Fumagiringillin, a New Fumagillin Derivative from a Strain of the Fungus *Aspergillus fumigatus*†

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A new fumagillin derivative, fumagiringillin (**1**), has been isolated together with fumagillin (**2**) and 12α-fumitremorgin C (**3**) from a strain of *Aspergillus fumigatus*. The structures were elucidated by spectral analyses.

The anticancer activity of fumagillin has long been recognized.[1] As a potent angiogenesis (new blood vessel formation) inhibitor, fumagillin suppresses the growth of a wide variety of tumors,[2,3] but because of toxic side-effects cannot be utilized. Derivatives with fewer side-effects have been synthesized, and one of these, TNP470, is now undergoing clinical trials for the treatment of a variety of cancers.[4,5] Fumagillin and TNP470 are currently the only treatment for cryptosporidiosis and microsporidiosis caused by *Enterocytozoon bieneusi*. These are common and frequently fatal infections in AIDS sufferers.[6,7] Fumagillin has also acquired importance in veterinary medicine against microsporidiosis of bees and fish.[8,9] Fumagillin is also a strong antiparasitic and amoebicidal compound[10] and is one of just two chemotherapeutics capable of controlling the proliferative kidney disease of salmonid fish infected by an unclassified myxosporean parasite, the so-called 'PKX'.[11]

During studies on the detection and isolation of bioactive metabolites from marine and saline fungi, the culture extract of one isolate, *Aspergillus fumigatus*, showed moderate cytotoxicity against the P388 murine leukemia cell line. Further purification led to the isolation of fumagiringillin (**1**) and two previously identified fungal metabolites: fumagillin[12] (**2**) and 12α-fumitremorgin C[13] (**3**).

The fungal strain (CANU A151) was grown in a liquid medium of half-strength potato dextrose broth for four weeks at 26 °C. The EtOAc extract of the filtrate was separated using bioassay-guided fractionation (cytotoxicity against the P388 murine leukemia cell line). Active fractions were purified by a combination of reverse-phase (C18) and normal-phase (Diol) column chromatography to afford **1**−**3**.

The molecular formula of **1** was deduced as $C_{26}H_{36}O_8$ from the HRESMS and was supported by the ^{13}C NMR spectrum, which showed all 26 carbons. HSQC and APT spectra defined four methyls, four methylenes, four aliphatic methines, nine olefinic methines, three quaternary carbons, and two carbonyl carbons. The $^1H-^1H$ COSY spectrum revealed three partial structural units (shown by bold-faced lines in Figure 1). From the CIGAR data the connectivity of the methoxy group (δ_C 57.1, δ_H 3.39) to C-1 was established by the observation of correlations of H-1 to C-17 and H-17 to C-1, while the linkage of C-4 to C-9 through C-5 was concluded by the observation of correlation peaks from H-4β and H-1 to C-5 and from H-4β to C-9. Correlation peaks between the H-6 protons and C-5 and from H-6a to C-9 connected C-5 to C-6. The connectivity of C-8 and C-6 through oxygen was assumed on the basis of chemical shifts (δ_C 85.3 and 77.1, respectively) and supported by the correlation of H-6a to C-8. The series of correlations observed from H-9 and H-10 to C-14 and from H-9, H-10, and H-14 to C-8 defined that section of the molecule, while the connectivity of C-16 and C-15 to C-12

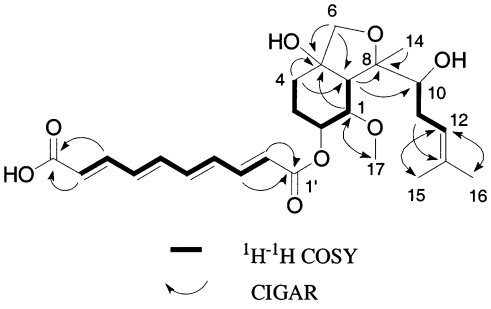

Figure 1. Selected 2D NMR correlations for fumagiringillin (**1**).

