

Communication

Two New Metabolites from the Endophytic Fungus *Alternaria* sp. A744 Derived from *Morinda officinalis*

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Abstract: Two new compounds isobenzofuranone A (1) and indandione B (2), together with eleven known compounds (3–13) were isolated from liquid cultures of an endophytic fungus *Alternaria* sp., which was obtained from the medicinal plant *Morinda officinalis*. Among them, the indandione (2) showed a rarely occurring indanone skeleton in natural products. Their structures were elucidated mainly on the basis of extensive spectroscopic data analysis. All of the compounds were evaluated with cytotoxic and α -glucosidase inhibitory activity assays. Compounds 11 and 12 showed significant inhibitory activities against four tumor cell lines; MCF-7, HepG-2, NCI-H460 and SF-268, with IC₅₀ values in the range of 1.91–9.67 μ M, and compounds 4, 5, 9, 10, 12 and 13 showed excellent inhibitory activities against α -glucosidase with IC₅₀ values in the range of 12.05–166.13 μ M.

Keywords: *Alternaria* sp.; endophytic fungus; *Morinda officinalis*; cytotoxicity; α -glucosidase inhibitory

1. Introduction

Endophytic fungi have been considered a rich source of structurally novel and bioactive diverse metabolites that have become interesting and significant resources for drug discovery [1–3]. *Morinda officinalis*, known as one of the ‘top four south authentic traditional Chinese medicines’, has obvious regional characteristics. Its roots contain plant sterols, anthraquinones, flavonoids, vitamin C, sugar, resin and other ingredients, and they are widely used to treat impotence, spermatorrhea, rheumatism and female infertility [4]. However, there are few systematic reports on endophytic fungus resources from this plant and their active components. During our ongoing search aimed at structurally unique and bioactive substances from endophytic fungi [5–8], we conducted a chemical analysis on a fraction of the broth extract of the fungus *Alternaria* sp. A744 derived from *M. officinalis*, which led to the isolation of two new secondary metabolites along with eleven known compounds (Figure 1). All the compounds were evaluated for their cytotoxic and α -glucosidase inhibitory activities via assays. Herein, the details of the isolation, structural elucidation and bioassay are described.

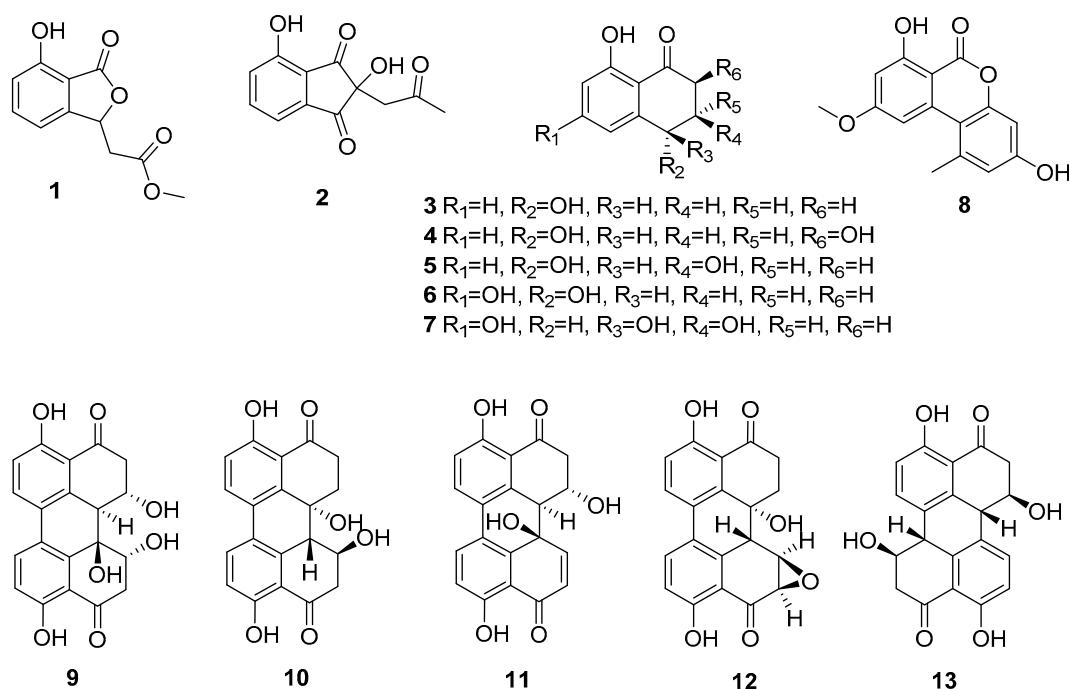


Figure 1. Structures of compounds 1–13 isolated from *Alternaria* sp. A744.

