Crossbyanols A−D, Toxic Brominated Polyphenyl Ethers from the Hawai'ian Bloom-Forming Cyanobacterium *Leptolyngbya crossbyana*

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Periodically, the marine cyanobacterium *Leptolyngbya crossbyana* forms extensive blooms on Hawai'ian coral reefs and results in significant damage to the subtending corals. Additionally, corals near mats of this cyanobacterium, but not directly overgrown, have been observed to undergo bleaching. Therefore, samples of this cyanobacterium were chemically investigated for bioactive secondary metabolites that might underlie this toxicity phenomenon. ^{1}H NMR spectroscopy-guided fractionation led to the isolation of four heptabrominated polyphenolic ethers, crossbyanols A−D (**1**−**4**). Structure elucidation of these compounds was made challenging by their very low proton to carbon (H/C) ratio, but was completed by combining standard NMR and MS data with 2 Hz-optimized HMBC data. Derivatization of crossbyanol A as the diacetate assisted in the assignment of its structure. Crossbyanol B (**2**) showed antibiotic activity with an MIC value of 2.0−3.9 μg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA) and relatively potent brine shrimp toxicity (IC$_{50}$ 2.8 ppm).

Cyanobacteria are well known for their interesting natural products chemistry, in part because some of these are environmental toxins with noxious properties to humans and domesticated animals and in part because they have provided a wealth of structurally diverse natural products with potent pharmacological activities and potential biomedical utility.[1] In 2008, there was an unusually extensive bloom of the cyanobacterium *Leptolyngbya crossbyana* (Tilden) Anagnostidis et Komárek 1988 reported on Honaunau reef in Hawai'i.[2] Dense colonies of *L. crossbyana* were observed to overgrow coral branches and apparently caused the subtending corals to die. Additionally, coral branches near these mats but not directly overgrown were also observed to undergo bleaching (release of symbiotic zooxanthellae) and showed general signs of stress. To date, however, there have been no chemical investigations of this bloom-forming cyanobacterium from Hawai'i or any other location. However, another species of *Leptolyngbya* collected from Panama was recently reported to produce an unusual depsipeptide, coibamide A, which has potent cancer cell antiproliferative properties.[3] Additionally, a congener of microcystin and its biosynthetic gene were reported from a *Leptolyngbya* sp. isolated from a cyanobacterial mat responsible for black band disease on Florida Coral reefs.[4] Therefore, we undertook the chemical investigation of *L. crossbyana* to identify its toxic constituents, which might underlie its invasiveness on Hawai'ian reefs as well as provide new pharmacologically active compounds for our drug discovery program. Herein, we report the isolation, structure elucidation, and bioactivity of several new secondary metabolites from Hawai'ian collections of *L. crossbyana*.

Samples of *L. crossbyana* were collected by scuba at Honaunau reef off the island of Hawai'i in January 2009. It was found growing as extensive mats with large colonies covering corals of the genus *Porites*.[2] The cyanobacterial brown-red thalli were smooth, firm, and large in size (sometimes exceeding 50 cm in diameter). Microscopically, the colonies were composed of mats of entangled, fine and slightly curved filaments. The filaments were thin, approximately 2 μm wide, and enclosed by thin sheaths. The cells were approximately 3 μm long and 1.5 μm wide with constrictions

crossbyanol A (**1**), R$_1$ = R$_2$ = H
crossbyanol B (**2**), R$_1$ = R$_2$ = SO$_3$H
crossbyanol C (**3**), R$_1$ = SO$_3$H, R$_2$ = H
crossbyanol D (**4**), R$_1$ = H, R$_2$ = SO$_3$H
crossbyanol A diacetate (**5**), R$_1$ = R$_2$ = COCH$_3$
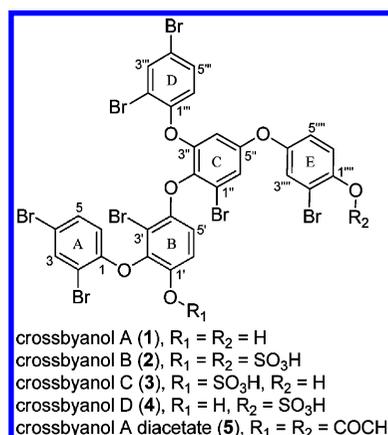
at their cross-walls. A phylogenetic inference using partial SSU (16S) rRNA gene sequences revealed relatedness and shared evolutionary history with other stromatolite-forming or calcium carbonate-encrusting cyanobacteria of the order Oscillatoriales (Figure 1). This phylogenetic group is composed of morphologically similar members of the former *Lyngbya-Plectonema-Phormidium* (LPP) group, which includes the genus *Leptolyngbya*. Morphologically, this sample was consistent with the morphospecies *Leptolyngbya crossbyana*.[5]

