Two New Malyngamides from a Madagascan *Lyngbya majuscula*

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The lipid extract of a Madagascan *Lyngbya majuscula* has yielded malyngamides Q and R, both amides of 7-methoxytetradec-4-enoic acid. The isolation of these metabolites was accomplished using preparative liquid chromatography, with final purification through repetitive reversed-phase HPLC. Structure elucidation was accomplished utilizing 1D and 2D NMR spectroscopic characterization of the natural products and comparisons with malyngamides A and B. DPFGSE 1D NOE data suggested a different geometrical stereochemistry at C-6 in malyngamides Q and R from that observed for malyngamide A, as well as the other known malyngamides. The *Z* stereochemistry was confirmed for malyngamide R by measurement of key diagnostic $^3J_{CH}$ couplings utilizing the HSQMBC pulse sequence. The absolute stereochemistry of C-4″ of the pyrrolidone ring was defined by chiral GCMS analysis of serine released by ozonolysis and acid hydrolysis.

The marine cyanobacterium *Lyngbya majuscula* Gomont (Oscillatoriaceae) occurs pantropically and is a prolific producer of many interesting secondary metabolites, yielding no fewer than 100 reported compounds. Many of these metabolites possess interesting biological activities, such as microcolin A (antiproliferative/immunosuppressant),[1] ypaoamide (antifeedant),[2] and barbamide (molluscicidal).[3] For others, despite their intriguing structures, significant biological properties have not yet been detected. The malyngamides, a well-represented class of *L. majuscula* natural product with more than 16 members, are examples of this latter group. These have been isolated from *L. majuscula* collected in many distinct marine habitats and from locations spanning the globe in tropical locations. In this regard, we have previously identified malyngamides H,[4] I,[5] J,[6] K,[6] and L[6] from Caribbean and Indonesian samples. We now report the isolation and structure elucidation of malyngamides Q and R, components of the organic extract of a shallow-water Madagascan *Lyngbya majuscula*.

Results and Discussion

Malyngamide Q (**1**) was isolated as a pale yellow oil from the lipid extract of *L. majuscula*, collected near Sakatia Island, Madagascar. HRFABMS showed an $[M + H]^+$ ion at *m/z* 569.3053 consistent with a molecular formula $C_{29}H_{46}O_7N_2Cl$, indicating a structure with eight degrees of unsaturation. The IR spectrum possessed absorptions for NH or OH protons (3313 cm^{-1}) and amide carbonyl groups (1713, 1630 cm^{-1}). A UV maximum was observed at 264 nm, suggestive of one or more conjugated enone systems.

Diagnostic resonances in the ^1H NMR spectra strongly suggested that compound **1** was a metabolite of the malyngamide class. In particular, resonances assignable to an aliphatic carbon chain, an exomethylene possessing a vinyl chloride (^1H δ 6.03; ^{13}C δ 118.28), three methoxy groups, a disubstituted olefin (^1H δ 5.40 2H; ^{13}C δ 127.33 and 130.58), and five resonances in the amide/ester region of the carbon spectrum (see Table 1), were consistent with structural features of this compound class. Additionally, a pair of unusual proton shifts (^1H δ 6.88 and 5.10) were found to correlate to two equally intriguing carbon

Table 1. NMR Data for Malyngamides Q (**1**) and R (**2**)[a]

