Characterization of the Degradation Products of Luteinizing Hormone Releasing Hormone

MICHAEL G. MOTTO[x], PATRICIA F. HAMBURG, DAVID A. GRADEN, CHARLES J. SHAW, AND MARY LOU COTTER

Received October 27, 1989, from *The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ 08869-0602.* Accepted for publication July 5, 1990.

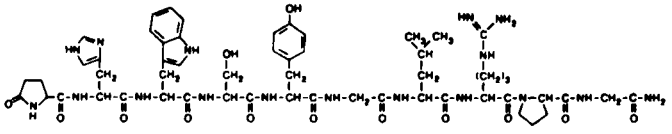
Abstract □ The degradation products of luteinizing hormone releasing hormone [LH/RH; **1**; gonadorelin releasing hormone (GnRH); <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2] were determined in aqueous solution (pH 6.5) at 25, 37, 50, and 80 °C. The predominant route of degradation involved the cleavage of the <Glu-His and Trp-Ser peptide bonds to give peptides **5–9** and hydrolysis of the terminal Gly-NH_2 to the free acid form in peptides **4** and **10**. Racemization of the serine and histidine residues to give peptides **2** and **3** was a second route of degradation.

The decapeptide luteinizing hormone releasing hormone [LH/RH; **1**; gonadorelin releasing hormone (GnRH); <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2] was first isolated by Schally et al.[1] and was found to release luteinizing hormone and follicle-stimulating hormone from the pituitary. The natural peptide has found various therapeutic applications, including the treatment of hypothalamic amenorrhea and hypogonadotropia.[2–4] The stability of the peptide in aqueous solutions has been described by Shi et al.,[5] although in that study the identity of the degradation products was not determined. The present report describes the isolation and characterization of the degradation products of LH/RH that were relevant to the development of a parenteral formulation in dilute aqueous solutions of the peptide at its native pH (6.5), and provides information as to the rate of LH/RH degradation under these conditions.

Experimental Section

Materials and Reagents—Luteinizing hormone releasing hormone (LH/RH; GnRH; peptide content 90% by weight; material supplied as the diacetate, 99.1%) was obtained from Ferring AG (Kiel, Germany). Authentic samples of peptides **4–9** were obtained from commercial sources. All other chemicals were reagent or HPLC grade. Water was obtained from a Millipore Milli-Q purification system.

Instruments—The positive ion fast atom bombardment (FAB) mass spectra (FAB-MS) were obtained either on a Finnigan-MAT 8230 or a VG 7070 EQ mass spectrometer with 8 KeV xenon atoms. The sample matrix consisted of dithioerythritol and dithiothreitol with 0.01 M *p*-toluenesulfonic acid or glycerol and thioglycerol. The ^1H NMR spectra (400 MHz) and the ^{13}C NMR spectra (100 MHz) spectra were obtained with a Varian XL-400 spectrometer. The solvent was either deuterium oxide or methanol-d_4, with sodium 3-trimethylsilyl-1-propanesulfonate (DSS) or tetramethylsilane (TMS) as internal standard, respectively. Amino acid analysis was performed with a Beckman System 6300 analyzer.



Luteinizing hormone releasing hormone

High-Performance Liquid Chromatography Analysis—The HPLC instrument consisted of a Perkin-Elmer Series 4 solvent delivery system, a Waters WISP autosampler, a DuPont SP-150 C-8 column (4.6 mm × 25 cm, 5-μm particle size, 150 Å pore size), and a Hewlett-Packard 1040A diode array UV detector with multichannel integration capability that was set to monitor 210 and 280 nm. The mobile phase consisted of triethylammonium phosphate buffer[6] (0.52 M, pH 2.35):water:acetonitrile in the ratio 43.5:43.5:13. The buffer was prepared by adding triethylamine (900 mL) and phosphoric acid (85%, 610 mL) to 11 L of water. The pH of the buffer was then adjusted with phosphoric acid to a value of 2.35. The flow rate was 1.6 mL/min. Chromatographic peak shape was much improved at elevated temperature (60 °C) when compared with the same method run at ambient temperature, and no peptide degradation was observed during the chromatography; therefore, the column was maintained at 60 °C during the analysis. A calibration curve was prepared for LH/RH at 210 nm ($r^2 = 0.9999$), and response factors at 210 nm were assumed to be 1 for all degradation products.

Determination of Configuration at Chiral Centers of Isolated Degradation Products—A literature[7] method was used with slight modifications. The peptide was hydrolyzed to its constituent amino acids which were converted to *N*-pentafluoropropionyl:isopropyl ester derivatives. These amino acid derivatives were then analyzed by capillary GC using a chiral column. The hydrolysis involved treating ~1 mg of peptide with 0.5 mL of acetic acid, 0.5 mL of 12 M HCl, and two drops of 1% aqueous phenol solution. This mixture was heated for 16 h at 110 °C. The final volume of methylene chloride solution which was injected into the GC was 0.5 mL or less. The GC temperature program included a 1.5-min initial hold at 90 °C and then a 4 °C/min rise to 200 °C.

