Characterization of Novel Macrolide Toxins, Mycolactones A and B, from a Human Pathogen, *Mycobacterium ulcerans*

Geewananda Gunawardana,[†] Delphi Chatterjee,[§] Kathleen M. George,[‡] Patrick Brennan,[§] David Whittern,[†] and P. L. C. Small*,[‡]

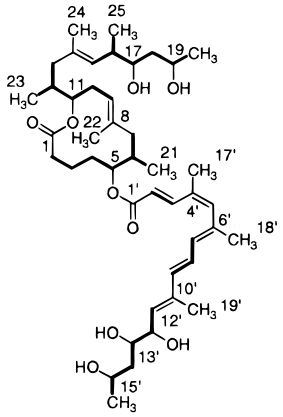
Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60046-3500, Rocky Mountain Laboratories, NIH, NIAID, 903 South Fourth Street, Hamilton, Montana 59840, and Colorado State University, Department of Microbiology, Fort Collins, Colorado 80523

Mycobacterium ulcerans causes a severe skin disease, Buruli ulcer characterized by extensive necrosis in the absence of an acute inflammatory response. Even though the better known pathogenic members of the genus *Mycobacterium*, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are not associated with toxins, the possible presence of a toxin in *M. ulcerans*, which is an extracellular pathogen, has been hypothesized for a number of years.[1] Despite several attempts, no compound responsible for the cytopathic effect of this organizm has been identified. Partial purification of the toxin and evidence that it was a lipophilic molecule were reported earlier.[2] The spectral analyses of the toxin lead to the identification of two compounds which were named mycolactones A (**1**) and B (**2**) (Chart 1). The structure elucidation of the two compounds is described in this paper. This is the first identification of a macrolide produced by a human pathogen, as well as the only macrolide identified in the genus *Mycobacterium*.

The cells were cultured in modified Middlebrook 7H9 medium, harvested, extracted with chloroform/methanol (2:1), and evaporated to produce a crude extract. The crude extract was triturated with ice-cold acetone; the soluble fraction was concentrated and separated by preparative TLC on silica gel developed in chloroform/methanol/water (90:10:1). The examination of the TLC band responsible for major biological activity, by ^{1}H NMR, revealed it to be a mixture of two compounds. The two compounds could be separated by HPLC using aqueous ammonium acetate (10 mM)/acetonitrile gradient (50−100% over 8 min) on a C18 RP column. However, when the two peaks were isolated and the material re-analyzed by HPLC under the same conditions given above, the same two peaks were found in both fractions. Similarly, the examination of the fractions by ^{1}H NMR revealed them to be mixtures similar to the starting material. The HPLC conditions produced baseline separation of the two compounds, and the eluent between the two peaks contained no compounds. This evidence suggests that the two compounds exist as an equilibrium mixture under these conditions.

The extraction of the TLC band produced a yellow glass: UV (MeOH) λ_{max} 375 (log ϵ 3.25); IR (film) ν_{max} 1726, 1706 cm^{-1}. The molecular formula of the compound was deduced as $C_{44}H_{70}O_9$ (found, *m*/*z* 765.4912, M + Na$^+$; calcd 765.4912, Δ 0.1 ppm) by HRMS. The molecular ion itself was observed at *m*/*z* 743 (M^+ + H) as a weak ion; however, the ready loss of H_2O from M^+ + H gave a major peak at *m*/*z* 725 (found 725.4988; calcd for $C_{44}H_{69}O_8$, 725.4988; Δ 0.1 ppm). The per-O-acetylation of the compound (Py/Ac_2O, r.t.) gave a penta-acetate (*m*/*z* 975, M^+ + Na), while the catalytic hydrogenation produced a compound with a *m*/*z* ion at 779 (M^+ + Na), indicating the presence of 5 hydroxyl functions and 7 double bonds, respectively. The presence of two absorption bands at 1726 and 1706 cm^{-1} in the IR spectrum and two signals at δ 173.3 and 166.9 ppm in the ^{13}C spectrum suggested the presence of two ester carbonyl functions. To satisfy the degree of unsaturation, the compound must have a cyclic structure.

