Design of Monocyclic (1−3) and Dicyclic (1−3/4−10) Gonadotropin Releasing Hormone (GnRH) Antagonists†

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Careful analysis of the NMR structures of cyclo(4−10)[Ac-Δ^{3}Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]-GnRH, dicyclo(4−10/5−8)[Ac-DNal^1,DCpa^2,DTrp^3, Asp^4,Glu^5,DArg^6,Lys^8,Dpr^{10}]GnRH, and 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 showed that, in the N-terminal tripeptide, a type II β-turn around residues 1 and 2 was probable along with a γ-turn around DTrp^3/DPal^3. This suggested the possibility of constraining the N-terminus by the introduction of a cyclo(1−3) scaffold. Optimization of ring size and composition led to the discovery of cyclo(1−3)[Ac-DAsp^1,DCpa^2,DLys^3,DNal^6,DAla^{10}]GnRH (**5**, K_i = 0.82 nM), cyclo(1,1′−3)[Ac-DAsp^1(Gly),DCpa^2,DOrn^3,DNal^6,DAla^{10}]GnRH (**13**, K_i = 0.34 nM), cyclo(1,1′−3)[Ac-DAsp^1-(Gly),DCpa^2,DLys^3,DNal^6,DAla^{10}]GnRH (**20**, K_i = 0.14 nM), and cyclo(1,1′−3)[Ac-DAsp^1(βAla), DCpa^2,DOrn^3,DNal^6,DAla^{10}]GnRH (**21**, K_i = 0.17 nM), which inhibited ovulation significantly at doses equal to or lower than 25 μg/rat. These results were particularly unexpected in view of the critical role(s) originally ascribed to the side chains of residues 1 and 3.[1] Other closely related analogues, such as those where the [DAsp^1(βAla), DOrn^3] cycle of **21** was changed to [DOrn^1(βAla), DAsp^3] of cyclo(1,1′−3)[Ac-DOrn^1(βAla),DCpa^2,DAsp^3,DNal^6,DAla^{10}]GnRH (**22**, K_i = 2.2 nM) or where the size of the cycle was conserved and [DAsp^1(βAla), DOrn^3] was replaced by [DGlu^1(Gly), DOrn^3] as in cyclo(1,1′−3)[Ac-DGlu^1(Gly),DCpa^2,DOrn^3,DNal^6,DAla^{10}]GnRH (**23**, K_i = 4.2 nM), were approximately 100 and 25 times less potent in vivo, respectively. Analogues with ring sizes of 18 {cyclo(1,1′−3)[Ac-DGlu^1(Gly),DCpa^2,DLys^3,DNal^6,DAla^{10}]GnRH (**24**)} and 19 {cyclo(1,1′−3)[Ac-DGlu^1(βAla),DCpa^2,DLys^3,DNal^6,DAla^{10}]GnRH (**25**)} atoms were also less potent than **21** with slightly higher K_i values (1.5 and 2.2 nM, respectively). These results suggested that the N-terminal tripeptide was likely to assume a folded conformation favoring the close proximity of the side chains of residues 1 and 3. The dicyclic analogue dicyclo(1−3/4−10)[Ac-DAsp^1,DCpa^2,DLys^3,Asp^4,DNal^6,Dpr^{10}]GnRH (**26**) was fully active at 500 μg, with a K_i value of 1 nM. The in vivo potency of **26** was at least 10-fold less than that of monocyclic cyclo(1−3)[Ac-DAsp^1,DCpa^2,DLys^3,DNal^6,DAla^{10}]GnRH (**5**); this suggested the existence of unfavorable interactions between the now optimized and constrained (1−3) and (4−10) cyclic moieties that must interact as originally hypothesized. Tricyclo(1−3/4−10/5−8)[Ac-DGlu^1,DCpa^2, DLys^3,Asp^4,Glu^5,DNal^6,Lys^8,Dpr^{10}] GnRH (**27**) was inactive at 500 μg/rat with a corresponding low affinity (K_i = 4.6 nM) when compared to those of the most potent analogues (K_i < 0.5 nM).

Introduction

The rationale for the synthesis of constrained gonadotropin releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) antagonists was discussed in the first paper in this series. In summary, it was hypothesized that constrained antagonists of the decapeptide amide characterized by Matsuo et al.[2] and Burgus et al.[3] might be orally active contraceptives. Additionally, their conformational stability would allow analysis of their conformation through structural evaluation in solution using NMR spectroscopy. The ultimate goal would be a template useful for the design of nonpeptide GnRH-like drugs. Like many peptide hormones, GnRH is a highly flexible molecule that exists in solution as an equilibrium mixture of multiple conformers.[4−6] Nonetheless, working models of its preferred conformation that may coincide with its receptor-bound conformation have been proposed. For example, a feature common to many models is a β-turn at Gly^6-Leu^7 originally suggested by empirical conformational energy calculations.[7,8] Support for the presence of this turn has been provided by D-amino acid substitution studies,[9,10] an analogue incorporating *N*-methyl Leu^7,[10,11] 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.[10] Evidence for the presence of such a turn in bioactive GnRH analogues was provided by the study of cyclic GnRH antagonists reviewed in part in the previous paper.[12]

Two clues to the conformation of potent GnRH antagonists were found from the NMR studies[13−15] of the dicyclo(4−10/5−8)[Ac-DNal^1,DCpa^2,DTrp^3,Asp^4,Glu^5, DArg^6,Lys^8,Dpr^{10}]GnRH and 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.[12] First, we observed that the N-terminal tripeptide exhibited some structural stability in solution and, second, that it could assume two preferred loci in space relative to the monocyclic or dicylic C-terminal heptapeptides

