Notes

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Studies on Peptides. CXLV.[1,2] Synthesis of a 27-Residue Peptide Amide Corresponding to the Entire Amino Acid Sequence of Canine Gastrin-Releasing Polypeptide (cGRP)

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Canine gastrin-releasing polypeptide (cGRP, characterized as a bombesin-like 27-residue peptide) was synthesized by successive azide condensations of 7 peptide fragments of established purity, followed by deprotection with trifluoromethanesulfonic acid in trifluoroacetic acid. Prior to deprotection, the Met(O) residue in the protected cGRP was reduced by brief treatment with phenylthiotrimethylsilane in the presence of a catalytic amount of trimethylsilyl trifluoromethanesulfonate. The homogeneous HPLC-purified product induced a significant increase of immunoreactive gastrin level in rat plasma, like synthetic human GRP.

Keywords——canine gastrin-releasing polypeptide synthesis; Met(O) reduction; phenylthiotrimethylsilane; trimethylsilyl trifluoromethanesulfonate; trifluoromethanesulfonic acid deprotection; immunoreactive gastrin release

Following our synthesis of human gastrin-releasing polypeptide (hGRP),[3] we wish to report the synthesis of a similar 27-residue peptide amide, which was isolated from the canine intestine by Reeve *et al.*[4] in 1983. This peptide (cGRP) was characterized as the largest form of bombesin-like peptide, together with two shorter forms (23 and 10 residues), the sequences of which were identical with the corresponding carboxyl-terminal segments of the 27-residue peptide. The sequence of cGRP is different from that of hGRP[5] in the replacement of 5 amino acid residues at positions 1,3,5,7 and 12: Val, Leu, Ala, Gly and Thr in hGRP are substituted by Ala, Val, Gly, Gln and Asp in cGRP. The synthetic route to cGRP that we have employed is shown in Fig. 1. Of seven fragments used in our present synthesis, three fragments, [1] to [3], are identical with those employed for the hGRP synthesis. The other four fragments which cover the areas of species variation were newly synthesized.

Fragment [4], Z(OMe)–Asp(OBzl)–Lys(Z)–Met(O)–$NHNH_2$, was prepared in a stepwise manner starting with H–Met(O)–NHNH–Troc.[6] The respective amino acids were introduced by the Su procedure[7] and the Troc group was removed from the resulting tripeptide derivative by treatment with Zn.[8] Fragment [5], Z(OMe)–Thr–Val–Leu–$NHNH_2$, was obtained easily by the usual hydrazine treatment of the corresponding methyl ester, an intermediate of our porcine GRP synthesis.[9] Fragment [6], Z(OMe)–Gly–Gln–Gly–$NHNH_2$, was prepared by the Su condensation of Z(OMe)–Gly–OH with a TFA-treated sample of Z(OMe)–Gln–Gly–OMe[10] followed by the usual hydrazine treatment. The N-terminal fragment, Z(OMe)–Ala–Pro–Val–Pro–Gly–$NHNH_2$ [7], was prepared in a stepwise manner, as shown in Fig. 2, using the mixed anhydride (MA)[11] or the active ester procedure, such as

