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Phomopsidone A, a novel depsidone metabolite from the mangrove endophytic fungus *Phomopsis* sp. A123

Wei Zhang [a,b,1], Liyan Xu [b,c,1], Lishan Yang [b], Yaojian Huang [b], Shengying Li [a], Yuemao Shen [d,*]

[a] *Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Qingdao, Shandong 266101, PR China*
[b] *School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, PR China*
[c] *Basic Medicine College, Putian University, Putian, Fujian 351100, PR China*
[d] *School of Life Sciences, Shandong University, Jinan, Shandong 250100, PR China*

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ABSTRACT

One novel pentacyclic depsidone containing an oxetane unit, phomopsidone A (**1**), together with the reported excelsione (also named as phomopsidone) (**2**), and four known isobenzofuranones (**3**–**6**) were isolated from the mangrove endophytic fungus *Phomopsis* sp. A123. Their structures were elucidated by 1D and 2D NMR spectroscopic analysis and high resolution mass spectrometry. The bioactivity assays showed that these compounds possess cytotoxic, antioxidant, and antifungal activities.

1. Introduction

A great number of compounds with novel structures and diverse bioactivities have been isolated and identified from marine-derived endophytic fungi in the past decades [1,2]. During our ongoing search for chemical constituents produced by mangrove endophytic fungi, a novel pentacyclic depsidone, phomopsidone A (**1**), together with the known depsidone compound excelsione (**2**) [3] (*i.e.* phomopsidone [4]), and four known isobenzofuranones including 7-methoxy-6-methyl-3-oxo-1,3-dihydroisobenzofuran-4-carboxylic acid (**3**) [5], diaporthelactone (**4**) [6], 7-hydroxy-4,6-dimethy-3H-isobenzofuran-1-one (**5**) [7], and 7-methoxy-4,6-dimethyl-3H-isobenzofuran-1-one (**6**) [8] were obtained from the metabolites of the mangrove endophytic fungal strain *Phomopsis* sp. A123.

Phomopsis sp. A123 was isolated from the foliage of mangrove plant *Kandelia candel* (*L.*) Druce in 2003, which was previously known as the producer of cytotoxic deacetylmycoepoxydiene and mycoepoxydiene [9,10]. Herein, we report the isolation and structural elucidation of phomopsidoneA (**1**). The cytotoxic activity, radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazinyl (DPPH), and antimicrobial activity of compounds **1**–**6** were also described.

2. Experimental

2.1. General procedures

UV spectra were recorded by UNICO single-beam 210A spectral photometer. The IR spectra were measured in KBr on a Nicolet FT-IR 360. NMR spectra were taken by a Bruker Avance III-600 NMR spectrometer with TMS as an internal standard, δ in ppm relative to Me_4Si, and J in Hz. HRESI-MS data were acquired using BioTOFTM-Q mass spectrometer (Bruker). Column chromatography was performed with silica

* Corresponding author. Tel./fax: +86 531 88382548.
E-mail address: yshen@sdu.edu.cn (Y. Shen).
[1] These authors contributed equally.

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gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), silica gel GF_{254} (Merck), Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and reversed-phase RP-18 (40–63 μm, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was carried out on pre-coated silica gel GF_{254} plates (0.20–0.25 mm, Qingdao, China).

2.2. Fungal material

The fungal strain *Phomopsis* sp. A123 was isolated from the foliage of the plant, *K. candel* (*L.*) Druce, which was collected from the mangrove nature conservation area of Fugong, Fujian, China. It was identified as a non-sporulating fungus by traditional morphology. By sequencing the ITS rDNA and comparing it to the sequence database in GenBank, A123 was assigned as a *Phomopsis* species, being closely related to *Phomopsis liquidambari* (Accession No. AY 601919), with a 98% identity [9,10].

2.3. Culture conditions and extraction

Phomopsis sp. A123 was cultured at 28 °C with 10 L of PDA medium (20% stored seawater) for 14 d. The agar cultures were diced and extracted with EtOAc/MeOH/AcOH (80/15/5, *V/V/V*). The crude extract was partitioned between EtOAc and H_2O. The organic solution was collected by filtration, and evaporated giving rise to crude extract, which was further partitioned with an equal volume of EtOAc and H_2O. The organic phase was dried over Na_2SO_4 (*anh.*) and concentrated *in vacuo* to yield 11.6 g extract (dark oil). This extract was purified by repeated column chromatography (RP-18, Sephadex LH-20, and silica gel) to afford one new and five known compounds, whose structures were elucidated by HRESI-MS, 1D and 2D NMR.

