Geodin A Magnesium Salt: A Novel Nematocide from a Southern Australian Marine Sponge, *Geodia*

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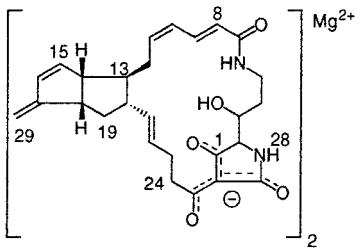
A *Geodia* species collected from southern Australian waters of the Great Australian Bight has yielded a potent new in vitro nematocidal agent identified as geodin A Mg salt (**1**), a new macrocyclic polyketide lactam tetramic acid magnesium salt. The structure for **1** was assigned on the basis of detailed spectroscopic analysis.

Marine invertebrates and algae are well documented in the scientific literature as excellent sources of novel bioactive (antibiotic, anticancer, antiviral, etc.) metabolites. Our investigations into the chemistry of southern Australian marine organisms has featured an interdisciplinary approach, drawing on academic and industrial expertise, with high-throughput biological screens and bioassay-directed fractionation strategies aimed at detecting metabolites with agrochemical potential. Bioassay-directed fractionation of extracts targeted during this program have returned an array of novel in vitro nematocides. Two recent examples include epoxylipids[1] from the brown alga *Notheia anomala* and the amphilactams[2] from two marine sponges, *Amphimedon* spp. In this report we describe the outcome of our investigations into a *Geodia* sp. collected in the Great Australian Bight. Bioassay-directed fractionation of this sponge yielded a single potent nematocidal agent identified as a new macrocyclic polyketide lactam tetramic acid magnesium salt, to which we have assigned the structure and trivial name geodin A Mg salt (**1**).



geodin A Mg salt (**1**)

Results and Discussion

The EtOH extract of a *Geodia* sp. collected in July 1995 at a depth of 55 m in the waters of the Great Australian Bight, Australia, was found to inhibit significantly larval development of the nematode *Haemonchus contortus* (LD_{99} = 14 μg/mL). The EtOH extract of this sponge was decanted, concentrated in vacuo, and the CH_2Cl_2-insoluble portion subsequently partitioned between n-BuOH and H_2O. The n-BuOH-soluble portion, which accounted for 5% of the total extract biomass and all of the nematocidal activity (LD_{99} = 4 μg/mL), was further purified by elution through Sephadex LH-20 (MeOH) followed by sequential trituration of the combined active fractions with petroleum spirits and CH_2Cl_2. The resulting bioactive residue was precipitated from H_2O/MeOH to yield the pure nematocidal agent **1** (LD_{99} = 1 μg/mL). Despite being extracted from the sponge with aqueous EtOH, **1** was only sparingly soluble in MeOH.

Initial low-resolution ESI (-ve) mass spectrometry of **1** suggested an M−H ion at *m/z* 463. Subsequent high-resolution ESI (−ve) mass spectrometry on an FT instrument confirmed a composition for this ion ($C_{27}H_{31}N_2O_5$, Δmmu = −0.2), but more significantly revealed higher mass ions at *m/z* 949 ($[C_{27}H_{31}N_2O_5]_2Mg$ − H, Δmmu = −4.7) and *m/z* 1413 ($[C_{27}H_{31}N_2O_5)_3Mg$, Δmmu = +4.2). Furthermore, although initial attempts at low resolution ESI (+ve) mass spectrometry did not return measurable ions, when performed on the high-resolution FT instrument a significant ion was observed at *m/z* 487 ($C_{27}H_{31}N_2O_5Mg^+$, Δmmu = +0.5). These analyses confirmed that **1** occurred naturally as a Mg salt, although given the appearance of in situ-generated R_3Mg^- ions in the ESI mass spectrometer these experiments did not unambiguously establish whether **1** satisfied the formula R_2Mg rather than RMgX. To determine this required an independent elemental analysis. Energy dispersive spectroscopy (EDS) confirmed the presence of high levels of Mg and the absence of other significant inorganic counterions, while atomic absorption spectroscopy (AAS) established the ratio of Mg to organic content as consistent with $[C_{27}H_{31}N_2O_5]_2Mg$. Though armed with the molecular formula it remained for us to solve the structure of the organic anion subunit $C_{27}H_{31}N_2O_5$.

