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Secondary metabolites from the roots of *Engelhardia roxburghiana* and their antitubercular activities

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Abstract

Engelharquinone (**1**), engelharquinone epoxide (**2**), engelharolide (**3**), and engelhardic acid (**4**), were isolated as naturally occurring products from a plant source, *Engelhardia roxburghiana* together with 20 previously known compounds, four of which were hitherto not known as plant constituents. Their structures were identified by means of spectroscopic analysis. A biological evaluation showed that three of the previously isolated antitubercular constituents [(−)-4-hydroxy-1-tetralone, 3-methoxyjuglone and engelhardione] and engelharquinone (**1**) exhibited moderate antitubercular activity against *Mycobacterium tuberculosis* 90-221387.

Keywords: *Engelhardia roxburghiana*; Juglandaceae; Root; Naphthoquinones; Engelharquinone; Engelharquinone epoxide; Butanolide; Engelharolide; Sesquiterpene; Engelhardic acid; Antitubercular activity

1. Introduction

Engelhardia roxburghiana Wall. (Juglandaceae) has been shown to have potent antitubercular activity (Lin et al., 2005). In our previous study, three new compounds and three antitubercular constituents [engelhardione, 3-methoxyjuglone, and (−)-4-hydroxy-1-tetralone] were isolated from the $CHCl_3$-soluble layer of the root of *E. roxburghiana*. Further phytochemical investigation of this extract has led to isolation of two new naphthoquinones (**1** and **2**), a new butanolide **3**, and a new sesquiterpene **4**, along with four compounds (**5**–**8**) that are isolated for the first time from a natural source, as well as 16 previously known compounds (**9**–**24**). Their structures were established by spectroscopic analyses and through comparison with the relevant data in the literature. The isolation, and structural elucidation of these new compounds and these antitubercular activity of some of the isolates are described herein.

2. Results and discussion

Extensive chromatographic purification of the $CHCl_3$-soluble layer of the root of *E. roxburghiana* on silica gel and Sephadex LH-20 columns afforded four new compounds (**1**–**4**). A further four compounds were isolated for the first time from a natural source, and were identified as methyl-4-(butyryloxy)benzoate (**5**) (Sakai et al., 1956), 4-(2-hydroxyphenyl)-4-oxobutyric acid (**6**) (Wenkert et al., 1977), 5-methoxy-1-naphthalenol (**7**) (Jung and Hagenah, 1987), and 5-hydroxy-2-hydroxymethyl-1,4-naphthoquinone (**8**) (Wurm and Baumann, 1981). Moreover, the following 16 previously known compounds were also isolated: 5,8-dihydroxy-4-methoxy-1-tetralone (**9**) (Machida et al., 2005); β-sitostenone (**10**) (Suga and Kondo, 1974); a mixture of docosyl *trans*-ferulate (**11**), tricosyl *trans*-ferulate (**12**), tetracosyl *trans*-ferulate (**13**), and

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hexacosyl *trans*-ferulate (**14**) (Ulubelen et al., 1994; Kuo and Chen, 1999), 3-*epi*-betulinic acid (**15**) (Herz et al., 1972); acetovanillone (**16**) (Crestini and D'kAuria, 1997); vanillin (**17**) (Ito et al., 2001); 4-hydroxybenzaldehyde (**18**) (Fujimoto et al., 1998); 2,6-dimethoxy-1,4-benzoquinone (**19**) (Bozell et al., 1995); 1-methoxynaphthalene (**20**) (Pouchert and Behnke, 1993); 2-(4-hydroxyphenyl)ethanol (**21**) (Baraldi et al., 2002); cinnamic acid (**22**) (Miyazawa et al., 1998); 3,4,5-trimethoxybenzoic acid (**23**) (Chang et al., 2000); and vanillic acid (**24**) (Harrison et al., 1995).

