Phytochemistry, Vol. 26, No. 6, pp. 1701–1704, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

TWO MALYNGAMIDES FROM THE CARIBBEAN CYANOBACTERIUM *LYNGBYA MAJUSCULA*

WILLIAM H. GERWICK,* SANYA REYES and BELINDA ALVARADO

Department of Chemistry, University of Puerto Rico, Rio Piedras, Puerto Rico 00931; *College of Pharmacy, Oregon State University, Corvallis, OR 97331, U.S.A.

(*Received* 12 *October* 1986)

Key Word Index—*Lyngbya majuscula*; Cyanobacteria; fatty acid amides; antimicrobial agents.

Abstract—Chemical examination of two collections of the Caribbean cyanobacterium, *Lyngbya majuscula*, led us to isolate several antimicrobial agents. The most active was elemental sulphur, followed by the previously reported fatty acid (−)-7(*S*)-methoxytetradec-4(*E*)-enoate, and then by two new amides of this fatty acid, malyngamide D and malyngamide D acetate. The new compounds possess vinyl chloride and α,β-unsaturated enone functional groups. The structures of the new compounds are based on spectroscopic evidence including several 2D NMR techniques at high field as well as chemical interconversion.

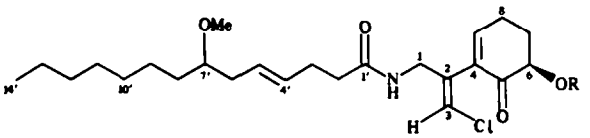
INTRODUCTION

Collections of the marine cyanobacterium *Lyngbya majuscula* from various Pacific locations have yielded a wide variety of novel lipids, many of which show interesting biological properties [1, 2]. Hence, as part of a survey of seaweed chemistry from Puerto Rico in the Caribbean [3], we were interested to examine and contrast the chemistry of this species of blue-green with that of Pacific collections. From both very shallow water and deeper water collections we have found Caribbean *L. majuscula* to contain chlorine containing amides of (−)-7(*S*)-methoxytetradec-4(*E*)-enoic acid (**3**), most closely related in structure to deoxymalyngamide C, a previously described metabolite from a Pacific collection of *L. majuscula* [4]. The structures of two of these (**1**, **2**) are described here and are based on classical spectroscopic data interpretation and are confirmed by use of several 2D NMR techniques. Additionally, one of the collections contained substantial amounts of the methoxylated fatty acid in its free acid form (**3**) as well as elemental sulphur.

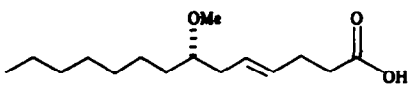
RESULTS AND DISCUSSION

L. majuscula is a year round mat-former on mangrove branches along the southern coast of Puerto Rico. Collections of this alga from very shallow water and high sunlight conditions in June 1984 yielded, upon vacuum chromatography, first elemental sulphur, which by bioautography was responsible for the most of the antimicrobial activity in the crude extract, followed by the previously reported methoxylated fatty acid (**3**). A new compound, **2**, eluted with a more polar solvent mixture and it was further purified using a combination of normal and reverse phase HPLC techniques. Similar isolation methodology was used to purify second new metabolite (**1**), closely related to **2**, from an earlier and deeper water collection of *L. majuscula* found in a nearby location. The structure of the previously reported fatty acid, (−)-7(*S*)-methoxytetradec-4(*E*)-enoate (**3**) was established by comparison of the relevant spectroscopic data with that in the literature [5] (see Experimental). The major one of the two new compounds, **2**, is structurally defined below, utilizing several 2D NMR methods. The related compound **1** was less well characterized; however, its structure is secure based on comparisons with the data for **2** and chemical interconversion of **2** into **1**.

