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Biosynthetically Intriguing Chlorinated Lipophilic Metabolites from Geographically Distant Tropical Marine Cyanobacteria

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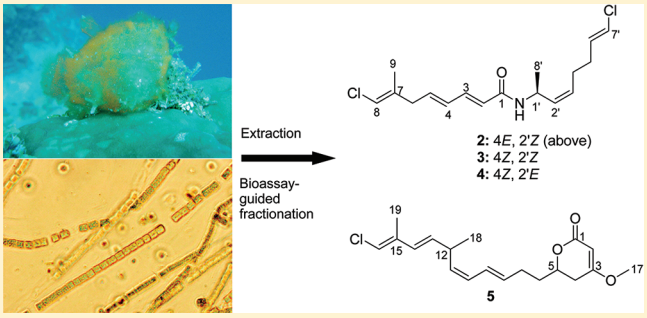
Supporting Information

ABSTRACT: Five new vinylchlorine-containing metabolites, the lipoamides janthielamide A and kimbeamides A–C and the ketide-extended pyranone kimbelactone A, have been isolated from collections of marine cyanobacteria made in Curaçao and Papua New Guinea. Both janthielamide A and kimbeamide A exhibited moderate sodium channel blocking activity in murine Neuro-2a cells. Consistent with this activity, janthielamide A was also found to antagonize veratridine-induced sodium influx in murine cerebrocortical neurons. These lipoamides represent the newest additions to a relatively rare family of marine cyanobacterial-derived lipoamides and a new structural class of compounds exhibiting neuromodulatory activities from marine cyanobacteria.



INTRODUCTION

Marine cyanobacteria are prolific producers of diverse bioactive secondary metabolites with many exhibiting anticancer activity largely owing to interference with tubulin (e.g., dolastatin 10,[1] curacin A[2]) or actin (e.g., lyngbyastatin 1,[3] hectochlorin,[4] lyngbyabellin A,[5] desmethoxymajusculamide C[6]) formation and function. While freshwater and terrestrial cyanobacteria have long been recognized as producers of potent neurotoxins, such as anatoxin-*a*,[7] *β*-methylaminoalanine,[8] and saxitoxin,[9] marine cyanobacteria have only recently garnered attention for their potential to produce neuromodulatory compounds.[10−12] Metabolites exhibiting such activity, including voltage-gated sodium channel (VGSC) activation or blocking effects and/or intracellular calcium ion modulation, have potential therapeutic utility in the treatment of spinal cord injury, chronic pain, CNS disorders including stroke and epilepsy, and treatment of cardiovascular, inflammatory, and neurodegenerative disorders.[13,14] With the discovery of potent VGSC activation for antillatoxin A[15] and nanomolar VGSC blocking activity for kalkitoxin,[15] two metabolites obtained from collections of marine cyanobacteria, the capacity of these oceanic prokaryotes to produce neuroactive compounds was realized.

Halogen atom incorporation is common in natural products from the marine realm in part owing to the relatively high concentrations of bromide, chloride, and iodide present in seawater.[16,17] Chloride, the most common seawater halide incorporated into marine cyanobacterial natural products,[18,19] is present in several linear lipophilic metabolites which feature terminal or pendant vinyl chloride functionalities, including (−)-(*E*)-1-chlorotridec-1-ene-6,8-diol,[20] malyngamide A[21] and additional malyngamides,[10] pitiamide A,[22] jamaicamides,[23] the taveuniamides,[24] and grenadamides B and C,[25] among others. These vinyl chloride-containing cyanobacterial compounds exhibit a variety of biological activities, such as VGSC blocking activity,[23] cytotoxicity,[10,23] toxicity,[24] and insecticidal activity.[25]

In the present work, two independent collections of cyanobacteria from Caribbean and Indo-Pacific locations yielded chromatographic fractions that had similar ^{1}H NMR profiles, molecular weights, and isotopic patterns. Additionally, fractions from both extracts exhibited similar neuromodulatory activity profiles in both a sodium channel blocking assay in murine Neuro-2a cells and a calcium ion modulation assay in murine neocortical neurons. At 20 μg/mL in Neuro-2a cells, the Curaçao-derived chromatographic fraction inhibited the ouabain/veratradine-induced toxicity by 129%, while the Papua New Guinea-derived fraction inhibited this toxicity by 86%. Subsequently, bioassay-guided fractionation of these chromatographic fractions employing the murine Neuro-2a sodium channel blocking assay yielded a new lipoamide, janthielamide

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Table 1. NMR Spectroscopic Data for Janthielamide A (1) in $CDCl_3$ (^1H at 500 MHz, ^{13}C at 75 MHz)

position	δ_C[a,b]	δ_H mult (J in Hz)[c]	COSY	HMBC
1	116.3 CH	5.88 d (7.0)	2	2, 3
2	130.1 CH	6.25 dd (10.4, 7.0)	1, 3	1, 4
3	122.3 CH	6.39 dd (15.4, 10.4)	2, 4	1, 2, 5
4	144.1 CH	5.76 dd (15.4, 7.6)	3, 5	2, 3, 12
5	37.4 CH	2.29 m	4, 6, 12	3, 4, 6, 7, 12
6	39.8 CH_2	2.03 m	5, 7	4, 5, 7, 8, 9, 12
7	127.2 CH	5.33 dt (15.3, 6.7)	6, 8, 11a/b	5, 6, 8
8	137.5 CH	5.25 dd (15.3, 7.9)	7, 9	5, 6, 7, 13
9	19.9 CH	2.14 m	8, 10a/b, 13	7, 8, 10, 13
10a	36.9 CH_2	1.53 m	9, 10b, 11a	8, 11, 13
10b		1.40 m	9, 10a, 11b	8, 11, 13
11a	37.7 CH_2	3.27 m	7, 10a	10, 1′
11b		3.19 m	7, 10b	10, 1′
12	19.8 CH_3	1.02 d (6.7)	5	4, 5, 6
13	21.2 CH_3	0.98 d (6.7)	9	8, 10
NH		5.35		2′, 4′
1′	167.0 qC			
2′	118.8 CH	5.51 s	4′, 5′	12, 1′, 3′, 5′
3′	150.5 qC			
4′	35.2 CH_3	2.14 s	2′	1′, 2′, 3′, 4′, 5′
5′	27.2 CH_3	1.82 s	2′	1′, 2′, 3′, 12

[a]Recorded at 75 MHz. [b]Multiplicity deduced from HSQC. [c]Recorded at 500 MHz.

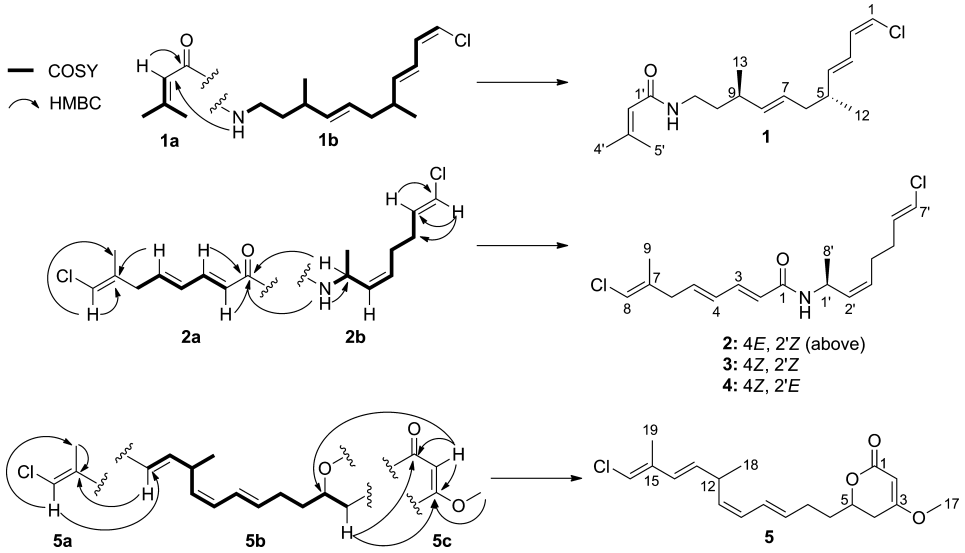

Figure 1. Partial and full structures for janthielamide A (**1**), kimbeamide A (**2**), and kimbelactone A (**5**) with COSY and key HMBC correlations shown.

