Design of Potent Dicyclic (4−10/5−8) Gonadotropin Releasing Hormone (GnRH) Antagonists

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Received March 15, 1999

With the ultimate goal of identifying a consensus bioactive conformation of GnRH antagonists, the compatibility of a number of side chain to side chain bridges in bioactive analogues was systematically explored. In an earlier publication, cyclo[Asp^4-Dpr^{10}]GnRH antagonists with high potencies in vitro and in vivo had been identified.[1] Independently from Dutta et al.[2] and based on structural considerations, the cyclic [Glu^5-Lys^8] constraint was also found to be tolerated in GnRH antagonists. We describe here a large number of cyclic (4−10) and (5−8) and dicyclic (4−10/5−8) GnRH antagonists optimized for affinity to the rat GnRH receptor and in vivo antiovulatory potency. The most potent monocyclic analogues were cyclo(4−10)-[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Asp^4,D-Arg^6,Xaa^{10}]GnRH with Xaa = D/LAgl (**1**, K_i = 1.3 nM) or Dpr (**2**, K_i = 0.36 nM), which completely blocked ovulation in cycling rats after sc administration of 2.5 μg at noon of proestrus. Much less potent were the closely related analogues with Xaa = Dbu (**3**, K_i = 10 nM) or cyclo(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Glu^4,D-Arg^6,D/L-Agl^{10}]GnRH (**4**, K_i = 1.3 nM). Cyclo(5−8)[Ac-D-Nal^1,D-Cpa^2,D-Trp^3,Glu^5,D-Arg^6,Lys^8,D-Ala^{10}]GnRH (**13**), although at least 20 times less potent in the AOA than **1** or **2** with similar GnRHR affinity (K_i = 0.84 nM), was found to be one of the most potent in a series of closely related cyclo(5−8) analogues with different bridge lengths and bridgehead chirality. The very high affinity of cyclo(5,5′−8)[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Glu^5(βAla),D-Arg^6,(D or L)Agl,^{8}D-Ala^{10}]GnRH **14** (K_i = 0.15 nM) correlates well with its high potency in vivo (full inhibition of ovulation at 25 μg/rat). 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 (**24**, K_i = 0.32 nM) is one-fourth as potent as **1** or **2**, in the AOA; this suggests that the introduction of the (4−10) bridge in **13**, while having little effect on affinity, restores functional/conformational features favorable for stability and distribution. To further increase potency of dicyclic antagonists, the size and composition of the (5−8) bridge was varied. For example, the substitution of Xbb$^{5'}$ by Gly (**30**, K_i = 0.16 nM), Sar (**31**, K_i = 0.20 nM), Phe (**32**, K_i = 0.23 nM), D-Phe (**33**, K_i = 120 nM), Arg (**36**, K_i = 0.20 nM), Nal (**37**, K_i = 4.2 nM), His (**38**, K_i = 0.10 nM), and Cpa (**39**, K_i = 0.23 nM) in cyclo-(4−10/5,5′−8)[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Asp^4,Glu^5(Xbb$^{5'}$),D-Arg^6,Dbu,^{8}Dpr^{10}]GnRH yielded several very high affinity analogues that were 10, ca. 10, 4, >200, 1, ca. 4, >2, and 2 times less potent than **1** or **2**, respectively. Other scaffolds constrained by disulfide (**7**, K_i = 2.4 nM; and **8**, K_i = 450 nM), cyclo[Glu^5-Aph^8] (**16**, K_i = 20 nM; and **17**, K_i = 0.28 nM), or cyclo[Asp^5-/Glu^5-/Asp^5-(Gly$^{5'}$)-Amp^8] (**19**, K_i = 1.3 nM; **22**, K_i = 3.3 nM; and **23**, K_i = 3.6 nM) bridges yielded analogues that were less potent in vivo and had a wide range of affinities. The effects on biological activity of substituting D-Cpa or D-Fpa at position 2, D-Pal or D-Trp at position 3, and D-Arg, D-Nal, or D-Cit at position 6 are also discussed. Interestingly, monocyclo(5−8)[Glu^5,D-Nal^6,Lys^8]GnRH (**18**, K_i = 1.0 nM) and dicyclo(4−10/5−8)[Asp^4,Glu^5,D-Nal^6,Lys^8,Dpr^{10}]GnRH (**28**, K_i = 1.2 nM) contain the native N-terminal pGlu-His-Trp- and are antagonists with relatively high affinity but very low antagonist potency in vivo, illustrating an earlier observation that structural constraints alone may lead to partial agonism or competitive antagonism. All of these observations suggest very rigorous requirements for ligand/receptor recognition and binding as well as a distinct effect of some substitutions on pharmacokinetics.

Introduction

The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is under the stimulatory control of gonadotropin releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), the decapeptide amide characterized by Matsuo et al.[3] and Burgus et al.[4] With the availability of a synthetic replica, it was quickly found that GnRH played a major role in triggering the onset of ovulation and spermatogenesis in mammals. Therefore, it was proposed that a competitive antagonist of GnRH could be used to inhibit reproductive processes by preventing the GnRH-stimulated LH surge in proestrus or disrupting spermatogenesis in the male. It was not before 1983, however, that potent GnRH antagonists allowed the demonstration of the validity of the concept and the efficacy of such analogues in both male[5] and female rats[6] and later in men[7−10] and women.[11,12]

10.1021/jm990115h CCC: $19.00
Published on Web 02/23/2000

We have used structure−activity relationship (SAR) studies to constrain the structure of GnRH antagonists with the goal of defining a consensus model of the conformation of potent GnRH antagonists, as indicated by NMR spectroscopy. Because there is such a large number of GnRH antagonists that are highly potent yet very different in primary structures, it is unlikely that they would all interact with the GnRH receptor by interacting with the same contact points and assuming the same conformation. Yet kinetic arguments suggest that both GnRH agonists and antagonists interact with the receptor at similar loci.[13] A consensus model of the bioactive conformation of GnRH antagonists would therefore encompass the common conformational features found in several potent and constrained analogues. Such a model could then serve as a template for the design of non-peptide GnRH antagonist mimetics. How we arrived at a working model of the consensus conformation of potent GnRH antagonists is the subject of this and the three following reports.

In this paper, the synthesis, biological activities, and potencies of monocyclic (4−10), (5−8) and dicyclic (4−10/5−8) GnRH antagonists (see representative structure in Figure 1) are described. In the second paper, the synthesis, biological activities, and potencies of monocyclic (1−3) and dicyclic (1−3/4−10) GnRH antagonists are described. In the third paper, the synthesis, biological activities, and potencies of several monocyclic (0−8), (1−8), (2−8), (3−8) and dicyclic (1−8/4−10), (2−6/4−10), (2−8/4−10), (3−8/4−10) and (1−5/4−10) GnRH antagonists are described. In the fourth paper, the NMR structure of a potent dicyclic (1−5/4−10) GnRH antagonist and how the data together with prior structural data[14−17] were used to arrive at a consensus model of the conformation of potent GnRH antagonists are described.

