Aurilides B and C, Cancer Cell Toxins from a Papua New Guinea Collection of the Marine Cyanobacterium *Lyngbya majuscula*

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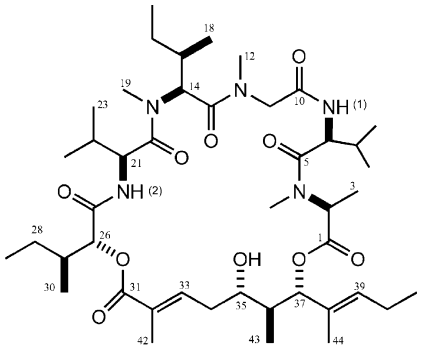
Cytotoxicity-guided fractionation of a strain of the marine cyanobacterium *Lyngbya majuscula* collected from Papua New Guinea led to the isolation of aurilides B (**1**) and C (**2**). The planar structures of **1** and **2** were established by spectroscopic analysis, including HR-FABMS, 1D ^{1}H and ^{13}C NMR, and 2D COSY, HSQC, HSQC-TOCSY, and HMBC spectra. The absolute configuration was determined by spectroscopic analysis and chiral HPLC analysis of acid hydrolysates of **1** and **2**. Both aurilides B and C showed in vitro cytotoxicity toward NCI-H460 human lung tumor and the neuro-2a mouse neuroblastoma cell lines, with LC_{50} values between 0.01 and 0.13 μM. Aurilide B (**1**) was evaluated in the NCI 60 cell line panel and found to exhibit a high level of cytotoxicity (the mean panel GI_{50} concentration was less than 10 nM) and to be particularly active against leukemia, renal, and prostate cancer cell lines.

Cyanobacteria are phenomenal producers of structurally intriguing and biologically active secondary metabolites,[1] including such important molecules as curacin A and the cryptophycins.[2,3] In our ongoing program to explore these organisms as sources of novel anticancer leads, we recently discovered dolabellin and lyngbyabellins E−I from this Papua New Guinea collection of the marine cyanobacterium *Lyngbya majuscula* Gomont (Oscillatoriaceae).[4] In addition, we have now identified two new cytotoxins from this collection, aurilides B (**1**) and C (**2**), which are closely related to aurilide (**3**),[5] originally isolated from the sea hare *Dolabella auricularia*. Herein, we describe the isolation, structure determination, and biological activities of aurilides B (**1**) and C (**2**).

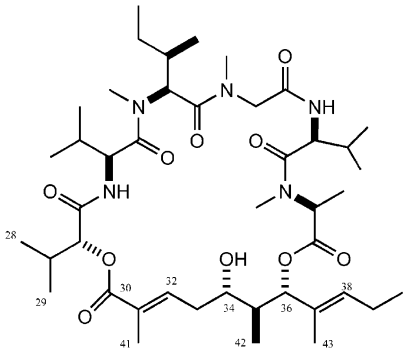
Results and Discussion

Collections of a shallow water (1−3 m) strain of *L. majuscula* were made from Alotau Bay, Papua New Guinea, in 2002. The alga was extracted with CH_2Cl_2/MeOH (2:1) and fractionated by silica gel vacuum liquid chromatography. A preliminary bioassay of the relatively polar EtOAc/hexanes eluted fraction showed toxicity in the brine shrimp model ($LD_{50} \approx 1$ ppm). Guided by this assay, this fraction was further chromatographed over a Mega Bond RP_{18} solid-phase extraction (SPE) cartridge and then reversed-phase HPLC to afford two new metabolites, aurilides B (**1**) and C (**2**).

