Published in final edited form as:

Org Lett. 2008 December 4; 10(23): 5385–5388. doi:10.1021/ol802233f.

Synthesis and Structure of Mycolactone E isolated from Frog Mycobacterium

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Abstract

The structure of mycolactone E, isolated from the frog pathogen *Mycobacterium liflandii*, was established via organic synthesis. Within the mycolactone family of metabolites, a structural variation has been seen only at the unsaturated fatty acid moiety thus far, and mycolactone E follows this observation. Interestingly, the absolute configuration of its unsaturated fatty acid matches that of the mycolactones from human mycobacteria, rather than the structurally more closely related mycolactone F from fish mycobacteria.

Buruli ulcer is a severe necrotizing skin disease caused by *Mycobacterium ulcerans*, but it is one of the most neglected diseases.¹ Infection with *M. ulcerans*, probably carried by aquatic insects,² results in progressive necrotic lesions that, if untreated, can extend to 15% of a patient's skin surface. Currently, surgical intervention is the only realistic therapy.³ In 1999, Small and co-workers isolated the toxic metabolites, named mycolactones A and B, from *M. ulcerans*. Evidence from animal studies suggested that mycolactones A and B are directly responsible for the observed pathology.⁴ The gross structure of mycolactones A and B was elucidated primarily through 2-D NMR experiments.⁵ Their stereochemistry was predicted via the NMR database approach and then confirmed by total synthesis.^{6,7} Through these studies, mycolactones A and B are now described as a mixture of *Z*- $\Delta^{4',5'}$ - and *E*- $\Delta^{4',5'}$ -geometric isomers (Scheme 1). Mycolactones A and B constitute the major metabolites produced by West African strains of *M. ulcerans*. However, several mycolactone congeners,¹ including mycolactone C,⁸ were recently isolated from clinical isolates of *M. ulcerans* from Africa, Malaysia, Asia, Australia, and Mexico. With use of organic synthesis, the structure of mycolactone C was also elucidated (Scheme 1).⁹

Interestingly, a mycolactone-like metabolite, named mycolactone F, was isolated from the fish pathogen *M. marinum* as well as *M. pseudoshottsii*,¹⁰ and its complete structure was established with use of organic synthesis as the major tool.¹¹ Intriguingly, the absolute configuration of the 1,3-diol present in the unsaturated fatty acid of mycolactone F corresponds to the antipode of that present in the mycolactones isolated from the human mycobacteria.

A frog pathogenic mycobacterium was discovered and named *M. liflandii*.¹² Following importation of the West African clawed frog *Xenopus tropicalis*, a lethal frog disease, caused via infection of *M. liflandii*, appeared in the United States.¹² From lipid extracts, Small and co-workers succeeded in isolation of a partially purified mycolactone, called mycolactone E, and proposed the structure **II** (Scheme 2).¹³ Based on MS analysis in combination with deuterium exchange and chemical transformation, Leadlay and co-workers suggested the alternative structure **I** (Scheme 2).¹⁴ In this paper, we report a structure elucidation of mycolactone E with use of organic synthesis as the major tool.

Between the two proposed structures, the MS/MS fragmentation pattern, in particular a fragment ion corresponding to $[(M+Na)-102\text{ Da}]^+$ reported by Leadlay and coworkers,¹⁴ supports **I** over **II**. For this reason, we chose first to focus on the proposed structure **I**. It is worthwhile to note that the gross structure difference between **I** and mycolactone F is only Me- vs. Et-group at the terminal position of unsaturated fatty acid. Therefore, we recognized the possibility that the complete structure of mycolactone E could be established via a direct extension of the strategy used for the case of mycolactone F. More specifically, we anticipated that two remote diastereomers **9a** and **9b** (Scheme 3) possible for **I** should play a central role for this work, and began the synthesis of **9a** and **9b** via the synthetic route used for mycolactone F.¹¹ Specifically, we first synthesized the unsaturated fatty acids **4a** and **4b** (Scheme 4)¹⁵ and then coupled **4a** and **4b** with the core alcohol **5**,^{7, 16} (Scheme 5), to furnish the two remote diastereomers **9a** and **9b**, respectively.

Determination of Gross Structure

Overall, we adopted the strategy precedent in the mycolactone F series. In the mycolactone E series, however, we faced a new challenge; mycolactone E was available only in a very minute amount,¹⁷ which presented a severe limitation in choosing an analytical method for comparison of the natural and synthetic materials. With this limitation, we first recorded the MS/MS spectra for the synthetic and natural materials under the identical conditions in a side-by-side manner; although the relative peak-intensity of $[(M+Na)-102\text{ Da}]^+/[M+Na]^+$ was found delicately to depend on each measurement, the synthetic material gave the MS/MS profile very similar to that of the natural mycolactone E.¹⁸