A (**1**), from the Curaçao collection and the novel lipoamide kimbeamide A (**2**), two *cis*–*trans* isomers, kimbeamides B (**3**) and C (**4**), and the polyketide kimbelactone A (**5**) from the Papua New Guinea collections. Despite geographical distance and morphological differences, both collections were found to contain evolutionarily closely related cyanobacteria based on phylogenetic analyses. Herein, we report the isolation, structure elucidation and bioactivity of these metabolites, and a thorough characterization of the putative marine cyanobacterial producers.

RESULTS AND DISCUSSION

Collection and Isolation. A green-pigmented filamentous cyanobacterial mat was collected by hand from Jan Thiel Bay in Curaçao. The cyanobacterial tissue was extracted repeatedly with CH_2Cl_2/MeOH (2:1) and then fractioned by silica gel vacuum column chromatography to produce nine subfractions (A–I). Bioassay-guided fractionation of the main bioactive fraction (D) utilizing normal-phase column chromatography led to the isolation of janthielamide A (**1**) as a yellow, optically active amorphous solid [34.2 mg, 1.54%, $[\alpha]_D$ 10.2 (c 0.60, $CHCl_3$)]. The LR-ESI-MS spectrum of **1** revealed a 3:1 ratio at m/z 310/312 for the $[M + H]^+$ pseudomolecular ion, consistent with the presence of a single chlorine atom. HR-ESI-TOFMS established the molecular formula as $C_{18}H_{28}ClNO$, revealing that **1** contained five double bond equivalents. The IR spectrum displayed absorptions characteristic for NH or OH protons (3297 cm^{-1}) and an amide carbonyl group (1732 cm^{-1}), while UV showed a maximum at 224 nm (log ε = 4.4) consistent with the presence of at least

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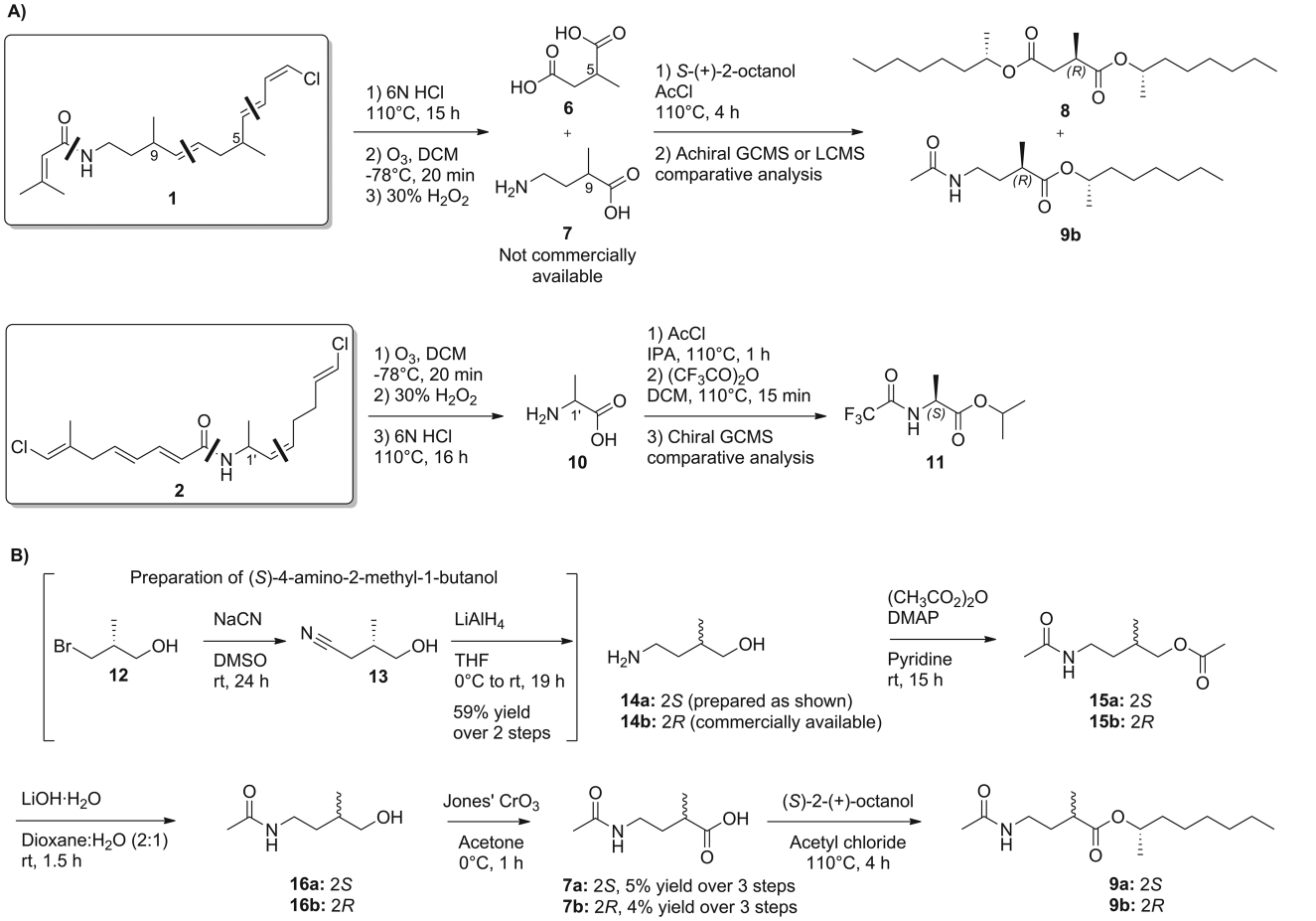
Scheme 1. (A) Fragmentation Strategy for Janthielamide A (1) and Kimbeamide A (2). (B) Preparation of Enantiomerically Pure 2-Methyl-γ-aminobutyric Acid Standards for Determining the Absolute Configuration of Janthielamide A (1)



one conjugated diene or enone. The ^{1}H NMR spectrum of **1** (Table 1) contained two doublet methyls (δ_H 0.98 and 1.02), two singlet methyls (δ_H 1.82 and 2.14), a diastereotopic deshielded methylene (δ_H 3.19 and 3.27), two upfield shifted methines (δ_H 2.14 and 2.29), three resonances at 1−2 ppm accounting for two methylenes, one NH proton at δ_H 5.35, and seven olefinic resonances between 5 and 7 ppm. The ^{13}C NMR spectrum of **1** included two quaternary (δ_C 167.0 and 150.5), nine methine (δ_C 144.1, 137.5, 130.1, 127.2, 122.3, 118.8, 116.3, 37.4 and 19.9), three methylene (δ_C 39.8, 37.7 and 36.9), and four methyl carbons (δ_C 35.2, 27.2, 21.2 and 19.8), accounting for all 18 carbon atoms in the molecular formula. The quaternary and methine carbons were further assigned as four double bonds and one carbonyl moiety according to their chemical shifts.

Two ^{1}H spin systems were assembled by COSY (Figure 1); fragment **1a** featured an interesting vinylic *gem*-dimethyl functionality, while fragment **1b** contained a conjugated diene, two methyl-substituted methine carbons, a third olefin group, and two adjacent methylene groups. The lone carbonyl carbon observed in the ^{13}C NMR spectrum was assigned to fragment **1a** based on an HMBC correlation from H2′ to C1′. Subsequently, spin systems **1a** and **1b** were connected through an amide bond based on a correlation from the amide proton to C2′ in the HMBC spectrum. Lastly, the chlorine atom was attached to the C1 position (δ_C 116.3) on the basis of chemical shift arguments and comparison with literature values for similar systems.[20,22,25]

The configurations of the double bonds were determined via coupling constant data. The vicinal coupling constant for H1/H2 was 7.0 Hz, a value consistent with a polarized *Z* double bond.[26] The C3−C4 and C7−C8 olefins were both assigned as *E* on the basis of vicinal coupling constants of 15.4 and 15.3 Hz for H3/H4 and H7/H8, respectively.

