Chem. Pharm. Bull. 30(3)1043—1047(1982)

Human Chorionic Gonadotropin. VII.[1,2] Preparation and Immunocharacteristics of Carboxyl-terminal Peptides of the β-Subunit of Human Chorionic Gonadotropin

Koichi Kawasaki,*,[a] Shin Iguchi,[a] Chiye Kawasaki,[a] Mitsuko Maeda,[a] Yoshio Okada,[a] Kenji Yamaji,[b] Tetsu Takagi,[b] Nagatoshi Sugita,[b] and Osamu Tanizawa[b]

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, Tarumi-ku, Kobe, 673, Japan[a] and Osaka University Medical School, Fukushima-ku, Fukushima, Osaka, 553, Japan[b]

(Received August 28, 1981)

Various carboxyl-terminal peptides of hCG-β were prepared from the corresponding blocked peptides by TFA treatment followed by hydrogenation. The prepared peptides were tested for inhibitory action on the binding between ^{125}I-hCG and the antiserum against the carboxyl-terminal portion of hCG-β. The results suggest that an antigenic site of this antiserum may exist around the amino-terminal portion of the carboxyl-terminal hexadecapeptide.

Keywords——human chorionic gonadotropin; β-subunit of hCG; antibody against C-terminal portion of hCG-β; synthesis of C-terminal peptide of hCG-β; antigenic site

In the preceding paper,[1] we reported the synthesis of a carboxyl-terminal peptide corresponding to positions 112—145 of hCG-β and the preparation of an antiserum against the synthetic peptide. We describe here the preparation of various carboxyl-terminal peptides of hCG-β and their inhibitory action on binding between ^{125}I-hCG and the antiserum.

Two different amino acid sequences of the carboxyl-terminal portion of hCG-β were proposed by Carlsen and Bahl *et al.*[3] and Morgan *et al.*[4] as shown in Fig. 1. We synthesized the carboxyl-terminal Asn^{138}-dotriacontapeptide[5] of Carlsen and Bahl and the carboxyl-terminal tetratriacontapeptide[1,6] of Morgan. Later Bahl *et al.*[7] corrected their structure to that of Morgan.

To prepare various carboxyl-terminal peptides, we treated the synthetic intermediates[1,6] to the tetratriacontapeptide of the Morgan structure with TFA to remove the Bu^t groups on Gln (position 145) and Thr (position 140, in fragments II—IV), and the Boc group on Lys (position 122, in fragments I—III) followed by hydrogenation to remove the Z group on the amino-terminal amino acid. Carboxyl-terminal Asn^{138}-peptide derivatives of the Carlsen and Bahl structure were also prepared in the same way. The prepared peptides are shown in Fig. 1.

Since position 138 of the Carlsen and Bahl structure was reported as Asx, we also synthesized an Asp^{138}-decapeptide (XVII) to compare its binding activity to the antiserum with that of Asn^{138}-decapeptide (XIV). The synthetic scheme is shown in Fig. 2. Z–Thr(Bu^t)–OSu[8] was coupled with H–Pro–OH to afford Z–Thr–(Bu^t)Pro–OH, which was hydrogenated over Pd catalyst to remove the Z group. The resulting peptide was coupled with Z–Asp(OBzl)–ONp[9] to give Z–Asp(OBzl)–Thr(Bu^t)–Pro–OH followed by condensation with H–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t [5] by the DPPA method[10] to afford Z–Asp(OBzl)–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t. This decapeptide was treated with 90% TFA followed by hydrogenation to afford XVII.

