# Barbamide, a Chlorinated Metabolite with Molluscicidal Activity from the Caribbean Cyanobacterium *Lyngbya majuscula*

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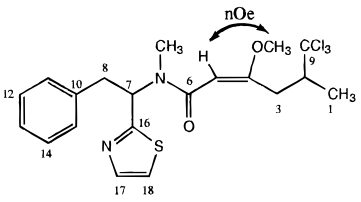
The lipid extract from a Curaçao collection of the marine cyanobacterium *Lyngbya majuscula* was toxic to the mollusc *Biomphalaria glabrata*. Subsequent bioassay-guided fractionation of this extract yielded a novel lipopeptide, barbamide, as the active compound ($LC_{100}$ = 10 μg/mL). The structure of barbamide was determined by spectroscopic methods and was found to contain several unique structural features, including a trichloromethyl group and the methyl enol ether of a β-keto amide.

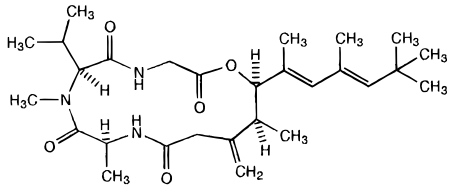
In our continuing search for bioactive secondary metabolites from marine cyanobacteria, we have undertaken an in depth investigation of a Caribbean variety of *Lyngbya majuscula* (family Oscillatoriaceae). The lipid extract of *L. majuscula* collected from Curaçao possessed several bioactivities (Table 1), including potent brine shrimp toxicity (*Artemia salina*, $LC_{50}$ = 25 ng/mL), goldfish toxicity (*Carassius auratus*, $LC_{50}$ = 25 μg/mL), and molluscicidal activity (*Biomphalaria glabrata*, $LC_{100}$ < 100 μg/mL). Bioguided fractionation of the crude extract using each of the above bioassays led to the isolation of four different natural products (**1**−**4**). The molluscicidal effects were largely due to a new compound, barbamide (**1**), which is the subject of this report. The ichthyotoxic effect was due to two distinctly different components, antillatoxin (**2**) and malyngamide H (**3**).[1,2] Finally, the brine shrimp toxicity was traced to an unusual lipid, curacin A (**4**), which also proved to be potently antiproliferative to cancer cells in vitro.[3] Each of these metabolites possesses only one of the above bioactivities (Table 1), leading to our speculation that they represent separate adaptations to a predator-rich environment.[1] Herein we report the structure elucidation of barbamide (**1**) and discuss further the implications of the co-occurrence of these bioactive components in this extract of *L. majuscula*.

Barbamide (**1**) was obtained as a pale yellow oil from *L. majuscula*. It displayed a 100:96:31:3 $MH^+$ ion cluster at *m/z* 461/463/465/467 on FABMS (3-NBA/2% TFA matrix), indicating that **1** possessed three chlorine atoms. An HRFABMS measurement gave a $MH^+$ ion at *m/z* 461.0621 consistent with the molecular formula $C_{20}H_{24}Cl_3N_2SO_2$. The IR spectrum of **1** showed the presence of a tertiary amide (1647 $cm^{-1}$), while the UV spectrum showed an absorption maximum at 240 nm, indicative of an α,β-unsaturated carbonyl moiety.

The $^1$H-NMR and $^{13}$C-NMR spectra were complicated by the presence of two conformers in a ratio of approximately 2:1. However, at elevated temperature (97 °C) the NMR bands of the two conformers coalesced. Unfortunately, the sample was unstable at these elevated temperatures and required that all NMR experiments be conducted at room temperature. Hence, the structure of barbamide (**1**) was determined using the resonances of the major conformer (Table 2).




**1**

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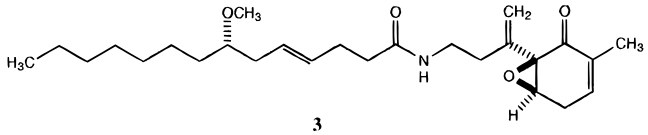


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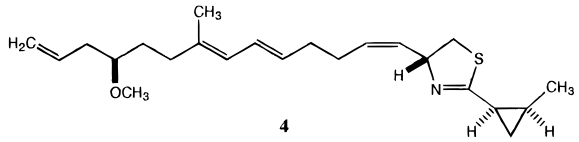


**4**

The $^{13}$C-NMR spectrum of **1** contained an amide carbonyl signal at δ 166.8 (s) together with bands at δ 169.4 (s), 141.9 (d) and 120.6 (d), resonances that were characteristic of a 2-substituted thiazole ring.[4] Furthermore, a polarized conjugated double bond [δ 94.0 (d) and δ 167.0 (s)] and a monosubstituted phenyl moiety [δ 137.7 (s), 128.8 (2C, d), 128.1 (2C, d), and 126.3 (d)] were evident. This accounted for all nine degrees of unsaturation implied by the molecular formula, and thus, barbamide contained only the phenyl and thiazole rings.

