

# Polyhydroxyanthraquinones as Quorum Sensing Inhibitors from the Guttates of *Penicillium restrictum* and Their Analysis by Desorption Electrospray Ionization Mass Spectrometry

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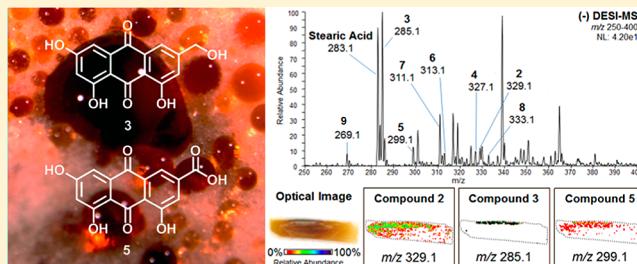
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## S Supporting Information

**ABSTRACT:** The endophytic fungus *Penicillium restrictum* was isolated from the stems of a milk thistle (*Silybum marianum*) plant. In culture, the fungus produced distinct red guttates, which have been virtually uninvestigated, particularly from the standpoint of chemistry. Hence, this study examined the chemical mycology of *P. restrictum* and, in doing so, uncovered a series of both known and new polyhydroxyanthraquinones (1–9). These compounds were quorum sensing inhibitors in a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA), with IC<sub>50</sub> values ranging from 8 to 120 μM, suggesting antivirulence potential for the compounds. Moreover, the spatial and temporal distribution of the polyhydroxyanthraquinones was examined *in situ* via desorption electrospray ionization–mass spectrometry (DESI-MS) imaging, demonstrating the first application of this technique to a guttate-forming fungus and revealing both the concentration of secondary metabolites at the ventral surface of the fungus and their variance in colonies of differing ages.



Infections from drug-resistant strains of the bacterium *Staphylococcus aureus* have reached pandemic proportions.<sup>1</sup> In the U.S. alone, methicillin-resistant *S. aureus* (MRSA) causes more fatalities on an annual basis than all other bacterial pathogens or even HIV/AIDS.<sup>1</sup> Perhaps even more alarming, new MRSA strains have spread beyond hospital settings and into surrounding communities,<sup>2</sup> infecting otherwise healthy individuals.

A potential new strategy to combat infections is the development of “antivirulence” therapies, which target nonessential pathways in bacteria related to pathogenesis. By disarming the bacterial pathogen, it has been proposed that infections could be managed without creating environmental pressure to develop resistance.<sup>3</sup> The potential effectiveness of antivirulence strategies as a way to fight bacterial infections, including MRSA, has been a topic of growing interest in the microbiology community.<sup>4,5</sup> However, there are currently a lack of small-molecule drug leads for such treatments, and to date, no antivirulence drugs have been approved for clinical use.

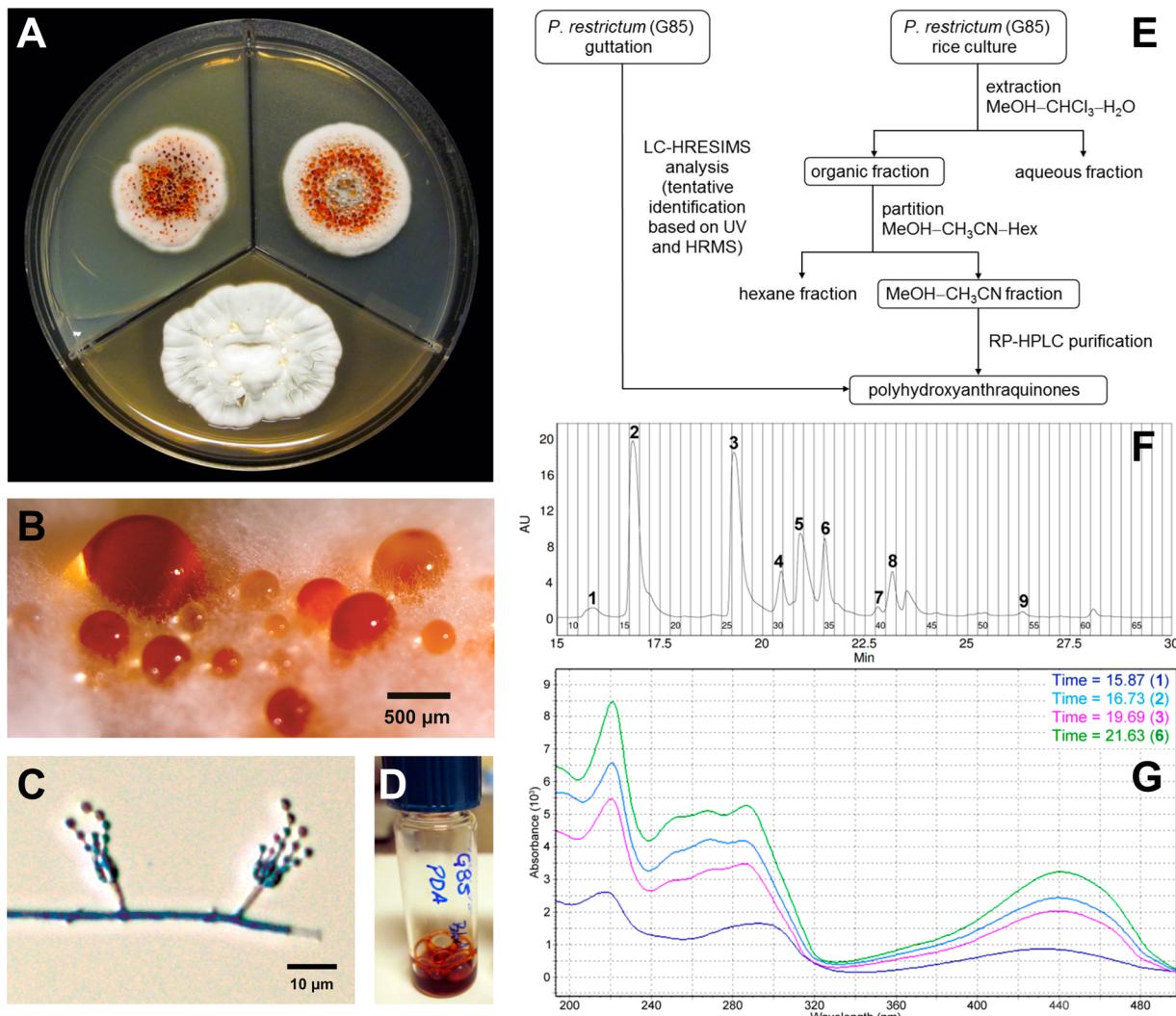
In ongoing studies to discover compounds from nature that target virulence in MRSA,<sup>6</sup> an endophytic fungus (coded “G85”) was isolated from the stems of milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)] and determined to be

*Penicillium restrictum*. This fungus produced distinct red guttates (also known as exudates),<sup>7</sup> which upon characterization yielded a series of nine polyhydroxyanthraquinones, including five that were new to the literature. The compounds were evaluated for their ability to modulate MRSA pathogenesis regulation. Interestingly, several of them inhibited peptide quorum sensing function, a regulatory cascade essential for the production of virulence factors that cause acute complications, such as skin and soft tissue infections and pneumonia.<sup>8</sup> The terminology “quorum sensing inhibitors” has been utilized for such compounds, consistent with recent literature.<sup>9</sup> Thus, from a biomedical perspective, this research reports on new molecules with potential therapeutic relevance for managing infections.

