# Carriebowlinol, an Antimicrobial Tetrahydroquinolinol from an Assemblage of Marine Cyanobacteria Containing a Novel Taxon

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# Carriebowlinol, an Antimicrobial Tetrahydroquinolinol from an Assemblage of Marine Cyanobacteria Containing a Novel Taxon

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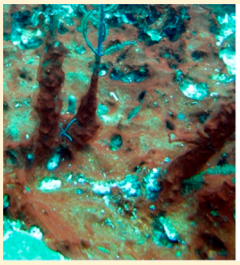
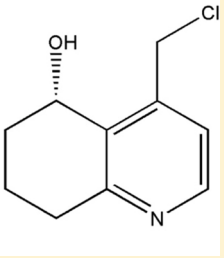
Supporting Information

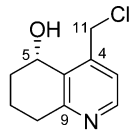
**ABSTRACT:** A combined biodiversity- and bioassay-guided natural products discovery approach was used to explore new groups of marine cyanobacteria for novel secondary metabolites with ecologically relevant bioactivities. Phylogenetic analysis of cyanobacterial collections from Belize revealed a new taxon not previously well explored for natural products. The new alkaloid 5-hydroxy-4-(chloromethyl)-5,6,7,8-tetrahydroquinoline (**1**), named carriebowlinol, and the known compound lyngbic acid (**2**) were isolated from a nonpolar extract and identified by NMR and MS techniques. Compounds **1** and **2** inhibited the growth of pathogenic and saprophytic marine fungi, and **1** inhibited the growth of marine bacteria, suggesting an antimicrobial ecological function.



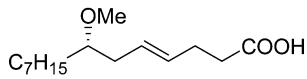



Cyanobacteria have emerged as an important source of biologically active natural products (NPs) with potential benefits against human diseases.[1] Numerous structurally diverse molecules such as polyketide macrolides, linear and cyclic peptides, fatty acids, and alkaloids have been isolated from marine collections of cyanobacteria.[2] Despite the prolific structural diversity among the molecules biosynthesized by marine cyanobacteria, this chemical diversity has not been matched with a corresponding richness in biological diversity of the producing strains. In fact, NPs isolated from marine cyanobacteria have been attributed to a limited number of taxa.[3] However, recent molecular-based phylogenetic inferences of NP-producing marine cyanobacteria have revealed a large degree of novel biodiversity that was overlooked by traditional morphologically based classification systems.[4] Furthermore, phylogenetic analyses of natural product-producing strains of cyanobacteria have revealed correlations between phylogenetic position and production of NPs.[4,5] Herein, we take advantage of the ability of phylogenetic analyses to target taxonomic groups not yet explored for NP discoveries. Although their pharmacological importance has been well explored, the ecological functions of a proportionally low number of cyanobacterial NPs have been documented.[6,7] There is evidence that the NPs from marine cyanobacteria can serve as chemical defenses or may be toxic to grazers and competitors.[7] The chemical defenses of cyanobacteria may play a critical role in bloom formation and persistence under appropriate environmental conditions by limiting the grazing activity of some potential consumers.[7,8] In addition, cyanobacteria have been the source of numerous antimicrobial metabolites,[9] which may provide competitive advantages to producers in their interactions with fungi and other microorganisms.[10]

In our quest to discover new secondary metabolites and to better understand their ecological functions, we isolated a new compound (**1**) and the known lyngbic acid (**2**)[11] from an abundant cyanobacterial mat collected on the coral reef at Carrie Bow Cay, Belize, and assessed antimicrobial activities against ecologically relevant microorganisms from tropical marine environments.




Carriebowlinol (**1**)




Lyngbic acid (**2**)

A thin, reddish mat-forming cyanobacterial bloom (BCBC12-12) was found partially covering the coral reefs near Carrie Bow Cay, Belize (Figure 1A). The environmental specimen was found microscopically to be a mixed assemblage composed of two different types of filamentous cyanobacteria: a larger type

