BIOFILM PRODUCTION BY CLINICAL STAPHYLOCOCCUS AUREUS
AND ITS INHIBITION BY *HYPERICUM* METABOLITES

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BIOFILM PRODUCTION BY CLINICAL

STAPHYLOCOCCUS AUREUS AND ITS INHIBITION

BY HYPERICUM METABOLITES

BY

SIMON ANTRANIK SARKISIAN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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OF

SIMON ANTRANIK SARKISIAN

APPROVED:

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________________Nasser Zawia_______________________

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND
2011
ABSTRACT

Evolution has allowed bacteria to develop sophisticated methods of survival. One of these methods is biofilm production. Biofilms can be best described as complex bacterial communities embedded in a self-producing slime. Once bacteria form these biofilm communities, they become very difficult to treat with antibiotics. Along with biofilm production, another rising concern is antibiotic resistance. Bacterial resistance to many of our current antibiotics is sharply increasing, thereby creating a critical need to develop novel antimicrobial drugs.

The most successful strategy for the discovery of new antibacterial agents has been the study of molecules from nature. Most of our current clinical antibiotics derive from metabolites produced by bacteria. Antimicrobial compounds of plant origin also have enormous therapeutic potential. Previous studies have demonstrated that certain plant metabolites have potent inhibitory effects on the growth of pathogenic bacteria. Not only might metabolites from plants help mitigate infectious diseases, but they may also lack adverse side effects often associated with existing antimicrobial agents, including hypersensitivity, allergic reaction, and immunosuppression. Therefore, future efforts to discover new antimicrobial drugs should also include the evaluation of new natural products from both microbes and plants.

In this thesis, Chapter 1 describes an investigation to classify and quantify biofilm production in unique clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA). *Staphylococcus aureus* bacteria are responsible for causing a wide range of diseases and many are capable of biofilm production. To date, the largest focus of
biofilm research has been on *S. epidermidis* and *Pseudomonas aeruginosa*. Few investigators have addressed the basic and central question: “What percentage of our clinical *S. aureus* bacteria produce biofilms and from what patient source are they most likely identified?” Two hundred and nineteen (n = 219) clinical methicillin-resistant *S. aureus* (MRSA) isolates obtained from patients at the Veterans Affairs Medical Center (VAMC) in Providence, Rhode Island were evaluated. I evaluated biofilm formation using a modified microtiter-plate assay, and used a statistical approach to quantifying biofilm production. The results indicate that biofilm production is most frequently encountered in clinical MRSA from catheter sources. The surface area of these catheters may provide the ideal conditions for biofilm growth, especially in urine. Interestingly, a lesser incidence of biofilm production was observed by MRSA isolates obtained in the nares.

Chapter 2 describes a phytochemical investigation of plant metabolites from *Hypericum* species that inhibit bacterial growth as well as biofilm production in Gram-positive bacteria. For this study, seven acylphloroglucinol metabolites were obtained from Dr. Geneive Henry at Susquehanna University. I tested each compound in assays that measure both bacterial growth and biofilm production. The results showed that not only do some of these metabolites inhibit bacterial growth, but they also inhibit biofilm growth at sub-MIC concentrations. Important findings from this investigation included new structure-activity relationships demonstrating the importance of certain functional groups to the antibacterial nature of these metabolites. These results suggest that *Hypericum* spp. deserve further attention as a source of new antimicrobial agents.
ACKNOWLEDGMENTS

First and foremost, I thank my parents, my sisters, and the rest of my family for always pushing me to reach my goals and to never give up. I especially thank my father, Antranik, for raising me to believe that with hard work, any dream is possible.

I thank my advisor David Rowley for his guidance throughout my graduate years at URI as well as my summers with INBRE. When you accepted me into your lab, I told you that I would do the impossible and graduate with a Masters Degree in a year. We did it, Dr. Rowley.

I would like to thank Kerry LaPlante for inspiring me to conduct research with infectious diseases. One summer with Dr. LaPlante allowed me to witness a whole new aspect of medicine. Thank you for being patient with me as an undergraduate student in your lab so that I could confidently come back as a graduate student. I would also like to thank the Veterans Affairs Medical Center of Providence, Rhode Island as well as the manager of the LaPlante laboratory, Suzanne Woodmanee. Sue would not hesitate at all to help me learn all the techniques I needed to master my work. More than just a lab manager, Sue is a friend who I will always remember.

