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Synthesis of a 37-Residue Peptide Amide Corresponding to the Entire Amino Acid Sequence of α-Form of Rat Calcitonin Gene-Related Peptide (α-rCGRP)[1)]

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The heptatriacontapeptide amide corresponding to the entire amino acid sequence of α-form of rat calcitonin gene-related peptide (α-rCGRP) was synthesized by the conventional solution method. All protecting groups employed were removed by treatment with 1 M trifluoromethanesulfonic acid–thioanisole–trifluoroacetic acid, and the deprotected peptide was subjected to air-oxidation to form the intramolecular disulfide bond. After purification by gel-filtration on Sephadex G-50, followed by reversed-phase high perfórmance liquid chromatography, a highly purified sample of synthetic α-rCGRP was obtained. In terms of suppression of bone ^{45}Ca-release stimulated by synthetic human parathyroid hormone (1—34), synthetic α-rCGRP was as active as synthetic human CGRP.

Keywords——rat calcitonin gene-related peptide synthesis; aminosuccinimide formation; β-cycloheptylaspartate; thioanisole-mediated acidolysis; trifluoromethanesulfonic acid deprotection; intramolecular disulfide bond formation; ^{45}Ca-release suppression

The existence of a novel peptide, referred to as calcitonin gene-related peptide (CGRP), was predicted on the basis of structural analysis of the rat calcitonin gene by Rosenfeld *et al.*[2)] Subsequently, human CGRP (hCGRP)[3)] and porcine CGRP[4)] were isolated from human medullary thyroid carcinoma and porcine spinal cord, respectively. It has become evident that the calcitonin gene generates at least two messenger ribonucleic acids (mRNAs), one encoding the precursor of calcitonin in thyroid C-cells and the second encoding the precursor of a novel structurally related neuropeptide, CGRP, which predominates in the brain. In 1984, Rosenfeld *et al.*,[5)] detected the presence of yet another CGRP, referred to as β-CGRP in the rat, and thus raised the possibility that a single neuropeptide gene generates multiple RNA products. This β-form of rat CGRP (β-rCGRP) differs in sequence from α-rCGRP by an internal amino acid residue at position 35 (Lys instead of Glu), as shown in Fig. 1.

Following the synthesis of hCGRP,[6)] which was conducted in collaboration with Fujii *et al.*, we wish to report the solution synthesis of a 37-residue peptide corresponding to the entire amino acid sequence of α-rCGRP, the sequence of which was first deduced from the rat gene by Rosenfeld *et al.*, in 1983.[2d)]

The TFA-labile Z(OMe) or Boc group was employed for N^{α}-protection, and amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[7)] were employed, *i.e.*, Asp(OChp),[8)] Glu(OBzl), Ser(Bzl), Lys(Z), Arg(Mts),[9)] and Cys(MBzl). Structurally different from hCGRP, rCGRP contains the Asp–Asn linkage at position 25–26, which is known to be particularly susceptible to base-catalyzed succinimide formation.[10)] Thus, Asp(OChp), which is known to suppress this side reaction, was employed. In the

H- 1 -Cys- 3 -Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-
Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys- 25 -Asn-
Phe-Val-Pro-Thr-Asn-Val-Gly-Ser- 35 -Ala-Phe-NH_2

	1	3	25	35
rat α	Ser	Asn	Asp	Glu
rat β	Ser	Asn	Asp	Lys
human	Ala	Asp	Asn	Lys

Fig. 1. Amino Acid Sequences of Rat and Human CGRPs

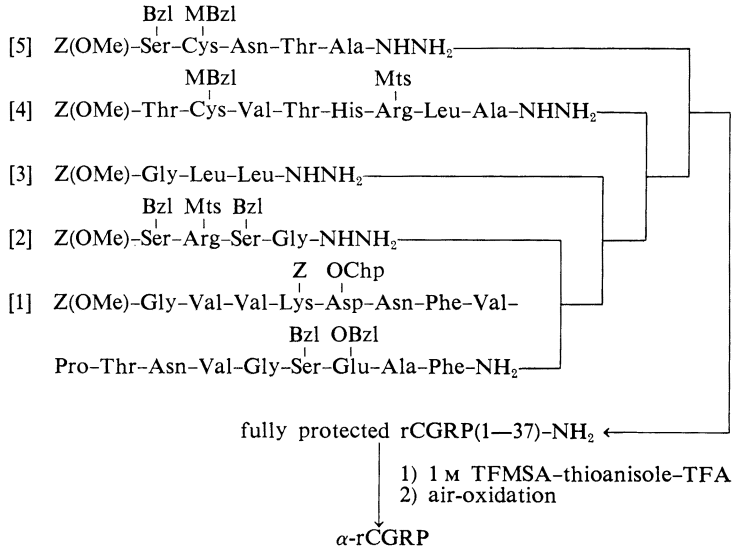

Fig. 2. Synthetic Route to Rat α-CGRP

hCGRP synthesis, the S-adamantyl group[11] was employed for the protection of the sulfhydryl function of Cys. However, in the present synthesis, a readily available derivative, Cys(MBzl),[12] was employed.

In order to construct the entire peptide backbone of α-rCGRP, five fragments were prepared as building blocks, as shown in Fig. 2. Of these, fragments [2] and [3] are identical with those employed for the synthesis of hCGRP.[6] Fragments [1], [4] and [5], which cover the areas of species variation between these CGRPs (positions 1, 3, 25, and 35 in Fig. 1), were newly synthesized.

Fragment [1] was prepared according to the scheme shown in Fig. 3. First, the C-terminal dodecapeptide amide was prepared by the azide condensation[13] of two subunits, the hepta- and pentapeptide units. The former heptapeptide amide, Boc–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2, was prepared in a stepwise manner starting with H–Phe–NH_2.[14] The active ester procedure, such as Su[15] or Np[16] procedure, was employed for elongation of the peptide chain. To prepare the latter peptide unit, Boc–Phe–Val–Pro–Thr–OMe was first synthesized by the DCC + HOBt[17] condensation of the known tripeptide, Boc–Phe–Val–Pro–OH[18] and H–Thr–OMe. After the usual TFA treatment, Boc–Asn–OH was introduced by the Np method to afford Boc–Asn–Phe–Val–Pro–Thr–OMe, which was converted to the corresponding hydrazide by the usual hydrazinolysis. The dodecapeptide amide thus constructed was further elongated to [1] in a stepwise manner by the active ester procedure.

