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NOTE

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Induced Production of *N*-Formyl Alkaloids from *Aspergillus fumigatus* by Co-culture with *Streptomyces peucetius*

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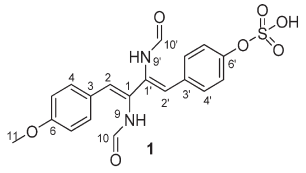
Supporting Information

ABSTRACT: Co-culture of the fungus *Aspergillus fumigatus* with the bacteria *Streptomyces peucetius* led to the induction of production of formyl xanthocillin analogues. This mixed fermentation yielded two new metabolites, fumiformamide (**1**) and *N*,*N*′-((1*Z*,3*Z*)-1,4-bis(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide (**2**), together with two known *N*-formyl derivatives and the xanthocillin analogue BU-4704. The structures were determined by spectroscopic methods and by comparison with literature. Cytotoxic activity of all the analogues was tested on the NCI-60 cell line screen, and compound **2** exhibited significant activity against several cell lines. The analogues did not show antimicrobial activity.

Natural products continue being an incredible resource for drug discovery.[1] However finding new microbial metabolites is becoming increasingly difficult. Different approaches have been taken to stimulate the production of microbial secondary metabolites.[2] One of them is mixed fermentation, where the co-culture of two or more microbes may induce secondary metabolite synthesis. The idea behind this approach is to mimic a more natural microbial ecosystem, where the organisms produce bioactive secondary metabolites to survive in a competitive environment. Examples of the production of previously unknown secondary metabolites by co-fermentation include the emericellamides,[3] libertellenones,[4] pestalone,[5] and marinamide.[6]

With the goal of isolation and identification of new metabolites produced by induction of unexpressed pathways, we started the process of screening mixed fermentations. Co-culture of *Aspergillus fumigatus* with several other organisms on agar plates showed different degrees of inhibition between the various surface cultures. The most significant effect was observed with *Streptomyces peucetius* (Figure 1). A follow-up study of the mixed fermentation of *A. fumigatus* with *S. peucetius* led to the isolation of two new natural products, the sulfated formyl xanthocillin analogue fumiformamide (**1**) and *N*,*N*′-((1*Z*,3*Z*)-1,4-bis(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide (**2**), together with two previously described *N*-formyl derivatives (**3**[7] and **4**[8]) and the xanthocillin analogue BU-4704 (**5**).[9] Compound **2** has been obtained as a synthetic product,[8] but has never been reported as a natural product. The isocyano analogues BU-4704 (**5**),[9] xanthocillin X mono- (**6**)[10] and dimethyl ether (**7**),[10,11] methoxy xanthocillin X dimethyl ether (**8**),[10] and xanthoascin[12] have been isolated from *Aspergillus*; however no formyl analogues have been reported from this genus.



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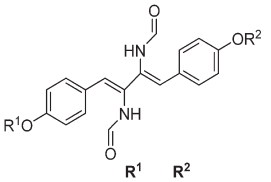
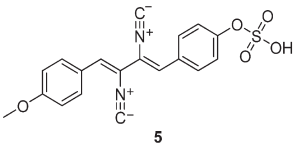


	R^1	R^2
2	CH_3	CH_3
3	H	H
4	CH_3	H



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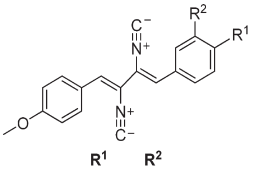

	R^1	R^2
6	OH	H
7	OCH_3	H
8	OCH_3	OCH_3

The study of the mixed culture of *A. fumigatus* and *S. peucetius* was undertaken in ISP2 liquid media at three different fermentation times (5, 10, and 14 days). For each of the conditions single cultures of *A. fumigatus* and *S. peucetius* were carried as controls. After extraction with BuOH, the samples were analyzed by LCMS. The chromatographic profiles of two of the co-cultures (ISP2 media for 10 and 14 days) were significantly different when

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Figure 1. Co-culture of *Aspergillus fumigatus* with different microorganisms on an agar plate. *A. fumigatus* is in the center and *S. peucetius* is at the bottom right of the plate. A zone of inhibition is observed between both microorganisms.

