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)–NHNNH 2 [3], Boc–(42–45)–NHNNH 2 [4], Boc–(39–41)–NHNNH 2 [5], Boc–(37–38)–NHNNH 2 [6], Boc–(34–36)–NHNNH 2 [7], and Boc–(30–33)–NHNNH 2 [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 reaction 8) of the N-terminal fragment, Z(OMe)–His–Ser–Asp–Ala–NHNNH 2 , did not proceed satisfactorily,
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, 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 procedure 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(OMe)-Asp(OBu')-Ser(Bzl)-Tyr-OMe, the Z(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, Boc−Leu−Asp−Ser(Bzl)−Tyr−OMe, which was converted to the corresponding hydrazide [9] by the usual hydrazine treatment.

As the next fragment, we first prepared Boc−His−Ile−Glu(OBzl)−Ser(Bzl)−NHNH₂. 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 Cambie 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 Boc−Leu−Ser(Bu')−His−Asp(OBu')−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 Ser(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 Boc−Ile−Glu(OBzl)−Ser(Bzl)−NHNH₂ and Boc−His−NHNH₂. From the synthetic viewpoint, we concluded that the His residue at position 22 in EGF indeed has a unique character.

Thus, fragment [10], Boc−Ile−Glu(OBzl)−Ser(Bzl)−NHNH₂, was prepared in a stepwise manner starting with H−Ser(Bzl)−NHNH−Troc as shown in Fig. 3. After two successive introductions of Z(OMe)−Glu(OBzl)−OH and Boc−Ile−OH by the active ester procedure,12,13) the Troc group was removed from the resulting tripeptide derivative, Boc−Ile−Glu(OBzl)−Ser(Bzl)−NHNH−Troc by Zn treatment.14)

The next fragment, Boc−Gly−Val−Cys(MBzl)−Met−NHNH₂ [11], was also prepared in a

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**Fig. 3. Synthetic Scheme for the Protected Tripeptide Hydrazide, Boc−(EGF 23−25)−NHNH₂**

**Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Boc−(EGF 18−21)−NHNH₂**
stepwise manner, as shown in Fig. 4, using the Su procedure for introduction of the amino acids. In this synthesis, we decided to use Met without its S-protection, in view of the reduction step of the Cys(MBzl) sulfoxide, which might form partially during the synthesis. By this treatment, the Met sulfoxide, if formed, can be reduced together with Cys(MBzl) sulfoxide prior to the final deprotection of all protecting groups. The S-alkylation observable during the N₂-deprotection by TFA can be suppressed by the use of anisole containing ethanedithiol (EDT). This fragment [11] was prepared without particular difficulty.

Fragment [12]. Boc–Cys(MBzl)–Leu–Asn–Gly–NHNH₂, possesses the Asn–Gly sequence, which from a synthetic viewpoint needs some care, since the β-amide of Asn linked to Gly is known to be labile to base. Thus, this fragment was prepared with the aid of Troc–NHNH₂, as with hydrazides containing Glu(OBzl) or Asp(OBzl). Z(OMe)–Asn–Gly–NHNH–Troc is a known compound used in our previous synthesis of RNase A. Z(OMe)–Leu–OH and Boc–Cys(MBzl)–OH were successively introduced by the active ester procedure as shown in Fig. 5. From the resulting tetrapeptide derivative, Boc–Cys(MBzl)–Leu–Asn–Gly–NHNH–Troc, the Troc group was removed by treatment with Zn in a mixture of DMF–AcOH.

The next fragment, Boc–Tyr–Asp–Gly–Tyr–NHNH₂ [13], had to be synthesized through a base-sensitive Asp(OBzl)–Gly intermediate. Z(OMe)–Asp(OBzl)–Gly–Tyr–OMe was prepared in a stepwise manner as shown in Fig. 6. The Bzl group was first removed by hydrogenolysis, then the Z(OMe) group by TFA treatment. The resulting tripeptide, H–Asp–Gly–Tyr–OMe, was coupled with Boc–Tyr–NHNH₂, via the azide, to give Boc–Tyr–Asp–Gly–Tyr–OMe, which was then smoothly converted to the corresponding hydrazide [13].

