Structure−Activity Relationship Studies of Gonadotropin-Releasing Hormone Antagonists Containing *S*-Aryl/Alkyl Norcysteines and Their Oxidized Derivatives

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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 dioxidized to sulfoxides and sulfones. All of the analogues of acyline were screened for the antagonism of the 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 antagonistic 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 the most potent in suppressing testosterone levels.

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 relationship (SAR) studies. 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-hydrogens 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[a]),[1−6] α-hydroxyglycine,[7] and α-alkoxyglycine[8−10] scaffolds to mimic natural and unnatural amino acids in biologically active peptides.

Norcysteine (Ncy) or α-thiolglycine ($H_2NCH(SH)COOH$) 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 the 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 the 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 (NHCO) 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 sulfoxide/sulfone 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 helicity[16] or forces the peptide to adopt a β-turn conformation[17] or (ii) decrease hydrophobicities in amphiphilic helices, causing them to revert to the 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 thioethers or sulfides (**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-

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[a] Abbreviations: Ac, acetyl; Agl, α-aminoglycine; 4Aph, 4-aminophenylalanine; Atz, 5′-(3′-amino-1*H*-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.

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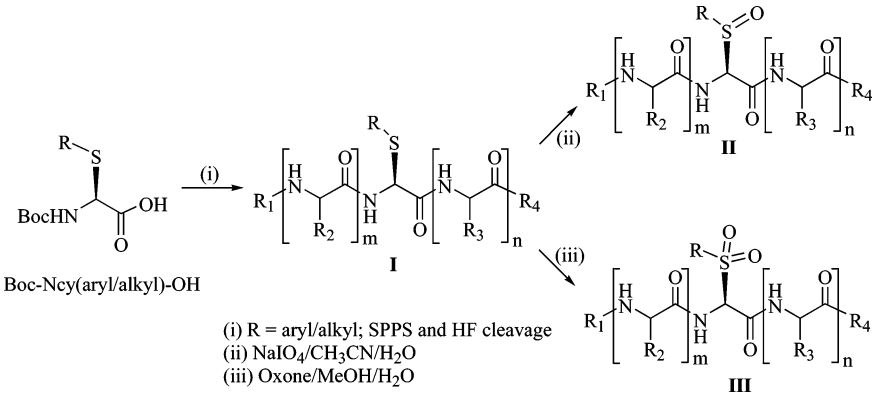
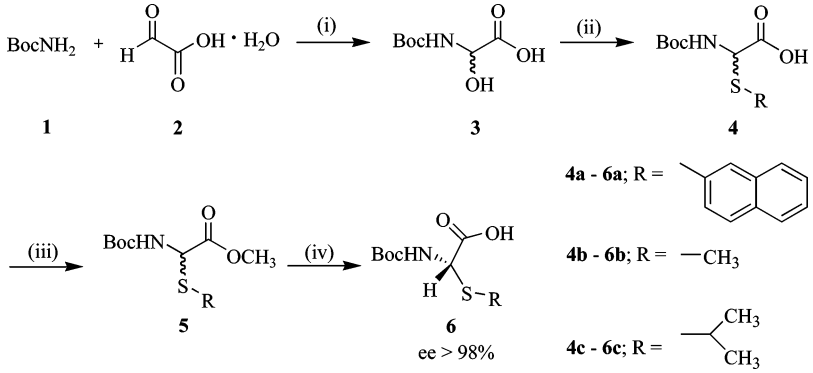


Figure 1. Use of Ncy(aryl/alkyl) amino acids as "switchable" residues in peptides.

Scheme 1. Synthesis of Boc-D,L-Ncy(aryl/alkyl)-OH and Enzymatic Resolution of Their Methyl Esters[a]



[a] Reagents and conditions: (i) acetone, reflux, 5 h, or diethyl ether, rt, overnight; (ii) 2-naphthalenethiol/2-propanethiol, toluene, PTSA, Dean−Stark trap, reflux, or methanethiol, molecular sieves (3 Å), PTSA, diethyl ether, rt, 96 h, pressure bottle; (iii) Me_3SiCHN_2, benzene/methanol (4:1); (iv) papain, CH_3CN/buffer (3:2), pH 6.2.

