Analogues of thiolactomycin: potential drugs with enhanced anti-mycobacterial activity

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Analogues of the antibiotic thiolactomycin (TLM) have been synthesized and have been shown to have enhanced activity against whole cells of Mycobacterium tuberculosis H37Rv and against mycolic acid biosynthesis in cell extracts of Mycobacterium smegmatis. TLM has a methyl-branched butadienyl side chain attached at position 5 on a ‘thiolactone’ ring, namely 4-hydroxy-3,5-dimethyl-5H-thiophen-2-one. Various combinations of strong bases were explored to create a reactive anion at position 5 on the thiolactone ring which could react with halides to produce 5-substituted derivatives; the best reagent was two equivalents of lithium-bis-(trimethylsilyl)amide in tetrahydrofuran.

The analogue with a 5-tetrahydrogeranyl substituent showed the best biological activity with an MIC90 for M. tuberculosis of 29 µM and 92% mycolate inhibition in extracts of M. smegmatis, as compared to 125 µM and 54%, respectively, for TLM; other related C10 and C15 isoprenoid derivatives had similar biological activity. These isoprenoid-based derivatives did not inhibit type II fatty acid synthase from M. smegmatis, but compounds with iso-butyl and iso-butenyl side chains did show some inhibitory activity against this enzyme. These short-chain derivatives did not inhibit mycolate synthesis or have significant antibiotic activity. Treatment of the thiolactone with a weaker base, sodium hydride in tetrahydrofuran, gave 3-alkyl-3,5-dimethyl-thiophene-2,4-dione analogues, which had no effect on fatty acid or mycolate synthesis. However, the geranyl derivative had an MIC99 of 60 µM for M. tuberculosis, one quarter that (240 µM) of TLM, demonstrating its excellent antibiotic potential against an unknown cellular target.

Keywords: mycobacteria, mycolic acids, antibiotics, tuberculosis

INTRODUCTION

The structure and antibiotic properties of thiolactomycin (TLM) (1) were first reported by Oishi and co-workers (Oishi et al., 1982; Noto et al., 1982; Sasaki et al., 1982; Nawata et al., 1989). TLM was the first example of a naturally occurring thiolactone to exhibit antibiotic activity. The compound has moderate in vitro activity against a broad spectrum of pathogens, including Gram-positive and Gram-negative bacteria (Noto et al., 1982; Hamada et al., 1990) and Mycobacterium tuberculosis (Slayden et al., 1996; Choi et al., 2000; Kremer et al., 2000). TLM has also shown encouraging anti-malarial activity, involving inhibition of the type II fatty acid biosynthetic pathway in apicoplasts (Waller et al., 1998; Morita et al., 2000). TLM has chemotherapeutic potential, as it is non-toxic to mice and affords significant protection against urinary tract and intraperitoneal bacterial infections (Miyakawa et al., 1982). A series of 3-acetyl analogues of TLM have been prepared (Sakya et al., 2001), with several having activity against Staphylococcus aureus and Pasteurella multocida.
Methods

Chemical methods and materials. 1H and 13C NMR spectra (δH and δC, chemical-shift data are expressed as p.p.m.) were recorded on Bruker WP 200 or WM 300-WB and JEOL Lambda 500 instruments. J values for coupling constants are in Hz; singlets, doublets, triplets, quartets and multiplets are signified by s, d, t, q and m, respectively. Melting points (uncorrected) were determined on a Linkam HFS91 heating stage, using a TC92 controller; recrystallization was not performed due to unusual difficulties for these compounds. Electron impact (EI) mass spectra were recorded on a Kratos Lambda 500 instruments.

