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β-Sulfonamido gonadotropin-releasing hormone analogs: synthesis and evaluation of several parent hormone properties‡

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Abstract: With the aim of producing long-acting analogs of gonadotropin releasing hormone (GnRH), four analogs, containing -$X^6{}_{aa}\psi(CH_2SO_2NH)$-$Leu^7$ building unit (X_{aa}=Gly, Ala, Val or Phe), and a reduced-size analog [Des-Tyr^5]-GnRH which includes the unit $Phe^5\psi(CH_2SO_2NH)$-Leu^6, and [β-Ala^6]-GnRH were synthesized. The peptides were evaluated for their capacity to induce LH-release from rat pituitary cells and to withstand proteolysis by pituitary-derived enzymes, compared with the parent peptide GnRH. Albeit stable toward enzymatic degradation, the sulfonamido containing peptides were only marginally bioactive. [β-Ala^6]-GnRH, however, induced LH-release and bound to pituitary receptors nearly as efficiently as GnRH. This analog was also highly stable toward proteolysis suggesting that it may serve as a long-acting GnRH-analog. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GnRH analogs; β-sulfonamido surrogates

INTRODUCTION

The decapeptide gonadotropin-releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a key integrator between the nervous and endocrine systems and plays a central role in regulating the reproductive system. GnRH controls the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which, in turn, stimulate gonadal steroidogenesis and gametogenesis [1,2]. Chronic administration of GnRH or its agonists leads to desensitization of GnRH receptors with the consequent suppression of gonadotropin secretion [3,4]. This finding has led to the development and investigation of numerous peptidic and non-peptidic GnRH-related molecules to be employed for the therapy of various hormone-dependent diseases such as prostate and breast cancer [5], as well as for contraception [6]. Many of these compounds are GnRH-agonists possessing modifications at the Gly^6-Leu^7 domain known to be associated with the proteolysis of GnRH by hypothalamic and pituitary endopeptidases [7,8]. Stabilization of GnRH, as well as other peptides, toward enzymatic degradation might have a marked importance for the production of metabolically stable, long-acting relevant drugs. To this end, the present study initiated the synthesis of GnRH-analogs in which a β-sulfonamido moiety, i.e. -CONH-, is at the 6-position of GnRH.

The synthesis is reported of four analogs of GnRH containing the -$X_{aa}{}^6\psi(CH_2SO_2NH)Leu^7$-sequence ($X_{aa}$ = Gly, Ala, Val and Phe), a reduced-size, i.e. [Des-Tyr^5]-GnRH analog of GnRH Phe^5 possessing the =$Phe^5\psi(CH_2SO_2NH)Leu^6$ unit, and [β-Ala^6]-GnRH. The latter analog was prepared for the purpose of comparison with [$Gly^6\psi(CH_2SO_2NH)$]-GnRH, i.e. possessing -CH_2CH_2CONH- and -$CH_2CH_2SO_2NH$- moieties, respectively, and for examining the net effect of GnRH-chain extension by a methylene group, i.e. -CH_2CH_2CONH- in [β-Ala^6]-GnRH vs -CH_2COHN, in GnRH. The capacity of these analogs to induce LH-release from rat pituitary as well as their stability toward pituitary-mediated proteolysis is reported.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade. Trifluoroacetic acid (TFA) used for the high performance liquid chromatography (HPLC) and N,N^1-dimethylformamide (DMF) used

Abbreviations: DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; GnRH, gonadotropin-releasing hormone; HPLC, high pressure liquid chromatography; NMM, N-methylmorpholine; PyBOP, benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium-hexafluorophosphate; TES, triethyl silane; TFA, trifluoroacetic acid

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‡ This paper is dedicated to the memory of Professor Arno Spatola, a dear friend and a leader in peptide science

for the peptide synthesis were obtained from Merck (Darmstadt, Germany); N-α-9-fluorenyl-methoxycarbonyl (Fmoc)-protected amino acid derivatives and Rink-amide resin were purchased from Novabiochem (Laufelfingen, Switzerland). Fmoc-amino acids were side chain protected as follows: Arg, 2, 2, 5, 7, 8-pentamethyl-chromane-6-sulfonyl chloride (Pmc); His, trityl (Trt); Trp *tert*-butyloxycarbonyl (Boc); Ser *tert*-butyl (tBu); Tyr *tert*-butyl (*t*Bu).

Melting points (Buchi 540 apparatus) were uncorrected. Spectra were recorded as follows: IR spectra ($CHCl_3$) on a Perkin-Elmer 'Spectrum RX I' spectrophotometer; nanospray ionization mass spectra performed on LCQ system (Finnigan, Bremen, Germany); ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra on a Varian XL300 spectrometer (the spectra were recorded in $CDCl_3$ and assignments are relative to TMS as internal standard. Optical rotations, $[\alpha]_D$, were determined using a Schmidt-Haensch 1604 polarimeter. TLC analysis was performed on Merck pre-coated silica gel 60 F-264 (0.25 mm) plates. Column chromatography was performed on Merck Kieselgel 60 (40–60 μm). HPLC purification of peptides was performed using a Spectra-Physics SP8800 liquid chromatography system, and the column effluents were monitored with an Applied Biosystem 757 variable wavelength absorbance detector. The columns used were: HPLC prepacked Lichrocart containing Lichrosorb 100 RP-18 (250-10 mm, 7 μm, Merck, Darmstadt, Germany) for semipreparative purifications, and Lichrospher 100 RP-18, 250-4 mm (5 μm) and wide pore butyl (C4, 5 μm), 250-4.6 mm (J. T. Baker Inc., Phillipsburg, NJ) for analytical separations. Lyophilized peptides were hydrolysed in vacuum with a 6N HCl solution at 110 °C for 22 h. Amino acid analysis was performed using a Dionex automatic amino acid analyser.