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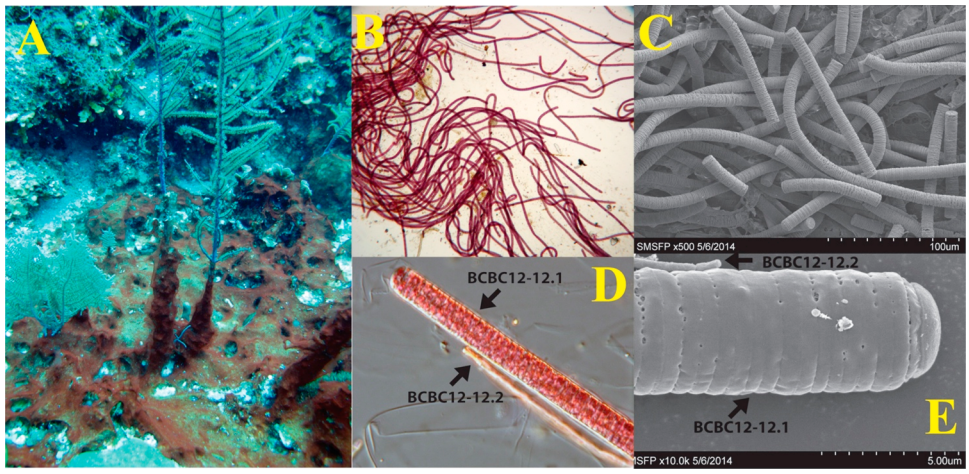


**Figure 1.** Morphological images of the carriebowlinol-producing cyanobacterial specimen. (A) Cyanobacterial mat covering the coral reefs of Carrie Bow Cay, Belize. (B) Microscopic image of the cyanobacterial mat revealing that the larger filamentous type with phenotypic similarities to *Lyngbya majuscula* was the predominant component. (C) Scanning electron microscope (SEM) image revealing that the surfaces of the filaments were free from associated bacterial biofilm. (D, E) Microphotographs highlighting the two major types of filamentous cyanobacteria, BCBC12-12.1 and BCBC12-12.2 by (D) light microscopy and (E) SEM.

**Table 1. NMR Spectroscopic Data for Carriebowlinol (1) in $CD_3OD$ and $CD_3CN$ ($^1H$ 600 MHz, $^{13}C$ 150 MHz)**

| position | $\delta_C$, mult (in $CD_3OD$) | $\delta_H$ ($J$ in Hz), in $CD_3OD$ | $\delta_H$ ($J$ in Hz), in $CD_3CN$ | COSY[a] (in $CD_3OD$) | COSY[a] (in $CD_3CN$) | HMBC[b] (in $CD_3OD$) |
|---|---|---|---|---|---|---|
| 2 | 149.3, CH | 8.37, d (4.8) | 8.40, d (4.8) | 3 | 3 | 3, 4, 9, 10, |
| 3 | 123.7, CH | 7.39, d (4.8) | 7.26, d (4.8) | 2 | 2 | 4, 10, 11 |
| 4 | 149.0, C | | | | | |
| 5 | 63.8, CH | 5.08, dd (3.4, 3.4) | 5.01, ddd (6.2, 3.4, 3.4) | 6a, 6b | 6a, 6b, 5-OH | 4, 7, 9, 10 |
| 6a | 32.6, $CH_2$ | 2.06, m | 1.99, m | 5, 7a, 7b | 5, 7a, 7b | 5, 7, 8, 10 |
| 6b | | 1.86, m | 1.80, m | 5, 7a, 7b | 5, 7a, 7b | 5, 7, 8, 10 |
| 7a | 17.9, $CH_2$ | 2.06, m | 1.99, m | 6a, 6b, 8a, 8b | 6a, 6b, 8a, 8b | 5, 6, 8, 9 |
| 7b | | 1.86, m | 1.80, m | 6a, 6b, 8a, 8b | 6a, 6b, 8a, 8b | 5, 6, 8, 9 |
| 8a | 33.3, $CH_2$ | 2.95, ddd (17.2, 5.5, 5.5) | 2.91, ddd (17.2, 6.8, 4.1) | 7a, 7b, 8b | 7a, 7b, 8b | 6, 7, 9, 10 |
| 8b | | 2.82, ddd (17.2, 9.8, 6.6) | 2.76, ddd (17.2, 6.2, 5.4) | 7a, 7b, 8a | 7a, 7b, 8a | 6, 7, 9, 10 |
| 9 | 158.8, C | | | | | |
| 10 | 132.9, C | | | | | |
| 11a | 42.2, $CH_2$ | 5.04, d (12.4) | 4.98, d (12.3) | 11b | 11b | 3, 4, 10 |
| 11b | | 4.73, d (12.4) | 4.69, d (12.3) | 11a | 11a | 3, 4, 10 |
| 5-OH | | 3.29, s | 3.21, d (6.2) | | 5 | |

