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Studies on Peptides. CXLII.[1,2] Synthesis of Des-1-Ala-des-α-amino-Human Calcitonin Gene-Related Peptide

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Subsequent to our synthesis of human calcitonin gene-related peptide (hCGRP) (*J. Chem. Soc., Chem. Commun.*, **1985**, 602), its analog, des-1-Ala-des-α-amino-hCGRP, was synthesized. This peptide, prepared by replacement of the N-terminal Ala–Cys residues with 3-mercaptopropionic acid, followed by air oxidation, was more active than the parent peptide in respect of Ca-lowering and Pi-lowering activities in rat serum.

Keywords——human CGRP; hCGRP analog; *S-p*-methoxybenzyl-3-thiopropionic acid; thioanisole-mediated deprotection; serum Ca-lowering activity; serum Pi-lowering activity

Human calcitonin gene related peptide (hCGRP) is a 37-residue peptide amide with one disulfide bridge, characterized by Morris *et al.*[3] in 1984. Recently we reported a solution synthesis of hCGRP,[4] for which seven peptide fragments were selected as building blocks to construct the entire amino acid sequence of this new peptide. Using available fragments [1] to [6] and a newly prepared N-terminal fragment, we have synthesized des-1-Ala-des-α-amino-hCGRP (Fig. 1), which may be resistant to degradation by the action of aminopeptidase. As expected, this analog of hCGRP exhibited much higher activity than the parent molecule in respect of Ca-lowering activity in rat serum.

The N-terminal fragment [7], MBzl–S–CH_2–CH_2–CO–Asp–Thr–Ala–$NHNH_2$, was syn-

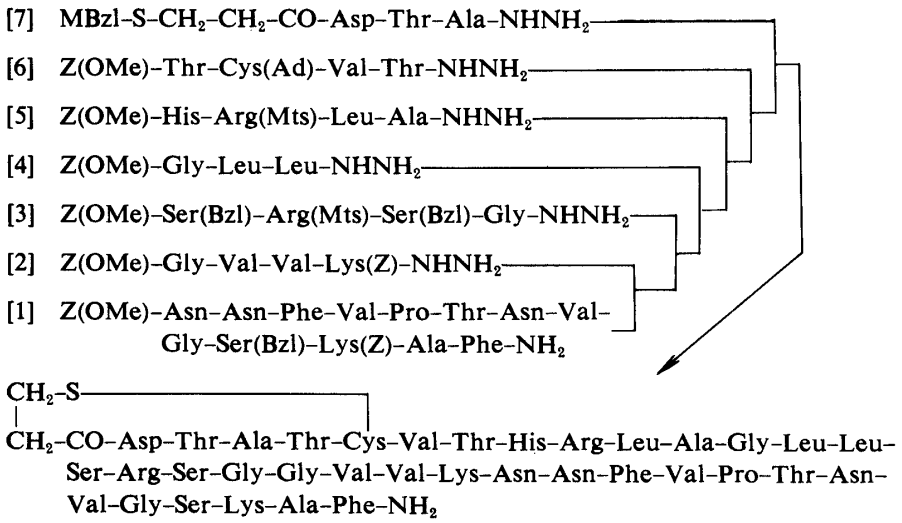

Fig. 1. Synthetic Route to Des-1-Ala-des-α-amino-hCGRP

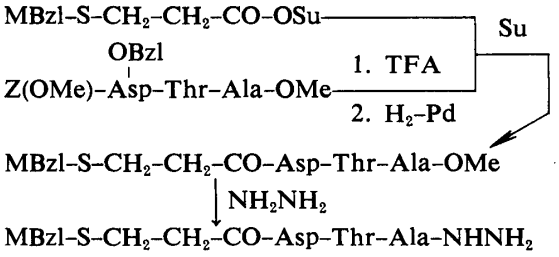

Fig. 2. Synthetic Scheme for the N-Terminal Fragment [7]

