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NOTE

Diapolic acid A–B from an endophytic fungus, *Diaporthe terebinthifolii* depicting antimicrobial and cytotoxic activity

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Endophytes are diverse microbial communities including bacteria, fungi and actinomycetes that live within the plant tissues, at least for a part of their life cycle, without causing any symptoms of disease in the host under normal conditions.[1,2] Plants have proven to be a treasure trove of diverse fungal endophytes, with an estimated range of 30–150 species per host, and subset of this endophytic fungi may be host-species specific.[3,4] Many reports have shown that plants have substantial influence on the metabolic processes of endophytes leading to a variety of biosynthetic capabilities.[4] These repertoires of their biosynthetic capacities made them one of the rich sources of novel secondary metabolites with enormous biological diversity that can serve as an invaluable source of lead compounds against a plethora of diseases.[5–8] The proficiency of endophytes to produce diverse secondary metabolites inspired us to explore the isolation of bioactive metabolites from the endophytic fungus, *Diaporthe terebinthifolii* GG3F6. Here, in our continuing search for biologically active secondary metabolites from endophytic fungi harbored in traditionally used Indian medicinal plants,[9] we investigated the fungal endophyte isolated from the rhizomes of *Glycyrrhiza glabra* Linn. The plant, *G. glabra* (licorice), a tall shrub of the Leguminosae family is used in Indian traditional medicine for treating a variety of ailments including coughs and colds.[10] The rhizomes of licorice have been used worldwide as herbal medicine and natural sweetener (30–50 times sweeter than sucrose).[11] In the preliminary screening, the EtOAc extract of *D. terebinthifolii* showed prominent anticancer activity against human breast cancer cell line (T47D) with $IC_{50} < 1\ \mu g\ ml^{-1}$; in addition, the recent literature search (Natural product database, DNP) indicated that no phytochemical study has been carried out on *D. terebinthifolii*, which prompted further investigation of the bioactive constituents of this endophyte. In the current study, we report the isolation and structural elucidation of two new fatty acid derived metabolites Diapolic acid A–B (**1–2**), from the crude extract of GG3F6. Further, these compounds (**1–4**; Figure 1a) were evaluated for cytotoxic and antimicrobial activities in which the xylarolide (**3**) was proven to be most bioactive among the isolated metabolites.

The isolation of the endophyte, sequencing of its ITS1-5.8S-ITS2 ribosomal gene, phylogenetic analysis, determination of antimicrobial and cytotoxic activity were carried out as described in our previous work.[8,9]

Fungal endophyte, GG3F6, was isolated from the rhizome of *G. glabra* collected from Jammu, J&K, India. Acquisition of the ITS1-5.8S-ITS2 ribosomal gene sequence (GenBank Acc. No. KU168142) showed that the organism is a strain of *D. terebinthifolii*. The nucleotide sequence showed >99% homology with *D. terebinthifolii*. The evolutionary position of GG3F6 is presented in Supplementary Figure S2 (Supplementary Information). The culture was also submitted to the National Fungal Culture Collection of India under the Voucher No. NFCCI 3711. The 5 l culture of GG3F6 was extracted three times with EtOAc; the resulting extract was separated via column chromatography and semi-preparative HPLC, affording two new fatty acids (**1–2**) along with two known compounds xylarolide (**3**) and phomolide G (**4**).

Compound **1** was obtained as colorless crystal from $CHCl_3$. The molecular formula was determined as $C_9H_{14}O_3$ by HRESIMS and NMR data, accounting for three indices of hydrogen deficiency. The IR spectrum suggested the presence of a conjugated acid (3344, 1691 cm^{-1}; Supplementary Figure S4). Analysis of the ^1H, ^{13}C and DEPT NMR (Table 1) together with HSQC (Supplementary Figure S9) data indicated the presence of one methyl (δ_H 0.86, δ_C 13.9, CH_3-9), two methylenes (δ_H 1.45–1.50, δ_C 39.0, CH_2-7; δ_H 1.28–1.37, δ_C 18.5, CH_2-8), one oxygenated sp^3 methine (δ_H 4.19, δ_C 71.7, CH-6), four sp^2 methines (δ_H 5.80, δ_C 120.8, CH-2; δ_H

