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Three New Malyngamides from the Marine Cyanobacterium *Lyngbya majuscula*

Min Wu, Kenneth E. Milligan, and William H. Gerwick*

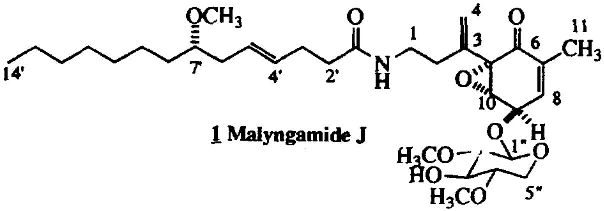
College of Pharmacy, Oregon State University, Corvallis, OR 97331 USA

Abstract: **Three new malyngamides, J (1), K (2) and L (3), all amides of 7(*S*)-methoxytetradec-4(*E*)-enoic acid, were isolated from Curaçao collections of *Lyngbya majuscula*. The structures of these new malyngamides were elucidated on the basis of 1D and 2D NMR spectroscopic analysis of the natural products and key derivatives. The stereochemistry of malyngamide J (1) was probed by NMR and exciton chirality circular dichroism spectroscopy.** © 1997 Elsevier Science Ltd.

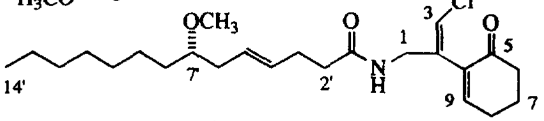
Our collections of the marine cyanobacterium *Lyngbya majuscula* from Curaçao in the Southern Caribbean have provided a wide variety of novel secondary metabolites, many of which show diverse biological activities.[1-3] Our continuing investigation of these collections for their biomedical potential have yielded three new secondary metabolites. The crude lipid extracts of several collections of this organism displayed brine shrimp and fish toxicity as well as a variety of potentially unusual metabolites by 2D-TLC. Fractionation of these crude extracts by silica gel, reversed phase and high performance liquid chromatography yielded three new metabolites, malyngamide J (1), malyngamide K (2), and malyngamide L (3) as well as two known malyngamides (H, 4;I, 5) which had been previously isolated from Japanese and Indonesian collections of *L. majuscula*.[3,4] The malyngamides are a structurally intriguing class of secondary metabolite which combine fatty acid and presumed amino acid subunits, and which occur in nearly all tropical marine collections of *L. majuscula*. The five malyngamides isolated from these most recent collections were responsible for part of the brine shrimp and fish toxicities associated with the crude extracts. Moreover, one of these, malyngamide J (1), has been selected for *in vivo* evaluation by the NCI because of its unusual profile of cytotoxicity in the 60 cell line *in vitro* assay and its structural novelty.

High resolution FAB MS of compound 1 (malyngamide J) gave an $[M+H]^+$ peak at *m/z* 608.3798 for a molecular formula of $C_{33}H_{53}NO_9$, indicating that it possessed eight degrees of unsaturation. Analysis of UV (MeOH, λ_{max} 240 nm, ϵ = 5600), ^{1}H NMR and ^{13}C NMR data (table 1) revealed the presence of three double bonds, a carbonyl in conjugation with one of the double bonds, a second carbonyl with a ^{13}C shift

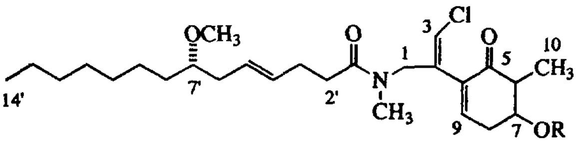
for e-mail correspondance: gerwickw@ccmail.orst.edu



