Solution Syntheses of Two Enkephalin-Containing Peptides, Peptide E and Dynorphin(1—24), Using N^{in}-(2,4,6-Triisopropylphenylsulfonyl)tryptophan[1)]

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Two enkephalin-containing peptides, peptide E and dynorphin (1—24), were synthesized by conventional solution methods employing a new Trp derivative, N^{in}-(2,4,6-triisopropylphenylsulfonyl)tryptophan [Trp(Tps)]. All protecting groups employed including the Tps group were removed by treatment with 1 M trifluoromethanesulfonic acid (TFMSA)–thioanisole in trifluoroacetic acid (TFA) at the final steps of these syntheses. Subsequent purifications by Sephadex G-25 chromatography, CM-Biogel A ion exchange chromatography, and reversed-phase high-performance liquid chromatography afforded highly purified samples. Both synthetic peptide E and dynorphin (1—24) exhibited high *in vitro* opioid activity. The usefulness of this new tryptophan derivative for practical peptide synthesis was established through these syntheses of complex Trp-containing peptides.

Keywords tryptophan protecting group; opioid peptide; enkephalin-containing peptide; peptide E solution synthesis; dynorphin (1—24) solution synthesis; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; opioid activity *in vitro*

The indole moiety of tryptophan (Trp) is susceptible to oxidation and is easily alkylated by cations during the acid treatment for removal of N^{α}-protecting groups.[2)] These side reactions can be minimized to an acceptable extent by selecting an effective scavenger, such as 2-methylindole,[3)] dimethylsulfide[4)] and anisole containing ethanedithiol.[5)] However, for the synthesis of complex Trp-containing peptides, employment of a protecting group at the indole nitrogen atom seems highly favorable. Since Illi's method[6)] is widely applicable for the introduction of an electron-withdrawing group at the indole nitrogen atom, various arylsulfonyl-type protecting groups, such as 2,4,6-trimethoxybenzenesulfonyl (Mtb),[7)] 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr),[8)] and mesitylenesulfonyl (Mts),[9)] have been developed. These protecting groups are stable toward N^{α}-deprotection with TFA and are readily removable by the final acidolytic deprotecting reagents, such as HF,[10)] MSA[11)] and 1 M trifluoromethanesulfonic acid–thioanisole in TFA.[12)] The 2,4,6-triisopropylphenylsulfonyl (Tps) group, introduced by Kiso *et al.*,[13)] has similar properties as an indole-protecting group and its utility was successfully demonstrated by the synthesis of δ-sleep inducing peptide (DSIP). To extend its utility to more complex peptides, we applied this new Trp protecting group for the syntheses of two enkephalin-containing peptides (ECPs), peptide E and dynorphin (1—24).

Among the various ECPs isolated by several research groups,[14)] peptide E and dynorphin (1—24) were reported to have potent *in vitro* opioid activities. The former peptide was originally isolated from bovine adrenal medulla by Udenfriend's group in 1981[15)] and the latter one, regarded as a C-terminally extended variant of dynorphin (1—17), was isolated from porcine pituitary by Goldstein's group in 1982.[16)] As shown in Fig. 1, both peptides contain two Met- or Leu-enkephalin sequences at their amino- and carboxy-terminii and a considerable sequence homology exists between them, although they were derived from different precursor proteins,[17)] preproenkephalin A and preproenkephalin B, respectively. The solid-phase synthesis of peptide E was achieved in 1983 by Meienhofer *et al.*,[18)] who used the formyl (For) group[19)] for the protection of Trp, while the synthesis of the latter has not been reported so far.

Our synthetic routes to peptide E and dynorphin (1—24) are shown in Figs. 2 and 5, respectively. The TFA-labile Boc or Z(OMe) group was employed for N^{α}-protection, and amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole/TFA[12)] were employed, *i.e.*, Arg(Mts),[20)] Lys(Z), Asp(OChp),[21)] Glu(OBzl), Tyr(Cl_2Bzl),[22)] and Trp(Tps). Of these, Asp(OChp) is a derivative recently devised by Fujii *et al.*[21)] to suppress base-catalyzed succinimide formation[23)] during the peptide assembly. The Met^{15} residue in peptide E was introduced as its sulfoxide[24)] to prevent air-oxidation during the synthesis.

Synthesis of Peptide E Four peptide fragments ([1] to [4]) were selected as building blocks to construct the entire peptide backbone of peptide E as shown in Fig. 2. Of these, fragment [2] containing the Glu(OBzl) residue was prepared with the aid of Troc-$NHNH_2$,[25)] which is known to be cleaved by Zn[26)] or Cd[27)] in AcOH without affecting the other side-chain protecting groups employed.

First, the C-terminal protected undecapeptide benzyl

peptide E : H-Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-
Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-OH

dynorphin (1—24): H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-
Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-OH

Fig. 1. Structures of Peptide E and Dynorphin (1—24)

This paper is dedicated to Professor Haruaki Yajima on the occasion of his retirement from Kyoto University in March 1989.

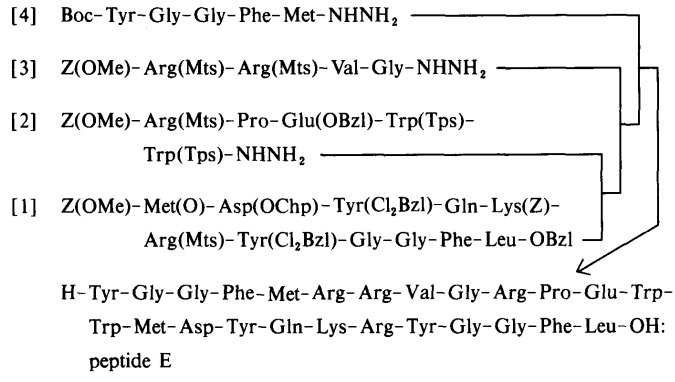

Fig. 2. Synthetic Route to Peptide E

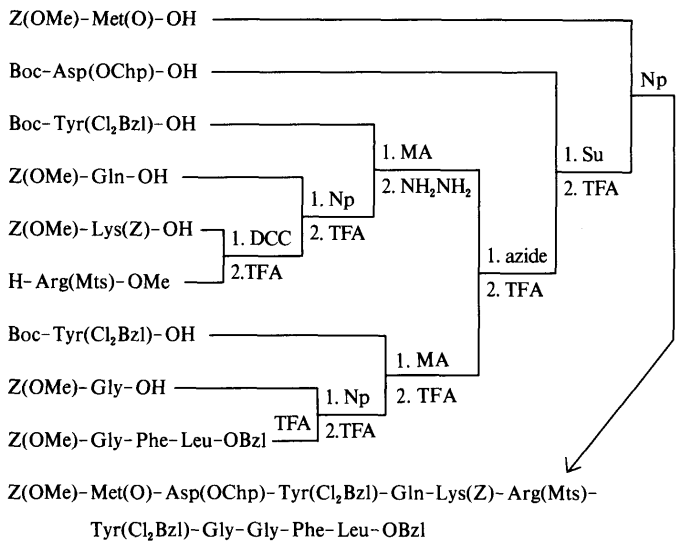

Fig. 3. Synthetic Scheme to the Protected Undecapeptide Benzyl Ester [1]

ester [1], Z(OMe)–Met(O)–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl, was prepared according to the scheme illustrated in Fig. 3. The pentapeptide ester corresponding to the Leu-enkephalin sequence was prepared in a stepwise manner starting with the known tripeptide, Z(OMe)–Gly–Phe–Leu–OBzl,[28] by the Np active ester[29] and the mixed anhydride (MA)[30] methods. Next, Boc–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–OMe was prepared in a stepwise manner starting with H–Arg(Mts)–OMe by the MA and the Np methods, then converted to the corresponding hydrazide by the usual hydrazinolysis. The resulting hydrazide was condensed *via* the azide[31] with a TFA-treated sample of the above-described C-terminal pentapeptide ester to afford Boc–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl. The peptide chain of the resulting nonapeptide ester was elongated to obtain [1] by successive condensations with Boc–Asp(OChp)–OH and Z(OMe)–Met(O)–OH *via* the Su[32] or the Np active ester method. The homogeneity of [1] was ascertained by thin layer chromatography (TLC), amino acid analysis after acid hydrolysis and elemental analysis, as was also done with other fragments.

