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Polyketides and nitrogenous metabolites from the endophytic fungus *Phomopsis* sp. D15a2a

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ABSTRACT

Three new polyketides, phomopones A–C (**1**–**3**), one new cyclic tetrapeptide, 18-hydroxydihydrotentoxin (**4**), and a new amide, 6-hydroxyenamidin (**5**) together with a known derivative, enamindin (**6**) were obtained from the endophytic fungus *Phomopsis* sp. D15a2a isolated from the plant *Alternanthera bettzickiana*. The structures of the new compounds were elucidated by 1D, 2D NMR and HRMS data. The absolute configurations of the isolated metabolites were determined either by X-ray crystallography, Marfey's method or by converting the compounds to Mosher esters.

Introduction

Fungi of genus *Phomopsis* have been reported as endophytes, saprobes, plant pathogens, animal pathogens and are even known to infect humans [1]. They have been rather well investigated due to their production of structurally diverse metabolites. *Phomopsis.* sp. TJ507A, an endophyte obtained from the medicinal plant *Phyllanthus glaucus* (Phyllanthaceae), yielded a series of protoilludane, illudalane and botryane sesquiterpenoids, of which some showed β-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibitory activities [2]. From the endophytic fungus *Phomopsis* sp. YE3250, seven polyoxygenated cyclohexenoids, phomopoxides A–G, were isolated with promising α-glycosidase inhibitory activity [3]. Four furanones, phomopsolidones A–D, were isolated from *Phomopsis* sp. DC275 and two of them exhibited antibacterial activity against *Bacillus subtilis* with MIC values of 0.1 ng [4]. In this study, the endophytic fungus *Phomopsis* sp. D15a2a, that was isolated from leaves of *Alternanthera bettzickiana* (Amaranthaceae) collected in Anambra state of Nigeria, was investigated with regard to its secondary metabolites. The EtOAc extract of *Phomopsis* sp. following fermentation on solid rice medium yielded three new polyketides, phomopones A–C (**1**–**3**), a new cyclic tetrapeptide, 18-hydroxydihydrotentoxin (**4**), and a new amide, 6-hydroxyenamidin (**5**) in addition to a known derivative, enamindin (**6**) (Figure 1) [5].

Results and discussion

Compound **1** had the molecular formula of $C_{10}H_{14}O_3$ with four degrees of unsaturation as established from the HRESIMS data. The ^{13}C NMR data of **1** (Table 1) showed one carbonyl at δ_C 199.9 (C-1), two olefinic carbons at δ_C 173.6 (C-5) and 109.6 (C-6), two oxygenated methines at δ_C 75.5 (C-9) and 72.4 (C-2), a methyl at δ_C 20.5 (C-10) and four aliphatic methylenes, accounting for two degrees of unsaturation. The remaining two degrees of unsaturation suggested a bicyclic structure for compound **1**. Two spin systems from C-2 to C-4 and from C-7 to C-10 were established from the COSY correlations between H-2/ H-3ab/H-4ab and between H-7ab/H-8ab/H-9/Me-10 (Figure 2). The HMBC correlations from H-2 and H-3ab to C-1, from H-3ab to C-5, from H-4ab to C-5 and C-6, from H-7ab to C-1, C-5 and C-6, and from H-8ab to C-6 indicated the presence of an α,β-unsaturated ketone and linkages between C-1/C-2, C-4/C-5, and C-6/C-7. The location of a hydroxy group at

