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Riley D. Kirk
University of Rhode Island

Margaret E. Rosario
University of Rhode Island

Nana Obie
University of Rhode Island

Terra Marie M. Jouaneh
University of Rhode Island

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Screening the PRISM library against *Staphylococcus aureus* reveals a sesquiterpene lactone from *Liriodendron tulipifera* with inhibitory activity

Riley D. Kirk,† Margaret E. Rosario,‡ Nana Oblie,† Terra Marie M. Jouaneh,‡ Marina A. Carro,‡ Christine Wu,‡ Andrew M. Kim, † Elizabeth Leibovitz,‡ Elizabeth Sage Hunter,‡ Robert Literman,‡ Sara M. Handy,‡ David C. Rowley,† Matthew J. Bertin†,*

†Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States

‡Center for Food Safety and Applied Nutrition, Office of Regulatory Science, U.S. Food and Drug Administration, College Park, Maryland 20740, United States
ABSTRACT

Infections caused by the bacterium *Staphylococcus aureus* continue to pose threats to human health and put a financial burden on the healthcare system. The overuse of antibiotics has contributed to mutations leading to the emergence of methicillin-resistant *Staphylococcus aureus*, and there is a critical need for the discovery and development of new antibiotics to evade drug resistant bacteria. Medicinal plants have shown promise as sources of new small molecule therapeutics with potential uses against pathogenic infections. The Principal Rhode Island Secondary Metabolite (PRISM) library is a botanical extract library generated from specimens in the URI Heber W. Youngken Jr. Medicinal Garden by upper-division undergraduate students. PRISM extracts were screened for activity against strains of methicillin-susceptible *S. aureus* (MSSA). An extract generated from the tulip tree (*Liriodendron tulipifera*) demonstrated growth inhibition against MSSA, and a bioassay-guided approach identified a sesquiterpene lactone, laurenobiolide, as the active constituent. Intriguingly, its isomers tulipinolide and epi-tulipinolide lacked potent activity against MSSA. Laurenobiolide also proved to be more potent against MSSA than the structurally similar sesquiterpene lactones costunolide and dehydrocostus lactone. Laurenobiolide was most abundant in the twig bark of the tulip tree, supporting the twig bark’s historical and cultural usage in poultices and teas.
INTRODUCTION

Infections caused by pathogenic bacteria claim millions of lives yearly and accrue billions of dollars in healthcare costs. The discovery and implementation of antibiotics to combat pathogens have saved millions of lives worldwide since the discovery of penicillin by Sir Alexander Fleming in 1928. Apart from inhibitors of cell wall biosynthesis like the β-lactam antibiotics, other classes of antibiotic molecules with distinct mechanisms of action such as the inhibition of protein biosynthesis, the inhibition of DNA synthesis, or the inhibition of folic acid metabolism are now routinely utilized in the clinic. The pharmacophores for the majority of these drugs were initially identified from natural products, primarily from microbial sources. However, the emergence and spread of antibiotic drug resistance among bacterial pathogens now represent an existential threat to our healthcare systems.

*Staphylococcus aureus* causes severe health and economic problems associated with morbidity, mortality, and extended hospital stays due to invasive infections. While methicillin-resistant *S. aureus* (MRSA) is of major concern in hospital settings, methicillin-susceptible *S. aureus* (MSSA) can be the primary cause of invasive *S. aureus* infections in the hospital. Examining an infant cohort with invasive *S. aureus* infections from 1997-2012 showed that infant mortality after infection was similar for MRSA and MSSA, and MSSA actually caused more infant infections and more deaths than MRSA. Methicillin-susceptible *Staphylococcus aureus* (MSSA) can infect multiple human organs, including the skin which is the most prevalent. Untreated MSSA can lead to invasive infections in the bloodstream that lead to life threatening conditions such as sepsis.

Plant metabolites offer promise in providing new molecular scaffolds to use as antibiotics either in isolation or in combination with currently used treatments to increase efficacy.
same specialized metabolites that a plant may use to combat microbes in the environment can potentially be harnessed to combat human pathogens. The tulip tree (Liriodendron tulipifera) has been of interest to medicinal chemists for its purported biological properties and cultural and historical use. Liriodendron tulipifera is a large flowering deciduous tree endemic across the eastern United States. Indigenous Americans valued the tulip tree for its timber. Medicinally it was utilized as a tonic, antipyretic, and anti-malarial agent. During the Civil War, Samuel Preston Moore, Surgeon General of the Confederacy, responded to the blockade of medicines by the Union Army by building new field hospitals and commissioning a study of medicinal plants of the Southeast to treat soldiers. Francis Porcher led the search for alternative plant medicines, conducting a deep study of the Southeast principally based on the cultural legacy of Cherokee healers. He ultimately compiled a book containing plant remedies native to the Southern States to help soldiers deal with injuries. One of the plants he highlighted was the tulip tree which was used for rheumatism, gout, laxative, headache, malaria and other purposes. The described bioactivities were later validated by discovery of anti-plasmodial aporphine alkaloids and sesquiterpene lactones from the tree. While antibacterial activity has been demonstrated historically from plant preparations and recently from extracts, no studies have yet identified the antibacterial constituents of the tulip tree.

