Design and Synthesis of Potent Hexapeptide and Heptapeptide Gonadotropin-Releasing Hormone Antagonists by Truncation of a Decapeptide Analogue Sequence

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A novel strategy for designing reduced-size analogues of the decapeptide gonadotropin-releasing hormone (GnRH) was developed. As opposed to previous attempts to delete residues from either of the peptide's termini, our approach is based upon the known importance of both C- and N-terminals of GnRH analogues for receptor recognition, whereas the central part of the molecule is replaced by a short spacer. The present truncation strategy was successful for generation of reduced-size hexapeptide and heptapeptide antagonists possessing potent antagonistic capacity. The same methodology was not suitable for the generation of reduced-size agonists, suggesting different conformational characteristics for GnRH agonists and antagonists. A heptapeptide antagonist designed by this method was shown to inhibit serum levels of luteinizing hormone in castrated rats in vivo. Structure−activity studies suggested that the structural preferences for GnRH receptor recognition are similar to those reported for decapeptide antagonists. Our studies resulted in a heptapeptide GnRH antagonist (Ac-D-Nal2-D-Cpa-D-Pal-Gly-Arg-Pro-D-Ala-NH_2) with high receptor binding affinity (IC_{50} = 7 nM), as compared to that of GnRH itself (IC_{50} = 2 nM). The highest affinity of a hexapeptide antagonist that we have synthesized was somewhat lower (IC_{50} = 45 nM).

Introduction

Gonadotropin-releasing hormone (GnRH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is secreted from the hypothalamus in a pulsatile pattern and regulates the reproductive system by controlling the secretion of the gonadotropic hormones, luteinizing hormone (LH), and follicle stimulating hormone (FSH) from the anterior pituitary.[1,2] These hormones, in turn, stimulate gonadal steroidogenesis and gametogenesis. Continuous administration of GnRH results in down-regulation and receptor desensitization, leading to an inhibition of pituitary gonadotropin secretion. Therefore, synthetic GnRH analogues, both agonists and antagonists, may be used for contraception and for the treatment of various hormone-dependent diseases, including prostate and breast cancer.[3]

GnRH agonists which were approved for clinical use are deca- or nonapeptides, whereas all the antagonists in clinical trials are decapeptides. Development of reduced-size GnRH analogues is important for the characterization of the interactions of the natural peptide with its receptor. Furthermore, such analogues may also be useful as drugs or as intermediates in the search for non-peptide ligands.

The reported studies of reduced-size GnRH analogues were traditionaly based on the gradual deletions of residues from either the C- or the N-termini of GnRH or its decapeptide/nonapeptide analogues.[4−7] There is also a report of a cyclic hexapeptide, a weak GnRH antagonist, which is based solely on the central 5−8 residues.[8] These strategies are not in line with numerous studies which suggested that both termini of the GnRH molecule are involved in direct interactions with the GnRH receptor (for review, see refs 9 and 10). On the basis of conformational energy calculations[11] and various physicochemical methods,[9] it was suggested that the bioactive conformation of GnRH include a type II′ β turn involving residues 5−8 {Tyr^5-Gly^6-Leu^7-Arg^8}. There are indications that the dominant conformation of GnRH antagonists is similar, but not identical, to that of GnRH.[12]

Our approach for developing reduced-size GnRH analogues is based on replacing several residues from the central part of the peptide by a short spacer, while the terminal residues remain unchanged. A similar truncation strategy was employed successfully to form potent truncated analogues of the 36-amino acid neuropeptide Y (NPY), e.g., NPY (1−4)-Aca-(25−36) (Aca = amino caproic acid).[13] The success of this strategy for the establishment of novel GnRH antagonists was followed by structure−activity studies.