Degradation of Luteinizing Hormone Releasing Hormone—The LH/RH (41.6 mg) was dissolved in 130 mL of water to provide a solution of 0.32 mg/mL at pH 6.5. The solution was divided into thirteen 10-mL portions. The 10-mL samples were sealed into glass vials with rubber septa and were placed in constant temperature chambers set to maintain temperatures of 4 ± 1, 25 ± 1, 37 ± 1, and 50 ± 1 °C. Three vials were placed at each temperature. The remaining vial was used as the initial time point. Vials were removed and the solutions analyzed by HPLC after 2 weeks, 1 month, and 3 months. The 4 °C sample was used as an HPLC control to assure that no peptide loss had occurred due to adsorption to the vials. No change was observed in the 4 °C samples over the 3-month analysis time.

The LH/RH (5.02 g) was dissolved in 490 mL of water and placed in an amber bottle equipped with a rubber septum; the pH of the solution was ~6.5. Approximately 20 mL of this solution was removed and stored at 4 ± 1 °C as a control. The remaining solution was purged with argon and placed into a constant temperature bath maintained at 80 ± 5 °C. Aliquots were removed from this sample and monitored daily by HPLC. After 10 days, the sample was removed from the bath and used for the isolation of degradation products.

Isolation of Degradation Products by Preparative High-Performance Liquid Chromatography—The HPLC system consisted of a Perkin-Elmer Series 10 solvent delivery system equipped with preparative pump heads, a preparative C-18 column (2.5 × 50 cm; 10-μm particle size; Partisil-10 ODS-3; Whatman, Clifton, NJ), and an LDC UV-III UV detector (λ = 214 nm). The degradation product mixture was initially fractionated by concentrating the

0022-3549/91/0500-0419$01.00/0

volume to ~250 mL and injecting 10–20-mL portions onto the column (maintained at 50 °C) until the entire sample was fractionated. The column was eluted with the following mobile phase: triethylammonium phosphate buffer (0.52 M, pH 2.35), Milli-Q water, and acetonitrile in the ratio 2:1.6:0.4 at a flow rate of 19 mL/min. Fractions were collected and analyzed by HPLC. Appropriate fractions which contained the desired peptide products in the triethylammonium phosphate:acetonitrile mobile phase were combined and concentrated on a rotary evaporator to remove most of the acetonitrile. Rechromatography of these fractions was effected, if necessary, by injecting the concentrated solutions onto the same preparative column or a semi-preparative column (C-8, 9.4 mm × 25 cm, 5-μm particle size, ES Industries, Marlton, NJ; or a C-18, 10 mm × 25 cm, 5-μm particle size, 330 Å, Vydac, Hesperia, CA) and eluting the peptides under appropriate conditions. Typical conditions for the semi-preparative column were mixtures of the TEAP buffer (0.2 M, pH 2.0, prepared by diluting the 0.52 M buffer with water and adjusting the pH with phosphoric acid) with acetonitrile (88:12) or THF (90:10) at a flow rate of 6 mL/min.

The final combined fractions which contained pure peptide degradation products in triethylammonium phosphate:acetonitrile or THF solution were then subjected to a chromatographic procedure to remove the nonvolatile salts. A fraction was first concentrated on a rotary evaporator to remove the acetonitrile or THF. The resulting concentrate was then pumped onto a semi-preparative HPLC column (C-18, M9-ODS 3, Whatman) which had previously been equilibrated with ammonium formate solution. This mobile phase was an ammonium formate:formic acid solution (pH 2.35) that was prepared by adding ammonium hydroxide (96.5 mL) and formic acid (636 mL) to 9 L of water. The peptide was then eluted with a solvent gradient [100% ammonium formate solution to 70% formate solution:30% acetonitrile over a 25-min (linear) period at a flow rate of 8 mL/min]. The fractions which contained the peptide in ammonium formate solution were concentrated on a rotary evaporator to remove the acetonitrile and lyophilized. The residue was then diluted with water and lyophilized again. This process of dilution/lyophilization was usually repeated at least six times in order to remove most of the ammonia, formic acid, and triethylamine. The degradation product materials that were isolated according to these procedures typically amounted to 10–100 mg.