Competitive binding activities of these peptides in an RIA system with ^{125}I-hCG and the antiserum against I are summarized in Fig. 3. The ordinate indicates moles of unlabeled hCG required to inhibit 50% of ^{125}I-hCG binding to the antiserum per mol of peptide. The abscissa gives the chain length of peptides from the carboxyl-terminus. As shown in the

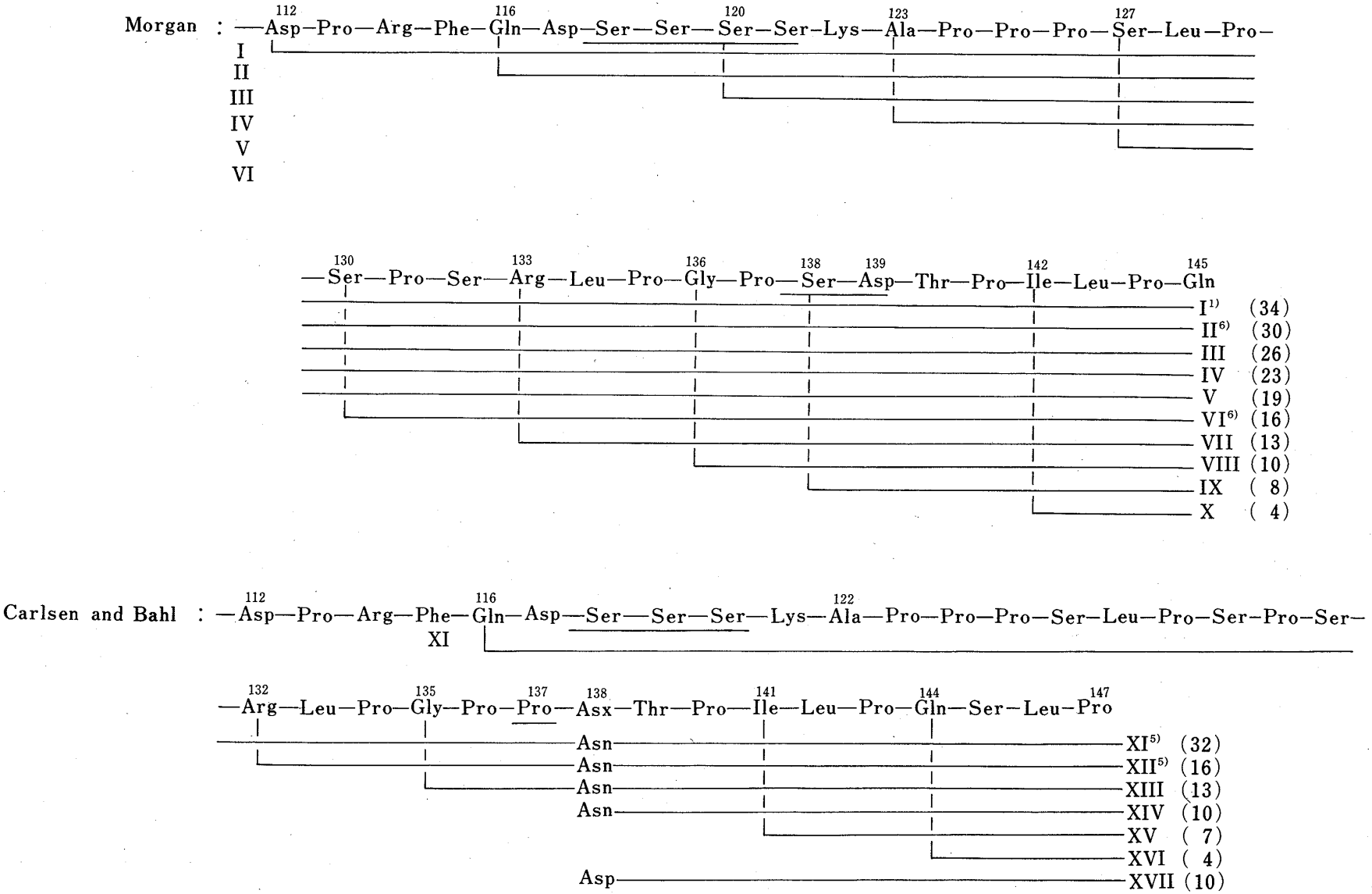

Fig. 1. Comparison of the Carboxyl-Terminal Sequences of hCG-β and Prepared Peptides

Numbers in parentheses indicate numbers of amino acid residues of peptides.

