Betidamino Acid Scan of the GnRH Antagonist Acyline†

Guangcheng Jiang,‡ Charleen Miller, Steven C. Koerber, John Porter,§ A. Grey Craig, Sheela Bhattacharjee,|| Patricia Kraft,|| Thomas P. Burris,|| Carolyn A. Campen,|| Catherine L. Rivier, and Jean E. Rivier*

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, Ferring Research Institute Inc., Bldg 2, Rm 434, 3550 General Atomics Court, San Diego, California 92121, Alanex Corp., 3550 General Atomics Court, San Diego, California 92121, R. W. Johnson Pharmaceutical Research Institute, 1000 Rt. P.O. Box 300, Raritan, New Jersey 08869

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Strong clinical evidence suggests that GnRH antagonists will replace GnRH agonists in a number of indications because of their ability to inhibit gonadotropin secretion as long as an adequate concentration of the analogue is present in the circulation whereas superagonists will take approximately 2 weeks to desensitize the gonadotrophs. Until recently, antagonists were either too weak and/or would release histamine. Azaline B {[Ac-D2Nal[1],D4Cpa[2],D3Pal[3],4Aph[5](atz),D4Aph[6](atz),ILys[8],DAla[10]]GnRH} and long-acting members of the azaline family {Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(X)-D4Aph(Y)-Leu-ILys-Pro-DAla-NH_2}, however, appear to be promising drug candidates. Because these antagonists tend to form gels (due to the formation of β-sheet structures) and, as a result, are not readily amenable to formulation for long-term delivery, we have investigated ways of increasing hydrophilicity while retaining high potency and lack of histamine releasing activity. Betidamino acids (a contraction of "**bet**a" position and "am**ide**") are N'-monoacylated (optionally, N'-monoacylated and N-mono- or N,N'-dialkylated) aminoglycine derivatives in which each N'-acyl/alkyl group may mimic naturally occurring amino acid side chains or introduce novel functionalities. We have used unresolved N^{α}-Boc,N'^{α}-Fmoc-aminoglycine, and N^{α}-Boc,N'^{α}-(CH_3)Fmoc-aminoglycine as templates for the introduction of betidamino acids in acyline (Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Ac)-D4Aph(Ac)-Leu-Ilys-Pro-DAla-NH_2), a long acting member of the azaline B family, to test biocompatibility of these betide derivatives. Diastereomeric peptides could be separated using RP-HPLC in most cases. Biological results obtained in vitro (binding affinity to rat pituitary gland membranes) and in vivo (rat antiovulatory assay, AOA) indicate in most cases small differences in relative potencies (<5-fold) between the D- and L-nonalkylated betidamino acid-containing acylines. Importantly, most betide diastereomers have high affinity for the GnRH receptor and were equipotent with acyline in the AOA. Greater differences in affinity and potency between diastereomers were observed after introduction of a methyl group on the side chain nitrogen ("beta" position) of the same analogues, with one of the diastereomer having an affinity and a potency in the AOA equivalent to that of acyline. These results suggest that chirality at the α-carbon coupled to side chain orientation is important for receptor recognition. The duration of action of some of the most potent analogues was also determined in the castrated male rat in order to measure the extent (efficacy and duration of action) of inhibition of luteinizing hormone release. Data suggest that introduction of a betidamino acid results in reduction of duration of action. Also, introduction of betidamino acids results in peptides with increased hydrophilicity (as determined by elution times on C_{18} silicas at pH 7.3) compared to that of the parent compound. N'-Methyl substitution results in parallel increase in retention times on C_{18} silicas as expected.

Introduction

GnRH antagonists are now recognized as potential drugs for the management of sex steroid-dependent pathophysiologies including cancers of the breast and prostate, induction of ovulation, and male contraception.[1−5] Currently, most available long-term studies were carried out with the Nal-Glu antagonist {[Ac-D2Nal[1],D4Cpa[2],D3Pal[3],Arg[5],4-(*p*-methoxybenzoyl)-D-2-Abu[6],DAla[10]]GnRH}[6] and more recently cetrorelix {[Ac-D2Nal[1],D4Cpa[2],D3Pal[3],DCit[6],DAla[10]]GnRH}[6,7] which, although very potent in inhibiting gonadotropin secretion, also stimulate the release of histamine in vitro (ED_{50} ca. 1.8 and 3.5 μg/mL respectively).[4] Whereas the Nal-Glu antagonist is relatively short acting, Cetrorelix is unexpectedly long acting when compared to similar analogues with an aromatic ring at position 6.[8] These preliminary studies, however, suggest that a GnRH antagonist will ultimately be used to transiently inhibit

* To whom reprint requests should be addressed.

† Abbreviations: The abbreviations for amino acids are in accord with the recommendations of the IUPAC−IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9−37). Betidamino acids, where the corresponding amino acids (Xaa) are known, are abbreviated bXaa or otherwise defined. Additional abbreviations: b2Nal, Agl(2-naphthoyl); Apc, 3-amino-4-pyrazolecarboxylic acid; 2Nal, 2-naphthylalanine; b3Pal, Agl(nicotinoyl); 3Pal, 3-pyridylalanine; 4Aph, 4-aminophenylalanine; b4Aph, Agl(4-aminobenzoyl); b4Cpa, Agl(4-Cl-benzoyl); 4Cpa, 4-chlorophenylalanine; Ac, acetyl; Agl, aminoglycine; AOA, antiovulatory assay; bAla, Agl(formyl); bnArg, Agl(guanidinoacetyl); bIOrn, Agl(isopropyl-β-Ala); bTyr, Agl(4-hydroxybenzoyl); Boc = Me_3CO_2C; CZE, capillary zone electrophoresis; DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; GnRH, gonadotropin releasing hormone; bhSer, Agl(hydroxyacetic acid); IC_{50}, inhibitory concentration for 50% response; ILys, N^{ϵ}-isopropylysine; LSIMS, liquid secondary ion mass spectrometry; Pca, 2-pyrazinecarboxylic acid; SAR, structure activity relationships; SPPS, solid-phase peptide synthesis; TBTU, *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid.

‡ Ferring Research Institute, Inc.
§ Alanex Corp.
|| R. W. Johnson Pharmaceutical Research Institute.

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gonadotropin secretion as is needed for example in in vitro fertilization protocols or for the diagnosis of gonadotropin-dependent gonadal dysfunction. Furthermore, a potent and safe preparation of a GnRH antagonist may be found advantageous for chronic administration in the management/treatment of endometriosis, precocious puberty, uterine myoma, ovarian hyperandrogenism and hirsutism, premenstrual syndrome, and breast and gynecological cancers.[5] Most of these disorders were originally studied and are presently treated with long-acting formulations of the superagonists which desensitize the gonadotrophs after an undesired stimulatory phase that lasts approximately 2 weeks. These formulations, that may last as long as 3 months, were made possible for essentially two reasons: relatively small quantities of the superagonist need to be delivered (<5 mg for 1 month treatment), and the superagonists are readily bioavailable and very water soluble even in the presence of physiological concentration of salts. Doses of GnRH antagonists, as we know them now, need to be significantly larger (≥10-fold), and these peptides are much less soluble with a tendency to form gels and are therefore difficult to formulate.

