

## Incorporation of Radiolabelled Substrates into Mycobacterial Lipids of *M. Bovis* BCG and *M. Kansasii*

Nirmala Deo, Divakar Sharma, Deepa Bisht, Krishnamurthy Venkatesan\*

Department of Biochemistry, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra, India

\*Corresponding author: Krishnamurthy Venkatesan, Department of Biochemistry, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Tajganj, Agra 282004, India, Tel: 91-562-2331751/ Fax: 0562-2331755;  
E-mail: [drvenkatesan1952@gmail.com](mailto:drvenkatesan1952@gmail.com)

### Abstract

**Background:** Diseases caused by microorganisms of the genus Mycobacterium continue to be a serious problem. The cell envelope constitutes up to 60% of the dry weight of lipids. The cell wall of the mycobacterium is essential for their growth and survival in the infected host. The cell wall lipids have been isolated from *M. bovis* BCG and *M. kansasii* mycobacterial strains and studied the incorporation of radiolabelled substrates for exploring the potential involvement of lipid biosynthetic pathways.

**Methods:** We have incubated *M. bovis*, BCG and *M. kansasii* with radiolabelled substrates-1-C<sup>14</sup>-palmitic acid, 1-C<sup>14</sup>-sodium-n-valerate, 1-C<sup>14</sup>-sodium-n-acetate and 1-C<sup>14</sup>-sodium-n-propionate and calculated the incorporation in PGL, PDIM and mycolates by Liquid Scintillation Counter.

**Results:** We have found the percentage of assimilation of 1-C<sup>14</sup>-palmitic acid into lipids of *M. bovis* BCG and *M. kansasii* were PGL: 0.39%, PDIM: 0.70% & Mycolates: 0.30% and PGL: 0.42%, PDIM: 0.81% & Mycolates: 1.45% respectively. The percentage of assimilation of 1-C<sup>14</sup>-sodium-n-valerate into lipids of *M. bovis* BCG and *M. kansasii* were PGL: 0.28%, PDIM: 0.58% & Mycolates: 1.05% and PGL: 0.35%, PDIM: 0.68% & Mycolates: 0.90% respectively. The percentage of assimilation of 1-C<sup>14</sup>-sodium-n-acetate into lipids of *M. bovis* BCG and *M. kansasii* were PGL: 0.45%, PDIM: 0.80% & Mycolates: 1.45% and PGL: 0.48%, PDIM: 0.90% & Mycolates: 1.50% respectively. The percentage of assimilation of 1-C<sup>14</sup>-sodium-n-propionate into lipids of *M. bovis* BCG & *M. kansasii* were PGL: 0.48%, PDIM: 0.85% & Mycolates: 1.40% and PGL: 0.50%, PDIM: 0.90% and Mycolates: 1.48% respectively.

**Conclusion:** The findings of this study suggest the usefulness of radiolabelled substrate incorporation approach for exploring the involvement of lipid biosynthetic pathway.

### Introduction

Mycobacteria synthesize a wide variety of complex lipids to maintain very unusual structures. Mycobacterial waxes are composed of mycocerosic acid diesters of three closely related diols: phthiocerol A, phthiocerol B, and phthiodiolone A<sup>[1-3]</sup>. Dimycocerosates were first characterized in *M. tuberculosis* and detected by infra-red spectroscopy in lipid extracts from *M. tuberculosis*, *M. bovis*<sup>[4]</sup>, *M. marinum* and *M. leprae*<sup>[5]</sup>. The presence of phthiocerol dimycocerosates was reported by Goren

et al<sup>[6]</sup> in virulent strains of *M. tuberculosis* but not attenuated strains.

