Consensus Bioactive Conformation of Cyclic GnRH Antagonists Defined by NMR and Molecular Modeling†

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Little is known of the conformation of peptide hormones as they interact with their receptors for a number of reasons: peptide hormones are notoriously flexible in solution, their receptors are particularly complex, and there is strong evidence that receptor−ligand interaction leading to activation is a dynamic process. Insights into the active conformation of the decapeptide gonadotropin releasing hormone (GnRH) have been obtained previously from the solution structures of four constrained GnRH antagonists {cyclo(1−10)[Ac-Δ^3-Pro^1,D-Cpa^2,D-Trp^{3,6},-NMeLeu^7,βAla^{10}]GnRH (**1**), cyclo(4−10)[Ac-Δ^3Pro^1,D-Fpa^2,D-Trp^3,Asp^4,D-Nal^6,Dpr^{10}]GnRH (**2**), dicyclo(4−10/5−8)[Ac-D-Nal^1,D-Cpa^2,D-Trp^3,Asp^4,Glu^5,D-Arg^6,Lys^8,Dpr^{10}]GnRH (**3**), and dicyclo(4−10/5−5′−8)[Ac-D-Nal^1,D-Cpa^2,D-Pal^3,Asp^4,Glu^5(Gly),D-Arg^6,Dbu^8,Dpr^{10}]GnRH (**4**)}. However, the precise location of the N-terminal tripeptide in the highly potent (K_i < 0.4 nM) **2**−**4** remained unclear due to the lack of constraints in this region. The NMR structure of the newly discovered and potent (K_i = 0.24 nM) dicyclo(1−1′−5/4−10)[Ac-Glu^1(Gly),D-Cpa^2,D-Trp^3,Asp^4,Dbu^5,D-Nal^6,-Dpr^{10}]GnRH (**5**) now allows the definition of the conformation of this region. A combined computational analysis (consensus forcing) of compounds **2**−**5**, designed to explore the common conformations available to them that are simultaneously consistent with the NMR data corresponding to each compound, leads to a consensus structural model for the GnRH pharmacophore. This model shares some common features with the structure of the nonpeptidic GnRH mimetic T-98475. In the course of that comparative study, two additional contact points to those proposed by the authors are identified, suggesting that this model has predictive value.

Introduction

The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is under the positive control of the decapeptide amide gonadotropin-releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, GnRH). Since the elucidation of its primary structure in 1971,[1,2] GnRH has been the subject of intense structure−activity relationship (SAR) studies aimed at elucidating its mechanism of action and identifying drug candidates (agonists and antagonists), which subsequently have been studied extensively in the clinic.[3−5]

Given the flexibility inherent to peptides, the unambiguous assignment of a single bioactive conformation of any linear GnRH antagonist is problematic due in part to the fact that such a conformation may reside in equilibrium with a manifold of similar structures. Further, the propensity to assume a single bioactive conformation may be facilitated by receptor binding, which may favor a conformation not observed in solution. Noncovalent modifications such as D-amino acid substitution[6] and backbone alkylation[7,8] have been used to stabilize the structure of GnRH agonists and have been reviewed recently by Kutscher et al.[9]

Extensive structural studies have not yet yielded an appreciation of the three-dimensional structure of GnRH, as seen at the receptor site; furthermore, there is no current evidence that GnRH or GnRH analogues have been crystallized. The GnRH receptor has been cloned in a number of species including mouse,[10,11] rat,[11−14] sheep,[15,16] human,[17,18] cow,[19] pig,[20] and catfish[21] and belongs to the seven transmembrane receptor family for which limited experimental structural information is available. Transmembrane domains TM2, TM3, TM5, TM6, and TM7 and extracellular loop E1 are most highly conserved (>90% homology). By comparing mammalian receptor sequences, Illing et al. identified those residues that may be responsible for the subtle differences in pharmacology.[16] What is known of the structure of the GnRH receptor is inferred from mutagenesis and homology studies carried out mostly by the groups of Sealfon and Millar.[22−32]

It is because of the complexity of this system that our long-term strategy has been to employ covalently constrained GnRH antagonists that are highly potent in

† 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; Cpa, 4-chlorophenylalanine; Dpr, 2,3-diaminopropionic acid; Fpa, 4-fluorophenylalanine; GnRH, gonadotropin releasing hormone; HPLC, high performance liquid chromatography; LH, luteinizing hormone; MeLeu, methylleucine; MES, minimum energy structure; Nal, 3-(2′-naphthyl)-alanine; Pal, 3-(3′-pyridyl)- alanine; RMS, root-mean-square; SA, simulated annealing; SAR, structure−activity relationships; sc, subcutaneous; Xaa, any amino acid.

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Table 1. Structure, Affinity, and Antiovulatory Activity of Cyclic GnRH-like Molecules[a]

no.	structure of cyclic analogues[a]	K_i (nM)[b]	AOA[c] dose	AOA[c] no. rats	ref
1	cyclo(1−10)[-Δ³-Pro-DCpa-DTrp-Ser-Tyr-DTrp-NMeLeu-Arg-Pro-βAla-]	14 ± 0.99	1000	5/8	34
2	cyclo(4−10)[Ac-Δ³Pro-DFpa-DTrp-c[Asp-Tyr-D2Nal-Leu-Arg-Pro-Dpr-NH_2]	0.27 ± 0.03	10	2/20	35
3	dicyclo(4−10/5−8)[Ac-D2Nal-DCpa-DTrp-Asp-Glu-DArg-Leu-Lys-Pro-Dpr-NH_2]	0.32 ± 0.06	5.0	3/20	36
4	dicyclo(4−10/5,5′−8)[Ac-D2Nal-DCpa-DPal-Asp-Glu(Gly)-DArg-Leu-Dbu-Pro-Dpr-NH_2]	0.14 ± 0.03	5.0	2/8	37
5	dicyclo(1,1′−5/4−10)[Ac-Glu(Gly)-DCpa-DTrp-Asp-Dbu-D2Nal-Leu-Arg-Pro-Dpr-NH_2]	0.24 ± 0.03	2.5	2/8	58

[a] Structure of GnRH is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. [b] See Experimental Section of paper no. 1 in this series for assay details. Average ± SEM of at least three independent determinations is reported. [c] See Experimental Section of paper no. 1 in this series for assay details. Dose is in μg/rat; no. rats is the number of rats ovulating over total.

