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Human Chorionic Gonadotropin. II.[1,2] Synthesis of a Hexadecapeptide corresponding to the C-Terminal Sequence 132—147 of the β-Subunit of Human Chorionic Gonadotropin (hCG)

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A hexadecapeptide, H–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH, corresponding to the C-terminal sequence 132—147 of hCG-β proposed by Carlsen *et al.*, was synthesized stepwise by fragment condensation, assembling five fragments [Z–Arg(NO_2)–Leu–Pro–NHNHBoc, Z–Gly–Pro–Pro–NHNHBoc, Z–Asn–Thr–Pro–NHNHBoc, Z–Ile–Leu–Pro–NHNHBoc, and Z–Gln–Ser–Leu–Pro–OBu^t].

Keywords——human chorionic gonadotropin; synthesis of the C-terminal sequence of hCG-β; synthesis of a hexadecapeptide; peptide synthesis by fragment condensation; β-subunit of hCG

Human chorionic gonadotropin (hCG) is a glycoprotein hormone which consists of two peptide chains, the α-subunit(hCG-α) and the β-subunit(hCG-β). hCG shows structural similarity to other glycoprotein hormones such as human luteinizing hormone(hLH),[4] human follicle stimulating hormone(hFSH)[5] and human thyrotropin(hTSH).[6] Among these proteins, hCG showed remarkable structural similarity to hLH. The amino acid sequence of hCG-α and hLH-α are almost the same except for a few amino acids, and hCG-β also has a similar amino acid sequence to hLH-β. However, hCG-β possesses a characteristic amino acid sequence, which is not present in the β-subunit of hLH or in other glycoprotein hormones, at its carboxyl-terminal region. The amino acid sequence of hCG-β was elucidated by Carlsen *et al.*[7] and Morgan *et al.*[8] The amino acid sequence proposed by the former group consists of 147 amino acids, while the sequence proposed by the latter group consists of 145 amino acids. The amino acid sequence of the carboxyl-terminal region of hLH-β and the two proposed sequences of the carboxyl-terminal region of hCG-β are shown in Fig. 1.

The characteristic tail portion of hCG-β is of interest for immunologic studies to distinguish hCG from other glycoprotein hormones, especially from hLH. Namely, the antibody against

1) Part I: Y. Okada, K. Kawasaki, S. Iguchi, C. Kawasaki, Y. Tsuda, M. Yagyu, K. Yamaji, T. Takagi, and O. Tanizawa, *Chem. Pharm. Bull.*, **28**, 359 (1980).
2) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1976). Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, ONp=*p*-nitrophenyl ester, OBu^t=*tert*-butyl ester. Other abbreviations used in this paper are: DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, THF=tetrahydrofuran, TFA=trifluoroacetic acid.
3) Location: *a) Ikawadani-cho, Tarumi-ku, Kobe, 673, Japan; b) Fukushima, Fukushima-ku, Osaka, 553, Japan.*
4) H.T. Keutman, R.W. Williams, and R.J. Ryan, *Biochem. Biophys. Res. Commun.*, **90**, 842 (1979).
5) B.B. Saxena and P. Rathnam, *J. Biol. Chem.*, **251**, 993 (1976).
6) M.R. Sairam and C.H. Li, *Biochem. Biophys. Res. Commun.*, **54**, 426 (1973).
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hCG-β (Carlsen *et al.*[7]) $-\overset{109}{\text{Thr}}-\text{Cys}-\text{Asp}-\text{Asp}-\text{Pro}-\text{Arg}-\text{Phe}-\overset{116}{\text{Gln}}-\text{Asp}-\text{Ser}-\text{Ser}-\text{Ser}-\!\!-\!\!-\text{Lys}\cdot\text{Ala}-\text{Pro}-\text{Pro}-\overset{125}{\text{Pro}}-$

hCG-β (Morgan *et al.*[8]) $-\overset{109}{\text{Thr}}-\text{Cys}-\text{Asp}-\text{Asp}-\text{Pro}-\text{Arg}-\text{Phe}-\overset{116}{\text{Gln}}-\text{Asp}-\text{Ser}-\text{Ser}-\text{Ser}-\overset{121}{\underline{\text{Ser}}}-\text{Lys}-\text{Ala}-\text{Pro}-\text{Pro}-\overset{126}{\text{Pro}}-$

hLH-β (Keutmann *et al.*[4]) $-\overset{97}{\text{Thr}}-\text{Cys}-\text{Asp}-\text{His}-\text{Pro}-\overset{102}{\text{Gln}}$

