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Human Chorionic Gonadotropin. III.[1,2] Synthesis of a Dotriacontapeptide corresponding to the C-Terminal Sequence 116—147 of the β-Subunit of Human Chorionic Gonadotropin (hCG)

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A dotriacontapeptide, H–Gln–Asp–Ser–Ser–Ser–Lys–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH, corresponding to the C-terminal sequence 116—147 of hCG-β proposed by Carlsen *et al.* was synthesized by stepwise fragment condensation, linking five fragments [Z–Gln–Asp(OBzl)–OH, Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–$NHNH_2$, Z–Ala–Pro–Pro–Pro–NHNHBoc, Z–Ser(Bzl)–Leu–Pro–NHNHBoc, and Z–Ser(Bzl)–Pro–Ser–$NHNH_2$] to the hexadecapeptide reported in the preceding paper.

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

We describe here the synthesis of a dotriacontapeptide, H–Gln–Asp–Ser–Ser–Ser–Lys–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH (I), corresponding to the carboxyl-terminal sequence 116—147 of the β-subunit of human chorionic gonadotropin(hCG-β) proposed by Carlsen *et al.*[4] In the preceding paper,[1] we reported the synthesis of a hexadecapeptide, Z–Arg(NO_2)–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (II), corresponding to the sequence 132—147 of hCG-β proposed by Carlsen *et al.*[4] We used the hexadecapeptide II as the starting material for construction of the dotriacontapeptide I. An outline for the synthesis of I is shown in Fig. 1.

The principle for the synthesis of I was the same as that of the synthesis of II reported in the preceding paper.[1] The peptide I was constructed by stepwise fragment condensations using the azide procedure[5] or the DPPA procedure,[6] linking five fragments [Z–Ser(Bzl)–Pro–Ser–$NHNH_2$ (III), Z–Ser(Bzl)–Leu–Pro–NHNHBoc (IV), Z–Ala–Pro–Pro–Pro–NHNHBoc (V), Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–$NHNH_2$ (VI), and Z–Gln–Asp(OBzl)–OH (VII)] to the hexadecapeptide II. α-Amino groups of acylating amino acids and peptides were protected with the

1) Part II: K. Kawasaki, C. Kawasaki, Y. Tsuda, M. Maeda, Y. Okada, K. Yamaji, T. Takagi, and O. Tanizawa, *Chem. Pharm. Bull.*, **28**, 2692 (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, DMSO=dimethylsulfoxide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, DPPA=diphenylphosphoryl azide.
3) Location: *a) Ikawadani-cho, Tarumi-ku, Kobe, 673, Japan; b) Fukushima, Fukushima-ku, Osaka, 553, Japan.*
4) K.B. Carlsen, O.P. Bahl, and N. Swaminathan, *J. Biol. Chem.*, **248**, 9810 (1973).
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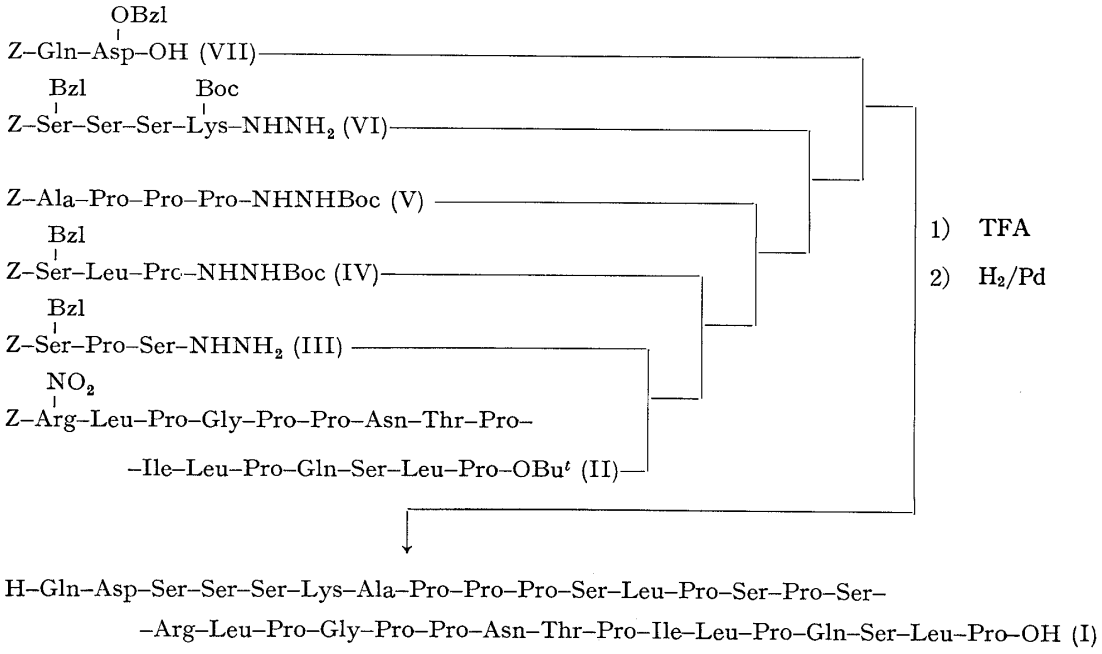

