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Asperpyrone D and other metabolites of the plant-associated fungal strain *Aspergillus tubingensis*[☆]

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Abstract

Bioactivity-guided fractionation of a cytotoxic extract of *Aspergillus tubingensis*, a fungal strain occurring in the rhizosphere of the Sonoran desert plant, *Fallugia paradoxa*, afforded a new dimeric naphtho- γ -pyrone asperpyrone D, nine known naphtho- γ -pyrones, funalenone, and the cytotoxic cyclic penta-peptide, malformin A₁.

Keywords

Aspergillus tubingensis; Rhizosphere fungus; *Fallugia paradoxa*; Secondary metabolites; Asperpyrone D; Naphthopyrones; Malformin A₁; Cytotoxic activity

1. Introduction

Plant-associated microorganisms are known to produce a variety of metabolites with novel structures and interesting biological activities (Tan and Zou, 2001; Schulz et al., 2002; Strobel et al., 2004; Gunatilaka, 2006) and the claimed medicinal properties and biological activities of some plant species have been attributed to the microorganisms living in association with these plants (Kettering et al., 2004; Chomcheon et al., 2005). As part of our ongoing efforts to understand plant-microbial interactions and to discover small molecule natural products with novel structures and/or biological activities from plant-associated microorganisms of the Sonoran desert (Turbyville et al., 2006; Wijeratne et al., 2006), we have investigated the fungal strain *Aspergillus tubingensis* (Trichocomaceae) occurring in the rhizosphere of *Fallugia paradoxa* (Apache plume; Rosaceae). An EtOAc extract of *A. tubingensis* was found to exhibit strong cytotoxic activity against several cancer cell lines. Bioassay-guided fractionation of this extract resulted in the isolation of a new dimeric naphtho- γ -pyrone named asperpyrone D (**1**), a strongly cytotoxic cyclic peptide, malformin A₁ (**2**), funalenone (**3**) and nine known naphtho- γ -pyrones (**4–12**). A previous investigation of the sclerotia of *A. tubingensis* (NRRL 4700) has afforded three new aflavinines, one of which has shown insecticidal activity (TePaske et al., 1989). Monomeric and dimeric naphtho- γ -pyrones with a broad range of biological activities have been found to occur in

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higher plants belonging to the genera *Cassia* (Li et al., 2001), *Paepalanthus* (Coelho et al., 2000), and *Senna* (Barbosa et al., 2004), and in filamentous fungal genera, *Aspergillus* (Galmarini and Stodola, 1965; Wang and Tanaka, 1966; Sakurai et al., 2002; Akiyama et al., 2003) and *Fusarium* (Singh et al., 2003). Malformin A₁ has previously been reported from several *Aspergillus* strains including *A. niger* (Sugawara et al., 1990), *A. ficuum*, *A. awamori*, and *A. phoenicis* (Iriuchijima and Curtis, 1969).

2. Results and discussion

The cytotoxic EtOAc extract of *A. tubingensis*, collected from the rhizosphere of *F. paradoxa*, on fractionation using a combination of size-exclusion, normal phase, and reversed phase chromatography afforded compounds **1–12**. Asperpyrone D (**1**) was determined to have the molecular formula C₃₁H₂₄O₁₀ by HR-FAB-MS and ¹³C NMR spectroscopy and indicated 20 degrees of unsaturation. The bands at 3425 and 1655 cm⁻¹ in its IR spectrum suggested the presence of hydroxyl and conjugated carbonyl functionalities. The UV spectrum of **1** showed strong absorption bands at 384, 281, 248 and 225 nm, and was found to be similar to that of asperpyrone C (**13**) (Akiyama et al., 2003), suggesting dimeric naphtho- γ -pyrone chromophore for **1**. This was further supported by the diagnostic signals at δ 14.80 and δ 13.40 for the two phenolic hydroxyl groups in its ¹H NMR spectrum (Gorst-Allman et al., 1980). The ¹³C NMR spectrum of **1** displayed 30 signals for 31 carbons which consisted of two methyl, three methoxy, six methine, and 20 quaternary carbons. The ¹H NMR spectrum of **1** also showed two methyl singlets (δ _H 2.37 and 2.54), three methoxy singlets (δ _H 3.60, 3.62 and 3.99), six aromatic protons (two *meta*-coupled doublets at δ _H 6.27 and 6.45, and four singlets at δ _H 6.00, 6.33, 7.07 and 7.14), and two phenolic hydroxyls (see above). Both ¹H and ¹³C NMR data closely resembled those of asperpyrone C (**13**) (Akiyama et al., 2003) except for the presence of an OH group in **1** compared with an OCH₃ in **13**. This OH group in **1** was located at C-8 by the HMBC correlations of H-9 with C-8 (δ _C 155.9). The correlations of H-7' (δ _H 6.27) with C-6' (δ _C 106.2), and H-9 (δ _H 7.14) with C-7 (δ _C 116.4) in the HMBC spectrum of **1** confirmed the C-7–C-6' linkage of the two naphthopyrone moieties (Fig. 1). Thus, the structure of asperpyrone D was established as the dimeric naphtho- γ -pyrone **1** with a linear and angular monomeric moieties linked via C-7 and C-6'.

