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Isobornyloxycarbonyl Function, a New Convenient Amino-Protecting Group in Peptide Synthesis. IV.[1] Synthesis of Gonadotropin-Releasing Hormone (Gn-RH or LH-RH/FSH-RH)[2]

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Gonadotropin-releasing hormone (Gn-RH or LH-RH/FSH-RH), a decapeptide amide (Pyr)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, was synthesized by the use of the *d*-isobornyloxycarbonyl-protercting group for temporary protection of the all amino groups of the intermediary peptides. The synthetic Gn-RH possessed full biological activity (LH-releasing and FSH-releasing activity) when compared with natural highly purified porcine Gn-RH, and appeared to be pure by various chemical criteria.

We have recently reported the preparation of N-isobornyloxycarbonyl (IBOC)-amino acids,[4] and its application to the synthesis of peptides.[5] The IBOC-group is easily removable by the treatment with trifluoroacetic acid as is known with the *t*-butoxycarbonyl-group which has wide application in some peptide syntheses.

With an aim to see if this new protecting group is applicable to other peptide syntheses, we have now synthesized a decapeptide amide, gonadotropin-releasing hormone (Gn-RH or leuteinizing hormone-releasing hormone/follicle-stimulating hormone-releasing hormone, LH-RH/FSH-RH), which was first isolated from porcine hypothalami by Schally, *et al.*[6] and later on synthesized by Matsuo, *et al.*[7]

Recently, some other syntheses of this releasing hormone by the solid-phase method[8] and the classical method[9] have been reported.

The method for the synthesis of the releasing hormone by the present authors is outlined in Fig. 1.

d-IBOC-N^G-nitro-arginyl-proline benzyl ester[5] was saponified by the usual manner to obtain the free acid (I), which was coupled with glycine amide *via* the corresponding pentachlorophenyl ester which was prepared by the trichloroacetate method[10] to give *d*-IBOC-N^G-nitro-arginyl-prolyl-glycine amide (II). This tripeptide amide was treated with trifluoroacetic acid for 30 min at room temperature to remove the *d*-IBOC-protecting group and the

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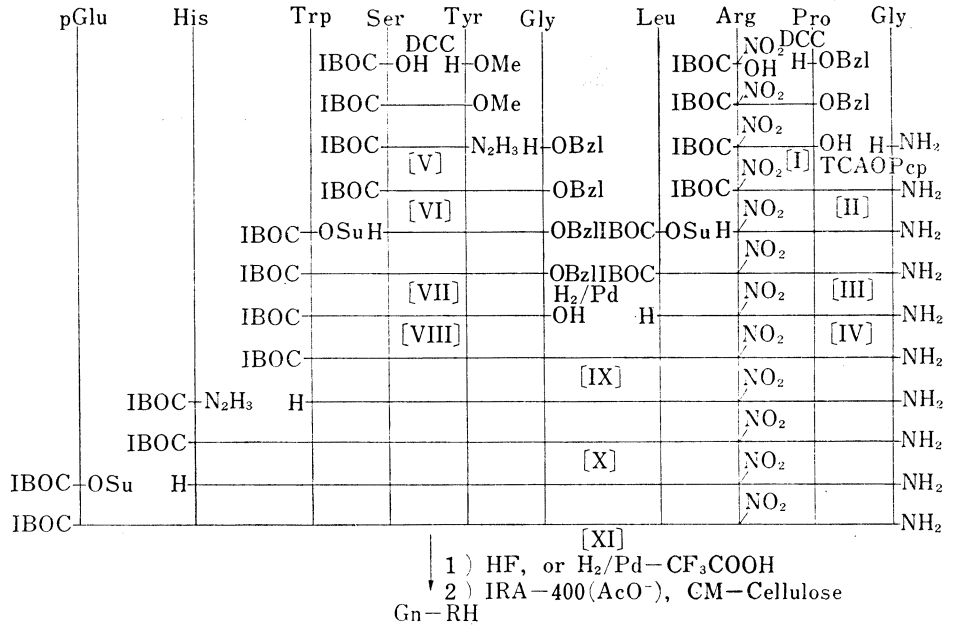