hCG-β (Carlsen *et al.*) $-\overset{126}{\text{Ser}}-\text{Leu}-\text{Pro}-\text{Ser}-\text{Pro}-\text{Ser}-\overset{132}{\text{Arg}}-\text{Leu}-\text{Pro}-\text{Gly}-\text{Pro}-\overset{137}{\underline{\text{Pro}}}-\overset{138}{\underline{\text{Asn}}}-\text{Thr}-\text{Pro}-\text{Ile}-\text{Leu}-\text{Pro}-\overset{144}{\text{Gln}}-$

hCG-β (Morgan *et al.*) $-\overset{127}{\text{Ser}}-\text{Leu}-\text{Pro}-\text{Ser}-\text{Pro}-\text{Ser}-\overset{133}{\text{Arg}}-\text{Leu}-\text{Pro}-\text{Gly}-\text{Pro}-\overset{138}{\underline{\text{Ser}}}-\overset{139}{\underline{\text{Asp}}}-\text{Thr}-\text{Pro}-\text{Ile}-\text{Leu}-\text{Pro}-\overset{145}{\text{Gln}}$

hCG-β (Carlsen *et al.*) $-\text{Ser}-\text{Leu}-\overset{147}{\text{Pro}}$

Fig. 1. Comparison of the Carboxyl-Terminal Sequences of hCG-β and hLH-β

this characteristic tail portion of hCG-β might react with hCG but not with the other glycoproteins described above. However, a specific antibody to hCG which has sufficient binding activity for practical use has not been prepared yet, and remains an interesting target for research in the fields of diagnostic studies by radioimmunoassay and fertility regulation. In view of these considerations, we started the synthesis of the characteristic tail portion of the two proposed sequences of hCG-β with the eventual aim of preparing a specific antibody to hCG. To date, solid phase syntheses of carboxyl-terminal fragments of the two proposed structures of hCG-β and some immunologic studies on the synthetic peptides have been reported by Folkers *et al.*[9] and Matsuura *et al.*[10] Here we wish to describe the synthesis of a hexadecapeptide, H–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH (I), corresponding to the sequence 132—147 of hCG-β proposed by Carlsen *et al.*[7] The synthetic scheme for I is illustrated in Fig. 2. Stepwise fragment condensation by the azide procedure[11] was employed to extend the peptide chain. The α-amino groups of acylating

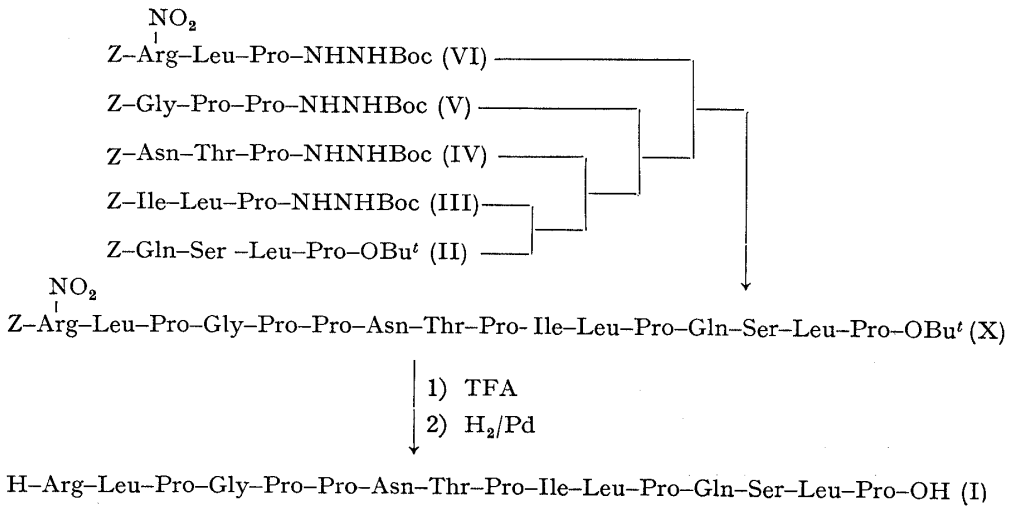

Fig. 2. Synthetic Scheme for the Peptide I

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10) S. Matsuura, M. Ohashi, H-C. Chen, and G.D. Hodgen, *Endocrinol.*, **104**, 396 (1979).
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amino acids and peptides were protected by the Z group[12] and the carboxyl group of carboxyl-terminal proline at position 147 was protected as the *tert*-butyl ester.[13] Carboxyl groups of carboxyl-terminal amino acids of acylating fragments were protected as the Boc-hydrazide.[14] Five fragments [Z–Gln–Ser–Leu–Pro–OBu^t (II), Z–Ile–Leu–Pro–NHNHBoc (III), Z–Asn–Thr–Pro–NHNHBoc (IV), Z–Gly–Pro–Pro–NHNHBoc (V), and Z–Arg(NO_2)–Leu–Pro–NHNHBoc (VI)] were assembled to construct the hexadecapeptide (I).

