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Ancorinoside A Mg salt from the marine sponge, *Ancorina* sp., which specifically inhibits blastulation of starfish embryos

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Abstract—A new tetramic acid Mg salt, ancorinoside A Mg salt, has been isolated from the marine sponge *Ancorina* sp. together with the known ancorinoside A. The structure of ancorinoside A Mg salt has been established on the basis of spectroscopic and chemical investigations and chemical correlations. Ancorinoside A Mg salt potently inhibited blastulation of the starfish (*Asterina pectinifera*) embryo.

1. Introduction

In fertilized eggs of the starfish (*Asterina pectinifera*), cell division proceeds rapidly and almost synchronously without growth for a total of eight or nine cleavages. Completion of this rapid cleavage period is followed by the immediate activation of a new developmental program, blastulation. Studies on the molecular mechanisms of these developmental changes would be facilitated by the availability of chemicals having various effects on development. During the course of our search[1–7] for substances capable of arresting the embryonic development of the starfish (*A. pectinifera*) at a specific stage, we found that the MeOH extract of the marine sponge *Ancorina* sp. inhibited starfish embryogenesis specifically at the stage prior to blastulation. A preliminary examination led to the isolation of a new tetramic acid designated ancorinoside A (**1**, Fig. 1) which inhibited blastulation of the starfish embryo.[8] Further investigation of the extract of *Ancorina* sp. led to the isolation of ancorinoside A Mg salt (**2**, Fig. 2). This paper deals with the isolation, structure elucidation, and biological activities of **2**.

2. Results and discussion

A marine sponge *Ancorina* sp. (129 g, wet weight), collected off the coast of Tokushima Prefecture, Japan, was extracted with MeOH. The MeOH extract was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble

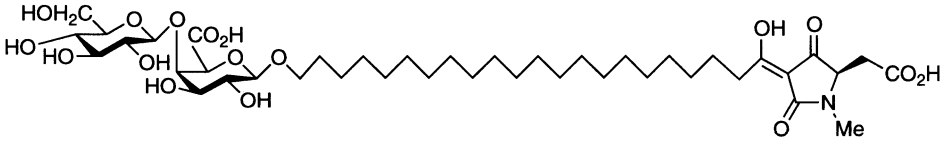

Figure 1. Structure of ancorinoside A (**1**).

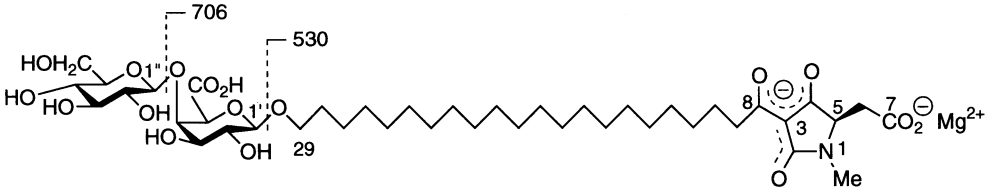

Figure 2. Structure and (−)FABMS fragmentation pattern of ancorinoside A Mg salt (**2**).

Keywords: biologically active compounds; magnesium and compounds; marine metabolites; pyrrolidines/pyrrolidinones.
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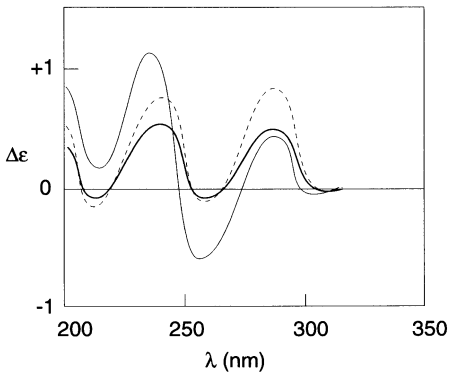

Figure 3. CD spectra of ancorinoside A Mg salt (**2**) (thin line) in H_2O solution, the Mg-free form of **2** (heavy line) in MeOH solution, and ancorinoside A (**1**) (dotted line) in MeOH solution at room temperature.

fraction was separated and subjected to ODS chromatography to afford a biologically active fraction. Concentration of the fraction gave ancorinoside A Mg salt (**2**) as a white powder. From the mother liquor ancorinoside A (**1**)[8] was obtained by ODS HPLC.

