Published in final edited form as:

J Med Chem. 2007 May 3; 50(9): 2067–2077. doi:10.1021/jm0613931.

Structure-Activity Relationship Studies of Gonadotropin Releasing Hormone Antagonists Containing S-aryl/alkyl Norcysteines and their Oxidized Derivatives

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Abstract

A series of acyline analogues incorporating L- and D- isomers of S-arylated/alkylated norcysteines [Ncy(R), where R is 2-naphthyl, methyl and isopropyl] at positions 1, 4, 7 and 10 were synthesized. Some of these analogues were mono- and di-oxidized to sulfoxides and sulfones. All of the analogues of acyline were screened for the antagonism of GnRH-induced response in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor. Nine of the analogues (**9**, **11**, **15**, **16**, **17**, **19**, **20**, **21**, and **22**) had an antagonist potency ($IC_{50} < 2$ nM) similar to that of acyline ($IC_{50} = 0.52$ nM) in this assay. Selected analogues (**9**, **11**, **15**, **16**, **19** and **21**) were tested *in vitro* for their antagonism at the rat GnRH-R in a reporter gene assay as well as in an *in vivo* intact male rat assay. Analogues **9** and **15** were most potent in suppressing testosterone levels.

Keywords

Norcysteine scaffold; acyline; structure activity relationship studies; GnRH antagonists; biological activities

Introduction

The use of unnatural amino acids to design peptides with new or improved properties is a well-established approach referred to as structure activity relationships studies (SAR). Unnatural amino acids have been extensively used in peptide analogues to limit conformational flexibility, enhance enzymatic stability, and improve pharmacodynamics and bioavailability. In this respect, a large variety of amino acid isosteres have been designed and synthesized. The substitution of one of the α -hydrogen of glycine by a heteroatom is an interesting approach to increase the structural diversity and functional versatility of amino acids. We and others have used α -aminoglycine (Agl),¹⁻⁶ α -hydroxyglycine⁷ and α -alkoxyglycine⁸⁻¹⁰ scaffolds to mimic natural and unnatural amino acids in biologically active peptides.

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Norcysteine (Ncy) or α -thiolglycine ($\text{H}_2\text{N}-\text{CH}(\text{SH})-\text{COOH}$) is an unnatural amino acid possessing an electronegative sulfur atom attached directly to the α -carbon atom. The synthesis of racemic N^α -protected and S-substituted Ncy derivatives was first reported by Zoller et al.¹¹ Qasmi et al.¹² used tert-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc) protected-Ncy(isopropyl)-OH as an intermediate for the synthesis of α -aminoglycine derivatives. A dipeptide, L-alanyl-D, L-2-thiophenylglycine synthesized by solution phase peptide synthesis, was found to be a novel peptide substrate in the study of microbial peptide transport.^{13,14} However, there are no reports on the incorporation of Ncy(aryl/alkyl) in bioactive peptides by solid phase peptide synthesis (SPPS). Recently, we described the synthesis of Boc-D,L-Ncy(Mob)-OH, the resolution of its methyl ester, and the introduction of both D- and L-Ncy in cyclic gonadotropin-releasing hormone (GnRH) analogues.¹⁵ Boc-Ncy(Mob)-OH is compatible with SPPS and can be introduced in peptides as a bridge head to constrain peptide conformation via norcysteine-containing disulfide bridges that are shorter in ring size than the cystine bridges by one or two methylene groups.

The successful use of Boc-Ncy(Mob)-OH in cyclic GnRH antagonists led us to investigate the SAR of linear GnRH antagonist containing S-substituted Ncy derivatives, i.e., Ncy(aryl/alkyl). We hypothesized that an aryl/alkyl moiety on the sulfur atom would mimic natural or unnatural amino acid side chains in peptides. Ncy(aryl/alkyl) residues would correspond to β -sulfa derivatives of natural or unnatural amino acids, in which a methylene group is replaced by a sulfur atom. Structurally, the lever arm bearing the side chain in Ncy(aryl/alkyl) is longer than that (CH_2) of the corresponding amino acids, but shorter than that (NH-CO) found in betidamino acids.¹ In addition, we hypothesized that Ncy(aryl/alkyl) would have unique properties that could be used to modulate the hydrophobicity and the conformation of the peptides. It has been well demonstrated that oxidation of sulfur to sulfoxides/sulfones functionalities in peptides can modulate their conformations. For instance, this type of transformation may (i) establish an intramolecular hydrogen bond to a main-chain amide-NH that either disrupts helicity¹⁶ or forces the peptide to adopt a β -turn conformation,¹⁷ (ii) or may decrease hydrophobicities in amphiphilic helices causing them to revert to β -sheet conformation.^{18,19} Ncy(aryl/alkyl) amino acids can thus be used as “switchable” residues in peptides that can be converted from hydrophobic thioether or sulfide (**I**, Figure 1) to hydrophilic sulfoxides (**II**, Figure 1) and sulfones (**III**, Figure 1) upon oxidation.

Our laboratory has contributed significantly to the development of GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) analogues with tailored structural, physicochemical and biological properties and developed several GnRH antagonists for clinical investigations.²⁰⁻²⁴ GnRH antagonists, in addition to GnRH superagonists, are now recognized as potential drugs for the management of sex steroid-dependent pathologies.^{25,26} GnRH superagonists initiate a gonadal hormone surge, termed the flare effect²⁷ that may last >2 weeks before their inhibitory action resulting from desensitization of the pituitary GnRH receptors. GnRH antagonists competitively block the action of endogenous GnRH and have an immediate inhibitory effect that lasts as long as they are present in sufficient concentrations in the circulation. For this reason, in order for the preparations of GnRH antagonists to be used successfully in humans, they need to be long acting to avoid daily administration and exhibit negligible side effects such as stimulating histamine release.²⁸

One of our analogues, acyline [$\text{Ac-D-2NaI}^1, \text{D-4Cpa}^2, \text{D-3Pal}^3, \text{4Aph}(\text{Ac})^5, \text{D-4Aph}(\text{Ac})^6, \text{Ilys}^8, \text{D-Ala}^{10}$]GnRH,²⁴ (**8**; Table 1) is a potent and long acting GnRH antagonist distributed by the National Institutes of Health (NIH) to academic researchers.^{29,30} However, acyline readily forms gels in aqueous buffers at concentrations ≥ 10 mg/mL and cannot be formulated for very long duration of action. Since a sulfur atom is more polarizable³¹ than a methylene group, we hypothesized that by replacing selected amino acids in acyline with Ncy(aryl/alkyl) and their

oxidation to sulfoxides/sulfones, we could generate biologically active analogues with the desired pharmacokinetic properties (improved water solubility and potency).

We report here, the synthesis and resolution of three differentially derivatized norcysteines (which correspond to *N*^α-Boc protected β-sulfa analogues of 2-naphthylalanine, 2-aminobutyric acid and leucine) compatible with SPPS and their use in the design of analogues of acyline. The biological activities of Ncy(aryl/alkyl)-containing acyline analogues and their corresponding mono-oxidized (sulfoxide) and di-oxidized (sulfone) derivatives were investigated *in vitro* for their ability to antagonize the GnRH-induced response in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor (GnRH-R). Selected analogues were tested in an *in vitro* antagonist potency assay for rat GnRH-R and in an intact male rat model for *in vivo* efficacy in suppressing testosterone levels.

Results and Discussion

Chemistry

The racemic Boc-Ncy(aryl/alkyl)-OH (**4a-c**) were synthesized by a modified procedure reported for the synthesis of α-isopropylthiohyppuric acid by Zoller et al.¹¹ and for the synthesis of Boc-Agl(Fmoc)-OH by Quasmi et al.¹² In short, refluxing *tert*-butyl carbamate (**1**) and glyoxylic acid monohydrate (**2**) in acetone for 5 h or stirring in diethylether at room temperature (RT) overnight yielded the α-hydroxy intermediate (**3**). The reaction of 2-naphthalenethiol, methanethiol and 2-propanethiol with **3** in the presence of PTSA afforded the racemic Boc-Ncy(aryl/alkyl)-OH (**4a-c**) in a 43–75% yield, Scheme 1. The racemic Boc-Ncy(aryl/alkyl)-OH (**4a-c**) were converted into their methyl esters (**5a-c**) and then resolved by enzymatic hydrolysis with papain.¹⁵ The enantiomeric excess of the resolved amino acids (**6a-c**) was found to be greater than 98% according to chiral HPLC.

All of the Ncy(aryl/alkyl)-containing GnRH analogues shown in Table 1 were synthesized either manually or automatically on a *p*-methylbenzhydrylamine resin (MBHA-resin) using the Boc strategy. The protected peptido-resins were cleaved and deprotected in anhydrous HF (1.5 h at 0–5 °C) in the presence of scavenger anisole (10% v/v) and methyl sulfide (5% v/v). The crude peptides were purified by RP-HPLC in at least two different solvent systems (TEAP pH 2.25 and 0.1% TFA on C₁₈ silica). The analytical techniques used for the characterization of the analogues in Table 1 included RP-HPLC with two different solvent systems (0.1% TFA and TEAP pH 2.30) and capillary zone electrophoresis (CZE). Mass spectrometric analysis supported the identity of the intended structures. Since the *L*- or *D*- enantiomers of Boc-Ncy(aryl/alkyl)-OH used for the synthesis of peptides in Table 1 were not resolved initially, two diastereomers were synthesized by incorporating Boc-*D,L*-Ncy(aryl/alkyl)-OH (**4a-c**) and separated by RP-HPLC. The absolute stereochemistry at the Ncy(aryl/alkyl) residue in GnRH analogues was determined by coelution of each diastereomer with those analogues synthesized with the resolved Boc-Ncy(aryl/alkyl)-OH (**6a-c**), (see Experimental Section).

