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Human Chorionic Gonadotropin. IV.[1,2] Synthesis of a Hexadecapeptide corresponding to the C-Terminal Sequence 130—145 of the β-Subunit of Human Chorionic Gonadotropin (hCG)

Yoshio Okada, Shin Iguchi, Masashi Mimura, Koichi Kawasaki,[3a)]
Kenji Yamaji, Tetsu Takagi, and Osamu Tanizawa[3b)]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University[3a)] and Osaka University Medical School[3b)]

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The hexadecapeptide corresponding to sequence 130—145 of the β-subunit of human chorionic gonadotropin (hCG) according to the amino acid sequence proposed by Morgan *et al.* was synthesized by successive azide condensation of 4 peptide fragments, followed by TFA treatment and catalytic hydrogenation. This peptide was conjugated with bovine serum albumin (BSA). Antiserum produced against this conjugate did not bind with ^{125}I-hCG.

Keywords——human chorionic gonadotropin; β-subunit; C-terminal hexadecapeptide; chemical synthesis; antiserum

In our previous reports,[1,4] we described the synthesis of the C-terminal hexadecapeptide and dotriacontapeptide corresponding to sequences 132—147 and 116—147 of the β-subunit of hCG according to the amino acid sequence elucidated by Carlsen *et al.*,[5] and reported their immunological properties. This report deals with the synthesis of the hexadecapeptide corresponding to sequence 130—145 of the β-subunit of hCG according to the amino acid sequence

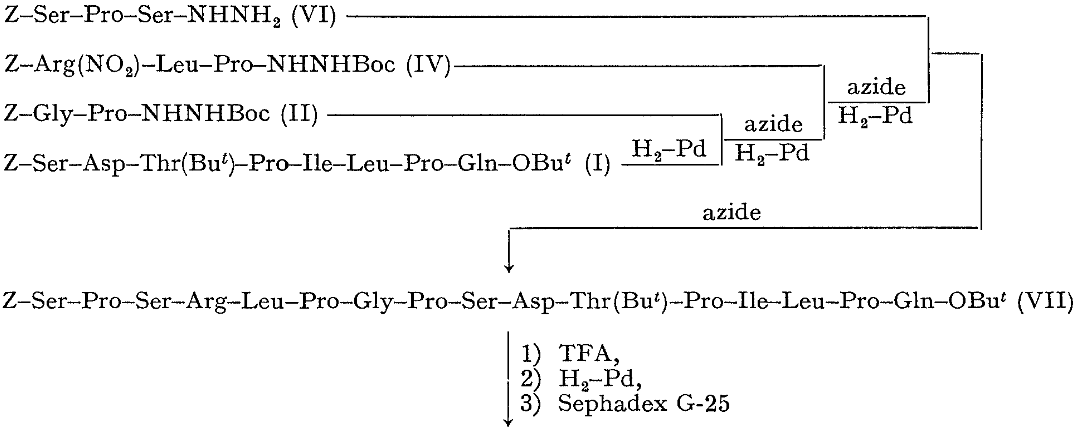

Fig. 1. Synthetic Scheme for the Hexadecapeptide corresponding to Sequence 130—145 of the β-Subunit of hCG

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2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration except in the case of glycine.

3) Location: *a) Ikawadani-machi, Tarumi-ku, Kobe, 673, Japan*; *b) Fukushima-ku, Osaka, 553, Japan.*

4) K. Kawasaki, C. Kawasaki, Y. Tsuda, M. Maeda, Y. Okada, K. Yamaji, T. Takagi, and O. Tanizawa, *Chem. Pharm. Bull.*, **28**, 2692 (1980).

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elucidated by Morgan *et al.*[6] and later confirmed by Birken and Canfield[7] and Keutman and Williams,[8] as well as its immunological properties.

The hexadecapeptide was synthesized by the azide coupling[9] of four fragments as shown in Fig. 1. The α-amino function of amino acids was protected with the benzyloxycarbonyl (Z) group. The C-terminal carboxyl group and hydroxyl group of Thr residue were protected with *t*-butyl derivatives during the synthesis. The β-carboxyl group of Asp residue and the guanidino group of Arg residue were protected with a benzyl group and nitro group respectively, which were removed by hydrogenation immediately after their introduction. The hydroxyl group of the Ser residue was not protected.

