HETEROCYCLES, Vol. 53, No. 3, 2000, pp. 723 - 728, Received, 11th November, 1999

TWO ISOCOUMARINS FROM THE CULTURED LICHEN MYCOBIONT OF *GRAPHIS* SP.

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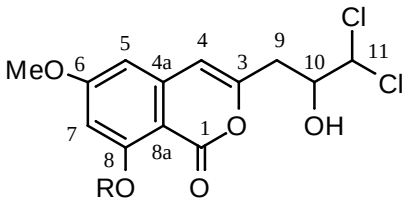
Abstract --- From the cultures of spore-derived mycobionts of lichen *Graphis* sp., two isocoumarins, 8-methyldichlorodiaportin (**1**) and 6,8-dimethyl-citreoisocoumarin (**2**), were isolated. Their structures were determined by spectroscopic and chemical methods. This is the first instance of isolation of isocoumarin metabolites from lichen mycobiont.

Lichens, a symbiotic association, produce a variety of characteristic secondary metabolites, some of which have been found to exhibit a wide range of potentially useful biological activities.[1] The roles of mycobiont and photobiont partners in the secondary metabolism have been discussed[2] and still remain to be elucidated. Recent studies demonstrated that cultures of spore-derived lichen mycobionts have an ability to produce certain lichen substances or novel metabolites in large amounts under osmotically stressed conditions.[3-6] It was pointed out that cultures of lichen mycobionts could be new sources of bioactive compounds and also good tools for investigating the symbiotic mechanism in lichens. In the course of our studies on cultured lichen mycobionts, we cultivated the spore-derived mycobiont *Graphis* sp. and isolated two isocoumarin derivatives from its cultures. In this paper, we report the isolation and characterization of the new compounds.

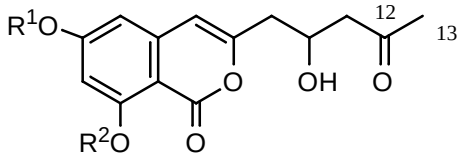
Specimens of *Graphis* sp. were collected from the bark of trees in the Philippines. The polyspore-derived mycobionts were cultured on conventional malt-yeast extract medium supplemented with 10% sucrose at 18°C in the dark. After 7 months, the cultivated colonies were harvested and extracted with ether and then with acetone. Subsequent purification of the extract by preparative TLC and HPLC afforded two isocoumarins (**1**) and (**2**).

Compound (**1**) was isolated as colorless needles. The HR-EIMS spectrum of **1** exhibited a strong peak at *m/z* 332.0206 $(M)^+$, indicating a molecular formula of $C_{14}H_{14}O_5Cl_2$ for **1**. It showed UV maxima at 243, 269.5, 279, 290 and 323 nm, and IR bands at 3408 (OH), 1703, 1668 (conjugated ester), and 1607, 1574 cm^{-1} (substituted aromatic system). Its ^{1}H-NMR spectrum exhibited signals for two aromatic methoxy groups at δ 3.87 and 3.88 (each s), a pair of *meta*-coupled aromatic protons at δ 6.59 and 6.62 (each d,

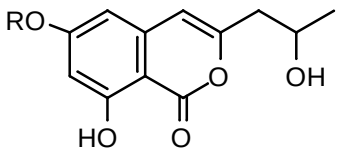
J=2.0 Hz) and an olefinic proton at δ 6.45 (br s). It showed further signals for a methylene at δ 2.58 (dd, *J*=14.5, 9.5 Hz) and δ 2.83 (dd, *J*=14.5, 3.0 Hz), a methine at δ 4.17 and another methine at δ 6.28, which were connected in sequence in the ^{1}H-^{1}H COSY spectrum. These ^{1}H-NMR spectral features as well as its ^{13}C-NMR spectral data were closely similar to those of dichlorodiaportin (**3**), a metabolite recently isolated from the cultures of *Penicillium nalgiovense*,[7] except that **1** showed one more methoxyl signal than **3**, suggesting **1** to be a 8-methylated compound of **3**. All spectral data including 2D-NMR studies, i.e. NOESY, HMQC and HMBC techniques, were fully consistent with the structure (**1**) of the isolated compound.



