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Studies on Peptides. CXXVII.^{1,2)} Synthesis of a Tripentacontapeptide with Epidermal Growth Factor Activity

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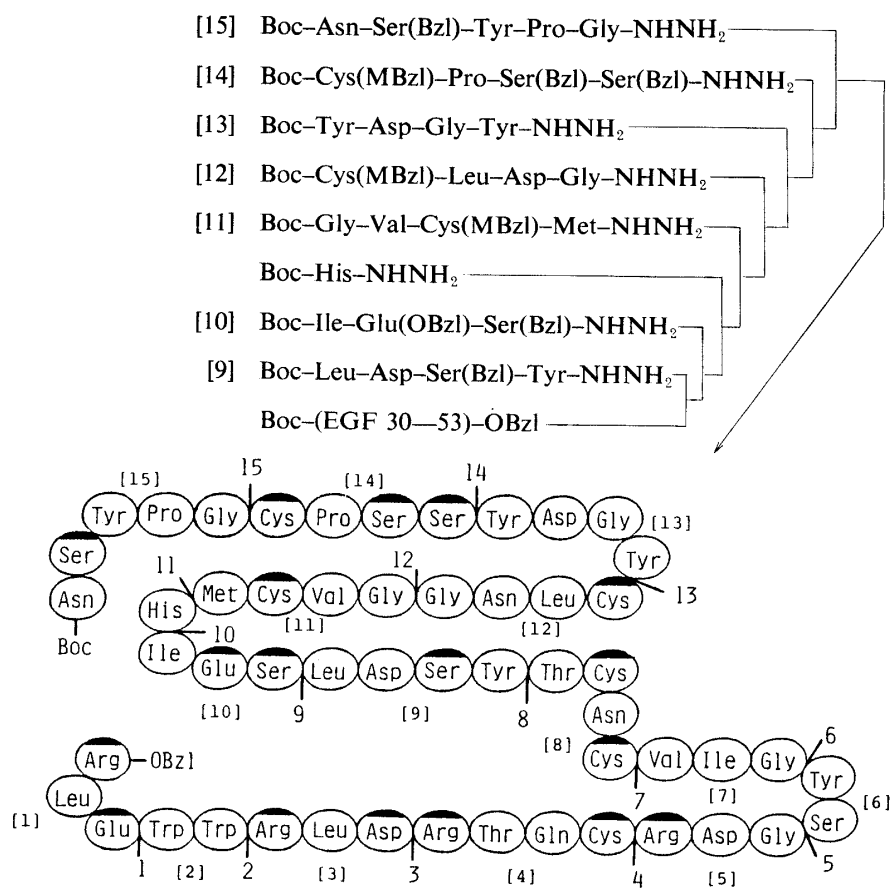
The tripentacontapeptide corresponding to the entire linear sequence of epidermal growth factor was synthesized by assembling 15 peptide fragments and one His residue (position 22), followed by deprotection with trifluoromethanesulfonic acid-thioanisole in trifluoroacetic acid. The deprotected peptide was subjected to air-oxidation. After purification by ion-exchange chromatography on diethyl aminoethyl cellulose followed by high performance liquid chromatography, a peptide with powerful anti-gastric activity was obtained.

Keywords—epidermal growth factor (EGF) solution synthesis; methionine sulfoxide reduction; cysteine sulfoxide reduction; TFMSA deprotection; thioanisole-mediated deprotection; DEAE purification; HPLC purification; synthetic EGF immunodiffusion test; histamine-stimulated gastric acid secretion inhibition

As described in the preceding paper,¹⁾ epidermal growth factor (EGF)³⁾ isolated from mouse submaxillary gland was found to possess powerful anti-gastric activity.⁴⁾ Because of this significant physiological activity, we began a program to synthesize this submaxillary principle, which consists of 53 amino acids with 3 disulfide bridges. As reported,¹⁾ a half of the molecule, the protected tetracosapeptide corresponding to positions 30 to 53 of EGF, was synthesized by assembling 8 fragments, Z(OMe)-(51-53)-OBzl [1], Boc-(49-50)-OH [2], Boc-(46-48)-NHNH₂ [3], Boc-(42-45)-NHNH₂ [4], Boc-(39-41)-NHNH₂ [5], Boc-(37-38)-NHNH₂ [6], Boc-(34-36)-NHNH₂ [7], and Boc-(30-33)-NHNH₂ [8].

We wish to report in this paper the synthesis of the tripentacontapeptide corresponding to the linear sequence of EGF by further chain elongation of the above intermediate followed by deprotection with trifluoromethanesulfonic acid (TFMSA) in TFA.⁵⁾ We succeeded in isolating, after air-oxidation, a peptide with anti-gastric activity equivalent to that of natural EGF.

As shown in Fig. 1, seven hydrazides, [9] to [15], were newly synthesized using amino acid derivatives bearing protecting groups removable by TFMSA; *i.e.*, Glu(OBzl), Ser(Bzl), and Cys(MBzl). The His residue (position 22) was introduced as a single amino acid for reasons that we will discuss in a latter section of this paper. The protecting groups of the Asp residues at particular positions (11 and 27) were deliberately removed in the synthesis of the corresponding fragments, since base-catalyzed ring-closure of Asp(OBzl) is reported to be sequence-dependent,⁶⁾ as mentioned in the preceding paper. In this connection, the hydroxyl group of the Ser residue was protected as its Bzl ether, referring to our previous synthesis of chicken VIP (vasoactive intestinal polypeptide).⁷⁾ For some reason, the azide reaction⁸⁾ of the N-terminal fragment, Z(OMe)-His-Ser-Asp-Ala-NHNH₂, did not proceed satisfactorily,



, protected amino acids: Ser(Bzl), Cys(MBzl), Glu(OBzl), Arg(Mts), Asp(OBzl).

| , positions of condensation.

Fig. 1. Synthetic Route to the Protected Tripentacosapeptide Corresponding to EGF

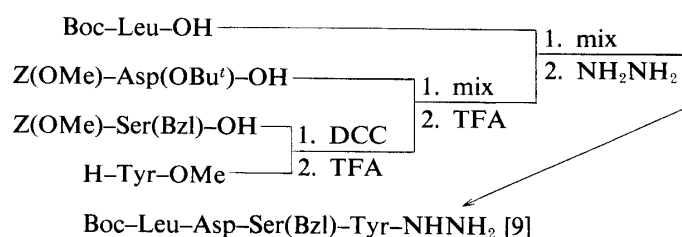


Fig. 2. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Boc-(EGF 26—29)-NHNH₂.

unless the hydroxyl group of the Ser residue was protected. We thought that since the combination of His, Ser and Asp is an active triad of serine proteases,⁹⁾ interaction of these three functional groups might participate in this unusual phenomenon of the above azide reaction. EGF possesses one His residue at position 22, near Ser and Asp residues.

Taking the precaution stated above, fragment [9], Boc-Leu-Asp-Ser(Bzl)-Tyr-NHNH₂, was synthesized according to the scheme illustrated in Fig. 2. Z(OMe)-Ser(Bzl)-Tyr-OMe was prepared by the DCC procedure¹⁰⁾ and this, after the usual TFA treatment, was condensed with Z(OMe)-Asp(OBu')-OH, instead of Z(OMe)-Asp(OBzl)-OH, by the mixed

anhydride method. From the resulting tripeptide ester, $Z(\text{OMe})\text{-Asp}(\text{OBu}')\text{-Ser}(\text{Bzl})\text{-Tyr-OMe}$, the $Z(\text{OMe})$ and the Bu' groups were removed simultaneously by TFA treatment. Next, Boc-Leu-OH was introduced by the mixed anhydride method to give the protected tetrapeptide ester, $\text{Boc-Leu-Asp-Ser}(\text{Bzl})\text{-Tyr-OMe}$, which was converted to the corresponding hydrazide [9] by the usual hydrazine treatment.

As the next fragment, we first prepared $\text{Boc-His-Ile-Glu}(\text{OBzl})\text{-Ser}(\text{Bzl})\text{-NHNH}_2$. In a preliminary test, an attempt to condense this fragment, *via* the azide, with the amino component (EGF 26—53) was unsatisfactory, despite the use of 15 equivalents of the acyl component. Acid hydrolysis of the product indicated that only 30% of the acyl component was incorporated into the chain. We recalled the earlier observations by Camble and Petter,¹¹⁾ made during synthetic studies on urogastrone, a peptide structurally related to EGF that was isolated from human urine; they reported that condensations of His-containing fragments, such as $\text{Boc-Leu-Ser}(\text{Bu}')\text{-His-Asp}(\text{OBu}')\text{-Gly-OH}$ (positions 8—12) or larger N-terminal fragments, with the amino component (13—47) by DCC or other means were disappointing in both solid and solution syntheses, because of poor incorporations of acyl components. Thus, we adopted $\text{Ser}(\text{Bzl})$ in fragment [9] as stated above. However, we encountered the same kind of difficulty as reported in the urogastrone synthesis. This implies that besides the functional interrelationship of the particular amino acids mentioned above, conformational elements of an amino component in organic solvents may contribute to this unusual phenomenon. After several trials, we reached the conclusion that this His-containing segment could be introduced in two steps, *i.e.*, by two successive azide condensations of $\text{Boc-Ile-Glu}(\text{OBzl})\text{-Ser}(\text{Bzl})\text{-NHNH}_2$ and Boc-His-NHNH_2 . From the synthetic viewpoint, we concluded that the His residue at position 22 in EGF indeed has a unique character.