Results and Discussion

Peptides were synthesized by the solid phase method on a methyl benzhydrylamine resin using techniques previously reported by this laboratory.[18] Unusual amino acids, such as orthogonally protected Boc-3-aminophenylalanine(Fmoc) [Boc-Aph(Fmoc)], Boc-4-aminomethylphenylalanine(Fmoc) [Boc-Amp(Fmoc)], and Boc-aminoglycine(Fmoc) [Boc-Agl(Fmoc)] were synthesized as reported earlier.[19,20] 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.[21] The (4−10) 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 peptide was then cleaved with HF and concomitantly deprotected. The peptide hydrazide was then converted to the corresponding azide using isoamyl nitrite and HCl in dioxane and cyclized in DMF under dilute conditions with the free side chain amino groups of D/LAgl, L-Dpr, or L-Dbu at position 10 to give the crude cyclized peptide (see Experimental Section). Purification was carried out by HPLC.[22,23] Retention times using isocratic conditions and purities, determined to be >80% in most cases by HPLC and CZE, are shown in Table 1. The question of how meaningful can antiovulatory data be for cyclic peptides that are 70−80% pure is one that has been raised in our laboratory. The conclusion always has been that, in view of all other errors and parameters to be considered in an in vivo assay, the final result will not be statistically different from the one that has been obtained. More to the point is the question as to whether the impurities are made up of one single component or several closely related ones and whether it is likely that the impurity(ies) will be more potent, toxic, or antagonistic of the activity that is measured than the desired product. We have, over the years, isolated impurities and tested them after characterization; these generally resulted from deletions, trifluoroacetylation, and rearrangement of the aspartic residue to yield the corresponding beta-peptides. None of these peptides were as potent as the desired molecules, although they showed in most cases some antagonistic activity.

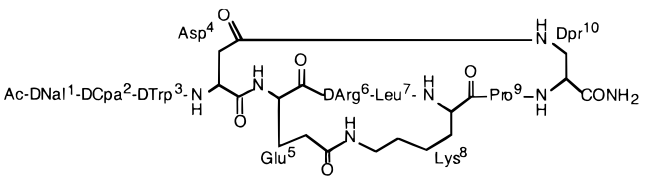

Figure 1. Structure of **24**.

Amino acid analyses[24] including those of Pal, Cpa, Fpa, Nal, and Dpr were consistent with expected results. Calculated values for protonated molecular ions were within 100 ppm of those obtained using FAB mass spectrometry (see Table 1).

Biological evaluation was conducted using an in vivo antiovulatory assay (AOA),[25] found to be more discriminatory than the in vitro binding and functional assays originally used to identify lead compounds yet inappropriate for a rigorous discussion of SAR.[7,26−28] Consequently, we also report binding affinities to rat GnRH receptor (rGnRHR) expressed as K_i. The relative potencies discussed in this and the following papers were derived from the ratio of doses that gave equivalent responses in the AOA and are approximate. Ideally, peptides would be tested at different doses yielding no inhibition, partial inhibition, and complete inhibition of ovulation, respectively, in order to obtain a dose−response curve from which relative potencies could be derived.[29] This requires a large number of animals (8−10 per dose) to reach statistical significance; therefore,

† 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: Agl, aminoglycine; Amp, 4-aminomethylphenylalanine; AOA, antiovulatory assay; Aph, 4-aminophenylalanine; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; Cpa, 4-chlorophenylalanine; Dbu, 2,4-diaminobutyric acid; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Dbu, 2,4-diaminooctanoic acid; DIC, diisopropycarbodiimide; Dpr, 2,3-diaminopropionic acid; EDT, ethanedithiol; Fmoc, fluorenylmethyloxycarbonyl; Fpa, 4-fluorophenylalanine; GnRH, gonadotropin releasing hormone; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; LH, luteinizing hormone; Nal, 3-(2′-naphthyl)-alanine; OFm, fluorenylmethyl ester; Pal, 3-(3′-pyridyl)-alanine; sc, subcutaneous; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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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 MH^+	MS[d] found MH^+	K_i (nM)[e]	AOA dose/rat in μg	AOA rats ovulating/total
	Cyclo(4−10) Antagonists									
1	cyclo(4−10)[Ac-DNal[1],DFpa[2],DTrp[3],Asp[4],DArg[6],(D/L)Agl[10]]GnRH	22	N/A	95[II]	80	1462.72	1462.7	1.3 ± 0.50	1.0	9/17
									2.5	0/4
2	cyclo (4−10)[Ac-DNal[1],DFpa[2],DTrp[3],Asp[4],DArg[6],Dpr[10]]GnRH	23	4.9 (30.6)	>98[II]	84	1476.74	1476.7	0.36 ± 0.11	1.0	2/10
									2.5	0/10
3	cyclo (4−10)[Ac-DNal[1],DFpa[2],DTrp[3],Asp[4],DArg[6],Dbu[10]]GnRH	24	4.11 (41.6)	96[I]	97	1490.75	1490.7	10 ± 1.2	250	4/4
									500	4/5
4	cyclo(4−10)[Ac-DNal[1],DFpa[2],DTrp[3],Glu[4],DArg[6],(D/L)Agl[10]]GnRH	23	4.4 (33)	>98[I]	>98	1476.73	1476.7	1.3 ± 0.17	10	7/13
									25	1/7
5	cyclo(4−10)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],DArg[6],(D/L)Agl[10]]GnRH	22	3.5 (30.0)	90[II]	>90	1440.67	1440.7	0.32 ± 0.04	10	2/8
									25	3/8
									50	2/9
6	cyclo (4−10)[Ac-Δ[3]Pro[1],DFpa[2],DTrp[3],Asp[4],MeTyr[5],DNal[6],Dpr[10]]GnRH	23	4.4 (43.2)	91[II]	N/A	1429.69	1429.7	0.74 ± 0.19	5.0	0/5
	Cyclo(5−8) Antagonists									
7	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],Cys[5],DArg[6],Cys[8]]GnRH	14	3.89 (30)	97[II]	70	1300.52	1300.6	2.4 ± 0.44	1000	4/6
8	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],DCys[5],DArg[6],DHCys[8],DAla[10]]GnRH	15	4.49 (41.4)	92[I]	>98	1366.57	1366.6	450 ± 44	250	4/4
9	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],DAsp[5],DArg[6],DLys[8],DAla[10]]GnRH	17	3.2 (33)	98[I]	98	1335.64	1335.6	130 ± 41	500	4/4
10	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],DGlu[5],DArg[6],DOrn[8],DAla[10]]GnRH	17	4.20 (28.2)	90[II]	>98	1349.66	1349.6	8.5 ± 1.7	250	5/5
									1000	0/4
11	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],DGlu[5],DArg[6],DLys[8],DAla[10]]GnRH	18	5.49 (37.2)	97[II]	N/A	1349.65	1349.8	720 ± 66	1000	4/4
12	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],DGlu[5],DArg[6],DLys[8],DAla[10]]GnRH	18	4.10 (28.8)	90[II]	91	1335.64	1335.5	120 ± 7.2	1000	2/3
13	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],Glu[5],DArg[6],Lys[8],DAla[10]]GnRH	18	4.3 (39.0)	88[II]	98	1387.67	1387.6	0.84 ± 0.22	25	4/5
									50	0/7
14	cyclo(5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5](βAla),DArg[6],(D or L)Agl[8],DAla[10]]GnRH	18	3.91 (29.4)	96[I]	>98	1364.63	1364.6	0.15 ± 0.02	10	3/8
									25	0/8
15	cyclo(5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5](βAla),DArg[6],(L or D)Agl[8],DAla[10]]GnRH	18	5.06 (29.4)	94[I]	96	1364.63	1364.6	1.8 ± 0.40	25	7/8
									100	1/5
16	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],Glu[5],DArg[6],Aph[8],DAla[10]]GnRH	18	6.13 (39.0)	98[I]	98	1421.66	1421.7	20 ± 6.2	500	5/5
17	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5],DArg[6],Aph[8],DAla[10]]GnRH	18	7.20 (30.6)	97[I]	>98	1383.64	1383.4	0.28 ± 0.03	100	4/4
									250	0/4
18	cyclo(5−8)[Glu[5],DNal[6],Lys[8]]GnRH	18	3.70 (33.6)	96[I]	97	1242.78	1242.8	1.0 ± 0.30	500	5/5
									1000	0/4
19	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[5],DPal[6],Amp[8],DAla[10]]GnRH	19	5.10 (31.8)	93[I]	94	1375.60	1375.4	1.3 ± 0.34	500	1/6
20	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],Glu[5],DArg[6],(D or L)Doc[8],DAla[10]]GnRH	20	4.00 (39.0)	98[I]	94	1415.70	1415.7	21 ± 2.4	250	3/3
									1000	0/4
21	cyclo(5−8)[Ac-DNal[1],DCpa[2],DTrp[3],Glu[5],DArg[6],(L or D)Doc[8],DAla[10]]GnRH	20	4.50 (39.0)	95[I]	95	1415.70	1415.7	2.3 ± 0.76	50	3/3
									250	0/4
22	cyclo(5−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5],DPal[6],Amp[8],DAla[10]]GnRH	20	5.00 (31.2)	97[I]	97	1389.62	1389.6	3.3 ± 0.50	100	7/7
									250	3/4
23	cyclo(5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[5](Gly),DPal[6],Amp[8],DAla[10]]GnRH	22	3.50 (33.0)	>98[I]	97	1432.62	1432.5	3.6 ± 0.52	50	7/7
									250	1/6
	Dicyclo(4−10/5−8) Antagonists									
24	dicyclo(4−10/5−8)[Ac-DNal[1],DCpa[2],DTrp[3],Asp[4],Glu[5],DArg[6],Lys[8],Dpr[10]]GnRH	23/18	4.50 (31.2)	83[I]	83	1412.66	1412.6	0.32 ± 0.06	2.5	9/10
									5.0	3/20
									10	0/10
25	dicyclo(4−10/5−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5],DNal[6],Lys[8],Dpr[10]]GnRH	23/18	5.81 (40.8)	>98[II]	N/A	1415.64	1415.6	1.3 ± 0.23	25	2/5
									50	2/8
									100	1/8

