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Synthesis and Structure of Two New Mycolactones Isolated from *M. ulcerans* subsp. *shinshuense*

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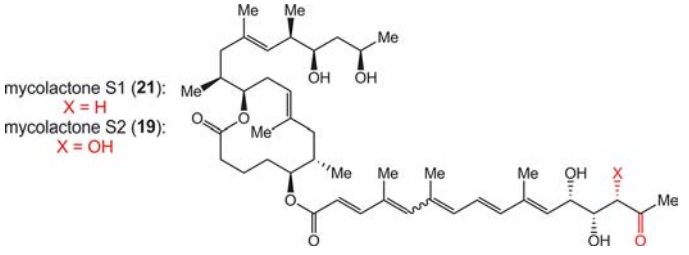
ABSTRACT



Two new mycolactones, mycolactones S1 and S2, were isolated from culture agar of *Mycobacterium ulcerans* subsp. *shinshuense*. Their structures were established in a three-step procedure: (1) probable structures were speculated from MS analysis; (2) candidates were synthesized; (3) HPLC profiles were established for identification of the natural products. Newly isolated mycolactones correspond to the "oxidized forms" of mycolactone A/B, the causative toxin of Buruli ulcer, isolated from *Mycobacterium ulcerans*.

Buruli ulcer is a severe and devastating skin disease caused by *Mycobacterium ulcerans* infection, yet it is one of the most neglected diseases.[1] Infection with *M. ulcerans* results in painless necrotic lesions that, if untreated, can extend to 15% of a patient's skin surface. Surgical excision/skin grafting has been the only method for treatment of Buruli ulcer. Encouragingly, most patients have been reported to respond to combination treatments with rifampin and streptomycin.[2]

In 1999, Small and co-workers isolated polyketide-derived macrolide mycolactones A and B from West African strains of *M. ulcerans*.[3] Various in vitro and in vivo studies demonstrated that mycolactones A and B are the causative toxins of Buruli ulcer.[4]

The gross structure of mycolactones A and B was elucidated with use of spectroscopic methods, and the stereochemistry was predicted via the NMR database approach and confirmed by total synthesis.[5−7] Under standard laboratory conditions, mycolactones A and B exist as a 3:2 equilibrating mixture and are referred to as mycolactone A/B in this paper. Mycolactones C and D

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(1) For general reviews on Buruli ulcer and mycolactones, see: (a) Asiedu, K., Scherpbier, R., Raviglione, M., Ed. *Buruli ulcer: Mycobacterium ulcerans infection*; World Health Organization: Geneva, Switzerland, 2000. (b) Hong, H.; Demangel, C.; Pidot, S. J.; Leadlay, P. F.; Stinear, T. *Nat. Prod. Rep.* **2008**, *25*, 447. (c) Kishi, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 6703.

(2) For example, see a review: Converse, P. J.; Nuermberger, E. L.; Almeida, D. V.; Grosset, J. H. *Future Microbiol.* **2011**, *6*, 1185.

(3) George, K. M.; Chatterjee, D.; Gunawardana, G.; Welty, D.; Hayman, J.; Lee, R.; Small, P. L. C. *Science* **1999**, *283*, 854.

(4) George, K. M.; Pascopella, L.; Welty, D. M.; Small, P. L. C. *Infect. Immun.* **2000**, *68*, 877.

(5) Gunawardana, G.; Chatterjee, D.; George, K. M.; Brennan, P.; Whittern, D.; Small, P. L. C. *J. Am. Chem. Soc.* **1999**, *121*, 6092.

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were isolated from different strains of human *M. ulcerans* (Figure 1).[8,9] Fish and frog pathogenic mycobacteria are also known to produce mycolactone-like metabolites.