Early studies showed that TLM activity was directed at type II fatty acid synthases (Hayashi et al., 1983, 1984). The use of [1-14C]acetate labelling in pea-leaf chloroplasts confirmed that TLM, and some of its analogues, inhibit type II dissociable fatty acid synthases (Jones et al., 1994, 2000). Through the use of in vivo [1,2-14C]acetate labelling of Mycobacterium smegmatis, TLM was shown to inhibit the biosynthesis of both fatty acids and mycolic acids (Slayden et al., 1996), which are the characteristic major 2-alkyl-branched 3-hydroxy fatty acids in mycobacteria. Synthesis of the shorter-chain mono-unsaturated ω-mycolates of M. smegmatis was not affected by TLM, but TLM did inhibit the synthesis of the longer chain di-unsaturated ω-mycolates and epoxymycolates.

The use of M. smegmatis cell extracts confirmed that TLM specifically inhibited the mycobacterial acyl-carrier-protein-dependent type II fatty acid synthase (FAS-II), but it did not inhibit the multifunctional type I fatty acid synthase (FAS-I) (Slayden et al., 1996). Analysis of the in vivo and in vitro data has suggested two separate sites of action for TLM: the β-ketoacyl-acyl carrier protein synthase in FAS-II and the elongation step involved in the synthesis of ω-mycolates and oxygenated mycolates. In a recent study (Kremer et al., 2000), it was shown that the enzymes targeted by TLM are KasA and KasB, which are involved in fatty acid and mycolic acid biosynthesis in M. tuberculosis. It was also indicated that a number of TLM analogues with different side chains in position 5 of the thiolactone ring also gave enhanced activity. This study presents details of the synthesis and additional properties of these and other analogues of TLM.
Table 1. Synthesis of 5-substituted analogues of TLM and their activity against M. tuberculosis and extracts of M. smegmatis

The biological data have been reported previously (Kremer et al., 2000).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Synthetic route</th>
<th>Yield (%)</th>
<th>Inhibition of activity in extracts from M. smegmatis (%)*</th>
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<tr>
<td></td>
<td></td>
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<td>MIC90</td>
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<tr>
<td>TLM</td>
<td>–</td>
<td>56</td>
<td>125</td>
</tr>
<tr>
<td>3</td>
<td>A, B</td>
<td>20, 53</td>
<td>96</td>
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<tr>
<td>4</td>
<td>C</td>
<td>22</td>
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<td>5</td>
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<tr>
<td>10</td>
<td>A</td>
<td>11</td>
<td>50</td>
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</table>

*Negative values indicate stimulation; results are shown ±SD.

acetate. The organic layers were combined, washed with saturated brine solution, dried over MgSO₄ and reduced in vacuo to give the crude product as a yellow oil. Flash column chromatography, eluting with 2% ethyl acetate in petrol, afforded the pure product as a yellow oil in a 53% yield (52 mg) and with analytical data identical to that recorded above.

4-Hydroxy-3,5-dimethyl-5-octyl-5H-thiophene-2-one

Following Procedure C, thiolactone (16) (250 mg, 1.7 mmol), t-butyllithium in hexanes (2.04 ml, 3.4 mmol) and 1-bromo-octane (0.45 ml, 2.6 mmol) in anhydrous THF (10 ml) afforded the crude product as a yellow oil. Flash column chromatography, eluting with 5% up to 20% ethyl acetate in petrol (5% increments), yielded the racemic title compound (110 mg, 22% yield) as a sticky, yellow solid; νmax (cap film)/cm⁻¹ 1611m and 1076s; δH (200 MHz CDCl₃) 0.80 (t, J 6-6, 3H, 15-H), 1.10–1.41 (m, 12H, 9-H, 10-H, 11-H, 12-H, 13-H and 14-H), 1.59 (s, 3H, 6-H), 1.67 (s, 3H, 7-H), 1.74–1.82 (m, 2H, 8-H); δC (500 MHz CDCl₃) 7.6 (C-6), 14.2 (C-15), 22.7 (C-14), 25.3 (C-13), 26.1 (C-7), 29.3 (C-12), 29.4 (C-11), 29.7 (C-10), 31.9 (C-9), 38.7 (C-8), 57.7 (C-5), 110.7 (C-3), 179.1 (C-4), 196.3 (C-2); m/z (EI) 256:1494 (M⁺ 5% C₁₅H₂₅O₄S requires 256:1497), 144 [MH⁺–(CH₃)₂CH₃ 100%].

