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Studies on Peptides. CLXII.[1,2] Synthesis of Chicken Calcitonin-Gene-Related Peptide (cCGRP) by Application of Sulfoxide-Directed Disulfide-Bond-Forming Reaction

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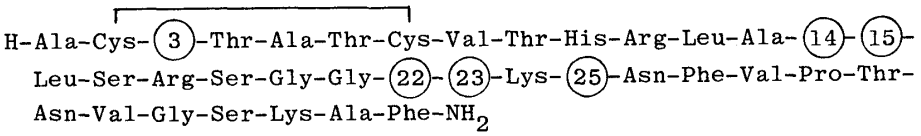
A 37-residue peptide corresponding to the entire amino acid sequence of chicken calcitonin-gene-related peptide (cCGRP) was synthesized by successive assembling of seven peptide fragments *via* the azide, followed by acid treatments of protected cCGRP in two steps, *i.e.*, first with trifluoroacetic acid (TFA) in the presence of diphenylsulfide to establish the disulfide bond between the Cys(MBzl) sulfoxide and the Cys(MBzl) residues (positions 2 and 7, respectively) and then with 1 M trimethylsilyl trifluoromethanesulfonate in TFA to remove the rest of the protecting groups employed. The synthetic peptide suppressed the ^{45}Ca release from mouse calvaria.

Keywords——chicken calcitonin-gene-related peptide (cCGRP) synthesis; sulfoxide-directed disulfide-bond-forming reaction; *p*-methoxybenzylcysteine sulfoxide; hard acid deprotection; trimethylsilyl trifluoromethanesulfonate; soft base; diphenylsulfide; calcium release

Following the syntheses[3,4] of α- and β-human calcitonin-gene-related peptides (α- and β-hCGRPs),[5,6] we wish to report the synthesis of a 37-residue peptide corresponding to the entire amino acid sequence of chicken CGRP (cCGRP), the sequence of which was elucidated by Minvielle *et al.*[7] (Fig. 1). In the former syntheses, the disulfide bonds were established between the two Cys(Ad) residues[8] by oxidation with thallium trifluoroacetate [$Tl(CF_3COO)_3$].[9] In the present synthesis, we applied a newly found reaction, named the sulfoxide-directed disulfide-bond-forming reaction,[10] to establish the disulfide bond between the Cys(MBzl)(O)[11] and the Cys(MBzl) residues (positions 2 and 7, respectively).

First, protected cCGRP was prepared by assembling seven peptide fragments of established purity (Fig. 2). Of these fragments, three fragments, [1], [3], and [5], are those used for our previous syntheses of α- and β-hCGRPs. Thus, two fragments, [2] and [4] which cover the area of species variation, were newly synthesized. In addition, the previously employed Cys(Ad) in fragments [6] and [7] was replaced by Cys(MBzl) and Cys(MBzl)(O), respectively.



	3	14	15	22	23	25
chicken	Asn	Asp	Phe	Val	Gly	Asn
α-human	Asp	Gly	Leu	Val	Val	Asn
β-human	Asn	Gly	Leu	Met	Val	Ser