2.4. Fractionation and isolation

The crude extract (11.6 g) was subjected to silica gel column (100 g), eluted with a gradient of $CHCl_3$-acetone to yield 9 fractions: Fra.1–9. Fra.1 (0.7 g) was further subjected to silica gel column (10 g), eluted with a gradient of petroleum ether-EtOAc to yield Fra.1-1, Fra.1-2 and Fra. 1-3. Fra.1-1 (65.3 mg) was chromatographed on Sephadex LH-20 (140 g) and eluted with MeOH to give three compounds: compound **3** (5.3 mg), compound **4** (13.4 mg) and compound **5** (3.9 mg); Fra.1-2 (22.8 mg) was subjected to silica gel column (1 g) using petroleum ether-EtOAc to yield compound **1** (4.8 mg) and compound **2** (5.7 mg); Fra.1-3 (5.1 mg) was chromatographed on Sephadex LH-20 (10 g) and eluted with MeOH to provide compound **6** (3.0 mg).

Phomopsidone A (**1**):

White powder, m. p. 198 °C;IR (KBr): ν max = 3430, 1766, 1610, 1495, 1257, 1149, 1018, 880, and 720 cm^{-1}; UV (MeOH) λ_{max} ($\log\varepsilon_{max}$) = 330 nm. HRESI-MS:*m/z* = 341.0630 (*calc.* 341.0656 for $[C_{18}H_{12}O_7 + H]^+$).

2.5. Cell culture and cytotoxicity study

Human tumor cell lines Raji and MDA-MB-435 were donated by the Anticancer Center, Xiamen University and cultured in a RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg mL^{-1} penicillin, 80 μg mL^{-1} kanamycin and 100 μg mL^{-1} streptomycin. Cultures were maintained in a humidified incubator at 37 °C under an atmosphere of 5% CO_2. The cytotoxic activity of purified compounds was determined following the MTT assay developed by Mosmann et al. [11]. The optical density (OD) of the samples was measured with a microplate reader (M-3350, Bio-Rad) at 595 nm with $OD_{655\ nm}$ as a reference. Growth inhibition rate was calculated with the following equation:

$$\text{Inhibition rate} = (OD_{\text{control well}} - OD_{\text{treated well}})/(OD_{\text{control well}} - OD_{\text{blank well}}) \times 100\%$$

IC_{50} is defined as the needed concentration of a compound that results in at least 50% inhibition of growth.

2.6. Antioxidant assay

Quantitative measurement of radical scavenging properties was carried out by following the previous protocol [12] with slight modifications. The 20 mL reaction mixture contains 10 mL of 10 mM testing compound (or 80% MeOH as a blank) and 10 mL of 1 mM solution of DPPH in MeOH. As a positive control, 10 mL of 10 mM ascorbic acid was used instead of the sample compound. Decoloration was measured at 517 nm after incubation at 25 °C for 20 min. All measurements were performed in triplicate. The actual decrease in absorption induced by the tested compounds was compared to that of the positive control. The IC_{50} value calculated denotes the concentration (mg mL^{-1} in 80% MeOH) of the sample required to scavenge 50% of DPPH radicals.

2.7. Detection of antimicrobial activities

The antimicrobial activity was determined by measuring the minimal inhibitory concentration (MIC). Approximate 10^5 mL^{-1} cells or conidia suspension of the selected bacteria or fungi was inoculated into LB or PD liquid medium in each well of a 96-well microtiter plate, respectively. Each compound was dissolved in methanol at the concentration of 1 mg mL^{-1}, followed by two-fold serial dilutions on 96-well plates. Methanol was used as a negative control. Kanamycin and amphotericin B served as antibacterial and antifungal positive control, respectively. *Bacillus subtilis* CMCC(B)63501, *Staphylococcus aureus* CMCC(B)26003, *Escherichia coli* CMCC (B)44103, *Aspergillus niger*ACCC30005, and *Candida albicans* AS2.538 were used as indicator strains. Microbial growth was evaluated after incubation at 37 °C for bacteria (24 h) and at 28 °C for fungi (48 h).