The organic extract (1.7 g) was subjected to silica gel vacuum-liquid chromatography (VLC, stepwise gradient of hexanes/EtOAc/MeOH) to produce nine fractions (A−I). Fraction H was found to possess a natural product profile of interest and was thus subjected to ^{1}H NMR-guided fractionation using repetitive silica Sep-Pak chromatography followed by RP HPLC to afford four pure aromatic compounds, crossbyanols A (**1**, 48 mg, 2.8%), B (**2**, 56 mg, 3.3%), C (**3**, 7.9 mg, 0.5%), and D (**4**, 4.9 mg, 0.3%).

EIMS of crossbyanol A (**1**) showed a complex molecular ion cluster at *m/z* 1023.5, 1025.5, 1027.4, 1029.4, 1031.4, 1033.4, 1035.4, and 1037.4 in a ratio of 1:7:21:35:35:21:7:1, indicating the presence of seven bromine atoms in the molecule. The molecular formula of **1** was determined as C$_{30}$H$_{15}$^{79}Br$_7$O$_6$, on the basis of HRESIMS (obsd *m/z* [M + Na]$^+$ 1046.5045; calcd for *m/z* 1046.5044). The ^{1}H and ^{13}C NMR spectra showed all peaks to be in the aromatic range (δ_H 6.27−7.72; δ_C 108.5−155.8 ppm, Table 1; see Supporting Information) and was composed of 13 aromatic

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Figure 1. Maximum-likelihood (PhyML) phylogenetic analysis of bloom-forming Hawai'ian *Leptolyngbya crossbyana* (arrow) based on 16S rRNA nucleotide sequences. The specimens are indicated as species, strain, and acc. nr in brackets. Specimens designated with an asterisk represent type-strains obtained from *Bergey's Manual*. The support values of important nodes are indicated as boot-strap (ML), posterior probability (MrBayes), and boot-strap (MP).

methines and 17 aromatic quaternary carbons, 10 of which were oxygenated. Coupling patterns and constants for each proton resonance as well as COSY correlations indicated the presence of three 1,2,4-trisubstituted phenyl rings, one 1,2,3,4-tetrasubstituted phenyl ring, and one 1,2,3,5-tetrasubstituted phenyl ring (Table 1). One- and two-dimensional NMR analysis, including 8 Hz-optimized HMBC, allowed assignment of all carbon atoms in the five phenyl rings (Table 1 and Figure 2). These were composed of two 2,4-dibromo-1-oxygenated phenyl rings, a 2-bromo-1,3-dioxygenated phenyl ring, a 3-bromo-1,2,4-trioxygenated phenyl ring, and one 1-bromo-2,3,5-trioxygenated phenyl ring. The remaining two protons implicit in the molecular formula were not observed in the CD_3OD ^{1}H NMR spectrum and, thus, were suspected to represent two exchangeable hydroxy protons. This was confirmed by an IR absorption band at 3354 cm^{-1} and the formation of diacetate derivative **5** upon acetylation (*m/z* 1152 for the formic acid adduct ion peak by ESIMS).

Theoretically, on the basis of the above partial structures with unassigned connectivities through six oxygen atoms, the number of possible arrangements was calculated at 144. The locations of the two free phenol groups were determined by the NMR-based assignment of the aromatic protons in **5** combined with the shifts in key protons following acetylation. Specifically, the proton signals of H-6′ and H-6″″ were significantly downfield shifted by 0.38 and 0.34 ppm, respectively (Figure 3), and therefore, the acetate functionalities were localized to C-1′ and C-1″″. As a result, the theoretical number of possible structures for crossbyanol A was reduced to 18.

