

Synthesis of a Highly Potent Analog of Luteinizing Hormone Releasing Hormone (LH-RH): [Des-Gly-NH₂¹⁰, Pro-NH-Et⁹]-LH-RH¹⁾

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A highly potent analog of luteinizing hormone releasing hormone (LH-RH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH₂-CH₃ was synthesized by the conventional solution method. The key intermediate, H-Leu-Arg(NO₂)-Pro-NHCH₂-CH₃, which was prepared by the stepwise manner using the activated esters of the corresponding protected amino acids, was coupled with N-terminal hexapeptide, pGlu-His-Trp-Ser-Tyr-Gly-OH, by the HONB/DCC method to give the mono-protected nonapeptide ethylamide. The protected peptide was treated with stannous chloride in 60% aqueous formic acid to remove the nitro group. The purification of the resulting peptide was carried out by a column chromatography on Amberlite XAD-2 and followed by a column chromatography on carboxymethylcellulose. The synthetic method described is promising for preparing this analog of LH-RH in large quantities and good quality.

Recently we³⁾ have communicated that an analog of luteinizing hormone releasing hormone (LH-RH), [Des-Gly-NH₂¹⁰, Pro-NH-Et⁹]-LH-RH (I), was found to be five times as potent as the original hormone, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂,⁴⁾ in inducing ovulation in the diestrous rat and the constant estrous rabbit.

Since we reported the synthesis of this analog³⁾ without detailed description, and moreover the analog have been gradually become important in a clinical use,⁵⁾ we now wish to describe an improve synthesis of this peptide in detail.

For the synthesis of I the desired mono-protected nonapeptide ethylamide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg(NO₂)-Pro-NH-Et (XI) was prepared by the conventional solution method, as shown in Chart 1. The key intermediate Z-Leu-Arg(NO₂)-Pro-NH-Et (IV) was prepared by the stepwise elongation starting from ethylamine. The intermediate was then treated with 25% hydrogen bromide in acetic acid to remove the Z-protecting group, and the corresponding free base (V) was obtained by treatment with Amberlite IR-45 (OH⁻). The other intermediate pGlu-His-Trp-Ser-Tyr-Gly-OH (X) was prepared by the coupling of a crystalline tripeptide pGlu-His-Trp-OH (VIII)⁶⁾ and H-Ser-Tyr-Gly-OEt (VII) by the HONB/DCC method⁶⁾ to minimize undesirable racemization and followed by saponification of the ethyl ester (IV).

- 1) Amino acids, peptides and their derivatives mentioned in this communication are L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemistry Nomenclature in July 1965 and July 1966; *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967). Z=benzyloxycarbonyl, OEt=ethyl ester, NH-Et=ethylamide, NO₂=nitro, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, DCC=N,N'-dicyclohexylcarbodiimide.
- 2) Location: Juso-Honmachi, Yodogawa-ku, Osaka.
- 3) M. Fujino, S. Shinagawa, I. Yamazaki, S. Kobayashi, M. Obayashi, T. Fukuda, R. Nakayama, W.F. White and R.H. Rippel, *Arch. Biochem. Biophys.*, **154**, 488 (1973).
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pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	HN-Et
								Z-OH H-	
								DCC	
								Z-	
									II
								H ₂ /Pd	
			Z	NHNH ₂ H	OEt		Z-NO ₂	ODNP H	
			Z		OEt		Z-NO ₂		
			H ₂ /Pd	VI					III
					OEt	Z-OSu	H-NO ₂	HBr	
	VIII	OH H	DCC/HONB	VII	OEt	Z	H-NO ₂		
				IX	OEt	Z			IV
				NaOH			H-NO ₂	HBr	
				X	OH H		H-NO ₂		V
					DCC-HONB				

Coupling of N-terminal hexapeptide X and C-terminal tripeptide ethylamide V was effected by the HONB/DCC method to give the crude monoprotected nonapeptide XI which was purified by column chromatography on silica gel using a mixture of ethylacetate, pyridine, acetic acid and water as a eluting solvent. The purified mono-protected peptide XI having a nitro group as a protecting group was subjected to a reduction with stannous chloride in 60% aqueous formic acid for 120 min at 80–85°, which is a modification of the method of Hayakawa, *et al.*⁷⁾ The crude peptide thus obtained was purified by a column chromatography on Amberlite XAD-2 and followed by column chromatography on carboxymethylcellulose in a manner similar to that described for the purification of synthetic LH-RH.⁶⁾ The final product was found to be homogeneous by thin-layer (silica gel) chromatography (TLC) and paper electrophoresis to Ehrlich, Sakaguchi and Pauly reagents and the ultraviolet (UV) spectrum of this material was identical with that of LH-RH itself. The ovulation-inducing activity of the product obtained was as much higher as that described previously⁸⁾ ($ED_{50}=32.0\pm 8$; the ED_{50} of LH-RH= 215 ± 15), determined by the methods of Yamazaki and Nakayama.⁹⁾

The synthetic methodology described in this report is promising for preparing I in large quantities with excellent quality.

