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Cytosporones O, P and Q from an endophytic *Cytospora* sp.

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ABSTRACT

Cytosporones O, P and Q, together with the known compounds cytosporones B, C, D, E and dothiorelones A, B, C, and H were isolated from the ascomycete fungus *Cytospora* sp. during a chemotaxonomic study of fungal endophytes belonging to the related genera *Cytospora* and *Phomopsis* from Brazil. The structures were determined by NMR spectroscopy and mass spectrometry. With exception of cytosporones D, E, Q, and dothiorelone B, all compounds were consistently detected in the metabolite profiles of eight *Cytospora* isolates investigated; and were also produced by a distinct chemotype of *Phomopsis*.

Fungal endophytes comprise a heterogeneous and diverse group of species capable of colonizing asymptomatically the interior of plants and are considered as an important part of global fungal diversity.[1] The taxonomy of several genera of fungi occurring as common endophytes (e.g., *Colletotrichum*, *Phomopsis*, *Xylaria*) is quite complex due to the lack of good morphological markers for in vitro species recognition and differentiation among isolates in the same genus are generally indicated by distinct morphotypes.[2]

Endophytes are also recognized as producers of a vast array of secondary metabolites, many of them with promising bioactivities.[3] Chemotaxonomic studies of morphologically related endophytes using secondary metabolite profiling of fungal cultures by LC–UV–MS can be used as support for species delimitation as well as for compound dereplication and discovery of new metabolites.[4]

During a chemotaxonomic study of fungal endophytes belonging to the related ascomycete genera *Cytospora* and *Phomopsis* isolated from native and cultivated plants in Brazil, we have cultivated one isolate of *Cytospora* sp. in large scale and purified the known natural products cytosporones B, C, D, E and the structurally related dothiorelones A, B, C, and H, together with three new cytosporone analogues described herein this Letter.

The fungus *Cytospora* sp. CML 1841(=IBT 41593)[5] isolated as an endophyte of the mistletoe *Phoradendron perrottetii* in Brazil[6] was three-point inoculated in 200 plates containing MEA medium[7] and cultivated for 20 days at 25 °C. The contents of the plates were extracted twice with ethyl acetate (5 L) and the dried extract (3.6 g) was subjected to reversed phase (C18) followed by stepwise normal phase (Diol) flash chromatography.[8] Target compounds were identified based on their retention times and UV spectra under standard analytical HPLC conditions[9] in comparison to data of the compounds most commonly found in the metabolite profiles of eight *Cytospora* isolates cultivated under the same growth conditions on MEA plates and subjected to micro-scale extraction of metabolites[10] followed by analytical HPLC profiling. Final purification of compounds was accomplished by semi-preparative HPLC.[11]

Eleven compounds were purified and eight were identified as the known compounds cytosporones B, C, D, E and the dothiorelones A, B, C, and H after comparison of their HRESIMS and NMR data[12] to those reported in the literature.[13–15]

Compound **1** was purified from the same fraction containing dothiorelone H (**2**) and HRESIMS data indicated that both compounds have the same molecular formula $C_{16}H_{22}O_5$ (*m/z* calcd for $C_{16}H_{23}O_5$ 295.1545; found 295.1523 for **1** and 295.1520 for **2**) as well as identical UV spectra (λ_{max} 204, 282 nm; CH_3CN–H_2O plus 50 ppm TFA). ^1H and ^{13}C NMR data confirmed the structural similarity of **1** and **2** (Table 1) and also indicated a shift in the position of the hydroxyl group attached to the saturated side chain in **1**, as revealed with the replacement of a methyl doublet at δ_H 1.14, in the ^1H NMR of **2** (H-16), by a triple doublet at δ_H 0.93 in **1**. HMBCs from this methyl triple doublet to an oxygenated carbon signal at δ_C 73.9, in ^{13}C NMR, supported the attachment of a hydroxyl at

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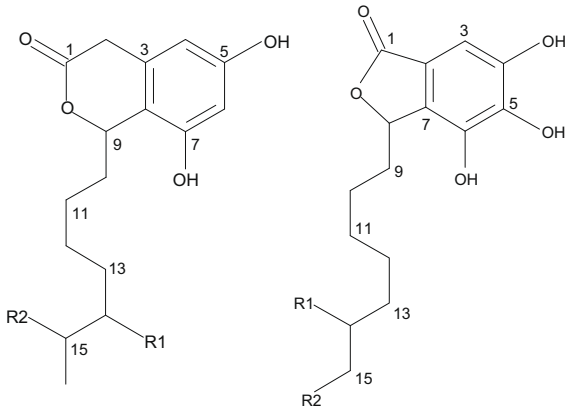
Table 1
NMR data for **1** (^{1}H at 800 MHz, ^{13}C at 200 MHz; CD_3OD) and **2** (^{1}H at 500 MHz, ^{13}C at 125 MHz; CD_3OD)