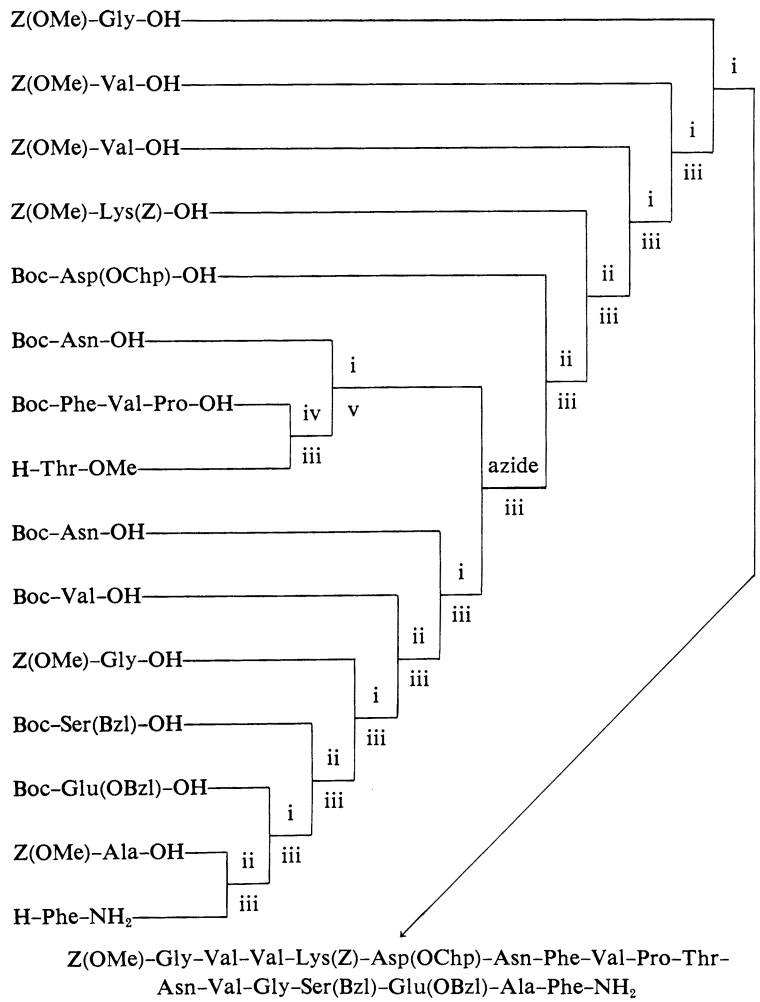

Fig. 3. Synthetic Scheme for the Protected Heptapeptide Amide; Fragment [1]
i, Np; ii, Su; iii, TFA; iv, DCC+HOBt; v, NH_2NH_2.

Preliminarily, after introduction of the Asp(OChp) residue, we tried the azide condensation of Z(OMe)–Gly–Val–Val–Lys(Z)–$NHNH_2$, a fragment used in the hCGRP synthesis,[6] with this tridecapeptide amide. However, despite the use of the acyl component in excess (10 to 15 eq) and a long reaction time (7 d), amino acid analysis of the product showed an insufficient incorporation of the acyl component (*ca.* 65%). The bulkiness of the Chp group at the N-terminal residue of the amino component may account for this unsatisfactory azide reaction. On the other hand, the satisfactory incorporation of Z(OMe)–Lys(Z)–OH into the tridecapeptide amide by the Su method was ascertained by thin layer chromatography (TLC) and amino acid analysis. Each product was purified by reprecipitation from DMSO–DMF with MeOH and its homogeneity was confirmed by TLC, elemental analysis, and amino acid analysis. In the latter instance, after the incorporation of the bulky Val–Val sequence (positions 22–23), the recovery of Val on amino acid analysis became slightly low, due to the resistance to acid hydrolysis.

Fragment [4] was synthesized according to the scheme shown in Fig. 4. In the hCGRP synthesis, to construct the peptide backbone corresponding to positions 6 to 13, two

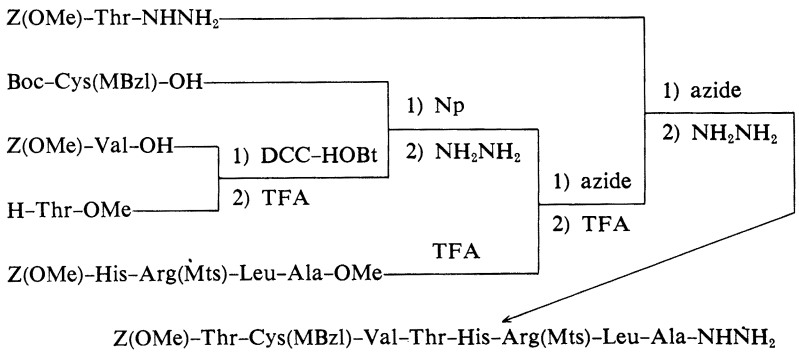

Fig. 4. Synthetic Scheme for the Protected Octapeptide Hydrazide; Fragment [4]

fragments, (6—9) and (10—13), were employed.[6] Considering the good solubility of Z(OMe)–His–Arg(Mts)–Leu–Ala–OMe,[6] we decided to elongate this peptide chain and establish the peptide backbone by condensation of one fragment, [4]. Z(OMe)–Cys(MBzl)–Val–Thr–OMe, prepared by DCC+HOBt condensation followed by the Np method, was converted to the corresponding hydrazide and the resulting hydrazide was next condensed with a TFA-treated sample of Z(OMe)–His–Arg(Mts)–Leu–Ala–OMe[6] by the azide method to afford Boc–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–OMe. Subsequent introduction of Z(OMe)–Thr–$NHNH_2$, *via* the azide, afforded Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–OMe, which was smoothly converted to [4] by the usual hydrazine treatment. In the previous hCGRP synthesis, the azide condensation of Z(OMe)–Thr–Cys(Ad)–Val–Thr–$NHNH_2$ with the relatively large amino component (positions 10—37) was performed at lower temperature (−15 °C) than usual (−4 °C) in order to minimize the Curtius rearrangement.[19] In this fragment synthesis, the condensation between Thr (position 9) and His (position 10) could be performed as usual without particular attention. The employment of the altered fragment, namely that corresponding to positions 6 to 13 of the peptide backbone, was effective to avoid the problem of the Curtius rearrangement at the larger peptide level.

The synthetic scheme for fragment [5] is shown in Fig. 5. Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–OMe, prepared in a stepwise manner by the Np or the Su active ester procedures, was converted to the corresponding hydrazide. The azide condensation of the resulting hydrazide with H–Ala–OMe afforded Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–OMe, which was converted to the corresponding hydrazide [5] as usual.

The five fragments thus obtained, [1] to [5], were successively assembled by the azide procedure according to the scheme shown in Fig. 2. Each condensation was performed in a mixture of HMPA–DMF (5 : 1) at −4 °C and the amount of acyl component was increased from 6 to 10 eq as the peptide chain was elongated in order to ensure completion of the coupling reaction. Each product was purified by precipitation from HMPA–DMF (5 : 1) with MeOH and its homogeneity was confirmed by amino acid analysis after 6 N HCl hydrolysis, in which Phe was selected as a diagnostic amino acid. By comparison of the recovery of Phe with those of newly incorporated amino acids, satisfactory incorporation of each fragment was ascertained in each condensation step. The results of amino acid analyses are listed in Table I.