Whereas it became apparent that opportunities to improve potency and duration of action were relatively limited, we pursued an approach directed at increasing water solubility and decreasing the propensity of these analogues to form less soluble gels resulting from the formation of beta sheets.[9] The effect on biological activity of the introduction of betidamino acids and methylated betidamino acids[10] suspected to increase solubility was therefore investigated. Betidamino acids are *N′*-monoacylated (optionally, *N′*-monoacylated and *N*-mono- or *N*,*N′*-dialkylated) aminoglycine derivatives in which each *N′*-acyl/alkyl group may mimic naturally occurring amino acid side chains or introduce novel functionalities. α-Aminoglycine and α,α-diamino carboxylic acids have been employed frequently in the construction of retro-inverso peptides to increase proteolytic stability of peptide-derived drugs, as building blocks in heterocyclic synthesis and in the study of peptide delivery systems.[11−13] The description of the synthesis of racemic N^{α}-Fmoc, $N^{\alpha'}$-Boc-aminoglycine by Qasmi et al.[14] was key to the development of the concept of using one of the two nitrogen as a functional group onto which we would graft an acyl group mimicking an amino acid side chain. Acyline was selected as the parent compound in this study because it is the most accessible member of the azaline B family with long duration of action (identical with that of azaline B) in the castrated male rat assay.[15] Peptides were tested both in vitro for affinity and in vivo for efficacy in the AOA and duration of action.

Synthesis, Purification and Chemical Characterization (Table 1).

We have described synthetic pathways to orthogonally protected betidamino acid and methylbetidamino acid scaffolds (*N′*-monoacylated aminoglycine derivatives)[10,16,17] for solid-phase peptide synthesis and their use in the design of selected bioactive GnRH analogues. In short, $FmocNR^1CH(NR^2Boc)CO_2H$ (R^1 = H, Me; R^2 = H, Me), useful as templates for the introduction of desired functionalities into peptides, were prepared by condensation of glyoxylic acid and carbamates Fmoc-NH−R^1 (R^1 = H, Me), followed by conversion with Me_2-CHSH of the resulting $FmocNR^1CH(OH)CO_2H$ to $FmocNR^1CH(SCHMe_2)CO_2H$ (R^1 = H, Me) which were further treated with Boc-NHR^2 (R^2 = H, Me) in the presence of *N*-bromosuccinimide[17] to give the desired products.

Analogues shown in Table 1 were synthesized by the SPPS methodology on a *p*-methylbenzhydrylamine resin (MBHA-resin) using protocols previously described.[15] In most cases, analogues were obtained by acylation (acid anhydrides, active esters or acid chlorides depending on availability) of the side chain of the aminoglycine (Agl) or methylaminoglycine (Me, Agl) on the partially deprotected (removal of the Fmoc protection), fully built peptido-resin. We found this procedure to give relatively pure crude preparations of the peptides and to be simpler than synthesizing separately the desired betidamino acids prior to their introduction in the elongating peptide chain. The protected peptide-resins were cleaved in anhydrous HF in the presence of a scavenger (anisole), precipitated, extracted, and lyophilized. The crude peptides were purified by reversed-phase HPLC.[18] Introduction of the isopropyl group on β-amino of β-alanine (used for **39** and **40**) was achieved via a reductive isopropylation reaction as previously described,[15] starting from β-alanine, acetone, acetic acid, and H_2 in the presence of Pd/C as a catalyst, and followed by the Z-protection of the resulting secondary amino function. The analytical techniques used for the characterization of the analogues included HPLC with two different solvent systems (acidic and neutral), optical rotation (when diastereomers could be separated), CZE, and LSIMS. Results from these studies support the identity of the intended structures (Table 1). Diastereomers when isolated were generally greater than 95% pure. When we encountered difficulties associated with their separation on a preparative scale, the ratio of the two diastereomers could be determined and the purification carried out in such a way as to yield an approximate 1:1 mixture of the two compounds in order to get consistent/interpretable biological results (see Table 1 for actual ratios in the column entitled "purity"). Compounds **2**, **15**, and **43** were synthesized for comparison purposes as they are diastereomers of **1**.

Results and Discussion

The structural preferences of betidamino acids and their corresponding mono- and bismethylated derivatives (as compared to those of amino acids and β-methyl amino acids) were investigated using molecular mechanics in combination with a continuum solvation model[19] to calculate the total energy of the Ac-Xaa-methyl amides as a function of backbone dihedral angle.[10] We describe here a systematic study whereby each residue of acyline (Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Ac)-D4Aph(Ac)-Leu-ILys-Pro-DAla-NH_2) (except for proline at position 9) was substituted by its corresponding betidamino acid or closely related betidamino acid. The use of an unlimited number of betidamino acids that can be readily obtained by substitution of the aminoglycine scaffold and its mono- or dialkylated derivatives with acylating and other reactive agents needed broad validation as a general approach to SAR. Preliminary results have been presented elsewhere.[10,16,20] Because a number of amino acids in acyline are unnatural and not readily available, we thought that a scan of acyline with the corresponding betidamino acids, *N′*-monomethyl betidamino acids and other closely

related structures, would be a good testing ground for this new technology. One of the main considerations was also to obtain one or more GnRH analogues that would be equivalent in terms of low histamine release, and better than azaline B or acyline in terms of biological (more potent and longer acting) and physicochemical properties (more soluble in aqueous buffers). Earlier encouraging results leading to the discovery of azaline B and acyline in fact paved the way for the present study,[15] in that unpredictable results had been obtained with respect to the effect of the composition of the acyl group of the amino functions of 4Aph and D4Aph at positions 5 and 6, respectively, on duration of action. We wished to further explore the effect of side chain diversity in modulating biological activities.

Compounds **3**−**14**, **16**−**36**, **38**−**42**, **44**−**48** (Table 1), all containing an aminoglycine or methylated aminoglycine residue (betidamino acid), are resolved diastereomers or 50/50 mixtures of diastereomers. When a methylaminoglycine derivative is present, the methyl group is located on the same nitrogen as the acyl group mimicking the side chain of the corresponding amino acids. Compounds **2**, **15**, and **43** were synthesized for comparison purposes to further define the chiral requirements at positions 1, 3, and 10, respectively. Clearly, introduction of an L-residue at position 1 and 3 leads to a significant decrease in potency (compare AOA results for **2** and **15** with those for **1**) whereas chiral inversion has minimal effect at position 10 (compare the AOA results for **43** with those for **1**), as expected from earlier studies[21] and further documented here (peptides **44**−**48** with D/L-Agl, D/L-Agl(Me), D- or L-Agl(For) and D/L-Agl(Me,For) at position 10. This will be discussed further later in the context of position 10 substitutions and their effect on potency.

Data presented in Table 1 are of unusual interest for several reasons which will be identified first and then further discussed in detail.

Observation Number 1. Substitution of each residue of acyline (with the exception of proline at residue 9 which was not synthesized) by the corresponding betidamino acid yields in most cases at least one diastereomer that is equipotent with acyline in the AOA. (See **3**, **17**, **22**, **30**, **36**, **44**, **46**.)