Engelharquinone (**1**) was obtained as yellow needles, and displayed a molecular ion at *m/z* 348.0633 (calcd for 348.0634) that was established by HR-EI-MS corresponding to the formula $C_{20}H_{12}O_6$ (14 degrees of unsaturation). The UV spectrum exhibited bands at 258, 293 sh, 322 sh, 432 nm and a bathochromic shift upon addition of KOH suggested the presence of a phenolic naphthoquinone with hydrogen bonding (Ferreira et al., 1977). This was supported by IR bands at 1669, 1633 cm^{-1} for carbonyl absorptions and 3523 cm^{-1} due to hydroxyl group. The ^1H NMR spectrum of compound **1** (Table 1) indicated the existence of two chelated hydroxyls and one aliphatic alcohol at δ 11.52, 11.49, and 4.80, respectively (each 1H, *s*, exchangeable with D_2O), suggesting the presence of a naphthoquinone ring system. The COSY results revealed two sets of 1,2,3-trisubstituted phenyl units. The first ABC spin system of three aromatic protons at δ_H 6.93 (1H, *dd*, $J = 8.4$, 1.2 Hz, H-2), 7.48 (1H, *dd*, $J = 8.4$, 7.6 Hz, H-3), and 7.14 (1H, *dd*, $J = 7.6$, 1.2 Hz, H-4), and the chemical shifts at δ_H 7.24 (1H, *dd*, $J = 7.6$, 2.0 Hz, H-8), 7.62 (1H, *dd*, $J = 7.6$, 7.2 Hz, H-9), and 7.65 (1H, *dd*, $J = 7.2$, 2.0 Hz, H-10), were attributed to a second ABC spin system indicating the partial structure of a naphthoquinone ring. Also, the COSY analysis revealed the presence of a methine proton at δ 4.23 (1H, *dd*, $J = 4.0$, 1.2 Hz, H-12) coupled to methylene protons [δ 3.04 (1H, *dd*, $J = 10.8$, 1.2 Hz, H-14a) and δ 3.07 (1H, *dd*, $J = 10.8$, 4.0 Hz, H-14b)]. The ^{13}C NMR spectroscopic assignments of **1** (Table 1) were based on the analysis of the ^{13}C, DEPT, and HMQC NMR spectra, which showed signals for one methylene at δ_C 54.0 (C-14), one methine at δ_C 52.6 (C-12), and twelve quaternary carbons, including six aromatic tertiary carbons, two phenolic carbons, one tertiary alcoholic carbon, and three carbonyl groups at δ_C 188.8 (C-6), 180.5 (C-11), and 198.1 (C-13). H_2-14 (δ_H 3.04, 3.07) showed an HMBC correlation with C-5 (δ_C 81.9), C-5a (154.4), C-11a (148.6), and C-4a (146.2). The aliphatic hydroxyl group gave cross-peaks corresponding to a correlation with C-5 (δ_C 81.9), C-5a (154.4), and C-4a (146.2). The CIGAR-HMBC (Table 2 in pyridine-d_5) experiment confirmed a correlation between H-12 (δ_H 4.44) and C-11 (δ_C 181.8). The chemical shifts at H-12, H-14, and C-5 (δ_C 81.9) of **1** were similar to those of the naphthoquinone dimer, natalenone (Ferreira et al., 1977), which was

Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data for **1** and **2** ($CDCl_3$)

Position	^1H		^{13}C	
	1	**2**	**1**	**2**
1			163.5	163.4
2	6.93 (*dd*, 8.4, 1.2)	6.93 (*dd*, 8.4, 1.2)	119.3	119.0
3	7.48 (*dd*, 8.4, 7.6)	7.53 (*dd*, 8.4, 7.6)	136.8	136.9
4	7.14 (*dd*, 7.6, 1.2)	7.25 (*dd*, 7.6, 1.2)	113.3	115.5
4a			146.2	144.5
5			81.9	77.8
5a			154.4	66.9
6			188.8	194.7
6a			115.2	114.9
7			162.0	162.4
8	7.24 (*dd*, 7.6, 2.0)	7.23 (*dd*, 8.2, 1.2)	125.2	125.1
9	7.62 (*dd*, 7.6, 7.2)	7.63 (*dd*, 8.2, 7.6)	137.3	137.9
10	7.65 (*dd*, 7.2, 2.0)	7.57 (*dd*, 7.6, 1.2)	120.4	120.5
10a			132.6	132.9
11			180.5	186.3
11a			148.6	64.4
12	4.23 (*dd*, 4.0, 1.2)	3.93 (*dd*, 4.4, 0.8)	52.6	48.6
13			198.1	199.6
13a			110.5	112.7
	3.04 (*dd*, 10.8, 1.2)	2.35 (*dd*, 11.6, 0.8)		
14			54.0	38.6
	3.07 (*dd*, 10.8, 4.0)	2.41 (*dd*, 11.6, 4.4)		
OH-1	11.49 (*s*)	11.65 (*s*)		
OH-5	4.80 (*s*)	3.78 (*s*)		
OH-7	11.52 (*s*)	11.02 (*s*)		

The coupling constants in Hz are shown in parentheses; the chemical shifts are expressed as δ values.