The stereochemistry of Ncy(aryl/alkyl) residues in acyline analogues being assigned, we directed our efforts to oxidise the thioethers (S-aryl/alkyl) to sulfoxides and sulfones. Control oxidation experiments were performed on acyline (**8**) and monitored by RP-HPLC and ESI-MS. The reaction of acyline with sodium periodate (NaIO₄)¹⁷ didn't show any oxidized product on RP-HPLC. However, reacting acyline with oxone³² or H₂O₂¹⁷ resulted in a new product with a mass (*M* + *H*⁺ = 1548.7) higher than that of acyline (*M* + *H*⁺ = 1532.7) by 16 atomic mass units (amu). This higher mass could be assigned to the formation of the *N*-oxide^{33,34} of *D*-3Pal at position 3. These results clearly indicated that desired sulfoxides can be prepared from Ncy(aryl/alkyl)-containing acyline analogues by oxidation with NaIO₄ without formation of any other oxidized products like sulfones or *N*-oxide of *D*-3Pal. We have previously demonstrated that the non-aromatic substitution of *D*-3Pal by *D*-glutamine in acyline was

compatible with high antagonistic potency as well as long duration of action.³⁵ In a control experiment, the addition of NaIO₄ and oxone to [D-Gln³]acyline didn't show any oxidation products on RP-HPLC indicating that the substitution with Ncy(aryl/alkyl)-OH in this analogue would generate only the desired sulfoxides and sulfones upon oxidation. Since the introduction of Ncy(isopropyl) at position 7 in acyline led to the most potent analogues (**15** and **17**) in an *in vitro* assay, we synthesized [D-Gln³]acyline analogues **23** and **26** incorporating L- and D-Ncy(isopropyl) at position 7, respectively. The analogues **11**, **13**, **15**, **17**, **19**, **21**, **23** and **26** were oxidized with NaIO₄¹⁷ in H₂O/CH₃CN (3:2) to yield the sulfoxides **12**, **14**, **16**, **18**, **20**, **22**, **24** and **27**, respectively. The sulfoxides have a chiral center at the sulfur atom. It was difficult to separate the diastereomers by preparative RP-HPLC, however analogues **18**, **27** and **14** were detected as diastereomeric mixtures on analytical HPLC or CZE (see Table 1 for actual ratios in the column entitled "purity"). L- and D-Ncy(2-naphthyl) introduced at position 1 in analogues **9** and **10** did not react with NaIO₄, and no oxidation product was identified on RP-HPLC. The di-oxidation of analogues **23** and **26** with oxone³² in MeOH/H₂O (1:1) gave sulfones **25** and **28**, respectively. The fragmentation of -SO-R or SO₂-R groups (where R is methyl/isopropyl) was observed in MALDI-MS, and molecular weights of sulfoxides/sulfones were determined by ESI-MS.

***In vitro* Biological Evaluation (Table 1)**

All of the analogues in Table 1 were tested *in vitro* for their antagonist activity in a reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a stably integrated luciferase reporter gene.³⁶ The sulfoxides, which exist as a mixture of diastereomer were difficult to separate by RP-HPLC and were tested as mixtures. The antagonism of the GnRH agonist-induced response by each analogue was obtained at several concentrations to determine the IC₅₀, the concentration required to suppress the response in the reporter gene assay by 50%. Average IC₅₀s in multiple experiments are reported in Table 1.

The overall rationale for the synthesis of the analogues described in Table 1 is presented in our introduction and consisted predominantly of obtaining one or more GnRH analogues better than acyline in terms of biological activity (more potent) and physicochemical (more soluble in aqueous buffers) property. Earlier encouraging results from the betidamino acid scan³ of acyline paved the way for the present study and we wished to further explore the effect of side chain diversity in modulating biological activities. The structures of different substitutions incorporated in acyline at positions 1, 4, 7 and 10 are presented in Figure 2. The general observations from the *in vitro* antagonist potency data of these analogues (Table 1) for the human GnRH-R will be presented first and then further discussed in detail.

Observation Number 1—Nine of the analogues (**9**, **11**, **15**, **16**, **17**, **19**, **20**, **21**, and **22**) presented here had an antagonist potency (IC₅₀ < 2 nM) similar to that of acyline (IC₅₀ = 0.52 nM) in a reporter gene assay, demonstrating compatibility of Ncy(aryl/alkyl)-containing acyline analogues for human GnRH receptor.

Observation Number 2—Analogues containing D-isomer at position 1 (**9**) and L-isomer at positions 4 (**11**) and 7 (**15**) had higher antagonist potency than their corresponding diastereomers (**10**, **13**, and **17**, respectively). However, the chiral inversion has minimal effect at position 10 (**19** and **21**). This observation is consistent with the previous report²⁵ and supports the selection of D-residues (at position 1) and L-residues (at positions 4 and 7) in acyline as being those that favored increased affinity and potency.

Observation Number 3—Mono-oxidized (sulfoxides) and di-oxidized (sulfones) analogues eluted earlier on RP-HPLC than the corresponding parent analogues and remained in solution at a concentration of 50 mg/mL in 5% mannitol upon standing at room temperature

for 24 h, when Acyline, azaline B and Ncy(aryl/alkyl)-containing GnRH antagonists formed gels.

Observation Number 4—Whereas some of the sulfoxides (**16**, **20** and **22**) are potent in the *in vitro* assay with $IC_{50} < 2$ nM, the sulfones are significantly less potent ($IC_{50} > 2$ μ M), suggesting that the mono-oxidation of sulfur is preferred over the di-oxidation for the peptide-receptor interaction.

Analysis of each entry in Table 1 shows that analogue **9** ($IC_{50} = 0.73$ nM) is 3 times more potent than its diastereomer **10** ($IC_{50} = 2.2$ nM). In GnRH antagonists, D-isomers of amino acids are favored over the L-isomers at the N-terminus (i.e., at positions 1, 2 and 3) and it was therefore expected that inversion from the D- to L-isomer would result in decrease in potency and affinity at these positions.^{3,25} The preference for D-isomers at the N-terminus may be explained in terms of stabilization of peptide conformation for receptor binding and/or stabilization against proteolysis. L- and D-Ncy(2-naphthyl) residues at position 1 in acyline are sterically hindered and our attempts to mono-oxidize these residues failed.

Over the past thirty years, no better substitution than serine at position 4 has been identified in the search for improved GnRH agonists and antagonists. Among those amino acids that were tried in a number of antagonists series are the aliphatic amino acids glycine, alanine, methionine, and proline, the charged amino acids diaminopropionic acid (Dpr), ornithine, lysine, arginine, and aspartic acid, and threonine.³⁷ On the other hand, it is well documented that a lactam ring spanning Asp⁴ to Dpr¹⁰ is compatible with high affinity and biological activity of the resulting antagonists.^{38,39} In this series, we first synthesized two diastereomers, **11** and **13**, by incorporating racemic Ncy(methyl) at position 4 in acyline, which were separated by RP-HPLC and then further mono-oxidized with NaIO₄ to analogues **12** and **14**, respectively. In the first pair, analogue **11** containing L-isomer of Ncy(methyl) was 21 times ($IC_{50} = 1.20$ nM) more potent than the corresponding diastereomer **13** ($IC_{50} = 25$ nM). Mono-oxidation of both diastereomers resulted in analogues (**12** and **14**) with decreased antagonist potency (9–13 times) compared to acyline. Structurally, mono-oxidized Ncy(methyl) residues in acyline provide the closest mimic for the serine residue and this is evident from the observation that significant gain in antagonist potency (4-fold) for the mono-oxidized analogue **14** ($IC_{50} = 6.9$ nM) was found over its parent analogue **13** ($IC_{50} = 25$ nM). However, this notion did not materialize in the case of analogue **12** and may be attributed to the orientation of the side-chain.

Several different amino acids have been introduced at position 7 of GnRH agonists and antagonists in the past.²⁵ Interestingly, leucine, found in mammalian GnRH, is not a conserved amino acid in many other species: tryptophan is found in salmon GnRH,⁴⁰ dogfish GnRH,⁴¹ chicken II GnRH,⁴² lamprey I GnRH,⁴³ and lamprey III GnRH;⁴⁴ phenylalanine is found in tunicate GnRH-1,⁴⁵ and tunicate GnRH-3;⁴⁶ histidine is found in tunicate GnRH-2,⁴⁵ and tunicate GnRH-3; and tyrosine is found in tunicate GnRH-5 and –6.⁴⁶ It is evident from the earlier studies^{36,47,48} that the pharmacophore that recognized GnRH antagonists is not very discriminatory when it comes to substitutions at position 7. The amino acids such as phenylalanine, 4-fluorophenylalanine, 4-chlorophenylalanine, tryptophan, N^α-methyl leucine, norvaline, methionine, tyrosine, norleucine, propargylglycine, asparagine, N^γ-methyl asparagine and N^γ, N^γ-dimethyl asparagine are generally tolerated with the exception of cysteine and histidine.³⁶

The incorporation of Ncy(isopropyl) at position 7 resulted in two diastereomers, **15** and **17**, which were essentially equipotent to acyline. Interestingly, **15** is the analogue with the highest antagonist potency ($IC_{50} = 0.31$ nM) of all the analogues shown here. Extension of the side chain via mono-oxidation (analogues **16** and **18**) resulted in a 4-fold decrease in antagonist potencies *in vitro*. This observation is complementary to that of Hocart et al.⁴⁷ who observed

a 2-fold decrease in potency for hydrophilic substitution at position 7. In fact, diastereomeric analogues **16** ($t_R = 20.3$ min) and **18** ($t_R = 20.1$ min and 20.8 min) were found to be more hydrophilic than the parents **15** ($t_R = 22.7$ min) and **17** ($t_R = 23.4$ min) respectively, as determined by their retention times on RP-HPLC (Table 1).

Whereas the deletion of residue 10 and its replacement by an ethylamide results in significant increase of potency *in vitro* in GnRH agonist series,⁴⁹ the selection of D-Ala¹⁰ by most groups developing GnRH antagonists was based on the fact that it extended duration of action *in vivo*. In the present study, we mimicked the alanine at position 10 with Ncy(methyl) and its mono-oxidized derivatives. All of the Ncy(methyl)-containing acyline analogues (**19** – **22**) showed high antagonist potencies ($IC_{50} < 1.0$ nM) and were essentially equipotent with acyline ($IC_{50} = 0.52$ nM) *in vitro*. This observation complements our previous study,³ wherein side chain modification of acyline with betides [Agl, Agl(Me), Agl(formyl), Agl(Me, formyl)] at position 10 did not produce a significant difference in binding affinity.

