Gonadotropin-Releasing Hormone Antagonists with N^ω-Triazolylornithine, -lysine, or -p-aminophenylalanine Residues at Positions 5 and 6[†]

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In order to be used as fertility regulators in humans, gonadotropin releasing hormone (GnRH) antagonists must be extremely potent and long acting and exhibit negligible side effects such as stimulating histamine release. To this aim, we have recently synthesized a series of analogues with the standard $\text{Ac-DNal}^1\text{-DCpa}^2\text{-DPal}^3$ substitutions, where the N^ω-amino function of ornithine, lysine, or p-aminophenylalanine (Aph) was converted to the aminotriazolyl (atz) derivatives at positions 5 and 6 with further modifications at positions 7 and 10. The analogues were tested for their ability to bind to pituitary cell membranes, to release histamine in a mast cell assay, to inhibit luteinizing hormone (LH) secretion by castrated male rats or cultured pituitary cells, and to interfere with the ovulation in intact female rats. While the subcutaneous (sc) injection of 50 μg of Azaline A {7, $[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{Lys}^5(\text{atz}),\text{DLys}^6(\text{atz}),\text{ILys}^8,\text{DAla}^{10}]\text{GnRH}$} dissolved in 0.2 mL of an aqueous media significantly inhibited LH release in the castrated male rat for 24 h, the same dose of Azaline B (11), $[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{Aph}^5(\text{atz}),\text{DAph}^6(\text{atz}),\text{ILys}^8,\text{DAla}^{10}]\text{GnRH}$, inhibited LH release for 72 h. A similar long duration of action was observed for Antide {$[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{Lys}^5(\text{Nic}),\text{DLys}^6(\text{Nic}),\text{ILys}^8,\text{DAla}^{10}]\text{GnRH}$} but not for Nal-Glu {$[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{Arg}^5,4\text{-}(p\text{-methoxybenzoyl})\text{-D-2-Abu}^6,\text{DAla}^{10}]\text{GnRH}$}. In the same paradigm, a 5-fold dilution of the peptide (50 μg in 1 mL) and the use of three injection sites rather than one resulted in significantly shorter duration of action for most of the peptides tested. This suggested that long duration of action might be the result of slow release from the injection site(s). In order to investigate this possibility, Nal-Glu and Azaline B were injected intravenously (iv) at three doses (10, 50, 250 μg) to castrated male rats. At all doses, both peptides significantly lowered LH levels for 8 h. By 24 h, Nal-Glu (250 μg) and Azaline B (50 and 250 μg) still measurably inhibited LH secretion. Finally, only Azaline B (250 μg) was still active at 48 h. These findings demonstrate that subtle structural modifications will yield peptides with different half-lives after iv administration. These findings led us to investigate the effects of other structural modifications on duration of action. We observed that systematic substitutions at positions 7 (NMeLeu) and 10 ($\text{Pro}^9\text{-NHEt}$, and Gly-NH_2) were found to be deleterious. Of interest was the observation that only the $\text{DAla}^{10}\text{-NH}_2$ substitution led to long duration of action and enzymatic stability under the conditions tested. Most analogues {excluding $[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{DCit}^6,\text{DAla}^{10}]\text{GnRH}$ (SB-75), $[\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{DHar}^6\text{-}(N^g,N^g\text{-Et}_2),\text{Har}^8(N^g,N^g\text{-Et}_2),\text{DAla}^{10}]\text{GnRH}$ (RS-26306) and $([\text{Ac-DNal}^1,\text{DCpa}^2,\text{DPal}^3,\text{NMeTyr}^5,\text{DLys}^6\text{-}(\text{Nic}),\text{ILys}^8,\text{DAla}^{10}]\text{GnRH}$ (A-75998) which were recently reported to be long acting} were tested for binding affinity to pituitary cell membranes. On the basis of the limited data obtained in that assay, a correlation may exist between nonparallelism {with the standard $[\text{DAla}^6,\text{NMeLeu}^7,\text{Pro}^9\text{-NHEt}]\text{GnRH}$} in the dose–response curve and long duration of action. In an in vitro histamine release assay and the rat antiovulatory assay (AOA), Azaline B ($\text{ED}_{50} = 224 \pm 23$ μg/mL: AOA $\text{ED}_{100} = 1.0$ μg) compared favorably against Nal-Glu ($\text{ED}_{50} = 1.8 \pm 0.66$ μg/mL: AOA $\text{ED}_{100} = 1.5$ μg), SB-75 ($\text{ED}_{50} = 2.1 \pm 0.3$ μg/mL: AOA $\text{ED}_{100} = 2.0$ μg), RS-26306 ($\text{ED}_{50} = 11 \pm 1.1$ μg/mL: AOA $\text{ED}_{100} \geq 2.5$ μg) or A-75998 ($\text{ED}_{50} = 22 \pm 3.2$ μg/mL: AOA $\text{ED}_{100} \gg 1.0$ μg). Azaline B is readily soluble in water/3% mannitol/5% ethanol (≥ 20 mg/mL) and is readily produced synthetically.

