Grenadadiene and Grenadamide, Cyclopropyl-Containing Fatty Acid Metabolites from the Marine Cyanobacterium *Lyngbya majuscula*

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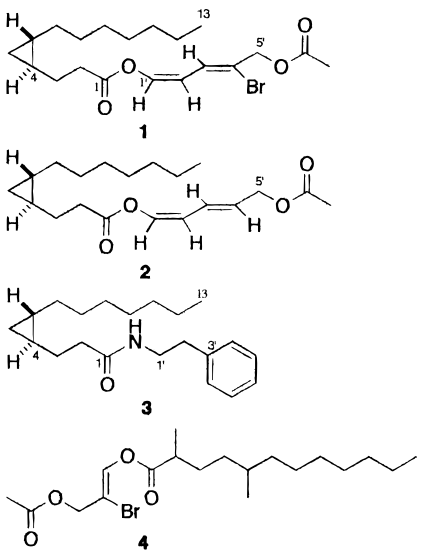
Grenadadiene (**1**), debromogrenadiene (**2**), and grenadamide (**3**), three structurally unique cyclopropyl-containing metabolites, were isolated from the organic extract of a Grenada collection of the marine cyanobacterium *Lyngbya majuscula*. The structures and the relative stereochemistries of these compounds were determined using spectroscopic methods. These are the first reported cyclopropyl-containing fatty acid derivatives from a *Lyngbya* sp. Grenadadiene (**1**) has an interesting profile of cytotoxicity in the NCI 60 cell line assay, while grenadamide (**2**) exhibited modest brine shrimp toxicity (LD_{50} = 5 μg/mL) and cannabinoid receptor binding activity (K_i = 4.7 μM).

The mat-forming marine cyanobacterium (blue-green alga) *Lyngbya majuscula* Gomont (Oscillatoriaceae) is widely recognized as a rich producer of biologically active and structurally unique secondary metabolites. Nearly half of the natural products isolated from *L. majuscula* have fatty acid/polyketide-derived biogenetic subunits that are found in combination with amino acid-derived components. Examples of these "lipopeptides" include the potent fish toxin antillatoxin[1] and the powerful immunosuppressants microcolins A and B.[2] Herein, we report the isolation of three structurally unique cyclopropyl-containing fatty acid-derived metabolites, grenadadiene (**1**), debromogrenadiene (**2**), and grenadamide (**3**), from *L. majuscula* collected in Grenada in the Southern Caribbean. Grenadadiene (**1**) is unusual in several respects, including the incorporation of a bromine atom, a feature rarely observed in cyanobacterial metabolites. Grenadamide (**3**) is intriguing because it contains a β-phenylethylamine substructure, a motif associated with numerous sympathomimetic agents.[3] Grenadamide (**3**) exhibited modest cannabinoid receptor-binding activity (K_i = 4.7 μM) and brine shrimp toxicity (LD_{50} = 5 μg/mL). Grenadadiene has shown an interesting profile of cytotoxicity to cancer cells in the NCI's in vitro 60 cell line assay and has been selected for *in vivo* evaluation.

The cyanobacterium *L. majuscula* was collected from Grenada in July 1995 and kept cold in 2-propanol until extracted. A portion of the crude lipid extract (6 g) was subjected to silica gel vacuum liquid chromatography (VLC) using a mixture of hexanes and EtOAc as eluent. Using the brine shrimp assay and TLC to guide purification, grenadadiene (**1**) and debromogrenadadiene (**2**) were isolated as inactive metabolites from a nonpolar fraction (ca. 2% EtOAc in hexanes), while a moderately polar fraction (ca. 50% EtOAc in hexanes) yielded a mixture of grenadamide (**3**) and the previously described 7-methoxytetradec-4(*E*)-enoic acid as the brine shrimp active material.[4] These latter components were separated by sequential Sephadex and NP-HPLC (Experimental Section).



1

2

3

4

The EIMS of **1** displayed equal intensity $[M]^+$ and $[M + 2]^+$ ions at *m/z* 414 and 416, respectively, indicating that **1** contained one bromine atom; this was confirmed by HREIMS measurement (*m/z* at 414.1405 = $C_{20}H_{31}{}^{79}BrO_4$). This molecular composition required grenadadiene (**1**) to have five double-bond equivalents, two of which were due to ester carbonyls (^{13}C NMR δ 170.1, 169.6; IR $\nu_{C=O}$ 1759 cm^{-1}). Furthermore, four sp^2-hybridized carbons at δ 108.7, 121.3, 124.3, and 137.5 indicated the presence of two double bonds, and therefore, grenadadiene (**1**) contained one ring.

