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Putative Mycobacterial Efflux Inhibitors from the Seeds of *Aframomum melegueta*

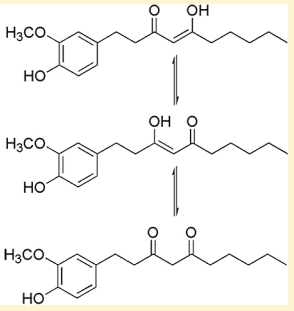
Barbara Gröblacher,[†] Veronika Maier,[†] Olaf Kunert,[‡] and Franz Bucar*[,†]

[†]Institute of Pharmaceutical Sciences, Department of Pharmacognosy, Karl-Franzens University Graz, Universitätsplatz 4, 8010 Graz, Austria

[‡]Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, Karl-Franzens University Graz, Heinrichstraße 28, 8010 Graz, Austria

Supporting Information

ABSTRACT: In order to identify new putative efflux pump inhibitors that represent an appropriate target in antimycobacterial chemotherapy, nine paradol- and gingerol-related compounds (**1**–**9**) isolated from the seeds of *Aframomum melegueta* were assessed for their potential to inhibit ethidium bromide (EtBr) efflux in a *Mycobacterium smegmatis* model. Five of the compounds from *A. melegueta* and NMR spectroscopic data of the diketone 6-gingerdione (**2**) and its enolic tautomers, methyl-6-gingerol (**5**) and *rac*-6-dihydroparadol (**7**), are presented herein for the first time. After determination of their antimycobacterial activities and modulatory effects on the MIC of antibiotics as well as their synergistic effects in combination with antibiotics against *M. smegmatis* mc^2 155, their impact on EtBr accumulation and efflux was evaluated using a microtiter plate-based fluorometric assay. The compounds exhibited moderate to weak antimycobacterial activities, and the best modulators induced a 4- to 16-fold decrease of the MICs of EtBr and rifampicin as well as a reduction of the MIC of isoniazid with fractional inhibitory concentration index values indicating synergistic activities in some cases. 6-Paradol (**3**), 8-gingerol (**6**), and *rac*-6-dihydroparadol (**7**) were the most potent EtBr efflux inhibitors in *M. smegmatis* mc^2 155, displaying EtBr efflux inhibiting activities comparable to reference inhibitors.



Multidrug resistance (MDR) remains a major challenge in the chemotherapy of infectious diseases such as tuberculosis (TB), one of the leading causes of mortality worldwide.[1,2] The most recent WHO report 2011 has revealed approximately 8.8 million incident cases, 12 million prevalent cases, and 1.4 million deaths. Especially in immune-deficient patients, including HIV-positive cases, a rising incidence of MDR and XDR (extensively drug resistant) TB has been estimated for 2010.[3] Intrinsic drug resistance of bacteria such as mycobacteria is attributed to the hydrophobic cell wall barrier and MDR efflux pumps.[2,4] Efflux pumps serve as transporters for various structurally dissimilar, noxious compounds including antibiotics, thereby reducing their intracellular concentration and contributing to MDR.[2,5] Thus, identification and characterization of efflux pump inhibitors (EPIs) that thwart these resistance mechanisms represent indispensible steps to combat the development and emergence of drug resistance. The use of an antibiotic, initially ineffective against MDR strains, together with an EPI as adjuvant to restore antibiotic activity is regarded as a possible alternative in the treatment of infectious diseases.[1,6] The nonpathogenic strain *Mycobacterium smegmatis* mc^2 155 (wild-type), which expresses several different efflux pumps, is considered as a suitable model to study efflux and identify new putative EPIs.[1,7,8] The fluorescent dye ethidium bromide (EtBr), which is a substrate for various efflux pumps, is used to evaluate efflux activity. Due to its low fluorescence signal outside cells and its concentration-dependent increase inside cells, it is possible to register efflux pump activity in a microtiter plate-based assay.[7,8] The following compounds have displayed activities as EPIs: verapamil,[2] chlorpromazine,[9] carbonyl cyanide *m*-chlorophenylhydrazone (CCCP),[2] as well as reserpine[2] and biochanin A,[10] the latter two derived from natural sources.

