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Article

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# Semisynthetic Antimycobacterial C-3 Silicate and C-3/ C-21 Ester Derivatives of Fusidic Acid: Pharmacological Evaluation and Stability Studies in Liver Microsomes, Rat Plasma and *Mycobacterium tuberculosis* culture

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Fusidic acid (FA), a natural product fusidane triterpene-based antibiotic with unique structural features, is active *in vitro* against *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). While possessing good pharmacokinetics in man, FA is rapidly metabolized in rodents – thus complicating proof-of-concept studies in this model. Towards the repositioning of FA as an anti-TB agent, we herein describe the synthesis, activity and metabolism of FA and semi-synthesized ester derivatives in rat liver microsomes, rat plasma and mycobacterial cell culture. FA and derivative molecules with a free C-3 OH underwent species-specific metabolism to the corresponding 3-OH epimer, 3-epifusidic acid (3-epiFA). FA was also metabolized in rat plasma to form FA lactone. These additional routes of metabolism may contribute to the more rapid clearance of FA observed in rodents. C-3 alkyl and aryl esters functioned as classic prodrugs of FA, being hydrolyzed to FA in microsomes, plasma and *Mycobacterium tuberculosis* culture. In contrast, C-3 silicate esters and C-21 esters were inert to hydrolysis and so did not act as prodrugs. The antimycobacterial activity of the C-3 silicate esters was comparable to that of FA and these compounds were stable in microsomes and plasma, identifying them as potential candidates for evaluation in a rodent model of tuberculosis.

Keywords: Tuberculosis, Fusidic acid, Metabolism, Prodrug, Silicate, Hydrolysis

The widespread emergence of drug-resistant *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), has exacerbated the global threat of TB to public health.[1]The acute need for novel anti-TB drugs has prompted a surge in investment in various stages of anti-TB drug discovery and development.[2] However, the high attrition rates of candidate drugs, especially in clinical phases, necessitate a constant supply of new chemical matter into the development pipeline as well as an in-depth understanding of the pharmacokinetics and preclinical predictors of absorption, distribution, metabolism and excretion (ADME) of these drug candidates.

There are numerous reports of natural product-derived antibacterial molecules with inhibitory activity against *Mtb*,[3–6] underscoring the potential of these compounds in anti-TB drug discovery. An example is fusidic acid (FA) (**1** ), (Figure 1), a naturally occurring fusidane antibiotic derived from *Fusidium coccineum*[7], which has been used clinically for the management of Gram-positive infections and is especially useful in the treatment of methicillin-resistant *Staphylococcus aureus*.[8,9]

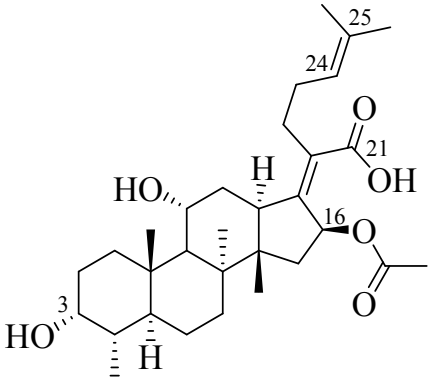



Figure 1: Fusidic acid (**1**)

FA has also been shown to have potent *in vitro* activity against *Mtb.*[10–12] It had an $MIC_{90} \leq 16$ mg/L (30 μM) across 167 of 170 clinical *M.tb* isolates tested – well within the achievable plasma

concentrations in man – and showed no cross resistance with other first line TB drugs.[10] Its general antibacterial mechanism of action (MoA) involves inhibition of protein synthesis by stabilizing the elongation factor-guanosine diphosphate complex, thus impeding peptide elongation[13,14] Notably, a similar MoA has been reported for the anti-plasmodium activity of the drug in *Plasmodium falciparum*, which causes malaria.[15,16] Given the notoriously slow progress in anti-TB drug development, the lack of cross resistance between FA and other antimycobacterial agents,[17] and its clinical tolerance and good pharmacokinetic profile in the management of bacterial infections,[18,19] the repositioning of FA and/or its active derivatives is an attractive 'low-hanging fruit' in TB drug discovery.

A complication hampering pre-clinical development of FA is that its pharmacokinetic profile in rodents – a key model in the early phase drug discovery pipeline – appears peculiar to these animals: various investigators have reported that it is poorly absorbed in mice and rats when administered orally, shows poor exposure, and is rapidly cleared.[20,21] This is a particularly significant problem in anti-TB drug development, where mouse models are commonly used to evaluate new drugs.[22,23] Our own unpublished investigations of the efficacy of FA in acute and chronic mouse models of TB initially found no *in vivo* activity up to a dose of 200 mg/kg (unpublished data). Therefore, there is a need to understand the metabolism of FA in rodents and find derivatives that allow for the required proof-of-concept studies so that the potential utility of this compound as an anti-TB antibiotic can be better evaluated. We hypothesized that lack of *in vivo* activity, despite the good *in vitro* activity, was likely due to differences in metabolism and clearance in mice, relative to man. Further, we hypothesized that using prodrugs of FA would improve the exposure of the compound in mice, thus allowing proof-of-concept studies in a mouse model.

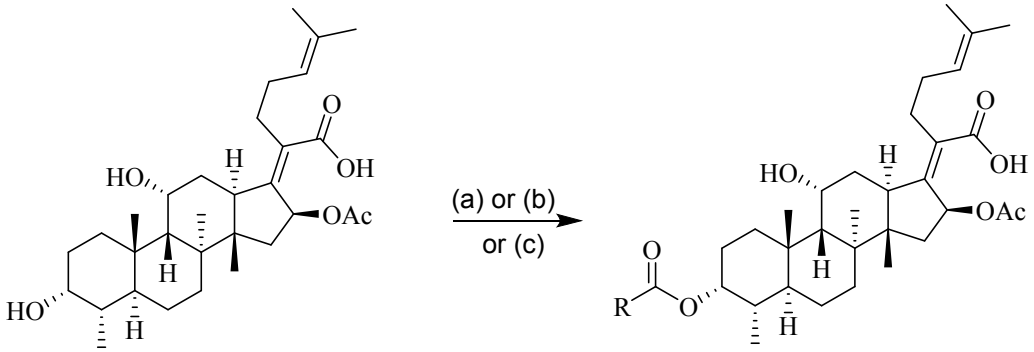
Prodrugs are derivatives of a compound designed to be converted *in vivo* to the active drug. This strategy is used to improve delivery across membranes by altering the physicochemical properties of the active drug.[24] Various chemical modifications can be considered based on the desired ease of active compound release and synthetic accessibility. The work presented here focuses on the carboxylic acid and silicate esters of FA as the former group is commonly known to increase lipophilicity by masking polar groups to which they are attached. These compounds can also be added to ionizable groups to control ionization in different pH environments. Silicate esters have recently been reported as prodrugs.[25,26] The size of the substituents can be used to influence steric hindrance on the silicon thus controlling the rate of hydrolysis.[26]

FA can be easily esterified at either the C-3 hydroxyl group or at the C-21 carboxylic acid. We have previously described the synthesis and antiplasmodium activity of C-21 FA esters and amides, and the antimycobacterial activity of C-21 amides.[27,28] Herein, we report the chemical and biological characterization of C-3 and C-21 FA esters, and C-3 silicate ester derivatives.