Observation Number 2. Substitution of each residue of acyline by the corresponding *N*′-methylated betidamino acid yields in most cases at least one diastereomer that is equipotent with acyline in the AOA. (See **5**, **19**, **25**, **29**, **38**, **40**, **48**.)

Observation Number 3. One of the betidamino containing diastereomeric pair is equipotent with acyline and the other is generally at most one-fifth to one-half as potent (see **4**, **16**, **23**, **47**) whereas available results with regular amino acids are more likely to show that the corresponding pairs of regular analogues are only one-fifth to one tenth as potent. (See **2**, **15**.)

Observation Number 4. Whereas one of the *N*′-methylated betidamino acid containing diastereomers is equipotent with acyline, the other is, in general significantly less potent (see **6**, **18**, **24**, **32**, **41**), suggesting that a methyl group on the side chain of betidamino acids introduces conformational constraints similar in their effects to what is seen with equivalent amino acids.

While it is understood that these results compare activities in vivo where other factors (i.e. distribution/depot formation, degradation, and elimination) than those pertaining solely to the receptor ligand interaction such as affinity and occupancy are not taken into consideration, it is important to note that there is a relatively good correlation between high affinity for membranes derived from rat pituitary and in vivo potency in the AOA.[22] All compounds cited above that are comparable in efficacy in the AOA with acyline have affinities that are also comparable with that of acyline. Two analogues with position 7 substitutions (**34** and **35**) also have high affinities that are consistent with their in vivo potency which are at most 5 times less than that of acyline.

Analysis of each entry of Table 1 shows that **2** (the diastereomer of **1**) is one-tenth as efficient at inhibiting ovulation in the rat, since it takes approximately 10 times more of **2** as of **1** to achieve the same level of inhibition (100%). Whereas the observation that chiral inversion of an amino acid from L to D in a bioactive peptide will generally drastically lower its potency,[23,24] there are also cases where increases in potency were observed;[24−26] these were explained in terms of β-turn stabilization and/or stabilization against enzymatic degradation. It is clear that the D residues in acyline at positions 1, 2, 3, 6, and 10 have been selected as being those that favored increased affinity and potency. It is therefore expected that inversion from the D- to the L-isomer will result in loss of potency and affinity at these positions. Remarkably the difference in potency upon incorporation of either D- or L-b2Nal at position 1 (diastereomers **3** and **4**) is only 2-fold with a 4-fold increase in IC_{50}. We have suggested on the basis of molecular modeling calculations that the side chains of betidamino acids explore a larger conformational volume than do the side chains of equivalent amino acids, resulting in a greater overlap of the volume spanned by D- and L-betidamino acids. If this expanded volume coincides with that recognized by the receptor (i.e. is part of the pharmacophore), we would expect both diastereomers to have similar potencies (see observation 1 above). Methylation of the side chain nitrogen (as in **5** and **6**) does not seem to influence the overall efficacy of one of the two diastereomers (**5**, 75% inhibition of ovulation at 1.0 μg and full inhibition of ovulation at 2.5 μg, observation 2), whereas the other has drastically reduced potency (100% inhibition of ovulation at 25 μg, observation 4); similarly there is an almost 30-fold increase in IC_{50} (0.3 versus 9.2 nM, respectively). This suggests that introduction of the *N*′-methyl group at D- or L-b2Nal differentially impairs side chain mobility of one diastereomer. Examination of molecular models of analogues containing Ac(D- or L)-b2Nal at position 1, with and without *N*′-methylation, which were constructed by homology to the structures of cyclic (4−10) and dicyclic (4−10,5−8) antagonists of GnRH[27,28] suggests several possible explanations for a selective modulation of the structure. In the L-b(Me)2Nal model, the *N*′-methyl function could interfere sterically with placement of the D3Pal side chain in position 3. Conversely, in the D-b(Me)2Nal model, steric interactions of the *N*′-methyl with either of the backbone amide proton or carbonyl of that residue could be accommodated by slight rotations of the ϕ and ψ angles of residue 1, respectively, resulting in negligible perturbation in the positioning of the naphthalene side chain as well as that of the rest of the molecule. This would be compatible with the biological results that were obtained if we were

Table 1. Physicochemical Properties and Selected Biological Activities of Members of a Betide Scan of Acyline

