Microbial Transformation of Benzosampangine

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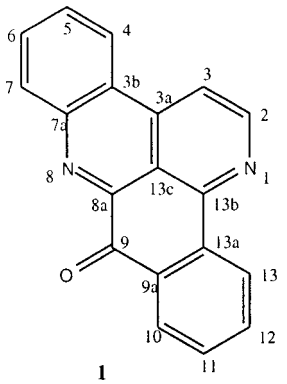
Microbial transformation studies of the synthetic antifungal alkaloid benzosampangine (**1**) have revealed that **1** is metabolized by a number of microorganisms. Using a standard two-stage fermentation technique *Absidia glauca* (ATCC 22752), *Cunninghamella blakesleeana* (ATCC 8688a), *Cunninghamella* species (NRRL 5695), *Fusarium solani* f. sp. *cucurbitae* (CSIH #C-5), and *Rhizopogon* species (ATCC 36060) each produced a β-glucopyranose conjugate of benzosampangine (**2**). The identity of **2** was established on the basis of spectroscopic data.

As part of an extensive program aimed at the discovery and development of prototype antibiotics from higher plants, we previously reported the isolation, structural characterization, and in vitro antifungal activity of the copyrine alkaloid, 3-methoxysampangine, from the root bark of the west African tree, *Cleistopholis patens* (Benth) Engl. and Diels (Annonaceae).[1] The synthesis, ^{13}C NMR assignments, and antimycotic/antimycobacterial activities of several analogues of the sampangines, including benzosampangine (**1**), were previously reported.[2] Benzosampangine (**1**), which has not been reported as a natural product, was shown to possess good in vitro antifungal activity against *Candida albicans* and *Aspergillus fumigatus*, (MIC = 0.39 μg/mL; amphotericin B: 0.78 μg/mL and 0.39 μg/mL, respectively), and *Cryptococcus neoformans* (MIC = 1.56 μg/mL; amphotercin B, 0.39 μg/mL).

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.[3,4] This method produces significant quantities of metabolites that would be difficult to obtain from either animal systems or chemical synthesis. Furthermore, the results from this method often parallel very closely those obtained from human biotransformation and thus can be predictive. The microbial transformation of sampangine resulted in the production of conjugate metabolites.[5]

A total of 54 microorganisms was evaluated for the ability to metabolize benzosampangine (**1**) using the standard two-stage screening/fermentation procedure.[6] Thin layer chromatographic (TLC) analyses of the culture extracts were used to detect organisms capable of metabolizing **1**. Appropriate controls were utilized to ensure that the metabolites were a result of enzymatic activity and not a consequence of degradation or other nonmetabolic changes. Based on TLC analyses and control studies, several microorganisms [*Absidia glauca* (ATCC 22752), *Cunninghamella blakesleeana* (ATCC 8688a), *Cunninghamella* species (NRRL 5695), *Fusasium solani* f. sp. *cucurbitae* (CSIH #C-5), and *Rhizopogon* species (ATCC 36060)] were found to be capable of converting **1** to a more polar compound. *C. blakesleeana* (ATCC 8688a) was chosen for preparative scale fermentation.



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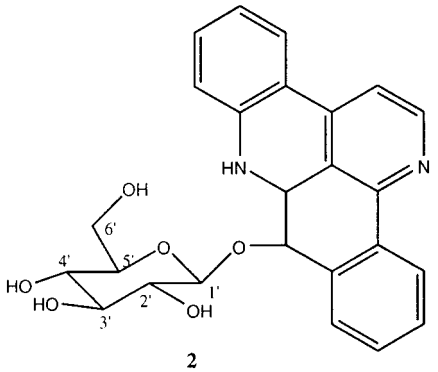

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A preparative scale biotransformation of **1** by *C. blakesleeana* (ATCC 8688a) afforded one major metabolite (**2**) in 2% isolated yield. The thermospray mass spectrum of **2** showed no molecular ion peak, indicating that the conjugate decomposed thermally during analysis. A HRFABMS revealed a molecular formula of $C_{25}H_{22}O_6N_2$. The molecular weight was the sum of benzosampangine (282) and an additional moiety of 164 mass units (consistent with glucose).

The ^{13}C NMR spectrum (75 and 125 MHz) of **2** showed 25 carbon resonances—one triplet, 15 doublets, and nine singlets—none of which was a carbonyl carbon. Five of the resonances were in the aliphatic oxygenated region (δ 60−80). The carbon resonating at δ 106.6 as a doublet was determined from the HMQC spectrum to be the anomeric carbon, because it had a one-bond correlation with the anomeric proton (δ 4.62 d J = 7.9 Hz). A three-bond correlation (HMBC) between the anomeric proton and a singlet carbon at δ 128.9, which shows another three-bond

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correlation to H-10 (δ 8.48 d, $J = 7.8$ Hz), led us to conclude that **2** is an *O*-glycoside in which the glycone part is coupled with benzosampangine at C-9.