There are only few studies on the uptake and incorporation of radiolabelled substrates into one or other specific cell wall lipid of mycobacteria grown in culture medium or macrophage/Schwannoma cell lines (with *M. leprae*) *in vitro*. The present study was designed to study the incorporation of the radiolabelled substrates, 1-C<sup>14</sup>-palmitate, 1-C<sup>14</sup>-valerate, 1-C<sup>14</sup>-sodium-n-acetate and 1-C<sup>14</sup>-sodium-n-propionate into a host of cell wall lipid fractions- phenolic mycosides (PGLs), phthiocerol

Received Date: January 31, 2017

Accepted Date: February 17, 2017

Published Date: February 27, 2017

**Citation:** Nirmala Deo., et al. Incorporation of Radiolabelled Substrates into Mycobacterial Lipids of *M. Bovis* BCG and *M. Kansasii*. (2017) J Anal Bioanal Sep Tech 2(1): 58- 62.

DOI: 10.15436/2476-1869.17.1324

**Keywords:** Phenolic glycolipid; Phthiocerol dimycocerosate; Mycolates; Mycobacteria; Palmitic acid; Sodium-n-valerate; Sodium-n-acetate; Sodium-n-propionate

**Abbreviations:** PGL: Phenolic glycolipids; PDIM: Phthiocerol dimycocerosates; cpm: counts per minute; TLC: Thin layer chromatography



rol based wax esters (PDIMs) and mycolates of *M. bovis* BCG and *M. kansasii*. The research gained from incorporation of radiolabelled substrates might help in exploring the potential involvement of lipid biosynthetic pathways to maintain the cell wall/rigidity of *M. bovis* BCG and *M. kansasii*. Further research in this direction might be used for studying the antimycobacterial efficacy of various drugs.

## Materials and Methods

### Extraction of free non-polar lipids

The extraction method involves the treatment of organisms with a biphasic mixture of methanolic saline and petroleum ether (b.p. 60 - 80°C) as described by Dobson *et al*<sup>[3]</sup>. Harvested *M. bovis* BCG/ *M. kansasii* organisms were suspended in 3 ml of aqueous methanol in 8.5 ml test tubes screw capped with PTFE (poly tetra flour ethylene) lined caps. Free non-polar lipids were extracted by partitioning between the organisms in aqueous methanol and 3ml of petroleum ether (b.p. 60 - 80°C) for 15 min. The mixture was centrifuged for 5 min at 2000 rpm at room temperature. The upper petroleum ether layer were separated and stored in small screw capped vial. The lower layer was re-extracted with a further aliquot of 2 ml petroleum ether for 15 min. The combined petroleum ether extracts was evaporated to dryness under a stream of nitrogen at 37°C and examined for phenolic glycolipids and wax esters (phthiocerol dimycocerosates).

### TLC analysis of phenolic glycolipids extracted from cells

The non-polar lipids extracted with petroleum ether was dissolved in a small volume of chloroform and spotted on the corner of a 10 x 10 cm silica gel G-precoated aluminium plates and developed with chloroform-methanol (96:4, v/v) once in the first direction followed by toluene-acetone (80:20, v/v) thrice in the second direction. The plates were sprayed with  $\alpha$ -naphthol-sulphuric acid reagent and heated at 120°C for 10 min. The presence of PGL was confirmed by comparing the mobility of authentic mycoside available in the Department.

### TLC analysis of wax esters ( Phthiocerol Dimycocerosates -PDIMs)

The other half of the non-polar lipid extract was deposited on Silica gel G – precoated plate (10 x 10 cm, Merck) which was run in Petroleum Ether-Diethyl ether (90:10, v/v) for the detection of phthiocerol dimycocerosates (wax esters). The characteristic lipids were visualized by spraying plate with H<sub>2</sub>SO<sub>4</sub> followed by heating.

