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NOTE

Antioxidant activity of red pigments from the lichens *Lethariella sernanderi*, *L. cashmeriana*, and *L. sinensis*

Kaoru Kinoshita · Tadayasu Togawa · Akira Hiraishi · Yuko Nakajima · Kiyotaka Koyama · Takao Narui · Li-song Wang · Kunio Takahashi

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Abstract A yellow and new dark red pigments were isolated from *Lethariella sernanderi*, *L. cashmeriana*, and *L. sinensis* as antioxidant components. The yellow pigment was identified as canarione (**1**), and the others were determined to be 1,2-quinone derivatives, rubrocashmeriquinone (**2**) and 7-chlororubrocashmeriquinone (**3**), and 7-chlorocanarione (**4**) by analysis of their spectroscopic data.

Keywords Lichens · *Lethariella sernanderi* · *L. cashmeriana* · *L. sinensis* · Canarione · 7-Chlorocanarione · Rubrocashmeriquinone · 7-Chlororubrocashmeriquinone · NMR

Introduction

The lichens *Lethariella* spp. were used for tea named 紅雪茶 or 鹿心茶 in Yunnan, China. In recent years, this tea has also been consumed in Japan owing to its associated health benefits. During our studies on secondary metabolites from *Lethariella* spp., we isolated sernandrin and known compounds from *Lethariella sernanderi* [1, 2]. In this paper, we report the antioxidant activity of hot water extracts of *L. sernanderi*, *L. sinensis*, and *L. cashmeriana*. Each extract was red in color, and we found some pigments from each methanol extract of the aforementioned thalli.

K. Kinoshita · T. Togawa · A. Hiraishi · Y. Nakajima · K. Koyama · T. Narui · K. Takahashi (✉)
Meiji Pharmaceutical University,
2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan
e-mail: diamonds@my-pharm.ac.jp

L. Wang
Kunming Institute of Botany,
Chinese Academy of Science,
Heilongtan, 650204 Kunming, China

Results and discussion

The hot water extracts of *L. sinensis* and *L. cashmeriana* were developed on TLC and produced two red spots. These spots guided the separation that was successfully performed for each MeOH extract of lichen thalli. One of the red spots on TLC from both extracts was elucidated to be due to the same compound (**3**); the other red spot was due to a different compound. In the case of *L. sernanderi*, the red compounds were identified in the acetone extract on TLC and isolated from acetone extracts. From the acetone extract of *L. sernanderi*, a yellow pigment (**1**) was isolated together with dark red compounds (**2** and **3**). The two new red pigments (**2** and **3**) were also isolated from the insoluble part of the MeOH extract of *L. sinensis*. Moreover, **3** and **4** were isolated from the MeOH extract of *L. cashmeriana*. Compound **1** was identified to be canarione, and compound **4** was determined to a derivative of **1**, namely 7-chlorocanarione. The structures of **2** and **3** (Fig. 1) were determined to be 1,2-quinone derivatives by analysis of their spectroscopic data.

Compound **1**, yellow pigment, had 14 carbons (one methyl group, three methines, and ten quaternary carbons) on ^{13}C NMR (Table 1) and DEPT spectra (in DMSO-d_6). From high resolution EIMS, the molecular formula was determined to $C_{14}H_8O_6$. The ^1H NMR spectrum of **1** showed a methyl group at δ 2.42 and three aromatic protons at δ 6.08 (s), 6.39 (s), and 7.41 (s). From HMBC correlations, the structure of **1** was considered to canarione. Compound **1** was then methylated by using CH_2N_2 to give the corresponding dimethyl ether (**1a**). The two new

methoxyl proton signals at δ 3.62 and 3.80 (each 3H, s) in the ^{1}H NMR spectra of **1a** were assigned as C-5 and C-8. The methoxyl protons at δ 3.62 showed an NOE to the methine proton at δ 5.96 (CH-7). Thus compound **1** was identified as canarione [3]. Although canarione has already been identified in *L. sinensis*, *L. cashmeriana*, and *L. sernanderi* by TLC [4], this is the first isolation of the compound as a component from the thallus.