Fig. 1. Amino Acid Sequences of Chicken and α- and β-Human CGRP

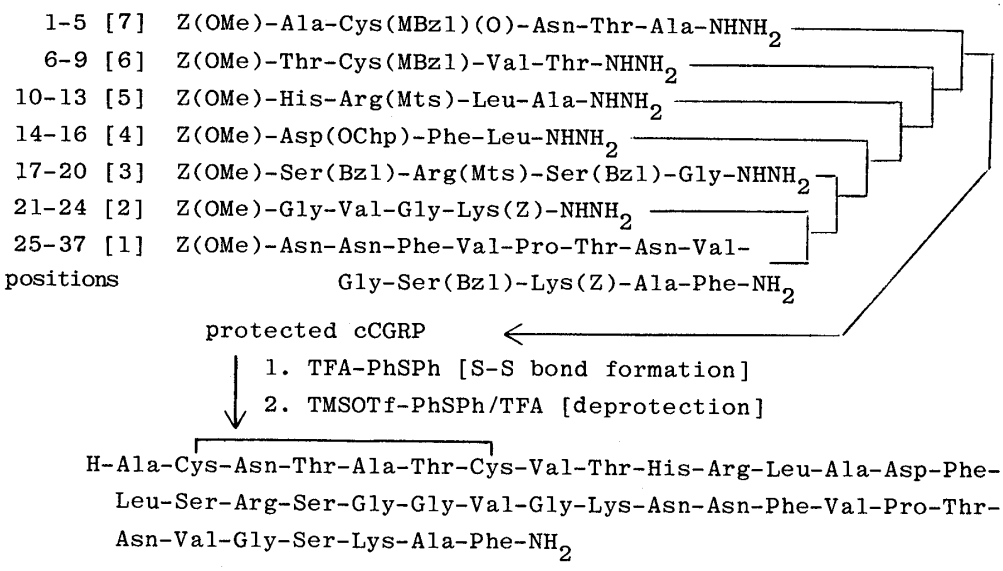


Fig. 2. Synthetic Route to Chicken CGRP

Fragment [2], Z(OMe)–Gly–Val–Gly–Lys(Z)–$NHNH_2$, was prepared in a stepwise manner starting with a known dipeptide, Z(OMe)–Gly–Lys(Z)–OMe.[3] Prior to each condensation reaction, the N^{α}-protecting Z(OMe) group was cleaved by TFA in the presence of anisole. The Su active ester procedure[12] was employed to introduce the respective amino acid residues, Val and Gly, to give a protected tetrapeptide ester, which was converted to [2] by the usual hydrazine treatment.

Fragment [4], Z(OMe)–Asp(OChp)–Phe–Leu–$NHNH_2$, was prepared by Su condensation of Z(OMe)–Asp(OChp)–OH with a TFA-treated sample of Z(OMe)–Phe–Leu–NHNH–Troc.[3] As expected, the Chp ester[13] suppressed well the base-catalyzed succinimide formation of the Asp residue[14] in this coupling step. From the resulting tripeptide derivative, the Troc group was removed by treatment with Zn in AcOH[15] to give [4].

Fragment [6], Z(OMe)–Thr–Cys(MBzl)–Val–Thr–$NHNH_2$, was prepared in a stepwise manner starting with Z(OMe)–Val–Thr–OMe.[3] Z(OMe)–Cys(MBzl)–OH was condensed by the Su procedure, then Z(OMe)–Thr–$NHNH_2$ by the azide procedure.[16] The resulting tetrapeptide ester was converted to [6] by hydrazine treatment as usual.

Fragment [7], Z(OMe)–Ala–Cys(MBzl)(O)–Asn–Thr–Ala–$NHNH_2$, was prepared starting with a known tripeptide, Z(OMe)–Asn–Thr–Ala–OMe.[3] For condensation of Z(OMe)–Cys(MBzl)(O)–OH, DPPA[17] gave the best result. Next, Z(OMe)–Ala–OH was introduced by the Su procedure to give a protected pentapeptide ester, which was converted to [7] by the usual hydrazine treatment.

According to the route illustrated in Fig. 2, the seven fragments thus obtained were successively assembled *via* the azide to give protected cCGRP. Every reaction proceeded smoothly, without any solubility problem, when DMF or a mixture of DMF and DMSO was employed as a solvent and the amount of the acyl component was increased from 1.8 to 5.0 eq as the chain elongation progressed. Throughout this synthesis, every protected peptide, including protected cCGRP, could be purified by precipitation from DMSO with MeOH, and Phe was used as a diagnostic amino acid in amino acid analysis. The recovery of Phe after acid hydrolysis was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each fragment condensation (Table I). The homogeneity of every protected peptide was further ascertained by elemental analysis and thin layer chromatography (TLC).

Next, protected cCGRP was converted to a free form of cCGRP by two steps of acid

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

	Protected peptides						cCGRP	No.
	21—37	17—37	14—37	10—37	6—37	1—37		
Asp	3.15	3.27	4.42	4.30	4.34	5.21	5.34	(5)
Thr	0.81	0.91	1.03	1.02	2.66	3.23	3.72	(4)
Ser	0.83	2.78	3.11	3.07	2.95	3.07	3.05	(3)
Pro	0.95	1.13	1.06	1.05	0.98	0.95	1.08	(1)
Gly	2.94	4.00	3.89	3.92	4.26	4.33	4.17	(4)
Ala	1.21	1.30	1.27	2.24	2.07	3.72	4.16	(4)
Cys							0.87	(1)
Val	2.84	2.86	2.66	2.71	3.93	3.91	4.04	(4)
Leu			0.86	1.96	2.03	2.09	2.07	(2)
Phe	2.00	2.00	3.00	3.00	3.00	3.00	3.00	(3)
Lys	1.87	1.97	2.17	2.04	2.01	2.23	2.22	(2)
His				0.89	0.96	1.09	1.00	(1)
Arg		0.87	0.99	1.85	2.07	2.25	2.18	(2)
Recov. (%)	77	82	75	77	85	84	93	

