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Compounds of *Alpinia katsumadai* as potential efflux inhibitors in *Mycobacterium smegmatis*

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ABSTRACT

Efflux pumps are one of the well established mechanisms that contribute to antibiotic resistance in bacteria, such as mycobacteria. As a result, the identification of efflux pump inhibitors is an attractive target in antimicrobial therapy. The isolated compounds, three diarylheptanoids, *trans,trans*-1,7-diphenylhepta-4,6-dien-3-one (**1**), (5*R*)-*trans*-1,7-diphenyl-5-hydroxyhept-6-en-3-one (**2**), (3*S*,5*S*)-*trans*-1,7-diphenylhept-1-ene-3,5-diol (**3**) and the flavonoid pinocembrin (**4**), from *Alpinia katsumadai*, Zingiberaceae, were examined for their antimycobacterial activity and their synergistic effects with different antibiotics against *M. smegmatis* mc^2 155. Furthermore, these compounds were evaluated as potential EtBr efflux inhibitors. Although they showed weak antimycobacterial activities (MIC $\geqslant$ 64 mg/L), especially compound **1** revealed a significant activity on the EtBr accumulation and efflux as well as a synergistic effect in combination with rifampicin.

1. Introduction

The *Mycobacterium tuberculosis complex* and *Mycobacterium leprae* are considered to be one of the most serious disease causing pathogens in humans.[1] According the annual report on global control of tuberculosis (TB) published by the WHO 2010, approximately 1.7 million deaths from TB, 9.4 million incident cases and an estimated 14 million prevalent cases of TB have been reported in 2009, including HIV-positive cases, cases of MDR (multidrug-resistant) and XDR (extensively drug-resistant)—TB. A major problem represents the increasing rates of MDR and XDR strains that fail to treatment with typically standard first-line drugs.[2] As a result, the rapidly emergence of antibiotic resistance acquires the development of new antibacterial agents with new mechanisms of action.[3–5] Acquired drug resistance in *M. tuberculosis* occurs as a consequence of chromosomal mutations in genes encoding drug targets or drug activating enzymes, whereas intrinsic drug resistance is referred to the hydrophobic cell wall barrier and multidrug efflux pumps.[6–8] MDR efflux pumps have the ability to transport a wide range of chemically and structurally unrelated compounds, which also implicates different antibiotics.[9–11] The identification of new efflux-pump inhibitors (EPIs) could therefore be an attractive target to prevent the distribution of antibiotic resistance in bacteria.[1,5,11,12] To date, a few compounds have already been characterised as EPIs like chlorpromazine,[13] verapamil,[8] CCCP,[8] the plant alkaloid reserpine[8] and the flavonoid biochanin A.[14] In this context, plants seem to be an enormous source for the discovery of new lead compounds.[3,5,15] The seeds of *Alpinia katsumadai* are widely recognised in traditional Chinese medicine for the treatment of stomach diseases and emesis.[16,17] The main constituents, diarylheptanoids and flavonoids,[17,18] have shown anti-inflammatory,[19] antioxidant,[20] antiemetic[21,22] and antiviral[16] activities. Recently, there are no studies about their antimycobacterial activity as well as their ability to inhibit efflux pumps. An assay for ethidium bromide (EtBr) accumulation and efflux was used to determine the effect of these compounds on the efflux in *M. smegmatis* mc^2 155, a strain which provides several efflux pumps.[14,23,24] EtBr, a fluorescent dye and a substrate for numerous efflux pumps, is considered to be the best candidate for recording efflux pump activity. As soon as EtBr enters the cell, its fluorescence increases dramatically in a concentration dependent manner. Based on this property the accumulation and efflux of EtBr can be assessed fluorometically by monitoring the increase or the loss of fluorescence over time.[24] Regarding the high amount of lipids, mainly fatty acids that are obtained during the isolation process of a variety of plants, the investigations included the evaluation of fatty acids concerning their antimycobacterial activity and modulating effects on the MICs of antibiotics. This study presents the identification of new putative EPIs from *A. katsumadai* and the evaluation of their impact on the EtBr efflux in *M. smegmatis* mc^2 155.