3. Results

3.1. Structure elucidation

Compound **1** was isolated as white powder. The molecular formula was determined to be $C_{18}H_{12}O_7$ by HRESI-MS based on the protonated molecular ion ($[M + H]^+$) at *m/z* 341.0630 (*calc.* 341.0656 for $[C_{18}H_{12}O_7 + H]^+$), indicating twelve

degrees° of unsaturation. The detailed analysis of ^{1}H and ^{13}C NMR spectra clearly indicated the presence of a penta-substituted phenyl moiety. DEPT analysis indicated that compound **1** contains two methyl groups (δ_C 22.8, *q*; and 12.1, *q*). The down field chemical shift of C-8 (δ_C 163.5, *s*), together with the HMBC correlations from H-9 to C-7, C-7a, C-8, C-10a and C-11; and from H-7a to C-6, C-7 and C-8, depicted the penta-substituted benzene ring as the ring A of this molecule (Fig. 1). The HMBC correlations from H-10a (δ_C 22.8, *q*) to C-6, C-9, C-10, and C-11 confirmed the position of one benzyl group (ring A). The HMBC correlations between H-7a and C-6, C-7 and C-8 positioned one oxygenated methylene group (δ_C 55.2, *t*). Six more quaternary carbons (δ_C 145.5, *s*; 114.7, *s*; 150.5, *s*; 141.6, *s*; 146.8, *s*; and 111.9, *s*) were observed on the DEPT spectrum, which were attributed to a second phenyl ring B (Table 1). The HMBC correlations from H-15a to C-14, C-15 and C-16 provided the clue for this methyl (δ_C 12.1, *q*) substitution on the phenyl ring B, while the correlations from H-17 to C-1, C-2, C-4, C-14, C-15, and C-16 indicated the existence of a $-CH_2-O-CO-$ group.

Interestingly, in the HMBC spectrum of compound **1**, a remarkable number of $^{n}J_{CH}$ couplings ($n > 3$) were observed (Table 1; Fig. 2). It is well-known that $^{n}J_{CH}$ correlations for $n > 3$ are normally very weak or non-observable [13] because the H–H coupling would cause significant attenuation on C–H correlation intensities. The observation of longer range $^{n}J_{CH}$ couplings could be possible for compounds with either no or few H–H couplings. As compound **1** has no H–H couplings, the observation of longer range $^{n}J_{CH}$ coupling becomes reasonable. Similar to this study, long-range couplings have also been reported in compound **2** [3], corynesidones [14], mollocellins [15], and others.

No further HMBC information was available for determination of the connection pattern between ring A and ring B. However, the 1D and 2D NMR spectra of **1** were found to be highly similar to those of compound **2**, except the chemical shift of proton 7a at δ_H 4.77 in **2** (Table S1) dramatically shifted to δ_H 5.48 in **1**, while compound **2** was identified as excelsione

Table 1
^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) data for Compound **1** in [D6] Pyridine (TMS, δ values).

Compound 1			
No.	δ_H (mult., *J* in Hz)	δ_C	HMBC
1		170.5 (*s*)	
2		111.9 (*s*)	
3		146.8 (*s*)	
4		141.6 (*s*)	
6		164.5 (*s*)	
7		118.7 (*s*)	
8		163.5 (*s*)	
9	6.91 (*s,1H*)	117.9 (*d*)	7, 7a, 8 10a, 11
10		145.5 (*s*)	
11		113.4 (*s*)	
12		163.0 (*s*)	
14		150.5 (*s*)	
15		114.7 (*s*)	
16		145.5 (*s*)	
17	5.03 (*s, 2H*)	69.2 (*t*)	1, 2, 4, 14, 15, 16
7a	5.48 (*s, 2H*)	55.2 (*t*)	6, 7, 8
10a	2.57 (*s, 3H*)	22.8 (*q*)	6, 9, 10, 11
15a	2.01 (*s, 3H*)	12.1 (*q*)	14, 15, 16

by an X-ray crystal structure [3] (*i.e.* phomopsidone [4]). Mass spectrometric analysis revealed that the molecular weight difference is 18 amu between compounds **1** and **2**, suggesting that compound **1** might be a dehydrated product of **2**. Taking the differences of chemical shifts and molecular weights into consideration, an ether group in **1** should be formed via a dehydrolytic condensation between the two hydroxy groups tethered to C-8 and C-7a in **2**. Collectively, the structure of compound **1** was determined to be a new depsidone derivative and named as phomopsidone A (Fig. 1).