Peptide Synthesis

GnRH and all GnRH modified analogs were prepared by manual solid-phase peptide synthesis. The peptides were synthesized on Rink-amide resin (25 μmole scale; 53 mg) using Fmoc chemistry [9]. The chemical operations were performed in sintered plastic syringes at room temperature. Couplings were achieved by reacting 4 Fmoc-AA-OH equivalents with 4 equiv. of PyBOP and 8 equiv. of NMM in DMF. The same strategy was used for the coupling of Fmoc-sulfonamido dipeptide derivatives. The reaction mixtures were shaken in a mechanical shaker for 50 min at room temperature and the procedure was repeated twice for each coupling step. After 2 h, the resins were washed with DMF and DCM. The completion of the coupling reaction was indicated by a negative ninhydrin test. A solution of piperidine (20%) in DMF was used to remove the Fmoc. The final decapeptides were deprotected and cleaved from the resin using a solution of TFA/phenol/H_2O/thioanisole/1,2-ethanedithiol/TES (81.5 : 5 : 5 : 2.5 : 1, v/v). After 2 h at room temperature, the cleavage solution was collected and the crude peptides were precipitated from the solution with peroxide-free dry ether at 0 °C. After several washings with ether, the precipitated peptides were dissolved in a solution of water or water/acetonitrile with 1% of TFA and then lyophilized.

HPLC purifications and analyses were achieved by using a linear gradient established between 0.1% TFA in water as buffer A and 0.1% TFA in 75% aqueous acetonitrile as buffer B. The eluent composition was 20%–80% B over 40 min using the RP-18 column. Fractions containing the GnRH analogs were lyophilized. Mass spectrometry: ψGly [GnRH] (**1**) m/z 1232.89 (MH+), (calculated 1231.56, C_{55}, $H_{77}N_{17}O_{14}S$) Ser: 0.93; Glu: 0.92; Gly: 1; His: 0.96; Arg: 1.01; Pro: 0.89; Tyr: 0.85; Leu: 0.10; ψAla[GnRH] (**2**) m/z 1246.87 (MH+), (calculated 1245.57, C_{56}, $H_{79}N_{17}O_{14}S$); Ser: 0.81; Glu: 0.88; Gly: 1; His: 1.03; Arg: 1.01; Pro: 0.88; Tyr: 0.84; Leu: 0.030; ψVal[GnRH] (**3**) m/z 1274.83 (MH+), (calculated 1273.60 C_{58}, $H_{83}N_{17}O_{14}S$) Ser: 0.85; Glu: 0.93; Gly: 1; His: 0.95; Arg: 1.03; Pro: 0.92; Tyr: 0.85; Leu: 0.05; ψPhe[GnRH] (**4**) m/z 1322.89 (MH+), (calculated 1321.60 C_{62}, $H_{83}N_{17}O_{14}S$) Ser: 0.82; Glu: 0.90; Gly: 1; His: 0.93; Arg: 1.00; Pro: 0.89; Tyr: 0.79; Leu: 0.120; βAla[GnRH] (**5**) m/z 1196.81 (MH+), (calculated 1195.59 C_{56}, $H_{77}N_{16}O_{13}$) Ser: 1.06; Glu: 0.91; Gly: 1; His: 0.96; Arg: 1.05; Pro: 0.87; Tyr: 0.85; Leu: 0.87; (-Tyr)ψPhe[GnRH] (**6**) m/z 1159.86 (MH+), (calculated 1158.54 $C_{53}H_{74}N_{17}O_{14}S$) Ser: 0.85; Glu: 0.93; Gly: 1; His: 0.95; Arg: 1.03; Pro: 0.92; Leu: 0.02.

Preparation of pseudopeptides* 2a–d*; general procedure. Under nitrogen, the corresponding sulfonyl chloride (10) (1.0 mmol) was dissolved in CH_2Cl_2 (5 ml) and the solution was cooled to 0 °C. Next, a suspension of leucine *tert*-butylester hydrochloride (2.0 mmol) in CH_2Cl_2 (5 ml) containing triethylamine (2.0 mmol) was added and the resulting mixture was stirred overnight. After dilution with CH_2Cl_2 (25 ml), the mixture was washed with 1M $KHSO_4$ (2 × 30 ml), saturated $NaHCO_3$ (2 × 30 ml) and brine (30 ml). The organic phase, dried over Na_2SO_4, was filtered, concentrated to a small volume under reduced pressure and the product was crystallized by the addition of hexane. All compounds were obtained as colourless solids.

Fmoc-GlyΨ(CH$_2$SO$_2$NH)Leu-OBu^t **2a.** From 1.50 g (4.10 mmol) of **1a**; 2.0 g (94%); mp = 85.9–86.7 °C; $[\alpha]_D^{20} = -11$ (1, MeCN); IR main peaks at 3446, 2960, 1723, 1517, 1236, 1138 cm^{-1}; ^{1}H-NMR δ 0.94, 0.96 [two s, 6H, CH(C*H*$_3$)$_2$], 1.46 [m, 11H, C(CH$_3$)$_3$ and Leu βCH$_2$], 1.82 (m, 1H, Leu γCH), 3.18 (m, 2H, CH$_2$S, main peak of two conformers), 3.70 (bs, 2H, CH$_2$N, main peak of two conformers), 3.98 (m, 1H, Leu αCH), 4.20 (t, 1H, J = 6.5 Hz, Fmoc CH), 4.35 (m, 2H, Fmoc CH$_2$), 5.13 (d, 1H, J = 9.5 Hz, CONH), 5.67 (bs, 1H, SO$_2$NH), 7.23–7.73 (m, 8H, aromatic rings); ^{13}C-NMR δ 21.5, 22.7 [CH(*C*H$_3$)$_2$], 24.5 (Leu γCH), 27.8 [C(*C*H$_3$)$_3$], 35.8 (CH$_2$N) 42.3 (Leu βCH$_2$), 47.1 (Fmoc CH), 53.2 (CH$_2$S), 55.2 (Leu αCH), 67.0 (Fmoc CH$_2$), 82.7 [*C*(CH$_3$)$_3$], 119.8, 124.9, 126.8, 127.5, 141.1, 143.6 (aromatic rings) 156.1 (Fmoc CO), 171.9 (Leu CO).

