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Microbial Transformation of Sampangine

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Microbial transformation studies of the antifungal alkaloid sampangine (**2**) have revealed that it is metabolized by a number of microorganisms. Using a standard two-stage fermentation technique, *Beauvaria bassiana* (ATCC 7159), *Doratomyces microsporus* (ATCC 16225), and *Filobasidiella neoformans* (ATCC 10226) produced the 4′-*O*-methyl-*β*-glucopyranose conjugate (**3**), while *Absidia glauca* (ATCC 22752), *Cunninghamella elegans* (ATCC 9245), *Cunninghamella* species (NRRL 5695), and *Rhizopus arrhizus* (ATCC 11145) produced the *β*-glucopyranose conjugate (**4**). Metabolites **3** and **4** have been characterized on the basis of spectral data. Both **3** and **4** had significant in vitro activity against *Cryptococcus neoformans* but were inactive against *Candida albicans*. Metabolite **4** was inactive in vivo in a mouse model of cryptococcosis.

Due to the high mortality and morbidity rates of AIDS,[1] special attention has been, and is still being, given to the discovery and development of therapeutic agents to either cure the cause or alleviate the symptoms of this syndrome. The characteristic breakdown in the immune system that is associated with AIDS is often manifested in the form of serious opportunistic infections (OI). The most common disseminated fungal OI in AIDS patients are cryptococcosis and candidiasis.[2] Unfortunately, treatment of fungal OI in AIDS patients poses many problems, including the required long duration of therapy, more frequent and severe drug toxicities, the relative ineffectiveness of the available systemic antifungal agents, and the development of resistance to the existing agents. These significant shortcomings have intensified the search for new, more effective, and less toxic prototype antifungal agents.

As part of an extensive program aimed at the discovery and development of prototype antifungal antibiotics from higher plants, we previously reported the isolation, structure elucidation, and in vitro antifungal activity of the copyrine alkaloid, 3-methoxysampangine (**1**) from the root bark of the West African tree, *Cleistophathis patens* (Benth) Engl. and Diels (Annonaceae).[3] Due to the scarcity of **1** (0.000156% yield), our attention was directed to sampangine (**2**), the parent alkaloid. Prior to discovery of **1**, the unsubstituted parent compound had also been reported as a natural product in the stem bark of *Cananga odorata* Hook, F. and Thomas (Annonaceae)[4] and had been previously synthesized (26% yield).[5] Sampangine (**2**) was synthesized in our laboratory and shown to be as active as **1** against *Candida albicans* and *Cryptococcus neoformans*.[2,5] The immediate synthetic accessibility of **2** made it a more desirable candidate for further drug metabolism studies.

One of the important methods used to establish the metabolic fate of new drug candidates is the use of microorganisms, because they can serve as convenient, reliable, and predictive models for mammalian drug metabolism.[6,7] This method produces significant quantities of metabolites that would be difficult to obtain from either animal systems or chemical synthesis. The results from this method often parallel those obtained from human biotransformation and, thus, can be predictive. This work reports on the isolation and characterization of the two sampangine metabolites, **3** and **4**, and results of their in vitro and in vivo antifungal evaluation.

Results and Discussion

Of 70 microbial cultures screened, only seven showed definite biotransformation of **2**. *Beauvaria bassiana* ATCC 7159, *Doratomyces microsporus* ATCC 16225, and *Filobasidiella neoformans* ATCC 10226 produced one main metabolite designated SAMM1 (**3**), while *Absidia glauca* ATCC 22752, *Cunninghamella elegans* ATCC 9245, *Cunninghamella* species NRRL 5695, and *Rhizopus arrhizus* ATCC 11145 produced another metabolite designated SAMM2 (**4**). *B. bassiana* ATCC 7159 and *R. arrhizus* ATCC 11145 were chosen for preparative scale fermentation to produce sufficient quantities of these metabolites for structure elucidation and other biological studies. It is noteworthy that all cultures that demonstrated the ability to metabolize sampangine turned bright red within a few days after sampangine was added.

