Majusculoic Acid, a Brominated Cyclopropyl Fatty Acid from a Marine Cyanobacterial Mat Assemblage

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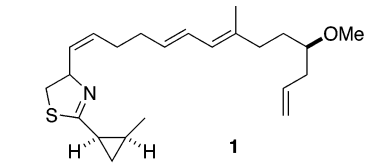
The novel antifungal compound majusculoic acid was isolated from a cyanobacterial mat microbial community. The structure of majusculoic acid was solved by interpretation of mass spectrometric and NMR data and conversion to the corresponding methyl ester. Majusculoic acid exhibits antifungal activity against *Candida albicans* ATCC 14503 (MIC 8 μM).

Most natural products reported from collections of free-living cyanobacteria (mainly *Lyngbya majuscula*) are obtained from filamentous varieties. Marine cyanobacteria drape over coral reef substrates as diaphanous films or cover submerged mangrove roots in coral lagoons or coastal waterways in thick mats. The metabolites from cyanobacteria are mainly cyclic peptides and cyclodepsipeptides,[1] but occasionally polyketides, fatty acids, and highly modified lipids are found. For example, samples of *Lyngbya majuscula* from mangroves in Curaçao provided the lipid curacin A (**1**), a potent antimitotic agent.[2] In constrast to filamentous forms, stromatolithic cyanobacteria, and other tropical "sand-mat" forms that cover the marine benthos between reefs, appear as pigmented patches that are overlaid upon complex microbial communities. Tropical benthic cyanobacterial sand mats are vertically stratified microbial consortia consisting of layers of cyanobacteria, nonphotosynthetic eubacteria, diatoms, and other microbes held together within a mucilaginous mat of polysaccharide.[3a] Although the cyanobacteria that live within these microbial consortia belong to only a few genera (usually *Lyngbya* spp. but occasionally *Anabaena, Phormidium*, and others) and show limited genetic diversity by 16S rRNA,[3b] they have broad chemical diversity and produce a variety of metabolites with molecular structures that may vary greatly with geographic location. Our survey of antifungal activity of extracts of cyanobacterial sand mats, collected in the Bahamas during 2000−2003, revealed a significant "hit rate" against fluconazole-resistant strains of *Candida* species.[4] We now report a brominated cyclopropyl carboxylic acid, majusculoic acid (**2**), from a second cyanobacterial mat with antifungal activity against *Candida* spp. Our previous investigations of a cyanobacterial sand mat collected at Cay Lobos, Southern Bahamas, led to the isolation of a series of antifungal cyclic peptides (e.g., lobocyclamide C[5]); however, none of these compounds were detected in the present sample.

The CH_2Cl_2−MeOH-soluble extract of a single sand−cyanobacterial mat assemblage, collected in a mangrove inlet at Sweetings Cay, Bahamas, was partitioned between EtOAc and H_2O. The organic fraction was passed through a Sephadex LH-20 column (elution with MeOH) with monitoring of antifungal activity of the eluted fractions. Majusculoic acid (**2**) ($[\alpha]_D$ −15.8°, *c* 0.1, MeOH) was isolated as a solid after purification by HPLC. HREIMS of **2** ([M + NH_4^+] *m/z* 314.0888/316.0867) showed the presence of one Br atom (equal intensity $^{79}Br/^{81}Br$ isotope peaks) and established the molecular formula as $C_{15}H_{23}BrO_2$. IR bands observed for **2** (ν 3450, 3070, 1705, 1620 cm^{-1}) and a single C=O signal in the ^{13}C NMR spectrum (δ 178.2 ppm) suggested a carboxylic acid. This assignment was supported by conversion of majusculoic acid into the corresponding methyl ester **3** (CH_2N_2, diethyl ether−MeOH, 0 °C). Majusculoic acid (**2**) was assigned a 4,5-methano-substituted fatty acid structure by interpretation of 2D NMR spectra and identification of the spin systems as follows.



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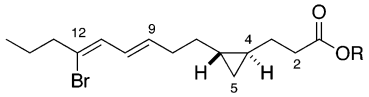
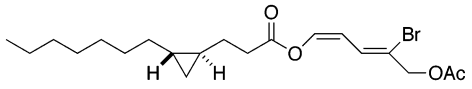


2 R = H
3 R = Me



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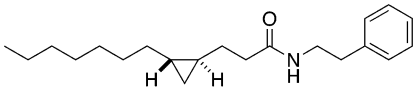

