Studies on Peptides. CXXXVI.\textsuperscript{1,2} Solution-Phase Synthesis of a 37-Residue Peptide Amide Corresponding to the Entire Amino Acid Sequence of Human Calcitonin Gene-Related Peptide (hCGRP)

Nobutaka Fuji,\textsuperscript{a} Akira Otaka,\textsuperscript{a} Susumu Funakoshi,\textsuperscript{a} Motoyoshi Nomizu,\textsuperscript{a} Kenichi Akaji,\textsuperscript{a} Haruaki Yajima,\textsuperscript{a, \textasteriskcentered} Itsuo Yamamoto,\textsuperscript{b} Kanji Torizuka,\textsuperscript{b} Kouki Kitagawa,\textsuperscript{c} Tadashi Akita,\textsuperscript{c} Kenji Ando,\textsuperscript{c} Tatsuhiko Kawamoto,\textsuperscript{c} Yasutsugu Shimomishii,\textsuperscript{d} and Toshifumi Takao\textsuperscript{d}

Faculty of Pharmaceutical Sciences,\textsuperscript{a} and School of Medicine, Kyoto University,\textsuperscript{b} Sakyoku, Kyoto 606, Japan, Faculty of Pharmaceutical Sciences, Tokushima University,\textsuperscript{c} Tokushima 743, Japan, and Institute for Protein Research, Osaka University,\textsuperscript{d} Suita, Osaka 565, Japan

(Received July 15, 1985)

The heptatriacontapeptide amide corresponding to the entire amino acid sequence of human calcitonin gene-related peptide (hCGRP) was synthesized by assembling seven peptide fragments in solution, followed by deprotection with 1 M trifluromethanesulfonic acid-thioanisole in trifluoroacetic acid and subsequent air-oxidation to establish the disulfide bridge. The S-1-adamantyl group of the Cys residue was removed in two ways; one was by treatment with the above acid, together with the other protecting groups employed, and the other was selectively by treatment with (CF\textsubscript{3}COO)\textsubscript{2}TI. The synthetic peptide produced a significant increase of cyclic adenosine monophosphate in calvaria and brain of a newborn rat, but suppressed \textsuperscript{45}Ca-release from mouse calvaria stimulated by parathyroid hormone.

\textbf{Keywords}—S-1-adamantylcysteine; thallium trifluoroacetate; human calcitonin gene-related peptide synthesis; cysteine sulfoxide; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; cAMP increase; \textsuperscript{45}Ca release

As described in the preceding paper, Morris \textit{et al.}\textsuperscript{3} determined the structure of human calcitonin gene-related peptide (hCGRP) isolated from tissues of patients with medullary thyroid carcinoma. Because of the great physiological interest of this newly found neuropeptide, we undertook the synthesis of the heptatriacontapeptide amide corresponding to the entire amino acid sequence of hCGRP and reported in the preceding paper\textsuperscript{1} the synthesis of seven peptide fragments, [I]—[7], which we selected as the building blocks. Cys(Ad)\textsuperscript{4,5} was applied to the present synthesis for the first time. We wish to report in this paper that we succeeded in obtaining the biologically active peptide with a high degree of purity by successive azide condensation of the above seven peptide fragments, followed by thioanisole-mediated deprotection\textsuperscript{9} of all protecting groups employed with TFMSA in TFA\textsuperscript{7} and subsequent air oxidation to establish the disulfide bridge. Alternatively, the S-Ad group was selectively removed by using (CF\textsubscript{3}COO)\textsubscript{2}TI, after removing other protecting groups by treatment with TFA–thioanisole.\textsuperscript{8} A detailed account of these investigations is presented herein.