Fragment [2], Z(OMe)–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–$NHNH_2$, was prepared according to the scheme illustrated in Fig. 4. The tripeptide unit,

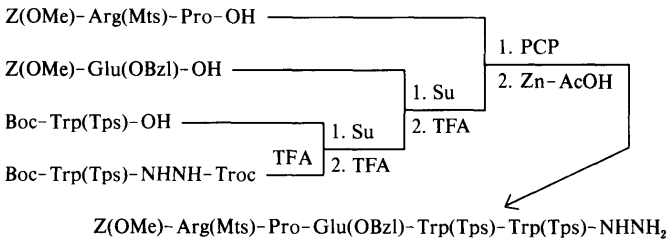

Fig. 4. Synthetic Scheme to the Protected Pentapeptide Hydrazide [2]

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

	Protected peptides				Synthetic peptide E
	15—25	10—25	6—25	1—25	
Asp	1.00	1.01	0.97	1.00	1.13 (1)
Glu	1.07	2.01	2.00	1.99	1.98 (2)
Pro		0.85	0.86	0.79	1.02 (1)
Gly	2.04	2.08	2.80	5.05	5.44 (5)
Val			0.98	0.96	1.11 (1)
Met[a]	0.75	0.58	0.70	1.25	1.62 (2)
Leu	1.00	1.00	1.00	1.00	1.00 (1)
Tyr	1.84	2.07	1.71	2.66	2.68 (3)
Phe	1.00	1.00	0.99	1.90	1.93 (2)
Lys	1.00	1.00	0.99	1.00	1.02 (1)
Trp		N.D.	N.D.	N.D.	N.D. (2)
Arg	1.06	1.79	3.42	3.55	4.26 (4)
Recovery (%)	99	70	93	98	90

a) Calcd as Met + Met(O).

Z(OMe)–Glu(OBzl)–Trp(Tps)–Trp(Tps)–NHNH-Troc, was prepared in a stepwise manner starting with Boc–Trp(Tps)–NHNH–Troc. Then, a TFA-treated sample of the tripeptide derivative was condensed with the known dipeptide derivative, Z(OMe)–Arg(Mts)–Pro–OH,[33] *via* the PCP ester[34] method. From the resulting pentapeptide derivative, after purification by silica gel column chromatography, the Troc group was removed by treatment with Zn–AcOH to give fragment [2].

Fragment [3], Z(OMe)–Arg(Mts)–Arg(Mts)–Val–Gly–$NHNH_2$, was prepared from two known dipeptide units. Namely, Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$[35] was condensed *via* the azide with a TFA-treated sample of Z(OMe)–Val–Gly–OMe[36] to give the protected pentapeptide ester, which was converted to [3] by treatment with hydrazine.

Fragment [4], which corresponds to the Met-enkephalin sequence, was prepared by stepwise condensation procedures starting with the known dipeptide derivative, Z(OMe)–Phe–Met–OMe,[28] *via* the MA and the Nb active ester,[37] respectively. Boc–Tyr–Gly–Gly–Phe–Met–OMe thus obtained was converted to [4] by the usual hydrazine treatment.

The four fragments thus obtained were assembled successively by the azide method as shown in Fig. 2. Every condensation was carried out in DMF until the solution became negative to ninhydrin. The amount of each acyl component was increased from 1.5 to 2 eq as the chain elongation progressed. The protected intermediates and protected peptide E were purified by either column chromatography on silica gel, or gel-filtration on Sephadex LH-20 using DMF as an eluant. Throughout this synthesis and

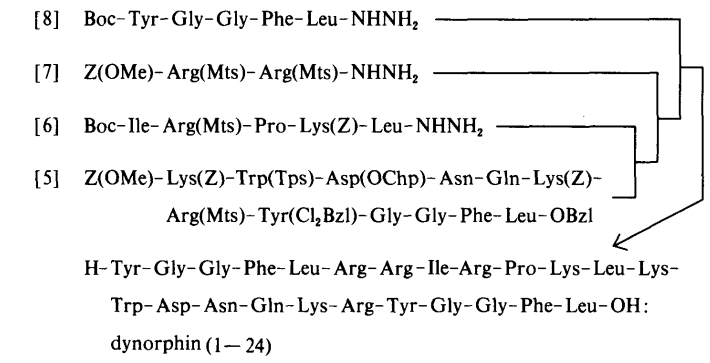

Fig. 5. Synthetic Route to Dynorphin (1—24)

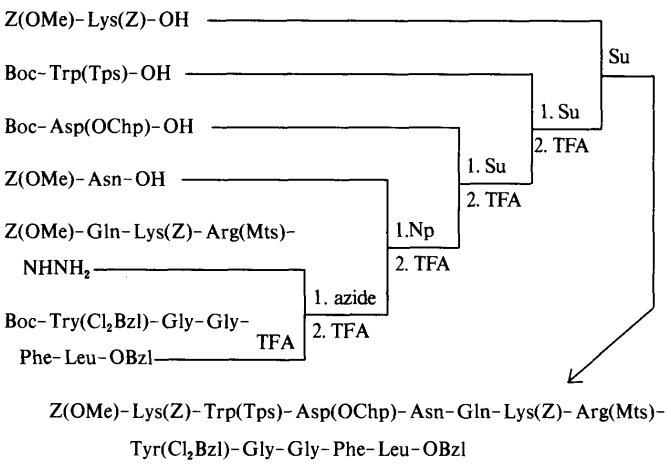

Fig. 6. Synthetic Scheme to the Protected Dodecapeptide Ester [5]

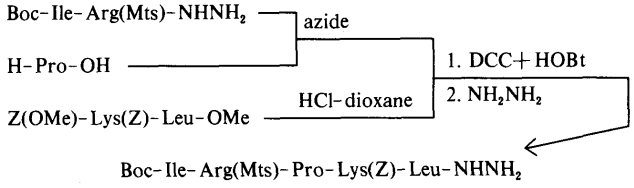

Fig. 7. Synthetic Scheme to the Protected Pentapeptide Hydrazide [6]

in the case of dynorphin (1—24) described later, Leu was selected 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 in each condensation was ascertained, as shown in Table I.

Prior to the final deprotection, the fully protected 25-peptide ester was treated with phenylthiotrimethylsilane[38] in DMF to reduce the Met(O) residue back to Met. The progress of the reduction was monitored on TLC. Next, all protecting groups were removed from the reduced peptide by treatment with 1 M TFMSA–thioanisole/TFA in the presence of *m*-cresol and dimethylsulfide in an ice-bath for 4 h. Dimethylsulfide was used as an additional scavenger to suppress the indole modifications during the final acidolytic deprotection, as recommended by Sakakibara *et al.*[4] The deprotected peptide was then converted to the corresponding acetate by Amberlite IR-400 (acetate form) and lyophilized. The lyophilized product was purified by gel-filtration on Sephadex G-25 (3% AcOH as an eluant), followed by high-performance liquid chromatography (HPLC) on a column of Chemcopak (Nucleosil $7C_{18}$) (Fig. 8). The ion-exchange chromatography on CM-Biogel A was not effective for further purification. The HPLC-purified product exhibited a well-defined single spot on TLC in three different solvent systems and a single peak on analytical HPLC (Fig. 8b). The purity of this sample was further confirmed by amino acid analyses after acid hydrolysis (Table I) and enzymic hydrolysis (papain + LAP). One of the reasons for the low isolation yield from the deprotection may be the oxidation-sensitive Met residues. Also, this peptide has a tendency to be adsorbed easily on the walls of glassware. Consequently, to obtain this peptide in a pure form was not an easy task.

Synthesis of Dynorphin (1—24) Four peptide fragments ([5] to [8]) were selected as building blocks to construct the entire peptide backbone of dynorphin (1—24) as shown in Fig. 5.

First, the C-terminal-protected dodecapeptide benzyl ester [5], Z(OMe)–Lys(Z)–Trp(Tps)–Asp(OChp)–Asn–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl, was prepared according to the scheme illustrated in Fig. 6. Z(OMe)–Gln-Lys(Z)–Arg(Mts)–OMe, the intermediate tripeptide unit in fragment [1] of peptide E, was converted to the hydrazide in the usual manner, then condensed with a TFA-treated sample of Boc-Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl *via* the azide. The peptide chain of the resulting octapeptide ester was elongated to [5] by successive condensations with Boc–Asn–OH, Boc–Asp(OChp)–OH, Boc–Trp(Tps)–OH, and Z(OMe)–Lys(Z)–OH *via* the Np or the Su active ester and the MA method, respectively. The base-catalyzed succinimide formation, which is known to be sequence-dependent,[23] is usually high risk in the case of the Asp–Asn sequence. To suppress this side reaction, employment of β-cycloheptyl ester protection at the Asp residue was effective.

