Design, Synthesis, and Molecular Modeling of a Novel Amide-Linked Cyclic GnRH Analogue Cyclo(4−9)[Lys^4,D-Trp^6,Glu^9]GnRH: Stimulation of Gonadotropin Gene Expression

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This report describes the rational design, synthesis, and pharmacological properties of an amide-linked cyclic analogue of gonadotropin-releasing hormone (GnRH) namely Cyclo(4−9)[Lys^4,D-Trp^6,Glu^9]GnRH. The conformationally restricted analogue is characterized by reduced flexibility of the peptide strand due to the introduction of a β-turn mimetic through 4,9 residue amide cyclization. The cyclic analogue was found to stimulate gonadotropin gene expression in the goldfish pituitary with similar potency compared to two native forms of GnRH. Simulation studies based on ROE connectivities in linear GnRH and potency of cyclic analogue supports the His^2, Trp^3, Tyr^5 clustering considered important for triggering receptor activation.

Gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH) stimulates production of gonadotropin hormones through interaction with specific receptors triggering important physiological functions.[1] A vast number of linear analogues has been synthesized and tested for several medicinal uses. Several linear analogues, triptorelin, leuprolide, buserelin, goserelin, nafarelin, histrelin, are known in clinical use for cancer treatment and reproductive disorders. The number of reported synthetic cyclic GnRH analogues is however limited.[2−4] Our experience in cyclization procedures has previously been applied to developing novel analogues of angiotensin,[5−8] myelin basic protein,[9−14] and thrombin receptor peptides (TRAPS),[15−18] which led us to pursue the synthesis of a rationally designed cyclic GnRH analogue. In this study we used linear salmon and chicken GnRH as control to test the activity of the synthesized GnRH analogue in goldfish in terms of gonadotropin subunit gene expression. This well characterized model system has previously been used to evaluate the activity of various GnRH analogues.[19−23]

Development of alternative molecules that mimic the activity of GnRH and analogues for clinical use provides an advantage over linear GnRH peptides in terms of stability and is a necessary step to improve their therapeutic efficacy. Two approaches have been used in the past to develop potent GnRH analogues. One is based on the design and synthesis of nonpeptides or small semimimetic peptides. This design is based on the knowledge of the amino acid residues of GnRH and involves the construction of a chemical moiety in which the important pharmacophoric groups are incorporated. The other approach is based on the design and synthesis of potent cyclic GnRH analogues, which offer several advantages such as increased resistance to metabolic degradation, and restriction of conformational flexibility. Constrained peptide analogues of high potency offer important information regarding the bioactive conformation assumed by the peptide which would be important in further development of peptide mimetics.

In this report, the rational design, synthesis, and biological activity of a cyclic peptide analogue, namely cyclo (4−9) [Lys^4,D-Trp^6,Glu^9]GnRH, derived from GnRH with D-Trp at position 6, is described. D-amino acids at position 6 are known to stabilize a β-turn between residues 5 to 8. The cyclic analogue was designed based on the conformational features of GnRH which show a His^2/Trp^3/Tyr^5 ring cluster[20,21] and the need of a β-turn at the sequence Tyr^5-Gly^6-Leu^7-Arg^8. Incorporation of a β-turn mimetic in the position of the bend leads to a peptide analogue in which the amino acids Gly^6 and Leu^7 have been replaced by a γ-lactam.[24] This information has enabled us to design and synthesize a cyclic analogue with the linkage between residues 4 (Lys) and 9 (Glu) and with D-Trp at position 6.

The utility of backbone cyclization has been well established in peptides, and it has been demonstrated to increase biological activity and in vivo stability.[25−27] The advantages of using cyclic analogues over linear counterparts include the following: (i) A lack of conformational flexibility which is an important characteristic of linear counterpart; cyclization confirms or eliminates suggested active conformations. (ii) Greater stability and higher resistance to enzymatic degradation makes cyclic analogues better candidates for therapeutic drugs. (iii) More precise conformation and increased receptor selectivity and specificity due to cyclization of amino acid sequences. This is important as there is clear evidence for the presence of GnRH receptor subtypes in humans and other animals with different ligand selectivity.[28] (iv) Constitutes a prephase intermediate step toward the rational design and development of a nonpeptide drug for oral administration.

