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Polyketides and Nitrogenous Metabolites from the Endophytic Fungus *Phomopsis* sp. D15a2a

Haiqian Yu [a], Simon-Patrick Höfert [b], Mariam Moussa [a], Christoph Janiak [b], Werner E. G. Müller [c], Blessing O. Umeokoli [a d], Haofu Dai [e], Zhen Liu [a,*], Peter Proksch [a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany

[b] Institute of Inorganic and Structural Chemistry, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany

[c] Institute of Physiological Chemistry, Universitätsmedizin der Johannes-Gutenberg-Universität Mainz, 55128 Mainz, Germany

[d] Department of Pharmaceutical and Medicinal Chemistry, Nnamdi Azikiwe University, Awka, Nigeria

[e] Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, China

*Corresponding authors.

Tel.: +49 211 81 14163; fax: +49 211 81 11923;

e-mail: zhenfeizi0@sina.com (Z. Liu),

proksch@uni-duesseldorf.de (P. Proksch)

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.

Keywords: *Phomopsis* sp.; endophytic fungus; polyketide; peptide.

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]

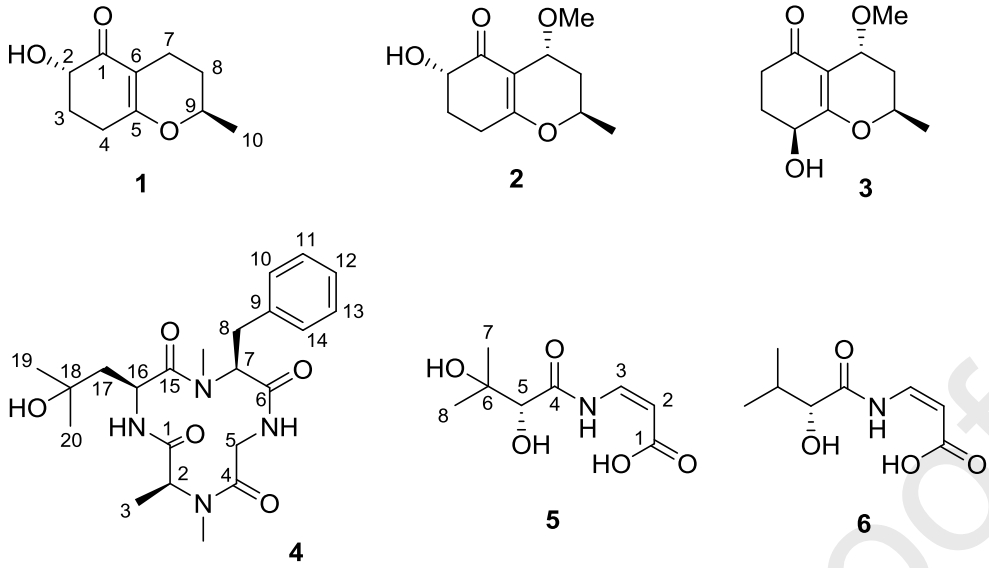

Figure 1. Structures of isolated compounds from *Phomopsis* sp.

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

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).

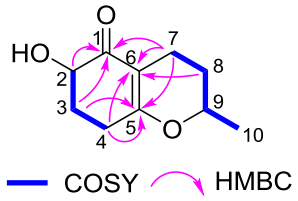

Figure 2. COSY and key HMBC correlations of compound **1**.

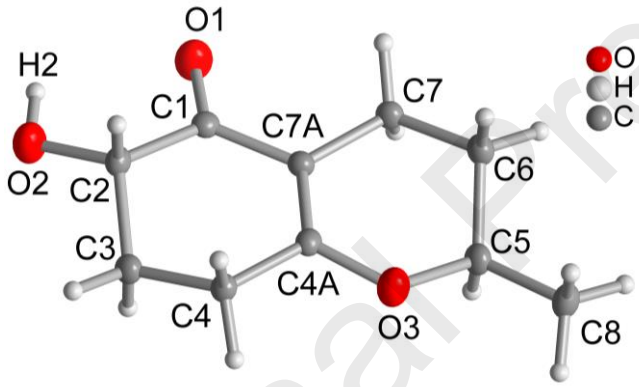

Figure 3. Molecular structure of compound **1** from X-ray diffraction analysis.

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 $^{3}J_{\text{H-9/H-8b}}$ (12.6 Hz) and the small values of $^{3}J_{\text{H-9/H-8a}}$ (2.4 Hz), $^{3}J_{\text{H-7/H-8a}}$ (2.4 Hz) and $^{3}J_{\text{H-7/H-8b}}$ (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**.

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)	30.1, CH_2	2.23, dddd (12.0, 5.0, 5.0, 3.3)	30.6, CH_2	2.20, dddd (13.3, 7.9, 4.9, 4.4)
		1.84, dddd (12.0, 12.0, 12.0, 5.0)		1.86, dddd (12.0, 12.0, 12.0, 5.0)		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)	28.4, CH_2	2.64, ddd (17.7, 12.0, 5.0)	67.2, CH	4.38, dd (7.2, 4.4)
		2.42, br ddd (17.5, 5.0, 3.2)		2.48, ddd (17.7, 5.0, 3.3)		
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	67.6, CH	4.14, dd (3.0, 2.4)	67.4, CH	4.22, dd (3.1, 2.3)
		2.22, m				
8	28.6, CH_2	1.95, dddd (13.8, 5.8, 4.6, 2.8)	34.0, CH_2	2.12, ddd (14.5, 2.4, 2.4)	33.9, CH_2	2.13, ddd (14.5, 2.3, 2.3)
		1.53, dddd (13.8, 9.3, 9.3, 5.6)		1.39, ddd (14.5, 12.6, 3.0)		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).

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. 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.

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 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 ^1H 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.

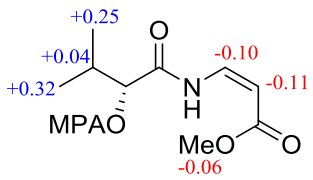

Figure 4. $\Delta\delta^{RS}$ ($\delta_R - \delta_S$) values of methylated (*R*)- and (*S*)-MPA esters of **6**.

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

	$\delta_{C,}$ 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).

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

Acknowledgements

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Supplementary Data

Supplementary data (UV, MS and NMR spectra of **1**−**5** as well as X-ray data of **1**) associated with this article can be found in the online version.

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- The endophytic fungus *Phomopsis* sp. yielded five new compounds.
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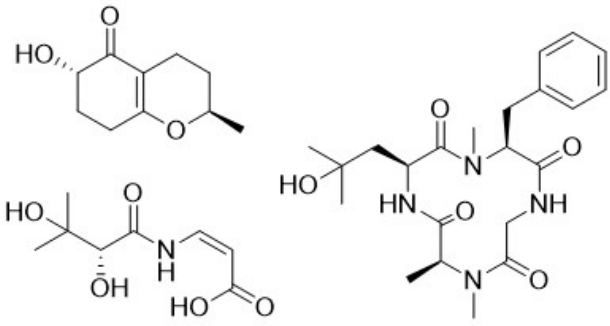
- Marfey's method was applied for **4**, a new cyclic tetrapeptide.
- The absolute configurations of amides were determined by Mosher's reaction.

Phomopsis sp.



Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: