

**3,4-DIHYDRO-2*H*,5*H*-PYRANO[3,2-*c*]CHROMENE AND  
BENZOPHENONE DERIVATIVES FROM CULTURED LICHEN  
MYCOBIONTS OF VIETNAMESE *GRAPHIS* SP.**

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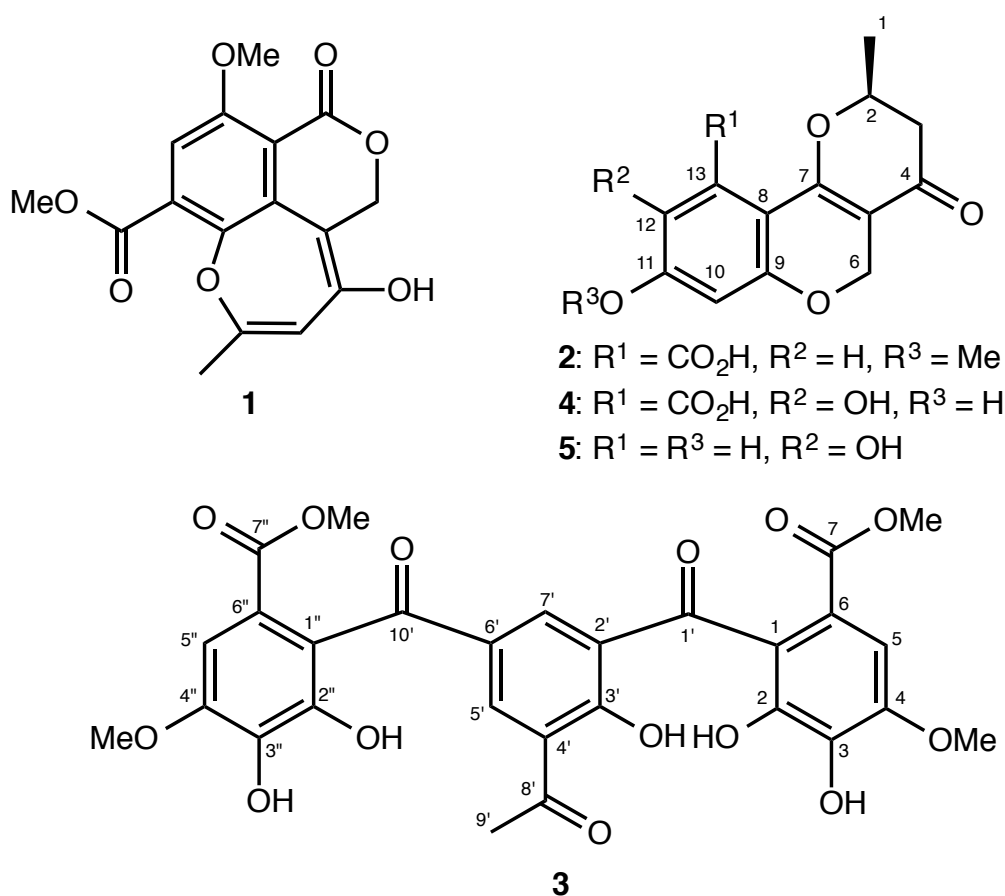
**Abstract** – Spore-derived mycobionts of the lichen *Graphis* sp. collected in Vietnam were cultivated on a malt-yeast extract medium supplemented with 10% sucrose and their metabolites were investigated. Two new phenolic compounds **2** and **3** were isolated along with proserin A (**1**). Their structures were determined by spectroscopic methods.