Compound **1** was only sparingly soluble in CD_3OD, and complete NMR characterization was carried out in DMSO-d_6 (see Table 1). Although the ^{1}H NMR data for **1** were broad, preliminary examination revealed resonances and correlations consistent with the structure fragment C7 to C12, where C7 was substituted by either oxygen (ester or lactone) or nitrogen (amide or lactam). The ^{13}C NMR data for **1** also revealed resonances for a total of 15 sp^2 hybridized carbons, attributed to six double bonds and three carbonyl carbons. This analysis accounted for nine of the 13 available double-bond equivalents, and required that the $C_{27}H_{31}N_2O_5$ anion subunit in **1** be tetracyclic. Searching the marine natural products literature for "acids" with 27 carbons and molecular weights >450, that also incorporated the C7 to C12 structure fragment as

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Table 1. NMR (DMSO-d_6, 400 MHz) Data for Geodin A Mg Salt (**1**)

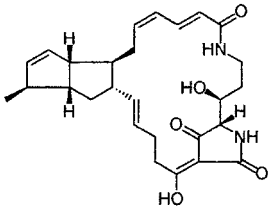
	^{13}C δ (m)	^{1}H δ (m, J Hz)	COSY	HMBC (^{1}H−^{13}C)
1	192.1 (s)			
2	66.7 (d)	3.60 (s)		C3, C4, C25, C27
3	68.9 (d)	3.81 (br dd)	H4a, 28NH	
3-OH		7.42 (s)		C2, C1, C26
4a	30.1 (t)[a]	1.15 (m)	H3, H4b	C3
4b		1.40 (t, 11.9)	H4a, H5a	C5
5a	35.4 (t)	2.75 (br t)	H4b, H5b, 6NH	C7
5b		3.35 (obscured)	H5a, 6NH	
6-NH		8.15 (t, 5.6)	H5a, H5b	C7
7	165.2 (s)			
8	125.1 (d)	5.92 (d, 14.6)	H9	C7, C10[e]
9	133.7 (d)[d]	7.20 (dd, 12.1, 14.6)	H8, H10	C7, C10, C11
10	128.3 (d)	6.24 (m)	H9, H11	C8, C12
11	133.5 (d)[d]	5.92 (m)	H10, H12a, H12b	C10[e]
12a	26.4 (t)	2.24 (br d, 12.0)	H11, H12b	
12b		2.58 (m)	H11, H12a, H13	
13	50.8 (d)	1.25 (m)	H12b, H14, H20	
14	53.0 (d)	3.15 (m)	H13, H15, H18	C12, C13, C15, C16, C18
15	141.5 (d)	6.24 (m)	H14, H16	C14, C17, C18
16	132.1 (d)[c]	6.04 (d, 5.5)	H15	C14, C17, C18
17	158.8 (s)			
18	44.6 (d)	3.00 (m)	H14, H19b, H20	C14, C17, C19, C29
19a	39.3 (t)[b]	1.89 (m)		C21[f]
19b		1.05 (m)	H18, H20	C17, C18, C20
20	48.6 (d)	2.06 (m)	H13, H18, H19b, H21	C13, C14, C22[g]
21	130.7 (d)	5.08 (dd, 8.1, 15.0)	H20, H22	C20, C22, C23
22	132.6 (d)[c]	5.19 (dt, 7.3, 15.0)	H23a, H21	C19, C20, C21, C23
23a	30.2 (t)[a]	1.89 (m)	H22, H23b, H24b	C21[f], C22, C24
23b		1.72 (m)	H23a, H24b	C25
24a	39.5 (t)[b]	2.06 (m)		C22[g]
24b		3.15 (m)	H23a, H23b	C25
25	193.7 (s)			
26	99.6 (s)			
27	177.7 (s)			
28-NH		4.60 (d, 5.5)	H3	
29a	103.6 (t)	4.86 (s)		C16, C17, C18
29b		4.70 (s)		C16, C17, C18

[a−d] Entries marked with the same symbol are interchangeable. [e] Correlation from H8 and/or H11 to C10. [f] Correlation from H19a and/or H23a to C21. [g] Correlation from H20 and/or H24a to C22.