The C-terminal protected octapeptide, Z–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (I) was synthesized in a stepwise manner as illustrated in Fig. 2. Z–Pro–OH and H–Gln–OBu^t[10] were coupled by the *p*-nitrophenyl ester method[11] to afford Z–Pro–Gln–OBu^t (**1**). **1** was hydrogenated to furnish the corresponding amine (**1a**). **1a** was coupled with Z–Leu–ONp[12] to afford Z–Leu–Pro–Gln–OBu^t (**2**). **2** was converted to the corresponding amine (**2a**) and linked with Z–Ile–ONp[13] to give Z–Ile–Leu–Pro–Gln–OBu^t (**3**). After removal of the Z group of **3**, **3a** and Z–Pro–ONp[14] were coupled to give Z–Pro–Ile–Leu–Pro–Gln–OBu^t (**4**). The pentapeptide (**4**) was converted to the corresponding amine (**4a**) and coupled with

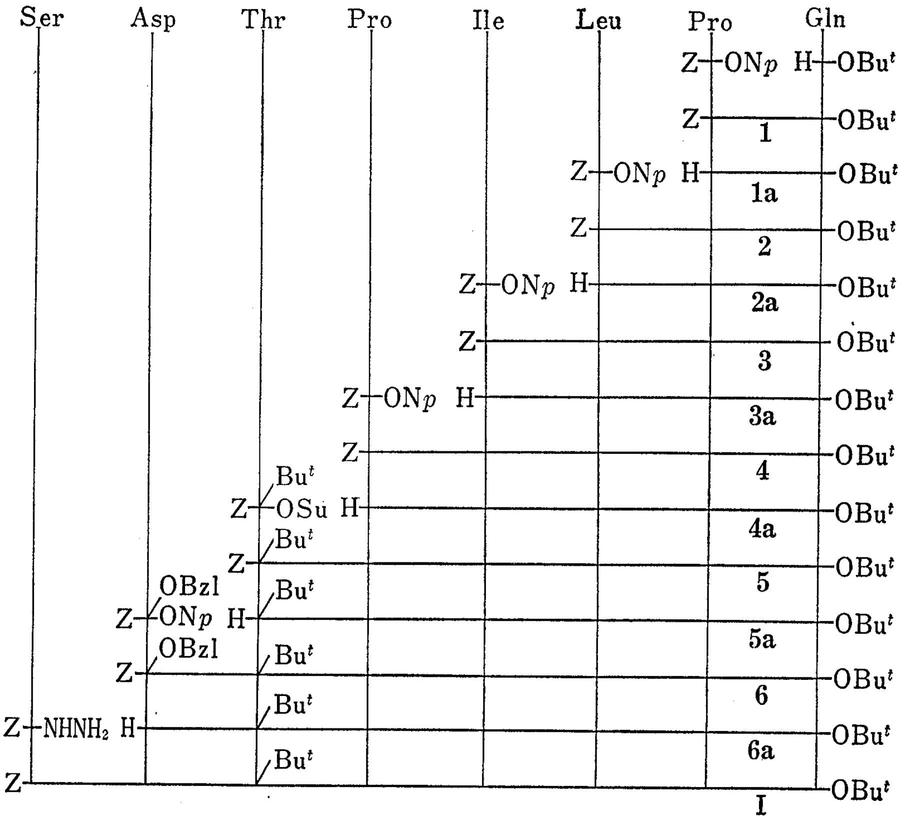

Fig. 2. Synthetic Scheme for the Protected Octapeptide (I)

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6) F.J. Morgan, S. Birken, and R.E. Canfield, *J. Biol. Chem.*, **250**, 5247 (1975).
7) S. Birken and R.E. Canfield, *J. Biol. Chem.*, **252**, 5386 (1977).
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Z–Thr(Bu^t)–OSu[15] to provide Z–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (**5**). The hexapeptide amine (**5a**) was linked with Z–Asp(OBzl)–ON*p*[16] to give Z–Asp(OBzl)–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (**6**). **6** was hydrogenated to give **6a** and coupled with Z–Ser–$NHNH_2$[17] to give Z–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (**I**). The dipeptide fragment, Z–Gly–Pro–NHNHBoc (II) was prepared by the condensation of Z–Gly–Pro–OH[18] and *t*-butylcarbazate using the DCC coupling method.