### Mycolic acid extraction and preparation of methyl esters

The aqueous portion containing cells left out after extraction of non-polar lipids was centrifuged and the supernatant was collected and saved for radioactive counting. The residual cell material was saponified to release cell-wall 'bound lipids' like mycolic acids by employing the procedure described by Dobson *et al*<sup>[3]</sup>. The material was subjected to alkaline methanolysis by treating with a mixture of methanol (1 ml), 30% aqueous KOH (1 ml) and toluene (0.1 ml) and heating in an oven at 75°C for 18 hrs. The alkaline contents were cooled and acidified to pH 2.0 with 6N HCl and extracted with diethyl ether (3 x 1 ml aliquots). The combined extracts collected in an 8.5 ml screw

capped tube were evaporated to dryness under a stream of nitrogen at 37°C. The dried lipid material supposedly containing mycolic acids was dissolved in 1 ml of dichloromethane and treated with 1 ml of catalyst solution and 0.5 ml of iodomethane. The contents were mixed for 30 minutes using tube rotor, centrifuged for 5 minutes and upper layer was saved for counting. The lower layer was dried under a stream of nitrogen at 37°C. The dried material was supposedly composed of methyl esters of mycolic acids and other fatty acids.

### Detection of mycolates by unidimensional TLC

The mycolate fraction was dissolved in a small volume of petroleum ether (b.p. 40 - 60°C) and spotted on a 10 x 10 silica gel G-precoated aluminium plate and developed with petroleum ether: diethyl ether (90:10) five times followed by 100% methanol once. The plate was sprayed with 0.5% potassium dichromate in 10% sulphuric acid and heated at 120°C for 10 min. The mycolates were identified using standard mycolates from *M. tuberculosis* and the spots on the TLC plates were scrapped and collected in scintillation vial. 10 ml of scintillation fluid was added and the radioactive counts were read by Liquid Scintillation Counter (Rackbeta-LKB, Wallac). The radioactivity in cell free-Sauton's medium and the supernatants saved at every stage of experiment was measured by diluting a suitable aliquot with scintillation fluid and counting in a Liquid Scintillation Counter.

### Computation of radioactivity into various lipid fractions

The percent incorporations of the radioactive 1-C<sup>14</sup>-palmitic acid, 1-C<sup>14</sup>-sodium-n-valerate, 1-C<sup>14</sup>-sodium-n-acetate and 1-C<sup>14</sup>-sodium-n-propionate into phenolic glycolipids, wax ester (PDIMs) and mycolates were calculated from the counts per minute (cpm) of the individual lipid fractions and the total counts and radioactivity incorporated into each lipid fraction was expressed as percent of the total radioactivity assimilated.

## Results

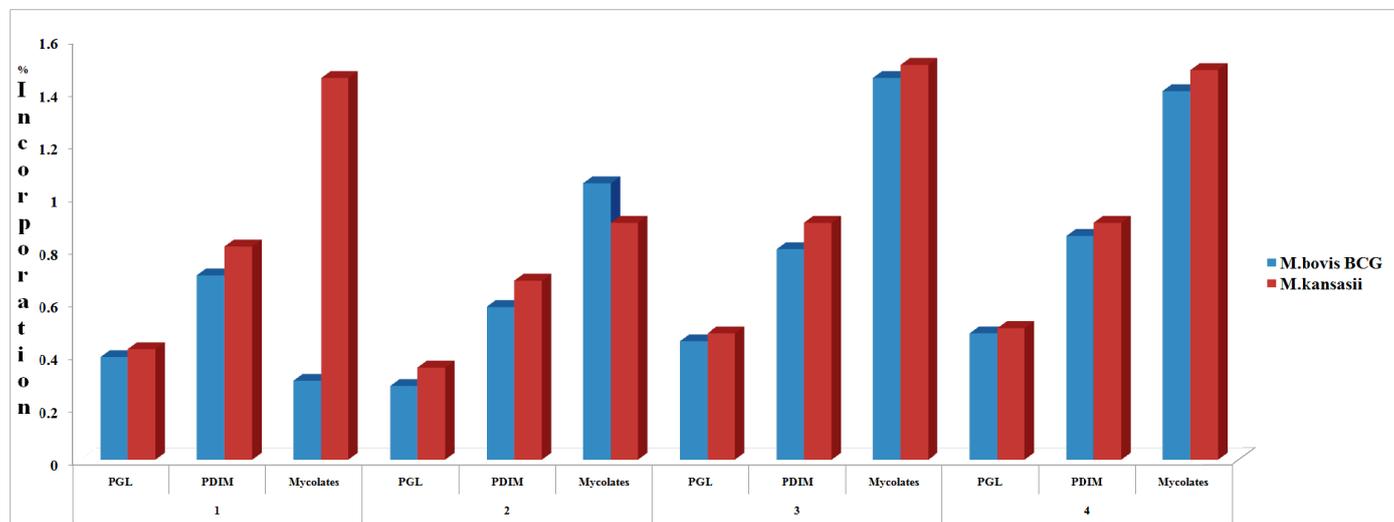
### Incorporation of labeled 1-C<sup>14</sup>-palmitic acid into lipid of *M. bovis* BCG and *M. kansasii*

