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NAPHTHOQUINONES AND PHENALENONE DERIVATIVES FROM THE CULTURED LICHEN MYCOBIONTS OF *TRYPETHELIUM* SP.

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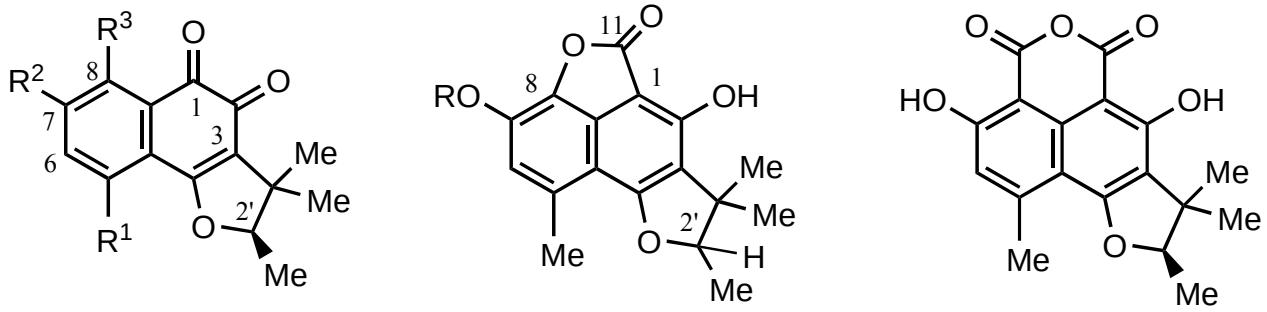
Abstract – Spore-derived mycobionts of the crustose lichen *Trypethelium* sp. collected in Vietnam were cultivated on a malt-yeast extract medium supplemented with 10% sucrose. The investigation of their metabolites resulted in isolation of a new naphthoquinone and a new phenalenone derivative, together with (+)-trypethelone methyl ether and (+)-sclerodin. Their structures were determined by spectroscopic methods.

Lichens are symbiotic organisms of fungi (mycobionts) and photoautotrophic algal partners, namely, green algae and/or cyanobacteria. Lichens produce many unique compounds, which are considered to have important biological and ecological functions, such as antimicrobial activity.[1] Most of these metabolites are produced by the fungal partner, in symbiosis or in the aposymbiotic state. Cultures of isolated lichen mycobionts, however, often exhibit the ability under osmotically stressed conditions to produce substances that have never been detected in the lichenized state.[2] These findings suggested that cultures of lichen mycobionts could be a new source of bioactive compounds. In the course of our studies on cultured mycobionts of Vietnamese lichens,[3] we cultivated the spore-derived mycobiont *Trypethelium* sp. and isolated a novel naphthoquinone and a new phenalenone derivative along with two known compounds from its cultures. In this paper, we report the isolation and characterization of the new compounds.

Specimens of *Trypethelium* sp. were collected from tree bark in Ba Ria-Vung Tau Province, Vietnam in 2009. The polyspore-derived mycobionts were cultivated on a malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After 3-4 months, the cultures were harvested and extracted with

ether, acetone and then with MeOH. Subsequent purification of the extracts by a combination of column chromatography and preparative TLC gave four metabolites **1**—**4**.

Compound **1** was isolated as a purple-red solid. The HR-ESIMS of **1** established the molecular formula of $C_{17}H_{18}O_4$. On the basis of its UV and IR spectra as well as ^{1}H- and ^{13}C-NMR spectral features, it was identified as (+)-trypethelone methyl ether, which was previously isolated from the cultured mycobionts of *Trypethelium eluteriae*.[4]



1: R^1 = Me , R^2 = OMe, R^3 = H
2: R^1 = Me, R^2 = OMe , R^3 = OH
5: R^1 = R^2 = R^3 = H
6: R^1 = Me, R^2 = OH, R^3 = OMe
7: R^1 = Me, R^2 = R^3 = OH

3: R = Me, H-2'α
8: R = H, H-2'β

4

Figure 1. Structures of isolated compounds **1**—**4** and their related compounds