Table 1. (Continued)

no.	compound	ring size	RT isocratic conditions[a]	purity HPLC[b]	purity CZE[c]	MS[d] calc MH^+	MS[d] found MH^+	K_i (nM)[e]	AOA dose/rat in μg	AOA rats ovulating/ total
	Dicyclo(4−10/5−8) Antagonists (continued)									
26	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Asp[5](Gly),DArg[6],Dbu[8],Dpr[10]]GnRH	23/18	3.92 (35.4)	84[II]	85	1389.63	1389.9	0.35 ± 0.11	5.0	3/3
									10	4/6
									25	0/5
27	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Asp[5](Phe),DArg[6],Dbu[8],Dpr[10]]GnRH	23/18	4.22 (34.8)	90[I]	95	1479.67	1479.9	12 ± 3.4	25	4/4
28	dicyclo(4−10/5−8)[Asp[4],Glu[5],DNal[6],Lys[8],Dpr[10]]GnRH	23/18	4.33 (34.2)	98[II]	95	1281.63	1281.6	1.2 ± 0.18	500	3/8
29	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Asp[5](βAla),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	3.82 (33.0)	90[II]	85	1403.64	1403.6	0.37 ± 0.08	5.0	4/7
30	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	23/19	4.10 (31.2)	81[II]	80	1403.68	1403.7	0.16 ± 0.04	2.5	14/16
									5.0	2/8
									10	1/6
									25	0/3
31	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Sar),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.06 (31.2)	90[II]	82	1417.66	1417.7	0.20 ±0.03	10	4/9
32	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Phe),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.17 (36.0)	70[II]	77	1493.88	1493.9	0.23 ± 0.03	1.0	11/15
									2.5	3/10
									5.0	1/8)
									10	0/9)
33	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](DPhe),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.00 (37.2)	>98[II]	95	1493.69	1493.8	120 ± 36	100	5/5
									500	3/8
34	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Asp[5](Phe),DArg[6],Orn[8],Dpr[10]]GnRH	23/19	4.36 (37.8)	>98[II]	82	1493.69	1493.8	0.75 ± 0.04	2.5	3/3
									5.0	2/6
35	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Phe),DCit[6],Dbu[8],Dpr[10]]GnRH	23/19	3.92 (36.6)	90[II]	N/A	1494.87	1494.7	0.20 ± 0.03	10	5/5
									25	0/4
36	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Arg),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.42 (33.0)	71[I]	70	1502.72	1502.7	0.20 ± 0.05	0.5	5/5
									1.0	2/11
									2.5	1/8
37	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Nal),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.75 (37.2)	>98[II]	88	1543.70	1543.7	4.2 ± 0.64	2.5	5/5
									5.0	1/7
									10	0/4
38	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](His),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	4.05 (33.0)	90[II]	85	1483.68	1483.7	0.10 ± 0.04	1.0	4/4
									2.5	3/8
39	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Cpa),DArg[6],Dbu[8],Dpr[10]]GnRH	23/19	3.92 (33.0)	90[II]	>98	1527.65	1527.7	0.23 ± 0.04	2.5	7/8
									5.0	0/6
40	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Arg),DPal[6],Dbu[8],Dpr[10]]GnRH	23/19	3.5 (30)	90[II]	97	1494.68	1494.7	0.59 ± 0.08	10	3/3
									100	1/3
									250	0/6
41	dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5](Phe),DArg[6],Orn[8],Dpr[10]]GnRH	23/20	3.96 (36.6)	>98[II]	N/A	1507.70	1507.7	0.27 ± 0.06	10	5/5
									25	0/8

[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.

the curves were generated only with a limited number of key compounds.[29] Rats were administered doses of 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500, and 1000 μg/rat until a dose exhibiting partial inhibition was found. We observed, with few exceptions, that if a peptide was partially active at a given dose (i.e., 3−6 rats ovulated out of 10), it was fully active (100% inhibition) at twice that dose and inactive at half that dose (see data for analogues **24**, **26**, **30**, **32**, **36**, **37**, and **40** in this paper as examples). Therefore, in the present discussions we assumed that if a peptide was partially active, for example at 10 μg/rat, it was unlikely to be active at 5.0 μg/rat and likely to be fully active at 25 μg/rat. In a few cases which will be pointed out, a dose−response curve with a different slope was found for which we have no explanation (see data for analogues **5** and **25** in this paper as examples). Additionally, statistical analysis of assay variability requires that at any given dose of an antagonist, 8 out of 10, 9 out of 10, or 4 out of 5 ovulating rats indicated an inactive compound; conversely, 1 or 2 animals ovulating out of 8 or 10 was not statistically different from 100% inhibition. Therefore, when we state that a peptide inhibits ovulation at a given dose, we mean that the inhibition is statistically significant and that it is complete (100% unless stated otherwise). When a dose resulted in partial inhibition (3−6 rats ovulated out of 10), further testing was only rarely pursued. These assumptions, although not rigorous, were helpful in attributing relative potencies to a large number of GnRH antagonists and directed our research. The question remains whether the AOA is an adequate biological test for SAR studies meant to identify analogues with high affinity for the GnRH receptor, a necessary condition when it comes to identifying structural requirements for binding. In a recent article we reported both AOA and IC_{50} data on rat pituitary cell membranes using labeled histrelin as a tracer and found a few analogues that were impotent in vivo (no inhibition of ovulation at 5 μg/rat) and yet had high affinity (IC_{50} < 1.5 nM). On the other hand, we did not find potent analogues (100% inhibition of ovulation at 2.5 μg or less) with affinities (IC_{50}) > 3.0 nM.[30] In this same paper we reported the spread of affinities for compounds with very similar potencies in vivo to be on the order of one log unit, i.e., the free energy of a hydrogen bond. Similar observations are reported here and in two following papers in this series.