I would like to thank my undergraduate research professor, Yinsheng Wan, who sparked my interest in research with his work in UV radiation and skin cancer.

Thank you to all my committee members for their help and insight with this research.

Lastly, I would like to thank all those who I collaborated with as well as all the friends that I made during my time at the University of Rhode Island.
Dedicated to

My parents, Antranik and Lucy,

&

My sisters, Anahid and Elizabeth

Thank you for always believing in me. I love you all.

“Choose a job you love, and you will never work a day in your life.”

- Confucius
PREFACE

This thesis was formatted in accordance with the manuscript format guidelines established by the Graduate School of the University of Rhode Island.
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CHAPTER 1

The following manuscript has been formatted for submission to the journal Diagnostic Microbiology and Infectious Disease.

Biofilm-Producing Strains of Clinical Methicillin-Resistant *Staphylococcus aureus* are Most Frequently Encountered from Catheters

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University of Rhode Island, Department of Pharmacy Practice\textsuperscript{1}

Infectious Diseases Research Laboratory, Providence Veterans Affairs Medical Center\textsuperscript{2}
ABSTRACT

We classified and quantified biofilm production in clinical methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained from patient blood, nares, tissue, urine and catheter sources. Overall, 30.6% of the MRSA isolates were biofilm producers. Cultures obtained from catheter sources were significantly more likely to produce biofilm (52.3%, n=44, P=0.0005) compared to the other sources. Interestingly, cultures obtained from patient nares were significantly less likely to produce biofilm (18.6%, n=59, P=0.0198).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) produce an extracellular mucoid substance often referred to as a biofilm (Arciola, Campoccia, & Montanaro, 2002). The main component is polysaccharidic in nature and consists of glycosaminoglycans (Arciola, Baldassarri, & Montanaro, 2001). Biofilm formation is linked with the pathogenicity of most bacteria, including the *Staphylococcus* species, allowing them to better persist in the normally hostile environment of tissue and blood. Additionally, the physical barrier imposed by biofilms impedes antibiotic effectiveness and host defenses (Ceri, et al., 1999; Donlan, 2000; Oie, Huang, Kamiya, Konishi, & Nakazawa, 1996).

It is well known that *Staphylococcus epidermidis* produce biofilm, and it is estimated that greater than 60% of these isolates are capable of this production (Arciola, et al., 2001; Arciola, et al., 2002; Christensen, Simpson, Bisno, & Beachey, 1982; Cramton, Gerke, & Gotz, 2001; Cramton, Ulrich, Gotz, & Doring, 2001; de
Araujo, et al., 2006; Donlan, et al., 2001). However, much less is known about biofilm producing abilities in MRSA, particularly with clinical isolates. Therefore, it was the intent of this project to 1) measure biofilm formation by clinical strains of MRSA obtained from patients at the Veteran Affairs Medical Center (VAMC) in Providence, Rhode Island, 2) assess the frequency of biofilm production in clinical MRSA isolates, and 3) classify the relationship of biofilm producing MRSA to the source of patient obtainment (blood, nares, tissue, urine, and catheters).

MATERIALS AND METHODS

Biofilm production in methicillin-resistant *Staphylococcus aureus* (MRSA) was measured and classified in 219 unique clinical MRSA isolates obtained from patients at the Providence VAMC. The strains were collected from patient blood, nares, tissue, urine, and catheters from January 2004 to January 2009. Known biofilm producing *S. aureus* (ATCC 35556) and *S. epidermidis* (RP62A; ATCC 35984) were used as positive controls. A stable biofilm-negative mutant, M7, from the wild-type *S. epidermidis* RP62A served as the non-biofilm control.