Compound **3** was obtained as white powder. Its molecular formula was assigned to be $C_{11}H_{10}O_5$ by HRESI-MS ($[M + H]^+$: *obs.* 223.0595, *calc.* 223.0606). The structure of **3** was identified to be 7-methoxy-6-methyl-3-oxo-1,3-dihydroisobenzofuran-4-carboxylic acid, which was previously synthesized by condensation of 3-hydroxy-4-methylbenzoic acid with aqueous

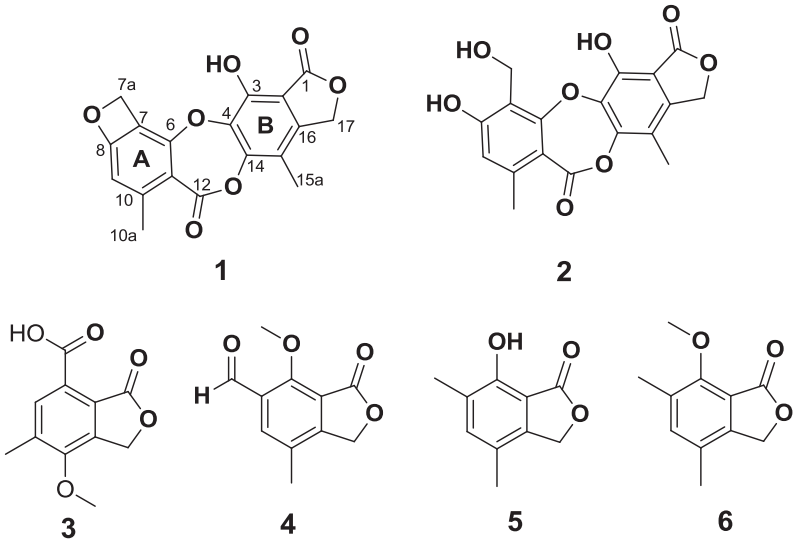

Fig. 1. Structure of compounds **1**–**6**.

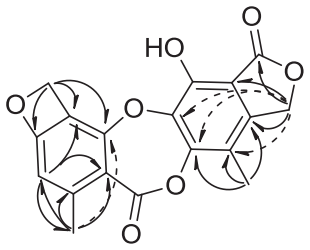

Fig. 2. Key HMBC ^{2}J and ^{3}J correlations (solid arrows) and ^{4}J correlations (dashed arrows).

formaldehyde and hydrochloric acid in early 1950s [5]. However, this is the first time that compound **3** was reported as a natural product.

3.2. Bioactivity assay

In MTT assay [11], compound **1** gave an IC_{50} of 63 μM against MDA-MB-435 cell line, while other compounds showed weaker inhibition with IC_{50} values higher than 150 μM. Compounds **4**, **5** and **6** also displayed cytotoxic activities against Raji cell line with IC_{50} values of 27 μM, 47 μM and 18 μM, respectively, while **2** showed lower cytotoxicity with an IC_{50} of 160 μM.

Compounds **1**–**6** also showed weak antioxidant activity as scavengers of DPPH radicals [12] (data not shown). In addition, compounds **4** and **5** displayed antifungal bioactivity against *A. niger* with MIC values of 243 μM and 485 μM, respectively. Compound **6** inhibited the growth of *Alternaria alternaria* with an MIC value of 500 μM. However, all these compounds did not display antibacterial activities against the tested strains including *B. subtilis*, *S. aureus*, and *E. coli*.

4. Conclusion

During our continuing search for bioactive natural products from marine-derived endophytic fungi, a novel depsidone, phomopsidone A (**1**), together with excelsione (**2**) [3] (*i.e.* phomopsidone[4]), and four known isobenzofuranones (**3**–**6**) were isolated from the mangrove endophytic fungus *Phomopsis*sp. A123. The bioactivity assays demonstrated that these compounds possess cytotoxic, antioxidant, and antifungal activities.