In the final step, the fully protected α-rCGRP was treated with 1 M TFMSA–thioanisole in TFA in the presence of *m*-cresol[20] (0 °C, 4 h) to remove all protecting groups. The deprotected peptide was reduced with 2-mercaptoethanol in Tris–HCl buffer containing 6 M guanidine–HCl (pH 8.0) and applied to a column of Sephadex G-25. The main fraction was submitted to air-oxidation to form the intramolecular disulfide bond as follows: a diluted

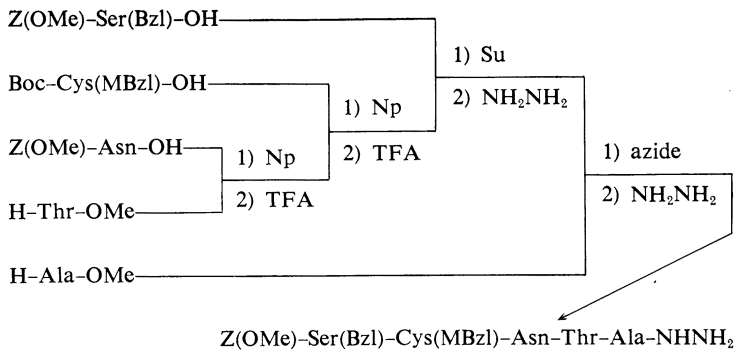

Fig. 5. Synthetic Scheme for the Protected Pentapeptide Hydrazide; Fragment [5]

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

Amino acid	Protected peptides					Synthetic α-rCGRP
	20—37	17—37	14—37	6—37	1—37	
Asp	2.73 (3)	2.93 (3)	3.00 (3)	3.00 (3)	4.09 (4)	4.02 (4)
Thr	0.77 (1)	0.94 (1)	0.87 (1)	2.76 (3)	3.82 (4)	3.85 (4)
Ser	0.73 (1)	2.23 (3)	2.19 (3)	2.68 (3)	3.56 (4)	3.89 (4)
Glu	1.06 (1)	1.05 (1)	1.04 (1)	1.06 (1)	1.22 (1)	1.18 (1)
Pro	1.00 (1)	0.97 (1)	0.92 (1)	0.90 (1)	1.05 (1)	1.08 (1)
Gly	1.89 (2)	2.61 (3)	3.59 (4)	4.00 (4)	4.22 (4)	4.37 (4)
Ala	1.00 (1)	1.04 (1)	1.03 (1)	2.25 (2)	3.40 (3)	3.23 (3)
Cys					N.D.	0.75 (1)
Val	3.34 (4)	3.32 (4)	3.63 (4)	4.64 (5)	4.74 (5)	4.48 (5)
Leu			1.76 (2)	3.32 (3)	3.44 (3)	3.17 (3)
Phe	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
Lys	0.88 (1)	0.95 (1)	0.87 (1)	0.95 (1)	1.00 (1)	1.07 (1)
His				1.20 (1)	1.18 (1)	1.05 (1)
Arg		0.87 (1)	0.90 (1)	2.33 (2)	2.09 (2)	2.05 (2)
Recovery (%)	88	83	83	85	82	90

solution of the desired eluate (1 l) in 5% $AcONH_4$ (pH 7.5) was kept standing at room temperature for 4 d, while the progress of air-oxidation was monitored by the use of Ellman's reagent.[21] The oxidized product, after removal of the solvent by lyophilization, was purified by gel-filtration on Sephadex G-50, followed by reversed-phase high performance liquid chromatography (HPLC) on a Nucleosil $7C_{18}$ column using gradient elution with CH_3CN (30% to 40% for 50 min) in 0.2% TFA (Fig. 7-a). The purity of the product thus obtained was confirmed by analytical HPLC (Fig. 7-b), TLC in three different solvent systems, amino acid analyses after acid hydrolysis (Table I) and enzymic hydrolysis (papain + LAP). The excellent recoveries of Asp (1.02) and Asn (3.03) in enzymic hydrolysate confirm the absence of the contaminant derived from ring closure at the Asp^{25}–Asn^{26} linkage. In addition, our synthetic α-rCGRP was confirmed to be monomeric by HPLC using a TSKgel G 2000SW column (Fig. 8).

Synthetic α-rCGRP (1×10^{-7} M) suppressed the ^{45}Ca-release from bone stimulated by a synthetic sample of human parathyroid hormone (1—34),[22] purchased from Toyo Jozo Co. Its potency was estimated to be at the same level as that of synthetic hCGRP.

Experimental

General experimental procedures employed in this study were as follows.

N^{α}-Deprotection——The N^{α}-protecting group, Z(OMe) or Boc, was removed 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[14] using isoamyl nitrite with stirring in a cold room (−4 °C). Mixed anhydrides were prepared using ethyl 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, then crystallized or precipitated from appropriate solvents.

The melting points are uncorrected. Optical rotations were determined with a Union PM-201 polarimeter. Acid hydrolyses were carried out in 6 N HCl in a sealed tube, and amino acid analyses were 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) or cellulose (precoated Cellulose F, Merck). Solvent systems used were as follows; Rf_1 = $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_2 = *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_3 = *n*-BuOH–AcOH–pyridine–H_2O (30 : 20 : 6 : 24), and Rf_4 = *n*-BuOH–AcOH–pyridine–H_2O (30 : 6 : 20 : 24).

HPLC was conducted with a Shimadzu LC 4A instrument equipped with a Chemopak column (Nucleosil $7C_{18}$, 4.8 × 250 mm).

Z(OMe)–Ala–Phe–NH_2——A TFA-treated sample of Z(OMe)–Phe–NH_2 (6.60 g, 20 mmol) was dissolved in DMF (100 ml), together with Et_3N (5.60 ml, 40 mmol) and Z(OMe)–Ala–OSu (7.70 g, 22 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with MeOH and the resulting solid was purified by procedure B, followed by recrystallization from MeOH with ether. Yield 6.70 g (84%), mp 220—222 °C, $[\alpha]_D^{25}$ −20.2° (*c* = 1.0, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{21}H_{25}N_3O_5$: C, 63.14; H, 6.31; N, 10.52. Found: C, 62.79; H, 6.28; N, 10.47.

Boc–Glu(OBzl)–Ala–Phe–NH_2——A TFA-treated sample of Z(OMe)–Ala–Phe–NH_2 (8.00 g, 20 mmol) was dissolved in DMF (100 ml), together with Et_3N (6.20 ml, 44 mmol) and Boc–Glu(OBzl)–ONp (11.0 g, 24 mmol). The mixture was stirred for 48 h, and DMF was removed by evaporation. Addition of MeOH to the residue afforded a powder, which was purified by procedure B, followed by reprecipitation from DMF with ether. Yield 10.0 g (90%), mp 174—177 °C, $[\alpha]_D^{25}$ −30.0° (*c* = 1.0, MeOH), Rf_1 0.58. *Anal.* Calcd for $C_{29}H_{38}N_4O_7$: C, 62.80; H, 6.91; N, 10.10. Found: C, 62.70; H, 6.97; N, 10.28.

