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Trypethelone and phenalenone derivatives isolated from the mycobiont culture of *Trypethelium eluteriae* Spreng. and their anti-mycobacterial properties

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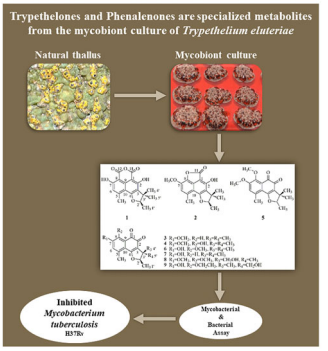
ABSTRACT

The metabolites of the mycobiont culture of the lichen *Trypethelium eluteriae* were isolated by column chromatography and preparative TLC. Nine compounds (**1**–**9**) including two new trypethelones, 8-methoxytrypethelone (**6**) and 5′-hydroxy-8-ethoxytrypethelone (**9**), together with four known trypethelones (**3**–**4**, **7**–**8**), and two known phenalenones (**1**–**2**) were characterized. It is the first report of 8-methoxytrypethelone methyl ether (**5**) purification as a racemic mixture in *T. eluteriae*. Earlier, 7-hydroxyl-8-methoxyltrypethelone (**10**) was reported as new compound with erroneous spectroscopic data. This compound was identified later as 8-hydroxytrypethelone methyl ether (**4**). X-ray crystallographic structures of compounds **5**–**7** were elucidated for the first time. Phenalenones (**1**–**2**) and trypethelones (**5**–**6** and **9**) were the additional compounds discovered in the cultured mycobiont of *T. eluteriae*. Six compounds (**1**–**2**, **5**–**8**) were screened against *Mycobacterium tuberculosis* H37Rv and two compounds (**7**–**8**) against non-tuberculosis mycobacteria and other human pathogenic bacteria. Compound (**7**) inhibited *M. tuberculosis* H37Rv strain with an MIC of 12.5 μg/mL.



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1. Introduction

Lichens are fungi (mycobionts) which form obligate symbiotic associations with green algae or cyanobacteria (photobionts) to fulfill their nutritional requirements. The mycobiont produces several specialized metabolites, both in its natural as well as cultured states, through the polymalonate, shikimate and mevalonate pathways. A total of 1050 compounds were isolated and characterized (Stocker-Wörgötter 2008), which displayed a range of biological activities (Huneck 1999, 2001; Huneck and Yoshimura 1996). Interestingly, the cultured mycobionts produce known and novel compounds in addition to those present in the intact thallus (Valarmathi and Hariharan 2007; Shanmugam et al. 2016). The lichen genus *Trypethelium* Spreng. (Trypetheliaceae) consists of 16 species worldwide (Aptroot and Lücking 2016). *Trypethelium eluteriae* Spreng. is a pyrenocarpous, corticolous, crustose lichen, predominantly found in tropical to subtropical regions of the world (Lambright and Tucker 1980; Luangsuphabool et al. 2016). The intact thallus of this lichen growing in the wild contains compounds like emodin, physcion and secalonic acid (Mathey et al. 2002; Luangsuphabool et al. 2016).

The mycobiont cultures of *Trypethelium* spp. are known to synthesize a number of trypethelone and phenalenone derivatives (Mathey et al. 1980; Takenaka et al. 2013; Basnet et al. 2018) which shows significant to moderate activities against bacterial pathogens including mycobacterial strains, RKO cell line and human leukocyte elastase (Sun et al. 2010; Elsebai et al. 2011a, 2011b; Basnet et al. 2018). We cultured *T. eluteriae* mycobiont, isolated and characterized the nine compounds (**1**–**9**) including two new trypethelone derivatives, 8-methoxytrypethelone (**6**) and 5′-hydroxy-8-ethoxytrypethelone (**9**), and a racemic 8-methoxytrypethelone methyl ether (**5**). These compounds were screened against *Mycobacterium tuberculosis* H37Rv strain, non-tuberculosis mycobacterial strains, and other human bacterial pathogens.