Preparative scale biotransformation of **2** by *R. arrhizus* ATCC 11145 afforded **4** in 83.5% yield. Thermospray MS of **4** indicated that its molecular weight (*m*/*z* 396; M^+) was the sum of **2** (232) and a moiety of 164 mass units. HRFABMS of SAMM2 showed the exact molecular weight, which was consistent with the molecular formula $C_{21}H_{20}N_2O_6$. The IR spectrum showed a broad absorption at 3300 cm^{-1}, which suggested the presence of at least one hydroxyl group, while the absence of any strong absorption around the 1670 cm^{-1} region indicated the loss of the carbonyl group of sampangine. The ^{13}C NMR spectrum (75 and 125 MHz) of **4** showed 21 carbon resonances, none of which was a carbonyl carbon (one triplet, 13 doublets, and seven singlets). Five of those resonances were in the region for aliphatic oxygenated carbons (*δ* 60−80) (Table 2) and were consistent with the sugar glycoside. The carbon resonating at *δ* 107.7 as a doublet was determined from HETCOR and HMBC spectra to be consistent with an anomeric carbon. The other resonances were characteristic of the sampangine moiety, with all original proton signals for sampangine intact (Tables 1 and 2). It was especially noteworthy, however, that upfield shifts were observed for C-4 (*δ* 22.4), C-5 (*δ* 11.6), C-6a (*δ* 17.7), C-7 (*δ* 51.6), H-4 (*δ* 2.15), and H-5 (*δ* 2.14), which indicated that the moiety

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Table 1. Comparative ^{1}H NMR Assignments of **2**−**4**[a]

H	**2**[b] δ_H (J in Hz)	**3**[c] δ_H (J in Hz)	**4**[c] δ_H (J in Hz)
2	8.84, d (5.8)	8.29, d (5.0)	8.27, d (5.0)
3	7.69, d (5.8)	6.68, d (5.0)	6.66, d (5.0)
4	7.90, d (5.5)	5.76, d (7.2)	5.75, d (7.2)
5	9.11, d (5.5)	6.97, d (7.2)	6.97, d (7.2)
8	8.43, dd (7.8, 1.0)	8.46, dd (8.0, 0.8)	8.46, d (8.0)
9	7.67, ddd (7.8, 7.8, 1.3)	7.56, ddd (8.0, 8.0, 0.8)	7.56, ddd (8.0, 8.0, 1.2)
10	7.81, ddd (7.8, 7.8, 1.0)	7.36, ddd (8.0, 8.0, 0.8)	7.36, ddd (8.0, 8.0, 1.2)
11	8.78, dd (7.8, 1.3)	8.70, dd (8.0, 0.8)	8.70, d (8.0)
1′		4.67, d (7.8)	4.69, d, (7.9)
2′		3.67, dd (9.3, 7.8)	3.68, dd (9.1, 7.9)
3′		3.55, dd (9.3, 9.7)	3.47, dd (9.1, 9.1)
4′		3.34, dd (9.7, 9.7)	3.57, dd (9.1, 9.1)
5′		3.22, dt (9.7, 3.2)	3.25, m
6′		3.73, m	3.78, m
OCH_3		3.59, s	

[a] Chemical shifts (δ) are reported in parts per million, J values are in Hertz. [b] Spectra recorded in $CDCl_3$. [c] Spectra recorded in CD_3OD.

Table 2. Comparative ^{13}C NMR Assignments of **2**−**4**

carbon	**2**[a] δ_C	**3**[b] δ_C	**4**[b] δ_C
2	147.1, d[c]	150.2, d	150.7, d
3	118.9, d	111.8, d	111.9, d
3a	138.3, s	145.8, s	145.9, s
4	123.2, d	100.9, d	100.8, d
5	148.1, d	136.7, d	136.5, d
6a	147.4, s	129.7, s	129.7, s
7	181.5, s	129.8, s	129.9, s
7a	131.9, s	131.3, s	131.4, s
8	128.1, d	122.2, d	122.2, d
9	131.1, d	129.4, d	129.3, d
10	134.3, d	123.9, d	123.9, d
11	125.1, d	124.5, d	124.6, d
11a	135.0, s	125.3, s	125.8, s
11b	150.6, s	145.3, s	145.7, s
11c	119.2, s	122.1, s	122.3, s
1′		107.6, d	107.7, d
2′		75.7, d	75.6, d
3′		78.1, d	78.0, d
4′		80.1, d	70.8, d
5′		77.1, d	78.1, d
6′		61.8, t	62.1, t
OCH_3		60.8, q	

