BOC-Sta-Ile-Phe-OCH_3, 103372-21-0; BOC-His(Tos)-OH, 35899-43-5; BOC-His(Tos)-Sta-Ile-Phe-OCH_3, 103372-22-1; BOC-Proψ[CH_2O]Phe-OH, 103336-10-3; BOC-His(Tos)-Proψ-[CH_2O]Phe-His(Tos)-Sta-Ile-Phe-OCH_3, 112374-76-2; BOC-Trp(CHO)-OH, 47355-10-2; BOC-Sta-Ile-Amp, 103372-24-3; BOC-His(Tos)-Sta-Ile-Amp, 103372-25-4; BOC-His(Tos)-Proψ-[CH_2O]Phe-His(Tos)-Sta-Ile-Amp, 112374-79-5; BOC-Leuψ[CH-(O-*t*-BDMS)CH_2]Val-Ile-Amp, 103372-32-3; Leuψ[CH(O-*t*-BDMS)CH_2]Val-Ile-Amp, 103406-77-5; BOC-His(Tos)-Leuψ-[CH(O-*t*-BDMS)CH_2]Val-Ile-Amp, 103372-33-4; His(Tos)-Leuψ-[CH(OH)CH_2]Val-Ile-Amp, 103372-31-2; BOC-His(Tos)-Proψ-[CH_2O]Phe-His(Tos)-Leuψ[CH(OH)CH_2]Val-Ile-Amp, 112374-80-8; BOC-Phe-OH, 13734-34-4; BOC-Phe-His(Tos)-Sta-Ile-Amp, 112374-82-0; BOC-Phe-His-Sta-Ile-Amp, 97920-11-1; BOC-Ao-OH, 15761-39-4; BOC-Pro-Phe-OH, 52071-65-5; His(Tos)-Sta-Ile-Phe-OCH_3, 112374-84-2; renin, 9015-94-5.

Design of Potent Cyclic Gonadotropin Releasing Hormone Antagonists†

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In order to improve the biological potency of cyclic gonadotropin releasing hormone (GnRH) antagonists, we have synthesized analogues, the conformations of which were restrained through internal side chain/side chain amide bridges linking aspartic acid or glutamic acid and L-2,3-diaminopropionic acid or L-ornithine. A disulfide bridge linking L-cysteine residues was also introduced. Residues belonging to the bridge spanned from position 4 to positions 9 or 10. Two series of analogues were synthesized and are characterized by residues at positions 1 [Ac-D-3-(2′-naphthyl)alanine], 2 [D-(4-chlorophenyl)alanine or D-(4-fluorophenyl)alanine], 3 [D-3-(3′-pyridyl)alanine or D-tryptophan], 5 (arginine or tyrosine), and 6 [D-3-(3′-pyridyl)alanine or D-arginine], respectively. These substitutions were selected in an effort to optimize high biopotency for inhibition of luteinizing hormone secretion, minimization of histamine release activity, and high (relative) hydrophilicity. The most potent analogues in the antiovulatory assay were *cyclo*(4–10)[Ac-DNal[1],DCpa[2],DPal[3],(Asp[4] or Glu[4]),Arg[5],DPal][6],Dpr[10]]GnRH (compounds **5** and **7**), which were fully active at ca. 12.5 μg/rat in the first series, and *cyclo*(4–10)[Ac-DNal[1],DFpa[2],DTrp[3],Asp[4],DArg[6],Dpr[10]]GnRH (compound **12**), which was fully active at 2.5 μg/rat in the second.

Several techniques, ranging from binding studies and structure–activity relationships (SAR) to conformational study of the ligand using both theoretical and spectroscopic approaches, are available to gain an understanding of the nature of the interaction of a ligand with its receptor. However, when the structure of the receptor is unknown (as in the case for releasing factors) and the ligands are relatively small linear peptides with virtually unlimited conformational freedom, any conclusion concerning the nature of their interaction is speculative at best. In order to gain insight into the nature of the interactions of GnRH (the structure of which is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) and its pituitary receptor, extensive SAR studies using receptor binding assays, in vitro and in vivo assays, and some spectroscopic measurements have been carried out by us and others.[1] Potent and long-acting GnRH agonists were prepared and, because of the desensitizing effects resulting from long term administration, have been used in clinical studies where suppression of sex steroid secretion is desired.[2] However, the administration of these agonists to humans is complicated by the fact that an early stimulatory phase exists, which is never desired.