Unfortunately, no correlations between protons and carbons in adjacent rings were observed by the 8 Hz-optimized HMBC experiment nor by either NOESY or ROESY experiments. Additionally, because all of the partial structures in crossbyanol A (**1**) were similar or identical in mass, sequencing by EIMS fragmentation was not possible. Moreover, efforts to grow crystals suitable for X-ray diffraction analysis were unsuccessful from a variety of solvents. Resolution of this dilemma was finally obtained by measuring a mixture of crucial four- and five-bond J_{C-H} correlations by a modified HMBC experiment, optimized for $^nJ_{H-C}$ of 2 Hz.[6] As shown in Figure 2, this latter experiment provided a series of inter-residue correlations between four of the five partial structures, and a single conceivable ether linkage remained by deduction between oxygenated quaternary carbons C-4′ and C-2″. Owing to the congested and highly substituted aromatic ring system represented by structure **1**, its optical rotary properties were evaluated, but found to be zero.

Crossbyanol B (**2**) showed a similar isotope pattern to **1** by negative-ion LRESIMS, and by negative-ion HRESIMS an *m/z* of 1182.4172 was observed for a molecular formula of $C_{30}H_{15}{}^{79}Br_7O_{12}S_2$. The ^{1}H and ^{13}C NMR spectra of **2** also showed that all of the proton and carbon signals were in the aromatic region (Table 1). Analysis of 1D and 2D NMR data confirmed that **2** had the five aromatic rings substituted identically to that for crossbyanol A (**1**), namely, three 1,2,4-trisubstituted phenyl rings, one 1,2,3,4-tetrasubstituted phenyl ring, and one 1,2,3,5-tetrasubstituted phenyl ring. Once again, the sequencing of these five substructures was accomplished by the observation of four- and five-bond inter-ring correlations using a 2 Hz-optimized HMBC experiment (Figure 2).[6] Finally, on the basis of the molecular formula and NMR analysis, two sulfate groups were deduced, and these could be placed at oxygenated carbons C-1′ and C-1″″ by chemical shift reasoning in comparison to **1**.

Crossbyanol C (**3**) and crossbyanol D (**4**) both showed essentially identical molecular ion peaks by negative-ion HRESIMS at *m/z* values of 1102.4703 and 1102.4642, indicating they share a common molecular formula of $C_{30}H_{15}{}^{79}Br_7O_9S$. The ^{1}H NMR and COSY data showed that **3** and **4** possess the same five substructures with identical substitution patterns to that for crossbyanols A and B. Hence, from their molecular formulas, it became apparent that each had one sulfate group and one hydroxyl group and, thus, were positional isomers at these two substituents. Finally, the position

Table 1. NMR Data of Crossbyanols A–D (**1**–**4**) in CD$_3$OD[a]