Biofilm formation was evaluated and quantified using a modified microtiter-plate assay (Christensen, et al., 1985; Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000) and growth conditions were optimized for biofilm production in staphylococci (Cramton, Gerke, et al., 2001; Cramton, Ulrich, et al., 2001; LaPlante & Woodmansee, 2009). Based on optical density measurements (OD$_{610}$), bacterial films were classified into the following categories: biofilm production or no biofilm production using the method established by Stepanovic *et al.* (Stepanovic, et al.,
2000). Statistical Analysis Software (SAS) (Version 9.1) was used for all analyses, and a P-value of ≤ 0.05 was considered statistically significant.

RESULTS

Assays conducted with known biofilm producing strains *S. aureus* (ATCC 35556) and *S. epidermidis* (RP62A; ATCC 35984) resulted in average OD$_{610}$ readings of 1.87±0.61 and 1.76±0.64, respectively. The non-biofilm producing staphylococci produced with an average OD$_{610}$ reading of 0.26±0.09.

Overall, 30.6% (67/219) of the MRSA isolates were determined to be biofilm producers. Biofilm production was most common among isolates obtained from catheter sources (52.3%, P=0.0005). Of interest, isolates obtained from the nares were significantly less likely to produce biofilm (18.6% formed biofilm; P=0.0198).

Table 1: Biofilm Production Based on Isolate Source

<table>
<thead>
<tr>
<th>Source (n = 219)</th>
<th>Biofilm Production %, (n)</th>
<th>No Biofilm Production %, (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter (n = 44)</td>
<td>52.3%, (23)</td>
<td>47.7%, (21)</td>
</tr>
<tr>
<td>Urine (n = 13)</td>
<td>30.8%, (4)</td>
<td>69.2%, (9)</td>
</tr>
<tr>
<td>Tissue (n = 56)</td>
<td>30.4%, (17)</td>
<td>69.6%, (39)</td>
</tr>
<tr>
<td>Blood (n = 47)</td>
<td>25.5%, (12)</td>
<td>74.5%, (35)</td>
</tr>
<tr>
<td>Nares (n = 59)</td>
<td>18.6%, (11)</td>
<td>81.4%, (48)</td>
</tr>
<tr>
<td>Total:</td>
<td>30.6%, (67)</td>
<td>69.4%, (152)</td>
</tr>
</tbody>
</table>
Figure 1: Biofilm Production Based on Isolate Source (Presented as % Biofilm Production)

**DISCUSSION**

*Staphylococcus aureus* are the leading cause of bacterial infections in the United States and cause a wide range of diseases from simple skin infections to potentially fatal systemic diseases (Suzuki, Swoboda, Campbell, Walker, & Gilmore, 2010). Many staphylococcal infections, such as endocarditis and osteomyelitis, are caused by biofilm producing strains (Donlan, 2000; Donlan, et al., 2001). In total, it is estimated that biofilms are associated with 65% of all hospital acquired infections in the United States and account for health care costs exceeding one billion dollars annually (Licking, 1999; Martone, Jarvis, Culver, & Haley, 1992).

* P value is source versus others, i.e. blood versus non-blood.
To date, the largest focus of biofilm research has been on *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Fey & Olson, 2010; Jensen, Givskov, Bjarnsholt, & Moser, 2010). Fewer studies have examined biofilm formation by *S. aureus*. Thus, knowledge is currently lacking regarding the percentage of clinical MRSA that produce biofilms and from what patient sources they are most likely encountered.

Interestingly, MRSA isolates obtained from patients’ nares were significantly less likely to be biofilm formers compared to isolates obtained from the other patient sources. We are not aware of this finding previously discussed in the literature. Another study found that 60% of methicillin-resistant *Staphylococcus epidermidis* (MRSE) isolates obtained from the nares were able to produce biofilm (de Araujo, et al., 2006).