The necessary six fragments were assembled successively onto a TFA-treated sample of
Ad
Boc-Ala-Cys-Asp-Thr-Ala-NHNH₂
Ad
Z(OMe)-Thr-Cys-Val-Thr-NHNH₂

Mts
Z(OMe)-His-Arg-Leu-Ala-NHNH₂

Bzl Mts Bzl
Z(OMe)-Ser-Arg-Ser-Gly-NHNH₂

Z
Z(OMe)-Gly-Val-Val-Lys-NHNH₂

Z(OMe)-Asn-Asn-Phe-Val-Pro-Thr-Asn-

Val-Gly-Ser-Lys-Ala-Phe-NH₂

H-Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-
Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-
Asn-Val-Gly-Ser-Lys-Ala-Phe-NH₂

Fig. 1. Synthetic Route to hCGRP

<table>
<thead>
<tr>
<th>Protected peptide</th>
<th>Synthetic hCGRP</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>25—37</td>
<td>21—37</td>
<td>17—37</td>
</tr>
<tr>
<td>Asp</td>
<td>3.15</td>
<td>2.96</td>
</tr>
<tr>
<td>Thr</td>
<td>0.97</td>
<td>0.93</td>
</tr>
<tr>
<td>Ser</td>
<td>1.04</td>
<td>0.96</td>
</tr>
<tr>
<td>Pro</td>
<td>1.10</td>
<td>1.08</td>
</tr>
<tr>
<td>Gly</td>
<td>1.05</td>
<td>1.99</td>
</tr>
<tr>
<td>Ala</td>
<td>1.08</td>
<td>1.00</td>
</tr>
<tr>
<td>Cys</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Val</td>
<td>1.81</td>
<td>2.54</td>
</tr>
<tr>
<td>Leu</td>
<td>2.11</td>
<td>3.41</td>
</tr>
<tr>
<td>Phe</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Lys</td>
<td>1.13</td>
<td>2.04</td>
</tr>
<tr>
<td>His</td>
<td>1.04</td>
<td>1.09</td>
</tr>
<tr>
<td>Arg</td>
<td>0.98</td>
<td>0.96</td>
</tr>
<tr>
<td>Rec. (%)</td>
<td>92.4</td>
<td>93.5</td>
</tr>
</tbody>
</table>

a) Cysteic acid was not calculated.  b) Phe: diagnostic amino acid.

the C-terminal fragment [1] via the azide⁹⁻ as shown in Fig. 1. The amount of the acyl component was increased from 1.5 to 5 eq as the chain elongation progressed. A mixture of DMF-DMSO-HMPA was employed as a solvent in most cases, because of the increasing insolubility of protected intermediates in DMF. The fragment condensation reactions, except for fragment [6], proceeded smoothly as usual without particular difficulty. Condensation of fragment [6] had to be performed at lower temperature (−15 °C) than usual (+4 °C) in order
Boc-(hCGRP 1—37)-NH<sub>2</sub>

1. 1 M TFMSA–thioanisole in TFA, m-cresol (or i) TFA–thioanisole, ii) (CF<sub>3</sub>COO)<sub>Tl</sub>
2. reduction with 2-mercaptoethanol
3. Sephadex G-25 (1 M AcOH)

Crude reduced H–(hCGRP 1—37)-NH<sub>2</sub>

4. air-oxidation at pH 7.5
5. Sephadex G-50 (1 M AcOH)
6. CM-Biogel A
   [gradient elution with 0.02—0.2 M AcONH<sub>4</sub> (pH 5.8—6.8)]
7. HPLC on Nucleosil 5C<sub>18</sub>
   [gradient elution with CH<sub>3</sub>CN (30—35%) in 0.2% TFA]

Purified H–(hCGRP 1—37)-NH<sub>2</sub>

Fig. 2. Deprotection and Purification of H–(hCGRP 1—37)-NH<sub>2</sub>

to minimize Curtius rearrangement<sup>10</sup> of the corresponding azide. Completion of each coupling reaction was checked by means of the ninhydrin test, and protected products, except for two compounds, were purified by precipitation from DMSO with appropriate solvents, such as DMF or MeOH. Gel-filtration on Sephadex LH-60 was found to be effective to purify the two protected peptides, i.e., the protected hCGRP and the protected dotriacontapeptide amide [Z(OMe)—(hCGRP 6—37)-NH<sub>2</sub>]. Throughout this synthesis, Phe was used as a diagnostic amino acid in amino acid analysis. Each intermediate was subjected to acid hydrolysis and the recovery of Phe was compared with those of newly added amino acids in order to ascertain satisfactory incorporation, after each condensation (Table I). The homogeneity of every intermediate was further ascertained by elemental analysis and thin layer chromatography (TLC).