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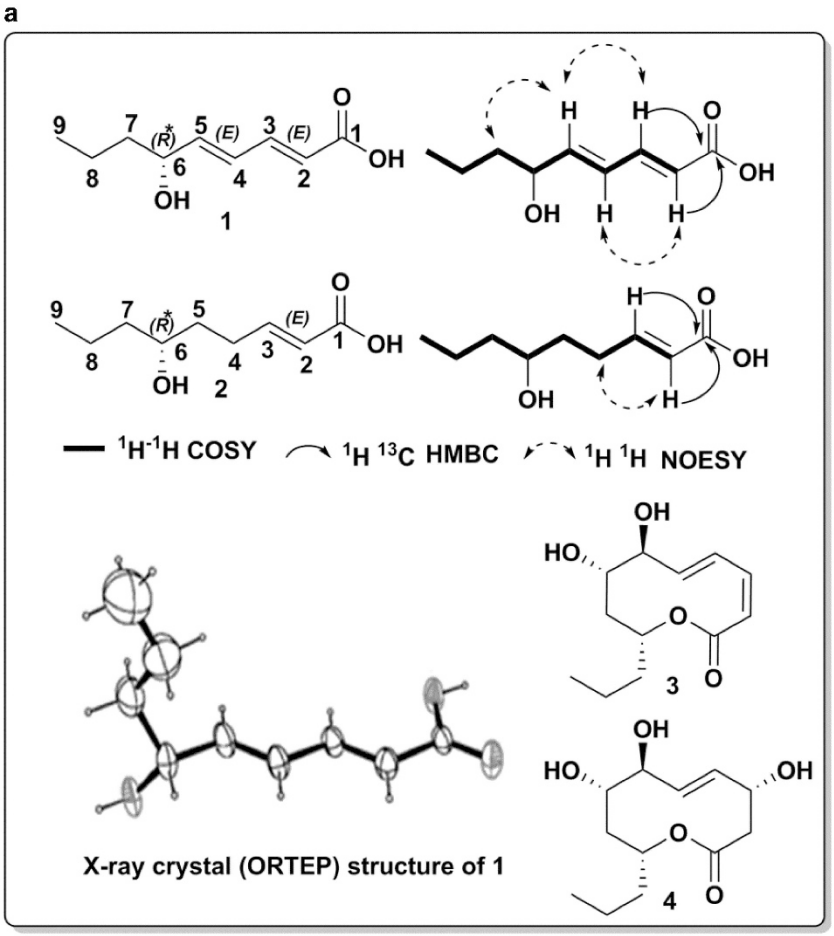

Figure 1 (**a**) Chemical structures of isolated compounds **1–4** with key COSY, HMBC and NOESY correlations of **1–2**, (**b**) Colony formation assay of T47D cells were treated with (**3**) at different concentrations for 48 h. Cells were trypsinized and 1000 viable cells were seeded in 6-well plate. Cells were allowed to form colonies for 14 days, stained with 1% crystal violet and counted manually. Columns, mean; bars, s.d.; with ***$P<0.001$, **$P<0.01$, *$P<0.05$. A full color version of this figure is available at the *The Journal of Antibiotics* journal online.

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of 1 and 2 recorded in $CDCl_3$