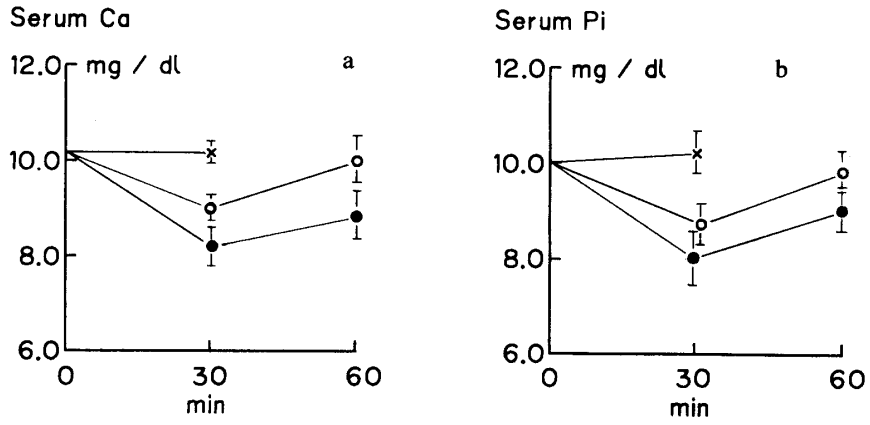

Fig. 3. Biological Activities of the Synthetic hCGRP Analog

a: Effect on Ca in serum.
b: Effect on Pi in serum.
—○—, synthetic hCGRP (80 μg/kg i.v.); —●—, des-Ala-des-amino analog (80 μg/kg i.v.); —×—, vehicle (pH 6.0, 0.38 mmol citric acid buffer containing 0.1% BSA).

thesized according to the scheme shown in Fig. 2. First, a TFA-treated sample of Z(OMe)–Asp(OBzl)–Thr–Ala–OMe[4] was subjected to hydrogenolysis in order to remove the Bzl ester. Thr, was avoided. The resulting tripeptide ester, H–Asp–Thr–Ala–OMe, was allowed to react with MBzl–S–CH_2–CH_2–COOSu[6] to give MBzl–S–CH_2–CH_2–CO–Asp–Thr–Ala– with MBzl–S–CH_2–CH_2–COOSu[6] to give MBzl–S–CH_2–CH_2–CH_2–CO–Asp–Thr–Ala–OMe, which was converted to [7] by the usual hydrazine treatment. Next, as an amino component, the protected dotriacontapeptide amide, Z(OMe)–(hCGRP 6—37)–NH_2, was prepared by condensations of fragments [1] to [6] as reported previously.[4] This, after TFA treatment, was condensed with fragment [7] by the azide procedure[7] and the product was purified by gel-filtration on Sephadex LH-60 using DMF–DMSO (7 : 3) as an eluant.

The protected peptide amide thus obtained was treated with 1 M TFMSA–thioanisole in TFA[8] in the presence of *m*-cresol and the desired product was isolated as described in the case of hCGRP synthesis,[4] *i.e.*, as follows. 1. Reduction with 2-mercaptoethanol. 2. Incubation at pH 8.0 to reverse a possible N→O shift.[9] 3. Gel-filtration on Sephadex G-25 to remove the reducing reagent. 4. Air-oxidation in a dilute solution at pH 7.5 to establish the intramolecular disulfide bond. 5. Repeated lyophilization to obtain a crude product. 6. Purification by ion-exchange chromatography on CM-Biogel A using ammonium acetate buffer. 7. Purification by high performance liquid chromatography (HPLC) using gradient elution with acetonitrile (25—40%) in 0.1% TFA. The hCGRP analog thus purified exhibited a single spot on thin layer chromatography (TLC) and a single peak in analytical HPLC. Its acid hydrolysate gave the amino acids in ratios predicted by theory.

In respect of the Ca-lowering activity in rat serum, this hCGRP analog (80 μg/kg) exhibited much higher activity than the synthetic hCGRP. A similar tendency was also observed in respect of Pi-lowering activity in rat serum (Fig. 3).

Experimental

General experimental procedures described herein are essentially the same as described in the previous synthesis of hCGRP.[4] *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: 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–AcOEt–H_2O (1:1:1:1). HPLC was conducted with a Waters 204 compact model.