The Heber W. Youngken Jr. Medicinal Garden at the University of Rhode Island is a botanical collection of nearly 300 medicinal plant species. In 2019, we developed an extract library of aqueous and organic extracts of the aerial and root portions of specimens found in the garden. This resource, the Principal Rhode Island Secondary Metabolite (PRISM) library, was screened for antibiotic activity against MSSA. The library had 110 extracts from 26 plants at the time this work was carried out.
Herein, we identified the organic (methanol) extract of the tulip tree leaves \((Liriodendron tulipera)\) as having growth inhibitory properties against \(S.\ aureus\) DMS 1104, an MSSA strain. Using bioassay-guided isolation, the bioactive constituent was identified as the sesquiterpene lactone laurenobiolide. We further investigated the relative abundance of this compound in various parts of the tulip tree demonstrating that the compound was present in higher concentrations in the twig bark when compared to other plant parts. Additionally, we examined other Magnoliaceae specimens to determine if they produced laurenobiolide and other related sesquiterpene lactones. Finally, we evaluated a panel of structurally similar sesquiterpene lactones (Figure 1) for their ability to inhibit MSSA.

**Figure 1.** The primary active compound against MSSA was determined to be the sesquiterpene lactone laurenobiolide in this study. Other sesquiterpene lactones identified from \(Liriodendron tulipifera\) and magnolias were tulipinolide, epi-tulipinolide, costunolide, and dehydrocostus lactone.

**RESULTS AND DISCUSSION**
Screening the PRISM library for anti-MSSA activity revealed five active extracts from the 110 tested. These were the methanol extracts of *Amorpha fruticosa* flowers and leaves, a combined leaf and flower methanol extract from *Isatis tinctora*, a methanol extract from the aerial parts of a *Datura* sp., and a methanol extract derived from *L. tulipifera* leaves that provided reproducible activity against methicillin-susceptible *S. aureus* (Figure S1). This *L. tulipifera* extract was subsequently prioritized for further study due to its documented historical uses as an antimicrobial treatment. High performance liquid chromatography (HPLC) (Figure S2) was used to visualize the complex metabolite profile leading to our strategy for bioassay-guided fractionation. The tulip tree extract was first fractionated on a C18 column using a stepwise gradient of CH₃OH in H₂O. Five fractions were generated, and the most robust activity against MSSA was observed from constituents that eluted with 60% and 80% CH₃OH (Figure S3). A more polar fraction also initially displayed antibacterial activity, but upon retesting individually isolated components, it seemed to be an additive effect of multiple compounds rather than a single potent entity responsible for the activity. Upon visual analysis of the HPLC chromatograms for each fraction, the most active fractions contained two large peaks that eluted at 30 minutes from the column (Figure S3). We speculated that a compound in one of these peaks could be responsible for the observed activity.

The two prominent peaks were isolated using semi-preparative HPLC and the latter eluting peak exhibited the largest zone of inhibition in the MSSA assay (Figure S4A). The peak purity was checked using a phenyl-hexyl column paired with HPLC-DAD analysis. We determined that the earlier eluting peak (peak 1) was a single pure compound while the later eluting peak was two separate analytes (Figure S4B). Upon repurification and retesting of the three compounds, it was determined that only peak 2.1 had antimicrobial activity, while peaks 1
and 2.2 did not show any observable activity in the disk diffusion assay (Figure S4C). Analysis and dereplication via 1D and 2D NMR (Figure S5 – S9 and S11) and HRESIMS (Figure S10) revealed that these three metabolites were isomers and the active peak 2.1 was determined to be laurenobiolide. HRESIMS of 2.1 gave an m/z of 313.1418 ([M+Na]+, calcd: 313.1416 for C₁₇H₂₂O₄Na). The specific rotation measured for peak 2.1: [α]²³D 16.8 (c 1.0, EtOH) closely matched the literature value of [α]²⁵D 17.1 (EtOH) for laurenobiolide.21 Additionally, examination of the ¹H NMR spectra of peak 2.1 showed an envelope of olefinic protons from δ 4.70 to 5.50 consistent with the original characterization of this metabolite by Tada and Takeda in 1971,²² and the ¹H NMR spectrum clearly showed the presence of conformational isomers, a phenomenon previously described for laurenobiolide during NMR analysis (Figure S6).²³,²⁴ Examination of correlations from COSY, HSQC, and HMBC NMR data confirmed the identification (Figures S7-S9). Examination of ¹H NMR spectra identified peak 1 as epi-tulipinolide (Figure S5), and peak 2.2 as tulipinolide (Figure S11). Evidence included a multiplet resonance at δ 5.72 in peak 1 corresponding to H-8, which was in a cluster of resonances between δ 4.76 and 5.03 in peak 2.2. Furthermore, the acetyl methyl groups at δ 2.09 and 2.06 were consistent with those of tulipinolide and epi-tulipinolide, respectively, as detailed in the original isolation and elucidation work, as were the olefinic methyl resonances.²⁵ Interestingly, among these three sesquiterpene lactones that are extremely similar in structure (Figure 1), only laurenobiolide displayed antibiotic activity towards S. aureus DMS 1104. The minimum inhibitory concentration (MIC) for laurenobiolide and a panel of sesquiterpene lactones was determined via broth dilution assay (Table 1). Laurenobiolide showed the strongest potency with an MIC of 7.8 µg/mL. Costunolide, one of the terpenoids devoid of an acetyl group, had an MIC
value of 31 µg/mL. Epi-tulipinolide, tulipinolide and dehydrocostus lactone had MIC values of 62 µg/mL, 250 µg/mL, and 62 µg/mL, respectively.