At the outset of this project we were concerned by the possible contamination of our low potency cyclic analogues with small amounts of very potent linear analogues; we became less concerned as new cyclic antagonists were more potent. Indeed, because some of the monocyclic and dicyclic analogues presented here are equipotent to the most potent linear analogues described in the literature, it is unlikely that they would be contaminated by linear intermediates or linear analogues that would have had to be present in easily detectable (HPLC, CZE, and MS) quantities.

Systematic and empirical amino acid substitutions used in the design and synthesis of GnRH agonists and antagonists have resulted in linear analogues with high potencies and relatively long duration of action.[7] Conformational analysis of GnRH, and the conclusion that it may assume a folded conformation with a type II′ β-bend encompassing residues 5 to 8, led to the synthesis of several homodetic and heterodetic cyclic competitive antagonists to GnRH.[1,32] One of these analogues, cyclo(1−10)[Δ^3Pro^1,D-Cpa^2,D-Trp^{3,6},MeLeu^7,βAla^{10}]-GnRH, was active albeit with low potency in vivo as well as low affinity in a cell membrane binding assay.[31,33] An extensive study of the conformation of this constrained analogue was carried out using both computer analysis[34,35] and NMR spectroscopy.[36] Results showed that, while GnRH exhibited a wide range of conformations, this analogue assumed one of two closely related conformations that was distinguishable by a *cis*−*trans* isomerization of the βAla-Δ^3Pro amide bond. This isomerization had little or no effect on the conformation of residues 2 to 9. In this model, the Ser^4 and Pro^9 C_α-hydrogens (roughly 2.5 Å apart) pointed toward each other, suggesting that further stabilization of this conformation could be achieved by the introduction of a bridge of appropriate length. This was best simulated by two or three methylene groups in a lactam bridge between residues 4 and 9.[35] However, we rapidly found that significantly more potent analogues than those having lactam bridges between residue 4 and 9 could be obtained by bridging residues 4 to 10 with an (Asp^4, Dpr^{10}) lactam ring.[1] The resultant, potent antagonist cyclo(4−10)[Ac-Δ^3Pro^1,D-Fpa^2,D-Trp^3,Asp^4,D-Nal^6,Dpr^{10}]-GnRH (full inhibition of ovulation at 5 μg/rat) was then selected for further conformational studies.[14,15]

The following two examples from Table 1 show that lactam ring size of the bridging element is critical for high affinity and in vivo potency as well as is the positioning of the ring amide with respect to the rest of the molecule. In the first case, cyclo(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Asp^4,D-Arg^6,D/L-Agl^{10}]GnRH (**1**) contains a 22-membered bridging element and is equipotent to the 23-membered lactam analogue **2** with about the same affinity; however, increasing the lactam ring size to 24 atoms (**3**) decreases affinity 20-fold and potency more than 500-fold as compared to that of **2**. As an example of the second case, cyclo(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Glu^4,D-Arg^6,Dpr^{10}]GnRH contains a 24-membered ring analogous to that of **3** and inhibits ovulation by 82.5% at 10 μg/rat, a 50-fold improvement in potency versus that of **3**.[1] On the other hand, cyclo-(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Glu^4,D-Arg^6,D/L-Agl^{10}]-GnRH (**4**, K_i = 1.3 nM) contains a 23-membered ring analogous to that of **2**, yet is about 25 times less potent than **2**. Therefore, we conclude that the positioning of the ring amide with respect to the rest of the molecule is critical, which is a distinction that could not be predicted by computer modeling because no model for the receptor is available.

Data also show that substitutions of [D-Fpa^2, D-Trp^3] by [D-Cpa^2, D-Pal^3], found to be optimal in a number of linear structures, has deleterious effects in the (Asp^4, D/L-Agl^{10})-bridged decapeptides (**5** is more than 25 times less potent than **1** in the AOA yet its K_i is only 3-fold lower). This discrepancy is worthwhile noting as it emphasizes that factors in addition to high affinity are required for biological efficacy. Additionally, it points to the fact that both substitutions are well tolerated with respect to the rest of the molecule in the active conformation. This is supported by data presented in the fourth paper of this series.[31] Molecular modeling

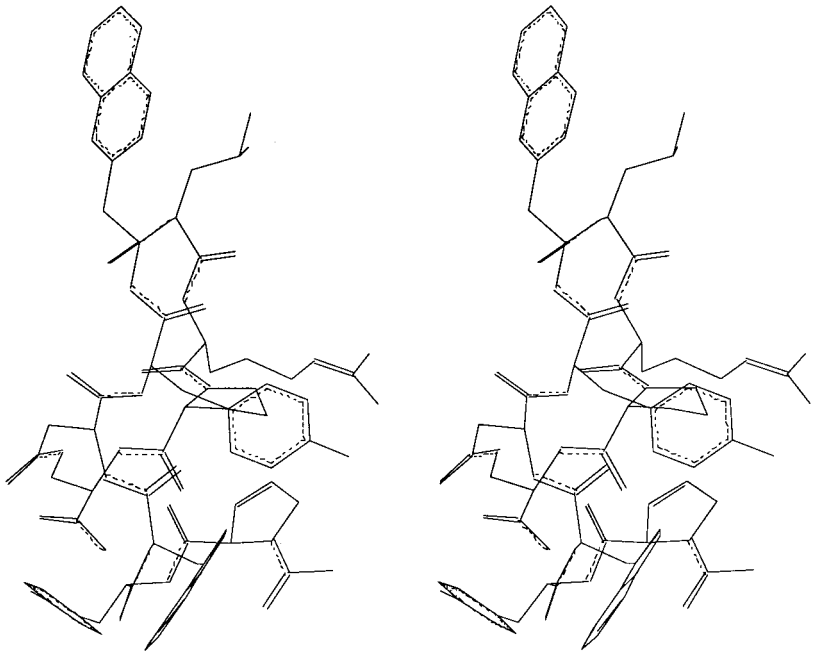

Figure 2. Stereo rendering of NMR-derived structure of cyclo(4−10)-[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH. The N-terminus is at the bottom in the near field.

provides further evidence suggesting that the potencies of **1**−**4** are modulated by the positioning of the 4−10 bridge amide. On the basis of the model of **2** from the NMR study,[14] the backbone of residues 5−8 lies in a fairly well-defined energetic minimum. As the size of the ring formed by residues 4−10 is varied to give **1**, **3**, or **4**, the bridge atoms rearrange with little perturbation of the backbone atoms of residues 5−8. Another complementarity in function similar to that reported above is illustrated by the fact that substitution of [DNal^1] by [Δ^3Pro^1] and [DArg^6] by [DNal^6] yielded **6**, which is fully active at 5 μg/rat or less when earlier studies in linear analogues had shown that the [Δ^3Pro^1] substitution was not well tolerated with the [DArg^6] substitution.[37] Although **6** (K_i = 0.74 nM) has an additional N-MeTyr at position 5, it is unlikely that this substitution, known to interfere with stabilization of the 5−8 type II′ β-turn, would enhance potency. In fact, it has been shown to decrease potency while increasing solubility and histamine releasing activity.[19]