In our facility, we found that cultures taken from catheter samples produced significantly more biofilm than other sources. These results are similar to those found by a Japanese study, which concluded that the biofilm-forming capacities of MRSA isolates from catheter-related cases were significantly greater than those from catheter-unrelated cases (Ando, Monden, Mitsuhata, Kariyama, & Kumon, 2004). Their studies suggest that MRSA colonization and infection of the urinary tract may be promoted by *hla, hlb*, and *fnbA* gene products (Ando, et al., 2004). However, many genes can be responsible for biofilm growth in *S. aureus* (Houston, Rowe, Pozzi, Waters, & O’Gara, 2011; Kaito, et al., 2011). Our clinical MRSA isolates were not tested for biofilm-producing genes because of this lack of standardization.
Urinary surfaces provide attractive sites for bacterial colonization as well as antibacterial resistance due to the gentle flow of warm nutritious urine (Stickler, 2008). The capacity of a microorganism to establish and form a biofilm on a given surface depends on the nature of the surface in question (Donlan & Costerton, 2002). When a device such as a urinary catheter is exposed to bodily fluids such as urine, various components adsorb onto the surface and form a conditioning film. This film essentially covers the surface and becomes the bona fide interface where microbial interaction takes place. In the case of biofilm formation the steps are bacterial attachment, microcolony formation and build up of biofilm. It follows that the nature of the material constituting the catheter determines the composition of the conditioning film, which in turn influences which microorganisms can attach (Ferrieres, Hancock, & Klemm, 2007).

In conclusion, based on the data from our study, a correlation may exist between biofilm production and catheters as the source. This may be explained by the high likelihood of S. aureus cultures that are taken from urinary and central venous catheters. Of equal interest is the lesser incidence of biofilm formation by MRSA isolates derived from patient nares.

ACKNOWLEDGEMENTS

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do they necessarily reflect the position or policy of the United States Department of Veterans Affairs. We gratefully acknowledge Suzanne Woodmansee of the LaPlante Infectious Diseases laboratory for assay assistance and interpretation.
REFERENCES


CHAPTER 2

The following manuscript has been formatted for submission to the journal Phytomedicine.

Inhibition of Bacterial Growth and Biofilm Production by Metabolites from Hypericum spp.

S.A. Sarkisian \textsuperscript{a}, G.E. Henry \textsuperscript{b}, K.L. LaPlante \textsuperscript{a,c}, D.C. Rowley \textsuperscript{a,*}

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\textsuperscript{b}Susquehanna University, Selinsgrove, PA
\textsuperscript{c}VA Medical Center Infectious Diseases Research Laboratory, Providence, RI
ABSTRACT

Biofilm embedded bacterial pathogens such as *Staphylococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are difficult to eradicate and are major sources of bacterial infections. New drugs are needed to combat these pathogens. *Hypericum* is a plant genus that contains species known to have antimicrobial properties. However, the specific metabolites responsible for the antimicrobial properties are not entirely known, nor have most compounds been tested as inhibitors of biofilm development. The investigation presented here tested seven secondary metabolites isolated from the species *Hypericum densiflorum*, *Hypericum ellipticum*, *Hypericum prolificum*, and *Hypericum punctatum* as inhibitors of bacterial growth and biofilm production. Assays were conducted against *Staphylococcus epidermidis*, *Staphylococcus aureus*, clinical methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*. Five of the seven metabolites demonstrated growth inhibition against the Gram-positive bacteria with minimum inhibitory concentrations (MIC) ranging from 1.95 µg/mL to 7.81 µg/mL. Four of the metabolites inhibited biofilm production by certain Gram-positive strains at sub-MIC concentrations.

INTRODUCTION

Bacterial resistance to many of our current antibiotics is sharply increasing, thereby creating a critical need to develop novel antimicrobial drugs (Spellberg et al., 2004). Antimicrobials of plant origin have enormous therapeutic potential. Not only could they help mitigate infectious diseases, but they may also lack adverse side
effects often associated with existing antimicrobial agents, including hypersensitivity, allergic reaction, and immunosuppression (Iwu et al., 1999; Mukherjee et al., 2002). Previous investigations have demonstrated the effectiveness of plant metabolites from traditional herbs against Gram-positive and Gram-negative microorganisms (Dall'agnol et al., 2005; Gibbons, 2004; Mukherjee et al., 2002). In particular, studies involving members of the genus Hypericum have suggested that their metabolites possess promising antimicrobial properties.

