A 14-Membered Macrolide and Isocoumarin Derivatives from the Cultured Lichen Mycobionts of Graphis vestitoides

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Spore-derived mycobionts of the crustose lichen Graphis vestitoides 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 novel 14-membered macrolide and a new isocoumarin, together with five known isocoumarin derivatives. Their structures were determined by spectroscopic and chemical means. This is the first instance of isolation of a 14-membered macrolide from a lichen mycobiont.

Key words Graphis vestitoides; lichen; mycobiont; 14-membered macrolide; isocoumarin

Lichens, symbiotic associations between fungi (mycobionts) and photoautotrophic algae or cyanobacteria (photobionts), produce a wide range of characteristic secondary metabolites, namely, lichen substances, some of which are potentially useful and biologically active compounds.1–3) Most of these metabolites are produced by the fungus, in symbiosis or in the aposymbiotic state. However, previous studies demonstrated that the secondary metabolites in cultured mycobionts are often structurally unique and similar to fungal metabolites.4–6) These findings suggested that laboratory cultures of lichen mycobionts could provide a potential source of novel secondary metabolites to be produced for pharmaceutical purposes. In continuing our chemical studies on the cultured mycobionts of lichens,7) we cultivated the spore-derived mycobionts of Graphis vestitoides collected in Vietnam and isolated a novel 14-membered macrolide and a new isocoumarin as well as five known isocoumarin derivatives from their cultures. In this paper, the isolation and characterization of these new metabolites are described.

Results and Discussion

Specimens of Graphis vestitoides (Fink) Staiger were collected from the bark of trees in Dong Nai Province, Vietnam, in 2008. The polyspore-derived mycobionts were cultivated on a malt-yeast extract medium supplemented with 10% sucrose at 18°C in the dark. After three months, the cultures were harvested and extracted with Et2O, acetone and then with MeOH. Subsequent purification of the extracts by a combination of column chromatography, preparative TLC and HPLC gave new compounds 1 and 2, together with five known compounds: 6,8-dihydroxy-3-(hydroxymethyl)isocoumarin (3),8) 4,6-dihydroxy-3,9-dehydromellein,9) cis-4,6-dihydroxymellein,10,11) trans-5,7-dihydroxy-3-(1-hydroxyethyl)phthalide12) and cis-5,7-dihydroxy-3-(1-hydroxyethyl)phthalide.12)

Compound 1 was isolated as a white crystalline solid, mp >300°C. The high resolution electrospray ionization mass spectrometry (HR-ESI-MS) of 1 established the molecular formula of C14H18O5. It showed UV maxima at 251.5, 289, 300, 324.5 and 336.5 nm, and IR bands at 3393, 3245, 1679, 1637 and 1609 cm−1, representing the hydroxy and carbonyl groups and an aromatic ring. Its 1H-NMR spectrum showed the signals for a pair of meta-coupled aromatic protons at δ 6.43 and 6.59 (each 1H, d, J = 2.0 Hz), and an olefinic proton at δ 7.23 (s). The 13C-NMR spectrum exhibited the signals for three sp3 CH carbons and seven sp2 quaternary carbons including three oxygenated and two carbonyl carbons. The 1H-detected heteronuclear multiple-bond connectivity (HMBC) correlations from an olefinic proton (H-4) to three carbon signals at δ 99.0 (C-8a), 138.8 (C-4a) and 150.0 (C-3), from an aromatic proton (H-5) to carbon signals at δ 108.5 (C-4), 165.8 (C-6) and C-8a, and from another aromatic proton (H-7) to C-6, C-8 (δ 162.5) and C-8a (Fig. 2), as well as a nuclear Overhauser effect spectroscopy (NOESY) cross peak between H-4 and H-5 suggested a 3,6,8-substituted isocoumarin ring system as in 6,8-dihydroxy-3-(hydroxymethyl)isocoumarin (3), a major constituent of this culture. The downfield resonance of H-4 and C-4 relative to those of 3 and HMBC correlation from H-4 to a carboxyl carbon at δ 161.8 indicated that 1 possessed a carboxyl group at C-3 instead of a hydroxymethyl group as in 3. These spectral features were similar to those of 6-methoxy-8-hydroxisocoumarin-3-carboxylic acid (4),13) except for the absence of the methoxy group in 1. Methylation of 1 with TMS-CH2N2 afforded 5, which demonstrated the signals for three methoxyl groups at δ 3.93, 3.94 and 3.99 in the 1H-NMR spectrum. Accordingly, the structure of 1 was characterized as 6,8-dihydroxyisocoumarin-3-carboxylic acid.

