Decreased Histamine Release by Luteinizing Hormone-Releasing Hormone Antagonists Obtained upon Translocation of the Cationic Amino Acid from Position 8 to Position 7†

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We report analogues of *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Ilys-Pro-D-Ala-NH_2, the parent antagonist (PA), which is a potent antagonist of LHRH. To simplify future radioactive labeling we prepared *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Arg-Pro-D-Ala-NH_2 (**4**), [Arg^8]PA, which had good activity in the antiovulatory assay (AOA). Other analogues were designed at first by substituting with Arg at positions 5, 6, 7, 9, and 10, and Trp or Leu at position 8. Subsequent analogues were prepared in attempts to improve the AOA of the initial ones. Substitutions with Arg^9 or Arg^{10} led to analogues **1-3** with no AOA activity at 5 μg/rat. However, substitution with Arg^7 gave **9**, [Arg^7,Leu^8]PA, with significant activity in the AOA at 5 μg/rat and borderline activity at 2.5 μg/rat, and substitution with $Ilys^7$ gave **13**, [$Ilys^7$,Leu^8]PA, with borderline activity at 2 μg/rat, both analogues showing much weaker activity than PA in the histamine release assay (HRA) and therefore being potentially safer. Substitutions with D-Arg^8 or Arg^5 led to analogues with either good AO activity at 5 μg/rat (analogue **7**) or with borderline activity at 5 μg/rat (analogue **8**), although both were more potent than **6** in the HRA. Combinations of Ilys or Arg at positions 7 and 8 led to **10** and **11**, both of which were tested at 2 μg/rat and found to have either good AO activity (analogue **10**) or borderline activity (analogue **11**) but unsuitably potent in HR. Substitutions using $Ilys^7$ and neutral amino acids at position 8 led to **14-17** which were inactive in the AOA. Of great significance is the substitution with Arg^7 yielding analogue **9**, which was much safer in the HRA than analogue **4**, [Arg^8]PA. Analogues **9** and **13**, featuring substitutions with the Arg^7-Leu^8 or $Ilys^7$-Leu^8 sequences were even safer than PA or **6** in the HRA. Analogue **12**, [D-Trp^3,Tyr^5,D-Arg^6,Arg^7,Leu^8]PA, featuring the Arg^7-Leu^8 sequence, had much lower potency in the HRA than [D-Trp^3,Tyr^5,D-Arg^6,Leu^7,Arg^8]PA, which has the normal Leu^7-Arg^8 sequence. $Ilys^7$ together with neutral amino acids at position 8 led to analogues **14-17** which were also very weak (safer) in the HRA, with the smaller amino acids Ala^8 and Abu^8 being the weakest of all analogues prepared. The translocation of the strongly basic amino acid, as developed for analogues **9** and **13**, may be an attractive new approach to the design of safer AO analogues which low histamine release.

Introduction

There is great interest in designing highly potent and reversible antagonists of the luteinizing hormone-releasing hormone (LHRH) to inhibit the release of pituitary gonadotropins for the regulation of reproductive function, as recently reviewed by Karten and Rivier.[1] Most of the analogues prepared to date feature a strongly cationic amino acid at position 8, since it has been suggested that this is an important requirement for interaction of LHRH with its anterior pituitary cell receptors.[2] The development of potent LHRH antagonists led to analogues which have four or five D-amino acids, e.g. *N*-Ac-D-Nal-D-4-F-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-Gly-NH_2, Nal-Arg,[3] and *N*-Ac-D-Nal-D-Cpa-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2, Antag.[4] However, this type of analogue featuring a sequence of lipophilic N-terminal D-amino acids, and the two cationic charges of D-Arg^6 and Arg^8, caused transient edema of the face and extremities in the rat,[5] and later studies showed that these side effects were caused by histamine release (HR) from mast cells.[6,7]

Attempts to maintain antiovulatory (AO) potency and to decrease the side effect of histamine release led to the discovery that substitution with *N*-ε-alkyllysines, especially $Ilys^8$, resulted in antagonists with decreased release of histamine as a side effect.[8] Antagonists were later de-