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HMBC correlations were observed from the carboxyl signal (δ 178.2 ppm) to the α-methylene signal of H-2 (δ 2.40, t, J = 6.4 Hz) and the β-methylene signal H-3 (δ 1.22 (m, 2H). Three high-field signals in the ^1H NMR spectrum [δ 0.91, (m, 1H, H-4), 0.22 (m, 2H), and 0.42 (m, 1H, H-6)] were mutually coupled (gCOSY) and assigned to a 1,2-disubstituted cyclopropane ring. The *trans* stereochemistry of the three-membered ring was confirmed by comparison of ^1H and ^{13}C chemical shift comparisons of C-4, C-5, and C-6 with those of related compounds (vide infra) based upon symmetry arguments. The two diastereotopic H-5 methylene ^1H NMR signals (δ 0.22, m, 2H) were almost coincident due to the local pseudo-C_{2v} environment of a *trans*-4,6-dialkyl cyclopropane as noted for **4** (δ 0.19 m, $CDCl_3$).[9] Additionally, the ^{13}C NMR chemical shifts for the C-5 methylene group in **2** (δ 12.1) and **4** (δ 11.7) were

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virtually identical. A conjugated diene was suggested by observation of a weak chromophore (UV λ_{max} 220 nm) and gCOSY cross-peaks for sequential vicinal couplings between H-9 (δ 5.70, 1H ddd, J = 14.8, 7.0 Hz), H-10 (δ 6.15, dd, 1H, J = 14.8, 9.5 Hz), and H-11 (δ 6.10, d, 1H, J = 9.5 Hz). The large H-9−H-10 vicinal coupling constant ($J_{H9,10}$ = 14.8 Hz) and NOE correlation between H-9 and H-11 establish an *E*-C-9/C-10 double bond. The unusually high-field ^{13}C chemical shift of the quaternary sp^2 carbon C-12 (δ 127) in the trisubstituted 1,3-diene, compared with the corresponding signal in a simple model ((3*E*,5*E*)-6-methylnona-3,5-diene; calculated δ 143.7 ppm, ChemDraw Ultra), is reconciled by placement of Br at C-12 (calculated δ 121.4 ppm) and invocation of the "heavy atom effect".[6] The *E*-C-11/C-12 double bond was assigned on the basis of an NOE effect observed from H-11 to H_2-13. Further inspection of gCOSY, TOCSY, and HMBC cross-peaks, originating from the diene and cyclopropyl NMR signals, revealed two intervening CH_2 units between the two groups and termination of the chain by an *n*-propyl group, which completed the structure of **2** (note, the depicted absolute configuration is arbitrary).

Although medium chain fatty acids have been isolated from *L. majuscula* (e.g., the C_{14} "lyngbic acid",[7] and the lower C_{12} homologue, (*E*)-(−)-7-methoxydodec-4-enoic acid[8]), the C_{15} acid **2** appears to be more closely related to the C_{13} cyclopropyl fatty acyl derivatives grenadadiene (**4**) and grenadamide (**5**) obtained from a sample of *L. majuscula* from Grenada.[9] Surprisingly, few other brominated compounds have been reported from cyanobacteria,[10] despite their widespread occurrence in marine phyla, notably Rhodophyta and Porifera.[11] These findings consolidate an emerging theme; small-chain cyclopropyl fatty acids are frequently encountered marker constituents in marine cyanobacteria. Furthermore, the isolation of **2** illustrates that benthic cyanobacterial sand-mat microbial consortia, like their filamentous counterparts that overlay coral and mangroves, are significant sources of natural products.

Majusculoic acid (**2**) exhibited antifungal activity against *C. albicans* ATCC 14503 (minimum inhibitory concentration, MIC 8 μM; fluconazole, MIC 1 μM) and *C. glabrata* (19.3 μM) but was inactive against the fluconazole-resistant strain, *C. albicans* UCD-FR1.

Table 1. ^{1}H, ^{13}C, TOCSY, and HMBC Data for Majusculoic Acid (**2**) in $CDCl_3$

#	^{1}H δ (m, J (Hz), integ.)	^{13}C	HMBC[b]	TOCSY
1		178.2[a]	2,3	
2	2.40 (t, 6.4 Hz, 2H)	29.7	1,3	3,4,5,6
3	1.22 (m, 2H)	29.8	1,2,4	2,4,5,6
4	0.91 (m, 1H)	14.4		2,3,5,6,7
5	0.22 (m, 2H)	12.1		2,3,4,6,7
6	0.42 (m, 1H)	18.7	3,7,8	2,3,4,5,7,8,9
7a	1.28 (m, 1H)	33.9	6,9	3,4,5,6,8,9
7b	1.32 (m, 1H)			
8	2.17 (dd, 7.0, 6.6, 1H)	33.2	7,9	4,5,6,7,9,10
9	5.70 (ddd, 14.8, 7.0, 1H)	127.7	8,11	6,7,8,10,11
10	6.15 (dd, 14.8, 9.5, 1H)	128.0	8,9,11	8,9,11
11	6.10 (d, 9.5, 1H)	136.7	10,13	9,10,13,14,15
12		127.0	10,11,13,14	
13	2.41 (t, 6.7, 2H)	43.8	11,14,15	11,14,15
14	1.56 (tt, 6.7, 6.4, 2H)	21.7	13,15	11,13,15
15	0.93 (t, 6.4, 3H)	13.2	13,14	11,13,14

[a] Detected in the HMBC experiment (J = 8 Hz). [b] $^{2,3}J_{CH}$ filter = 8 Hz.