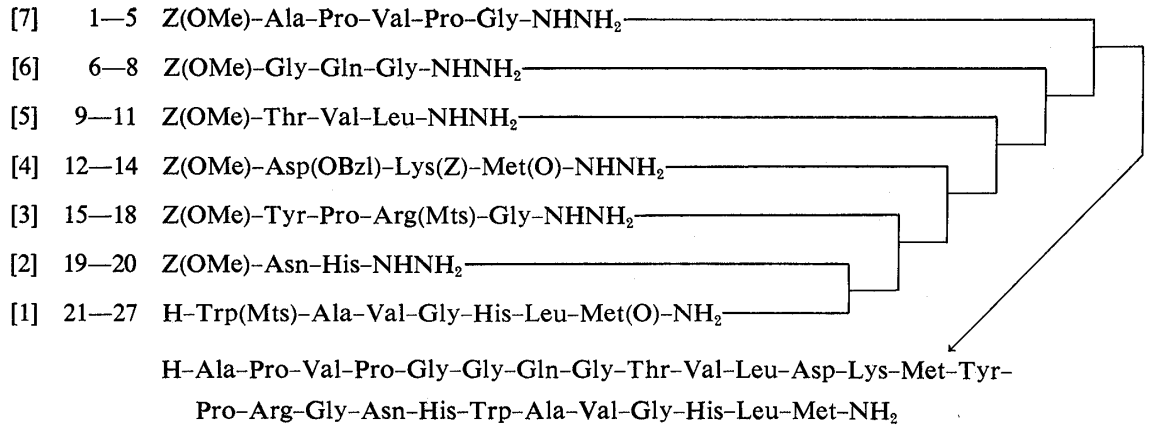

Fig. 1. Synthetic Route to Canine GRP

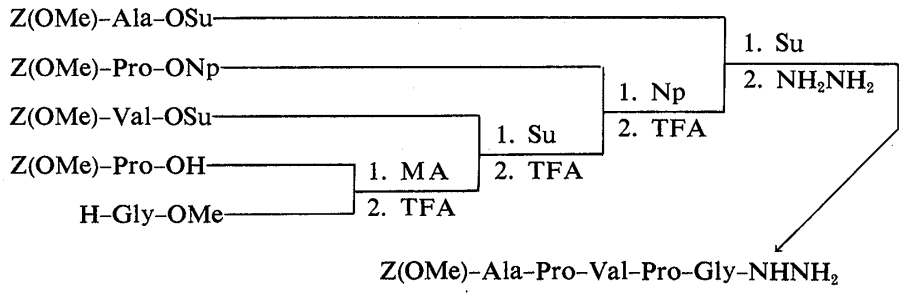

Fig. 2. Synthetic Scheme for the N-Terminal Pentapeptide Hydrazide [7]

the Su and the Np procedure.[12] Establishment of the Pro–Val bond required a much longer time than usual because of the steric hindrance between these two amino acids. Recrystallization of Pro-containing peptides is often a difficult task. Thus, each product was purified by silica gel chromatography. The protected pentapeptide ester thus obtained was smoothly converted to [7] by the usual hydrazine treatment. This hydrazide and all intermediates, including two oily derivatives, Z(OMe)–Pro–Gly–OMe and Z(OMe)–Val–Pro–Gly–OMe, were characterized by elemental analysis.

The seven fragments thus obtained were then condensed successively by the azide procedure[13] (Fig. 1) to minimize racemization. The amount of the acyl component was increased from 3 to 4 eq as chain elongation progressed in order to secure complete condensation. All products, including the protected cGRP, were purified by gel-filtration on Sephadex LH-60 using DMF as an eluant. Throughout this synthesis, Leu was selected as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Leu with the recoveries of newly added amino acids, satisfactory incorporation of each fragment was ascertained (Table I).

The protected cGRP thus obtained was treated with phenylthiotrimethylsilane[14] in the presence of trimethylsilyl trifluoromethanesulfonate to reduce Met(O) as described in the hGRP synthesis,[3] then it was exposed to 1 M TFMSA–thioanisole in TFA[15] in the presence of *m*-cresol and EDT to remove all the protecting groups employed. The deprotected peptide was treated with dil. ammonia to reverse a possible N→O shift[16] and then purified by gel-filtration on Sephadex G-25, followed by high performance liquid chromatography (HPLC). The purity of synthetic cGRP was ascertained by thin-layer chromatography (TLC),analytical HPLC, isoelectrofocusing and amino acid analyses, after acid hydrolysis and enzymatic digestion.

Upon i.v. injection into rats, the synthetic cGRP raised plasma immunoreactive gastrin

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

	Protected intermediates					Synthetic cGRP	Theory
	15—27	12—27	9—27	6—27	1—27		
Asp	1.04	2.12	2.29	2.17	2.15	1.98	(2)
Thr			0.91	0.97	0.97	0.93	(1)
Glu				1.00	1.03	1.01	(1)
Pro	0.95	0.99	1.03	0.97	2.95	3.05	(3)
Gly	1.95	1.99	2.15	3.91	4.96	4.93	(5)
Ala	0.99	1.01	1.08	1.03	2.01	2.15	(2)
Val	0.96	0.99	2.04	2.00	2.97	2.71	(3)
Met[a]	0.91	1.80	1.74	1.74	1.84	1.79	(2)
Leu	1.00	1.00	2.00	2.00	2.00	2.00	(2)
Tyr	0.96	0.95	1.05	0.96	0.97	0.95	(1)
Trp	N.D.	N.D.	N.D.	N.D.	N.D.	0.86[b]	(1)
Lys		1.03	1.07	1.06	1.04	0.98	(1)
His	1.93	1.96	2.09	1.93	1.89	1.91	(2)
Arg	0.96	1.01	1.07	1.00	0.97	0.96	(1)
Recov. (%)	75	93	78	88	93	80	

a) Met + Met(O). *b*) 4 N MSA hydrolysis.

level, and its potency was judged to be equivalent to that of synthetic hGRP.

