Synthesis of Non-Toxic Compounds and the Inhibition of Oral Bacteria

Monica Patel

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SYNTHESIS OF NON-TOXIC COMPOUNDS AND THE INHIBITION OF ORAL
BACTERIA

A Thesis
Submitted to the School of Graduate Studies and Research
in Partial Fulfillment of the
Requirements for the Degree
Master of Science

Monica Bhupendra Patel
Indiana University of Pennsylvania
May 2014
We hereby approve the thesis of

Monica Bhupendra Patel

Candidate for the degree of Master of Science

Keith Kyler, Ph.D.
Professor of Chemistry, Chair

Jonathan Southard, Ph.D.
Professor of Chemistry

Nathan McElroy, Ph.D.
Associate Professor of Chemistry

Avijita Jain, Ph.D.
Professor of Chemistry

ACCEPTED

Timothy P. Mack, Ph.D.
Dean
School of Graduate Studies and Research
Title: Synthesis of Non-Toxic Compounds and the Inhibition of Oral Bacteria

Author: Monica Bhupendra Patel

Thesis Chair: Dr. Keith Kyler

Thesis Committee Members: Dr. Jonathan Southard
Dr. Nathan McElroy
Dr. Avijita Jain

In recent years, research has shown that dental care correlates with many health factors due to the amount of bacteria present in the mouth. Thus, it is important to create more efficient compounds to fight oral bacteria, specifically Streptococci mutans. This research chose cost effective compounds to inhibit the cavity causing S. mutans.

Based on a study on phenolic antibacterial compounds against oral bacteria, 4-hexylphenol had reasonable germ-kill activity and log P values. By modeling this compound with one alcohol group and a long hydrophobic carbon chain, Geraniol was chosen as a starting material.

Three compounds were synthesized and confirmed through $^1$Hydrogen Nuclear Magnetic Resonance spectroscopy. All three compounds showed over 50% inhibition of Streptococci mutans within a 24-hr period when comparing Colony Forming Units/mL.

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<tr>
<th>Structure</th>
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<tr>
<td></td>
<td></td>
<td>(C$<em>{19}$H$</em>{24}$O$_2$)</td>
<td>(C$<em>{19}$H$</em>{30}$O$_2$)</td>
<td>(C$<em>{17}$H$</em>{28}$O)</td>
</tr>
</tbody>
</table>

**Figure 1.** Summarization of our compounds containing key features.
I would like to thank Dr. Keith Kyler and Dr. Narathan Bharathan for all the support and guidance throughout this research, and also I would like to acknowledge the Indiana University of Pennsylvania’s Chemistry Department Faculty for their advice and input. I would like to share my gratitude to my family and friends for their never ending encouragement and support. Most importantly, I would like to thank my love, Michael C. Hummel who has been the greatest support system and best partner I could ever ask for.
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$^1$HNMR of compound 2
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</tr>
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CHAPTER 1
THE PROBLEM

Statement of the Problem

Dental plaque and tooth decay are major continuous infections that occur in human beings. Dental plaque formation may lead to tooth decay because the constant acidic environment enables decay of the tooth surface minerals. This event is a serious problem because the oral bacteria strain, *Streptococci mutans* (*S. mutans*), is the leading cause of these infections and are constantly present in the mouth. S. mutans take effect after ingestion of sugars and carbohydrates, and essentially cause dental plaque. In order to stop dental plaque buildup, dental care physicians recommend removing plaque with materials like toothbrushes and toothpastes. These techniques remove the foods that *S. mutans* react with. The problem with this method is that removing the foods does not inhibit the bacteria; it only temporarily takes away the source of activation. There needs to be compounds that inhibit the growth of the *S. mutans* bacteria strain so that continuous inhibition occurs throughout the day.

Furthermore, dental care treatments need to be safe and accessible for people all over the world. There needs to be effective dental hygiene materials that can inhibit *S. mutans* with minimal amounts and that the materials can be easy to manufacture in order to make them accessible to all populations. The compounds that are synthesized in this research were chosen in order to fulfill these certain characteristics.

Currently, toothpaste containing sodium fluoride, and brushing are the common methods to inhibit S. mutan growth. Most dental health care physicians recommend
these treatments to stop plaque buildup and to maintain a healthy mouth. However, 95% of toothpastes in the United States contain fluoride as its inhibitory compound against *S. mutans* and if ingested, it can present health risks. These risks include permanent tooth discoloration, stomach ailments, acute toxicity, skin rashes, and glucose metabolism impairment. Furthermore, in 1997, the Food and Drug Administration added poison warnings on toothpaste bottles to notify poison control in case of ingestion.

The reason for this toxicity is due to the amount of fluoride being used. In the U.S., fluoride toothpastes contain approximately 1 mg of fluoride for each gram of toothpaste. Some instruction labels do state to only use about a pea-sized amount and not to ingest, however, many do not follow this warning because physical examples in advertisements are using much more than that.

Additionally, children are drawn to flavored toothpastes. This causes them to be the leading demographics of ingestion since they enjoy the taste and are unable to determine the difference between the paste and its resembled food product. The amount of fluoride that is present, 1 mg, is an extremely high amount for a compound that is known to be toxic if ingested.