The molecular formula of **2** was provided by high resolution EIMS analysis of a small parent ion at 481.2556 (1.9‰, $C_{26}H_{40}N_1O_5Cl_1$), thus yielding 7 degrees of unsaturation. The 100 MHz ^{13}C NMR spectrum of **2** showed an α,β-unsaturated carbonyl (δ193.04), ester or amide carbonyls (δ172.39, 169.88) and six olefin carbons forming three double bonds (see Table 1), and hence, **2** was monocyclic. Furthermore, the high field (400 MHz ^1H NMR spectrum of **2** showed all of the bands ascribable to the protons in the methoxylated fatty acid (**3**) [5]. Thus, by analogy with findings for Pacific collections of this organism, **2** was deduced to be an amide derivative of **3**. An inventory of atoms at this point suggested that **3** was attached to a $C_{11}H_{12}O_3Cl_1$ unit, containing 5 degrees of unsaturation. The enone olefin was trisubstituted as indicated by the β-proton appearing as a double doublet (δ6.95) coupled only to a pair of allylic protons at δ2.67. Consequently, the other olefin in this portion of the



1 R = H
2 R = CMe
O



3

Table 1. ^1H and ^{13}C NMR chemical shift and coupling data for malyngamide D (1) and malyngamide D acetate (2)

C No.	Compound 1 ^1H NMR*	^{13}C NMR†	Compound 2 ^1H NMR‡	^{13}C NMR‡	LR HETCOSY‡
1	4.04 (*ddd*, J = 14.8, 6.2, 1.2)	43.31 *t*	3.95 (2H, *m*)	43.21	H-3, NH
	3.90 (*ddd*, J = 14.8, 6.2, 1.2)				
2	—	135.31 *s*	—	135.59	H-1, H-3, H-9
3	6.31 *br s*	119.65 *d*	6.31 (*t*, J = 1.1)	119.73	H-1
4	—	133.57 *s*	—	134.92	H-8, H-1, H-3
5	—	199.37 *s*	—	193.15	H-9, H-7
6	4.29 (*dd*, J = 13.7, 5.5)	72.72 *d*	5.41 (*dd*, J = 13.6, 5.5 Hz)	73.63	H-8, H-7
7	1.95 (*dddd*, J = 19.4, 10.3, 5.5, 5.5)		2.31 (1H, *m*)		
	2.45 (*m*)	31.03	2.22 (1H, *m*)	28.49	H-9
8	2.62 (*m*)	25.11	2.67 (2H, *m*)	25.06	H-7
9	6.95 (*t*, J = 3.5)	151.93 *d*	7.28 (*t*, J = 4.0)	150.53	H-7, H-8
1′	—	172.56 *s*	—	172.49	H-1, H-2′(3′), N–H
2′	2.23	36.27	2.30 (2H, *m*)	36.23‖	H-3′
3′	2.30 (*m*)				
	2.48 (*m*)	28.49	2.30 (2H, *m*)	28.21	H-4′(5′)
4′	5.47 (*m*)	130.58	5.47 (*m*)	130.64	H-6′, H-3′
5′	5.47 (*m*)	127.65	5.47 (*m*)	127.62	H-3′, H-6′
6′	2.18 (*m*)	36.27	2.22 (*m*)	36.25‖	H-5′(4′)
7′	3.16(*t*, J = 5.6, 5.6)	80.66 *d*	3.15 (*tt*, J = 5.7, 5.7)	80.71	H-6′
8′	1.44 (2H, *m*)	33.28	1.42 (2H, *m*)	33.25	H-6′
9′	1.27 (*m*)	25.11	1.27 (*m*)	25.20	—
10′	1.27 (*m*)	29.67	1.27 (*m*)	29.65§	—
11′	1.27 (*m*)	29.22	1.27 (*m*)	29.21§	—
12′	1.27 (*m*)	31.76	1.27 (*m*)	31.73	H-14′
13′	1.32 (2H, *m*)	22.57	1.27 (*m*)	22.55	H-14′
14′	0.89 (3H, *t*, J = 6.4)	14.01	0.88 (*t*, J = 6.7)	14.00	
15′ (OMe)	3.32 (3H, *s*)	56.38 *q*	3.32 (3H, *s*)	56.48	—
16′	—	—	—	169.99	H-17′
17′	—	—	2.19	20.68	—
NH	6.01 (*br*, *t*, J = 6.2)	—	6.09 (*br*, *t* J = 6)	—	—

*Recorded on a Bruker HX 500 in $CDCl_3$.
†Recorded on a JEOL FX90Q in $CDCl_3$.
‡Recorded on a Bruker AM400 in $CDCl_3$.
§‖Could be reversed.

molecule was also required to be trisubstituted, the proton of which appeared as a broadened singlet at δ6.31. This compared favorably with the chemical shift of the α-chloro vinyl proton at C-3 in several malyngamide C natural products and derivatives [4]. Further, this proton showed a small coupling (1.1 Hz) to a pair of protons at δ3.95, which from chemical shift considerations must be on a carbon atom bonded to a nitrogen atom. Indeed, they were coupled by 6.0 Hz to a triplet proton at δ6.08, assigned as the amide proton. These coupling and chemical shift values closely match those of the identical system (C-1–C-3) in several of the malyngamide C-related compounds [4], and the geometry of the C-2–C-3 olefin in 2 is assigned as in malyngamide C based on the close comparability of the relevant NMR data.

