Natural Products Chemistry and Taxonomy of the Marine Cyanobacterium *Blennothrix cantharidosmum*

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A Papua New Guinea field collection of the marine cyanobacterium *Blennothrix cantharidosmum* was investigated for its cytotoxic constituents. Bioassay-guided isolation defined the cytotoxic components as the known compounds lyngbyastatins 1 and 3. However, six new acyl proline derivatives, tumonoic acids D−I, plus the known tumonoic acid A were also isolated. Their planar structures were defined from NMR and MS data, while their stereostructures followed from a series of chiral chromatographies, degradation sequences, and synthetic approaches. The new compounds were tested in an array of assays, but showed only modest antimalarial and inhibition of quorum sensing activities. Nevertheless, these are the first natural products to be reported from this genus, and this inspired a detailed morphologic and 16S rDNA-based phylogenetic analysis of the producing organism.

Cyanobacteria are an ancient group of organisms well known for their prolific production of natural products.[1,2] Several genera, such as *Lyngbya* and *Microcystis*, have been well investigated for production of both dangerous toxins and drug leads.[3,4] However, there are numerous other cyanobacterial genera that have not been studied in such detail. Recent advances in the molecular taxonomy of cyanobacteria have allowed more rapid and precise identification of cyanobacterial specimens and have the potential to assist in the discovery of new natural products from some of these lesser-known genera.

Blennothrix cantharidosmum Komárek (formerly *Hydrocoleum* Gomont) is one of the most abundant species of mat-forming benthic cyanobacteria in the tropics[5] and an important contributor to nitrogen fixation.[6] Under bacteriological systems, *Blennothrix* has been assigned as a subgenus of *Microcoleus* on the basis of several common morphological features, most distinctively, the presence of multiple trichomes within a single sheath.[7] However, recent phylogenetic studies have shown that *Blennothrix* species are more closely related to the common planktonic genus *Trichodesmium*.[5,8] Despite their widespread occurrence, very few studies have been carried out on the chemistry of *Blennothrix*, and these studies have focused solely on their pigments.[5,9] In this article, we describe the first secondary metabolites reported from a *Blennothrix* species and further investigate the taxonomy of this important genus of marine cyanobacterium.

Results and Discussion

Natural Products Isolation and Structure Elucidation. The strain was collected near Duke of York Island in Papua New Guinea in 2005. The specimen was extracted with CH_2Cl_2−MeOH and fractionated by silica vacuum liquid chromatography. Initial assays revealed activity against an H-460 cell line in several of the polar fractions, which were subsequently fractionated by reversed-phase solid-phase extraction (SPE) and HPLC to yield the known metabolites lyngbyastatins 1[10] and 3[11] as the active metabolites, in addition to six new acylproline derivatives, the tumonoic acids D−I (**1**−**6**), as well as the known compound tumonoic acid A (**7**).[12] Structures of the tumonoic acids are given in Figure 1.

The molecular formula of tumonoic acid D (**1**) was determined to be $C_{16}H_{29}NO_3$ by HRESIMS (*m*/*z* 284.2211, $[M + H]^+$). Analysis of ^{1}H NMR data (Table 1) revealed downfield resonances (δ_H 4.64, d; 3.62, ddd; 3.51, ddd) characteristic of a proline residue, while the large methylene envelope (δ_H 1.24−1.28) and methyl signals (δ_H 1.15, d; 0.88, t) suggested a branched fatty acid. HMBC correlations from the methyl doublet to carbons at 179.9, 38.1, and 34.1 ppm confirmed its location at C-2. 2D NMR analysis (Figure 2) enabled the assignment of all protons and carbons in the proline residue and the terminal regions of the fatty acid moiety. The three remaining carbons (δ_C 29.2, 29.4, 29.6) were assigned as the methylene carbons in the central portion of the fatty acid, which was consistent with the observed molecular formula. However, no HMBC correlations were observed linking the proline and fatty acid portions of **1**, but on the basis of the downfield shifts of H-2′ and H-5′, it was clear that the proline nitrogen was part of an amide group, leading to the structure shown.

Tumonoic acid E (**2**) ($C_{18}H_{31}NO_4$, *m*/*z* 326.2321, $[M + H]^+$) possessed more complex ^{1}H and ^{13}C NMR spectra than **1**. While resonances consistent with a proline residue were present, it was clear that the fatty acid moiety of **2** was more highly functionalized. The presence of a downfield doublet (δ_H 5.23, d), along with two sp^2 carbon resonances (δ_C 137.0, 132.3), suggested the presence of a double bond. Also observed were resonances consistent with an oxygenated methine (δ_H 4.15, d; δ_C 80.6) and two additional methyls. A broad IR stretch at 3381 cm^{-1} confirmed the presence of a free hydroxy group. COSY and HMBC correlations (Figure 2) situated the oxygenated methine at H-3, while HMBC correlations from the three methyl groups (δ_H 1.05, d; δ_C 14.3, δ_H 1.63, br s; δ_C 10.9, and δ_H 0.95, d; δ_C 20.9) confirmed their positions at C-2, C-4, and C-6, respectively. Likewise, HMBC correlations situated the vinylic proton at H-5, while ROESY correlations between H-5 and H-3 confirmed the *E*-geometry of the double bond. The H-2 pentet (δ_H 2.82, dq, J = 6.8, 6.5) of the fatty acid moiety was characteristic for an

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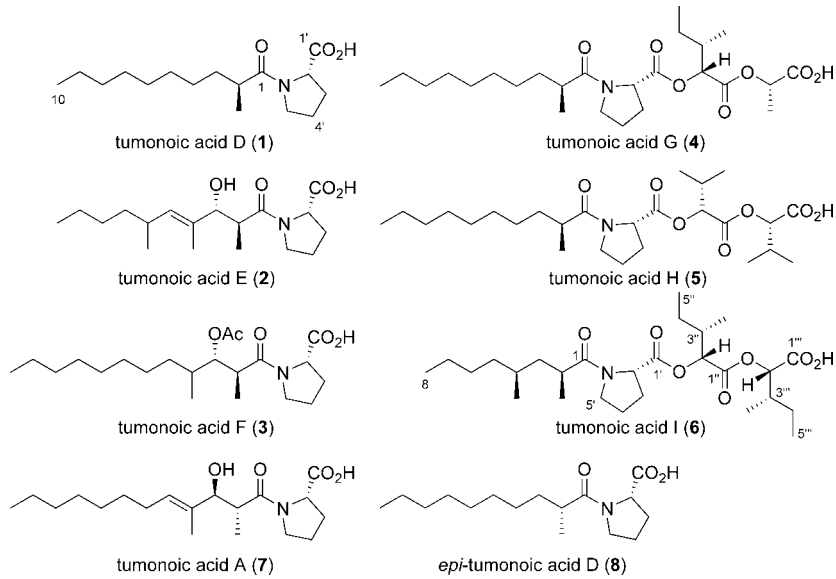

Figure 1. Structures of tumonoic acids D−I (**1**−**6**) and A (**7**) and epi-tumonoic acid D (**8**). Configurations for the fatty acid portions of **2**−**6** are relative only.