Seeds of *Aframomum melegueta* (Roscoe) K. Schum. (Zingiberaceae), commonly known as grains of paradise, Guinea grains, alligator pepper, melegueta pepper, or Guinea pepper, are mainly used in traditional West African medicine.[11−13] Extracts of the seeds are employed as a remedy against various ailments including inflammatory disorders, stomachic diseases, diarrhea,[14] measles, hemorrhage, and leprosy,[15] as well as tuberculosis and snakebites.[13] Previous investigations resulted in the isolation and characterization of the volatile oil[12] and the pungent principles of *A. melegueta*, namely, paradols, shogaols, and gingerols.[16−18] Studies of these metabolites have reported anti-inflammatory,[13] antinocicep-

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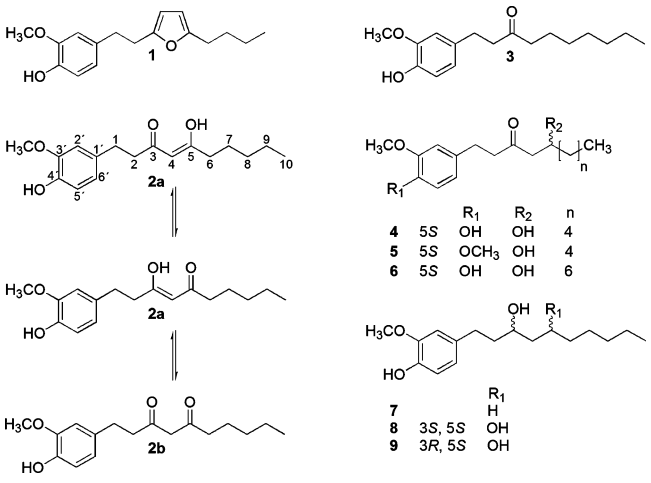
tive,[14] antioxidant,[19,20] antitumor-promoting,[20] antifeedant,[12] cytotoxic,[21] and antimicrobial activities.[22,23] Until now, there have been no studies on the modulating and synergistic activities of these compounds with antibiotics and their ability to inhibit EtBr efflux in mycobacteria. The present paper describes the isolation and characterization of nine constituents in the seeds of *A. melegueta*, of which five (**1**, **5**, **7**, **8**, **9**) are newly described from this species. It also reports the first NMR-based structure elucidation of 6-gingerdione and its two enolic forms (**2**), methyl-6-gingerol (**5**) and *rac*-6-dihydroparadol (**7**). For compound **2** the presence of enolic tautomers together with their respective diketone (6-gingerdione) was documented through the assignment of two sets of NMR spectroscopic data and by GC-MS derivatization experiments. For all compounds, the antimycobacterial together with modulatory and synergistic activities toward antibiotics as well as their potential as EtBr efflux inhibitors in *M. smegmatis* mc^2 155 were evaluated.



RESULTS AND DISCUSSION

Screening of plants belonging to the Zingiberaceae family revealed that the *n*-hexane extract of *A. melegueta* possesses promising antimycobacterial and modulating activities on the MIC of EtBr. Activity-guided fractionation of the extract resulted in the isolation of nine different paradol- and gingerol-related compounds. Five of these compounds (**1**, **5**, **7**, **8**, **9**) had not been described previously from *A. melegueta*, but were known from *Zingiber officinale*. Compounds **1**, **3**, **4**, and **6** were the known compounds 4-[2-(5-butylfuran-2-yl)ethyl]-2-methoxyphenol,[24] 6-paradol,[25] 6-gingerol,[25] and 8-gingerol,[25] as determined from NMR and GC-MS data.[26] The known compounds **8** and **9** were identified as the 6-gingerdiols 3*S*,5*S*-1-(4-hydroxy-3-methoxyphenyl)decane-3,5-diol and 3*R*,5*S*-1-(4-hydroxy-3-methoxyphenyl)decane-3,5-diol by comparing their NMR-spectroscopic data and the values for optical rotation to literature data.[27] The structures of methyl-6-gingerol (**5**) and *rac*-6-dihydroparadol (**7**) were established on the basis of NMR spectroscopy together with their characteristic MS fragmentation patterns.

For compound **2**, the HPLC chromatogram showed two clearly separated peaks. However, within a few hours after isolation of either of the peaks the initial mixture of isomers was again obtained. In addition, the NMR spectra of **2** revealed two sets of resonances for most of the protons and carbons of the side chain. The NMR resonance set showing the lower intensity (22%) was completely assigned by means of 1D ^{1}H and 2D DQF-COSY, HMBC and HSQC experiments. The general skeleton was comprised of a 4-hydroxy-3-methoxyphenyl moiety with an aliphatic C_{10}-carbon chain attached in position 1′. Carbon resonances at δ 204.3 and 203.4 were assigned by HMBC correlations to keto groups at positions C-3 and C-5, respectively, separated by a methylene group. Therefore, the minor component of the mixture was identified as 1-(4-hydroxy-3-methoxyphenyl)decane-3,5-dione (**2b**), also called 6-gingerdione. The second set of NMR resonances (78%) pointed to the presence of an enol form of **2b** with an olefinic methine group at C-4. The absence of an observable OH proton resonance together with the very small difference of the carbon shift values of C-3 and C-5 ($\Delta\delta_C$ = 0.8 ppm) and the shift values of C-2 and C-6 implied that the observed NMR resonances represent averaged signals of two compounds in rapid exchange. This confirmed the presence of both enolic tautomers **2a**, namely, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)dec-3-en-5-one and 5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-dec-4-en-3-one. The existence of three different isomers was also proved by GC-MS, in which after trimethylsilylation of the free OH groups three peaks were clearly visible, two of them originating from the tautomeric enol forms (*m*/*z* 436) and one from the unchanged dione (*m*/*z* 364). To the best of our knowledge, NMR data for either the keto or the enol forms of compound **2** were not reported previously. The structures of compounds **1**−**9** are as shown, and the ^{1}H and ^{13}C NMR spectroscopic data for compounds **2a**, **2b**, **5**, and **7** are provided in Table 1.