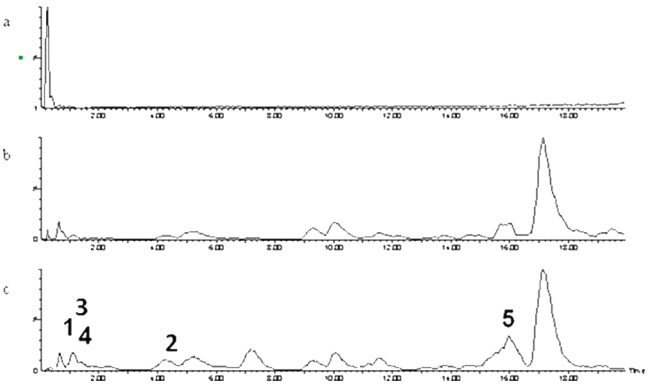

Figure 2. Chromatogram of the extracts after 14 days of incubation (negative ion mode) of (a) *Streptomyces peucetius*, (b) *Aspergillus fumigatus*, and (c) co-culture.

compared with their corresponding single culture controls (Figure 2). The main differences were observed in the negative ion mass spectra and corresponded to two compounds with t_R 1.2 min, mass 418 ($[M - H]^-$ = 417) and t_R 1.3 min, mass 338 ($[M - H]^-$ = 337). These two compounds exhibited a maximum UV absorbance at 334 nm. There was also a noticeable increase of production of a less polar compound (t_R 15.9 min) with mass 382 ($[M - H]^-$ = 381). It had a maximum UV absorbance at 354 nm. An increase of the peak at t_R 7.2 was also observed, but it was assigned as fumitremorgin A[13] by comparison with an authentic sample.

Fumiformamide (**1**) was isolated as a yellow solid. The ESI mass spectrum (negative mode) showed a peak with *m/z* 417 and a fragment at *m/z* 337. The loss of 80 uma from the molecular ion suggested the presence of a sulfate group. The sulfur atom was confirmed by its molecular formula, assigned as $C_{19}H_{18}N_2O_7S$ by HRMS of its pseudomolecular ion $[M - H]^-$. The UV−vis spectra showed absorption at 334 nm, suggesting the presence of a conjugated system.

The chromatographically homogeneous compound unexpectedly showed very complex NMR spectra. The ^{1}H NMR spectra recorded in DMSO-d_6 (Table 1) showed eight signals for exchangeable protons, four of which appeared as singlets (δ_H 9.63, 9.60, 9.51, and 9.49) and four as doublets (δ_H 9.42, 9.39, 9.36, and 9.32). The integration for the doublets was slightly smaller than the one obtained for the singlets, with a ratio of the areas of singlet/doublet of approximately 6:4. Additional signals included several superimposed singlets (δ_H 8.19−8.22), four doublets (δ_H 7.89, 7.88, 7.83, and 7.81), three very complex multiplets centered at δ_H 7.46, 7.15, and 6.94, and several singlets between δ_H 6.58 and 6.50. The only signals in the upfield region were two very close singlets assigned as methoxyl groups (δ_H 3.784 and 3.779). The ^{13}C NMR spectrum of **1** showed 54 signals, significantly more than the 19 carbons expected according to its molecular formula. With the exception of seven of the signals resonating between δ_C 165 and 160 (assigned as CH by HSQC) indicating formyl carbonyl groups and four of the signals between δ_C 55.58 and 55.64 confirming methoxyl groups, the signals resonating between δ_C 114 and 133 supported that compound **1** was fully conjugated.

COSY correlations between the protons at δ_H 8.20 and the exchangeable protons at δ_H 9.63, 9.60, 9.51, and 9.49; between δ_H 7.83 and the exchangeable proton at δ_H 9.36; and between δ_H 7.81 and the exchangeable proton at 9.32 ppm together with HMBC correlations between H-9 cis (δ_H 9.63 and 9.60) and C-10 cis (δ_C 160.84 and 160.79); between H-9 trans (δ_H 9.36) and C-10 trans (δ_C 164.99, 164.96); between H-9′ cis (δ_H 9.51 and 9.49) and C-10′ cis (δ_C 160.72 and 160.66); and between H-9′ trans (δ_H 9.32) and C-10′ trans (δ_C 164.95) allowed us to assign the signals at lower field as belonging to two formamide groups. Furthermore, the formyl groups were confirmed by the J_1(CH-10) of 194 Hz obtained in a F2-decoupled HSQC experiment. The presence of *N*-formyl groups suggested the existence of conformers with rotational hindrance and explained the highly complex NMR spectra, as previously described.[8,14,15] These data suggested that fumiformamide (**1**) was a 1,4-diphenylbuta-1,3-diene-2,3-diyl-diformamide system with one methoxyl and one sulfate group.