Compound **2** was soluble in H_2O but insoluble in MeOH, and showed a single peak on reversed-phase HPLC using an ODS column (60–80% CH_3CN in H_2O). The UV and IR spectra of **2** were similar to those of ancorinoside A (**1**). The pseudomolecular ion peaks at *m/z* 870 ($[M+H]^+$) and 868 ($[M-H]^-$) were observed in the positive and negative ion mode FABMS of **2**, respectively. The ion peaks were also observed in ESIMS of **2**. The findings indicated that the molecular weight of **2** is 869, which was larger than that of ancorinoside A (**1**) by 22 amu. A molecular formula of $C_{41}H_{67}NO_{17}Mg$ is established by the HR-FAB mass spectrometry. X-Ray fluorescence spectroscopy supported the presence of magnesium. The ratio of magnesium to the organic anion unit $[C_{41}H_{67}NO_{17}]^{2-}$ was determined to be 1:1 by atomic absorption spectrometry. In agreement with the presence of Mg in **2**, the ^1H and ^{13}C NMR spectra of **2** showed very broad signals.

The treatment of **2** with a cation-exchange resin afforded a Mg-free form of **2**. The UV, IR, ^1H and ^{13}C NMR spectra of the Mg-free form of **2** were identical to those of ancorinoside A (**1**), suggesting that **2** is a Mg salt of **1**. The interpretation was supported by the observation of fragment ions in the negative ion mode FABMS of **2**, as shown in Fig. 2. The CD spectrum (Fig. 3)of the Mg-free form of **2** was also identical to that of **1**, indicating that the chirality at C-5 of **2** is the same as for **1**. To confirm this, **2** was oxidized with

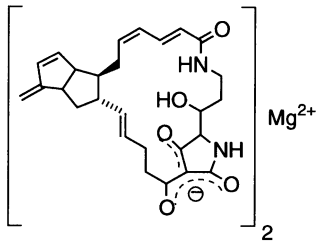

Figure 5. Structure of geodin A Mg salt isolated from the marine sponge, *Geodia* sp.

$NaIO_4/KMnO_4$, followed by acid hydrolysis, to afford *N*-methyl-D-aspartic acid, which was identified by HPLC after derivatization with Marfey's reagent.[9,10] These results clearly showed that **2** had 5*R* configuration. The absolute configuration of β-glucopyranose and β-galactopyranosyluronic acid of **2** was confirmed to be D by GLC analysis of the trimethylsilyl (TMS) ethers of 1-(L-α-methylbenzylamino)-1-deoxyalditols[11] which were derived from the hydrolysis product of **2**. From these data, the structure of **2** was determined to be a Mg salt of ancorinoside A (**1**).

Tenuazonic acid,[12] a simple tetramic acid from the fungus *Alternaria tenuis*, was shown to be biosynthesized from one molecule of L-isoleucine and two molecules of acetate.[13] The tetramic acid ring of equisetin, a metabolite of the fungus *Fusarium equiseti*, was suggested to be derived biosynthetically from L-serine.[14] Many other tetramic acids isolated from the marine organisms, e.g. aurantosides,[15–17] alteramide A,[18] cylindramide,[19] and rubrosides,[20] have been reported to afford the corresponding L-amino acids upon oxidative degradation. Ancorinoside A Mg salt (**2**) and ancorinoside A (**1**) have a (5*R*)-tetramic acid ring the precursor of which is considered to be a D-amino acid. There are very few tetramic acids having 5*R* configuration.[21] Recently, a few bacterial tetramic acids, such as phomasetin[22] from *Phoma* sp., aflastatin A[23,24] from *Streptomyces* sp. and reutericyclin[25] from *Lactobacillus reuteri* LTH2584, have been proven to have 5*R* configuration. Very few naturally occurring tetramic acid Mg salts have been obtained from microorganisms. These are an antibiotic designated magnesidin[26] from *Pseudomonas magnesiorubra* nov. sp. (ATCC No. 21856), magnesidin A[27] from *Vibrio gazogenes* ATCC29988, and a mixture of Mg and Ca tenuazonates[28] from *Phoma sorghina* (Fig. 4). Geodin A Mg salt (Fig. 5), a nematocide, has been isolated from the southern Australian marine sponge, *Geodia* sp.[29] Tetramic acid metal salts have been reported to be easily converted into the corresponding free acids by use of silica gel as chromatographic media and/or acids as solvent during isolation.[27,29] It is noted that