Table 2
^1H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for **1** and **2** (pyridine-d_5)

Position	^1H		^{13}C	
	1	**2**	**1**	**2**
1			164.0	163.2
2	7.03 (*dd*, 8.0, 1.0)	6.94 (*dd*, 8.0, 0.8)	119.3	118.4
3	7.46 (*t*, 8.0)	7.58 (*dd*, 8.4, 7.6)	136.9	136.9
4	7.89 (*dd*, 8.0, 1.0)	8.11 (*dd*, 7.6, 1.2)	115.4	117.5
4a			149.0	147.3
5			82.6	78.0
5a			156.9	68.7
6			188.4	193.2
6a			116.9	116.7
7			162.2	161.6
8	7.58 (br *d*, 8.0)	7.14 (*dd*, 8.4, 1.2)	125.3	124.9
9	7.62 (*t*, 8.0)	7.37 (*dd*, 8.4, 8.0)	137.4	136.9
10	7.65 (*d*, 8.0)	7.47 (*dd*, 8.0, 1.2)	119.6	119.3
10a			133.7	133.8
11			181.8	187.8
11a			149.8	65.2
12	4.44 (br *d*, 4.0)	4.17 (*d*, 4.8)	53.6	49.2
13			200.3	200.5
13a			112.1	114.0
	3.22 (*d*, 10.5)	2.61 (*dd*, 11.6, 4.8)		
14			56.3	40.7
	3.16 (*dd*, 10.5, 4.0)	2.49 (*d*, 11.6)		
OH-1	12.32 (*s*)			
OH-5	5.03 (*s*)			
OH-7	12.01 (*s*)			

The coupling constants in Hz are shown in parentheses; the chemical shifts are expressed as δ values.

isolated from the roots of *Euclea natalensis* (Ebenaceae), suggesting that **1** had a configuration similar to that of natalenone.

Engelharquinone epoxide (**2**) was obtained as an orange-red powder. The EI-MS results identified a molecular ion peak $[M]^+$ at *m/z* 364, which was compatible with the molecular formula $C_{20}H_{12}O_7$ (14 degrees of unsaturation) deduced in combination with the ^1H NMR, ^{13}C NMR, and DEPT spectroscopic analyses. The UV and IR spectra were similar to those of **1**, and also suggested the presence of a phenolic naphthoquinone in **2**. The ^{13}C NMR spectrum of **2** (Table 1 in $CDCl_3$) revealed 20 signals, which were identified through DEPT experiments into 12 quaternary carbon atoms [9 sp^2, including three carbonyl groups at δ_C (199.6), (194.7) and (186.3), plus three sp^3 due to oxygenated carbons at δ_C (77.8), (66.9) and (64.4)], one methine (sp^3 at δ_C 48.6), and a methylene at δ_C (38.6). Careful investigation showed that the ^1H NMR spectrum of **2** (Table 1 in $CDCl_3$) was similar to that of **1**. Moreover, the ^{13}C NMR spectrum of **2** indicated the presence of an oxygen bearing two carbons at δ_C 66.9 (C-5a) and 64.4 (C-11a), suggesting that an epoxide ring was present instead of the double bond between the C-5a (δ_C 154.4) and C-11a (δ_C 148.6) of **1**. Furthermore, the methylene protons at δ_H 3.04 (H-14a) and 3.07 (H-14b) in **1** were shifted upfield to δ_H 2.35 (H-14a) and 2.41 (H-14b) in **2**. This information suggested that the relative configurations of the epoxide ring and the methylene bridge were on the same side. According to the abovementioned observations, the structure of engelharquinone epoxide was elucidated as **2**; this was further supported by the COSY, HSQC, NOESY, and HMBC ($CDCl_3$) analyses. The correlation between H-12 (δ_H 4.17) and C-11 (δ_C 187.8) was also further confirmed by the CIGAR-HMBC (Table 2 in pyridine-d_5) results.