The potential of GnRH antagonists as contraceptive agents was realized even prior to the elucidation of GnRH's structure, and an extensive program sponsored by the National Institutes of Health Contraceptive Development Branch was initiated in 1972. Some 15 years and approximately 3000 analogues later, extremely potent and long-acting GnRH antagonists have been designed, and preliminary data from clinical investigators are very encouraging. Despite this, little was known regarding the role of the backbone and side-chain conformations or flexibility of GnRH when interacting with its receptor. In order to address this problem, we applied an integrated approach combining theoretical techniques, including molecular dynamics, energy minimization, template forcing and interactive graphics[3] with peptide synthesis,[4] NMR spectroscopy,[5] receptor binding studies,[6] and several biological

† 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: Dpr, 2,3-diaminopropionic acid; Nal, 3-(2′-naphthyl)alanine; Pal, 3-(3′-pyridyl)alanine; Cpa, (4-chlorophenyl)alanine; Fpa, (4-fluorophenyl)alanine; MeLeu, *N*-methylleucine; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; AOA, antiovulatory assay; DCC, *N,N′*-dicyclohexylcarbodiimide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; EDT, ethanedithiol; DMF, dimethylformamide; TEA, triethylamine; sc, subcutaneous.

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assays,[7,8] to the design of two new families of conformationally constrained GnRH antagonists.[3] Earlier attempts at cyclizing GnRH had led to compounds with low potency as agonists or partial agonists.[9]

Peptide Synthesis. Peptides were synthesized by the solid-phase method on a methylbenzhydrylamine resin by use of techniques previously reported by this laboratory.[10] Cyclization was achieved by generating the hydrazide of the β-carboxyl of the aspartic or γ-carboxyl of the glutamic acid residue protected as the benzyl ester by stirring the peptide resin with hydrazine at 20 °C for 100 h in CH_2Cl_2. The peptide was then cleaved with HF and concomitantly deprotected. The peptide hydrazides were converted to the corresponding azide with isoamyl nitrite and HCl in dioxane and reacted in DMF under dilute conditions with the β-amino group of L-Dpr or the δ-amino group of L-Orn residues to give the crude cyclized peptides (see the Experimental Section). Final purification was carried out with HPLC.[11,12] Peptides were judged to be greater than 95% pure by HPLC in two systems. Retention times, with use of isocratic conditions and specific rotations, are given in Table I. Amino acid analyses[13] including those of Pal, Cpa, Fpa, Nal, and Dpr were consistent with expected results. Calculated values for protonated molecular ions were in good agreement with those obtained from FAB mass spectrometry.

Bioassays. Biological evaluation included an in vitro assay, a receptor binding assay, administration to castrated male rats, and an in vivo antiovulatory assay (AOA) (see the Experimental Section).

Results and Discussion

Systematic and empirical substitution in the design and synthesis of GnRH agonists and antagonists have resulted in linear analogues with high potency and relatively long duration of action.[1] Conformational analysis of GnRH, and the conclusion that it may assume a folded conformation with a β-type bend encompassing residues 5 to 8, led to the synthesis of several homodetic and heterodetic cyclic competitive antagonists to GnRH.[9,13,14] One of these analogues, *cyclo*(1–10)[Δ^3Pro^1,DCpa^2,DTrp^{3,6},MeLeu^7,βAla^{10}]GnRH, was active although with low potency. We carried out an extensive study of the conformation of this constrained analogue by using both computer analysis[3] and NMR spectroscopy.[5,16] Results showed that while GnRH exhibited a wide range of conformations, this analogue assumed one of two closely related conformations distinguishable by a cis–trans isomerization of the βAla-Δ^3Pro amide bond. This isomerization had little or no effect on the conformation of residues 2 to 9. Given these results, we investigated the possibility of designing new families of conformationally constrained analogues targeted to this region of conformational space. Observation that the Ser^4 and Pro^9 α-hydrogens pointed toward each other, were directed toward the center of the ring, and were roughly 2.5 Å apart in the preferred minimized structure of *cyclo*(1–10)[Δ^3Pro^1,DCpa^2,DTrp^{3,6},MeLeu^7,βAla^{10}]GnRH suggested the possibility of further stabilization by the introduction of an intramolecular bond of appropriate length best simulated by two or three methylene groups.[3] As an alternative to this synthetic challenge of bridging two α-carbons six residues apart within a decapeptide, a bridge generated by the coupling of the side chains of Dpr^4 and Asp^9 was synthesized in order to restrain the conformation of the GnRH antagonist and mimic the putative active conformation arrived at on the basis of computer simultations and subsequently supported by NMR studies.[3,16] Several 4–9 side chain bridged analogues were synthesized, tested, and found to be essentially equipotent to analogue *cyclo*(1–10)[Δ^3Pro^1,DCpa^2,DTrp^{3,6},NMeLeu^7,βAla^{10}]GnRH,[14] thus suggesting that the molecules that had been designed had similar conformational properties (unpublished results and ref 14). Whereas all cyclo(1–10) structures that had been synthesized and tested showed low potency, this new family of cyclo(4–9) analogues suggested new bridging possibilities. Earlier results with cyclic GnRH agonists suggested the possibility of bridging residues 4 to 10, although biological potency was minimal.[14] Our results with the 4–9 bridged antagonist led us to reexamine these results and to synthesize [Ac-Δ^3Pro^1,DFpa^2,DTrp^3,Dpr^4,DNal^6,Asp^{10}]GnRH. It was found to be 7 times less potent in vitro than the corresponding linear [Ac-Δ^3Pro^1,DFpa^2,DTrp^3,DNal^6]GnRH, to have a similar affinity for the receptor (K_D = 0.80 nM versus 0.64 nM for the linear peptide), and to fully inhibit ovulation at 100 μg/rat. Because this compound, bridged at positions 4 and 10, was found to be significantly more potent than any other cyclic GnRH analogue known at that time, we introduced this bridging constraint, as well as similar ones, into three families of GnRH antagonists. The first series alluded to above was based on [Ac-Δ^3Pro^1,DFpa^2,DTrp^3,DNal^6]GnRH because of its high in vitro relative potency (1.8), its low K_D (0.64 nM), and its potent inhibitory activity in vivo (complete inhibition of ovulation at 5.0 μg/rat). In addition, this analogue exhibited a comparatively low potency at releasing histamine from rat mast cells (ref 18 and unpublished data). A second series was based on [Ac-DNal^1,DCpa^2,DTrp^3,DArg^6]GnRH because similar analogues had been found to be the most potent in the AOA[1] (exhibiting 100% inhibition at 1 μg or less per rat). Yet these analogues were later found to be strong releasers of hist-