Fig. 1. Synthesis of Gn-RH

resulting free base was acylated with *d*-IBOC-leucine *via* the corresponding N-hydroxysuccinimide ester[11] to give *d*-IBOC-leucyl-N^G-nitro-arginyl-prolyl-glycine amide (III). The free base (IV) was obtained by the treatment of III with trifluoroacetic acid followed by neutralization with triethylamine, and was coupled with *d*-IBOC-tryptophyl-seryl-tyrosyl-glycine (VIII), which was prepared from *d*-IBOC-tryptophyl-seryl-tryosyl-glycine benzyl ester (VII) by the catalytic hydrogenation, by the dicyclohexylcarbodi-imide method[12] to yield *d*-IBOC-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^G-nitro-arginyl-prolyl-glycine amide (IX).

The protected heptapeptide (IX) was treated with trifluoroacetic acid to give the free base of IX which was acylated by the stepwise manner, with *d*-IBOC-histidine hydrazide (*via* azide)[1] and *d*-IBOC-pyroglutamic acid (*via* N-hydroxysuccinimide ester),[1] to give the protected decapeptide amide (XI), *d*-IBOC-pyroglutamyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^G-nitro-arginyl-prolyl-glycine amide.

The removal of the protecting group of the protected decapeptide amide XI at the final step was achieved by the following two methods: the one is the hydrogen fluoride (Sakakibara's) method[13] and the other the hydrogenation followed by teratment with trifluoroacetic acid.

The resulting hydrofluoride and trifluoroacetate were converted to the corresponding acetate by the treatment with Amberlite CG-400 (acetate form) and then purified by chromatography on carboxymethylcellulose using a gradient elution with an ammonium acetate buffer.

The final product thus obtained was found to be homogeneous and identical with an authentic synthetic Gn-RH[14] by paper chromatography, thin-layer (silica gel) chromato-

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14) We are indebted to Prof. N. Yanaihara, Shizuoka College of Pharmacy, for the gift of synthetic Gn-RH (Ref. 8).

graphy and paper electrophoresis to Ehrlich, Sakaguchi and Pauly reagents (but negative to ninhydrin), and the amino acid analysis and elemental analysis were also in good accordance with theoretical values.

The synthetic decapeptide amide thus obtained was subjected to the biological evaluation;[15] the peptide was incubated with hemisected male rat pituitaries and the total media were subjected to the assessment for the FSH and LH content.[16] In different four point assays the synthetic peptide was found to be 120% in the FSH releasing activity and 110% in the LH releasing activity, when compared with a highly purified porcine Gn-RH.[17]

Experimental

All melting points were taken by the capillary method and are uncorrected. Evaporations were all carried out with a rotary evaporator. The purity of products was tested by thin-layer chromatography. Solvent systems used were: $CHCl_3$–MeOH–AcOH (9:1:0.5, Rf^1)), AcOEt–pyridine–AcOH–H_2O (60:20:6:10, Rf^2)), *n*-BuOH–AcOH–H_2O (4:1:1, Rf^3)), *n*-BuOH–pyridine–AcOH–H_2O (30:20:6:24, Rf^4)).

***d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-proline (I)**——*d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-proline benzyl ester[5] (11.7 g, 20 mmole) was dissolved in acetone (150 ml) and to this was added 1N NaOH (26 ml) in an ice-bath. After stirring for 2 hr at room temperature, the mixture was acidified by addition of 1N HCl (27 ml) and was concentrated by evaporation *in vacuo* to remove the acetone. The concentrated solution was diluted with 5% $NaHCO_3$ (110 ml), and the mixture was extracted with AcOEt (100 ml×2). The aqueous layer was then acidified again to pH 2 with 1N HCl and was extracted with AcOEt (100 ml×3). The AcOEt-extracts were combined and washed with water, dried over anhydr. Na_2SO_4, and evaporated to dryness. The residue was triturated with pet. ether to give a solid which was collected by filtration, and purified by reprecipitation from AcOEt–ether to give the pure acid; 7.0 g (70.3%), mp 129° (decomp.), $[\alpha]_D^{23}$ −66.4° (c=0.51 in EtOH), Rf^1=0.41. *Anal.* Calcd. for $C_{22}H_{36}O_7N_6 \cdot C_2H_5OC_2H_5$: C, 54.72; H, 8.13; N, 14.73. Found: C, 54.42; H, 8.12; N, 14.75.