Compound 2 was obtained as a colorless solid. The molecular formula of 2 was established by HR-ESI-MS as C14H14O5, implying six degrees of unsaturation. The presence of hydroxy and carbonyl groups as well as double bonds was shown by IR absorption bands at 3423, 1710 and 1598 cm−1. The 1H-NMR spectrum of 2 exhibited the signals for eight methylene protons, six olefinic methine protons and two oxygenated methine protons at δ 4.70 (brd, J = 9.0 Hz) and 5.24 (m) (Table 1). The 13C-NMR spectrum of 2 showed 14 signals, which were assigned by distortionless enhancement by polarization transfer (DEPT) experiments as four methylenes, two oxygenated methines and eight sp2 carbons including six CH carbons and
two carbonyl carbons at δ 168.2 and 177.5 (Table 1). The sequence from olefinic proton at δ 5.83 to oxygenated methine proton at δ 4.70 was revealed by a series of 1H–1H shift correlation spectroscopy (1H–1H COSY) correlations as drawn in bold lines and was further defined by the HMBC correlations as shown in Fig. 2. Furthermore, significant HMBC correlations from olefinic protons H-3 and H-4 (δ 7.42) to a carbonyl carbon (C-2, δ 168.2), and from oxygenated methine proton H-14 to C-2 and a carboxyl carbon (C-15, δ 177.5) established a 14-membered lactone ring with a carboxyl group at C-14. The presence of a hydroxy group at C-5 was deduced from the molecular formula and characteristic chemical shifts of H-5 (δ 5.24) and C-5 (δ 68.8). The geometry of the double bonds C-3/C-4, C-6/C-7 and C-9/C-10 was assigned as E, Z and E from the 1H–1H coupling constants of 15.5, 11.0 and 15.0 Hz, respectively. These spectral data demonstrated that 2 was closely related to mutolide (6), a fungal macrolide isolated from the culture broth of the fungus Sphaeropsidales sp. (strain F-24707), but except that 6 possessed a hydroxyl group at C-5, a methyl group at C-14 and an E configuration of double bond C-6/C-7. Accordingly, the planar structure of 2 was elucidated and designated graphilide.

The absolute configuration of C-14 in 2 was determined by the 1H-NMR analyses of its (R)- and (S)-phenylglycine methyl esters (PGME) amides. Treatment of 2 with (S)- and (R)-PGME in N,N-dimethylformamide (DMF) in the presence of benzotriazololxytr(pyrrolinyl)phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBT) and Et3N yielded the corresponding PGME amides 7 and 8, respectively. The differences in the chemical shifts Δδ (δS−δR) of the two amides led to the assignment of the S configuration at C-14 of 2 (Fig. 3).