The rationale for additional biocompatible bridging opportunities, exemplified by the synthesis of dicyclic and tricyclic analogues to be discussed in this and the following papers, was derived from the determination of the structure of the potent cyclo(4−10)[Ac-Δ^3-Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH antagonist.[14,15,17] The best defined feature of that molecule is the presence of a β-hairpin conformation within residues 5−8, stabilized by two transannular hydrogen bonds. This structure includes a type II′ β-turn around positions 6 and 7. Conformational averaging was observed in the (Asp^4, Dpr^{10}) bridge, most likely favored by the presence of two methylene groups. The linear N-terminal tripeptide was found to lie above the ring structure on the same side as the arginine side chain at position 8 and appeared to be somewhat structured, although some flexibility and sensitivity to the environment was observed for these three residues. In the absence of the structure of the GnRH receptor, considerable synthetic efforts were expended around this model to identify those favorable modifications yielding analogues with high affinity for rGnRHR and high potency as measured by ovulation inhibition in the cycling rat.[25]

According to our model of the structure of cyclo(4−10)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH (Figure 2), the most promising side chain to side chain interactions were between Tyr^5 and Arg^8. However, earlier SAR had definitively shown that both residues were sensitive to minor modifications in that [Phe^5]-GnRH,[38,39] [Ser^5]GnRH,[40] [Lys^8]GnRH,[41] and [NorVal^8]-GnRH[41] are ca. 50, 0.5, 2, and <0.5% as potent as GnRH, respectively. Despite those examples, the possibility of bridging the side chains of these two residues was investigated, based on the fact that the above analogues still showed full intrinsic activity, albeit low potency, and based on observations derived from the structures of two native molecules that contain amino acids at positions 5 and 8 other than those found in mammalian GnRH. Indeed, native chicken II GnRH (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 = [His^5, Trp^7, Tyr^8]GnRH) is as much as $^1/_3$ as potent as GnRH,[42] and *brain* tunicate I GnRH (pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly-NH_2) and its cyclic 5−8 analogue are biologically active,[43] suggesting that the structure of tunicate I GnRH is stabilized by ion pairing between the side chains of Asp^5 and Lys^8.

Our first bridging attempt was with a cystine bridge spanning residues 5 and 8 to yield **7** (K_i = 2.4 nM), which inhibited ovulation only partially (four out of six rats ovulated) at 1 mg/rat. Changing chirality of the bridgeheads and increasing the size of the ring by one methylene as in **8** also yielded an inactive analogue at 250 μg/rat with a much higher K_i (450 nM). Linking the side chains of residues 5 and 8 by means of an amide bond became the next priority. In the series in which the bridgeheads were D-amino acids, cyclo[DAsp^5, DLys^8] (**9,** K_i = 130 nM) and cyclo[DGlu^5, DOrn^8] (**10**, K_i = 8.5 nM) were found to be inactive at 500 μg/rat and achieve full inhibition of ovulation at 1000 μg/rat, respectively. Increasing the size of the ring with DXaa bridgeheads

by one methylene as in **11** and **12** yielded inactive analogues at 1 mg/rat with high K_i values (720 and 120 nM, respectively). The effect of substitution by all L-amino acids at positions 5 and 8 was then investigated, and the [Glu[5], Lys[8]] bridge in **13** (K_i = 0.84 nM) and the [Glu[5](βAla[5′]), Agl[8]] bridge in **14** (K_i = 0.15 nM) were found to yield analogues that inhibited ovulation at 50 and 25 μg/rat. The diastereomers **14** (K_i = 0.15 nM) and **15** (K_i = 1.8 nM) differed only in the chirality of the aminoglycine residue at position 8, and a good parallelism is seen between affinity and potency in the AOA. Although the absolute stereochemistry of aminoglycine has not yet been determined, it is noteworthy that **14** is 4 times more potent than **15** with a 10-fold difference in affinities. An aromatic ring in the bridge was introduced next with the use of 3-aminophenylalanine (Aph), and we found no increase in potency as **17** (K_i = 0.28 nM) is fully active at 250 μg/rat and **16**, with a much higher K_i (K_i = 20 nM), is inactive at twice that dose. Substitution of DTrp[3] by DPal[3] in **11** and **12** resulted in a 5-fold increase in affinity; the identical substitution in **16** and **17** resulted in a greater than 2-fold increase in potency and an 80-fold increase in affinity.

Realizing that the cyclo[Glu[5], Lys[8]] bridge was favorable in structures with the N-terminal tripeptide(s) optimized for GnRH antagonism (Ac-DNal-DCpa-DTrp of **13**), we hypothesized that the native tripeptide sequence pGlu-His-Trp would also be compatible with this constraint. We first tested **18** for agonism and found it inactive. Surprisingly, but with precedent,[44] **18** was found to have high affinity for rGnRHR (K_i = 1.0 nM) and to inhibit ovulation (no rat ovulated out of four), albeit at high doses (1 mg). Indeed, we had shown that structural constraints in a peptide may lead to partial agonism or competitive antagonism.[44] When the size of the 5−8 bridge was further increased using 4-aminomethylphenylalanine (4-Amp) as in **19** (K_i = 1.3 nM), **22** (K_i = 3.3 nM), and **23** (K_i = 3.6 nM) or using 2,8-diaminooctanoic acid (Doc) as in **20** (K_i = 21 nM) and **21** (K_i = 2.3 nM), analogues became 5 (**21**, **23**), 10 (**19**), or 20 (**20**) times less potent than **13** at inhibiting ovulation, following the order of potency defined by affinity (see Table 1).

Neither we (see above) nor Dutta et al.[2] could reach the level of potency with the cyclo(5−8) bridge that had been reached with the cyclo(4−10) bridges, although several analogues (**14** and **17**) had affinities for rGnRHR comparable to those of the most potent agonists and antagonists ever published. The high affinities of some of these analogues and their comparatively poor potency in vivo suggest that they may be unstable, readily eliminated or unable to distribute from the site of injection. To test the compatibility of the cyclo(5−8) and cyclo(4−10) bridges in the same molecule and generate even more constrained analogues for structural studies, we synthesized a number of dicyclic analogues.

Dicyclic analogues are presented in Table 1 in order of increasing size of the 5−8 ring. While our study was in progress, Dutta et al.[2] identified an 18-atom [Glu[5], Lys[8]] bridge which yielded their most potent analogue. Since that analogue was at least 50-fold less potent than our best 4−10 cyclic analogue (such as **2**), the effect of a dicyclic (5−8/4−10) configuration was explored. Therefore, improvements in the 5−8 bridge composition were attempted while retaining a constant 4−10 cycle. Our data (**13** is fully active at 50 μg/rat) corroborated that of Dutta et al.[2] and suggested that the [Glu[5], Lys[8]] and Glu[5](β-Ala),D/LAgl[8]) bridges were optimal. The first bridge was therefore used in the dicyclic analogues **24** and **25.** To our surprise, although not as potent as the corresponding and closely related monocyclic 4−10 analogue **2** (DFpa and DCpa at position 2 are considered equivalent), **24** had high affinity (K_i = 0.32 nM) comparable to those of **13** and **2**, although it was approximately 5 times more potent than **13** and 4 times less potent than **2** in vivo. Compound **24** was therefore chosen for high resolution NMR investigation[17] to complement our earlier structural work on **2**.[14,15] The observed structures of **2** and **24** are very similar, characterized by a RMS deviation over the position of the backbone atoms of the superposed structures of less than 0.5 Å. Our conclusions differed from those of Paul et al.,[45] who reported a conformational search and molecular dynamics study of cyclo(5−8)[Ac-DPhe[1,2],DTrp[3],Glu[5],DArg[6],Lys[8],DAla[10]]GnRH described originally by Dutta et al.[2] The dominating features of this molecule are an N-terminal tripeptide that lies below the plane of the other residues, and the presence of a β-turn involving residues 3−6 that was never seen in any of our analogues.[14,15] Conformational analyses were also conducted for **24** as previously described.[17] Because the Asp[4]-Dpr[10] lactam forming the 4−10 bridge is highly planar, the Cα−Cα distances of residues 4 and 10 remain about 5.2 Å apart through most of these trajectories. However, if that bond is broken by the incorporation of the naturally occurring residues in positions 4 and 10, the Cα−Cα distances are no longer constrained. In a short (2 ps) 300 K molecular dynamics trajectory starting with the minimum energy structure of **24**, which had been mutated into **13**, the 4−10 Cα−Cα distance spanned the range 4.6−5.9 Å. With **13** and **24** having about the same affinity for rGnRHR, we propose that while the monocyclic (5−8) compound **13** is quite flexible and the incorporation of the second bridge results in a decrease in flexibility, such a constraint has little effect on the ability of the receptor to recognize and bind these ligands. Compound **25**, with the same (4−10/5−8) scaffold as **24** but different substitutions at positions 3 (DPal) and 6 (DNal), had a lower affinity (K_i = 1.3 nM) and was more than 10 times less potent. This agrees with earlier observations that [Ac-DNal[1], DNal[6]] analogues are generally less potent than [Ac-DNal[1], DArg[6]] analogues,[46] an effect that is probably independent of the substitution at position 3 (DTrp versus DPal).