† All symbols and abbreviations used follow guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1989**, *264*, 688-673). Unless otherwise noted, all amino acids have the L-configuration. Other abbreviations used are as follows: LHRH, luteinizing hormone-releasing hormone; Antide, *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Nic)-D-Lys(Nic)-Leu-Ilys-Pro-D-Ala-NH_2; Nal-Arg, *N*-Ac-D-Nal-D-4-F-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-Gly-NH_2; Antag, *N*-Ac-D-Nal-D-Cpa-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2; PA, *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Ilys-Pro-D-Ala-NH_2; histamide, histamine amide; MBHA, 4-methylbenzhydrylamine; Nal, 3-(2-naphthyl)alanine; Cpa, 4-chlorophenylalanine; Pal, 3-(3-pyridyl)alanine; Lys(Pic), lysine(*N*-ε-picolinoyl); Lys(Nic), lysine(*N*-ε-nicotinoyl); Ilys, lysine(*N*-ε-isopropyl); Abu, 2-aminobutyric acid; DCHA, dicyclohexylamine; DCM, dichloromethane; Boc, *tert*-butyloxycarbonyl; Ac, acetyl; Bzl, benzyl; Brz, 2-bromobenzyloxycarbonyl; Tos *p*-tolylsulfonyl; For, formyl; ONp, 4-nitrophenyl ester; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; CHL, chloroform; Pyr, pyridine; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamyl.

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Table I. Physicochemical Characteristics of LHRH Antagonists[a]

analogue no.	MW	yield,[c] mg (%)	OR,[d] deg	TLC[b] R_fA	R_fB	R_fC	R_fD	HPLC retention time, min[e]
1	1692	45 (24)	+25	0.49	0.20	0.68	0.48	8.6
2	1666	40 (12)	+7	0.57	0.29	0.74	0.49	8.4
3	1689	60 (10)	−12	0.60	0.34	0.77	0.54	10.8
4	1577	85 (35)	−26	0.42	0.16	0.69	0.44	6.0
5	1692	140 (50)	−37	0.57	0.26	0.74	0.53	9.2
6	1650	95 (32)	−8	0.45	0.17	0.68	0.47	6.8
7	1530	105 (51)	−9	0.52	0.23	0.70	0.50	6.4
8	1530	80 (36)	−9	0.55	0.25	0.75	0.53	7.2
9	1577	50 (37)	−21	0.39	0.18	0.64	0.42	6.0
10	1634	55 (35)	−23	0.06	0.04	0.41	0.26	3.6
11	1648	60 (34)	−13	0.09	0.04	0.40	0.25	3.8
12	1468	85 (43)	−25	0.56	0.30	0.74	0.46	5.8
13	1591	80 (45)	−16	0.13	0.06	0.54	0.35	3.4
14	1563	70 (33)	−40	0.13	0.06	0.60	0.38	3.2
15	1549	50 (24)	−44	0.10	0.04	0.51	0.33	3.0
16	1711	65 (28)	−13	0.04	0.01	0.26	0.28	3.6
17	1711	60 (23)	−30	0.04	0.01	0.26	0.28	2.8

[a] The analogues made include as follows: *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Pro-D-Arg-NH_2, [Trp^8, D-Arg^{10}]PA (1); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Arg-D-Ala-NH_2, [Trp^8,Arg^9]PA (2); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Arg-histamide, [Trp^8,Arg^9-histamide]PA (3); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Arg-Pro-D-Ala-NH_2, [Arg^8]PA (4); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Arg-Pro-D-Trp-NH_2, [Arg^8,D-Trp^{10}]PA (5); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Arg-Trp-Pro-D-Ala-NH_2, [Arg^7,Trp^8]PA (6); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Arg-Leu-Trp-Pro-D-Ala-NH_2, [D-Arg^8,Trp^8]PA (7); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Arg-D-Lys(Pic)-Leu-Trp-Pro-D-Ala-NH_2, [Arg^5,Trp^8]PA (8); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Arg-Leu-Pro-D-Ala-NH_2, [Arg^7,Leu^8]PA (9); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Arg-Ilys-Pro-D-Ala-NH_2, [Arg^7,$Ilys^8$]PA (10); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Ilys-Pro-D-Ala-NH_2, [$Ilys^{7,8}$]PA (11); *N*-Ac-D-Nal-D-Cpa-D-Trp-Ser-Tyr-D-Arg-Arg-Leu-Pro-D-Ala-NH_2, [D-Trp^3,Tyr^5,D-Arg^8,Arg^7,Leu^8]PA (12); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Leu-Pro-D-Ala-NH_2, [$Ilys^7$,Leu^8]PA (13); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Abu-Pro-D-Ala-NH_2, [$Ilys^7$,Abu^8]PA (14); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Ala-Pro-D-Ala-NH_2, [$Ilys^7$,Ala^8]PA (15); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Lys(Pic)-Pro-D-Ala-NH_2, [$Ilys^7$,Lys(Pic)^8]PA (16); *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Ilys-Lys(Nic)-Pro-D-Ala-NH_2, [$Ilys^7$,Lys(Nic)^8]PA (17). [b] The composition of solvents A–D is described in the Experimental Section. [c] The yields reported are based on milliequivalents of starting amino acid-resin or on milliequivalents of amino groups for MBHA resin. [d] OR = optical rotation. OR was determined as $[\alpha]^{27}_D$, in degrees (*c* 1, 1 N AcOH). [e] The solvent composition was 65% solvent B = 60% acetonitrile–40% solvent A; solvent A = 0.05% trifluoroacetic acid; flow rate 1.8 mL/min. From the HPLC patterns, it was estimated that the analogues had a purity of at least 95%.