[D-Ser^4]-LH/RH (2)—FAB-MS *m/z* (relative intensity): 1182 (MH^+, 100), 661 (10), 274 (38), 249 (66), 221 (64); ^{1}H NMR (methanol-d_6): δ 0.80 (3 H, d, J = 6.2 Hz, Leu δ CH_3), 0.84 (3 H, d, J = 6.2 Hz, Leu δ CH_3), 6.61 (2 H, d, J = 8.3 Hz, Tyr meta), 6.93 (1 H, t, J = 7.8 Hz, Trp $H_5^{\dagger}$), 6.97 (1 H, s, His H_5), 6.98 (2 H, d, J = 8.3 Hz, Tyr ortho), 7.01 (1 H, t, J = 7.8 Hz, Trp $H_6^{\dagger}$), 7.17 (1 H, s, Trp H_2), 7.26 (1 H, d, J = 8.1 Hz, Trp H_7), 7.49 (1 H, s, His H_2), 7.50 (1H, d, J = 8.1 Hz, Trp H_4) (†assignments may be reversed); ^{13}C NMR (methanol-d_6): δ 21.96, 23.52, 25.73, 25.86, 26.21, 26.53, 28.42, 29.31, 29.34, 30.42, 30.50, 37.13, 41.58, 42.08, 43.40, 43.75, 52.01, 53.36 (2 C), 56.60, 57.56 (2 C), 57.88, 62.11, 62.61, 110.60, 112.30, 116.20 (2 C), 119.19, 119.76, 124.88, 127.59, 129.07, 131.20 (2 C), 132.92, 137.83 (2 C), 157.09, 158.36, 171.51, 171.92, 172.82, 174.01, 174.33, 174.42, 174.74, 174.87, 181.32; amino acid analysis (relative molar amount): Glu (1.01), His (0.96), Ser (0.75), Tyr (1.03), Gly (1.90), Leu (0.97), Arg (0.95), Pro (0.96), (Trp is oxidized during hydrolysis and is not seen and Ser normally shows a lower response); chiral analysis: L-Glu, L-His, L-Trp, D-Ser, L-Tyr, L-Leu, L-Arg, L-Pro, Gly.

[D-His^2]-LH/RH (3)—FAB-MS *m/z* (relative intensity): 1182 (MH^+, 100), 661 (12), 274 (41), 249 (65), 221 (68); ^{1}H NMR (methanol-d_6): δ 0.86 (3 H, d, J = 5.4 Hz, Leu δ CH_3), 0.92 (3 H, d, J = 5.4 Hz, Leu δ CH_3), 6.81 (2 H, d, J = 8.5 Hz, Tyr meta), 6.93 (1 H, s, His H_2), 7.10 (2 H, d, J = 8.5 Hz, Tyr ortho), 7.13 (1 H, t, J = 7.6 Hz, Trp H_7), 7.15 (1 H, s, Trp H_2), 7.23 (1 H, t, J = 7.6 Hz, Trp $H_6^{\dagger}$), 7.47 (1 H, s, Trp H_8), 7.60 (1 H, s, Trp $H_5^{\dagger}$), 8.24 (1 H, s, His H_5) (†assignments may be reversed); ^{13}C NMR (methanol-d_6): δ 23.79, 25.14, 26.98, 27.30,. 27.69, 28.21, 29.22, 30.04, 30.57, 32.25, 33.19, 38.80, 42.72, 43.53, 45.09, 45.41, 53.98, 55.23, 55.39, 55.42, 58.46 (2 C), 59.57, 63.66, 63.88, 111.59, 114.73, 118.26, 118.30 (2 C), 119.69, 121.09, 122.21, 124.83, 127.25, 129.60, 131.04, 133.31 (2 C), 136.21, 138.44, 157.30, 159.42, 172.2, 173.8, 174.1, 176.4, 177.3, 177.8, 178.2, 184.73; amino acid analysis (relative molar amount): Glu (1.01), His (0.97), Ser (0.74), Tyr (1.01), Gly (1.94), Leu (0.99), Arg (0.97), Pro (0.98), (Trp is oxidized during hydrolysis and is not seen and Ser normally shows lower response); chiral analysis: L-Glu, L-Trp, L-Ser, L-Tyr, D-His, L-Leu, L-Arg, L-Pro, Gly.

[Gly-OH^{10}]-LH/RH (4)—FAB-MS *m/z* (relative intensity): 1183.4 (MH^+, 100), 274 (61), 249 (80), 221 (77); ^{1}H NMR (methanol-d_6): δ 0.87 (3 H, brs, Leu δ CH_3), 0.93 (3 H, brs, Leu δ CH_3), 6.83 (2 H, d, J = 7.8 Hz, Tyr meta), 7.10 (2 H, d, J = 7.8 Hz, Tyr ortho), 7.17 (1 H, s, Trp H_2), 7.17 (1 H, s, His H_2), 7.23 (1 H, t, J = 7.2 Hz, Trp $H_6^{\dagger}$), 7.48 (1 H, d, J = 8.1 Hz, Trp $H_5^{\dagger}$), 7.58 (1 H, d, J = 8.1 Hz, Trp $H_5^{\dagger}$), 7.77 (1 H, s, His H_5) (†assignments may be reversed); ^{13}C NMR (methanol-d_6): δ 23.83, 25.21, 26.97, 27.29, 27.53, 28.08, 29.12, 30.21, 30.59, 32.16, 32.38, 38.78, 41.26, 42.76, 43.53, 45.50, 50.84, 53.95, 55.26, 57.28, 58.27, 58.48, 59.47, 63.40, 64.04, 111.51, 114.72, 118.30 (2 C), 118.40, 119.96, 121.06, 124.78, 127.26, 129.07, 130.75, 133.34 (2 C), 134.17, 138.93, 157.30, 159.41, 174.2, 174.4, 176.3, 176.5, 177.2, 177.4, 177.6, 189.79; amino acid analysis (relative molar amount): Glu (1.02), His (1.03), Ser (0.71), Tyr (1.05), Gly (1.94), Leu (0.95), Arg (1.07), Pro (1.10), (Trp is oxidized during hydrolysis and is not seen and Ser normally shows lower response); chiral analysis: L-His, L-Trp, L-Ser, L-Tyr, L-His, L-Leu, L-Arg, L-Pro, Gly. The sample had the same HPLC retention time and spectral data as authentic synthesized material.