## RESULTS AND DISCUSSION

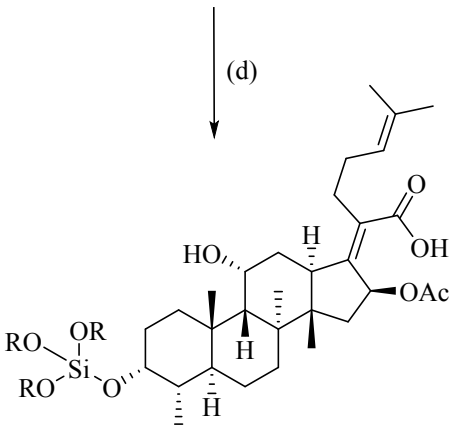
**Chemistry:** Synthetic routes to C-3 ester derivatives of FA are outlined in Scheme 1. Short, medium and long chain aliphatic C-3 carboxylic esters of FA were synthesized under two different conditions depending upon availability of the starting materials. Ester derivatives **1.2** and **1.4** were synthesized by the reaction of FA (**1**) with the corresponding anhydride using pyridine as base and solvent, while **1.3** and **1.5** were synthesized by the reaction of FA with the corresponding carboxylic acid using the coupling reagent T3P in the presence of pyridine. Aromatic C-3 carboxylic esters were synthesized either through EDCI-DMAP-mediated (**1.12 – 1.13**) or T3P-mediated (**1.9 – 1.11**) coupling of FA with the respective carboxylic acid.

Three silicate esters of FA (**1.6 – 1.8**) were synthesized by reacting the relevant trialkoxychlorosilane with FA at 0 °C for 1h in the presence of imidazole as base and DMF as solvent (Scheme 1). Trialkoxychlorosilanes were prepared in-house by dropwise addition of the respective alcohols to a solution of $SiCl_4$ in pentane at 25 °C for 1h and used without purification. All target compounds were purified using column chromatography and fully characterized by analytical and spectroscopic techniques. The synthesis and characterization of C-21 ester derivatives has been previously reported.[27]




**1** **1.2 - 1.13**

**1.2**: R=$CH_3$
**1.3**: R=$C_2H_5$
**1.4**: R=$C_3H_7$
**1.5**: R=$C_4H_9$
**1.9** : R = $C_6H_5$
**1.10** : R = 4-OMe $C_6H_5$
**1.11** : R = 4-CN $C_6H_5$
**1.12** : R = 4-Cl $C_6H_5$
**1.13** : R =4-nBu $C_6H_5$




**1.6 - 1.8**

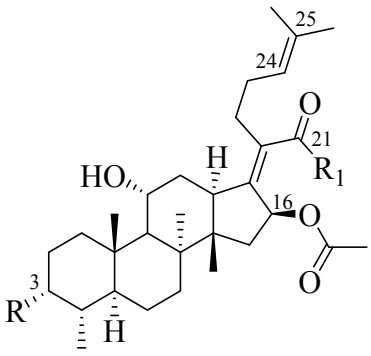
**1.6**: R= $C_2H_5$
**1.7**: R= $CH(CH_3)_2$
**1.8**: R= $C_8H_{17}$

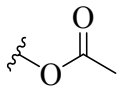
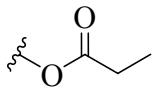
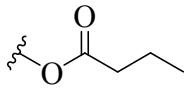
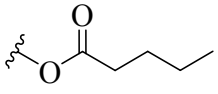
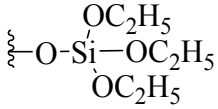
$$SiCl_4 + 3\ ROH \xrightarrow[25\ ^oC,\ 1h]{pentane} ClSi(OR)_3$$

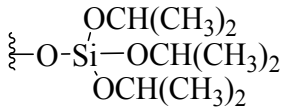
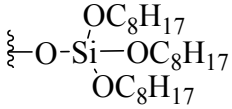
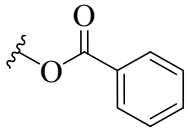
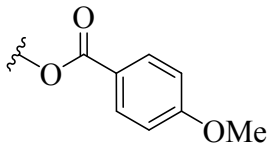
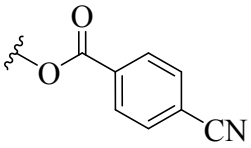
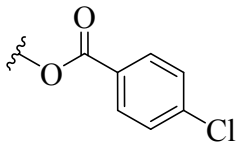
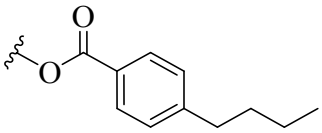
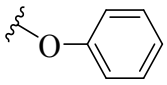
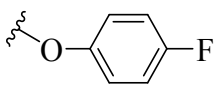
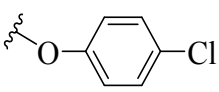
**Scheme 1**: *Reagents and conditions*: (a) $(RCO)_2O$, pyridine, 25 $^oC$, 3 h for compounds **1.2** and **1.4**; (b) RCOOH, T3P (50% w/v solution in DMF), pyridine, 0 $^oC$ – 25 $^oC$, 16 h for compounds **1.3**, **1.5** and **1.9 – 1.11**; (c) RCOOH, EDCI-DMAP, DCM, 25 $^oC$, 16 h for compounds **1.12 – 1.13**; (d) $ClSi(OR)_3$, Imidazole, DMF, 0 $^oC$, 1 h

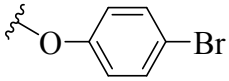
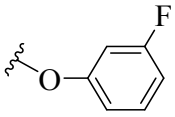
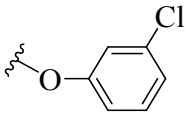
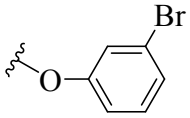
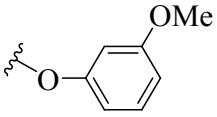
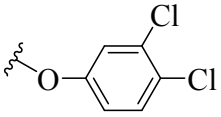
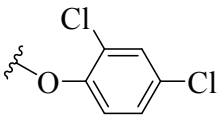
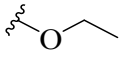
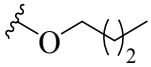
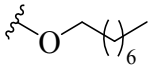
**Antimycobacterial activity and cytotoxicity:** Synthesized derivatives were screened *in vitro* against a drug sensitive *Mtb* H37RvMa (ATCC 27294 virulent laboratory strain) strain cultured in minimal media (GAST-Fe). The $MIC_{99}$ (minimum concentration required to inhibit the growth of 99% of the bacterial population) was determined for each compound and a maximum concentration limit was set at 160 μM (Table 1). Potent derivatives were also evaluated for cytotoxicity against the Chinese Hamster Ovarian (CHO) mammalian cell line, and their half minimal inhibitory concentration ($IC_{50}$) determined. Emetine, a potent inhibitor of eukaryotic protein synthesis, was used as the positive control in this assay.[29]

**Table 1**: Antimycobacterial activities, cytotoxicities and metabolic stabilities of FA and related derivatives




| Compound | R | $R_1$ | $MIC_{99}$ (µM) | CHO $IC_{50}$ (µM) | T½ (min) | |
|---|---|---|---|---|---|---|
| | | | | | RLM[a] | Rat plasma |
| **FA (1)** |  |  | <0.15 | >50 | 84 | 414 |
| **3-epiFA** |  |  | 11.4 | >50 | >200 | nd[b] |
| **1.1** |  |  | 1.25 | >50 | <20 | 414 |
| **1.2** |  |  | 20 | nd | 20 | nd |
| **1.3** |  |  | 2.50 | >50 | <20 | 529 |
| **1.4** |  |  | 1.25 | >50 | 22 | 414 |
| **1.5** |  |  | 2.50 | >50 | 26 | 396 |
| **1.6** |  |  | 0.2 | >50 | >200 | >414 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **1.7** |  |  | 2.5 | >50 | >200 | >400 |
| **1.8** |  |  | 0.3 | >50 | nd | nd |
| **1.9** |  |  | 20 | >50 | 108 | 529 |
| **1.10** |  |  | 20 | >50 | >200 | >540 |
| **1.11** |  |  | >160 | >50 | 117 | >540 |
| **1.12** |  |  | >160 | >50 | >200 | >540 |
| **1.13** |  |  | >160 | >50 | >200 | 414 |
| **1.14** |  |  | >160 | >50 | 22 | >540 |
| **1.15** |  |  | >160 | >50 | 21 | >540 |
| **1.16** |  |  | >160 | >50 | 108 | >540 |

