Chemical constituents of the lichen *Roccella sinensis* growing in Binh Thuan province

Duong Thuc Huy, Bui Xuan Hao

**Abstract** – The lichen *Roccella sinensis* has not been studied chemically. This research described the isolation and elucidation of compounds isolated from the lichen *Roccella sinensis* collected in Binh Thuan. Phytochemistry investigation of this lichen was carried out by using normal phase silica gel column chromatography and thin-layer chromatography. Six compounds were isolated. Their structures were established by extensively spectroscopic analysis as well as comparison with NMR data in the literatures. They are (+)-D-montagnetol (1), (+)-D-erythrin (2), lecanorin (3), 1-acetylerythritol (4), (E)-nostodione A (5), and 2,4-dihydroxyphthalide (6). This is the first time compounds 3-6 were found in the *Roccella* genus. Compounds 1, 2, and 6 were evaluated for their cytotoxic activities against HepG2 (liver hepatocellular carcinoma), NCI-H460 (human lung cancer), MCF-7 (human breast cancer), and HeLa (human epithelial cancer) and all of them showed no activity.

**Keyword** – *Roccella sinensis*, lichen, erythrin, montagnetol

1. INTRODUCTION

Lichens are symbiotic products of the mycobiont (fungal partner) and photobiont (algal partner) [1]. The genus *Roccella* includes 24 species which are restricted to coastal habitats [2]. Phytochemical studies on lichens *Roccella* spp. have been conducted over forty years on seven *Roccella* lichens [1] and the results showed that these lichens produced a diverse range of metabolites as meso-erythritol, dibenzofurans, chromones, amino acids, carbohydrates, ergosterol, and β-carotene. Among them, erythrin and lecanoric acid were found in most of *Roccella* species [2] and the former was reported as a principal secondary product [3, 4] and the content of this compound was 7.3% in *R. montagnei* [5]. The lichen *Roccella sinensis* is distributed commonly in Binh Thuan province, Vietnam. This lichen has not yet been chemically studied. The phytochemical study on the lichen *R. sinensis* led to the isolation of six compounds, including (+)-D-montagnetol (1), (+)-D-erythrin (2), lecanorin (3), 1-acetylerythritol (4), (E)-nostodione A (5), and 2,4-dihydroxyphthalide (6) (Fig. 1). Herein we describe the isolation and structural elucidation of these compounds as well as the cytotoxic evaluation of some isolated compounds.

2. MATERIALS AND METHODS

**Plant material**

*Roccella sinensis* was collected on the unknown trees in Lien Huong temple, Lien Huong district, Binh Thuan province. A voucher specimen (No US-B024) was deposited in the herbarium of the Department of Organic Chemistry, University of Science. Its scientific name was determined Dr. Wetchasart Polyiam, Lichen Research Unit, Department of Biology, Faculty of Science, Ramkhamhaeng University, Thailand.

**General experimental procedures**

The NMR spectra were recorded on a Bruker Avance III (500 MHz for 1H NMR and 125 MHz for 13C NMR) spectrometer. Proton chemical shifts were referenced to the solvent residual signal of CD3COCD3 at δH 2.05 and of CD3SOCD3 at δH 2.50. The 13C–NMR spectra were referenced to the central peak of CD3COCD3 at δc 29.4 and of CD3SOCD3 at δc 39.5. The HR–ESI–MS were recorded on a HR–ESI–MS Bruker microOTOF Q-II. Gravity
column chromatography was performed with Silica gel 60 (0.040–0.063 mm, Himedia).

