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ANTINEOPLASTIC AGENTS. 575. THE FUNGUS *Aspergillus phoenicis.*[1]

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Abstract – Cancer cell line bioassay-guided separation of an extract from the fungus *Aspergillus phoenicis* collected in Saskatchewan, Canada, resulted in the isolation of three new constituents designated asperlactone (**1**), aspergillol A (**2**), and aspergillol B (**3**), accompanied by four previously known constituents: asperic acid (**4**), hexylitaconic acid (**5**), methyl 2-hydroxyphenylacetate (**6**), and methyl 4-hydroxyphenylacetate (**7**). The structure of each was determined by analyses of high-resolution mass spectra and high-field NMR data. Asperic acid (**4**) was found to inhibit growth of the murine lymphocytic leukemia P388 (ED_{50} 0.18 μg/mL) and a panel of human cancer cell lines (GI_{50} 1.7–2.0 μg/mL; pancreas, breast, CNS, lung, colon, and prostate), while aspergillol A (**2**) showed moderate inhibition against the breast adenocarcinoma MCF-7 (GI_{50} 7.2 μg/mL).

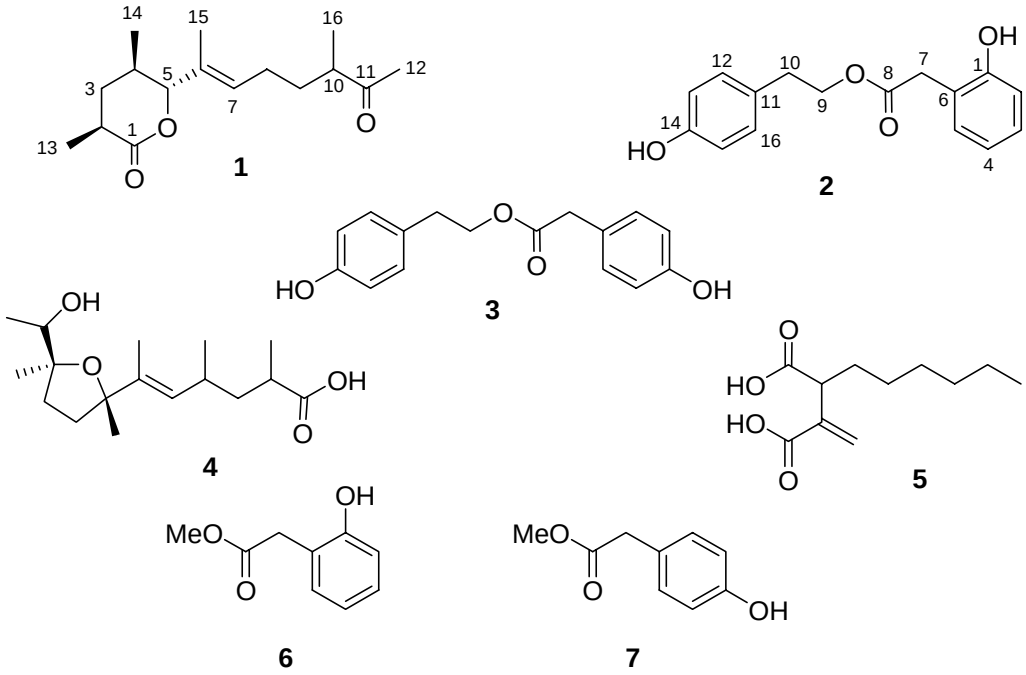
INTRODUCTION

Fortunately, the realization is rapidly gaining momentum that naturally occurring substances,[2] especially from microorganisms as well as their recombinant modifications,[3] will increasingly allow discovery of ever more useful anticancer, antiinfective, and other drugs for a broad spectrum of medical applications. Illustrative are some recent examples of substances isolated from fungi that include the antiviral anthraquinone hypericin from an endophytic fungus,[4a] the strong cancer cell growth inhibitory (GI_{50} 0.039 μg, 41 mM/mL) cyclodepsipeptides pteratides 1 and 2 from a Malaysian Basidiomycete,[4b] and the cancer cell selective (against PTEN-negative cells) peptide, culicinin D, from an entomopathogenic fungus.[4c]