Position	1			2		
	δ_C	δ_H mult. (*J* in Hz)	HMBC	δ_C	δ_H mult. (*J* in Hz)	HMBC
1	174.2			174.1		
2	35.7	3.80 d (19.5), 3.48 d (19.6)	C1, C3, C4, C8	35.4	3.79 d (19.5), 3.48 d (19.6)	C1, C3, C4, C5, C7
3	132.9			132.8		
4	106.3	6.13 br s	C2, C5, C6, C8	106.2	6.13 br s	C2, C5, C6, C8
5	159.7			159.7		
6	102.1	6.22 d (1.6)	C4, C5, C7, C8	102.2	6.23 d (1.5)	C4, C5, C7, C8
7	155.4			155.3		
8	114.0			113.9		
9	80.2	5.61 dd (8.6, 5.1)	C1, C3, C7, C8, C10, C11	80.2	5.61 dd (8.6, 5.2)	C1, C3, C7, C8, C10, C11
10	36.9	1.87 m, 1.82 m	C8, C9, C11	36.6	1.84 m	C8, C9, C11, C12
11	26.9	1.49 m, 1.41 m		26.6	1.55 m, 1.39 m	
12	26.5	1.48 m, 1.40 m		30.3	1.37 m	C13
13	37.9	1.48 m, 1.40 m		26.7	1.39 m	
14	73.9	3.43 m	C12, C16	40.0	1.41 m	
15	31.2	1.48 m, 1.40 m	C13, C14, C16	68.6	3.70 m	C13
16	10.5	0.93 td (7.4, 1.5)	C14, C15	23.4	1.14 d (6.2)	C14, C15

C-14, thus defining the structure of a new cytosporone analogue, cytosporone O. Detailed analysis of NMR data for **2** also supported the reassignment of ^{13}C signals for C-13 at δ_C 26.7 and C-14 at δ_C 40.0, instead of δ_C 30.3 and δ_C 31.1 originally described.[15] Dothiorelone H has recently been isolated from an endophytic *Pestalotiopsis* fungus and named as cytosporone J,[16] but no ^{13}C NMR data were provided by the authors for this compound.

Two compounds, **3** and **4**, were purified and exhibited UV spectra (λ_{max} 220, 276 nm; CH_3CN–H_2O plus 50 ppm TFA) almost identical to that of cytosporone E. Comparative analysis of ^{1}H and ^{13}C NMR data for **3**, **4** (Table 2), and cytosporone E[13] confirmed their core structure as trihydroxy benzene γ-lactones attached to a heptane side chain, except for the presence of one extra oxygen in the structures of **3** and **4**, as indicated by their molecular formula $C_{15}H_{20}O_6$ derived from HRESIMS (*m/z* calcd for $C_{15}H_{21}O_6$ 297.1338; found 297.1327 for **3** and 297.1334 for **4**).



1: R_1 = OH, R_2 = H **3**: R_1 = OH, R_2 = H

2: R_1 = H, R_2 = OH **4**: R_1 = H, R_2 = OH

Correlation of a methyl doublet at δ_H 1.13 (H-15) in the ^{1}H NMR of **3** to a carbinolic proton at δ_H 3.69 (m) in ^{1}H–^{1}H COSY and to an oxygenated carbon signal at δ_C 68.7 in HMBC indicated the attachment of a hydroxyl group at C-14, thus identifying **3** as a new compound, cytosporone P.

No methyl signal was observed in the ^{1}H NMR spectrum of **4**, instead a downfield methylene triplet at δ_H 3.53 indicated the presence of a primary alcohol moiety. Further correlations from this methylene signal to another methylene at δ_H 1.51 (H-14) in ^{1}H–^{1}H COSY and to C-13 and C-14 in HMBC supported the attachment of a hydroxyl group at C-15. This new compound was named cytosporone Q.[17]

Table 2
NMR data for **3** and **4** (^{1}H at 800 MHz, ^{13}C at 200 MHz; CD_3OD)