### Table 1. MIC values for sesquiterpene lactones tested against *S. aureus* DSM 1104

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laurenobiolide</td>
<td>7.8</td>
</tr>
<tr>
<td>Epi-tulipinolide</td>
<td>62</td>
</tr>
<tr>
<td>Tulipinolide</td>
<td>250</td>
</tr>
<tr>
<td>Costunolide</td>
<td>31</td>
</tr>
<tr>
<td>Dehydrocostus lactone</td>
<td>62</td>
</tr>
<tr>
<td>Honokiol</td>
<td>7.8</td>
</tr>
<tr>
<td>Gentamicin sulfate (positive control)</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Laurenobiolide was evaluated for its cytotoxic effects to HaCaT cells, a commonly studied human keratinocyte cell line. The compound demonstrated cytotoxic effects at 2.9 µg/mL showing 13% cell viability as compared to vehicle control (Figure S12). Epi-tulipinolide and tulipinolide showed less cytotoxic activity against the HaCaT cells at 29 µg/mL with cell viability of 66% and 84%, respectively (Figure S12). Doses at 0.29 µg/mL and lower did not show cytotoxicity. These observations are consistent with those of Dettweiler and coworkers, who showed that branch bark extracts of *L. tulipifera* possessed some mammalian toxicity when tested against cells.15

Next, we investigated which parts of the tulip tree contained the highest concentrations of laurenobiolide. HPLC-DAD and LC-MS/MS analysis were performed to evaluate the presence of this constituent in the trunk bark, leaves, and twigs of the tulip tree. Laurenobiolide was most abundant in the twigs, and low amounts were detected in the leaves, but no laurenobiolide was detected in the trunk bark (Figure S13). These findings could be an artifact of the sampling approach where outer bark pieces were removed non-invasively so as to not damage the tree. Further analysis showed the presence of laurenobiolide in the twig bark, but not the inner twig tissue (Figure S14). These observations are consistent with some of the documented traditional
Cherokee uses of the twig bark for medicine, and these data will now inform future investigations of plant parts while provoking intriguing questions about the ecological role of laurenobiolide. Additionally, the twig itself showed zones of inhibition when tested against MSSA, while other plant parts tested did not show activity (Figure 2). A poultice-like preparation made with twigs and jojoba oil showed a zone of inhibition, while a portion of ‘stripped twig’ with the outer covering removed did not (Figure S15). This helps connect the historic use of the twig bark poultices with the data on laurenobiolide discovered in this report.

Figure 2. MSSA inhibition assay with *L. tulipifera* plant parts. Twig, trunk bark, flower, and leaf were directly placed onto MSSA plates. Plant parts were surface sterilized with methanol and dried prior to evaluation. The zone of inhibition can be seen for the twig specimen. Gentamicin sulfate was used as the positive control.

We also examined several magnolia species for the presence of laurenobiolide and other sesquiterpene lactones. Laurenobiolide, epi-tulipinolide, and tulipinolide were only detected in *L. tulipifera* (Figure 3A and 3B). However, costunolide was detected in *L. tulipifera*, *Magnolia virginiana*, and *Magnolia acuminata* (Figure 3C and Figure S16), and dehydrocostus lactone was detected in *M. macrophylla* and *M. virginiana* (Figure S17). This was confirmed by comparison of the extracts to authentic standards of dehydrocostus lactone and costunolide. Additionally,
costunolide and dehydrocostus lactone were identified as library hits on the GNPS Social Molecular Networking website following library analysis (Figures S16 and S17).