The next fragment, Boc–Cys(MBzl)–Pro–Ser(Bzl)–Ser(Bzl)–NHNH₂ [14], was prepared in a stepwise manner also starting with H–Ser(Bzl)–OH, as shown in Fig. 7. In order to make the purification easier, Z(OMe)–Ser(Bzl)–Ser(Bzl)–OH was first prepared by the Su method and then converted to the corresponding methyl ester by treatment with diazomethane. Next, Z(OMe)–Pro–OH was best introduced by the mixed anhydride procedure, then Boc–

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**Fig. 5. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Boc–(EGF 14–17)–NHNH₂**

**Fig. 6. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Boc–(EGF 10–13)–NHNH₂**
Cys(MBzl)–OH by the Su procedure. The resulting oily protected tetrapeptide ester was converted to the corresponding hydrazide [14], which was purified by column chromatography on silica gel.

The N-terminal pentapeptide hydrazide, Boc–Asn–Ser(Bzl)–Tyr–Pro–Gly–NHNH₂ [15], was prepared according to the scheme illustrated in Fig. 8. The known dipeptide, Boc–Tyr–Pro–OH, was condensed with H–Gly–OMe by the Su method to give Boc–Tyr–Pro–Gly–OMe. Next, Boc–Asn–Ser(Bzl)–NHNH₂ was prepared by the Np condensation of Boc–Asn–OH and H–Ser(Bzl)–OH, followed by methylation and then hydrazine treatment. The azide condensation of the resulting hydrazide, Boc–Asn–Ser(Bzl)–NHNH₂, with the TFA-treated sample of Boc–Tyr–Pro–Gly–OMe afforded, in quantitative yield, the protected pentapeptide ester, Boc–Asn–Ser(Bzl)–Tyr–Pro–Gly–OMe, which was smoothly converted to the corresponding hydrazide [15].

The seven fragments thus obtained, [9] to [15], and Boc–His–NHNH₂ were successively assembled onto the TFA-treated sample of Boc–(EGF 30–53)–OBzl, synthesis of which was reported in the preceding paper. Prior to each condensation, the N²-Boc group was removed by TFA in the presence of anisole and EDT²⁶ at the Trp residues (positions 49 and 50) and partial S-alkylation²⁷ at the Met residue (position 21); and a mixture of DMF–DMSO (1:1) was employed as a solvent for each condensation reaction. For chain elongation, two to three additions of the azide (total 3 to 10 equivalents) were required to bring each condensation reaction to a completely ninhydrin-negative state, including the introduction of the single His residue at position 22. Each product was purified by repeated precipitation from DMF–DMSO (1:1) with methanol and the final protected tripeptidpeptide ester (protected EGF) was purified by gel-filtration on Sephadex LH-60 using DMSO–DMF (3:7) as an eluant.

Throughout this synthesis, Leu was used as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Leu with those of newly incorporated amino acids, satisfactory incorporation of each fragment and the His residue, after each coupling reaction, was ascertained (Table I). After overcoming several difficulties encountered during the course of construction of the EGF peptide backbone, we were able to obtain the protected EGF with satisfactory amino acid composition, except for cysteine.

The Cys(MBzl) residues of protected EGF thus obtained were found to be partially oxidized to the corresponding sulfoxides. When protected EGF was hydrolyzed with 6N HCl
### Table 1. Amino Acid Ratios in 6N HCl Hydrolysates of Synthetic EGF and Its Intermediates

<table>
<thead>
<tr>
<th>A.A.</th>
<th>Protected peptides</th>
<th>Syn. EGF</th>
<th>Natural EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>4.17 (4)</td>
<td>4.35 (4)</td>
<td>4.23 (4)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.76 (2)</td>
<td>1.91 (2)</td>
<td>1.87 (2)</td>
</tr>
<tr>
<td>Ser</td>
<td>1.69 (2)</td>
<td>2.56 (3)</td>
<td>2.38 (3)</td>
</tr>
<tr>
<td>Glu</td>
<td>2.15 (2)</td>
<td>3.31 (3)</td>
<td>3.18 (3)</td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td>1.29 (1)</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>2.13 (2)</td>
<td>2.12 (2)</td>
<td>2.15 (2)</td>
</tr>
<tr>
<td>Cys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>0.91 (1)</td>
<td>0.71 (1)</td>
<td>0.80 (1)</td>
</tr>
<tr>
<td>Met</td>
<td></td>
<td>0.75 (1)</td>
<td>0.76 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>0.86 (1)</td>
<td>1.65 (2)</td>
<td>1.71 (2)</td>
</tr>
<tr>
<td>Leu</td>
<td>3.00 (3)</td>
<td>3.00 (3)</td>
<td>3.00 (3)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.77 (2)</td>
<td>1.91 (2)</td>
<td>1.89 (2)</td>
</tr>
<tr>
<td>Trp*</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>His</td>
<td></td>
<td>0.87 (1)</td>
<td>0.85 (1)</td>
</tr>
<tr>
<td>Arg</td>
<td>4.28 (4)</td>
<td>4.36 (4)</td>
<td>4.26 (4)</td>
</tr>
<tr>
<td>Recov.</td>
<td>88%</td>
<td>77%</td>
<td>91%</td>
</tr>
</tbody>
</table>