Mammalian gonadotropin-releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) plays a major role in the modulation of reproductive functions; as a result, a concerted effort directed toward the devel-

opment of potent and long-acting agonists and antagonists has been sustained by many laboratories.[1,2] Whereas some of the superagonists are now available for therapeutic use, the relatively low potency of the competitive antagonists coupled with the finding that some of them release histamine in a variety of tests[3,4] including in human,[5] have

been the main roadblock to their acceptance and use. Because the histamine-releasing property of these analogues was correlated to both their overall hydrophobicity and the presence of strongly basic side chains, analogues such as the Nal-Glu antagonist [Ac-DNal^1,DCpa^2,DPal^3,-Arg^5,4-(p-methoxybenzoyl)-D-2-Abu^6,DAla^{10}]GnRH^6 (1) were developed and extensively studied in humans.[7] It was recognized however that for contraceptive as well as other purposes that require sustained administration, the therapeutic index (relative potency at inhibiting gonadotropin secretion over relative potency at stimulating histamine release) of Nal-Glu is still too low. In a series of recent papers,[8-10] we have described an approach to generate trifunctional, moderately basic, novel amino acids by the selective modification of the ω-amino function of ornithine, lysine, and p-aminophenylalanine. The resulting peptides had the desired low potency in releasing histamine[9] or in a test for anaphylactoid activity[10] while being considerably more soluble in aqueous buffers and remaining very effective in inhibiting ovulation in the rat. Because the potency of these peptides was still at least 1 order of magnitude less than needed for them to be economically viable (assuming a duration of action comparable to that of Nal-Glu), the search for better analogues was pursued. Since another way to increase efficiency over potency is to increase duration of action and because Antide (3) ([Ac-DNal^1,DCpa^2,DPal^3,Lys^5(Nic),DLys^6-(Nic),ILys^8,DAla^{10}]GnRH),[11-13] SB-75 (4) ([Ac-D-Nal^1,DCpa^2,DPal^3,DCit^6,DAla^{10}]GnRH),[14,15] RS-26306 (5) [Ac-DNal^1,DCpa^2,DPal^3,DHar^6(N^g,$N^{g'}$-Et$_2$),DAla^{10}]-

GnRH,[16] and A-75998 (17) ([Ac-DNal^1,DCpa^2,DPal^3,-NMeTyr^5,DLys^6(Nic),ILys^8,DAla^{10}]GnRH)[17] were reported to have an unusually long duration of action while Nal-Glu was reported to have a comparatively short duration of action, we have compared the effectiveness of a series of analogues in vivo after sc and iv delivery and tried to better define those parameters responsible for the desired bioactivities (including histamine release in vitro) and pharmacokinetics (long duration of action). Nal-Glu (1), Antide (3), SB-75 (4), RS-26306 (5), and Azaline A (7) and B (11) (see Table I) as well as some newly reported analogues (including A-75998 (17) from TAP Pharmaceuticals Inc. and the Pharmaceutical Products Division of Abbott Laboratories) were tested in a number of assays. The structures of these newly developed analogues were derived from substitutions known to increase potency as well as enzymatic resistance to degradation in the agonist series. These substitutions include NMeLeu in position 7[18] and Pro-NHEt in positions 9 and 10.[19] Other modifications known to be biologically active include the native C-terminal Gly-NH$_2$ and the DAla-NH$_2$ first reported by Erchegyi et al. in an antagonist.[20] The amino acid residues with substituted ω-NH$_2$ functions were derivatized on a partially protected resin-bound peptide by modifying ornithine, lysine, or p-aminophenylalanine residues which had been temporarily, orthogonally protected with the 9H-fluorenylmethoxycarbonyl (Fmoc) group.[21] These new analogues are equipotent (gonadotropin suppression) to some of the most potent antagonists prepared to date, as determined in several bioassays including the rat antiovulatory assay (AOA),[22] in vitro cell culture assay,[23] and binding assay[24] and significantly less potent in a histamine release assay.[4] Yet the biological profile of these new analogues can be distinguished by the observation that

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some analogues inhibit LH secretion for significantly longer periods of time in the castrated male rat model than others when given at a dose 10–50 times that shown to be effective to acutely inhibit ovulation in the AOA.

Synthesis, Purification and Characterization (See Table I)

The syntheses of 1 and 2 were reported in an earlier paper, which described the relatively low histamine-releasing potency of these analogues.[6] Similarly, the synthesis of Antide (3) was first reported by Ljungqvist et al.[11] while large quantities of this peptide were synthesized in our laboratory for its evaluation in two Phase I clinical settings (Pavlou et al. and Bremner et al. personal communication). Here we describe a more general method for the synthesis of this peptide and related ones such as Azaline A and B (see Table I). It involves direct modification of the unprotected ω-amino function of a variety of amino acids (ornithine, lysine, or p-aminophenylalanine) directly on the otherwise fully protected resin. Antide synthesized following this procedure is identical (in both its physical and biological properties) to that prepared via the direct introduction of the N^ϵ-nicotinyllysines, with comparable yields (see Experimental Section). For the synthesis of 3 and 7–16, the distal amino functions of the ornithine, lysine, or p-aminophenylalanine residues to be modified were protected as their Fmoc derivatives. The fully assembled peptide resin was treated with a freshly prepared solution of piperidine in dimethylformamide (DMF) or N-methylpyrrolidinone (NMP) to remove the Fmoc group; the nicotinyl or triazolyl functions (with the exception of 16 which was not derivatized) were introduced as previously described.[8–10] Nicotinic acid was reacted with the partially deblocked peptido-resin in the presence of diisopropylcarbodiimide (DIC) until a negative ninhidrin test was obtained. Similarly, the triazolyl function was introduced by first reacting the resin-bound partially deprotected peptide with diphenyl cyanocarbonimidate (PCI) to obtain a reactive N-cyanoisourea intermediate which is then reacted with an excess of hydrazine hydrate to give, in one step, the desired triazolyl derivatives in high yields.[9] This overall strategy to generate the nicotinyl and triazolyl derivatives was found to be clearly advantageous in a research setting because of its versatility, facility, and flexibility.

The synthesis of 4 as a 1/1 mixture of the D- and LCit[6] analogue was originally reported by Folkers et al.[25] Later, Bajusz et al.[14] reported the synthesis and characterization of diastereomerically pure 4 which they named SB-75. The synthesis of 4 using Boc-D-citrulline in position 6 or of RS-26306 using Boc-D- and L-Har(N^g,$N^{g'}$-Et$_2$) at positions 6 and 8, respectively (Bop coupling), and of A-75998 was straightforward.

