Design of Potent Dicyclic (1−5/4−10) Gonadotropin Releasing Hormone (GnRH) Antagonists†

Jean E. Rivier,*,‡ Guangcheng Jiang,‡ R. Scott Struthers,§ Steven C. Koerber,‡ John Porter,‡ Laura A. Cervini,‡ Dean A. Kirby,‡ A. Grey Craig,‡ and Catherine L. Rivier

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, and Peptide Chemistry Department, Neurocrine Biosciences Inc., 10555 Science Center Drive, San Diego, California 92121

Received March 15, 1999

In three earlier papers, the structures and biological potencies of numerous mono- and dicyclic antagonists of GnRH were reported. Among these, two families, each containing two to four members were identified that had very high antagonist potencies in an antiovulatory assay (within a factor of 2 of those of the most potent linear analogues) and high affinities (K_i < 0.5 nM) for the rat GnRH receptor (rGnRHR). The most favored cycles bridged the side chains of residues (4−10),[1,2] (5−8),[2] (4−10/5−8),[2] (1−3),[3] and (1−3/4−10).[3] Our goal was to identify a consensus model of bioactive conformations of GnRH antagonists, yet these biocompatible constraints did not sufficiently restrain the spatial location of the N-terminal tripeptide with respect to the C-terminal heptapeptide, due largely to the rotational freedom about the bonds connecting these regions. Examination of models derived from NMR studies of cyclo(4−10) analogues suggested a large number of possible cyclic constraints such as cyclo (0−8), (1−8), or (2−8). All analogues tested with these substitutions were inactive as antiovulatory agents at 1 mg/rat (**5**−**9**) and had low affinity for rGnRHR. On the other hand, bridging positions 3 and 8 with a [DAsp^3] to [Dbu^8] (**12**, K_i = 13 nM) or [Orn^8] (**13**, K_i = 14 nM) in the parent compound cyclo(3−8)[Ac-DNal^1,DCpa^2,DXaa^3,Arg^5,DNal^6,Xbb^8,DAla^{10}]GnRH yielded analogues that blocked ovulation at 250 μg/rat. Analogue **14** (K_i = 2.3 nM), with a [DAsp^3, Lys^8] bridge, was fully active at 50 μg/rat. Loss of potency (>20-fold) was observed with the substitution of [DAsp^3] in **14** by [DGlu^3] in **15** (K_i = 23 nM). Dicyclic analogues possessing the (4−10) cycle and selected (1−6), (2−6), and (2−8) cycles led to analogues that were inactive at doses of 500 μg/rat or larger. Two analogues with (1−8/4−10) cycles (**16**, K_i = 1.1 nM) or (3−8/4−10) cycles (**22**, K_i = 17 nM) showed full antiovulatory potency at 250 μg/rat. None of these substitutions yielded analogues potent enough (>80% inhibition of ovulation at 5 μg/rat or less and K_i < 0.5 nM) to be candidates for structural analysis by NMR. On the other hand, four dicyclic (1,1′−5/4−10) analogues met this criterion: dicyclo(1,1′−5/4−10)[Ac-Asp^1(Gly),DCpa^2,DTrp^3,Asp^4,Dbu^5,DNal^6,Dpr^{10}]GnRH (**32**, K_i = 0.22 nM), dicyclo(1,1′−5/4−10)[Ac-Asp^1(Gly),DCpa^2,DNal^3,Asp^4,Dbu^5,DNal^6,Dpr^{10}]GnRH (**34**, K_i = 0.38 nM), dicyclo(1,1′−5/4−10)[Ac-Asp^1(βAla),DCpa^2,DTrp^3,Asp^4,Dbu^5,DNal^6,Dpr^{10}]GnRH (**40**, K_i = 0.15 nM), and dicyclo(1,1′−5/4−10)[Ac-Glu^1(Gly),DCpa^2,DTrp^3,Asp^4,Dbu^5,DNal^6,Dpr^{10}]GnRH (**41**, K_i = 0.24 nM). Since they differed slightly in terms of the (1,1′−5) bridge length (21 and 22 atoms) and bridgehead configuration, we may hypothesize that they assume similar bioactive conformations that satisfy a very discriminating receptor, since many other closely related analogues were significantly less potent.

Introduction

Prior to combinatorial chemistry and high throughput screening, molecules that bound to a given receptor were generally derived from a native ligand. Although the structure of GnRH has been studied extensively by NMR[4−13] and some aspects of the interaction of GnRH with its receptor are known,[14] the bioactive conformation of GnRH has not been rigorously defined because of its flexibility in solution. Our approach to defining such a conformation has been to design a highly constrained molecule with affinity equal to or greater than that of GnRH (K_i = 0.5 nM) for its rat receptor and derive the bioactive conformation of this analogue from NMR analysis. Defining the bioactive conformation of GnRH or of one of its analogues would aid in the design of low molecular weight (<500 Da) nonpeptide mimetics.[15]

In three earlier papers, the structures and biological potencies of numerous mono- and dicyclic antagonists of GnRH were reported. Among these, a few were identified with very high antagonist potencies in an antiovulatory assay, often within a factor of 2 of those of the most potent linear analogues. The most favored cycles bridged the side chains of residues (4−10),[1,2] (5−8),[2] (4−10/5−8),[2] (1−3),[3] and (1−3/4−10).[3] Our interest being in identifying a model of the consensus conformations of potent GnRH antagonists, these biocompatible constraints did not allow the rigorous spatial location of the N-terminal three amino acids with respect to the C-terminal heptapeptide, due to the relative rotational freedom of these subsequences.

Like many peptide hormones, GnRH is a highly flexible molecule that exists in solution as an equilibrium mixture of multiple conformers.[13,16,17] Nonetheless,

10.1021/jm990116+ CCC: $19.00
Published on Web 02/23/2000

working models of its preferred conformation have been proposed that may coincide with its receptor-bound conformation (see Kutscher et al. for a recent review of the field[18]). For example, a feature common to many models is a type II′ β-turn at Gly^6-Leu^7 that was originally suggested from empirical conformational energy calculations.[11,12] Support for the presence of this turn has been provided by D-amino acid substitution studies,[19,20] an analogue incorporating *N*-methyl Leu^7,[21,22] and a lactam-bridged analogue that forced the ψ angle of Gly^6 and the ϕ angle of Leu^7 to values characteristic of a β-turn.[23]

We review below and chronologically the development of potent cyclic GnRH antagonists and the study of their secondary structure in our laboratory. As a member of the head-to-tail cyclic family of GnRH antagonists, cyclo(1−10)[Δ^3Pro^1,DCpa^2,DTrp^3,DTrp^6,MeLeu^7,βAla^{10}]-GnRH (**1**) was among the most potent analogues in a rat pituitary cell culture assay yet was inactive in the AOA at 1.5 mg/rat. This analogue was found to exhibit a type II′ β-turn involving residues 6−7 and a type II β-turn involving residues 1−2 that are connected by extended antiparallel β-like strands.[1,24−26] Detailed theoretical and experimental analysis of the conformation of **1** revealed a close proximity of residues Ser^4 and Pro^9 with their Cα protons facing each other.[27,28] In fact, cyclo(4−9)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Dpr^4,DTrp^6,Asp^9]-GnRH was found to be equipotent with **1** in vitro and also inactive in the AOA at 1.5 mg/rat (data not shown). Further synthetic efforts finally led to the discovery of cyclo(4−10)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]-GnRH (**2**), which inhibited ovulation by 100% at 10 μg/rat.[1] Extensive NMR and theoretical analysis of the conformation of **2** revealed a type II′ β-turn involving residues 6−7, a γ turn at DTrp^3, and a likely type II β-turn around residues 1−2.[29−31] Upon the discovery of the bio-compatibility of a bridge spanning residues 5 to 8, this second cycle was incorporated in **2** to yield dicyclo(4−10/5−8)[Ac-DNal^1,DCpa^2,DTrp^3,Asp^4,Glu^5,-DArg^6,Lys^8, Dpr^{10}]GnRH (**3**) that inhibited ovulation by 85% at 5 μg/rat and by 100% at 10 μg/rat. It is worthwhile mentioning that at a dose of 5 μg/rat two sets of data (1 and 2 rats out of 10 ovulated, i.e., 3 rats out of 20 ovulated) were obtained that are not statistically different, suggesting repeatability of the assay. The high-resolution NMR and theoretical analysis of **3** suggested a solution conformation very similar to that of **2**.[31] Structure−activity relationship studies conducted on the family of dicyclic GnRH antagonists represented by **3** suggested that optimization of the (5−8) bridge could lead to similarly potent compounds. The discovery of dicyclo(4−10/5,5′−8)[Ac-DNal^1,DCpa^2,DPal^3,Asp^4,Glu^5-(Gly),DArg^6,Dbu^8,Dpr^{10}]GnRH (**4**), which inhibits ovulation at 25 μg/rat by 100%, was described in the preceding paper.[3] To our knowledge, **4** is the first highly potent, constrained GnRH antagonist that has been observed to form a type I′ β-turn, rather than a type II′ β-turn, around residues 6−7. Although it could be that **4** changes conformation upon binding to the GnRH receptor to form a type II′ β-turn, it is also possible that the presence of a (6−7) turn, rather than the turn type per se, is important for biological activity. From the point of view of GnRH antagonist design, it is more puzzling that the tail formed by residues 1−3 of **3** and **4** assumes radically different orientations with respect to the rest of the molecule. Indeed, the N-terminal tripeptide is somewhat structured but does not populate a single major conformation. However, the orientation of the tripeptide on the same side as the 5−8 bridge could favor interactions with this bridge, or, more specifically, with residues 5 or 8.[31] We present here the results of our investigation carried out to test this hypothesis.

Results and Discussion

Peptides were synthesized by the solid phase method on a methyl benzhydrylamine resin as described in accompanying papers.[2,3] In the case of dicyclic analogues, the first cycle (other than that linking the side chains of residues 4 and 10) was formed on the resin using the orthogonal protection provided by the Fmoc and OFm protecting groups.[32] The 4 to 10 cycle was formed in three steps by first generating the β-Asp- or γ-Glu-hydrazides from the corresponding benzyl esters at position 4 by stirring the peptide resin with hydrazine at 20 °C for 100 h in DMF.[1] The peptides were then cleaved with HF and concomitantly deprotected. The peptide hydrazides were then converted to the corresponding azides using isoamyl nitrite and HCl in dioxane and cyclized in DMF under dilute conditions with the free side chain amino group of Dpr at position 10 to give the crude cyclized peptides (see Experimental Section). Purification was carried out by HPLC.[33,34] Purities were determined to be >80% in most cases by HPLC and CZE (Table 1). Retention times using isocratic conditions and specific rotations are given in Table 1. Amino acid analyses[35] including those of Pal, Cpa, Fpa, Nal and Dpr were carried out on most analogues and were found to be consistent with expected values. Calculated values for protonated molecular ions were in agreement with those obtained using FAB mass spectrometry (see Table 1).