The bioactive compound **2** was identified as malformin A₁ (*cyclo*-Leu-Ile-Cys-Cys-Val) by comparison of NMR spectral data with those reported in the literature (Sugawara et al., 1990). Compounds **3–12** were characterized by comparison of their mass and NMR spectral data with those reported in the literature. Compound **3** was identified as funalenone, a rare phenalene with MMP-1 inhibitory activity previously encountered in *A. niger* IFO-5904 (Inokoshi et al., 1999). It is noteworthy that funalenone (**3**) has recently been reported to inhibit HIV-1 integrase (Shiomi et al., 2005). The remaining metabolites were identified as the monomeric naphtho- γ -pyrones, TMC-256A1 (**4**), rubrofusarin B (**5**), fonsecin B (**6**) and fonsecin (**7**), and the naphtho- γ -pyrone dimers, fonsecinone A (**8**), asperpyrone A (**9**), aurasperone A (**10**), dianhydro-aurasperone C (**11**) and aurasperone E (**12**).

Malformin A₁ (**2**) has been reported to possess a variety of biological activities including plant growth stimulation (John and Curtis, 1974), prevention of interleukin-1 (IL-1) induced endothelial changes by inhibition of protein synthesis (Dawes, 1994), phytochrome-mediated response modulation of *Phaseolus vulgaris* (Curtis and John, 1975), as well as mycotoxic (Franck, 1984), and antibacterial activities (Suda and Curtis, 1966). In the present study malformin A₁ (**2**) was found to be strongly cytotoxic against the human cancer cell lines NCI-H460 (non-small cell lung carcinoma), MIA Pa Ca-2 (pancreatic cancer), MCF-7 (breast cancer), and SF-268 (CNS cancer; glioma) with slight selectivity towards the pancreatic cancer cell line (MIA Pa Ca-2) compared with the normal human primary

fibroblast cells WI-38 (Table 1). None of the other metabolites (**1** and **3 – 12**) encountered in this study showed cytotoxicity towards any of the cell lines used when tested at a concentration of 5 µg/ml. However, it is noteworthy that some naphtho- γ -pyrones have been reported to reverse drug resistance in human KB cells (Ikeda et al., 1990).

3. Experimental

3.1. General

Melting points were determined on an Electrothermal micromelting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8300 spectrometer in KBr disks, 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 ^1H NMR and 125 MHz for ^{13}C NMR. The Chemical shift values (δ) are given in parts per million (ppm) and the coupling constants in Hz. Low-resolution APCI mass spectra were measured on a Shimadzu LCMS-8000QPa HPLC-MS system. High-resolution FAB-MS were obtained with a JEOL HX110A spectrometer.

3.2. Cytotoxicity assays

The tetrazolium-based colorimetric assay (MTT assay) (Rubinstein et al., 1990) was used for the in vitro assay of cytotoxicity to human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), human glioma (SF-268), human pancreatic cancer (MIA Pa Ca-2) cell lines, and normal human fibroblast (WI-38) cells as previously reported (Wijeratne et al., 2003). All samples for cytotoxicity assays were dissolved in DMSO. During bioassay-guided fractionation, cytotoxicity of fractions was monitored using the NCI-H460 cell line.