The protected tripeptide, Z–Arg(NO_2)–Leu–Pro–NHNHBoc (IV) was synthesized as illustrated in Fig. 3. Z–Leu–ON*p* and H–Pro–NHNHBoc[16] were coupled to afford Z–Leu–Pro–NHNHBoc (**7**). **7** was converted to the corresponding amine (**7a**) and coupled with Z–Arg(NO_2)–OH[19] by the diphenylphosphorazidate (DPPA) method[20] to give the tripeptide (IV). The protected tripeptide hydrazide, Z–Ser–Pro–Ser–$NHNH_2$ (VI), was synthesized by two different methods as shown in Fig. 4. One involved DCC coupling of Z–Ser–Pro–OH[21] and H–Ser–OMe,[22] and the other involved azide coupling of Z–Ser–$NHNH_2$ and H–Pro–Ser–OMe (**9a**) to give Z–Ser–Pro–Ser–OMe (**8**), which was converted to VI.

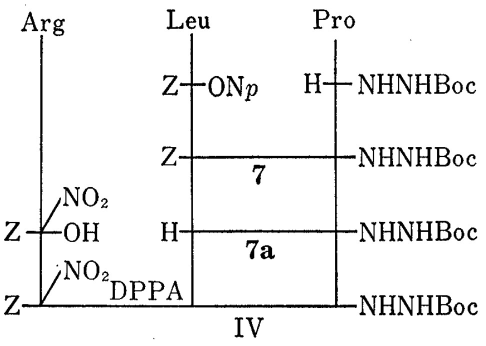

Fig. 3. Synthetic Scheme for the Protected Tripeptide (IV)

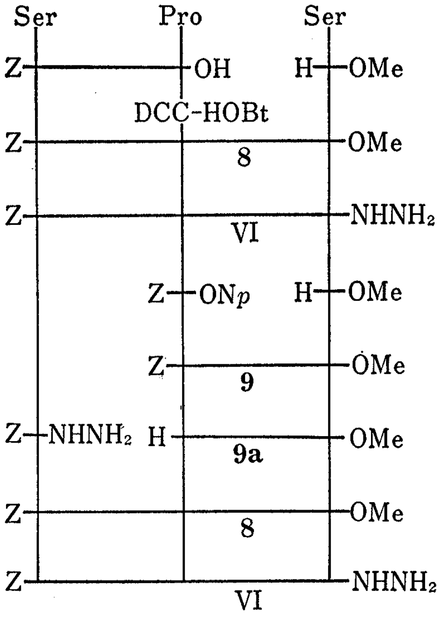

Fig. 4. Synthetic Scheme for the Tripeptide Hydrazide (VI)

As shown in Fig. 1, the four fragments prepared above were assembled by the azide method. The protected octapeptide (I) was hydrogenated and the resulting amine was coupled with Z–Gly–Pro–N_3 prepared from II by treatment with HCl followed by isopentylnitrite, to give Z–Gly–Pro–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (III), which was purified by gel-filtration on Sephadex LH-20 using EtOH and by silica gel column chromatography. III, after removal of the Z group, was coupled with Z–Arg(NO_2)–Leu–Pro–N_3 prepared from IV to give Z–Arg(NO_2)–Leu–Pro–Gly–Pro–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (V), which was purified by gel-filtration on Sephadex LH-20. The peptide (V)