While retaining the size of the ring at 18 atoms, the possibility of adding an amide bond in the cycle was also investigated. From data shown here and in Rivier et al.,[1] it was clear that the amide bond in the (4−10) cycle played an important role during binding to the receptor; it was therefore conceivable that an additional amide bond in the (5−8) cycle as in **14** and **15** would increase chances for additional contact points with the receptor. This could be achieved by shortening the side chains of residues 5 and 8 while inserting an additional residue such as glycine in **26** (K_i = 0.35 nM) or Phe in **27** (K_i = 12 nM), forming an undecapeptide. This approach

opened the possibility of introducing another functional group that could hypothetically replace either of the two original residues (Tyr[5] and Arg[8]), which had been substituted for the sake of constructing the bridge. While **26** was ca. 2.5 times less potent than **24**, introduction of the phenylalanine side chain in **27** resulted in significant loss of affinity and potency since it was inactive at 25 μg/rat. In view of the relatively high potencies of **24** (with a DArg[6]) and **25** (with a DNal[6]) and the precedence that those potencies are influenced by the nature of the N-terminal tripeptide, a chimera of the native hydrophilic tripeptide sequence pGlu-His-Trp and **25** yielded **28** with the same affinity (K_i = 1.2 nM) and a 20-fold loss of potency (partial inhibition of ovulation at 500 μg/rat) as compared to that of **25**.

The compatibility of 19-member rings in GnRH antagonists was then explored. Compound **29** is homologous to **26**, has matching affinity (K_i = 0.37 and 0.35 nM, respectively), and is approximately 2 times more potent in vivo. Shifting the position of the lactam amide bond from β[Asp[5]] in **29** to γ[Glu[5]] in **30** and substituting [βAla] by [Gly] influenced affinity (2-fold increase) and potency only marginally, as **30** inhibited ovulation by 75% and **29** by 43% at 5.0 μg/rat. Interestingly, methylation of the γ-amide nitrogen in **31** (Sar) resulted in some loss of potency (ca. 2-fold) but not affinity (K_i = 0.20 nM). Having identified a scaffold that allowed an eleventh residue in the GnRH antagonist sequence with retention of biopotency, we decided to explore whether the nature of the side chain of this amino acid at position 5′ would influence potency in vivo. Unlike the [DPhe[5′]] in **33** (K_i = 120 nM), the [Phe[5′]] in **32** (K_i = 0.23 nM) retained high potency (88% inhibition at 5.0 μg/rat); the same substitution [Phe[5′]] in **34**, where the bridgeheads are [Asp[5]] and [Orn[8]], was marginally less favorable (66% inhibition at 5 μg/rat) with parallel loss of affinity (K_i = 0.75 nM). In fact, **32** was the first dicyclic analogue of GnRH that showed statistically significant inhibition of ovulation at 2.5 μg/rat.

On the basis of the fact that DCit at position 6[47] is among the favorable (along with [DNal[6]] and [DArg[6]]) substitutions in GnRH antagonists, we substituted this amino acid in **32** to yield **35.** As expected, very high affinity (K_i = 0.20 nM) was retained, yet **35** is about 3-fold less potent (full inhibition of ovulation at 25 μg/rat but not at 10 μg) than **32**. Potent antagonists of GnRH had also been obtained with a DArg at position 6 or an Arg at position 5 with a hydrophobic D-residue at position 6;[48] we therefore hypothesized that the additional guanidino function of an Arg at position 5′ would be favorable. Analogue **36**, whereby Arg was introduced at position 5′ along with DArg at position 6, was found to be the most potent dicyclic antagonist of GnRH, by inhibiting ovulation by 82% at 1 μg/rat and having high affinity (K_i = 0.20 nM) for rGnRHR. We acknowledge that the preparations of both **32** and **36** were estimated to be only 70% pure. It is, however, doubtful that the potencies and high affinities of these analogues are due to any of the multiple closely related impurities for reasons stated earlier. It is noteworthy that some of these dicyclic analogues chromatographed very poorly, giving rise to broad, yet symmetrical, chromatographic profiles that made preparative purification and subsequent analysis quite challenging. We hypothesize that a slow conformational equilibrium is responsible for poor resolution. We have, in some cases, remedied this problem in CZE using mixed organic/aqueous buffers.[49]

Three additional compounds with Nal (**37**), His (**38**), and Cpa (**39**) at position 5′ were synthesized to evaluate the level of receptor specificity at that position. All three antagonists were found to be more than twice less potent than **36** with poor affinity of **37** and high affinities of **38** and **39.**

Although a rigorous description of the multiple biologically active conformations of GnRH antagonists remains unavailable (there are hundreds of GnRH antagonists with similarly high affinities for the GnRH receptor), the physicochemical studies that have been conducted to date begin to allow the description of the SAR in three-dimensional terms. The high resolution NMR investigations of the monocyclic (4−10) antagonist **2**[14] and the dicyclic (4−10/5−8) antagonist **24**[17] suggest common features that are shared by these potent antagonists. These include a turn (type II′ β) involving residues 5−8 and some local structure in the N-terminal tripeptide. As a consequence of this type of turn, the side chains of residues 5 and 8 point out and away from the same face of the plane in which the backbone atoms of the residues of the turn lie. Consequently, bridging of residues 5 and 8 can occur without severe disruption of the turn topography. However, our subsequent investigation of the NMR structure 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[16] revealed a type I′ β-turn at residues 6−7. The picture that emerges of the potent GnRH antagonists' conformations depends not so much on a specific turn type in the region of residues 5−8 but rather on the presentation of a turn in combination with the proper orientation of backbone and side chain functionalities. It appears that the receptor is relatively insensitive to the chemical nature of the side chain of the inserted residue 5′ in the (5,5′−8) bridge; compare the affinity of **32** with that of **36**, which differ by a Phe or Arg at 5′, respectively. In fact, of the 17 compounds (**14**, **15**, **26**−**27**, **29**−**41**) in which the size of the 5−8 bridge is incrementally increased, 12 induce full inhibition of ovulation at 25 μg/rat or less and have K_i values < 0.5 nM. This is consistent with the observation that substitutions at residue 5 (Arg and substituted Phe) and, to a lesser extent, at residue 8 (Arg, diethyl-homo-Arg and ILys) may yield very potent compounds. More enigmatic is the influence of DXaa[6] on potency since **36** presents DArg[6] and inhibits ovulation at >2.5 μg/rat; while **40**, with DPal[6], is ca. 100 times less potent. Although the role of the N-terminal tripeptide in the potent antagonists' conformations is less obvious, owing to the lack of detailed structural information, direct interaction between this region and residues 5 and/or 8 is a clear feature of most molecular modeling studies of the constrained antagonists. We describe in the following two papers specific analogues that were designed and synthesized to uncover such interactions.