***d*-Isobornyloxycarbonyl-N^G-nitro-arginyl-prolyl-glycine Amide (II)**——To a solution of the compound I (6.5 g, 13.05 mmole) and triethylamine (1.83 ml, 13.05 mmole) in dimethylformamide (DMF, 10 ml) was added pentachlorophenyl trichloroacetate (6.5 g, 15.7 mmole) with stirring at 0°, and the mixture was stirred 1 hr at room temperature. To the reaction mixture was added ice-cold water (50 ml) and the resulting precipitate was collected filtration and washed well with water. After being dired *in vacuo*, the product was purified by trituration with boiling EtOH. The purified ester (8.20 g, 11 mmole) and glycine amide (9.8 g, 13.2 mmole) were dissolved in DMF (20 ml) and the mixture was stirred at room temperature for 18 hr. The reaction mixture was diluted with $CHCl_3$ (200 ml), and the solution was washed with 5% $NaHCO_3$, 1N HCl and water. dried over anhydr. $MgSO_4$ and evaporated. The resulting residue was triturated under ether to give a fine white solid which was purified by reprecipitation from $CHCl_3$–ether to give the pure product; 4.30 g (59.3%), mp 143°, $[\alpha]_D^{23}$ −48.0° (c=0.53 in EtOH), Rf^1=0.16. *Anal.* Calcd. for $C_{24}H_{40}O_7N_8 \cdot H_2O$: C, 50.51; H, 7.42; N, 19.63. Found: C, 50.30; H, 7.21; N, 19.60.

***d*-Isobornyloxycarbonyl-leucyl-N^G-nitro-arginyl-prolyl-glycine Amide (III)**——The compound II (3.10 g, 5.62 mmole) was treated with trifluoroacetic acid (15 ml) for 45 min at room temperature and to this was added ether (200 ml) to give a fine white solid which was collected by filtration and washed well with dry ether. The dried solid was dissolved in DMF (20 ml), and to this solution was added N-ethylmorpholine (0.74 ml) and *d*-isobornyloxycarbonyl-lecuine N-hydroxysuccinimide ester (oil, 2.75 g, 6.5 mmole: prepared by the DCC method). The mixture was stirred for 46 hr at room temperature and was diluted with water (100 ml) to give oily substance which was extracted with $CHCl_3$ (100 ml×3). The $CHCl_3$-extracts were combined, washed with 5% $NaHCO_3$ and 1N HCl, dried over anhydr. $MgSO_4$, and evaporated. The syrupy residue was soon solidified by the addition of ether. The solid was collected by filtration and reprecipitated from $CHCl_3$–ether to give the pure product; 2.60 g (70%), mp 140—143°, $[\alpha]_D^{23}$ −50.0° (c=0.5 in EtOH), Rf^1=0.33. *Anal.* Calcd. for $C_{30}H_{51}O_8N_9 \cdot H_2O$: C, 52.87; H, 7.80; N, 18.43. Found: C, 52.76; H, 7.79; N, 18.02.

***d*-Isobornyloxycarbonyl-seryl-tyrosine Hydrazide (V)**——To a solution of *d*-isobornyloxycarbonyl-serine dicyclohexylammonium salt[4] (29.2 g, 62 mmole) and tyrosine ethyl ester hydrochloride (16.7 g, 68 mmole) in a mixture of THF (300 ml) and CH_2Cl_2 (100 ml) was added DCC (14.1 g, 68 mmole), and the mixture was stirred for 12 hr at room temperature. The reaction mixture was filtered off to remove dicyclohexylurea and the filtrate was evaporated *in vacuo* to dryness. The residue was dissolved in AcOEt (300 ml), washed with 1N H_2SO_4 and 5% $NaHCO_3$, dried over anhydr. Na_2SO_4 and evaporated to give *d*-isobornyloxycarbonyl-

