# Structural Biochemistry XIII: Synthesis of Luteinizing Hormone-Releasing Hormone Modification [Trp$^8$]-LH-RH

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**Abstract** □ A fragment condensation method was utilized for synthesis of the Trp$^8$-substituted luteinizing hormone-releasing hormone (LH-RH). *tert*-Butoxycarbonyl protection was employed for the α-amino positions, and benzyl protection was used for the phenol group of Tyr and the imidazole nitrogen of His. Peptide bond-forming reactions were performed using *N*-hydroxysuccinimide (for Trp), dicyclohexylcarbodiimide-1-hydroxybenzotriazole, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride, or mixed carbonic anhydride methods. Biological evaluation of [Trp$^8$]-LH-RH indicated no luteinizing hormone-releasing activity or inhibition of luteinizing hormone release over the dose ranges studied.

**Keyphrases** □ Luteinizing hormone-releasing hormone—derivatives, synthesis, structure-activity relationships □ Chemical synthesis—luteinizing hormone-releasing hormone derivatives, structure-activity relationships □ Hormones—luteinizing hormone-releasing hormone, derivatives, synthesis, structure-activity relationships □ Structure-activity relationships—luteinizing hormone-releasing hormone derivatives

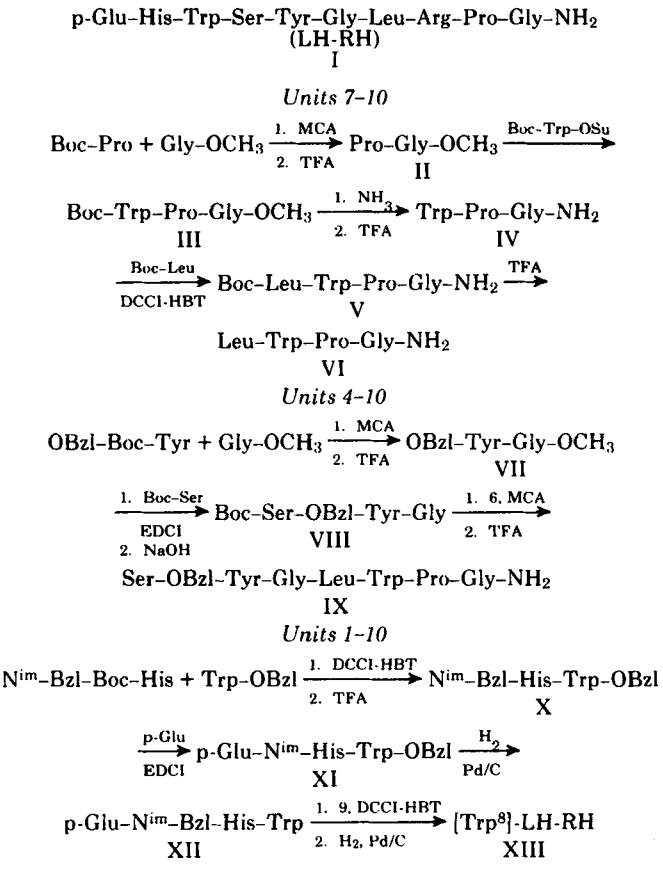
In humans, 1 μg of the hypothalamus decapeptide luteinizing hormone-releasing hormone (LH-RH, I) stimulates the pituitary to synthesize and release the luteinizing hormone (LH). Isolation of this decapeptide hormone from porcine hypothalami was first reported in 1971 (1, 2). This discovery has impressive potential for solving certain medical problems, from overcoming a cause of female sterility to opening new approaches to contraception. Therefore, synthetic efforts directed at the decapeptide hormone and related substances have been undertaken and presently underlie the major effort in decapeptide chemistry (3–15).

## BACKGROUND

One facet of the LH-RH potential that seemed particularly attractive was that a suitable modification of this hormone might inhibit certain cancer types, *e.g.*, of the ovary. In 1968, certain tryptophan dipeptides were observed[1] to have antineoplastic activity. This discovery led to attempts to modify LH-RH by replacement of Arg at position 8 with Trp. This amino acid replacement modification also was selected because the hypothalamus growth hormone release-inhibiting tetradecapeptide hormone, somatostatin (16–18), contains Trp at position 8. In addition, LH-RH activity was known to be profoundly affected by modification at the Arg position (19–21).