The MS/MS profile discussed supported the proposed gross structure **I**, but we wished to have more direct, unambiguous evidence to establish the gross structure. An obvious choice was to rely on the NMR spectroscopy, but the amount of natural mycolactone E available was unfortunately insufficient to measure a proton NMR spectrum.¹⁹ Fortuitously, however, a ¹H NMR spectrum was recorded in one of our laboratories in 2005 (Figure 1).^{13,20}

At first glance, we felt that the ¹H-NMR spectrum of natural mycolactone E was not consistent with the proposed gross structure **I**; more specifically, the H5' and H7' resonances stood out as a singlet at 6.43 and 6.05 ppm in the ¹H-NMR spectrum of **9a**, but no corresponding resonances were easily recognized in the ¹H-NMR spectrum of natural mycolactone E. However, on the basis of the knowledge gained in the mycolactone F series, we soon realized the possibility that this ¹H-NMR spectrum might not totally deny the proposed structure **I**.

As mentioned, the structural difference between mycolactone F and mycolactone E is minimal. Therefore, we anticipate the chemical behaviors of mycolactone E to be parallel with those

observed on mycolactone F or its remote diastereomer. Although slow, we observed the geometric isomerism of mycolactone F under the standard laboratory conditions, to yield a mixture of three predominant geometric isomers. Thus, an aged sample of mycolactone E should be composed of a mixture of the corresponding geometric isomers. Then, there is a possibility that the H5' and H7' protons might give six independent singlets and therefore it could not be straightforward to detect these signals in the ^1H -NMR spectrum of natural mycolactone E.

In order to experimentally test this possibility, we subjected synthetic **9a** to a photochemically-induced isomerization and obtained an approximately 1:1:1 mixture of the three dominant geometric isomers. With the use of the diagnostic chemical shifts identified in the mycolactone F series,¹¹ we assigned these products as *all-trans*-, *Z*- $\Delta^{4,5}$ - and *Z*- $\Delta^{6,7}$ -geometric isomers (Scheme 6). The ^1H -NMR spectrum of the sample thus obtained is shown in Figure 1. Amazingly, all the signals present in this spectrum were detected in the ^1H -NMR spectrum of natural mycolactone E, thereby confirming the proposed gross structure **I**.

A parallel experiment was conducted on **9b**, a remote diastereomer of **9a**. As expected from our previous work,^{11, 21} **9b** exhibited the ^1H -NMR spectrum virtually identical to that of **9a**.¹⁸ These experiments have now established that the structure of mycolactone E is represented by either **9a** or **9b**.

Determination of Stereochemistry

As before,¹¹ we relied on the chiral HPLC profile, to differentiate **9a** and its remote diastereomer **9b**. For the HPLC experiments, we purposely used the photochemically-equilibrated mixture of **9a** and **9b**, with the hope that each of their geometric isomers should give a distinct retention-time and therefore the HPLC comparison could be performed on the basis of six, instead of two, distinct retention times. Experimentally, we found that a Chiralpak IA chiral column in a mixture of benzene and ethanol meets our need well; with a fine-tuning on the ratio of two solvents, all of the six remote diastereomers, i.e., *all trans*-, *Z*- $\Delta^{4,5}$ -, and *Z*- $\Delta^{6,7}$ -isomers of both **9a** and **9b**, gave a distinct retention time (Figure 2).²² We then applied this HPLC condition to natural mycolactone E (Figure 2, Panel a), thereby showing that natural mycolactone E and the 1:1:1 mixture of three geometric isomers of **9a** exhibit the identical HPLC profile. This conclusion was further confirmed via a co-injection of the natural and synthetic materials (Figure 2, Panels b and c). These experiments have established that the natural product used for the comparison is a mixture of the three major geometric isomers of **9a**, but not **9b**. At present, we have not established whether the intact natural product is a single geometric isomer or a mixture of the three geometric isomers.

In conclusion, we have established the complete structure of mycolactone E isolated from the frog pathogen *M. liflandii* in a stepwise manner. First, the proposed gross structure **I** was confirmed via (1) the synthesis of **9a** and its remote diastereomer **9b**, (2) the photochemical isomerization of **9a** and **9b** into a 1:1:1 mixture of the three major geometric isomers, and (3) the ^1H NMR comparison of these mixtures with that of natural mycolactone E, recorded in 2005. Second, the stereochemistry was deduced via (1) the establishment of a HPLC analytical method to differentiate **9a** from **9b** and (2) the established analytical method was applied to the natural product, thereby establishing the complete structure of mycolactone E as **9a**. Within mycolactones isolated from the *human* and *fish* mycobacteria, a structural variation has been seen only at the unsaturated fatty acid moiety thus far, and mycolactone E isolated from the *frog* mycobacterium follows this observation. Interestingly, the absolute configuration of its unsaturated fatty acid matches that of the mycolactones from the human mycobacteria, rather than the structurally more closely related mycolactone from the fish mycobacteria. Lastly, we

should note that the supply of structurally well-defined mycolactone E and its remote diastereomer for biological tests is now secured via organic synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Dr. Han-Je Kim in one of our laboratories for valuable discussion. We thank Drs. Phil Saxton and Nancy Wong at Eisai Research Institute for performing MS experiments. We are grateful to the National Institutes of Health (YK: CA 22215; PS: R01-1015-084) and to the Eisai Research Institute (YK) for generous financial support. SA gratefully acknowledges a postdoctoral fellowship from Association pour la Recherche sur le Cancer (ARC).