signed with no arginine at position 8, e.g. *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Nic)-D-Lys(Nic)-Leu-Ilys-Pro-D-Ala-NH_2, Antide.[9] This antagonist is potent in the AO assay (AOA),[10] showing partial inhibition of ovulation in rats at 0.5 μg and full inhibition at 1.0 μg, and is among the weakest in the HR assay (HRA),[7] having an ED_{50} of >300 μg/mL for histamine release from rat mast cells in vitro. Because of these favorable characteristics, Antide is currently undergoing further clinical investigations (M. Karten, personal communication).

In order to study the effect of translocation of the strongly basic amino acid on the susceptibility of LHRH antagonists to enzymatic attack, we prepared analogues of *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Ilys-Pro-D-Ala-NH_2 (parent antagonist, PA). PA has similar AO activity as Antide, being significantly antiovulatory at doses at 0.5 μg, although featuring higher HR potency, and in addition it has the same 5 position substituted with D-amino acids.[9] To facilitate the synthesis of radioactively labeled analogues for enzymatic studies, we used Arg^8 instead of $Ilys^8$, thus avoiding extra blocking schemes for the side chain imino group of Ilys, and prepared *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Arg-Pro-D-Ala-NH_2. In an attempt to maintain low histamine release we prepared analogues in which Arg was translocated to positions other than 8, including positions 5, 6, 7, 9, or 10, and introduced Trp at position 8, so that there would only be one strongly basic amino acid per analogue at physiological pH. Since we found what might be a suggestion of AO activity in the analogue featuring Arg^7,Trp^8 substitution, we prepared other analogues substituting with Leu^8 or other amino acids instead of Trp^8 in an attempt to enhance the antiovulatory effect. We report here the physicochemical properties (Tables I and II) for 17 new analogues (1–17) of LHRH and biological data on their antiovulatory and histamine releasing properties (Table III).

Results and Discussion

Peptide Synthesis. All peptides were assembled manually by the solid-phase (SP) method,[11,12] with modifications previously described.[13] The starting resins were prepared by esterification of a chloromethylated polystyrene resin, 1% cross-linked with divinylbenzene, with the cesium salt of the appropriate Boc-amino acid,[14] or on a 4-methylbenzhydrylamine resin (MBHA).[15] Boc-amino

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Table II. Amino Acid Analyses of LHRH Antagonists

compd no.[c]	amino acid												
	Nal[a]	Cpa[a]	Pal[a]	Ser	Lys	Leu	Arg	Ilys[a]	Pro	Ala	Trp[b]	Abu[a]	Tyr
1	1.05	1.03	1.05	0.90	1.92	1.04	1.00		0.91		0.80		
2	0.93	1.00	1.05	0.90	2.03	1.06	1.01			0.99	0.77		
3	1.01	1.01	1.00	0.98	1.90	1.00	0.90				0.88		
4	0.90	0.99	1.04	1.01	2.07	1.05	1.05		1.00	0.98			
5	1.03	0.97	0.90	0.90	1.80	1.03	1.02		1.01		0.70		
6	1.00	1.00	1.00	1.04	1.85		1.00		1.01	0.98	0.81		
7	0.90	0.97	0.99	0.92	0.97	1.05	1.08		0.98	0.93	0.70		
8	1.03	0.98	1.03	0.93	0.89	1.10	1.05		0.93	0.98	0.81		
9	1.08	1.03	0.98	1.00	1.80	1.08	1.08		0.98	0.92			
10	1.00	1.03	1.03	0.90	1.87		1.10	0.96	1.02	0.98			
11	1.08	1.03	1.00	0.96	1.91			2.02	1.00	1.00			
12	1.09	1.06		1.00		1.00	2.06		1.05	0.98	0.65		0.99
13	1.01	0.98	0.98	0.95	1.80	1.04		0.97	0.97	1.00			
14	1.01	0.98	1.04	0.97	1.80			1.03	1.03	1.06		1.00	
15	0.98	1.05	1.01	0.93	1.80			1.01	1.00	2.15			
16	0.92	0.90	0.90	0.97	3.04			1.04	1.04	1.00			
17	0.93	0.99	0.90	0.98	3.20			0.98	1.05	1.00			

