Tanikolide, a Toxic and Antifungal Lactone from the Marine Cyanobacterium *Lyngbya majuscula*

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A new brine-shrimp toxic and antifungal compound, tanikolide **1**, has been isolated from the lipid extract of a Madagascan collection of the marine cyanobacterium *Lyngbya majuscula*. The structure of tanikolide was determined by spectroscopic methods, relying heavily on 2D NMR spectroscopy. The absolute configuration at C-5 of tanikolide was established as *R* by oxidizing the primary alcohol to an acid and analyzing the corresponding (*R*)- and (*S*)-PGME amide derivatives by ^{1}H NMR.

Ongoing efforts in our laboratory[1−5] and elsewhere[6] have demonstrated that cyanobacteria (blue-green algae) are an exciting source of novel bioactive natural products. Many structurally diverse metabolites have been isolated from this group of photosynthetic microorganisms, and these exhibit a rich variety of biological activities. Of all the marine cyanobacteria, *Lyngbya majuscula* Gomont (Oscillatoriaceae) has been the richest source, yielding more than 100 different secondary metabolites, nearly half of which are of a lipopeptide nature. For example, from a single Curaçao collection of *L. majuscula*, we isolated curacin A (cytotoxic),[1] malyngamide H (ichthyotoxic),[2] barbamide (molluscicidal)[3], antillatoxin (ichthyotoxic),[4] and the carmabins.[5] In continuation of our chemical studies of tropical *Lyngbya* collections, we have investigated the lipid extract of *L. majuscula* collected from Tanikeli Island, Madagascar. This collection yielded an extract that possessed strong brine shrimp toxicity (100% at 10 ppm). Brine shrimp bioassay-guided fractionation resulted in the isolation of a new toxic and antifungal metabolite, tanikolide (**1**), which was characterized by various spectroscopic methods and chemical derivatization.

Using a brine shrimp bioassay to guide the process, the lipid extract of *L. majuscula* collected from Madagascar was fractionated (see Experimental Section) to yield a new toxin, tanikolide (**1**, 70 mg, 8.1% of crude extract). High-resolution mass measurement of **1** defined a molecular formula of $C_{17}H_{32}O_3$. The CI/MS gave an $[M + H]^+$ peak at *m*/*z* 285 and a base peak at *m*/*z* 253, indicating the facile loss of a $-CH_2OH$ fragment. The IR spectrum indicated the presence of an ester or a lactone (1715 cm^{-1}) and a primary alcohol (3425, 1050 cm^{-1}). The two degrees of unsaturation implied by the formula were accounted for by the presence of one carbonyl (δ171.6) and one ring.

The ^{13}C NMR and DEPT spectrum of **1** indicated the presence of 17 carbons and 31 carbon-bound hydrogens. These were present as a carbonyl of an ester or a lactone, a methylene carbon attached to an oxygen (δ 67.5), a quaternary carbon attached to an oxygen (δ 86.4), a methyl group (δ 14.1), and 13 highfield methylene carbons. A survey of ^{13}C NMR shifts for γ- and δ-lactone rings indicated that the ring in **1** was six-membered (e.g., C=O for δ lactones at δ 171; C=O for γ lactones at δ 177; C-1 in **1** at δ 171.6).[7]

The ^{1}H NMR of **1** showed the presence of a pair of double doublets (δ 3.66 and 3.56) for a methylene attached to oxygen and a two-proton multiplet (δ 2.48) assigned to a methylene adjacent to carbonyl group. Five methylene groups appeared as multiplets (see Experimental Section) and another broad singlet (δ 1.26) accounted for 14 unresolved protons. A three-proton triplet at δ 0.88 was assigned to a terminal methyl group.

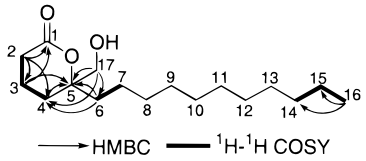

Figure 1. Summary of important connectivities observed in **1** by HMBC and ^{1}H−^{1}H COSY.