	malyngamide Q (**1**)			malyngamide R (**2**, major confomer)	
C-atom	^1H ppm (mult, *J* in Hz)	13C (ppm)	C-atom	^1H ppm (mult, *J* in Hz)	13C (ppm)
1		165.61	1		164.52
2	6.88 (s)	95.38	2	6.82 (s)	95.10
3		171.53	3		170.90
4a	3.07 (d,14.5)	36.55	4a	2.90 (d, 14.7)	36.71
b	4.16 (d, 14.5)		b	4.44 (m)	
5		135.20	5		133.52
6	6.03 (s)	118.28	6	6.16 (s)	119.97
7a	3.94 (d, 14.2)	37.59	7a	3.96 (d, 13.5)	44.62
b	4.10 (d, 14.2)		b	4.53 (m)	
8	3.67 (s)	55.99	8	3.65 (s)	55.76
1′		172.73	1′		173.65
2′	2.15 (m)	36.21	2′ a	2.21 (m)	33.37
			b	2.29 (m)	
3′	2.24 (m)	28.35	3′ a	2.16 (m)	28.12
			b	2.20 (m)	
4′	5.40 (m)	130.58	4′	5.41 (m)	130.80
5′	5.40 (m)	127.33	5′	5.41 (m)	127.37
6′	2.13 (m)	36.25	6′ a	2.12 (m)	36.98
			b	2.17 (m)	
7′	3.10 (m)	80.62	7′	3.10 (m)	80.76
8′	1.37 (m)	33.24	8′	1.39 (m)	33.39
9′ a	1.23 (m)	25.29	9′ a	1.24 (m)	25.33
b	1.29 (m)		b	1.32 (m)	
10′	1.22 (m)	29.68	10′ a	1.24 (m)	29.58
			b	1.32 (m)	
11′	1.22 (m)	29.25	11′	1.24 (m)	31.86
12′	1.23 (m)	22.60	12′	1.25 (m)	22.72
13′	1.25 (m)	31.77	13′	1.24 (m)	31.84
14′	0.84 (t, 7.0)	14.06	14′	0.85 (s)	14.18
15′	3.26 (s)	56.40	15′	3.29 (s)	56.29
1″		170.80	1″		171.03
2″	5.10 (s)	95.11	2″	5.09 (s)	95.09
3″		176.66	3″		176.98
4″	4.58 (t, 2.4)	62.35	4″	4.56 (m)	62.52
5″ a	3.79 (m)	59.51	5″ a	3.80 (m)	59.16
b	4.35 (m)		b	4.46 (m)	
6″	3.86 (s)	58.67	6″	3.86 (s)	58.65
NH	6.01 (dd, 6.5)		NCH_3	2.82 (s)	33.88
OH	4.64 (dd, 6.5)		OH	4.65 (brs)	

[a] All ^1H and ^{13}C chemical shifts referenced to residual $CHCl_3$ (δ 7.24 and δ 77.00, respectively).

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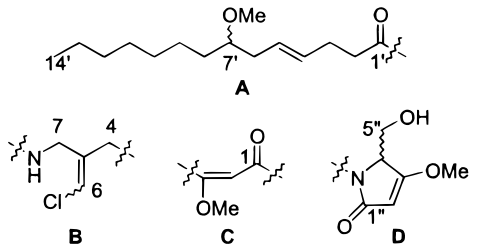


Figure 1. Partial structures of malyngamide Q (**1**).

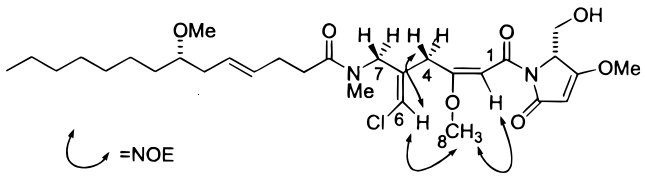


Figure 2. Malyngamide R (**2**) with important NOE enhancements shown.

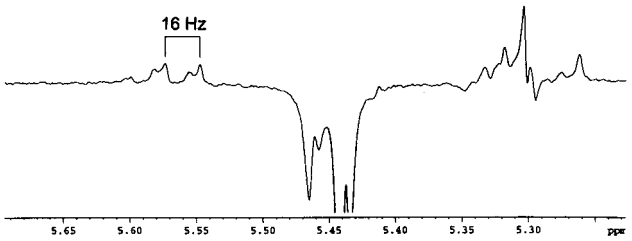


Figure 3. Suppression of signals arising from $^1H-^{12}C$ by BIRD NMR pulse sequence allowing observation of $^3J_{H4'-H5'} = 16$ Hz, therefore defining the C4′−C5′ olefin as *trans*.

resonances (^{13}C δ 95.10 and 95.38, respectively) by HMQC. The assignment of these to components of conjugated enone systems corresponded well with chemical shift data reported in the literature for malyngamide A,[7] and began to define **1** as a member of the pyrrolidone-type subclass of malyngamides.

