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Quercitrin and Afzelin Isolation: Chemical Synthesis of a Novel Methicillin-Resistant Staphylococcus Aureus (MRSA) Antibiotic Analogue

Taylor Ramsaroop

University of Mississippi. Sally McDonnell Barksdale Honors College

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QUERCITRIN AND AFZELIN ISOLATION:
CHEMICAL SYNTHESIS OF A NOVEL METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) ANTIBIOTIC ANALOGUE

by Taylor Nichole Ramsaroop

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2017

Approved by

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Acknowledgements

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Abstract

The increasing prevalence of bacterial resistance to antibiotics is an epidemic where once easily treated infectious illnesses are becoming more difficult to treat, leading to prolonged suffering, exacerbated medical costs, and sometimes death. Methicillin-Resistant S. aureus (MRSA) is a strain of the Staph Infection causing bacteria that is resistant to penicillin-like beta-lactam antibiotics and occurs at a high incidence in several countries in Europe, the Americas, and the Asia-Pacific region. In a routine screening of plant extracts, an extract of American Sycamore leaves (Plantus occidentalis) was noted to show promising activity against the experimental MRSA strain included in the screening panel. However, the identified active components known as plantanosides [kaempferol 3-O-α-l-(2″,3″-di-E-p-coumaroyl)rhamnoside, kaempferol 3-O-α-l-(2″-E-p-coumaroyl-3″-Z-p-coumaroyl)rhamnoside, kaempferol 3-O-α-l-(2″-Z-p-coumaroyl-3″-E-p-coumaroyl)rhamnoside, kaempferol 3-O-α-l-(2″,3″-di-Z-p-coumaroyl)rhamnoside)] obtained by isolation from their natural source are not a commercially viable solution as large scale preparation is uneconomic. For this reason, synthetic routes will be more efficient for the commercial development of novel treatments. This study details the isolation of two natural products which 1) contain the kaempferol and rhamnose portions of the plantanosides (afzelin) and 2) a closely related quercetin rhamnoside derivative (quercitrin, which would allow evaluation of important structure-activity-relationship for antibiotic activity) and exploratory synthesis efforts to derivatize these natural products into analogs of the antibiotic plantanosides.
Analogue synthesis was not achieved, but this synthetic strategy to achieve esterification at the hydroxyls of the rutinose portion of rutin (a model compound chosen for quercitrin) shed light into the behavior of the kaempferol core and rhamnose portions of the antibiotic plantanosides; namely, it was concluded that the phenolic hydroxyls of the core have the greatest reactivity which causes complications when attempting esterification at the sugar moiety hydroxyls. This knowledge contributes to the foundation for a possible solution to the MRSA epidemic.
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Introduction

The increasing prevalence of bacterial resistance to antibiotics is an epidemic where once easily treated infectious illnesses are becoming more difficult to treat, leading to prolonged suffering, exacerbated medical costs, and sometimes death. According to the CDC, a minimum of 2,049,442 illnesses are caused in the U.S. by antibiotic resistance and leads to a minimum of 23,000 deaths each year. Estimates regarding the medical cost per patient with an antibiotic-resistant infection range from $18,588 to $29,069 (CDC 2017). The total economic burden placed on the U.S. economy by antibiotic-resistant infections has been estimated to be as high as $20 billion in health care costs and $35 billion a year in lost productivity (Ventola 2015, WHO 2017).

Antibiotic resistance occurs when microbes adapt their biomolecular processes to avoid being killed by antimicrobial agents. Resistant bacterial populations may grow by passing genes that allow these biomolecular processes to occur from one microorganism to another via horizontal gene transfer or outcompeting other antimicrobial susceptible strains. Adapting gene expression to environmental pressures is a naturally occurring mechanism in all living entities and is the underlying mechanism of Darwin’s theory of evolution by natural selection, but the rate at which bacteria are acquiring these mechanisms is extremely high. This high rate can be attributed to widespread overuse and misuse of antibiotics (“Resisting Our Drugs” 2017).
*Staphylococcus aureus* species are among the most frequently occurring of all antibiotic-resistant threats. Methicillin-Resistant *S. aureus* (MRSA) is a strain of the Staph Infection causing bacteria that is resistant to penicillin-like beta-lactam antibiotics and occurs at a high incidence in several countries in Europe, the Americas, and the Asia-Pacific region (MRSA Research Center 2017). This bacterium causes a range of illnesses, from skin and wound infections to pneumonia and bloodstream infections that can cause sepsis and death. MRSA affects 90,000 Americans each year, 80,000 of which are classified as severe, leading to the death of more than 20,000. To put that in perspective, MRSA kills more Americans each year than HIV/AIDS, Parkinson’s disease, emphysema, and homicide combined.

A great number of these incidences are nosocomial (hospital acquired) in nature, thereby increasing healthcare costs, antimicrobial resistance, and unnecessary deaths. This subsequently strains the entire healthcare system. The current treatment of choice for MRSA is administration of the glycopeptide antibiotic vancomycin. However, this strategy has a low success rate of only 35-57% and has several undesirable side effects. For these reasons, the need to discover novel treatments is great.