Compound **2** was isolated as a purple-red solid. The HR-ESIMS of **2** established the molecular formula of $C_{17}H_{18}O_5$, that is, one oxygen atom more than trypethelone methyl ether (**1**). Its UV spectrum showed characteristic absorptions of 1,2-naphthoquinone at 214, 278, 322, 432 and 520 nm as **1**.[4] The ^{1}H-NMR spectrum of **2** was very similar to that of **1**, except that **2** exhibited the signals for a hydrogen-bonded hydroxyl group at δ 13.15 and one aromatic proton at δ 6.71 instead of two doublets for *meta*-coupled aromatic protons in **1**. These findings suggested **2** to be an 8-hydroxylated derivative of **1**. This assumption was verified by the NOESY interactions between the aromatic proton (H-6) and a methoxy group and between the aromatic proton and an aromatic methyl group as well as HMBC correlations from the aromatic proton to two oxygenated carbon signals at δ 152.5 (C-7) and 156.0 (C-8) and aromatic methyl carbon at δ 22.2. The positive specific optical rotation of this compound suggested the *R*-configuration at C-2', the same as in **1** ($[\alpha]_{578}$ +110, $[\alpha]_{546}$ +137 (MeOH))[4] and dunnione (**5**) ($[\alpha]_D$ +310 ($CHCl_3$)).[5] Accordingly, compound **2** was characterized as (+)-8-hydroxy-7-methoxytrypethelone. Its related compound, 7-hydroxy-8-methoxytrypethelone (**6**), has been isolated together with trypethelone (**7**) and trypethelone methyl ether (**1**) from cultured mycobiont of the lichen *Astrothelium* sp.[6]

Compound **3**, obtained as an orange solid, had the molecular formula of $C_{18}H_{18}O_5$ as established by

HR-ESIMS. Its ^{1}H-NMR spectrum showed signals for four methyl groups at δ 1.28 (s), 1.52 (s), 1.47 (d) and 2.66 (d), a methoxyl at δ 4.19 (s), an aromatic proton at δ 6.70 (q) and a hydroxyl group at δ 7.44. These spectral features implied its structural similarity to **2**. However, compound **3** exhibited UV maxima at 234, 264 and 345 nm, which were not consistent with characteristics for 1,2-naphthoquinones such as **1** and **2**. Its ^{13}C-NMR spectrum showed a signal for a carbonyl group at δ 167.5 and four oxygenated quaternary carbons at δ 129.5, 140.7, 155.9 and 164.6, while **2** revealed two carbonyl and three oxygenated quaternary carbon signals. These findings suggested that the compound possessed a skeleton analogous to (-)-7,8-dihydro-3,6-dihydroxy-1,7,7,8-tetramethyl-5*H*-furo[2',3':5,6]naphtho[1,8-*bc*]furan-5-one (**8**) isolated from marine-derived fungus, *Coniothyrium cereal*.[7] The ^{13}C-NMR spectrum of **3** differed from that of **8** by the presence of an additional methoxy signal and downfield shift of C-7, indicating the substitution of a methoxy group at C-7. Positioning of the hydroxyl group at C-2 could be inferred from an HMBC correlation of the hydroxyl proton with an aromatic quaternary carbon at δ 121.0, which was assigned to C-3 by HMBC interaction with a methyl signal at δ 1.52. The *R*-configuration of C-2' in **3** was deduced from a positive sign of its specific optical rotation opposite to that of **8** ($[\alpha]_D$ -55 ($CHCl_3$)).[7] Thus, compound **3** was elucidated to be (+)-7,8-dihydro-6-hydroxy-3-methoxy-1,7,7,8-tetramethyl-5*H*-furo[2',3':5,6]naphtho[1,8-*bc*]furan-5-one.

Compound **4** was identified as (+)-sclerodin by comparison of its spectroscopic data with those reported in the literature.[8,9]