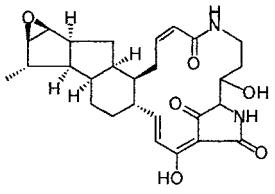
indicated, drew our attention to the cytotoxic tetramic acid cylindramide (**2**),[3] isolated by Fusetani et al. from the Japanese marine sponge *Halichondria cylindrata*. The nematocide **1** appeared to possess an anion subunit that was the 17,29-didehydro conjugate base analogue of cylindramide (**2**). Given the salt character of **1** it was perhaps not surprising that the published NMR data for cylindramide (**2**) did not correlate well. It should also be noted that the reported NMR data for cylindramide were acquired in a 1:1 mixture of $CD_3OD/CDCl_3$, a solvent system that was incapable of dissolving **1**. Despite ^{1}H NMR chemical shift differences, detailed analysis of the NMR data for **1** (see Table 1) established unequivocal correlations consistent with the gross structure for the $C_{27}H_{31}N_2O_5$ substructure as shown. Complete connectivity sequences could be established from C1 through to C27 and out to C29. Furthermore, examination of the ROESY NMR data identified a series of correlations (including H11 to H14, H18, and H20; H18 to H20; H13 to H15) that defined the relative stereochemistry about C13, C14, C18, and C20 as shown, common with that previously assigned to cylindramide (**2**).[3] The *E* stereochemistry about $\Delta^{8,9}$ and $\Delta^{21,22}$ was readily determined from $J_{8,9} = 14.6$ Hz and $J_{21,22} = 15.0$ Hz, respectively. Overlap between ^{1}H NMR (DMSO-d_6) resonances for H10 and H15, as well as H11 and H8, prevented a measure of $J_{10,11}$ and hence assignment of the $\Delta^{10,11}$ stereochemistry. Fortunately this measurement was secured ($J_{10,11} = 10.8$ Hz) when the data were acquired in CD_3OD (low concentration), and supported a *Z* $\Delta_{10,11}$ stereochemistry. In our hands, spectroscopic methods could not be used to unambiguously assign relative stereochemistry about C2 and C3. Based on the arguments detailed above we propose the structure and trivial name for the nematocidal agent present in this *Geodia* sp. to be geodin A Mg salt (**1**), as shown (relative stereochemistry only).

Several macrocyclic lactam tetramic acids have been isolated from marine sources. In addition to cylindramide (**2**),[3] which is cytotoxic to B16 melanoma cells (IC_{50} 0.8 μg/mL), the related metabolite discodermide (**3**)[4] has been reported from a Caribbean marine sponge, *Discodermia dissoluta*. Discodermide (**3**) inhibits the in vitro proliferation of cultured murine P388 leukemia cells (IC_{50} 0.3 μg/mL), and growth of *Candida albicans* (MIC 12.5 μg/mL). The discovery of **2** and **3** from taxonomically unrelated sponges allows for speculation that such metabolites are biosynthetically derived from symbiotic and/or associated bacteria. Support for this hypothesis can be found in the discovery of alteramide A (**4**)[5] from the marine bacterium *Altermonas* sp. associated with the Japanese marine sponge *Halichondria okadai* and aburatubolactam A (**5**)[6] from the culture broth of a *Streptomyces* sp. separated from a Japanese marine mollusk. Alteramide A (**4**) demonstrated in vitro cytotoxicity against murine leukemia P388 cells (IC_{50} 0.1 μg/mL), murine lymphoma L1210 cells (IC_{50} 1.7 μg/mL), and human epidermoid carcinoma KB cells (IC_{50} 5.0 μg/mL), while aburatubolactam A (**5**) was reported to inhibit TPA-induced superoxide anion generation by human neutrophils. Although none of these marine tetramic acids **2**, **3**, **4**, or **5** was reported to occur naturally as a salt, it should be noted that in their respective isolations aburatubolactam A (**5**)[6] was eluted with $MeOH/CHCl_3$ through silica gel, and cylindramide (**2**)[3] was subjected to