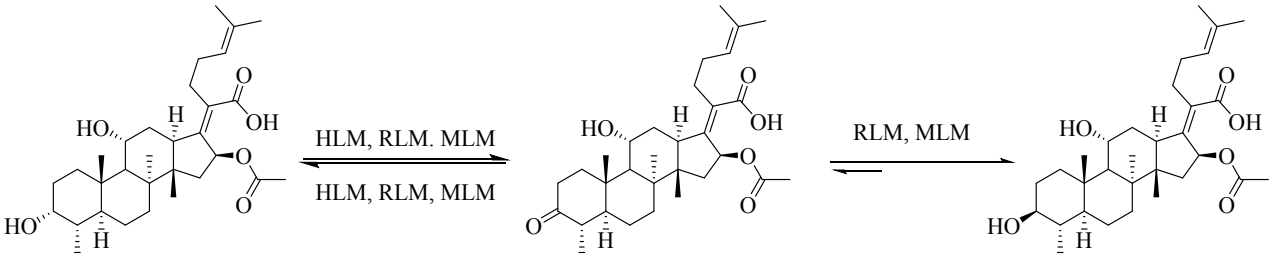
| | | | | | | |
|---|---|---|---|---|---|---|
| **1.17** |  |  | >160 | >50 | 108 | 311 |
| **1.18** |  |  | >160 | >50 | 27 | >540 |
| **1.19** |  |  | 160 | >50 | 90 | >540 |
| **1.20** |  |  | >160 | >50 | 64 | >540 |
| **1.21** |  |  | >160 | >50 | <20 | >540 |
| **1.22** |  |  | >160 | >50 | 186 | >540 |
| **1.23** |  |  | >160 | >50 | 52 | >540 |
| **1.24** |  |  | 40 | >50 | <20 | 290 |
| **1.25** |  |  | >160 | >50 | <20 | 323 |
| **1.26** |  |  | >160 | 34 | <20 | >540 |
| **1.27** |  |  | >160 | >50 | <20 | 323 |
| **Rifampicin** | | - | 0.005 | nd | nd | nd |
| **Kanamycin** | | - | 3.2 | nd | nd | nd |

| | | | | | |
|---|---|---|---|---|---|
| **Emetine -** | - | nd | 0.4 | nd | nd |

[a]Rat Liver Microsomes [b]not determined

The antimycobacterial activities of the C-3 alkyl esters improved with increasing chain length with the optimal chain size being the butyrate (**1.4**); longer alkyl chains exhibited progressively reduced activities. Two of the three silicate esters, triethoxy silicate ester **1.6** and trioctyloxy silicate ester **1.8**, exhibited antimycobacterial activities comparable to FA. Triisopropoxy silicate ester **1.7** exhibited relatively lower activity ($MIC_{99}$ 2.5 μM) compared to the above silicate derivatives. Further, all these silicate esters were found to be relatively non-cytotoxic against the CHO cell line (Table 1). Only two C-3 aryl esters, **1.9** and **1.10**, had some antimycobacterial activity with the rest having no activity at the highest measured concentration. All C-21 esters were inactive.

**Microsomal metabolism of FA:** As seen in Table 1, FA had a moderate half-life in rat liver microsomes (RLM). Metabolite identification studies revealed that the main metabolites were 3-ketofusidic acid (3-ketoFA), **1.1,** and a metabolite with the same mass and fragmentation pattern as FA but which eluted at a different retention time. This metabolite was also formed as the main metabolite of 3-ketoFA metabolism in rat and mouse but not in human liver microsomes (HLM), and its formation was NADPH-dependent. Its identity was deduced as 3-epifusidic acid (3-epiFA) and this was confirmed by comparison with LC-MS/MS of a synthetic standard of the metabolite. 3-ketoFA is the main metabolite of FA detected in humans and its formation by HLM was therefore expected.[19] Our data showed that the formation of 3-ketoFA was metabolically reversible in man, since it was metabolized to form FA (Figure S1). However, as shown in Figure 2, the stereoselectivity of the reaction changes in mouse liver microsomes (MLM) and RLM where 3-epiFA was formed as the main metabolite (Figure S2). 3-EpiFA was stable in microsomes under the incubation conditions used.

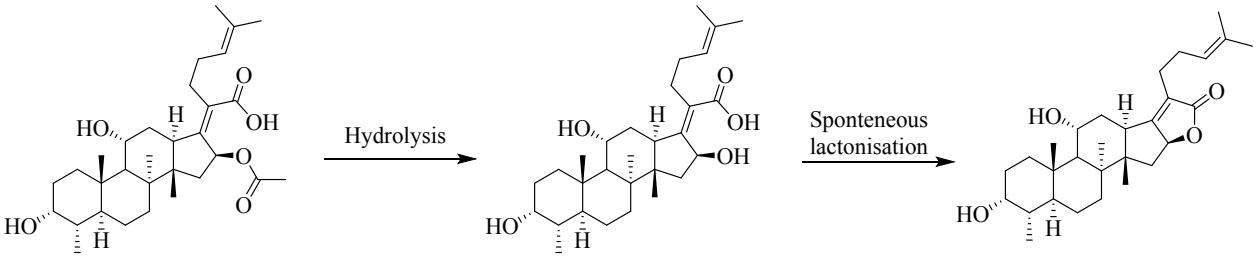



**Fusidic acid (1)** **3-ketofusidic acid** **3-epifusidic acid (M1)**

**Figure 2**: Proposed pathway for metabolism of FA and 3-ketoFA to 3-epiFA in HLM, MLM and RLM.

**Microsomal metabolism of FA esters:** Both the alkyl and aryl C-3 esters were metabolized in RLM almost exclusively to FA. In general, the alkyl esters were less stable than their aryl counterparts, with stability increasing almost linearly with chain length and lipophilicity (**Table 1**). In contrast, C-21 esters were stable to hydrolysis in RLM and instead showed metabolic breakdown similar to FA with the corresponding 3-keto and 3-epi metabolites detected in the incubations. The C-3 silicates were also stable to hydrolysis and no metabolites could be identified in RLM.

**Plasma stability of FA and its esters:** FA was stable in rat plasma. However, the FA lactone, which was likely generated through hydrolysis of the 16-acetyl group followed by spontaneous lactonization (**Figure 3**), was observed as a minor metabolite. The lactone was not detected in human plasma and has also not been reported as a metabolite in man.




**Fusidic acid (1)** **Deacetylfusidic acid** **Fusidic acid lactone**

**Figure 3**: Proposed C-16 hydrolysis pathway in FA in rat plasma.

C-3 alkyl and aryl esters and C-21 alkyl esters were hydrolyzed to FA in rat plasma. In contrast, C-21 aryl esters and C-3 silicate esters were stable to hydrolysis.

**Mycobacterial metabolism:** Mycobacteria contain numerous enzymes that can metabolize drugs and drug-like compounds, producing metabolites that could contribute to *in vitro* activity or form part of intrinsic resistance.[30] We therefore investigated the metabolism of selected compounds representing C-3 esters, C-3 silicates and C-21 esters in *Mtb* H37Rv cultures during 48-hours exposure. Control incubations were performed using heat-killed (HK) cells in order to distinguish between enzymatic metabolism and aqueous degradation. Figure 4 shows a typical set of results for the live and HK cells.

