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An Acetophenone Derivative, Clavatol, and a Benzodiazepine Alkaloid, Circumdatin A, from the Marine-Derived Fungus *Cladosporium*

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Abstract – The crude extract of the mycelium of *Cladosporium* was found to exhibit antimicrobial activity against the *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus*. Bioassay-guided fractionation of an organic extract led to the isolation of an acetophenone derivative, clavatol (2',4'-dihydroxy-3',5'-dimethylacetophenone) (**1**), and a benzodiazepine alkaloid, circumdatin A (**2**). Compound **1** showed moderate antibacterial activity against *S. aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* with minimum inhibitory concentration (MIC) values of 62.5, 62.5, 31.0 μg/mL, respectively, but compound **2** was inactive. Compounds **1** and **2** exhibited UV-A protection activity with ED_{50} values of 227.0 and 82.0 μM, respectively, indicating that they were more potent than the positive control, oxybenzone (ED_{50} 350 μM), a common sunscreen agent.
Keywords – *Cladosporium* sp., Acetophenone, Clavatol, Benzodiazepine alkaloid, Circumdatin A

Introduction

Multidrug-resistant strains of many clinically important pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Staphylococcus aureus* (MDRSA) strains, are posing a worldwide health problem (Doern *et al*., 1998; Spencer *et al*., 1997). There is an urgent need to discover new agents to treat the patients infected with methicillin-resistant and multidrug-resistant bacteria (Jones, 1996). Since the discovery of penicillin from *Penicillium notatum* in the 1940s, terrestrial microorganisms have been a key source of many important products in the drug industry. These encouraging results obtained from terrestrial microorganisms suggest that their marine counterparts might also have the potential to be useful sources of new drug leads. Our screening assays were designed to identify antimicrobial compounds that are active against *S. aureus*, MRSA and MDRSA (Zhang *et al*., 2008a). We investigated antimicrobial activity from the fungal extracts, and a crude mycelium extract of *Cladosporium* sp. was found to be active in the *S. aureus*, MRSA, and MDRSA screen. Bioassay-guided fractionation led to the isolation of clavatol (**1**) (Astudillo *et al*., 2000; Weber *et al*., 2005) as an active component. Also isolated from the active fraction was the benzodiazepine alkaloid, circumdatin A (**2**) (Rahbaek *et al*., 1999). This paper describes the isolation of an acetophenone derivative, clavatol (**1**), a benzodiazepine alkaloid, circumdatin A (**2**), and the evaluation of **1** and **2** for their antibacterial and UV-A blocking properties.

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Experimental

Fungal isolation and culture – The fungal strain was isolated from the surface of the marine red alga *Chondria crassicualis* (Korean name: Seosil), collected at Yokji Island, Kyeongnam Province, Korea in 2008, and identified as a *Cladosporium* sp. based on fatty acid methyl ester analysis (Korean Culture Center of Microorganisms, Seoul, Korea, similarity index of 0.638). A voucher specimen is deposited at Pukyong National University with the code MFC353-b. The fungus was cultured (10 L) for 3 weeks (static) at 29 °C in SWS medium consisting of soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and isolation – The mycelium and broth were separated by filtration, and the freeze-dried mycelium

cake was extracted with CH_2Cl_2-MeOH (1 : 1) to afford crude extract (1.8 g), which was subjected to silica gel flash chromatography. Elution was performed with *n*-hexane-EtOAc (stepwise, 0 - 100% EtOAc) to yield four fractions. Fractions 2 and 3, which were active in antibacterial assay, were separated by medium-pressure liquid chromatography (MPLC) (ODS) using a H_2O-MeOH gradient elution to afford crude compounds **1** and **2**, respectively. These were further purified by HPLC (ODS-A, 10×250 mm, 1 mL/min) utilizing a 30 min gradient program of 50% to 100% MeOH in H_2O to furnish **1** (9.0 mg) and **2** (7.0 mg), respectively.

Clavatol (1) : a colorless oil; UV (MeOH) λ_{max} nm (log ε) 215 (1.2), 270 (1.1); IR (KBr) ν_{max} 3402, 1692, 1633, 1594, 1216, 1187, 1105 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 2.50 (3H, s, H_3-1), 7.47 (1H, s, H-6'), 2.00 (3H, s, H_3-7'), 2.12 (3H, s, H_3-8'), 12.91 (1H, s, 2'-OH), 9.49 (1H, s, 4'-OH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 26.2 (CH_3, C-1), 203.1 (qC, C-2), 112.2 (qC, C-1'), 160.6 (qC, C-2'), 110.3 (qC, C-3'), 160.6 (qC, C-4'), 115.9 (qC, C-5'), 130.3 (CH, C-6'), 8.2 (CH_3, C-7'), 16.2 (CH_3, C-8'); LREIMS *m/z* 180 $[M]^+$ (9), 165 $[M-CH_3]^+$ (22), 118 (2), 109 (1), 87 (12), 83 (100).