Regarding the absolute configuration of **1**, we envisioned that hydrolysis and ozonolysis followed by oxidative workup would yield 2-methylsuccinic acid (**6**) and 2-methyl-γ-aminobutyric acid (GABA, **7**) as key degradation products containing the stereocenters in question (C5 and C9, Scheme 1). Enantiomerically pure (*R*)- and (*S*)-**6** are commercially available, and thus, were initially derivatized to afford methyl or isopropyl esters; however, these proved inseparable on chiral GCMS. Ultimately, esterification with (*S*)-2-(+)-octanol[27] afforded dioctan-2-yl 2-methylsuccinate diastereomers (**8**) that separated well on achiral GCMS. The products of hydrolysis and ozonolysis of janthielamide A (**1**) were similarly esterified with (*S*)-2-(+)-octanol. Comparison of the retention times for (*R*)- and (*S*)-**8** with janthielamide A derived **8** allowed for assignment of the absolute configuration at C5 as *R*.

Unfortunately, in the case of 2-methyl-GABA, enantiomerically pure standards were not commercially available. This analogue of the neurotransmitter GABA has previously been synthesized;[28] however, the starting material is a controlled substance, and the synthetic route is complex. We envisioned a more facile synthetic route whereby the desired standards could be afforded via oxidation of the corresponding amino alcohol.

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Table 2. NMR Spectroscopic Data for Kimbeamide A (2) in C_6D_6 (^{1}H at 600 MHz, ^{13}C at 125 MHz)

position	δ_C[a,b]	δ_H (J in Hz)[c]	COSY	HMBC	NOESY
1	164.1 qC				
2	124.0 CH	5.32 d (14.9)	3	1, 3, 4, 5	4, NH
3	140.5 CH	7.45 dd (14.9, 11.2)	2, 4	1, 2, 4, 5	5
4	130.6 CH	5.90 dd (15.0, 11.2)	3, 5	2, 3, 6	2, 6, 9
5	137.5 CH	5.43 dt (15.0, 7.0)	4, 6	3, 6, 7	3, 9
6	40.1 CH_2	2.31 d (7.0)	5	2, 3, 5, 7, 8, 9	4
7	136.4 qC				
8	114.2 CH	5.53 bs		6, 7, 9	6
9	16.6 CH_3	1.54 s		6, 7, 8	4, 5
NH		4.70 d (7.4)		1, 1′	2
1′	42.4 CH	5.0 m	8′	1, 2′, 3′, 8′	4′a/b
2′	132.6 CH	5.07 dd (10.5, 9.2)	1′, 3′	3′, 4′, 8′	
3′	130.3 CH	5.17 dt (10.5, 7.5)	2′, 4′a/b	1′, 2′, 4′, 5′	5′a
4′a	27.1 CH_2	2.10 m	3′, 4′b	3′, 5′, 6′	1′
4′b		1.96 m	3′, 4′a	3′, 5′, 6′	1′
5′a	30.8 CH_2	1.76 m	4′b, 6′	3′, 4′, 6′, 7′	
5′b		1.71 m	4′b, 6′	3′, 4′, 6′, 7′	
6′	133.3 CH	5.69 m	5′a/b	5′, 7′	
7′	117.8 CH	5.67 d (13.4)		5′, 6′	4′a/b, 5′a
8′	21.7 CH_3	1.01 d (6.6)	1′	1′ 2′	NH

[a]Recorded at 125 MHz. [b]Multiplicity deduced from HSQC. [c]Recorded at 600 MHz.

The *N*-protected standards were prepared as follows: the (*S*)-*N*-acetyl-2-methyl-GABA standard (**7a**) was obtained from commercially available (*R*)-(−)-3-bromo-2-methyl-1-propanol (**12**) via an S_N2 substitution of bromine with cyanide to form the nitrile (**13**), followed by reduction with $LiAlH_4$ to give (*S*)-4-amino-2-methyl-1-butanol (**14a**). This was *N*- and *O*-protected with acetyl groups (**15a**), and then the hydroxyl group was deprotected (**16a**) and oxidized to the carboxylic acid (**7a**) using Jones' reagent. To obtain the (*R*)-*N*-acetyl-2-methyl-GABA standard (**7b**), the amine and hydroxyl groups of commercially available (*R*)-4-amino-2-methyl-1-butanol (**14b**) were protected, and then the hydroxyl group was deprotected and oxidized as described above for **7a**. Both **7a** and **7b** were then esterified with (*S*)-2-(+)-octanol. These diastereomers (**9a**, **9b**) were successfully resolved utilizing achiral reverse phase LCMS, and comparison of their retention times with that of the corresponding janthielamide A (**1**) derivative led us to assign the absolute configuration at C9 as *R*, completing the structure elucidation of compound **1**.

In parallel with the above study, two extracts from Papua New Guinea collections of orange-colored cyanobacterial consortia were combined (see the Experimental Section) and subjected to a similar bioassay-guided protocol employing normal-phase column chromatography and HPLC to yield kimbeamide A (**2**) as an optically active pale yellow oil [1.6 mg, 0.10%, $[\alpha]_D$ +44 (*c* 0.05, MeOH)]. The LR-ESI-MS of **2** revealed an isotopic pattern for the $[M + H]^+$ pseudomolecular ion cluster consistent with the presence of two chlorine atoms [328/330/332 = 1:0.5:0.15]. The HR-ESI-TOFMS of **2** gave an $[M + H]^+$ at *m/z* 328.1234 (calcd for $C_{17}H_{24}Cl_2NO$, 328.1229) in agreement with a molecular formula of $C_{17}H_{23}Cl_2NO$, therefore requiring 6 degrees of unsaturation. The IR spectrum featured absorptions for NH or OH protons (3290 cm^{-1}) and an amide carbonyl group (1718 cm^{-1}), while UV showed a maximum at 250 nm (log ε = 4.0) suggesting the presence of a conjugated dienone system.

The ^{1}H NMR spectrum of compound **2** (Table 2) had resonances for a methyl doublet at δ_H 1.01, a methyl singlet at δ_H 1.54, a doublet at δ_H 2.31 integrating for two protons, annotated as a *bis*-allylic methylene, four resonances between 1 and 2.5 ppm accounting for two diastereotopic methylenes, an NH proton at δ_H 4.70, nine resonances from 5 to 6 ppm characteristic of olefinic protons, and a doublet of doublets at δ_H 7.45 for the β proton in a conjugated dienone system. Analysis of the ^{13}C NMR and HSQC spectra revealed two quaternary (δ_C 164.1 and 136.4), 10 methine (δ_C 140.5, 137.5, 133.3, 132.6, 130.6, 130.3, 124.0, 117.8, 114.2 and 42.4), three methylene (δ_C 40.1, 30.8 and 27.1), and two methyl (δ_C 21.7 and 16.6) carbons, accounting for all 17 carbon atoms required by the molecular formula. Two spin systems were assembled by COSY (Figure 1); fragment **2a** featured a conjugated diene whereas fragment **2b** featured two isolated alkene functionalities separated by two methylene groups. The lone carbonyl carbon was assigned to fragment **2a** at position C1 based on correlations in the HMBC from H2 to C1 and H3 to C1, respectively. Spin systems **2a** and **2b** were connected through an amide bond based on HMBC data including correlations from NH to C1 and C1′ as well as from H1′ to C1. HMBC correlations from H8 to C6, C7 and C9 allowed for placement of a methyl substituted alkene on the distal side of fragment **2a**. Additionally, correlations from H7′ to C5′ and C6′, as well as from H5′ and H6′ to C7′, allowed for assignment of the remaining proton of the terminating alkene of fragment **2b**. As with janthielamide A (**1**), the relatively unique chemical shifts exhibited by C8 (δ_C 114.2) and C7′ (δ_C 117.8) indicated that chlorine atoms were appended to these vinylic carbons.

The configurations of the double bonds were determined on the basis of vicinal coupling constants and NOE correlations. The C2−C3, C4−C5, and C6′−C7′ olefins were assigned as *E* according to coupling constants of 14.9, 15.0, and 13.4 Hz for H2/H3, H4/H5, and H6′/H7′, respectively. The C7−C8 olefin, in turn, was also assigned as *E* based on an NOE correlation between H8 and H6. Lastly, the C2′-C3′ olefin was determined to be *Z* given a vicinal coupling constant for H2′/H3′ of 10.5 Hz.

