryness. The resulting red residue was dissolved in water (0.3 L), acidified to pH 6 with 3 N HCl, and then refrigerated overnight. The crude product was collected by filtration and washed with ice water to remove color. Drying overnight under vacuum gave 133 g (0.43 mol, 71%); an aliquot was recrystallized from water; mp 94–95° dec.

***N*-Acetyl-3-(3-pyridyl)-DL-alanine ethyl ester** was prepared from diethyl acetamido(3-pyridylmethyl)malonate (132 g, 0.42 mol) as described by Hoes et al.[27] for *N*-acetyl-3-(2-pyridyl)-DL-alanine ethyl ester to yield 65 g (0.277 mol, 66%); recrystallization was done in ethyl acetate/hexane; mp 118–121 °C.

***N*-Acetyl-3-(3-pyridyl)-D-alanine Ethyl Ester.** The racemic acetyl ethyl ester (65 g, 0.27 mol) was dissolved in water (0.5 L) and treated with potassium chloride (22 g, 0.29 mol). The pH was adjusted to 7 with 2 N potassium hydroxide, and α-chymotrypsin (200 mg) was added. The resolution mixture was stirred overnight at room temperature with the pH kept constant at 7.2 by use of an automatic titrator.

The solvent was concentrated to 200 mL by rotary evaporation and extracted with ethyl acetate (3 × 200 mL). The combined extracts were dried ($MgSO_4$), filtered, and rotary evaporated to yield the resolved compound. Drying overnight under vacuum gave 28 g (0.12 mol, 87%): mp 122 °C; $[\alpha]_D$ −6.8° (c 1, MeOH).

3-(3-Pyridyl)-D-alanine was prepared from *N*-acetyl-3-(3-pyridyl)-D-alanine ethyl ester (128 g, 0.12 mol) as described by Hoes et al.[27] for 3-(2-pyridyl)-D-alanine to yield 16.1 g (0.097 mol, 81%): mp 253–256 °C; $[\alpha]_D$ −26.1° (c 1, 1 N HCl).

Boc-3-(3-pyridyl)-D-alanine. D3Pal (21 g, 0.126 mol) was dissolved in H_2O (100 mL) and *tert*-butyl alcohol (100 mL); pH was adjusted to 8.6. Boc-carbonate (1.2 equiv, 30.2 g) was added dropwise over 30 min at constant pH with use of an automatic titrator. After 3 h of stirring, H_2O (200 mL) was added and the mixture extracted with hexane (2 × 200 mL). After acidification of the aqueous phase with sodium hydrogen sulfate to pH 3.8, the mixture was rotary evaporated to dryness. The resulting solid was dried under high vacuum overnight and then extracted with hot ethyl acetate (3 × 250 mL). The combined extracts were concentrated to dryness by rotary evaporation. The Boc-amino acid was again extracted with hot ethyl acetate (300 mL) and filtered. The filtrate was concentrated by half and petroleum ether (300 mL) added. Filtration of the product and drying under vacuum gave 29 g (0.11 mol, 88%): mp 135–136 °C; $[\alpha]^{22}_D$ −17.8° (c 1, 95% EtOH).

Peptide Synthesis. TFA treatment was extended to two times 15 min. Coupling time was 90–120 min; no recouplings were performed during automatic synthesis. Threefold-excess protected amino acid was used based on the original substitution of the methyl benzhydrylamine–resin. N-Terminal acetylation was performed with a large excess of acetic anhydride in CH_2Cl_2 for 15 min.

Preparation of Anisole Adduct 9. Ac-D2Nal-D4ClPhe-D3Pal-(OBzl)Ser-(Tos)Arg-(OBzl)DGlu-Leu-(Tos)Arg-Pro-DAla-MBHA-resin (5.0 g) assembled on the Beckman 990 synthesizer was concomitantly deprotected and cleaved in the presence of HF (180 mL) and anisole (20 mL). The reaction mixture was stirred for 3 h at room temperature. The HF was removed from the reaction vessel by use of high vacuum and the residue treated with ether (250 mL) and filtered. The peptide was extracted from the resin with dilute AcOH and lyophilized to yield a fluffy white solid (2.4 g) consisting of two major peaks, **8** (38%) and the anisole adduct **9** (18%).