The plants of the Clusiaceae (Guttiferae) family consist of more than 1000 species of which Hypericum is the most widely studied genus (Dall'agnol et al., 2005). These plants are commonly found as herbs, shrubs or small trees and are distributed primarily in the temperate regions of the world. Hypericum has been used in folk medicine dating back more than 2400 years, thereby suggesting the therapeutic potential of many species. Records indicate that Cherokee, Iroquois, and Montagnais American Indian tribes used Hypericum perforatum, also known as St. John’s wort, as a febrifuge/cough medicine (Saddiqe et al., 2010).

There is a growing interest in Hypericum metabolites because of their wide range of biological activities. Phytochemical investigations on Hypericum perforatum, for instance, have led to the isolation of active antimicrobial compounds, which include naphthodianthrones and phloroglucinols (Saddiqe et al., 2010). Owing to these findings, we were inspired to expand the search for new antibacterial metabolites to include other Hypericum species. Here we report the biological evaluation of seven phloroglucinol derivatives from H. densiflorum, H. ellipticum, H. prolificum and H. punctatum for their antibacterial properties. The compounds were
tested for inhibition of bacterial growth and biofilm formation against a panel of clinically relevant pathogens. The panel was comprised of *Staphylococcus epidermidis, Staphylococcus aureus, clinical methicillin-resistant S. aureus* (MRSA), *Pseudomonas aeruginosa, Escherichia coli*, and *Acinetobacter baumannii*. Five of the compounds demonstrated potent antimicrobial effects against the Gram-positive pathogens. Additionally, four of the metabolites attenuated biofilm formation at concentrations below the minimum inhibitory concentration.

**Figure 1: Structures of Hypericum metabolites.**

---

**MATERIALS AND METHODS**

**Chemicals and Instrumentation.** NMR spectra were recorded on a JEOL ECP 400 MHz spectrometer (\(^1\)H, 400 MHz; \(^{13}\)C, 100 MHz) using CDCl\(_3\), (CD\(_3\))\(_2\)CO and DMSO-d\(_6\) as solvents and TMS as internal standard. Gradient HMQC and HMBC
data were obtained using standard pulse programs. MS analyses were carried out on a Q-Star Elite (Applied Biosystems MDS) mass spectrometer equipped with a Turbo Ionspray source (University of Rhode Island). HRMS data were acquired using electrospray ionization on an Agilent G6520A Q-TOF high resolution mass spectrometer (CUNY, Hunter College). Preparative HPLC was performed using a Waters Delta 600 system equipped with a Waters 2487 dual wavelength absorbance detector. Column chromatography (CC) was performed using Fisher Scientific silica gel (230-400 mesh), and analytical TLC was performed using Sigma-Aldrich polyester backed plates precoated with silica gel UV254. All solvents were HPLC grade and were obtained from Fisher Scientific and Aldrich Chemical Co.

**Plant material.** All plant samples were collected and identified by Joseph A. Isaac of Civil and Environmental Consultants, Pittsburgh, Pennsylvania (PA), USA. The aerial portions (leaves and stems) were collected and voucher specimens have been deposited at the Carnegie Museum Herbarium in Pittsburgh, PA. *Hypericum densiflorum* Pursh. was collected in Westmoreland County, PA, in June 2005 (voucher #18640) and September 2007 (voucher # 19442). *Hypericum ellipticum* (voucher #20714) was collected in Clearfield County, PA, in August 2009. *Hypericum prolificum* L. (voucher #18641) was collected in Lawrence County, PA, in June 2005 and *H. punctatum* Lam., spotted St. John's wort, (voucher #18944) was collected in Lawrence County, PA, in September 2005.

**Extraction and isolation procedures.** Compounds 3, 4, and 5 were isolated from hexanes and acetone extracts of *H. densiflorum* as previously described (Henry et al., 2009). Compound 7 was isolated from the hexanes extract of *H. prolificum* as
previously reported (Henry et al., 2006). Aerial parts of *H. ellipticum* were oven dried at 38°C and ground to a fine powder using a coffee grinder. The plant material (459 g) was extracted sequentially at room temperature using hexanes (3 × 1.0 L), acetone (3 × 1.0 L) and methanol (3 × 1.0 L). The solvents were allowed to percolate for 24 h each time. The combined extracts for each solvent were concentrated *in vacuo*. The acetone extract (14.6 g) was fractionated using silica gel CC to afford fractions A1-A11, eluted with a solvent gradient of acetone-hexanes (5:95 to 100:0, v/v). Fraction A3 (509 mg), eluted with acetone-hexanes (1:3, v/v), was further fractionated with ethyl acetate-hexanes (1:4 to 1:3, v/v) to afford a brown solid (145 mg), which was purified by semi-preparative HPLC (Atlantis dC18 column, 5μm, 19 × 150 mm, methanol-water (4:1, v/v), isocratic elution, flow rate 10 mL/min) to afford compound 6 (28.0 mg). The structure of compound 6 was established based on NMR spectroscopic and mass spectrometric data (Manning et al., 2011).