Thus, fragment [10], $\text{Boc-Ile-Glu}(\text{OBzl})\text{-Ser}(\text{Bzl})\text{-NHNH}_2$, was prepared in a stepwise manner starting with $\text{H-Ser}(\text{Bzl})\text{-NHNH-Troc}$ as shown in Fig. 3. After two successive introductions of $Z(\text{OMe})\text{-Glu}(\text{OBzl})\text{-OH}$ and Boc-Ile-OH by the active ester procedure,^{12,13)} the Troc group was removed from the resulting tripeptide derivative, $\text{Boc-Ile-Glu}(\text{OBzl})\text{-Ser}(\text{Bzl})\text{-NHNH-Troc}$ by Zn treatment.¹⁴⁾

The next fragment, $\text{Boc-Gly-Val-Cys}(\text{MBzl})\text{-Met-NHNH}_2$ [11], was also prepared in a

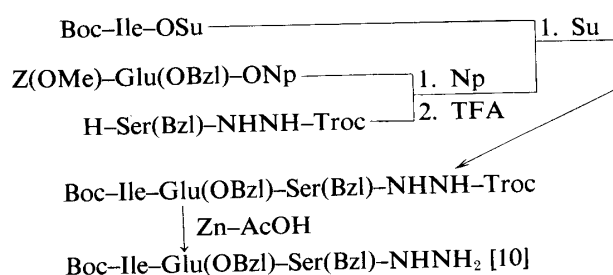


Fig. 3. Synthetic Scheme for the Protected Tripeptide Hydrazide, $\text{Boc-(EGF 23—25)-NHNH}_2$

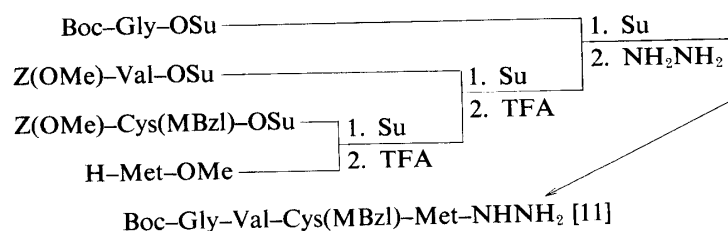


Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, $\text{Boc-(EGF 18—21)-NHNH}_2$

TABLE I. Amino Acid Ratios in 6N HCl Hydrolysates of Synthetic EGF and Its Intermediates

A.A.	Protected peptides								Syn. EGF	Natural EGF
	26—53 (28)	23—53 (31)	22—53 (32)	18—53 (36)	14—53 (40)	10—53 (44)	6—53 (48)	1—53 (53)		
Asp	4.17 (4)	4.35 (4)	4.23 (4)	4.16 (4)	5.25 (5)	6.35 (6)	6.27 (6)	7.31 (7)	7.08 (7)	6.55 (7)
Thr	1.76 (2)	1.91 (2)	1.87 (2)	1.76 (2)	1.92 (2)	2.07 (2)	1.87 (2)	1.84 (2)	2.08 (2)	1.96 (2)
Ser	1.69 (2)	2.56 (3)	2.38 (3)	2.46 (3)	2.51 (3)	2.62 (3)	4.34 (5)	4.82 (6)	5.32 (6)	5.82 (6)
Glu	2.15 (2)	3.31 (3)	3.18 (3)	3.13 (3)	3.00 (3)	3.17 (3)	3.19 (3)	3.20 (3)	3.26 (3)	2.92 (3)
Pro							1.29 (1)	2.32 (2)	2.04 (2)	2.16 (2)
Gly	2.13 (2)	2.12 (2)	2.15 (2)	3.12 (3)	3.99 (4)	4.82 (5)	4.77 (5)	5.74 (6)	5.71 (6)	6.26 (6)
Cys								2.20 (3)	2.17 (3)	2.46 (3)
Val	0.91 (1)	0.71 (1)	0.80 (1)	1.90 (2)	1.63 (2)	1.48 (2)	1.60 (2)	1.57 (2)	1.24 (2)	1.14 (2)
Met				0.75 (1)	0.76 (1)	0.54 (1)	0.63 (1)	0.49 (1)	0.66 (1)	0.90 (1)
Ile	0.86 (1)	1.65 (2)	1.71 (2)	1.77 (2)	1.67 (2)	1.58 (2)	1.61 (2)	1.63 (2)	1.54 (2)	1.37 (2)
Leu	3.00 (3)	3.00 (3)	3.00 (3)	3.00 (3)	4.00 (4)	4.00 (4)	4.00 (4)	4.00 (4)	4.00 (4)	4.00 (4)
Tyr	1.77 (2)	1.91 (2)	1.89 (2)	1.74 (2)	1.83 (2)	3.86 (4)	3.82 (4)	4.68 (5)	4.59 (5)	4.59 (5)
Trp ^{a)}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.05 (2)	N.D.
His			0.87 (1)	0.85 (1)	1.13 (1)	0.67 (1)	0.56 (1)	0.62 (1)	0.76 (1)	0.95 (1)
Arg	4.28 (4)	4.36 (4)	4.26 (4)	4.31 (4)	4.20 (4)	4.16 (4)	4.25 (4)	4.30 (4)	4.11 (4)	4.11 (4)
Recov.	88%	77%	91%	90%	88%	82%	75%	79%	73%	85%

a) Trp was determined by 4N MSA hydrolysis.

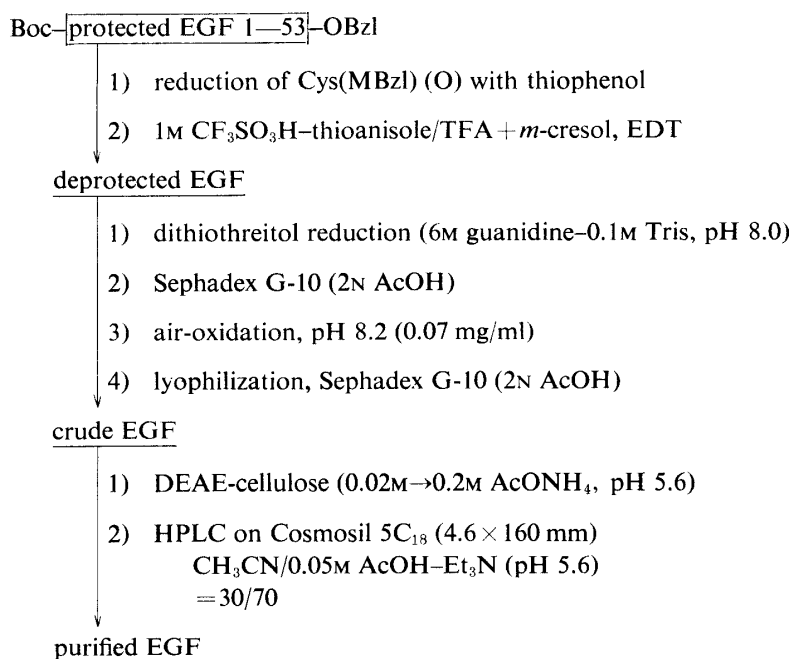


Fig. 9. Deprotection and Purification of EGF

in the presence of phenol, *p*-hydroxyphenyl-cysteine was detected on a short column of an amino acid analyzer (retention time 26 min, Hitachi KLA-5 analyzer). As examined previously,¹⁵⁾ satisfactory recovery of cysteine cannot be expected from Cys(MBzl) sulfoxide unless the sulfoxide is reduced before deprotection. Thus, the protected EGF was incubated with thiophenol as performed in our previous RNase A synthesis.²²⁾ This treatment is

effective to reduce the sulfoxide of the Met residue, which might be partially formed during the synthesis as described above.

Deprotection and subsequent purification were carried out according to the procedures illustrated in Fig. 9. The reduced form of protected EGF was treated with 1 M TFMSA-thioanisole (molar ratio 1 : 1) in TFA^{5b,23)} in the presence of *m*-cresol and EDT in an ice-bath for 120 min. This system has the ability to cleave the Mts²⁴⁾ group from Arg residues within 60 min at 0 °C, as well as other protecting groups based on benzyl alcohol, without accompanying marked side reactions, such as succinimide formation of the Asp residue.^{5b)} The roles of thioanisole and *m*-cresol were recently reviewed.²⁵⁾ The former has been proven to have the ability to accelerate the cleavage of protecting groups through the S_N2 type mechanism.^{5b,23)} The latter traps alkyl cations effectively and suppresses O-sulfonylation at the Tyr residue as well.²⁴⁾ An additional scavenger, EDT, also effectively suppresses indole-alkylation at the Trp residue²¹⁾ in this deprotection step. This thioanisole mediated treatment was repeated twice more to ensure complete deprotection. The deprotected peptide was then reduced with dithiothreitol in 0.1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine-HCl. These conditions are the same as those selected by Taylor *et al.*²⁷⁾ for reductive cleavage of the disulfides of natural EGF. This basic condition also seemed suitable to us for the reversal of any possible N→O shift at the Ser and Thr residues.²⁸⁾ Any aggregated disulfide complex, if formed, could be reduced during this treatment. This treatment was also expected to be effective for the reduction of any remaining Met(O) residue, if present, and for the removal of the S-alkyl group from the Met residue,²⁹⁾ if such an S-sulfonium product was formed during the deprotection. The reduced product was next separated from the reducing reagent by gel-filtration on Sephadex G-10 with 2 N AcOH as an eluant and the desired fractions were diluted with water to a concentration of 0.07 mg/ml. The pH was adjusted to 8.2 with conc. ammonia, then the dilute solution was kept at 25 °C for 7 d to establish three disulfide bridges, the progress of air-oxidation being monitored by the use of Ellman's reagent³⁰⁾ (Fig. 10-a). The Ellman color intensity dropped slowly and after 5 d, reached a constant value. Taylor *et al.*²⁷⁾ dialyzed reduced EGF against water, then took the solution to dryness by air-drying to regenerate EGF. Full recovery of the EGF activity was recorded, but the re-oxidized product was not chemically characterized in their work. After several trials, we concluded that more prolonged air-oxidation was necessary to obtain reproducible results, namely to give a product with the same retention time as natural EGF in high performance liquid chromatography (HPLC). After 5 d' standing, the solution was gently stirred for an additional 2 d to

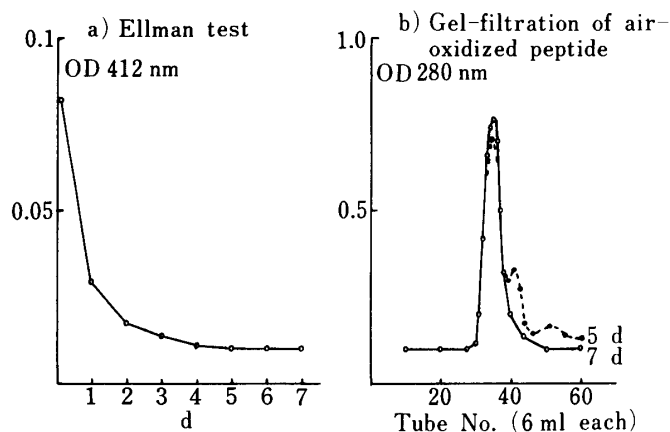


Fig. 10. Air-Oxidation of Deprotected EGF

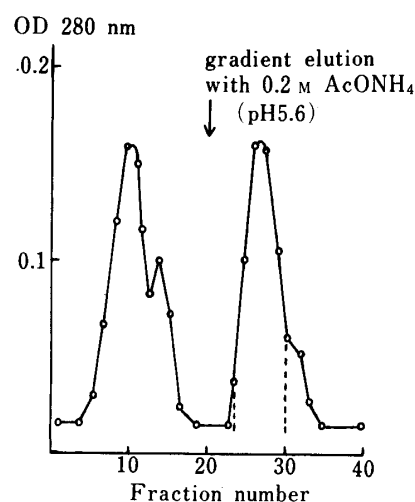


Fig. 11. DEAE-Cellulose Purification of the Air-Oxidized Product

ensure the disulfide bond formation. The entire solution was lyophilized and the oxidized product was subjected to gel-filtration on Sephadex G-10 with 2 N AcOH as an eluant. Some insoluble material, presumably highly aggregated products, was removed prior to this gel-filtration. The 7 d oxidized product was eluted as a sharp single peak in 24% yield, but the 5 d' oxidized product gave a peak with two shoulders (Fig. 10-b).