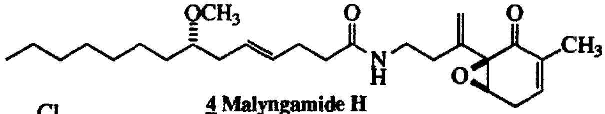
1 Malyngamide J



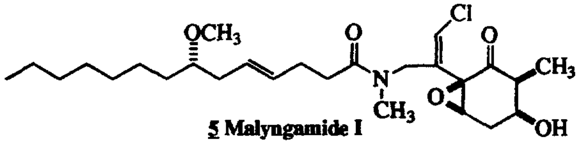
2 Malyngamide K



3 R = H Malyngamide L
6 R = COCH3



4 Malyngamide H



5 Malyngamide I

consistent with an amide, and by subtraction, three ring structures. Analysis by ^{1}H-^{1}H COSY and ^{1}H-^{13}C HETCOR led to the formation of six distinct spin systems (figure 1). Fragment **a** was assigned by recognition of several ^{1}H and ^{13}C NMR resonances typical for a 7-methoxytetradec-4(*E*)-enoate moiety, a common feature of the known malyngamides. Partial structure **b**, indicated by ^{1}H-^{1}H COSY, placed two consecutive methylene groups adjacent to a secondary amide N-atom. This was confirmed by HMBC correlation between the methylene protons $H_{a,b}$-1 (δ3.43, δ3.37) and the carbonyl carbon C-1' (δ172.7). Partial structure **c**, a 1,1-disubstituted double bond, was readily formulated by characteristic ^{13}C (δ117.6 and 139.9) and ^{1}H NMR (δ5.22 and 5.28) shifts. Additional ^{1}H-^{1}H COSY correlations gave fragment **d** as a deshielded oxymethylene contiguous to four consecutive deshielded oxymethines. The most deshielded methine C-1" (^{1}H δ4.48, ^{13}C δ103.15) was consistent with an acetal. At one end of partial structure **e** an olefinic methyl group at δ1.88 was long range coupled to a deshielded olefinic proton δ6.42 which in turn was adjacent to two consecutive deshielded methines at δ4.68 (H-9) and δ3.70 (H-10). As this latter proton was attached to a carbon at δ63.8 and also showed an HMBC correlation to a quaternary carbon at δ62.3, it was identified as part of a trisubstituted epoxide. The olefinic methyl group was also coupled by HMBC to a conjugated carbonyl at δ198.6, completing partial structure **e**. In a biogenetic fashion consistent with other malyngamides, this carbonyl carbon was connected to the quaternary epoxide carbon to form a highly oxidized cyclohexenone ring.

Table 1. NMR Data for Malyngamide J (1), Malyngamide K (2) and Malyngamide L (3) (all in $CDCl_3$).