Decapeptide synthesis was performed by a fragment condensation, as outlined in Scheme I. The *tert*-butoxycarbonyl (Boc) group was used for α-amino protection, and its removal was performed employing 50% trifluoroacetic acid in methylene chloride. With peptides containing a Trp unit, 2-mercaptoethanol was added to prevent oxidative degradation (22). The phenolic group of Tyr was protected as the *O*-benzyl ether, and the imidazole nitrogen of His was protected as the benzylamine derivative. In both cases, deprotection was achieved by hydrogenolysis using palladium-on-carbon.

Methyl ester saponification was effected employing aqueous sodium hydroxide in methanol. Peptide bond-forming reactions were efficiently realized employing the *N*-hydroxysuccinimide (NHS) active ester for coupling Trp (II → III), and either dicyclohexylcarbodiimide 1-hydroxybenzotriazole (DCCI-HBT), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), or mixed carbonic anhydride (MCA) techniques at the other positions (13). By this combination of methods, the protected decapeptide was obtained in reasonable yield. After hydrogenolysis, [Trp$^8$]-LH-RH was isolated and evaluated[2].




*Scheme I*

The luteinizing hormone-releasing biological assay was performed with 200-g female rats, which were ovariectomized and allowed to rest 6 weeks. Three days following treatment with progesterone and estradiol benzoate, the animals were treated intravenously with 10–1000-ng doses of [Trp$^8$]-LH-RH with standard LH-RH as a control. Samples were collected 15 min later and analyzed for luteinizing hormone content. While the standard LH-RH produced significant dose-related luteinizing hormone release as expected, the [Trp$^8$]-LH-RH showed no significant luteinizing hormone release over the same dose range. Also, at doses of 50–200 μg, [Trp$^8$]-LH-RH showed no inhibition of luteinizing hormone release when given to 25-day-old male rats (23).

Based on these results, preparation of a modified LH-RH decapeptide bearing D-Trp at positions 8 and/or 2 is of interest with respect (24) to influencing luteinizing hormone release (5, 8, 9). Unfortunately, the limited amount of [Trp$^8$]-LH-RH available did not allow a definitive assessment of antineoplastic activity.

## EXPERIMENTAL

All of the precursor L-amino acids and derivatives were employed as

[1] G. R. Pettit and S. K. Gupta, unpublished results.

[2] Dr. B. H. Vickery, Institute of Biological Sciences, Syntex, Palo Alto, Calif.

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received[3]. Solvent extracts containing completely protected peptides were washed consecutively with water, 0.5 *M* citric acid, water, 5% aqueous sodium bicarbonate, and saturated aqueous sodium chloride. Each extract was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure at 35–40° using a rotating evaporator. Dimethylformamide was distilled (under reduced pressure) from calcium oxide, while tetrahydrofuran was distilled from lithium aluminum hydride just prior to use.

All melting points were recorded using a hot-stage apparatus[4] and are uncorrected. Each analytical specimen gave one spot on TLC and gave IR, PMR, and mass spectral data in accord with the assigned structure[5].

**Boc-Pro-Gly-$OCH_3$**—A solution of Boc-Pro (10.75 g, 50 mmoles) in tetrahydrofuran (250 ml) was cooled to −15°, and *N*-methylmorpholine (5.5 ml, 50 mmoles) followed by isobutyl chlorocarbonate (6.95 ml, 50 mmoles) was added. Before Gly-$OCH_3$ hydrochloride (6.6 g, 50 mmoles) and *N*-methylmorpholine (5.5 ml, 50 mmoles) were added, the mixture was stirred for 15 min at −15°. Stirring was continued for 2 hr at 0° and for 48 hr at 23°. The solvent was removed, and the residue was dissolved in ethyl acetate, washed, and dried. Solvent evaporation yielded a pink solid, which was recrystallized from ether to afford 11.8 g (78%), mp 70–72°; $[\alpha]_D^{25}$ −138° (methanol).