Table 2. Resonance Assignments of **5** in $CDCl_3$/DMSO-d_6 1:1 (v/v) at 12 °C Referenced to the Residual DMSO Solvent Signal at 2.49 ppm; Uncertainty ± 0.02 ppm

residue	NH	Hα	Hβ	others
Ac^0				Me 1.80
Glu^1	7.91	4.31	1.84, 1.64	Hγ 2.09, 1.84
$Gly^{1'}$	8.20	3.74		
		3.53		
D-Cpa^2	8.16	4.38	2.95, 2.75	Hδ 7.05
D-Trp^3	8.08	4.47	3.15, 3.04	Hδ1 7.12, NHε1 10.67, Hζ2 7.29, Hη2 7.02, Hζ3 6.93, Hε3 7.51
Asp^4	8.44	4.50	2.52	
Dbu^5	7.88	3.97	1.80	Hγ 3.00, NHδ 7.73
D-Nal^6	8.51	4.40	3.13, 3.04	Hδ1 7.69, Hζ1 7.77, Hη1 7.44, Hθ 7.46, Hη2 7.84, Hε2 7.79, Hδ2 7.39
Leu^7	8.21	4.04	1.41	Hγ 1.07, Hδ 0.60
Arg^8	7.30	4.38	1.76, 1.53	Hγ 1.51, Hδ 3.08, NHε 7.37
Pro^9		4.28	2.09, 1.87	Hγ 1.99, 1.88, Hδ 3.75, 3.48
Dpr^{10}	8.20	4.42	3.73, 3.07	NHγ 7.41
NH_2	7.30			
	7.10			

vivo or have low K_d in vitro, in our search for common structural features defining the GnRH pharmacophore.[33] NMR investigations of four structurally distinct cyclic GnRH antagonists have been reported (see Table 1): cyclo(1−10)[Ac-Δ³Pro^1,DCpa²,DTrp^{3,6},NMeLeu⁷,-βAla¹⁰]-GnRH (**1**),[34] cyclo(4−10)[Ac-Δ³Pro^1,DFpa²,DTrp³,-Asp^4,DNal⁶,Dpr^{10}]-GnRH (**2**),[35] dicyclo(4−10/5−8)[Ac-DNal¹,DCpa²,DTrp³,Asp^4,Glu^5,DArg⁶,Lys^8,Dpr^{10}]GnRH (**3**),[36] and dicyclo(4−10/5−5′−8)[Ac-DNal¹,DCpa²,DPal³,-Asp^4,Glu(Gly)⁵,DArg⁶,Dbu^8,Dpr^{10}]GnRH (**4**).[37] A distinguishing feature of the three most potent compounds in this series (**2**−**4**) is that they have high affinity for the GnRH receptor (<0.4 nM) and inhibit ovulation in rats at doses not exceeding 10 μg/rat (about 2−5 times the dose at which the parent linear compounds are active). The bridges linking residues 5 and 8 in the dicyclic compounds **3** and **4** were designed based on systematic substitutions as described earlier.[38] However, the N-terminal tripeptide was still unrestricted in these analogues. To extend the C-terminal rigidity of these analogues to the whole molecule, possible constraints involving the first three residues were investigated.[39] Since in **2** the N-terminus was observed to be proximal to residues 5 and 8,[40] a significant number of antagonists incorporating bridges between the N-terminus and residue 5 were synthesized and tested with several being highly potent antagonists.[39,41] Here we describe the solution structure of one of the most potent (K_i = 0.24 nM) compounds, dicyclo(1,1′−5/4−10)[Ac-Glu^1(Gly),DCpa²,DTrp³,Asp^4,Dbu^5,DNal⁶,Dpr^{10}]-GnRH (**5**),[41] determined by nuclear magnetic resonance (NMR) spectroscopy. Our results yield the first well-defined structural information for the three N-terminal residues of a highly potent GnRH antagonist and have allowed us to develop a consensus structural model for GnRH through combined molecular dynamics analysis of **2**−**5**. The comparison of this consensus model with energetically reasonable conformations of the nonpeptidic GnRH mimetic T-98475 reveals common features that may underlie the affinity of T-98475 and **2**−**5** for the GnRH receptor.

Results and Discussion

The syntheses of **1**−**5** and their characterization were described in the references listed in Table 1. The structure in solution of **5** was studied by two-dimensional homonuclear NMR techniques using methodology analogous to that described for **2**−**4**.[35−37] The resonance assignments are given in Table 2, and a list of conformationally meaningful restraints derived from the NMR data is included in the Supporting Information. The restraints, which included 42 interproton distances, one hydrogen bond, and four dihedral angles, were incorporated into simulated annealing calculations that yielded 50 structures of **5**.