We examined whether or not the two Cys(Ad) residues were partially oxidized to the corresponding sulfoxides during the synthesis. The protected hCGRP was subjected to 6 N HCl hydrolysis in the presence of phenol. No S-p-hydroxyphenylcysteine<sup>11</sup> was detected on a short column of an amino acid analyzer indicating that no air-oxidation had taken place at the Cys residues in the protected hCGRP. In the final step of the synthesis, deprotection was carried out in two ways as described above, i.e., in one step by 1 M TFMSA–thioanisole in TFA, or in two steps by TFA–thioanisole, followed by (CF<sub>3</sub>COO)<sub>Tl</sub>.

In the first instance, deprotection and subsequent purification were carried out according to the following scheme (Fig. 2). The protected hCGRP was treated with 1 M TFMSA–thioanisole (molar ratios 1 : 1) in TFA in the presence of m-cresol in an ice-bath for 3 h, a little longer time than usual, to ensure the complete deprotection of all protecting groups employed. The deprotected peptide was reduced with 2-mercaptoethanol in 6 M guanidine–HCl in 0.1 M Tris–HCl buffer at pH 8.0. This thiol treatment seems to be effective to prevent the possible disulfide aggregation and the above basic conditions are considered to reverse the possible N→O shift at the Ser and Thr residues.<sup>12</sup> After removal of the reducing reagent and salts by gel-filtration on Sephadex G-25, the product was subjected to air-oxidation to form a disulfide bridge between the two Cys residues. A diluted solution (peptide concentration 74.5 mg/l) in 0.08 M AcONH<sub>4</sub> buffer (pH 7.5) was kept standing at 25°C and the progress of the reaction was monitored by using the Ellman test.<sup>13</sup> After 2 d, the values became constant. The crude product obtained after lyophilization was purified by gel-filtration on Sephadex G-50 using 1 M AcOH as an eluant. The chromatographic pattern indicated that little polymer
formed during the air oxidation. The product was next purified by ion-exchange chromatography on CM-Biogel A using gradient elution with 0.02—0.2 M AcONH₄ (pH 5.8—6.8) and finally by reversed-phase high performance liquid chromatography (HPLC) on a Nucleosil 5C₁₈ column using gradient elution with acetonitrile in 0.2% 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 purified 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.

In the 2nd experiment, the protected hCGRP was first treated with TFA-thioanisole in the presence of m-cresol at 20°C for 24 h to remove the Bzl-based protecting groups (Z and Bzl) and the Mts group from the Arg residues. This thioanisole-mediated deprotection was repeated once more to ensure complete deprotection. The S-Ad group was next removed by treatment with (CF₃COO)₂TI in TFA in an ice-bath for 90 min. The deprotected peptide was incubated with 2-mercaptoethanol as described above to generate the SH group from the S-TI salt. Subsequent air-oxidation and purification were carried out in essentially the same manner as described in the former experiment. The yield obtained here (8.3% from the protected peptide) was somewhat less than that of the former experiment (17.3%).

We suppose that the TFA–thioanisole deprotection, even though it was repeated twice, was not acidic enough to cleave the many protecting groups employed. From the yields obtained here, it seems premature to conclude that the S-Ad group has superior properties to the S-MBzl group in all respects. We intend to continue further evaluation of the S-Ad group by synthesizing several other Cys-containing peptides.

Synthetic hCGRP (2 × 10⁻⁷ M) increased cyclic adenosine monophosphate (cAMP) in monolayer-cultured cells from kidney, liver, calvaria and brain of newborn rats. The cells from brain and calvaria showed significantly higher response (approximately 5 times) than was obtained with [Asu¹⁷]-eel calcitonin (Fig. 3). Next, the effects on ⁴⁵Ca release from mouse calvaria were examined. Synthetic hCGRP (1 × 10⁻⁷ M) suppressed the ⁴⁵Ca release induced by human parathyroid hormone (hPTH, 1—34) (Fig. 4).

