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ANTAGONISTS OF LHRH SUPERIOR TO ANTIDE; EFFECTIVE SEQUENCE/ACTIVITY RELATIONSHIPS

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Twenty new analogs of LHRH featured acylated aminocyclohexylalanines and acylated lysines in positions 5,6. (N-Ac-D-2-Nal[1],DpClPhe[2],D-3-Pal[3],PicLys[5],c-D-PzACAla[6],Val[7],Ilys[8],D-Ala[10])-LHRH (100% AOA/0.5ug) and (N-Ac-D-2-Nal[1],DpClPhe[2] D-3-Pal,PicLys[5],D-Piclys[6],Abu[7],D-Ala[10])-LHRH (50% AOA/0.25ug) were most potent.

Dedication and Acknowledgement

We are very pleased and extraordinarily honored to join in the Celebration of the 80th birthday of Professor Yu Wang, of the Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. We dedicate this manuscript to him. Professor Yu Wang was very courteous and constructive on both organic chemical research and personnel matters for the Institute for Biomedical Research, University of Texas at Austin, in behalf of Professor Karl Folkers, and the staff of the Institute. Appreciation is expressed to the Robert A. Welch Foundation for their respective support of this research.

The leutinizing hormone releasing hormone (LHRH) is pGlu, His, Trp, Ser, Tyr, Gly, Leu, Arg, Pro, Gly-NH_2.

Since the discovery that the LHRH antagonist (N-Ac-D-2-Nal[1], D-4-FPhe[2], D-Trp[3], D-Arg[6])-LHRH caused transient edema of the face and extremities of rats when injected subcutaneously[1], attention has been focused on lowering the histamine release as well as on increasing antiovulatory, AOA, potency. The release of histamine has been ascribed to the presence of strongly basic residues in positions 6 and 8, e.g. D-Arg[6], Arg[8], and a cluster of hydrophobic amino acids at the N-terminal, that is the structural features which are characteristic of many potent LHRH antagonists[2]. It is, however, most likely that the structural features that favour high AOA potency are unrelated to those favouring histamine release[2,3].

Recently, several reports have appeared on attempts to reduce histamine release by modifications and substitutitions in the critical positions[4-8].

We have developed an antagonist, N-Ac-D-2-Nal[1],D-pClPhe[2],D-3-Pal[3], NicLys[5], D-NicLys[6], ILys[8], D-Ala[10])-LHRH, which released negligible histamine[9]. The AOA of this analog, which was named Antide, was 36% at 0.5 ug and 100% at 1

ug. Antide is presently being pharmacologically evaluated for possible clinical studies. Replacement of NicLys^5, D-NicLys^6 by PicLys^5, D-PicLys^6, increased the AOA two-fold to 40% at 0.25 ug and 100% at 0.5 ug. The ED_{50} for histamine release decreased from > 300 to 93 + 11[9] (analog 12), respectively, which includes variability in the histamine assay. Recently, we reported that substitution of Leu in analog 12 by alloisoleucine, Val or Abu (analog 16) resulted in ED_{50}'s of > 300, 213 + 30 and 273 + 27 respectively[10]. In the same paper, we reported on a substantial increase in AOA, when D-PicLys^6 of analog 12 was replaced by an acylated cis-4-aminocyclohexylalanine, c-D-PzACAla. The resulting analog, (N-Ac-D-2-Nal^1, D-pClPhe^2, D-3-Pal^3, PicLys^5, c-PzACAla^6, ILys^8, D-Ala^{10})-LHRH, (analog), showed 73% AOA at 0.25 ug and 29% at 0.125 ug. The ED_{50} was 28 + 7.5.

We now describe some results on further variations in positions 1,2,3,5,6,7,8 of analogs with acylated aminocyclohexylalanines or acylated Lys^5 / D-Lys^6 residues.

Experimental

MATERIALS: The natural amino acids were obtained from Peninsula Laboratories, San Carlos, CA. BOC-D-2-Nal, BOC-D-pClPhe, BOC-D-3-Pal and BOC-ILys(Z) dicyclohexylammonium salt were provided by the courtesy of Dr Narasimha Rao of the Southwest Foundation for Biomedical Research, San Antonio, TX. The Abu and Cit were obtained from Sigma Chemical Co., St. Louis, Mo. They were converted to the Boc derivatives using di-t-butyl dicarbonate as described[11]. The unnatural amino acids were prepared using standard procedures. The hydroxyl groups of Ser and HOBLys were protected as benzyl derivatives and the guanidino group of Arg as the tosyl derivative. The benzhydrylamine resin was from Beckman Bioproducts, Palo Alto, CA. The methylene chloride was distilled from sodium carbonate. The dicyclohexylcarbodiimide was from Aldrich Chemical Co., Milwaukee, Wis. and was distilled *in vacuo* before use. All other solvents and reagents were reagent grade.