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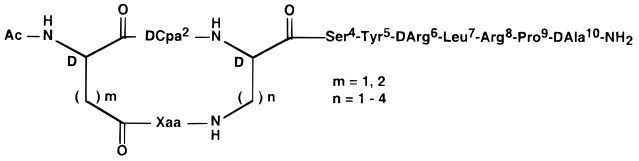
(i.e., above or below the plane defined by the (4−10) cycle, corresponding to the same side as that of the side chains of residues 5 and 8 and, therefore, of the (5−8) bridge, or the opposite side, respectively). Similarly, in fitting the observed NMR data of the cyclic(4−10) antagonist[16] via molecular modeling techniques, we had described two conformational families differing in the position of the N-terminal tripeptide with respect to the cyclic heptapeptide as either above (i.e., "up," on the same side of the ring as the Arg^8 side chain) or below (i.e., "down," opposite side with respect to Arg^8 side chain). The in vacuo energies of representative energy-minimized conformers of these families suggested a clear preference for the down structure; this conclusion for the in vacuo case was subsequently supported by extensive theoretical calculations employing the method of local states.[17] However, solvated molecular mechanics studies showed the tail-up structure to be much more favorably solvated. The proper balance of solvation and entropic terms could be achieved by slight modulation of the force field describing the solvent−solute interaction to shift the free-energy of either conformer to a more negative, hence favored, value. The NMR data did not yield any conclusive data to either validate or rule out either position of the N-terminal tail. We hypothesized that a constrained N-terminal tripeptide in a potent (1−3/4−10) dicyclic analogue would have limited rotational freedom with respect to the C-terminal cycle, and as a result, one of the two main conformations of the N-terminal tail would be favored and detectable by NMR. We present here the results of an extensive synthetic effort aimed at identifying potent (1−3) cyclic antagonists of GnRH of the type shown in the following bridging scheme.



Bridging scheme of most analogues presented in **Table 1**.

Results and Discussion

Most peptides were synthesized by the solid phase method on a methyl benzhydrylamine resin at room temperature using techniques previously reported by this laboratory.[12,18] However, three analogues (**8**, **9**, **17**) were synthesized using high temperature protocols, which validates this methodology for more complex structures than those that were presented in the original papers.[19−21] Commercially available trifunctional amino acids used for bridge formation were orthogonally protected using the acid labile Boc and the base labile Fmoc and OFm protecting groups [Boc-Xaa-(Fmoc) and Boc-Xbb(OFm)].[12,22] Although cyclization on the resin uses simpler protocols than those followed for azide coupling in solution, early efforts to achieve such cycles between residues 4−10 with carbodiimides and different additives seemed to fail. It was assumed that this was due to steric effects resulting from the proximity of residue 10 to the support. Only in a few cases did we introduce the (4−10) cycle with BOP. In most cases, residues 4 to 10 were cyclized in three steps by first generating the hydrazide of the β-carboxyl of the aspartic at position 4, protected as the benzyl ester, by stirring the peptide resin with hydrazine at 20 °C for 100 h in DMF.[23] The peptides were then cleaved with HF and concomitantly deprotected. The peptide-hydrazide intermediate was converted to the corresponding azide using isoamyl nitrite and HCl in dioxane and reacted in DMF under dilute conditions with the β-amino group of Dpr^{10} to give the crude cyclized peptides. Purification was carried out by HPLC.[24,25] The majority of peptides were greater than 90% pure as determined by HPLC and CZE (Table 1). Amino acid analyses,[26] including those of Pal, Cpa, Fpa, Nal, and Dpr, were consistent with expected results (not shown). Calculated values for protonated molecular ions were within 100 ppm of those obtained using LSI mass spectrometry (see Table 1).

Biological evaluation was conducted using an in vivo antiovulatory assay (AOA)[27] to determine in vivo potency and a competitive radioligand binding assay to determine receptor affinity (see Experimental Section). The relative in vivo potencies discussed in this paper were derived from the ratio of doses of an antagonist that gave equivalent percent inhibition in the antiovulatory test to that of another. Potencies relative to a standard would have necessitated an inordinate amount of animals, as each analogue would have had to be tested at several doses in order to obtain dose−response curves from which a relative potency could be calculated.

Results presented in Table 1 are discussed in terms of ring size, chirality of the bridgeheads, and composition of the elements forming the (1−3) bridge. Antagonists using a disulfide bond to bridge the side chains of residues 1 and 3 were originally synthesized to identify a preferred chirality at the bridgeheads. For example, a preparation of [Ac-^{D}Cys^1,^{D}Cpa^2,^{D}Cys^3,NicLys^5,^{D}NicLys^6,ILys^8,^{D}Ala^{10}]GnRH ([^{D}Cys^{1,3}]antide) showed significant inhibition of ovulation at 50 μg/rat (data not shown), whereas a homologue with an [^{L}Cys^1, ^{D}Cys^3] substitution was inactive at 1 mg/rat. These early results suggested that the D-configuration at positions 1 and 3 was preferred.

Cyclization of the N-terminus tripeptide raised a design challenge. We had observed that a hydrophilic N-terminus (such as [Δ^3Pro^1]) must be paired with a hydrophobic residue at position 6 (such as [^{D}Trp^6]) or that a hydrophobic residue at position 1 (such as [^{D}Nal^1]) must be paired with a hydrophilic residue at