[a] Spectra recorded in $CDCl_3$ with TMS as internal standard. [b] Spectra recorded in CD_3OD with TMS as internal standard. [c] Number of attached protons determined by DEPTGL.

added via biotransformations resulted in profound magnetic and electronic effects on ring B and C-7 in ring C of **2**. A NOE (NOESY and ROESY) was observed between the anomeric proton and H-8 (δ 8.46, d, J = 8.3 Hz). In addition, three-bond correlations between the anomeric proton and a singlet carbon at δ 129.9, which shows another three-bond correlation to H-8, led us to conclude that **4** is an *O*-glycoside in which the glycone part is coupled with **2** at C-7. From the COSY spectrum, the proton NMR assignments for H-9, H-10, and H-11 were determined. H-11 has a three-bond correlation to two singlet carbons (δ 131.4 and 145.7), one of which (δ 131.4) was determined to be C-7a, because it shows a three-bond correlation to H-9. Therefore, the remaining singlet carbon (δ 145.7) should be C-11b. C-11b shows another three-bond correlation to a proton resonating at δ 8.27 (d, J = 5.0 Hz), which was assigned to H-2. Consequently, H-3 was determined from the COSY spectrum. The NOE (NOESY and ROESY) connected H-3 to H-4, was assigned to the peak at δ 5.75 (d, J = 7.2 Hz). Finally, H-5 was assigned from the COSY spectrum.

The unambiguous assignments of H-2 and H-5 were crucial in distinguishing between the two tautomeric forms that can be drawn for **4**. H-1′ resonated at δ 4.69 (d, J = 7.9 Hz), the large coupling constant indicating a 1,2-diaxial relationship with H-2′, thus establishing the β-glycosidic linkage. A careful comparison of the ^{13}C NMR data for the sugar moiety in **4** with those of other β-D-glucopyranose systems revealed identical resonances for each carbon;[8−10] thus, it was concluded that the sugar part is β-glucopyranose. An additional piece of supportive evidence was drawn from the coupling constants. Coupling constants $J_{2',3'}$, $J_{3',2'}$, $J_{3',4'}$, $J_{4',3'}$, $J_{4',5'}$, and $J_{5',4'}$ were larger than 9.0 Hz (Table 1), supporting the 1,2-trans-diaxial relationship between each consecutive pair of protons, and ultimately confirming the identity of the hexose as β-glucopyranose. This was further supported by the existence of NOEs between protons 1′ and 3′, 1′ and 5′, 3′ and 5′, and 2′ and 4′, supporting the 1,3-cis-diaxial interactions between these protons. An ^{1}H NMR spin simulation was run using NMRSIM, and identical chemical shifts, coupling patterns, and coupling constants were obtained for each proton.

The complete unambiguous assignments of other carbon resonances, particularly the singlets, were made possible from HMBC and HMQC spectra. Independent assignments of proton and carbon resonances and cross-peaks in HMBC, HMQC, COSY, and ROESY were also performed using "AURELIA", which is a 2D and 3D analysis package to conduct such assignments. These assignments were consistent with those obtained using conventional assignment methods.