The structure of tanikolide (**1**) was established by using ^{1}H−^{1}H COSY and HMBC experiments as shown in Figure 1. An oxymethylene at δ 3.66/3.56 showed HMBC correlations with carbons at δ 86.4 (C-5), 26.6 (C-4), and 36.6 (C-6), whereas the methylene protons at δ 2.48 showed HMBC correlations with carbons at δ 171.6 (C-1), 16.6 (C-3), and 26.6 (C-4). ^{1}H−^{1}H COSY delineated a connected spin system for methylenes at positions 2, 3, and 4, which, taken together with the above HMBC correlations, confirmed the presence of a six-membered lactone with substitutions at C-5. A combination of ^{1}H NMR, ^{13}C NMR, and DEPT spectra suggested that the two substituents at C-5 were a hydroxymethylene group and a saturated alkyl chain of 11 carbons. The length of alkyl chain was confirmed by CIMS, which showed a diagnostic fragment peak at *m*/*z* 129 (10%, $M - C_{11}H_{23}$). A methyl group signal at δ 0.88 showed HMBC correlation with two methylene carbons at δ 22.6 (C-15) and 31.8 (C-14), a sequence confirmed by ^{1}H−^{1}H COSY analysis. The above data led to the formulation of three distinct spin systems; a δ lactone, a hydroxymethylene group, and an undecyl chain. These three groups were unequivocally connected through the C-5 quaternary carbon by HMBC correlations (Figure 1).

The absolute configuration at C-5 was determined by using newly developed methods for determining the absolute stereochemistry of tertiary carboxylic acids. A recent report has shown that the anisotropic effects of chiral reagents can be used to determine the absolute configuration of tertiary carboxylic acids via their amide derivatives.[8] Oxidation of tanikolide with pyridinium dichromate gave tanikolide acid (**2**) in high yield.[9] Both the (*R*)-amide (**3**) and (*S*)-amide (**4**) of tanikolide acid (**2**) were prepared using (*R*)- and (*S*)-phenylglycinyl methyl ester hydrochlo-

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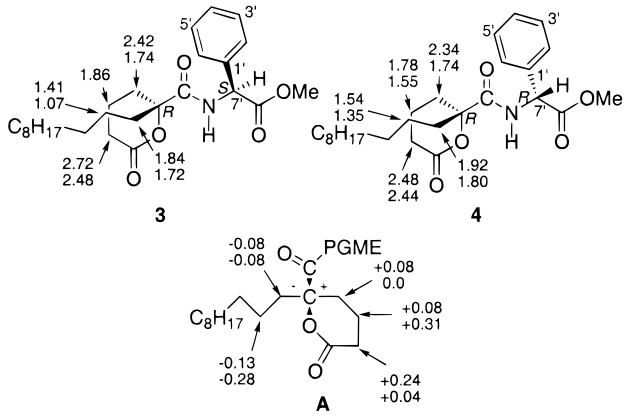
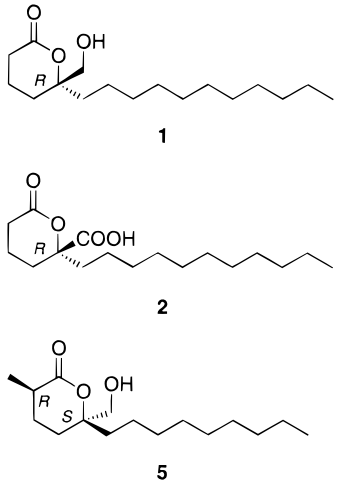


Figure 2. Selected ^{1}H NMR data for (*S*)- and (*R*)-PGME amide derivatives of tanikolide (**3**, **4**).

ride and purified by HPLC.[10] GC−MS of these two amide derivatives showed M^+ values at *m*/*z* 445, which confirmed their formation. All of the protons of these two derivatives were assigned using 2D NMR experiments, including HSQC and HMBC. Selected ^{1}H NMR ($CDCl_3$, 600 MHz) data for the (*S*)-amide (**3**) and (*R*)-amide (**4**) are shown in Figure 2. A model summarizing ^{1}H NMR data from these two derivatives in which substituents having positive $\Delta\delta$ ($\delta_S-\delta_R$) values were placed on the right-hand side and those with negative $\Delta\delta$ ($\delta_S-\delta_R$) values were placed on the left-hand side,[10] proved that the absolute configuration at C-5 was *R* (**A**, Figure 2). However, reaction of **2** with (*S*)-PGME yielded a minor product (ca. 10% of the major amide product) that was chromatographically indistinguishable from the (*R*)-amide (HPLC, GC−EIMS) and vice versa, indicating that tanikolide was partially racemic (see Experimental Section).