† 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: AOA, antiovulatory assay; Aph, 4-aminophenylalanine; Boc, *tert*-butoxycarbonyl; Cpa, 4-chlorophenylalanine; Dbu, 2,4-diaminobutyric acid; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Dpr, 2,3-diaminopropionic acid; EDT, ethanedithiol; 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; Nal, 3-(2′-naphthyl)-alanine; NMP, *N*-methylpyrrolidinone; OFm, fluorenylmethyl ester; Pal, 3-(3′-pyridyl)-alanine; 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 (% Acn)[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
1	cyclo(1−3)[Ac-Asp[1],DCpa[2],Dpr[3],DNal[6],DAla[10]]GnRH	11	4.10 (34.8)	88[II]	85	1308.60	1308.6	31 ± 2.1	1000	7/8
2	cyclo(1−3)[Ac-DDpr[1],DCpa[2],DAsp[3],DNal[6],DAla[10]]GnRH	11	4.56 (32.4)	92[II]	N/A	1308.60	1308.6	1.7 ± 0.27	250	6/6
									1000	0/8
3	cyclo(1−3)[Ac-DAsp[1],DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	13	4.0 (34.8)	>95[II]	>95	1336.63	1336.6	1.6 ± 0.06	N/A	N/A
4	cyclo(1−3)[Ac-DGlu[1],DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	13	4.40 (34.8)	>98[I]	>98	1336.62	1336.6	0.31 ± 0.07	25	3/3
									50	2/6
									100	0/3
5	cyclo(1−3)[Ac-DAsp[1],DCpa[2],DLys[3],DNal[6],DAla[10]]GnRH	14	4.10 (34.8)	90[II]	N/A	1350.64	1350.6	0.82 ± 0.11	5.0	4/5
									10	2/8
									25	1/11
6	cyclo(1−3)[Ac-DGlu[1],DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	14	4.4 (35.4)	93[I]	91	1350.64	1350.6	5.2 ± 0.26	250	5/6
									500	4/6
7	cyclo(1,1′−3)[Ac-DAsp[1](Gly),DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	15	4.03 (34.8)	94[I]	98	1379.62	1379.5	0.70 ± 0.07	50	4/6
									100	0/4
8	cyclo(1,1′−3)[Ac-DAsp[1](Ala),DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	15	4.52 (34.2)	91[I]	98	1393.65	1393.8	1.8 ± 0.24	50	8/8
									100	1/6
9	cyclo(1−3)[Ac-DAsp[1](DAla),DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	15	3.57 (34.2)	96[I]	>98	1393.85	1393.8	0.57 ± 0.11	100	2/7
10	cyclo(1,1′−3)[Ac-DAsp[1](βAla),DCpa[2],DDpr[3],DNal[6],DAla[10]]GnRH	15	4.10 (36.0)	98[I]	97	1379.68	1379.7	0.35 ± 0.03	25	3/4
									50	0/6
11	cyclo(1−3)[Ac-DGlu[1],DCpa[2],DLys[3],DNal[6],DAla[10]]GnRH	15	4.11 (34.2)	96[II]	N/A	1364.66	1364.7	0.47 ± 0.01	50	1/4
									100	0/5
12	cyclo(1−3)[Ac-DGlu[1],DCpa[2],DLys[3],DArg[6],DAla[10]]GnRH	15	4.41 (22.8)	93[I]	90	1323.67	1323.7	31 ± 4.4	500	8/8
13	cyclo(1,1′−3)[Ac-DAsp[1](Gly),DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	16	4.16 (36.6)	>98[I]	N/A	1393.65	1393.6	0.34 ± 0.03	10	10/10
									25	0/6
14	cyclo(1,1′−3)[Ac-DAsp[1](Ala),DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	16	4.14 (35.4)	93[I]	91	1407.66	1407.5	3.3 ± 0.23	100	2/9
									250	0/6
15	cyclo(1,1′−3)[Ac-DAsp[1](Ala),DCpa[2],DOrn[3],DArg[6],DAla[10]]GnRH	16	3.39 (22.8)	>98[I]	93	1366.68	1366.7	350 ± 70	500	7/8
16	cyclo(1,1′−3)[Ac-DAsp[1](DAla),DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	16	4.14 (36.0)	92[I]	98	1407.66	1407.6	2.4 ± 0.35	250	5/9
					borate				500	4/8
17	cyclo(1,1′−3)[Ac-DAsp[1](βAla),DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	16	4.93 (34.2)	>98[I]	>98	1393.65	1393.7	0.32 ± 0.005	50	2/8
									100	2/6
18	cyclo(1,1′−3)[Ac-DAsp[1](Gaba),DCpa[2],DDpr[3],DNal[6],DAla[10]]GnRH	16	4.30 (36.0)	97[I]	N/A	1393.65	1393.6	0.18 ± 0.02	25	5/6
									100	6/11
19	cyclo(1,1′−3)[Ac-DGlu[1](Gly),DCpa[2],DDbu[3],DNal[6],DAla[10]]GnRH	16	3.91 (35.4)	92[I]	93	1393.68	1393.7	20.3 ± 2.4	250	4/4
20	cyclo(1,1′−3)[Ac-DAsp[1](Gly),DCpa[2],DLys[3],DNal[6],DAla[10]]GnRH	17	4.00 (35.4)	96[I]	95	1407.66	1407.7	0.14 ± 0.01	10	4/4
									25	0/6
21	cyclo(1,1′−3)[Ac-DAsp[1](βAla),DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	17	4.38 (36.6)	96[I]	83	1407.66	1407.5	0.17 ± 0.02	10	3/11
					borate				25	0/7
22	cyclo(1,1′−3)[Ac-DOrn[1](βAla),DCpa[2],DAsp[3],DNal[6],DAla[10]]GnRH	17	3.99 (36.0)	96[I]	N/A	1407.66	1407.5	2.2 ± 0.35	1000	3/8
23	cyclo(1,1′−3)[Ac-DGlu[1](Gly),DCpa[2],DOrn[3],DNal[6],DAla[10]]GnRH	17	4.00 (36.0)	93[II]	69	1407.72	1407.7	4.2 ± 0.09	100	4/5
									250	2/5
24	cyclo(1,1′−3)[Ac-DGlu[1](Gly),DCpa[2],DLys[3],DNal[6],DAla[10]]GnRH	18	4.64 (34.8)	>98[I]	>98	1421.68	1421.8	1.5 ± 0.48	50	6/7
									100	3/6
25	cyclo(1,1′−3)[Ac-DGlu[1](βAla),DCpa[2],DLys[3],DNal[6],DAla[10]]GnRH	19	4.39 (35.4)	97[I]	N/A	1435.70	1435.7	2.2 ± 0.35	50	6/8
									100	0/10
26	dicyclo(1−3,4−10)[Ac-DAsp[1],DCpa[2],DLys[3],Asp[4],DNal[6],Dpr[10]]GnRH	14/23	3.22 (36.6)	93[I]	N/A	1375.64	1375.7	1.2 ± 0.15	100	4/4
									200	1/8
27	tricyclo(1−3/4−10/5−8)[Ac-DGlu[1],DCpa[2],DLys[3],Asp[4],Glu[5],DNal[6],Lys[8],Dpr[10]]GnRH	15/23/18	4.35 (35.4)	97[I]	N/A	1309.66	1309.61	4.6 ± 0.49	500	4/5