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Table I. Physical Constants, Biological Activities, and Potencies of Cyclic GnRH Analogues

compound	$[\alpha]^{20}_D$[a]	t_R,[b] min	relative potencies in vitro[e]	K_D, nM	AOA[f]
1, Ac-Δ^3Pro-DFpa-DTrp-Ser-Tyr-DTrp-Leu-Arg-Pro-Gly-NH_2			1.0 standard	0.27	7.5 (0/10)
2, Ac-DNal -DCpa-DPal -Ser-Arg-DPal -Leu-Arg-Pro-DAla-NH_2	−29.8°	4.5 (43% 8)[c]	0.91 (0.76-1.10)	0.24 (0.17-0.34)	0.5 (5/10)[g] 1.0 (0/10)[g]
3, Dpr—Asp-NH_2	−27.5°	3.6 (40% B)[c]	0.013 (0.01-0.02)	4.5 (3.4-5.8)	25 (5/10) 50 (0/10)
4, Dpr—DAsp-NH_2	−27.0°	3.5 (40% B)[c]	0.004 (0.003-0.005)	10 (7.4-13)	50 (2/9)
5, Asp—Dpr-NH_2	−45.1°	3.5 (50% B)[d]	0.09 (0.06-0.14)	1.1 (0.73-1.6)	10 (4/19) 25 (0/10)
6, Asp—Orn-NH_2	−25.2°	5.1 (38% B)[c]	0.001 (0.0001-0.002)	11 (7.0-1.6)	25 (4/4) 50 (8/10)
7, Glu—Dpr-NH_2	−23.8°	3.3 (45% B)[d]	0.16 (0.12-0.21)	1.0 (0.83-1.2)	10 (2/8) 12.5 (0/10)
8, Cys—Cys-NH_2	−37.8°	4.7 (37% B)[c]	0.04 (0.03-0.05)	0.80 (0.4-1.4)	10 (7/10) 25 (0/15)
9, Dpr(Ac)- Asn-NH_2	−20.0°	3.5 (39% B)[c]	0.003 (0.003-0.004)	1.0 (0.64-1.6)	50 (3/7)
10, Ac-DNal-DFpa-DTrp-Ser-Try-DArg- Leu-Arg-Pro-Gly-NH_2	−29.1°	3.7 (60% B)[d]	0.6 (0.4-0.8)	0.64 (0.38-1.0)	1.0 (0/10)
11, Dpr Asp-NH_2	−25.8°[h]	4.5 (45% B)[c]	0.014 (0.01-0.02)	10 (8.4-13)	10 (7/10) 25 (1/11)
12, Asp—Dpr-NH_2	−28.3°	4.9 (51% B)[c]	0.3 (0.2-0.4)	1.9 (0.9-3.7)	1.0 (2/10) 2.5 (0/10)
13, Asp— Orn-NH_2	−14.9°	3.7 (68% B)[d]	0.003 (0.002-0.005)	41 (20-83)	100 (8/8)
14, Glu—Dpr-NH_2	−20.2°	4.3 (58% B)[d]	0.15 (0.12-0.18)	1.1 (0.6-1.9)	2.5 (3/4) 10 (1/8)
15, Cys—Cys-NH_2	−8.7°	5.1 (54% B)[c]	0.02 (0.02-0.03)	6.0 (4.0-9.2)	50 (5/9) 100 (0/7)
16, Dpr— Asp-Gly-NH_2	−12.9°	5.2 (52% B)[c]	<0.001	>100	2000 (10/10)[g]
17, Asp—Dpr-Gly-NH_2	−15.0	4.4 (58% B)[d]	<0.001		1000 (4/4)