C-terminally amidated analogues (1–9, 11, 12, 14, 16, and 17) were synthesized by the SPPS methodology either manually or on a Beckman 990 peptide synthesizer with use of previously described protocols on a methylbenzhydryl amine (MBHA) resin using the *tert*-butyloxycarbonyl (Boc) group for N^α-amino protection.[6] C-terminally, N-ethylamidated peptides (10, 13, 15) were synthesized on the (N-ethylamino)methyl (NEAM) resin as previously reported.[26] Whereas the peptide amides were

cleaved in HF for 1–1.5 h at 0 °C, the C-terminally, N-ethylamidated peptides were cleaved and deprotected at room temperature for at least 3 h. This longer treatment in HF was not found to be deleterious to the stability of the triazolyl moiety. Amino acid analyses which could quantitate Cpa, Cit, NMeTyr, diethylhomoarginine, and Nal but not Pal, the ω-triazolyl amino acids, or aminophenylalanine, were consistent with expected values. Calculated values for protonated molecular ions were in agreement with those obtained using liquid secondary ion mass spectrometry (LSIMS) (see supplemental material). Analogues reported in Table I were all greater than 92% pure as determined by RPHPLC under acidic (0.1% TFA) and neutral (TEAP pH 7.0) conditions.

Stability toward Pronase and Subtilisin

Five peptides, 1, 3–5, and 11 were subjected to digestion with pronase (a mixture of several nonspecific endogenous and exogenous proteases from *Streptomyces griseus*) and subtilisin, a serine protease from *Bacillus subtilis* (a nonspecific endopeptidase which acts on internal peptide bonds and which is generally used for the total hydrolysis of peptide/proteins). Under conditions that usually lead to protein cleavage or complete hydrolysis, all peptides were stable with the exception of 1 which, overnight, in the presence of pronase, yielded two fragments (30% conversion) which were isolated by RPHPLC. Mass spectrometric analysis of the isolated fragments indicated that MH$^+$ = 656.3 and MH$^+$ = 830.0 correspond to the mass of the N-terminal tetrapeptide Ac-Nal-DCpa-DPal-Ser minus 1 mole of H$_2$O and to the mass of the C-terminal hexapeptide, respectively. A tentative correlation between long duration of action and stability to enzymatic digestion (pronase and subtilisin) is still under investigation.

Results and Discussion

The purpose of this study was 3-fold. First, to evaluate in several in vivo systems the duration of action of related families of analogues with different solubilities in aqueous buffers at physiologic pHs. Second, to correlate these results with data derived from in vitro systems and third, to investigate which physicochemical properties were responsible for such differences if observed. The families of peptides tested here were those of analogues that have either entered clinical testing or are being considered for such studies.

On the basis of results indicating that the Arg[5] substitution was compatible with retention of considerable potency in the AOA, we synthesized a series of GnRH analogues. One of these, the Nal-Glu antagonist 1 (presently under clinical investigation in humans[27]), still suffers from residual histamine-releasing activity, with an in vitro histamine release ED$_{50}$ of 1.8 μg/mL. Results obtained by Ljungqvist et al.[11,12] indicated that substitution of positions 5 and 6 with weakly basic acylated lysine residues (NicLys), an isopropyllysine in position 8,[11] and DAla in position 10[20] in combination with a hydrophobic N-terminus (Ac-DNal-DCpa-DPal-) resulted

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Table I. Physicochemical and Biological Characterization of GnRH Antagonists with Ac-DNal^1-DCpa^2-DPal^3 Substitutions Abbreviated as X