Interpretation of the ^{1}H and ^{13}C NMR (Table 1), ^{1}H−^{1}H COSY, and ^{1}H−^{13}C COSY data generated partial structures **1a**−**1c** (Figure 1) for grenadadiene (**1**). Partial structure **1a** was composed of a fatty acid ester with a 1,2-disubstituted cyclopropane intervening between C-7 (δ33.9) and C-3 (δ29.2). Characteristically shielded methylene protons (H_2-5) and two methine protons (H-4,6) (Table 1) were diagnostic for the cyclopropyl ring. HREIMS of a fragment ion at *m/z* 195.1748 (Figure 1), analyzing for $C_{13}H_{23}O$, further confirmed

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Table 1. ^{1}H and ^{13}C NMR Assignments for Grenadadiene (1), Debromogrenadadiene (2), and Grenadamide (3)[a]

C atom	grenadadiene (1)			debromogrenadadiene (2)			grenadamide (3)		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1		169.6			170.5			171.5	
2	2.49 (t, 7.4)	34.0	17.8, 29.2, 169.6	2.52 (t, 7.3)	34.1	170.5, 26.9, 18.4	2.18 (t, 7.4)	36.8	18.8, 30.3, 171.5
3	1.55 (m)	29.2	18.8	1.60 (m)	29.6		1.50 (brq, 8.5)	30.3	11.7, 36.8, 171.5
4	0.42 (m)	17.8	33.9	0.50 (m)	18.4		0.35 (m)	18.8	
5	0.19 (m)	11.7	18.8, 29.2, 33.9	0.22 (m)	11.7	29.6, 34.1, 34.0	0.16 (m)	11.7	18.8, 30.3, 34.1
6	0.42 (m)	18.8		0.50 (m)	18.0		0.35 (m)	18.1	
7	1.20 (m)	33.9	29.0	1.22 (m)	34.0		1.09 (m)	34.1	29.0
8−10	1.23 (m)	29.0	29.0	1.29 (m)	29.0		1.25 (m)	29.0	
11	1.23 (m)	31.8	29.0	1.29 (m)	31.8		1.25 (m)	31.8	22.6, 14.0
12	1.23 (m)	22.5	14.0	1.29 (m)	29.0		1.25 (m)	22.6	14.0
13	0.90 (t, 7.5)	14.0	31.8	0.88 (t, 6.7)	14.0	31.8	0.87 (t, 6.5)	14.0	22.6, 31.8
1'	7.27 (d, 6.5)	137.5	124.3, 108.7, 169.6	7.12 (d, 6.2)	134.9	111.3, 126.3, 170.5	3.53 (p, 6.6)	40.4	139.0, 171.5
2'	5.75 (dd, 10.5, 6.5)	108.7	121.3, 137.5	5.49 (dd, 10.5, 6.2)	111.3	126.7, 134.9	2.81 (t, 6.6)	35.6	40.4, 128.6, 139.0
3'	6.96 (d, 10.5)	124.3	68.9, 137.5	6.67 (dd, 15.5, 10.5)	126.3	134.9		139.0	
4', (8')		121.3		5.78 (td, 15.5, 6.7)	126.7	111.3	7.20 (m)	128.6	35.6, 126.4
5', (7')	4.79 (brs)	68.9	121.3, 124.3, 170.1	4.62 (d, 6.7)	64.7	171.0	7.31 (m)	128.5	139.0
6'							7.25 (m)	126.4	128.6
OAc	2.08 (s)	20.7	170.1	2.10 (s)	22.6	171.0			
		170.1			171.0				
NH							5.43 (NH, brs)		

[a] All spectra in $CDCl_3$; ^{1}H at 300 MHz, ^{13}C at 75 MHz; assignments by ^{1}H−^{1}H COSY, ^{1}H−^{13}C COSY, and HMBC experiments.

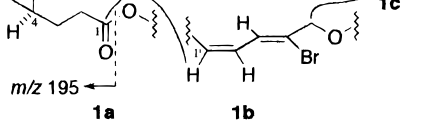

Figure 1. Partial structures **1a**−**c** generated from interpretation of NMR data, shown with HMBC interactions (curved lines), which provided connections between these units.

partial structure **1a**. Partial structure **1b** was partially defined by sequential ^{1}H−^{1}H COSY correlations between δ 5.75 (H-2′), its olefinic partner δ 7.27 (H-1′), and a third olefinic proton at δ 7.0 (H-3′), indicating that the two double bonds in **1** formed a conjugated diene (λ_{max} = 254 nm, MeOH). Two additional deshielded methylene protons at δ 4.79 (H_2-5′) displayed a weak correlation to the deshielded olefinic proton (H-3′), confirming partial structure **1b**. Partial structure **1c** was readily generated as an acetate ester from observation of an ester carbonyl (δ170.1) and a deshielded methyl group (^{1}H δ2.08; ^{13}C δ20.7).