A ^1H–^1H COSY experiment allowed for formulation of the remaining atoms in the molecule ($C_8H_9O_3$). The enone proton (H-9) was correlated to an allylic methylene at δ2.67 (H-8), which was in turn correlated to another multiplet methylene at δ2.30 and 2.22. These were in turn correlated to a deshielded double doublet proton at δ5.41, which from a HETCOR experiment, was attached to a carbon bearing an oxygen functionality (δ73.53). A three-proton singlet at δ2.19 readily identified this oxygen functionality as a secondary acetate. The observed coupling pattern and unique chemical shift of the C-6 proton is explained by its position adjacent to the enone carbonyl, thus forming a six-membered ring.

The limited amount of malyngamide D acetate (2) precluded a confirmation of its structure via classical chemical degradations and ensuing spectroscopic analysis, as was performed for malyngamide C [4]. As an alternative, we have applied a variety of 2D NMR techniques aimed at explaining all proton and carbon chemical shifts and coupling patterns. Proton and carbon assignments were developed from $^1H-^1H$ COSY and HETCOR experiments. These assignments were then employed in the interpretation of an extensively coupled LR ^1H–^{13}C HETCOSY experiment [6] (Table 1). A final pictorial analysis of these long range ^1H–^{13}C correlations is given in Fig. 1.

An earlier collection of *L. majuscula* from March 1983, made in approximately 8 m water depth, did not contain detectable quantities of the methoxylated fatty acid (3); however, an amide of 3, malyngamide D (1), was isolated as a minor natural product of this collection. An HR EIMS

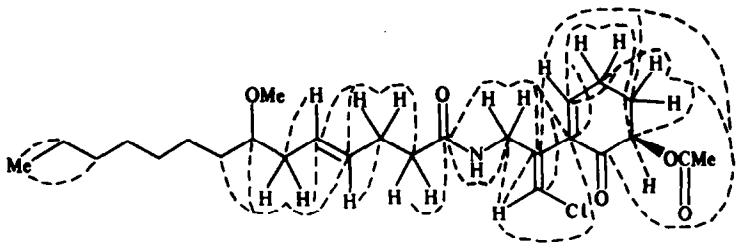


Fig. 1. Pictorial representation of long range couplings observed in a LR ^1H–^{13}C HETCOSY experiment [6] of malyngamide D acetate (2).

analysis of 1 gave a small parent at 439.2509, which calculated for a molecular formula of $C_{24}H_{38}N_1O_4Cl_1$. A substantial fragment at 143.1437 ($C_9H_{19}O_1$, 15%) was diagnostic for the methoxylated fatty acid (3) portion of the structure [5]. Further, all of the ^1H NMR bands assigned to 3 were also present in the spectrum of 1. Additional high resolution mass spectral cleavages gave insight into the linear array of atoms in malyngamide D. A $C_{11}H_{14}N_1O_3Cl_1$ fragment was produced as a major ion by McClafferty rearrangement to fragment the C-2′–C-3′ bond. By high field (500 MHz) ^1H NMR analysis, including extensive ^1H homonuclear decoupling, this functional group laden end of the molecule contained most of the same groups and in the same positions as observed for 2. The key differences were that: (a) the acetate methyl group was absent and (b) the corresponding alpha proton at C-6 was shifted upfield by over 1.0 ppm (δ4.29). The high degree of similarity of 1 and 2 was also indicated by the close correlation in carbon resonance lines (Table 1), differing only in the absence of those bands assigned to the acetate group in 2 in the spectrum of 1. To prove this close relationship of 1 and 2, the acetate ester in 2 was selectively removed by mild base treatment to produce 1 in moderate yield. The high field ^1H NMR of the product was superimposable with that of natural malygamide D (1), and the rotations were of the same sign and within experimental error. Thus, 1 was defined as the deacetylated analogue of 2, and for nomenclatural simplicity, is considered herein as the parent structure, malyngamide D.