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2. Results and discussion

2.1. Structure elucidation

The isolated compounds from *A. katsumadai* were identified by NMR spectroscopy, LC–MS analysis and comparison with literature data as *trans,trans*-1,7-diphenylhepta-4,6-dien-3-one (**1**), (5*R*)-*trans*-1,7-diphenyl-5-hydroxyhept-6-en-3-one (**2**), (3*S*,5*S*)-*trans*-1,7-diphenylhept-1-ene-3,5-diol (**3**) and as the flavanone pinocembrin (dihydrochrysin) (**4**).[18] The optical rotation of **4** indicated no active stereo centre α_D^{20} −3.5 (*c* 0.31, MeOH). NMR data including ^{1}H-spectra as well as HPLC profiles of compounds **1**–**4** are available as Supplementary data, for structures see Figure 1.

2.2. MIC and modulation assay

The four isolated compounds and the fatty acids (oleic acid, linoleic acid, linolenic acid, palmitic acid and stearic acid) were assessed for their antimycobacterial activity against *M. smegmatis* mc^2 155. Whereas the compounds of *A. katsumadai* and the saturated fatty acids (palmitic acid, stearic acid) exhibited weak antimycobacterial activities (MIC $\geqslant$ 64 mg/L), the unsaturated fatty acids (oleic acid, linoleic acid, linolenic acid) exerted significant activities (MIC $\leqslant$ 8 mg/L). These results reported here are consistent with similar studies conducted by others.[25,26] Furthermore, the isolated compounds and the unsaturated fatty acids were tested for their modulating activities on the MICs of various antibiotics as well as EtBr. The three diarylheptanoids (**1**–**3**) were the best modulators causing a four to eightfold reduction of the MIC of EtBr and rifampicin. However, they showed only minor modulating effects on the MIC of isoniazid, ciprofloxacin and ethambutol. In contrast to the diarylheptanoids, pinocembrin (**4**) not only induced a fourfold decrease of the MIC of EtBr, it also revealed a two to fourfold reduction of the MIC of ethambutol. From all fatty acids tested, linolenic acid was the best modulator and could modulate the MIC of rifampicin and EtBr at least to a factor of 4. None of the investigated compounds indicated any activity on the MIC of isoniazid. Resistance to isoniazid is known to be mediated by mutations of the katG genes, whereas efflux only plays a subordinate role.[7] This is supported by the present results, which showed our compounds to be efflux inhibitors that are devoid of resistance modulating effects to isoniazid. The correlation between the modulation factors of EtBr and rifampicin concerning the tested compounds suggests that efflux pumps probably confer resistance to rifampicin. These findings are in accordance with previously published data.[6] Compound **1** achieved good results as a modulator and when combined with EtBr or rifampicin even indicated a synergistic activity. The fractional inhibitory concentration index (FICI)[27] of **2** and **3** revealed a synergistic activity in combination with EtBr and an additive effect with rifampicin. For compound **4** the FICI showed a synergistic effect with EtBr, while the combination with rifampicin was indifferent. In general, it should be taken into account that it is difficult to distinguish between an indifferent and an additive effect when using doubling dilutions of antibiotics.[27] MIC values, modulation factors as well as FICI are listed in Table 1.

