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A Novel Conformation in a Highly Potent, Constrained Gonadotropin-Releasing Hormone Antagonist

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Abstract: Through design, synthesis, and biological testing of constrained gonadotropin releasing hormone (GnRH) antagonists, we are studying the structural requirements for biological activity. Here we describe the conformational analysis in solution of a highly potent, dicyclic GnRH antagonist, dicyclo(4-10/5,5′-8)[Ac-D-2Nal[1],D-pClPhe[2],D-3Pal[3],Asp[4],Glu[5](Gly),D-Arg[6],Dbu[8],Dpr[10]]GnRH (**1**), using NMR spectroscopy. The dicyclic part of this molecule adopts a preferred conformation 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, which is stabilized by hydrogen bonds between Leu[7] NH/Asp[4] CO, Dbu[8] NH$^{\delta}$/Glu[5] CO, and Dpr[10] NH$^{\gamma}$/Dbu[8] CO. This is a novel conformation that had not been observed previously in any constrained GnRH antagonist and is remarkably different from that found for another dicyclic (4-10/5-8) GnRH antagonist with very similar sequence, dicyclo(4-10/5-8)[Ac-D-2Nal[1],D-pClPhe[2],D-Trp[3],Asp[4],Glu[5],D-Arg[6],Lys[8],Dpr[10]]GnRH (**2**) (Bienstock et al. *J. Med. Chem.* **1993**, *36*, 3265−3273). The conformation of **2** contains a type II′ β turn around residues 6−7, which had been proposed to be essential for GnRH activity. These results are important for our general understanding of polypeptide conformation, since they show that the dicyclo(4-10/5-8) backbone can adopt more than one family of conformations despite its dicyclic nature, and from the point of view of the design of GnRH antagonists, since they suggest that the presence of a turn around residues 6−7, rather than the type of β turn, may be necessary for biological activity.

Naturally occurring peptides regulate a large diversity of biological functions and thus are logical targets for the design of drugs with clinical applications. Drug design based on such peptides is commonly hindered by difficulties in obtaining information on the structural requirements for biological activity: The peptides are usually flexible and unstructured in solution, while direct determination of receptor bound conformations is hampered because most receptors are integral membrane proteins. A powerful strategy to overcome these problems involves the use of covalent constraints designed to induce particular conformational features that are suspected to be important for biological activity.[1] The constraints can increase biological activity by reducing the entropy loss upon receptor binding, if they preinduce correct conformations in solution. In addition, such conformations can then be studied directly in the constrained analogs, in the absence of receptor. Even if the conformations forced by the constraints are not optimal for biological activity, the increased likelihood that the structures adopted by the constrained analogs in solvent are analogous to their receptor-bound structures makes conformational analyses in solution more meaningful. The results of such analyses can thus be used to refine putative binding conformations, to suggest alternative or additional constraints, and, in general, to rationalize the observed activities on structural grounds.[1d]

Key considerations in the strategy outlined above are as follows. (i) To what extent do the constraints introduced limit the conformational possibilities of the resulting analogs? (ii) Do the conformations in solution of the constrained analogs indeed correspond to their biologically active conformations? (iii) Do analogous constraints always result in the same preferred conformations? To shed light on these questions, we have analyzed the conformation in solution of a highly potent, dicyclic antagonist of gonadotropin-releasing hormone (GnRH), dicyclo(4-10/5,5′-8)[Ac-D-2Nal[1],D-pClPhe[2],D-3Pal[3],Asp[4],Glu[5](Gly),D-Arg[6],Dbu[8],Dpr[10]]GnRH (**1**) (Figure 1, Table 1). Here we describe the results of this analysis, and compare them with those obtained previously[2] for an equipotent GnRH antagonist incorporating the same type of constraints, dicyclo(4-10/5-8)-[Ac-D-2Nal[1],D-pClPhe[2],D-Trp[3],Asp[4],Glu[5],D-Arg[6],Lys[8],Dpr[10]]-GnRH (**2**).

GnRH is a linear decapeptide hormone involved in the regulation of ovulation and spermatogenesis.[3] Both **1** and **2** were designed in the context of ongoing efforts in our

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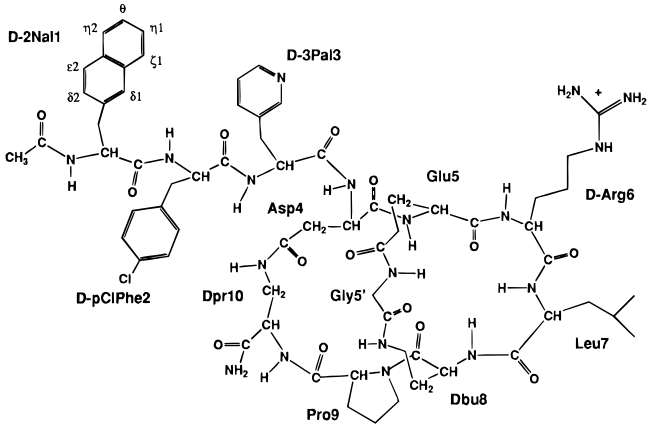

Figure 1. Schematic representation of the dicyclic GnRH antagonist **1**. Nomenclature and abbreviations for amino acid residues throughout the text follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). The symbols represent the L-isomer except when indicated otherwise. Other abbreviations used are as follows: 2Nal, 3-(2′-naphthyl)alanine; pClPhe, 4-chlorophenylalanine; 3Pal, 3-(3′-pyridyl)-alanine; Dbu, 2,4-diaminobutyric acid; Dpr, 2,3-diaminopropionic acid. The nomenclature used to designate the aromatic positions in D-2Nal[1] is indicated.