Compounds for this thesis project were chosen in order to use very minimal concentrations. A solution was made that contained approximately 10 mg of compound per 4 mL of the control, Methanol. Then 10 µL of the solution was added to a 10⁻⁵ dilution. Given the extremely low amount of compounds used, we assumed toxicity should be less of an issue.

Another problem that arises in dental care around the world is that basic dental materials are found to be too expensive. In Africa, Asia and the Middle East, people use
chewing sticks to inhibit bacteria on their teeth since many do not have access to modern dental care equipment. These chewing sticks are made from roots, stems or twigs of the local trees and shrubs. Specifically, in Namibia, about 20% of 2394 subjects’ ages 12-44 used Diospyros lycioides chewing sticks for cleaning and that particular 20% generally have shown low dental decay rates. The World Health Organization has stated that these chewing sticks have been effective in dental care. However, these chewing sticks are recommended as an alternative tool if toothbrushes and pastes are unavailable and that is why they are used in some parts of developing countries.

Many residents in the United States cannot access dental care because it is too expensive. 47 million people live in areas where it is difficult to access dental care and about 17 million low-income children do not visit the dentist every year. This is due to high dental costs and inability to attain dental insurance. A cost effective household solution is needed that will inhibit S. mutan growth in order to lower dental decay rates.
Significance of the Problem, Thesis Statement, Hypotheses, and Questions to be Researched

Dental decay is a continuously occurring infection that is caused by the bacteria strain \textit{S. mutans}. As one of the most common infections in the world, there is a need for an accessible and efficient compound that inhibits the bacteria. If compounds can target this particular strain, it is expected that there would be lower rates of dental decay and essentially lower rates of problems that arise from the decay, including root canals and tooth degradation.

Three compounds were synthesized and modeled after previously researched organic compounds that were reported to inhibit \textit{S. mutans}. Previously researched compounds that have shown low Minimum Inhibitory Concentrations (MIC) have hydrophobic and hydrophilic characteristics which were measured with a log P lower than 7. The similarities taken for the compounds in this research include a long hydrophobic carbon chain with a hydrophilic end para on the benzene ring. With these characteristics, three compounds were modeled and with low concentrations, inhibition of \textit{S. mutans} took place. The three compounds are seen in Figure 1.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Molecular Formula</th>
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</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>Compound 1 (1) ( (C_{19}H_{24}O_2) )</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>Compound 2 (2) ( (C_{19}H_{30}O_2) )</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>Compound 3 (3) ( (C_{17}H_{28}O) )</td>
</tr>
</tbody>
</table>

\textbf{Figure 1.} Summarization of our compounds containing key features (repeated).

The main hypothesis was that \textbf{3} was most likely to inhibit \textit{S. mutans} with the highest percentages of inhibition than the other two compounds. This is deduced from
previous research where 3 contains a long hydrophobic carbon chain para to a phenol group on the benzene ring. Previous work by other researchers has shown similar structures inhibiting *S. mutans* with minimal amounts of the compound. The findings particularly pointed out a significance of a carbon chain and phenol group. With comparison to those findings, it was expected for 2 to also show significant inhibition. Background research is further explained in Chapter 2.

For this project, we evaluated various hydrophobic compounds that were known to inhibit *S. mutans* in order to find those molecular structural characteristics among the most effective inhibitors. After evaluating these features, we were able to establish a fundamental structure type of compound to investigate as an inhibitor. After synthesis and confirmation of the compounds through $^1$Hydrogen Nuclear Magnetic Resonance Spectroscopy, *S. mutan* inhibition testing was conducted by counting Colony Forming Units/milliliter (CFU/mL).
CHAPTER 2
REVIEW OF RELATED LITERATURE

Review of Related Literature for Synthesizing Compounds

In recent years, dental hygiene materials have become more prominent in chemical research. Many oral care materials are too expensive to be used by populations around the world. Researchers have been attempting to find less costly and more effective oral care products that inhibit the cause of the oral diseases.

Tooth decay and periodontal disease are one of the most common bacterial infections. There are over 200 to 300 types of bacteria that occur in the mouth; however, *Streptococci mutans* are the main cause of dental decay. Figure 2 shows the steps of tooth decay and its factors. Step 1 shows that 3 main factors cause tooth decay; the teeth, bacteria, and food ingestion. In Step 2, dental plaque occurs when *S. mutans* accumulate on the tooth surface. When the bacterial communities are formed, they feed off of carbohydrates and sugars from ingested foods and produce an acidic byproduct which is seen in Step 3. Then in Step 4, acidity on the tooth surface causes a decrease in the pH and will eventually lead to tooth mineral loss causing tooth erosion. Furthermore, *S. mutans* buildup can become a potential problem because this strain of bacteria can store polysaccharides from foods for long periods of time after ingestion. This causes a continuation of secretion of the acidic byproduct for hours after eating and more tooth decay.
**Figure 2.** Steps of tooth decay.\(^7\)
Over the years, researchers have tried to inhibit *S. mutans* because of the diseases it causes in the mouth. Numerous researchers have particularly focused on antimicrobial agents found in plants, food spices and different beverages because they are usually low in toxicity. For example, Greenberg et al. reported that bark extract taken from the stem bark of *Magnolia officinali*, showed strong anti-microbial effects against oral bacteria. During that study, they found other phenolic natural extracts and flavors that had similar effects, including eugenol, magnolol, and thymol. Another study conducted by Kubo et al. found anti-microbial effects against *S. mutans* with α-terpineol from green tea flavor and α-terpinyl and linalyl acetates from cardamom seed flavor.