Peptide cyclization has proved to be a very valuable tool in the design of analogues with resistance to metabolic degradation in other systems.[29] However, this approach requires at least two structural changes within a single analogue and connection of two residues which have minimal influence on biological activity. Therefore, in this study we have replaced Ser^4 and Pro^9 with Lys and Glu respectively, which allow an amide bond

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Table 1. Primary Structure of Tested GnRH (LHRH) Analogues in Gonadotropin Gene Expression

	amino acid sequence									
name	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
GnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$GlyNH_2$
GnRH salmon							Trp	Leu		
GnRH II chicken (GnRH−II)					His		Trp	Tyr		
cyclo(4−9)[Lys^4,D-Trp^6,Glu^9]GnRH (**1**)				Lys		D-Trp			Glu	

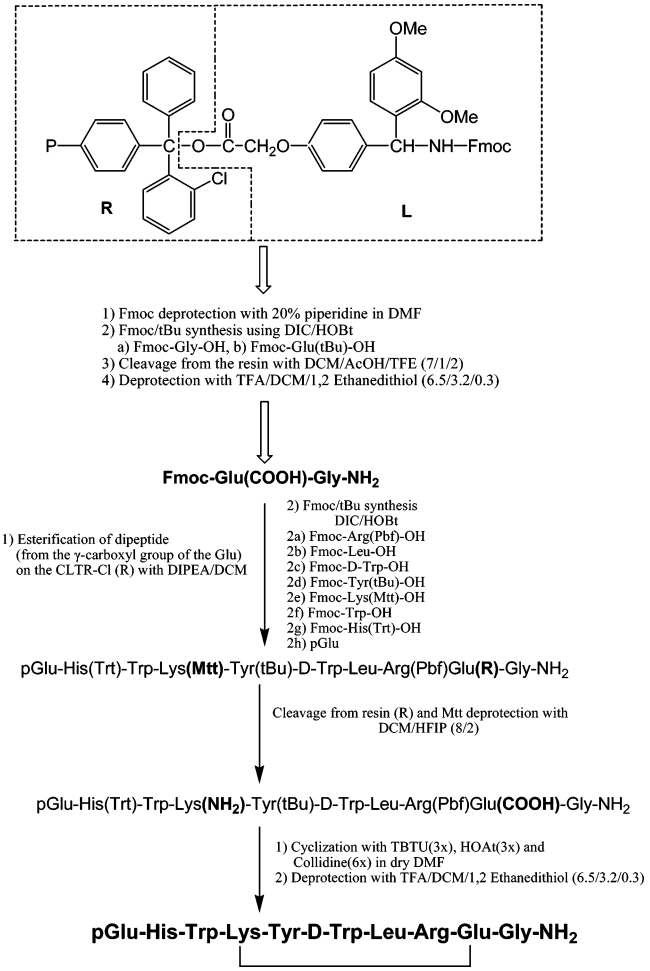

Figure 1. Synthetic procedure of cyclic LHRH analogue (**1**).

linkage between two residues not needed for activity. The potency of the cyclic analogue confirms the validity of our choice. In particular, the cyclic analogue has been demonstrated to stimulate gonadotropin subunit gene expression with potency comparable to that exerted by linear salmon and chicken GnRH forms.

The amino acid sequence of linear and cyclic GnRH analogues used in this study is shown in Table 1 in comparison to mammalian and fish GnRH molecules. It should be noted that chicken GnRH-II tested in goldfish is the second form of GnRH molecule present in mammals including humans.[30,31] The chicken GnRH-II is also referred to as GnRH-II or GnRH type II.

