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Phytochemistry 65 (2004) 3119–3123
PHYTOCHEMISTRY
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Three isocoumarins and a benzofuran from the cultured lichen mycobionts of *Pyrenula* sp.

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Received 31 March 2004; received in revised form 18 August 2004
Available online 6 November 2004

Abstract

The spore-derived mycobionts of the lichen *Pyrenula* sp. were cultivated on a malt–yeast extract medium supplemented with 10% sucrose. The investigation of their metabolites resulted in isolation of four compounds, three isocoumarins and a biogenetically related benzofuran; their structures were determined by spectroscopic methods.

Keywords: *Pyrenula* sp.; Lichen; Isolated mycobiont; Isocoumarin; Benzofuran

1. Introduction

Lichens, symbiotic associations of mycobiont and photobiont partners, are well known to produce a variety of characteristic secondary metabolites with manifold biological activities (Huneck and Yoshimura, 1996; Huneck, 1999). On the other hand, cultures of spore-derived lichen mycobionts under osmotically stressed conditions are capable of producing certain lichen substances such as anthraquinones (Takenaka et al., 2002) or novel metabolites, e.g., isofuranonaphthoquinones (Yamamoto et al., 2002). We have recently isolated and characterized new xanthones from the cultured mycobionts of *Pyrenula japnica* and *Pyrenula pseudobufonia* (Tanahashi et al., 1999; Takenaka et al., 2000). In continuation of our studies on cultured lichen mycobionts (Takenaka et al., 2003), we cultivated the spore-derived mycobionts of *Pyrenula* sp. and isolated four phenolics from their cultures. In this paper, we describe the isolation and characterization of these compounds.

2. Results and discussion

The polyspore-derived mycobionts of *Pyrenula* sp. collected in Tsushima, Nagasaki Prefecture, Japan was cultured on the conventional malt–yeast extract medium supplemented with 10% sucrose at 18 °C in the dark. After seven months, the cultivated colonies were harvested and extracted with acetone. Subsequent purification of the extract by preparative TLC and preparative HPLC afforded compounds **1**–**4**.

Compound **1** was isolated as yellow crystals. The HR-EIMS spectrum of **1** exhibited a strong peak at *m/z* 246.0893 $[M]^+$, indicating a molecular formula of $C_{14}H_{14}O_4$ for **1**. It showed UV maxima at 245.5, 252.5, 297, 307.5, 389.5, and 405.5 *sh* nm, and IR bands at 3234, 1663, 1641, 1620, 1601, 1583, and 1479 cm^{-1}, suggesting the presence of hydroxyl group(s), a chelated carbonyl group and substituted aromatic system. The

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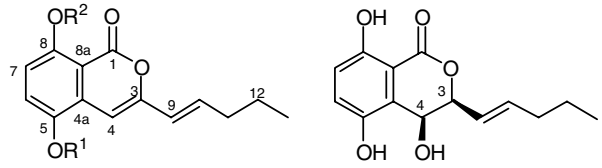
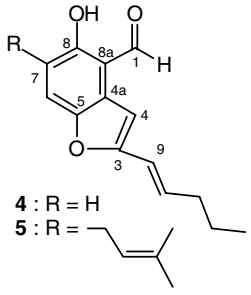
doi:10.1016/j.phytochem.2004.09.011