Boc–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2——A TFA-treated sample of Boc–Glu(OBzl)–Ala–Phe–NH_2 (8.90 g, 16 mmol) was dissolved in DMF (100 ml), together with Et_3N (5.00 ml, 35.2 mmol) and Boc–Ser(Bzl)–OSu (7.53 g, 19.2 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by reprecipitation from DMF with ether. Yield 11.12 g (95%), mp 199—201 °C, $[\alpha]_D^{25}$ −14.0° (*c* = 1.0, MeOH), Rf_1 0.74. *Anal.* Calcd for $C_{39}H_{49}N_5O_9$: C, 64.00; H, 6.75; N, 9.57. Found: C, 63.77; H, 6.65; N, 9.40.

Z(OMe)–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2——A TFA-treated sample of Boc–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2 (9.60 g, 13 mmol) was dissolved in DMF (200 ml), together with Et_3N (4.00 ml, 28.6 mmol) and Z(OMe)–Gly–ONp (5.60 g, 15.6 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by reprecipitation from DMF with ether. Yield 10.42 g (94%), mp 203—206 °C, $[\alpha]_D^{25}$ −16.1° (*c* = 1.1, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{45}H_{52}N_6O_{11} \cdot 1/2H_2O$: C, 62.70; H, 6.20; N, 9.75. Found: C, 62.66; H, 6.04; N, 9.47.

Boc–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2——A TFA-treated sample of Z(OMe)–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2 (9.00 g, 11 mmol) was dissolved in DMF (150 ml), together with Et_3N (3.40 ml, 25.3 mmol) and Boc–Val–OSu (4.30 g, 14.3 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by reprecipitation from DMF with ether. Yield 8.50 g (91%), mp 231—234 °C, $[\alpha]_D^{25}$ −5.5° (*c* = 1.3, DMF), Rf_1 0.85. *Anal.* Calcd for $C_{46}H_{61}N_7O_{11}$: C, 62.21; H, 6.92; N, 10.93. Found: C, 62.06; H, 6.86; N, 11.26.

Boc–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2——A TFA-treated sample of Boc–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2 (4.50 g 5 mmol) was dissolved in DMF (100 ml), together with Et_3N (0.70 ml, 5 mmol), Boc–Asn–ONp (2.30 g, 6.5 mmol), HOBt (0.68 g, 5 mmol), and NMM (1.27 ml, 11.5 mmol). The mixture was stirred

for 5 h, and the solution was poured into cold MeOH (500 ml). The precipitate was purified by procedure B, followed by reprecipitation from DMF with cold MeOH. Yield 4.00 g (80%), mp >240 °C (dec.), $[\alpha]_D^{25}$ −14.0° (c=0.6, DMF), Rf_1 0.58. *Anal.* Calcd for $C_{50}H_{67}N_9O_{13}$: C, 59.92; H, 6.74; N, 12.58. Found: C, 59.61; H, 6.68; N, 12.21. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.98, Ser 0.97, Glu 1.00, Gly 0.93, Ala 1.00, Val 0.87, Phe 1.09 (recovery of Ala; 92%).

Boc–Phe–Val–Pro–Thr–OMe——Boc–Phe–Val–Pro–OH[18] (3.30 g, 7.1 mmol) was dissolved in AcOEt (50 ml) and, to this ice-chilled solution, DCC (1.70 g, 8.17 mmol), HOBt (0.92 g, 7.1 mmol) and H–Thr–OMe [prepared from 1.50 g (8.52 mmol) of the hydrochloride with Et_3N (1.20 ml, 8.52 mmol) in DMF (20 ml)] were added. The mixture was stirred at room temperature for 24 h, the precipitated urea derivative was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was purified by procedure A, followed by recrystallization from AcOEt with petroleum ether. Yield 3.50 g (87%), mp 96—101 °C, $[\alpha]_D^{25}$ −85.0° (c=1.0, MeOH), Rf_1 0.73. *Anal.* Calcd for $C_{29}H_{44}N_4O_8$: C, 60.40; H, 7.69; N, 9.72. Found: C, 60.27; H, 7.91; N, 9.64.

Boc–Asn–Phe–Val–Pro–Thr–OMe——A TFA-treated sample of Boc–Phe–Val–Pro–Thr–OMe (10.90 g, 17 mmol) was dissolved in DMF (100 ml), together with Et_3N (2.40 ml, 17 mmol), Boc–Asn–ONp (7.20 g, 20.4 mmol), HOBt (2.20 g, 17 mmol) and NMM (4.00 ml, 37.4 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The product was purified by procedure A, followed by recrystallization from AcOEt with ether twice. Yield 9.70 g (83%), mp 124—134 °C, $[\alpha]_D^{25}$ −75.0° (c=1.0, MeOH), Rf_1 0.73. *Anal.* Calcd for $C_{33}H_{50}N_6O_{10}\cdot 1/2H_2O$: C, 56.64; H, 7.35; N, 12.01. Found: C, 56.79; H, 7.30; N, 12.00.

Boc–Asn–Phe–Val–Pro–Thr–NHNH$_2$——Boc–Asn–Phe–Val–Pro–Thr–OMe (6.90 g, 10 mmol) dissolved in MeOH (100 ml) was treated with 80% hydrazine hydrate (6.20 ml, 100 ml) at room temperature for 24 h. The MeOH was evaporated off *in vacuo* and the residue was dissolved in *n*-BuOH and washed with H_2O–NaCl. The solution was dried over $MgSO_4$, and *n*-BuOH was removed by evaporation. The residue was triturated with ether and the resulting solid was recrystallized from MeOH with ether twice. Yield 4.00 g (59%), mp 198—201 °C, $[\alpha]_D^{25}$ −44.0° (c=1.0, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{32}H_{50}N_8O_9\cdot H_2O$: C, 54.22; H, 7.40; N, 15.81. Found: C, 54.10; H, 7.52; N, 15.89. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.95, Thr 0.92, Pro 0.90, Val 0.83, Phe 1.00 (recovery of Phe; 87%).

Boc–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$——A TFA-treated sample of Boc–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2 (4.00 g, 4 mmol) was dissolved in DMF–DMSO (2 : 1, 50 ml) containing Et_3N (0.56 ml, 4 mmol). To this ice-chilled solution, the azide [prepared from 5.60 g (8 mmol) of Boc–Asn–Phe–Val–Pro–Thr–$NHNH_2$ in DMF (50 ml)] and NMM (1.30 ml, 12 mmol) were added. The mixture was stirred at −4 °C for 48 h, then additional azide (2 mmol) and NMM (3.6 mmol) were added and the reaction mixture was further stirred at −4 °C for 48 h. DMF was removed by evaporation and the residue was poured into H_2O (500 ml). The precipitate was collected and washed with hot MeOH well, then reprecipitated from DMSO with MeOH. Yield 5.20 g (83%), mp >200 °C (dec.), $[\alpha]_D^{25}$ −13.5° (c=0.5, DMSO), Rf_1 0.44. *Anal.* Calcd for $C_{77}H_{105}N_{15}O_{20}\cdot 2H_2O$: C, 57.92; H, 6.88; N, 13.16. Found: C, 58.18; H, 6.99; N, 13.12. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.97, Thr 0.95, Ser 0.99, Glu 1.06, Pro 1.01, Gly 1.04, Ala 1.06, Val 1.84, Phe 2.00 (recovery of Phe, 89%).

