SUPPLEMENTARY MATERIAL

A new secondary metabolite of the crinoid (*Comanthina schlegeli*) associated fungus *Alternaria brassicae* 93.

Liu-Hong Zhang^a, Hong-Wei Wang^b, Jia-Yi Xu^a, Jing Li^{a, c} *, Lan Liu^{a, d} *

^a School of Marine Sciences, Sun Yat-Sen University, Guangzhou 510275, P.R. China;

^b School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, P.R. China

^c South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, Guangzhou 510275, P.R. China

^d Key Laboratory of Functional Molecules from Oceanic Microorganisms (Sun Yat-Sen University), Department of Education of Guangdong Province, Guangzhou 510275, P.R. China

*Corresponding authors. Jing Li: Tel: 020-84114834, Email: zsulijing@163.com;

Lan Liu: Tel: 020-84114834, Email: cesllan@mail.sysu.edu.cn.

Fungus *Alternaria brassicae* 93 isolated from crinoid (*Comanthina schlegeli*), which was collected from the South China Sea. Six compounds were isolated from *Alternaria brassicae* 93, including one new compound (**1**), along with five known compounds, ochratoxin A methyl ester (**2**), *cis*-4-hydroxymellein (**3**), (R)-7-hydroxymellein (**4**), trans-2-anhydromevalonic (**5**) and protocatechuic acid (**6**). Their structures were determined by spectroscopic methods and comparison with reported data. Cytotoxicity against two human cancer cell lines and antibacterial activity against twelve aquatic bacteria of compound **1** were also tested.

Keywords: crinoid; *Alternaria brassicae*; associated fungus; secondary metabolites

Experimental section

1. General experimental procedures

The NMR spectra were recorded on Bruker AVANCE 400 and AVANCE 500 (Bruker Co. Ltd., Zurich, Switzerland). TLC was carried out on precoated silica gel GF-254 plates (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China) and chromatography was performed over silica gel (200–300 mesh and 300–400 mesh; Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), Sephadex LH-20 (GE healthcare, Buckinghamshire, UK) and reversed-phase HPLC (Waters 1525, Waters Co. Ltd., Massachusetts, USA). The ESIMS data were measured on Thermo LCQ DECA XP plus Mass Spectrometer (Thermo Co., Massachusetts, USA). Optical rotations were recorded using ADP 440 automatic rotation (Bellingham+Stanley Ltd., Wales, UK). UV spectra were obtained with UV BlueStar A (Lab Tech Inc., Beijing, China).

2. Source and fermentation of fungus

The strain 93 was isolated from a Crinoid (*Comanthina schlegeli*) sample collected from the South China Sea and was identified as *Alternaria brassicae* by ITS sequence analysis. A stock culture of strain 93 was inoculated in 1000 mL baffled flasks containing 300 mL PDB seed medium (30% potatoes extract, 2% dextrose, 3% sea salt, pH 5.6±0.2) and was cultured at 28°C for 72 h on a rotary shaker (150 rpm). Then, 3 mL of the resulting culture was respectively transferred into 130 bottles of 1000 mL Erlenmeyer flasks containing rice medium (60 g rice, sea salt 3% in 80 mL water, pH 7.0) and was fermented by static culturing for 30 days at 28°C.

3. Extraction and isolation

Each flask was immersed in methanol (300 mL × 3) for three days and filtered through filter to obtain methanol extracts, which were concentrated under reduced pressure and extracted with ethyl acetate (300 mL × 4). The ethyl acetate crude extract (58 g) was loaded on silica gel column, eluting with PE (petroleum ether)-EA (ethyl acetate) (100:0-0:100) and seven fractions were collected. Fraction B (0.7 g) was purified by silica gel column with PE-EA (100:0-0:100) to give fraction B-1 and fraction B-2. Fraction B-1 was further purified by semipreparative HPLC with C₁₈