Table 1. NMR Data ($CDCl_3$) for Tumonoic Acids D−F (**1**−**3**)[a]

	tumonoic acid D (**1**)		tumonoic acid E (**2**)		tumonoic acid F (**3**)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
fatty acid						
1	179.9		178.2		175.6	
2	38.1	2.59 dq (6.8, 6.8)	41.3	2.82 dq (6.8, 6.5)	40.0	2.96 dq (9.6, 6.8)
3	34.1	1.69 m, 1.43 m	80.6	4.15 d (6.5)	77.5	5.21 dd (9.6, 1.5)
4	27.5	1.26 m	132.3		33.9	1.75 br tq (5.9, 6.7)
5	29.6	1.25 m	137.0	5.23 d (9.3)	33.6	1.31 m
6	29.4	1.25 m	32.1	2.38 m	27.3	1.25 m
7	29.2	1.25 m	37.1	1.33 m, 1.21 m	29.7	1.25 m
8	31.8	1.24 m	28.8	1.22 m	29.2	1.25 m
9	22.6	1.28 m	22.7	1.29 m	29.5	1.25 m
10	14.1	0.88 t (6.8)	14.1	0.87 t (7.2)	31.8	1.25 m
11					22.6	1.28 m
12					14.1	0.88 t (6.9)
2-Me	16.8	1.15 d (6.8)	14.3	1.05 d (6.8)	13.7	1.14 d (6.8)
4-Me			10.9	1.63 br s	12.9	0.91 d (6.8)
6-Me			20.9	0.95 d (6.6)		
OAc					169.9	
					20.8	1.99 s
proline						
1′	171.4		n/o[b]		172.8	
2′	60.5	4.64 d (7.6)	60.2	4.62 d (9.5)	59.7	4.55 dd (8.1, 2.5)
3′	26.6	2.56 m 1.94 m	27.3	2.51 m 2.03 m	27.8	2.41 ddd (2.5, 6.1, 10.0)
						2.00 m
4′	24.8	2.03 m	24.8	2.04 m	24.6	2.10 m
5′	48.0	3.62 ddd (9.5, 7.8, 3.2)	48.0	3.71 ddd (9.2, 7.6, 7.6)	47.7	3.64 dd (8.4, 5.1)
		3.51 ddd (9.8, 9.5, 7.1)		3.60 ddd (9.2, 7.0, 3.6)		

[a] ^1H NMR spectra were recorded at 500 MHz, and ^{13}C spectra at 75 MHz. [b] Not observed.

anti-configuration of the 2-Me and 3-OH substituents.[12,13] The relative configuration at C-6 was not assigned.

Tumonoic acid F (**3**) ($C_{21}H_{37}NO_5$, *m*/*z* 384.2736, $[M + H]^+$) also possessed a complex fatty acid moiety, two secondary methyls (δ_H 1.14, d and 0.91, d), and an oxygenated methine proton (δ_H 5.21, d) shifted further downfield than that of **2**. In combination with the presence of a deshielded methyl group (δ_H 1.99, s; δ_C 20.8) and an additional carbonyl resonance (δ_C 169.9), this suggested the presence of an *O*-acetyl group at C-3, which was confirmed by 2D NMR analysis (Figure 2). HMBC correlations also positioned the secondary methyls at C-2 and C-4, respectively. As for **2**, the H-2 pentet of the fatty acid moiety suggested an *anti*-arrangement for the 2-Me and 3-OAc moieties. In an attempt to determine the absolute configuration at C-3, a small sample of **3** was subjected to base hydrolysis and derivatization with Mosher's acid chloride (both *R* and *S*). However, this proved unsuccessful. Prior research has suggested that reduction of the C-1 amide to an alcohol is necessary before successful esterification can occur.[12] Given the small amount of material available, further attempts were not made.

The molecular formula of tumonoic acid G (**4**) was determined to be $C_{25}H_{43}NO_7$ (*m*/*z* 470.3106, $[M + H]^+$) by HRESIMS. While ^1H and ^{13}C NMR analyses revealed the presence of the same proline and 2-methyldecanoyl moieties as tumonoic acid D (**1**), the spectra were significantly more complex, with additional downfield methine and methyl resonances being the most distinctive new elements. COSY analysis established the presence of a spin system incorporating two methines (δ_H 5.05, d; δ_H 2.05, m), two methyls (δ_H 0.91, t; δ_H 0.96, d), and a diastereotopic methylene unit (δ_H 1.44, m; δ_H 1.28, m), consistent

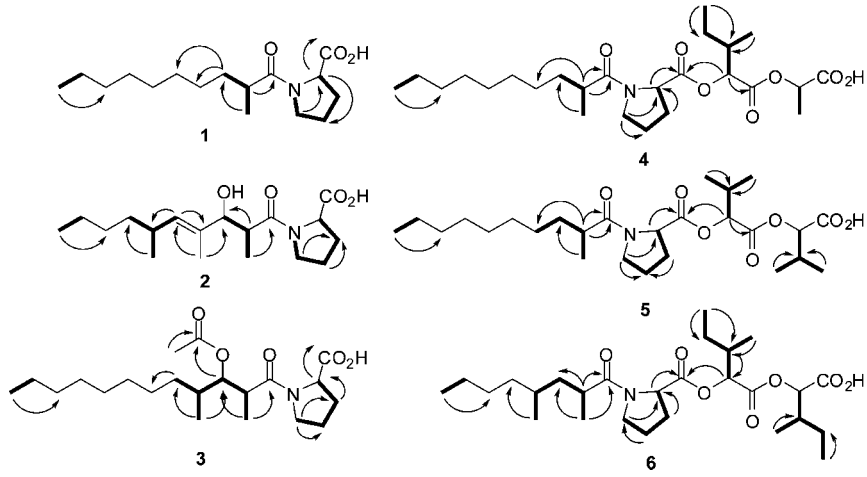

Figure 2. Elucidation of planar structures of tumonoic acids D−I (**1**−**6**). COSY correlations are indicated by bolded bonds, while key HMBC correlations are designated by arrows (proton to carbon).