Chart 1



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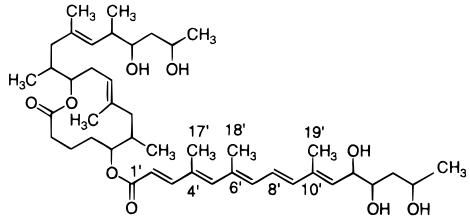

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The examination of ^{1}H and ^{13}C spectra for the compound (Table 1) revealed that the toxin is a mixture of two isomeric compounds present in about a 3:2 ratio. The structure of the major compound, designated mycolactone A (**1**) was elucidated as follows: The proton spin systems shown in emboldened bonds could be identified from the GMQCOSY, TOCSY, and HSQC spectra.[4] The individual moieties identified can be connected using the information from HMQC and ROESY experiments[5] to complete the molecule. The long-range correlations between the proton signals at 2.02 and 2.41 ppm (2-CH_2) and the carbon signal at 173.3 ppm confirmed the position of one of the ester carbonyls at C1. The long-range coupling of olefinic methyl protons to the olefinic carbons 3 bonds away allowed the joining of spin systems separated by methyl-bearing quaternary carbons. The long-range correlations between the methyl proton signal at 1.71 ppm (22-CH_3) and the carbon signals at 46.4 (C7) and 123.8 ppm (C9) and between the methyl proton signal at 1.64 ppm (24-CH_3) and the carbon signals at 44.3 (C12) and 133.9 ppm (C14) allowed the establishment of the dodecanoic chain. The long-range

* Corresponding author: (tel.) 406-363-9280; (fax) 406-363-9371; (e-mail) psmall@nih.gov.
† Abbott Laboratories.
‡ Rocky Mountain Laboratories.
§ Colorado State University.

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Table 1. NMR Data[a] for Mycolactones A and B

	1 and **2**			**1**		**2**	
C no.	^{13}C shift	^{1}H shift	C no.	^{13}C shift	^{1}H shift	^{13}C shift	^{1}H shift
1	173.3		1′	166.9			
2	35.9	2.02,2.41	2′	119.6	5.94	117.4	5.89
3	20.8	1.58	3′	143.1	7.92	151.1	7.36
4	31.4	1.71	4′	132.1		133.2	
5	79.3	4.71	5′	141.8	6.35	144.3	6.46
6	32.8	1.96	6′	134.7		135.3	
7	46.4	2.05	7′	134.8	6.16	136.1	6.37
8	137.2		8′	125.1	6.64		
9	123.8	5.13	9′	139.9	6.40		
10	29.3	2.08,2.50	10′	137.2			
11	76.3	4.90	11′	134.6	5.60		
12	35.4	1.99	12′	72.4	4.28		
13	44.3	1.83,2.13	13′	75.7	3.68		
14	133.9		14′	41.9	1.55		
15	131.2	5.04	15′	67.7	3.99		
16	40.5	2.39	16′	24.2	1.12		
17	76.9	3.50	17′	21.0	1.97	14.3	2.05
18	43.8	1.39,1.66	18′	17.6	2.01	17.1	2.01
19	68.9	3.96	19′	13.3	1.91		
20	24.6	1.14					
21	20.5	0.90					
22	15.9	1.71					
23	15.0	0.89					
24	16.2	1.64					
25	17.1	0.98					

[a] The NMR data were recorded in CD_3COCD_3 solution at 500 MHz for ^{1}H and at 125 MHz for ^{13}C. The chemical shifts are recorded as δ ppm.