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Table 3. NMR spectroscopic data for kimbelactone A (5) in C_6D_6 (^{1}H at 600 MHz, ^{13}C at 125 MHz)

position	δ_C [a,b]	δ_H (J in Hz)[c]	COSY	HMBC	NOESY
1	165.8 qC				
2	91.2 CH	4.97 s		1, 3, 4, 5	17
3	171.7 qC				
4a	33.0 CH_2	1.84 dd (16.6, 12.1)	4b, 5	1, 2, 3, 5, 6	6a/b
4b		1.61 dd (16.8, 3.4)	4a, 5	2, 3, 5	6b
5	74.2 CH	3.85 dddd (16.0, 11.9, 8.0, 4.0)	4a, 6a/b	3, 6, 7	7
6a	34.4 CH_2	1.51 m	5, 6b, 7	4, 5, 7, 8	4a
6b		1.14 m	5, 6a, 7	4, 5, 7, 8	4a
7	28.5 CH_2	2.12 m	6a/b, 8	5, 6, 8, 9	5, 9
8	133.8 CH	5.46 dt (14.8, 7.4)	7, 9	6, 7, 10	6a/b
9	127.2 CH	6.41 dd (14.8, 11.4)	8, 10	6, 7, 8, 10	7, 12
10	128.1 CH	6.01 t (10.9)	9, 11	9, 11, 12	8
11	133.9 CH	5.16 t (10.1)	10, 12	9, 12, 13, 18	18
12	35.6 CH	3.32 ddd (14.5, 11.6, 7.2)	11, 18	13, 14, 18	9, 18
13	134.5 CH	5.51 dd (15.6, 6.6)	12, 14	11, 12, 15, 16, 18	18, 19
14	128.5 CH	5.94 d (15.6)	13	12, 15, 16, 18, 19	12, 18
15	137.1 qC				
16	118.5 CH	5.89 s		14, 15, 19	
17	55.1 CH_3	2.91 s		2, 3	2
18	21.2 CH_3	1.02 d (6.7)	12	11, 12	11, 13, 14
19	12.9 CH_3	1.76 s		14, 15, 16	13, 18

[a]Recorded at 125 MHz. [b]Multiplicity deduced from HSQC. [c]Recorded at 600 MHz.

The absolute configuration of kimbeamide (**2**) at C1′ was determined by cleaving the C2′−C3′ olefin and the amide bond via ozonolysis followed by oxidative workup and hydrolysis to produce alanine as a degradation product (Scheme 1). This residue, as well as enantiomerically pure L- and D-alanine standards, were esterified with 2-propanol and acylated with trifluoroacetic anhydride to furnish volatile derivatives for chiral GCMS analysis.[29,30] By comparison of retention times of the cleaved product and standards, metabolite **2** was found to possess the *S* configuration at C1′, thus completing the structure elucidation of kimbeamide A (**2**).

Two *cis*−*trans* isomers of **2** were also isolated from the orange cyanobacterial collections from Kimbe Bay. Kimbeamide B (**3**) differed from **2** in the geometry of the C4−C5 olefin (*Z*, vicinal coupling constant for H4/H5 = 10.9 Hz), while kimbeamide C (**4**) differed from **3** in the geometry of the C2′−C3′ olefin (*E*, vicinal coupling constant for H2′/H3′ = 15.5 Hz). Unfortunately, because compounds **3** and **4** were isolated in very low yields and were unstable, we were unable to complete the analysis of their stereochemistry. Nevertheless, we hypothesize that C1′ of compounds **3** and **4** is *S* based on their similarity to and co-occurrence with kimbeamide A (**1**).

Kimbelactone A (**5**) was isolated as an optically active pale yellow oil [3.3 mg, 0.20%, $[\alpha]_D$ −164.7 (*c* 0.17, CH_2Cl_2)] from a slightly more polar chromatographic fraction of the Papua New Guinea cyanobacterial collections. The LR-ESI-MS spectrum of **5** revealed a 3:1 ratio at *m*/*z* 359/361 for the $[M + Na]^+$ pseudomolecular ion, consistent with one chlorine atom. The HR-ESI-TOFMS established the molecular formula as $C_{19}H_{25}ClO_3$ revealing that **5** contained 7 double bond equivalents. A UV maximum of 227 nm suggested the presence of one or more conjugated diene or enone systems.

Examination of the ^{1}H NMR spectrum of **5** (Table 3) revealed a methyl doublet at δ_H 1.02, a methyl singlet at δ_H 1.76, a slightly upfield-shifted *O*-methyl at δ_H 2.91, five resonances between 1 and 2.5 ppm accounting for six methylene protons, two midfield shifted methine protons at δ_H 3.32 and δ_H 3.85, as well as eight resonances between 5 and 6.5 ppm characteristic of olefinic protons. The ^{13}C NMR and HSQC spectra revealed three quaternary (δ_C 171.7, 165.8 and 137.1), 10 methine (δ_C 134.5, 133.9, 133.8, 128.5, 128.1, 127.2, 118.5, 91.2, 74.2 and 35.6), three methylene (δ_C 34.4, 33.0 and 28.5), one *O*-methyl (δ_C 55.1), and two aliphatic methyl (δ_C 21.2 and 12.9) carbons accounting for all 19 carbons required by the molecular formula.

One large ^{1}H NMR spin system (fragment **5b**) featuring a conjugated diene, a methyl-substituted methine, an oxygen-substituted methine (based on a chemical shift of δ_C 74.2), and an additional alkene moiety was assembled by COSY data (Figure 1). A second fragment, **5a**, contained a methyl-substituted vinyl chloride functionality and was assembled on the basis of HMBC correlations from H19 to C15 and H16 to C19. The chemical shift of C16 at δ_C 118.5 allowed for placement of the chlorine atom at this position. Fragment **5c** possessed an enone with *O*-Me substitution at the β carbon as revealed by HMBC correlations from H2 to C1 and C3 and H17 to C2 and C3. Fragment **5a** was connected to the distal side of fragment **5b** based on HMBC correlations from H14 to C15, C16, and C19 and from H16 to C14. Reciprocal HMBC correlations from H19 to C14 and C16 further corroborated this connection. Fragment **5c** was connected to the proximal side of fragment **5b** forming a β-methoxy-substituted pyranone ring based on HMBC correlations from H5 to C3 and from H4 to C1, C2 and C3, as well as an HMBC correlation from H2 to C5.

As before, the geometries of the double bonds were determined on the basis of vicinal coupling constants and NOE correlations. The C8−C9 and C13−C14 olefins were found to be *E* on the basis of large coupling constants (14.8 and 15.6 Hz, respectively), whereas the C10−C11 olefin was determined to be *Z* (coupling constant for H10/H11 = ∼10.5 Hz). Lastly, the configuration of the C15−C16 olefin was assigned as *E* on the basis of similarity of carbon chemical shifts in comparison with the kimbeamide A olefin at C7−C8. The