As one of our initiatives to discover new anticancer substances produced by marine[5a] and terrestrial[5b] microorganisms, we continued to evaluate specimens collected (2001) in Saskatchewan, Canada. That led to isolation of a fungus later identified as *Aspergillus phoenicis* (formerly known as *Aspergillus saitoi*)[6] from a plant collected in September, 2001 near Mechem, Saskatchewan. The plant was found growing in a dry lake whose surface was covered with precipitated salts. Fungi of the genus *Aspergillus* such as *A.*

flavus are well known producers of the powerful carcinogenic aflatoxins that are natural contaminants of human and animal foods. In general, *Aspergillus* species produce both very toxic[6a] and potentially useful[7] (as well as valuable, e.g., for food processing) metabolites, such as hydroxyflavanone antioxidants[7a,b] and enzymes. The latter group includes a variety of important enzymes produced by *A. phoenicis* such as α-D-mannosidases,[8a] β-glucosidases,[9a-d] catalases,[10] xylanases,[11] aspartic proteinases,[12] and a carboxypeptidase used for peptide sequencing.[13]

An example of some starkly contrasting biological properties of *A. phoenicis* components was displayed by the recent isolation of α-cyclopiazonic acid, a very potent indole alkaloid carcinogen/neurotoxin (seizures/death),[6b] and of the antioxidant mixture (FSAP) that in part reduced the liver fibrosis induced by CCl_4 in rats.[6c] Hence, it was clear, as usual, from the outset that cancer cell line (P388 murine leukemia and human cancer cell lines) bioassay-guided isolation would be essential to direct separations. The result was isolation of three new constituents named asperlactone (**1**), aspergillol A (**2**), and aspergillol B (**3**), of which phenol **2** showed moderate cancer cell inhibitory activity. In addition, four previously known compounds were isolated: asperic acid (**4**), hexylitaconic acid (**5**), and methyl 2-hydroxy- and 4-hydroxyphenylacetates (**6** and **7**). Of these, tetrahydrofuran **4** was found to exhibit significant activity against P388 and a panel of human cancer cell lines.

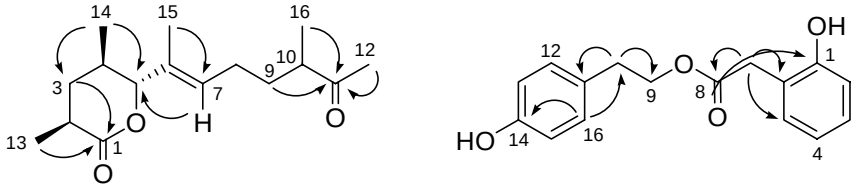


RESULTS AND DISCUSSION

The scale-up production of *A. phoenicis* by fermentation (4 × 97-L batches) provided 388 L of broth that was extracted with ethyl acetate. The solvent was evaporated and the residue was initially separated by a solvent-partitioning sequence (hexane/9:1 MeOH–H_2O → CH_2Cl_2/3:2 MeOH–H_2O), followed by

separation of the marginally active (P388 lymphocytic leukemia, ED_{50} 10 μg/mL) CH_2Cl_2 fraction by a gel permeation → partition chromatographic → HPLC sequence to afford pure **1** (10.6 mg), **2** (5.3 mg), **3** (3.2 mg), **4** (6.5 mg), **5** (2.7 mg), **6** (2.0 mg) and **7** (3.4 mg).

