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2,3-DIALKYLCHROMONES FROM MYCOBIONT CULTURES OF THE LICHEN *GRAPHIS SCRIPTA*

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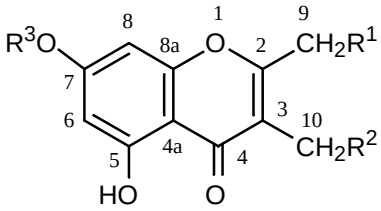
Abstract --- From the cultures of spore-derived mycobionts of lichen *Graphis scripta*, three chromones, 5-hydroxy-2,3-dimethyl-7-methoxychromone (**1**), 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone (**2**), 5-hydroxy-2-hydroxymethyl-3-methyl-7-methoxychromone (**3**), were isolated. Compound (**1**) has been characterized as a natural product for the first time. The structures of two new chromones (**2**) and (**3**) were established on the basis of spectroscopic evidences. This is the first instance of isolation of 2,3-dialkylated chromones from lichen mycobionts.

One characteristic of lichens, a symbiotic association, is the production of diverse secondary metabolites, some of which show a wide range of potentially useful biological activities.[1] An intriguing question concerning the formation of lichen substances is the role of mycobiont and photobiont partners in the biosynthesis of these substances. In some cases, the formation of lichen substances has definitely been ascribed to the mycobiont partner.[2,3] On the other hand, our recent studies demonstrated that cultures of lichen mycobionts have an ability under osmotically stressed conditions to produce substances which have never been detected in the lichenized state but were structurally related to fungal metabolites.[4-6] These results indicated that the normal metabolic pathways of lichen association were not operative but the dormant fungal metabolism was induced in the cultures of the isolated mycobiont. It was pointed out that cultures of lichen mycobionts could be new sources of bioactive compounds. In the course of our studies on cultured lichen mycobionts, we cultivated the spore-derived mycobiont *Graphis scripta* and isolated from its cultures three chromone derivatives, two of which were new. In this note, we report the isolation and characterization of these compounds.

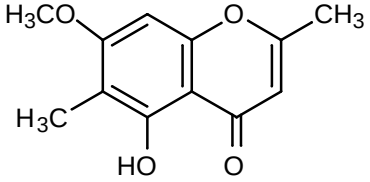
Specimens of *Graphis scripta* were collected from the bark of trees in Tochigi, Japan. The mycobiont cultures were prepared from spores discharged from apothecia and cultivated on conventional malt-yeast extract medium supplemented with 10% sucrose at 18° in the dark. After 6 months, the colonies were harvested and extracted with Et_2O and then with acetone. Subsequent purification of the extracts by

preparative TLC and HPLC afforded three chromones (**1**, **2** and **3**).

Compound (**1**) was isolated as colorless needles. The HR-EIMS spectrum of **1** exhibited a strong peak at *m/z* 220.0743 $(M)^+$, indicating a molecular formula of $C_{12}H_{12}O_4$ for **1**. It showed UV maxima at 204, 244.5, 255sh, 289, 314 nm, and IR bands at 1668 (conjugated carbonyl) and 1626, 1589 cm^{-1} (substituted aromatic system), indicating characteristics for chromone derivatives.[7] Its ^{1}H-NMR spectrum exhibited signals for two methyl groups at δ 2.00 and 2.38, an aromatic methoxy group at δ 3.84 (s), a pair of *meta*-coupled aromatic protons at δ 6.31 and 6.32 (each d, *J*=2.0 Hz), and a chelated phenolic hydroxyl group at δ 12.95. The ^{13}C-NMR spectrum of **1** showed twelve carbon resonances including a carbonyl carbon, a methoxyl, two methyls, two aromatic carbons and six quaternary carbons (four deshielded by oxygenation). These observations suggested the isolated compound to be 5-hydroxy-2,3-dimethyl-7-methoxychromone. The HMQC and HMBC experiments with **1** as well as comparison of its NMR spectral data with those of 5,7-dihydroxy-2,3-dimethylchromone (**4**)[8,9] supported this proposal. Although compound (**1**) has already been known as a synthetic product,[10] this constitutes the first instance of the isolation and characterization of **1** as a natural product.