Because the goal was to identify potent, orally active contraceptive agents, all antagonists were tested for antiovulatory activity in the rat. Because an understanding of their bioactive conformation was fundamental to our design strategy, binding affinities were also determined. Biological evaluation was conducted using an in vivo antiovulatory assay (AOA)[36] and a binding

† Abbreviations: The abbreviations for the amino acids are in accord 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. In addition: AAA, amino acid analysis; Agl, aminoglycine; Amp, 4-aminomethylphenylalanine; AOA, antiovulatory assay; Aph, 4-aminophenylalanine; Boc, *tert*-butoxycarbonyl; BOP, benzotriazolyloxy tris-(dimethylamino) phosphonium hexafluoro-phosphate; Cpa, 4-chlorophenylalanine; Dbu, 2,4-diaminobutyric acid; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Doc, 2,8-diaminooctanoic acid; Dpr, 2,3-diaminopropionic acid; EDT, ethanedithiol; Fpa, 4-fluorophenylalanine; GnRH, gonadotropin releasing hormone; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; HPLC, high performance liquid chromatography; ILys = N^{ϵ}-isopropylysine; LH, luteinizing hormone; Nac, 2-naphthyl-acetic acid; Nal, 3-(2′-naphthyl)-alanine; NMP, *N*-methylpyrrolidinone; Ofm, fluorenylmethyl ester; Pal, 3-(3′-pyridyl)-alanine; rGnRHR = rat GnRH receptor; TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

* To whom correspondence should be addressed. Tel: (858) 453-4100. Fax: (858) 552-1546. E-mail: jrivier@salk.edu.

‡ The Salk Institute.

§ Neurocrine Biosciences Inc.

Table 1. Characterization of Cyclic GnRH Antagonists by HPLC, CZE, MS and in an Antiovulatory Assay

no.	compound	ring size	RT isocratic conditions[a]	purity HPLC[b]	purity CZE[c]	MS[d] calc	MS[d] found	K_i[e] (nM)	AOA dose/rat in μg	AOA rats ovulating/ total
1	cyclo(1−10)[Δ^{3}Pro^1,D-Cpa^2,D-Trp^3,D-Trp^6,MeLeu^7,β-Ala^{10}]GnRH	31	N/A	>97^{II}	N/A	1350.62	1352.7	14 ± 0.99	1500	9/10
2	cyclo(4−10)[Ac-Δ^{3}Pro^1,D-Fpa^2,D-Trp^3,Asp^4,D-Nal^6,Dpr^{10}]GnRH	23	3.8 (37.2)^{I}	>97^{II}	N/A	1415.67	1415.7	0.27 ± 0.03	5.0	4/10
									10	0/10
3	dicyclo(4−10/5−8)[Ac-D-Nal^1,D-Cpa^2,D-Trp^3,Asp^4,Glu^5,D-Arg^6,Lys^8,Dpr^{10}]GnRH	23/18	4.50 (31.2)	>97^{II}	83	1412.66	1412.6	0.32 ± 0.06	5.0	3/20
									10	0/10
4	dicyclo(4−10/5,5′−8)[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Asp^4,Glu^5(Gly),D-Arg^6,Dbu^8,Dpr^{10}]GnRH	23/19	4.10 (31.2)	81^{II}	80	1403.08	1403.7	0.12 ± 0.04	5.0	2/8
									10	1/6
									25	0/3
5	cyclo(0−8)[Suc-D-Nal^1,D-Cpa^2,D-Pal^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	32	3.99 (42.0)	98^{I}	98	1475.8	1475.8	21 ± 4.1	1000	5/5
6	cyclo(1−8)[Ac-Glu^1,D-Cpa^2,D-Pal^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	30	4.60 (32.4)	>98^{I}	98	1349.66	1349.6	62 ± 5.0	1000	4/4
7	cyclo(1−8)[Ac-D-Glu^1,D-Cpa^2,D-Pal^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	30	3.88 (31.8)	97^{I}	98	1349.66	1349.6	55 ± 10	1000	4/4
8	cyclo(2−8)[Ac-D-Nal^1,D-Asp^2,D-Pal^3,Arg^5,D-Nal^6,Dpr^8,D-Ala^{10}]GnRH	23	4.00 (34.8)	98^{I}	>98	1309.65	1309.6	67 ± 21	1000	4/4
9	cyclo(2−8)[Ac-D-Nal^1,D-Glu^2,D-Pal^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	27	4.11 (34.8)	>98^{I}	>98	1365.71	1365.8	110 ± 11	1000	3/3
10	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Cys^3,Arg^5,D-Nal^6,Cys^8,D-Ala^{10}]GnRH	20	5.15 (45.0)	81^{II}	84	1363.55	1363.5	9.9 ± 2.2	50	5/5
					borate				250	5/6
11	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Arg^5,D-Nal^6,Dpr^8,D-Ala^{10}]GnRH	20	5.28 (41.4)	97^{I}		1348.68	1348.7	21 ± 4.8	1000	4/4
12	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Arg^5,D-Nal^6,Dbu^8,D-Ala^{10}]GnRH	21	5.0 (39.0)	>98^{I}	>98	1356.63	1356.7	13 ± 4.8	100	14/14
									250	0/4
13	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Arg^5,D-Nal^6,Orn^8,D-Ala^{10}]GnRH	22	5.07 (41.4)	94^{II}	85	1370.64	1370.6	14 ± 0.67	100	5/5
					borate				250	0/5
14	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	23	3.93 (43.8)	90^{II}	87	1384.66	1384.7	2.3 ± 0.58	10	5/5
									25	3/7
									50	0/5
15	cyclo(3−8)[Ac-D-Nal^1,D-Cpa^2,D-Glu^3,Arg^5,D-Nal^6,Lys^8,D-Ala^{10}]GnRH	24	4.16 (42.6)	95^{I}	N/A	1398.68	1398.7	23 ± 3.5	1000	3/3
16	dicyclo(1−8/4−10)[Ac-Glu^1,D-Cpa^2,D-Pal^3,Asp^4,Arg^5,D-Nal^6,Lys^8,Dpr^{10}]GnRH	30/23		95^{I}	N/A	1374.65	1374.7	1.1 ± 0.32	50	7/7
									250	0/5
17	dicyclo(1−8/4−10)[Ac-D-Glu^1,D-Cpa^2,D-Pal^3,Asp^4,Arg^5,D-Nal^6,Lys^8,Dpr^{10}]GnRH	30/23	3.16 (34.5)	98^{I}	97	1374.81	1374.7	1.8 ± 0.44	100	3/6
									250	2/6
18	dicyclo(2−6/4−10)[Ac-D-Nal^1,Cys^2,D-Trp^3,Asp^4,D-Cys^6,Dpr^{10}]GnRH	17/23	4.40 (36.0)	91^{II}	N/A	1359.58	1359.5	13 ± 1.1	500	5/7
19	dicyclo(2,2′-6/4−10)[Ac-D-Nal^1,D-Asp^2(Gly),D-Pal^3,Asp^4,D-Dpr^6,Dpr^{10}]GnRH	20/23		97^{I}	>98	1357.64	1357.8	490 ± 195	1000	4/4
20	dicyclo(2−8/4−10)[Ac-D-Nal^1,D-Glu^2,D-Pal^3,Asp^4,Arg^5,D-Nal^6,Lys^8,Dpr^{10}]GnRH	27/23	4.83 (35.4)	92^{II}	98	1390.71	1390.6	15 ± 4.2	1000	4/4
21	dicyclo(3−8/4−10)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Asp^4,Arg^5,D-Nal^6,Lys^8,Dpr^{10}]GnRH	23/23	3.35 (42.0)	93^{I}	>98	1409.66	1409.8	11 ± 1.7	100	4/4
									250	4/7
22	dicyclo(3−8/4−10)[Ac-D-Nal^1,D-Cpa^2,D-Asp^3,Asp^4,D-Arg^6,Lys^8,Dpr^{10}]GnRH	23/23	3.6 (35.4)	94^{I}	96	1375.64	1375.5	17 ± 3.7	100	5/5
									250	0/4
23	dicyclo(1−5/4−10)[Ac-Cys^1,D-Cpa^2,D-Trp^3,Asp^4,Cys^5,D-Nal^6,Dpr^{10}]GnRH	17/23	4.36 (39.6)	92^{II}	N/A	1377.54	1377.5	5.7 ± 0.68	100	7/12
24	dicyclo(1−5/4−10)[Ac-D-Cys^1,D-Cpa^2,D-Trp^3,Asp^4,D-Cys^5,D-Arg^6,Dpr^{10}]GnRH	17/23	4.52 (31.2)	90^{II}	86	1336.56	1336.5	89 ± 11	500	6/6
25	dicyclo(1−5/4−10)[Ac-D-Cys^1,D-Cpa^2,D-Trp^3,Asp^4,Cys^5,D-Nal^6,Dpr^{10}]GnRH	17/23	4.20 (40.8)	94^{II}	N/A	1377.54	1377.5	0.25 ± 0.06	100	8/8
									500	4/10
26	dicyclo(1−5/4−10)[Ac-Asp^1,D-Cpa^2,D-Trp^3,Asp^4,D-Dpr^5,D-Nal^6,Dpr^{10}]GnRH	17/23	5.30 (40.8)	92^{I}	95	1356.60	1356.6	4.8 ± 1.7	250	5/9
									500	1/5
27	dicyclo(1−5/4−10)[Ac-Glu^1,D-Cpa^2,D-Trp^3,Asp^4,Dbu^5,D-Nal^6,Dpr^{10}]GnRH	19/23	5.75 (41.4)	>98^{I}	97	1384.64	1384.7	1.7 ± 0.32	100	1/8
									250	0/5
28	dicyclo(1−5/4−10)[Ac-Glu^1,D-Cpa^2,D-Trp^3,Asp^4,Orn^5,D-Nal^6,Dpr^{10}]GnRH	20/23	4.0 (44.4)	98^{I}	98	1398.65	1398.6	0.86 ± 0.15	10	4/4
									25	4/8
29	dicyclo(1,1′−5/4−10)[Ac-Asp^1(Gly),D-Cpa^2,D-Trp^3,Asp^4,Dpr^5,D-Nal^6,Dpr^{10}]GnRH	20/23	3.92 (44.4)	79^{I}	N/A	1413.63	1413.6	0.49 ± 0.10	10	2/8
									25	0/3
30	dicyclo(1−5/4−10)[Ac-Glu^1,D-Cpa^2,D-Trp^3,Asp^4,Lys^5,D-Nal^6,Dpr^{10}]GnRH	21/23	3.70 (42.4)	>98^{I}	>98	1412.67	1412.7	0.26 ± 0.09	10	4/5
									25	3/8
									50	0/3

