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Synthesis, in vivo and in vitro biological activity of novel azaline B analogs

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Abstract—Several azaline B analogs (**2**–**10**) were synthesized and evaluated for their ability to antagonize GnRH in vitro and for duration of action in inhibiting luteinizing hormone secretion in a castrated male rat assay in vivo. Analogs, **8** (IC_{50} = 1.85 nM), and **9** (IC_{50} = 1.78 nM), are equipotent with azaline B (**1**, IC_{50} = 1.36 nM) in vitro. Whereas **9** is short acting, **8** is as long acting as azaline B. Other analogs have IC_{50} greater than 2.0 nM and are all short acting.

Gonadotropin-releasing hormone (GnRH) is a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) isolated and characterized[1–3] by the groups of the 1977 Nobel laureates, Schally and Guillemin. GnRH is synthesized in the hypothalamus and acts upon the receptors in the anterior pituitary where it triggers the synthesis and release of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH).[4] GnRH superagonists and antagonists have been widely studied[5] for the treatment of prostate and breast tumors, uterine fibroids, precocious puberty, endometriosis, premenstrual syndrome, contraception and infertility. GnRH antagonists may have a clinical advantage over superagonists because they lower gonadal sex hormone levels almost immediately and avoid the initial upregulation of the gonadotropin-gonadal axis (flare effect) caused by the use of superagonists.[6]

Azaline B (**1**), discovered in our laboratory,[7] is one of the most potent and long-acting GnRH antagonists. Because of its limited solubility in aqueous buffers, its clinical development was abandoned generating a need for more potent antagonists with improved physicochemical properties, such as ease of formulation for acute or slow release, extended duration of action and economical simpler synthesis. All of these properties may be fulfilled by closely related analogs of azaline B. Herein we describe a series of azaline B derivatives (Ac-D-2Nal[1]-D-4Cpa[2]-D-3Pal[3]-Ser[4]-4Aph(X)[5]-D-Aph(Cbm)[6]-Leu[7]-ILys[8]-Pro[9]-D-Ala[10]-NH_2) wherein the ω-amino function of the 4-aminophenylalanine (Aph) at position 5 was acylated with N^{α}-carbamoylated amino acids, dicarbamoylated diamino acids and with different carboxylic acids bearing lactam/urea functionalities. The acylated groups (X) at position 5 were introduced to (i) increase the number of potential intra- and intermolecular hydrogen bonding sites on the peptide side chains for structural stabilization and peptide/receptor/plasma protein interactions, (ii) increase solubility in aqueous buffers, and (iii) decrease the propensity of azaline B and congeners to form gels under certain conditions.[8] The effects of these substitutions on the biological activity were evaluated in vitro and in vivo.

Azaline B (**1**) was synthesized as previously described.[7] All of the other analogs (**2**–**10**, Table 1) were derived from the resin-bound peptide precursor [Ac-D-Nal-D-Cpa-D-Pal-Ser(Bzl)-Aph(N^{ω}-Fmoc)-D-Aph(Cbm)-Leu-ILys(N^{α}-Z)-Pro-D-Ala]-MBHA resin, synthesized manually on *p*-methylbenzhydrylamine resin (1 g, 0.33 mequiv NH_2/g) using solid phase peptide synthesis (SPPS) techniques[9] and N^{α}-Boc strategy. In the synthesis of **2**–**4**, the Fmoc protection on Aph at position 5 was removed by the treatment with 30% piperidine in *N*-methylpyrrolidone

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Table 1. Physicochemical characterization and biological activities of GnRH antagonists

