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NEW RED PIGMENTS FROM LICHEN *LETHARIELLA SINENSIS*

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Abstract – New red pigments were isolated from a lichen thallus, *Lethariella sinensis* by Wei & Jiang. The structures of the red pigments, rubrosinensiquinones A (**1**), B (**2**), and C (**3**), were determined by spectroscopic analyses.

The lichens *Lethariella* spp. are used for a health-promoting tea named Lu xing cha or Hong xue cha in Yunnan, China and Tibet.¹ During our studies on secondary metabolites from *Lethariella* spp., we discovered three new red pigments, rubrocashmeriquinone (**4**), 7-chlororubrocashmeriquinone (**5**), and 7-chlorocanarione (**6**), along with one known yellow pigment, canarione (**7**) (Figure 1), and studied their antioxidant activity.² In this paper, we report additional new red pigments, named rubrosinensiquinones A (**1**), B (**2**), and C (**3**) from *Lethariella sinensis*.

The MeOH and H₂O extracts of *L. sinensis* were analyzed by TLC to reveal the presence of several red spots, which successfully guided the subsequent chromatographic separation of each extract. The two new red pigments (**1** and **2**) were isolated from the MeOH extract. Whereas, **3** was isolated from the H₂O extract. The structures of **1**, **2**, and **3** (Figure 2) were determined to be 1,2-quinone derivatives based on the following spectroscopic data. Compound **1**, dark red amorphous, was suggested to have the molecular formula C₂₀H₁₇NO₈ by its positive HR-ESI (electrospray ionization) MS spectral data *m/z* 400.1014 [M+H]⁺, (calcd for C₂₀H₁₈NO₈, [M+H]⁺ 400.1032). The IR spectrum exhibited absorptions for hydroxy