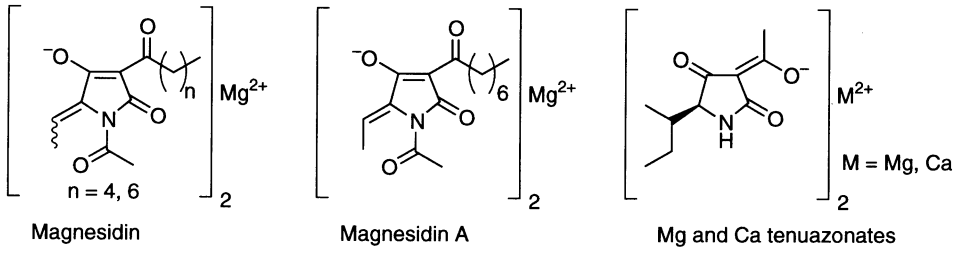

Figure 4. Naturally occurring tetramic acid Mg salts from microorganisms.

careful selection of chromatographic media and solvents is important during isolation of naturally occurring tetramic acids. To the best of our knowledge, ancorinoside A Mg salt (**2**) is the second example of a naturally occurring tetramic acid Mg salt isolated from marine sponges.

When fertilized starfish eggs were cultured from fertilization in the presence of ancorinoside A Mg salt (**2**) at a concentration of 0.4 μg/mL or greater, the development proceeded normally to 256–512 cell stage after encompassing 8–9 cycles of cleavage, and the embryos ceased to develop further without exhibiting any sign of blastulation. The inhibitory activity of ancorinoside A Mg salt (**2**) against starfish eggs was almost identical to that of ancorinoside A (**1**), even though metal ions such as Mg (II) have been reported to act as transmembrane transports and influence the fluidity and permeability of membrane.[30] Interestingly, the related compounds of ancorinoside A (**1**), ancorinosides B–D isolated from the marine sponge *Penares sollasi* Thiele, have been reported to inhibit membrane type 1 matrix metalloproteinase (MT1-MMP).[31]

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 3.30 (residual CHD_2OD) and δ_C 49.8 for CD_3OD, and δ_H 7.55 (residual C_5HD_4N) and δ_C 135.5 for C_5D_5N. FABMS, HR-FABMS, and GC-EIMS were measured on a JEOL SX102A spectrometer. UV and IR spectra were recorded on a Shimadzu UV-160A and a JASCO FT/IR-5300 spectrometer, respectively. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. CD spectra were recorded with a JASCO J-600 circular dichroic spectropolarimeter. Atomic absorption spectral measurements were performed using a Nippon Jarrell Ash atomic absorption and flame emission spectrophotometer AA-8200. The atomization temperature was 1500°C and argon gas purge was used. 1-Methyladenine was purchased from Sigma, St Louis, Missouri, USA.

3.2. Bioassays

Specimens of *A. pectinifera* were collected from the coastal waters off Japan during their breeding season and kept in sea water at 15°C in laboratory aquaria. Oocytes and sperm were removed from ovarian and testicular fragments, respectively. Experiments were performed at 20°C and artificial seawater (Jamarin Laboratory, Osaka, Japan) was used throughout. Oocytes were induced to mature by the treatment with 1 μM 1-methyladenine.[32] Maturing oocytes were fertilized at 40 min after the start of 1-methyladenine treatment. Stock solutions of compounds to be tested in MeOH were added to the suspensions of fertilized eggs to give final concentrations of MeOH less than 0.2% in sea water. MeOH at the concentrations used had no effect on embryonic development. To assay for the developmental arrest of embryos, a small number of fertilized eggs were added to serially diluted sample solutions within 30 min after insemination. They were periodically observed for any cytological changes.