In the literature, two effective procedures were described by Savage *et al.*³¹⁾ for the isolation of natural EGF, one by the Bio-gel procedure and the other by the diethyl aminoethyl (DEAE)-cellulose procedure. We purified the above air-oxidized product by these procedures and compared the purity of each product by disk isoelectrofocusing (pH 3—10). It was found that the latter procedure was far more effective than the former procedure in our hands. Thus, the curde air-oxidized product, isolated by gel-filtration on Sephadex G-10, was next purified by ion-exchange chromatography on DEAE-cellulose. The material eluted from the column with the starting buffer, pH 5.6, 0.02 M AcONH₄ buffer, was inactive. However, the product that emerged from the column at the same retention volume as natural EGF on gradient elution from the starting buffer to pH 5.6, 0.2 M AcONH₄ buffer (Fig. 11) exhibited about 80% of the anti-gastric activity of natural EGF. The yield of this purification step was 16%. Thus, to reach this stage approximately 95% of the material was lost during the

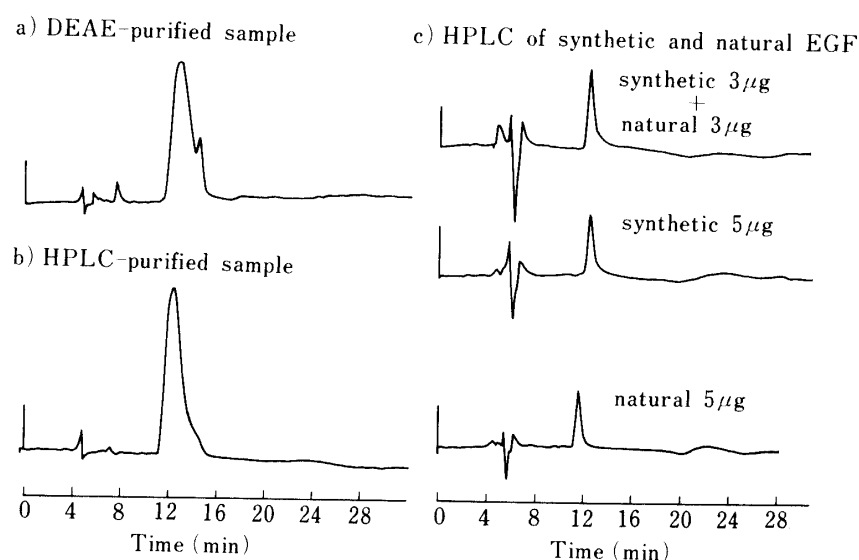


Fig. 12. HPLC of Synthetic EGF on Cosmosil 5C₁₈

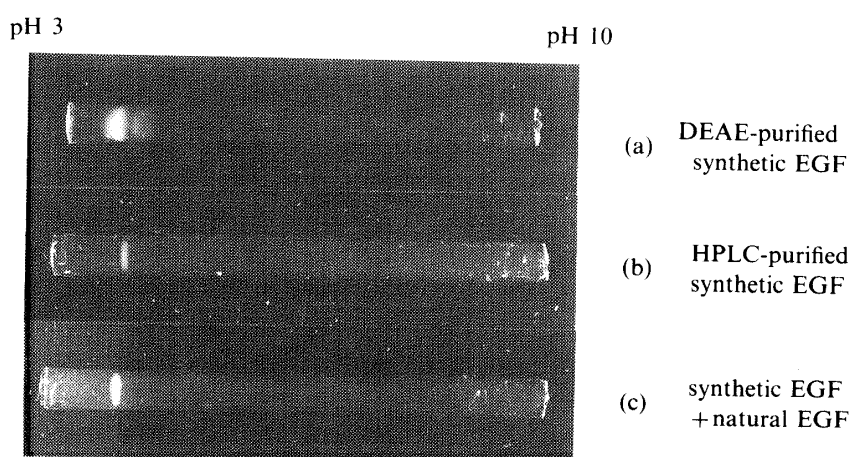


Fig. 13. Disk Isoelectrofocusing of Synthetic EGF

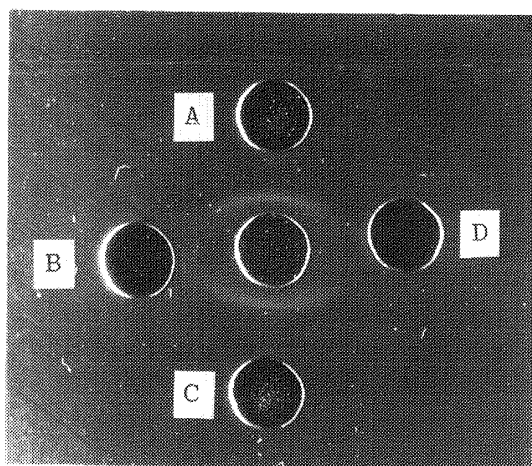


Fig. 14. Immunodiffusion of Synthetic EGF in Agar Gel

A: synthetic EGF 5 μ g. C: synthetic EGF 10 μ g. B, D: blank. Central well: anti EGF sera.

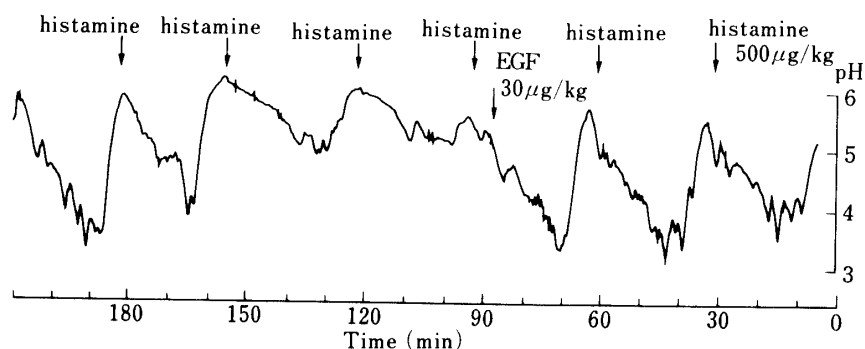


Fig. 15. Effect of Synthetic EGF on Histamine-Stimulated Gastric Acid Secretion in the Rat

conversion of the inactive protected EGF into the active product. Various unsuppressed side reactions involved in the deprotection and the occurrence of inappropriate disulfide bond formation may be the main reasons for the low yield. In addition, we noted that the isolated product has a tendency to self-aggregate in a salt-free state. For example, repeated lyophilization produced more water-insoluble material.

It seems noteworthy that when oxidation was carried out in the presence of reduced and oxidized glutathione, based on the disulfide exchange reaction of RNase A reported by Scheraga *et al.*,³²⁾ a relatively stable glutathione adduct (2 moles of glutathione per mole of EGF) was isolated as a main product at this DEAE-purification step. The anti-gastric activity of this adduct was 1/5 of that of natural EGF.

Examination of the DEAE-purified product by disk isoelectrofocusing (pH 3–10) revealed the presence of a main band matched with that of natural EGF, together with a few adjacent faint bands. In order to remove these impurities, the DEAE-purified product was subjected to reverse phase HPLC on Cosmosil 5C₁₈, according to the method of Matrisian *et al.*³³⁾ When eluted with the solvent system of acetonitrile–triethylammonium acetate buffer, the DEAE-purified product emerged from the column as a single peak with a small shoulder (Fig. 12-a), as revealed by disk isoelectrofocusing. The main product was collected by repeated HPLC under identical conditions and finally lyophilized to give a fluffy powder which showed a single peak on HPLC (Fig. 12-b). The main product thus obtained exhibited the same retention time in HPLC (Fig. 12-c) and the same mobility in disk isoelectrofocusing (Fig. 13) as natural EGF. The product also exhibited a sharp immunodiffusion band against anti-natural EGF sera (Fig. 14). Its amino acid ratios in 6 N HCl hydrolysate were in excellent

agreement with those of natural EGF hydrolyzed under identical conditions (Table I). Its ultraviolet (UV) extinction coefficient coincided with the literature value²⁷⁾ for natural EGF.

When tested in Shild rats³⁴⁾ ($n=6$), our synthetic peptide (30 $\mu\text{g}/\text{kg}$) suppressed the acid output (taken as 100%) stimulated by histamine (500 $\mu\text{g}/\text{kg}$) to 66.5 (± 11.5)%. A typical example is shown in Fig. 15. As a standard, natural EGF (per kg) suppressed the acid output to 78.8% at a dose of 16 μg , 68.6% at 30 μg , and 43.8% at 64 μg .

From the experimental data cited above, we concluded that the air-oxidized product of the synthetic tripentacontapeptide was obtained with a high degree of homogeneity, and showed physicochemical properties and anti-gastric activity comparable to those of natural EGF.

Experimental

General experimental procedures employed in this investigation are essentially the same as described in Part LXXXVIII³⁵⁾ of this series. Unless otherwise mentioned, products were purified by one of the procedures described in the preceding paper.¹⁾ Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck). R_f values refer to the following solvent systems (v/v): R_{f1} CHCl_3 -MeOH (10:0.5), R_{f2} CHCl_3 -MeOH- H_2O (8:3:1), R_{f3} CHCl_3 -MeOH-AcOH (9:1:0.5).

HPLC was conducted with a Waters 204 compact model. Rotation and UV absorption were measured with a Union PM 101 instrument and a Hitachi model 100-20 spectrometer respectively. Natural EGF (lot. 4112) was purchased from Toyobo Co., Ltd. Pharmalyte (pH 3–10) for disc isoelectrofocusing and the wet cellulose dialysis tubing (m.w. cutoff: 2000) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and Spectrum Medical Industries Inc., Los Angeles U.S.A., respectively. Anti-EGF sera were prepared by K. Hayashi³⁶⁾ using mouse EGF isolated according to Savage *et al.*³¹⁾

Z(OMe)-Ser(Bzl)-Tyr-OMe—DCC (7.43 g, 36 mmol) was added to a mixture of H-Tyr-OMe [prepared from 6.95 g (30 mmol) of the hydrochloride] and Z(OMe)-Ser(Bzl)-OH [prepared from 13.76 g (30 mmol) of the CHA salt] in DMF-AcOEt (1:1, 200 ml) and the mixture was stirred for 12 h at room temperature. After filtration, the filtrate was concentrated and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 9.75 g (61%), mp 85–88°C, $[\alpha]_D^{20} +10.8^\circ$ ($c=0.7$, MeOH), R_{f1} 0.58. *Anal.* Calcd for $\text{C}_{29}\text{H}_{32}\text{N}_2\text{O}_8$: C, 64.91; H, 6.01; N, 5.22. Found: C, 65.19; H, 6.09; N, 5.30.

Z(OMe)-Asp(OBu')-Ser(Bzl)-Tyr-OMe—Z(OMe)-Ser(Bzl)-Tyr-OMe (4.20 g, 7.8 mmol) was treated with TFA-anisole (8.5 ml–1.7 ml) in an ice-bath for 60 min, then *n*-hexane was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) containing Et_3N (1.1 ml, 7.8 mmol). A mixed anhydride [prepared from 4.39 g (8.2 mmol) of Z(OMe)-Asp(OBu')-OH·DCHA] in tetrahydrofuran (THF) (30 ml) was added to the above ice-chilled solution and the solution, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure A, followed by recrystallization from AcOEt and isopropyl ether; yield 4.15 g (75%), mp 54–56°C, $[\alpha]_D^{20} -2.7^\circ$ ($c=0.7$, MeOH), R_{f1} 0.33. *Anal.* Calcd for $\text{C}_{37}\text{H}_{45}\text{N}_3\text{O}_{11}$: C, 62.79; H, 6.41; N, 5.94. Found: C, 63.06; H, 6.54; N, 5.94.

