Novel, Potent Luteinizing Hormone-Releasing Hormone Antagonists with Improved Solubility in Water§

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A series of luteinizing hormone-releasing hormone antagonists with new substitutions in position 6 or positions 5 and 6 that included lysine acylated at the ϵ-amino group with different heterocyclic carboxylic acids or amino-substituted heterocyclic carboxylic acids was synthesized. These novel analogs were synthesized on a solid-phase support via the acylation of lysine residue in otherwise protected resin-bound peptides. All analogs were tested in the rat antiovulatory assay (AOA) and the best of them in in vitro histamine release assay. Introduction of lysine acylated with amino-substituted heterocyclic carboxylic acids yielded several water-soluble antagonists with good therapeutic ratio (high AOA to low histamine releasing activity). The best antagonist in terms of activity, histamine release, and solubility was nictide: NAcDNal-DCpa-DPal-Ser-PicLys-D(6ANic)-Orn-Leu-ILys-Pro-DAlaNH$_2$ (6ANic = 6-aminonicotinoyl).

Introduction

Competitive antagonists of luteinizing hormone-releasing hormone (LHRH) have the potential of being therapeutic drugs for endocrine diseases as well as nonsteroidal contraceptive agents.[1,2] The requirements of safety for contraception are extremely high. The ideal LHRH antagonist for contraception should have high potency, release negligible histamine, be stable toward enzymatic degradation for oral administration, be readily formulated, and be relatively inexpensive. So far the complete inhibition of ovulation by LHRH antagonists was achieved at the dose of 0.5 μg/rat,[3,4] and the best ED_{50} value for histamine release reported was over 300 μg/mL.[5] Unfortunately none of the antagonist known today can be characterized by both these high values.

Antide (1) was the first peptide of a new generation of LHRH antagonists with a significantly improved safety margin[5,6] (ED_{50} for histamine release over 300 μg/mL, complete inhibition of ovulation at 1 μg/rat). However, its poor water solubility caused problems with formulation.[7,8]

Recently, we reported on increasing hydrophilicity by introducing acylated DThr in position 1.[9] The resulting antagonists had good solubility and negligible histamine release (ED_{50} = 300 μg/mL), but their antiovulatory ED_{100} was only 2.5 μg/rat.

We report here on the synthesis of more potent LHRH antagonists which are modified in position 6. The new amino acid residues were synthesized on a partially protected resin-bound peptide by modifying lysine that had been orthogonally protected in the prior steps of the synthesis. After deprotection, the ϵ-NH_2 group of Lys was acylated with different heterocyclic carboxylic acids and amino substituted heterocyclic carboxylic acids. Increased water solubility was achieved, and several very potent antagonists with low histamine release activity were identified.

Results and Discussion

Our earlier results[5,10] indicated that substitution of positions 5 and 6 with lysine acylated at the ϵ-amino group with heterocyclic carboxylic acids such as nicotinic acid, 2-pyridinecarboxylic acid (picolinic acid), or 2-pyrazinecarboxylic acid in different combinations resulted in potent antagonists with low histamine-release activity (analogs **1**, **4**, **5**, and **9** in Table 1). Particularly active was analog **5** with PicLys[5], DPicLys[6] (100% AOA at 0.5 μg/rat). Via this route, we synthesized five more analogs (**2**, **3**, **6**, **7**, and **8** in Table 1) so that all nine possible combinations for the three carboxylic acids mentioned above were tested. All new analogs were either partially active or not active at 0.5 μg. ED_{50} values for all compounds in Table 1 ranged from 39 to over 300 μg/mL. It was found that very small structural changes can elicit quite meaningful differences in biological responses. A straightforward relationship between the structure of the residues in positions 5 and 6 and biological activity is not evident. Analogs **1**, **3**, and **9** with NicLys[5] or PzcLys[5] have the highest ED_{50} values, while the best combination for potency was PicLys[5] and DPicLys[6] in analog **5**. However, all analogs in Table 1 are very lipophilic, which causes their poor solubility in water at pH = 7 (<1 mg/mL) and can make their administration difficult. For this reason we decided to introduce a free amino group to the heterocyclic carboxylic acids used for acylation in order to increase solubility while maintaining a good therapeutic ratio (low histamine-release activity to high in vivo potency). Modified carboxylic acids used for acylation of lysine were 6-aminonicotinic acid (6ANic), 2-aminonicotinic acid (2ANic), 6-amino-2-pyridinecarboxylic acid (6APic), and 3-amino-2-pyrazinecarboxylic acid (3APzc). Two series of analogs of general sequence NAcDNal-DCpa-DPal-Ser-( )[5]-( )[6]-Leu-( )[8]-Pro-DAlaNH$_2$ were synthesized, one with ILys, and the other with Arg in position 8. The analogs in each series had the new substituents either in position 6 (with PicLys, our best choice for potency, in position 5) or in positions 5 and 6