Next, fragment [6], Boc–Ile–Arg(Mts)–Pro–Lys(Z)–Leu–$NHNH_2$, was prepared by the DCC + HOBt[39] condensation of Boc–Ile–Arg(Mts)–Pro–OH and the known dipeptide derivative, H-Lys(Z)–Leu–OMe,[40] followed by the usual hydrazine treatment of the resulting pentapeptide ester as shown in Fig. 7. The former tripeptide unit was prepared by the azide condensation of the known dipeptide, Boc–Ile–Arg(Mts)–$NHNH_2$,[41] and H–Pro–OH.

Fragment [7], Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$, is a known dipeptide derivative which was used to prepare fragment [3] in peptide E. Next, the N-terminal fragment [8], corresponding to the Leu-enkephalin sequence, was prepared by the condensation of Boc–Tyr–ONb and a TFA–treated sample of Z(OMe)–Gly–Gly–Phe–Leu–OBzl, followed by the usual hydrazine treatment of the resulting pentapeptide ester.

The four fragments thus obtained were assembled successively by the azide method as shown in Fig. 5. Each intermediate was purified by either reprecipitation from DMF with AcOEt or by column chromatography on silica gel. The results of amino acid analysis after acid hydrolysis are listed in Table II.

In the final step of the synthesis, the protected 24-peptide ester thus obtained was treated with 1 M TFMSA–thioanisole/TFA in the presence of *m*-cresol and dimethylsulfide essentially in the same manner as described for the synthesis of peptide E. The deprotected peptide was also

Table II. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic Dynorphin (1—24) and Its Protected Intermediates

	Protected peptides				Synthetic dynorphin
	13—24	8—24	6—24	1—24	(1—24)
Asp	2.13	2.10	1.95	2.13	2.11 (2)
Glu	1.15	1.12	1.00	1.10	1.11 (1)
Pro		0.97	1.14	1.23	1.09 (1)
Gly	2.00	2.00	1.95	4.49	4.07 (4)
Ile		0.94	1.14	0.96	1.00 (1)
Leu	1.00	2.00	2.00	3.00	3.00 (3)
Tyr	0.98	0.98	0.91	1.70	1.97 (2)
Phe	1.03	1.06	0.98	2.00	2.03 (2)
Lys	1.99	3.08	3.29	3.22	3.06 (3)
Trp	N.D.	N.D.	N.D.	N.D.	N.D. (1)
Arg	1.03	2.03	4.10	4.39	4.05 (4)
Recovery (%)	95	98	91	82	85

similarly purified, *i.e.*, gel-filtration on Sephadex G-25 (3% AcOH as an eluant), ion-exchange chromatography on CM-Biogel A (Fig. 9), and HPLC on a column of Chemcopak (Nucleosil $7C_{18}$) (Fig. 10a). The purity of this product was ascertained on TLC, analytical HPLC (Fig. 10b), and by amino acid analyses after acid hydrolysis (Table II) and enzymic hydrolysis. The satisfactory recovery of Asp (0.91) in the enzymic hydrolysate confirms the absence of a contaminant derived from ring closure at the Asp–Asn linkage.

The biological activity was determined by measuring the inhibition of electrically evoked contraction of the isolated guinea-pig ileum to give ED_{50} of 17.8 nM for peptide E and 0.8 nM for dynorphin (1—24), in reasonable agreement with the literature values for the natural peptides.[15,16,42]

As demonstrated in these syntheses, the Tps group could be successfully employed for the syntheses of complex Trp-containing peptides. This protecting group fulfills two important criteria as a protecting group for practical peptide synthesis: stability to N^{α}-deprotection with TFA, and susceptibility to the final deprotection with 1 M TFMSA–thioanisole/TFA. Along with other Trp protecting groups, such as Mds or Mts, the acidolytically removable Tps group is promising as a protecting group of choice for the synthesis of Trp-containing peptides.

Experimental

General experimental procedures employed in this study are as follows.

N^{α}-Deprotection The N^{α}-protecting group, Z(OMe) or Boc, was treated with TFA (*ca.* 2—3 ml per 1 g of the protected peptide) in the presence of anisole (2 mol eq or more) under ice-cooling for 60 min. After evaporation of TFA *in vacuo* at 30 °C or less, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* and used for the next coupling reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* and used for the coupling reaction.

Coupling Reactions The DCC and the active ester couplings were carried out at room temperature. The azide coupling was carried out according to the method of Honzl and Rudinger[31] using isoamyl nitrite with stirring in a cold room (4 °C). Mixed anhydrides were prepared using isobutyl chloroformate.

Purification Unless otherwise mentioned, products were purified by one of the following procedures.

Procedure A: For the purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized or precipitated from appropriate solvents.

Procedure B: For the purification of protected peptides less soluble in AcOEt, the crude product was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, in a batchwise manner, then crystallized or precipitated from appropriate solvents.

The melting points are uncorrected. The optical rotation was determined with a Union PM-201 polarimeter. Acid hydrolysis with 6 N HCl was carried out in a sealed tube, and amino acid analysis was performed on an IRICA model A-3300 amino acid analyzer. LAP (Lot. 15F-0402) and papain (Lot. 102F-8160) were purchased from Sigma Chemical Co.

TLC was carried out on silica gel (precoated Silica gel 60 F_{254}, Merck) and the solvent system used were as follows; Rf_1=$CHCl_3$–MeOH–H_2O (8:3:1), Rf_2=$CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_3=*n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_4=*n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24), Rf_5=*n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24). HPLC was conducted with a Shimadzu LC-4A model equipped with a Chemcopak column (Nucleosil $7C_{18}$, 10.0 × 250 mm).

Synthesis of Peptide E Z(OMe)–Gly–Gly–Phe–Leu–OBzl (**1**): A mixture of a TFA-treated sample of Z(OMe)–Gly–Phe–Leu–OBzl[28] (23.60 g, 40.0 mmol), Z(OMe)–Gly–ONp (12.61 g, 35.0 mmol) and Et_3N (10.64 ml, 76.0 mmol) in DMF (80 ml) was stirred for 24 h. The solvent was removed by evaporation and the residue was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 18.60 g (72%), mp 139—142 °C, $[\alpha]_D^{25}$ −16.0° (*c*=1.0, AcOEt), Rf_1 0.76. *Anal.* Calcd for $C_{35}H_{42}N_4O_8 \cdot 0.5H_2O$: C, 64.10; H, 6.61; N, 8.55. Found: C, 64.33; H, 6.54; N, 8.61.

Boc–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**2**): A mixture of a TFA-treated sample of **1** (3.60 g, 5.60 mmol), Boc–Tyr(Cl_2Bzl)–ONp (3.10 g, 5.60 mmol) and Et_3N (1.68 ml, 12.0 mmol) in DMF (30 ml) was stirred for 48 h at room temperature and the product was purified by procedure A, followed by recrystallization from MeOH; yield 4.72 g (52%), mp 164—165 °C, $[\alpha]_D^{25}$ −6.0° (*c*=1.0, DMF), Rf_1 0.79. *Anal.* Calcd for $C_{47}H_{55}Cl_2N_5O_9$: C, 62.38; H, 6.13; N, 7.74. Found: C, 62.42; H, 6.21; N, 7.52.

Z(OMe)–Lys(Z)–Arg(Mts)–OMe (**3**): The title compound was prepared by the DCC method and purified by procedure A, followed by recrystallization from MeOH with ether; yield 77%, mp 103—105 °C, $[\alpha]_D^{25}$ −14.0° (*c*=0.5, MeOH), Rf_1 0.82. *Anal.* Calcd for $C_{39}H_{52}N_6O_{10}S$: C, 58.78; H, 6.58; N, 10.55. Found: C, 58.68; H, 6.60; N, 10.39.

