Synthesis of μ-Conotoxin GIIIA: A Chemical Probe for Sodium Channels[1)]

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μ-Conotoxin GIIIA, a 22 amino acid peptide paralytic toxin which inhibits the muscle voltage-activated sodium channels, was synthesized by a solid phase method. No purification of intermediates was necessary for the synthesis, and a simple air oxidation of the deprotected crude peptide gave the desired toxin. By all criteria applied, the synthetic material was indistinguishable from the authentic natural toxin.

Keywords μ-conotoxin; peptide toxin; sodium channel; peptide synthesis; disulfide bond formation

Voltage-sensitive sodium channels are found in virtually all multicellular animals and play a key role in signal transmission along nerve and muscle membranes. Since the complete amino acid sequence of eel sodium channel was first elucidated,[2)] more defined approaches at the molecular level have become feasible to investigate the structural features which are essential for the expression of functions. A number of specific neurotoxins are useful molecular probes for chemical studies of the sodium channels.[3)] Tetrodotoxin and saxitoxin bind to the sodium channel with high affinity and block the entry of sodium ions through the membrane. We already have demonstrated the specific photolabeling of eel sodium channel protein with a tetrodotoxin derivative.[4)] However, difficulty in the derivatization of the toxin[5)] and low yields of the photolabeling[6)] seriously hampered the chemical use of these toxins on a relatively large scale. μ-Conotoxins (geographutoxins) are peptide toxins isolated from *Conus geographus*[7)] and share a common binding site with tetrodotoxin and saxitoxin on muscle sodium channels.[8)] The availability of the toxin has been limited because it has to be purified from the venom of small snails. It will therefore provide a useful and powerful probe for sodium channel research if μ-conotoxins can be synthesized in quantity. In the present paper, we describe the synthesis and characterization of μ-conotoxin GIIIA (geographutoxin I), which is usually the major component of the μ-conotoxin family.

μ-Conotoxin GIIIA **1** is a peptide amide with 22 amino acid residues, strongly basic (with a net ionic charge of +6), and cross-linked by three disulfide bridges whose structure is not yet known (Chart 1). Solid-phase synthesis of **1** was performed by using the conventional *tert*-butyloxycarbonyl (Boc) protocol. Cleavage from the resin with hydrogen fluoride gave a crude peptide with acetamidomethyl protection on Cys in 89% yield and of 67% purity as judged by high performance liquid chromatography (HPLC) (Fig. 1a). Amino acid analysis of the main peak component gave a result consistent with the composition expected. The crude peptide could be stored at least for two months at room temperature, and used in the next step without further purification. Removal of the acetamidomethyl protecting group was achieved in the usual way.[9)] A mild air oxidation of the deprotected crude peptide at room temperature at pH 7.5 for 50 h yielded a crude mixture of oxidized peptides (Fig. 1b). Isolation and purification of the main peak eluting at *ca.* 20 min gave a highly purified peptide (>98%) in 11% yield from the S-protected crude peptide. The positive ion fast atom bombardment mass spectrum (FAB-MS) of this peptide showed the expected molecular ion peak. In order to detect cysteine residues within the peptide,[10)] this compound was then pyridylethylated. The result of amino acid analysis of the pyridylethylated derivative was consistent with the theoretical values (Asp, 2.09; Thr, 0.99; Glu, 2.13; Ala, 1.07; Lys, 3.76; Cys, 5.51; Arg, 3.00; Hyp, 2.95). Sequence analysis of this derivative confirmed the proposed primary structure of **1**. The synthetic toxin **1**[11)] was shown to be identical with natural μ-conotoxin GIIIA by HPLC (Fig. 1c). Finally, binding activity of the synthetic material to eel electroplax membrane was indistinguishable from that of the authentic