position	1 δ$_C$, mult	1 δ$_H$, mult., J (Hz)	1 8 Hz HMBC	1 2 Hz HMBC	2 δ$_C$	2 δ$_H$, mult., J (Hz)	2 8 Hz HMBC	2 2 Hz HMBC	3 δ$_C$	3 δ$_H$	4 δ$_C$	4 δ$_H$
1	154.5, qC				153.9				154.3		154.7	
2	113.5, qC				112.7				113.5		113.5	
3	136.7, CH	7.71, d (2.3)	1, 2, 4, 5	1, 2, 4, 5, 6, 2'	135.9	7.77, d (2.2)	1, 2, 4, 5	1, 2, 4, 5, 6, 2'	136.8	7.77	136.8	7.79
4	115.5, qC				114.9				115.5		115.5	
5	132.4, CH	7.23, dd (2.3, 8.8)	1, 3, 4	1, 3, 4	131.7	7.34, dd (2.2, 8.8)	1, 3, 4	1, 3, 4	132.3	7.31	132.5	7.35
6	117.0, qC	6.46, d (8.8)	1, 2, 4	1, 2, 3, 4, 2'	117.0	6.56, d (8.8)	1, 2, 4	1, 2, 3, 4, 2'	117.7	6.55	117.1	6.50
1'	149.7, qC				144.3				144.9		150.1	
2'	141.6, qC				145.1				145.7		141.8	
3'	113.2, qC				111.9				112.4		113.3	
4'	147.0, qC				150.6				151.6		146.9	
5'	121.0, CH	6.91, d (8.9)	1', 3', 4'	1', 2', 3', 4', 6'	119.1	7.11, d (9.2)	1', 3', 4'	1', 2', 3', 4', 6'	119.4	7.06	121.26	7.04
6'	117.7, qC	6.98, d (8.9)	1', 2', 4'	1', 2', 3', 4', 5'	122.5	7.83, d (9.2)	1', 2', 3', 4'	1', 2', 3', 4', 5'	123.1	7.80	117.8	7.06
1''	112.8, qC				120.5				112.7		121.3	
2''	142.9, qC				137.1				142.9		137.9	
3''	146.4, qC				152.3				146.5		153.2	
4''	108.9, CH	6.27, d (2.7)	2'', 3'', 5'', 6''	1'', 2'', 3'', 4'', 6'', 1'''	106.3	6.09, d (2.8)	2'', 3'', 5'', 6''	1'', 2'', 3'', 4'', 6'', 4'''	108.5	6.25	107.0	6.07
5''	151.5, qC				155.7				151.5		156.7	
6''	118.12, CH	6.80, d (2.7)	1'', 2'', 4'', 5''	1'', 2'', 3'', 4'', 5''	115.9	6.87, d (2.8)	1'', 2'', 4'', 5''	1'', 2'', 3'', 4'', 5'', 4'	118.3	6.86	116.7	6.85
1'''	153.8, qC				153.0				153.9		153.9	
2'''	116.2, qC				116.3				116.2		117.2	
3'''	137.1, CH	7.70, d (2.2)	1''', 2''', 4''', 5'''	1''', 2''', 4''', 5''', 6''', 3'',	136.0	7.75, d (2.2)	1''', 2''', 4''', 5'''	1''', 2''', 4''', 5''', 6''', 3'',	136.6	7.75	136.9	7.74
4'''	118.05, qC				117.7				118.0		118.5	
5'''	133.2, CH	7.33, dd (2.2, 8.7)	1''', 3''', 4'''	1''', 2''', 3''', 4''', 6'''	132.4	7.44, dd (2.2, 8.7)	1''', 3''', 4'''	1''', 2''', 3''', 4''', 6'''	133.1	7.42	133.2	7.43
6'''	122.3, CH	6.81, d (8.7)	1''', 2''', 4'''	1''', 2''', 3''', 4''', 5''', 3''	123.4	7.06, d (8.7)	1''', 2''', 4'''	1''', 2''', 3''', 4''', 5''', 3''	122.0	6.87	124.2	7.05
1''''	149.5, qC				155.3				150.0		157.1	
2''''	116.5, qC				116.6				116.5		117.4	
3''''	122.7, CH	7.08, d (2.9)	1'''', 2'''', 4'''', 5''''	1'''', 2'''', 4'''', 5'''', 6'''', 5''	122.4	7.22, d (2.8)	1'''', 2'''', 4'''', 5''''	1'''', 2'''', 4'''', 5'''', 6''''	123.3	7.21	122.5	7.13
4''''	156.0, qC				148.0				155.3		148.3	
5''''	117.8, CH	6.72, dd (2.9, 8.9)	1'''', 3'''', 4''''	1'''', 2'''', 3'''', 4'''', 6''''	117.9	6.93, dd (2.8, 8.9)	1'''', 3'''', 4'''', 6''''	1'''', 2'''', 3'''', 4'''', 6''''	118.6	6.88	117.9	6.84
6''''	122.9, CH	6.85, d (8.9)	1'''', 2'''', 4'''', 5''''	1'''', 2'''', 3'''', 4'''', 5'''', 5''	123.6	7.10, d (8.9)	1'''', 2'''', 4'''', 5''''	1'''', 2'''', 3'''', 4'''', 5''''	122.9	6.97	124.5	7.03