Consistent with other malyngamides, the 1D NMR data and DQF−COSY couplings described an aliphatic chain consistent with a 7-methoxytetradec-4(*E*)-enoic acid (fragment **A**, Figure 1). DQF−COSY and HMBC connectivities were used to define fragment structure **B**. In this partial structure, $^1H-^1H$ coupling could be observed from the NH proton at δ 6.01 to the C-7 methylene protons (δ 3.94 and 4.10), which, in turn, showed HMBC cross-peaks to the quaternary olefinic carbon (C-5, δ 135.20), the vinyl chloride functionalized carbon (C-6, δ 118.28), and the methylene carbon (C-4, δ 36.55). The conjugated enone systems of fragments **C** and **D** were supported by chemical shift comparisons with the known natural product malyngamide A.[7] In fragment **C**, this was further delineated by HMBC connectivities from H-2 (δ 6.88) to C-1 (δ 165.60) and C-3 (δ 171.53), the latter of which was also attached to a methoxy group, H-8 (δ 3.67, HMBC from H-8 to C-3). Similarly, the pyrrolidone fragment **D** assignments were corroborated by HMBC correlations from H-2″ (δ 5.10) to C-1″ (δ 170.80), C-3″ (δ 176.66), and the methine carbon C-4″ (δ 62.35). This methine carbon (C-4″) was shown to be adjacent to a primary alcohol (^{13}C δ 59.51; ^1H δ 4.35 and 3.79) by DQF−COSY correlations to H-4″. Summation of fragments **A**−**D** accounted for all components of the molecular formula (Figure 1).

Proton H-4″ of fragment **D** (Figure 4) showed HMBC connectivity to C-1 of fragment **C**, establishing the C-1 amide bond as present in malyngamide A. Attachment of fragment **C** to C-4 was indicated by the observation of

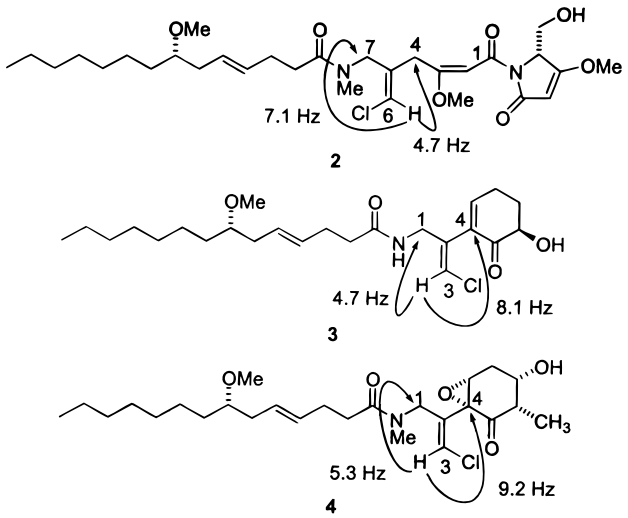


Figure 4. HSQMBC correlations for malyngamide R (**2**), F (**3**), and I (**4**), confirming the *Z* geometry of the C5−C6 olefin in malyngamide R and the *E* geometry of the comparable olefins (C2−C3) in malyngamides F and I.

HMBC connectivities between H-2 to C-4 and H-$4_{a,b}$ to C-3. The linking of the aliphatic chain of fragment **A** to the rest of the structure was supported by HMBC connectivities between the exchangeable NH proton and C-1′ as well as from H-$7_{a,b}$ to C-1′, thus completing the planar structure of malyngamide Q.

Malyngamide R (**2**) was also isolated as a pale yellow oil from the lipid extract of *L. majuscula*. FABMS of **2** revealed an $[M + H]^+$ ion cluster characteristic of a molecule containing a single chlorine atom. HRFABMS described a compound with a pseudomolecular weight ($M + H^+$ at *m/z* 583.3150) consistent with a molecular formula of $C_{30}H_{48}O_7N_2Cl$, again indicating eight degrees of unsaturation. The IR spectrum indicated an exchangeable proton (3385 cm^{-1}) and amide/ester carbonyl groups (1713 and 1630 cm^{-1}). A UV maximum was observed at 258 nm.