Position	3			4		
	δ_C	δ_H mult. (*J* in Hz)	HMBC	δ_C	δ_H mult. (*J* in Hz)	HMBC
1	174.3			174.3		
2	131.2			131.3		
3	103.1	6.78 s	C1, C2, C4, C5, C7	103.1	6.78 s	C1, C2, C4, C5
4	148.8			148.8		
5	141.4[a]			141.4[c]		
6	141.2[b]			141.2[d]		
7	117.4			117.4		
8	82.0	5.47 dd (7.4, 3.1)	C1, C2, C6, C7, C9, C10	82.0	5.47 dd (7.4, 3.1)	C1, C2, C9, C10
9	34.0	2.25 m, 1.74 m	C8, C10, C11	34.0	2.23 m, 1.74 m	C8, C10, C11
10	25.7	1.35 m		25.7	1.33 m	
11	30.6	1.35 m		30.5[e]	1.33 m	
12	26.9	1.35 m		30.6[f]	1.33 m	
13	40.2	1.39 m	C11, C12, C14	26.9	1.33 m	
14	68.7	3.69 m	C12, C13	33.7	1.51 m	C12, C13, C15
15	23.6	1.13 d (6.2)	C13, C14	63.1	3.53 t (6.7)	C13, C14

[a–f] Assignment of carbons can be interchanged.

Cytosporone O (**1**) and the dothiorelones A, C and H (**2**) were detected in the metabolite profiles of seven out of eight *Cytospora* isolates investigated by HPLC-DAD and always occurred together. Cytosporone C was produced by seven isolates; cytosporone P (**3**) and cytosporone B were produced by five isolates. These seven compounds were also consistently detected in the metabolite profiles of a distinct group of *Phomopsis* isolates in agreement with findings of Brady et al.[13] that cytosporones were produced by fungi of both genera. Cytosporone Q was detected in the metabolite profiles of three *Cytospora* isolates only. Investigations are underway to determine the chemotaxonomic relationships of a group of endophytic *Cytospora* and *Phomopsis* isolated from tropical plants in Brazil and to assess the possible role of the isolated cytosporones and dothiorelones as chemotaxonomic markers.

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7. Malt Extract Agar (MEA)—malt extract (Difco) 20 g, peptone (Difco) 1 g, glucose (BHD) 20 g, distilled water 750 mL.
8. The crude extract was loaded onto a C18 Snap cartridge (60 g, Biotage) and subjected to reversed phase flash chromatography on an Isolera One system (Biotage) using a water–methanol gradient (H_2O–MeOH 90:10, 3 fractions; 90:10–0:100, 9 fractions; 0:100, 4 fractions) to give 16 fractions. Fractions 09 (316 mg), 10 (281 mg), and 11 (440 mg) were individually submitted to stepwise normal phase flash chromatography over 10 g diol cartridges (Biotage) eluting from 100% heptane to 100% dichloromethane in 50% steps, and from dichloromethane to 100% ethyl acetate in 10% steps, with two additional elutions with ethyl acetate–methanol (90:10 and 80:20) and a final wash with 100% methanol to afford another 16 sub fractions each (09_01 to 09_16, 10_01 to 10_16, and 11_01 to 11_16). All solvents were HPLC grade and were purchased from Merck.
9. Analytical HPLC was performed on a HP 1100 system equipped with a diode array detector (Agilent) using a Luna II C18 column (100 × 2 mm, 3 μm; Phenomenex) and an elution gradient of water–acetonitrile from 85:15 to 0:100 in 20 min plus 5 min of pure acetonitrile; both solvents containing 50 ppm of trifluoroacetic acid.
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11. Semi-preparative purification of target compounds was performed on a Waters 600 HPLC system with a 996 PDA detector using a Luna II C18 (250 × 10 mm, 5 μm) column (Phenomenex). Fractions 11_05, 11_06, 11_08, and 11_10 were chromatographed using water–methanol containing 100 ppm of trifluoroacetic acid as eluents (H_2O–MeOH 30:70–06:94 in 20 min, plus 5 min of 100% MeOH) to afford cytosporone B (12.8 mg), cytosporone D (0.6 mg), cytosporone C (13.7 mg), and cytosporone E (0.5 mg). Fractions 09_09, 09_12 and 10_07 were submitted to semi preparative HPLC using water–acetonitrile containing 50 ppm of TFA as eluents (H_2O–ACN 68:32–66:34 in 12 min, plus 5 min of 100% ACN) to afford dothiorelones A–C and H (**2**), and compounds **1**, **3,** and **4**.
12. HRESIMS was obtained on a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray ESI source. ^{1}H and 2D NMR spectra for known cytosporones and dothiorelones were acquired in Varian Unity Inova 500 MHz spectrometer using standard pulse sequences. ^{1}H, ^{13}C and 2D NMR spectra for compounds **1**, **3**, and **4** were acquired in Bruker Avance 800 MHz using standard pulse sequences.
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17. Optical rotations were measured on a Perkin Elmer 341 polarimeter; **1**: $[\alpha]_D^{20}$ −1.9 (*c* 0.005, MeOH), **3**: $[\alpha]_D^{20}$ −25.0 (*c* 0.003, MeOH), **4**: $[\alpha]_D^{20}$ −52.1 (*c* 0.001, MeOH).