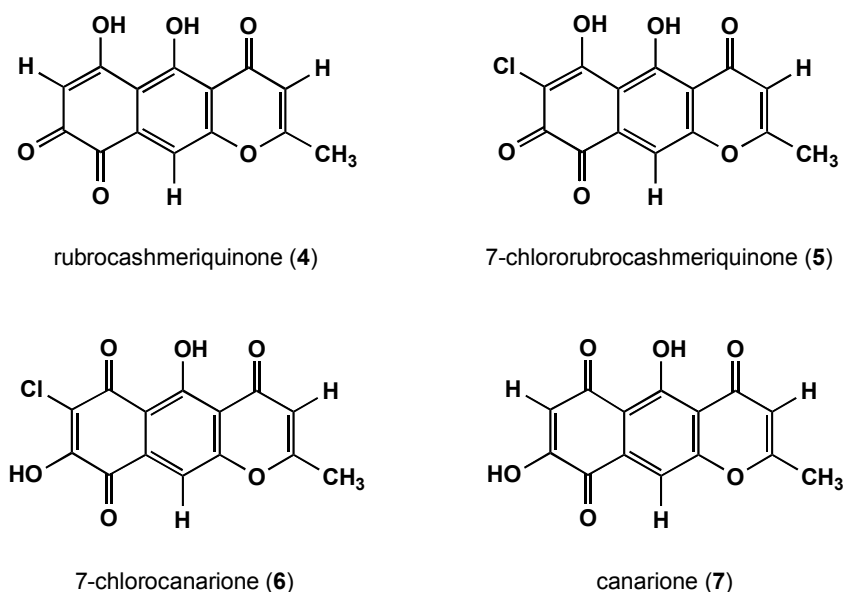


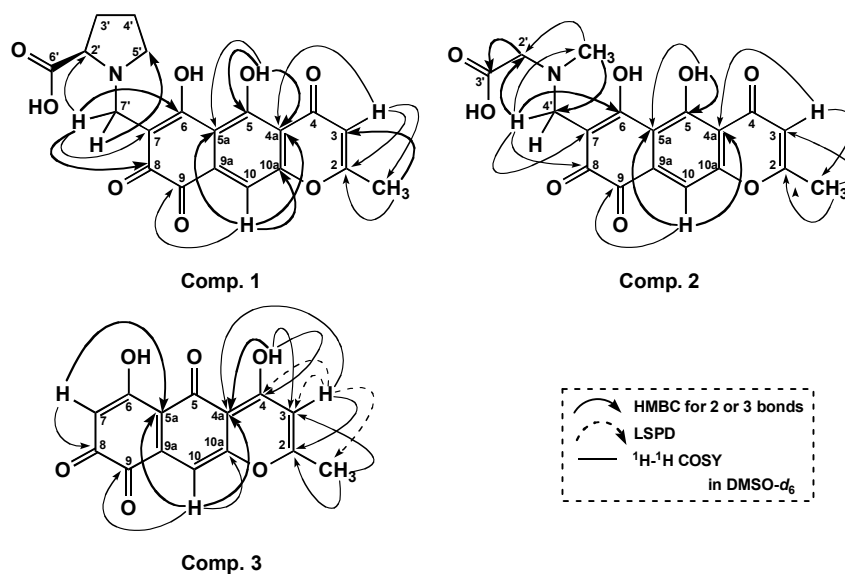
Figure 1. Structures of isolated compounds from *Lethariella* spp. (4-7)

(3440 cm^{-1}) and carbonyl ($1650, 1630\text{ cm}^{-1}$) groups. The ^1H and ^{13}C NMR data of **1** are shown in Table 1. Both spectroscopic signal patterns of **1** are similar to those of **4–7**. Therefore, we suggest that these are similar naphthoquinone derivatives. In addition, the UV spectrum (MeOH) of **1** showed a longer wavelength absorption (515 nm) than that of 1,4-naphthoquinone derivative **7**. Generally, the UV absorptions of 1,2-quinones tend to be at longer wavelengths than 1,4-quinones. For example 1,4-benzoquinone absorbs at 242, 281, and 434 nm , whereas 1,2-benzoquinone absorbs at 390 and 610 nm .³ Thus, the structure of **1** was determined to be a 1,2-quinone derivative. HMBC (Hetero-nuclear Multiple-Bond Connectivity) and ^1H - ^1H COSY data are presented in Figure 2. The methylene proton at $\delta 3.97$ (H-7'), with an HMQC (Hetero-nuclear Multiple Quantum Coherence) correlation with the $\delta 47.5$ (C-7') methylene carbon, showed HMBC correlations with the aromatic quaternary carbons at $\delta 106.3$ (C-7), 172.9 (C-6), 183.7 (C-8), the methylene carbon at $\delta 52.6$ (C-5'), and the methine carbon at $\delta 67.7$ (C-2'). HMBC and ^1H - ^1H COSY data of **1** indicated the presence of the fragment, $\delta 67.7$ (C-2') - $\delta 28.3$ (C-3') - $\delta 23.3$ (C-4') - $\delta 52.6$ (C-5'). Additionally, the structure of **1** contains nitrogen and carboxylic acid moieties according to the molecular formula of $\text{C}_{20}\text{H}_{17}\text{NO}_8$. The methine carbon at $\delta 67.7$ (C-2') and methylene carbon at $\delta 52.6$ (C-5') shifted downfield because these carbons were near the nitrogen. As a result, the structure of the proline (amino acid) with a methylene group on the nitrogen atom was considered. Furthermore this proline moiety was connected via a methylene group to C-7 of the quinone moiety. Comparing to the optical rotation data of proline derivatives,^{4,5} it was indicated that compound **1** with negative rotation was a derivative of (-)-*S*-proline. The IR data of **1** (1620 cm^{-1}) suggested the presence of a zwitter ion (COO^- and NH^+) on the proline moiety. Compound **1** was a new pigment, which we named rubrosinensiquinone A.