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was converted to the corresponding amine by catalytic hydrogenation and coupled with Z–Ser–Pro–Ser–N_3 prepared from VI to afford the protected hexadecapeptide, Z–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (VII), which was purified by gel-filtration on Sephadex LH-20 using EtOH and then by silica gel column chromatography. The peptide (VII) was treated with trifluoroacetic acid (TFA) and hydrogenated. The deblocked peptide was purified by gel-filtration on Sephadex G-25 using 5% AcOH. The purified product was homogeneous upon thin-layer chromatography on silica gel. The 6 N HCl hydrolysate contained the constituent amino acids in the expected ratios. Despite the presence of the Pro residues,[23] complete digestion of the peptide with aminopeptidase (AP–M)[24] made it possible to identify the presence of Gln residue in this peptide. Judging from the experimental results cited above, it can be concluded that the synthetic hexadecapeptide possesses a high degree of homogeneity and the L-configuration of constituent amino acid residues.

The hexadecapeptide was converted to the corresponding hydrochloride and conjugated with bovine serum albumin (BSA) in the usual manner.[25] Antiserum was produced in New Zealand White rabbits by multiple intradermal injections of the conjugate in Freund's Complete adjuvant. Little binding of labeled hCG (^{125}I-hCG) was observed to the antiserum obtained above.

The protected hexadecapeptide (VII) was also used for the synthesis of the triacontapeptide corresponding to sequence 116—145 of the β-subunit of human chorionic gonadotropin, as will be reported in an accompanying paper.

Experimental

Thin–layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf^1=*n*-butanol, AcOH and H_2O (4: 1: 5), Rf^2=*n*-butanol, pyridine, AcOH and H_2O (4: 1: 1: 2), Rf^3=$CHCl_3$, MeOH and AcOH (90: 8: 2), Rf^4=$CHCl_3$, MeOH and H_2O (8: 3: 1), Rf^5=*n*-butanol, AcOEt, AcOH and H_2O (1: 1: 1: 1), Rf^6=*n*-butanol, pyridine, AcOH and H_2O (30: 20: 6: 24), Rf^7=*n*-butanol, pyridine, AcOH and H_2O (2: 1: 1: 2). Aminopeptidase (3501, Aminopeptidase 191226) was purchased from the Protein Research Foundation, Osaka, Japan.

Z-Pro-Gln-OBu*t* (1)——A solution of H–Gln–OBu^t (prepared from 11.0 g of H–Gln–$OBu^t \cdot H_3PO_3$ and 11.2 ml of triethylamine) and Z–Pro–ON*p* (15.9 g) in 25% aqueous dioxane (40 ml) was stirred for 2 days at room temperature. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford a white crystalline material, which was collected by filtration and recrystallized from AcOEt and ether; yield 13.0 g (75.1%), mp 65—70°, $[\alpha]^{23}_D$ −65.5° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{22}H_{31}N_3O_6$: C, 61.0; H, 7.21; N, 9.7. Found: C, 60.9; H, 7.22; N, 9.7.

Z-Leu-Pro-Gln-OBu*t* (2)——H–Pro–Gln–OBu^t (**1a**) (prepared from 10.7 g of **1** by catalytic hydrogenation) and Z–Leu–ON*p* (11.3 g) were dissolved in dioxane (50 ml) containing triethylamine (4.1 ml). After 2 days, the solvent was removed by evaporation and the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. Petroleum ether was added to the residue to give an amorphous powder, which was applied to a silica gel column (3×60 cm). The column was eluted with $CHCl_3$ (1800 ml) and 3% MeOH in $CHCl_3$ (1400 ml). With 3% MeOH in $CHCl_3$, the desired peptide (**2**) was obtained; yield 13.5 g (84.0%), amorphous powder, $[\alpha]^{25}_D$ −83.8° (*c*=1.0, MeOH). *Anal.* Calcd for $C_{28}H_{42}N_4O_7 \cdot 1/2\ H_2O$: C, 60.5; H, 7.80; N, 10.1. Found: C, 60.5; H, 7.89; N, 10.0.

Z-Ile-Leu-Pro-Gln-OBu*t* (3)——**2a** (prepared from 13.5 g of **2** by catalytic hydrogenation) was coupled with Z–Ile–ON*p* (9.5 g) in dioxane (50 ml) containing triethylamine (3.45 ml). After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and evaporated to dryness. The residue was applied to a column of silica gel (3×45 cm), which was first eluted with $CHCl_3$ (800 ml) and then with 3% MeOH in $CHCl_3$ (800 ml). From the latter eluate, the peptide (**3**) was obtained as a solid; yield 12.0 g (74.0%), mp 85—95°, $[\alpha]^{25}_D$ −99.2° (*c*=1.0, MeOH).