Figure 1 shows the novel strategic synthesis of cyclic analogue, cyclo (4−9) [Lys^4,D-Trp^6,Glu^9]GnRH, in which residues at positions 4 (Ser) and 9 (Pro) were replaced by Lys and Glu, respectively. Initially the dipeptide Fmoc-Glu-Gly-NH_2 was synthesized by Fmoc/tBu synthetic methodology using "linker"(Rink-Bernatowitz)-resin (2-chlorotrityl chloride). The obtained peptide was then attached to 2-chlorotrityl chloride resin through the γ carboxyl group of Glu. Stepwise peptide synthesis led to the linear protected decapeptide attached to resin through the γ carboxyl group of Glu. Cleavage of protected peptide from the resin and Mtt deprotection using 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) released the Glu^9 side-chain carboxyl group and the Lys^4 side-chain amino group. These groups need to be available for the next coupling−cyclization step. The amide bond was established using cyclization reagents *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxy-7-azabenzotriazole (HOAt), and 2,4,6-collidine in DMF for highest yield (80%). The use of HOAt speeds up the reaction rate of the cyclization when compared to common cyclization using HOBt. Usage of 2,4,6-collidine ensures that the final product will be free of racemization, not feasible if other bases such as diisopropylethylamine (DIPEA) were used. The free cyclic analogue was purified by semi preperative reverse phase high performance liquid chromatography (RP-HPLC), and its structure was confirmed by electron spray ionization mass spectrometry (ESI-MS, $M + H^+ = 1365.67$). This synthetic procedure leads to high yield and free racemization final cyclic product as mild conditions for the coupling steps, removal of peptide from resin, cyclization step, and the final deprotection were used. Following this procedure we avoided to use the Boc/Bzl peptide methodology.

In a previous publication[20] the conformational analysis of GnRH was achieved, using a combination of 1D-NOE, 2D-NOESY NMR experiments and molecular modeling. These

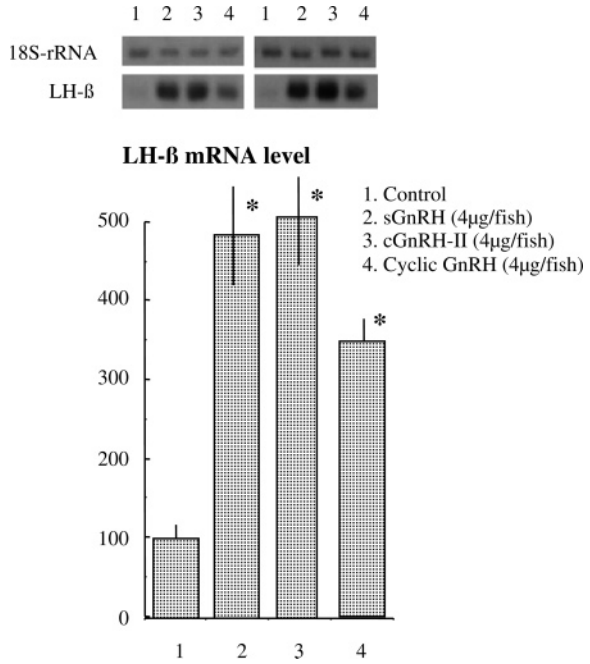

Figure 2. The effects of cyclic GnRH, sGnRH, and cGnRH-II on LH-β mRNA levels in the goldfish pituitary in vivo. Goldfish were injected with 4 μg/fish of each compound dissolved in 100 μL of saline for 24 h. Results (means ± SE, $n = 3$) reflect the percent change with respect to the control. The results were analyzed by a one-way ANOVA followed by the Student−Newman−Keuls test for multiple comparisons of means. Means were considered statistically different if $P < 0.05$ and are indicated by an asterisk.

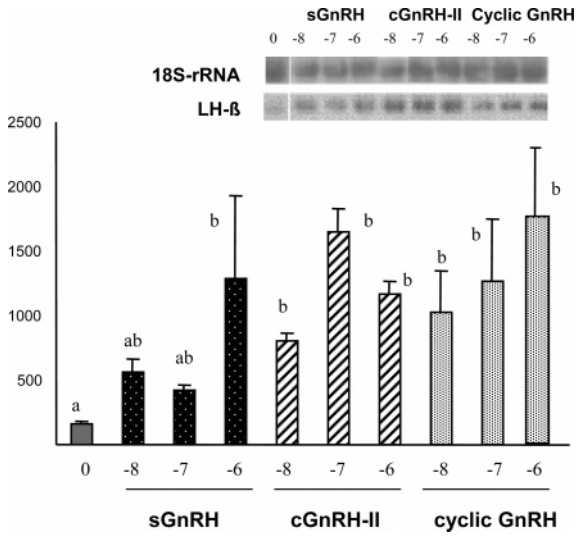

Figure 3. Dose-related effects of cyclic GnRH, sGnRH, and cGnRH-II on LH-β mRNA levels in primary cultures of dispersed goldfish pituitary cells. Goldfish pituitary cells were treated continuously for 12 h with various concentrations of cyclic GnRH, sGnRH, and cGnRH-II at concentrations of 10^{-8}, 10^{-7}, or 10^{-6} M. Results (mean ± SE, $n = 3-4$), reflecting the percent change with respect to control. The results were analyzed as described in Figure 2, by a one-way ANOVA followed by the Student−Newman−Keuls. Means were considered statistically different if $P < 0.05$ and are indicated by different letters.