[a] $c = 1$, AcOH-H_2O (1:1). [b] Retention times under isocratic conditions, buffer system A was TEAP, pH 2.25. [c] 0.1% TFA. [d] B was 60% CH_3CN in A. [e] [Ac-Δ^3Pro^1,DFpa^2,DTrp^{3,6}]GnRH = 1. [f] AOA-antiovulatory assay: dosage in micrograms (rats ovulating/total). [g] Administered in corn oil. [h] $c = 1$, DMF.

Table II. Inhibitory Effect of Compounds **3** and **8** on GnRH-Induced LH Secretion in Intact Male Rats[a]

treatment	ng of LH/mL ± SEM
control	1.27 ± 0.39
20 ng of GnRH	1.84 ± 0.13[b]
50 ng of GnRH	2.42 ± 0.24[c]
25 μg of **3** + 50 ng of GnRH	1.69 ± 0.07[d]
100 μg of **3** + 50 ng of GnRH	1.18 ± 0.08[d]
25 μg of **8** + 50 ng of GnRH	1.62 ± 0.12[d]
100 μg of **8** + 50 ng of GnRH	1.40 ± 0.100[d]

[a] The antagonists were injected iv immediately prior to GnRH. Each treatment was given to six rats. Blood samples were obtained 10 min after injection. [b] $p > 0.05$ from control. [c] $p \leq 0.01$ from control. [d] $p \leq 0.01$ from 50 ng of GnRH alone.

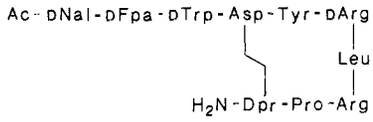
Table III. Effect of the Oral Administration of GnRH Antagonists on LH Secretion by Castrated Male Rats[a]

treatment	ng of LH/mL
control	9.10 ± 0.74
1 mg of [Ac-DNal^1,DFpa^2,DTrp^3,DArg^6]GnRH	6.40 ± 0.84[c]
3 mg of **3**	8.19 ± 0.84[b]
3 mg of **9**	8.22 ± 0.80[b]
3 mg of **8**	8.31 ± 0.84[b]

[a] Five rats were used per treatment. Blood samples were obtained 5 h after treatment. Results are expressed as means ± SEM. [b] $p > 0.05$. [c] $p \leq 0.05$.

amine both in vitro[18] and in vivo.[17] The third series based on [Ac-DNal[1],DCpa[2],DPal[3],Arg[5],DPal[6],DAla[10]]GnRH combined the high potency in vivo and the hydrophilicity of the members of the second family while also being less potent in the rat mast cell histamine release assay. We report here on the biological profile of cyclic analogues from the latter two series.

As can be seen in Table I, the linear compounds **2** and **10** were essentially equipotent in vitro to **1** (our standard), had similar K_D values of 0.24 and 0.64 nM, respectively, and yet were approximately 7–10 times more potent than **1** in the AOA. Since both in vitro tests showed similar high potencies for **1**, **2**, and **10**, the 7–10-fold increased potency of **2** and **10** in vivo is probably not the result of a better interaction with the receptor; rather, it may be the result of greater resistance to enzymatic degradation or a difference in compartmentalization in vivo. The observation, however, that [Ac-DNal[1],DCpa[2],DPal[3],DArg[6],DAla[10]]GnRH or **10** were potent releasers of histamine, both in vitro and in vivo,[18] and thus unsuitable for clinical applications led to the synthesis of compounds related to peptide **2**.[4] Important structural features for histamine release have been discussed elsewhere[1,4,18] and encompass a hydrophobic N-terminal and a basic D-amino acid residue in position 6. Therefore, we can suggest that the low potency of **2** and congeners in the histamine release assay[19] resulted from shifting the basic side chain of DArg in position 6 to a position further away from that of Arg[8] since the proximity of these two side chains had been found to be, at least in part, responsible for the deleterious activity. It was therefore desirable to investigate the effect of cyclization on the potency of the parent analogues (Table I) and also to find out whether cyclization of peptides modeled after **10** would retain high biological potency in the AOA while being inactive in releasing histamine. This did not turn out to be the case as both **11** and **12** have ED_{50} values (<0.5 μg/mL) similar to that of [Ac-DNal[1],DFpa[2],DTrp[3],DArg[6]]-GnRH (0.25 μg/mL) for release of histamine from rat mast cells while GnRH itself has an ED_{50} of 185 μg/mL.