Table 2. NMR Data ($CDCl_3$) for Tumonoic Acids G−I (**4**−**6**)[a]

	tumonoic acid G (**4**)		tumonoic acid H (**5**)		tumonoic acid I (**6**)	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
fatty acid						
1	176.1		175.9		175.9	
2	37.8	2.56 dq (6.7, 6.7)	37.7	2.55 tq (6.8, 6.7)	35.2	2.67 dq (6.8, 6.8)
3	33.8	1.64 m 1.37 m	33.8	1.64 m, 1.36 m	41.2	1.64 ddd (13.5, 6.8, 6.8) 1.19 m
4	27.3	1.31 m	27.4	1.30 m	30.1	1.48 m
5	29.1	1.27 m	29.5	1.26 m	36.1	1.32 m, 1.02 m
6	29.3	1.27 m	29.3	1.26 m	29.0	1.31 m, 1.21 m
7	29.7	1.27 m	29.7	1.26 m	22.9	1.28 m
8	31.8	1.24 m	31.8	1.26 m	14.4	0.88 t (6.7)
9	22.6	1.28 m	22.6	1.28 m		
10	14.1	0.87 t (6.8)	14.0	0.89 t (6.7)		
2-Me	16.7	1.11 d (6.7)	16.8	1.10 d (6.7)	16.9	1.08 d (6.7)
4-Me					20.1	0.88 d (6.8)
proline						
1′	172.3		172.2		172.1	
2′	59.1	4.50 dd (7.7, 4.5)	59.2	4.51 dd (8.1, 4.9)	59.2	4.52 dd (7.3, 4.5)
3′	29.5	2.23 m, 2.05 m	29.2	2.24 m, 2.08 m	29.2	2.21 m, 2.11 m
4′	25.0	2.18 m, 2.02 m	25.0	2.18 m, 2.01 m	24.9	2.20 m, 2.02 m
5′	47.1	3.69 ddd (9.8, 7.4, 6.0)	47.1	3.70 ddd (9.9, 7.1, 6.0)	47.0	3.68 ddd (9.4, 7.3, 6.8)
		3.60 ddd (9.8, 6.5, 6.5)		3.59 ddd (9.9, 9.6, 6.7)		3.61 ddd (9.4, 7.1, 6.9)
hydroxy acid 1						
1″	169		168.9		169.5	
2″	75.5	5.05 d (3.3)	77.2	5.06 d (4.1)	75.0	5.20 d (3.3)
3″	36.6	2.05 m	29.8	2.33 m	36.6	2.05 m
4″	25.8	1.44 m, 1.28 m	18.6	1.00 d (7.0)	25.8	1.41 m, 1.25 m
5″	11.5	0.91 t (7.4)			11.6	0.92 t (7.3)
3″-Me	14.2	0.96 d (6.8)	17.0	0.98 d (6.8)	14.2	0.95 d (7.0)
hydroxy acid 2						
1‴	n/o[b]		n/o[b]		n/o[b]	
2‴	71.1	5.27 brq (5.1)	79.0	5.04 d (5.4)	77.2	5.09 d (4.6)
3‴	16.7	1.50 d (5.1)	30.2	2.27 m	36.4	2.04 m
4‴			18.5	1.00 d (7.0)	24.5	1.55 m, 1.30 m
5‴					11.4	0.92 t (7.3)
3‴-Me			17.7	0.99 d (7.1)	15.2	0.98 d (6.8)

[a] ^1H NMR spectra were recorded at 500 MHz, and ^{13}C spectra at 75 MHz. [b] Not observed.

with the presence of an isoleucic acid residue. ^{13}C NMR shifts (Table 2) and HMBC correlations (Figure 2) confirmed this assignment, and a strong correlation from H-2″ to C-1 of the proline residue was used to connect the isoleucic acid to the proline unit. The second new spin system consisted of another downfield methine connected to a deshielded methyl resonance. While no HMBC correlations were observed from these protons, the distinctive chemical shifts (δ_H 5.27, brq; δ_C 71.1 and δ_H 1.50, d; δ_C 16.7) were indicative of a lactic acid residue, which was later confirmed by HPLC analysis as described below. The terminal carboxylic acid group was not observed in the ^{13}C NMR spectrum, likely due to broadening caused by partial deprotonation of this functionality.

The ^1H and ^{13}C NMR spectra of tumonoic acid H (**5**) ($C_{26}H_{45}NO_7$, *m/z* 484.3254, $[M + H]^+$) were very similar to those of **4**, with the same proline and 2-methyldecanoyl units again present; however, the abundance of secondary methyls (δ_H 1.00, d; 1.00, d; 0.99, d; 0.98, d) indicated the presence of two 2-hydroxyisovaleric acid residues in place of the isoleucic and lactic acids of **4**. 2D NMR analysis (Figure 2) was consistent with such a structure, and later chiral HPLC analysis confirmed the presence of the hydroxyisovaleric acid residues.