1 : $R^1 = R^2 = H$
1a : $R^1 = CH_3$, $R^2 = H$
2 : $R^1 = H$, $R^2 = CH_3$

3

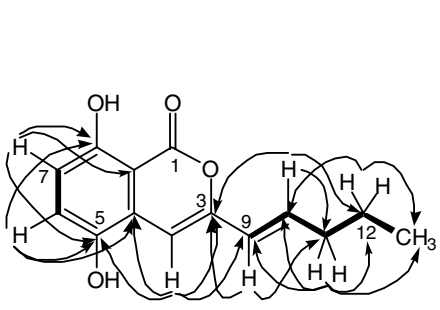


4 : R = H
5 : R =

^{1}H NMR spectrum exhibited signals for a pair of *ortho*-coupled aromatic protons at δ 6.76 and 7.11 (each *d*, $J = 9.0$ Hz) and an olefinic proton at δ 6.68 (*br s*). It showed further signals for a methyl at δ 0.98 (*t*, $J = 7.5$ Hz), two methylene groups at δ 1.53 (2H, *sext*, $J = 7.5$ Hz) and δ 2.23 (2H, *qd*, $J = 7.5$, 1.5 Hz), a pair of *trans*-oriented olefinic protons at δ 6.49 (*dt*, $J = 16.0$, 7.5 Hz) and δ 6.18 (*dt*, $J = 16.0$, 1.5 Hz), which were connected in sequence in the ^{1}H–^{1}H COSY spectrum, suggesting the presence of 1*E*-pentenyl group. Its ^{13}C NMR spectrum showed, besides signals due to a pentenyl group, nine sp^2 carbon signals, which were assigned by DEPT as three CH carbons and six quaternary carbons including an ester carbonyl carbon at δ 167.5 and three oxygenated carbons at δ 146.0, 152.2 and 155.6. The HMBC interactions from an olefinic proton (H-4) to three carbon signals at δ 126.3 (C-4a), 146.0 (C-5) and 152.2 (C-3) and from two aromatic protons to two carbon signals of C-5 and C-8 (δ 155.6) suggested 3,5,8-substituted isocoumarin ring system (Fig. 1). Further HMBC correlations from two olefinic protons at δ 6.18 and 6.49 to C-3 indicated the substitution of the pentenyl group at C-3. The molecular formula of **1** and chemical shifts of ^{13}C NMR spectrum indicated two *para*-substituted hydroxyl groups at C-5 and C-8. Thus, compound **1** was characterized as (*E*)-5,8-dihydroxy-3-(1-pentenyl)isocoumarin.

The MS spectrum of compound **2** established the composition $C_{15}H_{16}O_4$. Its ^{1}H and ^{13}C NMR spectra displayed a methoxy signal in addition to signals characteristic to the 5,8-dioxy-3-pentenyl-isocoumarin ring system as seen in **1**. The methoxyl group was correlated with an aromatic proton signal at δ 6.92 in the NOESY spectrum. These findings suggested **2** to be 5- or 8-*O*-methylated compound of **1**. The methoxyl group was suggested to be located at C-8 by the upfield shift of C-1 (+6.4 ppm) and downfield shift of C-8a (−2.8 ppm), and by an IR band at 1711 cm^{-1} ascribed to a non-chelated carbonyl group. Further support was obtained by the fact that **2** was discriminated from **1a**, a mono-methylated compound derived from **1**. Compound **1a**, which showed a ^{1}H NMR signal of a chelated hydroxyl group at δ 10.51 and a NOESY cross peak between a methoxyl group and an aromatic proton signal due to H-6, was assigned to be (*E*)-8-hydroxy-5-methoxy-3-(1-pentenyl)isocoumarin. Accordingly, **2** was determined to be (*E*)-5-hydroxy-8-methoxy-3-(1-pentenyl)isocoumarin.

The HR-EI mass spectrum of **3** exhibited a peak at *m/z* 264.1000 $[M]^+$, indicating a molecular formula of $C_{14}H_{16}O_5$. Its ^{1}H and ^{13}C NMR spectra displayed its structural similarity to **1**. The marked differences between **1** and **3** were accounted for by the hydrogenation of C-3–C-4 double bond and hydroxylation at C-4 in **3**. In the ^{1}H NMR spectrum of **3**, the signals of two oxygenated methine protons were observed at δ 4.87 and 4.93 instead of an olefinic proton signal as seen in **1**. The observations, together with ^{13}C NMR signals at δ 85.0 and 62.8, implied a 3,4-dihydro-4-hydroxyisocoumarin skeleton. This assumption was consistent with the results of the HMBC experiments with **3**. The relative configuration at C-3 and C-4 was suggested to be *cis* by the comparison of $J_{3,4}$ (2.0 Hz) of **3** with those of similar isocoumarins. The $J_{3,4}$ values of *cis*-4-hydroxymellein



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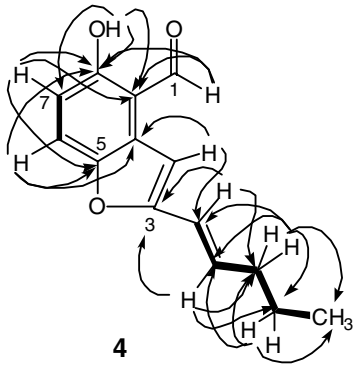

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Fig. 1. ^{1}H–^{1}H COSY (bold lines) and HMBC (arrows) correlations observed for **1** and **4**.