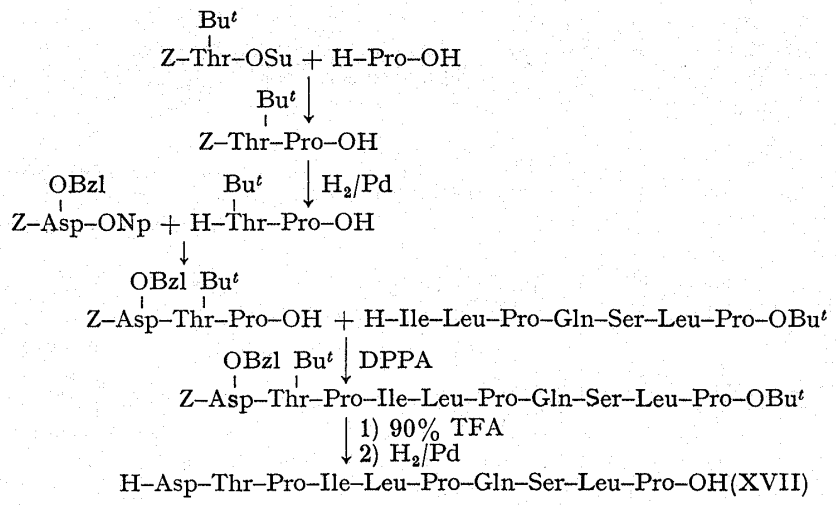

Fig. 2. Synthetic Scheme for XVII

figure, elongation of the chain length from the tetrapeptide to the hexadecapeptide of the Morgan structure significantly increased the cross-reactivity, which reached a plateau after the hexadecapeptide. This result suggests that an antigenic site may exist around the amino-terminal portion of VI. Chen *et al.*[11] reported that Arg–Leu–Pro–Gly-(positions 133—136) was one of the antigenic sites for antiserum against the carboxyl-terminal triacontapeptide (positions 116—145) of hCG-β. The specificities of their antiserum and our antiserum to the carboxyl-terminal peptide of hCG-β are similar.

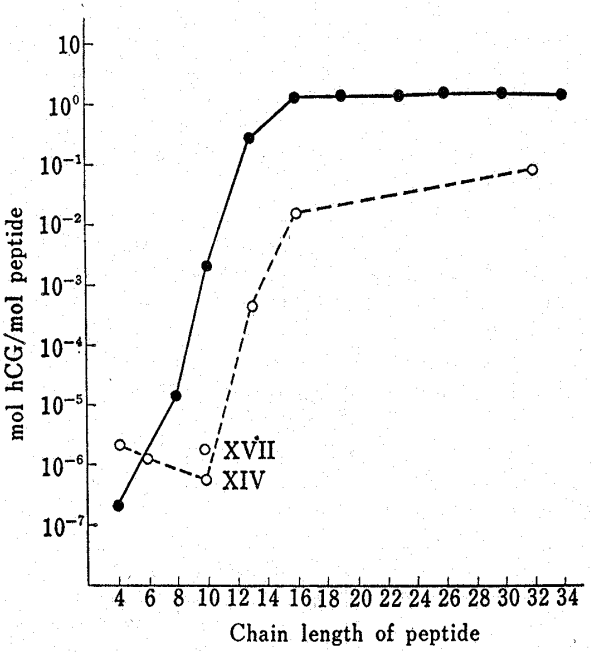

Fig. 3. Competitive Binding Activities of Peptides in an RIA System with ^{125}I-hCG and the Antiserum

●—●: Morgan structure.
○—○: Carlsen and Bahl structure.

The peptides based on the Carlsen and Bahl structure showed that elongation of the chain from the tetrapeptide to the decapeptide did not increase binding activity to the antiserum. However, increasing chain length from the decapeptide to the hexadecapeptide (positions 132—147) significantly increased the cross activity. The 50% intercept-doses are 100-fold larger than those of peptides based on the Morgan structure. The Asp^{138}-decapeptide(XVII) showed slightly stronger competitive binding activity with respect to the antiserum that the Asn^{138}-decapeptide.