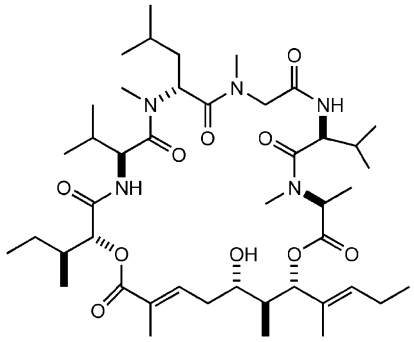
The molecular formula of aurilide B (**1**) was established as $C_{44}H_{75}N_5O_{10}$ on the basis of HR-FABMS [*m/z* 834.5603 $(M + H)^+$ (Δ −1.1 mmu)]. The ^{1}H NMR data showed the presence of two amide NH groups at δ 7.69 and 6.75 and three *N*-methylamide groups at δ 3.23, 2.88, and 2.63, suggesting the peptidic nature of **1**. Detailed analysis of the 2D NMR data permitted assignment of all signals from the proton and carbon NMR spectra (Table 1) and revealed a structural framework consisting of peptide and polyketide sections (substructures **a** and **b**, respectively) (Figure 1). Substructure **a** was composed of six amino acid residues on the basis of interpretation of 2D NMR data. Elucidation of the three *N*-methylated residues (*N*-methylglycine, *N*-methylalanine, and *N*-methylisoleucine) began with HMBC correlations from the α-proton



Aurilide B (**1**)



Aurilide C (**2**)



Aurilide (**3**)

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Table 1. NMR Data (400 MHz, C_6D_6) for Aurilides B (**1**) and C (**2**)

	aurilide B (**1**)			aurilide C (**2**)	
position	δ_C	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)
1	170.2			170.2	
2	58.9	3.23, m	1, 3, 4, 5	59.6	3.08, m
3	13.8	1.21, d (7.1)	1, 2	14.0	1.25, d (7.1)
4	36.1	2.63, s	2, 5	36.8	2.55, s
5	172.1			172.1	
6	54.3	5.12, dd (9.0, 7.4)	5, 7, 9	54.4	5.15, dd (9.0, 5.0)
7	31.0	1.97, m		32.0	1.98, m
8	20.1	1.15, d (7.0)	6, 7, 9	20.4	1.17, d (7.0)
9	17.3	1.25, d (7.0)	6, 7, 8	17.5	1.28, d (7.0)
10	169.9			170.11	
11	51.8	4.40, d (18.0)	10, 12, 13	51.9	4.39, d (18.0)
		3.80, d (18.0)			3.80, d (18.0)
12	36.8	3.23, s	11, 13	37.1	3.22, s
13	170.0			170.14	
14	58.6	5.24, d (10.0)	13, 18, 19, 20	58.7	5.26, d (10.0)
15	33.9	2.48, m	14, 16, 18	34.1	2.49, m
16	27.4	1.86, 1.30, m	14, 15, 17	27.6	1.89, 1.30, m
17	12.1	1.03, t (7.1)		12.2	1.03, t (6.9)
18	14.8	0.85, d (7.0)	15, 16	15.1	0.86, d (7.0)
19	30.7	2.88, s	20	30.6	2.85, s
20	173.1			173.2	
21	54.7	4.78, dd (8.8, 8.8)	20, 22	54.9	4.75, dd (8.6, 7.5)
22	31.7	1.98, m		31.0	1.95, m
23	18.1	0.89, d (6.0)	21, 22, 24	18.9	0.88, d (6.0)
24	20.2	0.90, d (6.0)	23	20.3	0.90, d (6.0)
25	170.3			170.3	
26	78.5	4.90, d (6.1)	25, 27, 31	80.4	4.54, d (7.5)
27	37.2	2.17, m	26, 30	30.5	2.36, m
28	26.1	1.50, 1.14, m	29	18.7	1.00, d (7.0)
29	11.8	0.83, t (7.7)	27, 28	18.4	0.88, d (7.0)
30	14.9	1.03, d (6.0)	26, 27, 28	169.7	
31	169.3			128.3	
32	128.0			146.0	7.75, t (9.0)
33	145.3	7.74, t (9.0)	31, 42	30.9	2.14, m
34	30.9	2.19, m	32, 33, 42	71.2	3.98, m
35	71.0	3.97, m	34	41.2	2.02, m
36	41.1	2.07, m	43	82.6	5.17, d (11.2)
37	82.5	5.18, d (11.2)	1, 36, 38, 44	132.1	
38	131.4			134.6	5.62, t (7.7)
39	134.2	5.61, t (7.7)	37, 44	21.4	1.95, 1.92, m
40	21.4	1.95, 1.92, m	38, 39, 41	14.3	0.89, t (obsc)
41	14.1	0.89, t (obsc)	39, 40	12.8	1.95, s
42	12.7	1.95, s	31, 32, 33	10.1	0.66, (7.0)
43	10.2	0.64, d (7.0)	35, 36, 37	11.4	1.54, s
44	11.3	1.54, s	37, 38, 39		
NH (1)		7.69 br, d (9.1)	10		7.66 br, d (9.1)
NH (2)		6.75 br, d (8.8)	25		6.70 br, d (8.8)

