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Human Chorionic Gonadotropin. VI.[1,2] Synthesis of a Tetratriacontapeptide corresponding to the C-Terminal Sequence 112—145 of the β-Subunit of Human Chorionic Gonadotropin (hCG)

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The tetratriacontapeptide corresponding to sequence 112—145 of the β-subunit of human chorionic gonadotropin (hCG) was synthesized by assembling peptide fragments by the azide procedure, followed by TFA treatment and catalytic hydrogenation. This peptide was conjugated with bovine serum albumin (BSA). New Zealand white rabbits were immunized with the conjugated antigen in Freund's complete adjuvant. Antiserum capable of 30% specific binding of ^{125}I-hCG or ^{125}I-β-subunit of hCG at a dilution of 1:5000 was produced.

Keywords——human chorionic gonadotropin; β-subunit; C-terminal tetratriacontapeptide; chemical synthesis; solution method; antiserum; specific binding with hCG; 1:5000 dilution.

The antibody to the tail portion of the β-subunit of human chorionic gonadotropin (hCG) is expected to be specific to hCG and to offer an excellent tool for the discrimination of hCG from other human glycoprotein hormones, because the unique C-terminal portion of hCGβ[3–5] (approximately 30 amino acid residues) is not present in β-subunits of other human glycoprotein hormones (human luteinizing hormone, hLH,[6] human follicle-stimulating hormone, hFSH,[7] and human thyroid-stimulating hormone, hTSH[8]). Fig. 1 shows the amino acid sequences of the C-terminal regions of hCGβ and hLHβ. To date, syntheses of C-terminal peptides of the β-subunit of hCG by the solid phase method[9,10] and the solution method[11,12] and the production of antisera against those synthetic peptide antigens have been described. A program has been initiated in our laboratories directed to the synthesis of C-terminal peptides of hCGβ and to the production of such antisera as described above. This report deals with the synthesis of a tetratriacontapeptide (I) corresponding to sequence 112—145 of the β-subunit of hCG, as well as its immunological properties.

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        110                                120
hCGβ   –Cys–Asp–Asp–Pro–Arg–Phe–Gln–Asp–Ser–Ser–Ser–Ser–Lys–Ala–Pro–Pro–Pro–Ser–
                            112
hLHβ   –Cys–Asp–His–Pro–Gln–OH
             130                                    140                 145
hCGβ   Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr–Pro–Ile–Leu–Pro–Gln–OH
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Fig. 1. Amino Acid Sequences of the C-Terminal Region of hCGβ and hLHβ

As illustrated in Fig. 2, starting with the C-terminal hexadecapeptide amine (II),[1] seven peptide fragments were coupled successively by the azide procedure[13] in order to minimize racemization and to avoid the need for the protection of side chain functional groups of amino acid residues as far as possible during the synthesis. The α-amino function of amino acids was protected by the Z group. The Bzl group of the β-carboxyl function of aspartic acid and the NO_2 group of arginine were removed by catalytic hydrogenation over palladium immedi-