des-<Glu^1-LH/RH (5)—FAB-MS *m/z* (relative intensity): 1071.6 (MH^+, 100); ^{1}H NMR (methanol-d_6): δ 0.85 (3 H, d, J = 5.2 Hz, Leu δ CH_3), 0.91 (3 H, d, J = 5.2 Hz, Leu δ CH_3), 6.44 (1 H, s, His H_2), 6.80 (2 H, d, J = 8.3 Hz, Tyr meta), 7.11 (1 H, s, Trp H_2), 7.12 (2 H, d, J = 8.3 Hz, Tyr ortho), 7.23 (1 H, t, J = 7.0 Hz, Trp $H_6^{\dagger}$), 7.47 (1 H, d, J = 8.4 Hz, Trp $H_5^{\dagger}$), 7.53 (1 H, d, J = 8.4 Hz, Trp H_8), 8.50 (1 H, s, His H_5) (†assignments may be reversed); ^{13}C NMR (methanol-d_6): δ 16.43, 21.85, 23.20, 25.03, 25.34, 25.75, 27.99, 28.60, 30.30 (2 C), 36.75, 40.77, 41.58, 43.14, 43.44, 52.05 (2 C), 53.27, 55.68, 56.44, 56.53, 61.71, 62.00, 109.42, 112.80, 116.37 (2 C), 117.38, 119.09, 120.24, 122.88, 125.30, 127.76, 128.80, 129.64, 131.31 (2 C), 136.94, 155.36, 157.46, 171.78, 171.88, 172.27, 173.98, 174.22, 174.92, 175.03, 175.50; amino acid analysis (relative molar amount): Glu (0.03), His (0.94), Ser (0.76), Tyr (1.01), Gly (1.93), Leu (0.99), Arg (0.97), Pro (0.96), (Trp is oxidized during hydrolysis and is not seen and Ser normally shows lower response); chiral analysis: L-His, L-Trp, L-Ser, L-Tyr, L-His, L-Leu, L-Arg, L-Pro, Gly. The sample was spectroscopically identical with an authentic standard.

<Glu-His-Trp (6)—FAB-MS *m/z* (relative intensity): 453 (MH^+, 100); ^{1}H NMR (D_2O): δ 1.19 (2 H, t, J = 7.3 Hz, <Glu H^{γ}), 1.55 (2 H, m, <Glu H^{β}), 4.12 (1 H, m, <Glu H^{α}), 4.55 (2 H, m, His H^{α}, Trp H^{α}), 7.01 (1 H, dd, J = 8.1 Hz, Trp $H_5^{\dagger}$), 7.02 (1 H, s, His H_5), 7.11 (1 H, dd, J = 8.1 Hz, Trp $H_6^{\dagger}$), 7.11 (1 H, s, Trp H_2), 7.35 (1 H, d, J = 8.1 Hz, Trp $H_7^{\ddagger}$), 7.53 (1 H, d, J = 8.1 Hz, Trp $H_4^{\ddagger}$), 8.23 (1 H, s, His H_2), (†,‡assignments may be reversed); ^{13}C NMR (D_2O): δ 28.00 (<Glu $C^{\beta\dagger}$), 29.45 (Trp $C^{\beta\dagger}$), 30.19 (His $C^{\beta\dagger}$), 32.09 (<Glu C^{γ}), 49.57 (His C^{α}), 49.57 (Trp C^{α}), 59.44 (<Glu C^{α}), 112.84 (Trp C_3), 114.51 (Trp C_7), 119.52 (His C_5), 121.95 (Trp $C_4^{\ddagger}$), 121.22 (Trp $C_6^{\ddagger}$), 126.87 (Trp C_2), 129.92 (Trp C_{3a}), 136.10 (His C_2), 130.68 (His C_4), 138.80 (Trp C_{7a}), 176.81 (C=O), 173.03 (C=O), 184.67 (<Glu^5) (†,‡assignments may be reversed); amino acid analysis (relative molar amount): Glu (1.03), His (1.00), (Trp is oxidized during hydrolysis); chiral analysis: L-Glu, L-His, L-Trp. The sample had the same spectral data as authentic synthesized material and the assignments were consistent with that for the similar peptide <Glu-His-Trp-NH_2 reported in the literature.[8]