Experimental Section

General Experimental Procedures. Optical rotation was measured with a Jasco DIP 370 polarimeter. The infrared (IR) spectrum was recorded on a Mattson Galaxy FTIR using neat film deposited on a ZnSe plate. NMR spectra were recorded on a Varian INOVA spectrometer at a proton frequency of 399.77 MHz and a carbon frequency of 100.53 MHz, with solvent used as an internal standard ($CDCl_3$, ^{1}H, δ 7.24 ppm; ^{13}C, 77.00 ppm). Multiplicities of ^{13}C spectra were assigned by DEPT experiments. Mass spectra were recorded on a VG ZAB mass spectrometer. HPLC separations were performed with a Rainin pump with serial detectors (refractive index, Waters R401 differential refractometer; UV, Dynamax UA-5 detector). High-resolution mass spectrometric measurements were provided by the University of California, Riverside Mass Spectrometry Facility.

Material. A single sand mat overlaid with cyanobacteria (02−04−026) was removed to a depth of approximately 2−3 cm by hand from a shallow inlet at −3 m at Sweetings Cay, Bahamas (26°33.100′ N, 77°52.403′ W) in June 2002 and kept at −20 °C until required. A voucher specimen stored in EtOH is archived in the Department of Chemistry, UC Davis.

Isolation. The sand−cyanobacteria (6.2 kg) was extracted with 2:1 CH_2Cl_2−MeOH for several hours with agitation assisted by a direct-drive motorized stirrer. The mixture was filtered and the dark green filtrate concentrated to an oily residue that was partitioned between EtOAc and H_2O. The EtOAc-soluble fraction was passed through a Sephadex LH-20 column (MeOH), and eluted fractions were monitored for antifungal activity against *Candida albicans*. Bioactive fractions were pooled and further separated by flash chromatography (silica, gradient elution with 1:9 EtOAc in *n*-hexane to 100% EtOAc). The fraction eluting in 40% EtOAc was purified by reversed-phase HPLC [C_8 Microsorb 3 μm, 10 × 250 mm, 3 mL/min, 85:15 MeOH−H_2O (0.1% aqueous TFA)] to give **2** (1.8 mg, 2.9 × 10^{-5}% based on wet wt).

Majusculoic acid (2): white amorphous solid; $[\alpha]_D$ −15.8° (*c* 0.1, MeOH); IR (ZnSe) ν 1708, 2800−3250 cm^{-1}; ^{1}H and ^{13}C NMR (see Table 1); HREIMS $[M + H]^+$ *m/z* 314.0888/316.0867 (calcd for $C_{15}H_{23}{}^{79}BrO_2$, 314.0881).

Methyl Majusculoate (3). A sample of **2** (ca. 0.6 mg, R_f 0.23, 2:7 EtOAc−hexane) was treated with ethereal diazomethane (1.0 mL) at 0 °C and gently agitated for 30 min. The volatiles were removed under a stream of N_2 to give **3**, as a nonpolar oil (R_f 0.63). MS (M^+): *m/z* 328.1.

Antifungal Bioassay. Preliminary survey of cyanobacteria−sand extracts was carried out by disk-diffusion assay. Briefly, 98 samples (2002−2003 collection) were suspended in MeOH for >24 h. Each supernatant was evaporated to dryness and reconstituted in 1:1 $CHCl_3$−MeOH at 20 mg/mL (sonication), then centrifuged. The supernatant (15 μL) was applied to a sterile paper disk (6.5 mm diameter) and allowed to air-dry. Disks were placed on plates containing agar-Saboraub media that were previously coated with a lawn of *Candida* (see species below) and the plates grown overnight at 37 °C. Zones of inhibition were measured to the nearest 0.5 mm, and zones of >7 mm were scored as significant. Antifungal susceptibility assays for minimum inhibitory concentrations (MICs) were performed as previously described using minor modifications of published procedures.[12] *C. albicans* ATCC 14503 was obtained from the American Type Culture Collection. *C. glabrata* was obtained from the University of Texas Medical Center. *C. albicans* UCD FR1 was a strain raised in-house by repetitive passage through Saboraub media containing inhibitory concentrations of fluconazole.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and HMBC spectra ($CDCl_3$) for **2.** This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Faulkner, D. J. *Nat. Prod. Rep.* **2002**, *19*, 1−46. (b) Burja, M. A.; Banaigs, B.; Abou-Manjour, E.; Burgess, J. G.; Wright, P. C. *Tetrahedron* **2001**, *57*, 9347−9377. (c) Gerwick, W. H.; Tan, L. T.; Sitachitta, N. In *The Alkaloids: Chemistry and Biology*; Cordell, G. A., Ed.; Academic Press: San Diego, 2001; Vol. 57, pp 75−184.