Table 1. Biological Potency of Constrained GnRH Antagonists in the Antiovulatory Assay

	compound	AOA[a]
(**1**)	dicyclo(4-10/5,5′-8)[Ac-D-2Nal[1], D-pClPhe[2],D-3Pal[3],Asp[4],Glu[5](Gly), D-Arg[6],Dbu[8],Dpr[10]]GnRH	5 (2/8)[b]
(**2**)	dicyclo(4-10/5-8)[Ac-D-2Nal[1], D-pClPhe[2],D-Trp[3],Asp[4],Glu[5], D-Arg[6],Lys[8],Dpr[10]]GnRH	5 (2/10)[c]
(**3**)	cyclo(1−10)[Δ^3Pro[1],D-pClPhe[2], D-Trp[3],D-Trp[6],NMeLeu[7],βAla[10]]GnRH	1000 (5/8)[d]
(**4**)	cyclo(4-10)[Ac-Δ^3Pro[1],D-pFPhe[2], D-Trp[3],Asp[4],D-2Nal[6],Dpr[10]]GnRH	10 (2/10)[c]

[a] AOA−antiovulatory assay in the rat: dosage in μg (rats ovulating/total rats). [b] Reference 8b. [c] Reference 8a. [d] Reference 1b.

laboratories to obtain GnRH analogs with clinical applications in contraception and reproductive therapies.[1d] A central theme in this research has been the stabilization of a type II′ β-turn conformation around residues 6−7, which was proposed to be important for the biological activity of GnRH.[4] Conformational analysis of a moderately active cyclic decapeptide antagonist, cyclo(1-10)[Δ^3Pro[1],D-pClPhe[2],D-Trp[3],D-Trp[6],NMeLeu[7], βAla[10]]-GnRH (**3**),[5] led to the design of highly potent GnRH antagonists with a bridge between residues 4 and 10.[1d,6] In turn, the conformational behavior observed for one of these compounds, cyclo(4-10)[Ac-Δ^3Pro[1],D-pFPhe[2],D-Trp[3],Asp[4],D-2Nal[6],Dpr[10]]-GnRH (**4**), suggested the possibility of adding a bridge between residues 5 and 8 to further constrain the conformation of the analogs.[7] The resulting dicyclic analogs (e.g., **1** and **2**) have similar activities to those of the parent monocyclic (4-10) antagonists (Table 1).[8] The conformations observed in solution for **2**−**4** are very similar and all include a type II′ β turn around residues 6−7,[2,5,7] supporting the hypothesis that this conformation is essential for GnRH antagonist activity. The results we describe below show that, in contrast, the conformation in solution of the dicyclic analog **1** is strikingly different from those of **2**−**4** and includes a type I′ β turn around residues 6−7 nested with a type II β turn around residues 5−6. The observation that two small dicyclic peptides with analogous constraints and very similar sequence can adopt such different conformations has fundamental implications for our general understanding of peptide conformation. Our results also reveal a novel conformation in a constrained GnRH antagonist and suggest that the presence of a turn around residues 6−7, regardless of the type of turn, may be important for biological activity.

Experimental Procedures

Synthesis. Dicyclo(4-10/5,5′-8)[Ac-D-2Nal[1],D-pClPhe[2],D-3Pal[3], Asp[4],Glu[5](Gly), D-Arg[6],Dbu[8],Dpr[10]]GnRH (**1**) was assembled on a methylbenzhydrylamine resin (MBHA-resin) (2.0 g) containing ca. 0.8 mequiv of amino group/g with the protecting groups shown in parentheses [Ac-D-2Nal[1]-D-pClPhe[2]-D-3Pal[3]-Asp(OBzl)[4]-Glu(OFm)[5]-D-Arg(Tos)[6]-Leu[7]-Dbu(Fmoc-Gly)[8]-Pro[9]-Dpr(Z)[10]][9] and cyclized in two steps. Standard protocols for *tert*-butyloxycarbonyl (Boc)-protected amino acids were used. In summary, the Boc group was removed with trifluoroacetic acid (60%) in CH_2Cl_2 (DCM) containing 2−5% ethanedithiol for 20 min followed by neutralization with 10% triethylamine (TEA) in DCM. Couplings were performed in DCM or DCM/dimethylformamide (DMF) (1:1) using dicyclohexylcarbodiimide for 2 h or less. A 2-fold excess of protected amino acids was used. N-Terminal acetylation was performed with a large excess of acetic anhydride in DCM in 15 min. Extension of the side chain of Dbu in position 8 with Gly was achieved prior to the introduction of Boc-Glu(OFm) at position 5. The first cyclization (5-8) was achieved on the solid phase after deprotection of the OFm/Fmoc groups by 25% piperidine in DMF (1 × 1 min and 2 × 10 min) using a 3-fold excess of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) in the presence of excess of diisopropylethylamine (DIEA) in *N*-methylpyrrolidone.[10] The cyclization step (4 h) was repeated until a negative Kaiser ninhydrin test[11] was obtained. Three to four recouplings were necessary.

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 48 h. After washing with MeOH and DCM and drying, 3.2 g of protected peptide hydrazide MBHA-resin was obtained. The Ac-D-2Nal[1]-D-pClPhe[2]-D-3Pal[3]-Asp(NHNH$_2$)[4]-Glu[5]-D-Arg(Tos)[6]-Leu[7]-Dbu(Gly)[8]-Pro[9]-Dpr(Z)[10]-MBHA-resin was treated with liquid HF (60 mL) at 20 °C (20 min) and 0 °C (60 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 10% aqueous acetonitrile and lyophilized to yield a fluffy, crude peptide hydrazine (1.3 g) which exhibited a major component (50%) by high-performance liquid chromatography (HPLC).