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Anal. Calcd for $C_{34}H_{53}N_5O_8$: C, 61.9; H, 8.10; N, 10.6. Found: C, 62.1; H, 8.21; N, 10.6.

Z-Pro-Ile-Leu-Pro-Gln-OBu^t (4)——**3a** (prepared from 10.2 g of **3** by catalytic hydrogenation) was coupled with Z–Pro-ON*p* (6.0 g) in dioxane (70 ml) containing triethylamine (2.18 ml). After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column (3×45 cm) chromatography in the manner described above to give **4** as an amorphous powder; yield 9.5 g (81.0%), $[\alpha]_D^{25}$ −122.3° (c=1.0, MeOH). *Anal.* Calcd for $C_{39}H_{60}N_6O_9 \cdot 1/2H_2O$: C, 61.2; H, 8.03; N, 11.0. Found: C, 61.0; H, 7.96; N, 10.9.

Z-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (5)——Z–Thr(Bu^t)–OSu (5.0 g) and **4a** (prepared from 8.1 g of **4** by catalytic hydrogenation) were dissolved in dioxane (50 ml) containing triethylamine (1.5 ml). After 2 days, the solvent was removed by evaporation and the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue and the resulting solid mass was purified by silica gel column (3×45 cm) chromatography in the manner described above to give the peptide (**5**); yield 6.8 g (70.0%), mp 60—65°, $[\alpha]_D^{25}$ −109.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{47}H_{75}N_7O_{11} \cdot 1/2H_2O$: C, 61.2; H, 8.41; N, 10.6. Found: C, 61.0; H, 8.34; N, 10.6.

Z-Asp(OBzl)-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (6)——A solution of **5a** (prepared from 6.0 g of **5** by catalytic hydrogenation) and Z–Asp(OBzl)–ON*p* (2.92 g) in dioxane (50 ml) containing triethylamine (0.92 ml) was stirred for 2 days at room temperature. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to form a white precipitate, which was collected by filtration and recrystallized from AcOEt and ether; yield 5.4 g (74.0%), mp 75—85°, $[\alpha]_D^{25}$ −84.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{58}H_{86}N_8O_{14}$: C, 62.2; H, 7.75; N, 10.0. Found: C, 62.1; H, 7.74; N, 9.8.

Z-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (**I**)——Z–Ser–N_3 (prepared from 0.45 g of Z–Ser–$NHNH_2$ in 10 ml of DMF) was added to the DMF solution (10 ml) of **6a** (prepared from 1.0 g of **6** by catalytic hydrogenation) containing triethylamine (0.25 ml) under cooling at −20°. This reaction mixture was stirred in a cold room (4°) for 2 days. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and concentrated. The residue in EtOH (3 ml) was applied to a column of Sephadex LH-20 (1×170 cm), which was equilibrated and eluted with EtOH. Individual fractions (3 ml each) were collected. The eluate (tube Nos. 32—46) was collected and concentrated. Ether was added to the residue to give a white powder, which was collected by filtration; yield 0.65 g (70.0%), mp 80—90°, $[\alpha]_D^{25}$ −90.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{54}H_{85}N_9O_{16} \cdot 2\frac{1}{2}H_2O$: C, 55.8; H, 7.81; N, 10.9. Found: C, 55.8; H, 7.68; N, 10.9.

Z-Gly-Pro-NHNHBoc (**II**)——N,N′-Dicyclohexylcarbodiimide (DCC) (2.48 g) was added to a cold solution of Z–Gly–Pro–OH[21] (3.0 g) and NH_2NHBoc (1.3 g) in DMF (30 ml). The reaction mixture was stirred overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Petroleum ether was added to the residue to yield an amorphous powder, which was collected by filtration and recrystallized from AcOEt and petroleum ether; yield 3.5 g (83.0%), mp 40—45°, $[\alpha]_D^{25}$ −90.2° (c=1.0, MeOH). *Anal.* Calcd for $C_{20}H_{28}N_4O_6 \cdot 2/3H_2O$: C, 55.5; H, 6.83; N, 13.0. Found: C, 55.4; H, 6.85; N, 12.8.