no.	compound[a]	$[\alpha]^{20}_D$[b]	Δt_R[c]	purity[d]	MS[e]	AOA[f] dose (μg)	AOA[f] ov.rat/ total	IC_{50}[g] (nM)	duration of action[h]
1	[Ac-D2Nal[1],D4Cpa[2],D3Pal[3],4Aph[5](Ac),D4Aph[6](Ac),ILys[8],DAla[10]]GnRH (acyline)	−38	0	>97	1532.7	2.5 1.0 0.5	(0/7) (5/13) (6/7)	0.3	long acting
2	[Ac-L-2Nal[1]]acyline	−19	+2.4	>97	1532.9	25.0 10.0	(0/3) (2/6)	0.64	
3	[Ac-D or LAgl (2-naphthoyl)[1]]acyline	−31	−2.8	96	1561.8	2.5 1.0	(0/8) (2/7)	3.0	intermediate
4	[Ac-L or DAgl (2-naphthoyl)[1]]acyline	−1.2	−1.5	93	1561.7	5.0 2.5	(0/8) (5/5)	0.88	
5	[Ac-D or LAgl (Me,2-naphthoyl)[1]]acyline	−44	−2.0	>97	1576.04	2.5 1.0	(0/8) (2/8)	0.30	very short
6	[Ac-L or DAgl (Me,2-naphthoyl)[1]]acyline	+14	−0.2	>97	1576.02	25.0 10.0	(0/8) (78)	9.2	
7	[D or LAgl (4-Cl-benzoyl)[2]]acyline	−28	−1.1	88	1561.8	50	(7/8)	NA	
8	[L or DAgl (4-Cl-benzoyl)[2]]acyline	−23	−0.8	91 94/6	1561.8	50	(8/8)	NA	
9	[D or LAgl (Me,4-Cl-benzoyl)[2]]acyline	−53	−2.4	>97	1575.8	250	(6/8)	280	
10	[L or DAgl (Me,4-Cl-benzoyl)[2]]acyline	+12	+0.7	>97	1575.8	100 50	(0/8) (3/8)	1.2	
11	[D or LAgl (Pca)[2]]acyline	−22	−10.4	96	1529.9	250	(3/3)	1700	
12	[L or DAgl (Pca)[2]]acyline	−13	−10.3	>97	1529.9	250	(2/2)	2000	
13	[D or LAgL (Apc)[2]]acyline	−29	−4.1	93	1532.8	250	(6/8)	2500	
14	[L or DAgl (Apc)[2]]acyline	−10	−4.3	88	1532.8	250	(8/9)	>1000	
15	[L-3Pal[3]]acyline	−39	+0.6	>97	1532.7	10.0 5.0	(0/8) (1/8)	0.23	
16	[D or LAgl (nicotinoyl)[3]]acyline	−34	−3.3	95	1561.8	2.5 1.0	(0/7) (3/3)	0.76	
17	[L or DAgl (nicotinoyl)[3]]acyline	−24	−0.7	92	1561.8	2.5 1.0	(0/5) (6/14)	0.63	intermediate
18	[D or LAgl (Me,nicotinoyl)[3]]acyline	−28	−2.8	>97	1575.8	25.0 10.0	(6/8) (8/8)	36	
19	[L or DAgl (Me,nicotinoyl)[3]]acyline	−41	+0.4	>97	1575.8	2.5	(3/8)	NA	
20	[D or LAgl (isonicotinoyl)[3]]acyline	−32	−5.8	65	1561.8	10.0	(3/7)	220	
21	[L or DAgl (isonicotinoyl)[3]]acyline	−25	−1.0	94	1561.8	10.0 2.5	(0/8) (2/8)	1.4	
22	[D or LAgl (formyl)[4]]acyline	−31	−0.4	>97	1545.8	5.0 1.0	(0/8) (2/8)	0.63	
23	[L or LAgl (formyl)[4]]acyline	−30	−0.1	>97	1545.8	5.0 1.0	(0/8) (6/8)	0.84	
24	[D or LAgl (Me,formyl)[4]]acyline	−19	−0.8	>97	1559.8	2.5 10.0	(8/8) (1/8)	0.90	
25	[L or DAgl (Me,formyl)[4]]acyline	−35	+0.5	>97	1559.8	2.5	(5/8)	5.5	
26	[D or LAgl (hydroxyacetic acid)[4]]acyline	−34	−0.9	>97	1575.8	5.0	(2/8)	0.056	
27	[L or DAgl (hydroxyacetic acid)[4]]acyline	−30	−0.5	>97	1575.8	5.0	(4/8)	0.30	
28	[D/LAgl (4-(acetylamino)benzoyl)[5]]acyline		−1.0	88	1561.8	5.0	(2/8)	0.51	
29	[D/LAgl (Me, 4-(acetylamino)benzoyl)[5]]acyline		−0.6	>97 35/65	1575.9	2.5 1.0	(0/8) (5/8)	0.43	very short acting
30	[D/LAgl (4-hydroxybenzoyl)[5]]acyline		−0.5	>97	1521.8	2.5 1.0	(0/8) (5/8)	0.30	
31	[D/LAgl (4-(acetylamino)benzoyl)[6]]acyline		−7.1	97	1561.8	5.0 2.5	(1/8) (2/8)	NA	
32	[D or LAgl (Me, 4-(acetylamino)benzoyl)[6]]acyline	−45	−1.0	>97	1575.8	25.0 10.0	(4/8) (8/8)	5.3	
33	[L or DAgl (Me, 4-(acetylamino)benzoyl)[6]]acyline	−2.0	+0.2	97	1575.8	2.5	(2/8)	1.9	
34	[D/LAgl (Ac)[7]]acyline		−4.1	92	1534.7	10.0 5.0	(0/8) (5/8)	5.0	
35	[D/LAgl (Me,Ac)[7]]acyline		−4.1/−3.9	>97 43/55	1547.7	5.0 2.5	(1/8) (2/8)	1.7	
36	[D/LAgl (isobutyryl)[7]]acyline		−1.8/−1.6	97 47/50	1561.8	2.5 1.0	(0/8) (6/8)	2.0	short acting
37	[Dpr (isobutyryl)[7]]acyline	−26	−2.0	>97	1575.8	2.5	(0/8)	2.0	
38	[D/LAgl (Me/isobutyryl)[7]]acyline		−1.4	>97 60/40	1575.8	2.5 1.0	(0/8) (2/8)	0.53	short acting
39	[D/LAgl (isopropyl-β-Ala)[8]]acyline		−1.9	>97	1505.7	5.0 2.5	(0/11) (3/9)	NA	
40	[D or LAgl (Me, isopropyl-β-Ala)[8]]acyline	−5	+0.7	>97	1561.8	2.5 1.0 0.5	(0/8) (0/8) (5/8)	0.50	medium acting
41	[L or DAgl (Me, isopropyl-β-Ala)[8]]acyline	−15	+1.5	>97	1561.8	10.0 2.5	(4/8) (7/8)	2.4	
42	[D/LAgl (guanidinoacetyl)[8]]acyline		−1.9/−1.6	>97 45/55	1534.0	5.0 2.5 1.0	(0/4) (1/7) (3/4)	0.74	
43	[L-Ala[10]]acyline	−48	−0.8	96	1532.8	2.5 1.0	(0/8) (8/8)	0.38	intermediate
44	[D/LAgl[10]]acyline		−1.9	>97	1533.9	2.5 1.0	(0/8) (3/8)	0.48	
45	[D/LAgl (Me)[10]]acyline		−1.0	>97	1547.7	2.5 1.0	(0/8) (8/8)	0.90	

Table 1 (Continued)

no.	compound[a]	$[\alpha]^{20}_D$[b]	Δt_R[c]	purity[d]	MS[e]	AOA[f] dose (μg)	AOA[f] ov.rat/ total	IC_{50}[g] (nM)	duration of action[h]
46	[D or LAgl (formyl)^{10}]acyline	−43	−1.9	>97	1561.7	1.0	(6/17)	0.59	long
						0.5	(9/11)		
47	[L or DAgl (formyl)^{10}]acyline	−57	−1.7	>97	1561.7	2.5	(0/4)	0.62	
						1.0	(3/3)		
48	[D/LAgl (Me,formyl)^{10}]acyline		−1.0	>97	1575.9	2.5	(0/8)	0.67	intermediate
						1.0	(1/8)		

[a] When the diastereomers were isolated, they are labeled D or L, when the diastereomers could not be separated preparatively, the mixture of the two was determined to be approximately 50/50 (as specified in the purity column) and was labeled D/L. [b] Corrected to $c = 1$ in 50% AcOH/H_2O; values are not given when diastereomers were not separated. [c] Retention times (min) under gradient conditions. Buffer system A: TEAP pH 7.30. Buffer system B: 60% CH_3CN/40% A. **1** was used as an internal standard throughout the study. Δt_R is retention time (t_R) of compound minus that of the standard. [d] Purity by HPLC as previous ratios indicate the presence and amount of diastereomers. [e] MS observed $[M + H^+]$ using a JEOL HX-110 MS were consistent with calculated $[M + H^+]$. [f] AOA-antiovulatory assay: dosage in micrograms (rats ovulating/total). [g] Binding assay uses rat pituitary gland membrane preparations. Values are IC_{50}s expressed in [nM]. Compounds are competing with radiolabeled histrelin ([ImBzlDHis^6,Pro^9-NHEt)]GnRH) and tested in duplicate one time ($n = 1$). These assays were performed on a robotic system with automated data reduction. [h] Measurement of circulating LH levels in castrated rats treated subcutaneously with the peptides (50 μg) was carried out over a period of 72 h or more as reported earlier.[8] Long duration of action = fully active after 72 h; intermediate = fully active at 48 h but only marginally so at 65 h. Medium acting = active at 48 h but not at 65. Short acting = fully active at 24 h. Very short acting = fully active at 12 h but not beyond.

to assume that it is the D-b(Me)2Nal-containing analogue (**5**) that was the most potent.