compounds	RT (%CH$_3$CN)^a	$[\alpha]_D{}^b$ (deg)	RT^c	in vitro relative potencies^d	AOA^e	in vitro histamine release:^{f} ED$_{50}$ ± SEM, μg/mL	K_D (nM)^g
1 [X,Arg^5,DGlu^6(AA),DAla^{10}]GnRH; Nal-Glu	6.20 (36.0)	−28	22.8	1.0 (0.8–1.3)	0.5 (8/16), 1.5 (0/10)	1.8 ± 0.66	0.67 (0.43–1.0)
2 [X,Arg^5,DPal^6,DAla^{10}]GnRH	3.20 (36.0)	−30	14.1	0.91 (0.76–1.10)	0.5 (5/10)*, 1.0 (0/10)*	2.9 ± 0.33	0.24 (0.17–0.34)
3 [X,Lys^5(Nic),DLys^6(Nic),ILys^8,DAla^{10}]GnRH; Antide	4.00 (30.0)	−31	13.6	0.66 (0.4–1.0)	1.0 (2/10)*, 1.5 (2/20)*, 8.0 (2/8)	≥300	0.35 (0.25–0.47)
4 [X,DCit^6,DAla^{10}]GnRH; SB-75	3.65 (31.8)	−22	10.1	1.56 (1.00–2.45)	1.0 (1/10)*, 2.0 (0/10)*	2.1 ± 0.3	
5 [X,DHar^6(N^g,N$^{g'}$-Et$_2$),Har^8(N^g,N$^{g'}$-Et$_2$),DAla^{10}]GnRH; RS-26306	4.05 (34.8)	−4		2.17 (1.54–3.05)	2.5 (1/8)*, 2.5 (1/8)	11 ± 1.1	
6 [X,DPal^6,ILys^8,DAla^{10}]GnRH	4.61 (27.6)	−23	16.3	1.3 (0.9–1.9)	1.0 (8/14), 2.5 (0/6)	34 ± 7.3	not //
7 [X,Lys^5(atz),DLys^6(atz),ILys^8,DAla^{10}]GnRH; Azaline A	3.72 (27.0)	−34	7.08	0.23 (0.15–0.36)	2.0 (1/10), 2.0 (8/20)*	139 ± 8.7	0.48 (0.38–0.57)
8 [X,Orn^5(atz),DOrn^6(atz),ILys^8,DAla^{10}]GnRH	4.93 (25.8)	−43	6.20	0.2 (0.1–0.3)	2.0 (1/10)	158 ± 10	0.21 (0.14–0.32)
9 [X,Lys^5(atz),DLys^6(atz),ILys^8]GnRH	3.99 (27.6)	−27	5.53	0.12 (0.07–0.23)	1.0 (10/19), 2.5 (0/7), 3.0 (5/8)*	66 ± 3.9	0.86 (0.51–1.5)
10 [X,Lys^5(atz),DLys^6(atz),ILys^8,Pro^9-NHEt]GnRH	4.77 (33.3)	−24	7.58	0.36 (0.23–0.49)	1.0 (7/15), 2.5 (3.9)	64 ± 7.9	1.5 (0.13–18)
11 [X,Aph^5(atz),DAph^6(atz),ILys^8,DAla^{10}]GnRH; Azaline B	4.48 (30.6)	−33	9.00	1.3 (0.8–2.0)	0.5 (7/9), 1.0 (0/7)	224 ± 23	not //
12 [X,Aph^5(atz),DAph^6(atz),ILys^8]GnRH	4.01 (37.2)	−33	8.45	1.0 (0.65–1.5)	1.0 (9/16)	28 ± 5.9)	not //
13 [X,Aph^5(atz),DAph^6(atz),ILys^6,Pro^9-NHEt]GnRH	4.00 (32.4)	−30	11.9	1.6 (1.0–2.5)	1.0 (8/9), 2.5 (0.8)	83 ± 13	not //
14 [X,Aph^5(atz),DAph^6(atz),NMeLeu^7,ILys^8,DAla^{10}]GnRH	4.18 (31.0)	−29	10.6	1.6 (1.0–2.5)	0.5 (6/10), 1.0 (0/8)	34 ± 2.1	
15 [X,Aph^5(atz),DAph^6(atz),NMeLeu^7,ILys^8,Pro^9-NHEt]GnRH	4.24 (32.4)	−33	14.1	1.8 (1.1–3.0)	5.0 (5/5)		
16 [X,Aph^5,DAph^6,ILys^8]GnRH	4.62 (30.0)	−24		1.20 (0.78–1.84)	1.0 (6/7), 2.5 (0/6)	23 ± 4.5	
17 [X,NMeTyr^5,DLys^6(Nic),ILys^8,DAla^{10}]GnRH; A-75998	3.50 (30.0)	−51		1.42 (0.87–2.35)	4.0 (3/8), 2.0 (7/8)*, 4.0 (0/8)*	22 ± 3.2	

^{a} RT = Retention time in min, %CH$_3$CN. Peptides (10 μg/10 μL) dissolved in 0.1% TFA were applied to a Vydac C$_{18}$ column (5 mm, 300-Å pore size; 4.5 × 250 mm) under isocratic conditions, 0.1% TFA/H$_2$O with %CH$_3$CN shown, at a flow rate of 2.0 mL/min. UV detection was 0.1 AUFS at 210 nm. ^{b} c = 1 (weight of lyophilized peptide in 50% HOAc/H$_2$O). ^{c} Retention time in minutes. Peptides (0.5 mg/mL, 10 μL) dissolved in TEAP pH 7.0/CH$_3$CN (76%/24%) were applied to the same Vydac C$_{18}$ column described above. All peptides were soluble in the above buffer except for Antide (3) which was loaded in an acidified (H$_3$PO$_4$) buffer. A gradient was run between 30% and 48% CH$_3$CN in 30 min at a flow rate of 1.5 mL/min. UV detection was 0.1 AUFS at 210 nm. Under these conditions, the agonists [DTrp^6,Pro^9-NHEt]GnRH and [DHis(N^{im}Bzl)^6,Pro^9-NHEt]GnRH had retention times of 7.55 and 7.85 min, respectively. ^{d} Relative to [Ac-Δ^{3}Pro^1,DFpa^2,DTrp^{3,6}]GnRH = 1.0. ^{e} AOA = antiovulatory assay: dosage in μg/rat (rats ovulating/total). (*) Vehicle was corn oil. Other wise peptides were dissolved in ca. 1% DMSO/saline. ^{f} ED$_{50}$ for [Ac-DNal^1,DFpa^2,DTrp^3,DArg^6]GnRH (internal standard) was 0.17 ± 0.01 μg/mL. ^{g} In binding studies, the K_D for the potent agonist [DAla^6,NMeLeu^7,Pro^9-NHEt]GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_D values were calculated from the potencies of the analogues (relative to the standard) determined from displacement data.^{24} Not // = not parallel.

in analogues (Antide being the prototype) with a desirable therapeutic ratio (high potency in the AOA and an in vitro histamine release $ED_{50} \geq 300$ $\mu g/mL$ for Antide (3) when a value of ca. 150 $\mu g/mL$ was found for GnRH itself).[4] This led us to synthesize a similar series of position 5,6-substituted cyanoguanidino- and N-triazolyl-containing analogues[9] which were readily soluble in aqueous solutions at pH 6.4. Results presented in that report did not address the issue of duration of action. SB-75 (similar in structure to a large family of analogues that include the Nal-Arg antagonist[28]) and Antide were both reported to be longer acting than Nal-Glu;[15,29] SB-75 and Antide are also being studied in a clinical setting.

If more effective GnRH antagonists are to be designed, a clear knowledge of those structural elements responsible for binding to the GnRH receptor, those which conferred enzymatic stability and those which retarded elimination, is to be obtained. First, an order of hydrophobicity was derived from chromatographic studies (see Table I) for possible correlation purposes with biopotencies or duration of action. Second, intrinsic binding affinity and relative ability to inhibit release of LH were measured in vitro (Table I). Third, the castrated male rat was chosen as the animal model, and levels of LH were measured at different times after injection of the peptide under different regimes of administration in order to identify the best protocol for the in vivo testing of these analogues. Indeed, if all these analogues had different pharmacokinetics in vivo, it would be desirable to identify those parameters that determine long duration of action as they may be crucial to the successful commercial development of these already acknowledged potent and versatile therapeutic and contraceptive agents.