cyclo-[His-Trp] (7)—FAB-MS *m/z* (relative intensity): 324 (MH^+, 100), 194 (80), 130 (90); ^{1}H NMR (D_2O): δ 1.05 (1 H, dd, His H^{β}), 2.41 (1 H, dd, His H^{β}), 3.1 (1 H, dd, Trp H^{β}), 3.2 (1H, m, Trp H^{β}), 3.85 (1 H, dd, His H^{α}), 4.28 (1 H, dd, Trp H^{α}), 5.82 (1 H, s, His H_5), 7.08 (1 H, dd, Trp $H_5^{\dagger}$), 7.10 (1 H, s, Trp H_2), 7.17 (1 H, dd, Trp $H_6^{\dagger}$), 7.38 (1 H, d, Trp H_7), 7.48 (1 H, d, Trp H_4), 8.07 (1 H, s, His H_2) (†assignments may be reversed); ^{13}C NMR (D_2O): δ 29.74 (t, Trp $C^{\beta\dagger}$), 29.55 (t, His $C^{\beta\dagger}$), 56.52 (d, Trp $C^{\alpha\ddagger}$), 54.08 (d, His $C^{\alpha\ddagger}$), 108.37 (s, Trp C_3), 112.80 (d, Trp C_6), 118.59 (d, His C_5), 119.66 (s, Trp C_{3a}), 120.40 (d, Trp C_5), 122.74 (d, Trp C_4), 126.33 (d, Trp C_2), 128.08 d, Trp C_7), 134.50 (s, His C_4), 136.55 (d, His C_2), 136.59 (s, Trp C_{7a}), 168.87 (s, His C=O††), 170.15 (s, Trp C=O††), (†,‡,††assignments may be reversed); amino acid analysis: His (Trp is oxidized during hydrolysis); chiral analysis: L-His, L-Trp. The sample had the same spectral data as authentic synthesized material, and the assignments were consistent with the spectral data published in the literature.[9]

Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (8)—FAB-MS *m/z* (relative intensity): 748.57 (MH^+, 100); ^{1}H NMR (methanol-d_6): δ 0.64 (3 H, d, J = 4.6 Hz, Leu δ CH_3), 0.69 (3 H, d, J = 4.6 Hz, Leu δ CH_3), 6.61 (2 H, d, J = 8.3 Hz, Tyr meta), 6.91 (2 H, d, J = 8.3 Hz, Tyr ortho); ^{13}C NMR (methanol-d_6): δ 21.71, 22.98, 24.93, 25.18, 25.60, 28.44, 30.41, 36.97, 40.61, 41.41, 42.98, 43.23, 51.90, 53.09, 55.50, 56.32, 61.57,

61.73, 116.20 (2 C), 128.65, 131.20 (2 C), 155.19, 157.35, 171.45, 171.55, 172.15, 173.91, 174.74, 174.95, 175.32; amino acid analysis (relative molar amount): Ser (0.78), Tyr (1.00), Gly (2.08), Leu (1.06), Arg (1.05), Pro (1.00), (Ser normally shows lower response); chiral analysis: L-Ser, L-Tyr, L-Leu, L-Arg, L-Pro, Gly. The sample was spectroscopically identical to an authentic standard.

Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (9)—FAB-MS *m/z* (relative intensity): 934.65 (MH^+, 100); ^1H NMR (methanol-d_6): δ 0.79 (3 H, d, J = 5.4 Hz Leu δ CH_3), 0.84 (3 H, d, J = 5.4 Hz, Leu δ CH_3), 6.77 (2 H, d, J = 8.3 Hz, Tyr meta), 6.99 (1 H, t, J = 7.5 Hz, Trp $H_5^{\ddagger}$), 7.06 (2 H, d, J = 8.3 Hz, Tyr ortho), 7.11 (1 H, s, Trp H_2), 7.16 (1 H, t, J = 7.5 Hz, Trp $H_6^{\ddagger}$), 7.43 (1 H, d, J = 8.4 Hz, Trp $H_7^{\dagger}$), 7.48 (1 H, d, J = 8.4 Hz, Trp $H_4^{\dagger}$) (†,‡assignments may be reversed); amino acid analysis (relative molar amount): Ser (0.62), Tyr (0.85), Gly (1.69), Leu (0.86), Arg (0.89), Pro (1.00), (Ser normally shows lower response); chiral analysis: L-Ser, L-Tyr, L-Leu, L-Arg, L-Pro, Gly. The sample was spectroscopically identical to an authentic standard.

