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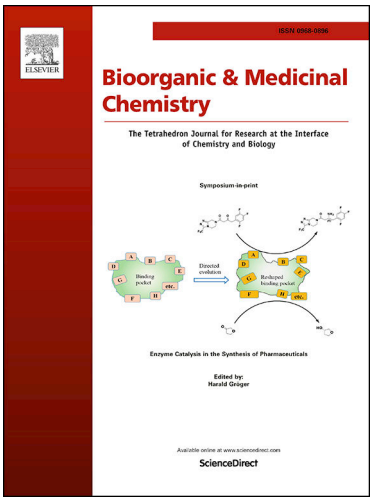

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Structure-Activity Relationship Analyses of Fusidic Acid Derivatives Highlight Crucial Role of the C-21 Carboxylic Acid Moiety to Its Anti-mycobacterial Activity

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ABSTRACT

Fusidic acid (FA) is a potent congener of the fusidane triterpenoid class of antibiotics. Structure-activity relationship (SAR) studies suggest the chemical structure of FA is optimal for its antibacterial activity. SAR studies from our group within the context of a drug repositioning approach in tuberculosis (TB) suggest that, as with its antibacterial activity, the C-21 carboxylic acid group is indispensable for its anti-mycobacterial activity. Further studies have led to the identification of 16-deacetoxy-16β-ethoxyfusidic acid (**58**), an analog which exhibited comparable activity to FA with an *in vitro* MIC_{99} value of 0.8 µM. Preliminary SAR studies around the FA scaffold suggested that the hydrophobic side chain at C-20, like the C-11 OH group, was required for activity. The C-3 OH group, however, can be functionalized to obtain more potent compounds.

KEYWORDS

Fusidic acid, anti-mycobacterial activity, cytotoxicity, bioisosteres

1. INTRODUCTION

Globally, there were 10 million tuberculosis (TB) infections in 2018, from which 1.5 million people died.[1] This public health concern has been exacerbated by the evolution of multidrug-resistant TB (MDR-TB) as the World Health Organization recently estimated ~484 000 new cases with resistance to rifampicin – the most effective first-line drug, of which 78% had MDR-TB. Therefore, the importance of exploring new treatment options for TB cannot be overemphasized. One potential candidate compound for anti-TB drug development is the triterpenoid antibiotic, fusidic acid (FA (**1**); **Fig. 1**), which is used in the treatment of infections caused by methicillin-resistant *Staphylococcus aureus*[2,3] and for which empirical evidence regarding safety and tolerability in humans[4] as well as *in vitro* potency against various strains of *Mycobacterium tuberculosis* (*M.tb*) exists.[5–7] However, preclinical anti-TB development of FA is curtailed by pharmacokinetic limitations in the rodent model of *M.tb* characterized by rapid clearance leading to poor exposure.[8]

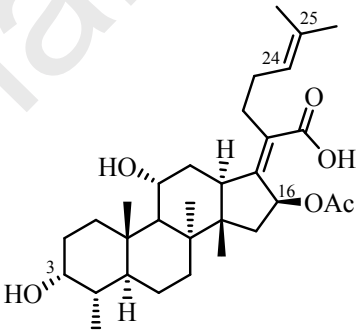


Fig. 1: Chemical structure of fusidic acid (FA, **1**) showing the substitutions at positions C-3, C-16 and C-21

The antibacterial structure-activity relationship (SAR) studies of FA have revealed that the *trans–syn–trans* conformation of the tetracyclic triterpene backbone, the acetyl-oxy group at C-16 and

the carboxylic acid group at C-21 are requisite for activity. Furthermore, the orientation of the lipophilic side chain and the carboxylic acid group around the Δ17,20 bond, rather than the double bond, have been demonstrated to be essential for antibacterial activity.[9–11]