From a basic science perspective, the observation that these compounds could be isolated from fungal guttates was intriguing. Although the phenomenon of fungal guttations was first documented over a century ago,<sup>10,11</sup> their ecological role remains poorly understood.<sup>7,12</sup> It has been suggested that they may serve a variety of functions, such as a reservoir for

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**Figure 1.** (A) Ten-day-old *P. restrictum* colonies grown on different nutrient media: top left panel PDA, top right panel MEA, and bottom panel YESD. (B) Close-up of exudate droplets on PDA. (C) Cotton blue stain of conidiophore and conidia of monoverticillate *P. restrictum*. (D) Exudate (50  $\mu$ L) collected from the surface of the fungal colony via micropipet. (E) Flowchart for the isolation of polyhydroxyanthraquinones from the solid-state culture extract (right) and guttates (left). (F) Preparative HPLC chromatogram ( $\lambda = 254$  nm) of the MeOH-CH<sub>3</sub>CN fraction used to purify compounds 1–9. (G) UV profiles (190 to 500 nm) for compounds 1–3 and 6, as examples.

water during periods of active growth,<sup>13</sup> as an excretion system for waste products, metabolic byproducts, inorganic substances, secondary metabolites, and/or enzymes,<sup>7</sup> and as a means to degrade plant tissues<sup>14</sup> and/or to condition the composition of the surrounding soil for bacterial community associations.<sup>7</sup> Previously, a few investigators have reported the presence of secondary metabolites in fungal guttates, including pyrrolopyrazine, lolaine, and ergot alkaloids from grass-associated endophytes belonging to *Neotyphodium* spp.,<sup>15</sup> ochratoxins A and B from *P. nordicum* and *P. verrucosum*,<sup>16</sup> destruxins A, B, and E from cultures of *Metarrhizium anisopliae*,<sup>7</sup> and azaphilones and meroterpenes from *P. citreonigrum*.<sup>17</sup> Herein, desorption electrospray ionization mass spectrometry (DESI-MS)<sup>18,19</sup> was employed to investigate the spatial and temporal distribution of bioactive secondary metabolites for the first time in a guttate-forming fungus.

DESI-MS enables the sampling, analysis, and imaging of molecules from biological surfaces under ambient conditions.<sup>20</sup> To conduct DESI-MS, a spray of charged droplets is focused onto the surface of a sample. Compounds are solvated in a thin

film, desorbed by subsequent droplet impact, and ions are formed upon droplet drying and Coulombic fission. These ions then enter the vacuum region of the MS, where *m/z* values are measured.<sup>21,22</sup> Ambient ionization by DESI permits the 2D imaging of a biological surface, so as to ascertain the location, relative quantification, and maturation of key compounds.<sup>23,24</sup> DESI-MS has been applied to several fields, including embryology,<sup>25</sup> forensics,<sup>26</sup> and cancer diagnostics.<sup>27</sup> With respect to natural products, Kubanek and co-workers employed this technique in the characterization of antifungal compounds from a marine alga.<sup>28,29</sup> Alternative ambient ionization methods have been applied in the study of natural products, namely, imaging of bacteria from culture by nano-DESI.<sup>30,31</sup> The current studies report DESI-MS imaging of secondary metabolites on the surface of an intact endophytic fungus growing in axenic culture.

In short, this study pursued the chemical mycology of an endophytic fungus, *P. restrictum*, from both biomedical and basic science perspectives. In doing so, a series of small-molecule quorum sensing inhibitors were identified, their

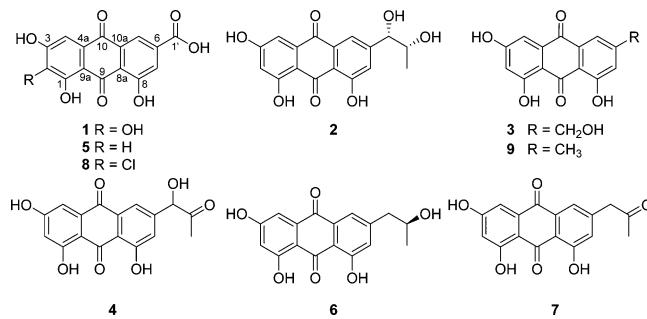
mechanism of antivirulence activity was investigated, and their production and native distribution were examined.

## RESULTS AND DISCUSSION

**Polyhydroxyanthraquinones Are the Major Constituents of the Guttates of Endophytic *Penicillium restrictum*.** A fungal endophyte (coded “G85”) was isolated from the stems of a healthy milk thistle plant (*Silybum marianum*). Using morphological characteristics and molecular studies (based on ITS1-5.8S-ITS2 and RPB2 sequence data; Figures S2 and S3, Supporting Information), G85 was identified as *Penicillium restrictum* (Eurotiales, Ascomycota). When grown on either potato dextrose agar (PDA) or malt extract agar (MEA) medium, this isolate produced striking red guttates on 10-day-old cultures that resembled droplets of blood (Figures 1 and S1). Interestingly, when grown on 2% soy peptone, 2% dextrose, and 1% yeast extract (YESD), a few guttates were noted, but they lacked the deep red coloring seen on the other two media. Since YESD was the most nutrient rich of the three media, we hypothesized that the biosynthesis of compounds responsible for the red coloring was stimulated by nutrient stress.<sup>32</sup> Regardless, the red guttates from G85 grown on PDA were sampled using a micropipet and analyzed directly by high-resolution LC-MS, revealing the presence of several polyhydroxyanthraquinones. Due to the paucity of material obtained from the guttates (Figure 1D), scale-up studies were conducted to provide reference materials for biological testing and to structurally elucidate the polyhydroxyanthraquinones.