Fig. 1. Synthetic Scheme for the Peptide I

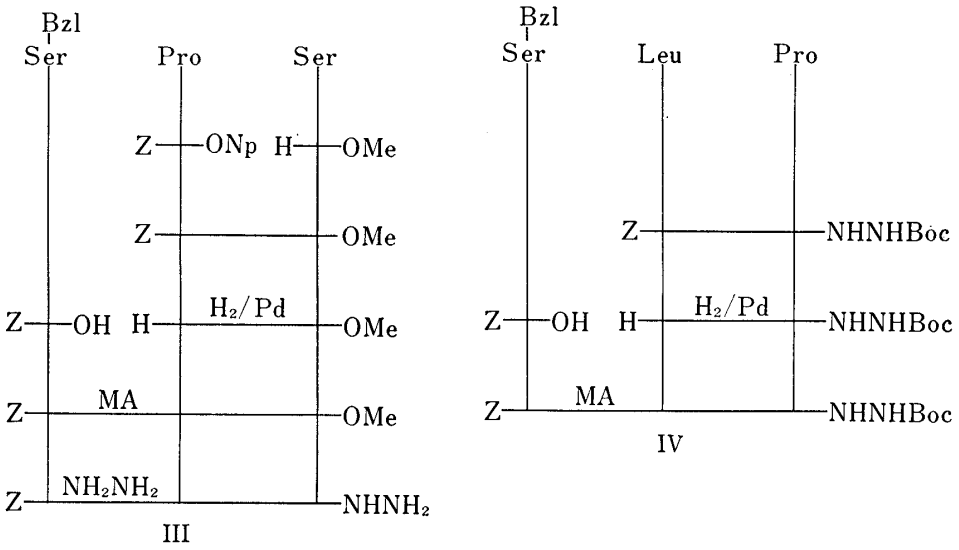

Fig. 2. Synthetic Schema for the Peptides III and IV

MA: mixed anhydride method.

Z group.[7]

Fragments III and IV were synthesized as shown in Fig. 2. The hydroxyl groups of serine residues in fragment III and IV were protected with Bzl groups. Benzylation of Z–Ser–OH was performed with benzyl bromide and sodium hydride according to the procedure for benzylation of Boc–Ser–OH reported by Sugano *et al.*[8] Protection of the hydroxyl group of serine made purification of the product easier and increased the coupling yield. Z–Pro–ONp[9] was coupled with H–Ser–OMe[10] to afford Z–Pro–Ser–OMe,[11] which was hydrogenated over a

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Pd catalyst. The resulting dipeptide amine was coupled by the mixed anhydride method[12] with Z–Ser(Bzl)–OH to afford Z–Ser(Bzl)–Pro–Ser–OMe, which was converted to the corresponding hydrazide (III). Fragment IV, Z–Ser(Bzl)–Leu–Pro–NHNHBoc, was synthesized by the mixed anhydride method by coupling Z–Ser(Bzl)–OH with H–Leu–Pro–NHNHBoc.[1]

Fragments V, VI and VII were synthesized as shown in Fig. 3. Boc–Hydrazide[13] of the fragment V was formed on the carboxyl group of the carboxyl-terminal proline after formation of the tetrapeptide to avoid formation of the diketopiperazine of prolylproline. Z–Ala–ONp[14] was coupled with H–Pro–OH to afford Z–Ala–Pro–OH,[15] which was converted to the corresponding methyl ester with diazomethane, followed by hydrazinolysis. The resulting Z–Ala–Pro–$NHNH_2$ was coupled by the azide method with H–Pro–Pro–OH[16] to afford Z–Ala–Pro–Pro–Pro–OH which in turn was converted to the corresponding Boc–hydrazide, Z–Ala–Pro–Pro–Pro–NHNHBoc (V) by the DCC procedure.[17]