12

Overall, **5** and **7** in one series and **12** in the other (Table I) were the most potent cyclic analogues in the AOA. Interestingly, the only "structural" difference between the three analogues is an extra methylene group in the ring of **7**, a modification that is unfavorable in the second series since **14** (homologous to **7**) is considerably less potent. Presently we have no explanation for this observation nor for the fact that **8**, which has a disulfide bridge, comes in a close second in terms of potency, in this series, while the equivalent **15**, for example, is at least 5 times less potent in the AOA than **12**. This observation suggests that the GnRH receptor may accommodate several closely related backbone conformations provided that adequate compensation in terms of charge or bulk is provided by the different amino acid side chains. A clue to the ability of some of the amide bonds to form stabilizing hydrogen bonding during interaction with the receptor is indicated by the fact that **5** and **12** have apparent affinities for the GnRH receptor approximately 4 times greater than those of **3** or **11**, respectively, and yet the only difference between

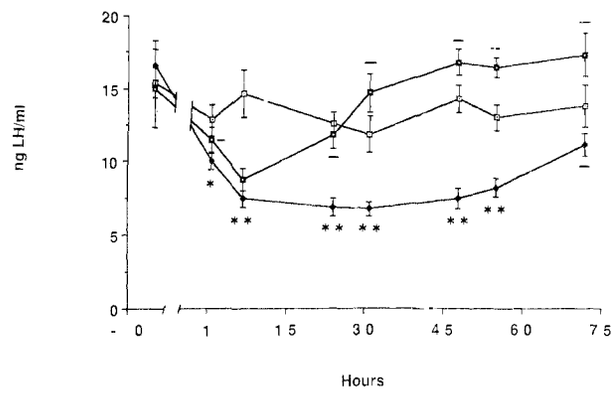


Figure 1. Effect of the sc administration of the vehicle (control = ⊡), 100 μg of [Ac-Δ[3]Pro[1],DFpa[2],DNal[3,6]]GnRH (●), or 100 μg of **3** (□) on plasma LH levels in castrated male rats. Blood samples were obtained under light ether anesthesia and taken at 0, 1, 7, 24, 31, 48, 55, and 72 h. Each point represents the mean ± SEM of five animals. –, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$.

the two families of structures is the orientation of the amide bond linking the Asp/Dpr side chains. The observation that the introduction of an S–S bond did not decrease the relative high potency, both in vivo and in terms of affinity to the receptor (**8**), is less evident for **15**; this indicates that steric effects, or the possibility of generating hydrogen bonds with the receptors, may not be the only parameters crucial for receptor interaction.

It should also be noted that when the first series of analogues was compared to the series based on [Ac-DNal[1],DCpa[2],DTrp[3],DArg[6]]GnRH, **7** (Glu[4] Dpr[10]), was found to be as potent as **5** (Asp[4] Dpr[10]) in vivo. This suggested that minor changes in the size of the ring will be integrated within the overall tertiary structure of the analogue when interacting with its receptor, carrier proteins, etc., in such a way as to improve the biological end effects. Why **6** or **13**, which have the same bridge length as peptides **7** or **14**, were significantly less potent remains to be studied. In this case, the only difference between **6** and **13** versus **7** and **14** is the location of the amide linkage that has been shifted one carbon atom away from the C-terminus. The fact that **9** with blocked bridge heads was significantly less potent than either of the cyclic analogues reported here was expected from earlier studies where substitutions at Ser[4] and Gly[10] by bulkier residues gave a GnRH antagonist less potent[20] than the parent compound. These results are consistent with our conformational hypothesis, which requires these residues to be in close proximity;[3] to be noted is the tolerance by the receptor of the change in chirality at Xaa[10] between **3** and **4**. Indeed, replacement of Asp[10] (**3**) by DAsp[10] (**4**) resulted in a binding affinity in vitro and a potency in vivo (AOA) reduced by a factor of ca. 2. The effect on conformation of such substitutions is now being investigated. Consistent with our hypothesis,[3,5,16] **16–17**, with backbone conformations significantly altered in comparison to those of the Xaa[4] Xbb[10] cycle, are at least 2 orders of magnitude less potent.