The above deductions were further substantiated by the interpretation of the $^1$H NMR, $^1$H-$^1$H COSY, and $^1$H-$^{13}$C COSY, from which it was possible to establish seven partial structures (Figure 1). From the $^1$H-NMR spectrum, the phenyl group (partial structure A) appeared as a five-proton overlapping multiplet in the δ 7.15−7.35 region, whereas the protons of the thiazole moiety (partial structure B) appeared at δ 7.79 (d, *J* =

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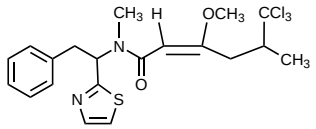
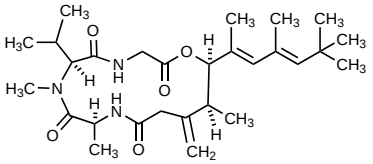
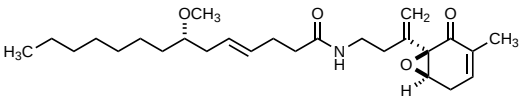
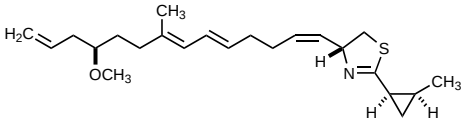
**Table 1.** Biological Evaluation of Crude *L. majuscula Extract* and Pure Compounds **1**−**4**

| material | molluscicidal activity (*B. glabrata*) | Ichthyotoxicity (*C. auratus*) | brine shrimp toxicity (*A. salina*) |
|---|---|---|---|
| crude curaçao *Lyngbya majuscula* extract | $LC_{100}$ < 100 μg/mL | $LC_{50}$ = 25.0 μg/mL | $LC_{50}$ = 0.025 μg/mL |
|  **1** barbamide | $LC_{100}$ = 100 μg/mL | ND[a] | ND[a] |
|  **2** antillatoxin | ND[a] | $LC_{50}$ = 0.05 μg/mL | ND[a] |
|  **3** malyngamide H | ND[a] | $LC_{50}$ = 5.0 μg/mL | ND[a] |
|  **4** curacin A | ND[a] | ND[a] | $LC_{50}$ = 0.0025 μg/mL |

[a] ND = not detected at 25 μg/mL. [b] ND* = not detected in VLC fraction at 100 μg/mL.

**Table 2.** $^1$H- (400 MHz, DMSO) and $^{13}$C-NMR (100 MHz, DMSO) Data for the Major Conformer of Barbamide (**1**)

| C-atom | $^1$H δ(mult, *J* in Hz) | $^{13}$C δ (mult[a]) | HMBC (optimized for 7 Hz) |
|---|---|---|---|
| 1 | 1.10 (d, 6.3) | 15.2 (q) | 34.0, 52.0, 105.6 |
| 2 | 2.84 (obscured) | 52.0 (d) | |
| 3a | 2.74 (obscured) | 34.0 (t) | 15.2, 52.0, 94.0, 105.6, 167.0 |
| b | 3.02 (dd, 13.1, 12.4) | | |
| 4 | | 167.0 (s) | |
| 5 | 5.37 (br. s) | 94.0 (d) | 34.0, 166.8, 167.0 |
| 6 | | 166.8 (s) | |
| 7 | 6.31 (dd, 10.3, 5.5) | 54.3 (d) | 28.2, 35.8, 166.8, 169.4 |
| 8a | 3.25 (dd, 14.3, 10.3) | 35.8 (t) | 54.3, 128.8, 137.7, 169.4 |
| b | 3.56 (obscured) | | |
| 9 | | 105.6 (s) | |
| 10 | | 137.7 (s) | |
| 11 | 7.28 (m) | 128.8 (d) | |
| 12 | 7.28 (m) | 128.1 (d) | |
| 13 | 7.28 (m) | 126.3 (d) | |
| 14 | 7.28 (m) | 128.1 (d) | |
| 15 | 7.28 (m) | 128.8 (d) | |
| 16 | | 169.4 (s) | |
| 17 | 7.79 (d, 3.2) | 141.9 (d) | |
| 18 | 7.71 (d, 3.2) | 120.6 (d) | 141.9, 169.4 |
| N$CH_3$ | 2.88 (s) | 28.2 (q) | 54.3, 166.8 |
| O$CH_3$ | 3.60 (s) | 55.4 (q) | 167.0 |

[a] Multiplicity was determined using the DEPT sequence.