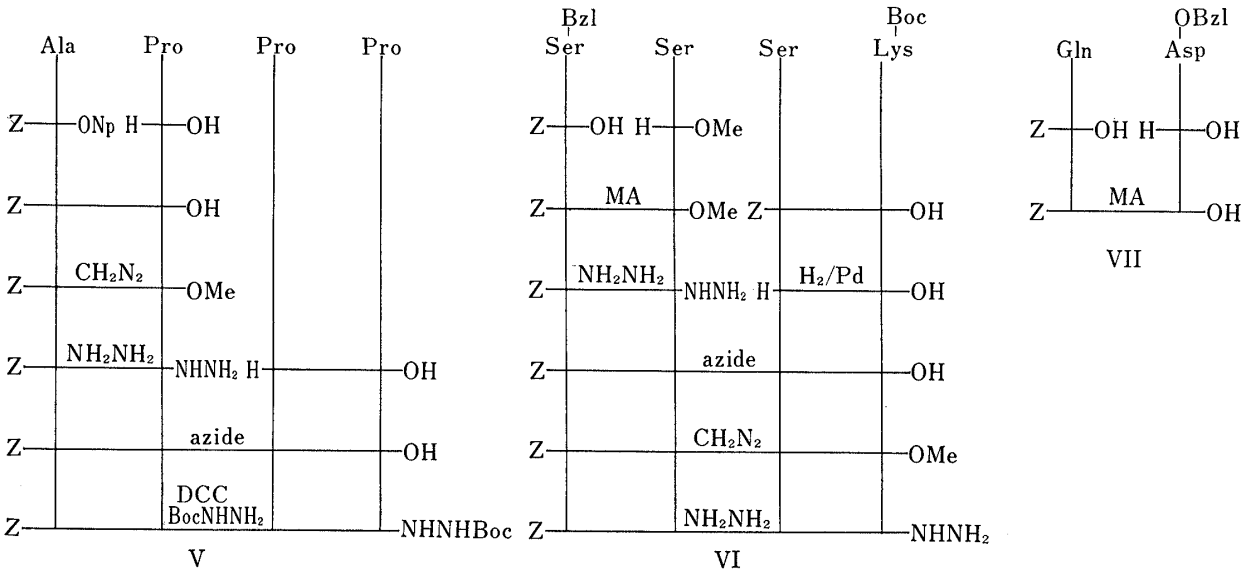

Fig. 3. Synthetic Schema for the Peptides V, VI, and VII

Fragment VI was synthesized as follows. The ε-amino group of lysine was protected with a Boc group and the hydroxyl group of amino-terminal serine was protected with a Bzl group. Z–Ser(Bzl)–OH was coupled by the mixed anhydride method with H–Ser–OMe[10] to afford Z–Ser(Bzl)–Ser–OMe, which was converted to the corresponding hydrazide. The dipeptide hydrazide was coupled by the azide method with H–Ser–Lys(Boc)–OH(prepared by hydrogenation from the corresponding Z derivative[19]). The resulting tetrapeptide, Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–OH, was treated with diazomethane followed by hydrazinolysis to afford the fragment VI, Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–$NHNH_2$.

Fragment VII was prepared by the mixed anhydride method from Z–Gln–OH[20] and H–Asp(Bzl)–OH.[21]

12) T. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951).
13) *a*) R.A. Boissonas, 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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The five fragments (III, IV, V, VI, and VII) thus synthesized were linked one by one by the azide method and the DPPA method[6] to the hexadecapeptide II, Z–Arg(NO_2)–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t. II was hydrogenated over a Pd catalyst and the resulting hexadecapeptide amine was coupled by the azide method with the fragment III to afford the nonadecapeptide, Z–Ser(Bzl)–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (VIII). VIII was purified by Sephadex LH-20 column chromatography using methanol as an eluent and hydrogenated over a Pd catalyst. The resulting nonadecapeptide amine was coupled by the azide method with Z–Ser(Bzl)–Leu–Pro–$NHNH_2$ (prepared by TFA treatment from the fragment IV) to afford the docosapeptide, Z–Ser(Bzl)–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (IX). IX was purified by Sephadex LH-20 column chromatography and hydrogenated over a Pd catalyst. The deblocked IX was coupled by the azide method with Z–Ala–Pro–Pro–Pro–$NHNH_2$ (prepared by TFA treatment from the fragment V) to afford the hexacosapeptide, Z–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (X). X was purified by Sephadex LH-20 column chromatography followed by partition chromatography between *n*-butanol and 10% acetic acid on Sephadex G-50. X was hydrogenated and the resulting hexacosapeptide amine was coupled by the azide method with the fragment VI to afford the triacontapeptide, Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (XI), which was purified according to the procedure described for X. XI was hydrogenated and the resulting triacontapeptide amine was coupled by the DPPA method[6] with the fragment VII to afford the dotriacontapeptide, Z–Gln–Asp(Bzl)–Ser–Ser–Ser–Lys(Boc)–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OBu^t (XII), which was purified by the procedure described for X. XII was treated with TFA, followed by hydrogenation to remove all protecting groups. The resulting peptide I, H–Gln–Asp–Ser–Ser–Ser–Lys–Ala–Pro–Pro–Pro–Ser–Leu–Pro–Ser–Pro–Ser–Arg–Leu–Pro–Gly–Pro–Pro–Asn–Thr–Pro–Ile–Leu–Pro–Gln–Ser–Leu–Pro–OH (I), was purified by Sephadex G-50 column chromatography using 5% acetic acid as an eluent. The peptide I showed a single spot on thin-layer chromatography, and the amino acid ratios in an acid hydrolysate showed good agreement with the theoretical values. Aminopeptidase M digestion of the peptide I was performed according to the procedure reported by Hofmann *et al.*[22] The peptide I was not digested completely within 24 hr and a few unidentified peaks were observed on the amino acid analyzer chart. Judging from the absorbance at 440 nm, one of them might correspond to a peptide with a proline residue at the amino-terminus. The peptide I contains 11 proline residues, and hydrolysis of some of the proline bonds by the enzyme might be slow.

The peptide I was converted to the hydrochloride and conjugated with bovine serum albumin by the water-soluble DCC procedure in the usual manner.[23] An antiserum was produced in New Zealand white rabbits by means of multiple intradermal injections of the conjugate in Freund's Complete adjuvant. Little binding of labeled hCG(^{125}I-hCG) was observed with the antiserum to I. Folkers *et al.*[9] prepared a hentriacontapeptide corresponding to the sequence 117—147 of hCG-β proposed by Carlsen *et al.*[7] by the solid phase procedure. They reported that the peptide produced an antibody which had weak binding activity (less than 1%) to the labeled hCG. There are some differences between our results and those of Folker's group, and the details of our immunologic studies of I will be reported elsewhere.

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

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 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 *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); Rf^5 *n*-BuOH, AcOH, pyridine, H_2O (2: 1: 1: 2).