Of all the single betidamino acid substitutions in acyline, position 2 substitutions are the only ones that result in a drastic loss of potency and affinity and are exceptions to our general observations 1−4. Substitution of D4Cpa at position 2 by bD4Cpa yielded **10** which has an IC_{50} 230 times lower than that of **9** and is ca. 10 times more potent than **9** in the AOA. In comparison with acyline, **10** is also 50 times less potent than acyline with an affinity that is only 4 times less than that of acyline. To understand this unexpected result and explore the role of different functionalities at position 2, we made another two diastereomeric pairs, **11** and **12**, and **13** and **14**, where Pca (2-pyrazine carboxylic acid) and Apc (3-amino-4-pyrazolecarboxylic acid) were coupled to the aminoglycine side chain. All four compounds were essentially inactive at 250 μg with very poor affinity. Although a supporting argument based on steric hindrance could be proposed, we cannot exclude an electronic density-based argument resulting from the introduction of the added conjugated carbonyl function of the 4-chlorobenzoyl moiety. Interestingly, substitutions such as the introduction of X = 4-methyl, 4-methoxy, 4-fluoro, 4-chloro, 4-bromo, 4-trifluoromethyl, 4-nitro, 3,4-dichloro or 2,4-dichloro on the phenylalanine at position 2 of [Ac-Δ^3-Pro^1,DPhe(X)^2,DTrp^{3,6}]-GnRH according to the principles of Topliss's manual approach to Hansch's quantitative structure activity relationship studies had shown relatively little effect on potency[29] suggesting that other effects than the $2\pi-\pi^2$ or $\pi + \sigma$ determinants were effective.

Position 3 acyl groups of aminoglycine included nicotinoyl as well as the related isonicotinoyl. From the original studies of Folkers et al.,[30] we were expecting the nicotinoyl pair of analogues (**16**, **17**) to be significantly more potent than the isonicotinoyl pair (**20**, **21**) since [DCpa^{1,2},D3Pal^3,DArg^6,DAla^{10}]Ac-GnRH, when tested in the AOA, is close to 10 times more potent than [DCpa^{1,2},D4Pal^3,DArg^6,DAla^{10}]Ac-GnRH. In the betide series, one of the two diastereomers of [b3Pal^3]acyline (**17**) is at least as potent as acyline in the AOA while the other (**16**) is probably also equipotent in the AOA. While early investigations suggested that the D-isomer at position 3 led to loss of residual intrinsic activity of the given antagonists and was therefore a favorable substitution,[21] this was not confirmed in the most potent and recent GnRH antagonists. The observation that **16** and **17** are equipotent may therefore be of great significance as it suggests that the introduction of an L-amino acid at position 3 may be compatible with high potency. On the other hand **21**, the best of the two [b4Pal^3]GnRH diastereomers, is approximately half as potent as acyline, and **20** about 4−5 times less potent than **21**. AOA data resulting from *N*-methylation of [b3Pal^3]acyline {[b(Me)3Pal^3]acyline: **18**, **19**} supports observations 2 and 4 in that **19** is about half as potent as acyline and **18** half as potent. What should be noted here is the parallelism that exists between the data obtained with each of the isosteres in both betide and peptide series.

No better substitution than serine at position 4 has been identified in the search for improved GnRH antagonists. Among those amino acids that were tried in a number of antagonist series are the aliphatic amino acids glycine, alanine, methionine, and proline, the charged amino acids diaminopropionic acid, ornithine, lysine, arginine, and aspartic acid, and threonine.[31] The only substitution that led to a compound as potent as its parent, and that inhibits ovulation at a dose below 2.5 μg, was ornithine (but not lysine or diaminopropionic acid) in cases where the D-amino acid in positions 1 and 6 were hydrophobic (i.e. D2Nal, Rivier et al., unpublished results). On the other hand, it is well documented that a lactam ring spanning Asp^4 to Dpr^{10} is compatible with high affinity and biological activity of the resulting antagonists.[27,28,32] In this series, our expectations were therefore limited and three related pairs of diastereomers were synthesized, [bAla^4]acyline (**22** and **23**), [bhomoSer^4]acyline (**26** and **27**), and [b(Me)-Ala^4]acyline (**24** and **25**). None of the substitutions really mimicked a serine residue. In the first pair, we were surprised to find that **22** was essentially equipotent to acyline, and its diastereomer **23** was 5 times less potent with both analogues having IC_{50}s 2−3-fold that of acyline. Extension of the side chain to the homoserine mimic resulted in a significant loss of potency in vivo (ca. 5-fold) as expected with both diastereomers being essentially equipotent. Interestingly, **26** is the analogue with the highest affinity of all analogues shown here. We had observed earlier that some selected hydrophilic analogues had unusually high affinities for the GnRH receptor and **26** may be a representative of such a class of analogues.[33] Its relatively low potency

in vivo may be explained by a number of independent or combined effects such as a greater susceptibility to be metabolized or a faster release from the injection site than the corresponding acyline which has been recognized to form a gel at the site of action. Methylation of the side chain of [bAla^4]acyline (**24** and **25**), as in most other cases, resulted in one analogue retaining some potency (**25**) and the other being somewhat less potent. The loss of potency of one analogue (**24**) relative to the other (**25**) cannot be determined on the basis of the available data although **25** which may be the most potent of the two analogues in vivo is surprisingly the one with the highest IC_{50}.

D- or L-betide 4-acetamidophenylalanine {same as D- or L-Agl(4-acetamidobenzoyl), D- or L-b4Aph(Ac)} is the perfect betide mimic of D- or L-4Aph(Ac). Unfortunately, we were unable to separate the two diastereomers of [D- or L-b4Aph(Ac)^5]acyline (**28**) in pure form and therefore tested them as a 50/50 mixture as shown by CZE (which could analytically separate the two isomers). The fact that only partial inhibition could be achieved at 5 μg (two rats out of eight ovulated) was unexpected, yet [D/L-bTyr^5] (**30**) is as potent as acyline with full inhibition of ovulation at 2.5 μg. Finally, methylation of the side chain also yielded a diastereomeric pair (**29**) which could not be satisfactorily separated preparatively. Whereas for all other cases shown above methylation of the side chain resulted in one analogue being equipotent with acyline and the other being ca. 10 times less potent, AOA data of **29** suggest that both analogues are equipotent with acyline or, if a factor of 10 has to be introduced between the potency of the two diastereomers, one of them should be significantly more potent than acyline in order to compensate for the low potency of the other. Noteworthy is that all these analogues have IC_{50}s comparable to that of acyline.