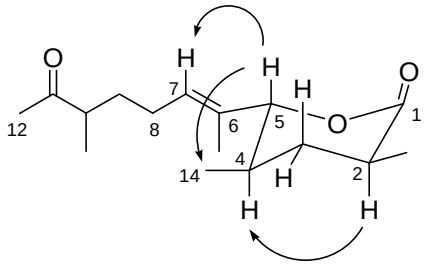
Asperlactone (**1**) was obtained as a colorless oil, and its molecular formula was determined to be $C_{16}H_{26}O_4$ by HRAPCIMS. The ^{1}H NMR spectrum of **1** showed signals corresponding to five methyl groups at δ 0.86 (3H, d, *J* = 6.0 Hz), 1.10 (3H, d, *J* = 7.0 Hz), 1.29 (3H, d, *J* = 7.0 Hz), 1.60 (3H, s), and 2.14 (3H, s) and one olefinic proton at 5.40 (1H, t, *J* = 7.0 Hz). By employment of ^{1}H–^{1}H COSY techniques, two proton-relayed spin-spin systems [A: CH-CH_2-CH_2-CH (Me); B: O-CH-CH (Me)-CH_2-CH(Me)-] were revealed. HMBC experiments allowed us to determine the linkages of the spin-spin systems. In system A, the signals corresponding to the methyl protons at δ 1.10 (H-16), the tertiary proton at δ 2.55 (H-10) and the methylene proton at δ 1.38, 1.75 (H-9) showed HMBC cross-peaks with a carbonyl carbon (C-11, δ 212.3). Another methyl proton at δ 2.14 also showed correlations with C-11. Based on its chemical shift and these HMBC correlations, the carbon link from C-7 to C-12 was determined. HMBC correlations were observed from the methyl protons at δ 1.60 (H-15) and the tertiary proton at δ 4.21 (1H, d, *J* = 10.5 Hz, H-5) to the olefinic carbon (C-7, δ 131.1), confirming a double bond linkage between system A and B. The correlations between the methyl signal at δ 1.29 (H-13) and the methylene at δ 1.40, 1.98 (H-3) to the carbonyl carbon (C-1, δ 174.5) indicated that the other end of system A was linked to a carbonyl carbon (Figure 1).



1 **2**

Figure 1. HMBC correlations for **1** and **2**

According to the molecular formula and degrees of unsaturation, the structure of **1** should be a δ lactone. 2D-ROESY experiments afforded a valuable NOE correlation from δ 4.21 (H-5) to δ 5.40 (H-7). That showed that the double bond between C-6 and C-7 should have *E* geometry. The NOE correlation of δ 0.86 (H-14) and δ 4.21 (H-5) indicated the methyl at C-4 and proton at C-5 to be oriented in a *cis* relationship. Another NOE correlation between H-2 and H-4 suggested the 2-Me and 4-Me groups to be in a *cis* configuration also. Both H-4 and H-5 were assigned as axial in a partial chair conformation, based on the coupling constant of H-5 (*J* = 10.5 Hz). Thus, the overall structure and relative configuration of **1** was assigned as shown in Figure 2.



1

Figure 2. NOE correlations for **1**

Aspergillol A (**2**) was also obtained as an oil (yellow). The molecular formula was found to be $C_{16}H_{16}O_4$ by HRAPCIMS. The ^{1}H spectrum of **2** revealed eight aromatic proton signals at δ 7.10 (1H, m), 7.04 (1H, m), 6.96 (2H, d, *J* = 7.6 Hz), 6.78 (1H, m), 6.76 (1H, m), and 6.67 (2H, d, *J* = 7.6 Hz). Three methylene proton signals were observed at δ 4.19 (2H, t, *J* = 6.8 Hz), 3.57 (2H, s), and 2.76 (2H, t, *J* = 6.8 Hz) in the ^{1}H spectrum. The ^{13}C APT spectrum showed a signal at δ 174.3 corresponding to a carbonyl carbon, aromatic carbons at δ 156.8, 156.6, 132.1, 131 (2C), 130.2, 129.4, 122.5, 120.5, 116.2 (2C), and 115.9 and three methylene carbons at δ 66.9, 36.8, and 35.1. Based on the ^{1}H and APT spectra, it was concluded that the structure contained two aromatic rings: one *o*-substituted and the other *p*-substituted. The ^{1}H-^{1}H COSY and HMBC data indicated a -CH_2-CH_2-O-CO-CH_2- unit. HMBC correlations of H-7 (δ 3.57) to C-6 (122.5), C-1 (156.8), and C-8 (174.3) suggested that H-7 was bonded to the *o*-substituted aromatic ring. The H-10 signal (2.76) showed HMBC cross-peaks with C-9 (66.9) and C-11 (130.2), while H-12 and H-16 (6.96) showed HMBC correlations to C-11 (130.2), C-10 (35.1), and C-14 (156.6), which confirmed that C-10 was bonded to the *p*-substituted aromatic ring. From the spectral data analyses, it was clear that aspergillol A (**2**) corresponded to structure **2**.