C-atom	^{13}C for 1[a]	^{1}H for 1[a]	HMBC for 1	^{13}C for 2[a]	^{1}H for 2[a]	^{13}C for 3[b]	^{1}H for 3[b]
1	37.5	3.37 m 3.43 m	1', 3, 2 2	44.0	3.94 d (6.1)	52.49	4.0 d (14.3) 4.1 d (14.4)
2	33.1	2.44 m 2.35 m	3, 4, 5	136.7		136.45	
3	139.9			119.7	6.2 bs	118.72	6.2 bs
4	117.6	5.22 s 5.28 s	5,2 5, 2, 3	136.5		135.71	
5	62.3			198.6		197.3	
6	194.2			38.6	2.54 ddd (6.5, 13.5)	48.20	2.65 m
7	136.1			22.9	2.08 ddd (6.5, 13.2, 10.3)	71.66	4.13 m
8	136.9	6.42 bs	11, 10	26.3	2.50 ddd (10.2, 4.3)	34.68	2.66 m 2.78 m
9	70.4	4.68 bd (4.4)	7, 12, 5, 10	151.5	6.95 t (4.1)	143.70	6.57 m
10	63.8	3.7 bs	8, 5, 9			11.25	1.25 m
11	16.5	1.88 s	6, 7, 8				
1'	172.7			172.5		173.76	
2'	36.8	2.28 m	1', 3' 4'	36.6	2.24 dd (5.8, 7.5)	34.94	2.26 m
3'	28.9	2.35 m	4' 5'	29.5	2.32 m	27.82	2.28 m
4'	131.2	5.48 m	3', 6'	130.9	5.47 m	131.05	5.45 m
5'	127.6	5.48 m	3', 6'	127.9	5.47 m	127.21	5.45 m
6'	36.6	2.18 m	5', 7'	36.6	2.20 m	36.42	2.15 dd (5.8, 5.5)
7'	81.0	3.15 m	6', 15'	80.9	3.15 dddd (5.8, 11.4)	80.80	3.15 m
8'	33.4	1.43 m		33.6	1.43 m	33.37	1.41 m
9'	32.0	1.28 m		32.0	1.28 m	25.29	1.27 m
10'	28.9	1.28 m		25.5	1.28 m	29.76	1.27 m
11'	29.5	1.28 m		29.9	1.28 m	29.31	1.27 m
12'	30.0	1.28 m		30.0	1.28 m	31.85	1.27 m
13'	22.9	1.28 m		22.9	1.28 m	22.66	1.27 m
14'	14.3	0.89 t (6.7)	13', 12'	14.3	0.88 t	14.11	88.0 t (6.8)
NH		6.17 m	1'		6.10 m		
OH		2.78 bs	14, 15, 13				3.26 d (9.6)
7' OMe	56.7	3.32 s	7'	56.7	3.34 s	56.54	3.29 bs
NMe						33.48	2.97 bs
1"	103.15	4.48 d (7.4)	9, 17				
2"	82.8	3.02 dd (9.2, 7.4)	17, 14, 12				
3"	75.5	3.53 dd (9.2, 10.6)	13, 15				
4"	78.9	3.25 dddm (5.0, 8.9, 10.6)	18, 14				
5"	63.4	3.15 bdd (8.9, 11.5) 4.04 dd (5.0, 11.5)	14, 15, 12 14, 12				
2" OMe	58.9	3.6 s	13				
4" OMe	61.0	3.5 s	15, 14				

[a] 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR.

[b] 300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR.

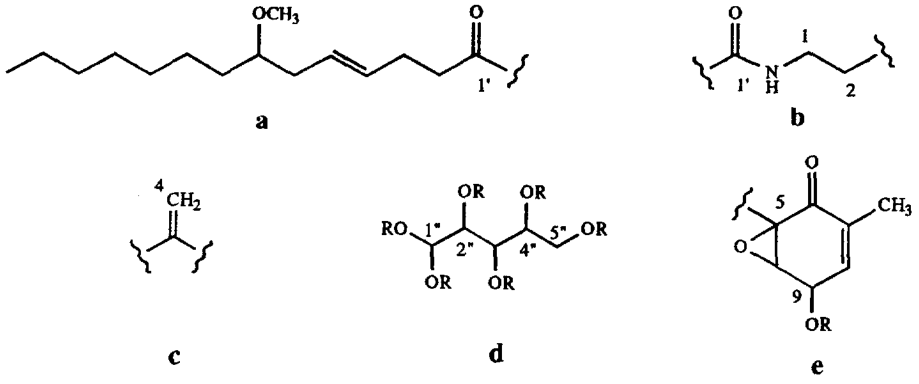


Figure 1. Partial structures a-e forming malyngamide J (1).

These structural fragments were connected by interpretation of HMBC correlations. A correlation between H$_{a,b}$-1 to C-1' linked partial structures **a** and **b**. The terminal olefin protons H$_{a,b}$-4 were long range correlated to both C-5 and C-2, thereby connecting sub-structures **b**, **c** and **e**. Correlations between H$_{a,b}$-5" and C-1" were consistent with a six-membered heterocyclic ring. The correlation observed from H-1" to C-9, as well as the reciprocal H-9 to C-1" correlation, confirmed the linkage between the cyclohexenone and heterocyclic ring. HMBC correlations seen from the C-2" methoxyl group (δ3.6) to C-2" at δ82.8 and from the C-4" methoxyl to C-4" (δ78.9) placed these two methoxyl groups.