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§ **Abbreviations of the Unnatural Amino Acids.** Nal, 3-(2-naphthyl)alanine; Cpa, 3-(4-chlorophenyl)alanine; Pal, 3-(3-pyridyl)alanine; ILys, *N*ϵ-isopropyllysine; NicLys, *N*ϵ-nicotinoyllysine; PicLys, *N*ϵ-(2-pyridylcarbonyl)lysine (*N*ϵ-picolinoyllysine); PzcLys, *N*ϵ-pyrazinylcarbonyllysine; (2ANic)Lys, *N*ϵ-(2-aminonicotinoyl)lysine; (6ANic)Lys, *N*ϵ-(6-aminonicotinoyl)lysine; (6Apic)Lys, *N*ϵ-(6-aminopicolinoyl)lysine; (3Apzc)Lys, *N*ϵ-(3-amino-2-pyrazinylcarbonyl)lysine.
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Table 1. Biological Data for LHRH Analogs of General Sequence NAcDNal-DCpa-DPal-Ser-( )[5]-( )[6]-Leu-ILys-Pro-DAlaNH$_2$

analog	position 5	position 6	AOA[a] (rats ovul/total rats) dose in μg/rat 0.25	0.5	1.0	μg/mL
1[c]	NicLys	DNicLys		9/25	0/10	>300
2	NicLys	DPicLys		5/8		60 ± 1.4
3	NicLys	DPzcLys		2/9	1/12	263 ± 23
4[c]	PicLys	DNicLys		3/8	0/8	39 ± 1
5[d]	PicLys	DPicLys	6/10	0/10		93 ± 11
6	PicLys	DPzcLys		6/6		
7	PzcLys	DNicLys		6/6		
8	PzcLys	DPicLys		3/6		
9[d]	PzcLys	DPzcLys		5/6		288 ± 30

[a] Antiovulatory activity. [b] Histamine-release activity. [c] 1 and 4 are from ref 5. [d] 5 and 9 are from ref 3.

Table 2. Biological Data for LHRH Analogs of General Sequence AcDNal-DCpa-DPal-Ser-( )[5]-( )[6]-Leu-( )[8]-Pro-DAlaNH$_2$