The aerial parts of *H. punctatum* were dried and ground to a fine powder using a coffee grinder. The powdered plant material (255 g) was extracted with acetone (3 × 1.1 L) followed by methanol (3 × 1.0 L) at room temperature. The solvents were allowed to percolate for one night each time. The acetone and methanol extracts were concentrated *in vacuo*. The acetone extract (15.5 g) was subjected to silica gel CC, eluting with an acetone-hexanes stepwise solvent gradient (5:95 to 100:0, v/v) to afford nine fractions (A1-A9). Fraction A5 (520 mg), eluted with acetone-hexanes (1:4, v/v), was chromatographed further by silica gel CC using ethyl acetate-hexanes (1:9 to 1:4, v/v) to afford sub-fractions B1-B6. Subfraction B3 (294.2 mg) was purified by semi-preparative HPLC on an Atlantis dC18 column (19 × 150 mm,
methanol:water (85:15, v/v), isocratic elution, flow 10 mL/min), to afford compound 1 (40.2 mg) and compound 2 (55.1 mg) as yellow solids. The NMR spectroscopic data of 1 and 2 are consistent with those reported in the literature (Rios and Delgado, 1992).

**Microorganisms and culture conditions.** *Staphylococcus epidermidis* (RP62A; ATCC 35984), *S. aureus* (ATCC 35556), clinical methicillin-resistant *S. aureus*, *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and *A. baumannii* (ATCC 17978) were obtained from the Veterans Affairs Medical Center (VAMC) in Providence, Rhode Island and maintained in tryptic soy broth (Difco, Becton Dickinson) at -80 ºC. All strains were incubated on tryptic soy agar (Difco, Becton Dickinson) at 37 ºC for 24 h. The clinical MRSA isolate is a biofilm-producing strain that was obtained in 2004 from the blood of an infected patient at the VAMC in Providence, RI.

**Antimicrobial activity.** Bacterial cells (10⁵ CFU/mL) were inoculated into Mueller-Hinton broth (Difco, Becton Dickinson) at 200 µL/well in 96-well microtiter plates. The minimum inhibitory concentration (MIC) of each pure metabolite was determined following methods recommended by the Clinical and Laboratory Standards Institute (CLSI) (2006a; 2006b). Briefly, two-fold serial dilutions of each compound were added to wells containing bacterial cells. After 24 h of incubation at 37 ºC, MICs were determined by visually inspecting each well for bacterial growth. The minimum bactericidal concentration (MBC), defined as the minimum concentration required to kill 99.9% of a bacteria inoculums, was determined by re-inoculating 20 µL of each culture medium from the microtiter plate wells onto tryptic soy agar plates. After 24 h of incubation at 37 ºC, MBCs were determined by visually inspecting the agar plates.
for bacterial growth. All MIC and MBC measurements were performed at least in duplicate.