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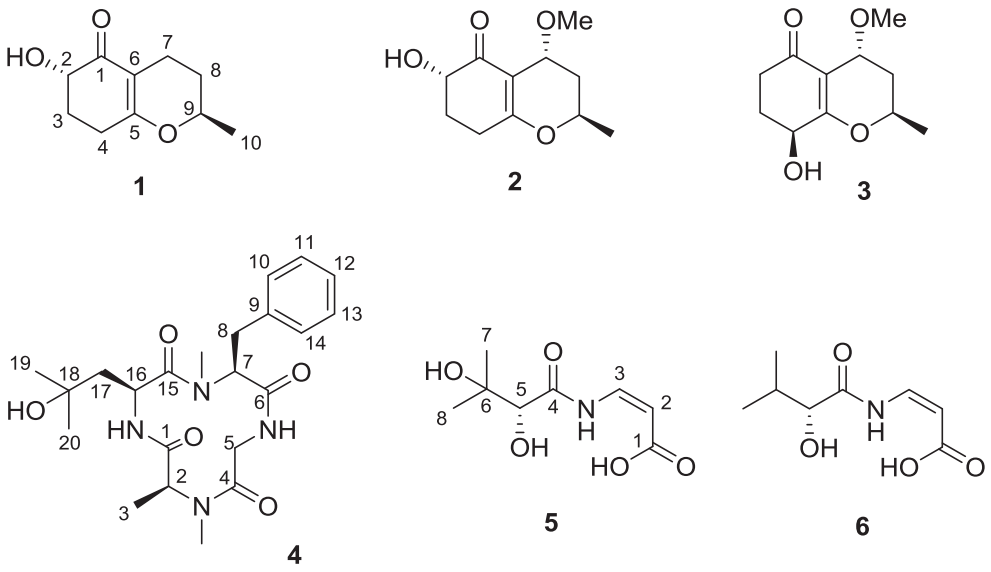

Figure 1. Structures of isolated compounds from *Phomopsis* sp.

Table 1
^{1}H and ^{13}C NMR Data of Compounds 1–3.

position	**1** [a]		**2** [b]		**3** [a]	
	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)	δ_C, type	δ_H, (J in Hz)
1	199.9, C		199.1, C		199.3, C	
2	72.4, CH	4.03, dd (12.0, 5.2)	72.5, CH	4.01, dd (12.0, 5.0)	34.4, C	2.54, ddd (16.9, 7.9, 4.7) 2.38, ddd (16.9, 8.8, 4.9)
3	30.8, CH_2	2.23, dddd (12.0, 5.2, 5.2, 3.2) 1.84, dddd (12.0, 12.0, 12.0, 5.0)	30.1, CH_2	2.23, dddd (12.0, 5.0, 5.0, 3.3) 1.86, dddd (12.0, 12.0, 12.0, 5.0)	30.6, CH_2	2.20, dddd (13.3, 7.9, 4.9, 4.4) 1.92, dddd (13.3, 8.8, 7.2, 4.7)
4	28.3, CH_2	2.59, br ddd (17.5, 12.0, 5.2) 2.42, br ddd (17.5, 5.0, 3.2)	28.4, CH_2	2.64, ddd (17.7, 12.0, 5.0) 2.48, ddd (17.7, 5.0, 3.3)	67.2, CH	4.38, dd (7.2, 4.4)
5	173.6, C		176.2, C		175.4, C	
6	109.6, C		110.9, C		112.7, C	
7	18.2, CH_2	2.25, m 2.22, m	67.6, CH	4.14, dd (3.0, 2.4)	67.4, CH	4.22, dd (3.1, 2.3)
8	28.6, CH_2	1.95, dddd (13.8, 5.8, 4.6, 2.8) 1.53, dddd (13.8, 9.3, 9.3, 5.6)	34.0, CH_2	2.12, ddd (14.5, 2.4, 2.4) 1.39, ddd (14.5, 12.6, 3.0)	33.9, CH_2	2.13, ddd (14.5, 2.3, 2.3) 1.40, ddd (14.5, 12.6, 3.1)
9	75.5, CH	4.23, dqd (9.3, 6.4, 2.8)	71.8, CH	4.34, dqd (12.6, 6.3, 2.4)	71.8, CH	4.30, dqd (12.6, 6.3, 2.3)
10	20.5, CH_3	1.33, d (6.4)	20.8, CH_3	1.39, d (6.3)	20.7, CH_3	1.45, d (6.3)
7-OMe			56.6, CH_3	3.37, s	56.8, CH_3	3.36, s,

[a] Measured in CD_3OD at 600 (^{1}H) and 150 MHz (^{13}C); [b] Measured in CD_3OD at 300 (^{1}H) and 75 MHz (^{13}C).