analog no.	position 5	position 6	position 8	AOA[a] (rats ovul/total rats) dose in μg/rat 0.25	0.5	1.0	HRA[b] ED_{50} + SEM, μg/mL
10	PicLys	D(2ANic)Lys	ILys		2/5		
11	PicLys	D(6ANic)Lys	ILys	3/7	2/12	0/6	96 ± 0
12	PicLys	D(6APic)Lys	ILys		4/6		
13	PicLys	D(3APzc)Lys	ILys		3/13	1/6	100 ± 0
14	(2ANic)Lys	D(2ANic)Lys	ILys		7/7		
15	(6ANic)Lys	D(6ANic)Lys	ILys		7/7		
16	(6APic)Lys	D(6APic)Lys	Ilys		7/7		
17	(3APzc)Lys	D(3APzc)Lys	ILys		6/7		
18	PicLys	D(2ANic)Lys	Arg		7/7		
19	PicLys	D(6ANic)Lys	Arg	2/6	2/12	0/6	8 ± 3
20	PicLys	D(6APic)Lys	Arg		2/6		
21	PicLys	D(3APzc)Lys	Arg		6/6		
22	(2ANic)Lys	D(2ANic)Lys	Arg		6/6		
23	(6ANic)Lys	D(6ANic)Lys	Arg		6/6		
24	(6APic)Lys	D(6APic)Lys	Arg		6/6		
25	(3APzc)Lys	D(3APzc)Lys	Arg		4/6		

[a] Antiovulatory activity. [b] Histamine-releasing activity.

Table 3. Biological Data for LHRH Analogs of General Sequence NAc( )[1]-DCpa-DPal-( )[5]-( )[6]-( )[7]-ILys-Pro-( )[10]NH$_2$

analog no.	position 1	position 4	position 5	position 6	position 7	position 10	AOA[a] (rats ovul/total rats) dose in μg/rat 0.25	0.5	HRA[b] ED_{50} ± SEM, μg/mL
26	DNal	Ser	PicLys	D(6ANic)Orn	Leu	DAla	6/8	0/8	100 ± 0
27	DNal	Ser	PicLys	D(6ANic)Lys	Leu	Sar	6/8	0/8	31 ± 0.8
28	DNal	Ser	PicLys	D(6ANic)Lys	His	DAla		6/6	
29	DNal	Ser	Tyr	D(6ANic)Lys	Leu	DAla		3/5	
30	DNal	Thr	PicLys	D(6ANic)Lys	Leu	DAla		5/5	
31	DNal	Ser	PzcOrn	D(6ANic)Lys	Leu	DAla		6/6	
32	DNal	Ser	PzcOrn	D(6ANic)Orn	Leu	DAla		4/5	
33	DQal	Ser	PzcOrn	D(6ANic)Orn	Leu	DAla		5/5	

[a] Antiovulatory activity. [b] Histamine-releasing activity.

simultaneously, in L and D configurations, respectively (Table 2). In all cases introduction of the amino group improved solubility in water (>8 mg/mL), but none of the analogs was fully active at the dose of 0.5 μg/rat. However, partial inhibition of ovulation at this dose was observed for several peptides, mainly those with the amino-substituted ring in position 6 and PicLys in position 5. D(6ANic)Lys was the most effective substituent in both ILys and Arg series (peptides **11** and **19**, respectively, 100% AOA at 1 μg/rat). Another good substituent was D(3APzc)-Lys (peptide **13**, AOA 1/6 rats at 1 μg/rat). D(6APic)Lys and D(2ANic)Lys in position 6 were less active. As expected, histamine release was quite different in both series. ED_{50} values of 96 and 100 μg/mL were achieved for peptides **11** and **13**, with ILys in position 8, but the value was only 8 ± 3 μg/mL for peptide **19** with Arg in this position.

The best of the series, peptide **11**, was subjected to modifications in order to still increase its activity, while maintaining good ED_{50} value and water solubility. Changes were made in positions 1, 4, 5, 6, 7, and 10 (Table 3). No improvement in AOA value was observed for peptides **28–33**. However, peptides **26** and **27** with D(6ANic)Orn in position 6 and Sar in position 10, respectively, showed increased activity (total inhibition at 0.5 μg/rat dose), with the former maintaining very good ED_{50} value of 100 μg/mL. These data as well as good water solubility place peptide **26** among the best antagonists of LHRH synthesized so far and make it an excellent candidate for further biological tests. It was named nictide.