2.3. Accumulation assay

Accumulation and efflux assays were validated using known EPIs, such as verapamil, CCCP and chlorpromazine as reference inhibitors. As pre-screening prior to efflux assays, compounds were screened for their ability to increase the EtBr accumulation in *M. smegmatis* mc^2 155. The accumulation of EtBr increases in the presence of a potential EPI and thus, indicates efflux pump inhibition. [5] Compounds were tested at concentrations half their MIC in the presence of 0.4% glucose and 0.5 mg/L EtBr (conditions that resulted in minimal accumulation of EtBr). The reference inhibitor verapamil indicated the highest EtBr accumulation, whereas CCCP showed the weakest level of EtBr accumulation. In general, all isolated compounds from *A. katsumadai* considerably increased the accumulation of EtBr in relation to the reference inhibitors and the EtBr control (no EPI included), which served as negative control. With compound **1** a higher EtBr accumulation than with chlorpromazine could be achieved, but not as high as with verapamil. Compounds **2** and **3** induced modest levels of EtBr accumulation below chlorpromazine, but higher than CCCP. With the exception of compound **4**, all compounds revealed levels of EtBr accumulation that were at least higher than the EtBr accumulation of CCCP. Results are shown in Figure 2. However, a clear steady state at the end of each experiment could be observed. In order to allow a comparative analysis of the EtBr accumulation induced by the four isolated compounds, mean values and their standard deviations of the last 10 min are given in Table 2. Moreover, various concentrations of each test compound that ranged from the MIC to 1/16 of the MIC were tested. Hence, we could demonstrate that compound **1** showed similar levels of EtBr accumulation between one fourth of the MIC and the MIC value itself, indicating a saturation effect. However, at lower concentrations of **1** a clear dose dependency was observed when comparing results at 1/4, 1/8 and 1/16 MIC. Even a concentration of 1/8 of the MIC exerted levels of EtBr accumulation comparable to CCCP. Results are presented in Figure 3.

2.4. Efflux assay

Following conditions were ascertained for loading the cells with EtBr: the use of EtBr and verapamil at half their MIC, the absence of glucose and shaking for 1 h at 37 °C. Potential EPIs produced minor

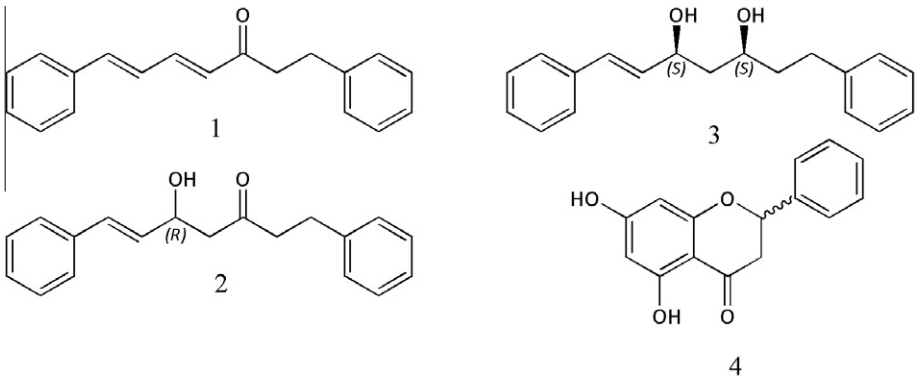

Figure 1. Chemical structures of compounds **1**–**4**.

Table 1
MIC values,[a] modulation factors[b] and FICI for *M. smegmatis* mc^2 155

Compound	MIC (mg/L)	[c] as modulator (mg/L)	MF (EtBr)	MF (CIP)	MF (EB)	MF (INH)	MF (RIF)	FICI (EtBr)	FICI (RIF)
Compound **1**	⩾128	64	4	1	1	1	4–8	0.27	0.28
Compound **2**	64	32	8	2	1–2	1	4	0.25	0.75
Compound **3**	⩾128	64	8	2	2	2	4	0.25	0.75
Compound **4**	128	64	4	1–2	2–4	1	2	0.5	1.5
Linoleic acid	4	2	2	—	—	1	2	—	—
Linolenic acid	8	4	4–8	—	—	1	4	—	—
Oleic acid	8	4	1–2	—	—	1	4–8	—	—
Palmitin acid	128	64	1	—	—	0.5	—	—	—
Stearic acid	⩾128	64	1	—	—	0.5	—	—	—
CCCP	32	—	—	—	—	—	—	—	—
Chlorpromazine	64	—	—	—	—	—	—	—	—
Verapamil	512	—	—	—	—	—	—	—	—

[a] MIC of EtBr = 8 mg/L, MIC of CIP, ciprofloxacin = 0.125 mg/L, MIC of EB, ethambutol = 1 mg/L, MIC of INH, isoniazid = 4 mg/L, MIC of RIF, rifampicin = 32 mg/L, — = not tested.
[b] MF = Modulation factor, n = 4–8.