Fig. 3. Effects of Synthetic hCGRP on Adenyl Cyclase Activity
1, hCGRP; 2, Asu¹⁷-eel calcitonin; 3, hPTH (1—34)

Fig. 4. Effects of Synthetic hCGRP on ⁴⁵Ca Release
1, hPTH(1—34); 2, hCGRP; 3, hPTH(1—34)+hCGRP; 4, hPTH(1—34)+Asu¹⁷-eel calcitonin.
Experimental

General experimental procedures employed in this investigation were essentially the same as described in the preceding paper. TLC was performed on silica gel (Kieselgel G, Merck). Rf values refer to the following (v/v) solvent systems: Rf1, CHCl3-MeOH-H2O (8:3:1); Rf2, n-BuOH-AcOH-pyridine-H2O (4:1:1:2); Rf3, n-BuOH-AcOH-pyridine-H2O (3:6:20:24). HPLC was conducted with a Waters 204 compact model. FAB-MS spectra were obtained on a JEOL JMS-HX 100 double-focus mass spectrometer equipped with a FAB ion source, and a data processor (JEOL JMA-3100). Leucine aminopeptidase (LAP, Lot No. 1-6607) was purchased from Sigma.

Z(O-Me)-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH2, Z(O-Me)-(hCGRP 21—37)-NH2—Z(O-Me)-(hCGRP 25—37)-NH2 (1.67 g, 0.94 mmol) was treated with TFA (5.0 ml) in the presence of anisole (1.0 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 then dissolved in DMF-DMSO (1:1, 10 ml) containing Et3N (0.13 ml, 0.94 mmol). The azide [prepared from 1.21 g (1.69 mmol) of Z(O-Me)-(hCGRP 21—24)-NHNH2 in DMF-DMSO (1:1, 5.0 ml) and Et3N (0.24 ml, 1.69 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° C for 14 h. H2O (100 ml) was added to form a powder, which was washed with H2O and precipitated from DMSO with DMF; yield 1.66 g (77%), mp 289—291° C, [α]D20 +17.4° (c=0.9, HMPA), Rf0 = 0, Rf5 = 0. Anal. Caled for C111H155N32O26: C, 59.02; H, 6.79; N, 14.01. Found: C, 58.72; H, 7.01; N, 14.11.

Z(O-Me)-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH2 —Z(O-Me)-(hCGRP 17—37)-NH2 —The above protected heptadecapeptide amide, Z(O-Me)-(hCGRP 21—37)-NH2 (1.66 g, 0.72 mmol) was treated with TFA-anisole (10 ml-1.5 ml) in an ice-bath for 2 h, then dry ether was added. The resulting powder isolated as described above was dissolved in HMPA-DMF-DMSO (1:1:1, 20 ml) containing Et3N (0.1 ml, 0.72 mmol). The azide [prepared from 1.37 g (1.45 mmol) of Z(O-Me)-(hCGRP 17—20)-NHNH2 in DMF (10 ml) and Et3N (0.20 ml, 1.45 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4° C for 14 h, was mixed with H2O (100 ml). The resulting powder isolated as described above and finally precipitated from DMSO with MeOH; yield 2.10 g (97%), mp 284—286° C, [α]D20 —14.0° (c=0.7, HMPA), Rf0 = 0.49. Anal. Caled for C150H232N38O57S: C, 59.08; H, 6.68; N, 13.78. Found: C, 58.78; H, 6.87; N, 13.68.