From coupling constants (ranging from 7.4 Hz to 10.6 Hz), the oxymethine protons of the heterocyclic ring were all *anti* to one another (figure 2); this was confirmed from nOe data showing H-1", H-3", and H-5" all to be on one face of the ring, and H-2" and H-4" to be on the other (see table 1). Therefore, partial structure **d** was shown to be a 2,4-dimethoxyxylose residue. A small coupling constant between H-9 and H-10 (1.7 Hz) in the cyclohexenone ring (partial structure **e**) required a dihedral angle between these protons to be around 60°-70°. By molecular modeling (Chem3D-Plus), two candidate structures were identified (A and B, figure 3) in which the H-9 and H-10 protons were *trans* to one another. The absolute stereochemistry of the epoxycyclohexenone ring was determined by observation of nOe between H-10 and both H-4a and H-9 in combination with a negative split Cotton effect in the CD spectrum of compound **1**.[5] While both enantiomeric possibilities (A and B, figure 3) could reasonably be expected to display nOe between H-10 and H-4a, only B would also have a negative first Cotton effect (MeOH $\Delta\epsilon_{247}$ max = -12.5), thus establishing the absolute stereochemistry of metabolite **1** as 5*S*,9*R*,10*S*. It should be noted that the absolute stereochemistry of the C-5-C-10 epoxide in malyngamide J (**1**) is opposite that found for malyngamide H (**4**)(5*R*,10*R*),[3] a finding which raises the interesting possibility that the bulky *B*-oriented sugar in **1** prevents enzymatic epoxidation from this face.

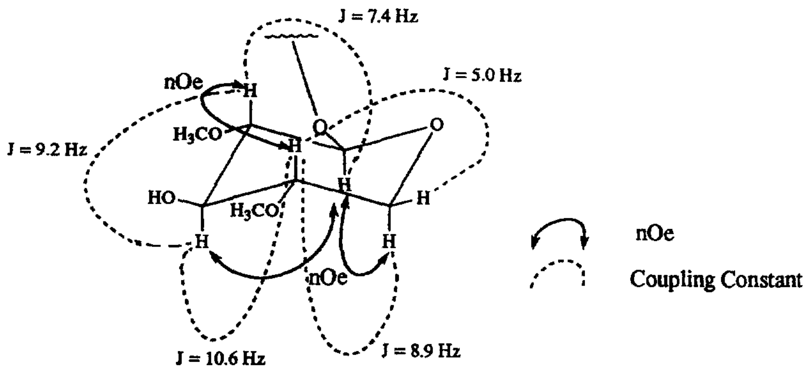
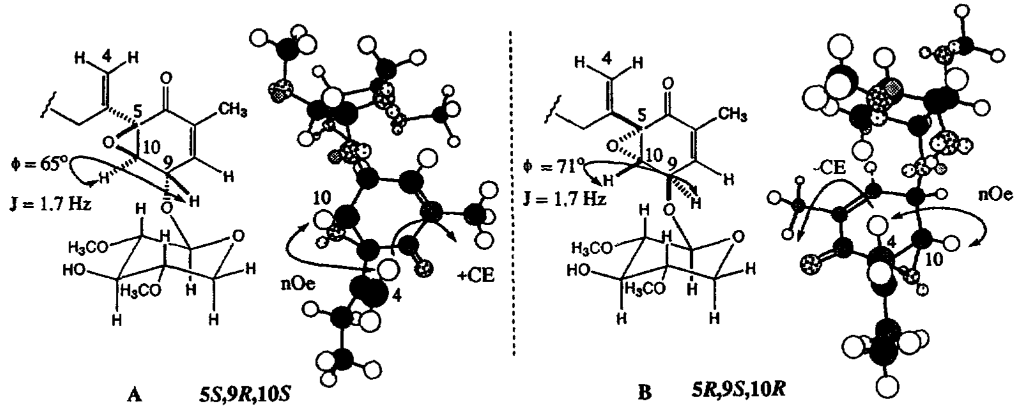


Figure 2. Stereochemistry of dimethoxylated xylose residue in malyngamide J (1).