2. Results and discussion

A mycobiont culture of *T. eluteriae* was established in malt-yeast extract medium supplemented with 6% sucrose. After 240 days, the cultures were harvested, dried and extracted with acetone, and the metabolite profile was analyzed using HPLC. The compounds were separated by a combination of column chromatography and preparative TLC. The structures of the isolated compounds (**1**–**9**) (Figure 1) were characterized using spectroscopic techniques including UV, FT-IR, 1D, 2D NMR, HRESIMS, ECD and X-ray crystallography.

Compound **5** was obtained in the form of red plates, and the molecular formula was interpreted as $C_{18}H_{20}O_5$ based on HRESIMS (*m/z* 317.1378 $[M + H]^+$) and NMR data. It gave a negative ferric reaction, indicating the absence of phenol. The UV spectrum showed λ_{max} at 277, 369 and 476 nm suggesting a trypethelone skeleton. IR spectrum demonstrated the absence of a hydroxy group and showed peaks at ν_{max} 1691 cm^{-1} (quinone carbonyl), 1636, 1567, 928, 900 and 874 cm^{-1} (aromatic system). The ^1H and ^{13}C NMR spectroscopic data (Table S1 in supplementary material) were reminiscent of 8-methoxytrypethelone methyl ether (Mathey et al. 1980; Guay and Brassard 1984). However, the specific rotation was slightly negative ($[\alpha]^{25}_D$ −5°), which

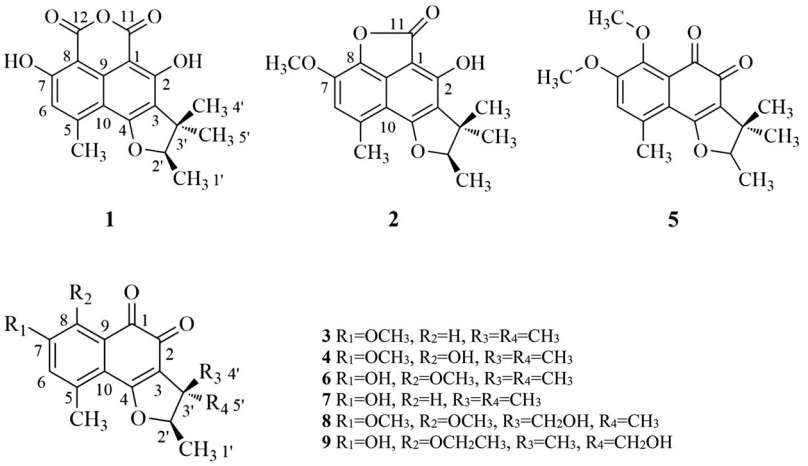

Figure 1. Chemical structures of isolated compounds **1**–**9**.

is in contrast to the positive rotation of 8-methoxytrypethelone methyl ether ($[\alpha]^{25}_{D}$ +62°) reported by Mathey et al. (1980). In addition, the X-ray diffraction analysis indicated a centrosymmetric (*P*–1) space group (CCDC 1411890) (Figure S2 in supplementary material). Therefore, we concluded that compound **5** (8-methoxytrypethelone methyl ether) was purified as racemic mixture.

