Agelastatins C and D, New Pentacyclic Bromopyrroles from the Sponge *Cymbastela* sp., and Potent Arthropod Toxicity of (−)-Agelastatin A

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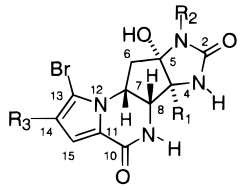
Two new agelastatins, C (**3**) and D (**4**), along with the known compound (−)-agelastatin A (**1**), were isolated from the Indian Ocean sponge *Cymbastela* sp. The structures were determined by analysis of spectroscopic data and chemical correlation of **4** with a common derivative **5**, also prepared from **1**. Agelastatin A (**1**) exhibited potent activity against brine shrimp (LC_{50} 1.7 ppm) in addition to insecticidal activity against larvae of beet army worm, *Spodoptera exigua*, and corn rootworm, *Diabrotica undecimpunctata*.

Pyrroloaminopropylimidazole alkaloids are characteristically found in sponges from the family Axinellidae. *Agelas dendromorpha* (Axinellida) was shown to contain two cytoxtoxic pyrroloaminopropylimidazoles, (−)-agelastatins A (**1**) and B (**2**).[1] Many pyrroloaminopropylimidazoles have been described from sponges; however, the novel, highly fused tetracyclic pyrrole skeleton represented by **1** is unique to the agelastatins. We report here that the West Australian sponge *Cymbastela* sp. (Axinellida) contains additional examples of this heterocyclic system—two minor congeners that we have named agelastatins C (**3**) and D (**4**). Previous reports of secondary metabolites from *Cymbastela* spp. include diterpene and sesquiterpene hydrocarbons, isonitriles, isothiocyanates and isocyanates,[2a−c] polar sterols,[2d] peptides,[2e] and 3-pyridyl guanidine,[2f] but no pyrroles. In addition, we have found **1** exhibits toxicity toward brine shrimp larvae (nauplii) with an LC_{50} of 1.7 ppm and also shows potent insecticidal activity.

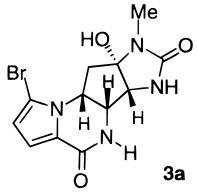
The methanolic extract from a lyophilized sample of *Cymbastela* sp., collected at Muiron Island, WA, was fractionated by sequential solvent partitioning, silica chromatography ($CHCl_3$−MeOH), and finally purification by reversed-phase HPLC (70:30 MeOH−H_2O) to provide (−)-agelastatin A (**1**),[1a] and the new compound, (−)-agelastatin C (**3**). A second sample of *Cymbastela*, collected nearby, also yielded **1** and **3** and a third compound, agelastatin D (**4**). (−)-Agelastatin A was identified by comparison of ^{1}H- and ^{13}C-NMR data with those of reported values,[1a] while the structures of the new compounds were derived as follows.

The formula of **3**, $C_{12}H_{13}BrN_4O_4$ (HRFABMS), indicated the incorporation of one additional oxygen atom into the agelastatin A structure ($C_{12}H_{13}BrN_4O_3$). The ^{1}H-NMR spectrum of **3** was very similar to that of **1**, but lacked an H-4 methine signal at δ 3.88 ppm. Comparison of the ^{13}C-NMR spectra of **1** and **3** revealed



1 R_1= H ; R_2 = Me ; R_3 = H *Agelastatin A*
2 R_1 = H ; R_2 = Me ; R_3 = Br *Agelastatin B*
3 R_1 = OH; R_2 = Me ; R_3 = H *Agelastatin C*
4 R_1 = H ; R_2 = H ; R_3 = H *Agelastatin D*

the expected differences—absence of the doublet due to C-4 (δ 67.4, d) in **1** and replacement by a quaternary aminal signal (δ 89.9 s). All other signals were similar to those of **1** and consistent with C-4 substitution by an hydroxyl group. Assuming the 5-OH group is α, as found in **1**, the new 4-OH group is also likely to be α because the alternate 4β,5α-*trans* configuration is highly disfavored by ring strain.[3] Hemi-aminals such as **1** and **3** can epimerize in hydroxylic solvents (*cf.* mutarotation in aldopentoses and corresponding azasugars). Our MM2 calculations (Chem3D Pro) show the trans isomer **3a** would be ca. 20 kcal·mol^{-1} higher in strain energy than structure **3**; therefore, we have assigned agelastatin C the more stable *cis*-fused imidazolone ring (4α-hydroxyagelastatin A). This configuration is also supported by CD spectra as revealed below.