Compound **2** was obtained as a dark red amorphous solid which had the same Rf value on TLC ($CHCl_3$/MeOH/H_2O 8:4:0.5) as **1**. Compound **2** was assigned the molecular formula $C_{14}H_8O_6$ according to the HREIMS spectra data *m/z* 272.0318 [M^+] (calcd 272.0321), which was same as for compound **1**. Moreover, the ^{1}H and ^{13}C NMR spectral pattern (Table 1) of **2** was similar to that of **1**. The ^{1}H NMR spectrum of **2** showed a methyl group at δ 2.32, three aromatic protons at δ 6.11 (s), 5.36 (s), and 7.13 (s) and a hydrogen-bonded hydroxy group at δ 17.24 (brs). On the other hand, the UV spectrum of **2** showed a longer wavelength absorption (496 nm) than **1**. Generally, the UV spectrum of 1,2-quinones exhibit longer wavelength absorption than 1,4-quinone, e.g., 1,4-benzoquinone has absorptions at 242, 281, and 434 nm, whereas 1,2-benzoquinone has absorptions at 390 and 610 nm. From the above results and HMBC correlations (Fig. 2), the structure of **2** was therefore deduced to have a 1,2-quinone moiety instead of a 1,4-quinone as in canarione (**1**) and was determined as shown in Fig. 1. Compound **2** is a new compound named rubrocashmeriquinone.

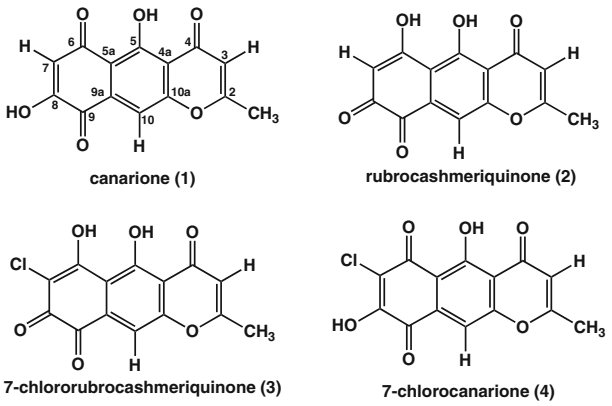

Fig. 1 The structures of isolated compounds (**1**–**4**)

Compound **3** was obtained as dark purple amorphous solid. The molecular formula, $C_{14}H_7O_6Cl$, was established by ESI (positive) V mode TofMS measured on the ion at *m/z* 306.9999 ([M + H]$^+$, calcd for $C_{14}H_8O_6^{35}Cl$, 307.0009) and ESI (negative) V mode TofMS measured on the ion *m/z* 304.9846 ([M − H]$^-$, calcd for $C_{14}H_6O_6^{35}Cl$, 304.9853). The ^{1}H NMR spectrum of **3** showed a methyl group at δ 2.31, two aromatic protons at δ 6.11 (s), and 7.18 (s) and hydrogen-bonded hydroxy group at δ 16.33 (brs). These chemical shifts were almost the same as with **2**, but one aromatic proton signal of **2** at δ 5.36 (s) (7-H) did not appear in the ^{1}H NMR spectrum of **3**. From the above, we deduced that the 7-H aromatic proton of **2** was replaced by 7-Cl in **3**. The structure of **3** was thus determined as shown as Fig. 1.