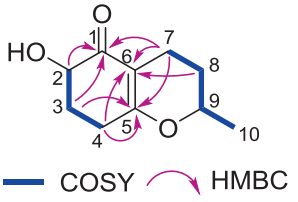

Figure 2. COSY and key HMBC correlations of compound **1**.

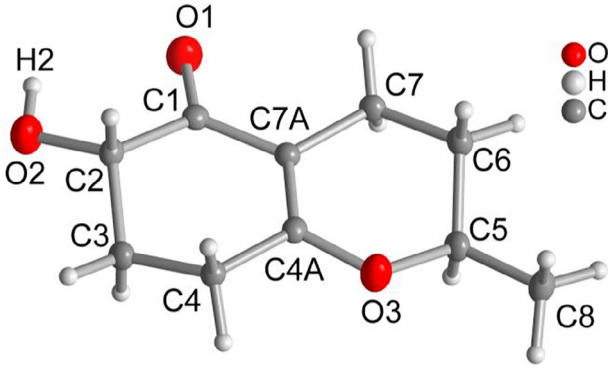

Figure 3. Molecular structure of compound **1** from X-ray diffraction analysis.

C-2 and an ether linkage between C-5 and C-9 were deduced from the molecular formula of **1**, the chemical shifts of C-2, C-5 and C-9 in addition to the weak HMBC correlation from Me-10 to C-5. Thus, the planar structure of **1** was elucidated as shown and the trivial name phomopone A was given to **1**. The relative and absolute configuration of **1** was determined by X-ray diffraction analysis (Figure 3).

The molecular formula of compound **2** was determined as $C_{11}H_{16}O_4$ by the HRESIMS data. The NMR data of **2** (Table 1) were similar to those of **1** except for the replacement of an aliphatic methylene by an oxygenated methine (δ_C 67.6 and δ_H 4.14) and an additional methoxy group (δ_C 56.6 and δ_H 3.37) in **2**. The COSY correlations between the protons of this additional methine and H-8ab, H-8ab/H-9, and between H-9/Me-10, together with the HMBC correlations from the protons of this additional methine to C-1 (δ_C

199.1), C-5 (δ_C 176.2) and C-6 (δ_C 110.9) indicated the location of this additional oxygenated methine group at C-7. Furthermore, the attachment of the additional methoxy group at C-7 was confirmed by the HMBC correlations from the protons of the methoxy group to C-7 and in turn from H-7 to the carbon of the methoxy group. The remaining structure of **2** was elucidated to be identical to that of **1** after detailed analysis of the 2D NMR spectra of **2**. Compound **2** is suggested to share the same absolute configurations at C-2 and C-9 as **1** in consideration of the close biogenetic relationship between both compounds. The large value of $^3J_{H\text{-}9/H\text{-}8b}$ (12.6 Hz) and the small values of $^3J_{H\text{-}9/H\text{-}8a}$ (2.4 Hz), $^3J_{H\text{-}7/H\text{-}8a}$ (2.4 Hz) and $^3J_{H\text{-}7/H\text{-}8b}$ (3.0 Hz) indicated diaxial orientation of H-9 and H-8b and equatorial orientation of H-8a and H-7, implying that H-7 and H-9 were on different sides of the ring. Thus, the configuration of **2** was tentatively assigned as 2*S**,7*R**,9*R**.