Since the discovery of penicillin in 1928, natural products have played a major role in antibiotic development (Moloney 2016). In the face of today’s resistance epidemic, they have made resurgence as an important source for promising drug candidates. Drug discovery from plant sources is advantageous because species for investigation may be chosen from ones that have been used long-term in ethnomedicine, thus isolated compounds from these plants are likely
to be safer and based on their history of use, they may have a higher likelihood of yielding an active component (Katiyar et al 2012). Additionally, isolated active compounds from the plant can serve either as a direct drug candidate or as a model for semi-synthetic drug development. A major disadvantage to the natural products approach is that intellectual property of natural compounds becomes convoluted, making them undesirable investments for pharmaceutical companies. Semi-synthesis is one possible solution to make proprietary drug candidates marketable, using the natural products as a model for novel compounds.

In a routine screening of plant extracts, an extract of American Sycamore leaves (Plantus occidentalis) was noted to show promising activity against the experimental MRSA strain included in the screening panel (Ibrahim et al 2009). All isolated metabolites were assayed in vitro, four of which were highly active and selective against MRSA (Figure 1). All four had a mass of m/z 747.1647 as determined by (+)-ESIMS; $^1$H and $^{13}$C NMR data revealed the metabolite to be L-rhamnose glycosides, each with a kaempferol nucleus and α-configuration of the sugar. The major of these four glycosides to be isolated by bioassay-guided fractionation and purification of fractions was a flavonoid known as plantanoside.
The structure-activity relationship of the four closely related compounds showed that the presence of the flavonoid moiety connected to the p-coumaroyl functionality through the sugar, the configuration about the double bonds of p-coumaroyl units, and the presence of hydroxy groups at positions 5, 7, and 4’ were important for anti-MRSA activity.

However, despite having shown biological activity against MRSA these compounds obtained by isolation from their natural source are not a commercially viable solution as it requires significant time and resources to prepare quantities of these active materials due to the relatively small amount of compound present in the plant extract, thus making commercialization uneconomic.

**Figure 1:** Structures of the four plantanosides biologically active against MRSA; basis of this study’s analogue synthetic endeavors.

1. $R^1 = R^2 = E\text{-}p\text{-cumaroyl}$
2. $R^1 = E\text{-}p\text{-cumaroyl}, R^2 = Z\text{-}p\text{-cumaroyl}$
3. $R^1 = Z\text{-}p\text{-cumaroyl}, R^2 = E\text{-}p\text{-cumaroyl}$
4. $R^1 = R^2 = Z\text{-}p\text{-cumaroyl}$
For this reason, synthetic routes will be more efficient for the commercial development of novel treatments. This study details the isolation of two natural products which 1) contain the kaempferol and rhamnose portions of the plantanosides (afzelin) and 2) a closely related quercetin rhamnoside derivative (quercitrin, which would allow evaluation of important structure-activity-relationship for antibiotic activity) and initial synthesis efforts to derivatize these natural products into analogs of the antibiotic plantanosides (Figure 2).

Figure 2: Structure of afzelin and quercitrin, the *Solanum Cernuum* isolates of interest.
Before beginning the isolation process for preparation of quantities of afzelin and quercitrin, an analytical HPLC method had to be developed that would adequately separate the compounds of interest and guarantee indication of target product or lack thereof during synthetic reactions.

Analyses were performed with a Hewlett Packard Agilent 1100 Series equipped with a G1311A quaternary pump, a G1322 degasser, a G1316A oven, G1313A autosampler and a G1315A diode array detector. Acetonitrile and water for HPLC (high performance liquid chromatography) were purchased from EMD Millipore (Cincinnati, Ohio). The elution was performed with 0.01 mol L⁻¹ phosphoric acid and acetonitrile.

A 3.9 mg sample of the 10 grams of Solanum cernuum crude extract (see Plant Material below) was analyzed in 1 mL of methanol by an existing HPLC method, the Plantanoside method, on a 4.6x150mm Synergi Reverse Phase Polar column. This method was chosen as a starting point as it had been developed for the purpose of analyzing plantanoside isomers, and our target(s) (if achieved) should possess similar polarity and thus similar retention times. At 1.5 mL/minute, a gradient of 15/85 acetonitrile/acidified water to 95/5 over 15 minutes, to 35/65 over 2 minutes, to 95/5 wash over 2 minutes, to column re-equilibration at 15/85 for 4 minutes. The gradient is outlined in Table 1 below.
The sample was detected at 254 nm as well as 318 nm at 30°C with 3µL injections. Wavelength 315 nm was added because the literature reports this to be the optimal wavelength for observing flavonoids. The method was further modified to a flow rate of 1 mL/minute due to leakage of column fittings at the high pressure caused by the 1.5 mL/minute flow rate. The gradient was also later modified to 15/85 ACN/H2O for 5 minutes, to 50/50 over 12 minutes, to 95/5 wash over 1 minute for 2 minutes, to re-equilibration at 15/85 over 1 minute for 2 minutes. An isocratic step followed by a much slower gradient was added in hopes to increase separation as all components were eluting within two minutes of each other.