The chemical profiles of the guttates and the extract of the fungus grown in solid-state culture were nearly identical (Figure S4). The MeOH–CHCl<sub>3</sub> extract of the solid-state cultures of *P. restrictum* was purified using well-described natural product protocols<sup>33–39</sup> (Figure 1 and Supporting Information). This led to the isolation of a series of polyhydroxyanthraquinones, including the known compounds  $\omega$ -hydroxyemodin (3),<sup>40–43</sup> emodic acid (5),<sup>40–42</sup> (+)-2'S-isorhodoptilometrin (6),<sup>43–45</sup> and emodin (9)<sup>43,46</sup> and five new compounds (1, 2, 4, 7, and 8); their numbering refers to elution order via preparative HPLC (Figure 1F). Full isolation and characterization details are delineated in the Supporting Information (Table S1 and Figures S4–S7), and although 6 was known, this represented the first characterization of its absolute configuration (via Mosher's esters),<sup>47</sup> resulting in a configuration opposite of literature reports.<sup>45</sup> The compounds displayed UV/vis spectra (Figure 1G) characteristic of polyhydroxyanthraquinones,<sup>41,48–50</sup> and the most notable difference in the structures was the nature of the side chain at the 6 position. A number of polyhydroxyanthraquinones, including compounds 3, 5, and 9, have been reported as major pigments of *Penicillium*<sup>40,51</sup> and other fungal species,<sup>43,45,52–54</sup> mushrooms,<sup>55,56</sup> lichens,<sup>42,57,58</sup> marine animals,<sup>59</sup> and plants.<sup>60–67</sup> However, they have never been described in guttates.

**Polyhydroxyanthraquinones Are Quorum Sensing Inhibitors.** The ability of MRSA to cause an infection requires a functional accessory gene regulator (*agr*) quorum-sensing system.<sup>8</sup> This regulatory system is responsible for the production of toxins and exoenzymes that play a major role in the pathogenesis of acute infections. To explore potential antivirulence activity, the ability of the polyhydroxyanthraquinones (1–9) to suppress (or quench) the *agr* quorum-sensing system was evaluated. For these experiments reporter strain AH2759, which was derived from community-associated MRSA (CA-MRSA) strain LAC of the USA300 pulse-field gel type,<sup>68</sup>



was utilized. This strain is clinically relevant due to the emergence of USA300 in community and hospital settings, their aggressive nature, and their ability to cause skin and soft tissue infections in otherwise healthy subjects.<sup>1</sup> Strain AH2759 contains a plasmid with the *agr* P3 promoter driving transcription of a modified *luxABCDE* operon from *Photobacterium luminescens*,<sup>69</sup> thereby coupling quorum-sensing function with bioluminescence expression. Compounds 1–9 were tested as quorum sensing inhibitors against AH2759 at sub-growth-inhibitory concentrations; a representative dose-response curve is shown in Figure S8.

The polyhydroxyanthraquinones (1–9) suppressed quorum sensing with IC<sub>50</sub> values in the 8–120 μM range (Table 1). A

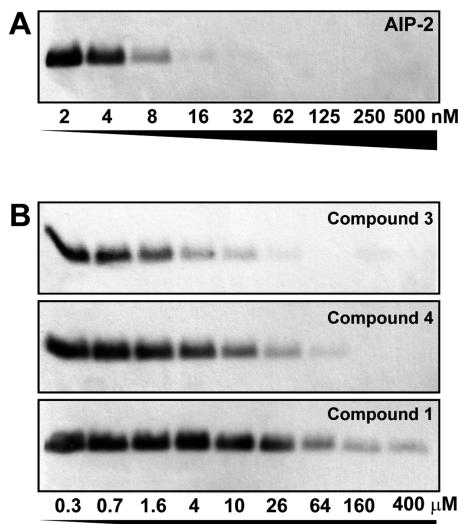
**Table 1. Activity of Compounds 1–9 as Quorum Sensing Inhibitors**

compound	<i>agr</i> P3 <sub>lux</sub> IC <sub>50</sub> (μM) <sup>a</sup>
AIP-2 <sup>b</sup>	0.012 ± 0.004
3	8.1 ± 1.4
6	8.9 ± 1.6
9	17.1 ± 3.1
7	19.8 ± 6.8
2	26.5 ± 7.5
4	30.1 ± 5.4
5	30.6 ± 4.9
8	37.8 ± 8.8
1	120 ± 18

<sup>a</sup>Standard error for IC<sub>50</sub>'s were from a four-parameter logistic fit.  
<sup>b</sup>Positive control.

preliminary structure–activity relationship suggested three categories. The most potent activity was observed for compounds 3 and 6, which had side chains at the 6 position containing either a primary alcohol (3) or a secondary propanol (6) moiety. The least potent activity was observed for compound 1, which had a carboxylic acid side chain at the 6 position and was the only compound with a phenolic OH at the 2 position. The remaining compounds were essentially equipotent within the error of the experiment, with IC<sub>50</sub> values ranging from 17 to 37 μM; the side chain at the 6 position varied in all of them, and 8 was the only other compound with a substituent at the 2 position, a chlorine.

As a parallel test of antivirulence activity and to corroborate the results in Table 1, compounds were evaluated for suppression of the production of delta toxin, a hemolytic peptide encoded in the *agr* RNAIII transcript of *S. aureus*,<sup>8</sup> by the same MRSA strain (AH2759); compounds 3, 4, and 1 were chosen as representative quorum sensing inhibitors with high, medium, and low activity, respectively. An immunoblot (Figure 2) indicated dose-dependent suppression in toxin production by all three compounds, consistent with the IC<sub>50</sub> values (Table



**Figure 2.** Inhibition of delta toxin production by polyhydroxyanthraquinones. Reporter strain AH2759 was grown in TSB with a dose response of control AIP-2 (2–500 nM; panel A) and compounds 1, 3, and 4 (0.3–400  $\mu$ M; panel B). Spent media was collected, and immunoblots were performed for delta toxin.

1). The positive control, AIP-2, a peptide known to target the *agr* system, also demonstrated dose-dependent suppression of delta toxin production. This peptide was more potent than the most active polyhydroxyanthraquinones (3 and 6). However, AIP-2 is a labile thiolactone, which imparts several challenges with respect to drug development. Moreover, only a limited number of small molecules with activity as quorum sensing inhibitors in *S. aureus* have been reported,<sup>5,9</sup> most of which are AIP peptide analogues. Those few that are small molecules have IC<sub>50</sub> values similar to 3 and 6.<sup>5,9</sup> Thus, these polyhydroxyanthraquinones may provide new leads for MRSA antivirulence drug development.