Table 1. (Continued)

no.	compound	ring size	RT isocratic conditions[a]	purity HPLC[b]	purity CZE[c]	MS[d] calc	MS[d] found	K_i[e] (nM)	AOA dose/rat in μg	AOA rats ovulating/ total
31	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Gly),DCpa[2],DTrp[3],Asp[4],Dpr[5],DNal[6],Dpr[10]]GnRH	21/23	3.95 (40.2)	98[I]	97	1427.64	1427.8	0.44 ± 0.12	5.0	3/3
									10	2/7
32	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DTrp[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	21/23	4.19 (41.5)	98[I]	>98	1427.64	1427.8	0.22 ± 0.06	1.0	5/7
									2.5	4/16
									5.0	0/5
33	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DPal[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	21/23	3.60 (35.2)	93[I]	92	1389.63	1389.5	0.12 ± 0.03	1.0	4/4
									2.5	3/5
									5.0	3/5
34	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DNal[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	21/23	3.30 (45.2)	>98[I]	>98	1438.65	1438.7	0.38 ± 0.16	1.0	5/7
									2.5	2/8
35	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DPal[3],Asp[4],Dbu[5],DPal[6],Dpr[10]]GnRH	21/23	3.70 (22.6)	97[I]	>98	1340.61	1340.6	1.6 ± 0.39	250	3/3
36	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DPal[3],Asp[4],Dbu[5],DPal[6],ILys[8],Dpr[10]]GnRH	21/23	3.40 (22.4)	97[I]	>98	1354.65	1354.7	2.8 ± 0.41	50	3/3
37	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DTrp[3],Asp[4],DDbu[5],DNal[6],Dpr[10]]GnRH	21/23	3.8 (42)	95[I]	95	1427.64	1427.8	3.1 ± 1.1	25	4/4
									50	4/8
									100	0/8
38	dicyclo(1,1′−5/4−10)[Ac-Asp[1](Gly),DCpa[2],DTrp[3],Asp[4],Orn[5],DNal[6],Dpr[10]]GnRH	22/23	4.40 (40.2)	95[I]	>98	1441.66	1441.8	0.31 ± 0.06	10	3/3
									25	3/6
									50	2/5
									100	0/3
39	dicyclo(1,1′−5/4−10)[Ac-Glu[1](βAla),DCpa[2],DTrp[3],Asp[4],Dpr[5],DNal[6],Dpr[10]]GnRH	22/23	4.10 (42.3)	95[I]	98	1441.66	1441.8	0.76 ± 0.23	5.0	3/4
									10	4/8
									25	1/5
40	dicyclo(1,1′−5/4−10)[Ac-Asp[1](βAla),DCpa[2],DTrp[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	22/23	4.00 (42.0)	>98[I]	>98	1441.66	1441.7	0.15 ± 0.01	2.5	1/8
									5.0	0/5
41	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Gly),DCpa[2],DTrp[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	22/23	4.60 (43.0)	98[I]	92	1441.66	1441.6	0.24 ± 0.03	2.5	2/8
									5.0	1/7
									10	0/6
42	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Gly),DCpa[2],DTrp[3],Asp[4],DDbu[5],DNal[6],Dpr[10]]GnRH	22/23	3.8 (42.0)	94[I]	95	1441.66	1441.9	2.9 ± 0.44	25	5/5
									50	1/8
									100	0/8
43	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Gly),DCpa[2],DTrp[3],Asp[4],Dbu[5],DArg[6],Dpr[10]]GnRH	22/23	4.90 (30.5)	98[I]	98	1400.67	1400.7	17 ± 5.7	5.0	2/3
									50	3/3
44	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Ala),DCpa[2],DTrp[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	22/23	3.90 (41.9)	86[I]	93	1455.67	1455.6	1.6 ± 0.46	10	5/5
45	dicyclo(1,1′−5/4−10)[Ac-Glu[1](DAla),DCpa[2],DTrp[3],Asp[4],Dbu[5],DNal[6],Dpr[10]]GnRH	22/23	3.75 (40.4)	93[I]	>98	1455.67	1455.7	0.22 ± 0.04	10	2/4
									25	0/5
46	dicyclo(1,1′−5/4−10)[Ac-Glu[1](Gly),DCpa[2],DTrp[3],Asp[4],Orn[5],DNal[6],Dpr[10]]GnRH	23/23	2.90 (38.9)	96[I]	96	1455.67	1455.6	1.1 ± 0.00	10	5/6
									25	7/8
									50	0/6

[a] Retention times under isocratic conditions, buffer 0.1% TFA. [b] Percent purity was determined by HPLC using one of two buffer systems selected for giving the best resolution. Conditions are described in the Experimental Section. I: A = TEAP (pH 2.5); II: A = 0.1% TFA. [c] Conditions for capillary zone electrophoresis (CZE) are described in the Experimental Section. N/A peptides did not elute under standard conditions. [d] All observed *m/z* were measured using LSI-MS. Calculated and observed *m/z* values of the $[M + H]^+$ monoisotopes are reported. [e] Average ± SEM of at least three independent determinations is reported.

Table 2. Cα−Cα Distance Matrix of the NMR-Derived Model of the Cyclo (1−4) Analogue

	0	1	2	3	4	5	6	7	8	9	10
0	0.00	3.91	6.25	6.42	9.24	8.29	11.63	12.99	10.28	12.05	11.88
1	3.91	0.00	3.91	6.06	8.22	6.68	10.42	12.80	10.78	13.05	12.19
2	6.25	3.91	0.00	3.90	6.38	6.71	10.53	12.65	10.31	11.75	9.83
3	6.42	6.06	3.90	0.00	3.91	5.82	9.10	10.09	7.07	7.93	6.24
4	9.24	8.22	6.38	3.91	0.00	3.93	6.01	7.00	5.00	6.67	5.42
5	8.29	6.68	6.71	5.82	3.93	0.00	3.89	6.62	5.93	9.20	9.10
6	11.63	10.42	10.53	9.10	6.01	3.89	0.00	3.91	5.73	9.38	10.25
7	12.99	12.80	12.65	10.09	7.00	6.62	3.91	0.00	3.90	7.03	9.12
8	10.28	10.78	10.31	7.07	5.00	5.93	5.73	3.90	0.00	3.97	6.14
9	12.05	13.05	11.75	7.93	6.67	9.20	9.38	7.03	3.97	0.00	3.89
10	11.88	12.19	9.83	6.24	5.42	9.10	10.25	9.12	6.14	3.89	0.00

assay to rat GnRH receptor (rGnRHR) cloned in human embrionic kidney (Hex 293) cells (see Experimental Section). The relative potencies discussed in this paper were derived from the ratio of doses that gave equivalent responses in the AOA and are approximate. K_i values are the average of three or more independent assays.

Bridging opportunities between the side chains of the three N-terminal amino acids and those of any of the C-terminal amino acids (5−9) could not be rigorously defined from NMR analysis of the structures of either **2**, **3**, or **4**. Positions of the eleven Cα carbons (i.e., 10 residues + *N*-acetyl function) of the minimum energy structure of the NMR-restrained model of the cyclo(4−10) antagonist **2**[37,38] were extracted and used to form the Cα−Cα distance matrix given in Table 2. On the basis of Cα−Cα distance matrix, certain side chain bridges were precluded from consideration because the distance (>8.5 Å) could not be spanned easily by an Asp/Glu to Dpr/Dbu/Orn/Lys bridge. For example, the Cα−Cα distance of residues 3 and 6 is 9.1 Å which was judged as too large to be bridged successfully, especially in view of the requirement for a Gly or D-amino acid at position 6. After examination of the structure of **3** (interactions between the N-terminal three amino acids with the 5−8 bridge, consequently with residues 5 or 8, had been postulated[31]), two exceptions to this preclusion were made, and bridged residue 1 to 8 (**6**, **7**, **16**, **17**) and residue 2 to 8 (**8**, **9**, **20**) where the Cα−Cα distances are 10.78 and 10.31 Å, respectively. The Cα−Cα distances of residues 3 and 8 (7.1 Å) did not preclude formation of a 3−8 cycle (**10**−**15**, **21**, **22**). Most other analogues described here were designed to test the compatibility of a bridge between residues 1 and 5 (6.7 Å). Because the three-dimensional structure of the (4−10) cycle in **2** and **3** was stable and yielded potent analogues, it was used in most of the analogues presented here (**16**−**46**).

The synthesis and characterization of the reference compounds **1**−**4** were described earlier;[1,2] their structures are presented in Table 1 for added clarity. In **5**, the N^{α} of DNal^1 was bridged to the N^{ϵ} of Lys^8 with succinic acid. In **6** and **7**, the role of chirality at position 1 was explored by bridging the side chain of Glu and DGlu to that of Lys at position 8. In **8** and **9**, the side chains of DAsp^2 to Dpr^8 and DGlu^2 to Lys^8 were bridged, thus generating two cycles of easily accessible minimal (23 atoms) and maximal (27 atoms) length. All of these monocyclic analogues (**5**−**9**) were inactive at 1 mg/rat, which was expected based on the Cα−Cα distances between residues 1−8 and 2−8 being greater than 8.5 Å, and all had low binding affinities for rGnRHR (K_i > 20 nM). Attention was then focused on the theoretically more promising (3−8) cycle (**10**−**15**, **21**−**22**). Our first attempt using the [DCys^3, Cys^8] cycle yielded **10** (K_i = 9.9 nM), which was essentially inactive at 250 μg/rat. Yet, when position 8 of cyclo(3−8)[Ac-DNal^1,DCpa^2, DAsp^3,Arg^5,DNal^6,Xaa^8,DAla^{10}]GnRH was substituted with Dpr, Dbu, Orn, and Lys, the corresponding analogues **11**−**14** were found to be increasingly active as the ring size was expanded. Analogue **11** (K_i = 21 nM) was inactive at 1 mg/rat, **12** (K_i = 13 nM) and **13** (K_i = 14 nM) were fully active at 250 μg/rat, and **14** (K_i = 2.3 nM) was fully active at 50 μg/rat. Binding affinity also followed incrementally. It should be noted that the corresponding linear analogue of **14** ([Ac-DNal^1,DCpa^2, DPal^3,Arg^5,DNal^6,DAla^{10}]GnRH) was fully active at 2.5 μg/rat or about 20 times more potent than **14**.[39] Significant loss of potency (>20-fold) was observed with the substitution of DAsp^3 in **14** by DGlu^3 in **15** (K_i = 23 nM), a substitution that had been found to also be deleterious when introduced in the 4−10 cycle[1] and the (1−3) cycle[3] but not in the (5−8) cycle.[2]

The first two dicyclic (1−8/4−10) analogues differed in the chirality of residue 1 only. Interestingly, **16** (K_i = 1.1 nM) with Glu^1 was more potent (100% inhibition of ovulation at 250 μg/rat) than **17** (K_i = 1.8 nM) with DGlu^1 (67% inhibition of ovulation at 250 μg/rat). Although these differences in potency and affinity may not be statistically significant, they were an indication of a trend which was later exploited. Arg substitution at position 5 had been shown to be compatible with high potency;[40] it was therefore introduced in most analogues in which position 8 is a bridgehead in an effort to compensate for the loss of the basic charge (thought to be important) at that position. The NMR structure of **17** was determined.[41] The conclusion of that study was that the 4−10 cycle was almost unchanged as compared to that of **2**. Residue 2 was then bridged to residues 6 (**18**, **19**) and 8 (**20**). In the case of **18** (K_i = 13 nM), a 17-atom [Cys^2, DCys^6] cycle was introduced, and in the case of **19** (K_i = 490 nM), it was a 20-atom [DAsp^1(Gly), DDpr^6] cycle. In that way D and L configuration were explored at position 2 while maintaining a D-residue at position 6 (a prerequisite for high potency).[20] Both analogues had low binding affinity.

Attempts at bridging residues 2 to 7 with a [Cys^2, Cys^7] and a [DCys^2, Cys^7], which link two of the most distant residues as measured by a Cα−Cα distance of 12.65 Å (see Table 2), yielded the desired products (shown by MS analysis) with a number of impurities that could not be separated. These mixtures had low binding affinity (K_i > 30 nM). Maximizing the ring at 27 atoms gave **20** which had poor affinity (K_i = 15 nM) and was inactive at 1 mg/rat. Although we were

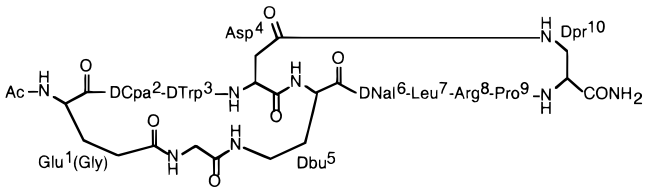

Figure 1. Bridging configuration of **41**.

discouraged to pursue these ring configurations, our limited data is insufficient to exclude the possibility that (2−7) or (2−8) cycles could be optimized to produce potent analogues.