Results

To test the truncation strategy, we have synthesized the two agonists and two antagonists that are listed in Table 1. The residues 4−8 or 4−7 of the parent corresponding analogues, GnRH and antide (a potent GnRH antagonist), were replaced by D-Ala as a spacer. Thus, in each pair of analogues (peptides 1,2 and peptides 3,4), there is one in which the original Arg residue in position 8 of GnRH is conserved and one which lacks this residue. The side chain of Arg^8 of GnRH was previously suggested to stabilize the active conformation of the

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Table 1. Sequences and Mass Spectra Analysis of GnRH, of Antide, and of Their Reduced-Size Analogues

peptide	sequence[a]	MH^+ obsd (calcd)[b]
GnRH	**pGlu^1-His^2-Trp^3**-Ser-Tyr-Gly-Leu-**Arg^8-Pro^9-Gly^{10}-NH$_2$**	
1	**pGlu-His-Trp**-D-Ala-**Arg-Pro-Gly-NH$_2$**	834.2 (833.9)
2	**pGlu-His-Trp**-D-Ala-**Pro-Gly-NH$_2$**	677.7 (677.4)
antide	**Ac-D-Nal2^1-D-Cpa^2-D-Pal^3**-Ser-Lys(Nic)-D-Lys(Nic)-Leu-Lys(Isp)-**Pro^9-D-Ala^{10}-NH$_2$**	
3	**Ac-D-Nal2-D-Cpa-D-Pal**-D-Ala-Arg-**Pro-D-Ala-NH$_2$**	983.6 (982.6)
4	**Ac-D-Nal2-D-Cpa-D-Pal**-D-Ala-**Pro-D-Ala-NH$_2$**	827.4 (826.4)

[a] **Bold** letters represent the residues of GnRH or of antide, which were preserved in the respective reduced-size analogues. D-Nal2 = β-[2-naphthyl]-D-Ala; D-Cpa = D-*p*-chloro-Phe; D-Pal = β-[2-pyridyl]-D-Ala; Lys(Nic) = N^ϵ-[nicotinoyl]; Lys(Isp) = N^ϵ-[isopropyl]Lys. [b] Observed (obsd) and calculated (calcd) *m/z* values of MH^+ monoisotopes. Purities of the reduced-size analogues 1−4 were >99%, according to two analytical HPLC solvent systems that are detailed in Table 2.

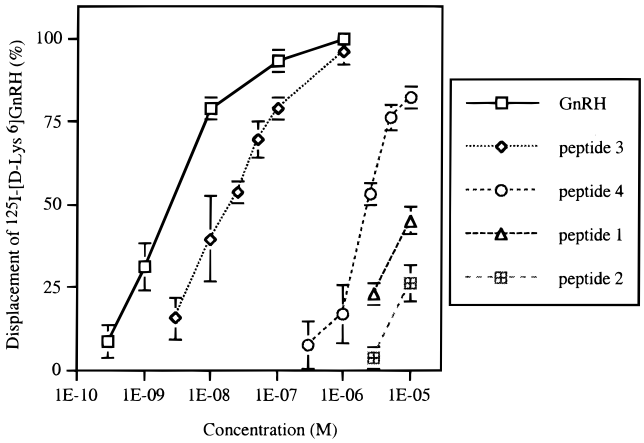

Figure 1. Dose−response curves for the displacement (%) of specific binding of ^{125}I-[D-Lys^6]GnRH to pituitary membranes of proestrous rats, by GnRH and by reduced-size analogues. Membranes were incubated for 90 min at 4 °C with ^{125}I-[D-Lys^6]GnRH and with the unlabeled peptides. Nonspecific binding was determined by the presence of 1 μM of [D-Lys^6]-GnRH, and it was subtracted from the total binding for the calculation of specific binding. Results are the mean ± SEM of two experiments carried out in triplicate.

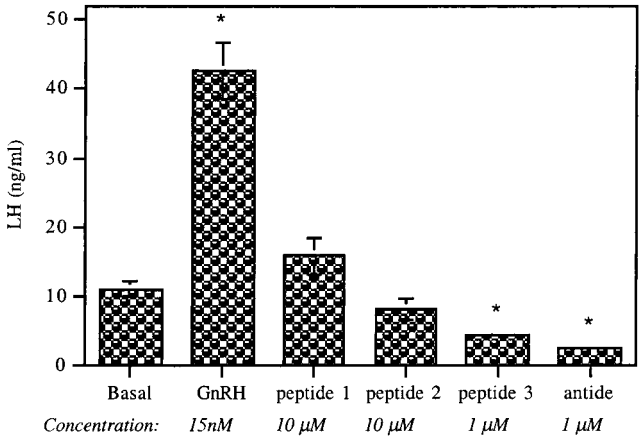

Figure 2. Induction of LH release from dispersed rat pituitary cells by GnRH and its reduced-size analogues. At 48 h after dispersion, the medium was changed, and the cells were incubated for 4 h at 37 °C with the examined peptides. The medium was collected, and LH concentration was determined by RIA. Results are the mean ± SEM of LH concentrations in two experiments (four wells/experimental group). The LH concentration in each well was determined using triplicates. *LH release significantly different ($p < 0.01$) from the control group (basal).