Aspergillol B (**3**) was obtained as a yellow oil corresponding to molecular formula $C_{16}H_{16}O_4$, as determined by HREIMS. The ^{1}H NMR spectrum of **3** was very similar to that of **2**, except for the coupling constants of the aromatic protons. The ^{1}H NMR spectrum of **3** showed four doublets at δ 6.68, 6.72, 6.96 and 7.02, which confirmed that both aromatic rings of **3** were *p*-substituted and **3** was simply an isomer of **2**.

Further separation (by HPLC) of cancer cell line active fractions led to the isolation of asperic acid (**4**),[14] hexylitaconic acid (**5**),[14] 2-hydroxyphenylacetic acid methyl ester (**6**),[15] and 4-hydroxyphenylacetic acid methyl ester (**7**).[16] With each of these previously known compounds, the characterization and final identification was determined by spectroscopic analyses and comparison of physical properties.

Asperic acid (**4**) exhibited significant cancer cell growth inhibitory activity when evaluated against the murine P388 lymphocytic leukemia cell line (ED_{50} 0.16 μg/ml) and a minipanel of six human tumor cell lines: pancreas adenocarcinoma (BXPC-3 GI_{50} 1.7 μg/mL), CNS glioblastoma (SF-268 GI_{50} 1.9 μg/mL),

lung large cell (NCI-H460 GI_{50} 1.9 μg/mL), colon adenocarcinoma (KM 20L2 GI_{50} 1.8 μg/mL), and prostate adenocarcinoma (DU-145 GI_{50} 2.0 μg/mL). Aspergillol A (**2**) showed moderate inhibitory activity against breast adenocarcinoma (MCF-7 GI_{50} 7.2 μg/mL).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Assignments for **1**[a], **2**[b] and **3**[c]

Position	**1**		**2**		**3**	
	δ_H (*J* Hz)	δ_c	δ_H(*J* Hz)	δ_c	δ_H (*J* Hz)	δ_c
1		174.5		156.8	7.02 (d,7.5)	130.9
2	2.51 (m)	36.4	6.78 (m)	115.9	6.72 (d,7.5)	116.3
3	1.40 (m), 1.98 (m)	37.3	7.10 (m)	129.4		156.7
4	1.95 (m)	31.1	6.76 (m)	120.5	6.72 (d, 7.5)	116.3
5	4.21 (d, 10.5)	93.8	7.04 (m)	132.1	7.02 (d ,7.5)	130.9
6		132.2		122.5		126.3
7	5.40 (t, 7.0)	131.1	3.57 (s)	36.8	3.48 (s)	41.3
8	2.04 (m)	25.3		174.3		174.5
9	1.38 (m), 1.75 (m)	32.0	4.19 (t, 6.8)	66.9	4.20 (t, 7.0)	66.9
10	2.55 (m)	46.5	2.76 (t, 6.8)	35.1	2.78 (t, 7.0)	35.1
11		212.3		130.2		130.0
12	2.14 (s)	28.2	6.96 (d, 7.6)	131.0	6.96 (d, 7.5)	131.0
13	1.29 (d, 7.0)	17.3	6.67 (d, 7.6)	116.2	6.68 (d, 7.5)	116.2
14	0.86 (d, 6.0)	17.0		156.6		156.6
15	1.60 (s)	10.7	6.67 (d, 7.6)	116.2	6.68 (d, 7.5)	116.2
16	1.10 (d, 7.0)	16.2	6.96 (d, 7.6)	131.0	6.96 (d, 7.5)	131.0

[a] 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR (recorded in $CDCl_3$)

[b] 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR (recorded in MeOH-d_4)

[c] 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR (recorded in MeOH-d_4)

EXPERIMENTAL

Solvents used for column chromatography were distilled. Sephadex LH-20 (particle size 25-100 μm) used for gel permeation and partition column chromatographic separations was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The TLC plates were viewed under shortwave UV light and then developed by I_2 or 3% ceric sulfate in 3*N* sulfuric acid spray reagent followed by heating at approximately 150 °C. Analytical HPLC was conducted with a Hewlett-Packard Model 1100 HPLC.