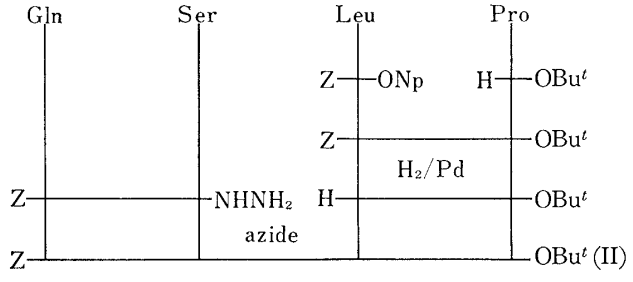

Fig. 3. Synthetic Scheme for the Peptide II

Z–Gln–Ser–Leu–Pro–OBu^t (II) was synthesized as illustrated in Fig. 3. Z–Leu–ONp[15] was coupled with H–Pro–OBu^t[16] to afford Z–Leu–Pro–OBu^t, which was hydrogenated over a Pd catalyst. The resulting amine was coupled by the azide procedure with Z–Gln–Ser–$NHNH_2$[17] to afford the fragment II.

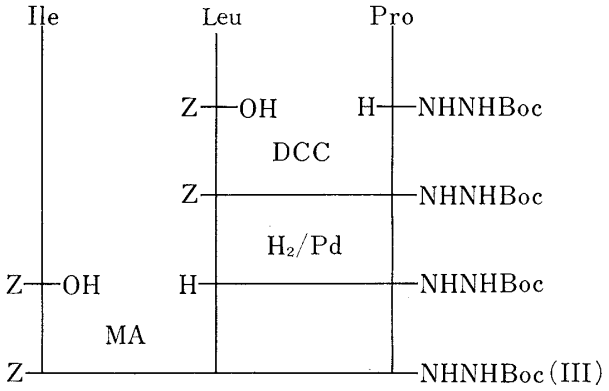

Fig. 4. Synthetic Scheme for the Peptide III

MA: mixed anhydride method.

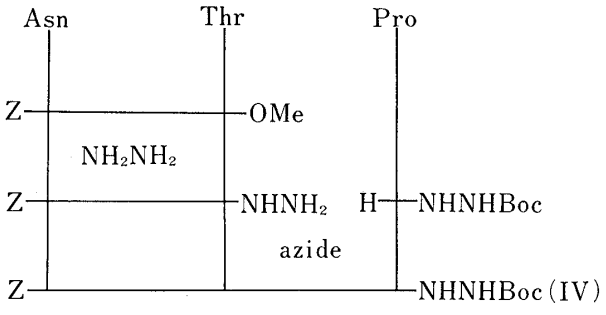

Fig. 5. Synthetic Scheme for the Peptide IV

Z–Ile–Leu–Pro–NHNHBoc (III) was synthesized as shown in Fig. 4. Z–Leu–OH[18] was coupled by the DCC procedure[19] with H–Pro–NHNHBoc[20] to afford Z–Leu–Pro–NHNHBoc, followed by hydrogenation over a Pd catalyst. The resulting dipeptide amine was coupled by the mixed anhydride method[21] with Z–Ile–OH[22] to afford the fragment III. The DCC procedure was also attempted for the formation of the fragment III but the procedure was not suitable because of the formation of large amounts of acylurea.

Z–Asn–Thr–Pro–NHNHBoc (IV) was synthesized as shown in Fig. 5. Z–Asn–Thr–

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13) G.W. Anderson and F.M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).
14) *a*) R.A. Boissonnas, S. Guttmann, and P.A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960); *b*) R. Schwyzer, E. Surbeck-Wegman, and H. Dietrich, *Chimia*, **14**, 366 (1960).
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OMe[23] was converted to the corresponding hydrazide, which was coupled by the azide procedure with H–Pro–NHNHBoc to afford the fragment IV.

Z–Gly–Pro–Pro–NHNHBoc (V) was synthesized as shown in Fig. 6. To avoid formation of the diketopiperazine of prolylproline, Boc–$NHNH_2$ was attached on the carboxyl group after formation of the tripeptide. Z–Gly–ONp[24] was coupled with H–Pro–Pro–OH[25] to afford Z–Gly–Pro–Pro–OH, which was converted to the corresponding Boc–hydrazide (V) by the DCC procedure.