Trp was determined by 4N MSA hydrolysis.

**Scheme:**

Boc-protected EGF 1—53-OBzl

1) reduction of Cys(MBzl) (O) with thiophenol
2) 1M CF₃SO₃H-thioanisole/TFA + m-cresol, EDT

Deprotected EGF

1) dithiothreitol reduction (6M guanidine-0.1M Tris, pH 8.0)
2) Sephadex G-10 (2N AcOH)
3) air-oxidation, pH 8.2 (0.07 mg/ml)
4) lyophilization, Sephadex G-10 (2N AcOH)

Crude EGF

1) DEAE-cellulose (0.02M~0.2M AcONH₄, pH 5.6)
2) HPLC on Cosmosil 5C₁₈ (4.6 × 160 mm)
   CH₃CN/0.05M AcOH—Et₃N (pH 5.6) = 30/70

Purified EGF

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\(^{2b,23}\) in the presence of 4 - cresol and EDT in an ice-bath for 120 min. This system has the ability to cleave the Met\(^{24}\) 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 4 - 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 residue\(^{21}\) 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,\(^{20}\) if such an S-sulfoxonium 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 reagent\(^{30}\) (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](image)

![Fig. 11. DEAE-Cellulose Purification of the Air-Oxidized Product](image)
ensure the disulfide bond formation. The entire solution was lyophilized and the oxidized product was subjected to gel-filtration on Sephadex G-10 with 2N AcOH as an eluant. Some insoluble material, presumably highly aggregated products, was removed prior to this gel-filtration. The 7d oxidized product was eluted as a sharp single peak in 24% yield, but the 5d oxidized product gave a peak with two shoulders (Fig. 10-b).

In the literature, two effective procedures were described by Savage et al.,\textsuperscript{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 crude 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, 0.02M AcONH\textsubscript{4} 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, 0.2M AcONH\textsubscript{4} 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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure12}
\caption{HPLC of Synthetic EGF on Cosmosil 5C\textsubscript{18}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13}
\caption{Disk Isoelectrofocusing of Synthetic EGF}
\end{figure}
conversion of the inactive protected EGF into the active product. Various un suppressed 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., 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 5C_{18}, according to the method of Matrisian et al. 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 value for natural EGF.

When tested in Shild rats (n = 6), our synthetic peptide (30 μg/kg) suppressed the acid output (taken as 100%), stimulated by histamine (500 μg/kg) to 66.5 ± (11.5)%o. 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 LXXXVIII of this series. Unless otherwise mentioned, products were purified by one of the procedures described in the preceding paper. Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck). Rf values refer to the following solvent systems (v/v): Rf_1 CHCl_3–MeOH (10:0.5), Rf_2 CHCl_3–MeOH–H_2O (8:3:1), Rf_3 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. Hayashi using mouse EGF isolated according to Savage et al.

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–AcOH (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, [α]_D^20 +10.8° (c=0.7, MeOH), Rf_1 0.58. Anal. Calcd for C_{20}H_{21}N_{2}O_{4}: C, 66.91; 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,N (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 AcOH and isopropyl ether; yield 4.15 g (75%), mp 54–56 °C, [α]_D^20 -2.7° (c=0.7, MeOH), Rf_1 0.33. Anal. Calcd for C_{27}H_{33}N_{2}O_{8}: 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,N (0.6 ml, 4.5 mmol). A mixed anhydride [prepared from 1.96 g (4.8 mmol) of Boc-Leu-OMe–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 × 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, [α]_D^20 -21.3°, Rf_5 0.57. Anal. Calcd for C_{35}H_{48}N_{6}O_{15}: 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–NHH_2, [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-ButOH. 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, [α]_D^20 -30.5° (c=0.8, DMF), Rf_5 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 C_{35}H_{48}N_{6}O_{15}: C, 58.27; H, 6.90; N, 11.99. Found: C, 58.30; H, 6.99; N, 11.86.