Table 1. Physicochemical and Biological Characterization of GnRH Antagonists

		purity (%)			MS[d] $(M + H)^+$		human GnRH-R		rat GnRH-R		
no.	compound	HPLC[a]	CZE[b]	t_R[c] (min)	calcd	obsd	pIC_{50}[e] (av ± SEM)	IC_{50}[f] (nM)	pIC_{50}[e] (av ± SEM)	IC_{50}[f] (nM)	species selectivity[g]
7	[Ac-D-2Nal[1],D-4Cpa[2],D-3Pal[3],4Aph(Atz)[5],D-4Aph(Atz)[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
8	[Ac-D-2Nal[1],D-4Cpa[2],D-3Pal[3],4Aph(Ac)[5],D-4Aph(Ac)[6],Ilys[8],D-Ala[10]]GnRH (acyline)	98	98	22.9	1532.7	1532.7	9.28 ± 0.17	0.52	8.80 ± 0.06	1.50	2.89
9	[D-Ncy(2-naphthyl)[1]]acyline	98	99	23.1	1550.7	1550.8	9.13 ± 0.33	0.73	8.40 ± 0.03	4.40	6.03
10	[Ncy(2-naphthyl)[1]]acyline	99	98	27.2	1550.7	1550.5	8.66 ± 0.24	2.20			
11	[Ncy(methyl)[4]]acyline	98	97	24.4	1548.7	1548.7	8.90 ± 0.14	1.20	8.60 ± 0.07	2.40	2.00
12	[Ncy(SO,methyl)[4]]acyline	93	96	21.5	1564.7	1564.4	8.34 ± 0.04	4.60			
13	[D-Ncy(methyl)[4]]acyline	96	97	25.6	1548.7	1548.5	7.59 ± 0.07	25			
14	[D-Ncy(SO,methyl)[4]]acyline	99	67:33	23.1	1564.7	1564.4	8.16 ± 0.05	6.90			
15	[Ncy(isopropyl)[7]]acyline	99	99	22.7	1550.7	1550.8	9.50 ± 0.21	0.31	8.60 ± 0.08	2.50	8.07
16	[Ncy(SO,isopropyl)[7]]acyline	99	99	20.3	1566.7	1566.5	8.90 ± 0.02	1.20	8.50 ± 0.06	3.20	2.67
17	[D-Ncy(isopropyl)[7]]acyline	96	99	23.4	1550.7	1550.7	9.09 ± 0.13	0.81			
18	[D-Ncy(SO,isopropyl)[7]]acyline	37:63	60:40	20.1, 20.8	1566.7	1566.4	8.52 ± 0.16	3.00			
19	[Ncy(methyl)[10]]acyline	94	99	23.4	1564.7	1564.8	9.09 ± 0.11	0.80	8.80 ± 0.06	1.70	2.13
20	[Ncy(SO,methyl)[10]]acyline	99	99	21.3	1580.7	1580.4	9.05 ± 0.15	0.87			
21	[D-Ncy(methyl)[10]] acyline	97	99	24.2	1564.7	1564.9	9.21 ± 0.17	0.60	8.80 ± 0.05	1.70	2.83
22	[D-Ncy(SO,methyl)[10]]acyline	99	99	21.3	1580.7	1580.4	9.08 ± 0.17	0.83			
23	[D-Gln[3],Ncy(isopropyl)[7]]acyline	99	99	20.9	1530.7	1530.8	8.20 ± 0.08	6.20			
24	[D-Gln[3],Ncy(SO,isopropyl)[7]]acyline	91	99	18.0	1546.7	1546.5	7.88 ± 0.20	13			
25	[D-Gln[3],Ncy(SO_2,isopropyl)[7]]acyline	96	99	16.2	1562.7	1562.3	5.41 ± 0.15	3815			
26	[D-Gln[3],D-Ncy(isopropyl)[7]]acyline	98	95	20.9	1530.7	1530.8	7.40 ± 0.26	39			
27	[D-Gln[3],D-Ncy(SO,isopropyl)[7]]acyline	44:56	95	17.8, 18.6	1546.7	1546.5	7.07 ± 0.07	85			
28	[D-Gln[3],D-Ncy(SO_2,isopropyl)[7]]acyline	90	95	23.1	1562.7	1562.3	5.69 ± 0.33	2004			

[a] Percentage purity determined by HPLC using buffer system A (TEAP, pH 2.30) and buffer system B (60% CH_3CN/40% A) under gradient conditions (from 30% to 80% B over 50 min) at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm. [b] Percentage purity determined by CZE. Buffer 100 mM sodium phosphate (85:15 H_2O/CH_3CN) and detection at 214 nm. [c] Retention times under gradient conditions (from 40% to 70% B over 30 min). Buffer system A: TEAP, pH 7.0. Buffer system B: 60% CH_3CN/40% A. [d] MALDI or ESI mass spectral analyses (*m*/*z*). Calculated $[M + H]^+$ of the monoisotope compared with the observed $[M + H]^+$ monoisotopic mass. [e] pIC_{50} is the negative log of IC_{50} (M), as determined in human and rat GnRH reporter gene assays. [f] IC_{50} is the concentration 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 receptors and a GnRH-responsive stably integrated luciferase reporter gene assay. [g] Ratio of IC_{50}(rat) to IC_{50}(human).