Experimental

General experimental procedures described herein are essentially the same as described in connection with the synthesis of hGRP.[3] The active ester reaction was performed at room temperature. Azides were prepared according to Honzl and Rudinger[13] with isoamyl nitrite and mixed anhydrides were prepared according to Vaughan and Osato[11] with isobutyl chloroformate. Unless otherwise stated, products were purified by either one of the following procedures. A (extraction procedure): The product was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and concentrated. The residue was recrystallized or precipitated from appropriate solvent. B (washing procedure): After evaporation of the solvent, the residue was treated with 5% citric acid and ether and the resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and recrystallized or precipitated from appropriate solvents.

The *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (10:0.5), Rf_2 $CHCl_3$–MeOH–H_2O(8:3:1), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_4 *n*-BuOH–AcOH–AcOEt–H_2O (1:1:1:1). HPLC was conducted with a Waters 204 compact model.

Z(OMe)–Lys(Z)–Met(O)–NHNH–Troc——A mixture of Z(OMe)–Lys(Z)–OSu (5.77 g, 11.15 mmol), Et_3N (2.78 ml, 20.14 mmol) and a TFA-treated sample of Z(OMe)–Met(O)–NHNH–Troc[6] (4.82 g, 9.29 mmol) in DMF (30 ml) was stirred overnight and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.94 g (81%), mp 153—156 °C, $[\alpha]_D^{20}$ −16.2° (c=1.0, DMF), Rf_2 0.68. *Anal.* Calcd for $C_{31}H_{40}Cl_3N_5O_{10}S$: C, 47.67; H, 5.16; N, 8.97. Found: C, 47.95; H, 5.11; N, 9.27.

Z(OMe)–Asp(OBzl)–Lys(Z)–Met(O)–NHNH–Troc——A mixture of a TFA-treated sample of the above dipeptide derivative (5.94 g, 7.60 mmol), Et_3N (2.25 ml, 16.30 mmol) and Z(OMe)–Asp(OBzl)–OSu (5.53 g, 11.14 mmol) in DMF (50 ml) was stirred overnight and concentrated. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 5.80 g (77%), mp 125—129 °C, $[\alpha]_D^{20}$ −1.0° (c=1.0, DMF), Rf_2 0.59. *Anal.* Calcd for $C_{42}H_{51}Cl_3N_6O_{13}$: C, 51.14; H, 5.21; N, 8.52. Found: C, 51.01; H, 5.20; N, 8.70.

Z(OMe)–Asp(OBzl)–Lys(Z)–Met(O)–NHNH$_2$ [4]——The above protected tripeptide derivative (1.72 g, 1.74 mmol) in DMF–AcOH (10 ml–2 ml) was treated with Zn powder (2.28 g, 20 eq) at room temperature for 2 h. The solution was filtered and the filtrate was concentrated. The residue was treated with 2% EDTA and the resulting powder was purified by washing with 5% $NaHCO_3$ and H_2O, followed by precipitation from DMSO with MeOH; yield 0.87 g (62%), mp 159—162 °C, $[\alpha]_D^{20}$ +11.9° (c=1.0, DMF), Rf_2 0.64. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.99, Met + Met(O) 0.76, Lys 1.00 (recovery of Lys 74%). *Anal.* Calcd for $C_{39}H_{50}N_6O_{11}S \cdot 1/2H_2O$: C, 57.13; H. 6.27; N, 10.25. Found: C, 57.33; H, 6.15; N, 10.10.

Z(OMe)–Thr–Val–Leu–NHNH$_2$ [5]——Z(OMe)–Thr–Val–Leu–OMe[9] (2.53 g, 4.96 mmol) in DMF (30 ml) was treated with 80% hydrazine hydrate (2.49 ml, 10 eq) for 24 h and the solvent was removed by evaporation. Treatment of the residue with MeOH afforded a powder, which was precipitated from DMSO with MeOH; yield 1.85 g (73%), mp 242—246 °C, $[\alpha]_D^{20}$ −12.3° (c=1.0, DMSO), Rf_2 0.59. Amino acid ratios in a 6 N HCl hydrolysate: Thr 0.95, Val 0.99, Leu 1.00 (recovery of Leu 86%). *Anal.* Calcd for $C_{24}H_{39}N_5O_7$: C, 56.56; H, 7.71; N, 13.74. Found: C, 56.71; H, 7.80; N, 13.44.

