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Biphenyl-Based Analogues of Thiolactomycin, Active against *Mycobacterium tuberculosis* mtFabH Fatty Acid Condensing Enzyme

Suzanne J. Senior,[a,b] Petr A. Illarionov,[a] Sudagar S. Gurcha,[a] Ian B. Campbell,[c] Merrill L. Schaeffer,[d] David E. Minnikin[a] and Gurdyal S. Besra[a,*]

[a]*School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[b]*Department of Microbiology & Immunology, University of Newcastle, Newcastle upon Tyne NE2 4HH, UK*
[c]*GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, UK*
[d]*GlaxoSmithKline, Collegeville, Pennsylvania, PA 19426, USA*

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Abstract—Analogues of the antibiotic thiolactomycin, with biphenyl-based 5-substituents, were found to have excellent in vitro inhibitory activity against the recombinant *Mycobacterium tuberculosis* β-ketoacyl-ACP synthase mtFabH condensing enzyme. In particular, 5-(4′-benzyloxy-biphen-4-ylmethyl)-4-hydroxy-3,5-dimethyl-5*H*-thiophen-2-one exhibited approximately a 4-fold increased potency against this key condensing enzyme involved in *M. tuberculosis* mycolic acid biosynthesis, compared to thiolactomycin.

Mycobacterium tuberculosis continues to be a primary cause of morbidity and mortality worldwide; it is currently estimated that one-third of the world's population is infected with the bacillus.[1] The emergence of bacterial resistance to existing antitubercular agents has become a significant concern for the effective treatment of tuberculosis. The determination of the whole-genome sequence of *M. tuberculosis*[2] has pinpointed targets that potentially offer improved therapy.[3]

Thiolactomycin (TLM, **2**, Table 1) is a thiolactone antibiotic isolated from a soil *Nocardia* spp.[4] TLM exhibits potent in vivo activity against many pathogenic bacteria, including Gram-negative and Gram-positive bacteria and *M. tuberculosis*.[5–7] It is of relevance in the present context that TLM inhibits bacterial and plant type II fatty acid synthases (FAS-II) but not mammalian or yeast type I fatty acid synthases (FAS-I).[8] For instance, in *Escherichia coli*, TLM inhibits both β-ketoacyl-ACP synthase I to III and acetyl coenzyme A (CoA):ACP transacylase activities in vivo and in vitro.[9,10] In addition, TLM possesses encouraging antimalarial activity, involving inhibition of the type II fatty acid biosynthetic pathway in apicoplasts.[11]

TLM inhibits *M. tuberculosis* FAS-II through inhibition of both β-ketoacyl-ACP synthase condensing enzymes, mtFabH and KasA, in vitro and in vivo leading to inhibition of cell wall mycolic acid biosynthesis and to cell death.[12–15] Previous studies have shown that a number of TLM analogues, with aliphatic substituents at the 5-position of the thiolactone ring, have significantly enhanced activity against mycolate synthesis.[13,16] The best results were obtained with 10-carbon isoprenoid-based side chains, but the use of more chemically stable aromatic substituents is an attractive alternative. It is shown here that a single benzyl **3** group has little activity against the key condensing enzyme mtFabH, but addition of *para*-substituents leads to a range of encouraging activities (Table 1). In particular, analogues incorporating a biphenyl unit with a distal *para*-oxygen function had excellent activity (Table 1). These latter analogues were conveniently prepared by Suzuki coupling protocols.