peptide by hydrogen bonding with the side chains of His^2 and Tyr^5.[14] We have hypothesized that, in reduced-size analogues lacking the Tyr residue, the Arg residue may be less important for attaining GnRH receptor binding affinity. A representative sequence of an antagonist that is now in clinical trials, antide,[15] is shown in Table 1. All of the currently used decapeptide GnRH antagonists possess identical substitutions at residues 1, 2, 3, and 10, while Pro^9 is conserved. Residue Arg^8 is generally replaced by a polar, noncharged residue (except for cetrorelix, which contains Arg^8).[16] Peptides 3 and 4 are therefore the reduced form of antide or of any of the other clinically tested GnRH antagonists. The GnRH receptor binding affinity of antide is about 1 order of magnitude higher than that of GnRH (in our system, IC_{50} values are 0.3 and 2 nM, respectively).

The two reduced-size GnRH agonists (peptides 1 and 2) were found to have very low GnRH receptor binding affinities (Figure 1). Consequently, the agonistic activity of these peptides was weak or negligible, even at 10 μM (Figure 2). On the other hand, peptide 3 is a high affinity GnRH analogue (Figure 1). Thus, the size-reduction strategy employed, i.e., the elimination of the central region of the decapeptide GnRH analogue, is successful only for the generation of potent antagonists. The presence of an Arg residue increases the binding affinity of agonists (peptide 1 versus peptide 2) and, more crucially, of antagonists (peptide 3 versus peptide 4) (Table 1). To exclude the option that peptide 3 is a partial agonist, we performed in vitro studies using dispersed cells of rat pituitaries. At 1 μM, peptide 3 was not only inactive as an agonist but like antide it actually inhibited basal release of LH (Figure 2). No agonistic activity was observed at lower concentrations of peptide 3 (data not shown).

To further demonstrate the antagonistic activity of peptide 3, we have tested it for its in vivo inhibition of LH release in castrated rats (Figure 3). While the inhibition of LH release by peptide 3 is significantly weaker than that of antide, this experiment suggests that peptide 3 is a rather long-acting GnRH antagonist.

Structure−activity studies of the reduced-size antagonists, peptide 3 and peptide 4, are summarized in Table 2. The results of the GnRH receptor binding studies suggest that (I) Arg in position Y (peptide 3, Table 2) can be replaced by the positively charged Lys (peptide 5) or by polar residues (peptides 7, 8) without substantial change in GnRH receptor affinity, while its substitution by a hydrophobic residue results in much lower receptor affinity (peptides 6, 9). These preferences are very similar to those of the respective position in decapeptide agonists[17] and antagonists.[18] It seems that there is not much preference for L-configuration over D-configuration (e.g., peptide 11 versus peptide 22). (II) Position X (the spacer residue, see Table 2) is very

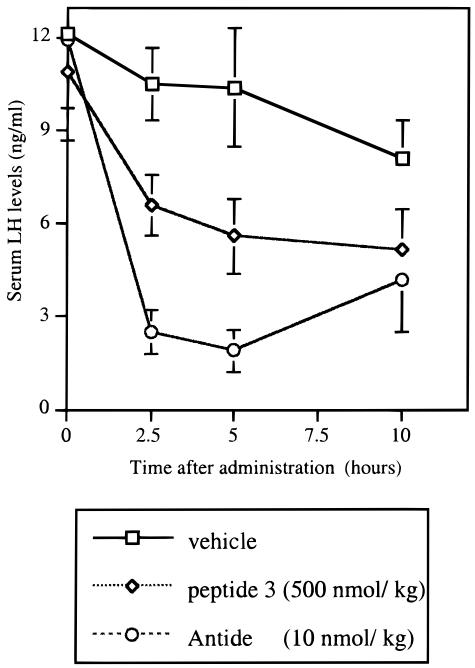

Figure 3. Effect of peptide 3 or antide on serum LH levels in castrated rats following intraperitoneal administration of the peptides. Blood samples were taken from each rat at the indicated time. Control animals were injected 0.1 M PBS, which was also used for dissolving the examined peptides. The serum samples were assayed for LH using RIA. Results are the mean ± SEM of LH concentrations in the serum of five animals/experimental group. The LH concentrations were determined using three different dilutions of each serum sample. LH release in the presence of both peptides is significantly lower ($p < 0.05$) than in the respective vehicle control group (at all time intervals except for time 0).