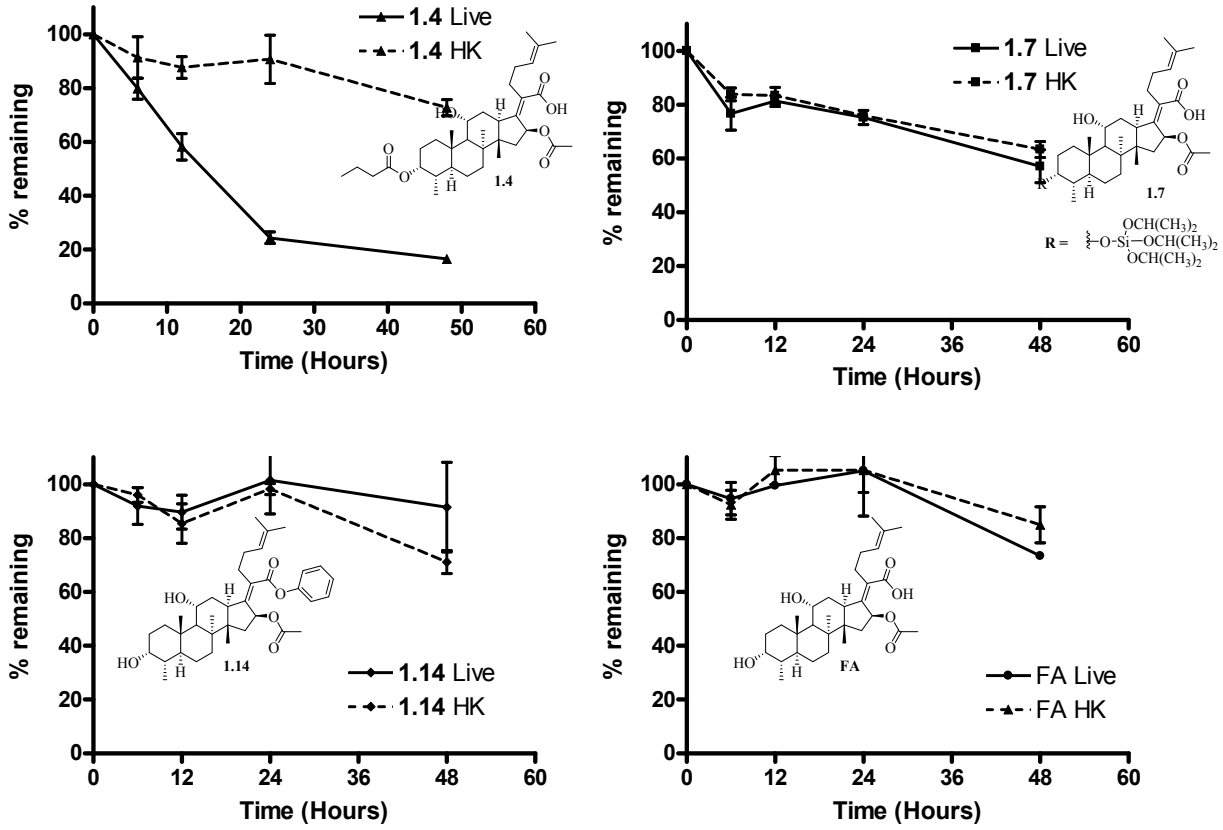


**Figure 4**: Stability of FA and selected derivatives in live and heat-killed (HK) cultures of *Mtb* H37Rv

**1.4** was less stable in the presence of live *Mtb* H37Rv cells than in the HK cells. A similar observation was made for **1.5** (data not shown). FA was the only metabolite detected for both compounds and it was formed at levels more than 500 times higher in the presence of live cells compared to HK cells. This indicates that the hydrolysis of these C-3 esters is enzymatic. In contrast, the amounts of **1.7**, a C-3 silicate, were similar in both the HK and live cells, hinting at a non-specific degradation of this compound. Notably, FA was not formed, and no metabolites were identified from the incubations; **1.14**, a C-21 aryl ester, behaved similarly.

**Discussion:** FA is a clinically important, naturally occurring fusidane antibiotic. It has good oral bioavailability in patients (approximately 90 % with film-coated tablets) and a long half-life ( > 10 h), which makes it an attractive choice for oral therapy.[19,31] FA is also well tolerated in both acute and chronic treatment with few patients experiencing adverse events that require discontinuation of therapy.[18,32,33] Since it is also active against *Mtb*, it could potentially be repositioned for TB therapy. However, preliminary investigations in acute and chronic mouse models of TB showed no *in vivo* activity despite the good *in vitro* activity. Pharmacokinetic studies show that FA has lower exposure and is cleared more rapidly in rodents relative to humans.[20,21] When used clinically, FA is primarily cleared by metabolism in the liver, with less than 1% of the drug excreted unchanged in bile and urine.[34] We therefore used liver microsomes to investigate the species difference in clearance. The *in vitro* data we present suggests that the more rapid rodent clearance is due, at least in part, to faster hepatic metabolism leading to formation of 3-epiFA, a

metabolite not reported in humans. The microsomal data also show that the metabolism of FA to 3-ketoFA, the main metabolite in humans, is a reversible reaction which would lead to a lower *in vivo* clearance of FA in humans. In contrast, mouse and rat microsomes convert 3-ketoFA to 3-epiFA, moreover this reaction was not reversible under the conditions tested. An analysis of data reported by Lakshminayarana *et al.* shows that the microsomal half-life was > 200 min ($Cl_{int}$ < 7 µl/min/mg) for more than half of the TB compound panel tested, including for recent drug candidates such as bedaquiline and PA-824.[35] The microsomal metabolism of FA (t½ 84 min) and 3-ketoFA (t½ < 20 min) is much faster and would be expected to translate to higher *in vivo* clearance. FA was stable in rat plasma, but a lactone metabolite was observed. This metabolite has not been observed in humans. The poor exposure of FA in rodent models appears, therefore, to be partly due to these additional routes of metabolism. It would therefore be difficult to observe the *in vivo* efficacy of FA in a mouse model unless modifications were made to the dosing to give sufficient exposure despite the rapid metabolism. It is unclear from the current data whether or not CYP450 enzymes are a major contributor to the rodent-specific routes of metabolism. If they are, co-administration of FA with an inhibitor of CYP450 metabolism like 1-aminobenzotriazole, may reduce the metabolic clearance enough to allow for a proof-of-concept study in mice.[36] It would also be of interest to evaluate the pharmacokinetics of FA in non-rodent models of tuberculosis, for example in the marmoset model, where exposure might be better and/or more similar to that observed in humans.[31]

The work in this manuscript revolves around potential prodrugs of FA, which could be used to deliver FA *in vivo*. C-3 esters act as classic prodrugs and are metabolized to FA in liver microsomes and plasma. In addition, these compounds are hydrolyzed to FA in *Mtb* H37Rv cultures, suggesting that they might act as prodrugs. Interestingly, C-3 silicates and C-21 esters are stable to hydrolysis

in microsomes, plasma and *Mtb* culture, with only the alkyl esters showing some hydrolysis in plasma. They are therefore not prodrugs of FA.

Studies in other bacteria suggest that a free C-21 carboxylic acid group is required for activity.[14,37,38] C-21 esters would therefore be expected to be inactive, unless they can be hydrolyzed to release FA. The data from the *in vitro* mycobacterial incubations suggest that C-21 esters are resistant to hydrolysis and should therefore be inactive. We have previously reported that C-21 esters and amides of FA have anti-plasmodium activity.[27] As part of that work, their stability in *P. falciparum* cultures was assessed and they were similarly found to be stable to hydrolysis.