of each amino acid residue to the corresponding *N*-methyl carbon (Figure 1). Extension of the spin system of each residue by $^1H-^1H$ COSY (Figure 1) and HSQC-TOCSY experiments completed their construction. The presence of two valine residues was shown by $^1H-^1H$ correlations of their amide NH protons at δ 7.69 and 6.75 to the corresponding α-protons at δ 5.12 and 4.78. These in turn showed $^1H-^1H$ correlations to the appropriate remaining protons within each unit. A 2-hydroxyisoleucic acid residue (Hila) was suggested by a proton at δ 4.90 (H-26), which was attached to a downfield carbon at 78.5 ppm. TOCSY correlations linked the H-26 to H-30 spin system. The sequence of these six residues (two valines, *N*-methylglycine, *N*-methylalanine, *N*-methylisoleucine, and 2-hydroxyisoleucic acid residue) was deduced from HMBC correlations between H_3-4/C-5, NH(1)/C-10, H_3-12/C-13, H_3-19/C-20, and NH(2)/C-25 (Table 1) to generate substructure **a** (Figure 1).

Substructure **b** was elucidated as follows. COSY analysis connected proton signals from the olefinic proton H-33, via the allylic methylene protons at H_2-34 and H-35 oxymethine signal, to the methine proton at H-36, which in turn showed correlations to the methyl group at C-43 and the oxymethine proton at H-37 (Figure 1). A second spin system could be traced from CH_3-41 to the allylic methylene protons at H_2-40 and then to the other olefinic proton at H-39. HMBC long-range correlations between the resonance of H_3-44 and those of C-37, C-38, and C-39 revealed that CH_3-44 was attached to C-38, which in turn was connected to C-37 and C-39 (Figure 1). In a similar manner, the HMBC correlations observed between H_3-42 and C-31, C-32, and C-33 completed substructure **b** (Table 1). The *E*-geometry of both double bonds $\Delta^{32,33}$ and $\Delta^{38,39}$ was assigned on the basis of the ^{13}C NMR chemical shifts of the two methyl groups (CH_3-42 at δ 12.7 and CH_3-44 at δ 11.3).[6]

Substructures **a** and **b** were connected on the basis of HMBC data. The α-proton (H-26) of Hila showed a cross-peak to the C-31 carbonyl carbon of substructure **b**, and H-37 of **b** correlated with the C-1 carbonyl carbon of substructure **a** to complete a 26-membered ring (Figure 1).

Diagnostic NOEs from H_3-43 to H-35 and H-37, as well as the close similarity of ^1H and ^{13}C NMR shifts and $J_{H,H}$ values between **1** and the known compound aurilide (**3**), were indicative of the relative configuration of the polyketide portion of **1** as 35*S**, 36*S**, 37*S**. The absolute configuration of C-35 through C-37 was defined via the *S*- and *R*-MTPA esters of the C-35 hydroxyl group of **1**.[7] The $\Delta\delta_{(S-R)}$ values for H-33 through H-44 were indicative of a 35*S* configuration (Figure 2), and hence a 36*S*, 37*S* configuration was indicated as found for aurilide (**3**).

