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An Endophytic Fungus from *Hypericum perforatum* that Produces Hypericin

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Abstract: For the first time, an endophytic fungus has been isolated from the stems of the medicinal herb *Hypericum perforatum* (St. John's Wort). The fungus produced the naphthodianthrone derivative hypericin (**1**) in rich mycological medium (potato dextrose broth) under shake flask and bench scale fermentation conditions. Emodin (**2**) was also produced simultaneously by the fungus under the same culture conditions. We propose **2** as the main precursor in the microbial metabolic pathway to **1**. The fungus was identified by morphology and authenticated by 28S (LSU) rDNA sequencing. Compounds **1** and **2** were identified by LC-HRMS, LC-MS/MS, and LC-HRMS/MS and confirmed by comparison with authentic standards. In bioassays with a panel of laboratory standard pathogenic control strains, including fungi and bacteria, both fungal **1** and **2** possessed antimicrobial activity comparable to authentic standards. This endophytic fungus has significant scientific and industrial potential to meet the pharmaceutical demands for **1** in a cost-effective, easily accessible, and reproducible way.

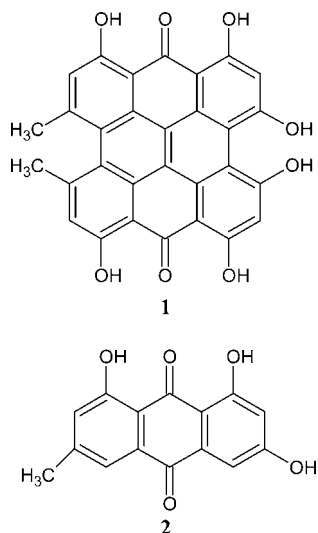
Hypericin (**1**), a naphthodianthrone derivative (2,2'-dimethyl-4,4',5,5',7,7'-hexahydroxy-mesonaphthodianthrone), is a plant-derived substance of high medicinal value. Compound **1** is one of the main constituents of *Hypericum* species and was first isolated from the medicinal herb *Hypericum perforatum* L., commonly called St. John's Wort.^{1,2} The chemical formula of **1** was reported in 1942,³ and eight years later the finalized structure was published.⁴ *H. perforatum* (Clusiaceae) is a pseudogamous, facultatively apomictic, perennial medicinal plant that is native to Europe, West and South Asia, North Africa, North America, and Australia.^{5,6} It has long been in use, at least from the time of ancient Greece,⁷ as an antidepressant due to the unique monoamine oxidase (MAO) inhibiting capacity of **1**, having effects similar to bupropion¹ and

imipramine.⁸ In addition to this, potential uses of **1** extend to improved wound healing, anti-inflammatory effects,⁹ antimicrobial and antioxidant activity,¹⁰ sinusitis relief,¹¹ and seasonal affective disorder (SAD) relief.¹² Compound **1** also has remarkable antiviral activity against a plethora of enveloped viruses including HIV-1, HSV-1, HSV-2, BVDV, BIV, and influenza A either by inhibiting viral infectivity in a hypericin (**1**) preincubation and light-dependent inactivation reaction or by inhibiting viral replication in cell cultures.¹³ Several *in vitro* studies have revealed the multifaceted cytotoxic activity of compound **1** as a result of photodynamic activity.^{13–16}

Little is known about the biosynthesis of **1** other than that it may presumably be formed via emodin (**2**) as the initial precursor and with protohypericin as the penultimate precursor.^{17–19} Synthetically, it is prepared readily by treating emodin dianthrone with ferrous sulfate and pyridine-*N*-oxide in pyridine, followed by light irradiation of the protohypericin formed.¹⁷ However, nothing is known about the sequence of steps in *H. perforatum* or other plants that lead to protohypericin and finally to **1**, though the gene *hyp-1* responsible for this pathway has been studied in cell cultures.¹⁹ Hypericin (**1**) is not abundant and is only available in the plants of *Hypericum* species, which unfortunately demands the uprooting of comparatively rare, perennial plants from forests. As a result, there is a problem in sourcing **1** to meet the projected demands available from the natural sources. Therefore, it is essential to find alternative sources of hypericin (**1**) to meet pharmaceutical demand by establishing an inexhaustible, cost-effective, and renewable resource of this compound using fermentation technology (involving a microbe) that promises reproducible and dependable productivity.

