A New Anthraquinone and Cytotoxic Curvularins of a *Penicillium* sp. from the Rhizosphere of *Fallugia paradoxa* of the Sonoran Desert

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In the course of screening rhizosphere microflora of Sonoran desert plants for potential anticancer agents[1,2], an unidentified *Penicillium* sp. (Trichonaceae) isolated from the rhizosphere of the apache plume (*Fallugia paradoxa* D. Don; Rosaceae) was found to produce several cytotoxic substances. Fractionation of the cytotoxic EtOAc extract led to the isolation of a new anthraquinone (**1**), a known anthraquinone (**2**), and cytotoxic curvularins, **3**～**5**. This is the first report of the natural occurrence of **2**. Curvularins **3**～**5** have been reported as metabolites of several fungi including *Penicillium* sp.[3,4] This paper briefly describes the isolation of **1**～**5**, structure elucidation of **1**, and evaluation of **1**～**5** for their ability to inhibit the growth of three sentinel tumor cell lines.

Materials and Methods

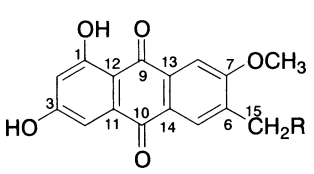
General Experimental Procedures

Physical and Spectral Data

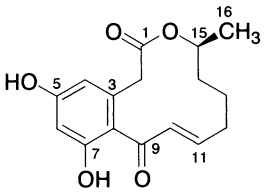
Melting points were determined on a Gallenkamp micromelting point apparatus and are uncorrected. IR spectra for KBr disks were recorded on a Shimadzu FTIR-8300 spectrometer and UV spectra in MeOH on a Shimadzu UV-1601 spectrometer. 1D and 2D NMR spectra were recorded on a Bruker DRX-500 instrument at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Low- and high-resolution mass spectra were recorded respectively on Shimadzu LCMS-QP8000α and JEOL HX110A spectrometers.

Fungal Isolation, Identification and Cultivation

The fungal strain was isolated from the rhizosphere of the apache plume [*Fallugia paradoxa* D. Don] growing in Chiricahua Mountains in southern Arizona, and was recognized as *Penicillium* by Dr. ELIZABETH E. PIERSON and Ms. JUN ZHANG of the Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona. However, the characteristic features of this species did not match those of any known *Penicillium*, leading to its classification as a new, previously undescribed species, and the analysis of the ITS regions of the ribosomal DNA was inconclusive. Excised roots of *F. paradoxa* (1 cm long sections; *ca.* 5 g) were placed in 5 ml phosphate buffered saline (PBS, 0.1 M, pH=7.4) and microorganisms were detached from the root surface by vortexing and sonication. A serial dilution of the suspension was placed on potato dextrose agar (PDA, Difco, Plymouth, MN) supplemented with chloramphenicol and streptomycin. After 4 days of incubation at 25°C, single colonies were transferred to water agar containing the same antibiotics and after 3 days a pure culture of *Penicillium* sp. was obtained by hyphal



1 R = OH
2 R = H



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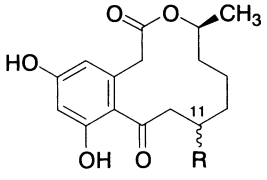

4 R = OCH_3
5 R = OH

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tipping. The strain is deposited in the Division of Plant Pathology and Microbiology and Southwest Center for Natural Products Research and Commercialization of the University of Arizona microbial culture collections under the code name AH-00-89-F6. The organism was subcultured on PDA slants, overlaid with 40% glycerol and stored at −80°C. For isolation of secondary metabolites the fungus was cultured in sixty T-flasks (800 ml) each containing 135 ml of PDA coated on 5 sides of the flasks (total surface area per flask *ca.* 460 cm^2), for 30 days at 27°C.