This thesis project focused on evaluating molecular structural features in compounds from previous studies that showed inhibition against *S. mutans*. Those particular characteristics were factored into this research for modeling a new set of compounds to inhibit the *S. mutans* bacterial strain.

We first briefly focused on investigating the lipophilic characteristics of compounds proven to inhibit *S. mutans*. Lipophilicity helps determine how well compounds distribute throughout the body and relate with membranes. Lipophilic compounds are able to penetrate the lipid bilayer membrane easily because the compound can fit into the hydrophobic portion of the membrane. However, because membranes contain molecules that have hydrophobic chains with a polar group at the end, it seemed reasonable to design molecules which also have both hydrophobic chains and a small polar functional group at one end. A compound that has both hydrophobic and hydrophilic portions is able to interact with the lipid bilayer.
Kubo et al. previously investigated molecules with both hydrophilic and hydrophobic portions because they are simple and easy to acquire. As seen in Figure 3, the compounds of interest in Kubo et al.’s research contained a long hydrophobic carbon chain and a hydrophilic hydroxyl group on the end.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>MIC (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>1-Tridecanol (4)</td>
<td><img src="image" alt="Structure" /></td>
<td>6.25</td>
</tr>
<tr>
<td>1-Tetradecanol (5)</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt;800</td>
</tr>
<tr>
<td>Geraniol (6)</td>
<td><img src="image" alt="Structure" /></td>
<td>400</td>
</tr>
<tr>
<td>Farnesol (7)</td>
<td><img src="image" alt="Structure" /></td>
<td>12.5</td>
</tr>
<tr>
<td>Nerolidol (8)</td>
<td><img src="image" alt="Structure" /></td>
<td>25</td>
</tr>
</tbody>
</table>

**Figure 3.** Structure-antibacterial activity relationship of long-chain alcohols against *S. mutans.*

In Kubo et al.’s research, various molecules with hydrophobic and hydrophilic portions were tested against *S. mutans* to determine if there was a correlation between carbon chain lengths and the Minimal Inhibitive Concentrations (MIC) values at µg/mL. MICs represent the least amount of a compound needed to inhibit a microbe and that is an interest for this thesis research because we want to be able to use very minimal amounts of the compounds against the bacteria. **Figure 3** shows 4 having a MIC of 6.25 µg/mL, and then 5 having over 800 µg/mL. Kubo et al. noticed a correlation that shows low MIC values of compounds against *S. mutans* with carbon chains containing approximately 14 carbon atoms.
Kubo et al. saw a similar pattern with naturally occurring isoprenes. **Figure 3** shows that terpenes, like 7 and 8 have relatively low MICs’ with 12.5 μg/mL and 25 μg/mL respectively. 6 had a much higher MIC value because it contained a shorter hydrophobic chain. With Kubo et al.’s research, compounds with a fundamental terpene skeleton seemed to show good inhibitory effects against *S. mutans* with low MIC values, and containing both hydrophobic and hydrophilic components. 12

Since we noted from Kubo’s research that compounds with a long hydrophobic carbon chain and hydrophilic components were effective against *S. mutans*, we proposed that compounds with a particular level of lipophilicity would be most effective in inhibiting *S. mutans*. Log P values measure lipophilicity (partition coefficient in octanol-water). 13 As log P values increase, compounds are considered more hydrophobic. Greenberg et al. found that compounds with log P greater than 7 had poor water solubility and were harder to investigate so they focused on log P values lower than that in their research. 13 From Greenberg’s research results, we particularly looked at compounds that had log P values close to 7 with low MIC values, and compounds that contained hydrophobic and hydrophilic components. **Figure 4** shows the compounds that were particularly important for this thesis research. 14, 15, 16

In **Figure 4**, the log P values for Greenberg et al. phenolic compounds were displayed and they are low enough to have good distribution, while close enough to the optimum log P of 7. 14 Furthermore, the MICs’ are all pretty low and show that there is strong germ-kill activity with MIC values less than 50 μg/mL. 14
<table>
<thead>
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<th>Name</th>
<th>Structure</th>
<th>Log P</th>
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<td>4-Hexylphenol</td>
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<td>4.62</td>
<td>25</td>
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<td>(9)</td>
<td><img src="image" alt="Structure" /></td>
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<td>4-Heptylphenol</td>
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<td>(10)</td>
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<td>4-Nonylphenol</td>
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<td>6.21</td>
<td>5</td>
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<td>(11)</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>Xanthorrhizol</td>
<td></td>
<td>5.57</td>
<td>7.8</td>
</tr>
<tr>
<td>(12)</td>
<td><img src="image" alt="Structure" /></td>
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</table>

**Figure 4.** Compounds of interest from Greenberg et al.\textsuperscript{14, 15, 16}

Given that both hydrophobicity and the polar end phenol group seemingly being key features, we summarized our compounds with said characteristics in **Figure 1**.