Boc-Leu-Asp-Ser(Bzl)-Tyr-OMe—Z(OMe)-Asp(OBu')-Ser(Bzl)-Tyr-OMe (3.20 g, 4.5 mmol) was treated with TFA-anisole (7.3 ml–1.5 ml) in an ice-bath for 30 min, then at room temperature for 3 h to remove the Z(OMe) group first, then the Bu' ether completely. The resulting peptide, H-Asp-Ser(Bzl)-Tyr-OMe, isolated as usual, was dissolved in DMF (20 ml) containing Et_3N (0.6 ml, 4.5 mmol). A mixed anhydride [prepared from 1.96 g (4.8 mmol) of Boc-Leu-OH·DCHA] in THF (20 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure C, followed by column chromatography on silica (3.0 $\times$ 20 cm) using CHCl_3 -MeOH-AcOH (9:1:0.5) as an eluant. The solvent of the desired fraction was removed by evaporation and the residue was triturated with ether to afford a powder; yield 1.91 g (60%), mp 98–101°C, $[\alpha]_D^{20} -21.3^\circ$, R_{f3} 0.57. *Anal.* Calcd for $\text{C}_{35}\text{H}_{48}\text{N}_4\text{O}_{11}$: C, 59.98; H, 6.90; N, 8.00. Found: C, 59.72; H, 7.14; N, 7.80.

Boc-Leu-Asp-Ser(Bzl)-Tyr-NHNH₂ [9]—Boc-Leu-Asp-Ser(Bzl)-Tyr-OMe (1.58 g, 2.3 mmol) in MeOH (15 ml) was treated with 80% hydrazine hydrate (1.4 ml, 23 mmol) overnight. The solvent was evaporated off and the residue was extracted with *n*-BuOH. The organic phase was washed with 5% AcOH and H_2O , and concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from MeOH and isobutyl alcohol; yield 1.30 g (83%), mp 168–170°C, $[\alpha]_D^{26} -30.0^\circ$ ($c=0.8$, DMF), R_{f2} 0.35. Amino acid ratios in 6N HCl hydrolysate: Asp 1.07, Ser 0.80, Leu 0.95, Tyr 1.00 (recovery of Tyr, 92%). *Anal.* Calcd for $\text{C}_{34}\text{H}_{48}\text{N}_6\text{O}_{10}$: C, 58.27; H, 6.90; N, 11.99. Found: C, 58.30; H, 6.99; N, 11.86.

Z(OMe)-Ser(Bzl)-NHNH-Troc—This derivative was prepared by the mixed anhydride procedure from Z(OMe)-Ser(Bzl)-OH [prepared from 15.0 g (32.7 mmol) of the CHA salt] and Troc-NHNH₂ (7.43 g, 35.9 mmol) and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 13.5 g

(75%), mp 91–92 °C, $[\alpha]_D^{15} -3.5^\circ$ ($c=0.6$, MeOH), R_f 0.76. *Anal.* Calcd for $C_{22}H_{24}Cl_3N_3O_7$: C, 48.14; H, 4.41; N, 7.66. Found: C, 48.06; H, 4.23; N, 7.75.

Z(OMe)-Glu(OBzl)-Ser(Bzl)-NHNH-Troc—Z(OMe)-Ser(Bzl)-NHNH-Troc (6.78 g, 12.4 mmol) was treated with TFA-anisole (16.0 ml–4.0 ml), then TFA was removed by evaporation. The oily residue was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (50 ml) together with Et_3N (1.7 ml, 12.4 mmol), NMM (1.4 ml, 13.8 mmol) and Z(OMe)-Glu(OBzl)-ONp (7.10 g, 13.6 mmol). After being stirred for 12 h, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH and ether; yield 7.68 g (81%), mp 73–75 °C, $[\alpha]_D^{15} -11.9^\circ$ ($c=0.6$, MeOH) R_f 0.41. *Anal.* Calcd for $C_{34}H_{37}Cl_3N_4O_{10}$: C, 53.17; H, 4.86; N, 7.30. Found: C, 53.40; H, 4.77; N, 7.44.

Boc-Ile-Glu(OBzl)-Ser(Bzl)-NHNH-Troc—Z(OMe)-Glu(OBzl)-Ser(Bzl)-NHNH-Troc (7.50 g, 9.8 mmol) was treated with TFA-anisole (16 ml–3.2 ml) and the N^{α} -deprotected peptide isolated as stated above was dissolved in DMF (50 ml) together with Et_3N (1.4 ml, 9.8 mmol), NMM (1.1 ml, 10.8 mmol) and Boc-Ile-OSu (3.53 g, 10.8 mmol). After being stirred for 12 h, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH and ether; yield 4.80 g (60%), mp 178–180 °C, $[\alpha]_D^{15} -9.4^\circ$ ($c=0.4$, DMF) R_f 0.35. *Anal.* Calcd for $C_{36}H_{48}Cl_3N_5O_{10}$: C, 52.91; H, 5.92; N, 8.57. Found: C, 53.05; H, 5.89; N, 8.80.

Boc-Ile-Glu(OBzl)-Ser(Bzl)-NHNH₂ [10]—The above Troc derivative (4.00 g, 4.9 mmol) in DMF-AcOH (1:1, 40 ml) was treated with Zn powder (3.20 g, 10 eq) at room temperature overnight. After filtration, the filtrate was concentrated and the residue was treated with 5% EDTA to afford a powder, which was washed with 5% EDTA, 5% $NaHCO_3$ and H_2O , and recrystallized from MeOH and ether; yield 1.71 g (54%), mp 153–155 °C, $[\alpha]_D^{26} -13.9^\circ$ ($c=0.8$, DMF). R_f 0.30. Amino acid ratios in 6N HCl hydrolysate: Ile 1.00, Glu 0.98, Ser 0.84 (recovery of Ile 86%). *Anal.* Calcd for $C_{33}H_{47}N_5O_8 \cdot 2.5H_2O$: C, 57.71; H, 7.63; N, 10.20. Found: C, 57.81; H, 7.15; N, 10.22.

Z(OMe)-Cys(MBzl)-Met-OMe—A mixture of Z(OMe)-Cys(MBzl)-OSu (5.03 g, 10 mmol) and H-Met-OMe [prepared from 3.0 g (15 mmol) of the HCl salt] in DMF (50 ml) was stirred in the presence of Et_3N (3.5 ml, 25 mmol) at room temperature for 12 h. After evaporation of the solvent, the product was purified by procedure A and recrystallized from MeOH and ether; yield 3.71 g (67%), mp 97–99 °C, $[\alpha]_D^{15} -26.1^\circ$ ($c=0.5$, MeOH), R_f 0.78. *Anal.* Calcd for $C_{26}H_{34}N_2O_7S_2$: C, 56.70; H, 6.22; N, 5.09. Found: C, 56.96; H, 5.96; N, 5.06.

Z(OMe)-Val-Cys(MBzl)-Met-OMe—Z(OMe)-Cys(MBzl)-Met-OMe (3.15 g, 5.7 mmol) was treated with TFA-anisole-EDT (8 ml–1.2 ml–0.5 ml) as usual, then excess TFA was removed by evaporation and the residue was washed with *n*-hexane. The oily residue was dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (30 ml) together with Et_3N (1.7 ml, 12.0 mmol) and Z(OMe)-Val-OSu (2.38 g, 6.3 mmol). The mixture was stirred at room temperature for 12 h, then the solvent was removed by evaporation. The residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 2.90 g (78%), mp 169–170 °C, $[\alpha]_D^{15} -40.3^\circ$ ($c=0.6$, DMF), R_f 0.90. *Anal.* Calcd for $C_{31}H_{43}N_3O_8S_2$: C, 57.30; H, 6.67; N, 6.47. Found: C, 57.55; H, 6.54; N, 6.34.

Boc-Gly-Val-Cys(MBzl)-Met-OMe—Z(OMe)-Val-Cys(MBzl)-Met-OMe (2.48 g, 3.8 mmol) was treated with TFA-anisole-EDT (6.0 ml–0.8 ml–0.3 ml) as stated above, then *n*-hexane was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h, then dissolved in DMF (10 ml) together with Et_3N (1.1 ml, 7.9 mmol) and Boc-Gly-OSu (1.14 g, 4.2 mmol). After being stirred at room temperature overnight, the solution was concentrated and the product was purified by procedure B followed by precipitation from MeOH and ether; yield 1.91 g (78%), mp 122–123 °C, $[\alpha]_D^{15} -48.0^\circ$ ($c=0.6$, DMF), R_f 0.29. *Anal.* Calcd for $C_{29}H_{46}N_4O_8S_2$: C, 54.18; H, 7.21; N, 8.72. Found: C, 54.26; H, 7.26; N, 8.71.

Boc-Gly-Val-Cys(MBzl)-Met-NHNH₂ [11]—Boc-Gly-Val-Cys(MBzl)-Met-OMe (1.54 g, 2.4 mmol) in a mixture of DMF and MeOH (1:1, 10 ml) was treated with 80% hydrazine hydrate (1.5 ml, 24 mmol) at room temperature overnight. The solid formed on standing overnight was precipitated from DMF with MeOH; yield 1.31 g (85%), mp 209–212 °C, $[\alpha]_D^{26} -18.1^\circ$ ($c=0.5$, DMSO). R_f 0.68. Amino acid ratios in 6N HCl hydrolysate: Gly 1.00, Val 1.06, Met 0.90 (recovery of Gly 88%). *Anal.* Calcd for $C_{28}H_{46}N_6O_7S_2$: C, 52.31; H, 7.21; N, 13.07. Found: C, 52.56; H, 7.02; N, 13.28.

Z(OMe)-Leu-Asn-Gly-NHNH-Troc—Z(OMe)-Asn-Gly-NHNH-Troc¹⁹⁾ (3.03 g, 5.6 mmol) was treated with TFA-anisole (6.0 ml–1.2 ml) and the N^{α} -deprotected peptide isolated as stated above was dissolved in DMF (20 ml) together with Et_3N (1.7 ml, 11.9 mmol) and Z(OMe)-Leu-ONp (2.56 g, 6.2 mmol). After being stirred for 12 h, the mixture was concentrated and the product was purified by procedure B followed by recrystallization from MeOH and ether; yield 3.38 g (93%), mp 146–149 °C, $[\alpha]_D^{15} -14.5^\circ$ ($c=0.8$, DMSO), R_f 0.68. *Anal.* Calcd for $C_{24}H_{33}Cl_3N_6O_9$: C, 43.94; H, 5.07; N, 12.81. Found: C, 44.13; H, 5.04; N, 12.82.