The stability of the C-3 silicates in *Mtb* culture indicates that their antimycobacterial activity does not require biotransformation to the parental compound, FA. Given that these compounds show similar activities to FA, but, by contrast, are stable in rodent microsomes and plasma, they appear to be promising compounds for evaluation in a mouse model of TB infection.

Further exploration of C-3 silicates and C-3 alkyl esters would also require a determination of their MoA and whether they are cross-resistant with FA.

## Conclusion

FA and its main metabolite in man, 3-ketoFA, undergo species-specific biotransformation in MLM and RLM, leading to formation of 3-epiFA. In addition, FA is hydrolyzed in rat plasma to its lactone. These metabolic pathways may explain the more rapid clearance of FA in rodents. C-3 alkyl and aryl esters of FA act as prodrugs and are hydrolyzed in microsomes and plasma to release

FA. These compounds are also hydrolyzed to FA by *Mtb* H37Rv. C-21 esters and C-3 silicate esters are stable to hydrolysis in microsomes, plasma and *Mtb* cultures. Given their potent activities and stabilities in microsomes and plasma, the C-3 silicates represent attractive compounds for *in vivo* antimycobacterial activity evaluation.

## EXPERIMENTAL PROCEDURES:

**General Method:** FA was purchased from Avachem Scientific while all other reagents and solvents (anhydrous) used for reactions were purchased from Sigma Aldrich. $^{1}$H NMR spectra were recorded on a Varian Mercury Spectrometer at 300 MHz or a Varian Unity Spectrometer at 400 MHz. $^{13}$C NMR spectra were recorded at 75 MHz on a Varian Mercury Spectrometer or at 100 MHz on a Varian Unity Spectrometer. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constants, *J*, are recorded in Hertz (Hz). Melting points were obtained from a Reichert-Jung Thermovar hot-stage microscope apparatus and are uncorrected. Thin layer chromatographic (TLC) was performed on aluminium-backed pre-coated silica gel 60 plates purchased from Merck. Spots were visualised using UV light (λ 254/366 nm) as well as anisaldehyde stain reagent. Column chromatography was performed with Merck silica-gel 60 (70−230 mesh). LC-MS analysis was performed using an Agilent® 1260 Infinity Binary Pump, Agilent® 1260 Infinity Diode Array Detector (DAD), Agilent® 1290 Infinity Column Compartment, Agilent® 1260 Infinity Standard Autosampler, and a Agilent® 6120 Quadrupole (single) mass spectrometer, equipped with APCI and ESI multimode ionisation source. All compounds were confirmed to have >95% purity. The chemical characterization of compounds 3-epiFA and **1.1** – **1.8** are shown below. Data on all other compounds have already been reported elsewhere.[27]

**3-Epifusidic acid**

$NaBH_4$ (0.0220 g, 0.582 mmol) was added portion wise to a solution of **1.1** (0.100 g, 0.194 mmol) in methanol (3 ml) at 25°C. Reaction mixture was then stirred for 3h. After completion of the reaction (TLC), reaction mixture was acidified to pH = 1 by dropwise addition of 1N aq. HCl. Methanol was then removed *in vacuo* and residue was dissolved in EtOAc (15 ml). The EtOAc layer was washed with water (10 ml), dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography on 100-200 silica gel using EtOAc:DCM as eluent, affording **3-epiFA** as a white solid (0.025 g, 25%); $R_f$ 0.5 (60% EtOAc/DCM); Mp. 211-213°C; $^1$H NMR (400 MHz, $CD_3OD$) δ 5.81 (d, *J* = 8.5 Hz, 1H), 5.14 (t, *J* = 7.2 Hz, 1H), 4.31 (m, 1H), 3.07-2.98 (m, 2H), 2.59-2.50 (m, 1H), 2.42-2.35 (m, 1H), 2.32-2.27 (m, 1H), 2.19-2.01 (m, 5H), 1.96 (s, 3H), 1.91-1.72 (m, 4H), 1.67 (s, 3H), 1.61 (s, 3H), 1.61-1.57 (m, 3H), 1.40-1.29 (m, 1H), 1.33 (s, 3H), 1.26-1.22 (m, 1H), 1.19-1.10 (m, 2H), 1.02 (s, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.93 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 172.8, 171.2, 147.2, 131.9, 130.9, 122.9, 75.9, 74.3, 67.0, 49.0, 48.5, 43.6, 42.6, 39.7, 39.2, 38.6, 36.2, 36.1, 33.7, 32.0, 31.2, 28.5, 27.9, 24.5, 23.0, 22.7, 21.1, 19.3, 16.6, 16.4 and 14.5; LC-MS (ESI): *m/z* 457 $[M\text{-}OAc]^+$, 539 $[M+23]^+$; purity (LC-MS): 98% ($t_r$ = 4.71 min.).

**General Procedure for the synthesis of 3-ketofusidic acid 1.1**

Jones' reagent (0.8 ml) was added dropwise over 30 min. to an ice cooled solution of fusidic acid (**1**) (2.00 g, 3.87 mmol) in acetone (20 ml). Reaction was monitored by TLC. Reaction mixture was stirred at 0 °C for additional 10 min. Water (40 ml) was then added to the reaction mixture and aqueous layer was extracted with EtOAc (80 ml). Organic layer was then dried over anhydrous

sodium sulphate, filtered and concentrated *in vacuo*. The residue was purified by column chromatography on 100-200 silica gel using EtOAc:hexane as eluent, affording **1.1**.

3-Ketofusidic acid **1.1**

White solid (33%); R*f* 0.4 (60% EtOAc:hexane); Mp. 119-121 $^{o}$C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.91 (d, *J* = 8.3 Hz, 1H), 5.10 (t, *J* = 7.2 Hz, 1H), 4.40 (m, 1H), 3.07-3.03 (m, 1H), 2.51-2.42 (m, 4H,), 2.41-2.04 (m, 7H), 1.97 (s, 3H), 2.01-1.86 (m, 3H), 1.68 (s, 3H), 1.67-1.63 (m, 2H), 1.60 (s, 3H), 1.35-1.33 (m, 1H), 1.31 (s, 3H), 1.27-1.13 (m, 2H), 1.16 (s, 3H), 1.02 (d, *J* = 6.6 Hz, 3H), 0.95 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 212.4, 173.5, 170.4, 151.0, 132.7, 129.7, 122.9, 74.3, 68.1, 48.8, 48.4, 45.7, 45.4, 44.3, 39.4, 39.0, 38.0, 36.9, 36.1, 35.4, 33.5, 28.7, 28.3, 25.7, 24.7, 22.6, 21.9, 20.6, 18.0, 17.7 and 12.3; LC-MS (ESI): *m/z* 515 $[M+H]^+$, 537 $[M+23]^+$, 455 $[M-OAc]^+$; purity (LC-MS): 97% (tr = 3.89 min.).

**General Procedure for the synthesis of C-3 alkyl esters 1.2 – 1.5**

T3P (2 eq., 50% w/v in DMF, d=1.09 g/ml) was added dropwise to an ice cooled solution of FA (**1**) (1 eq.) and respective carboxylic acid (1.1 eq.) in pyridine. Reaction mixture was then slowly warmed to 25 $^{o}$C and stirred for16 h. After completion of the reaction (TLC), water (25 ml) was added to the reaction mixture and aqueous layer was extracted with EtOAc (2×15 ml). Organic layer was washed with aq 0.500 M HCl (2×15 ml) to remove excess of pyridine. Organic layer was dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo*. The residue was purified by column chromatography on 100-200 silica gel using EtOAc:hexane as eluent to afford compound **1.2 – 1.5**.