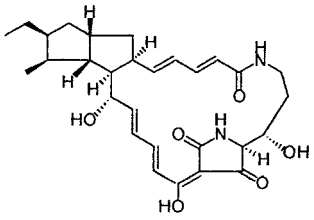
ODS−HPLC using 80% MeOH/0.05% TFA, both conditions capable of transforming salts to conjugate acids. For example, silica chromatography of the crude solvent extract from an Australian *Spongia* sp. has been reported to efficiently transform natural phenolic potassium salts into conjugate acids,[7] while ODS−HPLC chromatography with MeOH/H_2O/TFA (90:10:0.1) resulted in quantitative conversion of the naturally occurring tetramic acid magnesium salt magnesidin A (**6**) into its corresponding conjugate acid.[8] To the best of our knowledge the magnesidins (**7**), reported as an inseparable mixture in 1974[9] from *Pseudomonas magnesiorubra* cultured from washings of the green marine alga *Caulerpa peltata*, were the first recorded occurrence in the primary scientific literature of naturally occurring tetramic acid Mg salts. Their rediscovery 20 years later as the antibiotic agents in *Vibrio gazogenes* cultured from marine mud[8] represents the only other report of naturally occurring tetramic acid Mg salts in the scientific literature.



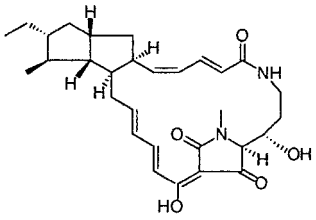
cylindramide (**2**)



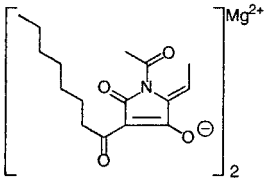
discodermide (**3**)



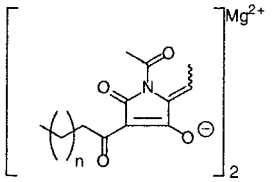
alteramide A (**4**)



aburatubolactam (**5**)

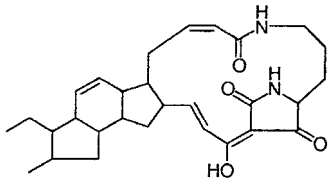


magnesidin A (**6**)

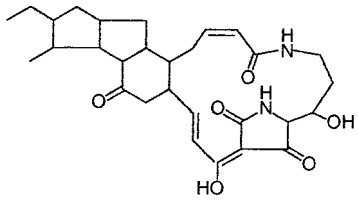


magnesidins (**7**)
n=3,5 (1:1)

This is not to say that macrocyclic lactam tetramic acids have not been featured in the natural products literature. On the contrary, examples of this structure class have been isolated from terrestrially sourced microbes. For example, *Streptomyces phaechromogenes* var. ikaruganensis has been reported to yield (after chromatography on silicic acid) the antibiotic macrocyclic lactam tetramic acid ikarugamycin (**8**),[10] while *Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia*) yielded the antifungal agent maltophilin (**9**) after extraction of the acidified (pH 4) culture filtrate and ODS−HPLC with CH_3CN/H_2O/TFA (60:40:0.08).[11] Curiously, this latter microbe which is an opportunist pathogen of humans, when isolated from the rhizosphere of oilseed rape (*Brassica napus* L. ssp. oleifera Metzg. Sinsk) was reported to yield both maltophilin (**9**) and alteramide A (**4**), drawing an even closer biosynthetic link between marine and terrestrially sourced tetramic acids.



ikarugamycin (**8**)



maltophilin (**9**)

Whether one or more of the known marine or terrestrial macrocyclic lactam tetramic acids listed above occurred naturally as Mg salts is impossible to say. Clearly, the ease with which the salt character can be lost during isolation should encourage a cautious approach to the selection of extraction procedures, as well as chromatographic media and solvents, when dealing with this structure class.

To date we have encountered geodin A Mg salt (**1**) in three separate specimens of *Geodia* sp. collected in the Great Australian Bight. Attempts to convert geodin A Mg salt (**1**) into its conjugate acid by treatment with either HCl or TFA yielded a product that even after removal of acid underwent facile oxidation. Attempts to resolve the resulting oxidative products were compromised by instability and ongoing transformations. Mass spectral analysis of this mixture revealed ions consistent with single and double additions of H_2O to geodin A, while NMR analyses confirmed that these additions were localized about $\Delta^{15,16}$ and $\Delta^{17,29}$. It would appear that the organic anion subunit (geodin A) is acid labile to the point that the tetramic acid functionality induces autooxidation. Consistent with this hypothesis is the observation that no such oxidative decomposition was observed for the naturally occurring Mg salt **1**. It is noteworthy that among known macrocyclic lactam tetramic acids the oxidatively sensitive cyclopentadiene moiety is unique to geodin A Mg salt (**1**). These observations support our proposition that geodin A only occurs naturally as the Mg salt.