Antimycobacterial and Modulatory Activity. All isolated compounds were examined for their antimycobacterial activity and modulating activities on the MICs of different antibiotics against *M. smegmatis* mc^2 155 using Mueller-Hinton broth as culture media. Except for compounds **1**, **3**, **6**, and **7**, with MIC values in the range 32−64 mg/L, they exhibited weak antimycobacterial activities, with MIC values ≥ 128 mg/L. Taking their structural similarity and previously published data into consideration, certain structural features seem to be important for this class of compounds to exert antimycobacterial activity. The length of the aliphatic chain as well as a single OH or keto group at position 3 of the chain apparently enhances the antimycobacterial activity, whereas the presence of a keto group at position 3 and an OH group at position 5 or two OH or two keto groups at positions 3 and 5 results in a reduction of the activity. The introduction of a methoxy group instead of the OH group at position 4 on the aromatic ring causes neither a decrease nor an increase of activity. As modulators, the most active compounds (**1**, **2**, **3**, and **6**) induced an 8-fold decrease, followed by **7**, with a 4-fold decrease of the MIC of EtBr, rendering them putative EPIs. In particular, compound **1** was the best modulator, causing an 8-fold reduction of the MIC of EtBr, a 16-fold reduction of rifampicin, and a 4-fold reduction of isoniazid (INH) with a fractional inhibitory concentration index (FICI) indicating a synergistic activity when combined with EtBr or rifampicin. Compound **3** also achieved substantial results as a modulator and reduced the MIC of EtBr by a factor of 8 and of rifampicin and INH by a factor of 4. Compound **7** showed a potentiating activity on the MIC of EtBr and rifampicin; however, the effect on the MIC of INH, ethambutol, and ciprofloxacin was rather low. The correlation of the modulation factors ascertained for EtBr and rifampicin, which was reported previously for compounds isolated from *Alpinia katsumadai*, indicates that

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Table 1. NMR Spectroscopic Data for Compounds 2a, 2b, 5, and 7[a],

	2a[b]		2b[b]		5[c]		7[b]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.86, t (8.4)	31.2, CH_2	2.83, m	29.4, CH_2	2.81, s	29.9, CH_2		143.6, C
2	2.56, t (8.4)	40.6, CH_2	2.82, m	45.6, CH_2	2.79, s	46.3, CH_2		146.4, C
3		193.5, C		203.4, C		211.5, C	6.70, s	120.9, CH
4	5.54, s/5.55, s	99.5, CH	3.53, s	57.4, CH_2	2.50, dd (16.0, 4.7)	51.4, CH_2		134.1, C
					2.56, dd (16.0, 8.2)			
5		194.3, C		204.3, C	4.00 brs	68.9, CH	6.69	111.0, CH
6	2.25, t (7.6)	38.3, CH_2	2.44, t (6.8)	43.9, CH_2	1.40, m	38.5, CH_2	6.83, d (8.0)	114.2, CH
7	1.59, m	25.4, CH_2	1.53, m	23.0, CH_2	1.31, m	26.4, CH_2		
					1.42, m			
8	1.30, m	31.4, CH_2	1.25, m	31.4, CH_2	1.27, m	32.9, CH_2		
					1.29, m			
9	1.31, m	22.4, CH_2	1.31, m	22.4, CH_2	1.32, m	23.6, CH_2		
10	0.89, t (6.6)	13.9, CH_3	0.89, t (6.6)	13.9, CH_3	0.91, t (7.4)	14.4, CH_3		
1′		132.7, C		132.7, C		135.2, C	2.60, m	31.8, CH_2
							2.72, m	
2′	6.69	111.1, CH	6.69	111.1, CH	6.81, d (1.8)	113.6, CH	1.69, m	39.4, CH_2
							1.74, m	
3′		147.0, C		147.0, C		150.0, C	3.61 brs	71.4, CH
4′		144.4, C		144.4, C		148.6, C	1.45, m	37.6, CH_2
5′	6.83	114.2, CH	6.83	114.2, CH	6.84, d (8.4)	113.4, CH	1.31, m	25.6, CH_2
							1.41, m	
6′	6.68	120.8, CH	6.68	120.8, CH	6.73, dd (8.4, 1.8)	121.7, CH	1.27, m	29.3, CH_2
7′							1.25, m	29.6, CH_2
8′							1.25, m	31.8, CH_2
9′							1.28, m	22.6, CH_2
10′							0.87, t (6.9)	14.1, CH_3
3′-OCH_3	3.86, s	55.9	3.86, s	55.9	3.82, s	56.5		
4′-OCH_3					3.78, s	56.7		
2-OCH_3							3.87, s	55.9

