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Synthesis of Luteinizing Hormone Releasing Hormone (LH-RH)*,[1]

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Pyroglutamylhistidyltryptophyl-(O–Bzl)seryl-(O–Bzl) tyrosylglycylleucyl-(N^G-nitro)-arginylprolylglycinamide was synthesized by successive fragment condensations of three peptide derivatives, pGlu–His–$NHNH_2$, Boc–Trp–(O–Bzl)Ser–(O–Bzl)Tyr–Gly–$NHNH_2$ and Boc–Leu–(N^G-nitro)Arg–Pro–Gly–NH_2; the latter two were prepared by the modified solid phase method using bromoacetylpolystyrene resin as carrier.

Removal of all the protecting groups from the decapeptide derivative was easily effected by hydrogenolysis over a new type of catalyst, colloidal palladium on PVP. Thus the present reduction method made the large scale production of the peptide quite easy.

Since Matsuo, *et al.*[3] determined in 1971 the structure of luteinizing hormone releasing hormone (LH-RH, I) isolated from porcine hypothalami by Schally and his collaborators,[4] numerous syntheses of this hormone utilizing the solid phase,[5–10] classical[9a,12–18] or their combination[6,11] method have been reported in the last few years.

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* Dedicated to the memory of Prof. Eiji Ochiai.

1) Most part of this work is included in Japanese patent applications, 47-34956 (Apr., 6, 1972) and 47-106882 (Oct., 24, 1972). Abbreviations adopted in this communication are: Boc=tert.-butyloxycarbonyl; Bzl=benzyl; DCC=dicyclohexylcarbodiimide; Cbz=benzyloxycarbonyl; DCHA=dicyclohexylamine.

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pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I)

For synthesis of this decapeptide sequence, some authors[12-15] proposed to use unprotected peptide fragments so as to make the subsequent purification after each coupling step easy, while most other papers, especially in the case of the solid phase method, described syntheses of fully protected decapeptide derivatives, in which a variety of protecting groups was carefully chosen for respective deprotection methods.

For removal of the protecting groups from the decapeptide derivatives, liquid hydrogen fluoride[19] is most widely used[5,6b,7-11,17,18] as the reagent of choice. However, catalytic hydrogenolysis which is considered to be the most preferable method for large scale synthesis was applied only in a limited number of cases. Although some protected LH-RH derivatives were reported to undergo hydrogenolysis in the presence of 5% Pd on $BaSO_4$ or Pd black,[16,17] Sievertsson, *et al.*[6b] stated that the reduction of the protected decapeptide VIII over Pd on $BaSO_4$ required a long reaction time (40 hr) and was also accompanied by some side reactions under the conditions in which all the protecting groups could be removed without affecting the tryptophan moiety.

This paper describes an alternative synthesis of VIII in which the solid phase method with bromoacetyl polystyrene[20] was successfully employed, and it also describes hydrogenolysis of VIII to LH-RH with a new efficient catalyst, colloidal Pd on polyvinylpyrrolidinone (PVP).