Figures 1 and 2 illustrate the effect of given doses of cyclic antagonists on LH secretion in the castrated rat over a period of several hours. Whereas linear standards [Ac-Δ[3]Pro[1],DFpa[2],DNal[3,6]]GnRH (Figure 1) and **1** (Figure 2)

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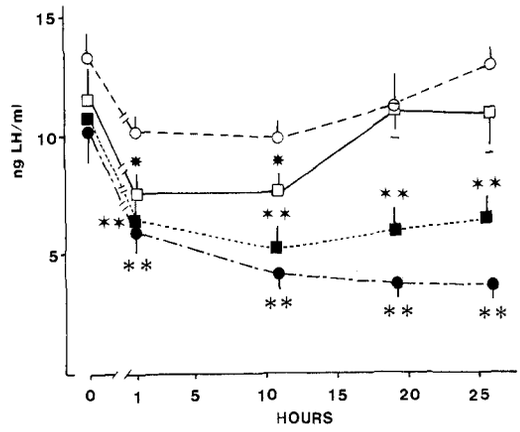

Figure 2. Effect of the sc administration of the vehicle (control = ○), 100 μg of [Ac-Δ^3Pro^1,DFpa^2,DTrp^{3,6}]GnRH (●), or 100 μg of **9** (□) or **8** (■) on plasma LH levels in castrated male rats. Legend as in Figure 1. Blood samples were obtained at 0, 1, 11, 20, and 26 h.

are both potent and long acting at the doses tested, **3** and **4** are only active for 10–15 h. Compound **8**, one of the three most potent analogues of this series, compares favorably with the linear analogue (Figure 2), suggesting that stabilization of conformation may not affect duration of action.

Table II shows the inhibitory effect of **3** and **8** on GnRH-induced LH secretion in intact male rats. In this in vivo system, the ability of the antagonists to inhibit the stimulatory effect of exogenously administered GnRH (versus endogenously released LH in the AOA or castrated male rat model) was tested. Both peptides were active at the doses tested. A dose response was observed since the levels of LH at the 100 μg doses of the antagonists were lower than those observed after administration of the 25 μg dose. It should be noted that a 1000-fold excess of the antagonist was needed to block the effect of 50 ng of GnRH. Of interest is the fact that the K_D for GnRH is about 4 nM and very similar to that of **3** (see Table I). Yet in order to be effective, the analogue has to saturate all of the GnRH receptors, since it is known that partial occupancy by the agonist can lead to full expression of biological activity.[6]

Finally, the effect of oral administration of the peptide on LH secretion in the castrated male rat was investigated (Table III). One could indeed suggest that a cyclic antagonist of GnRH would offer a more compact conformation than a linear decapeptide and, therefore, may be more effective at crossing the stomach linings into the blood stream. Whereas [Ac-DNal^1,DFpa^2,DTrp^3,DArg^6]-GnRH (**10**), at a dose 1000 times that at which it is fully active in the AOA assay, will only be active at the $P \leq 0.05$ level to inhibit LH secretion, **3**, **8**, and **9** were found inactive at the 3-mg dose. Yet, because the cyclic analogues are about 10–20 times less potent in vivo after subcutaneous administration than the linear standard reported here, for example, it cannot be concluded that cyclization is detrimental to oral activity.

In summary, potent cyclic GnRH antagonists have been designed and synthesized, and their biological activities were tested. Of note is the fact that particular care was taken to exclude contamination of these preparations by very potent linear analogues (either agonists or antagonists). Some of the hydrazide intermediates were found to have very low potencies in the tests described here (unpublished results). Furthermore, contamination of the cyclic analogues by linear peptides such as **2** should have been at the level of 5–15% to affect the results presented here. We have no indication of such contamination. The argument that **8** or **15** may act as a linear peptide upon reduction of the disulfide bridge (in the blood stream) can also be rejected since **9** is very impotent, and furthermore, **5**, **7** and **12**, with structures that cannot open under physiological conditions, are at least as potent if not more potent than **8** and **15**. We have shown that at least one cyclic analogue (**12**) can display biological activities and potencies within a factor of 2 of that of the corresponding linear analogue (**10**). The significance of this observation in terms of the conformation assumed by the ligand during binding to its receptor has not escaped our attention and is the subject of further studies.