Chemical degradation of **4** was achieved by simple acid hydrolysis (H_2SO_4) of the glucosidic bond followed by chromatographic identification of the hydrolysate. Sampangine (**2**) and glucose were identified as hydrolsis products. On the other hand, HCl hydrolysis of **4** produced **2**, glucose, and 4-chlorosampangine. The nucleophilic chlorination of **2** at position 4 was expected, for **2** was left in contact with HCl for about 50 h, and such a reaction has been observed previously.[11]

Preparative scale biotransformation of **2** by *B. bassiana* ATCC 7159 afforded **3** in a 9.7% yield. Thermospray MS of **3** indicated a molecular weight of 410, and a HRFABMS gave the exact molecular weight to be consistent with the molecular formula $C_{22}H_{22}N_2O_6$. The ^{13}C NMR spectrum of **3** showed all 22 carbon resonances (no carbonyls). Six resonances were in the region for aliphatic oxygenated carbons (δ 60−δ 80, Table 2), one of which could be assigned as a methoxyl group (δ 60.8). The other resonances were characteristic of the sampangine moiety with no substitution at any of its protons. The data were consistent with the assignment of metabolite **3** as the *O*-methylhexose conjugate of **2**. The resonance at δ 107.6

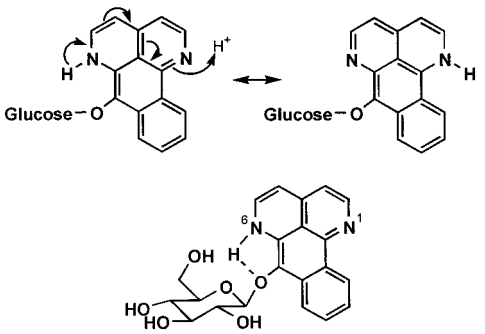


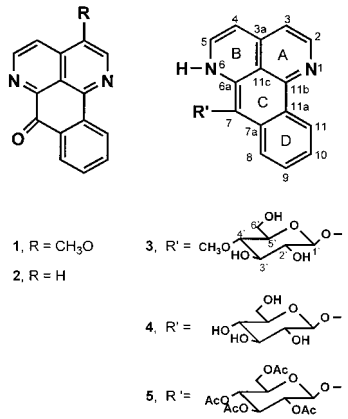
Figure 1. The possible vs existing tautomeric structures of **4**.

was determined from the HETCOR spectrum to be an anomeric carbon. The H-1′ resonated at δ 4.67 (d, J = 7.8 Hz Table 1). This large coupling constant indicated a 1,2-diaxial relationship between H-1′ and H-2′ and established the β-glycosidic linkage. Coupling constants $J_{2',3'}$, $J_{3',2'}$, $J_{3',4'}$, $J_{4',3'}$, $J_{4',5'}$, and $J_{5',4'}$ were larger than 9.0 Hz (Table 1), which indicated the 1,2-diaxial relationships between each of the consecutive pairs of protons. This ultimately established the identity of this hexose as a glucopyranose derivative. A careful comparison of the ^{13}C NMR data for the glucose part in **3** with those of **4** revealed nearly identical resonances except for C-4′, which in **3** showed a downfield shift (10 ppm) in relation to **4**. This downfield shift was attributed to the presence of an *O*-methyl group on C-4′, which resonated in the ^{13}C NMR spectrum at δ 60.8 and in the ^{1}H NMR spectrum at δ 3.6 (s, 3H). This conclusion was further confirmed by comparing the ^{13}C resonances of the sugar part with those reported for the corresponding carbons in similar 4′-*O*-methylglucopyranoside.[12]

The complete unambiguous assignments of all carbon resonances, particularly the singlets, were aided and confirmed by the complete unambiguous assignments of all carbon resonances of **4** because they are almost identical metabolites. From the above data, **3** can be represented as sampangine-4′-*O*-methyl-β-glucopyranoside.

The mass spectra for both metabolites **3** and **4** revealed the presence of a common molecular ion at *m*/*z* 235 (M^+ + 1). The presence of such a common molecular ion in both samples led us to the conclusion that dihydrosampangine (MW = 234) is the common intermediate in the pathway leading to **3** and **4**. Thus, the proposed mechanism involved in the formation of **3** and **4** is as follows: (i) reduction of **2** into dihydrosampangine, possibly through reductase or hydrogenase enzyme catalysis; (ii) coupling of this "fragile" moiety through the newly formed hydroxyl group with glucose to form the more stable *O*-glucoside; (iii) tautomerism of H-7 to N-6 either before or after *O*-glucosylation; (iv) *O*-methylation of C-4′ of glucose could take place either before or after the coupling process to yield **3**.