Circumdatin A (2) : a colorless oil; UV (MeOH) λ_{max} nm (log ε) 289 (3.6), 348 (3.6), 365 (3.6), 373 (3.5); IR (film) ν_{max} 2958, 2934, 1672, 1649, 1607, 1589, 1546, 1437, 1386, 1289, 1239, 1218, 1192, 1143, 1051, 779 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 7.26 (1H, d, J = 2.6 Hz, H-4), 7.19 (1H, dd, J = 9.2, 2.6 Hz, H-6), 7.48 (1H, d, J = 9.2 Hz, H-7), 5.73 (1H, br d, J = 2.0 Hz, H-13), 5.69 (1H, dd, J = 5.9, 2.0 Hz, H-15), 6.40 (1H, d, J = 5.9 Hz, H-16), 4.54 (1H, dd, J = 7.1, 2.0 Hz, H-19), 2.63 (1H, m, H_a-20), 2.04 (2H, m, H_b-20, H_a-21), 1.93 (1H, m, H_b-21), 3.37 (1H, m, H_a-22), 3.61 (1H, m, H_b-22), 3.64 (3H, s, H_3-23), 3.86 (3H, s, H_3-24); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 162.9 (qC, C-2), 133.0 (qC, C-3), 112.3 (CH, C-4), 158.7 (qC, C-5), 116.9 (CH, C-6), 130.1 (CH, C-7), 125.2 (qC, C-8), 161.9 (qC, C-10), 158.1 (qC, C-11), 109.7 (qC, C-12), 95.1 (CH, C-13), 156.5 (qC, C-14), 115.3 (CH, C-15), 145.1 (CH, C-16), 157.1 (qC, C-18), 57.9 (CH, C-19), 26.1 (CH_2, C-20), 23.1 (CH_2, C-21), 46.1 (CH_2, C-22), 54.9 (CH_3, 14-OMe), 55.6 (CH_3, 5-OMe); LREIMS *m/z* 393 $[M]^+$ (100), 378 $[M-CH_3]^+$ (26), 364 $[M+H-OCH_3]^+$ (26), 350 (11), 349 $[M+H-OCH_3-CH_3]^+$ (10), 322 (10), 281 (4), 229 (4), 210 (1), 202 (1), 196 (2), 183 (1), 176 (15), 160 (19), 146 (4), 135 (27), 117 (19), 102 (10), 77 (22).

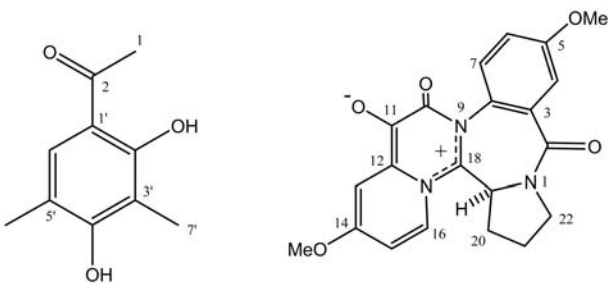
Antibacterial assay – The *in vitro* antibiotic activity in fermentation broth and purification samples was evaluated by the conventional a 2-fold serial dilution method using *S. aureus*, methicillin-resistant *S. aureus*, and multidurg-resistant *S. aureus* as indicator strains. A 5-mL suspension containing 10^5 cells per mL was used as inoculum of the test organism. The MIC values were determined after the inoculation for 18 hours at 37 °C (Li *et al*., 2003).

Ultraviolet-A protecting assay – Samples to be tested were dissolved in MeOH, and the solution (200 μL) was dispensed into wells of a 96-well microtiter tray. The absorbance of the sample solution was measured at 340 nm with microplate reader (Packard Co., Spectra Count™). The ultraviolet-A protecting activity was expressed as ED_{50}, which is the concentration of the tested compound required to give a 50% increase of the absorbance from that of the blank solution [MeOH (200 μL)]. The ED_{50} value was determined by linear regression of data plotted on a semi-log scale.

Results and Discussion

Clavatol (**1**) is a colorless oil isolated from the mycelium extract. The IR spectrum of **1** suggested the presence of hydroxyl (3402 cm^{-1}), conjugated ketone (1692 cm^{-1}), and aromatic (1633 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra, including DEPT, showed one acetyl, two singlet methyls, two aromatic hydroxyls, one aromatic methine, and five quaternary sp^2-hybridized carbons, including two oxygenated functional groups. Detailed analysis of the ^1H and ^{13}C NMR spectra of **1**, including the results from HMQC and HMBC experiments, suggested the presence of a 1,2,3,4-tetrasubstituted acetophenone. The presence of an acetophenone chromophore was further supported by the UV spectral data [215 nm (log ε 1.2), 270 (1.1)]. Diagnostic HMBC correlations, from H_3-1 to C-2 and C-1', from H_3-7' to C-2' and C-3', from H_3-8' to C-4', C-5', and C-6', and from H-6' to C-2, C-2', and C-8', showed the connection between C–2 and C–1' and the positions of the two hydroxyl and two methyl groups. Based on this evidence, we propose that compound **1** is clavatol (2',4'-dihydroxy-3',5'-dimethylacetophenone) (Astudillo *et al*., 2000; Weber *et al*., 2005). Clavatol (**1**) has been detected in the endophytic fungi, *Aspergillus clavatus* (Hassal and Todd, 1947), *Phompsis* sp. isolated from the medicinal plant *Erythrina crista-galli* (Weber *et al*., 2005), and *Trichoderma pseudokoningii* isolated from the sunflower *Helianthus annus* L. (Astudillo *et al*., 2000). However, isolation of clavatol (**1**) from the genus of *Cladosporium*, reported here, is the first such description.