Comparison of the root-mean-square (RMS) deviations among the structures obtained revealed three different structural families. Most structures in the three families are characterized by small deviations from the NMR restraints and relatively low energies (within 30 kcal/mol of the minimum energy structure, MES). About 75% of the structures belong to the first family, which includes the lowest energy structure and has a conformation in the 4−10 ring similar to that observed previously in **2** and **3**. In contrast, the structures in the two least populated families exhibit an unusual conformation in the 4−10 ring which places the side chains of Asp^4 and Arg^8 in very close proximity. Since very strong nuclear Overhauser effects (NOEs) between protons in these side chains would be expected from these structures, but such NOEs were either much weaker or nonobservable, these two structural families were not considered further. The 11 structures with the lowest energy in the major family (≤5.0 kcal/mol above

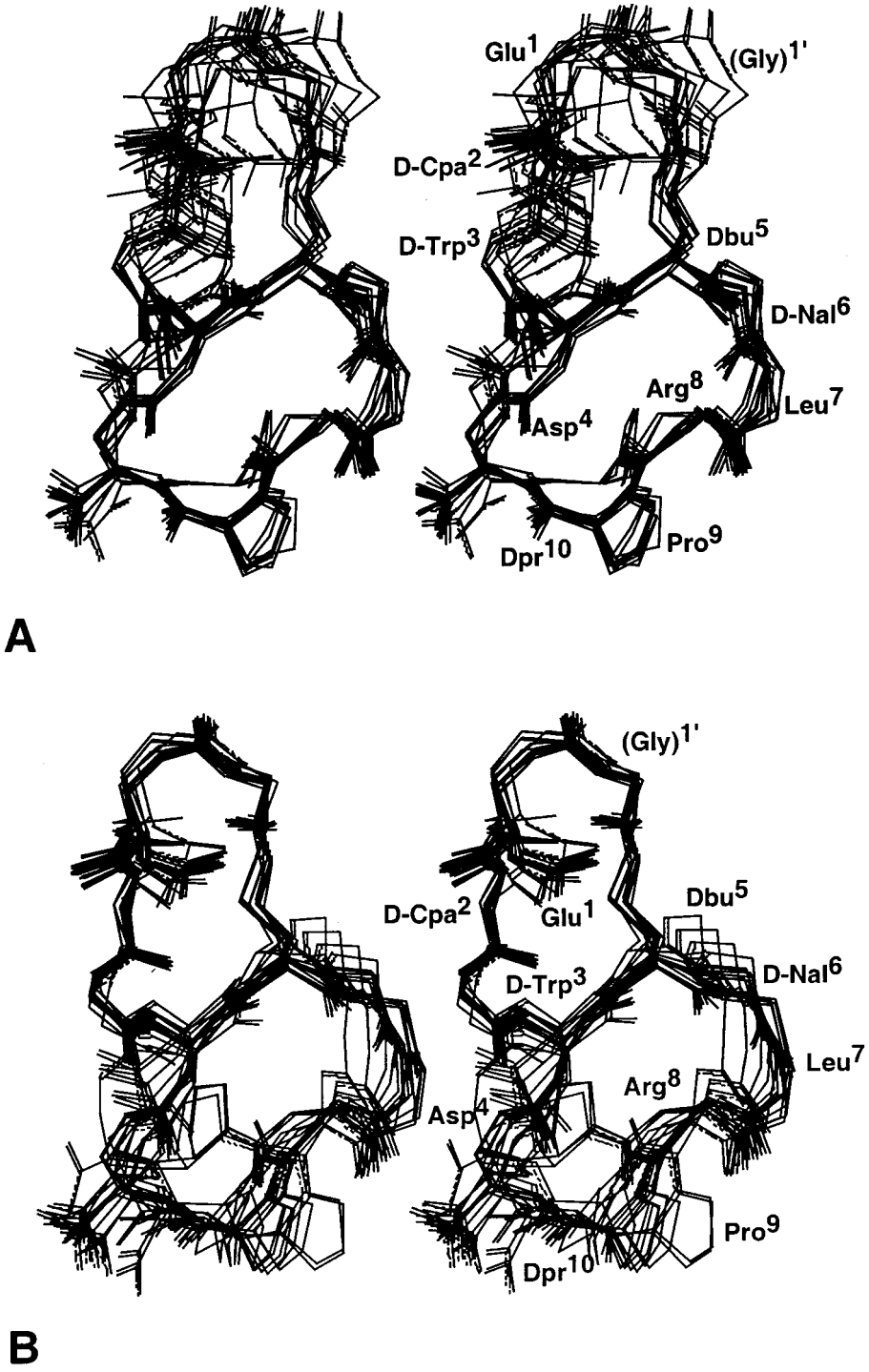

Figure 1. Stereo superpositions of 11 structures of **5**, obtained by NMR and simulated annealing; only backbone and bridge heavy atoms are shown. Conformations were superimposed using the backbone atoms of residues 4−10 (A) or the backbone atoms of residues 1−5 (B).

the MES) were selected as a representative ensemble for the structure of **5**. All these structures had either 0 or 1 violation from the distance restraints above 0.2 Å and no violation of the torsion angle restraints greater than 5°.

Stereoviews of two superpositions of the 11 structures are shown in Figure 1, and the minimum energy structures of these resultant families are shown in Figure 2. The two superpositions of Figure 1, which were performed using either the backbone and bridge heavy atoms of the 4−10 ring (A) or the backbone and bridge heavy atoms of the 1,1′−5 ring (B), show that the structure of both rings is well-defined but there is a noticeable degree of libration of one ring with respect to the other. Thus, while the average RMS deviation for all backbone and bridge heavy atoms among the 11 structures is 1.01 ± 0.42 Å, RMS deviations of 0.63 ± 0.31 Å and 0.43 ± 0.26 Å were obtained when either the 4−10 ring or the 1,1′−5 ring were superimposed, respectively.