Significant radioactivity has been found in phenolic glycolipids (PGL), wax esters (PDIMs) and mycolates of *M. bovis* BCG and *M. kansasii* incubated into 1-C<sup>14</sup>-palmitic acid (2 $\mu$ Ci, specific activity 23.3 mCi/mmol). The counts per minute ranged from 3950 in case of PGL to 14000 for mycolates through a count of 8400 in wax esters, in the case of *M. bovis* BCG while the radioactivity as per cpm in case of *M. kansasii* phenolic glycolipids, wax esters and mycolate fractions were 4872, 8872 and 11543 respectively. The percentage of total radioactivity assimilated into lipids of *M. bovis* BCG was: PGL: 0.39%, PDIM: 0.70% and Mycolates: 0.30%. In *M. kansasii* the values were: PGL: 0.42%, PDIM: 0.81% and Mycolates: 1.45%. (Table 1, Figure 1). The incorporation, when expressed as percentage of total radioactivity, ranged from 0.39 % to 1.45 %.

### Incorporation of labeled 1-C<sup>14</sup>-valerate into lipids of *M. bovis* BCG and *M. kansasii*

Significant radioactivity has been found in phenolic glycolipids (PGL), wax esters (PDIMs) and mycolates of *M. bovis* BCG and *M. kansasii* incubated into 1-C<sup>14</sup>-sodium n-valerate (1 $\mu$ Ci/ml, specific activity 10.14 mCi/mmol). The counts

per minute ranged from 3600 in case of PGL to 12010 for mycolates through a count of 6600 in wax esters, in the case of *M. bovis* BCG while the radioactivity incorporated as per cpm in the case of *M. kansasii* Phenolic Glycolipids, wax esters and mycolate fractions were 3800, 6600 and 11543 respectively. The percentage of assimilation of  $^{14}\text{C}$ - sodium-n-valerate into lipids of *M. bovis* BCG as; PGL: 0.28%, PDIM: 0.58% and Mycolates: 1.05%. In *M. kansasii* the values were: PGL: 0.35%, PDIM: 0.68% and Mycolates:0.90% (Table:1, Figure.1). The incorporation, when expressed as percentage of total radioactivity, ranged from 0.28 % to 1.05 %.



**Figure 1:** Incorporation of radioactively labeled 1-C<sup>14</sup>-palmitate, 1-C<sup>14</sup>-valerate, 1-C<sup>14</sup>-acetate and 1-C<sup>14</sup>-propionate into cell wall lipids of *M. bovis* BCG and *M. kansasii*.

- 1: 1-C<sup>14</sup>-palmitate      2: 1-C<sup>14</sup>-valerate
- 3: 1-C<sup>14</sup>-acetate      4: 1-C<sup>14</sup>-propionate

**Table 1:** Incorporation of 1-C<sup>14</sup> labeled substrates into cell wall lipids of *M. bovis* BCG and *M. kansasii*.