treatment. Our model experiments[10] showed that the disulfide bond is formed intramolecularly at the sulfur atom of Cys(MBzl)(O), following liberation of the SH group from the coexisting Cys(MBzl) by TFA with the aid of a suitable soft base, such as diphenyl sulfide (PhSPh). Thus, to establish the disulfide bond of cCGRP, the above protected 37-residue peptide was treated with TFA in the presence of PhSPh (at the concentration of 0.2 M) in an ice-bath for 30 min, then at room temperature for 120 min. During this treatment, the relatively acid-labile Bzl-type protecting groups were judged to be partially cleaved. To achieve complete deprotection, TMSOTf[18] was added to a concentration of 1 M in TFA and the concentration of PhSPh was increased to a total of 0.5 M in TFA. Thus, the above TFA-treated peptide was further exposed to 1 M TMSOTf/TFA in the presence of PhSPh in an ice-bath for 120 min. By this treatment, the Mts[19] and the Chp groups were judged to be completely cleaved from the Arg and Asp residues, respectively, together with the Bzl-type protecting groups. Since this deprotection was conducted in the presence of the disulfide bond, we selected PhSPh as a soft base, rather than thioanisole. As noted previously,[7] the recovery of cystine decreased when 1 M TMSOTf–thioanisole/TFA was used.

The deprotected peptide was briefly treated with diluted ammonia. This treatment seems to be effective to reverse the possible N→O shift at the Thr and Ser residues[20] and ensure complete hydrolysis of the resulting trimethylsilyl moieties. The treated product was subjected to gel-filtration on Sephadex G-15 and purified first by ion-exchange chromatography on CM-Trisacryl M using gradient elution with 0.2 M NaCl in 0.01 M $AcONH_4$ buffer (pH 6.8). After desalting by gel-filtration on Sephadex G-15, the product was finally purified by reversed-phase high-performance liquid chromatography (HPLC) on a Cosmosil 5C18 column using gradient elution with MeCN in 0.1% TFA. The desired product emerged from the HPLC column as a main peak, well separated from other impurities which are often observed in the synthesis of Cys-containing peptides.

The main product thus obtained exhibited a sharp single spot on TLC and gave a single band on disc-isoelectrofocusing (Pharmalyte pH 3—10). It gave the expected mass ion peak as a monomer in fast atom bombardment mass spectrometry (FAB-MS). Its acid hydrolysate gave the amino acid ratios predicted by theory and its purity was further ascertained by enzymatic digestion.

As described above, we were able to establish the disulfide bond of cCGRP by the new sulfoxide-directed disulfide-bond-forming reaction. Besides the $Tl(CF_3COO)_3$ oxidation procedure, another new route has thus been opened to synthesize peptides containing one disulfide bond without laborious and time-consuming air-oxidation. However, in the present synthesis, the total yield from protected cCGRP was less than 10%, *i.e.*, 8.8%. The purification yields of α-hCGRP and β-hCGRP prepared by the $Tl(CF_3COO)_3$ oxidation procedure were 11% and 12%, respectively. We intend to continue seeking further chemical improvements for the synthesis of cystine-containing peptides.

When the effect on ^{45}Ca release from mouse calvaria were examined,[3] the synthetic peptide (2×10^{-6} M) suppressed the ^{45}Ca release induced by human parathyroid hormone (hPTH 1—34) and its potency was judged to be slightly higher than that of β-hCGRP.

Experimental

General experimental procedures employed in this investigation were essentially the same as described in connection with our previous syntheses of α- and β-hCGRP.[3,4] TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following (v/v) solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH–AcOH (9:1:0.5), Rf_3 $CHCl_3$–MeOH (10:0.5), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30:20:6:24). HPLC was conducted with a Waters 204 compact model. FAB-MS spectra were obtained on a ZAB SE instrument (VG Analytical Co., England). Leucine aminopeptidase (LAP, lot No. L-6007) was purchased from Sigma.