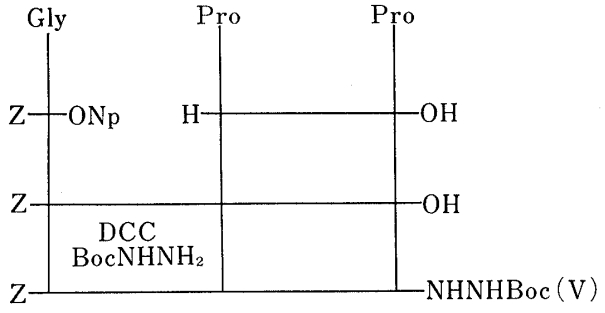

Fig. 6. Synthetic Scheme for the Peptide V

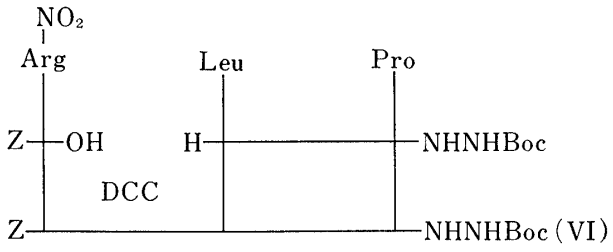

Fig. 7. Synthetic Scheme for the Peptide VI

Z–Arg(NO_2)–Leu–Pro–NHNHBoc was synthesized as shown in Fig. 7. The guanidino group of arginine was protected with a nitro group.[26] Z–Arg(NO_2)–OH[27] was coupled by the DCC method with H–Leu–Pro–NHNHBoc to afford VI, which was purified by silica gel column chromatography.

The five fragments thus obtained were assembled one by one by the azide method. Z–Ile–Leu–Pro–NHNHBoc (III) was treated with HCl and the resulting tripeptide hydrazide was linked by the azide procedure to H–Gln–Ser–Leu–Pro–OBu^t (prepared by hydrogenation from the fragment II). The resulting heptapeptide, Z–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (VII), was hydrogenated over a Pd catalyst and the resulting heptapeptide amine was coupled by the azide procedure with Z–Asn–Thr–Pro–$NHNH_2$ (prepared by TFA treatment from the fragment IV) to afford the decapeptide, Z–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (VIII). VIII was hydrogenated and the resulting decapeptide amine was coupled by the azide method with Z–Gly–Pro–Pro–$NHNH_2$ (prepared by HCl treatment from the fragment V) to afford the tridecapeptide, Z–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (IX). The fragment IX was hydrogenated and coupled by the azide procedure with Z–Arg(NO_2)–Leu–Pro–$NHNH_2$ (prepared by TFA treatment from the fragment VI) to afford the hexadecapeptide, Z–Arg(NO_2)–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (X). X was purified by extraction with n-butanol, followed by Sephadex LH-20 column chromatography using ethanol as an eluent. X was treated with TFA, followed by hydrogenation to remove all protecting groups. The resulting peptide I, H–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH, was purified by Sephadex G-50 column chromatography using 5% acetic acid as an eluent. Amino acid ratios in an acid hydrolysate of I were as expected, and aminopeptidase M[28] digested I completely in spite of the presence of proline residues.

The hexadecapeptide acetate was converted to the corresponding hydrochloride and conjugated with bovine serum albumin by the water-soluble DCC procedure in the usual man-

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28) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

ner.[29] An antiserum was produced in New Zealand white rabbits by multiple intradermal injection of the conjugate in Freund's Complete adjuvant. Little binding of labeled hCG(^{125}I-hCG) was observed with the antiserum to I. The details of the preparation of the antiserum and its cross-reaction with the labeled hCG will be reported elswhere. The blocked hexadecapeptide (X) was used to construct a dotriacontapeptide corresponding to the sequence 116—147 of hCG-β proposed by Carlsen *et al.*[7] The details of the synthesis of the dotriacontapeptide will be described in the following paper.

Experimental

Melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). The amino acid compositions of acid hydrolysates and aminopeptidase M digests were determined with a JEOL JLC-6AH amino acid analyzer (one-column system). Solvents were evaporated off *in vacuo* at a temperature of 40°. Solvent systems for ascending thin-layer chromatography on siliac gel G (type 60, E. Merck) are indicated as follows: Rf^1 *n*-BuOH, AcOH, H_2O (4: 1: 5, upper phase); Rf^2 *n*-BuOH, AcOH, pyridine, H_2O (4: 1: 1: 2); Rf^3 $CHCl_3$, MeOH, H_2O (8: 3: 1, lower phase), Rf^4 AcOEt, benzene (1: 1).

Z-Leu-Pro-OBu^t——Z–Leu–ONp[15] (30.4 g) was added to a solution of H–Pro–OBu^t[16] (prepared from 23.5 g of Z–Pro–OBu^t by hydrogenolysis over a Pd catalyst) in DMF (30 ml). The reaction mixture was stirred at room temperature for 12 hr and the solvent was evaporated off. The residue was extracted with AcOEt and the AcOEt layer was washed successively with 5% Na_2CO_3, 1 M NH_4OH and H_2O. After drying over Na_2SO_4, the AcOEt was evaporated off to give an oily material; yield 28 g (86%), Rf^1 0.87. After deblocking by hydrogenolysis; Rf^1 0.60, Rf^2 0.75.