(Okuno et al., 1986) and 4-chloro-5,6,8-trihydroxy-3-methylisochroman-1-one with 3,4-*cis* configuration (Stadler et al., 1995) are ≈2 Hz, while $J_{3,4}$ in *trans*-4-hydroxymellein (Aldridge et al., 1971) has been reported to be 4 Hz. Chiral HPLC analysis of **3** demonstrated that the isolated compound was not a single isomer but an enantiomeric mixture with the ratio of 9:11, although it remained to be determined which enantiomer is predominant. Accordingly, compound **3** was determined to be a mixture of (3*R*,4*R*)- and (3*S*,4*S*)-3,4-dihydro-4,5,8-trihydroxy-3-(1-pentenyl)isocoumarins.

Compound **4** was also obtained as a yellow crystalline solid. The MS spectrum of compound **4** established the composition $C_{14}H_{14}O_3$. Its ^{1}H NMR spectrum displayed signals for *ortho*-coupled aromatic protons at δ 6.81 and 7.54 (each *d*, J = 9.0 Hz) and an olefinic proton at δ 6.78, and signals characteristic to the 1-pentenyl group as seen in **1**. However, the ^{1}H NMR spectrum exhibited additionally a signal due to an aldehydic group at δ 10.27, which could not be observed in **1**. The ^{13}C NMR spectrum showed also an aldehydic carbonyl carbon at δ 192.9 instead of an ester carbonyl. The location of the aldehydic group was determined to be at an *ortho* position of the phenolic hydroxyl group by the presence of a hydrogen bonded proton at δ 11.34 and HMBC correlations from the hydroxyl and aldehydic protons to the oxygenated carbon (δ 111.3) (Fig. 1). Furthermore, the HMBC spectrum of **4** showed similar correlations to those of **1** (H-4 to C-4a and C-9, H-6 to C-4a, C-5 and C-8, H-7 to C-5, C-8 and C-8a) and the ^{13}C NMR spectrum displayed three oxygenated carbon signals in addition to a carbonyl carbon, although the molecule possesses only three oxygen atoms. These findings led us to situate the hydroxyl group at C-8 and to assume an furan ring consisted of C-3 and C-5. [1] The structure was consistent with the biogenesis that the carbonyl group at C-3, which was connected as an enol form to the carboxyl group at C-8a in **1**, was attacked by the phenolic hydroxyl group at C-5 to construct a furan ring in **4**. Accordingly, the structure of compound **4** was determined as shown and designated as pyrenulafuran. Although **4** has already been reported as a synthetic intermediate in the total synthesis of E-1 (**5**) isolated from the mycelium of *Aspergillus amstelodami* IFO-6667 (Inoue et al., 1977), the present study constitutes the first isolation of **4** as a natural product.

Isocoumarins have not been isolated from the thalli of lichens, but from the cultured lichen mycobionts of *Graphis s*p. (Tanahashi et al., 2000). This is the first instance of the isolation of 5,8-dihydroxyisocoumarin derivatives and a benzofuran from lichen mycobionts.

[1] For convenience and in order to facilitate biogenetic discussion, the numbering chosen is not in accord with the IUPAC systematic numbering for dibenzofuran.

3. Experimental

3.1. General

Melting point was measured on a Yanaco micro melting point apparatus and are reported uncorrected. The UV spectra were recorded on a Shimadzu UV-240 spectrophotometer and the IR spectra on a Shimadzu FTIR-8200 infrared spectrophotometer. HR-EIMS were obtained with a Hitachi M-4100 mass spectrometer. The NMR experiments were performed with Varian VXR-500, Varian Gemini-300 and Varian Gemini-200 spectrometers, with tetramethylsilane as internal standard. HPLC was performed using a Waters system (600E Multisolvent Delivery System, 486 Tunable Absorbance Detector). Thin-layer chromatography was performed on precoated Kieselgel 60F_{254} plates (Merck) and spots were visualized under UV light.

3.2. Plant material

Specimens of *Pyrenula* sp. were collected from the bark of trees in Tsushima, Nagasaki Prefecture, Japan (350m alt.). The voucher specimens were identified by Professor Hiromi Miyawaki of Saga University, Japan and were deposited at Osaka City Institute of Public Health and Environmental Sciences with the Registration No. NH9941514. Mycobionts of *Pyrenula* sp. were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 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 for seven months.

3.3. Extraction and isolation

The harvested colonies (94 test tubes, freeze-dried weight 16.91 g) were extracted with acetone at room temperature, and the combined extracts were concentrated under reduced pressure to give a residue (418 mg). The extracts were repeatedly subjected to preparative TLC (toluene–acetone, 9:1 or $CHCl_3$) and preparative HPLC (μBondasphere 5μC18–100 Å, MeCN–H_2O, 7:3), giving rise to **1** (16.0 mg), **2** (1.2 mg), **3** (2.0 mg) and **4** (2.4 mg).

