Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7638–7646
Bioorganic & Medicinal Chemistry

Activity of 7-methyljuglone derivatives against *Mycobacterium tuberculosis* and as subversive substrates for mycothiol disulfide reductase

Anita Mahapatra,[a,†] Sannah P. N. Mativandlela,[a] B. Binneman,[a] P. B. Fourie,[b] Chris J. Hamilton,[c,*,‡] J. J. M. Meyer,[a] F. van der Kooy,[a] Peter Houghton[d] and Namrita Lall[a,*]

[a]*Department of Botany, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa*
[b]*Medical Research Council, Pretoria, South Africa*
[c]*School of Chemistry and Chemical Engineering, David Keir Building, Queen's University Belfast, Stranmillis Road, Belfast BT9 5AG, UK*
[d]*Department of Pharmacy, Franklin-Wilkins Building, 150 Stamford Street, King's College London, London SE1 9NN, UK*

Received 23 July 2007; revised 23 August 2007; accepted 31 August 2007
Available online 6 September 2007

Abstract—The naphthoquinone 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone) has previously been isolated and identified as an active component of root extracts of *Euclea natalensis* which displays antitubercular activity. Herein, a series of synthetic and plant-derived naphthoquinone derivates of the 7-methyljuglone scaffold have been prepared and evaluated for antibacterial activity against *Mycobacterium tuberculosis*. Several of these compounds have been shown to operate as subversive substrates with mycothiol disulfide reductase. The absence of a direct correlation between antitubercular activity and subversive substrate efficiency with mycothiol disulfide reductase, might be a consequence of their non-specific reactivity with multiple biological targets (e.g. other disulfide reductases).
© 2007 Elsevier Ltd. All rights reserved.

1. Introduction

Tuberculosis (TB) is a contagious disease caused by respiratory infection from the Gram positive bacteria *Mycobacterium tuberculosis*. In recent years, TB has re-emerged as a major world health problem with an estimated annual death toll of 2 million. With approximately one-third of the world population currently infected, increased prevalence of the disease in HIV-infected patients and the emergence of multi-drug-resistant bacteria, TB remains a major world health problem. Hence there is a continuing need to find additional lead compounds and biological targets for novel antitubercular chemotherapies.

Naphthoquinones are widely distributed in plants, fungi and some animals[1] and many are found to exhibit an interesting range of pharmacological properties including antibacterial,[2,3] antiviral,[4] trypanocidal,[5] anticancer,[6] antimalarial,[7,8] and antifungal[9] activity. Other quinone-related scaffolds such as 2-*H*-pyran-3(6*H*)-one derivatives[10] and 2,3-dideoxyhexenopyranosides[11] are also known for their biological activity against Gram positive bacteria such as *Staphylococcus aureus* and *M. tuberculosis*. During the investigation of South African medicinal plants, traditionally used in treatment of chest complaints, significant activity of the crude extract of *Euclea natalensis*[12] and that of diospyrin **22**, isolated from this plant, was observed against drug-resistant strains of *M. tuberculosis*.[13] It has since been shown that a 2-aminoacetate derivative

Keywords: Naphthoquinones; Subversive substrates; Disulfide reductase; Mycothiol; Tuberculosis.
* Corresponding authors. E-mail addresses: c.hamilton@uea.ac.uk; namrita.lall@up.ac.za
† Present address: Herbal Drugs & Bio-Remedies Cell, Regional Research Laboratory (CSIR), Bhubaneswar 751 013, Orissa, India.
‡ Present address: School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, Norfolk NR4 7TJ, UK.

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.08.064

of dimethylether-diospyrin has enhanced antituberculosis activity.[14] The monomer of diospyrin, (7-methyljuglone, **6**), exhibited intracellular and extracellular inhibition of *M. tuberculosis* comparable to streptomycin and ethambutol.[15] As the yield of 7-methyljuglone **6** from the plant *E. natalensis* is low (0.03%),[15] it has now been synthesised along with some of its derivatives (Fig. 1). Previous studies have shown that mono- or dihydroxy substitution (at C_5 or C_5 & C_8 positions in the aromatic ring) of naphthoquinones results in higher toxicity as compared to the parent 1,4-naphthoquinone due to increased efficiency of redox cycling.[16] The introduction of a fluorine atom into antibiotic quinolones has been shown to enhance their activity.[17,18] In view of these previous observations, it was decided to synthesise derivatives of 7-methyljuglone in order to establish structure–activity relationship.

Towards this goal, this paper reports the synthesis, antibacterial activity and cytotoxicity of a series of 5-hydroxy-, 5-alkoxy- and 5-acetoxy-8-substituted-naphthoquinones (Fig. 1). To provide insight into possible mode(s) of action, the subversive substrate properties of some of these compounds with *M. tuberculosis* mycothiol disulfide reductase are also reported.

2. Results and discussion

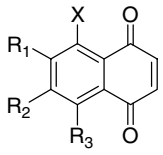
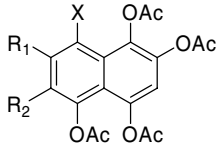
2.1. Synthesis

The 8-fluoronaphthoquinone **2** was prepared by the reaction of 4-fluoro-3-methylphenol with maleic anhydride under Friedel Crafts acylation conditions. Compounds **3**,[19] **4**[20] and **5**,[21,22] were prepared in a similar manner. Reductive cleavage of the chlorine substituent of **3** and **5** was achieved by treatment with tin(II) chloride to access **6**[19] and **7**[21] (Scheme 1).

The 5-*O*-acyl naphthoquinones **8**–**10** and the 5-*O*-alkyl derivatives **11**–**16** were prepared by 5-*O*-acylation/alkylation of the 5-hydroxynaphthoquinone precursors **3**, **5**–**6** (Scheme 2). The 1,2,4,5-tetra-acetoxy derivatives **17**–**19** were prepared by treatment of appropriate naphthoquinones (**3**, **5**–**6**) with acetic anhydride under strongly acidic conditions (Scheme 2). Hydrolysis of **17**[21] with methanolic HCl afforded the 2-hydroxynaphthoquinone derivative **20**.