Fmoc-AlaΨ(CH$_2$SO$_2$NH)Leu-OBu^t **2b.** From 1.54 g (4.06 mmol) of **1b**; white solid (1.61 g, 76%); mp = 103.0–104.2 °C; $[\alpha]_D^{20} = -8$ (1, MeCN); IR main peaks at 3437, 3009, 1703, 1515, 1418, 1363, 1220, 1137 cm^{-1}; ^{1}H-NMR δ 0.96, 0.98 [two s, 6H, CH(*C*H$_3$)$_2$], 1.26 (d, 3H, J = 6.0 Hz, NCHC*H*$_3$), 1.50 [s, 9H, C(CH$_3$)$_3$], 1.58 (m, 2H, Leu βCH$_2$), 1.86 (m, 1H, Leu γCH), 3.20 (m, 2H, CH$_2$S, main peaks of two conformers), 4.05 (m, 1H, Leu αCH), 4.26 (t, 1H, J = 6.5 Hz, Fmoc CH), 4.35 (m, 1H, C*H*CH$_2$S), 4.46 (m, 2H, Fmoc CH$_2$), 5.28 (d, 1H, J = 8.5 Hz, CONH), 5.68 (bs, 1H, SO$_2$NH), 7.28–7.78 (m, 8H, aromatic rings); ^{13}C-NMR δ 20.3, 21.0 [CH(*C*H$_3$)$_2$], 23.5 (NCH*C*H$_3$), 24.0 (Leu γCH), 27.4 [C(*C*H$_3$)$_3$], 42.9 (Leu βCH$_2$), 44.3 (CHCH$_2$S), 47.9 (Fmoc CH), 54.8 (Leu αCH), 58.5 (CH$_2$S), 67.2 (Fmoc CH$_2$), 82.6 [*C*(CH$_3$)$_3$], 120.1, 125.2, 127.1, 127.7, 141.2, 143.6 (aromatic rings), 156.4 (Fmoc CO), 172.4 (Leu CO).

***Fmoc-ValΨ(CH$_2$SO$_2$NH)Leu-OBu^t* 2c.** From 1.00 g (2.45 mmol) of **1c**; white solid (1.10 g, 75%); mp = 131.5–134.5 °C; $[\alpha]_D^{20} = +17$ (1, $CHCl_3$); IR main peaks at 3438, 3009, 1706, 1517, 1446, 1363, 1236, 1136 cm^{-1}; ^{1}H-NMR δ 0.96 [m, 12H, 2 × CH(CH_3)$_2$], 1.50 [s, 9H, C(CH_3)$_3$], 1.60 (m, 2H, Leu βCH_2), 1.88 [m, 2H, 2 × CH(CH$_3$)$_2$], 3.13 (d, 2H, J = 6.0 Hz, CH_2S), 4.07 (m, 1H, Leu αCH), 4.29 (t, 1H, J = 6.0 Hz, Fmoc CH), 4.38 (m, 2H, Fmoc CH_2), 4.47 (m, 1H, CHCH$_2$S), 5.09 (d, 1H, J = 10.0 Hz, CONH), 5.83 (d, 1H, J = 9.0 Hz, SO$_2$NH), 7.28–7.79 (m, 8H, aromatic rings); ^{13}C-NMR ($CDCl_3$) δ 18.2, 19.1, 22.3, 23.4 [2 × CH(*C*H$_3$)$_2$], 24.7 (Leu γCH), 27.5 [C(*C*H$_3$)$_3$], 32.9 [*C*H(CH$_3$)$_2$], 41.7 (Leu βCH_2), 47.9 (Fmoc CH), 54.9 (*C*HCH$_2$S), 55.9 (Leu αCH), 56.3 (CH_2S), 67.4 (Fmoc CH_2), 82.4 [*C*(CH$_3$)$_3$], 120.2, 125.3, 127.3, 128.0, 141.5, 143.8, (aromatic rings), 157.6 (Fmoc CO), 176.7 (Leu CO).

***Fmoc-PheΨ(CH$_2$SO$_2$NH)Leu-OBu^t* 2d.** From 1.10 g (2.40 mmol) of **1d**; 1.18 g (81%); mp = 151.6°–152.3 °C; $[\alpha]_D^{20} = +10$ (1, $CHCl_3$); IR main peaks at 3407, 3006, 1710, 1690, 1357, 1238, 1138 cm^{-1}; ^{1}H-NMR δ 0.92, 0.94 [two s, 6H, CH(CH_3)$_2$], 1.45 [s, 9H, C(CH$_3$)$_3$], 1.54 (m, 2H, Leu βCH_2), 1.81 (m, 1H, Leu γCH), 2.96 (m, 2H, CH_2Ph), 3.14 (d, 2H, J = 6.0 Hz, CH_2S), 4.00 (m, 1H, Leu αCH), 4.19 (t, 1H, J = 6.5 Hz, Fmoc CH), 4.31 (m, 2H, Fmoc CH_2), 4.71 (m, 1H, CHCH$_2$S), 5.13 (d, 1H, J = 8.5 Hz, CONH), 5.54 (d, 1H, J = 10.0 Hz, SO$_2$NH), 7.21–7.74 (m, 13H, aromatic rings); ^{13}C-NMR δ 21.5, 22.8 (Leu CH$_3$), 24.5 (Leu γCH), 27.9 [C(*C*H$_3$)$_3$], 40.3 (CH_2Ph), 42.2 (Leu βCH_2), 47.1 (Fmoc CH), 48.5 (*C*HCH$_2$S), 55.3 (Leu αCH), 56.1 (CH_2S), 67.1 (Fmoc CH_2), 82.3 [*C*(CH$_3$)$_3$], 119.7, 124.9, 126.8, 127.4, 128.5, 129.2, 136.2, 141.0, 143.5 (aromatic rings) 156.1 (Fmoc CO), 172.0 (Leu CO).