tolerant for a variety of residues (peptides 10−20). Substitution by the Gly residue, enabling maximal freedom of bond angles, resulted in the peptide with the highest GnRH receptor affinity in this series ($IC_{50} = 7$ nM), only 3−4 times lower than that of GnRH. The analogue containing the stretched aminocaproic acid (Aca) residue in the X position has high GnRH receptor affinity (peptide 18), which suggests that the distance between the two parts of the molecule can be varied without disturbing the GnRH receptor binding affinity. (III) High affinity hexapeptides can also be produced by the size-reduction strategy (the last five peptides shown in Table 2 are hexapeptides). The preferences for the residue in position X of these hexapeptides (Table 2) are not clear from our limited results. A Gly residue substitution (peptide 24) resulted in the hexapeptide with the highest GnRH receptor affinity, similar to the results concerning the heptapeptides (peptide 11). The preferences as to D- versus L-configurations are unclear: while D-Arg is preferred over Arg or Lys, D-Ala seems to be much inferior (peptides 23, 25, 26, and 4, respectively).

The employment of Gly as a spacer was preferred in both hexapeptide (peptide 24) and heptapeptide (peptide 11) antagonists. To apply this substitution for agonists, we have synthesized two reduced-size agonists: pGlu-His-Trp-Gly-Arg-Pro-Gly-NH_2 and pGlu-His-Trp-Gly-Pro-Gly-NH_2. These peptides are based on the sequences of peptides 1 and 2 (Table 1), respectively, with D-Ala substituted by Gly. Both of these peptides were found to have very low GnRH receptor binding affinities (data not shown), similar to the affinities of peptides 1 and 2, respectively. These results suggest that the low affinities of peptides 1 and 2 are not the result of the use of a specific spacer (D-Ala) but rather a consequence of fundamental conformational attributes of GnRH agonists.

To test the preferences in the N-terminal tripeptide of heptapeptide antagonists, we have modified these residues, using peptide 11 as a template (Table 3). Substitution of positions 2 and 3 by D-Phe resulted in a fairly potent antagonist (peptide 27, Table 3). However, while the peptides shown in Tables 1 and 2 are all highly water-soluble, this peptide has a much lower solubility. Thus, solubility can be varied according to needs, without major changes in receptor binding affinity. Various aromatic D-amino acid substitutions in position 1 of peptide 27 resulted in immensely decreased binding affinity (peptide 28−32, Table 3). These include substitution of the D-Nal2 residue (D-Nal2 = β-[2-naphthyl]-D-Ala) by the related D-Nal1 residue (D-Nal1 = β-[1-naphthyl]-D-Ala, peptide 29), as well as deletion of the terminal acetyl (peptide 31) or its replacement by an Fmoc group (peptide 32). The preferences in the N-terminal tripeptide of the heptapeptide antagonists are therefore similar to those of the relevant decapeptide antagonists,[19] i.e., positions 2 and 3 may be accommodated by various aromatic D-amino acids (polar or nonpolar), while Ac-D-Nal2 in position 1 is clearly superior to other aromatic D-amino acids, including residues as similar as Ac-D-Nal1.

Discussion

Reduced-size high affinity GnRH antagonists were rationally designed based on previous structure−activity studies of GnRH analogues. Substitution of the four central residues by a single amino acid resulted in heptapetides with GnRH receptor binding affinities in the nanomolar range. These results emphasize the role of the two termini of GnRH antagonists for receptor recognition. When the same methodology was employed for the GnRH molecule itself, it resulted in peptides which recognize the GnRH receptor only at the micromolar range. These findings reflect fundamental differences between the conformations of GnRH agonists and antagonists. It was previously suggested by Nikiforovich and Marshall[12] that the β-II′ turn in the central region of GnRH agonists maintains the proper spatial arrangement of the N-terminal tripeptide, which presumably participates in a direct interaction with the GnRH receptor. This model explains the loss of receptor affinity in the reduced-size agonists described above, which lack the central β-II′ turn region. As for GnRH antagonists, it was suggested that they differ from GnRH agonists with respect to the conformation of the central 5−8 residues, while the spatial arrangement of the N-terminal tripeptide is very similar to that of the agonists.[12] Our detailed structure−activity studies, directed at the spacer connecting the two termini of the reduced-size antagonists, demonstrate high tolerance of this position. Substitution by Ala, D-Ala, D-Tyr, Sar, βAla, and even aminocaproic acid (Aca) and 1-amino cyclobutane-1-carboxylic acid (MePro) all resulted in fairly similar affinities. The latter two substitutions indicate