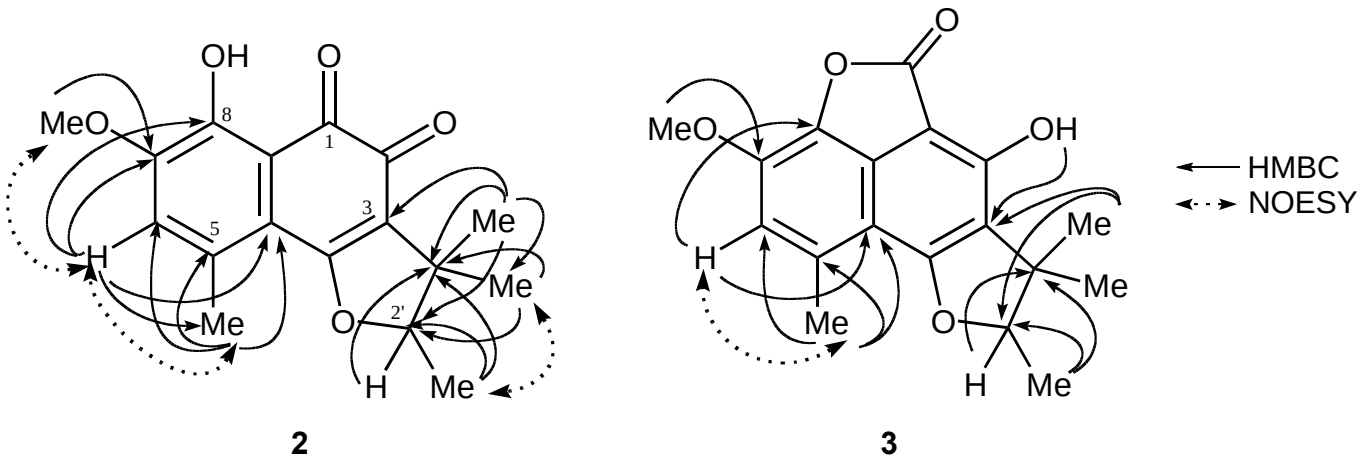


Figure 2. HMBC and NOESY correlations of **2** and **3**

The present study demonstrated that the cultured mycobionts of *Trypethelium* sp. are capable of producing novel metabolites, which have never been detected in lichen thalli but are structurally related to metabolites found in fungi. 1,2-Naphthoquinone-type metabolites have already been isolated from the

cultured mycobionts of lichens,[4,6] but phenalenone related compounds such as lactone **3** and sclerodin (**4**) were isolated for the first time from the cultured lichen mycobionts. Previous study reported the co-occurrence of sclerodin (**4**) with trypethelone (**7**) and lactone **8** in a plant disease causing fungus, *Gremmeniella abientina*.[10] It is noted that the optical purity of sclerodin isolated from several strains of this fungus was varied in a manner dependent on the culture conditions.[10] Therefore, chiral HPLC analyses of **4** as well as **1**—**3** using several kinds of chiral column and solvent system[11] were performed to confirm their optical purity. However, there was no indication of mixture with their enantiomers. All of the isolated metabolites might be pure or almost pure 2'*R*-isomers.

Table 1. ^{13}C-NMR Spectral Data of **1**—**4** and **8** in $CDCl_3$

C	**1**	**2**	**3**	**4**	**8**[a]
1	182.2	186.9	95.0	93.4	94.9
2	175.3	174.4	155.9	164.2	156.3
3	122.0	122.8	121.0	119.1	120.9
4	171.5	170.6	164.6	166.2	164.9
5	140.4	132.6	132.4	149.9	132.5
6	123.4	119.8	118.5	117.2	118.3
7	161.8	152.5	140.7	165.9	137.1
8	113.2	156.0	129.5	97.2	128.9
9	134.2	114.1	134.7	135.3	133.8
10	118.7	115.6	107.8	108.5	107.3
11	---	---	167.5	164.8[b]	167.4
12	---	---	---	165.4[b]	---
1'	14.7	14.7	14.5	14.5	14.4
2'	92.5	92.4	92.0	92.1	92.0
3'	43.0	43.1	43.4	43.5	43.3
4'	20.3	20.2	21.0	20.7	20.0
5'	25.8	25.7	25.7	25.5	25.5
5-CH_3	22.2	22.2	20.1	23.7	20.9
7-OCH_3	55.8	56.2	58.9	---	---

[a]Data taken from ref. 7. [b]Assignments may be interchanged.

EXPERIMENTAL

General Procedures. Melting points were measured on a Yanaco micro melting point apparatus and are not corrected. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. The optical rotations were measured on a Jasco DIP-370 digital polarimeter. HR-ESIMS were obtained with a Thermo Scientific Q Exactive. The NMR experiments were performed with Varian NMR System-500 and Varian UNITY INOVA (500

MHz) spectrometers, with TMS as internal standard. Silica gel 60 (Merck) was used for column chromatography. Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60F_{254} plates (Merck) and spots were visualized under UV light.