Experimental Section

Amino acid derivatives Boc-Arg(Tos), Boc-Asp(OBzl), Boc-Cys(MeOBzl), Boc-Glu(OBzl), Boc-Gly, Boc-Leu, Boc-Orn(Z), Boc-Pro, Boc-Ser(Bzl), Boc-DTrp, and Boc-Tyr(2,6-Cl_2-Bzl) were obtained from Bachem Inc. (Torrance, CA). Boc-D-Nal, Boc-D-Fpa and Boc-D-Cpa were synthesized in our laboratory[13] or obtained from the Contraceptive Development Branch, Center for Population Research at NIH. L-2,3-Diaminopropionic acid was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and derivatized [Boc-Dpr(Z)] by using known procedures.[21-23] *p*-Methylbenzhydrylamine-resin (MBHA-resin) with a capacity of 0.6 mequiv/g was obtained from a polystyrene cross-linked with 1% divinylbenzene (Biobeads SX-1, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA) by use of a previously published procedure.[10] Cation-exchange resin (H^+-form) was prepared from analytical grade resin (Bio-Rex 70, 100–200 mesh, Na^+ form, Bio-Rad Laboratories) by washing with 0.5 M HCl. EDT, DMF, TEA, anhydrous hydrazine, isoamyl nitrite, and diisopropylethylamine were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). TFA was obtained from Halocarbon Products Corp. (Hackensack, NJ). DCC was purchased from Pierce Chemical Co. (Rockford, IL). Methylene chloride and methanol were obtained from Fisher Scientific Co. Fair Lawn, NJ). Acetonitrile for preparative HPLC was obtained from E. M. Science (Cherry Hill, NJ) and for analytical HPLC from Burdick and Jackson (Muskegon, MI). 5,5′-Dithiobis(2-nitrobenzoic acid)[24] was purchased from Sigma Chemical Co. (St. Louis, MO).

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 with *o*-phthalaldehyde post-column derivatization and fluorescence detection. Preparative HPLC were run with a Waters Associates (Milford, MA) Prep LC/System 500A and Model 450 variable-wavelength UV detector, a Fisher (Lexington, MA) Recordall Model 5000 strip-chart recorder, and an Eldex Laboratories (San Carlos, CA) Chromat-A-Trol Model II gradient maker. Analytical HPLC were run on a Hitachi Ltd. (Tokyo, Japan) liquid chromatograph Model 655A with processor Model 655-61. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter.

Synthesis of *cyclo*(4–10)[Ac-DNal^1,DFpa^2,DTrp^3,Asp^4,-DArg^6,Dpr^{10}]GnRH (Ac-DNal-DFpa-DTrp-Asp-Tyr-DArg-Leu-Arg-Pro-Dpr-NH_2) (12). Linear Peptide Hydrazide. MBHA-resin (3.0 g) was used for manual solid-phase synthesis. Previously reported protocols were used.[25] Couplings in CH_2Cl_2 or DMF-CH_2Cl_2 (1:1) were performed with DCC (1 M in CH_2Cl_2) for 90–120 min. A twofold excess of protected amino acids was used on the basis of the original substitution of the MBHA-resin. Deblockings were performed with TFA-CH_2Cl_2 (3:2) containing 2.5% EDT for 20 min followed by neutralization with 10% TEA in CH_2Cl_2. N-Terminal acetylation of Nal^1 or Δ^3Pro^1 was performed with a large excess of acetic anhydride in CH_2Cl_2 for 15

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min. Hydrazinolysis of aspartic β-benzyl ester residue was performed with a large excess of anhydrous hydrazine in CH_2Cl_2 for 100 h. After the residue was washed with methanol and CH_2Cl_2 and dried, 4.5 g of protected peptide hydrazide MBHA-resin was obtained. The Ac-DNal-DFpa-DTrp-Asp($NHNH_2$)-Tyr(2,6-Cl_2-Bzl)-DArg(Tos)-Leu-Arg(Tos)-Pro-Dpr(Z)-MBHA-resin (4.5 g) was treated with 60 mL of HF at 0 °C for 60 min in the presence of 6 mL of anisole. The HF was removed from the reaction vessel under vacuum, and the residue was treated with anhydrous ether (100 mL) and filtered. The peptide hydrazide was extracted from the resin with 10% aqueous acetic acid and lyophilized to yield crude peptide hydrazide (1.60 g). HPLC analysis indicated one major peak (ca. 50%).

Cyclization. Crude peptide hydrazide (1.00 g, 0.67 mmol) was dissolved in 40 mL of DMF and 4 N HCl in dioxane (0.85 mL, 3.4 mmol) at −25 °C, and isoamyl nitrite (0.14 mL, 1.0 mmol) was added with stirring over 10 min. Stirring was continued at −25 °C for 3 h. The solution of peptide azide was diluted with 680 mL of precooled DMF (−25 °C), and diisopropylethylamine was added until a pH of 7.6 was reached as measured on a wet pH indicator strip. The solution was stored for 24 h at −25 °C and for 72 h at 5 °C. Then, the solvent was removed with a rotary evaporator to yield a crude cyclic peptide (0.92 g). HPLC analysis showed one major component (ca. 40%).