Circumdatin A (**2**) was isolated as a colorless oil. The



1 2

Fig. 1. Chemical structures of clavatol (**1**) and circumdatin A (**2**).

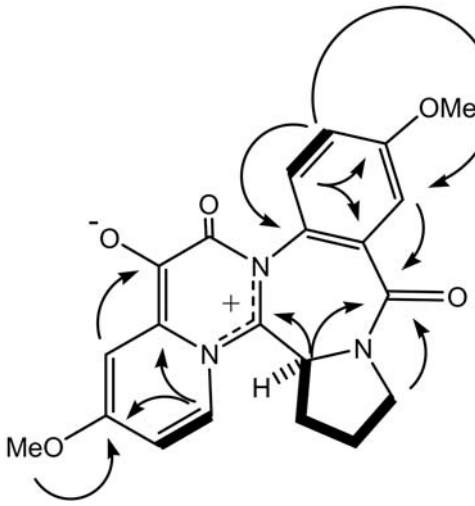


Fig. 2. Structure of circumdatin A (**2**) was elucidated by ^{1}H - ^{1}H COSY (▬) and HMBC (→) correlations.

IR spectrum of **2** exhibited bands characteristic of amide (1672 cm^{-1}) and double bond (1649 cm^{-1}) functionalities. The ^{1}H and ^{13}C NMR data for benzodiazepine **2** showed the presence of two 1,2,4-trisubstituted aromatic rings, two methoxy groups, one deshielded sp^3 methine, three sp^3 methylenes, two amides, and two deshielded sp^2 quaternary carbons bonded to hetero atoms (Fig. 1).

Detailed analysis of the COSY, TOCSY, HMQC, and HMBC spectra of **2** suggests the presence of 1,2,2,3,4-pentasubstituted-7-methoxy-1,4-benzodiazepine and 3,4-disubstituted 8-methoxy-3-azaquinazolin-1,2-dione (Fig. 1). The presence of 3,4-disubstituted 8-methoxy-3-azaquinazolin-1,2-dione was further supported by UV spectral data [348 nm (log ε 3.6), 365 (3.6), 373 (3.5)].

The connectivities and assignments of the functional groups of **2**, which suggested a planar structure for this metabolite, were made by interpretation of COSY and HMBC data (Fig. 2).

Thus, compound **2** was characterized as circumdatin A based on direct comparison with the physicochemical data of circumdatin A (Rahbaek *et al*., 1999). Isolation of circumdatin A from the genus of *Cladosporium* is the first report.

Benzodiazepine alkaloids [e.g., circumdatins A-I (Rahbaek *et al*., 1999; Dai *et al*., 2001; Rahbaek and Breinholt, 1999; Lopez-Gresa *et al*., 2005; Zhang *et al*., 2008b), *epi*-aszonalenins A-C (Rank *et al*., 2006), benzodiazepinedione (Barrow and Sun, 1994), and asperlicins (Sun *et al*., 1994)] are microbial products commonly found in nutrient-rich cultures of both terrestrial and marine fungi. They exhibit interesting biological activities, such as treatment of gastrointestinal and CNS disorder (Rahbaek and Breinholt, 1999; Sun *et al*., 1994), inhibition of mitochondrial NADH oxidase (Lopez-Gresa *et al*., 2005), psychoactive properties (Rank *et al*., 2006), and UV-A protection activity (Zhang *et al*., 2008b). Circumdatins A-H have been previously isolated from *Aspergillus ochraceus* and appear to be good chemotaxonomic markers for this species (Rahbaek *et al*., 1999; Dai *et al*., 2001; Rahbaek and Breinholt, 1999; Lopez-Gresa *et al*., 2005). Circumdatin I was isolated from a marine isolate of the fungus *Exophiala* (Zhang *et al*., 2008b).

Clavatol (**1**) showed moderate antibacterial activity against *S. aureus*, MRSA, and MDRSA with MIC values of 62.5, 62.5, 31.0 μg/mL, respectively. Compounds **1** and **2** exhibited UV-A protection activity with ED_{50} values of 227.0 and 82.0 μM, respectively, indicating that they were more potent than the positive control, oxybenzone (ED_{50} 350 μM), a common sunscreen agent.

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