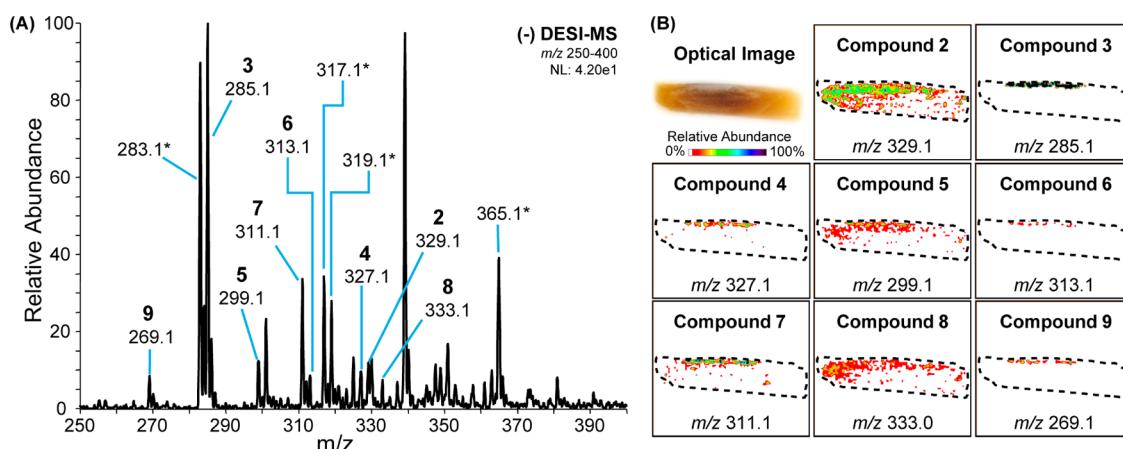
**DESI-MS Imaging of *P. restrictum* Reveals the Spatiotemporal Relationships of Polyhydroxyanthraquinones.** The above studies demonstrated biological activity of polyhydroxyanthraquinones isolated from fungal guttates and

from whole fungal extracts. However, the analysis of such extracts inherently destroys biologically relevant data in regard to the location and timing of secondary metabolite production. From the extract data, it was not possible to confirm that 1–9 were concentrated in fungal guttates, to explore spatial relationships, or to assess temporal expression *in situ*. DESI-MS imaging was implemented to capture such data *in situ*.

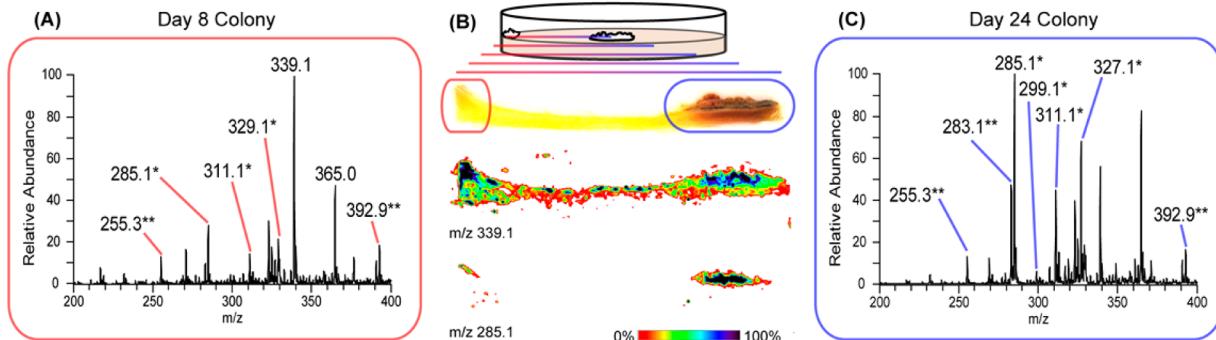
**MS Imaging of Imprinted Guttates.** The distribution and relative concentrations of compounds 1–9 within guttates were explored by imprinting fungal plates, transferring chemical and spatial information onto a suitable surface for DESI-MS imaging. Imprinting fungi prior to imaging was necessary, as both the fungal surface and guttates were easily disrupted by the pneumatic pressure of DESI. Optical and DESI-MS ion images of fungal imprints indicated successful transfer of chemical and spatial information (Figures S10 and S11). A small compromise in spatial resolution during the imprinting process was justified to preserve the chemical composition of the guttates. The detected polyhydroxyanthraquinones were most abundant within regions corresponding to the locations of guttate transfer.

**MS Imaging of Cryosectioned Culture.** The spatial distribution of the polyhydroxyanthraquinones across the fungal surface was performed by sectioning a fungal colony perpendicular to the surface (Figure S9), yielding sections comprising the depth of the culture medium (~3 mm). The negative ion mass spectra were highly selective for 1–9, due to the phenolic moieties. Further, the ionization efficiencies of 1–9 were likely similar, and thus, relative MS abundance reflected concentration. A representative DESI-MS spectrum, corresponding to fungal mycelium (Figure 3A), shows compounds 2–9 in the *m/z* range 250–400. DESI imaging revealed a number of additional ions, presumably of fungal origin, as they were not observed to an appreciable extent in the mass spectrometric analysis of guttates and were absent in nonfungal regions of DESI-MS ion images (Figure S12).

The spatial distribution of polyhydroxyanthraquinones (Figure 3B) indicated localization of compounds 3, 4, 6, 7, and 9 at the fungal surface. Additional endogenous compounds (e.g., *m/z* 283.1, stearic acid; Figure S12) also appeared localized at the surface. Co-localization of these compounds



**Figure 3.** (A) Representative negative mode DESI mass spectrum (*m/z* 250–400). All polyhydroxyanthraquinones isolated by extraction were detected, except for compound 1, *m/z* 315. Single asterisks (\*) denote ions that display co-localization with *P. restrictum*. The ion with *m/z* 283.1 was tentatively identified as stearic acid. (B) Ion images for detected compounds are displayed in false color, reflecting differences in relative mass spectral abundance. MS was acquired once per pixel for a total analysis time of 0.61 s/pixel. An optical image of an adjacent section is also shown, and a colony along the upper-middle edge was confirmed by staining. Optical image size is 13 × 3 mm (w × h).



**Figure 4.** (A) Normalized negative mode DESI-MS of day 8 colony; polyhydroxyanthraquinones are noted by a single asterisk (\*), and endogenous compounds are tentatively identified with double asterisks (\*\*). (B) Optical image (optical image size is  $35 \times 4$  mm (w  $\times$  h)) of *P. restrictum* with illustration of day 8 colony (left) and day 24 colony (right) location on the culture plate. Ion images corresponding to selected ions,  $m/z$  339.1 and 285.1, are shown. MS was acquired once per pixel for a total analysis time of 0.65 s/pixel. 100% abundance in the ion images shown is 23.5 counts. (C) Normalized negative mode DESI-MS of day 24 colony.

with fungal mycelia (Figure S13) provided evidence of fungal origin. Furthermore, the polyhydroxyanthraquinones that were found to be concentrated on the fungal surface were the most active in the quorum sensing assays, with compounds 3 and 6 possessing  $IC_{50}$  values of  $<10 \mu\text{M}$  (Table 1). Compounds 2, 5, and 8 were distributed relatively uniformly throughout the section.

The temporal distribution of polyhydroxyanthraquinones was observed to differ substantially between 8- and 24-day-old colonies. The optical and DESI ion images, comprising the radius of the culture, are displayed in Figure 4b with a 24-day-old colony on the right (i.e., center of plate) and an 8-day-old colony on the left (i.e., circumference of plate). An unknown ion detected at  $m/z$  339.1, attributed to fungal growth in the culture, was distributed homogeneously between the day 8 and day 24 colonies, whereas compound 3 ( $m/z$  285.1) was detected in greater relative abundance in the established colony, as indicated by the black coloration in the ion images (Figure 4b). This finding was supported by normalized mass spectra obtained from day 8 and day 24 regions (Figure 4a and c). The mass spectra indicated differences in the polyhydroxyanthraquinones being produced and their relative concentration. For example compound 3 was detected in both day 8 and day 24 colonies, and its concentration increased with colony age by ~4-fold.