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Crude peptide hydrazide (1.3 g, 0.9 mmol) was dissolved in DMF (30 mL) at −25 °C; 4 N HCl in dioxane (1.02 mL, 4.1 mmol) and isoamyl nitrite in three aliquots (0.2 mL, 1.5 mmol) were added with stirring over 15 min. Stirring at −25 °C was continued for 3 h. The solution of peptide azide was diluted with DMF (600 mL precooled to −25 °C), and DIEA was added until the pH on moistened pH indicator gave a value of 7.6. The solution was stored at −25 °C (24 h) and 5 °C (72 h). The solvent was evaporated under vacuum to yield crude cyclic peptide [1.3 g after lyophilization from water/acetonitrile (3:1)], which exhibited a major component by HPLC.

Peptide Purification by Reversed-Phase HPLC. Crude cyclic peptide (1.0 g) was dissolved in 0.25 M triethylammonium phosphate (50 mL) buffer, pH 2.25 (TEAP 2.25), containing acetonitrile and purified according to published procedures.[12] Peptide was loaded onto a preparative reversed-phase HPLC cartridge (5 × 30 cm) packed with Vydac C_{18} silica gel (15−20 μm particle size) (The Separations Group, Hesperia, CA). The peptide was eluted under gradient conditions (40−60% B in 60 min), with solvent A being TEAP 2.25 and solvent B being 60% acetonitrile and 40% A, at a flow rate of 100 mL/min. Analytical control of individual fractions was carried out using reversed-phase analytical HPLC (Vydac C_{18}) under isocratic conditions (56% B, t_R = 4.1 min). The selected fractions were diluted (1:1) with water and further purified preparatively using different solvents, A (0.1% TFA) and B (60% acetonitrile/40% A), under gradient conditions (30−70% B in 40 min). Selected fractions were lyophilized to yield 50 mg of cyclic peptide of high purity. The observed mass spectral value of 1403.7 for the monoisotopic protonated molecular ion was in agreement with the calculated value of 1403.68. $[\alpha]_D$ is −36° (c = 1 in AcOH).

Nuclear Magnetic Resonance. NMR studies were performed on a 5 mM solution of **1** in 650 μL of $CDCl_3$/DMSO-d_6 (2:1, v/v). All spectra were acquired at 500 MHz on a Varian VXR500 spectrometer. Resonance assignments were obtained at 5 and 25 °C from two-dimensional (2D) correlated spectroscopy (COSY),[13] 2D total correlation spectroscopy (TOCSY),[14] and 2D nuclear Overhauser spectroscopy (NOESY)[15] experiments. Coupling constants were measured from a one-dimensional (1D) spectrum and a primitive E-COSY (PE COSY) experiment[16] acquired at 35 °C. Amide proton temperature coefficients were obtained from five 1D spectra and five TOCSY spectra acquired at temperatures ranging from 5 to 45 °C. Interproton distances were calculated from measurement of cross-peak volumes in a series of NOESY spectra acquired at 5 °C with mixing times of 75, 100, 125, and 200 ms. The methodology and parameters used for acquisition of the spectra, data processing, and calculation of interproton distances are analogous to those described previously for the analysis of the monocyclic (4-10) GnRH antagonist **4**.[7a]

Computational Analysis. Structures of **1** were obtained using the DISCOVER and INSIGHTII programs (Biosym Technologies Inc., San Diego, CA) on a Silicon Graphics 4D/25 Personal Iris workstation and a CRAY Y-MP8/864 supercomputer from the Center for High Performance Computing in Austin, TX. A full consistent valence force field with the parameters described in ref 17 was used in the calculations. Initial structures compatible with sets of NMR restraints were generated with the standard simulated annealing protocol implemented in INSIGHTII/DISCOVER, which is adapted from ref 18. To release additional strain energy, the structures with the lowest violations were subjected to a 10 ps molecular dynamics simulation at 300 K and to energy minimization until the maximum derivative was 0.1 kcal/(mol Å). The methodology used in molecular dynamics simulations and energy minimizations, as well as that used to implement the NMR restraints in all calculations, is analogous to that described for the analysis of **4**.[7b] The set of NMR restraints used in the calculations was incremented gradually as a function of the results obtained, as described in the Results section.

Table 2. ^{1}H NMR Chemical Shifts for the Dicyclic GnRH Analog **1**[a]

residue	NH	H^α	$H^{\beta1}$	$H^{\beta2}$	others
Ac^0					CH_3 1.76
D-2Nal[1]	8.07	4.59	3.09[b]	2.86[c]	$H^{\delta1}$ 7.60, $H^{\delta2}$ 7.30, $H^{\epsilon2}$ 7.65, $H^{\zeta1}/H^{\eta2}$ 7.72, $H^{\eta1}/H^{\theta}$ 7.38
D-pClPhe[2]	8.15	4.47	2.97[b]	2.76[c]	$H^{\delta}{}_2/H^{\epsilon}{}_2$ 7.16
D-3Pal[3]	7.76	4.66	3.10[b]	2.83[c]	$H^{\delta1}$ 8.59, $H^{\delta2}$ 8.12, $H^{\epsilon2}$ 7.66, H^{ζ} 8.56
Asp[4]	8.61	4.79	2.92[c]	2.43[b]	
Glu[5]	9.03	4.23	2.41[b]	1.80[c]	$H^{\gamma1}$ 2.65,[b] $H^{\gamma2}$ 2.10[c]
Gly[5′]	8.36	4.04, 3.08			
D-Arg[6]	9.08	3.51	1.90	1.60	$H^{\gamma}{}_2$ 1.68, $H^{\delta1}$ 3.15, $H^{\delta2}$ 3.11, NH^{ϵ} 7.50, $H^{\eta1}{}_2$ 7.24, $H^{\eta2}{}_2$ 6.75
Leu[7]	8.26	3.72	1.88	1.62	H^{γ} 1.48, $H^{\delta1}{}_3$ 0.84, $H^{\delta2}{}_3$ 0.78
Dbu[8]	6.47	4.08	2.14[c]	1.31[b]	$H^{\gamma1}$ 3.87,[c] $H^{\gamma2}$ 2.59,[b] NH^{δ} 7.30
Pro[9]		4.21	2.10	1.81	$H^{\gamma1}$ 2.04, $H^{\gamma2}$ 1.88, $H^{\delta1}$ 3.70, $H^{\delta2}$ 3.46
Dpr[10]	8.54	4.45	4.18[b]	2.71[c]	NH^{γ} 7.58
NH_2					$NH_2$1 7.55, $NH_2$2 7.11

[a] Obtained in $CDCl_3$/DMSO-d_6 (2:1, v/v) at 5 °C. Chemical shifts were referenced to the residual DMSO solvent signal (at 2.49 ppm); uncertainty ±0.02 ppm. [b] *Pro-R*. [c] *Pro-S*.