Z(OMe)-Ser(Bzl)-NHH–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–NHH_2 (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, [a]D 3.5° (c = 0.6, MeOH). RFv 0.76. Anal. Caled for C32H32Cl3N6O2: C, 48.14; H, 4.41; N, 7.66. Found: C, 48.06; H, 4.23; N, 7.75.

Z(OMe)–Glut(OBzl)–Ser(Bz)–NHNN–Troc — Z(OMe)–Ser(Bz)–NHNN–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 Et3N (1.7 ml, 12.4 mmol), NMM (1.4 ml, 13.8 mmol) and Z(OMe)–Glut(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, [a]D 11.9° (c = 0.6, MeOH). RFv 0.41. Anal. Caled for C34H38Cl3N6O16: C, 53.17; H, 4.86; N, 7.30. Found: C, 53.40; H, 4.77; N, 7.44.

Boc–Ile–Glu(OBzl)–Ser(Bz)–NHNN–Troc — Z(OMe)–Glu(OBzl)–Ser(Bz)–NHNN–Troc (7.50 g, 9.88 mmol) was treated with TFA-anisole (16 ml–3.2 ml) and the N-deprotected peptide isolated as stated above was dissolved in DMF (50 ml) together with Et3N (1.4 ml, 13.8 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, [a]D 9.4° (c = 0.4, DMF). RFv 0.35. Anal. Caled for C36H44Cl3N6O18: C, 52.91; H, 5.92; N, 8.57. Found: C, 53.05; H, 5.89; N, 8.80.

Boc–Ile–Glu(OBzl)–Ser(Bz)–NHNN, [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% NaHCO3 and H2O, and recrystallized from MeOH and ether; yield 1.71 g (54%), mp 153–155°C, [a]D 13.9° (c = 0.8, DMF). RFv 0.30. Amino acid ratios in 6 N HCl hydrolysate: Ile 1.00, Glu 0.98, Ser 0.84 (recrystallization of Ile 86%). Anal. Caled for C31H38N6O6: 2.5H2O: 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 Et3N (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, [a]D 26.1° (c = 0.5, MeOH). RFv 0.78. Anal. Caled for C35H38N6O18S2: 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 Et3N (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, [a]D 40.3° (c = 0.6, DMF). RFv 0.90. Anal. Caled for C40H40N8O16S2: 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–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 Et3N (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, [a]D 48.0° (c = 0.6, DMF). RFv 0.29. Anal. Caled for C49H47N10O18S2: 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–NHNN, [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, [a]D −18.1° (c = 0.5, DMSO). RFv 0.68. Amino acid ratios in 6 N HCl hydrolysate: Gly 1.00, Val 1.06, Met 0.90 (recovery of Gly 88%). Anal. Caled for C42H42N10O18S2: C, 52.31; H, 7.21; N, 13.07. Found: C, 52.56; H, 7.02; N, 13.28.

Z(OMe)–Leu–Asn–Gly–NHNN–Troc — Z(OMe)–Asn–Gly–NHNN–Troc (3.03 g, 5.6 mmol) was treated with TFA-anisole (6.0 ml–1.2 ml) and the N-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) together with Et3N (1.7 ml, 11.9 mmol) and Z(OMe)–Leu–ONp (2.56 g, 6.2 mmol). After being stirred for 12 h, the solution 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, [a]D −14.5° (c = 0.8, DMSO). RFv 0.68. Anal. Caled for C43H36N11O21S: 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–NHNN–Troc — Z(OMe)–Leu–Asn–Gly–NHNN–Troc (3.38 g, 5.2 mmol) was treated with TFA-anisole (8.4 ml–1.7 ml) and the N-deprotected peptide isolated as stated above was dissolved in DMF (20 ml) together with Et3N (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. [α]D^20 = -23.4° (c = 0.8, DMSO). Rf_2 0.71. Anal. Caled for C_{23}H_{46}Cl_{3}N_{10}O_{10}S·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-NHNH_2 [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% NaHCO_3 and H_2O. The powder was purified by precipitation from DMF with MeOH; yield: 2.15 g (78%), mp 196—198 °C. [α]D^20 = -19.1° (c = 0.7, DMSO). Rf_2 0.47. Amino acid ratios in 6N HCl hydrolysate: Asp 1.04, Gly 1.00, Leu 1.04 (recovery of Gly, 84%). Anal. Caled for C_{29}H_{45}N_{10}O_{10}S·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. [α]D^20 = +9.8° (c = 0.6, DMF). Rf_2 0.27. Anal. Caled for C_{21}H_{34}N_{2}O_{10}: 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 N^4-deprotected peptide isolated as stated above was dissolved in DMF (60 ml) containing Et,N (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. [α]D^20 = -3.2° (c = 0.6, DMF). Rf_2 0.28. Anal. Caled for C_{21}H_{34}N_{2}O_{10}: C, 61.83; H, 5.68; N, 6.76. Found: C, 61.78; H, 5.96; N, 6.58.