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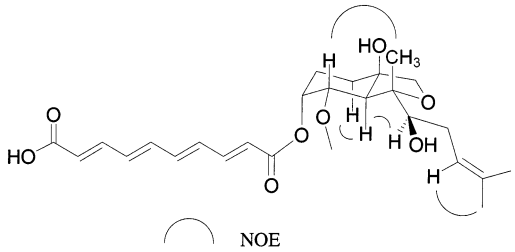

Figure 2. Selected NOE correlations in the NOESY spectrum of **1**.

through C-13 was deduced by the correlations from H-12 and H-15 to C-16, from H-12 and H-16 to C-15, and from H-11a, H-15 and H-16 to C-13. The connectivity of the two carbonyl carbons with C-2′ and C-9′ was made on the basis of the correlations of H-2′ and H-3′ to C-1′ and H-8′ and H-9′ to C-10′. No CIGAR correlations linked the unsaturated side chain, C-1′−C10′, with the skeleton, so attachment at C-2 is reliant on chemical shift arguments at the 2-position (δ_C 66.8, δ_H 5.67) and by comparison with comparable shifts for fumagillin (**2**) (δ_C 66.6, δ_H 5.71).[14] The assignment of C-1′ and C-10′ was also based on comparison with the relevant chemical shifts of **1** with those of fumagillin (**2**). The IR spectrum of compound **1** showed absorptions at 1706 and 1627 cm^{-1}, confirming the presence of carbonyl functionalities in **1**. The planar structure was determined as shown in Figure 1.

The relative stereochemistry of **1** was assigned on the basis of coupling constants and a 2D NOESY experiment. The large coupling constant between H-1 and H-9 (12.0 Hz) and the small coupling constant between H-2 and H-1 (3.0 Hz) was the starting point and suggested axial or pseudoaxial orientations for H-1 and H-9. It was not possible to probe the C5/C9 ring junction directly, but a combination of factors suggests *trans* stereochemistry. The assignment of stereochemistry was guided by the observation of strong positive NOE correlations between H-4α and H-9 and between H-1 and the H-14 protons. This clearly positioned each of these groups on the same side of the molecule, respectively (Figure 2). However, no NOE correlations were observed from H-1 and/or H-3 to H-6. Such additional correlations would have been expected for the *cis* but not the *trans* stereoisomer. The two possible stereoisomers were modeled using the Chem 3D program, when, after energy minimization using the MM2 option (minimum RMS gradient = 0.01), these predictions were confirmed. The calculated interproton distances were less than or approximately 3 Å for H-9/H-4α and H-1/H-14 for **both** stereoisomers. In the case of the *cis* stereoisomer the H-1/H-6β and H-3β/H-6β calculated interproton distances were estimated to be ~2.51 Å, but in the *trans* stereoisomer the H3/H6 and H-1/H-6 interproton distances were all significantly greater than 4 Å. As **no** NOE enhancements were observed from H-1 and H-3 protons to the H-6 protons, the C5/C9 ring junction stereochemistry was assigned as *trans*.

An NOE correlation between H-12 and H-16 established a *Z* relationship between these two protons. The stereochemistries of the four conjugated double bonds were determined as all-*E* on the basis of the coupling constants ($J_{2',3'}$ = 15.5 Hz, $J_{8',9'}$ = 15.5 Hz) and the NOE correlations between H-2′ and H-4′, H-3′ and H-5′, H-4′ and H-6′, H-5′ and H-7′, H-6′ and H-8′, and H-7′ and H-9′ (Figure 2). The stereochemistry of the hydroxyl group at C-10 could not be assigned with any certainty. However, a positive NOE correlation was observed between H-9 and H-10. Together, these observations led to assignment of the relative stereochemistry for fumagiringillin as **1**.

There is a close structural relationship between fumagillin (**2**) and fumagiringillin (**1**), so the possibility that **1** was an artifact was carefully considered. By checking the retention times and UV spectra from the HPLC profiles for compound **1** against the crude extract, it was determined that fumagiringillin (**1**) was present in crude extracts of the fungus that had been prepared under neutral conditions by solvent extraction. Furthermore, fumagiringillin (**1**) was not present in trace quantities, but was isolated in amounts comparable to that of fumagillin (**2**).

Biological testing of fumagiringillin (**1**) against the murine leukemia cell line P388 established that the compound was not cytotoxic (IC_{50} >125 μg/mL).

The identities of fumagillin (**2**) and 12α-fumitermorgin (**3**), the other two compounds isolated and characterized from this strain of *Aspergillus fumigatus*, were established by comparison of spectroscopic data with those previously reported for these compounds.[14,15]

Experimental Section

General Experimental Procedures. Optical rotations were recorded using a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a GBC UV/vis 920 spectrometer and IR spectra on a Shimadzu FTIR-8201 PC spectrometer. NMR spectra were recorded on a Varian UNITY INOVA-500 spectrometer, operating at 500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively. HRESMS were determined using a Micromass LCT mass spectrometer. Column chromatography used J.T. Baker 40 μM Prep LC Bakerbond Octadecyl (C_{18}) and 40 μM Prep LC Bakerbond Diol (COHCOH). All solvents were distilled prior to being used.