SYNTHESIS: The peptides were synthesized by the solid-phase method on benzhydrylamine resin using a Beckman 990 peptide synthesizer, as described[12].

PURIFICATION AND PURITY: Two methods of purification have been used. The first method consisted of chromatography on silica gel (EM 9385) with the solvent system n-butanol-acetic acid-water 4:1:2 or 4:1:5 upper phase followed by gelfiltration on Sephadex G 25 with 6% acetic acid as the eluant. The second method employed gelfiltration as above followed by chromatography on Sephadex LH 20 with the solvent system n-butanol-acetic acid-water-methanol 10:10:90:8. If a peptide was not sufficiently pure by either of these two methods, final purification was effected by preparative HPLC, as described[12].

The purified peptides showed single spots on TLC on EM 0.25 mm silica gel plates in at least four of the following solvent systems: 1:n-butanol-pyridine-acetic acid-water = 4:1:1:2, 2: n-butanol-acetic acid-water = 4:1:2, 3: n-butanol-pyridine-acetic acid-water = 40:1:10:20, 4: n-butanol-pyridine-acetic acid-water = 5:3.3:1:4, 5: n-butanol-pyridine-acetic acid-water = 30:10:3:12 and 6: ethyl acetate-pyridine-acetic acid-water = 5:5:1:3. The TLC data are in Table I.

The purity was further checked by analytical HPLC using a Waters instrument with a 660 solvent programmer and a Vydac C_{18} column. Solvent A was 0.01M KH_2PO_4 adjusted to pH 3 with phosphoric acid and solvent B was 80% acetonitrile and 20%A. Different linear gradients of increasing amounts of B

were used. The flow rate was 1.5 m./min and the absorbance was measured at 210 nm. The purities of the peptides was estimated at 97-99%. The HPLC data are in Table I.

Amino acid analyses were carried out on a Beckman 118 CL Amino Acid Analyzer following standard procedures[12]. The results were in agreement with theory within the limits of experimental error (data not shown). Unnatural amino acids were detected qualitatively.

BIOLOGICAL ASSAYS: The AOA was determined in rats as previously reported[13]. The wheal area was calculated as described[9]. The in vitro histamine release test in rat mast cells was performed, as described [14,15] and the results are reported as ED_{50} values which is the concentration in ug/ml that releases 50% of the total releasable histamine.

TABLE I. CHROMATOGRAPHIC DATA

	HPLC		TLC					
	Gradient	Ret. time						
#	% CH_3CN	min	R_f1	R_f2	R_f3	R_f4	R_f5	R_f6
1.	32 to 48/15 min*	7.6	0.73	0.39	0.50	0.81	0.60	
2.	24 to 80/15 min	8.1	0.76	0.42	0.43	0.76	0.64	
3.	"	8.0	0.64	0.47	0.46	0.79	0.68	
4.	"	7.8	0.72	0.40	0.43	0.78	0.63	
5.	"**	8.0	0.76	0.39		0.73	0.64	
6.	24 to 64/20 min***	10.4	0.70	0.40			0.54	0.76
7.	24 to 80/15 min	8.4	0.72	0.39	0.43	0.78	0.62	
8.	"	7.8	0.70	0.40	0.44	0.76	0.58	
9.	24 to 64/20 min	11.6	0.73	0.43			0.57	0.77
10.	24 to 80/15 min	8.2	0.74	0.38	0.41	0.76	0.63	
11.	"**	7.5	0.71	0.34		0.69	0.58	
12.	24 to 64/20 min	14.0	0.75	0.40			0.61	0.85
13.	"	14.6	0.76	0.42			0.61	0.78
14.	"	15.1	0.77	0.48			0.68	0.84
15.	"	12.7	0.67	0.36			0.52	0.80
16.	"	10.0	0.72	0.39			0.54	0.80
17.	"	8.7	0.63	0.34			0.45	0.76
18.	"	11.1	0.69	0.40			0.55	0.76
19.	"	13.6	0.77	0.41			0.58	0.79
20.	24 to 80/15 min	9.8	0.77	0.42	0.43	0.77	0.66	
21.	24 to 64/20 min	12.5	0.75	0.39			0.63	0.79
22.	"	12.2	0.74	0.41			0.62	0.79
23.	24 to 80/15 min*	7.8	0.73	0.39	0.46	0.77	0.61	
24.	24 to 64/20 min	9.0	0.65	0.39			0.51	0.79

* Flow rate 2 ml/min
** Flow rate 1.8 ml/min
*** Solvent A = 0.1% trifluoroacetic acid; solvent B = 0.1% trifluoroacetic acid in 80% aqueous acetonitrile

The data from the determinations of AOA, wheal area and ED_{50} are in Table II.