Z-Gln-Ser-Leu-Pro-OBu^t (II)——Z–Leu–Pro–OBu^t (8.36 g) dissolved in MeOH (100 ml) was hydrogenated over a Pd catalyst for 3 hr. Pd was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in a mixture of DMF (20 ml) and Et_3N (2.8 ml).

Next, Z–Gln–Ser–$NHNH_2$[17] (7.64 g) was dissolved in a mixture of DMF (40 ml), DMSO (5 ml) and 5.5 N HCl/dioxane (8 ml). Isopentyl nitrite (2.8 ml) was then added at −15° and the mixture was stirred for 5 min. Et_3N (6 ml) was added and the mixture was combined with the solution of H–Leu–Pro–OBu^t described above. The reaction mixture was stirred for 3 days in a cold room and the solvent was evaporated off. The residue was dissolved in AcOEt and H_2O, and the crystalline material in the AcOEt layer was collected by filtration, washed with H_2O and recrystallized from MeOH/AcOEt; yield 7.8 g (61%), mp 168—170°, $[\alpha]_D^{21}$ −80.1° (c=1.0, MeOH), Rf^1 0.80. After deblocking by hydrogenolysis; Rf^1 0.42, Rf^2 0.72. *Anal.* Calcd for $C_{31}H_{47}N_5O_9$: C, 58.8; H, 7.5; N, 11.1. Found: C, 58.5; H, 7.5; N, 11.1. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Glu_{1.10}Ser_{0.89}Pro_{1.15}Leu_{1.00}$ (average recovery 95%).

Z-Leu-Pro-NHNHBoc——DCC (1.36 g) was added to a solution of Z–Leu–OH[18] (1.75 g) and H–Pro–NHNHBoc[20] (1.52 g) in AcOEt (20 ml) at −10° and the reaction mixture was stirred for 2 days in a cold room. Dicyclohexylurea was removed by filtration and the filtrate was washed successively with 5% Na_2CO_3, 5% citric acid, 3% NH_4OH and H_2O. After drying over Na_2SO_4, the AcOEt was evaporated off and the residue was precipitated from AcOEt/petro·ether; yield 2.5 g (80%), amorphous powder, $[\alpha]_D^{22}$ −116.3° (c=1.0, MeOH), Rf^1 0.92, Rf^3 0.90, Rf^4 0.40. *Anal.* Calcd for $C_{24}H_{36}N_4O_6$: C, 60.5; H, 7.6; N, 11.8. Found: C, 60.4; H, 7.6; N, 11.6.

H-Leu-Pro-NHNHBoc——Z–Leu–Pro–NHNHBoc (5.5 g) was hydrogenated over a Pd catalyst in MeOH in the usual manner. The deblocked material was recrystallized from MeOH/ether; yield 3.1 g (79%), mp 132—134°, $[\alpha]_D^{24}$ −114.7° (c=1.0, MeOH), Rf^1 0.77, Rf^3 0.46. *Anal.* Calcd for $C_{16}H_{30}N_4O_4$: C, 56.1; H, 8.8; N, 16.3. Found: C, 56.1; H, 9.0; N, 16.0.

Z-Ile-Leu-Pro-NHNHBoc (III)——Ethylchloroformate (1.9 ml) was added to a solution of Z–Ile–OH[22] (5.27 g) and Et_3N (2.76 ml) in THF (50 ml) at −15°, and the mixture was stirred for 10 min. The mixture was added to a solution of H–Leu–Pro–NHNHBoc (4 g) in DMF (10 ml) and the reaction mixture was stirred overnight. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 5% Na_2CO_3, 5% citric acid, 3% NH_4OH and H_2O, and dried over Na_2SO_4. The AcOEt was evaporated off and the residue was precipitated from AcOEt/petro·ether; yield 4.5 g (70%), mp 92—95°, $[\alpha]_D^{24}$ −112.2° (c=1.0, MeOH), Rf^3 0.85, Rf^4 0.40. *Anal.* Calcd for $C_{30}H_{47}N_5O_7$: C, 61.1; H, 8.0; N, 11.9. Found: C, 61.2; H, 8.2; N, 11.7.

Z-Asn-Thr-$NHNH_2$——Hydrazine hydrate (80%, 8 ml) was added to a solution of Z–Asn–Thr–OMe[23] (10.0 g) in DMF (50 ml). After 20 hr at room temperature, MeOH (50 ml) was added to the solution to form a solid precipitate. The material was collected by filtration, washed with MeOH and 50% aqueous MeOH;

29) J. Vaneckova, J. Barthova, T. Barth, I. Krejci, and I. Rychlick, *Collect. Czech. Chem. Commun.*, **40**, 1461 (1975).

yield 8.5 g (85%), mp 215—217°, $[\alpha]_D^{23}$ −10.9° (c=1.0, DMF). Rf^1 0.54. *Anal.* Calcd for $C_{16}H_{23}N_5O_6$: C, 50.4; H, 6.1; N, 18.4. Found: C, 50.3; H, 6.0; N, 18.5.