[a]COSY correlations are from proton(s) stated to the indicated proton(s). [b]HMBC correlations are from proton(s) stated to the indicated carbon.

that was clearly the most dominant in terms of biomass (strain BCBC12-12.1) and a finer type (strain BCBC12-12.2) tightly associated with strain BCBC12-12.1 (Figure 1B–E). Phylogenetic inference of the two cyanobacterial strains showed that they fell into distinct lineages clustering with morphologically similar specimens (Figure S1). However, due to the tight association between the two filaments and the difficulties to separate them, the correlations between respective filament and gene sequence were assumed based on phenotypic similarities with related strains of cyanobacteria.

The strain BCBC12-12.1 (GenBank KJ766311), despite sharing phenotypic similarities with *Lyngbya majuscula*, formed an independent lineage, which was evolutionarily distinct from the genus *Lyngbya* as well as any known cyanobacterial groups (Figure S1). This phylogenetic lineage corresponded with clade IV proposed in Engene *et al.*,[4] which has been shown to be one of the most chemically rich marine cyanobacterial groups. NP-producing strains within clade IV include the credneramides A and B producing strain PNG-05-19-05-13 (GenBank KC222263), the malyngolide-producing strain FFP12-4 (GenBank KC207937), and the lyngbyoic acid-producing strain IRL-1 (GenBank KC222261). Strain BCBC12-12.1 was genetically distinct within the NP-rich clade IV, suggesting that this specimen would be a potential target for the discovery of novel NPs. On the other hand, strain BCBC12-12.2 was most closely related to specimens of the genus *Hormoscilla*, a cyanobacterial lineage that has not yet yielded any NPs and is commonly associated with other marine cyanobacteria.

Freeze-dried BCBC12-12 was extracted with a mixture of EtOAc–MeOH (1:1) to afford a lipophilic extract, which was evaluated against three species of marine fungi, *Lindra thalassiae*, the saprophytic *Dendryphiella salina*, and *Fusarium* sp. The extract showed potent antifungal activity against all the species and was thus subjected to $^1H$ NMR analyses and antifungal bioassay-guided fractionation to identify the active compounds.

A portion of the extract was subjected to solvent partitioning, and the EtOAc-soluble fraction was fractionated by $SiO_2$ chromatography to give two antifungal metabolites, the new compound **1**, obtained as a white, amorphous solid after recrystallization with EtOAc, and the known compound lyngbic acid (**2**), which was identified by comparison of $^1H$ NMR, $^{13}C$