Boc-Tyr-Gly-Ser-OMe —— Z(OMe)-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 N^4-deprotected peptide, precipitated with n-hexane, was dissolved in DMF (80 ml) containing Et,N (1.5 ml, 12.9 mmol). The azide [prepared from 3.81 g (12.9 mmol) of Boc-Tyr-NHNH_2 in DMF (30 ml) and Et,N (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% NaHCO_3. 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 H_2O and concentrated. Trituration of the residue with ether afforded a powder, which was recrystallized from MeOH and CHCl_3; yield 5.01 g (62%), mp 127—130 °C. [α]D^20 = -9.3° (c = 0.5, DMF). Rf_2 0.35. Anal. Caled for C_{39}H_{49}N_{10}O_{10}: C, 55.55; H, 6.22; N, 8.64. Found: C, 55.37; H, 6.47; N, 8.80.

Boc-Tyr-Ser-OMe-NHNH_2 [13] —— Boc-Tyr-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 H_2O, then concentrated. The residue was triturated with ether to afford a powder; yield 3.11 g (64%), mp 205 °C (dec.). [α]D^20 = -147.0° (c = 0.5, DMF). Rf_2 0.013. Amino acid ratios in 6N HCl hydrolysate: Asp 1.01, Gly 1.00, Tyr 1.93 (recovery of Gly, 86%). Anal. Caled for C_{29}H_{38}N_{10}O_{10}·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 Et,N (2.4 ml, 17.1 mmol) were added to a solution of H-Ser(Bzl)-OH (2.99 g, 15.3 mmol) and Et,N (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. [α]D^20 = +14.1° (c = 0.3, DMF). Rf_2 0.75. Anal. Caled for C_{41}H_{44}N_{2}O_{10}: 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. [α]D^20 = +13.8° (c = 0.7, DMF). Rf_2 0.85. Anal. Caled for C_{30}H_{44}N_{2}O_{4}: 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 N^4-deprotected peptide isolated as usual was dissolved in DMF (30 ml) containing Et,N (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. [α]D20 16 2 (c=1.0, DMF), Rf 0.61. Anal. Calcd for C35H34N4O8: 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. [α]D20 -4.0 (c=0.5, DMF), Rf 0.24. Amino acid ratios in 6 N HCl hydrolysate: Pro 1.00, Ser 1.81 (recovery of Pro 77%). Anal. Calcd for C31H32N6O8S·0.5H2O: 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) 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. [α]D20 -39.5 (c=0.4, DMF), Rf 0.28. Anal. Calcd for C22H31N4O5·0.5H2O: 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-OPn (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. [α]D20 4.9 (c=0.4, DMF). Rf 0.44. Anal. Calcd for C36H37N5O8: 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. [α]D20 14.4 (c=0.6, DMF). Rf 0.66. Anal. Calcd for C37H42N5O8: 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. [α]D20 -15.0 (c=0.4, DMSO), Rf 0.56. Anal. Calcd for C36H38N6O8: 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—7 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. [α]D20 41.4 (c=0.3, DMF). Rf 0.59. Anal. Calcd for C38H48N10O11·0.5H2O: 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. [α]D20 10.6 (c=0.9, DMF), Rf 0.42. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.09, Ser 0.96, Pro 1.00, Gly 1.00, Tyr 0.94 (recovery of Gly, 74%). Anal. Calcd for C34H46N4O10·3H2O: 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 tetracaspase peptide (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—7 C for 12 h. Additional azide [prepared from 0.28 g (0.40 mmol) of Boc-Leu-Asp-Ser(Bzl)-Tyr-
NHNH$_2$ and Et$_3$N (56 µl, 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.). [α]$_D^{25}$ = 36.5 (c = 0.2, DMSO). R$_f$$_2$ origin. Anal. Calcd for C$_{25}$H$_{30}$N$_4$O$_5$S$_7$: H$_2$O; C, 56.84; H, 6.55; N, 12.21. Found: C, 56.93; H, 6.48; N, 12.27.