MBzl-S-CH_2-CH_2-CO-Asp-Thr-Ala-OMe——A TFA-treated sample of Z(OMe)-Asp(OBzl)-Thr-Ala-OMe[4] (1.00 g, 1.74 mmol) in DMF (30 ml) was hydrogenated over a Pd catalyst for 3 h and the catalyst was removed by filtration. Et_3N (0.53 ml, 3.83 mmol) and MBzl-S-CH_2-CH_2-COOSu (0.68 g, 2.09 mmol) were added to the filtrate and the mixture was stirred for 12 h, then concentrated. The residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. Trituration of the residue with ether afforded a powder which was recrystallized from AcOEt and ether; yield 0.56 g (61%), mp 140—142 °C, $[\alpha]_D^{18}$ −10.2° (c=0.6, DMF), Rf_1 0.47. *Anal.* Calcd for $C_{23}H_{33}N_3O_9S$: C, 52.36; H, 6.30; N, 7.97. Found: C, 52.09; H, 6.44; N, 7.70.

MBzl-S-CH_2-CH_2-CO-Asp-Thr-Ala-$NHNH_2$——The above ester (0.55 g, 0.95 mmol) in DMF–MeOH (1:1, 50 ml) was treated with 80% hydrazine hydrate (0.57 ml, 10 eq) at room temperature for 24 h. The solvent was removed by evaporation and the residue was treated with EtOH to afford a powder, which was recrystallized from DMF and EtOH; yield 0.44 g (87%), mp 178—180 °C, $[\alpha]_D^{18}$ −16.0° (c=1.0, DMF), Rf_1 0.21. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.06, Thr 0.97, Ala 1.00 (recovery of Ala 81%). *Anal.* Calcd for $C_{22}H_{33}N_5O_8S \cdot H_2O$: C, 48.43; H, 6.47; N, 12.84. Found: C, 48.69; H, 6.46; N, 13.17.

Protected Des-1-Ala-des-α-amino-hCGRP, MBzl-S-CH_2-CH_2-CO-(hCGRP 3—37)-NH_2——Z(OMe)-(hCGRP 6—37)-NH_2 (270 mg, 60 μmol) was treated with TFA (3.0 ml) in the presence of anisole (0.3 ml) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF–DMSO (2:1, 5 ml) containing Et_3N (17 μl, 120 μmol). The azide [prepared from 157 mg (298 μmol) of MBzl-S-CH_2-CH_2-CO-Asp-Thr-Ala-$NHNH_2$] in DMF (5 ml) and Et_3N (124 μl, 894 μmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 48 h, was poured into H_2O (200 ml). The resulting powder was washed with MeOH and purified by gel-filtration on Sephadex LH-60 (3.2 × 136 cm) using DMF–DMSO (7:3) as an eluant. The desired fractions corresponding to the front peak (tube Nos. 50—76, 10 ml each, determined by ultraviolet (UV) absorption measurement at 275 nm) were combined and the solvent was removed by evaporation. The residue was treated with AcOEt to form a powder; yield 182 mg (62%), mp 271—273 °C, $[\alpha]_D^{18}$ −19.7° (c=0.3, DMSO), Rf_1 0.42. Amino acid ratios in a 6 N HCl hydrolysate: Asp 4.37, Thr 3.78, Ser 3.10, Pro 0.98, Gly 4.33, Ala 3.67, Val 3.46, Leu 3.36, Phe 2.00, Lys 2.01, His 1.00, Arg 2.11 (recovery of Phe 81%). *Anal.* Calcd for $C_{233}H_{335}N_{49}O_{57}S_4 \cdot 7H_2O$: C, 56.09; H, 7.05; N, 13.76. Found: C, 56.18; H, 6.97; N, 13.96.

Des-1-Ala-des-α-amino-hCGRP——The above protected peptide (80 mg, 16.5 μmol) was treated with 1 M TFMSA-thioanisole in TFA (6.6 ml) in the presence of *m*-cresol (0.34 ml, 200 eq) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 1 h and dissolved in 6 M guanidine–HCl in 0.1 M Tris–HCl buffer (pH 8.0, 4.0 ml) containing 2-mercaptoethanol (0.2 ml). The pH of the solution was adjusted to 8.0 with $MeNH_2$ and the solution was incubated under an argon atmosphere at room temperature for 24 h. This solution was applied to a column of Sephadex G-25 (2.8 × 138 cm), which was eluted with 1 N AcOH. The fractions corresponding to the main peak (tube Nos. 47—70, 9 ml each, determined by UV absorption measurement at 206 nm) were combined and the entire solution was diluted with ice-chilled H_2O to 1000 ml. The pH of the solution was adjusted to 7.5 with 5% NH_4OH and the solution was kept standing at 23 °C for 7 d, during which time the Ellman test values (412 nm)[10] dropped from 0.091 to 0.019. The pH of the solution was adjusted to 5.0 with AcOH and then the solvent and the salt were removed by repeated lyophilization to give a powder; yield 40.2 mg (66%).