2.2. Antimycobacterial activity

The minimum inhibitory concentrations (MIC) of compounds **2**–**19** against *M. tuberculosis* H37Rv were deter-

Structure	NQ	X	R_1	R_2	R_3
	**1**	H	H	H	OH
	2	F	Me	H	OH
	3	Cl	Me	H	OH
	4	Br	Me	H	OH
	5	Cl	H	Me	OH
	6	H	Me	H	OH
	7	H	H	Me	OH
	8	H	Me	H	OAc
	9	Cl	Me	H	OAc
	10	Cl	H	Me	OAc
	11	H	Me	H	OMe
	12	Cl	Me	H	OMe
	13	Cl	H	Me	OMe
	14	H	Me	H	OEt
	15	Cl	Me	H	OEt
	16	Cl	H	Me	OEt
	**17**	H	Me	H	
	18	Cl	Me	H	
	19	Cl	H	Me	

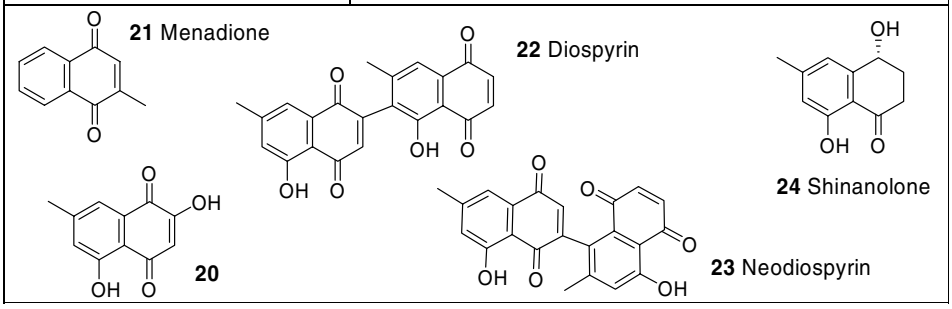
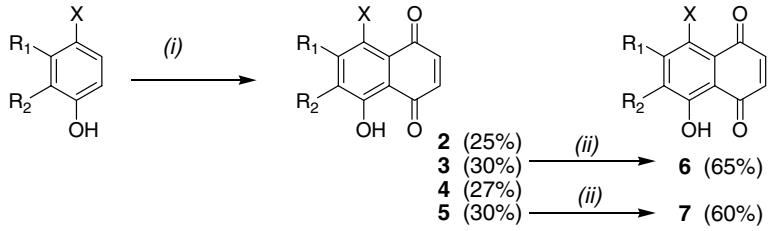
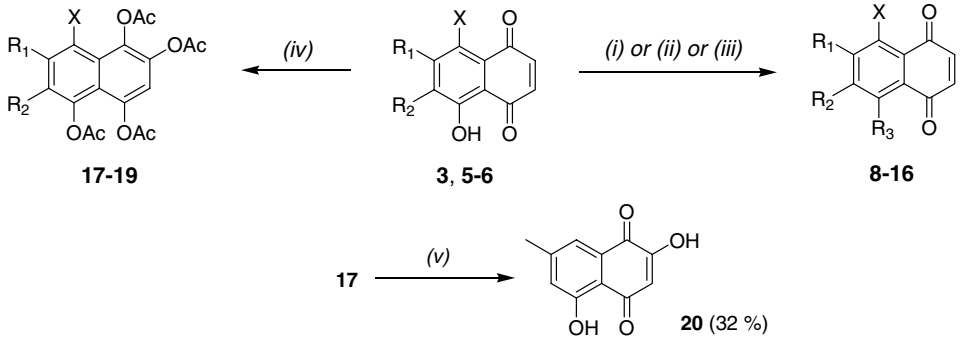


Figure 1. List of naphthoquinones studied for antimycobacterial and disulfide reductase activity.



Scheme 1. Reagents and conditions: (i) maleic anhydride, $AlCl_3$, NaCl, 180 °C, 2 min; (ii) $SnCl_2$, 4 M HCl/THF, 60 °C, 2–4 h.



Starting material	Reagents	Product	Yield (%)
6	*(i)*	**8**[23]	70
3	*(i)*	**9**[19]	98
5	*(i)*	**10**	74
6	*(ii)*	**11**[24]	96
3	*(ii)*	**12**	70
5	*(ii)*	**13**	75
6	*(iii)*	**14**	98
3	*(iii)*	**15**	70
5	*(iii)*	**16**	72
6	*(iv)*	**17**[21]	60
3	*(iv)*	**18**	70
5	*(iv)*	**19**	70

Scheme 2. Reagents and conditions: (i) Ac_2O, NaOAc, reflux, 2 h; (ii) Ag_2O, MeI, Me_2CO, 60 °C, 2–4 h; (iii) Ag_2O, EtI, Me_2CO, 60 °C, 2–4 h; (iv) Ac_2O, concd H_2SO_4, (v) methanolic 2 M HCl, 30 min, reflux. See above-mentioned references for further information.

mined in liquid media (Table 1). The naphthoquinones **4**–**8** and **20** exhibited the greatest activity among all the derivatives screened. Of these, 7-methyljuglone **6** was the most potent followed by its 5-acetoxy derivative **8**, which was 5-fold less active. In the halide series **2**–**4**, the activity increased with an increase in halide bulkiness and decrease in halide electronegativity. Earlier studies into the antibacterial activity of a family of isoxazolenaphthoquinones demonstrated that a hydroxyl substituent at C-2 enhanced the antibacterial activity.[25] However, for the naphthoquinone series in Table 1, it is evident that incorporation of the 2-hydroxyl substituent in compound **20** does not enhance its antitubercular activity relative to its unsubstituted precursor **6**. Interestingly, the 7-methyl substituted compound **6** is more potent than its 6-methyl isomer **7**, but the opposite trend is observed for the 8-chloro derivatives **3** and **5**. The low activities in the tetra-acetate series (**17**–**19**) are likely due to the lack of the quinone motif. The alkoxy derivatives (**11**–**16**) were found to be less active than their hydroxyl precursors **3**, **5** and **6**.