Engelharolide (**3**) was obtained as a pale-brown solid, the molecular formula of which was assigned as $C_{14}H_{16}O_5$ on the basis of the HR-EI-MS, ^{13}C NMR and DEPT spectroscopic analyses, with a molecular ion at *m/z* 264.0997 that indicated seven degrees of unsaturation. In addition, the ^1H NMR (see Section 3) and COSY spectra of **3** showed different spin systems, which began with olefinic methine protons at δ_H 6.21 (1H, *d*, $J = 5.7$ Hz, H-3) coupled to 7.05 (1H, *d*, $J = 5.7$ Hz, H-4)] acting as an AB spin-coupling system, two methylene protons at δ_H 2.19 coupled to the other methylene protons at 2.69 that were attributable to the ethylene groups H_2-6 and H_2-7, respectively, and an ABM spin-coupling system with 6.65 (1H, br *d*, $J = 9.0$ Hz, H-13) coupled to 6.83 (1H, *d*, $J = 9.0$ Hz, H-12) and 6.66 (1H, br *s*, H-9). The ^1H NMR spectrum displayed signals for a methoxy group [δ 3.25 (3H, *s*, OCH_3-5)], suggesting the presence of an α,β-unsaturated γ-lactone system, which was confirmed by the presence in the ^{13}C NMR spectrum of signals at δ_C 169.9 (C-2), 124.8 (C-3), 153.5 (C-4), and 110.7 (C-5), the methoxy group at δ_H 3.25 (OCH_3-5) correlated with the oxygenated quaternary carbon atom at δ_C 110.7 (C-5), and the olefinic carbon δ_C 153.5 (C-4) in the HMBC spectrum. The IR spectrum displayed strong absorption bands at 1766 and 1735 cm^{-1}. These data led us to assign a structure containing a methoxy butenolide moiety linked to an unbranched alkyl chain, as formulated in **3**. The ^{13}C NMR spectrum (see Section 3) contained, in addition to the signals that were attributable to the butenolide moiety, resonances at δ_C 110.8 (C-9), 114.3 (C-12), and 120.8 (C-13) due to the three aromatic carbons, three quaternary carbons at 132.5 (C-8), 148.1 (C-10), and 143.9 (C-11), and one methoxyl at 55.8 (OCH_3-10). Engelharolide (**3**) showed a dextrorotatory optical activity with $[\alpha]_D^{23}$: +11.39° (*c* 0.13, MeOH) in comparison with (−)-4-methoxy-2-eicosen-4-olide {$[\alpha]_D^{25}$: −34.7° (*c* 0.6, CH_2Cl_2)} with an *R*-configuration (Miller and Hegedus, 1993), suggesting an *S*-configuration of C-5 in **3**.

The HR-EI-MS analysis of angelhardic acid (**4**), which was obtained as a colorless oil, showed a molecular ion peak $[M]^+$ at *m/z* 234.1618 (calcd for 234.1619), which corresponded to the molecular formula $C_{15}H_{22}O_2$, indicating the existence of five degrees of unsaturation. The structure of **4** was determined from careful investigation of the 1D and 2D NMR spectroscopic measurements. The ^1H NMR spectrum (see Section 3) established the presence of two signals for δ_H 4.66 (1H, *m*, H-14a) and 4.70 (1H, br *s*, H-14b), which were characteristic of an exomethylene group. Additionally, the ^1H NMR spectrum showed the presence of a downfield olefinic proton at δ_H 7.24 (1H, br *d*, $J = 5.6$ Hz, H-5), which suggested that compound **4** had an α,β-unsaturated carbonyl moiety (C=O, IR: 1685 cm^{-1}); this was identified in the COSY correlations with a multiplet signal at δ_H 2.22 (1H, H-6_{eq}). An allylic coupling between H-3_{axi} at δ_H 2.50 and an olefinic proton at δ_H 7.24 (H-5) was observed in the COSY analysis. Furthermore, in the ^1H NMR spectrum, the two methyl doublet signals at δ_H 0.85 (3H, *d*, $J = 6.8$ Hz, H-12) and 0.93 (3H, *d*, $J = 6.8$ Hz, H-13) were coupled in the COSY spectrum with a resonance at δ_H 1.98 (1H, *m*, H-11), which indicated an isopropyl moiety. The ^{13}C NMR spectroscopic data for **4** (see Section 3) exhibited 15 carbon signals that were resolved by the DEPT experiment as follows: five methines, one of which was the olefinic carbon [δ_C 145.1 (C-5)]; five methylenes including an exomethylene at δ_C 107.7 (C-14); two methyls of an isopropyl group at δ_C 15.8 (C-12) and 21.5 (C-13); and three quaternary carbons, one of which was typical for a carbonyl group at δ_C 171.4 (C-15). In an HMBC experiment, the H-5 at δ_H 7.24 showed a cross-peak with the carbon signal δ_C 171.4 (C-15) that supported the location of the carboxyl group at C-4, and a cross-peak for a secondary carbon at δ_C 44.7 (C-7). Additionally, the exomethylene protons (H_2-14) at δ_H 4.66 and 4.70 correlated with two carbons at δ_C 42.3 (C-1) and 31.1 (C-9) in the HMBC spectrum. Furthermore, the two methyl signals at δ_H 0.85 and 0.93 showed a cross-peak correlation with the secondary carbon at δ_C 44.7 (C-7), which was observed in the HMBC spectrum. Based on the abovementioned data, compound **4** was proposed to be

a bicyclic sesquiterpenoid, which is a candinane derivative. The NOESY (Fig. 1) established a cross-peak between H-6_{eq} (δ_H 2.22) and H-1_{axi} (δ_H 2.40), H-7_{axi} (δ_H 1.52), and H-5 (δ_H 7.24), suggesting that **4** is a *cis*-fused amorphane derivative (Paul et al., 2001).