3.3. Animal material, extraction, and isolation

The marine sponge *Ancorina* sp. (129 g, wet weight) was collected off the coast of Tokushima Prefecture, Japan, and frozen immediately after collection. The marine sponge was identified by Professor Patricia R. Bergquist, The University of Auckland, New Zealand. A voucher specimen is kept in the laboratory of one of the authors (S. O.). The MeOH extract of the sponge was concentrated to afford an aqueous solution, which was extracted with EtOAc. The aqueous layer was then extracted with *n*-BuOH. The *n*-BuOH-soluble fraction was subjected to low-pressure column chromatography on ODS using 0–100% MeOH in H_2O as eluent. The biologically active fraction (80% MeOH in H_2O) was concentrated to ca. 2 mL, resulting in precipitation of pure ancorinoside A Mg salt (**2**) (8.0 mg, 0.0062% yield based on the wet weight), which was collected on a glass filter by filtration of the suspension. The filtrate was subjected to reversed-phase HPLC (ODS) using 60–80% CH_3CN in H_2O as eluent to afford ancorinoside A (**1**) (2.0 mg, 0.0016% yield based on the wet weight) as a viscous colorless oil.

3.3.1. Ancorinoside A Mg salt (2). A white powder; mp 200–208°C (dec); $[\alpha]_D^{25}$ −7.7° (*c* 0.18, H_2O); UV (H_2O) λ_{max} 285 (ε 9300), 243 nm (ε 8900); CD (H_2O) 305 (Δε −0.09), 278 (Δε +0.44), 256 (Δε −0.52), 232 nm (Δε +1.10); IR (KBr) 3400, 1701 (sh), 1661 (sh), 1649 (sh), 1624 (sh), 1605, 1489 cm^{-1}; HR-FABMS (positive, glycerol matrix) *m/z* 870.4380 $[M+H]^+$ (calcd for $C_{41}H_{68}NO_{17}Mg$, 870.4338); HR-FABMS (negative, glycerol matrix) *m/z* 868.4167 $[M-H]^-$ (calcd for $C_{41}H_{66}NO_{17}Mg$, 868.4181); FABMS (positive, glycerol matrix) *m/z* 870 $[M+H]^+$; FABMS (negative, glycerol matrix) *m/z* 868 $[M-H]^-$, 706 [M-163]$^-$, 530 [M-339]$^-$; ESIMS (positive) *m/z* 870 $[M+H]^+$; ESIMS (negative) *m/z* 868 $[M-H]^-$.

3.4. Mg analyses

X-Ray fluorescence spectroscopy indicated the presence of high levels of Mg and the absence of other significant inorganic counterions. The magnesium content in **2** was determined to be 2.78% (calcd for $C_{41}H_{67}NO_{17}Mg$, 2.79%) by atomic absorption spectrometry (AAS).

3.5. Preparation of the Mg-free form of 2

An aqueous solution of **2** (4 mg) was subjected to a column on cation exchange resin (Dowex 50W-x8; H^+-form) using H_2O to afford the Mg-free form of **2** (3 mg). The absence of Mg in the compound was confirmed by AAS.

3.5.1. The Mg-free form of 2. A viscous colorless oil; UV (MeOH) 283 (ε7700), 230 nm (ε4500); CD (MeOH) 290 (Δε +0.41), 245 nm (Δε +0.46); IR (film) 3370, 1715, 1660 (sh), 1640 (sh), 1615 cm^{-1}; HR-FABMS (negative, glycerol matrix) *m/z* 846.4479 $[M-H]^-$ (calcd for $C_{41}H_{68}NO_{17}$, 846.4487); FABMS (negative, glycerol matrix) *m/z* 846 $[M-H]^-$; FABMS (positive, glycerol matrix) *m/z* 848