Analogues **23**–**28** were synthesized to study the biological effects of mono- and dioxidation of L- or D- Ncy(isopropyl) residues in [D-Gln³]acyline. Analogue **23** ($IC_{50} = 6.20$ nM) is 6 times more potent than its diastereomer **26** ($IC_{50} = 39$ nM), which is consistent with the data obtained for an earlier series (analogues **15** and **17**). However, we were surprised to see significantly decreased antagonist potencies for mono- and di-oxidized analogues. Whereas mono-oxidized analogues **24** ($IC_{50} = 13$ nM) and **27** ($IC_{50} = 85$ nM) were half as potent as the parent analogues **23** ($IC_{50} = 6.2$ nM) and **26** ($IC_{50} = 39$ nM), the di-oxidized analogues **25** and **28** lost antagonist potency *in vitro* ($IC_{50} = > 2$ μ M). The decrease in hydrophobicity (by analogy with Hocart's observation, see above) or steric hindrance may explain the loss of potency upon mono- and di-oxidation.

To confirm and extend the above data in a parallel assay, analogues **9**, **10**, **11**, **13**, **15**, **17**, **19**, and **21** were tested in an *in vitro* inositol phosphate (IP) assay.⁵⁰ This assay measures functional receptor by assessing coupling to effector protein. A very similar trend in antagonist potency was observed: analogue **13** being the least potent in the series (Figure 3), whereas all other analogs had similar affinity to that of acyline. This is consistent with the early observation that a D-residue at position 4 is incompatible with significant potency.

In addition, selected analogues (**7**, **8**, **9**, **11**, **15**, **16**, **19**, and **21**) were tested *in vitro* for their antagonism at the rat GnRH-R in a reporter gene assay. Like the native GnRH [K_i (human) = 0.8 nM and K_i (rat) = 26 nM],⁵¹ all the analogues tested showed a decrease in antagonist potencies for rat GnRH-R over the human GnRH-R with **9** and **15** showing the greatest species selectivity (Table 1).

Inhibitory Efficacy of GnRH antagonists in Intact Male Rats (Figure 4 and Table 2)

Selected analogues (**9**, **11**, **15**, **16**, **19** and **21**) were tested in an intact male rat assay. This assay is designed to achieve a steady-state concentration of plasma testosterone after 3–4 days of continuous administration of GnRH antagonist which provides a direct measure of *in vivo* efficacy to suppress testosterone in an intact animal model. All infused analogues produced approximately steady state concentrations of testosterone between days 4–7 (Figure 4). Varying levels of testosterone inhibition were seen at the same dose (Table 2). Within the tested series, analogues **9** and **15** were the most efficacious at inhibiting testosterone (1.03 and 1.59 ng/mL) although both azaline B (0.32 ng/mL) and acyline (0.69 ng/mL) achieved greater testosterone inhibition. While **9** and **15** were not as efficacious in the rat model as the two reference compounds, their higher antagonist potencies (6-fold and 8-fold, respectively) for hGnRH-R compared to rGnRH-R suggests that they could be significantly more efficacious in human and thus be good candidates for the therapy of sex hormone-dependent cancers. The question remains as to why **19** and **21** were not as efficacious as **9** in this assay despite the fact

that **19** and **21** were more potent than **9** in the reporter gene assay? Our results from earlier publications^{36,52} suggested that a correlation between *in vitro* potency and *in vivo* efficacy of GnRH antagonists may not exist. The *in vivo* efficacy of the GnRH antagonist depends on unique properties of distribution, binding to plasma proteins, plasma clearance, and also enzymatic stability. Because testosterone suppression for **19** and **21** remained stable over time, we assume that compound instability is a less likely explanation.

To conclude, we have successfully synthesized, enzymatically resolved and incorporated using the Boc-strategy both D- and L- isomers of three S-substituted norcysteines [Ncy(aryl/alkyl)] in acyline. These three unnatural amino acids are β -sulfa derivatives of 2-naphthylalanine, 2-aminobutyric acid and leucine. Additionally, upon partial (sulfoxide) or total (sulfone) oxidation of the sulfur atom in these amino acids in acyline we could modulate hydrophobicity and generate analogues that were readily soluble in aqueous 5% mannitol. Most of the new acyline analogues substituted with Ncy(aryl/alkyl) were potent antagonists of the human GnRH receptor with lack of discrimination for L- and D- isomers at positions 1, 7 and 10 but some discrimination at position 4. This parallels results obtained with betidaminoacids¹ and additional data will be needed to fully understand the potential and limitations of this novel class of amino acids in biologically active peptides.

Experimental Section

Instruments

Melting points (mp) were determined on a Thomas-Hoover Uni-Melt capillary melting point apparatus and were uncorrected. Optical rotations were measured on a Perkin Elmer polarimeter (Model 241) in a 1 dm microcell at 25 °C at the concentration indicated (w/v %). Thin-layer chromatography (TLC) was performed in a solvent vapor saturated chamber on Merck silica gel 60 F₂₅₄ plates using the following solvent systems: (A) EtOAc:hexane = 30:70 and (B) CH₂Cl₂:MeOH:AcOH = 90:8:2. The plates were visualized by UV absorption, I₂, and ninhydrin spray. Silica gel flash column chromatography was performed on Geduran® Silica Gel 60 (40–63 μ m) purchased from EMD Chemicals Inc. (Gibbstown, NJ). All ¹H and ¹³C NMR spectra were recorded at 60 °C on a Varian 500 MHz spectrometer in DMSO-d₆ as a solvent. Chemical shifts (δ) were expressed in parts per million referenced to the solvent peaks, either 2.50 ppm or 40.51 ppm for DMSO-d₆ (¹H and ¹³C signals respectively). Coupling constants (J) are reported in Hz. Electrospray ionization mass spectrometry (ESI-MS) for the amino acid derivatives and for sulfoxides/sulfones of the peptides were performed on a Bruker Esquire 3000 Plus instrument using nitrogen/helium gas. The sample solution was prepared in 1% AcOH in methanol. Enantiomeric purity was determined by HPLC (Agilent 1100) equipped with Agilent Technologies LC/MSD Trap XCT ion trap mass spectrometer. Chiral analytical chromatography employed CHIRALCEL OD-RH reversed-phase column (150 mm $\times$ 4.6 mm, 5 μ m particle size) purchased from Daicel USA (Fort Lee, NJ) with matching guard cartridge. Mobile Phase A: 0.1% HCO₂H in H₂O, B: 0.1% HCO₂H in CH₃CN; Gradient: 1% B - 99% B in 50 minutes; flow rate of 0.5 mL/min A/B with post-column injection of C: 20 mM NH₄OAc in H₂O; flow rate of 0.1 mL/min. The absorbency was monitored at 215 nm and 254 nm using Diode-array detector (DAD). Enantiopure standards: Fmoc-D-Trp(Boc)-OH (*t*_R = 18.6 min) and Fmoc-Trp(Boc)-OH (*t*_R = 19.0 min) were used for method calibration and standardization. The Agilent Data Analysis™ Software was used for data analysis.

Peptides were synthesized by the solid-phase approach either manually or on a CS-Bio peptide synthesizer (Model CS536). Peptides were purified by preparative RP-HPLC on a 5 cm $\times$ 30 cm cartridge, packed in the laboratory with reversed-phase Vydac C₁₈ silica (15–20 μ m particle size, 300 Å) using a Waters Prep LC 4000 preparative chromatograph system, with a Waters 486 tunable absorbance UV detector and Linseis L250E (Linseis Inc., NJ) chart recorder. The collected fractions were screened by analytical RP-HPLC on a system using two Waters 501

HPLC pumps, a Shimadzu SPD-6A UV detector, Rheodyne Model 7125 injector, Linseis L250E chart recorder and a Vydac C₁₈ column (0.46 cm × 25 cm, 5 μm particle size, 300 Å pore size). The purity of the final peptides was determined by analytical RP-HPLC performed on a Hewlett-Packard Series II 1090 liquid chromatograph using a Vydac C₁₈ column (0.21 cm × 15 cm, 5 μm particle size, 300 Å pore size) at 40 °C. The capillary zone electrophoresis (CZE) analysis of the peptides was performed on a Beckman P/ACE System 2050; field strength of 15 kV at 30 °C on an Agilent μSil bare fused-silica capillary (75 μm i.d. × 40 cm length). Matrix assisted laser desorption ionization mass spectroscopy (MALDI-MS) of the peptide analogues were measured on an ABI-PerSeptive DE-STR instrument. The instrument employs a nitrogen laser (337 nm) at a repetition rate of 20 Hz. The applied accelerating voltage was 20 kV. Spectra were recorded in delayed extraction mode (300 ns delay). All spectra were recorded in the positive reflector mode. Spectra were sums of 100 laser shots. Matrix alpha-cyano-4-hydroxycinnamic acid was prepared as saturated solutions in 0.3% TFA in 50% CH₃CN. The observed monoisotopic (M + H)⁺ values of each peptide corresponded with the calculated (M + H)⁺ values.

