Bisanthraquinone Metabolites Produced by the Endophytic Fungus *Diaporthe* sp.

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Two bisanthraquinones named (+)-epicytoskyrin (1) and (+)-1,1′-bislunatin (2) were produced by the endophytic fungus from a tea plant, which is a species closely related to *Diaporthe phaseolorum* strain sw-93-13. The chemical structures of the metabolites were elucidated on the basis of the physicochemical properties including the circular dichroism (CD) spectrum.

Key words endophyte; *Diaporthe* sp.; bisanthraquinone; (+)-epicytoskyrin; (+)-1,1′-bislunatin; circular dichroism (CD)

We reported that 6 species of endophytic fungi were obtained from the young stems of the tea plant *Camellia sinensis* (L.) O.K. (Theaceae). And it was found that one filamentous fungus *Diaporthe* sp., which is closely related to *D. phaseolorum* var. *sojae*, has a capacity to transform catechins possessing 2*R*-phenyl substitution into the corresponding 3,4-*cis*-dihydroxyflavan derivatives.[1,2] In this paper, we deal with the chemical structure elucidation of the two metabolites produced by another *Diaporthe* sp. filamentous fungus from the tea plant.

We conducted rDNA analysis for the 18S and ITS1-5.8S-ITS2 regions and comparison with those of *Diaporthe phaseolorum* var. *sojae*, analyzed by us,[2] for 18S and ITS1-5.8S-ITS2 regions, and those of *D. phaseolorum* strain sw-93-13 (AF001018)[3] for ITS1-5.8S-ITS2 region. The results showed that the similarity for the 18S region is 98.2% with *D. phaseolorum* var. *sojae*, and the similarities for the ITS1, 5.8S and ITS2 regions are 87.8%, 100% and 98.7% respectively (the total similarity is 94.4%) with *D. phaseolorum* strain sw-93-13 as shown in Table 1. These findings indicated that the fungus (AB245447) may be a species closely related to *Diaporthe phaseolorum* strain sw-93-13.[4]

The endophytic filamentous fungus *Diaporthe* sp. was cultivated in potato dextrose broth (PDB) with rotary shaking at 90 rpm at 27 °C. After the cultivation for 2 weeks, the whole culture medium including the fungus bodies was extracted with EtOAc. The evaporated extract was separated by Sephadex LH-20 (eluted with EtOH) and Sep-Pak C18 (eluted with aq. THF) to afford a yellowish metabolite (**1**, 47.0 mg/l) and a reddish metabolite (**2**, 10.3 mg/l), which were also yielded by the cultivation of the fungus on potato dextrose agar (PDA).

The yellowish metabolite (**1**), named (+)-epicytoskyrin, showed a quasi-molecular ion peak at *m*/*z* 575 to be $C_{30}H_{23}O_{12}$ in the FAB-MS. The IR spectrum exhibited the absorptions due to hydroxyl, carbonyl and aromatic functions and the UV spectrum showed the characteristic absorption pattern of anthraquinone derivatives. The complete decoupled ^{13}C-NMR of **1** showed totally fifteen carbon signals, which is only half the number of the molecular formula determined by the high-resolution FAB-MS. Therefore, it has been found that **1** is a bis-type compound.

The ^{13}C distortionless enhancement by polarization transfer (DEPT) spectrum revealed the presence of fourteen quaternary carbons, ten tertiary carbons, two methoxyl carbons, and four carbonyl carbons. And four aromatic protons (6- and 6′-H, 8- and 8′-H), six methoxyl protons (7- and 7′-OCH_3), six methine protons (1- and 1′-H, 2- and 2′-H, 3- and 3′-H) and six hydroxyl protons (2- and 2′-OH, 4- and 4′-OH, 5- and 5′-OH) were observed in the ^{1}H-NMR spectrum in THF-d_6.