Experimental

All melting points were taken by the capillary method and are uncorrected. Evaporations were carried out with a rotary evaporator. The purity of product was tested by thin-layer chromatography using Merck's precoated silica gel plate 60 F₂₅₄. Solvent systems used were: *Rf*¹, CHCl₃-MeOH-AcOH (9:1:0.5); *Rf*², AcOEt-pyridine-AcOH-H₂O (60:20:6:10); *Rf*³, *n*-BuOH-pyridine-AcOH-H₂O (30:20:6:24); *Rf*⁴, *n*-BuOH-AcOEt-AcOH-H₂O (1:1:1:1).

Z-Pro-NH-Et (II)—To a solution of Z-Pro-OH (124.6 g, 0.5 mole) and HONB (90 g) in tetrahydrofuran (2 liters) was added DCC (120 g) at 0°. After stirring at 0° for 3 hr and additional 2 hr at 10°, the reaction mixture was filtered to remove the formed dicyclohexylurea, and the filtrate was concentrated to *ca.* 1 liter.

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To this solution was added ethylamine (70% solution, 50 ml), and the mixture was stirred for 3 hr at room temperature. The solution was evaporated to dryness *in vacuo* and the resulting residue was dissolved in AcOEt (600 ml), washed with 5% NaHCO₃ and water, and evaporated to dryness to give crystals (needles), which were collected by filtration and recrystallized from AcOEt-pet.ether: Yield, 128 g (92.7%), mp 104–105°, $[\alpha]_D^{25}$ –38.6° ($c=0.94$ in EtOH), $Rf^1=0.69$. Anal. Calcd. for C₁₅H₂₀O₃N₂: C, 65.19; H, 7.30; N, 10.14. Found: C, 65.20; H, 7.48; N, 10.18.

Z-Arg(NO₂)-Pro-NH-Et (III)—Compound II (83 g, 0.3 mole) was hydrogenated over a Pd-black in MeOH (1.5 liters). The reaction mixture was filtered to remove the catalyst and the filtrate was evaporated to dryness *in vacuo*. The resulting residue was dissolved in tetrahydrofuran (500 ml) together with 2,4-dinitrophenyl Z-nitroarginate¹⁰ (prepared from 100.6 g of Z-Arg(NO₂)-OH and 2,4-dinitrophenol by the DCC-method). After stirring for 10 hr at room temperature, the reaction mixture was evaporated to dryness. The residue was dissolved in CHCl₃ (2 liters) and the solution was washed with 1N aqueous ammonia and 1N HCl, and dried over MgSO₄. The solution was then evaporated to dryness. The residue was triturated with ether to give solid which was collected by filtration and crystallized from EtOH; yield, 130.8 g (91.4%), mp 143–146°, $[\alpha]_D^{25}$ –45.5° ($c=1.0$ in MeOH), $Rf^1=0.55$. Anal. Calcd. for C₂₁H₃₁O₆N₇: C, 52.82; H, 6.54; N, 20.53. Found: C, 52.95; H, 6.77; N, 19.65.

Z-Leu-Arg(NO₂)-Pro-NH-Et (IV)—A solution of the compound III (130 g) in 25% HBr-acetic acid (1.2 liters) was allowed to stand at room temperature for 50 min. The product was precipitated by addition of dry ether to the reaction mixture, and the precipitate was collected, washed well with dry ether and dried over NaOH-pellets *in vacuo*. The dried product was dissolved in dimethylformamide (DMF) (1 liter) containing triethylamine (40.1 ml). To this was added Z-Leu-OSu (97.7 g, 0.27 mole), and the mixture was stirred for 8 hr at room temperature. The reaction mixture was then evaporated *in vacuo* to a small volume, and to this was added CHCl₃ (2 liters). The solution was washed with water (1 liter \times 3), dried over anhydrous MgSO₄, and evaporated to dryness to give a fine powder. The powder was triturated with ether and collected by filtration, and purified by reprecipitation from MeOH-ether; yield, 142 g (89.9%), mp 144–146°, $[\alpha]_D^{25}$ –59.0° ($c=1.0$ in MeOH), $Rf^1=0.50$. Anal. Calcd. for C₂₇H₄₂O₇N₈·H₂O: C, 54.28; H, 7.28; N, 18.41. Found: C, 54.12; H, 7.30; N, 18.23.