Herein we report, for the first time, the production of hypericin (**1**) by an endophytic fungus, INFU/Hp/KF/34B, isolated from *H. perforatum* L. (Figure S1, Supporting Information), a plant from Harwan, Jammu and Kashmir, India. Emodin (**2**), postulated as the main precursor in the endophytic biochemical pathway to **1**, has also been found to be produced by the same endophyte. This is also the first report of isolation of an endophytic fungus from *Hypericum* species. Molecular analysis of the fungus based on a large subunit (LSU) rRNA gene revealed 99% similarity to another fungal isolate, 9097 (accession number EF420068), that itself is a new unidentified fungus, similarly to other related taxa, for example, *Chaetomium globosum* (98%, accession number AY545729) and unidentified fungal isolate 9038 (98%, accession number EF420066). Further studies of this nature are currently underway, in addition to detailed morphological characterization. The DNA sequence (S2, Supporting Information) obtained has been deposited into EMBL-

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Bank (European Molecular Biology Laboratory) under accession number AM909688. The endophytic fungus has been deposited at the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ), Braunschweig, Germany (accession number DSM 21024). A literature survey shows that antineoplastic Taxol, camptothecin, and podophyllotoxin have been reported as being produced by endophytes from *Taxus brevifolia*,²⁰ *Nothapodytes foetida*,²¹ and *Podophyllum peltatum*,²² respectively. It has not been documented that **1** might be produced by any microorganism associated with a plant species or if **2** is the main precursor in the microbial metabolism of **1**.

Both the fungal biomass and the culture media from grown cultures were assessed for the presence of **1** and **2**. The culture media did not yield any trace of these compounds. The identification of **1** and **2** in the fungal biomass was achieved by comparison with authentic reference standards using LC-HRMS, LC-MS/MS, and LC-HRMS/MS. The quantitative analysis by LC-MS/MS indicated a yield over a range of $35 \pm 2 \mu\text{g}/100 \text{ g}$ (for **1**) and $113 \pm 1 \mu\text{g}/100 \text{ g}$ (for **2**) dry weight of fungal mycelia under shake flask conditions after 6–7 days of incubation of the isolated microorganism (subsequent repetitions). The retention times and the ESI-MS/MS spectra (Figure 1) of fungal **1** and **2** were identical to the data obtained for the authentic standards. The high-resolution measurement confirmed the molecular formulas of the compounds: **1** [$\text{M} - \text{H}$][−] 503.07724 ($\text{C}_{30}\text{H}_{16}\text{O}_8$); **2** [$\text{M} - \text{H}$][−] 269.04555 ($\text{C}_{15}\text{H}_{10}\text{O}_5$), and the characteristic fragments. Antimicrobial assays of fungal **1** and **2** were conducted against a number of laboratory standard pathogenic control strains in comparison with the standards **1** and **2**, resulting in comparable activities.

The discovery of a fungal endophyte producing **1** may have significant scientific and industrial implications. The fungal culture could be scaled up to provide adequate commercial production of **1** to satisfy new drug development and clinical needs. This production reduces the need to harvest wild populations of the source plants, preserving these species from becoming endangered. Biologically, the production of bioactive metabolites by fungal endophytes poses some interesting questions. Whether the origins of the biosynthetic pathways of endophytic metabolites such as **1**, Taxol, podophyllotoxin, or camptothecin originate in the plant or the endophytic microflora are important questions that still remain unanswered. It is not yet clear if horizontal transfer of the genes that support biosynthesis is a common occurrence in either normal or specialized conditions or a genetic serendipity. The actual source organism of **1** (fungus or its host plant) is a fascinating puzzle that remains to be solved, since production of **1** in culture by the endophytic fungus from *H. perforatum* does not ratify the endophyte as the source of the **1** in the aerial host tissues. Moreover, metabolic

regulation of an endophytic fungus in axenic culture is likely to be substantially different from that which occurs in the host plant.²³ It is highly probable that this endophytic fungus follows the same hypothetical polyketide pathway for biosynthesis of **1**, via **2**, that has long been proposed in its host *H. perforatum*,^{4,19,24,25} since the main precursor **2** has also been obtained simultaneously from the endophyte as an intracellular metabolite. The role of the fungus in the production of **1**, the detailed biochemical pathway (presumably through **2**), and regulation of that production inside and outside its host need further investigation. Additionally, along with optimization studies to increase the production of compound **1** by the cultured fungal endophyte, we are attempting to answer some of the intriguing molecular and genetic questions that remain unsolved.