Table 1: HPLC analytical method for Plantanosides, used to establish a base for method development
Gradient changes were made according to peaks in areas of interest that suggested coelution or inadequate separation. Crude extract samples were run against commercially acquired quercitrin standards, so the area of interest in terms of extract was known. It was predicted that a slower gradient would achieve better separation, so the gradient was modified again to 20/80 ACN/H2O for 2 minutes, to 35/65 over 12 minutes, to 95/5 wash over 1 minute for 2 minutes, to re-equilibration at 20/80 over 1 minute for 3 minutes.
To be sure that all materials were being observed 220 nm and 270 nm detector wavelength was added to the method, as well. Most materials were captured at 270 nm, so this was the final wavelength chosen for the analytical procedure. Adding a short isocratic step to the gradient where resolution needed improvement as evidenced by previous runs (ten to fifteen minute time frame in method) allowed adequate separation of quercitrin and afzelin from their neighboring peaks. Thus, the HPLC method was finalized and named Platsolanum (from the Plantanoside HPLC method from which it was derived for this solanum extract). This gradient is outlined in the table below: it includes 20/80 ACN/H2O for 2 minutes, to 21/79 over 4 minutes, to 28/72 over 5 minutes for 1 minute, to 35/65 over 12 minutes, to 95/5 wash over 0.5 minutes for 2 minutes, to column re-equilibration at 20/80 over 0.5 minutes for 3 minutes. Low variability in the results of duplicate analyses demonstrates adequate performance of the method and equipment.

<table>
<thead>
<tr>
<th>Time in Method (minutes)</th>
<th>% Acetonitrile in MP</th>
<th>% H2O in MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>14</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>15</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>21</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 3: Third trial gradient in finding an efficient and successful HPLC analytical method to separate the compounds of Solanum butanolic extract, named Platsolanum.
<table>
<thead>
<tr>
<th>Time in Method (minutes)</th>
<th>% Acetonitrile in MP</th>
<th>% H2O in MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>79</td>
</tr>
<tr>
<td>11</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>12</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>24</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>24.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>26.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>27</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

**Table 4**: HPLC analytical method Platsolanum gradient used for the duration of quercitrin and afzelin isolation process from *Solanum cernuum*.

**Figure 4**: Chromatogram of *S. cernuum* butanoic extract given by the finalized HPLC analytical method Platsolanum used for the duration of the quercitrin/afzelin isolation process.
While this gradient proved to be successful for its purpose and was used for the duration of the isolation process, later during a series of synthesis reactions it was adjusted using this same process to accommodate the reaction components and compounds produced in the synthesis reactions which eluted with longer retention times (Table 5 and Table 12).

<table>
<thead>
<tr>
<th>Time in Method (minutes)</th>
<th>% Acetonitrile in MP</th>
<th>% H2O in MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>24.5</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>24.5</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>29.5</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>

*Table 5: HPLC analytical method Platsolanum gradient used for synthesis reactions.*
Figure 5: HPLC chromatogram of Quercitrin/Afzelin/Rutin/Propionic Acid Derivatives Mix used as standard for synthetic reactions analyzed by modified Platsolanum method. (Quercitrin 8.426 min, Afzelin 10.571 min, Rutin 7.195 min)
Plant Material

The aim for this stage was to collect enough quantity of quercitrin and afzelin to use later for analogue synthesis reactions and then improve efficiency of chromatography. We chose the Brazilian plant *Solanum cernuum* for its quercitrin and afzelin content after review of published natural sources of the compounds identified the plant as a relatively good yielding source. Plant material was provided as a partially processed extract through collaboration with Dr. Jairo K. Bastos, University of Sao Paulo, Ribeirão Preto, São Paulo, Brazil. The leaves of *Solanum cernuum* were collected in the city of Teresópolis, state of Rio de Janeiro, Brazil in January 2012. The dried leaves were powdered using a knife mill and extracted sequentially seven times for 48 hours with 70% aqueous ethanol by maceration followed by percolation. The filtered extract was concentrated under vacuum, furnishing the hydroethanolic crude extract, which was further lyophilized and stored as a dry powder. The ethanoic extract was then dissolved in methanol-water (50:50) and partitioned with different organic solvents in the sequence: hexane, dichloromethane, ethyl acetate and *n*-butanol. Each partition was undertaken three times with each solvent, the various specific partition layers combined and the solvent was removed under vacuum for the final crude partition solvent extract. Of 10 grams of *S. cernuum* crude extract butanol partition fraction, 100 mg was set aside to retain and 3.9 mg was used for HPLC analysis.

Solid phase extraction was performed on 9.8943g of Solanum cernuum crude extract, butanol partition fraction. Solid phase extraction was selected over a direct chromatographic separation so should any sample remain on the column, it could be recovered. Regular chromatography offers precision, an unnecessary provision at the time as the crude extract
required only an estimate of which solid phase extraction fractions to pool in order to maximize quantity and purity of quercitrin and afzelin. A gradient of increasing polarity with heptane, ethyl acetate, and methanol was chosen based on reported solid phase extractions from this plant; however, the gradient was adjusted as performed based on observations of what was visible on the column and in collected fractions. Fortunately, the products of interest have a visible absorbance such that the method could be modified contemporaneously to ensure their elution from the silica stationary phase.