Experimental Section

General Experimental Procedures. See ref 12.

Collection, Extraction, and Isolation. A sponge specimen, identified as a *Geodia* sp. (Museum of Victoria Registry Number: F79999), was obtained during a scientific expedition in the Great Australian Bight aboard the RV Franklin in July 1995. The specimen was collected by epibenthic sled at a depth of 51 meters at position 34′ 02″S: 114′ 48″E. A description of the specimen is as follows: growth form macrobenthic, massive; color in life pink brown with a white interior, white in EtOH; texture firm, compressible, harsh; surface opaque, irregular, minutely tuberculose; oscules scattered, inconspicuous; spicules megascleres plagio-orthotriaenes (680−920 × 15 mm), oxeas curved, hastate (950−1100 × 10−30 mm); microscleres sterraster euasters (50 mm), oxyaster euasters (35 mm), tylaster euasters (12 mm); ectosome a distinct, dense

crust of sterraster euasters 300 mm in width; choanosome whispy bundles of oxeas and asterose microscleres scattered throughout abundant interstitial collagen and well-developed subectosomal spaces obvious between plumose bundles of triaenes, clads directed outward, supporting the ectosomal crust.

The frozen sponge was transported to the laboratory where it was thawed, documented, diced, and steeped in EtOH at −20 °C, prior to biological screening and chemical analysis. A portion of the EtOH extract was decanted and concentrated in vacuo to yield a viscous gum (11.3 g) that was triturated with CH_2Cl_2. The CH_2Cl_2-insoluble residue (10.74 g) was subsequently partitioned between n-BuOH (510 mg, 4.5%) and H_2O (9.8 g). Since all the nematocidal activity was concentrated in the n-BuOH solubles, this material was fractionated by gel permeation chromatography (elution with MeOH through Sephadex LH-20, 2.5 × 80 cm column). Fractions containing the bioactive agent geodin A Mg salt (**1**) were initially identified by UV−vis monitoring and thin-layer chromatography (geodin A Mg salt is UV active at 254 nm and returns a characteristic dark blue spot on TLC after developing with vanillin/HOAc/H_2SO_4), and subsequently confirmed by in vitro nematocidal screening. The combined bioactive material was concentrated in vacuo and triturated (defatted) successively with petroleum spirits and CH_2Cl_2 to yield an insoluble residue that was precipitated from MeOH/H_2O to give pure geodin A Mg salt (**1**) (18.4 mg, 0.20% of the EtOH extractables, 0.012% of the sponge dry weight).

Geodin A Mg salt (1): amorphous white solid; mp 173° (dec), $[\alpha]^{20}_D$ +179° (*c* 1.0, DMSO); IR (KBr) ν_{max} 3345, 1610, 1535, 1480 cm^{-1}; UV (DMSO) λ_{max} (ϵ) 264 nm (19 100); ^1H and ^{13}C NMR data, see Table 1; ESIMS (−ve) *m*/*z* 463 (100); HRESIMS (+ve) *m*/*z* 487.2076 (calcd for $C_{27}H_{31}N_2O_5Mg^+$, 487.2081); HRESIMS (−ve) *m*/*z* 1413.6507 (calcd for $(C_{27}H_{31}N_2O_5)_3Mg^-$, 1413.6549), 949.4284 (calcd for $[(C_{27}H_{31}N_2O_5)_2Mg - H]^-$, 949.4237), 463.2234 (calcd for $C_{27}H_{31}N_2O_5^-$, 463.2231).

Mg Analyses on 1. A sample of geodin A Mg salt (**1**) (1−2 mg) was carbon-coated and its X-ray spectrum analyzed by an Oxford Isis EDS (energy dispersive spectrometry) system using a Phillips XL30 scanning electron microscope as the electron source. Semiquantitative measurement of X-ray intensity indicated a molar O:Mg ratio of approximately 10:1, consistent with two tetramic acid ligands per Mg atom, $(C_{27}H_{31}N_2O_5)_2Mg$.

Geodin A Mg salt (**1**) (1.02 mg) was acid-digested with concentrated nitric acid (10 drops, 160°, 30 min) and made up to 25 mL with 0.1 M HCl, and the magnesium concentration determined by AAS to be 0.80 ppm which equates to 2.0% Mg [calculated for $(C_{27}H_{31}N_2O_5)_2Mg$, 2.5%].

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