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16. The core alcohol 5 was synthesized by Dr. Han-Je Kim in this laboratory.
17. The lipid extract from fermentation broth of *M. liflandii* was used for this work. The amount of mycolactone E in this extract was estimated to be equivalent with ~10 injections for HPLC analysis.
18. See Supporting Information.

19. Chronologically, we performed the HPLC comparison before the ^1H NMR comparison (*vide infra*).
20. To the best of our knowledge, this is the only ^1H NMR spectrum ever recorded on natural mycolactone E.
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22. For the mycolactone F case, a mixture of isopropanol and toluene was used as an eluting solvent. The HPLC profile in two solvent systems is significantly different. For the details, see Supporting Information.

Figure 1.

^1H NMR spectrum (acetone- d_6) of natural and synthetic mycolactone E. **Panel A:** natural mycolactone E (500 MHz). **Panels D and C:** before and after photochemical isomerization of synthetic mycolactone E **9a** (600 MHz). **Panel B:** after photochemical isomerization of **9a** (500 MHz). For the entire spectra shown in Panels A–D and the corresponding spectra in the **9b** series, see Supporting Information.

Figure 2.

HPLC analysis of synthetic photochemically-isomerized mycolactone E (**9a**), its remote diastereomer **9b**, and natural mycolactone E.²² Column: Chiral Tech, Chiralpak IA (5 μ m), 250 $\times$ 4.6 mm; Solvent (isocratic): EtOH/PhH = 1.5/98.5; flow rate 1 mL/min; detection: absorption at 323 nm. **Panel A: 9a. Panel B: 9b. Panel C:** a ca. 1:1 mixture of **9a** and **9b**. **Panel a:** the lipid extract containing natural mycolactone E.¹⁷ Peak indicated by * corresponds to the C13'-ketone. **Panel b:** a ca. 1:1 mixture of mycolactone E and synthetic **9a**. **Panel c:** a ca. 1:1 mixture of natural mycolactone E and synthetic **9b**.

**Isolated from human
pathogen *M. ulcerans***

Mycolactone A: X = OH, $\Delta^{4',5'}$ = Z;

Mycolactone B: X = OH, $\Delta^{4',5'}$ = E

Mycolactone C-Z: X = H, $\Delta^{4',5'}$ = Z;

Mycolactone C-E: X = H, $\Delta^{4',5'}$ = E

**Isolated from fish pathogen
M. marinum and *M. pseudoshottsii***

Mycolactone F

Scheme 1.

Structures of the mycolactones isolated from human and fish mycobacteria.

Scheme 2.

Proposed structures for mycolactone E isolated from frog pathogen mycobacterium.

Scheme 3.
Two remote diastereomers possible for **I**.

Scheme 4.

Synthesis of Side-chain. Reagents and Conditions: (a) (1) $\text{CH}_2=\text{CHMgBr}$, CuI , THF, followed by addition of TMSCl , $i\text{-Pr}_2\text{NEt}$. (2) OsO_4 , NMO , followed by $\text{Pb}(\text{OAc})_4$ treatment; 67% overall yield. (b) Follow the Cr-mediated catalytic asymmetric allylation reported in Reference 15. (c) Stepwise chain elongations consisting of Horner-Emmons olefination, DIBAL reduction, and MnO_2 oxidation. For the details, see Supporting Information and Reference 11.

Scheme 5.

Completion of total synthesis. Reagents and Conditions: Yamaguchi esterification: (a) $\text{Cl}_3\text{C}_6\text{H}_2\text{COCl}$, $i\text{-Pr}_2\text{NEt}$, DMAP, PhH, rt, 24 h, 74%. (b) TBAF, THF, rt, 18 h, 76%.

Scheme 6.

Photochemical isomerism of 9a,b. Photochemical isomerization was done in acetone with a Reonet photoreactor at 300 nm for 16 minutes, to furnish a 1:1:1 mixture of the three geometric isomers as the major products. The diagnostic chemical shifts (^1H NMR in acetone- d_6) are: 6.43 ppm (H-5') and 7.38 (H-3') for *all trans* isomer; 6.66 (H-5') and 7.39 (H-3') for *Z*- $\Delta^{6',7'}$ isomer; **5c**: 6.32 (H-5') and 7.93 (H-3') for *Z*- $\Delta^{4',5'}$ isomer.¹¹ For the time-course of photochemistry, see Supporting Information.