Fungal Identification. The producing organism was isolated from saline lake sand from Western Australia. A reference sample of the strain (CANU A151) has been deposited in the culture collection of the School of Biological Sciences, University of Canterbury. The identification of the strain was made according to Pitt and Hocking.[16]

Morphological and cultural characteristics: Colonies on Czapek yeast extract agar (CYA) 48−55 mm in diameter (7 days, 25 °C), wrinkled, dense, and velutinous, white at first and becomes green with the forming of conidial heads, reverse pale. Colonies on malt extract agar (MEA) 50−61 mm in diameter (7 days, 25 °C), similar to those on CYA but less dense and with conidia in duller colors, reverse greenish. Colonies on 25% glycerol nitrate agar (G25N) 8−10 mm diameter (7 days, 25 °C). No growth at 5 °C. Growth at 37 °C is exceptionally rapid, colonies on CYA 29−35 mm in diameter in 2 days. This strain can grow at 50 °C.

Conidial apparatus develops as erect conidiophores. Tips of conidiophores enlarge and form vesicles with many phialides producing conidia in long dry chains. Conidial heads are compactly columnar, 33−47 μm in diameter, green to dark green. Conidiphores are unbranched, smooth, and pale green, stipes 210−330 μm × 4−5 μm in size. Vesicles are flask-shaped, 14−25 μm in diameter, fertile over half or more of the enlarged area, bearing phialides only, the lateral ones characteristically bent so that the tips are approximately parallel to the stipe axis. Phialides crowded, pale green, 6−8 μm long. Conidia are globose to subglobose, roughened, green in mass, and 2−3 μm in diameter.

The characteristics of strain CANU A151 are consistent with the descriptions of *Aspergillus fumigatus* given by Pitt and Hocking,[16] Kozakiewicz,[17] and Samson.[18]

Fermentation and Isolation. The fungus was grown on potato dextrose agar (GibcoBRL) plates for 7 days at 25 °C. Mycelial disks (8 mm in diameter) were transferred into 5 L of liquid medium consisting of half-strength potato dextrose broth (Difco) and incubated for four weeks at 26 °C on a rotary shaker (180 rpm). The culture was filtered under suction, and the mycelium and filtrate were extracted separately with

Table 1. ^{1}H and ^{13}C NMR Data for Fumagiringillin (**1**)[a]

position	δ_C[b]	δ_H[c]	^{1}H−^{1}H COSY	NOESY	CIGAR[d]
1	76.9 (CH)	3.68 (1H, dd, 12.0, 3.0)	H-2, H-9	H-2, H-9, H-14	C-2, C-5, C-9, C-17
2	66.8 (CH)	5.67 (1H, m)	H-1, H-3β	H-1, H-3β, H-17	
3	25.9 (CH_2)	H3β: 1.93 (1H, m)	H-2, H-3α, H-4α	H-3α	
		H3α: 2.07 (1H, m)	H-3β, H-4α, H-4β	H-3β	
4	27.9 (CH_2)	H4α: 1.63 (1H, m)	H-3α, H-3β	H-9	C-3
		H4β: 1.71 (1H, m)	H-3α, H-3β	H-3β	C-2, C-5, C-9
5	81.0 (C)				
6	77.1 (CH_2)	H6a: 3.81 (1H, d, 9.5)	H-6b	H-6b	C-5, C-8, C-9
		H6b: 3.65 (1H, d, 9.5)	H-6a	H-6a	C-5
8	85.3 (C)				
9	54.1 (CH)	2.19 (1H, d, 12.0)	H-1	H-1, H-4α, H-10	C-1, C-8, C-10, C-14
10	78.7 (CH)	3.37 (1H, m)	H-11a	H-9, H-11a, H-11b	C-8, C-12, C-14
11	30.4 (CH_2)	H11a: 2.09 (1H, m)	H-10, H-11b, H-12	H-10, H-11b, H-12, H-14	C-10, C-12, C-13
		H11b: 2.39 (1H, m)	H-11a, H-12	H-10, H-11a, H-12, H-15	C-12
12	121.9 (CH)	5.29 (1H, m)	H-11a, H-11b, H-15, H-16	H-11a, H-11b, H-16	C-15, C-16
13	133.3 (C)				
14	15.7 (CH_3)	1.29 (3H, s)		H-1, H-11a	C-8, C-9, C-10
15	18.1 (CH_3)	1.64 (3H, s)	H-12	H-11b	C-12, C-13, C-16
16	26.1 (CH_3)	1.71 (3H, s)	H-12	H-12	C-12, C-13, C-15
17	57.1 (CH_3)	3.39 (3H, s)		H-2	C-1
1′	166.3 (C)				
2′	123.1 (CH)	5.98 (1H, d, 15.5)	H-3′	H-3′, H-4′	C-1′, C-4′
3′	144.0 (CH)	7.30 (1H, dd, 15.5, 11.5)	H-2′, H-4′	H-2′, H-5′	C-1′
4′	134.1 (CH)	6.47 (1H, m)	H-3′, H-5′	H-2′, H-6′	C-6′
5′	140.0 (CH)	6.64 (1H, m)	H-4′	H-3′, H-7′	C-3′, C-7′
6′	139.3 (CH)	6.63 (1H, m)	H-7′	H-4′, H-8′	C-4′, C-5′, C-8′
7′	133.6 (CH)	6.49 (1H, m)	H-6′, H-8′	H-5′, H-9′	C-5′
8′	145.4 (CH)	7.36 (1H, dd, 15.5, 11.0)	H-7′, H-9′	H-6′, H-9′	C-10′
9′	122.3 (CH)	5.95 (1H, d, 17.0)	H-8′	H-7′, H-8′	C-7′, C-10′
10′	170.7 (C)				