studies revealed that His and Trp remain in spatial proximity, while Tyr shares its spatial interactions with both Arg and His side chains. Arg and His interact in space with pGlu and Trp side chains. In addition, pGlu interacts with Gly, imposing a cyclic conformation on GnRH. The study demonstrated that there is clustering of three aromatic rings His, Trp and Tyr in GnRH which seems to be important for activity. On the other hand the bioactive conformation of cyclic GnRH antagonist is characterized by Asp[4] and Arg[8] proximity seen in NOE experiments.[32]

Molecular dynamics studies carried out for cyclo(4−9)[Lys[4],D-Trp[6],Glu[9]]GnRH revealed four discreet family groups. From each family the minimum energy conformation was studied (Figure 4). In conformations **A** and **D**, two clusters are evident, one containing the aromatic rings of Trp[3] and Tyr[5] (presented yellow) and one containing pGlu[1] and His[2] (presented cyan). D-Trp[6] in **A** seems to be in spatial proximity with the first cluster, and pGlu[1] approaches Arg[8]. In conformer **D** one more cluster is present, that of D-Trp[6] indole with guanidino group of Arg[8] (presented purple). In **B** and **C** the proximity between Trp[3] and Tyr[5] is absent, but the second cluster involving residues pGlu[1] and His[2] is still formed. A new family appears: that of aromatic rings of D-Trp[6] and Tyr[5] (presented yellow). In conformer **A** four hydrogen bonds can be observed (green dots): one intraresidue between CO of His[2] and Hδ_1 of His[2], and three interresidue between CO of His[2] and NH of Lys[4], CO of Tyr[5] and Hϵ_1 of Trp[3], and CO of Tyr[5] and NH of Leu[7]. In conformer **B** only one hydrogen bond is seen between CO of Leu[7] and NH of Glu[9], same as in conformer **C**, where Arg[8] moves toward the cluster of the aromatic rings.

The present conformational analysis reveals conformations with high tendency of clustering between the key amino acids Trp[3], Tyr[5], and D-Trp[6] as well as a closeness between pGlu[1] and Arg[8]. Such clustering, with the additional spatial proximity of His[2], Trp[3], and Tyr[5], was observed in GnRH itself.[19,20] As can be seen (Figure 4), some contacts are lost whereas some new are created and stabilized by hydrogen bonds; this net constrained formation, which presents both conformational similarities and dissimilarities between the synthetic cyclic analogue under study and its linear counterpart, may explain in part the high biological activity of the former, as stated by the biological tests performed.