3a

Agelastatin D (**4**), $C_{11}H_{11}BrN_4O_3$, was isolated in low amount (0.5 mg) from the larger of the two samples of *Cymbastela* sp. collected, although it was probably present in both. Compound **4** is a lower homologue of **1** and lacks an *N*-methyl group. Although insufficient amounts of **4** were available to record a ^{13}C-NMR spectrum, evidence for the relationship between the two

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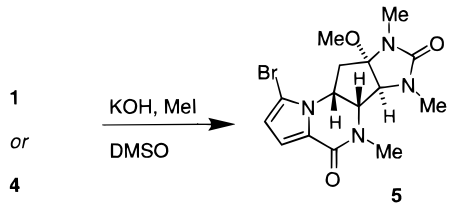
compounds was provided by methylation (KOH, MeI, DMSO) of **1** and **4**; both compounds gave the same trimethyl compound **5**. Given the small quantities of **4** available for chemical correlation, the identification of the two methylation products as **5** was made by MS and matching of retention times by reversed-phase HPLC retention times in two different solvents.



The similarity in chemical shifts and ^{1}H-NMR coupling constants for **1**−**4** indicates they share the same relative configuration. The CD spectra for **1**, **3**, and **4** are essentially superimposable (negative Cotton effect at λ 219 nm and a positive Cotton effect at 246 nm); therefore, the absolute configuration of the agelastatins must be the same. Because the absolute configuration of **1** was firmly established by exciton coupling in the CD spectra of *p*-*N*,*N*-dimethylaminobenzoyl derivatives,[1b] the configuration of **3** is 4*R*,5*S*,7*R*,8*R* and **4** is 4*S*,5*S*,-7*R*,8*S*.[4]

Agelastatin A was highly toxic to brine shrimp. Dose-response measurements of **1** against newly hatched brine shrimp gave an LC_{50} of 1.7 ppm (5.0 μM).[5] Agelastatin C (**3**) was much less toxic (LC_{50} ca. 200 ppm), but insufficient amounts of **4** were available for testing. Nevertheless, it appears that the addition of a 4α-OH group renders agelastatin C less toxic, possibly due to hydrolytic instability of the bis-aminal groups on the imidazolone ring. Agelastatin A was found to be insecticidal against beet army worm, *Spodoptera exigua*, and corn root worm, *Diabrotica undecimpunctata*. Larvae of both insects were fed on a diet treated with either **1** or solvent (control) and monitored for mortality over 4, 5, or 7 days. The 4-day and 7-day LC_{50}s of **1** against beet army worm were 26 μg/mL and 20.3 μg/mL, respectively, within 95% confidence limits, while for corn root worm 5-day and 7-day LC_{50}s were 37 μg/mL and 32 μg/mL. These values are comparable to a commercial preparation of the biopesticide *Bacillus thuringiensis* tested against beet army worm under the same conditions (JavelinWG, LD_{50} 24 μg/mL and 12 μg/mL for 4-day and 7-day trials, respectively).

In summary, we have identified two new agelastatins, **3** and **4**, and shown that agelastatin A (**1**) is significantly toxic toward three arthropods: brine shrimp *Artemia franciscana*, and the insects *Spodoptera exigua* and *Diabrotica undecimpunctata*.