3-Acetoxyfusidic acid **1.2**

White solid (58%); R*f* 0.4 (50% EtOAc:hexane); Mp. 112-114 $^{o}$C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.91 (d, *J* = 8.4 Hz, 1H), 5.11 (t, *J* =7.2 Hz, 1H), 4.94 (m, 1H), 4.34 (m, 1H), 3.08-3.04 (m, 1H), 2.53-2.42 (m, 2H), 2.35-2.30 (m, 1H), 2.22-2.05 (m, 5H), 2.07 (s, 3H), 1.97 (s, 3H), 1.91-1.76 (m, 4H), 1.68 (s, 3H), 1.63-1.53 (m, 4H), 1.61 (s, 3H), 1.38 (s, 3H), 1.36-1.32 (m, 1H), 1.20-1.02 (m, 2H), 0.99 (s, 3H), 0.93 (s, 3H), 0.83 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.3, 170.9, 170.5, 151.1, 132.6, 129.6, 123.0, 74.4, 74.1, 68.3, 49.1, 48.8, 44.3, 39.5, 39.0, 37.8, 36.9, 35.8, 34.8, 32.7, 31.1, 28.7, 28.4, 27.4, 25.7, 24.3, 22.5, 21.3, 20.6 (2C), 18.1, 17.8 and 15.5; LC-MS (ESI): *m/z* 559 $[M+1]^+$, 581 $[M+23]^+$, 499 $[M-OAc]^+$; purity (HPLC): 98% (tr = 12.56 min.).

3-Propionyloxyfusidic acid **1.3**

White solid (56%); R*f* 0.5 (50% EtOAc:hexane); Mp. 93-95 $^{o}$C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.91 (d, *J* = 8.3 Hz, 1H), 5.12 (t, *J* = 7.2 Hz, 1H), 4.95 (m, 1H), 4.34 (m, 1H), 3.08-3.04 (m, 1H), 2.51-2.42 (m, 2H), 2.36 (q, *J* = 7.6 Hz, 2H), 2.35-2.30 (m, 1H), 2.22-2.06 (m, 5H), 1.97 (s, 3H), 1.89-1.78 (m, 4H), 1.73-1.52 (m, 4H), 1.68 (s, 3H), 1.60 (s, 3H), 1.38 (s, 3H), 1.35-1.32 (m, 1H), 1.19-1.03 (m, 2H), 1.16 (t, *J* = 7.6 Hz, 3H), 0.99 (s, 3H), 0.93 (s, 3H), 0.83 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.1, 173.2, 170.5, 151.0, 132.6, 129.5, 123.0, 74.3, 73.8, 68.2, 49.1, 48.8, 44.3, 39.4, 39.0, 37.8, 37.0, 35.7, 34.8, 32.8, 31.0, 28.8, 28.3, 28.1, 27.4, 25.7, 24.3, 22.5, 20.6, 20.5, 18.1, 17.7, 15.5 and 9.4; LC-MS (ESI): *m/z* 573 $[M+1]^+$, 595 $[M+23]^+$, 513 $[M-OAc]^+$; purity (HPLC): 98% (tr = 13.20 min.).

3-Butyryloxyfusidic acid **1.4**

White solid (66%); R*f* 0.5 (50% EtOAc:hexane) Mp. 162- 164 $^{o}$C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.90 (d, *J* = 8.3 Hz, 1H), 5.10 (t, *J* = 7.2 Hz, 1H), 4.95 (m, 1H), 4.34 (m, 1H), 3.07-3.03 (m, 1H), 2.53-2.41 (m, 2H), 2.35- 2.29 (m, 1H), 2.32 (t, *J* = 7.3 Hz, 2H,), 2.22-2.03 (m, 5H), 1.97 (s, 3H), 1.91-1.76 (m, 4H), 1.72-1.52 (m, 6H), 1.68 (s, 3H), 1.60 (s, 3H), 1.38 (s, 3H), 1.35-1.32 (m, 1H),

1.19-1.05 (m, 2H), 0.99 (s, 3H), 0.97 (t, *J* = 7.4 Hz, 3H), 0.93 (s, 3H), 0.83 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.5, 173.4, 170.4, 151.2, 132.6, 129.6, 123.0, 74.4, 73.8, 68.2, 49.1, 48.8, 44.3, 39.4, 39.0, 37.8, 36.9, 36.7, 35.7, 34.8, 32.7, 31.0, 28.7, 28.3, 27.4, 25.6, 24.3, 22.5, 20.5 (2C), 18.6, 18.0, 17.7, 15.6 and 13.7; LC-MS (ESI): *m/z* 587 $[M+1]^+$, 609 $[M+23]^+$, 527 $[M\text{-}OAc]^+$; purity (HPLC): 99% (tr = 13.94 min.).

3-Pentanoyloxyfusidic acid **1.5**

White solid (56%); R*f* 0.6 (50% EtOAc:hexane) Mp. 118- 120 $^{o}$C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.90 (d, *J* = 8.4 Hz, 1H), 5.11 (t, *J* = 7.1 Hz, 1H), 4.94 (m, 1H), 4.34 (m, 1H), 3.07-3.03 (m, 1H), 2.49-2.45 (m, 2H), 2.35-2.29 (m, 1H), 2.33 (t, *J* = 7.4 Hz, 2H), 2.22-2.04 (m, 5H), 1.96 (s, 3H), 1.91-1.77 (m, 4H), 1.64-1.53 (m, 6H), 1.67 (s, 3H), 1.60 (s, 3H), 1.40-1.32 (m, 3H), 1.38 (s, 3H), 1.19-1.04 (m, 2H), 0.99 (s, 3H), 0.97-0.93 (m, 6H), 0.82 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.2, 173.5, 170.4, 151.2, 132.6, 129.5, 123.0, 74.4, 73.8, 68.2, 49.1, 48.8, 44.3, 39.4, 39.0, 37.8, 37.0, 35.7, 34.8, 34.5, 32.8, 31.0, 28.7, 28.3, 27.4, 27.3, 25.6, 24.3, 22.5, 22.3, 20.5 (2C), 18.1, 17.7, 15.6 and 13.7; LC-MS (ESI): *m/z* 601 $[M+1]^+$, 623 $[M+23]^+$, 541 $[M\text{-}OAc]^+$; purity (HPLC): 99% (tr = 14.37 min.).

**General Procedure for the synthesis of C-3 silicate esters 1.6 – 1.8**.

Corresponding alcohol (3 eq.) was added dropwise to a solution of $SiCl_4$ (1 eq.) in *n*-pentane at 25 $^{o}$C under nitrogen atmosphere. Addition of alcohol was accompanied by frothing in the reaction indicating a rapid reaction between the alcohol and $SiCl_4$. The reaction was stirred for 1 h. The clear solution formed was concentrated *in vacuo* to obtain crude trialkoxychlorosilane which was used as such for next step.

A solution of fusidic acid (1 eq.) and imidazole (2 eq.) in DMF (5 ml) was cooled to 0 $^{o}$C under nitrogen atmosphere and trialkoxychlorosilane (3 eq, crude obtained above) was added. Reaction

was stirred at 0 °C for 1 h and monitored by TLC. After completion of reaction (TLC), reaction mixture was diluted with EtOAc (25 ml) and washed with water (3×15 ml). EtOAc layer was then dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. Product was purified by column chromatography on 100-200 size silica gel using EtOAc:hexane as eluent, affording the target compounds (**1.6** – **1.8**).