Z-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (**III**)——The peptide (I) (820 mg) in MeOH (20 ml) and water (5 ml) containing 0.5 ml of AcOH was hydrogenated over a palladium catalyst. After removal of the palladium and the solvent, the residue was lyophilized and the resulting white fluffy powder was used for coupling with dipeptide azide. Z–Gly–Pro–N_3 prepared as follows was added to the solution of octapeptide amine (Ia) in DMF (10 ml) containing 0.1 ml of triethylamine. Z–Gly–Pro–NHNHBoc (II) (620 mg) was dissolved in 3 N HCl/dioxane (3.0 ml). After 30 min, this solution was diluted with DMF (10 ml) and cooled to −10° followed by addition of isopentylnitrite (0.20 ml). This reaction mixture was stirred for 5 min and triethylamine (1.24 ml) was added. This solution was added to the ice-cooled solution of Ia prepared above and the reaction mixture was stirred for 2 days in a cold room (4°). After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford a white precipitate, which was collected by filtration and purified by column chromatography on Sephadex LH-20 (1×170 cm), equilibrated and eluted with EtOH. Individual fractions (3 ml each) were collected and the eluate (tube Nos. 28—37) was concentrated. Ether was added to the residue to give a white powder, which was collected by filtration; yield 800 mg (82%), mp 135—150°, $[\alpha]_D^{25}$ −108.1° (c=1.0, MeOH). *Anal.* Calcd for $C_{61}H_{95}N_{11}O_{18} \cdot 3H_2O$: C, 55.3; H, 7.69; N, 11.6. Found: C, 55.3; H, 7.49; N, 11.8. Amino acid ratios in an acid hydrolysate: Asp 1.00; Thr 1.00; Ser 0.87; Glu 0.94; Pro 3.38; Gly 1.02; Ile 0.83; Leu 0.85 (average recovery 85%).

Z-Leu-Pro-NHNHBoc (7)——**7** was prepared from H–Pro–NHNHBoc (5.0 g) and Z–Leu–ON*p* (8.5 g) and purified by silica gel column (3×40 cm) chromatography; yield 8.6 g (82.0%), amorphous powder, $[\alpha]_D^{25}$ −112.9° (c=1.0, MeOH). *Anal.* Calcd for $C_{24}H_{36}N_4O_6$: C, 60.5; H, 7.61; N, 11.8. Found: C, 60.7; H, 7.77; N, 11.6.

Z-Arg(NO_2)-Leu-Pro-NHNHBoc (**IV**)——**7a** (prepared from 6.2 g of **7** by catalytic hydrogenation) and Z–Arg(NO_2)–OH (3.75 g) were dissolved in DMF (50 ml). The solution was cooled with ice-salt and diphenylphosphorazidate (DPPA) (3.5 g) was added, followed by triethylamine (3.12 ml). The reaction mixture was stirred in a cold room for 24 hr. After removal of the solvent, the residue was dissolved in AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to give a white crystalline material, which was collected by filtration; yield 6.2 g (86.0%), mp 123—125°, $[\alpha]_D^{25}$ $-89.8°$ ($c=1.0$, MeOH). *Anal.* Calcd for $C_{30}H_{47}N_9O_9$: C, 53.2; H, 6.99; N, 18.6. Found: C, 53.0; H, 6.98; N, 18.7.