Figure 3. Sesquiterpene lactones in *Liriodendron tulipifera* and three other *Magnolia* spp. A) Epi-tulipinolide (E), laurenobiolide (L) and tulipinolide (T) were only detected in *L. tulipifera* extracts. B) An extracted ion chromatogram (m/z 231) shows the base peak of the three acetylated sesquiterpene lactones in *L. tulipifera*, and an unidentified metabolite in *M. virginiana* and *M. acuminata*. C) An extracted ion chromatogram (m/z 233) shows the presence of costunolide in *L. tulipifera*, *M. virginiana* and *M. acuminata*. D) Amongst the magnolias tested, only the *M. macrophylla* extract showed anti-MSSA activity. Gentamicin sulfate, an aminoglycoside antibiotic, was used as the positive control (20 µg/disk; upper left quadrant on each plate). The test concentration of the extracts was 200 µg/disk, and each extract was tested in duplicate. Negative controls are in the bottom left quadrant of each plate.

Interestingly, other than *L. tulipifera*, only the *M. macrophylla* extract showed inhibition of MSSA amongst the magnolia extracts tested (Figure 3D). To determine the active components in the *M. macrophylla* extract, we conducted bioassay-guided isolation. Following additional inhibition tests, we characterized honokiol as the major antibacterial component in the *M.*
*macrophylla* extract (MIC = 7.8 µg/mL against MSSA). Honokiol has reported potent anti-MSSA activity with MIC values as low as 2.5 µg/mL. The *M. denudata* extract did not show the presence of any of the known sesquiterpene lactones on which we focused (Figure S18) and its extract was inactive against MSSA (Figure 2D). Even though *M. virginiana* and *M. acuminata* extracts contained costunolide, which showed modest inhibitory activity against MSSA, the concentration was likely too low to show antibacterial activity.

While *Magnolia* and *Liriodendron* are both genera in the magnolia family, *Liriodendron* is the only genus in the Liriodendroidae subfamily, which consists of two species: *L. tulipifera* and *L. chinense*. Single nucleotide polymorphism analysis from DNA sequencing results of the specimen used in the current report clearly showed that the specimen was a *Liriodendron* and specifically *L. tulipifera* (Figure S19). It will be intriguing to examine authentic specimens of *L. chinense* to compare the metabolite profile and sesquiterpene lactone composition between the two *Liriodendron* species.

We searched for biosynthetic genes that could putatively generate sesquiterpene lactones such as laurenobiolide in publicly available *L. tulipifera* RNAseq data. The search was based on the pathway identified in *Helianthus annuus*. This pathway synthesizes a sesquiterpene lactone from inunolide or eupatolide using germacrene-A synthase/hydroxylase, and a cytochrome P450 enzyme. This pathway is predicted to occur widely in Viridiplantae, and germacrene-A has been identified in the closely related genus *Magnolia*. We recovered *L. tulipifera* transcripts for farnesyl pyrophosphate synthase, cytochrome P450 (E-class, group IV), acetyl-CoA acetyltransferase (cytosolic), and multiple terpene cyclases including germacrene D synthase (Table S1). Although we found seven distinct terpene cyclases, we did not find a transcript with sufficient homology to indicate the presence of germacrene A synthase or germacrene-A
hydroxylase in the *L. tulipifera* transcriptome. We would predict that laurenobiolide would derive from germacrene A acid. Terpene cyclases can have wide substrate affinity, and it is possible that laurenobiolide is being synthesized via a novel biosynthetic pathway. However, due to the lack of a reference genome (this analysis utilized RNAseq data) it is also possible these germacrene A genes were simply not expressed in the tissue at the time of collection. Further genomic and transcriptomic investigations are needed to discern whether this compound is being synthesized via an undescribed biosynthetic pathway, or with germacrene-A, as in *Helianthus annuus*.

There has been a consistent effort to develop drugs with unique chemical scaffolds and new mechanisms of action to combat pathogen resistance. While relatively few antibacterial compounds have been identified in the terpene class, terpenes and terpenoids are an incredibly diverse group of phytochemicals that possess potential as potent antimicrobials. The exact mechanism of action of many terpene antibiotics is unknown, but Griffin and coworkers identified that certain terpene metabolites inhibit two essential processes, which include oxygen uptake and oxidative phosphorylation.

**CONCLUSION**

The tulip tree extract has documented antimicrobial activity, yet the constituents responsible for the activity had not been identified until now. In this report, we have determined for the first time that the metabolite laurenobiolide isolated from *Liriodendron tulipifera* possesses potent anti-MSSA activity and is the likely component responsible for the antiseptic activity ascribed to the tulip tree. This work sheds light on the intriguing story of the cultural and historical use of tulip tree preparations. The structural features that allow laurenobiolide to inhibit MSSA growth, while very closely related structures do not inhibit growth, remains an
intriguing observation that we plan to explore in future studies. The most likely anti-MSSA structural features would appear to be the position of double bonds and/or the absolute configuration about the ring juncture. Furthermore, we have demonstrated the utility of the PRISM library as a source of molecular potential in therapeutic areas, encouraging its future expansion and use in screening efforts. Future research will focus on the mechanism of action of laurenobiolide and its ecological role for the tree. Additionally, we will continue to search for antibacterial components from magnolias and other botanical specimens.