2.3. Bactericidal activity

The bactericidal activities of derivatives exhibiting MICs of less than 20 μg/ml were determined (Fig. 2). Of the naphthoquinones tested, derivatives **8** and **1** showed the highest bactericidal activity at their respective MIC concentrations. The bactericidal activity of **8** was comparable to that of streptomycin, within experimental error. Apart from compounds **2**–**4**, **6**–**7** and **22**–**23**, all the remaining compounds killed at least 99.9% of the initial bacterial inoculum. These observations are consistent with the previously observed influence of similar structural modifications on the biological activity of diospyrin **22**.[14]

2.4. Cytotoxicity on Vero cells

Cytotoxicity results for the Vero cell line indicate that the least toxic derivative (IC_{50} of 21.1 μg/ml) with good antituberculosis activity is derivative **10** (Table 1). Derivatives **17**–**19** exhibit reduced activity and cytotox-

Table 1. Antituberculosis activity and cytotoxicity of 7-methyljuglone **6** and its derivatives

Compound	MIC[a] (μg/ml)	IC_{50}[b] (μg/ml)	SI[c]
2	10.0	7.6	0.76
3	10.0	2.5	0.25
4	5.0	3.6	0.71
5	5.0	4.6	0.92
6	0.5	15.1	30.22
7	5.0	3.1	0.63
8	2.5	6.0	2.40
9	10.0	6.3	0.63
10	10.0	21.1	2.1
11	15.0	5.8	0.39
12	>20.0	7.7	—
13	15.0	8.0	0.54
14	15.0	6.3	0.42
15	>20.0	36.1	—
16	15.0	12.5	0.83
17	>20.0	17.2	—
18	>20.0	27.8	—
19	>20.0	149.4	—
20	5.0	3.1	0.63
Menadione **21**	5.0	6.5	1.30
Diospyrin **22**	8.0	17.8	2.2
Neodiospyrin **23**	10.0	32.0	3.2
Shinanalone **24**	>100	—	—
Juglone **1**	1.0	1.2	1.19
Isoniazid	0.2	>200	>1000
Streptomycin	1.0	>120	>120
Ethambutol	2.0	>150	>75

[a] Minimum inhibitory concentration.
[b] Fifty percentage inhibitory concentration.
[c] Selectivity index (IC_{50}/MIC).

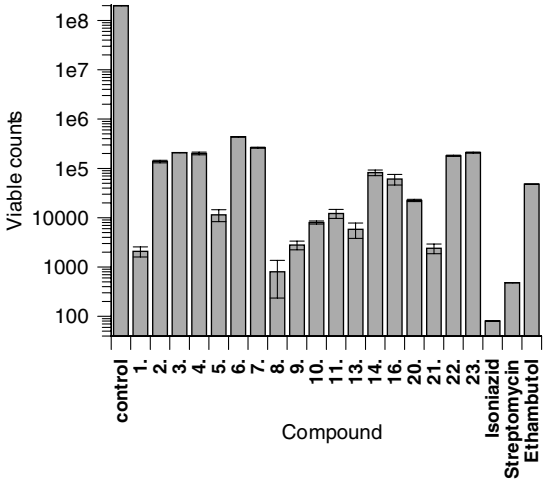


Figure 2. Comparative bactericidal effect of synthesised 7-methyljuglone derivatives against H37Rv strain of *M. tuberculosis*. Compounds were screened at their individual MIC concentrations as indicated in Table 1.

icity, which suggests that the quinone motif contributes both to biological activity and cytotoxicity. This is probably due to these naphthoquinones acting as non-specific subversive substrates in both bacteria and mammalian cells.

2.5. Subversive substrate properties with Mtr

Many naphthoquinones are known to operate as subversive substrates with flavoprotein disulfide reductases such as glutathione reductase, trypanothione reductase and lipoamide dehydrogenase.[5,26] The native functions of these enzymes involve the NAD(P)H-dependent reduction of disulfide bonds in proteins or oxidised versions of low molecular weight thiols such as glutathione. The enzyme-mediated toxicity of quinones/naphthoquinones is a consequence of their enzymatic reduction to semiquinone radicals (Fig. 3b). The naphthoquinone is then regenerated via the concomitant reduction of oxygen to toxic superoxide anion radicals. In this manner the naphthoquinone substrate is regenerated and the futile redox cycle continues. It seems plausible that some of compounds **2**–**19** could be exerting their biological activity as subversive substrates with similar disulfide reductases found in *M. tuberculosis*.

Mycobacterium tuberculosis lacks glutathione, instead it maintains millimolar concentrations of the structurally distinct low molecular weight thiol mycothiol (MSH) (Fig. 3a).[27–29] Analogous to glutathione, MSH plays an important role in oxidative stress management and is oxidised to the symmetrical disulfide (MSSM) in the process. The NADPH-dependent enzyme mycothiol disulfide reductase (Mtr)[30] helps to maintain an intracellular reducing environment by reducing MSSM back to MSH. MSH is essential for the growth of *M. tuberculosis*[31] and MSH-deficient mycobacteria exhibit increased sensitivity to oxidative stress,[32,33] making this redox pathway a potential biological target for novel antitubercular chemotherapies. The catalytic properties of Mtr were originally reported using both MSSM and some commercial naphthoquinones (e.g. menadione **21**) as substrates.[30] The biological importance of Mtr and its ability to turnover other naphthoquinone substrates prompted a study of the substrate properties of some of the aforementioned naphthoquinones with *M. tuberculosis*.

The subversive substrate properties of some of the naphthoquinones with Mtr and comparisons with their MIC values (expressed as μM concentrations) in whole cell assays are summarised in Table 2. Whilst K_m values show

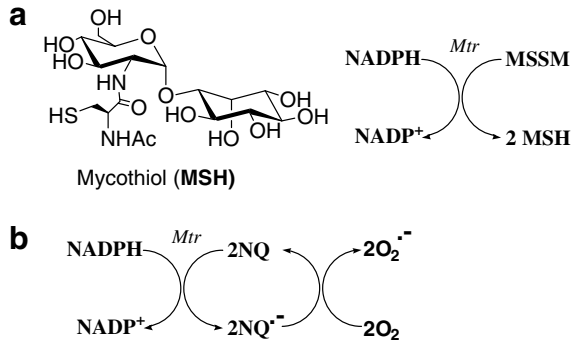


Figure 3. (a) Mycothiol and its disulfide reductase; (b) futile enzymatic redox cycle of naphthoquinones.

