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NAPHTHOPYRONES FROM CULTURED LICHEN MYCOBIONTS OF *PYRENULA* SP.

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Abstract – Spore-derived mycobionts of the lichen *Pyrenula* sp. were cultivated on a malt-yeast extract medium supplemented with 10% sucrose and their metabolites were investigated. A new naphthopyrone derivative, pyrenulin, as well as toralactone were isolated. Their structures were determined by spectroscopic methods. This is the first instance of the isolation of naphthalene derivatives from lichen mycobionts.

Lichens, which exhibit 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.[1,2] Recent studies demonstrated that cultures of spore-derived lichen mycobionts have the ability to produce certain lichen substances[3-5] or novel metabolites that are structurally related to fungal metabolites[6-10] under osmotically stressed conditions. These findings suggested that cultures of lichen mycobionts could be a new source of bioactive compounds. In the course of our studies on cultured lichen mycobionts, we cultivated the spore-derived mycobiont *Pyrenula* sp. and isolated a novel naphthopyrone derivative along with toralactone from its cultures. In this paper, we report the isolation and characterization of the new compound.

Specimens of *Pyrenula* sp. were collected from the bark of trees in Miyazaki. The polyspore-derived mycobionts were cultured on conventional malt-yeast extract medium supplemented with 10% sucrose at 18˚C in the dark. After 14 months, the cultivated colonies were harvested and extracted with ether and then with acetone. Subsequent purification of the extracts by preparative TLC afforded two compounds **1** and **2**.

Compound **1**, obtained as an unstable substance, had the molecular formula of $C_{15}H_{12}O_5$ as established by

HREIMS. Its ^{1}H NMR spectrum showed signals for a methyl, a methoxyl, a hydrogen-bonded hydroxy group, and four sp^2 methine protons. Acetylation of **1** gave a diacetate, suggesting the presence of two hydroxy groups in **1**. Detailed 2D-NMR studies of **1** and its acetate indicated **1** to be a naphtho-α-pyrone derivative, toralactone. Toralactone is known as a constituent of the plant species *Cassia tora* L.,[11,12] but has not been isolated from either lichens or cultured mycobionts.