NH_2) 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 effect,[27] 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, 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 stimulation of histamine release.[28]

One of our analogues, acyline [Ac-D-2Nal[1],D-4Cpa[2],D-3Pal[3],-4Aph(Ac)[5],D-4Aph(Ac)[6],Ilys[8],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 a very long duration of action. Since a sulfur atom is more polarizable[31] 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 monooxidized (sulfoxide) and dioxidized (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 diethyl ether 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 *p*-toluenesulfonic acid (PTSA) afforded the racemic **4a**−**c** in 43−75% yield, Scheme 1. The racemic **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 peptidoresins 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 C_{18} 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 **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 the Experimental Section).

The stereochemistry of Ncy(aryl/alkyl) residues in acyline analogues being assigned, we directed our efforts to oxidize 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 ($NaIO_4$)[17] did not show any oxidized product on RP-HPLC. However, reacting acyline with oxone[32] or H_2O_2[17] 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 the desired sulfoxides can be prepared from Ncy(aryl/alkyl)-containing acyline analogues by oxidation with $NaIO_4$ without formation of any other oxidized products such as sulfones or the *N*-oxide of D-3Pal. We have previously demonstrated that the nonaromatic substitution of D-3Pal by D-glutamine in acyline was compatible with high antagonistic potency as well as a long duration of action.[35] In a control experiment, the addition of $NaIO_4$ and oxone to [D-Gln[3]]acyline did not 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-Gln[3]]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 $NaIO_4$[17] in H_2O/CH_3CN (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 **14**, **18**, and **27** 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 $NaIO_4$, and no oxidation product was identified on RP-HPLC. The dioxidation of analogues **23** and **26** with oxone[32] in MeOH/H_2O (1:1) gave sulfones **25** and **28**, respectively. The fragmentation of −SOR or $-SO_2R$ groups (where R is methyl/isopropyl) was observed in MALDI-MS, and the 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 antagonistic 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 diastereomers, 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 concentra-