Z(OMe)–Gly–Gln–Gly–OMe——A mixture of Z(OMe)–Gly–OSu (5.97 g, 17.75 mmol), Et_3N (3.60 ml, 26.09 mmol) and a TFA-treated sample of Z(OMe)–Gln–Gly–OMe[10] (4.51 g, 11.83 mmol) in DMF (60 ml) was stirred overnight and concentrated. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.97 g (96%), mp 176—179 °C, $[\alpha]_D^{18}$ −1.0° (c=1.0, DMF), Rf_2 0.58. *Anal.* Calcd for $C_{19}H_{26}N_4O_8$: C, 52.05; H, 5.98; N, 12.78. Found: C, 52.00; H, 5.94; N, 13.05.

Z(OMe)–Gly–Gln–Gly–NHNH$_2$ [6]——The above protected tripeptide ester (3.65 g, 8.33 mmol) in DMF (20 ml) was treated with 80% hydrazine hydrate (2 ml, 5 eq) at room temperature overnight and the solution was concentrated. Treatment of the residue with EtOH afforded a powder, which was precipitated from DMSO–DMF (1 : 1) with MeOH; yield 2.43 g (67%), mp 196—199 °C, $[\alpha]_D^{18}$ −7.2° (c=1.0, DMSO), Rf_2 0.21. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.01, Gly 2.00 (recovery of Gly 90%). *Anal.* Calcd for $C_{18}H_{26}N_6O_7 \cdot 1/2H_2O$: C, 48.31; H, 6.08; N, 18.78. Found: C, 48.33; H, 6.04; N, 18.81.

Z(OMe)–Pro–Gly–OMe——This dipeptide ester was synthesized by the mixed anhydride procedure and purified by procedure A; yield 5.50 g (89%), oil, $[\alpha]_D^{20}$ −51.1° (c=1.1, MeOH), Rf_1 0.35. *Anal.* Calcd for $C_{17}H_{22}N_2O_6 \cdot 1/2H_2O$: C, 56.81; H, 6.45; N, 7.80. Found: C, 56.59; H, 6.64; N, 7.59.

Z(OMe)–Val–Pro–Gly–OMe——A mixture of a TFA-treated sample of the above dipeptide ester (5.06 g, 14.44 mmol), Et_3N (3.99 ml, 28.91 mmol) and Z(OMe)–Val–OSu (6.56 g, 17.34 mmol) in DMF (30 ml) was stirred overnight. An additional portion of active ester (1 eq) was added and the mixture was stirred for a further 48 h until the solution became ninhydrin-negative. The solvent was removed by evaporation and the product was purified by procedure A followed by column chromatography on silica gel (4.5 × 12 cm), which was eluted with AcOEt–*n*-hexane (from 1 : 1 to 2 : 1 v/v). The desired eluates (monitored by $Ce(SO_4)_2$ test on TLC) were concentrated to give an oily product; yield 3.63 g (56%), $[\alpha]_D^{20}$ −102.2° (c=1.1, MeOH), Rf_1 0.31. *Anal.* Calcd for $C_{22}H_{31}N_3O_7$: C, 58.78; H, 6.95; N, 9.35. Found: C, 58.88; H, 7.17; N, 9.26.

Z(OMe)–Pro–Val–Pro–Gly–OMe——A mixture of a TFA-treated sample of the above tripeptide ester (3.50 g, 7.78 mmol), Et_3N (3.80 ml, 27.54 mmol) and Z(OMe)–Pro–ONp (8.97 g, 19.47 mmol) in DMF (60 ml) was stirred for 24 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica gel (3.5 × 13 cm), which was eluted with $CHCl_3$–MeOH (20 : 0.5). The desired eluates (monitored by means of the $Ce(SO_4)_2$ test on TLC) were concentrated and the residue was triturated with *n*-hexane to afford a powder; yield 3.00 g (71%), mp 46—50 °C, $[\alpha]_D^{20}$ −142.2° (c=1.0, MeOH), Rf_1 0.31. *Anal.* Calcd for $C_{27}H_{38}N_4O_8$: C, 59.32; H, 7.01; N, 10.25. Found: C, 59.59; H, 7.19; N, 9.95.