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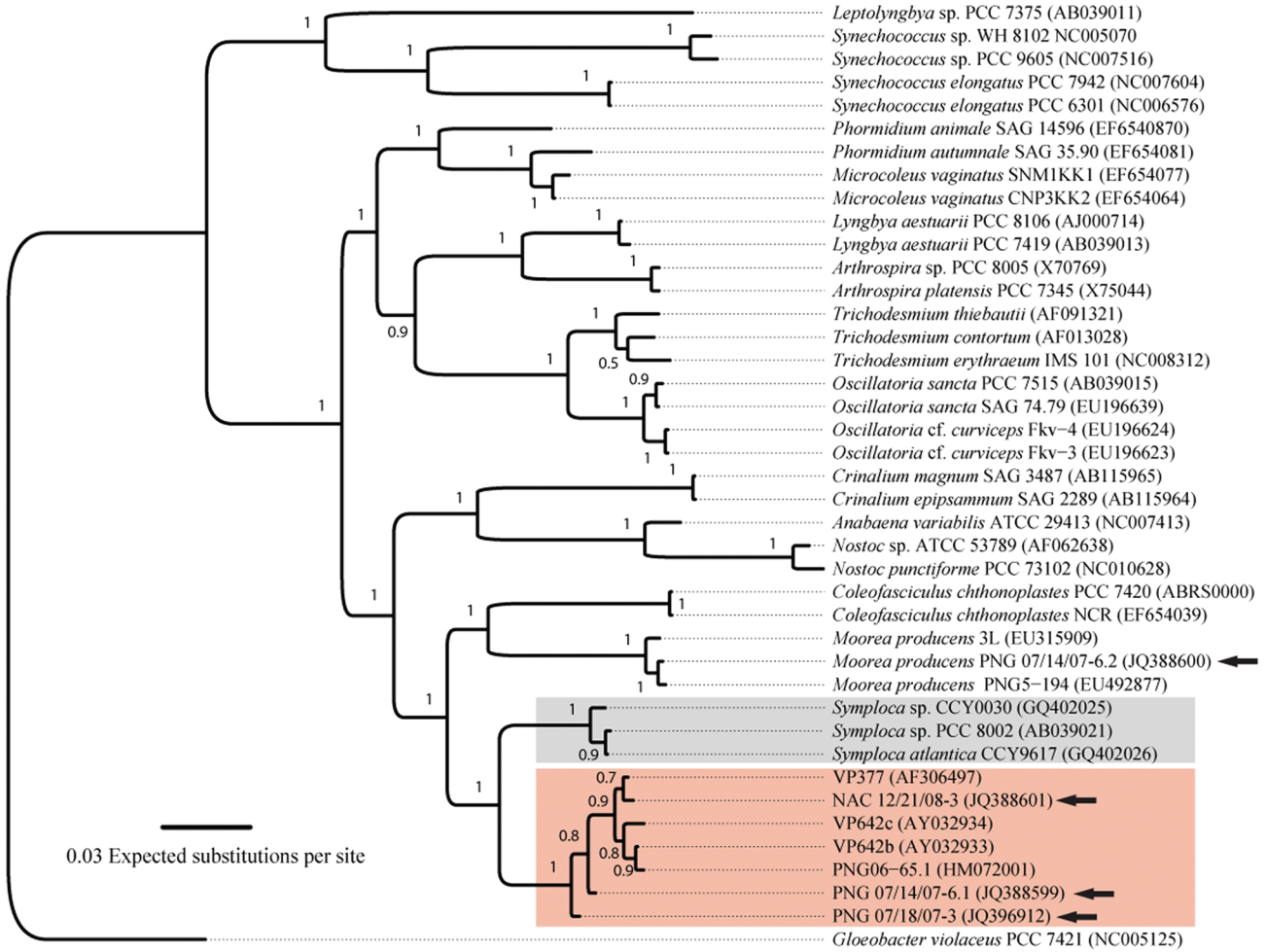

Figure 2. Molecular-phylogenetic inference of the kimbeamide-producing strains PNG 07/14/07-6.1 (JQ388599) and PNG 07/18/07-3 (JQ396912) from Papua New Guinea as well the janthielamide-producing strain NAC 12/21/08-3 (JQ388601). The clade that includes the NP-producing strains is highlighted with a red box. The closest related group is the genus *Symploca* (reference strain: PCC 8002^{T}, GenBank acc. no. AB039021) with a p-distance of 4.9% based on the SSU rRNA gene sequence divergence (highlighted with a gray box). The cladogram is based on SSU (16S) rRNA gene sequences using the bayesian (MrBayes) and maximum likelihood (PhyML) methods, and the support values are indicated as posterior probability at the nodes. The specimens are indicated as species, strain and access number in brackets. Specimens designated with (^{T}) represent type-strains obtained from Bergey's Manual.[36] The scale bar is indicated at 0.03 expected nucleotide substitutions per site, corrected using the general time reversal (GTR) model.

absolute configurations at C5 and C12 of kimbelactone A (**5**) remain unknown as the compound decomposed before such studies could be undertaken.

Biological Activity. Biological evaluation of fractions arising from extracts of these Curaçao and Papua New Guinea collections of tropical marine cyanobacteria revealed similar neuromodulatory activity profiles. The most potent bioactive fractions from both collections exhibited sodium channel blocking activity in murine Neuro-2a cells at 20 μg/mL and suppressed spontaneous calcium oscillations in murine neocortical neurons at 5 μg/mL. The lipoamide janthielamide A (**1**), obtained as the main bioactive constituent of the Curaçao collection, exhibited sodium channel blocking activity in murine Neuro-2a cells with an IC_{50} value of 11.5 μM. Janthielamide A was also found to antagonize veratridine-induced sodium influx in cerebrocortical neurons with an IC_{50} value of 5.2 μM (95% confidence interval = 2.67−10.1 μM; n = 6). From the Papua New Guinea collections, pure kimbeamide A (**2**) was found to exhibit sodium channel blocking activity in murine Neuro-2a cells at 20 μg/mL; however, further biological evaluation of these latter lipoamides was prevented by the rapid decomposition of **2**-**5** under either dry or organic solvent storage. Decomposition due to autoxidation is common in polyunsaturated compounds such as fatty acids, and thus, it is not surprising that these lipoamides readily decomposed despite efforts to prevent autoxidation by cold storage in benzene.[31,32]

Characterization of the Cyanobacterial Collections. The similar neuromodulatory activity profiles and related natural product skeletal types from these two collections suggested that the producing cyanobacteria might be closely related, despite their geographic distance and macroscopic morphological differences. On the basis of their growth morphologies as well as their filament and cell morphologies, the kimbeamide- and the janthielamide-producing strains best fell under the taxonomic definition of the genus *Symploca*. However, phylogenetic inferences of the 16S rRNA gene sequenced from each collection revealed that these two natural product-producing strains form a separate clade that is evolutionarily distant (p-distance = ∼5% gene sequence divergence) from the genus *Symploca* (reference strain: PCC

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8002[T], GenBank acc. no. AB039021). This clade of "tropical marine *Symploca*" has yielded several important natural products, including dolastatin 10 (e.g., strain VP377; GenBank acc. no. AF306497),[33] symplostatin 1 (e.g., strain VP642b; GenBank acc. no. AY032933),[33] and the hoiamides (strain PNG06−65.1; GenBank acc. no. HM072001)[34] (Figure 2). Although strains of this group have been published as *Symploca*, the evolutionary divergence together with the distinct ecological habitats of these two groups suggests that this clade of "tropical marine *Symploca*" needs to be described as a new genus.

In addition, microscopic morphological and phylogenetic analyses indicated that the cyanobacterial collections from Kimbe Bay, Papua New Guinea, consisted of a consortium of *Moorea producens*[35] (formerly *Lyngbya majuscula*) and "tropical marine *Symploca*". However, because the biomass was primarily composed of the finer "*Symploca*" filaments, and the fact that the *M. producens* was only found in one of the kimbeamide-producing specimens, it is likely that these are metabolites of the "tropical marine *Symploca*" clade.

■ CONCLUSION

The lipoamides janthielamide A and kimbeamides A−C, as well as the pyranone kimbelactone A, were isolated from independent collections of marine cyanobacteria from Curaçao and Papua New Guinea. All of these new cyanobacterial natural products are characterized by multiple unsaturations and an intriguing terminal vinyl chloride moiety. Both janthielamide A (**1**) and kimbeamide A (**2**) exhibited modest sodium channel blocking activity in murine Neuro-2a cells. Additionally, compound **1** antagonized veratridine-induced sodium influx in murine cerebrocortical neurons with an IC_{50} value of 5.2 μM. Thus, these metabolites constitute new lead molecules in the development of potential neuromodulatory agents.

Similar compounds in this structure class, such as pitiamide A,[22] grenadamide B, and grenadamide C,[25] were initially reported without characterization of their absolute configurations, likely due to the difficulties in determining the chirality of remote methyl substituents and the small quantities isolated. In the case of janthielamide A (**1**), hydrolysis and ozonolysis afforded two fragments, 2-methylsuccinic acid and 2-methyl-γ-aminobutyric acid, for which standards could be obtained commercially or via synthesis. Following optimization, our synthetic methodology to enantioselectively produce (*R*)- and (*S*)-*N*-acetyl-2-methyl-GABA could provide a facile route for generation of GABA analogues from the corresponding amino alcohols for further biological evaluations of these neurotransmitter-like compounds. Similarly, ozonolysis and hydrolysis of kimbeamide A (**2**) yielded alanine, which was readily analyzed and compared with amino acid standards. Thus, the determination of absolute configuration of these compounds was accomplished using a variety of approaches including fragmentation and derivatization followed by chiral or achiral GCMS or achiral LCMS analyses. The generation of diasteromers via esterification of carboxylic acids with (*S*)-2-(+)-octanol, as previously explored by our group,[27] has proven to be a robust method for determining the absolute configurations of small chiral fragments of natural products. Implementation of this methodology was key in the assignment of the chiral centers at C5 and C9 in **1**, which had proven difficult to solve using standard chiral GCMS and LCMS methodologies.

