Four Phenolics from the Cultured Lichen Mycobiont of *Graphis scripta* var. *pulverulenta*

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From the cultures of the spore-derived mycobionts of the lichen *Graphis scripta* var. *pulverulenta*, four phenolic compounds, graphislactones A—D, were isolated. Their structures were determined by spectroscopic methods. This is the first instance of the isolation of 6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives from the lichen mycobiont.

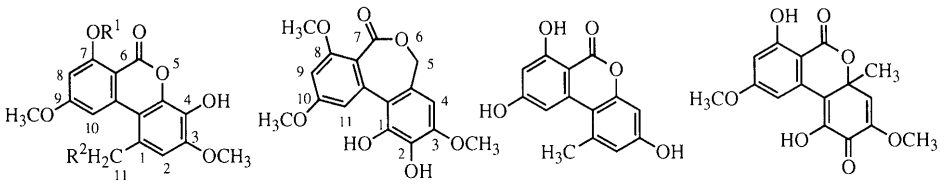
Key words *Graphis scripta* var. *pulverulenta*; lichen; isolated mycobiont; 6*H*-dibenzo[*b*,*d*]pyran-6-one derivative; graphislactone

Lichens, several of which have important medicinal usages as crude drugs, produce a variety of characteristic secondary metabolites. Some lichen substances have been found to exhibit a wide range of potentially useful biological activities, *e.g.*, antibiotic and anticancer activities, and monoamine oxidase inhibitory effect.[1] Recently, cultures of spore-derived lichen mycobionts were shown to be capable of producing certain lichen substances or novel metabolites in large amounts under osmotically stressed conditions.[2] In the course of our search for new bioactive compounds, we cultivated the spore-derived mycobiont *Graphis scripta* var. *pulverulenta* and isolated four phenolics from its cultures. In this paper, we report the isolation and characterization of these new compounds.

Specimens of *Graphis scripta* (L.) Ach. var. *pulverulenta* Ach. were collected from the bark of trees at Mt. Koya, Wakayama Prefecture, Japan. No depsidone was detected in the thallus. The polyspore-derived mycobionts were cultured on the conventional malt-yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After 7 months, the cultivated colonies were harvested and extracted with cold acetone. Subsequent purification of the extract by preparative TLC afforded four compounds, **1**, **2**, **3** and **4** in respective amounts equivalent to 0.20, 0.087, 0.14 and 0.050% of the fresh weight of the cultures.

Compound **1**, called graphislactone A, was isolated as a colorless crystalline solid. The HR-EI mass spectrum of **1** exhibited a strong peak at *m/z* 302.0794 $(M)^+$, indicating a molecular formula of $C_{16}H_{14}O_6$ for **1**. It showed IR bands at 3452, 1659, 1626, 1605 and 1574 cm^{-1}, suggesting the presence of hydroxyl group(s), a chelated carbonyl group and substituted aromatic system(s). Its ^{1}H-NMR spectrum exhibited signals for a methyl group at δ 2.69 (s), two methoxy groups at δ 3.89 and 3.90 (each s), a pair of meta-coupled aromatic protons at δ 6.60 and 7.20 (each d, J = 2.0 Hz), an aromatic proton at δ 6.91 (s), a phenolic hydroxyl group at δ 9.24 and a chelated phenolic hydroxyl group at δ 11.88. The NOESY spectrum showed through-space connectivities between the OMe at δ 3.90 and two doublets resonating at δ 6.60 and 7.20, between the OMe at δ 3.89 and a singlet resonating at δ 6.91, and between the CMe and a doublet (δ 7.20)/a singlet (δ 6.91). The ^{13}C-NMR spectrum of **1** (Table 1) showed sixteen carbon resonances, almost all of which were assigned by heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) (Fig. 1) experiments with **1** as well as comparison of ^{13}C-NMR data with a fungal metabolite alternariol (**5**).[3] These observations allowed us to depict the structure **1** for the isolated compound. Our supposition was finally confirmed by the fact that the spectral data of graphislactone A were in good accordance with those described for a chemically reduced product (**1**) of botrallin (**6**), a metabolite of *Botrytis allii*.[4] This constitutes the first instance of the isolation of **1** as a natural product.