| Radio labelled substrate               | Lipid Fractions | <i>M. bovis</i> BCG | <i>M. kansasii</i> |
|--|-----------------|---------------------|--------------------|
| 1-C <sup>14</sup> -palmitic acid       | PGL             | 0.39                | 0.42               |
|  | PDIM            | 0.70                | 0.81               |
|  | Mycolates       | 0.30                | 1.45               |
| 1-C <sup>14</sup> -sodium-n-valerate   | PGL             | 0.28                | 0.35               |
|  | PDIM            | 0.58                | 0.68               |
|  | Mycolates       | 1.05                | 0.90               |
| 1-C <sup>14</sup> -sodium n-acetate    | PGL             | 0.45                | 0.48               |
|  | PDIM            | 0.80                | 0.90               |
|  | Mycolates       | 1.45                | 1.50               |
| 1-C <sup>14</sup> -sodium-n-propionate | PGL             | 0.48                | 0.50               |
|  | PDIM            | 0.85                | 0.90               |
|  | Mycolates       | 1.40                | 1.48               |

**Incorporation of labeled 1-C<sup>14</sup>-acetate into lipids of *M. bovis* BCG and *M. kansasii***

Significant radioactivity has been found in phenolic glycolipids (PGL), wax esters (PDIMs) and mycolates of *M. bovis* BCG and *M. kansasii* incubated into 1-C<sup>14</sup>-sodium n-acetate (2μCi/ml, specific activity 40 mCi/mmol). The percentage of assimilation of  $^{14}\text{C}$ - sodium-n- acetate into lipids of *M. bovis* BCG was: PGL: 0.45%, PDIM: 0.80% and Mycolates: 1.45%. In *M. kansasii* the values were: PGL: 0.48%, PDIM: 0.90% and Mycolates: 1.50% (Table: 1, Figure.1). The incorporation, when expressed as percentage of total radioactivity, ranged from 0.45 % to 1.50 %.

**Incorporation of labeled 1-C<sup>14</sup>-propionate into lipids of *M. bovis* BCG and *M. kansasii***

Significant radioactivity has been found in Phenolic Glycolipids (PGL), wax esters (PDIMs) and mycolates of *M. bovis* BCG and *M. kansasii* incubated into 1-C<sup>14</sup>-sodium n-propionate (2μCi/ml, specific activity 5-20 mCi/mmol). The percentage of assimilation of  $^{14}\text{C}$ - sodium-n-propionate into lipids of *M. bovis* BCG was: PGL: 0.48%, PDIM: 0.85% and Mycolates: 1.40%. In *M. kansasii* the values were: PGL: 0.50%, PDIM: 0.90% and Mycolates: 1.48%.(Table:1, Figure.1). The incorporation, when expressed as percentage of total radioactivity, ranged from 0.48 % to 1.48%.

## Discussion

Lipid profiles of mycobacteria have been increasingly used in the chemotaxonomy of mycobacteria<sup>[7]</sup>. Phthiocerol Dimycocerosates (PDIMs) are proving to be powerful characters for the classification and identification of a limited number of mycobacteria. It is present only in very limited number of mycobacterial species like *M. tuberculosis*, *M. bovis*, *M. marinum*, *M. microti*, *M. kansasii* and *M. leprae*<sup>[3,8]</sup>. *M. bovis* has shown dimycocerosates of all three members of phthiocerol family, viz., phthiocerol A, phthiocerol B and phthiodiolone A. The phthiocerol A derived ester is the major constituent in both *M. bovis* and *M. tuberculosis* strains. Therefore, in species identification, PDIM can give supportive role to phenolic glycolipids and mycolates.