The structures of **3** and **4** suggested a possible tautomerism of the proton on N-6 to N-1 and vice versa (Figure 1). To test the validity of this concept, **4**, which was available in larger quantities, was acetylated to afford **5**. The ^{13}C NMR spectrum of **5** showed four carbons resonating at δ 21.8, 21.9, 22.0, and 22.2 (each with 3H) and another four carbons resonating at δ 170.6, 170.7, 171.5, and 172.0 (each with no H). Other carbon resonances were comparable to those of **4** (Table 3). Likewise, the ^{1}H NMR spectrum revealed three peaks, two of which integrated for three protons each, and the other one integrated for six protons (Table 3). The HRFABMS showed a molecular weight (*m*/*z* 565.1838; M + H^+) consistent with the molecular formula $C_{29}H_{28}O_{10}N_2$. This molecular weight was the sum of **4** (396) and a moiety of 168 mass units (consistent with four units of CH_2CO). These findings proved the presence of four acetate groups, presumably on the four hydroxyl groups of glucose. Because H-2 and H-5 were unambiguously assigned, the splitting of H-5 into a double doublet suggested its coupling to an adjacent proton with a large coupling constant (J = 6.5 Hz). The only possibility for such coupling would be the proton on N-6, which resonates at δ 8.07 (d, J = 6.5 Hz). This coupling pattern was confirmed by the collapse of H-5 into a doublet (J = 7.2 Hz) and the disappearance of H-6 upon D_2O exchange. Especially noteworthy were the downfield shifts of H-2′ (δ 1.78), H-3′ (δ 1.83), H-4′ (δ 1.62), and H-6′ (δ 0.47) due to the deshielding effects of the acetyl groups. The resonances of the other protons were largely the same as **4**, except for H-8, which showed an upfield shift (0.57 ppm), as a result of the shielding effect of the carbonyl group of the C-2′ acetate. These results also revealed the inaccessibility of the N−H group for acetylation, and suggest that **4** exists as one tautomer, with the proton only on N-6. This is most likely due to hydrogen bonding between N−H and an oxygen atom (Figure 1).



Both **3** and **4** demonstrated significant in vitro activity against *C. neoformans*, but were inactive against *C. albicans*. The MIC of **3** against *C. neoformans* is 0.2 μg/mL and of **4** is 0.78 μg/mL. By comparison, the MIC of **2** ranges from 3.12 μg/mL for *C. albicans* to 0.2 μg/mL for *Cr. neoformans*. Unfortunately, **4** is inactive in vivo in a mouse model of cryptococcosis (no protection from lethality as compared to untreated controls).

Experimental Section

General Experimental Procedures. Melting points were determined in open capillary tubes using Thomas−Hoover capillary melting point apparatus and are uncorrected. The IR spectra were recorded in KBr using either a Perkin−Elmer 281 B IR spectrophotometer or a ATI Mattson Genesis Series FTIR spectrophotometer. ^{1}H and ^{13}C NMR were obtained on either a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively, or a Bruker DRX-500 spectrometer operating at 500 and 125 MHz, respectively. Both ^{1}H and ^{13}C NMR spectra were recorded in deuterated MeOH, and the chemical shift values are expressed in δ (ppm) relative to the internal standard, TMS. For the ^{13}C NMR spectra, the number of attached protons were determined by the Attached Proton Test (APT) and DEPT. 2D NMR data were obtained using the standard pulse sequences of either the VXR-300 for COSY, HETCOR, LR−HETCOR, and NOESY or the Bruker DRX-500 for COSY, phase-sensitive DQ-filtered COSY, HMQC, HMBC, NOESY, and ROESY. Some spectra were assigned using the program AURELIA (Bruker Instruments, Inc.) and NMRSIM by Dr. Clemens Anklin (Bruker Instruments, Bellerica, MA).