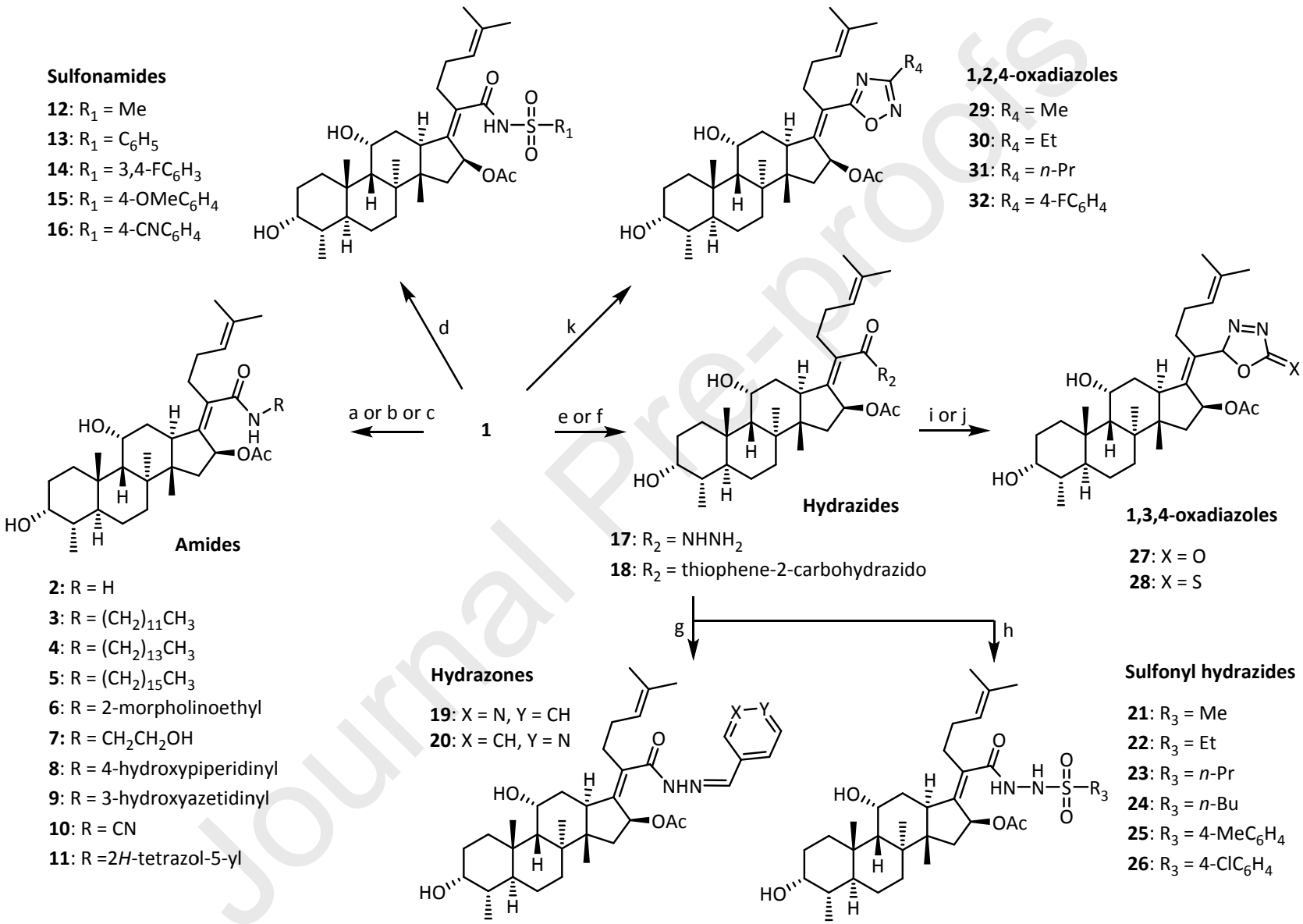
In a drug repositioning endeavor, our group has in the recent past interrogated the SAR of FA with respect to structural modifications that influence its metabolic profile, pharmacokinetics, and antiplasmodium activity.[12–16] Even more recently, we described a prodrug approach involving masking the metabolically labile C-3 position through esterification and demonstrated that this strategy can improve FA concentration and tissue distribution.[17] In preliminary studies, we have previously reported on the anti-mycobacterial activity of C-21 esters and amides and showed that the C-21 carboxylic acid group appears indispensable for anti-mycobacterial activity.[12,13] In an attempt to expand the SAR and further validate the essentiality of the C-21 carboxylic acid group to anti-mycobacterial activity, we invoked bioisosteric replacement of the C-21 carboxylic acid group and other subtle chemical modifications on the FA triterpenoid structure.

In medicinal chemistry, often the design of bioisosteres envisages structural modifications that are likely to be beneficial, with shape, size, electronic distribution, polarizability, polarity, lipophilicity, and pKa potentially playing crucial contributing roles in molecular recognition and mimicry.[18] Among the functional groups we explored as bioisosteres of the carboxylic acid group include amides, sulfonamides, hydrazides, sulfonyl hydrazides, and oxadiazoles.