Due to the complex ^{1}H NMR spectrum, the positions of the substituents could not be deduced by analysis of the typical patterns observed on substituted aromatic rings. HMBC correlations between the signals for methoxyl groups (δ_H 3.784 and 3.779), δ_H 7.47 (H-4), and δ_H 6.94 (H-5) and C-6 (δ_C 159.3, 159.08, 159.06, 158.8); between H-4 and C-2 (δ_C 124.1, 123.6) and C-5 (δ_C 114.63, 114.55, 114.4, 114.3); and between H-5 and C-3 (δ_C 128.5, 128.20, 128.16, 127.9) located the methoxyl group at C-6. Similar analysis allowed us to assign the sulfate group to C-6′: HMBC correlations between H-4′ (δ_H 7.42) and H-5′ (δ_H 7.15) and the downfield quaternary carbon C-6′ (δ_C 153.6, 153.4, 153.2); between H-5′ and C3′ (δ_C 130.6, 130.5, 130.4, 130.24); and between H-2′ (δ_H 6.554, 6.549, 6.50) and C-4′ (δ_C 130.3, 130.2, 130.1, 129.9). The coupling constant between the amine and the formyl protons allowed us to assign the cis and trans conformations for the formaldehyde group. The signals of H-9 and H-10 were observed as singlets when these protons were in the cis conformation, while they were doublets with *J* values between 11.0 and 11.7 in the trans conformation. The difference in the coupling constant was due to the dihedral angle between the protons of the amine and the CH-9. Similar results were shown in the data published by Quintanilla-Licea et al.,[15] where a coupling constant (*J* value) of ∼11−12 Hz

Table 1. NMR Data of Compound 1 in DMSO-d_6

position	δ_C,[a] type	δ_H, mult. (J in Hz)	HMBC
1	132.2, 132.1, 132.0, 131.4, C		9′cis, 9′trans, 10cis
2	124.1, 123.6, CH	6.58, 6.51, s	4, 8, 9cis
3	128.5, 128.20, 128.16, 127.9, C		5, 7
4, 8	131.2, 131.05, 130.96, 130.8, CH	7.47, m[d]	2
5, 7	114.63, 114.55, 114.4, 114.3, CH	6.94, m	4, 8
6	159.3, 159.08, 159.06, 158.8, C		4, 5, 7, 8, 11
9		9.63, 9.60, s (cis)	
		9.42 d (11.0), 9.36 d (11.7) (trans)	
10	160.84, 160.79, CH (cis)[b]	8.22−8.19, br s (cis)	9cis
	164.99, 164.96, CH (trans)[c]	7.89 d (11.0), 7.83 d (11.7) (trans)	9trans
11	55.64, 55.62, 55.60, 55.58, CH_3	3.784, 3.779, s	
1′	132.8, 132.7, C		9cis, 9trans, 2
2′	122.9, 122.6, 122.5, 122.1, CH	6.554, 6.549, 6.50, s	9′cis, 4′, 8′
3′	130.6, 130.5, 130.4, 130.24, C		5′, 7′
4′, 8′	130.3, 130.2, 130.1, 129.9, CH	7.42, m[d]	2′
5′, 7′	120.72, 120.67, 120.6, 120.5 CH	7.15, m	
6′	153.6, 153.4, 153.2, C		4′, 5′, 7′, 8′
9′		9.51, 9.49, s (cis)	
		9.39 d (11.0), 9.32 d (11.3) (trans)	
10′	160.72, 160.66, CH (cis)[b,c]b	8.22−8.19, br s (cis)	9′cis
	164.95, CH (trans)[b,c]c	7.88 d (11.0), 7.81 d (11.3) (trans)	9′trans

[a] δ_C deduced by gHSQC and gHMBC experiments. [b,c] Assignment can be interchanged. [d] The multiplicity of this signal was unresolved due to peak overlapping, and the chemical shift was assigned by interpretation of HSQC and HMBC data.