[a] Measured at 600 MHz for ¹H NMR and 150 MHz for ¹³C NMR.
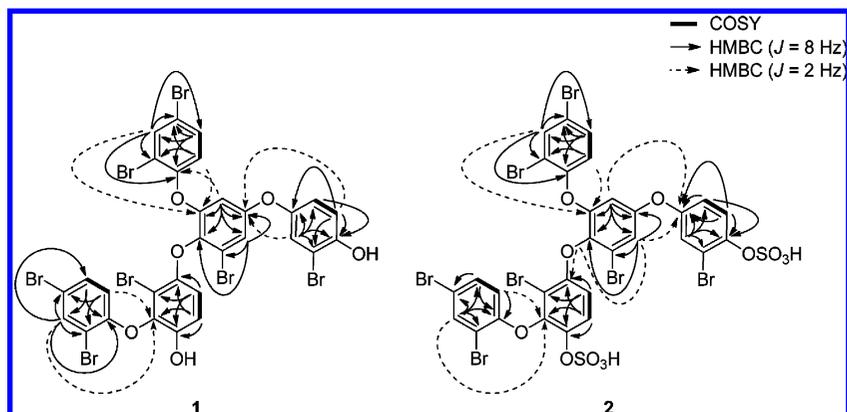
Figure 2. Structure elucidation of compounds **1** and **2** based on key COSY and HMBC correlations.
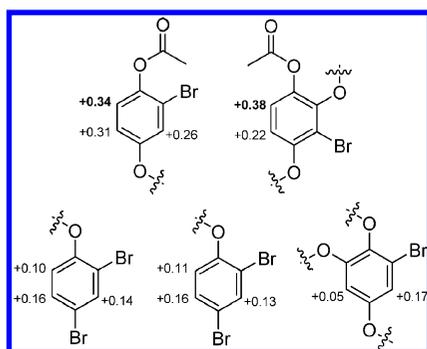
Figure 3. Assignment of the hydroxy group positions based on $\Delta\delta_H$ values ($\delta_5 - \delta_1$) of adjacent protons for compound **1** and diacetate **5**.

of the sulfate residue was assigned by the comparison of chemical shifts for the carbons in rings B and E in **1**–**4**. Specifically, the chemical shifts of C-1′, C-2′, and C-4′ in **3** were similar to those of **2**, while the chemical shifts of C-1′′′′ and C-4′′′′ were similar to those of **1**. Therefore, the sulfate group of **3** was placed on C-1′ and hydroxy group on C-1′′′′. A similar logic was used to deduce that the sulfate in crossbyanol D (**4**) was located at C-1′′′′ and the hydroxy group at C-1′,

While a coral toxicity assay was unavailable to us, crossbyanols A–D (**1**–**4**) were assayed for antibacterial, cytotoxicity, and voltage-gated sodium channel activation and inhibition activity. Additionally, the two major compounds, crossbyanols A (**1**) and B (**2**), were assayed for brine shrimp lethality. Crossbyanol B (**2**) showed the most potent antibacterial activity with an MIC value of 2.0–3.9 μg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA) as well as potent toxicity to brine shrimp with an IC$_{50}$ value of 2.8 μg/mL. Crossbyanol A (**1**) displayed weak cytotoxicity against H-460 human lung cancer cells (IC$_{50}$ 30 μg/mL) and activated the voltage-gated sodium channel in Neuro-2a cells (IC$_{50}$ 20 μg/mL), while crossbyanols C (**3**) and D (**4**) showed no activity in these assays at the maximum test concentration of 20 μg/mL.

A number of polybrominated diphenyl ethers have been reported from diverse marine life, and a strategy for their structural elucidation was recently suggested.[7] However, crossbyanols A–D (**1**–**4**) are unique in that they are of a branched oligomeric structure and contain five bromophenol subunits. While the substructures composing crossbyanols A–D were relatively easily determined despite a low H/C ratio, the ring connections were challenging and only accomplished by use of a 2 Hz-optimized HMBC experiment. Thus, in spite of nearly 150 possible structures from various arrangements of the five polybromophenol subunits, a single skeletal backbone was found to be produced by this marine cyanobacterium. This latter finding supports the occurrence of a highly specific and

potentially canonical crossbyanol biosynthetic pathway that may possess a biosynthetic logic comparable to that of the polyketide synthase (PKS) or non-ribosomal peptide synthetase (NRPS) pathways.[8,9]