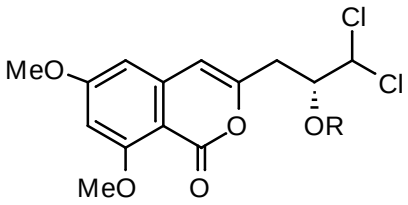
1: R = Me, 10*R* :10*S* =19 : 1
3: R = H, 10*R*



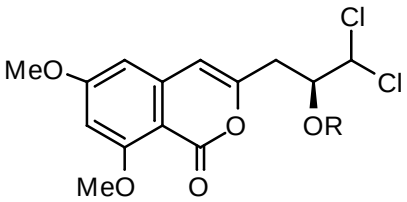
2: R^1 = R^2 = Me, 10*R* :10*S* =18 : 7
4: R^1 = R^2 = H, 10*S*
5: R^1 = Me, R^2 = H



6: R = Me
7: R = H

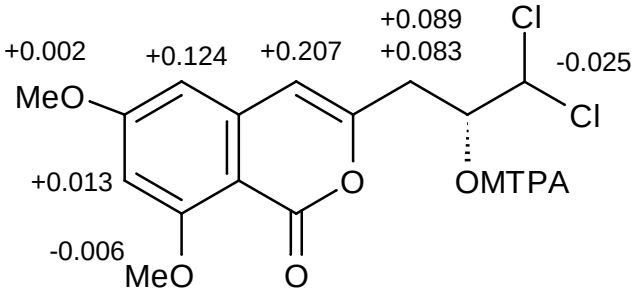


(*R*)-1a: R = (*R*)-MTPA
(*S*)-1a: R = (*S*)-MTPA



(*R*)-1b: R = (*R*)-MTPA
(*S*)-1b: R = (*S*)-MTPA

To determine the absolute configuration of C-10 in **1** by a modification of Mosher's method,[8] compound (**1**) was esterified with (*R*)- and (*S*)-MTPAs. The ^{1}H-NMR analyses demonstrated that respective products were mixtures of **(*R*)-1a** and **(*R*)-1b** and of **(*S*)-1a** and **(*S*)-1b** in a ratio of 19:1 and therefore indicated that compound (**1**) is a mixture of enatiomeric isomers. This was further supported by chiral HPLC analysis of **1**. Differences in the chemical shifts of the corresponding proton signals between **(*R*)-1a** and **(*S*)-1a** indicated the absolute configuration of C-10 in the predominant enantiomer of **1** to be *R*. Accordingly, compound (**1**) was determined to be a mixture of (10*R*)- and (10*S*)-8-methyldichlorodiaportins in a ratio of 19:1.



Δδ Values obtained from **(*S*)-1a** and **(*R*)-1a**

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

C	**1**[a]				**2**[b]			
	δ_H			δ_C	δ_H			δ_C
1				157.57				159.26
3				154.25				154.98
4	6.45	br s		105.03	6.24	br s		105.42
4a				141.56				141.99
5	6.62	d	(2.0)	100.38	6.34	d	(2.0)	99.85
6				165.09				165.47
7	6.59	d	(2.0)	98.35	6.44	d	(2.0)	98.57
8				162.67				163.30
8a				101.86				103.10
9	2.58	dd	(14.5, 9.5)	36.23	2.60	dd	(14.5, 5.0)	40.08
9	2.83	dd	(14.5, 3.0)		2.68	dd	(14.5, 7.5)	
10	4.17	ddt	(9.5, 6.0, 3.0)	72.10	4.47	m		65.45
11	6.28	d	(3.0)	76.94	2.65	dd	(18.0, 9.0)	48.91
11					2.76	dd	(18.0, 3.0)	
12								209.33
13					2.20	s		30.74
6-OMe	3.88	s		55.69	3.89	s		56.30
8-OMe	3.87	s		55.99	3.96	s		55.62
10-OH	6.09	br d	(6.0)		n.d.[c]			

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

The MS spectrum of compound (**2**) established the composition $C_{16}H_{18}O_6$. Its ^{1}H- and ^{13}C-NMR spectra displayed signals characteristic to the 3,6-dimethoxy-8-substituted isocoumarin ring system as seen in **2**. The marked differences between **1** and **2** were caused by the nature of their side chain. The signals of aliphatic protons in **2** [two sets of methylene at δ 2.60 (dd, *J*=14.5, 5.0 Hz) and δ 2.68 (dd, *J*=14.5, 7.5 Hz), and δ 2.65 (dd, *J*=18.0, 9.0 Hz) and δ 2.76 (dd, *J*=18.0, 3.0 Hz), a methine at δ 4.47 and a methyl group at δ 2.20] formulated a 2-hydroxy-4-oxopentyl group, the same side chain as in the fungal metabolites, citreoisocoumarin (**4**)[9] and 6-methylcitreoisocoumarin (**5**).[7,10] Accordingly, compound (**2**) was characterized as 6,8-dimethylcitreoisocoumarin. The MTPA esters of **2** could not be prepared owing to the small amount isolated. Chiral HPLC analysis and comparison of the specific optical rotation of **2** with that reported for **4** suggested that the isolated compound was again an enantiomeric mixture (10*R*:10*S* = 18:7).