To assign the absolute configuration of the amino acid residues in aurilide B, **1** was hydrolyzed with acid and analyzed by chiral

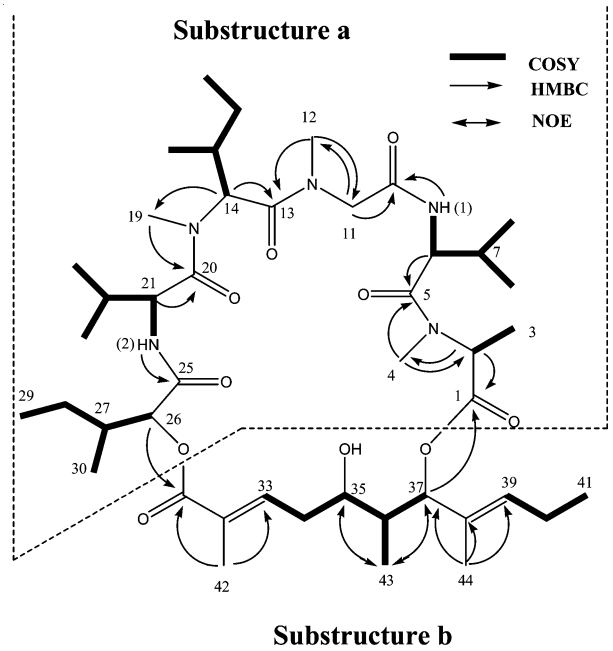

Figure 1. Key COSY, HMBC, and NOE correlations for **1**.

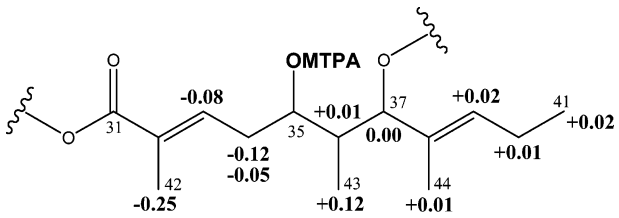

Figure 2. $\Delta\delta_{(S-R)}$ values (ppm) of MTPA esters of aurilide B (**1**) obtained in CD_3CN.

HPLC and chiral GC-MS analysis. The absolute configurations of the three components, Val, MeIle, and isoleucic acid, were determined to be L, allo-L, and allo-D, respectively. An additional portion of the hydrolysate of **1** was used to determine the absolute configuration of MeAla by Marfey analysis;[8] this residue was shown to be L, thus completing the structure of aurilide B (**1**).

Aurilide C (**2**) was isolated by RP-HPLC from the same fraction containing aurilide B (**1**). High-resolution FABMS analysis of aurilide C (**2**) revealed an $[M + H]^+$ ion (*m/z* 820.5449) consistent with a molecular formula of $C_{43}H_{74}N_5O_{10}$. Aurilide C had high structural homology to **1**, as evidenced by nearly identical ^1H and ^{13}C NMR chemical shifts for most of the molecule (Table 1). However, it displayed subtle differences for the isoleucic acid unit of **1**. 2D NMR and mass spectrometric analysis revealed that the isoleucic acid residue was replaced by a 2-hydroxyisovaleric acid residue (Hiva). Hydrolysis and stereoanalysis of the peptide portion of **2** were undertaken as described above for aurilide B (**1**). The absolute configurations of the four components, Val, MeAla, MeIle, and Hiva, were determined to be L-, L-, allo-L-, and D-, respectively. We propose that the polyketide portion is of the same configuration as that of **1** on the basis of their highly comparable spectroscopic features.