[a] Standards for Nal, Cpa, Pal, Ilys, and Abu were prepared by derivatization with PITC. [b] Tryptophan in peptides was estimated by its UV absorption at 280 nm as we have reported.[24,26] The values found for tryptophan are somewhat low and suggest that the peptide has several moles of AcOH, TFA, and/or H_2O, as has been our experience with tryptophyl-peptides.[24,26] [c] The sequences for analogues 1–17 are given as a footnote to Table I.

Table III. Biological Activities of LHRH Analogues in the Antiovulatory Assay (AOA) and in the Histamine Release Assay (HRA)[a]

amino acid substituents in PA,[b] and reference analogues[h]	peptide no.[i]	AOA (rats ovulating/10 rats) dose in μg				HRA ED_{50}, μg/mL ± SEM[c]
		1	2	2.5	5	
Trp^8,D-Arg^{10}-NH_2	1				10/10	72 ± 24[d]
Trp^8,Arg^9	2				10/10	39 ± 2.9[d]
Trp^8,Arg^9-histamide	3				9/10	220 ± 44[d]
Arg^8	4	4/10	1/10			2.7 ± 0.65[e]
Arg^8,D-Trp^{10}-NH_2	5	10/10				6.5 ± 0.61[e]
Arg^7,Trp^8	6	10/10			8/10	92 ± 9.2[e]
D-Arg^8,Trp^8	7	10/10			0/10	11 ± 1.3[e]
Arg^5,Trp^8	8	10/10			7/10	17 ± 0.79[e]
Arg^7,Leu^8	9			6/10	1/10	210 ± 75[d]
Arg^7,$Ilys^8$	10	9/10	1/10		0/10	8.3 ± 1.7[f]
$Ilys^7$,$Ilys^8$	11		7/10			19 ± 2.6[f]
D-Trp^3,Tyr^5,D-Arg^6,Arg^7,Leu^8	12		10/10			2.4 ± 0.17[f]
$Ilys^7$,Leu^8	13		6/10			186 ± 46[g]
$Ilys^7$,Abu^8	14		10/10			>300[g]
$Ilys^7$,Ala^8	15		10/10			>300[g]
$Ilys^7$,Lys(Pic)^8	16		10/10			87 ± 6.7[g]
$Ilys^7$,Lys(Nic)^8	17		8/10			70 ± 4.2[e]
Antide[h]						>300
PA[h]						93 ± 11
Antag[h]						0.1 ± 0.2

[a] Preliminary partial results were presented at the 12th American Peptide Symposium held on June 16–21, 1991 in Boston, MA.[27] [b] PA = *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Ilys-Pro-D-Ala-NH_2; Nal-Arg = *N*-Ac-D-Nal-D-4-F-Phe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-Gly-NH_2; Antide = *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Nic)-D-Lys(Nic)-Leu-Ilys-Pro-D-Ala-NH_2; Antag = *N*-Ac-D-Nal-D-Cpa-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2. [c] The HRA assays were run with four sets of compounds, d–g, corresponding to footnotes *d*–*g*, respectively. A standard of LHRH and a standard of Nal-Arg were evaluated for each set of analogues. The results are given as the mean ± standard error of the mean (SEM). [d] ED_{50} ± SEM for LHRH = 185 ± 20, Nal-Arg = 0.19 ± 0.02. Peptides **2** and **3** were not completely soluble in water at 2000 μg/mL; hence, the reproducibility of the assay was greatly affected. As part of set e, ED_{50} ± SEM for analogue **2** = 230 ± 44 and for analogue **3** = 133 ± 36. [e] ED_{50} ± SEM for LHRH = 182 ± 13, Nal-Arg = 0.19 ± 0.01. [f] ED_{50} ± SEM for LHRH = 196 ± 20, Nal-Arg = 0.17 ± 0.01. [g] ED_{50} ± SEM for LHRH = 169 ± 6.9, Nal-Arg = 0.16 ± 0.01. [h] Published values for HRA are given for Antide and PA[9] and for Antag[7] for comparison. In the AOA, at a dose of 1 μg, Antide and Antag show 100% inhibition and PA shows 90% inhibition of ovulation, whereas at a dose of 0.5 μg, Antide shows 36% inhibition, PA shows 100% inhibition, and Nal-Arg shows 62% inhibition. [i] The sequences for analogues 1–17 are given as a footnote to Table I.