Experimental

Melting points are uncorrected. Solvent systems for ascending thin-layer chromatography on silica gel G (type 60, E. Merck) are indicated as follows: Rf^1=*n*-BuOH–AcOH–H_2O (4: 1: 5, upper phase), Rf^2= pyridine–AcOH–*n*-BuOH–H_2O/(1: 1: 4: 2), Rf^3=$CHCl_3$–MeOH–H_2O (8: 3: 1, lower phase). Acid hydrolyses were performed in constant-boiling HCl at 110°C for 24 h in evacuated tubes.

General Procedure for Deblocking of the Blocked Peptides——The blocked peptides[5,6] were treated with 90% TFA or TFA containing anisole at room temperature for 1 h. For Thr(Bu^t)-containing peptides, TFA treatment was performed for 3 h. Ether was added to give a precipitate, which was collected by filtration or

centrifugation. The precipitate was washed with ether and dried. The material was then hydrogenated over Pd catalyst in MeOH in a usual manner. The deblocked material was purified by Sephadex G-25 column chromatography using 5% AcOH as an eluent. Some peptides (XIII—XVII) were converted to their hydrochlorides by adding 1 N HCl followed by lyophilization. Yields in the deblocking procedure were 80—90%.

III: $[\alpha]_D^{28}$ −182.5° (c=0.2, H_2O), Rf^1 0.01, Rf^2 0.17. *Anal.* Calcd for $C_{117}H_{191}O_{37}N_{31}\cdot 2CF_3COOH\cdot HCl\cdot 15H_2O$: C, 46.0; H, 7.1; N, 13.8. Found: C, 45.8; H, 6.9; N, 13.8. Amino acid ratios in an acid hydrolysate: Asp 0.99; Thr 1.08; Ser 5.53; Glu 1.18; Pro 10.47; Gly 1.00; Ala 1.03; Ile 0.89; Leu 3.02; Lys 1.00; Arg 0.92 (average recovery 87%).

IV: $[\alpha]_D^{28}$ −186.1° (c=0.2, H_2O), Rf^1 0.05, Rf^2 0.24. *Anal.* Calcd for $C_{105}H_{169}N_{27}O_{32}\cdot CF_3COOH\cdot HCl\cdot 10H_2O$: C, 48.5; H, 7.3; N, 14.3. Found: C, 48.2; H, 6.9; N, 14.4. Amino acid ratios in acid hydrolysate: Asp 1.08; Thr 1.04; Ser 3.74; Glu 1.21; Pro 10.30; Gly 1.00; Ala 0.98; Ile 1.15; Leu 3.18; Arg 1.07 (average recovery 85%).

V: $[\alpha]_D^{27}$ −162.5° (c=0.2, H_2O), Rf^1 0.09, Rf^2 0.34. *Anal.* Calcd for $C_{89}H_{143}N_{23}O_{28}\cdot CF_3COOH\cdot HCl\cdot 9H_2O$: C, 47.1; H, 7.2; N, 14.2. Found: C, 47.0; H, 6.8; N, 14.5. Amino acid ratios in an acid hydrolysate: Asp 1.04; Thr 1.06; Ser 3.61; Glu 1.05; Pro 6.85; Gly 1.00; Ile 0.99; Leu 3.07; Arg 1.05 (average recovery 83%).

VII: $[\alpha]_D^{27}$ −139.2° (c=0.2, H_2O), Rf^1 0.09, Rf^2 0.36. *Anal.* Calcd for $C_{62}H_{103}N_{17}O_{19}\cdot 2CF_3COOH$: C, 49.0; H, 6.5; N, 14.7. Found: C, 48.7; H, 6.8; N, 15.0. Amino acid ratios in an acid hydrolysate: Asp 0.89; Thr 0.99; Ser 0.95; Glu 1.01; Pro 4.59; Gly 1.00; Ile 1.04; Leu 2.13; Arg 1.19 (average recovery 84%).