Compound 2, malyngamide K, ($[\alpha]_D$ = -8.4°, c = 0.28, $CHCl_3$) gave by HR FABMS an $[M+H]^+$ ion at m/z 424.2618, consistent with a molecular formula of $C_{24}H_{38}NO_3Cl$. UV (λ_{max} 223 nm, ϵ = 8900) and ^{13}C NMR data accounted for five of the six degrees of unsaturation inherent in this formula as an α,β-unsaturated carbonyl (δ198.6), an amide carbonyl (δ172.5), and six olefinic carbons forming three double bonds (table 1). Hence, malyngamide K was monocyclic. Comparison of ^1H and ^{13}C NMR data for malyngamide K with that of other malyngamides, including the other new ones reported herein, supported the presence of a 7(*S*)-methoxytetradec-4(*E*)-enoic acid residue in compound 2. By ^1H-^1H COSY, an NH proton was adjacent to a methylene that in turn was allylically coupled to a deshielded olefinic proton. Comparison of ^1H and ^{13}C NMR data for this system showed it highly comparable to the vinyl chloride typical of many malyngamides. The remainder of the ^1H and ^{13}C signals for compound 2 were assigned to a cyclohexenone ring attached as in malyngamide F to the remainder of the molecule.[6] These three partial structures could be assembled in only one way, thus yielding the complete structure of malyngamide K (2).



A 5*S*,9*R*,10*S*

B 5*R*,9*S*,10*R*

Figure 3. Proposed relative configuration of malyngamide J (1) based on nOe, and absolute configuration based on CD, is 5*R*,9*S*,10*R* (stereoisomer B).

Malyngamide L (3), of molecular formula $C_{26}H_{42}NO_4Cl$ by LR FABMS and HR EIMS, was isolated as an optically active oil ($[\alpha]_D$ = +17.3°, c=0.1, EtOH). The NMR features of malyngamide L were remarkably similar to those of malyngamide K (2) except for three aspects: 1) the NH of malyngamide K was replaced in malyngamide L by an N-methyl group, 2) malyngamide L possessed an extra secondary methyl group [^{1}H NMR δ1.25 (3H, m), ^{13}C NMR δ11.25 (CH_3)], and 3) malyngamide L possessed a secondary hydroxyl function [^{1}H NMR δ4.13 (1H, m), ^{13}C NMR δ71.66 (CH)]. The α-hydroxyl proton underwent a diagnostic downfield shift (δ4.13 to δ5.32) upon acetylation. The new hydroxyl and methyl groups in malyngamide L (3) were located by ^{1}H-^{1}H COSY adjacent to one another and bordered on one side by a quaternary center (C-5) and on the other by a methylene group (C-8). Location of the secondary hydroxyl and methyl groups within the cyclohexenone ring of malyngamide L was firmly established by observing connectivity from the characteristically deshielded C-9 olefin proton (δ6.57) to the C-8 methylene pair (δ2.66 and 2.78) and then to the C-7 α-hydroxy proton (δ4.13). Assembly of these various partial structures allowed formulation of malyngamide L (3) as shown. Owing to the unresolved multiplet nature of the proton resonances for C-6 and C-7, we were unable to determine the relative stereochemistry of these hydroxyl and methyl substituents. As has been shown for all malyngamides isolated to date, it is likely that malyngamide L (3), as well as malyngamide K (2), have *S* stereochemistry at C-7'.

All three of these new malyngamides were both brine shrimp and fish toxic [malyngamide J, brine shrimp LC_{50} = 18 μg/ml, gold fish LC_{50} = 40 μg/ml; malyngamide k, brine shrimp LC_{50} = 6 μg/ml, gold fish LC_{50} = 7 μg/ml; malyngamide L, brine shrimp LC_{50} = 8 μg/ml, gold fish LC_{50} = 15 μg/ml].