In conclusion, a novel constraint [cyclo(Asp[4], D/LAgl[10])] was identified in **1** that is shorter by one methylene group than the previously identified cyclo(Asp[4], Dpr[10]) constraint used in **2,** the structure of which was

extensively studied by NMR and computer simulations.[1,14] Both **1** and **2** have high affinities for rGnRHR and inhibit ovulation at 2.5 μg/rat. Although **1** and **2** are essentially equipotent, it is worthwhile mentioning that **1** is a mixture of two diastereomers with the strong possibility that one of them is less potent than the other. Using aminoglycine as a bridgehead at position 8, a novel constraint [Glu^5(βAla), D or LAgl^8] was found in **14** that is identical in size to the (Glu^5, Lys^8) cycle originally identified in **13**. Although **14** is twice as potent as **13**, we have not been able to identify in this series (**7**−**23**) antagonists that would be fully active at less than 10 μg/rat. On the other hand, four dicyclic analogues (**32**, **36**, **37**, and **39**) have been identified that inhibit ovulation at ca. 5 μg/rat or less, and **38** inhibits ovulation only partially (63%) at 2.5 μg/rat. With the exception of **37**, all analogues in this series have K_i values < 0.3 nM. The relatively high in vivo potency of **37** is unexpected given its relatively weak binding affinity for GnRHR (K_i = 4.2 nM). These five analogues have an inserted amino acid in the ring (5′ position) which encompasses 19 atoms as compared to 18 atoms in the most potent cyclo(5−8)-containing analogues **13**-**15**. These five dicyclo(4−10/5−8) analogues have binding affinities similar to those of the monocyclic (5−8) analogues, which suggests that the (4−10) bridge is not critical for maintenance of residues 4 to 10 in conformations favorable for receptor interaction and that the 5−8 cycle, by itself, is able to induce the optimal structure for receptor binding. The fact that this does not translate into equivalent in vivo potencies (these five analogues are at least five times more potent than the monocyclic (5−8) analogues) may be due to unique pharmacokinetic properties of the monocyclic analogues. Such a constraint has proven critical in preventing **18**, a weak antagonist despite the pGlu-His-Trp N-terminus (a hallmark of GnRH agonists), from activating signal transduction by the receptor. Derivatives of this study were extensive NMR investigations of [Ac-Δ^3-Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH[14,15] (analogous to **2**) and **24**[17] that led to the identification of a β-hairpin turn conformation between residues 5−8, which is stabilized by two transannular hydrogen bonds. These structures also include a type II′ β-turn around positions 6 and 7. The tripeptide N-terminus is located above the ring on the same side as the (5−8) bridge and appears to be somewhat structured. The NMR study of **30**[16] shows a somewhat different preferred conformation (stabilized by several hydrogen bonds) containing a type II β-turn around residues 5−6, nested with a type I′ β-turn around residues 6−7, and a type II β-turn-like structure involving residue 9 and the side chain of residue 10.

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 Assoc. (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 Laboratories, Richmond, CA) as previously published.[50] 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-Z), 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[19,20,24] 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[51] 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.[52] 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.[53] 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 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.[22,23]

As a final step, all peptides were rechromatographed in a 0.1% TFA solution with 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.

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). 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.[49,54]

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 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[55−57] were harvested by striking the culture flask against the palm of the hand, resuspended in 5% sucrose, and homogenized 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,D-Ala^6,NMeLeu^7,Pro^9-NHEt]GnRH, ∼100 000 cpm)[13] 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).

6. Antiovulatory Assay (AOA). The AOA was carried out as described by Corbin and Beattie.[25] 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 were expressed in terms of number of rats ovulating over the number of animals receiving excipient for each experiment. Results were 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. For example, if a dose of 5 μg/rat is required to obtain inhibition in 7 rats out of 10 (70% inhibition) for a given peptide, and if a 25 μg dose of another peptide is required 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 since 5 times less material of the former is needed 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.[58,59] Molecular modeling and visualization were performed using Insight II (MSI, Inc., San Diego, CA) on a Silicon Graphics Iris Crimson workstation.

Cyclo(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Asp^4,D-Arg^6,(D/L)-Agl^{10}]-GnRH (1). Analogue **1** was manually synthesized on 1.0 g of 1.0 mequiv/g-substituted MBHA resin using DIC as the coupling reagent in DCM. After the solid phase synthesis was completed, the peptide-resin (2.6 g) was cleaved and deprotected in HF (40 min, 0 °C), as described, to yield 0.93 g (0.60 mmol) of crude linear peptide.

The (4−10) cyclization step was carried out on the crude intermediate. Diphenylphosphoryl azide (DPPA, 0.38 mL, 1.7 mmol, Aldrich) and diisopropylethylamine (0.34 mL, 2 mmol, Fluka) were added to the peptide (0.90 mg, 0.58 mmol) dissolved in 1 L of DMF at −5 °C. After 24 h at −10 °C, the DMF was removed by rotary evaporation and the residue was reconstituted in 50% CH_3CN/H_2O (200 mL), shell-frozen, and lyophilized to a powder. Crude, cyclized peptide was purified by preparative HPLC in TEAP 2.25, TEAP 5.0, and 0.1% TFA to yield 32 mg (18 μmol, 2%) of **1**.

Analogue **5** ($[\alpha]_D = -45°$) was obtained using this general procedure in a comparable yield.

Cyclo(4−10)[Ac-D-Nal^1,D-Fpa^2,D-Trp^3,Glu^4,D-Arg^6,(D/L)-Agl^{10}]-GnRH (4). Resin-bound peptide [Boc-Glu^4(γ-OFm),Tyr^5-(2BrZ),D-Arg^6(Tos),Arg^8(Tos), (D/L)-Agl^{10}(α'-Fmoc)]-GnRH(4−10)-MBHA was first assembled manually from 1 g of 1.0 mequiv/g-substituted MBHA resin. The Glu(γ-OFm) and Agl(α'-Fmoc) side chains were deprotected with 20% piperidine in NMP (2 × 10 min). Cyclization then proceeded at pH 9 in NMP with a 3-fold molar excess of BOP/DIPEA (1:4) for 24 h. The procedure was repeated for 18 h with fresh reagents, at which time the Kaiser test was negative. The peptide chain was then completed in the usual fashion. Peptide-resin (2.5 g) was cleaved and deprotected in HF (90 min, 0 °C), as described, to yield 1.30 g (0.80 mmol) of crude peptide that was purified in TEAP 2.25, TEAP 5.6, and 0.1% TFA, as described, to yield 170 mg (94 μmol, 9%) of **4** ($[\alpha]_D = -20°$).

Analogues **9** ($[\alpha]_D = -56°$), **10** ($[\alpha]_D = -29°$), **11** (cyclization conditions: TBTU/DIPEA 1:3, 22 °C, 40 h), **12** ($[\alpha]_D = -26°$, $c = 0.62$), **16**, **18**, and **19** were obtained using this general procedure in comparable yields.