Results and Discussion

Policy of Design. Table II consists of analog 1[10] and eight analogs in which one residue has been exchanged in the sequence of 1.

Table II. ANALOGS WITH ACYLATED AMINOCYCLOHEXYLALANINES. SEQUENCE: N-Ac-D-2-Nal[1], DpClPhe2, ( )3, Ser4, ( )5, ( )6, ( )7, ( )8, Pro9,D-Ala-NH210.

#	3	5	6	7	8	AOA %/ug 0.125	0.25	0.5	In Vitro Histamine Release ED50+SEM ug/ml	Wheal area mm2/10 ug
1.*	D-3-Pal	PicLys	c-D-PzACAla	Leu	Ilys	29	73	100	28+7.5	122.8+5.7
2.	D-PzAla	"	"	"	"	-	22	100		127.8+4.9
3.	D-TinGly	"	"	"	"	-	0	-		84.6+3.9
4.	D-3-Pal	c-PzACAla	"	"	"	-	57	100	33+1.4	115.5+2.4
5.	"	Tyr	"	"	"	-	22	11		95.0+0
6.	"	PicLys	c-D-PmACAla	"	"	-	-	9		120.4+4.7
7.	"	"	c-D-PzACAla	Val	"	14	73	100	84+5.6	127.8+4.9
8.	"	"	"	Phe	"	-	0	-		116.2+3,2
9.	"	"	"	Leu	Arg	-	14	-		119.7+8.5
10.	"	c-PzACAla	D-PicLys	"	Ilys	-	64	90	49+4.8	99.5+4.5
11.	"	"	"	Val	"	-	12	-		103.9+6.2

*From reference 10

Compounds 2 and 3 have D-PzAla and D-TinGly, respectively, instead of D-3-Pal in position 3. Both analogs showed substantial loss of antiovulatory activity, from 73 to 22 and 0% respectively at 0.25 ug. As was observed earlier[9], balanced basicity/lipophilicity is essential in this series of antagonists. D-PzAla has a pyrazine ring instead of a pyridine ring in the side chain. The pK_a values for the conjugate acids of pyridine and pyrazine are 5.2 and 0.6 respectively[16] which means that D-PzAla is an extremely weak base, much weaker than D-3-Pal, but since it has two ring nitrogens it should be more hydrophilic than D-3-Pal. It may be that D-3-Pal has just the right balance of basicity/hydrophilicity. On the other hand, the very lipophilic D-TinGly residue is clearly unsuitable. Most often, in analogs with very lipophilic residues in position 3, notably D-Trp, the lipophilicity is balanced by $D\text{-}Arg^6$ and/or Arg^8 which confer hydrophilicity[17]. The wheal areas of analog 3, 84.6 compared to 122.8 for the $D\text{-}3\text{-}Pal^3$ analog is noteworthy.

Analogs 4 and 5 have $PicLys^5$ replaced by $\underline{c}\text{-}D\text{-}PzACAla^5$ and the natural Tyr^5 respectively. Interestingly, analog 4 retained substantial activity, 57% AOA at 0.25 ug in spite of the relative closeness of the large, rigid, acylated aminocyclohexane rings. This may be considered further evidence for a B-turn

involving residues 5-8[18] since in such a conformation the two sterically demanding side chains of residues 5 and 6 would occupy different sides of the backbone with residue 6 in the D configuration. The anaphylactoid activity of analog 4 are essentially the same as for analog 1.

Substitution of PicLys^5 in analog 1 for Tyr^5 (analog 5), diminished potency, from 73 to 22% at 0.25 ug. This result is in contrast to the case for Antide where substitution of Tyr^5 gave an equipotent analog[9]. The interaction between the electron poor pyrazine ring of c-D-PzACAla^6 and the electron-rich phenolic side chain of Tyr^5 possibly causes unfavourable changes in conformation.