Table 2. Substrate properties of substituted naphthoquinones with *M. tuberculosis* Mtr

Compound	K_m (μM)	k_{cat} s^{-1}	k_{cat}/K_m ($\times 10^5$)	MIC (μM)
1	324 (±50)	1.088 (±0.079)	336	6
3	233 (±45)	0.212 (±0.021)	91	45
5	36 (±5)	0.592 (±0.017)	1644	22
6	254 (±23)	2.365 (±0.214)	931	3
8	389 (±59)	3.169 (±0.168)	815	11
12	435 (±112)	0.142 (±0.018)	33	85
21	483 (±146)	0.732 (±0.140)	152	29
22	33 (±4)	0.118 (±0.005)	357	21
23	63 (±8)	0.308 (±0.010)	488	27
24	—not active-up to 800 μM—			>530[34]
MSSM	54[a]	64[b]	118519[b]	—
Isoniazid				0.2

[a] As reported in Ref. 40.
[b] As reported in Ref. 35.

the substrate binding affinities and the k_{cat} values express the maximum turnover rates, the overall catalytic efficiency (k_{cat}/K_m) best expresses the futile substrate efficiency of these substrates. It is the latter value that was used to look for a direct correlation between futile substrate properties (in vitro) and the whole cell antibacterial activities of these compounds.

The K_m values of compounds **1**, **3**, **6**, **8**, **12** and **21** are all in the 200–500 μM range, whereas **5**, **22** and **23** are significantly lower (30–60 μM). The previously reported K_m value of **21** with Mtr is 540 μM,[30] which is comparable to the value determined herein. All of the naphthoquinones in Table 1 can be viewed as structural elaborations of the basic juglone scaffold **1** and in this context, it appears that methylation of the C-2 position **21** or the 5-hydroxyl group **12** is the most detrimental to substrate binding with Mtr. Depending on which naphthoquinone motif acts as the electron acceptor during enzymatic turnover, diospyrin **22** can be viewed as either a 2- or a 6-substituted derivative of 7-methyljuglone **6**; similar observations can be made for neodiospyrin **23**. These dimeric versions of **6** exhibit a 5- to 10-fold reduction in K_m. The turnover rates (k_{cat}) of the compounds in Table 1 are also reported. 7-Methyljuglone **6** and its 5-acetoxy derivative **8** have the fastest turnover rate at substrate saturation followed by **1** and **21**. The addition of the 8-chloro substituent (compounds **3**, **5** and **12**) is notably detrimental to the turnover rate at a level which is comparable to that observed for the naphthoquinone dimers **22** and **23**. In terms of substrate efficiency (k_{cat}/K_m), **5** is the most efficient subversive substrate with Mtr followed by **6** and **8**. There is no direct correlation between the antibacterial activity in whole cell assays (MIC) and the k_{cat}/K_m values of these compounds with Mtr. Shinanolone **24** lacks the conjugated benzoquinone motif that is required for subversive substrate activity hence it is not a substrate for Mtr. It also displays significantly weaker antibacterial activity than any of the other naphthoquinones in whole cell assays as do compounds **17**–**19** which also lack the quinone motif. Comparing the Mtr substrate properties of the naphthoquinones (Table 2) with those observed for (MSSM),[35,40] it is evident that MSSM is turned over more efficiently.

The absence of a direct correlation between the subversive substrate efficiency of these naphthoquinones and their MIC values is probably because their antibacterial activity in whole cells is the accumulative consequence of their non-specific reactivity with multiple biological targets. In *M. tuberculosis* one of these targets could plausibly be Mtr. Additional targets could include other flavoprotein oxidoreductases such as lipoamide dehydrogenase and thioredoxin reductase, which unlike Mtr are also found in eukaryotes. It has also been suggested that naphthoquinone structures such as **6** could behave as non-functional ubiquinone and/or menaquinone surrogates, which may perturb electron transfer in respiratory chain processes.[35] Some of these biochemical functions are shared by the mammalian hosts which may explain the low selectivity index for these simple naphthoquinones. However, the mycothiol disulfide redox pathway is unique to actinomycetes (e.g. *M. tuberculosis*) and substitutes the glutathione reductase pathway utilised in mammals. There is scope for narrowing the target specificity of these naphthoquinones by appropriate structural modifications (e.g. carbohydrate/polyol motifs) so as to tailor their specificity for Mtr. Such an approach has previously been demonstrated by modification of simple naphthoquinone scaffolds with polyamine motifs to enhance their specificity for trypanothione reductase versus mammalian glutathione reductase and LADH enzymes.[5]

3. Conclusions

The results presented here show that 7-methyljuglone **6** exhibits the most potent and the most selective antitubercular activity of compounds **1**–**19**. However, in terms of substrate efficiency, compound **6** was not the most potent subversive substrate for Mtr, which is one of several potential biological targets for the antitubercular activity of naphthoquinones such as **6**. The poor selectivity of these compounds is possibly due to their non-specific activity with other disulfide reductases (e.g. thioredoxin reductase, lipoamide dehydrogenase) which are also found in mammalian cells. It will be interesting to see if it is possible to further elaborate the 7-methyljuglone scaffold in order to optimise its subversive substrate properties and specificity for Mtr.

4. Materials and methods

4.1. General

Melting points were determined on a Buchi apparatus and are uncorrected. The IR spectra were recorded on a Perkin-Elmer Spectrum RXI FT-IR spectrometer. ^1H and ^{13}C NMR spectra were measured in $CDCl_3$ with a Varian 200 MHz NMR spectrometer with chemical shift values being represented in ppm relative to the internal standard TMS. High-resolution EI mass spectra were obtained using a JEOL JMS—AX505 double

focussing mass spectrometer. *M. tuberculosis*, H37Rv (ATCC 27294) and Vero cells (African green monkey kidney cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). Recombinant *M. tuberculosis* mycothiol disulfide reductase was over-expressed and purified from an *Mycobacterium smegmatis* mc^2155 transformant as previously described.[35] Porcine LADH, Juglone **1** and menadione **21** were purchased from Sigma. Diospyrin **22**, neodiospyrin **23** and shinanalone **24** were purified from *E. natalensis* plant extracts as previously described.[36] Spectrophotometric assays of Mtr and LADH were carried out in 1 ml cuvettes on a temperature controlled Perkin-Elmer UV Lambda 25 spectrophotometer. Kinetic data were analysed (by non-linear regression) using Grafit Version 5 (Erithacus Software Ltd).