Semipreparative HPLC was performed with a Waters Delta 600 coupled with a Waters 2487 double λ absorbance detector. High-resolution mass spectra were obtained using a JEOL LCMate magnetic sector instrument by APCI with a polyethylene glycol reference, or using a JEOL GCMate double-focusing mass spectrometer by EI. The ^{1}H and ^{13}C NMR, APT, ^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY spectral data were recorded using a Varian Unity INOVA-500 spectrometer operating at 500 MHz or 400 MHz for ^{1}H NMR as well as for 2D NMR and at 125 MHz or 100 MHz for ^{13}C NMR, all referenced to tetramethylsilane (TMS).

COLLECTION AND FERMENTATION

Plant samples were collected (September, 2001) in clean plastic bags from dry lakes near Mechem, Saskatchewan, and shipped to our laboratory. Plant samples were rinsed in sterile H_2O and disinfected in 70% EtOH prior to dilution-plating. Prior to large-scale fermentation (6-L flasks containing 4 L media), the human cancer cell line activity of the fungus was determined to be optimum in half-strength malt extract broth (final concentration: malt extract, 3g/L; maltose, 0.9 g/L; dextrose, 3g/L; yeast extract, 0.6g/L) containing 14g/L Instant Ocean, for 21 days at rt with shaking.

The fungus was identified by large subunit rRNA gene sequencing (Accugenix, Newark, DE). Results from the MicroSeq database indicated that the fungus was *Aspergillus phoenicis* (% difference=0, confidence level to species). Voucher specimens (ASU-B901528) are available from RKP.

EXTRACTION AND ISOLATION

The culture broth (388 L) was extracted three times with EtOAc. The combined extract was removed from the rotavapor flasks with EtOAc–water and partitioned (× 4) with EtOAc. After evaporation of solvent, the residue was dissolved in 9:1 MeOH–H_2O and extracted with hexane. After separation, the MeOH–H_2O layer was diluted with H_2O to a ratio of 3:2. The resulting aqueous solution was extracted with CH_2Cl_2. The cancer cell line moderately active (P388 murine lymphocytic leukemia ED_{50} 10 μg/mL) CH_2Cl_2 fraction (13.42 g) was triturated with MeOH to yield soluble and sparingly soluble phases. Successive Sephadex LH-20 column chromatographic separations employing MeOH → hexane → CH_2Cl_2–MeOH (5:1:1) followed by separation of active fractions using a C_{18} column with a linear gradient of H_2O–MeCN (95:5 60 min to 50:50) as mobile phase afforded **1** (10.6 mg), **2** (5.3 mg), **3** (3.2 mg), **4** (6.5 mg), **5** (2.7 mg), **6** (2.0 mg) and **7** (3.4 mg).

Asperlactone (**1**): colorless oil; IR ν_{max} 2965, 2930, 1727, 1713, 1219, 772 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) assignments have been summarized in Table 1; HRAPCIMS *m/z* 267.1726 $[M+H]^+$ (calcd 267.1960).

Aspergillol A (**2**): yellow oil; ^{1}H-NMR (MeOH-d_4, 400 MHz) and ^{13}C-NMR (MeOH-d_4, 100 MHz)

assignments have been recorded in Table 1; HRAPCIMS *m/z* 273.1140 $[M+H]^+$ (calcd 273.1127).

Aspergillol B (**3**): yellow oil; for the ^{1}H-NMR (MeOH-d_4, 500 MHz) and ^{13}C-NMR (MeOH-d_4, 125 MHz) assignments, refer to Table 1; HREIMS *m/z* 272.1141 $[M+H]^+$ (calcd 272.1048).

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