Table 3. Amide Temperature Coefficients of the Dicyclic GnRH Analog **1**[a]

amide proton	$\Delta\delta/\Delta T$	amide proton	$\Delta\delta/\Delta T$
D-2Nal[1] NH	5.4	D-Arg[6] NH	4.0
D-pClPhe[2] NH	6.0	Leu[7] NH	1.0
D-3Pal[3] NH	3.2	Dbu[8] NH	0.5
Asp[4] NH	7.8	Dbu[8] NH^{δ}	0.0
Glu[5] NH	6.4	Dpr[10] NH	5.8
Gly[5′] NH	3.5	Dpr[10] NH^{γ}	3.5

[a] $\Delta\delta/\Delta T$ (ppb/K) in $CDCl_3$/DMSO-d_6 (2:1, v/v).

Results

Nuclear Magnetic Resonance. Study of the conformational behavior in solution of the dicyclic GnRH analog **1** was performed using NMR spectroscopy and computational techniques, in analogous manner to that followed previously to study the monocyclic analog **4**[7] and the dicyclic analog **2**,[2] and employing also a similar solvent mixture, $CDCl_3$/DMSO-d_6 (2:1, v/v). Sequential assignment[19] of all the proton resonances in the molecule (Table 2) was accomplished using 2D COSY, TOCSY, and NOESY data. The conformational analysis was based on three different types of parameters: (i) amide proton accessibility data deduced from chemical shift temperature coefficients (Table 3), (ii) torsion angle information derived from vicinal coupling constants (Table 4), and (iii) interproton distances calculated from nuclear Overhauser effects (NOEs). Out of more than 150 interproton distances quantitated, 74 were selected to be used as restraints in the subsequent computational analysis (supporting information). The most conformationally relevant interproton distances are summarized in Table 5, and Figure 2 shows expansions of a 2D NOESY spectrum where the cross-peaks corresponding to most of these interactions can be observed.

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Table 4. Coupling Constants in the Dicyclic GnRH Analogue **1**[a]

residue	$^3J_{HN\alpha}$	$^3J_{\alpha\beta1}$	$^3J_{\alpha\beta2}$	residue	$^3J_{HN\alpha}$
D-2Nal[1]	7.3	5.1	9.2	D-Arg[6]	5.8
D-pClPhe[2]	8.5	4.5	9.3	Leu[7]	7.3
D-3Pal[3]	8.4	4.6	5.4	Dbu[8]	6.7
Asp[4]	7.3	11.5	3.3	Dpr[10]	8.5

[a] Coupling constants (Hz) measured in $CDCl_3$/DMSO-d_6 (2:1, v/v) at 35 °C. The Glu[5] $^3J_{HN\alpha}$ coupling could not be measured due to line broadening.

Table 5. Interproton Distances with Most Conformational Relevance Measured for the Dicyclic GnRH Analogue **1**[a]

interproton distance		measured	limits
D-2Nal[1] H^α	D-pClPhe[2] NH	2.45	2.25−2.75
D-pClPhe[2] H^α	D-3Pal[3] NH	2.45	2.25−2.75
D-pClPhe[2] $H^{\beta1}$	D-3Pal[3] NH	3.3	3.0−3.7
D-pClPhe[2] $H^{\beta2}$	D-3Pal[3] NH	3.4	3.1−3.9
D-pClPhe[2] NH	D-3Pal[3] NH	2.75	2.45−3.05
D-3Pal[3] H^α	Asp[4] NH	2.4	2.2−2.7
D-3Pal[3] NH	Asp[4] NH	2.9	2.6−3.3
Asp[4] H^α	Glu[5] NH	2.35	2.15−2.65
Asp[4] $H^{\beta1}$	Dbu[8] NH	3.35	3.05−3.75
Glu[5] H^α	D-Arg[6] NH	2.25	2.05−2.45
Glu[5] H^α	Leu[7] NH	3.3	3.0−3.6
Gly[5′] NH	Dbu[8] NH^δ	2.65	2.45−2.95
D-Arg[6] H^α	Leu[7] NH	3.25	2.95−3.55
D-Arg[6] NH	Leu[7] NH	2.7	2.5−3.0
D-Arg[6] H^α	Dbu[8] NH	3.9	3.3−4.5
D-Arg[6] H^α	Dbu[8] $H^{\gamma2}$	2.8	2.5−3.1
D-Arg[6] H^α	Dbu[8] NH^δ	3.1	2.8−3.4
Leu[7] H^α	Dbu[8] NH	2.85	2.55−3.15
Leu[7] NH	Dbu[8] NH	2.65	2.45−2.95
Dbu[8] NH	Dbu[8] NH^δ	3.25	2.95−3.55
Dbu[8] H^α	Pro[9] $H^{\delta*}$	2.35	2.15−3.35
Pro[9] H^α	Dpr[10] NH	2.2	2.0−2.4
Dpr[10] NH	Dpr[10] NH^γ	2.6	2.4−2.9

[a] Measured in $CDCl_3$/DMSO-d_6 (2:1, v/v) at 5 °C. Distances are given in Å. Limits correspond to estimated error ranges that were used as restraints in the computational analysis. Pseudoatoms representing methylene protons that have not been assigned stereospecifically are indicated by an asterisk.