Z(O-Me)-Gly-Leu-Leu-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH2 —Z(O-Me)-(hCGRP 14—37)-NH2 —The above protected eicosapeptide amide, Z(O-Me)-(hCGRP 17—37)-NH2 (2.14 g, 0.70 mmol) was treated with TFA-anisole (10 ml-2.0 ml) and the Nα-deprotected peptide isolated as described above was dissolved in HMPA-DMF-DMSO (1:1:1, 20 ml) containing Et3N (98 µl, 0.70 mmol). The azide [prepared from 0.84 g (1.75 mmol) of Z(O-Me)-(hCGRP 14—16)-NHNH2 in DMF (8 ml) and Et3N (0.24 ml, 1.75 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° C for 18 h. H2O (200 ml) was added to form a powder, which was washed with H2O and precipitated from DMSO with MeOH; yield 2.71 g (93%), mp 284—287° C, [α]D20 —22.9° (c=0.5, DMSO), Rf0 = 0.66, Rf5 = 0.79. Anal. Caled for C164H225N35O56S: C, 58.16; H, 6.89; N, 13.65. Found: C, 58.04; H, 7.07; N, 13.71.

Z(O-Me)-His-Arg(Mts)-Leu-Ala-Gly-Leu-Leu-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH2 —Z(O-Me)-(hCGRP 10—37)-NH2 —The above protected tetrasacopeptide amide, Z(O-Me)-(hCGRP 14—37)-NH2 (2.17 g, 0.65 mmol) was treated with TFA-anisole (10 ml-2.0 ml) and the Nα-deprotected peptide isolated as described above was dissolved in HMPA-DMF-DMSO (1:1:1, 20 ml) containing Et3N (91 µl, 0.65 mmol). The azide [prepared from 1.67 g (1.96 mmol) of Z(O-Me)-(hCGRP 10—13)-NHNH2 in DMF (5 ml) and Et3N (0.27 ml, 1.96 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° C for 14 h. H2O (100 ml) was added and the resulting powder was purified as described above by precipitation from DMSO with MeOH; yield 2.49 g (96%), mp 275—274° C, [α]D20 —19.7° (c=0.8, DMSO), Rf0 = 0.42, Rf5 = 0.79. Anal. Caled for C194H271N43O46S2·14H2O: C, 54.89; H, 7.16; N, 13.86. Found: C, 55.05; H, 7.16; N, 13.48.

Z(O-Me)-Thr-Cys(Ad)-Val-Thr-His-Arg(Mts)-Leu-Ala-Gly-Leu-Leu-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-Phe-NH2 —Z(O-Me)-(hCGRP 6—37)-NH2 —The above protected octacapeptide amide, Z(O-Me)-(hCGRP 10—37)-NH2 (1.02 g, 0.26 mmol) was treated with TFA-anisole (10 ml-1.0 ml) and the Nα-deprotected peptide isolated as described above was dissolved in DMF-DMSO (2:1, 10 ml) containing Et3N (71 µl, 0.51 mmol). The azide [prepared from 0.57 g (0.77 mmol) of Z(O-Me)-(hCGRP 6—9)-NHNH2 in DMF (5 ml) and Et3N (0.11 ml, 0.77 mmol) were added to the above ice-chilled solution and the mixture was stirred at −15° C for 48 h. H2O (200 ml) was added to form a powder, which was purified by gel-filtration on Sephadex LH-60 (3.2 x 136 cm) with DMF-DMSO (7:3) as an eluant. The fractions corresponding to the main peak (9 ml each, identified by measuring the ultraviolet (UV) absorption at 275 nm) were combined and the solvent was removed by evaporation. The residue was treated with AcOEt to form a powder; yield 0.93 g (80%), mp 300° C (dec), [α]D20 —10.1° (c=0.5, DMSO), Rf5 = 0.51. Anal. Caled for C226H331N45O53S2·6H2O: C, 56.95; H, 7.08; N, 13.89. Found: C, 57.13; H, 7.13; N, 13.66.