Aurilides B (**1**) and C (**2**) were tested for cytotoxicity to NCI-H460 human lung tumor and neuro-2a mouse neuroblastoma cells. Intriguingly, aurilide B was approximately 4-fold more toxic than C to these cell lines. The LC_{50} for aurilide B was 0.01 and 0.04 μM for neuro-2a and H460 cells, respectively, and 0.05 and 0.13 μM for aurilide C. Hence, aurilide B (**1**) was evaluated in the NCI 60 cell line panel and found to exhibit a high level of growth inhibition in leukemia, renal, and prostate cancer cell lines with a GI_{50} less than 10 nM (see Supporting Information). As a result, aurilide B (**1**) was evaluated in the NCI's hollow fiber assay, an in vivo model for assessing potential anticancer activity, and showed net tumor cell killing activity.[9] In the microfilament disruption assay, compound **1** induced loss of the microfilament network when tested against A-10 smooth muscle cells at 2.9 μM. However, given the compound's much more potent cytotoxic properties, it likely possesses multiple modes of cytotoxic action. Given its profile of potent in vitro and in vivo cytotoxicity to cancer cells, aurilide B adds to the growing list of promising marine-derived clinical and preclinical anticancer lead compounds.[10]

Biosynthetically, the aurilides likely derive from an assembly by nonribosomal polypeptide synthetases (NRPS) and polyketide synthases (PKS),[1] and thus, the structural variation of the aurilides might be due to adenylation domains with relaxed substrate specificities. Recently, another cytotoxic depsipeptide, kulokekahilide-2,[11] was isolated from a cephalaspidean mollusk and is also closely related to aurilide (**3**). Observation that identical metabolites occur in unrelated genera of marine invertebrates, as well as the isolation of closely related compounds from microbial sources, provides further evidence that many natural products are actually produced by dietary or symbiotic sources rather than by the invertebrate associants.[12,13] For example, the isolation here of aurilides B (**1**) and C (**2**) from the marine cyanobacterium *L. majuscula* clearly indicates that the sea hare *D. auricularia* obtains and accumulates aurilide (**3**)[5] from its diet of marine cyanobacteria.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR and UV spectra were recorded on Nicolet 510 and Beckman DU640B spectrophotometers, respectively. NMR spectra were recorded on Bruker Avance DPX 400 MHz and Bruker Avance DRX 300 MHz spectrometers with the solvent C_6D_6 (δ_H at 7.16, δ_C at 128.4) or CD_3CN (δ_H at 1.94, δ_C at 118.3) used as an internal standard. High-resolution mass spectra were recorded on a Kratos MS-50 TC mass spectrometer. Chiral GC-MS analysis was accomplished on a Hewlett-Packard gas chromatograph 5890 Series II with a Hewlett-Packard 5971 mass selective detector using an Alltech capillary column (Chirasil-VAL phase 25 m × 0.25 mm). HPLC was performed using Waters 515 HPLC pumps and a Waters 996 photodiode array detector. Chemical standards were obtained from Sigma-Aldrich except for isoleucic acid, which was a gift of the R. E. Moore laboratory, University of Hawaii, Manoa.

Collection. The marine cyanobacterium *Lyngbya majuscula* (voucher specimen available from WHG as collection number PNG5-27-02-1) was collected from shallow waters (1−3 m) in Alotau Bay, Papua New Guinea, on May 7, 2002. The material was stored in 2-propanol at −20 °C until extraction.

Extraction and Isolation. Approximately 138 g (dry wt) of the alga was extracted repeatedly with CH_2Cl_2/MeOH (2:1) to produce 3.05 g of crude organic extract. The extract (3.0 g) was fractionated by silica gel vacuum liquid chromatography using a stepwise gradient solvent system of increasing polarity starting from 10% EtOAc in hexanes to 100% MeOH. The fraction eluting with 90% EtOAc was found to be active at 1 ppm in the brine shrimp toxicity assay. This fraction was further chromatographed on Mega Bond RP_{18} solid-phase extraction (SPE) cartridges using a stepwise gradient solvent system of decreasing polarity starting with 80% MeOH in H_2O and ending with 100% MeOH. The most active fractions after SPE (85% toxicity at 1 ppm to brine shrimp) were then purified by HPLC [Phenomenex Sphereclone 5 μ ODS (250 × 10 mm), 9:1 MeOH/H_2O, detection at 211 nm], giving compounds **1** (80.5 mg) and **2** (5.1 mg).