As mentioned above, the conformation of the 4−10 ring of **5** is similar to those observed for **2** and **3**.[35,36] This conformation is characterized by a type II′ β-turn around D-Nal[6] and Leu[7], which is closed by a hydrogen

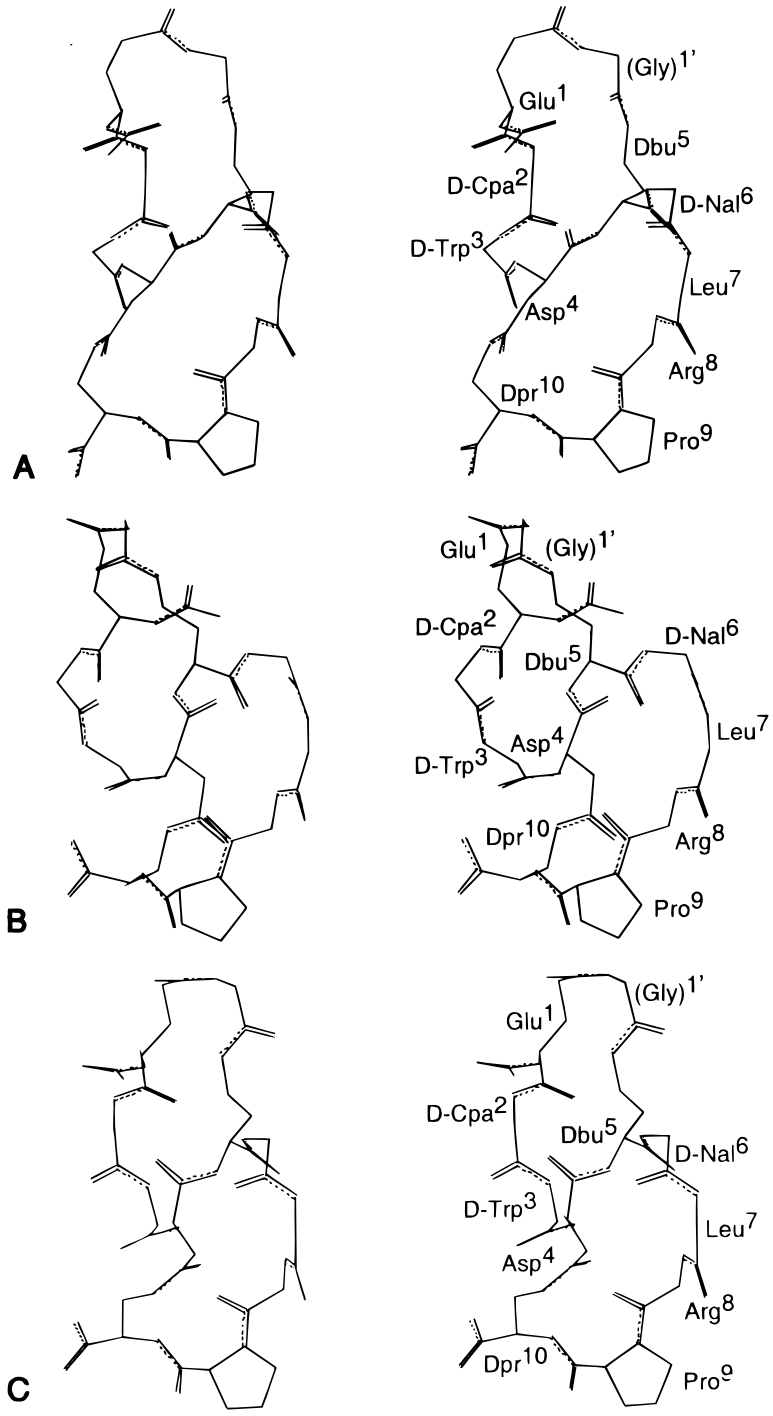

Figure 2. Stereofigures of the minimum energy structures of three families of conformers of **5** resulting from an RMS deviation-based family clustering procedure. (A) Minimum energy family; (B, C) higher energy families which were not considered further due to hypothesized, yet missing NMR features involving residues 4 and 8.

bond between Arg^8 NH and Dbu^5 CO. This turn conformation is defined unambiguously by diagnostic NOEs between D-Nal^6 Hα and Leu^7 NH, and between Leu^7 NH and Arg^8 NH, and by the low temperature coefficient of Arg^8 NH (-0.0002 ppm/K). However, some freedom of movement of the central peptide bond in the turn is observed in the structures. A turn-like structure formed by Pro^9 and the side chain of Dpr^{10} exists at the opposite end of the 4$-$10 ring although alternative conformations are observed in the 4$-$10 bridge similar to those found previously for **2** and **3**.

The observation of a well-defined conformation in the 1,1′$-$5 ring allows us to define, for the first time, the structure of the three N-terminal residues in a highly potent GnRH antagonist. The structure is characterized by an extended conformation in D-Cpa^2 and a type II′ β-turn about residues D-Trp^3 and Asp^4. This turn is evidenced by diagnostic D-Trp^3 Hα/Asp^4 NH and Asp^4 NH/Dbu^5 NH NOEs. A Dbu^5 NH/D-Cpa^2 CO hydrogen bond closing this turn is observed in only a few of the structures, consistent with only a moderately low temperature coefficient for Dbu^5 NH (0.0037 ppm/K). An additional feature of the structure of the 1,1′$-$5 ring is a turn-like conformation encompassing the side chain of Glu^1 and the bridging glycine residue (Gly$^{1'}$), which includes a hydrogen bond between D-Cpa^2 NH and Gly$^{1'}$

CO. This hydrogen bond is supported by the low temperature coefficient of D-Cpa[2] NH (0.0025 ppm/K). Although the relative orientation of the two rings is somewhat variable, overall the 1,1′−5 ring emerges above the plane formed by the 4−10 ring.

Consensus Conformation of Potent GnRH Antagonists. The identification of the consensus conformation of potent GnRH antagonists resulted from a detailed comparison of the previously identified NMR-derived structures of **2**−**5** analyzed en masse by the computer modeling method of consensus forcing (vide infra). Candidate structures for each of these constrained analogues had been calculated from the NMR-derived interproton distances and were used as the starting structures in the consensus forcing procedure. The assumption is made that, because **2**−**5** are very potent, there is a common conformation available to all of these compounds, and that this conformation may encompass that of the most active GnRH antagonists.

