Naphthoquinones From Cultured Mycobiont of *Marcelaria cumingii* (Mont.) and Their Cytotoxicity

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**Abstract**

Five naphthoquinones including 4 compounds with new absolute configurations, (–)-2'5'-trypethelone methyl ether (1), (–)-2'5'-8-methoxytrypethelone methyl ether (2), (–)-(2',3',5')-4'-hydroxytrypethelone (3), and (–)-(2',3',5'R)-4'-hydroxy-8-methoxy trypethelone methyl ether (5), together with a known compound, (–)-2'5'-trypethelone (4), were isolated from cultured mycobiont of *Marcelaria cumingii*. These compounds were structurally elucidated by high-resolution mass spectra, nuclear magnetic resonance, circular dichroism, optical rotation, and single-crystal X-ray analysis. The cytotoxicity against several cancer cell lines of the isolated compounds were tested. (–)-2'5'-Trypethelone methyl ether (1) showed selective inhibition of HCT116 and A549 cell lines with half-maximal inhibitory concentration values of 0.32 ± 0.03 and 1.05 ± 0.12 μM, respectively. The binding conformation and molecular interactions including the effect of substituent modification were also revealed.

**Keywords**

*Marcelaria cumingii*, naphthoquinone, mycobiont, Trypetheliaceae, cytotoxicity

Cultured lichen mycobionts have shown to be capable of producing diverse metabolites under stressed conditions that were not detectable in natural lichens, but are structurally related to fungal metabolites.¹⁻³ Trypetheliaceae is a family of crustose lichens with approximately 15 genera and 418 species around the world recorded.⁴⁻⁵ Thailand is located in the tropical region with diverse distribution of lichens. In 2017, the total Thai lichens were reported to be at least 1292 species and, currently, Trypetheliaceae crustose lichens were found in 44 species.⁶⁻⁸ The genus *Marcelaria*, especially *Marcelaria cumingii*, possesses dominant morphological characteristics and is an abundant source of secondary metabolites, comprising anthraquinones and xanthones.¹⁰⁻¹² Nevertheless, no chemical data on the cultured mycobiont of this species have been found.

1,2-Naphthoquinone derivatives were regarded as major components of mycobionts derived from some lichens of Trypetheliaceae family, such as *Astrothelium* sp and *Trypethelium* sp.¹³⁻¹⁵ Several mycobiont-derived 8-naphthoquinones were isolated; nonetheless, the absolute stereochemistry of these metabolites was not completely assigned.³⁻¹³,¹⁶ Using the ascospore discharge technique,¹⁵ *M. cumingii* was successfully cultured. Herein we reported the separation of the methanol extract of this cultured mycobiont (Figure 1) resulting in the isolation of 5 naphthoquinones. The structural elucidation and their cytotoxicity against several cancer cell lines were conducted.
Results and Discussion

Compound 1 (Figure 2) was isolated as dark violet-red crystalline solid. The high-resolution electrospray ionization mass spectra (HRESIMS) positive-ion peak [M + Na]+ at m/z 309.1108 established the molecular formula of C17H18O4. Comparison of the nuclear magnetic resonance (NMR) data of 1 with that of trypethelone methyl ether, a known naphthoquinone,13-15 indicated the similarity in the structures. To define the absolute configuration of C-2’, the circular dichroism (CD), the optical rotation, as well as single-crystal X-ray analysis were applied. Elsabai et al reported the characteristic Cotton effect (CE) of mycobiont-derived naphthoquinone scaffold at λ max (Δε) 480 nm (–1.1) in accordance with a levorotatory-specific rotation. In case of 1, its negative CE at λ max (Δε) 492 nm (–1.1) in accordance with a levorotatory-specific rotation can be described with the reference (–)-trypethelone, indicating the 2’S configuration of 1. Accordingly, 1 was defined as (–)-2’S-trypethelone methyl ether. The absolute configuration of 1 was unequivocally evidenced by single-crystal X-ray crystallography (Figure 3) as shown in CheckCIF 1.