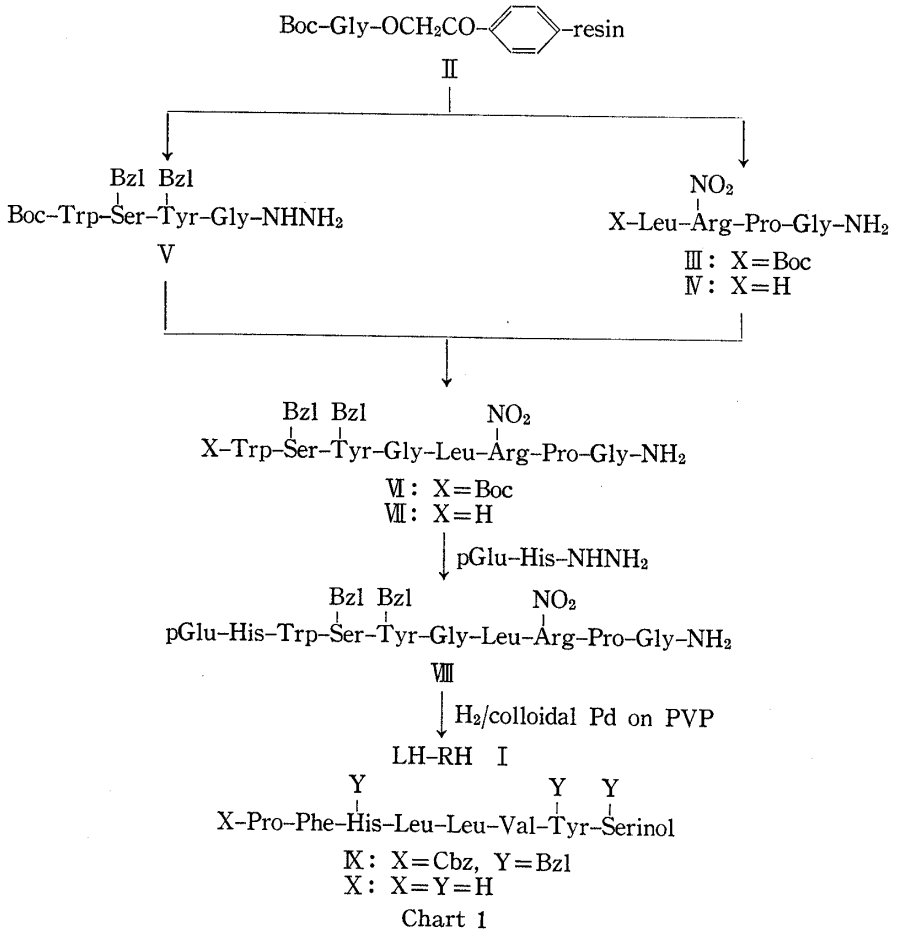

Chart 1

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The synthesis was so designed as to make the method versatile for synthesizing other peptides analogous to LH-RH (Chart 1).

As was reported previously,[20b,c] the solid phase method with bromoacetyl polystyrene has been found, in this laboratory, to be well suited for rapid synthesis of peptide fragments as either C-terminal amide, hydrazide or carboxylic acid derivatives with all the protecting groups intact. Thus, Boc-leucyl-(N^G-nitro)arginylprolylglycinamide (III) was prepared from the Boc-glycyloxyacetyl resin (II) by repeating a three step cycle of deblocking, neutralization and coupling, and the final cleavage of the resulting protected peptide resin with gaseous ammonia in methanol. This ammonolysis was smooth enough to minimize the side reaction which was reported to cause decomposition of the nitro-arginine side chain.[21] Similarly, Boc-tryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycine hydrazide (V) was prepared from the same starting material *via* hydrazinolysis[20c,d] for the final step. In these solid phase syntheses, hydrogen chloride in ethyl acetate was used for removal of the Boc group and a two-fold excess of Boc-amino acid and DCC except for Boc-proline (2.5-fold excess) was used in each coupling reaction. The overall yields of III and V from II were 49 and 40%, respectively. Crude III obtained as a powder was directly used for the next step without purification. After removal of the Boc group, III was coupled with V by the method of Rudinger, *et al.*[22] to yield the corresponding octapeptide derivative (VI). Some tryptophan-containing peptide hydrazides were reported to give N-nitroso byproducts when they were submitted to the azide method with an excess of sodium nitrite,[23] but in the present case, Rudinger's method with 1.1 equimolar iso-amyl nitrite gave a rather better yield of the product than in the coupling of the corresponding carboxylic acid derivative of V with IV by the DCC method.[9a] The hydrazide (V) was also readily purifiable as compared with the corresponding carboxylic acid derivative. VI was then deblocked to VII with dry hydrogen chloride in acetic acid containing thioglycolic acid which was added to protect the tryptophan residue. Pyroglutamylhistidine hydrazide was prepared *via* the known route described by Gillessen, *et al.*[24] with slight modification and coupled with VII in the same manner as for VI to furnish the desired decapeptide derivative (VIII). This reaction required triethylamine as a base, an excess of the hydrazide and a relatively long reaction time for completion, but optical rotations of this and the final products indicated that racemization at the histidine residue[25] was negligible as in the synthesis of thyrotropin releasing hormone.[24]

Catalytic hydrogenolysis of VIII over Pd black or Pd on $BaSO_4$ was unsatisfactory; *i.e.*, the reaction took an extremely long time and gave only a poor yield of the product LH-RH. Therefore, the reduction was tried with various types of palladium catalyst, of which Skita's colloidal Pd on gum arabic[26] and, more preferably, Pd on PVP were found to be suitable for this hydrogenolysis. The latter was prepared according to Skita's procedure from $PdCl_2$ and PVP in a mixture of methanol and diluted acetic acid and the black colloidal solution obtained was directly used for the reduction. This catalyst was found to hold better colloidal state than Skita's catalyst in several solvent systems tested and was also found to cleave the benzyl groups on the model compound Cbz-prolylphenylalanyl-(N^{im}-Bzl)histidylleucylleucylvalyl-(O-Bzl)tyrosyl-(O-Bzl) serinol (IX, X=Cbz, Y=Bzl)[20c] which was totally resistant to hydrogenolysis over Pd black even under a high pressure of hydrogen in our experiments. The