Starting Materials

Most amino acid derivatives were obtained from Reanal Fine Chemical Co. (Budapest, Hungary), including Boc-Leu, Boc-Pro, and Boc-Ser(Bzl). Boc-D-Ala and Boc-D-Gln were obtained from Bachem Inc. (Torrance, CA). Boc-D-4Cpa, Boc-D-2Nal, and Boc-D-3Pal were synthesized at the Southwest Foundation for Biomedical Research (under NIH Contract NO1-HD-6-2928) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. Boc-L- and Boc-D-4Aph(Fmoc) were synthesized according to the published procedure.²² Boc-ILys(N^ω-Z)⁵³ was obtained according to the published procedure. The two enantiopure standards for chiral HPLC: Fmoc-D-Trp(Boc)-OH and Fmoc-Trp(Boc)-OH (ee > 99.9%) were purchased from EMD Biosciences Inc. (San Diego, CA). Methanethiol, 2-naphthalenethiol, 2-propanethiol, *tert*-butylcarbamate, trimethylsilyldiazomethane (2 M solution in hexanes) and *p*-toluenesulfonic acid (PTSA) were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Glyoxylic acid monohydrate, sodium periodate (NaIO₄), oxone and papain were purchased from Acros (Fisher Scientific, Pittsburgh, PA). The methylbenzhydrylamine (MBHA) resin⁵⁴ with substitution of 0.40 mequiv/g was obtained according to the published procedure of Rivier et al. using *p*-toluoyl chloride in lieu of benzoyl chloride in the Friedel and Crafts step.⁵⁵ All solvents were reagent grade or better.

Synthesis of Boc-D,L-Ncy(2-naphthyl)-OH (4a)

A mixture of *tert*-butyl carbamate (**1**, 50 g, 0.43 mol) and glyoxylic acid monohydrate (**2**, 43.24 g, 0.47 mol) were refluxed for 5 h in acetone (300 mL) or stirred at RT overnight in diethyl ether (300 mL). Insolubles were filtered and filtrate was concentrated in vacuum to yield racemic α-(hydroxy)-*N*-(*tert*-butoxycarbonyl)glycine (**3**) as a gummy solid in quantitative yield. The gummy solid was dissolved in EtOAc and precipitated as white solid by adding hexanes. mp = 90 – 91 °C; ESI-MS Calcd for C₇H₁₃NO₅: 192.08 (M + H)⁺, Found: 192.10. Without further purification the racemic α-(hydroxy)-*N*-(*tert*-butoxycarbonyl)glycine (**3**, 15 g, 68.43 mmol) was mixed with 2-naphthalenethiol (27.40 g, 171 mmol) and PTSA (0.5 g) in dry toluene (200 mL) and the mixture was refluxed under a Dean-Stark trap for 6 h. The solvent was evaporated and the resulting solid was dissolved in a mixture of EtOAc (300 mL) and H₂O (400 mL) and the pH was adjusted to 8.5 using saturated sodium carbonate. The aqueous layer was washed with additional EtOAc (2 × 100 mL) and acidified to pH 3.0 with slow addition of solid potassium hydrogen sulfate. The resulting white suspension was extracted with EtOAc (3 × 150 mL), and the combined organic extracts were dried over sodium sulfate and rotoevaporated to give Boc-D,L-Ncy(2-naphthyl)-OH (**4a**, 9.82 g, 43%).

Synthesis of Boc-D,L-Ncy(methyl)-OH (4b)

This compound was prepared by condensing excess of methanethiol gas (18 g, 375 mmol) at -40°C in a pressure bottle containing a solution of **3** (10 g, 45.62 mmol), molecular sieves 3 Å and PTSA (0.3 g) in diethylether (100 mL). The pressure bottle was closed and the reaction mixture stirred for 96 h at RT. The workup was performed as described above to give Boc-D,L-Ncy(methyl)-OH (**4b**, 6.88 g, 68%).

Synthesis of Boc-D,L-Ncy(isopropyl)-OH (4c)

This compound was prepared from **3** and 2-propanethiol in 75% yield by using the procedure described for **4a**.

Synthesis of Boc-D,L-Ncy(aryl/alkyl)-OCH₃ (5a-c)

To a solution of Boc-D,L-Ncy(aryl/alkyl)-OH (**4a-c**, 10 mmol) in benzene (24 mL) and methanol (6 mL) was added 2 M solution of trimethylsilyldiazomethane in hexane (6 mL, 12 mmol) dropwise at RT. The reaction mixture was stirred for 45 min and then quenched with drop wise addition of AcOH till bubbling of N₂ ceased. Then a mixture of EtOAc (250 mL) and H₂O (50 mL) was added and the reaction mixture was stirred for 5 min. EtOAc layer was separated, dried over sodium sulfate and evaporated under vacuum. The crude product was purified by flash chromatography using a mixture of EtOAc:hexane (20:80). The concentration of fractions containing the product gave Boc-D,L-Ncy(aryl/alkyl)-OCH₃ (**5a-c**) in 90% to 95% yield.

5a. white solid; mp = $73-76^{\circ}\text{C}$; TLC (A): $R_f = 0.65$; HPLC assay: C₁₈ silica column (0.21 cm $\times$ 15 cm), buffer A: TEAP, pH 2.30, buffer B: 60% CH₃CN/40% A, gradient condition: 25% – 95% buffer B over 42 min at a flow rate of 0.2 mL/min, UV detection: 0.1 AUFS at 214 nm, $t_R = 37.3$ min, purity = 99%; ¹H NMR (500 MHz, DMSO-d₆, 60°C): 8.04 (1H, s), 8.21 (3H, m), 7.88 (3H, m), 7.55 (1H, br), 5.65 (1H, d, $J = 6.4$), 3.68 (3H, s), 1.28 (9H, s); ¹³C NMR (125 MHz, DMSO-d₆, 60°C): 169.06, 156.88, 133.90, 133.09, 132.96, 130.84, 130.13, 129.13, 128.25, 128.20, 127.37, 127.32, 80.00, 59.93, 53.27, 28.92; ESI-MS Calcd for C₁₈H₂₁NO₄S: 348.1192 (M + H)⁺, Found: 347.9245.

5b. white solid; mp = $47-50^{\circ}\text{C}$; TLC: R_f (A) = 0.60; HPLC assay: conditions same as described for **5a**, $t_R = 16.4$ min, purity = 99%; ¹H NMR (500 MHz, DMSO-d₆, 60°C): 7.58 (1H, br), 5.11 (1H, d, $J = 5.7$), 3.69 (3H, s), 2.12 (3H, s), 1.40 (9H, s); ¹³C NMR (125 MHz, DMSO-d₆, 60°C): 169.25, 155.37, 79.86, 56.70, 53.0, 28.78, 13.19; ESI-MS Calcd for C₉H₁₇NO₄S: 236.0879 (M + H)⁺, Found: 235.8817.

5c. oil; TLC: R_f (A) = 0.71; HPLC assay: conditions same as described for **5a**, $t_R = 28.8$ min, purity = 99%; ¹H NMR (500 MHz, DMSO-d₆, 60°C): 7.55 (1H, br), 5.20 (1H, d, $J = 5.5$), 3.68 (3H, s), 3.13 (1H, septet, $J = 6.9$), 1.40 (9H, s), 1.24 (6H, d, $J = 6.9$); ¹³C NMR (125 MHz, DMSO-d₆, 60°C): 170.16, 155.06, 79.80, 55.79, 53.05, 35.56, 28.78, 24.28, 23.94; ESI-MS Calcd for C₁₁H₂₁NO₄S: 264.1192 (M + H)⁺, Found: 263.8817.

Enzymatic resolution of Boc-D,L-Ncy(alkyl/aryl)-OCH₃ (5a-c)

Preparation of buffer: 0.1 M Na₂HPO₄·7H₂O, 0.1 M citric acid and 1.5 mM EDTA·Na₂ were dissolved in deionized water (1000 mL). pH of the solution was adjusted to 6.20 by slow addition of 2N NaOH; Activation of papain: papain (50 mg) and dithiotreitol (100 mg) were dissolved in the buffer (1 mL).

Boc-D,L-Ncy(alkyl/aryl)-OMe (**5a-c**, 3 g) were suspended in a mixture of CH₃CN (15 mL)/buffer (10 mL) and an activated aliquot of papain solution (500 μL) was added. The reaction mixtures were stirred at RT and pH was maintained at 6.20 by the addition of 0.1 M NaOH. The reactions were carried out until about 50% of the methylesters were hydrolyzed, which

took 24 h, 10 h and 12 h for **5a**, **5b** and **5c**, respectively, as monitored by RP-HPLC. Then, the reactions were quenched by adding AcOH (5 mL) and the solvents were evaporated under vacuum. The resulting residues were dissolved in EtOAc (100 mL) and the EtOAc layer was washed with H₂O (3 × 100 mL), dried over Na₂SO₄ and evaporated under vacuum. The crude products containing unreacted D-methyl esters and the resolved Boc-Ncy(aryl/alkyl)-OH (**6a-c**) were chromatographed on a silica gel column. The unreacted D-methyl esters were eluted with a mixture of DCM:MeOH (95:5) and the resolved Boc-Ncy(aryl/alkyl)-OH were eluted with DCM:MeOH:AcOH (85:10:5). The concentration of the fractions containing resolved Boc-Ncy(aryl/alkyl)-OH (**6a-c**) gave gummy solids, which were precipitated by dropwise addition of water/methanol and scratching the walls of the flask with a glass rod.

6a. white solid; mp = 95 – 96 °C; Yield: 1.35 g, 84% yield with respect to D-isomer; TLC: *R_f* (B) = 0.64; $[\alpha]_D^{25} = +154.50$ (c = 1.0, MeOH); HPLC assay: conditions same as described for **5a**, *t_R* = 30.6 min, purity = 98%; ¹H NMR (500 MHz, DMSO-d₆, 60 °C): 8.37 (1H, s), 8.21 (3H, m), 7.87 (3H, m), 7.78 (1H, br), 5.91 (1H, d, J = 5.4), 1.62 (9H, s); ¹³C NMR (125 MHz, DMSO-d₆, 60 °C): 170.11, 155.25, 134.20, 133.29, 132.82, 131.03, 131.64, 129.34, 128.57, 128.46, 127.59, 127.56, 80.12, 60.64, 28.96; ESI-MS Calcd for C₁₇H₁₉NO₄S: 334.1035 (M + H)⁺, Found: 333.9211.