4-Hydroxy-3,5-dimethyl-5-(3-methyl-butyl)-5H-thiophene-2-one

As the sole example of Procedure D, thiolactone (2) (310 mg, 2.2 mmol) was dissolved in anhydrous THF (10 ml) under nitrogen at −78 °C. Sodium bis-trimethylsilyl amide (1 M, 2.2 ml, 2.2 mmol) was added slowly to the mixture followed by the drop-wise addition of t-butyllithium in hexanes (1.7 M, 1.3 ml, 2.2 mmol). After stirring for 30 min at −78 °C, 1-bromo-3-methylbutane (290 µl, 2.4 mmol) was added dropwise to the reaction mixture, which was allowed to stir at room temperature for 16 h. The reaction mixture was then
acidified with 2 M aqueous acetic acid and partitioned between saturated brine (10 ml) and ethyl acetate (20 ml). The aqueous layer was re-extracted with ethyl acetate (2 × 20 ml), and the organic phases were combined, dried (MgSO₄), and reduced in vacuo to yield the crude product as a yellow oil. Flash column chromatography, eluting with 10% up to 50% ethyl acetate in petrol (5% increments), yielded the racemic title compound (270 mg, 57% yield) as an off-white solid; melting point 107–109 °C; v_max (KBr disc)/cm⁻¹ 1599s, 1092s; δ_H (200 MHz CDCl₃) 0.89 (6H, d, J 6.5, 11-H and 12-H), 1.26–1.61 (3H, m, 8-H, 9-H and 10-H), 1.67 (3H, s, 6-H), 1.75 (3H, s, 7-H), 7.95 (1H, s, OH); δ_C (125.7 MHz CDCl₃) 7.5 (C-6), 22.5, 28.2 and 34.1 (C-9, C-10, C-11 and C-12), 26.0 (C-7), 36.4 (C-8), 57.9 (C-5), 110.1 (C-33), 178.0 (C-4), 206.2 (C-2); m/z (EI) 214/1023 (M⁺ 60% C₁₁H₁₀O₅S requires 214/1028).

3,3,5-Trimethyl-thiophene-2,4-dione

According to Procedure E, thiolactone (2) (290 mg, 2 mmol) was dissolved in anhydrous THF (10 ml) under nitrogen at 0 °C. In a separate flask, sodium hydride (100 mg, 4 mmol) was washed with hexanes (× 3) and added in small portions to the reaction mixture. The reaction mixture was stirred for 30 min before the drop-wise addition of iodomethane (140 µl, 2.5 equiv.). The reaction mixture was washed with hexanes (30 ml), dried (MgSO₄) and the supernatant was centrifuged at 10000 g for 21 days and the viable number of bacteria was determined by counting colonies. MICs were also defined as the lowest concentration of material required to produce 50, 90 or 99% end-points in broth media.

Incorporation of [1,2-14C]acetate into fatty acids and mycolic acids. M. smegmatis was grown in a broth medium in 20 tubes to the mid-exponential phase, at which point TLM (75 µg ml⁻¹), for example, was added to half of the tubes. Further incubation followed at 37 °C for 1 h. Uniformly labelled [1,2-14C]acetate (2-11 GBq mmol⁻¹; Amersham Biosciences) was added at 37 kBq mmol⁻¹ to both sets of tubes and they were further incubated at 37 °C, with gentle agitation, for 16 h. The 14C-labelled cells were harvested by centrifugation at 2000 g and washed twice with 0.9% aqueous sodium chloride and once with water.