The ^1H and ^{13}C NMR spectra of **2** displayed signals very closely related to **1**; however, these were complicated by the presence of a 3:1 ratio of two conformers caused by a tertiary NCH_3 amide moiety in **2**. As a result, proton signals were doubled for H-2, H-4, H-6, and H-7 and carbons C-2, C-4, C-5, C-6, C-7, and C-1′, as well as for the protons and carbon of the NCH_3 group. Despite this complexity, HMBC and HMQC allowed facile interpretation of these spin systems and, used in conjunction with the data and structure of **1**, allowed the complete structural assignment of **2** as shown (Table 1).

To determine the geometry of the C4′−C5′ disubstituted olefin in the aliphatic chain of **2**, a modified BIRD NMR pulse sequence was employed.[8] By suppressing signals arising from ^1Hs bound to ^{12}C, a 1D ^1H NMR spectrum was created that clearly allowed observation of only ^1Hs bound to ^{13}C. This greatly deconvoluted spectrum allowed measurement of the $^3J_{H4'-H5'}$ as 16.0 Hz. By this method, the C4′−C5′ olefin was demonstrated to exist in the trans configuration (Figure 3).

Investigation of the geometry of the C5−C6 olefin in **2**, as well as of the acyclic methoxy enone (C2−C3), was accomplished utilizing DPFGSE 1D NOE.[9] NOE enhancements were seen from H-2 to the protons of the adjacent OMe (H-8), thus defining the C2−C3 olefin to possess an *E* configuration. Dipolar couplings were also observed between H-6 and H-4_a and H-6 and H_3-8. Malyngamide Q

(1) shared these same NOE correlations. From these data, we deduced a 5(*Z*) stereochemistry in both **1** and **2**. This geometry differs from that reported for malyngamide A, which possesses an *E* olefin at this position. In the latter case, a 5(*E*) geometry was demonstrated by observation of NOE between the NCH_3 functionality and the C-6 vinyl proton.[7]

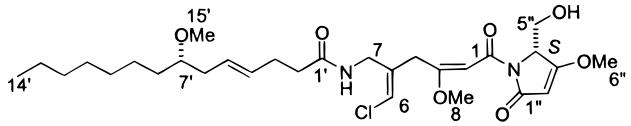
Because malyngamide R (**2**) possessed an unprecedented 5(*Z*) geometry, we sought to confirm this result through the use of $^1H-^{13}C$ long-range heteronuclear coupling constants. In the past, these coupling constants were difficult to obtain on small samples for a variety of reasons.[10−12] To simplify the detection and measurement of these couplings, we have developed a new pulse sequence based on the well-known HSQC sequence. This new pulse sequence provides pure absorption line shapes and eliminates the phase problems associated with the evolution of $^1H-^1H$ couplings during the pulse sequence.[10] Our method is similar to that presented by Sklenár and co-workers but requires fewer gradient pulses and can be set up with only simple modifications to existing HSQC pulse sequences.[10,13] Using the HSQMBC pulse sequence on malyngamide R, we observed a 7.1 Hz ^3J coupling from H-6 to C-7 and a 4.7 Hz ^3J coupling from H-6 to C-4. These coupling constants, obtained directly from the HSQMBC spectrum, were identical to those calculated by the procedure described by Titman and Keeler for the quantitative analysis of coupling constants (data not shown),[12] and unambiguously verified our observations concerning the geometry of C-6.

With the availability of this new methodology for the determination of the $^1H-^{13}C$ heteronuclear coupling constants, we verified the geometry of comparable olefins in two additional malyngamides available from our repository of pure compounds. When the new pulse sequence was applied to malyngamide F (**3**), a $^3J_{H3-C1}$ coupling constant equal to 4.7 Hz and a $^3J_{H3-C4}$ coupling constant of 8.1 Hz were observed.[14] Similarly, in malyngamide I (**4**), a $^3J_{H3-C1}$ coupling equal to 5.3 Hz and a $^3J_{H3-C4}$ coupling of 9.2 Hz were observed.[5] Thus, for both malyngamides F and I, the reported *E* geometry was confirmed by these experiments.