These physicochemical data were similar to those for cytoskyrin A (**3**), which is a bisanthraquinone metabolite produced by the endophytic fungus *Cytospora* sp. from *Conocarpus erectra*,[5] except for the ^{1}H- and ^{13}C-NMR signals (in THF-d_6) at 1,1′-, 2,2′- and 3,3′-positions (Table 2). Meanwhile, the chemical shifts and coupling patterns of 1,1′-, 2,2′-, and 3,3′-positions resemble closely to those for (+)-

Table 1. Comparison on rDNA Base Sequences of the Endophytic Fungus, *Diaporthe phaseolorum* var. *sojae* and *Diaporthe phaseolorum* strain sw-93-13

	18S	Similarity	ITS1-5.8S-ITS2						
			ITS1	Similarity	5.8S	Similarity	ITS2	Similarity	Total similarity
The endophytic fungus	1736		182		153		157		
Diaporthe phaseolorum var. *sojae*	1736	98.2	177	69.3	153	100.0	158	80.9	82.4
Insert	2		4		0		1		5
Different	7		35		0		14		49
Gap	2		1		0		2		3
Diaporthe phaseolorum strain sw-93-13			180	87.8	153	100.0	156	98.7	94.4
Insert			3		0		0		3
Different			5		0		2		7
Gap			1		0		1		2

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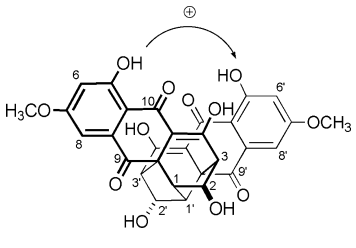
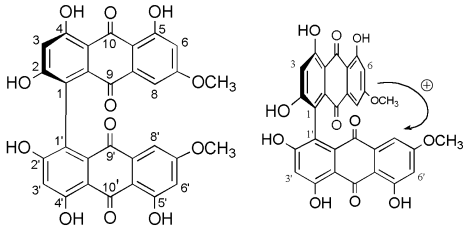


(+)-epicytoskyrin (**1**)



(+)-1,1'-bislunatin (**2**)

Fig. 1

Tabel 2. ^{13}C- and ^{1}H-NMR Data for (+)-Epicytoskyrin (**1**) and Cytoskyrin A (**3**) in THF-d_8

	(+)-Epicytoskyrin (**1**)		Cytoskyrin A (**3**)[5]	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1, 1′	49.2	3.41 (2H, s)	51.8	4.04 (2H, s)
2, 2′	69.8	4.49 (2H, br d, J=5.3 Hz)	74.8	4.00 (2H, d, J=4 Hz)
2, 2′-OH		4.57 (2H, br s)		4.40 (2H, d, J=4 Hz)
3, 3′	58.8	2.86 (2H, d, J=5.3 Hz)	61.9	2.85 (2H, s)
4, 4′	182.3		183.5	
4, 4′-OH		14.60 (2H, br s)		14.38 (2H, s)
4a, 4a′	106.9		106.2	
5, 5′	165.4		165.4	
5, 5′-OH		12.12 (2H, s)		11.88 (2H, s)
6, 6′	107.2	6.78 (2H, d, J=2.4 Hz)	107.1	6.75 (2H, d, J=2.5 Hz)
7, 7′	166.9		167.6	
8, 8′	107.9	7.18 (2H, d, J=2.4 Hz)	108.0	7.07 (2H, d, J=2.5 Hz)
8a, 8a′	135.1		137.1	
9, 9′	194.2		194.4	
9a, 9a′	57.5		61.0	
10, 10′	186.4		188.5	
10a, 10a′	111.7		111.7	
7, 7′-OCH_3	56.3	3.92 (6H, s)	56.7	3.92 (6H, s)

Tabel 3. ^{13}C- and ^{1}H-NMR Data for (+)-Epicytoskyrin (**1**) and (+)-Rugulosin (**4**) in DMSO-d_6