**Extraction and isolation**

The clean, air-dried and ground material (1.7 kg) was extracted by maceration with methanol (3×5 L) at the ambient temperature, and the filtrated solution was concentrated under the reduced pressure to afford the crude extract (450 g). This crude extract was applied to normal phase silica gel column chromatography, eluting with the solvent system n-hexane: ethyl acetate (9:1 to 0:10) to afford the extracts, H (10.2 g), EA1 (21.1 g), EA2 (66.1 g), EA3 (29.8 g) and EA4 (64.2 g). The extract EA1 (21.1 g) was applied to silica gel column chromatography (CC), eluted stepwise with n-hexane: ethyl acetate: acetic acid (9:1:0.2) to (0:10:0.02) to give three fractions EA1.1 (2.8 g), EA1.2 (15.1 g) and EA1.3 (1.1 g). Fraction EA1.1 (2.8 g) was fractionated, eluting with n-hexane: ethyl acetate: acetic acid (9:1:0.05) to afford three subfractions, EA1.1.1 (298.8 mg), EA1.1.2 (1.2 g) and EA1.1.3 (0.9 g). A part of subfraction EA1.1.2 (300 mg) was rechromatographed, eluting with n-hexane: ethyl acetate: acetic acid (9:1:0.04), then applied to preparative thin-layer chromatography to afford 6 (6.9 mg). Fraction EA1.2 (15.1 g) was rechromatographed, eluting with chloroform: ethyl acetate: acetonitrile (9:1:3) to afford two compounds, 1 (1.5 g) and 2 (1.0 g). Extract EA2 (66.1 g) was applied to silica gel column chromatography, eluting with n-hexane: ethyl acetate: acetic acid from (9:1:0.02) to (0:10:0.02) to give two fractions EA2.1 (22.1 g) and EA2.2 (33.9 g). Subfraction EA2.1 was rechromatographed, eluting with chloroform: ethyl acetate: acetonitrile (9:1:3) to afford two compounds, 3 (9.9 mg) and 4 (79.7 mg). Fraction EA4 was applied to silica gel CC to provide three subfractions EA4.1 (16.1 g), EA4.2 (25.3 g) and EA4.3 (15.2 g). Subfraction EA4.1 was further chromatographed, eluting with chloroform: ethyl acetate: acetonitrile (9:1:3) to afford 5 (6.1 mg).

**(+)--D-Montagnetol (1)**

White crystals (methanol). $[\alpha]_{D}^{25}+27.0$ (c 0.1, methanol). The ¹H-NMR data (Acetone-d$_{6}$): 4.62 (dd, 11.5, 3.0 Hz, H-1a), 4.45 (dd, 11.5, 6.5 Hz, H-1b), 4.00 (td, 7.0, 2.7 Hz, H-2), 3.72 (m, H-3), 3.84 (m, H-4a), 3.74 (m, H-4b), 6.23 (d, 2.0 Hz, H-3’), 6.27 (d, 2.0 Hz, H-5’), 2.51 (s, H-8’). The ¹³C-NMR data (Acetone-d$_{6}$): 67.8 (C-1), 71.0 (C-2), 73.1 (C-3), 64.3 (C-4), 105.5 (C-1’), 165.9 (C-2’), 101.5 (C-3’), 163.1 (C-4’), 112.2 (C-5’), 144.6 (C-6’), 172.5 (C-7’), 24.4 (C-8’). These spectroscopic data were suitable with those reported in the literature [6].

**(+)--D-Erythrin (2)**

White crystals (methanol). $[\alpha]_{D}^{25}+27.0$ (c 0.1, methanol). The ¹H-NMR data (Acetone-d$_{6}$): 4.70 (dd, 11.5, 3.0 Hz, H-1a), 4.53 (dd, 11.5, 6.5 Hz, H-1b), 4.00 (td, 6.5, 2.5 Hz, H-2), 3.70 (m, H-3), 3.82 (m, H-4a), 3.74 (m, H-4b), 6.75 (d, 2.5 Hz, H-3’), 6.79 (d, 2.5 Hz, H-5’), 2.59 (s, H-8’), 6.30 (d, 2.5 Hz, H-3’), 6.37 (d, 2.5 Hz, H-5’), 2.62 (s, H-8’). The ¹³C-NMR data (Acetone-d$_{6}$): 67.6 (C-1), 70.2 (C-2), 72.3 (C-3), 63.7 (C-4), 112.7 (C-1’), 163.8 (C-2’), 109.8 (C-3’), 154.8 (C-4’), 117.3 (C-5’), 144.0 (C-6’), 169.5 (C-7’), 23.7 (C-8’), 104.8 (C-1”), 164.2 (C-2”), 101.8 (C-3”), 166.7 (C-4”), 112.9 (C-5”), 144.7 (C-6”), 170.5 (C-7”), 24.3 (C-8”). These spectroscopic data were suitable with those reported in the literature [6].