In summary, we have successfully designed new antagonists of LHRH with increased water solubility by introducing an amino group to the heterocyclic carboxylic acids used for acylation of ε-amino group of lysine in position 6. Further manipulation of the substituents in the best antagonist thus obtained gave a very promising analog, nictide, which combines satisfactory water solubility with good therapeutic ratio.

Experimental Section

Instruments. Amino acid analyses were carried out on a Beckman 118-CL Amino Acid Analyzer after hydrolysis in constant boiling HCl for 24 h using standard procedures.[11] The unnatural amino acids were qualitatively determined with the exception of Pal which was quantified. Analytical reversed-phase HPLC was run on a Waters instrument and a Vydac C_{18} column, 25 × 3.6-mm i.d. The peptide synthesizer used was Beckman Model 990. ^1H NMR spectra were run on a Nicolet NT-360 instrument. Mass spectra were taken on a 5995 Hewlett-Packard instrument.

Starting Materials. The amino acid derivatives: Boc-DAla, Boc-Arg(Tos), Boc-Leu, Boc-Pro, Boc-D- and -L-Lys(Fmoc), and Boc-Ser(Bzl) and benzhydrylamine (BHA) resin were purchased from Advanced ChemTech. Boc-DNal, Boc-DCpa, Boc-DPal, and Boc-ILys were synthesized at the Southwest Foundation for Biomedical Research and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. The 2- and 6-aminonicotinic acids and 3-amino-2-pyrazinecarboxylic acid were purchased from Aldrich. All solvents were reagent grade.

Peptide Synthesis. The resin-bound peptides containing Fmoc-protected amino functions at the side chains were synthesized by the SPPS method either manually or on a Beckman 990 peptide synthesizer by the described protocols[11] on a benzhydrylamine (BHA) resin (0.4 g per peptide) using *tert*-butyloxycarbonyl (Boc) group for N^{α}-amino protection. The coupling time was 180 min. A 3-fold excess of protected amino acid was used. Terminal acetylation was performed for 15 min with 20% acetic anhydride in CH_2Cl_2.

N^{ϵ}-(2-Pyridylcarbonyl)lysine (N^{α}-Picolinoyllysine). Boc-Lys (4.94 g, 20 mmol) and 4-nitrophenyl 2-pyridinecarboxylate (4.36 g, 18 mmol), prepared according to the known procedure,[12] were stirred together in DMF (100 mL) for 3 days at room temperature. After evaporation of DMF in high vacuum, the reaction mixture was dissolved in ethyl acetate (60 mL) and extracted with saturated sodium bicarbonate solution (2 × 15 mL). The combined aqueous layers were acidified with 10% HCl to pH = 3 and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with water (2 × 15 mL) and brine (15 mL), dried, and evaporated to give a crude product (5.7 g, 81%). This product was used without further purification. For analytical purposes it was purified by column chromatography on silica gel (eluant $CHCl_3$:MeOH:AcOH, 94:5:1): R_{f_1} = 0.36, $CHCl_3$:MeOH:AcOH, 94:5:1; R_{f_2} = 0.71, *n*-BuOH:py:AcOH:H_2O, 30:10:3:12; mp 112–114 °C; ^1H NMR ($CDCl_3$) δ 1.43 (s, 9H), 1.73–1.45 (m, 6H), 3.45 (m, 2H), 4.32 (m, 1H), 7.43 (m, 1H), 7.85 (m, 1 H), 8.2 (m, 1H), 8.55 (m, 1H). Anal. Calcd for $C_{12}H_{17}N_3O_3$: C, 57.36; H, 6.82; N, 16.72. Found: C, 57.28; H, 6.70; N, 16.48.