***Preparation of Fmoc-βAla-Leu-OBu^t* 4.** To a stirred solution of Fmoc-β-alanine (1.02 g, 3.30 mmol) and *N*-methylmorpholine (0.33 g, 3.30 mmol) in dry THF (20 ml), cooled to −15 °C, *i*-butylchloroformate (0.45 g, 3.30 mmol) was added. After 20 min, a suspension of leucine *tert*-butylester hydrochloride (0.74 g, 3.30 mmol) in THF (10 ml) containing N-methylmorpholine (0.33 g, 3.30 mmol) was added and the resulting reaction mixture was stirred at −15 °C for 1 h and kept at 4 °C for 16 h. After evaporation of the solvent to a small volume, ethyl acetate (50 ml) was added and the mixture was washed with 1 M $KHSO_4$ (2 × 30 ml), saturated $NaHCO_3$ (2 × 30 ml) and brine (30 ml). The organic phase, dried over Na_2SO_4, was filtered, concentrated to a small volume under reduced pressure and the product was crystallized by addition of hexane; white solid, 1.45 g (91%); mp = 98.4–99.1 °C; $[\alpha]_D^{20} = +5$ (2, $CHCl_3$); IR main peaks at 3436, 2962, 1719, 1672, 1507, 1450, 1236, 1151 cm^{-1}; ^{1}H-NMR δ 0.95, 0.97 [two s, 6H, CH(*C*H$_3$)$_2$], 1.49 [s, 9H, C(*C*H$_3$)$_3$], 1.60 (m, 3H, Leu βCH$_2\gamma$CH), 2.48 (t, 2H, J = 5.5 Hz, βAla αCH$_2$), 3.53 (m, 2H, βAla βCH$_2$), 4.21 (t, 1H, J = 7.0 Hz, Fmoc CH), 4.36 (d, 2H, J = 7.0 Hz, Fmoc CH_2), 4.52 (m, 1H, Leu αCH), 5.70 (bs, 1H, CONH), 6.12 (d, 1H, J = 8.0 Hz, OCONH), 7.28–7.78 (m, 8H, aromatic rings); ^{13}C-NMR δ 21.5, 22.2 [CH(*C*H$_3$)$_2$], 25.7 (Leu γCH), 27.7 [C(*C*H$_3$)$_3$], 35.9 (βAla αCH$_2$), 37.3 (βAla βCH$_2$), 41.8 (Leu βCH_2), 47.9 (Fmoc CH), 52.3 (Leu αCH), 66.9 (Fmoc CH_2), 82.1 [*C*(CH$_3$)$_3$], 119.2, 124.5, 126.3, 127.9, 141.4, 144.1 (aromatic rings), 156.6 (Fmoc CO), 171.3 (CONH), 172.4 (Leu CO).

***Preparation of pseudopeptides* 3a–d *and* 5; *general procedure*.** The corresponding *tert*-butylester (1.00 mmol) was treated with trifluoroacetic acid (3.0 ml) and the mixture was allowed to stand for 1 h at room temperature. After evaporation to dryness under reduced pressure, the residue was repeatedly co-evaporated with anhydrous diethylether and kept overnight under high vacuum. All compounds were crystallized from Et_2O/hexane and obtained as a colourless solid.

***Fmoc-GlyΨ(CH$_2$SO$_2$NH)Leu-OH* 3a.** From 1.80 g (3.47 mmol) of **2a**; 1.39 g (87%); mp = 91.6–92.4 °C; $[\alpha]_D^{20} = -9$ (1, MeCN); IR main peaks at 3444, 2960, 1719, 1518, 1450, 1214, 1141 cm^{-1}; ^{1}H-NMR δ 0.90, 0.94 [two s, 6H, CH(CH_3)$_2$], 1.60 (m, 2H, Leu βCH_2), 1.81 (m, 1H, Leu γCH), 3.21 (m, 2H, CH_2S, main peak of two conformers), 3.68 (bs, 2H, CH_2N, main peak of two conformers), 4.12 (m, 2H, Leu αCH and Fmoc CH), 4.32 (m, 2H, Fmoc CH_2), 5.52 (d, 1H, J = 9.5 Hz, CONH), 5.74 (t, 1H, J = 6.5 Hz, SO$_2$NH), 7.22–7.71 (m, 8H, aromatic rings); ^{13}C-NMR δ 21.2, 22.8 [CH(*C*H$_3$)$_2$], 24.4 (Leu γCH), 35.8 (CH_2N) 41.8 (Leu βCH_2), 46.9 (Fmoc CH), 53.1 (CH_2S), 54.3 (Leu αCH), 67.3 (Fmoc CH_2), 119.8, 124.8, 126.9, 127.5, 141.0, 143.4 (aromatic rings) 156.6 (Fmoc CO), 175.7 (Leu CO).

***Fmoc-AlaΨ(CH$_2$SO$_2$NH)Leu-OH* 3b.** From **2b** (1.35 g, 2.54 mmol); 1.10 g (91%); mp = 129.6–130.8 °C; $[\alpha]_D^{20} = +9$ (1, $CHCl_3$); IR main peaks at 3436, 2960, 1717, 1517, 1450, 1334, 1236, 1139 cm^{-1}; ^{1}H-NMR δ 0.94, 0.96 [two s, 6H, CH(CH_3)$_2$], 1.26 (m, 3H, NCHCH_3), 1.60 (m, 2H, Leu βCH_2), 1.82 (m, 1H, Leu γCH), 3.20 (m, 2H, CH_2S), 4.20 (m, 2H, Leu αCH and Fmoc CH), 4.30 (m, 1H, CHCH$_2$S), 4.45 (m, 2H, Fmoc CH_2), 5.28 (d, 1H, J = 8.5 Hz, CONH), 5.70 (d, 1H, J = 8.5 Hz, SO$_2$NH), 7.25–7.78 (m, 8H, aromatic rings); ^{13}C-NMR δ 20.9, 21.5 [CH(CH_3)$_2$], 23.1 (NCH*C*H$_3$), 24.6 (Leu γCH), 41.9 (Leu βCH_2), 43.9 (*C*HCH$_2$S), 47.2 (Fmoc CH), 54.7 (Leu αCH), 58.5 (CH_2S), 67.6 (Fmoc CH_2), 120.2, 125.3, 127.4, 128.0, 141.5, 143.8, (aromatic rings), 156.8 (Fmoc CO), 176.7 (Leu CO).