The extract, dissolved in methanol, was dried onto 25g of Flash Silica (purchased from Sorbent Technologies) and packed as a dry powder into a 100 mL column (1CV) to form a separation bed for the solid phase separation; Flash Silica consists of 40-63 micron irregular silica particles which allow for rapid chromatography. The gradient consisted of about 2 column volumes (CV) of n-heptane, 8 CVs 50/50 ethyl acetate/n-heptanes, 10 CVs ethyl acetate, 8 CVs 95/5 ethyl acetate/methanol, 3 CVs 80/20 ethyl acetate/methanol, 3 CVs 50/50 ethyl acetate/methanol, 4 CVs 25/75 ethyl acetate/methanol, 4 CVs methanol, 6 CVs 90/10 methanol/water (Table 6).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent</th>
<th>Fraction Volume (mL)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-heptanes</td>
<td>300</td>
<td>At 175mL fraction turned from clear to yellow</td>
</tr>
<tr>
<td>2-12</td>
<td>50/50 n-heptane/EtOAc</td>
<td>200 (Fr 2), 100 (Fr 3), 50 (Fr 4-12)</td>
<td>Fr 3: dark green Fr 6-12: increasingly clearer green</td>
</tr>
<tr>
<td>13-32</td>
<td>EtOAc</td>
<td>50</td>
<td>Fr 14-19: characteristic yellow-green color of flavonoids Fr 20-32: increasingly clearer yellow</td>
</tr>
<tr>
<td>33-46</td>
<td>95/5 EtOAc/MeOH</td>
<td>50</td>
<td>Fr 39: color began darkening again</td>
</tr>
<tr>
<td>47-52</td>
<td>80/20 EtOAc/MeOH</td>
<td>50</td>
<td>Fr 50: brown, indicating presence of alkaloids</td>
</tr>
<tr>
<td>53-59</td>
<td>50/50 EtOAc/MeOH</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>60-63</td>
<td>25/75 EtOAc/MeOH</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>64-66</td>
<td>MeOH</td>
<td>100 (Fr 64), 200 (Fr 65), 150 (Fr 66)</td>
<td></td>
</tr>
<tr>
<td>67-70</td>
<td>90/10 MeOH/H2O</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: *S. cernuum* solid phase extraction mobile phase gradient and fraction details.

The next step in the purification process was to collect pools and increase efficiency of chromatography as compared to previous studies for maximum quercitrin/afzelin purity and yield. TLC plates were used for analysis of each fraction, as well as determination of the mobile phase (MP) to be used for large-scale preparative chromatography. Pooling by fraction color would have been sufficient for approximate compound separation, but TLC analysis allowed a closer look into their contents. The first MP was initially chosen based on what is typical for flavonoids, then adjusted based on quercitrin standard and crude extract RF values. The following table outlines the TLC MP selection.
<table>
<thead>
<tr>
<th>MP Solvent</th>
<th>Quercitrin RF</th>
<th>Crude Extract RF</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:8:0.05 mL (CH₂Cl₂:EtOAc:MeOH)</td>
<td>0.07</td>
<td>0.06, 0.17</td>
<td>Chosen because usually works with flavonoids, but results indicate need for greater polarity</td>
</tr>
<tr>
<td>1:8:1 mL (CH₂Cl₂:EtOAc:MeOH)</td>
<td>0.10</td>
<td>0.07, 0.11</td>
<td>RF too small to translate to chromatography, need greater polarity still</td>
</tr>
<tr>
<td>4:4:1 mL (Acetone:EtOAc:H₂O)</td>
<td>0.41</td>
<td>0.51</td>
<td>Too polar</td>
</tr>
<tr>
<td>4:4:0.3 mL (Acetone:EtOAc:H₂O)</td>
<td>0.41</td>
<td>0.36, 0.50, 0.72, 0.87</td>
<td>Best yet, quercitrin and first two extract compounds visible at 254ƛ, last two extract compounds at 365ƛ, extract RF values may be low due to sample running slightly to left from origin (Figure 6)</td>
</tr>
</tbody>
</table>

**Table 7:** Mobile Phase selection by TLC for quercitrin and afzelin preparative chromatography. Dichloromethane was used to explore relative polarities but was not considered as MP candidate.

Every third fraction of the 70 total fractions was run on TLC using this 4:4:0.3mL (Acetone:EtOAc:H₂O) along with quercitrin and afzelin standards. Analysis revealed fractions 15-36 to contain the most quercitrin/afzelin, so every third fraction in this range was again assayed but with just water-acidified (3% and 1%) EtOAc as acetone was likely too reactive to serve as a preparative chromatographic solvent.
Using this culmination of analyses, six pools were chosen to be combined, dried, and weighed.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Fractions</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-17</td>
<td>1.2250</td>
</tr>
<tr>
<td>2</td>
<td>18-34</td>
<td>3.4580</td>
</tr>
<tr>
<td>3</td>
<td>35-45</td>
<td>1.0126</td>
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<tr>
<td>4</td>
<td>46-49</td>
<td>0.2847</td>
</tr>
<tr>
<td>5</td>
<td>50-57</td>
<td>1.4921</td>
</tr>
<tr>
<td>6</td>
<td>58-60</td>
<td>0.8407</td>
</tr>
</tbody>
</table>

**Table 8:** Pools from *S. cernuum* solid phase

The HPLC method described previously described, Platsolanum, showed that Pool 2 contained mostly quercitrin and Pool 3 contained relatively low concentrations of both quercitrin and
afzelin, too little to justify preparative chromatography. The remaining pools were discarded as they contained no compounds of interest.

