A sterol biosynthetic pathway in *Mycobacterium*

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Abstract **The genome sequence of *Mycobacterium tuberculosis* (and also *M. leprae*) revealed a significant number of homologies to *Saccharomyces cerevisiae* sterol biosynthetic enzymes. We addressed the hypothesis of a potential sterol biosynthetic pathway existing in *Mycobacterium* using cultures of *Mycobacterum smegmatis*. Non-saponifiable lipid extracts subjected to analysis by gas chromatography-mass spectrometry (GC-MS) showed cholesterol was present. Sterol synthesis by *M. smegmatis* was confirmed using ^{14}C-radiolabelled mevalonic acid and incorporation into C4-desmethyl sterol co-migrating with authentic cholesterol on TLC. The sterol biosynthetic pathway has provided a rich source of targets for commercially important bioactive molecules and such agents represent new opportunities for *Mycobacteria* chemotherapy.**

Key words: Sterol; Biosynthesis; Inhibition; *Mycobacterium*

1. Introduction

The genus *Mycobacterium* comprises a wide array of pathogenic species, which includes *Mycobacterium tuberculosis* (tuberculosis), *Mycobacterium leprae* (leprosy), and *Mycobacterium bovis*. Additionally, a number of *Mycobacterium* spp. found in soil and water can cause systemic disease in severely immuno-compromised people, especially AIDS patients.

Sterols in all living organisms are synthesised from acetic acid via mevalonic acid and squalene (as key intermediates) [1]. Lanosterol is the first cyclisation product in mammals and fungi, while plants produce cycloartenol instead of lanosterol. The final sterol products are cholesterol in mammals, ergosterol in fungi and phytosterols in plants [2]. Sterols are indispensable components in eukaryotic organisms for stabilising membranes composed of phospholipid bilayers. They have also been implicated as having a hormonal role, the so-called 'sparking role' in yeast proliferation [3] and act as meiosis activating factor (MAS) in mammals [4]. Prokaryotic organisms, on the other hand, do not contain sterols in their membranes, although previous studies indicated that the bacterium *Methylococcus capsulatus* produces sterols [5,6] and many strains of *Mycoplasma*, bacteria without cell walls, require sterols as an essential growth factor [6,7]. A report of sterols occurring in *Escherichia coli* has been made erroneously since it has no cytochrome P450 [8]. Some bacterial species synthesise hopanoids, which are thought to function like sterols in eukaryotic organisms [9].

The recently completed genome sequence of *Mycobacterium tuberculosis* [10] revealed one gene to be homologous to the sterol 14α-demethylase biosynthetic enzyme (34% homologous at the amino acid level). Recently an activity of this enzyme as a sterol demethylase has been reported in abstract form [11]. Our interest in sterol biosynthesis coupled to the knowledge that the most complete set of genes encoding structural enzymes of sterol biosynthesis exists for *Saccharomyces cerevisiae* led us to search for homologies in *M. tuberculosis*. This revealed a significant number of homologies in the *M. tuberculosis* genome to the yeast sterol biosynthetic enzymes. These included Rv 3823 (50% to HMG CoA reductase); Rv 1745 (46.9% to isopentenyl pyrophosphate isomerase); Rv 3383c (39.1% to farnesyl pyrophosphate synthetase); Rv 3397 (32.9% to squalene synthase) and Rv 1814 (24% to sterol C5-desaturase). In the present paper, we addressed the hypothesis of a sterol biosynthetic pathway in *Mycobacterium* using cultures of *Mycobacterium smegmatis*, which can be cultured in volume more safely.

2. Materials and methods

2.1. Strains

Mycobacterium smegmatis (Trevisan) Lehmann and Neumann (strain NCTC 10265 (09)) was obtained from the National Collection of Type Cultures, The Public Health Laboratory Service, London.

2.2. Chemicals

Unless specified all chemicals were obtained from Sigma, Poole, Dorset, UK.

2.3. Growth conditions

A single isolated colony of *M. smegmatis*, obtained from plate culture, was placed into 10 ml modified LB medium consisting of 2% (w/v) Difco Bactopeptone, 0.5% (w/v) Difco yeast extract and 0.5% (w/v) sodium chloride and incubated at 37°C with shaking (225 rpm) overnight. Subsequently, 500 ml of the modified medium contained in a 2-l flask was inoculated with 5 ml of the overnight culture and grown at 37°C with shaking to saturation.