The order of elution under neutral conditions on a C_{18} RPHPLC column is as follows: 9 < 8 < 7 < 10 < 12 < 11 < 4 < 14 < 5 < 13 < 3 < 15 = 2 < 6 < 1 with 9 being the most hydrophilic analogue. Interestingly, all analogues reported here are equipotent in the pituitary cell culture assay, with the exception of 7–10 which are approximately 3–5 times less potent. Affinity of these analogues for their receptor are also all within the same range, with the exception of 6 and 11–13 which show nonparallel but internally consistent dose–response curves. Similarly in the AOA, all compounds are equipotent showing 100% inhibition in the range of 1.0–3.0 $\mu g/rat$ with the exception of 15 and 17. The fact that some of these analogues have different ED_{100} in the AOA depending on whether they were injected in an aqueous buffer or in corn oil should be noted. These results make it clearly impossible to draw a correlation between hydrophobicity as measured by RPHPLC retention times and observed biological potencies. We also compared the ability of the various analogues to inhibit LH secretion in castrated rats. As shown in Figure 1, panel A, Antide (3) was found to be significantly longer acting than Nal-Glu (1) and Azaline A (7). Indeed, 10 μg of the Nal-Glu antagonist injected sc in 0.2 mL lowered LH levels significantly for only 10 h whereas increasing the dose to 50 μg extended the duration of action to 24 h. Azaline A was even shorter acting than Nal-Glu,

and substitution of the C-terminal DAla-NH_2 by a Gly-NH_2 or an N-ethylamide (Figure 1, panel B) did not make any significant difference in either duration of action or hydrophobicity. We then tested the hypothesis that the long duration of action of Antide might be due to its hydrophobicity and propensity to depot after sc injection. As shown in Table I, the most hydrophobic of all peptides in this series was the Nal-Glu antagonist, which had a relatively short duration of action. Unexpectedly, and yet to be explained, was the long duration of action of Azaline B and its modified C-terminal homologue 12 which were more hydrophilic than Antide (Figure 1, panel B). In a second experiment (Figure 1, panels C and D), we investigated the duration of action of Antide, (3), Azaline B (11), and [NMeLeu⁷]Azaline B (14) at two dosages (10 and 50 $\mu g/rat$) and of 6 and 13 at a single dose (50 $\mu g/rat$). Both Antide and Azaline B were equipotent at both doses; interestingly, the addition of the NMeLeu⁷ substitution which was recognized in some agonists to confer enzymatic stability,[30] had a slight but significantly detrimental effect on duration of action. [Pro⁹-NHEt]Azaline B (13) also had a duration of action shorter than that of the parent Azaline B. Another compound, SB-75 (4), reported by Bokser et al.[15] to be long acting, was indeed found to have a very long duration of action (Figure 1, panel E). This analogue is closely related to 6 except for the substitutions at positions 6 and 8. Since the ILys residue in position 8 was unlikely to be detrimental to long duration of action (see 3 and 11), we suggest in agreement with Bokser et al.[15] that the observed long duration of action of 4 as compared to that of 6 (Figure 1, panel C) was due to an as yet undefined and favorable role played by the citrulline residue in position 6. Finally, 2 was also found to be relatively short acting (data not shown).

These results led us to investigate the possibility that the concentration at which any one of these peptides was injected might play a role in modulating the duration of action of these analogues. In Figure 1, panel F, we present data on three peptides injected in 1 mL of 0.04 M phosphate buffer containing 0.1% BSA (instead of 200 μL) at three loci instead of one (total amount of 10 or 50 μg). Interestingly, comparison of the data for Azaline B in this assay with those obtained in previous assays (see Figure 1, panels B, D, and E) indicated that the dilution had no measurable effect on the duration of action. On the other hand, comparison of the results on SB-75 under these dilute conditions and the concentrated conditions shown in Figure 1, panel E, indicated that the duration of action of this analogue appeared to be a function of its concentration. Indeed, SB-75 (4) injected at 50 μg (single 200-μL injection at one site only) significantly ($P < 0.01$) lowered plasma LH levels for at least 72 h. In contrast, this analogue injected at a total dose of 50 μg administered in three equal injections of 330 μL each at three different sites only altered LH secretion for a very short time. We therefore investigated the issue of duration of action after iv administration (Figure 2) to eliminate factors associated with depot formation and differential release from the injection sites if such was the problem. Under these conditions, major differences were found in the duration of action of the analogues tested. Azaline B, injected iv at 50 μg provided a significant ($P \leq 0.01$) inhibition of LH