All isolated compounds were preliminarily evaluated for cytotoxic activity against KB and HeLaS3 cell lines. (–)-2’S-trypethelone methyl ether (1) displayed potent cytotoxic activity with half-maximal inhibitory concentration (IC50) of 9.87 ± 0.80 and 5.15 ± 0.87 µM, respectively, compared with the positive control doxorubicin (IC50 values between 0.12 ± 0.05 and 0.06 ± 0.03 µM) (Table 1). Accordingly, (–)-2’S-trypethelone methyl ether (1) was set to be tested further. It exhibited cytotoxicity against HCT116 and A549 with IC50 of 0.32 ± 0.03 and 1.05 ± 0.12 µM, respectively (Table 2).

Table 1. In Vitro Cytotoxicity of 1 to 5 Against KB and HeLaS3 Cell Lines.

| Compound | IC50 (µM) KB | IC50 (µM) HeLaS3 |
|----------|-------------|-----------------|
| 1        | 9.87 ± 0.80 | 5.15 ± 0.87     |
| 2        | 41.29 ± 3.12| 20.09 ± 1.08    |
| 3        | 53.78 ± 1.06| 51.33 ± 1.84    |
| 4        | 34.80 ± 2.59| 36.04 ± 2.12    |
| 5        | >100        | >100            |
| Doxorubicin* | 0.12 ± 0.05 | 0.06 ± 0.03     |

IC50, half-maximal inhibitory concentration.

*Positive control.

Figure 1. Cultured mycobiont of Marcelaria cumingii.

Figure 2. Chemical structures of 1 to 5.

Figure 3. Oak Ridge Thermal Ellipsoid Plot of 1 (20% probability level; atom colors: C, cyan; O, red; H, white).
Table 2. In Vitro Cytotoxicity of 1 Against Vero, MRC-5, HT29, HCT116, and A549 Cell Lines.

| Cells       | IC₅₀ (µM)       | Doxorubicin |
|-------------|-----------------|-------------|
| Vero        | 1.87 ± 0.22     | 2.06 ± 0.31 |
| MRC-5       | 14.28 ± 2.33    | 2.74 ± 0.23 |
| HT29        | 6.13 ± 0.15     | 0.30 ± 0.06 |
| HCT116      | 0.32 ± 0.03     | <0.1        |
| A549        | 1.05 ± 0.12     | 0.24 ± 0.05 |

IC₅₀, half-maximal inhibitory concentration.

The binding region was found to be similar to S1. All compounds bound at the same site and laid on the surface. The structure–activity relationship (SAR) of those 1,2-naphthoquinone derivatives, (–)-2’S-trypethelone methyl ether (1), having the methoxy group at C-7 showed the strongest activity. Moreover, 3 methyl substituents at C-2’ and C-3’ of (–)-2’S-trypethelone methyl ether (1), (–)-2’S-8-methoxytrypethelone methyl ether (2), and (–)-2’S-trypethelone (4) have been suggested to improve the cytotoxic potency.

Computational chemistry was applied to clarify the SAR and showed the comparison of molecular binding pose between doxorubicin and all inhibitors (supplemental Figure S1). All compounds bound at the same site and laid on the protein surface. The binding region was found to be similar to that reported by Kongkathip. Doxorubicin was shown to be the most active compound against KB and HeLaS3. This corresponded with the docking result, which showed that it formed H-bond with Arg99 (2.05 Å), Met101 (2.18 and 2.25 Å), Val103 (1.75 Å), Gly105 (1.68 Å), Ile130 (2.83 Å), and Asp515 (1.86 Å). (–)-2’S-Trypethelone methyl ether (1) exhibited lower activity than doxorubicin. However, it still formed the H-bond with Ile130 with the distance of 1.99 Å. Moreover, oxygen atom of carbonyl group at C-1 formed H-bond with NH (backbone) of Lys129 at 1.92 Å (supplemental Figure S2).