**Figure 1.** Summarization of our compounds containing key features (repeated).

Greenberg’s research differed from Kubo’s with both the hydrophobic and hydrophilic portions. Kubo’s research contained some double bonds in the long carbon chain while the compounds of interest from Greenberg’s research were all saturated carbon chains. Furthermore, Greenberg had phenol groups on the end of the carbon chain.
while Kubo had only hydroxyl groups. Since Greenberg et al.’s research had reasonable
log P values, we decided to model the phenol group aspect for our compounds, but also
decided to model both saturated and unsaturated hydrophobic portions since the MIC
values were low for both research publications.\textsuperscript{12, 14, 15, 16}

The data for the phenolic compounds from Greenberg’s research and the data for
the terpenes in Kubo’s research are shown in Figure 5. The molecules shown are 6, 7,
12, 10, 11, and cholesterol.\textsuperscript{14, 17, 18}

Based on our working hypothesis that these type of compounds would inhibit \textit{S.}
\textit{mutans} by interacting with the phospholipid membrane, we also considered the length of
each molecule to evaluate how extensively it could insert into the membrane. Because it
is well known that a very common substance, cholesterol, has a large hydrophobic group
and a small polar end group, we elected to use the length of cholesterol as a guide in
designing our compounds. We decided to compare cholesterol values to Figure 5
because it regularly acts in membrane interactions.\textsuperscript{19} Cholesterol is able to insert into
bilayer membranes because of its hydroxyl group located on the end of the molecule. It is
able to form hydrogen bonds with the phospholipid groups in the membrane.\textsuperscript{19} Since
cholesterol is able to have membrane interactions, we proposed that the length of the
molecule may be an ideal length to interact with the membranes of the bacteria. We also
wanted to investigate if the log P and length of cholesterol was similar to the previously
studied compounds in Kubo and Greenberg’s research. By considering previous research
and factors in inhibition, the compounds in this thesis project should have similar
lipophilicity and lengths to the compounds in Figure 5. It is assumed that the similarities
would help the compounds interact with the bacteria by penetrating the lipid bilayer and cause more inhibition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log P</th>
<th>Length (Angstroms)</th>
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<tbody>
<tr>
<td>Geraniol (6)</td>
<td>2.9</td>
<td>9.297</td>
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<td>Farnesol (7)</td>
<td>4.8</td>
<td>15.022</td>
</tr>
<tr>
<td>Xanthorrhizol (12)</td>
<td>5.57</td>
<td>12.337</td>
</tr>
<tr>
<td>4-Heptylphenol (10)</td>
<td>5.15</td>
<td>13.016</td>
</tr>
<tr>
<td>4-Nonylphenol (11)</td>
<td>6.21</td>
<td>15.871</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.7</td>
<td>16.501</td>
</tr>
</tbody>
</table>

**Figure 5.** Comparison of log P values and lengths (Angstroms) of compounds of interest. 

Figure 5 helped determine that synthesis of compounds with log P values close to 6 and overall lengths of molecules close to 16 Angstroms are necessary for this thesis research because we propose those characteristics would be most effective in inhibiting S. mutans. If the reader refers back to the compounds listed in Figure 1, one will see that our compounds meet the prescribed criteria with respect to hydrophobicity, polar end group, and length.

The particular characteristics for modeling our compounds was drawn by seeing that the length of cholesterol is around 16 Angstroms and the log P values of 10, 12, 11, and cholesterol range from 5.15-8.7. 

6 was a good starting material for the synthesized part of this research because it contains a hydrophobic carbon chain and we anticipated that it could be easily connected to a phenol group. Synthesis was completed of three compounds that have a hydrophobic carbon chain and a phenol group located on the end of structure; the experimental for which is given in Chapter 3. One compound has an unsaturated carbon chain while the other 2 compounds are saturated. The compounds are drawn in Figure 6 and labeled accordingly.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Molecular Formula</th>
<th>Name</th>
<th>Molecular Weight (amu)</th>
<th>Log P</th>
<th>Length (Angstroms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>(C₁₉H₂₄O₂)</td>
<td>4-(4,8-dimethylnona-1,3,7-trienyl)phenyl acetate</td>
<td>284.399</td>
<td>3.56</td>
<td>About 15.502 Angstroms from C₁ to C₁₈</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>(C₁₉H₃₀O₂)</td>
<td>4-(4,8-dimethylnonyl)phenyl acetate</td>
<td>290.447</td>
<td>5.421</td>
<td>About 15.632 Angstroms from C₁ to C₁₈</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>(C₁₇H₂₈O)</td>
<td>4-(4,8-dimethylnonyl)phenol</td>
<td>248.41</td>
<td>5.195</td>
<td>About 15.032 Angstroms from O₁ to C₁₈</td>
</tr>
</tbody>
</table>

**Figure 6.** Compounds synthesized for Monica Bhupendra Patel thesis research.¹⁸,²⁰
Review of Related Literature for Testing Compounds

Once our designed inhibiting Compounds 1, 2 and 3 were synthesized, a decision had to be made on how they should be tested on the bacteria *Streptococci mutans*. There are many ways to test compounds on bacterial agents and a few methods were considered. These included turbidity measurements, the Kirby-Bauer disk diffusion susceptibility test, and a comparison of Colony Forming Units/milliliter (CFU/mL) on agar plates.