This is the first instance of the isolation of isocoumarin derivatives from lichen mycobionts. It is of great interest from the viewpoint of their physiological and biological significances that **1** and **2** were found in the cultures of isolated lichen mycobionts but not in the lichen itself. Diaportin (**6**)[11] and orthosporin (**7**),[12] metabolites of this type, had so far been isolated from fungi as phytotoxic metabolites. It might be postulated that the metabolic pathways of the isocoumarin derivatives could be expressed for survival of the mycobiont in pre-lichenized condition but supressed in associated lichens because of their toxicity to photobiont.

EXPERIMENTAL

Melting points were measured on a Yanaco micro melting point apparatus and are not corrected. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. The optical rotations were measured on a JASCO DIP-370 digital polarimeter. 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* sp. were collected by N. A. Lam from the bark of trees at Mt. Sto Tomas, Tuba Benquet in the Philippines (120˚E, 16˚N, *ca.* 1300 m alt.) in May 1997. The voucher specimen was identified by H. Miyawaki and was deposited at Osaka City Institute of Public Health and Environmental Sciences with registration No. NH 9752573. 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 57 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. After cultivation for 7 months, the colonies and slants with crystals were harvested. The harvested colonies (fresh weight 67.1 g, dry weight 22.0 g) were continuously extracted with ether (50 mL x 5, 1 h each) and then with acetone (50 mL x 5, 1 h each) at rt, and the combined extracts were concentrated under reduced pressure to give residues (ether ext. 135.1 mg; acetone ext. 763.2 mg). The respective residues were repeatedly subjected to preparative TLC with C_6H_6-acetone (9:1) or toluene-acetone (4:1), and preparative HPLC (μBondasphere 5μC18-100 Å) with H_2O-CH_3CN (11:9 or 13:7), giving rise to **1** (55.2 mg) and **2** (3.3 mg).

8-Methyldichlorodiaportin (1)

Colorless needles, mp 155˚C (MeOH). $[\alpha]_D^{25}$ + 36˚ (*c*=0.26, $CHCl_3$). UV (EtOH) λ_{max} (log ε): 243 (4.69), 269.5 (3.83), 279 (3.85), 290 (3.75), 323 (3.85) nm. IR (KBr) ν_{max}: 3408, 1703, 1668, 1607, 1574 cm^{-1}. ^1H- and ^{13}C-NMR: Table 1. NOESY correlations: H-4/H-5, H-4/H-9 (δ 2.58), H-4/H-11, H-5/6-OMe, H-7/6-OMe, H-7/8-OMe, H_2-9/H-11, 10-OH/H-11. HMBC correlations: H-4→C-3, 4a, 5, 9; H-5→C-6, 7; 6-OMe→C-6; H-7→C-5, 6, 8; 8-OMe→C-8; H_2-9→C-4, 10, 11; H-10→C-3; 10-OH→C-9, 10, 11; H-11→C-9. EIMS *m/z* 332 $(M)^+$, 249, 220, 191. HR-EIMS *m/z* Calcd for $C_{14}H_{14}O_5Cl_2$ $(M)^+$: 332.0219. Found: 332.0206.

6,8-Dimethylcitreoisocoumarin (2)

Colorless needles, mp 142-143˚C (MeOH). $[\alpha]_D^{24}$ + 3˚ (*c*=0.20, $CHCl_3$). UV (EtOH) λ_{max} (log ε): 244.5 (4.67), 279 (3.78), 290.5 (3.68), 323.5 (3.79) nm. IR (KBr) ν_{max}: 3437, 1705, 1670, 1607, 1572 cm^{-1}. ^1H- and ^{13}C-NMR: Table 1. NOESY correlations: H-4/H-5, H-4/H-9 (δ 2.60), H-4/H-10, H-4/H-11 (δ 2.65), H-5/6-OMe, H-7/6-OMe, H-7/8-OMe, H_3-13/H-9 (δ 2.60), H_3-13/H-11 (δ 2.65). HMBC correlations:

H-4→C-3, 4a, 5, 8a, 9; H-5→C-4, 6, 7, 8a; 6-OMe→C-6; H-7→C-5, 6, 8, 8a; 8-OMe→C-8; H_2-9→C-3 4, 10, 11; H-10→C-3; H_2-11→C-9, 10, 12; H_3-13→C-11, 12. EIMS *m/z* 306 $(M)^+$, 288, 248, 220, 191. HR-EIMS *m/z* Calcd for $C_{16}H_{18}O_6$ $(M)^+$: 306.1104. Found: 306.1107.