Tanikolide was tested for brine shrimp toxicity (*Artemia salina*), molluscicidal activity (*Biomphalaria glabrata*), and ichthyotoxicity (*Carassius auratus*). It showed an LD_{50} of 3.6 μg/mL against brine shrimp and 9.0 μg/mL against the snail. Although it did not show overt toxicity to goldfish, a narcotic effect was observed at 10 μg/mL. To the fungus *Candida albicans*, tanikolide gave a 13-mm diameter zone of inhibition (100 μg/disk) using paper disk−agar plate methodology.[11] To date, only one compound of a related nature, malyngolide (**5**), has been reported from *L. majuscula*.[12] Interestingly, the stereoconfiguration at C-5 in malyngolide (2*R*,5*S*) is opposite that of tanikolide (5*R*). (−)-Malyngolide shows no activity to *C. albicans*.



Experimental Section

General Experimental Procedures. UV spectra were recorded on a Hewlett−Packard 8452A diode array spectrophotometer and IR on a Nicolet 510 spectrophotometer. NMR spectra were recorded on a 600 MHz (Bruker DRX 600) or a 300 MHz (Bruker AC 300) spectrometer in $CDCl_3$; ^{13}C NMR was recorded at 75 MHz (Bruker AC 300). All chemical shifts are reported relative to TMS as internal standard. LRMS were obtained using a Hewlett−Packard GC (model 5810) connected to a Hewlett−Packard mass selective detector (model 5971), while HRMS were obtained on a Kratos MS 50 TC. Optical rotations were measured with a Perkin−Elmer model 141 polarimeter. HPLC was performed using a Waters 6000A pump and either a R 401 differential refractometer or a Lambda-Max 480 lc spectrophotometer. TLC grade (10−40 μm) Si gel was used for vacuum chromatography, and Merck aluminum-backed TLC sheets (Si gel 60 F_{254}) were used for TLC.

Biological Material. The marine cyanobacterium *L. majuscula* (voucher specimen available from W.H.G. as MNT-6 Apr 97−01) was collected by hand from shallow water (20 ft) on 6 April 1997, at Tanikeli Island, Madagascar, and stored in 2-propanol at low temperature until workup.

Extraction and Isolation. A total of 1 kg (dry wt) of the alga was extracted twice with CH_2Cl_2−MeOH (2:1) to yield 940 mg of crude extract. A portion of this (860 mg) was fractionated using vacuum liquid chromatography (VLC) on Si gel by a stepwise gradient of hexane−EtOAc and EtOAc−MeOH to give 11 fractions of 200 mL each. Fraction 4 (229 mg, hexanes−EtOAc, 1:1) showed brine shrimp toxicity at 10 ppm and was further subjected to VLC on Si gel (CH_2Cl_2−MeOH) to yield six fractions (4A−4F, 100 mL each). Fraction 4C was purified by HPLC (Versapak Si 10 μ, 300 × 4.1 mm, 1 mL/min, RI detection, hexanes−EtOAc−IPA, 80:15:5) to yield 70 mg (8.1% of crude extract) of tanikolide (**1**, t_R = 22 min) as a colorless oil.

Tanikolide (1): $[\alpha]^{25}_D$ +2.3° (*c* 0.65, $CHCl_3$); ORD $[\alpha]_{589}$ +1.35°, $[\alpha]_{578}$ +0.9°, $[\alpha]_{546}$ +1.25°, $[\alpha]_{436}$ −210°, and $[\alpha]_{365}$ +0.7° (*c* 0.2, $CHCl_3$); no UV absorbance; IR ν_{max} (neat) 3425, 1715, 1250, 1050, 940 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 3.66 (1H, dd, *J* = 12, 6.4 Hz, H-17), 3.56 (1H, dd, *J* = 12, 6.4 Hz, H-17), 2.48 (2H, m, H-2), 1.94−1.85 (3H, m, H-3, H-4a), 1.75−1.71 (2H, m, H-4b, H-6a), 1.63 (1H, m, H-6b) 1.34 (1H, m, H-7), 1.29 (1H, m, H-7), 1.28 (2H, m, H-15), 1.26 (14H, bs, H-8−14), 0.88 (3H, t, *J* = 7.1 Hz, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ171.6 (s, C-1), 86.4 (s, C-5), 67.5 (t, C-17), 36.6 (t, C-6), 31.8 (t, C-14), 29.7 (t, C-2), 29.9 t, 29.6 t, 29.5 t, 29.5 t, 29.4 t, 29.3 t (C-8−13), 26.6 (t, C-4), 23.4 (t, C-7), 22.6 (t, C-15), 16.6 (t, C-3), 14.1 (q, C-16); GC−EIMS (70 eV) t_R = 7.91 min, obs *m*/*z* (rel int) 253 (48), 169 (100), 154 (81), 57 (37); LRCIMS (CH_4, pos. ion) obs *m*/*z* (rel int) 285 $[M + H]^+$ (6), 268 (7), 267 (33), 254 (18), 253 (100), 249 (15), 231 (7), 225 (25), 129 (10), 123 (5), 109 (6), 95 (7); HRCIMS obs $[M + H]^+$ *m*/*z* 285.2430 for $C_{17}H_{33}O_3$ (−0.3 mmu).