Z-Arg(NO_2)-Leu-Pro-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (V)——The decapeptide (III) (650 mg) in EtOH (8 ml) and water (5 ml) containing AcOH (0.5 ml) was hydrogenated over a palladium catalyst. The resulting peptide amine acetate was converted to the corresponding hydrochloride (IIIa). IIIa was dissolved in DMF (10 ml) containing triethylamine (0.14 ml). Z–Arg(NO_2)–Leu–Pro–N_3 prepared as follows was added to the cold solution of IIIa obtained above. Z–Arg(NO_2)–Leu–Pro–$NHNH_2$ (prepared from 1.02 g of IV and 2 ml of TFA containing 0.3 ml of anisole) was dissolved in DMF (15 ml), which was cooled to $-15°$. Next, 6 N HCl in dioxane (0.5 ml) was added to the cold solution, followed by isopentylnitrite (0.2 ml). After 5 min, the pH of the solution was adjusted to 8 with triethylamine (0.42 ml). The solution was added to a cold DMF solution of IIIa. This reaction mixture was stirred in a cold room for 2 days. After removal of the solvent, the residue was extracted with *n*-butanol. The extract was washed with water and concentrated. Ether was added to the residue and the resulting precipitate was collected by filtration and purified by gel-filtration on Sephadex LH-20 (3 × 87 cm), equilibrated and eluted with EtOH. The eluate (tube Nos. 30—38, 6 g each) was collected and concentrated. Ether was added to the residue to give a white precipitate. The peptide (V) was further purified by column chromatography on silica gel (2.3 × 30 cm), eluting with $CHCl_3$–MeOH–H_2O (16 : 3 : 1). Individual fractions (3 g each) were collected. The eluate (tube Nos. 17—21) was combined and the solvent was evaporated off. Ether was added to the residue to give a white powder; yield 590 mg (69.0%), mp 153—157°, $[\alpha]_D^{25}$ $-102.7°$ ($c=1.0$, MeOH). *Anal.* Calcd for $C_{78}H_{124}N_{18}O_{23}\cdot 3H_2O$: C, 54.0; H, 7.55; N, 14.5. Found: C, 53.9; H, 7.43; N, 14.6. Amino acid ratios in an acid hydrolysate: Asp 1.01; Thr 1.04; Ser 0.91; Glu 1.10; Pro 3.74; Gly 1.00; Ile 0.95; Leu 2.03; Arg 0.60 (average recovery 87%). The value for Arg is not corrected.

Z-Ser-Pro-Ser-OMe (8)——DCC (3.5 g) and N-hydroxysuccinimide (1.96 g) were added to a solution of Z–Ser–Pro–OH (4.7 g) and H–Ser–OMe (prepared from 2.46 g of H–Ser–OMe·HCl and 2.38 ml of triethylamine) in DMF (30 ml) cooled with ice-salt. This reaction mixture was stirred in a cold room overnight. After removal of the urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 1 N HCl and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford a crystalline material, which was collected by filtration; yield 1.74 g (28.5%), mp 113—116°, $[\alpha]_D^{23}$ $-76.9°$ ($c=1.0$, MeOH). *Anal.* Calcd for $C_{20}H_{27}N_3O_8$: C, 54.9; H, 6.22; N, 9.6. Found: C, 54.9; H, 6.33; N, 9.6.

Z-Ser-Pro-Ser-$NHNH_2$ (VI)——a) Hydrazine hydrate (80%, 0.1 ml) was added to a solution of **8** (450 mg) in MeOH (8 ml). The solution was kept at room temperature overnight. The resulting precipitate was collected by filtration and recrystallized from MeOH; yield 350 mg (82.0%), mp 165—167°, $[\alpha]_D^{23}$ $-31.5°$ ($c=1.0$, DMF). *Anal.* Calcd for $C_{19}H_{27}N_5O_7$: C, 52.2; H, 6.22; N, 16.0. Found: C, 52.0; H, 6.12; N, 16.0.

b) Z–Ser–N_3 (prepared from 1.0 g of Z–Ser–$NHNH_2$ in the usual manner) in DMF (10 ml) was added to a cold DMF solution of H–Pro–Ser–OMe (**9a**) (prepared from 1.0 g of **9**[1] by catalytic hydrogenation). The reaction mixture was stirred in a cold room for 2 days and the solvent was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. The oily residue was dissolved in MeOH (15 ml) containing hydrazine hydrate (80%, 1.5 ml). The solution was kept at room temperature overnight, then concentrated to a small volume. Upon addition of ether, a white crystalline material appeared, which was collected by filtration and recrystallized from EtOH; yield 500 mg (40% based on Z–Pro–Ser–OMe), mp 159—160°, $[\alpha]_D^{23}$ $-30.5°$ ($c=1.0$, DMF). *Anal.* Calcd for $C_{19}H_{27}N_5O_7$: C, 52.2; H, 6.22; N, 16.0. Found: C, 52.0; H, 6.12; N, 16.0.

