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Structure and Biosynthesis of the Jamaicamides, New Mixed Polyketide-Peptide Neurotoxins from the Marine Cyanobacterium *Lyngbya majuscula*

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Summary

A screening program for bioactive compounds from marine cyanobacteria led to the isolation of jamaicamides A–C. Jamaicamide A is a novel and highly functionalized lipopeptide containing an alkynyl bromide, vinyl chloride, β-methoxy eneone system, and pyrrolinone ring. The jamaicamides show sodium channelblocking activity and fish toxicity. Precursor feeding to jamaicamide-producing cultures mapped out the series of acetate and amino acid residues and helped develop an effective cloning strategy for the biosynthetic gene cluster. The 58 kbp gene cluster is composed of 17 open reading frames that show an exact colinearity with their expected utilization. A novel cassette of genes appears to form a pendent carbon atom possessing the vinyl chloride functionality; at its core this contains an HMG-CoA synthase-like motif, giving insight into the mechanism by which this functional group is created.

Introduction

Marine life forms continue to provide some of the most structurally intriguing and biologically active natural products, several of which are contributing to a very active pipeline of pharmaceutical agents [1]. Among these organisms, marine cyanobacteria (blue-green algae) are quite possibly the most exciting in their production of new chemotypes with important biological activity [2]. The major metabolic theme employed by marine cyanobacteria to construct these natural products integrates two modular biosynthetic systems, nonribosomal peptide synthetases (NRPSs), which are responsible for assembling amino acids, and polyketide synthases (PKSs), for linking together acetate as the primary building block. Inherent to this mixed NRPS/PKS biosynthesis is great structural diversity, and this is further compounded by a truly extraordinary number of secondary tailoring manipulations, including oxidation, methylation, and diverse forms of halogenation. The study of these cyanobacterial metabolites and their biosynthetic origins has great value not only for drug discovery but also for harnessing these pathways to create new molecules of biomedical utility [3].

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While the biological activities reported for marine cyanobacterial metabolites vary widely, three major trends emerge. A number of these metabolites target either the polymerization of tubulin (e.g., dolastatin 10 [4], curacin A [5]) or the polymerization of actin (e.g., hectochlorin [6], majusculamide C [7]). Additionally, a growing number of potently bioactive metabolites from cyanobacteria target the mammalian voltage-gated sodium channel, either as blockers (kalkitoxin [8]) or activators (antillatoxin [9]). Hence, we have employed a simple cell-based screen of marine cyanobacterial extracts and compounds for detecting new neurotoxins that modulate the activity of this important ion channel [10]. This approach has been fruitful, and we report here the results that followed from initial detection of neurotoxic activity in a Jamaican collection of *Lyngbya majuscula*. Because of the unusual structures of the isolated compounds, jamaicamides A–C (Figure 1), we have been motivated to examine their biosynthetic origins in considerable detail. Isotope-labeling experiments with producing laboratory cultures identified the component building blocks, whereas their assembly has been partially elucidated by a molecular genetics approach. In this work, we report the discovery, structure elucidation, biological properties, biosynthetic subunits, and sequence of the gene cluster encoding for the biosynthesis of jamaicamides A–C.

Results and Discussion

Isolation and Structure Elucidation of the Jamaicamides A, B, and C

A dark green strain of *Lyngbya majuscula* was found growing in low abundance in Hector's Bay, Jamaica (designated strain JHB). A small sample preserved at the time of collection was extracted and shown to possess potent brine shrimp toxicity. Additional effort ultimately was successful in adapting this organism to laboratory culture. Pan cultures yielded approximately 10 g/l biomass after 6 weeks, and this was extracted by standard methods for lipid natural products. Again, the extract was strongly bioactive in the brine shrimp model, and this was used to direct the isolation of hectochlorin (**1**), a novel cyclic peptide with potent actin-polymerizing properties [6]. Further evaluation of the extract and its primary vacuum liquid chromatographic (VLC) fractions identified a midpolarity mixture with activity in a cellular assay designed to detect mammalian voltage-gated sodium channel-blocking substances [10]. This fraction was also identified as possessing potentially novel substances by TLC and ^{1}H NMR analysis. Subsequent HPLC of this fraction led to the isolation of three new lipopeptides with low micromolar levels of channel-blocking activity.

The HRFABMS of jamaicamide A (**2**) yielded an $[M + H]^+$ for a molecular formula of $C_{27}H_{37}N_2O_4ClBr$ (*m/z* 567.1625, −0.7 mmu dev.). The isotope peaks at *m/z* 567/569/571 (4:5:1.5 ratio) were consistent with the pres-

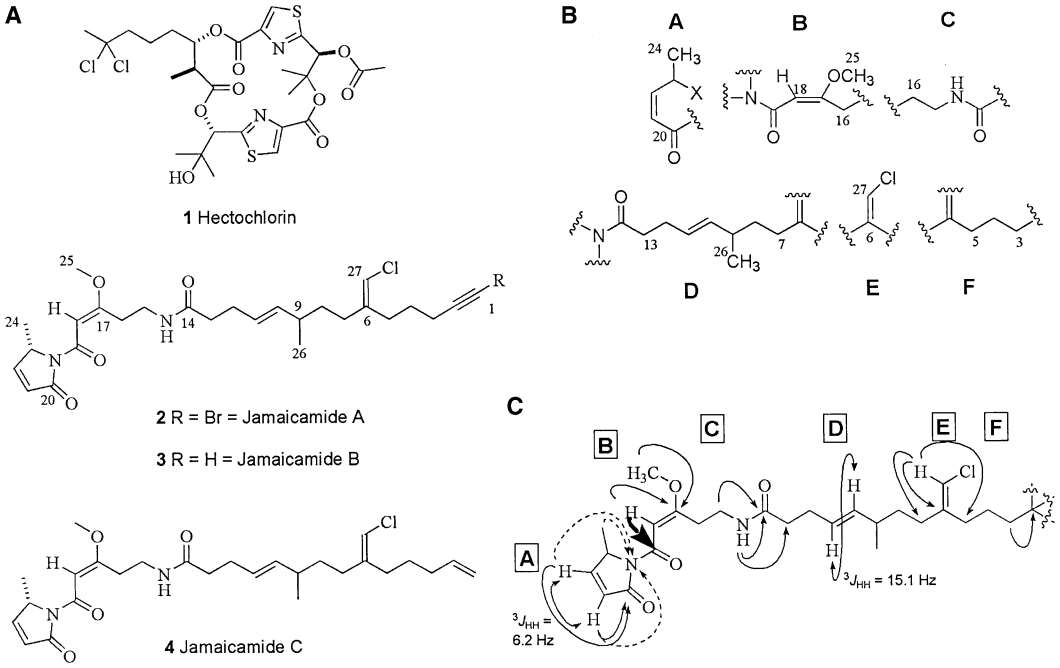

Figure 1. Structures of Metabolites Isolated from Jamaican Strain of *Lyngbya majuscula* and Assembly of Partial Structures A–F of Jamaicamide A

(A) Structures of hectochlorin and jamaicamide A–C, four metabolites isolated from this Jamaican strain of *Lyngbya majuscula*.
(B) Partial structures A–F of jamaicamide A (**2**) derived from ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, HSQC-COSY, and ^{1}H-^{13}C HMBC data.
(C) Assembly of partial structures A–F in jamaicamide A (**2**). Double-headed arrows represent $^3J_{HH}$ couplings, single-headed arrows represent important $^{2,3}J_{CH}$ couplings from HMBC, dashed arrows represent key ^{1}H-^{15}N HMBC correlations, and bolded arrow represents a key ACCORD-1, 1-ADEQUATE correlation (H-18 to C-19). The boxed letters indicate the original partial structure designations.

ence of one chlorine and one bromine atom in the molecule, and the molecular formula indicated 10 degrees of unsaturation. Comparison of the ^{13}C NMR and DEPT spectra identified jamaicamide A to possess 3 methyl groups, 9 methylenes, 8 methines, and 7 quaternary carbon atoms.

Analysis of the HSQC [11] and HSQC-COSY (HSQC-TOCSY with 12 ms DIPSI mixing time) [12] spectra allowed construction of six partial structures (A–F, Figure 1). By ^{1}H-^{1}H COSY, partial structure A was composed of a modestly deshielded doublet methyl group (δ1.46) adjacent to three contiguous methine protons. The first of these was at a shift (δ4.87) consistent with its being attached to a carbon bearing a heteroatom (δ_C 58.1) and showed both vinylic and allylic couplings to an adjacent polarized double bond (δ7.22 and 6.08), the shifts of which were indicative of an enone functionality. This was confirmed by HMBC, which identified an adjacent carbonyl carbon at δ170.0, a chemical shift consistent with an amide or ester group. Partial structure B was composed of a deshielded singlet proton (H-18, δ_H 6.73; δ_C 95.0), which showed reciprocal HMBC correlations to an *O*-methyl group (δ_H 3.75; δ_C 56.1) and an allylic methylene group (δ_H 2.84, 3.00). This proton, H-18, also showed an HMBC correlation to a deshielded olefinic carbon (δ_C 175.3), and this latter signal showed HMBC correlations from both the *O*-methyl group and H_2-16 methylene. The extreme level of double bond polarization was explained by the combination of vinyl methoxy group and adjacent amide-type carbonyl (C-19 δ_C 166.0), the latter of which was located proximately, as revealed by a correlation observed by Accordion 1,1-ADEQUATE [13]. This variant of the ADEQUATE experiment, created in the course of the present investigation, comprehensively detects ^{1}H-^{13}C-^{13}C spin systems for a wide range of ^{13}C-^{13}C coupling values. Moreover, when this data set is used in conjunction with that obtained from HMBC, it allows for unequivocal discrimination between 2- and 3-bond HMBC correlations. The shifts for this highly polarized and substituted system closely match those for a similar arrangement of atoms observed in the cyanobacterial metabolite barbamide [14]. Selective irradiation of the protons at δ3.75 (H_3-25) using the DPFGSE 1D NOE experiment resulted in a strong NOE enhancement of H-18 (δ6.73), indicating 17(*E*) geometry in jamaicamide A (**2**, Figure 1) [15, 16].

