Production of Xanthones with Free Radical Scavenging Properties, Emodin and Sclerotiorin by the Cultured Lichen Mycobionts of *Pyrenula japonica*

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From the cultures of the spore-derived mycobionts of the lichen *Pyrenula japonica,* two new xanthones, 1,8-dihydroxy-3-hydroxymethyl-5-methoxyxanthone and 1,2,8-trihydroxy-5-methoxy-3-methylxanthone, were isolated along with 1,7-dihydroxy-3-methylxanthone, 1,5,8-trihydroxy-3-methylxanthone, 1,8-dihydroxy-5-methoxy-3-methylxanthone, emodin and sclerotiorin. Their structures were determined by spectroscopic methods. Sclerotiorin was isolated for the first time from lichen mycobionts. Radical scavenging activities of the isolated xanthones were also studied.

Introduction

Lichens, several of which have important medicinal usages as crude drugs, produce diverse secondary metabolites. Some of lichen substances have been found to exhibit a wide range of potentially useful biological activities (Yamamoto, 1991). Recent studies demonstrated that cultures of spore-derived lichen mycobionts have an ability to produce novel metabolites under osmotically stressed conditions (Tanahashi *et al.*, 1997; Tanahashi *et al.*, 2000; Takenaka *et al.*, 2000). 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 have recently isolated from the cultured mycobionts of *Pyrenula japonica* and *P. pseudobufonia*, three xanthones, 1,8-dihydroxy-5-methoxy-3-methylxanthone (**1**), 1,5,8-trihydroxy-3-methylxanthone (**2**) and 1,7-dihydroxy-3-methylxanthone (**3**) (Tanahashi *et al.*, 1999). As continuation of this study, we cultivated the spore-derived mycobionts *P. japonica* collected in Shiga, Japan, and isolated from the cultures two new xanthones, **4** and **5**, along with five known compounds. In this paper, we report the structure determination of the new compounds and the evaluation of radical scavenging activities of the isolated xanthones.

Materials and Methods

General experimental procedures

Melting points were measured on a Yanaco 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 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

Specimens of *Pyrenula japonica* Kurok. were collected from the bark in Shiga Prefecture, Japan (200 m alt.) in 1996. The voucher specimen was identified by Dr. H. Miyawaki, Saga University, Japan and was deposited at Osaka City Institute of Public Health and Environmental Sciences with the registration No. NH9672154. Mycobionts of *P. japonica* were obtained from the spores discharged from apothecia of a thallus, and were cultivated in 102 test tubes containing modified

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MY 10 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. Black compact colonies with yellow crystals which covered large areas of colonies and surrounding agar were found in each test tube. After cultivation for 5 months, the colonies and slants with crystals were harvested.

Extraction and isolation

The harvested colonies (dry weight 75.5 g) were extracted with Et_2O. The combined extracts were concentrated *in vacuo* to give a residue of 2.62 g, which was suspended in $CHCl_3$ and the $CHCl_3$-soluble materials were submitted to CC on Sephadex LH-20 CC ($CHCl_3$–MeOH 6:4 v/v). The fractions, which contained yellow compounds, were combined and repeatedly subjected to preparative TLC with C_6H_6-acetone (9:1) and toluene-acetone (95:5), giving rise to **1** (133.3 mg), **2** (212.0 mg), a mixture of **2** and **3** (248.4 mg), **4** (8.2 mg), **5** (27.0 mg), **6** (6.1 mg) and **7** (184.1 mg). A mixture of **2** and **3** was further purified by preparative TLC (toluene-AcOH, 20:3), yielding **2** (181.3 mg) and **3** (33.1 mg).

1,8-Dihydroxy-3-hydroxymethyl-5-methoxyxanthone (**4**): Yellow needles, m.p. 221–221.3 °C ($CHCl_3$–MeOH). UV λ_{max}^{MeOH} nm (log ε): 236 (4.17), 255 (4.29), 263 sh (4.24), 271 sh (4.16), 341 (3.84), 387.5 (3.27). IR ν_{max}^{KBr} cm^{-1}: 3533, 1659, 1634, 1609, 1589, 1493. ^1H NMR (DMSO-d_6): δ 3.86 (3H, s, 5-OCH_3), 4.57 (2H, br s, 3-CH_2OH), 5.58 (1H, br s, 3-CH_2OH), 6.71 (1H, d, *J*=9.0 Hz, H-7), 6.75 (1H, br s, H-4), 6.98 (1H, br s, H-2), 7.45 (1H, d, *J*=9.0 Hz, H-6), 11.10, 11.61 (each 1H, br s, OH). ^{13}C NMR: Table I. HMBC correlations: H-2→C-1, 3-CH_2OH, C-4, C-9, C-9a, 3-CH_2OH→C-2, C-3, C-4, H-4→C-4a, 3-CH_2OH, C-9a, 5-OCH_3→C-5, C-6, H-6→C-4b, C-5, C-7, C-8, H-7→C-5, C-6, C-8, C-8a. HR-EIMS *m/z*: Calcd for $C_{15}H_{12}O_6$ $[M]^+$: 288.0634. Found: 288.0652.