Cyclo(5−8)[Ac-D-Nal^1,D-Cpa^2,D-Trp^3,D-Cys^5,D-Arg^6,D-HCys^8,D-Ala^{10}]GnRH (8). The peptide was synthesized automatically on 2 g of 0.76 mequiv/g-substituted MBHA resin to yield 4 g of completed peptide-resin. Peptide-resin was cleaved and deprotected in HF (40 min, 0 °C), as described above. Peptide was extracted from the resin with 200 mL of CH_3CN/H_2O and acidified with 4 mL of AcOH. The solution was diluted to 4 L with 30% CH_3CN/H_2O, adjusted to pH 6.8 with 28% NH_4OH, and stirred at 22 °C for 2 days. At that time a negative Elman test indicated complete cyclization, and the solution was directly loaded onto the preparative HPLC for purification in TEAP 2.25 and 0.1% TFA. Final yield of lyophilized peptide **8** was 88 mg (56 μmol, 4%, $[\alpha]_D = -5°$).

Analogue **7** ($[\alpha]_D = -5°$; $c = 0.39$) was obtained using this general procedure in a comparable yield.

Cyclo(5−8)[Ac-D-Nal^1,D-Cpa^2,D-Trp^3,Glu^5,D-Arg^6,Lys^8,D-Ala^{10}]-GnRH (13). Peptide **13** was automatically synthesized on 1.5 g of 0.76 mequiv/g-substituted MBHA resin using DIC as the coupling reagent in DCM. After the solid phase synthesis was completed, the resin was prepared for (5−8) lactam formation

by first deprotecting the side chain groups of Glu[5](γ-OFm) and Lys[8](ϵ-Fmoc) with 20% piperidine in DMF (2 × 15 min). Cyclization then proceeded at pH 9 in 20 mL of DMF with a 2.5 molar excess of BOP/HOBt/DIPEA (1:1:3) at 65 °C in an orbital shaker for 65 h. After drying, the peptide-resin (2.8 g) was cleaved and deprotected in HF (90 min, 0 °C). After workup and extraction with 10% CH_3CN/H_2O, the solution of crude, cyclized peptide was purified directly by preparative HPLC in TEAP 2.25 and 0.1% TFA to yield 100 mg (63 μmol, 6%) of **13** ($[\alpha]_D = -32°$).

Analogues **3** ($[\alpha]_D = -25°$), **17**, **20** ($[\alpha]_D = -18°$), **21** (separated from **20** during HPLC purification), **22**, and **23** were obtained using this general procedure.

Cyclo(5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5](β-Ala[5′]), DArg[6],(D or L)Agl[8], DAla[10]]GnRH (14) and cyclo-(5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Glu[5](β-Ala[5′]),DArg[6],(L or D)Agl[8],DAla[10]]GnRH (15). The peptides were synthesized manually on 1 g of 0.4 mequiv/g-substituted MBHA resin. The Fmoc-protecting group of [Boc-DArg[6],D/L-Agl[8](Fmoc),DAla[10]]-GnRH(6−10)-MBHA was removed with 30% piperidine in DMF (2 × 10 min), whereupon N_α-Fmoc-β-Ala was coupled to the freed amino function of Agl using HBTU in DCM/DMF and 3 equiv of TEA. The N-terminal Boc group of the growing peptide chain was deblocked with TFA as described, and Boc-Glu(γ-OFm) was coupled with DIC/DCM. The OFm/Fmoc groups of Glu and β-Ala were removed with piperidine as above, and cyclization proceeded in DMF with HBTU/3 equiv TEA for 2 h to give a negative Kaiser test. Completion of the peptide chain yielded the final peptide-resin (3.2 g). After HF cleavage and purification in TEAP 2.25, TEAP 5.4, and 0.1% TFA, the two diastereoisomeric Agl-peptides **14** ($[\alpha]_D = -10°$) and **15** ($[\alpha]_D = -34°$) were separated using HPLC and obtained in 8 mg (5 μmol, 3%) and 22 mg (14 μmol, 7%) yields, respectively.

Dicyclo(4−10/5,5′−8)[Ac-DNal[1],DCpa[2],DPal[3],Asp[4],Glu[5]-(DPhe[5′]),DArg[6],Dbu[8],Dpr[10]]GnRH (33). Analogue **33** was assembled automatically on 2.0 g of 0.76 mequiv/g-substituted MBHA resin and cyclized in two steps. Extension of the side chain of Dbu in position 8 with DPhe was achieved prior to the introduction of Boc-Glu(γ-OFm) at position 5. The first cyclization (5−8) was performed on the solid phase after deprotection of the OFm/Fmoc groups by 25% piperidine in DMF (2 × 10 min). The lactam was formed using a 3-fold excess of BOP in the presence of excess DIPEA in DMF over a 15 h period at 65 °C. Completion of the peptide chain yielded 3.9 g of cyclo (5−8) peptide-resin that was prepared for the 4−10 cyclization step.

Hydrazinolysis of the aspartic acid β-benzyl ester was carried out at the last stage of the synthesis with a large excess of anhydrous hydrazine in DMF for 4 days. After washing with DMF, MeOH, and DCM and drying, the protected peptide hydrazide-MBHA resin was obtained. The cyclo(5,5′−8)Ac-DNal-DCpa-DPal-Asp($NHNH_2$)-Glu-DArg(Tos)-Leu-Dbu(DPhe)-Pro-Dpr(Z)-MBHA-resin was treated with liquid HF (50 mL) at 0 °C (40 min) in the presence of anisole (5 mL). The HF was removed from the reaction vessel under vacuum, and the solid residue was triturated in anhydrous ether (100 mL) and filtered. The peptide hydrazide was extracted from the resin with 50% aqueous acetonitrile and lyophilized to yield a fluffy, crude peptide hydrazide (1.6 g) which exhibited a major component (>75%) by high performance liquid chromatography (HPLC).

Crude peptide hydrazide (1.6 g, 0.9 mmol) was dissolved in dry DMF (40 mL) at −20 °C and acidified with 4 N HCl in dioxane (1.4 mL, 5.6 mmol). After 10 min, isoamyl nitrite was added in three aliquots (0.30 mL, 2.2 mmol total) with stirring over 20 min. Stirring at −20 °C was continued for 3 h. The solution of peptide azide was diluted with DMF (800 mL precooled to −20 °C), and the pH was adjusted to 7 with TEA. The solution was stored at −5 °C (72 h). The solvent was evaporated under vacuum to yield crude cyclic peptide which was purified directly in TEAP 2.25 and 0.1% TFA to yield 78 mg (50 μmol, 3%) of cyclic peptide **33**.

This two step cyclization procedure was utilized for dicyclic analogues **24** ($[\alpha]_D = -52°$, $c = 0.64$), **25**, **26** ($[\alpha]_D = -48°$), **27**, **28**, **29**, **30** ($[\alpha]_D = -36°$, $c = 0.68$), **31**, **32** ($[\alpha]_D = -52°$), **34**, **35**, **36** ($[\alpha]_D = -42°$, $c = 0.72$), **37**−**39**, **40** (3.6 equiv of isoamyl nitrite), and **41** with comparable yields.

The (4−10) cyclization method of **33** above was utilized for monocyclic (4−10) analogues **2** ($[\alpha]_D = -20°$) and **6** with similar results.

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, Georgia Morgan, Mary Tam, Susanne Johnson, Duane Pantoja, Charleen Miller, Greg Reinhart, Qiu Xie, and Yaira Haas for excellent technical assistance and Debbie Doan for manuscript preparation. We are indebted to Dr. T. Lee and Dr. 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.

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JM990115H