Z(OMe)–Val–Gly–Lys(Z)–OMe (Positions 22—24)——A mixture of a TFA-treated sample of Z(OMe)–Gly–Lys(Z)–OMe (6.60 g, 12.8 mmol), TEA (3.9 ml, 2.2 eq) and Z(OMe)–Val–OSu (5.80 g, 1.2 eq) in DMF (50 ml) was stirred at room temperature overnight and the solvent was removed by evaporation. For purification, the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4, and concentrated (named the extraction procedure hereafter). The residue was recrystallized from MeOH

TABLE II. Physical Constants and Analytical Data of Fragments and Intermediates

Protected peptides	mp (°C)	$[\alpha]_D^{19}$ (°)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
Z(OMe)–(22—24)–OMe	137—140	−96.2 (MeOH)	$C_{31}H_{42}N_4O_9$	60.57 (60.65	6.89 7.00	9.11 9.23)
Z(OMe)–(21—24)–OMe	148—150	−29.9 (MeOH)	$C_{33}H_{45}N_5O_{10}$	59.00 (58.83	6.75 6.79	10.43 10.55)
Z(OMe)–(21—24)–$NHNH_2$ [2]	212—214	−20.9 (DMF)	$C_{32}H_{45}N_7O_9$	57.22 (57.47	6.75 6.66	14.60 14.67)
Z(OMe)–(14—16)–NHNH–Troc	102—104	−21.2 (MeOH)	$C_{38}H_{50}Cl_3N_5O_{10}$	54.13 (54.07	5.98 6.06	8.31 8.14)
Z(OMe)–(14—16)–$NHNH_2$ [4]	176—178	−35.4 (MeOH)	$C_{35}H_{49}N_5O_8$	62.95 (62.81	7.40 7.33	10.49 10.50)
Z(OMe)–(7—9)–OMe	171—178	−28.5 (DMF)	$C_{30}H_{41}N_3O_9S$	58.14 (58.40	6.67 6.62	6.78 6.91)
Z(OMe)–(6—9)–OMe	223—227	−8.1 (DMF)	$C_{34}H_{48}N_4O_{11}S \cdot 0.5H_2O$	55.95 (56.11	6.63 6.92	7.68 7.65)
Z(OMe)–(6—9)–$NHNH_2$ [6]	263—265	−27.6 (DMF)	$C_{33}H_{48}N_6O_{10}S$	54.99 (54.74	6.71 6.70	11.66 11.64)
Z(OMe)–(2—5)–OMe	195—199	−2.4 (DMF)	$C_{32}H_{43}N_5O_{12}S \cdot 0.5H_2O$	52.59 (52.39	6.07 6.06	9.58 9.35)
Z(OMe)–(1—5)–OMe	207—211	−11.3 (DMF)	$C_{35}H_{48}N_6O_{13}S \cdot 1.5H_2O$	51.27 (51.18	6.27 6.18	10.25 9.93)
Z(OMe)–(1—5)–$NHNH_2$ [7]	220—224	−51.3 (DMSO)	$C_{34}H_{48}N_8O_{12}S \cdot H_2O$	50.36 (50.37	6.22 6.33	13.82 13.75)

and ether; yield 5.25 g (67%), Rf_2 0.84. Physical constants and analytical data are listed in Table II, together with those of other intermediates.

Z(OMe)–Gly–Val–Gly–Lys(Z)–OMe (Positions 21—24)——A mixture of a TFA-treated sample of the above tripeptide ester (5.00 g, 8.13 mmol), TEA (2.49 ml, 2.2 eq) and Z(OMe)–Gly–OSu (3.28 g, 1.2 eq) in DMF (40 ml) was stirred overnight and the solvent was removed by evaporation. For purification, the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, and dried over P_2O_5 *in vacuo* (named the washing procedure hereafter). The dried powder was recrystallized from MeOH and ether; yield 2.60 g (48%), Rf_2 0.62.