Collectively, the DESI-MS imaging suggested that the polyhydroxyanthraquinones were produced by fungal mycelia and were expressed differentially over time. Interestingly, the polyhydroxyanthraquinones that were more potent quorum sensing inhibitors were concentrated at the fungal surface, while less active compounds were diffused through the culture medium. These findings may have biological relevance, as production or concentration of bioactive secondary metabolites at the fungal surface could facilitate interactions with the surrounding environment.<sup>70</sup> However, these data are only correlative, and it is also possible that the different distributions are a result of varying diffusivities of the compounds.

Overall, the results demonstrate that fungal endophytes, and guttate-forming fungi in particular, are a potentially useful source of biologically active compounds. The small-molecule quorum sensing inhibitors identified from *P. restrictum* could serve as lead compounds for the development of new treatments for MRSA infections. Importantly, this study also illustrates the power of DESI-MS as a means to obtain spatial

and temporal information about the production of fungal secondary metabolites.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** UV, IR, and CD spectra were obtained on a Varian Cary 100 Bio UV-vis spectrophotometer (Varian Inc.), a PerkinElmer Spectrum One with Universal ATR attachment (PerkinElmer), and an Olym DSM 17 CD spectrophotometer (Olym, Inc.), respectively. NMR experiments were conducted in methanol- $d_4$  or DMSO- $d_6$  using a JEOL ECA-500 (operating at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ ; JEOL Ltd.). HRMS data were measured using an electrospray ionization (ESI) source coupled to an LTQ Orbitrap XL system (Thermo) in both positive and negative ionization modes and by a liquid chromatographic/autosampler system that consisted of an Acuity UPLC system (Waters Corp.). HPLC was carried out on Varian Prostar HPLC systems equipped with Prostar 210 pumps and a Prostar 335 photodiode array detector, with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2, Varian Inc.). For preparative HPLC, a Gemini-NX (5  $\mu\text{m}$ ; 250  $\times$  21 mm; Phenomenex) column was used. For semipreparative HPLC, a Gemini-NX (5  $\mu\text{m}$ , 250  $\times$  10 mm; Phenomenex) column was used. For analytical HPLC, a Gemini-NX (5  $\mu\text{m}$ , 250  $\times$  4.6 mm; Phenomenex) column was used. For UPLC analysis, a BEH C<sub>18</sub> (1.7  $\mu\text{m}$ ; 50  $\times$  2.1 mm; Waters Corp.) column was used.

**Isolation and Morphological and Molecular Identification of the Fungal Strain.** A healthy asymptomatic plant of *Silybum marianum* (milk thistle) was obtained from Horizon Herbs (lot #6510), a private seed company located in Williams, OR, USA, in August 2011. The stem and leaves of the plant were cut into small pieces (approximately 2–5 mm in length) and washed in distilled H<sub>2</sub>O. Subsequently, the segments were surface-sterilized by sequential immersion in 95% EtOH (10 s), sodium hypochlorite (10–15% available chlorine; 2 min), and 70% EtOH (2 min). The surface-sterilized segments were transferred under aseptic conditions onto 2% malt extract agar [MEA; Difco, 20 g of MEA, 1 L of sterile distilled H<sub>2</sub>O amended with streptomycin sulfate (250 mg/L) and penicillin G (250 mg/L)]. To test the efficacy of the surface-sterilization procedure and to confirm that emergent fungi were endophytic and not of epiphytic origin, the individual surface-sterilized leaf and stem segments were spread and then removed on separate MEA plates with antibiotics; the absence of fungal growth on the nutrient medium confirmed the effectiveness of the sterilization procedure.<sup>71</sup> Plates were sealed with Parafilm and incubated at room temperature in 12 h dark/light cycles until the emergence of fungal colonies was observed. One of the endophytes from milk thistle stems was assigned the accession number G85. The cultures of G85 were subsequently grown on 2% MEA, PDA (Difco), and YESD. The fungal culture is maintained at the University of North Carolina at Greensboro, Department of

Chemistry and Biochemistry Fungal Culture Collection. The macro-morphology and micromorphology of the fungus are described in detail in the Supporting Information. For the molecular identification of G85, the complete internal transcribed spacer regions 1 and 2 and 5.8S nrDNA (ITS), along with the D1/D2 variable domains (partial region of large subunit of the 28S nuclear rDNA, LSU), were sequenced using methods described previously<sup>35</sup> and outlined in the Supporting Information; the ITS methodology has been proposed as a molecular barcode for fungi.<sup>72</sup> We also sequenced the partial ribosomal polymerase II subunit 2 region (RPB2; Supporting Information), as it has been used to demonstrate phylogenetic relationships among species currently recognized within *Penicillium*.<sup>73</sup> The combined ITS and LSU sequence (KF367458) and the partial RPB2 sequences (AB860248 and AB860249) were deposited in GenBank. A herbarium voucher of the plant was generated from milk thistle seeds harvested from the same plot in Oregon (lot #6462), and this was deposited in the Herbarium of the University of North Carolina at Chapel Hill (NCU602014).

**Organism Culture Methods and Extract Preparation.** The red guttates observed on 10-day-old cultures on a Petri dish with MEA medium were collected with a micropipet (approximately 150 µL of guttate). After collection, the same volume of MeOH was added, and then the solution was filtered using 0.45 mm Teflon filters and dried *in vacuo*; approximately 10 mg of dry material (red solid) was obtained. For the scale-up, the fungus was grown as a solid phase culture on rice using methods described previously (Supporting Information).<sup>74</sup> For extraction, 60 mL of 1:1 MeOH–CHCl<sub>3</sub> was added, and the mixture was shaken for 16 h on a reciprocating shaker. The solution was filtered, and equal volumes of H<sub>2</sub>O and CHCl<sub>3</sub> were added to the filtrate to bring the total volume to 250 mL. The solution was stirred vigorously for 1 h and partitioned in a separatory funnel, and the bottom, organic layers were concentrated by rotary evaporation. The resulting sample was defatted by stirring for 1 h in a mixture of 50 mL of MeOH, 50 mL of CH<sub>3</sub>CN, and 100 mL of hexane, and the biphasic solution was partitioned in a separatory funnel. The bottom layer was collected and evaporated to dryness, resulting in the MeOH–CH<sub>3</sub>CN fraction (~1 g of a red, solid material).