Incorporation of labeled substrates like acetate, propionate, palmitate into various mycobacterial cell wall lipid fractions has been studied. These include the studies on biosynthesis of lipids, development of sensitive tools for antimicrobial screening etc. There is limited information on the biosynthesis of phenolic glycolipids and their components<sup>[9-11]</sup>. The carbon atoms in the methyl branched structure in mycocerosic acids has been shown to be derived from propionate<sup>[12,13]</sup>. Phenolphthiocerol dimycocerosates were identified as the intermediates in the biosynthesis of phenolic glycolipids<sup>[11]</sup>. Two steps in the biosynthesis of mycocerosic acid have been demonstrated and the enzymes isolated by Rainwater and Kolattukudy<sup>[14-16]</sup> the biosynthetic steps leading to phenolphthiocerol itself are not known. *In vitro* biosynthesis of PGL-I from 1-C<sup>14</sup>-acetate by *M. leprae* within Schwannoma cell lines has been reported by Mukherjee *et al*<sup>[7]</sup>. Incorporation of [U-<sup>14</sup>C] palmitic acid into phenolic lipid-I (PGL-I) fraction of *M. leprae* in murine macrophage system *in vitro* was found useful in quantitating the metabolism of *M. leprae* in macrophages Ramasesh *et al*<sup>[18]</sup>. These workers reported that the percent incorporation of labeled palmitic acid into PGL-I depended on experimental condition but did not exceed 1% (range 0.4 to 0.6 %). It has also been reported by them that treatment of infected macrophages with rifampicin caused a significant reduction in the incorporation of 1-C<sup>14</sup>-palmitic acid into PGL-I fraction. In our study we have found that the percent incorporation of labeled 1-C<sup>14</sup>-palmitic acid, 1-C<sup>14</sup>-valerate, 1-C<sup>14</sup>-acetate and 1-C<sup>14</sup>-propionate into phenolic glycolipids in *M. bovis* BCG and *M. kansasii* ranged from 0.28% to 0.50%. The incorporation of labeled PGL was least in 1-C<sup>14</sup>-valerate of *M. bovis* BCG and highest in 1-C<sup>14</sup>-propionate of *M. kansasii*. The percent incorporation into wax esters fraction ranged from 0.58 to 0.90 %. The incorporation of labeled PDIM (wax esters) was least in 1-C<sup>14</sup>-valerate of *M. bovis* BCG and highest in 1-C<sup>14</sup>-propionate of *M. kansasii*. The percent incorporation into mycolate fraction ranged from 0.30 to 1.50 %. The incorporation of mycolate was least in 1-C<sup>14</sup>-palmitic acid of *M. bovis* BCG and highest in 1-C<sup>14</sup>-acetate of *M. kansasii*.

The findings of the present study suggest that uptake of radiolabeled substrates like 1-C<sup>14</sup>-palmitic acid, 1-C<sup>14</sup>-valerate, 1-C<sup>14</sup>-acetate and 1-C<sup>14</sup>-propionate and their incorporation into various cell wall lipids will be helpful in studies on viability, lipid biosynthesis and screening of antimicrobials.

## Conclusion

The incorporation of radiolabelled substrates like 1-C<sup>14</sup>-palmitate, 1-C<sup>14</sup>-valerate, 1-C<sup>14</sup>-acetate and 1-C<sup>14</sup>-propionate into PGLs, PDIMs and mycolates was adequate in *M. bovis* BCG and *M. kansasii*. The incorporation of radioactivity into specific lipid fraction as a percentage of total radioactivity assimilated varied from 0.28% in the case of PGL to 1.5% in mycolates. This study suggests the usefulness of radiolabelled substrate incorporation approach in determining the viability of the bacilli, probe into biosynthesis of lipids and screening chemotherapeutic agents for their antimycobacterial efficacy.

**Conflict of interest:** There is no conflict of interest among the authors.

**Acknowledgements:** The authors are grateful to Director, NJIL & OMD for the support. The research was supported by ICMR, New Delhi. Authors are also thankful to Dr. Pradeep Dagur, Mr. Neeraj Dubey, Mr. Vijay Dua and staff of Biochemistry Division.