Z-Pro-Ser-OMe——A mixture of Z–Pro–ONp[8] (7.4 g), H–Ser–OMe·HCl[9] (3.9 g) and Et_3N (3.5 ml) in dioxane (60 ml) was stirred at room temperature overnight. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 1 N HCl, 5% Na_2CO_3 and H_2O, then dried over Na_2SO_4. The AcOEt was evaporated off and the residue was recrystallized from AcOEt/ether; yield 6.03 g (82%), mp 104—105°, $[\alpha]_D^{25}$ −55.8° (c=1.0, MeOH), $[\alpha]_D^{22}$ −55.4° (c=1.0, AcOH) [lit. 10): Z–Pro–Ser–OMe was prepared by the mixed anhydride method, mp 103—104°, $[\alpha]_D^{22}$ −56.8° (c=1.0, AcOH)], Rf^1 0.81, *Anal.* Calcd for $C_{17}H_{22}N_2O_6$: C, 58.3; H, 6.3; N, 8.0. Found: C, 58.6; H, 6.4; N, 8.0.

Z-Ser(Bzl)-OH——Sodium hydride (57%, 2.78 g) in Bayol 85 was added to a solution of Z–Ser–OH (7.17 g) in DMF (60 ml) and the mixture was stirred at 0° for 15 min. Benzyl bromide (4 ml) was then added and the mixture was stirred for 4 hr in an ice-bath. The DMF was evaporated off and the residue was dissolved in H_2O. The solution was washed with ether and acidified with conc. HCl. The resulting precipitate was extracted with ether and the ether layer was washed with H_2O until Z–Ser–OH was no longer present in the ether layer. After drying over Na_2SO_4, the ether was evaporated off and the residue was recrystallized from benzene; yield 4 g (40%), mp 112°, $[\alpha]_D^{23}$ +11.3° (c=1.0, MeOH), Rf^3 0.85, Rf^4 0.67. *Anal.* Calcd for $C_{18}H_{19}NO_5$: C, 65.6; H, 5.8; N, 4.3. Found: C, 65.9; H, 5.8; N, 4.2.

Z-Ser(Bzl)-Pro-Ser-NHNH$_2$ (III)——A mixed anhydride, prepared from Z–Ser(Bzl)–OH (1.1 g) with Et_3N (0.46 ml) and ethylchloroformate (0.33 ml) in THF (15 ml) at −10°, was added to a solution of H–Pro–Ser–OMe (prepared by hydrogenation from 1.13 g of Z–Pro–Ser–OMe) in DMF (10 ml) and the mixture was stirred overnight. The solvent was evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed with 0.2 N HCl, 10% Na_2CO_3 and H_2O and dried over Na_2SO_4. The AcOEt was condensed and petro. ether was added to precipitate an amorphous powder; 1.41 g, Rf^3 0.87. This powder was dissolved in MeOH (12 ml) and $NH_2NH_2 \cdot H_2O$ (0.4 ml) was added. The mixture was kept at room temperature overnight and the resulting precipitate was collected by filtration. Recrystallized from MeOH; yield 1.13 g (64%), mp 152°, Rf^3 0.70, $[\alpha]_D^{25}$ −38.5° (c=1.0, DMF). *Anal.* Calcd for $C_{26}H_{33}N_5O_7$: C, 59.2; H, 6.3; N, 13.3. Found: C, 59.1; H, 6.2; N, 13.5.

Z-Ser-(Bzl)-Leu-Pro-NHNHBoc (IV)——A mixed anhydride, prepared from Z–Ser(Bzl)–OH (1.1 g) in the manner described above, was added to a solution of H–Leu–Pro–NHNHBoc[1] (1.13 g) in DMF (10 ml) and the mixture was stirred overnight. The solvent was evaporated off and the residue was extracted with ether. The ether layer was washed successively with 5% Na_2CO_3, 5% citric acid, 3% NH_4OH and H_2O, then dried over Na_2SO_4. The ether was evaporated off and the residue was precipitated from ether/petro. ether; yield 1.51 g (70%), amorphous powder, $[\alpha]_D^{25}$ −87.8° (c=1.0, MeOH), Rf^3 0.85, Rf^4 0.52. *Anal.* Calcd for $C_{34}H_{47}N_5O_8$: C, 62.5; H, 7.3; N, 10.7. Found: C, 62.3; H, 7.3; N, 10.6.

Z-Ala-Pro-OH——A solution of H–Pro–OH (6.1 g) and Z–Ala–ONp (19.2 g) in 50% aqueous dioxane (100 ml) containing Et_3N (7.4 ml) was stirred at room temperature overnight. The solvent was evaporated off and the residue was dissolved in 5% Na_2CO_3 (100 ml). The solution was washed with AcOEt and acidified with conc. HCl. The resulting oily precipitate was extracted with AcOEt and the extract was washed with water, dried over Na_2SO_4, then evaporated down. Ether was added to the residue to give a white precipitate, which was collected by filtration and recrystallized from AcOEt; yield 13.8 g (77%), mp 117—119° [Ref. 14): mp 120—122° (prepared by saponification from Z–Ala–Pro–OMe)], $[\alpha]_D^{24}$ −83.2° (c=1.0, MeOH), Rf^1 0.68. *Anal.* Calcd for $C_{16}H_{20}N_2O_5$: C, 60.0; H, 6.3; N, 8.6. Found: C, 59.8; H, 6.2; N, 8.6.