Compound **6** was obtained as dark purple-red crystals and its molecular formula of $C_{17}H_{18}O_5$ was established using HRESIMS *m/z* 303.1228 $[M + H]^+$ (calcd. for $C_{17}H_{19}O_5$, 303.1227). It gave a positive ferric reaction for phenol and the trypethelone skeleton was recognized in the UV spectrum at λ_{max} 276, 307, 367 and 487 nm. The IR spectrum showed significant absorption of a broad peak at ν_{max} 3247 cm^{-1} indicating the presence of hydroxy group followed by a typical carbonyl at ν_{max} 1690 cm^{-1}. ^{1}H NMR and HSQC experiments suggested one aromatic proton (δ_H 6.99, s, H-6), one oxygenated methine (δ_H 4.63, q, *J* = 6.5 Hz, H-2′), four methyls [(δ_H 1.27, s, H-4′), (δ_H 1.45, s, H-5′), (δ_H 1.46, d, *J* = 6.5 Hz, H-1′) and (δ_H 2.57, s, C5-CH_3)], and one methoxy group (δ_H 3.92, s, 8-OCH_3). The ^{13}C NMR spectrum showed 17 carbon signals in accordance with a molecular formula of two carbonyls, two methine carbons, eight quaternary carbons, four methyls and a methoxy group (Table S1 in supplementary material). This NMR data suggested that the trypethelone skeleton had C-7 and C-8 in oxygenated positions in the aromatic ring carrying one hydroxy and one methoxy group. The absence of downfield signal around δ_H 12.0 in ^{1}H NMR spectrum indicated a hydroxy group at C-7 which was unchelated with the carbonyl at C-1, and a methoxy group at C-8. The HMBC correlation of methoxy proton to C-8 also confirmed the position of the methoxy group (Figure S1 in supplementary material). Furthermore, the aromatic proton (H-6) showed correlations to two oxygenated carbons (C-7 and C-8), an aromatic methyl carbon (5-CH_3), and an aromatic carbon (C-10). Methyl proton (5-CH_3) exhibited correlations to C-4, C-5, C-6 and C-10. In addition, a number of correlations were observed at the furan ring: H-4′ interacted with C-2′, C-3, C-3′ and C-5′, H-5′ with C-2′, C-3, C-3′ and C-4′, H-1′ with C-2′ and C-3′, and H-2′ with C-4′ and C-5′. NOE interaction was observed at the furan ring between H-2′ and H-5′ (Figure S1 in supplementary material). The absolute configuration at C-2′ as 2′R was determined by the

positive specific rotation ($[\alpha]^{25}_{D}$ + 502°) and the positive Cotton effect at 303 and 495 nm (Figure S3 in supplementary material) (Mathey et al. 1980; Li et al. 2018). The structure of compound **6** is illustrated in the X-ray ORTEP diagram (Figure 2) (CCDC 1411971). Hence compound **6** is a new trypethelone derivative, we named 8-methoxytrypethelone.

During the structure elucidation of compound **6**, we observed that the planar structure of **6** was identical to that of 7-hydroxyl-8-methoxyltrypethelone (**10**), which was isolated from the mycobiont culture of *Astrothelium* sp. by Sun et al. (2010). However, the methoxy group δ_C 56.8/C-8 in **10** displayed significant difference from that of **6** (62.1/C-8) (Figure S15 in supplementary material). It should be noted that the methoxy group (δ_C 56.8/C-8) in **10** was flanked by two ortho substituents C-7 and C-9 hence the downfield signal expected around δc 60 instead of δ_C 56.8. This was evident with the structural assignments of other compounds **4** and **5**. The ^{13}C NMR data of compound **5** (8-methoxytrypethelone methyl ether) indicating a methoxy signal at δc 61.3 (Figure S7 in supplementary material) that was assigned to C-8 based on HMBC correlation (Figure S1 in supplementary material), which was flanked by two ortho substituents C-7 and C-9 (Mathey et al. 1980) (Table S1 in supplementary material). Furthermore, compound **4** (8-hydroxytrypethelone methyl ether) was structurally identical to that of compound **5** except an addition of hydroxy instead of methoxy group at C-8 (methoxy at C-7 and hydroxy at C-8) (Figure 1). The NMR data of **4** showed a downfield signal at δ_H 13.1 (Figures S30 and S32 in supplementary material) denoting the chelation of the hydroxy group with a carbonyl at C-1, indicating a hydroxy group at C-8 and consequently the methoxy group (δc 56.2) at C-7 (Figure S33, Table S1 in supplementary material) (Takenaka et al. 2013; Basnet et al. 2018 – erroneously named as 8-hydroxy-7-methoxytrypethelone).