3.3. Fermentation of *A. tubingensis*, extraction and isolation of the metabolites

A. tubingensis (Trichocomaceae) was isolated from the rhizosphere of *Fallugia paradoxa* (D. Don) Endl. Ponil. (Apache plume) (Rosaceae) collected in Greasewood Mountains of Tucson, Arizona. The plant identification was made by Dr. Annita Harlan and a voucher specimen was deposited at the University of Arizona Herbarium. The fungal strain was identified by Ms. Donna Bigelow by the analysis of the ITS regions of the ribosomal DNA as previously described (Wijeratne et al., 2003). The fungal strain is deposited in the Division of Plant Pathology, Department of Plant Sciences and the Southwest Center for Natural Products Research and Commercialization of the University of Arizona microbial culture collection under the accession number AH-02-35-F5. The detailed general procedures of isolation, identification and cultivation of rhizosphere fungal strains have been described previously (Wijeratne et al., 2003; Zhan et al., 2004). Methanol (200 ml) was added to each of the 44 T-flasks of a solid culture of *A. tubingensis* in potato dextrose agar (PDA), and the flasks were shaken overnight at room temperature, filtered, and the residue washed with MeOH (50 ml for each flask) yielding a total of 9.9 l of the MeOH extract which was concentrated to 2.4 l by evaporation under reduced pressure. The concentrated MeOH extract was extracted with EtOAc (2×3). Evaporation of EtOAc under reduced pressure afforded a dark yellow solid (2.5023 g). A portion (1.9 g) of the EtOAc extract was subjected to size-exclusion chromatography on Sephadex LH-20 (60.0 g) and eluted with CH_2Cl_2 -hexane (1:1, 500 ml), CH_2Cl_2 -hexane (2:1, 1000 ml), CH_2Cl_2 -hexane (4:1, 500 ml), CH_2Cl_2 -acetone (1:1, 250 ml), acetone (250 ml), and MeOH (1000 ml). Twenty two fractions were collected and combined based on their TLC profiles to afford nine combined fractions F₁-F₉ [F₁ (15.3 mg), F₂ (79.8 mg), F₃ (209.4 mg), F₄ (131.0 mg), F₅ (570.5 mg), F₆ (129.4 mg), F₇ (376.2 mg), F₈ (70.5 mg) and F₉ (309.2 mg)]. Of these, only F₃ was found to be cytotoxic and it was separated on a column of silica gel (6.0 g) and by elution with hexane-acetone (4:1, 150 ml), hexane-acetone (3:1, 400 ml), hexane-acetone (2:1, 100 ml),

hexane–acetone (1:1, 100 ml), acetone (25 ml) and MeOH (50 ml). Thirty-three fractions were collected and were combined based on their TLC profiles to yield ten sub-fractions [F_{3A} (11.7 mg), F_{3B} (14.5 mg), F_{3C} (10.5 mg), F_{3D} (10.6 mg), F_{3E} (12.9 mg), F_{3F} (52.7 mg), F_{3G} (39.6 mg), F_{3H} (13.4 mg), F_{3I} (3.3 mg) and F_{3J} (14.3 mg)]. Of these, sub-fractions F_{3H}, F_{3I} and F_{3J} were found to be cytotoxic. These were combined and further separated by silica gel preparative TLC (developer solvent: hexane–acetone, 1:1) followed by size-exclusion chromatography on Sephadex LH-20 by elution with MeOH, yielding malformin A₁ (**2**) (4.0 mg) as a white powder. Sub-fraction F_{3G} was subjected to reversed phase (RP-18) preparative TLC (80% MeOH–H₂O) affording **1** (0.9 mg) and **9** (4.5 mg). Compounds **6** (3.0 mg) and **7** (5.6 mg) were isolated from sub-fraction F_{3C} using reversed phase (RP-18) preparative TLC (75% MeOH–H₂O). Sub-fraction F_{3D} was separated on silica gel preparative TLC (hexane–acetone, 3:2) followed by reversed phase (RP-18) preparative TLC (80% MeOH–H₂O), yielding **8** (0.5 mg) and **10** (2.2 mg). Purification of sub-fraction F_{3F} following a procedure identical with that used for sub-fraction F_{3D} above, afforded **12** (4.5 mg). Compound **3** (57.3 mg) was obtained by silica gel (8.0 g) column chromatography of fraction F₉ and elution with MeOH–CH₂Cl₂ (2:8) followed by purification by silica gel preparative TLC (MeOH–CH₂Cl₂, 3:7). Separation of fraction F₅ on a column of silica gel (15.0 g) and elution with hexane–acetone (2:1) afforded **11** (127.6 mg) and the sub-fractions F_{5A} (31.4 mg), F_{5B} (33.9 mg) and F_{5C} (168.5 mg). TLC analysis of fraction F₄ and the sub-fractions F_{3B} and F_{5C} indicated them to be similar and therefore these were combined and subjected to silica gel preparative TLC (hexane–acetone, 3:2), yielding **4** (101.2 mg) and **5** (101.3 mg).