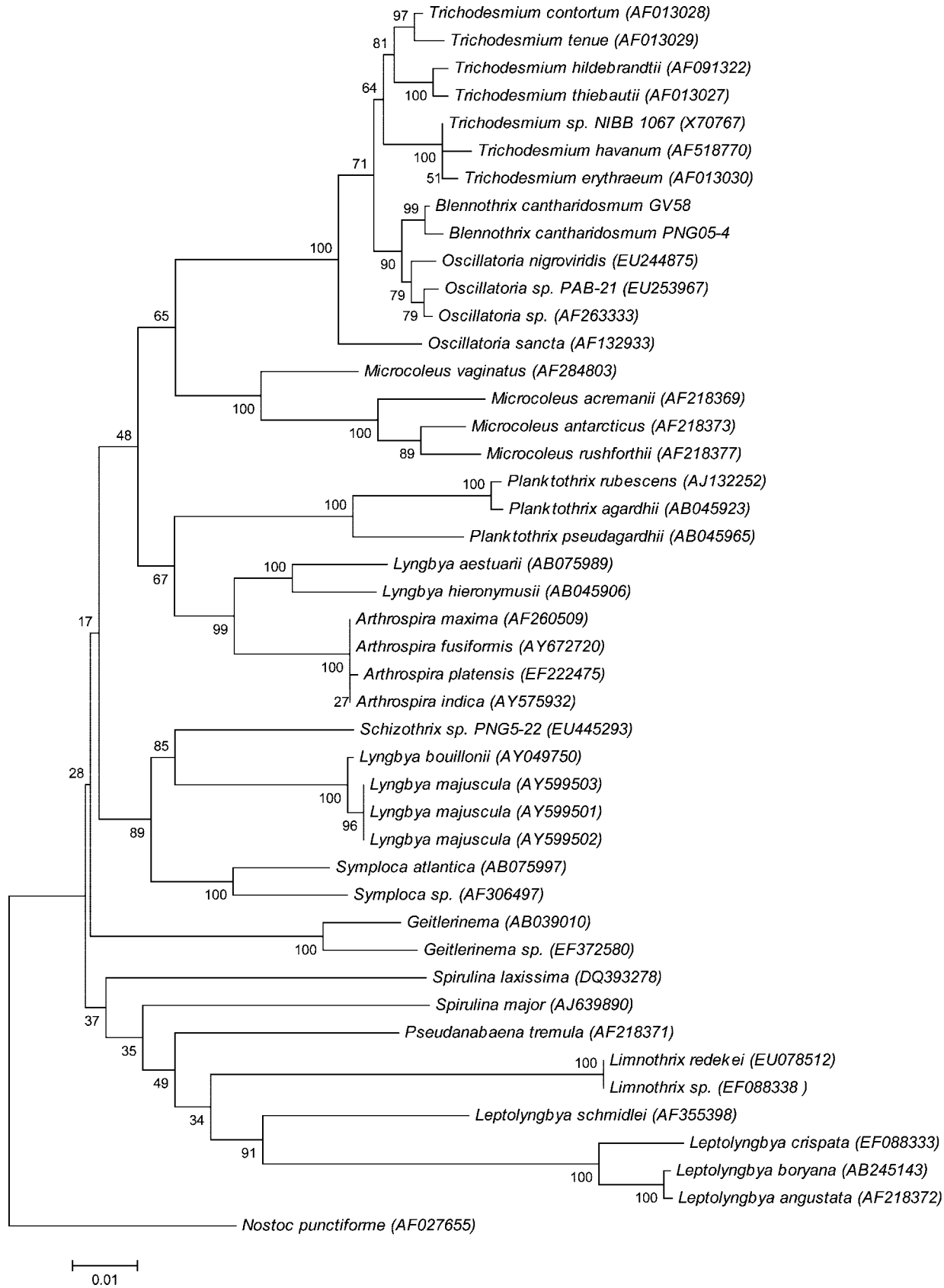

Figure 3. Phylogenetic relationships of filamentous marine cyanobacteria from 16S rRNA nucleotide sequences, including new sequences from *Blennothrix cantharidosmum* and *Schizothrix* sp., determined in this study (performed using partial 1082-nucleotide sequence).

Likewise, tumonoic acid I (**6**) ($C_{27}H_{47}NO_7$, *m*/*z* 498.3413, [M + H]$^+$) was very similar to both **4** and **5**. A second set of resonances characteristic of isoleucic acid (Table 2) indicated the replacement of the lactic acid residue of **4** with an additional isoleucic acid residue. Compound **6** showed a much smaller methylene envelope than that of the other tumonoic acids, indicating the presence of a shorter fatty acid chain. Additionally, a distinctive secondary methyl resonance (δ_H 0.88, d; δ_C 20.1) indicated the presence of a second methyl branch along the fatty acid chain; this could be positioned at C-4 using 2D NMR

correlations to give the structure shown (Figure 2). On the basis of the wide separation of the ^{1}H NMR resonances (δ_H 1.64, 1.19) of H-3 of the dimethyloctanoic acid moiety, and comparison with literature examples, we propose that the C-2 and C-4 methyl groups are in a *syn*-orientation.[14]

Stereochemical Investigations. After solving the planar structures of **1**−**6**, the next issues to be addressed were their absolute configurations. This was determined for the proline residues of **1**−**7** by acid hydrolysis followed by derivatization with Marfey's reagent (FDVA) and subsequent LCMS analysis. This analysis revealed that the proline unit in all seven compounds was L. The configurations of the α-hydroxy acids of **4**−**6** were determined by chiral HPLC. Analysis of the acid hydrolysates of **4**−**6** and comparison with known standards revealed the following compositions: **4**, D-*allo*-isoleucic acid, L-lactic acid; **5**, D-2-hydroxyisovaleric acid, L-2-hydroxyisovaleric acid; **6**, D-*allo*-isoleucic acid, L-isoleucic acid. In order to determine the positions of the isomeric hydroxy acids in **5** and **6**, the natural products were derivatized with α-methylbenzylamine and the products subjected to base hydrolysis, yielding only one free hydroxy acid. Analysis of these hydrolysates revealed only D-2-hydroxyisovaleric acid for **5** and D-*allo*-isoleucic acid for **6**, enabling the configurations of the hydroxy acids to be assigned as shown.

The C-2 configuration of the fatty acid moiety of tumonoic acid D (**1**) was determined by synthesis. Racemic 2-methyldecanoic acid was first derivatized with L-proline methyl ester. The methyl ester present in each component of the mixture was hydrolyzed under basic conditions to yield two epimeric acids, **1** and **8**. These were separated using reversed-phase (RP) HPLC and characterized using standard spectroscopic techniques. ^{1}H and ^{13}C NMR, IR, and $[\alpha]_D$ data were very similar for both epimers, and it was not possible to conclusively distinguish the two solely on this basis. However, co-injections of the synthetic epimers with natural **1** and analysis by LCMS conclusively showed that the earlier eluting peak was the natural epimer. Finally, each of the two synthetic tumonoic acids was hydrolyzed under acidic conditions to give free 2-methyldecanoic acid, which was purified by HPLC and its $[\alpha]_D$ value measured and compared with literature values.[15] This sequence revealed that **1** contained (2*S*)-2-methyldecanoic acid, whereas the unnatural epimer **8** incorporated (2*R*)-2-methyldecanoic acid. Although we did not conduct similar reaction sequences on the other new tumonoic acids, we propose on biogenetic grounds that **4** and **5** share the same absolute configuration at C-2. As the known tumonoic acid A (**7**) possesses the opposite 2*R* configuration, we have not assigned the absolute configurations of the fatty acid portions for **2**, **3**, or **6**. Intriguingly, our isolate of tumonoic acid A (**7**) was of 2*R* configuration, as in the original report {$[\alpha]_D$ −79.4 (*c* 0.3, $CHCl_3$); lit. $[\alpha]_D$ −79 (*c* 1.1, $CHCl_3$)[12]}, and this is opposite the configuration at C-2 in compound **1**.