***Fmoc-ValΨ(CH$_2$SO$_2$NH)Leu-OH* 3c.** From **2c** (0.85 g, 1.43 mmol); 0.68 g (89%); mp = 82.5–83.2 °C; $[\alpha]_D^{20} = +3$ (1, MeCN); IR main peaks at 3437, 2963, 1716, 1517, 1450, 1333, 1255, 1138 cm^{-1}; ^{1}H-NMR δ 0.98 [m, 12H, 2 × CH(CH_3)$_2$], 1.60 (m, 2H, Leu βCH_2), 1.83 [m, 2H, 2 × CH(CH$_3$)$_2$], 3.19 (d, 2H, J = 6.0 Hz, CH_2S), 4.22 (m, 2H, Leu αCH and Fmoc CH), 4.35 (m, 2H, Fmoc CH_2), 4.43 (m, 1H, CHCH$_2$S), 5.20 (bs, 1H, CONH), 5.81 (d, 1H, J = 9.0 Hz, SO$_2$NH), 7.26–7.80 (m, 8H, aromatic rings); ^{13}C-NMR δ 18.1, 19.3, 21.5, 23.1 [CH(*C*H$_3$)$_2$], 24.6 (Leu γCH), 32.3 [*C*H(CH$_3$)$_2$], 41.9 (Leu βCH_2), 47.3 (Fmoc CH), 52.6 (CHCH$_2$S), 54.7 (Leu αCH), 55.5 (CH_2S), 66.7 (Fmoc CH_2), 120.2, 125.3, 127.3, 128.0, 141.5, 143.8, (aromatic rings), 157.6 (Fmoc CO), 176.7 (Leu CO).

***Fmoc-PheΨ(CH$_2$SO$_2$NH)Leu-OH* 3d.** From **2d** (1.05 g, 1.72 mmol); 0.81 g (85%); mp = 131.7–132.2 °C; $[\alpha]_D^{20} = +9$ (1, $CHCl_3$); IR main peaks at 3432, 2960, 1711, 1515, 1450, 1362, 1234, 1141 cm^{-1}; ^{1}H-NMR δ 0.87, 0.90 [two s, 6H, CH(CH_3)$_2$], 1.59 (m, 2H, Leu βCH_2), 1.80 (m, 1H, Leu γCH), 2.90 (m, 2H, CH_2Ph), 3.22 (m, 2H, CH_2S), 4.12 (m, 2H, Leu αCH and Fmoc CH), 4.27 (m, 2H, Fmoc CH_2), 4.58 (m, 1H, CHCH$_2$S), 5.31 (d, 1H, J = 8.5 Hz, CONH), 5.78 (d, 1H, J = 8.5 Hz, SO$_2$NH), 7.09–7.71 (m, 13H, aromatic rings); ^{13}C-NMR δ 21.3, 22.8 [CH(*C*H$_3$)$_2$], 24.4 (Leu γCH), 40.1 (CH_2Ph), 41.7 (Leu βCH_2), 46.9 (Fmoc CH), 48.5 (*C*HCH$_2$S),

54.4 (Leu αCH), 56.2 (CH_2S), 67.2 (Fmoc CH_2), 119.7, 124.9, 126.8, 127.5, 128.5, 129.1, 136.1, 141.0, 143.3, (aromatic rings), 157.3 (Fmoc CO), 172.6 (Leu CO).

***Fmoc-βAla-Leu-OH* 5.** From **4** (1.3g, 2.70 mmol); 0.80 g (92%); mp = 157.5–158.2 °C; $[\alpha]_D^{20} = -9$ (1, MeCN); IR main peaks at 3437, 2960, 1717, 1680, 1509, 1450, 1236, cm^{-1}; ^{1}H-NMR δ 0.92 [two s, 6H, $CH(CH_3)_2$], 1.60 (m, 3H, Leu $\beta CH_2\gamma CH$), 2.42 (m, 2H, βAla αCH_2), 3.45 (m, 2H, βAla βCH_2), 4.15 (t, 1H, $J = 7.0$ Hz, Fmoc CH), 4.29 (d, 2H, $J = 7.0$ Hz, Fmoc CH_2), 4.50 (m, 1H, Leu αCH), 5.75 (bs, 1H, CONH), 6.52 (d, 1H, $J = 8.0$ Hz, OCONH), 7.22–7.71 (m, 8H, aromatic rings); ^{13}C-NMR ($CDCl_3$) δ 21.7, 22.8 [$CH(CH_3)_2$], 24.9 (Leu γCH), 36.1 (βAla αCH_2), 37.2 (βAla βCH_2), 40.7 (Leu βCH_2), 47.1 (Fmoc CH), 51.0 (Leu αCH), 66.9 (Fmoc CH_2), 119.8, 124.9, 126.8, 127.5, 141.0, 143.6 (aromatic rings), 156.6 (Fmoc CO), 172.0 (CONH), 175.4 (Leu CO).

Animals

Wistar derived rats were purchased from and maintained by Harlen Laboratories Ltd, Rehovot, Israel. Experiments were carried out in compliance with the regulations of The Weizmann Institute of Science.

Enzyme Degradation and Fragment Analysis

Female rats, after long-term ovariectomy, were used as a source of pituitary enzymes. Anterior pituitaries from three animals were detached immediately after decapitation and homogenized (10 strokes) in a glass teflon homogenizing vessel containing phosphate-buffered saline (PBS, pH = 7.4). About 50 μg of GnRH or its analogs were incubated at 37 °C in PBS with pituitary homogenates (~40 mg protein). At the indicated time intervals (see Figure 1), a sample of each incubation mixture was withdrawn and placed in a boiling-water bath for 10 min to terminate the reaction. Each sample (20 μl) was diluted with water (containing 0.1% of TFA) to a final volume of 100 μl and spun for 10 min at 3000 × **g**. Aliquots of the resulting supernatant were then injected for HPLC analysis. Elution was carried out for 40 min with a linear gradient ranging from 0% to 80% of system B (75% ACN, 25% water, 0.1% TFA).

Identification of unchanged peptides and their fragments was obtained by mass spectrometry analysis of the recovered HPLC peaks. Results are summarized in Table 2.