Before choosing a method for testing, a medium needed to be found that the *S. mutans* bacterium grows the best in. This is important because different strains of bacteria have different broths and agar mediums that it can survive in and be worked with in laboratory testing. Specifically, oral streptococci strains are biochemically and physiologically influenced by its environment.\(^{21}\)

In previous work, Emilson et al. tested various media on *Streptococcus mutans* to see the best growth.\(^{22}\) Mitis-salivarius (MS) agar had been used in culture testing, but they wanted to view the changes of growth of MS with MC agar, Mitis-sucrose-bacitracin (MSB), BCY agar, and MM10 sucrose agar. *S. mutans* were grown in a broth solution and incubated at 37° C for 15 hours. Then the agar plates were streaked by plating 100 μL of the 10\(^{-4}\) and 10\(^{-5}\) dilutions of the incubated broth solution. Once streaking was completed, the agar plates were incubated for 48 hours at 37 ° C to see bacteria growth. The researchers found that MC, BCY and MM10 sucrose agar had approximately 10% higher values of expressed *S. mutans* than MC and MSB agar.\(^{22}\) A concern of this study is that the samples were taken from oral specimens and consisted of various types of *S.*
mutans. Furthermore, it was done many years ago in 1975 and recent research with more current results does exist.\textsuperscript{22}

Another study used trypticase soy broth (TSB) for growth of \textit{Streptococcus mutans} 6715. This type of broth had been used by others for \textit{S. mutans} growth and was being evaluated to a chemically defined medium. Turchi et al. found that \textit{S. mutans} adapted quickly to the TSB medium with only 2 hours of lag phase than the chemically defined medium with a lag phase of 4 hours.\textsuperscript{21} TSB broth was found to be a good medium for the thesis project because the bacteria grew quickly and adapted well to the medium, and the materials were already available.

After choosing the medium, the determination of Colony Forming Units/milliliter (CFU/mL) was used in order to measure the inhibition of \textit{S. mutans}. There are numerous ways to test inhibition for \textit{S. mutans}; however, this method is cost effective and the materials were already accessible. This technique involves plating the bacteria with the compounds already present in the bacteria and broth dilutions. First, the broth and bacteria solution are incubated until it reaches optical growth. Then, dilutions are done to the power of $10^{-5}$ with 900 µL of water and 100 µL of broth. The compound is added to the final dilution and plated every 4 hours starting at 0 hour to 24 hours. The plates are counted to find the CFU/mL and a graph is charted to show the comparison of inhibition at different hours.\textsuperscript{23} The materials needed included the trypticase soy broth, agar, and sterile loops and plates.

Turbidity and the Kirby-Bauer disc diffusion susceptibility test were considered for measuring inhibition of \textit{S. mutans} with the compounds but were ultimately not used. Turbidity is usually used for water quality measurements.\textsuperscript{24} It measures the loss of
transparency in the water due to suspended particles. In a turbidimeter, a light beam passes through the sample. Turbidity is then measured by how much the sample scatters light. The more solids in the water results in more scattered light from the sample and essentially a higher turbidity reading.\textsuperscript{24}

Bacteria samples are placed in a broth medium instead of water, and turbidity measurements “determine the amount of light scattered by a suspension of cells.”\textsuperscript{24} Bacteria scatters the light by a proportion of the turbidity (optical density) of the suspension of cells. It is then displayed on a standard curve. For bacteria cells, turbidity can estimate the number of bacteria in the broth medium.\textsuperscript{25} With the addition of the compounds, it can be seen if the amount of colonies of cells (cells/mL) decreases. It would result in a decrease in turbidity. This is a fast and non-destructive method of testing bacteria inhibition. However, the issue with this application is that the instrument is only sensitive to cell counts of greater than $10^7$ cells/mL.\textsuperscript{25} The compounds in this project are aiming to inhibit the bacteria. If the cell count decreases significantly, cell densities less than $10^7$ cells/mL would not detected.\textsuperscript{25}

The Kirby-Bauer disc diffusion susceptibility test was another method that was considered. This test determines the sensitivity of bacteria to various antimicrobial compounds. The Kirby-Bauer test is used in most antibiotic testing to be able to visually see various compounds in one bacteria culture. Bacteria is grown on an agar plate that is specific to the strain. Then, antibiotic disks are placed onto the agar surface and it is incubated. The zones on the plates are measured after a certain time frame to see if there was inhibition of the bacteria around the compound’s discs.\textsuperscript{26} A concern with this method was obtaining some of the costly materials.
CHAPTER 3
EXPERIMENTAL

Materials and Instruments

The following materials were purchased from Sigma-Aldrich Chemical Company. All materials were used without further purification from the material: ethyl acetate, hexane, geraniol, anous aldehyde stain, thionyl chloride, methanesulfonic acid, dichloromethane, methanesulfonyl chloride, sodium chloride, diethyl ether, acetonitrile, triphenylphosphine, pyridine, acetic anhydride, hydrochloric acid, magnesium sulfate anhydrous, butyl lithium, nitrogen gas, hydrogen gas, silica gel, 5% palladium on carbon, deuterated chloroform, potassium carbonate, methanol, trypticase soy broth, and trypticase soy agar. The bacteria used is from ATCC and it is *Streptococcus mutans* ATCC 25175.

The purity of 6 was checked with Thin Layer Chromatography (TLC; silica gel 60 G F254, 1:5 ethyl acetate: hexane). The TLC indicated that 6 was approximately greater than 95% pure with the only detected impurity being the corresponding aldehyde. This molecule was used without further purification. A simple distillation of triethylamine was conducted by boiling between 89-95 °C to clean it up.