No	Compound	Purity		t_R[c] (min)	MS[d] (M + H)$^+$		pIC$_{50}$[e] Ave ± SEM	IC$_{50}$ (nM)[f]	Duration of action[g]
		HPLC[a]	CZE[b]		Calc.	Obs.			
1	[Ac-D-2Nal^1, D-4Cpa^2, D-3Pal^3, 4Aph(Atz)^5, D-4Aph(Atz)^6, ILys^8, D-Ala^{10}]GnRH (azaline B)	98	99	27.7	1612.8	1612.7	8.9 ± 0.07	1.36	Long
2	[Aph(N^{α}-Cbm-Asn)^5, D-Aph(Cbm)^6]azaline B	95	98	26.7	1648.8	1648.8	8.7 ± 0.17	2.22	Short
3	[Aph(N^{α}-Cbm-DAsn)^5, D-Aph(Cbm)^6]azaline B	96	95	26.8	1648.8	1648.8	8.6 ± 0.10	2.37	Short
4	[Aph(N^{α}-Cbm-Gln)^5, D-Aph(Cbm)^6]azaline B	98	96	26.6	1662.8	1662.8	8.2 ± 0.02	6.86	Short
5	[Aph(N^{α},N^{β}-di-(Cbm)Dap)^5, D-Aph(Cbm)^6]azaline B	95	95	27.2	1663.8	1663.8	8.2 ± 0.01	6.37	Short
6	[Aph(N^{α},N^{β}-di-(Cbm)D-Dap)^5, D-Aph(Cbm)^6]azaline B	96	97	27.3	1663.8	1663.7	8.5 ± 0.19	2.82	Short
7	[Aph(N^{α},N^{γ}-di-(Cbm)Dab)^5, D-Aph(Cbm)^6]azaline B	95	95	27.1	1677.8	1677.8	8.5 ± 0.08	3.53	Short
8	[Aph(L-pGlu)^5, D-Aph(Cbm)^6]azaline B	99	97	28.1	1602.8	1602.8	8.7 ± 0.11	1.85	Long
9	[Aph((L-tetrahydro-pyrimidine-2-one)-6-carboxyl)^5, D-Aph(Cbm)^6]azaline B	95	98	28.3	1617.8	1617.8	8.7 ± 0.00	1.78	Short
10	[Aph(thymine-1-acetyl)^5, D-Aph(Cbm)^6]azaline B	99	98	30.2	1657.8	1657.7	8.6 ± 0.5	2.46	Short

Abbreviations: Ac, acetyl; Aph, 4-aminophenylalanine; Atz, [5′-(3′-amino-1*H*-1′,2′,4′-triazolyl)]; Cbm, carbamoyl; 4Cpa, 4-chlorophenylalanine; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; ILys, N^{ε}-isopropyllysine; 2Nal, 3-(2-naphthyl)alanine; 3Pal, 3-(3-pyridyl)alanine, pGlu, pyroglutamic.

[a] Percentage purity determined by HPLC using buffer system A; TEAP, pH 2.30, buffer system B, 60% CH_3CN/40% A under gradient conditions (30–80% B over 50 min), at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5 μm particle size, 300 Å pore size). Detection at 214 nm.

[b] Percentage purity determined by capillary zone electrophoresis (CZE) using a Beckman P/ACE System 2050 controlled by an IBM Personal system/2 model 50Z; field strength of 15 kV at 30 °C. Buffer, 100 mM sodium phosphate (85:15, H_2O:CH_3CN), pH 2.50, on a Agilent μSil bare fused-silica capillary (75 μm i.d. × 40 cm length). Detection at 214 nm.

[c] Retention times under gradient conditions (30–80% B over 50 min); buffer system A; TEAP, pH 7.0, buffer system B, 60% CH_3CN/40% A.

[d] Mass spectra (MALDI-MS) were measured on an ABI-Voyager DESTR instrument using saturated solution of α-cyano-4-hydroxycinnamic acid in 0.3% trifluoroacetic acid and 50% acetonitrile as matrix. The calculated $[M+H]^+$ of the monoisotope compared with the observed $[M+H]^+$ monoisotopic mass.

[e] The pIC$_{50}$ is the negative log of the IC$_{50}$ in molar, as determined in the GnRH reporter gene assay.

[f] IC$_{50}$ is the concentration of antagonist required to repress the GnRH induced response by 50% in the reporter gene assay in HEK-293 cells expressing the human GnRH receptor and a GnRH-responsive stably integrated luciferase reporter gene. The values shown represent the geometric mean from two independent experiments.

[g] Castrated male rat assay. Duration of action: long = over 80% inhibition of LH release at 72 h but not at 96 h; short = over 80% inhibition of LH release at 3 h but not at 72 h.