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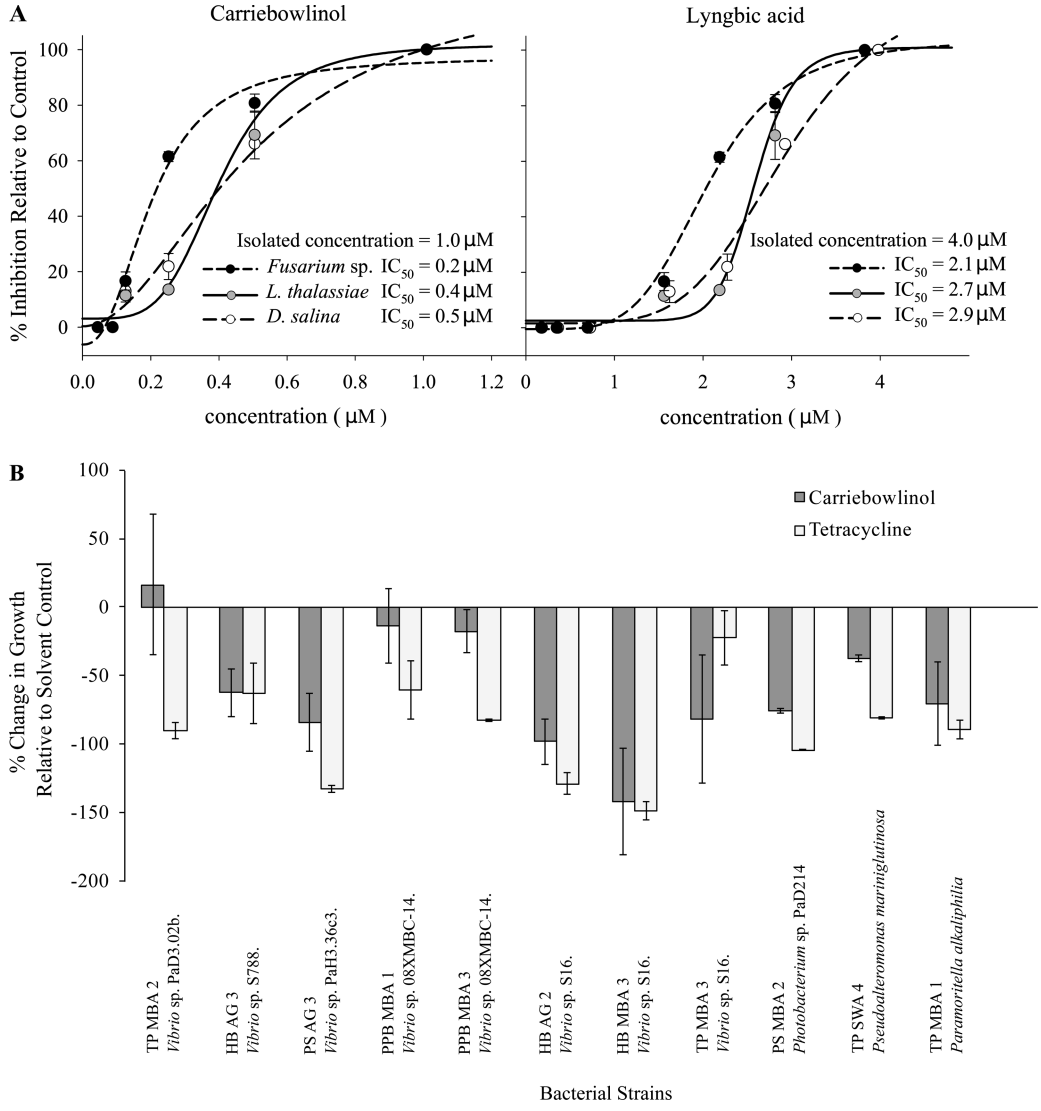


**Figure 2.** Ecological antimicrobial activity: (A) Growth inhibition curves of carriebowlinol (**1**) and lyngbic acid (**2**) on marine fungi *Fusarium* sp., *Lindra thalassiae*, and *Dendryphiella salina* relative to solvent controls. $IC_{50}$ values and isolated concentrations (as the molar amount of isolated compound per algae wet mass) are given. (B) Growth inhibitory effects of carriebowlinol (**1**) at 1 $\mu$M and tetracycline (positive control at 0.225 $\mu$M) against 11 strains of marine bacteria. Bars represent mean percent change in absorbance relative to solvent controls across three replicate 96-well plates; error bars denote the standard error ($n$ = 3). For details about the bacterial strains, see Table S1.

NMR, and MS data reported in the literature.[11,12] The observed specific rotation of lyngbic acid, $[\alpha]^{26}_{D}$ −9.3 ($c$ 0.6, $CHCl_3$), was comparable to the reported value for 7(*S*)-methoxytetradec-4(*E*)-enoic acid ($[\alpha]^{26}_{D}$ −9.0 ($c$ 1.07, $CHCl_3$)[13]).