**Lecanorin (3)**

White amorphous powder. The ¹H-NMR data (Acetone-d$_{6}$): 6.28 (d, 2.5 Hz, H-3), 6.37 (d, 2.5 Hz, H-5), 2.59 (s, H-8), 6.57 (d, 2.0 Hz, H-3’), 6.57 (d, 2.0 Hz, H-5’), 2.29 (s, H-7’). The ¹³C-NMR data (Acetone-d$_{6}$): 110.6 (C-1), 164.2 (C-2), 101.9 (C-3), 159.1 (C-4), 107.4 (C-5), 144.7 (C-6), 174.4 (C-7), 24.4 (C-8), 114.7 (C-1’), 154.5 (C-2’), 114.5 (C-3’), 152.0 (C-4’), 112.9 (C-5’), 141.1 (C-6’), 21.4 (C-7’). These spectroscopic data were suitable with those reported in the literature [7].

**1-Acetylerythritol (4)**

Colorless oil. The ¹H-NMR data (D$_{2}$O): 4.33 (dd, 12.0, 3.0 Hz, H-1a), 4.18 (dd, 12.0, 6.5 Hz, H-1b), 3.87 (dd, 7.5, 6.5, 3.0 Hz, H-2), 3.73 (dd, 7.5, 6.5, 3.0 Hz, H-3), 3.80 (dd, 12.0, 3.0 Hz, H-4a), 3.65 (dd, 11.5, 6.5 Hz, H-4b), 2.00 (s, AcO-). The ¹³C-NMR data (D$_{2}$O): 66.9 (C-1), 72.9 (C-2), 71.1 (C-3), 64.2 (C-4), 171.5 (AcO-), 20.8 (AcO-). These spectroscopic data were suitable with those reported in the literature [8].

**(E)-Nostodione A (5)**

Red amorphous powder. HR-ESI-MS, positive mode: m/z 312.0653 [M+Na]⁺ (calcd. for C$_{18}$H$_{31}$NO$_{5}$Na, 312.0637). The ¹H-NMR data
(DMSO-d$_6$): 7.66 (d, 8.0 Hz, H-4), 7.42 (td, 7.0, 2.5 Hz, H-5), 7.33 (t, 7.0 Hz, H-6), 7.84 (brd, 8.0 Hz, H-7), 7.31 (brs, H-9), 7.70 (d, 8.5 Hz, H-11/15), 6.96 (d, 8.5 Hz, H-12/14), 10.29 (s, 13-OH). The $^{13}$C-NMR data (DMSO-d$_6$): 193.3 (C-1), 119.4 (C-2), 158.6 (C-2a), 140.7 (C-3a), 114.7 (C-4), 125.2 (C-5), 113.9 (C-6), 120.8 (C-7), 120.9 (C-7a), 123.6 (C-7b), 177.0 (C-8), 128.8 (C-9), 124.5 (C-10), 131.7 (C-11/15), 116.4 (C-12/14), 159.8 (C-13). HMBC data: H-9 to C-1, C-2a, C-11; H-11/15 to C-9, C-14; H-7 to C-5, C-3a; H-4 to C-6, C-3a, C-7a. These spectroscopic data were suitable with those reported in the literature [9].

2,4-Dihydroxyphthalide (6)

White amorphous powder. The $^1$H-NMR data (Acetone-d$_6$): 6.38 (brs, H-3), 6.53 (brs, H-5), 5.22 (s, H-8). These spectroscopic data were suitable with those reported in the literature [10].