Compound **4** was obtained as a dark red powder, which showed the same Rf value on TLC ($CHCl_3$/MeOH/H_2O 8:4:0.5) as **3**. Its molecular formula, $C_{14}H_7O_6^{35}Cl$, was established by high resolution EIMS spectrometry. The ^{1}H and ^{13}C NMR signals were almost the same as with **1**, but one aromatic proton signal of **1** at δ 6.08 (s) (7-H) did not appear in the ^{1}H NMR spectrum of **4**. From the above, we

Table 1 ^{13}C and ^{1}H NMR spectral data for **1**–**4** (in DMSO-d_6)

Position	**1**		**2**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	168.3		165.1		164.9		167.9	
3	110.9	6.39 (s)	111.7	6.11 (s)	112.1	6.11 (s)	110.0	6.39 (s)
4	180.0[a]		176.7		176.0[a]		179.6	
4a	114.4		116.5		116.6		114.7	
5	161.9		159.0		161.9		159.3[a]	
5a	111.5		111.8		111.2		111.4	
6	185.4[a]		163.3		167.8		177.1[b]	
7	112.8	6.08 (s)	105.1	5.36 (s)	110.2		116.6	
8	159.0		184.2		178.1[a]		159.0	
9	180.0		184.0		182.0		179.4[b]	
9a	135.4		134.6		133.6		134.7	
10	106.4	7.41 (s)	104.9	7.13 (s)	106.0	7.18 (s)	106.4	7.39 (s)
10a	159.0		159.0		158.9		158.8[a]	
2-CH_3	19.7	2.42 (s)	19.3	2.32 (s)	19.3	2.31 (s)	19.7	2.42 (s)
5-OH				17.24 (s)		16.33 (s)		

^{1}H NMR spectral data (400 MHz) and ^{13}C NMR spectral data (100 MHz). δ in ppm from TMS

[a,b] Assignments may be interchanged

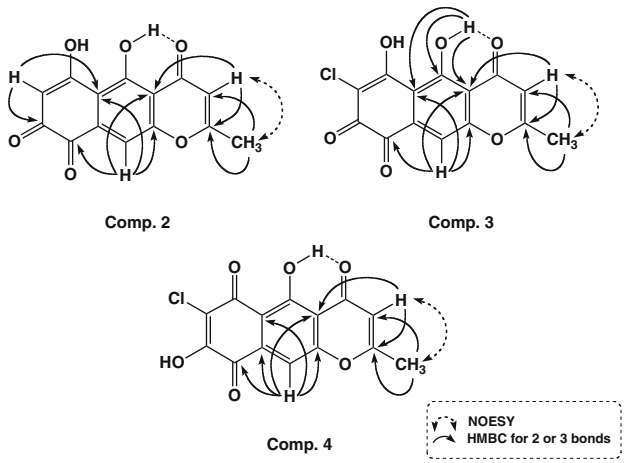

Fig. 2 HMBC correlation of compounds **2–4**

Table 2 Antioxidant activity of hot water extracts and compounds **1–4**

Sample	Concentration	Activity[a]
L. sernanderi hot water extract	100 mg/mL[b]	163.9
L. cashmeriana hot water extract	10 mg/mL	35.4
L. sinensis hot water extract	10 mg/mL	23.8
1	3.6×10^{-2} M[c]	137.2
2	3.6×10^{-2} M	87.2
3	3.3×10^{-2} M	277.4
4	3.3×10^{-2} M	142.7
Quercetin	1 mM	100.0

[a] Activity of 1 mM quercetin was arbitrarily taken as 100

[b] Sample was dissolved in DMSO

[c] Sample was dissolved in MeOH

deduced that the 7-H aromatic proton of **1** was replaced by 7-Cl in **4**. Together with HMBC correlations (shown in Fig. 2), compound **4** was deduced to be a new compound, 7-chlorocanarione.

The antioxidant activities of hot water extracts of *L. sernanderi*, *L. sinensis*, and *L. cashmeriana* and the isolated compounds were examined by using a commercial assay kit based on the Cu(II) reducing activity. The activity was expressed relative to that shown by 1 mM quercetin, the latter arbitrarily taken as 100 [5]. Compound **3** showed the strongest activity among the four isolated compounds (Table 2), which suggested that the 1,2-quinone and 7-Cl moieties were important for antioxidant activity. The hot water extracts contained the antioxidant compounds of different polarity. It was thought the combination of various compounds has an important significance for overall antioxidant effect. Daily intake of *Lethariella* spp. may therefore exert higher health benefits owing to its antioxidant effect.