In total, 18 isolates, including several compounds that had been isolated in a previous study, were tested, but only engelharquinone (**1**) and 3-methoxyjuglone showed potent antitubercular activity (Table 3). 3-Methoxyjuglone, which had a minimal inhibitory concentration (MIC) of 3.125 as previously reported (Lin et al., 2005), showed stronger antitubercular activity than 2-methoxyjuglone. Due to the small amounts present, the antitubercular activity of **1** could not be further defined.

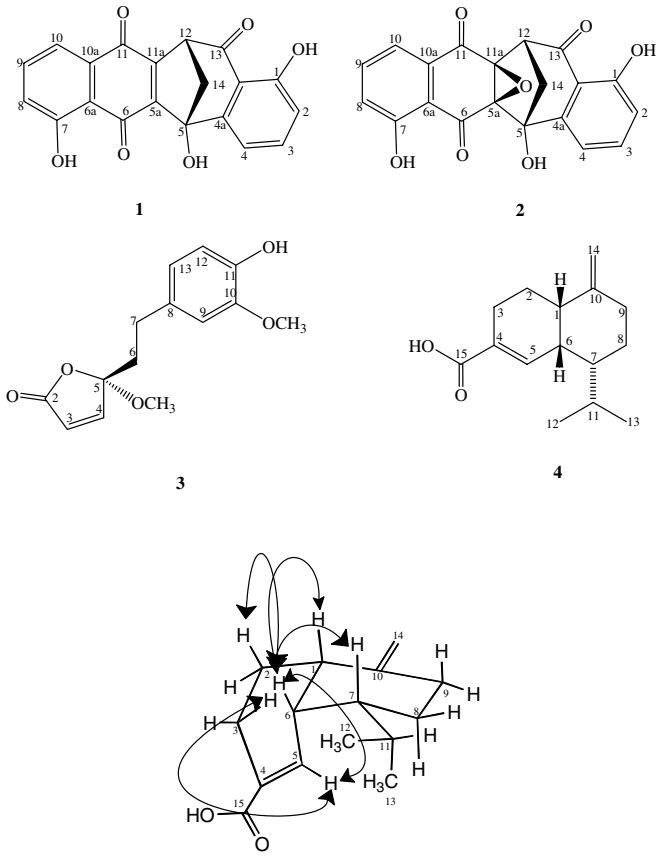

Fig. 1. Selected NOESY correlations of **4**.

Table 3
Antitubercular activity of additional isolates from the root of *E. roxburghiana* on *M. tuberculosis* 90-221387

Compound	MICs (μg/mL)
Engelharquinone (**1**)	⩽20
Engelharolide (**3**)	⩾100
2-Methoxyjuglone	30
3-Methoxyjuglone (Lin et al., 2005)	3.125

3. Experimental

3.1. General

All melting points were determined on a Yanaco micro-melting apparatus and were uncorrected. Optical rotations were measured on a Jasco P-1020 polarimeter. UV spectra were measured on a Jasco V-530 UV/VIS spectrophotometer. IR spectra (KBr or neat) were recorded on a Genesis II FTIR spectrophotometer. 1D and 2D NMR spectra were recorded on a Varian Unity-plus 400, Varian Unity Inova-500, and Varian Unity Inova-600 MHz FT-NMR system. Chemical shifts were given in ppm (δ), with TMS as an internal standard. EI-MS spectra were recorded on a Micromass Trio-2000 GC/MS spectrometer. HR-EI-MS spectra were recorded on a Finnigan MAT-95XL high-resolution mass spectrometer. Silica gel (70–230, 230–400 mesh) (Merck) was used for CC, and silica gel 60 F-254 (Merck) was used for TLC and prep. TLC. Sephadex LH-20 gel (Pharmacia) was also used for CC.

3.2. Plant material

The roots of *E. roxburghiana* was collected from Lai-I, Pingtung County, Taiwan, in September 2001, and identified by I. S. Chen. A voucher specimen (Chen 6043) was deposited in the herbarium of the School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China.