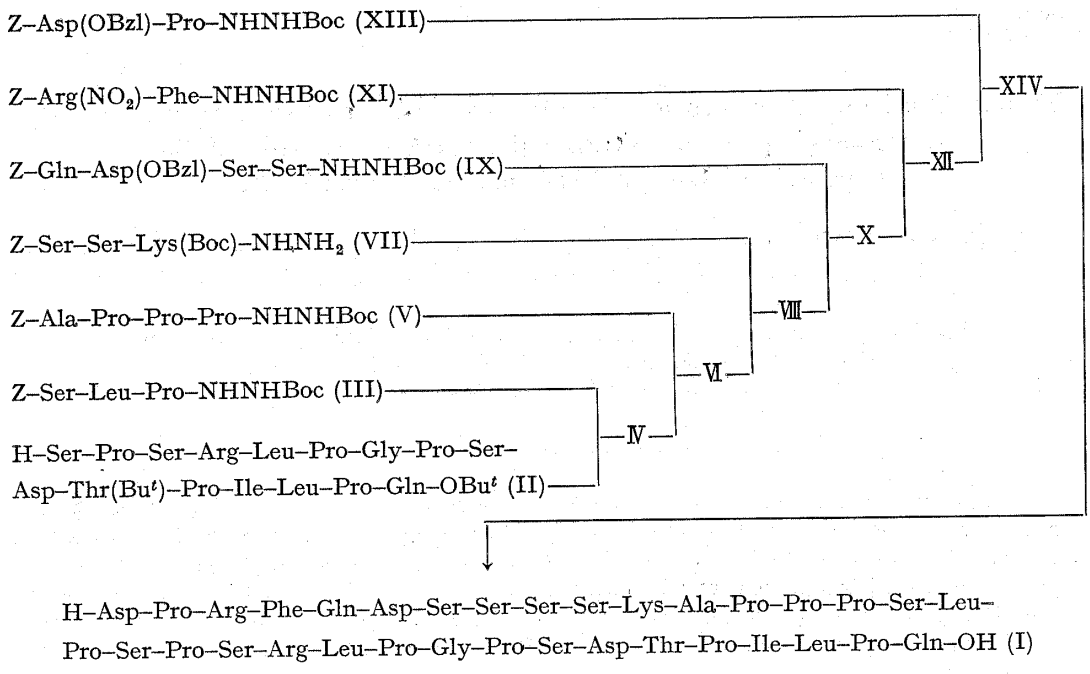

Fig. 2. Synthetic Scheme for the Tetratriacontapeptide (I)

ately after their introduction. The regenerated guanidino group was protected as its hydrochloride during the synthesis.[14] Hydroxyl groups of serine residues were not protected. The carboxyl group of the C-terminal glutamine residue and the hydroxyl group of the sole threonine residue were protected by *t*-butyl ester and *t*-butyl ether, respectively, and the *ε*-amino group of lysine was protected by *t*-butyloxycarbonyl (Boc) during the synthesis.

The protected tripeptide, Z–Ser–Leu–Pro–NHNHBoc (III) was synthesized by coupling of H–Leu–Pro–NHNHBoc[1] and Z–Ser–$NHNH_2$[15] by the azide method. Z–Arg(NO_2)–OH and H–Phe–NHNHBoc[16] were coupled by the DCC–HOBt method[17] to afford the peptide fragment, Z–Arg(NO_2)–Phe–NHNHBoc (XI). Z–Asp(OBzl)–Pro–NHNHBoc (XIII) was prepared from Z–Asp(OBzl)–ONp and H–Pro–NHNHBoc.[18]

As shown in Fig. 2, the seven peptide fragments were assembled by the azide method. H–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(But)–Pro–Ile–Leu–Pro–Gln–OBut (II) was coupled with Z–Ser–Leu–Pro–N_3 prepared from III by treatment with trifluoroacetic acid (TFA) followed by isopentylnitrite to give Z–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(But)–Pro–Ile–Leu–Pro–Gln–OBut (IV). IV was purified by gel-filtration on Sephadex LH-20 using EtOH as an eluent. After removal of the Z-group from IV by catalytic hydrogenation, the resulting amine was coupled with Z–Ala–Pro–Pro–Pro–NHNHBoc (V),[1] Z–Ser–Ser–Lys(Boc)–$NHNH_2$ (VII)[1] and Z–Gln–Asp(OBzl)–Ser–Ser–NHNHBoc (IX)[1] suc-cessively by the azide method to give Z–Gln–Asp(OBzl)–Ser–Ser–Ser–Ser–Lys(Boc)–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(But)–Pro–Ile–Leu–Pro–Gln–OBut (X)[1] in the same manner as described previously.[1] X was hydrogenated over a palladium catalyst and the resulting amine was coupled with Z–Arg(NO_2)–Phe–N_3, prepared from XI by treatment with TFA followed by isopentylnitrite, to afford Z–Arg(NO_2)–Phe–Gln–Asp–Ser–Ser–Ser–Ser–Lys(Boc)–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(But)–Pro–Ile–Leu–Pro–Gln–OBut (XII). XII was purified by gel-filtr-ation on Sephadex LH-20 using DMF as an eluent and by silica gel column chromatography with *n*-butanol, AcOH and H_2O (4: 1: 5, upper phase). XII was converted to the corresponding amine by catalytic hydrogenation and coupled with Z–Asp(OBzl)–Pro–NHNHBoc (XIII) by the azide procedure to afford a protected tetratriacontapeptide, Z–Asp(OBzl)–Pro–Arg–Phe–Gln–Asp–Ser–Ser–Ser–Ser–Lys(Boc)–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–

Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (XIV), which was purified by gel-filtration on Sephadex LH-20 and silica gel column chromatography in the same way as described above. XIV was treated with TFA containing anisole at room temperature for 3 h, hydrogenated over a palladium catalyst and purified by gel-filtration on Sephadex G-25 using 5% AcOH as an eluent. The location of the eluted material was detected by the method of Lowry *et al.*[19] The desired peptide was converted to the corresponding hydrochloride by lyophilization from water containing 0.1 N HCl. The purified product thus obtained was homogeneous upon thin-layer chromatography on silica gel. Its purity was further assessed by amino acid analysis of the 6 N HCl hydrolysate and the aminopeptidase (AP-M) digest.[20] In the latter case, AP-M digested the peptide (I) reasonably well, although I contains 10 proline residues.[21] Amino acid analyses of both hydrolysates gave molar ratios in good agreement with the expected values.

I was conjugated with bovine serum albumin (BSA) in the usual manner.[22] New Zealand white rabbits were immunized with the conjugated antigen in Freund's complete adjuvant by multiple intra-dermal and intra-spleen injections. Eight weeks after the first immunization, antiserum capable of 30% binding of ^{125}I-hCG or ^{125}I-β-subunit of hCG at a dilution of 1:5000 was produced. These values were similar to those of antiserum against a synthetic peptide (residues 116—145 of hCGβ) reported by Matsuura *et al.*[10] (30% binding of ^{125}I-hCG at a dilution of $1:4\times10^3$). These bindings were inhibited by hCG as well as by the peptide (I) prepared above in a dose-dependent manner and at least 500 mIU of hCG was needed for the inhibition reaction. The cross-reactivity of the antiserum with α-subunit of hCG or hLH was negligible. Thus, the antiserum against the peptide (I) was proved to be specific to hCG, as expected. Furthermore, this antiserum did not inhibit the biological activity of hCG in a mouse uterine weight bioassey. This finding is consistent with the fact that the antiserum described above[10] did not neutralize the biological activity of hCG *in vivo*,[10] although Stevens[23] reported that antiserum against a synthetic peptide (residues 111—145 of hCGβ) inhibited the biological activity of CGs from man and baboon in the same test.

Experimental

Melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). The amino acid composition of the acid hydrolysate (6 N HCl, 110°C, 20 h) and the aminopeptidase M digest were determined with a JEOL JLC-6AH amino acid analyzer (one-column system). Solvents were evaporated off *in vacuo* at a temperature of 40°C, Solvent systems for ascending thin-layer chromatography on silica gel G (type 60, Merck) are indicated as follows: Rf^1=$CHCl_3$, MeOH and AcOH (90:8:2), Rf^2=$CHCl_3$, MeOH and H_2O (8:3:1, lower phase), Rf^3=*n*-butanol, pyridine, AcOH and H_2O (30:20:6:24).