Experimental Section

General Experimental Procedures. Quantitation of the compounds **1** and **2** was performed by using a Thermo Finnigan Surveyor HPLC system (Ringoes, NJ) consisting of Surveyor MS-pump and Surveyor Autosampler-Plus (injection volume $5 \mu\text{L}$) (Thermo Scientific). The compounds were separated on a Luna C₁₈ ($50 \times 2 \text{ mm}$, $3 \mu\text{m}$ particle size) column from Phenomenex (Torrance, CA). The mobile phase consisted of water containing 10 mM ammonium acetate (pH 5.0) (A) and acetonitrile–methanol, 9:1 (B). Samples were separated using a gradient program as follows: (flow rate of $250 \mu\text{L min}^{-1}$) 55% A isocratic for 2 min, linear gradient to 100% B over 6 min (flow rate of $300 \mu\text{L min}^{-1}$). After 100% B isocratic for 7 min, the system was returned to its initial conditions (55% A) within 1 min and was equilibrated for 4 min before the next run was started. MS detection (multiple reaction monitoring mode) was performed by using a TSQ Quantum Ultra AM (Thermo Scientific) equipped with an ESI ion source (Ion Max) operating in negative mode. Nitrogen was employed as both the sheath (50 arbitrary units) and auxiliary (8 arbitrary units) gas, and argon served as the collision gas with a pressure of 1.5 mTorr. The capillary temperature was set to 250°C .

Compounds **1** and **2** were identified by HRMS fragment spectra (LTQ-Orbitrap Spectrometer, Thermo Scientific), which were consistent with authentic standards (**1** from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, and **2** from AppliChem GmbH, Darmstadt, Germany). The spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of pump, flow manager, and autosampler (injection volume $1 \mu\text{L}$). Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. The separations were performed by using a Phenomenex Gemini C₁₈ column ($3 \mu\text{m}$, $0.3 \times 150 \text{ mm}$) (Torrance, CA) with a H_2O (+0.1% HCOOH , +1 mM NH_4Ac) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate $4 \mu\text{L min}^{-1}$). Samples were analyzed by using a gradient program as follows: 30% A isocratic for 1 min, linear gradient to 100% B over 10 min; after 100% B isocratic for 60 min, the system returned to its initial condition (30% A) within 1 min and was equilibrated for 9 min. The spectrometer was operated in negative mode (1 spectrum s^{-1} ; mass range 50–1000) with nominal mass resolving power of 60 000 at m/z 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass ($m/z = 386.7149314$; CsI_2^-).

Isolation and Culture of Endophytic Fungi. As part of an effort to identify endophytic fungi that produce **1**, wild specimens of *Hypericum perforatum* L. were collected at the bloom stage from natural populations at Harwan ($34^\circ 07' \text{ N}$, $74^\circ 52' \text{ E}$, 10 km from Srinagar), Jammu and Kashmir, India, at an altitude of 1587 m. A specimen from the population has been deposited in the herbarium of Indian Institute of Integrative Medicine (IIIM), Jammu Tawi, India (formerly Regional Research Laboratory, RRL), under accession number 112/IIIM-S. The plants were removed from the soil and transported to the Institute of Environmental Research (INFU), University of Dortmund, Germany, for processing within 24 h of collection. The plants were washed thoroughly in running tap water followed by deionized (DI) water to remove any soil and dirt adhering to the plant parts. The stems were cut for the isolation of endophytic fungi. Surface sterilization of the stems was carried out following the methods of Lodge et al. (1996),²⁶ Strobel et al. (2004),²³ and Puri et al. (2005),²¹ suitably modified. Briefly, the stems were thoroughly washed in running tap water, and small fragments of ca. 10 mm (length) by 5 mm (breadth) were cut