	(+)-Epicytoskyrin (**1**)		(+)-Rugulosin (**3**)[6,7]	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1, 1′	47.7	3.34 (2H, br s)	47.8	3.38 (2H, br s)
2, 2′	68.3	4.37 (2H, br d, J=5.0 Hz)	68.5	4.38 (2H, br d, J=5.5 Hz)
2, 2′-OH		5.44 (2H, br s)		5.38 (2H, br s)
3, 3′	57.8	2.79 (2H, d, J=5.0 Hz)	58.4	2.78 (2H, d, J=5.5 Hz)
4, 4′	182.2		180.8	
4, 4′-OH		14.52 (2H, br s)		14.54 (2H, s)
4a, 4a′	105.6		106.2	
5, 5′	163.0		160.2	
5, 5′-OH		11.92 (2H, br s)		11.37 (2H, s)
6, 6′	106.8	6.89 (2H, d, J=2.3 Hz)	124.1	7.16 (2H, d, J=1 Hz)
7, 7′	165.2		147.6	
8, 8′	107.0	7.14 (2H, d, J=2.3 Hz)	120.6	7.43 (2H, d, J=1 Hz)
8a, 8a′	133.8		132.1	
9, 9′	193.5		194.0	
9a, 9a′	56.2		55.7	
10, 10′	183.0		186.0	
10a, 10a′	110.3		114.2	
7, 7′-OCH_3	55.8	3.92 (6H, s)		
7, 7′-CH_3			21.5	2.42 (6H, s)

rugulosin (**4**), which is a dimeric anthraquinone metabolite produced by *Penicilium islandicum*[6,7] (Table 3). Therefore, the chemical structure of (+)-epicytoskyrin (**1**) was determined to be a respective epimer at 2- and 2′-positions of cytoskyrin A (**3**). Recently, Nicolaou *et al.* reported chemical synthesis of compound **1**.[8]

The absolute configuration of (+)-epicytoskyrin (**1**) was clarified as shown in Fig. 1 by application of the exciton chirality method by Harada *et al.*[9] Namely, a positive maximum due to the molecular chirality was observed at 260 nm ([θ]

+10700) in the circular dichroism (CD) spectrum.

The second reddish metabolite (**2**), named (+)-1,1′-bislunatin, showed a quasi-molecular ion peak at *m*/*z* 571, $C_{30}H_{19}O_{12}$, in the FAB-MS, and the IR spectrum showed absorption band due to hydroxyl, carbonyl and aromatic groups. The UV spectrum showed characteristic absorption bands of anthraquinone skeleton.

(+)-1,1′-Bislunatin (**2**) was assumed to be a bis-type compound like **1**, since the number of carbon signals in the ^{13}C-NMR is half to the molecular formula determined by the high-resolution FAB-MS.

The ^{13}C-DEPT spectrum showed eighteen quaternary carbons, six tertiary carbons, two methoxyl carbons, and four carbonyl carbons. And six aromatic protons (3- and 3′-H, 6- and 6′-H, 8- and 8″-H), six methoxyl protons (7- and 7′-OCH_3), and four chelated hydroxyl protons (4- and 4′-OH, 5- and 5′-OH) were observed in the ^{1}H-NMR spectrum in pyridine-d_5. Furthermore, conspicuous cross-peaks in the heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum were observed between 3-H and three carbons (1-C, 2-C, 4a-C), 6-H and two carbons (8-C, 10a-C), 8-H and three carbons (6-C, 9-C, 10a-C), and between 7-OCH_3 protons and 7-C. From these findings, it was deduced that **2** was a bis-type compound of lunatin (**5**) produced by the fungus *Curvularia lunata*,[10] joining together at C-1 and C-1′ or at C-3 and C-3′. Finally the joining position was determined at C-1 and C-1′ by comparison on the chemical shifts at C-1 (-1′), C-3 (-3′) and C-9a (-9a′) with those of lunatin (**5**) in the ^{13}C- and ^{1}H-NMR spectra in DMSO-d_6 (Table 4). As to the absolute configuration, it was clarified as shown in Fig. 1 since the CD spectrum showed a positive maximum due to the molecular chirality[11] at 269 nm ([θ] +14500).