3. RESULTS AND DISCUSSION

Compounds 1-4 and 6 were identified as (+)-d-montagnetol (1), (+)-d-erythrin (2), lecanorin (3), 1-acetylerthritol (4), and 2,4-dihydroxyphthalide (6) by comparison of their $^1$H and $^{13}$C NMR spectroscopic data as well as specific rotations with those reported in the literature. Compounds 1 and 2 were common lichen metabolites from the lichens Roccella spp. whereas compounds 3, 4, and 6 were known for the first time from this Roccella genus.

Compound 1 was obtained as white crystals (in methanol). The $^1$H NMR spectrum showed two oxygenated methylenes and two oxygenated methines in the zone of 3.70–4.70 ppm. Additionally, 1 contained one orcinol unit, including one aromatic methyl at $\delta_H$ 2.51 and two meta-coupled protons at $\delta_H$ 6.23 and 6.27. The $^{13}$C NMR spectrum revealed two oxygenated methines ($\delta_C$ 73.1 and 71.0), two oxygenated methylenes ($\delta_C$ 67.8 and 64.3), two aromatic methines ($\delta_C$ 101.5 and 112.2), two quaternary aromatic carbons ($\delta_C$ 105.5 and 144.6), two oxygenated aromatic carbons ($\delta_C$ 163.1 and 165.9), one carboxyl carbon at $\delta_C$ 172.3, and one methyl group at $\delta_C$ 24.4. $^1$H NMR chemical shifts of the methylene protons H$_2$-1 shifted to low-field indicating that this group was esterificated. These findings implied that 1 possessed a butane-1,2,3,4-tetraol pattern and an orcinol unit and these two moieties were linked together via an ester linkage. The coupling constant values of H-1 (11.5, 3.0 Hz and 11.5, 6.5 Hz) and H-2 (7.0, 2.7 Hz) supported the erythro configuration of the butane-1,2,3,4-tetraol pattern of 1. Comparison of the NMR data and the specific rotation of 1 ($[a]_{D}^{25}$+59.0, c 1.0, CH$_3$OH) with those of d(+)-montagnetol [6] showed good compatibility. Altogether, 1 was elucidated as (+)-(2R,3S)-1-(2,4-dihydroxy-6-methylbenzoyl)butan-1,2,3,4-tetraol or (+)-d-montagnetol as shown in Fig. 1.

Compound 2 was isolated as white crystals (in methanol). The 1D-NMR spectroscopic data of 2 resembled those of lecanoric acid [1], except for the presence of signals of two oxygenated methylenes ($\delta_C$ 67.1 and 63.0) and two oxygenated methines ($\delta_C$ 72.4 and 69.4). Comparison of the NMR data of the erythritol moiety of 1 and those of 2 suggested that they
possessed the same pattern (or erythritol derivative). Detailed analysis of the coupling constant of H-2 (δ 3.91, td, 7.0, 3.0 Hz) indicated that 2 also possessed the same erythro configuration as 1. Furthermore, the optical rotation of 2 was dextrorotatory [6]. Accordingly, the absolute configuration (2R,3R) was assigned to 2. Consequently, 2 was elucidated to be (+)-(2R,3R)-1-[4-(2,4-dihydroxy-6-methylbenzoyl)-2-hydroxy-6-methyl]butan-1,2,3,4-tetraol or (+)-D-erythrin as shown in Fig. 1.