**S. Cernuum Quercitrin Chromatography:** 3.458g of the isolated quercitrin containing pool was dissolved in 50 mL wet acidified (3% water and 1% HOAc) EtOAc (w.a.EtOAc) for load on a 3” Normal Phase column. Mobile phase, based on the final TLC plate, was chosen as 90/10 w.a. EtOAC/Heptane (Wet acidified solvents are used on Normal Phase chromatography because dehydration of the silica adsorbent degrades column bed integrity) and pumped at a flow rate of 200mL/minute. UV detection was not utilized due to the EtOAc being UV adsorbent at 270 nm. Instead, elution progress was monitored by periodic fraction spotting on TLC. When sprayed with H2SO4 and heated quercitrin on the plate appears yellow, so fraction collection continued until there was no longer yellow visible on the plates. A total of 46 125mL fractions were collected and sampled (500 µL each, dried, redissolved in 1mL MeOH) for HPLC analysis by Platsolanuum method. These chromatograms were used to conduct a column analysis (Figure 7). Fractions were pooled and the pools dried and weighed for a total weight of 3.431g, 99.2% recovery.
Column analysis revealed that Fractions 38-46 (0.5680g) contained 96% purity of quercitrin but the dried product had a slight yellow-green color, rather than yellow. To obtain the flavonoid characteristic yellow crystallization was necessary because otherwise biological activity against MRSA may be affected by any impurities. The quercitrin was dissolved in 10mL/g 95% EtOH,
heated at 70°C, sonicated, set in the freezer for an hour, then left to sit at room temperature for 12 days; crystals were visible within minutes of sitting at room temperature but was still left for some time to assure complete crystallization. At this time, the liquid phase was removed and the crystals washed three times in cold MeOH (to discourage dissolution) and vacuum-oven dried overnight. The final color of the crystals was dark yellow.

**S. Cernuum Afzelin Chromatography:** Although this particular *S. cernuum* extract contained too little afzelin to justify preparative chromatography, we had in our possession impure afzelin and impure afzelin/quercitrin samples from a prior project from a different *S. cernuum* extract on which preparative chromatography was possible. The impure afzelin were dissolved in w.a. EtOAc for load on 3” NP column, mobile phase 90/10 w.a. EtOAC/heptane at flow rate 250 mL/minute without UV detection. The same TLC spotting technique used for quercitrin chromatography was used here; when fractions spotted on the plates were no longer visible, fraction collection ceased. A total of 41 125mL fractions were collected and sampled (500 µL each, dried, redissolved in 1mL MeOH) for HPLC analysis by Platsolanuum method. These chromatograms were used to conduct a column analysis (Figure 8). Fractions were pooled and dried accordingly.
Column analysis revealed that Fractions 30-36 of TR10 chromatography contained 100% pure afzelin but the dried product’s color indicated the need for crystallization. All isolated quercitrin and afzelin from these three chromatographies were combined respectively and underwent the crystallization process described above, except afzelin required an extra step after one week of showing few crystals: the afzelin was vacuum filtered and concentrated to about 50%, then left to sit further for another five days. It was at this point the crystals were washed as described above. The final color was pale yellow.

In summary, this method of quercitrin isolation from *S. cernuum* was successful with high yield at 96% purity. The plant extract portion we obtained did not obtain enough afzelin to
attempt preparative chromatography, however, this process was effective for the isolation of afzelin from previously processed extract fractions. Afzelin was slower to crystallize than quercitrin, which was completed in less than ninety minutes. It is clear from these column analyses the extremely success of these purification processes. Overall, these preparative strategies of *S. cernuum* plant material can be taken forward for high purity and high yield of quercitrin and afzelin.

**Synthesis Reactions**

This section details the initial synthesis efforts to derivatize the natural products quercitrin and afzelin into analogs of the antibiotic plantanosides. Anhydrous rutin, a glycoside of the flavanol quercitrin and the disaccharide rutinose, was chosen as a model compound to substitute for quercitrin and afzelin during trial synthesis reactions as it is very readily available commercially and quite inexpensive. Several types of reactions with varying conditions were attempted to achieve esterification at the hydroxyls of the rutinose portion of the rutin. If successful, such a structure may behave similarly to the plantanoside natural products and show antibiotic activity against MRSA.

The general strategy chosen to create a plantanoside analog from rutin was to conduct straightforward reactions with relatively simple conditions in the interest of creating a commercially viable method. Each reaction is further detailed below and grouped by a larger overarching strategy, namely (1) transcinnamic acid esterifications at the hydroxyls of the carbohydrate portion of the rutin, (2) 3-phenylpropionic acid esterifications at the hydroxyls of
the carbohydrate portion of the rutin, (3) variation in temperature and acidic conditions based on observed selectivity of 3-phenylpropionic acid reactions, (4) sequential adding of reagents based on observed selectivity of 3-phenylpropionic acid reactions, and (5) rutin hydrolysis by HCl and enzyme.