The methoxylated fatty acid (3) displayed antimicrobial activity to the gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilus* while malyngamide D acetate (2) showed slight activity to *Staphylococcus aureus*. Malyngamide D (1) was mildly cytotoxic (ID_{50} < 30 μg/ml) to KB cells in tissue culture.

EXPERIMENTAL

General. All NMR shifts are reported relative to TMS ($\delta = 0$) as an internal standard. Low resolution mass spectra were obtained on Hewlett Packard 5995 A and Varian Mat CH7 spectrometers and high resolution spectra on a Kratos MS 50 mass spectrometer. All solvents were distilled from glass prior to use.

Collection, extraction, chromatography. L. majuscula was collected in March 1983 from 7–10 m water depth *ca* 1 km southwest of Isla Guayacan, and again from shallow water (0.1–1.0 m) in June 1984 on the southeast shore of Isla Guayacan, Puerto Rico. In each case, the fresh material was frozen *in situ* with CO_2 (s) and stored frozen until workup. Voucher material is on deposit at the herbarium at the University of Puerto Rico, Mayaguez. The material was extracted repeatedly with $CHCl_3$–MeOH (2:1), which, following solvent reduction *in vacuo*, was partitioned between $CHCl_3$ and saturated brine. The organic layer in each case was dried ($MgSO_4$), filtered and reduced *in vacuo*. The March 1983 collection yielded 5.8 g from 342.0 g dry wt (1.7%) while the June 1984 collection gave 15.5 g from 806 g (1.9%).

The March 1983 extract was chromatographed over silica gel in the vacuum mode using a gradient of pentane, CH_2Cl_2, EtOAc and MeOH. A polar fraction eluting with 100% EtOAc was mainly a single compound (1) that was effectively purified employing normal phase HPLC (μ-Porasil, 3.9 × 25 cm, 75% EtOAc in hexane; 53 mg, 0.92% of extract). The June 1984 extract was also chromatographed over silica gel in the vacuum mode employing a gradient of isooctane, CH_2Cl_2, EtOAc and MeOH. The least polar material, eluting in fractions 1–4, also contained the greatest amount of antimicrobial activity. The active compound was identified as elemental sulphur by TLC and MS comparisons with authentic material. Fractions 5–10, eluting with 60% CH_2Cl_2 in isooctane to 40% EtOAc in CH_2Cl_2, were largely the previously described methoxy fatty acid (3, *ca* 3.4 g, 22% of extract). Fraction 11 contained the majority of the new compound (2) which could be purified by a combination of normal (8 mm × 10 cm μ-Porasil, Z-module, 40% EtOAc–isooctane) and reverse phase HPLC separtions (3.9 mm × 25 cm μ-Bondapak, 35% H_2O–MeOH) and was a colourless oil (48.5 mg, 0.31%).

Malyngamide D (1). Pure 1 showed the following: optical rotation $[\alpha]_D^{25} = +17.0°$ ($c = 0.90$, $CHCl_3$); IR ($CHCl_3$) 3660, 3340, 2930, 1725, 1660, 1510, 1465, 1390, 1260, 1080, 970, 935, 905, 885, 817 cm^{-1}; UV (MeOH) λ_{max} = 208, 239, 258, 273; ε = 9350, 3850, 2300, 1600; LR EIMS (70 eV) 441 (0.7), 439 (1.8), 404 (5.2), 386 (1.6), 372 (3.5), 336 (2.4), 299 (3.4), 297 (11), 279 (4.3), 262 (9.8), 243 (5.8), 208 (7.8), 183 (6.8), 165 (5.0), 148 (8.5), 143 (24), 130 (10), 121 (4.6), 111 (20), 103 (3.4), 91 (6.9), 77 (8.2), 69 (100); HR EIMS (70 eV) 439.2509 (0.5%, $C_{24}H_{38}N_1O_4Cl_1$, 2.0 mamu dev.), 407.2242 (2.0%, $C_{23}H_{34}NO_3Cl_1$, 1.5 mamu dev.), 404.2799 (7.1%, $C_{24}H_{38}N_1O_4$, 0.2 mamu dev.), 372.2535 (2.1%, $C_{23}H_{34}N_1O_3$, 0.1 mamu dev.), 297.1130 (14.4%, $C_{15}H_{20}N_1O_3Cl_1$, 0.1 mamu dev.), 262.1453 (15.2%, $C_{15}H_{20}N_1O_3$, 1.0 mamu dev.), 243.0671 (8.0%, $C_{11}H_{14}N_1O_3Cl_1$, 0.9 mamu dev.), 208.0979 (13.2%, $C_{11}H_{14}N_1O_3$, 0.5 mamu dev.), 143.1437 (14.6%, $C_9H_{19}O_1$, 0.1 mamu dev.), 111.1171 (28.2%, C_8H_{15}, 0.3 mamu dev.).