Boc-Ile-Glu(OBzI)-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(OBzI)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzI)-Leu-Arg(Mts)-OBzI [Boc-(EGF 23–53)-OBzI] — 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- deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 30 ml) containing Et$_3$N (87 µl, 0.62 mmol). The azide [prepared from 0.68 g (1.06 mmol) of Boc-Ile-Glu(OBzI)-Ser(Bzl)-NHNH$_2$] in DMSO (2 ml) and Et$_3$N (163 µl, 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 DMSO (1 ml) and Et$_3$N (87 µl, 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.). [α]$_D^{25}$ = 23.7 (c = 0.3, DMSO). R$_f$$_2$ origin. Anal. Calcd for C$_{26}$H$_{33}$N$_4$O$_4$S$_7$: H$_2$O; C, 57.12; H, 6.62; N, 11.74. Found: C, 57.08; H, 6.56; N, 11.79.

Boc-His-Ile-Glu(OBzI)-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(OBzI)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzI)-Leu-Arg(Mts)-OBzI [Boc-(EGF 22–53)-OBzI] — The above protected triacontapeptide 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- deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 20 ml) containing Et$_3$N (77 µl, 0.55 mmol). The azide [prepared from 0.30 g (1.10 mmol) of Boc-His-NHNH$_2$] in DMSO (3 ml) and Et$_3$N (169 µl, 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 Et$_3$N (77 µl, 0.55 mmol) were added and the reaction mixture, after being stirred for an additional 12 h, was concentrated. Treatment of the residue with H$_2$O afforded a powder, which was purified by repeated precipitation from DMSO-DMF (1:1) with MeOH; yield 2.90 g (97%), mp 254 °C (dec.). [α]$_D^{25}$ = 25.5 (c = 0.4, DMSO). R$_f$$_2$ origin. Anal. Calcd for C$_{27}$H$_{36}$N$_4$O$_4$S$_7$: H$_2$O; 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(OBzI)-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(OBzI)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzI)-Leu-Arg(Mts)-OBzI [Boc-(EGF 18–53)-OBzI] — The above protected hexatriacontapeptide Boc (EGF 18–53)-OBzI (3.27 g, 0.54 mmol), was treated with TFA-anisole-EDT (10 ml-1.6 ml-0.31 ml) and the N-deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 20 ml) containing Et$_3$N (73 µl, 0.52 mmol). The azide [prepared from 0.67 g (1.04 mmol) of Boc-Gly-Val-Cys(MBzl)-Met-NHNH$_2$] in DMF (6 ml) and Et$_3$N (161 µl, 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 Et$_3$N (73 µl, 0.52 mmol) were added and the mixture, after being stirred for an additional 12 h, was concentrated. Trituration of the residue with H$_2$O afforded a powder, which was precipitated twice from DMSO-DMF (1:1) with MeOH; yield 3.10 g (97%), mp 268 °C (dec.). [α]$_D^{25}$ = 24.8 (c = 0.4, DMSO). R$_f$$_2$ origin. Anal. Calcd for C$_{32}$H$_{39}$N$_4$O$_4$S$_7$: H$_2$O; C, 56.94; H, 6.59; N, 12.62. Found: C, 56.90; H, 6.62; N, 12.03.