**Biofilm Inhibition.** *Hypericum* metabolites that demonstrated growth inhibitory properties against staphylococci were tested for their ability to prevent biofilm formation by planktonic *S. aureus* and *S. epidermidis*. Biofilm production was quantified using a microtiter plate assay (Stepanovic et al., 2000). After overnight growth on tryptic soy agar (Difco, Becton Dickinson), stationary cultures of the biofilm-producing reference strains *S. aureus* (ATCC 35556), *S. epidermidis* (RP62A; ATCC 35984), and non-biofilm producing staphylococci were evaluated. A stable biofilm-negative mutant, M7, from the wild-type *S. epidermidis* RP62A served as the non-biofilm control. After 24 h of incubation at 37 °C, tryptic soy broth (Bacto, Becton Dickinson) and planktonic bacteria were removed by gently washing with sterile normal saline. The minimum biofilm inhibitory concentration (MBIC), which is the lowest concentration of an antimicrobial agent that results in no detectable biofilm growth, was quantified by staining the bacteria with 2% crystal violet and measuring optical density at 570 nm (Synergy 2, Bio-Tek Instruments, Inc, Winooski, VT). Wells containing sterile medium served as blanks for all absorbance readings and the non-biofilm isolate was used as a negative control. All tests were conducted in quadruplicate and the results were averaged.

**RESULTS AND DISCUSSION**

Each of the seven metabolites was first tested for growth inhibitory properties against the panel of pathogens. Compounds **1-5** demonstrated potent growth inhibition
of the Gram-positive bacteria (Table 1), with MIC and MBC values ranging from 1.95 
µg/mL to 7.81 µg/mL. The MBC values closely mirrored the MIC values, suggesting
a bactericidal mechanism of action. A comparison of MIC values for metabolites 3-5
shows that the acyl group modulates potency only minimally. The benzoyl derivative
5, isolated from *H. densiflorum*, is the most potent congener in this series, registering a
MIC of 1.95 µg/mL against biofilm-producing *S. aureus*.

Additional structure-activity relationships are apparent from the variable
activities of the metabolites. Attachment of the geranyl group to either C3 of the
phloroglucinol ring (1 and 2) or the C2-oxygen (3-5) had little effect on the
antibacterial properties of the *Hypericum* metabolites. However, a previous study
reported that similar acylphloroglucinol compounds from *Hypericum beania* lacking
the geranyl chain were 2-4 fold less active against *S. aureus* (Shiu and Gibbons, 2006),
highlighting the importance of this structural feature. Compounds 6 and 7 did not
produce any measurable activity against the Gram-positive bacteria. Introduction of a
methyl group and a second prenyl group (compound 6), leading to loss of aromaticity
in the phloroglucinol ring, resulted in loss of antibacterial activity. Elaboration of the
terpene moiety to include an additional two isoprene units (compound 7) also
abolished the antibacterial effects. The additional cyclization would limit
conformational flexibility of the terpene unit. The additional prenyl groups also
increase the lipophilicity, which would likely limit aqueous solubility and therefore
decrease intracellular concentrations (Appendino et al., 2008). Winkelmann *et al.*
reported modest anti-staphylococcal activity (32 µg/mL) for a related
Table 1: MIC/MBC and MBIC activities of *Hypericum* metabolites. All values are reported in μg/mL.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Biofilm Producing S. epidermidis (RP62A; ATCC 35984)</th>
<th>Non-Biofilm Producing S. epidermidis (M7)</th>
<th>Biofilm Producing S. aureus (ATCC 35556)</th>
<th>Clinical MRSA (L32)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MBIC</td>
<td>MIC</td>
</tr>
<tr>
<td>1</td>
<td>7.81</td>
<td>7.81</td>
<td>1.95</td>
<td>7.81</td>
</tr>
<tr>
<td>2</td>
<td>3.91</td>
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1 represents 3-geranyl-1-(2-methylpropanoyl)phloroglucinol, 2 represents 3-geranyl-1-(2-methylbutanoyl)phloroglucinol, 3 represents 2-geranyloxy-1-(2-methylpropanoyl)phloroglucinol, 4 represents 2-geranyloxy-1-(2-methylbutanoyl)phloroglucinol, 5 represents 2-geranyloxy-4,6-dihydroxybenzophenone, 6 represents elliptophenone A, 7 represents prolificin A.
acylphloroglucinol lacking one of the additional isoprene units of 7, further demonstrating the detrimental effects of the cyclization and/or increased lipophilicity (Winkelmann et al., 2003).