Z(OMe)–Ala–Pro–Val–Pro–Gly–OMe——A mixture of a TFA-treated sample of the above tetrapeptide ester (2.81 g, 5.14 mmol), Et_3N (1.77 ml, 12.83 mmol) and Z(OMe)–Ala–OSu (2.70 g, 7.71 mmol) in DMF (40 ml) was stirred overnight and concentrated, and the product was purified by procedure A, followed by column chromatography on silica gel (3.5 × 14 cm), which was eluted with $CHCl_3$–MeOH (20 : 0.5). The desired product was isolated as stated above; yield 2.47 g (78%), mp 66—70 °C, $[\alpha]_D^{15}$ −158.6° (c=1.0, MeOH), Rf_1 0.22. *Anal.* Calcd for $C_{30}H_{43}N_5O_9$: C, 57.49; H, 7.08; N, 11.18. Found: C, 57.55; H, 6.99; N, 10.95.

Z(OMe)–Ala–Pro–Val–Pro–Gly–NHNH$_2$ [7]——The above pentapeptide ester (2.43 g, 3.93 mmol) in MeOH (30 ml) was treated with 80% hydrazine hydrate (1 ml, 5 eq) for 2 d and the solvent was evaporated off. The residue was extracted with *n*-BuOH. The organic phase was washed with H_2O and concentrated. Treatment of the residue with ether afforded a powder, which was recrystallized from $CHCl_3$ and ether; yield 2.31 g (95%), mp 199—203 °C, $[\alpha]_D^{20}$ −117.5° (c=1.0, MeOH), Rf_2 0.64. Amino acid ratios in a 6 N HCl hydrolysate: Pro 1.97, Gly 1.00, Ala 1.02, Val 1.00 (recovery of Gly 89%). *Anal.* Calcd for $C_{29}H_{43}N_7O_8 \cdot 1/2H_2O$: C, 55.58; H, 7.08; N, 15.64. Found: C, 55.61; H, 7.08; N, 15.40.

Synthesis of the Protected cGRP——Successive azide condensations of seven fragments were carried out according to the route shown in Fig. 1. Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 1 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated by addition of dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF containing Et_3N (1 eq). The corresponding azide (the amount was increased from 3 to 4 eq as the chain was elongated) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at −4 °C, except for the condensation of fragment [4]. In this case, the reaction was performed at −15 °C in order to suppress Curtius rearrangement.[17] When the solution became negative to the ninhydrin test, H_2O was added and the resulting powder was purified by gel-filtration on Sephadex LH-60 using DMF as an eluant. Fractions (10 ml each) were examined by measuring the ultraviolet (UV) absorption at 280 nm, and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to form a powder. Of these products, the C-terminal protected nona- and trideca-peptides are

TABLE II. Physical Constants and Analytical Data of Protected cGRP and Its Intermediates

Compounds	Yield (%)	Rf_1	$[\alpha]_D^{20}$ (DMF)	mp (°C)	Formula	Analysis (%) Calcd (Found)		
						C	H	N
Z(OMe)-(12—27)-NH_2	59	0.21	+7.5°	187—191	$C_{127}H_{167}N_{27}O_{31}S_4 \cdot 4H_2O$	55.10 (55.11)	6.37 6.23	13.06 13.59)
Z(OMe)-(9—27)-NH_2	58	0.25	−13.0°	176—180	$C_{142}H_{194}N_{30}O_{35}S_4 \cdot 3.5H_2O$	55.51 (55.51	6.59 6.50	13.68 13.72)
Z(OMe)-(6—27)-NH_2	63	0.16	−23.6°	204—208	$C_{151}H_{208}N_{34}O_{39}S_4 \cdot 8.5H_2O$	53.26 (53.21	6.66 6.31	13.99 13.80)
Z(OMe)-(1—27)-NH_2	61	0.18	−13.7°	189—193	$C_{171}H_{239}N_{39}O_{44}S_4 \cdot 9H_2O$	53.55 (53.56	6.75 6.63	14.24 13.75)

identical with those employed for the hGRP synthesis. Yields, physical constants and analytical data of the protected cGRP and other intermediates are listed in Table II.

H-Ala-Pro-Val-Pro-Gly-Gly-Gln-Gly-Thr-Val-Leu-Asp-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (cGRP)——The protected cGRP (45 mg, 12.3 μmol) in DMF (0.5 ml) was treated with phenylthiotrimethylsilane (224 μl, 100 eq) and trimethylsilyl trifluoromethanesulfonate (10.9 μl, 4 eq) at room temperature for 30 min under an Ar atmosphere, while the starting material disappeared and a new spot, Rf_4 0.45, was detected on TLC. The solution, after being neutralized with Et_3N, was concentrated and the residue was treated with ether to form a powder; yield 45 mg (100%), Rf_4 0.45.