Since the only difference between residues 5 and 6 in acyline is the chirality of the 4Aph(Ac), it is interesting to see that conclusions derived from biological data resulting from substitutions in 5 may be different from conclusions derived from data found for identical substitutions at position 6. This is quite common in peptide chemistry, where two identical and consecutive amino acids in a sequence will have quite different sensitivity to substitutions; a good example is the biological consequences of substituting a DAla for glycine residues at positions 2 and 3 of [Met]enkephalin. At position 2, the substitution results in significant increase in potency (10-fold) while the same substitution at position 3 yields an analogue that is considerably less potent.[34]

Observations 1−4 also apply at position 6. Although the two diastereomers of [b4Aph(Ac)^6]acyline could not be separated preparatively (**31**), their mixture inhibited ovulation (two rats out of eight ovulating) at a dose (2.5 μg) that suggests that one of the diastereoisomer is at least as potent as acyline while the other may be somewhat less potent (within a factor of 2−3). On the other hand methylation of the side chain resulted in one analogue being almost as potent as acyline (**33**) and the other (**32**) being around 10 times less potent (partial inhibition at 25 μg). This is again consistent with the binding assay data that show a 2.5-fold difference in affinities.

It is known that the pharmacophore that recognizes GnRH antagonists is not very discriminatory when it comes to substitutions at position 7 where amino acids such as Phe, 4Fpa, 4Cpa, Trp, NαMeLeu, Nva, Met, and Nle are generally tolerated (Rivier et al., unpublished results[35,36]). Here we demonstrate that, although substitution of Leu^7 by D/L-Agl(Ac)^7 (**34**) in acyline is deleterious as it lowers potency by a factor of 5−10 compared to acyline, methylation of the side chain (**35**) restores some activity. While Agl(Ac) is isosteric to norvaline, the methylated derivative (betidevaline), is isosteric to Ile. We show (compare AOA results of **34** and **35**) that the addition of the methyl group in **35** is important for potency as it contributes to mimicking leucine more closely. In fact [bLeu^7]acyline (**36**) and [b(Me)Leu^7]acyline (**38**) as diastereomeric mixtures are equipotent with acyline in the AOA. Also noteworthy is that extension of the isopropyl side chain by one methylene using diaminopropionic acid in lieu of Agl to yield **37** is still well tolerated by the pharmacophore. Unfortunately, for no analogue in this series were we able to quantitatively separate the different diastereomeric pairs on a preparative scale, thus limiting our ability to draw sharp conclusions. Nevertheless, the biological results are consistent with our original premises with good correlation between in vivo and in vitro data.

We then synthesized [bIOrn^8]acyline (**39**) (which is isosteric with Ilys^8) and [b(Me)IOrn^8]acyline (**40**, **41**) (which is the methylated isostere of ILys^8) as well as [bnorArg^8]acyline (**42**) (which is the isostere of Arg^8). As a 1:1 mixture of the two diastereomers, **39** inhibited ovulation at 5 μg/rat and was partially active at 2.5 μg, suggesting that one of the two diastereoisomers may indeed be as potent as acyline. As two separated diastereomers, the methylated derivatives show significantly different potencies (**40** being possibly more potent than acyline while **41** is almost twenty times less potent with a corresponding loss of affinity for the GnRH receptor). As a 50/50 mixture of two diastereomers, **42** is almost as potent as acyline in the AOA. These results are consistent with what has been observed in this series whereby substitution of an amino acid by a betidamino acid of the equivalent chirality, overall size, and functionality in a bioactive molecule will result in an equivalently potent analogue.

Substitution of DAla^{10} in acyline by Agl (**44**), (Me)-Agl (**45**), bAla (**46** and **47**), and b(Me)Ala (**48**) was of particular interest for several reasons. Whereas deletion of residue 10 and its replacement by an ethylamide results in a significant increase of potency in vitro in the GnRH agonist series,[37] it was shown that this modification has little effect on the affinity as measured in the cultured rat pituitary cell assay, but yields analogues that are less potent and with shorter duration of action in vivo.[8] Of all substitutions that were published,[21] DAla^{10} was ultimately selected by most groups developing GnRH antagonists because it seemed to confer extended duration of action in vivo, an observation also confirmed here (compare duration of action of **43** which is intermediate with that of the long acting acyline). At the same time it confers increased duration of action, this substitution also increases the overall hydrophobicity of the antagonists, a less favorable property when it comes to trying to formulate these analogues in depot preparations for extended delivery. It was therefore of interest to investigate the effect of such substitutions as the introduction of Agl and its

derivatives on potency and duration of action. Resolved [bAla^{10}]acyline (**46** and **47**) were found to be either as potent or almost as potent as acyline. Similarly, racemic mixtures of [b(Me)Ala^{10}]acyline (**48**), [Agl^{10}]acyline (**44**), and [Agl(Me)^{10}]acyline (**45**) are virtually equipotent with acyline (**1**). Never observed before is the fact that a residue containing a primary amine (in **44**) or a secondary amine (in **45**) at position 10 is compatible with unexpectedly high potency as compared to the introduction of a closely related D-serine residue which resulted in a 10 fold loss of potency when comparing [Ac-D2Nal^1,D4Fpa^2,DTrp^3,DArg^6,DSer^{10}]GnRH (partially potent at 10 μg/rat) with [Ac-D2Nal^1,D4Fpa^2,-DTrp^3,DArg^6,DAla^{10}]GnRH and [Ac-D2Nal^1,D4Fpa^2,-DTrp^3,DArg^6]GnRH which are fully active at 1 μg (0 rat ovulating out of 8 and 10 respectively, data not shown). Because an argument based on steric hindrance may not apply here if one were to assume that Ser and MeAgl are approximately isosteric, one explanation for these results may be a low nucleophilicity of the β-amino function. All analogues with substitutions at the 10 position have comparable IC_{50}'s compatible with their in vivo potency.

Two questions remain as to whether our ability to separate easily the different diastereomers has a bearing on the secondary structure assumed by the different analogues, and whether the solubility of these analogues as measured by their relative retention times on reverse phase supports (C_{18}) at neutral pH can be correlated with duration of action.

Because diastereomeric pairs **1** and **2** and **1** and **15** clearly demonstrate that the D-isomer is more hydrophilic than the L-isomer (positive values for Δt_R), we have organized Table 1 in such a way as to prejudice the assignment of the D or L versus the L- or D-diastereomers on the basis of their relative retention times with the D or L assignment for the analogue having the shortest retention time and the L or D assignment for the analogue with the longer retention time. Importantly, we have no evidence demonstrating that any of these assignments are correct although in most cases the expected order of relative potencies is confirmed (see below); however it is also clear that the diastereomeric pair **1** and **43** have an opposite order of elution.