Effects of substrates on fatty acid and mycolic acid metabolism. One set of 14C-labelled control and substrate-treated cells, for example, was subjected to alkaline hydrolysis in 15% aqueous tetrabutylammonium hydroxide at 100 °C overnight followed, after cooling to room temperature, by the addition of 2 ml CH₃Cl and 100 µl CH₃I. The entire reaction mixture was mixed on a shaking table for 30 min, centrifuged and the upper aqueous phase discarded. The lower organic phase was washed with 10% aqueous HCl followed by water; it was then dried. A known aliquot (~ 100000 c.p.m.) of the resultant mixture of fatty acid methyl esters (FAMEs) and α, ω- and epoxy-mycolic acid methyl esters (MAMEs) was analysed by TLC (Merck 5737 silica gel 60F254), developing twice in petroleum ether/acetone (95:5). Autoradiograms of the TLC plates were produced by overnight exposure of the plates to Kodak X-Omat AR film at −70 °C. This revealed the 14C-labelled FAMEs (R_f 0.9) and α, ω- and epoxy-MAMEs (R_f 0.4–0.5), which were counted directly to estimate the total synthesis and the degree of inhibition of synthesis of FAMEs and MAMEs.

Preparation of soluble cytosolic and particulate cell-wall fractions. M. smegmatis (30 g, wet wt) was washed and resuspended in a buffer (30 ml) containing 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM DTT, 5 mM MgCl₂ and 2 mM PMSF at 4 °C and subjected to probe sonication for 10 min in 10 × 60 s pulses with 90 s cooling intervals between pulses. The sonicate was centrifuged at 27000 g for 30 min at 4 °C and the supernatant was centrifuged at 100000 g for 1 h to yield the soluble pale-yellow enzyme fraction, with a typical 140730
protein concentration of 8–10 mg ml$^{-1}$ and containing FAS-I and FAS-II enzyme activities. The particulate (P60) cell-wall enzyme fraction with mycolate-synthesizing activity was prepared as described previously (Wheeler et al., 1993a).

Assays for FAS-I, FAS-II and mycolate-synthesizing activities.

The standard reaction mixture for the incorporation of radioactivity from [2-14C]malonyl-CoA (1887 GBq mmol$^{-1}$; NEN Life Science Products) into C$_{16}$ and C$_{34}$ fatty acids catalysed by FAS-I was composed as follows: 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM DTT, 300 µM acetyl-CoA, 100 µM NADPH, 100 µM NADH, 1 µM FMN, 500 µM α-cyclodextrin, 20 µM malonyl-CoA, 100000 c.p.m. of [2-14C]malonyl-CoA and 100 µl of the cytosolic enzyme preparation (1–2 mg of protein) in a total volume of 500 µl. Similarly, the standard reaction mixture for the incorporation of radioactivity from [2-14C]malonyl-CoA into C$_{24}$ to C$_{32}$ fatty acids catalysed by FAS-II contained the following: 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM DTT, 100 µM palmitoyl-CoA, 140 µM NADPH, 140 µM NADH, 180 µg acyl carrier protein (ACP), 40 µM malonyl-CoA, 200000 c.p.m. of [2-14C]malonyl-CoA and 100 µl of the cytosolic enzyme preparation (1–2 mg of protein) in a total volume of 500 µl. For both assays, TLM (200 µM) was mixed, as were the other analogues, with the other assay components prior to the addition of protein. In both the FAS-I and the FAS-II assay, reactions were terminated by the addition of 500 µl of 20% potassium hydroxide in 50% methanol at 100 °C for 30 min. Following acidification with 300 µl of 6 M HCl, the resultant 14C-labelled fatty acids were extracted three times with petroleum ether. The organic extracts from both assays were pooled, washed once with an equal volume of water and dried in a scintillation vial prior to counting. It should be noted that FAS-II activity was identified as the label that appeared in all fatty acids, as a result of adding palmitoyl-CoA to the assay system; individual products were not identified.

Incubations utilizing the mycolate-synthesizing P60 cell-wall fraction were performed as described by Wheeler et al. (1993a). The reaction mixture contained 50 mM potassium phosphate (pH 6.0), 10 mM KHCO$_3$, 37 kBq of [1,2-14C]acetate and 440 µl of the P60 preparation (2.0–3.5 mg of protein) in a total volume of 1 ml. TLM (200 µM) was mixed, as were the other analogues, with the other assay components prior to the addition of protein. Reaction mixtures, which were prepared in quadruplicate at each concentration, were incubated at 37 °C for 1 h. Reactions were terminated by the addition of 2 ml of 15% aqueous tetrabutylammonium hydroxide to the reaction mixtures; the corresponding FAMEs and MAMEs were isolated and counted as described previously.