His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH (10)—FAB-MS *m/z* (relative intensity): 1072.87 (MH^+, 100); ^1H MNR (methanol-d_6): δ 0.66 (3 H, brs, Leu δ CH_3), 0.71 (3 H, brs, Leu δ CH_3), 6.62 (2 H, brs, Tyr meta), 6.91 (1 H, s, His H_2), 6.91 (1 H, br, Trp $H_5^{\ddagger}$), 6.91 (2 H, br, Tyr ortho), 7.02 (1 H, br, Trp $H_6^{\ddagger}$), 7.11 (1 H, s, Trp H_2), 7.29 (1 H, br, Trp $H_7^{\ddagger}$), 7.36 (1 H, brs, Trp $H_4^{\ddagger}$), 7.75 (1 H, s, His H_5) (†,‡assignments may be reversed); chiral analysis: L-His, L-Trp, L-Ser, L-Tyr, L-Leu, L-Arg, L-Pro, Gly.

Results and Discussion

The degradation of LH/RH at 25, 37, 50, and 80 °C in an aqueous solution of pH 6.5 was monitored by HPLC (Figure 1, Table I). At 50 °C, ~80% of the original LH/RH remained after 3 months. Nine predominant products were observed (Figure 1) at all degradation temperatures and these products were subsequently isolated by preparative HPLC. The analytical and preparative HPLC methods were based on a triethylammonium phosphate buffer which was reported to provide good chromatographic resolution and recovery of LH/RH peptides.[6]

The isolated peptides **2–10** (Table II) were characterized by FAB-MS, NMR, chiral analysis,[7] and amino acid analysis. Authentic samples of peptides **4–9** were used to confirm the structural assignments of these peptides.

The isolated sample of the cyclo[His-Trp] (**7**), showed an MH^+ ion at *m/z* 324 by FAB-MS and ^1H and ^{13}C NMR spectra that were consistent with the structure. A detailed study of the conformation of this molecule by NMR has been reported.[8,10] The highfield resonances of the His H_5 proton (5.7 ppm) and the His H^{β} protons (1.05 and 2.41 ppm) are distinguishing spectral characteristics of this molecule.[8,10] Chiral analysis of the sample showed that the diketopiperazine was composed of L-His and L-Trp. Final confirmation of the structural assignment was obtained by comparing the spectral properties of an authentic synthesized sample with those of the isolated sample.

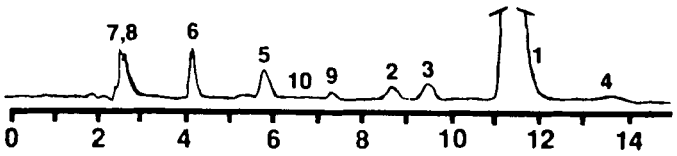


Figure 1—The HPLC profile (λ 210 nm) of a product mixture that was obtained after treatment of a 8.67 × 10^{-3} M solution of LH/RH (pH 6.5) for 10 days at 80 °C.

The peptide fragments des-[<Glu[1]]-LH/RH (**5**), <Glu-His-Trp (**6**), Ser-Tyr-Gly-His-Leu-Arg-Pro-Gly-NH_2 (**8**), Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (**9**), and the LH/RH free acid (**4**) were isolated and characterized by spectral interpretation with comparison to similar peptides reported in the literature.[9,11] Amino acid analysis along with chiral GC analysis[7] confirmed the amino acid composition and the chirality of each isolated peptide. The structural assignments for these peptides were also confirmed by comparison of the spectral data (NMR and FAB-MS) with that of authentic material. The peptide fragment His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH (**10**) was isolated and characterized in a similar manner to the other peptide fragments, although no authentic standard was available for confirmation of the structure.

The isolated peptides **2** and **3** had HPLC retention times and exhibited spectral characteristics that were similar to the data for LH/RH itself. The FAB-MS of these peptides indicated that they were isobaric with LH/RH, suggesting that these two peptides were isomers of LH/RH. The amino acid analysis indicated that both of the isolated peptides contained the same amino acids as those of LH/RH. The chiral analysis of **2**, however, showed the presence of D-Ser instead of L-Ser. Likewise, **3** was shown to contain a D-His instead of L-His. These results were not unexpected since racemization of the Ser[4] and His[2] residues of other LH/RH analogues in alkaline media has been reported.[12]

The identified products accounted for >90% of the total product material after ~20% of the original LH/RH drug substance had been degraded (Table I, 50 °C). The remaining product material was probably represented by the small unidentified peaks that appeared in the chromatogram of the product mixture (Figure 1). These products could be smaller fragments, such as free amino acids (e.g., <Glu), since both **5** and **10** have lost the terminal amino acid by hydrolysis. The remaining products could also be fragments of the racemized peptides (**2**, **3**) since hydrolysis of these peptides would also be expected. Mass accountability was somewhat lower in reactions run at 80 °C, where the degradation proceeded much more rapidly (~30% degradation after ~1 week), and many more secondary reactions might be expected to occur.