Preparation of Anisole Adduct 9 Using 8 as Starting Material. Compound **8** (5 g) was HF treated as described above for treatment of the resin–peptide. The reaction product was lyophilized to dryness. Analysis by HPLC demonstrated two major peaks consisting of **8** (26%) and **9** (35%). Purification was carried out as described below.

Purification. Crude peptides **8** and **9** (1.5 g) were dissolved in 0.25 M triethylammonium phosphate (150 mL), pH 2.25 (TEAP 2.25), and loaded onto a 5 × 30 cm preparative reversed-phase HPLC cartridge packed by us using a Vydac C_{18} (330-Å pore size, 15–20-μm particle size).[15] The peptide was eluted with use of a flow rate of 125 mL/min on a Waters Prep 500 system with a mixture of A (TEAP 2.25) and B (50% CH_3CN, 50% A) with use of a gradient (35–65% B in 90 min). The unreacted DGlu[6] antagonist **8**, which eluted first, was collected for retreatment with HF anisole for ultimate total conversion to the desired anisole adduct.

The collected fractions were converted to the TFA salt by loading on a preparative reversed-phase HPLC cartridge as above and eluted with use of a mixture of solvents A (0.1% TFA) and B (60% CH_3CN, 40% 0.1% TFA) and the following gradient: (30 (10′) → 30 (30′) → 75% B).

Biological Testing. Edematogenic test,[9] histamine release in vitro,[10] in vitro dispersed pituitary cell culture assay,[16] binding assay,[17] AOA,[18] and the in vivo measure of LH inhibition after administration of the peptides in the castrated male rat[28] were carried out as described in the respective references.

Acknowledgment. Research was supported by NIH Contract N01-HD-4-2833 and NIH Grant HD-1-3527. Research was conducted in part by The Clayton Foundation for Research, California Division. Catherine Rivier and Wylie Vale are Clayton Foundation Investigators. We thank Ron Kaiser, John Dykert, Georgia Morgan, Mary Tam, and Yaira Haas for excellent technical assistance and Rebecca Hensley for manuscript preparation. We are indebted to Dr. Marvin Karten for his invaluable assistance in this effort. We thank Drs. Naqvi and Lindberg for some of the antiovulatory data obtained at the Mason Research Institute under Contract N01-HD-0-2846 with the Contraceptive Development Branch, Center for Population Research, NICHD.

Registry No. **1**, 101685-07-8; **2**, 101685-05-6; **3**, 103732-99-6; **4**, 101707-87-3; **5**, 103733-00-2; **6**, 101685-06-7; **7**, 101685-08-9; **8**, 103733-01-3; **9**, 103733-02-4; **10**, 103733-03-5; **11**, 103733-04-6; **12**, 103733-05-7; Boc-(1-formyl)DTrp, 64905-10-8; Boc-DTrp, 5241-64-5; Boc-(imBzl)DHis, 65717-64-8; Boc-(OBzl)Ser, 23680-31-1; Boc-(2,6-Cl_2Bzl)Tyr, 40298-71-3; Boc-(Tos)Arg, 13836-37-8; Boc-(NMe)Leu, 53363-89-6; Boc-DAla, 7764-95-6; Boc-Leu, 13139-15-6; Boc-Pro, 15761-39-4; DL-2Nal, 14108-60-2; L-2Nal, 58438-03-2; D-2Nal, 76985-09-6; Boc-carbonate, 24424-99-5; $6NO_2$DTrp, 56937-50-9; gonadotropin releasing hormone, 33515-09-2; hist-

(27) Hoes, C.; Raap, J.; Bloemhoff, W.; Kerling, K. E. T. *Recl. Trav. Chim. Pays-Bas* **1980**, *99*(3), 99–104.