Table 2. Significant IC_{50} (μM) Data for Compounds 2 and 4 Obtained on the NCI-60 Cell Line Screen[a]

panel	cell line	2	4
non-small-cell lung	HOP-92	0.89	>2.50
	NCI-H460	1.12	1.84
CNS	SF-295	0.70	>2.50
	U251	0.92	>2.50
melanoma	MALME-3M	0.65	>2.50
	SK-MEL-28	0.98	2.03
	SK-MEL-5	0.81	>2.50
	UACC-62	0.75	>2.50
ovarian	SK-OV-3	0.84	2.13
renal	A498	0.86	1.72
	ACHN	>2.50	1.37
leukemia	MOLT-4	>2.50	1.81

[a] Complete NCI-60 cell line data in Supporting Information

indicates a trans conformation, while a J value of less than 2 Hz, a cis rotamer. It is also known that the carbonyl group in the trans conformation resonates at lower field than those of the equivalent cis isomers.[8,15] The ratio of the cis:trans isomers, as deduced from the integration of the formyl proton signals, was approximately 6:4. The preference for the cis isomer is congruent with similar findings reported for *N*-alkyl or *N*-benzyl formamides, where the cis isomer predominates up to 80% over the trans isomer.[15]

Compound **3** was previously isolated from *Penicillium notatum*,[8] but only limited spectroscopic data were described. A standard battery of NMR experiments provided assignments for the signals in the ^1H and ^{13}C NMR spectra (Experimental Section).

Of the few formaldehyde analogues of xathocillins that have been reported, three bearing two formyl groups were isolated from the fungi *Hamigera avellanea*,[8] *Cordyceps brunnearubra* BCC1395,[14] and *Penicillium notatum*.[7] Melanocins A and B, with only one formyl group, were isolated from *Eupenicillium shearii*.[16] However, all of these metabolites contained hydroxyl and/or methoxyl groups, whereas this is the first report of a formyl-substituted xanthocillin analogue with a sulfate functional group.

Fumiformamide (**1**) and compounds **2** and **3** are the formyl analogues of the three previously isolated metabolites from *Aspergillus*[17] BU-4704 (**5**), xanthocillin X dimethyl ether (**7**), and xanthocillin X monomethyl ether (**6**), respectively. Since no formyl analogues have ever been described for this genus, it is possible that the co-culture with *Streptomyces* led to the induction or enhanced production of these analogues. Subsequently, a careful analysis of the LCMS traces of the extracts of the single control culture of *A. fumigatus* confirmed the presence of these metabolites in very low proportions. Higher production of metabolites by mixed culture was also described on the co-culture of *Emerycella* sp. with *Salinispora arenicola*.[3]

The cytotoxicity of all the compounds was assessed on the NCI-60 cell line screen.[18,19] Compound **2** showed submicromolar activity against several cell lines, while compound **4** exhibited only moderate toxicity against fewer cell lines (Table 2). The rest of the compounds were inactive. Fumiformamide (**1**) and BU-4704 (**5**) were also tested using a Kirby-Bauer disk diffusion assay[20] against *Escherichia coli*, *Candida albicans*, *Staphylococcus aureus*, *Burkholderia thailandesis*, and *Fusarium pallidoroseum*, but were inactive at 50 μg/disk.

While several biological properties have been reported for xanthocillin analogues with isocyano functional groups, including antibiotic,[9,10,21,22] antifungal,[9,21] antiviral,[10,21] inhibition

of melanin biosynthesis,[23] thrombopoietin receptor activators,[17,24,25] and cytotoxicity,[9,12] the formyl analogues showed only modest activity against the malarial parasite *Plasmodium falciparum* K1 and weak cytotoxicity.[14]

EXPERIMENTAL SECTION

General Experimental Procedures. UV spectra were acquired in spectroscopy grade MeOH using a Varian Cary 50 UV−vis spectrophotometer. NMR spectra were collected using a Bruker Avance III 600 NMR spectrometer (Bruker Biospin), referenced to residual solvent (δ_H 2.50 and δ_C 39.5 for DMSO-d_6) and on a Varian Inova 500 MHz in DMSO-d_6 with TMS as internal standard. High-resolution mass spectra were obtained on a Waters LCT Premier TOF mass spectrometer. HPLC-MS consisted of a Waters 600 pump, a Waters ZQ electrospray mass spectrometer, a Waters 996 photodiode array spectrometer, and a Sedex 75 evaporative laser light scattering detector. Purification was done with Sephadex LH-20 (Pharmacia) and Dynamax 60A C-18 (4.6 × 250 mm, Varian). Analytical HPLC was performed with an XTerra C-18 HPLC column (Waters).

