Abstract: The EtOAc extract of the liquid fermentation of Alternaria sp. Samif01, an endophytic fungus obtained from Salvia miltiorrhiza Bunge, showed antibacterial activity against several tested bacterial pathogens. Fractionation of this extract led to the isolation of seven dibenzo-α-pyrones (1-7), including one new compound, 2-acetoxy-2-epi-altenuene (1) and one new natural product, 3-epi-dihydroaltenuene A (2). The structures of the new metabolites were elucidated by comprehensive analysis of the spectroscopic data including (1D-, 2D-) NMR, and HRESIMS, while the absolute configuration of 1 was determined by TDDFT ECD computation. Altenisol (5), 4-hydroxyalternariol-9-methyl ether (6), and alternariol (7) showed inhibitory activities against the tested bacteria with MIC values in the range of 86.7-364.7 µM. A preliminary structure-antibacterial activity relationship was discussed. In addition, compounds 2, 5 and 6 displayed promising antioxidant effects by using DPPH and hydroxyl radical assays. The cytotoxicity of the isolated compounds was evaluated as well.

Keywords: endophytic fungus; dibenzo-α-pyrones; antibacterial; antioxidant; Alternaria; Salvia miltiorrhiza
Contents

Experimental Section ........................................................................................................... 3

General experimental procedure ....................................................................................... 3
Extraction and isolation ....................................................................................................... 3
DPPH radical scavenging assay .......................................................................................... 4
Hydroxyl radical scavenging assay ..................................................................................... 5

References ......................................................................................................................... 5

Table S1. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data of 1 and 2 (CD$_3$OD) ............... 6

Figure S1. Key $^1$H-$^1$H COSY and HMBC correlations of 1 ........................................ 7

Figure S2. Key NOESY correlations of 1 ............................................................................. 7

Figure S3. Low-energy conformers ($\geq$1%) of 2R, 3S, 4aS-1 obtained by optimization with B3LYP/6-31G (d) with CPCM for MeOH ................................................................. 8

Figure S4. Key NOESY correlations of 2 ............................................................................. 9

Figure S5. Comparison of the CD spectra of 1 and 4 measured in MeOH ......................... 9

Figure S6. $^1$H NMR spectrum of 1 (CD$_3$OD, 400MHz) ................................................ 10

Figure S7. $^{13}$C NMR spectrum of 1 (CD$_3$OD, 100MHz) ................................................. 10

Figure S8. HMBC spectrum of 1 (CD$_3$OD) ...................................................................... 11

Figure S9. NOESY spectrum of 1 (CD$_3$OD) .................................................................... 11

Figure S10. HRESIMS spectrum of 1 ................................................................................. 12

Figure S11. IR spectrum of 1 ............................................................................................. 12

Figure S12. $^1$H NMR spectrum of 2 (CD$_3$OD, 400MHz) ............................................. 13

Figure S13. $^{13}$C NMR spectrum of 2 (CD$_3$OD, 100MHz) ............................................. 13

Figure S14. NOESY spectrum of 2 (CD$_3$OD) ................................................................. 14

Figure S15. HRESIMS spectrum of 2 ................................................................................. 14
Experimental Section

General experimental procedure

Optical rotations were recorded on a Rudolph Autopol IV automatic polarimeter (Rudolph Research Analytical, New Jersey). Circular dichroism (CD) spectra were recorded on a JASCO J-810 CD spectrometer (JASCO Corp., Tokyo, Japan). Infrared (IR) spectra were measured on a Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Electron Scientific Instrument Crop., Wisconsin). High-resolution electrospray ionization–mass spectrometry (HR-ESI–MS) spectra were recorded on a Bruker Apex IV FTMS instrument (Bruker Daltonics, Bremen, Germany). $^1$H, $^{13}$C, and 2D NMR (HSQC, COSY, HMBC) spectra were measured on Avance 400 NMR spectrometers (Bruker BioSpin, Zürich, Switzerland). Chemical shifts were expressed in δ (ppm) referring to the solvent residual peaks at δ$_H$ 3.31, δ$_C$ 49.0 for CD$_3$OD, and coupling constants (J) in Hertz. Silica gel (200–300 mesh) for column chromatography and GF254 silica gel for TLC was purchased from the Qingdao Marine Chemical Company (China). Semi-preparative high performance liquid chromatography (HPLC) separation was carried out on a Lumtech instrument (Lumiere Tech. Ltd., Beijing, China) equipped with a K-501 pump (flow rate: 3 mL/min) and a K-2501 UV detector using a Luna-C18 column (250 mm×10 mm i.d., 5 μm, Phenomenex Inc., California).