Tumonoic acids A−C were originally isolated in 1999 along with two methyl esters and were not reported to possess any significant biological activities.[12] The new tumonoic acids differ from these previously isolated examples in the greater diversity of fatty acids incorporated. All three tumonoic acids in the original isolation possess the same 2,4-dimethyl-3-hydroxydodec-4-enoic acid moiety, while those in the current study include a range of unusual fatty acids.

Biological Activities. The tumonoic acids A (**7**) and D−I (**1**−**6**) were assayed for anticancer, antimalarial, anti-Chagas, antileishmania, and antimicrobial (MRSA) activity. Tumonoic acid I (**6**) displayed moderate activity in the antimalarial assay ($IC_{50} = 2$ μM), whereas none of the other naturally occurring analogues showed any activity in this assay at a concentration of 10 μg/mL. It was interesting to observe only **6** displaying such activity given the strong structural similarities between **6** and the other isolated acids, especially **4** and **5**. It was also noted that several of these tumonoic acids bore a close resemblance to homoserine lactones involved in bacterial communication. Thus, investigations were carried out into the possible effects of these compounds on bacterial quorum sensing and biofilm formation. The tumonoic acids showed no activity in an assay utilizing a mutated *E. coli* strain that produces green fluorescence protein in response to 3-oxohexanoyl homoserine lactone addition.[16] However, in an assay using a wild-type strain of *Vibrio harveyi* that regulates bioluminescence via quorum sensing, several of these tumonoic acids (**2**−**5**) displayed modest inhibition of bioluminescence without affecting bacterial growth, with tumonoic acid F (**3**) being the most active (IC_{50} of 62 μM).

Morphological and Phylogenetic Analysis. Given the difficulties in the taxonomic classification of cyanobacteria and relative paucity of 16S rRNA sequences for *Blennothrix* species in the literature, a detailed morphological and phylogenetic analysis of *Blennothrix* strain PNG05-4 (PNG-5/21/05-4) was carried out. The strain was morphologically identified as *B.* (*Hydrocoleum) cantharidosmum* Komárek & Anagnostidis. The thallus formed a soft, gelatinous, thick, 2 cm high, brown-gray mat covering the reef substrate. The cyanobacterium was filamentous, and filaments were solitary or several were enclosed together in common sheaths. Furthermore, the trichomes were cylindrical and slightly constricted at the crosswalls. Cells were disk-shaped, 21 μm wide and 3.1 μm long. The end cells were cylindrical, with thickened outer cell walls.

The almost complete 16S rRNA gene sequence (1414 nucleotides) was PCR-amplified and sequenced (GenBank Acc. No. EU253968). BLAST analysis of the 16S rRNA gene sequence showed highest sequence identity (99.1%) with the *B.* (*Hydrocoleum) cantharidosmum* strain GV58 from New Caledonia (GenBank Acc. No. DQ883636).[5] Due to the lack of full 16S rRNA sequences for related species available in databases, including the *Blennothrix* sp. from New Caledonia, a 1082-nucleotide stretch of the 16S rRNA genes was used in the final multiple sequence alignment and phylogenetic tree construction. The three main kinds of reconstruction methods [(1) the distance method by neighbor-joining (NJ), (2) maximum parsimony (MP), and (3) likelihood method by maximum-likelihood (ML)] were compared for their predicted relationships. The overall topography of the different phylogenetic trees was consistent with the ML method giving the most robust bootstrap values; hence, this tree is displayed in Figure 3. The clading was also stable using either the 1414- or the 599-nucleotide stretches (see Supporting Information); however, use of the 1414 base pair stretch excluded several species of interest in the study. In the final phylogenetic analysis, 43 16S rRNA cyanobacterial gene sequences were included, including four from *Microcoleus* species. Consistent with previous findings,[5] species of *Blennothrix* and *Microcoleus* are genetically very different and form separate clades in the phylogenetic tree.

Schizothrix is a former genus that has also been assigned as a subgenus of *Microcoleus* on the basis of the presence of multiple trichomes within a sheath,[7] and thus it bears a close resemblance to *Blennothrix*. However, the 16S rRNA gene data for *Schizothrix* species available in GenBank is even less extensive than that for *Blennothrix*, with only a 599-nucleotide stretch being currently available (*Schizothrix calcicola* UTEX, GenBank Acc. No. AY271841). Hence, in the interests of further clarifying the taxonomic relationships between these genera, a *Schizothrix* sp. PNG5-22 (PNG-5/20/05-2) from our own collection was morphologically and phylogenetically identified according to previously described taxonomic methods, and its 16S rRNA gene sequence was also included in this study. The thallus of this specimen was soft, mucilaginous, hemispherical, 4 cm tall, and dark yellowish-brown. The cyanobacterium was filamentous, with several cylindrical trichomes within the same sheaths. The cells were isodiametric, 1.2 μm wide by 3.2 μm long.

A 1370-nucleotide stretch of the *Schizothrix* PNG5-22 16S rRNA gene was PCR-amplified and sequenced (GenBank Acc. No. 1060443), and a 1082-nucleotide stretch included in the phyloge-

netic analysis described above. Interestingly, this *Schizothrix* sp. PNG5-22 appeared genetically very different from members of both *Microcoleus* and *Blennothrix* (Figure 3). Thus, it appears that the taxonomic groupings originally created using phycological criteria and nomenclature are supported by this genetic-based analysis, and this raises concerns about the recent regrouping of genera under the bacteriological system.[7]

Conclusions. The tumonoic acids were originally isolated from a *Lyngbya majuscula*/*Schizothrix calcicola* assemblage, as well as a collection of *L. majuscula*, all collected in Guam.[12] Given the uncertainties involved in cyanobacterial taxonomy and the overall morphological resemblance between *Blennothrix*, *Schizothrix*, and *Lyngbya*, it is possible that the original tumonoic acid isolation was in fact from a *Lyngbya*/*Blennothrix* assemblage.[17] However, in the absence of 16S rRNA data from the previous work it is impossible to make a definitive assignment. The current study illustrates the inadequacy of morphological identification in cyanobacterial taxonomy and demonstrates the complementary power of molecular taxonomy in the classification of these organisms. Precise taxonomic investigations are important in guiding chemical investigations of some of the less well studied cyanobacteria, and these can lead to the isolation of new metabolites such as those described herein.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P1010 polarimeter. IR and UV spectra were recorded on a Nicolet IR-100 FT-IR and Beckman-Coulter DU800 spectrophotometer, respectively. NMR spectra were recorded on Varian Unity 300 and 500 MHz spectrometers with the solvent $CDCl_3$ (δ_H at 7.26, δ_C at 77.0) used as an internal standard. High-resolution mass spectra were acquired using an Agilent ESI-TOF mass spectrometer. LCMS analyses were carried out on a Finnigan LCQ advantage mass spectrometer, attached to a Finnigan Surveyor HPLC system. HPLC was performed using Waters 515 pumps and a Waters 996 photodiode array detector, under computer control using Empower software. Chemicals were acquired from VWR, except for 2-methyldecanoic acid, which was purchased from Narchem, and 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC·HCl), purchased from Chem-Impex International.