	1			*2*		
Position	δ_H, *mult. (J in Hz)*	δ_C, *type*	*HMBC*	δ_H, *mult. (J in Hz)*	δ_C, *type*	*HMBC*
1	—	171.9, C	—	—	171.1, C	—
2	5.80 d (15.1)	120.8, CH	1, 3, 4	5.87 d (15.4)	121.0, CH	1, 4
3	7.41–7.13 m	146.0, CH	1, 5, 4, 2	7.18–6.89 m	151.4, CH	1, 4, 5
4	6.42–6.19 m	127.2, CH	3, 5, 2, 6	2.60–2.25 m	28.5, CH_2	2, 3, 5, 6
5	6.05–6.10 m	146.0, CH	3, 4, 6, 7	1.58–1.63 m	35.3, CH_2	3, 4, 6, 7
6	4.19 d (5.5)	71.7, CH	5, 4, 7, 8	3.66 *br*s	70.9, CH	8, 4
7	1.45–1.50 m	39.0, CH_2	5, 6, 8, 9	1.44–1.50 m	39.6, CH_2	5, 6, 8, 9
8	1.28–1.37 m	18.5, CH_2	6, 7, 9	1.45–1.25 m	18.7, CH_2	6, 7, 9
9	0.86 t (7.2)	13.9, CH_3	7, 8	0.95 t (6.6)	14.0, CH_3	7, 8

Table 2 Antimicrobial activity and *in vitro* cytotoxicity of compounds 1–4

	IC_{50} (μM)					
	Human pathogens		*Cancer cell lines*			
S.No	*C. albicans*	*Y. enterocolitica*	*MIAPaCa2*	*A549*	*HCT-116*	*T47D*
1	ND	78.4 ± 1.46	ND	ND	ND	ND
2	ND	73.4 ± 0.9	ND	ND	ND	ND
3	78.8 ± 0.4	72.1 ± 1.2	38 ± 0.018	ND	100 ± 0.033	**7** ± 0.004
4	ND	69.2 ± 0.8	ND	ND	ND	ND
+Con.	1.5 ± 0.022	3.5 ± 0.202	0.0068 ± 1.19	0.0025 ± 3.82	0.006 ± 0.69	0.011 ± 2.46

Results are expressed as the mean values of IC_{50} ± s.d. in μM of three individual experiments. Ciprofloxacin, nystatin and paclitaxel were used as (+Con.) positive controls for bacterial cultures, fungal pathogens and cancer cell lines, respectively. 'ND' means no activity detected.

7.41–7.13, δ_C 146.0, CH-3; δ_H 6.42–6.19, δ_C 127.2, CH-4; δ_H 6.05–6.10, δ_C 146.0, CH-5) and one carbonyl carbon (δ_C 171.9, C-1). The ^{1}H–^{1}H COSY spectrum led to identify a coupling system H-2 ↔ H-3 ↔ H-4 ↔ H-5 ↔ H-6 ↔ H-7 ↔ H-8 ↔ H-9, which determined the structure of the unsaturated aliphatic chain as shown by bold lines in Figure 1a. In the HMBC spectrum, key correlations of H-2 and H-3/C-1(δ_C 171.9), of H-6/C-4, C-5, C-7 and C-8 suggested 6-hydroxy, $\Delta^{2,3}$ and $\Delta^{4,5}$ unsaturated carbonyl moiety. The *E* geometries of the C-2/C-3 and C-4/C-5 double bonds were assigned on the basis of the large coupling constants between H-2 and H-3 ($J = 15.1$ Hz), and between H-4 and H-5 ($J = 15.0$ Hz; Table 1) and were confirmed by NOESY correlations of H-2/H-4, of H-5/ H-3 and H-7 (Figure 1). Finally, detailed analysis of NMR spectra (^{1}H, ^{13}C NMR and 2D) along with X-ray crystallography (Figure 1a) data leads to the identification of compound **1** as (2*E*,4*E*)-6-hydroxynona-2, 4-dienoic acid. Compound **1** was named Diapolic acid A.

Compound **2** was isolated as a white powder. The molecular formula was determined as $C_9H_{16}O_3$ by HRESIMS and NMR data, accounting for two indices of hydrogen deficiency. The ^{1}H and ^{13}C NMR data (Table 1) of **2** resembled those of **1**, except for the disappearance of pair of double bond signals in **1** and the presence of pair of methylene signals in **2**. These characteristics implied that the one of the double bond group in **1** was reduced in **2**. Further, the HMBC correlations (Figure 1a) of H-2 and H-3/C-1 confirmed that the double bond between C-4 and C-5 was reduced. Detailed analysis of 2D NMR (HSQC, ^{1}H–^{1}H COSY and HMBC) spectra confirmed that the other parts of the compound **2** were the same as those of **1**. The optical rotation value of compound **2** is similar to that of compound **1**, however, precise relative configuration of compound **2** at C-6 will be determined in near future.[12] Thus, the structure of **2** was identified as (*E*)-6-hydroxynon-2-enoic acid and named Diapolic acid B.