Despite macroscopic morphological differences, the putative producing organisms were revealed by microscopic and phylogenetic analyses to be closely related, belonging to an as yet undescribed genus of tropical marine cyanobacteria that is most similar to the extant genus *Symploca*. The similarities in the biological activity profiles, secondary metabolite chemical structure classes, and microscopic morphological characters all suggested that these organisms may be evolutionarily closely related. Ongoing investigation of the chemistry of morphologically similar and phylogenetically related organisms will certainly continue to reveal intriguing chemotaxonomic relationships in marine cyanobacteria.

Janthielamide A (**1**) and kimbeamides A−C (**2**−**4**), presumably of a mixed PKS/NRPS biosynthetic origin, further expand the halogenated lipoamide chemotype previously found in marine cyanobacteria which includes pitiamide A[22] and grenadamides B and C.[25] On the other hand, kimbelactone A (**5**) is most closely structurally related to fuligoic acid, a chlorinated polyene-pyrone from a myxomycete.[37] It is tempting to speculate on the biogenesis of the two different types of vinyl chloride moieties that are featured in these new lipoamides. With regard to kimbeamides A−C (**2**−**4**) and kimbelactone A (**5**), which possess a methyl-substituted vinyl chloride moiety, and grenadamides B and C,[25] which feature a propyl-substituted vinyl chloride, we hypothesize that this functionality arises in a fashion similar to that of the pendant vinyl chloride present in the jamaicamides A−C.[23,38] This is likely occurring through an hydroxymethylglutaryl CoA synthase (HMGCS) like addition of acetate to a β-keto-thioester intermediate, followed by chlorination, dehydration and decarboxylation. As for the terminal vinyl chloride functionality present in janthielamide A (**1**), kimbeamides A−C (**2**−**4**), grenadamide C[25] and pitiamide A,[22] it is conceivable that this moiety arises first via radical chlorination at the penultimate carbon of a β-hydroxy ACP thioester as well as sulfation of the β-hydroxy group. This functionally dense system is then predicted to undergo a concerted thioester-mediated hydrolysis, decarboxylation and sulfate elimination similar to the mechanism shown for terminal alkene formation in curacin A.[39,40]

■ EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were acquired at 75 or 500 MHz with a 5 mm probe, at 600 MHz with a 1.7 mm cryoprobe, or at 125 MHz with a direct observe ^{13}C 5 mm cold probe. NMR spectra were referenced to residual solvent ^{1}H and ^{13}C signals (δ_H 7.26, δ_C 77.16 for $CDCl_3$ and δ_H 7.16, δ_C 128.62 for C_6D_6). HPLC separation was accomplished with UV detection using HPLC grade solvent and a Phenomenex Maxil Silica column (10 μm, 10 × 500 mm). GCMS analysis was accomplished using a DSQ single quadrupole mass spectrometer, and either a chiral Cyclocil B Alltech capillary column (25 m × 0.25 mm) or an achiral DB-5 Agilent capillary column (30 m × 0.25 mm). LCMS analysis was accomplished using a Phenomenex Luna 5 μ C18(2) (250 × 4.60 mm).

Biological Material Collection and Identification. For the Curaçao collection, approximately 3 L of a lime green filamentous cyanobacterial mat was collected by hand at 1 m depth from a sandy reef substrate in Jan Thiel Bay on the leeward side of Curaçao (N 12°07.634′, W 68°88.088′). Following collection, the cyanobacterium was stored in 1:1 EtOH:seawater and stored at −20 °C; prior to shipping, the samples were thawed and the supernatant was discarded. For longer storage, EtOH was added and the samples were stored at −20 °C until workup. A voucher specimen (NAC 12/21/08-3) is maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA.

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For the Papua New Guinea collections, approximately 1.5 L of bright orange cyanobacterial puffballs (PNG 07/14/07-6) were collected by hand at 20 m depth from at least eight sites located throughout Kimbe Bay off the North coast of New Britain, Papua New Guinea (S 5°26.192′, E 150°40.813′). Field notes identified the puffballs as a consortium of *Schizothrix* sp. with a minor amount of *Lyngbya* sp. present. A separate collection of 250 mL of the cyanobacterium (PNG 07/18/07-3) was made at 25 m depth in Kimbe Bay (S 5°19.588′, E 150°18.034′). The collected samples were soaked in 1:1 EtOH:seawater in the field; the supernatant was decanted and discarded before shipment. For longer storage, EtOH was added, and the samples were stored at −20 °C until workup. Voucher specimens [collection numbers PNG 07/14/07-6 (1.5 L collection) and PNG 07/18/07-3 (250 mL collection)] are maintained at the University of California San Diego, Scripps Institution of Oceanography, La Jolla, CA.

Morphological Characterization. Morphological characterization was performed using an epifluorescent microscope (1000×). Morphological comparison and putative taxonomic identification of the cyanobacterial specimens were performed in accordance with modern classification systems.

DNA Extraction, PCR Amplification, and Cloning. Algal biomass (∼50 mg) was partly cleaned under a dissecting microscope. The biomass was 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) following the manufacturer's specifications. DNA concentration and purity were measured using a spectrophotometer. The 16S rRNA genes were PCR-amplified from isolated DNA using the modified lineage-specific primers, CYA106F 5′-CGGACGGGTGAGTAACGCGTGA-3′ and CSL1445R 5′-GGTAACGACTTCGGGCGTG-3′. 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 dH_2O. The PCR reactions were performed using the following gradient: initial denaturation for 2 min at 95 °C, 25 cycles of amplification, followed by 20 s at 95 °C, 20 s at 55 °C, 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 M13 primers. The 16S rRNA gene sequences are available in the DDBJ/EMBL/GenBank databases under acc. No. JQ388601 (NAC 12/21/08-3), JQ396912 (PNG 07/18/07-3), JQ388599 (PNG 07/14/07-6.1), and JQ388600 (PNG 07/14/07-6.2).

Phylogenetic Inference. The 16S rRNA gene sequences were aligned with evolutionary informative cyanobacteria using the L-INS-I algorithm in MAFFT 6.717[41] and refined using the SSU secondary structures model for *Escherichia coli* J01695[42] without data exclusion. The best-fitting nucleotide substitution model optimized by maximum likelihood was selected using corrected Akaike/Bayesian Information Criterion (AIC_C/BIC) in jModeltest 0.1.1.[43] The evolutionary histories of the cyanobacterial genes were inferred using Maximum likelihood (ML) and Bayesian inference algorithms. The ML inference was performed using GARLI 1.0[44] for the GTR+I+G model assuming heterogeneous substitution rates and gamma substitution of variable sites [proportion of invariable sites (pINV) = 0.494, shape parameter (α) = 0.485, number of rate categories = 4] with 1000 bootstrap replicates. Bayesian inference was conducted using MrBayes 3.1[45] with four Metropolis-coupled MCMC chains (one cold and three heated) run for 3000000 generations. The first 25% were discarded as burn-in, and the following data set was sampled with a frequency of every 100 generations. The MCMC convergence was detected by AWTY.

Extraction and Isolation. Two of the three liters of EtOH-preserved biomass (884.6 g, dry weight) of the Curaçao collection of "tropical marine *Symploca*" (NAC 12/21/08-3) were extracted with 2:1 CH_2Cl_2/MeOH eight times to give 2.77 g of crude extract (extract no. 1869). A portion of the crude extract (2.22 g) was fractionated using vacuum liquid chromatography (VLC) on silica gel (type H, 10−40 μM) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A−I). Fraction D, eluted with 40% EtOAc in hexanes (307 mg), was subjected to two iterations of normal-phase chromatography using Si Varian Bond Elut Sep-Paks. During the first NP-SPE cartridge chromatography step, the initial condition of 2.5% EtOAc in hexanes eluted 185 mg of material. A second NP-SPE cartridge chromatography, employing a stepwise gradient of 100% hexanes to 100% EtOAc, a fraction (D1F) eluting with 5% EtOAc in hexanes gave compound **1** (34.2 mg, 1.5% of extract). The surrounding subfractions D1E (22.4 mg) and D1G (6.2 mg) eluted with 2% and 10% EtOAc in hexanes, respectively, also contained **1**; however, these fractions contained additional impurities.