15) We thank Dr. W.F. White, and Dr. R.H. Rippel, Abbott Laboratories for the biological assays.
16) Ovarian weight method and ascorbic acid depression method.
17) AVS-77-33 #215—269.

seryl-tyrosine ethyl ester (IV) as an oil. The oil was dissolved in MeOH (300 ml) and to this was added hydrazine hydrate (10 ml). The solution was kept at room temperature for 50 hr to form crystals which were collected by filtration and recrystallized from MeOH–ether; 25.2 g (97.7%), mp 158—160°, $[\alpha]_D^{23}$ −29.0° (c=0.53 in EtOH), Rf^1=0.24. *Anal.* Calcd. for $C_{23}H_{31}O_6N_4\cdot\frac{1}{2}H_2O$: C, 59.46; H, 7.81; N, 12.06. Found: C, 59.19; H, 7.53; N, 11.93.

***d*-Isobornyloxycarbonyl-seryl-tyrosyl-glycine Benzyl Ester (VI)**——The compound V (16.7 g, 40 mmole) was dissolved is a mixture of DMF (100 ml) and 1N HCl (120 ml), and to this solution was added 2N sodium nitrite (22 ml) at −5°. After the mixture was stirred for 10 min at −5°, the reaction mixture was diluted with aqueous NaCl (saturated, 30° ml) and the azide was extracted with AcOEt (100 ml×2). The extracts were combined and washed with ice-cold water, dried over anhydr. Na_2SO_4 for 20 min and filtered.

To a solution of glycine benzyl ester *p*-toluenesulfonate (44 mmole) and triethylamine (6.72 ml, 44 mmole) in a mixture of AcOEt (50 ml) and DMF (20 ml) was added the above mentioned azide solution, and the mixture was stirred at 0° for 50 hr. The reaction mixture was washed with 5% $NaHCO_3$ and 1N HCl, dried over anhydr. Na_2SO_4, and evaporated. The residue was triturated under pet. ether to give the pure product; 14.0 g (59.4%), mp 66—67°, $[\alpha]_D^{23}$ −28.5° (c=0.54 in EtOH), Rf^1=0.69. *Anal.* Calcd. for $C_{32}H_{41}O_8N_3$: C, 64.52; H, 6.94; N, 7.05. Found: C, 64.32; H, 7.13; N, 6.90.

***d*-Isobornyloxycarbonyl-tryptophyl-seryl-tyrosyl-glycine Benzyl Ester (VII)**——The compound VI (5.90 g, 10 mmole) was treated with trifluoroacetic acid (15 ml) at room temperature for 20 min, to this solution was added dry ether (100 ml) to yield a fine white solid which was washed with ether by decantation, and dried over NaOH-pelletes *in vacuo* to give the free base of VI as trifluoroacetate.

The trifluoroacetate was dissolved in DMF (40 ml), neutralized with N-ethylmorpholine (1.28 ml), and to this was added *d*-isobornyloxycarbonyl-tryptophan N-hydroxysuccinimide ester which was prepared from the acyl amino acid (4.2 g, 11 mmole) by the DCC method. The mixture was stirred for 50 hr at 5°, and was diluted with water (100 ml) to give oily precipitate which was extracted with AcOEt (80 ml×3). The AcOEt-extracts were combined, washed with 5% $NaHCO_3$ and 1N HCl, dried over anhydr. Na_2SO_4, and evaporated. The residue was triturated under pet. ether to give a solid which was reprecipitated from AcOEt–pet. ether to give the pure product; 6.90 g (89%), mp 122°, $[\alpha]_D^{23}$ −27.9° (c=0.50 in EtOH), Rf^1=0.63. *Anal.* Calcd. for $C_{43}H_{51}O_9N_5\cdot\frac{1}{2}H_2O$: C, 65.29; H, 6.63; N, 8.86. Found: C, 65.38; H, 6.92; N, 8.70.