3-Triethoxysilyloxyfusidic acid **1.6**

White solid (50%); R*f* 0.5 (60% EtOAc:hexane); Mp. 72-74 °C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.89 (d, *J* = 8.3 Hz, 1H), 5.10 (t, *J* = 7.2 Hz, 1H), 4.34 (m, 1H), 4.00 (m, 1H), 3.85 (q, *J* = 7.0 Hz, 6H), 3.07-3.04 (m, 1H), 2.51-2.42 (m, 2H), 2.36-2.31 (m, 1H), 2.25-2.02 (m, 5H), 1.96 (s, 3H), 1.87-1.76 (m, 4H), 1.67 (s, 3H), 1.60 (s, 3H), 1.57-1.45 (m, 4H), 1.36 (s, 3H), 1.34-1.30 (m, 1H), 1.23 (t, *J* = 7.0 Hz, 9H), 1.16-1.04 (m, 2H), 0.97 (s, 3H), 0.92 (s, 3H), 0.90 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.3, 170.4, 151.3, 132.6, 129.5, 123.0, 74.4, 73.1, 68.3, 59.2 (3C), 49.1, 48.8, 44.4, 39.4, 39.0, 37.1, 36.4, 36.3, 35.4, 33.0, 30.5, 30.2, 28.7, 28.4, 25.6, 24.2, 22.5, 20.5, 20.4, 18.2(3C), 18.1, 17.7 and 16.2. LC-MS (ESI): *m/z* 619 $[M\text{-}OAc]^{+}$; purity (LC-MS): 98% (tr = 5.17 min.).

3-Triisopropoxysilyloxyfusidic acid **1.7**

White solid (35%); R*f* 0.5 (60% EtOAc:hexane); Mp. 70-72 °C; $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.89 (d, *J* = 8.3 Hz, 1H), 5.10 (t, *J* = 7.2 Hz, 1H), 4.34 (m, 1H), 4.23 (h, *J* = 6.1 Hz, 3H), 3.99 (m, 1H), 3.07-3.04 (m, 1H), 2.51-2.42 (m, 2H), 2.36-2.31 (m, 1H), 2.25- 2.02 (m, 5H), 1.96 (s, 3H), 1.91-1.73 (m, 4H), 1.67 (s, 3H), 1.60 (s, 3H), 1.57-1.45 (m, 4H), 1.36 (s, 3H), 1.34-1.30 (m, 1H), 1.19 (d, *J* = 6.1 Hz, 18H), 1.14-1.03 (m, 2H), 0.96 (s, 3H), 0.92 (s, 3H), 0.90 (d, *J* = 6.7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 174.2, 170.4, 151.4, 132.6, 129.4, 123.0, 74.4, 72.9, 68.4, 65.7 (3C), 49.2, 48.8, 44.4, 39.4, 39.0, 37.1, 36.4 (2C), 35.3, 33.1, 30.6, 30.0, 28.7, 28.4, 25.6, 25.4

(6C), 24.2, 22.5, 20.6, 20.4, 18.1, 17.7 and 16.3; LC-MS (ESI): *m/z* 661 $[M\text{-}OAc]^+$; purity (LC-MS): 99% (tr = 6.87 min.).

3-Trisoctyloxysilyloxyfusidic acid **1.8**

Colourless viscous oil (23%); R*f* 0.7 (50% EtOAc:hexane); $^1$H NMR (400 MHz, $CDCl_3$) δ 5.90 (d, *J* = 8.3 Hz, 1H), 5.10 (t, *J* = 7.2 Hz, 1H), 4.34 (m, 1H), 3.75 (t, *J* = 6.7 Hz, 6H), 3.99 (m, 1H), 3.07-3.04 (m, 1H), 2.51-2.42 (m, 2H), 2.36-2.31 (m, 1H), 2.22-2.02 (m, 5H), 1.96 (s, 3H), 1.87-1.75 (m, 4H), 1.67 (s, 3H), 1.60 (s, 3H), 1.57-1.45 (m, 10H), 1.36 (s, 3H), 1.34-1.25 (m, 31H), 1.14-1.03 (m, 2H), 0.96 (s, 3H), 0.92 (s, 3H), 0.90-0.87 (m, 12H); $^{13}$C NMR (100 MHz, $CDCl_3$) δ 173.9, 170.4, 151.4, 132.6, 129.4, 123.0, 74.4, 73.0, 68.3, 63.6 (3C), 49.1, 48.8, 44.4, 39.4, 39.0, 37.2, 36.4, 36.3, 35.3, 33.2, 32.4 (3C), 31.8 (3C), 30.6, 30.2, 29.4 (3C), 29.3 (3C), 28.7, 28.4, 25.7 (3C), 25.6, 24.4, 22.6 (3C), 22.4, 20.6, 20.4, 18.1, 17.7, 16.3 and 14.1 (3C).

## MATERIALS AND METHODS:

**Compounds and Reagents:** Fusidic acid was purchased from Avachem Scientific (Texas, USA). Microsomal preparations (Human, Rat, Mouse) were purchased from XenoTech (Kansas, USA) through their African distributor, African Institute of Biomedical Sciences and Technology (Harare, Zimbabwe). The human liver microsomal preparations were pooled (50 individuals) mixed gender, and the rat liver microsomes were from male IGS rats (pool of 433), while the mouse liver microsomes were from male BALB/c mice (pool of 800). All microsomal preparations were provided as 20 mg/ml suspensions transported in liquid nitrogen and were stored at -80 °C until use. Each individual vial was used for a maximum of three times to eliminate any variation due to freeze-thaw stability. NADPH (as β-Nicotinamide adenine dinucleotide 2′-phosphate

reduced tetrasodium salt hydrate) was purchased from Merck (Johannesburg, South Africa) and was divided to 5-10 mg aliquots and stored at -80 °C until use. All solvents and additives for chromatography work were purchased from Merck (Johannesburg, South Africa) or from Sigma-Aldrich (Johannesburg, South Africa) and were of HPLC grade. Deionized water (18Ω) for buffer preparation and HPLC mobile phases was collected from a Millipore Synergy water purification system (Microsep, Tygervalley, South Africa).

**LC-MS/MS:** All LC-MS/MS analysis was performed on an Agilent 1200 Rapid Resolution (600 bar) HPLC system consisting of a binary pump, degasser, auto sampler and temperature-controlled column compartment coupled to an AB SCIEX 4000 QTRAP mass spectrometer. Analyst (v1.4, 1.5, 1.6, AbSciex, Johannesburg, South Africa) was used for LC-MS control and data acquisition. The mobile phase used was 5 mM ammonium formate containing 5% acetonitrile (A) and 5 mM ammonium formate in 95% acetonitrile (B). The column used for the metabolic stability work was a Kinetex C18 2.1 mm x 50 mm, with 2.6 μM particles, while a Kinetex C18 2.1 mm x 150 mm, with 2.6 μM particles was used for the metabolite identification work. The mobile phase flow rate was 500 μl/min and the run time was 3 minutes for metabolic stability methods and 400μl/min with a run time of 15 minutes for metabolite identification methods.

Purified air was used as the nebulizer gas (gas 1) and heater gas (gas 2) and both were in general set to 50 psi and 60 psi, respectively. Curtain gas ($N_2$) was generally set at 30 psi and collision gas ($N_2$) was set to 'medium' for multiple reaction monitoring (MRM) experiments. In general, ion spray voltage was set at 5000 V and source temperature at 500 °C. Compound dependent parameters were optimized by infusing at 10 μl/min, a 500 nM solution of the compound in 50% mobile phase A/B. Significant source fragmentation was observed and flow injection analysis (FIA) experiments were set up to optimize all source parameters.