Table 3. ^{1}H and ^{13}C NMR Assignments of **5**[a]

carbon	δ_H (J in Hz)[b]	δ_C
2	8.52, d (4.9)	151.9, d[c]
3	6.69, d (4.9)	112.9, d
3a		147.2, s
4	5.74, d (7.2)	101.9, d
5	6.89, dd (7.2, 6.5)	135.3, d
6a		130.3, s
7		130.8, s
7a		127.8, s
8	7.89, d (8.1)	119.6, d
9	7.57, ddd (8.1, 8.1, 1.2)	129.8, d
10	7.43, ddd (8.1, 8.1, 1.2)	124.4, d
11	8.97, d (8.1)	126.2, d
11a		127.2, s
11b		143.8, s
11c		121.7, s
1′	5.02, d (8.1)	104.5, d
2′	5.46, dd (9.5, 8.1)	72.8, d
3′	5.30, dd (9.5, 9.5)	74.0, d
4′	5.19, dd (9.5, 9.5)	69.5, d
5′	3.68, m	74.1, d
6′	4.25, m	62.6, t
N−H	8.07, d (6.5)[d]	
CH$_3$COO	2.29, s	21.8, q
CH$_3$COO	2.08, s	21.9, q
CH$_3$COO	2.05, s	22.0, q
CH$_3$COO	2.05, s	22.2, q
CH$_3$*C*OO		170.6, s
CH$_3$*C*OO		170.7, s
CH$_3$*C*OO		171.5, s
CH$_3$*C*OO		172.0, s

[a] Spectra recorded in $CDCl_3$ with TMS as internal standard. [b] Chemical shifts (δ) are reported in parts per million, J values are in Hertz. [c] Number of attached protons determined by DEPT-GL. [d] Exchangeable proton.

Low-resolution MS were obtained using LC/MS (Vestec model 201 mass spectrometer with a Technivent data system with thermospray interface). High-resolution FABMS and EIMS were carried out on a ZAB HS mass spectrometer (VG Analytical Ltd., Manchester, U.K.) equipped with an 11/250 data system. The MS were recorded in multichannel analyzer mode accumulating four to six scans while scanning from 2500 to 1100 at 30 s/degree. Ionization was achieved using a xenon gun operated at 8 keV energy and 0.8-mA emission. The sample in MeOH (1 mg/mL) was added to thioglycerol−glycerol (2:1) as the matrix. This analysis was performed at the MS Laboratory, Department of Chemistry, the University of Kansas, Lawrence, Kansas.

Cultures and Fermentation Screening Procedure. The microbial cultures were originally obtained from The American Type Culture Collection (ATCC), Rockville, Maryland, or from the USDA Northern Regional Research Laboratories (NRRL), Peoria, Illinois, and are maintained in The University of Mississippi, Department of Pharmacognosy Culture Collection. *Fusarium solani f. sp. cucurbitae* CSIH# c-5 and *Mucor ramannianus* 1839 were obtained from Dr. Charles Sih, Department of Pharmaceutical Biochemistry, University of Wisconsin, Madison, Wisconsin. Stock cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

All the preliminary screening and preparative-scale experiments were carried out in a medium (designated medium α) consisting of (per liter of distilled water): glucose, 20 g; NaCl, 5 g; K_2HPO_4, 5 g; yeast extract (BBL, Cockeysville, MD), 5 g; peptone (Difco, Detroit, MI), 5 g. Preliminary screening experiments were carried out in 125-mL stainless steel-capped Delong culture flasks containing 25 mL of medium. The media were sterilized at 121 °C and 78 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol.[13] Sampangine (**2**) was prepared as a 10% solution in DMSO and added to the 24-h-old stage II culture medium of the microorganism at a concentration of 0.16 mg/mL of medium (at this concentration culture growth persisted and no inhibition was observed). Cultures were shaken at room temperature on a model G-10 Gyrotory shaker (New Brunswick Scientific Co., NJ), operating at 250 rpm. Substrate controls were composed of sterile medium to which the substrate (4 mg substrate/100 μL DMSO) was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate addition. After two weeks of incubation, each control was harvested and analyzed by TLC.