HMBC data (Table 1 and Figure 1) were used to assemble these three partial structures as well as confirm the above structural assignments. The correlation observed from the ester carbonyl (C-1) to the olefinic proton (H-1′) connected partial structure **1a** and **1b**. The long-range correlation between the acetate ester carbonyl and the allylic methylene protons (H_2-5′) enabled connection of the acetate (**1c**) unit to partial structure **1b**. The remaining bromine atom could only be attached to the molecule at the olefinic carbon, C-4′, thereby completing the structure of grenadadiene (**1**).

The almost identical chemical shifts observed for the cyclopropyl ring methylene protons (H_2-5, δ0.19) revealed that they were in a similar chemical environment, indicating that the relative stereochemistry of the ring is trans-1,2-disubstituted. This was confirmed by comparison of ^{1}H NMR chemical shifts at H_2-5 with those of synthetic reference compounds.[5] The double bond geometries in **1** were determined by NOE difference spectroscopy. A *Z* geometry for the H-1′−H-2′ double bond was suggested by seeing an enhancement in H-2′ upon irradiation of H-1′, and this was further supported by a distinctive 6.5 Hz coupling constant between these protons.[6] Similarly, a *Z* geometry for C3′−C4′ was indicated by observing enhancement of H-3′ when the H_2-5′ allylic methylene protons were irradiated.

A minor compound, **2**, isolated by HPLC of the same column fraction that yielded grenadadiene (**1**), analyzed by HREIMS as a debromo analogue of grenadadiene ($C_{20}H_{32}O_4$). Moreover, both molecules possessed very similar ^{1}H and ^{13}C NMR spectra, with the main difference being an additional olefinic proton in the spectrum of **2** that was coupled to another olefinic proton (H-3′) and a deshielded methylene (H-5′). By ^{1}H−^{1}H COSY, this new olefinic proton was easily placed at C-4′, the position bearing bromine in grenadadiene (**1**). The geometry of the C-3′−C-4′ disubstituted olefin was established as *E* by measurement of a 15.5 Hz coupling between C-3′ and C-4′. All other features of the structure and stereochemistry of this minor metabolite, debromogrenadadiene (**2**) (except for the sign of optical rotation, a finding that we are hesitant to interpret), were essentially identical to those of grenadadiene (**1**).

Grenadamide (**3**) displayed a $[M]^+$ at *m*/*z* 315.2563, consistent with a molecular formula of $C_{21}H_{33}NO$. The ^{13}C NMR spectrum of **3** indicated the presence of a monosubstituted phenyl moiety [δ139.0 (s), 128.6 (2C, d), 128.5 (2C, d), 126.4 (1C, d)] and an amide carbonyl (δ 171.5), which accounted for five of the six required degrees of unsaturation. For the remaining unsaturation, the shielded proton signals at δ 0.38 and 0.16 indicated that grenadamide (**3**) also contained a 1,2-disubstituted cyclopropane ring.

Data from ^{1}H and ^{13}C NMR (Table 1), ^{1}H−^{1}H COSY, and ^{1}H−^{13}C COSY were again used to generate two partial structures (Figure 2). Partial structure **3a** was the same fatty acyl group as found in grenadadiene (**1**). The second spin system (**3b**) was a phenyl ring monosubstituted with a β-ethylamine group. HMBC correlations from the H_2-1′ protons to the amide carbonyl connected the two spin systems, completing the structure of grenadamide (**3**).

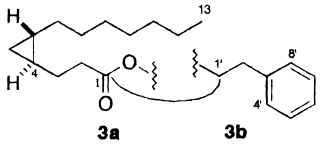

Figure 2. Partial structures **3a** and **3b** generated from interpretation of NMR data, shown with HMBC interactions (curved lines), which provided connections between these units.

Besides grenadadiene (**1**), the only other examples of bromine-containing metabolites from *Lyngbya* sp. are the 2-bromopropenyl ester of 2,5-dimethyldodecanoate (**4**) from a *Lyngbya* species collected from Victoria, Australia,[7] and the aplysiatoxins.[8] The former compound (**4**) is biogenetically related to **1** in that it derives from a fatty acid that is esterified with a bromine- and olefin-containing carbon unit. However, metabolites **1**−**3** are the only reported cyclopropyl-containing fatty acids from a *Lyngbya* species. However, it is perhaps noteworthy that a C_{20} cyclopropane-containing fatty acid was isolated from the digestive gland of the sea hare *Bursatella leachii*.[9] Sea hares are well-known to incorporate secondary metabolites from their algal diet, including mat-forming cyanobacteria.[10] Thus, our finding of structurally similar fatty acids in *L. majuscula* supports the concept of a cyanobacterial origin for the *B. leachii* metabolite.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker AC 300 spectrometer operating at a proton frequency of 300 MHz and a carbon frequency of 75 MHz with the solvent used as an internal standard ($CDCl_3$ at δ 7.26 and 77.0). LR- and HR-EIMS were recorded on a Kratos MS50TC mass spectrometer. UV and IR were recorded on Hewlett-Packard 8452A UV−vis and Nicolet 510 spectrophotometers, respectively. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. HPLC separations were performed with a Waters M-6000A pump, a Rheodyne 7010 injector, and a Waters Lambda-Max 480 spectrophotometer. Merck aluminum-backed thin-layer chromatography sheets were used for TLC, and all solvents were distilled from glass prior to use.