The reduced peptide (44 mg, 12.0 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3 ml) in the presence of *m*-cresol (125 μl, 100 eq) and EDT (50 μl, 50 eq) in an ice-bath for 180 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O (5 ml). The pH of the solution was adjusted to 8.0 with 5% NH_4OH, and after 30 min to 5.0 with 1 N AcOH. The solution was applied to a column of Sephadex G-25 (1.5 × 100 cm), which was eluted with 1 N AcOH. The fractions (2.5 ml each, monitored by UV absorption measurement at 280 nm) corresponding to the front main peak (tube Nos 32—43) were combined and the solvent was removed by lyophilization to give a powder; yield 37 mg (106%). The crude product thus obtained (18.0 mg) was purified by HPLC on a Cosmosil 5C18 column (1.0 × 25 cm) using isocratic elution with 24%

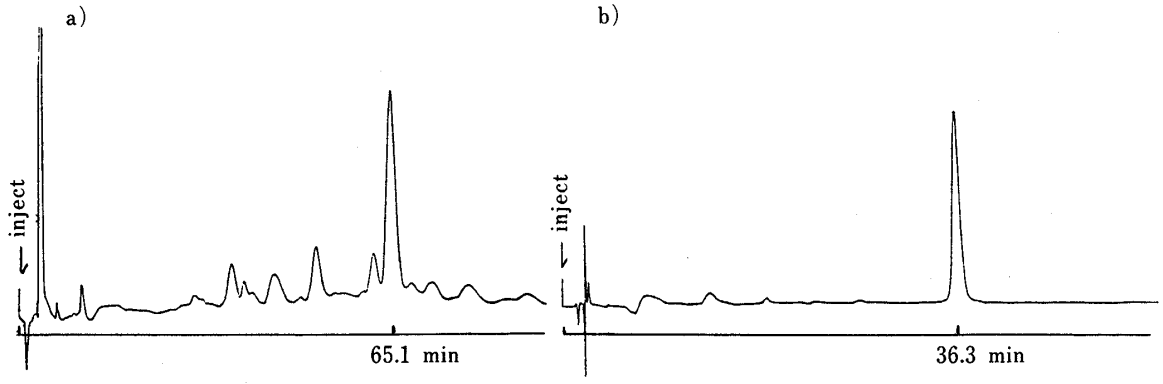

Fig. 3. HPLC of Synthetic cGRP

a: Crude sample, OD_{280} (preparative column). b: Purified sample, OD_{280} (analytical column).

Fig. 4. Disk Isoelectrofocusing of Synthetic cGRP

acetonitrile in 0.1% TFA at a flow rate of 1.5 ml per min (Fig. 3-a). The eluate corresponding to the main peak (retention time 65.1 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 2.8 mg. The rest of the sample was similarly purified; yield 5.7 mg. Overall yield from the protected cGRP was 16.3%. $[\alpha]_D^{20}$ −90.7° (c=0.1, 0.1 N AcOH); Rf_3 0.34; retention time, 36.3 min in HPLC on a analytical Nucleosil 5C18 column (4×150 mm) by gradient elution with acetonitrile (20—25%, 30 min) in 0.1% TFA at a flow rate of 0.8 ml per min (Fig. 3-b); a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5×6.5 cm) containing Pharmalyte (pH 3.0—10.0): mobility 5.9 cm (stained with Coomassie Brilliant Blue G-250, Sigma) from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (Fig. 4). Amino acid ratios in a 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in an aminopeptidase digest (numbers in parentheses are theoretical): Asp 0.98 (1), Thr 1.06 (1), Pro 2.95 (3), Gly 4.79 (5), Ala 2.13 (2), Val 2.85 (3), Met 1.78 (2), Leu 2.00 (2), Tyr 0.96 (1), Lys 1.00 (1), His 1.72 (2), Arg 0.98 (1), Trp 0.80 (1), Asn and Gln were not determined (recovery of Leu 84%).

References and Notes

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, Bzl=benzyl, Mts=mesitylenesulfonyl, Troc=2,2,2-trichloroethyloxycarbonyl, TFMSA=trifluoromethanesulfonic acid, MSA=methanesulfonic acid, TFA=trifluoroacetic acid, DMF=dimethylformamide, DMSO=dimethylsulfoxide, EDT=ethanedithiol, EDTA=ethylenediaminetetraacetic acid disodium salt.
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