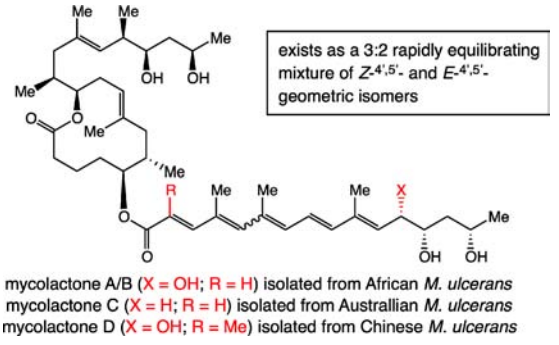

Figure 1. Structures of the mycolactones isolated from different strains of human pathogen *M. ulcerans*.

Structurally, all of the mycolactones reported to date are composed of a 12-membered macrolactone, referred to as mycolatone core, and a highly unsaturated fatty acid side chain. Interestingly, the macrolactone core is conserved in all of the members in the mycolactone class of natural products. On the other hand, there is a remarkable structural diversity observed in the unsaturated fatty acid portion, including the length of fatty acid backbone, the degree of unsaturation, the degree of hydroxylation, the stereochemistry of hydroxylation, and others.[1c]

We recently reported a fluorogenic chemosensor that allows us to detect as low as 2 ng of mycolactone A/B in a semiquantitative manner.[10] We are interested in developing this method as (1) a cost and time effective kit to detect *M. ulcerans* infection and (2) a simple tool for mycolactone-based chemotaxonomy of mycobacteria. As the first case study of exploring the second possibility, we chose *M. ulcerans* subsp. *shinshuense*. This strain was isolated from a skin ulcer patient, identified as *M. ulcerans* subsp. *M. shinshuens*e, registered as JATA 753, maintained on Middlebrook 7H9 broth at −80 °C at Mycobacterium Reference Center, Research Institute of Tuberculosis, Tokyo, Japan, and used for the present study.[11]

Liquid inocula were prepared with Middlebrook 7H10 medium (OADC) and used to seed soft agar in a culture dish (Φ = 9.0 cm). The mycobacteria was grown at 28 °C for 6−8 weeks and harvested. After colonies were put into distilled water, harvested bacteria were warmed at 98 °C for 15 min and cooled. After a 2:1 mixture of chloroform and methanol was added, the mixture was stirred for 30 min at rt and centrifuged (3500 rpm). The organic layer was separated and concentrated under reduced pressure to give a lipid extract.

On fluorogenic TLC analysis of the lipid extract, two distinct greenish-blue fluorescent spots, characteristic to mycolactone A/B, were detected; one of them, designated as mycolactone S1, had an R_f value higher than mycolactone A/B, whereas the other, designated as mycolatone S2, had an R_f value very similar to mycolactone A/B.[12] Two metabolites were isolated by preparative TLC (approximately 0.6 and 0.4 μg from one Φ = 9.0 cm culture dish, respectively) and subjected to the structure study.

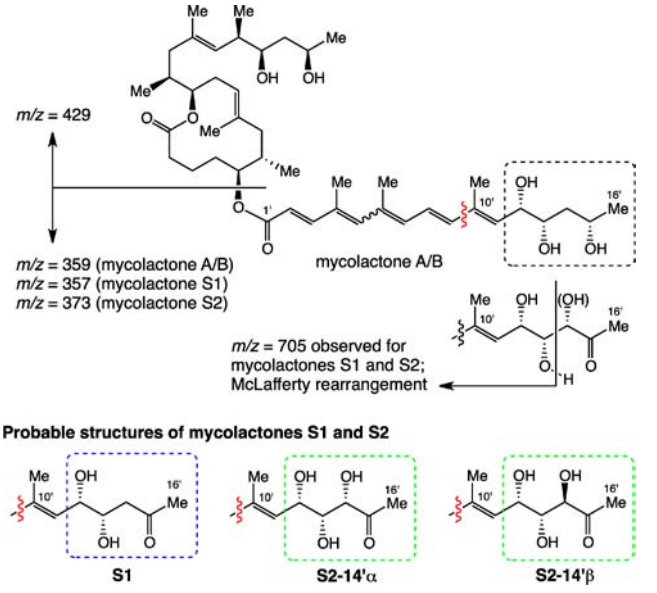

Figure 2. Distinct ions found in the MS/MS spectrum of mycolactones S1 and S2 and probable structures.[12]