Extraction and isolation

The fungal mycelia and broth were separated by filtration, then extracted with methanol and ethyl acetate, respectively. The ethyl acetate extract of the broth and the methanolic extract of the mycelia were combined for having similar constituents as revealed by TLC and HPLC analysis. The mixture (30 g) was subjected to vacuum liquid chromatography (VLC) (10 cm×8cm, L×i.d) over silica gel eluting with a gradient of dichloromethane-methanol (100:0, 99:1, 98:2, 97:3, 95:5, 90:10, 80:20, 50:50, 0:100) to obtain nine fractions (Fr. A-I).

Fr. C (4 g) was further subjected to VLC (i.d×L, 4 cm×25 cm) using a mixture of petroleum ether and acetone with increasing polarity (50:1, 20:1, 15:1, 10:1, 8:1, 5:1, 3:1, 1:1, 1:3, 0:1) as eluents to yield eight subfractions (Fr.C-1~C-8). Fr. C-3 was
chromatographed over Sephadex LH-20 (chloroform-methanol, 1:1) to afford a mixture of 5 (4 mg) and 7 (5 mg), which was further purified by semi-preparative HPLC eluting with 55% of methanol-water. Similarly, Fr. C-6 was subjected to gel permeation over Sephadex LH-20 (chloroform-methanol, 1:1), and followed by purification using semi-preparative HPLC (40% of methanol-water as the eluent) to yield 4 (16 mg), 3 (6 mg) and 2 (3.2 mg).

Fr. E (1.0 g) was processed in a similar way by MPLC over silica gel (i.d×L, 1.5 cm×50 cm) eluting with a gradient of petroleum ether-acetone (50:1, 20:1, 15:1, 10:1, 8:1, 5:1, 3:1, 1:1, 1:3, 0:1) to obtain eight subfractions (Fr. E-1~E-8). Subfraction E-7 was subjected to gel permeation over Sephadex LH-20 (chloroform-methanol, 1:1), and further purified by semi-preparative HPLC (60% MeOH-H$_2$O) to yield 6 (4 mg).

Fr. G (2.0 g) was subjected to MPLC over silica gel (i.d×L, 3.0 cm×50 cm) eluting with a gradient of petroleum ether-acetone as described for Fr. E to yield eight subfractions (Fr. G-1~G-8). Subfraction G-5 was purified in a similar manner as described for Fr. E-7, however, a different solvent system (45% MeOH-H$_2$O) was used in the semi-preparative HPLC, to afford 1 (1.3 mg).

DPPH radical scavenging assay

DPPH radical scavenging activity was determined based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reported previously (Wang et al. 2012). Briefly, 80 μL of DPPH solution (0.2 mg/mL) and 20 μL of each tested compound of various concentrations were added into each well of a 96-well microplate. The microplate was shaken vigorously for complete mixing of the solution and incubated at 37°C for 30 min in the dark. The absorbance of the solution was then measured at 517 nm. The experiments were performed in triplicate. Butylated hydroxytoluene (BHT) was used as the positive control. Percentage (%) of DPPH radical scavenging activity was calculated by \[\frac{(A_c - A_s)}{A_c}\] × 100, where $A_c$ is the absorbance of the reaction mixture without test sample, and $A_s$ is the absorbance of the tested sample. The median effective concentration (EC$_{50}$) was calculated using the linear relation between the inhibitory probability and concentration logarithm.
**Hydroxyl radical scavenging assay**

The hydroxyl radical scavenging activity was determined as described previously with some modifications (Li et al. 2012). Stock solutions of FeSO$_4$ (2.0 mg/ml) and 1% H$_2$O$_2$ in distilled de-ionized water, and salicylic acid (SA) (1.5 mg/ml) in ethanol were prepared.

Briefly, 25 μl of FeSO$_4$, 50 μl of H$_2$O$_2$ was mixed in 96-well microplate to generate hydroxyl radical. Then 50 μl of SA and 50 μl of sample with various concentrations were added to each well. The reaction mixtures were shaken and incubated at 37°C for 1 h. The absorbance at 526 nm was then recorded. All the tests were performed in triplicate. Ascorbic acid was used as the positive control.