Of all the monocyclic and dicyclic compounds described so far (**5**−**20**), the most potent (**14**) was fully active at 50 μg/rat with partial inhibition of ovulation (57%) at 25 μg/rat and a K_i value of 2.3 nM. Defining the structure of **14** was the [DAsp^3, Lys^8] cycle, a hydrophobic N-terminus [Ac-DNal^1, DCpa^2] and the [Arg^5, DNal^6] substitutions. Adding the [Asp^4, Dpr^{10}] cycle to the [DAsp^3, Lys^8] constraint yielded dicyclic **21** (K_i = 11 nM) which showed partial inhibition at 250 μg/rat (4 rats out of 7 ovulated) which was a >10-fold loss of potency versus **14**. The corresponding [Tyr^5, DArg^6] analogue (**22**, K_i = 17 nM) was only marginally more potent as it completely inhibited ovulation at 250 μg/rat but not at 100 μg/rat. Whereas we had previously discovered potent dicyclic analogues in other ring combinations,[2] in this case the two combined constraints were deleterious.

Among other possible bridges, cyclo (1−5) was potentially a better lead. Indeed, we realized that if we could find an optimum bridge between these residues, we would likely be able to suggest a consensus model of the conformations of potent GnRH antagonists by locking the N-terminal residues into a loop (see Figure 1 for illustration). Also, precedence indicated that both residues 1 and 5 could be involved in successful ring formation, and therefore these side chains were not necessary for receptor interaction.[2,3] Also, the Cα−Cα distance between residues 1 and 5 (6.7 Å) is not very different from that of residues 1 and 3 (6.1 Å) or that of residues 5 and 8 (5.9 Å), which had been successfully bridged.

We then explored the effect of chirality at the 1−5 bridgeheads using a cystine bridge in **23**−**25**. Antagonists **23** (K_i = 5.7 nM) and **25** (K_i = 0.25 nM) were partially active at 100 and 500 μg/rat (7 and 4 rats out of 12 and 10 ovulated, respectively). Analogue **24** (K_i = 89 nM) with the [DCys^1, DCys^5] configuration was inactive at 500 μg/rat. The corresponding [Cys^1, DCys^5] configuration (dicyclo(1−5/4−10)[Ac-Cys^1,DCpa^2,DTrp^3, Asp^4,DCys^5,DNal^6,Dpr^{10}]GnRH) was also synthesized but could not be purified adequately. A 50% pure preparation of this analogue had a 2 nM affinity for rGnRHR and was partially active in the AOA at 500 μg/rat (3/12 rats ovulated). These results were somewhat unexpected in view of the fact that residue 1 was traditionally of the D-configuration in potent antagonists. However, the Xaa^1 → DXaa^1 transition can be considered as a structurally conservative exchange of the N-terminal acetyl and Cα proton functionalities. None of the molecular models, based on available NMR investigations of the GnRH analogues, suggested that this switching of N-terminal functionalities should be energetically forbidden. Nor are there any apparent gross structural changes accompanying the Xaa^1 → DXaa^1 transition. The modulation in activity observed with the change in chirality of Xaa^1 in the linear antagonists may therefore reflect a specific interaction with the receptor rather than a conformational perturbation of the analogue's structure. Substituting the [Cys^1, DCys^5] bridge by the comparable [Asp^1, DDpr^5] bridge yielded **26** (K_i = 4.8 nM), which inhibited ovulation by 45% at 250 μg/rat. This was about the same potency as that observed for a 70% pure preparation of dicyclo(1−5/4−10)[Ac-Cys^1,DCpa^2,DTrp^3,Asp^4,DCys^5, DNal^6,Dpr^{10}]GnRH and suggested comparable compatibility of the lactam and disulfide bridges. This was an important observation as it encouraged us to use lactam rings that could easily be enlarged as shown earlier. Two extra methylene groups were added to the (1−5) bridge of **26** (Glu^1, instead of Asp^1; and Dbu^5, instead of Dpr^5) to yield **27** (with a 19-atom ring and K_i = 1.7 nM), which inhibited ovulation by 88% at 100 μg/rat, a 5-fold increase in potency as compared to **26** and a 2-fold increase in affinity. Therefore, the L^{1}L^5 configuration was compatible with biological activity, and enlarging the size of the [Xaa^1-Xaa^5] ring beyond 17 atoms appeared to be favorable.

Indeed, further increasing the ring size to 20 atoms yielded **28** (K_i = 0.86 nM), which showed 50% inhibition at 25 μg/rat. Relying on our observation that an Asp bridgehead is often more favorable than a Glu bridgehead, **29** was synthesized with an inserted Gly residue in the bridge while keeping the ring size constant at 20 atoms. This antagonist **29** (K_i = 0.49 nM) was even more potent than **28** and inhibited ovulation by 100% at 25 μg/rat and by 75% at 10 μg/rat. Increasing the size of the ring by one additional atom (21-membered ring) was achieved in three ways. The [Asp^1] in **29** was replaced by [Glu^1] to give **30** (K_i = 0.26 nM), and a Gly residue was inserted between [Glu^1] and [Dpr^5] (**31**, K_i = 0.44 nM) and between [Asp^1] and [D and LDbu^5] (**32**−**37**). It is in this series that two antagonists, **32** (K_i = 0.22 nM) and **34** (K_i = 0.38 nM) were identified that were potent enough to meet criteria for structural analysis (active in the AOA at 5 μg/rat or less). Ring expansion from 20 to 21 atoms in **30** as compared to **28** with a [Glu^1] bridgehead had no significant effect on potency or binding affinity. However, the same ring expansion conserving the [Asp^1] bridgehead yielded **32**, which is 4 times more potent than **29** as they both inhibited ovulation to the same extent (75% at 10 and 2.5 μg/rat, respectively). Comparison of the potencies of **28**, **29**, and **31**−**34** showed that increasing the ring size from 20 to 21 atoms is best achieved with Asp at position 1 (as compared to Glu), as has been seen in other bridging motifs.[2] Taken together, the data for **28** to **34** suggest that minor structural changes will have significant biological consequences in vivo yet limited influence on binding affinity (K_i values = 0.12−0.86 nM).

Compounds **35** and **36** were synthesized to test the general applicability of the (1−5/4−10) scaffold to other potent linear GnRH antagonists. We and others[42] had found that DPal at positions 3 and 6 was favorable in that it generally yielded analogues with increased potency; the use of isopropyl lysine at position 8 was also favorable in that it reduced histamine releasing

activity while retaining high potency.[43] The fact that these substitutions in dicyclo (1−5/4−10) GnRH antagonists had deleterious effects (**35** is significantly less potent than **33**, and **36** is inactive at 50 μg/rat) was unexpected and can only be explained by structural considerations. In fact, it was surprising to see that the introduction of [DPal[3,6]] in **35** was equally to more deleterious than changing chirality of Dbu[5] in **32** to DDbu in **37**, depending on whether we rely on binding affinity or antiovulatory activity.

A further increase in ring size to 22 atoms was achieved in several ways with [Asp[1](Gly), Orn[5]] (**38**), [Glu[1](βAla), Dpr[5]] (**39**), [Asp[1](βAla), Dbu[5]] (**40**), and [Glu[1](Xaa), D and LDbu[5]] (**41**−**45**). The in vivo potencies of **38** (K_i = 0.31 nM) and **39** (K_i = 0.76 nM), compared to that of **32** (K_i = 0.22 nM), showed significant losses (20- and 5-fold, respectively), suggesting that the optimal ring size was exceeded despite the high binding affinities of these analogues. However, varying the [Asp[1](Gly), Dbu[5]] motif of **32** to the [Asp[1](βAla), Dbu[5]] and [Glu[1](Gly), Dbu[5]] motifs in **40** (K_i = 0.15 nM) and **41** (K_i = 0.24 nM) yielded two of the four most potent analogues reported here with full inhibition of ovulation at 5 μg/rat. These results suggest that both amide linkages in the (1−1′,5) bridge (especially the Gly/βAla amide bond to Dbu[5]) interact with the GnRH receptor. Additionally, when these bonds are shifted by one methylene, less potent analogues result as may be seen when comparing the potency of **32** with that of **38** or the potency of **40** with those of **38** or **39**. The change in chirality of [Dbu[5]] in **41** to [DDbu[5]] in **42** resulted in a 10-fold loss of potency and affinity, documenting the sensitivity to chirality at position 5, as it had been shown at other positions. Substitution of [DNal[6]] in **42** by [DArg[6]] in **43** resulted in concomitant losses of potency and affinity, reinforcing the importance of functionality at position 6 while the rest of the molecule is constrained. This distinction was much less apparent in the linear analogues, whereby the pairs of amino acids [Arg[5]-DNal[6]], [Tyr[5]-DArg[6]], or [Tyr[5]-DNal[6]] were easily accommodated with retention of high potency. It could be possible that the GnRH receptor recognizes linear GnRH antagonists in multiple modes, a subset of which may be available to the cyclic antagonists identified so far. If this were the case, such analogues could be used to discriminate between GnRH receptors if more than one were present in the mammal,[44] which is a hypothesis supported by the presence of a second gene for GnRH in human.[45,46]

Compounds **44** (K_i = 1.6 nM) and **45** (K_i = 0.2 nM) differ from **41** by the introduction of a methyl group in the side chain loop (Ala[1′] and DAla[1′] for Gly[1′]). The fact that **41** and **45** have the same high binding affinity for rGnRHR, which is 7-fold higher than that of **44**, suggests a close steric interaction of this part of the molecule with the receptor. Increasing the 1−5 ring size to 23 atoms gave **46** that bound with reduced affinity (5-fold as compared to that of **41**) and inhibited ovulation at a dose of 50 μg/rat, which is 5 times the dose at which comparable inhibition was found for **41**. This suggested that the optimal (1−5) ring size was reached at 21−22 atoms, although further studies may be warranted to further document this observation.

Led by hypotheses drawn from structural analysis of the potent constrained analogue **2**[37,38] and by means of a significant synthetic effort, a new family of constrained dicyclic (1−5/4−10) GnRH analogues was identified. Four members of that family (**32**, **34**, **40**, and **41**) differed slightly in terms of the (1−5) bridge length (21 and 22 atoms) and inhibited ovulation by 100% at 5 μg/rat or less, although data for **34** at 5.0 μg/rat is not available. All contained the favorable [Asp[4], Dpr[10]] bridge and compared favorably with linear analogues that inhibit ovulation at doses ranging from 1 to 2.5 μg/rat.