Z(OMe)–Gln–Lys(Z)–Arg(Mts)–OMe (**4**): A mixture of a TFA-treated sample of **3** (18.0 g, 23.0 mmol), Z(OMe)–Gln–ONp (8.00 g, 19.0 mmol) and Et_3N (6.02 ml, 43.0 mmol) in DMF (100 ml) was stirred for 48 h at room temperature and the product was purified by procedure B, followed by reprecipitation from DMF with AcOEt twice; yield 13.0 g (73%), mp 173—175 °C, $[\alpha]_D^{25}$ −4.0° (*c*=1.0, DMF), Rf_1 0.54. *Anal.* Calcd for $C_{44}H_{60}N_8O_{12}S \cdot H_2O$:C, 53.31; H, 6.71; N, 14.13. Found: C, 53.49; H, 7.00; N, 13.71.

Boc–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–OMe (**5**): An MA [prepared from Boc-Tyr(Cl_2Bzl)-OH (2.50 g, 5.70 mmol)] was allowed to react with a TFA-treated sample of **4** (5.00 g, 5.40 mmol) in THF (30 ml) containing Et_3N (0.76 ml, 5.40 mmol) and the mixture was stirred for 5 h. The product was purified by procedure B, followed by reprecipitation from MeOH with ether; yield 6.05 g (94%), mp 108—110 °C, $[\alpha]_D^{25}$ −6.0° (*c*=1.0, MeOH), Rf_1 0.79. *Anal.* Calcd for $C_{56}H_{73}Cl_2N_9O_{13}S \cdot 2H_2O$: C, 55.16; H, 6.37; N, 10.34. Found: C, 55.28; H, 6.80; N, 10.24.

Boc–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–$NHNH_2$ (**6**): The above-prepared tetrapeptide ester **5** (5.00 g, 4.20 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (2.10 ml, 42.0 mmol) for 48 h. The solvent was removed by evaporation and the residue was triturated with H_2O to give a solid, which was washed well with H_2O and reprecipitated from DMF with EtOH; yield 4.50 g (90%), mp 125—128 °C, $[\alpha]_D^{25}$ −2.0° (*c*=1.0, DMF), Rf_1 0.45. *Anal.* Calcd for $C_{55}H_{73}Cl_2N_{11}O_{12}S$: C, 55.82; H, 6.22; N, 13.02. Found: C, 55.46; H, 6.34; N, 12.90.

Boc–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**7**): The azide [prepared from **6** (4.90 g, 4.14 mmol)] in DMF (50 ml) and Et_3N (0.58 ml, 4.14 mmol) were added to a solution of a TFA-treated sample of **2** (2.50 g, 2.76 mmol) in DMF (30 ml) containing Et_3N (0.39 ml, 2.76 mmol). The reaction mixture was stirred for 48 h, then the DMF was removed by evaporation *in vacuo*. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 4.50 g (83%), mp 184—187 °C, $[\alpha]_D^{25}$ −14.0° (c=0.5, DMF), Rf_1 0.65. *Anal.* Calcd for $C_{97}H_{116}Cl_4N_{14}O_{19}S \cdot 2H_2O$: C, 58.48; H, 6.07; N, 9.85. Found: C, 58.60; H, 5.85; N, 9.70.

Boc–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**8**): A mixture of a TFA-treated sample of **7** (3.80 g, 1.94 mmol), Boc–Asp(OChp)–OSu (1.65 g, 3.88 mmol), and NMM (0.64 ml, 6.00 mmol) in DMF (30 ml) was stirred for 24 h and the product was purified by procedure B, followed by reprecipitation from DMF with MeOH; yield 3.70 g (88%), mp 194—196 °C, $[\alpha]_D^{25}$ −2.0° (*c*=0.5, DMF),

Rf_1 0.63. *Anal.* Calcd for $C_{108}H_{133}Cl_4N_{15}O_{22}S \cdot 3H_2O$: C, 58.40; H, 6.31; N, 9.46. Found: C, 58.39; H, 6.28; N, 9.89.

Z(OMe)–Met(O)–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl [1]: A mixture of a TFA-treated sample of **8** (3.20 g, 1.48 mmol), Z(OMe)–Met(O)–ONp (0.80 g, 1.78 mmol), and NMM (0.43 ml, 3.88 mmol) in DMF (50 ml) was stirred for 24 h and the product was purified by procedure B, followed by reprecipitation from DMF with MeOH; yield 3.00 g (85%), mp 253—255 °C, $[\alpha]_D^{25}$ −20.0° (c = 0.5, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{117}H_{142}Cl_4N_{16}O_{25}S_2 \cdot 2H_2O$: 58.20; H, 6.10; N, 9.28. Found: C, 58.32; N, 5.94; N, 9.32.

Boc–Trp(Tps)–NHNH–Troc (**9**): The title compound was prepared by the DCC + HOBt method and purified by procedure A, followed by recrystallization from AcOEt with *n*-hexane; yield 90%, mp 114—117 °C, $[\alpha]_D^{25}$ −4.0° (c = 1.0, AcOEt), Rf_1 0.71. *Anal.* Calcd for $C_{34}H_{45}Cl_3N_4O_7S$: C, 59.42; H, 6.40; N, 6.93. Found: C, 59.20; H, 6.50; N, 6.63.

Boc–Trp(Tps)–Trp(Tps)–NHNH–Troc (**10**): The title compound was prepared by the MA method and purified by procedure A, followed by column chromatography on silica gel using $CHCl_3$–MeOH (30 : 1) as an eluant; yield 66%, mp 104–107 °C, $[\alpha]_D^{25}$ −23.0° (c = 1.0, AcOEt), Rf_1 0.89. *Anal.* Calcd for $C_{60}H_{77}Cl_3N_6O_{16}S_2$: C, 53.72; H, 5.97; N, 7.37. Found: C, 54.06; H, 6.23; N, 7.06.

Z(OMe)–Glu(OBzl)–Trp(Tps)–Trp(Tps)–NHNH–Troc (**11**): An MA [prepared from Z(OMe)–Glu(OBzl)–OH (1.65 g, 4.12 mmol)] was allowed to react with a TFA-treated sample of **10** (5.00 g, 4.12 mmol) in THF (70 ml) containing Et_3N (0.58 ml, 4.12 mmol) and the mixture was stirred for 5 h. The product was purified by procedure A, followed by column chromatography on silica gel using $CHCl_3$–MeOH (30 : 1) as an eluant; yield 4.50 g (73%), mp 98—100 °C, $[\alpha]_D^{25}$ −14.0° (c = 1.0, AcOEt), Rf_1 0.78. *Anal.* Calcd for $C_{76}H_{90}Cl_3N_7O_{14}S_2$: C, 61.01; H, 6.06; N, 6.55. Found: C, 61.08; H, 6.14; H, 6.60.

Z(OMe)–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–NHNH–Troc (**12**): Z(OMe)–Arg(Mts)–Pro–OH[33] (1.20 g, 2.00 mmol) was converted to its PCP ester by the DCC method and, without isolation, allowed to react with a TFA-treated sample of **11** (2.00 g, 1.34 mmol) in DMF (40 ml) containing Et_3N (0.19 ml, 1.34 mmol) and the mixture was stirred for 12 h. The product was purified by procedure A, followed by column chromatography on silica gel using $CHCl_3$–MeOH (30 : 1) as an eluant; yield 1.60 g (62%), mp 110—112 °C, $[\alpha]_D^{25}$ −18.0° (c = 1.0, AcOEt), Rf_1 0.82. *Anal.* Calcd for $C_{96}H_{119}Cl_3N_{12}O_{18}S_3$: C, 59.69; H, 6.21; N, 8.70. Found: C, 59.56; H, 6.39; N, 8.22.

Z(OMe)–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–$NHNH_2$ [2]: The above-prepared pentapeptide derivative **12** (1.93 g, 1.00 mmol) in MeOH–AcOH (10 ml–2 ml) was treated with Zn powder (1.31 g) for 1 h at room temperature. The solution was filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in AcOEt, washed well with 3% ethylenediaminetetraacetic acid (EDTA) solution and H_2O, and dried over Na_2SO_4. The AcOEt was removed *in vacuo*. The residue was recrystallized twice from AcOEt with ether; yield 1.20 g (68%), mp 118—121 °C, $[\alpha]_D^{25}$ −17.0° (c = 1.0, MeOH), Rf_1 0.73. *Anal.* Calcd for $C_{93}H_{118}N_{12}O_{16}S_3$: C, 63.60; H, 6.77; N, 9.57. Found: C, 63.51; H, 6.84; N, 9.12. Amino acid ratios in a 6 N HCl hydrolysate; Arg 0.90, Pro 0.99, Glu 1.00, Trp N. D. (recovery of Glu, 90%).