*Corresponding author. Fax: + 44-121-414-5925; e-mail: g.besra@bham.ac.uk

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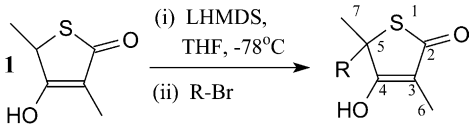
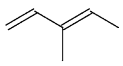
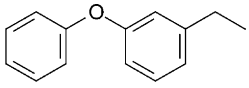
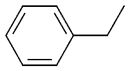
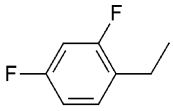
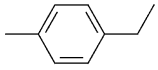
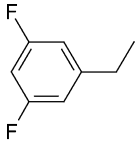
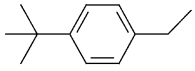
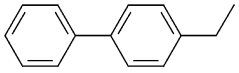
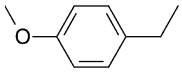
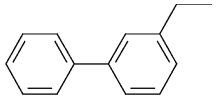
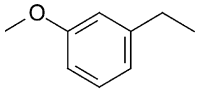
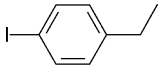
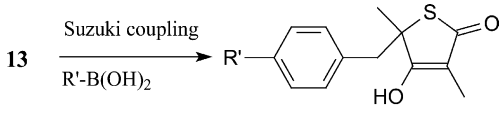
Table 1. Structures and in vitro mtFabH activity: (a) directly coupled substituents, (b) Suzuki coupled substituents

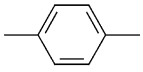
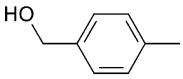
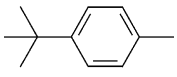
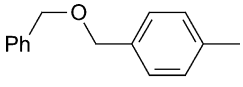
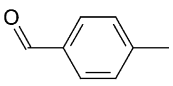
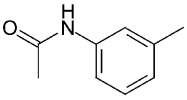
(a)



	R	IC_{50}, μM		R	IC_{50}, μM
2		74.9	**8**		52±8.9
3		>250	**9**		246±170
4		112±15	**10**		263±86
5		43±6.7	**11**		51±6.2
6		155±26	**12**		215±77
7		>500	**13**		153±54

(b)



	R′	IC_{50}, μM		R′	IC_{50}, μM
14		52±8.9	**17**		19±2.0
15		25±3.1	**18**		17±1.3
16		29±4.8	**19**		119±17

Chemical Strategy

TLM was synthesized by Wang and Salvino[17] using sodium hydride and *n*-butyl lithium to deprotonate the key thiolactone (**1**, Table 1a) at C-5, followed by reaction with a halide. Previous studies[13,17] showed that other base combinations were possible, with lithium hexamethyldisilazane (LHMDS) being the most promising.[17] These studies showed that a number of aliphatic and aromatic analogues had encouraging activity against fatty acid synthesis.[11,13,17] These results demonstrated the importance of side-chain structure in TLM analogues, in accordance with previous predictions.[9,10] Aromatic substituents are attractive for their stability and the potential for systematic modification in structure–activity studies.

A range of available arylmethyl bromides (Table 1a) were linked at position 5 on the thiolactone ring **1**. The optimised conditions involved the addition of three equivalents of LHMDS to 4-hydroxy-3,5-dimethyl-5*H*-thiophen-2-one **1** under nitrogen, followed by cooling to −78 °C for 30 min and addition of aryl bromide. Stirring at room temperature for 3 h, gave rise to selective nucleophilic addition at position-5. All compounds were completely characterized, as shown for **11**.[18]

Suzuki reactions (Table 1b) were chosen to allow systematic modification of the substitution pattern, using aryl-boronic acids according to Miyaura et al.[19] Suzuki coupling reactions were performed by heating the iodo intermediate, bis(triphenylphosphine) palladium (II) chloride (5 mol %), dimethoxyethane and aqueous sodium carbonate under reflux for 6 h before quenching with acid. Six novel analogues with varying substituents were isolated and characterized[20] and are shown in Table 1b.