4.2. Chemistry

4.2.1. General procedure for the preparation of 8-halogen derivatives (2–5). A mixture of anhydrous $AlCl_3$ (40 g, 300 mmol) and NaCl (8 g, 137 mmol) were heated to 180 °C. A mixture of appropriate 4-halo-3-methyl phenol (10.7 mmol) or 4-halo-2-methyl phenol and maleic anhydride (4 g, 40.8 mmol) was added to the above melt with vigorous stirring for 2 min, and then poured into a mixture of ice and 12 M HCl. The mixture was kept for 30 min, and the precipitate was filtered and dried at room temperature overnight. The residue obtained was powdered and extracted with *n*-hexane with vigorous stirring at 50 °C. The extract was concentrated under reduced pressure and crystallised from chloroform to afford the corresponding halogenated products.

4.2.1.1. 8-Fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone (2). The compound was obtained as dark orange needles from $CHCl_3$, yield 25%, mp 150 °C; IR (KBr, cm^{-1}): 1663, 1642 (C=O); δ_H ($CDCl_3$, 200 MHz); 12.59 (1H, s, 5-OH), 7.31 (1H, s, H-6), 6.95 (2H, s, H-2, H-3), 2.53 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz): 189.51 (C-4), 183.22 (C-1), 160.54 (C-5), 148.99 (C-8), 140.85 (C-2), 140.36 (C-3), 137.61 (C-7), 136.62 (C-9), 125.89 (C-6), 114.53 (C-10), 21.89 (C-11); found: (EI) 206.0372, $C_{11}H_7FO_3$ requires 206.0379.

4.2.1.2. 8-Bromo-5-hydroxy-7-methyl-1,4-naphthoquinone (4). The compound was obtained as dark red needles from $CHCl_3$, yield 27% mp 154 °C; IR (KBr, cm^{-1}): 1663, 1642 (C=O); δ_H ($CDCl_3$, 200 MHz); 12.54 (1H, s, 5-OH), 7.18 (1H, s, H-6), 6.88 (2H, s, H-2, H-3), 2.47 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz); 189.50 (C-4), 183.21 (C-1), 160.54 (C-5), 148.99 (C-8), 140.84 (C-2), 140.69 (C-3), 136.61 (C-7), 136.52 (C-9), 125.88 (C-6), 114.53 (C-10), 21.89 (C-11); found: (EI) 265.9574, $C_{11}H_7BrO_3$ requires 265.9579.

4.2.2. General procedure for the preparation of 5-hydroxy-7- and 6-methyl-1,4-naphthoquinones (6 and 7). A solution of appropriate 8-chloro-1,4-naphthoquinones (200 mg, 0.90 mmol) in THF (20 ml) was added dropwise to a solution of $SnCl_2$ (1.0 g, 51 mmol) in 4 M HCl (70 ml) and THF (20 ml) at 60 °C and stirred for 3 h. It was then cooled and filtered into a solution of $FeCl_3$. The resulting precipitate was filtered and dried to afford the required products.

4.2.2.1. 5-Hydroxy-7-methyl-1,4-naphthoquinone (6). The compound was obtained as orange needles from $CHCl_3$, yield 65%, mp 125 °C (lit.[37] 126 °C); IR(KBr, cm^{-1}): 1670, 1645 (C=O); δ_H ($CDCl_3$, 200 MHz); 11.84 (1H, s, 5-OH), 7.42 (1H, s, H-8), 7.06 (1H, s, H-6), 6.89 (2H, s, H-2, H-3), 2.41 (3H, s, CH_3); found: (EI) 188.0475, $C_{11}H_8O_3$ requires 188.0473.

4.2.2.2. 5-Hydroxy-6-methyl-1,4-naphthoquinone (7). The compound was obtained as dark orange needles from $CHCl_3$, yield 60%, mp 104 °C (lit.[21] 108 °C); IR (KBr, cm^{-1}): 1663, 1655 (C=O); δ_H ($CDCl_3$, 200 MHz); 13.0 (1H, s, 5-OH), 7.48 (1H, s, H-7), 7.23 (1H, s, H-8), 6.89 (2H, s, H-2, H-3), 2.32 (3H, s, CH_3). δ_C ($CDCl_3$, 50 MHz); 15.81 (CH_3), 115.00, 124.64, 126.04, 136.60, 136.76, 140.24, 140.94, 160.22 (C-5), 182.62 (C=O), 190.10 (C=O); found: (EI) 188.0462, $C_{11}H_8O_3$ requires 188.0473.

4.2.3. General procedure for the preparation of 5-acetoxy derivatives (8–10). A mixture of appropriate naphthoquinone (0.45 mmol), anhydrous sodium acetate (40 mg, 0.49 mmol) and acetic anhydride (2 ml, 19.59 mmol) was heated under reflux for 3 h. The reaction mixture was poured into hot water. It was allowed to cool and extracted with $CHCl_3$, dried ($MgSO_4$), concentrated and crystallised from chloroform–hexane to yield the corresponding 5-acetoxy derivatives.

4.2.3.1. 5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone (10). The compound was prepared from **5** as a greyish amorphous powder, yield 74%, mp 230 °C; IR (KBr, cm^{-1}): 1770 (aryl acetate C=O), 1660 (quinone C=O); δ_H ($CDCl_3$, 200 MHz); 7.63 (1H, s, H-6), 6.73 (1H, d, *J* 10.0, H-2), 6.90 (1H, d, *J* 10.0, H-3), 2.44 (3H, s, CH_3), 2.27 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz); 190.97 (C=O), 182.28 (C=O), 168.89 (C=O), 159.54 (C-5), 140.12, 139.25, 138.80, 137.88, 131.85, 130.88, 128.80, 20.82 (CH_3), 16.14 (CH_3); found: (EI) 264.0182, $C_{13}H_9ClO_4$ requires 264.0189.

4.2.4. General procedure for the preparation of 5-alkoxyderivatives (11–16). A mixture of appropriate naphthoquinone (0.45 mmol), Ag_2O (130 mg, 0.56 mmol) and either methyl- or ethyl-iodide (48.19 mmol) in acetone (3 ml) was refluxed at 60 °C for 2–4 h. The reaction mixture was then filtered and concentrated under reduced pressure. It was purified by silica gel chromatography (eluted with a gradient of ethyl acetate in hexane) and then crystallised (from hexane/chloroform) to afford the respective 5-methoxy- and 5-ethoxy-1,4-naphthoquinone derivatives.