Malyngamide D acetate (2). Pure 2 showed the following: $[\alpha]_D^{25} = -15.1$ ($c = 3.6$, $CHCl_3$); IR ($CHCl_3$) 3230, 2975, 2940, 1760, 1705, 1680, 1525, 1395, 1255, 1110, 985 cm^{-1}; UV (MeOH) λ_{max} = 252, ε = 1760; LR EIMS (70 eV), 481 (2.4), 446 (23), 388 (19), 339 (100), 304 (15), 285 (17), 281 (67), 250 (37), 192 (36), 190 (25), 143 (92); HR EIMS (70 eV) obs. M + 481.2556 (1.9%, $C_{26}H_{40}N_1O_5Cl_1$, 3.9 mamu dev.), (M^+ − Cl) 446.2948 (13.6%, $C_{26}H_{40}N_1O_5$, 4.2 mamu dev.).

(−)-7(S)-*Methoxytetradec*-4(E)-*enoic acid* (3) $[\alpha]_D^{25} = -9.0$

(c = 1.07, $CHCl_3$), LR EIMS and LR ^1H NMR as previously reported in [5].

Deacetylation of **2** *to* **1**. Pure malyngamide D acetate (**2**, 28.2 mg) in 1.0 ml MeOH at room temp. was treated with 1 ml 5% K_2CO_3 in MeOH. The soln rapidly became coloured and was dark brown within 5 min, at which time it was acidified to pH 4 with 5% HCl in MeOH. This acidified soln, a golden yellow colour, was diluted further with 10 ml H_2O and the MeOH reduced *in vacuo* (40°). The resultant soln was partitioned (2x) between $CHCl_3$–H_2O and the pooled $CHCl_3$ extracts reduced *in vacuo* to yield 25.3 mg of crude product, a complex mixture by TLC analysis. Prep. TLC (100% Et_2O) gave 7 bands, the 5th of which (R_f = 0.10) was pure malyngamide D (5.8 mg, 0.0132 mm, 22.6%) which showed the following: $[\alpha]_D^{25}$ = +21.7 (c = 0.58, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) as reported above for naturally occurring **1**.

Acknowledgements—We thank Dr. Adriana Baez at the University of Puerto Rico Medical School for conducting cytotoxicity assays, and Dr. David Ballantine of the Department of Marine Sciences at the University of Puerto Rico for the taxonomic identifications of our collections. We thank Mr. Roger Kohnert for help obtaining NMR spectra on the OSU Bruker AM 400 spectrometer, purchased in part through grants from the National Science Foundation (CHE 82-16190) and from the M. J. Murdock Charitable Trust, and Mr. Peter Demou at Yale University for help in obtaining NMR spectra on the Bruker HX 500, supported in part by NSF Grant CHE 79-16210. This work was supported by the Puerto Rico and Oregon Sea Grant Programs and the National Institutes of Health (CA 42850-01).

REFERENCES

1. Kashiwagi, M., Mynderse, J. S., Moore, R. E. and Norton, T. R. (1980) *J. Pharm. Sci.* **69**, 735.
2. Mynderse, J. S., Moore, R. E., Ksahiwagi, M. and Norton, T. R. (1977) *Science* **196**, 538.
3. Ballantine, D. L., Gerwick, W. H., Velez, S. M., Alexander, E. and Guevara, P. (1987) *Hydrobiologia* (in press).
4. Ainslie, R. D., Barchi, J. J., Jr., Kuniyoshi, M., Moore, R. E. and Mynderse, J. S. (1985) *J. Org. Chem.* **50**, 2859.
5. Cardellina, J. H., II, Marner, F.-J., Mynderse, J. S. and Moore, R. E. (1978) *Phytochemistry* **17**, 2091.
6. Sato, Y., Gould, S. J. and Kohnert, R. (1986) *Tetrahedron Letters* **27**, 143.