Boc–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$——A TFA-treated sample of the above-prepared dodecapeptide amide (4.20 g, 2.7 mmol) was dissolved in DMF–DMSO (1 : 1, 100 ml), together with Et_3N (0.38 ml, 2.7 mmol), Boc–Asp(OChp)–OSu (1.60 g, 3.7 mmol), HOBt (0.35 g, 2.7 mmol) and NMM (0.75 ml, 6.8 mmol). The mixture was stirred for 48 h, and this solution was poured into H_2O (500 ml). The precipitate was collected and washed with hot MeOH, then reprecipitated from DMSO with MeOH. Yield 4.20 g (89%), mp >230 °C (dec.), $[\alpha]_D^{25}$ −7.8° (c=0.6, DMSO), Rf_1 0.53. *Anal.* Calcd for $C_{88}H_{122}N_{16}O_{23}\cdot 2H_2O$: C, 58.46; H, 7.02; N, 12.40. Found: C, 58.15; H, 7.02; N, 12.63. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.75, Thr 0.92, Ser 0.98, Glu 1.07, Pro 1.03, Gly 0.98, Ala 1.04, Val 2.14, Phe 2.00 (recovery of Phe, 86%).

Z(OMe)–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$——A TFA-treated sample of the above-prepared tridecapeptide amide (4.20 g, 2.4 mmol) was dissolved in DMF–DMSO (1 : 4, 100 ml), together with Et_3N (0.34 ml, 2.4 mmol), Z(OMe)–Lys(Z)–OSu (2.00 g, 3.6 mmol) and NMM (0.60 ml, 3.6 mmol). The mixture was stirred for 48 h, and the solution was poured into H_2O (200 ml). The precipitate was collected and washed with hot MeOH, then reprecipitated from DMSO with MeOH. Yield 4.00 g (80%), mp >250 °C (dec.), $[\alpha]_D^{25}$ +90.8° (c=0.8, DMSO), Rf_1 0.64. *Anal.* Calcd for $C_{106}H_{140}N_{18}O_{27}\cdot 5H_2O$: C, 58.17; H, 6.91; N, 11.53. Found: C, 58.09; H, 6.66; N, 11.64. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.71, Thr 0.86, Ser 0.89, Glu 1.08, Pro 1.07, Gly 1.05, Ala 1.05, Val 2.00, Phe 2.00, Lys 0.89 (recovery of Phe, 90%).

Z(OMe)–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$——A TFA-treated sample of the above-prepared tetradecapeptide amide (4.00 g, 1.9 mmol) was dissolved in DMF–DMSO (1 : 4, 100 ml), together with Et_3N (0.80 ml, 5.7 mmol) and Z(OMe)–Val–ONp (1.60 g, 3.8 mmol). The mixture was stirred for 48 h, and the solution was poured into H_2O (500 ml). The precipitate was collected and washed well with hot MeOH, then reprecipitated from DMSO with MeOH. Yield 3.20 g (79%), mp >250 °C (dec.), $[\alpha]_D^{25}$ +112.1° (c=0.6, DMSO), Rf_1 0.59. *Anal.* Calcd for $C_{111}H_{149}N_{19}O_{28}\cdot 3H_2O$: C, 59.21; H, 6.94; N, 11.82. Found: C, 58.80; H, 6.63; N, 11.95. Amino acid ratios in 6 N HCl hydrolysate: Asp 2.92, Thr 1.01, Ser 1.01, Glu 1.07, Pro 1.09, Gly 0.98, Ala 1.09, Val 2.85, Phe 2.00, Lys 0.91 (recovery of Phe, 87%).

Z(OMe)–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–

NH_2——A TFA-treated sample of the above-prepared pentadecapeptide amide (3.20 g, 1.5 mmol) was dissolved in DMF–DMSO (1 : 4, 100 ml), together with Et_3N (0.63 ml, 4.5 mmol) and Z(OMe)–Val–ONp (1.20 g, 3.0 mmol). The mixture was stirred for 48 h, and the solution was poured into H_2O (500 ml). The precipitate was collected and washed well with hot MeOH, then reprecipitated from DMSO with MeOH. Yield 2.70 g (80%), mp >250 °C (dec.), $[\alpha]_D^{25}$ +71.4° (c=0.2, DMSO), Rf_1 0.80. *Anal.* Calcd for $C_{116}H_{158}N_{20}O_{29}\cdot 3\,1/2H_2O$: C, 59.04; H, 7.05; N, 11.87. Found: C, 59.09; H, 6.93; N, 12.05. Amino acid ratios in 6 N HCl hydrolysate; Asp 2.80, Thr 0.92, Ser 0.83, Glu 1.00, Pro 1.17, Gly 1.00, Ala 0.94, Val 3.40, Phe 2.00, Lys 0.76 (recovery of Phe, 86%).

Z(OMe)–Gly–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH_2; [1]——A TFA-treated sample of the above-prepared hexadecapeptide amide (2.70 g, 1.2 mmol) was dissolved in DMF–DMSO (1 : 4, 100 ml), together with Et_3N (0.67 ml, 4.8 mmol) and Z(OMe)–Gly–ONp (1.30 g, 3.6 mmol). The mixture was stirred for 48 h, and the solution was poured into MeOH (500 ml). The precipitate was collected and washed well with hot MeOH, then reprecipitated from DMSO with MeOH. Yield 2.20 g (78%), mp >250 °C (dec.), $[\alpha]_D^{25}$ +60.1° (c=0.3, DMSO), Rf_1 0.60. *Anal.* Calcd for $C_{118}H_{161}N_{21}O_{30}\cdot 3H_2O$: C, 58.86; H, 6.94; N, 12.22. Found: C, 58.73; H, 6.96; N, 12.26.

Boc–Cys(MBzl)–Val–Thr–OMe——A TFA-treated sample of Z(OMe)–Val–Thr–OMe[6] (4.80 g, 12 mmol) was dissolved in DMF (30 ml), together with Et_3N (3.36 ml, 24 mmol) and Boc–Cys(MBzl)–ONp (5.36 g, 12 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The product was purified by procedure A, followed by recrystallization from AcOEt with petroleum ether. Yield 5.75 g (86%), mp 130—132 °C, $[\alpha]_D^{25}$ −8.2° (c=0.9, DMF), Rf_1 0.69. *Anal.* Calcd for $C_{26}H_{41}N_3O_8S$: C, 56.19; H, 7.43; N, 7.56. Found: C, 56.24; H, 7.54; N, 7.55.

Boc–Cys(MBzl)–Val–Thr–$NHNH_2$——Boc–Cys(MBzl)–Val–Thr–OMe (9.50 g, 17 mmol) dissolved in MeOH (150 ml) was treated with 80% hydrazine hydrate (10.0 ml, 170 mmol) at room temperature for 72 h. The precipitate was collected and washed well with cold MeOH. Yield 8.50 g (89%), mp 205—207 °C, $[\alpha]_D^{25}$ −2.0° (c=1.0, DMSO), Rf_1 0.68. *Anal.* Calcd for $C_{25}H_{41}N_5O_7S\cdot 1/2H_2O$: C, 53.17; H, 7.50; N, 12.40. Found: C, 53.55; H, 7.46; N, 12.35.