The EI-MS of the second new compound, graphislactone B (**2**), showed $(M)^+$ at *m/z* 316, indicating an increase of 14 mass units more than **1**. A comparison of the ^{1}H- and ^{13}C-NMR spectra of **2** with those of **1** suggested a close relationship between their structures. The ^{1}H-NMR spectral features of **2** resembled those of **1** except for the absence of a chelated phenolic hydroxyl group and the presence of an additional methoxy signal. These findings



1: $R^1 = R^2 = H$
2: $R^1 = CH_3$, $R^2 = H$
3: $R^1 = H$, $R^2 = OH$

Chart 1

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Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1**, **2**, **3** and **5** in DMSO-d_6

C	**1**		**2**		**3**		**5**[a]
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_C
1	—	126.1	—	125.7	—	129.3	138.3
2	6.91 s	112.9	6.88 s	112.3	7.16 s	112.0	117.6
3	—	148.1	—	148.0	—	148.2	158.4
4	—	140.5	—	140.1[d]	—	140.5	101.6
4a	—	137.9	—	141.6[d]	—	137.0	152.6
6	—	164.4	—	156.0	—	164.4	164.7
6a	—	98.4	—	102.3	—	98.7	97.4
7	—	164.0	—	163.5	—	163.8	164.1
8	6.60 d[b]	99.4	6.75 d[b]	97.8	6.64 d[b]	100.0	100.9
9	—	165.9	—	164.5	—	166.5	165.5
10	7.20 d[b]	103.7	7.30 d[b]	102.7	7.60 d[b]	104.7	104.4
10a	—	132.2	—	131.6	—	133.5	138.1
10b	—	110.4	—	110.8	—	111.3	109.0
11	2.69 s	24.6	2.75 s	24.6	4.77 s	63.2	25.3
3-OMe	3.89 s	55.9[c]	3.87 s	56.0	3.91 s	56.1[e]	
7-OMe	—	—	3.92 s	56.2	—	—	
9-OMe	3.90 s	55.7[c]	3.96 s	55.7	3.92 s	55.9[e]	
OH	9.24 br 11.88 br s		9.05 br s		5.65 br 9.47 br 11.75 br s		

a) Data taken from Ref 3. *b*) $J=2.0$ Hz. *c*—*e*) Values with the same superscript are interchangeable.

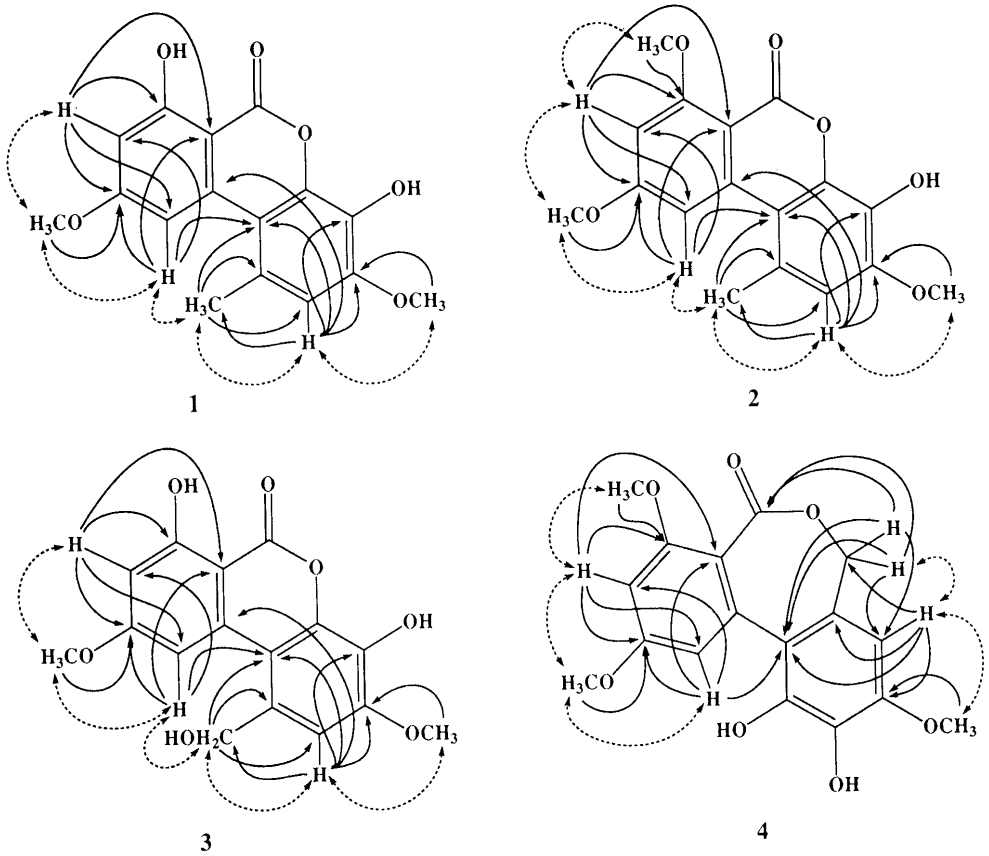

Fig. 1. HMBC Correlations (Bold Arrows) and NOEs (Dotted Arrows) Observed for Compounds **1**—**4**