All 1HNMR spectrums were performed on a Bruker 300 MHz Spectrometer. All spectra were obtained on solution in CDCl₃. Evaporation occurred on a Buchi Rotavapor with a Buchi Heating Bath, weight measurements were on an Adventurer Pro Ohaus balance, and a Barnstead 1 Thermolyne hot plate and SRS DigiMelt melting point meter were used. All TLC were performed as Silica Gel 60 G F254 coated on glass plates from
Thomas Scientific. Then for testing inhibition, sterilization occurred with an autoclave, incubator and all performed under a vented hood.
Method of Obtaining the Compounds

1. Preparation of methanesulfonyl chloride (13) according to the published procedure in Organic Syntheses, Coll. Vol. 4, p. 571 (1963); Vol. 30, p. 58 (1950) by C. R. Noller, Peter J. Hearst. 27

![Diagram showing the conversion of methanesulfonic acid to methanesulfonyl chloride]

Figure 7. Preparation of methanesulfonyl chloride (13). 27

A three necked round bottom flask with a magnetic stir bar was connected to a reflux condenser, a thermometer, and a separatory funnel under the hood. Then 25 mL of thionyl chloride was added drop-wise for a 2 hour period to approximately 18 mL of 14. This reaction was kept in between 80-95º C. During this time, the solution was bubbling slightly. After the addition of thionyl chloride, the flask was kept at 90º C for approximately 2-3 hours. A distillation was conducted where the solution boiled in a 100 mL round bottom flask. Approximately 75 mL of 13 was obtained with a boiling point of 163 º C which compared favorably with reported literature values.27,28

2. Preparation of geranyl chloride (15) according to the following laboratory notes performed by K.S. Kyler (KSK-IUP-II-87).29 (TLC; silica gel 60 G F254, 1:5 ethyl acetate: hexane)
**Figure 8.** Preparation of geranyl chloride (15). 29

In a round bottom flask with stir bar and stopper, 6.1 mL of 6 was added, to 100 mL of CH₂Cl₂. The reaction was kept at -20º C under nitrogen. Then, 9.7 mL of Et₃N was added to the flask followed by a drop-wise addition of 4.0 mL of 13. Stirring occurred for 1 hour at -20 º C. Then, 10 grams of Et₄N⁺Cl⁻ was added and the reaction was warmed to 23º C and stirred for 3 hours. The reaction was then diluted with Et₂O and washed with H₂O and a brine solution (approximately 10 mL of sodium chloride with 50 mL of water). The solvent was evaporated in a rotary evaporator. It gave a yellow oil which was passed through a short column of silica gel. The purity of the product, 15, was confirmed by TLC whose R_f value matched the R_f reported in KSK-IUP-II-87, which was approximately greater than 95% pure. 29 This molecule was used without further purification.

3. Preparation of geranyl-triphenyl-phosphonium chloride (16) according to the following laboratory notes performed by K.S. Kyler (KSK-IUP-I-91). 30
Figure 9. Preparation of geranyl-triphenyl-phosphonium chloride (16).⁴⁰

First, 4 mL of CH₃CN and 3.44 grams of 15 were combined in a round bottom flask. Under nitrogen, 10.48 grams of Ph₃P was added. This stirred and heated for 2 hours between 75 °C and 83°C. The resulting brown/yellow mixture was poured into 400 mL of diethyl ether which turned the solution into a white color. This solution was poured into a vacuum filtration (suction filtration with a glass funnel). This was washed twice in 300 mL of ether and dried under vacuum filtration.³⁰ Approximately 5.13 grams of the phosphonium salt was obtained and characterized by a melting point of 188° C which compared favorable to reported literature values.³¹

4. Preparation of p-acetobenzylaldehyde (17). (TLC; silica gel 60 G F254, 1:4 ethyl acetate: hexane)
Figure 10. Preparation of p-acetobenzylaldehyde (17).

In a 100 mL round bottom flask with stir bar, 2.38 grams (30 mmole) of pyridine was added to 20 mL of CH$_2$Cl$_2$ and 2.44 grams (20 mmole) of 18. That stirred at room temperature and a drop-wise addition of 3.06 grams (30 mmole) of acetic anhydride occurred for about 10-15 minutes. This solution turned into a transparent yellow color. Then a TLC was taken that showed that the reaction still needed to be continued. After another 30 minutes of stirring and continuing the reaction, another TLC was done.

The solution was placed in a separatory funnel and rinsed with ethyl acetate. The solution was then washed with approximately 20 mL of hydrochloric acid and that allowed it to separate. A few more mL of ethyl acetate was added to allow it further separation. It was rinsed with a brine solution (10 mL of sodium chloride with 50 mL of water). The entire solution was given time to separate and then the water was poured out. Lastly, 1 gram of magnesium sulfate anhydrous was added. A TLC was taken of the solution to check the purity of the product 17 and showed that the reaction was completed with approximately greater than 95% purity. This molecule was used without further purification. The solvent was evaporated off in a rotary evaporator and produced 4.28 grams of 17.
5. Preparation of 1 according to the following laboratory notes performed by K.S. Kyler (KSK-IUP-I-94).  