6b. white solid; mp = 123 – 124 °C; Yield: 86 % yield with respect to D-isomer; TLC: *R_f* (B) = 0.61; $[\alpha]_D^{25} = +205.77$ (c = 1.0, MeOH); HPLC assay: conditions same as described for **5a**, *t_R* = 9.2 min, purity = 99%; ¹H NMR (500 MHz, DMSO-d₆, 60 °C): 7.34 (1H, br), 5.02 (1H, d, J = 5.7), 2.11 (3H, s), 1.40 (9H, s); ¹³C NMR (125 MHz, DMSO-d₆, 60 °C): 170.08, 155.11, 79.66, 56.74, 28.81, 13.10; ESI-MS Calcd for C₈H₁₅NO₄S: 222.0722 (M + H)⁺, Found: 221.8468.

6c. gummy; Yield: 90% yield with respect to D-isomer; TLC: *R_f* (B) = 0.69; $[\alpha]_D^{25} = +115.39$ (c = 1.0, MeOH); HPLC assay: conditions same as described for **5a**, *t_R* = 18.5 min, purity = 98%; ¹H NMR (500 MHz, DMSO-d₆, 60 °C): 7.26 (1H, br), 5.12 (1H, d, J = 5.7), 3.13 (1H, septet, J = 6.9), 1.40 (9H, s), 1.24 (6H, d, 6.9); ¹³C NMR (125 MHz, DMSO-d₆, 60 °C): 170.87, 155.45, 79.62, 55.97, 35.42, 28.81, 24.38, 23.96; ESI-MS Calcd for C₁₀H₁₉NO₄S: 250.1035 (M + H)⁺, Found: 249.8480.

Peptide Synthesis

All of the analogues in Table 1 were synthesized either manually or on a CS-Bio peptide synthesizer on a *p*-methylbenzhydrylamine (MBHA) resin (1 g, 0.40 mmol/g substitution) using the solid-phase peptide synthesis (SPPS) methodology (Boc strategy) and protocols previously described.⁵⁶ A 3-equiv excess of Boc-amino acid (1.2 mmol) based on the original substitution of the resin was used for each coupling. Peptide couplings were mediated for 1 h by DIC/HOBt (1.2 mmol/1.8 mmol) in DMF and monitored by the qualitative ninhydrin test. Boc removal was achieved with TFA (60% in DCM, 1–2% *m*-cresol) for 20 min. An isopropyl alcohol (1% *m*-cresol) wash followed TFA treatment, and then successive washes with triethylamine solution (10% in DCM), methanol, triethylamine solution, methanol, and DCM completed the neutralization sequence. The fully protected resin bound decapeptides were acetylated as described below. The Fmoc-protecting groups on 4Aph⁵ and D-4Aph⁶ were removed by treatment with 30% piperidine in DMF. Then the Boc-protection on N-terminal amino acid [D-2-Nal or D,L-Ncy(2-naphthyl)] was removed by TFA treatment. Acetylation of 4Aph⁵, D-4Aph⁶ and the N-terminus was performed by using excess of acetic anhydride in DCM for 30 min. HF treatment (anhydrous) of the completed peptide-resin for 75 min at 0–5 °C in the presence of anisole (10% v/v) and methyl sulfide (5% v/v) yielded the desired crude peptide after elimination of HF under vacuum, diethyl ether wash, extraction with 0.1% TFA in 40% CH₃CN/H₂O and lyophilization. All Ncy(aryl/alkyl)-containing peptides were initially synthesized with Boc-D,L-Ncy(aryl/alkyl)-OH (**4a-c**). Additionally analogues **10**, **11**, **15**, **19** and

23 were synthesized with the resolved Boc-Ncy(aryl/alkyl)-OH (**6a-c**). Azaline B (**7**)⁵⁷ and acyline (**8**)²⁴ were synthesized and purified as previously described.

Peptide Purification

The crude lyophilized peptide was dissolved in a minimum amount of 0.25 N TEAP pH 2.25/30% CH₃CN and loaded onto the HPLC cartridge described earlier. The peptides eluted with a flow rate of 100 mL/min using a linear gradient of 1% B per 3 min increase from the baseline % B (eluent A = 0.25 N TEAP pH 2.25, eluent B = 60% CH₃CN, 40% A). The fractions containing the product were subjected to a second purification step carried out with eluent A = 0.1% TFA in water and B = 60% CH₃CN/40% A on the same cartridge, using a linear gradient of 1% B per min increase from the baseline % B. The fractions containing the product were pooled and subjected to lyophilization. The final yield for these purified peptides was about 20%.

Determination of the Stereochemistry of Ncy(aryl/alkyl) in the Peptides

Since the L- and D-enantiomers of Boc-Ncy(aryl/alkyl)-OH used for the synthesis of peptides, were not resolved initially, two diastereomers were synthesized by incorporating Boc-D,L-Ncy(aryl/alkyl)-OH (**4a-c**) and separated by RP-HPLC. The absolute stereochemistry at the Ncy(aryl/alkyl) residue in GnRH analogues was confirmed by comparison of RP-HPLC retention times (Table 1) of each diastereomer with those analogues synthesized with the resolved Boc-Ncy(aryl/alkyl)-OH (**6a-c**). Analogues **11**, **15**, **19** and **23** synthesized with resolved Boc-Ncy(aryl/alkyl)-OH (**6b-c**) coeluted on HPLC with the first eluting diastereomer of the pairs (**11** + **13**, **15** + **17**, **19** + **21** and **23** + **26**). Analogue **10** synthesized with resolved Boc-Ncy(2-naphthyl)-OH (**6a**) coeluted on HPLC with the second eluting diastereomer of the pairs (**9** + **10**). Thus analogues **10**, **11**, **15**, **19** and **23** contained L-enantiomer of Ncy(aryl/alkyl) in their sequence. The results of these coelution studies are consistent with our findings that analogues containing L-isomer at positions 4 and 7 and D-isomer at positions 1 and 10 show higher antagonist potency *in vivo* than the analogues with opposite stereochemistry at the same position in the sequence.

Synthesis of [_D-Gln³, Ncy(O, isopropyl)⁷]acyline (**24**)

Analogue [_D-Gln³, Ncy(O, isopropyl)⁷]acyline (**23**, 12.40 mg, 8.10 μmol) was dissolved in 5 mL H₂O/CH₃CN (3:2 v/v) and NaIO₄ (8.67 mg, 40.53 μmol) was added. The reaction mixture was stirred at RT and the progress of the reaction was monitored by RP-HPLC. After 16 h, RP-HPLC analysis showed a new peak eluting earlier (*t_R* = 18.0 min) than **23** (*t_R* = 20.9 min). The reaction mixture was diluted with H₂O (10 mL) and purified by preparative RP-HPLC as described above. Yield = 4.5 mg (35.91%); ESI-MS (M + H)⁺ calcd. = 1546.7, found = 1546.5. Using this procedure, analogues **12**, **14**, **16**, **18**, **20**, **22** and **27** were obtained in comparable yields from analogues **11**, **13**, **15**, **17**, **19**, **21** and **26**, respectively. Acyline was used as an experimental control and no oxidized products were detected on RP-HPLC under the same conditions.

Synthesis of [_D-Gln³, Ncy(O₂, isopropyl)⁷]acyline (**25**)

Analogue [_D-Gln³, Ncy(O, isopropyl)⁷]acyline (**23**, 10 mg, 6.53 μmol) was dissolved in 5 mL H₂O/MeOH (2.5:2.5 v/v) and oxone (20 mg, 32.65 μmol) was added. The reaction mixture was stirred at RT until no starting material was detected (ca 3h) on RP-HPLC. The reaction mixture was then diluted with H₂O (10 mL) and the single major peak eluting (*t_R* = 16.2 min) earlier than **23** (*t_R* = 20.9 min) was purified by preparative RP-HPLC as described above. Yield = 3.5 mg (34.28%); ESI-MS (M + H)⁺ Calcd. = 1562.7, found = 1562.3. Using this procedure, analogue **28** was obtained in comparable yield from analogues **26**. [_D-Gln³]acyline⁵⁸ was used

as an experimental control and no oxidized product was detected on HPLC under the same conditions.

Peptide Characterization (Table 1)

Purity of the peptides was assessed using RP-HPLC and CZE under conditions reported in the legend of Table 1. The composition of the analogues was confirmed by mass spectrometric analysis.

Solubility studies

We studied the ability of GnRH antagonists to dissolve and to form gels upon standing in 5% mannitol. All GnRH analogues were lyophilized from a 0.5% trifluoroacetic acid/acetonitrile solution and were handled similarly. Dissolution was achieved with gentle swirling using the same protocol, avoiding vortexing and the formation of foam. All the peptides reported in Table 1 dissolved in 5% mannitol within minutes at a concentration of 50 mg/mL. However, upon standing at RT for 24 h, these solutions behaved differently in that some formed gels and other remained unchanged. By our definition, a gel had formed when the tube in which the original solution had been made could be turned upside down without a change of shape of the content of the tube. While acyline, azaline B and Ncy(aryl/alkyl)-containing GnRH antagonists had formed gels within 24 h, all the sulfoxides and sulfones remained in solution and did not form gels. We concluded from these experiments that sulfoxides/sulfones were more soluble than acyline, azaline B and Ncy(aryl/alkyl)-containing GnRH antagonists.

Biological Testing. Cell Culture

For the human GnRH-receptor reporter gene assay, human embryonic kidney cells (HEK293 cells) genetically modified to stably express a cloned human GnRH receptor (Larry Jameson, Northwestern University, IL) and a luciferase reporter gene under the control of LH alpha subunit promoter,⁵⁹ were cultured in phenol red free DMEM containing 10% (v/v) FBS, G418 (0.4 mg/mL), penicillin/streptomycin solution (100 units penicillin and 100 µg streptomycin per mL medium), and L-glutamine (2 mM). For the rat GnRH-receptor reporter gene assay, human embryonic kidney cells (HEK293 cells), genetically modified to stably express a cloned rat GnRH receptor were cultured in phenol red free DMEM containing 10% (v/v) FBS, hygromycin (0.04 mg/mL), penicillin/streptomycin solution (100 units penicillin and 100 µg streptomycin per mL medium), and L-glutamine (2 mM). Prior to use in functional assays these rGnRH cells were transiently transfected with a luciferase reporter gene in which luciferase transcription is driven by NFAT-responsive promoter elements (lipofectamine 2000 transfection reagent). After 16 h, cell lines were trypsinized and plated at 50,000 cells per well in a volume of 80 µL per well in white 96 well culture plates. The cells were incubated at 37 °C under 5% CO₂ overnight for assay the next day.