**MATERIALS AND METHODS**

**General Experimental Procedures.** Optical rotations were measured using a Jasco P-2000 polarimeter. NMR spectra were recorded using an Agilent NMR 500 MHz spectrometer with (CD$_3$)$_2$SO (referenced to residual DMSO at $\delta_H$ 2.50 and $\delta_C$ 39.5) or CDCl$_3$ (referenced to residual CHCl$_3$ at $\delta_H$ 7.26 and $\delta_C$ 77.2) at 25 °C. HRESIMS analysis was performed using an AB SCIEX TripleTOF 4600 mass spectrometer with Analyst software. MS/MS data were recorded on this same instrument using the product ion function. LC-MS/MS was performed using a ThermoFisher LTQ XL mass spectrometer with an electrospray ionization (ESI) source, and certain mass spectrometry experiments (standard analysis) were completed using a ThermoFisher ISQ mass spectrometer. Both the LTQ and ISQ were coupled to Dionex Ultimate 3000 HPLC systems equipped with a micro vacuum degasser, an autosampler, and diode-array detector (DAD). Semi-preparative and analytical HPLC was carried out using a Dionex UltiMate 3000 HPLC system equipped with a micro vacuum degasser, an autosampler, and a DAD. Standard compounds, costunolide and dehydrocostus lactone, were purchased from Sigma-Aldrich.

**Plant Harvesting and Preparation of Plant Extracts.** Specimens of the tulip tree (*Liriodendron tulipifera* L., Magnoliaceae), the yulan magnolia (*Magnolia denudata* Desr.,
Magnoliaceae, Kiri-01292), the sweetbay magnolia (Magnolia virginiana L., Magnoliaceae, Kiri-01276), the cucumber tree (Magnolia acuminata L., Magnoliaceae, Kiri-01293), and the bigleaf magnolia (Magnolia macrophylla Michx., Magnoliaceae, Kiri-01288) were harvested from the University of Rhode Island campus, and voucher specimens of the magnolias can be found in the KIRI herbarium. Biological material from the L. tulipifera specimen is held in the lab of the corresponding author (voucher 8038051126). Plant extracts in the PRISM library were prepared as previously described from specimens in the Heber W. Youngken Jr. Medicinal Garden. Briefly, plants were separated into aerial and underground portions, then further divided (twigs, leaves, etc.) for extraction in both aqueous and organic (methanol) solvents. Each solvent was vacuum filtered to remove particulates, then either freeze-dried or evaporated under reduced pressure to remove remaining solvent. Dried product was reconstituted at 10 mg/mL in DMSO or CH$_3$OH depending on solubility.

**Methicillin-Susceptible Staphylococcus aureus Inhibition Assays.** S. aureus strain DSM 1104 was cryopreserved and stored at -80 °C. The strain was grown in Luria Bertani (LB) broth (Difco, Fisher scientific, Waltham, MA) at 37 °C, 150 rpm for 20 h. Plant extracts were prepared as described above and 20 µL were loaded onto sterile antimicrobial susceptibility test disks (Oxoid, Thermo Fisher, Franklin, MA). The disks were air dried for 30 min. Control disks were prepared with 20 µL MeOH and similarly dried. The disks were then added to Petri dishes (100 × 15 mm) containing 15 mL of LB agar (Alpha biosciences, Baltimore, MD) inoculated with DSM 1104. Plant parts that were directly tested against DSM 1104 were first sterilized by wiping with CH$_3$OH and then dried before testing. Gentamicin sulfate (20 µg/disk) was the positive control for MSSA. Negative vehicle controls were DMSO or CH$_3$OH (20 µL). Plates
were incubated at 37 °C for 18 h after which the diameters of the zones of growth inhibition were measured to the nearest mm.

**Minimum Inhibitory Concentration (MIC) of Sesquiterpene Lactones.** Minimum inhibitory concentrations (MICs) were determined in duplicate by broth microdilution in LB medium as previously described, and in accordance with Clinical Laboratory Standards Institute (CLSI) standards. Staphylococcus aureus DSM 1104 was obtained from the American Type Culture Collection (ATCC 25923™). Test samples were prepared at 10 mg/mL in DMSO. Gentamicin sulfate (2 mg/mL) in H2O was the positive control and DMSO was the negative control.