[a]Chemical shifts (δ) in ppm at 600 MHz (^1H) and 150 MHz (^{13}C), multiplicities, J values (in parentheses) in Hz. [b]In $CDCl_3$. [c]In MeOD.

Table 2. MIC Values,[a] Modulation Factors,[b] and FICIs for Compounds 1−9 in *M. smegmatis* mc^2 155

compd	MIC in 7H9 T ADC (mg/L)	MIC in MHB (mg/L)	[c] as modulator (mg/L)	MF (EtBr)	MF (CIP)	MF (EB)	MF (INH)	MF (RIF)	FICI (EtBr)	FICI (RIF)
1	≥128	32	16	8	2	2	4	16	0.19	0.19
2	≥128	128	64	8	2	1−2	2	4	0.25	0.5
3	≥128	32	16	8	1	1	4	4	0.25	0.5
4	≥128	≥128	64	2	1	1	1	1	1	2
5	≥128	≥128	64	2	1	1	1	1	0.63	2
6	≥128	64	32	8	2	2	2	2	0.19	1
7	128	32	16	4	2	2	1	4	0.38	0.75
8	≥128	≥128	64	2	1	1	1	1	1	2
9	≥128	≥128	64	2	1	1	1	1	1	2

[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, = 32−64 mg/L, MIC of CCCP = 32 mg/L, MIC of CP, chlorpromazine, = 64 mg/L, MIC of VER, verapamil, = 512 mg/L. [b]MF = modulation factor, n = 4−8.

resistance to rifampicin is due to the presence of efflux pumps.[4,28] In contrast to the diarylheptanoids from *A. katsumadai*, some of the compounds from *A. melegueta* revealed modulating effects on the MIC of INH. Resistance to INH emerges through mutations in the katG enzymes, while it is also evident that other mechanisms such as low cell wall permeability and efflux pumps can be involved in the development of INH resistance.[29,30] As a result, it is possible that each compound has its own mechanism of action and may affect efflux in a different way.

The observed discrepancy between our MIC values and those reported by other authors may be a result of the use of different methods for determination of MIC including different culture media and *M. smegmatis* strains.[23] Therefore, the impact of culture media on the MIC of compounds from *A. melegueta* was determined by the broth dilution method with the following results: in Mueller-Hinton broth (MHB) compounds showed MIC values ranging from 32 to 128 mg/L, whereas in Middlebrook 7H9 broth supplemented with ADC enrichment (7H9 T ADC) MIC values were ≥128 mg/L. These decreased antimycobacterial activities reported for 7H9 T ADC may

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primarily be referred to as nondenatured proteins that after preparation still remain present in 7H9 T ADC. Bovine albumin, as a main component of the ADC enrichment, acts as a protective agent by binding free fatty acids and may contribute to the MIC values found in our experiments.[31] For accumulation and efflux experiments compounds were tested at concentrations correlating to one-half of their MIC determined in 7H9 T ADC. MIC values, modulation factors, and FICIs are listed in Table 2.

EtBr Accumulation and Efflux Inhibition. The compounds isolated from *A. melegueta* were assessed for their potential to increase the accumulation of EtBr in mycobacterial cells, which in the presence of a putative EPI is regarded as evidence for efflux pump inhibition.[6] Conditions that enable a minimal accumulation of EtBr were chosen to screen reference inhibitors and test compounds: compounds at concentrations corresponding to half their MIC and the use of 0.5 mg/L EtBr in the presence of 0.4% glucose. The reference inhibitor verapamil induced the highest EtBr accumulation, while CCCP resulted in the lowest level of EtBr accumulation. Almost all compounds from *A. melegueta* considerably increased the accumulation of EtBr in comparison to the EtBr control, which contains no EPI (data not shown). In particular, compounds **1**, **2**, **3**, **5**, **6**, and **7** were able to enhance the EtBr accumulation in relation to the reference inhibitors and the EtBr control. Results are presented in Figure 1. In this