To elucidate the chirality of the stereogenic C-4″ carbon, we used chiral GCMS analysis of the fragment amino acid produced from compound **2** by degradative methods.[15] Sequential ozonolysis, hydrolysis, and derivitization of malyngamide R led to the formation of a pentafluoropropyl serine methyl ester fragment (PFPSME) from C3″-C5″. This derivatized fragment coeluted with a similarly derivatized L-serine methyl ester standard (t_R = 16.32 min)-(pentafluoropropyl D-serine methyl ester standard eluted at t_R =16.81 min), and thus defined a 4″(*S*) stereochemistry in malyngamide R.

The stereochemistries at C-7′ of the fatty acid chains in malyngamides Q and R were implied by the isolation of co-occurring 7′(*S*)-methoxy fatty acid (**5**) as a free acid. The acid isolated from this collection of *L. majuscula* displayed an optical rotation ($[\alpha]^{25}_D = -8.3°$, CH_2Cl_2, *c* 0.8) similar to that previously reported for the 7(*S*) acid ($[\alpha]^{25}_D = -11.1°$, $CHCl_3$, *c* 3.9),[16] thus supporting a 7′(*S*) stereochemistry in **1** and **2**, as found in all other described malyngamides.

Malyngamide R (**2**) was modestly brine-shrimp toxic (LD_{50} 18 ppm). Malyngamide Q decomposed soon after it was defined, such that it could not be evaluated for biological properties. Malyngamides Q and R represent the newest members of the malyngamide class of *L. majuscula* metabolites, and of that class, the first with altered



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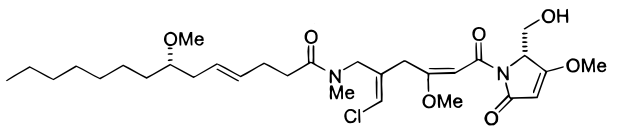

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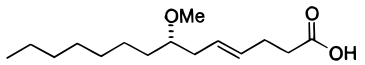

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geometry about C-6. This represents a subtle yet fundamental difference in the biogenetic pathway giving rise to these natural products. Overall, the biogenesis of the malyngamides appears to be of mixed polyketide/amino acid origin, a signature of cyanobacterial metabolism.[17] In this regard, a Claisen-type condensation of serine and malonyl CoA (from acetate) may be responsible for formation of the pyrrolidone ring in malyngamides Q (**1**) and R (**2**).

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker DRX 600 MHz spectrometer. Mass spectra were recorded on a Kratos MS50TC mass spectrometer. UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer, while FT−IR spectra were recorded on a Nicolet 510 spectrophotometer. Chiral GCMS 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 separation was accomplished with a Waters M-6000A pump, a Rheodyne 7010 injector, and a Waters Lambda-Max 480 spectrophotometer. Optical rotation measurements were recorded on a Perkin-Elmer model 141 polarimeter.

Collection. The marine cyanobacterium, *L. majuscula*, was collected by hand from shallow water (2 m) on April 8, 1997, at Sakatia Island, Madagascar (48°9′E/13°18′S), and stored at −20 °C in IPA until workup. A voucher sample is available from W.H.G. as collection number MNS-8 APR 97-2.

Extraction and Isolation of Malyngamides Q and R. The IPA-preserved alga (16.1 g dry wt) was extracted with CH_2Cl_2/MeOH (2:1) two times to give a crude extract of 750 mg. A portion of this (700 mg) was fractionated using vacuum liquid chromatography (VLC) on Si gel with a stepwise gradient of hexanes/EtOAc and EtOAc/MeOH to give nine fractions. Because fractions seven and eight (eluted 70−100% hexanes/EtOAc) both contained **1** and **2**, they were recombined and subjected to reversed-phase chromatography using an C18 Waters Sep-Pak (85% MeOH/H_2O) and then reversed-phase HPLC (Phenomonex ODS, 250 × 10 mm, 5 μ) in 80% MeOH/H_2O to yield pure **1** (peak centered at 6 min, 32 mg, 4.0% of extract) and **2** (peak centered at 12 min, 9.1 mg, 1.1% of extract).