3.4. (*E*)-5,8-dihydroxy-3-(1-pentenyl)isocoumarin (*1*)

Yellow crystals, m.p. 184° (MeOH). UV λ_{max}^{EtOH} nm (log ε): 245.5 (4.39), 252.5 (4.31), 297 (4.02), 307.5 (4.03), 389.5 (4.03), 405.5 *sh* (3.98); IR ν_{max}^{KBr} cm^{-1}: 3234, 1663, 1641, 1620, 1601, 1583, 1479; for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2; HR-EIMS *m*/*z*, Calcd for $C_{14}H_{14}O_4$ $[M]^+$: 246.0893. Found: 246.0893.

Table 1
^{1}H NMR spectral data of compounds **1**–**3**

H	1	2	3
3			4.87 *br d* (7.0)
4	6.68 *br s*	6.68 *br s*	4.93 *d* (2.0)
6	7.11 *d* (9.0)	7.13 *d* (9.0)	7.10 *d* (9.0)
7	6.76 *d* (9.0)	6.92 *d* (9.0)	6.84 *d* (9.0)
9	6.18 *dt* (16.0, 1.5)	6.15 *dt* (15.5, 1.5)	5.89 *ddt* (15.0, 7.0, 1.5)
10	6.49 *dt* (16.0, 7.5)	6.52 *dt* (15.5, 7.0)	6.00 *ddt* (15.0, 7.0, 1.0)
11	2.23 *qd* (7.5, 1.5)	2.23 *qd* (7.0, 1.5)	2.15 *qd* (6.5, 1.0)
12	1.53 *sext* (7.5)	1.53 *br sext* (7.5)	1.50 *br sext* (7.5)
13	0.98 *t* (7.5)	0.98 *t* (7.5)	0.97 *t* (7.5)
OMe		3.87 *s*	

Table 2
^{13}C NMR spectral data of compounds **1**–**4** and **1a**

C	1[a]	1a[b]	2[a]	3[a]	4[b]
1	167.5	166.1	161.1	171.3	192.9
3	152.2	151.6	152.4	85.0	158.7
4	101.0	99.3	99.4	62.8	99.0
4a	126.3	127.2	129.3	126.9	131.0
5	146.0	146.5	146.6	147.6	148.5
6	124.4	121.9	121.5	125.9	119.8
7	115.8	114.2	111.4	118.8	113.2
8	155.6	155.0	155.4	156.1	159.5
8a	106.7	106.2	109.5	108.5	111.3
9	123.5	119.1	123.1	125.9	118.3
10	136.4	136.7	136.0	137.9	136.4
11	35.8	34.9	35.2	35.5	35.1
12	23.3	22.0	22.7	23.1	22.1
13	14.1	13.7	13.5	14.0	13.7

[a] Measured in CD_3OD.
[b] Measured in $CDCl_3$.

3.5. (*E*)-5-hydroxy-8-methoxy-3-(1-pentenyl) isocoumarin (*2*)

Yellow crystalline solid, UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 245.5 (4.27), 253 *sh* (4.25), 288 *sh* (3.83), 299.5 (3.87), 311.5 *sh* (3.76), 383 (3.76), 403 *sh* (3.66); IR ν_{max}^{KBr} cm^{-1}: 3248, 1711, 1654, 1595; for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2. NOESY correlations: H-7 ↔ OCH_3, H-4 ↔ H-9; HR-EIMS *m*/*z*, Calcd for $C_{15}H_{16}O_4$ $[M]^+$: 260.1049. Found: 260.1056.

3.6. cis-3,4-Dihydro-4,5,8-trihydroxy-3-(1-pentenyl) isocoumarin (*3*)

Colorless crystalline solid, UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 220 (4.12), 247.5 *sh* (3.67), 297 *sh* (3.25), 348 (3.53); IR ν_{max}^{KBr} cm^{-1}: 3200, 1645, 1587, 1479. CD λ_{max} nm ($\Delta\varepsilon$): 217 (−3.36), 237 (−0.90), 257 (−1.92), 279 (+0.01), 341 (+0.44), 384 (+0.14); for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2. HMBC correlations: H-3 → C-9, C-10, H-4 → C-3, C-4a, C-8a, H-6 → C-4a, C-5, C-8, H-7 → C-5, C-8a, H-9 → C-10, C-11, H-10 → C-3, C-9, C-11, H_2-11 → C-9, C-10, C-12, C-13, H_2-12 C-10, C-11, C-13, H_3-13 → C-11, C-12; HR-EIMS *m*/*z*, Calcd for $C_{14}H_{16}O_5$ $[M]^+$: 264.0998. Found: 264.1000.