Table 2. Analytical Data and GnRH Receptor Binding Affinities of Peptides of the Form: Ac-D-Nal2-D-Cpa-D-Pal-X-Y-Pro-D-Ala-NH_2

peptide	X	Y	purity (%) HPLC-1[a]	purity (%) HPLC-2[b]	MH^+ obsd (calcd)[c]	IC_{50} (nM)[d]
3	D-Ala	Arg	>99	>98	983.6 (982.6)	17 ± 3
5	D-Ala	Lys	>99	>98	955.6 (954.6)	20 ± 3
6	D-Ala	Lys(Isp)	>99	>99	1001.6 (1000.6)	130 ± 40
7	D-Ala	Lys(Nic)	>99	>99	1059.7 (1058.0)	14 ± 3
8	D-Ala	Cit	>99	>99	981.6 (981.2)	23 ± 7
9	D-Ala	Nle	>97	>99	942.1 (941.6)	88 ± 28
10	Ala	Arg	>97	>98	982.6 (981.5)	28 ± 7
11	Gly	Arg	>99	>99	968.6 (967.4)	7 ± 2
12	Sar	Arg	>99	>99	982.6 (981.4)	31 ± 11
13	βAla	Arg	>99	>99	982.6 (981.5)	38 ± 15
14	Leu	Arg	>99	>99	1024.7 (1023.1)	81 ± 17
15	D-Leu	Arg	>99	>99	1024.7 (1023.3)	74 ± 21
16	Pro	Arg	>99	>99	1008.6 (1008.5)	110 ± 25
17	D-Tyr	Arg	>99	>99	1074.7 (1074.5)	25 ± 2
18	Aca	Arg	>99	>97	1024.7 (1023.5)	11 ± 2
19	MePro	Arg	>99	>99	1008.6 (1007.5)	42 ± 13
20	Ana	Arg	>99	>95	1030.6 (1029.4)	140 ± 40
21	D-Arg	Cit	>99	>99	1065.7 (1065.6)	72 ± 20
22	Gly	D-Arg	>99	>99	968.6 (967.4)	16 ± 4
4[e]	D-Ala	-	>97	>95	827.4 (825.3)	1500 ± 600
23	D-Arg	-	>99	>99	911.5 (910.5)	70 ± 5
24	Gly	-	>99	>99	812.5 (811.3)	45 ± 5
25	Arg	-	>95	>98	911.5 (911.1)	130 ± 50
26	Lys	-	>98	>99	883.5 (883.2)	550 ± 70

[a] The purity of the peptides was assessed by two HPLC systems. (1) A 0.1% TFA in water, B 0.1% TFA in 75% acetonitrile: 20% B to 80% B at 1%/min. (2) A 0.1% TFA in water, B 0.1% TFA in 75% 2-propanol: 0% B to 50% B at 1%/min, using Lichrosorb RP-18 column. [b] Elution system: A 0.1% TFA in water, B 0.1% TFA in 75% acetonitrile: 0% B to 100% B at 2.5%/min employing a wide pore butyl (C4) column. [c] Observed and calculated *m/z* values of MH^+ monoisotopes are reported. [d] IC_{50} = Concentration of peptide which displaces 50% of ^{125}I-[D-Lys^6]GnRH bound to rat pituitary membranes; values ± SEM are based on displacement curves obtained by incubating rat pituitary membranes for 90 min at 4 °C with ^{125}I-[D-Lys^6]GnRH and increasing concentrations of the indicated unlabeled peptides. The IC_{50} of GnRH at the same experimental conditions was found to be 2 nM. [e] This peptide and the following peptides 23−26 are hexapeptide (no Y residue). Aca = aminocaproic acid; Ana = anthranilic acid; Cit = citrulline; Isp = isopropyl; MePro = 1-amino cyclobutane-1-carboxylic acid; Nle = norleucine; Sar = sarcosine.