(28) Rivier, C.; Rivier, J.; Perrin, M.; Vale, W. *Biol. Reprod.* **1983**, *29*, 374–378.

(29) Karten, M. J.; Hook, W. A.; Siraganian, R. P.; Coy, D. H.; Folkers, K.; Rivier, J. E.; Roeske, R. W. In *LHRH and Its Analogs, Vol. II*; Nestor, J., Vickery, B., Eds.; MTP: Lancaster, in press.

amine, 51-45-6; *N*-(trifluoroacetyl)-3-(2-naphthyl)-DL-alanine, 103733-06-8; trifluoroacetic anhydride, 407-25-0; *N*-(trifluoroacetyl)-3-(2-naphthyl)-D-alanine, 103774-97-6; Boc-3-(2-naphthyl)-D-alanine, 76985-10-9; Boc-6-nitro-D-tryptophan, 103733-07-9; diethyl acetamidomalonate, 1068-90-2; 3-picolyl-chloride hydrochloride, 39901-94-5; diethyl acetamido(3-pyridylmethyl)malonate, 103733-08-0; 3-(3-pyridyl)-D-alanine, 70702-47-5; Boc-3-(3-pyridyl)-D-alanine, 98266-33-2; *N*-acetyl-3-(3-pyridyl)-DL-alanine ethyl ester, 103733-09-1; *N*-acetyl-3-(3-pyridyl)-D-alanine ethyl ester, 103774-98-7.

Mechanism of Action of the Marine Natural Product Stypoldione: Evidence for Reaction with Sulfhydryl Groups

E. Timothy O'Brien,[†,‡] David J. Asai,[†,§] Amiram Groweiss,[∥] Bruce H. Lipshutz,[⊥] William Fenical,[∥] Robert S. Jacobs,[†,#] and Leslie Wilson*[†]

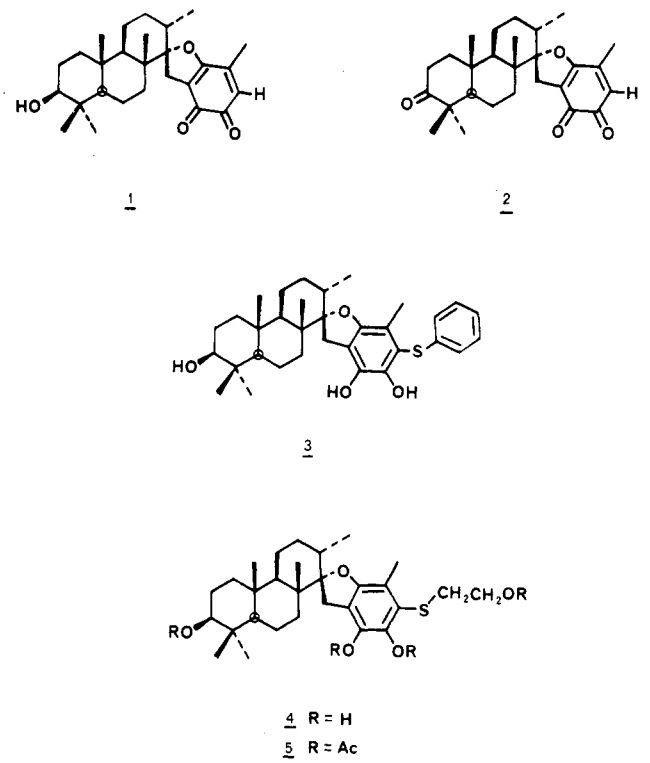
Departments of Biological Sciences and Chemistry and The Marine Science Institute, University of California, Santa Barbara, California 93106, and The Institute of Marine Resources, Scripps Institution of Oceanography, La Jolla, California 92093.
Received July 22, 1985