Z(OMe)–Gly–Val–Gly–Lys(Z)–$NHNH_2$ [2] (Positions 21—24)——The above protected tetrapeptide ester (2.60 g, 3.88 mmol) in DMF (5 ml) was treated with 80% hydrazine hydrate (0.97 ml, 5 eq) at room temperature for 24 h, then the solvent was removed by evaporation and the residue was treated with H_2O. The resulting powder was precipitated from DMF with MeOH; yield 1.73 g (66%), Rf_2 0.28. Amino acid ratios in a 6 N HCl hydrolysate: Gly 2.09, Val 0.97, Lys 1.00 (recovery of Lys, 78%).

Z(OMe)–Asp(OChp)–Phe–Leu–NHNH–Troc (Positions 14—16)——A mixture of a TFA-treated sample of Z(OMe)–Phe–Leu–NHNH–Troc[4] (5.03 g, 7.96 mmol), TEA (1.2 ml, 1.2 eq), and Z(OMe)–Asp(OChp)–OSu (4.68 g, 1.2 eq) in DMF (20 ml) was stirred at 4 °C for 14 h. The product was purified by the extraction procedure, followed by recrystallization from AcOEt and ether; yield 4.74 g (71%), Rf_3 0.68.

Z(OMe)–Asp(OChp)–Phe–Leu–$NHNH_2$ [4] (Positions 14—16)——The above Troc-derivative (2.92 g, 3.46 mmol) in AcOH–DMF (2 ml–5 ml) was treated with Zn powder (3.0 g) in an ice-bath for 14 h. The solution was filtered, then the filtrate was concentrated *in vacuo* and the residue was treated with 3% EDTA. The resulting powder was washed with H_2O and recrystallized from MeOH and ether; yield 1.25 g (54%), Rf_3 0.24. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.01, Phe 0.96, Leu 1.00 (recovery of Leu, 75%).

Z(OMe)–Cys(MBzl)–Val–Thr–OMe (Positions 7—9)——A mixture of a TFA-treated sample of Z(OMe)–Val–Thr–OMe[3] (2.00 g, 5.04 mmol), TEA (1.55 ml, 2.2 eq), and Z(OMe)–Cys(MBzl)–OSu (3.04 g, 1.2 eq) in DMF 20 ml was stirred in an ice-bath for 14 h, then the solvent was removed by evaporation. The product was purified by the washing procedure, followed by precipitation from DMF with MeOH; yield 2.03 g (65%), Rf_2 0.83.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–OMe (Positions 6—9)——The azide [prepared from 1.12 g (1.8 eq) of Z(OMe)–Thr–$NHNH_2$] in DMF (5 ml) and TEA (0.63 ml, 2.2 eq) were added to an ice-chilled solution of a TFA-treated sample of the above tripeptide (1.30 g, 2.1 mmol) in DMF (5 ml) containing TEA (67 μl, 1 eq) and the mixture was stirred at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMF with MeOH; yield 1.24 g (82%), Rf_1 0.87.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–$NHNH_2$ [6] (Positions 6—9)——The above tetrapeptide ester (1.23 g, 1.71 mmol) in HMPA (4 ml) was treated with 80% hydrazine hydrate (0.85 ml, 10 eq) at room temperature for 24 h, then H_2O was added. The resulting powder was precipitated from DMF with MeOH; yield 1.21 g (98%), Rf_2 0.32. Amino acid ratios in a 6 N HCl hydrolysate: Thr 1.80, Val 1.00 (recovery of Val, 92%).

Z(OMe)–Cys(MBzl)(O)–Asn–Thr–Ala–OMe (Positions 2—5)——Z(OMe)–Cys(MBzl)(O)–OH (1.97 g, 1.5 eq) and then DPPA (1.0 ml, 1.5 eq) were added to a solution of a TFA-treated sample of Z(OMe)–Asn–Thr–Ala–OMe (1.50 g, 3.11 mmol) in DMF (5 ml) containing TEA (1.1 ml, 2.5 eq) and the mixture, after being stirred at room temperature for 14 h, was concentrated. The product was purified by the washing procedure, followed by precipitation from DMF with MeOH; yield 1.41 g (63%), Rf_1 0.67.