H-Leu-Arg(NO₂)-Pro-NH-Et (V)—Compound IV (80 g) was dissolved in 25% HBr-acetic acid (400 ml) and the solution was stirred at 20° for 70 min. The product was precipitated by addition of dry ether to the reaction mixture, and the precipitate was collected, washed well with dry ether and dried over NaOH-pellets *in vacuo*. The dried powder was dissolved in 10% aqueous MeOH (1 liter) and the solution was passed through a column of Amberlite IRA-4B (OH[–]) to remove HBr. The eluate and washings were combined and evaporated to a small volume and then lyophilized to give the desired free base; yield, 47 g (75.8%), $[\alpha]_D^{25}$ –51.2° ($c=0.5$ in 5% AcOH), $Rf^2=0.31$. Anal. Calcd. for C₁₉H₃₆O₅N₈·H₂O: C, 48.08; H, 8.06; N, 23.61. Found: C, 47.97; H, 7.81; N, 23.55.

Z-Ser-Tyr-Gly-OEt (VI)—Z-Ser-Tyr-NHNH₂¹¹ (41.6 g, 0.1 mole) was dissolved in a mixture of DMF (250 ml) and 3N HCl (100 ml), and to this solution was added 2N sodium nitrite (55 ml) at –5°. After the mixture was stirred for 10 min at –5°, the reaction mixture was diluted with aqueous NaCl (saturated, 400 ml) and the azide produced was extracted with AcOEt (250 ml \times 2). The extracts were combined and washed cold water, dried over anhydrous Na₂SO₄ for 30 min and filtrated to give the azide solution. To a solution of glycine ethyl ester hydrochloride (16.8 g, 0.12 mole) and triethylamine (16.8 ml) in a mixture of AcOEt (100 ml) and DMF (50 ml) was added the above mentioned azide solution. The mixture was stirred at 0° for 2 days. The reaction mixture was washed with 5% NaHCO₃ and 1N HCl, dried over anhydrous Na₂SO₄, and evaporated. The resulting solid was collected by filtration and purified by recrystallization from EtOH; yield, 20.0 g (41%), mp 162–163°, $[\alpha]_D^{25}$ –16.9° ($c=1.0$ in MeOH), $Rf^1=0.64$. Anal. Calcd. for C₂₄H₂₉O₈N₃: C, 59.13; H, 6.00; N, 8.62. Found: C, 58.93; H, 6.12; N, 8.45.

H-Ser-Tyr-Gly-OEt (VII)—Compound VI (13.2 g, 27 mmole) was dissolved in MeOH (200 ml) and was hydrogenated over a Pd-black as a catalyst for 5 hr. The solution was filtered to remove the catalyst and evaporated to give crystals, which was recrystallized from EtOH; yield, 9.2 g (96%), mp 171–172°, $[\alpha]_D^{25}$ 17.8° ($c=0.5$ in 5% AcOH), $Rf^2=0.31$. Anal. Calcd. for C₁₆H₂₃O₆N₃: C, 54.38; H, 6.56; N, 11.89. Found: C, 54.24; H, 6.48; N, 11.94.

pGlu-His-Trp-Ser-Tyr-Gly-OEt (IX)—Compound VII (8.8 g, 25 mmole) and pGlu-His-Trp-OH⁹ (VIII) (9.92 g, 22 mmole) were dissolved in DMF (200 ml), and to this was added HONB (5.5 g) and DCC (6.35 g) at 0°. The mixture was stirred at 0° for 2 hr and at room temperature overnight. The resulting dicyclohexylurea was filtered off and the filtrate was evaporated to dryness, and the resulting residue was triturated with ether to give a fine solid which was collected by filtration and purified by reprecipitation from DMF-AcOEt; Yield, 17.4 g (98%), mp 175–178 (decomp), $[\alpha]_D^{25}$ –15.5° ($c=1.0$ in DMF), $Rf^2=0.17$, $Rf^4=0.71$. Anal. Calcd. for C₃₈H₄₅O₁₀N₉·H₂O: C, 56.63; H, 5.87; N, 15.64. Found: C, 56.54; H, 5.76; N, 14.92.