HPLC analysis (CHIRALCEL OB-H 0.46 × 25 mm, *n*-hexane–2-propanol, 4:1, 0.6 ml/min) showed two peaks at R_t 10 and 12 min in the ratio of 9:11.

3.7. Pyrenulafuran (*4*)

Yellow crystalline solid, UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 250.5 (3.73), 281 *sh* (3.49), 317.5 (3.64), 328 *sh* (3.61), 375.5 *sh* (3.57); IR ν_{max}^{KBr} cm^{-1}: 3432, 1641, 1560, 1414; ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, *t*, *J* = 7.5 Hz, H_3-13), 1.55 (2H, *sext*, *J* = 7.5 Hz, H_2-12), 2.26 (2H, *qd*, *J* = 7.5, 1.5 Hz, H_2-11), 6.35 (1H, *dt*, *J* = 16.0, 1.5 Hz, H-9), 6.57 (1H, *dt*, *J* = 16.0, 7.5 Hz, H-10), 6.78 (1H, *br s*, H-4), 6.81 (1H, *d*, *J* = 9.0 Hz, H-7), 7.54 (1H, *d*, *J* = 9.0 Hz, H-6), 10.27 (1H, *br s*, H-1), 11.34 (1H, *s*, 8-OH); ^{13}C NMR: see Table 2. NOESY correlations: H-7 ↔ OH, H-4 ↔ H-9, H-9 ↔ H_2-11, H-10 ↔ H_2-12; HR-EIMS *m*/*z*, Calcd for $C_{14}H_{14}O_3$ $[M]^+$: 230.0944. Found: 230.0936.

3.8. Methylation of *1*

To a solution of **1** (6.0 mg) in acetone (10 ml) were added K_2CO_3 (110 mg) and CH_3I (0.01 ml), with the whole heated under reflux for 1.5 h. After removal of K_2CO_3 by filtration, the reaction mixture was concentrated in vacuo and the residue was purified by preparative HPLC (μBondasphere 5μC18–100 Å, H_2O–MeCN, 3:17) to yield **1a** (2.0 mg).

(*E*)-8-hydroxy-5-methoxy-3-(1-pentenyl)isocoumarin (**1a**): colorless crystals, UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 244.5 (4.27), 253 (4.25), 264 *sh* (3.77), 285 *sh* (3.89), 297 (4.00), 308.5 (3.96), 367 *sh* (3.83), 386 (3.96), 405 *sh* (3.85); IR ν_{max}^{KBr} cm^{-1}: 1686, 1649, 1620, 1580, 1478; ^{1}H NMR ($CDCl_3$): δ 0.96 (3H, *t*, *J* = 7.5 Hz, H_3-13), 1.51 (2H, *sext*, *J* = 7.5 Hz, H_2-12), 2.22 (2H, *qd*, *J* = 7.5, 1.0 Hz, H_2-11), 3.87 (3H, *s*, 5-OCH_3), 6.05 (1H, *dt*, *J* = 15.5, 1.0 Hz, H-9), 6.59 (1H, *dt*, *J* = 15.5, 7.5 Hz, H-10), 6.63 (1H, *br s*, H-4), 6.87 (1H, *d*, *J* = 9.0 Hz, H-7), 7.13 (1H, *d*, *J* = 9.0 Hz, H-6), 10.51 (1H, *br s*, 8-OH); ^{13}C NMR: see Table 2. NOESY correlations: H-6 ↔ OCH_3, H-9 ↔ H_2-11; HR-EIMS *m*/*z*, Calcd for $C_{15}H_{16}O_4$ $[M]^+$: 260.1049. Found: 260.1045.

Acknowledgements

This research was financially supported by Grant-in-Aid (C)(# 13836009 and #15510186) from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan, the Kobe Pharmaceutical University Collaboration Fund and a grant of the Fujisawa Foundation to Y.T. We are grateful to Professor H. Miyawaki (Saga University, Japan) for identification of the voucher specimen. Thanks are also due to Dr. M. Sugiura (Kobe Pharmaceutical University) for ^{1}H and ^{13}C NMR spec-

tra, and to Dr. K. Saiki (Kobe Pharmaceutical University) for mass spectra measurements.

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