Partial structure C was composed of two mutually coupled and therefore adjacent midfield methylenes, one of which was also a portion of partial structure B (H_2-16), and the other (δ3.49 and 3.53) was coupled to an amide NH proton (δ6.68). A series of contiguous methylene and methine groups, plus one branching methyl group, delineated partial structure D. A triplet-appearing methylene group at δ2.18, a chemical shift consistent with an adjacent carbonyl, was sequentially adjacent to an allylic methylene (δ2.28), a *trans* disubstituted double bond (J = 15.1 Hz), a methine (δ2.02) possessing a branching doublet methyl group (δ0.94), and then two additional methylene groups (δ1.34 and 2.00). The modestly deshielded nature of this latter resonance (C-7)

Table 1. ^{1}H and ^{13}C NMR Spectral Data (in ppm) for Jamaicamide A (**1**) with HMBC and ACCORD 1,1-ADEQUATE Correlations

Position No.	δ ^{1}H (*J* in Hz)[a]	*m*	δ ^{13}C (mult.)[b,c]	HMBC[d]	Accordion 1,1-ADEQUATE[e]
1	–	–	38.17 C	–	–
2	–	–	79.9 C	–	–
3	2.22 (7.2)	dd	19.6 CH_2	C-2, C-4, C-5	C-2, C-4
4	1.63	m	25.8 CH_2	C-2, C-3, C-5, C-6	C-3, C-5
5	2.25	m	29.2 CH_2	C-3, C-4, C-6, C-7, C-27	C-4, C-6
6	–	–	141.7 C	–	–
7	2.00	m	32.6 CH_2	C-5, C-6, C-8, C-9, C-27	C-6, C-8
8	1.34	m	34.7 CH_2	C-6, C-7, C-9, C-10, C-26	C-7, C-9
9	2.02	obs	36.3 CH	C-7, C-8, C-10, C-11, C-26	C-8, C-10, C-26
10	5.26 (15.1, 7.8)	dd	136.6 CH	C-8, C-9, C-11, C-12, C-26	C-9, C-11
11	5.35 (15.1, 6.4)	dt	127.5 CH	C-9, C-10, C-12, C-13	C-10, C-12
12	2.28	m	28.5 CH_2	C-10, C-11, C-13, C-14	C-11, C-13
13	2.18 (7.9)	t	36.7 CH_2	C-11, C-12, C-14	C-12, C-14
14	–	–	172.4 C	–	–
15	3.49	m	38.20 CH_2	C-14, C-16, C-17	C-16
	3.53	m		C-14, C-16, C-17	C-16
16	2.84	m	32.3 CH_2	C-15, C-17, C-18	C-15, C-17
	3.00	m		C-15, C-17, C-18	C-15, C-17
17	–	–	175.3 C	–	–
18	6.73	s	95.0 CH	C-16, C-17, C-15	C-17, C-19
19	–	–	166.0 C	–	–
20	–	–	170.0 C	–	–
21	6.08 (6.2, 1.4)	dd	125.8 CH	C-20, C-22, C-23	C-20, C-22
22	7.22 (6.2, 1.9)	dd	153.1 CH	C-20, C-21, C-23, C-24	C-21, C-23
23	4.87 (6.8, 1.8, 1.4)	ddd	58.1 CH	C-21, C-22, C-24	C-21, C-22, C-24
24	1.46 (6.7)	d	17.9 CH_3	C-22, C-23	C-23
25	3.75	s	56.1 CH_3	C-17, C-18	–
26	0.94 (6.8)	d	20.8 CH_3	C-8, C-9, C-10	C-9
27	5.79	s	112.8 CH	C-4, C-5, C-6, C-7	C-6
NH	6.68	m	–	C-14	–

[a] Recorded at 500.17 MHz.
[b] Recorded at 125 MHz.
[c] Multiplicity deduced by multiplicity edited HSQC.
[d] Carbons showing long-range correlation with indicated proton.
[e] Carbons with $^{2}J_{CH}$ correlations to indicated proton via $^{1}J_{CC}$ coupling.

was consistent with its being adjacent to a double bond. HMBC confirmed the presence of a carbonyl adjacent to the initial methylene group (C-13) of partial structure D, and by chemical shift (δ172.4), this carbonyl was present as an amide group.

Partial structure E was composed of a singlet methine proton at δ5.79 with an associated carbon resonance at δ112.8. By both HMBC and the Accordion 1,1-ADEQUATE experiment, this was adjacent to a moderately deshielded olefinic carbon at δ141.7 and yielded a grouping of chemical shifts characteristic for a chlorine-substituted terminal olefin. This functionality has been widely observed in diverse marine cyanobacterial metabolites, most notably the malyngamides [3].

Partial structure F consisted of three consecutive and coupled methylene groups (see Table 1). One of the two terminal methylenes of substructure F was at a shift (δ_H 2.25; δ_C 29.2) suggestive of its being allylic. This was confirmed by measurement of both HMBC and Accordion 1,1-ADEQUATE correlations to the vinyl chloride-containing terminal olefin group (partial structure E). The other terminal methylene group of partial structure F was slightly more shielded (δ_H 2.22; δ_C 19.6), the protons of which showed HMBC correlations to a singlet ^{13}C resonance at δ79.9 and ostensibly to a carbon at δ38.2 (assigned above to the C-15 methylene in partial structure C). This latter connection was internally incompatable with other features of the structure assignment and made us query the data set more carefully. As described below, only following partial assembly of the substructures of jamaicamide A (**2**) and spectroscopic investigations with model compounds was it possible to derive the correct interpretation of these data.

Partial structure A was shown to be a 5-membered heterocylic ring (pyrrolinone or butenolide) by virtue of an HMBC correlation between the H-23 methine and C-20 carbonyl at δ170.0. A ^{1}H-^{15}N HMBC [17] provided crucial correlations (^{3}J between N and H-18, H-21, H-22, and H_3-24) between partial structures A and B and showed that the ring was nitrogen containing (this was also required to provide the necessary branch point from the fully assigned pyrrolinone ring [Figure 1]). Partial structures B and C were connected above through HMBC correlations between H-18 and C-16, while structure C was connected to D via HMBC correlations between the solitary NH proton of C to the C-14 amide-type carbonyl in D as well as the adjacent C-13 methylene carbon. The H-27 singlet resonance of partial structure E displayed key HMBC interconnections with both D and F (e.g., H-27 to C-5 and C-7).

With this partial assembly complete, it became appropriate to take stock of the assigned atoms. Subtraction

of the atoms assigned to partial structures A–F from the molecular formula ($C_{27}H_{37}N_2O_4ClBr - C_{25}H_{37}N_2O_4Cl$) yielded only the fragment "C_2Br" unassigned, and this must be connected to the terminus of partial structure F. Although considered initially implausible, only a single molecular constitution was possible for this fragment: a terminal alkynyl bromide. In order to confirm the presence of this unprecedented fragment, the ^{13}C NMR spectrum of a model compound, 11-bromo-undec-10-ynoic acid amide (Sigma-Aldrich Library of Rare Chemicals, catalog), was acquired. C-11 and C-10 of this model compound resonate at δ37.4 and δ80.3, respectively (see Supplemental Data). Upon reinspection of the ^{13}C NMR spectrum for jamaicamide A, a small shoulder on the C-15 (δ 38.2) resonance could be observed at δ38.17. Moreover, a careful plot expansion showed the HMBC correlations from the H_2-3 protons were to the two carbon signals of this unusual acetylenic bond at δ79.9 and δ38.17. With this final moiety confirmed, the planar structure of jamaicamide A (**2**) was complete.

The geometry of the C-6/C-27 vinyl chloride moiety was determined by measuring the $^3J_{CH}$ coupling constants using the HSQMBC experiment [18]. Coupling constant values of $^3J_{C5\text{-}H27} = 6.7$ Hz and $^3J_{C7\text{-}H27} = 4.9$ Hz dictated an *E* geometry for this double bond. The absolute stereochemistry of the pyrrolinone methyl (C-23) in jamaicamide A was determined by Marfey's analysis [19]. L-Ala was released from jamaicamide A following ozonolysis, acid hydrolysis, derivatization with FDAA, and HPLC in comparison with the appropriately derivatized L- and D-Ala standards. Determination of the other stereocenter in **2**, the secondary methyl group at C-9, has proven problematic. Several attempts at oxidative ozonolysis of the C-10/C-11 olefin did not yield an isolable product, presumably due to additional oxidation of the terminal alkyne (C-1) and olefin (C-6). Hence, the stereochemistry at C-9 remains unknown at present.

Jamaicamide B (**3**) was also isolated as a pale yellow oil from the lipid extract of this cultured *L. majuscula* JHB. Jamaicamide B was slightly more polar than jamaicamide A, eluting before jamaicamide A under RPHPLC conditions. The observed ratio of the $[M + H]^+$ isotope peak cluster in the FABMS was consistent with the presence of a single chlorine atom (*m/z* 489/491 in an approximate 3:1 ratio). HRFABMS revealed a molecular formula of $C_{27}H_{37}O_4N_2Cl$, again indicating 10 degrees of unsaturation. The ^1H and ^{13}C resonances for jamaicamide B were highly similar to those of jamaicamide A. However, the absence of a ^{13}C signal at δ38.17 (C-1 in **2**) and the appearance of a new signal at δ68.6 with an associated proton at δ1.98, taken in concert with the absence of a bromine atom evident from mass spectrometry, suggested that jamaicamide B was the debromo analog of jamaicamide A. This assignment was further supported by the downfield shift of C-2 (from δ79.9 in **2** to δ84.1 in **3**) and by HMBC correlations from the C-3 methylene group at δ2.20 to both C-1 and C-2.

Jamaicamide C (**4**) was isolated in small quantity (0.5% of crude extract) as a pale yellow oil and was slightly more hydrophobic than either jamaicamide A or B. As observed for jamaicamide B (**3**), the isotope pattern of the molecular ion cluster clearly indicated the presence of a single chlorine atom (*m/z* 491/493 in an approximate 1:0.4 ratio). HRFABMS yielded a molecular formula of $C_{27}H_{39}O_4N_2Cl$ for **4**, indicating 9 degrees of unsaturation, one less than either jamaicamide A or B. ^1H and ^{13}C NMR clearly demonstrated that the two additional hydrogen atoms in jamaicamide C relative to jamaicamide B (**3**) resulted from partial saturation of the terminal alkyne, forming a terminal olefin in **4**. Specifically, new ^1H NMR signals were observed at δ5.02 and 4.97 (δ_C 114.7), and these were coupled to an additional new resonance at δ5.82 (δ_C 138.4). Hence, jamaicamide C (**4**) was defined as 1,2-dihydrojamaicamide B (**3**).

The jamaicamides have several structural features of novelty and biosynthetic interest. First, it can be predicted that they result from alternating PKS and NRPS assembly units; this hypothesis was confirmed as described below. Second, jamaicamide A has a structural feature rarely observed in nature, an alkynyl bromide moiety. This feature, as far as we know, has only been observed in fatty acid derivates isolated from lichens collected in Central Asia [20, 21]. While the vinyl chloride of all three jamaicamides has previously been observed in various *Lyngbya* metabolites [3], this is the first time that this functionality has been found in the middle of an extended polyketide chain. For example, in the malyngamides, this distinctive appendage is always found either one or two carbons distant from an amide functionality [22, 23]. Finally, the pyrrolinone functionality has been observed in several other *Lyngbya* metabolites and is believed to be important to the biological activities of some of these natural products (e.g., the microcolins) [24]. However, the biosynthetic origins and mechanism of formation of this ring system are unknown. As described below, a combined precursor feeding approach and molecular gene cloning and sequencing approach has given considerable insight into the biosynthetic origins of these unusual features of jamaicamide A.

Biological Properties of the Jamaicamides

Jamaicamides A, B, and C exhibited cytotoxicity to both the H-460 human lung and Neuro-2a mouse neuroblastoma cell lines. The LC_{50}s were approximately 15 μM for all three compounds to both cell lines. All three compounds also exhibited sodium channelblocking activity at 5 μM, producing approximately half the response of saxitoxin applied at 0.15 μM. None of the jamaicamides exhibited sodium channel-activating activity at the concentrations tested. In the goldfish toxicity assay, a system that has been useful for the detection of neurotoxic activity in crude extracts as well as purified compounds [9, 25], jamaicamide B (**3**) was the most active (100% lethality at 5 ppm after 90 min), followed by jamaicamide C (**4**) (100% lethality at 10 ppm after 90 min). Interestingly, jamaicamide A (**1**) was the least active fish toxin (sublethal toxicity at 10 ppm after 90 min). Neither jamaicamide A (**2**) nor B (**3**) showed significant brine shrimp toxicity, while jamaicamide C was only modestly active at 10 ppm (25% lethality) [26].