*Anal.*—Calc. for $C_{13}H_{22}N_2O_5$: C, 54.53; H, 7.74; N, 9.78. Found: C, 54.40; H, 7.86; N, 9.85.

**Pro-Gly-$OCH_3$ (II) Hydrochloride**—After 1 hr at room temperature, the solvent was removed from a solution of Boc-Pro-Gly-$OCH_3$ (102.8 mg, 0.358 mmole) in 50% trifluoroacetic acid (TFA)–methylene chloride (4 ml). The oily residue was treated with cold ethereal hydrogen chloride to form an oily precipitate. The solvent was removed, and the residue was dissolved in water (5 ml) and chromatographed on a column of XAD-2 resin (75 ml). Elution with water (300 ml), concentration to 15 ml, and lyophilization yielded 79.5 mg (100%) of the dipeptide II as a viscous oil.

**Boc-Trp-Pro-Gly-$OCH_3$ (III)**—A solution of Pro-Gly-$OCH_3$ hydrochloride (0.82 g, 3.71 mmoles), Boc-Trp-OSu (1.49 g, 3.71 mmoles), *N*-methylmorpholine (0.41 ml, 3.71 mmoles), and dimethylformamide (6 ml) was stirred at 23° for 8 hr. Ethyl acetate was added, and the mixture was washed and dried. The solvent was removed, and the residue was purified by column chromatography on silica gel (100 g, elution with ethyl acetate) and crystallized from ether–ligroin to afford 0.98 g (56%) of the tripeptide III, mp 86–88°; $[\alpha]_D^{25}$ −112° (methanol).

*Anal.*—Calc. for $C_{24}H_{32}N_4O_6$: C, 61.00; H, 6.83; N, 11.86. Found: C, 60.86; H, 7.00; N, 11.67.

**Boc-Trp-Pro-Gly-$NH_2$**—Boc-Trp-Pro-Gly-$OCH_3$ (0.82 g, 1.73 mmoles) was dissolved in methanol (40 ml) and cooled to 0°. Ammonia was passed into the solution for 1.5 hr. The reaction flask was tightly stoppered and allowed to stand at room temperature for 24 hr. Following solvent removal, the residue was crystallized from ethyl acetate–ligroin to afford 0.788 g (100%) of amide, mp 120–121°; $[\alpha]_D^{25}$ −49° (methanol).

*Anal.*—Calc. for $C_{23}H_{31}N_5O_5 \cdot H_2O$: C, 57.98; H, 6.93; N, 14.71. Found: C, 58.38; H, 6.66; N, 14.86.

**Trp-Pro-Gly-$NH_2$ (IV) Trifluoroacetate**—A solution of Boc-Trp-Pro-Gly-$NH_2$ (0.65 g, 1.42 mmoles) in 50% trifluoroacetic acid–methylene chloride (20 ml) containing 1% 2-mercaptoethanol was maintained at room temperature for 1 hr. The solvent was removed, and the residue was triturated with ether. The colorless precipitate was collected, and this hygroscopic solid was dissolved in water (20 ml) and lyophilized to yield 0.65 g (97%) of the amide IV as the trifluoroacetate.

**Boc-Leu-Trp-Pro-Gly-$NH_2$ (V)**—Dicyclohexylcarbodiimide (1.33 g, 6.48 mmoles) was added to a solution prepared from Trp-Pro-Gly-$NH_2$ trifluoroacetate (2.77 g, 5.89 mmoles), Boc-Leu (1.37 g, 5.89 mmoles)[1], 1-hydroxybenzotriazole (1.59 g, 11.8 mmoles), and *N*-methylmorpholine (0.66 ml, 5.89 mmoles) in tetrahydrofuran (80 ml) cooled to 0°. The mixture was stirred at 0° for 1 hr and at 23° for 3 hr. The solution was filtered, the solvent was removed, and the residue was dissolved in ethyl acetate, washed, and dried. After the solvent was evaporated, the residue was recrystallized from ethyl acetate ligroin to afford 2.37 g (70%) of the tetrapeptide amide V, mp 119–120°; $[\alpha]_D^{25}$ −37° (methanol).

*Anal.*—Calc. for $C_{29}H_{42}N_6O_6 \cdot H_2O$: C, 59.18; H, 7.48; N, 14.29. Found: C, 59.04; H, 7.52; N, 14.48.