EXPERIMENTAL

General Experimental Procedures. Ultraviolet spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on either Bruker AM 400 or ACP 300 NMR spectrometers. All ^{1}H NMR chemical shifts are reported relative to an internal tetramethylsilane (TMS) standard and ^{13}C spectra are referenced to the center line $CDCl_3$ at 77.25 ppm. CD measurements were obtained on a Jasco 41A spectropolarimeter. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, while high resolution mass spectra (HRMS) were obtained on a Kratos MS 50 TC. High performance liquid chromatography (HPLC) was done using an M-6000 pump, U6K injector and either R 401 differential refractometer or a Lambda-Max 480 lc spectrophotometer. TLC-grade (10-40 mm) silica gel was used for vacuum chromatography, Kieselgel 60 silica (40-63 mm) was used for flash chromatography, and Merck aluminum-backed TLC sheets (silica gel 60 F_{254}) were used for thin layer chromatography.

Collection. The *Lyngbya majuscula* (voucher specimen available from WHG as NAK-11 Aug 94-3) which yielded metabolites 1 and 2 was hand collected using SCUBA from Playa Kalki, Curaçao (-5 to -8 m) on 11 August 1994 and preserved in isopropyl alcohol at low temperature until work-up. Metabolite 3 was isolated from a Curaçao collection of *L. majuscula* (voucher specimen available from WHG as NSB-9 Aug 94-3) from Santa Barbara Beach, Curaçao (-0.1 to -0.5 m) on 9 August 1994 and preserved in isopropyl alcohol until work-up.

Extraction and Isolation of Metabolites 1 and 2. The defrosted alga was homogenized in CH_2Cl_2/MeOH (2:1, v/v), filtered, and the solvents removed *in vacuo* to yield a residue which was partitioned between CH_2Cl_2 and H_2O. The algal residue was then repetitively extracted with CH_2Cl_2/MeOH (2:1, v/v) and the CH_2Cl_2 layers combined to give 4.32 g of a dark-green extract. Fractionation of the crude extract was achieved by silica gel vacuum chromatography using a step-wise gradient from 4% to 100% (v/v) EtOAc in hexanes, gradient flash chromatography (MeOH/$CHCl_3$, 0%-2%) and reversed-phase C-18 flash chromatography to remove most of pigments. The final purification of malyngamide J (1) was completed using HPLC (10-mm Alltech Lichrosorb Diol column, MeOH/EtOAc/hexanes 1/3/16) to yield 14.5 mg. Malyngamide K (2) was purified from another column fraction by HPLC (10-mm Alltech Lichrosorb Diol column, MeOH/EtOAc/hexanes 1.5/9/120) to yield 6.6 mg.

Extraction and Isolation of Metabolite 3. The defrosted alga was homogenized in CH_2Cl_2/MeOH (2:1, v/v) and extracted as detailed above to give 1.08 g of dark-green organic extract. This was sequentially fractionated using vacuum liquid chromatography (1% to 100% EtOAc in hexanes (v/v), Sephadex LH-20 column chromatography (50% EtOAc/MeOH, v/v), and silica gel column chromatography (80% EtOAc/hexanes, v/v). Final purification was obtained using HPLC (10-mm Alltech Lichrosorb Diol column, MeOH/EtOAc/hexanes 0.5/1.5/8) to yield pure malyngamide L (3)(3.9 mg).