1,2,8-Trihydroxy-5-methoxy-3-methylxanthone (**5**): Yellow needles, m.p. 214.4–214.8 °C (MeOH–DMSO). UV λ_{max}^{MeOH} nm (log ε): 206 (4.23), 243 (4.17), 263 (4.18), 280 (4.27), 351.5 sh (3.58), 420.5 sh (3.42). IR ν_{max}^{KBr} cm^{-1}: 3487, 1663, 1636, 1607, 1576, 1489. ^1H NMR (DMSO-d_6): δ 2.30 (3H, s, 3-CH_3), 3.88 (3H, s, 5-OCH_3), 6.70 (1H, d, *J*=9.0 Hz, H-7), 6.95 (1H, br s, H-2), 7.45 (1H, d, *J*=9.0 Hz, H-6). ^{13}C NMR: Table I. NOESY correlations: 3-CH_3↔H-4, 5-OCH_3↔H-6. HMBC correlations: H-2→C-1, 3-CH_3, C-4, C-9, C-9a, 3-CH_3→C-2, C-3, C-4, 5-OCH_3→C-5, H-6→C-4b, C-5, C-8, H-7→C-5, C-6, C-8, C-8a. HR-EIMS *m/z*: Calcd for $C_{15}H_{12}O_6$ $[M]^+$: 288.0634. Found: 288.0629.

Sclerotiorin (**7**): Orange needles, m.p. 188–190 °C (MeOH). $[\alpha]_D$ + 409° (*c* = 0.54, $CHCl_3$). UV λ_{max}^{MeOH} nm (log ε): 202 (4.01), 223 sh (3.99), 266 sh (3.90), 274 (3.94), 285.5 (3.93), 296 sh (3.90), 364 (4.29), 419 sh (4.04), 447 sh (3.88), 482 sh (3.52). IR ν_{max}^{KBr} cm^{-1}: 1738, 1722, 1661, 1631, 1522. ^1H NMR (CD_3OD): δ 0.88 (3H, t, *J*=7.5 Hz, 7′-CH_3), 1.02 (3H, d, *J*=6.5 Hz, 5′-CH_3), 1.33, 1.46 (each 1H, m, H_2-6′), 1.53 (3H, s, 7-CH_3), 1.88 (3H, d, *J*=1.5 Hz, 3′-CH_3), 2.12 (3H, s, 7-OAc), 2.53 (1H, m, H-5′), 5.74 (1H, br d, *J*=10.0 Hz, H-4′), 6.36 (1H, d, *J*=16.0 Hz, H-1′), 6.86 (1H, s, H-4), 7.18 (1H, d, *J*=16.0 Hz, H-2′), 8.19 (1H, s, H-1). ^{13}C NMR (CD_3OD): δ 12.3 (7′-CH_3), 12.5 (3′-CH_3), 19.9 (7-$OCOCH_3$), 20.6 (5′-CH_3), 22.9 (7-CH_3), 31.2 (C-6′), 36.3 (C-5′), 86.2 (C-7), 107.3 (C-4), 110.6 (C-5), 115.8 (C-8a), 117.4 (C-1′), 133.9 (C-3′), 141.5 (C-4a), 144.1 (C-2′), 149.4 (C-4′), 155.2 (C-1), 160.5 (C-3), 170.0 (7-$OCOCH_3$), 188.0 (C-6), 192.8 (C-8). EIMS *m/z* (%): 392 $[^{37}ClM]^+$ (27), 390 $[M]^+$ (72), 348 (100), 306 (66).

Methylation of **5**

To a solution of compound **5** (5.0 mg) in acetone (30 ml) were added K_2CO_3 (120 mg) and Me_2SO_4 (11 ml), and the whole was heated under reflux for 7 h. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The washed and dried organic layer was concentrated *in vacuo* and the residue was purified by preparative TLC (C_6H_6-acetone, 9:1) to yield **8** (1.1 mg) as a crystalline solid. ^1H NMR ($CDCl_3$): δ 2.37 (3H, s, 3-CH_3), 3.89 (3H, s, 2-OCH_3), 3.94 (3H, s, 8-OCH_3), 3.95 (3H, s, 5-OCH_3), 4.02 (3H, s, 1-OCH_3), 6.67 (1H, d, *J*=9.0 Hz, H-7), 7.11 (1H, d, *J*=9.0 Hz, H-6), 7.13 (1H, br s, H-4). NOESY correlations: 2-OCH_3↔3-CH_3, 3-CH_3↔H-4, 5-OCH_3↔H-6, H-7↔8-OCH_3. EIMS *m/z* (%): 330 $[M]^+$ (35), 315 (100), 300 (7.3), 285 (28). HR-EIMS *m/z*: Calcd for $C_{18}H_{18}O_6$ $[M]^+$: 330.1104. Found: 330.1091.

