Mycolactone Purification from M. ulcerans Cultures and HPLC-Based Approaches for Mycolactone Quantification in Biological Samples

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Abstract

Mycolactones are a family of polyketide synthase products made by the human pathogen *Mycobacterium ulcerans*. The mycolactones were recently identified as novel inhibitors of the host membrane translocation complex (Sec61). Here, we provide protocols for the purification of mycolactones from bacterial cultures, and for their quantitative assessment in biological samples.

Keywords: Mycolactone, *Mycobacterium ulcerans*, Folch's extraction, TLC, LC-MS/MS

1 Introduction

Mycobacterium ulcerans, the causative agent of a chronic necrotizing skin disease called Buruli ulcer (BU), is unique amongst human pathogens in its capacity to produce a polyketide synthase-derived cytotoxin called mycolactone [1, 2]. Mycolactone of African *M. ulcerans* isolates is naturally produced as a 3:2 mixture of cis/trans stereoisomers (mycolactones A and B, referred to as mycolactone A/B), of a 12-membered lactone ring to which a C5-O-linked polyunsaturated acyl side chain and a C-linked upper side chain comprising C12–C20 are appended (Figure 1) [1–3]. Since its initial discovery in a Malaysian isolate (*M. ulcerans* 1615) [1], eight mycolactone variants have been identified that are produced by *M. ulcerans* strains of different geographical origins, or by *M. ulcerans* lineages causing a BU-like ulcerative disease in fish and frogs [3–5]. Natural mycolactones differ in the length, number and localization of hydroxyl groups and the number of double bonds of the lower side chain [3] and these alterations were shown to result in differences in cytotoxicity [6–8]. Mycolactone A/B is the major form produced by African strains of *M. ulcerans.* It is the most biologically active natural mycolactone [7]. Recently, mycolactone was shown to cause cell death by inhibiting the host membrane translocation complex (Sec61) [9, 10]. Consequently,

assays of Sec61-mediated protein translocation were developed to detect and quantify mycolactone (see "**Biochemical and biological assays of mycolactone-mediated inhibition of Sec61**" chapter). While such bioassays are reliable and sensitive, they can neither be used to assess the structural integrity of mycolactone nor to identify new mycolactone variants. Notably, the distinctive mass spectrometric signature of mycolactone A/B was identified in perilesional skin and serum of patients with BU disease [11]. The presence of mycolactone A/B could still be detected several weeks after completion of antibiotic therapy [12], suggesting a slow elimination rate. Therefore, quantitative assessment of mycolactone A/B in patient samples has the potential to inform on both the development and clinical resolution of BU disease. Here, we provide a protocol that was optimized for the extraction and purification of mycolactones from *M. ulcerans* cultures, where total lipids are extracted with the Folch procedure [13] and mycolactones are then isolated by thin layer chromatography (TLC). This protocol is applicable to all mycolactone congeners, and allows to constitute long-term storage solutions that can serve as standards in bioassays. We also describe a procedure for the quantitative assessment of mycolactone A/B in biological samples that uses high performance liquid chromatography (HPLC) combined with mass spectrometry (MS).

2 Materials

2.1 Mycolactone extraction from *M. ulcerans* cultures

1. Confluent culture of *M. ulcerans* (see **Note 1**). Bacteria are collected when the yellow colonies are well formed (see **Note 2**).
2. 12 mL glass tubes.
3. 100 mL glass flasks.
4. 50 ml conical tubes.

5. Magnetic stirrer and stir bar.
6. Chemical fume hood.
7. Cold room (at 4°C).
8. Water bath (at 50°C).
9. Freezer (at -20°C).
10. Phosphate buffered saline (PBS).
11. Incline plane (45-60°).
12. Absolute ethanol.
13. Ice-cold acetone.
14. Opaque glass tubes.
15. Chloroform, methanol mixture 2:1 (v/v).
16. Deionized water.
17. Glass Pasteur pipette.
18. Centrifuge with swirling buckets.
19. Running buffer for TLC: mixture of Chloroform, absolute Ethanol and deionized water 90:10:1 (v/v) (see **Note 3**).
20. Silica-gel, thin layer chromatography (TLC).
21. Glass TLC developing chamber.
22. Scalpel.
23. Ultrasonic bath sonicator.
24. Rotating wheel.
25. Nitrogen evaporator.
26. Opaque glass tube
27. UV-spectrophotometer.