(2) Gerwick, W. H.; Proteau, P. J.; Nagle, D. G.; Hamel, E.; Blokhin, A. V.; Slate, D. L. *J. Org. Chem.* **1994**, *59*, 1243−1245.

(3) (a) Stal, L. J. *New Phytol.* **1995**, *131*, 1−32. (b) Thacker, R. W.; Paul, V. J. *Appl. Environ. Microbiol.* **2004**, *70*, 3305−3312.

(4) Molinski, T. F. Unpublished results, from samples of cyanobacteria collected in 2002 ($N = 52$). Preliminary testing was conducted using a simple agar disk diffusion assay (300 μg/disk). Strain (% actives): fluconazole-sensitive *Candida albicans* ATCC 14503 (23%), and the fluconazole-resistant strains *C. krusei* (21%), *C. glabrata* (12%), *C. albicans* UCD-FR1 (9.6%).

(5) (a) MacMillan, J. B.; Molinski, T. F. *Org. Lett.* **2002**, *4*, 1883−1886. (b) MacMillan, J. B.; Ernst-Russell, M. A.; de Ropp, J. S.; Molinski, T. F. *J. Org. Chem.* **2002**, *67*, 8210−8215.

(6) Wehrli, F. W.; Marchand, A. P.; Wehrli, S. *Interpretation of Carbon-13 NMR Spectra*; John Wiley & Sons: Chichester, 1983; p 49.

(7) Cardellina, J. H., II; Dalietos, D.; Marner, F.-J.; Mynderse, J. S.; Moore, R. E. *Phytochemistry* **1978**, *17*, 2091. The trivial name lyngbic acid [(*E*)-7-methoxy-4-tetradecenoic acid] was coined by Gerwick et al. (ref 1c).

(8) Mesguiche, V.; Valls, R.; Piovetti, L.; Pfeiffer, G. *Tetrahedron Lett.* **1999**, *40*, 7473−7476.

(9) Sitachitta, N.; Gerwick, W. H. *J. Nat. Prod.* **1998**, *61*, 681−684.

(10) The exceptions, to date, are **6**, a related fatty acid metabolite from *L. majuscula*, collected in Victoria, Australia, (a) Hodder, A. R.; Capon, R. J. *J. Nat. Prod.* **1991**, *54*, 1668−1671, a series of dimeric polybrominated indoles from *Rivularia firma*, (b) Norton, R. J.; Wells, R. J. *J. Am. Chem. Soc.* **1982**, *104*, 3628−3635), and the following macrolides which contain a terminal vinyl bromide: lyngbyallosides from *Lyngbya bouilloni*, (c) Klein, D.; Braekman, J. C.; Daloze, D.; Hoffmann, L.; Demoulin, V. *J. Nat. Prod.* **1997**, *60*, 1057−1059. (d) Luesch, H.; Yoshida, W. Y.; Harigan, C. G.; Doom, J. P.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2002**, *65*, 1945−1948, oscillariolide from *Oscillatoria* sp., (e) Murakami, M.; Matsuda, H.; Makabe, K.; Yamaguchi, K. *Tetrahedron Lett.* **1991**, *32*, 2391−2394, and phormidolide from *Phormidium* sp., (f) Williamson, R. T.; Boulanger, A.; Vulpanovici, A.; Roberts, M. A.; Gerwick, W. H. *J. Org. Chem.* **2002**, *67*, 7927−7936.

(11) Gribble, G. W. In *Progress in the Chemistry of Organic Natural Products*; Herz, W., Kirby, G. W., Moore, R. E., Steglich, W., Tamm, C., Eds.; Springer: Vienna, 1996; Vol. 68.

(12) Jones, R. N.; Barry, A. L.; Gavan, T. L.; Washington, J. A., III. In *Manual of Clinical Microbiology*, 4th ed.; Lennette, A., Balows, A., Hausler, W. J., Shadomy, H. J., Eds.; American Society of Microbiology: Washington, D.C., 1985.

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