Experimental Section

General. General procedures are described elsewhere.[6] FABMS measurements were performed at University of California, Riverside Mass Spectrometry facility. MALDI FTMS measurements were carried out on a custom-built instrument at the University of California, Davis.[7]

Collection, Extraction, and Description of *Cymbastela* sp. Two samples of *Cymbastela* sp. (order Halichondrida, family Axinellidae; accession numbers 93-04-037 and 93-06-055) were collected by hand using scuba near South Muiron Island (21° 43′ S, 114° 20′ E), Western Australia, at −10 meters in January 1993. Sponge samples were identified by Mary K. Harper. Sample 93-06-055 is a lamellate cup with an orange exterior and yellow interior. Spicules are centrally curved oxea (210 μM average) with sharply pointed ends, sometimes slightly telescoped. The sponge shares morphological characteristics with several other Australasian *Cymbastela* species but clearly differs in secondary-metabolite content from *Cymbastela* sp. containing criamides,[2e] *C. hooperi* (isonitriles),[2a−c] and *C. cantharella* (alkaloid).[2f] The two samples of *Cymbastela* (93-06-055 and 93-04-037) are taxonomically indistinguishable. Both samples are deposited in the department of chemistry, UC Davis, and sample 93-06-055 is placed in the Scripps Institution of Oceanography benthic invertebrate collection (#P1169).

The animals were immediately frozen at −20 °C until required and were extracted using two different protocols. (a) *Sample 93-04-037*: The lyophilized sponge (6.73 g) was extracted with MeOH (3 × 180 mL) and filtered. The combined extracts were concentrated to approximately 200 mL and the extract separated using sequential solvent partitioning against *n*-hexane, $CHCl_3$, and *n*-BuOH.[8] The *n*-hexane layer was separated by HPLC (silica, *n*-hexanes−EtOAc) to give a series of known A-ring norsterols.[9] The $CHCl_3$ (188 mg) extract was purified using flash chromatography on Si gel with stepwise gradient elution from 95:5 $CHCl_3$−MeOH to 100% MeOH, followed by reversed-phase chromatography (C_{18} silica cartridge, 70:30 MeOH−H_2O), and reversed-phase HPLC (Dynamax C_{18} column, 10 × 300 mm, 30:70 MeOH−H_2O) to afford agelastatin A (**1**, 4.0 mg, 0.059% dry wt) and agelastatin C (**3**, 1.0 mg, 0.015%). (b) *Sample 93-06-055*: The lyophilized sponge (27.1 g) was extracted with 1:1 MeOH−CH_2Cl_2. The extract was concentrated to dryness, dissolved in H_2O, and partitioned with EtOAc. The aqueous extract was fractionated by reversed-phase chromatography (C_{18} silica cartridge, stepwise gradient elution H_2O to MeOH), and reversed-phase HPLC (Microsorb C_{18} column, 10 × 300 mm, MeOH−H_2O, 15:85) to afford **1**, **3**, and agelastatin D (**4**, 0.5 mg, 0.002%).

(−)-Agelastatin A (1): colorless solid; $[\alpha]_D$ −59.3° (*c* 0.13, MeOH) (lit.[1a] −84.3° (*c* 0.3, EtOH)); CD (MeOH) $\Delta\epsilon_{219}$ −7.2, $\Delta\epsilon_{246}$ +6.6; ^{1}H NMR and ^{13}C NMR identical with literature values[1a] (see Table 1).

(−)-Agelastatin C (3): 1.0 mg (0.015% dry wt); colorless solid; $C_{12}H_{13}BrN_4O_4$; $[\alpha]_D$ −5° (*c* 0.06, MeOH); UV (MeOH) λ_{max} 278 nm (ϵ 11 000), 228 nm (ϵ 7900); CD (MeOH) $\Delta\epsilon_{219}$ −6.8, $\Delta\epsilon_{246}$ +6.6; IR (ZnSe film) ν_{max} 3322 (br), 2926, 1705, 1654, 1554, 1424, 1334, 1272, 1146, 1126, 744 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; HRFABMS found *m*/*z* 357.0180 (M + H$^+$), calcd for $C_{12}H_{14}{}^{79}BrN_4O_4$, 357.0198.