**Isolation and Structure Elucidation of Polyhydroxyanthraquinones.** The instrumentation and methods utilized to isolate and structurally elucidate compounds **1–9** followed well-established protocols (Supporting Information).<sup>35,37,75</sup>

**Transverse Sectioning of *P. restrictum*.** Culturing dishes containing *P. restrictum* raised on PDA were selected at maturity (57 days). A distinct colony was excised using a razor blade, containing the full depth of the culture medium (~3 mm), consisting of PDA medium with filamentous fungal growth along the ventral surface. The excised colony was submerged in liquid nitrogen for flash freezing. The frozen colony was then halved using a cryotome blade in a ventral-to-dorsal direction, yielding the transverse planar surface (Figure S9). The halved colony was then embedded in optimal cutting temperature (OCT) matrix, preserving the transverse plane orientation, in preparation for cryosectioning. The embedded colony was sectioned at a thickness of 15 µm and, subsequently, thaw mounted onto glass microscope slides in preparation for mass spectrometric analysis. The embedded colony sections were retained at –80 °C until the time of analysis.

**DESI-MS Imaging of Transverse Sections of *P. restrictum*.** The sections were analyzed by DESI using a laboratory-built prototype<sup>19</sup> coupled to a linear ion trap mass spectrometer (LTQ, Thermo). DESI-MS imaging was carried out in the negative ion mode using the following parameters: 5 kV spray voltage, incident spray angle ( $\alpha$ ) 52°, spray-to-MS inlet distance ~8 mm, spray-to-surface distance 1–2 mm, 180 psi N<sub>2(g)</sub>, and 0.7 µL/min DMF–CH<sub>3</sub>CN (1:1). Sections were analyzed using a 2D moving stage in horizontal rows separated by a 200 µm vertical step and subsequently converted into spatially accurate images. Post hoc processing of the hyperdimensional data provided the 2D ion images, retaining spatial relationships and displaying relative mass spectral abundances of particular ions. An in-house program was used for converting acquired XCalibur 2.0 mass

spectral files (.raw) into a format compatible with Biomap software (<http://www.maldi-msi.org>).

**Imprinting of *P. restrictum*.** A polytetrafluoroethylene (PTFE) surface was cleaned with MeOH, allowed to dry, and dried further under an electronic desiccator for 10 min. The PTFE surface was then mounted onto the bottom of a 100 mL beaker using double-sided adhesive tape. A culturing dish (Ø 35 mm) was inverted, lowered, and touched to the PTFE surface; the touch consisted of contact with no additional applied pressure. The PTFE surface was removed from the glass beaker, adhered to a glass microscope slide using double-sided adhesive tape, and dried in the electronic desiccator for 15 min or until completely dry. Imprints were stored at 4 °C until analysis; however, it was later determined that dry, imprinted samples yielded detectable mass spectral signal in ambient conditions for several weeks.

**DESI-MS Imaging of Imprints of *P. restrictum*.** Imprints were analyzed as described above. DESI-MS imaging was carried out in the negative mode using the following major parameters: 5 kV spray voltage, incident spray angle ( $\alpha$ ) 52°, spray-to-MS inlet distance ~8 mm, spray-to-surface distance 1–2 mm, 180 psi N<sub>2(g)</sub>, and 1.2 µL/min MeOH–H<sub>2</sub>O (1:1) pH 10 by addition of NH<sub>4</sub>OH.

**DESI-MS Time Course Study.** A culture of *P. restrictum* was maintained at ambient conditions for 16 days, and fungal growth was apparent in the center of the culture plate. At day 16, the plate was opened, and a small amount of material was used to inoculate the edge of the plate (via a sterile inoculation loop). The plate was resealed with Parafilm and maintained at room temperature (rt) for another 8 days. Subsequently, the culture was flash frozen, sectioned, and analyzed by DESI-MS imaging as described above.

**Reporter Strain Assay for Quorum Sensing Inhibition.** The *agr* P3<sub>lux</sub> reporter strain AH2759 was created by transduction of plasmid pAmiAgrP3<sup>69</sup> using bacteriophage 80 $\alpha$  as described previously.<sup>68,76</sup> The plasmid was crossed into strain AH1263,<sup>68</sup> which is the USA300 CA-MRSA strain LAC cured of the native plasmid pUSA03 that confers erythromycin resistance. Overnight cultures of AH2759 grown in tryptic soy broth (TSB) supplemented with chloramphenicol at 10 µg/mL were inoculated at a dilution of 1:250 into fresh TSB containing antibiotic. Bacterial aliquots (100 µL) were added to 96-well microtiter plates (Costar 3603), where each well contained 100 µL aliquots of TSB with antibiotic and the polyhydroxyanthraquinones at concentrations ranging from 0.2 to 2000 µM. After mixing, the effective inoculum dilution was 1:500 and the polyhydroxyanthraquinone concentrations ranged from 0.1 to 1000 µM. Wells contained a final, constant DMSO concentration of 1%. Plates were incubated at 37 °C with shaking at 250 rpm, and a Tecan Infinite M200 plate reader was used to measure turbidity (OD<sub>600</sub>) and luminescence at 1 h intervals beginning at 15 h of incubation. Dose–response curves were generated with cell-density-normalized luminescence values, and IC<sub>50</sub> values were obtained by a weighted, four-parameter logistic fit using KaleidaGraph v4.1.3 (Synergy Software). Except for compounds **5** and **7**, reported IC<sub>50</sub>'s were the average of two experiments, one with  $n = 3$  and the other with  $n = 4$ . IC<sub>50</sub>'s for **5** and **7** were from the experiment with  $n = 4$ . For some of the compounds, there was a significant growth delay at the 1000 µM concentration; data from those wells were excluded from the analysis. Wells containing synthetic AIP-2 (Anaspec) at concentrations from 2 to 500 nM were included as a positive control.

**Quorum Sensing Inhibition Observed by Delta Toxin Immunoblot.** After collecting data in the *agr* P3<sub>lux</sub> assay, the cultures from the microtiter plate wells were pooled and filter sterilized using SpinX 0.22 µm filters. The media (2.5 µL) from cultures treated with compounds **1**, **3**, and **4**, as well as from cultures treated with AIP-2, were subjected to SDS-PAGE on 15% gels. Following electrophoresis, gels were washed for 20 min in transfer buffer and proteins were transferred to Immobilon-PSQ polyvinylidene difluoride (Millipore) membranes for 90 min at a constant current of 160 mA. Membranes were soaked overnight with blocking solution [consisting of 4.25% nonfat milk and 0.75% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST)] at 4 °C with gentle agitation. Membranes were washed three times for 10 min with TBST at rt and probed for 1 h at rt with rabbit anti-delta toxin polyclonal antibody

(Abgen) diluted 1:2000 in blocking buffer. Membranes were washed again briefly with TBST and probed for 1 h at rt with goat anti-rabbit antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories) diluted 1:10 000 in blocking buffer. Membranes were briefly washed with TBST, and bound conjugate was detected using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) followed by exposure to Classic X-ray film from Research Product International.