Pituitary Cell Culture and LH Determination

Primary rat pituitary cell cultures were prepared from 21-day-old Wistar-derived female rats as described [11]. All cell culture compounds were purchased from Biological Industries (Beit Haemek, Israel). The cells were maintained (50 000 cell/well) in 96-well plates containing M-199, 10% horse serum and antibiotics at 37 °C in a humidified incubator. After 48 h, the cells were washed with M-199 medium containing 0.1% BSA and incubated for 4 h at 37 °C in the dark, as described [11], with M-199/0.1% BSA (0.25 ml) containing the desired concentrations (10^{-10} M, 10^{-9} M, 10^{-8} M) of the various peptides (four wells/experimental group) without serum and antibiotics. The incubation was terminated by removing the medium and diluting it with an equal volume of 1% BSA in PBS (pH = 7.4) solution. Four different aliquots from each sample were analysed for LH concentration by double-antibody radioimmunoassay (RIA) [12] using a kit kindly supplied by The National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). Results are expressed in terms of the LH-RP-3 rat reference preparation.

RESULTS

Chemistry

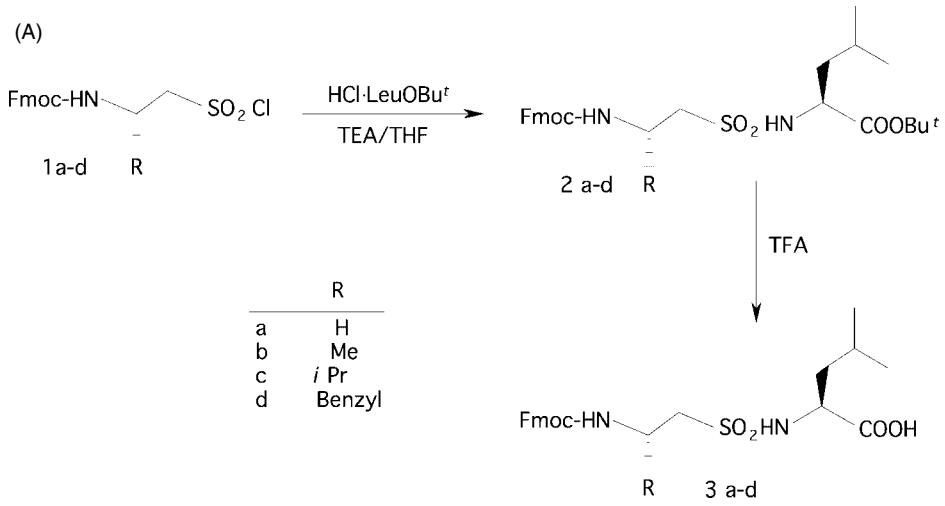
All pseudo GnRH-related peptides (Table 1) as well as GnRH were prepared by solid phase synthesis carried out manually, using Fmoc-chemistry. The sulfonamido units were inserted into the peptide chain through coupling of the corresponding Fmoc-protected pseudodipeptides **3a–d** (Scheme 1A). The key synthons used to obtain these sulfonamido isosteres were the Fmoc-protected aminoethanesulfonylchlorides **1a–d**, prepared from the respective Fmoc-protected amino-acids via mesylate-thioacetate (Scheme 1B), according to the published protocol [10].

Coupling of **1a–d** to leucine *tert*-butylester led to the pseudodipeptide esters **2a–d** and subsequent acidolysis with trifluoroacetic acid effected removal of the *tert*-butylester group to yield **3a–d**. The Fmoc-protected β-alanine leucine isostere 5 was obtained by coupling

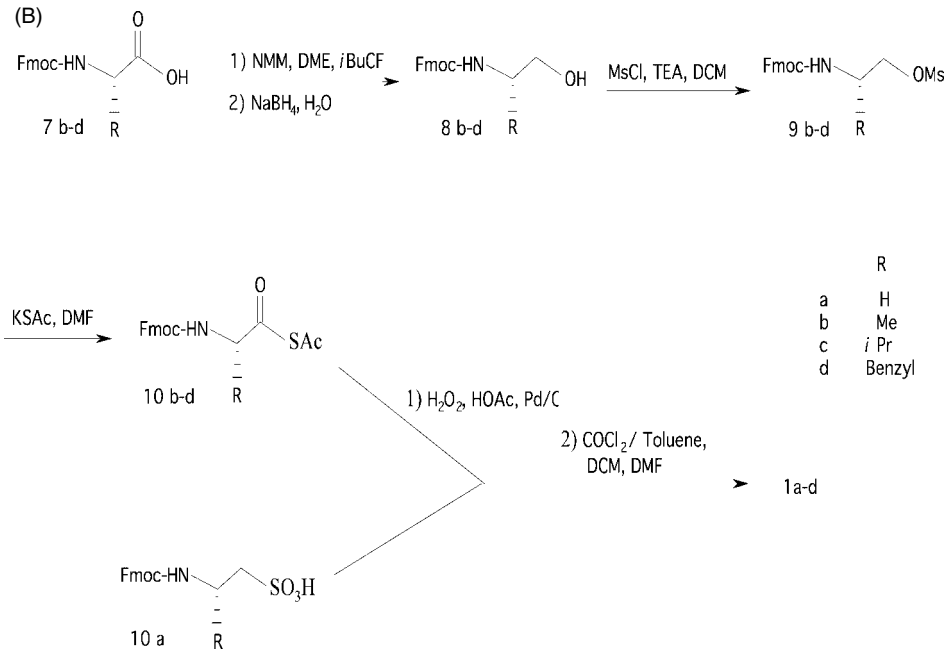
Table 1 Sequences and Mass Spectra Analysis of GnRH and its Synthetic Analogs

Peptide	Sequence[a]	MH^+ obsd (MW calcd)
GnRH	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	
1	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-**Gly^{6}Ψ(CH_2SO_2NH)**Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	1232.89 (1231.56)
2	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-**Ala^{6}Ψ(CH_2SO_2NH)**Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	1246.87 (1245.57)
3	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-**Val^{6}Ψ(CH_2SO_2NH)**Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	1274.83 (1273.60)
4	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-**Phe^{6}Ψ(CH_2SO_2NH)**Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	1322.89 (1321.60)
5	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-**β-Ala^6**-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	1196.81 (1195.59)
6	pGlu^1-His^2-Trp^3-Ser^4-**Phe^{5}Ψ(CH_2SO_2NH)**Leu^6-Arg^7-Pro^8-Gly^9-NH_2	1159.86 (1158.54)

[a] Modifications are shown in bold letters.