Z-Asn-Thr-Pro-NHNHBoc (IV)——Isopentyl nitrite (0.7 ml) was added to a solution of Z–Asn–Thr–$NHNH_2$ (1.9 g) in a mixture of DMF (10 ml) and 6 N HCl/dioxane (1.4 ml) at −20°, and the mixture was stirred for 5 min. Et_3N (1.4 ml) was added and the whole was combined with a solution of H–Pro–NHNH-Boc[20] (1.6 g) in DMF (10 ml) containing Et_3N (1.18 ml). The reaction mixture was stirred for 3 days in a cold room and the solvent was evaporated off. The residue was dissolved in AcOEt and the AcOEt layer was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in 50% aqueous MeOH and treated with Dowex 50 (×2, H^+ form) at −10°. After removal of the resin, the solvent was evaporated off and ether was added to the residue to afford a solid mass; yield 1.63 g (60%), mp 140° with sintering at 80°, $[\alpha]_D^{21}$ −79.8° (c=1.0, MeOH), Rf^1 0.64. *Anal.* Calcd for $C_{26}H_{38}N_6O_9 \cdot 1/2H_2O$: C, 53.1; H, 6.7; N, 14.3. Found: C, 52.9; H, 6.8; N, 14.1.

Z-Asn-Thr-Pro-$NHNH_2$•TFA——Anisole (0.5 ml) and TFA (2 ml) were added to Z–Asn–Thr–Pro–NHNHBoc (0.67 g). After 1 hr at 0°, ether was added to give a precipitate, which was collected by filtration, washed with ether and dried over KOH pellets *in vacuo*; yield 0.6 g (90%), mp 65—70°, $[\alpha]_D^{21}$ −46.8° (c=1.0, MeOH). *Anal.* Calcd for $C_{21}H_{30}N_6O_7 \cdot CF_3COOH \cdot H_2O$: C, 45.2; H, 5.5; N, 13.8. Found: C, 45.2; H, 5.3; N, 13.8.

Z-Gly-Pro-Pro-OH——A solution of H–Pro–Pro–OH[25] (5.4 g) and Z–Gly–ONp[24] (6.6 g) in 50% aqueous dioxane (100 ml) containing triethylamine (7 ml) was stirred at room temperature overnight. The solvent was evaporated off and the residue was dissolved in 5% $NaHCO_3$ (100 ml). The solution was washed with AcOEt and acidified (Congo red) with conc. HCl. The resulting oily material was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated down. Petro·ether was added to the residue to give a precipitate; yield 7.5 g (90%), mp 74—90°, $[\alpha]_D^{20}$ −134.6° (c=1.0, MeOH), Rf^1 0.59. *Anal.* Calcd for $C_{20}H_{25}N_3O_6 \cdot H_2O$: C, 57.0; H, 6.5; N, 10.0. Found: C, 57.2; H, 6.4; N, 9.8.

Z-Gly-Pro-Pro-NHNHBoc (V)——DCC (4.1 g) was added to a solution of Z–Gly–Pro–Pro–OH (7.5 g) and Boc-$NHNH_2$ (2.6 g) in acetonitrile (100 ml) at 0°, and the mixture was stirred at room temperature overnight. After removal of the urea derivative, the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed successively with 5% $NaHCO_3$, 10% citric acid and H_2O. After drying over Na_2SO_4, the solvent was evaporated off and the residue was recrystallized from a small amount of AcOEt; yield 2.9 g (31%), mp 166—169°, $[\alpha]_D^{20}$ −162.4° (c=1.0, MeOH), Rf^1 0.70. *Anal.* Calcd for $C_{25}H_{35}N_5O_7$: C, 58.0; H, 6.8; N, 13.5. Found: C, 58.0; H, 6.9; N, 13.3. Amino acid ratios in an acid hydrolysate (6 N HCl, 18 hr): $Gly_{1.00}Pro_{1.93}$ (average recovery 83%).