Collection. 2-Propanol preserved *L. majuscula* (voucher specimen available from WHG as GGA-29/Jul/94-02) was collected by hand from shallow water (4−6 m) in July 1995 from Grand Anse Beach, Grenada, and preserved in 2-propanol at low temperature until extraction.

Isolation and Purification. Following filtration of the 2-propanol preservative, the alga (741 g, dry wt) was extracted with CH_2Cl_2/MeOH (2:1) twice, combined with the preservative, and evaporated in vacuo to give the crude extract (11.3 g). A portion of the crude extract (6 g) was fractionated using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH. Eluted material was collected in 14 × 200-mL fractions and monitored by TLC. Similar fractions were combined to give eight fractions. Fraction 2 (1.2 g, eluted with 2% EtOAc/hexanes) was further fractionated twice on Si gel column chromatography and a final purification on NP-HPLC (500 × 10 mm Maxsil 10 μM) with 3% EtOAc/hexanes to give grenadadiene (**1**, t_R = 8−9 min, 58.5 mg, 0.9% of extract) and debromogrenadadiene (**2**, t_R = 12−13 min, 3.2 mg, 0.05% of extract). Fraction 5 (1.2 g, eluted with 50% EtOAc/hexanes) showed brine shrimp toxicity (LD_{50} = 50 μg/mL) and was further fractionated over Sephadex LH-20 using EtOAc/MeOH (1:1) as eluent, followed by a final purification on NP-HPLC (500 × 10 mm Maxsil 10 μM) to give grenadamide (**3**) (30% EtOAc/hexanes, 13.2 mg, 0.2% of the extract).

Grenadadiene (1). Pure grenadadiene showed: $[\alpha]_D$ −8° ($CHCl_3$, c = 0.1); UV (MeOH) λ_{max} 252 nm (ϵ 12 200); IR (neat) ν_{max} 2924, 2855, 1759, 1219, 1115, 1026 cm^{-1}; ^1H and ^{13}C NMR see Table 1; LR EIMS (70 eV) *m/z* 416 (20), 414 (22), 222 (18), 220 (18), 195 (90), 177 (34), 141 (100), 135 (24), 121 (38), 99 (58), 97 (50), 95 (45), 83 (56), 69 (60); HREIMS $[M]^+$ *m/z* 414.1405 (calcd for $C_{20}H_{31}O_4{}^{79}Br$, 414.1406), 416.1385 (calcd for $C_{20}H_{31}O_4{}^{81}Br$, 416.1386).

Debromogrenadadiene (2). Pure debromogrenadiene showed: $[\alpha]_D$ +5° ($CHCl_3$, c = 0.1); UV (hexanes) λ_{max} 232 nm (ϵ 8000); IR (neat) ν_{max} 2954, 2850, 1746, 1221, 1020 cm^{-1}; ^1H and ^{13}C NMR see Table 1; LR EIMS (70 eV) *m/z* 336 (8), 330 (13), 315 (28), 195 (100), 177 (38), 142 (40), 135 (24), 121 (35), 107 (22); HREIMS $[M]^+$ *m/z* 336.2301 (calcd for $C_{20}H_{32}O_4$, 336.2300).

Grenadamide (3). Pure grenadamide showed: $[\alpha]_D$ −11° ($CHCl_3$, c = 0.1); UV (MeOH) λ_{max} 206 nm (ϵ 2600); IR (neat) ν_{max} 3300, 2924, 1645, 1552, 1456, 700 cm^{-1}; ^1H and ^{13}C NMR see Table 1; LR EIMS (70 eV) *m/z* 315 (38), 230 (45), 163 (40), 105 (60), 104 (100), 91 (10), 72 (25); HREIMS $[M]^+$ *m/z* 315.2563 (calcd for $C_{21}H_{33}NO$, 315.2562).

Bioassays for Brine Shrimp Toxicity and Cannabinoid Receptor Binding Activity. Evaluation of the crude extract, chromatography fractions, and pure compounds for brine shrimp (*Artemia salina*) toxicity was determined as detailed in ref 11. The assay of compounds for cannabinomimetic activity was performed as described in ref 12.

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