Boc-Cys(MBzl)-Leu-Asn-Gly-NHNH-Troc—Z(OMe)-Leu-Asn-Gly-NHNH-Troc (3.38 g, 5.2 mmol) was treated with TFA-anisole (8.4 ml–1.7 ml) and the N^{α} -deprotected peptide isolated as stated above was dissolved in DMF (20 ml) together with Et_3N (1.5 ml, 10.6 mmol) and Boc-Cys(MBzl)-OSu (2.49 g, 5.7 mmol). After being stirred for 12 h, the solution was concentrated and the product was purified by procedure B

followed by precipitation from DMF with AcOEt; yield 3.01 g (72%), mp 142–144 °C, $[\alpha]_D^{15} -23.4^\circ$ ($c=0.8$, DMSO), R_f 0.71. *Anal.* Calcd for $C_{31}H_{46}Cl_3N_7O_{10}S \cdot 0.5H_2O$: C, 45.17; H, 5.75; N, 11.90. Found: C, 45.15; H, 5.73; N, 12.11.

Boc-Cys(MBzl)-Leu-Asn-Gly-NHNH₂ [12]—The above protected tetrapeptide derivative (3.51 g, 4.3 mmol) in a mixture of DMF-AcOH (1:1, 40 ml) was treated with Zn powder (2.81 g, 43 mmol) at room temperature overnight. After filtration of the mixture, the filtrate was concentrated down. The residue was treated with 5% EDTA and the resulting powder was washed with 5% EDTA, 5% NaHCO₃ and H₂O. The powder was purified by precipitation from DMF with MeOH; yield 2.15 g (78%), mp 196–198 °C, $[\alpha]_D^{26} -19.1^\circ$ ($c=0.7$, DMSO). R_f 0.47. Amino acid ratios in 6N HCl hydrolysate: Asp 1.04, Gly 1.00, Leu 1.04 (recovery of Gly, 84%). *Anal.* Calcd for $C_{28}H_{45}N_7O_8S \cdot 0.5H_2O$: C, 51.83; H, 7.15; N, 15.11. Found: C, 52.09; H, 7.17; N, 15.14.

Z(OMe)-Gly-Tyr-OMe—DCC (13.62 g, 66 mmol) was added to a mixture of Z(OMe)-Gly-OH (14.35 g, 60 mmol) and H-Tyr-OMe [prepared from 20.85 g (90 mmol) of the HCl salt] in DMF (300 ml). After being stirred overnight, the solution was filtered and the filtrate was concentrated. The product was purified by procedure A followed by recrystallization twice from MeOH and ether; yield 20.10 g (81%), mp 88–90 °C, $[\alpha]_D^{15} +9.8^\circ$ ($c=0.6$, DMF). R_f 0.27. *Anal.* Calcd for $C_{21}H_{24}N_2O_7$: C, 60.57; H, 5.81; N, 6.73. Found: C, 60.72; H, 6.02; N, 7.03.

Z(OMe)-Asp(OBzl)-Gly-Tyr-OMe—Z(OMe)-Gly-Tyr-OMe (7.0 g, 16.8 mmol) was treated with TFA-anisole (18 ml–3.6 ml) and the N²-deprotected peptide isolated as stated above was dissolved in DMF (60 ml) containing Et₃N (2.4 ml, 16.8 mmol). A mixed anhydride of Z(OMe)-Asp(OBzl)-OH [prepared from 10.0 g (17.6 mmol) of the DCHA salt] in THF (100 ml) was added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 4 h, was concentrated. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 8.45 g (81%), mp 84–86 °C, $[\alpha]_D^{15} -3.2^\circ$ ($c=0.6$, DMF) R_f 0.28. *Anal.* Calcd for $C_{32}H_{35}N_3O_{10}$: C, 61.83; H, 5.68; N, 6.76. Found: C, 61.78; H, 5.96; N, 6.58.

Boc-Tyr-Asp-Gly-Tyr-OMe—Z(OMe)-Asp(OBzl)-Gly-Tyr-OMe (8.03 g, 12.9 mmol) in MeOH (80 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 60 min, then the solution was filtered. The filtrate was concentrated and the residue was treated with TFA-anisole (17 ml–4.2 ml) as usual. The N²-deprotected peptide, precipitated with *n*-hexane, was dissolved in DMF (80 ml) containing Et₃N (1.8 ml, 12.9 mmol). The azide [prepared from 3.81 g (12.9 mmol) of Boc-Tyr-NHNH₂] in DMF (30 ml) and Et₃N (2.0 ml, 14.3 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was concentrated. The residue was dissolved in 5% NaHCO₃. The aqueous phase, after being washed with AcOEt, was acidified with citric acid, and the resulting precipitate was extracted with *n*-BuOH. The organic phase was washed with H₂O and concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from MeOH and CHCl₃; yield 5.01 g (62%), mp 127–130 °C, $[\alpha]_D^{15} -9.3^\circ$ ($c=0.5$, DMF), R_f 0.35. *Anal.* Calcd for $C_{30}H_{38}N_4O_{11} \cdot H_2O$: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.37; H, 6.47; N, 8.80.

Boc-Tyr-Asp-Gly-Tyr-NHNH₂ [13]—Boc-Tyr-Asp-Gly-Tyr-OMe (4.85 g, 7.7 mmol) in MeOH (50 ml) was treated with 80% hydrazine hydrate (2.5 ml, 77.0 mmol) for 24 h. After evaporation of the solvent, the residue was extracted with *n*-BuOH. The organic phase was washed with 5% AcOH and H₂O, then concentrated. The residue was triturated with ether to afford a powder; yield 3.11 g (64%), mp 205 °C (dec.), $[\alpha]_D^{26} -147.0^\circ$ ($c=0.5$, DMF), R_f 0.013. Amino acid ratios in 6N HCl hydrolysate: Asp 1.01, Gly 1.00, Tyr 1.93 (recovery of Gly, 86%). *Anal.* Calcd for $C_{29}H_{38}N_6O_{10} \cdot 4H_2O$: C, 49.56; H, 6.60; N, 11.96. Found: C, 49.26; H, 6.59; N, 11.74.

Z(OMe)-Ser(Bzl)-Ser(Bzl)-OH · DCHA—Z(OMe)-Ser(Bzl)-OSu (7.66 g, 16.8 mmol) and Et₃N (2.4 ml, 17.1 mmol) were added to a solution of H-Ser(Bzl)-OH (2.99 g, 15.3 mmol) and Et₃N (2.1 ml, 15.3 mmol) in DMF (50 ml). After being stirred at room temperature for 12 h, the solution was concentrated and the product was purified by procedure C, followed by conversion to the corresponding DCHA salt as usual. The salt was recrystallized from MeOH and ether; yield 7.10 g (87%), mp 117–120 °C, $[\alpha]_D^{15} +14.1^\circ$ ($c=0.3$, DMF), R_f 0.75. *Anal.* Calcd for $C_{41}H_{55}N_3O_8$: C, 68.59; H, 7.72; N, 5.85. Found: C, 68.60; H, 7.80; N, 5.83.

Z(OMe)-Ser(Bzl)-Ser(Bzl)-OMe—Z(OMe)-Ser(Bzl)-Ser(Bzl)-OH [derived from 5.00 g (7.0 mmol) of the DCHA salt] in MeOH-DMF (2:1, 30 ml) was treated with ethereal diazomethane as usual. After addition of a few drops of AcOH, the solvent was evaporated off and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 3.35 g (87%), mp 109–111 °C, $[\alpha]_D^{15} +13.8^\circ$ ($c=0.7$, DMF), R_f 0.85. *Anal.* Calcd for $C_{30}H_{34}N_2O_8$: C, 65.44; H, 6.22; N, 5.09. Found: C, 65.47; H, 6.16; N, 5.11.

Z(OMe)-Pro-Ser(Bzl)-Ser(Bzl)-OMe—Z(OMe)-Ser(Bzl)-Ser(Bzl)-OMe (4.30 g, 7.8 mmol) was treated with TFA-anisole (8.4 ml–1.7 ml) and the N²-deprotected peptide isolated as usual was dissolved in DMF (30 ml) containing Et₃N (1.1 ml, 7.8 mmol). A mixed anhydride [prepared from 4.30 g (9.3 mmol) of Z(OMe)-Pro-OH · DCHA salt] in THF (30 ml) was added to the above ice-chilled solution, and the mixture, after

being stirred in an ice-bath for 5 h, was concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 3.90 g (77%), mp 125–127°C, $[\alpha]_D^{15} -16.2^\circ$ ($c=1.0$, DMF), Rf_1 0.61. *Anal.* Calcd for $C_{35}H_{41}N_3O_9$: C, 64.90; H, 6.38; N, 6.49. Found: C, 64.96; H, 6.36; N, 6.55.

Boc-Cys(MBzl)-Pro-Ser(Bzl)-Ser(Bzl)-NHNH₂ [14]—Z(OMe)-Pro-Ser(Bzl)-Ser(Bzl)-OMe (2.31 g, 3.6 mmol) was treated with TFA-anisole (5.8 ml–1.2 ml) and the N²-deprotected peptide isolated as usual was dissolved in DMF (20 ml) together with Et₃N (1.1 ml, 7.5 mmol) and Boc-Cys(MBzl)-OSu (1.72 g, 3.9 mmol). The mixture was stirred overnight and the solvent was removed by evaporation. The product was purified by procedure A. The oily product was dissolved in MeOH (20 ml) and treated with 80% hydrazine hydrate (2.2 ml, 36 mmol) for 24 h. The solvent was evaporated off and the residue was extracted with *n*-BuOH. The organic phase was washed with H₂O and concentrated. The residue was purified by column chromatography on silica (3×15 cm) using CHCl₃-MeOH (10:0.5) as an eluant. The solvent of the desired fractions was removed by evaporation and the residue was treated with isopropyl ether to afford a powder; yield 1.46 g (51%), mp 96–99°C, $[\alpha]_D^{26} -4.0^\circ$ ($c=0.5$, DMF), Rf_1 0.24. Amino acid ratios in 6N HCl hydrolysate: Pro 1.00, Ser 1.81 (recovery of Pro 77%). *Anal.* Calcd for $C_{41}H_{54}N_6O_9S \cdot 0.5H_2O$: C, 60.35; H, 6.79; N, 10.30. Found: C, 60.25; H, 6.70; N, 10.21.

Boc-Tyr-Pro-Gly-OMe—DCC (4.77 g, 23.1 mmol) was added to a mixture of Boc-Tyr-Pro-OH⁽²⁰⁾ (7.95 g, 21 mmol) and HOSu (2.66 g, 23.1 mmol) in THF (100 ml). The mixture was stirred at room temperature overnight and filtered. To this filtrate, H-Gly-OMe [prepared from 3.96 g (31.5 mmol) of the hydrochloride] in DMF (100 ml) and Et₃N (2.9 ml, 21 mmol) were added, and the mixture was stirred at room temperature overnight. The solvent was evaporated off and the residue was purified by procedure A followed by precipitation from MeOH and isopropyl ether; yield 6.41 g (68%), mp 74–76°C, $[\alpha]_D^{15} -39.5^\circ$ ($c=0.4$, DMF), Rf_1 0.28. *Anal.* Calcd for $C_{22}H_{31}N_3O_7 \cdot 0.5H_2O$: C, 57.63; H, 7.04; N, 9.17. Found: C, 57.96; H, 7.30; N, 8.57.