High-resolution mass spectroscopy has revealed the molecular formula of mycolactones S1 and S2 to be $C_{44}H_{68}O_9$ and $C_{44}H_{68}O_{10}$, which correspond to "mycolactone A/B − 2H" and "mycolactone A/B − 2H + O", respectively.[12] In the MS/MS analysis of [molecular ion + Na], both mycolactones S1 and S2 gave m/z = 429 ion as the base peak, indicating that the mycolactone core is conserved in these metabolites (Figure 2). Consistent with this conclusion, the ions representing the unsaturated fatty acid moieties changed from m/z = 359 (mycolactone A/B) to m/z = 357 (mycolactone S1) and m/z = 373 (mycolactone S2), respectively.

The MS/MS analysis gave additional information to speculate further the structure of mycolactones S1 and S2. Unlike mycolactone A/B, both metabolites gave a distinct ion at m/z = 705. We postulated that it was formed via a McLafferty rearrangement of the C15′-ketone with a H-shift from the C13′-OH (Figure 2). From these analyses, C15′-ketone of mycolactone A/B (**S1**) emerged as the likely

(8) For mycolactone C, see the following. (a) Isolation and gross structure: Mve-Obiang, A.; Lee, R. E.; Portaels, F.; Small, P. L. C. *Infect. Immun.* **2003**, *71*, 774. (b) Stereochemistry and total synthesis: Judd, T. C.; Bischoff, A.; Kishi, Y.; Adusumilli, S.; Small, P. L. C. *Org. Lett.* **2004**, *6*, 4901.

(9) For the structure of mycolactone D, see: Hong, H.; Spencer, J. B.; Porter, J. L.; Leadlay, P. F.; Stinear, T. *ChemBioChem* **2005**, *6*, 643.

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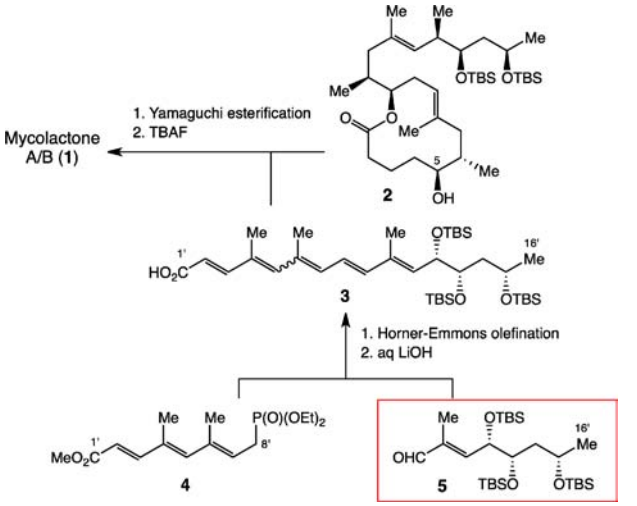
(12) For the details, see the Supporting Information.

candidate for the structure of mycolactone S1. Similarly, C16′-hydroxylated C15′-ketone of mycolactone A/B (**S2-14′α** or **S2-14′β**) appeared to be a probable candidate for mycolactone S2 (Figure 2).

At this stage, we opted to rely on the synthesis to establish the structure of mycolactones S1 and S2. Scheme 1 outlines our synthesis of mycolactone A/B.[13,14] In this synthesis, mycolactone A/B was assembled from mycolactone core **2** and unsaturated acid **3**, which was, in turn, prepared via Horner-Emmons olefination of aldehyde **5**. With this precedence, we recognize that the synthesis of candidates **S1** and **S2-14′α**/**-14′β** is reduced to the synthesis of their C9′−C16′ building blocks.