Z(OMe)–Arg(Mts)–Arg(Mts)–Val–Gly–OMe (**13**): The azide [prepared from Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$[35] (5.24 g, 6.00 mmol)] in DMF (50 ml) and Et_3N (0.84 ml, 6.00 mmol) were added to a solution of a TFA-treated sample of Z(OMe)–Val–Gly–OMe[36] (2.30 g, 8.00 mmol) in DMF (30 ml) containing Et_3N (1.12 ml, 8.00 mmol). The reaction mixture was stirred for 24 h, and the DMF was removed by evaporation *in vacuo*. The product was purified by procedure A, followed by column chromatography on silica gel using $CHCl_3$–MeOH (30 : 1) as an eluant; yield 4.90 g (60%), mp 108—110 °C, $[\alpha]_D^{25}$ −14.0° (c = 1.0, MeOH), Rf_1 0.79. *Anal.* Calcd for $C_{47}H_{68}N_{10}O_{12}S_2$: C, 54.84; H, 6.66; N, 13.61. Found: C, 54.45; H, 6.73; N, 13.31.

Z(OMe)–Arg(Mts)–Arg(Mts)–Val–Gly–$NHNH_2$ [3]: The above-prepared tetrapeptide derivative **13** (3.00 g, 3.0 mmol) in MeOH (50 ml) was treated with 80% hydrazine hydrate (3.00 ml, 20 mmol) for 48 h. The solvent was removed by evaporation *in vacuo* and the residue was triturated with H_2O to give a solid, which was washed well with H_2O and reprecipitated from MeOH with ether; yield 2.50 g (83%), mp 117—119 °C, $[\alpha]_D^{25}$ −3.0° (c = 1.0, MeOH), Rf_1 0.63. *Anal.* Calcd for $C_{46}H_{68}N_{12}O_{11}S_2 \cdot H_2O$: C, 52.75; H, 6.74; N, 16.05. Found: C, 53.07; H, 6.91; N, 16.15. Amino acid ratios in a 6 N HCl hydrolysate; Arg 2.07, Val 0.91, Gly 1.00 (recovery of Gly, 91%).

Z(OMe)–Gly–Phe–Met–OMe (**14**): An MA [prepared from Z(OMe)–Gly–OH (4.03 g, 17.0 mmol] was allowed to react with a TFA-treated sample of Z(OMe)–Phe–Met–OMe[28] (8.00 g, 17.0 mmol) in DMF (30 ml) containing Et_3N (2.38 ml, 17.0 mmol) and the mixture was stirred for 5 h. The product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 6.50 g (72%), mp 111—113 °C, $[\alpha]_D^{25}$ −21.4° (c = 1.3, MeOH), Rf_1 0.79. *Anal.* Calcd for $C_{26}H_{33}N_3O_7S$: C, 58.74; H, 6.26; N, 7.91. Found: C, 58.40; H, 6.25; N, 7.85.

Z(OMe)–Gly–Gly–Phe–Met–OMe (**15**) An MA [prepared from Z(OMe)–Gly–OH (2.15 g, 9.00 mmol)] was allowed to react with a TFA-treated sample of **14** (4.80 g, 9.00 mmol) in DMF (80 ml) containing Et_3N (1.26 ml, 9.00 mmol) and the mixture was stirred for 5 h. The product was purified by procedure B, followed by recrystallization from MeOH with ether; yield 3.40 g (65%), mp 161—163 °C, $[\alpha]_D^{25}$ −11.0° (c = 1.0, MeOH), Rf_1 0.70. *Anal.* Calcd for $C_{28}H_{36}N_4O_8S \cdot H_2O$: C, 55.42; H, 6.31; N, 9.24. Found: C, 55.87; H, 6.10; N, 9.25.

Boc–Tyr–Gly–Gly–Phe–Met–OMe (**16**): A mixture of a TFA-treated sample of **15** (2.20 g, 3.70 mmol), Boc–Tyr–ONb (1.65 g, 3.70 mmol), and Et_3N (1.12 ml, 8.0 mmol) in DMF (30 ml) was stirred for 24 h and the product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 1.94 g (76%), mp 193—195 °C, $[\alpha]_D^{25}$ −8.0° (c = 1.0, AcOEt), Rf_1 0.79. *Anal.* Calcd for $C_{33}H_{45}N_5O_9S \cdot H_2O$: C, 57.62; H, 6.59; N, 10.18. Found: C, 57.17; H, 6.56; N, 10.00.

Boc–Tyr–Gly–Gly–Phe–Met–$NHNH_2$ [4]: The above-prepared pentapeptide ester **16** (2.00 g, 2.90 mmol) in DMF (20 ml) was treated with 80% hydrazine hydrate (1.50 ml, 29.0 mmol) for 24 h. The solvent was removed by evaporation and the residue was triturated with H_2O. The resulting solid was reprecipitated from DMF with EtOH; yield 1.80 g (90%), mp 193—195 °C. $[\alpha]_D^{25}$ −2.0° (c = 0.5, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{32}H_{45}N_7O_8S \cdot H_2O$: C, 54.45; H, 6.71; N, 13.89. Found: C, 54.70; H, 6.86; N, 14.28. Amino acid ratios in a 6 N HCl hydrolysate: Tyr 0.95, Gly 1.95, Phe 1.00, Met 0.71 (recovery of Phe, 83%).

Z(OMe)–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–Met(O)–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**17**): A TFA-treated sample of [1] (1.00 g, 0.42 mmol) was dissolved in DMF (20 ml) containing Et_3N (59 μl, 0.42 mmol) and, to this ice-chilled solution, the azide [prepared from [2] (1.10 g, 0.63 mmol)] in DMF (20 ml) and Et_3N (89 μl, 0.63 mmol) were added. The reaction mixture was stirred for 48 h at 4 °C, the DMF was removed by evaporation, and the product was purified by procedure B, followed by column chromatography (3.5 × 35.0 cm) on silica gel using $CHCl_3$–MeOH–AcOH (18 : 4 : 1 v/v) as an eluant. Fractions containing the desired substance (Rf_2 = 0.60) were combined and the solvent was removed by evaporation. The residue was triturated with ether to afford a solid, which was reprecipitated from MeOH with ether twice; yield 1.00 g (61%), mp 227—230 °C, $[\alpha]_D^{25}$ −10.0° (c = 0.5, DMF), Rf_1 0.64, Rf_2 0.60. *Anal.* Calcd for $C_{201}H_{248}Cl_4N_{26}O_{38}S_5 \cdot 3H_2O$: C, 60.47; H, 6.41; N, 9.12. Found: C, 60.41; H, 6.36; N, 9.19.

Z(OMe)–Arg(Mts)–Arg(Mts)–Val–Gly–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–Met(O)–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**18**): A TFA-treated sample of **17** (0.90 g, 0.23 mmol) was dissolved in DMF (20 ml) containing Et_3N (32 μl, 0.23 mmol) and, to this ice-chilled solution, the azide [prepared from [3] (0.47 g, 0.46 mmol)] in DMF (30 ml) and Et_3N (65 μl, 0.46 mmol) were added. The reaction mixture was stirred for 48 h at 4 °C, the DMF was removed by evaporation, and the residue was triturated with ether. The resulting solid was purified by gle-filtration on Sephadex LH-20 (3.0 × 125.0 cm) using DMF as an eluant. The ultraviolet (UV) absorption at 275 nm was determined in each fraction (7 ml). The fractions corresponding to the first main peak (tube Nos. 28—35) were combined and the solvent was removed by evaporation. The residue was triturated with ether and the resulting powder was reprecipitated twice from DMF with ether; yield 0.92 g (83%), mp 185—187 °C, $[\alpha]_D^{25}$ −6.0° (c = 0.5, DMF), Rf_1 0.73, Rf_2 0.70. *Anal.* Calcd for $C_{238}H_{304}Cl_4N_{36}O_{46}S_7 \cdot 4H_2O$: C, 59.02; H, 6.49; N, 10.41. Found: C, 58.70; H, 6.48; N, 10.40.