Aurilide B (1): colorless, amorphous solid; $[\alpha]^{24}_D$ −17 (*c* 0.34, MeOH); UV (MeOH) λ_{max} 222 nm (log ϵ 4.65); IR (neat) 3481 (br), 3353 (br), 2964, 1740, 1687, 1646, 1251, 1205 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR FABMS *m/z* $[M + H]^+$ 834.5603 (calcd for $C_{44}H_{76}N_5O_{10}$, 834.5592).

Aurilide C (2): colorless, amorphous solid; $[\alpha]^{24}_D$ −19 (*c* 0.39, MeOH); UV (MeOH) λ_{max} 222 nm (log ϵ 4.56); IR (neat) 3487 (br), 3350 (br), 2962, 1740, 1687, 1647, 1251, 1207 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR FABMS *m/z* $[M + H]^+$ 820.5449 (calcd for $C_{43}H_{74}N_5O_{10}$, 820.5436).

MTPA Esters of 1. Two portions of aurilide B (**1**, 0.5 mg each) were reacted with *R*- or *S*-MTPACl (10 μL) in 300 μL of CH_2Cl_2 containing 10 mg of DMAP. The reaction mixtures were partitioned with EtOAc/0.1 M $NaHCO_3$, and the EtOAc layers washed with 0.1 M HCl and H_2O. The EtOAc layer was evaporated and then separated by ODS HPLC [Phenomenex Sphereclone 5 μ ODS (250 × 10 mm), 9:1 MeOH/H_2O, detection at 220 nm] to yield *S*- and *R*-MTPA esters.

S-MTPA ester: ^{1}H NMR (300 MHz, CD_3CN) δ 7.20 (H-33), 2.55 (H-34a), 2.19 (H-34b), 5.83 (H-35), 2.38 (H-36), 5.05 (H-37), 5.51 (H-39), 2.05 (H_2-40), 0.95 (H_3-41), 1.58 (H_3-42), 0.85 (H_3-43), 1.61 (H_3-44); FABMS *m*/*z* 1050.7 $(M + H)^+$.

R-MTPA ester: ^{1}H NMR (300 MHz, CD_3CN) δ 7.28 (H-33), 2.67 (H-34a), 2.24 (H-34b), 5.80 (H-35), 2.37 (H-36), 5.05 (H-37), 5.49 (H-39), 2.04 (H_2-40), 0.93 (H_3-41), 1.83 (H_3-42), 0.73 (H_3-43), 1.60 (H_3-44); FABMS *m*/*z* 1050.7 $(M + H)^+$.

Absolute Configuration of the Peptide Portion of Aurilide B (1). Aurilide B (**1**, 0.3 mg) was hydrolyzed in 6 N HCl at 110 °C for 24 h, then dried under a stream of N_2 and further dried under vacuum. The residue was reconstituted with 300 μL of H_2O prior to chiral HPLC analysis. Mobile phase I: 2 mM $CuSO_4$ in MeCN/H_2O (5:95), flow rate 1 mL/min [Phenomenex Chirex 3126 (D), 4.6 × 250 mm; detection at 254 nm]. Mobile phase I elution times (t_R, min) of authentic standards: allo-L-MeIle (16.8), L-MeIle (17.4), allo-D-MeIle (22.2), D-MeIle (23.0).