(–)-2’5-Trypethelone methyl ether (1) and (–)-2’S-8-methoxytrypethelone methyl ether (2) were different with an additional methoxy substituent at position C-8 for (–)-2’S-8-methoxytrypethelone methyl ether (2) and in their biological activity. (–)-2’S-Trypethelone methyl ether (1) and (–)-2’S-8-methoxytrypethelone methyl ether (2) formed equivalent hydrophobic interactions with Tyr100, Asp104, Ala128, and Phe513, while (–)-2’S-trypethelone methyl ether (1) also formed the hydrophobic interaction with Arg99, Met101, and Gly105 (supplemental Figure S3). The absence of methoxy substituent at C-8 caused the molecule to be deeper occupied and to form hydrophobic interactions with those amino acids. The effect of methoxy substituent at C-7 was evaluated due to its higher activity against KB than ones with a hydroxy substituent. The comparison of molecular binding of (–)-2’S-trypethelone methyl ether (1) and (–)-2’S-trypethelone (4) revealed that the methoxy substituent at C-7 of (–)-2’S-trypethelone methyl ether (1) formed hydrophobic interaction with Gly105 and the methyl substituent at C-5 formed hydrophobic interaction with Arg99, while (–)-2’S-trypethelone (4) lacked this interaction. This is due to the smaller molecular volume of (–)-2’S-trypethelone (4) so it can flip in a large pocket. The other 3 methyl substituents at C-2’ and C-3’ of (–)-2’S-trypethelone methyl ether (1) formed additional hydrophobic interactions with side-chain amino acids of Tyr100, Met101, and Phe513 as shown in supplemental Figure S4.

Conclusions

The methanol extract from cultured mycobiont of Marcelaria cumingii yielded 4 naphthoquinones with new absolute configurations: (–)-2’S-trypethelone methyl ether (1), (–)-2’S-8-methoxytrypethelone methyl ether (2), (–)-2’S,3’S,4’-hydroxytrypethelone (3), (–)-(2’S,3’S)-4’-hydroxy-8-methoxy trypethelone methyl ether (5), and a known naphthoquinone, (–)-2’S-trypethelone (4). The outcome confirmed a basis for secondary metabolites studies in Trypetheliciaceae cultured mycobiont and also suggests that this Marcelaria cultured mycobiont contains potent cytotoxic compounds.

Experimental

General Experimental Procedures

The NMR spectra were measured on a Bruker Avance III (400 MHz for 1H NMR and 100 MHz for 13C NMR) and Varian Mercury-400 Plus NMR (400 MHz for 1H NMR and 100 MHz for 13C NMR) spectrometers. The HRESIMS were recorded on a HRESIMS Bruker microTOF: Thin-layer chromatography was carried out on precoated silica gel 60 F254 or Sephadex LH-20 and spots were visualized by UV 254nm, UV365nm lamp. Column chromatography was performed with silica gel 60 (0.040-0.063 mm). Solvents used for isolation were n-hexane, dichloromethane, ethyl acetate, aceton, and methanol.

Fungal Isolation, Cultivation, and Identification

Specimen of M. cumingii was collected from tree bark at Pak Chong district, Nakhon Ratchasima, Thailand (700 m alt.). The mycobiont of M. cumingii was successfully isolated from perithelia of lichen thallus by the ascospore discharge technique17 and was cultivated in 90 mm Petri dishes containing Malt-Yeast-Extract agar at room temperature (30-32°C) for 9 weeks. The voucher specimen was identified by Dr Theerapat Luangsuphabool and was deposited at the Lichen Herbarium, Ramkhamhaeng University (voucher No. RAMK027993). The fungal culture is maintained in the lichen research unit at Ramkhamhaeng University, Thailand. The molecular data were analyzed to confirm the species identification. The genomic DNA was extracted from the mycobiont culture using CTAB precipitation protocol. The nuclear large subunit ribosomal DNA (mtLSU) and mitochondrial small subunit ribosomal DNA (mtSSU) loci were amplified using primer pairs LROR/
LR\textsuperscript{20} and mrSSU1/MSU\textsuperscript{21,22} respectively. Polymerase chain reaction conditions and DNA sequencing followed protocols previously described.\textsuperscript{23} The nuLSU and mrSSU sequences were deposited in DDBJ (accession number LC223104 and LC223105) and confirmed similarity sequences to \textit{M. cumingii} (KM453789; 99\%) and (LC034284; 99\%), respectively.