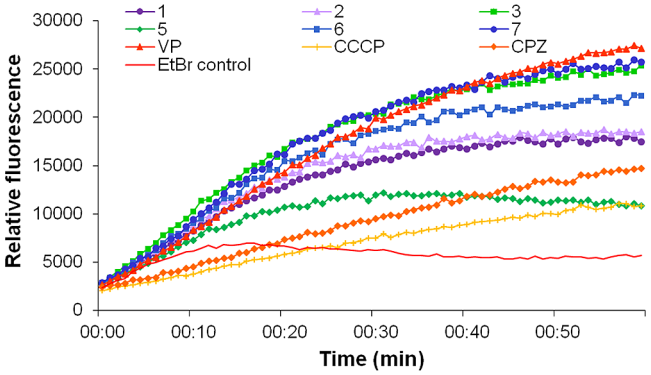

Figure 1. Effect of **1**–**3** and **5**–**7** at 64 mg/L and reference inhibitors on the EtBr accumulation in *M. smegmatis* mc^2 155. VP (verapamil), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), CPZ (chlorpromazine).

context, compounds **3**, **6**, and **7** achieved the highest EtBr accumulation at levels comparable to verapamil. Compounds **1** and **2** revealed moderate increases in EtBr accumulation, lower than verapamil but higher than chlorpromazine, whereas for compound **5** an EtBr accumulation similar to CCCP was observed. Compounds **4**, **8**, and **9** showed only slight increases of EtBr accumulation in relation to the EtBr control. However, to find out whether a concentration-dependent increase of the EtBr accumulation could be registered, a dilution series of compounds **3**, **6**, and **7** from 128–8 mg/L corresponding to concentrations of the MIC to 1/16 of the MIC was evaluated. Results of compound **3** are shown as an example in Figure 2. The steady state reached at the end of each assay allowed comparison of the EtBr accumulation caused by the best putative EPIs. Mean values including standard deviations of the relative fluorescence data are summarized in Table 3. It is well known that verapamil inhibits P-glycoprotein and bacterial efflux pumps.[2] Consequently, verapamil alone and in

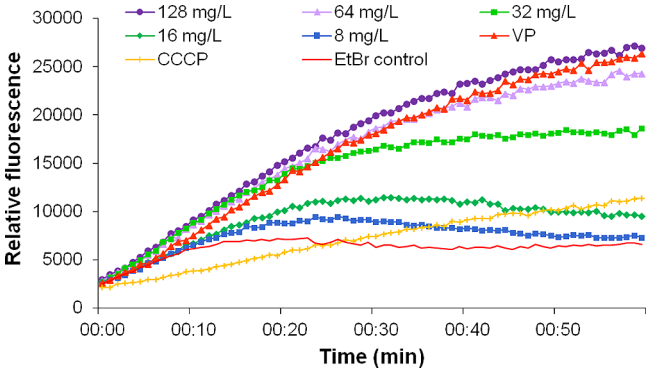

Figure 2. EtBr accumulation of a dilution series of **3** in *M. smegmatis* mc^2 155 in the presence of 0.5 mg/L EtBr and 0.4% glucose at 37 °C.

Table 3. Results of the Relative Fluorescence (Accumulation) and Normalized Fluorescence (Efflux) for Compounds 1–3 and 5–7 and the EtBr Control[a],

	accumulation		efflux	
compd	mean value	standard deviation	mean value	standard deviation
1	17180.3***	759.1	0.778***	0.012
2	17766.4***	1207.5	0.789***	0.019
3	24584.2***	735.5	0.793***	0.022
5	11764.3***	1052.6	0.746***	0.052
6	21917.0***	1451.3	0.795***	0.008
7	24671.7***	635.4	0.792***	0.020
EtBr control	6057.1	508.6	0.581	0.051
	n = 52		n = 84	

[a]Calculated as measured during the last 10 minutes of four assays. Compounds were compared to the EtBr control. The level of significance is indicated by asterisks. ***$p < 0.001$.