3.2 Hz) and 7.71 (d, *J* = 3.2 Hz). Furthermore, signals characteristic of a methoxyl (δ 3.60) and *N*-methyl amide (δ 2.88) were evident [corresponding $^{13}$C-NMR resonances at δ 55.4 (q) and 28.2 (q), respectively], giving rise to fragments C and D.

From the $^1$H-$^1$H and $^1$H-$^{13}$C COSY spectra two further fragments were obtained. Fragment E consisted of a deshielded methine group (δ 6.31, δ 54.3, d, C-7) connected to a methylene group (δ 3.25 and 3.56, δ 35.8, t, C-8). Fragment F was deduced to consist of a methyl group (δ 1.10, δ 15.2, q, C-1), connected to a methine (δ 2.84, δ 52.0, d, C-2), which in turn was correlated to the methylene protons at δ 2.74 and 3.02 (δ 34.0, t, C-3). This latter group showed an allylic coupling to the methine at δ 5.37 (δ 94.0, d, C-5) confirming its location next to the $\Delta^{4,5}$ double bond. The final fragment G was evident by consideration of the molecular formula, which required the addition of one carbon atom and three chlorine atoms, and a $^{13}$C-NMR spectrum resonance at δ 105.6 (s), which taken together, indicated the presence of a trichloromethyl group.[5,6]

These partial structures were connected through long range correlations observed by HMBC (Table 2 and Figure 1). The correlations observed from the methylene group ($H_2$-8) of partial structure E to C-10 and C-11 of partial structure A and from the methine group (H-7) of partial structure E to C-16 of partial structure B connected these three partial structures. Furthermore,

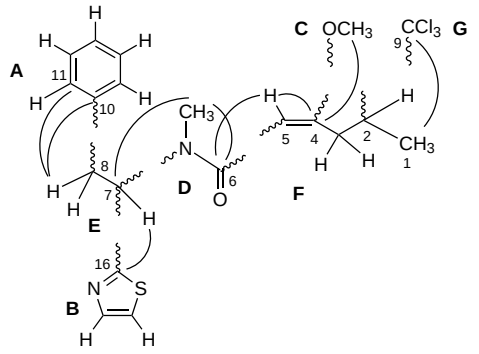



**Figure 1.** Partial structures A−G generated from interpretation of NMR data shown with HMBC connections (curved lines).

correlations between the *N*-methyl group protons and C-6 and C-7 enabled the connection of partial structures D and E. Correlations between the H-5 methine and carbons at C-6 and C-4, the methoxyl group and C-4, and from the $H_3$-1 methyl group to the trichloromethyl group (C-9), completed the structure assignment of barbamide. The stereochemistry at the $\Delta^{4,5}$ double bond was established as *E* by the observation of a strong NOE between the methoxyl group and H-5.

The trichloromethyl portion of barbamide closely resembles the trichloromethyl portion of dysidin, a polychlorinated amino acid derivative found in the sponge *Dysidea herbaceae*.[7] Microscopic investigations of *Dysidea* have shown it to be rich in symbiotic filamentous cyanobacteria. A flow-cytometric separation of the symbiont, *Oscillatoria spongeliae*, from the sponge cells suggested that the polychlorinated amino acid derivatives were associated with the cyanobacterial filaments.[8,9] Our finding of structurally similar components in the marine cyanobacterium *L. majuscula* provides further support for the cyanobacterial origin of these metabolites.

Interestingly, the amine portion of barbamide is the *N*-methyl equivalent of dolaphenine (*N*-methyldolaphenine), a structural component of the antineoplastic peptide dolastatin 10 isolated from the sea hare *Dolabella auricularia*.[10] Hence, it is conceivable that dolastatin 10, or at least a portion of it, also arises from cyanobacterial metabolism. Sea hares are well known to incorporate unique secondary metabolites from their algal diets, which include mat-forming cyanobacteria.[11]

The crude extract of this *L. majuscula* possessed a number of different biological properties (Table 1). It was toxic to brine shrimp, was ichthyotoxic, and also possessed molluscicidal activity. Using each of the above-mentioned bioassays to guide the fractionation of the extract, we were led to four distinctly different classes of natural products (Table 1), each of which was selective in its range of activity. The molluscicidal effects were due to barbamide (**1**), while the ichthyotoxicity was due to two components, antillatoxin (**2**) and malyngamide H (**3**).[1,2] Finally, the brine shrimp toxicity was traced to the potent new antimitotic agent curacin A (**4**).[3] The apparently selective activity of each of these compounds to only one class of animal is of considerable interest and leads to our speculation that compounds **1**−**4** represent separate adaptations by this cyanobacterium to these different classes of predators.