![Geranyl-triphenyl-phosphonium chloride (16)](image)

**Figure 11.** Preparation of compound 1; 4-(4,8-dimethylnona-1,3,7-trienyl)phenyl acetate.

First, 43 mL of THF was added to 4.49 grams of the 16 in a 250 mL round bottom flask with a stir bar and the reaction was setup under a nitrogen atmosphere. Then, the mixture cooled to -78 °C and 5.2 mL of butyl lithium was added and stirring occurred for one hour. The solution became orange. Next, 1.42 grams of 17 was added to the solution and it stirred while warming up to room temperature. After approximately 30 minutes, ethyl acetate was added and the entire solution was placed it into a separatory funnel and washed with brine. It was evaporated in the rotary evaporator.  

This procedure made approximately 5.8 grams of crude product. The crude material was purified by column chromatography on silica gel 60, with a 1:10 ethyl acetate: hexane solvent for elution. Approximately, 0.85 grams of the Wittig product, 1, was collected after evaporation of the solvent. This product was the first compound to be tested against the oral bacteria. 1 is a yellow type of substance that looks like an oil.

6. Characterization of 1 with 1HNMR

First, 1 mL of chloroform-d was added to 1 and then evaporated in a rotary evaporator. A few drops of chloroform-d was added again to 1 and placed into the NMR
tube. The Proton NMR spectrum was consistent with the structure assigned to 1. There was a slight mixture of trans and cis isomers but that was to be expected of this compound. 1 was taken out from the NMR tube with ethyl acetate and evaporated in a rotary evaporator to leave only Compound 1. The NMR of 1 is located in the results section in Chapter 4. Approximately 250 mg of 1 was saved for testing, and the rest of the product was used to make 2 and 3.

7. Preparation of 2. (TLC; silica gel 60 G F254, 1:5 ethyl acetate: hexane)

![Figure 12. Preparation of compound 2; 4-(4,8-dimethylnonyl)phenyl acetate.]

A hydrogenation reaction was conducted to convert 1 into 2. Approximately 600 mg of 1 was added to a 5 mL of THF in a 100 mL round bottom flask with a stir bar. Then, 500 mg of 5% palladium on carbon was added to the flask while stirring. A balloon filled with hydrogen gas was attached to a condenser. By turning the valve, the vacuum was turned on and off for about 30-60 seconds. This allowed the solution in the round bottom flask to boil on and off. Then, the solution stirred for 2 hours. The vacuum was turned on and off 3 more times and then turned off completely.

A TLC indicated the completion of the reaction with approximately greater than 95% purity. Gravity filtration of the solution eliminated the palladium. The round bottom flask was rinsed with ethyl acetate to retrieve the remaining product from the flask.
Then, evaporation of the solution left about 130 mg of 2, a clear oily substance. This molecule was used without further purification.

8. Characterization of 2 with $^1$HNMR

The procedure for Step 6 was repeated for 2. The NMR showed that all the double bonds from the carbon chain were gone and had successfully hydrogenated 1 to make 2. 2 was taken out from the NMR tube with ethyl acetate and evaporated in a rotary evaporator to leave only 2. The NMR of 2 is located in the results section in Chapter 4.

9. Preparation of 3 (TLC; silica gel 60 G F254, 1:10 ethyl acetate: hexane)

![Figure 13. Preparation of compound 3: 4-(4,8-dimethylnonyl)phenol.](image)

First, 110 mg of 2 was added to a round bottom flask with 5 mL of methanol, 100 mg of potassium carbonate and a stir bar. While stirring, a TLC was taken and indicated the completion of the reaction with approximately greater than 95% purity. Then, 10 mL of water and about 5 drops of 9 M hydrochloric acid was added to the product to acidify it. The product changed colors from a yellow solution to white. Ethyl acetate was added to the product and the solution was poured into a separatory funnel. It was stirred slightly by hand and the water was taken out. Then a slight amount of drying agent (magnesium sulfate anhydrous) was added and the solution was evaporated in a rotary evaporator. The procedure left approximately 30 mg of 3.

10. Characterization of 3 with $^1$HNMR

26
The procedure for Step 6 was repeated for 3. The NMR of 3 is located in the results section in Chapter 4.
Method of Testing the Compounds

Testing the compounds on the *Streptococci mutans* bacteria was preformed according to the procedure Culture Growth and Maintenance by N. Bharathan in Laboratory Research, Laboratory Techniques in Molecular Biology.\(^{23}\) This method involved diluting dense bacteria culture of ATCC 25175 *Streptococcus mutans* in trypticase soy broth media and spreading a small volume of the dilution culture onto a trypticase soy agar plate. The broth, agar, and water were autoclaved to ensure sterile materials. Also, all plating and streaking was conducted in sterile conditions under a sterile hood and cleaned with 70% ethanol.

1. Preparation of Initial Growth of *S. mutan* Culture

The bacterium was grown in order to ensure the bacteria contained viable colonies. A slight amount of the ATCC 25175 *Streptococci mutans* was added to 5.0 mL of trypticase soy broth under a sterile hood. This was incubated for optimal growth of 16-18 hours at 37 °C for 110 RPM.

2. Preparation of Initial Dilution of *S. mutans* Culture

Dilutions were done to 10\(^{-7}\) by adding 900 µL of sterile autoclaved water to each dilution in a total of 7 dilution tubes. Then 100 µL of culture was added to the first tube. The solution in the first tube was mixed and then 100 µL of the first tube was added to the second tube. This process was continued to the 7\(^{th}\) dilution (10\(^{-7}\)).