1 - Propionic Acid Approach: Transcinnamic Acid

A variety of propionic acids were chosen for attempted esterification at the hydroxyls of the carbohydrate portion of the rutin, the first of which being transcinnamic acid. This was chosen because it is a simple propionic acid (a close analog of the ester portion of the plantaniosides), inexpensive, and would give insight into how other substituted propionic acids will behave as reagents in the reaction conditions. The first series of these attempted reactions included transcinnamic acid, an acid chloride catalyst, intermediate base, and non-nucleophilic base in the aprotic solvent tetrahydrofuran (THF). Any presence of water had the potential to interfere with the reaction, so an anhydrous aprotic solvent and anhydrous rutin was necessary. Three reactions were conducted, each varying equivalents of each reactant.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rutin (equivalents)</th>
<th>pivaloyl Chloride (equivalents)</th>
<th>4-Dimethylaminopyridine (equivalents)</th>
<th>Transcinnamic Acid (equivalents)</th>
<th>N-methylmorphaline (equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1.5</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0.5</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>4.5</td>
<td>0.5</td>
<td>3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 9: First series of transcinnamic acid esterification reaction attempts.
A relatively dilute concentration (1mg rutin/mL THF) was chosen in hopes that esterification would be more selective than at higher concentrations and because beginning with a less concentrated mixture would indicate how to adjust reactant ratios. The products of each reaction were analyzed by the Platsolanum HPLC method after one hour, three hours, and one day to monitor the progress of the reaction. We hoped to see a product with retention time similar to that of plantanosides—this would mean that perhaps the desired esterified product was achieved. Only mixture three showed significant esterified products, but they were more polar than expected for the target molecule, so excess acid catalyst pivaloyl chloride was then added to each mixture (an additional 2.5 equivalents to Reaction 1, 5 equivalents to 2, 7.5 equivalents to 3). The progress of each reaction was again monitored over time.
Figure 9: TR 17-3 after one hour (top) and after one day plus addition of 7.5 equivalents of acid chloride (bottom). We expect retention time of about 15 min for desired product, this 12 min RT was short of that (more polar). In none of these was the rutin peak visible.
It was expected that the presence of rutin on the chromatograms would decrease over time as the reaction proceeded but the rutin peak was completely absent for each condition, suggesting it may have crystallized out of solution due to its relative insolubility in THF. Only Reaction 3 + 7.5 acid chloride equivalents showed any indication of a reaction of these acid additions, so the next series utilized 10 equivalents of the acid chloride catalyst.

2 - Propionic Acid Approach: 3-Phenylpropionic Acid

Based on the results of the above acid chloride additions the next series of reactions included increased equivalents of acid chloride catalyst, decreased reaction volume (rutin concentration remained at 1mg/mL, but now in 10mL THF rather than 25mL), and mixture temperature of 40℃ with stirring to address the issue of crystallization. Additionally, basic reagents were added before the acids with the acid chloride catalyst being the last addition which set the start of timing of the reactions. The progress of each reaction was again monitored over time.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rutin (equivalents)</th>
<th>Pivaloyl Chloride (equivalents)</th>
<th>DMAP (equivalents)</th>
<th>3-Phenylpropionic Acid (equivalents)</th>
<th>N-methylmorpholine (equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>10</td>
<td>0.5</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>30</td>
<td>0.5</td>
<td>3</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Table 10: First series of 3-phenylpropionic acid esterification*
Figure 10: TR 19-2 Reaction 2 after one hour (top), three hours (middle), one day (bottom).
It was suspected that the above conditions were not anhydrous enough and that the “wet” THF may be interfering with the reaction. This was explored in the following series of reactions. It was also suspected that conditions in the previous reactions may be too acidic for the alkaline conditions necessary for the ester formation, so in Reaction 3 of the following series, N-methylmorpholine concentration was doubled. Since there was no major difference between two and three equivalents of 3-phenylpropionic acid (Figure 10), two equivalents were chosen to elaborate upon (one equivalent showed not as significant an amount of products).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rutin (equivalents)</th>
<th>Pivaloyl Chloride (equivalents)</th>
<th>DMAP (equivalents)</th>
<th>3-Phenylpropionic Acid (equivalents)</th>
<th>N-methylmorpholine (equivalents)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (wet THF)</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>3</td>
<td>Clear yellow color</td>
</tr>
<tr>
<td>2 (dry THF)</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>3</td>
<td>Yellow with some yellow precipitate</td>
</tr>
<tr>
<td>3 (dry THF)</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>6</td>
<td>Opaque with some white precipitate</td>
</tr>
</tbody>
</table>

Table 11: Second series of 3-phenylpropionic acid esterification attempts.

Reactions 2 and 3 were the first set of conditions to result in the formation of a solid. Using the the Platsolanuum method for HPLC analysis of products after one hour, three hours, and one day revealed a single major peak with a retention time of 21 minutes for Reaction 3 (Figure 11).

While this peak was also present in Reactions 1 and 2, its intensity decreased over time while rutin’s peak increased in intensity. It was predicted that this may be an ester product at the phenol due to its instability over time. Reaction 3 was the only set of conditions where the 21 minute product concentration did not lessen over time, and thus was chosen to be repeated on a
larger scale and the product purified for structure characterization by Mass-Spectrometry (Figure 16). Analysis confirmed the rutin ester product at a phenol hydroxyl, but which position was unclear. Additionally, it was the trimethylacetyl acid that reacted with the rutin rather than the desired propionic acid.

Since ester formation is a condensation reaction, the HCl produced as a byproduct in this reaction would react with any water present to suppress the desired reaction. Despite using anhydrous THF and anhydrous rutin, it was possible a residual presence of water was interfering with product formation and/or stability. This suggested the addition of molecular sieves into the reaction mixtures to remove any residual water. Additionally, given the ratio of reactants to rutin

![Figure 11: (continued in appendix) TR 20-3 trimethylacetyl phenolic ester product chromatogram after one day, formed with 20 equivalents pivaloyl chloride per rutin eq., 0.5 DMAP eq., 2 3-phenylpropionic acid eq., 6 NMM eq.](image-url)
it seemed unusual that only one product was produced, so the next series of reactions involved tripling the reagents/rutin ratio in dry THF to overwhelm the molecule with reagents (Table 11).

Reaction 3 conditions were repeated as a standard.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rutin (equivalents)</th>
<th>pivaloyl Chloride (equivalents)</th>
<th>DMAP (equivalents)</th>
<th>3-Phenylpropionic Acid (equivalents)</th>
<th>N-methylmorpholine (equivalents)</th>
<th>Sives</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (dry THF)</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>6</td>
<td>No</td>
</tr>
<tr>
<td>2 (dry THF)</td>
<td>1</td>
<td>20</td>
<td>0.5</td>
<td>2</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>3 (dry THF)</td>
<td>1</td>
<td>60</td>
<td>1.5</td>
<td>6</td>
<td>18</td>
<td>No</td>
</tr>
</tbody>
</table>

*Table 12:* Third series of 3-phenylpropionic acid esterification reaction attempts.
These results showed a lot of peaks towards the end of the method, suggesting that there may not have been only one product as originally thought but that additional products were eluting during...
the wash cycle of the HPLC method. For this reason, the Platsolanum method for HPLC analysis was slightly altered accordingly:

<table>
<thead>
<tr>
<th>Time in Method (minutes)</th>
<th>% Acetonitrile in MP</th>
<th>% H2O in MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>25</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>28</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>28.5</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>31.5</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>

**Table 13:** Further modified Platsolanum gradient for synthesis.

This slowed the gradient and provided for more separation of products. Reaction 3 showed few peaks, none of which were of major intensity, so the reaction was repeated but with sieves and three staggered additions of reagents excluding rutin to better understand the effects the different quantities of reagents had during the progress of the reaction. Before adding the next increment of materials, a reaction mixture sample would be taken for analysis. Each sample was analyzed hourly and the following day to thoroughly observe what happens to the products over time. After about three hours the results of each were the same, with the excess products which begin to elute after the major 21-minute peak. Sequentiality did not seem to make a difference under these conditions as to the end products, but product formation over the time was explored in the next synthetic approach. All reactions would now contain both dry THF and molecular sieves as they had no effect on product (21-minute retention time) formation but did lessen the number of minor nonpolar products (appear at end of method, **Figure 16**).
3 - Temperature Selectivity Approach

This next approach explored the effect of temperature on rutin esterification selectivity. The sequential addition of six 3-phenylpropionic acid equivalents (two per addition, one hour between each addition) was repeated but at both room temperature and 40°C, all other reagent concentrations remained constant. Analysis by the hour did not show significant change in the reaction mixture until 24 later; the peak at about 20.7-minute retention time almost disappeared. At this point the room temperature reaction mixture was rotovaporated at room temperature, dissolved in EtOAc, and washed first with brine and then with water using a separatory funnel. The resulting organic phase was rotovaporated and left the oil product (98.3mg, original rutin concentration 50mg) which was washed with heptane and analyzed by HPLC (Figure 13).

![Figure 13: Results of sequential additions of 3-phenylpropionic acid while exploring the effect of temperature;](image-url)
Overnight of heated versus non-heated slightly different from each other with roughly the same contents but in slightly different ratios, so the reaction was repeated a third time but after reaching completion, reaction was removed from heat. After the final propionic acid addition, hourly analyses were taken (Figure 14). When the chromatograms began to become too complex, the reaction was quenched and washed as before (approximately four hours after the final addition, seven hours reaction time total).

![Figure 14: TR29.1 chromatogram, similar to TR29 after wash but with the appearance of two predominant peaks retention times 20.789 min and 22.020 min.](image)

To check for better selectivity at room temperature the following reactions were conducted, the first hour at 40°C and after at room temperature, analyzed hourly and overnight (Table 14).

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Table 14: Variable 3-phenylpropionic acid equivalents at one hour heated and after at room temperature.

Table 15 depicts a series of reactions in which the aim was to analyze time of reaction so as to take the 21-minute product consistent across multiple conditions and attach on more of the trimethyl acetyl; since the product degrades over time, this selectivity could be used to protect the more reactive phenols while working with the hydroxyls, then later removing the trimethyl acetyl protecting groups. Pyridine was chosen instead of THF as solvent.

Table 15: Series of temperature selectivity reactions.
4 - Sequential Reagents Approach

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reagent Order</th>
<th>Chromatogram Results</th>
</tr>
</thead>
</table>
| 1        | 1) 3-phenylpropionic acid, pivaloyl chloride  
2) NMM  
3) DMAP  
4) Rutin | New product retention time  
25.639 minutes |
| 2        | 1) 3-phenylpropionic acid, pivaloyl chloride  
2) Rutin  
3) DMAP  
4) NMM | New product retention time  
28.780 minutes |
| 3        | 1) 3-phenylpropionic acid, pivaloyl chloride, DMAP, NMM  
2) Rutin | 3 minor peaks |

Table 15: Series of sequential reagent reactions.

5 - Rutin Hydrolysis Approach: Acid and Enzymatic Hydrolysis

Rutin contains a disaccharide, rutinose, as part of its structure whereas quercitrin, afzelin and the plantanosides contain only a single carbohydrate molecule, rhamnose, within their structures. In order to create a closer molecular analog as a model compound to explore this chemistry, hydrolysis of rutin to a mono-carbohydrate containing structure was attempted. If HPLC analysis by the Platsolanum method revealed a peak with a similar retention time as quercitrin, the reaction was likely successful.
The first endeavor to hydrolyze rutin consisted of rutin in 1N HCl for 24 hours both at 40°C and room temperature. The product retention time (9.678 minutes) was inconsistent with that of quercitrin, so a variation of this reaction model was explored next (Table 16). These showed the same results.