2. RESULTS AND DISCUSSION

2.1: Chemistry

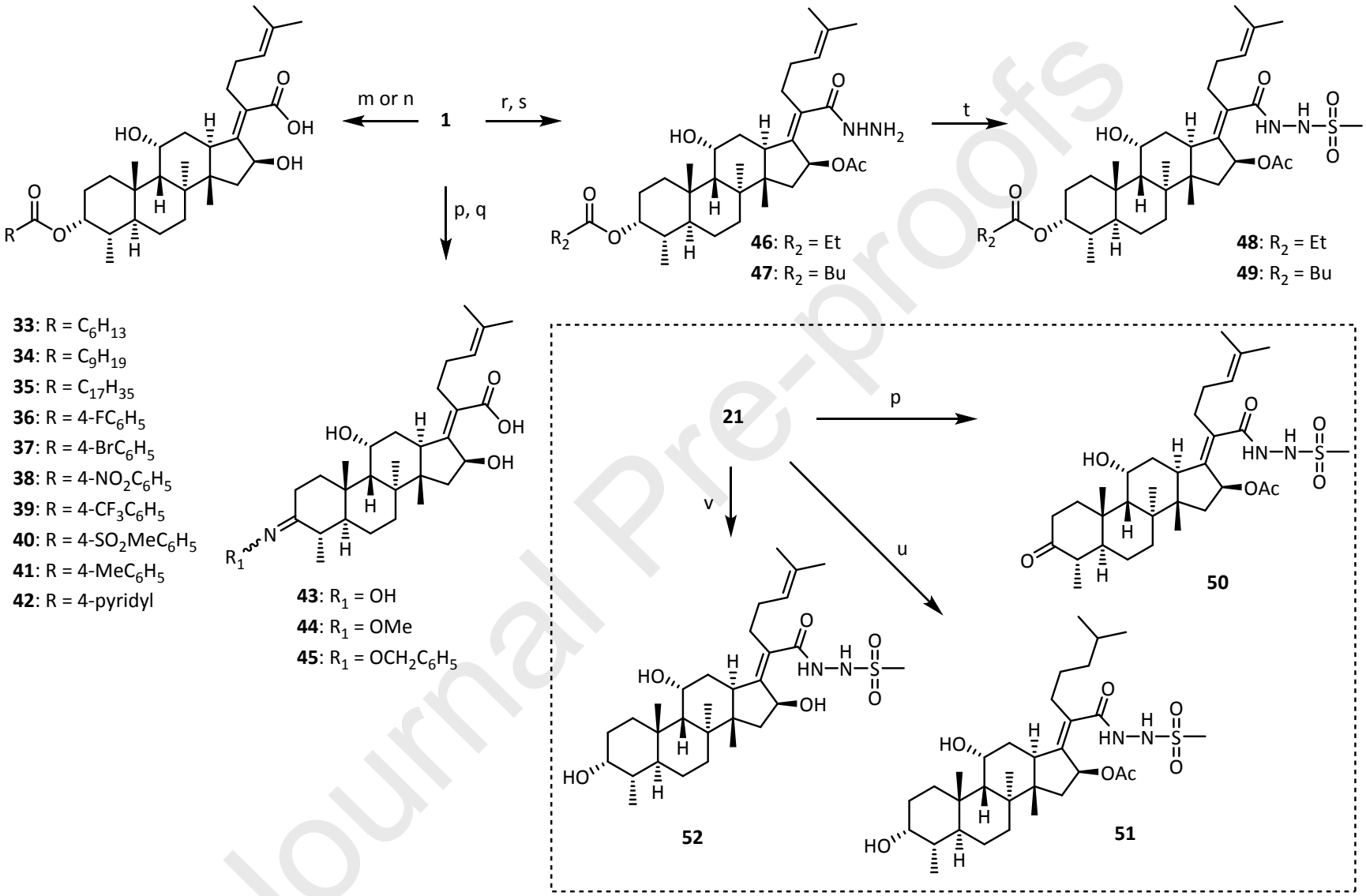
The syntheses of the analogs **2**–**32** have previously been described.[15,16] Briefly, the amide (**3**–**11**) and sulfonamide (**12**-**16**) derivatives were obtained through amide coupling reactions of the corresponding amines in the presence of the appropriate coupling reagents (Scheme 1). To **1** in acetonitrile at ambient temperature was added EDCI-HOBt coupling agent, followed by hydrazine monohydrate to afford **17**, from which the acylhydrazones, **19** and **20**, and the sulfonyl hydrazide analogs, **21**–**26**, were obtained. The *N*-(thiophene-2-carbonyl) fusidic acid hydrazide derivative, **18**, was obtained from a reaction of the thiophene-2-carbohydrazide with **1** in the presence of TBTU and DIPEA. [16] From a reaction of **17** with CDI and Et_3N in THF, the 2-oxo-1,3,4-oxadiazole derivative **27** was obtained while its 2-thioxo congener, **28**, was obtained from a reaction of CS_2 with **17** under ethanol reflux and KOH as a base. The four 3-substituted 1,2,4-oxadiazole derivatives, **29**–**32**, were obtained in two steps by first, reacting **1** with the respective amidoxime in the presence of EDCI and HOBt. The intermediate thus obtained was irradiated under microwave conditions at 100 ^{o}C in the presence of NaOAc and EtOH as a solvent to afford the target compounds.[16]



Scheme 1. Reagents and reaction conditions: (a) PyBOP, HOBt, DIPEA, NH_4Cl, DMF, 25 ^oC, 16 h for **2**; (b) RNH_2, EDCI, DMAP, DCM, 25 ^oC, 12 h, for **3** - **9**; (c) RNH_2, EDCI, HOBt, DIPEA, DCM, 25 ^oC, 24 h for **10** and **11**; (d) $R_1SO_2NH_2$, EDCI, DMAP, DCM, 25 ^oC, 48 h for **12** - **16**; (e) EDCI, HOBt, CH_3CN, 25 ^oC, 3 h, $NH_2NH_2.H_2O$, 25 ^oC, 16 h for **17**; (f) thiophene-2-carbohydrazide, TBTU, DIPEA, DMF, 25 ^oC, 16 h for **18**; (g) **17**, 3-or 4-pyridinecarboxaldehyde, ethanol, 85 ^oC, 8h for **19** and **20**; (h) **17**, RSO_2Cl, pyridine, 25 ^oC, 1–3 h for **21** - **26**; (i) **17**, CDI, Et_3N, THF, 0 – 25 ^oC, 16 h for **27**; (j) **17**, CS_2, KOH, EtOH, reflux, 16 h for **28**; (k) (1) EDCI, HOBt, CH_3CN, 25 °C, 3 h, amidoxime, 80 ^oC, 12 h, (2) NaOAc, EtOH, MW, 100 ^oC, 2 h for **29** - **32**.