The NMR data summarized above yields a clear picture of the conformational behavior of **1**. The dicyclic part of the molecule (residues 4-10) appears to be very rigid, as indicated by the observation of large chemical shift differences between pairs of diastereotopic methylene protons such as the H^α protons of Gly[5′] and protons in the side chains of Asp[4], Glu[5], and Dbu[8]. This conclusion is also supported by the large difference between the Asp[4] $^3J_{\alpha\beta1}$ and $^3J_{\alpha\beta2}$ coupling constants and the observation of several nonsequential NOEs (Asp[4] $H^{\beta1}$ to Dbu[8] NH, Glu[5] H^α to Leu[7] NH, and D-Arg[6] H^α to Dbu[8] NH, $H^{\gamma2}$, and NH^δ). The low temperature coefficient of Dbu[8] NH and the observation of a Leu[7] NH/Dbu[8] NH NOE is characteristic of a β-turn conformation around residues 6−7. However, the D-Arg[6] NH/Leu[7] NH NOE and the weak intensity of the D-Arg[6] H^α/Leu[7] NH NOE (Figure 2) show that the β-turn is type I′ rather than type II′, in contrast to what had been observed in all the constrained GnRH antagonists we had studied previously.[2,5,7] The β turn appears to be somewhat distorted, since the Leu[7] H^α/Dbu[8] NH distance (2.85 Å, Table 5) is shorter than that expected for a standard type I′ β turn (3.3 Å). A distorsion of the turn is also supported by the very upfield chemical shift of Dbu[8] NH (6.47 ppm, Table 2), which indicates that this amide proton is not hydrogen bonded despite being sequestered from the solvent. The computational analysis described below showed that this distorsion correlates with the formation of hydrogen bonds between Dbu[8] NH^δ and Glu[5] CO (the natural partner for Dbu[8] NH in a standard β turn) and between Leu[7] NH and Asp[4] CO. Note that Leu[7] NH and Dbu[8] NH^δ are more likely to be hydrogen bonded than Dbu[8] NH, since they are sequestered from the solvent (Table 3) without being shifted upfield as Dbu[8] NH (Table 2). The formation of a hydrogen bond involving Dbu[8] NH^δ suggests that the Glu(Gly)[5]−Dbu[8] bridge adopts a well-defined conformation, which is also supported by the observation of Gly[5′] NH/Dbu[8] NH^δ, D-Arg[6] H^α/Dbu[8] NH^δ, and Dbu[8] NH/NH^δ NOEs (Figure 2, Table 5).

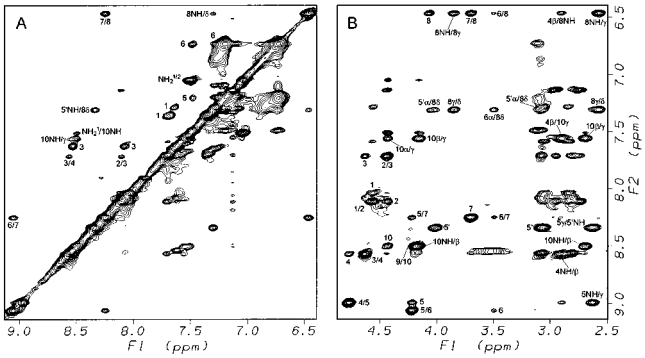

Figure 2. Contour maps corresponding to different regions of a NOESY spectrum of the dicyclic GnRH analog **1** in $CDCl_3$/DMSO-d_6 (2:1, v/v), recorded at 5 °C with a 200 ms mixing time. A: Amide/amide and aromatic/aromatic region. B: Amide, aromatic/aliphatic region. In A, intraresidue NOEs from the aromatic and guanidino groups in the side chains are labeled with the corresponding residue number and sequential amide/amide interactions have been labeled with the two residue numbers. In B, single numbers are used for intraresidue NH/H^α NOEs, and two residue numbers are used to indicate sequential $H^\alpha(i)$/NH($i+1$) interactions. Other NOEs in A and B have been labeled using both the residue numbers and the positions of the corresponding protons (one number for intraresidue and two numbers for interresidue NOEs).

The hydrogen bond between Leu[7] NH and Asp[4] CO, and all the interproton distances between the backbone protons of residues Glu[5]-D-Arg[6]-Leu[7] (Table 5), is typical of a standard type II β turn having residues 5−6 in the corner positions, another structural feature that was not observed in our previous studies of constrained GnRH antagonists.[2,5,7] The orientation of Asp[4] CO toward the inside of the molecule implies that Glu[5] NH is pointing outwards and exposed to the solvent (it has a high temperature coefficient, Table 3). Thus, a hydrogen bond between the NH of residue 5 and the CO of residue 8, characteristic of the β-hairpin-like conformation observed for **2**−**4**,[2,5,7] is not present in **1**. Model building to fit the NMR data corresponding to the Asp[4]−Dpr[10] bridge, and the computational analysis described below, showed that the CO of Dbu[8] is instead hydrogen bonded to Dpr[10] NH^γ, which has a relatively low temperature coefficient (Table 3). This hydrogen bond closes a type II β-turn-like structure around Pro[9] and the side chain of Dpr[10] and appears to confer higher rigidity to the Asp[4]−Dpr[10] bridge than observed for **2** and **4**. The interproton distances in this region and the very distinct Asp[4] $^3J_{\alpha\beta}$ coupling constants (Table 4) support this conclusion.