IC₅₀ Determination Using the Reporter Gene Assay

Each compound was assayed in duplicate at 11 descending concentrations in half log increments. Compounds in 1% DMSO (10 µL), or 1% DMSO alone as a control, were added to the hGnRH or rGnRH receptor-expressing HEK293 cells followed by gentle mixing and incubation for an additional 10 min at 37 °C under 5% CO₂. Following this, GnRH (10 µL) was added to a final concentration of 1 nM. Plates were then incubated for a minimum of 5 h at 37 °C under 5% CO₂ after which 100 µL of luciferase substrate mix was added to each well. Plates were sealed with Packard Topseal film and luminescence measured on a Molecular Devices Analyst after a 10 min incubation at RT in the absence of direct light. To derive IC₅₀ values, luciferase responses were plotted against the log of the concentration and data was submitted to nonlinear regression with a four-parameter logistic equation [sigmoidal dose-response (variable slope)] using the GraphPad Prism (version 2.01) curve fitting software

package. The geometric mean of the IC₅₀ from at least two independent experiments is reported for each compound.

Inositol Phosphate (IP) Assay

Peptides were diluted in PBS/10% dimethylsulfoxide at a 1 $\mu\text{g}/\mu\text{L}$ concentration. WT h-GnRH-R was transiently expressed in COS-7 cells. COS-7 cells were maintained at 37 °C in growth medium (DMEM) containing 10% fetal calf serum (FCS) (Gemini Bio-Products) and 20 $\mu\text{g}/\text{mL}$ gentamicin. The cells were seeded in 24-well plates (Costar, Cambridge, MA, USA). Twenty-four h after plating, the cells were transfected with 0.075 μg of cDNA/well using 2 μL lipofectamine in 0.25 mL OPTI-MEM. Five h later, 0.25 mL DMEM containing 20% FCS was added to each well. Twenty-four h after the start of transfection, the medium was replaced with fresh growth medium for 4 h. Twenty-eight h after the start of transfection, COS-7 cells transfected with the WT hGnRH-R cDNAs were washed twice with DMEM/0.1% BSA and intracellular inositol lipids were labelled with 4 $\mu\text{Ci}/\text{mL}$ [³H]myo-inositol in DMEM (inositol free) for 18 h at 37 °C. After the preloading period, the cells were washed twice in DMEM (inositol free) containing 5 mM LiCl and incubated for 2 h at 37 °C in the absence or presence of GnRH (10^{-10} , 10^{-8} and 10^{-6} M), buserelin (10^{-11} , 10^{-9} and 10^{-7} M) or increasing concentrations (10^{-11} to 10^{-5} M) of the different GnRH analogues in DMEM (inositol free)/LiCl prepared by serial dilutions of the original stocks in DMEM. Alternatively, transfected cells were co-incubated with 10^{-8} M GnRH and 10^{-8} to 10^{-5} M concentrations of the GnRH analogues. At the end of the incubation period, the medium was removed and 1 mL of 0.1 M formic acid was added to each well. The cells were frozen at -20 °C until assayed for IP production by Dowex anion-exchange chromatography and liquid scintillation spectroscopy as previously described.⁶⁰

Efficacy of Selected GnRH Analogues in Intact Male Rat Model

Stock solutions of all compounds were prepared at 1 mM in 1:3 (v/v) dimethyl sulfoxide (DMSO) in sterile water for injection and kept frozen at -80 °C until used. Working solutions were freshly prepared using the same solvent just prior to use. Alzet osmotic minipumps (Durect Corp, Model 2ML1, flow rate 9.8 $\mu\text{L}/\text{h}$) were filled according to manufacturer's instructions and primed overnight. Pumps to deliver reference GnRH antagonist azaline B were filled with solution at 19.6 μM to deliver ~ 0.66 nM/day/kg. This infusion rate was set to suppress testosterone to < 0.5 ng/mL. Azaline B was also given at a concentration of 9.8 μM , to deliver ~ 0.33 nM/day/kg. All test compounds were given at 19.6 μM (~ 0.66 nM/day/kg).

All experimental procedures in this study were done in accordance with the Guide for the Care and Use of Laboratory Animals; National Academy Press: Washington, DC, 1996 and the Animal Care and Use Committee at the Torrey Pines Institute for Molecular Studies protocol #FRI-05-016. Intact adult male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN), housed singly under controlled light (12 h light/12 h dark) and temperature (22–25 °C), and given water and food *ad libitum*. Animals were weighed on day 0, 2 and 7. Three rats were used for each compound tested.

On day 0, mini-pumps were implanted subcutaneously, just posterior to the scapulae, in the back of rats (275–310 g) under isoflurane anesthesia following manufacturer's guidelines. Sham operations were performed on control rats. Blood (200 μL) was collected from each animal on days 3, 4, 5, 6, and 7. Plasma was separated by centrifugation at 4,000 g for 10 min at 4 °C and stored at -80 °C until assayed for testosterone. Plasma samples were analyzed for testosterone using an enzyme-linked immunosorbent assay (DSL-10-4000, Diagnostic Systems Laboratories, Webster, TX) according to manufacturer's guidelines except that 25 μL plasma volume was used.

Acknowledgements

Work at the Salk Institute and ONPRC/OHSU were supported by NIH grants HD039899 and HD19899, respectively. We wish to thank Ron Kaiser and Charleen Miller for technical assistance and William Low and Dr. Wolfgang Fisher for MS analysis, Rika Shuto and Susan Song for *in vivo* testing, Thomas Baiga and Dr. Joseph Noel for ^1H , ^{13}C NMR and chiral HPLC analyses, Dorain Thompson and Monica Wolfe for construction and maintenance of stable cell lines for *in vitro* assays, Drs. Judit Erchegyi and David Chatenet for critical reading of the manuscript and Debbie Doan for manuscript preparation. *In vitro* and *in vivo* biological assays were carried out at FRI Inc. Inositol phosphate assay was carried out at the Oregon National Primate Research Center. J.R. is The Dr. Frederik Paulsen Chair in Neurosciences Professor.

Abbreviations

IUPAC rules are used for nomenclature except for the following:

Ac, acetyl
 Agl, α -aminoglycine
 4Aph, 4-aminophenylalanine
 Atz, [5'-(3'-amino-1H-1', 2', 4'-triazolyl)]
 Boc, tert-butoxycarbonyl
 Bzl, benzyl
 4Cpa, 4-chlorophenylalanine
 CZE, capillary zone electrophoresis
 DIC, *N,N'*-diisopropylcarbodiimide
 DIPEA, *N,N'*-diisopropylethylamine
 DMF, dimethylformamide
 Fmoc, 9-fluorenylmethoxycarbonyl
 GnRH, gonadotropin-releasing hormone
 GnRH-R, gonadotropin-releasing hormone receptor
 HF, hydrofluoric acid
 HOBT, 1-hydroxybenzotriazole
 ILys, *N*^ε-isopropyllysine
 LH, luteinizing hormone
 MBHA, *p*-methylbenzhydrylamine
 Mob, *p*-methoxybenzyl
 2Nal, 3-(2-naphthyl)-alanine
 Ncy, norcysteine
 3Pal, 3-(3-pyridyl)-alanine
 RP-HPLC, reversed phase high performance liquid chromatography
 RGA, Reporter Gene Assay
 TFA, trifluoroacetic acid
 Z, benzyloxycarbonyl.

References cited

1. Rivier JE, Jiang G, Koerber SC, Porter J, Simon L, Craig AG, Hoeger CA. Betidamino acids: Versatile and constrained scaffolds for drug discovery. *Proc. Natl. Acad. Sci. USA* 1996;93:2031–2036. [PubMed: 8700880]
2. Reubi JC, Schaer J-C, Wenger S, Hoeger C, Erchegyi J, Waser B, Rivier J. SST3-selective potent peptidic somatostatin receptor antagonists. *Proc. Natl. Acad. Sci. USA* 2000;97:13973–13978. [PubMed: 11095748]
3. Jiang G, Miller C, Koerber SC, Porter J, Craig AG, Bhattacharjee S, Kraft P, Burris TP, Campen CA, Rivier CL, Rivier JE. Betidamino acid scan of the GnRH antagonist Acylone. *J. Med. Chem* 1997;40:3739–3748. [PubMed: 9371239]