Boc-Cys(MBzl)-Leu-Asn-Gly-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzI)-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(OBzI)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzI)-Leu-Arg(Mts)-OBzI [Boc-(EGF 14–53)-OBzI] — The above protected hexatriacontapeptide Boc (EGF 18–53)-OBzI (3.27 g, 0.54 mmol), was treated with TFA-anisole-EDT (11 ml-1.9 ml-0.36 ml) and the N-deprotected peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 20 ml) containing Et$_3$N (75 µl, 0.54 mmol). The azide [prepared from 0.68 g (1.08 mmol) of Boc-Cys (MBzl)-Leu-Asn-Gly-NHNH$_2$] in DMSO (7 ml) and Et$_3$N (165 µl, 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 (0.50 mmol) of the hydrazide] was added. The whole was stirred for an additional 12 h, the solvent was evaporated off and H$_2$O 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 DMSO-DMF (1:1) with MeOH; yield 2.15 g (61%), mp 252 °C (dec.). [α]$_D^{25}$ = 17.8 (c = 0.3, DMSO). R$_f$$_2$ origin. Anal. Calcd for C$_{31}$H$_{42}$N$_5$O$_{10}$S$_{10}$: 12H$_2$O; 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-Val-Cys(MBzl)-Met-His-Ile-Glu(OBzI)-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(OBzI)-Leu-Arg(Mts)-Trp-Trp-Glu(OBzI)-Leu-Arg(Mts)-OBzI [Boc-(EGF 10–53)-OBzI] — The above peptide, Boc-(EGF 14–53)-OBzI (1.05 g, 0.16 mmol), was treated with TFA-anisole-EDT (5.0 ml-0.55 ml-0.11 ml) and the N-deprotected peptide isolated as stated above was dissolvent in DMSO-DMF (1:1, 10 ml) containing Et$_3$N (22 µl, 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 µl, 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 µl, 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.). [α]₂₀⁰⁰° = -32.3 (c = 0.1, DMSO), R₂₁₂ origin. Anal. Calcd for C₃₅₅H₅₅₅N₆O₂₅S₂₁: 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)-Glu-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 N'-depolymerized peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 10 ml) containing Et₃N (19 µl, 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 µl, 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 µl, 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.). [α]₂₀⁰° = -7.4 (c = 0.1, DMSO), R₂₁₂ origin. Anal. Calcd for C₃₇₃H₄₅₅N₁₅O₂₅S₁₅·16H₂O·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)-Glu-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 N'-depolymerized peptide isolated as stated above was dissolved in DMSO-DMF (1:1, 10 ml) containing Et₃N (18 µl, 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 µl, 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 µl, 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 from 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.). [α]₂₀⁰° = -40.0 (c = 0.2, DMSO), R₂₁₂ origin. Anal. Calcd for C₅₅₅H₆₅₅N₆O₂₅S₂₁·15H₂O·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—The 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)-OH 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.). [α]₂₀⁰° = -43.0 (c = 0.1, DMSO). Recoveries of cystine in an acidic 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 µmol) was treated with 1 M TFMSA-thioanisole (mole ratio 1:1) in TFA (4.2 ml) in the presence of mescol (249 µl, 200 eq) and EDT (50 µl, 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 23.5 mg (32%). In the preliminary run, the 5d 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 7d oxidized product (4.07 mg) was dissolved in 2 N AcOH (0.3 ml). After being adjusted to pH 7.0 with 5% NH$_4$OH, 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 x 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 5C$_{18}$ (4.6 x 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$_2$O (1.5 ml), the pH was adjusted to 5.8 with 1 N NH$_4$OH, 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 AcOH–Et$_3$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 5C$_{18}$ 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 x 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 4h (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: $E_{280}^{1%}$ at 280 nm 30.1 (lit. $^{27}$ 30.9), $[\beta]^{20}_{D} = -105.9$ (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](image-url)
tube (Spectrum), which was dialyzed against a solution of pH 1.5, 0.05 M 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 AcONa (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 AcONa; 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 ìmol) 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

2) Amino acids and peptide derivatives mentioned in this investigation were of the l-configuration. The following abbreviations are used: Z = benzoyloxy carbonyl, Z(OMe) = p-methoxybenzyl oxy carbonyl, Boc = tert-butoxycarbonyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, DCC = dicyclohexyl carbodiimide, Np = p-nitrophenyl, Su = N-hydroxysuccinimidyl, Troc = 2,2,2-trichloro ethyloxycarbonyl, CHA = cyclohexylamine, DCHA = dicyclohexylamine, NMM = N-methylmorpholine, EDTA = ethylenediaminetetraacetic acid disodium salt, DMF = dimethylformamide, TFA = trifluoroacetic acid, DMSO = dimethylsulfoxide.