$[M+H]^+$; ^{1}H NMR (CD_3OD, mult, J in Hz) δ 1.28 (30H, m, H_2-12–H_2-26), 1.37 (4H, m, H_2-11, H_2-27), 1.62 (2H, m, H_2-28), 1.64 (2H, m, H_2-10), 2.76 (1H, dd, J=16.5, 5.5 Hz, H-6b), 2.80 (2H, t, J=7.3 Hz, H_2-9), 2.87 (1H, dd, J=16.5, 4.6 Hz, H-6a), 2.93 (3H, s, H_3-30), 3.20 (1H, m, H-5″), 3.21 (1H, dd, J=9.2, 8.3 Hz, H-2″), 3.27 (1H, m, H-4″), 3.33 (1H, m, H-3″), 3.54 (1H, dt, J=9.2, 6.4 Hz, H-29b), 3.56 (1H, dd, J=10.1, 7.3 Hz, H-2′), 3.62 (1H, dd, J= 10.1 Hz, 2.8, H-3′), 3.64 (1H, dd, J=11.9, 5.5 Hz, H-6″b), 3.84 (1H, dd, J=11.9, 2.3 Hz, H-6″a), 3.94 (1H, dt, J=9.2, 6.9 Hz, H-29a), 4.01 (1H, dd, J=5.5, 4.6 Hz, H-5), 4.18 (1H, br s, H-5′), 4.26 (1H, d, J=7.3 Hz, H-1′), 4.33 (1H, br d, J= 2.8 Hz, H-4′), 4.47 (1H, d, J=8.3 Hz, H-1″); ^{13}C NMR (CD_3OD) δ 197.0 (s, C-4), 190.9 (s, C-8), 174.9 (s, C-2), 174.4 (s, C-7), 173.1 (s, C-6′), 106.7 (d, C-1″), 105.5 (d, C-1′), 103.8 (s, C-3), 81.7 (d, C-4′), 78.8 (d, C-3″), 78.8 (d, C-5″), 76.4 (d, C-2″), 75.8 (d, C-3′), 75.7 (d, C-5′), 73.4 (d, C-2′), 72.1 (d, C-4″), 72.1 (t, C-29), 64.8 (d, C-5), 63.4 (t, C-6″), 35.9 (t, C-6), 35.8 (t, C-9), 31.6 (17C, t, C-11–C-26, C-28), 27.9 (2C, t, C-10, C-27), 27.7 (q, C-30); ^{1}H NMR (C_5D_5N, mult, J in Hz) δ 1.27 (30H, m, H_2-12–H_2-26), 1.30 (2H, m, H_2-27), 1.40 (2H, m, H_2-11), 1.65 (2H, m, H_2-28), 1.81 (2H, m, H_2-10), 2.97 (1H, dd, J=16.5, 6.9 Hz, H-6b), 3.07 (3H, s, H_3-30), 3.21 (2H, m, H_2-9), 3.26 (1H, dd, J= 16.5, 5.2 Hz, H-6a), 3.70 (1H, dt, J=8.8, 7.1 Hz, H-29b), 3.87 (1H, ddd, J=9.1, 6.3, 2.8 Hz, H-5″), 3.95 (1H, dd, J=9.1, 7.7 Hz, H-2″), 4.01 (1H, t, J=9.1 Hz, H-4″), 4.05 (1H, dd, J=11.3, 6.3 Hz, H-6″b), 4.16 (1H, t, J=9.1 Hz, H-3″), 4.19 (1H, dt, J=8.8, 7.1 Hz, H-29a), 4.27 (1H, m, H-5), 4.30 (1H, dd, J=11.3, 2.8 Hz, H-6″a), 4.31 (1H, dd, J=9.3, 3.0 Hz, H-3′), 4.45 (1H, dd, J=9.3, 7.7 Hz, H-2′), 4.74 (1H, br s, H-5′), 4.80 (1H, d, J=7.7 Hz, H-1′), 5.06 (1H, br d, J=3.0 Hz, H-4′), 5.29 (1H, d, J=7.7 Hz, H-1″); ^{13}C NMR (C_5D_5N) δ 194.1 (s, C-4), 191.5 (s, C-8), 174.4 (s, C-2), 173.6 (s, C-7), 171.9 (s, C-6′), 106.9 (d, C-1″), 104.8 (d, C-1′), 101.8 (s, C-3), 81.8 (d, C-4′), 78.4 (d, C-3″), 78.1 (d, C-5″), 76.0 (d, C-2″), 75.3 (d, C-5′), 75.0 (d, C-3′), 72.7 (d, C-2′), 71.9 (d, C-4″), 70.1 (t, C-29), 63.2 (t, C-6″), 62.9 (d, C-5), 37.5 (t, C-9), 36.5 (t, C-6), 29.9 (17C, t, C-11–C-26, C-28), 27.1 (q, C-30), 26.4 (2C, t, C-10, C-27).