The long chain C-3 aliphatic ester derivatives, **33–35**, were synthesized in moderate yields (55 - 65%) by reacting **1** with the corresponding carboxylic acid using T3P as a coupling reagent in the presence of pyridine (Scheme 2). The aromatic ester congeners (**36**-**42**) were obtained in low yields (<50%) through a Steglich esterification reaction using DCC/DMAP as a coupling reagent in dry DCM (Scheme 2).[19] Substituents on the phenyl ring were selected based on the Craig plot to investigate both electronic and hydrophobic contributions to anti-mycobacterial activity.

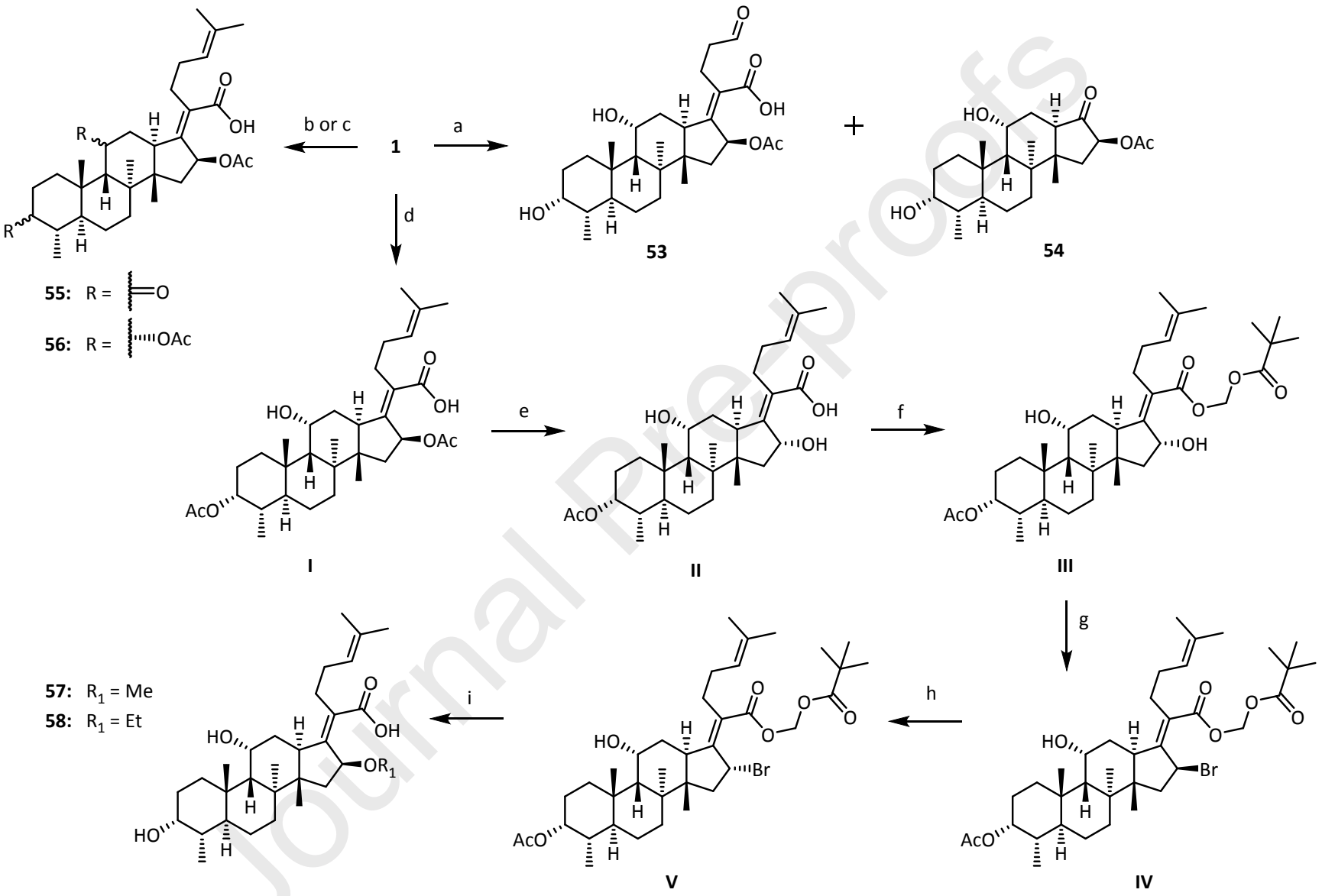
Oxidation of **1** with Jones reagent yielded both mono (3-keto) as well as disubstituted (3,11-diketo) derivatives. The C-3 oximes, **43–45**, were synthesized by irradiating a mixture of 3-ketofusidic acid and the respective hydroxylamine hydrochloride under microwave irradiation at 80 ^oC in the presence of NH_4OAc. The oximes were, as expected, obtained as a mixture of *E*- and *Z*-isomers.



Scheme 2. Reagents and reaction conditions: (m) ROOH, T3P (50% w/v solution in DMF), pyridine, 0 – 25 ^{o}C, 16 h for **33**–**35**; (n) ROOH, DCC, DMAP, DCM, N_2, 25 ^{o}C, 2-12 h for **36**–**42**; (p) Jones reagent, acetone, 0 ^{o}C, 40 min; (q) R_1ONH_2.HCl, NH_4OAc, EtOH, MW, 80 ^{o}C, 20 min for **43**-**45**; (r) propionoic acid, T3P (50% w/v solution in DMF), pyridine, 0 – 25 ^{o}C, 17 h, valeric acid anhydride, pyridine, 25 ^{o}C, 3 h; (s) EDCI, HOBt, CH_3CN, 25 ^{o}C, 3 h, $NH_2NH_2.H_2O$, 25 ^{o}C, 19-24 h for **46** and **47**; (t) MsCl, pyridine, 25 ^{o}C, 1-2 h for **48** and **49**; (u) H_2/Pd-C, EtOH, 25 ^{o}C, 16 h; (v) K_2CO_3, MeOH, 25 ^{o}C, 24 h.