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



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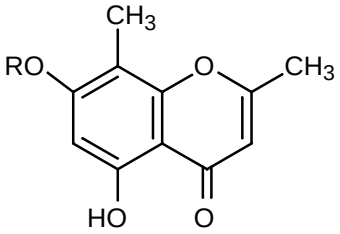

6: R =

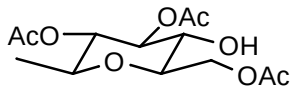

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of Compounds (**1**, **2** and **3**)

C	**1**[a]			**2**[a]			**3**[b]		
	δ_H		δ_C	δ_H		δ_C	δ_H		δ_C
2			162.50			164.58			164.05
3			115.12			118.28			114.80
4			181.96			182.29			181.80
4a			104.69			104.90			103.89
5			162.03			162.01			160.93
6	6.32	d (2.0)	97.63	6.34	d (2.0)	98.03	6.37	d (2.5)	97.67
7			165.06			165.58			165.03
8	6.31	d (2.0)	91.80	6.35	d (2.0)	92.30	6.58	d (2.5)	91.87
8a			157.57			157.66			157.05
9	2.38	s	18.45	2.46	s	17.95	4.48	br s	58.77
10	2.00	s	9.16	4.61	br s	56.68	1.98	s	7.9
5-OH	12.95	br s	--	12.52	br s	--	n.d.[c]		--
7-OMe	3.84	s	55.68	3.86	s	55.76	3.85	s	55.92

Values in parentheses are coupling constants in Hz.
[a,b] Measured in $CDCl_3$[a] or DMSO-d_6[b]. [c]Not detected.

The other chromones (**2**) and (**3**) were also isolated as crystalline compounds. Their HR-EIMS spectral measurements revealed the same composition $C_{12}H_{12}O_5$. The ^{1}H- and ^{13}C-NMR spectral features of both compounds were closely similar to those of **1**, except that the signal of a methyl group was replaced by that of a hydroxymethyl group in **2** and **3** (Table 1). The structural difference between these two could be accounted for by interchange of the position of methyl and hydroxymethyl groups. When their ^{1}H- and ^{13}C-NMR spectral data were compared with those of **1**, the chemical shifts of signals for the methyl group (**2**: δ_H 2.46, δ_C 17.95; **3**: δ_H 1.98, δ_C 7.93) suggested that a methyl group was placed at C-2 in **2** and at C-3 in **3**. This proposal was further comfirmed using the HMBC technique. In the HMBC spectrum of **2**, no interaction was observed between the proton signals for the methyl group and carbonyl carbon at δ_C 182.29, but a cross-peak from the proton signals for hydroxymethyl group to the carbonyl carbon was noted. On the other hand, a significant ^3J interaction was observed between the proton signals for the methyl group and carbonyl carbon in the HMBC experiments with **3**. Accordingly, compounds (**2**) and (**3**) were characterized as 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone and 5-hydroxy-2-hydroxymethyl-3-methyl-7-methoxychromone, respectively.

5,7-Dioxygenated chromone derivatives with 2, 6- or 2, 8-dialkyl substituents represented by eugenitin (**5**)[11] and lobodirin (**6**),[12] have so far been isolated from lichen in nature and lichen mycobiont. However, this is the first instance of the isolation of chromones with two alkyl groups at C-2 and C-3 from lichen mycobionts.

EXPERIMENTAL

Melting points were measured on a Yanaco micro melting point apparatus and were not corrected. The UV spectra were recorded on a Shimadzu UV-2500PC spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. HR-EIMS and EIMS 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. Thin-layer chromatography was performed on pre-coated Kieselgel 60F_{254} plates (Merck), and spots were visualized under UV light.