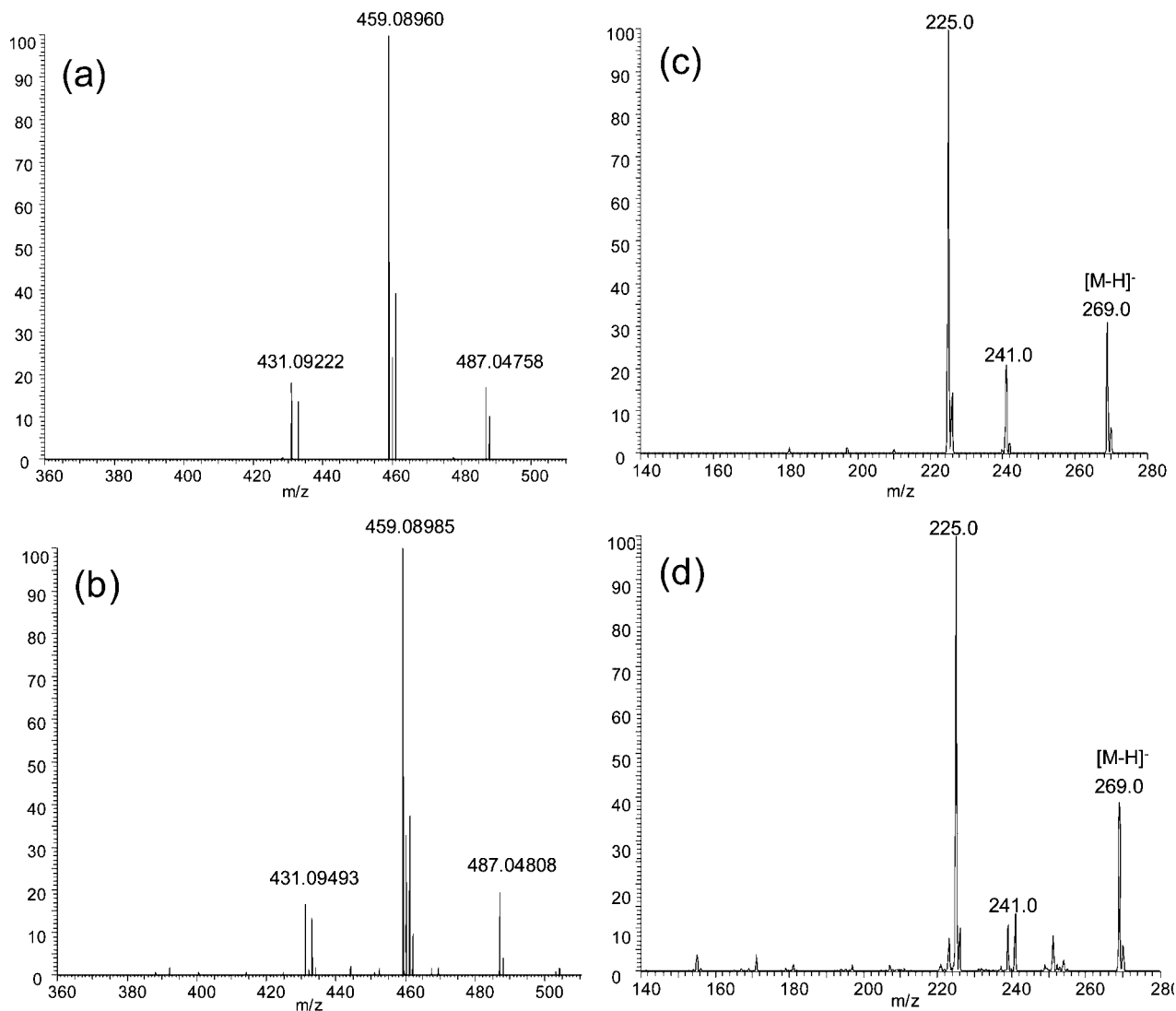


Figure 1. High-resolution MS/MS product ions of (a) standard hypericin (**1**) and (b) fungal hypericin (**1**) as well as nominal mass MS/MS product ions of (c) standard emodin (**2**) and (d) fungal emodin (**2**).