RESULTS AND DISCUSSION

Chemical synthetic strategy

The key intermediate thiolactone (2) was prepared according to Wang & Salvino (1984) and converted into a variety of derivatives. If the thiolactone (2) is treated with basic reagents, either one or two protons can be abstracted, as shown in the scheme in Fig. 1. A relatively moderate base, such as sodium hydride (Procedure E), removes only the enol proton, resulting in alkylation at ring position 3 on treatment with an alkyl halide (RX).

Very strong bases (Procedures A–D) will remove an additional proton from position 5 of the thiolactone ring, and this more reactive anionic centre in the dianion reacts preferentially with an RX to give 5-substituted derivatives (scheme in Fig. 1). A chiral synthesis of TLM was reported by Chambers & Thomas (1997).

Comparison of synthetic procedures for analogues substituted at position 4 on the thiolactone ring

Wang & Salvino (1984) produced the dianion by treating the thiolactone intermediate (2) with 1 equiv. of sodium hydride at 0 °C in 1:1 THF/hexamethylphosphoramide (HMPA), followed by 1 equiv. of n-butyl lithium at −20 °C. The necessity of using the potent carcinogen HMPA to produce the dianion was undesirable, so alternative ways of producing it were investigated. The first method (Procedure A) used THF solvent, with sodium hydride removing the enol proton assisted by t-butyl lithium to create the dianion (scheme in Fig. 1). This strategy proved effective in the synthesis of a number of analogues, but yields were disappointing (11–33%, compounds 3 and 6–10; Table 1). Consequently, the more straightforward use of 2 equivs of t-butyl lithium in THF (Procedure C) to produce the dianion was explored, but the yield (22%) for compound (4) was poor (Table 1). In one experiment for the production of the dianion from the thiolactone intermediate (2), a better combination appeared to be 1 equiv. of sodium-bis-(trimethylsilyl)amide (1 M in THF) and 1 equiv. of t-butyl lithium (Procedure D), producing a respectable yield (57%, compound 5; Table 1). Subsequently, it was found that 2 equivs of lithium-bis-(trimethylsilyl)amide in THF (Procedure B) was very convenient for this alkylation step, as demonstrated for an alternative preparation of compound (3) (53%, Table 1).

Effect of 5-substituted analogues on mycolate synthesis in M. smegmatis

Analogues of TLM, substituted at position 5 of the thiolactone ring, were assayed for their inhibition of mycolic acid biosynthesis in preparations of cell-wall material from M. smegmatis, prepared according to an established procedure (Wheeler et al., 1993a). TLM and its analogues were assayed at a concentration of 200 µM
and each experiment was repeated four times. On completion of the experiments, a combination of TLC and autoradiography was used to reveal the $^{14}$C-labelled FAMEs and MAMEs; these were counted directly to estimate the total synthesis and the degree of inhibition of synthesis of FAMEs and MAMEs. The values for the percentage inhibition of mycolic acid biosynthesis are shown in Table 1.

The 3-methyl-2-butenyl side chain (6) offered 51% inhibition of the synthesis of FAMEs and MAMEs, compared to an inhibition of 54% for TLM (1). An inhibition of 80% of the synthesis of these esters was observed for the trans-geranyl (3) and the trans-trans-farnesyl analogue (7). Saturating one double bond of the trans-geranyl side chain (8) increases inhibitory activity further (93%), as does saturating both of the double bonds (9) (92%). Decreasing the length of the side chain to a 3-methylbutyl group (5) reduces inhibitory activity considerably (45%), as does the introduction of a 3-methylbenzophenone side chain (10) at position 5 of the ring (21%). However, the benzophenone analogue is considered active enough for possible photoaffinity labelling investigations (Prestwich et al., 1997). The analogue with the octyl side chain (4) was made to investigate the importance of the C-3 methyl branch, as it had been reported previously (Jones et al., 1994, 2000) to have activity in pea extracts.