These analogues offer the unique advantage of being conformationally more stable than monocyclic (4−10) or dicyclic (4−10/5−8) analogues that exhibited an N-terminal tripeptide that was free to rotate relative to the cyclic moiety.[2] The present studies overturn some fundamental issues in GnRH analogue design in that certain side chain functionalities, often thought to be critical for affinity, may actually be replaced in pairs by adequate bridging elements. Surprising to us is the number of residues that could be replaced in pairs, suggesting innumerable possibilities in the case of proteins, where evolutionary pressures have created such diversity and yet maintained functions.[47] Additionally, these studies have provided suitable models for the investigation of possible consensus models of the conformations of potent GnRH antagonists. Recent studies indicate that agonists only bind to the fraction of receptors that are G-protein coupled, while antagonists bind to the G-protein coupled and uncoupled receptors.[48] The description of the consensus model of the binding conformations of GnRH antagonists to the GnRH receptor presented in the following paper[15] will help define that of GnRH and consequently that of the activated form of the receptor.

Experimental Procedures

Instruments. The HF cleavage line was designed in-house and allowed for HF distillation under high vacuum. Preparative RP-HPLC was accomplished using a Waters Associates (Milford, MA) Prep LC/System 500A and Model 450 variable wavelength UV detector, Fisher (Lexington, MA) Recordall Model 5000 strip chart recorder, and a Waters Prep LC 500A preparative gradient generator. The 5 × 30 cm cartridge was packed in the laboratory with reversed-phase 300 Å Vydac C_{18} silica (15−20 μm particle size). Analytical RP-HPLC screening was performed on a Vydac C_{18} column (0.46 × 25 cm, 5 μm particle size, 300 Å pore size) connected to a Rheodyne Model 7125 injector, an Altex 420 HPLC system using two Altex 100A pumps, a Kratos Spectroflow 757 UV detector set to 210 nm, and a Houston Instruments D-5000 strip chart recorder. Quality control HPLC was performed on one of two systems: (1) The Waters Associates HPLC system was comprised of two 6000A pumps, a WISP sample injector, a 300 Å Vydac C_{18} column as above, a Kratos Spectroflow Model 773 UV detector (at 210 nm), and a Waters Associates data module integrator/recorder. (2) The Hewlett-Packard Series II 1090 liquid chromatograph was connected to a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size), Controller Model 362, and a Think Jet printer. Capillary zone electrophoresis (CZE) analysis was performed on a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 Model 50Z connected to a ChromJet integrator. Optical rotations are uncorrected and were determined with a Perkin-Elmer Model 241 polarimeter in 50% AcOH and c = 1 unless noted otherwise.

Starting Materials. The *p*-Methylbenzhydrylamine resin (MBHA resin) with a capacity of 0.4−1.0 mequiv/g was obtained from a polystyrene resin cross-linked with 1% divinylbenzene (Biobeads SX-1, 200−400 mesh, Bio-Rad Labora-

tories, Richmond, CA) as previously published.[49] All *tert*-butyloxycarbonyl (Boc) N_α-protected amino acids with side chain protection were purchased from Bachem Inc. (Torrance, CA) or Chem-Impex Intl. (Wood Dale, IL). The side chain protection groups were as follows: Arg(Tos), Asp(β-OcHex or β-OFm), Cys(S-*p*-Mob), Dbu(γ-Fmoc), Dpr(β-Fmoc), Glu(γ-OcHex or γ-OFm), DHCys(S-*p*-Mob), His(Tos), ILys(Z)-DCHA, Lys(ϵ-2ClZ or ϵ-Fmoc), Orn(δ-Fmoc), Ser(OBzl), NMe-Tyr(2,6-diClBzl), Tyr(2BrZ). Boc-(D/L)-Agl(Fmoc), Boc-4Amp(Fmoc), Boc-3Aph(Fmoc), Boc-D4Cpa, Boc-Doc(Fmoc), Boc-D4Fpa, Boc-D2Nal, Boc-Δ^3Pro, and Boc-D3Pal were synthesized in our laboratory[35,50,51] or obtained from the Contraceptive Development Branch, Center for Population Research at NIH. Reagents and solvents were analytical reagent grade.

Peptide Synthesis. Peptides were made by the solid phase approach[52] either manually or on a Beckman 990 peptide synthesizer. Couplings on 1−2 grams of resin per peptide were mediated for 2 h by diisopropylcarbodiimide (DIC) in CH_2Cl_2, dimethylformamide (DMF), or *N*-methylpyrrolidinone (NMP) and monitored by the qualitative ninhydrin test.[53] Difficult couplings were mediated with BOP, HBTU, or TBTU in DMF or NMP; pH was adjusted to 9 with diisopropylethylamine (DIPEA). A 2.5 equiv excess of amino acid based on the original substitution of the resin was used in most cases. Coupling steps were followed by acetylation [10% $(CH_3CO)_2O$ in CH_2Cl_2 for 10−15 min] as necessary. Boc removal was achieved with trifluoroacetic acid (60% in CH_2Cl_2, 1−2% ethanedithiol, or *m*-cresol) for 20 min. An isopropyl alcohol (1% ethanedithiol or *m*-cresol) wash followed TFA treatment, and then successive washes with triethylamine solution (10% in CH_2Cl_2), methanol, triethylamine solution, methanol, and CH_2Cl_2 completed the neutralization sequence. The Fmoc groups were removed with 20% piperidine in DMF or NMP in two successive 10 min treatments. Lactam cyclization was performed after Fmoc deprotection of the side chains of the bridgehead residues by the method of Felix et al.[54] or by substituting HBTU or TBTU for BOP. HF cleavage occurred in the presence of 10% anisole and 2−5% dimethyl sulfide (for Trp- and Cys-containing peptides) for 40−90 min at 0 °C. After HF distillation, the crude peptide was precipitated with diethyl ether, filtered, and dissolved in 10% aqueous acetic acid or 25% aqueous acetonitrile. The product was then shell-frozen and lyophilized. Cystines were formed at room temperature by air oxidation in dilute 25% acetonitrile−water adjusted to pH 7 with NH_4-OH until a negative Elman test resulted.

Purification. The crude, lyophilized peptides (1−3 g) were dissolved in a minimum amount (300 mL) of 0.25 N TEAP pH 2.25 and acetonitrile, and the solution was loaded onto the HPLC. The peptides eluted with a flow rate of 100 mL/min using a linear gradient of 1% B per 3 min increase from the baseline % B. (Eluent A = 0.25 N TEAP pH 2.25, eluent B = 60% CH_3CN, 40% A). Occasionally, purifications in TEAP pH 2.25 followed by TEAP pH 5−7 were necessary to achieve the desired purity level.[33,34] As a final step, all peptides were rechromatographed in a 0.1% TFA solution and acetonitrile on the same cartridge at 100 mL/min (gradient of 0.6% acetonitrile/min).

Characterization of GnRH Analogues. Peptides were characterized as shown in Table 1. Analogues were greater than 90% pure in most cases using independent HPLC and CZE criteria. Conditions are outlined in the legend and below.

1. RP-HPLC. Peptide purity was determined by analytical HPLC in either 0.1% TFA or TEAP pH 2.5 buffer systems as indicated in Table 1. The TEAP pH 2.5 conditions were defined by a 1% B/min gradient slope from equilibrium A/B where A = 5% CH_3CN/95% 15 mM TEAP (pH 2.5) and B = 80% CH_3CN/20% A at 2 mL/min on the Waters Associates HPLC system; A = 15 mM TEAP (pH 2.5) and B = 60% CH_3CN/40% A at 0.2 mL/min on the Hewlett-Packard HPLC system. The 0.1% TFA conditions were defined by a 1% B/min gradient slope at 0.2 mL/min from equilibrium A/B where A = 0.1% TFA and B = 60% CH_3CN/0.1% TFA on the Hewlett-Packard HPLC system. Detection was set at 214 nm.[55]

2. Capillary Zone Electrophoresis (CZE). CZE analysis employed a field strength of 10−20 kV at 30 °C with a buffer of 15% CH_3CN/85% 100 mM sodium phosphate pH 2.5 on either a Beckman eCAP or a Supelco P15 fused silica capillary (363 μm o.d. × 75 μm i.d. × 50 cm length). A borate buffer consisting of 0.1 or 0.2 N sodium borate ± 15% CH_3CN was used in certain cases indicated in Table 1. For reasons unknown, some analogues could not be analyzed using CZE (N/A) despite our efforts at using different capillaries and buffer pHs or addition of acetonitrile.[55,56]

3. Amino Acid Analysis. Amino acid analyses [after 4 M methanesulfonic acid hydrolysis at 110 °C for 24 h] were performed on a Perkin-Elmer (Norwalk, CT) high-pressure liquid chromatograph using *o*-phthalaldehyde postcolumn derivatization and fluorescence detection.

4. Mass Spectroscopy. LSI-MS measurements were carried out with a JEOL JMS-HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and a Cs^+ gun voltage between 25 and 30 kV were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The mass of each analogue was measured, and the observed monoisotopic $(M + H)^+$ values were consistent with the calculated $(M + H)^+$ values.

5. GnRH Receptor Membrane Binding Assay. Human HEK-293 cells stably transfected with the rat GnRH receptor[57−59] were harvested by striking the culture flask against the palm of the hand, resuspending in 5% sucrose, and homogenizing using a polytron homogenizer (2 × 15 s). Nuclei were removed by centrifugation (3000*g* for 5 min), and the supernatant was centrifuged (20000*g* for 30 min, 4 °C) to collect the crude membrane fraction. The final membrane preparation was resuspended in binding buffer [10 mM Hepes (pH 7.5), 150 mM NaCl, and 0.1% BSA] and stored at −70 °C. Binding reactions were performed in a Millipore MultiScreen 96-well filtration plate assembly with polyethylenimine coated GF/C membranes. The reaction was initiated by adding membranes (7 μg of protein in 130 μL of binding buffer) to 50 μL of radioligand (des-Gly^{10}-[^{125}I-Tyr^5,DAla^6,NMeLeu^7,Pro^9-NHEt]GnRH, ~100,000 cpm)[60] and 20 μL of competitor at varying concentrations. The reaction was terminated after 90 min by application of vacuum and washing (2×) with phosphate buffered saline. Bound radioactivity was measured by removing the filters from the plate and direct γ counting. K_i values were calculated from competition binding data using nonlinear least squares regression using the Prism software package (GraphPad Software). Data are reported as the average ± SEM of three or more independent experiments.

6. Antiovulatory Assay (AOA). The AOA was carried out as described by Corbin and Beattie.[36] The peptides were first dissolved in 2 N HOAc, then brought to the appropriate concentration in 0.1% bovine serum albumin−0.04 M phosphate buffer, pH 7.4. Cycling rats were injected subcutaneously with the peptides (200 μL) at noon on proestrus. Results are expressed in terms of number of rats ovulating over the number of animals receiving excipient for each experiment. Results are discussed in terms of percent inhibition of ovulation (rats not ovulating over rats ovulating × 100) or in terms of relative potency derived from an approximate evaluation of doses at which a certain percent inhibition is reached (e.g., if it takes a dose of 5 μg/rat to obtain inhibition in 7 rats out of 10 (70% inhibition) for a given peptide and if it takes 25 μg of another peptide to obtain inhibition in 4 rats out of 7 (60% inhibition), the first peptide is reported as being ca. 5 times more potent than the second as it takes approximately 5 times less material of the former to attain the same level of inhibition of ovulation).

All protocols were approved by the Salk Institute Animal Welfare Committee.

7. Molecular Modeling. The potential energy parameters and functional forms were from the CVFF force field.[61,62] Molecular modeling and visualization were performed using Insight II (MSI, Inc., San Diego, CA) on a Silicon Graphics Iris Crimson workstation.