Malyngamide Q (1): pure malyngamide Q, $[\alpha]^{25}_D$ +2.1 (MeOH, *c* 0.8); UV λ_{max} (MeOH) 264 nm (log ϵ 4.18); IR ν_{max} (film) 3313, 2925, 2848, 1713, 1630, 1451, 1397, 1319, 1248, 1206, 1164, 1069, 961, 854, 776 cm^{-1}; FABMS (3-NBA) obsd *m/z* (rel int) 571 (20), 569 (60), 537 (80), 507 (25), 426 (55), 395 (70), 299 (100); HRFABMS (3-NBA) obsd $[M + H]^+$ *m/z* 569.3053 for $C_{29}H_{45}O_7N_2Cl$ (Δ −5.9 mmu); obs. $[M - CH_4O]^+$

at *m/z* 537.2731 for $C_{28}H_{41}O_6N_2Cl$ (Δ 0.0 mmu); ^1H and ^{13}C NMR data, see Table 1.

Malyngamide R (**2**)**:** pure malyngamide R, $[\alpha]^{25}_D$ +2.0 (MeOH, *c* 0.9); UV λ_{max} (MeOH) 258 nm (log ϵ 4.18); IR ν_{max} (film) 3385, 2913, 2854, 1713, 1630, 1451, 1385, 1319, 1242, 1200, 1158, 1069, 997, 848, 770 cm^{-1}; FABMS (3-NBA) *m/z* (rel int) 585 (25), 583 (70), 440 (100), 202 (90), 171 (35); HRFABMS (3-NBA) obsd $[M + H]^+$ at *m/z* 583.3150 for $C_{30}H_{47}O_7N_2Cl$ (Δ 0.0 mmu); ^1H and ^{13}C NMR data, see Table 1.

Stereoanalysis of C-4″ in Malyngamide R (2). Malyngamide R was ozonized (1.0 mg of **2** in 1.0 mL CH_2Cl_2, 3 min, ambient temperature), immediately dried *in vacuo*, and hydrolyzed (1 mL of 6 N HCl, 110 °C, 18 h). The hydrolysate was dried under a constant stream of nitrogen and derivatized using an Alltech PFA-IPA Amino Acid Kit (#18093). The dried hydrolysate was treated with 0.2 N HCl (5 min at 110 °C) and then dried under a constant stream of nitrogen. To this vial, 150 μL of acetyl chloride and 500 μL of IPA was added and heated at 110 °C for 45 min. After drying with a constant stream of nitrogen, the derivatizing agent, pentafluoropropyl isopropionic acid (1 mL dissolved in 2 mL CH_2Cl_2), was added and the solution heated at 115 °C for 15 min, blown dry with nitrogen, and then solubilized in hexanes. For standards, 1 mg each of the serine methyl ester enantiomers (L and D) were subjected to the identical derivitization sequence as the ozonized hydrolysate of **2**. All samples were analyzed by gas chromatography under identical conditions, beginning with a sustained initial oven temperature of 50 °C (4 min), a 3 °C/min ramp from 50 °C to 150 °C, and concluding with a 20 °C/min ramp from 150 °C to 180 °C. The derivitized malyngamide R fragment and the derivatized L-serine methyl ester standard both eluted at 16.32 min. The derivitized D-serine methyl ester eluted at 16.81 min.

Brine Shrimp Toxicity Bioassay. Evaluation for brine shrimp toxicity was performed as previously described using *Artemia salina* as the test organism. [18,19] After a 24-h hatching period, aliquots of a 10-mg/mL stock solution of **2** were added to test wells containing 5 mL of artificial seawater and brine shrimp to achieve a range of final concentrations from 0.05 to 100 ppm. After 24-h the live and dead shrimp were tallied.

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

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