The aforementioned evidence clearly indicates that the proposed structure 7-hydroxyl-8-methoxyltrypethelone (**10**) by Sun et al. (2010) was incorrect; the methoxy group (δc 56.8) in **10** should have been placed at C-7 rather than C-8, and therefore 7-hydroxyl-8-methoxyltrypethelone (**10**) should actually be 8-hydroxytrypethelone methyl ether (**4**) (Takenaka et al. 2013; Basnet et al. 2018). Hence, we conclude that compound **6** is a new trypethelone derivative, 8-methoxytrypethelone isolated and characterized in this study.

Compound **7** was identified as trypethelone based on spectroscopic data and relevant literatures (Mathey et al. 1980; Ayer et al. 1989; Basnet et al. 2018). Elsebai et al. (2011b) reported (−)-trypethelone ($[\alpha]^{23}_{D}$ −355° *c* 0.10, CH_3OH) and elucidated its

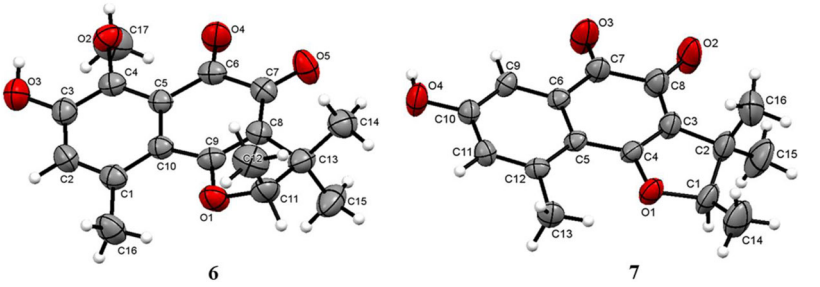

Figure 2. X-ray ORTEP drawings of compounds **6** and **7**.

crystal structure. In our study, the trypethelone indicated positive specific rotation ($[\alpha]^{25}_{D}$ +394° *c* 0.0020, CH_3OH), and we report the X-ray crystallographic structure of (+)-trypethelone (Figure 2) for the first time (CCDC 1520256).

Compound **9** was isolated as a red amorphous powder with spectral data almost identical to compound **6** with an additional ethoxy and hydroxylated methylene against the loss of methoxy and a methyl signal. The molecular formula $C_{18}H_{20}O_6$ was deduced from HRESIMS based on ion peak at *m/z* 333.1315 $[M + H]^+$ and the ferric reaction indicated phenol. The UV spectrum showed maxima at λ_{max} 277, 310, 372 and 490 nm and IR spectrum exhibited key peaks at ν_{max} 3359 cm^{-1} (hydroxy), 1687, 1622 cm^{-1} (carbonyl) and 1596 cm^{-1} (aromatic system). The ^{13}C NMR spectrum indicated the presence of 18 carbons: ten quaternary, two methine, two methylene, and four methyl groups. Comparison of NMR data with that of compound **6** revealed an oxymethylene ($\delta_{C/H}$ 69.5/4.03, q, *J* = 7.0 Hz, H-11) and a methyl signal ($\delta_{C/H}$ 14.2/1.44, t, *J* = 7.0 Hz, H-12) with significant ^1H–^1H coupling, which substantiated the ethoxy group as a moiety of compound **9**. The absence of a downfield proton signal around δ_H 12.0 and an HMBC correlation of H-11 with C-8 confirmed the position of the ethoxy group at C-8 (Figure S1 in supplementary material), and consequently the hydroxy group was placed at C-7. In the furan ring, HSQC data suggested a correlation between doublet proton (δ_H 1.64, d, *J* = 7.0 Hz) and methyl carbon (δ_C 13.1) followed by HMBC cross peak with oxygenated methine ($\delta_{C/H}$ 92.3/4.77, q, *J* = 6.5 Hz), which indicated the methyl group (δ_C 13.1) at C-1′. This interpretation was also supported by ^1H–^1H COSY between H-1′ and H-2′. Typical geminal dimethyl resonances were usually seen at δ_C 20 (C-4′) and δ_C 25 (C-5′), respectively (Table S1 in supplementary material), indicating that the methyl signal (δ_C 19.4) was positioned at C-4′ based on the key HMBC correlations to C-2′, C-3′ and C-5′. The HSQC spectrum showed double doublet protons correlated with methylene carbon, which indicated hydroxylated methylene as a part of the molecule ($\delta_{C/H}$ 62.7/3.80 and 3.71, *J* = 11.5 Hz). The position of hydroxylated methylene was assigned to C-5′ owing to the absence of a methyl signal at δ_C 25, and also based on the HMBC correlations to C-2′, C-3′ and C-4′. The NOESY spectrum showed a single cross peak across H-1′ and H-4′ indicating methyl groups in beta orientation, which strongly points to the alpha orientation of the hydroxylated methylene attached to C-3′ (Figure S1 in supplementary material). The absolute configuration of compound **9** was determined by the specific rotation and ECD values. The specific rotation of **9** indicated the positive rotation ($[\alpha]^{25}_{D}$ +533°) and the ECD spectrum showed significant positive Cotton effects at 306 and 495 nm (Figure S3 in