4. Rivier J, Erchegyi J, Hoeger C, Miller C, Low W, Wenger S, Waser B, Schaer J-C, Reubi JC. Novel sst₄-selective somatostatin (SRIF) agonists. Part I: Lead identification using a betide scan. *J. Med. Chem* 2003;46:5579–5586. [PubMed: 14667212]
5. Kirby DA, Wang W, Gershengorn MC, Rivier JE. Analogs of thyrotropin-releasing hormone using an aminoglycine based template. *Peptides* 1998;19:1679–1683. [PubMed: 9880072]
6. Yaouancq L, Rene L, Tran Huu Dau ME, Badet B. Toward synthesis of alpha-alkyl amino glycines (A3G), new amino acid surrogates. *J. Org. Chem* 2002;67:5408–5411. [PubMed: 12126440]
7. Brown AR, Ramage R. Solid phase synthesis of α -hydroxyglycine extended peptides - biological precursors of peptide amides. *Tetrahedron Lett* 1994;35:789–792.
8. Kawai M, Omori Y, Yamamura H, Butsugan Y. Optical resolution of *N*-carbobenzoxy- α -methoxyglycine. *Tetrahedron: Asymmetry* 1992;3:1019–1020.
9. Miyazawa T, Iwanaga H, Yamada T, Kuwata S. Resolution of non-protein amino acids via the enantioselective hydrolysis of their esters mediated by sulfhydryl proteases. *Biotechnol. Lett* 1994;16:373–378.
10. Kawai M, Yamada K, Hosoda K, Omori Y, Kato S, Nagayama N, Masui S, Kamiya M, Yamamura H, Araki S, Butsugan Y. Optical resolution and absolute configuration of *N*-benzyloxycarbonyl- α -alkoxyglycines. *Chirality* 1999;11:561–568. [PubMed: 10423283]
11. Zoller U, Ben-Ishai D. Amidoalkylation of mercaptans with glyoxylic acid derivatives. *Tetrahedron* 1975;31:863–866.
12. Qasmi D, René L, Badet B. An α -aminoglycine derivative suitable for solid phase peptide synthesis using Fmoc strategy. *Tetrahedron Lett* 1993;34:3861–3862.
13. Kingsbury WD, Boehm JC, Perry D, Gilvarg C. Portage of various compounds into bacteria by attachment to glycine residues in peptides. *Proc. Natl. Acad. Sci. USA* 1984;81:4573–4576. [PubMed: 16593491]
14. Kingsbury WD, Boehm JC. Synthesis of alpha-thiophenylglycine peptides. Novel peptide substrates useful in the study of microbial peptide transport. *Int. J. Pept. Protein Res* 1986;27:659–665. [PubMed: 3531054]
15. Samant MP, Rivier JE. Norcystine, a new tool for the study of the structure-activity relationship of peptides. *Org. Lett* 2006;8:2361–2364. [PubMed: 16706526]
16. Watson AA, Fairlie DP, Craik DJ. Solution structure of methionine-oxidized amyloid beta-peptide (1–40). Does oxidation affect conformational switching? *Biochemistry* 1998;37:12700–12706. [PubMed: 9737846]
17. Jiang L, Burgess K. Oxidative induction of beta-turn conformations in cyclic peptidomimetics: conformational analyses as indicators of configuration. *J. Am. Chem. Soc* 2002;124:9028–9029. [PubMed: 12148988]
18. Schenck HL, Dado GP, Gellman SH. Redox-triggered secondary structure changes in the aggregated states of a designed methionine-rich peptide. *J. Am. Chem. Soc* 1996;118:12487–12494.
19. Dado GP, Gellman SH. Redox control of secondary structure in a designed peptide. *J. Am. Chem. Soc* 1993;115:12609.
20. Cetel NS, Rivier J, Vale W, Yen SSC. The dynamics of gonadotropin inhibition in women induced by an antagonistic analogue of gonadotropin-releasing hormone. *J. Clin. Endocrinol. Metab* 1983;57:62–65. [PubMed: 6406535]
21. Rivier J, Porter J, Rivier C, Perrin M, Corrigan A, Hook WA, Siraganian RP, Vale WW. New effective gonadotropin releasing hormone antagonists with minimal potency for histamine release *in vitro*. *J. Med. Chem* 1986;29:1846–1851. [PubMed: 2428978]
22. Theobald P, Porter J, Rivier C, Corrigan A, Perrin M, Vale W, Rivier J. Novel gonadotropin releasing hormone antagonist: Peptides incorporating modified N⁰-cyanoguanidino moieties. *J. Med. Chem* 1991;34:2395–2402. [PubMed: 1714956]
23. Rivier J, Porter J, Hoeger C, Theobald P, Craig AG, Dykert J, Corrigan A, Perrin M, Hook WA, Siraganian RP, Vale W, Rivier C. Gonadotropin releasing hormone antagonists with N⁰-triazolyl-ornithine, -lysine or -para-aminophenylalanine residues at positions 5 and 6. *J. Med. Chem* 1992;35:4270–4278. [PubMed: 1280300]

24. Rivier JE, Jiang G, Porter J, Hoeger C, Craig AG, Corrigan AZ, Vale WW, Rivier CL. GnRH antagonists: novel members of the azaline B family. *J. Med. Chem* 1995;38:2649–2662. [PubMed: 7629804]
25. Karten MJ, Rivier JE. GnRH analog design structure-function studies toward the development of agonists and antagonists: Rationale and perspective. *Endocr. Rev* 1986;7:44–66. [PubMed: 2420580]
26. Jiang G, Stalewski J, Galyean R, Dykert J, Schteingart C, Broqua P, Aebi A, Aubert ML, Semple G, Robson P, Akinsanya K, Haigh R, Riviere P, Trojnar J, Junien JL, Rivier JE. GnRH antagonists: A new generation of long acting analogues incorporating urea functions at positions 5 and 6. *J. Med. Chem* 2001;44:453–467. [PubMed: 11462984]
27. Goulet, MT. *Annual Reports in Medicinal Chemistry*. Academic Press; New York: 1995. Gonadotropin Releasing Hormone Antagonists.; p. 169
28. Sundaram K, Didolkar A, Thau R, Chaudhuri M, Schmidt F. Antagonists of luteinizing hormone releasing hormone bind to rat mast cells and induce histamine release. *Agents and Actions* 1988;25:307–313. [PubMed: 2464275]
29. Herbst KL, Coviello AD, Page S, Amory JK, Anawalt BD, Bremner WJ. A single dose of the potent gonadotropin-releasing hormone antagonist acyline suppresses gonadotropins and testosterone for 2 weeks in healthy young men. *J. Clin. Endocrinol. Metab* 2004;89:5959–5965. [PubMed: 15579744]
30. Herbst KL, Anawalt BD, Amory JK, Bremner WJ. Acyline: the first study in humans of a potent, new gonadotropin-releasing hormone antagonist. *J. Clin. Endocrinol. Metab* 2002;87:3215–3220. [PubMed: 12107227]
31. Fersht, A. *Enzyme Structure and Mechanism*. 2nd ed.. W. H. Freeman and Co.; New York: 1985.
32. McCarthy JR, Matthews DP, Paolini JP. Reaction of sulfoxides with diethylaminosulfur trifluoride: Fluoromethyl phenyl sulfone, a reagent for the synthesis of fluoroalkenes. *Org. Syn* 1993;72:209.
33. Boekelheide V, Linn WJ. Rearrangements of N-oxides. A novel synthesis of pyridyl carbinols and aldehydes. *J. Am. Chem. Soc* 1954;76:1286–1291.
34. Bell TW, Cho Y-M, Firestone A, Healy K, Liu J, Ludwig R, Rothenberger SD. 9-*n*-butyl-1,2,3,4,5,6,7,8-octahydroacridin-4-ol. *Org. Syn* 1990;69:226.
35. Jiang, G.; Rivier, J. Novel, water soluble and long acting GnRH antagonists.. 16th American Peptide Symposium; Minneapolis, MN. American Peptide Society; 1999. p. 286
36. Samant MP, Gulyas J, Hong DJ, Croston G, Rivier C, Rivier J. Iterative approach to the discovery of novel degarelix analogues: substitutions at positions 3, 7 and 8. Part II. *J. Med. Chem* 2005;48:4851–4860. [PubMed: 16033265]
37. Rivier, J.; Rivier, C.; Perrin, M.; Porter, J.; Vale, W. LHRH and Its Analogs - Contraceptive and Therapeutic Applications. *Advances in Reproduction*. MTP Press; Lancaster, Boston, The Hague: 1984. LHRH analogs as antioviulatory agents.; p. 11-22.
38. Rizo J, Koerber SC, Bienstock RJ, Rivier J, Gierasch LM, Hagler AT. Conformational analysis of a highly potent, constrained gonadotropin-releasing hormone antagonist. II. Molecular Dynamics simulations. *J. Amer. Chem. Soc* 1992;114:2860–2871.
39. Bienstock RJ, Rizo J, Koerber SC, Rivier JE, Hagler AT, Gierasch LM. Conformational analysis of a highly potent dicyclic gonadotropin-releasing hormone antagonist by nuclear magnetic resonance and molecular dynamics. *J. Med. Chem* 1993;36:3265–3273. [PubMed: 8230116]
40. Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J, Vale W. Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. USA* 1983;80:2794–2798. [PubMed: 6341999]
41. Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM. Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. *Proc. Natl. Acad. Sci. USA* 1992;89:6373–6377. [PubMed: 1631133]
42. Miyamoto K, Hasegawa Y, Minegishi T, Nomura M, Takahashi Y, Igarashi M, Kanagawa K, Matsuo H. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. *Biochem. Biophys. Res. Commun* 1982;106:820. [PubMed: 6814432]
43. Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ. Primary structure of gonadotropin-releasing hormone from lamprey brain. *J. Biol. Chem* 1986;261:4812–4819. [PubMed: 3514603]