Crossbyanols A (**1**) and B (**2**) showed quite different bioactivity profiles. In antibacterial and brine shrimp lethality assays, crossbyanol B (**2**) was by far the more potent, whereas in anticancer and sodium channel activation assays, crossbyanol A (**1**) displayed weak activity, but crossbyanol B (**2**) was inactive. Therefore, it appears that sulfation has a powerful effect on biological properties in this structure class. On the basis of these data we suspect that crossbyanol B (**2**) is likely important to the observed coral toxicity in these natural bloom situations, and assays to examine and confirm this speculation are ongoing.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P-2000 polarimeter, UV spectra on a Beckman Coulter DU800 spectrophotometer, and IR spectra on a Nicolet ThermoElectron Nicolet IR100 FT-IR spectrometer using KBr plates. NMR spectra were recorded with CD$_3$OD as an internal standard (δ_C 49.0, δ_H 3.31) on a Bruker 600 MHz spectrometer (600 and 150 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm MicroCryoProbe. LR EIMS and LR ESIMS were obtained on a ThermoFinnigan MAT900/XL mass spectrometer and a ThermoFinnigan LCQ Advantage Max mass detector, respectively. HRESIMS were obtained on an Agilent 6200 ESI-TOF mass spectrometer. HPLC was carried out using a Waters 515 pump system with a Waters 996 PDA detector.

Cyanobacterial Collection and Taxonomic Identification. The colonial cyanobacteria *L. crossbyana* (HI09-1) was collected in January 2009 by scuba from Honaunau reef, Hawai'i, at a depth of 20 m (GPS coordinates 19°42.338′ N, 155°91.292′ W). Morphological characterizations were performed using an Olympus IX51 epifluorescent microscope (100×) equipped with an Olympus U-CMAD3 camera. Taxonomic identification of cyanobacterial specimens was performed in accordance with current phycological systems.[5]

Polymerase Chain Reaction (PCR) and Cloning. Approximately 50 mg of algal biomass was cleaned and pretreated using TE (10 mM Tris; 0.1 M EDTA; 0.5% SDS; 20 μg/mL RNase)/lysozyme (1 mg/mL) at 37 °C for 30 min followed by incubation with proteinase K (0.5 mg/mL) at 50 °C for 1 h. Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega Inc., Madison, WI) following the manufacturer's specifications. DNA concentration and purity were measured on a DU 800 spectrophotometer (Beckman Coulter). The 16S rRNA genes were PCR-amplified from isolated DNA using the cyanobacteria-specific primers 359F and 781R, as previously described.[10] The PCR reaction volumes were 25 μL containing 0.5 μL (~50 ng) of DNA, 2.5 μL of 10× PfuUltra IV reaction buffer, 0.5 μL (25 mM) of dNTP mix, 0.5 μL of each primer (10 μM), 0.5 μL of PfuUltra IV fusion HS DNA polymerase, and 20.5 μL of dH$_2$O. The PCR reactions were performed in an Eppendorf Mastercycler gradient as follows: initial denaturation for 2 min at 95 °C, followed by 25 cycles of 20 s at 95 °C, 20 s at 50 °C, and 1.5 min at 72 °C, and final elongation for 3 min at 72 °C. PCR products were purified using a

MinElute PCR purification kit (Qiagen) before subcloning using the Zero Blunt TOPO PCR cloning kit (Invitrogen) following the manufacturer's specifications. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen) and sequenced with M13F/M13R primers. The 16S rRNA gene sequences are available in the DDBJ/EMBL/GenBank databases under acc. no. GU111930.

Phylogenetic Inference. Gene sequences were aligned with ClustalW XXL in MEGA 4.0 with standard gap opening and extension penalties without gaps.[11] The evolutionary distant γ-Proteobacter *Escherichia coli* J01695 (J01695) and the unicellular *Gloeobacter violaceus* PCC 7421 (NC005125) were included as appropriate out-groups. Representative type-strains were selected from *Bergey's Manual*.[12] The evolutionary histories of the cyanobacterial 16S rRNA genes were inferred using the maximum parsimony (MP), maximum likelihood (ML), and Bayesian algorithms. Appropriate nucleotide substitution models were selected using Akaike information criterion (AIC) and Bayesian information criterion (BIC) in Modeltest 3.7.[13] The ML inference was performed using PhyML v2.4.4.[14] The PhyML analysis was run using the K81+I+G model (selected by AIC and BIC criteria) assuming a heterogeneous substitution rate and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.455, shape parameter (α) = 0.659, number of rate categories = 4). Bootstrap resampling was performed on 500 replicates. Bayesian analysis was conducted using MrBayes 3.1.[15] The AIC and BIC criteria both selected K80+I+G as the optimum model (pINV = 0.454, α = 0.656, number of rate categories = 4). The Markov chains, one cold and three heated, were run for 3 000 000 generations. The first 25% were discarded as burn-in, and the following data set was sampled with a frequency of every 100 generations. The MP analysis was used to calculate the optimum tree (sum of branch lengths = 1.057 297 5) in MEGA 4.0. The evolutionary distances were computed using the maximum composite likelihood method. The ME tree was searched using the close-neighbor-interchange (CNI) algorithm at a search level of 1.[16] All positions containing gaps and missing data were eliminated from the data set (complete deletion option) for a total of 422 bp in the final data set.