2. Results

2.1. Structural Elucidation of New Compounds

Isobenzofuranone A (**1**), colorless oil, had a molecular formula of $C_{11}H_{10}O_5$ on the basis of negative high-resolution electrospray ionization mass spectra (HR-ESI-MS) ($[M - H]^-$ m/z 221.0460, calcd. for 221.0450), corresponding to seven degrees of unsaturation (see Figures S1–S9). The infrared spectroscopy (IR) spectrum exhibited absorption bands at 3435 (hydroxyl group) and 1732 (carbonyl group) cm^{-1} . The 1H -NMR spectroscopic data of **1** (Table 1) combined with heteronuclear multiple quantum coherence (HMQC) experiment implied one methoxy signal [δ_H 3.72 (s, $J = 1.1$, H₃-10)], one methylene signal [δ_H 3.08 (dd, $J = 16.6, 4.6$), 2.80 (dd, $J = 16.6, 8.0$)], one methine [δ_H 5.82 (dd, $J = 8.0, 4.6$, H-3)] and three aromatic protons [δ_H 6.90 (d, $J = 8.2$, H-6), 7.00 (d, $J = 7.4$, H-4), 7.55 (dd, $J = 8.2, 7.4$, H-5)]. The ^{13}C -NMR spectrum revealed 11 carbon resonances attributed to two carbonyl groups (δ_C 171.2, 171.5), a methoxyl carbon δ_C 52.4 (C-10), one methylene carbon δ_C 40.0 (C-8), three sp^2 quaternary carbons [δ_C 158.4 (C-7), 152.2 (C-3a), 112.5 (C-7a)], and three sp^2 methine carbons [δ_C 113.9 (C-4), 137.8 (C-5), 117.1 (C-6)]. The COSY correlations between H-4 (δ_H 7.00) and H-5 (δ_H 7.55), H-5 and H-6 (δ_H 6.9) confirmed the presence of 1,2,3-trisubstituted benzene moiety. The benzene ring along with two carbonyl groups accounted for six unsaturation degrees, while the remaining degrees of unsaturation indicated that an additional ring must be present in the molecule. Meanwhile, a signal of ^{13}C -NMR resonance at δ_C 171.5 and an absorption in the IR spectrum at 1732 cm^{-1} suggested the presence of a lactone carbonyl group. These data showed a great resemblance to the known compound isochracinic acid [9], the only difference between them is that a hydroxyl group at position C-10 in the known compound was replaced by a methoxy group in **1**. Furthermore, the heteronuclear multiple bond correlation (HMBC) correlations (Figure 2) from methine proton H-3 (δ_H 5.82) to C-1 and C-7a, H₂-8 to C-1 and C-3a, as well as H-6 to C-7a and C-1 secured the connection of C-3 and C-1 to the aromatic ring. Simultaneously, the relative downfield chemical shift of C-3 (δ_C 78.9) revealed a connection with oxygen, thereby forming a lactone ring. The HMBC cross-peaks from H-3 and the proton of methoxy H₃-10 (δ_H 3.72) to C-9 determined the presence of a methyl acetate unit in **1**. The critical COSY signals (Figure 2) from H-3 to H₂-8 suggested that the methylene group

was directly connected to the lactone ring at C-3. Thus, the planar structure of **1** was determined, as shown in Figure 1.

Table 1. $^1\text{H-NMR}$ (500 MHz) and $^{13}\text{C-NMR}$ (125 MHz) data for **1** and **2** in CD_3OD .

No.	1		2	
	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}
1		171.2, C		200.7, C
2				73.8, C
3	5.82 (dd, 8.0, 4.6)	78.4, CH		200.8, C
3a		152.2, C		143.4, C
4	7.00 (d, 7.4)	113.9, CH	7.44 (d, 7.4)	115.5, CH
5	7.55 (dd, 8.2, 7.4)	137.8, CH	7.77 (dd, 8.2, 7.4)	139.1, CH
6	6.90 (d, 8.2)	117.1, CH	7.28 (d, 8.2)	124.4, CH
7		158.4, C		158.3, C
7a		112.5, C		127.4, C
8	3.08 (dd, 16.6, 4.6) 2.80 (dd, 16.6, 8.0)	40.0, CH_2	3.37 (d, 2.5)	49.5, CH_2
9		171.5, C		208.1, C
10	3.72 (s)	52.4, CH_3	2.09 (s)	29.3, CH_3

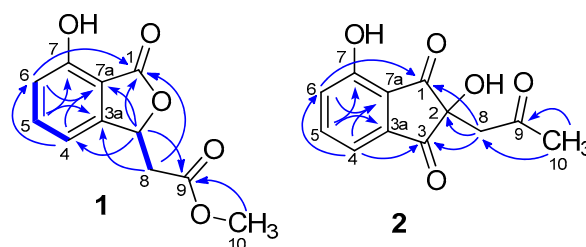


Figure 2. Key $^1\text{H-}^1\text{H}$ COSY (—) and HMBC (↷) correlations for compounds **1**, **2**.