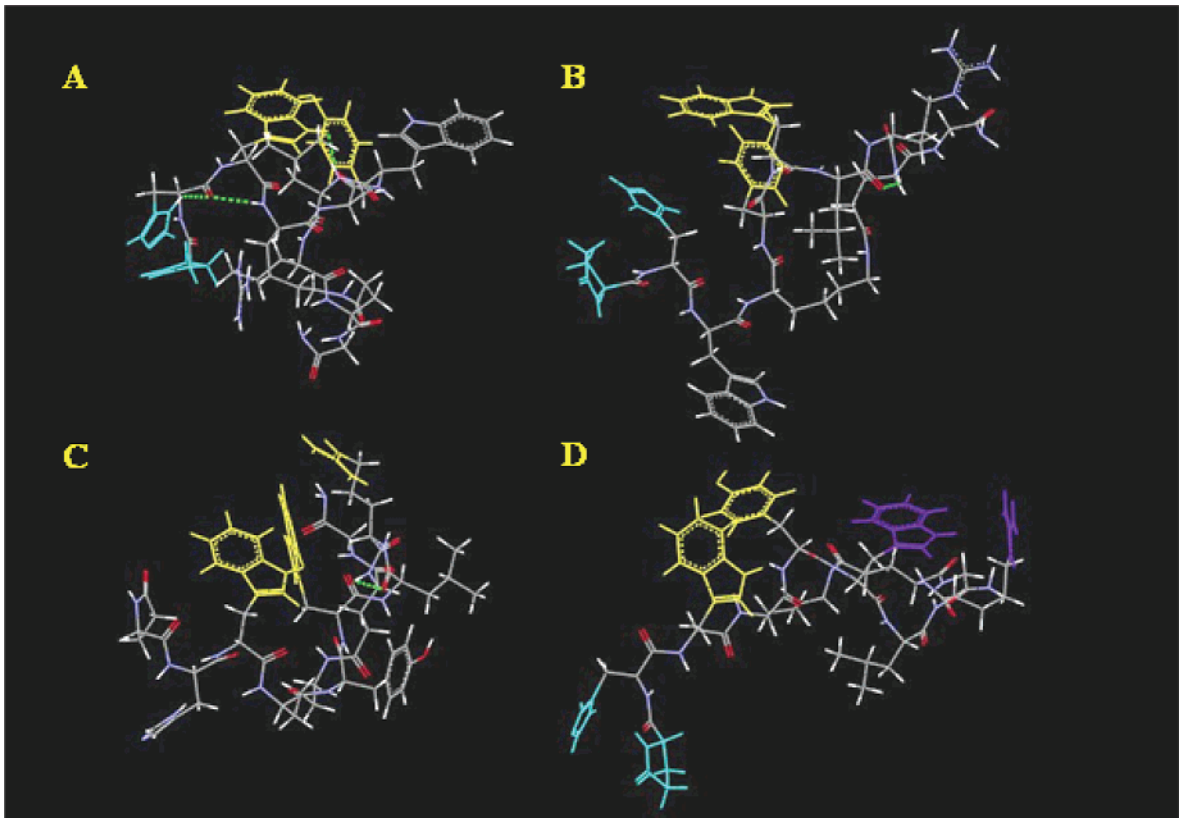

Figure 4. Representative lowest energy conformations of four discreet structure groups of cyclic analogue **1**, after simulated annealing experiments.

The results provide information on the activity of cyclic GnRH analogue in vivo and in vitro, in comparison with the native forms of GnRH in goldfish (salmon GnRH and chicken GnRH-II).[22,23] Injection of goldfish with 4 μg of cyclic GnRH significantly increased LH-β mRNA levels in the goldfish pituitary. The cyclic GnRH-induced LH-β mRNA increase was not significantly different from those induced by sGnRH and cGnRH-II (Figure 2). To further test the potency, the activity of cyclic GnRH was tested on cultured goldfish pituitary cells in vitro. Increasing concentrations of cyclic GnRH significantly increased the LH-β mRNA level with potency similar to chicken GnRH-II and higher than that of salmon GnRH (Figure 3). The observed difference in potency of salmon GnRH and chicken GnRH-II is consistent with earlier observations.[23,33,34]

In this work, cyclic GnRH analogue, (4,9)[Lys^4,D-Trp^6,Glu^9] is a rationally designed agonist, since cyclization was imposed at positions which are the least important for activity, without affecting position 2 which is responsible for antagonist activity. Furthermore, in this structure, important residues for activity such as Trp, His, Tyr remain intact and clustered for triggering agonist activity as native GnRH. Design was based on previous SAR studies and on the ring cluster conformational model suggested for this peptide.[20,21]

On the contrary cyclic analogues 4,10; 5,8; 1,3; 1,5, dicyclic 1,5−4,10; 1,3−4,10; and tricyclic 1,3−4,10−5,8 reported in the literature to exhibit antagonist activity bear structural characteristics at the backbone chain, which is the result of major modifications at crucial amino acids allowing affinity for GnRH receptor without triggering activity.[2−4] In all these analogues, residue at position 2 is a D amino acid required for antagonist activity. Major modifications imposed in GnRH at the backbone sequence may provide a locked conformation for optimal affinity and antagonist activity.