An EtOH-preserved consortium of "tropical marine *Symploca*" and *Moorea producens* (PNG 07/14/07-6, 101.7 g dry weight) was extracted with 2:1 CH_2Cl_2/MeOH six times to give 1.82 g of crude extract (extract no. 1707), while an additional sample of "tropical marine *Symploca*" (PNG 7/18/07-3, 5.97 g dry weight) was extracted with 2:1 CH_2Cl_2/MeOH five times to give 0.151 g of crude extract (extract no. 1708). On the basis of preliminary ^{1}H NMR and LCMS analyses indicating that the extracts were virtually identical, the crude extracts were combined and fractionated using vacuum liquid chromatography (VLC) on silica gel (type H, 10−40 μM) with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions (A−I). Fraction D, eluted with 40% EtOAc in hexanes (159 mg), was subjected to two iterations of normal-phase chromatography using Si Varian Bond Elut Sep-Paks. In the first round of NP-SPE column chromatography, the majority of the material (127 mg) eluted under the loading condition of 30% EtOAc in hexanes. The second round of NP-SPE cartridge chromatography utilizing a stepwise gradient of 100% hexanes to 100% EtOAc was more successful. Subfraction D1E (24.7 mg), eluted with 8% EtOAc in hexanes, was then fractionated using normal-phase HPLC with an isocratic condition of 18.5% EtOAc in hexanes at 6 mL/min on a Phenomenex Maxil 10 silica (500 × 10.0 mm, 10 μ) column to yield **2** (peak centered at 19.5 min, 1.6 mg, 0.10% of extract), **3** (peak centered at 15.5 min, 1.4 mg, 0.09% of extract) **4** (peak centered at 21 min, 0.8 mg, 0.05% of extract) and **5** (peak centered at 33 min, 0.7 mg, 0.04% of extract. Subfraction D1F (14.4 mg), eluted with 11% EtOAc in hexanes, was then fractionated using normal-phase HPLC with an isocratic condition of 30% EtOAc in hexanes at 6 mL/min on a Phenomenex Maxil 10 silica (500 × 10.0 mm, 10 μ) column to yield **5** (peak centered at 17.5 min, 2.6 mg, 0.16% of extract).

*Janthielamide A (**1**):* 34.2 mg (1.54%); yellow amorphous solid; $[\alpha]^{24}_D$ +10.2 (*c* 0.60, $CHCl_3$); UV (MeCN) λ_{max} (log ε) 224 (4.4) nm; IR ν_{max} (film) 3297, 2961, 2925, 1732, 1667, 1540, 1452, 1376, 1259, 1180, 973, 851, 755, 716 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 310 (100% rel abund) $[M + H]^+$; HRESI-TOFMS *m*/*z* $[M + H]^+$ 310.1934 (calcd for $C_{18}H_{29}ClNO$, 310.1932).

*Kimbeamide A (**2**):* 1.6 mg (0.10%); pale yellow oil; $[\alpha]^{24}_D$ +44.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 250 (4.0) nm; IR ν_{max} (film) 3290, 2926, 2855, 1718, 1660, 1541, 1449, 1373, 1255, 1140, 1115, 1078, 1001, 936 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m*/*z* 328 (28% rel abund) $[M + H]^+$, 373 (100% rel abund), 655 (5% rel abund) $[2 M + H]^+$, 677 (14% rel abund) $[2 M + Na]^+$, 700 (6% rel abund) $[2 M + 2 Na]^+$; HRESI-TOFMS *m*/*z* $[M + H]^+$ 328.1234 (calcd for $C_{17}H_{24}Cl_2NO$, 328.1229).

*Kimbeamide B (**3**):* 1.4 mg (0.09%) pale yellow oil $[\alpha]^{23}_D$ +33.4 (*c* 0.17, CH_2Cl_2); UV (obtained from UV trace during LCMS analysis) λ_{max} 246; ^{1}H and ^{13}C NMR data see the Supporting Information; ESIMS *m*/*z* 328 (13% rel abund) $[M + H]^+$, 373 (100% rel abund).

*Kimbeamide C (**4**):* 0.8 mg (0.05%); pale yellow oil; UV (obtained from UV trace during LCMS analysis) λ_{max} 258; ^{1}H NMR data, see Table S1 in Supporting Information; ESIMS *m*/*z* 328 (23% rel abund) $[M + H]^+$, 350 (11% rel abund) $[M + Na]^+$, 373 (100% rel abund), 677 (47% rel abund) $[2 M + Na]^+$.

*Kimbelactone A (**5**):* 3.3 mg (0.20%); pale yellow oil; $[\alpha]^{23}_D$ −164.7 (*c* 0.17, CH_2Cl_2); UV (MeCN) λ_{max} (log ε) 227 (4.2) nm; ^{1}H and ^{13}C NMR data, see Table 3; ESIMS *m*/*z* 301 (2% rel abund) $[M - Cl]^+$,

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337 (<1% rel abund) $[M + H]^+$, 359 (9% rel abund) $[M + Na]^+$, 695 (100% rel abund) $[2 M + Na]^+$; HRESI-TOFMS m/z $[M + Na]^+$ 359.1383 (calcd for $C_{19}H_{25}ClNaO_3$, 359.1384).

Stereoanalysis of C5 in Janthielamide A (1). Janthielamide A (**1**) was hydrolyzed (2.8 mg of **1** in 1 mL of 6 N HCl, 110 °C, 15 h), dried under a stream of N_2 gas, and then ozonized (1.5 mL CH_2Cl_2, 20 min, −78 °C) with oxidative workup (5 drops of 30% H_2O_2). The product was dried and then divided into two parts. The ozonized hydrosylate (0.8 mg) was treated with 300 μL of (*S*)-(+)-2-octanol and 150 μL of acetyl chloride and heated at 110 °C for 4 h to generate the dioctan-2-yl 2-methylsuccinate diastereomer. The excess reagent was evaporated under a constant stream of N_2 gas, and the dried residue was resuspended in CH_2Cl_2. Both (*R*)-(+)-methylsuccinic acid (2.1 mg) and (*S*)-(−)-methylsuccinic acid (1.3 mg) were derivatized using the same conditions as described for the reaction product of **1** to furnish the standards. All samples were analyzed by achiral GCMS under identical conditions; the initial oven temperature of 40 °C was held for 1 min, then the temperature was increased to 200 °C at a rate of 4.0 °C/min and held for 20 min. Co-injection of the derivatized (*R*)-methyl succinic acid standard with the derivatized fragment from **1** gave one peak at 46.06 min, while coinjection of the (*S*)-methyl succinic acid standard with the derivatized fragment from **1** gave two peaks at 44.93 min (*S*) and 46.05 min (janthielamide A). Thus, the absolute configuration at C5 was assigned as *R*.