Lichens are distinct symbiotic entities composed of an algal or cyanobacterial photobiont and a fungal mycobiont. Lichen thalli are well known to produce a great variety of compounds with different structures, that is, lichen substances, which are considered to have potential biological and ecological functions, such as antimicrobial and antiherbivore activities.<sup>1-3</sup> Most of these metabolites are produced by the fungal partner in symbiosis or in the aposymbiotic state. On the other hand, aposymbiotically cultured mycobionts under stressed conditions frequently biosynthesize unique substances that are not detectable in the lichen thalli but are structurally related to fungal metabolites.<sup>4,5</sup> From our interest in the metabolic capability of the isolated lichen mycobionts, we previously cultivated the mycobionts of many lichen species collected in Japan and other countries and isolated diverse metabolites from the cultures. In the course of our studies on cultured mycobionts of Vietnamese lichens,<sup>6,7</sup> we cultivated the spore-derived mycobionts of an unidentified *Graphis* sp., and isolated new benzophenone and 3,4-dihydro-2*H*,5*H*-pyrano[3,2-*c*]chromene derivatives from their cultures. We report here the isolation and characterization of these compounds.

Specimens of *Graphis* sp. were collected from tree bark in Ma Da Forest, Dong Nai Province in Sept.

2008. The polyspore-derived mycobionts were cultivated on a malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After 14 months, the cultures were harvested and extracted with ether, acetone and then with MeOH. The MeOH extract was partitioned between H<sub>2</sub>O and *n*-BuOH. Subsequent purification of the *n*-BuOH extract by a combination of column chromatography, preparative TLC and preparative HPLC gave three metabolites **1**–**3**.

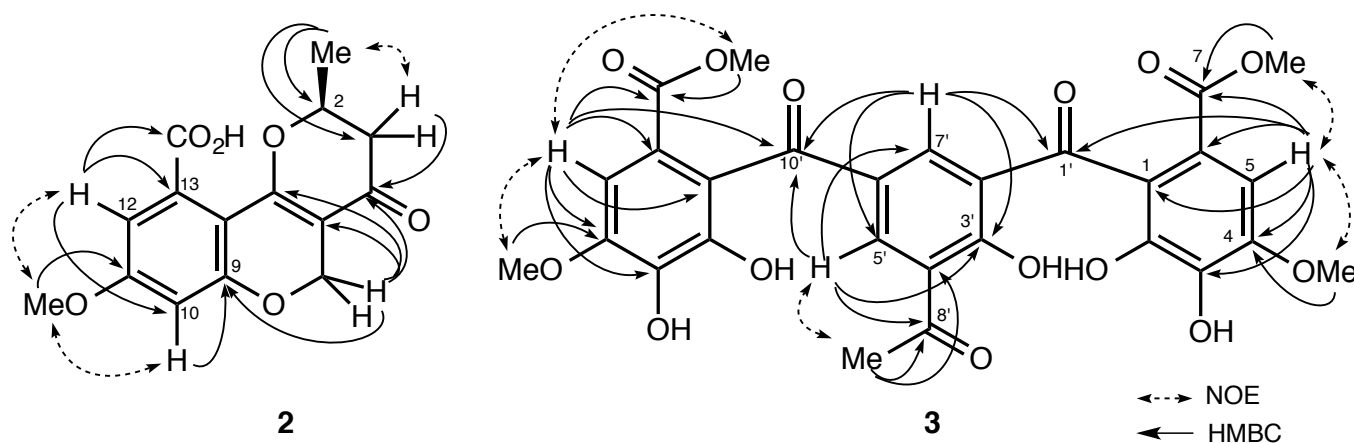
Compound **1** was identified as a 7-oxo-5,7-dihydrooxepino[4,3,2-*de*]isochromene derivative, proserin A, which has been isolated from the cultured mycobionts of *Graphis proserpens* Vain. collected in Japan.<sup>8</sup>