Plant material and isolation of compounds

Specimens of *Graphis scripta* were collected from the bark of trees at Kinugawa-onsen, Fujiwara-cho in Tochigi Prefecture, Japan (140° E, 37° N, *ca.* 400 m alt.) in September 1998. 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 registration No. NH 9893021. No depsidone was detected by TLC in the thallus used in this study. Mycobionts of *Graphis* sp. were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 81 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° in the dark. After cultivation for 6 months, the colonies were harvested. The harvested colonies (dry weight 87.5 g) were continuously extracted with Et_2O (20 mL × 5, 1 h each) and then with acetone (20 mL × 5,

1 h each) at rt, and the combined extracts were concentrated under reduced pressure to give residues (Et_2O ext., 70.3 mg; acetone ext. 165.6 mg)**.** The respective residues were repeatedly subjected to preparative TLC with toluene-acetone (4:1) and preparative HPLC (μBondasphere 5μC18-100 Å) with H_2O-CH_3CN (13:7), giving rise to **1** (5.8 mg), **2** (12.0 mg) and **3** (2.8 mg).

5-Hydroxy-2,3-dimethyl-7-methoxychromone (1)

Colorless needles, mp 120—121˚C (MeOH). UV (EtOH) λ_{max} (log ε): 204 (4.28), 244.5 (4.29), 255sh (4.26), 289 (3.85), 314 (3.70) nm. IR (KBr) ν_{max}: 1668, 1626, 1589, 1501, 822 cm^{-1}. ^1H- and ^{13}C-NMR: Table 1. NOESY correlations: H-6/7-OMe, H-8/7-OMe. HMBC correlations: H-6→C-4a, 5, 7, 8; H-8→C-4a, 6, 7, 8a; H_3-9→C-2, 3; H_3-10→C-2, 3, 4; 7-OMe→C-7; 5-OH→C-4a, 5, 6. EIMS *m/z* 220 $(M)^+$, 205, 191, 177, 167. HR-EIMS *m/z* Calcd for $C_{12}H_{12}O_4$ $(M)^+$: 220.0736. Found: 220.0743.

5-Hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone (2)

Colorless needles, mp 147˚C (MeOH). UV (EtOH) λmax (log ε): 206 (4.32), 234 (4.24), 249.5 (4.29), 256.5 (4.28), 290 (3.82), 320sh (3.66) nm. IR (KBr) ν_{max}: 3518, 1668, 1618, 1589, 1502, 820 cm^{-1}. ^1H- and ^{13}C-NMR: Table 1. NOESY correlations: H-6/7-OMe, H-8/7-OMe, H_3-9/H_2-10. HMBC correlations: H-6→C-4a, 5, 7, 8; H-8→C-4a, 6, 7, 8a; H_3-9→C-2, 3; H_2-10→C-2, 3, 4; 7-OMe→C-7; 5-OH→C-4a, 5, 6. EIMS *m/z* 236 $(M)^+$, 221, 218, 207, 205, 189, 175. HR-EIMS *m/z* Calcd for $C_{12}H_{12}O_5$ $(M)^+$: 236.0685. Found: 236.0707.

5-Hydroxy-2-hydroxymethyl-3-methyl-7-methoxychromone (3)

Colorless crystalline solid, mp 205˚C (decomp)(MeOH). UV (EtOH) λmax (log ε): 206.5, 233, 250.5, 258, 293, 325sh nm. IR (KBr) ν_{max}: 3393, 1666, 1618, 1582, 1506, 808 cm^{-1}. ^1H- and ^{13}C-NMR: Table 1. NOESY correlations: H-6/7-OMe, H-8/7-OMe, H_2-9/H_3-10. HMBC correlations: H-6→C-4a, 5, 7, 8; H-8→C-4a, 6, 7, 8a; H_3-9→C-2, 3; H_2-10→C-2, 3, 4; 7-OMe→C-7. EIMS *m/z* 236 $(M)^+$, 221, 207, 205, 191, 167. HR-EIMS *m/z* Calcd for $C_{12}H_{12}O_5$ $(M)^+$: 236.0685. Found: 236.0678.

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