acids were used for all syntheses.[16] The desired Boc-Lys(Pic) and Boc-D-Lys(Pic) were prepared by reacting Pic-ONp with the pertinent Boc-Lys isomer. The Boc group was removed at each cycle with 30% trifluoroacetic acid in DCM. After neutralization of the resin with 10% DIEA in DCM, coupling was performed with the appropriate Boc-amino acid and DCC[17] and was monitored by the ninhydrin test.[18] Introduction of Boc-D-Nal at position 1, acidolysis, neutralization, and acetylation with acetic anhydride gave the desired Ac-D-Nal substituent. Except for analogue **5**, peptides were removed from resins by ammonolysis[19] or aminolysis,[12] which results in the loss of the

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N-formyl group from Trp(For)-peptides,[20,21] and were freed from blocking groups with HF/anisole.[22] Peptide **5**, assembled on an MBHA resin, was treated with NH_3/MeOH to remove the D-Trp[10]-formyl group and then was treated with HF/anisole to liberate the free peptide. Analogues were purified by preparative HPLC, and purity was assessed by HPLC, TLC, and amino acid analysis (Tables I and II).

Bioassays. A standard AOA on 4-day cycling rats[10] was performed for each analogue (Table III) by Drs. Rehan Naqvi and Marjorie Lindberg of the TSI/Mason Research Institute, under contract NO1-HD-5-2934 with the Contraceptive Development Branch (CDB) of the National Institutes of Health (NIH). A standard HRA was performed for every analogue (Table III) by Dr. William A. Hook, of the Clinical Immunology Section, NIH, in duplicate with the mast cells from three different rats.[7]

Structure–Activity Relationships. Substitution of Ilys[8] of PA with Arg[8] led to analogue **4** which, as predicted, had substantial AO activity at a dose of 2 μg/rat. The substitution with D-Trp[10], led to analogue **5**, inactive at 1 μg/rat, which suggests that D-Ala[10] is a much better substituent than D-Trp[10]. Analogues **1–3**, with Arg[9]-histamide or Arg[10], had no apparent AO activity at doses of 5 μg. These finding are consistent with the previous report that the nature of the substituent at position 10 is critical for maintenance of AO activity.[23] However, substitution with Arg[7] led to **6**, which showed marginal AO activity at 5 μg. To eliminate the possibility that Trp[8] might be an undesirable substituent we prepared analogue **9**, featuring the Arg[7]-Leu[8] sequence, and found it to have significant activity at 5 μg. This is of especial interest, since most antagonists have a basic amino acid at position 8 which is thought to be important for binding to LHRH receptors, and also because this analogue was much weaker than analogue **4** or PA in the HRA in spite of containing an Arg residue as part of the sequence. The substitution with Ilys[7] led to analogue **13**, which shows borderline activity at 2 μg/rat. Analogue **7**, featuring D-Arg[6] inhibited ovulation fully at 5 μg/rat but was inactive at 1 μg/rat, whereas analogue **8**, featuring Arg[5], showed a hint of AO activity at 5 μg. Other substitutions were made including combinations of Ilys and Arg at positions 7 and 8, which led to analogues **10** and **11**. Of these, only analogue **10** appeared to be a potent antagonist at 2 μg/rat, although, both **10** and **11** had high HR potency very likely due to their dual cationic charges. Substitutios with Ilys[7] and neutral amino acids at position 8 led to **14–17** which showed no significant AO activity at doses at 2 μg.