Stereoanalysis of C9 in Janthielamide A (1). The desired fragment for stereoanalysis of C9, *N*-acetyl-protected and (*S*)-2-octanol esterified 2-methyl-γ-aminobutyric acid (GABA), was obtained in addition to the derivatized 2-methyl-succinic acid under the conditions employed for stereoanalysis of C5 from the initial aliquot of **1** {HR-ESI-TOFMS m/z 294.2035 $[M + Na]^+$ (calcd $C_{15}H_{29}NO_3Na$, 294.2040)}. The derivatized (*R*)-2-methyl-GABA standard was obtained via oxidation of *N*-acetyl protected (*R*)-4-amino-2-methyl-1-butanol (**16b**). To (*R*)-4-amino-2-methyl-1-butanol (**14b**, 100 mg, 106.4 μL) was added 4-dimethylaminopyridine (11.8 mg, 0.097 mmol, 0.1 equiv), pyridine (3.34 mL) and acetic anhydride (3.34 mL). The reaction mixture was stirred 15 h at rt and then dried under a stream of N_2 gas to afford the diprotected (amine and alcohol) fragment (**15b**). The residue was resuspended in 2:1 1,4-dioxane/H_2O (8.66 mL), and $LiOH·H_2O$ (407 mg, 9.7 mmol, 10 equiv) was added. After 1.5 h, solvents were removed under vacuum, and the residue was resuspended in H_2O (2 mL), acidified to pH 1 with 6 N HCl, and extracted with EtOAc (3 × 3 mL). The combined organic layer was dried over Na_2SO_4 and evaporated to dryness under a stream of nitrogen to give the *N*-acetyl-protected fragment (**16b**). To *N*-acetyl-protected (*R*)-4-amino-2-methyl-1-butanol (**16b**) in acetone (1 mL) at 0 °C was added dropwise Jones' reagent (400 μL, prepared by dissolving 2.7 g CrO_3 in 2.3 mL of concd H_2SO_4 and diluting to 10 mL at 0 °C). After 1 h at 0 °C, 2-propanol (800 μL) was added, and the mixture was stirred for 15 min. A small volume of H_2O was added, and the organic solvents were removed via a stream of nitrogen. A small volume of acetonitrile was added, the solution was vortexed for 5 s, the organic layer was removed, and the process was repeated two additional times until the chromium residue appeared dry. The combined acetonitrile layers were dried under N_2 gas to give *N*-protected 2-methyl-GABA (**7b**). (*S*)-2-Octanol (200 μL) and acetyl chloride (100 μL) were added to the *N*-protected 2-methyl-GABA, and the reaction was sealed and warmed to 110 °C. After 4 h, the reaction was cooled and then dried under N_2 gas, thus affording the *N*-acetyl and (*S*)-2-octanol esterified (*R*)-2-methyl-GABA standard [**9b**, HR-ESI-TOFMS m/z 294.2039 $[M + Na]^+$ (calcd for $C_{15}H_{29}NO_3Na$, 294.2040)].

(*S*)-4-Amino-2-methyl-1-butanol was obtained from (*R*)-(−)-3-bromo-2-methyl-1-propanol (**12**) via an S_N2 reaction in which the bromine was replaced with a nitrile, followed by reduction with $LiAlH_4$. To sodium cyanide (0.076 g, 1.55 mmol, 1.1 equiv) were added DMSO (2.24 mL) and (*R*)-(−)-3-bromo-2-methyl-1-propanol (**12**, 148 μL, 1.41 mmol). After 24 h, the reaction was quenched with H_2O (2.2 mL) and extracted with EtOAc (3 × 3 mL). The aqueous layer was acidified to pH 3 with 10% H_2SO_4 and extracted with EtOAc (3 × 3 mL). The organic layers were combined, washed with brine, dried over Na_2SO_4, and evaporated to dryness under N_2 gas to give (*S*)-4-hydroxy-3-methylbutanenitrile (**13**). This residue was resuspended in THF (3.5 mL) and added dropwise to a solution of $LiAlH_4$ (0.271 g, 7.40 mmol, 5.25 equiv) in THF (3.7 mL) at 0 °C. The reaction was allowed to warm to rt and stirred 19 h. The reaction was cooled to 0 °C and quenched with H_2O (3 mL) and a few drops of 10% NaOH. After the mixture was stirred 30 min, a small amount of Na_2SO_3 was added, and the mixture was stirred for 10 min and then filtered over qualitative paper #1. The filtrate was dried under N_2 to yield (*S*)-4-amino-2-methyl-1-butanol [**14a**, HR-ESI-TOFMS m/z 104.1072 $[M + H]^+$ (calcd for $C_5H_{14}NO$, 104.1070)]. The *N*-acetyl and (*S*)-2-octanol esterified (*S*)-2-methyl-GABA standard [**9a**, HR-ESI-TOFMS m/z 294.2042 $[M + Na]^+$ (calcd for $C_{15}H_{29}NO_3Na$, 294.2040)] was obtained as described above for the derivatized (*R*)-2-methyl-GABA standard from (*S*)-4-amino-2-methyl-1-butanol (**14a**). The standards and natural product derived fragment were resuspended in MeCN at a concentration of 1 mg/mL and subjected to analysis by LCMS. At a 0.4 mL/min flow rate, the run conditions were as follows: 60% acidified water (0.1% formic acid in 99.9% H_2O):40% MeCN held for 15 min, then ramped to 35% acidified water:65% MeCN over 90 min, then ramped to 100% MeCN over 10 min and held for 15 min, then ramped back to 60% acidified water:40% MeCN over 10 min and held 10 min. Co-injection of the derivatized (*R*)-2-methyl-GABA standard with the derivatized fragment from **1** gave one peak at 71.47 min, while coinjection of the derivatized (*S*)-2-methyl-GABA standard with the derivatized fragment from **1** gave two peaks at 70.47 min (*S*) and 71.51 min (janthielamide A). Thus, the absolute configuration at C9 was assigned as *R*.

Stereoanalysis of C-1′ in Kimbeamide A (2). Kimbeamide A (**2**) was ozonized (0.2 mg of **2** in 400 μL CH_2Cl_2, 5 min, −78 °C) with oxidative workup, dried under a stream of N_2 gas, and hydrolyzed (1 mL of 6 N HCl, 110 °C, 16 h). The hydrolysate was dried under a constant stream of N_2 gas and treated with 150 μL of acetyl chloride and 500 μL of IPA and heated at 110 °C for 1 h to generate the isopropyl ester of alanine. The excess reagent was evaporated under a constant stream of N_2 gas, and the dried residue was derivatized with trifluoroacetic anhydride (400 μL) in CH_2Cl_2 (400 μL) at 110 °C for 15 min. The *N*-(trifluoroaceto)isopropyl ester of the alanine residue was solubilized in EtOAc. Both L- and D-alanine (0.5 mg each) were derivatized using the same conditions as described for the ozonized hydrolysate of **2** to furnish standards. All samples were analyzed by chiral GCMS under identical conditions; the initial oven temperature of 50 °C was held for 3 min, increased to 90 °C at a rate of 1 °C/min, and held for 10 min. Co-injection of the derivatized L-alanine standard with the derivatized fragment from **2** gave one peak at 18.82 min, while coinjection of the D-alanine standard with the derivatized fragment from **2** gave two peaks at 16.87 min (D-alanine) and 18.82 min (kimbeamide A).

Sodium Channel Activation and Blocking Assay. Neuro-2a 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. 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 16 wells designated as negative controls for each plate. Brevetoxin-2, at a final concentration of 0.435 μg/mL (486 nM), was used as the positive control for the sodium channel activating assay (e.g., 100% activation = complete cell death) and tetrodotoxin, at a final concentration of 0.0435 μg/mL (136 nM), was the positive control for the blocking assay (e.g., 100% blocking = no cell death), as previously tested in our laboratory. Eight wells were used for each treatment. A mixture of ouabain, veratridine and HCl/PBS 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 of 5 mM ouabain/0.35 mM veratridine/0.75 mM HCl/PBS was added, and for the activating assay a solution of 5 mM ouabain/0.15 mM

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veratridine/1.75 mM HCl/PBS was added. A solution of PBS/5 mM HCl without ouabain or veratridine was added to the top half of each plate to give the general toxicity of the test compounds unrelated to sodium channel regulation. The final volume for each well was 230 μL. 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. The plates were read at 570 and 630 nm. IC_{50} determinations were the result of three technical replicates at each concentration and thus represent a single IC_{50} measurement. Under these conditions, tetrodotoxin gave an IC_{50} = 9.7 nM. Concentration−response graphs were generated using GraphPad Prism.

■ ASSOCIATED CONTENT

Supporting Information

Tabulated NMR spectroscopic data for **3** and tabulated ^{1}H NMR data for **2**−**4**; ^{1}H, ^{13}C, gCOSY, gHMBC, and gHSQC NMR spectra for **1**−**3** and **5**; NOESY spectra for **2**, **3**, and **5**; ^{1}H spectrum for **4**; GCMS and LCMS chromatograms from stereochemical analyses; CD spectra for **5**; macro- and microscopic images of the specimens; experimental data for neocortical neuron assays; dose−response curves for janthielamide A neuromodulatory activity; graphical data for neocortical neuron assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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