3.3. Extraction and isolation

Dried roots (11.4 kg) were extracted with 30 L of cold MeOH each time at 30 °C in three days, repeatly three times and the extract was concentrated under reduced pressure. The MeOH extract (2.05 kg), when partitioned between H_2O and $CHCl_3$ (1:1), afforded a $CHCl_3$-soluble fraction (Fr. A, 50.5 g). Fraction A (50.5 g) was subjected to silica gel CC and first eluted with *n*-hexane. The eluant polarity was then gradually increased with EtOAc and MeOH to furnish 14 fractions (A-1–A-14). Fraction A-5 (1.98 g) was subjected to Si gel chromatography eluted with *n*-hexane, and was then enriched with EtOAc to give seven fractions (A-5-1–A-5-7). Fraction A-5-5 (1.43 g) was resubjected to Si gel chromatography, and was purified by preparative TLC to yield β-sitostenone (**10**) (5.5 mg) and a mixture (24 mg) of docosyl *trans*-ferulate (**11**), tricosyl *trans*-ferulate (**12**), tetracosyl *trans*-ferulate (**13**), and hexacosyl *trans*-ferulate (**14**). Fraction A-8 (2.55 g) was subjected to Si gel chromatography and eluted with CH_2Cl_2-acetone step gradients to give six fractions (A-8-1–A-8-6). Fraction A-8-4 (335 mg) was resubjected to Si gel chromatography and purified by preparative TLC to afford 3-*epi*-betulinlic acid (**15**) (5.8 mg). Fraction A-9 (2.08 g) was applied to a Si gel column eluted with *n*-hexane-EtOAc (1:1) to give seven fractions (A-9-1–A-9-7).

Fraction A-9-2 (1.52 g) was subjected to Si gel chromatography and eluted with CH_2Cl_2-EtOAc step gradients to afford 12 fractions (A-9-2-1–A-9-2-12). The $CHCl_3$-soluble fraction (33 mg) of A-9-2-4 (90.1 mg) was purified by preparative TLC to give acetovanillone (**16**) (6.7 mg) and vanillin (**17**) (4.4 mg). Fraction A-9-2-5 (168 mg) was subjected to Si gel chromatography and eluted with *n*-hexane, and was enriched with acetone followed by MeOH to give 13 fractions (A-9-2-5-1–A-9-2-5-13). Fraction A-9-2-5-4 (39.7 mg) was purified by preparative TLC to produce engelhardic acid (**4**) (1.5 mg). Fraction A-9-2-8 (181 mg) was further separated on a silica gel column and eluted with CH_2Cl_2-EtOAc step gradients to give nine fractions (A-9-2-8-1–A-9-2-8-9). Fraction A-9-2-8-1 (5.7 mg) was purified by preparative TLC to give 4-hydroxybenzaldehyde (**18**) (3.3 mg). Fraction A-9-3 (206 mg) was resubjected to Si gel chromatography and eluted with CH_2Cl_2 to afford eight fractions (A-9-3-1–A-9-3-8). Fraction A-9-3-3 (14.1 mg) was purified by preparative TLC to give engelharolide (**3**) (2.7 mg). Fraction A-9-8-3-5 (8 mg) was purified by preparative TLC to afford methyl-4-(butyryloxy)benzoate (**5**) (1.5 mg) and 5-hydroxy-2-hydroxymethyl-1,4-naphthoquinone (**8**) (3.4 mg). Fraction A-10 (4.96 g) was subjected to Si gel chromatography and eluted with CH_2Cl_2-EtOAc step gradients to give 10 fractions (A-10-1–A-10-10). Fraction A-10-3 (100 mg) was resubjected to Si gel chromatography, eluted with *n*-hexane, and then enriched with EtOAc to afford five fractions (A-10-3-1–A-10-3-5). Fraction A-10-3-1 (24 mg) was purified by preparative TLC to give engelharquinone (**1**) (4.8 mg). Fraction A-10-3-2 (71 mg) was subjected to Sephadex LH-20, eluted with MeOH, and then purified by preparative TLC to obtain engelharquinone epoxide (**2**) (6.8 mg). The $CHCl_3$-soluble part (111 mg) of fraction A-10-4 (177 mg) was resubjected to Si gel chromatography and purified by preparative TLC to give 2,6-dimethoxy-1,4-benzoquinone (**19**) (5.2 mg). Fraction A-10-8 (2 g) was resubjected to Si gel chromatography and purified by preparative TLC to give 1-methoxynaphthalene (**20**) (6.5 mg), 5,8-dihydroxy-4-methoxy-1-tetralone (**9**) (2.0 mg), and 5-methoxy-1-naphthalenol (**7**) (12.8 mg). Fraction A-12 (5.64 g) was subjected to Si gel chromatography, eluted with CH_2Cl_2, and then enriched gradually with EtOAc and MeOH to obtain seven fractions (A-12-1–A-12-7). Fraction A-12-4 (595 mg) was applied to a Sephadex LH-20 column, eluted with MeOH, and purified by preparative TLC to afford 2-(4-hydroxyphenyl)-ethanol (**21**) (2.0 mg), 4-(2-hydroxyphenyl)-4-oxobutyric acid (**6**) (8.9 mg), cinnamic acid (**22**) (3.5 mg), 3,4,5-trimethoxybenzoic acid (**23**) (2.0 mg), and vanillic acid (**24**) (5.5 mg).