Stable Isotope Feeding Experiments to Jamaicamide A

Dissection of the chemical structure of jamaicamides A-C (**2–4**) suggested that these metabolites derive from

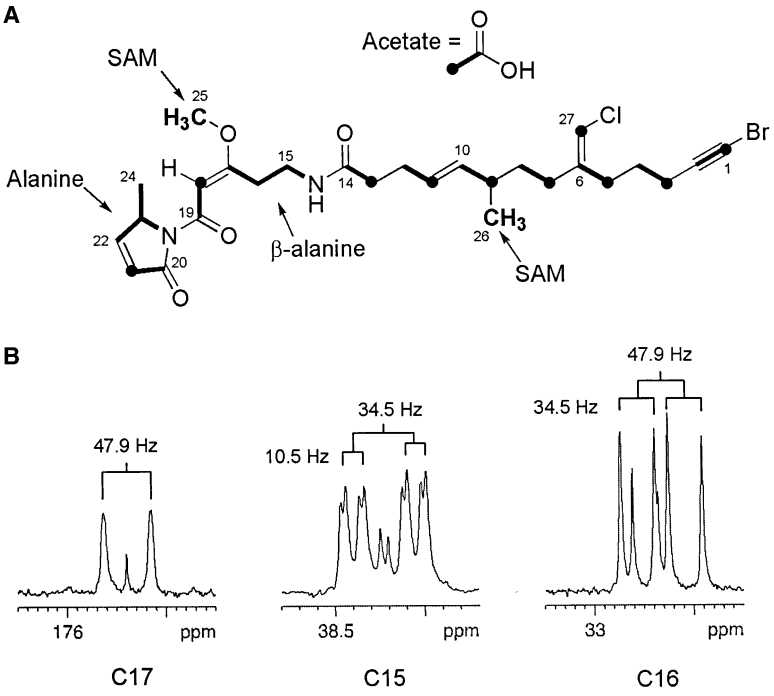

Figure 2. Biosynthetic Precursors of Jamaicamide A

(A) Biosynthetic precursors of jamaicamide A (**2**).

(B) Expansions of the ^{13}C NMR spectrum of jamaicamide A (**2**) isolated from cultures provided with [$^{13}C_3$,^{15}N]β-Ala. Coupling constants ($^2J_{CC}$ and $^2J_{NC}$) indicate intact incorporation of the labeled β-Ala precursor.

a mixture of polyketide (9 acetate units), amino acid (L-Ala and β-Ala), and methionine-derived methyl groups. However, their unique molecular features, such as the alkynyl bromide, vinyl chloride, and pyrrolinone ring, warranted a more in depth investigation. Indeed, the metabolic origin of the vinyl chloride pendent carbon (C-27) in the jamaicamides remained completely uncertain. Thus, to fully explore the biosynthetic origin of the carbon atoms within the jamaicamides, various isotopically labeled precursors were supplied to *L. majuscula* JHB, and labeling patterns were discerned by NMR spectroscopy (Figure 2).

Two separate isotope feeding experiments, [1-^{13}C] acetate and [2-^{13}C]acetate, were conducted initially (see Experimental Procedures) to explore the origin of the lipid chain of jamaicamide A (**2**). Compared to natural abundance, the ^{13}C NMR spectrum of compound **2** isolated from the [1-^{13}C]acetate feeding showed that carbons 2, 4, 6, 8, 10, 12, and 14, as well as carbons 19 and 20, all arise from C-1 of acetate (Table 2 and Supplemental Data). Similarly, analysis of the ^{13}C NMR spectrum from the [2-^{13}C]acetate feeding experiment established that carbons 1, 3, 5, 7, 9, 11, 13, 18, and 21 are all derived from C-2 of acetate (Table 2 and Supplemental Data). Interestingly, C-27 was also significantly labeled from this [2-^{13}C]acetate feeding, while the second branching carbon, C-26, showed no incorporation from acetate.

A doubly labeled [1,2-$^{13}C_2$]acetate feeding experiment was then performed to observe incorporation of intact acetate units into the jamaicamide A (**2**) backbone (Supplemental Data). Although a relatively high degree of incorporation from degraded acetate was observed, $^{13}J_{CC}$ coupling constant analysis indicated that intact acetates were incorporated into carbon pairs C-1/C-2 (J_{CC} = 170.7 Hz), C-3/C-4 (J_{CC} = 33.9 Hz), C-5/C-6 (J_{CC} = 43.3 Hz), C-7/C-8 (J_{CC} = 33.3 Hz), C-9/C-10 (J_{CC} = 40.3 Hz), C-11/C-12 (J_{CC} = 43.3 Hz), C-13/C-14 (J_{CC} = 49.7 Hz), C-18/C-19 (J_{CC} = 71.9 Hz), and C-20/C-21 (J_{CC} = 63.1 Hz). Results from these acetate experiments were confirmed by comparison with the labeling patterns observed for jamaicamide B (**3**) obtained from the same feeding experiment and clearly demonstrated that, starting from C-1, seven intact acetate units are linearly assembled to form the lipid portion of jamaicamide A. In addition, carbons C-18/C-19 and C-20/C-21 are also incorporated as intact acetate units. The enrichment of C-27 by C-2 of acetate is in keeping with previous biosynthetic findings for metabolites such as oncorhyncolide [27] and mupirocin [28]. In the jamaicamides, a 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS)-like mechanism is thought to be involved in the addition of a malonyl CoA- or acetyl CoA-derived acetate to the C-6 keto-carbon of the nascent 8-carbon polyketide (see cloning section below). The initially formed β-hydroxyacyl acid intermediate could then undergo decarboxylation and halogenation to form the vinyl chloride functionality observed in jamaicamides A–C.

Considering results obtained in our laboratory from biosynthetic studies with curacin A and barbamide [29, 30], we predicted that both the *O*-methyl carbon C-25 and the branching methyl group C-26 should originate from methionine via *S*-adenosyl methionine (SAM). Analysis of the ^{13}C NMR spectrum of jamaicamide A (**2**) isolated from an *S*-[methyl-^{13}C]methionine feeding experiment revealed that both C-25 and C-26 arise from the C1 pool via SAM, displaying incorporation enhancements of 2.6-fold and 2.5-fold, respectively (Table 2 and Supplemental Data).

The methyl pyrrolinone moiety of the jamaicamides was conceived to derive from a Claisen-like condensation and cyclization of an acetate unit (C-20/C-21) with

Table 2. Relative Enhancements of Carbon Resonances from Biosynthetic Feeding Experiments to Jamaicamide A (**2**)

Position	Chemical Shift (ppm)[a]	[1-^{13}C]Acetate	[2-^{13}C]Acetate	[CH_3-^{13}C]Methionine	[1-^{13}C]Alanine	[3-^{13}C]Alanine
1	38.17	1.0	*1.6*	1.1	0.9	*1.3*
2	79.9	*1.7*	1.1	1.2	1.0	0.6
3	19.6	0.9	*1.5*	0.9	1.0	*1.4*
4	25.8	*1.5*	0.9	1.0	1.0	1.0
5	29.2	1.0	*2.0*	1.0	1.0	*1.5*
6	141.7	*1.6*	0.9	1.2	1.0	0.9
7	32.6	0.9	*1.4*	0.9	1.0	*1.3*
8	34.7	*1.9*	1.0	1.0	0.9	1.0
9	36.3	1.0	*2.0*	0.9	1.0	*1.4*
10	136.6	*2.0*	0.9	1.0	0.9	1.0
11	127.5	0.9	*1.8*	1.0	0.9	*1.5*
12	28.5	*2.0*	1.0	1.0	1.0	1.0
13	36.7	1.1	*2.0*	1.0	1.0	*1.5*
14	172.4	*2.1*	1.0	1.1	1.1	0.9
15	38.2	1.1	1.3	1.0	0.9	1.0
16	32.3	1.1	1.1	1.0	1.0	1.1
17	175.3	1.1	1.2	1.1	1.1	1.0
18	95.0	1.1	*2.3*	1.0	1.0	*1.5*
19	166.0	*1.8*	0.9	1.3	1.1	0.8
20	170.0	*1.7*	0.9	1.1	1.0	0.7
21	125.8	1.1	*2.4*	1.0	1.0	*1.5*
22	153.1	1.1	1.0	1.0	*2.0*	1.0
23	58.1	1.0	1.0	0.9	0.9	0.9
24	17.9	1.3	1.2	1.0	1.0	*3.2*
25	56.1	1.3	1.2	*2.6*	1.1	1.1
26	20.8	1.1	1.0	*2.5*	1.0	1.0
27	112.8	1.1	*1.7*	1.0	1.1	*1.5*

Methods for quantitation are detailed in Experimental Procedures. Bolded/italicized numbers indicate ^{13}C enrichment.
[a] Referenced to $CDCl_3$ centerline, 77.0 ppm.

L-Ala (C-22/C-24 and the tertiary nitrogen). Results from the acetate feeding studies confirmed the acetate origin of the C-20/C-21 portion. Thus, to examine the origins of the C-22/C-24 of the jamaicamide pyrrolinone ring, both L-[1-^{13}C]Ala and L-[3-^{13}C]Ala were separately provided to jamaicamide-producing cultures. Analysis of the ^{13}C NMR spectra of isolated jamaicamide A (**2**) revealed a 2.0-fold enhancement of C-22 from the L-[1-^{13}C]Ala feeding and a 3.2-fold enhancement of C-24 from the L-[3-^{13}C]Ala feeding (Table 2 and Supplemental Data), supporting this hypothesis.

The ^{13}C NMR spectrum of jamaicamide A (**2**) isolated from the L-[3-^{13}C]Ala experiment displayed additional enrichment at carbon resonances 1, 3, 5, 7, 9, 11, 13, 18, 21, and 27 (Table 2). Pyridoxal phosphate-dependent transamination of alanine results in formation of pyruvate, which subsequently could undergo decarboxylation to produce acetate. Thus, a ^{13}C-labeled carbon at position 3 of alanine is converted into position 2 of acetate, providing a source of [2-^{13}C]acetate for incorporation into all acetate-derived subunits of jamaicamide A.

A major point of interest in the biosynthesis of the jamaicamides is the origin of the NH-C17 sector. Structurally, this unit appears to arise from β-Ala, and other biosynthetic schemes based on the utilization of this amino acid in cyanobacteria have been proposed [3]. However, no previous studies have demonstrated the incorporation of β-Ala into a marine cyanobacterial secondary metabolite. To directly evaluate whether β-Ala was incorporated into the NH/C-17 portion of **2**, [$^{13}C_3$,^{15}N]β-Ala was fed to the jamaicamide producer. Analysis of the ^{13}C NMR spectrum of jamaicamide A (Figure 2) showed intact incorporation of the nitrogen and all three isotopically labeled carbons, C-15/C-17, with coupling constants of $J_{C\text{-}15/N}$ = 10.5 Hz, $J_{C\text{-}15/C\text{-}16}$ = 34.5 Hz, and $J_{C\text{-}16/C\text{-}17}$ = 47.9 Hz. These results unequivocally confirmed that β-Ala is the precursor for this section of the jamaicamide A structure.