Compound **3** shared the same molecular formula as **2** as evident from the HRESIMS data. Comparison of the ^{1}H and ^{13}C NMR data of **2** and **3** (Table 1) revealed that both compounds exhibit almost identical signals in the cyclic ether ring whereas the chemical shifts of the oxygenated methine in the cyclohexenone ring in **3** were obviously shifted (δ_C 67.2 and δ_H 4.38 in **3** while δ_C 72.5 and δ_H 4.01 in **2**). In addition, the protons of this oxygenated methine showed HMBC correlations to C-5 and C-6 in **3** rather than to C-1 in **2**, confirming the attachment of a hydroxy group at C-4 in **3** rather than at C-2 in **2**. Based on the similar chemical shifts and coupling constants, **3** was suggested to share the same (7*R**,9*R**) configuration as **2**. The relatively small coupling constants between H-4 and H-3ab (7.2 and 4.4 Hz) in **3** were more comparable to those of H-4b (5.0 and 3.3 Hz) rather than to those of H-4a (12.0 and 5.0 Hz) in **2**, suggesting equatorial orientation of H-4 in **3** and hence (4*S**) configuration for **3**.

Compound **4** was isolated as a white powder. Its molecular formula of $C_{22}H_{32}N_4O_5$ with 9 degrees of unsaturation was deduced from the HRESIMS data. The ^{1}H NMR data of **4** displayed signals of five methyls, three methylenes, three aliphatic methines, and five aromatic methines. The presence of carbonyls at δ_C 169–172 together with α-protons/carbons at δ_H 4.78 (H-5a), 4.49 (H-16), 4.44 (H-7), 4.03 (H-2), 3.37 (H-5b), and δ_C 61.8 (C-7), 55.9 (C-2), 46.8 (C-16), 44.2 (C-5) suggested compound **4** to be a peptide.

Table 2
^{1}H and ^{13}C NMR Data of Compound 4.

amino acid	position	δ_C, type	δ_H, (*J* in Hz)
N-Methyl-Ala	1	170.4, C	
	2	55.9, C	4.03, q (7.1)
	3	15.4, CH_3	1.34, d (7.1)
	2-NCH_3	29.5, CH_3	2.65, s
Gly	4	170.4, C	
	5	44.2, CH_2	4.78, br d (15.4)
			3.37, d (15.4)
	5-NH		8.04, br s
N-Methyl-Phe	6	169.2, C	
	7	61.8, CH	4.44, d (11.0)
	8	33.7, CH_2	3.41, d (14.6)
			2.91, dd (14.6, 11.0)
	9	137.9, C	
	10, 14	128.1, CH	7.20, d (7.6)
	11, 13	128.6, CH	7.28, t (7.6)
	12	126.5, CH	7.19, d (7.6)
	7-NCH_3	30.6, CH_3	2.69, s
γ-OH-Leu	15	171.6, C	
	16	46.8, CH	4.49, m
	17	44.9, CH_2	2.11, dd (14.3, 4.9)
			1.35, m
	18	68.1, C	
	19	29.5, CH_3	0.92, s
	20	28.9, CH_3	0.90, s
	16-NH		8.14, br s
	18-OH		4.14, s

Measured in DMSO d_6 at 600 (^{1}H) and 150 MHz (^{13}C).

The ^{1}H and ^{13}C NMR data of **4** (Table 2) resembled those of dihydrotentoxin [6,7] except for the replacement of an aliphatic methine of the latter by an oxygenated carbon of the former at δ_C 68.1 (C-18) and an additional hydroxy group at δ_H 4.14 (18-OH). The HMBC correlations from Me-19 (δ_H 0.92) and Me-20 (δ_H 0.90) to C-18 and C-17 (δ_C 44.9), from 18-OH to C-17, C-18, C-19 (δ_C 29.5) and C-20 (δ_C 28.9), and from H-17ab (δ_H 2.11 and 1.35) to C-15 (δ_C 171.6), along with the COSY correlations between H-17ab/H-16/16-NH, indicated the replacement of leucine as in dihydrotentoxin by a γ-OH-Leu residue in **4**. Detailed analysis of 2D NMR data of **4** confirmed that the remaining structure of **4** was identical to that of dihydrotentoxin. Thus, compound **4** was identified as 18-hydroxydihydrotentoxin, representing a new cyclotetrapeptide. The absolute configuration of **4** was determined by Marfey's reaction employing the rule that D-FDAA-D-amino acid or L-FDAA-L-amino acid elute earlier during HPLC analysis compared to L-FDAA-D-amino acid or D-FDAA-L-amino acid. [8,9] Comparison of the retention time of the resulting L-FDAA- and D-FDAA-amino acid derivatives [L-FDAA-*N*-Methyl-Ala (35.66 min), D-FDAA-*N*-Methyl-Ala (38.86 min); L-FDAA-18-hydroxy-Leu (47.64 min), D-FDAA-18-hydroxy-Leu (56.15 min); L-FDAA-*N*-Methyl-Phe (85.17 min), D-FDAA-*N*-Methyl-Phe (85.80 min)] revealed that all amino acid residues in **4** were of the L-form.