[a] Retention times under isocratic conditions, buffer 0.1% TFA. Acn is acetonitrile. [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 or are not available. [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.

position 6 (such as [DArg^6]) in order to yield the most potent analogues. For example, [Ac-Δ^3Pro^1,DFpa^2, DTrp^{3,6}]GnRH inhibited ovulation at 7.5 μg/rat whereas the homologue [Ac-Δ^3Pro^1,DFpa^2,DTrp^3,DArg^6]GnRH inhibited ovulation by 60% at 20 μg/rat, and [Ac-DNal^1, DFpa^2,DTrp^3,DArg^6]GnRH inhibited ovulation by 62% at 0.5 μg/rat whereas the homologue [Ac-DNal^1,DFpa^2, DTrp^3,DNal^6,Pro^9-NHEt]GnRH was inactive at 25 μg/rat.[28] With DNal at position 6, the cyclo(1−3)[Asp^1, Dpr^3] cycle yielded an inactive analogue (**1**) at 1 mg/rat (K_i = 31 nM). Inverting the direction of the amide bond and changing the chirality of the bridgehead residues to match that of [DCys^{1,3}]antide yielded **2** (K_i = 1.7 nM), which is fully active at 1 mg/rat but not at 250 μg/rat. Analysis of these results with those presented in earlier papers[12,23] led to several observations that were taken into consideration in subsequent analogue design: (a) In modeling terms, a disulfide bridge is bulkier than an amide bond; therefore, a comparable lactam bridge would have to encompass a larger number of atoms than the 11 atoms used in the bridges of **1** and **2**. (b) All potent linear GnRH antagonists have D-residues at positions 1 and 3;[1,29] hence, it is likely that the D-configuration at those positions will be favored as it will orient the bridge in the same direction as the original side chains. (c) The direction of the amide bond forming the lactam bridge should be the same as that of the backbone.[23,30]

Whereas the most potent linear GnRH antagonists had a hydrophobic N-terminus and a neutral/basic, aromatic D-amino acid at position 6,[29,31−33] it was not clear whether the introduction of a lactam bridge would dramatically change the overall character of that part of the molecule. Due to the presence of an additional amide bond in the ring and deletion of two aromatic rings (naphthyl at position 1 and pyridyl/indolyl/naphthyl at position 3), we opted for the introduction of a hydrophobic residue at position 6 (DNal) in most of our analogues. The first analogues in which we tested these design protocols (**3** and **4**) gave encouraging results as they had low K_i values (1.6 and 0.31, respectively) and showed inhibition of ovulation at 50 μg/rat. Increasing the bridge size to 13 atoms in **3** and **4** was more favorable in **3** with cyclo(1−3)[DAsp^1, DOrn^3] than in **4** with cyclo(1−3)[DGlu^1, DDbu^3] since a partially purified (70% pure) **3** was fully active at 50 μg/rat (data not shown) and **4** was active at 100 μg/rat. It is difficult to explain the fact that the K_i of **4** is $^1/_5$ that of **3**. As shown in earlier papers, the positioning of the amide bond can be critical for high potency;[12,23] in this case, it appears to be advantageous for the amide bond to be proximal to the α-carbon of residue 1. Increasing the size of the ring by an additional atom such as in **5** (K_i = 0.82 nM) and **6** (K_i = 5.2 nM) yielded one of the most potent analogues of this series (**5**) that blocked ovulation by 75% and 90% at 10 and 25 μg/rat, respectively. As in **3**, the bridging amide bond is β to the α-carbon of the N-terminal residue, both bridgeheads are of the D-configuration, and residue 6 is the hydrophobic DNal residue. When comparing the affinity and potency of **5** to that of **1** (a factor greater than 40), it becomes evident that optimization of bridge size as well as its configuration (there are three additional methylene groups in **5** resulting from the substitution of [DDpr^3] by [DLys^3]) can be extremely critical and difficult to predict. Also, translating the amide bond by one methylene group from a β to γ position with respect to the α-carbon of residue 1 results in a loss of affinity (6-fold) and potency for **6** (>20-fold).

A new trend emerged whereby residue 1 was held constant and the lactam size was enlarged by increasing the length of the side chain of residue 3 that led to improved potencies. Further enlargement by one additional atom was achieved by inserting a residue between the side chains of [DAsp^1] and [DDbu^3] such as in **7**−**10** whereby [Gly$^{1'}$] was introduced in **7** (K_i = 0.70 nM), [LAla$^{1'}$] in **8** (K_i = 1.8 nM), and [DAla$^{1'}$] in **9** (K_i = 0.57 nM) or by introducing [βAla$^{1'}$] between the side chains of [DAsp^1] and [DDpr^3] such as in **10** (K_i = 0.35 nM). Intuitively, it was reasonable to increase the size of the bridging element that replaced the two bulky side chains (naphthyl and pyridyl) in the linear analogues. The most potent analogues in the AOA, **7** and **10**, had high binding affinities, and each contained an achiral bridging amino acid. Yet, when the potencies of **7** and **10** were compared to that of **5** they were found to be approximately 5 and 3 times less potent, respectively, with about the same or slightly lower K_i values. In contrast, **8** and **9** contain an inserted L- or D-Ala$^{1'}$ and are equipotent in the AOA with K_i values (1.8 and 0.57 nM) that are consistent with these potencies. This suggests that chirality at that position does not play a discriminating role, although introduction of the asymmetric center at the 1′ position (L- or D-Ala in lieu of Gly) resulted in significant loss of potency (**8** and **9** are ca. 10 times less potent than **5**). Two additional 15-membered ring analogues (**11** and **12**) with [DGlu^1] were also synthesized. We were not surprised to observe a similar loss of potency (5-fold and >50-fold) when comparing the potencies of **11** and **12** to that of **5**, reflecting the detrimental effect of shifting the amide bond γ to the α-carbon of residue 1 as shown earlier for **4**. Despite this unfavorable modification, **11** is the most potent cyclo(1−3) analogue with a DGlu in position 1 and with high affinity (K_i = 0.47 nM) for the GnRHR.