Experimental Section

1. Synthesis of Cyclic Analogue: Cyclo(4−9)[Lys^4,D-Trp^6,Glu^9]-GnRH (1). Synthesis of Dipeptide: Fmoc-Glu-GlyNH$_2$. Fmoc-linker(Rink)-2-chlorotrityl resin (2.5 g, 0.55 mmol Linker/g resin) was used for the synthesis of the dipeptide, following the protocol previously described using the Fmoc-Gly-OH and Fmoc-Glu(tBu)-OH protected amino acids.[9−11] The protected peptide, Fmoc-Glu-(tBu)-Gly-Linker-2-chlorotrityl resin, was treated with the splitting mixture dichloromethane (DCM)/acetic acid (AcOH)/2,2,2-trifluoroethanol (TFE) (15 mL, 7/1/2) for 1 h at RT, and the peptide was removed from the resin. The mixture was filtered off and the resin washed with the splitting mixture (10 mL) twice and DCM (10 mL) three times. The solvent was removed on the rotary evaporator, and the oily product was precipitated from dry diethyl ether as a white solid (930 mg, 90%). The above protected peptide was treated with 65% trifluoroacetic acid (TFA) and 3% 1,2 ethanedithiol in DCM for 4 h to deprotect Glu from tBu and liberate the amidated dipeptide fragment from linker. The solution was concentrated under vacuum to a small volume (0.5 mL). The final Fmoc dipeptide (Fmoc-Glu-GlyNH$_2$) was precipitated in white powder by the addition of dry/cold driethyl ether (Et$_2$O) (470 mg) (Figure 1).

Synthesis of Protected Linear Peptide: pGlu-His(Trt)-Trp-Lys(NH$_2$)-Tyr(tBu)-D-Trp-Leu-Arg(Pbf)-Glu(COOH)-GlyNH$_2$. Fmoc-Glu-GlyNH$_2$ was used for the synthesis of the linear protected peptide following the protocol previously described.[9−11] The dipeptide Fmoc-Glu-GlyNH$_2$ was attached to the 2-clorotrityl chloride resin through the γ-carboxyl group of Glu. Resin (1 g, 0.7 mequiv Cl$^-$/g resin) in dry DCM was stirred in a round-bottom flask. DIPEA (4.5 mmol, 0.75 mL) and Fmoc-Glu-GlyNH$_2$ (0.7 mmol, 287 mg) were added, and the solution was stirred for 45 min at RT. A mixture of DCM/methanol (MeOH) /DIPEA (7 mL, 85/10/5) was then added, and the mixture was stirred for another 10 min at RT. The Fmoc-Glu(resin)-GlyNH$_2$ was subsequently filtered and it was washed with DCM (3 × 10 mL), 2-propanol (^{i}PrOH) (2 × 10 mL), and *n*-hexane (2 × 10 mL) and dried in a vacuum for 24 h (1.26 g).

The remaining peptide chain was assembled by sequential couplings of the following Fmoc-protected amino acids (1.75 mmol), *N*,*N*′-diisopropylcarbodiimide (DIC) (2 mmol), and 1-hydroxybenzotriozole (HOBt) (2.6 mmol) in dimethylformamide (DMF) for 4−6 h. The completeness of each coupling was verified by the Kaiser test and thin layer chromatography (TLC). The protected amino acids which are used in synthesis were Fmoc-Arg(Pbf)-OH, Fmoc-Leu-OH, Fmoc-D-Trp-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Trp-OH, Fmoc-His(Trt)-OH, and pGlu-OH (Figure 1). Each time the Fmoc protecting group was removed by treatment with piperidine solution (25% in DMF, 2 × 15 min). The synthesized protected peptide on resin was dried in vacuo and then treated with the splitting mixture DCM/1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (8/2) for 8 h at RT to remove the peptide from the resin and deprotect the Lys from Mtt. The mixture was filtered, and the resin was washed with the splitting mixture (2 × 10 mL) and DCM (3 × 10 mL). The solvent was removed on a rotary evaporator, and the obtained oily product was precipitated from cold dry diethyl either as a white solid (0.95 g).