Preparation of (*R*)- and (*S*)-MTPA Esters of 1

To a solution of **1** (4.5 mg) in dry CH_2Cl_2 (1 mL) were added (*R*)-MTPA (20 mg), 4-DMAP (3 mg) and DCC (20 mg), and the whole was stirred at rt for 28 h. The reaction mixture was poured into 1 N HCl and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by preparative TLC (Et_2O) and preparative HPLC (MeOH-H_2O, 8:2) to yield a mixture (2.9 mg) of **(*R*)-1a** and **(*R*)-1b** in the ratio of 19:1. EIMS *m/z* 548 $(M)^+$, 314, 279. **(*R*)-1a**: ^{1}H-NMR: δ 2.925 (1H, dd, *J*=15.0, 9.0 Hz, H-9), 3.103 (1H, dd, *J*=15.0, 3.0 Hz, H-9), 3.565 (3H, d, *J*=1.0 Hz, MTPA-OMe), 3.890 (3H, s, 6-OMe), 3.991 (3H, s, 8-OMe), 5.788 (1H, dt, *J*=9.0, 3.0 Hz, H-10), 5.957 (1H, br s, H-4), 6.061 (1H, d, *J*=3.0 Hz, H-11), 6.149 (1H, d, *J*=2.0 Hz, H-5), 6.476 (1H, d, *J*=2.0 Hz, H-7), 7.147-7.416 (5H, m, MTPA-Ph). NOESY correlations: H-4/H-5, H-5/6-OMe, H-7/6-OMe, H-7/8-OMe. Compound **(1)** (4.8 mg) was treated with (*S*)-MTPA (20 mg) as described above to yield a mixture (3.2 mg) of **(*S*)-1a** and **(*S*)-1b** (19:1). EIMS *m/z* 548 $(M)^+$, 314, 279. **(*S*)-1a**: ^{1}H-NMR: δ 3.008 (1H, dd, *J*=15.0, 9.5 Hz, H-9), 3.192 (1H, dd, *J*=15.0, 3.5 Hz, H-9), 3.425 (3H, d, *J*=1.0 Hz, MTPA-OMe), 3.892 (3H, s, 6-OMe), 3.985 (3H, s, 8-OMe), 5.771 (1H, dt, *J*=9.5, 3.5 Hz, H-10), 6.164 (1H, br s, H-4), 6.036 (1H, d, *J*=3.5 Hz, H-11), 6.273 (1H, d, *J*=2.0 Hz, H-5), 6.489 (1H, d, *J*=2.0 Hz, H-7), 7.198-7.398 (5H, m, MTPA-Ph). NOESY correlations: H-4/H-5, H-5/6-OMe, H-7/6-OMe, H-7/8-OMe. The ^{1}H-NMR spectral data of **(*R*)-1b** and **(*S*)-1b** were identical with those of **(*S*)-1a** and **(*R*)-1a**, respectively.

HPLC Analysis of 1 and 2

Compound (**1**) was analysed by chiral HPLC [column, CHIRALCEL OB-H (4.6 i.d. x 250 mm, Daicel Chemical Industries, Ltd.); mobile phase, *n*-hexane-2-propanol (7:3); flow rate, 0.6 mL/min; detection, 254 nm] and demonstrated a major peak at 25.5 min and a minor one at 21 min. Compound (**2**) was subjected to chiral HPLC on the same column using *n*-hexane-2-propanol (3:2) at 1.0 mL/min flow rate. The major peak eluted at 23 min, and a minor one at 18 min.

ACKNOWLEDGEMENTS

We are grateful to Ms. Nadezna A. Lam, St. Louis University, Philippines for collection of the voucher specimen. Thanks are also due to Dr. M. Sugiura (Kobe Pharmaceutical University) for ^{1}H- and ^{13}C-NMR spectra, and to Dr. K. Saiki (Kobe Pharmaceutical University) for MS spectra measurements. This research was financially supported in part by a Grant-in-Aid for Scientific Research (C) (No. 11672129) from the Ministry of Education, Sciences, Sports and Culture of Japan and Kobe Pharmaceutical University Collaboration Fund.

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