The different conformation adopted by residues 4-10 in **1**, compared to **2** and **4**, has a dramatic effect on the orientation of the tail formed by residues 1−3 with respect to the rest of the molecule. The proximity of Asp[4] H^α to Glu[5] NH and of Asp[4] $H^{\beta1}$ to Dbu[8] NH (Table 5), together with the formation of the hydrogen bond between Asp[4] CO and Leu[7] NH (see above), implies that the tail is oriented below the ring formed by residues 4-10. In contrast, the tail was oriented above the ring in the monocyclic (4-10) analog **4**[7] and the dicyclic (4-10/5-8) analog **2**.[2] The conformational behavior of residues 1−3 of **1**, on the other hand, appears to be similar to that observed for **2** and **4**: The observation of sequential NH^i/NH^{i+1} interactions (Table 5) and of a low D-3Pal[3] NH temperature coefficient (Table 3)

suggest the presence of some preferred, turnlike structures, but the data do not seem to be compatible with a single conformation.

Computational Analysis. To develop a more quantitative picture of the conformational behavior of the dicyclic analog **1**, and to generate three-dimensional structures compatible with the NMR data, we used a combination of computational techniques based on a full consistent valence force field.[17] A set of restraints for interproton distances, torsion angles, and hydrogen bonds were derived from the NMR data. These restraints were introduced in a progressive fashion into simulated annealing calculations[18] to search the conformational space consistent with the data and test for the possibility of conformational averaging. Initially, only intraresidue and sequential interproton distances were restrained. The distances corresponding to nonsequential interactions fell within the ranges of values measured in almost all structures generated, showing the self-consistency of the data and supporting the conclusion that the conformation of the dicyclic part of the molecule is highly rigid. The nonsequential restraints, together with torsion angle restraints, were then introduced into additional calculations. Since hydrogen bonds between Leu^7 NH/Asp^4 CO, Dbu^8 NH$^\delta$/Glu^5 CO, and Dpr^{10} NH$^\gamma$/Dbu^8 CO were observed in many of the resulting structures, and such hydrogen bonds are consistent with the low temperature coefficients of the corresponding amide protons (Table 3), restraints for these hydrogen bonds were used in the final calculations. In all simulated annealing calculations, interproton distances involving methylene protons were introduced as floating restraints to test whether unique prochiral assignments were consistent with the data or not. Preliminary prochiral assignments for D-2Nal^1, D-pClPhe^2, D-3Pal^3, and Asp^4 H$^\beta$ protons were confirmed in this way, and prochiral assignments for Glu^5 and Dbu^8 H$^{\beta,\gamma}$ protons, as well as for Dpr^{10} H$^\beta$ protons, were obtained (see Table 1).

The gradual incorporation of restraints and careful analysis of the restraint violations showed that conformational averaging is only likely to occur in the tail formed by residues 1−3. Indeed, systematic violation of the D-pClPhe^2 H$^\alpha$/D-3Pal^3 NH restraint was observed since the short distance measured (2.4 Å) is incompatible with the proximity of the D-pClPhe^2 NH and H$^\beta$ protons to D-3Pal^3 NH (Table 5). Removal of the D-pClPhe^2 H$^\alpha$/D-Pal^3 NH restraint yielded lower restraint violations in the tail region and better convergence for its conformation, which often included β-turn-like structures around residues 1−2, closed by a hydrogen bond between D-3Pal^3 NH and Ac^0 CO. The relatively low temperature coefficient of D-3Pal^3 NH (Table 3) supports the presence of these turn conformations, but the short D-pClPhe^2 H$^\alpha$/D-3Pal^3 NH distance indicates that more extended tail conformations are also significantly populated.

A set of 94 restraints, 74 for interproton distances, seven for torsion angles (three from the backbone and four from the side chains), three for hydrogen bonds, and 10 to ensure the proper chirality of the C$^\alpha$ carbons, was used in our final simulated annealing calculations (supporting information). Out of 30 structures generated with these restraints, the 10 structures with the lowest violations were refined further by performing 10 ps molecular dynamics simulations followed by energy minimizations. The simulations allowed the release of strain energy and led to lower restraint violations and a better convergence of both the energies and the backbone conformations. While the energies of the 10 best simulated annealing structures ranged from 159 to 195 kcal/mol, those of the refined structures ranged from 156 to 167 kcal/mol.[20] The root mean square (rms) deviations between the backbone atoms of residues 4-10 of the 10 structures also decreased, from 0.8 Å or less to 0.4 Å or less, and were smaller than 0.2 Å in most cases. In the 10 final structures, there were only two to four interproton distance violations larger than 0.1 Å and none larger than 0.2 Å. A superposition of these 10 structures, where the rms deviation between the backbone atoms of residues 4-10 has been minimized, is shown in Figure 3A; the same superposition, displaying only the 4-10 backbone in a different orientation, is shown in Figure 3B. Note that the conformation of residues 4-10, including that of the 4-10 and 5-8 bridges, is very well defined. Residues 1−3 are more disordered, and part of the convergence observed in this region is artifactual (see above). A stereoview of the dicyclic region of the lowest energy structure obtained, which also contained the lowest violations, is shown in Figure 3C. The backbone torsion angles for this structure are listed in the legend of Figure 3; most of the torsion angles of the other 10 structures are within 10° of these values, and all are within 20°.

Discussion

The conformational analysis of the dicyclic GnRH antagonist **1** described above reveals that the dicyclic part of this molecule (residues 4-10) adopts a well-defined, rigid conformation under the conditions of this study. This conformation includes a type II β turn around residues 5−6, nested with a distorted 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. This conformation is substantially different from that adopted under analogous conditions by the equipotent dicyclic antagonist **2**, which contained a β-hairpin structure in residues 5-8, brought about by a type II′ β turn around residues 6−7.[2] Note that, although this compound exhibited some more flexibility in the 4-10 and 5-8 bridges than **1**, the backbone conformation of residues 4-10 was also well defined, in particular for residues 5-8.[2] A superposition of a representative structure of **2** with the structure of **1** depicted in Figure 3C, where the rms deviation for the backbone atoms of residues 4-10 has been minimized, is shown in Figure 4. The rms deviation among these atoms is 1.7 Å, while the minimum rms deviation for all backbone atoms is 3.8 Å. These deviations are quite large for molecules of this size.