44. Sower SA, Chiang YC, Lovas S, Conlon JM. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* 1993;132:1125–1131. [PubMed: 8440174]
45. Powell JFF, Reska-Skinner SM, Prakash OM, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM. Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. *Proc. Natl. Acad. Sci. USA* 1996;93:10461–10464. [PubMed: 8816823]
46. Adams BA, Tello J, Erchevyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM. Six novel GnRH hormones are encoded as triplets on each of two genes in protochordate *Ciona intestinalis*. *Endocrinology* 2003;144:1907–1919. [PubMed: 12697698]
47. Hocart SJ, Nekola MV, Coy DH. Improved antagonists of luteinizing hormone-releasing hormone modified in position 7. *J. Med. Chem* 1985;28:967. [PubMed: 3892004]
48. Rivier, J.; Varga, J.; Porter, J.; Perrin, M.; Rivier, C.; Vale, W.; Struthers, S.; Hagler, A. Design of cyclic GnRH antagonists.. The GnRH Satellite Symposium, 7th International Congress of Endocrinology; Quebec, Canada. June 28–30, 1984; *Endocrinology*; 1984.
49. Fujino M, Kobayashi S, Obayashi M, Sinagawa S, Fukuda T, Kitada C, Nakayama R, Yamazaki I, White WF, Rippel RH. Structure-activity relationships in the C-terminal part of luteinizing hormone releasing hormone (LH-RH). *Biochem. Biophys. Res. Commun* 1972;49:863–869. [PubMed: 4565128]
50. Knollman PE, Janovick JA, Brothers SP, Conn PM. Parallel regulation of membrane trafficking and dominant-negative effects by misrouted gonadotropin-releasing hormone receptor mutants. *J. Biol. Chem* 2005;280:24506–24514. [PubMed: 15886197]
51. Anderes KL, Luthin DR, Castillo R, Kraynov EA, Castro M, Nared-Hood K, Gregory ML, Pathak VP, Christie LC, Paderes G, Vazir H, Ye Q, Anderson MB, May JM. Biological characterization of a novel, orally active small molecule gonadotropin-releasing hormone (GnRH) antagonist using castrated and intact rats. *J. Pharmacol. Exper. Ther* 2003;305:688–695. [PubMed: 12606616]
52. Samant MP, Hong DJ, Croston G, Rivier C, Rivier J. Novel analogues of degarelix incorporating hydroxy-, methoxy- and pegylated- urea moieties at residues 3, 5, 6 and the N-terminus. Part III. *J. Med. Chem* 2006;49:3536–3543. [PubMed: 16759096]
53. Ljungqvist A, Feng D-M, Tang P-FL, Kubota M, Okamoto T, Zhang Y, Bowers CY, Hook WA, Folkers K. Design, synthesis and bioassays of antagonists of LHRH which have high antiovaratory activity and release negligible histamine. *Biochem. Biophys. Res. Commun* 1987;148:849–856. [PubMed: 2446602]
54. Stewart, J.; Pèna, C.; Matsueda, GR.; Haris, K. Some improvements in the solid phase synthesis of large peptides.. The 14th European Peptide Symposium; Wépion, Belgium. Editions de l'Université de Bruxelles, Bruxelles (Belgique); 1976. p. 285-290.
55. Rivier J, Vale W, Burgus R, Ling N, Amoss M, Blackwell R, Guillemin R. Synthetic luteinizing hormone releasing factor analogs Series of short chain amide LRF homologs converging to the amino terminus. *J. Med. Chem* 1973;16:545–549. [PubMed: 4577979]
56. Stewart, JM.; Young, JD. Solid Phase Peptide Synthesis. 2nd ed.. Pierce Chemical Co.; Rockford, IL: 1984. Solid Phase Peptide Synthesis.; p. 176
57. Rivier J, Porter J, Hoeger C, Theobald P, Craig AG, Dykert J, Corrigan A, Perrin M, Hook WA, Siraganian RP, Vale W, Rivier C. Gonadotropin releasing hormone antagonists with N⁰-triazolyl-ornithine, -lysine or -para-aminophenylalanine residues at positions 5 and 6. *J. Med. Chem* 1992;35:4270–4278. [PubMed: 1280300]
58. Jiang, G.; Stalewski, J.; Galyean, R.; C., S.; Broqua, P.; Aebi, A.; Aubert, ML.; Semple, G.; Akinsanya, K.; Haigh, R.; Riviere, P.; Trojnar, J.; Junien, JL.; Rivier, JE. Novel, water soluble and long acting GnRH antagonists.. Peptides for the New Millennium; Proceedings of the Sixteenth American Peptide Symposium; Minneapolis, MN. Kluwer Academic Publishers; 2000. p. 658-659.
59. Kay TW, Chedrese PJ, Jameson JL. Gonadotropin-releasing hormone causes transcriptional stimulation followed by desensitization of the glycoprotein hormone alpha promoter in transfected alpha T3 gonadotrope cells. *Endocrinology* 1994;134:568–573. [PubMed: 7507827]
60. Huckle WR, Conn PM. Use of lithium ion in measurement of stimulated pituitary inositol phospholipid turnover. *Methods Enzymol* 1987;141:149–155. [PubMed: 3110551]

Figure 1.

Use of Ncy(aryl/alkyl) amino acids as "switchable" residues in peptides.

(a)

(b)

At position 1

At position 3
and 10

At position 7

Figure 2.

(a) Chemical structure of acyl peptide (b) Structures of the L- or D-Ncy(aryl/alkyl) amino acids incorporated in acyl peptide at positions 1, 4, 7, and 10.

Figure 3. Inhibition of GnRH-stimulated inositol phosphate (IP) production by different peptides. The peptides (10^{-8} to 10^{-5} M) were preincubated with 10^{-8} M GnRH before stimulating COS-7 cells transiently transfected with the WT hGnRHR. IP production was determined after 2 h incubation. Error bars represent mean $\pm$ SEM of a representative experiment performed in triplicate and repeated three or more times.

Figure 4.

Plasma testosterone levels in intact male rats. GnRH antagonists were delivered for seven days by infusion pumps. All compounds were given at 19.6 μM (~ 0.66 nM/day/kg). Azaline B was also given at 0.5 x of 9.8 μM , to deliver ca. 0.33 nM/day/kg. The steady-state concentration of plasma testosterone was reached after 3–4 days of administration. ◆ vehicle; ■ azaline-B; □ azaline-B x 0.5; ■ 8; ● 9; ◆ 11; ● 15; ■ 16; ▲ 19; ■ 21.

(i) acetone, reflux, 5 h or diethylether, RT, overnight; (ii) 2-naphthalenethiol/2-propanethiol, toluene, PTSA, Dean-Stark, reflux or methanethiol, molecular sieves 3 Å PTSA, diethylether, RT, 96 h, pressure bottle; (iii) $\text{Me}_3\text{SiCHN}_2$, benzene:methanol (4:1); (iv) Papain, CH_3CN :buffer (3:2), pH 6.2.

Scheme 1.

Synthesis of Boc-D,L-Ncy(aryl/alkyl)-OH and enzymatic resolution of their methyl esters.

Table 1
Physicochemical and Biological Characterization of GnRH Antagonists

	purity		t_R^c (min)	MS ^d (M + H) ⁺		human GnRH-R		Rat GnRH-R		species selectivity ^g
	HPLC ^d	CZE ^b		calcd	obsd	pIC ₅₀ ^e avg ± SEM	IC ₅₀ ^f (nM)	pIC ₅₀ ^e avg ± SEM	IC ₅₀ ^f (nM)	
D-4Cpa ² -D-3Pal ³ -4Aph(Au) ⁵ -D-4Aph(Au) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (azaline B)]	98	99	17.7	1612.8	1612.7	8.90 ± 0.07	1.36	8.90 ± 0.04	1.40	1.03
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	98	98	22.9	1532.7	1532.7	9.28 ± 0.17	0.52	8.80 ± 0.06	1.50	2.89
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	98	99	23.1	1550.7	1550.8	9.13 ± 0.33	0.73	8.40 ± 0.03	4.40	6.03
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	98	27.2	1550.7	1550.5	8.66 ± 0.24	2.20			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	98	97	24.4	1548.7	1548.7	8.90 ± 0.14	1.20	8.60 ± 0.07	2.40	2.00
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	93	96	21.5	1564.7	1564.4	8.34 ± 0.04	4.60			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	96	97	25.6	1548.7	1548.5	7.59 ± 0.07	25			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	67:33	23.1	1564.7	1564.4	8.16 ± 0.05	6.90			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	99	22.7	1550.7	1550.8	9.50 ± 0.21	0.31	8.60 ± 0.08	2.50	8.07
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	99	20.3	1566.7	1566.5	8.90 ± 0.02	1.20	8.50 ± 0.06	3.20	2.67
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	96	99	23.4	1550.7	1550.7	9.09 ± 0.13	0.81			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	37:63	60:40	20.1, 20.8	1566.7	1566.4	8.52 ± 0.16	3.00			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	94	99	23.4	1564.7	1564.8	9.09 ± 0.11	0.80	8.80 ± 0.06	1.70	2.13
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	99	21.3	1580.7	1580.4	9.05 ± 0.15	0.87			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	97	99	24.2	1564.7	1564.9	9.21 ± 0.17	0.60	8.80 ± 0.05	1.70	2.83
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	99	21.3	1580.7	1580.4	9.08 ± 0.17	0.83			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	99	99	20.9	1530.7	1530.8	8.20 ± 0.08	6.20			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	91	99	18.0	1546.7	1546.5	7.88 ± 0.20	13			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	96	99	16.2	1562.7	1562.3	5.41 ± 0.15	3815			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	98	95	20.9	1530.7	1530.8	7.40 ± 0.26	39			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	44:56	95	17.8, 18.6	1546.7	1546.5	7.07 ± 0.07	85			
D-4Cpa ² -D-3Pal ³ -4Aph(Ac) ⁵ -D-4Aph(Ac) ⁶ -Ilys ⁸ -D-Ala ¹⁰ [GnRH (aevaline)]	90	95	23.1	1562.7	1562.3	5.69 ± 0.33	2004			

^a determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH₃CN/40% A under gradient conditions (30% to 80% B over 50 min), at flow rate of 0.2 mL/min, column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

^b determined by capillary zone electrophoresis (CZE). Buffer, 100 mM sodium phosphate (85:15, H₂O:CH₃CN) and detection at 214 nm.

^c or gradient conditions (40% to 70% B over 30 min); buffer system A; TEAP, pH 7.0, buffer system B, 60% CH₃CN/40% A.

^d spectral analyses. The calculated [M + H]⁺ of the monoisotope compared with the observed [M + H]⁺ monoisotopic mass.

^e negative log of the IC₅₀ in molar, as determined in human and rat GnRH reporter gene assay.

^f of antagonist required to repress the GnRH induced response by 50% in the reporter gene assay in HEK-293 cells expressing the human and rat GnRH receptor and a GnRH-regulated luciferase reporter gene assay.

C-50 (human)

Table 2

Mean and SEM of testosterone levels in intact male rats of averaged data points of days 4–7.

no	Testosterone (ng/mL) $\pm$ SEM (n = 3)
control	3.38 $\pm$ 0.69
azaline B	0.32 $\pm$ 0.20
azaline B $\times$ 0.5	1.39 $\pm$ 0.34
8 (acvline)	0.69 $\pm$ 0.29
9	1.03 $\pm$ 0.07
11	1.99 $\pm$ 0.09
15	1.59 $\pm$ 0.21
16	3.22 $\pm$ 0.34
19	2.21 $\pm$ 0.13
21	1.97 $\pm$ 0.46