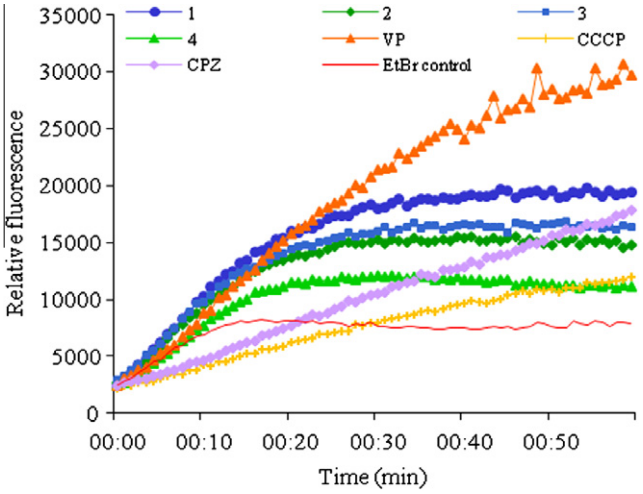

Figure 2. Effect of the potential EPIs from *A. katsumadai* and reference inhibitors on the EtBr accumulation at 0.5 mg/L EtBr in the presence of 0.4% glucose at 37 °C in *M. smegmatis* mc^2 155, OD 0.4. All compounds were tested at concentrations half their MIC. VP, verapamil (orange triangles), CCCP, carbonyl cyanide *m*-chlorophenylhydrazone (yellow crosses), CPZ, chlorpromazine (purple diamonds), EtBr control (red line), compound **1** (dark blue circles), compound **2** (dark green diamonds), compound **3** (blue squares), compound **4** (green triangles).

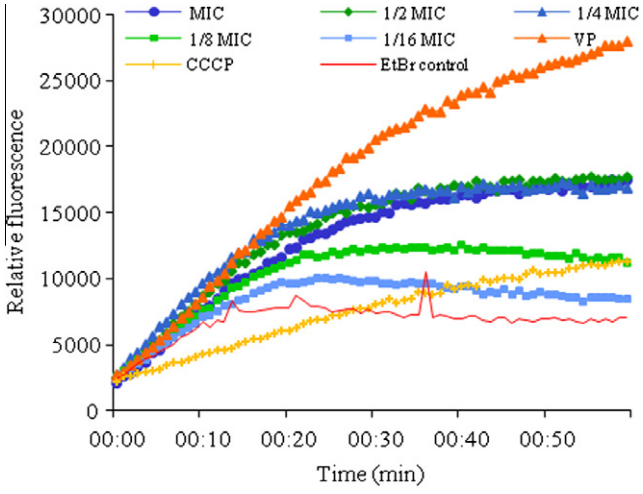

Figure 3. EtBr accumulation of the dilution series of compound **1** in *M. smegmatis* mc^2 155, OD 0.4, using concentrations from the MIC to 1/16 of the MIC. Assays were conducted at 0.5 mg/L EtBr with 0.4% glucose at 37 °C. VP, verapamil (orange triangles), CCCP, carbonyl cyanide *m*-chlorophenylhydrazone (yellow crosses), EtBr control (red line), MIC (dark blue circles), 1/2 MIC (dark green diamonds), 1/4 MIC (blue triangles), 1/8 MIC (green squares), 1/16 MIC (blue squares).