VIII: $[\alpha]_D^{27}$ −157.9° (c=0.2, H_2O), Rf^1 0.11, Rf^2 0.25. *Anal.* Calcd for $C_{45}H_{73}N_{11}O_{16}\cdot CF_3COOH\cdot H_2O$: C, 48.3; H, 6.6; N, 13.3. Found: C, 48.6; H, 6.9; N, 13.7. Amino acid ratios in an acid hydrolysate: Asp 1.03; Thr 1.01; Ser 0.96; Glu 0.94; Pro 3.18; Gly 1.00; Ile 0.97; Leu 0.99 (average recovery 95%).

IX: $[\alpha]_D^{28}$ −134.1° (c=0.2, H_2O), Rf^1 0.19, Rf^2 0.26. *Anal.* Calcd for $C_{38}H_{63}N_9O_{14}\cdot CF_3COOH$: C, 48.8; H, 6.5; N, 12.8. Found: C, 48.4; H, 6.9; N, 13.1. Amino acid ratios in an acid hydrolysate: Asp 1.07; Thr 1.04; Ser 0.91; Glu 1.00; Pro 2.14; Ile 0.93; Leu 0.95 (average recovery 90%).

X: $[\alpha]_D^{27}$ −63.3° (c=0.2, H_2O), Rf^1 0.35, Rf^2 0.53. *Anal.* Calcd for $C_{22}H_{39}N_5O_6\cdot CF_3COOH\cdot 2H_2O$: C, 46.5; H, 7.2; N, 11.3. Found: C, 46.9; H, 6.9; N, 11.4. Amino acid ratios in acid hydrolysate: Glu 1.00; Pro 1.16; Ile 0.95; Leu 0.96 (average recovery 87%).

XIII: $[\alpha]_D^{23}$ −187.9° (c=1.0, H_2O), Rf^1 0.02, Rf^2 0.37. *Anal.* Calcd for $C_{61}H_{99}H_{15}O_{18}\cdot HCl\cdot 4H_2O$: C, 50.9; H, 7.6; N, 14.6. Found: C, 51.2; H, 7.8; N, 14.2. Amino acid ratios in an acid hydrolysate: Gly 1.00; Pro 5.30; Asp 1.00; Thr 0.96; Ile 1.04; Leu 1.98; Glu 0.99; Ser 0.91 (average recovery 84%).

XIV: $[\alpha]_D^{23}$ −157.5° (c=0.5, H_2O), Rf^1 0.10, Rf^2 0.53. *Anal.* Calcd for $C_{49}H_{82}N_{12}O_{15}\cdot HCl\cdot 3H_2O$: C, 50.3; H, 7.7; N, 14.4. Found: C, 50.5; H, 7.6; N, 14.1. Amino acid ratios in an acid hydrolysate: Asp 0.99; Thr 0.94; Pro 3.14; Ile 1.00, Leu 1.95; Glu 1.01; Ser 0.91 (average recovery 88%).

XV: $[\alpha]_D^{23}$ −118.4° (c=0.5, H_2O), Rf^1 0.35, Rf^2 0.64. *Anal.* Calcd for $C_{36}H_{62}N_8O_{10}\cdot HCl\cdot 2H_2O$: C, 51.5; H, 8.0; N, 13.4. Found: C, 51.6; H, 7.9; N, 13.3. Amino acid ratios in an acid hydrolysate: Ile 1.00; Leu 1.89; Pro 2.05; Glu 0.99; Ser 0.90 (average recovery 86%).

XVI: $[\alpha]_D^{23}$ −75.5° (c=0.5, H_2O), Rf^1 0.32, Rf^2 0.62. *Anal.* Calcd for $C_{19}H_{33}N_5O_7\cdot HCl\cdot 1/2H_2O$: C, 46.2; H, 7.1; N, 14.2. Found: C, 45.9; H, 7.4; N, 14.4. Amino acid ratios in an acid hydrolysate: Glu 1.00; Ser 0.91; Leu 1.06; Pro 1.07 (average recovery 81%).