Table 16: Series of rutin hydrolysis reaction attempts.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rutin (in 14:3 THF:H2O)</th>
<th>HCl Concentration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2g</td>
<td>0.5mol/L</td>
<td>40°C</td>
</tr>
<tr>
<td>2</td>
<td>0.2g</td>
<td>0.5mol/L</td>
<td>RT</td>
</tr>
<tr>
<td>3</td>
<td>0.2g</td>
<td>0.1mol/L</td>
<td>40°C</td>
</tr>
<tr>
<td>4</td>
<td>0.2g</td>
<td>0.1mol/L</td>
<td>RT</td>
</tr>
</tbody>
</table>

Next was an attempted enzymatic hydrolysis. In 6:1 THF:H2O, 4:1 enzyme:rutin with pectinase (an enzyme that breaks down polysaccharides) and β-glucosidase (an enzyme that catalyzes hydrolysis of the glycosidic bonds to terminal non-reducing residues in beta-D-
glucosides and oligosaccharides with release of glucose) both at 40°C for 24 hours. No major product peaks appeared in either enzyme reaction besides rutin, suggesting no significant molecular changes occurred under these conditions.

In summary, after the several attempts detailed above, it became clear that synthetic preparation of plantanoside analogs will require more complicated conditions beyond the scope of this project. Analysis of the various reaction products by HPLC suggests the phenolic groups of the flavonoid core are more accessible and reactive than the hydroxyl groups of the carbohydrate portion. The approach for the next level of synthesis would be in three stages: (1) to react quercitrin (or rutin) with protecting groups to mask the more reactive phenolic groups, (2) to react the hydroxyl groups, (3) remove the protecting group(s) without disruption of the esters formed at the carbohydrate portion.

**Conclusion**

An HPLC analytical method (named Platsolanum) was successfully developed which included selection of detection wavelength, flow rate, and mobile phase gradient to analyze the contents of *S. cernuum* butanol partition extract and chromatographic fractions and pools, which ensured sufficient separation of target analytes. A new method was developed to easily obtain in significant quantities quercitrin and afzelin by solid phase extraction from *S. cernuum* plant material which involved selection of flash silica for solid phase extraction with a mobile phase
gradient of increasing polarity (n-heptane, EtOAc, MeOH, water), and preparative chromatography. Unique 90/10 wet acidified (3% water and 1% acetic acid) EtOAc/Heptane mobile phase selection and preparation along with Platsolanum HPLC analysis provided 1.4830g of quercitrin at 96% purity with high efficiency. Isolation of 0.982g of pure afzelin was also achieved. These extremely successful preparative techniques are replicable for obtaining extremely high purity isolates in significant quantity.

Synthesis was attempted to find conditions to derivatize the natural products quercitrin and afzelin into analogs of the antibiotic plantanosides using anhydrous rutin as a model compound to substitute for quercitrin during trial synthesis reactions. To achieve esterification at the hydroxyls of the rutinose portion of the rutin, varying conditions involving transcinnamic acid, 3-phenylpropionic acid, solvent, concentration, temperature, order of addition of reagents, and rutin hydrolysis by HCl, pectinase, and β-glucosidase were explored. While the reactions were not successful in the synthesis of quercitrin and afzelin analogs, it is now known that the phenolic groups are the most reactive constituents of the kaempferol core. Based on this conclusion for further exploration of plantanoside analog synthesis, a three step process (which was beyond the scope of this study) should be implemented: 1) phenolic hydroxyl protection, 2) glycosidic hydroxyl esterification, 3) removal of phenolic hydroxyl protecting groups. Further studies may also explore further the new products produced in the temperature selectivity reactions, others means of rutin hydrolysis, and the potential of trimethylacetyl phenolic hydroxyl protecting groups. Ultimately, these reaction attempts gave insight into the behavior and reactivity of the kaempferol core of the substrate, insight leading to a potential novel and commercially viable solution to the MRSA epidemic.
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**Figure 16:** NMR characterization of 21-minute product. formed by rutin, 2 equivalents of 3-phenylpropionic acid, 20 equivalents pivaloyl chloride, 6 equivalents NMM, and 0.5 equivalents DMAP all in THF. Reaction was quenched after 2 hours, dried on rotovaporator, washed with EtOAc then with brine using separatory funnel, dried for one hour with magnesium sulfate, then washed again with HCl 1N and water, but product was not solid and was not solid but liquid. Since there was an expected maximum of ~75mg of product (50mg rutin + 25mg acid), the majority of this weight was expected to be the excess products at the end of the method, and that further cleanup was required. Purification was carried out using semi-preparative C18 NP 250x10mm column (MP selection by TLC plate and mini-preparatory chromatography in Pasteur pipe), detection wavelength 270nm, flow rate 3.6mL/min, MP 80:20:0.01 MeOH:water:HOOAc. 40 1mL fractions were collected (after 30mL forerun). Fractions 4-8 were pooled (weight 2.4mg) and analyzed by an isocratic HPLC method with same MP as chromatography. The same process was repeated but with the presence of molecular sieves in reaction mixture, resulting in a much purer product of 26.6mg. It was this product that was characterized.