Compound **2**, dark red amorphous, was suggested to have the molecular formula $C_{18}H_{15}NO_8$ by its positive HR-ESIMS spectral data m/z 374.0856 $[M+H]^+$, (calcd for $C_{18}H_{16}NO_8$, $[M+H]^+$ 374.0876). The IR spectrum exhibited presences of hydroxy (3450 cm^{-1}) and carbonyl (1650 , 1640 , 1630 , and 1610 cm^{-1}) groups. The ^1H and ^{13}C NMR data of **2** are shown in Table 1. The UV spectrum (MeOH) of **2** was similar to that of **1**. HMBC data is presented in Figure 2. The methylene proton at δ 4.05 (H-4'), with an HMQC correlation with the δ 49.6 (C-4') methylene carbon, showed HMBC correlations with the aromatic quaternary carbons at δ 104.0 (C-7), 173.0 (C-6), 183.7 (C-8), the methylene carbon at δ 57.7 (C-2'), and the methyl carbon at δ 40.6 (1'- $\underline{\text{C}}\text{H}_3$). The methyl proton at δ 2.68 (1'- $\underline{\text{C}}\text{H}_3$) showed an HMBC correlation with the methylene carbons at δ 49.6 (C-4') and δ 57.7 (C-2'). The methylene proton at δ 3.45 (H-2') showed an HMBC correlation with the carbonyl carbon at δ 164.7 (C-3'). From the above, the 1-methylglycine with a methylene group on the nitrogen atom was considered as residual moiety. Furthermore 1-methylglycine moiety was connected via a methylene group to C-7 of the quinone moiety. Compound **2** was a new pigment, and named rubrosinensiquinone B.

Compound **3**, dark red amorphous, was suggested to have the molecular formula $C_{14}H_8O_6$ by its HR-EI (electron ionization) MS spectral data m/z 272.0319, (calcd for $C_{14}H_8O_6$, $[M]^+$ 272.0321). The IR spectrum exhibited presences of hydroxy (3440 cm^{-1}) and carbonyl (1660 cm^{-1}) groups. The ^1H and ^{13}C NMR data of **3** are shown in Table 1. Both spectroscopic signal patterns were similar to those of **4**. The UV spectrum (MeOH) of **3** showed almost the same absorption at 503 nm compared to **1** and **2**. HMBC and LSPD (long-range selective proton decoupling) data are presented in Figure 2. The aromatic methine proton of δ 6.06 (H-3) showed LSPD (long-range selective proton decoupling) correlations with the methyl carbon at δ 19.3 (2'- $\underline{\text{C}}\text{H}_3$), the aromatic methine carbon at δ 111.8 (C-3), and the aromatic quaternary carbon at δ 164.4 (C-4). The hydroxyl proton at δ 17.34 (4-OH) showed HMBC correlations with aromatic quaternary carbons at δ 116.6 (C-4a), 164.4 (C-4), and the aromatic methine carbon at δ 111.8 (C-3). From the above, we deduced that the 5-OH of **4** switched positions with the carbonyl group at position 4 of **4** in **3** (Figure 2). Compound **3** was a new pigment, and named rubrosinensiquinone C.

Antioxidant activities of the isolated compounds were determined by the CUPRAC-BCS (copper (II) reduction assay with bathocuproine disulfonic acid disodium salt) method.⁵ The principle of this assay is based on Cu(II) reducing activity. As a result, **2** was found to have antioxidant activity (0.19), **3** showed equivalent antioxidant activity (0.10) to canarione (0.06), and **1** showed no effect (activity is expressed as mM trolox equivalents /mM).