**Bioassay Guided Fractionation of Crude Plant Extracts.** We used 6.97 g of dried leaves for initial extraction in CH3OH, which resulted in 140.5 mg of extract. The tulip tree leaf organic extract was further fractionated using a C18 SPE cartridge to purify samples before additional testing. Five fractions were produced from the extract, beginning with the most polar (80% H2O: 20% CH3OH) and ending with the least polar (100% CH3OH). Following solvent removal, the chromatography fractions were reconstituted at 10 mg/mL in mobile phase followed by HPLC-DAD analysis using a Thermo Scientific Acclaim™ 3 µm C18 column (150 × 3 mm). A 70 min gradient method was utilized beginning with 15% acetonitrile (CH3CN) for 10 min, then increasing the concentration of CH3CN until 45 min, with a 20 min hold at 100% CH3CN, followed by a return to starting conditions for 5 min. Chromatograms were visually evaluated in parallel with repeated antimicrobial assays to identify potentially active compounds.

**Isolation of Sesquiterpene Lactones and Honokiol from Liriodendron tulipifera and Magnolia macrophylla.** Having identified the potential active components in the tulip tree chromatography fractions following initial chromatogram analysis, fractions 3 (60% H2O/40% CH3OH) and 4 (80% H2O/20% CH3OH) were combined and subjected to reversed-phase semi-
preparative HPLC using a Kinetex 5 μm C18 column (250 × 10 mm) and a gradient method. The mobile phase was 60% CH₃CN/40% H₂O with 0.05% formic acid (FA) added to each solvent with a flow rate of 3.0 mL/min. Initial conditions were held for 10.5 min followed by a linear gradient to 12.0 min reaching 100% CH₃CN and holding at this concentration for 4.0 min, ultimately returning to initial conditions from min 17-20. Two chromatographic peaks were collected at tᵣ 9.5 (peak 1, epi-tulipinolide, 3.3 mg) and 10.0 min (peak 2), respectively. Peak 2 was further purified using a Luna® phenyl-hexyl 5 μm column (250 × 10 mm) and an isocratic method. The mobile phase was 55% CH₃CN/45% H₂O with 0.05% FA added to each solvent and a flow rate of 3 mL/min. Peaks 2.1 (laurenobiolide, 1.7 mg; tᵣ 13.5 min) and 2.2 (tulipinolide, 1.5 mg; tᵣ 14.25 min) were isolated. The CH₃OH branch extract of *M. macrophylla* was subjected to RP-HPLC using the same isocratic method as described above. The mobile phase was 55% CH₃CN/45% H₂O with 0.05% FA added to each solvent and a flow rate of 3 mL/min. A single peak eluting at 18.25 min was collected and identified as honokiol (4 mg).

*Laurenobiolide (major conformer):* colorless oil; [α]²³° +16.8 (c 1.0, EtOH) ¹H NMR (DMSO-d₆, 500 MHz) δ 6.14 (1H, br s, H-13a), 5.86 (1H, br s, H-13b), 5.45 (1H, t, J = 9.9 Hz, H-6), 5.15 (1H, m, H-1), 4.72 (1H, t, J = 10.8 Hz, H-5), 4.24 (1H, dd, J = 9.5, 6.5 Hz, H-8), 3.27 (1H, m, H-7), 2.32 (1H, m, H-2a), 2.14 (1H, m, H-3a), 2.11 (2H, m, H-9), 2.10 (1H, m, H-2b), 1.98 (1H, m, H-3b), 2.03 (3H, s, H-17), 1.59 (3H, s, H-15), 1.58 (3H, s, H-14); ¹³C NMR (DMSO-d₆, 125 MHz) δ 169.7 (C, C-12), 169.6 (C, C-16), 139.3 (C, C-4), 138.1 (C, C-10), 130.3 (C, C-11), 128.9 (CH₂, C-13), 126.4 (CH, C-5), 125.4 (CH, C-1), 79.8 (CH, C-8), 71.8 (CH, C-6), 50.2 (CH, C-7), 38.4 (CH₂, C-3), 35.9 (CH₂, C-9), 24.7 (CH₂, C-2), 23.1 (CH₃, C-17), 21.3 (CH₃, C-15), 21.2 (CH₃, C-14); HRESIMS m/z 313.1418 ([M+Na]+ (calcd for C₁₇H₂₂O₄Na, 313.1416).
The laurenobiolide $^1$H NMR data have been deposited at NP-MRD (np-mrd.org/natural_products) (NP0048592).

**LC-MS/MS Analysis of *L. tulipifera* Plant Parts and Magnolia Specimens and Database Searching.** Samples of tulip tree trunk, twig, and leaf were taken from the *L. tulipifera* specimen on the URI campus and methanol extracts were made using 1.0 g portions of each *L. tulipifera* plant part. Additionally, twig extracts were generated from 1 g portions of *M. denudata, M. virginiana, M. acuminata,* and *M. macrophylla*. Each extract was subjected separately to HPLC-DAD and LC-MS/MS analysis with a specific scan event recording MS/MS spectra in data-dependent acquisition mode. A Kinetex 5 μm C18 column (150 × 4.6 mm) was used for separation of analytes in the extracts. The LC method consisted of a linear gradient from 15% to 100% CH$_3$CN in H$_2$O + 0.1% FA over 20 min, followed by an isocratic period at 100% CH$_3$CN of 5 min. The flow rate was held constant at 0.4 mL/min. The MS spray voltage was 3.5 kV with a capillary temperature of 325 °C. For the MS/MS component, the CID isolation width was 1.0 and the collision energy was 35.0 eV. Following acquisition, the raw data files were converted to .mgf format using MSConvert from the ProteoWizard suite (http://proteowizard.sourceforge.net/tools.shtml). Library searching was performed using the online platform at Global Natural Products Social Molecular Networking website (gnps.ucsd.edu). Both raw LC-MS/MS files and converted .mzXML files are available in a MassIVE dataset - accession MSV000089948.