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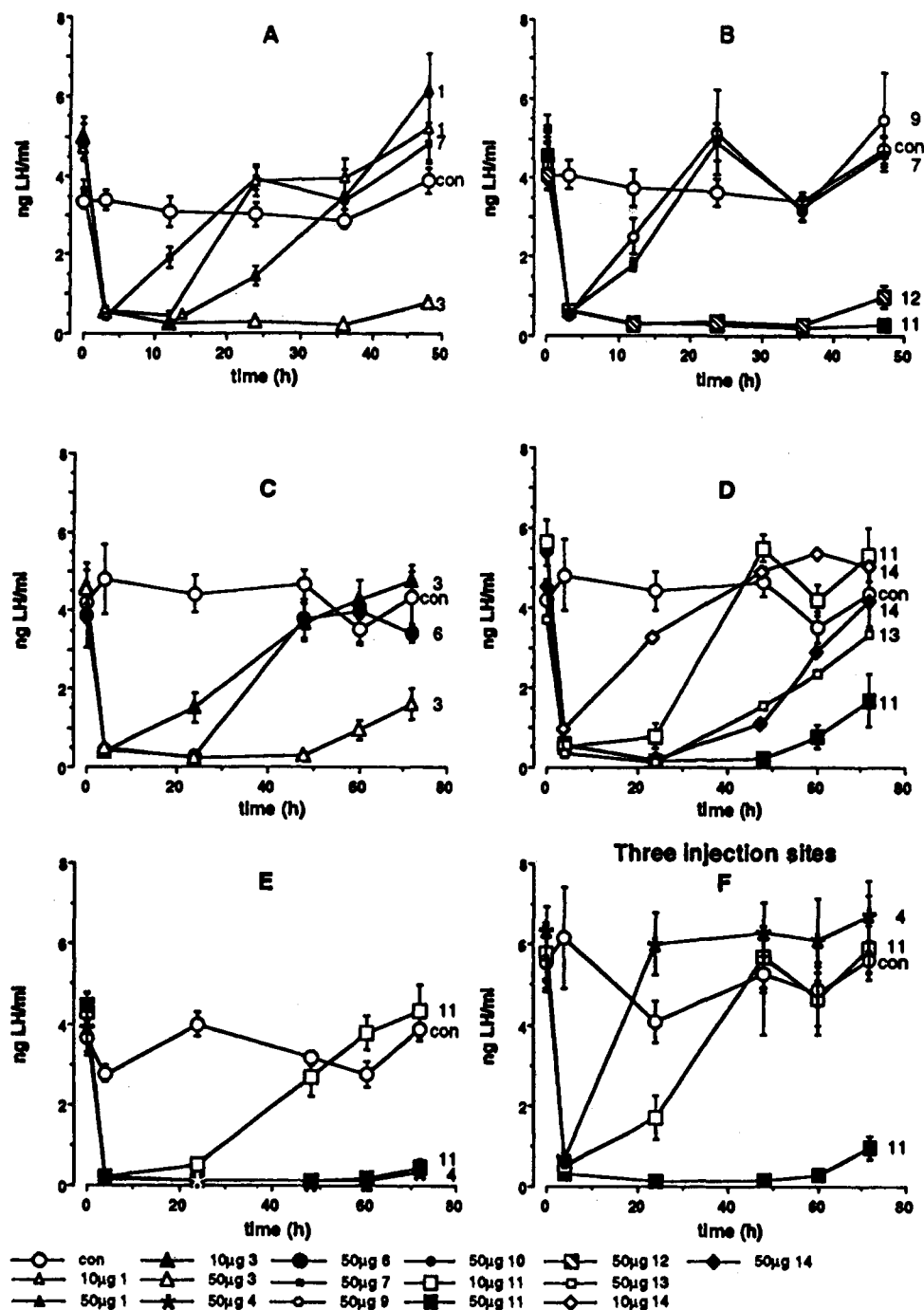
Figure 1. Inhibition of LH secretion after sc administration of analogues at one (panels A–E) or three injection sites (panel F). Total dose of 10 or 50 μg in all cases. Blood samples were collected at the times shown on the abscissa. The SEM, where not appearing, are encompassed within the size of the symbols in the graphs. Compound numbers as well as unique symbols are used to identify each response. Con = control. Mean weights of rats = 318 ± 17 g.

secretion for approximately 24 h (data not shown). At a dose of 250 μg, this analogue was active for ≥60 h. Antide (3) at 250 μg had lost its effectiveness within 24 h. RS-26306 (5) (250 μg), although significantly less effective at inhibiting the secretion of LH than Azaline B, partially suppressed LH secretion at 48 h and even less so at 60 h (Figure 2). SB-75 at 250 μg, on the other hand, was found to be inactive at 48 h with only partial inhibition at 24 h (Figure 2). Interestingly, all peptides tested for stability against pronase and subtilisin (1, 3–5, and 11) were found to be stable with the exception of Nal-Glu (1) which was less stable to pronase with ca. 30% conversion into two fragments overnight.

It should be noted that the values obtained for each point in the five assays presented or described here have very low SEM. In addition, in the assays where the same two compounds were tested under the same conditions (for example Azaline B in Figure 1, panels B, D, and E or Antide in Figure 1, panels A and C), almost identical results were obtained. This suggests that the observations made on the different analogues are intrinsic to the peptides and should be investigated further for the development of a rational SAR.

We have presented unusual and potentially important observations with regard to the duration of action of the different GnRH analogues. Binding to plasma proteins was suggested to play an important role in the differences in pharmacokinetics of GnRH and its potent agonist Nafarelin ([DNal[6]]GnRH)[31] yet there is no such evidence for GnRH antagonists. Similarly, a tentative correlation
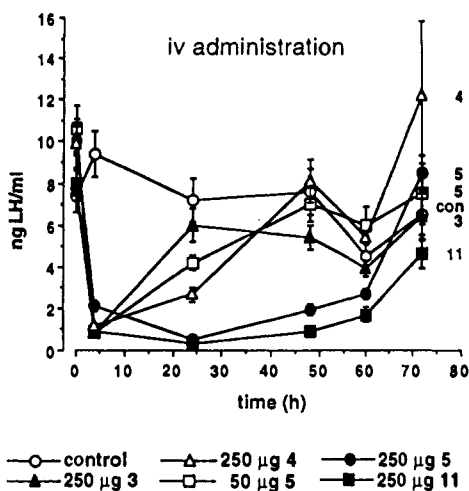
Figure 2. Inhibition of LH secretion after iv administration of analogues (50 or 250 μg). See legend of Figure 1 for other details.

between long duration of action and stability to enzymatic digestion (pronase and subtilisin) was not obtained.