Cyclo(0−8)[Suc-D-Nal[1],D-Cpa[2],D-Pal[3],Arg[5],D-Nal[6],Lys[8],D-Ala[10]]-GnRH (5). The peptide was synthesized automatically on 1.5 g of 0.76 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. The free N-terminus of the peptide-resin was succinylated with succinic anhydride (Sigma) in DCM for 45 min and repeated for another 30 min. The Lys-(ϵ-Fmoc) was then deprotected with 20% piperidine in DMF (2 × 10 min). Cyclization proceeded at pH 9 in DMF with BOP/HOBt/DIPEA (3:3:9 mmol) at 65 °C in an orbital shaker for 65 h. After drying, the peptide-resin (3.2 g) was cleaved and deprotected in HF (40 min, 0 °C), and the crude peptide (880 mg) was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 115 mg (74 μmol, 6%) of **5** ($[\alpha]_D = -29°$, $c = 0.67$).

With the exception of acetylation instead of succinylation, analogues **6** ($[\alpha]_D = -19°$, $c = 0.5$), **7** ($[\alpha]_D = -26°$, $c = 0.41$), **8** ($[\alpha]_D = -36°$, $c = 0.61$), **9** ($[\alpha]_D = -34°$, $c = 0.54$), **11** ($[\alpha]_D = -28°$, $c = 0.62$), **12**, and **15** ($[\alpha]_D = -36°$, $c = 0.41$) were obtained using the procedure for **5** in comparable yields.

Cyclo(3−8)[Ac-D-Nal[1],D-Cpa[2],D-Cys[3],Arg[5],D-Nal[6],Cys[8],D-Ala[10]]-GnRH (10). Analogue **10** was assembled automatically on 2.0 g of 0.76 mequiv/g-substituted MBHA resin. The completed peptide-resin was then cleaved and deprotected in HF (90 min, 0 °C), washed well with diethyl ether after HF removal, and extracted with 30:2:68 CH_3CN/AcOH/H_2O. The solution was diluted to 3 L with degassed water, adjusted to pH 7 with 28% NH_4OH, and stirred at 22 °C for 4 days for cystine formation. At that time, a negative Elman test indicated complete cyclization of the (3−8) bridge, and the solution was directly loaded on the prep HPLC for purification in TEAP 2.25 and 0.1% TFA, as described. After lyophilization of the desired fractions, 211 mg (146 μmol, 10%) of analogue **10** was obtained.

Cyclo(3−8)[Ac-D-Nal[1],D-Cpa[2],D-Asp[3],Arg[5],D-Nal[6],Lys[8],D-Ala[10]]-GnRH (14). The peptide was synthesized automatically on 2 g of 0.76 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. The (3−8) cyclization was performed on [Boc-D-Asp[3](β-OFm),Ser[4](OBzl),Arg[5](Tos),D-Nal[6],Lys[8](ϵ-Fmoc),D-Ala[10]]-GnRH(3−10)-MBHA after deprotection of the OFm/Fmoc groups by 20% piperidine in DMF (2 × 15 min). Cyclization then proceeded at pH 9 in DMF with BOP/DIPEA (3:9 mmol) at 65 °C in an orbital shaker (2 × 15 h). The final two amino acids were added to the peptide chain that was acetylated as the last solid-phase step. The peptide-resin was cleaved and deprotected in HF (90 min, 0 °C), and the crude peptide was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 108 mg (74 μmol, 5%) of **14**.

Analogue **13** was obtained using this general procedure in comparable yields.

Dicyclo(2−6/4−10)[Ac-D-Nal[1],Cys[2],D-Trp[3],Asp[4],D-Cys[6],Dpr[10]]-GnRH (18). Analogue **18** was assembled automatically on 2.0 g of 0.45 mequiv/g-substituted MBHA resin. The completed peptide-resin (3.2 g) was stirred for 100 h in DMF/hydrazine (40 mL) at 25 °C. The resin was collected by filtration and washed with DMF, DCM, and MeOH before drying under high vacuum. The peptide-resin was then cleaved and deprotected in HF (40 min, 0 °C). The peptide was extracted from the resin with 200 mL of 50% CH_3CN/H_2O and filtered. The filtrate was diluted to 4 L with degassed water, adjusted to pH 7.2 with 28% NH_4OH, and stirred at 22 °C for 7 days for the first cyclization. At that time, a negative Elman test indicated complete cystine formation to yield the (2−6) bridge, and the solution was acidified to pH 5.0 with AcOH. To reduce the volume, the solution was applied to a 4 × 8 cm column of Bio-Rex 70 (Bio-Rad) ion-exchange resin (H^+ form) at pH 5. The column was washed with 200 mL of water, and the peptide was eluted with 400 mL of 50% AcOH. Evaporation of the acetic acid and lyophilization of the ensuing yellow oil gave 520 mg of the intermediate, cyclo(2−6)[Ac-D-Nal[1],Cys[2],D-Trp[3],Asp[4](NHNH$_2$),D-Cys[6],Dpr[10]]GnRH.

The (4−10) bridge was formed via azide formation of the hydrazide intermediate followed by an intramolecular cyclization. First, the crude peptide hydrazide (0.36 mmol) was dissolved in dry DMF (40 mL) at −25 °C and acidified to pH 1 with 4.8 N HCl in dioxane (0.7 mL, 3.4 mmol). After 10 min, isoamyl nitrite was added in three aliquots (0.15 mL, 1.1 mmol total) with stirring over 30 min. Stirring at −25 °C was continued for 3 h. The solution of peptide azide was diluted to 1 L with DMF (precooled to −25 °C), and the pH was adjusted to 7 with TEA (0.6 mL). The solution was stored at −5 °C (48 h). The solvent was evaporated under vacuum to yield crude dicyclic peptide which was purified directly in TEAP 2.25 and 0.1% TFA to yield 12 mg (8 μmol, 1%) of dicyclic peptide **18**.

Analogues **23**−**25** were obtained using the procedure for **18** in comparable yields. Methods for dicyclic analogues **16**, **17**, and **20**: The (1−5), (1−8), or (2−8) cycles were formed on the resin following the procedure of **5**, and the (4−10) cyclization followed the procedure of **18**, including purification.

Dicyclo(3−8/4−10)[Ac-D-Nal[1],D-Cpa[2],D-Asp[3],Asp[4],Arg[5],D-Nal[6],Lys[8],Dpr[10]]GnRH (21). Analogue **21** was synthesized manually on 3.0 g of 0.76 mequiv/g-substituted MBHA resin. The peptide was completed to position 3, and then the (3−8) bridge was formed on the resin. The D-Asp[3](β-OFm) and Lys[8](ϵ-Fmoc) side chains of [Boc-D-Asp[3](β-OFm),Asp[4](β-OcHex),Arg[5](Tos),D-Nal[6],Lys[8](ϵ-Fmoc),Dpr[10](Z)]-GnRH(3−10)-MBHA were deprotected with 20% piperidine in DMF (2 × 15 min). Cyclization of the 3−8 bridge then proceeded at pH 9 in DMF with 2.5 molar excess of HBTU/HOBt/DIPEA (1:1:3) at 22 °C in for 16 h, after which the Kaiser test was negative. The final two amino acids were added to the peptide chain that was acetylated as the final peptide-building step.

The peptide was then prepared as the hydrazide for the (4−10) cyclization step. The completed peptide-resin was mixed for 22 h with a 30% anhydrous hydrazine/DMF solution, filtered, and washed with 50% DMF/DCM and MeOH to yield 6.3 g after drying. The peptide-resin was next cleaved and deprotected in HF (90 min, 0 °C), washed well with ether after HF removal, extracted with 30% CH_3CN/H_2O, shell-frozen, and lyophilized to yield 2.5 g (1.7 mmol) of crude cyclo(3−8)[Ac-D-Nal[1],D-Cpa[2],D-Asp[3],Asp[4](NHNH$_2$),Arg[5],D-Nal[6],Lys[8],Dpr[10]]-GnRH.

This intermediate was dissolved in dry DMF (70 mL) at −30 °C and acidified with 4 N HCl in dioxane (2.5 mL, 10 mmol). Isoamyl nitrite (0.80 mL, 6.0 mmol) was added dropwise over 1 h, while stirring, and the reaction proceeded until a positive starch-KI test remained for 30 min. The solution of peptide azide was diluted to 1.8 L with dry DMF (precooled to −30 °C, over 3 Å sieves), the pH was adjusted to 7 with diisopropylethylamine (3.5 mL, 21 mmol), and the solution was stirred at −30 °C for an additional hour. In this case, HPLC analysis indicated that no reaction took place; therefore, the DMF was removed and the procedure was repeated successfully. The solvent was evaporated under vacuum, the residue was dissolved in 30% CH_3CN/H_2O, shell-frozen, and lyophilized to yield 2.2 g of crude dicyclic peptide, which was seen by HPLC as two peaks with different retention times (0.1% TFA, 24%−40 min−48% CH_3CN gradient, $R_t = 23$ and 29 min). The hydrophilic product was the major entity, but both products were purified by preparative HPLC in TEAP 2.25 and 0.1% TFA. The yield of the major product was 18 mg (11 μmol, <1%) of **21** ($[\alpha]_D = -47°$, $c = 1$). It should be noted that both products were identical by mass spectroscopy and amino acid analysis and were deemed to be isomeric. The products were also distinguishable by the biological AOA test, since the major, hydrophilic product showed greater activity in all cases. Therefore the yield and data of the hydrophilic product is reported in Table 1.

Analogues **22** ($[\alpha]_D = -49°$, $c = 1$), **28** ($[\alpha]_D = -71°$, $c = 1$), and **30** ($[\alpha]_D = -63°$, $c = 1$) were obtained using the procedure for **21** in comparable yields.

Dicyclo(1−5/4−10)[Ac-Asp[1],D-Cpa[2],D-Trp[3],Asp[4],D-Dpr[5],D-Nal[6],Dpr[10]]GnRH (26). Peptide **26** was manually synthesized on 2.88 g of 0.43 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. After the solid phase synthesis was completed, the resin was prepared for (1−5) lactam formation by first deprotecting the side chain groups of Asp[1](β-OFm) and D-Dpr[5](β-Fmoc) with 20% piperidine in DMF (3 × 15 min). Cyclization then proceeded at pH 9 in 20 mL of NMP with a 2.5 molar excess of BOP/DIPEA (1:3) at 65 °C in an orbital shaker for 17 h. The procedure was repeated

with fresh reagents, once at 65 °C and three times at 22 °C, until a Kaiser test was negative. The resin was then treated with 20% acetic anhydride in DCM containing 2 drops of pyridine for 20 min to cap any unreacted amino groups. After drying, the peptide-resin was cleaved and deprotected in HF (90 min, 0 °C) to yield 1.53 g (1.1 mmol) of crude monocyclic peptide.