Z-Ser-Leu-Pro-NHNHBoc (III)——To a solution of H–Leu–Pro–NHNHBoc (prepared from 4.5 g of Z–Leu–Pro–NHNHBoc by catalytic hydrogenation) in DMF (20 ml), Z–Ser–N_3 prepared as follows was added. A solution of 6 N HCl in dioxane (3.0 ml) followed by isopentylnitrite (1.32 ml) was added to a solution of Z–Ser–$NHNH_2$ (2.4 g) in DMF (23 ml) cooled to −20°C. After 5 min, triethylamine (2.7 ml) was added. The solution was poured into the solution of amino component in DMF prepared above. The reaction mixture was stirred in a cold room (4°C) for 48 h. The solvent was removed by evaporation and the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a solid material. The product was dissolved in a small amount of $CHCl_3$ and applied to a silica gel column (2×20 cm). Elution was carried out with $CHCl_3$ (1000 ml) and then 2% MeOH in $CHCl_3$ (1500 ml). The latter effluent was collected and evaporated to dryness. Petroleum ether was added to the residue to afford a solid mass, yield 4.6 g (86%), mp 87—93°C, $[\alpha]_D^{23}$ −117.0° (c=1.0, MeOH), Rf^1 0.46, Rf^2 0.82. *Anal.* Calcd for $C_{27}H_{41}N_5O_8$: C, 57.5; H, 7.33; N, 12.4. Found: C, 57.4; H, 7.36; N, 12.3. Amino acid ratios in an acid hydrolysate: Ser 0.95; Pro 1.00; Leu 1.07 (average recovery 87%).

Z-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (IV)—— Z–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Ser–Asp–Thr(Bu^t)–Pro–Ile–Leu–Pro–Gln–OBu^t (2.08 g) was dissolved in EtOH (30 ml) and 5% aqueous AcOH (20 ml) and hydrogenated over a palladium catalyst to furnish

the corresponding amine (II). This amine was dissolved in DMF (10 ml) and the pH of the solution was adjusted to 8 with triethylamine (0.3 ml). To this cold solution, Z–Ser–Leu–Pro–N_3 prepared as follows was added. A solution of III (2.4 g) in TFA (4 ml) containing anisole (0.2 ml) was kept at room temperature for 30 min. Ether was added to form a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*. This hydrazide was dissolved in DMF (10 ml) and the solution was cooled to −20°C, then 7.5 N HCl in dioxane (1.12 ml) followed by isopentylnitrite (0.59 ml) was added. After stirring for 5 min, the pH of the solution was adjusted to 8 with triethylamine (1.18 ml) and this azide solution was poured into the DMF solution of II. The reaction mixture was stirred for 48 h in a cold room (4°C). 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 to give a white precipitate. This precipitate was dissolved in EtOH (3 ml) and the solution was applied to a column of Sephadex LH-20, which was eluted with the same solvent. Individual fractions (6 g each) were collected. The desired fractions (Rf^2 0.29, tube Nos. 35—45) were combined and the solvent was removed by evaporation. Ether was added to the residue to yield a white precipitate, which was collected by filtration, washed with ether and dried, yield 1.85 g (76%), mp 170—178°C, $[\alpha]_D^{27}$ −117.8° (c=0.3, MeOH), Rf^2 0.29. *Anal.* Calcd for $C_{103}H_{165}N_{23}O_{30}\cdot HCl\cdot 4H_2O$: C, 53.5; H, 7.58; N, 13.9. Found: C, 53.2; H, 7.61; N, 13.6. Amino acid ratios in an acid hydrolysate: Asp 0.99; Thr 0.99; Ser 3.55; Glu 1.07; Pro 6.61; Gly 1.00; Ile 0.93; Leu 2.91; NH_3 0.91; Arg 0.97 (average recovery 82.4%).

Z-Arg(NO_2)-Phe-NHNHBoc (XI)——H–Phe–NHNHBoc (prepared from 2.0 g of Z–Phe–NHNHBoc by catalytic hydrogenation) and Z–Arg(NO_2)–OH (1.7 g) were dissolved in DMF (20 ml). DCC (1.2 g) and HOBt (0.9 g) were added to the cold DMF solution prepared above. The reaction mixture was stirred in a cold room overnight. After removal of the urea derivative and the solvent, the residue was dissolved in AcOEt. This solution was washed with 5% aqueous Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated to dryness. Ether was added to the residue to afford a solid mass. It was purified by silica gel column chromatography using 3% MeOH in $CHCl_3$ as an eluent, yield 1.6 g (44.5%), mp 120—125°C, $[\alpha]_D^{27}$ −28.9° (c=1.0, MeOH), Rf^1 0.22. *Anal.* Calcd for $C_{28}H_{38}N_8O_8$: C, 54.7; H, 6.23; N, 18.8. Found: C, 54.5; H, 6.23; N, 18.5.