combination with one of the most active compounds was investigated according to its ability to increase the EtBr accumulation. Molar concentrations of verapamil corresponding to 1/4 of its MIC in combinations with test compounds that resulted in equal molar concentrations of verapamil at one-half of its MIC were used to examine the effect on the EtBr accumulation. All combinations induced an EtBr accumulation comparable to verapamil at one-half of its MIC (data not shown), and these findings indicate that verapamil as well as the test compounds from *A. melegueta* may affect the accumulation of EtBr through the same mechanism of action. In addition, compounds were examined for their ability to inhibit EtBr efflux in *M. smegmatis* mc^2 155. To gain the optimum conditions to measure efflux activity, conditions as follows were selected: incubation with EtBr and verapamil at half of their MICs in the absence of glucose for one hour under shaking at 37 °C. The reference inhibitors verapamil and chlorpromazine achieved similar activities as EtBr efflux inhibitors, and CCCP the weakest activity. Overall, all compounds from *A. melegueta* tested indicated potential to inhibit EtBr efflux related to the EtBr control (data not shown). According to the results obtained from the accumulation assay, compounds **3**, **6**, and **7** also showed to be the most potent EPIs from *A. melegueta* by causing an EtBr efflux inhibition comparable to chlorpromazine. In addition, with compound **2** a reduction of the EtBr efflux at levels of CCCP could be registered, while compound **5** was less active than CCCP. Results can be seen in Figure 3. None of the investigated

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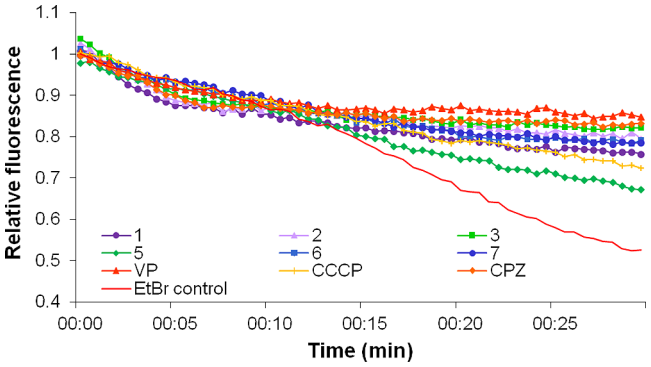

Figure 3. Effect of **1**–**3** and **5**–**7** at 64 mg/L and reference inhibitors on the EtBr efflux in *M. smegmatis* mc^2 155, after loading with 4 mg/L EtBr and 256 mg/L VP.

compounds achieved EtBr efflux inhibiting activity comparable to verapamil, but the most active compounds, **2**, **3**, **6**, and **7**, were able to decrease the EtBr efflux at levels of chlorpromazine or at least that of CCCP. In order to determine dose-dependent response, compounds were tested at concentrations of 128–8 mg/L (MIC to 1/16 of the MIC). The dose-dependent inhibition of the EtBr efflux displayed by compound **3** is shown in Figure 4. A comparative analysis of the efflux inhibition of

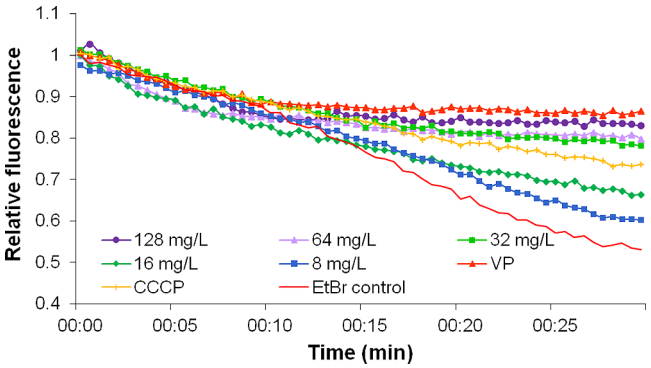

Figure 4. EtBr efflux inhibition of a dilution series of **3** in *M. smegmatis* mc^2 155. Cells were loaded with 4 mg/L EtBr plus 256 mg/L VP.

the most active compounds as compared to the EtBr control was generated on the basis of mean values and standard deviations of the normalized fluorescence data calculated for the last 10 minutes of four assays. Results are listed in Table 3. Moreover, IC_{50} values of the three most active compounds in the efflux assays, including the reference inhibitor verapamil, are summarized in Table 4.

In comparison to other putative EPIs tested, such as curcumin and its derivatives (unpublished data) and diaryl-

Table 4. IC_{50} Values of Compounds 2, 6, 7, and Verapamil[a]

	efflux	
compd	μg/mL	μM
3	17.7 ± 1.0	63.6 ± 3.7
6	26.8 ± 1.4	83.1 ± 4.5
7	19.4 ± 0.8	69.2 ± 2.7
VP	27.8 ± 2.0	56.6 ± 4.1

[a]IC_{50} values are expressed in μg/mL as well as μM. Data shown present the mean ± SEM of three independent experiments.

heptanoids from *A. katsumadai*,[28] the following structural requirements seem to be essential for these compounds to inhibit EtBr efflux in *M. smegmatis* mc^2 155. The basis represents an aliphatic chain including one substituent at position 3 or 5 attached to an aromatic ring. The length of the aliphatic chain and the substitution pattern of the aliphatic chain at position 3 or 5 are constitutive parameters that promote activity as EtBr efflux inhibitors. In particular, a single OH or carbonyl moiety at position 3 of the aliphatic chain induces a pronounced inhibition of the EtBr efflux in *M. smegmatis* mc^2 155.