3.3.1. Engelharquinone (*1*)

Yellow needles (MeOH); mp 247–250 °C; $[\alpha]_D^{25}$: −14.08 (*c* 0.07, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 258 (4.08), 293 sh (3.76), 322 sh (3.61), 432 (3.58) nm; UV (MeOH + KOH) λ_{max} (log ε): 275 sh (3.99), 356 (3.72), 553 (3.66) nm; IR (KBr) ν_{max}: 3523 (OH), 1669, 1633 (C⟝O), 1606 (benzene) cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; for ^1H NMR (Pyridine-d_5, 500 MHz) and ^{13}C NMR (Pyridine-d_5, 125 MHz), see Table 2; EI-MS *m/z* (rel. int.): 348 $[M]^+$ (40), 330 (58), 302 (100), 274 (36), 246 (14), 218 (11), 189 (44), 176 (7), 163 (10), 151 (11), 137 (18), 121 (20), 115 (37), 92 (32), 63 (28); HR-EI-MS *m/z* 348.0633 (calcd for $C_{20}H_{12}O_6$: 348.0634).

3.3.2. Engelharquinone epoxide (*2*)

Yellow powder; $[\alpha]_D^{25}$: −48.5 (*c* 0.026, $CHC1_3$); UV (MeOH) λ_{max} (log ε): 209 (4.03), 279 (3.75), 305 sh (3.54), 345 (2.74) nm; UV (MeOH + KOH) λ_{max} (log ε): 210 (4.15), 284 (3.77), 306 sh (3.54), 411 (2.59) nm; IR (KBr) ν_{max}: 3443 (OH), 1699, 1649 (C=O), 1576 (C=C of benzene) cm^{-1}; for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; for ^1H NMR (Pyridine-d_5, 500 MHz) and ^{13}C NMR (Pyridine-d_5, 125 MHz), see Table 2; EI-MS *m/z* (rel. int.): 364 $[M]^+$ (100), 318 (31), 290 (28), 188 (47), 176 (30), 149 (56), 121 (37), 119 (38), 93 (41), 91 (39), 69 (55); HR-EI-MS *m/z* 364.0584 (calcd for $C_{20}H_{12}O_6$: 364.0583).

3.3.3. Engelharolide (*3*)

Pale-brown solid; $[\alpha]_D^{25}$: +11.39 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε): 285 (3.08), 288 sh (2.88), 335 (2.30) nm; UV (MeOH + KOH) λ_{max} (log ε): 210 (4.56), 241 (3.93), 286 (3.71), 325 (3.22) nm; IR (KBr) ν_{max}: 3455 (OH), 1766, 1735 (α,β-unsaturated γ-lactone moiety), 1513, 1457 (benzene) cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz): δ 7.05 (1H, *d*, $J = 5.7$ Hz, H-4), 6.83 (1H, *d*, $J = 9.0$ Hz, H-12), 6.66 (1H, br *s*, H-9), 6.65 (1H, br *d*, $J = 9.0$ Hz, H-13), 6.21 (1H, *d*, $J = 5.7$ Hz, H-3), 5.47 (1H, *s*, OH-11, D_2O exchangeable), 3.87 (3H, *s*, OCH_3-10), 3.25 (3H, *s*, OCH_3-5), 2.69 (2H, *m*, H-7), 2.19 (2H, *m*, H-6); ^{13}C NMR ($CDCl_3$, 150 MHz): 169.9 (C-2), 153.5 (C-4), 148.1 (C-10), 143.9 (C-11), 132.5 (C-8), 124.8 (C-3), 120.8 (C-13), 114.3 (C-12), 110.8 (C-9), 110.7 (C-5), 55.8 (OCH_3-10), 51.2 (OCH_3-5), 39.2 (C-6), 29.2 (C-7); EI-MS *m/z* (rel. int.): 264 $[M]^+$ (84), 232 (65), 219 (18), 214 (12), 189 (11), 151 (22), 150 (89), 137 (100), 236 (13), 135 (24), 122 (15), 113 (25), 107 (15), 91 (10), 77 (10), 55 (13), 54 (18), 51 (17); HR-EI-MS *m/z* 264.0997 (calcd for $C_{14}H_{16}O_5$: 264.0997).