Z-Ala-Pro-NHNH$_2$——An ethereal solution of diazomethane was added to a solution of Z–Ala–Pro–OH (14.8 g) in MeOH (100 ml) until the solution became yellow. The solution was stored at 0° for 2 hr and the solvent was evaporated off. The residue was dissolved in MeOH (30 ml) and 80% $NH_2NH_2 \cdot H_2O$ (5.8 ml) was added. The mixture was kept at room temperature overnight, then the solvent was evaporated off. Addition of a small amount of EtOH to the residue and storage in a refrigerator overnight gave a crystalline material, which was recrystallized from MeOH/ether; yield 9.8 g (63%), mp 121—125°, $[\alpha]_D^{23}$ −93.2° (c=1.0, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{16}H_{22}N_4O_4$: C, 57.5; H, 6.6; N, 16.8. Found: C, 57.7; H, 6.6; N, 16.7.

Z-Ala-Pro-Pro-Pro-OH——5.85 N HCl/dioxane (6.8 ml) and isopentyl nitrite (2.7 ml) were added successively to a solution of Z–Ala–Pro–$NHNH_2$ (7 g) in DMF (20 ml) at −20° and the mixture was stirred for 5 min. The mixture was neutralized with Et_3N and combined with a solution of H–Pro–Pro–OH[15] (3.52 g) in H_2O (20 ml) containing Et_3N (2.8 ml). The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was dissolved in 5% $NaHCO_3$ (40 ml), and the solution was washed with AcOEt. The aqueous layer was acidified with conc. HCl and the resulting oily precipitate was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and evaporated down. Ether was added to

the residue to afford a solid mass which was collected by filtration, washed with ether and dried; yield 6.6 g (65%), amorphous powder, $[\alpha]_D^{20}$ −186.2° (c=1.0, MeOH), Rf^1 0.39. *Anal.* Calcd for $C_{26}H_{34}N_4O_7 \cdot 1.5H_2O$: C, 57.7; H, 6.8; N, 10.3. Found: C, 58.1; H, 7.1; N, 10.7. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Pro_{3.09}$ (average recovery 89%).

Z-Ala-Pro-Pro-Pro-NHNHBoc (V)——DCC (2.9 g) was added to a solution of Z–Ala–Pro–Pro–Pro–OH (5 g) and *tert*-butyl carbazate (1.6 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 and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 5% $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and evaporated down. The residue was solidified by addition of ether; yield 4.3 g (71%), $[\alpha]_D^{20}$ −206.5° (c=1.0, MeOH), Rf^1 0.53, Rf^3 0.80. *Anal.* Calcd for $C_{31}H_{44}N_6O_8$: C, 59.2; H, 7.1; N, 13.2. Found: C, 59.2; H, 7.1; 13.2. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}$ $Pro_{3.04}$ (average recovery 90%).

H-Ser-Lys(Boc)-OH——Z–Ser–Lys(Boc)–OH[18] (1.2 g) was hydrogenated over a Pd catalyst in 80% MeOH in the usual manner. After removal of the solvent, the residue was washed with AcOEt in a mortar and lyophilized from aqueous dioxane; yield 805 mg (94%), mp 219—221° (dec.), $[\alpha]_D^{24}$ −6.0° (c=1.0, 50% AcOH), Rf^1 0.50, Rf^3 0.15. *Anal.* Calcd for $C_{14}H_{27}N_3O_6$: C, 50.4; H, 8.1; N, 12.6. Found: C, 50.3; H, 8.2; N, 12.8.

Z-Ser(Bzl)-Ser-OMe——Et_3N (0.82 ml) and ethyl chloroformate (0.58 ml) were added successively at −10° to a solution of Z–Ser(Bzl)–OH (1.98 g) in THF (30 ml), and the mixture was stirred for 10 min. The mixture was then combined with a solution of H–Ser–OMe[9] (prepared from 0.93 g of the hydrochloride with 0.83 ml of Et_3N) in DMF (10 ml) and the reaction mixture was stirred for 3 hr in an ice-bath. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 5% Na_2CO_3 and H_2O, dried over Na_2SO_4 and evaporated down. The residue was recrystallized from AcOEt/ether; yield 1.99 g (77%), mp 108—109°, $[\alpha]_D^{23}$ +6.7° (c=1.0, MeOH), Rf^3 0.80, Rf^4 0.37. *Anal.* Calcd for $C_{22}H_{26}N_2O_7$: C, 61.4; H, 6.1; N, 6.5. Found: C, 61.4; H, 6.1; N, 6.5.

Z-Ser(Bzl)-Ser-NHNH$_2$——$NH_2NH_2 \cdot H_2O$ (0.7 ml) was added to a solution of Z–Ser(Bzl)–Ser–OMe (2.3 g) in EtOH (30 ml) and the mixture was stirred overnight. The resulting precipitate was collected by filtration and recrystallized from MeOH; yield 1.75 g (76%), mp 80—82°, $[\alpha]_D^{23}$ +9.7° (c=0.9, DMF), Rf^3 0.65. *Anal.* Calcd for $C_{21}H_{26}N_4O_6$: C, 58.6; H, 6.1; N, 13.0. Found: C, 58.7; H, 6.1; N, 12.8.