**Leu-Trp-Pro-Gly-$NH_2$ (VI) Trifluoroacetate**—Cleavage of the protecting group from Boc-Leu-Trp-Pro-Gly-$NH_2$ (26.8 mg, 0.047 mmole) was conducted with 50% trifluoroacetic acid–methylene chloride (4 ml) containing 1% 2-mercaptoethanol as described for preparation of IV to yield 32.8 mg (84%) of the amide VI as the trifluoroacetate.

**OBzl-Boc-Tyr-Gly-$OCH_3$**—To a solution of OBzl-Boc-Tyr (5.0 g, 16.2 mmoles) in tetrahydrofuran (90 ml), cooled to −15°, was added *N*-methylmorpholine (1.81 ml, 16.2 mmoles) and isobutyl chlorocarbonate (2.11 ml, 16.2 mmoles). Before Gly-$OCH_3$ hydrochloride (2.02 g, 16.2 mmoles) and *N*-methylmorpholine (1.81 ml, 16.2 mmoles) were added, the mixture was stirred for 15 min at −15°. Then the mixture was stirred for 2 hr at 0° and for 26 hr at 23°. The solvent was removed, and a solution of the residue in ethyl acetate was washed, dried, and concentrated to a solid. The solid was recrystallized from ether to provide 5.70 g (92%) of the dipeptide, mp 118–120°; $[\alpha]_D^{25}$ −11° (methanol). The dipeptide VII was previously prepared in a 90% yield (mp 118–120°) by a *p*-nitrophenyl ester peptide bond-forming method (25).

*Anal.*—Calc. for $C_{24}H_{30}N_2O_6$: C, 65.11; H, 6.78; N, 6.35. Found: C, 64.90; H, 6.96; N, 6.32.

**OBzl-Tyr-Gly-$OCH_3$ (VII) Hydrochloride**—Removal of the Boc group from OBzl-Boc-Tyr-Gly-$OCH_3$ (3.65 g, 8.26 mmoles) was achieved using 50% trifluoroacetic acid–methylene chloride (30 ml, for 1 hr at 23°). The solvent was removed, and the residue was treated with cold ethereal hydrogen chloride. The colorless precipitate was collected and washed with ether. The hygroscopic solid was dissolved in water (20 ml) and lyophilized to yield 3.02 g (97%) of dipeptide ester VII as the hydrochloride.

**Boc-Ser-OBzl-Tyr-Gly-$OCH_3$**—A solution prepared from Boc-Ser (0.27 g, 1.31 mmoles), OBzl-Tyr-Gly-$OCH_3$ hydrochloride (VII, 0.497 g, 1.31 mmoles), and *N*-methylmorpholine (0.15 ml, 1.31 mmoles) in ethyl acetate–dimethylformamide (2:1, 15 ml) was cooled to 0°. Following addition of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (0.27 g, 1.43 mmoles), the mixture was stirred at 0° for 1 hr and at 23° for 20 hr. The reaction mixture was diluted with ethyl acetate, washed, and dried; the solvent was removed to yield a yellow oil. The crude product was purified by column chromatography on silica gel (100 g, elution with 1:1 ethyl acetate–chloroform) and recrystallized from ethyl acetate–ligroin to afford 0.49 g (72%) of the tripeptide, mp 104–105°; $[\alpha]_D^{25}$ −26° (methanol).

*Anal.*—Calc. for $C_{27}H_{35}N_3O_8$: C, 61.25; H, 6.62; N, 7.94. Found: C, 61.15; H, 6.75; N, 8.07.

**Boc-Ser-OBzl-Tyr-Gly (VIII)**—Boc-Ser-OBzl-Tyr-Gly-$OCH_3$ (0.71 g, 1.34 mmoles) was dissolved in a solution of 0.86 *N* NaOH in 90% aqueous methanol (15 ml). After 45 min at room temperature, the methanol was removed and the residue was diluted with water (25 ml) and extracted with ether (10 ml). The aqueous layer was acidified with 1 *N* HCl, saturated with sodium chloride, and extracted with ethyl acetate (3 × 20 ml). The combined extract was concentrated to dryness, and the residue was recrystallized from ethyl acetate–ligroin to give 0.61 g (87%) of tripeptide VIII, mp 88–90°; $[\alpha]_D^{25}$ −24° (methanol).