3.6. The determination of absolute configuration of the sugar moiety of 2

After compound **2** (1 mg) was hydrolyzed with 2 M HCl (80°C, 3 h), the hydrolysate was partitioned between $CHCl_3$ and H_2O. Following the method described in the literature,[11] the water-soluble portion was dried in vacuo. The resulting residue was dissolved in H_2O (50 μL). To the solution in a screwed-cap vial was added an EtOH solution (50 μL) containing (*S*)-1-phenylethylamine (10 mg) and sodium cyanoborohydride (1 mg). After 3 h at 40°C, a few drops of acetic acid were added and the solvents in the mixture were evaporated to dryness. The residue was dissolved in MeOH and solution was concentrated in vacuo. To the residue, after being dried in vacuo, was added a solution of 25% *N*,*O*-bis(trimethylsilyl)acetamide in dry MeCN to give trimethylsilyl (TMS) ether derivatives of 1-(L-α-methylbenzylamino)-1-deoxyalditols (L-MBA-alditols). The mixture was analyzed by GLC using a Spelcowax 10 capillary column (0.20 mm×30 m) with or without coinjection of the TMS ethers of L- and DL-MBA-alditols derived from authentic D-sugars. The oven temperature was ramped from 150 to 250°C at 3°C/min. It was found that the sugar moiety of **2** is composed of D-glucose and D-galacturonic acid with the molar ratio of 1:1. Retention times in min were as follows: 26.68 and 26.81 for L- and D-glucose, respectively, 33.38 and 33.79 for D- and L-galacturonic acid, respectively. Identification of each GLC peak was performed on the basis of the GC-mass spectra.

3.7. The determination of absolute configuration of the tetramic acid moiety of 2

The $CHCl_3$ extract of the hydrolysate described above was dissolved in aqueous K_2CO_3 solution (6 mg/200 μL) and an aqueous $NaIO_4$ (40 mg/500 μL) was added over period of 1 h. To the mixture, an aqueous $KMnO_4$ solution (0.2 mg/20 μL) was added and the mixture was stirred for 2 h at room temperature. The mixture, after addition of an aqueous $NaHSO_3$ solution (20 mg/40 μL), was filtered through Celite and the filtrate was acidified with 10% H_2SO_4 (2 mL). The solution was extracted with EtOAc and the EtOAc extract was dried in vacuo. The residue was dissolved in 6 M HCl and heated to 100°C in a sealed tube for 24 h. The solvent, after being cooled, was removed in a stream of dry N_2, with heating, followed by high vacuum evacuation. To the acid hydrolysate of the degradation product was added a 50% acetone solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) in 1 M $NaHCO_3$. The mixture was kept at 40°C for 1 h with frequently mixing followed by neutralization with 100 μL of 0.2 M HCl. The reaction mixture, after degassing and filtration, was subjected to reversed-phase HPLC using a Wakopak 5C18AR column (4.6 mm× 25 cm) with 30% MeCN in 0.1% TFA. Elution was monitored at 340 nm. Retention times of standard specimens of *N*-Me-D-Asp and *N*-Me-L-Asp were 5.55 and 6.10 min, respectively.

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