To partially eliminate the influence of the side chain rotameric state of the residues of any particular compound on its local structure, and by extension to the ensemble consensus structure, only NOE distances involving backbone protons and those of the lactam bridges were included. The consensus NOE restraint file and the Discover Simulation Language (DSL) file for conducting the consensus forcing protocol are included in Supporting Information.

To find the consensus structure, the molecules were superposed but not allowed to interact intermolecularly. Three potential energy terms were then applied to the system simultaneously: (1) the standard CVFF potential energy function, (2) the NOE interproton distance restraints, and (3) a new restraining function V of the form

$$V = \sum_j \sum_i K_{ij}(\mathbf{r}_{ij} - \bar{\mathbf{r}}_j)^2$$

where $\mathbf{r}_{ij}$ is the position vector of the i-th atom in set j, $\mathbf{r}_j$ is the average position of the atoms forming set j, and K_{ij} (1.0 kcal/mol/Å^2) is the force constant restraining the atoms of set j to their average position. The sets j were defined as the main chain atoms of residues (1−5, 8−10). Residues 6 and 7 were held out because of the observation that **4** has a type I′ β-turn, rather than a type II′ β-turn shown by NMR to exist in analogues **2**, **3**, and **5**. In a typical search run, the system would be subjected to molecular dynamics at 1000 K for 1 ps, then the system would be allowed to cool during dynamics over a period of 5 ps ending at 300 K. Finally, minimization would be conducted to a final maximum derivative criterion of 1.0 kcal/mol/Å. This cycle was repeated 50 times with velocity rerandomization.

The consensus dynamics procedure exhibited excellent convergence. Of the 51 sets of structures (ensembles) generated, 30 had energies within a threshold of 30.0 kcal/mol above the minimum energy ensemble. All 30 ensembles were very similar. The average trace RMS deviation among the 30 ensembles is 1.57 Å and the average trace RMS deviation with respect to the minimum energy ensemble is 1.39 Å. In addition, the comparison of the conformations of **2**−**5** within each ensemble shows that the consensus dynamics procedure easily forced the compounds to adopt similar conformations. This is illustrated in Figure 3 where the minimum energy ensemble is shown. The average trace RMS deviation among **2**−**5** within each ensemble ranged from 0.76 to 1.24 Å, with an average of 0.98 Å.

The consensus forcing results demonstrate that **2**−**5** can assume strikingly similar conformations along the entire length of the decapeptides. The most prominent common feature observed in all analogues investigated thus far is a turn involving residues 5−8. This β-turn in GnRH had been predicted by the pioneering theoretical studies of Momany and co-workers.[42] In analogues **2**, **3**, and **5**, this type II′ β-turn is facilitated by a heterochiral [DXaa[6]-LXbb[7]] sequence; such a turn would be allowed by the naturally occurring [Gly[6]] in all GnRH sequences found to date, with the exception of lamprey and tunicate GnRHs that contain an L-residue at position 6. Within the set of five GnRH antagonists (**1**−**5**), for which high resolution NMR data is available, analogue **4** with a [Glu[5](Gly[5′]), Dbu[8]] bridge is an exception possessing a clearly defined type I′ β-turn at residues 6−7. The presence of a type I′ β-turn rather than a type II′ β-turn around residues 6−7 in **4** is likely due to the formation of hydrogen bonds between the 5−5′−8 bridge and the backbone. Notwithstanding the flip in the orientation of the amide bond joining residues 6−7 of a type I′ vis-à-vis type II′ β-turn differentiating **4** from **2**, **3**, and **5**, the three N terminal residues can achieve similar orientations with respect to the 4−10 ring in all compounds. Further, the side chains of all residues not involved in bridging emerge with similar orientations from the backbone in all compounds and can achieve superposition with a very low energy penalty. The observed rotameric states could be modulated easily upon receptor binding. Because of the similarities found during the consensus conformational search and because all compounds included in the search are constrained and highly potent GnRH antagonists, it is very likely that the consensus model for this family of peptides gives a faithful representation of the conformations adopted by the GnRH antagonists upon binding to the GnRH receptor. The backbone torsion angles of the four compounds in the minimum energy ensemble, which we take as the best representatives of the consensus model, are enumerated in Table 3.

To demonstrate that the consensus model allows the placement of GnRH with reasonable energy, an in vacuo model of GnRH was template-forced to the consensus model given in Table 3. GnRH was found to be able to assume this conformation with an overall RMS deviation of 0.17 Å over the backbone of residues 4−9 with an energetic penalty of 5.0 kcal/mol over the lowest energy GnRH conformer found to date. Consequently, there appears to be no structural pathology that would necessarily prevent GnRH from assuming the consensus model conformation, notwithstanding the D−L chiral inversion characterizing the N-terminal three residues of the antagonists.

T-98475: Application of Consensus Model. With the disclosure of a highly substituted thienopyridinone, Cho et al.[43] reported the first orally active nonpeptide GnRH antagonist of the human GnRH receptor. The starting point in their search for a lead compound was the directed screening of previously identified nonpeptide antagonists of G-protein coupled receptors with