Biological Activity

TLM **2** and analogues (**3**–**19**) were assayed to determine IC_{50} values against mtFabH using a well established assay protocol.[21] The aromatic side chain in compound **3**, demonstrated poor inhibition towards mtFabH with an IC_{50} value of >250 μM. Low activities were evident with the di-fluoro substituted analogues **9** and **10**, and also with the *meta*-linked di-aryl analogue **12**. Activity was increased however, by the introduction of a methyl substituent, **4** (IC_{50} 112 μM). The bulky *t*-butyl group **5** (IC_{50} 43 μM), demonstrated a further increase in activity that was comparable to the addition of an aromatic ring at the *para* position **11** (51 μM). Inhibitory properties increase when a methoxy group was introduced at the *para* position of the aromatic ring **6** (IC_{50} 155 μM), compared to **3**, but activity decreases when the methoxy group is attached at the *meta* position **7** (IC_{50} >500 μM), indicating the importance of *para* substitution. However, introduction of a *meta*-phenoxy group in **8** increases activity to 52 μM. TLM **2** shows good inhibitory properties towards mtFabH (IC_{50} 74.9 μM); however, analogues **8** (IC_{50} 52 μM) and **11** (IC_{50} 51 μM) demonstrate improved activities, suggesting that the presence of aryl rings enhance activity through an increased ability to undergo hydrophobic interactions in the hydrophobic pocket. Previous studies,[13,17] using different assays, showed enhanced activity for certain TLM analogues with lipophilic isoprenoid-based side chains.

The analogues, prepared by Suzuki coupling (Table 1b) were compared to the unsubstituted analogue **11** (IC_{50} 51 μM) (Table 1a). Introduction of a methyl group **14**, had little effect (IC_{50}, 52 μM), but a *para t*-butyl group 15 gave an improved IC_{50} value (IC_{50}, 25 μM). Compounds **16**–**18**, with a *para*-oxygen functionality, show an increase in activity (IC_{50}, 17–29 μM). This demonstrates the possible importance of a hydrogen bond accepting group, positioned in the active site and interacting with hydrogen bond donating amino acid residues.[22] It can be concluded that the presence of two aromatic rings, coupled with the substitution of the second aryl ring at the *para* position with a hydrogen bond acceptor group, greatly increases the inhibitory properties of the analogues compared to TLM.

In conclusion, new analogues of TLM have been found to have significantly enhanced activity against mtFabH. These types of condensing enzymes are of key importance in the synthesis of fatty acids in a number of microbial pathogens, as summarised above. It is now possible, therefore, to use such compounds to probe important biosynthetic mechanisms and pinpoint effective drug targets. The synthetic protocols, described, will readily allow systematic modifications to be made to effective lead compounds such as **17** (Table 1b). These stable aromatic compounds have an advantage over previous active, relatively labile, aliphatic-based analogues[13,17] in that their side chains are conformationally more predictable.

Experimental

General procedure A: Alkylation of thiolactone 1

To a stirred solution of 4-hydroxy-3,5-dimethyl-5*H*-thiophen-2-one 1 (50 mg, 0.347 mmol, 1 equiv) in anhydrous tetrahydrofuran (THF) (1 mL) −78 °C was added lithium bis(trimethylsilyl)amide, 20% in THF (LHMDS) (0.98 mL, 1.042 mmol, 3 equiv). The reaction mixture was stirred for 30 min at −78 °C before addition of the respective halide (0.382 mmol, 1.1 equiv). The mixture was allowed to warm to room temperature and stirred for a further 3 h, acidified to pH2 with 2 M aq acetic acid and extracted twice with dichloromethane. The organic layers were combined, washed with satd. brine, dried and reduced in vacuo to yield the crude product, which was purified by Fisher Matrix silica gel 60 flash chromatography (0–20% ethyl acetate in cyclohexane).

General procedure B: Suzuki cross coupling

A solution of **13** (60 mg, 0.167 mmol, 1 equiv), ethylenglycol dimethyl ether (DME) (1 mL), aq sodium carbonate (0.5 mL, 1 M) and the respective arylboronic

acid (0.183 mmol, 1.2 equiv) was degassed in a 'greenhouse' parallel synthesis reaction vessel for 10 min. Bis(triphenylphosphine) palladium (II) chloride (8 mg, 7×10^{-3} mmol, 5 mol %) was added and the mixture was heated under reflux for 6 h. The mixture was partitioned between water (10 mL) and ethyl acetate (10 mL) and separated. The aqueous layer was acidified to pH 2 with dilute hydrochloric acid (2 M) and the product was extracted with ethyl acetate (2×10 mL). The organic layers were combined, washed with satd. brine (3×10 mL), dried and reduced to give the crude product, which was purified as above.