Z-Ser(Bzl)-Ser-Ser-Lys(Boc)-OH——6 N HCl/dioxane (4.5 ml) and *tert*-butyl nitrite (1.06 ml) were added successively to a solution of Z–Ser(Bzl)–Ser–$NHNH_2$ (3.9 g) in DMF (40 ml) at −10°, and the mixture was stirred for 10 min. The mixture was then neutralized with Et_3N (1.26 ml) and combined with a solution of H–Ser–Lys(Boc)–OH (2 g) in 80% DMF (20 ml) containing Et_3N (0.83 ml). The reaction mixture was stirred for 40 hr in a cold room and the solvent was evaporated off. The residue was dissolved in H_2O and the solution was washed with AcOEt and acidified with citric acid. The resulting precipitate was collected by filtration, washed with H_2O and recrystallized from MeOH; yield 3.9 g (89%), mp 156—158°, $[\alpha]_D^{23}$ +6.8° (c=1.0, DMF), Rf^1 0.80, Rf^3 0.52. *Anal.* Calcd for $C_{35}H_{49}N_5O_{12}$: C, 57.4; H, 6.8; N, 9.6. Found: C, 57.3; H, 6.9; N, 9.4.

Z-Ser(Bzl)-Ser-Ser-Lys(Boc)-OMe——Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–OH (3 g) was treated with an ethereal solution of diazomethane in MeOH (20 ml) in the usual manner. After removal of the solvent by evaporation, the residue was recrystallized from EtOH; yield 2.85 g (90%), mp 117—120°, $[\alpha]_D^{24}$ +0.4° (c=1.0, DMF), Rf^3 0.70. *Anal.* Calcd for $C_{36}H_{51}N_5O_{12}$: C, 58.0; H, 6.9; N, 9.4. Found: C, 57.8; H, 7.0; N, 9.5.

Z-Ser(Bzl)-Ser-Ser-Lys(Boc)-NHNH$_2$ (VI)——$NH_2NH_2 \cdot H_2O$ (0.5 g) was added to a solution of Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–OMe (2.5 g) in MeOH (20 ml) and the mixture was stirred at room temperature overnight. The resulting precipitate was collected by filtration, washed with cold EtOH, and recrystallized from MeOH; yield 2.0 g (80%), mp 190—192° (dec.), $[\alpha]_D^{23}$ +4.8° (c=1.0, DMF), Rf^3 0.51. *Anal.* Calcd for $C_{35}H_{51}N_7O_{11}$: C, 56.3; H, 6.9; N, 13.2. Found: C, 56.3; H, 7.0; N, 13.0. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Ser_{3.64}Lys_{1.00}$ (average recovery 87%).

Z-Gln-Asp(OBzl)-OH (VII)——Et_3N (0.98 ml) and ethyl chloroformate (0.68 ml) were added successively to a solution of Z–Gln–OH (2 g) in DMF (12 ml) at −15°, and the mixture was stirred for 10 min. The mixture was combined with a solution of H–Asp(OBzl)–OH (1.58 g) in a mixture of 50% DMF and Et_3N (0.98 ml) and the whole was stirred for 3 hr. The solvent was evaporated off and the residue was washed with 0.5 N HCl (3 times) and H_2O (8 times) in a mortar. Recrystallized from MeOH; yield 2.1 g (61%), mp 186—189°, $[\alpha]_D^{22}$ −2.6° (c=1.0, DMF), Rf^1 0.92, Rf^3 0.41. *Anal.* Calcd for $C_{24}H_{27}N_3O_8$: C, 59.4; H, 5.6; N, 8.7. Found: C, 59.2; H, 5.8; N, 8.5.

Z-Ser(Bzl)-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (VIII)——The protected hexadecapeptide II (1 g) was hydrogenated over a Pd catalyst in a mixture of MeOH (10 ml) and 5% AcOH (10 ml) for 12 hr. The catalyst was filtered off and the solvent was removed by evaporation. The residue was lyophilized from a mixture of 1 N HCl (0.5 ml) and H_2O (20 ml). The resulting fluffy powder was dissolved in a mixture of DMF (10 ml) and Et_3N (0.07 ml).

Next, 6 N HCl/dioxane (0.75 ml) and *tert*-butyl nitrite (0.18 ml) were added successively to a solution of Z–Ser(Bzl)–Pro–Ser–$NHNH_2$ (III, 791 mg) in DMF (4 ml) at −10°, and the mixture was stirred for 5 min. The mixture was neutralized with Et_3N (0.65 ml) and combined with the solution of the hydrogenated hexa-

decapeptide described above. The whole was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was extracted with *n*-BuOH, and the extract was washed with 1% AcOH and H_2O. The *n*-BuOH was evaporated off and the residue was precipitated twice from EtOH/AcOEt; 1.05 g. The product was dissolved in MeOH (5 ml) and the solution was applied to a Sephadex LH-20 column (3×80 cm) equilibrated with MeOH. The column was developed with MeOH at a flow rate 7 g/10 min and fractions of 7 g were collected. Each fraction was tested with I_2 and fractions 30—34, which showed Rf^1 0.31, were pooled and evaporated down. The residue was lyophilized from aqueous dioxane; yield 980 mg (84%), fluffy powder, $[\alpha]_D^{22}$ −64.4° (c=0.5, DMF), Rf^1 0.31, Rf^2 0.71. *Anal.* Calcd for $C_{108}H_{166}N_{24}O_{28}\cdot HCl\cdot H_2O$: C, 56.3; H, 7.4; N, 14.6. Found: C, 56.2; H, 7.2; N, 14.3. Amino acid ratios in acid hydrolysate (6 N HCl, 24 hr): $Asp_{0.96}Thr_{0.89}Ser_{2.80}Glu_{1.04}Pro_{7.31}Gly_{1.00}Ile_{1.01}Leu_{3.08}Arg_{0.95}$ (average recovery 78%).