**Figure 1.** Structures of isolated compounds **1**–**3** and related compounds

Compound **2**, obtained as a colorless solid, was assigned the molecular formula of C<sub>15</sub>H<sub>14</sub>O<sub>6</sub> by HR-ESIMS. Its <sup>1</sup>H-NMR spectrum showed signals for a methyl group at δ<sub>H</sub> 1.51 (d), an oxygenated methine proton at δ<sub>H</sub> 4.68 (dq) and methylene protons at δ<sub>H</sub> 2.49 and 2.54 (each dd). The sequence from the methyl group to methylene protons was revealed by a series of <sup>1</sup>H-<sup>1</sup>H COSY correlations and was defined by the NOESY cross peak between the methyl and a proton signal at δ<sub>H</sub> 2.49. The <sup>1</sup>H-NMR spectrum, furthermore, revealed a methoxyl at δ<sub>H</sub> 3.82 (s), two oxygenated methylene protons, and a pair of *meta*-coupled aromatic protons at δ<sub>H</sub> 6.43 and 6.61 (each d, *J* = 2.5 Hz). The <sup>13</sup>C-NMR, DEPT and HSQC spectra of **2** showed the presence of a total of 15 carbon atoms, including a carbonyl at δ<sub>C</sub> 191.6, a

carboxyl at  $\delta_c$  176.0 and a methoxyl at  $\delta_c$  56.3, as well as one  $\text{CH}_3$ , two  $\text{CH}_2$ , and one  $\text{CH}$   $\text{sp}^3$  carbons and two  $\text{CH}$  and six quaternary  $\text{sp}^2$  carbons. These spectral features were similar to those of (-)-2,3-dihydrocitromyctin (**4**)<sup>9</sup> isolated from a marine-derived isolate of *Penicillium bilaii* and (-)-neuchromentin (**5**)<sup>10,11</sup> from the cultured broth of *Eupenicillium javanicum* var. *meloforme* PF1181. The  $^1\text{H}$ -NMR spectral data of **2** differed from those of **4** in terms of the presence of an additional methoxy signal and two doublets due to *meta*-coupled aromatic protons instead of a singlet of aromatic proton. In the  $^{13}\text{C}$ -NMR spectrum of **2**, an oxygenated aromatic carbon observed in **4** was replaced by an aromatic  $\text{CH}$  carbon. These findings suggested **2** to be a methylated and deoxygenated derivative of **4**. Detailed analysis of the HMBC spectrum of **2** showed the correlations of oxygenated methylene protons at  $\delta_{\text{H}}$  4.69 and 5.07 and an aromatic proton at  $\delta_{\text{H}}$  6.43 with a carbon at  $\delta_c$  162.4 (C-9) and another aromatic proton at  $\delta_{\text{H}}$  6.61 with carbons at  $\delta_c$  101.9 (C-10), 108.1 (C-13) and 176.0 (carboxyl), which revealed the location of the aromatic protons at C-10 and C-12, the methoxyl at C-11, and the carboxyl group at C-13. This substitution pattern was further supported by NOESY interactions between the methoxyl and two aromatic protons (Figure 2). The absolute configuration of C-2 in **2** could be tentatively assigned to *S* from the same negative sign of its specific optical rotation as that of **4** and **5**. Thus, compound **2** was elucidated to be (*S*)-(-)-8-methoxy-2-methyl-4-oxo-3,4-dihydro-2*H*,5*H*-pyrano[3,2-*c*]chromene-10-carboxylic acid.



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

Compound **3** was isolated as a colorless solid. Its molecular formula was established as  $\text{C}_{28}\text{H}_{24}\text{O}_{14}$  based on the  $[\text{M} + \text{H}]^+$  ion peak in the HR-ESIMS. Its IR absorptions at 3384, 1711, 1686, 1612 and  $1581\text{ cm}^{-1}$  showed the presence of hydroxyl, aromatic ketone and aromatic ester groups. Its  $^1\text{H}$ -NMR spectrum revealed signals for a methyl at  $\delta_{\text{H}}$  2.72, four methoxyls at  $\delta_{\text{H}}$  3.58, 3.66, 3.93 and 3.96 and four aromatic protons at  $\delta_{\text{H}}$  7.14 (s), 7.19 (s), 7.79 (d,  $J = 2.0\text{ Hz}$ ) and 8.35 (d,  $J = 2.0\text{ Hz}$ ), the latter two of which