To determine the absolute configuration of C-5, methylation of compound 2 followed by esterification with (R)- and (S)-methoxyphenylacetic acids (MPA) was undertaken. However, treatment of 2 with TMS-CHN2 failed to yield the desired methyl ester 9, but unexpectedly gave 10. The high resolution atmospheric pressure chemical ionization mass spectrometry (HR-APCI-MS) of 10 established the composition of C15H20O5, which was 14 mass units more than that of 2. The 1H- and 13C-NMR spectra of 10 showed signals for an oxygenated methine (δH 3.71, δC 49.2) and a methylene (δH 3.73, 4.10, δC 50.1), but no signals due to C-4/C-5 double bond as seen in 2. The 1H- and 13C-NMR spectra of 10 showed signals for an oxygenated methine (δH 3.71, δC 49.2) and a methylene (δH 3.73, 4.10, δC 50.1) in addition to a methoxy group (δH 3.75, δC 52.4), but no signals due to C-4/C-5 double bond as seen in 2. The HMBC experiments with 10 as well as its molecular formula indicated the formation of an epoxy ring between C-4 and C-5. The relative stereochemistry of the epoxy group was assumed to be cis from the large coupling constant (J=4.0Hz) between the vicinal protons H-4 and H-5. The formation of a cis epoxy ring was accounted for by a Michael-type addition of C-5 hydroxyl group to the double bond at C-4 in the favorable conformation of 9.

In a further effort to determine the absolute configuration of C-5, the amide 7 was esterified with (S)-MPA acid in dry CH2Cl2 in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and 4-pyrrolidinopyridine (PP) to yield (S)-MPA ester 11 but not the expected ester 12. The 1H- and 13C-NMR spectra of 11 showed the presence...
of a methylene group (C-3), an olefinic carbon (C-4) and an oxygenated sp² quaternary carbon (C-5) instead of two olefinic carbons and a hydroxyl methine as seen in 7. The assignments were clarified by HMBC correlations from H₂-3 to C-2, C-4 and C-5 and from H-4 to C-2, C-5 and C-6. The formation of 12 indicated that the core structure of 12 had undergone double-bond rearrangement in the same manner as occurred during the acylation of a 12-membered macrolide. Therefore, the absolute configuration at C-5 of 2 still remains to be elucidated.

In conclusion, the metabolites of the cultivated mycobiont colonies from G. vestitoides collected in Vietnam were investigated to isolate a novel 14-membered macrolide and six isocoumarin-related compounds, one of which was new. Isocoumarins have been detected in cultured lichen mycobionts. Several 14-membered macrolides were isolated from original marine sources and fungal origins, but have not been isolated from lichen thalli or cultured mycobionts. This is the first instance of isolation of a 14-membered macrolide from a lichen mycobiont.

Table 1. ¹H- (500 MHz) and ¹³C- (125 MHz) NMR Spectroscopic Data for 2 and 10

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<th>No.</th>
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<td>δH (J, Hz)</td>
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<td>4</td>
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<td>5</td>
<td>68.8</td>
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<td>6</td>
<td>133.6</td>
<td>5.71 br dd (11.0, 9.5)</td>
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<td>7</td>
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<tr>
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<td>9</td>
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(a) Measured in CD₃OD. (b) Measured in CDCl₃.

Fig. 3. Values of Δδ (δS−δR) Obtained from 7 and 8.
41.5 mg; acetonitrile extract: 12.8 mg) were fatty compounds. The more polar fractions around Rf/0.4 (Et2O extract: 48.9 mg; acetonitrile extract: 42.5 mg) were combined and repeatedly purified by preparative TLC (n-hexane–Et2O, 1:9; n-hexane–Et2O–AcOH, 40:60:1; CHCl3–MeOH, 9:1) to yield 3 (41.4 mg) and 4,6-dihydroxy-3,9-dehydrodelmelline (29.3 mg).

The MeOH extract was suspended in H2O and extracted with n-BuOH. The n-BuOH extract (2.46 g) was subjected to silica gel column chromatography and eluted by solvent system CHCl3–MeOH with increasing MeOH content to give three fractions, fr-I (CHCl3, 97.6 mg), fr-II (1% MeOH, 659 mg) and fr-III (1.2% MeOH, 125 mg). Fr-I was a mixture of fatty compounds. Fr-II was separated by preparative TLC (Et2O; CHCl3–MeOH, 8:2 or 6:4); giving 2 (57.0 mg), 3 (169.7 mg), 4,6-dihydroxy-3,9-dehydrodelmelline (173.3 mg) and cis-4,6-dihydroxydelmelline (2.3 mg). Fr-III was further purified by preparative TLC (Et2O; CHCl3–MeOH, 9:1 or 8:2) and preparative HPLC (µBondapak C18 5 µ, H2O–MeOH, 6:4; H2O–CH3CN, 9:1), giving 1 (20.3 mg), 3 (7.3 mg), trans-5,7-dihydroxy-3-(1-hydroxymethyl)phthalide (24.7 mg) and cis-5,7-dihydroxy-3-(1-hydroxymethyl)phthalide (7.7 mg).