Z(OMe)–Ala–Cys(MBzl)(O)–Asn–Thr–Ala–OMe (Positions 1—5)——A mixture of a TFA-treated sample of the above tetrapeptide ester (1.40 g, 1.94 mmol), TEA (600 μl, 2.2 eq), and Z(OMe)–Ala–OSu (0.82 g, 1.2 eq) in DMF (3 ml) was stirred for 14 h and the solvent was removed by evaporation. The residue was purified by the washing procedure, followed by precipitation from DMF with MeOH; yield 1.34 g (87%), Rf_1 0.63.

Z(OMe)–Ala–Cys(MBzl)(O)–Asn–Thr–Ala–$NHNH_2$ [7] (Positions 1—5)——The above pentapeptide ester (1.20 g, 1.51 mmol) in DMF–HMPA (1 : 1, 3 ml) was treated with 80% hydrazine hydrate (380 μl, 5 eq) at room temperature for 14 h and H_2O was added. The resulting powder was precipitated from a mixture of DMSO–DMF (1 : 1) with MeOH; yield 0.75 g (63%), Rf_1 0.34. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.00, Thr 0.95, Ala 1.96 (recovery of Asp, 67%).

Z(OMe)–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Z(OMe)–(cCGRP 21—37)–NH_2——The azide, prepared from fragment [2] (0.83 g, 1.8 eq), in DMF–DMSO (2 : 1, 5 ml) and TEA (0.21 ml, 2.2 eq) were added to an ice-chilled solution of a TFA-treated sample of fragment [1] (1.22 g, 0.69 mmol) in DMF–DMSO (1 : 1, 10 ml) and the mixture was stirred at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH; yield 1.34 g (86%), Rf_1 0.60. Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I and analytical data, as well as physical constants, in Table III, together with those of other fragment condensation products.

Z(OMe)–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Z(OMe)–(cCGRP 17—37)–NH_2——The azide, prepared from fragment [3] (0.63 g, 2.0 eq), in DMF (5 ml) and TEA (0.11 ml, 2.4 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(cCGRP 21—37)–NH_2 (0.75 g, 0.33 mmol) in DMF–DMSO (1 : 1, 5 ml) and the mixture was stirred at 4 °C

TABLE III. Physical Constants and Analytical Data of Fragment Condensation Products

Z(OMe)–(cCGRP)–NH_2 (Positions)	mp (°C)	$[\alpha]_D^{20}$ (°)	Formula	Analysis (%) Calcd (Found)		
				C	H	N
21—37	275—278	−52.6	$C_{110}H_{149}N_{23}O_{29}\cdot 1.5H_2O$	57.83	6.71	14.10
				(57.64	6.71	14.37)
17—37	277—280	−54.1	$C_{138}H_{186}N_{30}O_{35}\cdot 5H_2O$	56.85	6.78	14.42
				(56.83	6.60	14.42)
14—37	266—270	−59.0	$C_{173}H_{233}N_{33}O_{42}S\cdot 6H_2O$	57.92	6.88	12.88
				(57.78	6.90	13.46)
10—37	266—270	−58.7	$C_{203}H_{278}N_{42}O_{48}S_2\cdot 8H_2O$	56.92	6.92	13.74
				(56.47	6.86	14.21)
6—37	250—256	−24.8	$C_{227}H_{314}N_{46}O_{55}S_3\cdot 13H_2O$	55.67	7.00	13.16
				(55.48	6.72	13.21)
1—37	275—279	−18.2	$C_{252}H_{350}N_{52}O_{64}S_4\cdot 9.5H_2O$	55.71	6.85	13.41
				(55.40	6.89	13.79)

for 38 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH; yield 0.77 g (82%), Rf_1 0.57.

Z(OMe)–Asp(OChp)–Phe–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Z(OMe)–(cCGRP 14—37)–NH_2——The azide, prepared from fragment [4] (0.50 g, 5 eq), in DMF (3 ml) and TEA (0.13 ml, 6 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(cCGRP 17—37)–NH_2 (0.42 g, 0.15 mmol) in DMF–DMSO (1 : 1, 5 ml) and the mixture was stirred at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH as stated above; yield 0.44 g (85%), Rf_1 0.57.

Z(OMe)–His–Arg(Mts)–Leu–Ala–Asp(OChp)–Phe–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Z(OMe)–(cCGRP 10—37)–NH_2——The azide, prepared from fragment [5] (0.34 g, 3 eq), in DMF (2 ml) and TEA (67 μl, 3.6 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(cCGRP 14—37)–NH_2 (0.44 g, 0.13 mmol) in DMF (5 ml) and the mixture was stirred at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH as stated above; yield 0.49 g (95%), Rf_1 0.55.