suggested that the hydroxyl group at C-7 was methylated in **2**, and this received further support from an IR band at 1703 cm^{-1} ascribed to a non-chelated carbonyl group along with the upfield shift of C-6 and downfield shift of C-6a in the ^{13}C-NMR spectrum. The placement of the methoxyl groups at C-3, C-7 and C-9 was substantiated by its NOESY spectrum, which showed cross-peaks between H-2 and OMe at δ 3.87, between H-8 and OMe at δ 3.92, and between H-8 and OMe at δ 3.96 (Fig. 1). Thus, the structure of graphislactone B was elucidated as shown.

The mass spectrum of graphislactone C (**3**) established the composition $C_{16}H_{14}O_7$. The UV, IR and NMR spectral features of **3** were closely similar to those of

graphislactone A (**1**), the significant difference in their ^{1}H-NMR spectra being that **3** showed a two-proton singlet at δ 4.77 and an aliphatic hydroxyl group at δ 5.65 due to a hydroxymethyl group at C-1 instead of a methyl group as in **1**. The ^{13}C-NMR data of **3** were nearly identical with those of **1** except for the presence of the hydroxymethyl carbon signal resonating at δ 63.2 and downfield shift of C-1 relative to that of **1**. The positioning of the substituents on aromatic rings was established to be the same as in **1** by HMBC and NOESY correlations (Fig. 1). Accordingly, the compound was characterized as graphislactone C (**3**).

Graphislactone D (**4**) was a labile compound with the molecular formula $C_{17}H_{16}O_7$. Its ^{13}C-NMR spectrum displayed an oxygenated CH_2 in addition to three methoxyls, twelve aromatic carbons and a carbonyl carbon. When the ^{1}H-NMR data of **4** were compared with those of **3**, there were marked differences in the methylene signals, which appeared as a pair of doublets at δ 4.74 and 4.78 in **4** instead of as a two-proton singlet in **3**. This could be accounted for by assuming that the methylene protons are situated on a ring between two aromatic nuclei and their non-equivalence must result from a steric factor of the biphenyl system. An HMBC ^3J correlation of the methylene protons with the carbonyl carbon suggested that the carbon was not connected to the phenolic hydroxyl group as in **3**, but linked to the hydroxymethyl to construct a new seven-membered lactone ring in **4** (Fig. 1). The suggestion led us to situate the oxygenated substituents at C-1, C-2 and C-3. The location of a methoxyl at C-3 was determined by NOESY correlations between H-4 at δ 6.78 and methylene protons/OMe at δ 3.84, whereas the remaining two methoxyl groups were placed at C-8 and C-10 on the basis of HMBC and NOESY experiments. Consequently, the residual oxygenated substituents at C-1 and C-2 should be phenolic hydroxyl groups. Thus, the structure of graphislactone D was determined as **4** with a new basic skeleton.

This is the first instance of the isolation of 6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives from lichen mycobionts. Until now, this type of metabolites, as represented by alternariol (**5**), had been isolated from fungus *Alternaria tenuis* as mycotoxins[5] but not from lichen, a symbiotic association of mycobiont and phycobiont partners. The occurrence of graphislactones A—D in the cultures of lichen mycobionts is of great interest from the viewpoint of their physiological and biological significance, and may account for a better chance of survival of the mycobiont in pre-lichenized condition.

Experimental

Melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 IR spectrophotometer. HR-EI-MS were obtained with a Hitachi M-4100 mass spectrometer. The NMR experiments were performed with a Varian VXR-500 spectrometer with tetramethylsilane as an internal standard. TLC was performed on pre-coated Kieselgel $60F_{254}$ plates (Merck), and spots were visualized under UV light.

Plant Material Specimens of *Graphis scripta* (L.) Ach. var. *pulverulenta* Ach. were collected from the bark of trees at Mt. Koya, Wakayama Prefecture, Japan (35 °N, *ca.* 900 m alt.) in October 1993. The voucher specimen was identified by Prof. M. Nakanishi of Hiroshima University, Japan and was deposited at Osaka City Institute of Public Health and Environmental Sciences with the registration No. NH931038. No depsidone was detected by TLC in the thallus used in this study. Mycobionts of *G. scripta* var. *pulverulenta* were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 69 test tubes containing modified MY10 medium (malt extract 10 g, yeast extract 4 g, sucrose 100 g, agar 15 g, H_2O 1 l, pH 7) at 18 °C in the dark. Brown and elevated compact colonies with white crystals which covered large areas of colonies and surrounding agar were found in each test tube. After cultivation for 7 months, the colonies and slants with crystals were harvested.