MtFabH assay: determination of IC_{50} values

Direct, end-point scintillation proximity assays (SPA) were performed according to an established SPA assay.[21] The assay contained a mixture of recombinant *E. coli* (ec) produced mtFabH[12] (1.0 nM), biotinylated-malonyl-ecACP (2.5 μM),[21] and myristoyl-CoA (0.4 μM) in 100 mM sodium phosphate buffer with 0.01% CHAPS and 1 mM DTT. Compounds in DMSO were added to the assay plate to provide concentrations ranging from 500 to 0.98 μM, followed by recombinant mtFabH and biotinylated-malonyl-ecACP generated from malonyl-ecACP and NHS-biotin as described previously.[21] Following a 5-min preincubation at 30 °C, [^{3}H] myristoyl-CoA and unlabeled myristoyl-CoA were added to reach a final concentration of 0.4 μM containing approximately 30,000 cpm per well. Reactions were incubated at 30 °C and 100 μL of ethanol was added to quench the reaction after 20 min. Streptavidin-coated beads (40 μL) (10 mg/mL in DPBS) (Amersham) were added to each well, and the plates sealed with Topseal-A press-on, transparent adhesive sealing film (Packard) and shaken for 30 min. The plates were centrifuged for 1 min at 1700*g* to pellet the beads in each well and the incorporated radioactivity was counted using a 1450 Microbeta Trilux liquid scintillation counter (Wallac).

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16. Douglas, J. D.; Senior, S. J.; Morehouse, C.; Phetsukiri, B.; Campbell, I. B.; Besra, G. S.; Minnikin, D. E. *Microbiology* **2002**, *148*, 3101.
17. Wang, C. L. J.; Salvino, J. M. *Tetrahedron Lett.* **1984**, *25*, 5243.
18. Analytical data for compound **11**: ^{1}H NMR (D_3COD, 400 MHz) δ 1.54 (3H, s), 1.65 (3H, s), 3.00–3.15 (2H, m), 7.25 (2H, d, $J=5$ Hz), 7.28 (1H, m), 7.35 (2H, t, $J=8$ Hz), 7.42 (2H, d, $J=8$ Hz), 7.57 (2H, d, $J=8$ Hz); ^{13}C NMR (D_3COD, 100.6 MHz) δ 8.0 (C-6), 26.0 (C-7), 44.0, 59.0 (C-5), 110.0 (C-3), 126.0, 127.0, 128.0, 130.0, 132.0, 136.0, 140.0, 141.0, 181.0 (C-4), 194.0 (C-2); HRMS calcd for $C_{17}H_{22}O_2$ S (MH^+): 311.1098, found 311.1106 (−2.5 ppm).
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20. Analytical data for compound **17**: ^{1}H NMR (D_3COD, 400 MHz) δ 1.43 (9H, s), 1.52 (3H, s), 1.69 (3H, s), 2.97–3.08 (2H, m), 4.47 (2H, s), 7.13 (2H, d, $J=8$ Hz), 7.24 (2H, d, $J=8$ Hz), 7.34 (2H, d, $J=8$ Hz), 7.41 (2H, d, $J=8$ Hz); ^{13}C NMR (D_3COD, 100.6 MHz) δ 7.9 (C-6), 26.6 (C-7), 45.4, 59.8 (C-5), 65.3, 111.6 (C-3), 127.5, 128.2, 18.9, 132.5, 137.2, 141.2, 141.5, 142.1, 182.8 (C-4), 197.8 (C-2); HRMS calcd for $C_{21}H_{19}O_3S$ (MH+): 341.1211, found 341.1225 (4.0 ppm).
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