## ■ ASSOCIATED CONTENT

### § Supporting Information

Complete materials and methods for the macro, micro, and molecular identification of the fungal strain, methods and characterization of compounds, sample preparation for DESI-MS analysis, and Supplementary Figures S1–S26 and Table S1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Deleo, F. R.; Chambers, H. F. *J. Clin. Invest.* **2009**, *119*, 2464–2474.
- (2) Chambers, H. F.; Deleo, F. R. *Nat. Rev. Microbiol.* **2009**, *7*, 629–641.
- (3) Waldor, M. K. N. *Engl. J. Med.* **2006**, *354*, 296–297.
- (4) Gray, B.; Hall, P.; Gresham, H. *Sensors* **2013**, *13*, 5130–5166.
- (5) Gordon, C. P.; Williams, P.; Chan, W. C. *J. Med. Chem.* **2013**, *56*, 1389–1404.
- (6) Cech, N. B.; Junio, H. A.; Ackermann, L. W.; Kavanaugh, J. S.; Horswill, A. R. *Planta Med.* **2012**, *78*, 1556–1561.
- (7) Hutwimmer, S.; Wang, H.; Strasser, H.; Burgstaller, W. *Mycologia* **2010**, *102*, 1–10.
- (8) Thoendel, M.; Kavanaugh, J. S.; Flack, C. E.; Horswill, A. R. *Chem. Rev.* **2011**, *111*, 117–151.
- (9) Murray, E. J.; Crowley, R. C.; Truman, A.; Clarke, S. R.; Cottam, J. A.; Jadhav, G. P.; Steele, V. R.; O'Shea, P.; Lindholm, C.; Cockayne, A.; Chhabra, S. R.; Chan, W. C.; Williams, P. *J. Med. Chem.* **2014**, *57*, 2813–2819.
- (10) Colotelo, N. *Can. J. Microbiol.* **1978**, *24*, 1173–1181.
- (11) Wilson, J. K. *Mycologia* **1948**, *40*, 605–613.
- (12) Gareis, M.; Gareis, E. M. *Mycopathologia* **2007**, *163*, 207–214.
- (13) Jennings, D. H. *Mycol. Res.* **1991**, *95*, 883–884.
- (14) McPhee, W. J.; Colotelo, N. *Can. J. Bot.* **1977**, *55*, 358–365.
- (15) Koulman, A.; Lane, G. A.; Christensen, M. J.; Fraser, K.; Tapper, B. A. *Phytochemistry* **2007**, *68*, 355–360.
- (16) Gardes, M.; Bruns, T. D. *Mol. Ecol.* **1993**, *2*, 113–118.
- (17) Wang, X.; Sena Filho, J. G.; Hoover, A. R.; King, J. B.; Ellis, T. K.; Powell, D. R.; Cichewicz, R. H. *J. Nat. Prod.* **2010**, *73*, 942–948.
- (18) Takats, Z.; Wiseman, J. M.; Cooks, R. G. *J. Mass Spectrom.* **2005**, *40*, 1261–1275.
- (19) Wiseman, J. M.; Ifa, D. R.; Venter, A.; Cooks, R. G. *Nat. Protoc.* **2008**, *3*, 517–524.
- (20) Cooks, R. G.; Ouyang, Z.; Takats, Z.; Wiseman, J. M. *Science* **2006**, *311*, 1566–1570.
- (21) Venter, A.; Sojka, P. E.; Cooks, R. G. *Anal. Chem.* **2006**, *78*, 8549–8555.
- (22) Costa, A. B.; Cooks, R. G. *Chem. Phys. Lett.* **2008**, *464*, 1–8.
- (23) Wu, C.; Dill, A. L.; Eberlin, L. S.; Cooks, R. G.; Ifa, D. R. *Mass Spectrom. Rev.* **2013**, *32*, 218–243.
- (24) Dill, A. L.; Eberlin, L. S.; Ifa, D. R.; Cooks, R. G. *Chem. Commun.* **2011**, *47*, 2741–2746.
- (25) Ferreira, C. R.; Pirro, V.; Eberlin, L. S.; Hallett, J. E.; Cooks, R. G. *Anal. Bioanal. Chem.* **2012**, *404*, 2915–2926.
- (26) Morelato, M.; Beavis, A.; Kirkbride, P.; Roux, C. *Forensic Sci. Int.* **2013**, *226*, 10–21.
- (27) Eberlin, L. S.; Norton, I.; Orringer, D.; Dunn, I. F.; Liu, X.; Ide, J. L.; Jarmusch, A. K.; Ligon, K. L.; Jolesz, F. A.; Golby, A. J.; Santagata, S.; Agar, N. Y. R.; Cooks, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 1611–1616.
- (28) Lane, A. L.; Nyadong, L.; Galhena, A. S.; Shearer, T. L.; Stout, E. P.; Parry, R. M.; Kwasnik, M.; Wang, M. D.; Hay, M. E.; Fernandez, F. M.; Kubanek, J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 7314–7319.
- (29) Nyadong, L.; Hohenstein, E. G.; Galhena, A.; Lane, A. L.; Kubanek, J.; Sherrill, C. D.; Fernandez, F. M. *Anal. Bioanal. Chem.* **2009**, *394*, 245–254.
- (30) Watrous, J.; Roach, P.; Heath, B.; Alexandrov, T.; Laskin, J.; Dorrestein, P. C. *Anal. Chem.* **2013**, *85*, 10385–10391.
- (31) Lanekoff, I.; Geydebrekht, O.; Pinchuk, G. E.; Konopka, A. E.; Laskin, J. *Analyst* **2013**, *138*, 1971–1978.
- (32) Hanson, J. R. *The Chemistry of Fungi*; RSC Publishing: Cambridge, UK, 2008; p 240.
- (33) Ayers, S.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Matthew, S.; Carache de Blanco, E. J.; Shen, Q.; Swanson, S. M.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H. *J. Nat. Prod.* **2011**, *74*, 1126–1131.
- (34) Ayers, S.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Shen, Q.; Swanson, S. M.; Wani, M. C.; Darveaux, B. A.; Pearce, C. J.; Oberlies, N. H. *Tetrahedron Lett.* **2011**, *52*, 5128–5230.
- (35) El-Elimat, T.; Figueiroa, M.; Raja, H. A.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Day, C. S.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H. *J. Nat. Prod.* **2013**, *76*, 382–387.
- (36) El-Elimat, T.; Zhang, X.; Jarjoura, D.; Moy, F. J.; Orjala, J.; Kinghorn, A. D.; Pearce, C. J.; Oberlies, N. H. *ACS Med. Chem. Lett.* **2012**, *3*, 645–649.
- (37) Figueiroa, M.; Graf, T. N.; Ayers, S.; Adcock, A. F.; Kroll, D. J.; Yang, J.; Swanson, S. M.; Munoz-Acuna, U.; Carache de Blanco, E. J.; Agarwal, R.; Wani, M. C.; Darveaux, B. A.; Pearce, C. J.; Oberlies, N. H. *J. Antibiot.* **2012**, *65*, 559–564.
- (38) Sy-Cordero, A. A.; Graf, T. N.; Adcock, A. F.; Kroll, D. J.; Shen, Q.; Swanson, S. M.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H. *J. Nat. Prod.* **2011**, *74*, 2137–2142.
- (39) Sy-Cordero, A. A.; Pearce, C. J.; Oberlies, N. H. *J. Antibiot.* **2012**, *65*, 541–549.
- (40) Anslow, W. K.; Breen, J.; Raistrick, H. *Biochem. J.* **1940**, *34*, 159–168.
- (41) Birkinshaw, J. H. *Biochem. J.* **1955**, *59*, 485–486.
- (42) Piattelli, M.; Giudici de Nicola, M. *Phytochemistry* **1968**, *7*, 1183–1187.
- (43) Ren, H.; Tian, L.; Gu, Q.; Zhu, W. *Arch. Pharm. Res.* **2006**, *29*, 59–63.