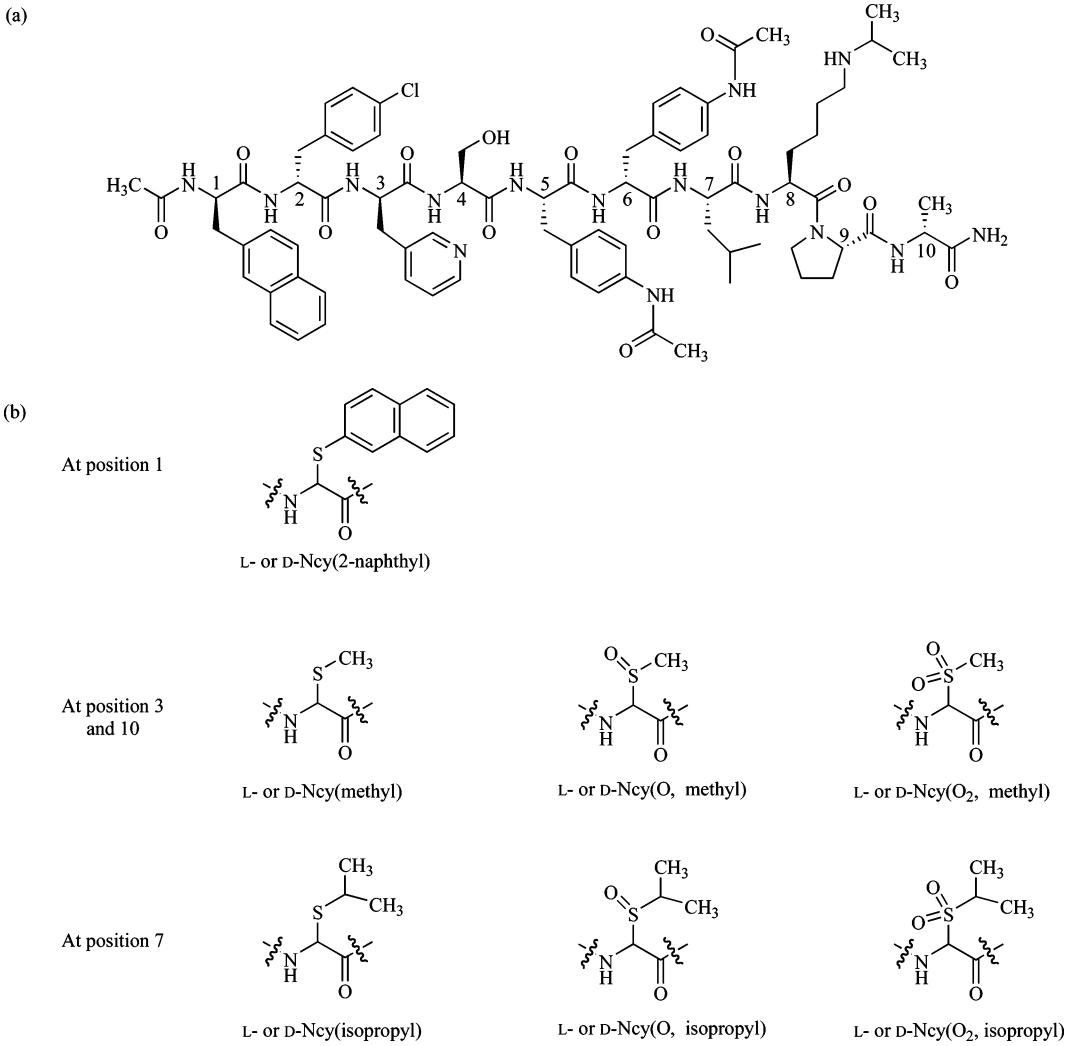


Figure 2. (a) Chemical structure of acyline. (b) Structures of the L- or D-Ncy(aryl/alkyl) amino acids incorporated in acyline at positions 1, 4, 7, and 10.

tions to determine the IC_{50}, the concentration required to suppress the response in the reporter gene assay by 50%. Average IC_{50} values 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 property (more soluble in aqueous buffers). Earlier encouraging results from the betidamino acid scan[3] of acyline paved the way for the present study, and we 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 antagonistic 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 antagonistic potency (IC_{50} < 2 nM) similar to that of acyline (IC_{50} = 0.52 nM) in a reporter gene assay, demonstrating compatibility of Ncy(aryl/alkyl)-containing acyline analogues for the human GnRH receptor.

Observation Number 2. Analogues containing the D-isomer at position 1 (**9**) and the L-isomer at positions 4 (**11**) and 7 (**15**) had higher antagonistic 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 report[25] 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. Monooxidized (sulfoxides) and dioxidized (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 μM), suggesting that the monooxidation of sulfur is preferred over the dioxidation 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 the L-isomer would result in a 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 the 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 monooxidize these residues failed.

Over the past 30 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 antagonist series are the aliphatic amino acids glycine, alanine, methionine, and proline, the charged amino acids diaminopropionic acid (Dpr), ornithine, lysine, arginine, aspartic acid, and threonine.[37] On the other hand, it is well documented that a lactam ring spanning Asp^4 to Dpr^{10} 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 monooxidized with $NaIO_4$ to analogues **12** and **14**, respectively. In the first pair, analogue **11** containing the L-isomer of Ncy(methyl) was 21 times (IC_{50} = 1.20 nM) more potent than the corresponding diastereomer **13** (IC_{50} = 25 nM). Monooxidation of both diastereomers resulted in analogues (**12** and **14**) with decreased antagonistic potency (9−13 times) compared to that of acyline. Structurally, monooxidized Ncy-(methyl) residues in acyline provide the closest mimic for the serine residue, and this is evident from the observation that a significant gain in antagonistic potency (4-fold) for the monooxidized analogue **14** (IC_{50} = 6.9 nM) was found over that of 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. Amino acids such as phenylalanine, 4-fluorophenylalanine, 4-chlorophenylalanine, tryptophan, N^{α}-methylleucine, norvaline, methionine, tyrosine, norleucine, propargylglycine, aspargine, N^{γ}-methylasparagine, and N^{γ},N^{γ}-dimethylasparagine 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 antagonistic potency (IC_{50} = 0.31 nM) of all the analogues shown here. Extension of the side chain via monooxidation (analogues **16** and **18**) resulted in a 4-fold decrease in antagonistic 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 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 in RP-HPLC (Table 1).

Whereas the deletion of residue 10 and its replacement by an ethylamide results in a significant increase of potency in vitro in GnRH agonist series,[49] the selection of D-Ala^{10} by most groups developing GnRH antagonists was based on the fact that it extended the duration of action in vivo. In the present study, we mimicked the alanine at position 10 with Ncy(methyl) and its monooxidized derivatives. All of the Ncy(methyl)-containing acyline analogues (**19**−**22**) showed high antagonistic 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.