decreases of the EtBr fluorescence relative to the EtBr control. The most potent reference EPIs were verapamil and chlorpromazine followed by CCCP. None of the isolated compounds achieved EtBr efflux inhibition levels higher than verapamil and chlorpromazine. Nevertheless, compound **1** revealed significant inhibition levels similar to chlorpromazine. With compound **4** an EtBr efflux inhibition comparable to CCCP could be observed. Compounds **2** and **3** were less active than the reference inhibitors, although they also indicated activities to inhibit EtBr efflux when compared to the EtBr control (no EPI). Results can be seen in Figure 4. The steady state at the end of the assay provides a comparison of the efflux inhibition caused by compounds **1–4**. Mean values including the standard deviations of the normalised fluorescence data for the last 10 min are summarised in Table 2. As in the accumulation assay, the efflux assay revealed no real dose depended response for compound **1**, until one fourth of its MIC is reached but showing dose dependency at lower concentrations of compound **1**. A concentration of one eighth of its MIC also resulted in inhibition levels comparable to CCCP. Dilution series of compound **1** and its effect on the EtBr efflux are

Table 2
Mean values and standard deviations of the relative fluorescence (accumulation) and normalised fluorescence data (efflux) of compounds **1**–**4** including the EtBr control[a,b]

Compound	Accumulation		Efflux	
	Mean value	Standard deviation	Mean value	Standard deviation
1	19356.6***	244.1	0.806***	0.006
2	15058.9***	269.3	0.720***	0.015
3	16515.3***	191.3	0.746***	0.017
4	11245.6***	146.9	0.765***	0.017
EtBr control	7832.3	223.2	0.607	0.052
	n = 13		n = 21	

[a] calculated as measured during the last 10 min of each assay.
[b] compounds **1**–**4** were compared to the EtBr control, level of significance is indicated by asterisks: $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

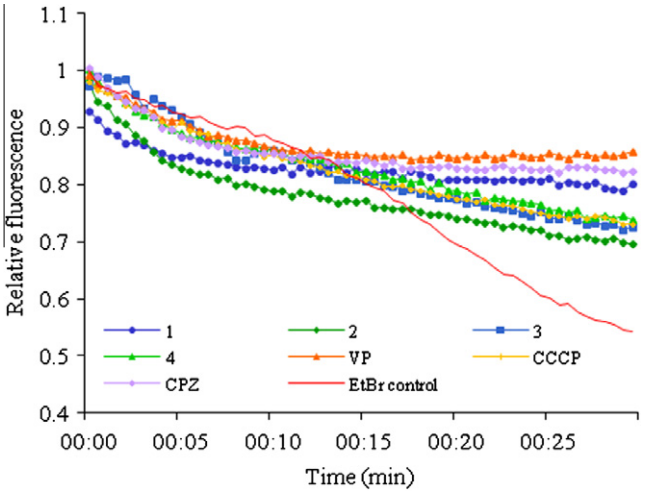

Figure 4. Effect of the potential EPIs from *A. katsumadai* and reference inhibitors on the EtBr efflux in *M. smegmatis* mc^2 155, OD 0.8, in the presence of 0.4% glucose at 37 °C, after loading with 4 mg/L EtBr and 256 mg/L VP. Compounds were tested at half their MIC. VP, verapamil (orange triangles), CCCP, carbonyl cyanide *m*-chlorophenylhydrazone (yellow crosses), CPZ, chlorpromazine (purple diamonds), EtBr control (red line), compound **1** (dark blue circles), compound **2** (dark green diamonds), compound **3** (blue squares), compound **4** (green triangles).