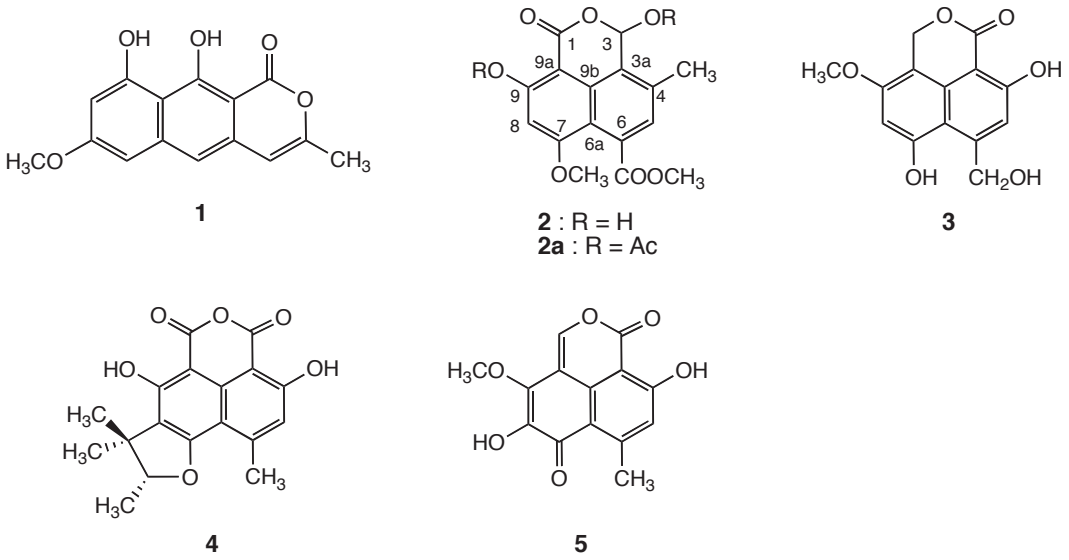

Figure 1. Structures of **1** and **2** and their related compounds

Compound **2** was isolated as colorless needles. The HREIMS spectrum of **2** established the composition $C_{16}H_{14}O_7$. It showed UV maxima at 225sh, 243.5, 301, 313, 342, 354, and 378sh nm, and IR bands at 3320 (OH), 1736, 1664 (conjugated ester), 1643, 1614, and 1508 cm^{-1}. Its ^{1}H NMR spectrum exhibited signals for an aromatic methyl group at δ_H 2.58, two methoxy groups at δ_H 3.91 and 4.09 (each s), two aromatic protons at δ_H 6.70 (s) and 7.26 (br s), and an acetalic proton at δ_H 6.98 (d, *J*=7.0 Hz). It showed further signals for two hydroxyls at δ_H 7.08 (d, *J*=7.0 Hz) and δ_H 12.37 (br s); the former was coupled with the acetalic proton and the latter was hydrogen-bonded with a carbonyl group. The ^{13}C NMR spectrum of **2** showed the signals for a methyl, two methoxyls, an acetalic carbon, two sp^2 methine carbons, and 10 sp^2 quaternary carbons including two ester carbonyls. These spectral features implied that **2** possessed a naphtho[1,8-*cd*]pyran-3-one skeleton related to corymbiferan lactone A (**3**)[13] from *Penicillium hordei* and (+)-sclerodin (**4**)[14] from *Aspergillus silvaticus*. Positioning of the functional substituents could be inferred by a series of COSY, NOESY, HMQC, and HMBC experiments. The HMBC correlation from an aromatic proton (H-5) at δ_H 7.26 to the methoxycarbonyl carbon and NOESY cross peak between the aromatic proton and the methyl group were indicative of the substitution of the methoxycarbonyl and methyl groups at *ortho* positions of H-5. The HMBC correlations were observed from another aromatic proton (H-8) at δ_H 6.70 to two oxygenated aromatic carbons, where methoxy and

hydrogen-bonded hydroxy groups were located from their HMBC interactions. Furthermore, the HMBC correlations from the signal due to a hydroxy group at δ_H 7.08 to an acetal carbon and from the acetal proton at δ_H 6.98 to a carbonyl carbon at δ_C 169.7 suggested the presence of a lactol bridge. Significant HMBC correlations from both aromatic protons to an aromatic quaternary carbon (C-6a) at δ_C 114.5, and NOESY cross peak between the acetal proton and the methyl group, together with the presence of a hydrogen-bonded hydroxy group adjacent to a carbonyl group, demonstrated the substitution of functional groups on the naphthalene ring as shown in Figure 2.

Table 1. ^{1}H and ^{13}C NMR Spectral Data of **2** (acetone-d_6) and **2a** ($CDCl_3$)

	2				**2a**		
C	δ_H			δ_C	δ_H		δ_C
1				169.7			159.1
3	6.98	d	(7.0)	95.4	7.80	br s	89.2
3a				126.3			123.7
4				137.5			136.6
5	7.26	br s		126.8	7.36	br s	128.3
6				132.1			131.4
6a				114.5			117.0
7				163.1			160.9
8	6.70	s		98.7	6.71	s	103.5
9				166.9			155.4
9a				93.8			102.8
9b				129.8			130.5
4-CH_3	2.58	br s		18.0	2.47	br s	18.2
6-COO$\underline{C}H_3$	3.91	s		52.6	3.96	s	52.7
6-$\underline{C}OOCH_3$				170.7			170.0
7-OCH_3	4.09	s		57.4	4.04	s	57.0
3-OH	7.08	d	(7.0)				
9-OH	12.37	br s					
3-OCO$\underline{C}H_3$					2.06	s	20.8
3-O$\underline{C}OCH_3$							168.8
9-OCO$\underline{C}H_3$					2.46	s	21.2
9-O$\underline{C}OCH_3$							169.0

Values in parentheses are coupling constants in Hz.