As can be observed in Figure 4, a good part of the conformational differences between **1** and **2** are concentrated in the region encompassing residues 5-8. This is remarkable since this is the region that is expected to be most constrained in these molecules. Note that the sequences of **1** and **2** are very similar and the 5-8 bridge is only one atom longer in **1** than in **2**. The observation that such a "small" change in the bridge length has such a dramatic structural effect is very interesting from the point of view of our general understanding of polypeptide conformation. Two basic arguments can be used to explain this result. On the one hand, it is possible that the two bridges do not constrain these molecules to the extent that one might expect, and many other conformations exist with energies only slightly higher than those of the conformations observed experimentally; a small change in chemical structure may then shift the equilibrium in favor of any of these conformations. In this case, rationalization of the biological significance of the results of conformational studies of con-

(20) Note that the convergence of the energies is very good considering that, for molecules of this size, energy differences of up to 30 kcal/mol can result from solvation energies and entropic effects that are not included in the calculations (see ref 7b).

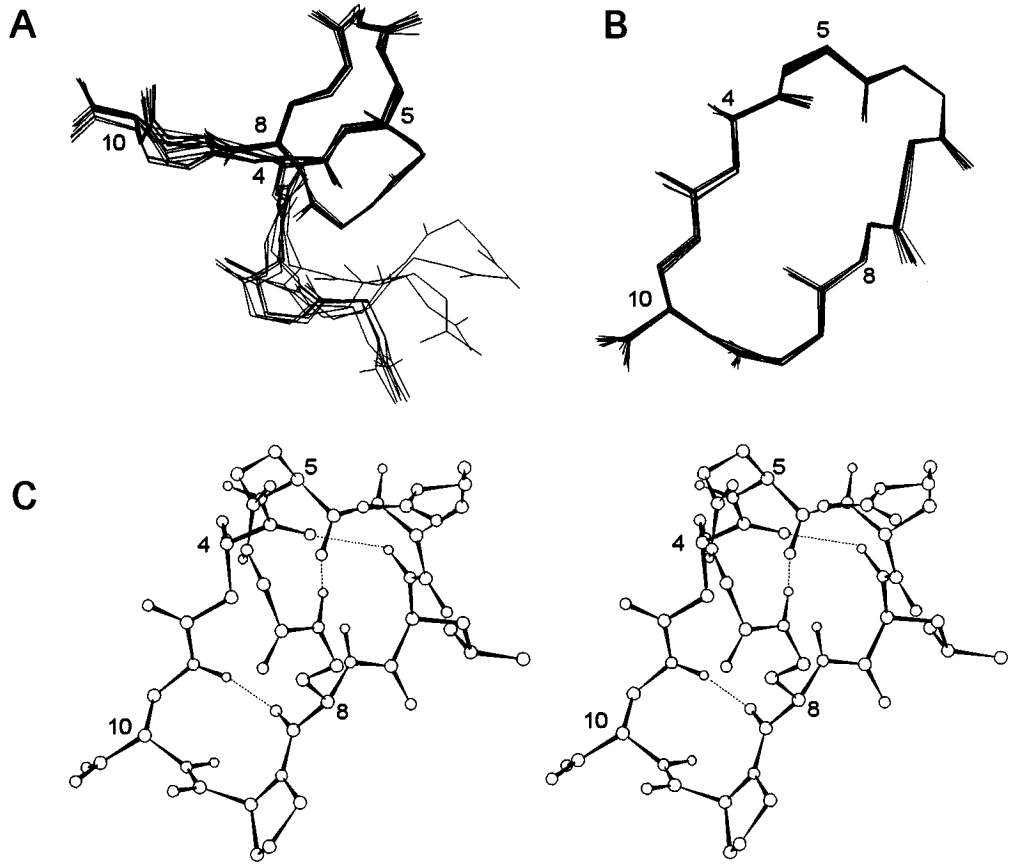

Figure 3. A: Superposition of 10 structures of the dicyclic analog **1** obtained with simulated annealing, restrained molecular dynamics simulations, and energy minimizations (see text). Only the backbone atoms and the heavy atoms in the 4-10 and 5-8 bridges are displayed. The superposition was obtained by minimizing the rms deviation between the backbone atoms of residues 4-10. B: Another view of the same structures in a different orientation, which illustrates the good definition of the conformation in residues 4-10; only the backbone atoms of residues 4-10 and the heavy atoms of the 4-10 bridge are displayed. C: Stereo view of the lowest energy structure of **1** obtained in our calculations (which is also the structure with the lowest restraint violations) in the same orientation used in B. All heavy atoms and amide hydrogens from residues 4-10 are displayed. Hydrogen bonds are indicated by dashed lines. The backbone dihedral angles (deg) of this structure are ϕ_1 85, ψ_1 −71; ϕ_2 80, ψ_2 30; ϕ_3 79, ψ_3 −38; ϕ_4 −73, ψ_4 111; ϕ_5 −51, ψ_5 134; ϕ_6 70, ψ_6 21; ϕ_7 52, ψ_7 45; ϕ_8 −93, ψ_8 157; ϕ_9 −68, ψ_9 108; ϕ_{10} −91, ψ_{10} 88. The positions of the bridged residues are indicated in A−C by the corresponding residue numbers beside their C^α carbons.

