SOP: PP032.2
Modified: 5/29/14

Purification of Mycobactin

Materials and Reagents

1. H37Rv γ-irradiated whole cells, 50 to 150 mg (wet weight)
2. Mettler-Toledo balance
3. Erlenmeyer flask, 2.0 L
4. Chloroform, Burdick & Jackson HPLC-grade
5. Methanol, Burdick & Jackson HPLC-grade
6. Ethanol, ACS/USP-grade
7. Graduated cylinder, glass, 100 ml
8. Glass funnel, large
9. Plastic funnels, small
10. Chemical fume hood
11. Magnetic stir bar, large
12. Magnetic stir plate
13. Glass bottle, 1 L
14. Round-bottom flask, 1 L, tared
15. Rotary evaporater (Rotovap)
16. Metal spatulas
17. Glass Pasteur pipet
18. NMR pipet (Wilmad #803A)
19. Rubber Pasteur pipet bulb
20. TLC reagents and equipment (note 1)
21. N_2 bath
22. Glass tubes, 16 x 100 mm + PTFE-lined lids
23. Glass syringe (10 ml)
24. Large filter paper (VWR funnel #413 Cat. No. 28333-123)
25. TLC plate, silica, glass-backed preparative (VWR #1.05715.0001, 0.25 mm)
26. HPTLC sheets, silica gel 60, alumina (VWR #5548.7)
27. TLC tanks (Kontes, large and small)
28. Ruler
29. Pencil
30. Aluminum foil
31. Teflon Oakridge tubes, 45 ml
32. Pipet, glass, (5 and 10 ml)
33. Rubber pipet bulb
34. Vortex
35. Benchtop centrifuge
36. $CDCl_3$, HPLC-grade (Aldrich 236896-1PAK)
37. NMR tube
38. ^1H NMR machine (note 2)

Protocol

1._____ Freeze dry H37Rv γ-irradiated cells by lyophilization (note 3).

2._____ Weigh dried cells and transfer to a 1.0 L glass bottle.

3._____ Suspend cells in 100% ethanol at a concentration of 30 ml/g of cells (note 4).

4._____ Add a large magnetic stir bar and cap bottle for overnight stir in fume hood.

5._____ Working in the hood, filter extract through large circular filter paper fitted into glass funnel, for capture in round-bottom flask.

6._____ Allow cells to dry overnight on filter (note 5).

7._____ Dry material in the round bottom flask on a rotary evaporator, resuspend in 5-10 ml EtOH, and aliquot 100-200 µl to tared glass vial. Use N_2 bath to dry, then weigh to estimate total crude lipid weight.

8._____ Apply total lipid to preparative TLC plates (note 6).

9._____ Develop preparative TLC plates in solvent system 95/5 $CHCl_3/CH_3OH$.

10._____ Working in the hood, use a glass slide to scrape non-specific areas below the doublet mycobactin bands. Discard silica in suitable bag or container to be transferred to hazardous waste container.

11._____ Scrape mycobactin-specific silica onto aluminum foil and divide between an appropriate number of Teflon Oakridge tubes for extraction, employing small funnels.

12._____ Add 10-20 ml of $CHCl_3/CH_3OH$ (2:1) to each tube and briefly vortex.

13._____ Balance and centrifuge at 3,000 rpm, 4°C for 10 minutes.

14._____ Transfer the supernatants to a fresh round-bottom flask *without filtering*.

15._____ Repeat steps 12-14 once more (note 7).

16._____ Dry combined extract via rotary evaporation, then resuspend in a small volume of 2:1 for transfer to 13 x 100 mm tube (note 8).

17._____ Completely dry under a stream of N_2 then resuspend in 100-200 µl $CHCl_3$.

18._____ Evaluate extract from first set of plates on analytical TLC using 2/3/3 petroleum ether/ n-butanol/ ethyl acetate (note 9).

19._____ Repeat steps 8-16 on several more batches of preparativeTLC plates until at least 10 mg is accumulated.