Isolation of Compounds The harvested colonies (fresh weight 18.5 g) were extracted with acetone at room temperature 4 times for 6 h each, and the combined extracts were concentrated under reduced pressure to give a residue (1.64 g). The slants with white crystals were collected and extracted in the same way to give a residue (50.1 g). The respective residues were suspended in H_2O and the resulting precipitates were collected by filtration. The filtrate was continuously extracted with Et_2O. The Et_2O extract and the precipitates, which contained phenolic compounds, were combined and repeatedly subjected to preparative TLC with $CHCl_3$–MeOH (9 : 1), AcOEt–C_6H_6–EtOH (4 : 1 : 0.5), or C_6H_6–acetone (3 : 1), giving rise to **1** (36.7 mg), **2** (16.1 mg), **3** (25.4 mg) and **4** (9.2 mg).

Graphislactone A (1) Colorless crystalline solid, mp 236—237 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 205 (4.19), 227 sh (4.31), 236 (4.38), 259 (4.47), 289sh (3.94), 296 (3.90), 328sh (3.85), 338 (3.87). IR ν_{max}^{KBr} cm^{-1}: 3452, 1659, 1626, 1605, 1574. ^{1}H- and ^{13}C-NMR: Table 1. HR-EI-MS *m*/*z*: Calcd for $C_{16}H_{14}O_6$ $(M)^+$: 302.0791. Found: 302.0794.

Graphislactone B (2) Colorless crystalline solid, mp 65—67 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 205 (4.27), 228 sh (4.30), 236 (4.32), 259 (4.34), 290 sh (3.85), 298 (3.82), 332 (3.80). IR ν_{max}^{KBr} cm^{-1}: 3427, 1703, 1618, 1606, 1587, 1572, 1520. ^{1}H- and ^{13}C-NMR: Table 1. HR-EI-MS *m*/*z*: Calcd for $C_{17}H_{16}O_6$ $(M)^+$: 316.0947. Found: 316.0965.

Graphislactone C (3) Colorless crystalline solid, mp 214—216 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 204 (4.21), 227 sh (4.40), 236 (4.49), 260 (4.48), 288 (3.98), 299 (3.96), 333 (3.95), 342 (3.95). IR λ_{max}^{KBr} cm^{-1}: 3429, 1668, 1616, 1591, 1574. ^{1}H- and ^{13}C-NMR: Table 1. HR-EI-MS *m*/*z*: Calcd for $C_{16}H_{14}O_7$ $(M)^+$: 318.0740. Found: 318.0746.

Graphislactone D (4) Colorless crystalline solid, mp 110—112 °C (MeOH). UV λ_{max}^{MeOH} nm (log ε): 209 (4.22), 236 (4.25), 254 sh (4.18), 278 sh (3.83), 303 sh (3.69). IR ν_{max}^{KBr} cm^{-1}: 3440, 1717, 1603, 1570. ^{1}H-NMR (DMSO-d_6): δ 3.81 (3H, s, 8-OMe), 3.82 (3H, s, 10-OMe), 3.84 (3H, s, 3-OMe), 4.74, 4.78 (each 1H, dd, J = 12.0 Hz, H_2-5), 6.66 (1H, d, J = 2.0 Hz, H-9), 6.78 (1H, s, H-4), 6.89 (1H, d, J = 2.0 Hz, H-11), 8.98, 9.10 (each 1H, br, 2 × OH). ^{13}C-NMR (DMSO-d_6): δ 55.3[a] (8-OCH_3), 55.8[a] (10-OCH_3), 56.0[a] (3-OCH_3), 68.3 (C-5), 97.7 (C-9), 103.9 (C-4), 106.8 (C-11), 113.2 (C-7a), 118.2 (C-11b), 127.3 (C-11a), 135.1 (C-4a), 135.7[b] (C-2), 143.8[b] (C-1), 147.6 (C-3), 158.6 (C-8), 160.6 (C-10), 166.1 (C-7). *a*, *b*) Values with the same superscript are interchangeable. HR-EI-MS *m*/*z*: Calcd for $C_{17}H_{16}O_7$ $(M)^+$: 332.0897. Found: 332.0892.

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