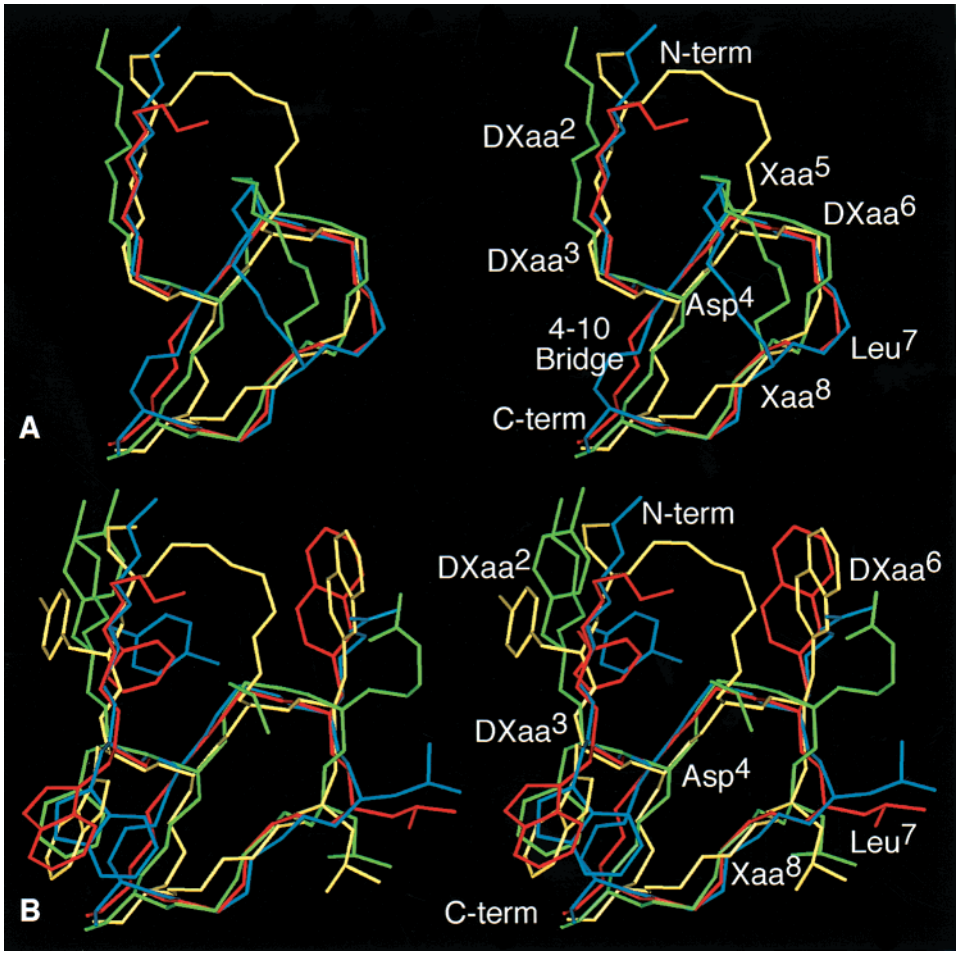

Figure 3. Minimum energy consensus model of GnRH antagonists. Compound **2**, red; **3**, blue; **4**, green; **5**, yellow. (A) Backbone and bridges only. (B) Backbone, 4−10 and 1,1′−5 bridges, and side chains of residues 2, 3, 6, and 7. Other side chains and bridges have been omitted for clarity.

Table 3. GnRH Antagonist Consensus Model

Antagonist **2**																					
Δ^3Pro^1		D-Fpa^2		D-Trp^3		Asp^4		Tyr^5			D-Nal^6		Leu^7		Arg^8		Pro^9		Dpr^{10}	
−66	155	153	−91	109	−75	−80	58	−94	108	176	88	−93	−138	78	−124	127	−51	115	−90	87
Antagonist **3**																					
D-Nal^1		D-Cpa^2		D-Trp^3		Asp^4		Glu^5			D-Arg^6		Leu^7		Lys^8		Pro^9		Dpr^0	
97	−120	126	−79	109	−47	−103	48	−97	101	−178	91	−104	−120	78	−131	131	−72	105	−78	90
Antagonist **4**																					
D-Nal^1		D-Cpa^2		D-Pal^3		Asp^4		Glu$^{5(Gly)}$			D-Arg^6		Leu^7		Dbu^8		Pro^9		Dpr^{10}	
84	−69	151	−79	138	−82	−77	49	−92	104	−164	83	43	52	62	−115	160	−53	107	−98	91
Antagonist **5**																					
Glu^1(Gly)		D-Cpa^2		D-Trp^3		Asp^4		Dbu^5			D-Nal^6		Leu^7		Arg^8		Pro^9		Dpr^{10}	
−120	−174	−66	48	82	−58	−66	80	−83	90	−177	87	−73	−115	−70	70	−160	−90	61	−89	82

[a] Backbone dihedral angles (ϕ and ψ) of minimum energy ensemble of **2**−**5**.

chemical features that could be functionalized to mimic the GnRH β-turn constituents. The thienopyridinone derivative, identified in the initial phase as antagonizing [I^{125}]leuprorelin at the hGnRH receptor in Chinese hamster ovary, was then functionalized in order to create one-for-one correspondences between the added moieties and residues Tyr^5, D-Xaa^6, Leu^7, and Arg^8. Thus, the design strategy of the Takeda group was to introduce critical functionalities into a rigid, bicyclic, heterocyclic scaffold proposed to mimic the relatively rigid main chain positioning of the residues of GnRH involved in the turn. Consequently, an appreciation of the placement of residues within a proposed conformation served to increase the efficiency of their design strategy.

The availability of a non-peptide GnRH mimetic (T-98475) and of the consensus GnRH antagonist model described above offers the opportunity to compare their structures in order to identify common modes of binding to the receptor. The question arises about a one-to-one correspondence between the functional groups of the two

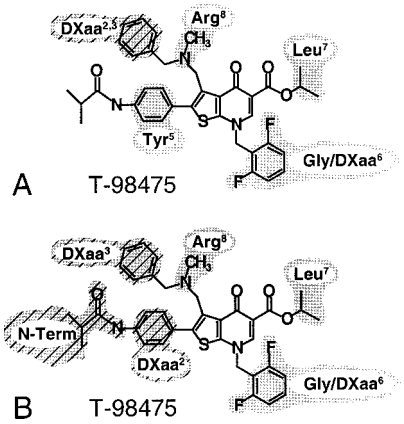

Figure 4. Structure of T-98475 and one of several possible correspondences of its functionalities to residues 5−8 of GnRH (solid gray)[43] and a proposed correspondence of the phenyl group (gray striped) to the aromatic DXaa side chain of residues 2 or 3 found important in peptide antagonists of GnRH.