Purification (Reversed-Phase HPLC). The crude cyclic peptide 0.92 g) was dissolved in 0.25 M triethylammonium phosphate (100 mL) buffer, pH 2.25 (TEAP 2.25), and loaded onto a (5 × 30 cm) preparative reversed-phase HPLC cartridge packed in this laboratory[11,12] with 15–20 μm Vydac C_{18} silica gel (The Separations Group, Hesperia, CA). The peptide was eluted with a mixture of solvents A (TEAP 2.25) and B (60% CH_3CN, 40% A) under gradient conditions (45–65% B in 60 min) at a flow rate of 100 mL/min. Analysis of individual fractions was performed by HPLC (5 μm Vydac C_{18} column) with isocratic elution with use of 51% B (TEAP system), t_R = 4.9 min. The selected fractions were diluted (1:1) with water and converted to the TFA salt by loading on a preparative cartridge as above and eluted with a mixture of solvents A (0.1% TFA) and B (60% CH_3CN, 40% A) under gradient (35–65% B in 40 min). Selected fractions were lyophilized to yield 100 mg of cyclic peptide determined by HPLC to be 98% pure: MH^+ 1476.6, calcd 1476.7.

Analogues **3–7**, **11–14**, and **16–17** were obtained by use of this general procedure.

Synthesis of *cyclo*(4–10)[Ac-DNal^1,DFpa^2,DTrp^3,Cys^{4,10},-DArg^6]GnRH (Ac-DNal-DFpa-DTrp-Cys-Tyr-DArg-Leu-Arg-Pro-Cys-NH_2), 15. Linear Peptide. The peptide was synthesized by use of a manual solid-phase procedure as described above but without hydrazinolysis; MBHA-resin (3.0 g) was used.

Formation of Disulfide Bridge by Air Oxidation. After cleavage by HF in the presence of anisole (10%) and their removal under vacuum, the product was washed with anhydrous ether and extracted from the resin with 10% aqueous acetic acid. The extract was quickly diluted to 2 L with water adjusted to pH 6.8 with NH_4OH. The solution was stirred slowly in an open breaker at room temperature for 120 h at which time all sulfhydryl had been oxidized as monitored by use of Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)].[23] The pH was adjusted with acetic acid to 5.0, and the solution was passed through a column (5 × 12 cm) containing Bio-Rex 70 (N^+-form). The resin was extensively washed with water and 5% aqueous acetic acid, and the oxidized peptide was removed from the resin with 250 mL of 50% aqueous acetic acid. This fraction was diluted with water and lyophilized, yielding the crude product (280 mg).

Purification. Purification was performed as described above by use of gradient of TEAP 2.25 (40–60% B in 60 min) and a gradient of 0.1% TFA (30–60% B in 40 min). Analytical control was performed under isocratic conditions 54% B (TEAP system), t_R = 5.1 min. Selected fractions were lyophilized to yield 60 mg of peptide determined to be 97.5% pure by HPLC: MH^+ 1497.60, calcd 1497.66.

Analogue **8** was obtained by use of this general procedure.

Amino acid analysis gave the expected ratios for all amino acids including Pal, Cpa, Fpa and Nal.[13]

Bioassays. In vitro, the peptides were tested for their ability to inhibit GnRH-stimulated LH secretion by cultured dispersed anterior pituitary cells.[7] The potency of GnRH antagonists was expressed relative to a standard ([Ac-Δ^3Pro^1,DFpa^2,DTrp^{3,6}]GnRH, potency = 1). In receptor binding studies, the K_D for the potent agonist [DAla^6,MeLeu^7,Pro^9-NHEt]GnRH (taken as standard) was determined from Scatchard analysis to be approximately 0.3 nM. A K_D for the antagonists was calculated from their potencies (relative to the standard) determined from displacement data.[6] Histamine release by rat mast cells has been reported.[18,19] The in vivo AOA were carried out as described by Corbin and Beattie;[8] cycling rats were injected subcutaneously with the peptides dissolved in saline (200 μL), unless otherwise mentioned, at noon on proestrus. The potencies obtained in the different biological assays are reported in Table I. The ability of GnRH analogues to modify LH secretion in vivo was evaluated in adult male Sprague–Dawley rats, which were kept under a standard feeding and lighting regimen. Castration, when appropriate, was performed under light ether anesthesia 7–10 days prior to the assay. On the day of the experiment, a first blood sample was obtained under ether anesthesia, immediately followed by administration of the treatment. Intravenous injections were done in the jugular vein in a volume of 1 mL. Subcutaneous injections were done, in a volume of 0.2 mL, in the neck area. Oral administration of the peptides (0.5 mL) was performed through a standard gavage tube.[26] These procedures, as well as subsequent blood sampling, were carried out under ether anesthesia. All protocols were approved by the Salk Institute Animal Welfare Committee. The peptides were first dissolved in 2 N HOAc and then brought to the appropriate concentration in 0.1% bovine serum albumin–0.04 M phosphate buffer, pH 7.4.

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