Compound 5 was isolated as a red amorphous powder. The HR-ESI-MS of 5 exhibited a peak at m/z 312.0653 [M+Na]+ indicating a molecular formula of C_{18}H_{11}NO_{5} with 14 degrees of unsaturation. The ^1H NMR, in accordance with HSQC spectra, revealed one broad singlet at δ_{H} 7.31, eight aromatic protons and one phenolic hydroxyl group at δ_{H} 10.29. In particular, two ortho coupled aromatic protons at δ 6.96 (2H, d, 8.5 Hz) and 7.70 (2H, d, 8.5 Hz) indicated the existence of 1,4-disubstituted D-ring. The HMBC correlation from H-12/H-14 to δ_{H} 6.96 to C-13 (δ_{C} 159.8) confirmed the position of the hydroxy group at C-13. Moreover, the four remaining aromatic protons at δ_{H} 7.84 (brd, 8.0 Hz), 7.66 (d, 8.0 Hz), 7.42 (td, 7.0, 2.5 Hz), and 7.33 (t, 7.0 Hz) were coupled with each other in an AAXX' system, confirming the 1,2-disubstituted A-ring. On the basis of HMBC, protons at δ_{H} 7.84 (H-7) and δ_{H} 7.33 (H-6) showed cross peaks to C-3a (δ_{C} 140.7), suggesting the presence of an indole-moiety. Furthermore, the ^{13}C NMR spectrum revealed two conjugated ketone signals at δ_{C} 193.3 and 177.0. The upfield chemical shift of C-8 comparing to C-1 confirmed the connectivity of this carbon to the indole moiety due to the resonance effect caused by the electron-donating nitrogen atom. The HMBC correlations showed cross peaks from H-9 (δ_{H} 7.31) to C-3a and C-1 and from protons H-11/H-15 (δ_{H} 7.70) to C-9, suggesting the attachment of the D-ring to the indole ring. The spectral data comparison of 5 and the two diastereomers of nostodione A [5] indicated that 5 was (E)-nostodione A. Moreover, careful observation of the ^{1}H NMR spectrum indicated that there were some minor peaks belonging to the minor compound (Z)-nostodione A [5] and the integrations of some protons indicated that the ratio of the E/Z isomers was 9/1. Up to now, nitrogen-containing compounds from lichens was very rare with reports of several compounds [1, 11] and only one nitrogen-containing compound, a cyclopeptide was found in the Roccella genus [12]. This was the first time alkaloid 5 was reported as a lichen metabolite.

Compounds 1, 2, and 6 (at the concentration of 100 µg/mL) were tested for cytotoxic activities against four cell lines MCF-7 (breast cancer cell line), HeLa (cervical cancer cell line), HepG2 (liver hepatocellular carcinoma cell line), and NCI-H460 (human lung cancer cell line) using sulforhodamine B colorimetric assay method (SRB assay) [20]. Their cytotoxic activities, expressed as a percentage of cell growth inhibition (I%), was presented in Table 1. These major compounds had not been tested the cytotoxicity toward cancer cell lines. All three compounds failed to show any cytotoxic activity. Common lichen substances 3 and 4 were not tested for these activities.

### Table 1. % Inhibition of cytotoxic activities against four cancer cell lines of isolated compounds

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounda</th>
<th>Inhibition of Cell Growth (I %)b</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>HeLa&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>D-Montagnetol (1)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>D-Erythrin (2)</td>
<td>19.07±5.07</td>
</tr>
<tr>
<td>3</td>
<td>2,4-Dihydroxyphthalide (6)</td>
<td>10.89±0.76</td>
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<tr>
<td></td>
<td>Camptothecin (positive control)c</td>
<td>58.2±3.3</td>
</tr>
</tbody>
</table>

*a) The compounds were tested at the concentration of 100 µg/mL. b) The presented data are means of three experiments ± S.D. c) Camptothecin was tested at the concentration of 0.01 µg/mL for MCF-7 and NCI-H 460, 0.07 µg/mL for HepG2, and of 1 µg/mL for HeLa.

4. CONCLUSION

Six known compounds were isolated from the lichen Roccella sinensis collected in Binh Thuan.
province. This is the first time compounds lecanorin (3), 1-acetylerythritol (4), (E)-nostodione A (5), and 2,4-dihydroxyphthalide (6) are reported in the genus Roccella. Compounds 1, 2, and 6 failed to reveal any cytotoxicities against four tested cancer cell lines. Further studies on this lichen are in progress.

TÀI LIỆU THAM KHẢO