Extraction and Isolation

Methanol (200 ml) was added to each of the sixty T-flasks after 30 days of cultivation. The flasks were shaken overnight at room temperature, and the resulting extract was filtered through a layer of Celite 545 on a Whatman No. 1 filter paper. The residual medium in each T-flask was washed with 50 ml of MeOH and the combined filtrate (8.6 liters) was concentrated to one forth of its volume and extracted with EtOAc (3×1000 ml). Combined EtOAc extracts were evaporated under reduced pressure to afford a dark brown solid (1.18 g), which was partitioned between hexane and 80% aqueous MeOH (MeOH/H_2O–80 : 20). The cytotoxic 80% aqueous MeOH fraction was evaporated under reduced pressure to obtain a dark brown solid (1.02 g) which was subjected to gel permeation chromatography on a column of Sephadex LH-20 (30.0 g) in hexane/CH_2Cl_2 (4 : 1) and eluted with hexane/CH_2Cl_2 (4 : 1) (800 ml), CH_2Cl_2/acetone (3 : 2) (600 ml), CH_2Cl_2/acetone (1 : 4) (300 ml), CH_2Cl_2/MeOH (1 : 1) (300 ml) and finally with MeOH (100 ml). Forty two fractions (50 ml each) were collected and combined on the basis of their TLC profiles to yield five major fractions [A (219.2 mg), B (153.6 mg), C (276.1 mg), D (72.2 mg), and E (45.7 mg)] of which the fractions B, C and D were found to be cytotoxic. Chromatography of fraction B (150.0 mg) on silica gel (4.0 g) by elution with hexane/acetone (4 : 1) afforded a yellow solid (36.5 mg) which was further separated by reversed phase TLC (eluant: 25% H_2O in MeOH) to furnish **2** (8.1 mg) and **3** (27.4 mg). Fraction C (270.0 mg) was further fractionated on silica gel (6.0 g) using CH_2Cl_2/MeOH (95 : 5) to furnish **1** (45.6 mg) and **5** (9.3 mg). Chromatography of fraction D (70.0 mg) on silica gel (2.2 g) by elution with CH_2Cl_2/MeOH (92 : 8) afforded **4** (7.9 mg).

1,3-Dihydroxy-6-hydroxymethyl-7-methoxyanthraquinone (**1**): Yellow powder; mp 272~274°C; UV λ_{max}^{MeOH} nm 218, 249, 432; IR ν_{max}^{KBr} cm^{-1} 3402, 2932, 1628, 1450, 1404, 1342, 1312, 1250, 1173, 1103; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.26 (1H, s, OH-1) 11.10 (1H, brs, OH-3), 7.76 (1H, s, H-5), 7.50 (1H, s, H-8), 7.05 (1H, d, J=2.0 Hz, H-4), 6.56 (1H, d, J=2.0 Hz, H-2), 5.58 (1H, brs, OH-15), 4.65 (2H, s, CH_2-15), 3.94 (3H, s, CH_3-16); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 186.3 (s, C-9), 182.3 (s, C-10), 164.4 (s, C-1), 164.2 (s, C-3), 160.6 (s, C-7), 151.4 (s, C-6), 134.7 (s, C-11), 134.1 (s, C-13), 118.3 (s, C-14), 116.8 (d, C-5), 116.2 (d, C-8), 110.1 (s, C-12), 108.2 (d, C-2), 107.0 (d, C-4), 62.2 (t, C-15), 56.4 (q, C-16); APCIMS+ve mode m/z 301 $[M+1]^+$; APCIMS−ve mode m/z 299 $[M-1]^+$; HRFAB-MS: calcd. for $C_{16}H_{13}O_6$ $[M^+ + H]$ 301.0712; found: m/z 301.0712.

1,3-Dihydroxy-6-methyl-7-methoxyanthraquinone (**2**): Yellow powder; ^{1}H NMR (acetone-d_6, 500 MHz) δ 7.66 (1H, s, H-5), 7.41 (1H, s, H-8), 7.16 (1H, d, J=2.4 Hz, H-4), 6.63 (1H, d, J=2.4 Hz, H-2), 3.93 (3H, s, OCH_3-7), 2.49 (3H, s, H-15); ^{13}C NMR (acetone-d_6, 125 MHz) δ 187.7 (s, C-9), 183.3 (s, C-10), 167.9 (s, C-1), 165.1 (s, C-3), 162.0 (s, C-7), 147.8 (s, C-6), 136.1 (s, C-11), 135.5 (s, C-13), 121.0 (d, C-5), 120.3 (d, C-8), 119.2 (s, C-14), 111.6 (s, C-12), 109.2 (d, C-2), 107.8 (d, C-4), 56.8 (q, OCH_3-7), 22.0 (t, C-15); APCIMS+ve mode m/z 285 $[M+1]^+$; APCIMS−ve mode m/z 283 $[M-1]^+$.