Cyclization of Protected Peptide. To a solution of the above linear protected peptide (0.1 mmol, 197 mg) in dry DMF (40 mL) were added 2,4,6-collidine (0.6 mmol, 0.079 mL) and HOAt (0.3 mmol, 40.83 mg). The resulting solution was being added dropwise to a solution of TBTU (0.3 mmol, 96.33 mg) in dry DMF (80 mL) for 3 h, and the solution was then stirred for a period of 10 h. Frequent controls were performed, every 2 h, by the ninhydrin test on TLC using *n*-butanol/acetic acid/water (BAW, 4/1/1) as an elutant and analytical RP-HPLC. The solvent was finally removed from the reaction mixture under reduced pressure affording a light-yellow oily residue. The cyclic protected peptide (136.9 mg) was precipitated from H$_2$O and was dried in a vacuo for 12 h.[9−11]

Preparation of Cyclo GnRH Analogue: Cyclo(4−9) pGlu-His-Trp-Lys^4-Tyr-D-Trp-Leu-Arg-Glu^9-GlyNH$_2$. The dried cyclic protected peptide (136.9 mg) was treated with 65% TFA in DCM in the presence of 0.3% 1,2-ethanedithiol for 5 h at room temperature. The final solution was concentrated under vacuum to a small volume (0.5 mL). Several drops of methanol were added, and the final free cyclic peptide was precipitated as a light -yellow amorphous solid by the addition of Et$_2$O (95 mg). The crude peptide product was further purified by semipreparative RP-HPLC (column: Nucleosil C$_{18}$ (5 μm, 4.6 × 250 mm), eluents: A, 0.08% TFA/H$_2$O, B, 0.08% TFA/CH$_3$CN, gradient, 10−100% B over 30 min, flow rate, 1 mL/min, detector, 214 nm, t_R, 15.8 min) and was identified by ESI-MS (M + H$^+$ = 1365.67).

2. Molecular Modeling. Molecular models of GnRH and analogue **1** were developed using SYBYL 6.8,[35] a general molecular modeling program, written by Tripos, running on an H/P workstation. MMFF94s force field was used for energy calculations.[36] GnRH was minimized with Powell algorithm, under constraints derived from NMR experiments on the peptide as reported in previous studies, and then a simulated annealing experiment was performed.

MMFF94s is the "s" (static) variant of MMFF94. It incorporates altered out-of-plane bending parameters that yield nearly planar energy-minimized geometries at unstrained delocalized trigonal nitrogen centers, such as amides. So the resultant MMFF94s geometries emulate the time-averaged structures typically observed in crystallographic and most other structure determinations.

Simulated annealing is a type of molecular dynamics experiment in which the temperature of the system is cycled over time with the goal of widely sampling conformational space. The additional force field of MMFF94 is not used in the dynamics experiment, to allow all possible conformations. We applied a temperature of 1000 K for a plateau time of 1000 fs to allow the system to rearrange from its present state and then lowered the temperature for a ramp time of 1000 fs to 200 K, to bring the system into a stable state. For the procedure, exponential ramping was followed. The cycle was repeated 50 times so that multiple conformations were obtained

and analyzed using the Molecular Spreadsheet. Conformations obtained were further minimized, to ensure the system was in a low energy state.

From all resulting conformers, we chose the 10 with the lower energy that fulfilled our constraints demand. The conformation with the lowest energy, retaining the desired conformation, was chosen as a scaffold from which to build analogue **1**. The resulting structures were then energy-minimized and subjected to a simulated annealing experiment using the same experimental conditions as with GnRH, but with no constraints applied. Resulting conformations were classified to four distinct family groups, accordingly to specific features observed, mainly hydrogen bonds and clustering between aromatic rings.

3. Biological Experiments with a Cyclic GnRH Analogue. The ability of cyclic GnRH to stimulate gonadotropin subunit (LH-β) mRNA level was tested in goldfish, in vivo and in vitro. Early-mid recrudescent goldfish, *Carassius auratus*, of mixed sex ranging from 8 to 12 cm in length were purchased from Aquatic Imports (Calgary, AB). The fish were maintained in semi-recirculating tanks at 17 °C on a 16L:8D photoperiod for acclimation prior to the experiments and fed a commercial fish diet. For in vivo experiments, goldfish (4−5 fish per group) were anesthetized lightly with 0.5% 3-aminobenzoic acid ethyl ester (Sigma) and injected intraperitoneally with 4 μg/fish salmon LHRH, 4 μg/fish chicken LHRH-II, 4 μg/fish of cyclic GnRH, or sham injected with the same volume (100 μL) of saline (0.9% sodium chloride) vehicle for 24 h. At each time point, control and treated fish were anesthetized and sacrificed in accordance with the principles and guidelines of the Canadian Council on Animal Care. The pituitaries were removed, and total RNA was extracted as described below.