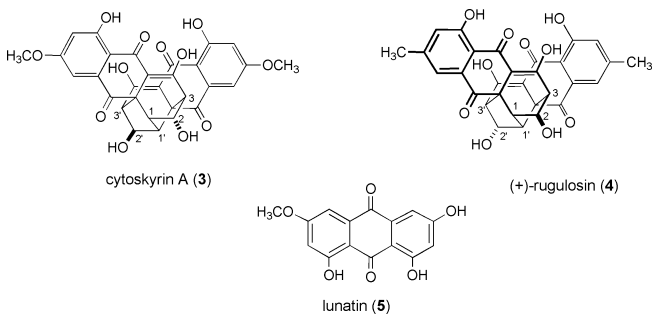


Fig. 2

The relative structure had been printed as a metabolite of *Verticillium lecanii* in the patent,[12] in which they measured NMR spectra in $CDCl_3$. However, it should be noted that (+)-1,1′-bislunatin (**2**) was insoluble and unstable in $CDCl_3$.

As a conclusion, we obtained two bisanthraquinone derivatives, (+)-epicytoskyrin (**1**) and (+)-1,1′-bislunatin (**2**) from the cultivation of the endophytic filamentous fungus closely related to *Diaporthe phaseolorum* strain sw-93-13 from a tea plant. In contrast, another endophytic filamentous fungus closely related to *D. phaseolorum* var. *sojae* from the same plant dose not produce them (**1**, **2**), but transforms catechins into the 3,4-*cis*-dihydroxyflavans.[1,2]

(+)-Epicytoskyrin (**1**) and (+)-1,1′-bislunatin (**2**) showed a moderate cytotoxic activity against KB cells at IC_{50} 0.5 μg/ml, IC_{50} 3.5 μg/ml, respectively.

Experimental

The instruments used to obtain physical data and experimental conditions for chromatography were the same as our previous paper.[2] CD spectra were recorded on a JASCO J-500A spectrometer. DNA extraction, PCR amplification and DNA sequencing were carried out using the same primer through the same procedure as described in our previous paper.[2,13]

Cultivation of the Fungus *Diaporthe* sp. in PDB The fungus was inoculated into the PDB (1 l) in five 500-ml Erlenmeyer flasks and shaken at 90 rpm at 27 °C. After two weeks, the whole including the fungus bodies was homogenized and extracted with EtOAc. The EtOAc extract was concentrated under reduced pressure to give a products (525 mg), which was purified by Sephadex LH-20 column chromatography (Sephadex LH-20 150 ml, eluted with EtOH) and reversed phase chromatography [Sep-Pak Vac C18 Cartridges, eluted with 50% aq. THF] to afford (+)-epicytoskyrin (**1**, 47.0 mg) and (+)-1,1′-bislunatin (**2**, 10.3 mg).

(+)-Epicytoskyrin (**1**): A yellowish powder, $[\alpha]_D$ +447° (c=0.53, in EtOH at 26 °C). IR (KBr) cm^{-1}: 3420, 1689, 1608, 1570. UV (EtOH) nm (ε): 255 (43900), 291 (38000), 337 (29200), 390 (44900). CD (EtOH) nm ([θ]): 232 (−2900), 240 (0), 260 (+10600), 263 (+10600), 285 (0), 299 (−5500), 353 (−6900), 386 (0), 416 (+3000). ^{1}H- and ^{13}C-NMR (in THF-d_8, DMSO-d_6): as given in Tables 2 and 3. FAB-MS *m*/*z*: 575 $[M+H]^+$. High-resolution FAB-MS *m*/*z*: Calcd for $C_{30}H_{23}O_{12}$: 575.1190 $[M+H]^+$. Found 575.1197.