The absolute configuration at C-6 of **1** and **2** was not determined. The known compounds xylarolide (**3**)[13] and phomolide G (**4**)[14] were authenticated by comparing their NMR data with those of reported one.

The compounds (**1–4**) were evaluated for antimicrobial activities against a panel of important bacterial and fungal pathogens (Supplementary Table S5). At 100 μM concentration, the compounds (**1–4**) were moderately active against several bacterial pathogens. Previously, no antimicrobial activity was reported with xylarolide and phomilide G against a few pathogens at different concentrations, using a plate assay.[13] However, the compounds showed maximum inhibition (⩾60%) against *Yersinia enterocolitica*, not used previously, and the IC_{50} values were 78.4, 73.4, 72.1 and 69.2 μM, respectively (Table 2). In case of the fungal pathogens, only xylarolide (**3**) displayed significant inhibitory effect against *Candida albicans*, corresponding to an IC_{50} value of 78.8 μM (Table 2). Further, all isolates (**1–4**) were screened for *in vitro* cytotoxicity against a panel of cancer cell lines, MIAPaCa-2, A549, HCT-116 and T47D using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Xylarolide (**3**) displayed potential growth inhibition of human mammary cells (T47D) with an IC_{50} of 7 μM (Table 2). Furthermore, the mechanistic studies revealed that the cell death induced by xylarolide (**3**) in T47D cells was apoptotic in nature. The apoptosis induction was confirmed by the colony formation assay (Figure 1b). There are only a few cells among the cancer cell population with an ability to form colonies, which defines the

clonogenic potential of a given type of cancer. Therefore, the ability of a chemotherapeutic agent to target these clonogenic cells is an essential feature of successful chemotherapy. With this view, we treated the T47D cells with doxorubicin (100 nM) in the presence or absence of xylarolide (**3**) (7 μM) for 48 h and then analyzed the formation of colonies. After 14 days of treatment, the number of colonies formed by the cells treated in combination with xylarolide (**3**) was significantly reduced as compared with the cells treated with doxorubicin alone. Doxorubicin is a topoisomerase-IIα inhibitor (**2**); however, it is also known to form an adduct with the DNA, resulting in induction of apoptosis, thus, leading apoptotic fragmentation of DNA. In this context, our data revealed that the potentiation of cytotoxicity of doxorubicin is enhanced by xylarolide (**3**) in T47D cells.

In summary, we report two new hydroxylated unsaturated fatty acids designated as Diapolic acid A–B (**1–2**) along with known compounds xylarolide (**3**) and phomolide G (**4**) from the EtOAc extract of an endophytic fungus *D. terebinthifolii*, associated with the medicinal plant *G. glabra*. The compound **3** was found to exhibit potential cytotoxic activity against the breast cancer cell line T47D with an IC_{50} of 7 μM and moderate antifungal activity against *C. albicans*.

Compound (**1**): colorless crystal from $CHCl_3$; $[\alpha]_D^{25}$+20 (c 0.10, $CHCl_3$); 1D and 2D NMR spectra (Table 1 and Supplementary Figures S5–S11, Supplementary Data); (+) – HRESIMS ion peak at *m/z* 171.1041 $[M+H]^+$ (calcd for $C_9H_{15}O_3$, 171.1016).

Compound (**2**): white powder; $[\alpha]_D^{25}$+5 (c 0.10, $CHCl_3$); 1D and 2D NMR data, (Table 1 and Supplementary Figures S13–S19, Supplementary Data); (+) – HRESIMS ion peak at *m/z* 173.1154 $[M+H]^+$ (calcd for $C_9H_{15}O_3$, 173.1177).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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