Effect of 5-substituted analogues on FAS-II from M. smegmatis

The effect of TLM analogues on mycobacterial FAS-II activity was studied in extracts of M. smegmatis. Details of the standard reaction mixture for incorporation of radioactivity from [2-$^{14}$C]malonyl-CoA into C$_{30}$ to C$_{40}$ fatty acids, catalysed by FAS-II, can be found in Methods. The reactions were performed in triplicate at concentrations of 1.0 mM substrate; the corresponding FAMEs and MAMEs were isolated and counted as described earlier. The more hydrophobic compounds with the longer side chains (compounds 3 and 7–9; Table 1) were completely inactive against FAS-II. The shorter-chain analogues (compounds 5 and 6), by contrast, did show activity against FAS-II.

Activity of 5-substituted compounds against M. tuberculosis H37Rv

MIC$_{50}$ and MIC$_{90}$ values for the analogues against M. tuberculosis H37Rv were determined (Table 1). Compared to TLM (1), the two four-carbon side-chain analogues (compounds 5 and 6) had much reduced activity against M. tuberculosis H37Rv (Table 1). The trans-geranyl analogue (compound 3) appears to be relatively inactive against the bacterium, but sequential saturation of one (8) or two (9) double bonds gives good activity (Table 1). Increasing the length of the side chain to trans-trans-farnesyl (7) also gives enhanced activity (Table 1). The benzophenone analogue (10) has activity equal to that shown for TLM (1) (Table 1).

Structure–activity conclusions for 5-substituted analogues

According to studies on the interaction of TLM with FAS-IIs from E. coli, TLM binds reversibly to the active sites of FabB, FabF and FabH (Heath et al., 2001; Price et al., 2001). This inhibition is competitive for malonyl acyl carrier protein but not for acetyl-CoA, suggesting that TLM mimics the binding of the former. Specifically, the crystal structure of the FabB/TLM adduct shows key binding of the carbonyl oxygen, at position 2 (1), with the two catalytic histidines and water-assisted hydrogen bonding of the enol group, at position 4, to amino acid residues 270 and 305. The thiolactone sulfur does not make any specific contacts. The two ring methyl group substituents, at positions 3 and 5, are accommodated in respective hydrophobic pockets formed from Phe229/Phe392 and Pro272/390. The unsaturated side chain, at position 5, specifically fits into a crevice between the two peptide bonds linking residues 391–392 and 271–272.

The present results (Table 1) show that modifications of TLM can provide analogues with enhanced activity against M. tuberculosis H37Rv and mycolic acid synthases from M. smegmatis; no analogues had increased activity against FAS-II in the latter species. The M. smegmatis FAS-II enzyme system may be the mycobacterial synthase most analogous to the E. coli type II enzyme described above (Heath et al., 2001; Price et al., 2001). It was argued that the planarity of the side chain in TLM (1) might be an important contributing factor for successful binding. This hypothesis is supported by the finding that for the only two TLM analogues (5 and 6) with any anti-FAS-II activity (Table 1) the most active (6) has a double bond. It is also notable that these two TLM analogues (5 and 6) have branched four-carbon side chains, as in TLM (1). Perhaps the mycobacterial FAS-II enzyme is more restricted beyond any binding cleft than that in the Pismum sativum type II synthase, against which TLM analogue (3) was particularly active (Jones et al., 1994, 2000).