Agelastatin D (4): 0.5 mg (0.002% dry wt); colorless solid; $C_{11}H_{11}BrN_4O_3$; UV (MeOH) λ_{max} 277 nm (10 800), 227 nm (7900); CD (MeOH) $\Delta\epsilon_{219}$ −6.2, $\Delta\epsilon_{246}$ +6.8; IR (ZnSe film) ν_{max} 3320 (br), 2923, 2852, 1672, 1637, 1597, 1420, 1364, 1350, 1260, 1112, 1072, 1037, 1022 cm^{-1}; ^{1}H NMR, see Table 1; negative ion MALDI FTMS found *m*/*z* 324.9977 (M − H), calcd for $C_{11}H_{10}{}^{79}BrN_4O_3$, 324.9936.

Table 1. ^{1}H- and ^{13}C-NMR Data for Agelastatins C (**3**) and D (**4**) Recorded in CD_3OD [^{13}C NMR multiplicities for **3** were assigned by DEPT and indicated by the number of attached H's (parentheses)]

		agelastatin C (**3**)		
no.	agelastatin A (**1**) ^{1}H NMR δ (mult, J Hz, int)	^{13}C NMR δ (mult)	^{1}H NMR δ (mult, J Hz, int)	agelastatin D (**4**) ^{1}H NMR δ (mult, J Hz, int)
2		160.26 (0)		
4	3.89 bs	89.85 (0)		3.91 (s, 1H)
5		93.78 (0)		
6a	2.65 (dd, J_{gem} 12.9, $J_{6b,7}$ 6.6, 1H)	40.96 (2)	2.68 (dd, J_{gem} 13.3, $J_{6a,7}$ 6.7, 1H)	2.54 (dd, J_{gem} 12.9, $J_{6a,7}$ 6.5, 1H)
6b	2.10 (dd, J_{gem} 12.9, $J_{6b,7}$ 12.3, 1H)		2.05 (dd, J_{gem} 13.3, $J_{6b,7}$ 11.9, 1H)	2.21 (br t, J_{gem} 12.9, $J_{6b,7}$ 12.4, 1H)
7	4.60 (m, $J_{7,6a}$ 6.6, $J_{7,6b}$ 12.3, $J_{7,8}$ 5.4, 1H)	51.97 (1)	4.56 (m, $J_{7,6a}$ 6.7, $J_{7,6b}$ 11.9, $J_{7,8}$ 5.1, 1H)	4.73 (m, $J_{7,6a}$ 6.5, $J_{7,6b}$ 12.4, $J_{7,8}$ 5.4, 1H)
8	4.09 (d, $J_{8,7}$ 5.4, 1H)	61.91 (1)	4.19 (d, $J_{8,7}$ 5.1, 1H)	4.09 (d, $J_{8,7}$ 5.4, 1H)
10		159.61 (0)		
11		124.00 (0)		
13		107.29 (0)		
14	6.33 (d, $J_{14,15}$ 4.2, 1H	113.90 (1)	6.33 (d, $J_{14,15}$ 4.1, 1H)	6.33 (d, $J_{14,15}$ 4.1, 1H)
15	6.92 (d, $J_{15,14}$ 4.2, 1H)	116.11 (1)	6.92 (d, $J_{15,14}$ 4.1, 1H)	6.91 (d, $J_{15,14}$ 4.1, 1H)
N-Me	2.81 (s, 3H)	24.47 (3)	2.78 (s, 3H)	