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product X was identified with an authentic sample prepared by treatment of IX with sodium in liquid ammonia.[20c]

Reduction of VIII in the presence of Pd on PVP was then carried out under 20 psi of hydrogen at room temperature. The reaction was completed in 19 hr to give crude LH-RH, which was slightly accompanied by a hardly separable byproduct, probably the dihydroindole derivative of LH-RH. On treatment of the reaction mixture with oxygen under cooling, this byproduct disappeared and the product gave practically a single spot on thin-layer chromatography (TLC).[27] Purification of this product was effected by Sephadex gel filtration yielding LH-RH (I) diacetate (48% yield from VIII) in high purity, which moved as a single component on TLC, in two solvent systems. Both the elemental and amino acid analyses gave satisfactory results and the optical rotation was also in good agreement with that reported for synthetic LH-RH.[12]

Experimental[28]

Boc-leucyl-(N^G-NO_2)arginylprolylglycine Amide (III)——Boc-glycyloxyacetyl copolystyrene-divinylbenzene[20b] (II: 2% DVB, 100 mesh, Gly: 10 mmoles, 12.80 g) suspended in 2.3N dry HCl/AcOEt (100 ml) was stirred at room temperature for 40 min, filtered and washed 6 times with AcOEt and 3 times with $CHCl_3$. The resulting resin was then treated with $CHCl_3$ (90 ml) containing Et_3N (10 ml) for 10 min, filtered and washed with $CHCl_3$ and CH_2Cl_2 (3 times each) to give the glycyloxyacetyl resin. This was mixed with Boc-proline (5.40 g, 25 mmoles) in CH_2Cl_2 (40 ml) and after a few min, DCC (5.15 g, 25 mmoles) in CH_2Cl_2 (40 ml) was added and the mixture was stirred for 3 hr at room temperature, filtered and the resin was successively washed with CH_2Cl_2, AcOH and AcOEt (3 times each) to give the Boc-prolylglycyloxyacetyl resin. In the same manner, Boc-(N^G-NO_2)arginine (4.38 g, 20 mmoles) and Boc-leucine·H_2O (5.00 g, 20 mmoles) were coupled successively to give Boc-leucyl-(N^G-NO_2) arginylprolylglycyloxyacetyl resin (13.80 g).

The tetrapeptide resin (11.42 g) thus obtained was suspended in MeOH (120 ml) and gaseous NH_3 was bubbled into the mixture for 6 hr under ice-cooling. The resin was filtered off and the filtrate was evaporated *in vacuo* to leave a residue, which was triturated with ether to give III as a white amorphous powder (2.36 g, 49% from II) which was directly used for the next step. A portion of the product was purified on preparative TLC (silica gel, eluent: CCl_4–AcOEt–MeOH (5: 5:2)) to give an analytical sample, mp 130—132° (decomp.), $[\alpha]_D^{24}$ −58.8° (c=3.4, MeOH), Rf^1 0.55. *Anal.* Calcd. for $C_{24}H_{43}O_8N_9$: C, 49.22: H, 7.40; N, 21.52. Found: C, 49.52; H, 7.53; N, 21.12.

Leucyl-(N^G-NO_2)arginylprolylglycine Amide (IV) Hydrochloride——A mixture of the protected peptide amide (III, 1.64 g, 2.80 mmoles) in 1.64N dry HCl/AcOH (12 ml) was kept to stand for 40 min at room temperature. To this was added ether (100 ml) and the precipitate separated was collected, washed thoroughly with ether and dried. Recrystallization from EtOH–isopropyl ether afforded IV·HCl as an amorphous powder (1.12 g, 77%), mp 219—222° (decomp.) $[\alpha]_D^{24}$ −25.5° (c=1.6, MeOH), Rf^2 0.37.

Boc-tryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycine Hydrazide (V)——Starting from Boc-glycyloxyacetyl copolystyrene-divinylbenzene[20b] (II: 2% DVB, 100 mesh, Gly: 10 mmoles, 12.80 g), the peptide was built up by successive couplings with Boc-(O-Bzl)tyrosine (7.45 g, 20 mmoles), Boc-(O-Bzl)serine (liberated from 10.50 g (22 mmoles) of its DCHA salt) and Boc-tryptophan (6.08 g, 20 mmoles) in the same manner as for III described above to give Boc-tryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycyloxyacetyl resin (15.71 g).