The P60 (particulate) cellular preparation from M. smegmatis, prepared according to Wheeler et al. (1993a), is a proven way of assessing mycolate biosynthesis in the presence of inhibitors that might be degraded by intact organisms or which might not gain ready access to the biosynthetic site. This was demonstrated very effectively by the specific inhibition of the mycolate Δ-5 desaturase by extremely labile cyclopropene fatty acids (Wheeler et al., 1993b). The two TLM analogues with branched four-carbon side chains (5 and 6) had comparable activity to TLM (1), with the presence of the double bond in (6) again appearing to be beneficial (Table 1). Therefore, analogue (6) appears to be a good surrogate for TLM (1) in studies of mycolate biosynthesis. The two fully trans unsaturated isoprenoid TLM analogues, with geranyl (3) and farnesyl (7) substituents, had essentially the same 80% inhibitory effect, showing that an additional five-carbon unit was unimportant (Table 1). In comparison with the geranyl analogue (3),
Table 2. Activity of 3-substituted analogues of TLM against M. tuberculosis and extracts of M. smegmatis

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Structure</th>
<th>Yield (%)</th>
<th>In vivo activity against M. tuberculosis (μM)</th>
<th>Inhibition of activity in extracts from M. smegmatis (%)†</th>
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*Analogues (11) and (12) were synthesized via route E.
†Negative values indicate stimulation; results are shown ±so.

saturation of one (dihydrogeranyl) (8) or both (tetrahydrogeranyl) (9) of the double bonds raises mycolate inhibition to over 90% (Table 1), strongly suggesting that unsaturation is unnecessary in the side chain. The TLM analogue with a benzophenone substituent (10) has sufficient anti-mycolate activity to allow its possible use as a photo-activated probe for the enzyme site, according to previous guidelines (Prestwich et al., 1997). The positive activity of (10) also shows that aromatic rings can be tolerated in the enzyme active site.

Interesting trends can also be discerned in the activity of the 5-substituted TLM analogues against M. tuberculosis H37Rv (Table 1). It is reasonable to assume that the prime site of mycobactericidal action remains mycolate synthesis, but additional factors must include cell-envelope permeability and degradative enzymes. The two TLM analogues (5) and (6) have little activity (Table 1) compared to TLM (1). As compounds (1), (5) and (6) have five-carbon 5-substituents, it is likely that cell-envelope penetration by these compounds is comparable. It can be speculated that the terminal double bond and perhaps the double bond conjugation in TLM (1) is less attractive to degradative enzymes than the exposed methyl groups in analogues (5) and (6). Such a hypothesis is perhaps not supported by the analogues with 10-carbon side chains (3, 8 and 9), though such generalizations are impossible without precise knowledge of the exact metabolic pathways for each compound. Compounds (3), (8) and (9) should be readily assimilated by M. tuberculosis, but the anti-mycobacterial activity increases with decreased unsaturation of the side chain; potent activity is given by the tetrahydrogeranyl compound (9) (Table 1). However, increased saturation of the side chain is not the only factor governing activity, as the farnesyl derivative (7) also shows excellent potency (Table 1). It is possible that the 15-carbon side chain in (7) allows enhanced delivery of the compound to the active site, overriding possible increased susceptibility to degradation as possibly shown by the geranyl derivative (3). In retrospect, it might have been very informative to prepare and test an analogue of (7) with a saturated 15-carbon isoprenoid 5-substituent. The benzophenone analogue of TLM (10) is as good an antibiotic against M. tuberculosis H37Rv as TLM (1) (Table 1). Since the activity of (10) against mycolate synthesis is roughly half that of TLM (1) (Table 1), it is likely that increased cell-envelope permeability and chemical stability are compensatory factors. This latter result indicates that the preparation and analysis of a range of TLM analogues with aromatic substituents at position 5 might be rewarding.

The findings summarized above show that some TLM analogues have activity profiles that are distinct from TLM itself. TLM (1) appears to be a general inhibitor of type II fatty acid condensing enzymes (e.g. FAS-II and mycolate synthases) but the analogues with longer side chains (3 and 7–9) inhibit mycolate synthases more strongly than TLM (1) and actually stimulate FAS-II (Table 1). Detailed knowledge of the individual enzymes will be necessary to provide an explanation of these facts. It proved impossible to evaluate the activity of the 5-octyl TLM analogue (4) (Table 1) to ascertain the necessity for chain branching. However, in studies on pea-leaf chloroplasts (Jones et al., 1994, 2000), it was found that TLM analogue (4) had similar activity to the geranyl derivative (3), suggesting that branching is not too important for activity.