Figure 2. HMBC correlations of **1-3**Table 1. ^1H and ^{13}C NMR spectral data of **1-3** in $\text{DMSO-}d_6$

Position	1		2		3	
	$^\circ\text{C}$	$^\circ\text{H}$ (mult., J in Hz)	$^\circ\text{C}$	$^\circ\text{H}$ (mult., J in Hz)	$^\circ\text{C}$	$^\circ\text{H}$ (mult., J in Hz)
2	164.8		163.2		163.1	
3	112.1	6.04 (1H, s)	112.1	6.10 (1H, s)	111.8	6.06 (1H, s)
4	175.9		175.8		164.4	
4a	116.6		116.5		116.6	
5	162.7		162.6		176.0*	
5a	111.3		111.3		111.8	
6	172.9		173.0		173.4*	
7	106.3		104.0		104.5	5.26 (1H, s)
8	183.7		183.7		184.2	
9	182.5		182.2		184.0	
9a	134.5		134.5		134.4	
10	105.7	7.13 (1H, s)	105.7	7.17 (1H, s)	104.7	7.09 (1H, s)
10a	159.1		159.0		158.8	
2'	67.7	3.70 (1H, dd, 3.7, 9.2)	57.7	3.45 (2H, s)		
3'	28.3	1.95 (1H, m), 2.06 (1H, m)	164.7			
4'	23.3	1.56 (1H, m), 1.80 (1H, m)	49.6	4.05 (2H, s)		
5'	52.6	2.94 (1H, m), 3.39 (1H, m)				
6'	169.6					
7'	47.5	3.97 (2H, s)				
2- CH_3	19.3	2.25 (3H, s)	19.4	2.31 (3H, s)	19.3	2.30 (3H, s)
1'- CH_3			40.6	2.68 (3H, s)		
4-OH						17.34 (1H, s)
5-OH		16.33 (1H, s)		16.34 (1H, s)		

*: may be interchanged

EXPERIMENTAL

General

The IR spectra were measured with a JASCO IR Report-100 infrared spectrophotometer. The ^1H and ^{13}C NMR spectra and HMQC, HMBC, COSY, and LSPD were recorded using a JEOL JNM-AL-400 (^1H 400 and ^{13}C 100 MHz) and a JEOL JNM-LA 500 (^1H 500 and ^{13}C 125 MHz) spectrometer in $\text{DMSO-}d_6$ using TMS as internal standard. The MS spectra were obtained using a JEOL JMS-700 and Thermo LTQ Orbitrap XL. Column chromatography was carried out on silica gel 60N (spherical, neutral, 63-210 μM , Kanto Chemical Co., Inc.) and PEGASIL PREP ODS-7515-12-A (Senshu Chemical Co., Inc.) and GE Healthcare Sephadex LH-20. HPLC was carried out using JASCO PU-2080 PLUS pump (flow rate, 1.5 mL/min), equipped with a JASCO UV-2075 PLUS UV/VIS Detector.

Plant material

The lichen thalli (Useaceae), *L. sinensis* Wei and Jiang, was collected and identified by Dr. Wang Li-Song in Yunnan, China, in 2005. A voucher specimen is deposited at the Department of Pharmacognosy, Meiji Pharmaceutical University.

Extraction and isolation

The lichen thallus (1.0 kg) was extracted with Et_2O , acetone, MeOH, and H_2O three times each. The MeOH extract (75.4 g) was subjected to column chromatography (C. C.) on silica gel using a CHCl_3 -MeOH stepwise gradient system (CHCl_3 -MeOH 1:0 \rightarrow 10:1 \rightarrow 5:1 \rightarrow 2:1 \rightarrow 1:1 \rightarrow 0:1 \rightarrow CHCl_3 -MeOH- H_2O 6:4:1) to yield Fraction 1 (CHCl_3 -MeOH 1:0), Fraction 2 (CHCl_3 -MeOH 10:1), Fraction 3 (CHCl_3 -MeOH 10:1), Fraction 4 (CHCl_3 -MeOH 5:1), Fraction 5 (CHCl_3 -MeOH 5:1 and 2:1), Fraction 6 (CHCl_3 -MeOH 2:1 and 1:1), Fraction 7 (CHCl_3 -MeOH 0:1), Fraction 8 (CHCl_3 -MeOH 0:1 and CHCl_3 -MeOH- H_2O 6:4:1), and Fractions 9 (CHCl_3 -MeOH- H_2O 6:4:1). Fr. 7 was repeatedly chromatographed on ODS using MeOH- H_2O to obtain compound **1** (10.4 mg). Compound **2** (2.6 mg) was isolated from Fr. 8 by the combination of ODS column chromatography (eluant: MeOH- H_2O 1:9 \rightarrow 3:7 \rightarrow 1:1 \rightarrow 7:3 \rightarrow 1:0) and ODS HPLC (eluant: 25% MeOH).