4.2.4.1. 8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone (12). The compound was prepared from **3** as a yellow amorphous powder; yield 70%, mp 142 °C; IR (KBr, cm^{-1}): 1657 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.28 (1H, s, H-6), 6.85 (1H, s, H-3), 6.83 (1H, s, H-2), 4.02 (3H, s, OCH_3), 2.56 (3H, s, CH_3); found: (EI) 236.0252, $C_{12}H_9ClO_3$ requires 236.0240.

4.2.4.2. 8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone (13). The compound was prepared from **5** as a brown needles, yield 75%; IR (KBr, cm^{-1}): 1658, (C=O); δ_H ($CDCl_3$, 200 MHz); 7.58 (1H, s, H-6), 6.85 (1H, s, H-3), 6.83 (1H, s, H-2), 3.85 (3H, s, OCH_3), 2.38 (3H, s, CH_3); δ_C ($CDCl_3$, 50 MHz); 188.27 (C=O), 183.23 (C=O), 159.58 (C-5), 142.85, 140.19, 133.44, 132.54, 129.30, 126.12, 118.02, 21.08 (CH_3), 16.13 (CH_3); found: (EI) 236.0453, $C_{12}H_9ClO_3$ requires 236.0240.

4.2.4.3. 5-Ethoxy-7-methyl-1,4-naphthoquinone (14). The compound was prepared from **6** as a brown semi-solid; yield 98%, IR (KBr, cm^{-1}): 1642 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.52 (1H, s, H-6), 7.07 (1H, s, H-8), 6.81 (2H, s, H-2, H-3), 4.20 (2H, q, *J* 7.5, OCH_2), 2.45 (3H, s, CH_3), 1.53 (3H, t, *J* 7.5, CH_2CH_3); found: (EI) 216.0577, $C_{13}H_{12}O_3$ requires 216.0786.

4.2.4.4. 5-Ethoxy-8-chloro-7-methyl-1,4-naphthoquinone (15). The compound was prepared from **3** as a brown semi-solid; yield 70%, IR (KBr, cm^{-1}): 1658 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.31 (1H, s, H-6), 6.85 (1H, s, H-3), 6.84 (1H, s, H-2), 4.24 (2H, q, *J*, 7.5, OCH_2), 2.56 (3H, s, 7-CH_3), 1.62 (3H, t, *J* 7.5, CH_2CH_3); δ_C ($CDCl_3$, 50 MHz); 185.58 (C=O), 183.92 (C=O), 159.18 (C-5), 146.17, 140.95, 140.94, 135.88, 133.70, 119.82, 119.43, 65.02 (OCH_2), 22.19 (CH_3), 14.61 (CH_3); found: (EI) 250.0137, $C_{13}H_{11}ClO_3$ requires 250.0397.

4.2.4.5. 5-Ethoxy-8-chloro-6-methyl-1,4-naphthoquinone (16). Was prepared from **5** as a brown semi-solid; yield 72%, IR (KBr, cm^{-1}): 1655 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.55 (1H, s, H-6), 6.82 (1H, s, H-3), 6.80 (1H, s, H-2), 3.94 (2H, q, *J* 7.5, OCH_2), 2.34 (3H, s, CH_3), 1.47 (3H, t, *J* 7.5, CH_2CH_3); δ_C ($CDCl_3$, 50 MHz); 183.90 (C=O), 183.58 (C=O), 156.94 (C-5), 141.74, 139.26, 138.33, 138.20, 129.29, 126.29, 114.30, 70.34 (OCH_2), 16.57 (CH_3), 14.07 (CH_3); found: (EI) 250.0564, $C_{13}H_{11}ClO_3$ requires 250.0397.

4.2.5. General Procedure for the preparation of 1,2,4,5-tetra-acetoxy derivatives (17–19). A mixture of appropriate naphthoquinone (100 mg), acetic anhydride (3 ml, 29.39 mmol) and concd H_2SO_4 (0.1 ml) was kept overnight then poured into ice. The reaction mixture was extracted with $CHCl_3$, dried ($MgSO_4$) and then concentrated under reduced pressure. The residue obtained was crystallised from chloroform–hexane to give the respective pentaacetate derivatives.

4.2.5.1. 8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-*O*-acetate (18). The compound was prepared from **3** as a brown semi-solid; yield 70%, mp 159 °C, IR (KBr, cm^{-1}): 1778 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.16 (1H, s H-6), 7.06 (1H, s, H-3), 2.48 (3H, s, OAc) 2.45 (3H, s, OAc), 2.38 (3H, s, OAc), 2.33 (3H, s, OAc), 2.25 ($ArCH_3$); δ_C ($CDCl_3$, 50 MHz); 169.30, 168.67, 168.39, 167.43 (4 × C=O), 145.81, 143.71, 139.60, 137.77, 136.70, 124.72, 123.90, 118.58, 116.93, 21.62, 21.52, 21.04, 20.62, 20.52 (5 × CH_3); found: (EI) 408.0561, $C_{19}H_{12}ClO_8$ requires 408.0612.

4.2.5.2. 8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-*O*-acetate (19). The compound was prepared from **5** as a brown solid; yield 70%, mp 137 °C, IR (KBr, cm^{-1}): 1772 (C=O); δ_H ($CDCl_3$, 200 MHz); 7.46 (1H, s, H-6), 7.11 (1H, s, H-3), 2.38 (3H, s, OAc) 2.37 (3H, s, OAc), 2.35 (3H, s, OAc), 2.29 (3H, s, OAc), 2.21 ($ArCH_3$); δ_C ($CDCl_3$, 50 MHz); 168.81, 168.32, 168.30, 167.67 (4 × C=O), 142.87, 140.61, 133.47, 132.54, 129.29, 126.12, 122.01, 118.02, 117.53, 21.06, 20.95, 20.74, 20.64, 16.64 (5 × CH_3); found: (EI) 408.0631, $C_{19}H_{12}ClO_8$ requires 408.0612.