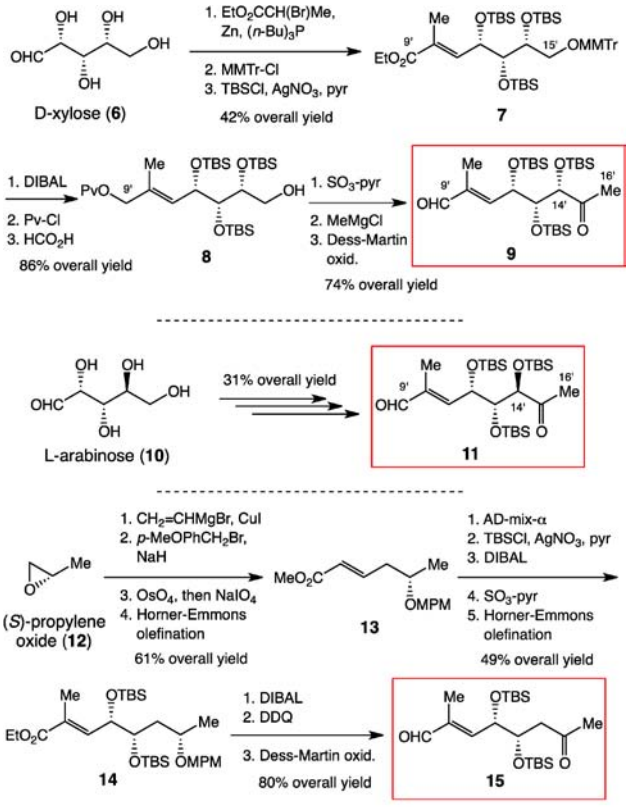
Scheme 1. Summary of the Synthesis of Mycolactone A/B



Scheme 2 summarizes the synthesis of the C9′−C16′ building blocks of candidates. For the **S2-14′α** series, the synthesis started with D-xylose, which was subjected to a coupling reaction with ethyl bromopropionate under the conditions reported by Demailly,[15] followed by protection of resultant primary and secondary alcohols, to give the unsaturated ester **7**, which was then converted to the desired ketoaldehyde **9** via **8**. Using the same synthetic route, the C9′−C16′ building block **11** in the **S2-14′β** series was synthesized from L-arabinose. The ^{1}H and ^{13}C NMR analysis demonstrated that **9** and **11** are distinctly different, thereby showing the C14′ stereochemistry kept intact throughout the synthesis.

The synthesis of the C9′−C16′ building block in the **S1** series was achieved with a slight modification of the previous synthesis.[13] In this synthesis, *p*-methoxyphenylmethyl (MPM) was used as the C15′ protecting group to introduce the C15′-ketone, cf., **14** → **15**. As demonstrated previously, the asymmetric dihydroxylation gave a ca. 8:1 diastereomeric mixture, but the undesired diastereomer was chromatographically removed after the DIBAL step.

Scheme 2. Synthesis of the C9′−C16′ Building Blocks[12]



With these three C9′−C16′ building blocks in hand, we studied the final assembly. Using the previous protocol, we were able to selectively transfer **9**, **11**, and **15** to **16**, **17**, and **18**, respectively (Scheme 3).

Our plan was to remove the TBS-protecting groups under standard TBAF conditions, which was effective for the mycolactone A/B series.[13b] For the present series, however, we had a concern about the stability of **S1**, **S2−14′α**, and **S2−14′β**; one could imagine possible side reactions such as a retro-aldol reaction to form a conjugated enediol anion.