Table 1. Antimycobacterial activity of compounds (**1**, **2**, **5**, **6**, **7** and **8**) against *Mycobacterium tuberculosis* H37Rv.

S. No.	Compound	MIC (μg/mL)
1	Compound **1**	>100
2	Compound **2**	50
3	Compound **5**	>100
4	Compound **6**	100
5	Compound **7**	12.5
6	Compound **8**	50

Compounds: **1**—sclerodin; **2**—lactone; **5**—8-methoxytrypethelone methyl ether; **6**—8-methoxytrypethelone; **7**—trypethelone; **8**—4′-hydroxy-8-methoxytrypethelone methyl ether.

supplementary material) which were superimposable on that of 5′-hydroxytrypethelone (Basnet et al. 2018). Based on these findings, compound **9** was named 5′-hydroxy-8-ethoxytrypethelone.

The following known compounds were identified based on their physical and spectroscopic data as reported in the literature: Sclerodin **1** (Homma et al. 1980; Ayer et al. 1986; Takenaka et al. 2013), lactone **2** (Takenaka et al. 2013), trypethelone methyl ether **3** (Mathey et al. 1980; Takenaka et al. 2013), 8-hydroxytrypethelone methyl ether **4** (Takenaka et al. 2013; Basnet et al. 2018), trypethelone **7** (Mathey et al. 1980; Ayer et al. 1989; Basnet et al. 2018), and 4′-hydroxy-8-methoxytrypethelone methyl ether **8** (Mathey et al. 1980).

Compounds **1**, **2**, **5**–**8** were tested against *M. tuberculosis* H37Rv strain and compounds **7** and **8** were screened against non-tuberculosis mycobacteria using the 7H11 Middlebrook agar dilution method (Hacek 1992). Further, compounds **7** and **8** were also tested against human pathogenic bacteria by the minimum inhibitory concentration (MIC) method (Wiegand et al. 2008) using Mueller Hinton agar. As a result, compound **7** showed a minimum inhibition concentration (MIC) of 12.5 μg/mL followed by compounds **2** and **8** with MIC of 50 μg/mL against *M. tuberculosis* H37Rv strain (Table 1). Compound **7** showed moderate MIC of 25 μg/mL against atypical *M. phlei*, *M. chitae*, *M. parafortuitum*, *M. flavescens*, *M. szulgai* and *M. kansasii* (Table S2 in supplementary material). Compounds **7** and **8** were also active against *Staphylococcus aureus* with an MIC of 25 μg/mL (Table S3 in supplementary material).

3. Conclusion

The mycobiont culture of *T. eluteriae* (KY418158) synthesized nine compounds (**1**–**9**) that were trypethelone and phenalenone derivatives; 8-methoxytrypethelone (**6**) and 5′-hydroxy-8-ethoxytrypethelone (**9**) were characterized as new compounds and 8-methoxytrypethelone methyl ether (**5**) was identified as a racemic mixture. Furthermore, compounds **1**, **2**, **5**, **6** and **9** were additional compounds discovered from the mycobiont culture of *T. eluteriae* in this study.

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Disclosure statement

No conflict of interest.

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