Material. *Streptomyces peucetius* was purchased from the American Type Culture Collection, number 29050, and maintained on ISP2 agar at room temperature. *Aspergillus fumigatus* was acquired from the Michigan Department of Health in 1993 and maintained on potato dextrose agar at room temperature

Fermentation and Isolation. A seed culture medium, International Streptomyces Project Medium 2 (ISP2), consisting of malt extract, 10 g/L; dextrose, 4 g/L; and yeast extract, 4 g/L, was sterilized in 500 mL Erlenmeyer flasks at 120 °C for 15 min. Agar plugs from each plated culture were inoculated into their respective flasks (100 mL). The flasks were incubated for 24 h at 26 °C on a rotary shaker (210 rpm). The resultant seed cultures were used to inoculate the production medium, also ISP2, in 1 L Erlenmeyer flasks (2 × 250 mL media) sterilized at 120 °C for 15 min. The inoculum per flask was as follows: 10 mL of *S. peucetius* and 1 mL of *A. fumigatus* with ratio packed cell volumes to media of <1 mL/15 mL and 2 mL/15 mL, respectively. Fermentation was carried out for 5, 10, and 14 days at 26 °C on a rotary shaker (210 rpm), having performed smaller scale pilot experiments demonstrating a potential time course of production.

Once the fermentation time point was reached, 100 mL of MeOH was added to 1 L of each culture. The samples were then homogenized with an Omni Macro homogenizer and extracted with BuOH (1 L) to obtain 545, 573, and 358 mg of extract, after 5, 10, and 14 days of incubation, respectively. Single control cultures of *S. peucetius* and *A. fumigatus* were grown under identical conditions. Each control fermentation was processed and extracted as described above.

The 10- and 14-day co-culture BuOH extracts were dissolved in 100 mL of MeOH−H_2O (9:1) and subjected to a solvent−solvent partition with hexane, CH_2Cl_2, and EtOAc. The CH_2Cl_2, EtOAc, and aqueous fractions were subjected to gel permeation on Sephadex LH-20 with MeOH. Samples found to contain the metabolites of interest by LCMS were combined in two fractions (A and B). Fraction A was purified by reversed-phase HPLC (Varian C18, 0.1% HCOOH in MeCN and 0.1% HCOOH, linear gradient 25−40 for 10 min, isocratic for 10 min, and linear gradient 40−100 for 35 min, at a flow rate of 1 mL/min), affording fumiformamide (**1**) (1.3 mg) and BU-4704 (**5**) (0.6 mg). In similar fashion, fraction B was purified by reversed-phase HPLC (Varian C18, 0.1% HCOOH in MeCN and 0.1% HCOOH, linear gradient 15−30 for 10 min and then isocratic for 20 min, at a flow rate of 1 mL/min) to afford 1.2 mg of **2**, 0.4 mg of **3**, and 2.3 mg of **4**.

Compound ***1****:* UV (MeOH) λ_{max} (log ε) 204 nm (3.85), 334 nm (3.64); IR (NaCl) ν_{max} 3393, 1671, 1599, 1509, 1356, 1253, 1054 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) see Table 1; HRESIMS m/z 417.0753 $[M - H]^-$ (calcd for $C_{19}H_{17}N_2O_7S$ 417.0756).

Compound ***3****:* ^1H NMR (500 MHz, DMSO-d_6) δ (J in Hz) 9.52, 9.40 (s, NH-9/9′ cis), 9.32, 9.23 (d, 11.3, NH-9/9′ trans), 8.20 and 8.19 (s and d, 1, H-10/10′ cis), 7.87, 7.80 (d, 11, H-10/10′ trans), 7.40−7.31 (m, H-4/4′), 6.80−6.73 (m, H-5/5′), 6.50, 6.49, 6.47, and 6.43 (s, H-2/2′); ^{13}C NMR (DMSO-d_6, δ deduced from HSQC and HMBC experiments) δ 164.4 (CH-10/10′ trans), 159.9 (CH-10/10′ cis), 157.4, 157.0 (C-6/6′), 130.2, 130.6 (C-4/4′), 126.2, 125.8 (C-3/3′), 122.8, 121.8, 120.4, 118.9 (CH-2/2′), 115.2, 115.0 (CH-5/5′). C-1/1′ could not be assigned.

ASSOCIATED CONTENT

Supporting Information. ^1H NMR, ^{13}C NMR, COSY, gHSQC, F2-decoupled HSQC, and gHMBC for fumiformamide (**1**) and in vitro testing results for compounds **2** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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