Oxidation of Tanikolide (1) with Pyridinium Dichromate. A solution of tanikolide (8.5 mg, 30 μmol) in DMF (200 μL) and pyridinium dichromate (51 mg, 135 μmol, 4.5 equiv) in DMF (500 μL) was stirred for 10 h. The reaction mixture was diluted with H_2O (5 mL) and extracted with Et_2O (3 × 5 mL). The combined organic extracts were washed with H_2O and dried over anhydrous Na_2SO_4. The solvent was evaporated to yield pure tanikolide acid **2** (7.0 mg): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.85 (s), 2.65−2.37 (2H, m), 2.22 (1H, m), 1.87 (4H, m), 1.43 (1H, m), 1.25 (18H, brs), 0.88 (3H, t, *J* = 6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 195.8, 170.4, 38.9, 32.1, 30.5, 29.8, 29.6, 29.1, 23.3, 22.9, 17.4, 14.3; GC−EIMS (of methyl ester derivative, CH_2N_2) t_R = 9.67 min, obs *m*/*z* 312 $[M]^+$ (0.5), 280 (4), 253 (100), 225 (40), 155 (10), 97 (16), 71 (10), 55 (24).

Condensation of Tanikolide Acid (2) with (*S*)-PGME. To a stirred solution containing a mixture of **2** (3.0 mg, 10 μmol) and (*S*)-PGME (2.0 mg, 10 μmol) in DMF (1.0 mL) were successively added PyBOP (5.2 mg, 10 μmol), HOBT(1.4 mg, 10 μmol), and *N*-methylmorphine (3.5 μL, 30 μmol) at 0 °C.

After the mixture was stirred at room temperature for 1.5 h, C_6H_6 (5 mL) and EtOAc (10 mL) were added, and the resulting solution was washed with H_2O and a saturated solution of NaCl. The organic phase was dried (anhyd Na_2SO_4) and concentrated in vacuo to yield a crude product (5.5 mg) that was subjected to HPLC (Versapak Si 10 μ, 300 × 4.1 mm, 2 mL/min, UV detection at 254 nm, hexane−EtOAc, 1:1) to yield **3** (1.5 mg, t_R= 7.0 min) and *ent*-tanikolide amide (150 μg, t_R= 6.0 min). Derivative **3**: IR ν_{max} (neat) 3425, 3340, 1751, 1685, 1506, 1230, 1040, 947 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.39−7.32 (5H, m, Ar−H), 7.18 (1H, d, J = 5.6 Hz, NH, $\Delta\delta$ 1.35 ppb/°C indicating it is H-bonded), 5.47 (1H, d, J = 5.6 Hz, H-7′), 3.73 (3H, s, OMe), 2.72 (1H, dt, J = 15.2, 3.5 Hz, H-2a), 2.48 (1H, dt, J = 15.2, 7.0 Hz, H-2b), 2.42 (1H, dt, J = 11.6, 3.9 Hz, H-4a), 1.86 (2H, m, H-3), 1.84 (1H, m, H-6a), 1.74 (1H, m, H-4b), 1.72 (1H, m, H-6b), 1.41 (1H, m, H-7a), 1.29−1.16 (16H, m, H-8−15), 1.07 (1H, m, H-7b), 0.88 (3H, t, J = 6 Hz, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ171.7 (C-17), 170.6 (COOMe), 170.0 (C-1), 135.3 (C-1′), 129.2 (C-3′, 5′), 129.0 (C-4′), 127.5 (C-2′, 6′), 88.3 (C-5), 56.9 (C-7′), 52.8 (OMe), 39.5 (C-6), 31.9 (C-14), 30.0 (C-4), 29.6 (C-2), 29.3 (C-8−13), 23.0 (C-7), 22.7 (C-15), 16.9 (C-3), 14.1 (C-16); GC−EIMS (70 eV) t_R = 20.62 min, m/z obs. 445 [M]$^+$ (1.5), 386 (100), 291 (28), 253 (92), 225 (50), 164 (42), 106 (90), 55 (48).