Structurally, phomopsidone A (**1**) belongs to depsidones, a group of secondary metabolites which were typically produced by lichens, such as neotricone [16], salazinic acid [17], and deoxystictic acid [18]. Recently, a growing number of depsidones have been isolated from various fungi, such as **2** from an unidentified endophytic fungus [3]; phomopsides from the mangrove endophytic fungus *Phomopsis* sp. ZZF08 [19], excelsional from *Phomopsis* sp. CAFT69 [20], corynesidones from the endophytic fungus *Corynespora cassiicola* L36 [14], chaetosidone A from the endophyte *Chaetomium* sp. [21], mollocellins from the fungal species of *Chaetomium brasiliense* [15], and botryorhodines from the endophytic fungus *Botryosphaeria rhodina* [22]. Among those reported depsidones, the substitution pattern of ring B in phomopsidoneA (**1**) is uncommon, with only **2** and excelsional sharing the same structural moiety. Particularly, the oxetane ring makes the structure of natural product **1** more unique.

Oxetanes have only been found in a few other natural products, a majority of which are terpenoids, such as oxetanocin A [23], dictyoxetane [24], merrilactone A [25], bradyoxetin [26], and taxol [27] (Fig. 3). Among these naturally occurring compounds, the oxetane unit is either tethered or fused to diverse ring systems. For instance, the benzo-condensed oxetanering (benzoxete) is often seen in some plant natural products, such as 5-aryl-2-hydroxybenzoxete from the stem of *Caesalpinia decapetala* [28], zizyberanone from the fruit of *Ziziphus jujuba* Mill [29], and amentotaxone from *Amentotaxus formosana* [30] (Fig. 3), while these oxetane units are substituted by hydroxy or dimethyl groups.

Phomopsidone A represents the first natural product containing non-substituted benzoxete unit. Benzoxete, due to its significant roles in chemical and biological approaches, has drawn great attentions to date [31,32]. However, the benzoxete-bearing structures were only reported as chemically synthetic products or intermediates in various reactions [33–36]. Due to high instability, the synthesized unsubstituted benzoxete, which was the parent compound for a number of benzo-condensed four-ring heterocycles, was only characterized by IR [37,38]. The first isolable benzoxete compound benzoxetene carrying methyl and acyl groups had to be prepared and characterized by the ^{1}H and ^{13}C-NMR spectra at low temperatures [34]. It was reported that methyl substituents on the benzene ring stabilized the benzoxete system only at a pure solid status, and the attempt to identify methylated benzoxetes by NMR in solution was unsuccessful [39]. Thus, in this work the isolation of phomopsidone A carrying an unsubstituted benzoxete unit might be helpful for understanding the nature's strategy for stabilization of this important structural moiety.

Although depsidones have been reported to possess antitumor [3,4,15,22], antibacterial [3,21], antifungal [15,22], radical scavenging [14], antimalarial [15], and aromatase inhibitory [14] activities, their pharmacological potential has not yet been fully explored. Meanwhile, oxetanes have been shown to be capable of improving key pharmaceutical properties when grafted onto the target molecular scaffolds [40]. Taking the antitumor drug taxol as an example, the oxetane unit leads to rigidification of the overall structure [41]; the replacement of the oxetane moiety resulted in significantly lower activity [42,43]. Thus, our identification of the oxetane containing phomopsidone A may suggest a new opportunity for further drug development based on depsidones.

In final, the putative biosynthetic route of compound **1** was proposed (Fig. 4). Specifically, two differently substituted benzoic acid derivatives with a triketide or tetraketide backbone might be synthesized after three or four time elongations with malonate two-carbon units by corresponding NR-PKS (non-reduced polyketide synthase). The extensive hydroxylations are likely mediated by multiple oxidative enzymes. The methyl groups on two aromatic rings might be installed by methyltransferases. A depside could be formed between two benzoic acid units by esterification followed by an ester bond formation between the two hydroxy groups on C-4 and C-6. Regarding the biosynthesis of benzoxete in ring A, a dehydration initiated electrocyclization likely takes