2.4. Identification of sterols by GC-MS

Samples for gas chromatography-mass spectrometry (GC-MS) were prepared from 500-ml cultures in growth conditions as described above. Cells were harvested by centrifugation at 1500×*g*. The cell pellet was saponified in 15% (w/v) KOH in 90% (w/v) ethanol at 80°C for 1 h. Non-saponifiable lipids (sterols and sterol precursors) were extracted with 3×5 ml heptane and dried under nitrogen. Following silylation for 1 h at 60°C with BSTFA (50 μl) in 50 μl of toluene, sterols were analysed by GC-MS (VG 12-250 (VG Biotech) using split injections with a split ratio of 20:1. Sterol identification was by reference to relative retention time and mass spectra as reported previously [2,12].

2.5. Synthesis of [^{14}C]cholesterol from [2-^{14}C]mevalonic acid

[2-^{14}C]Mevalonic acid (100 μl; 0.25 μCi) was added to 500 ml modified LB medium prior to the addition of the overnight culture of *M. smegmatis*. Cultures were incubated for 24 h at 37°C with shaking (150 rpm). Non-saponifiable lipids (sterols and sterol precursors) were isolated as described above, extracted with hexane and dried under nitrogen. The non-saponifiable lipid was applied to silica gel thin layer chromatography (TLC) plates (ART 573, Merck) and

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developed using toluene/diethyl ether, 9:1 (v/v). Radioactive sterol was located by autoradiography, identified by comparison following co-chromatography of known standards and excised for scintillation counting.

3. Results

Fig. 1 shows the GC-MS spectrum obtained following analysis of non-saponifiable lipid (sterol and sterol precursors) extracted from 500 ml culture of *M. smegmatis*. Surprisingly for this prokaryotic organism, a predominant sterol identified as cholesterol was observed in the extract, which was confirmed by mass spectral analysis compared to authentic cholesterol standard and cholesterol co-chromatographic studies. Minor peaks representing cholesterol derivatives, including dimethylcholesterol, were also observed. Cholesterol was found to be only a minor constituent of the bacterial cell representing 0.001 mg/g dry cell weight. These results confirm the hypothesis of the presence of a sterol biosynthetic pathway in *M. smegmatis* and are consistent with the presence of predicted *Mycobacterium* sterol biosynthetic enzymes.

Confirmatory evidence for the presence of a cholesterol biosynthetic pathway within *M. smegmatis* was also obtained from radiolabel feeding experiments utilising one of the earliest precursor molecules within the sterol pathway, namely [2-^{14}C]mevalonic acid. Fig. 2 shows the results of such experiments, which reveal that growing *M. smegmatis* could utilise [2-^{14}C]mevalonic acid, and incorporate radiolabel into a C4-desmethyl sterol ([^{14}C]cholesterol).

4. Discussion

The synthesis of cholesterol is a metabolically expensive process. Due to the metabolic cost to the organism, cholesterol is likely to be an essential cellular component for it to have emerged and persisted as a cellular constituent during the evolution of *Mycobacterium smegmatis*. Consequently, the ability of the *Mycobacteria* genus to synthesise sterol endogenously and its functional role within the bacterial cell is intriguing. Cholesterol is the predominant sterol in all mammalian cells, is a vital constituent of cell membranes and the precursor of steroid hormones and bile acids. However, cholesterol was shown to be only a minor sterol constituent within *M. smegmatis*. Thus, the role of cholesterol to the cell deserves further clarification.

Ergosterol, the principal sterol in most fungi, is an indispensable component in membrane structures [13]. The antifungals developed in the pharmaceutical and agrochemical sectors have targeted the ergosterol biosynthetic pathway to cause inhibition of ergosterol synthesis in fungi and ultimately cell growth arrest. Inhibitors of 14α-demethylation of lanosterol and 24-methylene-24,25-dihydrolanosterol are the most widely discovered class of sterol biosynthesis inhibitors [14]; they include substituted pyrimidines, pyridines, piperazines, triazoles and imidazoles. Morpholines are the second class of inhibitors; they inhibit $\Delta^8 \rightarrow \Delta^7$ isomerisation (the conversion of fecosterol to episterol) and block Δ^{14} reduction [15]. Further commercial antifungal drugs target squalene epoxidase (allyamines) [16]. Large efforts have also been made to