## Experimental Section

**General Experimental Procedures.** NMR spectra were recorded on a Bruker AM 400 spectrometer operating at a proton frequency of 400 MHz and a carbon frequency of 100 MHz with the solvent used as an internal standard (DMSO-$d_6$ at $\delta$ 2.49 and $\delta$ 39.5). MS were recorded on a Kratos MS50TC mass spectrometer. UV and IR spectra were recorded on Hewlett-Packard 8452A UV-vis and Nicolet 510 spectrophotometers, respectively. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. HPLC separation was 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.** The marine cyanobacterium *Lyngbya majuscula* (voucher specimen available from WHG as collection number NSB-15 Dec 91−2) was collected by hand from shallow water (0.1−1.0 m) on 15 December 1991, at Barbara Beach (Spanish Waters), Curaçao, Netherlands Antilles, and stored in 2-propanol at reduced temperature until workup.

**Bioassay for Mollusicidal Activity.** The screening for molluscicidal potential of the crude extract, chromatography fractions, and pure compounds was performed as previously detailed using the test organism *Biomphalaria glabrata*.[12] The extracts, fractions, or pure compounds were dissolved in 20 $\mu$L of EtOH and then diluted to 20 mL with distilled $H_2O$. The snails were observed after 24 h and considered dead when no heart beat could be detected upon microscopic investigation.

**Extraction and Isolation.** A total of 295 g (dry wt) of the alga was extracted with $CH_2Cl_2$/MeOH (2:1) two times to give the crude extract (3.3 g). A portion of the crude extract (3.0 g) was fractionated (Figure 1) using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexane/EtOAc and EtOAc/MeOH. Eluted material was collected in 15 200-mL fractions and monitored by TLC. Similar fractions were combined to give eight fractions. Fraction 5 (258 mg, eluted with 50% EtOAc/hexane) was molluscicidal at 25 $\mu$g/mL. After confirming ($^1$H NMR and molluscicdal activity) that the bioactive component was unreactive to $CH_2N_2$, the fraction was methylated and further fractionated by VLC on reversed phase (RP18) material using a MeOH/$H_2O$ gradient (60% MeOH−100% MeOH). Additionally, recent culture experiments with this isolate of *L. majuscula* conclusively show that the *O*-methyl and *N*-methyl groups in barbamide are not of artifactual occurrence. Fractions eluting with 80% MeOH showed a molluscicidal activity at 25 $\mu$g/mL and were combined. A final purification on RP-HPLC (ODS) with MeOH/$H_2O$ (4:1) as an eluent yielded barbamide (**1**, 18.9 mg, 0.6% of extract).

**Barbamide 1:** Pure barbamide showed $[\alpha]_D^{26}$ −89° (MeOH, *c* 1.9); UV $\lambda_{max}$ (MeOH) 240 nm ($\epsilon$ = 16 000); IR $\nu_{max}$ (film) 2937, 1647, 1605, 1455, 1441, 1248, 1114, 1094, 781, 759 cm$^{-1}$; FABMS (3-NBA/2% TFA) *m*/*z* 461/463/465/467 (100:96:31:3 $MH^+$ ion cluster), 243/245/247/249 (100:96:31:3 ion cluster); HRFABMS (3-NBA/2% TFA) 461.0621 ($C_{20}H_{24}Cl_3N_2SO_2$, $\Delta$ 0.3 mmu); for $^1$H and $^{13}$C NMR, see Table 2.

**Acknowledgment.** We are grateful to the CARMABI Tropical Research Center in Curaçao for their assistance in collection of the algal material and to M. A. Roberts for taxonomic identification. We thank D. G. Nagle for performing biological evaluations, C. Bayne for providing the *Biomphalaria glabrata*, G. Constantine and M. Graber for comments on the manuscript, and J. Rossi for obtaining UV data. We thank R. Kohnert, Oregon State University, Department of Chemistry, for assistance with NMR and B. Arbogast, Oregon State University, College of Agricultural Chemistry, for help with low and high resolution mass spectra. This work was supported by the National Cancer Institute (CA 52955).

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