2.2 Mycolactone extraction from biological samples

1. Frozen tissue samples or biological fluids from BU patients.
2. Balance
3. Scalpel.
4. 12 mL glass tubes.
5. Chloroform, methanol mixture 2:1 (v/v).
6. Tissue homogenizer.
7. Deionized water.
8. Centrifuge with swirling buckets.
9. Nylon filtration tissue.
10. Glass funnel.
11. Glass Pasteur pipette.
12. Water bath (at 50°C).
13. Ice-cold acetone.
14. Freezer (at -20°C).
15. Absolute ethanol.
16. Ultrasonic bath sonicator.
17. Nitrogen evaporator.
18. [22,22,22-$^{2}H_{3}$]-isotopologue of natural mycolactone is used as an internal standard for mass spectrometry (Figure 1) [14].

1. 2.3 Mycolactone analysis by LC-MS/MS.LC-MS system, *e.g.* Ultimate 3000 Dionex chromatographic system coupled to a Q-Orbitrap (Q-Exactive Focus, ThermoFisher Scientific) mass spectrometer equipped with electrospray ionization source (ESI).
2. Speedvac vacuum system.

3. Deionized water.
4. Acetonitrile.
5. Luna Omega PS C18 column, 150 x 2.1 mm, 1.6 μm.
6. Mobile phase A: Water (H_2O) containing 0.1% formic acid (HCOOH).
7. Mobile phase B: Acetonitrile (CH_3CN) containing 0.1% formic acid (HCOOH).
8. Injection vials.
9. Q-Orbitrap Mass spectrometer Q-Exactive Focus.

3 Methods

3.1 Extraction of mycolactone from bacterial cultures

This protocol is adapted from the original method described by George et al.[1], which combines lipid extraction using Folch's method and purification of mycolactone by TLC. Folch's method [13] consists of an initial lipid extraction step using a mixture of chloroform and methanol in volumetric ratios of 2:1. Addition of 0.2 volumes of deionized water in a second step results in a biphasic solution in which the upper phase is composed largely of water and methanol and is enriched with hydrophilic components; and a lower phase mainly containing lipids. Mycolactone is then further purified from the lower phase by TLC.

For all centrifugation steps, use a centrifuge with swirling buckets. For procedures involving solvents, use HPLC grade quality, glass materials and work under a chemical fume hood. Mycolactone exposure to light or UV triggers rapid degradation and loss of biological activity [15]. Therefore, samples must be protected from light during all steps of extraction as well as during storage.

Day 1

1. Collect bacteria from agar plates by carefully resuspending bacterial colonies in 50 mL of 1X PBS. Pour supernatant into 50 mL conical tubes (see **Note 4**).

2. Centrifuge for 20 min at 3200 x g.

3. Carefully remove as much of the supernatant as possible and let the pellet dry under a chemical fume hood for 90 min (see **Note 5**).

4. Resuspend the pellet with 60 mL of a 2:1 (v/v) mix of Chloroform and methanol and transfer to a foil-wrapped glass flask. Gently stir overnight with a magnetic stir bar at 4°C.

Day 2

5. Add 0.2 volumes (12 mL) of deionized water and stir for 1 h at room temperature to obtain an opaque emulsion.

6. Let sediment on an incline plane (45-60°) to get rid of bacterial debris and to facilitate separation. Samples will separate into three phases (Figure 2) with an upper water/methanol phase and a lower chloroform phase separated by an interphase. Using a glass Pasteur pipette, collect the bottom organic phase containing lipids by penetrating the interphase containing precipitated proteins and distribute into 12 mL glass tubes.

6. Centrifuge for 15 min at 500 x g at room temperature.

7. Once again, use a glass Pasteur pipette to carefully collect the bottom organic phase. Transfer into new glass tubes.

8. Place tubes containing the organic phase in a water bath at 50°C and dry under nitrogen (see **Note 6**). Pool fractions by adding the content of one tube to the evaporating one.

9. Add 10 mL of ice-cold acetone to precipitate phospholipids. Protect from light and store overnight at -20°C.

Day 3

10. Bring the frozen samples to room temperature, vortex and sonicate briefly to disrupt the pellet before centrifuging for 30 min at 500 x g at room temperature.

11. Collect the supernatant containing mycolactone, transfer it into a new glass tube (see **Note 7**) and dry under nitrogen.

12. Resuspend yellow pellet with 1 mL of absolute ethanol. Vortex well. (see **Note 8**).

13. Repeat step 11 to fully eliminate acetone and resuspend in 400 µl of absolute ethanol.

14. Drop by drop, using a glass pipette, apply the entire sample on the starting line drawn on the bottom of a silica TLC plate (Figure 3 and see **Note 9**). Rinse the tube with 400 µl of ethanol and apply to the starting line as well. Place the TLC plate vertically in the TLC chamber containing the TLC running buffer and cover to minimize evaporation (see **Note 10**). Migration occurs through capillary action. Remove the plate when the front of the migrating solvent is approximately 1 cm from the end and immediately draw a line on the solvent front. (see **Note 11** and **Figure 3**).