- (44) Powell, V. H.; Sutherland, M. D. *Aust. J. Chem.* **1967**, *20*, 541–553.
- (45) Khamthong, N.; Rukachaisirikul, V.; Tadpatch, K.; Kaewpet, M.; Phongpaichit, S.; Preedanon, S.; Sakayaroj, J. *Arch. Pharm. Res.* **2012**, *35*, 461–468.
- (46) Eder, R.; Widmer, C. *Helv. Chim. Acta* **1923**, *6*, 966–981.
- (47) Hoye, T. R.; Jeffrey, C. S.; Shao, F. *Nat. Protoc.* **2007**, *2*, 2451–2458.
- (48) Birkinshaw, J.; Gourlay, R. *Biochem. J.* **1961**, *81*, 618–622.
- (49) Song, R.; Lin, H.; Zhang, Z. J.; Li, Z.; Xu, L.; Dong, H. J.; Tian, Y. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 537–547.
- (50) Xu, L. J.; Chan, C. O.; Lau, C. C.; Yu, Z. L.; Mok, D. K. W.; Chen, S. B. *Phytochem. Anal.* **2012**, *23*, 110–116.
- (51) Alvi, K. A.; Nair, B.; Gallo, C.; Baker, D. *J. Antibiot.* **1997**, *50*, 264–266.
- (52) Qian, Z. J.; Zhang, C.; Li, Y. X.; Je, J. Y.; Kim, S. K.; Jung, W. K. *Evid.-Based Complementary Altern. Med.* **2011**, *2011*, 452621.
- (53) Fujimoto, H.; Nakamura, E.; Okuyama, E.; Ishibashi, M. *Chem. Pharm. Bull.* **2004**, *52*, 1005–1008.
- (54) Macias, M.; Ulloa, M.; Gamboa, A.; Mata, R. *J. Nat. Prod.* **2000**, *63*, 757–761.
- (55) Gill, M. *Nat. Prod. Rep.* **2003**, *20*, 615–639.
- (56) Gill, M.; Gimenez, A. *J. Chem. Soc., Perkin Trans. 1* **1990**, 2585–2591.
- (57) Cohen, P. A.; Hudson, J. B.; Towers, G. H. *Experientia* **1996**, *52*, 180–183.
- (58) Santesson, J. *Phytochemistry* **1970**, *9*, 2149–2166.
- (59) Kemami Wangun, H. V.; Wood, A.; Fiorilla, C.; Reed, J. K.; McCarthy, P. J.; Wright, A. E. *J. Nat. Prod.* **2010**, *73*, 712–715.
- (60) Lu, Y.; Suh, S.-J.; Li, X.; Hwang, S.-L.; Li, Y.; Hwangbo, K.; Park, S. J.; Murakami, M.; Lee, S. H.; Jahng, Y.; Son, J.-K.; Kim, C.-H.; Chang, H. W. *Food Chem. Toxicol.* **2012**, *50*, 913–919.
- (61) Hatano, T.; Uebayashi, H.; Ito, H.; Shiota, S.; Tsuchiya, T.; Yoshida, T. *Chem. Pharm. Bull.* **1999**, *47*, 1121–1127.
- (62) Kelly, T. R.; Chandrasekaran, N. S.; Walters, N.; Blancaflor, J. *J. Org. Chem.* **1983**, *48*, 3573–3574.
- (63) Kim, D. S. H. L.; Koonchanok, N. M.; Geahlen, R. L.; Ashendel, C. L.; Chang, C.-J. *Nat. Prod. Lett.* **1997**, *10*, 173–180.
- (64) Nikaido, T.; Ohmoto, T.; Sankawa, U.; Kitanaka, S.; Takido, M. *Chem. Pharm. Bull.* **1984**, *32*, 3075–3078.
- (65) Osman, C. P.; Ismail, N. H.; Ahmad, R.; Ahmat, N.; Awang, K.; Jaafar, F. M. *Molecules* **2010**, *15*, 7218–7226.
- (66) Yao, S.; Li, Y.; Kong, L. *J. Chromatogr. A* **2006**, *1115*, 64–71.
- (67) Zargar, B. A.; Masoodi, M. H.; Ahmed, B.; Ganie, S. A. *Food Chem.* **2011**, *128*, 585–589.
- (68) Boles, B. R.; Thoendel, M.; Roth, A. J.; Horswill, A. R. *PLoS One* **2010**, *5*, e10146.
- (69) Subrt, N.; Mesak, L. R.; Davies, J. *J. Antimicrob. Chemother.* **2011**, *66*, 979–984.
- (70) Höller, U.; Lenz, J. *Curr. Opin. Microbiol.* **2005**, *8*, 301–306.
- (71) Schulz, B.; Wanke, U.; Draeger, S.; Aust, H. J. *Mycol. Res.* **1993**, *97*, 1447–1450.
- (72) Schoch, C. L.; Seifert, K. A.; Huhndorf, S.; Robert, V.; Spouge, J. L.; Levesque, C. A.; Chen, W. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 6241–6246.
- (73) Houbraken, J.; Samson, R. A. *Stud. Mycol.* **2011**, *70*, 1–51.
- (74) Raja, H. A.; Oberlies, N. H.; El-Elimat, T.; Miller, A. N.; Zelski, S. E.; Shearer, C. A. *Mycoscience* **2013**, *54*, 353–361.
- (75) Figueroa, M.; Raja, H.; Falkingham, J. O., 3rd; Adcock, A. F.; Kroll, D. J.; Wani, M. C.; Pearce, C. J.; Oberlies, N. H. *J. Nat. Prod.* **2013**, *76*, 1007–1015.
- (76) Novick, R. P. *Methods Enzymol.* **1991**, *204*, 587–636.