In further SAR studies, we investigated the anti-mycobacterial activities of FA analogs bearing structural modifications at both C-3 and C-21 positions (**46**-**50**). The synthesis of target compounds commenced by coupling **1** with propionic acid and valeric anhydride, mediated by T3P, to afford the corresponding aliphatic esters,[12] which upon reaction with hydrazine monohydrate in the presence of EDCI-HOBt as a coupling reagent yielded the hydrazides **46** and **47**, respectively (Scheme 2).

Further reaction of compounds **46** and **47** with mesyl chloride at ambient temperature using pyridine as both base and solvent afforded the sulfonylhydrazinyl derivatives **48** and **49**, respectively. Meanwhile, the synthesis of **50** was accomplished through the Jones oxidation of **21**. Compound **51** was obtained by catalytic hydrogenation of the double bond between C-24 and C-25 of the mesyl hydrazide **21**. On the other hand, base-catalyzed hydrolysis of **21** with K_2CO_3 in methanol afforded its 16-deacetoxy-16α-hydroxy derivative **52**.



Scheme 3. Reagents and reaction conditions: (a) O_3, PPh_3, DCM, -78 ^oC to 25 ^oC, 2 h; (b) Jones reagent, acetone, 0 ^oC, 40 min for **55**; (c) acetic anhydride, pyridine, MW, 80 ^oC, 1.5 h for **56**; (d) acetic anhydride, pyridine, 25 ^oC, 3 h; (e) Sat. aq.$NaHCO_3$, MW, 100 ^oC, 10 min.; (f) $ClCH_2OCOC(CH_3)_3$, Et_3N, DMF, 25 ^oC, 16 h; (g) PPh_3, CBr_4, benzene, 25 ^oC, 1 h; (h) $Bu_4N^+Br^-$, MeCN, 25 ^oC, 65 h; (i) (1) R_1OH, Ag_2CO_3, 25 ^oC, 16 h, (2) 5N aq. NaOH, 80 ^oC, 1 h

Cleavage of the Δ24,25 and Δ17,20 bonds of FA by an ozonolysis reaction yielded low quantities (26-27%) of the aldehydic and keto products, **53** and **54**, respectively (Scheme 3).[20] The 3,11-diketo derivative, **55**, was obtained by Jones oxidation of FA, and the triacetoxy FA derivative, **56**, was obtained by reacting FA with acetic anhydride in the presence of pyridine under microwave irradiation at 80 ^oC.

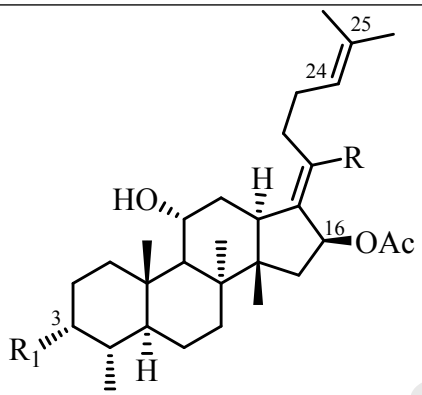
The synthesis of the C-16 derivatives of FA, **57** and **58**, was accomplished in six steps (Scheme 3).[21,22] First, the C-3 OH group of FA was protected as the acetoxy group by reacting FA with acetic anhydride in the presence of pyridine to obtain the C-3 acetoxy derivative (**I**). Then, intermediate **I** underwent hydrolysis of the C-16 acetoxy group in saturated aqueous $NaHCO_3$ solution under microwave irradiation at 100 ^oC to afford the intermediate **II** with inversion of configuration at C-16 (β-acetoxy to α-OH). The C-21 carboxylic acid group of **II** was then protected as the pivaloyloxymethyl ester (**III**) after a reaction with chloromethyl pivalate under basic conditions. This was followed by C-16 bromination with tetrabromomethane (CBr_4) in the presence of triphenylphosphine (PPh_3) to afford intermediate **IV** with inversion of configuration (α-OH to β-bromo). The C-16 β- bromo intermediate **IV** was converted to its α-bromo epimer **V** by reacting with tetrabutylammonium bromide in acetonitrile at ambient temperature. Finally, the intermediate **V** was reacted with the respective alcohols (MeOH or EtOH) in the presence of

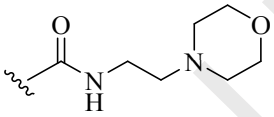
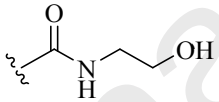
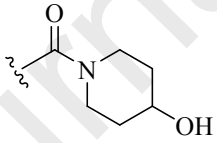
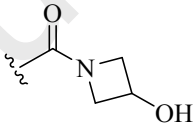
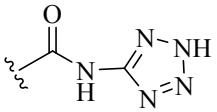
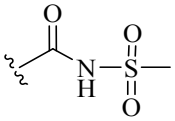
Ag_2CO_3 to afford the corresponding C-16 ether derivatives, which upon hydrolysis of the C-3 acetate and C-21 pivalate ester groups with 5 *N* NaOH solution afforded the target compounds **57** and **58**.