column (21.2×250 mm, $5\mu\text{m}$), eluting with MeOH-H₂O (50:50, v/v) to give compound **1** (7.8mg). Fraction B-2 was subjected to Sephadex LH-20 column equilibrated with MeOH-CH₂Cl₂ (1:1) to give fraction B-2-1 and fraction B-2-2. Fraction B-2-1 was further purified by semipreparative HPLC using MeOH-H₂O (80:20, v/v) as eluent to afford compound **2** (290.6mg). Fraction B-2-2 was isolated by semipreparative HPLC, eluting with MeOH-H₂O (60:40, v/v) to get compound **3** (19.5mg) and compound **4** (14.8mg). Fraction D (0.3 g) was purified by silica gel column with PE-EA (100:0-0:100) and MeOH-CH₃Cl (100:0-0:100) as well as by semipreparative HPLC and eluted with MeOH-H₂O (40:60, v/v) to obtain compound **5** and compound **6** (5.6mg).

4. Cytotoxicity assay

The cytotoxicity of compound **1** were evaluated against human breast carcinoma cell line (MDA-MB-435) and human lung cancer cell line (A549) by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide colorimetric assay. The assay was performed in 96-well tissue culture-treated plates as reported in the literature (Li et al. 2012). The epirubicin (EPI) was used as positive control, while DMSO was used as negative control.

5. Antibacterial activity assay

The antibacterial activity of compound **1** against twelve pathogenic bacteria (*Escherichia coli*, *Shigella castellani*, *Salmonella*, *Staphylococcus aureus*, *Vibrio parahemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio cholera*, *Citrobacter freundii*, *Exiguobacterium aurantiacum*, *Morganella morganii* and *Bacillus cereus*) were evaluated by using a whole-cell based assay in 96-well sterile polypropylene microtiter plates. Samples were serially diluted into eight concentrations (0.75 $\mu\text{g/ml}$, 1.50 $\mu\text{g/ml}$, 3.1 $\mu\text{g/ml}$, 6.25 $\mu\text{g/ml}$, 12.50 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$) in 20% DMSO/water and transferred in duplicate to 96-well flat bottom microplates. Bacterial inocula were prepared by correcting the OD₆₀₀ of bacterial suspensions in Mueller-Hinton broth to afford final target inocula. All bacteria were read at 600 nm using Multiskan Spectrum Microplate Reader (Thermo Scientific Inc., Shanghai, China) prior to and after incubation. The combination of several antibiotics was used as positive control, while DMSO was used as negative control.

6. The NMR data of compounds 1-6

Compound 1: ^1H NMR (400 MHz, Acetone- d_6) δ : 0.95(dd, $J = 7.4$ Hz, H-14), 1.84(dd, $J = 1.4$, 6.8 Hz, H-12), 1.88(dd, $J = 1.7$, 6.9 Hz, H-9), 1.89(s, H-13), 2.62(q, $J = 7.4$ Hz, H-5), 5.19(s, OH-4), 6.18(dq, $J = 1.7$, 15.7 Hz, H-10), 6.24(dq, $J = 1.5$, 15.2 Hz, H-7), 6.88(dq, $J = 6.8$, 15.7 Hz, H-11), 7.06(dq, $J = 7.0$, 15.2 Hz, H-8). ^{13}C NMR (100MHz, Acetone- d_6) δ : 8.9(C-14), 11.7(C-13), 18.6(C-9), 19.6(C-12), 54.6(C-5), 88.4(C-4), 121.3(C-10), 125.5(C-7), 133.9(C-11), 137.8(C-2), 147.1(C-8), 163.5(C-3), 198.2(C-6), 204.0(C-1).

Compound 2: ^1H NMR (400 MHz, Acetone- d_6) δ : 1.58(3H, d, $J = 6.3$ Hz, H-21), 2.93(1H, dd, $J = 11.9$, 17.2 Hz, H-4a), 3.21(1H, dd, $J = 7.1$, 13.9 Hz, H-14a), 3.26(1H, dd, $J = 5.5$, 13.8 Hz, H-14b), 3.32(1H, dd, $J = 3.0$, 17.2 Hz, H-4b), 3.74(3H, s, H-23), 4.95(1H, m, H-3), 4.96(1H, dt, $J = 6.9$, 7.1 Hz, H-13), 7.25(1H, m, H-18), 7.27(1H, m, H-16/H-20), 7.30(1H, m, H-17/H-19), 8.22(1H, s, H-6), 8.47(1H, d, $J = 6.9$ Hz, H-12), 12.91(1H, s, OH-8). ^{13}C NMR (100MHz, Acetone- d_6) δ : 20.8(C-21), 32.6(C-4), 38.3(C-14), 52.6(C-23), 55.2(C-13), 76.9(C-3), 111.7(C-9), 121.3(C-7), 123.4(C-5), 127.8(C-18), 129.3(C-17/C-19), 130.2(C-16/C-20), 137.5(C-15), 138.5(C-6), 142.7(C-10), 159.7(C-8), 162.8(C-1), 170.7(C-11), 172.3(C-22).