*Anal.*—Calc. for $C_{26}H_{33}N_3O_8$: C, 60.57; H, 6.45; N, 8.15. Found: C, 60.44; H, 6.35; N, 8.29.

**Boc-Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$**—By means of the mixed carbonic anhydride procedure described for the preparation of the dipeptide IV, Boc-Ser-OBzl-Tyr-Gly (VIII, 0.27 g, 0.521 mmole) was allowed to react in tetrahydrofuran (15 ml) with *N*-methylmorpholine (58 μl, 0.521 mmole) and isobutyl chlorocarbonate (75 μl, 0.573 mmole) followed by Leu-Trp-Pro-Gly-$NH_2$ trifluoroacetate (0.30 g, 0.521 mmole) and *N*-methylmorpholine (58 μl, 0.521 mmole) in tetrahydrofuran (2 ml). The product was recrystallized from chloroform–ligroin to afford 0.47 g (94%) of the heptapeptide amide, mp 136–138°; $[\alpha]_D^{25}$ −35° (methanol).

*Anal.*—Calc. for $C_{50}H_{65}N_9O_{11} \cdot H_2O$: C, 60.91; H, 6.80; N, 12.80. Found: C, 60.56; H, 6.61; N, 12.70.

**Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$ (IX) Hydrochloride**—A solution of Boc-Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$ (0.71 g, 0.735 mmole) in 50% trifluoroacetic acid–methylene chloride (40 ml) containing 1% 2-mercaptoethanol was allowed to remain for 1 hr at 23°. Solvent was removed, and the residue was treated with cold ethereal hydrochloric acid to form a colorless precipitate. The precipitate was collected, washed with ether, and dried to yield 0.55 g (85%) of the heptapeptide amide IX, mp 149–150°.

**$N^{im}$-Bzl-Boc-His-Trp-OBzl**—A solution prepared from $N^{im}$-Bzl-Boc-His (5.10 g, 14.8 mmoles) (26), Trp-OBzl hydrochloride (4.87 g, 14.8 mmoles) (27), 1-hydroxybenzotriazole (3.95 g, 29.6 mmoles), *N*-methylmorpholine (1.66 ml, 14.8 mmoles), and dimethylformamide (100 ml) was cooled to 0°, and dicyclohexylcarbodiimide (3.34 g, 16.3 mmoles)

[3] Nutritional Biochemicals Corp. or Koch-Light.

[4] Kofler.

[5] The amino acid analyses provided by Dr. R. H. Ode and Dr. J. Cronin were determined with a Beckman-Spinco 121 amino acid analyzer. Elemental microanalytical data were determined by Dr. A. Bernhardt, Mikroanalytisches Laboratorium, 5251 Elbach über Engelskirchen, West Germany.

was added. The mixture was stirred at 0° for 1 hr and at 23° for 16 hr. The solution was filtered, the solvent was removed, and the residue was triturated with chloroform. The chloroform solution was filtered, and the filtrate was washed, dried, and concentrated to dryness. Recrystallization of the residue from chloroform–ligroin afforded 6.27 g (68%) of the dipeptide, mp 67–68°; $[\alpha]_D^{25}$ +3° (methanol).

*Anal.*—Calc. for $C_{36}H_{39}N_5O_5$: C, 69.73; H, 6.28; N, 11.27. Found: C, 69.49; H, 6.18; N, 11.12.

**$N^{im}$-Bzl-His-Trp-OBzl (X) Hydrochloride**—Elimination of the Boc protecting group was realized as described for preparation of VII hydrochloride employing $N^{im}$-Bzl-Boc-His-Trp-OBzl (55.8 mg, 0.090 mmole) in 50% trifluoroacetic acid–methylene chloride (8 ml) containing 1% 2-mercaptoethanol. The hygroscopic product was dissolved in water (15 ml) and lyophilized to yield 45.9 mg (91%) of the dipeptide ester (X) hydrochloride.