Boc–Tyr–Gly–Gly–Phe–Met–Arg(Mts)–Arg(Mts)–Val–Gly–Arg(Mts)–Pro–Glu(OBzl)–Trp(Tps)–Trp(Tps)–Met(O)–Asp(OChp)–Tyr(Cl_2Bzl)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**19**): A TFA-treated sample of **18** (0.78 g, 0.16 mmol) was dissolved in DMF (20 ml) containing Et_3N (23 μl, 0.16 mmol) and, to this ice-chilled solution, the azide [prepared from [4] (0.23 g, 0.33 mmol)] in DMF (30 ml) and Et_3N (46 μl, 0.33 mmol) were added. The reaction mixture was stirred for 48 h at 4 °C, the DMF was removed by evaporation, and the residue was triturated with ether. The resulting powder was purified by gel-filtration on Sephadex LH-20 as described above. The solvent of the desired fractions (tube Nos. 29—36) was removed by evaporation and the residue was triturated with ether to afford the powder, which was reprecipitated twice

from DMF with ether; yield 0.65 g (68%), mp 228—230 °C, $[\alpha]_D^{25}$ −4.0° (c=0.5, DMF), Rf_1 0.63, Rf_2 0.60. *Anal.* Calcd for $C_{261}H_{337}Cl_4N_{41}O_{51}S_8 \cdot 2H_2O$: C, 59.15; H, 6.49; N, 10.84. Found: C, 58.90; H, 6.56; N, 10.81.

H–Tyr–Gly–Gly–Phe–Met–Arg–Arg–Val–Gly–Arg–Pro–Glu–Trp–Trp–Met–Asp–Tyr–Gln–Lys–Arg–Tyr–Gly–Gly–Phe–Leu–OH; Peptide E (**20**): The fully protected 25-residue peptide **19** (200 mg, 38 μmol) dissolved in distilled DMF (10 ml) was treated with $(CH_3)_3SiSPh$ (700 μl, 3.8 mmol) at 40 °C under an N_2 atmosphere. The reaction mixture was stirred for 30 min, the DMF was removed by evaporation *in vacuo*. The residue was triturated with dry ether and the resulting powder was collected by centrifugation and dried over KOH pellets *in vacuo*; yield 180 mg (91%), Rf_1 0.66. The dried product (170 mg, 32 μmol) was treated with 1 M TFMSA–thioanisole in TFA (17.8 ml) in the presence of *m*-cresol (0.75 ml, 220 eq) and dimethylsulfide (0.52 ml, 220 eq) in an ice-bath for 4 h. The mixture was washed twice with *n*-hexane and dry ether was added to the residue. The precipitate formed was collected by centrifugation and washed well with dry ether. This sample was dissolved in H_2O (20 ml) and stirred with Amberlite IR-400 (*ca.* 5 g, acetate form) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was dissolved in 3% AcOH (5 ml), applied to a column of Sephadex G-25 (2.5 × 105 cm) and eluted with the same solvent system. The UV absorption (at 275 nm) was determined for the individual fractions (6 ml each) and the fractions corresponding to the main peak (tube Nos. 45—67) were combined. The solvent was removed by lyophilization to afford a fluffy powder; 70.0 mg (68% from deprotection). A part of this sample (20 mg, *ca.* 2.0 mg each) was purified by RP-HPLC on a Nucleosil $7C_{18}$ (10.0 × 250 mm) column using a gradient of CH_3CN (from 25% to 45% in 50 min) in 0.1% TFA. The eluate corresponding to the main peak (t_R, 32.75 min, Fig. 8a) was pooled. The rest of the sample was similarly purified and the combined eluates were repeatedly lyophilized to afford a white fluffy powder; 6.0 mg (30% recovery on HPLC), $[\alpha]_D^{25}$ −73.1° (c=0.1, 1 N AcOH), Rf_4 0.19, Rf_5 0.70, Rf_6 0.20. HPLC: t_R, 32.75 min (Fig. 8b). Amino acid ratios in a papain plus LAP digest (numbers in parentheses are theoretical values): Asp 0.90 (1), Glu 1.18 (1), Pro 0.73 (1), Gly 5.40 (5), Val 0.75 (1), Met 1.80 (2), Leu 1.00 (1), Tyr 3.01 (3), Phe 2.00 (2), Lys 1.12 (1), Trp 2.06 (2), Arg 3.35 (4), Asn and Gln were not determined.

Synthesis of Dynorphin (1—24) Z(OMe)–Gln–Lys(Z)–Arg(Mts)–$NHNH_2$ (**21**): Z(OMe)–Gln–Lys(Z)–Arg(Mts)–OMe (**4**) (4.20 g, 4.50 mmol) dissolved in DMF (40 ml) was treated with 80% hydrazine hydrate (2.30 ml, 45 mmol) for 48 h. The DMF was removed by evaporation and the residue was triturated with H_2O to afford the solid, which was collected and washed well with H_2O; yield 4.00 g (95%), mp 208—210 °C, $[\alpha]_D^{25}$ −6.0° (c=1.0, DMF), Rf_1 0.40. *Anal.* Calcd for $C_{43}H_{60}N_{10}O_{11}S \cdot 0.5H_2O$: C, 55.29; H, 6.58; N, 15.00. Found: C, 55.26; H, 6.49; N, 14.61.

Z(OMe)–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**22**): The azide [prepared from **21** (4.16 g, 5.50 mmol)] in DMF (50 ml) and Et_3N (0.77 ml, 5.50 mmol) were added to a solution of a TFA-treated sample of **2** (3.62 g, 4.00 mmol) in DMF (30 ml) containing Et_3N (0.63 ml, 4.50 mmol). The reaction mixture was stirred for 48 h at 4 °C, then the solvent was removed by evaporation *in vacuo*. The product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 4.50 g (66%), mp 197—199 °C, $[\alpha]_D^{25}$ −15.0° (c=1.0, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{85}H_{103}Cl_2N_{13}O_{18}S \cdot 2H_2O$: C, 58.89; H, 6.22; N, 10.50. Found: C, 58.73; H, 6.24; N, 10.81.

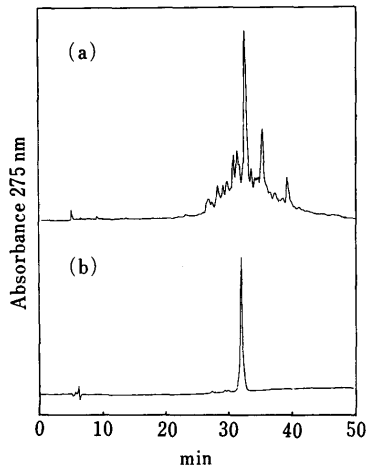

Fig. 8. HPLC of Sephadex G-25 Purified Product (a) and Finally Purified Peptide E (b)

Z(OMe)–Asn–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**23**): A mixture of a TFA-treated sample of **22** (7.00 g, 4.10 mmol), Z(OMe)–Asn–ONp (2.60 g, 6.20 mmol) and Et_3N (1.45 ml, 10.4 mmol) in DMF (70 ml) was stirred for 40 h and the product was purified by procedure B, followed by reprecipitation from DMF with AcOEt; yield 7.00 g, (94%), mp 205–207 °C, $[\alpha]_D^{25}$ −5.0° (c=1.0, DMF), Rf_1 0.75. *Anal.* Calcd for $C_{89}H_{109}Cl_2N_{15}O_{20}S \cdot 2H_2O$: C, 57.84; H, 6.16; N, 11.37. Found: C, 57.68; H, 6.02; N, 11.44.

Boc–Asp(OChp)–Asn–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**24**): A mixture of a TFA-treated sample of **23** (5.30 g, 2.90 mmol), Boc–Asp(OChp)–OSu (2.00 g, 4.70 mmol), Et_3N (0.41 ml, 2.90 mmol) and NMM (0.52 ml, 4.70 mmol) in DMF (60 ml) was stirred for 24 h and the product was purified by procedure B, followed by reprecipitation from DMF with ether; yield 5.10 g (89%), mp 200—204 °C, $[\alpha]_D^{25}$ −12.0° (c=0.5, DMF), Rf_1 0.78. *Anal.* Calcd for $C_{96}H_{126}Cl_2N_{16}O_{22}S \cdot 3H_2O$: C, 57.79; H, 6.67; N, 11.23. Found: C, 57.76; H, 6.42; N, 11.42.