Scheme 1A General scheme for the synthesis of Fmoc-protected pseudodipeptides.

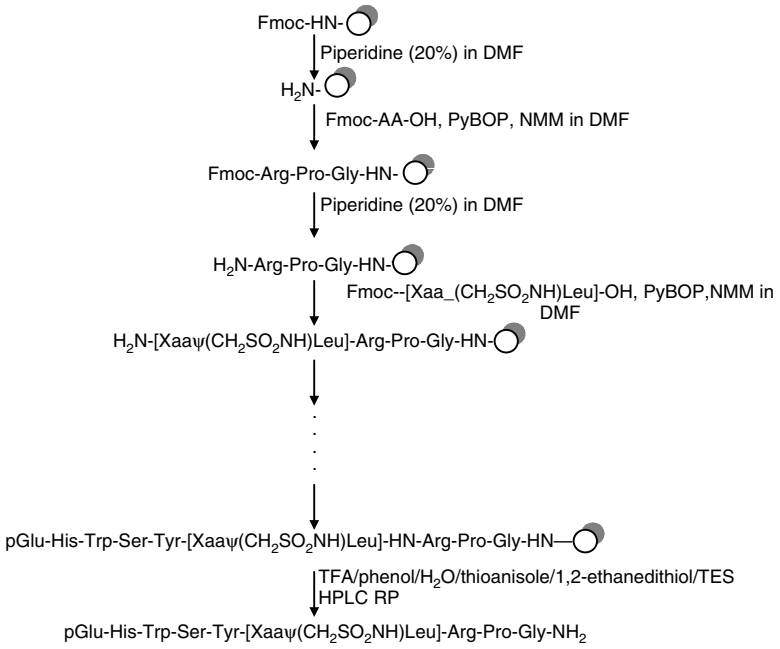


Scheme 1B General scheme for the synthesis of Fmoc-protected aminoethanesulfonylchlorides.

Fmoc-β-alanine4 to leucine *tert*-butylester, employing mixed anhydride procedure, and subsequent treatment with trifluoroacetic acid. The five Fmoc-protected pseudodipeptides were coupled to the free α-amino group of the resin-bound peptide (Scheme 2), i.e. H_2N-Arg-Pro-Gly-Rink amide, using standard coupling procedures (see Experimental Section). Fmoc-removal and further chain extension led to the desired polymer-bound peptides. Following acidolytic detachment from the insoluble support along with deprotection crude products were obtained, which were purified by semi-preparative HPLC. Purity and identity of the final products (>98%) was ascertained by analytical HPLC, amino-acid analysis and mass spectrometry.

Evaluation of Stability toward Proteolysis

Exposure of the GnRH-analogs to enzymatic degradation showed an increased stability relative to native GnRH (Figure 1). Thus, under the experimental conditions employed (see Experimental Section), after 3 h incubation at 37 °C with pituitary homogenate preparations only 22% of the native GnRH was detected, while the amount of the sulfonylated analogs remained practically unchanged. Only about 20% of [β-Ala^6]-GnRH was degraded. After 5 h incubation, native GnRH was nearly totally degraded while 80%–90% of the sulfonylated analogs and 60% of [β-Ala^6]-GnRH remained intact. After 7.5 h incubation, the degradation pattern of native GnRH and peptides 2, 3 and 4 was identified by HPLC-mass spectrometry (HPLC-MS) (Table 2).



Scheme 2 General scheme for solid-phase synthesis of GnRH analogs.

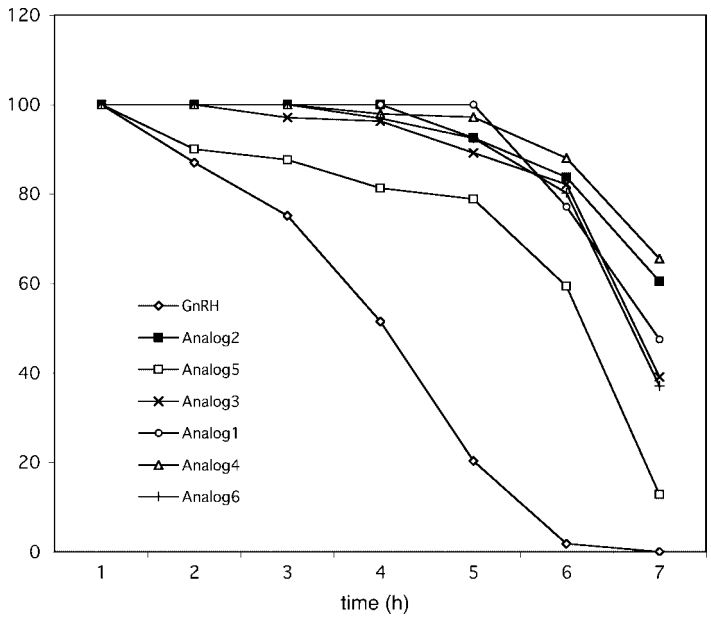


Figure 1 Patterns of proteolysis of GnRH and analogs by rat pituitary enzymes. The analogs corresponding to the peptide sequences are listed in Table 1.

In vitro LH-Release

The capacity of the newly synthesized GnRH analogs to induce LH-release from dispersed rat pituitary cells was evaluated in comparison with native GnRH. All the sulfonylated peptides manifested certain, though minor, activity. [β-Ala^6]-GnRH, however, was nearly as active as GnRH (Figure 2), and similarly capable of binding to the rat's pituitary GnRH receptor (i.e. $K_d \approx 5 \times 10^{-9}$ M), using a binding-competition assay.

DISCUSSION

In view of the short half-life of GnRH in circulation (~2–4 min), major efforts have been directed toward the design and synthesis of stable long-acting-peptides and peptidomimetics.