Experimental

General

Melting points were determined on a Yanagimoto MP micromelting point apparatus. The IR spectra were measured with a JASCO A-102 IR spectrophotometer or JASCO IR Report-100 infrared spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded using a JEOL JNM-AL-400 (^{1}H 400 and ^{13}C 100 MHz) and a JEOL JNM-LA500 (^{1}H 500 and ^{13}C 125 MHz) spectrometer in DMSO-d_6 using TMS as internal standard. The MS spectra were obtained using a JEOL JMS-700. Column chromatography was carried out on silica gel 60N (spherical, neutral, 63–210 μM, Kanto Chemical Co., Inc.) and GE Healthcare Sephadex LH-20.

Plant material

The lichen thalli (Useaceae), *L. sinensis* Wei and Jiang and *L. cashmeriana* Krog., were collected and identified by Dr. Wong Li-Song in Yunnan, China, in 2005. *Lethariella sernanderi* (Most.) Obermayer, was purchased at Kunming City market, Yunnan province, in the People's Republic of China in 2002 and identified by C. F. Culberson. A voucher specimen is deposited at the Department of Pharmacognosy, Meiji Pharmaceutical University.

Extraction and isolation

L. sernanderi (10 g) was extracted with hot water (150 mL) for 10 min, three times. The extract was lyophilized (0.9 g) and used for testing antioxidant activity. The ether extract (10.8 g), acetone extract (49.5 g), and MeOH extract (36.9 g) were already obtained from *L. sernanderi* (1 kg). Some compounds including one new one were isolated from fraction (Fr.) I to Fr. V of the acetone extract [2]. In this report the red-colored pigments of the hot water extract guided separation that was performed on Fr. IV and these were compared with the red spot of the hot water extract by TLC. Fraction IV was partitioned into a MeOH-soluble part (0.9 g) and an insoluble part (0.2 g). Antioxidant activities of both parts were examined. The MeOH-soluble part was subjected to Sephadex LH-20 column chromatography using MeOH to give compound **1** (canarione, 17.5 mg), **2** (14.1 mg), **3** (8.7 mg). The red pigments observed on TLC of the hot water extract were identified as two compounds, **2** and **3**.

Lethariella cashmeriana (512 g) was extracted with hot water (3 L) for 10 min, three times. The extract was lyophilized and extracted repeatedly with acetone and then MeOH (each 3 L, three times) to yield each extract (1.7 and 13.8 g), respectively. The MeOH extract was subjected to column

chromatography on silica gel using a $CHCl_3$/MeOH/H_2O stepwise gradient system (30:10:0.5 → 30:15:0.8 → 6:4:1) to yield five fractions, Fr. A to Fr. E. Fraction D was separated by Sephadex LH-20 column chromatography using MeOH to give Fr. D-1 to Fr. D-5. Compound **3** (11.7 mg) was isolated from Fr. D-3 by Sephadex LH-20 column chromatography using MeOH as eluant. Compound **4** (6.7 mg) and compound **3** (11.7 mg) were isolated from Fr. D-4 by the combination of HPLC (eluant $CHCl_3$/MeOH/H_2O) and Sephadex LH-20 column chromatography (eluant MeOH). One of the red pigments observed on TLC of the hot water extract was identified as compound **4**. Compound **3** is another novel red pigment.

L. sinensis (10 g) was extracted with hot water (150 mL) for 10 min, three times. The extract was lyophilized (0.7 g) and used for the antioxidant activity assay. *L. sinensis* was extracted with acetone (17 L × 3) and MeOH (15 L × 3) to yield the acetone extract (76.1 g) and MeOH extract (54.5 g). The separation of the red pigment was guided by comparison with the red spot of the hot water extract on TLC. The MeOH extract (1.56 g) was subjected to Sephadex LH-20 column chromatography using a MeOH eluant to give six fractions, Fr. 1 to Fr. 6. Fraction 4 was further separated by silica gel column chromatography using a $CHCl_3$/MeOH/H_2O stepwise gradient system (30:10:0.5 → 30:15:0.8 → 6:4:1) to yield four fractions, Fr. 4-1 to Fr. 4-4. Compound **2** was isolated from both Fr. 4-3 and Fr. 6 to yield 3.3 and 5.0 mg, respectively, by Sephadex LH-20 column chromatography using MeOH. Compound **3** was isolated from both Fr. 4-3 and Fr. 5 to yield 3.3 and 2.0 mg by Sephadex LH-20 column chromatography using MeOH. The red pigments observed on TLC of the hot water extract were identified as two compounds, **2** and **3**.