To address this question, we first tested the deprotection of **16**. On treatment with nonbuffered TBAF, **16** gave a complex mixture of products. After numerous attempts, we eventually found that TBAF buffered with 0.5 equiv

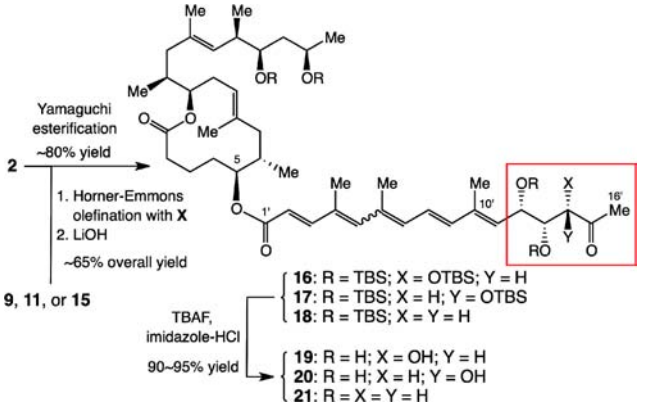
(13) (a) Song, F.; Fidanze, S.; Benowitz, A. B.; Kishi, Y *Org. Lett.* **2002**, *4*, 647. (b) Song, F.; Fidanze, S.; Benowitz, A. B.; Kishi, Y. *Tetrahedron* **2007**, *63*, 5739. (c) Jackson, K. L.; Li, W.; Chen, C.-L.; Kishi, Y. *Tetrahedron* **2010**, *66*, 2263.

(14) For synthetic studies of mycolactone A/B from other groups, see: (a) Yin, N.; Wang, G.; Qian, M.; Negishi, E. *Angew. Chem., Int. Ed.* **2006**, *45*, 2916. Wang, G.; Yin, N.; Negishi, E. *Chem.—Eur. J.* **2011**, *17*, 4118. (b) Feyen, F.; Jantsch, A.; Altmann, K.-H. *Synlett* **2007**, 415. Gersbach, P.; Jantsch, A.; Feyen, F.; Scherr, N.; Dangy, J.-P.; Pluschke, G.; Altmann, K.-H. *Chem.—Eur. J.* **2011**, *17*, 13017. Chany, A.-C.; Casarotto, V.; Schmitt, M.; Tarnus, C.; Guenin-Mace, L.; Demangel, C.; Mirguet, O.; Eustache, J.; Blanchard, N. *Chem.—Eur. J.* **2011**, *17*, 14413. (c) Alexander, M. D.; Fontaine, S. D.; La Clair, J. J.; DiPasquale, A. G.; Rheingold, A. L.; Burkart, M. D. *Chem. Commun.* **2006**, 4602. (d) van Summeren, R. P.; Feringa, B. L.; Minnaard, A. J. *Org Biomol. Chem.* **2005**, *3*, 2524.

(15) Le Mignot, V.; Lievre, C.; Frechon, C.; Demailly, G. *Tetrahedron lett.* **1998**, *39*, 983.

imidazole hydrochloride gave the desired product **19**. Although it was slow (5 days at rt), the deprotection under this condition was amazingly clean and furnished **19** in 95% isolated yield. With use of buffered TBAF, **17** and **18** were successfully deprotected to give **20** and **21**, respectively. Synthetic mycolactones were fully characterized and used to identify the HPLC profiles useful for the structure determination.

Scheme 3. Completion of the Synthesis of Structure Candidates for Mycolactones S1 and S2[12]



With synthetic mycolactones, we began to search for an analytical method to establish the structure of mycolactones S1 and S2. Given the fact that only a very minute amount of natural metabolites was available, we needed an analytical method with high sensitivity. In conjunction with the structure elucidation of fish and frog mycolactones, we demonstrated that the HPLC profile, composed of the peaks corresponding to the three major geometric isomers of the unsaturated fatty acid moiety, is a powerful analytical tool.[16] Like mycolactone A/B, the HPLC profile of mycolactones S1 and S2 should be composed of two peaks corresponding to Z-$\Delta^{4',5'}$- and E-$\Delta^{4',5'}$-geometric isomers (Figure 1).