Extraction and Isolation. The cyanobacterial tissue was extracted repetitively with 2:1 CH_2Cl_2/CH_3OH to yield 1.7 g of crude extract. A portion of the extract (1.4 g) was fractionated by silica gel VLC with a stepped gradient elution of hexanes, EtOAc, and MeOH. The fraction eluting with 75% EtOAc in CH_3OH was further fractionated using a Silica Sep-Pak column. The fraction eluting with 100% EtOAc was subsequently separated using RP-HPLC using 55% aqueous CH_3CN to give pure crossbyanol A (**1**, 48 mg, 2.8%). The VLC fraction eluting with 4:1 $CHCl_3/CH_3OH$ was subjected to RP-HPLC directly using 50% CH_3CN(aq) to give pure crossbyanols C (**3**, 7.9 mg, 0.5%) and D (**4**, 4.9 mg, 0.3%). Finally, the VLC fraction eluting with 2:1 $CHCl_3/CH_3OH$ was further purified using RP-HPLC and 40% CH_3CN(aq) to give pure crossbyanol B (**2**, 56 mg, 3.3%).

Crossbyanol A (1): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −0.002 (*c* 0.3, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 204 (5.25), 289 (4.21) nm; IR (KBr) ν_{max} 3354, 2365, 2330, 1698, 1582, 1465, 1224 cm⁻¹; ¹H, ¹³C, and 2D NMR data, Table 1; HRESIMS 1046.5045 ($C_{30}H_{15}{}^{79}Br_7NaO_6$, calcd $[M + Na]^+$ 1046.5044).

Crossbyanol B (2): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −0.14 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 206 (5.38), 281 (4.26) nm; IR (KBr) ν_{max} 3402, 2914, 2838, 2358, 2337, 1595, 1465, 1251, 1218 cm⁻¹; ¹H, ¹³C, and 2D NMR data, Table 1; HRESIMS *m/z* 1182.4172 ($C_{30}H_{14}{}^{79}Br_7O_{12}S_2$, calcd $[M − H]^-$ 1182.4210).

Crossbyanol C (3): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −0.001 (*c* 0.5, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 282 (4.33) nm; IR (KBr) ν_{max} 3382, 2365, 2324, 1698, 1580, 1458, 1224 cm⁻¹; ¹H and ¹³C NMR data, Table 1; HRESIMS *m/z* 1102.4703 ($C_{30}H_{14}{}^{79}Br_7O_9S$, calcd $[M − H]^-$ 1102.4648).

Crossbyanol D (4): pale yellow, amorphous solid; $[\alpha]^{25}_D$ +0.0012 (*c* 0.4, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 282 (4.30) nm; IR (KBr) ν_{max} 3279, 2364, 2317, 1458, 1238 cm⁻¹; ¹H and ¹³C NMR data, Table 1; HRESIMS *m/z* 1102.4642 ($C_{30}H_{14}{}^{79}Br_7O_9S$, calcd $[M − H]^-$ 1102.4648).

Crossbyanol A Diacetate (5). Crossbyanol A (**1**, 1 mg) was dissolved in 1 mL of anhydrous pyridine, and a catalytic amount of dimethylaminopyridine (DMAP) and 500 μL of acetic anhydride were added. The reaction was allowed to proceed at room temperature for 24 h with periodic evaluation by NP TLC. The excess reagent and solvents were removed under N_2 (g), and then the reaction product

was isolated by preparative NP TLC using 100% EtOAc as a developing and eluting solvent.