Malyngamide J (1). UV (MeOH) λ_{max} 240 nm (ϵ = 5600); IR (neat) 3323, 2921, 2848, 2353, 1680, 1652, 1535, 1440, 1268, 1096, 1040, 979 cm^{-1}; optical rotation $[\alpha]_D$ = +64° (*c* = 0.15, $CHCl_3$); CD (MeOH): $\Delta\epsilon$ = -12.5, +9.2 (λ_{max} 247, 217 nm); LR FABMS *m/z* obs. 608 (28), 448 (100), 416 (26), 192 (17), 175 (25), 163 (15), 136 (13), 101 (24), 95 (13), 87 (24), 81 (26), 75 (27), 69 (42), 59 (12), 55 (31), 45 (33); HR FABMS (positive ion, 3-nitrobenzyl alcohol) *m/z* obs. $[M+H]^+$ 608.3798 ($C_{33}H_{54}NO_9$, 0.0 mmu dev.); ^{1}H NMR and ^{13}C NMR data see table 1.

Malyngamide K (2). UV (MeOH) λ_{max} 223 nm (ϵ = 8900); IR (neat) 2926, 2848, 2347, 1741, 1652, 1541, 1462, 1229, 1084, 968 cm^{-1}; optical rotation $[\alpha]_D$ = -8.4° (*c* = 0.28, $CHCl_3$); LR FABMS *m/z* obs. 424 (78), 426 (32), 394 (20), 392 (57), 388 (8), 307 (17), 289 (15), 281 (14), 220 (15), 186 (10), 171 (18), 169 (51), 154 (100), 150 (17), 138 (30), 136 (82), 133 (10); HR FABMS (positive ion, 3-nitrobenzyl alcohol) *m/z* obs. $[M+H]^+$ 424.2618 ($C_{24}H_{39}NO_3Cl$, 0.0 mmu dev.); ^{1}H NMR and ^{13}C NMR data see table 1.

Malyngamide L (3). UV (MeOH) λ_{max} 206 nm (ϵ = 31,006); IR (neat) 3416, 2921, 2854, 1680, 1619, 1457, 1090 cm^{-1}; optical rotation $[\alpha]_D$ = +17.3° (*c* = 0.1, EtOH); LR FABMS [positive ion, 0.1 N oxalic acid in thioglycerol:glycerol (2:1)] obs. *m/z* $[M+H]^+$ 468 (100), 452 (10), 436 (80), 418 (7), 236 (20), 230 (75), 199 (40), 145 (28); HR EIMS *m/z* obs. $[M-Cl]^+$ 432.3096 ($C_{26}H_{42}NO_4$, 1.7 mmu dev.); ^{1}H NMR and ^{13}C NMR data see table 1.

Acetylation of Malyngamide L. Metabolite 3 was stirred overnight at RT in a solution containing 1 mL pyridine and 1 mL of acetic anhydride in a capped glass vial. Excess reagents were removed *in vacuo* to give an essentially quantitative yield of pure monoacetate derivative 6; IR (neat) 2926, 2854, 1735, 1685, 1652, 1457, 1090 cm^{-1}; ^{1}H NMR (CDCl$_3$) δ6.61 (1H, m H-9), 6.11 (1H, bs, H-3), 5.48 (2H, m, H-4' & H-5'), 5.32 (1H, m, H-7), 4.29-3.89 (2H, m, H$_2$-1), 3.3 (3H, s, OCH$_3$), 3.11 (1H, m, H-7'), 2.94 (major form) & 2.85 (minor form)(3H, s, NCH$_3$), 2.77 (3H, m, H-6 & H$_2$-8), 2.31 (4H, m, H$_2$-2' & H$_2$-3'), 2.17 (2H, dd, J = 5.5, 5.8 Hz, H$_2$-6'), 2.01 (minor) & 2.00 (major)(3H, s, OCOCH$_3$), 1.42 (2H, m, H$_2$-8'), 1.25 (10H, m, H$_2$-9' through H$_2$-13'), 1.24 (3H, m, H$_3$-11), 0.87 (3H, t, J = 6.8 Hz, H$_3$-14'); LR FABMS (positive ion, 3-nitrobenzyl alcohol) obs. *m/z* [M+H]$^+$ 510 (100), 367 (17), 270 (15), 212 (30).

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