**p-Glu-$N^{im}$-Bzl-His-Trp-OBzl (XI)**—To a dimethylformamide (40 ml) solution of p-Glu (0.45 g, 3.51 mmoles), $N^{im}$-Bzl-His-Trp-OBzl hydrochloride (1.95 g, 3.51 mmoles), and *N*-methylmorpholine (0.39 ml, 3.51 mmoles), cooled to 0°, was added 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (0.74 g, 3.86 mmoles). The mixture was stirred at 0° for 1 hr and at 23° for 12 hr. After concentration to 15 ml, the solution was poured into ice–5% sodium bicarbonate (100 ml). The solid was collected, washed with water, and recrystallized from dimethylformamide–water to afford 1.66 g (75%) of the tripeptide XI, mp 235–237°; $[\alpha]_D^{25}$ +13° (dimethylformamide).

*Anal.*—Calc. for $C_{36}H_{36}N_6O_5 \cdot H_2O$: C, 66.46; H, 5.85; N, 12.91. Found: C, 65.70; H, 5.64; N, 12.64.

**p-Glu-$N^{im}$-Bzl-His-Trp (XII)**—A mixture of p-Glu-$N^{im}$-Bzl-His-Trp-OBzl (XI, 1.55 g, 2.42 mmoles), 5% palladium-on-charcoal (1.0 g), and methanol–acetic acid (80 ml) (3:2) was stirred under 1 atmosphere of hydrogen at 23° for 8 hr. The solution was filtered through diatomaceous earth, and solvent was removed from the filtrate. The residue was recrystallized from methanol–ether to provide 0.64 g (48%) of the tripeptide XII, mp 165–167°; $[\alpha]_D^{25}$ +19° (methanol).

**p-Glu-His-Trp-Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$ ([$Trp^8$]-LH-RH) (XIII)**—A dimethylformamide (30 ml) solution of XII (0.335 g, 0.617 mmole), Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$ hydrochloride (0.55 g, 0.617 mmole), 1-hydroxybenzotriazole (0.17 g, 1.23 mmoles), and *N*-methylmorpholine (69 μl, 0.617 mmole) was cooled to 0°, and dicyclocarbodiimide (0.14 g, 0.679 mmole) was added. The mixture was stirred at 0° for 1 hr and at 23° for 7 hr. Then the solution was filtered and poured into ice–5% sodium bicarbonate (100 ml). Following collection of the solid and trituration with hot methanol, the resulting solution was filtered. The solvent was removed, and the residue was dissolved in acetic acid (20 ml) and lyophilized to yield 0.60 g (71%) of the protected decapeptide as a powder. Recrystallization from methylethyl ether gave a specimen of p-Glu-$N^{im}$-Bzl-His-Trp-Ser-OBzl-Tyr-Gly-Leu-Trp-Pro-Gly-$NH_2$, mp 245–248° dec.; $[\alpha]^{25}$ −19° (acetic acid).

A mixture composed of the decapeptide (89.7 mg, 0.0645 mmole), 5% palladium-on-charcoal (0.10 g), and methanol–acetic acid (40 ml) (4:1) was stirred under 1 atmosphere of hydrogen at 23° for 40 hr. The solution was filtered through diatomaceous earth, and the solvent was evaporated to a solid. The solid was purified by preparative TLC on silica gel with methanol–ethyl acetate (7:3) as the mobile phase. The product was eluted with methanol–acetic acid, dissolved in acetic acid, and lyophilized to yield 55.4 mg (71%) of [$Trp^8$]-LH-RH (XIII).

The decapeptide XIII (5 mg) was dissolved in 6 *N* HCl and hydrolyzed at 110° for 20 hr in a sealed tube. The solution was freeze dried, and the residue was dissolved in citrate buffer (1 ml). A 500-μl aliquot was diluted to 10.0 ml with the same buffer, and a 1-ml sample of this solution was used for amino acid analysis, which showed: His (0.6), Ser (1.0), Glu (1.0), Pro (1.0), Gly (2.0), Leu (1.0), and Tyr (1.0). Repeated amino acid analyses gave essentially the same results for His. Similar low values for this amino acid were noted with several other modified LH-RH decapeptides (6).

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