Z-Ser(Bzl)-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (IX)——The nonadecapeptide VIII (1 g) was hydrogenated over a Pd catalyst in a mixture of MeOH (20 ml) and 5% AcOH (20 ml) for 16 hr. The catalyst was removed by filtration and the filtrate was evaporated down. The residue was lyophilized from 1% AcOH to afford a fluffy powder which was dissolved in a mixture of DMF (5 ml) and Et_3N (0.06 ml).

Next, Z–Ser(Bzl)–Leu–Pro–NHNHBoc (IV, 981 mg) was treated with TFA (2 ml) containing anisole (0.1 ml) at room temperature for 40 min. Ether was added to afford a precipitate, which was collected by centrifugation, washed with ether and dried over KOH pellets. This tripeptide hydrazide trifluoroacetate was dissolved in DMF (5 ml) and the solution was cooled to −10°. 6 N HCl/dioxane (0.5 ml) and *tert*-butyl nitrite (0.18 ml) were added successively to this solution and the mixture was stirred for 5 min. The mixture was neutralized with Et_3N (0.7 ml) and then combined with the solution of the hydrogenated nonadecapeptide described above. The whole 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 1% AcOH and H_2O. After removal of the *n*-BuOH, the residue was precipitated 3 times from EtOH/AcOEt. This precipitate was dissolved in MeOH (6 ml) and the solution was applied to a Sephadex LH-20 column (3×80 cm) equilibrated with MeOH. The column was developed with MeOH at a flow rate of 7 g/10 min. Fractions of 7 g were collected and each fraction was checked with I_2. Fractions 29—34, which showed Rf^1 0.24, were pooled and the solvent was evaporated off. The residue was precipitated from EtOH/AcOEt and lyophilized from aqueous dioxane; yield 960 mg (79%), fluffy powder, $[\alpha]_D^{25}$ −79.8° (c=0.5, DMF), Rf^1 0.24, Rf^2 0.74. *Anal.* Calcd for $C_{122}H_{189}N_{27}O_{32}\cdot HCl\cdot 10H_2O$: C, 51.8; H, 7.5; N, 13.4. Found: C, 51.6; H, 7.3; N, 13.4. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{1.00}Thr_{0.96}Ser_{3.76}Glu_{1.05}Pro_{8.42}Gly_{1.04}Ile_{1.03}Leu_{4.15}Arg_{0.95}$ (average recovery 81%).

Z-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (X)——The docosapeptide IX (330 mg) was hydrogenated over a Pd catalyst in the manner described above for the peptide VIII. The hydrogenated docosapeptide was lyophilized from 1% AcOH to afford a fluffy powder, which was dissolved in a mixture of DMF and 10% Et_3N/DMF (0.15 ml).

Next, Z–Ala–Pro–Pro–Pro–NHNHBoc (V, 188 mg) was treated with TFA (1 ml) containing anisole (0.05 ml) at room temperature for 40 min. Ether was added to afford a precipitate, which was collected by centrifugation, washed with ether and dried; 190 mg, Rf^1 0.65. This tetrapeptide hydrazide trifluoroacetate was dissolved in DMF (1 ml) and the solution was cooled to −10°. 6 N HCl/dioxane (0.1 ml) and 10% *tert*-butyl nitrite/DMF (0.36 ml) were added successively and the mixture was stirred for 5 min. The mixture was neutralized with Et_3N and combined with the solution of the hydrogenated IX descrived above. The whole was stirred for 48 hr in a cold room. The solvent was evaporated off and the residue was dissolved in *n*-BuOH. The *n*-BuOH layer was washed with 1% AcOH and H_2O and evaporated down. The residue was precipitated twice from EtOH/AcOEt and dissolved in MeOH (3 ml). The solution was applied to a Sephadex LH-20 column (3×80 cm) equilibrated with MeOH. The column was developed with MeOH at a flow rate of 7 g/10 min and fractions of 7 g were collected. Each fraction was tested with I_2 and fractions 28—35, which showed Rf^1 0.10, were pooled and evaporated down. The residue was dissolved in *n*-BuOH (3 ml) and the solution was applied to a Sephadex G-50 column (3×80 cm) equilibrated with 10% AcOH. The column was developed with *n*-BuOH and fractions of 7 g were collected. Each fraction was tested with I_2 and fractions 29—36 were pooled and evaporated down. The residue was lyophilized from aqueous dioxane; yield 280 mg (78%), fluffy powder, $[\alpha]_D^{25}$ −98.3° (c=0.5, DMF), Rf^1 0.10, Rf^2 0.76. *Anal.* Calcd for $C_{133}H_{209}N_{31}O_{36}\cdot HCl\cdot 8H_2O$: C, 53.3; H, 7.6; N, 14.5. Found: C, 53.5; H, 7.4; N, 14.4. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{0.99}$ $Thr_{0.89}Ser_{3.71}Glu_{1.02}Pro_{11.61}Gly_{1.00}Ala_{1.04}Ile_{0.94}Leu_{4.11}Arg_{0.89}$ (average recovery 79%).

Z-Ser(Bzl)-Ser-Ser-Lys(Boc)-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (XI)——The hexacosapeptide X (200 mg) was hydrogenated over a Pd catalyst in a mixture of MeOH (5 ml) and 1% AcOH (5 ml) in the usual manner. The hydrogenated material was lyophilized from 1% AcOH to afford a fluffy powder, which was dissolved in a mixture of DMF (2 ml) and 10% Et_3N/DMF (0.09 ml).