Of great significance is that substitution with Arg[7], D-Arg[6], or Arg[5] led to **6**, **7**, and **8**, respectively, which are much weaker in the HRA than analogue **4**, analogue **6** being the weakest. It should be noted that a high value for an ED_{50} means a low potency in the HRA, hence a safer compound. To test the generality of the latter observation, we prepared analogue **12**, which has the same sequence as Antag but features the Leu[7]-Arg[8] sequence transposed to Arg[8]-Leu[7]. As predicted, **12** showed much lower potency than Antag in the HRA. Analogue **13**, featuring Ilys[7] is also very weak in the HRA. Ilys[7] together with neutral amino acids at position 8 led to analogues **14–17** which were also weak in HR, with Ala[8] and Abu[8] being the weakest, probably as weak as Antide itself.

The finding that analogues **9** and **13** may have AO activity in the range of 2–5 μg, together with lower than expected HR activity, indicates that the translocation of the strongly basic amino acid from position 8 to 7 may lead to therapeutically safer analogues which preserve in part AO activity but with decreased histamine release. Additionally, the use of smaller, less lipophilic amino acids at position 8 may lead to further lowering of histamine release. We are pursuing further studies of this new mode of substitution with the aim of enhancing the AO potency while maintaining a very low level of histamine release.

Conclusions. Transposition of cationic charge of an antagonist from position 8 to position 7, as in analogues **9**, featuring Arg[7], and **13**, featuring Ilys[7], and transposition of Leu[7] to position 8, may lead to safer analogues with partial retention of AO potency, but with lower HR potency. Additionally, the same transposition of cationic charge and the use of a smaller neutral amino acid at position 8, as in analogues **14** and **15**, may lead to lower HR, although with lower AO activity. These observations may be useful for the design of future LHRH antagonists endowed with low HR as a side effect.

Experimental Section

Boc-amino acids were purchased from Bachem Inc. Boc-D-Nal, Boc-D-Cpa, Boc-D-Pal, Boc-Lys(Nic), and Boc-Ilys were provided by P. N. Rao, of the Southwest Foundation for Biomedical Research, under Contract NO1-HD-6-2928 with the CDB, NIH. Additionally, Boc-Lys(Pic) and Boc-D-Lys(Pic) were synthesized in our laboratories and were found comparable on HPLC to a genuine sample of Boc-Lys(Pic) generously supplied by Drs. K. Folkers and A. Ljungqvist, of the Institute for Biomedical Research, University of Texas at Austin, Austin, TX. The MBHA resin was purchased from Applied Biosystems, and chloromethylated resins and ion-exchange resins were supplied by Bio-Rad. For all synthetic and chromatographic steps we used HPLC solvents supplied by Fisher Scientific. Water was purified by passing institutionally deionized and distilled water through a D5041 mixed ion-exchange cartridge (Barnstead Sybron Corp, Boston, MA) and distilling in an all-glass still. Other reagents were of analytical grade and were purchased from Aldrich Chemical Co., Pierce Chemical Co., or Chemical Dynamics. Peptides were treated with liquid HF in an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). Ammonia and HF were supplied in gas cylinders by Matheson. The purity of peptides was verified at 220 nm by analytical HPLC in a Millipore apparatus previously described[24] using a μBondapak (Millipore) C_{18} column (30 × 0.39 cm). The peptides were finally purified by preparative HPLC in a Rainin apparatus[24] using a column module, 2.14 × 25 cm, with a guard module, 2.14 × 5 cm, both packed with Dynamax-60A, 8 μm, C_{18} (Rainin). The solvent systems used both for analytical or preparative HPLC were as follows: (A) 0.05% TFA; (B) 60% MeCN–40% solvent A. Thin-layer chromatography (TLC) was performed on silica gel G precoated TLC plates (Analtech Uniplates, 0.25 mm). The following solvent systems were used (ratios given by volume): (A) 1-BuOH–AcOH–H_2O (3:1:1); (B) 1-BuOH–AcOH–H_2O (5:1:1); (C) 1-BuOH–AcOH–H_2O–Pyr (5:1:1:1); (D) 1-BuOH–AcOH–H_2O (4:1:5, upper phase). For analysis, peptides (40–80 μg) were applied

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in solution to the TLC plate and, after development, they were visualized with Ehrlich reagent or chlorine–tolidine.[12] For amino acid analysis, peptides were hydrolyzed with 6 N HCl for 24 h at 110 °C, and the amino acids in the hydrolysis product were derivatized with phenyl isothiocyanate. The resulting PTC-amino acids were determined by the Picotag method (Millipore),[25] using an amino acid standard (Pierce Chemical Co.) as reference, and an analytical HPLC set-up which we have described previously.[24] The optical rotations for amino acid derivatives and peptides were measured in a Rudolph polarimeter (precision ±0.01°). Microanalyses were performed by Galbraith Labs, Inc. (Knoxville, TN).