The structure of **1** was elucidated by interpreting the $^1$H NMR, $^{13}$C NMR, COSY, edited-HSQC, and HMBC spectra in $CD_3OD$ and in $CD_3CN$. Its molecular formula was determined as $C_{10}H_{12}ClNO$ on the basis of HREIMS/APCIMS and NMR spectroscopic data (Table 1). The ratio of $[M + H]^+$ isotope peaks, 3:1, at $m/z$ 198.0673/200.0642 clearly indicated the presence of one chlorine atom. Four carbon−carbon double-bond signals and another carbon−heteroatom double-bond signal in the $^{13}$C spectrum accounted for three of the five degrees of unsaturation implied by the molecular formula. Two mutually coupled low-field $^1$H doublets at $\delta_H$ 7.39 ($J$ = 4.8 Hz, $\delta_C$ 123.7) and $\delta_H$ 8.37 ($J$ = 4.8 Hz, $\delta_C$ 149.3) in $CD_3OD$ and three additional olefinic $^{13}$C signals at $\delta_C$ 158.8, 149.0, and 132.9 suggested the presence of one trisubstituted pyridine ring in the molecule. These data accounted for one of the remaining two degrees of unsaturation. The remaining degree of unsaturation could be accounted for by an additional ring within the structure of **1**, consistent with a 5,6,7,8-tetrahydroquinolinic system, which was confirmed by $^{13}$C, COSY, and HMBC correlations (Table 1).

The chemical shift values for geminally coupled C-11 methylene protons ($\delta_H$ 5.04, 4.73) and the $^{13}$C value of $\delta_C$ 42.2 indicated the presence of a chloromethyl moiety. HMBC correlations from hydrogens at H-11 ($\delta$ 5.04 and 4.73) to C-3 ($\delta_C$ 123.7), C-4 ($\delta_C$ 149.0), and C-10 ($\delta_C$ 132.9) established the position of this chloromethyl group in the pyridine ring. Strong absorption at 3216 cm$^{-1}$ in the IR spectrum and the $^{13}$C and $^1$H chemical shifts for C-5 in $CD_3OD$ at $\delta_C$ 63.8 and $\delta_H$ 5.08 (dd, $J$ = 3.4, 3.4 Hz) indicated the presence of a benzylic-like secondary hydroxy group in the molecule. The COSY spectrum in $CD_3CN$ indicated the coupling between the H-5 oxymethine at $\delta_H$ 5.01 and the hydroxy signal at $\delta_H$ 3.21 and thus confirmed the presence of a secondary hydroxy group in the molecule. The HMBC correlations in $CD_3OD$ from the


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oxymethine proton at $\delta_H$ 5.08 to the $sp^2$ carbons at $\delta_C$ 132.9, C-10; 149.0, C-4; and 158.8, C-9, in conjunction with the coupling of the oxymethine proton at $\delta$ 5.08 to $H_2$-6 observed in the COSY spectrum in $CD_3OD$, confirmed the C-5 position for the hydroxy group. This assigned position was supported by a correlation between H-5 at $\delta_H$ 5.13 and H-11 at $\delta_H$ 4.94 in the NOESY spectrum taken in $CDCl_3$. The COSY data indicated the connection of the hydroxy methine to three consecutive $CH_2$ groups. As seen in the NMR Table 1, the HMBC data connected the $-CHOH-CH_2-CH_2-CH_2-$ moiety to the C-9 and C-10 positions of the pyridine ring to complete the planar structure of the compound.

All efforts to determine the absolute configuration at C-5 using the modified Mosher's method were unsuccessful apparently due to the steric effect of the chlorine atom at C-11 and the instability of the tetrahydroquinoline ring system. The absolute configuration at C-5 was then proposed by the comparison of the observed positive specific rotation of **1** ($[\alpha]^{25}_D$ +51.3) with the previously reported values for the synthetic analogues (+)-(5*S*)-5-hydroxy-5,6,7,8-tetrahydroquinoline ($[\alpha]^{20}_D$ +44)[14] and (−)-(5*R*)-5-hydroxy-5,6,7,8-tetrahydroquinoline ($[\alpha]^{20}_D$ −40).[15] The positive specific rotation value of **1** was comparable to the former, thus suggesting a 5*S* configuration for compound **1**. These data support the structure of carriebowlinol (**1**) as (+)-5(*S*)-hydroxy-4-(chloromethyl)-5,6,7,8-tetrahydroquinoline.

Compound **1** is only the fourth quinoline alkaloid analogue from marine cyanobacteria. It is structurally different from other quinoline alkaloids previously isolated from two different collections of cyanobacteria identified as *Lyngbya majuscula* based on morphological features.[16] The morphological similarities between strain BCBC12-12.1 and *L. majuscula* suggest that these previous quinoline-producing cyanobacteria might be the same or related taxa. In general, quinoline alkaloids are relatively rare in marine organisms and are most commonly associated with sponge and bacterial metabolism.[3] In contrast, lyngbic acid (**2**) and structurally related metabolites have been isolated from geographically dispersed collections of cyanobacteria.[9] Moreover, lyngbic acid may serve as an important building block for the biosynthesis of some cyanobacterial metabolites, e.g., malyngamides;[12] however, no malyngamides were found in this sample.