Unfortunately, increasing the size of the lactam ring of **5** (14 atoms) to 15 atoms in **7**−**12** did not yield any antagonist that would inhibit ovulation at doses less than 10 μg/rat. We had shown earlier that an optimum cyclo(4−10) ring[12,23] could be obtained by gradually downsizing the bridge from [Asp^4, Orn^{10}]- and [Asp^4, Dbu^{10}]-containing analogues that were inactive at 500 μg/rat to [Asp^4, Dpr/aminoglycine^{10}]-containing analogues that inhibited ovulation at 2.5 μg/rat or less. We would reverse this strategy, since in the cyclo(1−3) ring system, increasing ring sizes as in **2**, **3**, and **5** resulted in graded increases in potency.

The most convenient way to enlarge the ring to 16 atoms was to insert an additional amino acid (**13**−**19**), despite the poor results seen with such an approach in 15-membered ring-containing analogues (**7**−**10**). Antagonist **13** inhibited ovulation at 25 μg/rat using a [DAsp^1(Gly$^{1'}$), DOrn^3] cycle and was an encouraging lead with remarkably high affinity (K_i = 0.34 nM). Further substitutions, such as the introduction of [Ala$^{1'}$] or [DAla$^{1'}$], resulted in **14** and **16** that were active or partially active at 250 and 500 μg/rat, respectively, and had higher K_i values (3.3 and 2.4 nM, respectively). This

was expected in view of the earlier hypothesis that such a substitution would increase hydrophobicity and hence might not be compatible with the [DNal^6] substitution that normally required a hydrophilic N-terminus in linear antagonists. Since at least one analogue with a [DNal^6] substitution in this series inhibited ovulation at about 25 μg/rat (**5**), we concluded that the (1−3) lactam ring was more likely to mimic the hydrophilic N-terminus of the earlier successful [Δ^3Pro^1] substitution.[28] It was therefore important to verify whether a DArg^6 substitution (as in **15**) would be compatible with high potency. The fact that **15** is >5 times less potent than **14** and its K_i is at least 100-fold greater suggests that the 1−3 lactam ring may be considered "hydrophilic" and is more likely to mimic [Ac-Δ^3Pro^1-DCpa/DFpa^2-DTrp^3] rather than the "hydrophobic" [Ac-DNal^1-DCpa/DFpa^2-DTrp/DPal^3] N-terminus. While keeping the ring size constant, the length of residue 1′ was increased [βAla (**17**) and Gaba (**18**)] with concomitant size reduction of the side chain of residue 3 [DDbu^3 and DDpr^3]; the resultant **17** and **18** inhibited ovulation by >50% at 100 μg/rat with remarkably low K_i values (0.32 and 0.18 nM). This alteration of the cycle was less drastic than that brought about by the introduction of either an L- or D-Ala$^{1'}$ as in **15**, which was inactive (K_i = 350 nM), and **16** which inhibited ovulation by 50% at 500 μg/rat (K_i = 2.4 nM). The complete loss of activity of **19** at 250 μg/rat (K_i = 20.3 nM) when [DAsp^1] of **7** was substituted by [DGlu^1] substantiated earlier results that showed the [DAsp^1] substitution to be favored over the [DGlu^1] substitutions; **4** and **6** were less potent than **3** and **5**, respectively, independently of the size of the ring.

The 17-membered ring size of [DAsp^1(Gly$^{1'}$), DLys^3] and [DAsp^1(βAla$^{1'}$), DOrn^3] was favorable in that 25 μg/rat of **20** and **21** inhibited ovulation by 100%. These are also the two analogues with the highest affinity for GnRHR in this series (K_i = 0.14 and 0.17 nM, respectively). Inverting the direction of the two lactam ring amide bonds of **21** (obtained by introducing DOrn at position 1 and DAsp at position 3) yielded **22** (K_i = 2.2 nM), which was only partially active (60% inhibition at 1 mg/rat) and therefore >40-fold less potent in vivo than **21**. This experimental result corroborated theoretical studies of α-helical structures, showing a reinforcement between the dipole moment of the bridge amide bond(s) and that of the backbone which adds structural stability as predicted by the helix-dipole model of Baldwin.[34] In this particular case, no evidence for hydrogen bonding between the amide bond(s) of the side chains and those of the backbone was seen; in fact, the only observed hydrogen bond involving a side chain atom in the NMR structures of the DTrp^3-containing analogue cyclo(4−10)-[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH occurs between DTrp^3:Hϵ and Pro^9:O. It is noteworthy that "reversing" the direction of the amide bond in lactam bridges very rarely resulted in moderately active analogues when introduced in other peptide hormones.[30]

The potency of **23** (60% inhibition at 250 μg/rat, K_i = 4.2 nM), which is to be compared to that of **20** (same ring size of 17 atoms, 100% inhibition at 25 μg/rat, K_i = 0.14 nM) or that of **11** (15-atom ring, 75% inhibition at 50 μg/rat, K_i = 0.47 nM), again exemplifies the deleterious effect of DGlu in position 1. Interestingly, increasing the size of the ring of **23** to 18 atoms ([DLys^3] instead of [DOrn^3]) to yield **24** is favorable. Finally, **25** with the 19-membered [DGlu^1(βAla),DLys^3] ring was more potent than either **23** or **24**, inhibiting ovulation by 100% at 100 μg/rat, although the K_i values of the three analogues, 2.2, 4.2, and 1.5 nM, respectively, are not statistically different.