4.2.6. 2,5-Dihydroxy-7-methyl-1,4-naphthoquinone (20). A mixture of tetra-acetate **17** (200 mg, 0.52 mmol) in methanolic HCl (2 M, 4 ml) was heated under reflux for 30 min. The reaction mixture was extracted with ether and evaporated. The residue was purified by column chromatography using chloroform–methanol (95:5) as eluent to obtain the required compound. This was then recrystallised from chloroform to obtain **20** as orange plates (34 mg, 32%); mp 198 °C (lit.[21] 208–210 °C decomp.); IR (KBr, cm^{-1}): 1640 (C=O); δ_H ($CDCl_3$, 200 MHz); 11.69 (1H, s, 5-OH), 7.42 (1H, s, H-8), 7.01 (1H, s, H-6), 6.08 (1H, s, H-3), 2.40 (3H, s, CH_3); found: (EI) 204.0425, $C_{11}H_8O_4$ requires 204.0423.

4.3. Enzyme assays

Subversive substrate assays with Mtr were carried out at 30 °C in 1 cm^3 of 50 mM Hepes (pH 7.6), 0.1 mM EDTA containing MyR (4.7 μg ml^{-1}), NADPH (70 μM) and varying concentrations of naphthoquinone substrate. The percentage of DMSO was kept constant (2% (v/v)) in all assay mixtures. Mtr was pre-incubated with NADPH for 5 min at 30 °C before initiating the reaction by addition of the naphthoquinone substrate. Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH consumption. Initial rates were measured from the linear region of the progress curve. K_m and V_{max} were determined by weighted non-linear regression analysis of the hyperbola plot of *v* against *s* using the Michaelis Menten equation. Stock solutions of most naphthoquinones were prepared in 100% DMSO. However, DMSO solutions of juglone and 7-methyljuglone degraded within hours at room temperature so they were prepared as 20% DMSO stock solutions in assay buffer, on the day of use.

4.4. Bioassays

The radiometric respiratory technique using the BACTEC system was used for susceptibility testing of *M. tuberculosis* H37Rv as described earlier.[38] DMSO solutions of test compounds were added to 4 ml of BACTEC 12B (7H12 medium) broth to achieve a range of assay concentrations (20, 15, 10, 5, 2.5, 0.5 and 0.2 μg/ml) together with the antimicrobial supplement PANTA (Becton Dickinson & Company). The 7H12 Middlebrook TB medium used during these studies consisted of an enriched Middlebrook 7H9 broth base supplemented with bovine serum albumin, catalase, casein hydrolysate and ^{14}C-labelled palmitic acid as a carbon source. All assay mixtures contained 1% DMSO which

did not affect cell growth (as determined in control experiments). Streptomycin, isoniazid and ethambutol were also tested at concentrations of 1.0, 0.2 and 2.0 μg/ml, respectively.

Samples of *M. tuberculosis*, cell culture (0.1 ml) yielding 1×10^4–1×10^5 (CFU/ml), were inoculated in the vials containing the test compounds. Two compound-free vials (vial-1 and vial-2) were used as controls (medium +1% DMSO): vial-1 was inoculated in the same way as the vials containing the compounds, and vial-2 was inoculated with a 1:100 dilution of the inoculum to produce an initial concentration representing 1% of the bacterial population (1×10^2–1×10^3 CFU/ml). The MIC was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

Growth of the mycobacterium leads to the consumption of the carbon source, with subsequent release of labelled $^{14}CO_2$. This labelled CO_2 moves into the atmosphere above the medium in the sealed vial and the BACTEC TB 460 instrument detects the amount of $^{14}CO_2$ and records it as a growth index (GI) on a scale of 0–999.[38] Inoculated vials were incubated at 38 °C and each vial was assayed daily at the same hour until cumulative results were interpretable. The GI value of the control vial was compared with the readings from the vials containing the compounds. The control vial contains a 1:100 dilution of the inoculum and when it reached a reading of 30 the readings were stopped. The difference in GI readings for the last two days (ΔGI) was used. If ΔGI readings of any of the compounds were less than the control vial, that compound was considered to be active. All compounds were tested in triplicate.

The bacterial viability was determined by plating the bacterial suspensions from the individual BACTEC vials at the beginning and at the end of the experiments from vials exhibiting MIC, on 7H11 agar for viable count measurements.[39] A total of 0.1 ml of culture from the BACTEC vials was removed and successively diluted 10-fold in sterile double-distilled water to give dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A total of 0.1 ml of each of these dilutions was plated onto 7H11 agar medium, and the resulting bacterial counts were measured after 21 days at 37 °C. The MBC was defined as the minimal concentration of compound which reduced the viable count by >99% compared with those in the initial inoculum.

4.5. Cytotoxicity assay

Vero cells were maintained in culture flasks in complete Minimum Essential Medium, Eagle. Subculture was done every 2–3 days after it had formed a confluent monolayer. During subculture, cells that attached to the culture flask were trypsinised (0.25% trypsin containing 0.01% EDTA) for 10 min at 37 °C then stopped by the addition of complete medium. About 10^5 of the viable cells were then re-suspended in complete medium.

Cytotoxicity was measured by the XTT (sodium 3′-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). Vero cells (100 μl) were seeded at 1×10^5 onto a microtiter plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. A dilution series were made of the compounds (0.2–200 μg/ml), added to the microtiter plate and incubated for 48 h. The XTT reagent was added to a final concentration of 0.3 mg/ml and incubated for 1–2 h. After incubation, the absorbance of the colour complex was quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm. IC_{50} was defined as the concentration of the compounds at which absorbance was reduced by 50%.

Acknowledgments

This work was supported by the National Research Foundation (South Africa) (AM) and Royal Society/National Research Foundation Programme Phase II (SPNM). We offer our sincere thanks to Mr. L. Govender (Traditional healer) and Jeannette Brand of the Medical Research Council (Pretoria) for their assistance and to Prof. J. Blanchard (Albert Einstein College of Medicine, New York) for generous provision of the plasmid encoding recombinant Mtr.