Most analogues were also tested for their histamine releasing activity. Since this is an in vitro assay, a correlation between histamine release potency and long duration of action cannot be addressed. Notable however, is the fact that Azaline B (a peptide that is both long acting under different sets of conditions and is readily soluble, at concentrations up to 20 mg/mL in aqueous solutions in the presence of 3–5% mannitol and 5% ethanol at pH 6.3) can be synthesized easily in large quantities in a manner similar to that used for Azaline A.[32] A clear advantage of Azaline B over Antide is its long duration of action after iv injection suggesting a very long half-life in biological fluids and its solubility under a number of quasi-physiological conditions. This desired property may allow its introduction in a number of different delivery systems and has facilitated its purification and characterization considerably. Finally, when compared to Nal-Arg (ED_{50} = 0.17 ± 0.01 μg/mL: AOA ED_{100} = 1.0 μg), the other analogues such as Nal-Glu (ED_{50} = 1.8 ± 0.66 μg/mL: AOA ED_{100} = 1.5 μg), SB-75 (ED_{50} = 2.1 ± 0.3 μg/mL: AOA ED_{100} = 2.0 μg), RS-26306 (ED_{50} = 11 ± 1.1 μg/mL: AOA ED_{100} ≥ 2.5 μg), or the recently disclosed A-75998 (ED_{50} = 22 ± 3.2 μg/mL: AOA ED_{100} = >4.0 μg) may seem to be significantly less potent at releasing histamine; yet we think that a greater therapeutic index than that obtained for these compounds should be sought. We have described here some of the pharmacologic properties (including long duration of action after iv administration) of Azaline B (ED_{50} = 224 ± 23 μg/mL: AOA ED_{100} = 1.0 μg) which is 10–100 times less potent at releasing histamine than most of the above compounds and either equipotent or twice as potent (when administered in aqueous solutions) at inhibiting ovulation in the rat as any of the other GnRH analogues being presently tested in the clinic.

Conclusion

GnRH analogues were synthesized on a solid phase support with a two-step modification of the N^ω-NH_2 of

ornithine, lysine, or *p*-aminophenylalanine residues in otherwise protected resin-bound peptides. Introduction of the N^ω-triazolyl moieties into GnRH analogues yielded several water-soluble antagonists which showed a desirable therapeutic ratio (low histamine release activity to high in vivo potency and duration of action). Introduction of the Aph(atz) at positions 5 and 6 selectively led to extended duration of action. Azaline B (11) inhibited ovulation in the rat by 100% at 1 μg/rat with an ED_{50} in the in vitro histamine release assay of 224 μg/mL while Azaline A (7) inhibited ovulation in the rat by 90% at 2 μg/rat with an ED_{50} in the in vitro histamine release assay comparable to that of GnRH itself (150 μg/mL). At the same dose of 50 μg, however, Azaline B was effective for 72 h as compared to 24 h for Azaline A in the castrated male rat. Such long duration of action for Azaline B was sustained either iv or sc, even after dilution prior to injection at three sites, a property that seems to be unique to this particular compound. Further substitutions in positions 7 or 10 of Azaline B (NMeLeu[7] and Gly[10]-NH_2 or Pro[9]-NHEt) were deleterious (short duration of action and comparatively low affinity and potency).

Experimental Section

Instruments. Preparative RPHPLC was accomplished using a Waters Assoc. (Milford, MA) Prep LC/System 500A and Model 450 variable wavelength UV detector, Fisher (Lexington, MA) Recordall Model 5000 strip-chart recorder and an Waters Prep LC 500A preparative gradient generator. Analytical RPHPLC 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-Arg(Tos), Boc-Leu, Boc-Orn(Fmoc), Boc-DOrn(Fmoc), Boc-Lys(Fmoc) and Boc-DLys(Fmoc), Boc-Pro, Boc-Ser(Bzl), Boc-DTrp, Boc-DCit, and Boc-Tyr(2-BrZ) were obtained from Bachem Inc. (Torrance, CA). Boc-DNal, Boc-DCpa, Boc-DPal, Boc-ILys(Z)·DCHA, Boc-NicLys, Boc-DNicLys, Boc D-Har($N^g,N^{g'}$-Et$_2$) acetate and L-Har($N^g,N^{g'}$-Et$_2$) acetate were synthesized at the Southwest Foundation for Biomedical Research (under Contract NO1-HD-6-2928 with NIH) and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. 1,1-Dichloro-1,1-diphenoxymethane, diphenyl cyanocarbonimidate (PCI), 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.[9] The methylbenzhydrylamine and (N-ethylamino)methyl resins used for peptide synthesis were obtained according to published procedures.[6,26] 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[33] on a Beckman 990 peptide synthesizer with use of previously described protocols on the methylbenzhydrylamine (MBHA) and (N-ethylamino)methyl (NEAM) resins (approximately 1

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g/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). 3-Fold excess protected amino acid was used based on the original substitution of the methylbenzhydrylamine or (*N*-ethylamino)methyl resins. N-Terminal acetylation was performed using the same protocol as that used for capping (excess acetic anhydride in DCM).

Compounds 1, 2, and 4–6 were synthesized and purified as previously described.[6]