Condensation of Tanikolide acid (2) with (*R*)-PGME. Compound **2** was condensed with (*R*)-PGME under the same conditions as above to yield **4** (1.5 mg, t_R = 6.0 min) and *ent*-tanikolide amide (150 μg, t_R= 7.0 min). Derivative **4**: IR ν_{max} (neat) 3421, 3348, 1754, 1685, 1504, 1245, 1051, 931 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.38−7.31 (6H, m, Ar−H and NH), 5.52 (1H, d, J = 6 Hz, H-7′), 3.85 (3H, s, OMe), 2.48 (1H, dt, J = 14.9, 4.0 Hz, H-2a), 2.44 (1H, m, H-2b), 2.34 (1H, dt, J = 10.7, 3.5 Hz, H-4a), 1.92 (1H, td, J = 11.0, 3.6 Hz, H-6a), 1.80 (1H, td, J = 11.0, 3.6 Hz, H-6b), 1.78 (1H, m, H-3a), 1.74 (1H, m, H-4a), 1.55 (1H, m, H-3b), 1.54 (1H, m, H-7a), 1.35 (1H, m, H-7b), 1.26 (16H, s, H-8−15), 0.88 (3H, t, J = 6 Hz, H-16); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.7 (C-17), 170.4 (COOMe), 169.8 (C-1), 135.9 (C-1′), 129.1 (C-3′, 5′), 128.8 (C-4′), 127.2 (C-2′, 6′), 88.2 (C-5), 56.6 (C-7′), 52.8 (OMe), 39.4 (C-6), 31.9 (C-14), 29.8 (C-2), 29.6 (C-4), 29.6−29.3(C-8−13), 22.9 (C-7), 22.7 (C-15), 16.9 (C-3), 14.1 (C-16); GC−EIMS (70 eV) t_R = 20.86 min, obs m/z 445 [M]$^+$ (1.2), 386 (95), 291 (33), 253 (100), 225 (55), 164 (45), 106 (87), 55 (50).

Brine Shrimp Toxicity Assay. The screening for brine shrimp toxicity of the crude extract, chromatography fractions, and pure compound was performed by a slight modification of the original method.[13] About 15 hatched brine shrimp (*Artemia salina*) in ca. 0.5 mL seawater were added to each well containing different concentrations of sample in 50 μL of EtOH and 4.5 mL of artificial seawater to make a total volume of ca. 5 mL. Samples and controls were run in duplicate. After 24 h at 28 °C, the number of alive and dead brine shrimp were counted under a dissecting light microscope.

Molluscicidal Assay. Molluscicidal assays were performed as previously detailed using the test organism *Biomphalaria glabrata*.[14] Appropriate amounts of sample stock solutions (10 mg/mL in EtOH) were diluted in 10 mL of distilled H_2O. The snails were observed after 24 h and considered dead when no heart beat could be detected by dissecting light microscopy.

Ichthyotoxicity Assay. A modification of the previously described method was used to assess toxicity to the goldfish *Carassius auratus*.[15] Dilutions of samples in 50 μL of EtOH were added to 50 mL of distilled H_2O in a 100-mL beaker. A single goldfish was added and observed for 1 h. Sample and controls were run in duplicate.

Antimicrobial Assay. Antimicrobial activity of tanikolide was evaluated using standard paper sensitivity disk−agar plate methodology (disk diameter, 6 mm).[11] Although tanikolide was found to give a 13-mm zone of inhibition at 100 μg/disk and a slight halo at 10 μg/disk to *Candida albicans* (ATCC 14053), it was inactive to *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC 11775), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 14028), *Bacillus subtilis* (ATCC 6051), and *Staphylococcus aureus* (ATCC 12600). (−)-Malyngolide obtained from a different collection of *L. majuscula* showed no antimicrobial activity to any of these test organisms at a concentration of 100 μg/disk.

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