Table 3. Analytical Data and GnRH Receptor Binding Affinities of Peptide 11 and Its N-Terminal Modified Analogues[a]

peptide	sequence	purity (%) HPLC-1	purity (%) HPLC-2	MH^+ obsd (calcd)	IC_{50} (nM)
11	Ac-D-Nal2-D-Cpa-D-Pal-Gly-Arg-Pro-D-Ala-NH_2	>99	>99	968.6 (967.4)	7 ± 2
27	Ac-D-Nal2-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>99	>98	933.4 (933.4)	28 ± 7
28	Ac-D-Phe-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>99	>99	880.6 (880.5)	230 ± 20
29	Ac-D-Nal1-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>98	>99	933.4 (933.2)	370 ± 90
30	Ac-D-Trp-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>99	>99	918.6 (918.1)	450 ± 100
31	D-Nal2-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>99	>98	892.0 (891.9)	280 ± 70
32	Fmoc-D-Nal2-D-Phe-D-Phe-Gly-Arg-Pro-D-Ala-NH_2	>98	>97	1113.4 (1112.8)	2200 ± 400

[a] For abbreviations and reference to HPLC systems, see legend to Table 2. D-Nal1 = β-[1-naphthyl]-D-Ala; D-Nal2 = β-[2-naphthyl]-D-Ala.

that both a long-chain spacer (Aca) and a residue that constrains the peptide conformation (MePro) can be fairly tolerated. On the basis of the high affinity and tolerability of the reduced-size antagonists, we suggest that the spatial arrangement of the N-terminal tripeptide tested (Ac-D-Nal2-D-Cpa-D-Pal) may be significantly based on stabilization by local interactions within this tripeptide, rather than the assumed stabilization of a similar conformation of the N-terminal tripeptide of agonists by the central region of these peptides.[12] This notion may open new directions for the design of novel GnRH antagonists.

The improved GnRH receptor affinities of peptide 3 over peptide 4 (Figure 1) and the structure−activity studies of peptide 3 (peptides 5−9, Table 2) suggest an important role for the Arg residue in GnRH receptor recognition by GnRH antagonists. Nevertheless, these results, as well as other observations following analogous structure−activity studies in decapeptide GnRH agonists[17] and antagonists,[18] still raise a debate as to whether the Arg residue interacts directly with the receptor or that it stabilizes the active conformation of the peptide by hydrogen bonding, as was suggested for GnRH itself.[14]

In all decapeptide GnRH antagonists that are currently in clinical trials, the positive charge of the eighth residue is masked by various groups (e.g., isopropyl attached to the Lys residue in antide, see Table 1). It was previously shown that the combination of positive charges in both the sixth and eighth residues, together with the aromatic N-terminus, result in potent GnRH antagonists. These basic antagonists, however, also cause side effects due to histamine release.[20] The potent reduced-size antagonists presented herein, although containing an Arg residue or a positively charged/polar substitution, may, potentially, be of low potency in inducing histamine release, due to the lack of a second positively charged/polar residue (found at the sixth position of most decapeptide antagonists).

In conclusion, our studies resulted in a novel class of reduced-size GnRH analogues which may contribute to the understanding of conformational differences be-

tween agonists and antagonists and to the structural requirements of GnRH antagonists. This class of GnRH antagonists may be further developed to form candidate drugs, which may have advantages over the currently available decapeptide antagonists.

Experimental Section

Materials. Unless otherwise stated, all chemicals and reagents were of analytical grade. Trifluoroacetic acid (TFA) for high performance liquid chromatography (HPLC) was obtained from Merck (Darmstadt, Germany). Commonly used 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acid derivatives and Rink-amide resin were purchased from Novabiochem (Laufelfingen, Switzerland). Ac-*β*-[2-Naphthyl]-D-Ala was purchased from Synthetech (Albany, OR). Fmoc-D-*p*-chloro-Phe and Fmoc-*β*-[2-pyridyl]-D-Ala were obtained from Bachem (Bubendorf, Switzerland). *N*,*N*′-disuccinimidyl carbonate, GnRH, and antide were acquired from Sigma (St. Louis, MO). 1-Aminocyclobutane-1-carboxylic acid (termed MePro; methano proline) was synthesized according to a published procedure.[21] The Fmoc derivative of this amino acid was synthesized in our laboratory according to a published procedure.[22]