Z-Arg(NO_2)-Leu-Pro-NHNHBoc (VI)——DCC (845 mg) was added to a solution of Z–Arg(NO_2)–OH[27] (0.9 g) and H–Leu–Pro–NHNHBoc (1.42 g) in DMF(20 ml) at −10°, and the reaction mixture was stirred for 48 hr in a cold room. The resulting precipitate was removed by filtration and the solvent was evaporated off. The residue was extracted with AcOEt and the AcOEt layer was washed successively with 5% Na_2CO_3, 5% citric acid, 3% NH_4OH and H_2O. After drying over Na_2SO_4, the solvent was evaporated off and the residue was purified by silica gel (Kieselgel 60, Merck) column chromatography (column: 2×20 cm). The column was developed with $CHCl_3$ (500 ml) and 3% MeOH/$CHCl_3$ (1000 ml) and the desired material was eluted in 3% MeOH/$CHCl_3$. The material was precipitated from AcOEt/petro·ether; yield 2.1 g (75%), mp 123—126°, $[\alpha]_D^{23}$ −89.8° (c=1.0, MeOH), Rf^3 0.65. *Anal.* Calcd for $C_{30}H_{47}N_9O_9$: C, 53.2; H, 7.0; N, 18.6. Found: C, 53.0; H, 7.0; N, 18.7.

Z-Ile-Leu-Pro-Gln-Ser-Leu-Pro-VBu^t (VII)——Z–Gln–Ser–Leu–Pro–OBu^t (II, 0.89 g) was hydrogenated over a Pd catalyst in MeOH in the usual manner. The deblocked material was dissolved in DMF (6 ml).

Next, Z–Ile–Leu–Pro–NHNHBoc (III, 0.97 g) was dissolved in 3 N HCl/dioxane (2.2 ml) and the solution was stirred at room temperature for 1 hr. The solution was diluted with DMF (10 ml) and combined with isopentyl nitrite (0.23 ml) at −20°. The mixture was stirred for 5 min and adjusted to pH 8 with Et_3N. This mixture was combined with the deblocked tetrapeptide solution described above. The reaction mixture was stirred for 3 days in a cold room and the solvent was evaporated off. The residue was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to the oily residue to afford a solid material, which was reprecipitated from AcOEt/ether; yield 1.2 g (89%), mp 170° with sintering at 95°, $[\alpha]_D^{21}$ −111.9° (c=1.0, MeOH), Rf^1 0.83, after deblocking by hydrogenolysis; Rf^1 0.46, Rf^2 0.70. *Anal.* Calcd for $C_{48}H_{76}N_8O_{12} \cdot H_2O$: C, 59.1; H, 8.1; N, 11.5. Found: C, 58.9; H, 8.0; N, 11.6. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Ile_{1.03}Leu_{1.88}Pro_{1.80}Glu_{1.00}Ser_{0.83}$ (average recovery 83%).

Z-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (VIII)——Z–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (VII, 0.56 g) was hydrogenated over a Pd catalyst in MeOH in the usual manner. The deblocked material was dissolved in DMF (5 ml).

Next, isopentyl nitrite (0.16 ml) was added to a solution of Z–Asn–Thr–Pro–$NHNH_2$·TFA (0.66 g) in a mixture of DMF (5 ml) and 6 N HCl/dioxane (0.39 ml) at −20° and the mixture was stirred for 5 min. The mixture was neutralized with Et_3N (0.33 ml) and combined with the deblocked heptapeptide solution described above. The reaction mixture was stirred for 3 days in a cold room and the solvent was evaporated off. AcOEt was added to the oily residue to afford a solid material, which was collected by filtration, washed with

AcOEt and H_2O. The material was reprecipitated from EtOH/AcOEt; yield 0.53 g (72%), mp 155—160°, $[\alpha]_D^{21}$ −124.9° (c=1.0, MeOH), Rf^1 0.58, Rf^2 0.82, after deblocking by hydrogenolysis; Rf^1 0.28, Rf^2 0.66. *Anal.* Calcd for $C_{61}H_{96}N_{12}O_{17}\cdot 4H_2O$: C, 54.6; H, 7.7; N, 12.5. Found: C, 54.7; H, 7.8; N, 12.9. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{1.00}Thr_{0.92}Ser_{0.81}Glu_{1.02}Pro_{2.95}Ile_{1.02}Leu_{1.88}$ (average recovery 97%).

Z-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (IX)——Z–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (VIII, 0.53 g) was hydrogenated over a Pd catalyst in MeOH in the usual manner. The deblocked material was dissolved in DMF (5 ml).