Mobile phase II: 2 mM $CuSO_4$ in MeCN/H_2O (15:85), flow rate 1 mL/min [column, Phenomenex chirex 3126 (D), 4.6 × 50 mm; detection at 254 nm]. Mobile phase II elution times (t_R, min) of authentic standards: allo-L-isoleucic acid (8.2), L-isoleucic acid (11.5), allo-D-isoleucic acid (14.2), d-isoleucic acid (18.2).

The hydrolysate of **1** was analyzed by HPLC as detailed above, both alone and by co-injection with standards, to confirm the assignments of allo-L-MeIle and allo-D-isoleucic acid. Following appropriate derivatization, the presence of 2 equiv of L-Val was confirmed by chiral GC/MS.[3]

The configuration of MeAla was determined by Marfey's analysis.[7] A portion of the hydrolysate was evaporated to dryness and resuspended in H_2O (100 μL). A 0.1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide solution in acetone (L-Marfey's reagent, 20 μL) and 1 N $NaHCO_3$ (10 μL) were added to a portion of the hydrolysate, and the mixture was heated at 40 °C for 1 h. The solution was cooled to room temperature, neutralized with 2 N HCl (5 μL), and evaporated to dryness. The residue was resuspended in H_2O (50 μL) and analyzed by reversed-phase HPLC [Microsob-MV C_{18}, 4.6 × 250 mm, UV detection at 340 nm] using a linear gradient of 9:1 50 mM triethylamine phosphate (TEAP) buffer (pH 3.1)/CH_3CN to 1:1 TEAP/CH_3CN over 60 min. The derivatized MeAla residue in the hydrolysate of **1** eluted at the same retention time as the derivatized standard L-MeAla (12.0 min) but not that of D-MeAla (13.7 min).

Absolute Configuration of the Peptide Portion of Aurilide C (2). The absolute configuration of aurilide C (**2**) was analyzed as described above for aurilide B (**1**). Allo-L-MeIle (16.8 min) and d-Hiva (9.2 min) were assigned to aurilide C (**2**) by chiral HPLC; L-MeAla (12.0 min) was determined on the basis of Marfey's analysis. The presence of 2 equiv of L-Val was confirmed by chiral GC/MS.[3]

Biological Activity. Brine shrimp (*Artemia salina*) toxicity was measured as previously described.[14] After a 24 h hatching period, aliquots of 10 mg/mL stock solutions of sample were added to test wells containing 5 mL of artificial seawater and brine shrimp to achieve a range of final concentrations from 0.1 to 100 ppm. After 24 h the live and dead shrimp were tallied.

Cytotoxicity was measured in NCI-H460 human lung tumor cells and neuro-2a mouse neuroblastoma cells using the method of Alley et al.,[15] with cell viability being determined by MTT reduction.[16] Cells were seeded in 96-well plates at 6000 cells/well in 180 μL of medium. Twenty-four hours later, the test chemicals were dissolved in DMSO and diluted into medium without fetal bovine serum and then added at 20 μg/well. DMSO was less than 0.5% of the final concentration. After 48 h, the medium was removed and cell viability determined.

Aurilide B (**1**) was tested for microfilament-disrupting activity using rhodamine-phalloidin. A-10 cells were grown on glass coverslips in Basal Medium Eagle (BME) containing 10% fetal calf serum. The cells were incubated with the test compound for 24 h and then fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 2 min, and chemically reduced with $NaBH_4$ [1 mg/mL in phosphate-buffered saline (PBS)] three times for 5 min each. Following a 45 min incubation with 100 nM TRITC-phalloidin in PBS (to visualize the actin cytoskeleton), the coverslips were washed, stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize DNA, mounted on microscope slides, and examined and photographed using a Nikon E800 Eclipse fluorescence microscope with a Photometrics Cool Snap FX3 camera. The images were colorized and overlayed using Metamorph software.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra in C_6D_6 for aurilide B (**1**) and ^{1}H and ^{13}C NMR for aurilide C (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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