Boc-Asn-Ser(Bzl)-OH—Boc-Asn-ONp (8.48 g, 24.0 mmol) and Et₃N (3.4 ml, 24.0 mmol) were added to a solution of H-Ser(Bzl)-OH (4.26 g, 21.8 mmol) in DMF (100 ml). After being stirred at room temperature for 12 h, the solvent was evaporated off and the product was purified by procedure C followed by precipitation from MeOH and ether; yield 6.05 g (68%), mp 182–184°C, $[\alpha]_D^{15} +4.9^\circ$ ($c=0.4$, DMF), Rf_3 0.44. *Anal.* Calcd for $C_{19}H_{27}N_3O_7$: C, 55.73; H, 6.65; N, 10.26. Found: C, 55.86; H, 6.65; N, 10.36.

Boc-Asn-Ser(Bzl)-OMe—Boc-Asn-Ser(Bzl)-OH (7.0 g, 17.1 mmol) in MeOH-DMF (1:1, 70 ml) was treated with ethereal diazomethane as usual. After addition of a few drops of AcOH, the solvent was evaporated off and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 6.60 g (91%), mp 97–100°C, $[\alpha]_D^{15} -14.4^\circ$ ($c=0.6$, DMF), Rf_3 0.66. *Anal.* Calcd for $C_{20}H_{29}N_3O_7$: C, 56.72; H, 6.90; N, 9.92. Found: C, 56.47; H, 6.99; N, 9.82.

Boc-Asn-Ser(Bzl)-NHNH₂—Boc-Asn-Ser(Bzl)-OMe (6.5 g, 15.3 mmol) in MeOH (60 ml) was treated with 80% hydrazine hydrate (9.6 ml, 10 eq) at room temperature overnight. The resulting solid was recrystallized from DMF with MeOH; yield 4.50 g (69%), mp 173–176°C, $[\alpha]_D^{15} -15.0^\circ$ ($c=0.4$, DMSO), Rf_2 0.56. *Anal.* Calcd for $C_{19}H_{29}N_5O_6$: C, 53.89; H, 6.90; N, 16.54. Found: C, 53.93; H, 7.11; N, 16.67.

Boc-Asn-Ser(Bzl)-Tyr-Pro-Gly-OMe—Boc-Tyr-Pro-Gly-OMe (2.50 g, 5.6 mmol) was treated with TFA-anisole (5.0 ml–1.2 ml) and the N²-deprotected peptide isolated as usual was dissolved in DMF (30 ml) containing Et₃N (0.78 ml, 5.6 mmol). The azide [prepared from 2.59 g (6.1 mmol) of Boc-Asn-Ser(Bzl)-NHNH₂] in DMF (20 ml) and Et₃N (0.94 ml, 6.7 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 12 h. The solvent was evaporated off and the residue was extracted with *n*-BuOH. The organic phase was washed with 5% citric acid and H₂O, and then concentrated. Trituration of the residue with ether afforded a powder which was precipitated from MeOH with isopropyl ether; yield 3.70 g (90%), mp 100–103°C, $[\alpha]_D^{15} -41.4^\circ$ ($c=0.3$, DMF), Rf_2 0.59. *Anal.* Calcd for $C_{36}H_{48}N_6O_{11} \cdot 0.5H_2O$: C, 57.66; H, 6.59; N, 11.21. Found: C, 57.59; H, 6.50; N, 11.30.

Boc-Asn-Ser(Bzl)-Tyr-Pro-Gly-NHNH₂ [15]—Boc-Asn-Ser(Bzl)-Tyr-Pro-Gly-OMe (2.70 g, 3.6 mmol) in MeOH (30 ml) was treated with 80% hydrazine hydrate (2.3 ml, 10 eq) at room temperature overnight. The solution was concentrated and the residue was extracted with *n*-BuOH. The organic phase was washed with H₂O and then concentrated. The resulting residue was precipitated from MeOH and ether; yield 2.52 g (93%), mp 130–133°C $[\alpha]_D^{26} -10.6^\circ$ ($c=0.9$, DMF), Rf_2 0.42. Amino acid ratios in 6N HCl hydrolysate: Asp 1.09, Ser 0.96, Pro 1.00, Gly 1.00, Tyr 0.94 (recovery of Gly, 74%). *Anal.* Calcd for $C_{35}H_{48}N_8O_{10} \cdot 3H_2O$: C, 52.88; H, 6.85; N, 14.10. Found: C, 52.57; H, 6.39; N, 14.15.

Boc-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 26–53)-OBzl]—The above tetracosapeptide ester (3.50 g, 0.80 mmol) was treated with TFA-anisole-EDT (10.0 ml–2.1 ml–0.40 ml) and the N²-deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 30 ml) containing Et₃N (112 μl, 0.80 mmol). The azide [prepared from 0.84 g (1.20 mmol) of Boc-Leu-Asp-Ser(Bzl)-Tyr-NHNH₂] in DMF (7 ml) and Et₃N (184 μl, 1.31 mmol) were added and the mixture was stirred at 4°C for 12 h. Additional azide [prepared from 0.28 g (0.40 mmol) of Boc-Leu-Asp-Ser(Bzl)-Tyr-

NHNH₂] and Et₃N (56 μ l, 0.40 mmol) were added. After being stirred for an additional 12 h, the solvent was evaporated off and the residue was purified by procedure B followed by precipitation twice from DMF–DMSO (1:1) with MeOH; yield 3.08 g (78%), mp 254 °C (dec.), $[\alpha]_D^{28}$ –36.5° (c =0.2, DMSO), R_f origin. *Anal.* Calcd for C₂₃₉H₃₁₈N₄₄O₅₈S₇·5H₂O: C, 56.84; H, 6.55; N, 12.21. Found: C, 56.93; H, 6.48; N, 12.27.

Boc-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 23–53)-OBzl]—The above protected octacosapeptide ester (3.08 g, 0.62 mmol) was treated with TFA–anisole–EDT (9 ml–1.6 ml–0.31 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1:1, 30 ml) containing Et₃N (87 μ l, 0.62 mmol). The azide [prepared from 0.68 g (1.06 mmol) of Boc-Ile-Glu(OBzl)-Ser(Bzl)-NHNH₂] in DMF (2 ml) and Et₃N (163 μ l, 1.16 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 12 h. Fresh azide [prepared from 0.40 g (0.62 mmol) of the hydrazide] in DMF (1 ml) and Et₃N (87 μ l, 0.62 mmol) were added. The whole was stirred for an additional 12 h, the solvent was removed by evaporation, and the residue was purified by procedure B followed by precipitation twice from a mixture of DMSO–DMF (1:1) with MeOH; yield 3.04 g (89%), mp 251 °C (dec.), $[\alpha]_D^{28}$ –23.7° (c =0.3, DMSO), R_f origin. *Anal.* Calcd for C₂₆₇H₃₅₃N₄₇O₆₄S₇·8H₂O: C, 57.12; H, 6.62; N, 11.74. Found: C, 57.08; H, 6.56; N, 11.79.

Boc-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 22–53)-OBzl]—The above protected untriacontapeptide ester (3.00 g, 0.55 mmol) was treated with TFA–anisole–EDT (8.5 ml–1.4 ml–0.28 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1:1, 20 ml) containing Et₃N (77 μ l, 0.55 mmol). The azide [prepared from 0.30 g (1.10 mmol) of Boc-His-NHNH₂] in DMF (3 ml) and Et₃N (169 μ l, 1.2 mmol) were added and the mixture was stirred at 4 °C for 12 h. Fresh azide [prepared from 0.15 g (0.55 mmol) of the hydrazide] and Et₃N (77 μ l, 0.55 mmol) were added and the reaction mixture, after being stirred for an additional 12 h, was concentrated. Treatment of the residue with H₂O afforded a powder, which was purified by repeated precipitation from DMSO–DMF (1:1) with MeOH; yield 2.99 g (97%), mp 254 °C (dec.), $[\alpha]_D^{28}$ –25.5° (c =0.4, DMSO), R_f origin. *Anal.* Calcd for C₂₇₃H₃₆₀N₅₀O₆₅S₇·7H₂O: C, 57.19; H, 6.58; N, 12.22. Found: C, 57.20; H, 6.54; N, 12.19.

Boc-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 18–53)-OBzl]—The above protected dotriacontapeptide ester (2.93 g, 0.52 mmol) was treated with TFA–anisole–EDT (10 ml–1.6 ml–0.31 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1:1, 20 ml) containing Et₃N (73 μ l, 0.52 mmol). The azide [prepared from 0.67 g (1.04 mmol) of Boc-Gly-Val-Cys(MBzl)-Met-NHNH₂] in DMF (6 ml) and Et₃N (161 μ l, 1.15 mmol) were added and the mixture was stirred at 4 °C for 12 h. Additional azide [prepared from 0.34 g (0.52 mmol) of the hydrazide] in DMF (3 ml) and Et₃N (73 μ l, 0.52 mmol) were added and the mixture, after being stirred for an additional 12 h, was concentrated. Trituration of the residue with H₂O afforded a powder, which was precipitated twice from DMSO–DMF (1:1) with MeOH; yield 3.10 g (97%), mp 268 °C (dec.), $[\alpha]_D^{28}$ –24.8° (c =0.4, DMSO), R_f origin. *Anal.* Calcd for C₂₉₆H₃₉₄N₅₄O₇₀S₉·7H₂O: C, 56.94; H, 6.59; N, 12.12. Found: C, 56.90; H, 6.62; N, 12.03.

Boc-Cys(MBzl)-Leu-Asn-Gly-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 14–53)-OBzl]—The above protected hexatriacontapeptide, Boc-(EGF 18–53)-OBzl (3.27 g, 0.54 mmol), was treated with TFA–anisole–EDT (11 ml–1.9 ml–0.36 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1:1, 20 ml) containing Et₃N (75 μ l, 0.54 mmol). The azide [prepared from 0.68 g (1.08 mmol) of Boc-Cys(MBzl)-Leu-Asn-Gly-NHNH₂] in DMF (7 ml) and Et₃N (165 μ l, 1.18 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 12 h. One equivalent of the azide [prepared from 0.34 g (0.54 mmol) of the hydrazide] was added. The whole was stirred for an additional 12 h, the solvent was evaporated off and H₂O was added to precipitate the product. The resulting powder was precipitated from DMSO–DMF (1:1) with MeOH and further purified by gel-filtration on Sephadex LH-60, using DMSO–DMF (3:7) as an eluant. The desired product was precipitated from DMF–DMSO (1:1) with MeOH; yield 2.15 g (61%), mp 252 °C (dec.), $[\alpha]_D^{28}$ –17.8° (c =0.3, DMSO), R_f origin. *Anal.* Calcd for C₃₁₉H₄₂₇N₅₉O₇₆S₁₀·12H₂O: C, 56.00; H, 6.65; N, 12.08. Found: C, 55.78; H, 6.51; N, 11.99.