Cloning and Identification of *jam* Gene Cluster from *L. majuscula*

The cloning, sequencing, and characterization of the jamaicamide biosynthetic gene cluster was undertaken to further investigate its assembly. Guided by the biosynthetic feeding experiments, which strongly supported a mixed PKS/NRPS assembly of jamaicamide A, we initially used a general PKS cloning strategy to isolate PKS-containing gene clusters from the jamaicamide A producer. This approach utilized PCR primers based on conserved regions of the β-ketosynthase domain (KS) of PKSs to amplify a 700 bp fragment mixture from *L. majuscula* JHB. These products were cloned into the pGEM-T vector, and 20 different clones were sequenced, yielding 9 distinct KS products. A DIG-labeled PKS probe was subsequently prepared from the PCR product mixture and used to screen a fosmid library of *L. majuscula* JHB. Colony hybridization of approximately 3500 colonies yielded a total of 28 fosmid clones that hybridized positively to the mixed PKS probe. These fosmids were verified to contain KS fragments by Southern hybridization of HindIII-digested fosmid DNA. From this restriction analysis, the 28 fosmids could be divided into at least four different groups, likely representing at least four distinct PKS-containing gene clusters. More-

over, using a mixed PKS probe, all four of these groups showed multiple signals by Southern hybridization of the HindIII-digested fosmids, suggesting that the pathways represented by these fosmids contained multiple PKS modules. Chemical analysis of *L. majuscula* JHB to date has revealed only the major metabolites jamaicamides A–C, hectochlorin [6], and 23-deoxyhectochlorin (K.M. and W.H.G, unpublished data).

To identify the jamaicamide gene cluster from these four groups, we focused on the potential genetic features that would lead to the incorporation of the novel pendent vinyl chloride group. The biosynthetic feeding experiments detailed above established that C-27 derived from C-2 of acetate. These results were suggestive of an HMGCS-like mechanism for acetate addition to a β-ketothioester polyketide intermediate, followed by decarboxylation, dehydration, and halogenation to form the vinyl chloride. Surprisingly, analysis of the GenBank database revealed a small subgroup of HMGCS-like proteins with high sequence identity to each other that are known to be associated with PKS gene clusters. These HMGCS-like proteins were found in the antibiotic TA [31], mupirocin [32], leinamycin [33], and PksX [34] pathways. The high level of similarity of these enzymes prompted us to design degenerate PCR primers for the amplification of a similar sequence from *L. majuscula* JHB. PCR with these primers yielded a distinct product of 680 bp, which was cloned into pGEM-T. Sequencing of five clones revealed the same PCR product in each. The translated amino acid sequence of this fragment showed about 70% identity to other PKS-associated HMGCS-like enzymes. With the expectation that the HMGCS fragment would provide a specific probe to target the jamaicamide gene cluster, we used this probe to screen the 28 positive PKS-containing fosmids by Southern hybridization. A single group of five overlapping fosmids probed positively with this fragment. Three of these overlapping fosmids, pJam1, pJam3, and pJam5, were chosen for DNA sequencing (Figure 3).

DNA Sequencing and Sequence Analysis

From fosmids pJam1, pJam3, and pJam5, a 70 kilobase pair (kbp) region of *L. majuscula* JHB was sequenced. The *jam* gene cluster was assigned to a 58 kbp region, with a flanking region of 6 kbp sequenced at both ends to confirm the boundaries of the *jam* gene cluster (Table 3). Directly upstream of the *jam* gene cluster is an apparent 1700 bp noncoding region that is followed by ORF1-3; these three ORFs are transcribed in the opposite direction to the *jam* gene cluster and show similarity to transposases. ORF2 and ORF3 show high similarity to the N and C terminus of ORF2 in the anabaenopeptilide biosynthetic gene cluster from the cyanobacterium *Anabaena* sp. [35]. ORF4 is highly similar to SusA from *Anabaena* sp., a sucrose synthase protein involved in carbon flux among nitrogen-fixing cyanobacteria [36, 37]. In the 6 kbp region immediately downstream of the *jam* gene cluster lies a 400 bp noncoding sequence followed by several more ORFs with similarity to transposases and a choloylglycine hydrolase (Table 3; ORF5-9). The total GC content of the *jam* gene cluster is 42%, comparable to other cyanobacteria as well as the barbamide gene sequence obtained from another strain of *Lyngbya majuscula* [38]. The *jam* gene cluster is comprised of 17 ORFs, with *jamA-jamP* transcribed in the same direction, while *jamQ* is transcribed in the reverse direction (Figure 3).

Located at the 5′ end of the cluster is *jam*A, which encodes for a protein with high similarity to a subclass of acyl-CoA synthetases that are involved in secondary metabolism. Next, *jamB* is located about 150 bp downstream of *jamA* and shows similarity to a large family of membrane-associated desaturases that utilize a di-iron active site to execute Δ5- or Δ9-fatty acid desaturation. The product of *jamC* is a small ACP protein of 100 amino acids with a signature phosphopantetheinyl binding site. Downstream of *jamC* is an intergenic gap of about 450 bp, which is followed by an unusual ORF, *jamD*, which encodes for a 683 amino acid protein. JamD has very weak similarity over a 250 amino acid region (21% identity) to an unknown flavoprotein from the planktonic cyanobacterium *Trichodesmium erythraeum* IMS101.

A small intergenic gap of about 80 bp precedes *jamE*, a sequence which encodes for a highly unusual PKS module containing a KS, AT, an unknown domain, and three consecutive ACP domains (see Figure 3 for domain abbreviations). The KS and AT regions are highly similar to the corresponding stigmatellin PKS domains from *Stigmatella aurantiaca* [39]. Between the AT and the first ACP is a novel domain which harbors a 215 amino acid region that shows weak homology to Fe^{2+}/α-ketoglutarate-dependent dioxygenases, including phytanoyl-CoA dioxygenase PhyH [40] and a putative hypodioxygenase from *Pseudomonas stutzeri* [41].

The three ACPs of JamE are all very similar to one another and show greatest similarity to other consecutively arranged ACP domains found in several PKS pathways (PksL from the "pksX" pathway in *Bacillus subtilis* [34], MmpB from the mupirocin (Mup) pathway in *Pseudomonas fluorescens* [32], and the last ACP in LnmJ of the leinamycin pathway [33]). Just downstream of *jamE* is a series of ORFs showing high similarity to corresponding genes in the antibiotic TA gene cluster (TA) from *Myxococcus xanthus* [31], the "pksX" pathway from *B. subtilis*, which is possibly involved in the biosynthesis of difficidin [34], and the mupirocin pathway from *P. fluorescens* [32]. All three of these pathways putatively encode for PKS metabolites that are modified with pendent carbons at C-1 (from acetate) positions of the backbone polyketide; this presumably occurs at the β-keto-*S*-ACP intermediate stage during polyketide assembly (Figure 3). This "β-keto modifying gene cassette" in the Jam pathway includes *jamF*, *jamG*, *jamH*, *jamI*, and possibly the N terminus of *jamJ*. The first gene, *jamF*, encodes for a 79 amino acid ACP that shows highest similarity to other small ACPs from the "pksX," antibiotic TA, and Mup pathways. Next, the gene product of *jamG* shows greatest similarity (about 40% identity) to PksF, TaK, and MupG and lower similarity (about 25%–30% identity) to FabB-type KS proteins involved in fatty acid synthesis [42]. It is noteworthy that JamG and the most closely related KSs all contain a serine at the normally highly conserved cysteine 163 residue. Next, *jamH* encodes for a protein with significant similarity to HMGCoA synthases (HMGCSs) that are found in association with

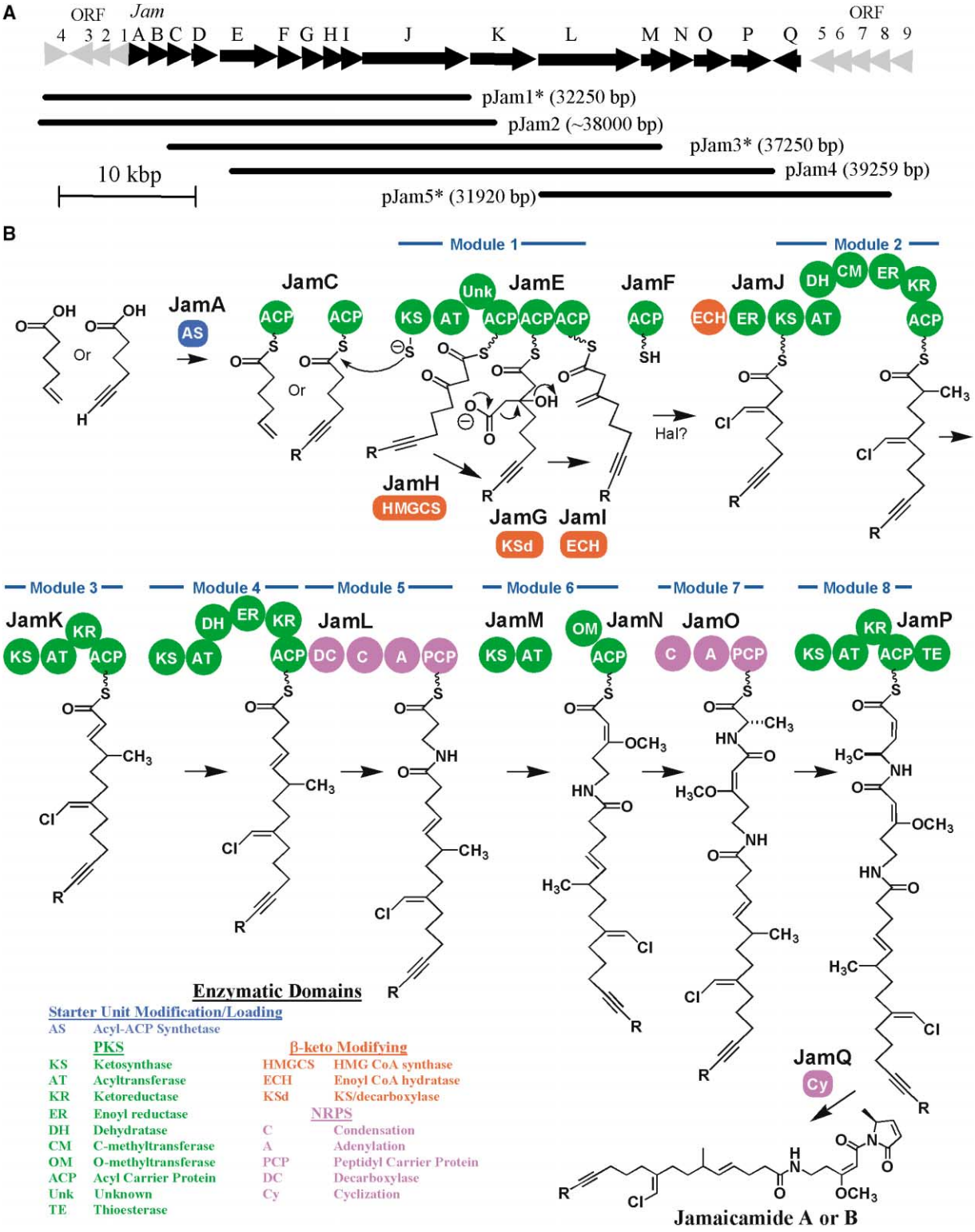

Figure 3. Biosynthetic Gene Cluster and Proposed Biosynthesis of Jamaicamides A and B

(A) Map and gene arrangement of the ~58 kbp *jam* gene cluster from *L. majuscula* JHB. Inserts of five fosmids containing the *jam* gene cluster are shown (asterisks indicate those fosmids sequenced during the current study; size of fosmid inserts indicated in parentheses).
(B) Proposed biosynthesis of jamaicamide A (**2**, R = Br) or B (**3**, R = H). The timing of bromination and chlorination is uncertain. JamB, a fatty acid desaturase, is likely involved in formation of the 5-hexenoic and 5-hexynoic acid precursors.