The H_2O extract (64.3 g) was further extracted with MeOH. This MeOH extract (1.9 g) was partitioned into a MeOH-soluble part (1.4 g) and an insoluble part (0.5 g). The MeOH-insoluble part (0.5 g) was subjected to column chromatography on ODS using a MeOH- H_2O stepwise gradient system (10% MeOH \rightarrow 30% MeOH \rightarrow 50% MeOH \rightarrow 70% MeOH \rightarrow 100% MeOH) to yield Fraction A (10% and 30% MeOH), Fraction B (30% MeOH), Fraction C (30% MeOH), Fraction D (30% and 50% MeOH), Fraction E (50% MeOH), and Fraction F (50 and 100% MeOH). Fr. E was subjected to Sephadex LH-20 column

chromatography using MeOH to give compound **3** (0.5 mg).

Compound 1: rubrosinensiquinone A

Dark red amorphous. $[\alpha]_D^{25} -941.2^\circ$ (c 0.0034, DMSO); UV (DMSO) λ_{\max} (log ϵ) nm: 281 (3.86), 299 (sh, 3.78), 380 (3.32), 436 (sh, 2.97), 515 (3.03); IR ν_{\max} (KBr) cm^{-1} : 3440, 1650, 1630, 1580, 1560, 1540, 1460, 1260, 1200, 1120, 1090, 960. Positive HR-ESIMS m/z 400.1014 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{20}\text{H}_{18}\text{NO}_8$, $[\text{M}+\text{H}]^+$ 400.1032). Positive ESIMS m/z 400 $[\text{M}+\text{H}]^+$ 285, 257. The ^1H and ^{13}C NMR data are presented in Table 1. HMBC and ^1H - ^1H COSY data are presented in Figure 2.

Compound 2: rubrosinensiquinone B

Dark red amorphous. UV (DMSO) λ_{\max} (log ϵ) nm: 277 (3.66), 378 (3.12), 524 (2.74); IR ν_{\max} (KBr) cm^{-1} : 3450, 2350, 1650, 1640, 1630, 1610, 1580, 1560, 1540, 1500, 1450, 1090. Positive HR-ESIMS m/z 374.0856 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{18}\text{H}_{16}\text{NO}_8$ $[\text{M}+\text{H}]^+$ 374.0876). Positive ESIMS m/z 374 $[\text{M}+\text{H}]^+$ 285, 257. The ^1H and ^{13}C NMR data are presented in Table 1. HMBC data is presented in Figure 2.

Compound 3: rubrosinensiquinone C

Dark red amorphous. UV (MeOH) λ_{\max} (log ϵ) nm: 227 (4.22), 279 (4.04), 378 (3.49), 503 (3.23); IR ν_{\max} (KBr) cm^{-1} : 3440, 1660, 1580, 1480, 1270, 1185, 1110, 1090, 960. HR-EIMS m/z 272.0319 (calcd for $\text{C}_{14}\text{H}_8\text{O}_6$ $[\text{M}]^+$ 272.0321). EIMS m/z (rel. int. %): 272 ($[\text{M}]^+$ 100), 244 (86), 216 (21). The ^1H and ^{13}C NMR data are presented in Table 1. HMBC and LSPD data are presented in Figure 2.

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