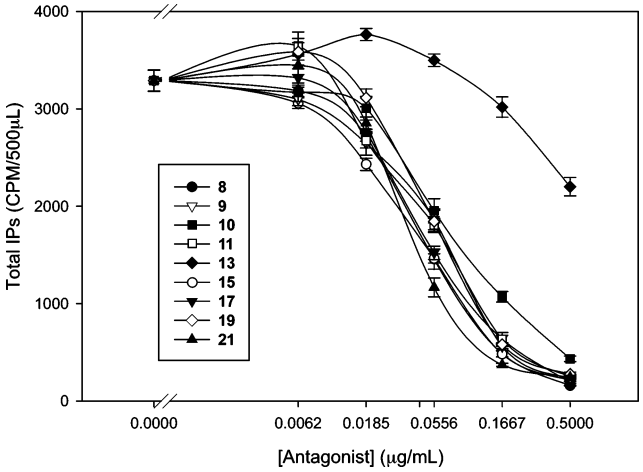


Figure 3. Inhibition of GnRH-stimulated IP production by different peptides. The peptides (10^{-8} to 10^{-5} M) were preincubated with 10^{-8} M GnRH before stimulation of COS-7 cells transiently transfected with the WT hGnRHR. IP production was determined after 2 h of incubation. Error bars represent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

Analogues **23**−**28** were synthesized to study the biological effects of mono- and dioxidation of L- or D-Ncy(isopropyl) residues in [D-Gln^3]acyline. Analogue **23** (IC_{50} = 6.2 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 antagonistic potencies for mono- and dioxidized analogues. Whereas monooxidized 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 dioxidized analogues **25** and **28** lost antagonistic potency in vitro (IC_{50} > 2 μM). The decrease in hydrophobicity (by analogy with Hocart's observation; see above) or steric hindrance may explain the loss of potency upon mono- and dioxidation.

To confirm and extend the above data in a parallel assay, analogues **8**, **9**, **10**, **11**, **13**, **15**, **17**, **19**, and **21** were tested in an in vitro inositol phosphate (IP) assay.[50] This assay measures the functional receptor by assessing coupling to the effector protein. A very similar trend in antagonistic potency was observed: analogue **13** was the least potent in the series (Figure 3), whereas all other analogues had affinity similar 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 of the analogues tested showed a decrease in antagonistic 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**,

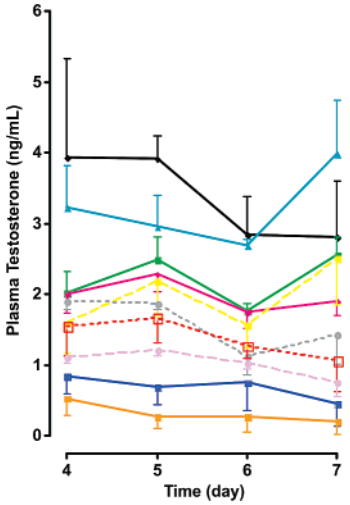


Figure 4. Plasma testosterone levels in intact male rats. GnRH antagonists were delivered for 7 days by infusion pumps. All compounds were given at 19.6 μM (~0.66 (nM/day)/kg). Azaline B was also given at 0.5 × 9.8 μM, to deliver ca. 0.33 (nM/day)/kg. The steady-state concentration of plasma testosterone was reached after 3−4 days of administration. Key: solid black tilted squares, vehicle; solid orange squares, azaline B; open red squares, azaline B × 0.5; solid blue squares, **8**; solid lavender circles, **9**; solid red-purple tilted squares, **11**; solid gray circles, **15**; solid gray-blue triangles, **16**; solid green squares, **19**; solid yellow squares, **21**.

Table 2. Mean ± SEM (n = 3) of Testosterone Levels in Intact Male Rats of Averaged Data Points of Days 4−7

no.	testosterone level (ng/mL)	no.	testosterone level (ng/mL)
control	3.38 ± 0.69	**11**	1.99 ± 0.09
azaline B	0.32 ± 0.20	**15**	1.59 ± 0.21
azaline B × 0.5	1.39 ± 0.34	**16**	3.22 ± 0.34
8 (acyline)	0.69 ± 0.29	**19**	2.21 ± 0.13
9	1.03 ± 0.07	**21**	1.97 ± 0.46