Table 3. Deduced Functions of the Open Reading Frames in the *jam* Gene Cluster

Protein	Amino Acids	Proposed Function	Sequence Similarity (Protein, Origin)	Identity/Similarity	Accession No.	Ref.
ORF4	~800	sucrose synthase	SusA, *Anabaena sp*.	81%, 91%	CAC87825	[36]
ORF3	117	unknown	ORF2, *Anabaena sp*. 90	73%, 88%	CAC01601	[35]
ORF2	207	transposase	ORF2, *Anabaena sp*. 90	63%, 80%	CAC01601	[35]
ORF1	191	transposase	alr1157, *Nostoc sp*. PCC 7120	54%, 60%	NP_485200	
JamA	592	hexanoyl-ACP synthetase	Mx1B, *Myxococcus xanthus*	47%, 64%	T18551	[70]
JamB	321	fatty acid desaturase	Ole1p, *Saccharomyces cerevisiae*	28%, 48%	NP_011460	[71]
JamC	100	ACP	Npun2294, *Nostoc punctiforme*	40%, 65%	ZP_00107884	
JamD	683	unknown	*Trichodesmium erythraeum* IMS101	13%, 32%	ZP_00074386	
JamE	1730	PKS				
		KS/AT 1–900	StiB, *Stigmatella aurantiaca*	52%, 66%	CAD19086	[39]
		unknown 901–1375	HtxA, *Pseudomonas stutzeri*	28%, 46%	AAC71711	[41]
		3 ACPs 1376–1730	PksL, *Bacillus subtilis*	34%, 54%	NP_389600	
JamF	79	ACP	TaB, *Myxococcus xanthus*	48%, 63%	CAB46501	[31]
JamG	408	ketosynthase/ decarboxylase	PksF, *Bacillus subtilus*	40%, 59%	NP_389594	
JamH	419	HMGCS-like	TaC, *Myxococcus xanthus*	64%, 77%	CAB46502	[31]
JamI	254	enoyl hydratase/ isomerase	PksH, *Bacillus subtilus*	40%, 56%	NP_389596	
JamJ	3302	enoyl hydratase/isomerase/PKS				
		1–250	PksI, *Bacillus subtilus*	48%, 65%	NP_389597	
		251–3302	MtaD, *Stigmatella aurantiaca*	41%, 59%	AAF19812	[44]
JamK	1705	PKS	StiB, *Stigmatella aurantiaca*	41%, 58%	CAD19086	[39]
JamL	3905	PKS/NRPS				
		PKS 1–2200	StiF, *Stigmatella aurantiaca*	41%, 59%	CAD19090	[39]
		NRPS 2201–3100 and 3601–3905	BarG, *Lyngbya majuscula*	51%, 65%	AAN32981	[38]
		decarboxylase 3101-3600	*Vibrio parahaemolyticus*	43%, 62%	NP_797616	
JamM	999	PKS	BarE, *Lyngbya majuscula*	56%, 74%	AAN32979	[38]
JamN	488	PKS	BarF, *Lyngbya majuscula*	58%, 72%	AAN32980	[38]
JamO	1158	NRPS	MxaA, *Stigmatella aurantiaca*	43%, 61%	AAK57184	[46]
JamP	1808	PKS/TE	NosB, *Nostoc sp*. GSV224	66%, 79%	AAF15892	[72]
JamQ	442	cyclization	Npun4108, *Nostoc punctiforme*	47%, 63%	ZP_00109659	
ORF5	238	unknown	blr2477, *Bradyrhizobium japonicum*	40%, 54%	NP_769117	
ORF6	207	transposase	alr1157, *Nostoc sp*. PCC 7120	48%, 63%	NP_485200	
ORF7	148	unknown				
ORF8	381	choloylglycine hydrolase	*Agrobacterium tumefaciens* str. C58	52%, 67%	NP_356074	
ORF9	146	unknown				

PKS pathways. JamH shows high identity to TaC, TaF, MupH, and PksG (60%–70%) and lower identity to HMGCSs involved in the mevalonate pathway (20%–35%). *JamI* is 270 bp downstream from *jamH* and encodes for an enoyl-CoA hydratase/isomerase (ECH) protein. JamI is most similar to pksH (40% identity) and MupJ (35% identity) and less similar to other ECHs (less than 25% identity).

Immediately downstream of *jamI* is a large ORF, *jamJ*, which encodes for a 3302 amino acid protein. The amino terminus of JamJ contains another ECH-like domain that is distinct from JamI and is most closely related to PksI and MupK (40% identity). Immediately following the ECH domain is an enoyl reductase (ER) domain that is similar to ER domains involved in modular PKS biosynthesis. The carboxyl terminus of JamJ contains a large seven domain PKS module (KS-AT-DH-CM-ER-KR-ACP; see Figure 3). The next ORF, *jamK*, encodes for a single module PKS protein with KS-AT-KR-ACP domain organization. The *jamL* gene encodes for a two-module mixed PKS/NRPS protein with a six domain PKS module (KS-AT-DH-ER-KR-ACP) and an unusual four domain NRPS module. Surprisingly, the NRPS module of JamL contains a 460 amino acid pyridoxal-dependent decarboxylase domain embedded between the A6 and A7 conserved motifs of the adenylation (A) domain as defined by Marahiel [43]. This novel domain shows high similarity to putative glutamate decarboxylases from bacterial genomic sequencing projects (40% identity). Consideration of the X-ray structure of the GrsA phenylalanine A domain shows that the DC domain of JamL is integrated into a flexible loop region between the conserved A6 and A7 motifs [43]. Similar integration of a monooxidase domain has been observed between the A4 and A5 motifs of the NRPS A domains of MtaG/MelG proteins in the myxothizol and melithiazol pathways, respectively, further illustrating the versatility of NRPS A domain structure to accommodate tailoring activities [44, 45]. Comparison of the amino acid binding pocket to known NRPS A domains showed that JamL has some similarity to other β-Ala activating A domains (Supplemental Data). Following *jamL* are two ORFs, *jamM* and

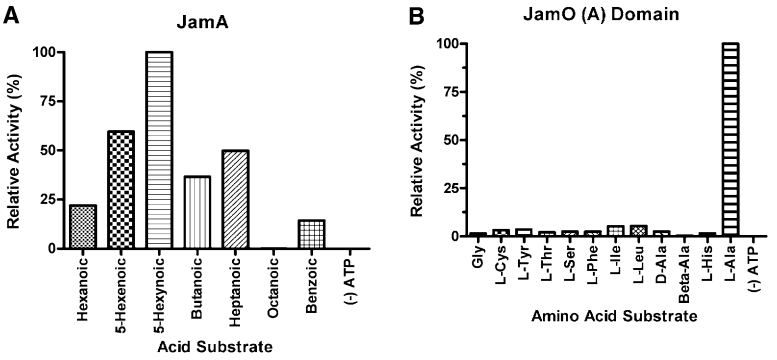

Figure 4. Substrate-Dependent ATP-PPi Exchange Activity for JamA and JamO(A) Domains

(A) JamA domain, (B) JamO(A) domain. Results presented represent the average from duplicate experiments. See Experimental Procedures for details.

jamN, which encode for proteins predicted to function as a single PKS module. Two proteins functioning as a single PKS module have also been found for $MxaB_1$ and $MxaB_2$ of the myxalamid pathway [46] and for the highly similar BarE and BarF proteins of the barbamide pathway [38].

The next ORF (*jamO)* encodes for a typical NRPS module with C, A, and PCP domains. The residues that line the amino acid binding pocket of the A domain of JamO show 100% identity to putative L-Ala activating A domains from the bleomycin (blmVII) [47], microcystin (McyA-2) [48], and myxalamid (MxaA) [46] pathways (Supplemental Data).

The penultimate module in the Jam pathway, *jamP*, codes for a single PKS module with a KS, AT, KR, ACP, and a terminal thioesterase (TE) domain. Although the C-terminal TE domain of JamP is preceded by a PKS module, it shows highest similarity to TE domains found at the end of NRPS enzyme systems, such as DhbF from *B. subtilis* [44]. Finally, *jamQ* is located at the end of the Jam pathway and is transcribed in the opposite direction of the rest of the pathway. It encodes for an unusual condensation domain that shows highest identity (47%) to an unknown protein from *Nostoc punctiforme* and weaker identity to more typical NRPS condensation domains (15%–20%).

The complete Jam pathway contains six PKS modules; JamE, JamJ, JamK, JamL (module 4), JamM/N, and JamP. Sequence analysis of the AT domains indicate that they are all specific for the loading of malonyl-CoA [49]. This is consistent with the precursor feeding experiments described above, which established the incorporation of nine intact acetate units into jamaicamide A. To date, all evidence from biochemical feeding experiments, as well as genetic analysis of several secondary metabolite pathways, indicates that methyl branching at the C-2 position of acetate-derived carbons in cyanobacteria derives from the addition of an SAM-derived methyl group. It is not known if cyanobacteria are capable of producing methylmalonyl-CoA.

Expression and Functional Analysis of JamA and the JamO Adenylation Domains

To determine the nature of the unusual starter unit incorporated into the Jam pathway, the activation enzyme JamA was overproduced as a 6×His fusion protein, purified, and analyzed using the substrate-dependent ATP-PPi exchange assay (Figure 4A). A variety of saturated and unsaturated fatty acid substrates were tested for activation by JamA. Initially, it was anticipated that JamA would activate a saturated fatty acid (e.g., hexanoic acid) and load this unit to JamC before desaturation by JamB to form the terminal alkene or alkyne functionalities of jamaicamide B and C, respectively. Surprisingly, JamA showed a marked preference for 5-hexynoic and 5-hexenoic acid over any of the saturated fatty acids (Figure 4A), suggesting that desaturation of free hexanoic acid by JamB (and possibly halogenation by Jam D) may occur prior to activation by JamA. However, because all desaturases studied to date either operate on an acyl-ACP as the catalytically relevant substrate or upon acyl chains attached to a variety of polar headgroups, including CoA, phospholipid, and diacylglycerols, this will require further experimental investigation [50]. Nevertheless, activation of the unusual starter units, 5-hexynoic and 5-hexenoic acid, by JamA supports the assignment of this being the *jam* gene cluster.

Recently, several adenylation domains specific for L-Ala have been assigned from the bleomycin [47], microcystin [48], and myxalamid [46] pathways. The binding pocket residues conferring specifity for L-Ala in these other pathways are identical to one another; however, biochemical proof of L-Ala activation has not yet been confirmed. Although the key residues of the JamO A domain precisely match these other A domains (Supporting Information), we sought biochemical confirmation of this proposed L-Ala specificity. The JamO A domain was overexpressed as a 6×His fusion construct, purified, and analyzed using the substrate-dependent ATP-PPi exchange experiment [51]. The JamO A domain activated L-Ala to a high level (=100%), whereas the other amino acids tested were activated to a much lower level (<5%; Figure 4B), confirming that JamO utilizes L-Ala as an extender unit, and biochemically supporting the binding pocket assignments made for other L-Ala A-domains.