Measurement of free radical scavenging activity

The EtOH solution (0.6 ml) of each test sample was added to a mixture of 0.1 M acetic acid buffer

(pH 5.5, 0.6 ml) and 0.25 mM DPPH EtOH solution (0.3 ml) in a test tube and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. α-Tocopherol and BHT were used as standard samples.

Results and Discussion

The polyspore-derived mycobionts of *Pyrenula japonica* Kurok. collected in Japan were cultured on conventional malt-yeast extract medium supplemented with 10% sucrose at 18° in the dark. After cultivation of 5 months, the colonies with yellow crystals and agar medium were extracted with Et_2O. The extract was separated by a combination of CC and preparative TLC to afford seven compounds, **1–7**.

Compounds **1–3** were identified as 1,8-dihydroxy-5-methoxy-3-methylxanthone, 1,5,8-trihydroxy-3-methylxanthone and 1,7-dihydroxy-3-methylxanthone by direct comparison with authentic samples isolated from the cultured mycobionts of *P. japonica* (Tanahashi *et al.*, 1999). Compound **6** was identified with an authentic emodin. Emodin has already been isolated from the cultured mycobionts of *Xanthoria fallax* and an unidentified *Caloplaca* spp. (Nakano *et al.*, 1972), but not from those of the genus *Pyrenula*. Compound **7** was determined to be sclerotiorin by its spectroscopic analysis. The sign of its optical rotation ($[\alpha]_D$ + 409°) was the same as a fungal metabolite, (+)-sclerotiorin (Curtin *et al.*, 1940; Whalley *et al.*, 1976), but the multitude was smaller than that ($[\alpha]_D$ + 500°) described in the literature (Birkshaw, 1952). This result could be accounted for by the possibility that compound **7** contained a stereoisomer such as 7-epi-sclerotiorin (Gregory *et al.*, 1963) but we could not come to a conclusion. Azaphilones such as sclerotiorin have never been reported as a constituent of the cultured mycobionts of lichens, and the present work constitutes the first instance.

Table I. ^{13}C NMR spectral data of compounds **1, 4** and **5**.

C	4[a]	5[a]	1[b]
1	160.0	145.8	161.0
2	107.7	138.4	111.7
3	154.8	137.0	149.9
4	104.2	107.6	108.0
4a	155.6	147.7	156.0
4b	144.8	145.0	145.6
5	139.7	139.7	140.0
6	121.1	120.5	108.0
7	108.9	108.2	109.1
8	152.7	152.6	154.2
8a	107.8	107.6	108.3
9	184.9	185.1	185.6
9a	106.1	105.9	105.9
3-CH_3	–	17.1	22.6
3-CH_2OH	62.2	–	–
5-OCH_3	56.7	56.6	57.4

[a] Measured in DMSO-d_6; [b] Measured in $CDCl_3$.

Compound **4** was isolated as yellow needles, m.p. 221–221.3 °C. Its HR-EI mass spectrum exhibited a strong peak at *m/z* 288.0652 $[M]^+$, indicating a molecular formula of $C_{15}H_{12}O_6$. It showed similar UV and IR spectral features to those of 1,8-dihydroxy-5-methoxy-3-methylxanthone (**1**).

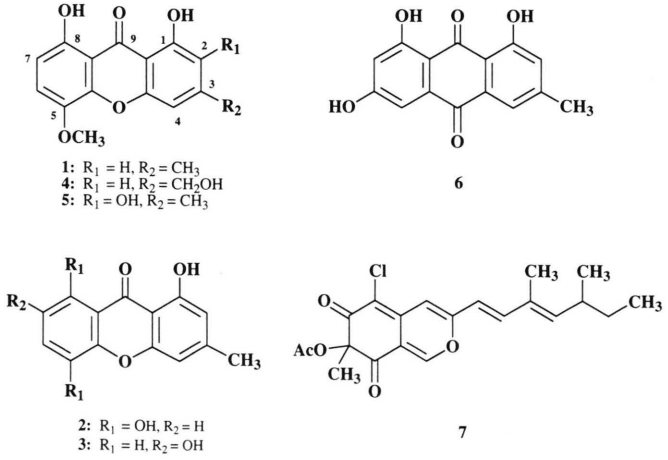

Fig. 1. Compounds **1–7** isolated from the cultured lichen mycobionts of *Pyrenula japonica*.