Exchange of c-D-PzACAla^6 for the isomeric c-D-PmACAla^6 (analog 6) led to a decrease in potency, from 100 to 9% AOA at 0.5 ug. This is remarkable since the only structrual difference between analogs 1 and 6 is a 1,3 relationship instead of a 1,4 relationship of the ring nitrogens of the heterocyclic acyl group in position 6. The pK_a for the conjugate acid of pyrimidine is 1.3[16], i.e. it is a very weak base, like pyrazine. The basicity of these heterocycles is further reduced in the residues by the presence of the CONH grouping on the rings. It is thus likely that these acylated aminocyclohexylalanines act as neutral hydrophilic sites capable of hydrogen bonding. It may be that the direction in which hydrogen bonding can take place is important and that this is the reason for the low activity of analog 6.

Analogs 7 and 8 have Val^7 and Phe^7 instead of the natural Leu^7. The first analog retained AOA at 0.5 and 0.25 ug (100 and 73%) while the second analog was inactive at 0.25 ug. This parallels earlier results with the PicLys^5, D-PicLys^6 analog, where Val^7 substitution gave an equipotent analog and Trp^7 subtstitution gave a peptide with very low activity[10]. Aromatic residues in position 7 do not seem to be effective in this series of peptides. In antagonists with D-Arg^6 , Arg^8, Trp^7, and Phe^7 substitutions gave analogs with 90 and 33%, respectively, at 0.25 ug[12]. A type of interaction similar to the one suggested for analog 5 could be invoked also for these analogs.

The Val^7 substitution caused an increase in ED_{50} for *in vitro* histamine release by a factor 3, from 28 to 84 ug/ml, which was also the case for the PicLys^5, D-PicLys^6 analog[10].

Replacement of ILys^8 by Arg^8 diminished potency to a large degree, from 73 to 14% at 0.25 ug (analog 9). ILys is usually the better substituent in position 8.

Compound 10 has c-PzACAla^5, D-PicLys^6, that is residues 5 and 6 are reversed in relation to analog 1. Analog 10 is equipotent with analog 1[10]. Unexpectedly, considering earlier results, exchange of Leu^7 for Val^7 in this peptide caused a large decrease in potency, from 64 to 12% at 0.25 ug (analog 11).

The first four peptides in Table III are the earlier reported[9] analog 12 and three analogs with variations in positions 1,2 and 3, all showing reduced potency.

TABLE III. ANALOGS WITH ACYLATED Lys^5 AND/OR $D\text{-}Lys^6$ RESIDUES. SEQUENCE: $(\ )^1, (\ )^2, (\ )^3, Ser^4, (\ )^5, (\ )^6, (\ )^7, (\ )^8, Pro^9, D\text{-}Ala\text{-}NH_2{}^{10}$.

#	1	2	3	5	6	7	8	AOA %/ug 0.25	0.5	1.0
12*	N-Ac-D-2-Nal	DpClPhe	D-3-Pal	PicLys	D-PicLys	Leu	ILys	40	100	90
13.	N-Ac-D-Cl_2Phe	"	"	"	"	"	"	-	38	-
14.	N-Ac-D-2-Nal	D-Cl_2Phe	"	"	"	"	"	-	64	90
15.	"	DpClPhe	D-Trp	"	"	"	"	-	20	-
16**	"	"	D-3-Pal	"	"	Abu	"	36	100	-
17.	"	"	"	HOBLys	"	"	"	11	-	-
18.	"	"	"	Cit	"	"	"	11	-	-
19.	"	"	"	PicLys	"	"	Arg	50	88	-
20**	"	"	"	"	"	Leu	IOrn	50	90	100
21.	"	"	D-PzAla	"	"	"	"	-	75	-
22.	"	D-3-Pal	DpClPhe	"	"	"	"	12	-	-
23.	"	DpClPhe	D-3-Pal	Tyr	"	"	"	0	-	-
24.	"	"	"	PzcLys	D-PzcLys	"	ILys	-	17	-
25.	"	"	"	PmcLys	D-PmcLys	"	"	-	0	-

*From reference 9
**From reference 10

In Vitro Hist. Rel. ED_{50}±SEM ug/ml	In vivo wheal area mm^2/10 ug
12. 93±11	123.0± 0
13.	116.2±3.2
14.	139.8±7.1
15.	71.0±4.3
16. 237±27	91.0±5.4
17.	95.1±5.0
18.	89.5±5.5
19. 27±3.3	113.2±5.4
20. 42±3.1	113.0±0
21.	103.9±3.7
22.	116.2±5.5
23.	113.2±5.5
24. 288±30	110.2±8.1
25.	130.2±2.5