Z-Arg(NO_2)-Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys(Boc)-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (XII)——The protected triacontapeptide (X)[1] (300 mg) was dissolved in EtOH (10 ml) and 5% aqueous AcOH (10 ml) and hydrogenated over a palladium catalyst. The resulting amine was dissolved in DMF (5 ml) and the pH of the solution was adjusted to 8 with triethylamine. Z–Arg(NO_2)–Phe–N_3 prepared as follows was added to the cold DMF solution prepared above. A solution of XI (316 mg) in TFA (0.5 ml) containing anisole (0.05 ml) was kept at room temperature for 40 min. Ether was added to the solution to afford a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*. This hydrazide was dissolved in DMF (4 ml) and cooled to −20°, then 6.4 N HCl in dioxane (0.16 ml) followed by isopentylnitrite (0.07 ml) was added. After 5 min, the pH of the solution was adjusted to 8 with triethylamine (0.15 ml) and this solution was mixed with the cold DMF solution of the triacontapeptide amine. This reaction mixture was stirred in a cold room for 48 h. The solution was applied to a column of Sephadex LH-20 (1.5×180 cm), equilibrated and eluted with DMF. Individual fractions (3 g each) were collected. The desired fractions (tube Nos. 25—30) were combined and evaporated to dryness. Ether was added to the residue to afford a white precipitate, which was further purified by chromatography on a silica gel column (1.3×35 cm), equilibrated and eluted with *n*-butanol, AcOH and H_2O (4: 1: 5, upper phase). The desired fractions (2 g each, tube Nos. 14—33) were collected and the solvent was removed by evaporation. Ether was added to the residue to afford a white precipitate, which was collected by centrifugation, yield 287 mg (88%), mp 187°C (dec.) with sintering at 160°C, $[\alpha]_D^{27}$ −63.0° (c=0.2, DMF), Rf^2 0.38. *Anal.* Calcd for $C_{175}H_{270}N_{42}O_{54}\cdot HCl\cdot 4H_2O$: C, 53.4; H, 7.15; N, 15.0. Found: C, 53.5; H, 7.32; N, 15.4. Amino acid ratios in an acid hydrolysate: Asp 2.03; Thr 0.95; Ser 7.77; Glu 2.17; Pro 8.96; Gly 1.00; Ala 1.01; Ile 0.93; Leu 2.96; Phe 1.20; Lys 1.05; NH_3 2.30; Arg 1.84 (average recovery 77.4%).

Z-Asp(OBzl)-Pro-NHNHBoc (XIII)——Z–Asp(OBzl)–ONp (4.8 g) and H–Pro–NHNHBoc (2.3 g) were dissolved in dioxane (22 ml) containing triethylamine (1.4 ml). The solution was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in AcOEt. The extract was washed with 10% citric acid, 5% aqueous Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Petroleum ether was added to the residue to give an amorphous powder, yield 4.45 g (78%), $[\alpha]_D^{27}$ −78.9° (c=1.0, MeOH), Rf^1 0.49, Rf^2 0.79. *Anal.* Calcd for $C_{29}H_{36}N_4O_8$: C, 61.3; H, 6.38; N, 9.9. Found: C, 61.2; H, 6.60; N, 9.6.