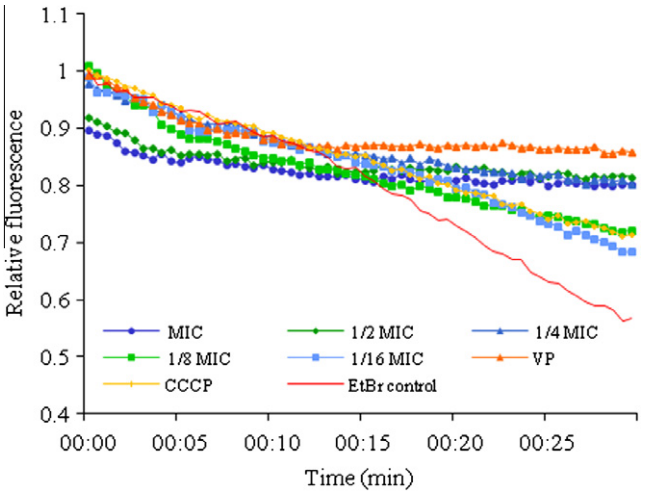

Figure 5. EtBr efflux of the dilution series of compound **1** in *M. smegmatis* mc^2 155, OD 0.8, using concentrations from the MIC to 1/16 of the MIC. Cells were loaded with 4 mg/L EtBr plus 256 mg/L VP and assays were conducted in the presence of 0.4% glucose at 37 °C. VP, verapamil (orange triangles), CCCP, carbonyl cyanide *m*-chlorophenylhydrazone (yellow crosses), EtBr control (red line), MIC (dark blue circles), 1/2 MIC (dark green diamonds), 1/4 MIC (blue triangles), 1/8 MIC (green squares), 1/16 MIC (blue squares).

represented in Figure 5. As far as there are just three diarylheptanoids investigated yet, conclusions about the structure–activity relationship of the isolated diarylheptanoids should be drawn with care. In comparison to curcumin and its derivatives (unpublished data), we assume that the following structural requirements seem to be essential for this group of diarylheptanoids to inhibit EtBr efflux: mainly the two double bonds as well as the presence of an un-substituted aromatic ring on each end of the heptyl chain.

3. Conclusion

In summary, the main constituents of the *n*-hexane extract of *A. katsumadai* were isolated and identified by 1D and 2D NMR spectroscopy as well as LC–MS analysis. The three diarylheptanoids and the flavonoid pinocembrin were screened for their antimycobacterial activity and their modulating activities on the MIC of different antibiotics. An EtBr accumulation assay and efflux assay were used to determine their potential to inhibit EtBr efflux. All isolated compounds exhibited weak antimycobacterial activities; however, as modulators they could induce a decrease of the MIC of EtBr and rifampicin. In addition, they revealed EtBr efflux inhibiting activities compared to the included controls. We successfully demonstrated that particularly compound **1** achieved considerable results as modulator and decreased the EtBr efflux in *M. smegmatis* mc^2 155 at levels comparable to known EPIs. Except the saturated fatty acids, all unsaturated fatty acids exerted significant antimycobacterial activities and showed substantial results as modulators of EtBr and rifampicin, which should be taken into consideration when screening lipophilic plant extracts for their antimycobacterial and modulating activities. Diarylheptanoids have not been described as bacterial efflux inhibitors before and represent a promising class of natural products to be investigated further.

4. Experimental

4.1. General experimental procedures

Silica gel 60 (0.043–0.063 mm, Merck, Germany) was used for column chromatography (32 × 7 cm). Semi-preparative HPLC was carried out on a Merck Hitachi connected to a Merck Hitachi Diode Array Detector with a LiChrospher Rp 18, 10 µm, 250 × 10 mm. Preparative HPLC was performed using a Varian PrepStar with a Dynamax solvent delivery system and Dynamax Absorbance Detector in combination with an UltraSep ES Rp 18, 10 µm, 250 × 20 mm. LC–MS analysis was conducted with a Thermo Finnigan LCQ Surveyor Liquid Chromatograph DECA XP plus mass detector (ESI) and PDA detector. Analytical HPLC was performed on an Agilent 1100 series instrument equipped with a quaternary pump, autosampler, diode array detector and a Phenomenex, Kinetex 2.6 µm, C 18, 100 × 2.10 mm as stationary phase. ^{1}H and ^{13}C spectra were recorded on a 600 Varian Unitylnova spectrometer using TMS as internal standard. The optical rotation was measured with a Jasco P-200 polarimeter. Statistical analyses were conducted using SigmaPlot 12.0. In order to test for statistical significance the t-test or if the normality test failed (p <0.05) the Mann-Whitney Rank Sum Test was performed to compare compounds **1**–**4** to the EtBr control.