Crossbyanol A diacetate (5): pale yellow, amorphous solid; ¹H NMR (CD_3OD, 600 MHz) δ_H 7.85 (d, *J* = 2.3 Hz, H-3), 7.83 (d, *J* = 2.2 Hz, H-3‴), 7.49 (dd, *J* = 2.2, 8.7 Hz, H-5‴), 7.39 (d, *J* = 2.3, 8.8 Hz, H-5), 7.36 (d, *J* = 8.9 Hz, H-6′), 7.34 (d, *J* = 2.9 Hz, H-3‴′), 7.19 (d, *J* = 8.9 Hz, H-6‴′), 7.13 (d, *J* = 8.9 Hz, H-5′), 7.03 (dd, *J* = 2.9, 8.9 Hz, H-5‴′), 6.97 (d, *J* = 2.7 Hz, H-6″), 6.92 (d, *J* = 8.7 Hz, H-6‴), 6.56 (d, *J* = 8.8 Hz, H-6), 6.32 (d, *J* = 2.7 Hz, H-4″); COSY spectrum, see Supporting Information; LR ESI-MS *m/z* 1152 [M + HCOO]⁻.

Brine Shrimp Toxicity Assay. Evaluation of brine shrimp (*Artemia salina*) toxicity was performed as previously reported.[17,18] Briefly, after incubation of the brine shrimp eggs for 24 h, about 20 hatched brine shrimp in ca. 0.5 mL of seawater were added to each well containing different concentrations of samples in 50 μL of EtOH and 4.5 mL of artificial seawater. The final sample concentrations of compounds **1** and **2** were 1, 5, and 25 ppm, and all control and sample experiments were run in triplicate. After 24 h incubation at 28 °C, the number of live and dead brine shrimp was counted.

Antibacterial Assay against MRSA. The minimum inhibitory concentration (MIC) values of the compounds were determined on methicillin-resistant *Staphylococcus aureus*. The bacterial strain was grown on GYT media for 24 h at 37 °C and then inoculated in GYT media with serially diluted samples for 24 h at 37 °C. Growth inhibition was evaluated by measuring optical density at 600 nm. The MIC of these samples was determined as the lowest concentration required to inhibit bacterial growth.

Cytotoxicity Assay. H-460 cells were added to 96-well plates at 3.33×10^4 cells/mL of Roswell Park Memorial Institute (RPMI) 1640 medium with fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells, in a volume of 180 μL per well, were incubated overnight (37 °C, 5% CO_2) to allow recovery before treatment with test compounds. Compounds were dissolved in DMSO to a stock concentration of 10 mg/mL. Working solutions of the compounds were made in RPMI 1640 medium without FBS, with a volume of 20 μL added to each well to give a final compound concentration of either 30 or 3 μg/mL. An equal volume of RPMI 1640 medium without FBS was added to wells designated as negative controls for each plate. Plates were incubated for approximately 48 h before staining with MTT. Using a ThermoElectron Multiskan Ascent plate reader, plates were read at 570 and 630 nm.

Sodium Channel Activation and Inhibition Assay. Neuro2a cells were added to 96-well plates at 3.0×10^5 cells/mL of RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin. The cells, in a volume of 200 μL per well, were incubated (37 °C, 5% CO_2) overnight to allow recovery before treatment with compounds. Test compounds were dissolved in DMSO to a stock concentration of 10 mg/mL. Working solutions of the compounds were made in RPMI 1640 medium without FBS, with a volume of 10 μL added to each well to give a final compound concentration of 20 μg/mL. An equal volume of RPMI 1640 medium without FBS was added to wells designated as the negative control for each plate. Brevetoxin-2, the positive control for the sodium channel activating assay, and saxitoxin, the positive control for the blocking assay, were added at a ratio of 1:100 of the concentration of the test compounds. A mixture of ouabain, veratridine, and 1× PBS/5 mM HCl was applied to the bottom half of each plate to cause sodium overload to varying degrees for the blocking and activating assays. For the blocking assay, a solution composed of 5 mM ouabain and 350 μM veratridine in PBS/HCl was used, and for the activating assay a solution composed of 5 mM ouabain and 150 μM veratridine in PBS/HCl was used. A solution of 1× PBS/5 mM HCl was added to the top half of each plate to give the general toxicity of the test compounds. Plates were incubated for approximately 16 h before staining with MTT for the activating assay. For the blocking assay, plates were incubated for approximately 24 h before MTT staining. Using a ThermoElectron Multiskan Ascent plate reader, plates were read at 570 and 630 nm.

Supporting Information Available: Microscopic and field photographs of *L. crossbyana*, LR and HR mass spectra, NMR spectra, and

bioactivity graphs of compounds **1−4**. These materials are available free of charge via the Internet at http://pubs.acs.org.

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