A lead point in the present study was the sensitivity of position-6 of GnRH toward proteolysis and the enhanced stability of analogs possessing modifications at this site. Thus, β-ethanesulfonylamino acid units were introduced at position-6, replacing the native

Table 2 Proteolysis Products of GnRH and Analogs[a]

GnRH	Mass obt[H]$^+$	Mass calc	Sequence
	441.2	440.29	Leu-Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp
	498.2	497.59	Gly-Leu-Arg-Pro-Gly-NH_2
	540.2	539.54	pGlu-His-Trp-Ser
	661.3	660.77	Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
	703.2	702.71	pGlu-His-Trp-Ser-Tyr
Main peak	760.3	759.77	pGlu-His-Trp-Ser-Tyr-Gly
	1182.6	1181.29	pGlu-His-Trp↓Ser↓Tyr↓Gly↓Leu-Arg-Pro-Gly-NH_2
Analog 1	441.2	440.29	Leu-Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp-
	540.2	539.21	pGlu-His-Trp-Ser
	540.2	539.21	pGlu-His-Trp-Ser
	548.3	547.29	ψGly-Leu-Arg-Pro-Gly-NH_2
Main peak	712.2	710.35	Tyr-ψGly-Leu-Arg-Pro-Gly-NH_2
	923.3	922.36	pGlu-His-Trp-Ser-Tyr-ψGly-Leu
	1231.6	1231.56	pGlu-His-Trp↓Ser↓Tyr-ψGly↓Leu-Arg-Pro-Gly-NH_2
Analog 2	441.2	440.29	Leu-Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp
	562.3	561.31	Ala-ψ-Leu-Arg-Pro-Gly-NH_2
Main peak	1246.6	1245.57	pGlu-His-Trp↓SerTyr↓-ψAla↓Leu-Arg-Pro-Gly-NH_2
Analog 3	441.2	440.29	Leu-Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp
	590.2		Val-ψ-Leu-Arg-Pro-Gly-NH_2
	753.3	752.40	Tyr-Val-ψ-Leu-Arg-Pro-Gly-NH_2
Main peak	1274.6	1273.6	pGlu-His-Trp↓Ser↓Tyr↓-ψVal↓Leu-Arg-Pro-Gly-NH_2
Analog 4	441.2	440.29	Leu-Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp
	639.2	637.34	Pheψ-Leu-Arg-Pro-Gly-NH_2
Main peak	1322.6	1321.6	pGlu-His-Trp↓Ser-Tyr↓-Pheψ-↓Leu-Arg-Pro-Gly-NH_2
Analog 5	328.1	327.20	Arg-Pro-Gly-NH_2
	453.1	452.18	pGlu-His-Trp
	512.3	511.32	βAla-Leu-Arg-Pro-Gly-NH_2
	540.2	539.21	pGlu-His-Trp-Ser
	887.3	886.40	pGlu-His-Trp-Ser-Tyr-βAla-Leu
	1043.4	1042.50	pGlu-His-Trp-Ser-Tyr-βAla-Leu-Arg
	1196.59	1195.59	pGlu-His-Trp↓Ser-↓Tyr↓-βAla-Leu-↓Arg-↓Pro-Gly-NH2\
Analog 6	850.3	849.35	pGlu-His-Trp-Ser—Pheψ-Leu
	1159.5	1158.54	pGlu-His-Trp-Ser-Pheψ-Leu-↓Arg-Pro-Gly-NH_2

[a] Fragments isolated by HPLC from the corresponding degradation mixtures of GnRH (3 hours) and analogs (Table 1) (7.5 h) were analysed by mass spectrometry. Arrows denote sites of enzymatic cleavage.

glycine. The new peptides, transition state isosteres analogs [13–15], contained two elements of modification: the replacement of the parent amide bond with a sulfonamidic bond and the addition of methylenic moiety. These modifications in the peptide backbone led to enhanced stability toward proteolysis by pituitary enzymes [16], pointing to the involvement of the Tyr^5-Gly^6-Leu^7 domain in the degradation of GnRH [7,8,17]. The contribution of this domain to the rigidity and resulting structural characteristics of GnRH was reported [18,19].

It is well documented that clearance of peptides by kidney and liver often leads to their enhanced removal from circulation. Our somewhat elaborated proteolysis study with pituitary, enzymes stem from their major involvement in modulating the pulsatile nature of GnRH action.

However, the activity of the obtained analogs, i.e. LH-releasing capacity of the parent peptide was nearly lost. The various analogs exhibited a marked propensity toward certain β-structure, compared with GnRH and revealed by CD [20,21]. This finding seems, however, of minor significance in view of the dramatic diminishment in activity.

The capacity of [β-Ala^6]-GnRH to bind to rat pituitary receptors and to induce LH-release similarly to GnRH

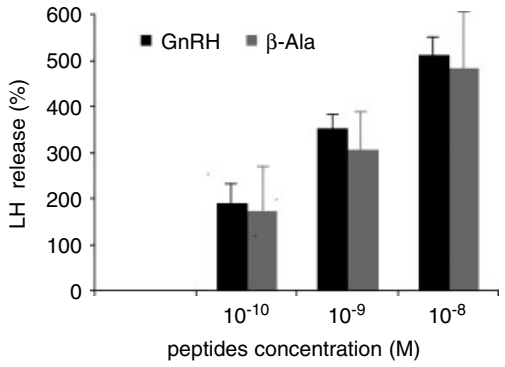


Figure 2 LH-release from dispersed rat pituitary cells.

is noteworthy. It suggests that the chain extension by a methylene moiety, i.e. -CH_2CH_2CONH- and CH_2CONH- in the two peptides, respectively, did not affect markedly the overall structure of GnRH. This is also evident from CD-spectrometry revealing that both peptides possess a negative band, near 190 nm, characteristic of a random coil conformation, while in the trifluoromethanol a negative peak around 220 nm and a positive peak around 195 nm, typical of a β-structure are observed (not shown).

The relative resistance of [β-Ala^6]-GnRH toward proteolysis compared with GnRH, coupled with its equal bioactivity, suggest that it might be a long acting analog. This, however, should be substantiated by *in vivo* studies.

The results obtained with [β-Ala^6]-GnRH should be viewed along with the increasing applications of β-amino acids in the design and synthesis of versatile bioactive peptides, e.g. receptor-mediated agonists and antagonists, antimicrobial derivatives and MHC-binders. The advantages gained by the incorporation of β-amino acids into peptide chains are due, primarily, to unique structural considerations and enhanced metabolic stability [22–25].

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