(+)-1,1′-Bislunatin (**2**): A reddish powder, $[\alpha]_D$ +1690° (c=0.25, in EtOH at 26 °C). IR (KBr) cm^{-1}: 3150, 1624, 1600. UV (EtOH) nm (ε): 224 (64400), 265 (38200), 299 (30500), 470 (15800). CD (EtOH) nm ([θ]): 219

Table 4. ^{13}C- and ^{1}H-NMR Data for (+)-1,1′-Bislunatin (**2**) and Lunatin (**5**) in DMSO-d_6

	(+)-1,1′-Bislunatin (**2**)			Lunatin (**5**)[10]	
	^{13}C-NMR	^{1}H-NMR		^{13}C-NMR	^{1}H-NMR
1, 1′	123.7		1	109.3	7.09 (1H, d, J=2.3 Hz)
2, 2′	164.0		2	165.9	
3, 3′	107.3	6.74 (2H, s)	3	108.1	6.56 (1H, d, J=2.3 Hz)
4, 4′	163.6		4	164.4	
4a, 4a′	108.6		4a	108.3	
5, 5′	164.1		5	164.1	
6, 6′	106.5	6.73 (2H, d, J=2.1 Hz)	6	106.6	6.82 (1H, d, J=2.5 Hz)
7, 7′	165.5		7	165.5	
8, 8′	106.9	6.96 (2H, d, J=2.1 Hz)	8	107.4	7.14 (1H, d, J=2.5 Hz)
8a, 8a′	134.9		8a	134.8	
9, 9′	181.4		9	181.1	
9a, 9a′	130.9		9a	134.6	
10, 10′	188.5		10	188.4	
10a, 10a′	109.2		10a	109.6	
7, 7′-OCH_3	56.0	3.81 (6H, s)	7-OCH_3	56.2	3.91 (3H, s)
OH		12.24, 12.82 (2H each, both s)	OH		12.22, 12.31 (1H each, both s)

(−4400), 227 (0), 230 (+900) 252 (−8700), 260 (0), 269 (14500). ^{1}H-NMR (pyridine-d_5) δ: 3.65 (6H, s, 7,7′-OCH_3), 6.84 (2H, d, J=2.4 Hz, 6,6′-H), 7.22 (2H, s, 3,3′-H), 7.34 (2H, d, J=2.4 Hz, 8,8′-H), 12.67, 13.33 (2H each, both s, 4,4′-OH, 5,5′-OH). ^{13}C-NMR (pyridine-d_5) δ: 55,9 (7,7′-OCH_3), 106.7 (6,6′-C), 107.5 (8,8′-C), 108.4 (3,3′-C), 110.1 (4a,4a′-C), 110.5 (10a,10a′-C), 125.5 (1,1′-C), 132.5 (9a,9a′-C), 136.3 (8a,8a′-C), 164.8 (4,4′-C), 165.8 (2,2′-C), 166.1 (5,5′-C), 166.2 (7,7′-C), 182.9 (9,9′-C), 189.8 (10,10′-C). ^{1}H- and ^{13}C-NMR (in DMSO-d_6): as given in Table 4. FAB-MS m/z: 571 $[M+H]^+$. High-resolution FAB-MS m/z: Calcd for $C_{30}H_{19}O_{12}$: 571.0876 $[M+H]^+$. Found 571.0873.

Cultivation of the Fungus *Diaporthe* sp. on PDA The fungus was inoculated onto ten plates of PDA (each 20 ml) and cultivated for two weeks at 27 °C. The whole including the fungus bodies was homogenized and then extracted with EtOAc. The EtOAc layer was evaporated *in vacuo* to give a crude product (80 mg). The product was purified by Sephadex LH-20 column chromatography (Sephadex LH-20 150 ml, eluted with EtOH) and reversed phase chromatography [Sep-Pak Vac C18 Cartridges, eluted with 50% aq. THF] to afford (+)-epicytoskyrin (**1**, 17.5 mg) and (+)-1,1′-bislunatin (**2**, 5.5 mg).

Acknowledgement This work was supported by the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from the Minister of Education, Culture, Sports, Science and Technology, 2004—2008.

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