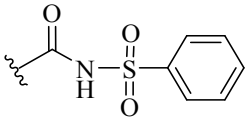
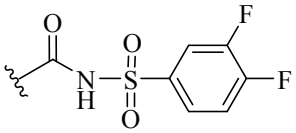
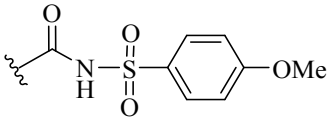
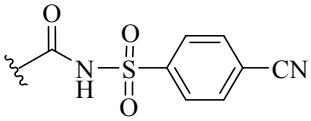
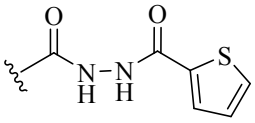
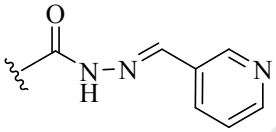
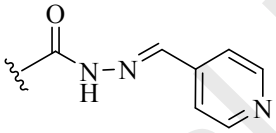
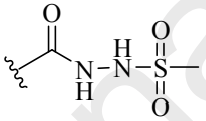
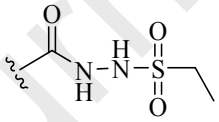
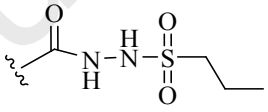
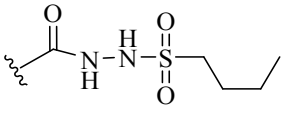
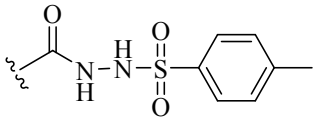
Detailed synthetic procedures are described in **Supplementary information S3-S30**. All target compounds were purified using column chromatography and fully characterized by analytical and spectroscopic techniques. The isolated yields, anti-mycobacterial and cytotoxic activities of the target compounds are reported in Table 1 (compounds **1**-**50**) and Table 2 (compounds **51**-**58**) below.

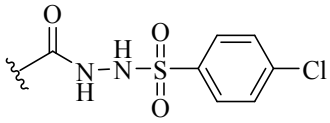
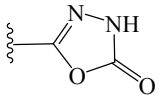
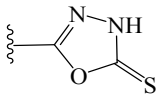
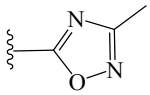
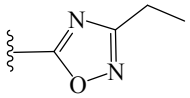
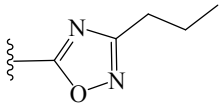
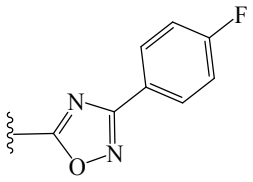
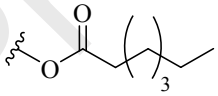
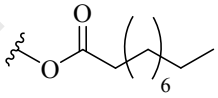
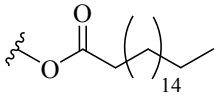
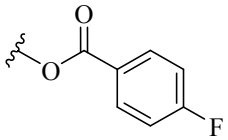
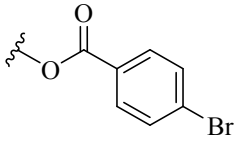
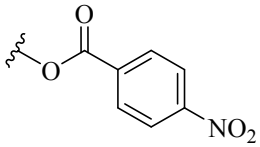
2.2: Biology

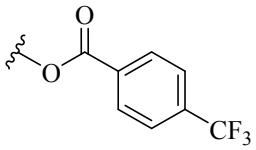
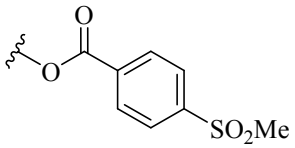
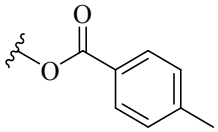
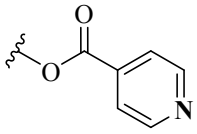
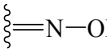
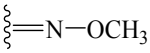
Table 1: Isolated yields, anti-mycobacterial and cytotoxic activities of compounds **1**-**50**



Compound	R	R_1	Yield (%)	H37Rv, MIC_{99}, µM	CHO*, IC_{50}, µM
1 (Fusidic acid)	—COOH	···OH	-	0.6	>194
2	—$CONH_2$	···OH	31	>125	113.0
3	—$CONH(CH_2)_{11}CH_3$	···OH	51	>125	>146
4	—$CONH(CH_2)_{13}CH_3$	···OH	71	>125	>140
5	—$CONH(CH_2)_{15}CH_3$	···OH	73	>125	>135
6		···OH	67	>125	-
7		···OH	25	>125	-
8		···OH	18	40	-
9		···OH	18	40	-
10	—CONHCN	···OH	53	40	>185
11		···OH	29	40	>171.0
12		···OH	55	80	>169.0

13		~OH	38	>125	96.1
14		~OH	30	20	81.4
15		~OH	15	31.3	-
16		~OH	30	125	-
17		~OH	98	40	112.0
18		~OH	50	40	78.8
19		~OH	63	20	-
20		~OH	63	20	-
21		~OH	90	10	92.2
22		~OH	45	40	77.3
23		~OH	25	40	79.7
24		~OH	71	>125	49.9
25		~OH	46	>125	48.2