***d*-Isobornyloxycarbonyl-trytophyl-seryl-glycine (VIII)**——The compound VII (5.0 g, 6.45 mmole) was dissolved in MeOH (70 ml) and was hydrogenated over palladium-black as a catalyst for 5 hr. The solution was filtered to remove the catalyst and evaporated to dryness. The residue was washed by decantation with ether and collected by filtration to give the pure acid; 4.46 g (98.3%), mp 161—163°, $[\alpha]_D^{23}$ −25.1° (c=0.50 in EtOH), Rf^1=0.19. *Anal.* Calcd. for $C_{36}H_{45}O_9N_5\cdot H_2O$: C, 60.91; H, 6.64; N, 9.87. Found: C, 60.65; H, 6.64; N, 9.47.

***d*-Isobornyloxycarbonyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^G-nitro-arginyl-prolyl-glycine Amide (IX)**——The compound VII (1.78 g, 2.94 mmole) was treated with trifluoroacetic acid (5 ml) at room temperature for 25 min. The excess of the acid was removed by evaporation *in vacuo*, and to this was added dry ether (50 ml) to yield a fine white solid which was collected by filtration and dried over NaOH-pelletes *in vacuo*.

The resulting powder was dissolved in DMF (10 ml) and to this was added N-ethylmorpholine (0.39 ml).

The compound VIII (1.68 g, 2.43 mmole) and N-hydroxysuccinimide (0.36 g, 2.91 mmole) were dissolved in acetonitrile (15 ml) and to this was added DCC (0.60 g, 2.91 mmole) at 0°. The mixture was stirred at 5° for 2 hr, and the reaction mixture was filtered to remove dicyclohexylurea and the filtrate was then added to the above DMF-solution which contained the free base of VII.

After the solution had been stirred for 24 hr at room temperature, the mixture was diluted with ether (50 ml) to give a fine solid which was collected by filtration and purified by reprecipitation from EtOH–AcOEt; 2.02 g (71.4%), mp 142°, $[\alpha]_D^{28}$ −37.7° (c=0.53 in EtOH), Rf^3=0.82. *Anal.* Calcd. for $C_{55}H_{75}O_{14}N_{14}\cdot 3H_2O$: C, 54.34; H, 6.96; N, 16.11. Found: C, 54.27; H, 6.92; N, 15.79.

***d*-Isobornyloxycarbonyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^G-nitro-arginyl-prolyl-glycine Amide (X)**——*d*-Isobornyloxycarbonyl-histidine hydrazide[1] (0.82 g, 2.5 mmole) was dissolved in a mixture of DMF (8 ml) and 1N HCl (10 ml) at −5°, and to this was added 2N sodium nitrite (1.5 ml). After stirring at 5° for 10 min, to this reaction mixture was added $NaHCO_3$ (0.84 g) and aqueous NaCl (saturated, 30 ml) and the mixture was extracted with AcOEt (20 ml×3). The AcOEt-layer was washed with 5% $NaHCO_3$ and dried over anhydr. Na_2SO_4 to give the azide solution.

Compound IX (2.02 g, 1.7 mmole) was treated with trifluoroacetic acid (5 ml) containing 0.5 ml of anisole for 30 min at room temperature, and to this was added dry ether (50 ml) under cooling.

The resulting white solid was collected by filtration, washed with ether, and dried over NaOH-pelletes *in vacuo*. The resulting powder was dissolved in DMF (15 ml) and the solution was neutralized with N-ethylmorpholine (0.22 ml) at −5°.

To the DMF-solution containing the amine component was added the above azide solution, and the solution was stirred for 50 hr at 0—5°. The solution was evaporated *in vacuo* to dryness and the residue was solidified by the addition of AcOEt to yield a precipitate which was collected by filtration to give the crude product.

The crude product was then purified by column chromatography on silica gel (250 g) with $CHCl_3$–MeOH–AcOH (7 : 3 : 0.1) as solvent. The eluate which containing the product were collected and evaporated to give the pure protected nonapeptide amide; 1.40 g (63.8%), mp 186°, $[\alpha]_D^{23}$ −26.5° (c=0.45 in EtOH), Rf^3=0.73. *Anal.* Calcd. for $C_{61}H_{95}O_{15}N_{17}\cdot 2CH_3COOH\cdot 4H_2O$: C, 52.45; H, 6.84; N, 15.99. Found: C, 52.24; H, 6.46; N, 16.07.