Boc-Ala-Cys(Ad)-Asp-Thr-Ala-Thr-Cys(Ad)-Val-Thr-His-Arg(Mts)-Leu-Ala-Gly-Leu-Leu-Ser(Bzl)-Arg(Mts)-Ser(Bzl)-Gly-Gly-Val-Val-Lys(Z)-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser(Bzl)-Lys(Z)-Ala-
Phe-\(\text{NH}_2\), Boc-(hGRP 1–37)-\(\text{NH}_2\) — The above protected dotriaccontapeptide amide, Z(OMe)-hGRP 6–37-\(\text{NH}_2\) (0.50 g, 0.11 mmol) was treated with TFA-anirole (5.0 ml–0.5 ml) and the N\(^{+}\)-deprotected peptide isolated as described above was dissolved in DMF-DMSO (2:1, 5 ml) containing Et\(_3\)N (46 \(\mu\)l, 0.33 mmol). The azide [prepared from 0.40 g (0.55 mmol) of Boc-(hGRP 1–5)-\(\text{NHNH}_2\)J in DMF (5 ml) and Et\(_3\)N (77 \(\mu\)l, 0.55 mmol) were added to the above ice-chilled solution. The mixture was stirred at 4 °C for 48 h, then \(\text{H}_2\)O (200 ml) was added. The resulting powder was purified by gel-filtration on Sephadex LH-60 (3.2 x 136 cm) with DMF-DMSO (7:3) as an eluant. The desired fractions were collected as described above and the solvent was removed by evaporation. The residue was treated with AcOEt to form a powder; yield 0.48 g (86%), mp 298–300 °C, \([\chi]^{\text{D}}_\text{D} +18.1^\circ\) (c = 0.5, DMSO), \(R_f\) 0.52. Anal. Calcd for C\(_{243}\)H\(_{339}\)N\(_5\)O\(_9\)S\(_4\)·7\(\text{H}_2\)O: C, 56.24; H, 7.17; N, 13.77. Found: C, 55.95; H, 7.04; N, 14.05.

H-(hGRP 1–37)-\(\text{NH}_2\) — (A) The above protected heptatriacontapeptide amide, Boc-(hGRP 1–37)-\(\text{NH}_2\) (100 mg, 19.7 \(\mu\)mol) was treated with 1 M TFMSA-thioanisole in TFA (7.9 ml) in the presence of \(m\)-cresol (0.41 ml, 200 eq) in an ice-bath for 3 h, then dry ether was added. The resulting powder was collected by centrifugation and dried over KOH pellets in vacuo for 30 min. The deprotected peptide thus isolated was dissolved in 6 M HCl-guanidine in 0.1 M Tris-HCl buffer (pH 8.0, 2.0 ml) containing 2-mercaptopethanol (0.2 ml). The pH of the solution was adjusted to 8.0 with 5% MeNH\(_2\) and the solution was incubated under an argon atmosphere at room temperature for 24 h. The solution was applied to a column of Sephadex G-25 (2.8 x 79 cm), which was eluted with 1 N AcOH. The ultraviolet (UV) absorption at 206 nm was determined in each fraction (6.2 ml each). The fractions corresponding to the main peak (tube Nos. 32–44, also checked by the Folin-Lowry test) were combined and the entire solution was diluted with ice-chilled \(\text{H}_2\)O to 1000 ml. The pH of the solution was adjusted to 7.5 with 5% \(\text{NH}_3\)OH and the solution was kept standing at 25 °C for 50 h, during which time, the Ellman test value (412 nm) dropped from 0.084 to 0.004. The pH of the solution was adjusted to 5.0 with conc. AcOH and the solvent was removed by lyophilization. The crude product thus obtained was dissolved in 1 N AcOH and the solution was applied to a column of Sephadex G-50 (2 x 116 cm), which was eluted with the same solvent. The UV absorption at 206 nm was determined in each fraction (7.2 ml). The fractions corresponding to the main peak (tube Nos. 41–52, also checked by the Folin-Lowry test) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 42.6 mg (57%).