26		~OH	65	>125	53.2
27		~OH	24	80	82.0
28		~OH	56	80	70.0
29		~OH	24	>125	47.8
30		~OH	23	>125	71.7
31		~OH	14	80	68.2
32		~OH	20	>125	77.4
33	~COOH		62	5.0	36.2
34	~COOH		63	>125	-
35	~COOH		57	>125	-
36	~COOH		6	>125	-
37	~COOH		24	>125	-
38	~COOH		14	>125	-

39			35	>125	-
40			39	31.3	-
41			12	31.3	-
42			11	31.3	-
43			23	2.5	164
44			59	20	-
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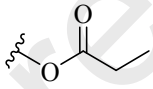
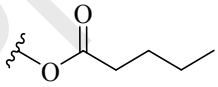
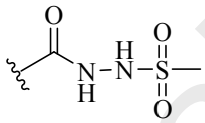
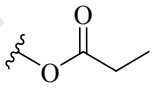
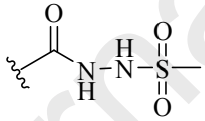
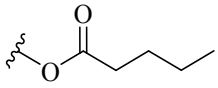
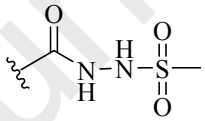
46			78	15.6	-
47			58	62.5	-
48			44	7.81	-
49			54	62.5	-
50			29	62.5	-
Rifampicin				0.005	
Emetine					0.4

*CHO: Chinese Hamster Ovarian cell line

The *in vitro* anti-mycobacterial activities of all synthesized compounds was investigated against the drug-sensitive *M.tb* H37Rv strain. The MIC_{99} (minimum concentration required to inhibit the growth of 99% of the bacterial population) was determined for each compound in the glycerol-

alanine-salt with Tween-80 and iron (GAST/Fe) media. The assay was performed at a maximum concentration limit of each test compound set at 125 µM (Table 1) and rifampicin was used as the reference drug (Table 1).

The anti-mycobacterial activity (MIC_{99}) of FA (**1**) was found to be 0.6 µM. To enable analysis of their SAR, all compounds with MIC_{99} ≤10 µM were regarded as active (relative to FA (**1**), Table 1); those with MIC_{99} ranging from 10 – 40 µM were classified as having moderate activity while compounds with MIC_{99} values in the range 40 – 125 µM were denoted as poorly active and compounds exhibiting MIC_{99} >125 µM being considered inactive.

The C-21 esters of FA have exhibited poor anti-mycobacterial activity[12] while the aromatic amides showed encouraging activity.[13] Replacing the C-21 carboxylic acid group with the primary and secondary aliphatic amide groups (**2**-**7,** MIC_{99} >125 µM) led to a loss of activity. However, the alicyclic amides **8** and **9** regained some activity with a MIC_{99} value of 40 µM. Likewise, the cyanoamide (**10**) and tetrazoloamide (**11**) bioisosteres showed moderate activity. The methane sulfonamide derivative (**12**) exhibited poor activity while the unsubstituted phenyl derivative (**13**) was inactive at the highest concentration tested. However, activity was regained following the introduction of various substitutions on the phenyl ring (**14–16**, Table 1). The 3,4-difluorophenylsulfonamide derivative **14** was found to be the most active compound among amide and sulfonamide derivatives, displaying moderate anti-mycobacterial activity with a MIC_{99} value of 20 µM.

The unsubstituted hydrazide analog **17** exhibited moderate activity (MIC_{99} = 40 µM). The pyridylhydrazone analogs, **19** and **20**, showed a 2-fold improvement in potency (MIC_{99} = 20 µM),

but the thiophene-2-carbonyl derivative, **18**, exhibited comparable activity to **17**. Among the sulfonyl hydrazides (**21-26**), the aromatic derivatives (**25** and **26**) were found to be inactive compared to their aliphatic congeners (**21**-**24**), which demonstrated a decrease in activity with increasing carbon chain length from the methyl to the butyl group (Table 1). The methane sulfonyl hydrazide derivative, **21**, was found to be the most active congener in this series (MIC_{99} = 10 μM).

Replacing the carboxylic acid group of FA with the oxadiazole bioisosteres was found to be detrimental to anti-mycobacterial activity (Table 1). The analogs **27** and **28** exhibited comparable activity (MIC_{99} = 80 μM). The 1,2,4-oxadiazole congeners were also found to be either poorly active (**31**, MIC_{99} = 80 μM) or inactive (**29**, **30**, and **32**, MIC_{99} >125 μM). In summary, bioisosteric replacement of the carboxylic acid group of FA resulted in poor anti-mycobacterial activity as compared to FA except for **21**, which showed moderate activity (MIC_{99} = 10 μM).

