

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. *sojiae*, 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. *sojiae*, 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. *sojiae*, 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 C₃₀H₂₃O₁₂ 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 ¹³C-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.

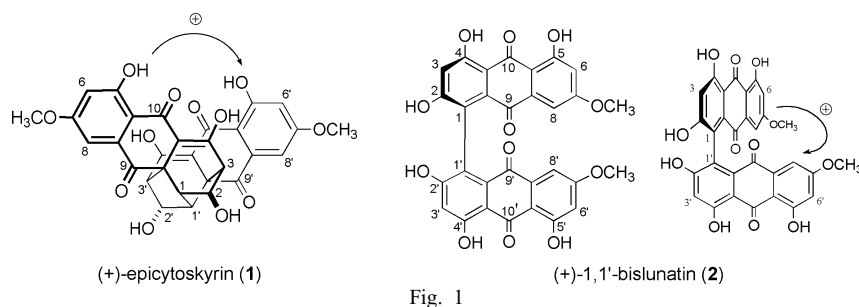
The ¹³C distortionless enhancement by polarization transfer (DEPT) spectrum revealed the presence of fourteen quaternary carbons, ten tertiary carbons, two methoxyl carbons, and four carbonyl carbons. And four aromatic protons (6- and 6'-H, 8- and 8'-H), six methoxyl protons (7- and 7'-OCH₃), six methine 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 ¹H-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 erecta*,⁵⁾ except for the ¹H- and ¹³C-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. *sojiae* 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		
<i>Diaporthe phaseolorum</i> var. <i>sojiae</i>	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
<i>Diaporthe phaseolorum</i> 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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Table 2. ^{13}C - and ^1H -NMR Data for (+)-Epicytoskyrin (1) and Cytoskyrin A (3) in $\text{THF-}d_8$

	(+)-Epicytoskyrin (1)		Cytoskyrin A (3) ⁵⁾	
	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -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)

Table 3. ^{13}C - and ^1H -NMR Data for (+)-Epicytoskyrin (1) and (+)-Rugulosin (4) in $\text{DMSO-}d_6$

	(+)-Epicytoskyrin (1)		(+)-Rugulosin (3) ^{6,7)}	
	^{13}C -NMR	^1H -NMR	^{13}C -NMR	^1H -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 *Penicillium 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 ($[\theta]$

+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'-OCH₃), and four chelated hydroxyl protons (4- and 4'-OH, 5- and 5'-OH) were observed in the 1H -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-OCH₃ 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

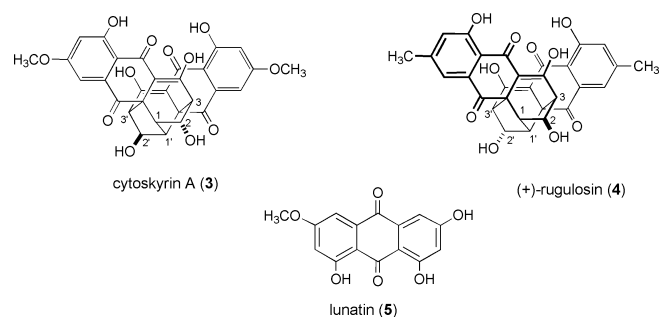


Fig. 2

the ^{13}C - and 1H -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 chirality¹¹ at 269 nm ($[\theta]$ +14500).

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. *sojiae* 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 IC₅₀ 0.5 $\mu g/ml$, IC₅₀ 3.5 $\mu g/ml$, 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, $[\alpha]_D^{25} +447^\circ$ ($c=0.53$, 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 ($[\theta]$): 232 (-2900), 240 (0), 260 (+10600), 263 (+10600), 285 (0), 299 (-5500), 353 (-6900), 386 (0), 416 (+3000). 1H - 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, $[\alpha]_D^{25} +1690^\circ$ ($c=0.25$, 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 ($[\theta]$): 219

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

	(+) -1,1'-Bislunatin (2)			Lunatin (5) ¹⁰	
	^{13}C -NMR	1H -NMR		^{13}C -NMR	1H -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). ¹H-NMR (pyridine-*d*₅) δ: 3.65 (6H, s, 7, 7'-OCH₃), 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). ¹³C-NMR (pyridine-*d*₅) δ: 55.9 (7, 7'-OCH₃), 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). ¹H- and ¹³C-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 C₃₀H₁₉O₁₂: 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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