The crude air-oxidized product thus obtained was dissolved in pH 5.8, 0.02 M AcON\(_2\)H buffer and the solution was applied to a column of CM-Biogel A (2 x 7 cm), which was first eluted with the same buffer (260 ml) and then with a linear gradient formed from pH 6.8, 0.2 M AcON\(_2\)H buffer (250 ml) through a mixing flask containing the starting buffer (250 ml). Each fraction (4 ml) was examined for UV absorption at 206 nm and by the Folin-Lowry test. A broad peak with shoulders was detected (Fig. 5). Fractions corresponding to the main peak (tube Nos. 96–105) were combined and the solvent and ammonium salt were removed by repeated lyophilization to give a fluffy powder; yield 25.7 mg (60%).

Subsequent purification was performed by reversed-phase HPLC on Nucleosil 5C\(_18\) (1.0 x 25 cm column). The above CM-purified sample (12 mg) was dissolved in 0.2% TFA. Portions of the solution (4 mg, 500 \(\mu\)l each) were applied to the above column, which was eluted with CH\(_3\)CN (30%) in 0.2% TFA for 50 min and then with a gradient of CH\(_3\)CN (30% to 35%) in 1 h at 0.2% TFA at a flow rate of 2.0 ml per min. (Fig. 6). The eluate corresponding to the main peak (retention time 100 min) was collected and the solvent was removed by lyophilization. The residue was dissolved in 0.5 N AcOH and the solution was applied to a column of Sephadex G-25, which was eluted with the same solvent. The desired fractions were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 5.97 mg (49%). The rest of the sample was similarly purified; total yield from the protected hGRP; 12.5 mg.

---

**Fig. 5.** Purification of Synthetic H-(hGRP 1–37)-\(\text{NH}_2\) by Ion-Exchange Chromatography on CM-Biogel A

**Fig. 6.** HPLC Purification of CM-Purified Sample
Fig. 7. HPLC and Disk Isoelectrofocusing of Purified Sample

a, HPLC of purified hCGRP; b, disk isoelectrofocusing of purified hCGRP.
Column: Nucleosil 5C18 (4×150 mm). Solvent: CH3CN:0.2% TFA (30:70).
Flow rate: 0.7 ml/min.

The synthetic peptide exhibited a single band in disk isoelectrofocusing (Fig. 7-b) on 7.5% polyacrylamide gel (0.5×6.3 cm) containing Pharmalyte (pH 3—10): mobility, 5.2 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 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 aminopeptidase digestion: Asp 0.95 (1), Thr 3.75 (4), Ser 2.87 (3), Pro 1.01 (1), Gly 3.77 (4), Ala 3.99 (4), Cys 0.66 (1), Val 4.20 (5), Leu 2.90 (3), Phe 2.00 (2), Lys 1.90 (2), His 0.81 (1), Arg 1.71 (2). A sample was not determined (recovery of 74%).

(B) Boc-(hCGRP 1—37)–NH₂ (50 mg, 9.8 μmol) was treated with TFA (7 ml) in the presence of thioanisole (0.46 ml, 400 eq) and m-cresol (0.41 ml, 400 eq) at 20°C for 24 h, then dry ether was added. The resulting powder was re-treated with TFA–thioanisole under identical conditions and precipitated with ether. The S-protected peptide thus obtained was next treated with (CF₃COO)₂Ti (26.8 mg, 5 eq) in TFA (2 ml) in the presence of m-cresol (0.21 ml, 20 eq) in an ice-bath for 90 min and dry ether was added. The deprotected peptide thus obtained was dissolved in 6 M HCl–guanidine in 0.1 M Tris–HCl buffer (pH 8.0, 2 ml) containing 2-mercaptoethanol (0.4 ml). The pH of the solution was adjusted to 8.0 with 5% MeNH₂ and the solution was incubated under an argon atmosphere at room temperature for 14 h as described above. The reduced product was separated from the reducing reagent by gel-filtration on Sephadex G-25 using 1 N AcOH as an eluant and then subjected to air-oxidation in dilute AcONH₄ buffer at pH 7.5. The solution was kept standing at 25°C for 3 days, during which time the Ellman test value dropped from 0.072 to 0.003. The solution, after being adjusted to pH 5.0 with conc. AcOH, was lyophilized.