using a flame-sterilized razor blade followed by surface sterilization by sequential immersion in 70% ethanol for 1 min, 1.3 M sodium hypochlorite for 3 min, and then 70% ethanol for 30 s. Finally, these surface-sterilized stem pieces were rinsed three times in sterile double-distilled water for 1 min each, to remove excess surface sterilants, and blotted in a sterile filter paper. The surface-sterilized stem fragments, thus obtained, were evenly spaced in sterile Petri dishes (TPP, Trasadingen, Switzerland) containing water agar (WA) medium (DIFCO, cat. no. 214530) amended with streptomycin 100 mg/L to eliminate any bacterial growth. Petri dishes were sealed using Parafilm (Pechiney, Chicago, IL) and incubated at 28 ± 2 °C in an incubator until fungal growth started. To ensure proper surface sterilization, unsterilized stem segments were prepared simultaneously and incubated under the same conditions in parallel, to isolate the surface-contaminating fungi. The hyphal tips, which grew out from sample segments over 4–6 weeks, were isolated and subcultured onto a rich mycological medium, potato dextrose agar (PDA) (DIFCO, cat. no. 213400), and brought into pure culture. The axenic culture, thus obtained, was coded as INFU/Hp/KF/34B and preserved by lyophilization, as well as by cryopreservation at -70 °C in the microbial library of our institute. The endophytic fungus growing on PDA was examined after 2, 3, 4, and 5 days, exhibiting unique morphology on the PDA medium (S3, Supporting Information).

Isolation and Analysis of Total Genomic DNA. Total DNA was isolated from the mycelial mass using the Macherey Nagel food DNA extraction kit strictly following manufacturer's guidelines²⁷ and subjected to PCR amplification (S4, Supporting Information). PCR product was sequenced using electrophoretic sequencing on an ABI

3730xl DNA analyzer (Applied Biosystems, Foster City, CA) using BigDye Terminator v 3.1 cycle sequencing kit (S4, Supporting Information). The sequences were matched against the nucleotide-nucleotide database (BLASTn) of the U.S. National Center for Biological Information (NCBI) for final identification of the endophytic isolate.

Preparation of Cell-Free Extract. The fungus was cultured under specific conditions wherein it exhibited some special morphological features (S5, Supporting Information). The cell-free extract was prepared by filtering the incubated culture through muslin cloth under vacuum. The mycelia and broth were treated separately. The mycelial pellet was dried in an oven to obtain the dry weight and was resuspended in deionized water (DI) and then sonicated in an ultrasonicator (Branson B-12, Danbury, Connecticut) under chilled conditions. The milky fluid was extracted three times with ethyl acetate (50 mL), followed by extraction (three times) with 50 mL of CHCl_3 –MeOH (4:1). The organic solvent was removed after each extraction by rotary evaporation in vacuum at 30 °C. The final dry organic extract was resuspended in 1 mL of HPLC grade methanol. The broth (100 mL) was extracted in a similar way.

Antimicrobial Assay. A disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI), 2006,²⁸ formerly known as National Committee for Clinical Laboratory Standards (NCCLS), was employed for the determination of the *in vitro* antimicrobial activity of the crude fungal extracts. A panel of laboratory standard pathogenic control strains belonging to the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, was used. Gram-positive bacterium *Staphylococcus aureus* ssp.

aureus (DSM 799), Gram-negative bacteria *Klebsiella pneumoniae* ssp. *ozaenae* (DSM 681), *Pseudomonas aeruginosa* (DSM 1128), *Salmonella enterica* ssp. *enterica* (DSM 9898), and *Escherichia coli* (DSM 682), and fungal organisms *Aspergillus niger* (DSM 1988) and *Candida albicans* (DSM 1386) were considerably susceptible to compounds **1** and **2**.