Boc–Trp(Tps)–Asp(OChp)–Asn–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl (**25**): A mixture of a TFA-treated sample of **24** (5.00 g, 2.60 mmol), Boc-Trp(Tps)-OSu (2.86 g, 4.30 mmol), Et_3N (0.36 ml, 2.60 mmol) and NMM (0.47 ml, 4.30 mmol) in DMF (50 ml) was stirred for 18 h and the product was purified by procedure B, followed by reprecipitation from DMF with ether; yield 4.80 g (78%), mp 196—198 °C, $[\alpha]_D^{25}$ −5.0° (c=1.0, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{122}H_{158}Cl_2N_{18}O_{25}S_2 \cdot 3H_2O$: C, 59.42; H, 6.70; N, 10.23. Found: C, 59.20; H, 6.44; N, 10.57.

Z(OMe)–Lys(Z)–Trp(Tps)–Asp(OChp)–Asn–Gln–Lys(Z)–Arg(Mts)–Tyr(Cl_2Bzl)–Gly–Gly–Phe–Leu–OBzl [5]: An MA [prepared from Z(OMe)–Lys(Z)-OH (0.56 g, 1.20 mmol)] was allowed to react with a TFA-treated sample of **25** (2.00 g, 0.83 mmol) in DMF (30 ml) containing Et_3N (0.12 ml, 0.83 mmol) and the mixture was stirred for 5 h. The product was purified by procedure B, followed by reprecipitation from DMF with ether; yield 2.00 g (88%), mp 230—235 °C, $[\alpha]_D^{25}$ −20.0° (c=0.5, DMF), Rf_1 0.65. *Anal.* Calcd for $C_{140}H_{176}Cl_2N_{20}O_{29}S_2 \cdot 2H_2O$: C, 60.61; H, 6.54; N, 10.10. Found: C, 60.40; H, 6.38; N, 10.31.

Boc–Ile–Arg(Mts)–Pro–OH (**26**): The azide [prepared from 5.83 g (10.0 mmol) of Boc–Ile–Arg(Mts)–$NHNH_2$[41]] was combined with an aqueous solution of H–Pro–OH (1.56 g, 13.0 mmol) containing Et_3N (3.80 ml, 26.0 mmol) and the reaction mixture was stirred for 24 h. The product was purified by the AcOEt extraction method, followed by recrystallization from AcOEt with petroleum ether; yield 6.50 g (98%), mp 130—134 °C, $[\alpha]_D^{25}$ −20.0° (c=1.0, AcOEt), Rf_1 0.42. *Anal.* Calcd for $C_{31}H_{50}N_6O_8S \cdot 0.5H_2O$: C, 55.09; H, 7.60; N, 12.44. Found: C, 54.99; H, 7.48; N, 12.48.

Boc–Ile–Arg(Mts)–Pro–Lys(Z)–Leu–OMe (**27**): The above-prepared tripeptide derivative **26** (5.00 g, 7.50 mmol) was allowed to react with the HCl salt of Z(OMe)–Lys(Z)–Leu–OMe[40] (4.29 g, 7.50 mmol)[obtained by deprotection with 4 N HCl–dioxane] in DMF (30 ml) containing Et_3N (1.05 ml, 7.50 mmol) by the DCC (1.70 g, 8.30 mmol) + HOBt (1.00 g, 7.50 mmol) method, and the mixture was stirred for 24 h. The product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 6.40 g (81%), mp 103—105 °C, $[\alpha]_D^{25}$ −18.0° (c=1.0, AcOEt), Rf_1 0.66. *Anal.* Calcd for $C_{52}H_{81}N_9O_{12}S \cdot H_2O$: C, 58.13; H, 7.79; N, 11.73. Found: C, 58.47; H, 7.96; N, 11.47.

Boc–Ile–Arg(Mts)–Pro–Lys(Z)–Leu–$NHNH_2$ [6]: The above-prepared pentapeptide ester **27** (4.10 g, 3.88 mmol) dissolved in DMF (40 ml) was treated with 80% hydrazine hydrate (3.8 ml, 78 mmol) for 48 h. The DMF was removed by evaporation and the product was purified by procedure A, followed by recrystallization twice from MeOH with ether; yield 4.00 g (98%), mp 204—207 °C, $[\alpha]_D^{25}$ −28.0° (c=1.0, AcOEt), Rf_1 0.63. *Anal.* Calcd for $C_{51}H_{81}N_{11}O_{11}S \cdot H_2O$: C, 57.01; H, 7.79; N, 14.34. Found: C, 57.12; H, 7.71; N, 14.75. Amino acid ratios in a 6 N HCl hydrolysate; Ile 1.07, Arg 1.12, Pro 1.06, Lys 1.29, Leu 1.00 (recovery of Leu, 76%).

Boc–Tyr–Gly–Gly–Phe–Leu–OBzl (**28**): A mixture of a TFA-treated sample of **1** (6.00 g, 10.5 mmol), Boc–Tyr–ONb (3.80 g, 8.50 mmol) and NMM (3.10 ml, 27.5 mmol) was stirred for 24 h and the product was purified by procedure A, followed by recrystallization from AcOEt with ether; yield 5.60 g (79%), mp 111—114 °C, $[\alpha]_D^{25}$ −18.0° (c=1.0, AcOEt), Rf_1 0.65. *Anal.* Calcd for $C_{34}H_{47}N_5O_9 \cdot H_2O$: C, 59.37; H, 7.18; N, 10.18. Found: C, 59.62; H, 7.10; N, 9.73.

Boc–Tyr–Gly–Gly–Phe–Leu–$NHNH_2$ [8]: The above-prepared pentapeptide ester **28** (4.60 g, 6.90 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (3.40 ml, 69 mmol) for 24 h. The DMF was removed by

evaporation and the residue was triturated with MeOH. The solid product was washed well with cold MeOH and recrystallized from DMF with MeOH; yield 4.50 g (97%), mp 214—216 °C, $[\alpha]_D^{25}$ −13.0° (c = 1.0, DMF), Rf_1 0.55. *Anal.* Calcd for $C_{33}H_{47}N_7O_8$: C, 59.17; H, 7.07; N, 14.64. Found: C, 59.10; H, 7.00; N, 14.38. Amino acid ratios in a 6 N HCl hydrolysate; Tyr 0.86, Gly 1.93, Phe 1.03, Leu 1.00 (recovery of Leu, 81%).

Boc-Ile-Arg(Mts)-Pro-Lys(Z)-Leu-Lys(Z)-Trp(Tps)-Asp(OChp)-Asn-Gln-Lys(Z)-Arg(Mts)-Tyr(Cl_2Bzl)-Gly-Gly-Phe-Leu-OBzl (**29**): A TFA-treated sample of [5] (1.70 g, 0.62 mmol) was dissolved in DMF (15 ml) containing Et_3N (87 μl, 0.62 mmol) and, to this ice-chilled solution, the azide [prepared from [6] (0.98 g, 0.93 mmol)] in DMF (15 ml) and Et_3N (0.13 ml, 0.93 mmol) were added. The reaction mixture was stirred for 48 h at −10 °C, additional azide (0.31 mmol) and Et_3N (0.31 mmol) were added, and the mixture was further stirred for 36 h. The DMF was removed by evaporation and the product was purified by procedure B, followed by reprecipitation from DMF with ether; yield 2.10 g (94%), mp 115—120 °C, $[\alpha]_D^{25}$ +4.0° (c = 1.0, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{182}H_{245}Cl_2N_{29}O_{37}S_3 \cdot 2H_2O$: C, 60.15; H, 6.91; N, 11.18. Found: C, 59.97; H, 7.05; N, 11.50.