Peptide Synthesis. All peptides, other than the above, were prepared in our laboratory by solid-phase peptide synthesis, with an AMS-422 multiple peptide synthesizer (ABIMED, Langenfeld, GmbH) using Fmoc chemistry,[22] following the company's protocols.[23] The unnatural amino acid residues of the GnRH antagonists were attached manually using 2 equiv of *N*,*N*′-dicyclohexylcarbodiimide/1-hydroxybenzotriazol (DCC/HOBT) in *N*-methyl pyroliddone (NMP). The reactions were performed in sintered plastic syringes which were shaken overnight in a mechanical shaker at room temperature. The solutions were than removed and the resin was washed three times with *N*,*N*′-dimethylformamide (DMF) and dichloromethane. A negative ninhydrin test indicated the completion of the reaction. Fmoc removal was achieved by 20% piperidine/DMF (3 × 5 min). All synthesized peptides were deprotected and cleaved from the resin using a solution of TFA:triethylsilane:anisole:water (17:1:1:1)[23] or reagent K solution (TFA:phenol:water:thioanisole:1,2-ethanedithiol; 33:2:2:2:1) for peptides containing Arg and Trp.[24] After 2 h at room temperature, the cleavage mixtures were filtered, and the peptides were precipitated with peroxide-free dry ether at 0 °C. Precipitated peptides were washed with cold dry ether, dissolved in water or water/acetonitrile solution, and lyophilized. Crude peptides were then subjected to semipreparative HPLC purification, performed on a Waters system composed of two model 510 pumps, a model 680 automated gradient controller, and a model 441 absorbance detector (Waters, Milford, MA). The column effluents were monitored by UV absorbance at 214/254 nm. HPLC prepacked columns employed (Merck, Darmstadt, Germany) were LichroCART 250−10 mm containing Lichrosorb RP-18 (7 *μ*m) for semipreparative purifications, and Lichrospher 100 RP-18, 250−4 mm (5 *μ*m) and wide pore butyl (C4, 5 *μ*m), 250−4.6 mm (J. T. Baker Inc. Phillipsburg, NJ) for analytical separations. Separations were achieved using gradients of acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). The homogeneity of the resulting peptides was tested by analytical HPLC using two solvent systems: (1) A 0.1% TFA in water, B 0.1% TFA in 75% acetonitrile; 20% B to 80% B at 1%/min; A 0.1% TFA in water, B 0.1% TFA in 75% 2-propanol; 0% B to 50% B at 1%/min, using Lichrosorb RP-18 column and (2) a gradient of 0% B to 100% B at 2.5%/min employing a wide pore butyl (C4) column. The purity of the peptides in both systems was usually higher than 98% (Table 2). Solutions containing purified peptides were lyophilized. Samples of each of the peptides were hydrolyzed (6 N HCl, 110 °C, 22 h, in a vacuum) and analyzed with a Dionex automatic amino acid analyzer. Quantification of *β*-[2-naphthyl]-D-Ala, D-*p*-Chloro-Phe, and *β*-[2-pyridyl]-D-Ala was achieved by using the respective standards. These results were also used for determination of the peptide content in each preparation. The peptides were also analyzed by an LCQ mass spectrometry system (Finnigan, Bremen, Germany) using a nanospray ionization technique. The latter two analyses further confirmed the composition and purity of the products. Pure peptides were dissolved in water, to obtain 1 mM concentration, and aliquots were kept frozen (−20 °C).

Side Chain Modifications. Two 10 mg aliquots of purified Ac-D-Nal2-D-Cpa-D-Pal-D-Ala-Lys-Pro-D-Ala-NH_2 (peptide 5) were used for modifications of the Lys residue: one aliquot was reacted with 5 equiv of nicotinic acid (BDH, Poole, U.K.) and 5 equiv of DCC/HOBT in DMF, resulting in Ac-D-Nal2-D-Cpa-D-Pal-D-Ala-Lys(Nic)-Pro-D-Ala-NH_2 (peptide 7). The other aliquot was subjected to reductive alkylation in solution using 10 equiv of $NaCNBH_3$ in a DMF/acetone/acetic acid (30:10:1) solution, according to a reported procedure,[25] resulting in Ac-D-Nal2-D-Cpa-D-Pal-D-Ala-Lys(Isp)-Pro-D-Ala-NH_2 (peptide 6). Both peptides were purified by HPLC and analyzed as described above.