Table I—Approximate Percentage of Degradation Products (2–10) in Stressed LH/RH Solutions (0.32 mg/mL) over a Period of Three Months

Compound	Percentage of Degradation Product									
	25 °C			37 °C			50 °C			4 °C
	14d	30d	90d	14d	30d	90d	14d	30d	90d	14d
1	99.4	99.2	98.6	98.8	97.8	94.9	96.7	92.8	81.1	99.4
2	0.01	0.01	0.09	0.09	0.30	0.72	0.56	1.38	4.12	—
3	<0.01	0.03	0.08	0.08	0.18	0.45	0.37	0.81	2.13	—
4	0.24	0.33	0.33	0.31	0.53	0.67	0.52	1.05	2.83	0.36
5	0.06	0.08	0.35	0.24	0.45	1.43	0.83	1.64	3.00	—
6	0.03	0.05	0.12	0.10	0.20	0.52	0.38	0.84	1.93	—
7	<0.01	<0.01	<0.01	<0.01	<0.01	0.20	<0.01	0.18	1.90	—
8	<0.01	<0.01	<0.01	<0.01	0.10	0.26	0.16	0.38	1.03	—
9	0.03	0.04	0.07	0.01	0.15	0.15	0.08	0.34	0.65	—
10	0.04	0.05	0.09	0.02	0.09	0.31	0.10	0.28	0.81	—

Table II—Amino Acid Sequence for Peptides 2–10

Compound	Amino Acid Sequence
2	<Glu-His-Trp-D-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
3	<Glu-D-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
4	<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH
5	His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
6	<Glu-His-Trp
7	cyclo-[His-Trp]
8	Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
9	Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
10	His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-OH

The degradation of LH/RH displayed pseudo first-order kinetics at the conditions studied since the plots of ln $\{([LH/RH]_{t=0}/([LH/RH]_{t=t})\}$ versus time were linear (Figure 2). The corresponding rate constants (k_{obs}) are also shown in Figure 2. These rate constants are empirically observed constants, since we know by the product distributions (Figure 3) that there are two distinct degradation mechanisms operating at the pH studied; that is, hydrolysis of the peptide bonds (and of the terminal amide) and racemization. Examination of the product distributions also indicates that the most prevalent sites of hydrolysis are between the <Glu-His and the Trp-Ser peptide bonds and at the terminal amide. Each of these hydrolysis reactions might be expected to have a different rate of reaction. The overall degradation reaction appears to be a series of simultaneous reactions with the proposed rate equation:

$$d[LH/RH]/dt = k_1 [LH/RH] + k_2 [LH/RH] + k_3 [LH/RH] + \ldots \quad (1)$$

where $k_1, k_2, k_3, \ldots$ are the individual reaction rates for each hydrolysis or racemization. Based on the product distributions (Figure 3, Table I) there appears to be no change in the reaction mechanism either with time or with temperature, which suggests that the reactions are essentially independent. Therefore, k_{obs} for the overall reaction is the summation of the reaction rates ($k_{obs} = k_1 + k_2 + k_3 + \ldots$) for each hydrolysis and racemization. An Arrhenius plot of the data is

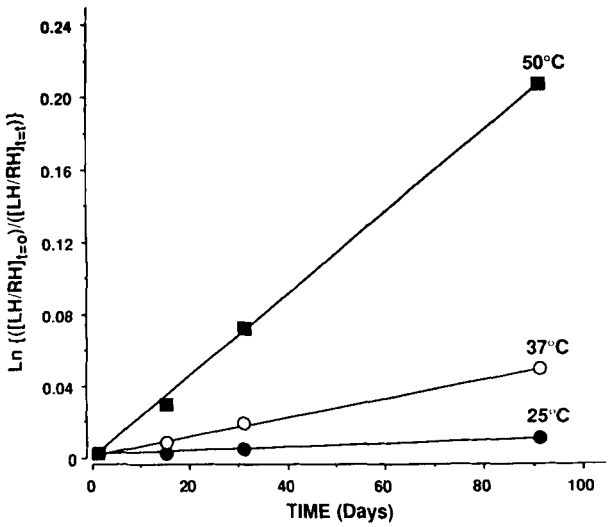


Figure 2—Plot of ln $\{([LH/RH]_{t=0})/([LH/RH]_{t=t})\}$ versus time at 25, 37, and 50 °C over a period of 90 days. The observed rate constants (k_{obs}, $days^{-1}$) at each temperature are as follows: 25 °C = 9.68×10^{-5}; 37 °C = 5.19×10^{-4}; 50 °C = 2.28×10^{-3}.