FA C-3 *n*-propyl, *n*-butyl and silicate esters have earlier been synthesized as potential prodrugs.[12] These ester analogs demonstrated anti-mycobacterial activity (MIC_{99} = 0.2 – 2.5 μM) comparable to FA (MIC_{99} = 0.6 μM). However, it was observed in the current work that the potency of the alkyl esters significantly decreased with increasing carbon chain length, as exemplified by **33**–**35** (MIC_{99} = 5 – >125 μM [**Table 1**]). The aryl ester analogs similarly showed poor activity as earlier observed in their C-21 counterparts.[12] However, the 4-mesylphenyl (**40**), 4-methylphenyl (**41**) and pyridyl congeners (**42**) were moderately active (MIC_{99} = 31.3 μM), suggesting that electron-withdrawing and hydrophobic groups were not tolerated. Meanwhile, functionalization of the C-3 OH group to the oxime resulted in a gain in potency which decreased with increasing size of

substituents from the unsubstituted oxime (**43**, MIC_{99} = 2.5 µM) to the benzyl-substituted oxime (**46**, MIC_{99} = 40 µM). The anti-mycobacterial activities of the two valeric esters, **47** and **49**, and the 3-keto sulfonyl hydrazide derivative, **50**, were similar with a MIC_{99} value of 62.5 µM. However, the propionic ester, **48**, showed about a two-fold increase in potency (MIC_{99} = 7.8 µM) relative to its hydrazide congener, **46** (Table 1).

We also undertook preliminary SAR studies, through subtle chemical modifications, to investigate the contributions of the C-16 acetyl group and the C-20 hydrophobic side chain on the anti-mycobacterial activity of FA (Scheme 3, Table 2). Briefly, a comparison of the activity of **51** to **21** seems to suggest that saturation of the Δ24,25 bond is well tolerated. However, the deacetylation of the C-16 acetoxy group reduced the activity by 4-fold, indicating the importance of the C-16 acetoxy group for anti-mycobacterial activity (**52,** MIC_{99} = 40 µM). Oxidation of the Δ24,25 (**53**) and Δ17,20 (**54**) bonds were detrimental to anti-mycobacterial activity, thus suggesting the importance of the C-17 side chain of FA.

The 3-keto derivative of FA exhibited low micromolar anti-mycobacterial activity (MIC_{99} = 1.25 µM), while its 3-acetoxy analog showed moderate activity (MIC_{99} = 20 µM).[12] However, in the current work, the C-3,11 diketo (**55**) and C-3,11-diacetoxy (**56**) derivatives showed reduced activity, indicating the significance of the C-11 OH group (Table 2). However, the 16-deacetoxy-16β-ethoxy derivative of FA (**58,** MIC_{99} = 0.8 µM) exhibited activity comparable to FA, indicating that modifications at the C-16 position are well tolerated.

Table 2: Isolated yields, anti-mycobacterial and cytotoxic activities of compounds **51**-**58**

Compound	Yield (%)	H37Rv, MIC_{99}, µM	CHO, IC_{50}, µM
51	58	10	97.8
52	22	40	32.0
53	27	40	
54	26	>125	
55	45	11.9	
56	35	52.5	
57	35	18.7	29.0
58	37	0.8	48.1

2.3: Cytotoxicity

Representative derivatives were evaluated for cytotoxicity against the Chinese Hamster Ovarian (CHO-K1) mammalian cell line, and their half minimal inhibitory concentration (IC_{50}) was determined (Table 1 and Table 2). Emetine was used as a reference drug in this assay. The amide analogs were generally non-cytotoxic. The cyanoamide, tetrazoloamide, and sulfonamide C-21 carboxylic acid surrogates were also non-cytotoxic except **13** and **14** (IC_{50} <100 µM). Similarly, all tested sulfonyl hydrazide and oxadiazole analogs exhibited cytotoxic activity with IC_{50} values <100 µM. The most potent analog, the 16-deacetoxy-16β-ethoxyfusidic acid (**58**) was cytotoxic at IC_{50} = 48.1 µM.

3. CONCLUSION

In the current work, the anti-mycobacterial activity of a series of FA C-21 carboxylic acid bioisosteres was evaluated towards expanding the SAR. None of these derivatives possessed superior *in vitro* activity to FA. Meanwhile, preliminary modifications of the C-11 OH, C-16 acetoxy and C-20 hydrophobic side chain groups led to a loss of activity, which seems to suggest their importance for anti-mycobacterial activity.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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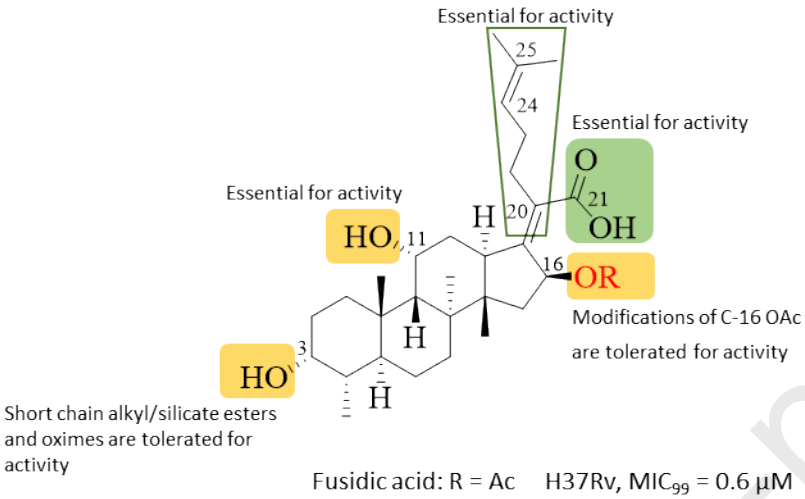
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Fusidic acid: R = Ac H37Rv, MIC_{99} = 0.6 µM

58: R = Et H37Rv, MIC_{99} = 0.8 µM