types of molecules; e.g., the isopropyl ester of T-98475 can be envisaged to substitute for the leucine side chain of the natural hormone. The correspondence of GnRH antagonist side chain functional groups with those of T-98475 as proposed by Cho et al.[43] are highlighted in Figure 4A in solid gray. According to their proposal, the side chains of residues 5−8 of GnRH are mimicked by the phenyl, difluorobenzyl isopropyl ester, and methylamine moieties of the thienopyridinone. Note that while Cho et al.[43] invoke specific roles for mimics of the side chains of residues 5 and 8 of GnRH antagonists, a large number of cyclic GnRH antagonists which feature residues 5 and/or 8 in the bridgehead (**3**−**5** and others[38,44]) which argues strongly against the *necessity* of these side chains. Consequently, the proposed correspondence of T-98475 with the current consensus model (see above) does not require residues 5 or 8.

Two proposals for the overlap of T-98475 and the GnRH antagonist consensus model were identified. In the first case, the difluorobenzyl and isopropyl ester moieties of T-98475 were hypothesized to correspond to the side chains of residues 6 and 7; the disubstituted phenyl assumes the position occupied by the side chain of residue 5, the tertiary amine mimics Arg[8], and the lone terminal phenyl assumes the role of the aromatic side chain of either residue 2 or 3. The role of this terminal phenyl functionality of T-98475, which was unascribed to a specific receptor interaction in the proposal of Cho et al., is diagrammed in Figure 4A with diagonally striped shading. This could represent a fifth contact point in addition to the side chains of residues 5−8. A stereo representation of the overlap which would be enjoyed by T-98475 and the consensus model with this hypothesized overlap scheme is presented in Figure 5A.

In the second proposal of binding commonality of T-98475 and the consensus model of GnRH antagonists, residues 6−8 are again mimicked by the difluorobenzyl, isopropyl ester and tertiary amine moieties; however, in this case the disubstituted and terminal phenyl groups of T-98475 are assumed to take the positions of the aromatic side chains of residues 2 and 3 (Figure 4B). This latter proposal also envisages a sixth contact point, the isopropylcarboxamide of T-98475 overlapping with the N-terminal acetyl of GnRH antagonists. A stereo depiction of the latter overlap scheme is shown in Figure 5B. It is worth noting that residue 6 contains an aromatic group only in **2** and **5**, while it is arginine in **3** and **4**. In the latter two compounds, residue 1 contains an aromatic ring which is oriented relatively close to residue 6 and could play the role of the difluorobenzyl group in binding. Alternatively, it is possible that the compounds may still bind in similar modes to the GnRH receptor but with the aromatic groups of residue 1 in **3** and **4** and residue 6 in **2** and **5** occupying different hydrophobic pockets. We cannot currently rule out or embrace either model of T-98475 mimicry. While further experimentation will be required to test these different possibilities, the significant similarity between T-98475 and **2**−**5** supports the predictive value of the consensus model.

Conclusion

With the identification of **5**, a highly constrained cyclo(1−5/4−10) potent GnRH antagonist[39] distinct from all earlier cyclic antagonists in that its N-terminus is now unequivocally locked as part of a cycle, we were able to conduct a precise NMR investigation of its structure in solution. NMR data confirmed the constrained nature of this analogue. With the disclosure of the NMR structure of **1**[34] more than a decade ago, this laboratory and collaborators began a long-term series of investigations on constrained GnRH antagonist analogues with the explicit purpose of defining the constituent underlying solution structure(s) available to GnRH antagonists. In retrospect, a series of different backbone and side chain bonding schemes was necessary because no single compound had been identified unambiguously locked in all parts of the molecule and was amenable to current NMR or other spectroscopic techniques. Consequently, the choice of substitution and covalent modification embodied in this study can be distinguished from that which would arise from more traditional methods of drug design. Whereas incremental optimization at each residue may have resulted in a marginally improved drug, our primary goal was not a drug but a constrained reporter of the conformation likely assumed by GnRH antagonists in solution and at the receptor.

Of the five candidate antagonists presented in Table 1, **1** stands out with in vitro K_i and in vivo AOA doses of 1−3 orders of magnitude greater than those of **2**−**5**. Alternatively, the K_i values of **2**−**5** are all subnanomolar, and the in vivo AOA results are within an order of magnitude of the best antagonists reported to date. Using a consensus structure derived from the NMR conformations of **5** and those of three additional GnRH antagonists described earlier (**2**−**4**),[35−37] a hypothetical consensus model conformation of potent GnRH antagonists was determined. The likely contact points were postulated and shown to be compatible with some of the functionalities found in T-98475, a nonpeptide GnRH ligand. Whereas the challenge of mapping/defining the putative GnRH/GnRH-receptor interactions was approached from the point of view of the ligand, recent complementary and equally successful approaches have employed mutagenesis of the receptor to gain similar information.[22,25,26] When the seven-transmembrane GnRH receptors themselves are finally modeled in detail, it is postulated that the definition of any struc-