[Ac-DNal[1],DCpa[2],DPal[3],Lys(nicotinyl)[5],DLys(nicotinyl)[6],ILys[8],DAla[10]]GnRH, Antide (3). The fully protected [Ac-DNal-DCpa-DPal-Ser(Bzl)-Lys(*N*ᵉ-Fmoc)-DLys(*N*ᵉ-Fmoc)-Leu-Lys(*N*ᵉ-isopropyl,*N*ᵉ-Z)-Pro-DAla]-MBHA resin was synthesized manually on 40 g of a methylbenzhydrylamine resin (0.76 meq NH_2/g) using solid phase peptide synthesis (SPPS) techniques[34] and an N^α-Boc strategy with the following side-chain protecting groups: Lys(Fmoc), Lys(*N*ᵉ-isopropyl,*N*ᵉ-Z), and Ser(Bzl). The individual amino acids were incorporated in a sequential manner utilizing either diisopropylcarbodiimide or BOP[35]-mediated activation 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.[36] The *tert*-butyloxycarbonyl (Boc) group was removed after each coupling cycle by treatment of the growing peptide-resin with 60% TFA in DCM in the presence of 1% ethanedithiol. This protected, resin-bound peptide was further derivatized to yield Antide as described below. The Fmoc-protecting groups were removed by treatment of the fully-protected, resin-bound Ac-DNal-DCpa-DPal-Ser(Bzl)-Lys(*N*ᵉ-Fmoc)-DLys(*N*ᵉ-Fmoc)-Leu-Lys(*N*ᵉ-isopropyl,*N*ᵉ-Z)-Pro-DAla- prepared above, with 20% piperidine in DMF (5 and 25 min). Acylation of the free *N*ᵉ-amino functions with nicotinic acid/diisopropyl-carbodiimide (110 mmol, 2.2 equiv of each) in DMF gave the protected and resin-bound Antide. Liberation of the desired peptide from the resin and its protecting groups through the action of anhydrous HF (ca. 300 mL) at 0 °C in the presence of anisole (30 mL) yielded, after concentration, extraction, and lyophilization, 35.5 g of crude Antide; this was subsequently purified by RPHPLC procedures as previously described[37] to give, after lyophilization, 11.3 g of highly purified Antide (3).

Compounds 7–9, 11, 12, 14, and 16 were synthesized and purified as previously described.[9]

[Ac-DNal[1],DCpa[2],DPal[3],Lys(atz)[5],DLys(atz)[6],ILys[8],Pro[9]-NHEt]GnRH (10). Ac-DNal-DCpa-DPal-Ser(Bzl)-Lys(Fmoc)-DLys(Fmoc)-Leu-ILys(Z)-Pro was assembled on an (*N*-ethylamino)methyl (NEAM) resin (1% cross-linked, 1 g) as described earlier.[26] After deprotection of the Fmoc protecting group as described above, the resin was treated with diphenyl cyanocarbonimidate (PCI, 2.4 g, 10 mmol) in enough DMF to obtain a free-flowing slurry. The mixture was agitated 12 h and washed. After

this step, the ninhydrin test was negative. The resin was then treated with 2 mL (excess) of hydrazine in DMF and then agitated for 8–12 h. The resin was washed, dried, and cleaved with HF in the presence of anisole at room temperature for 3–4 h. The peptide was extracted and lyophilized to yield 0.4 g of a white fluffy solid. Analogues 13 and 15 were similarly synthesized.

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 RPHPLC cartridge packed in our laboratory using a 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% A) with an appropriate gradient (90 min) such that retention time was ca. 45 min.[38] The collected fractions were screened by use of analytical RPHPLC under isocratic conditions, 0.1% TFA/H_2O/CH_3CN at a flow rate of 2.0 mL/min (Vydac C_{18} column, 5 μm, 300-Å pore size, 4.5 × 250 mm). Appropriate fractions were then combined and converted to the acetate salt by loading after dilution (1/1) in water on a preparative RPHPLC 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 RPHPLC. Purity of the peptides was assessed using RPHPLC under conditions reported in Table I.

Amino Acid Analysis. Amino acid analysis of the peptides was performed following hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h. A Perkin-Elmer LC system composed of two Series 10 LC pumps, an ISS-100 sample injector, a TRC 1 column oven, a Kratos Spectroflow 980 fluorescence detector, and an LCI-100 integrator were used as described earlier.[39]

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 25–30 kV was employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix.

Optical Rotations. Optical rotations were measured in 50% 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).

Enzymatic Digests. Pronase and subtilisin were purchased from Boehringer-Mannheim. Digests of 1, 3, 4, 11, and RS-26306 were carried out at an approximate molar ratio of pronase or subtilisin (0.25 μg)/peptides (3.0 μg) of ca. 1/12 in two buffer systems, 0.1 M phosphate buffer pH 8.0 and 0.1 M Tris buffer pH 6.0 (50 μL) at 37 °C for 24 h. Peptide digests were monitored by RPHPLC using a 0.1 % TFA/acetonitrile buffer system.

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Biological Testing. In vitro, the peptides were tested for their ability to inhibit GnRH-mediated LH secretion by cultured pituitary cells. Potencies of GnRH antagonists were expressed relative to a standard ([Ac-Δ^3-Pro^1,DFpa^2,DTrp^{3,6}]GnRH).[23] In binding studies, the K_D for the potent agonist [DAla^6,NMeLeu^7,Pro^9-NHEt]GnRH (taken as standard) was determined from a Scatchard analysis to be approximately 0.3 nM. All the other K_D values were calculated from the potencies of the analogues (relative to the standard) determined from displacement data.[24] The assay for histamine release by rat mast cells has been reported previously.[4,40] The AOA was carried out as described by Corbin and Beattie[22] using an aqueous vehicle unless stated otherwise; cycling rats (250–300 g at the time of the assay) were injected sc with the peptides dissolved in saline or 5% DMSO in saline (200 μL) at noon on proestrus. Results are expressed in terms of the dosage in μg/rat (rats ovulating/total number of treated rats). Measurement of circulating LH levels was similar to that reported earlier.[41–43] Male Sprague-Dawley rats were castrated 7–10 days prior to the start of the experiment.

The peptides were first dissolved at a concentration of 1.0 or 10 mg/mL in bacteriostatic water and then further diluted in 0.04 M phosphate buffer containing 0.1% BSA. Antide was first acidified with 2 N HOAc until in solution. Subsequent dilutions were made in phosphate buffer. The analogues were injected sc or iv, and blood samples (300 μL) were collected under metotane anesthesia (see Figure 1). Sera (50 μL) were tested for LH levels in duplicate using reagents provided by the National Pituitary and Hormone Distribution Program of the NIDDK.[44]

Supplementary Material Available: A table of mass spectral data for compounds 1–17 (1 page). Ordering information is given on any current masthead page.

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