**Extraction and Isolation**

The fungal biomass and agar were separately extracted. The mycobiont colonies were extracted with methanol at room temperature to yield the crude extract (0.45 g). This crude extract was applied to normal phase silica gel column, eluted with the solvent system of dichloromethane:methanol (25:1) to afford 5 fractions \textbf{C1} (50.2 mg), \textbf{C2} (41.3 mg), \textbf{C3} (101.8 mg), \textbf{C4} (98.4 mg), and \textbf{C5} (30.1 mg). Fraction \textbf{C1} was applied to silica gel column, eluted with dichloromethane:methanol (25:1) to give 2 fractions, \textbf{C1.1} (21.2 mg) and \textbf{C1.2} (15.8 mg). Purifying fraction \textbf{C1.1} by crystallizing in methanol yielded \textbf{1} (4.9 mg). Fraction \textbf{C1.2} was column chromatographed, eluted with hexane:ethyl acetate:acetone (8:3:1) to afford \textbf{2} (2.9 mg). Fraction \textbf{C3} was subjected to column chromatography, eluted with hexane:ethyl acetate:acetone (4:3:1) to afford \textbf{5} (1.1 mg) and \textbf{4} (3.0 mg). Fraction \textbf{C5} was washed 3 times with dichloromethane (5 mL each) to obtain the precipitate \textbf{C5.1} (10.1 mg), which was dissolved in methanol and then purified by column chromatography to yield \textbf{3} (4.1 mg).

\textbf{(-)-Trypethelone methyl ether (1)}. Dark violet-red crystalline solid (4.9 mg); [\alpha]\textsubscript{D}\textsuperscript{25} \textdegree –7 (c 0.13 mg/mL, acetone); CD (c 0.5 mg/mL, MeOH), \lambda\textsubscript{max}(\Delta\varepsilon) 492 nm (–1.1); \textit{H} and \textit{^{13}C} NMR (CDCl\textsubscript{3}); HRESIMS m/z 309.1108 [M + Na\textsuperscript{+}] (calculated for C\textsubscript{17}H\textsubscript{18}NaO\textsubscript{4}, m/z 309.1103) (Supplemental Tables S1, S2 and Figures S5-S10,S35).

\textbf{(-)-8-Methoxytropanyl methyl ether (2)}. Violet-red crystalline solid (2.9 mg); [\alpha]\textsubscript{D}\textsuperscript{25} –38 (c 0.50 mg/mL, acetone); CD (c 0.5 mg/mL, MeOH), \lambda\textsubscript{max}(\Delta\varepsilon) 492 nm (–0.8); \textit{H} and \textit{^{13}C} NMR (CDCl\textsubscript{3}); HRESIMS m/z 339.1213 [M + Na\textsuperscript{+}] (calculated for C\textsubscript{18}H\textsubscript{19}NaO\textsubscript{5}, m/z 339.1208) (supplemental Tables S1, S2 and Figures S11-S17,S36).

\textbf{(-)-4′-Hydroxy-8-methoxytropanyl methyl ether (3)}. Violet crystalline solid (4.1 mg); [\alpha]\textsubscript{D}\textsuperscript{25} –23 (c 0.47, acetone); CD (c 0.5 mg/mL, MeOH), \lambda\textsubscript{max}(\Delta\varepsilon) 490 nm (–0.3); \textit{H} and \textit{^{13}C} NMR (DMSO-d\textsubscript{6}); HRESIMS m/z 311.0900 [M + Na\textsuperscript{+}] (calculated for C\textsubscript{18}H\textsubscript{17}NaO\textsubscript{5}, m/z 311.0895) (supplemental Tables S1, S2 and Figures S18-S24,S37).