Indandione B (**2**) was obtained as yellow oil. HRESIMS analysis of **2** revealed a molecular formula $\text{C}_{12}\text{H}_{10}\text{O}_5$ ($[\text{M} - \text{H}]^-$ m/z 233.0467, calcd. for 233.0450), corresponding to eight degrees of unsaturation (see Figures S10–S17). The IR spectrum exhibited absorption bands at 3377 (hydroxyl group), 1743 and 1703 (carbonyl groups) cm^{-1} . The $^1\text{H-NMR}$ data of **2** (Table 1) revealed one methoxy signal [δ_{H} 2.09 (s, H_3 -10)], one methylene [δ_{H} 3.37 (d, $J = 2.5$, H_2 -8)], and one 1,2,3-trisubstituted benzene moiety [δ_{H} 7.28 (d, $J = 8.2$, H-6), 7.44 (d, $J = 7.4$, H-4), 7.77 (dd, $J = 7.4$, 8.2, H-5)]. The $^{13}\text{C-NMR}$ spectrum and the HMQC revealed 12 carbon resonances attributed to one methyl, one methylene, three methines, and seven quaternary carbons. The abovementioned information was quite similar to that of the known compound indanostatin, which was isolated from *Streptomyces* sp. [10]. They all have a typical indandione five-membered ring structure, except for the absence of a hydroxyl group at position C-4 and a methyl group at position C-5 on the benzene ring in **2**. The HMBC correlations from H-4 to C-3, C-6 and C-7a, H-5 to C-3a and C-7, H-6 to C-3a, C-7a, and C-1, along with the HMBC cross-peaks between H-8 to C-1, C-2, and C-3, secured the presence of an indandione five-membered ring. Moreover, the HMBC correlations (Figure 2) from H_2 -8 and H_3 -10 to C-9 implied the presence of a 2-oxopropyl unit in **2**. Finally, the HMBC cross-peaks of methylene protons with C-1, C-2 and C-3 suggested that the 2-oxopropyl group was connected with the indandione five-membered ring at C-2. Therefore, the planar structure of **2** was assigned, as shown in Figure 1.

The known compounds were determined as isosclerone (**3**) [11], 2,4,8-trihydroxy-1-tetralone (**4**) [9], 3,4-dihydro-3,4,8-trihydroxy-1[2H]-naphthalenone (**5**) [12], 6-hydroxyisosclerone (**6**) [13], *cis*-4-hydroxyscytalone (**7**) [14], alternariol-4-methyl ether (**8**) [15], 6-*epi*-stemphytriol (**9**) [16], dihydroalterperyleneol (**10**) [17], alterperyleneol (**11**) [16], altertoxin II (**12**) [18,19], and stemphyperyleneol (**13**) [20], by spectroscopic analysis and comparison with previous reports in literature.

2.2. In Vitro Cytotoxicity Assay

The in vitro cytotoxic activity of compounds **1–13** was investigated against four tumor cell lines, including MCF-7, HepG-2, NCI-H460 and SF-268, by the SRB (Sulforhodamine B) method with cisplatin as the positive control. As outlined in Table 2, compounds **11** and **12** showed significant inhibitory activities against the four tumor cell lines with IC₅₀ values in the range of 1.91–9.67 μM.

Table 2. Cytotoxic activity of compounds **1–13**.

Compounds	IC ₅₀ (μM)			
	MCF-7	HepG-2	NCI-H460	SF-268
1–8	≥100	≥100	≥100	≥100
9	35.73 ± 1.61	52.38 ± 2.46	43.31 ± 1.75	49.04 ± 1.84
10	≥100	≥100	≥100	≥100
11	3.73 ± 0.33	5.30 ± 0.95	5.47 ± 0.26	6.57 ± 0.35
12	1.91 ± 0.17	5.63 ± 0.10	9.67 ± 0.22	4.25 ± 0.01
13	≥100	≥100	≥100	≥100
Cisplatin	3.09 ± 0.27	1.39 ± 0.18	2.43 ± 0.15	2.37 ± 0.35

2.3. α-Glucosidase Inhibitory Activity Assay

Simultaneously, all compounds were further evaluated for their α-glucosidase inhibitory activity. Compounds **4**, **5**, **9**, **10**, **12** and **13** showed excellent inhibitory activity against α-glucosidase with IC₅₀ values in the range of 12.05–166.13 μM (Table 3), which was obviously stronger than the positive control of acarbose (IC₅₀ = 427.34 μM).