The (4−10) cyclization step was carried out on the crude monocyclic intermediate. A solution of diphenylphosphoryl azide (0.67 mL, 3.1 mmol) and TEA (0.35 mL, 2.5 mmol) in 400 mL of DMF (pH 8) was cooled to between −50 and −60 °C. To this solution was dropwise added the crude peptide in 50 mL of DMF over 3 h. The solution, at pH 6−7, was stored at −15 °C for 15 h and then was stirred at 22 °C for 24 h. The DMF was removed by rotary evaporation, and the residue was reconstituted in 25% CH_3CN/H_2O, shell-frozen, and lyophilized to a powder. HPLC analysis indicated an incomplete reaction, so the process was repeated with modifications. The powder was dissolved in 600 mL of DMF and cooled to 4 °C. Diphenylphosphoryl azide (0.54 mL, 2.5 mmol) and KH_2PO_4 (0.84 g, 6.2 mmol) were added, and the pH 6 reaction mixture was stirred at 4 °C while allowing to warm to 22 °C overnight. After 4 days, HPLC analysis showed the reaction was completed and the DMF was removed. The oily residue was dissolved in 40% CH_3CN/H_2O, shell-frozen, and lyophilized to a crude dicyclic product. The crude peptide was purified by preparative HPLC in TEAP 2.25 and 0.1% TFA to yield 17 mg (11 μmol, 1%) of **26**.

Dicyclo(1−5/4−10)[Ac-Glu^1,D-Cpa^2,D-Trp^3,Asp^4,Dbu^5,D-Nal^6,Dpr^{10}]GnRH (27). Peptide **27** was manually synthesized on 1.44 g of 0.43 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. The (1−5) cyclization proceeded on the resin using the cyclization method described for **26**. In preparation for the (4−10) cyclization, the hydrazide of Asp^4 was formed on the resin from hydrazine·monohydrate (6 mL, 124 mmol) in DMF (6 mL) over 72 h. The peptide-resin was then cleaved and deprotected in HF (90 min, 0 °C). Formation of the 4−10 bridge then proceeded according to the method described for **18** with 11 days of reaction before workup. The crude dicyclic peptide (600 mg) was purified by preparative HPLC in TEAP 2.25 and 0.1% TFA to yield 17 mg (11 μmol, 2%) of **27**.

Dicyclo(1,1′−5/4−10)[Ac-Asp^1(Gly),D-Cpa^2,D-Nal^3,Asp^4,Dbu^5,D-Nal^6,Dpr^{10}]GnRH (34). Peptide **34** was manually synthesized on 3 g of 0.76 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. The 1′ residue was introduced during the solid phase synthesis before the Asp^1-(β-OFm) residue was coupled. The Fmoc-protecting group of [Boc-Asp^4(β-OcHex),Dbu^5(γ-Fmoc),D-Nal^6,Arg^8(Tos),Dpr^{10}(β-Z)]-GnRH(4−10)-MBHA was first removed with 20% piperidine in DMF (2 × 10 min), whereupon N_α-Fmoc-Gly was coupled to the free Dbu-NH_2 side chain with DIC in DCM for 21 h. The N-terminal Boc group of the growing peptide chain was then deblocked with TFA, as described, and the synthesis was continued through the N-terminal acetylation. The Fmoc groups of Asp^1 and Gly$^{1'}$ were removed with piperidine as above, and cyclization proceeded in DMF with 3-fold molar excess of TBTU/HOBt/DIPEA (1:1:3) for 16 h at 22 °C and was repeated for 3 h. Unreacted free amino groups were then actetylated. The resin was then treated with 10 mL of 30% anhydrous hydrazine in DMF for 3 days and then washed with DMF (3×), methanol (2×), and DCM (3×). The peptide-hydrazide (6.2 g) was then cleaved and deprotected in anhydrous HF at 0 °C for 1.5 h, followed by the usual workup and lyophilization from 30% CH_3CN/H_2O to yield 2.50 g of crude cyclo(1,1′−5)[Ac-Asp^1(Gly),D-Cpa^2,D-Nal^3,Asp^4($NHNH_2$),Dbu^5,D-Nal^6,Dpr^{10}]-GnRH. The 4−10 bridge was formed in 30 min using the procedure reported for **21** and was seen as a single product, as monitored by HPLC. The crude dicyclic peptide was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 70 mg (43 μmol, 2%) of **34**.

Analogues **19**, **31** ($[\alpha]_D = -63°$, $c = 1$), **32** ($[\alpha]_D = -46°$, $c = 1$), **33**, **35**-**37**, **38** ($[\alpha]_D = -37°$, $c = 1$), **39** ($[\alpha]_D = -73°$, $c = 1$), **40** ($[\alpha]_D = -52°$, $c = 1$), **42, 43** ($[\alpha]_D = -43°$, $c = 1$), **44** ($[\alpha]_D = -85°$, $c = 1$), and **45** ($[\alpha]_D = -49°$, $c = 1$) were synthesized in a similar manner using the procedure described for **34**. The synthesis and (1,1′−5)-cyclization of analogues **29** ($[\alpha]_D = -49°$, $c = 1$), **41** ($[\alpha]_D = -67°$, $c = 1$), and **46** ($[\alpha]_D = -45°$, $c = 0.5$) proceeded according to the description for **34**; the (4−10) cycle was formed using the procedures of **18** and **27**.

Acknowledgment. This research was supported by NIH contract N01-HD-4-2833, NIH grant HD-1-3527, and the Hearst Foundation. Research was conducted in part by The Foundation for Research, California Division. C.R. is a Clayton Foundation Investigator. The authors thank Ron Kaiser, Charleen Miller, Duane Pantoja, Georgia Morgan, Mary Tam, Susanne Johnson, Duane Pantoja, Greg Reinhart, Qui Xie, and Yaira Haas for excellent technical assistance and Debbie Doan for manuscript preparation. We are indebted to Drs. T. Lee and A.G. Craig and T. Goedken for FAB mass spectral analyses. We thank Drs. Naqvi and Lindberg for some of the antiovulatory data obtained at the Mason Research Institute under contract N01-HD-0-2846 and Dr. P. N. Rao for the unnatural amino acids synthesized under contract NO1-HD-6-2928 with the Contraceptive Development Branch, Center for Population Research, NICHD.

References

(1) Rivier, J.; Kupryszewski, G.; Varga, J.; Porter, J.; Rivier, C.; Perrin, M.; Hagler, A.; Struthers, S.; Corrigan, A.; Vale, W. Design of potent cyclic gonadotropin releasing hormone (GnRH) antagonists. *J. Med. Chem.* **1988**, *31*, 677−682.

(2) Rivier, J. E.; Struthers, R. S.; Porter, J.; Lahrichi, S. L.; Jiang, G.; Cervini, L. A.; Ibea, M.; Kirby, D. A.; Koerber, S. C.; Rivier, C. L. Design of potent dicyclic (4−10/5−8) gonadotropin releasing hormone (GnRH) antagonists. *J. Med. Chem.* **2000**, *43*, 784−796.

(3) Rivier, J. E.; Porter, J.; Cervini, L. A.; Lahrichi, S. L.; Kirby, D. A.; Struthers, R. S.; Koerber, S. C.; Rivier, C. L. Design of monocyclic (1−3) and dicyclic (1−3/4−10) gonadotropin releasing hormone (GnRH) antagonists. *J. Med. Chem.* **2000**, *43*, 797−806.

(4) Chary, V. R.; Srivastava, S.; Hosur, R. V.; Roy, K. B.; Govil, G. Molecular conformation of gonadoliberin using two-dimensional NMR spectroscopy. *Eur. J. Biochem.* **1986**, *158*, 323−332.

(5) Deslauriers, R.; Levy, G. C.; McGregor, W. H.; Sarantakis, D.; Smith, I. C. P. Conformational flexibility of luteinizing hormone-releasing hormone in aqueous solution. A carbon-13 spin−lattice relaxation time study. *Biochemistry* **1975**, *14*, 4335−4343.

(6) Guarnieri, F.; Weinstein, H. Conformational memories and the exploration of biologically relevant peptide conformations: An illustration for the gonadotropin-releasing hormone. *J. Am. Chem. Soc.* **1996**, *118*, 5580−5589.

(7) Mabrey, S.; Klotz, I. M. Conformation of gonadotropin releasing hormone. *Biochemistry* **1976**, *15*, 234.

(8) Matsoukas, J. M.; Keramida, M.; Panagiotopoulos, D.; Mavromoustakos, T.; Maia, H. L. S.; Bigam, G.; Pati, D.; Habibi, H. R.; Moore, G. J. Structure elucidation and conformational analysis of gonadotropin releasing hormone and its novel synthetic analogue [Tyr(OMe)^5,D-Lys^6, Aze^{9}NHEt]GnRH: The importance of aromatic clustering in the receptor binding activity. *Eur. J. Med. Chem.* **1997**, *32*, 927−940.

(9) Nikiforovich, G. V.; Marshall, G. R. Conformation-function relationships in LHRH analogues. I. Conformation of LHRH peptide backbone. *Int. J. Pept. Protein Res.* **1993**, *42*, 171−180.

(10) Nikiforovich, G. V.; Marshall, G. R. Conformation-function relationships in LHRH analogues. II. Conformations of LHRH peptide agonists and antagonists. *Int. J. Pept. Protein Res.* **1993**, *42*, 181−193.

(11) Momany, F. A. Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. 1. Native decapeptide. *J. Am. Chem. Soc.* **1976**, *98*, 2990−2996.

(12) Momany, F. A. Conformational energy analysis of the molecule, luteinizing hormone-releasing hormone. 2. Tetrapeptide and decapeptide analogues. *J. Am. Chem. Soc.* **1976**, *98*, 2996−3000.

(13) Sprecher, R. F.; Momany, F. A. On the conformation of luteinizing hormone-releasing hormone, nuclear overhauser observations. *Biochem. Biophys. Res. Commun.* **1979**, *87*, 72−77.

(14) Sealfon, S. C.; Weinstein, H.; Millar, R. P. Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. *Endocrine Rev.* **1997**, *18*, 180−205.