\textbf{(-)-Trypethelone (4)}. Violet amorphous solid (3.0 mg); [\alpha]\textsubscript{D}\textsuperscript{25} –9 (c 0.22, acetone); CD (c 0.5 mg/mL, MeOH), \lambda\textsubscript{max}(\Delta\varepsilon) 490 nm (–0.9); \textit{H} and \textit{^{13}C} NMR (DMSO-d\textsubscript{6}); HRESIMS m/z 295.0949 [M + Na\textsuperscript{+}] (calculated for C\textsubscript{16}H\textsubscript{16}NaO\textsubscript{4}, m/z 295.0946) (supplemental Tables S1, S2 and Figures S25-S27,S38).

**Single-Crystal X-Ray Crystallography**

With the help of a Bruker X8 PROSPECTOR KAPPA CCD diffractometer equipped with an \textit{\lambda}\textsubscript{0} X-ray microfocus source operated at 45 kV, 0.65 mA, producing an intense monochromatic CuK\textsubscript{\alpha} radiation (\lambda = 1.54178 Å), the diffraction data of 1, 2, and 3 were collected at 296(2) K to atomic resolution of 0.83 Å and completeness of 96%-99%. Data processing covering integration, reduction together with correction of the absorption effects, and subsequent merging was carried out using SAINT, SADABS, and XPREF, respectively, in the APEX2 program suite (APEX2 v. 2014.9-0, Bruker AXS Inc., Madison, WI, USA). The 3 structures were solved by intrinsic phasing method with SHELXTL XT (SHELXTL XT, Program for crystal structure solution, v. 2014/4, Bruker AXS Inc., Madison, WI, USA), expanded using the difference Fourier technique, and were refined anisotropically by full-matrix least-squares on \textit{F}\textsuperscript{2} with SHELXL XLMP (SHELXTL XLMP, Program for crystal structure refinement—Multi-CPU, v. 2014/7, Bruker AXS Inc., Madison, WI, USA). All H-atom positions were placed geometrically and treated using a riding model: C–H = 0.93 Å, \textit{U}_{iso} = 1.2\textit{U}_eq(C)(aromatic); C–H = 0.96 Å, \textit{U}_{iso} = 1.5\textit{U}_eq(C)(methyl); C–H = 0.98 Å, \textit{U}_{iso} = 1.2\textit{U}_eq(C)(methylene); and O–H = 0.82 Å, \textit{U}_{iso} = 1.5\textit{U}_eq(hydroxyl). In 3, a cluster of disordered water sites was found as trace impurities of crystallization solvent (ethanol); the water H-atoms could not be determined. Absolute configurations of the 3 compounds were established by anomalous dispersion (Flack parameters \textit{x} close to zero). Crystal data of compounds 1, 2, and 3 (Figure 3, supplemental Figures S40–S41 and CheckCIF 1-3) have been deposited with the Cambridge Crystallographic Data Center (CCDC 1507772–1507774). They can be obtained free of charge via http://www.ccdc.cam.ac.uk/data_request/cif.

**Cytotoxicity Assay**

Cytotoxic activities of isolated compounds were tested using the standard MTT colorimetric method previously described.\textsuperscript{18}

**Molecular Docking Calculations**

\textbf{Protein and inhibitors preparation.} The protein structure of topoisomerase II was retrieved from the Protein Data Bank (pdb code: 1AB4).\textsuperscript{24} The crystallographic water molecules were deleted and the hydrogen atoms were added to protein by using AutoDockTools 1.5.6.\textsuperscript{26} The inhibitors were constructed and the geometry was optimized with PM6 level of theory by using MOPAC2009.\textsuperscript{27,28}
Molecular docking calculations. The atomic potential grid box for molecular docking was constructed with the grid size of 126 126 126 point. The distance spacing between each point is 0.375 Å. Subsequently, doxorubicin and all 5 inhibitors were docked into the protein by using AutoDock 4.2.6 program.26 The Lamarckian Genetic Algorithm was employed and the Gasteiger charge was employed to all inhibitors. The calculations of each inhibitor were set to 500 cycles while the other parameters were set as default. The most populous pose was selected for further molecular binding analyses.

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Supplemental Material

Supplemental material for this article is available online.

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