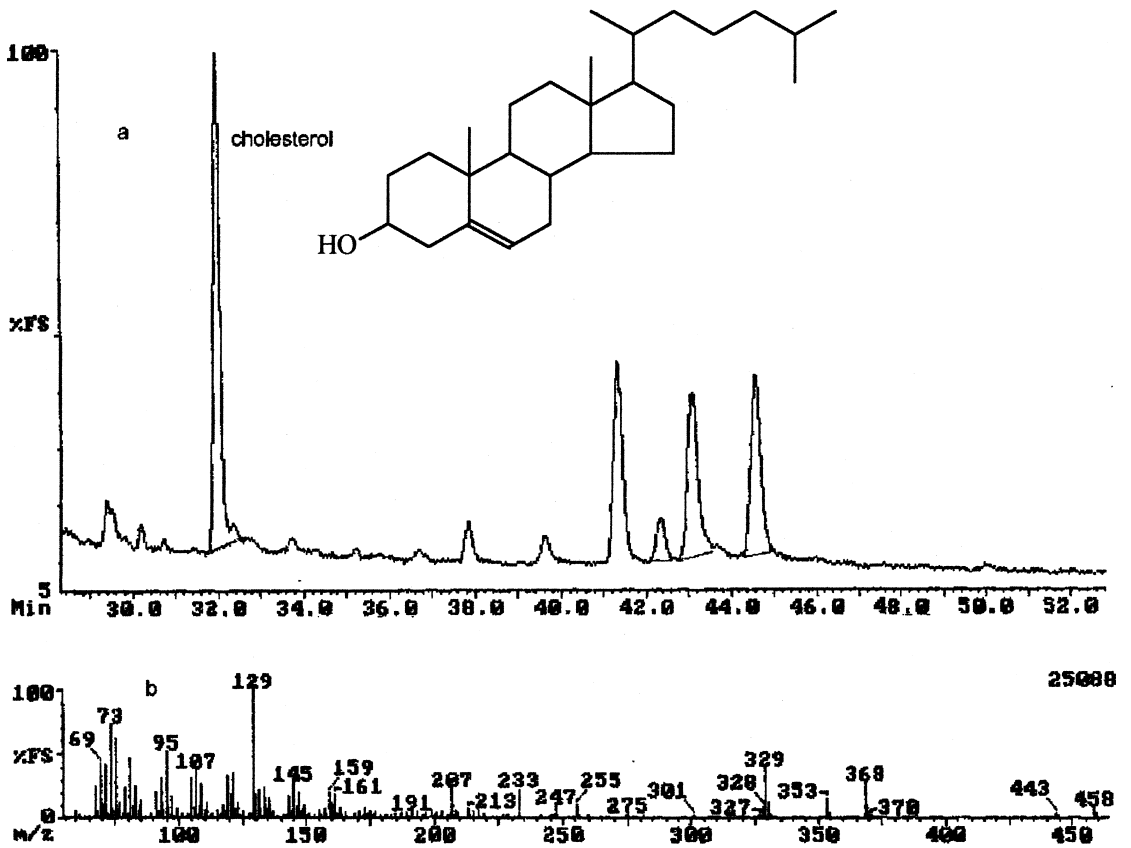

Fig. 1. GC-MS analysis of trimethylsilated derivatised non-saponifiable lipid extracted from *M. smegmatis* grown on modified L-broth, as described in Section 2. Cholesterol was the most abundant sterol detected by gas chromatography (a) and identity was confirmed from the mass spectrum (b).

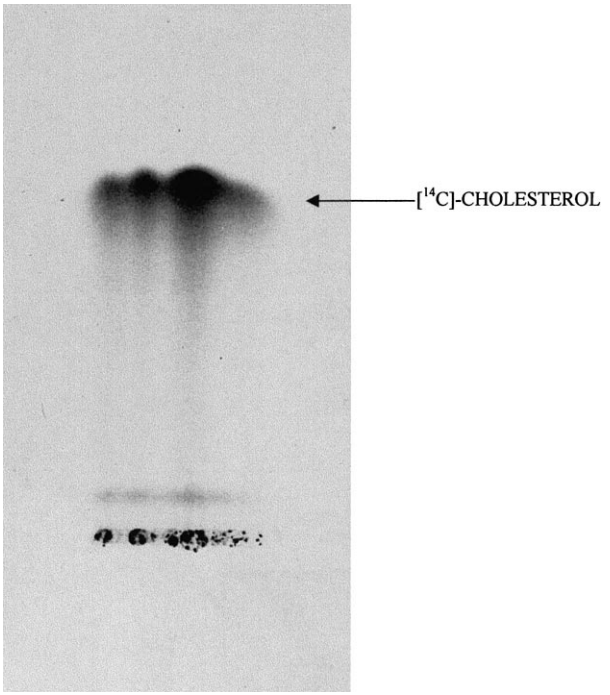

Fig. 2. Autoradiogram after TLC separation of non-saponifiable lipid extract of *M. smegmatis* utilising [2-^{14}C]mevalonic acid, and showing incorporation of radiolabel into a C4-desmethyl sterol ([^{14}C]cholesterol).

develop anti-cholesterol agents inhibiting HMG CoA reductase (pravastatin) [17] and other steps of sterol biosynthesis. New therapeutic compounds are needed urgently due to the emergence of drug resistant isolates of *M. tuberculosis* and the increased incidence of such infections in immunocompromised patients. Sterol biosynthesis inhibitors may represent an alternative strategy for combating this disease and the many existing drugs and experimental compounds are an attractive prospect for developing proof of this hypothesis.

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