Boc–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–OMe——A TFA-treated sample of Z(OMe)–His–Arg(Mts)–Leu–Ala–OMe[6] (4.30 g, 5 mmol) was dissolved in DMF (50 ml) containing Et_3N (0.70 ml, 5 mmol). To this ice-chilled solution, the azide [prepared from 2.50 g (5 mmol) of Boc–Cys(MBzl)–Val–Thr–$NHNH_2$ in DMF (30 ml)] and Et_3N (0.70 ml, 5.0 mmol) were added. The mixture was stirred at −4 °C for 48 h, and DMF was removed by evaporation. The residue was dissolved in *n*-BuOH and washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl. The solution was dried over $MgSO_4$, and *n*-BuOH was removed by evaporation. The residue was triturated with ether and the resulting solid was recrystallized from MeOH with ether twice. Yield 3.80 g (49%), mp 195—199 °C, $[\alpha]_D^{25}$ −6.9° (c=1.0, DMSO), Rf_1 0.68. *Anal.* Calcd for $C_{56}H_{86}N_{12}O_{16}S_2\cdot H_2O$: C, 54.53; H, 7.13; N, 13.63. Found: C, 54.44; H, 7.30; N, 12.98.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–OMe——A TFA-treated sample of Boc–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–OMe (7.00 g, 5.8 mmol) was dissolved in DMF (50 ml) containing Et_3N (0.81 ml, 5.8 mmol). To this ice-chilled solution, the azide [prepared from 2.10 g (14 mmol) of Z(OMe)–Thr–$NHNH_2$ in DMF (30 ml)] and Et_3N (2.00 ml, 14 mmol) were added. The mixture was stirred at −4 °C for 48 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by reprecipitation from DMF with ether. Yield 5.00 g (71%), mp 178—180 °C, $[\alpha]_D^{25}$ −1.4° (c=0.7, DMSO), Rf_1 0.69. *Anal.* Calcd for $C_{64}H_{93}N_{13}O_{17}S_2\cdot 4H_2O$: C, 52.91; H, 7.00; N, 12.54. Found: C, 53.05; H, 6.79; N, 12.35.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–$NHNH_2$; [4]——The above-prepared methyl ester (5.00 g, 3.6 mmol) dissolved in DMF (50 ml) was treated with 80% hydrazine hydrate (2.20 ml, 35 mmol) at room temperature for 24 h. DMF was removed by evaporation and the residue was triturated with H_2O. The resulting solid was collected and washed well with H_2O and cold MeOH. Yield 4.50 g (90%), mp 216—218 °C, $[\alpha]_D^{25}$ +3.9° (c=0.8, DMSO), Rf_1 0.49. *Anal.* Calcd for $C_{63}H_{93}N_{15}O_{16}S_2\cdot 2\,1/2H_2O$: C, 53.07; H, 6.92; N, 14.74. Found: C, 53.34; H, 6.83; N, 14.46. Amino acid ratios in 6 N HCl hydrolysate: Thr 1.55, Ala 1.00, Val 1.08, Leu 1.00, His 1.00, Arg 1.03 (recovery of Leu, 87%).

Z(OMe)–Asn–Thr–OMe——Z(OMe)–Asn–ONp (8.40 g, 20 mmol) and NMM (2.50 ml, 23.0 mmol) were added to a solution of H–Thr–OMe [prepared from 4.30 g (25 mmol) of the hydrochloride with 3.50 ml (25 mmol) of Et_3N in DMF (75 ml)], and the reaction mixture was stirred for 24 h. DMF was removed by evaporation and the residue was dissolved in *n*-BuOH. The organic layer was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl. The solution was dried over $MgSO_4$, and *n*-BuOH was removed by evaporation. The residue was triturated with ether and the resulting solid was recrystallized from MeOH with ether twice. Yield 4.60 g (55%), mp 136—137 °C, $[\alpha]_D^{25}$ −3.0° (c=1.0, MeOH), Rf_1 0.58. *Anal.* Calcd for $C_{18}H_{25}N_3O_8$: C, 52.55; H, 6.13; N, 10.21. Found: C, 52.26; H, 5.85; N, 10.16.

Boc–Cys(MBzl)–Asn–Thr–OMe——A TFA-treated sample of Z(OMe)–Asn–Thr–OMe (4.10 g, 10 mmol) was dissolved in DMF (50 ml), together with Et_3N (1.40 ml, 10 mmol), Boc–Cys(MBzl)–ONp (5.40 g, 10 mmol) and NMM (1.30 ml, 12 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by recrystallization from MeOH with ether. Yield 5.80 g (92%), mp 202—203 °C, $[\alpha]_D^{25}$ −19.0° (c=1.0, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{29}H_{38}N_4O_{10}S$: C, 54.88; H, 6.04; N, 8.83. Found: C, 54.99; H, 6.15; N, 8.83.

Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–OMe——A TFA-treated sample of Boc–Cys(MBzl)–Asn–Thr–OMe

(5.00 g, 8 mmol) was dissolved in DMF (50 ml), together with Et_3N (1.20 ml, 8 mmol), Z(OMe)–Ser(Bzl)–OSu (3.65 g, 8 mmol) and NMM (1.00 ml, 9.0 mmol). The mixture was stirred for 24 h, and DMF was removed by evaporation. The residue was triturated with ether and the resulting solid was purified by procedure B, followed by recrystallization from DMF with ether. Yield 5.20 g (94%), mp 196—199 °C, $[\alpha]_D^{25}$ −8.0° (c = 1.0, DMF), Rf_1 0.72. *Anal.* Calcd for $C_{39}H_{49}N_5O_{12}S \cdot 1/2H_2O$: C, 57.06; H, 6.14; N, 8.53. Found: C, 57.06; H, 6.13; N, 8.24.

Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–NHNH$_2$——The above-prepared methyl ester (4.50 g, 3.7 mmol) dissolved in DMF–DMSO (1 : 1, 70 ml) was treated with 80% hydrazine hydrate (3.50 ml, 37 mmol) at room temperature for 24 h. The solvent was concentrated *in vacuo* and poured into H_2O (200 ml). The precipitate was collected and washed well with H_2O and MeOH. Yield 4.00 g (89%), mp 219—221 °C, $[\alpha]_D^{25}$ +6.0° (c = 0.5, DMF), Rf_1 0.48. *Anal.* Calcd for $C_{38}H_{49}N_7O_{11}S \cdot 1/2H_2O$: C, 55.60; H, 6.14; N, 11.94. Found: C, 55.36; H, 6.19; N, 11.83.

Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–OMe——The azide [prepared from 2.00 g (2.5 mmol) of Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–NHNH$_2$ in DMF (30 ml)] and Et_3N (0.35 ml, 2.5 mmol) were added to an ice-chilled solution of H–Ala–OMe [prepared from 0.52 g (3.7 mmol) of the hydrochloride with Et_3N (0.52 ml, 3.7 mmol) in DMF (50 ml)]. The mixture was stirred at 4 °C for 24 h, and DMF was removed by evaporation. The residue was triturated with H_2O and the resulting solid was purified by procedure B, followed by recrystallization from DMF with ether. Yield 1.80 g (81%), mp 203—205 °C, $[\alpha]_D^{25}$ −13.9° (c = 1.0, DMSO), Rf_1 0.61. *Anal.* Calcd for $C_{42}H_{54}N_6O_{13}S$: C, 57.13; H, 6.16; N, 9.52. Found: C, 57.05; H, 6.40; N, 9.28.

Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–NHNH$_2$; [5]——Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–OMe (1.70 g, 2.1 mmol) dissolved in DMF–DMSO (1 : 1, 50 ml) was treated with 80% hydrazine hydrate (1.2 ml, 20 mmol) and the mixture was stirred at room temperature for 24 h, then concentrated *in vacuo*. The residue was poured into H_2O (200 ml). The precipitate was collected and washed well with H_2O and MeOH. Yield 1.50 g (88%), mp >225 °C (dec.), $[\alpha]_D^{25}$ −7.9° (c = 0.9, DMSO), Rf_1 0.49. *Anal.* Calcd for $C_{41}H_{54}N_8O_{12}S \cdot 3H_2O$: C, 52.55; H, 6.45; N, 11.96. Found: C, 52.38; H, 6.10; N, 11.77. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.97, Thr 0.90, Ser 0.75, Ala 1.00 (recovery of Ala, 85%).

Z(OMe)–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$; Z(OMe)–rCGRP(17—37)–NH$_2$——A TFA-treated sample of the protected heptadecapeptide amide [1] (1.00 g, 0.42 mmol) was dissolved in HMPA–DMF (5 : 1, 50 ml) containing Et_3N (0.06 ml, 0.42 mmol). To this ice-chilled solution, the azide [prepared from 1.10 g (1.3 mmol) of Z(OMe)–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–NHNH$_2$[6] in DMF (30 ml)] and Et_3N (0.18 ml, 1.4 mmol) were added. The mixture was stirred at −4 °C for 48 h, then additional azide (1.30 mmol) and Et_3N (1.4 mmol) were added, and stirring was continued for 48 h. The reaction mixture was poured into H_2O (300 ml) and the precipitate was collected and washed with hot MeOH, then reprecipitated from HMPA–DMF (5 : 1) with MeOH. Yield 1.10 g (83%), mp >230 °C (dec.), $[\alpha]_D^{25}$ −2.1° (c = 0.5, DMSO), Rf_1 0.60. *Anal.* Calcd for $C_{155}H_{208}N_{28}O_{38}S \cdot 3H_2O$: C, 58.96; H, 6.83; N, 12.46. Found: C, 59.03; H, 6.84; N, 12.29.

Z(OMe)–Gly–Leu–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$; Z(OMe)–rCGRP(14—37)–NH$_2$——A TFA-treated sample of Z(OMe)–rCGRP(17—37)–NH$_2$ (1.10 g, 0.35 mmol) was dissolved in HMPA–DMF (5 : 1, 50 ml) containing Et_3N (0.05 ml, 0.35 mmol). To this ice-chilled solution, the azide [prepared from 0.84 g (1.75 mmol) of Z(OMe)–Gly–Leu–Leu–NHNH$_2$[6] in DMF (20 ml)] and Et_3N (0.24 ml, 1.75 mmol) were added. The mixture was stirred at −4 °C for 48 h, additional azide (1.75 mmol) and Et_3N (1.75 mmol) were added, and stirring was continued for 48 h. The reaction mixture was poured into cold H_2O (300 ml) and the precipitate was collected and washed with hot MeOH, then reprecipitated from HMPA–DMF (5 : 1) with MeOH. Yield 1.00 g (84%), mp >235 °C (dec.), $[\alpha]_D^{25}$ +8.0° (c = 0.3, DMSO), Rf_1 0.65. *Anal.* Calcd for $C_{169}H_{233}N_{31}O_{41}S \cdot 3H_2O$: C, 58.99; H, 7.00; N, 12.62. Found: C, 58.79; H, 7.02; N, 12.84.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–Gly–Leu–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$; Z(OMe)–rCGRP(6—37)–NH$_2$——The above-prepared Z(OMe)–rCGRP(14—37)–NH$_2$ (0.35 g, 0.11 mmol) was treated with TFA–anisole (1.0 ml–0.2 ml) for 60 min with ice-cooling twice and dried over KOH pellets *in vacuo*. The dried TFA salt was dissolved in HMPA–DMF (5 : 1, 30 ml) containing Et_3N (15 μl, 0.11 mmol) and, to this ice-chilled solution, the azide [prepared from 0.69 g (0.5 mmol) of Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–NHNH$_2$ in DMF (10 ml)] and Et_3N (70 μl, 0.5 mmol) were added. The mixture was stirred at −4 C for 48 h, additional azide (0.5 mmol) and Et_3N (0.5 mmol) were added, and stirring was continued for 48 h. The reaction mixture was poured into cold H_2O (200 ml) and the precipitate was collected and washed with hot MeOH, then reprecipitated from HMPA–DMF (5 : 1) with MeOH. Yield 0.32 g (68%), mp >235 °C (dec.), $[\alpha]_D^{25}$ +25.0° (c = 0.4, DMSO), Rf_1 0.54, *Anal.* Calcd for $C_{223}H_{314}N_{44}O_{54}S_3 \cdot 10H_2O$: C, 56.37; H, 7.09; N, 12.97. Found: C, 56.72; H, 7.03; N, 12.89.

Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–Gly–Leu–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Val–Lys(Z)–Asp(OChp)–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Glu(OBzl)–Ala–Phe–NH$_2$; Z(OMe)–rCGRP(1—37)–NH$_2$——The above-prepared Z(OMe)–rCGRP(6—37)–NH$_2$ (0.30 g, 70 μmol) was treated with TFA–anisole (1.0 ml–0.1 ml) for 60 min with ice-cooling twice and

then dried over KOH pellets *in vacuo*. The dried TFA salt was dissolved in HMPA–DMF (5 : 1, 30 ml) containing Et_3N (9.8 μl, 70 μmol) and, to this ice-chilled solution, the azide [prepared from 0.29 g (350 μmol) of Z(OMe)–Ser(Bzl)–Cys(MBzl)–Asn–Thr–Ala–$NHNH_2$ in DMF (10 ml)] and Et_3N (50 μl, 350 μmol) were added. The mixture was stirred at −4 °C for 48 h, additional azide (350 μmol) and Et_3N (350 μmol) were added, and stirring was continued for 48 h. The reaction mixture was poured into H_2O (200 ml) and the precipitate was collected and washed well with hot MeOH. Yield 0.19 g (52%), mp >250 °C (dec.), $[\alpha]_D^{25}$ −12.1° (c=0.3, DMSO), Rf_1 0.59. *Anal.* Calcd for $C_{255}H_{356}N_{50}O_{63}S_4 \cdot 7\,1/2H_2O$: C, 56.79; H, 6.93; N, 12.99. Found: C, 56.77; H, 6.58; N, 12.81.