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 the 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 antagonistic potencies (6-fold and 8-fold, respectively) for hGnRH-R compared to rGnRH-R suggest 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 a 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 betidamino acids,[1] 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's) 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 F_{254} plates using the following solvent systems: (A) EtOAc:hexane = 30:70 and (B) CH_2Cl_2:MeOH:AcOH = 90:8:2. The plates were visualized by UV absorption, I_2, and ninhydrin spray. Silica gel flash column chromatography was performed on Geduran silica gel 60 (40−63 μm) purchased from EMD Chemicals Inc. (Gibbstown, NJ). All ^{1}H and ^{13}C NMR spectra were recorded at 60 °C on a Varian 500 MHz spectrometer in DMSO-d_6 (DMSO = dimethyl sulfoxide) as the solvent. Chemical shifts (δ) were expressed in parts per million referenced to the solvent peaks, either 2.50 or 40.51 ppm for DMSO-d_6 (^{1}H and ^{13}C signals, respectively). Coupling constants (J) are reported in hertz. 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 with an HPLC instrument (Agilent 1100) equipped with an Agilent Technologies LC/MSD Trap XCT ion trap mass spectrometer. Chiral analytical chromatography employed a CHIRALCEL OD-RH reversed-phase column (150 mm × 4.6 mm, 5 μm particle size) purchased from Daicel USA (Fort Lee, NJ) with a matching guard cartridge. Phases and conditions: mobile phase A, 0.1% HCO_2H in H_2O; mobile phase B, 0.1% HCO_2H in CH_3CN; gradient from 1% to 99% B in 50 min; flow rate of 0.5 mL/min A/B with postcolumn injection of mobile phase C (20 mM NH_4OAc in H_2O); flow rate of 0.1 mL/min. The absorbency was monitored at 215 and 254 nm using a diode-array detector (DAD). Enantiopure stardards 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 × 30 cm cartridge, packed in the laboratory with reversed-phase Vydac C_{18} silica (15−20 μM 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 Schimadzu SPD-6A UV detector, a Rheodyne model 7125 injector, a Linseis L250E chart recorder, and a Vydac C_{18} 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 C_{18} column (0.21 cm × 15 cm, 5 μm particle

size, 300 Å pore size) at 40 °C. The 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 was 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 α-cyano-4-hydroxycinnamic acid was prepared as a saturated solution in 0.3% TFA in 50% CH_3CN. 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(Xan) 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*-butyl carbamate, (trimethylsilyl)diazomethane (2 M solution in hexanes) and PTSA were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Glyoxylic acid monohydrate, sodium periodate ($NaIO_4$), oxone, and papain were purchased from Acros (Fisher Scientific, Pittsburgh, PA). The MBHA resin[54] 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) was refluxed for 5 h in acetone (300 mL) or stirred at rt overnight in diethyl ether (300 mL). Insolubles were filtered, and the filtrate was concentrated in vacuo 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 a white solid by adding hexanes. Mp: 90 − 91 °C. ESI-MS: *m*/*z* calcd for $C_7H_{13}NO_5$ 192.08 $(M + H)^+$, found 192.10. Without further purification the racemic **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 in a Dean−Stark trap for 6 h. The solvent was evaporated, the resulting solid was dissolved in a mixture of EtOAc (300 mL) and H_2O (400 mL), and the pH was adjusted to 8.5 using saturated sodium carbonate solution. 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 **4a** (9.82 g, 43%).

Synthesis of Boc-D,L-Ncy(methyl)-OH (4b). This compound was prepared by condensing an 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 diethyl ether (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 **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)-OCH$_3$ (5a−c). To a solution of **4a−c** (10 mmol) in benzene (24 mL) and methanol (6 mL) was added a 2 M solution of (trimethylsilyl)diazomethane in hexane (6 mL, 12 mmol) dropwise at rt. The reaction mixture was stirred for 45 min and then quenched with dropwise addition of AcOH till bubbling of N_2 ceased. Then a mixture of EtOAc (250 mL) and H_2O (50 mL) was added, and the reaction mixture was stirred for 5 min. The 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 **5a−c** in 90−95% yield.

Data for 5a. White solid. Mp: 73−76 °C. TLC: $R_f(A) = 0.65$. HPLC assay: C_{18} silica column (0.21 cm × 15 cm), buffer A, TEAP, pH 2.30; buffer B, 60% CH_3CN/40% A; gradient condition, from 25% to 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%. ^{1}H NMR (500 MHz, DMSO-d_6, 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). ^{13}C NMR (125 MHz, DMSO-d_6, 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: *m*/*z* calcd for $C_{18}H_{21}NO_4S$ 348.1192 $(M + H)^+$, found 347.9245.

Data for 5b. White solid. Mp: 47−50 °C. TLC: $R_f(A) = 0.60$. HPLC assay: conditions same as described for **5a**, $t_R = 16.4$ min, purity 99%. ^{1}H NMR (500 MHz, DMSO-d_6, 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). ^{13}C NMR (125 MHz, DMSO-d_6, 60 °C): 169.25, 155.37, 79.86, 56.70, 53.0, 28.78, 13.19. ESI-MS: *m*/*z* calcd for $C_9H_{17}NO_4S$ 236.0879 $(M + H)^+$, found 235.8817.