Also of interest is the observation that diastereomeric pairs at residues 1, 2, 3, 4 were easily separated, whereas those at residues 5−10 were significantly more difficult using triethylammonium phosphate buffers at different pHs or dilute TFA. This suggests that D- and L-betidamino acids at positions 5−10 do not alter the overall bioactive conformation in a way that is recognized by the chromatographic supports (at neutral pH) nor seemingly by the receptor since most of these analogues are very potent and have small IC_{50}s (with a couple of notable exceptions mentioned above). Easy separation of the diastereomeric pairs at residues 1−4 can be explained by the fact that these residues either are aromatic (residues 1−3) and are expected to show strong affinity for the C_{18} support or just precede (residue 4) a more rigid part of the molecule defined by a β-turn encompassing residues 5−8.[27,28,32] Another approach was to determine, on the basis of the biological results, whether there was a correlation between the relative retention times of the analogues and the chirality of the aminoglycine residue. We know from this work (see **2**, **15**, and **43**) and earlier data (unpublished) that a D residue at positions 1, 2, 3, 6, and 10 yields analogues that are more potent than those with an L residue at these positions.[38] From the HPLC data reported in Table 1, an L residue at position 1 or 3 but not 10 gives retention times that are longer than that of acyline (in which those residues are D). It is therefore tempting (as already discussed) to assign a chirality on the basis of biological activities and HPLC retention times. Unfortunately, there is no consistency within the data: as this could be true for compounds **3**, **5**, **7**, **46**, there may be just as many exceptions (**9**, **18**, **20**, **32**, **40**) or cases where we could not conclude on the basis of the fact that the two diastereomers are statistically equipotent or inactive at the doses tested (**11**, **16**, **22**, **24**, and **26**). Whereas we have been able to assign chirality in the case of at least one somatostatin analogue (Craig et al., in preparation), this was made possible by mass spectrometric analysis of fragments generated by enzymatic degradation (the L-Agl-containing diastereomer could be degraded whereas the D-Agl-containing diastereomer was resistant to degradation). This approach could not be used in this series of analogues because of the fact that both C- and N-termini of these GnRH antagonists are blocked and therefore resistant to enzymatic degradation. An alternative being studied at this time is to resolve the orthogonally blocked scaffold [Boc-Agl(Fmoc) or another equivalent intermediate], determine its chirality, and use it in an unequivocal synthesis of the analogues.

This project was initiated with the hope that increased numbers of amide bonds in the structure of betidamino-containing peptides would result in additional hydrogen-bonding opportunities that would in turn yield increased conformational stability in cases of intramolecular interactions, or increased affinity with the receptor or binding proteins in the case of intermolecular interactions. Both types of interactions were to result in increased duration of action unless other parameters such as biostability and biodistribution were to be negatively altered. The observation that the limited number of betidamino acid containing acylines all had shorter duration of action than acyline itself suggests that the original premises may be flawed or do not apply in these cases.

All betide acylines that are either equipotent to or more potent than acyline have a shorter chromatographic retention time than acyline, suggesting that the hydrophilicity of GnRH antagonists can be modulated by their conversion into betides with retention of potency in short term in vivo assays (AOA, Table 1). Additionally it was found that there is good correlation between in vivo and in vitro data although in vivo data (long duration of action in the castrate male rat assay) may be more discriminatory for the selection of a drug candidate. In long-term in vivo assays (Figures 1), it is apparent that, in the majority of the cases tested, introduction of a betide amino acid results in shortened duration of action. More specifically, while acyline shows full inhibition at, and beyond, the 72 h time point but not at the 120 h time point, secretion of LH is restored, at least in part, for all the other analogues tested in Table 1 at around 70 h, with the exception of compound **46** which contains a betide residue at position 10 (data not shown). This is significant because introduction of an L-Ala at that position (compound **43**)

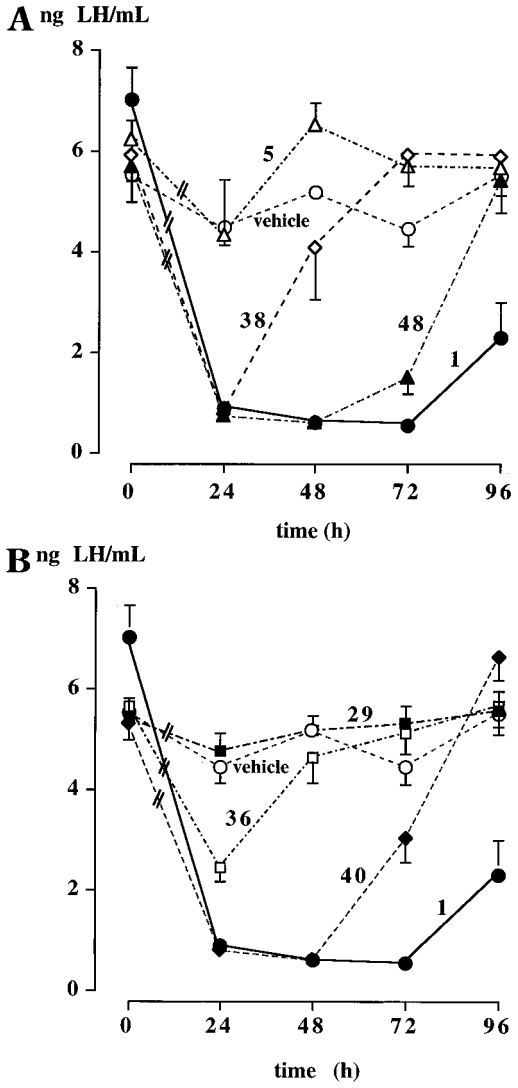

Figure 1. Inhibition of LH secretion after sc administration of analogues (50 μg in 200 μL). Blood samples were collected at the times shown on the abscissa. The SEM, where not appearing, are encompassed within the size of the symbols in the graphs. Compound numbers as well as unique symbols are used to identify each response. Con = control.

results in a significant loss of duration of action. These limited studies suggest that while hydrophilicity, as measured by retention times on reverse phase supports, is increased by the introduction of betidamino acids, more data will be needed to fully understand the potential and limitations of betidamino acids in biologically active peptides.

Materials and Methods

Instruments. Preparative RP-HPLC was accomplished using a Waters Associates (Milford, MA) Prep LC/System 500A and Model 450 variable-wavelength UV detector, Fisher (Lexington, MA) Recordall Model 5000 strip-chart recorder, and a Waters Prep LC 500A preparative gradient generator. Analytical RP-HPLC were run on a system using two Waters M-45 pumps, a Shimadzu Chromatopac EIA integrator, and a rheodyne Model 7125 injector. The peptide synthesizer used was Beckman Model 990. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. All melting points are uncorrected.

Starting Materials. Amino acid derivatives Boc-DAla, Boc-Leu, Boc-Pro, Boc-Ser(Bzl), and Boc-Tyr(2-BrZ) were obtained from Bachem Inc. (Torrance, CA). Boc-D2Nal, Boc-D4Cpa, Boc-D3Pal, and Boc-ILys(Z) were obtained from Synthetec (OR) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. N^{α}-Boc-4-nitro-L- and -D-phenylalanine, N^{α}-Boc-4-amino-L- and -D-phenylalanine, and N^{α}-Boc-[4-*N*-(9-fluorenylmethoxycarbonyl)amino]-L- and -D-phenylalanine were synthesized according to previously published procedures.[39] FmocNR^{1}CH(NR^{2}Boc)CO$_2$H (R^1 = H, Me; R^2 = H, Me) were obtained as described earlier.[10,17] The methyl benzhydrylamine resin used for peptide synthesis was obtained according to published procedures.[6,40] Resins with substitutions varying from 0.4 to 0.7 mequiv/g were used. All solvents were reagent grade or better.