XVII: $[\alpha]_D^{23}$ −160.6° (c=1.0, H_2O), Rf^1 0.12, Rf^2 0.55. *Anal.* Calcd for $C_{49}H_{81}N_{11}O_{16}\cdot HCl\cdot 2H_2O$: C, 51.1; H, 7.5; N, 13.4. Found: C, 51.1; H, 7.4; N, 13.0. Amino acid ratios in an acid hydrolysate: Asp 0.99; Thr 0.98; Pro 3.35; Ile 1.00, Leu 2.01; Glu 1.03; Ser 0.91 (average recovery 90%).

Z-Thr(But)-Pro-OH——Z–Thr(But)–OSu[8] (500 mg) dissolved in dioxane (2 ml) was added to a solution of H–Pro–OH (172 mg) in a mixture of H_2O (0.7 ml) and Et_3N (0.2 ml) and the whole was stirred at room temperature for 30 h. The solvent was evaporated off and the residue was extracted with 5% $NaHCO_3$. The aqueous layer was washed three times with AcOEt and acidified with citric acid. The resulting precipitate was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated down. The residue was precipitated from AcOEt/petro.ether; yield 412 mg (85%), mp 89°C, $[\alpha]_D^{23}$ −45.6° (c=0.5, MeOH), Rf^3 0.49. *Anal.* Calcd for $C_{21}H_{30}N_2O_6$: C, 62.1; H, 7.4; N, 6.9. Found: C, 62.0; H, 7.6; N, 6.6. Amino acid ratios in an acid hydrolysate: Thr 1.00; Pro 1.14 (average recovery 84%).

Z-Asp(OBzl)-Thr(But)-Pro-OH——H–Thr(But)–Pro–OH (prepared from 400 mg of the Z derivative by hydrogenation over Pd catalyst in a usual manner) dissolved in a mixture of DMF (4 ml) and Et_3N (0.14 ml) was combined with Z–Asp(OBzl)–ONp[9] (574 mg) and the whole was stirred at room temperature for 20 h. The solvent was evaporated off and the residue was extracted with 5% $NaHCO_3$. The aqueous layer was washed three times with AcOEt and acidified with citric acid. The resulting precipitate was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated down. The residue was dissolved in $CHCl_3$ and the solution was applied to a silica gel column (2×15 cm). The desired material was eluted with 3% MeOH/$CHCl_3$ and the solvent was evaporated off. The residue was dissolved in AcOEt and the solution was washed with H_2O, dried over Na_2SO_4 and evaporated down. The resulting residue was precipitated from AcOEt/petro.ether to give an amorphous powder; $[\alpha]_D^{22}$ −24.9° (c=1.0, MeOH), Rf^3 0.79. *Anal.* Calcd for $C_{32}H_{41}N_3O_9$: C, 62.8; H, 6.8; N, 6.9. Found: C, 62.5; H, 6.9; N, 6.6. Amino acid ratios in

an acid hydrolysate: Asp 1.00; Thr 0.96; Pro 0.99 (average recovery 88%).

Z-Asp(OBzl)-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t——DPPA (0.1 ml) was added to a solution of Z–Asp(Bzl)–Thr(Bu^t)–Pro–OH (294 mg) in a mixture of Et_3N (0.07 ml) and THF (4 ml) at −10°C, and the whole was stirred for 20 min. The mixture was then combined with a solution of H–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (prepared from 400 mg of the Z derivative by hydrogenation over Pd catalyst) in DMF (4 ml) and stirred for 48 h in a cold room. The solvent was evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed successively with H_2O, 5% citric acid, 5% Na_2CO_3 and H_2O, and evaporated down after being dried over Na_2SO_4. The residue was dissolved in MeOH and the solution was applied to a Sephadex LH-20 column (3×80 cm) equilibrated with MeOH. The column was developed with MeOH and fractions of 7 g were collected. Fractions 30—35 were pooled and evaporated down. The residue was precipitated from EtOH/ether; yield 282 mg (47%), amorphous powder, $[\alpha]_D^{23}$ −99.1° (c=1.0, MeOH), Rf^1 0.79. *Anal.* Calcd for $C_{72}H_{109}N_{11}O_{18}$: C, 61.0; H, 7.8; N, 10.9. Found: C, 60.8; H, 7.8; N, 10.8. Amino acid ratios in an acid hydrolysate: Asp 1.03; Thr 0.92; Pro 3.29; Ile 1.00; Leu 1.96; Glu 0.98; Ser 0.90 (average recovery 88%).