The hydroxyl radical scavenging activity was calculated by the following equation:

Scavenging activity (%) = \( \frac{D_0 - (D_1 - D_2)}{D_0} \times 100 \)

Where \( D_0 \) is the absorbance of reaction mixture without tested sample, \( D_1 \) is the absorbance of the sample and \( D_2 \) is the absorbance of the sample under identical condition as \( D_1 \) with ethanol instead of SA. The EC$_{50}$ value was calculated the same as that used for DPPH radical scavenging assay.

**References**

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Table S1. $^1$H (400 MHz) and $^{13}$C (100 MHz) NMR data of 1 and 2 (CD$_3$OD)

| Position | 1 $^{13}$C NMR | 1 $^1$H NMR | 2 $^{13}$C NMR | 2 $^1$H NMR |
|----------|----------------|-------------|----------------|-------------|
|          | $\delta$, type | $\delta$, mult. ($J$ in Hz) | $\delta$, type | $\delta$, mult. ($J$ in Hz) |
| 1        | 126.5, CH      | 6.18, d (3.0) | 31.6, CH$_2$   | 2.49, dt (13.1, 4.1) |
| 2        | 74.0, CH       | 5.28, dd (3.1, 5.6) | 75.7, CH | 3.59, m |
| 3        | 67.7, CH       | 3.97, ddd (8.4, 3.8, 5.6) | 72.8, CH | 3.56, m |
| 4        | 40.7, CH$_2$   | 2.40, dd (14.4, 3.8) | 45.6, CH$_2$ | 2.24, dd (12.2, 4.4) |
| 4a       | 82.1, C        | 84.2, C     | 170.3, C       | 170.3, C     |
| 6        | 170.1, C       | 102.2, C    | 165.9, C       | 165.9, C     |
| 6a       | 115.5, C       | 102.2, C    | 165.9, C       | 165.9, C     |
| 7        | 165.3, C       | 165.9, C    | 168.1, C       | 168.1, C     |
| 8        | 102.2, CH      | 6.50, d (2.3) | 105.2, CH | 6.40, d (1.9) |
| 9        | 168.0, C       | 168.1, C    | 99.9, CH       | 6.33, d (1.9) |
| 10       | 103.9, CH      | 6.67, d (2.3) | 99.9, CH | 6.33, d (1.9) |
| 10a      | 140.2, C       | 144.1, C    | 144.1, C       | 144.1, C     |
| 10b      | 137.1, C       | 42.7, CH    | 3.19, dd (12.9, 3.3) | 3.19, dd (12.9, 3.3) |
| 11       | 28.0, CH$_3$   | 1.55, s     | 19.1, CH$_3$   | 1.22, s     |
| 9-OCH$_3$ | 56.4, CH$_3$   | 3.88, s     | 56.2, CH$_3$   | 3.85, s     |
| 2-OCH$_3$ | 172.2, C       |             |               |             |
| 2-OOC$_H$ | 20.9, CH$_3$   | 2.10, s     |               |             |
Figure S1. Key $^1$H-$^1$H COSY and HMBC correlations of 1

Figure S2. Key NOESY correlations of 1
Figure S3. Low-energy conformers (≥1%) of 2R, 3S, 4aS-1 obtained by optimization with B3LYP/6-31G (d) with CPCM for MeOH
Figure S4. Key NOESY correlations of 2

Figure S5. Comparison of the CD spectra of 1 and 4 measured in MeOH

Note: The intensity of the spectrum of 4 was scaled by a factor of 2.
Figure S6. $^1$H NMR spectrum of 1 (CD$_3$OD, 400MHz)

Figure S7. $^{13}$C NMR spectrum of 1 (CD$_3$OD, 100MHz)
Figure S8. HMBC spectrum of 1 (CD$_3$OD)

Figure S9. NOESY spectrum of 1 (CD$_3$OD)
Figure S10. HRESIMS spectrum of 1

Figure S11. IR spectrum of 1
Figure S12. $^1$H NMR spectrum of 2 (CD$_3$OD, 400MHz)

Figure S13. $^{13}$C NMR spectrum of 2 (CD$_3$OD, 100MHz)
Figure S14. NOESY spectrum of 2 (CD$_3$OD)

Figure S15. HRESIMS spectrum of 2