4.2. Materials

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), chlorpromazine, verapamil, ciprofloxacin, ethambutol, isoniazid, rifampicin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), glucose, Tween 80, oleic acid, linoleic acid, linolenic acid, palmitic acid and stearic acid were obtained from Sigma-Aldrich (Vienna, Austria). Ethidium bromide (EtBr 1% solution) and PBS tablets (140 mM NaCl, 10 mM phosphate buffer and 3 mM KCl, pH 7.4 at 25 °C) were purchased from Calbiochem (Merck, Vienna, Austria). 7H9 broth and Middlebrook ADC enrichment were obtained from Difco (Becton, Dickinson and Company, England, UK). Mueller-Hinton broth, Columbia blood agar base and defibrinated horse blood were purchased from Oxoid (England, UK). Micro titre plates, cell culture dishes and cell culture flasks were purchased from TPP (Switzerland). Solutions of the test compounds were prepared freshly on the day of the experiment.

4.3. Plant material, extraction and isolation

The seeds of *Alpinia katsumadai* Hayata were obtained from Plantasia (Oberndorf, Austria). An amount of 900 g was crushed

and extracted successively with *n*-hexane using a Soxhlet apparatus for 24 h. 17 g of the extract were subjected to column chromatography on silica gel eluting with a step gradient of *n*-hexane, EtOAc and methanol (from 0% to 50% EtOAc in *n*-hexane, then adding increasing proportions of 2% to finally 100% MeOH) to yield 17 different fractions. Fraction 5, 14 and 16 were chromatographed on preparative or semi-preparative HPLC with various gradients consisting of MeCN/H_2O. Fraction 5 was purified by preparative HPLC with MeCN/H_2O (80:20 MeCN/H_2O for 5 min and within 15 min to 100% MeCN) and the mixture was partitioned with $CHCl_3$ to give **1** (52 mg). Semi-preparative HPLC was used to separate fraction 14 with MeCN/H_2O (60:40) to obtain compound **2** (43 mg) together with **4** (22 mg). Fraction 16 was fractionated via preparative HPLC by eluting with MeCN/H_2O (55:45) to afford **3** (20 mg). The purity of the compounds was checked by HPLC analysis and was 90.1% (**1**), 97.7% (**2**), 98.6% (**3**) and 97.4% (**4**) based on the peak area integration using a wavelength of 220 nm. Structure elucidation and identification was conducted on the basis of NMR spectroscopy (^1H, ^{13}C, HSQC, HMBC, H,H-COSY) in combination with mass spectroscopic data as well as in comparison with published data.[18]

4.4. Bacteria and growth conditions

Mycobacterium smegmatis mc^2 155 ATCC 700084 was obtained from the American type culture collection (LCG Promochem, Teddington, Middlesex, UK) and was used throughout the study. Prior to MIC and modulation assays, bacteria were grown on Columbia blood agar supplemented with 5% defibrinated horse blood under aerobic conditions at 37 °C for 72 h. A bacterial stock of *M. smegmatis* mc^2 155 was cultivated in 7H9 T supplemented with 10% ADC enrichment at 37 °C, 100 rpm under aerobic conditions until bacterial growth for accumulation and efflux assays.