Measurement of the competitive activities of prepared peptides in an RIA system with ^{125}I-hCG and the antiserum was performed according to a procedure reported by Matsuura *et al.*[12] The antiserum used in this experiment was CTP-34, which was reported in previous papers.[1,6]

References and Notes

1) Part VI: Y. Okada, K. Kawasaki, S. Iguchi, M. Yagyu, K. Yamaji, T. Takagi, N. Sugita, and O. Tanizawa, *Chem. Pharm. Bull.*, **29**, 2851 (1981).
2) Amino acids and peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used in this paper are: hCG-β=β-subunit of human chorionic gonadotropin, DMF=dimethylformamide, DPPA=diphenylphosphoryl azide, –OSu=*N*-hydroxysuccinimide ester, –OBu^t=*tert*-butyl ester, –OBzl=benzyl ester, Z=benzyloxycarbonyl, TFA=trifluoroacetic acid, THF=tetrahydrofuran, Boc=*tert*-butoxycarbonyl.
3) K.B. Carlsen, O.B. Bahl, and N. Swaminathan, *J. Biol. Chem.*, **248**, 9810 (1973).
4) F.J. Morgan, S. Birkin, and R.E. Canfield, *J. Biol. Chem.*, **250**, 5247 (1975).
5) K. Kawasaki, C. Kawasaki, Y. Tsuda, M. Maeda, Y. Okada, K. Yamaji, T. Takagi, and O. Tanizawa, *Chem. Pharm. Bull.*, **28**, 2692 (1980); K. Kawasaki, C. Kawasaki, Y. Tsuda, M. Yagyu, Y. Okada, K. Yamaji, T. Takagi, and O. Tanizawa, *ibid*, **28**, 2699 (1980).
6) Y. Okada, S. Iguchi, M. Mimura, K. Kawasaki, K. Yamaji, T. Takagi, and O. Tanizawa, *Chem. Pharm. Bull.*, **28**, 2707 (1980); Y. Okada, S. Iguchi, M. Yagyu, K. Kawasaki, K. Yamaji, T. Takagi, and O. Tanizawa, *ibid.*, **28**, 2714 (1980); Y. Okada, K. Kawasaki, S. Iguchi, M. Yagyu, K. Yamaji, T. Takagi, and O. Tanizawa, *J. Chem. Soc. Chem. Comm.*, **1981**, 357.
7) M.J. Kessler, T. Mise, R.D. Ghai, and O.P. Bahl, *J. Biol. Chem.*, **254**, 7909 (1979).
8) K.B. Mathur, H. Klostermeyer, and H. Zahn, *Z. Physiol. Chem.*, **346**, 60 (1966).
9) S. Guttman, *Helv. Chim. Acta*, **44**, 721 (1961).
10) T. Shioiri and S. Yamada, *Chem. Pharm. Bull.*, **22**, 849 (1974).
11) H.-A. Chen, S. Matsuura, and M. Ohashi, "Chorionic Gonadotropin," Proceedings of the conference on human chorionic gonadotropin ed. by S.G. Seel, Plenum Press, New York 1980, p. 231.
12) S. Matsuura, M. Ohashi, H.-C. Chen, and G.D. Hodgen, *Endocrinol.*, **104**, 396 (1979).