Dehydrocurvularin (**3**): Pale yellow needles; ^{1}H NMR (acetone-d_6, 500 MHz) δ 6.78 (1H, d, J=15.5 Hz, H-10), 6.57 (1H, dq, J=15.5, 4.8 Hz, H-11), 6.36 (1H, d, J=2.4 Hz, H-4), 6.31 (1H, d, J=2.4 Hz, H-6), 4.73 (1H, m, H-15), 4.08 (1H, d, J=17.3 Hz, H-2), 3.61 (1H, d, J=17.7 Hz, H-2), 2.42 (1H, m, H-12a), 2.35 (1H, m, H-12b), 1.99 (1H, m, H-13), 1.85 (1H, m, H-14), 1.67 (1H, m, H-13), 1.62 (1H, m, H-14), 1.19 (3H d, J=6.4 Hz, CH_3-16); ^{13}C NMR (d_6-acetone, 125 MHz) δ 197.7 (s, C-9), 172.3 (s, C-1), 166.5 (s, C-7), 163.6 (s, C-5), 150.1 (d, C-11), 140.0 (s, C-3), 133.1 (d, C-10), 116.2 (s, C-8), 114.2 (d, C-4), 103.3 (d, C-6), 73.4 (d, C-15), 44.2 (t, C-9), 35.3 (t, C-14), 33.7 (t, C-12), 25.5 (t, C-13), 22.8 (q, C-16); APCIMS+ve mode m/z 291 $[M+1]^+$; APCIMS−ve mode m/z 289 $[M-1]^+$.

11-Methoxycurvularin (**4**): Yellow oil; ^{1}H NMR (acetone-d_6, 500 MHz) δ 9.13 (2H, brs), 6.42 (1H, d, J=2.0 Hz), 6.41 (1H, d, J=2.0 Hz), 6.34 (2H, m), 4.94 (1H, m), 4.79 (1H, m), 3.93 (2H, d, J=15.5 Hz), 3.63 (2H, d, J=15.5 Hz), 3.66 (1H, m), 3.60 (1H, m), 3.40 (1H, m), 3.30 (3H, s), 3.25 (3H, s), 3.05 (2H, m), 2.95 (1H, m), 1.64 (2H, m), 1.27~1.53 (1H, m), 1.09 (6H, m); APCIMS+ve mode m/z 323 $[M+1]^+$; APCIMS−ve mode m/z 321 $[M-1]^+$.

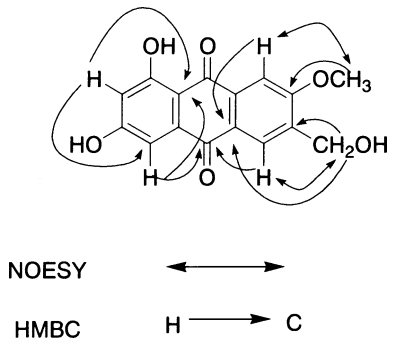
11-Hydroxycurvularin (**5**): Yellow oil; ^{1}H NMR (acetone-d_6, 500 MHz) δ 9.13 (2H, brs), 6.40 (1H, d, J=2.4 Hz), 6.39 (1H, d, J=2.4 Hz), 6.33 (2H, m), 4.94 (1H, m), 4.81 (1H, m), 4.09 (1H, m), 3.99 (1H, m), 3.83 (2H, d,

J=15.5 Hz), 3.67 (2H, d, J=15.5 Hz), 3.53 (1H, m), 3.29 (1H, dd, J=13.8, 3.0 Hz), 3.05 (3H, m), 2.87 (1H, m), 1.69 (2H, m), 1.27~1.60 (8H, m), 1.10 (6H, m); APCIMS+ve mode m/z 309 $[M+1]^+$; APCIMS−ve mode m/z 308 $[M-1]^+$.

Cytotoxicity Bioassays

The tetrazolium-based colorimetric assay (MTT assay)[5] was used for the *in vitro* evaluation of cytotoxicity to human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), and human glioma (SF-268) cells.

Fig. 1. Key NOESY and HMBC correlations for **1**.