15. Dry the plate. By scrapping the silica with a scalpel, recover the yellow band with a retention factor (R_f) of 0.23 [1] containing mycolactone in a new glass tube (see **Note 12**).

16. Separate mycolactone from silica by adding 8 mL of a mix of Chloroform and Methanol at a ratio of 2:1 (v/v). Rotate overnight at low speed protected from light, on a wheel at 4 °C.

Day 4

17. Vortex and then centrifuge for 15 min at 500 x g at room temperature.

18. Recover the yellow supernatant in a new glass tube. Rinse the silica again with the Chloroform: Methanol solution, centrifuge and recover the supernatant. Repeat if necessary (**Note 13**).

19. Dry the supernatant with nitrogen and resuspend mycolactone with 1 mL of absolute ethanol. Vortex and sonicate 1 min.

20. Centrifuge for 5 min at 500 x g then transfer mycolactone solution into an opaque glass tube.

21. Determine the concentration using a UV spectrophotometer (Mycolactone A/B, λmax: 362 nm, log ε : 4.29) [16]. Adjust to the desired concentration and keep the solution in amber glass vial at -20°C.

3.2 Extraction of mycolactone from tissues

Collected tissues should be snap frozen and kept at -80°C before the lipid extraction.

Day 1

1. Weigh and cut frozen tissues into small pieces with a scalpel.
2. Transfer 300mg of tissue into a 12 mL glass tube containing 6 mL (20 times the volume of the tissue sample) of a mixture of Chloroform, methanol at a ratio of 2:1 (v/v). (see **Note 14**).
3. Optional: In order to perform quantitative detection, a known and fixed quantity of the internal standard [22,22,22-$^{2}H_3$]-mycolactone [14] can be added to each sample at this step (see **Note 15**)
4. Use a tissue homogenizer to fully dissociate the tissue and homogenize the solution. Incubate overnight at 4°C, protected from light.

Day 2

5. Filtrate samples to eliminate tissue particles using a glass funnel covered with nylon filtration tissue and transfer into new glass tubes.
6. Add 0.2 volumes of deionized water and shake vigorously.
7. Centrifuge for 10 min at 500 x g at room temperature.
8. Use a glass Pasteur pipette to carefully collect the organic phase containing lipids by penetrating the interphase containing precipitated proteins (see Figure 2). Transfer the organic phase into new glass tubes.

9. Place tubes containing the organic phase in a water bath at 50°C and dry under nitrogen (see **Note 6**).
10. Add 2 mL of ice cold acetone to precipitate phospholipids. Protect from light and store overnight at -20°C.

Day 3

11. Bring the frozen samples to room temperature, vortex and sonicate briefly to disrupt the pellet.
12. Centrifuge for 15 min at 500 x g at room temperature.
13. Transfer supernatant into a new glass tube and dry under nitrogen.
14. Resuspend with 200 µl of absolute ethanol. Vortex well. Proceed to LC-MS/MS analysis.

3.3 Extraction of mycolactone A/B from biological fluids.

1. Measure sample volume.
2. Proceed to extraction by sequential addition of methanol (4:1; v/v), chloroform (1:1; v/v) and deionized water (3:1; v/v). Mix well after each step.
3. Optional: In order to perform quantitative detection, a known and fixed quantity of the internal standard [22,22,22-$^{2}H_{3}$]-mycolactone A/B [14] is added to each sample (see **Note 15**).
4. Carry out steps 5 to 14 described in § 3.2.

3.4 LC-MS/MS detection

3.4.1 Chromatographic separation

1. Dry samples in a Speedvac vacuum system.
2. Re-suspend in 100 µL of a mix of water and acetonitrile (phases A/B; 50:50; v/v).
3. Transfer into injection vials

4. Inject 20 µL of sample in LC-MS instrument. Mycolactone A/B and its internal standard are eluted from the C18 column (See **Note 16**) and maintained at 50°C, at a flow rate of 0.5 mL/min with a gradient of phase A and B as reported in Table 1.

3.4.2 Mass spectrometry method

The Q-Exactive Focus mass spectrometer is operated under electrospray ionization in positive ion mode. Ion source parameters are the following: capillary voltage, 3.5 kV; capillary temperature, 320 °C; sheath gas flow rate, 30 arbitrary unit and auxiliary gas flow rate, 15 (Figures 4 and 5).