6-Amino-2-pyridinecarboxylic Acid. Commercially available 6-amino-2-methylpyridine (10 g, 92 mmol) was refluxed with acetic anhydride (20.4 g, 200 mmol) in benzene (100 mL) for 3 h. The solvent and the excess acetic anhydride were then evaporated, and the crude product was crystallized from ethyl alcohol to give 6-(acetylamino)-2-methylpyridinium acetate (16 g, 83%). To a solution of 6-(acetylamino)-2-methylpyridinium acetate (15.8 g, 75 mmol) in water (100 mL) was added a solution of NaOH (3.0 g, 75 mmol) in water (20 mL), and the obtained solution of the free amine was oxidized with $KMnO_4$ (28 g, 177 mmol) according to Ferrari *et al.*[13] to give 6-(acetylamino)-2-pyridinecarboxylic acid (7.8 g, 58%). This acid was refluxed in 10% aqueous NaOH solution (34 mL) for 1 h, followed by acidification with concentrated HCl to yield crude 6-amino-2-pyridinecarboxylic acid, which was purified by crystallization from water (3.6 g, 61%): mp 316–318 °C; ^1H NMR (D_2O/NaOD) δ 6.59 (d, J = 8.0 Hz, 1H), 7.11 (d, J = 8.0 Hz, 1H), 7.49 (t, J = 8.0 Hz, 1H). Anal. Calcd for $C_6H_6N_2O_2$: C, 52.17; H, 4.38; N, 20.28. Found: C, 52.36; H, 4.21; N, 20.05.

NAcDNal-DCpa-DPal-Ser-PicLys-D(Acyl)Lys-Leu-ILys-Pro-DAlaNH$_2$. General Procedure. NAcDNal-DCpa-DPal-Ser-PicLys-D(Fmoc)Lys-Leu-ILys-Pro-DAla-BHA-resin (0.5 g, 0.48 mmol/g) was treated with solution of piperidine (10 mL) in DMF (10 mL) for 20 min to remove the Fmoc group. After sequential washes with DMF (2 × 1 min), 2-propanol (2 × 1 min), and CH_2Cl_2 (5 × 1 min), the appropriate aromatic carboxylic acid was coupled by the standard procedure with DCC/HOBT for 3 h or until there was a negative Kaiser test.[14] The amino groups on heterocyclic rings were not protected. The resin was washed with DMF (2 × 1 min) and CH_2Cl_2 (5 × 1 min). After vacuum drying, the peptide was cleaved from the resin by treatment with anhydrous HF (10 mL) and *p*-cresol (0.4 g) for 1 h at 0 °C. The HF was removed by high vacuum. The residue was washed with ethyl ether (3 × 20 mL), and the peptide was extracted with 20% acetic acid (4 × 20 mL). Lyophilization yielded a fluffy, white solid (100–150 mg).

NAcDNal-DCpa-DPal-Ser-(Acyl)Lys-D(Acyl)Lys-Leu-ILys-Pro-DAlaNH$_2$. General Procedure. The procedure, as above, was followed except that the starting resin-bound peptide was NAcDNal-DCpa-DPal-Ser-(Fmoc)Lys-D(Fmoc)Lys-Leu-ILys-Pro-DAlaNH$_2$.

Purification. The lyophilized crude peptides were gel filtered on Sephadex G-25 with 6% HOAc as the eluant, followed by chromatography on Sephadex LH-20 with the solvent system H_2O:*n*-BuOH:HOAc:MeOH, 90:10:10:8. The purity was checked by TLC and analytical reversed-phase HPLC. The structure was confirmed by amino acid analysis and FAB mass spectrometry.

Bioassays (see Tables 1–3). The AOA was carried out as described by Corbin and Beattie;[15] cycling rats (250–300 g) were injected subcutaneously with the peptides suspended in corn oil (0.3 mL) at noon on proestrus. Results are expressed in terms of rats ovulating/total number of treated rats. The histamine release was assayed with rat mast cells, as reported.[16] The ED_{50} value is the concentration of the analog (μg/mL) that releases 50% of total releasable histamine.

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Supplementary Material Available: FAB-MS spectrometry data, HPLC and TLC data, and amino acid analyses (4 pages). Ordering information is given on any current masthead page.

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