Plant Material. Specimens of *Trypethelium* sp. were collected from tree bark in Binh Chau, Ba Ria-Vung Tau Province, Vietnam (25 m alt.), in March 2009 by D. H. Le. The voucher specimen was identified by H. Miyawaki, and was deposited at Saga University, Japan (registration No. V355).

Cultivation of Mycobionts. Mycobionts were obtained from the spores discharged from apothecia of a thallus, and were cultivated in test tubes containing modified MY10 medium (10 g of malt extract, 4 g of yeast extract, 100 g of sucrose, 15 g of agar, 1l of H_2O, pH 7) at 18 °C in the dark.

Isolation of Metabolites. After cultivation for 3-4 months, the colonies were harvested. The harvested colonies (115 test tubes, dry weight of 51 g) were extracted with Et_2O, acetone and then MeOH at room temperature, and the combined extracts were concentrated under reduced pressure to give Et_2O (308 mg), acetone (316 mg) and MeOH (1.38 g) residues, respectively. The Et_2O and acetone extracts were subjected to silica gel column chromatography ($CHCl_3$-MeOH) followed by preparative TLC ($CHCl_3$ and/or toluene-acetone, 9:1). The Et_2O extract gave **2** (17.5 mg) and **4** (2.3 mg). The acetone extract furnished **1** (14.5 mg), **2** (81.8 mg), **3** (13.8 mg) and **4** (15.8 mg). The MeOH extract was separated by preparative TLC (toluene-acetone, 9:1) to give **2** (25.0 mg).

(+)-8-Hydroxy-7-methoxytrypethelone (2): purple-red solid, mp 153 °C; $[\alpha]_D^{25}$ +604 (*c* 0.01, $CHCl_3$); UV (EtOH) λ_{max} nm (log ε): 214 (4.41), 278 (4.52), 322 (3.73), 432 (3.61), 520 (3.72); IR (KBr) ν_{max} cm^{-1}: 1633, 1605, 1577, 1562; ^{1}H-NMR ($CDCl_3$): δ 1.25 and 1.43 (each 3H, s, H_3-4', H_3-5'), 1.45 (3H, d, *J* = 7.0 Hz, H_3-1'), 2.54 (3H, br s, 5-CH_3), 3.95 (3H, s, 7-OCH_3), 4.61 (1H, q, *J* = 7.0 Hz, H-2'), 6.71 (1H, br s, H-6), 13.15 (1H, br, 8-OH); ^{13}C-NMR: spectroscopic data see Table 1; HR-ESIMS *m/z*: Calcd for $C_{17}H_{19}O_5$ $[M+H]^+$: 303.1233. Found: 303.1228.

(+)-7,8-Dihydro-6-hydroxy-3-methoxy-1,7,7,8-tetramethyl-5*H*-furo[2',3':5,6]-naphtho[1,8-*bc*]furan-5-one (3): orange solid, mp 165-166 °C; $[\alpha]_D^{25}$ +56 (*c* 0.62, $CHCl_3$); UV (EtOH) λ_{max} nm (log ε): 234 (4.22), 264 (4.56), 345 (3.91); IR (KBr) ν_{max} cm^{-1}: 3356, 1721, 1636, 1632, 1604; ^{1}H-NMR ($CDCl_3$): δ 1.28 and 1.52 (each 3H, s, H_3-4', H_3-5'), 1.47 (3H, d, *J* = 6.5 Hz, H_3-1'), 2.66 (3H, d, *J* = 1.5 Hz, 5-CH_3), 4.19 (3H, s, 7-OCH_3), 4.68 (1H, q, *J* = 6.5 Hz, H-2'), 6.70 (1H, q, *J* = 1.5 Hz, H-6), 7.44 (1H, br s, 2-OH); ^{13}C-NMR: spectroscopic data see Table 1; HR-ESIMS *m/z*: Calcd for $C_{18}H_{19}O_5$ $[M+H]^+$: 315.1233. Found: 315.1230.

HPLC Analyses of 1—4. Analyses of each compound by chiral HPLC [column, CHIRALPAK AD-RH; mobile phase, H_2O-MeCN or H_2O-MeOH, column, CHIRALCEL OJ-RH; mobile phase, H_2O-MeCN or H_2O-MeOH] each showed one peak.

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