The air-oxidized product was next purified in essentially the same manner as described in A. 1. Gel-filtration on Sephadex G-50 (2×116 cm) using 1 N AcOH, followed by lyophilization; yield 19.8 mg (53%). 2. Ion-exchange
chromatography on CM-Biogel A (1 x 10.2 cm) by gradient elution with 0.02 M (pH 5.8) and 0.2 M (pH 6.8) AcONH$_4$ buffers, followed by repeated lyophilization; yield 4.8 mg (31%). 3. Reversed-phase HPLC on Nucleosil 5C$_{18}$ (1.0 x 2.5 cm column) using a gradient of CH$_3$CN (30% to 55% in 1 h) in 0.2% TFA, followed by gel-filtration on Sephadex G-25 using 0.5 M AcOH and subsequent lyophilization; yield 1.9 mg (51%), total yield from the protected hCGRP (8.4%). The product obtained here exhibited identical $R_f$ values on TLC and a retention time on HPLC with those of the product obtained in A. No thallium contamination in the synthetic peptide was detected by atomic absorption analysis.

**Bioassay**—Adenyl Cyclase Activity$^{46}$: Cells from the kidney, calvaria, liver and brain of a newborn rat were cultured in a multi-well plastic dish for 4 d. Increase in cAMP was examined after addition of synthetic hCGRP (2 x 10$^{-7}$ M), hPTH 1-34 (2 x 10$^{-7}$ M), or [Asu$_{1-7}$]-eel calcitonin (2 x 10$^{-7}$ M) in the presence of 1 mM isobutyl methylxanthine. Data are expressed as ratios to the basal value. Basal values for the kidney cells, calvaria cells, liver cells and brain cells were 1.06 ± 1.06, 0.55 ± 0.04, 0.45 ± 0.10 and 0.30 ± 0.15 pmol/well, respectively. hCGRP increased cAMP in all of these cells, as shown in Fig. 3.

$^{45}$Ca Release Assay$^{47}$: Effects on bone were assayed by measuring $^{45}$Ca release into the culture medium from mouse calvaria prelabeled with $^{45}$Ca. $^{45}$CaCl$_2$ (New England Nuclear, MA, U.S.A.) was injected into 2-d-old mouse and the parietal bones were dissected out at 48 h after the injection. These bones were incubated in a 24-well plastic dish (2 cm$^2$/well, Corning, NY, U.S.A.) with HAM F-12 medium (Nissui, Tokyo, Japan) supplemented with 10% horse serum (GIBCO, NY, U.S.A.). At 24 h, the medium was replaced with fresh medium containing peptides for the test and the culture was continued for a further 48 h. After the incubation, radioactivities of $^{45}$Ca released were measured in a $\beta$ counter (Packard, ILL, U.S.A.). Bone resorptive activity was expressed as percent $^{45}$Ca release from the mouse calvaria. As shown in Fig. 4, $^{45}$Ca release and hCGRP decreased $^{45}$Ca release to 11.8% compared to the value (17.0%) in the control, while $^{45}$Ca release (1-34) (Toyo Jozo, Shizuoka, Japan) increased $^{45}$Ca release to 38.6%. Furthermore, $^{45}$Ca release induced by PTH as potently as $^{45}$Ca release from $^{45}$Ca release analog (Asu$_{1-7}$-ecl calcitonin).

**Acknowledgement** The authors are grateful to Dr. Mitsunobu Kitamura, Environmental Preservation Center, Kyotou University, for atomic absorption of our synthetic peptide.

**References and Notes**


2) Amino acids and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z = benzoylcarbonyl, Z(OMe) = p-methoxybenzoylcarbonyl, Boc = tert-butoxycarbonyl, Ad = 1-adamantyl, MBzl = p-methoxybenzyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, DMF = dimethylformamide, DMSO = dimethylsulfoxide, HMPA = hexamethylenphosphoramid, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid.


15) [Asu$_{1-7}$]-ecl calcitonin was purchased from Toyo Jozo Ltd.