**Detection of Sesquiterpene Lactones in *L. tulipifera* Plant Parts and Magnolias.** *L. tulipifera* twig, leaf, and trunk bark CH$_3$OH extracts (10 mg/mL) were subjected to HPLC-DAD analysis to evaluate the presence of laurenobiolide. Branch CH$_3$OH extracts from *L. tulipifera, M. denudata, M. virginiana, M. acuminata,* and *M. macrophylla* were subjected to HPLC-DAD and
LC-MS/MS analysis to specifically investigate the presence of the sesquiterpene lactones epi-tulipinolide, laurenobiolide, and tulipinolide. The chromatography parameters for HPLC-DAD and LC-MS/MS were identical. The Luna® phenyl-hexyl 5 μm column (250 × 4.6 mm) was used for separation of analytes. The mobile phase was 55% CH₃CN/45% H₂O with 0.05% formic acid added to each solvent and flow rate of 0.6 mL/min. Following the HPLC-DAD and LC-MS/MS analysis, the presence of costunolide and dehydrocostus lactone was confirmed by chromatographic comparison to authentic standards using the method described above. These raw LC-MS/MS files and converted .mzXML files are also available in the same MassIVE dataset - accession MSV000089948.

**Culturing Human Keratinocytes (HaCaT).** Human keratinocytes (HaCaT) were a kind gift from the laboratory of Dr. Navindra Seeram at URI, and originally purchased from American Type Cell Culture Collection. The culture was maintained in Dulbecco’s modified Eagle’s medium (DMEM) and the DMEM was supplemented with 10% fetal bovine serum. The incubator was kept at 37 °C with 5% CO₂ at constant humidity. All test samples were dissolved in sterile DMSO for viability assessments.

**Cytotoxic Evaluation of Keratinocytes (HaCaT) in the Presence of L. tulipera Compounds.** The viability of HaCaT cells in the presence of laurenobiolide, tulipinolide, and epi-tulipinolide was evaluated using cell titer glo (CTG 2.0). HaCaT cells were seeded in a sterile 96-well plate at 5×10³ cells per well and incubated for 12 h. Post incubation, the media was removed, and compounds were added along with new media, followed by a 24 h incubation. The CTG 2.0 reagent was added to each well and shaken at 200 rpm for 2 min using an orbital shaker. The plate was allowed to equilibrate at room temperature for 10 min, and then the luminescence was recorded using a Spectramax M2 plate reader. Viability was determined by comparing
luminescence values of treated wells to those of the vehicle control (DMSO) (n = 4 per treatment). Cannabidiol (31 µg/mL) was used as a positive control.40

**Identifying Putatively Useful Nuclear Markers from Whole-Genome Sequence (WGS)**

**Data.** We used the SISRS bioinformatics package41 to develop nuclear genetic markers for the (1) delineation of *Liriodendron* spp. from other Magnoliaceae and (2) to delineate *L. chinese* and *L. tulipifera*. WGS data for six *L. tulipifera*, six *L. chinese*, and 83 specimens of *Magnolia* spp. were downloaded from the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). All read data were trimmed using the *bbmap* suite (https://sourceforge.net/projects/bbmap/) using a sliding-window with a Q10 cutoff and removing reads with a final average quality less than Q15 and/or length less than 50 bp. To enrich each dataset for nuclear data, we then used *bbmap* to remove any reads that mapped against a pooled set of 253 chloroplast assemblies and 5 mitochondrial assemblies downloaded from NCBI. Based on a nuclear genome size estimate for the group of 2.5 Gb, we subset reads from each species such that the final combined read depth was ~25 Gb (10X coverage), with reads sampled evenly across the 69 species and specimens therein. These subset reads were then pooled and used as input for a genome assembly using Ray with default parameters,42 with the resulting assembly representing loci that are conserved enough among species to be assembled and compared easily among taxa.