pGlu-His-Trp-Ser-Tyr-Gly-OH (X)—Compound IX (14.8 g, 18 mmole) was suspended in MeOH (50 ml)

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and to this was added 1N NaOH (54 ml) in an ice bath. After stirring for 90 min at room temperature, the clear solution obtained was then neutralized by addition of 1N HCl (54 ml) to give rise to precipitates, which were collected by filtration and washed with cold water and dried over P_2O_5 . The powder obtained was purified by reprecipitation from DMF-AcOEt; yield, 11.5 g (84%), mp 197–201° (decomp.), $[\alpha]_D^{25} -22.8^\circ$ ($c=0.5$ in DMF), $Rf^3=0.52$. Anal. Calcd. for $C_{36}H_{41}O_{10}N_9 \cdot 4H_2O$: C, 51.98; H, 5.93; N, 15.15. Found: C, 51.95; H, 5.39; N, 14.80.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg(NO₂)-Pro-NH-Et (XI)—Compound X (10 g, 13.3 mmoles) and IV (7.3 g, 16 mmoles) were dissolved in DMF (80 ml) in the presence of HONB (3.33 g), and to this was added DCC (3.48 g) at 0° with stirring. The mixture was stirred for 5 hr at 0° and for additional 12 hr at room temperature. The resulting dicyclohexylurea was filtered off and to this filtrate was added AcOEt (200 ml) to give precipitate; 21 g. The crude product thus obtained was purified by a column chromatography on silica gel (400 g, solvent system; Rf^2). The crude product was dissolved in 100 ml of Rf^2 -solvent and applied to the column, and then eluted with the same solvent. The fractions containing the desired pure product (checked by TLC) were combined and evaporated to dryness; yield, 8.6 g (54%), mp 183–186° (decomp.) $[\alpha]_D^{25} -47.4^\circ$ ($c=0.5$ in 5% AcOH), $Rf^2=0.23$, $Rf^4=0.66$. Anal. Calcd. for $C_{55}H_{75}O_{14}N_{17} \cdot 5H_2O$: C, 51.28; H, 6.65; N, 18.48. Found: C, 51.46; H, 6.37; N, 18.10.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NH-Et (I)—To a solution of compound XI (8.5 g, 7.2 mmoles) in 240 ml of 60% formic acid was added stannous chloride (13 g), and the mixture was then allowed to stand for 2 hr at 80°. The solution was cooled to 0° and the resulting precipitate was removed by filtration. The filtrate was diluted with water (500 ml) and the solution was applied to a column (8×15 cm) of Amberlite XAD-2, which was washed with 1N NH_4OAc (500 ml), and the product was then eluted with 60% aqueous EtOH (500 ml). The eluate were evaporated to a small volume *in vacuo* and then lyophilized to a constant weight; 7.8 g. The crude material thus obtained was dissolved in H_2O (50 ml) and applied to a column (8×40 cm) of carboxymethylcellulose, which was eluted with pH 6.8 NH_4OAc buffer by a gradient elution method (0.005N/0.15N=4 liters/4 liters). The fractions containing the pure peptide were combined and lyophilized to a constant weight to give a white fluffy powder; yield, 5.3 g (62%), $[\alpha]_D^{25} -53.6^\circ$ ($c=0.5$ in 5% AcOH). Anal. Calcd. for $C_{55}H_{76}O_{19}N_{16} \cdot CH_3COOH \cdot 5H_2O$: C, 52.52; H, 6.96; N, 17.19. Found: C, 52.60; H, 6.96; N, 16.83. Amino acid analysis: His, 0.94 (1); Arg, 0.96 (1); Ser, 0.90 (1); Glu, 0.94 (1); Pro, 1.00 (1); Gly, 1.00 (1); Leu, 1.02 (1); Tyr, 1.00 (1); ethylamine, 1.00 (1), Tyr/Trp=1.02 (UV). Compound I synthesized was homogeneous in thin-layer chromatography ($Rf^2=0.08$, $Rf^3=0.51$, $Rf^4=0.63$) and was also found to be homogeneous by electrophoresis (500 V, 3 hr) on paper at pH 6.5 (pyridine-acetate buffer); exactly same mobility as synthetic LH-RH.⁶⁾

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