6,8-Dihydroxyisorocoumarin-3-carboxylic Acid (1): White crystalline solid, mp >300°C (MeOH); UV (EtOH) λmax (log ε): 251.5 (4.48), 289 (3.67), 300 (3.67), 324.5 (3.70), 336.5 (3.75); IR (KBr) νmax cm⁻¹: 3393, 3245, 1679, 1637, 1609, 1498, 1391, 1327, 1240, 1181, 801, 689; ¹H-NMR (DMSO-d₆, 500 MHz) δ: 6.43 (1H, d, J=2.0 Hz, H-7), 6.59 (1H, d, J=2.0 Hz, H-5), 7.23 (1H, s, H-4); ¹H-NMR (CDCl3, 300 MHz) δ: 6.44 (1H, d, J=2.1 Hz, H-7), 6.53 (1H, d, J=2.1 Hz, H-5), 7.32 (1H, s, H-4); ¹C-NMR (DMSO-d₆, 125 MHz) δ: 99.0 (C-8a), 102.6 (C-7), 104.4 (C-5), 108.5 (C-4), 138.8 (C-4a), 150.0 (C-3), 161.8 (C-2, C-9), 162.5 (C-1), 165.5 (C-6); HR-ESI-MS m/z: 221.0085 (Calcd for C₇H₇O₃; 221.0086 [M+H]⁺).

Graphilide (2): Colorless solid; [α]D²⁰ +121° (c=1.06, MeOH); UV (EtOH) λmax (log ε): 215 sh (3.91); IR (KBr) νmax cm⁻¹: 3423, 1710, 1598, 1414, 1268; ¹H- and ¹³C-NMR spectroscopic data see Table 1; HR-ESI-MS m/z: 265.1084 (Calcd for C₁₅H₁₄O₆, 265.1077 [M+H]⁺).

Methylation of 1 To a solution of 1 (3.0 mg) in MeOH (1 mL) was added TMS-CH3Cl in n-hexane (0.20 mL), and the mixture was stirred at room temperature for 70 min. After termination by diluted acetic acid in MeOH, the reaction mixture was concentrated in vacuo and the residue was purified by preparative TLC (CHCl3–MeOH, 95:5) to yield methylated compound 5 (1.8 mg). ¹H-NMR (CDCl3, 300 MHz) δ: 3.93 (3H, s, –OCH3), 3.94 (3H, s, –OCH3), 3.99 (3H, s, –COOCH3), 6.56 (1H, d, J=2.1 Hz, H-7), 6.60 (1H, d, J=2.1 Hz, H-5), 7.31 (1H, s, H-4); HR-ESI-MS m/z: 265.0704 (Calcd for C₁₅H₁₄O₆; 265.0713 [M+H]⁺)

Preparation of (R)- and (S)-PMG Amides from 2 To a stirred solution of a mixture of 2 (2.0 mg) and (S)-PMG (5.0 mg) in DMF (1.0 mL) were successively added PyBOP (12.0 mg), HOBT (3.0 mg) and Et3N (10 µL) at 0°C. After the mixture was stirred at room temperature for 3 h, CHCl3 (15 ml) was added and the resulting diluted solution was successively washed with 1 equiv HCl and brine. The organic phase was dried (Na2SO4) and concentrated to give a residue that was preparative TLC (Et2O–EtOAc, 5:1) to afford 7 (1.0 mg). Compound 2 (2.0 mg) was treated with (R)-PMG (5.0 mg) as described above to yield 8 (0.9 mg).
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References