In order to evaluate the potential ecological function of **1** and **2**, the ability to inhibit the growth of three species of deleterious marine fungi (*D. salina*, *L. thalassiae*, and *Fusarium* sp.) and 11 strains of marine bacteria were investigated. Here, both compounds, at their isolated concentration (the molar amount of isolated compound per algae wet mass, 1 $\mu$M for **1** and 4 $\mu$M for **2**), completely inhibited the growth of all three fungal strains. Moreover, compound **1** was on average 9- to 10-fold more active than compound **2**, as indicated by their $IC_{50}$ values (Figure 2A). For both compounds, the isolated concentrations are higher than the observed $IC_{50}$ values for each marine pathogen (Figure 2A), indicating that they are present within the organism in sufficient concentrations to inhibit the growth of these fungi under natural conditions. Compound **1** also showed strong antibacterial activity (Figure 2B) against marine bacterial strains (Table S1). Although the antibacterial activity of compound **2** has not been tested in this work, its ability to disrupt quorum sensing in *Pseudomonas aeruginosa* has been reported.[9] Our results suggest an important ecological function of compounds **1** and **2** as antimicrobial agents. Scanning electron microscopy (SEM) revealed that the surface of the cyanobacterial filaments was completely clean from associated microbes (Figure 1C, E). This result reinforces the idea that the secondary metabolites could be biosynthesized by the cyanobacteria to inhibit microbial biofilm formation. Here, we demonstrated that ecologically driven studies can be useful to discover new active secondary metabolites and can help us to better understand the ecological function of NPs in marine environments.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** The melting point was measured using a Gallenkamp melting point apparatus. The optical rotation data for **1** and **2** were recorded on a Jasco P2000 polarimeter and on a Rudolph Research Analytical Autopol III automatic polarimeter, respectively. UV spectrophotometric data were acquired on a Shimadzu PharmaSpec UV−visible spectrophotometer. IR spectroscopic data were obtained on a Thermo Scientific iS5 FT-IR spectrometer. NMR data were collected on a JEOL ECA-600 spectrometer operating at 600.17 MHz for $^1H$ and 150.9 MHz for $^{13}C$. The edited-HSQC experiment was optimized for $J_{CH}$ = 140 Hz and the HMBC spectrum was optimized for $^{2/3}J_{CH}$ = 8 Hz. $^1H$ NMR chemical shifts (referenced to $CH_3CN$ observed at $\delta_H$ 1.93 and $CH_3OD$ at $\delta_H$ 3.30) were assigned using a combination of data from 2D DQF COSY and multiplicity-edited HSQC experiments. Similarly, $^{13}C$ NMR chemical shifts (referenced to $CD_3CN$ observed at $\delta_C$ 118.2 and $CD_3OD$ at $\delta_C$ 49.0) were assigned on the basis of multiplicity-edited HSQC experiments. The HRMS data were obtained using an Agilent 6210 LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector at the Mass Spectrometer Facility at the University of California, Riverside, USA. Silica gel 60 (EMD Chemicals, Inc. 230−400 mesh) and Varian BondElut octadecyl ($C_{18}$) were used for column chromatography. All solvents used were of HPLC grade (Fisher Scientific), and all NMR solvents were used as deuterium solvents.

**Collection and Morphological Characterization of Biological Material.** The cyanobacterial specimen BCBC12-12 was collected by scuba at a depth of 12−20 m on the east side of Carrie Bow Cay, Belize (16°80′261″ N; 88°08″188″ W). Collected biomass was cleaned under a dissecting microscope and preserved for (i) genetic analysis in 10 mL RNAlater (Ambion Inc.) and (ii) morphological analysis in seawater with 5% formalin, and was also frozen at −20 °C, and then the freeze-dried biomass was used for (iii) chemical analysis. Light microscopy was performed using a Leica epifluorescent microscope equipped with a Nikon Coolpix camera. Samples for SEM were placed on glass slides that had been coated with a drop of Tissue TAC slide adhesive (DADE) to facilitate adhesion. Samples were then fixed in 2.5% glutaraldehyde in 1× phosphate-buffered saline for 30 min and a secondary fix of 2% osmium tetroxide(aq) for 30 min. Dehydration was achieved with a graded (20%, 50%, 70%, 90%, 100%, 100%) ethanol series. Samples were then critical point dried and sputter coated (200 A) with a gold−palladium mixture. A Hitachi S-4800 SEM was used to image the samples.