Collection. The *Blennothrix* and *Schizothrix* specimens were collected by scuba in Papua New Guinea in May 2005. The *Blennothrix* sp. PNG05-4 (PNG-5/21/05-4) was collected to the west of Duke of York Island, near Kibil, at a depth of 30 ft (GPS coordinates S 04°07.270′, E 152°26.666′). The *Schizothrix* sp. PNG5-22 (PNG-5/20/05-2) was collected on a reef to the west of the Credner Islands, near Kerawara, at a depth of 40 ft (GPS coordinates S 04°12.922′, E 152°22.926′). The samples were stored in EtOH−seawater (1:1) at −20 °C until extraction, with a small (~100 mg) tissue sample preserved in 5 mL of RNA-later (Ambion) for genetic analysis. Voucher specimens are available from WHG.

Morphological Characterization. Preliminary morphological and taxonomic characterizations of the *Blennothrix* and *Schizothrix* specimens were performed using a field microscope (RFM-2 microscope from Richardson Technologies Inc.) and the taxonomic field guide by Littler and Littler,[18] while more accurate measurements and characterizations were made with an Olympus BH-2 light microscope. The following morphological parameters were evaluated: thallus morphology, cell length, cell width (and cell length/width ratios), constriction of cell walls, presence of specialized cells such as heterocysts, akinetes, or calyptra, size and shape of trichomes, and characteristics of end cells. Morphological identification was made in accordance with the phycological systems by Gomont,[19] Geitler,[20] and Komárek and Anagnostidis.[21]

DNA Extraction, 16S rDNA Gene PCR-Amplification, and Cloning. Genomic DNA was extracted from 40 mg of cleaned cyanobacterial tissues using the Wizard Genomic DNA purification kit (cat. A1120) according to the manufacturer's specifications (Promega, Madison, WI). The isolated genomic DNA was further purified using Genomic-tips 20/G from Qiagen (cat. 10223). The 16S rRNA genes were PCR-amplified from isolated DNA using the cyanobacteria-specific primers, 106F and 1509R, as previously described.[22] The reaction volumes were 25 μL containing 0.5 μL of DNA (50 ng), 2.5 μL of 10× PfuUltra II reaction buffer, 0.5 μL of dNTP mix (25 mM each of dATP, dTTP, dGTP, and dCTP), 0.5 μL of each primer (10 μM), 0.5 μL of PfuUltra II fusion HS DNA polymerase (cat. 600760), and 20.5 μL of dH_2O. The PCR reaction was performed in an Eppendorf Mastercycler gradient as follows: initial denaturation for 2 min at 95 °C, 30 cycles of amplification using 20 s at 95 °C, 20 s at 50 °C (16S rDNA)/56 °C (ITS), and 15 s at 72 °C, and final elongation for 3 min at 72 °C. PCR products were subcloned using the Zero Blunt TOPO PCR cloning kit (cat. K2800-20SC) from Invitrogen into pCR-Blunt II TOPO vector, transformed into TOPO cells, and cultured on LB-Kanamycin plates. Plasmid DNA was isolated using the QIAprep spin miniprep kit (cat. 27106) from Qiagen and sequenced in both directions using pCR-Blunt II TOPO vector specific primers M13F and M13R and the internal middle primers 359F and 758R as previously described.[22]

Phylogenetic Analysis. The 16S rRNA gene sequences were visually inspected, and a total DNA stretch of 1414/1082 nucleotides was aligned together with 43 cyanobacterial species representing 14 major genera of the order *Oscillatoriales*, obtained from GenBank (http://www.ncbi.nlm.nih.gov). The multiple sequence alignments were performed using ClustalX, with standard gap opening and extension penalties without gaps, and the aligned 16S rRNA gene sequences were used to generate phylogenetic trees in MEGA 4.0. The phylogenetic relations of the cyanobacterial 16S rRNA genes were compared by (1) the distance method by neighbor-joining (NJ), (2) maximum parsimony (MP), and (3) the likelihood method by maximum-likelihood (ML). The evolutionarily distant cyanobacterium *Nostoc punctiforme* PCC73102 (GenBank Acc. No. AF027655) from the order *Nostocales* was used as an outgroup.

Extraction and Isolation. Approximately 64.5 g (dry weight) of the alga was extracted repeatedly with CH_2Cl_2−MeOH (2:1) to yield 1.15 g of crude extract. The extract was fractionated by VLC using a stepwise gradient with solvents of increasing polarity, from 100% hexanes to 100% MeOH. The polar fractions eluting with 3:1 EtOAc−MeOH and 100% MeOH possessed activity against the H-460 cell line. These were further fractionated by RP solid-phase extraction on Strata C18-E 500 mg cartridges using a stepwise gradient from 10% MeOH−H_2O to 100% MeOH. Further purification was carried out via RP HPLC [Phenomenex Synergy-Hydro (4 μm particles, 21.2 × 250 mm), gradient elution from 45% CH_3CN−H_2O to 75% CH_3CN−H_2O over 30 min], yielding the tumonoic acids D (**1**, 19.6 min, 1.8 mg), E (**2**, 14.2 min, 1.7 mg), F (**3**, 24.5 min, 3.4 mg), G (**4**, 28.6 min, 8.0 mg), H (**5**, 31.1 min, 1.1 mg), I (**6**, 32.7 min, 5.5 mg), and A (**7**, 18.3 min, 7.6 mg) and a mixture containing lyngbyastatins 1 and 3 (33.8 mg, 15−17 min).

Tumonoic acid D (1): colorless oil; $[\alpha]_D$ −24.3 (*c* 0.81, MeOH); IR (neat) ν_{max} 2957, 2927, 2856, 1725, 1614, 1468, 1447, 1195 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 1; HRESI(+)MS *m/z* $[M + H]^+$ 284.2211 (calcd for $C_{16}H_{30}NO_3$, 284.2220).