3.3.4. Engelhardic acid (*4*)

Colorless oil; $[\alpha]_D^{25}$: +33.6 (*c* 0.055, MeOH); IR (KBr) ν_{max}: 3400–2500, 1686 (COOH), 1642, 888 (terminal methylene) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 7.24 (1H, br *d*, $J = 5.6$ Hz, H-5), 4.70 (1H, br *s*, H-14b), 4.46 (1H, *m*, H-14a), 2.50 (1H, br *dd*, $J = 17.6$, 6.0 Hz, H-3_{axi}), 2.40 (1H, br *ddd*, $J = 9.2$, 4.0, 4.0 Hz, H-1_{axi}), 2.28 (1H, *m*, H-2_{eq}), 2.22 (1H, *m*, H-6_{eq}), 2.14 (2H, *m*, H-9), 1.98 (1H, *m*, H-11), 1.92 (1H, *dddd*, $J = 13.2$, 12.0, 9.2, 6.0 Hz, H-2_{axi}), 1.72 (1H, *m*, H-8_{eq}), 1.57 (1H, *m*, H-3_{eq}), 1.52 (1H, *dddd*, $J = 12.4$, 11.6, 4.0, 4.0 Hz, H-7_{axi}), 1.10 (1H, *dddd*, $J = 12.4$, 12.4, 12.4, 4.0 Hz, H-8_{axi}), 0.93 (3H, *d*,

$J = 6.8$ Hz, H-13), 0.85 (3H, *d*, $J = 6.8$ Hz, H-12); ^{13}C NMR ($CDCl_3$, 100 MHz): 171.4 (C-15), 152.3 (C-10), 145.1 (C-5), 129.0 (C-4), 107.7 (C-14), 45.1 (C-6), 44.7 (C-7), 42.3 (C-1), 31.1 (C-9), 27.1 (C-11), 26.0 (C-8), 24.7 (C-3), 24.5 (C-2), 21.5 (C-13), 15.8 (C-12); EI-MS *m/z* (rel. int.): 234 $[M]^+$ (50), 219 (5), 206 (7), 191 (100), 173 (45), 149 (70), 145 (48), 123 (26), 105 (61), 91 (59), 79 (55), 69 (43), 55 (40), 41 (57); HR-EI-MS *m/z* 234.1618 (calcd for $C_{15}H_{22}O_2$: 234.1619).

3.3.5. Antitubercular activity assay

The antitubercular activity of each test compound was evaluated and compared with the MIC using a clinically susceptible isolate of *Mycobaterium tuberculosis* 90-221387. The agar-dilution method with Middlebrook 7H10 agar was used to determine the MICs, as described in Tentative Standard M24-T2 of the US National Committee of Clinical Laboratory Standards (Inderlied and Nash, 1996). Briefly, each test compound was added to Middlebrook 7H10 medium supplemented with oleic acid-albumin-dextrose-catalase at 50–56 °C in serial dilutions to yield a final concentration of 100–0.2 μg/mL. Then, 10 mL of each concentration of antimycobacterial agent-containing medium, as well as the drug-free control medium, was dispensed into plastic quadrant Petri dishes. The inoculum of the test isolate of *M. tuberculosis* 90-221387 was prepared by diluting the initial inoculum in Middlebrook 7H9 broth equivalent to the McFarland no.1 turbidity standard (10^7 CFU/mL). Final suspensions were achieved by adding Middlebrook 7H9 broth and preparing a 10^{-4} dilution of the standardized suspensions. After solidification of the Middlebrook 7H10 medium, 33 μL of each dilution was inoculated into each quadrant of the agar plates. The agar plates were then incubated at 35–37 °C with 10% CO_2 for 3 weeks. The MIC of each test compound was determined as the lowest concentration that inhibited macroscopic growth. The test compounds in this experiment also included ethambutol (Sigma, St. Louis, Missouri, USA) in order to compare the *in vitro* activity against *M. tuberculosis*. The MIC for the testing strain was 5 μg/mL.

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