Acetylation of **2** gave a diacetate **2a**, $C_{20}H_{18}O_9$. The results of detailed 2D-NMR experiments with **2a** were fully coincident with the proposed structure. Thus, the structure of the new compound was elucidated as **2** and designated pyrenulin. Metabolites with a naphthalene skeleton are rarely found as lichen substances, although simonyelline (**5**)[15] and euplectin[16] have so far been isolated from thalli of lichens. This is the first instance of isolation of this type of metabolite from cultured mycobionts of

lichen.

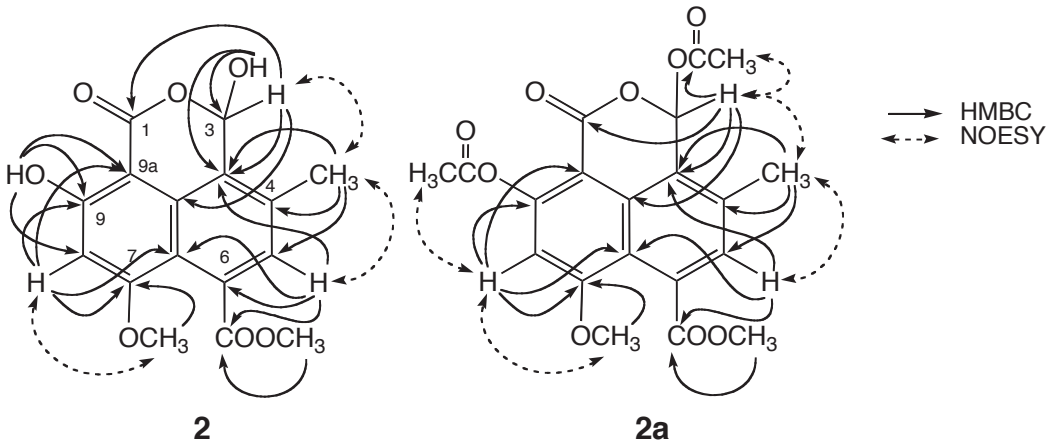

Figure 2. HMBC and NOESY correlations of **2** and **2a**

EXPERIMENTAL

General Procedures. 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. HREIMS 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 *Pyrenula* sp. were collected from the bark of trees in Hongo-cho, Miyazaki Pref., Japan (100m alt.) by N. Hamada. The voucher specimen (NH 96101336) was identified by Prof. H. Miyawaki, Saga University, Japan, and was deposited at Saga University. Mycobionts of *Pyrenula* sp. were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 7 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 14 months, the harvested colonies (dry weight 2.05 g) were continuously extracted with ether and then with acetone at rt, and the combined extracts were concentrated under reduced pressure to give residues (ether ext., 23.3 mg; acetone ext. 234.3 mg). The respective residues were repeatedly subjected to preparative TLC with toluene-acetone (19:1 or 9:1), giving rise to toralactone (**1**) (6.7 mg) and pyrenulin (**2**) (7.8 mg).

Pyrenulin (2): Colorless needles, mp 163-164 ˚C (MeOH). UV (EtOH) λ_{max} nm (log ε): 225sh (4.26), 243.5 (4.56), 301 (3.88), 313 (3.98), 342 (3.87), 354 (3.85), 378sh (3.23). IR (KBr) ν_{max} cm^{-1}: 3320, 1736, 1664, 1643, 1614, 1508. HREIMS *m/z*: Calcd for $C_{16}H_{14}O_7$ $[M]^+$: 318.0740. Found: 318.0718.

Acetylation of 2. Compound **2** (5.6 mg) was acetylated with Ac_2O-pyridine (each 0.1 mL) and the crude acetate was purified by preparative TLC (toluene-acetone, 19:1) to yield acetate (**2a**) (4.6 mg). **2a**: Pale yellow crystalline solid, mp 205 ˚C (MeOH). UV (EtOH) λ_{max} nm (log ε): 218 (3.87), 245.5 (4.30), 317sh

(3.65), 329.5 (3.71), 343sh (3.57). IR (KBr) ν_{max} cm^{-1}: 1771, 1740, 1719, 1601, 1522. HREIMS *m/z*: Calcd for $C_{20}H_{18}O_9$ $[M]^+$: 402.0951. Found: 402.0964.

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