Boc-Tyr-Asp-Gly-Tyr-Cys(MBzl)-Leu-Asn-Gly-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 10–53)-OBzl]—The above peptide, Boc-(EGF 14–53)-OBzl (1.05 g, 0.16 mmol), was treated with TFA–anisole–EDT (5.0 ml–0.55 ml–0.11 ml) and the N^α-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (1:1, 10 ml) containing Et₃N (22 μ l, 0.16 mmol). The azide [prepared from 0.40 g (0.64 mmol) of

Boc-Tyr-Asp-Gly-Tyr-NHNH₂] in DMF (4 ml) and Et₃N (98 μ l, 0.70 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 12 h. Additional azide [prepared from 0.30 g (0.48 mmol) of the hydrazide] and Et₃N (67 μ l, 0.48 mmol) were added and stirring was continued for an additional 12 h. The solvent was evaporated off and H₂O was added. The resulting powder was precipitated twice from DMSO-DMF (1:1) with MeOH; yield 1.11 g (98%), mp 250 °C (dec.), $[\alpha]_D^{28} -32.3^\circ$ ($c=0.1$, DMSO), R_f origin. *Anal.* Calcd for C₃₄₃H₄₅₃N₆₃O₈₄S₁₀ · 12H₂O: C, 56.10; H, 6.55; N, 12.02. Found: C, 55.84; H, 6.30; N, 11.96.

Boc-Cys(MBzl)-Pro-Ser(Bzl)-Ser(Bzl)-Tyr-Asp-Gly-Tyr-Cys-(MBzl)-Leu-Asn-Gly-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 6-53)-OBzl]—Boc-(EGF 10-53)-OBzl (0.95 g, 0.13 mmol) was treated with TFA-anisole-EDT (5.0 ml-0.52 ml-0.10 ml) and the N²-deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 10 ml) containing Et₃N (19 μ l, 0.13 mmol). The azide [prepared from 0.54 g (0.67 mmol) of Boc-Cys(MBzl)-Pro-Ser(Bzl)-Ser(Bzl)-NHNH₂] and Et₃N (103 μ l, 0.74 mmol) were added and the mixture was stirred at 4 °C for 12 h. Additional azide [prepared from 0.42 g (0.52 mmol) of the hydrazide] and Et₃N (73 μ l, 0.52 mmol) were added and the stirring was continued for a further 12 h. The solvent was evaporated off and the residue was treated with H₂O. The resulting precipitate was precipitated twice from DMSO-DMF (1:1) with MeOH; yield 1.02 g (98%), mp 242 °C (dec.), $[\alpha]_D^{28} -7.4^\circ$ ($c=0.1$, DMSO), R_f origin. *Anal.* Calcd for C₃₇₉H₄₉₅N₆₇O₉₁S₁₁ · 16H₂O: C, 56.29; H, 6.57; N, 11.61. Found: C, 56.05; H, 6.16; N, 11.75.

Boc-Asn-Ser(Bzl)-Tyr-Pro-Gly-Cys(MBzl)-Pro-Ser(Bzl)-Ser(Bzl)-Tyr-Asp-Gly-Tyr-Cys(MBzl)-Leu-Asn-Gly-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzl)-Ser(Bzl)-Leu-Asp-Ser(Bzl)-Tyr-Thr-Cys(MBzl)-Asn-Cys-(MBzl)-Val-Ile-Gly-Tyr-Ser-Gly-Asp-Arg(Mts)-Cys(MBzl)-Gln-Thr-Arg(Mts)-Asp(OBzl)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzl)-Leu-Arg(Mts)-OBzl [Boc-(EGF 1-53)-OBzl]—The above peptide, Boc-(EGF 6-53)-OBzl (1.02 g, 0.13 mmol) was treated with TFA-anisole-EDT (5.0 ml-0.57 ml-0.11 ml) and the N²-deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 10 ml) containing Et₃N (18 μ l, 0.13 mmol). The azide [prepared from 0.58 g (0.78 mmol) of Boc-Asn-Ser(Bzl)-Tyr-Pro-Gly-NHNH₂] and Et₃N (121 μ l, 0.86 mmol) were added and the mixture was stirred at 4 °C for 12 h. Additional azide [prepared from 0.39 g (0.52 mmol) of the hydrazide] and Et₃N (73 μ l, 0.52 mmol) were added and stirring was continued for a further 12 h. The solution was concentrated and H₂O was added. The resulting powder was precipitated from DMSO-DMF (1:1) with MeOH and further purified by gel-filtration on Sephadex LH-60 using the solvent system of DMSO-DMF (3:7) as an eluant. The desired fractions (9 ml each, front peak tube Nos. 53-70) were combined, the solvent was removed by evaporation and the residue was precipitated from DMSO-DMF (1:1) with MeOH; yield 0.94 g (85%), mp 251 °C (dec.), $[\alpha]_D^{28} -40.0^\circ$ ($c=0.2$, DMSO), R_f origin. *Anal.* Calcd for C₄₀₉H₅₃₁N₇₃O₉₉S₁₁ · 15H₂O: C, 56.61; H, 6.52; N, 11.79. Found: C, 56.34; H, 6.14; N, 11.90.

Reduction of the Sulfoxide of Cys(MBzl) residues of the Protected EGF—Thiophenol (6.9 ml, 600 eq) was added to a solution of the protected EGF (940 mg, 0.11 mmol) in HMPA-DMF (1:1, v/v, 10 ml) and the solution was incubated under an argon gas atmosphere at 40 °C for 4 d. A reference sample of Z(OMe)-Cys(MBzl)(O)-OH¹⁵ was completely reduced under identical conditions, as was Z(OMe)-Met(O)-OH. The solution was concentrated *in vacuo* and AcOEt was added. The resulting powder was precipitated from HMPA with AcOEt to give the reduced, protected EGF; yield 870 mg (93%), mp 255 °C (dec.), $[\alpha]_D^{20} -43.0^\circ$ ($c=0.1$, DMSO). Recoveries of cystine in an acid hydrolysate of the protected EGF, before and after reduction, were 1.91 and 2.20 (theoretical value 3), respectively.

Deprotection of the Reduced Protected EGF—The thiophenol-reduced form of the protected EGF (100 mg, 11.9 μ mol) was treated with 1 M TFMSA-thioanisole (mole ratio 1:1) in TFA (4.2 ml) in the presence of *m*-cresol (249 μ l, 200 eq) and EDT (50 μ l, 50 eq) in an ice-bath for 120 min, then dry ether was added and the precipitate was dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice more to ensure complete deprotection and the resulting deprotected peptide was dissolved in a solution of 0.1 M Tris-HCl buffer (5 ml) containing 6 M guanidine-HCl (5 ml). After being adjusted to pH 8.0 with 10% MeNH₂, the solution was incubated with dithiothreitol (DTT) (550 mg, 300 eq) under an argon gas atmosphere at 25 °C overnight.

Air-Oxidation of the Deprotected Peptide—The above solution was adjusted to pH 4 with 1 N HCl and applied to a column of Sephadex G-10 (2.8 × 68 cm), which was eluted with 2 N AcOH. The UV absorption at 280 nm was determined in each fraction (6 ml). The fractions corresponding to the front main peak (tube Nos. 22-40) were combined and the combined solution was diluted with ice-chilled water (700 ml). The pH was adjusted to 8.2 with conc. NH₄OH, and the solution was brought up to 1000 ml with ice-chilled water under gentle stirring in an ice-bath (final peptide concentration 0.07 mg per ml). The solution was kept standing at room temperature for 5 d, during which time the Ellman test value (absorption at 412 nm) dropped from 0.082 to a constant value of 0.014 (Fig. 10-a). The solution was then stirred gently for an additional 2 d. The pH was adjusted to 6.6 with 10% AcOH, and the entire solution was lyophilized. The residue was dissolved in 2 N AcOH (5 ml) and some insoluble material (*ca.* 7 mg) was removed by centrifugation. The supernatant solution was applied to a column of Sephadex G-10 (2.8 × 81 cm), which was eluted with the same solvent.

Individual fractions (each 6 ml) were collected and their absorptions at 280 nm were determined. The fractions corresponding to the main peak (Fig. 10-b, tube Nos. 31–48) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 23 mg (32%). In the preliminary run, the 5 d oxidized product gave a peak with 2 shoulders on Sephadex G-10 (Fig. 10-b, dotted line).

Purification of the G-10-Purified Product by Ion-exchange Chromatography on DEAE-Cellulose—The crude 7 d oxidized product (4.07 mg) was dissolved in 2 N AcOH (0.3 ml). After being adjusted to pH 7.0 with 5% NH_4OH , then diluted with pH 5.6, 0.02 M AcONH_4 buffer (30 ml), the solution was applied to a column of DEAE-cellulose (1.6 $\times$ 5 cm) equilibrated with the above pH 5.6, 0.02 M AcONH_4 buffer. The column was first eluted with the above starting buffer (75 ml) and then with a gradient formed from pH 5.6, 0.2 M AcONH_4 buffer (150 ml) through a mixing flask containing the starting buffer (50 ml). Individual fractions (5.0 ml each) were collected and the absorption at 280 nm was determined (Fig. 11). After the flow-through peak (peak I, tube Nos. 4–18), the desired peak (peak II, tube Nos. 23–26) with a small shoulder was detected in the gradient eluates. The maximum of this peak (tube No. 25) coincided with that of natural EGF under identical elution conditions. Fractions corresponding to peak II were combined and the solvent and AcONH_4 salt were removed by repeated lyophilization to give a white fluffy powder; yield 0.61 mg (15%). Lyophilization of the fractions corresponding to peak I gave a slightly yellowish powder; yield 0.69 mg. This material was devoid of antigastric activity.

The rest of the crude G-10 purified sample (18.9 mg) was similarly purified; total yield 3.70 mg from 23 mg (16%).

HPLC Purification of the DEAE-Purified Product—Subsequent purification was performed by reverse-phase HPLC on a Cosmosil 5C₁₈ (4.6 $\times$ 150 mm) column. The above DEAE-purified sample (3.65 mg) was dissolved in 1 N AcOH (0.5 ml). The solution was diluted with H_2O (1.5 ml), the pH was adjusted to 5.8 with 1 N NH_4OH , and a part of the solution (200 μl aliquot) was applied to the above column, which was eluted isocratically (flow rate, 0.4 ml per min) with 30% acetonitrile in 0.05 M $\text{AcOH-Et}_3\text{N}$ (pH 5.6) at 40 °C. The eluates corresponding to the main peak (retention time 12.5 min, Fig. 12-a) were collected. The rest of the sample was similarly purified and the combined eluates were lyophilized to give a white fluffy powder; yield 3.0 mg (80%) (overall yield from the protected EGF, 4.1%).