*Canarione (**1**)*

Yellow powder, mp 145.5–146.3°C. UV (MeOH) λ_{max} (log ε): 215 (4.07), 265 (3.94), 365 (3.34); IR ν_{max} (KBr) cm^{-1}: 3450, 1655, 1620, 1440, 1400, 1260, 1195, 1090. HREIMS *m/z*: 272.0319 (calcd for $C_{14}H_8O_6$, 272.0321). EIMS *m/z*: 272 (100), 244 (86), 216 (21). ^1H and ^{13}C NMR data are presented in Table 1.

*Rubrocashmeriquinone (**2**)*

Dark red amorphous solid, mp 174°C [decomposed (dec.)]. UV (MeOH) λ_{max} (log ε): 223 (4.28), 278 (4.11), 378 (3.60), 496 (3.17); IR ν_{max} (KBr) cm^{-1}: 3425, 1660, 1580, 1460, 1400, 1260, 1185, 1090, 1025, 960. HREIMS *m/z*: 272.0318 (calcd for $C_{14}H_8O_6$, 272.0321). EIMS *m/z*: 272 (100), 244 (73), 216 (20). ^1H and ^{13}C NMR data are presented in Table 1.

*7-chlororubrocashmeriquinone (**3**)*

Dark purple amorphous solid, mp 149°C (dec.). UV (MeOH) λ_{max}: 225 (4.19), 275 (3.98), 380 (3.38), 515 (2.96); IR ν_{max} (KBr) cm^{-1}: 3430, 1655, 1585, 1460, 1400, 1260, 1195, 1095, 1025, 960. ESI (positive) V mode Tof-MS *m/z*: 306.9999 ($[M + H]^+$, calcd for $C_{14}H_8O_6^{35}Cl$, 307.0009), ESI (negative) V mode TofMS *m/z*: 304.9846 ($[M - H]^-$, calcd for $C_{14}H_6O_6^{35}Cl$, 304.9853), ^1H and ^{13}C NMR data are presented in Table 1.

*7-Chlorocanarione (**4**)*

Dark red powder, mp 136–138°C. UV (MeOH) λ_{max} (log ε) nm: 225 (4.20), 281 (4.02), 384 (3.48), 513 (3.14); IR ν_{max} (KBr) cm^{-1}: 3450, 1655, 1645, 1620, 1560, 1460, 1260, 1190, 1095, 970. HREIMS *m/z*: 305.9933 (calcd for $C_{14}H_7O_6^{35}Cl$, 305.9931). EIMS *m/z* : 308 ($[M + 2]^+$, 35), 306 ($[M^+]$, 100), 278 (17), 280 (50), 250 (15). ^1H and ^{13}C NMR data are presented in Table 1.

Antioxidant activity assay

The antioxidant activities of hot water extracts and isolated compounds were determined by using a commercial assay kit for total antioxidant power (Oxford Biochemical Research, USA). The principle of this assay was based on the Cu(II) reducing activity. *L. sernanderi* hot water extract was dissolved in DMSO and compound **1** was dissolved in MeOH. Other extracts and compounds were dissolved in distilled water. Samples were diluted in the buffer supplied with the kit containing bathocuproine and placed on 96-well plates, and their absorbance at 490 nm was determined in a microplate reader (Bio-Rad, Tokyo, Japan) to obtain blank values. Cu^{2+} solution was added and incubated at room temperature for 3 min and then measured for absorbance at 490 nm. A standard curve was prepared using uric acid.

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