Peptide Synthesis. The resin-bound peptides incorporating the Fmoc-protected amino functions were synthesized by SPPS methodology[41] on a Beckman 990 peptide synthesizer with use of previously described protocols on the methylbenzhydrylamine (MBHA) and *N*-ethylaminomethyl (NEAM) resins (approximately 1 g per peptide) using *tert*-butyloxycarbonyl groups for N^{α}-amino protection. TFA treatment was extended to 2× 15 min. Coupling time was 90−120 min followed by acetylation (excess acetic anhydride in CH_2Cl_2 for 15 min). A 3-fold excess protected amino acid was used based on the original substitution of the methylbenzhydrylamine or *N*-ethylaminomethyl resins. N-Terminal acetylation was accomplished using the same protocol as that used for capping (excess acetic anhydride in DCM).

The individual amino acids were incorporated in a sequential manner utilizing either diisopropylcarbodiimide or BOP-mediated activation[42] of the carboxyl group. The extent to which individual couplings had proceeded was qualitatively determined by the ninhydrin test as described by Kaiser et al.[43] This protected, resin-bound peptide was further derivatized to yield the different analogues as described below. The Fmoc protecting groups were removed by treatment of the fully protected, Agl- or (Me)Agl-containing resin-bound peptide prepared above, with 20% piperidine in DMF (5 and 25 min). Acylation of the free N^{ϵ}-amino functions with the different acylating agents (acids, acid anhydrides, or chlorides) mimicking the different betidamino acid side chains was carried out. Liberation of the desired peptide from the resin and its protecting groups through the action of anhydrous HF (ca. 30 mL) at 0 °C in the presence of anisole (3 mL) yielded, after concentration, extraction, and lyophilization, ca. 1 g of the crude analogues; these were subsequently purified by RP-HPLC procedures as previously described[18] to give, after lyophilization, 20−50 mg of the desired diastereomers as individual components or as a 50/50 mixture.

Peptide Purification. The lyophilized, crude peptides (300 mg to 1.5 g) were dissolved in 0.25 M triethylammonium phosphate (200 mL), pH 2.25 (TEAP 2.25), and loaded onto a 5 × 30 cm preparative RP-HPLC cartridge packed in our laboratory using Vydac C_{18} silica (330-Å pore size, 15−20 μm particle size). The peptide was eluted using a flow rate of 100 mL/min on a Waters Prep 500 system with a mixture of A (TEAP 2.25) and B (60% CH_3CN, 40% TEAP 2.25) with an appropriate gradient (90 min) such that retention time was ca. 45 min.[44] The collected fractions were screened by use of analytical RP-HPLC under isocratic conditions, 0.1% TFA/H_2O at a flow rate of 2.0 mL/min (Vydac C_{18} column, 5 μm, 300 Å pore size; 4.6 × 250 mm). Appropriate fractions were then combined and converted to the trifluoroacetate salt by loading after dilution (1/1) in water, on a preparative RP-HPLC cartridge as described above and eluted with the use of a mixture of solvents A (0.1% TFA) and B (60% CH_3CN, 40% H_2O, 0.1% TFA) and the following gradient: 20 %B (10 min) followed by a 20 min gradient to 90% B.

Peptide Characterization. Analytical RP-HPLC. HPLC analyses were carried out using a Vydac C_{18} column (2.1 × 150 mm, 5 μm particle size, 300 Å pore size) at a flow rate of 0.2 mL/min. Differences in retention times (t_R) between that of acyline and that of the corresponding betide analogue are given in minutes (see conditions in legend Table 1). Buffer A was TEAP, pH 7.3, and buffer B was comprised of 60% acetonitrile and 40% buffer A. The gradient was from 40% to 75% buffer B in 30 min. The column temperature was maintained at 40 °C with UV detection at 214 nm.

LSIMS. Spectra were measured using a JEOL JMS−HX110 double-focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and Cs^+ gun voltage of 25 kV was employed. The samples were added directly to a glycerol matrix.

Optical Rotations. Optical rotations were measured in 1% acetic acid ($c = 1.0$; i.e., 10 mg/mL of peptide uncorrected for TFA counterions or water present after lyophilization). Values were obtained from the means of 10 successive 5 s integrations determined at room temperature (about 23 °C) on a Perkin-Elmer 241 polarimeter (using the D line of Na emission).

Biological Characterization (Table 1 and Figure 1). The AOA was carried out as described by Corbin and Beattie[45] using an aqueous vehicle (1−2% DMSO). Results are expressed in terms of the dosage in micrograms/rat (rats ovulating/total number of treated rats). Measurement of circulating LH levels in castrated rats injected subcutaneously with the peptides was carried out over a period of 96 h as reported earlier.[8,15,46−48]

The rat pituitary gland membrane preparations were obtained from an equal number of anterior pituitary glands from male and female rats (Pel-Freez Biologicals) which were collected and rapidly frozen until homogenization. Pituitary glands were homogenized in lots of 25 in 8 mL of homogenization buffer (0.32 M sucrose; 10 mg/mL aprotinin). Homogenization was performed in a Kontes tube (size 21) with 10 strokes from a Teflon pestle. The homogenate was then centrifuged at 635*g* for 6 min at 4 °C. The supernatant was collected, the resulting pellet was again homogenized, and the supernatant was collected as described above. The pooled supernatants were centrifuged at 40000*g* for 20 min at 4 °C, and the membrane pellet was collected and resuspended in 0.75 mL/25 pituitary glands of ice cold homogenization buffer. Pituitary gland membranes were prepared by Analytical Biological Services, Inc. This membrane preparation was frozen in liquid N_2 until use in the binding assay. Before use, the membrane preparation was thawed and diluted 15-fold with assay buffer (10 mM Hepes, 0.2% BSA (Intergen Co), pH 7.6). The competitive binding assay was performed utilizing [^{125}I]histrelin (Corning Hazelton) as the radioligand and rat anterior pituitary glands as a source of GnRH receptor. The binding assay was performed in a total volume of 150 μL within a 96-well microtiter plate. All reagents throughout the assay were maintained at 4 °C. Reagents were added in the following order: 26 μL of assay buffer (also containing 6.9% DMSO), 4 μL of test compound (dissolved in 50 mM Hepes, 30% DMSO) or cold histrelin (Bachem California; 300 nM final in assay buffer, 30% DMSO) for the NSB wells or assay buffer containing 30% DMSO for B0 wells, 20 μL of [^{125}I]histrelin (0.1 mCi), and 100 μL of diluted membrane preparation. The contents of the wells were mixed and then incubated for 2 h at 4 °C. The microtiter plates were then harvested using cold (4 °C) 0.9% NaCl with a Tomtec Mach II 96-well plate harvester (Wallac, Inc.) onto dry, polyethylenimine-treated glass filter mats (type B, Wallac, Inc.). After harvesting, the mats were dried in a microwave oven. The dried mats were treated with MeltiLex B/HS scintillator sheets (Wallac, Inc.) and counted on a Betaplate liquid scintillation counter (Wallac, Inc.).

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