20._____ Combine crude mycobactin and run 5 mg per preparative TLC plate, developing this time with 2/3/3 pet ether/ n-butanol/ ethyl acetate (note 10).

21._____ Evaluate cleaned mycobactin with TLC (note 11).

22._____ Evaluate with MALDI (note 12).

23._____ Once at least 3 mg clean mycobactin has been obtained, combine and dry completely under nitrogen. Resuspend in 1 ml deuterated chloroform ($CDCl_3$)

24._____ Dry completely and resuspend in 600 µl $CDCl_3$, then use NMR pipet to transfer to NMR tube.

25._____ Evaluate with ^{1}H-NMR (note 13).

26._____ Aliquot appropriately and include final TLC, MALDI, and NMR in QC data.

Notes:

1. See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.
2. An orientation class explaining basic NMR principles as well as how to log in and run your sample will be required. Contact the Central Instrument Facility in the basement of the Chemistry Building.
3. See Lyophilization SOP, SP004. Alternatively, one may use 10:10:3 $CHCl_3/CH_3OH/H_2O$ extract of Rv cells and start with step 8 of this protocol.
4. All organic solvents should be used in a chemical fume hood. *Make sure to use glass pipets with rubber bulbs for all work with organic solvents.*

5. A subsequent extraction can be performed with 10:10:3 $CHCl_3/CH_3OH/H_2O$ or 2:1 $CHCl_3/CH_3OH$, and the concentrated lipids developed on prep plates.
6. See Preparative Thin Layer Chromatography, SOP SP032, for directions on preparing the material for preparative TLC. Avoid overloading the plate (10 mg maximum) as this can compromise resolution of bands.
7. Extract until the amber/orange tint in the silica disappears. This may take 2 rounds of extraction if using 20 ml 2:1 per tube, and perhaps 3-4 rounds with smaller volumes.
8. Once transferred, use additional 1 ml 2:1 to rinse flask, adding to volume in tube prior to drying.
9. Follow SP033, using a 10 x 10 cm aluminum-backed sheet cut from a 20 x 20 cm sheet from the supplier. High Performance TLC sheets will lead to each amber band bifurcating into their own doublets. A line scored by spatula 1 cm from the top will help keep all analytical TLC runs consistent. Always run against previous sample or standard.
10. *Save 20 μl for TLC analysis later*. The "clean-up" plates employ the same solvent system used for analysis of each batch of crude mycobactin. It should help to clean the suspension of tetra-acylated PIM2, and other lipids which would be found in the vicinity of mycobactin on 95/5 $CHCl_3/CH_3OH$ prep plates.
11. As before, with 2/3/3 pet ether/ n-butanol/ ethyl acetate, but this time in duplicate, one to be charred with $CuSO_4$ to check for species close to the baseline (relatively polar) as well as above the mycobactin (relatively nonpolar). Use 100 μg of each sample, and check against the relatively crude sample prior to clean-up.
12. Submit 100 μl in $CHCl_3$, 2 μg/μl, and request negative mode analysis. Should see signature peaks at 983 and 1191 m/z. Ideally there should be nothing or very few peaks at 1400 and higher. Peaks in the ranges 1652-1694 and 1544-1586 likely represent PIM_2(4Ac) and PIM_6(1Ac), respectively.
13. Submit to 400 MHz instrument set up for autorun, specifying **[H]presat** (presaturated), 128 scans. Peaks in the 7-8 ppm range represent phenolic H, triplet at ~0.8 ppm terminal CH3group from the lipid tail, etc. Additional NMR shifts can be found in the reference, and in *Mycobactin Notebook II* p103.

References

R Barclay *et al.* (1985). Isolation, Identification, and Structural Analysis of the Mycbactins of *Mycobacterium avium, M. intracellulare, M. scrofulaceum,* and *M. paratuberculosis*. *Journal of Bacteriology* (Nov) 164(2):896-903.