showed *meta*-coupling. The  $^{13}\text{C}$ -NMR and DEPT spectra of **3** (Table 1) showed a total of 28 carbon signals. Of these, 18 carbon resonances were observed as duplicate signals, indicating the presence of two sets of the same partial structure. The partial structure consisted of two methoxyls ( $\delta_{\text{C}}$  56.80, 52.6/ $\delta_{\text{C}}$  56.75, 52.4), an ester carbonyl ( $\delta_{\text{C}}$  167.40/ $\delta_{\text{C}}$  167.45), and six aromatic carbons due to a pentasubstituted benzene ring. The chemical shift of three aromatic carbons in the region of  $\delta_{\text{C}}$  140-150 implied a 1,2,3-trioxygenated benzene core. The aromatic proton singlet at  $\delta_{\text{H}}$  7.19 showed an HSQC correlation with a carbon signal at  $\delta_{\text{C}}$  106.1 (C-5) and HMBC correlations with two quaternary carbons at  $\delta_{\text{C}}$  119.3 and 123.2 (C-1 and C-6), two oxygenated quaternary carbons at  $\delta_{\text{C}}$  140.5 (C-3) and 149.5 (C-4), and a carbonyl at 167.4 (C-7). The methoxyl signal at  $\delta_{\text{H}}$  3.96 showed an HMBC correlation with C-4, while the methoxyl signal at  $\delta_{\text{H}}$  3.66 showed an HMBC correlation with the carbonyl carbon C-7, confirming a methoxycarbonyl function. Furthermore, the NOESY interactions of the aromatic proton with the signals of the methoxyl and carbomethoxyl indicated both substituents to be situated at *ortho* positions of the aromatic proton. The other two oxygenated carbons were deduced to possess hydroxyl groups from the molecular formula of **3**. Another set of the signals of aromatic proton at  $\delta_{\text{H}}$  7.14, methoxyl at  $\delta_{\text{H}}$  3.93 and carbomethoxyl at  $\delta_{\text{H}}$  3.58 showed similar HSQC, HMBC and NOESY correlations to those described above (Figure 2). These findings suggested the partial structure to be a 2,3-dihydroxy-4-methoxy-6-methoxycarbonylphenyl group.

Table 1.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR Spectral Data of **3** in  $\text{DMSO-}d_6$

position	$\delta_{\text{H}}$	$\delta_{\text{C}}$	position	$\delta_{\text{H}}$	$\delta_{\text{C}}$	position	$\delta_{\text{H}}$	$\delta_{\text{C}}$
1		119.3 <sup>a</sup>	1'		202.6	1''		119.5 <sup>d</sup>
2		144.4 <sup>b</sup>	2'		124.3 <sup>c</sup>	2''		144.5 <sup>b</sup>
3		140.5	3'		166.1	3''		140.4
4		149.5	4'		127.1	4''		149.2
5	7.19 s	106.1	5'	8.35 d (2.0)	137.6	5''	7.14 s	106.1
6		123.2 <sup>a</sup>	6'		130.5 <sup>c</sup>	6''		123.9 <sup>d</sup>
7		167.40	7'	7.79 d (2.0)	139.3	7''		167.45
4-OMe	3.96 s	56.80	8'		200.8	4''-OMe	3.93 s	56.75
7-OMe	3.66 s	52.6	9'	2.72 s	31.1	7''-OMe	3.58 s	52.4
			10'		195.9			

Values in parentheses are coupling constants in Hz. <sup>a-d</sup>Assignments may be interchanged.