EXPERIMENTAL SECTION

General Experimental Procedures. Vacuum liquid chromatography (32 × 7 cm) was conducted on silica gel 60, particle size 0.043–0.063 mm (Merck, Germany). Preparative HPLC was carried out on a Varian PrepStar equipped with a Dynamax solvent delivery system and UltraSep ES RP_{18}, 10 μm, 250 × 20 mm as stationary phase. Semipreparative HPLC was performed using a Merck Hitachi instrument with a LiChrospher RP_{18}, 10 μm, 250 × 10 mm column. HPLC was conducted on an Agilent 1100 series (quaternary pump, autosampler, diode array detector) with a Phenomenex, Kinetex 2.6 μm, C_{18}, 100 × 2.10 mm column. GC-MS data were acquired without or after derivatization with Sigmasil A to trimethylsilyl (TMS) derivatives according to the method described by Jolad et al.[26] on an Agilent Technologies 7890A GC system including an Agilent Technologies 7683B Series injector and Agilent Technologies 5975C VL MSD with HP-5MS 5% phenylmethylsiloxane, 30 m × 250 μm × 0.25 μm, as stationary phase. Helium was used as carrier gas (flow 1.2 mL/min), and the temperature program was set as follows: 80 °C for 5 min, then 10 °C/min to 280 °C, and a final hold of 280 °C for 20 min. Optical rotations were measured on a Jasco P-200 polarimeter in $CHCl_3$. NMR spectra (1D ^1H, ^{13}C and 2D DQF-COSY, multiplicity-edited HSQC, HMBC) were recorded in MeOD or $CDCl_3$ on a 600 Varian Unity Inova spectrometer operating at 600 MHz using TMS as internal standard. Chemical shifts are given in δ (ppm); *J* values, in hertz (Hz). SigmaPlot 12.0 was used to perform statistical analyses. To evaluate the statistical significance, the *t*-test, or if the normality test failed ($p < 0.05$), the Mann–Whitney rank sum test, was conducted to allow comparison of the active test compounds to the EtBr control. IC_{50} values for the three most active compounds of the efflux assay were calculated after 30 min of three independent experiments using the four-parameter logistic curve, causing compound-specific half-maximum inhibition.

Plant Material, Extraction, and Isolation. Seeds (1 kg) of *A. melegueta* (grains of paradise) were purchased from Mag. Kottas (Vienna, Austria). A Soxhlet extraction of the crushed seeds with *n*-hexane yielded a final residue of 35 g. A portion of the extract (30 g) was applied to silica gel and eluted with a gradient of *n*-hexane, EtOAc, and MeOH mixtures of increasing polarities (starting with 100% *n*-hexane with a stepwise increase of 2% to 50% EtOAc, then additions of 2% to 75% MeOH, and finally washing with 100% MeOH) to give 15 fractions. Fractions 4, 6, 10, and 14 were chromatographed on preparative and semipreparative HPLC with gradients of MeCN/H_2O or MeOH/H_2O. Fraction 4 (110 mg) afforded compound **1** (11 mg) by preparative HPLC with a gradient of MeCN/H_2O (MeCN/H_2O, 70:30, to MeCN/H_2O, 90:10, and then to 100% MeCN). Fraction 6 (330 mg) was first subjected to preparative HPLC by eluting with MeCN/H_2O (MeCN/H_2O, 75:25, and finally 100% MeCN) to obtain compounds **2** and **3**. Compounds **2** (33.4 mg) and **3** (27.1 mg) were further purified by semipreparative HPLC with isocratic mixtures of MeCN/H_2O (70:30 and 75:25, respectively). Preparative HPLC of fraction 10 (355 mg) with a gradient of MeCN/H_2O (MeCN/H_2O, 65:35, then 100% MeCN) yielded compounds **4** (187.8 mg) and **5**, **6**, and **7** (57.4 mg). Semipreparative HPLC was performed to purify compounds **5** (18.9 mg) and **6** (30.6 mg) by eluting with an isocratic solvent system of MeOH/H_2O (75:25) or MeCN/H_2O (75:25). Compounds **8** (29.1 mg) and **9** (24.9 mg) from fraction 14 (210 mg)

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were obtained by preparative HPLC using MeCN/H_2O (MeCN/H_2O, 50:50, then 100% MeCN). Chemical and physical data including NMR spectra of compounds **2**, **5**, and **7**, NMR spectroscopic data for compounds **1**, **3**, **6**, **8**, and **9**, and optical rotation values are available as Supporting Information.