Proposed Biosynthesis of Jamaicamides A–C

With an ever-increasing number of NRPS- and PKS-containing gene clusters available in public databases, it has become possible to make reliable predictions about the function of new genes and even to predict the molecular structures of new natural products based on the DNA sequence of a gene cluster [52]. Indeed, in the case of jamaicamide A, the number of modules, and the activities encoded by each, are fully consistent with

its chemical structure and the biosynthetic feeding experiments (Table 3 and Figure 3). Similar to many other PKS and NRPS natural product gene clusters, the *jam* pathway is organized in a remarkably colinear arrangement with respect to its proposed biosynthesis (Figure 3). Based on sequence analysis and biochemical data for JamA, it is predicted that the JamA–JamC proteins comprise a system for the generation of activated 5-hexenoate and 5-hexynoate starter units. JamA, an acyl-CoA synthetase homolog, initiates Jam biosynthesis by the ATP-dependent activation of free 5-hexenoic or 5-hexynoic to the acyl-adenylate followed by subsequent loading to the ACP protein JamC. The terminally desaturated hexanoic acid precursors are likely generated by Jam B, a fatty acid desaturase. We speculate that a similar short chain acyl loading system (JamA–JamC) is involved in the biosynthetic initiation of a number of other cyanobacterial products that possess terminal hexynyl groups, including the dragonamides [53], antanapeptins [54], and carmabins [55]. The timing of the bromination is not clear at this time, but it most likely takes place on 5-hexynoate or after assembly of jamaicamide B (**3**). A possible candidate for the bromination is the functionally unassigned and novel protein JamD.

Next, the modified hexanoate, attached to JamC as a thioester, acts as a starter unit for module 1 of the PKS (JamE). JamE catalyzes one round of polyketide chain extension, generating an unreduced eight carbon β-ketothioester intermediate. This intermediate is then transformed, via an extensive array of modifications described below, to generate a pendent vinyl or possibly vinyl chloride functionality. The four consecutive ACPs, three on the C terminus of JamE plus the discrete ACP JamF, may be involved in channeling intermediates and providing enzyme docking stations for the various enzymes involved in vinyl or vinyl chloride formation. Consecutive ACPs are also present in PKS pathways containing β-keto-modifying gene cassettes for the production of "pksX" [34], mupirocin [32], and leinamycin [56]. Biochemical feeding experiments established that C-27, the vinyl chloride bearing carbon, is derived from C-2 of acetate. This labeling pattern is consistent with an HMGCS-like addition of acetate to a β-keto-polyketide intermediate. Because of the high sequence identity of JamH to HMGCSs, we propose that JamH adds acetate to the β-keto-S-ACP intermediate, thus generating an acyl-S-ACP intermediate reminiscent of HMG-CoA (Figure 3). Subsequent decarboxylation, dehydration of the tertiary alcohol, and possible isomerization of the resulting double bond by JamG, JamI, and/or the N-terminal ECH domain of JamJ is predicted to yield the pendent vinyl group. This sequence of reactions is similar to what has been proposed for pendent carbon formation in the mupirocin pathway [32]. Although speculative, the location and features of the domain found between the AT and the first ACP of JamE (e.g., modest sequence similarity to phytanoyl-CoA dioxygenase) suggests that it may be involved in chlorination of the pendent vinyl group. Putative chlorination enzymes in the barbamide (BarB1 and BarB2) [38], syringomycin (SyrB2) [57], and coronamic acid (CmaB) (GenBank accession number NP794454) also show similarity to the phytanoyl-CoA dioxygenase family of proteins. However, without experimental proof of the involvement of this domain, it cannot be ruled out that chlorination may take place as a post-PKS tailoring reaction catalyzed by an unidentified protein.

After vinyl or vinyl chloride formation, the nascent polyketide is predicted to undergo three more rounds of PKS chain elongation by modules 2, 3, and 4 that are found on JamJ, JamK, and JamL, respectively. JamJ contains unusual ECH and ER domains on the N terminus followed by the large PKS module 2, which contains the full complement of reductive domains and a C-methyltransferase (C-MT) domain. The C-MT domain in module 2 is consistent with feeding results that indicate that C-26 derives from SAM and not from methylmalonyl-CoA. JamK is comprised of a single PKS (module 3) with KS/AT/KR/ACP domain organization. However, based on the structure of jamaicamide A, a DH domain is also expected within module 3. Other characterized PKS pathways, such as the epothilone [58, 59], myxalamid [46], and stigmatellin [39] pathways, have also been shown to lack DH domains at expected positions in the corresponding PKS. In the epothilone pathway, the DH domain of module 5 acts twice during the biosynthesis to dehydrate the nascent polyketide after both the module 4 (missing the DH domain) and module 5 chain elongation events [60]. A similar mechanism may be at work in the Jam PKS with the DH domain in module 4 acting after chain elongation by both modules 3 and 4. JamL contains both the PKS module 4 for the incorporation of a fully reduced acetate unit and the novel NRPS module 5 responsible for incorporation of β-Ala. Not only does the A domain of JamL contain a binding pocket that resembles A domains that activate β-Ala (Supplemental Data), it also possesses an intergrated pyridoxal phosphate-dependent decarboxylation domain (DC) between the A6 and A7 core motifs with high sequence similarity to glutamate DC domains. In other NRPS pathways, such as the bleomycin and exochelin pathways, it has been proposed that β-Ala is directly activated and used as an extender unit [47, 61]. In jamaicamide A biosynthesis, this may also be the case such that the DC domain is inactive or unnecessary. However, it is tempting to speculate that the JamL adenlyation domain may have evolved to utilize β-Ala, or in cases of limiting β-Ala, it can decarboxylate aspartic acid to generate β-Ala within the modular biosynthetic environment. In this latter case, such a decarboxylation could occur prior to thiolation, or, alternatively, activation could occur at the γ-carboxylate and thus allow α-decarboxylation when attached to JamL (e.g., aspartate-*S*-PCP-JamL to β-Ala-*S*-PCP-JamL). In vitro biochemical investigations probing the substrate specificity and possible subsequent decarboxylation by the JamL NRPS module are underway.

After module 5, the biosynthetic pathway switches back to PKS with the two protein module 6 (JamM and JamN). Module 6 catalyzes another round of PKS chain extension followed by O-methylation (OM). The OM domain shows highest sequence identity with BarF and MtaF, both of which have been proposed to catalyze identical methyltransferase reactions to the enol form of the polyketide during barbamide [38] and myxothiazol

biosyntheses [44], respectively. Another switch point occurs between the PKS module 6 and the NRPS module 7 (JamO). Both sequence analysis of the A-domain binding pocket residues and in vitro biochemical characterization of the JamO A domain confirms its role in the incorporation of L-Ala during jamaicamide A biosynthesis.

Finally, the pathway terminates with PKS module 8 (JamP), which incorporates another malonyl-CoA extender unit, followed by chain termination and cyclization to yield jamaicamide A. Just as in module 3 (JamK), a KR domain is present in JamP, but the DH domain is notably absent. However, in contrast to module 3, module 8 is the last module in the pathway, leaving no obvious candidate to provide for this missing activity. Close inspection of the JamP KR domain using the relationships developed by Reid et al. and Caffrey supports that the ketoreduction proceeds to generate a 3*S*-configured product [62, 63]. Moreover, it has been reported that dehydration of the *S*-stereoconfigured β-alcohol generally leads to *cis* double-bond formation [62], a feature consistent with the C-21/C-22 double bond in the pyrrolinone ring of jamaicamide A. A thioesterase domain at the C terminus of JamP is possibly involved in acyl chain release from the JamP ACP, but the sequence of the JamP TE domain does not adequately explain pyrrolinone ring formation. A better candidate for this latter activity is JamQ, an NRPS condensation domain homolog, found at the very terminus of the gene cluster. JamQ shows weak similarity to NRPS condensation domains that are involved in amide bond formation in nonribosomal peptide assembly [64]. It is not clear if JamP first catalyzes the release of a linear jamaicamide precursor before cyclization, or if JamQ acts upon a JamP-tethered intermediate and is responsible for the simultaneous cyclization and thioester release. In either case, pyrrolinone ring formation clearly differs from normal condensation domain chemistry because it involves reaction between a much less nucleophilic amide nitrogen and the thioester carbon.

A curious insight resulting from consideration of the *jam* gene cluster sequence, as well as the gene clusters for three other pathways from another *L. majuscula* (L19 strain) (barbamide [38], carmabin, and curacin A, the latter two currently unpublished [Z. Chang, D. Sherman, W.H.G., and P. Flatt]), is that none appear to possess self-resistance, regulatory, or transport genes, a hallmark of the antibiotic biosynthetic gene clusters of actinomycetes. It is intriguing to speculate about the lack of these regulatory features in light of the likely biological role of these compounds in cyanobacteria [65]. Many of the compounds isolated from marine cyanobacteria have been shown to have specific targets in higher eukaryotic organisms (e.g., tubulin, actin) and have minimal or no antibacterial activity. Lack of genes encoding for small molecule transport proteins in these pathways suggests that these substances are accumulated within cyanobacterial cells, and that their natural roles are best accomplished through creation of elevated cellular concentrations. Export outside of the cellular environment may in fact diminish their natural efficacy by dilution. It is also intriguing to propose that continuous expression of these secondary metabolite pathways in *L. majuscula* poses little or no harm to the host organism (e.g., they lack the biochemical targets of their toxins) and thus provides constant protection against predation. Therefore, the pathways in *L. majuscula* do not appear to be under the rigorous regulatory mechanisms that govern the biosynthesis of antibiotics in actinomycetes.

The extraordinarily ordered nature of the jamaicamide gene cluster makes possible reasonable hypotheses concerning correlation of genetic and structural features, including those involved in novel steps in the pathway. Unfortunately, methods are not currently available for targeted gene disruption in *L. majuscula*, and the size of the *jam* gene cluster complicates its heterologous expression. Consequently, it cannot be unequivocally stated that all genes necessary for jamaicamide A production are present in this pathway, or that all of the components described here are necessarily required for jamaicamide A production. However, several lines of evidence provide compelling support that this gene cluster encodes for enzymes catalyzing most or all of jamaicamide biosynthesis. The composite of genetic evidence, including the extraordinary colinear organization of the gene cluster and several novel features that correlate well with the unusual structure motifs in the jamaicamides, strongly support this assignment. Moreover, all of these genetic features are in complete agreement with the extensive biosynthetic precursor feeding studies. Finally, the biochemical characterizations of the unusual acyl-activating enzyme JamA and the NRPS adenylation domain of JamO further support the assignment of this gene cluster to jamaicamide biosynthesis.

The jamaicamide biosynthetic pathway represents the most integrated mixed PKS/NRPS pathway identified to date, with two switch points between PKS and NRPS segments as well as two reverse switch points between NRPS and PKS segments. The remarkable capability of cyanobacteria to produce highly modified polyketide/peptide natural products is further exemplified by the rich array of novel modifications present in the jamaicamide pathway. These include the biosynthesis and incorporation of a novel alkynyl PKS starter unit, an HMGCS-like containing gene cassette for pendent vinyl or vinyl chloride formation, incorporation of a decarboxylase domain into an NRPS module, and chain termination resulting in pyrrolinone ring formation. All of these features require considerable additional biochemical characterization and will certainly be valuable additions to the molecular toolbox for creating structural diversity via biosynthetic pathway engineering.