Z(OMe)–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–Asp(OChp)–Phe–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Z(OMe)–(cCGRP 6—37)–NH_2——The azide, prepared from fragment [6] (0.35 g, 5 eq), in DMF (1 ml) and NMM (65 μl, 6 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(cCGRP 10—37)–NH_2 (0.41 g, 99.1 μmol) in DMF–DMSO (1 : 1, 2 ml) containing TEA (14 μl, 1 eq) and the mixture was stirred at −10 °C for 48 h, then at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH as stated above; yield 0.39 g (85%), Rf_1 0.78.

Z(OMe)–Ala–Cys(MBzl)(O)–Asn–Thr–Ala–Thr–Cys(MBzl)–Val–Thr–His–Arg(Mts)–Leu–Ala–Asp(OChp)–Phe–Leu–Ser(Bzl)–Arg(Mts)–Ser(Bzl)–Gly–Gly–Val–Gly–Lys(Z)–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser(Bzl)–Lys(Z)–Ala–Phe–NH_2, Protected cCGRP——The azide, prepared from fragment [7] (0.37 g, 5 eq), in DMF (2 ml) and NMM (61 μl, 6 eq) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–(cCGRP 6—37)–NH_2 (0.43 g, 92.2 μmol) in DMF–DMSO (2 : 1, 2 ml) containing TEA (13 μl, 1 eq) and the mixture was stirred at 4 °C for 14 h. After evaporation of the solvent, the product was purified by the washing procedure, followed by precipitation from DMSO with MeOH as stated above; yield 0.40 g (83%), Rf_1 0.74.

H–Ala–Cys–Asn–Thr–Ala–Thr–Cys–Val–Thr–His–Arg–Leu–Ala–Asp–Phe–Leu–Ser–Arg–Ser–Gly–Gly–Val–Gly–Lys–Asn–Asn–Phe–Val–Pro–Thr–Asn–Val–Gly–Ser–Lys–Ala–Phe–NH_2, cCGRP——Protected cCGRP (50 mg, 9.5 μmol) was treated with TFA (3.6 ml) in the presence of PhSPh (121 μl, 0.2 M concentration) in an ice-bath for 30 min, then at 25 °C for 120 min. TMSOTf (965 μl, to the concentration of 1 M) and PhSPh (297 μl, to the total concentration of 0.5 M) were added and the solution was further stirred in an ice-bath for an additional 120 min. After addition of dry ether, the resulting powder was collected by centrifugation and dissolved in MeOH–H_2O (1 : 1, 4 ml). The pH of the solution was adjusted to 8 with 5% NH_4OH and after 10 min, to 5.0 with 1 N AcOH. The solution was applied to a column of Sephadex G-15 (1.8 × 136 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak [5.8 ml each, tube Nos. 19—27, monitrored by ultraviolet (UV) absorption measurement at 227 nm] were collected and the solvent was removed by lyophilization to give a powder; yield 34.8 mg (95%).

The product was dissolved in H_2O (1 ml) and the solution was applied to a column of CM-Trisacryl M