In a previous study, Slayden et al. (1996) showed that TLM allows the synthesis of ~60-carbon mono-unsaturated α-mycolates in M. smegmatis, but inhibits the synthesis of ~70–80-carbon di-unsaturated α-mycolates in this organism. Detailed analyses of the mycolate profiles obtained after treatment of M. smegmatis extracts with TLM analogues were not recorded in the present study, so further explanation of the differential action of TLM on α- and α′-mycolates is not possible. Suffice it to say that α′-mycolate synthesis in M. smeg-
matis may involve a condensing enzyme that is quite distinct from FAS-II and mycolate synthases. Mono-
unsaturated ν’-mycolates are not produced by M. tuberculosis but they are major components of the
opportunist pathogen Mycobacterium chelonae (Min-
nikin et al., 1982). Therefore, it could be of immediate
value to carry out a detailed study of the effect of TLM
and its analogues (Table 1) on mycolic acid synthesis in
M. chelonae.

Synthesis and activity of ketothiolactone analogues
alkylated at position 3

The 3-alkylated 4-ketothiolactone analogues (11 and 12; 
Table 2) were synthesized by the addition of 1 equiv. of
sodium hydride to thiolactone (2), followed by an alkyl
iodide (see scheme in Fig. 1). The ketothiolactone
analogues were assayed for their activity against my-
colic acid biosynthesis and FAS-II in extracts from
M. smegmatis (Table 2). MIC_{99} values for these com-
pounds against M. tuberculosis H37Rv are also reported
(Table 2).

The ketothiolactone analogues (11 and 12) appear to
be inactive against both mycolic acid biosynthesis and
FAS-II, but they have promising effects against M. tu-
berculosis H37Rv.

The methyl analogue (11) had an MIC_{99} value equivalent
to that of TLM (1) (240 μM). The more hydrophobic
ketothiolactone analogue (12), with a trans-geranyl group, had
increased activity, as shown by an MIC_{99} value of 60 μM
(Table 2). The anti-mycobacterial activity of TLM
analogue (12) (Table 2) is, therefore, similar to that of
analogues (7) and (9) (Table 1). The site of action of
TLM analogues (11) and (12) remains to be pinpointed,
but potential new antibiotic principles have been high-
lighted by these compounds.

Conclusions

The principle that analogues of TLM (1) might have
enhanced activity against FAS-IIs was established by
Jones et al. (1994, 2000). In particular, it was found that
the TLM analogue with a 5-geranyl substituent (3)
showed greatly enhanced inhibition of fatty acid con-
densing enzymes in pea-leaf chloroplasts. The chemical
syntheses of the analogues studied by Jones et al. (1994,
2000) were not reported; hence, the study presented here
describes the development of the necessary synthetic
procedures for a range of such TLM analogues. The
essential biological activity of the majority of the
5-substituted TLM analogues (3 and 5–10; Table 1) has
been reported previously (Kremmer et al., 2000), but with
limited interpretation. The novel 3-substituted TLM
analogue (11 and 12) (Table 2) are reported for the first
time.

TLM (1) is an important anti-microbial agent of great
utility in studies of fatty acid biosynthesis (Heath et al.,
2001). Its synthesis is not facile, however, and some of
the TLM analogues (Table 1) can be recommended as
more accessible surrogates. As an agent against
M. tuberculosis and an inhibitor of mycolic acid syn-
thesis, the fully saturated tetrahydrogeranyl analogue
(9) recommends itself (Table 1); the lack of side chain
double bonds in this agent should enhance its chemical
stability. TLM remains the best inhibitor of mycobac-
erial FAS-II condensing enzymes, but the iso-pentenyl
analogue (6) would be a more accessible alternative.
The new antibiotic principles embodied in derivatives
(11) and (12) should be explored.

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