With use of synthetic materials, we first searched for, and identified, two orthogonal HPLC profiles of mycolactones S1 and S2, one with an achiral column and the other with a chiral column (Figure 3). We should note that the former column is sensitive to the gross structure of a given molecule, whereas the latter is to the absolute stereochemistry. We then subjected natural mycolactones S1 and S2 to the HPLC analysis under the identified conditions, thereby establishing that mycolactones S1 and S2 are **21** and **19**, respectively.

In conclusion, we reported the structure for two new mycolactones S1 and S2, isolated from the culture agar of *M. ulcerans* subsp. *shinshuense*. The structure elucidation was carried out in a three-step procedure: (1) probable structures were speculated from MS analysis; (2) candidates were synthesized; (3) HPLC profiles were established for identification of the natural products. Newly isolated mycolactones correspond to the "oxidized forms" of mycolactone A/B, thereby hinting probable metabolic and catabolic pathways of human mycolactones.

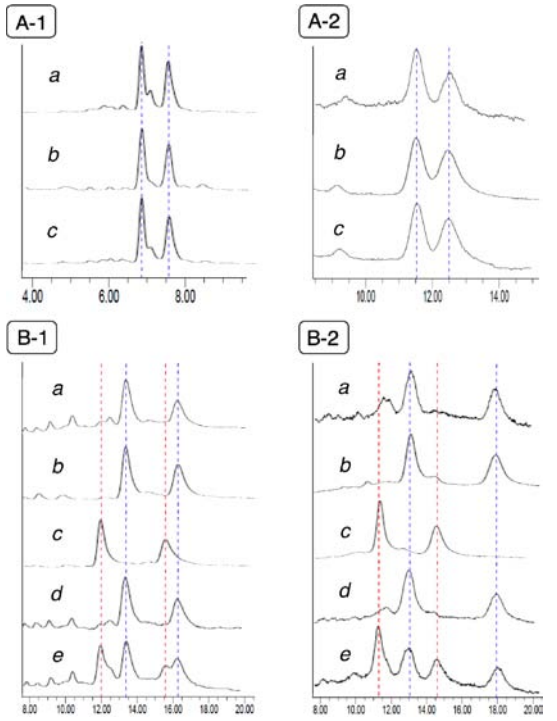


Figure 3. HPLC analysis of synthetic and natural mycolactones S1 and S2. Detection: UV absorption at 365 nm. Mycolactone S1 series: Panel A-1 (column: Keystone Scientific, Hypersil silica (3 μm silica, 120 Å) 250 × 4.6 mm; solvent (isocratic): $CHCl_3$/*i*-PrOH/H_2O/Et_3N = 93.3/6/0.2/0.5; flow rate: 1 mL/min. Key: (a) natural S1; (b) synthetic **21**; (c) a mixture of natural S1 and **21**. Panel A-2 (column: Chiral Tech, Chiralpak IA (5 μm) 250 × 4.6 mm; solvent (isocratic): *i*-PrOH/toluene = 6/94; flow rate: 0.9 mL/min. Key: (a) natural S1; (b) synthetic **21**; (c) a mixture of natural S1 and **21**. Mycolactone S2 series: Panel B-1 (column: Phenomex, Luna (5 μm silica, 100 Å) 250 × 4.6 mm; solvent (isocratic): $CHCl_3$/*i*-PrOH/H_2O/Et_3N = 93.3/6/0.2/0.5; flow rate: 1 mL/min. Key: (a) natural S2; (b) synthetic **19**; (c) synthetic **20**; (d) a mixture of natural S2 and **19**; (e) a mixture of natural S2 and **20**. Panel B-2 (column: Regis Tech, (S,S)-Whelk-01, 250 mm × 4.6 mm; solvent (isocratic): hexanes/CH_2Cl_2/EtOH/Et_3N = 65/27.5/7/0.5; flow rate: 0.6 mL/min. Key: (a) natural S2; (b) synthetic **19**; (c) synthetic **20**; (d) a mixture of natural S2 and **19**; (e) a mixture of natural S2 and **20**.

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Supporting Information Available. Experimental details and ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.