Its ^{1}H NMR spectrum exhibited signals for a methoxyl group at δ 3.86 (s), two aromatic protons at δ 6.46 and 6.71 (each br s), a pair of *ortho*-coupled doublets at δ 6.75 and 6.98 (each 1H, d, *J*=9.0 Hz) and two chelated hydroxyl groups at δ 11.10 and 11.61 as well as the signals assignable to a hydroxymethyl group at δ 4.57 (2H, br s) and δ 5.58 (br, OH). The ^{13}C NMR data of **4** were nearly identical with those of **1** except for the presence of the carbon signal resonating at δ 62.2 instead of the methyl carbon as in **1** and downfield shift of C-3. These findings indicated that compound **4** has a hydroxymethyl group at C-3 in place of a methyl group as in **1**. The position of the substituents on aromatic rings was further confirmed by its NOESY spectrum, which showed significant correlations between the methoxyl and a doublet at δ 7.45 and between the hydroxymethyl and two broad singlets resonating at δ 6.75 and 6.98. All spectral data of HMQC and HMBC experiments were fully consistent with the proposed structure for the isolated compound. Accordingly the structure of **4** was elucidated as 1,8-dihydroxy-3-hydroxymethyl-5-methoxyxanthone.

In the HR-MS, compound **5** showed a molecular ion at *m/z* 576.2619, corresponding to the molecular formula $C_{15}H_{12}O_6$, and the UV absorptions at 206, 243, 263, 280, 351.5 and 420.5 nm were indicative of a xanthone structure. Its ^{1}H NMR spectral features [a methyl group at δ 2.30, a methoxyl at δ 3.88, a pair of *ortho*-coupled aromatic protons at δ 6.70 and 7.45 (each d, *J*=9.0 Hz) and an aromatic proton at δ 6.95 (br s)] showed close similarity to those of **1**, the significant difference in their spectra being that **5** showed a broad singlet at δ 6.95 instead of a pair of *meta*-coupled aromatic proton signals as in **1**. These findings suggested that **5** possess an additional phenolic hydroxyl group in the ring A of **3**. The location of the hydroxyl group at C-2 was suggested by upfield shifts of C-1, C-3 and C-4a, relative to those of **1**, as well as the results of HMBC and NOESY experiments. In order to confirm the suggestion, compound **5** was methylated with Me_2SO_4 to give **8**. In the NOESY spectrum of **8**, the C-methyl group was correlated with an aromatic proton at δ 7.13 and OMe at δ 3.89, but no interaction between the aromatic proton and OMe at δ 4.02 was observed. These findings ruled out the possible structure with a hydroxyl group at C-4 for the new compound. Thus, compound **5** was characterized as 1,2,8-trihydroxy-5-methoxy-3-methylxanthone.

We have previously reported that xanthones **1–3** were commonly produced in lichen mycobionts of different species isolated from highly unrelated locations (Tanahashi *et al.*, 1999). In this study the same xanthones were isolated again from the cultured mycobionts of *P. japonica* from another collection site, although these compounds have not yet been found in lichenized condition. The occurrence of these xanthones in the cultures of lichen mycobionts is of great interest from the viewpoint of their physiological and biological significance, e.g. antioxidant and antibacterial activities, and may account for a better chance of survival of the mycobiont in prelichenized condition.

In this context, the antioxidant activities of compounds **1–3** and **5** were studied. Fig. 2 shows the dose-response curves for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the

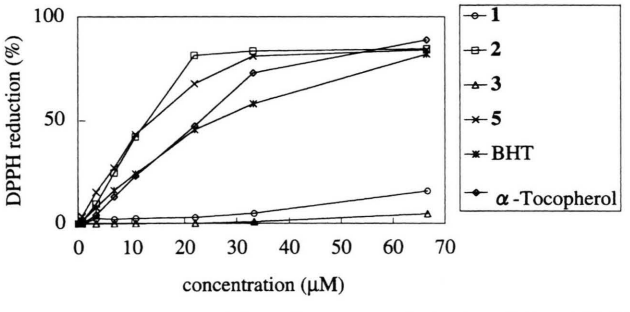

Fig. 2. Scavenging activity of compounds **1–3** and **5** on DPPH radical.

tested compounds. The radical scavenging activities of compounds **2** and **5** were higher than those of well-known antioxidants, α-tocopherol and 2,6-di(*tert*-butyl)-4-methylphenol (BHT), while compounds **1** and **3** showed only low activity. Considering the antioxidant activities of these xanthones, it is worth noting the importance of the two hydroxyl groups in the *ortho*-diphenolic arrangement or hydroquinone structure. Methylation of the hydroquinone in **3** reduces the activity and an isolated hydroxyl group in **5** is inefficient for the DPPH radical scavenging. Although further study is needed to know the radical scavenging activity against superoxide anion radical, hydroxy radical and peroxy radical, the present results demonstrated the possibility of the xanthones to serve as antioxidant in the cultured lichen mycobionts.

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