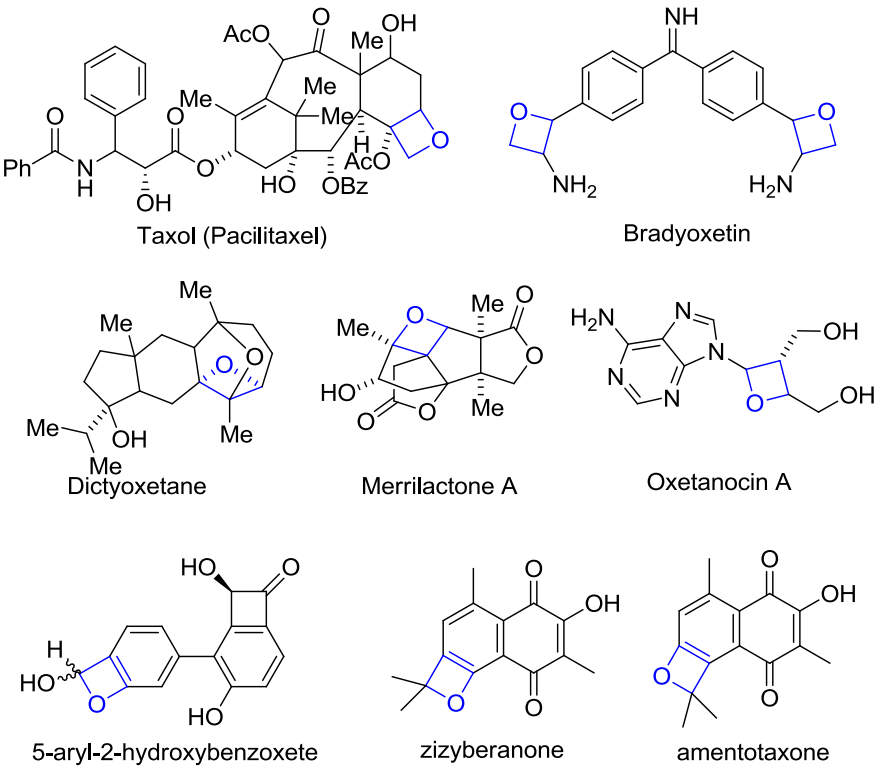

Fig. 3. Natural products containing oxetanes (blue: oxetanes unit).

place to form the four-ring heterocycle [39]. For the intramolecular lactonization in ring B, inspired by biosynthesis of the immunosuppressant mycophenolic acid [44,45], the hydroxylation of C-17 methyl group catalyzed by a P450 monooxygenase likely occurs first. Then, a hydrolase might be responsible for lactonization.

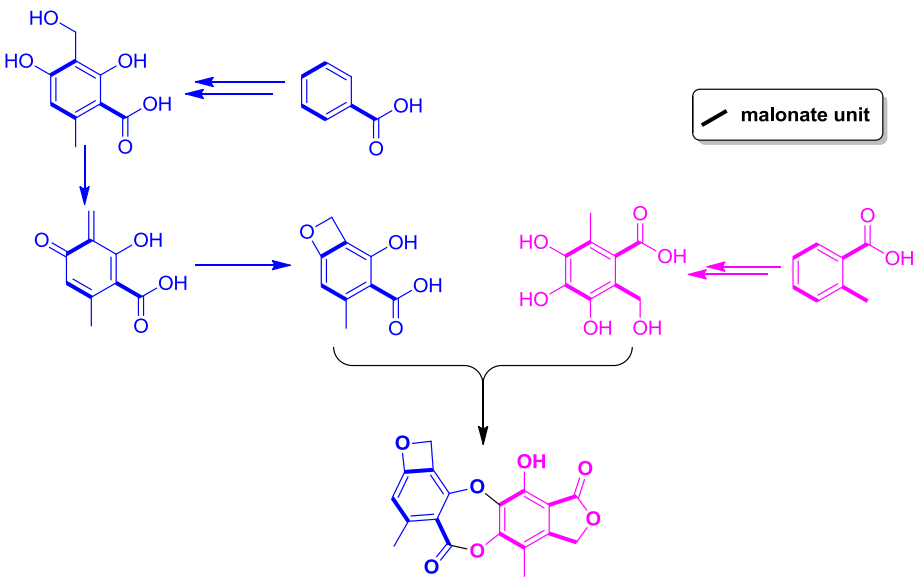

Fig. 4. A plausible biosynthetic pathway of Compound **1**.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.05.001.

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