[a] ^{1}H NMR spectra recorded at 500 MHz in $CDCl_3$, ^{13}C NMR spectra recorded at 125 MHz in $CDCl_3$. [b] Primary, secondary, tertiary, and quaternary carbons, assigned by APT. [c] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by number of protons, multiplicity, and coupling constant (J/Hz). [d] Long-range ^{1}H−^{13}C correlation from H to C observed in the CIGAR experiment.

EtOAc. The EtOAc extracts were then evaporated under reduced pressure and assayed for activity against the P388 murine leukemia cell line. The filtrate extract showed significant activity (IC_{50} 2.19 μg/mL), while the mycelial extract exhibited relatively weak activity (IC_{50} 23.35 μg/mL). The HPLC profiles of the two extracts were also different from each another. The filtrate extract was selected for chromatography.

The filtrate extract (591 mg) was separated on a C_{18} (50 g) column, eluted with a stepped gradient from 10% MeOH/H_2O to MeOH and then CH_2Cl_2. Fifteen fractions were collected, and fraction 7 was active against the P388 cell line. Fraction 7 (62.9 mg) was further chromatographed on Diol (20 g), using CH_2Cl_2/hexanes (50% to 100%), EtOAc/CH_2Cl_2 (20% to 100%), and MeOH/EtOAc (50% to 100%) as the eluent. Twenty-eight fractions were collected, and combinations were made mainly on the basis of comparisons of ^{1}H NMR spectra. Fumagiringillin (**1**) (6.9 mg), fumagillin (**2**) (9.2 mg), and fumitremorgin C (**3**) (4.4 mg) were isolated in fractions eluted with 20−40% EtOAc/CH_2Cl_2.

Fumagiringillin (1): pale yellow solid; $[\alpha]_D^{20}$ −12.5° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 317 (sh, 2.18), 332 (2.29), 348 (2.23) nm; IR ($CHCl_3$) ν_{max} 3687, 3398 (br), 2972, 1706, 1627, 1236, 1051, 1041 cm^{-1}; ^{13}C NMR and ^{1}H NMR, see Table 1; HRESMS *m*/*z* 475.2343 [M − H$^+$] (calcd for $C_{26}H_{36}O_8$, 475.2332).

P388 Murine Leukemia Bioassay. All extracts, fractions, and isolated compounds were submitted for biological activity against a P388 murine leukemia cell line using methodology adapted from Alley et al.[19] The samples were each prepared in MeOH at concentrations ranging between 1 and 10 mg/mL. A 2-fold dilution series of each sample for assay was then aliquoted into a 96-well microtiter plate and incubated for 72 h with P388 murine leukemia cells. Cell viability after incubation (expressed as an IC_{50} value) was determined colorimetrically by the addition of MTT tetrazolium (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide) to the wells, which were incubated for 90 min and analyzed at 540 nm on a Biotecksystems spectrophotometer.

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