The assignments for H-11, -12, and -13 were determined from the COSY spectrum. H-13 has a three-bond correlation to two singlet carbons (δ 130.4 and 145.1). One of these singlet resonances (δ 130.4) was determined to be C-9a based on a three-bond correlation to H-11. Therefore, the other singlet carbon (δ 145.1) is C-13b. C-13b has another three-bond correlation to a proton resonating at δ 8.77 (d, $J = 5.3$ Hz), which was assigned to H-2. The assignment for H-3 was determined from the COSY spectrum. H-3 has a three-bond correlation to two singlet carbons (δ 115.9 and 117.6). The singlet carbon at δ 115.9 was determined to be C-3b based on a three-bond correlation to H-5 (δ 6.98 ddd, $J = 7.8$, 7.8H, 0.5 Hz) and to H-7 (δ 7.06 br d, $J = 7.8$ Hz). The other singlet carbon (δ 117.6) was determined to be C-13c because it had a three-bond correlation to the proton on N-8 (ex D_2O, δ 10.3 s), which, in turn, had a three-bond correlation to C-3b.

The anomeric proton, which resonates at δ 4.62, shows a large coupling constant, $J = 7.9$ Hz, indicating that the glycone binds to the aglycon through a β-glycosidic linkage.[7] Other protons were assigned from the COSY spectrum. The signals for H-3′ and H-4′ were obscured by the DMSO proton impurity signal (δ 3.34) and were determined from the HMQC spectrum.

An additional piece of supportive evidence was drawn from the coupling constants, as $J_{2'-3'}$, $J_{3'-2'}$, $J_{3'-4'}$, $J_{4'-2'}$, $J_{4'-5'}$, and $J_{5'-4'}$ were all larger than 7.5 Hz, supporting the 1,2-*trans*-diaxial relationship between each consecutive pair of protons and ultimately confirming the identity of the hexose as β-glucose. Thus, **2** is benzosampangine-β-glucopyranoside. The spectroscopic data is very similar to that observed for the sampangine conjugate.[5] However, in marked contrast to the results seen with sampangine,[5] yields for this metabolite were low, the color of the media remained mostly unchanged, and methoxylated glucose conjugates were not observed.

Experimental Section

General Experimental Procedures. The ^1H and ^{13}C NMR were obtained on either a Varian VXR-300 FT spectrometer operating at 300 and 75 MHz, respectively, or a General Electric GN-Omega-500 spectrometer operating at 500 MHz for δ_H and 125 MHz for δ_C. For the ^{13}C NMR spectra, the number of attached protons were determined by the attached proton test and the distortionless enhancement through polarization transfer experiment. Two-dimensional NMR data were obtained using the standard pulse sequences of either the VXR-300 for homonuclear correlated spectroscopy (COSY) and nuclear Overhauser enhancement spectroscopy (NOESY) or the General Electric GN-Omega-500 heteronuclear multiple quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond correlation spectroscopy (HMBC).

LRMS were obtained using LC−MS (Vestec Model 201 mass spectrometer with a Technivent data system with thermospray interface). HRFABMS 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. HRESIMS analyses were carried out at Bruker Analytical Systems, Inc., Mass Spectrometry Division, Bellerica, MA.

Chromatographic Conditions. TLC analyses were performed on precoated Si gel G_{25} UV_{254} plates (0.25 mm, Machery-Nagel Duren) and visualized by exposure to short wavelength UV ($\lambda_{max} = 254$) 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 of the elution of solvent 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 YMC-Pack SIL (250 × 10 mm i.d., S-5 μm, 120 Å). All solvents used for chromatographic purposes were reagent grade except those for HPLC, which were HPLC grade.

Microorganisms. The cultures were obtained from The University of Mississippi, Department of Pharmacognosy Culture Collection, and were originally obtained from the American Type Culture Collection (ATCC), Rockville, MD, or from Northern Regional Research Laboratories (NRRL), Peoria, IL. *Mucor ramannianus* 1839 was obtained from Dr. Charles Sih, Department of Pharmaceutical Biochemistry, University of Wisconsin, Madison, WI. Stock cultures of fungi were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

Media. All the preliminary screening and preparative-scale experiments were carried out in medium α, which consists 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.