For mycolactone detection in complex biological matrix, data dependent acquisition FullMSddMS2 is done with the following parameters: Full scan (*m/z* 150 to 2,000), resolution 70,000 (Full Width at Half Maximum, FWHM) at *m/z* 200, with automatic gain control (AGC) target of $1x10^6$ ions and maximum time injection programmed in automatic mode. Data dependent MS/MS are acquired on "Top 3" data dependent parameters, which are resolution 17,500; AGC $1x10^5$ ions and isolation window 3 m/z; isolation offset 1 m/z. Normalized collision energy is fixed to 35%. Table 2 shows the LC-MS/MS parameters for identification of mycolactone and of its deuterated counterpart in complex biologic matrix (see **Note 17**).

4 Notes

1. For purification of mycolactone A/B, we use *M. ulcerans* strain 1615 originating from a Malaysian clinical isolate and known to produce relatively high amounts of mycolactone A/B. Culture medium is Middlebrook 7H9 supplemented with 0.2% (v/v) glycerol and 10% OADC. Bacteria are grown at 31°C. Starting from two confluent 175 cm^2 culture flasks we typically extract and purify 1 to 3 mg of mycolactone A/B.

2. Bacteria can be collected after 4 to 6 weeks of growth.
3. Prepare 100 mL of buffer by adding 90 mL of chloroform, 10 mL of ethanol and 1 mL of water.
4. *M. ulcerans* sticks to plastic, so rinse well to collect the maximum of colonies.
5. Pellet drying improves yield of mycolactone extraction.
6. Nitrogen is used to avoid lipid oxidation.
7. At this step, the supernatant should be light yellow.
8. There should be no residues left in the tube. If it's not the case, centrifuge again and collect the supernatant.
9. Using a pencil (never use a pen) gently draw a straight line 1 cm from the bottom, without removing the silica.
10. Place the plate into the TLC developing chamber as evenly as possible. There should be enough solvent to cover the bottom of the chamber, solvent should never rise above the drawn starting line.
11. In a recent paper, Kubicek-Sutherland *et al*., have observed that the MS profile of the ethanol extracts prior TLC purification was very similar to that of synthetic mycolactone A/B while TLC purification reduced the concentration of mycolactone [17]. While questioning the need for TLC purification, the authors have not tested the biological activity of mycolactone without TLC purification. In our hands, in the absence of TLC purification, the measure of absorbance of mycolactone A/B in bacterial lipid extracts by UV-spectrophotometry was hampered by contaminants, indicating the presence of a lot of other lipids in the solution. These contaminants disappear after TLC purification.

12. Retention factor (R_f) is calculated as follow: the distance moved by the compound divided by the distance moved by the solvent as measure from the starting line (see Figure 3).
13. Be careful to not aspirate the silica with the supernatant.
14. According to Folch's procedure, assuming that biological material has a weight of 1 g/mL, biological samples should be diluted 1:20 (v/v) with chloroform: methanol (2:1; v/v). Therefore, a sample of 300 mg should be extracted with 6 mL of chloroform: methanol (2:1; v/v).
15. Addition of a known quantity of internal standard allows for the precise determination of extraction efficacy in each sample and normalization. In our experiments, we have spiked samples with 25 ng of deuterated-mycolactone A/B.
16. Other columns such as a C8 column might be used. The elution gradient might be adjusted accordingly.
17. Identification of mycolactone A/B in complex matrix can be hampered by strong background staining (See Figure 6).

Time (min)	%B (CH_3CN with 0.1% HCOOH)	Flow mL/min	Oven T°C
0	50		
1	50		
7.5	95	0.5	50
12	95		
12.1	50		
20	50		

Table 1: Gradient conditions used for mycolactone A/B LC-MS analysis on the Luna C18 column.

						Ion fragments NCE 35%			
Compound	**Formula**	**RT min**	**Neutral mass**	**m/z obs.**	**$[M+Na]^+$**	**F1**	**F2**	**F3**	**F4**
Mycolactone A/B	$C_{44}H_{70}O_9$	5.78/5.90	742.5020	765.4898	765.4912	659.4120	429.2963	359.1815	315.1922
Mycolatone IS	$C_{44}H_{67}D_3O_9$	5.78/5.90	745.5208	768.5152	768.5100	662.4338	432.3150	359.1796	315.1945

Obs. = observed
IS = internal standard

Table 2: LC-MS/MS parameters for identification of mycolactone A/B and of its deuterated (Mycolactone IS) counterpart [18].

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