Results and Discussion

The molecular formula of compound **1** was determined to be $C_{16}H_{12}O_6$ on the basis of HRFABMS and NMR data. Its UV spectrum had absorption bands at 218, 249, 286 and 432 nm indicating **1** to be a 9,10-anthraquinone[6], which was further supported by its ^{13}C NMR spectrum with signals at δ 186.3 and 182.3 for quinone carbonyl carbons[7]. ^1H NMR spectrum of **1** had three D_2O exchangeable protons at δ 13.26, 11.10, and 5.58, two aromatic 1H singlets at δ 7.76 and 75.0, two aromatic *meta*-coupled 1H doublets (J=2.0 Hz) at δ 7.05 and 6.56, a 2H singlet at δ 4.65 and a singlet due to OCH_3 at δ 3.94. The chemical shift (δ 4.65) of the 2H singlet indicated that it may be due to the CH_2 of a CH_2OH group on an aromatic system. ^{13}C NMR spectrum of **1**, in addition to the two carbonyl carbon signals (see above) had signals due to 12 aromatic/olefinic carbons [of which 3 were oxygenated (δ 164.4, 164.2, and 160.6), and 4 were protonated (δ 116.8, 116.2, 108.2 and 107.0)], a OCH_3 carbon (δ 56.4), and a CH_2OH carbon (δ 62.2). These data suggested that **1** is a 9,10-anthraquinone bearing two OH, a OCH_3, and a CH_2OH substituent. The multiplicities of the aromatic protons suggested that one of the aromatic rings of the 9,10-anthraquinone system is *ortho* disubstituted and the other *meta* disubstituted. The substitution pattern was determined by the analysis of NOESY and HMBC spectra. In the NOESY spectrum, the OCH_3 protons and the CH_2 protons (of the CH_2OH) showed strong cross-peaks to aromatic singlets at δ 7.50 and 7.76, respectively, suggesting that these two substituents are in the *ortho*-disubstituted aromatic ring. In the HMBC spectrum of **1**, the carbonyl carbon at δ 182.3 showed strong correlations with the aromatic protons at δ 7.76 (s) and 7.05 (d, J=2.0 Hz). Other key HMBC and NOESY correlations are depicted in Fig. 1. On the basis of the foregoing evidence, the structure of **1** was determined to be 1,3-dihydroxy-6-hydroxymethyl-7-methoxyanthraquinone.

Comparison of the ^1H and ^{13}C NMR data of **2** with those of **1** indicated that they are structurally related, the major difference being the presence of a CH_3 in **2** in place of the CH_2OH in **1**. This was supported by mass spectral data, which showed that the M^+ of **2** is 16 mass units less than that of **1**. Identification of **2** as 1,3-dihydroxy-6-methoxy-7-methylanthraquinone was further supported by a detailed analysis of its HMQC and HMBC spectra. Although this anthraquinone has been synthesized previously[8] this is the first report of its natural occurrence. Comparison of the ^1H and ^{13}C NMR spectral data of compounds **3**~**5** with those reported in the literature allowed these to be identified as dehydrocurvularin (**3**)[9], 11-methoxycurvularin (**4**)[3], and 11-hydroxycurvularin (**5**)[3]. Co-occurrence of the anthraquinones **1** and **2**, and curvularins **3**~**5** in this *Penicillium* sp. is interesting as all these metabolites may arise from the same octa-ketide biogenetic precursor.

Compounds **1**~**5** were evaluated for *in vitro* cytotoxicity against a panel of three sentinel human cancer cell lines, NCI-H460 (non-small cell lung), MCF-7 (breast), and SF-268 (CNS glioma), recently recommended by the U.S. National Cancer Institute[10]. Cells were exposed to serial dilutions of test compounds for 72 hours in RPMI 1640 media supplemented with 10% fetal bovine serum, and cell viability was evaluated by the MTT assay[5]. As shown in Table 1, only curvularins **3**~**5** were found to be cytotoxic. As measured by the MTT assay, the concentrations resulting in 50% inhibition of cell proliferation/survival (IC_{50}) were found to range between 1.8 and 13.3 μM. Compounds **3**~**5** showed moderate cytotoxic activity toward NCI-H460, and MCF-7 cell lines, but were less active against the SF-268 cell line. Although compounds

Table 1. Cytotoxicities of the compounds **1**～**5** against a panel of human tumor cell lines[a].

Compound No.	Cell line[b]		
	NCI-H460	MCF-7	SF-268
1	NA[c]	NA[c]	NA[c]
2	NA[c]	NA[c]	NA[c]
3	1.8	2.2	5.2
4	4.5	3.3	13.3
5	7.6	3.6	11.7
Taxol	9.5	11.2	21.7

[a]Results are expressed as IC_{50} values in μM except for Taxol, which is in nM.
[b]Key: NCI-H460 = human non-small cell lung cancer; MCF-7 = human breast cancer; SF-268 = human CNS cancer (glioma). [c]NA = Not active at 10 μg/ml.

3～**5** have been reported to inhibit sea urchin embryogenesis by acting on components of the mitotic apparatus and to effectively inhibit cell division[4], this constitutes the first report of their ability to inhibit proliferation of human cancer cell lines. Studies to elucidate their molecular mechanism(s) of anticancer action are currently in progress.

Acknowledgments

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