(15) Koerber, S. C.; Rizo, J.; Struthers, R. S.; Rivier, J. E. Consensus bioactive conformation of cyclic GnRH antagonists defined by NMR and molecular modeling. *J. Med. Chem.* **2000**, *43*, 819−828.
(16) Kopple, K. D. Peptide backbone-folding in LHRH and analogues. In *Peptides: Synthesis-Structure−Function*; Rich, D. H., Gross, E., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 295−298.
(17) Kopple, K. D. Nitroxyl-induced T_1 relaxation rates as a probe of conformation in peptide hormones. *Int. J. Pept. Protein Res.* **1983**, *21*, 43−48.
(18) Kutscher, B.; Bernd, M.; Beckers, T.; Polymeropoulos, E. E.; Engel, J. Chemistry and molecular biology in the search for new LHRH antagonists. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2148−2161.
(19) Chandrasekaren, R.; Lakshminarayanan, A. V.; Pandya, U. V.; Ramachandran, G. N. Conformation of the LL and LD hairpin bends with internal hydrogen bonds in proteins and peptides. *Biochim. Biophys. Acta* **1973**, *303*, 14−27.
(20) Monahan, M.; Amoss, M.; Anderson, H.; Vale, W. Synthetic analogues of the hypothalamic luteinizing hormone releasing factor with increased agonist or antagonist properties. *Biochem.* **1973**, *12*, 4616−4620.
(21) Ling, N.; Vale, W. W. Analogues of luteinizing hormone releasing factor (LRF) synthesis and biologial activity of [(N-ME)Leu^7]-LRF and [D-Ala^6,(N-Me)-Leu^7]-LRF. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 801−806.
(22) Tonelli, A. E. The effects of isolated *N*-methylated residues on the conformational characteristics of polypeptides. *Biopolymers* **1976**, *15*, 1615−1622.
(23) Freidinger, R. M.; Veber, D. F.; Perlow, D. S.; Brooks, J. R.; Saperstein, R. Bioactive conformation of luteinizing hormone-releasing hormone: evidence from a conformationally constrained analogue. *Science* **1980**, *210*, 656−658.
(24) Rivier, J.; Koerber, S.; Struthers, S.; Tanaka, G.; Rivier, C.; Perrin, M.; Porter, J.; Corrigan, A.; Vale, W.; Hagler, A. Design of active multicyclic gonadotropin releasing hormone antagonists. In *Proc. Banbury Ctr. Conf. on Therapeutic Peptides & Proteins: Formulation, Delivery & Targetting*; Marshak, D., Liu, D., Eds.; Cold Spring Harbor Press: Cold Spring Harbor, New York, 1988; pp 85−91.
(25) Rivier, J.; Koerber, S.; Rivier, C.; Porter, J.; Hagler, A. Design, computer derived structure and biological activity of three bicyclic gonadotropin releasing hormone (GnRH) antagonists. In *Current Research in Protein Chemistry*; Villafranca, J., Ed.; Academic Press: 1990; pp 273−281.
(26) Rivier, J.; Kupryszewski, G.; Theobald, P.; Hoeger, C.; Porter, J.; Perrin, M.; Corrigan, A.; Struthers, S.; Koerber, S.; Hagler, A.; Vale, W.; Rivier, C. Two distinct approaches for the design of potent GnRH antagonists. In *Proc. of the 2nd Int'l. Symp. on GnRH Analogues in Cancer and Human Reproduction*; Lunenfeld, B., Eds.; Parthenon Publishing: Carnforth, Lancaster, U.K., 1991; pp 123−135.
(27) Struthers, R. S.; Rivier, J.; Hagler, A. T. Molecular dynamics and minimum energy conformations of GnRH and analogues: A methodology for computer aided drug design. In *Annals of New York Academy of Sciences. Macromolecular Structure and Specificity: Computer Assisted Modeling and Applications;* Venkataraghavan, B., Feldman, R. J., Eds.; 1985; Vol. 439, pp 81−96.
(28) Struthers, R. S.; Hagler, A. T.; Rivier, J. Theoretical stimulation of conformation, energetics, and dynamics. In *Conformationally Directed Drug Design: Peptides and Nucleic Acids as Templates or Targets*; Vida, J. A., Gordon, M., Eds.; American Chemical Society: Washington, DC, 1984; pp 239−261.
(29) Rizo, J.; Koerber, S. C.; Bienstock, R. J.; Rivier, J.; Hagler, A. T.; Gierasch, L. M. Conformational analysis of a highly potent, constrained gonadotropin-releasing hormone antagonist. 1. Nuclear magnetic resonance. *J. Am. Chem. Soc.* **1992**, *114*, 2852−2859.
(30) Rizo, J.; Koerber, S. C.; Bienstock, R. J.; Rivier, J.; Gierasch, L. M.; Hagler, A. T. Conformational analysis of a highly potent, constrained gonadotropin-releasing hormone antagonist. 2. Molecular dynamics simulations. *J. Am. Chem. Soc.* **1992**, *114*, 2860−2871.
(31) Bienstock, R. J.; Rizo, J.; Koerber, S. C.; Rivier, J. E.; Hagler, A. T.; Gierasch, L. M. Conformational analysis of a highly potent dicyclic gonadotropin-releasing hormone antagonist by nuclear magnetic resonance and molecular dynamics. *J. Med. Chem.* **1993**, *36*, 3265−3273.
(32) Felix, A. M.; Heimer, E. P.; Wang, C. T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. Synthesis, biological activity and conformational analysis of cyclic GRF analogues. *Int. J. Pept. Protein Res.* **1988**, *32*, 441−454.
(33) Rivier, J. Use of trialkylammonium phosphate (TAAP) buffers in reverse phase HPLC for high resolution and high recovery of peptides and proteins. *J. Liq. Chromatogr.* **1978**, *1*, 343−367.
(34) Hoeger, C. A.; Galyean, R. F.; Boublik, J.; McClintock, R. A.; Rivier, J. E. Preparative reversed phase high performance liquid chromatography. II. Effects of buffer pH on the purification of synthetic peptides. *Biochromatography* **1987**, *2*, 134−142.
(35) Porter, J.; Dykert, J.; Rivier, J. Synthesis, resolution and characterization of ring substituted phenylalanines and tryptophans. *Int. J. Pept. Protein Res.* **1987**, *30*, 13−21.
(36) Corbin, A.; Beattie, C. W. Inhibition of the pre-ovulatory proestrous gonadotropin surge, ovulation and pregnancy with a peptide analogue of luteinizing hormone releasing hormone. *Endocr. Res. Commun.* **1975**, *2*, 1−23.
(37) Rizo, J.; Koerber, S. C.; Bienstock, R. J.; Rivier, J.; Hagler, A. T.; Gierasch, L. M. Conformational analysis of a highly potent, constrained gonadotropin-releasing hormone antagonist. I. Nuclear magnetic resonance. *J. Am. Chem. Soc.* **1992**, *114*, 2852−2859.
(38) Rizo, J.; Koerber, S. C.; Bienstock, R. J.; Rivier, J.; Gierasch, L. M.; Hagler, A. T. Conformational analysis of a highly potent, constrained gonadotropin-releasing hormone antagonist. II. Molecular Dynamics simulations. *J. Am. Chem. Soc.* **1992**, *114*, 2860−2871.
(39) Rivier, J.; Porter, J.; Rivier, C.; Perrin, M.; Corrigan, A. Z.; Hook, W. A.; Siraganian, R. P.; Vale, W. W. New effective gonadotropin releasing hormone antagonists with minimal potency for histamine release in vitro. *J. Med. Chem.* **1986**, *29*, 1846−1851.
(40) Roeske, R. W.; Chaturvedi, N. C.; Rivier, J.; Vale, W.; Porter, J.; Perrin, M. Substitution of Arg^5 for Tyr^5 in GnRH antagonists. In *Peptides, Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: Rockford, IL, 1986; pp 561−564.
(41) Rizo, J.; Sutton, R. B.; Breslau, J.; Koerber, S. C.; Porter, J.; Rivier, J. E.; Hagler, A. T.; Gierasch, L. M. Defining the active conformation of gonadotropin releasing hormone (GnRH) through design and conformational analysis of constrained GnRH analogues. In *Peptides: Chemistry, Structure and Biology*; Hodges, R., Smith, J. A., Eds.; ESCOM Scientific Publishers: B. V./ Leiden, The Netherlands, 1994; pp 766−768.
(42) Folkers, K.; Bowers, C. Y.; Kubiak, T.; Stepinski, J. Antagonists of the luteinizing hormone releasing hormone with pyridylalanines which completely inhibit ovulation at nanogram dosage. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 1089−1095.
(43) Hocart, S. J.; Nekola, M. V.; Coy, D. H. Effect of reductive alkylation of lysine in positions 6 and/or 8 on the histamine-releasing activity of luteinizing hormone-releasing hormone antagonists. *J. Med. Chem.* **1987**, *30*, 1910−1914.
(44) Kaiser, U. B.; Conn, P. M.; Chin, W. W. Studies of gonadotropin-releasing hormone (GnRH) action using GnRH receptor-expressing pituitary cell lines. *Endocr. Rev.* **1997**, *18*, 46−70.
(45) White, R. B.; Eisen, J. A.; Kasten, T. L.; Fernald, R. D. Second gene for gonadotropin-releasing hormone in humans. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 305−309.
(46) Chen, A.; Yahalom, D.; Ben-Aroya, N.; Kaganovsky, E.; Okon, E.; Koch, Y. A second isoform of gonadotropin-releasing hormone is present in the brain of human and rodents. *FEBS Lett.* **1998**, *435*, 199−203.
(47) Olivera, B. M.; Rivier, J.; Clark, C.; Ramilo, C. A.; Corpuz, G. P.; Abogadie, F. C.; Mena, E. E.; Woodward, S. R.; Hillyard, D. R.; Cruz, L. J. Diversity of *Conus* Neuropeptides. *Science* **1990**, *249*, 257−263.
(48) Perrin, M. H.; Sutton, S. W.; Cervini, L.; Rivier, J. E.; Vale, W. W. Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of CRF receptors. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 729−734.
(49) Märki, W.; Spiess, J.; Taché, Y.; Brown, M.; Rivier, J. E. Total solid-phase synthesis of porcine gut gastrin releasing peptide (GRP), a mammalian bombesin. *J. Am. Chem. Soc.* **1981**, *103*, 3178−3185.
(50) Jiang, G.-C.; Simon, L.; Rivier, J. E. Orthogonally protected *N*-methyl-substituted α-aminoglycines. *Protein Pept. Lett.* **1996**, *3*, 219−224.
(51) Rivier, J. E.; Jiang, G.; Porter, J.; Hoeger, C.; Craig, A. G.; Corrigan, A. Z.; Vale, W. W.; Rivier, C. L. GnRH antagonists: novel members of the azaline B family. *J. Med. Chem.* **1995**, *38*, 2649−2662.
(52) Stewart, J. M.; Young, J. D. Solid-Phase Peptide Synthesis. Pierce Chemical Co.: Rockford, IL, 1969.
(53) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595−598.
(54) Felix, A. M.; Wang, C.-T.; Heimer, E. P.; Fournier, A. Applications of BOP reagent in solid-phase synthesis. II. Solid-phase side-chain to side-chain cyclizations using BOP reagent. *Intl. J. Pept. Protein Res.* **1988**, *31*, 231−238.
(55) Miller, C.; Rivier, J. Peptide chemistry: Development of high-performance liquid chromatography and capillary zone electrophoresis. *Biopolymers* **1996**, *40*, 265−317.

(56) Miller, C.; Rivier, J. Analysis of synthetic peptides by capillary zone electrophoresis in organic/aqueous buffers. *J. Pept. Res.* **1998**, *51*, 444−451.
(57) Eidne, K. A.; Sellar, R. E.; Couper, G.; Anderson, L.; Taylor, P. L. Molecular cloning and characterisation of the rat pituitary gonadotropin-releasing hormone (GnRH) receptor. *Mol. Cell. Endocrinol.* **1992**, *90*, R5−R9.
(58) Kaiser, U. B.; Zhao, D.; Cardone, G. R.; Chin, W. W. Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 1645−1652.
(59) Perrin, M. H.; Bilezikjian, L. M.; Hoeger, C.; Donaldson, C. J.; Rivier, J. E.; Haas, Y.; Vale, W. W. Molecular and functional characterization of GnRH receptors cloned from rat pituitary and a mouse pituitary tumor cell line. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 1139−1144.
(60) Perrin, M. H.; Haas, Y.; Rivier, J. E.; Vale, W. W. GnRH binding to rat anterior pituitary membrane homogenates: Comparison of antagonists and agonists using radiolabeled antagonist and agonist. *Mol. Pharmacol.* **1983**, *23*, 44−51.
(61) Maple, J.; Dinur, U.; Hagler, A. T. Derivation of force fields for molecular mechanics and dynamics from Ab Initio energy surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5350−5354.
(62) Maple, J. R.; Thacher, T. S.; Dinur, U.; Hagler, A. T. Biosym force field research results in new techniques for the extraction of inter- and intramolecular forces. *Chem. Des. Auto. News* **1990**, *5*, 5−10.

JM990116+