As mentioned earlier, our aim was to construct potent GnRH antagonists that would be structurally constrained enough to allow examination of the spatial relationship of the N-terminal tripeptide with respect to the rest of the molecule by NMR spectroscopy. Therefore, a cyclo(1−3) analogue was synthesized that contained the additional (4−10) cycle known to be compatible with high potency in a number of monocyclic and dicyclic antagonists. This resulted in a dicyclic analogue, **26**, that incorporated the favorable substitutions that were identified in potent **5** [DAsp^1, DLys^3]. Although **26** had high affinity for rGnRHR (K_i = 1.2 nM), it did not meet the potency requirements we had originally set (fully active at less than 5 μg/rat) to justify extended structural investigations by NMR. NMR investigations of cyclo(4−10)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH[13,14] and 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,[35] however, had shown that the N-terminal tripeptides were somewhat structured with no single conformation characterizing either analogue. The minimum energy NMR-derived structure of the N-terminal tripeptide of cyclo(4−10)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,DNal^6,Dpr^{10}]GnRH upon which we have superposed the corresponding residues of **21**, built by homology, is illustrated in Figure 1. This Figure illustrates the reproduction of the backbone conformation of the cyclo(4−10) antagonist NMR-derived structure obtainable with the [DAsp^1(βAla),DOrn^3] bridge motif. The resulting theoretical structure has no significant strain that would prevent its adoption in solution. Consequently, the [DAsp^1(βAla),DOrn^3] bridge motif appears to be compatible with the NMR conformation observed for cyclo(4−10)[Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Asp^4,-DNal^6,Dpr^{10}]GnRH.[13,14]

Studies carried out concomitantly with the introduction of other bridgeheads, as shown in the accompanying paper (next in this series), identified dicyclo(1−5/4−10) antagonists that were much better candidates for structural analysis and in which the N-terminus was unequivocally constrained. This did not deter us from combining the three best cycles identified so far in one single molecule, **27** {tricyclo(1−3/4−10/5−8)[Ac-DGlu^1, DCpa^2,DLys^3,Asp^4,Glu^5,DNal^6, Lys^8,Dpr^{10}]GnRH}. Compound **27** was inactive in vivo at 500 μg/rat and lacked the side chains of residues 1, 3−5, 8, and 10, which were formerly all thought to be critical for GnRH antagonist activity. Its relatively high affinity (4.6 nM), however, suggests that the remaining side chains, when held in the proper orientation, are sufficient to achieve high affinity binding.

In summary, we have identified analogues **5**, **13**, **20**, and **21** that differed only in the size of the (1−3) ring, ranging from 14 to 17 atoms, and that inhibited ovulation at 25 μg/rat with remarkably high affinity for GnRHR. Other structurally related analogues were less potent and had lower affinity, which suggests both tolerance and a certain degree of discrimination by the

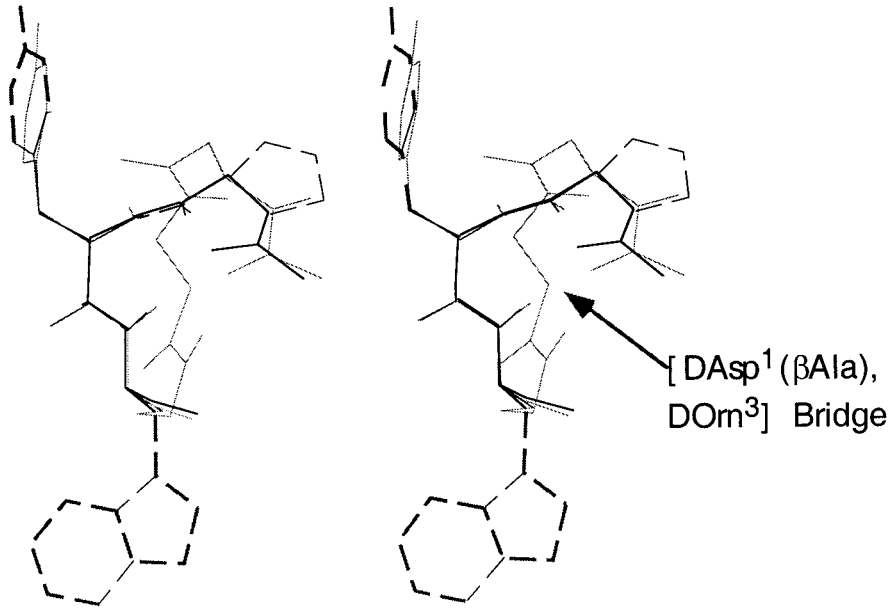

Figure 1. Overlaid stereoimages of N-terminal tripeptide sections of cyclo(4−10) antagonist (dark gray striped lines) and cyclo(1,1′−3) antagonist (solid gray). Common backbone region is shown in heavy black and [Asp[1](βAla), DOrn[3]] bridge is captioned.

GnRH receptor. A large (1−3) ring by itself may be quite flexible, and it is very difficult to propose a structural model of the receptor/antagonist interaction that would explain such results; all favorable substitutions may not induce the same conformation, nor is it excluded that antagonists may interact in different ways with the GnRH receptor.[35] Therefore, identification of an optimal mimic of the N-terminal three residues of potent GnRH antagonists may also benefit from the resources of combinatorial chemistry. With respect to our original goal, detailed information regarding the optimal positioning of the N-terminal tripeptide relative to the body of the molecule ultimately required (as shown in the following article)[36] the discovery of two families of GnRH analogues with cyclo(1,1′−5) and cyclo(1−8) constraints.

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, BioRad Laboratories, Richmond, CA) as previously published.[38] 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 protecting groups were as follows: Arg(Tos), Asp(β-OcHex or β-OFm), L- and DDbu(γ-Fmoc), Dpr(β-Fmoc), Glu(γ-OcHex or γ-OFm), Lys(ε-2ClZ or ε-Fmoc), Orn(δ-Fmoc), Ser(OBzl), NMe-Tyr(2,6-diClBzl), Tyr(2BrZ). Boc-D4Cpa, Boc-D2Nal, Boc-Δ[3]Pro and Boc-D3Pal were synthesized in our laboratory[26,33,39] 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[40] 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.[41] 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.[42] or by substituting HBTU or TBTU for BOP. HF cleavage occurred in the presence of 10% anisole and 2−5% dimethyl sulfide (for Trp-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.