The crude air-oxidized product thus obtained was dissolved in 0.02 M $AcONH_4$ buffer (pH 5.8, 3 ml) and the solution was applied to a column of CM-Biogel A (2 × 7 cm), which was eluted first with the same buffer (192 ml) and then with a linear gradient formed from 0.2 M $AcONH_4$ buffer (pH 6.8, 250 ml) through a mixing flask containing the starting buffer (250 ml). The fractions corresponding to the main peak (tube Nos. 69—84, 4 ml each, determined by measuring UV absorption at 206 nm and by the Folin–Lowry test[11]) were combined and the solvent and the ammonium salt were removed by repeated lyophilization to give a fluffy powder; yield 16.1 mg (40%). Subsequent purification was performed by reversed-phase HPLC on a Cosmosil $5C_{18}$ column (10 × 250 mm). A part of the above CM-purified sample (6.0 mg) was dissolved in 0.1% TFA (0.6 ml) and the solution was applied to the column, which was eluted with a gradient of acetonitrile (25 to 40% in 1 h) in 0.1% TFA at a flow rate of 1.8 ml per min (Fig. 4-a). The eluate corresponding to the main peak (retention time 44 min) was collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 1.78 mg. The rest of the sample was similarly purified; total yield 4.8 mg. The over all yield from the protected peptide was 7.8%. $[\alpha]_D^{20}$ −101.5° (c=0.1, 1 N AcOH), Rf_2 0.32, Rf_3 0.59. The retention time was 20 min in HPLC on an analytical Nucleosil $5C_{18}$ column (4 × 150 mm) on gradient elution with acetonitrile (25 to 40%) in 0.1% TFA at a flow rate of 1 ml per min (Fig. 4-b). Amino acid ratios in a 6 N HCl hydrolysate (numbers in parentheses are theoretical): Asp 3.71 (4), Thr 2.88 (4), Ser 2.79 (3), Pro 1.03 (1), Gly 4.35 (4),

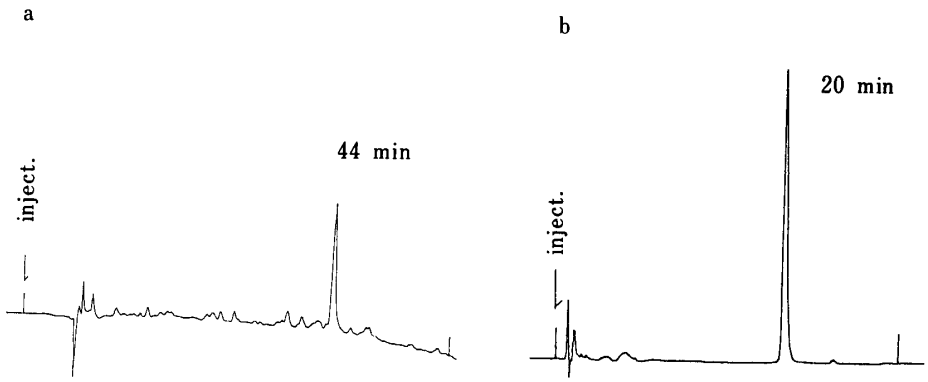

Fig. 4. HPLC of the Synthetic hCGRP Analog

a: CM-purified sample.
b: purified sample.

Cys N. D., Ala 3.13 (3), Val 3.97 (5), Leu 3.13 (3), Phe 2.00 (2), Lys 1.98 (2), His 0.89 (1), Arg 2.15 (2) (recovery of Phe 90%).

References and Notes

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used: Ad = 1-adamantyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Z = benzyloxycarbonyl, Mts = mesitylenesulfonyl, Bzl = benzyl, MBzl = *p*-methoxybenzyl, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid, Su = *N*-hydroxysuccinimidyl, DMSO = dimethylsulfoxide, DMF = dimethylformamide, BSA = bovine serum albumin.
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