Z(OMe)-Arg(Mts)-Arg(Mts)-Ile-Arg(Mts)-Pro-Lys(Z)-Leu-Lys(Z)-Trp(Tps)-Asp(OChp)-Asn-Gln-Lys(Z)-Arg(Mts)-Tyr(Cl_2Bzl)-Gly-Gly-Phe-Leu-OBzl (**30**): A TFA-treated sample of **29** (1.00 g, 0.28 mmol) was dissolved in DMF (20 ml) containing Et_3N (39 μl, 0.28 mmol) and, to this ice-chilled solution, the azide [prepared from Z(OMe)-Arg(Mts)-Arg(Mts)-$NHNH_2$ (0.29 g, 0.33 mmol)] in DMF (20 ml) and Et_3N (46 μl, 0.33 mmol) were added. The reaction mixture was stirred for 18 h at −10 °C, additional azide (0.08 mmol) and Et_3N (0.08 mmol) were added, and the mixture was further stirred for 30 h. The DMF was removed by evaporation and the product was purified by procedure B, followed by column chromatography on silica gel using $CHCl_3$–MeOH (20 : 1) as an eluant. The fractions containing the desired material were pooled and the solvent was removed by evaporation. The residue was triturated with ether to afford the powder, which was reprecipitated from MeOH with ether; yield 0.92 g (75%), mp 235—238 °C, $[\alpha]_D^{25}$ −12.0° (c = 0.5, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{216}H_{289}Cl_2N_{37}O_{44}S_5 \cdot 3H_2O$: C, 59.05; H, 6.77; N, 11.80. Found: C, 58.88; H, 6.96; N, 11.98.

Boc-Tyr-Gly-Gly-Phe-Leu-Arg(Mts)-Arg(Mts)-Ile-Arg(Mts)-Pro-Lys(Z)-Leu-Lys(Z)-Trp(Tps)-Asp(OChp)-Asn-Gln-Lys(Z)-Arg(Mts)-Tyr(Cl_2Bzl)-Gly-Gly-Phe-Leu-OBzl (**31**): A TFA-treated sample of **30** (1.00 g, 0.23 mmol) was dissolved in DMF (20 ml) containing Et_3N (32 μl, 0.23 mmol) and, to this ice-chilled solution, the azide [prepared from [8] (0.39 g, 0.58 mmol)] in DMF (15 ml) and Et_3N (0.16 ml, 0.12 mmol) were added. The reaction mixture was stirred for 48 h at 4 °C, and the DMF was removed by evaporation. The product was purified by procedure B, followed by column chromatography on silica gel using $CHCl_3$ as an eluant. The fractions containing the desired material were pooled and the solvent was removed by evaporation. The residue was triturated with ether to afford a powder, which was reprecipitated from MeOH with ether. Yield 0.67 g (60%), mp 127—130 °C, $[\alpha]_D^{25}$ −8.0° (c = 0.5, DMF), Rf_1 0.58, Rf_2 0.47. *Anal.* Calcd for $C_{240}H_{324}Cl_2N_{42}O_{49}S_5 \cdot 4H_2O$: C, 59.01; H, 6.85; N, 12.05. Found: C, 59.20; H, 6.88; N, 11.56.

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Leu-OH; Dynorphin (1—24) (**32**): The above-prepared fully protected 24-residue peptide **31** (200 mg, 42 μmol) was treated with 1 M TFMSA–thioanisole in TFA (12.5 ml) in the presence of *m*-cresol (0.44 ml, 100 eq) and dimethylsulfide (0.31 ml, 100 eq) in an ice-bath for 90 min. The mixture was washed with *n*-hexane twice and the oily residue was triturated with dry ether to afford a precipitate, which was collected by centrifugation and again treated with 1 M TFMSA–thioanisole–TFA under the same conditions as described above. The deprotected peptide, isolated by addition of ether, was dissolved in H_2O (20 ml) and stirred with Amberlite IR-400 (*ca.* 5 g, acetate form) for 30 min. The resin was removed by filtration, the pH of the filtrate was adjusted to 6.7 with cold 5% NH_4OH, and the solution was lyophilized. The residue was dissolved in 3% AcOH (5 ml) and applied to a column of Sephadex G-25 (2.5 × 105 cm), which was eluted with the same solvent. The UV absorption (at 275 nm) was determined for the individual fractions (6 ml) and the fractions corresponding to the main peak (tube Nos. 31—48) were combined. The solvent was removed by lyophilization to afford a white fluffy powder; 112 mg (91% from deprotection). The crude sample thus obtained (50 mg) was dissolved in 0.005 M $AcONH_4$ (pH 6.8, 2 ml) and applied to a column of CM Bio-Gel A (2.2 × 10 cm), which was eluted with a linear gradient of 0.6 M $AcONH_4$ (pH 6.8, 400 ml) through a mixing flask containing 0.005 M $AcONH_4$ (pH 6.8, 400 ml). The UV absorption of the individual fractions (10 ml) at 275 nm was determined. The fractions corresponding to the main peak (tube Nos. 31—42, Fig. 9) were collected and the solvent was removed by repeated lyophilization. For desalting, the lyophilized material was dissolved in 3% AcOH (3 ml) and passed through a column of Sephadex G-25 (2.5 × 105 cm). A white fluffy powder was recovered after lyophilization; 30.2 mg, 60% recovery on the ion-exchange chromatography. For further purification, a part of this sample (30 mg, *ca.* 2 mg each) was purified by HPLC on a Nucleosil 7C_{18} column (10 × 250 mm) using the solvent system of CH_3CN (from 25% to 45% in 40 min) in 0.1% TFA. The eluate corresponding to the main peak (t_R, 21.00 min; Fig. 10a) was pooled. The rest of the sample was similarly purified and the solvent of the combined eluates was removed by evaporation *in vacuo* and the product was lyophilized to afford a white fluffy powder; 15.0 mg (50% recovery on HPLC), $[\alpha]_D^{25}$ −40.0° (c = 1.0, 3% AcOH), Rf_4 0.35, Rf_5 0.76, Rf_6 0.36. HPLC: t_R, 21.00 min (Fig. 10b). Amino acid ratios in a papain + LAP digest (numbers in parentheses are theoretical values): Asp 0.91 (1), Pro 0.78 (1), Gly 4.28 (4), Ile 1.12 (1), Leu 3.00 (3), Tyr 2.10 (2), Phe 1.98 (2), Lys 2.88 (3), Arg 3.56 (4), Trp 0.87 (1), Asn + Gln were not determined, (recovery of Leu, 78%).

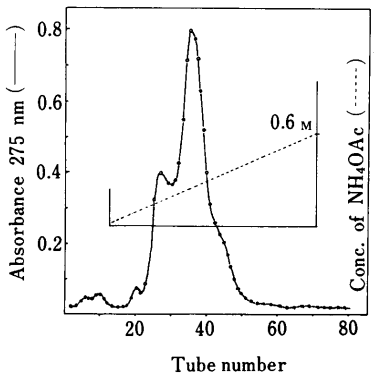

Fig. 9. Purification of Dynorphin (1—24) on CM-Biogel A

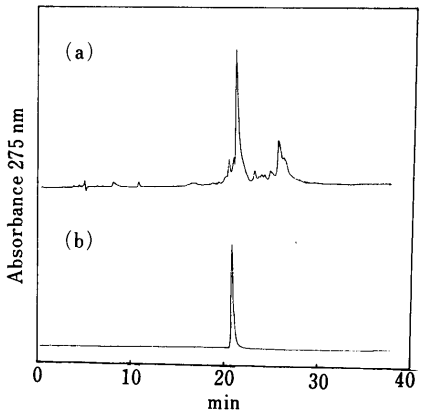

Fig. 10. HPLC of CM-Biogel A Purified Product (a) and Finally Purified Dynorphin (1—24) (b)

References and Notes

1) Amino acids, peptides and their derivatives in this paper are of L-configurations. Abbreviations used are those recommended by the I.U.P.A.C.–I.U.B. Joint Commission on Biochemical Nomenclature: *Eur. J. Biochem.*, **138**, 9 (1984). The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Bzl = benzyl, Cl_2Bzl = 2,6-dichlorobenzyl, Mts = mesitylenesulfonyl, Chp = cycloheptyl, Troc = 2,2,2-trichloroethoxycarbonyl, Tps = 2,4,6-triisopropylphenylsulfonyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccucinimidyl, Nb = *N*-hydroxy-5-norbornene-2,3-dicarboximidyl, PCP = pentachlorophenyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, MSA = methanesulfonic acid, TFMSA = trifluormethanesulfonic acid, Et_3N = triethylamine, NMM = *N*-methylmorpholine, AcOH = acetic acid, DMF = dimethylformamide, MeOH = methanol, AcOEt = ethyl acetate, LAP = leu-

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