Solid-Phase Synthesis of Protected Peptides. Boc-amino acids were used for the syntheses, and for protection of side chain functionalities, Boc-Arg(Tos), Boc-D-Arg(Tos), Boc-Ser(Bzl), Boc-Ilys(Z), Boc-Tyr(Brz), and Boc-Trp(For) or Boc-D-Trp(For) were used. The starting Boc-D-Ala-resin (0.64 mmol of D-Ala/g), Boc-Arg(Tos)-resin (0.56 mmol of Arg/g), or Boc-D-Arg(Tos)-resin (0.5 mmol of D-Arg/g), were prepared on a 200–400-mesh, chloromethylated resin (Bio-Rad), 1% cross-linked with divinylbenzene, by esterification with the cesium salt of the pertinent Boc-amino acid. For the synthesis of the D-Trp(For)[10] antagonist we coupled Boc-D-Trp(For) to an MBHA resin (0.75 mmol of D-Trp/g). The Boc-amino acid-resin (0.5–0.7 mmol) was subjected to the required number of coupling cycles by the SP method. In each cycle, resins were treated with 30% trifluoroacetic acid in DCM to remove Boc groups and, after neutralization with 10% DIEA in DCM, the resin was treated with a 3-fold excess of the appropriate Boc-amino acid and DCC. Where possible, completion of each coupling step was monitored by the ninhydrin test, which usually gave a negative response. If a test was positive, the coupling step was repeated, but if only fairly positive, the unreacted peptide was acetylated by treatment with Ac_2O–DIEA–DCM (1:1:8) for 10 min. After introduction of Boc-D-Nal at position 1, acidolysis and neutralization as for a normal peptide cycle, followed by acetylation with Ac_2O–DIEA–DCM (1:1:8), gave the desired *N*-Ac-D-Nal-substitution. The fully assembled peptides were removed from the resin either by ammonolysis with MeOH (25 mL) saturated with ammonia or by aminolysis with 20% histamine in methanol solution, both of which removed the formyl group from tryptophyl residues at the same time. After 3 days, the resin was removed by filtration and extracted three times with hot DMF. The peptide amide was isolated from the pooled extracts by precipitation with water or Et_2O–petroleum ether, usually yielding 400–600 mg of protected peptide. TLC analysis of protected peptides usually showed one major component with minor impurities and were used directly for preparation of the free analogues. All protected peptides were freed from blocking groups by treatment with liquid HF/anisole. Peptide **5** was prepared by coupling Boc-D-Trp(For) to an MBHA resin and then building up the peptide sequence on the resin by the usual methods. Treatment of the assembled peptide-resin with MeOH (25 mL) saturated with ammonia resulted in removal of the formyl group from D-Trp[10], and then treatment of the resulting peptide-resin with HF/anisole led to analogue **5**.

Picolinic Acid *p*-Nitrophenyl Ester (Pic-ONp). A magnetically stirred solution of picolinic acid (1.23 g, 10 mmol) and 4-nitrophenol (1.67 g, 12 mmol) in Pyr (10 mL) was cooled in ice to about 2–4 °C, and then it was treated with a solution of DCC (2.063 g, 10 mmol) in Pyr (5 mL). After 3h the suspension was brought to room temperature and was allowed to react for 1 additional hour. The dicyclohexylurea which had precipitated (1.87 g) was collected by filtration and washed with pyridine. The pooled filtrate was treated with water, and the crystals which formed were collected and washed with water, yielding 2.16 g: mp 154–155 °C. Anal. ($C_{12}H_8N_2O_5$): C, calcd 59.0, found, 59.5; H, N.