Bacterial 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 studies. Bacteria were cultivated either on Columbia blood agar supplemented with 5% defibrinated horse blood (MIC and modulation assay) or in 7H9 T supplemented with 10% Middlebrook ADC enrichment (accumulation and efflux assay) at 37 °C under aerobic conditions.

MIC Assay and Modulation Assay. MICs of the reference inhibitors and test compounds were determined by the broth dilution method as described previously.[7,10] Briefly, compounds were dissolved in DMSO, diluted either in Mueller-Hinton broth or in 7H9 T ADC, and serially diluted across a microtiter plate. A bacterial inoculum equal to the McFarland turbidity standard 0.5 was adjusted to a density of 5×10^5 cfu/mL. Aliquots of 0.125 mL of the bacterial suspension were transferred into the wells of a microtiter plate containing 0.125 mL aliquots of the serial dilution of each test compound. Using MTT as indicator, the MIC was observed after 72 h of incubation at 37 °C and defined as the lowest concentration that inhibited bacterial growth.

At concentrations corresponding to half of their MIC in MHB, compounds were examined for their modulatory activities on the MIC of EtBr and standard antibiotics as well as their combined effects with EtBr and rifampicin. For this reason, a solution of either the modulator or the antibiotic dissolved in DMSO and MHB was used as culture media instead of pure MHB, and according to this, the antibiotic or the modulator was serially diluted across a microtiter plate. The modulation factor was used to express the modulating effects on the MIC of the respective antibiotic, whereas the FICI was calculated to ascertain the effect of the combination of antibacterial agents.

$$\mathrm{MF} = (\mathrm{MIC\ antibiotic})/(\mathrm{MIC\ antibiotic} + \mathrm{modulator})$$

$$\mathrm{FICI} = \mathrm{FIC(A)} + \mathrm{FIC(B)}$$

$$\mathrm{FIC(A)} = \mathrm{MIC(A\ in\ the\ presence\ of\ B)}/\mathrm{MIC(A\ alone)}$$

$$\mathrm{FIC(B)} = \mathrm{MIC(B\ in\ the\ presence\ of\ A)}/\mathrm{MIC(B\ alone)}$$

(Synergy: FICI $\leq$ 0.5, additive: FICI > 0.5−1, indifference: FICI >1 to <2, antagonism: FICI $\geq$ 2.[32])

EtBr Accumulation and Efflux Assay. On the basis of the established procedure of Rodrigues et al.[8] accumulation and efflux assays were modified for microtiter plates.[28] For accumulation assays, a liquid overnight culture was adjusted with PBS + 0.05% Tween to an OD of 0.4. The following conditions were assessed to achieve a minimal accumulation of EtBr in mycobacterial cells: the use of an EtBr concentration of 0.5 mg/L in the presence of 0.4% glucose. All test compounds were screened at concentrations corresponding to half their MIC determined in 7H9 T ADC. For efflux assays, bacteria were adjusted with PBS + 0.05% Tween to an OD of 0.8. To ensure a maximum load of the cells with EtBr, cells were incubated with EtBr and verapamil at concentrations half their MIC in the absence of glucose at 37 °C for one hour. Cells were then resuspended in EtBr and verapamil-free PBS + 0.05% Tween containing 0.4% glucose. In general, 0.1 mL aliquots of each test solution and bacterial suspension were transferred into the wells of a microtiter plate. The loss or increase of fluorescence was monitored every 30 s (EtBr efflux) or every 50 s (EtBr accumulation) at 37 °C using a Wallac 1420 Victor2 multilabel counter (Perkin-Elmer Life Science) for 30 or 60 min using filters of excitation and emission wavelengths of 531 and 590 nm, respectively. To obtain a comparative analysis of the efflux, the fluorescence data of the EtBr-loaded cells were normalized to 1, thereby establishing them as maximum fluorescence value. The efflux was then expressed as the ratio between the fluorescence data from the respective test compound and the fluorescence data from the EtBr-loaded cells.

■ ASSOCIATED CONTENT

Supporting Information

Chemical and physical data including NMR spectra of compounds **2**, **5**, and **7**, NMR spectroscopic data for compounds **1**, **3**, **6**, **8**, and **9**, as well optical rotation values are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: + 43-316-3805531. Fax: + 43-316-3809860. E-mail: franz.bucar@uni-graz.at.

Notes

The authors declare no competing financial interest.

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