Significance

Marine cyanobacteria continue to be a source of novel natural product chemotypes, many of which possess interesting biological activities. This is amply illustrated in this report on the isolation and structure elucidation of the jamaicamides. Jamaicamide A represents a new chemotype of natural product, possessing several unusual functionalities, including an alkynyl bromide, vinyl chloride, and pyrrolinone ring. The jamaicamides show a spectrum of biological activities consistent with their functioning as defense metabo-

lites, including sodium channelblocking activity and arthropod and fish toxicity. In this regard, they join a growing number of cyanobacterial metabolites that possess neurotoxic properties. Despite their notoriously slow growth and sometimes difficult laboratory culture, this investigation was successful in mapping out the biosynthetic subunits using a classic precursor feeding and NMR detection approach. Great insight was achieved by these experiments, especially concerning the mechanism of formation of a pendent carbon atom that becomes functionalized to a vinyl chloride group. Based on these biosynthetic findings, a set of gene probes was constructed, which led to the efficient identification of fosmids containing portions of the jamaicamide gene cluster from a total genome library. Sequencing of these and assembly of the pathway revealed the remarkable set of colinear genes that codes for enzymes catalyzing jamaicamide biosynthesis. Several of these genes are deduced to program for novel tailoring enzymes, including those that create the various unusual functional groups in jamaicamide A. This report represents only the second natural product biosynthetic gene cluster to be described from a marine cyanobacterium, and illustrates the extent and breadth of new secondary metabolite genetic motifs awaiting discovery from these life forms.

Experimental Procedures

General Experimental Procedures

UV and IR spectra were recorded on a Beckman DU 640B UV spectrophotometer and a Nicolet 510 spectrophotometer, respectively. NMR spectra were recorded either at a ^{1}H resonance frequency of 600.04 MHz (Bruker DRX 600), 500.17 MHz (Bruker DRX 500), or 400.13 MHz (Bruker DPX 400). The Bruker DRX 600 was equipped with a Bruker Q-Switch TXI probe, the DRX 500 was equipped with a Bruker TXI CryoProbe, and the DPX 400 was equipped with a Bruker BBO probe. All chemical shifts are reported relative to residual $CHCl_3$ as internal standard (δ_H 7.26; δ_C 77.0). HRMS were obtained on a Kratos MS 50 TC mass spectrometer. Optical rotations were measured with a Perkin-Elmer model 243 polarimeter. HPLC was performed using Waters 515 pumps and a Waters 996 photodiode array spectrophotometer. TLC grade (10–40 m) silica gel was used for vacuum chromatography, and Merck aluminum-backed TLC sheets (silica gel 60 F_{254}) were used for TLC. All solvents were purchased as HPLC grade, and all stable isotope substrates were purchased from Cambridge Isotope Laboratories, Inc.

Collection and Culture Conditions for *Lyngbya majuscula* JHB

Lyngbya majuscula JHB was collected from Hector Bay, Jamaica at a depth of 2 m. Following transport to Oregon, *L. majuscula* JHB was isolated free of contaminating cyanobacteria and other microalgae using standard techniques [66]. Subsequently, the cultures have been maintained in SWBG11 and ESW media in a 28°C controlled temperature room with a 16 hr light/8 hr dark cycle provided by Sylvania 40 W cool white fluorescent lights (4.67 μmol photons s^{-1} m^{-2}).

Extraction and Isolation

A total of 114.2 g (wet weight) of the harvested alga was extracted twice with CH_2Cl_2/MeOH (2:1) at ambient temperature, followed by three extractions with heated CH_2Cl_2/MeOH (2:1) to yield 1.1 g of crude extract. The crude extract was then fractionated using vacuum liquid chromatography (VLC, 9.5 cm × 4 cm) on TLC grade silica gel with a stepwise gradient of hexane/EtOAc. Eluted material was collected, visualized by ^{1}H NMR, and similar fractions were recombined. VLC fractions eluting with a solvent concentration of 50%–80% EtOAc were further fractionated using a C_{18} SPE cartridge with a stepwise gradient of MeOH/H_2O. A fraction eluting in 80% MeOH in H_2O was subjected to RPHPLC. The final purification was achieved by ODS-HPLC (Phenomenex 250 mm × 10 mm, SPHERECLONE 5 m, PDA detection) using 82% MeOH in H_2O as eluent to give pure jamaicamide A (**2**, 4% of crude extract), and jamaicamide B (**3**, 2.8% of crude extract). Jamaicamide C (**4**) was isolated from a subsequent batch of harvested *L. majuscula* and represents 0.5% of the crude extract.

Jamaicamide A (2)

Jamaicamide A was isolated as a pale yellow oil having the following physical characteristics: UV (MeOH) λ_{max} 272 nm (log ϵ = 3.9); $[\alpha]_D^{25}$ = +44° (MeOH, *c* 1.48); IR (neat) 3314, 2933, 1718, 1666, 1599, 1543, 1431, 1395, 1136, 1080, 822 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS (3-NBA) obs. $[M + H]^+$ cluster at 567/569/571 (4:5:1.5); HRFABMS (3-NBA) 567.1625 (−0.7 mmu dev. for $C_{27}H_{37}O_4N_2ClBr$).

Jamaicamide B (3)

Jamaicamide B was isolated as a pale yellow oil having the following physical characteristics: UV (MeOH) λ_{max} 272 nm (log ϵ = 3.9); $[\alpha]_D^{25}$ = +53° (MeOH, *c* 0.61); IR (neat) 3300, 2931, 2865, 2115, 1718, 1659, 1599, 1544, 1435, 1395, 1136, 1080, 822 cm^{-1}; ^{1}H and ^{13}C NMR data, see Supplemental Data; FABMS (3-NBA) obs. $[M + H]^+$ cluster at 489/491 (1:0.4); HRFABMS (3-NBA) 489.2520 (−0.2 mmu dev. for $C_{27}H_{38}O_4N_2Cl$).

Jamaicamide C (4)

Jamaicamide C was isolated as a pale yellow oil having the following physical characteristics: UV (MeOH) λ_{max} 273 nm (log ϵ = 3.8); α_D^{25} = +49° (MeOH, *c* 0.39); IR (neat) 3303, 2928, 2857, 1721, 1660, 1601, 1550, 1437, 1397, 1202, 1171, 1082, 823 cm^{-1}; ^{1}H and ^{13}C NMR data, see Supplemental Data; FABMS (3-NBA) obs. $[M + H]^+$ cluster at 491/493 (1:0.4); HRFABMS (3-NBA) 491.2677 (0.3 mmu dev. for $C_{27}H_{40}O_4N_2Cl$).

Ozonolysis and Acid Hydrolysis of Jamaicamide A (2)

At room temperature, a slow stream of O_3 was bubbled into a 10 ml CH_2Cl_2 solution of jamaicamide A (**2**, 5 mg, 0.9 mM), which was then sealed in a reaction flask for approximately 5 min. The solution was then dried under a stream of N_2 and subjected to acid hydrolysis. Hydrolysis of the jamaicamide A ozonide was carried out in 1 ml of 6N constant boiling HCl under argon in a threaded Pyrex heavy wall tube sealed with a Teflon screw cap. The reaction vessel was then placed in a microwave oven (high power setting, 550 W) for 1 min [66]. The reaction mixture was dried under a stream of argon and derivatized with Marfey's reagent.

Amino Acid Analysis using Marfey's Reagent

To a vial containing 50 μl of a 50 mM solution of pure amino acid standard in H_2O was added 100 μl of a 36 mM solution of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine (FDAA) in $(CH_3)_2CO$ followed by 20 μl of 1 M $NaHCO_3$. The reaction mixture was stirred at room temperature for 1 hr, at which time 10 μl of 2N HCl was added and let stand for several minutes.

The jamaicamide A hydrolysate was derivatized by the addition of 100 μl of H_2O, followed by 500 μl of a 36 mM solution of FDAA in $(CH_3)_2CO$, followed by 100 μl of 1M $NaHCO_3$. The reaction mixture was stirred at room temperature for 1 hr, at which time 50 μl of 2N HCl was added and let stand for several minutes. The dried reaction mixture was dissolved in 500 μl of MeOH and analyzed by ODS-HPLC (Waters Nova-Pak C_{18} 3.9 mm × 150 mm 5μ, UV detection at 340 nm) with a linear gradient elution (9:1 triethylammonium phosphate [50 mM, pH 3.0]:CH_3CN to 1:1 triethylammonium phosphate [50 mM, pH 3.0]:CH_3CN over 60 min). The derivative of standard L-Ala showed t_R = 20.46 min, standard D-Ala showed t_R = 25.59 min, and L-Ala liberated from jamaicamide A (**2**) showed a t_R = 20.46 min, indicating that the stereochemistry at C-23 was of L-configuration.

General Culture Conditions and Isolation Procedure for Isotope-Labeled Feeding Studies

Approximately 2–3 g (total) of *L. majuscula* strain JHB-22/Aug/96-01C2 was inoculated into multiple Erlenmeyer flasks containing SWBG11 medium. Cultures were grown at 28°C under uniform illumination (4.67 μmol photon s^{-1} m^{-2}), aerated, and equilibrated 3 days

prior to the addition of isotopically labeled precursors on days 3, 6, and 8. The *L. majuscula* was harvested 10 days after inoculation, blotted dry, weighed, and repetitively extracted with 2:1 CH_2Cl_2/MeOH. The filtered lipid extracts were dried in vacuo and weighed, then applied to silica gel columns (approximately 1.5 × 4.5 cm) and eluted with a stepped gradient from 50% EtOAc/hexanes to 25% MeOH/EtOAc. The fractions were analyzed for jamaicamide content by TLC. Fractions eluting with 85% EtOAc/hexanes and 100% EtOAc were recombined and purified by RP HPLC (17:3 MeOH/H_2O) using a Phenomenex Sphereclone 5 μm ODS column (detection at 216 and 254 nm) to yield pure jamaicamide A (**2**). For each feeding experiment, compound purity was determined by HPLC and ^1H and ^{13}C NMR spectroscopy.

Quantitative Calculation of ^{13}C Enrichments from Isotope-Enriched Precursor Feeding Experiments

The relative ^{13}C enrichments from exogenously supplied isotopically labeled precursors were calculated as follows. All ^{13}C NMR spectra were recorded using inverse-gated decoupling and processed with 1.0 Hz line broadening (zgig Bruker pulse program). For each experiment, carbon resonance intensities for the natural abundance and enriched sample were tabulated, and an average normalization factor was calculated from carbon resonances expected to be unlabeled. Carbons 16, 22, and 26 were averaged for all jamaicamide A feeding experiments, with the exception of the *S*-[methyl-^{13}C]methionine feeding (C-24 replaced C-26) and the L-[1-^{13}C]Ala feeding (C-21 replaced C-22). An average normalization factor was calculated by dividing the average intensity of the selected resonances from the natural abundance spectrum by the average intensity of the same selected resonances from the ^{13}C-enriched spectrum. All resonances in the ^{13}C-enriched spectrum were then multiplied by this normalization value and rounded to the nearest tenth.

Sodium [1-^{13}C]Acetate and Sodium [2-^{13}C]Acetate Feedings

[1-^{13}C]acetate (150 mg total) was provided to 3 × 600 ml cultures on days 3, 6, and 8, and the cultures were harvested on day 10 (3.24 g wet wt., 0.34 g dry wt., 63.5 mg organic extract). A total of 1.4 mg of jamaicamide A (**2**) was isolated from VLC and HPLC purification of the crude extract. In a similar manner, [2-^{13}C]acetate (150 mg total) was provided to cultures (3.64 g wet wt., 0.29 g dry wt., 58.2 mg organic extract), yielding 0.8 mg of jamaicamide A (**2**).

Sodium [1,2-$^{13}C_2$]Acetate Feeding

[1,2-$^{13}C_2$]acetate (160.8 mg total) was diluted 1:2 with unlabeled sodium acetate and provided to 2 × 1 liter cultures, yielding 1.4 mg of jamaicamide A (**2**). Coupling constants for the intact ^{13}C-^{13}C units of **2** are given in the text and are reported in the Supplemental Data.