To a suspension of the completed peptide resin (4.62 g) in MeOH (9 ml) and DMF (37 ml) was added 90% hydrazine hydrate (1.64 g) and the mixture was stirred for 6 hr at room temperature. The resin was filtered, washed with MeOH and the combined filtrate and washings were evaporated to dryness to give a yellow residue. After trituration with H_2O, the pale yellow solid remained was collected and successively washed with MeOH, EtOH and ether to afford V as colorless needles (0.95 g, 40% from II), mp 195—196° (decomp.), $[\alpha]_D^{27}$ −18.9° (c=4.9, DMF), Rf^1 0.77. *Anal.* Calcd. for $C_{44}H_{51}O_8N_7$: C, 65.58; H, 6.38; N, 12.17. Found: C, 65.55; H, 6.52; N, 12.18.

Boc-tryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycylleucyl-(N^G-NO_2)arginylprolylglycine Amide (VI)——To a solution of V (0.59 g, 0.725 mmole) in DMF (20 ml) was added 2.1N dry HCl/THF (2.1 ml) followed by isoamyl

27) This byproduct gave yellow color with the Ehrlich reagent on TLC and after the re-oxidation of the reaction mixture, TLC gave a single spot which exhibited ordinary dark blue color reaction with the same reagent.

28) All the melting points are uncorrected. Optical rotations were measured with JASCO DIP 180 automatic polarimeter. Thin-layer chromatography (TLC) was performed on silica gel (Merck, TLC plate silica gel 60 F_{254} pre-coated, layer thickness 0.25 mm). Rf values refer to the following solvent systems: Rf^1, $CHCl_3$–MeOH (4: 1, v/v); Rf^2, n-BuOH–AcOH–H_2O (3: 1: 1); Rf^3, n-BuOH–AcOH–H_2O (4: 1: 2); Rf^4, n-BuOH–AcOH–pyridine–H_2O (5: 1: 3: 4).

nitrite (0.094 g, 0.80 mmole) at −18——22°. After stirring for 40 min at −25°, Et_3N (0.45 g) and then a solution of IV·HCl (0.38 g, 0.725 mmole) and Et_3N (0.15 g, 1.45 mmoles) in DMF (15 ml) were added dropwise at −50° and the whole was stirred for 45 hr at −18°. The resulting mixture was evaporated *in vacuo* and the residue obtained was treated with H_2O to give a pale yellow solid, which was washed with 0.5M citric acid, H_2O, 4% $NaHCO_3$ and H_2O, dried, and subsequently purified on preparative TLC (silica gel, $CHCl_3$–AcOH–MeOH) (95: 3: 10)) to afford VI as a slightly yellow amorphous powder (0.38 g, 41%), mp 145—147° (decomp.). Recrystallization from isopropanol afforded a pure sample, mp 151—153.5° (decomp.), $[\alpha]_D^{22}$ −73.8° (c=1.5, MeOH), Rf^1 0.62. *Anal.* Calcd. for $C_{63}H_{82}O_{14}N_{14}\cdot 2H_2O$: C, 58.37; H, 6.69; N, 15.13. Found: C, 58.46; H, 6.50; N, 14.94.

Tryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycylleucyl-(N^G-NO_2)arginylprolylglycine Amide (VII) Hydrochloride——To a suspension of VI (1.07 g, 0.85 mmole) in AcOH (4 ml) was added a few drops of thioglycolic acid and 1.64N HCl/AcOH (11 ml), and the mixture was kept for 40 min at room temperature under occasional shaking. The resulting mixture was lyophilized to leave a white powder, which was washed thoroughly with ether, dried and used for the coupling reaction described below. Yield 0.98 g (96%), Rf^2 0.66.