Tumonoic acid E (2): colorless oil; $[\alpha]_D$ −24.8 (*c* 0.94, MeOH); IR (neat) ν_{max} 3381, 2960, 2927, 2874, 1718, 1619, 1448, 1201 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 1; HRESI(+)MS *m/z* $[M + H]^+$ 326.2321 (calcd for $C_{18}H_{32}NO_4$, 326.2326).

Tumonoic acid F (3): colorless oil; $[\alpha]_D$ −173.3 (*c* 1.62, MeOH); IR (neat) ν_{max} 2959, 2927, 2856, 1746, 1626, 1461, 1461, 1373, 1238, 1195 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 1; HRESI(+)MS *m/z* $[M + H]^+$ 384.2736 (calcd for $C_{21}H_{38}NO_5$, 384.2744).

Tumonoic acid G (4): colorless oil; $[\alpha]_D$ −33.6 (*c* 0.69, MeOH); IR (neat) ν_{max} 2961, 2927, 2858, 1744, 1640, 1617, 1459, 1263, 1193, 1098 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2; HRESI(+)MS *m/z* $[M + H]^+$ 470.3106 (calcd for $C_{25}H_{44}NO_7$, 470.3112).

Tumonoic acid H (5): colorless oil; $[\alpha]_D$ −42.6 (*c* 0.69, MeOH); IR (neat) ν_{max} 2963, 2928, 2855, 1741, 1642, 1610, 1463, 1427, 1208, 1192, 1023 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2; HRESI(+)MS *m/z* $[M + H]^+$ 484.3254 (calcd for $C_{26}H_{46}NO_7$, 484.3269).

Tumonoic acid I (6): colorless oil; $[\alpha]_D$ −40.6 (*c* 3.0, MeOH); IR (neat) ν_{max} 2964, 2929, 2878, 1742, 1647, 1625, 1460, 1198, 1132 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 75 MHz) data, see Table 2; HRESI(+)MS *m/z* $[M + H]^+$ 498.3417 (calcd for $C_{27}H_{48}NO_7$, 498.3413).

It should be noted that for many of the tumonoic acids, a second, minor set of NMR signals was observed (<10% height of major peaks in all cases). These were attributed to minor isomers caused by *cis*−*trans* proline isomerization.

Absolute Configuration of the Proline Residues of Tumonoic Acids D−I. The tumonoic acids (~0.1 mg each) were hydrolyzed in 6 N HCl at 110 °C for 18 h and then extracted twice with EtOAc. The aqueous layers were dried under a stream of N_2 before derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-valerylamide (FDVA) in acetone (50 μL) and 0.1 M $NaHCO_3$ (100 μL) in sealed vials at 90 °C for 5 min. The resulting solutions were analyzed using RP HPLC using an HP LiChrospher 100 RP-18 (5 μm, 4 × 125 mm) column, eluting with 50% CH_3CN-H_2O at a flow rate of 0.8 mL/min. The L-proline derivative eluted at 7.7 min, with the D-proline derivative eluting at 8.9 min.

Chiral HPLC Analysis. Isoleucic acids were synthesized according to literature methods.[23] The organic layers from the acid hydrolysates of **4**, **5**, and **6** were evaporated to dryness and then redissolved in 2 mM $CuSO_4$. Analysis was carried out using three separate conditions. Condition 1: 100% 2 mM $CuSO_4$, 0.8 mL/min, Phenomenex Chirex 3126 (4.6 × 250 mm) column; elution times (t_R, min) for standards: L-lactic acid (22.1), D-lactic acid (29.2) min. Condition 2: 12.5% CH_3CN in 2 mM $CuSO_4$, 0.8 mL/min, Phenomenex Chirex 3126 (4.6 × 250 mm) column; elution times (t_R, min) for standards: L-2-hydroxyisovaleric acid (7.8), D-2-hydroxyisovaleric acid (12.1) min. Condition 3: 12.5% CH_3CN in 2 mM $CuSO_4$, 0.8 mL/min, Phenomenex Chirex 3126 (4.6 × 50 mm) column; elution times (t_R, min) for standards: L-*allo*-isoleucic acid (18.3), L-isoleucic acid (21.4), D-*allo*-isoleucic acid (28.5), D-isoleucic acid (34.1).

The hydrolysates of **4**, **5**, and **6** were analyzed by the methods above, revealing the presence of L-lactic acid and D-*allo*-isoleucic acid in **4**; L-2-hydroxyisovaleric acid and D-2-hydroxyisovaleric acid in **5**; and L-isoleucic acid and D-*allo*-isoleucic acid in **6**.

To define the positions of the hydroxy acids in **5** and **6**, both were separately derivatized using L-(−)-α-methylbenzylamine using the following method: 0.2 mg of the natural product was dissolved in 100 μL of CH_2Cl_2, the solution was cooled to 0 °C, and solutions of L-(−)-α-methylbenzylamine (12 μL), triethylamine (12 μL), EDAC·HCl (18 μL), and DMAP (12 μL) (all solutions 5 mg/mL) were added sequentially. The mixture was allowed to stir overnight, with LCMS analysis indicating complete conversion to the desired products. The crude products were evaporated to dryness, resuspended in CH_2Cl_2, and washed sequentially with 0.1 M HCl, H_2O, 1 M $NaHCO_3$, H_2O, and brine. The organic layers were evaporated to dryness and the residues resuspended in MeOH−1 M NaOH (1:1) and left to stir overnight at room temperature. The MeOH was removed under N_2, and the aqueous residue acidified with 2 M HCl and then extracted twice with EtOAc. The EtOAc was removed under N_2, and the samples were suspended in 2 mM $CuSO_4$ and analyzed by the relevant chiral HPLC methods as described above, revealing the presence of free D-2-hydroxyisovaleric acid in **5** and D-*allo*-isoleucic acid in **6**.

Synthesis of Tumonoic Acid D Epimers. Commercially available racemic 2-methyldecanoic acid (50 mg) was dissolved in 2 mL of CH_2Cl_2, the mixture was cooled to 0 °C, and L-proline methyl ester (30 mg), triethylamine (30 mg), EDAC·HCl (51 mg), and DMAP (33 mg) were added sequentially. The mixture was allowed warm to room temperature over 30 min and then stirred for 18 h, with LCMS analysis indicating complete conversion to the desired products. The crude products were evaporated to dryness and resuspended in EtOAc, the slurry was filtered, and then the organic solubles were washed as above to yield the crude product (52 mg). The mixture was then hydrolyzed in EtOH−1 M NaOH (1:1) overnight at room temperature. The aqueous layer was acidified with 1 M HCl and extracted twice with EtOAc, and the organic layer was evaporated to dryness to yield the epimeric acids **1** and **8** (40 mg).