Table 3. α-Glucosidase inhibitory activities of compounds **4**, **5**, **9**, **10**, **12** and **13**.

Compounds	α-glucosidase (IC ₅₀ , μM)
4	34.88 ± 1.59
5	102.34 ± 2.45
9	141.43 ± 7.66
10	74.94 ± 2.70
12	12.05 ± 2.06
13	166.13 ± 2.81
Acarbose	427.34 ± 12.03

3. Materials and Methods

3.1. General Experimental Material

Optical rotations were determined on an Anton Paar MCP-500 spectropolarimeter (Anton Paar, Graz, Austria) at room temperature. UV spectra were recorded on a Shimadzu UV-2600 spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured using a Shimadzu IR Affinity-1 spectrometer (Shimadzu, Kyoto, Japan). 1D and 2D NMR spectra were performed on a Bruker Avance-500 spectrometer with tetramethylsilane (TMS) as an internal standard (Bruker, Fällanden, Switzerland). ESIMS was measured by an Agilent Technologies 1290-6430A Triple Quad LC/MS (Agilent Technologies, Palo Alto, CA, USA). HRESIMS were done with a Thermo MAT95XP high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Shimadzu LC-20 AT (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A PDA detector was used for HPLC, and a YMC-pack ODS-A/AQ column (250 mm × 20 mm, 5 μm, 12 nm) was used for semi-preparative HPLC separation. Column chromatography (CC): silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), C-18 reversed phase silica gel (40–63 μm, Merck, Darmstadt, Germany), and Sephadex LH-20 gel (Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden). Silica gel

60 GF₂₅₄ glass plates (Merck, Darmstadt, Germany) were used for thin layer chromatography (TLC) spotting. All solvents used were of analytical grade (Guangzhou Chemical Regents Company, Ltd., Guangzhou, China). α -Glucosidase from *Saccharomyces cerevisiae* was purchased from Sigma (St. Louis, MO, USA).

3.2. Fungal Material

The endophytic fungal strain A744 was isolated from the twigs of *Morinda officinalis*, which was collected from Gaoyao city, Guangdong province of China, in January 2015. The strain A744 was identified by sequence analysis of rDNA ITS (internal transcribed spacer) region. The sequence of the ITS region of the strain has been submitted to GenBank (Accession No. KF706672). By using BLAST (nucleotide sequence comparison program) to search the GenBank database, A744 has 100% similarity to *Alternaria* sp. MY-2011 (Accession No. JN038490). The strain was preserved at the Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology.

3.3. Fermentation, Extraction and Compound Isolation

The fungal strain *Alternaria* sp. A744 was cultivated on potato-dextrose agar (PDA) medium at 28 °C for 5 days, and then plugs of agar supporting mycelial growth were cut into small pieces and transferred aseptically into five Erlenmeyer flasks (500 mL), each containing 250 mL potato-dextrose broth, and incubated on a rotary shaker for 5 days at 28 °C at 120 r/min to prepare the seed culture. Then, 10 mL of the seed culture was inoculated into a total of 600 Erlenmeyer flasks (500 mL) containing 250 mL culture broth for 7 days under the same conditions. The culture (150 L) was filtered to separate the broth and mycelia. The broth was extracted five times with EtOAc, while the mycelia were homogenized and saturated with MeOH by the ultrasonic extraction method. The EtOAc crude extract (7.1 g) was subjected to column chromatography (CC) on Sephadex LH-20 (CH₂Cl₂/MeOH, 1:1, *v/v*) to obtain four fractions (A–D) based on TLC monitoring.