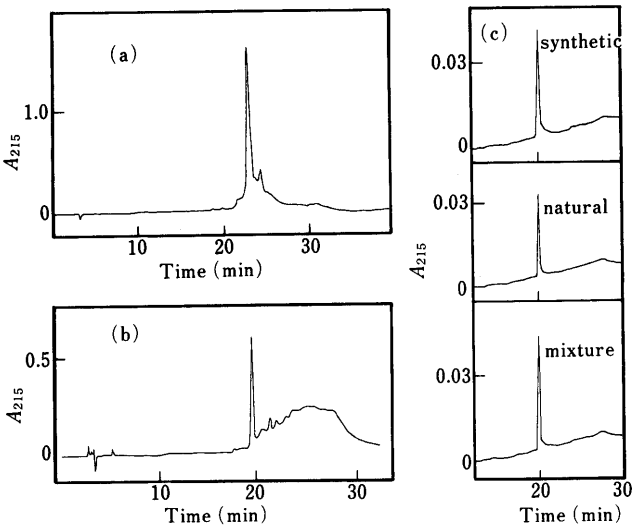

Fig. 1. HPLC Profiles of Synthetic Products

(a) HF-reaction products. (b) Air-oxidized products. (c) HPLC comparison of synthetic toxin, natural μ-conotoxin GIIIA and *ca.* 1 : 1 mixture of both samples. All HPLC analyses were performed using 0.1% trifluoroacetic acid as the solvent, applying a linear gradient of acetonitrile (0—15%) in 20 min at a flow rate of 1 ml/min.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
H–Arg–Asp–Cys–Cys–Thr–Hyp–Hyp–Lys–Lys–Cys–Lys–Asp–Arg–Gln–Cys–Lys–Hyp–Gln–Arg–Cys–Cys–Ala–NH_2

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Chart 1. Primary Structure of μ-Conotoxin GIIIA **1**

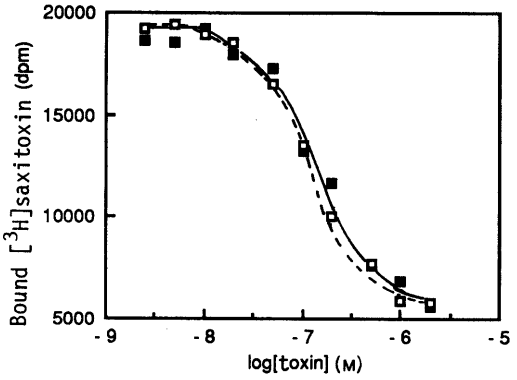

Fig. 2. Inhibition of [^{3}H]Saxitoxin Binding by Synthetic and Natural μ-Conotoxin GIIIA

—■— natural; ---□--- synthetic. The experiment was performed by the incubation of various concentrations of each toxin with 3 mg/ml of eel electroplax membrane protein and 4.4 nM [^{3}H]saxitoxin (specific activity 21 Ci/mmol) at 0°C for 30 min.

natural toxin (Fig. 2).

Thus, by all criteria applied, the synthetic material was shown to be identical with the authentic natural toxin. We conclude that μ-conotoxin GIIIA has been successfully synthesized in an overall yield of 10% from the starting resin.[12] Although the secondary structure of the toxin is unknown, it is worth noting that the natural toxin is the major product of the oxidation. This suggests that the thermodynamically favored conformer leads to the formation of the μ-conotoxin GIIIA by air oxidation in solution. Use of air oxidation to afford the desired product was reported for the synthesis of ω-conotoxin, which also contains three intramolecular disulfide bridges.[13] Natural μ-conotoxin GIIIA is relatively difficult to obtain because *Conus geographus* venom is not commercially available. The availability of the synthetic toxin described in the present paper will make it a much more generally useful probe for chemical studies of sodium channel structure. By using the toxin prepared by the present method, photoactivable derivatives of μ-conotoxin GIIIA are being synthesized as new photoaffinity labeling reagents for sodium channels, and its application for mapping the toxin binding site within the eel sodium channel is currently under way.

Experimental

Spectrometers used in this study were as follows; MS, JEOL JMS-HX 110; ^{1}H-nuclear magnetic resonance (^{1}H-NMR), JEOL JNM-GX 270. HPLC was performed with a Waters HPLC system using Chemcosorb columns (Chemco Scientific Co.; 7 μm particles, 80 Å pores, 20 × 250 mm for preparative separation, and 4.6 × 250 mm for analytical HPLC). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 20 h) were determined with a Hitachi 835 amino acid analyzer. Sequence analysis was carried out with an Applied Biosystems 477A sequencer equipped with an on-line PTH 120A analyzer.

Synthesis of μ-Conotoxin GIIIA Protection of α-amino groups was accomplished throughout synthesis with Boc. Amino acid side chains were protected as follows: Arg(tosyl), Asp(O-benzyl), Cys(S-acetamidomethyl), Hyp(O-benzyl), Lys(2-chlorobenzyloxycarbonyl), and Thr(O-benzyl). Glutamine was incorporated into the peptide with an unprotected side chain. Stepwise build-up of the peptide on paramethyl-benzhydrylamine resin (1.20 g, 0.42 m eq/g) was performed automatically on an Applied Biosystems model 430A peptide synthesizer, according to the manufacturer's instructions. A 4-fold excess of protected amino acids was used based on the original substitution of the resin for each coupling cycle, and recouplings were automatically performed at Arg and Gln. The amount of peptide resin finally obtained was 3.29 g.