Putative taxonomic identification was performed in accordance with modern taxonomic systems.[17,18] BCBC12-12 was deposited in the U.S. National Herbarium Algal Collection at the National Museum of National History (NMNH) and is available under the collection number 217967 (Barcoding number 01097574). The collection and strain information for BCBC12-12 can be accessed through the Research and Collections Information System (RCIS) database at the NMNH.

**Gene Sequencing.** All procedure details are reported in the Supporting Information.

**Extraction and Isolation.** The freeze-dried material (21.4 g) was extracted with EtOAc−MeOH (1:1) (2 × 1 L, 24 h each). The filtered extracts were combined and concentrated under reduced pressure, resulting in 3.05 g (yield 14.2%, dry wt) of the lipophilic extract. A portion (2.86 g) of this extract was partitioned between EtOAc and $H_2O$, and the aqueous portion subsequently partitioned between *n*-


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BuOH and $H_2O$. Concentration of these fractions by rotary evaporation at 45 °C under reduced pressure yielded 0.71 g of EtOAc-soluble fraction, 0.22 g of *n*-BuOH-soluble fraction, and 1.99 g of water-soluble fraction. The EtOAc-soluble fraction was chromatographed on a flash column of $SiO_2$ (11.0 g) using a hexanes−EtOAc−MeOH step gradient system to give six subfractions. Subfraction 4 (124.4 mg) eluted with EtOAc was obtained as an amorphous solid. The solid was recrystallized with EtOAc to give 40.0 mg of **1** (yield 0.20% dry wt and 0.02% wet wt). Subfraction 2 (205.5 mg) eluted with 25% EtOAc in hexanes was shown to be the known compound lyngbic acid (**2**, yield 1.02% dry wt, 0.102% wet wt).

**Carriebowlinol (1):** white, amorphous powder; mp 160−161 °C; $[\alpha]^{25}_D$ +51.3 (*c* 4.08, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 210 (5.04), 271 (2.65); IR $\nu_{max}$ 3216 (broad), 1589 and 1431 $cm^{-1}$; $^1H$ NMR ($CD_3OD$, $CD_3CN$, 600 MHz) and $^{13}C$ NMR ($CD_3OD$, 150 MHz) data see Table 1; HRESI/TOFMS *m*/*z* 198.0673/200.0642 $[M + H]^+$ (calcd for $C_{10}H_{13}ClNO$, 198.0680).

**Marine Antifungal Assay.** The growth inhibition assay described by Kubanek et al.[19] was used to assess the antifungal effects of extracts, fractions, and pure compounds (treatments) against the marine fungi *Lindra thalassiae* (MP005), *Dendryphiella salina* (EBGJ), and *Fusarium* sp. (MP012). The $IC_{50}$ and standard error values were computed for compounds **1** and **2**. Procedure details are reported in the Supporting Information.

**Marine Antibacterial Assay.** Compound **1** was tested at 1 $\mu$M against 11 marine bacterial strains (Table S1) using a 96-well assay modified from Morrow et al.[20] Lyngbic acid **2** was insoluble in the medium used and therefore was not considered for this work. The detailed procedure is described in the Supporting Information.

## ■ ASSOCIATED CONTENT

### Supporting Information

Supplementary Figure S1 and Table S1. Phylogenetic inference, gene sequencing, extraction and isolation procedures, and complete marine antifungal and antibacterial assays. $^1H$, $^{13}C$, DQF COSY, HSQC, HMBC, NMR spectra in $CD_3OD$, $^1H$, DQF COSY spectra in $CD_3CN$, and NOESY spectrum in $CDCl_3$ for compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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## ■ DEDICATION

Dedicated to Dr. William Fenical of Scripps Institution of Oceanography, University of California−San Diego, for his pioneering work on bioactive natural products.

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