Z-Asp(OBzl)-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys(Boc)-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr(Bu^t)-Pro-Ile-Leu-Pro-Gln-OBu^t (XIV)——XII (250 mg) was dissolved in EtOH (10 ml) and 5% AcOH (10 ml) and hydrogenated over a palladium catalyst. After removal of the palladium and the solvent, the residue was lyophilized from water containing 0.1 N HCl (0.19 ml) to afford the dotriacontapeptide amine trihydrochloride. This peptide was dissolved in DMF (3 ml) and the pH of the solution was adjusted to 8 with triethylamine. Z–Asp(OBzl)–Pro–N_3 prepared as follows was added to the cold DMF solution of amino component described above. XIII (183 mg) was dissolved in 6.4 N HCl in dioxane (0.21 ml). After 5 min, dioxane (0.2 ml) was added and the solution was kept at room temperature for 30

min, then diluted with DMF (5 ml). The solution was cooled to −20°, and isopentylnitrite (0.045 ml) was added. After 5 min, the pH of the solution was adjusted to 8 with triethylamine and the solution was combined with the cold DMF solution of the dotriacontapeptide amine. The reaction mixture was stirred in a cold room for 48 h and applied to a column of Sephadex LH-20 (1.8×190 cm), equilibrated and eluted with DMF. Individual fractions (3 g each) were collected and the desired fractions (tube Nos. 23—29) were combined and evaporated to dryness. Ether was added to the residue to afford a precipitate, which was collected by centrifugation. It was further purified by silica gel column chromatography using *n*-butanol, AcOH and H_2O (4: 1: 5, upper phase) as an eluent in the same manner as described previously, yield, 226 mg (82%), mp 191°C (dec.) with sintering at 178°C, $[\alpha]_D^{27}$ −69.5° (c=0.2, DMF), Rf^2 0.19. *Anal.* Calcd for $C_{191}H_{289}N_{43}O_{56}\cdot 2HCl\cdot 11H_2O$: C, 52.7; H, 7.25; N, 13.8. Found: C, 52.4; H, 7.20; N, 13.8. Amino acid ratios in an acid hydrolysate: Asp 3.20; Thr 1.02; Ser 7.41; Glu 2.12; Pro 10.38; Gly 1.00; Ala 1.03; Ile 0.93; Leu 2.89; Phe 1.16; Lys 1.08; NH_3 2.94; Arg 1.95 (average recovery 70%).

H-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Ser-Asp-Thr-Pro-Ile-Leu-Pro-Gln-OH (I)——A solution of XIV (150 mg) in TFA (3 ml) containing anisole (0.5 ml) was kept at room temperature for 3 h. Ether was added to the solution to give a white precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets *in vacuo*. The partially protected peptide in EtOH (7 ml) and H_2O (5 ml) was hydrogenated over a palladium catalyst. After 8 h, the palladium and the solvent were removed and the residue was lyophilized from water. The peptide in 5% AcOH (2 ml) was applied to a column of Sephadex G-25, equilibrated and eluted with 5% AcOH. Individual fractions (3 g each) were collected and the eluted material was detected by the method of Lowry *et al.* The desired fractions (tube Nos. 67—75) were combined, concentrated and lyophilized. This purification procedure was repeated to yield a fluffy powder (95 mg, 68%), which was converted to the corresponding tetrahydrochloride by lyophilization from water containing 0.1 N HCl (1.0 ml), $[\alpha]_D^{27}$ −153.8° (c=0.2, H_2O), Rf^3 0.27. *Anal.* Calcd for $C_{156}H_{247}H_{43}O_{52}\cdot 4HCl\cdot 20H_2O$: C, 46.1; H, 7.22; N, 14.8. Found: C, 46.0; H, 6.97; N, 14.4. Amino acid ratios in an acid hydrolysate: Asp 2.83; Thr 0.95; Ser 6.46; Glu 2.03; Pro 10.14; Gly 1.00; Ala 1.00; Ile 0.92; Leu 2.80; Phe 1.03; Lys 1.06; NH_3 2.50; Arg 1.93 (average recovery 87%); amino acid ratios in an AP-M[24] digest: Asp 2.67; Thr+Gln 2.20; Ser 6.67; Glu 0.96; Pro 8.85; Gly 1.00; Ala 0.86; Ile 0.97; Leu 2.80; Phe 1.01; Lys 0.85 (average recovery 91%). (Arg was not determined; Gln emerged at the same position as Thr, and was calculated as Thr).

References and Notes

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