(*tert*-Butyloxycarbonyl)lysine(picolinoyl) Dicyclohexylamine Salt, Boc-Lys(Pic)·DCHA. To a magnetically stirred solution of Pic-ONp (733 mg, 3 mmol) in DMF (7 mL) was added a solution of Boc-Lys (492 mg, 2 mmol) in 2 N NaOH (1 mL). After 7 h the solution was extracted with EtOAc (10 mL) three times. The aqueous solution was acidified with 10% citric acid (10 mL) and then extracted with DCM (10 mL) three times. The pooled DCM extracts were washed with water and saturated salt solution and dried ($MgSO_4$). Evaporation of the solvent left an oil which after treatment with DCHA (0.4 mL, 2 mmol), yielded crystals (770 mg, 72%): mp 138–140 °C. Recrystallization from EtOAc–petroleum ether gave the analytical sample: mp 138–141 °C; $[\alpha]^{27}_D$ +20° (c 2, EtOH). Anal. ($C_{29}H_{48}N_4O_5$): C, H, N.

(*tert*-Butyloxycarbonyl)-D-lysine(picolinoyl) Dicyclohexylamine Salt (Boc-D-Lys(Pic)·DCHA). This intermediate was prepared as described for the L isomer: mp 138–141 °C; $[\alpha]^{27}_D$ −20.5° (c 2, EtOH).

***N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Pro-D-Arg-NH_2, [Trp[8],Arg[10]]PA (1, Table I).** *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser(Bzl)-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Pro-D-Arg(Tos)-NH_2 (300 mg) prepared by the SP method, was treated with anisole (1 mL) and liquid HF (9 mL) for 60 min at 0 °C. After removal of HF under vacuum, the residue was extracted with 30% acetic acid (15 mL) three times, and the pooled extracts were washed with petroleum ether (50 mL) three times. The aqueous phase was treated with ion-exchange resin AG1-X2, acetate (2 g), the suspension was filtered, and the filtrate was lyophilized (265 mg). This material (145 mg) was purified by dissolving in water with enough acetic acid to dissolve the sample (5–10% acetic acid), applying the solution to a preparative C_{18} column, and eluting at 3 mL/min, with a gradient from 0 to 50% solvent B for 50 min. The fractions of the main peak were monitored by analytical HPLC. The desired fractions were pooled, concentrated in a vacuum to remove acetonitrile, and lyophilized, yielding the desired analogue (45 mg). All analogues were prepared by this method except for analogue **5**, which is described below. Analogues were homogeneous on TLC with four solvent systems and gave one single peak on analytical HPLC. Amino acid analyses were performed for each analogue by the HPLC-Picotag method,[25] using standards of Nal, Cpa, Pal, Ilys, and Abu. Trp in peptides was estimated at 280 nm by UV spectrophotometry.[24,26]

***N*-Ac-D-Nal-D-Cpa-D-Pal-Ser-Lys(Pic)-D-Lys(Pic)-Leu-Arg-Pro-D-Trp-NH_2, [Arg[8],Trp[10]]PA (5, Table I).** *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser(Bzl)-Lys(Pic)-D-Lys(Pic)-Leu-Arg(Tos)-Pro-D-Trp(For)-MBHA resin (0.35 mmol) was treated for 3 days with MeOH saturated with ammonia in order to remove the formyl group. The resulting resin was washed with MeOH to neutrality and air-dried, and the dry resin was treated with HF–anisole by the method described above, which yielded the free peptide (420 mg). Purification of a portion of the free peptide (200 mg) was accomplished by preparative HPLC as outlined above, yielding the desired analogue **5** (140 mg).

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Registry No. 1, 138207-84-8; **2**, 138207-85-9; **3**, 138207-86-0; **4**, 138207-87-1; **5**, 138207-88-2; **6**, 138207-89-3; **7**, 138207-90-6; **8**, 138207-91-7; **9**, 138207-92-8; **10**, 138207-93-9; 11, 138207-94-0; **12**, 138207-95-1; **13**, 138207-96-2; 14, 138207-97-3; **15**, 138207-98-4; **16**, 138207-99-5; **17**, 138208-00-1; Pic-OH, 98-98-6; Pic-ONp, 74104-89-5; Boc-Lys, 13734-28-6; Boc-D-Lys, 106719-44-2; Boc-Lys(Pic)·DCHA, 138208-03-4; Boc-D-Lys(Pic)·DCHA, 138208-04-5; *N*-Ac-D-Nal-D-Cpa-D-Pal-Ser(Bzl)-Lys(Pic)-D-Lys(Pic)-Leu-Trp-Pro-D-Arg(Tos)-NH_2, 138208-01-2; antide, 112568-12-4; histamine, 51-45-6.

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