Data for 5c. Oil. TLC: $R_f(A) = 0.71$. HPLC assay: conditions same as described for **5a**, $t_R = 28.8$ min, purity 99%. ^{1}H NMR (500 MHz, DMSO-d_6, 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, 6.9). ^{13}C NMR (125 MHz, DMSO-d_6, 60 °C): 170.16, 155.06, 79.80, 55.79, 53.05, 35.56, 28.78, 24.28, 23.94. ESI-MS: *m*/*z* calcd for $C_{11}H_{21}NO_4S$ 264.1192 $(M + H)^+$, found 263.8817.

Enzymatic Resolution of 5a−c. For the preparation of the buffer, 0.1 M $Na_2HPO_4 \cdot 7H_2O$, 0.1 M citric acid, and 1.5 mM EDTA·Na_2 were dissolved in deionized water (1000 mL). The pH of the solution was adjusted to 6.2 by slow addition of 2 N NaOH. For the activation of papain, papain (50 mg) and dithiotreitol (100 mg) were dissolved in the buffer (1 mL).

5a−c (3 g) were suspended in a mixture of CH_3CN (15 mL)/buffer (10 mL), and an activated aliquot of papain solution (500 μL) was added. The reaction mixtures were stirred at rt, and the pH was maintained at 6.2 by the addition of 0.1 M NaOH. The reactions were carried out until about 50% of the methyl esters were hydrolyzed, which took 24, 10, 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 H_2O (3 × 100 mL), dried over Na_2SO_4, 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 **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.

Data for 6a. White solid. Mp: 95−96 °C. Yield: 1.35 g, 84% with respect to the D-isomer. TLC: $R_f(B) = 0.64$. $[\alpha]^{25}_D = +154.50$ ($c = 1.0$, MeOH). HPLC assay: conditions same as described for **5a**, $t_R = 30.6$ min, purity 98%. ^{1}H NMR (500 MHz, DMSO-d_6, 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); ^{13}C NMR (125 MHz, DMSO-d_6, 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: *m*/*z* calcd for $C_{17}H_{19}NO_4S$ 334.1035 $(M + H)^+$, found 333.9211.

Data for 6b. White solid. Mp: 123 − 124 °C. Yield: 86% with respect to the D-isomer. TLC: $R_f(B) = 0.61$. $[\alpha]^{25}_D = +205.77$ (c = 1.0, MeOH). HPLC assay: conditions same as described for **5a**, t_R = 9.2 min, purity 99%. ^{1}H NMR (500 MHz, DMSO-d_6, 60 °C): 7.34 (1H, br), 5.02 (1H, d, J = 5.7), 2.11 (3H, s), 1.40 (9H, s). ^{13}C NMR (125 MHz, DMSO-d_6, 60 °C): 170.08, 155.11, 79.66, 56.74, 28.81, 13.10. ESI-MS: m/z calcd for $C_8H_{15}NO_4S$ 222.0722 $(M + H)^+$, found 221.8468.

Data for 6c. Gummy. Yield: 90% with respect to the D-isomer. TLC: $R_f(B) = 0.69$. $[\alpha]^{25}_D = +115.39$ (c = 1.0, MeOH). HPLC assay: conditions same as described for **5a**, t_R = 18.5 min, purity 98%. ^{1}H NMR (500 MHz, DMSO-d_6, 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). ^{13}C NMR (125 MHz, DMSO-d_6, 60 °C): 170.87, 155.45, 79.62, 55.97, 35.42, 28.81, 24.38, 23.96. ESI-MS: m/z calcd for $C_{10}H_{19}NO_4S$ 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 an MBHA resin (1 g, 0.40 mmol/g substitution) using the 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 4Aph^5 and D-4Aph^6 were removed by treatment with 30% piperidine in DMF. Then the Boc protection on the N-terminal amino acid [D-2-Nal or D,L-Ncy(2-naphthyl)] was removed by TFA treatment. Acetylation of 4Aph^5,D-4Aph^6 and the N-terminus was performed by using an 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% CH_3CN/H_2O, and lyophilization. All Ncy(aryl/alkyl)-containing peptides were initially synthesized with **4a**−**c**. Additionally, analogues **10**, **11**, **15**, **19**, and **23** were synthesized with the resolved **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% CH_3CN 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 percentage of B (eluent A, 0.25 N TEAP, pH 2.25; eluent B, 60% CH_3CN, 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% CH_3CN/40% A, on the same cartridge, using a linear gradient of 1% B per minute increase from the baseline percentage of 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 **4a**−**c** and separated by RP-HPLC. The absolute stereochemistry at the Ncy(aryl/alkyl) residue in GnRH analogues was confirmed by comparison of the RP-HPLC retention times (Table 1) of each diastereomer with those of the analogues synthesized with the resolved **6a**−**c**. Analogues **11**, **15**, **19**, and **23** synthesized with resolved **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 pair **9** + **10**. Thus, analogues **10**, **11**, **15**, **19**, and **23** contained the L-enantiomer of Ncy(aryl/alkyl) in their sequence. The results of these coelution studies are consistent with our findings that analogues containing the L-isomer at positions 4 and 7 and the 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-Gln^3,Ncy(SO,isopropyl)^7]acyline (24). Analogue [D-Gln^3,Ncy(SO,isopropyl)^7]acyline (**23**; 12.40 mg, 8.10 μmol) was dissolved in 5 mL of H_2O/CH_3CN (3:2, v/v), and $NaIO_4$ (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 that for **23** (t_R = 20.9 min). The reaction mixture was diluted with H_2O (10 mL) and purified by preparative RP-HPLC as described above. Yield: 4.5 mg (35.91%). ESI-MS: m/z calcd 1546.7 $(M + H)^+$, 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 in RP-HPLC under the same conditions.