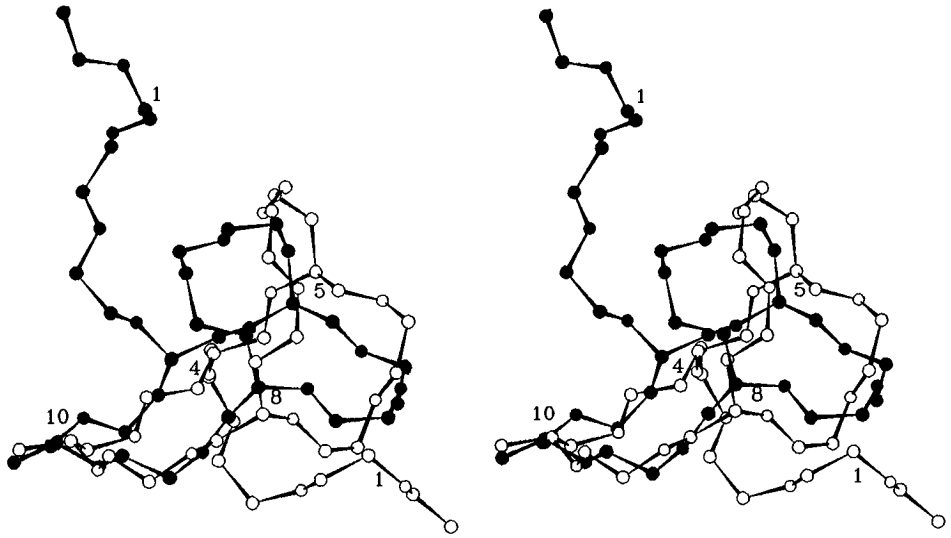

Figure 4. Stereoview of a superposition of a representative structure of the dicyclic GnRH antagonist **2** (●)[2] with the structure of the dicyclic antagonist **1** shown in Figure 3C (○). The rms deviation between the backbone atoms of residues 4-10 was minimized. Only C and N atoms in the backbone and the 4-10 and 5-8 bridges are shown. The positions of the C^α carbons of residue 1 and the bridged residues are indicated with the corresponding residue numbers. Note the large difference in the conformations of residues 5−8 and the completely different orientation of the N-terminus with respect to the rest of the molecule.

strained analogs in solution must be done with extreme caution, since there is a good likelihood that the analogs change conformation upon binding to their receptors. On the other hand, it is also possible that the two bridges in **1** and **2** do indeed constrain these molecules to a high degree, but a small, discrete number of conformations are still allowed. In such case,

conformational analysis of the constrained analogs in solution is much more likely to yield biologically relevant results, although caution must still be taken in their interpretation to account for the possibility that a conformational change occurs upon receptor binding. Careful theoretical analysis of the conformational space available to **1** and **2**, and of the energetic cost of forcing **1** to adopt the structure observed experimentally in **2** (and vice versa), should shed light on this issue. The conformational differences between **1** and **2** in residues 5-8 are also striking because the β-hairpin structure observed in **2** is the most conserved conformational feature in all the constrained GnRH antagonists we have studied so far.[2,5,7] The persistence of this β-hairpin structure in all the previously analyzed constrained antagonists supports the hypothesis that only a very limited number of conformations are possible in these constrained molecules, and the conformation observed for the dicyclic antagonist **1** may only be an exception caused by new hydrogen-bonding opportunities provided by the presence of a glycine residue in the 5-8 bridge.

The proposal that a type II′ β turn around residues 6−7 is important for the biological activity of GnRH arose from early theoretical calculations on GnRH[21] and from the observation of increased potency in linear GnRH analogs with a D-amino acid residue in position 6,[22] which should stabilize this type of turn.[23] The later development of biologically active GnRH analogs with a 6−7 γ-lactam, designed to stabilize the type II′ β-turn conformation,[4] has remained as a paradigm of the use of covalent constraints in peptide analog design. To our knowledge, the dicyclic compound **1** is the first highly potent, constrained GnRH antagonist that has been observed to form a type I′ β turn, rather than a type II′ turn, around residues 6−7. Athough it could be that **1** changes conformation upon binding to the GnRH receptor to form a type II′ β turn, it is also possible that the presence of a 6−7 turn, rather than the turn type, is important for biological activity. In fact, different types of turn may bring about two ends of a molecule in a similar fashion, so that analogous surfaces are offered for binding to the receptor. Yet another possibility is that the conformation observed for **1** is biologically relevant, and all other compounds that we have studied previously adopt such conformation upon receptor binding. From the point of view of the design of GnRH antagonists, more puzzling than the different type of turn around residues 6−7 is the radically different orientation of the tail formed by residues 1−3 with respect to the rest of the molecule observed in **1** and **2** (Figure 4). This observation, and the existence of conformational flexibility in the tail in all these constrained GnRH antagonists, underlines the necessity to introduce constraints in the N-terminus to fully define the conformational requirements for GnRH antagonist activity. To address this issue, we have developed a new series of GnRH antagonists with constraints involving residues 1−3 (ref 8b and our unpublished results) and are currently analyzing the conformations of the most potent of these compounds.

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Conclusions

Through design, synthesis, and analysis of constrained GnRH antagonists, we are developing a detailed model of the structural requirements for GnRH antagonist activity. A key question in this strategy is to what extent the covalent constraints introduced limit the conformational possibilities of the resulting analogs. The conformational analysis of the highly potent, dicyclic GnRH antagonist **1** presented here shows that the conformation of residues 4-10 in this compound contains a type II β turn around residues 5−6, a type I′ β turn around residues 6−7, and a type II β-turn-like structure around residue 9 and the side chain of residue 10. This is a novel conformation that had not been observed in any previously analyzed GnRH antagonist. The remarkable difference between this conformation and that adopted by the dicyclic antagonist **2**, which has very similar sequence and constraints, shows that the dicyclic (4-10/5-8) backbone can adopt more than one family of conformations. It is possible that either of these compounds changes conformation upon binding to the GnRH receptor or that both conformations are biologically relevant and they can offer a similar binding surface. Definition of the structural requirements for activity at the N-terminus will be necessary to resolve this issue.

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Supporting Information Available: Listing of all the restraints used to generate structures of the dicyclic GnRH antagonist **1** (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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