ABBREVIATIONS FOR THE UNNATURAL AMINO ACIDS
Abu = 2-aminobutyric acid
Cit = citrulline
Cl_2Phe = 3-(3,4-dichlorophenyl)alanine
HOBLys = Nε-(4-hydroxybenzoyl)lysine
ILys = Nε-isopropyllysine
3-Pal = 3-(3-pyridyl)alanine
pClPhe = 3-(4-chlorophenyl)alanine
PicLys = Nε-picoloyllysine
PmACAla = 3-(4-(4-pyrimidylcarbonyl)-aminocyclohexyl)alanine
PmcLys = Nε-(4-pyrimidylcarbonyl) lysine
PzACAla = 3-(4-pyrazinylcarbonyl-aminocyclohexyl)alanine
PzAla = 3-pyrazinylalanine
PzcLys = Nε-pyrazinylcarbonyllysine
TinGly = 2-thienylglycine

If D-Nal[1] is replaced by D-Cl_2Phe[1] (analog 13), the activity decreases from 100 to 38% AOA at 0.5 ug. D-Cl_2Phe is somewhat less sterically demanding than D-Nal and this might be the reason for the lower activity. Another concept is the lower electron density in the aromatic ring of D-Cl_2Phe compared to that of D-Nal.

If D-Cl_2Phe is placed in position 2 to replace D-pClPhe, the decrease in AOA is moderate, to 64% at 0.5 ug (analog 14). This is not surprising considering the similarity between pClPhe and Cl_2Phe. Evidently, decreasing the electron density in the N-terminal leads to lower potency. A Tyr and a Trp residue have both been proposed to be important in the active site of the LHRH receptor[19] and it seemed that decreasing the electron density in the aromatic rings would enhance pi-pi interaction between the antagonist and the electron rich Trp/Tyr residues in the receptor. Steric factors may, however, also be important.

Replacement of D-3-Pal by D-Trp in position 3 lowers potency considerably. Analog 15 has only 20% AOA at 0.5 ug. This peptide is most likely too lipophilic (cf. analog 3, Table II). It seems that in this series of antagonists, D-3-Pal is effective to achieve balanced lipophilicity. The very low wheal area value of 71.0 $\pm$ 4.3 of this analog is noted.

Compounds 17-19 are based upon analog 16[10]. In analog 17, PicLys^5 has been replaced by HOBLys^5, which can be considered an extended Tyr with a more acidic phenolic hydroxyl group. In compound 18, PicLys^5 has been replaced by Cit^5 which has been used by Bajusz *et al.*[8]. Both of these analogs showed markedly reduced potency, 11% at 0.25 ug as compared to 40% for the parent compound.

Analog 19 has Arg in position 8 instead of ILys. This analog retained its activity compared to the parent analog, 50 vs 40% at 0.25 ug. This is noteworthy in view of the results for analog 9, Table II. The ED_{50} value for histamine release decreased tenfold upon replacement of ILys^8 by Arg^8, which is in accordance with predictions.

Compounds 21-23 are all based on analog 20[10], which is a potent analog having IOrn in position 8.

Replacing D-3-Pal^3 by D-PzAla^3 (analog 21) caused only a minor decrease in activity, from 90 to 75% at 0.5 ug. This may mean that D-PzAla could be useful provided other changes are made in the sequence.

Since LHRH has the weakly basic His in position 2 and the lipophilic Trp in position 3, it was worthwhile to reverse the order of DpClPhe^2 and D-3-Pal^3 in order to have the basic and lipophilic residues in the same position as in LHRH. The resulting analog, 22, showed, however, greatly reduced activity, 12% at 0.25 ug. This is in accordance with a statement by Hocart *et al.* that position 2 requires a hydrophobic residue[20].

Analog 23 has Tyr^5 instead of PicLys^5. This change surprisingly caused loss of activity, 0% at 0.25 ug. The effect of this substitution was even more pronounced than for analog 5, Table II. It may be noted that for the analogs with NicLys^5,D-NicLys^6 replacement of NicLys^5 by Tyr gave an equipotent or

slightly more potent analog if ILys was in position 8 (45 vs 36% at 0.5 ug) whereas the same substitution with IOrn[8] caused a decrease in AOA from 88 to 22% at the same dose level[9].

The last analogs in Table III, 24 and 25 feature Lys[5],D-Lys[6] acylated by pyrazinylcarbonyl and 4-pyrimidinylcarbonyl groups. Both peptides are considerably less active than antagonists with acyl groups containing one nitrogen atom[9]. Analog 24 had 17% AOA at 0.5 ug and analog 25 was inactive at this dosage. Once again it is evident that the pyrimidine ring confers some property that is detrimental to antagonistic potency. The ED_{50} value of 288 ± 30 for analog 24 is noteworthy.

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