***d*-Isobornyloxycarbonyl-pyroglutamyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^G-nitro-arginyl-prolyl-glycine Amide (XI)**——The compound X (1.30 g, 1 mmole) was treated with trifluoroacetic acid (5 ml) containing 1% anisole for 30 min at room temperature. The excess of acid was removed off by evaporation *in vacuo* and to this was added dry ether (50 ml) to yield a fine white solid which was collected by filtration. The dried powder was dissolved in DMF (10 ml) and to this was added N-ethylmorpholine (0.26 ml) and *d*-isobornyloxycarbonyl-pyroglutamic acid N-hydroxysuccinimide ester[1] (0.53 g, 1.3 mmole). After the mixture was stirred at room temperature for 5 hr, to this reaction mixture was added AcOEt (80 ml) to give a precipitate which was collected by filtration and purified by reprecipitation from EtOH–AcOEt to give the pure product; 1.02 g (72.7%), mp 172°, $[\alpha]_D^{23}$ −28.1° (c=0.54 in EtOH), Rf^3=0.83, Rf^4=0.86. *Anal.* Calcd. for $C_{66}H_{90}O_{17}N_{18}\cdot 2.5CH_3COOH\cdot 6H_2O$: C, 51.19; H, 6.78; N, 15.14. Found: C, 50.73; H, 6.11; N, 15.45.

Pyroglutamyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-lecuyl-arginyl-prolyl-glycine Amide (Gn-RH)——a) The compound XI (200 mg) was treated with approximately 5 ml of hydrogen fluoride in the presence of anisole (0.11 ml) and mercaptoethanol at 0° for 50 min, and the hydrogen fluoride was removed *in vacuo*. The residue was dissolved in water (10 ml) and passed through a column of Amberlite CG-400 (AcO^-, 2×10 cm) and the column was washed well with water. The eluate and washings were combined and subjected to chromatography on a carboxymethylcellulose column with a gradient elution method (pH 6.8 ammonium acetate buffer, 0.005M/0.1M=500 ml/500 ml). The homogeneous Gn-RH was eluted in 180—365 ml fractions. The fractions were combined and lyophilized to a constat weight; 60 mg (36%), $[\alpha]_D^{23}$ −48.5° (c=0.5 in H_2O). *Anal.* Calcd. for $C_{55}H_{75}O_{13}N_{17}\cdot 2.5CH_3COOH\cdot 5H_2O$: C, 50.66; H, 6.73; N, 16.74. Found: C, 50.32; H, 6.81; N, 16.69. Amino acid anal.: His 0.96 (1), Arg 0.92 (1), Glu 0.92 (1), Ser 0.96 (1), Pro 1.12 (1), Gly 2.04 (2), Leu 1.08 (1), Tyr 1.08 (1). Tyr/Trp (UV)=1.10.

The compound synthesized was homogeneous and identical with an authentic preparation[14] in paper chromatography (Rf^4=0.70), thin-layer chromatography (Rf^4=0.72) and paper electrophoresis (R_{arg}=0.58, pH 6.5 pyridine–acetate buffer, 500 V, 3 hr).

b) A solution of the compound XI (300 mg) in MeOH (50 ml) and AcOH (0.05 ml) was hydrogenated for 24 hr over palladium-black. The mixture was filtered to remove the catalyst and evaporated. The residue was then treated with trifluoroacetic acid (3 ml) for 45 min at room temperature and to the reaction mixture was added dry ether to yield a fine white precipitate which was collected by filtration and dried over NaOH-pelletes *in vacuo*.

The resulting crude peptide was then purified by the same manner as described above (a) and the main fraction (180—370 ml) was lyophillized to a constant weight; 54 mg (24%).

This product was behaved exactly like the above-mentioned synthetic peptide in paper and thin layer chromatography and paper electrophoresis.

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