Exhaustive Methylation of Agelastatin A (1) and D (4). Agelastatin A (**1**, 3.5 mg) was dissolved in DMSO (75 μL), and powdered KOH (ca. 25 mg) was added to the stirred solution. Excess CH_3I was added after 10 min, and the mixture was stirred for an additional 30 min. The reaction mixture was then diluted with H_2O and neutralized with a saturated aqueous NaH_2PO_4. The mixture was eluted on a C_{18} Si cartridge with H_2O followed by MeOH. The MeOH fraction was concentrated and purified by reversed-phase HPLC (Microsorb C_{18} column, 10 × 300 mm, MeOH−H_2O, 67:33) to give **5**. ^{1}H NMR (CD_3OD) was identical with those of literature values.[1a] Positive FABMS showed *m/z* 383 (MH^+) $C_{15}H_{20}{}^{79}BrN_4O_3$. The product was analyzed by reversed-phase HPLC (C_{18} Microsorb, 4.6 mm × 300 mm), and the retention times of compound **5** in two solvent systems were 10.5 min (MeOH−H_2O, 45:55) and 11.8 min (CH_3CN−H_2O, 25:75), respectively.

Compound **4** (47 μg) was treated as for agelastatin A, above, to give **5**: positive ion mode MALDI FTMS *m/z* ($M + H^+$) 383.0714; calcd for $C_{15}H_{20}{}^{79}BrN_4O_3$, 383.0718; reversed-phase HPLC analysis (C_{18} Microsorb, 4.6 mm × 300 mm) in two solvent systems gave a single major compound, **5**, with retention times of 10.3 min (MeOH−H_2O, 45:55) or 11.8 min (CH_3CN−H_2O, 25:75).

Brine Shrimp Bioassay for Agelastatin A (1) and C (3).[10] Newly hatched nauplii of the brine shrimp, *Artemia franciscana*, were used in this bioassay.[11] The eggs were hatched in artificial seawater (37 g/L of Instant Ocean in deionized water) in a two-compartment Pyrex crystallizing dish divided by a vertical plastic strip that left a gap near the bottom of the dish. The eggs were placed in one compartment, and, after 24 h, the free-swimming nauplii swam were harvested from the second compartment after phototaxis in the direction of a 60 W incandescent light.[12]

The nauplii were used in the bioassay 24 to 36 h after the sowing of the eggs. The extracts to be tested were dissolved in 1 mL of artificial seawater containing 5% Tween 80, sonicated, and placed in a well of a 48-well tissue culture plate. A minimum of 25 nauplii were transferred to the well in the tip of a Pasteur pipet, using a minimal amount of hatching fluid in the transfer. Nauplii were also placed in six control wells of the culture plate that were filled with only seawater containing 5% Tween 80.

Compounds **1** and **3** were added to the wells at final concentrations from 0.1 to 1000 ppm. Dead animals present were counted after 24 h with the aid of a hand lens before the remaining live nauplii were killed by the addition of a few drops of 37% formaldehyde solution. The total number of dead animals in each well was counted, and the percent mortality was plotted against the log of the concentration in parts per million.[13] Interpolation of the plot provided LC_{50}s. Compound **1** had an LC_{50} of 1.6−1.7 ppm, and the LC_{50} for **3** was ~220 ppm.

Insect Mortality Assay. Agelastatin A (**1**) was incorporated into a proprietary solid agar diet, containing nutrients, antibiotics, and adjuvants, by serial dilution of a stock MeOH solution into 96-well microtiter plates (1.56−200 μg/mL, triplicate doses, final MeOH concentration, 5%). One neonate beet army worm (BAW) or two neonate corn root worm (CRM) larvae were placed in each well, the plates were covered with clear Mylar and incubated for 7 days under a 16:8 h photocycle at 28 ± 2 °C and 65% relative humidity. Controls were diet alone, 5% MeOH in diet, and JavelinWG (BAW only). BAW mortality was rated at 4 and 7 days after treatment, and CRW mortality was rated at 5 and 7 days. Each well was inspected under a dissecting microscope, and larvae were counted as dead if they remained immotile after stimulus with a needle probe. Mortality data were treated by probit analysis using POLO PC (LeOra Software, Berkeley, CA). BAW: LC_{50} 26 μg/mL (day 4), 20.3 μg/mL (day 7); CRW: LC_{50} 37 μg/mL (day 5), 32 μg/mL (day 7); for BAW and Javelin WG: LC_{50} 24 μg/mL (day 4), 12 (day 7).

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