The two epimeric products were separated by RP HPLC using a Phenomenex Jupiter (10 μm, 21 × 250 mm) column, eluting with 45% CH_3CN-H_2O at a flow rate of 4 mL/min to give tumonoic acid D (**1**, 14.2 mg) and the unnatural epimer **8** (13.8 mg). Co-injections with natural **1** were used to confirm which was the natural isomer, using the following HPLC conditions: HP LiChrospher 100 RP-18 (5 μm, 4 × 125 mm) column, eluting with 40% CH_3CN-H_2O at a flow rate of 0.8 mL/min. Elution times (t_R, min): **1** (27.5), **8** (31.7).

A small subsample of each of the synthetic tumonoic acids was hydrolyzed at 110 °C in dioxane−6 M HCl (1:1) for 18 h. The hydrolysate was extracted with EtOAc, the organic layer reduced under vacuum, and the residue purified by HPLC using a Phenomenex Synergi-Hydro (4 μm, 21 × 250 mm) column, eluting with a gradient from 50% CH_3CN-H_2O to 100% CH_3CN at a flow rate of 4 mL/min. The optical rotations of the purified methyldecanoic acids were measured and compared with literature values.[15] Compound **1** yielded (2*S*)-2-methyldecanoic acid ($[\alpha]_D$ +12.3 ($CHCl_3$, *c* 0.26); lit. +5.1), while the unnatural epimer **8** gave (2*R*)-2-methyldecanoic acid ($[\alpha]_D$ −9.8 ($CHCl_3$, *c* 0.32); lit. −5.1).

***epi*-Tumonoic acid D (8):** colorless oil; $[\alpha]_D$ −120 (*c* 0.67, $CDCl_3$); IR (neat) ν_{max} 2958, 2930, 2857, 1736, 1642, 1601, 1468, 1450, 1333, 1191 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 9.26 (br s, COO*H*), 4.60 (d, *J* = 4.7 Hz, H-2′), 3.63 (br t, *J* = 8.6 Hz, H-5′a), 3.53 (ddd, *J* = 9.2, 8.7, 6.1 Hz, H-5′b), 2.57 (dq, *J* = 6.8, 6.6 Hz, H-2), 2.41 (m, H-3′a), 2.03 (m, H-3′b), 2.01 (m, H-4′, 1.67 (m, H-3a), 1.41 (m, H-3b), 1.28−1.25 (m, H-4 − H-9), 1.15 (d, *J* = 6.6 Hz, 2-Me), 0.87 (t, *J* = 6.8 Hz, H-10); ^{13}C NMR ($CDCl_3$, 75 MHz) δ_C 179.1 (C-1), 172.8 (C-1′), 59.9 (C-2′), 47.7 (C-5′), 38.1 (C-2), 33.5 (C-3), 31.8 (C-8), 29.6, 29.4, 29.2 (C-5−C-7), 27.5 (C-4), 27.4 (C-3′), 24.8 (C-4′), 22.6 (C-9), 17.2 (2-Me), 14.0 (C-10); ESI(+)MS *m*/*z* 284 $[M + H]^+$.

Biological Activity. All metabolites were tested for activity in antimalarial,[24] antileishmania,[25] anti-Chagas,[26] cytotoxicity (NCI-H460 lung tumor,[27,28] L1210, colon 38, H-116, H-125M, CEM, and CFU-GM cell lines[29]), antibacterial (MRSA),[30] and quorum sensing assays. Procedures for all assays except quorum sensing are given in the appropriate references.

Quorum sensing assays were carried out using marine broth (MB) containing 1 g of yeast extract and 5 g of peptone per liter of synthetic seawater (Instant Ocean). For agar plates, 15 g of agar (USB) per liter of H_2O was used. 4-Bromo-5-(bromomethylene)-2-(5*H*)-furanone was synthesized as reported previously.[31] The 3-oxohexanoylhomoserine lactone (OHHL) is commercially available (Sigma-Aldrich, lot # 102K3851).

Vibrio harveyi BB120 produces bioluminescence in response to the autoinducers HAI-1, AI-2, and CAI-1. *V. harveyi* was cultured in MB at 28 °C while shaking. An overnight culture of V. *harveyi* BB120 in MB was diluted to an OD_{600} of 0.1. An aliquot (100 μL) of the diluted culture was added to 5 mL of MB. Test compounds dissolved in DMSO were added to the wells of an opaque microtiter plate (Nunc A/S, Denmark; 0.0076 to 250 μM final concentration; 0.5% DMSO concentration; negative control = DMSO alone). The synthesized brominated furanone was utilized as a positive control for inhibition of luminescence (0.0076 to 250 μM). The diluted *V. harveyi* culture was added to the wells and mixed thoroughly. The plates were incubated for 4 h and read on a Packard Lumicount microtiter plate reader (Packard, UK). Relative luminescence units were normalized by the optical density (600 nm) values obtained by transferring 100 μL to a clear-bottom microtiter plate.

Escherichia coli JB525 is *E. coli* MT102 harboring the *gfp* plasmid pJBA132. This mutant produces an unstable green fluorescent protein in response to a C6−C8 *N*-acyl-homoserine lactone autoinducer. *E. coli* JB525 was cultured in LB4 broth at 30 °C. Inhibition of fluorescence was determined using a method modified from Anderson et al.[16] An overnight culture of *E. coli* JB525 in LB4 broth was diluted to an OD_{450} of 0.25 with fresh media. Test compounds dissolved in DMSO, or DMSO alone, were added to the wells of an opaque microtiter plate (Nunc A/S, Denmark; 32 nM OHHL; 0.0076 to 250 μM test compounds; 1% DMSO concentration). The diluted *E. coli* culture was added to the wells and mixed thoroughly. The plates were incubated with shaking at 30 °C for 90 min. Fluorescence was determined using a Packard Fluorocount microtiter plate reader (λ = 480 nm excitation, λ = 515 nm emission). Relative fluorescence values were normalized by optical density values obtained by transferring 100 μL to a clear-bottom microtiter plate (λ = 450 nm, SpectraMax Multimode microplate reader, Molecular Devices).

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Supporting Information Available: Additional phylogenetic trees using the full 1400-nucleotide sequences and truncated 599-nucleotide sequence. NMR spectra showing all ^{1}H NMR, ^{13}C NMR, gCOSY, gHSQC, and gHMBC spectra for tumonoic acids D−I (**1**−**6**). This material is available free of charge via the Internet at http://pubs.acs.org.

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