Fraction D (3.5 g) was further purified on C₁₈ reversed phase silica gel and eluted with a gradient of MeOH/H₂O (*v/v*, 3:7→10:0) to yield eight major fractions (D1–D8). Subfraction D2 (1.7 g) was separated by a silica gel CC to give five fractions (D2.1–D2.5). D2.2 was subjected to a semi-preparative HPLC (ACN/H₂O, 30:70, 3 mL/min), then a secondary preparation HPLC (MeOH/H₂O, 40:60, 3 mL/min) to afford compounds **1** (4.6 mg) and **3** (8.0 mg). D2.3 was further separated by Sephadex LH-20 (CH₂Cl₂/MeOH, 1:1, *v/v*), following a silica gel CC (Hexane/Acetone, 8:1→2:1, *v/v*) and a semi-preparative HPLC (ACN/H₂O, 15:85, 3 mL/min) to obtain compounds **2** (6.0 mg), **4** (8.4 mg) and **5** (2.2 mg). D2.4 was separated by Sephadex LH-20 (Acetone), following a silica gel CC to yield compound **6** (9.2 mg). D2.5 was separated by a silica gel CC, following a Sephadex LH-20 (Acetone) and a preparation HPLC (ACN/H₂O, 10:90, 3 mL/min) to afford compound **7** (2.7 mg). D3 (125.3 mg) was further purified on Sephadex LH-20 (MeOH), following a semi-preparative HPLC (MeOH/H₂O, 55:45, 3 mL/min) to obtain compound **9** (17.0 mg). D4 (101.1 mg) was applied onto a semi-preparative HPLC (MeOH/H₂O, 45:55, 3 mL/min) to acquire compound **10** (13.4 mg). D5 (46.9 mg) was displayed in the same way as D4 to give compound **11** (20.5 mg). Fraction D7 (26.3 mg) was subjected to a silica gel CC, following a Sephadex LH-20 (Acetone) to obtain compound **12** (2.0 mg). Compound **8** (3.0 mg) was separated from D8 (66.1 mg) by a silica gel CC and a semi-preparative HPLC (MeOH/H₂O, 80:30, 3 mL/min). Finally, compound **13** (6.0 mg) was isolated from mycelia using a silica gel CC, following a semi-preparative HPLC (MeOH/H₂O, 50:50, 3 mL/min).

3.4. Spectroscopic Data

Isobenzofuranone A (**1**): colorless oil; $[\alpha]_D^{25} +2.9$ (*c* 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ) 211 (4.11), 233 (3.56), 299 (3.38) nm; IR ν_{\max} 3435, 2955, 2920, 2851, 1732, 1607, 1468, 1285, 1161, 1003 cm⁻¹; ESIMS negative *m/z* 221.0 [M – H]⁻; HRESIMS *m/z* 221.0460 [M – H]⁻ (calcd. for C₁₁H₉O₅, 221.0450); and ¹H (500 MHz) and ¹³C (125 MHz) NMR data, see Table 1.

Indandione B (2): yellow oil; $[\alpha]_D^{25}$ -4.3 (c 0.1, MeOH); UV (MeOH) λ_{\max} (log ϵ) 201 (4.03), 235 (3.02), 334 (3.57) nm; IR ν_{\max} 3377, 2924, 2853, 1744, 1703, 1601, 1464, 1290, 1177, 1020 cm^{-1} ; ESIMS negative m/z 233.0 $[M - H]^-$; HRESIMS m/z 233.0467 $[M - H]^-$ (calcd. for $\text{C}_{12}\text{H}_9\text{O}_5$, 233.0450); and ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1.

3.5. In Vitro Cytotoxicity Assay

The in vitro cytotoxic activities of compounds (1–13) were assayed against four human tumor cell lines MCF-7, HepG-2, NCI-H460 and SF-268, with cisplatin as a positive control. Assays were performed by the SRB method [21].

3.6. α -Glucosidase Inhibitory Activity Assay

An assay of α -glucosidase inhibitory activity was evaluated according the method previously published in Reference [22].

4. Conclusions

In this study, thirteen compounds, including two new ones, were isolated from *Alternaria* sp., an endophytic fungus from *Morinda officinalis*. All the structures were established by extensive spectroscopic analysis. The isolates were evaluated their cytotoxicities against four human tumor cell lines and α -glucosidase inhibitory activity assays. Compounds 11 and 12 exhibited significant inhibitory activities with IC_{50} values in the range of 1.91–9.67 μM . Compounds 4, 5, 9, 10, 12 and 13 showed excellent inhibitory activity against α -glucosidase, which might be useful for further developing α -glucosidase inhibitor.

Supplementary Materials: Supplementary material relating to this article can be accessed online.

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Author Contributions: Wei-Min Zhang conceived and designed the experiments and reviewed the manuscript; Ying Wang, Yu-Chan Chen, Hao-Hua Li, Sai-Ni Li and Ming-Li Yan performed the experiments; Hong-Xin Liu and Zhang-Hua Sun analyzed the data; Ying Wang and Hong-Xin Liu wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds of 1–13 are available from the authors.



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