Stypoldione, a marine natural product that possesses an *o*-quinone functional group, has been shown to inhibit a variety of biological processes including cell division. We found that stypoldione binds covalently to sulfhydryl groups of thiol-containing compounds via addition of sulfur to the C-4′ position of the quinone ring. We examined the ability of stypoldione to add to sulfhydryl groups of a number of thiol-containing substances, including glutathione, thiophenol, β-mercaptoethanol, and the protein tubulin. We suggest that the biological actions of stypoldione may be caused by the addition of this compound to thiol groups of biological molecules.

Stypoldione (1), an *o*-quinone derived from the tropical marine alga *Stypopodium zonale*, inhibits a large number of biological and biochemical processes.[1-9] Initially identified as one of the major products of the alga responsible for fish toxicity,[1,2] stypoldione subsequently was found to inhibit division of cultured mammalian cells, Ehrlich ascites tumor cells and P388 lymphocytic leukemia cells in mice, and fertilized sea urchin eggs.[3,4,6] More detailed analysis revealed that low concentrations of stypoldione could inhibit cell division in fertilized sea urchin eggs without affecting spindle organization or chromosome movement,[7] while higher concentrations inhibited spindle formation, amino acid uptake and incorporation into protein, and DNA synthesis.[4] Stypoldione was also found to inhibit polymerization of bovine brain tubulin into microtubules in vitro[5] and to inhibit flagellar movement of intact or demembranated sea urchin sperm.[8,9] Importantly, the presence of dithiothreitol in the demembranated sperm reactivation buffer prevented the inhibition of flagellar movement by stypoldione.[9]

The wide range of inhibitory activities indicated that the compound might be affecting a number of different receptor classes, while the ability of dithiothreitol to prevent the action of stypoldione on movement of demembranated sperm suggested that sulfhydryl groups might be involved in the action. Further, reactivity of *o*-quinones with sulfhydryl groups had been described previously.[10] In the present study, we determined that stypoldione can react with sulfhydryl groups from a number of different types of thiol-containing compounds including β-mercaptoethanol, cysteine, glutathione, and a number of proteins including tubulin. Our data suggest that the wide spectrum of biological activity of stypoldione is due to a common mechanism: adding to sulfhydryl groups at or near sensitive reactive sites on cellular proteins, and/or by adding to the sulfhydryl group of glutathione, thereby affecting the function of sensitive sulfhydryl-dependent proteins indirectly.

Scheme I



[†] Department of Biological Sciences, University of California.
[‡] Present address: Department of Anatomy, Duke University Medical Center, Durham, NC 27710.
[§] Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.
[∥] Scripps Institution of Oceanography.
[⊥] Department of Chemistry, University of California.
[#] Marine Science Institute, University of California.

(1) Gerwick, W. H.; Fenical, W.; Fritsch, N.; Clardy, J. *Tetrahedron Lett.* **1979**, 145.
(2) Gerwick, W. H.; Fenical, W. *J. Org. Chem.* **1981**, *46*, 22.
(3) Jacobs, R. S.; White, S.; Wilson, L. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1981**, *40*, 26.
(4) White, S. J.; Jacobs, R. S. *Mol. Pharmacol.* **1983**, *24*, 500.
(5) O'Brien, E. T.; Jacobs, R. S.; Wilson, L. *Mol. Pharmacol.* **1983**, *24*, 493.
(6) O'Brien, E. T.; White, S. J.; Jacobs, R. S.; Boder, G. B.; Wilson, L. In *Developments in Hydrobiology, 11th International Seaweed Symposium*; Bird, C. J., Ragan, P., Eds.; Dr. Junk: Dordrecht, The Netherlands, 1984; Vol. 22, p 141.
(7) O'Brien, E. T.; Asai, D. J.; Jacobs, R. S.; Wilson, L., unpublished data.

0022-2623/86/1829-1851$01.50/0