**Metabolic stability studies:** The microsomal stability of the test compounds was evaluated using a single point metabolic stability assay.[39] Incubation mixtures contained microsomes and 1 μM compound in 0.1 M phosphate buffer and were pre-incubated at 37 °C for 3 minutes. The reactions were started by adding 1 mM NADPH and were incubated at 37 °C for 60 minutes. Ice-cold acetonitrile (-20 °C) containing the internal standard was added to stop the reaction at T0 (before addition of NADPH) and at T30 minutes. The samples were then centrifuged at 4 °C, 4000g for 30 minutes and then filtered through a 0.2 μM polyvinylidene difluoride (PVDF) filter. The decrease in parent concentration was monitored using MRM methods. Data were processed on Analyst v1.5 and analyzed on Microsoft Excel 2007. Half-life was calculated using the % remaining at 60-minutes, using the equation below, which assumes first order kinetics;

$$T_{½}\,(\text{min}) = -\frac{\text{In 2 x Incubation time (60 min)}}{\text{In (\% remaining/100)}}$$

Based on the assay reproducibility, half-life was reported as < 20 min for all compounds with < 15 % remaining at 60-minutes and as > 200 min for all compounds with > 80 % remaining. Propranolol ($T_{½}$ < 20 min and Midazolam ($T_{½}$ < 20 min) and MMV390048 ($T_{½}$ > 200 min) were used as controls.

**Metabolite identification studies:** The test compound (10 μM) was incubated at 37 °C with 1mg/ml microsomes (MLM, male mouse CD1, Xenotech; RLM; male rat IGS, Xenotech; HLM; human mixed gender, Xenotech) in phosphate buffer (100 mM, pH 7.4) which contained magnesium chloride (5 mM). Reactions were started after a 5-minute pre-incubation by adding 1 mM NADPH (1 mM) and were incubated with shaking in a water bath at 37°C for 1 hour. An equal volume of ice-cold acetonitrile was added to stop the reaction and to precipitate the proteins. After centrifuging the mixture at 14000 rpm for 30 minutes the supernatant was transferred to a

HPLC vial. Control samples with no NADPH, no microsomes and a T0 sample were also included and processed in a similar way to the samples.

The LC-MS method was created using Lightsight v2.1 which relied on Analyst method files created during direct infusion of the compounds.[40] The methods generally consisted of survey scans were coupled through information dependent acquisition to enhanced product ion scans. For all compounds, a method with an enhanced mass spectrum scan coupled to an enhanced product ion scan (EMS-IDA-EPI) was used as the method for the primary data acquisition and subsequently, MRM, neutral loss, or precursor ion scans, with or without dependent EPI scans were used to get more data on the metabolites. Where synthetic references of the metabolites were available, they were analyzed by the same LC-MS/MS method and in the same batch as the incubation samples to allow for a good comparison of retention time and fragmentation.

**Plasma stability experiments:** Rat plasma was obtained from Sprague Dawley rats, courtesy of the Division of Clinical Pharmacology, University of Cape Town. The assay set-up was largely based on previously described procedure.[41] Compounds were tested at 1 μM in a 50% dilution of plasma in phosphate buffered saline (PBS, 0.1 M, pH 7.4) and the negative controls in PBS only. Incubations were performed at 37 °C for 3 hours and stopped by addition of ice-cold acetonitrile containing the internal standard. The processing and analysis were then carried out as for metabolic stability above. Albendazole ($T_{½}$ > 540 min) and Benfluorex ($T_{½}$ < 20 min) were used as positive controls.[41]

**Antimycobacterial evaluation experiments:** The minimum inhibitory concentration (MIC) was determined using the standard broth micro dilution method, where a 10 ml culture of *M. tuberculosis* pMSp12::GFP,[35] was grown to an optical density (OD600) of 0.6 – 0.7 in GAST-Fe (glycerol–alanine–salts) medium pH 6.6, supplemented with 0.05% Tween-80.[42,43] The culture

was then diluted 1:100 in GAST-Fe. The compounds to be tested were reconstituted to a concentration of 10 mM in DMSO. Two-fold serial dilutions of the test compound were prepared in GAST-Fe, across a 96-well microtitre plate, after which, 50 µl of the 1:100 diluted M. tuberculosis culture was added to each well in the serial dilution. The concentration range assayed was 20 – 0.039 µM. The plate layout was a modification of the method previously described.[38] Controls used were a minimum growth control (Rifampicin at 2xMIC), and a maximum growth control (5% DMSO in GAST-Fe). The micro titre plate was sealed in a secondary container and incubated at 37 °C with 5% $CO_2$ and humidification. Relative fluorescence (excitation 485 nM; emission 520 nM) was measured using a plate reader (FLUOstar OPTIMA, BMG LABTECH), at day 7 and day 14. In the absence of a MIC shift between day 7 and day 14, day 14 data were analyzed and reported. The raw fluorescence data were archived and analyzed using the CDD Vault from Collaborative Drug Discovery, in which, data were normalized to the minimum and maximum inhibition controls to generate a dose response curve (% inhibition), using the Levenberg-Marquardt damped least-squares method, from which the $MIC_{90}$ was calculated (Burlingame, CA www.collaborativedrug.com).[39] The lowest concentration of drug that inhibits growth of more than 90%, and 99%, of the bacterial population was considered to be the $MIC_{90}$ and $MIC_{99}$ respectively.

***M. tuberculosis* H37Rv experiments:** To assess metabolism of the compound in *Mtb* H37RvMa, cells were grown in GAST/Fe to $O.D_{600}$ 0.5 and harvested by centrifugation at 4000 rpm for 10 minutes at room temperature. The cell pellets were resuspended in PBS (0.05% $Tween_{80}$) pH 7.4. The compounds were added at half the MIC and the incubations conducted at 37 °C with sampling at 0, 6, 12, 24 and 48 hours. At each time-point, an aliquot was taken from the incubation and added to a tube containing acetonitrile and internal standard. The samples were then centrifuged

for 5 minutes at 13000 rpm, filtered using 0.2µm filters and the supernatants transferred to a 96 well plate for analysis by LC-MS. The % compound remaining was calculated by comparing the instrument response (analyte:internal standard ratio) at the sample time points to that at T0. Control incubations were performed with HK cells (heated at 80 $^{o}$C in a water bath for 1 hour).

**Cytotoxicity experiments:** The MTT-assay is used as a colorimetric assay for cellular growth and survival, and compares well with other available assays.[44,45] The reduction of tetrazolium salt MTT to a formazan salt was used to measure all growth and chemosensitivity. The test samples were tested in triplicate on one occasion using Chinese Hamster ovary cells (CHO-K1, ATCC, Manassas VA, USA) using the methods of Mosmann.[44] Cells were cultured in Hams/F12 media supplemented with 10% v/v fetal bovine serum (Thermo Fisher Scientific; origin South America). The test samples were prepared to a 10 mM stock solution in DMSO and were tested as a suspension if not properly dissolved. Emetine was used as the reference drug in all experiments. The highest concentration of solvent to which the cells were exposed to had no measurable effect on the cell viability (data not shown). Plates were developed after 44 hours of exposure to the drug by the addition of a solution of MTT. After four hours further incubation at 37 °C, the supernatant was removed from the cells via suction and DMSO was added to each well to dissolve the reduced dye crystals. Plates were analyzed at 540 nM wavelength using a spectrophotometer to determine the relative amount of formazan in each well. The 50% inhibitory concentration ($IC_{50}$) values were obtained from full dose-response curves, using a non-linear dose-response curve fitting analysis via GraphPad Prism v.4 software.

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## Supporting Information

The supporting information includes LC-MS/MS chromatograms showing the metabolism of FA and 3-ketoFA in HLM and MLM, as well as NMR spectra of selected compounds reported in this manuscript.

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