Compound 3: ^1H NMR (400 MHz, Acetone- d_6) δ : 1.51(3H, d, $J = 6.6$ Hz, H-9), 4.66(1H, d, $J = 1.5$ Hz, H-4), 4.79(1H, m, H-3), 4.83(1H, s, OH-4), 6.95(1H, d, $J = 8.5$ Hz, H-7), 6.99(1H, d, $J = 7.4$ Hz, H-5), 7.59(1H, t, $J = 7.8$ Hz, H-6), 11.1(1H, s, OH-8). ^{13}C NMR (100MHz, Acetone- d_6) δ : 16.3(C-9), 67.1(C-4), 79.5(C-3), 108.3(C-8a), 118.0(C-7), 119.5(C-5), 137.3(C-6), 143.3(C-4a), 162.5(C-8), 170.4(C-1).

Compound 4: ^1H NMR (400 MHz, Acetone- d_6) δ : 1.50(3H, d, $J = 6.2$ Hz, H-9), 2.65(1H, dd, $J = 11.6$, 16.7 Hz, H-4), 3.19(1H, dd, $J = 2.9$, 16.8 Hz, H-4), 4.75(1H, m, H-3), 6.71(1H, d, $J = 8.4$ Hz, H-5), 7.12(1H, d, $J = 8.5$ Hz, H-6), 8.37(1H, s, OH-7), 10.57(1H, s, OH-8). ^{13}C NMR (100MHz, Acetone- d_6) δ : 21.1(C-9), 29.1(C-4), 77.0(C-3), 109.2(C-8a), 116.2(C-5), 124.8(C-6), 125.6(C-4a), 146.3(C-7), 156.2(C-8), 170.9(C-1).

Compound 5: ^1H NMR (500 MHz, $\text{CD}_3\text{OD}-\text{d}_4$) δ : 2.16(3H, s, H-6), 2.38(2H, t, $J = 6.5$ Hz, H-5), 3.71(2H, t, $J = 6.5$ Hz, H-4), 5.72(1H, s, H-6). ^{13}C NMR (125MHz, $\text{CD}_3\text{OD}-\text{d}_4$) δ : 19.0(C-6), 44.9(C-5), 60.9(C-4), 118.7(C-3), 158.1(C-2), 170.4(C-1).

Compound 6: ^1H NMR (500 MHz, $\text{CD}_3\text{OD}-\text{d}_4$) δ : 6.80(1H, d, $J = 8.1$ Hz, H-6), 7.42(1H, dd, $J = 1.8$, 8.1 Hz, H-4), 7.44(1H, d, $J = 1.8$ Hz, H-7). ^{13}C NMR (125MHz,

CD₃OD-d₄) δ : 115.9(C-7), 117.9(C-6), 123.5(C-5), 124.0(C-4), 146.2(C-3),
151.6(C-2), 170.6(C-1).

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

The MS and NMR spectra of compound 1

Figure S2. The ESI-MS spectrum of compound 1

Figure S3. The HRESI-MS spectrum of compound 1

Figure S4. The ^{13}C NMR spectrum of compound 1

Figure S5. The ^1H NMR spectrum of compound 1

Figure S6. The DEPT (90°) spectrum of compound 1

Figure S7. The DEPT (135°) spectrum of compound 1

Figure S8. The HSQC spectrum of compound 1

Figure S9. The ^1H - ^1H COSY spectrum of compound 1

Figure S10. The HMBC spectrum of compound **1**

Figure S11. The NOESY spectrum of compound **1**