Cleavage from the Resin The peptide resin (3.29 g) was treated with 30 ml of distilled anhydrous HF for 1 h at 0 °C in the presence of 3 ml of anisole. After removal of HF under reduced pressure, the resin was washed with 600 ml of anhydrous diethyl ether. The peptide was extracted from the resin with 1% acetic acid. After gel filtration on a 1.6 × 50 cm column of Sephadex G-10 using 0.1% acetic acid as the eluent, fractions containing the peptide were combined and lyophilized to give 1.36 g of crude S-protected peptide. Part of this sample was purified on an analytical C_{18} HPLC column using the conditions described in Fig. 1. Amino acid analysis gave the following ratios with expected values in parentheses: Asp (2), 1.99; Thr (1), 0.95; Glu (2), 2.11; Ala (1), 1.05; Lys (4), 4.09; Arg (3), 3.00; Hyp (3), 2.99. Acetamidomethyl-Cys was not detected.

Removal of Acetamidomethyl Group and Disulfide Bridge Formation The crude S-protected peptide (304 mg) was dissolved in 10 ml of 50% aqueous acetic acid and crystalline mercuric acetate (1.91 g, 6 mmol) was added to this solution. After standing for 24 h at room temperature, β-mercaptoethanol (23.4 g, 0.3 mol) was added. The mixture was stirred for 24 h at room temperature in an argon atmosphere. The mixture was washed three times with benzene (100 ml × 3) to remove most of the excess β-mercaptoethanol and the benzene layer was removed by decantation. The aqueous layer was centrifuged (3000 rpm, 5 min) to obtain a clear solution. The remaining mercuric ion and β-mercaptoethanol in the solution were then removed by chromatography on a 1.6 × 50 cm column of Sephadex G-10 using 0.1% acetic acid as the eluent. The fractions containing peptide were combined and lyophilized to give a crude deprotected peptide (227 mg). The crude deprotected peptide (52 mg) was dissolved in H_2O (10 ml) containing β-mercaptoethanol (938 mg, 12 mmol) and the solution was adjusted to pH 8.5 with aqueous ammonium hydroxide. The solution was stirred for 14 h at room temperature in an argon atmosphere.[14] After evaporation of β-mercaptoethanol under reduced pressure, the residue was dissolved in 200 ml of H_2O and the solution was adjusted to pH 7.5 with aqueous ammonium hydroxide. The solution was allowed to air-oxidize with stirring at room temperature for 50 h. The reaction mixture was concentrated and subjected to purification by HPLC. The crude product was first partially purified on a preparative chromatographic system using 0.1% trifluoroacetic acid as a solvent, applying a linear gradient of acetonitrile (10—12.5%) in 20 min at a flow rate of 5 ml/min. The toxin-enriched fraction was further purified on an analytical C_{18} HPLC column using the conditions described in Fig. 1 to yield 6.8 mg of μ-conotoxin GIIIA. FAB-MS *m/z*: 2609 (MH^+).

Preparation of S-Pyridylethyl Derivative of μ-Conotoxin The purified synthetic toxin (1 mg, 0.4 μmol) was dissolved in 100 μl of 0.1 M Tris–HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.5, containing 1 M β-mercaptoethanol (0.1 mmol). After reduction for 17 h at room temperature, 4-vinylpyridine (108 μl, 1 mmol) was added and the reaction mixture was mixed occasionally for 3 h at room temperature. Excess reagent was extracted with benzene and the aqueous layer was chromatographed on a 0.5 × 8 cm column of Sephadex G-10 using 0.1% acetic acid as the eluent. The crude pyridylethylated product was further purified by analytical HPLC using 0.1% trifluoroacetic acid as a solvent, applying a linear gradient of acetonitrile (5—15%) in 15 min at a flow rate of 1 ml/min. The pure sample obtained was subjected to amino acid analysis and sequence determination. The yield of pyridylethylation was determined as 32% from the result of amino acid analysis.

Binding Experiment Preparation of the membrane suspension from *Electrophorus electricus* electric organ was carried out as described.[15] The membrane suspension was diluted four times with 50 mM potassium phosphate buffer, 5 mM EDTA, pH 7.5 and used for binding studies. Assays were performed by incubation of the membrane (450 μl, 3 mg of protein/ml) with 4.4 nM [^{3}H]saxitoxin (Amersham, specific activity 21 Ci/mmol) at 0 °C for 30 min, in the presence of various concentrations of natural or synthetic μ-conotoxin. After centrifugation (12000 rpm) of the incubation mixture for 5 min at 4 °C, 0.2 ml aliquots were taken from supernatants for counting of the radioactivity of unbound [^{3}H]saxitoxin. Amount of the bound [^{3}H]saxitoxin was obtained by subtracting the amount of unbound toxin from the total [^{3}H]saxitoxin present.

Acknowledgements We are grateful to Drs. T. Abe and M. Satake, Brain Research Institute, Niigata University, for a generous gift of natural toxin for comparison. This work was supported by grants from the Ministry of Education, Science and Culture, the Mitsubishi Foundation, the Toray Science Foundation, and the Fujisawa Foundation.

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