## References:

1. Board, R.G., Goodfellow M. Microbiological classification and identification. (1980) Academic Press, London 184-256.
2. Ratledge, C., Stanford, J.L. The Biology of Mycobacteria. (1982) Academic Press, London (1): 95-184.
3. Dobson, G., Minnikin, D.E., Minnikin, S.M., et al. 'Chemical methods in bacterial systemics' (Goodfellow, M. and Minnikin, D.E., eds.) (1985) Academic Press, London 237-265.
4. Randall, H.M., Smith, D.W. Characterization of mycobacteria by infrared spectroscopic examination of their lipid fractions. Zentrablatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. (1964) I. Abteilung. 194: 190-201.
5. Draper, P., Payne, S., Dobson, G., et al. Isolation of a characteristic phthiocerol dimycocerosate from Mycobacterium leprae. (1983) J Gen Microbiol 129(2): 859-863.  
[Pubmed](#) | [Crossref](#)
6. Goren, M.B., Brokl, O., Schaefer, W.B., Lipids of putative relevance to virulence in Mycobacterium tuberculosis: Phthiocerol dimycocerosate and the attenuation indicator lipid. (1974) Infect Immun 9(1): 150-158.  
[Pubmed](#)
7. Minnikin, D.E., Lipids, their chemistry, biosynthesis and roles. "The Biology of Mycobacteria" Ratledge C and Stanford J. (1982) London Academic Press 95-184.
8. Dandapat, P., Verma, R., Venkatesan, K., et al. Rapid detection of Mycobacterium bovis on its lipid profile by thin layer chromatography. (1999) Vet Microbiol 65(2): 145-151.  
[Pubmed](#) | [Crossref](#)
9. Gastembide-Odier, M., Sarda, P. Contribution a l'étude de la structure et de la biosynthese de glycolipides spécifiques isolés de mycobactéries, les mycosides A& B. (1970) Pneumology 142(2): 241-255.  
[Pubmed](#) | [Crossref](#)
10. Thurman, P., Draper, P. Biosynthesis of Phenolic glycolipids in *M. microti*. (1989) Acta Leprologica 7 (Suppl 1): 74-76.  
[Pubmed](#)
11. Thurman, P., Chai, W., Rosankiewicz, J.R., et al. Possible intermediates in the biosynthesis of mycoside B by Mycobacterium microti. (1993) Eur J Biochem 212(3): 705-711.  
[Pubmed](#)
12. Gastambide-Odier, M., Delaumeny, J.M., Lederer E. Biosynthese de l'acide C32-mycocerosique: incorporation d'acide propionique.

(1963) *Biochem Biophys Acta* 70: 670-678.

[Pubmed](#) | [Crossref](#)

13. Yano, I., Kusunose, M. Propionate incorporation into mycocerosic acid by resting cells of *Mycobacterium tuberculosis bovis*. (1966) *Biochim Biophys Acta* 116(3): 593-596.

[Pubmed](#) | [Crossref](#)

14. Rainwater, D.L., Kolattukudy P.E. Isolation and characterization of acyl coenzyme A carboxylase from *Mycobacterium tuberculosis* and *Mycobacterium bovis* which produce multiple methyl branched mycocerosic acids. (1982) *J Bacteriol* 151(2): 905-911.

[Pubmed](#) | [Crossref](#)

15. Rainwater, D.L., Kolattukudy, P.E. Synthesis of mycocerosic acids from methylmalonyl A coenzyme by cell-free extracts of *Mycobacterium tuberculosis* var *bovis* BCG. (1983) *J Biol Chem* 258(5): 2979-2985.

[Pubmed](#) | [Crossref](#)

16. Rainwater, D.L., Kolattukudy, P.E. Fatty acid synthesis in *Mycobacterium tuberculosis* var *bovis* Bacillus Calmette-Guerin. Purification and characterization of a novel fatty acid synthase, mycocerosic acid synthase, which elongates n-fatty acyl-CoA with methylmalonyl-CoA. (1985) *J Biol Chem* 260(1): 616-623.

[Pubmed](#) | [Crossref](#)

17. Mukherjee, R., Mistry, V., Antia, N.H., et al. Incorporation of <sup>14</sup>C acetate into specific phenolic glycolipid of *M. leprae*, maintained within cultured cells. (1985) *IRCS Med Sci* 13: 203-204.

18. Ramashesh, N., Hastings, R.C., Krahenbuhl, J.L. Metabolism of *Mycobacterium leprae* in macrophages. (1987) *Infect Immun* 55(5): 1203-1206.

[Pubmed](#)