The purity of the product obtained here was estimated by HPLC to be more than 98% (Fig. 12-b) and the purified peptide exhibited the same retention time as natural EGF in HPLC on Cosmosil 5C₁₈ under identical elution conditions (Fig. 12-c). The HPLC-purified sample thus obtained behaved as a single component in disk isoelectrofocusing on 7.5% polyacrylamide (0.5 $\times$ 7.0 cm) containing Pharmalyte (pH 3–10): mobility, 1.0 cm from the origin toward the cathodic end of the gel (isoelectric point 4.6), after running at 200 V for 4 h (Fig. 13). The sample was fixed with 10% trichloroacetic acid as a white band. Staining with Coomassie Brilliant Blue G-250 gave a faint band, because EGF contains no Lys residue. Disk isoelectrofocusing of a mixture of synthetic EGF and natural EGF (20 μg each) in the same tube confirmed that they had identical mobility (Fig. 13). UV: $E_{1\text{cm}}^{1\%}$ at 280 nm 30.1 (lit.²⁷⁾ 30.9), $[\alpha]_{\text{D}}^{20}$ -105.9° ($c=0.2$, 2 N AcOH). Amino acid ratios in 6 N HCl hydrolysate are shown in Table I.

Trials of Air-Oxidation of the Deprotected Peptide—a) Air-Oxidation through Dialysis: The thiophenol-treated, protected EGF (30 mg, 3.57 μmol) was deprotected and reduced with DTT (165 mg, 300 eq) in 0.1 M Tris-HCl buffer (3 ml) containing 6 M guanidine-HCl at pH 8.0 overnight as described above. After being diluted with the same buffer (3 ml), then adjusted to pH 4.0 with 1 N HCl, the solution was placed in a wet cellulose

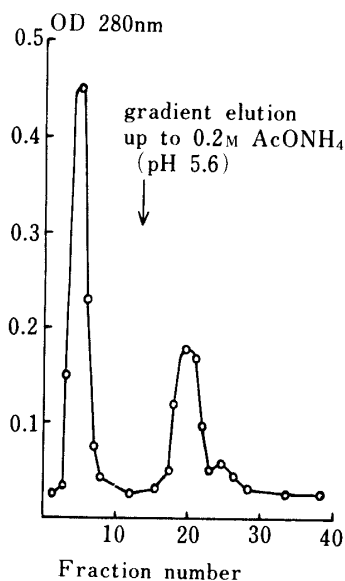


Fig. 16. DEAE Purification of the EGF-Glutathione Adduct

tube (Spectrum), which was dialyzed against a solution of pH 1.5, 0.05 N HCl–0.15 M NaCl (100 ml) at 4 °C for 2 h. Dialysis was repeated twice more. During that time, the solution became pasty due to the formation of an insoluble material. The content of the tube was poured into 0.01 M AcONH₄ (850 ml), then the pH was adjusted to 8.0 with 5% NH₄OH. The Ellman test value (412 nm) dropped from 0.070 to 0.043, while the solution was kept standing at 25 °C for 2 d. The entire solution was lyophilized to give a powder, which was mostly insoluble in pH 6.5, 0.02 M AcONH₄; the insoluble material amounted to 26 mg. Purification of this product was abandoned.

b) **Air-Oxidation in the Presence of Glutathione:** The thiophenol-reduced form of protected EGF (100 mg, 11.9 μmol) was deprotected and incubated with DTT (550 mg, 300 eq) in 0.1 M Tris-HCl buffer (5 ml) containing 6 M guanidine-HCl at pH 8.0 as described above. After being adjusted to pH 4.0 with 1 N HCl, the solution was gel-filtered on Sephadex G-10 (2.8 × 61 cm) using 2 N AcOH as an eluant. The fractions corresponding to the front main peak determined by UV absorption measurement (280 nm) were combined and the combined solution was diluted with ice-chilled water to 300 ml (final protein concentration, 0.24 mg per ml). After addition of reduced glutathione (37 mg, 10 eq) and oxidized glutathione (73 mg, 10 eq), the solution was adjusted to pH 8.2 with conc. NH₄OH in an ice-bath and left to stand at 25 °C for 5 d. Some insoluble material, deposited from the solution, was removed by centrifugation and the entire solution was lyophilized. The residue was dissolved in 2 N AcOH (2 ml) and applied to a column of Sephadex G-10 (2.8 × 61 cm), which was eluted with the same solvent. Individual fractions (6.2 ml each) were collected and their absorptions at 280 nm were determined. The fractions corresponding to the main peak (tube Nos. 24–32) were combined and the solvent was removed by lyophilization to give a hygroscopic powder, which was rechromatographed on the same column to remove contaminating glutathione. Lyophilization of the main fractions gave a fluffy powder; yield 8 mg (11%). The air-oxidized product thus obtained (4.6 mg) was purified by ion-exchange chromatography on DEAE-cellulose as described above. The main product emerged from the column at a retention volume similar to that of natural EGF (Fig. 16), but its retention time in HPLC was 39 min (natural EGF 12.5 min) under the conditions described above; yield 0.64 mg (14%). Amino acid ratios in 6 N HCl hydrolysate: Asp 6.95 (7), Thr 2.13 (2), Ser 5.43 (6), Glu 5.05 (3+2), Pro 1.48 (2), Gly 7.10 (6+2), Cys 3.10 (3+1), Val 1.20 (2), Met 0.51 (1), Ile 1.53 (2), Leu 4.00 (4), Tyr 4.08 (5), His 0.72 (1), Arg 4.52 (4), recovery of Leu 53% (numbers in parentheses are theoretical values).

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References and Notes

- 1) Part CXXVI: K. Akaji, N. Fujii, and H. Yajima, *Chem. Pharm. Bull.*, **33**, 173 (1985).
- 2) Amino acids and peptide derivatives mentioned in this investigation were of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, DCC = dicyclohexylcarbodiimide, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, Troc = 2,2,2-trichloroethyloxycarbonyl, CHA = cyclohexylamine, DCHA = dicyclohexylamine, NMM = *N*-methylmorpholine, EDTA = ethylenediaminetetraacetic acid disodium salt, DMF = dimethylformamide, TFA = trifluoroacetic acid, DMSO = dimethylsulfoxide.
- 3) C. R. Savage, Jr., J. H. Hash, and S. Cohen, *J. Biol. Chem.*, **248**, 7669 (1973).
- 4) J. M. Bower, R. Camble, H. Gregory, E. L. Gerring, and I. R. Willshire, *Experientia*, **31**, 825 (1975).
- 5) a) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, **1974**, 107; b) H. Yajima and N. Fujii, *J. Am. Chem. Soc.*, **103**, 5867 (1981).
- 6) J. Martinez and M. Bodanszky, *Int. J. Peptide Protein Res.*, **12**, 277 (1978) and references cited therein.
- 7) H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano, and H. Adachi, *Int. J. Peptide Protein Res.*, **16**, 33 (1980).
- 8) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, **26**, 2333 (1961).
- 9) W. W. Bachovchin and J. D. Roberts, *J. Am. Chem. Soc.*, **100**, 8041 (1978) and references cited therein.
- 10) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
- 11) R. Camble and N. N. Petter, "Peptide 1976" Proceedings of the Fourteenth European Peptide Symposium, Ed. by A. Loffet, 1976, p. 299.
- 12) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
- 13) G. W. Anderson, J. E. Zimmerman, and F. Callahan, *J. Am. Chem. Soc.*, **85**, 3039 (1963); *idem, ibid.*, **86**, 1839 (1964).
- 14) R. B. Woodward, K. Heusler, J. Gosteli, P. Naegeli, W. Oppolzer, R. Ramage, S. Ranganathan, and H. Vorbrüggen, *J. Am. Chem. Soc.*, **88**, 852 (1966); H. Yajima and Y. Kiso, *Chem. Pharm. Bull.*, **19**, 420 (1971).
- 15) H. Yajima, S. Funakoshi, N. Fujii, K. Akaji, and H. Irie, *Chem. Pharm. Bull.*, **27**, 1060 (1979).
- 16) B. Iselin, *Helv. Chim. Acta*, **44**, 61 (1961).

- 17) K. Okamoto, K. Yasumura, K. Fujitani, S. Katakura, K. Akaji, H. Yajima, Y. Nakata, A. Inoue, and T. Segawa, *Chem. Pharm. Bull.*, **32**, 430 (1984); B. F. Lundt, N. L. Johansen, A. Vølund, and J. Markussen, *Int. J. Peptide Protein Res.*, **12**, 258 (1978).
- 18) L. Gráf, S. Bajusz, A. Patthy, E. Barát, and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **6**, 415 (1971); R. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature New Biol.*, **235**, 114 (1972).
- 19) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 811.
- 20) K. Akaji, N. Fujii, H. Yajima, M. Moriga, M. Aono, and A. Takagi, *Int. J. Peptide Protein Res.*, **18**, 180 (1981).
- 21) M. Löw, L. Kisfaludy, E. Jaeger, P. Tham, S. Knof, and E. Wünsch, *Z. Physiol. Chem.*, **359**, 1637 (1978); H. Ogawa, T. Sasaki, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 3144 (1978); Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **53**, 464 (1980) and references cited therein.
- 22) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 831.
- 23) Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and H. Moritoki, *J. Chem. Soc., Chem. Commun.*, **1979**, 971.
- 24) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, **1978**, 482; H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3752 (1978).
- 25) H. Yajima and N. Fujii, "The Peptides," Vol. 5, Ed. by E. Gross and J. Meienhofer, Academic Press, 1983, p. 65.
- 26) J. J. Sharp, A. B. Robinson, and M. D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).
- 27) J. M. Taylor, W. M. Mitchell, and S. Cohen, *J. Biol. Chem.*, **247**, 5928 (1972).
- 28) S. Sakakibara, "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 1, Ed. by B. Weinstein, Marcel Dekker, New York, 1971, p. 51.
- 29) W. C. Jones, Jr., T. M. Rothgeb, and F. R. N. Gurd, *J. Biol. Chem.*, **251**, 7452 (1976); T. M. Rothgeb, B. N. Jones, D. F. Hayes, and R. S. Gurd, *Biochemistry*, **16**, 5813 (1977).
- 30) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
- 31) C. R. Savage, Jr. and S. Cohen, *J. Biol. Chem.*, **247**, 7609 (1972).
- 32) L. G. Chavez, Jr. and H. A. Scheraga, *Biochemistry*, **19**, 996 (1980); Y. Konishi and H. A. Scheraga, *ibid.*, **19**, 1308 (1980).
- 33) L. M. Matrisian, B. R. Larsen, J. S. Finch, and B. E. Magun, *Anal. Biochem.*, **125**, 339 (1982).
- 34) M. N. Ghosh and H. O. Schild, *Br. J. Pharmacol.*, **13**, 54 (1958).
- 35) N. Fujii and H. Yajima, *J. Chem. Soc., Perkin Trans. 1*, **1981**, 789.
- 36) K. Hayashi, *Igaku No Ayumi*, **127**, 574 (1983).