Fermentation Procedures. 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.[6]

In general, the substrate 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 incubated at room temperature with shaking on a model G-10 gyrotory shaker (New Brunswick Scientific Co., NJ), operating at 250 rpm. Cultures were harvested after 14 days of incubation by filtering the mycelia off and extracting the filtrate three times with an equal volume of water-saturated *n*-butanol. The concentrated organic phase was analyzed by TLC for the presence of metabolites. 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 addition of substrate. After two weeks of incubation, each control was harvested and analyzed by TLC.

Preparative Scale Transformation of Benzosampangine by *C. blakesleeana* (ATCC 8688a). *C. blakesleeana* (ATCC 8688a) was grown in four 2-L culture flasks, each containing 400 mL of medium α. A total of 192 mg of benzosampangine (in 4.8 mL of DMSO) was evenly distributed among 25-h-old stage II culture. After 14 days, there was no starting material (**1**) detected by TLC analyses. The incubation mixtures were combined and filtered to remove the mycelia, and the filtrate (1.6 L) was extracted with H_2O-saturated BuOH (1 L × 4). The combined extracts were evaporated to dryness in vacuo at 40 °C to afford an orange-brown residue (3.91 g).

Isolation and Characterization of 2. A portion of this residue (1.19 g) was purified by MPLC over an octadecyl reversed-phase silica column (55 g, 5 × 12.5 cm), using H_2O−MeOH (10:0−4.5:5.5) mixture as eluent. Fractions of 20 mL were collected and combined based on TLC analyses. Fractions 10−15 yielded a partially pure metabolite (70.8 mg), which was further purified by HPLC over Si gel (YMC-Pack SIL, 250 × 10 mm i.d., S-5 μm, 120 Å) using $CHCl_3$−MeOH (8:2) mixture as eluent, flow rate, 2 mL/min; UV and refractive index detection. Fractions were collected and combined based

on retention time (t_R) and TLC analysis. Fractions at $t_R = 12.8$ min were combined and evaporated to dryness to afford pure **2** (6 mg, 2% yield). TLC analysis using MeOH−$CHCl_3$ (1:9) revealed a single spot with $R_f = 0.23$. ^{1}H NMR (DMSO, 500 MHz) δ 10.3 (1 H, s, NH), 8.91 (1 H, d, $J = 7.8$ Hz, H-13), 8.77 (1 H, d, $J = 5.3$ Hz, H-2), 8.48 (1 H, d, $J = 7.8$ Hz, H-10), 8.07 (1 H, d, $J = 7.8$ Hz, H-4), 7.83 (1 H, d, $J = 5.3$ Hz, H-3), 7.59 (1 H, ddd, $J = 7.8$, 7.8, 1.0 Hz, H-11), 7.39 (1 H, ddd, $J = 7.8$, 7.8, 1.0 Hz, H-12), 7.38 (1 H, ddd, $J = 7.8$, 7.8, 1.0 Hz, H 6), 7.06 (1 H, d, $J = 7.8$ Hz, H-7), 6.98 (1 H, ddd, $J = 7.8$, 7.8, 0.5 Hz, H-5), 6.8 (1 H, br s, OH) 5.3 (1 H, s, OH), 5.1 (1 H, s, OH), 4.62 (1 H, d, $J = 7.9$ Hz, H-1′), 4.50 (1 H, s, OH), 3.64 (1 H, d, $J = 11.6$ Hz, H-6′), 3.58 (1 H, d, $J = 11.6$ Hz, H-6′), 3.57 (1 H, m, H-2′), 3.35 (1 H, m, H-4′), 3.34 (1 H, m, H-3′), 3.18 (1 H, m, H-5′); ^{13}C NMR (DMSO, 125 MHz) δ 149.9 (d, C-2), 145.1 (s, C-13b), 139.6 (s, C-7a), 138.6 (s, C-3a), 131.9 (d, C-6), 130.4 (s, C-9a), 128.9 (s, C-9), 128.3 (d, C-11), 126.8 (s, C-8a), 124.4 (d, C-4), 124.3 (s, C-13a), 123.5 (d, C-13), 122.6 (d, C-12), 121.3 (d, C-10), 120.4 (d, C-5), 117.6 (s, C-13c), 115.9 (s, C-3b), 115.6 (d, C-7), 110.5 (d, C-3), 106.6 (d, C-1′), 77.0 (d, C-5′), 76.4 (d, C-3′), 74.3 (d, C-2′), 69.4 (d, C-4′), 60.8 (t, C-6′); HRFABMS [M + H^+] 447.1553 (calcd for $C_{22}H_{22}N_2O_6$+H^+, 447.1556).

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