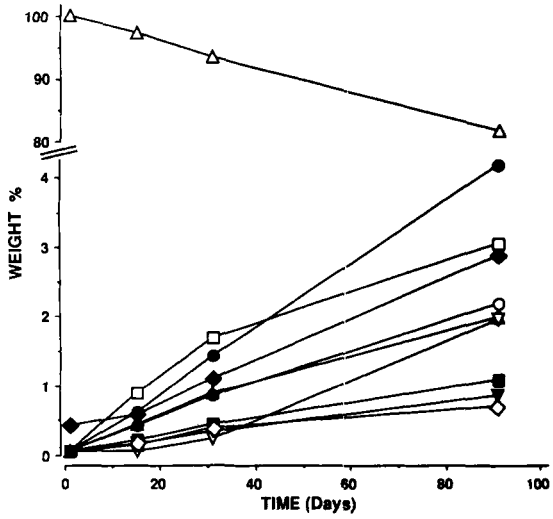


Figure 3—Variation of LH/RH and its degradation products with time at 80 °C and pH 6.5. Key: **1** (△); **2** (●); **3** (○); **4** (◆); **5** (□); **6** (▲); **7** (▽); **8** (■); **9** (◇); **10** (▼).

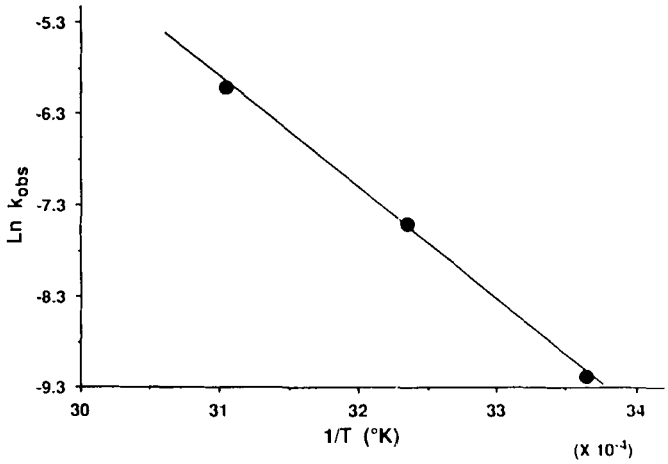


Figure 4—Arrhenius plot of ln k_{obs} versus $1/T$ for the degradation of LH/RH in aqueous solution at pH 6.5.

reasonably linear (Figure 4) and the slope of the line gives an activation energy for the overall reaction of 24.14 ± 10 kcal/mol. This is similar to that reported for an LH/RH analogue in aqueous solution at pH 5.4.[13]

This study shows that the peptide LH/RH is relatively stable in solution at its native pH (6.5) and that at 25 °C, <3% degradation occurs over a 90-day time period.

References and Notes

1. Schally, A. V.; Arimura, A.; Baba, Y.; Nair, R. M. G.; Matsuo, H.; Redding, T. W.; Debeljuk, L.; White, W. F. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 393–395.
2. Brodie, T. D.; Crowley, W. F. *Int. J. Fertility* **1985**, *30*, 66–75.
3. Corbin, A.; Bex, F. J.; Jones, R. C. *Int. J. Fertility* **1985**, *30*, 57–65.
4. Hafez, E. S. E.; Reel, J. R. *Hypothalamic Hormones*, Vol. 1; Ann Arbor Science: Ann Arbor, MI, 1981.
5. Shi, Y-F.; Sherins, R. J.; Brightwell. D.; Gallelli, J. F.; Chatterji, D. C. *J. Pharm. Sci.* **1984**, *73(6)*, 819–821.
6. Rivier, J. *J. Liq. Chromatogr.* **1978**, *1*, 343–366.
7. Shaw, C.; Cotter, M. L. *Chromatographia* **1986**, *21(4)*, 197–200.
8. Deslauries, R.; Levy, G. C.; McGregor, W. H.; Sarantakis, D.; Smith, I. C. P. *Biochemistry*, **1975**, *14(19)*, 4335–4343.
9. Sheinblatt, M.; Andorn, M.; Rudi, A. *Int. J. Pept. Protein Res.* **1988**, *31*, 373–387.

10. Wessels, P. L.; Feeney, J.; Gregory, H.; Gormley, J. J. *J. Chem. Soc., Perkin Trans. 2* **1973**, 1691–1698.
11. Oyler, A. R.; Naldi, R. E.; Lloyd, J. R.; Graden, D. A.; Shaw, C. J.; Cotter, M. L. *J. Pharm. Sci.* **1991**, *80*, 271–275.
12. Nishi, K.; Ito, H.; Shinagawa, S.; Hatanaka, C.; Funino, M.; Hattori, M. *Peptide Chemistry 1979;* Yonehara, H., Ed.; Protein Research Foundation: Osaka, Japan, 1980; pp 175–181.
13. Johnson, D. M.; Pritchard, R. A.; Taylor, W. F.; Conley, D.; Zuniga, G.; McGreevy, K. G. *Int. J. Pharm.* **1986**, *31*, 125–129.

Acknowledgments

The authors acknowledge Mr. Joseph Perry and Dr. Anneli DePaolis for performing the amino acid analysis, Dr. Robert T. Rosen of Rutgers University for providing the FAB mass spectra of the isolated peptides, Dr. David Burinsky and Mr. Richard Dunphy for providing the FAB mass spectra of the authentic peptides, and Dr. Kevin Facchine and Ms. Madeline Cozine for providing the NMR spectra of the authentic peptides.