Animal Studies. Wistar-derived rats were obtained from the Weizmann Institute Animal Resource Center. Experiments were carried out in compliance with the regulations of the Weizmann Institute of Science.

Binding to the Pituitary GnRH Receptor. [D-Lys^6]-GnRH (synthesized in our laboratory) was iodinated by the chloramine T method,[26] and ^{125}I-[D-Lys^6]GnRH was purified (1700 *μ*Ci nmol^{-1}) by an analytic HPLC system as described above. The binding assay was conducted as previously described.[26] In brief, pituitary membranes (25 *μ*g protein/tube, prepared from Wistar-derived proestrous rats) were incubated for 90 min at 4 °C with 50 000 cpm (23.5 pM) ^{125}I-[D-Lys^6]-GnRH, alone or in the presence of various concentrations of the unlabeled peptides, in a total volume of 0.5 mL of the assay buffer (10 mM Tris-HCl containing 0.1% bovine serum albumin [BSA]). The reaction was terminated by rapid filtration through Whatman GF/C filters. The filters were washed three times with cold assay buffer and counted in an Auto-Gamma Counting System (Packard, Meriden, CT). The experiments were performed in triplicate. Nonspecific binding was defined as the residual binding in the presence of excess of [D-Lys^6]-GnRH (1 *μ*M). Specific binding was calculated by subtracting the nonspecific binding from the maximal binding, determined in the absence of any competing peptide. IC_{50} values were calculated using the curve-fitting software program Enzfitter (Elsevier Biosoft, Cambridge, U.K.).

LH Release from Cultured, Dispersed, Pituitary Cells. Cells from 21-day-old Wistar-derived female rats were dispersed as previously described[27] and incubated in 96-well plates (50 000 cells/well) at 37 °C in M-199 medium containing 5% horse serum. After 48 h the cells were washed with M-199 medium containing 0.1% BSA and incubated for 4 h at 37 °C with M-199/0.1% BSA (0.25 mL) containing the desired concentrations of the various peptides (four wells/experimental group). The incubation was terminated by removing the medium and diluting it by an equal volume of 1% BSA in phosphate buffered saline (PBS) solution. Three different aliquots from each sample were analyzed for LH concentration by radioimmunoassay (RIA)[28] using the kit kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Program. Results are expressed in terms of the RP-3 reference preparation.

In Vivo Bioactivity of GnRH Antagonists. A total of 0.5 mL of 0.1 M PBS containing peptide 3 or antide was injected intraperitoneally to castrated rats (five per group). The control group was injected with the vehicle (0.5 mL of 0.1 M PBS). Blood samples were withdrawn by cardiac puncture under light ether anesthesia at 0, 2.5, 5, and 10 h after drug administration,. The serum samples were assayed for LH content as described above.

Statistical Analysis. Results are expressed as the mean ± SEM. Comparisons were made using one-way analysis of variance (Instat 2.01, GraphPad Software, CA). $P < 0.05$ was taken as significant.

Abbreviations. Abbreviations of common amino acids are in accordance with the recommendations of IUPAC. Additional

abbreviations: Aca = 6-aminocaproic acid; BSA, bovine serum albumin; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; D-Nal2, β-[2-naphthyl]-D-Ala; D-Pal = β-[2-pyridyl]-D-Ala; GnRH, gonadotropin-releasing hormone; HPLC, high performance liquid chromatography; LH, luteinizing hormone; IC_{50}, concentration of ligand which displaces 50% of bound tracer; Lys(Isp), N^{ϵ}-[isopropyl]Lys; Lys(Nic), N^{ϵ}-[nicotinoyl]Lys; MePro, 1-aminocyclobutane-1-carboxylic acid; PBS, phosphate buffered saline; D-Cpa, D-*p*-chloro-Phe; pGlu, pyroglutamic acid; RIA, radioimmunoassay; SAR, structure−activity relationship; SEM, standard error of the mean.

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Supporting Information Available: Amino acid analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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