Asperpyrone D (1). Yellow powder, m.p. 200 °C (dec.); UV nm (MeOH) $\lambda_{\max}$ (log ϵ): 384 (3.86), 281 (4.68), 248 (4.62), 225 (4.52); IR $\nu_{\max}$ (KBr) cm⁻¹: 3425, 2928, 2858, 2365, 2338, 1655, 1616, 1570, 1427, 1380, 1261, 1204, 1165 and 1065; ¹H NMR (500 MHz, CDCl₃): δ 14.80 (1H, s, OH-5), 13.40 (1H, s, OH-5'), 7.14 (1H, s, H-9), 7.07 (1H, s, H-10), 6.45 (1H, d, J = 2.2 Hz, H-9'), 6.33 (1H, s, H-3'), 6.27 (1H, d, J = 2.2 Hz, H-7'), 6.00 (1H, s, H-3), 3.99 (3H, s, OCH₃-10'), 3.62 (3H, s, OCH₃-6), 3.60 (3H, s, OCH₃-8'), 2.54 (3H, s, CH₃-2'), 2.37 (3H, s, CH₃-2); ¹³C NMR (125 MHz, CDCl₃): δ 184.5 (s, C-4), 182.7 (s, C-4'), 167.6 (s, C-2), 166.9 (s, C-2'), 162.2 (s, C-5 and C-8'), 159.5 (s, C-10'), 158.6 (s, C-6), 156.6 (s, C-5'), 155.9 (s, C-8), 155.5 (s, C-10b'), 153.1 (s, C-10a), 140.7 (s, C-6a'), 140.6 (s, C-9a), 116.4 (s, C-7), 111.5 (s, C-5a), 110.4 (d, C-3'), 108.0 (s, C-4a'), 107.2 (d, C-3), 106.2 (s, C-6'), 106.0 (d, C-9), 105.2 (s, C-10a'), 104.4 (s, C-4a), 100.9 (d, C-10), 97.4 (d, C-9'), 96.6 (d, C-7'), 62.4 (q, OCH₃-6), 56.1 (q, OCH₃-10'), 55.3 (q, OCH₃-8'), 20.8 (q, CH₃-2), 20.6 (q, CH₃-2'); HR-FAB-MS: m/z 557.1431 [M+1]⁺ (C₃₁H₂₅O₁₀ requires 557.1448).

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Fig. 1.
Selected HMBC correlations of **1**.

1 R=H
13 R=CH₃

2

3

4 R=CH₃
5 R=H

6 R=CH₃
7 R=H

8 R=CH₃
9 R=H

10 R=CH₃
11 R=H

12

Table 1

Cytotoxic data for malformin A₁ (**2**) against a panel of cancer cell lines and normal human primary fibroblast cells^a

Compounds	Cell lines ^b				
	NCI-H460	MIA Pa Ca-2	MCF-7	SF-268	WI-38
2	0.07	0.05	0.10	0.07	0.10
doxorubicin	0.01	0.07	0.04	0.05	0.30

^aResults are expressed as IC₅₀ values in μ M;

^bNCI-H460 = human non-small cell lung cancer; MIA Pa Ca-2 = human pancreatic cancer; MCF-7 = human breast cancer; SF-268 = human CNS cancer (glioma); WI-38 = normal human primary fibroblast cells.