Pyroglutamylhistidyltryptophyl-(O-Bzl)seryl-(O-Bzl)tyrosylglycylleucyl-(N^G-NO_2)arginylprolylglycine Amide (VIII)——A stirred suspension of pyroglutamylhistidine hydrazide[24] (2.04 g, 7.26 mmoles) in DMF (100 ml) was acidified with 2.3N dry HCl/AcOEt (32 ml) and then treated with isoamyl nitrite (0.94 g, 8 mmoles) with cooling at −20——22°. After stirring for 40 min at the same temperature, the mixture was neutralized by addition of Et_3N (7.00 g) at −45°. A 40 ml portion of the reaction mixture was stored at −20° and to the remaining solution was added a solution of VII·HCl (2.89 g, 2.42 mmoles) and Et_3N (0.49 g, 4.84 mmoles) in DMF (26 ml) and the whole was stirred for 16.5 hr at −18°. To the mixture was added 20 ml of the stored solution mentioned above and the whole was stirred for 8 hr. The remaining azide solution (20 ml) was added and stirring was continued for 16 hr at −18° and for 24 hr at −8°. The resulting mixture was concentrated *in vacuo* and the residue was treated with satd. KCl (100 ml) to precipitate a yellow mass, which was washed with satd. KCl and H_2O and dried. Recrystallization from MeOH afforded a pale yellow crystalline powder (2.71 g, 80%), mp 170—171°, $[\alpha]_D^{18}$ −23.0 (c=2.6, AcOH), Rf^3 0.40. *Anal.* Calcd. for $C_{69}H_{86}O_{15}N_{18}\cdot 5H_2O$[29]: C, 55.34; H, 6.46; N, 16.84. Found: C, 55.53; H, 5.98; N, 17.09. Lit.[9a] mp 166—169° (decomp.), $[\alpha]_D^{24}$ −25.2° (c=1, AcOH); Lit.[9c] mp 169° (decomp.), $[\alpha]_D^{25}$ −26.9° (c=1, AcOH).

Pyroglutamylhistidyltryptophylseryltyrosylglycylleucylarginylprolylglycine Amide (LH-RH, I) Diacetate——A mixture of $PdCl_2\cdot 2H_2O$ (0.60 g) and polyvinylpyrrolidinone (0.60 g, average Mw, 40000) in MeOH (90 ml) and 0.2N AcOH (90 ml) was shaken for 30 min at room temperature. To this was added hydrazine hydrate (0.05 g) and the whole mixture was shaken again under hydrogen atmosphere for 60 min at room temperature to give colloidal Pd. VIII (3.00 g, 2.13 mmoles) was then added to this colloidal mixture and hydrogenated for 19 hr at 20 psi. To the resulting mixture were introduced successively nitrogen (10 min) and oxygen (90 min) with cooling in an ice-water bath. The mixture was then concentrated *in vacuo* and filtered with the aid of celite to remove the catalyst. The filtrate was lyophilized to afford a reddish powder, which was dissolved in water and passed through a column of Dowex 1×4 (OH type, 15 ml, eluent: H_2O) and lyophilized after addition of acetic acid. The product thus obtained was dissolved in 0.2N AcOH and applied to a Sephadex G-15 column (5.5×97 cm), which was eluted with 0.2N AcOH. Each fraction (10 ml) was checked with spot test (Ehrlich reagent). The fractions (tube No. 166—185) were pooled and lyophilized to give a white fluffy powder (1.42 g, 48% from VIII), $[\alpha]_D^{25}$ −51.6° (c=0.68, H_2O), −51.2° (c=1.0, 1% AcOH), Rf^3 0.31, Rf^4 0.48. *Anal.* Calcd. for $C_{55}H_{75}O_{13}N_{17}\cdot 2AcOH\cdot 5H_2O$: C, 50.89; H, 6.73; N, 17.10. Found: C, 50.83; H, 6.46; N, 16.88. Amino Acid Analysis: Glu, 1.13; His, 1.04; Trp, 0.94; Ser, 0.94; Tyr, 1.00; Gly, 1.95; Leu, 1.00; Arg, 1.05; Pro, 0.98 (hydrolyzed in 6N HCl containing thioglycolic acid, 110°, 24 hr).

Prolylphenylalanylhistidylleucylleucylvalyltyrosylserinol (X) Dihydrochloride——A mixture of $PdCl_2\cdot 2H_2O$ (50 mg) and polyvinylpyrrolidinone (50 mg, average Mw, 40000) in MeOH (12 ml) and 50% AcOH (12 ml) was treated in a similar manner to that described above to give the colloidal catalyst solution. IX (50 mg) was added to this solution and the mixture was hydrogenated for 34 hr at 32—40 psi and filtered with the aid of celite. The filtrate was evaporated to dryness to leave a residue, which was successively washed with cold H_2O, MeOH–AcOEt (1: 1) and ether to give a white powder (39 mg), mp 212—214° (decomp.). Recrystallization from DMF–H_2O–AcOEt afforded a pure sample (27 mg), mp 213—214° (decomp.), $[\alpha]_D^{24}$ −39.0 (c=0.47, DMF) Rf^2 0.51. Amino Acid Analysis: Pro, 1.00; Phe, 1.03; His, 1.02; Leu, 2.09; Val, 0.91; Tyr, 1.00 (hydrolyzed in 6N HCl, 110°, 48 hr). This product had practically the same physical data as well as an identical Rf with those of an authentic sample of X reported.[20c]

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29) This sample was slightly hygroscopic.