The molecular formula of compound **5** was determined to be $C_8H_{13}NO_5$ according to the HRESIMS data, indicating the presence of an additional oxygen atom when compared to the co-isolated known compound, enamindin (**6**). [5] The ^{1}H and ^{13}C NMR data of **5** (Table 3) were similar to those of enamindin (**6**). However, the presence of an oxygenated carbon at δ_C 73.3 (C-6) and the observation that the methyl groups (Me-7 and Me-8) resonated as a singlet in **5** rather than as a doublet in **6** indicated the presence of an additional hydroxy group at C-6. This was further confirmed by the HMBC correlations from Me-7 and Me-8 (both at δ_H 1.23) to C-6 and C-5 (δ_C 78.9), and from H-5 (δ_H 3.92) to C-7 (δ_C 25.9), C-8 (δ_C 25.5), and C-4 (δ_C 173.9). The remaining structure of **5** including the configuration of the double bond was elucidated to be identical to enamindin (**6**) after detailed analysis of the 2D NMR spectra. Thus, compound **5** was determined to be 6-hydroxyenamidin. Due to the limited amount of **5**, Mosher's reaction was carried out for **6**, whose absolute configuration had not been reported in the previous publication. [5] Calculation of differences of chemical shifts between the resulting methylated (*R*)- and (*S*)-MPA esters of **6** led to the assignment of the absolute configuration of C-5 as *R* (Figure 4). Considering the close biogenetic relationship and similarity of optical rotation between **5** and **6**, compound **5** was suggested to have the same *R* configuration at C-5.

All isolated compounds were tested for their cytotoxicity against the L5178Y mouse lymphoma cell line but proved to be inactive when tested at a concentration of 10 μM.

In conclusion, three new polyketides, phomopones A – C (**1**–**3**), a new cyclic tetrapeptide, 18-hydroxydihydrotentoxin (**4**), and a new amide, 6-hydroxyenamidin (**5**) together with a known derivative, enamindin (**6**) were isolated from the rice medium fermenta-

Table 3
^{1}H and ^{13}C NMR Data of Compound 5.

	δ_C, type	δ_H, (*J* in Hz)
1	171.1, C	
2	99.0, CH	5.18, d (9.0)
3	137.3, CH	7.44, d (9.0)
4	173.9, C	
5	78.9, CH	3.92, s
6	73.3, C	
7	25.9, CH_3	1.23, s
8	25.5, CH_3	1.23, s

Measured in CD_3OD at 300 (^{1}H) and 75 MHz (^{13}C).

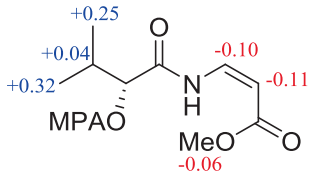

Figure 4. $\Delta\delta^{RS}$ ($\delta_R - \delta_S$) values of methylated (*R*)- and (*S*)-MPA esters of **6**.

tion of *Phomopsis* sp. The absolute configurations of those compounds were determined by X-ray crystallography, Marfey's method or Mosher's reaction.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2019.151325.

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