**Identifying Fixed Sites for Use in Species and Genus Delineation Assays.** To identify genomic sites that would be useful for species delineation between *Liriodendron* species, we mapped the trimmed reads from all twelve *Liriodendron* specimen against the SISRS composite genome using *bbmap* and identified all sites that were (1) fixed and identical within species for all six *L. tulipifera* or *L. chinese* specimens and (2) variable between species. A total of 164,710 species-specific SNPs were identified.
To identify SNPs that can delineate Liriodendron from Magnolia, we first identified all sites in the composite genome that were fixed and identical among all twelve Liriodendron specimens. Next, after setting aside eight Magnolia specimens for downstream testing, we removed any sites where those fixed Liriodendron alleles were found in any of the remaining 73 Magnolia samples. Magnolia samples had variable read coverage, and not finding a Liriodendron specific allele could be due to either (1) the allele not being present or (2) the allele not being sampled. To make a more conservate estimate of robust genus-specific SNPs, we only considered SNPs where there was read coverage in at least half (n=38) of the Magnolia specimens. A total of 630,741 Liriodendron-specific SNPs were identified.

**Testing Genus- and Species-Specific Genetic Markers.** To assess the ability of the genetic SNP markers to identify samples to the genus and species level, we mapped read data from eight Magnolia specimens, two L. tulipifera samples, and one L. chinese sample against the SISRS composite genome. Alleles at sites identified above were queried for all samples, and the proportion of sites matching either (1) the Liriodendron genus-specific allele or (2) the L. tulipifera or L. chinese specific allele was tabulated.

For genus-level screening, individual samples had coverage for 83,908 – 594,416 genus SNPs per specimen. The three Liriodendron samples had the matching Liriodendron allele at 99.2% - 99.9% of surveyed sites, while no more than 0.44% of sites had the corresponding allele across the eight Magnolia samples. For species-level screening, the three Liriodendron samples had coverage for 22,094 – 152,985 sites per specimen. The L. chinese-specific allele was detected at over 99% of sites in the L. chinese sample, while fewer than 2% of sites contained the allele in either L. tulipifera sample. In parallel, 97.5% - 99.4% of sites in the two L. tulipifera
samples had the *L. tulipifera*-specific allele, while it was found in only 0.21% of sites for the *L. chinese* sample.

**Evaluation of Potential Biosynthetic Genes.** Publicly available *Liriodendron tulipifera* RNAseq data originally collected from apical shoots was retrieved from SRA (SRR16546781, SRR16546782, SRR16546783, SRR16546784, SRR16546785, SRR16546803, SRR16546804), trimmed with Trimmomatic (v0.39), and assembled using rnaSPAdes (v3.15.3). This resulted in 332,056 transcripts, with an average length of 1,011 bp. Transdecoder (https://github.com/TransDecoder/TransDecoder) was then run to predict reading frames of the resulting transcripts, resulting in 189,683 predicted proteins. These proteins were annotated with KEGG, Interpro, and a custom BLAST database comprised of biosynthetic genes that produced sesquiterpenes such as costunolide in other plant species.

**ASSOCIATED CONTENT**

**Supporting Information.** The supporting information is available free of charge on the ACS publications website and includes: HPLC chromatograms of tulip tree extracts and chromatography fractions and bioassay-guided isolation information, NMR and MS data for epitulipinolide, laurenobiolide, tulipinolide, and honokiol, cytotoxicity data for sesquiterpene lactones to HaCaT cells, and chromatography data on distribution of metabolite in plant parts and magnolia specimens. Additionally, information on sequence analysis and putative biosynthetic enzymes are included.

**AUTHOR INFORMATION**

**Corresponding Author**
Matthew J. Bertin – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; orcid.org/0000-0002-2200-0277; Phone: Tel: +1-401-874-5016; Email: mbertin@uri.edu

Authors

Riley D. Kirk – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; orcid.org/0000-0001-5324-7857; Email: rileykirk@uri.edu

Margaret E. Rosario – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: mer008@uri.edu

Nana Oblie – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: nana_oblie@uri.edu

Terra Marie Jouaneh – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; orcid.org/0000-0002-5898-0107; Email: tjouaneh@uri.edu

Marina A. Carro – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: marinaacarro@gmail.com

Christine Wu – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: christine_wu@my.uri.edu
Andrew M. Kim – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: kim2267@uri.edu

Elizabeth Leibovitz – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: elizabeth_leibo@uri.edu

Elizabeth Sage Hunter – Center for Food Safety and Applied Nutrition, Office of Regulatory Science, U.S. Food and Drug Administration, College Park, Maryland 20740, United States; orcid.org/0000-0002-5628-1281; Email: Elizabeth.Hunter@fda.hhs.gov

Robert Literman – Center for Food Safety and Applied Nutrition, Office of Regulatory Science, U.S. Food and Drug Administration, College Park, Maryland 20740, United States; Email: Robert.Literman@fda.hhs.gov

Sara M. Handy – Center for Food Safety and Applied Nutrition, Office of Regulatory Science, U.S. Food and Drug Administration, College Park, Maryland 20740, United States; orcid.org/0000-0003-3861-4609; Email: Sara.Handy@fda.hhs.gov

David C. Rowley – Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island 02881, United States; Email: drowley@uri.edu

Notes

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