The remaining structure of **3** corresponded to  $\text{C}_{10}\text{H}_6\text{O}_4$ . The  $^{13}\text{C}$ -NMR and DEPT spectra revealed that ten carbons comprised a methyl ( $\delta_{\text{C}}$  31.1), two aromatic CH carbons, four aromatic quaternary carbons

including a hydroxylated carbon, and three carbonyls. These carbon signals were assigned by correlations in the HSQC, HMBC, and NOESY spectra as follows: the methyl signal at  $\delta_{\text{H}}$  2.72 showed HMBC correlations with a carbonyl at  $\delta_{\text{C}}$  200.8 and a quaternary carbon at  $\delta_{\text{C}}$  127.1, indicating substitution of an acetyl group on the benzene ring. A *meta*-coupled aromatic proton at  $\delta_{\text{H}}$  8.35 showed an HSQC correlation with a carbon signal at  $\delta_{\text{C}}$  137.6 (C-5') and HMBC correlations with another CH carbon at  $\delta_{\text{C}}$  139.3 (C-7'), two carbonyl carbons at  $\delta_{\text{C}}$  200.8 (C-8') and  $\delta_{\text{C}}$  195.9 (C-10'), and an oxygenated aromatic carbon at  $\delta_{\text{C}}$  166.1 (C-3') and NOESY correlation with the methyl signal at  $\delta_{\text{H}}$  2.72; another *meta*-coupled methine proton at  $\delta_{\text{H}}$  7.79 showed an HSQC correlation with C-7' and HMBC correlations with C-3', C-5', and two carbonyl carbons at  $\delta_{\text{C}}$  195.9 (C-10') and 202.6 (C-1'), which confirmed the substitution pattern of the benzene ring. Each of two carbonyl groups C-1' and C-10' could be connected to the partial structure, 2,3-dihydroxy-4-methoxy-6-methoxycarbonylphenyl group by significant 4-bond HMBC correlations from H-5 to C-1' and from H-5'' to C-10'. Thus, the structure of compound **3** was determined as shown and designated graphisidin. The related metabolites with benzophenone moieties, disulochrin<sup>12</sup> and acremonidin E<sup>13</sup> were isolated from the fermentation of fungi.

For the genus *Graphis*, our previous cultivation and chemical investigations have been carried out on *G. scripta* var. *pulverulenta*,<sup>5</sup> *G. scripta*,<sup>14,15</sup> *G. prunicola*,<sup>14</sup> *G. cognata*,<sup>14</sup> *G. scripta* var. *serpentina*,<sup>16</sup> *G. rikuzensis*,<sup>16</sup> *G. apriens*,<sup>17</sup> *G. handelii*,<sup>17</sup> *G. awaensis*,<sup>17</sup> *G. proserpens*,<sup>8</sup> *G. vestitoides*<sup>18</sup> and unidentified *Graphis* sp.,<sup>19</sup> resulting in the isolation of 6*H*-dihydro[*b,d*]pyran-6-one derivatives, isocoumarins, thiophene derivative, chromones, phenyl ethers, 7-oxo-5,7-dihydrooxepino[4,3,2-*de*]isochromenes, and a 14-membered macrolide. In this study, we have cultivated the spore-derived mycobionts of an unidentified *Graphis* sp. collected in Vietnam and isolated two new compounds, 3,4-dihydro-2*H*,5*H*-pyrano[3,2-*c*]chromene derivative **2** and benzophenone **3**. These types of metabolites have never been detected in the thalli of lichens and lichen mycobionts. The present study constitutes an example to show the diversity of metabolites produced by the cultured mycobionts of *Graphis* sp.

## EXPERIMENTAL

**General Procedures.** The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. The optical rotation was 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 tetramethylsilane as an internal standard. HPLC was performed using a Shimadzu system (LC-10AD *VP* Liquid Chromatograph, SPD-10A *VP* UV-VIS Detector). Thin-layer chromatography was performed on pre-coated Kieselgel 60F254 plates (Merck), and spots were visualized under UV light.