Next, 6 N HCl/dioxane (0.17 ml) and 10% *tert*-butyl nitrite/DMF (0.38 ml) were added successively to a solution of Z–Ser(Bzl)–Ser–Ser–Lys(Boc)–$NHNH_2$ (VI, 242 mg) in a mixture of DMF (2 ml) and DMSO (1 ml) at −20°, and the mixture was stirred for 10 min. Et_3N (0.15 ml) was added and the mixture was combined

with the solution of the hydrogenated hexacosapeptide described above. The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The product was purified according to the procedure described for the fragment X. The product was lyophilized from aqueous dioxane; yield 205 mg (86%), fluffy powder, $[\alpha]_D^{26}$ −87.3° (c=0.5, DMF), Rf^1 0.20, Rf^2 0.72. *Anal.* Calcd for $C_{160}H_{250}N_{36}O_{45}\cdot HCl\cdot 8H_2O$: C, 53.7; H, 7.5; N, 14.1. Found: C, 53.6; H, 7.2; N, 14.0. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{1.00}Thr_{0.89}Ser_{6.59}Glu_{1.10}Pro_{11.43}Gly_{1.00}Ala_{1.06}Ile_{1.00}Leu_{4.21}Lys_{1.01}Arg_{0.99}$ (average recovery 86%).

Z-Gln-Asp(OBzl)-Ser-Ser-Ser-Lys(Boc)-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OBu^t (XII)——The triacontapeptide XI (120 mg) was hydrogenated over a Pd catalyst in the manner described for VIII. The hydrogenated material was lyophilized from a mixture of H_2O (10 ml) and 0.1 N HCl (0.3 ml) to afford a fluffy powder, which was dissolved in DMF (2 ml).

Next, 10% Et_3N/DMF (0.4 ml) and DPPA (83 mg) were added successively to a solution of Z–Gln–Asp-(OBzl)–OH (145 mg) in DMF (2 ml) at −20°, and the mixture was stirred for 30 min between −15 to −20°. The mixture was then combined with the solution of the hydrogenated triacontapeptide described above and the whole was stirred for 48 hr in a cold room. The solvent was evaporated off and the product was purified according to the procedure described for X. The product was lyophilized from aqueous dioxane; yield 108 mg (89%), fluffy powder, $[\alpha]_D^{25}$ −75.9° (c=0.4, DMF), Rf^1 0.21, Rf^2 0.71. *Anal.* Calcd for $C_{169}H_{263}N_{39}O_{50}\cdot HCl\cdot 4H_2O$: C, 54.1; H, 7.3; N, 14.6. Found: C, 54.4; H, 7.2; N, 14.3. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{2.12}Thr_{0.88}Ser_{6.55}Glu_{2.10}Pro_{11.42}Gly_{1.00}Ala_{1.07}Ile_{0.90}Leu_{3.83}Lys_{0.95}Arg_{0.89}$ (average recovery 85%).

H-Gln-Asp-Ser-Ser-Ser-Lys-Ala-Pro-Pro-Pro-Ser-Leu-Pro-Ser-Pro-Ser-Arg-Leu-Pro-Gly-Pro-Pro-Asn-Thr-Pro-Ile-Leu-Pro-Gln-Ser-Leu-Pro-OH (I)——The dotriacontapeptide XII (108 mg) was dissolved in TFA (1 ml) containing anisole (0.01 ml) and the solution was stirred at room temperature for 40 min. Ether was added and the resulting precipitate was collected by centrifugation, washed with ether and dried. The material was lyophilized from a mixture of H_2O (10 ml) and 0.1 N HCl (0.1 ml) to afford powder; 110 mg, Rf^1 0.00, Rf^2 0.12. This fluffy powder was hydrogenated over Pd catalyst in 5% AcOH (15 ml) for 7 hr, then the catalyst was filtered off. The solvent was removed and the residue was lyophilized from H_2O to afford a fluffy powder. The powder was dissolved in 50% AcOH (1 ml) and the solution was applied to a Sephadex G-50 column (1×170 cm) equilibrated with 5% AcOH. The column was developed with 5% AcOH at a flow rate of 2 ml/10 min. Fractions of 2 ml were collected and each fraction was tested by means of the Sakaguchi reaction. Fractions 29—33 were pooled and the solvent was evaporated off. The residue was lyophilized from H_2O; 78 mg (87%), fluffy hygroscopic powder, $[\alpha]_D^{28}$ −173.4° (c=0.2, H_2O), Rf^1 0.00, Rf^2 0.05, Rf^5 0.53. *Anal.* Calcd for $C_{145}H_{235}N_{39}O_{46}\cdot HCl\cdot 13H_2O$: C, 49.3; H, 7.5; N, 15.5. Found: C, 49.1; H, 7.8; N, 15.2. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Asp_{2.09}Thr_{0.86}Ser_{6.63}Glu_{2.11}Pro_{11.45}Gly_{1.00}Ala_{0.97}Ile_{0.90}Leu_{3.86}Lys_{0.95}Arg_{0.90}$ (average recovery 79%). Amino acid ratios in an aminopeptidase M digest:[22,24] $Asp_{0.87}(Asn+Gln+Thr)^{25}{}_{4.53}Ser_{7.50}Glu_{0.50}Pro_{9.97}Gly_{1.00}Ala_{0.80}Ile_{1.00}Leu_{4.03}Lys_{1.06}$ (average recovery 72%).

24) 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; $(NH_4)_2SO_4$ interfered with the analysis of Arg.
25) Calculated as Thr.