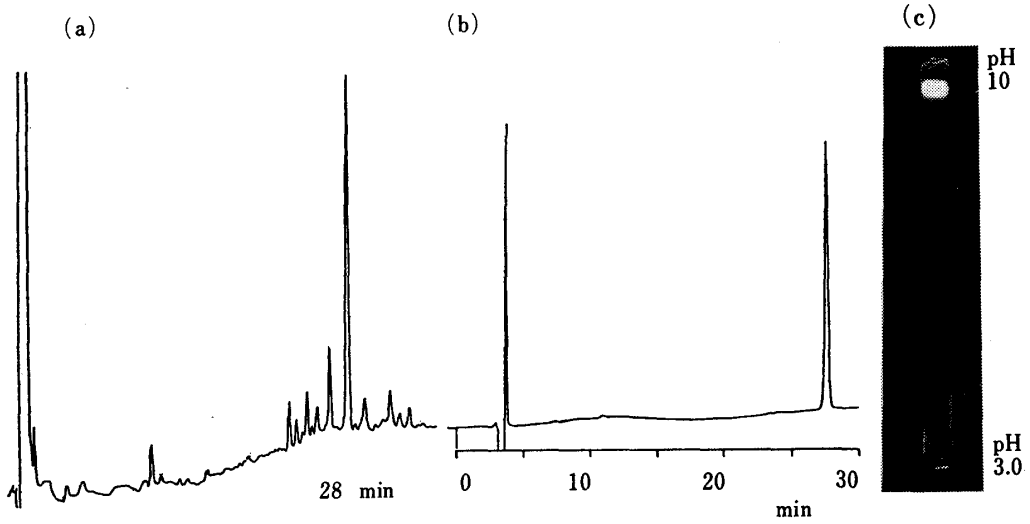


Fig. 3. HPLC (YMC R-ODS-5) and Disk Isoelectrofocusing of Synthetic cCGRP
(a) CM-Purified sample. (b) Purified sample.

(2 × 13.5 cm), which was eluted first with pH 6.8, 0.01 M $AcONH_4$ buffer (80 ml) and then with the same buffer (300 ml) containing NaCl (at the concentration of 0.2 M) through a mixing flask containing the starting buffer (150 ml). Individual fractions (5.8 ml each) were collected and UV absorption at 227 nm was measured. After elution of three small peaks, the desired product emerged from the column as a main peak. The fractions corresponding to this peak (tube Nos. 32—39) were collected and the solvent was removed by lyophilization. For desalting, the residue was applied to a column of Sephadex G-15 (1.8 × 136 cm), which was eluted with 1 N AcOH as described above. Lyophilization of the desired eluates gave a fluffy powder; yield 8.2 mg (22.3%). The HPLC elution pattern of this CM-purified sample on an analytical YMC R-ODS-5 column [eluted with MeCN (25—40%, 30 min) in 0.1% TFA at a flow rate 1 ml/min] is shown in Fig. 3a.

Subsequent purification was performed by reversed-phase HPLC on a Cosmosil 5C18 column (10 × 250 mm). The above CM-purified sample was dissolved in 0.1% TFA and portions of the solution were applied to the above column, which was eluted with a gradient of MeCN (25—40%, 60 min) in 0.1% TFA at a flow rate of 1.8 ml/min. The desired eluate (monitored by UV absorption measurement at 230 nm) was taken and the solvent was removed by lyophilization to give a white fluffy powder; 3.2 mg (8.8% from protected cCGRP); $[\alpha]_D^{23}$ −59.3° (c=0.2, 0.5 N AcOH), Rf_4 0.49, Rf_5 0.69. FAB-MS m/z: 3839.3 $(M+H)^+$. A single peak (retention time 28 min, monitored by UV absorption measurement at 230 nm) on HPLC [analytical YMC R-ODS-5 column (4.6 × 250 mm)] was obtained by gradient elution with MeCN (25—40%, 30 min) in 0.1% TFA at a flow rate of 1.0 ml/min (Fig. 3b). The synthetic cCGRP exhibited a single band in disk isoelectrofocusing (Fig. 3c) on 7.5% polyacrylamide gel (0.5 × 6.8 cm) containing Pharmalyte (pH 3—10); mobility 6.3 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4.5 h (stained with Coomassie Brilliant Blue G-250, Sigma). Amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in LAP digestion (numbers in parentheses are theoretical): Asp 1.17(1), Thr 4.50(4), Ser 3.26(3), Pro 1.07(1), Gly 4.11(4), Ala 4.18(4), Cys 0.97(1), Val 4.11(4), Leu 2.15(2), Phe 3.00(3), Lys 2.07(2), His 1.00(1), Arg 2.06(2), Asn was not determined (recovery of Phe, 66%).

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References and Notes

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2) Amino acids and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylenesulfonyl, Chp = cycloheptyl, Ad = 1-adamantyl, MBzl = *p*-methoxybenzyl, (O) = sulfoxide, Su = *N*-hydroxysuccinimidyl, DPPA = diphenylphosphoryl azide, DMF = dimethylformamide, DMSO = dimethylsulfoxide, HMPA = hexamethylphosphoramide, TFA = trifluoroacetic acid, TEA = triethylamine, TMSOTf = trimethylsilyl trifluoromethanesulfonate, EDTA = ethylenediaminetetraacetic acid disodium salt,

NMM = *N*-methylmorpholine.

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