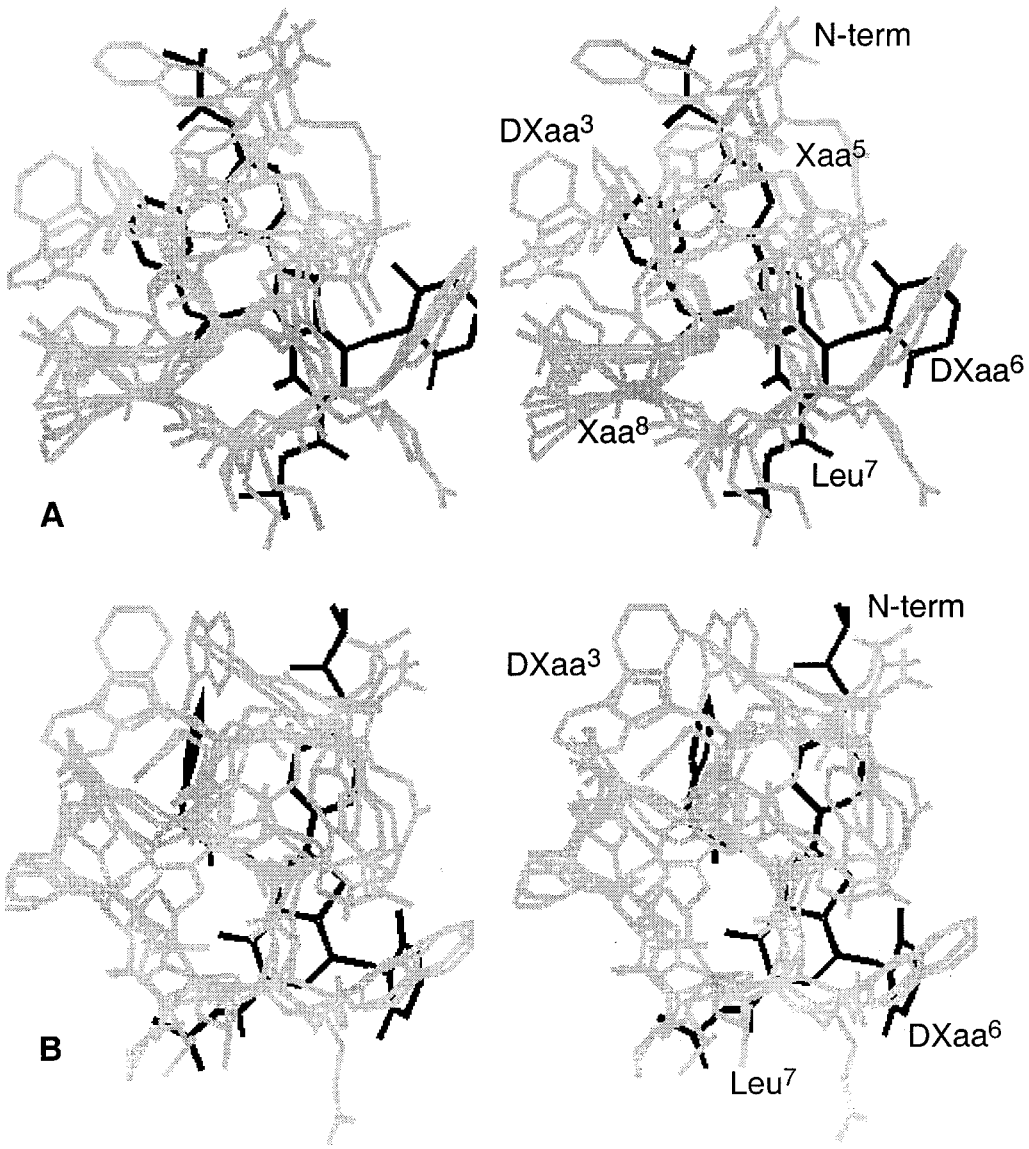

Figure 5. Stereo rendering of the overlap of T-98475 with the consensus model: T-98475 in solid black and consensus model in gray. Orientation: Pro^9 is in the near field and T-98475 is in the far field in this orientation.

turally based model of a bioactive conformation will help in generating readily testable docking hypotheses and will help in the design of new ligands.

Experimental Section

Peptide Synthesis. Peptides were synthesized manually by the solid phase approach.[38,39,45] The synthesis and characterization of peptides **1**−**5** are reported in the references listed in Table 1.

Nuclear Magnetic Resonance Spectroscopy. NMR analysis of **5** was performed following the methodology analogous to that used for compounds **2**−**4**.[35−37] All NMR experiments were performed on a Varian Unity 500 spectrometer with a 5 mM sample of **5** dissolved in $CDCl_3$/DMSO-d_6 1:1 (v/v). Resonance assignments were obtained at 12 °C using 2D COSY,[46,47] TOCSY,[48] and NOESY[49−51] experiments. Interproton distances were derived from quantification of NOE cross-peak volumes in NOESY experiments acquired with mixing times ranging from 75 to 200 ms. Coupling constants were measured from a PE-COSY experiment.[52] Amide protection from the solvent was deduced from chemical shift temperature coefficients measured over a range of 5 to 45 °C using TOCSY experiments. The set of restraints derived from these data included 42 interproton distances, four torsion angles, and one hydrogen bond.

Molecular Modeling. The potential energy parameters and functional forms were from the CVFF force field.[53,54] Molecular modeling and visualization were performed using Discover/Insight II (MSI, Inc., San Diego, CA) on a Silicon Graphics Iris Crimson workstation. Simulated annealing procedures have been reported.[35−37,40]

Biological Activities (Affinities on Pituitary Cell Membranes and AOA). In binding studies, the K_i for the potent [DAla^6,NMeLeu^7,Pro^9-NHEt]GnRH[55] taken as standard was determined to be approximately 0.3 nM. All of the other K_i values were calculated from the potencies of the analogues (relative to the standard) determined from displacement data.[55] Affinities are shown in Table 1 (see paper 1 in this series for assay details). The in vivo AOA was carried out as described by Corbin and Beattie[56] using an aqueous vehicle containing 1−2% DMSO. Results are expressed in terms of the dosage in micrograms per rat (rats ovulating/total number of treated rats). The most potent linear antagonists inhibit ovulation by 100% at 1 μg/rat.[57]

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Supporting Information Available: DISCOVER Simulation Language (DSL) NMR restraint file for **5**; DSL restraint file for consensus forcing procedure; DSL script for consensus forcing. This material is available free of charge via the Internet at http://pubs.acs.org.

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