Next, Z–Gly–Pro–Pro–NHNHBoc (V, 0.54 g) was deblocked with 6 N HCl/dioxane (1 ml) in DMF (1 ml) according to the procedure described for deblocking of III in the synthesis of VIII. The hydrazide was then converted to the azide with isopentyl nitrite (0.15 ml) at −20° in the usual manner and the azide solution, which was first adjusted to pH 8 with Et_3N, was combined with the deblocked decapeptide solution described above. The reaction mixture was stirred for 3 days in a cold room and the solvent was evaporated off. The residue was dissolved in *n*-BuOH and the *n*-BuOH layer was washed with H_2O. The solvent was evaporated off and AcOEt was added to the oily residue to afford a solid material, which was precipitated from EtOH/AcOEt; yield 0.32 g (51%), mp 155—160°, $[\alpha]_D^{21}$ −143.3° (c=0.5, MeOH), Rf^1 0.38, Rf^2 0.83. *Anal.* Calcd for $C_{73}H_{113}N_{15}O_{20}\cdot 3H_2O$: C, 55.7; H, 7.6; N, 13.3. Found: C, 55.9; H, 7.6; N, 13.7. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{0.99}Thr_{0.88}Ser_{0.80}Glu_{1.00}Pro_{5.24}Gly_{1.09}Ile_{1.01}Leu_{2.05}$ (average recovery 100%).

Z-Arg(NO$_2$)-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (X)——Z–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (IX, 250 mg) was hydrogenated over a Pd catalyst in 80% aqueous MeOH (25 ml) in the usual manner. The deblocked material was dissolved in DMF (2.5 ml).

Next, Z–Arg(NO$_2$)–Leu–Pro–NHNHBoc (VI, 407 mg) was deblocked with TFA (1 ml) in the presence of anisole (0.05 ml) in the usual manner. The deblocked material was converted to the corresponding azide with *tert*-butyl nitrite (0.07 ml) in a mixture of DMF (4 ml) and 6 N HCl/dioxane (0.2 ml) at −20°. The azide solution was neutralized with Et_3N and combined with the solution of the deblocked tridecapeptide described above. The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was dissolved in *n*-BuOH and the *n*-BuOH layer was washed with H_2O and 1% AcOH. After removal of the *n*-BuOH, the residue was dissolved in EtOH (5 ml) and the solution was applied to a Sephadex LH-20 column (3×60 cm). The column was developed with EtOH and fractions of 3 g were collected. Fractions 41—47 which had Rf^1 0.40, were pooled and the solvent was evaporated off. The residue was precipitated from EtOH/AcOEt; yield 250 mg (77%), mp 165—171°, $[\alpha]_D^{22}$ −133.0° (c=1.1, MeOH), Rf^1 0.40, Rf^2 0.71. *Anal.* Calcd for $C_{90}H_{142}N_{22}O_{25}\cdot 3H_2O$: C, 54.4; H, 7.5; N, 15.5. Found: C, 54.1; H, 7.3; N, 15.3. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{0.95}Thr_{0.89}Ser_{0.92}Glu_{1.06}Pro_{6.10}Gly_{0.94}Ile_{1.07}Leu_{3.00}Arg_{0.67}$ (average recovery, excluding Arg. 77%).

Z-Arg(NO$_2$)-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OH——The fragment X (200 mg) was deblocked with TFA (1 ml) in the presence of anisole (0.01 ml) at room temperature in the usual manner; yield 168 mg (87%), hygroscopic powder, $[\alpha]_D^{25}$ −127.0° (c=1.0, DMF), Rf^1 0.21, Ef^2 0.48. *Anal.* Calcd for $C_{86}H_{134}N_{22}O_{25}\cdot 3H_2O$: C, 53.5; H, 7.3; N, 16.0. Found: C, 53.4; H, 7.0; N, 15.8.

H-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OH (I)——Z–Arg(NO$_2$)–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH (145 mg) was hydrogenated over a Pd catalyst in a mixture of MeOH (3 ml) and 10% AcOH in the usual manner. The hydrogenated material was lyophilized from 0.1 N HCl; yield 122 mg (82%), hydroscopic and fluffy powder, $[\alpha]_D^{25}$ −158.7° (c=0.5, H_2O), Rf^1 0.05, Rf^2 0.45. *Anal.* Calcd for $C_{78}H_{129}N_{21}O_{21}\cdot 2HCl\cdot 13H_2O$: C, 47.5; H, 7.9; N, 14.9. Found: C, 47.2; H, 7.6; N, 14.7. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{1.00}Thr_{0.88}Ser_{0.94}Glu_{1.08}Pro_{6.13}Gly_{0.95}Ile_{0.98}Leu_{3.06}Arg_{0.92}$ (average recovery 79%). Amino acid ratios in an aminopeptidase M digest[28,30] (24 hr): $Asp_{0.04}(Asn+Gln+Thr)_{2.52}Ser_{1.22}Glu_{0.73}Pro_{5.99}Gly_{1.00}Ile_{1.07}Leu_{3.08}$ (recovery of Gly 74%).

30) Aminopeptidase M (Pierce, lot No. 08307.33) was suspended in 3.2 M $(NH_4)_2SO_4$. The enzyme had the ability to convert Gln to Glu, and $(NH_4)_2SO_4$ disturbed the analysis of Arg.