Z-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (VII)——Z–Ser–Pro–Ser–N_3 (prepared from 237 mg of VI in the usual manner) in DMF (5 ml) was added to a cold solution of the partially protected tridecapeptide (Va) (prepared from 300 mg of V by catalytic hydrogenation) in DMF (10 ml) containing triethylamine (0.05 ml). The reaction mixture was stirred in a cold room for 2 days. After removal of the solvent, the residue was extracted with *n*-butanol. The extract was washed with water and concentrated. Ether was added to the residue, providing a white precipitate, which was collected by filtration and purified by gel-filtration on Sephadex LH-20 (1 × 180 cm), equilibrated and eluted with EtOH. Individual fractions (3 g each) were collected. The eluate (tube Nos. 24—29) was concentrated and ether was added to the residue to yield a white precipitate. This was further purified by column chromatography on silica gel (2.5 × 11 cm), eluting with the solvent system of $CHCl_3$–MeOH–H_2O (8 : 3 : 1). The eluate (tube Nos. 10—13, 3 g each) was concentrated. Ether was added to the residue to give purified VII; yield 250 mg (71.2%), mp 240° (dec.) with sintering at 165°, $[\alpha]_D^{23}$ $-107.8°$ ($c=1.0$, MeOH). *Anal.* Calcd for $C_{89}H_{142}N_{20}O_{26}\cdot HCl\cdot 2H_2O$: C, 54.0; H, 7.48; N, 14.1. Found: C, 53.8; H, 7.49; N, 14.1. Amino acid ratios in an acid hydrolysate: Asp 0.97; Thr 1.06; Ser 2.97; Glu 1.06; Pro 5.28; Gly 1.00; Ile 0.92; Leu 1.87; Arg 0.89 (average recovery 95%).

H-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln-OH——The protected hexadecapeptide (VII) (120 mg) was dissolved in TFA (1 ml) containing anisole (0.2 ml). The solution was kept at room temperature for 3 hr. Addition of ether to the solution gave a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets, yield 110 mg, Rf^2 0.58 (I_2 and Sakaguchi stain). The peptide obtained above was dissolved in EtOH (5 ml) and water (4 ml) containing AcOH (0.2 ml), then hydrogenated over a palladium catalyst. After removal of the palladium and the solvent, the residue was lyophilized to give a white fluffy powder, which was purified by gel-filtration on Sephadex G-25 (3×140 cm), equilibrated and eluted with 5% AcOH. Individual fractions (3 ml each) were collected. Lyophilization of the effluent (fraction Nos. 74—80) afforded the desired peptide, which was converted to the corresponding hydrochloride; yield 57 mg (48.7%), $[\alpha]_D^{23}$ $-163.0°$ ($c=0.3$, H_2O), Rf^1 0.05, Rf^2 0.38 (ninhydrin and Sakaguchi stain). *Anal.* Calcd for $C_{73}H_{120}N_{20}O_{24} \cdot 2HCl \cdot 11H_2O$: C, 45.4; H, 7.50; N, 14.5. Found: C, 45.1; H, 7.09; N, 13.9. Amino acid ratios in an acid hydrolysate: Asp 0.95; Thr 1.05; Ser 2.90; Glu 1.06; Pro 5.17; Gly 1.00; Ile 0.86; Leu 1.81; Arg 0.91 (average recovery 88%). Amino acid ratios in an AP–M digest: Asp 0.91; (Thr+Gln) 1.93; Ser 3.05; Pro 4.97; Gly 1.02; Ile 1.00; Leu 1.92; Arg 0.92 (average recovery 95%) (Gln emerged at the same position as Thr and was calculated as Thr).