Fermentation Sampling and Chromatographic Conditions. The fermentations were sampled by withdrawing 5 mL of culture and extracting it with 5 mL of either EtOAc or H_2O-saturated butanol. The concentrated organic phase was analyzed by TLC for the presence of metabolites.

TLC analyses were performed on precoated Si gel G-25 UV_{254} plates (0.25 mm, Machery−Nagel Duren) using either 9.5 or 19.5% MeOH and 0.5% ammonium hydroxide in $CHCl_3$ as the solvent system. Visualization was accomplished by exposure to short wavelength UV (λ_{max} 254) and/or by spraying with Dragendorff's spray reagent. The adsorbent used for column chromatography was either Si gel 60/230−400 mesh (EM Science) or reversed-phase silica (C-18) (40−63 μm, EM Science). Low- or medium-pressure elution was achieved using either a pump (constant flow rate) or a regulated air/nitrogen flow. HPLC was conducted using Waters 600 Automated Gradient Controller equipped with Waters 486 tunable absorbance detector and Waters 510 HPLC pumps. Columns used were either YMC−Pack SIL (250 × 10 mm i.d., S-5 μm, 120 Å) or Phenomenex, Ultra Carb 5 ODS-30 (250 × 10 mm i.d.). All solvents used for chromatographic purposes were reagent grade except those for HPLC, which were HPLC grade.

Preparative Scale Production of Compound 3 by *Beauvaria bassiana*. *B. bassiana* ATCC 7159 was grown in three 2-L culture flasks each containing 400 mL of medium α. A total of 192 mg of **2** (in 4.8 mL DMSO) was evenly distributed among the 24-h-old stage II culture. After 5 days, complete conversion of **2** into **3** was detected by TLC. The incubation mixtures were combined and filtered to remove the mycelia, and the filtrate (1.2 L) was extracted with EtOAc (1 L × 4). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo at 40 °C to afford a red residue (1.19 g), which was purified by column chromatography over Si gel (110 g, 2.5 × 50 cm) using CH_3CN−$CHCl_3$−NH_4OH (6.9: 3: 0.1) as the eluting solvent system. Fractions of 20 mL each were collected and pooled on the basis of TLC analyses. Fractions 69−87 yielded a semi-pure red metabolite (40 mg), which was further purified by chromatography on reversed-phase silica (C-18; 16 g, 1 × 18 cm) using MeOH−H_2O (4:6−6:4) as eluent. Fractions (5 mL each) were collected and pooled, and fractions 45−57 (one spot; $R_f = 0.19$; MeOH− $CHCl_3$ 1:9) were combined and evaporated to dryness to give 33 mg of **3** as a red coarse powder (9.7% yield), which darkens on heating and begins to melt about 114 °C (dec). LC/MS $[M + H]^+$ 411; HRFABMS m/z calcd for $C_{22}H_{23}N_2O_6$ $[M + H]^+$ 411.1556, found 411.1570; IR (KBr) ν_{max} (cm^{-1}), 3280; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

Preparative Scale Production of Compound 4 by *Rhizopus arrhizus* (ATCC 11145). A total of 640 mg of **2** was dissolved in 16.0 mL of DMSO and distributed equally among 10 2-L culture flasks each containing 400 mL of 24-h-old *R. arrhizus* (ATCC 11145) stage II culture. After 5 days, the entire incubation mixture was combined and filtered to remove the mycelia, and the filtrate (4 L) was extracted with H_2O-saturated butanol (4 L × 4). The combined extracts were evaporated to dryness in vacuo at 40 °C to afford a red residue (11.54 g), which was purified over Si gel (800 g, 8 × 48 cm) using $CHCl_3$−MeOH−NH_4OH (8.7: 1.25: 0.05) as eluent. Fractions of 50 mL each were collected and pooled. Fractions 7−30 (single spot on TLC; $R_f = 0.33$; $CHCl_3$−MeOH−NH_4OH 8:1.95:0.05) were combined and evaporated to dryness to afford pure metabolite **4** as a red amorphous powder (913 mg, 83.5% yield). Metabolite **4** darkens near 138 °C and begins to melt about 160 °C (dec). LC/MS $[M + H]^+$ 397; HRFABMS m/z calcd for $C_{21}H_{21}N_2O_6$ $[M + H]^+$ 397.1400, found 397.1408; IR (KBr) ν_{max} (cm^{-1}), 3300; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