References and notes

1. Thomson, R. H. *Naturally Occurring Quinones*; Academic Press: London, UK, 1971.
2. Osman, S. A. A.; Abdalla, A. A.; Alaib, M. O. *J. Pharm. Sci.* **1983**, *72*, 68–71.
3. Roushdi, I. M.; Ibrahim, E. S. A.; Habib, N. S. *Pharmazie* **1976**, *31*, 856–859.
4. Brinkworth, R. I.; Fairlie, D. P. *Biochim. Biophys. Acta* **1995**, *1253*, 5–8.
5. Salmon-Chemin, L.; Buisine, E.; Yardley, V.; Kohler, S.; Debreu, M. A.; Landry, V.; Sergheraert, C.; Croft, S. L.; Krauth-Siegel, L. R.; Davioud-Charvet, E. *J. Med. Chem.* **2001**, *44*, 548.
6. Hazra, B.; Sur, P.; Roy, D. K.; Sur, B.; Banerjee, A. *Planta Med.* **1984**, *51*, 295–297.
7. Yardley, V.; Snowdon, D.; Croft, S.; Hazra, B. *Phytother. Res.* **1996**, *10*, 559–562.
8. Bullock, F. J.; Tweedie, J. F.; McRitchie, D. D.; McRitchi, D. D.; Tucker, M. A. *J. Med. Chem.* **1970**, *13*, 550–552.
9. Perry, N. B.; Blunt, J. W. *J. Nat. Prod.* **1991**, *54*, 978–985.
10. Georgiadis, M. P.; Couladouros, E. A.; Delitheos, A. K. *J. Pharm. Sci.* **1992**, *81*, 1126–1131.
11. Saquib, M.; Gupta, M. K.; Sagar, R.; Prabhakar, T. s.; Shaw, A. K.; Kumar, R.; Maulik, P. R.; Gaikwad, A. N.; Sinha, S.; Srivastava, A. K.; Chaturvedi, V.; Srivastava, R.; Srivastava, B. S. *J. Med. Chem.* **2007**, *50*, 2942–2950.
12. Lall, N.; Meyer, J. J. M. *J. Ethnopharmacol.* **1999**, *66*, 347–354.
13. Lall, N.; Meyer, J. J. M. *J. Ethnopharmacol.* **2001**, *78*, 213–216.
14. Lall, N.; Das Sharma, M.; Hazra, B.; Meyer, J. J. M. *J. Antimicrob. Chemother.* **2003**, *51*, 435–438.
15. Lall, N.; Meyer, J. J. M.; Wang, Y.; Bapela, N. B.; van Rensburg, C. E. J.; Fourie, B.; Franzblau, S. G. *Pharm. Biol.* **2005**, *43*, 353.

16. Ollinger, K.; Brunmark, A. *J. Biol. Chem.* **1991**, *266*, 21496–21503.
17. Domagala, J. M.; Hana, L. D.; Heifets, C. L., et al. *J. Med. Chem.* **1986**, *29*, 394–404.
18. Miyamoto, T.; Matsumoto, J.; Chiba, K.; Egawa, H.; Shibamori, K.; Minamida, A.; Nishimura, Y.; Okada, H.; Kataoka, M.; Fujita, M.; Hirose, T.; Nakano, J. *J. Med. Chem.* **1990**, *33*, 1645–1656.
19. Musgrave, O. C.; Skoyles, D. *J. Chem. Soc. Perkin Trans.* **2001**, *1*, 1318–1320.
20. Laatsch, H. *Z. Naturforsch, B. Anorg. Chem. Org.* **1986**, *41*, 377–385.
21. Lillie, T. J.; Musgrave, O. C. *J. Chem. Soc. Perkin Trans.* **1977**, *1*, 355–359.
22. Branville, J.; Grandmaison, J.-L.; Lang, G.; Brassard, P. *Can. J. Chem.* **1974**, *52*, 80–87.
23. Parker, K. A.; Tallman, E. A. *Tetrahedron* **1984**, *40*, 4781–4788.
24. Maiti, B. C.; Musgrave, O. C.; Skoyles, D. *Tetrahedron* **2005**, *61*, 1765–1771.
25. Bogdanov, P. M.; Albesa, I.; Sperandeo, N. R.; De Bertorello, M. M. *Rev. Argent. Microbiol.* **1993**, *25*, 119–128.
26. Biot, C.; Bauer, H.; Schirmer, R. H.; Davioud-Charvet, E. *J. Med. Chem.* **2004**, *47*, 5972.
27. (a) Newton, G. L.; Bewley, C. A.; Dwyer, T. J.; Horn, R.; Aharonowitx, Y.; Cohen, G.; Davies, J.; Faulkner, D. J.; Fahey, R. C. *Eur. J. Biochem.* **1995**, *230*, 821; (b) Fahey, R. C. *Annu. Rev. Microbiol.* **2001**, *55*, 333.
28. Spies, H. S. C.; Steenkamp, D. J. *Eur. J. Biochem.* **1994**, *224*, 203–213.
29. Sakuda, S.; Zhou, Z. Y.; Amada, Y. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 1347.
30. Patel, M. P.; Blanchard, J. S. *Biochemistry* **1999**, *38*, 11827.
31. Sareen, D.; Newton, G. L.; Fahey, R. C.; Buchmeier, N. A. *J. Bacteriol.* **2003**, *185*, 6737.
32. Rawat, M.; Newton, G. L.; Ko, M.; Martinez, G. J.; Fahey, R. C.; Av-Gay, Y. *Antimicrob. Agents Chemother.* **2002**, *46*, 3348.
33. Newton, G. L.; Unson, M. D.; Anderberg, S. J.; Aguilera, J. A.; Oh, N. N.; delCardayre, S. B.; Av-Gay, Y.; Fahey, R. C. *Biochem. Biophys. Acta, Res. Commun.* **1999**, *255*, 239.
34. Weigenand, O.; Hussein, A. A.; Lall, N.; Meyer, J. J. M. *J. Nat. Prod.* **2004**, *67*, 1936–1938.
35. Patel, M. P.; Blanchard, J. S. *Biochemistry* **2001**, *40*, 5119.
36. Van der Kooy, F.; Meyer, J. J. M.; Lall, N. *S. African J. Botany* **2006**, *72*, 349.
37. Cooke, R. G.; Dowd, H. *Aust. J. Chem.* **1953**, *6*, 53–57.
38. Heifets, L. B.; Good, R. C. Current Laboratory Methods for the Diagnoses of Tuberculosis. In *Tuberculosis: Pathogenesis, Prevention and Control*; Bloom, B. R., Ed.; ASM Press: Washington DC, ISBN 1-55581-072-1, 1994; pp 85–108.
39. Rastogi, N.; Goh, K. S. *Antimicrob. Agents Chemother.* **1991**, *35*, 1933–1936.
40. Newton, G. L.; Ta, P.; Fahey, R. C. *J. Bacteriol.* **2005**, *187*, 7309.