Although five of the Hypericum compounds displayed growth inhibitory effects against the Staphylococcus test strains, all were inactive against the Gram-negative bacteria (E. coli, P. aeruginosa, A. baumanii) at the highest test concentration of 125 µg/mL. Structural differences between Gram-positive and Gram-negative bacterial cell walls may account for the variable activities. Gram-negative bacteria possess an outer lipopolysaccharide membrane surrounding the cell wall whereas Gram-positive bacteria do not. The lack of an outer lipopolysaccharide membrane in the Gram-positive bacteria may allow increased permeability of Hypericum metabolites into cells (Tian et al., 2009). These results are in agreement with previous studies of Hypericum extracts that have shown weak to no activity against E. coli (Bussmann et al., 2010; Jayasuriya et al., 1989; Rabanal et al., 2002; Schwob et al., 2002; Vajs et al., 2003).

Due to growing interest in antimicrobial agents that can treat infections caused by biofilm forming bacteria, the active metabolites (1-5) were tested for their ability to attenuate biofilm production by each of the Staphylococcus species. The MBIC values of the Hypericum metabolites ranged from 1.95 µg/mL to 7.81 µg/mL. Metabolite 2, isolated from H. punctatum, displayed the most potent biofilm inhibition against S. aureus and S. epidermidis at an MBIC of 1.95 µg/mL. Compounds 1, 2, and 3 also inhibited biofilm formation at concentrations below their respective MIC and MBC values against some test strains. Compounds with 2-methylpropanoyl groups (1 and 3)
consistently demonstrated MBIC values at or below their respective MIC values. Interestingly, a study by Socolsky et al. showed that other acylphloroglucinols isolated from *Elaphoglossum yungense* produced a modest increase in the amount of biofilm of *S. aureus* and *P. aeruginosa* at 10 µg/mL, despite reducing growth (Socolsky et al., 2010).

The results of these studies add to growing knowledge of the antimicrobial properties of acylphloroglucinol metabolites. Other studies have reported acylphloroglucinol derivatives possessing antimicrobial activity against staphylococci (Gibbons et al., 2005; Henry et al., 2009; Ishiguro et al., 1994; Pecchio et al., 2006; Schempp et al., 1999; Winkelmann et al., 2001; Winkelmann et al., 2003). A study by Socolsky et al. reported that acylphloroglucinols containing an additional chromene substituent possess antimicrobial activity against *P. aeruginosa*, indicating that certain acylphloroglucinols may possess useful antimicrobial effects against Gram-negative pathogens (Socolsky et al., 2010). While a detailed mechanism of action for the antimicrobial activities of acylphloroglucinols remains to be determined, Hubner and colleagues showed that reduced sensitivity of *S. aureus* to hyperforin did not lead to a cross resistance against clinically used antibiotics (Hubner, 2003), therefore suggesting a possible unique mechanism of action. Shiu et al. found that certain acylphloroglucinols retained activities against tetracycline, fluoroquinolone, and macrolide drug-resistant strains, thereby also suggesting alternate mechanisms of action (Shiu and Gibbons, 2006). These studies further highlight the promise of investigating acylphloroglucinol derivatives as novel agents to treat bacterial disease.
Several of the metabolites in this study have previously demonstrated biological activities in other medically relevant assays. Compounds 3, 4, 5 and 7 are reported to have antitumor activity with IC$_{50}$ values ranging from 4.1 to 36 µM (Henry et al., 2009; Henry et al., 2006). Additionally, compounds 3, 4, and 5 display antioxidant activity and cyclooxygenase (COX) inhibition (Henry et al., 2006).

The potent anti-staphylococcal activity of the Hypericum metabolites suggests that these deserve further consideration as novel antibacterial agents. Staphylococcus aureus is recognized as an important human pathogen able to adapt and evolve in terms of its resistance traits and virulence factors; it is among the most important causes of human infections in both the hospital and community settings (Tang and Stratton, 2010). Clinical antibiotics currently used to treat MRSA infections include daptomycin, linezolid, and vancomycin. It is interesting to note that these clinically used antibiotics have shown MIC values that are similar to our active Hypericum spp. metabolites (Brunton et al., 2006). These bioactive metabolites further highlight the potential for finding structurally new antibacterial agents from plants, and add to our understanding of structure-activity relationships for phloroglucinols that limit growth and biofilm production by pathogenic bacteria.

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