correlations between the olefinic protons at 5.94 (2′-CH) and 7.92 ppm (3′-H) and the carbonyl signal at 166.0 ppm confirmed the α,β-unsaturated ester carbonyl at 1′. The olefinic proton signal at 6.35 ppm (5′-H), which is directly attached to the carbon resonating at 141.8 ppm (C5′), showed long-range correlations to carbon signals at 143.1 (C3′) and 134.8 ppm (C7′), while the methyl proton signal at 1.91 ppm (19′-CH_3) showed long-range correlations to carbon signals at 139.9 (C9′) and 134.6 ppm (C11′), respectively completing the hexadecanoic chain. The proton signal at 4.71 ppm (5-CH) showed long-range coupling to the carbon signal at 166.9 ppm (C1′), indicating that the hydroxyl function at C5 is acylated with the hexadecyl moiety. The protons on the remaining 6 hydroxyl-bearing methine functions resonated at 4.9 (C11), 4.28 (C12′), 3.99 (C15′), 3.96 (C19), 3.68 (C13′), and 3.5 (C17) ppm. On the basis of chemical shift considerations it is evident that the hydroxyl function at C11 is involved in the formation of the lactone. This was further confirmed by the presence of NOE correlations between the 11-H (4.9 ppm) and 2-CH_2 (2.02, 2.41 ppm) proton signals. The olefinic proton signal at 6.35 (5′-H) ppm showed NOE correlation to proton signals due to both 17′-CH_3 and 18′-CH_3, but not to any other olefinic protons, suggesting that the olefin at 4′ has *Z* configuration. Similarly, *Z* configuration was assigned to the 8-en, as no NOE correlation was observed between the 22-CH_3 methyl protons (1.71 ppm) and the 9-H olefinic proton (5.13 ppm). All other olefinic bonds were found be of *E* configuration on the basis of NOE information. Therefore, structure **1** ([12-[(3*E*)-6,8-dihydroxy-1,3,5-trimethyl-3-nonenyl]-(9*Z*)-7,9-dimethyl-2-oxooxacyclododec-9-en-6-yl] (2*E*,4*Z*,6*E*,8*E*,10*E*)-12,13,15-trihydroxy-4,6,10-trimethyl-2,4,6,8,10-hexadecapentaenoate) was assigned to mycolactone A.

The two isomers appeared to differ in the configuration of olefin at 4′ and this was reflected in the chemical shifts of the carbons and protons in the region from carbon 2′ to 7′. The proton signal at 6.46 ppm (5′-H) showed NOE to signals at 7.36 (3′-H) and 6.37 (7′-H) indicating that the 4′ olefin has the E configuration in the minor isomer. Therefore structure **2** ([12-[(3E)-6,8-dihydroxy-1,3,5-trimethyl-3-nonenyl]-(9*Z*)-7,9-dimethyl-2-oxoox-acyclododec-9-en-6-yl] (2E,4E,6E,8E,10E)-12,13,15-trihydroxy-4,6,10-trimethyl-2,4,6,8,10-hexadecapentaenoate) was assigned to mycolactone B.

Macrolides are an important class of complex polyketides produced primarily by soil bacteria in the order Actinomycetales.[6] None of the known macrolide producers are human pathogens. The isolation of mycolactones is the first report of macrolides from a *Mycobacteria* species as well as the first identification of a macrolide from any bacterial pathogen. Recent evidence suggests that mycolactones play a major role in the pathology of Buruli ulcer by causing necrosis and immunosuppression.[3] The significance of this finding may extend far beyond the role of these compounds in the virulence of Buruli ulcer. The analysis of the *M. tuberculosis* genome reveals a large number of genes which encode complex polyketides.[7] Even though none of these gene products have yet been identified, such compounds could play an important role not only in the immunosuppression and tissue destruction which occurs in tuberculosis but also in the ability of *M. tuberculosis* to survive in an intracellular environment. Thus, the identification of mycolactones establishes a new role for macrolides as virulence determinants in bacterial pathogens.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, COSY, HMBC, HMQC, ROESY and MS data for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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