**Plant Material.** Specimens of *Graphis* sp. were collected from the bark of trees in Ma Da Forest, Dong Nai Province (ca 90 m alt.) in Sept. 2008. The voucher specimens were identified by Prof. H. Miyawaki, Saga University, and were deposited at Saga University, Japan, with the registration No. V62. Mycobionts were obtained from spores discharged from apothecia of a thallus and were cultivated in test tubes containing modified MY10 medium (malt extract 10 g, yeast extract 4 g, sucrose 100 g, agar 15 g, H<sub>2</sub>O 1 l, pH 7) at 18 °C in the dark for 14 months.

**Isolation of Metabolites.** The harvested colonies (131 test tubes, freeze-dried weight 68 g) were extracted with Et<sub>2</sub>O, acetone and then with MeOH at room temperature. The Et<sub>2</sub>O extract (72 mg) and acetone extract (404 mg) were mainly mixtures of fatty acids and glycerides. After concentration, the MeOH extract was suspended in H<sub>2</sub>O and extracted with *n*-BuOH. The *n*-BuOH extract (1.838 g) was chromatographed on a Wakosil 40C18 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) column (H<sub>2</sub>O-MeOH). The eluate with 30% MeOH was concentrated and the residue was then repeatedly subjected to preparative TLC (CHCl<sub>3</sub>-MeOH, 4:1) and preparative HPLC ( $\mu$ Bondasphere 5 $\mu$ C18-100Å, H<sub>2</sub>O-MeCN, 4:1, 13:7 or 3:2), giving rise to **1** (36.6 mg), **2** (5.8 mg) and **3** (20.9 mg).

**(S)-(-)-8-Methoxy-2-methyl-4-oxo-3,4-dihydro-2H,5H-pyrano[3,2-c]chromene-10-carboxylic acid (2):**  $[\alpha]_D^{29}$  -18° (*c* 0.57, MeOH); UV (MeOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ): 211 (3.96), 230 sh (3.86), 250 (3.76), 309 (3.64), 357 (3.75); IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3451, 1711, 1642, 1598; <sup>1</sup>H-NMR (CD<sub>3</sub>OD):  $\delta$  1.51 (3H, d, *J* = 6.0 Hz, H<sub>3-1</sub>), 2.49 (1H, dd, *J* = 17.0, 4.5 Hz, H-3), 2.54 (1H, dd, *J* = 17.0, 12.5 Hz, H-3), 3.82 (3H, s, 11-OCH<sub>3</sub>), 4.68 (1H, dqd, *J* = 12.5, 6.0, 4.5 Hz, H-2), 4.69, 5.07 (each 1H, d, *J* = 12.5 Hz, H<sub>2-6</sub>), 6.43 (1H, d, *J* = 2.5 Hz, H-10), 6.61 (1H, d, *J* = 2.5 Hz, H-12); <sup>13</sup>C-NMR (CD<sub>3</sub>OD):  $\delta$  20.4 (C-1), 43.7 (C-3), 56.3 (OCH<sub>3</sub>), 63.9 (C-6), 77.5 (C-2), 101.9 (C-10), 103.7 (C-5), 108.1 (C-13), 109.2 (C-12), 143.1 (C-8), 162.4 (C-9), 165.7 (C-11), 166.3 (C-7), 176.0 (COOH), 191.6 (C-4); HR-ESIMS *m/z*: Calcd for C<sub>15</sub>H<sub>15</sub>O<sub>6</sub> [M+H]<sup>+</sup>: 291.0869. Found : 291.0867.

**Graphisidin (3):** UV (EtOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ): 217 (4.66), 238 (4.57), 276 (4.33), 340 (3.88); IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3384, 1711, 1686, 1612, 1581; <sup>1</sup>H- and <sup>13</sup>C-NMR: spectroscopic data see Table 1; HR-ESIMS *m/z*: Calcd for C<sub>28</sub>H<sub>25</sub>O<sub>14</sub> [M+H]<sup>+</sup>: 585.1245. Found: 585.1233.

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