Acetylation of 4. Compound **4** (20 mg) was dissolved in 1 mL Ac_2O, and the reaction was allowed to stand for 24 h at ambient temperature. H_2O (10 mL) was added, and the acetate was extracted with H_2O-saturated butanol. The combined extracts were washed with a saturated solution of $NaHCO_3$ (2 × 30 mL) and H_2O (3 × 30 mL) and evaporated to dryness in vacuo at 40 °C to give a deep orange residue (32.2 mg). TLC analysis ($CHCl_3$−MeOH−NH_4OH, 8: 1.95: 0.05) of the residue indicated complete conversion of **4** to one major product with $R_f = 0.92$. The residue (32.2 mg) was purified by HPLC using C8 reversed-phase silica (Phenomenex 250 × 10 mm i.d., 5 μm) with MeOH−H_2O (8: 2) as eluent. The flow rate was adjusted to 2 mL/min, and fractions were collected and combined based on retention time (t_R) and TLC analysis. Fractions at t_R = 20 min. were combined and evaporated to dryness to afford 5.2 mg of pure **5** as a greenish powder. HRFABMS *m/z* calcd for $C_{29}H_{29}N_2O_{10}$ $[M + H]^+$ 565.1822, found 565.1838; ^1H and ^{13}C NMR, Table 3.

Hydrolysis of 4 using H_2SO_4.[15] A solution of **4** (10 mg) in MeOH (6 mL) was treated with 0.1M H_2SO_4 (1 mL), and the mixture was kept at ambient temperature for 12 h. H_2O (6 mL) was added, and whole mixture was concentrated to 6 mL and extracted with $CHCl_3$ (3 × 10 mL). The combined extracts were washed with 5% $NaHCO_3$ and saturated NaCl solutions (2 × 10 mL), then evaporated to dryness. TLC analysis (EtOAc−$CHCl_3$, 1: 9) revealed the complete hydrolysis of **4** to **2** ($R_f = 0.21$).

The aqueous layer was neutralized with saturated $Ba(OH)_2$, the resulting precipitate was filtered off, and the solution was evaporated to dryness. TLC analysis (iPrOH−EtOAc−H_2O, 7:2:1) of the aqueous residue revealed the presence of single compound ($R_f = 0.40$), the identity of which was confirmed to be glucose by comparison with an authentic sample of D-glucose ($R_f = 0.40$).

The hydrolysis of **4** using 6M HCl (room temperature, 2 days) and usual workup[14] yielded a residue ($CHCl_3$ extract) that by TLC showed **2** ($R_f = 0.21$) and 4-chlorosampangine ($R_f = 0.49$). These compounds were isolated, and their identities were confirmed by comparison of spectral data.[2,11]

Biological Methods. Evaluation of in vitro activity against *C. albicans* NIH B311 and *C. neoformans* ATCC 52657 was accomplished using bioautography and agar well-diffusion assay with **2** as a positive control.[16,17]

The method used to determine the minimum inhibitory concentration (MIC) was the twofold serial broth macrodilution assay.[18] After incubation (37 °C, 48 h for *Cr. neoformans*; 24 h for *C. albicans*), the MIC was taken as the lowest concentration inhibiting growth of organism based on visual assessment. Sampangine was used as positive control.

In vivo efficacy was determined by comparison of lethality in treated vs nontreated infected mice.[19] Nine ICR Swiss female mice were heavily infected with 1×10^6 cfu of *Cr. neoformans* (ATCC 52657). Metabolite **3** (dissolved in 10% DMSO/saline) was administered intraperitoneally at a dose of 50 mg/kg body weight daily, beginning 24 h post-infection and continuing for 12 days. The mice were monitored for morbidity and mortality as compared to a vehicle-treated control group.

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