Dispersed Cell Experiments. The in vitro experiments were performed on late recrudescent goldfish (April). The fish were anesthetized and sacrificed and their pituitaries removed and placed in dispersion media (Medium 199 with Hank's salts (Gibco, Grand Island, NY), 25 mM HEPES, 2.2 g/L sodium bicarbonate, 0.3% bovine serum albumin, 100000U/L penicillin, 100 mg/L streptomycin, pH 7.2). The pituitaries were washed and diced into fragments. The pituitaries were treated with trypsin, trypsin inhibitor, DNAse II, and EGTA (Sigma Chemical Company). Fragments were dispersed by gentle trituration in calcium-free Hank's Balanced Salt Solution (Gibco) with 25 mM HEPES, 2.2 g/L sodium bicarbonate, 0.3% bovine serum albumin, 100000U/L penicillin, and100 mg/L streptomycin (pH 7.2). Cells were harvested and cell yield and viability determined using Trypan Blue exclusion. Cells were resuspended in culture media (Medium 199 with Earle's salts (Gibco), 25 mM HEPES, 2.2 g/L sodium bicarbonate, 100,000U/L penicillin, and 100 mg/L streptomycin, pH 7.2.) and plated (4−5 million cells per well) for 2 h at 28 °C, 5% CO_2 and saturated humidity. 1% horse serum (Gibco) was added to each well, and the cells were cultured overnight at 28 °C, 5% CO_2, and saturated humidity. The following day the culture media was replaced with testing media (Medium 199 with Hank's salts, 25 mM HEPES, 2.2 g/L sodium bicarbonate, 0.1% bovine serum albumin, 100000U/L penicillin, 100 mg/L streptomycin, pH 7.2). Salmon LHRH, chicken LHRH-II, and cyclic analogue **1** were diluted in testing media and added to the wells to achieve final concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M. The cells were incubated for 12 h at 28 °C, 5% CO_2, and saturated humidity afterwhich the media was removed and total RNA was extracted.

Determination of mRNA Levels. RNA extraction and Northern analysis was performed as described.[37] Briefly, total RNA was extracted from the pituitaries using Trizol Reagent (Gibco), based on the acid−guanidinium thiocyanate phenol−chloroform extraction method.[38] Sample purity was determined from ratios of the sample absorbances at 260:280 nm. The ratios ranged from 1.6 to 2.1. RNA was then loaded and resolved on a 1.2% agarose/formaldehyde gel at 90 V for 1.5−2 h with 5 μg of RNA per lane. Using the capillary transfer method in the presence of 20XSSPE, the RNA was transferred to a Hybond-N+ membrane (Amersham, Arlington Heights, IL). Alternatively, RNA was transferred to a Hybond-N+ membrane using a Bio-Dot dot blot apparatus (Bio-Rad Laboratories, Hercules, CA) as described. The membrane was then baked for 2 h at 80°C.

cDNA fragments were labeled via the random primer method with [α-32P]-deoxycytidine 5′-triphosphate (dCTP) 3000 Ci/mmol (Amersham). The membranes were prehybridized for 1 h and hybridized for 2 h in 30 mL of Amersham's Rapid Hybridization Buffer with the specific probe of interest. The membranes were washed in a series of increasing stringency washes up to 0.1 × SSC in the presence of 0.1% sodium dodecyl sulfate (SDS) and exposed to film. The probes could not all be hybridized at once so the membrane was stripped with boiling 0.1% SDS and rehybridized. To control for possible loading errors, the 18s rRNA subunit was used as an internal standard. The gels were stained with ethidium bromide to ensure equal loading of RNA in all lanes. The autoradiograms were scanned and quantified with a computerized densitometry program (Image 1.54, NIH, Bethesda, MD). mRNA levels were expressed with respect to the 18s rRNA levels for that lane.

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Supporting Information Available: Compound structure (Table A), ESI-MS (Figure A), and analytical RP-HPLC (Figure B) for cyclo(4−9)[Lys^4,D-Trp^6,Glu^9]GnRH analogue are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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