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.[24,25] 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.[43] 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). 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.[43,44]

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[45−47] 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 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)[48] 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 averate + SEM of three or more independent experiments.

6. Antiovulatory Assay (AOA). The AOA was carried out as described by Corbin and Beattie.[27] 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), then 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.[49,50] Molecular modeling and visualization were performed using Insight II (MSI, Inc., San Diego, CA) on a Silicon Graphics Iris Crimson workstation.

All peptides were made according to the examples that follow as representatives of the various cyclic motifs.

Cyclo(1−3)[Ac-Asp^1,D-Cpa^2,Dpr^3,D-Nal^6,D-Ala^{10}]GnRH (1). The peptide was synthesized automatically on 2 g of 0.45 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. The completed peptide-resin (3.6 g) was stirred for 75 h in DMF/hydrazine, filtered, and washed with 50% DMF/DCM and MeOH. After drying, the peptide-resin was cleaved and deprotected in HF (40 min, 0 °C). The peptide was extracted from the resin with 50% aqueous acetonitrile, shell-frozen, and lyophilized to give the crude hydrazide (860 mg, 0.57 mmol). This intermediate was dissolved in dry DMF (40 mL) at −20 °C and acidified with 4 N HCl in dioxane (0.6 mL, 2.4 mmol). After 10 min, isoamyl nitrite was added in three aliquots (0.15 mL, 1.1 mmol total) with stirring over 15 min. Stirring at −20 °C was continued for 3 h. The solution of peptide azide was diluted to 1 L with DMF (precooled to −20 °C), and the pH was adjusted to 7 with TEA. The solution was stored at −5 °C (48 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 39 mg (ca. 26 μmol, 3%) of cyclic peptide **1**.

Analogues **2** and **3** ($[\alpha]_D = -8°$, $c = 0.58$) were obtained using this general procedure in comparable yields.

Cyclo(1−3)[Ac-D-Asp^1,D-Cpa^2,D-Lys^3,D-Nal^6,D-Ala^{10}]GnRH (5). The peptide was synthesized automatically on 1.5 g of 0.45 mmol/g-substituted MBHA resin using DIC as the coupling reagent in DCM. After the peptide was completely assembled, the D-Asp(β-OFm) and D-Lys(ϵ-Fmoc) side chains were deprotected with two, 15 min treatments of 20% piperidine in DMF. Cyclization then proceeded at pH 9 in DMF using 2.5 molar excess of BOP reagent and 4 equiv of DIPEA and was carried out for three 2 h periods. Since a Kaiser test gave a positive result for free amine functionality, a subsequent treatment with 20% DIC in NMP/DMSO for 34 h (or >15 h with BOP for **4**, **6**, and **11**) drove the cyclization to completion as evidenced by the Kaiser test. The peptide-resin was cleaved and deprotected in HF (40 min, 0 °C), and the crude peptide (519 mg) was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 50 mg (36 μmol, 5%) of **5**.

Analogues **4**, **6**, **11**, and **12** ($[\alpha]_D = -15°$, $c = 0.84$) were obtained using this general procedure in comparable yields.

Cyclo(1−3)[Ac-D-Asp^1(Gly),D-Cpa^2,D-Dbu^3,D-Nal^6,D-Ala^{10}]-GnRH (7). Analogue **7** was assembled manually on 1.3 g of 0.76 mequiv/g-substituted MBHA resin. Extension of the side chain of D-Dbu in position 3 with Gly was achieved prior to

the introduction of Boc-DAsp(β-OFm) at position 1. The Fmoc-protecting group of [Boc-DCpa^2,DDbu^3(γ-Fmoc),Ser^4(OBzl),Tyr^5-(2BrZ),DNal^6,Arg^8(Tos),DAla^{10}]-GnRH(2−10)-MBHA was first removed with 20% piperidine in DMF (2 ×10 min), whereupon Nα-Fmoc-Gly was coupled to the free DDbu-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 OFm/Fmoc groups of Asp^1 and Gly$^{1'}$ were removed with piperidine as above, and cyclization proceeded in NMP with one molar equivalent of BOP/DIPEA (1:3) at pH 9 for 15 h at 22 °C. The peptide-resin (2.8 g) was cleaved and deprotected in HF (90 min, 0 °C), and the crude peptide (1.3 g) was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 90 mg (57 μmol, 6%) of **7**.

The analogues listed below were obtained using this general procedure in comparable yields; exceptions in cyclizing reagents are as follows: 3 equiv of BOP/DIPEA (1:3) was used for **10**; 3 equiv of BOP/HOBt/DIPEA (1:1:3) was used for **13** ($[\alpha]_D = -11°$, $c = 0.47$), **14** ($[\alpha]_D = -6°$, $c = 0.71$), **15** ($[\alpha]_D = +3.4°$, $c = 0.51$), **16** ($[\alpha]_D = -8°$, $c = 0.79$), **18** ($[\alpha]_D = -14°$), **21**, **22** ($[\alpha]_D = -32°$), and **25** ($[\alpha]_D = -19°$); 1 equiv of TBTU/DIPEA (1:3) for 24 h was used for **19**, **20**, **23**, and **24**.

Cyclo(1−3)[Ac-DAsp^1(Ala),DCpa^2,DDbu^3,DNal^6,DAla^{10}]-GnRH (8). Analogue **8** was synthesized manually using the Boc-strategy from 1.3 g of 0.76 mequiv/g-substituted MBHA resin. The synthesis was carried out at 70 °C in a water-jacketed synthesis vessel that was kept at constant temperature by a circulating water bath.[19] Boc-amino acids were coupled as usual with a 2 molar excess of DIC in 25% DMSO/toluene for 10−30 min. The resin was washed after couplings with 25% DMSO/toluene, CH_3OH, and toluene (2 × 30 mL each). Boc-groups were removed in TFA/toluene/1,2-ethanedithiol (58:40:2) for 3 min. The resin was neutralized by washing with 10% 1,2-ethanedithiol/2-propanol, 10% TEA/toluene, CH_3OH, 10% TEA/toluene, CH_3OH, 25% DMSO/toluene (2 × 30 mL each). The extension of DDbu^3 with Ala$^{1'}$ followed the same procedure as that for **7**, except that DDbu^3(β-Fmoc) was deprotected with 20% piperidine/toluene for 3 min. Acetylation of the N-terminus occurred with 20% Ac_2O/toluene for 3 min. To cyclize DAsp^1 to Ala$^{1'}$, the OFm/Fmoc groups were deblocked as described, and 1.5 equiv of BOP/DIPEA (1:3) was added to the resin in NMP for 24 h at 65 °C. With unsatisfactory Kaiser test results, the cyclization was repeated with fresh reagents at 22 °C (5 × 24 h), until the test was negative. The peptide-resin was cleaved and deprotected in HF (90 min, 0 °C), and the crude peptide (0.8 g) was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 15 mg (9 μmol, 1%) of **8**.