Synthesis of [D-Gln^3,Ncy(SO$_2$,isopropyl)^7]acyline (25). Analogue **23** (10 mg, 6.53 μmol) was dissolved in 5 mL of H_2O/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. 3 h) in RP-HPLC. The reaction mixture was then diluted with H_2O (10 mL), and the single major peak eluting (t_R = 16.2 min) earlier than that for **23** (t_R = 20.9 min) was purified by preparative RP-HPLC as described above. Yield: 3.5 mg (34.28%). ESI-MS: m/z calcd 1562.7 $(M + H)^+$, found 1562.3. Using this procedure, analogue **28** was obtained in comparable yield from analogue **26**. [D-Gln^3]acyline[58] was used as an experimental control, and no oxidized product was detected in HPLC under the same conditions.

Peptide Characterization (Table 1). The purity of the peptides was assessed using RP-HPLC and CZE under conditions reported in the footnotes 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 others 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 the shape of the content of the tube. While acyline, azaline B, and Ncy(aryl/alky)-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, Illinois) and a luciferase reporter gene under the control of the LH α 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 of penicillin and 100 μg of streptomycin/mL of 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 of penicillin and 100 μg of streptomycin/mL of 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, the cell lines were trypsinized and plated at 50000 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% CO_2 overnight for assay the next day.

IC_{50} 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% CO_2. Following this, GnRH (10 μL) was added to a final concentration of 1 nM. The plates were then incubated for a minimum of 5 h at 37 °C under 5% CO_2, after which 100 μL of a luciferase substrate mixture was added to each well. The plates were sealed with Packard Topseal film, and luminescence was measured with a Molecular Devices Analyst after a 10 min incubation at rt in the absence of direct light. To derive IC_{50} values, luciferase responses were plotted against the log of the concentration, and the data were 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 IC_{50} from at least two independent experiments is reported for each compound.

IP Assay. Peptides were diluted in PBS/10% dimethyl sulfoxide at a 1 μg/μL concentration. WT hGnRH-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 μg/mL gentamicin. The cells were seeded in 24-well plates (Costar, Cambridge, MA). Twenty-four hours after plating, the cells were transfected with 0.075 μg of cDNA per well using 2 μL of lipofectamine in 0.25 mL of OPTI-MEM. Five hours later, 0.25 mL of DMEM containing 20% FCS was added to each well. Twenty-four hours after the start of transfection, the medium was replaced with fresh growth medium for 4 h. Twenty-eight hours 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 labeled with 4 μCi/mL [^{3}H]-myoinositol 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 they were assayed for IP production by Dowex anion-exchange chromatography and liquid scintillation spectroscopy as previously described.[60]

Efficacy of Selected GnRH Analogues in the Intact Male Rat Model. Stock solutions of all compounds were prepared at 1 mM in 1:3 (v/v) DMSO in sterile water for injection and kept frozen at −80 °C until they were 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 μL/h) were filled according to the 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 No. FRI-05-016). Intact adult male Sprague−Dawley rats were purchased from Harlan (Indianapolis, IN), housed singly under controlled light (12 h of light/12 h of dark) and temperature (22−25 °C), and given water and food ad libitum. Animals were weighed on days 0, 2, and 7. Three rats were used for each compound tested.

On day 0, minipumps were implanted subcutaneously, just posterior to the scapulae, in the backs of rats (275−310 g) under isoflurane anesthesia following the 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 4000*g* for 10 min at 4 °C and stored at −80 °C until it was 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 the manufacturer's guidelines except that a 25 μL plasma volume was used.

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