S-[Methyl-^{13}C]Methionine Feeding

S-[methyl-^{13}C]methionine (54 mg total) was provided to 3 × 600 ml cultures (2.76 g wet wt., 0.24 g dry wt., 67.2 mg organic extract), providing 0.9 mg of jamaicamide A (**2**) for ^{13}C enrichment analysis.

L-[1-^{13}C]Alanine and L-[3-^{13}C]Alanine Feedings

L-[1-^{13}C]Ala (300 mg total) was administered to 2 × 1.5 liter cultures (2.15g wet wt., 0.26 g dry wt., 28.5 mg organic extract), yielding 1.2 mg of jamaicamide A (**2**). Simlarly, 195 mg of L-[3-^{13}C]Ala was provided to 3 × 600 ml cultures (5.09 g wet wt., 0.64 g dry wt., 49.1 mg organic extract) to produce 2.0 mg of jamaicamide A (**2**).

[$^{13}C_3$,^{15}N]β-Alanine Feeding

[$^{13}C_3$,^{15}N]β-Ala (150 mg total) was provided to 3 × 600 ml cultures (4.48 g wet wt., 0.49 g dry wt., 34.2 mg organic extract), yielding 1.4 mg of pure jamaicamide A (**2**). An expanded region from the ^{13}C NMR spectrum of labeled jamaicamide A (**2**) is presented in Figure 2. Coupling constants for the intact ^{13}C-^{13}C and ^{13}C-^{15}N units are as follows: $^1J_{C15\text{-}C16}$ = 34.5 Hz, $^1J_{C16\text{-}C17}$ = 47.9 Hz, $^1J_{C15\text{-}N}$ = 10.5 Hz.

Bioassay Methods

Chemicals were evaluated for their capacity to either activate or block sodium channels using the following modifications to the cell-based bioassay of Manger et. al [10]. Twenty-four hours prior to chemical testing, cells were seeded in 96-well plates at 8 × 10^4 cells/well in a volume of 200 μl. Test chemicals dissolved in DMSO were serially diluted with medium and added at 10 μl/well, resulting in concentrations of 15, 5, and 1.5 μM. DMSO was less than 1% final concentration. Plates to evaluate sodium channel-activating activity received 20 μl/well of either a mixture of 3 mM ouabain and 0.3 mM veratridine (Sigma Chemical Co.) in 5 mM HCl or 5 mM HCl alone in addition to the test chemical. Plates were incubated for 18 hr, and results were compared to similarly treated solvent controls with 10 μl medium added in lieu of the test chemical. The sodium channel activator brevetoxin PbTx-1 (Calbiochem) was added at 10 ng/well in 10 μl medium and used as the positive control. Sodium channel-blocking activity was assessed in a similar manner except that the ouabain and veratridine stock solution was 5.0 and 0.5 mM, respectively, and the sodium channel blocker saxitoxin (Calbiochem) was used as the positive control. Plates were incubated for approximately 22 hr.

Cytotoxicity was measured in NCI-H460 lung tumor cells and neuro-2a cells using the method of Alley et al. [67], with cell viability being determined by MTT reduction [10]. Cells were seeded in 96-well plates at 5000 and 8000 cells/well in 180 μl for H460 and neuro-2a cells, respectively. Twenty-four hours later, the test chemical dissolved in DMSO and diluted into medium was added at 20 μg/well, producing concentrations between 15 and 0.5 μM. DMSO was less than 1% final concentration. After 48 hr, the medium was removed and cell viability was determined. Ichthyotoxicity was determined as previously described [23] using goldfish (*Carassius auratus*) and an exposure time of 90 min. Brine shrimp toxicity was determined by the method of Meyer et. al [26] using an exposure time of 24 hr. For both assays, the test chemical was dissolved in 100% ethanol and added at less than 1% final concentration.

Bacterial Strains and Growth Conditions

Escherichia coli strain DH10b was routinely used in this study as a host for DNA cloning and sequencing. *Escherichia coli* strain EPI300 was used in this study as a host for fosmid library construction and amplification of fosmid clones. The DH10b strain of *E. coli* containing pBluescript or pGEM3ZF was grown overnight in LB (Luria-Bertani) medium with ampicillin at a final concentration of 100 μg/ml. The EPI300 strain of *E. coli* harboring fosmid vector was grown overnight in LB medium with chloramphenicol at a final concentration of 12 μg/ml.

DNA from *Lyngbya majuscula* JHB used for PCR amplification was isolated with a Wizard Kit (Promega, Madison, WI). High molecular weight DNA from *Lyngbya majuscula* JHB used for library construction was purified as previously described [38]. The high molecular weight DNA was end repaired, size selected, and ligated into the copy control fosmid vector pCC1FOS using protocols provided with the CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI). Plasmid preparations were carried out using commercial kits (Qiagen, Santa Clarita, CA). Other standard DNA manipulations, such as restriction digests and ligations, were done using standard methods [68]. Southern analysis, digoxigenin labeling of probes, hybridization, and detection procedures were carried out according to the directions provided with the DIG high prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals, Mannheim, Germany).

PCR Cloning of HMGCS-like and PKS Probe Fragments, Isolation, and Sequencing of the *jam* Biosynthetic Gene Cluster

PCR amplification of probe fragments used in this study was performed with *Taq* DNA-polymerase (Promega) with the manufacturer's suggested concentration of template and primers in an Eppendorf Mastercycler gradient system. Conditions used were as follows: denaturation, 30 s at 94°C; annealing, 30 s at 48°C; extension, 60 s at 72°C; 30 cycles. Degenerate PCR primers were designed based on two conserved sequences found in HMGCS-like genes from PKS pathways (LPYEDPV and MVKGAHR) (forward primer 5′-CTNCCNTAYGAYGAYCCCGT-3′ and reverse primer 5′-NCKRTGN GCNCCYTTNACCAT-3′). These primers were used to amplify a 650 bp fragment from *Lyngbya majuscula* JHB genomic DNA. The 650 bp products were gel purified, cloned into pGEM-T, and five of the resulting pGEM-T clones were DNA sequenced.

For amplification of the β-ketosynthase domain (KS) fragments from the *L. majuscula* JHB genomic DNA, previously designed primers KS1Up: 5′-MGI GAR GCI HWI SMI ATG GAY CCI CAR CAI MG-3′ and KSD1: 5′-GGR TCI CCI ARI SWI GTI CCI GTI CCR TG-3′ were used [69]. These primers were used to amplify an approximately 700 bp fragment. The 700 bp products were gel purified, cloned

into pGEM-T, and 20 of the resulting pGEM-T clones were DNA sequenced. For mixed KS probe generation, the KS fragment amplified from genomic DNA was purified from an agarose gel and labeled with DIG High Prime mix.

Fosmid Sequencing

Sequencing of the fosmids pJam1, pJam3, and pJam5 was performed by a shotgun cloning approach. The fosmid DNA was partially digested with AluI or Sau3AI (~0.25–0.125 units/μg of fosmid DNA for 1 hr). The partially digested DNA was separated on an agarose gel, and the 1.5–3 kb fragments were purified from each digest. SmaI/CIAP (calf intestinal alkaline phosphatase)-treated 3ZF was used to clone the AluI fragments, and BamHI/CIAP-treated pBluescript was used to clone the Sau3AI fragments. The random subclones were miniprepped and sequenced using Big Dye III terminator cycle sequencing (PE biosystems) at the Center for Gene Research and Biotechnology at Oregon State University. Sequence-specific primers were synthesized (Invitrogen, Carlsbad, CA) and used as sequencing primers to fill any resulting gaps in the sequence. The sequence data was edited and assembled using VectorNTI software (InforMax Inc., Frederick, MD). Database searches to assign putative translated protein function were performed by using the NCBI (National Center for Biotechnology Information) BLAST server.

Cloning, Expression, Purification, and Assay of JamA and JamO (A) Activity

The entire ORF encoding for JamA was amplified with primers JamA-For 5′-GGA ATT CAT ATG AGC AAG CCA GAA TTT TC-3′ and JamA-Rev 5′-CCG CTC GAG ACT GGA GAC TGC TGC TCC-3′ from fosmid pJam1 with PfuI polymerase according to the manufacturer's directions. The adenylation domain of JamO (amino acids 514–1053) was amplified with primers JamO (A)-For 5′-GGA ATT CAT ATG AGT CGC TTG CCA CTG CTT-3′ and JamO (A)-Rev 5′-CCG CTC GAG TTG CTC TGT TTC CGT CTG-3′ from fosmid pJam5 with PfuI polymerase according to the manufacturer's directions. The resulting PCR products were both cloned in-frame with a carboxy-terminal 6×His fusion at the NdeI and XhoI restriction sites of pET20b expression vector (Novagen). The JamA construct (pDJE-Jam01) and the JamO construct (pDJE-Jam02) were both verified by DNA sequencing. Overproduction of recombinant proteins in *E. coli* BL21 (DE-3) and subsequent purification of both JamA and JamO (A) proteins were done in identical fashion. Briefly, overnight cultures were diluted 1:100 in fresh LB-broth containing ampicillin (100 μg/ml) and grown at 37°C until OD = 0.6. Cultures were then induced with 0.4 mM IPTG and grown for an additional 6 hr at 25°C. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris [pH 8.0], 300 mM NaCl, and 10 mM imidazole), and lysed by sonication with five 10 s bursts using a UPC 2000U sonicator at 75 mAmp. Recombinant protein was purified by nickel chelate chromatography. Purified proteins were dialyzed overnight at 4°C against storage buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 10% glycerol), using Fisher dialysis membrane (Fisher Scientific, Pittsburgh, PA) with a molecular weight cutoff of 12,000–14,000 Da, aliquoted, and flash frozen at −80°C.

Substrate-dependent ATP-PPi exchange was assayed (100 ml) by incubating 2 mM of JamA or JamO(A) (protein concentrations determined by Bradford assay) in reaction buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 10 mM Mg Cl_2, 1 mM EDTA) containing 2 mM ATP, 0.2 mM tetrasodium pyrophosphate, and 0.15 μCi [^{32}P] tetrasodium pyrophosphate (Perkin Elmer, Boston, MA) and 0.5 mM of fatty acid (Lancaster or Sigma) or amino acid (Sigma) substrate, respectively. Reactions were incubated for 20 min at 30°C. The reaction was terminated by the addition of stop mix (500 ml, 1.2% w/v activated charcoal, 0.1 M tetrasodium pyrophosphate, and 0.35 M perchloric acid). Free [^{32}P]pyrophosphate was removed by centrifugation of the sample and washing the charcoal pellet three times with wash buffer (0.1 M tetrasodium pyrophosphate and 0.35 M perchloric acid). The final wash solution was aspirated, and the bound radioactivity was determined by scintillation counting on a Beckman LS 6800 (Beckman, Fullerton, CA).

Supplemental Data

Tables of ^{1}H and ^{13}C NMR spectral data for jamaicamides B and C, ^{13}C NMR spectra for acetylene model compound 11-bromo-undec-10-ynoic acid, ACCORD-ADEQUATE spectrum of jamaicamide A, ^{13}C NMR spectrum for biosynthetic feeding experiments, and a table with analysis of the two Jam NRPS adenylation domain binding pockets are available as Supplemental Data at http://www.chembiol.com/cgi/content/full/11/6/817/DC1.

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Accession Numbers

The nucleotide sequence of the jamaicamide genes described in this study has been submitted to GenBank under accession number AY522504.