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Supporting Information Available: *Hypericum perforatum* L. plant and its endophytic fungus INFU/Hp/KF/34B growing out from cut areas of surface-sterilized stem segments on a streptomycin-supplemented WA plate and on rich mycological medium, morphological descriptions of the isolated endophytic fungus on solid rich media (PDA), in culture (PDB) including culture conditions, isolation of total genomic DNA, PCR amplification of LSU (28S) rDNA and sequencing, and the LSU (28S) rDNA sequence. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- (1) Nahrstedt, A.; Butterweck, V. *Pharmacopsychiatry* **1997**, *30*, 129–134.
- (2) Brockmann, H.; Haschad, M. N.; Maier, K.; Pohl, F. *Naturwissenschaften* **1939**, *32*, 550–550.
- (3) Brockmann, H.; Pohl, F.; Maier, K.; Haschad, M. N. *Ann. Chem.* **1942**, *553*, 1–52.
- (4) Brockmann, H.; Falkenhausen, E. H.; Dorlaes, A. *Naturwissenschaften* **1950**, *37*, 540–540.
- (5) Hickey, M.; King, C. *100 Families of Flowering Plants*, 2nd ed.; Cambridge University Press: Cambridge, 1981.
- (6) Wichtl, M. *Z. Phytother.* **1986**, *3*, 87–90.
- (7) Tammamo, F.; Xepapadakis, G. *J. Ethnopharmacol.* **1986**, *16*, 167–174.
- (8) Raffa, R. B. *Life Sci.* **1998**, *62*, 265–270.
- (9) Zaichikova, S. G.; Grinkevich, N. I.; Barabanov, E. I. *Farmatsiya* **1985**, *34*, 62–64.
- (10) Radulovic, N.; Stankov-Jovanovic, V.; Stojanovic, G.; Smelcerovic, A.; Spiteller, M.; Asakawa, Y. *Food Chem.* **2007**, *130*, 15–21.
- (11) Razinkov, S. P.; Yerofeyeva, L. N.; Khovrina, M. P.; Lazarev, A. I. *Zh. Ushn. Nos. Gorl. Bolezn.* **1989**, *49*, 43–46.
- (12) Martinez, B.; Kasper, S.; Ruhmann, S.; Moller, H. J. *Nervenheilkunde* **1993**, *36*, 103–108.
- (13) Kubin, A.; Wierrani, F.; Burner, U.; Alth, G.; Grunberger, W. *Curr. Pharm. Des.* **2005**, *11*, 233–253.
- (14) Kamuhabwa, A. R.; Agostinis, P. M.; D'Hallewin, M. A.; Baert, L.; de Witte, P. A. *Photochem. Photobiol.* **2001**, *74*, 126–132.
- (15) Delaey, E. M.; Obermueller, R.; Zupko, I.; De Vos, D.; Falk, H.; de Witte, P. A. *Photochem. Photobiol.* **2001**, *74*, 164–171.
- (16) Hadjur, C.; Richard, M. J.; Parat, M. O.; Jardon, P.; Favier, A. *Photochem. Photobiol.* **1996**, *64*, 375–381.
- (17) Mazur, Y. U. S. Patent 5,120,412, 1992.
- (18) Falk, H.; Meyer, J.; Oberreiter, M. *Monatsh. Chem.* **1993**, *124*, 339–341.
- (19) Bais, H. P.; Vepachedu, R.; Lawrence, C. B.; Stermitz, F. R.; Vivance, J. M. *J. Biol. Chem.* **2003**, *278*, 32413–32422.
- (20) Stierle, A.; Strobel, G.; Stierle, D. *Science* **1993**, *260*, 214–216.
- (21) Puri, S. C.; Verma, V.; Amna, T.; Qazi, G. N.; Spiteller, M. *J. Nat. Prod.* **2005**, *68*, 1717–1719.
- (22) Eyberger, A. L.; Dondapati, R.; Porter, J. R. *J. Nat. Prod.* **2006**, *69*, 1121–1124.
- (23) Strobel, G.; Daisy, B.; Castillo, U.; Harper, J. *J. Nat. Prod.* **2004**, *67*, 257–268.
- (24) Birch, A. J. *Science* **1967**, *156*, 202–206.
- (25) Thomson, R. H. *Naturally Occurring Quinones*; Butterworths Scientific Publications: London, UK, 1957.
- (26) Lodge, D. J.; Fisher, P. J.; Sutton, B. C. *Mycologia* **1996**, *88*, 733–738.
- (27) Anonymous. *Genomic DNA from Food, Nucleospin 8, Nucleospin 96* (Rev.02); Macherey-Nagel: Duren, Germany, 2007.
- (28) Wikler, M. A. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-Ninth Edition (M2-A9)*; CLSI (Clinical and Laboratory Standards Institute): Wayne, PA, 2006.

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