4.5. Bioassay methods

4.5.1. MIC assay and modulation assay

MIC determination of the reference inhibitors, antibiotics as well as the isolated compounds and fatty acids was conducted by broth dilution method and was done as described previously.[14,23] Briefly, test compounds were dissolved in DMSO and diluted in MHB to reach particular start concentrations. A bacterial inoculum was adjusted equal to the McFarland turbidity standard 0.5 and diluted to yield a final bacterial density of 5×10^5 cfu/mL. 0.125 mL aliquots of the bacterial suspension were transferred into the wells containing 0.125 mL aliquots of MHB with the two-fold serially dilutions of each test compound. Plates were incubated at 37 °C for 72 h and the MIC was registered after adding MTT and was defined as the lowest concentration that inhibited bacterial growth.[27]

Additionally, all compounds were assessed for their modulating activities and synergistic effects with different antibiotics and EtBr. Compounds were solved in DMSO and diluted in MHB to achieve concentrations in the assay, which were correlating to one half of their MIC. On the one hand constant concentrations of the modulators were used and the antibiotics were serially diluted, on the other hand the antibiotics were held at the same concentration and the modulators were serially diluted. The modulation factor (MF) was used to quantify the modulating effect of the test compounds on the MIC of antibiotics and EtBr and the fractional inhibitory concentration index (FICI) to evaluate the effect of the combination of antibacterial agents.

MF = (MIC antibiotic)/(MIC antibiotic + modulator)
FICI = FIC(A) + FIC(B)
FIC(A) = MIC(A in the presence of B)/MIC(A alone)
FIC(B) = MIC(B in the presence of A)/MIC(B alone)
(Synergy: FICI $\leqslant$ 0.5, additive: FICI >0.5–1, indifference: FICI >1 to <2, antagonism: FICI $\geqslant$ 2)[27]

4.5.2. EtBr accumulation assay

EtBr accumulation and efflux assays with some modifications for micro titre plates were adapted from the established protocol of Rodrigues et.al.[24] A liquid overnight culture of *M. smegmatis* mc^2 155 was cultivated in 7H9 T ADC at 37 °C and 100 rpm. The bacterial suspension was centrifuged at 4000 rpm for 5 min. The supernatant was discarded and adjusted in PBS containing 0.05% Tween 80 to an OD_{600} of 0.4. To determine the conditions that caused minimal accumulation of EtBr in *M. smegmatis* mc^2 155, various concentrations of EtBr that ranged from 8–0.125 mg/L were tested with and without 0.4% glucose. Reference inhibitors and test compounds were tested in the presence of 0.4% glucose and 0.5 mg/L EtBr at concentrations half their MIC. 0.1 mL aliquots of each test solution and 0.1 mL bacterial suspension were transferred into the wells. The increase of fluorescence was monitored every 50 seconds at 37 °C for 60 min with a Wallac 1420 Victor$^2{}_{TM}$ multilabel counter (Perkin Elmer life science) using a wavelength excitation of 531 nm and an emission of 590 nm.

4.5.3. EtBr efflux assay

An overnight culture of *M. smegmatis* mc^2 155 was adjusted with PBS containing 0.05% Tween 80 to an OD_{600} of 0.8. In the absence of glucose bacterial cells were loaded with EtBr (half MIC) and verapamil (half MIC), the reference inhibitor that caused the highest EtBr accumulation, for 1 h under shaking at 37 °C. The EtBr loaded cells were spun down at 4000 rpm and the supernatant was removed. After adjusting the OD_{600} to 0.8 with PBS containing 0.4% glucose, aliquots of 0.1 mL were transferred into the wells containing the dilutions of the test compounds and controls. The loss of fluorescence was recorded every 30 s at 37 °C for 30 min with a Wallac 1420 Victor$^2{}_{TM}$ multilabel counter. The excitation and emission wavelengths were 531 and 590 nm, respectively. The efflux was expressed as the ratio between the raw data and the data from the EtBr loaded cells, which were normalised to 1 establishing the EtBr loaded cells as the maximum fluorescence. Accumulation and efflux assays were repeated at least three times with reproducible results.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.039.

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