H–Ser–Cys–Asn–Thr–Ala–Thr–Cys–Val–Thr–His–Arg–Leu–Ala–Gly–Leu–Leu–Ser–Arg–Ser–Gly–Gly–Val–Val–Lys–Asp–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser–Glu–Ala–Phe–NH_2; α-rCGRP——The above-prepared Z(OMe)-rCGRP(1—37)–NH_2 (95 mg, 18 μmol) was treated with 1 M TFMSA–thioanisole in TFA (7.30 ml) in the presence of *m*-cresol (0.38 ml, 200 eq) for 4 h with ice-cooling, then dry ether was added. The precipitate was collected by centrifugation, washed with ether three times and dissolved in a solution of 0.1 M Tris–HCl buffer containing 6 M guanidine–HCl (pH 8.0, 4 ml). After the pH was adjusted to 8.0 with 5% methylamine, the solution was incubated with 2-mercaptoethanol (0.20 ml) at 30 °C for 24 h under an N_2 gas atmosphere. The insoluble material formed during incubation was removed by centrifugation and the supernatant solution was applied to a column of Sephadex G-25 (2.2 × 110 cm), which was eluted with 1 N AcOH. The ultraviolet (UV) absorption at 275 nm was determined in each fraction (7.0 ml) and the fractions corresponding to the main peak (tube Nos. 32—43) were combined. The combined solution was diluted with ice-chilled H_2O (1000 ml, peptide concentration 0.07 mg/ml). The pH of this solution was adjusted to 7.5 with 5% NH_4OH and the solution was kept standing at room temperature for 4 d; during this time, the Ellman test value (UV absorption at 412 nm) dropped from 0.153 to a constant value of 0.013. The solution was then stirred gently for an additional 24 h. The pH was adjusted to 6.6 with 1 N AcOH, and the solution was lyophilized. The residue was dissolved in 1 N AcOH (4.0 ml) and applied to a column of Sephadex G-50 (2.7 × 100 cm), which was eluted with 1 N AcOH. The UV absorption at 275 nm was determined in each fraction (6.0 ml), then the fractions corresponding to the main peak (tube Nos. 66—97, Fig. 6) were combined and the solvent was removed by lyophilization to afford a white fluffy powder, 33 mg (48% from deprotection).

A part of this sample (25 mg, *ca.* 0.7 mg each) was purified by HPLC on a Nucleosil $7C_{18}$ (4.8 × 250 mm) column using a gradient of CH_3CN (from 30% to 40% in 50 min) in 0.2% TFA. The eluate corresponding to the main peak (t_R: 39.80 min, Fig. 7-a) was pooled. The rest of the sample was similarly purified and the combined eluates were repeatedly lyophilized to afford a white fluffy powder, 7.5 mg (30% recovery on HPLC). $[\alpha]_D^{25}$ −44.0° (c=0.4, 5% AcOH), Rf_2 0.21, Rf_3 0.41, Rf_4 0.84. Amino acid ratios in the 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in papain plus LAP digest (numbers in parentheses are theoretical values): Asp 1.02 (1), Thr 3.73 (4), Ser 3.54 (4), Asn 3.03 (3), Glu 1.00 (1), Gly 4.38 (4), Ala 3.15 (3), Val 4.41 (5), Leu 3.38 (3), Phe 2.00 (2), Lys 1.03 (1), His 0.94 (1), Arg 2.18 (2), Pro 0.76 (1), Cys was not determined (recovery of Phe, 75%). HPLC t_R: 39.80 min (Fig. 7-b). Molecular weight estimation was conducted by HPLC on a TSKgel G 2000SW column (7.5 × 600 mm), upon which

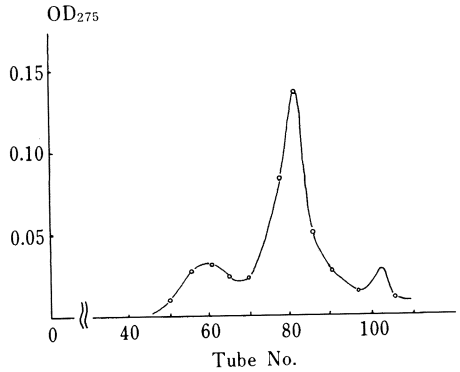

Fig. 6. Purification of the Air-Oxidized Crude Peptide on Sephadex G-50

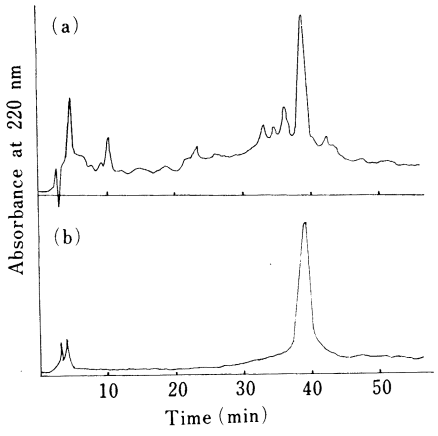

Fig. 7. HPLC of Sephadex G-50 Purified Product (a) and Finally Purified α-rCGRP (b)

HPLC was performed on a reversed-phase column (Chemopak, Nucleosil $7C_{18}$, 4.8 × 250 mm). Isocratic elution with (A) (10 min) was followed by linear gradient elution from (A) to (B) (50 min) at a flow rate 1 ml/min. (A): 30% CH_3CN (0.2% TFA). (B): 40% CH_3CN (0.2% TFA).

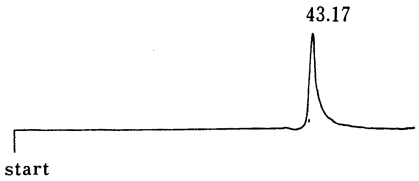

Fig. 8. Elution of Synthetic α-rCGRP from a TSKgel G 2000SW Column (7.5 × 600 mm)

Sample, synthetic α-rCGRP (M_r = *ca.* 3800, 50 μg); eluent, 0.1 M $AcONH_4$ (pH 4.2); flow rate, 0.5 ml/min; absorbance, 230 nm.

synthetic α-rCGRP (M_r = *ca.* 3800, t_R 43.17 min) was eluted between human growth hormone releasing factor (hGRF) derivative (M_r = *ca.* 4500, t_R 42.18 min) and human atrial natriuretic peptide (hANP) derivative (M_r = *ca.* 2000, t_R 49.72 min) (Fig. 8).

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of L-configuration. Abbreviations used are those recommended by the I.U.P.A.C.-I.U.B. Commission on Biochemical Nomenclature: *J. Biol. Chem.*, **247**, 977 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, Chp = cycloheptyl, MBzl = *p*-methoxybenzyl, Ad = adamantyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, DCC = *N*,*N'*-dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, Et_3N = triethylamine, NMM = *N*-methylmorpholine, DMF = dimethylformamide, DMSO = dimethylsulfoxide, HMPA = hexamethylphosphoramide, MeOH = methanol, AcOEt = ethyl acetate, AcOH = acetic acid, LAP = leucine aminopeptidase.
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