Analogues **9** and **17** were obtained using this high temperature procedure in 3% yields.

Dicyclo(1−3/4−10)[Ac-DAsp^1,DCpa^2,DLys^3,Asp^4,DNal^6,Dpr^{10}]GnRH (26). Analogue **26** was assembled automatically on 2.0 g of 0.76 mequiv/g-substituted MBHA resin. Both lactam cyclizations occurred separately on the resin using modified versions of the method of Felix et al.[22] Resin-bound peptide (1.5 mmol) [Boc-Asp^4(β-OFm),Tyr^5(2BrZ),DNal^6,Arg^8-(Tos),Dpr^{10}(β-Fmoc)]-GnRH(4−10)-MBHA was first assembled. The Asp(β-OFm) and Dpr(β-Fmoc) side chains were deprotected with two, 15 min treatments of 20% piperidine in DMF. Cyclization of the 4−10 bridge then proceeded at pH 9 in DMF with BOP/HOBt/DIPEA (3:3:9 mmol) at 65 °C in an orbital shaker for 15 h. The procedure was repeated for additional 15 and 72 h periods. A third, 15 h recoupling included the addition of a catalytic amount of (dimethylamino)pyridine, after which the Kaiser test was negative. The final three amino acids were added to the peptide chain that was acetylated as the final elongation step.

The second cyclization (1−3) proceeded in a similar manner, whereby the Fmoc/OFm groups were deblocked and coupling reagents were applied for two 15 min periods. The completed peptide-resin (2.7 g) was then cleaved and deprotected in HF (40 min, 0 °C), and the crude peptide (619 mg) was purified in TEAP 2.25 and 0.1% TFA, as described, to yield 11 mg (8 μmol, 0.5%) of **26** ($[\alpha]_D = -6$ °C, $c = 0.85$).

Tricyclo(1−3/4−10/5−8)[Ac-DGlu^1,DCpa^2,DLys^3,Asp^4,Glu^5,DNal^6,Lys^8,Dpr^{10}]GnRH (27). Analogue **27** was assembled automatically on 3 g of 0.45 mequiv/g-substituted MBHA resin. Cycles (1−3) and (5−8) were formed separately on the resin during the synthesis of the peptide, whereas the (4−10) cycle was formed after peptide completion in two steps via the Curtius reaction of the peptide-hydrazide. In detail, resin-bound peptide (1.35 mmol) [Boc-DGlu^5(γ-OFm),DNal^6,Lys^8(ϵ-Fmoc),Dpr^{10}(Z)]-GnRH(5−10)-MBHA was first assembled. The DGlu^5(γ-OFm) and Lys^8(ϵ-Fmoc) side chains were deprotected with two, 30 min treatments of 20% piperidine in DMF. Cyclization of the 5−8 bridge then proceeded at pH 9 in DMF with BOP/DIPEA (5:15 mmol) for 15 h at 22 °C. The procedure was repeated for an additional 15 min at 65 °C followed by 2 h at 22 °C. Unreacted amino groups were then acetylated (20% Ac_2O/DCM, 15 min), and the remaining four amino acids of the sequence were coupled. In a similar manner, treatment with 20% piperidine/DMF (2 × 30 min) removed the OFm and Fmoc protecting groups of DGlu^1 and DLys^3 in cyclo(5−8)[Ac-DGlu^1(γ-OFm),DCpa^2,DLys^3(ϵ-Fmoc),Asp^4(β-OcHex),DGlu^5,DNal^6,Lys^8,Dpr^{10}(Z)]-GnRH(1−10)-MBHA. Cyclization of the 1−3 bridge then proceeded at pH 9 in DMF with BOP/DIPEA (5:15 mmol) for 3 h at 22 °C (repeated for 15 h).

The final (4−10) cyclization occurred in a three-step process, including HF cleavage. The completed peptide-resin (4.8 g) was stirred for 100 h in DMF/hydrazine at 22 °C, filtered, and washed with 50% DMF/DCM and MeOH. After drying, the peptide-resin was cleaved and deprotected in HF (40 min, 0 °C). The peptide was extracted from the resin with 50% aqueous acetonitrile, shell-frozen, and lyophilized to give crude dicyclo(1−3/5−8)[Ac-DGlu^1,DCpa^2,DLys^3,Asp^4(NHNH$_2$),DGlu^5,DNal^6,Lys^8,Dpr^{10}]-GnRH (1.55 g, 1.1 mmol). This intermediate hydrazide was dissolved in dry DMF (40 mL) at −25 °C and acidified with 4 N HCl in dioxane (1.3 mL, 5.2 mmol). After 10 min, isoamyl nitrite was added in three aliquots (0.3 mL, 2.2 mmol total) with stirring over 15 min. Stirring at −20 °C was continued for 3 h. The solution of peptide azide was diluted to 1 L with dry DMF (precooled to −20 °C), and the pH was adjusted to 7 with TEA. The solution was stored at −10 °C for 48 h. The solvent was evaporated under vacuum to yield crude tricyclic peptide which was purified directly in TEAP 2.25 and 0.1% TFA to yield 18 mg (13 μmol, 1%) of tricyclic peptide **27** ($[\alpha]_D = -45$ °C).

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