3. Plating Initial Dilutions of *S. mutans*

After the dilutions were made, 100 µL of dilution 10\(^{-2}\) was plated onto a trypticase soy agar plate. This step of plating dilutions was then repeated for dilutions 10\(^{-3}\), 10\(^{-4}\) and 10\(^{-5}\). The plating was done in a streaking manner under a sterile hood, with sterile agar
plates and sterile plastic loops. After some time of incubation of the plates, the $10^{-5}$ dilution agar plate had growth of bacteria colonies.

4. Preparation of S. mutans Used For Testing

The bacteria colonies were taken with a sterile plastic loop off of the $10^{-5}$ dilution agar plate and placed into three tubes with 5 mL of trypticase soy broth. The tubes were incubated at 37 °C at 110 RPM for 16-18 hours.

5. Preparation of Dilutions Used For Testing

Then, similarly to step 1, each of the bacteria culture tubes were diluted down to $10^{-5}$. Since the three compounds were going to be dissolved with methanol for testing, methanol also needed to be plated as a control to make sure it did not inhibit the bacteria itself. Then four $10^{-5}$ dilutions were made for each of the three bacteria culture tubes by following Step 1. This was done so that each of the three compounds would be tested in triplicate to analyze results.

6. Preparation of Solutions Containing Methanol and Compounds

Solutions were made with methanol and each of the compounds. This was done so the compound would be dissolved in some control solution that would not affect the results. Approximately 10 mg of each of the compounds were added to 4 mL of Methanol. The 1 solution contained 9.99 mg of 1 with 4 mL of Methanol, the 2 solution contained 10.2 mg of 2 with 4 mL of Methanol, and the 3 solution contained 10.1 mg of 3 with 4 mL of Methanol.

7. Addition of Compound Solutions to S. mutans

Once the $10^{-5}$ dilution tubes were made, the compound solutions were added to that last dilution. Since there were four $10^{-5}$ dilution tubes per culture, each culture was
exposed to **1**, **2** and **3** and the first culture was additionally exposed to our control. 10 µL of each of the compound and methanol solution were added to the $10^{-5}$ dilutions. This step made a total of ten $10^{-5}$ dilution tubes, three containing **1** solutions, three containing **2** solutions, three containing **3** solutions, and one with the control.

8. Plating of Compound Solutions and *S. mutans*

When the dilution tubes contained the compounds, plating was done every 4 hours starting at 0 hour, right after the compounds were added to the bacteria, to 24 hours. Plating was done under a sterile hood with sterile equipment. 100 µL were taken from the $10^{-5}$ dilutions and pipetted onto a trypticase soy agar plate. Then the plate was streaked with a sterile plastic loop until the liquid did not move on the agar. The plates were incubated at 37 °C at 110 RPM for 24 hours.

9. Counting Bacteria Colonies

Counting the bacteria colonies and seeing if those colonies decreased after exposure to the compound showed if there was inhibition. Colony Forming Units/milliliter (CFU/mL) were counted 24 hours after streaking. CFU/mL is determined by the number of bacteria colonies multiplied by the dilution factor. Since the $10^{-5}$ dilutions were plated, the dilution factor equals $10^6$ because 100 µL of the $10^{-5}$ dilution tube were placed onto the agar plate. The three CFU/mL results for each of the compounds were then averaged together to determine Colony Forming Units/mL versus time.

To count the colonies, a template was made to split the agar plate into squares. (Figure 14) One of the squares was counted and then multiplied by 22, the total number of squares on the plate. This is a technique used in counting bacteria colonies since there
are sometimes too many colonies to count. It provides a reasonable estimate of the total number of colonies on an agar plate.

![Agar plate template](image)

**Figure 14.** Agar plate template.

10. Repeated Testing for 3

The entire testing procedure was repeated again for 3 because the first results barely had any bacteria colonies on the zero hour agar plates. Hypothetically, the zero hour plates should have the most bacteria colonies because the compound needs time to work. We assumed over time the compounds would inhibit the bacteria, so it is assumed that the less amount of colonies may have been due to rapid inhibition or contamination error.
CHAPTER 4
DATA AND ANALYSIS

Nuclear Magnetic Resonance Spectroscopy Results

The three compounds were characterized by Nuclear Magnetic Resonance Spectroscopy (1H NMR). In all three compounds, there is a peak shown around 7.26 ppm which represents the solvent used for the NMR, chloroform-d, deuterated chloroform. Furthermore, the NMRs’ were interpreted by referring to the textbook Organic Structural Spectroscopy, Second Edition, by Joseph B. Lambert et. al.

The NMR for 1 is shown in Figure 15. Compound 1 was confirmed by peaks around 7.05 ppm and 7.30 ppm for the benzene ring, a strong singlet at 2.25 ppm representing the acetate group, and various peaks ranging from 1.00 ppm to 2.00 ppm representing the methyl groups and the saturated and unsaturated carbon chain. Also, there was a mixture of cis and trans isomers for the carbon chain, but that was expected since there are multiple double bonds. The signal at 5.20 ppm corresponds to the H7 on the nonatrienyl chain. Also, the signals at 6.40 ppm to 6.60 ppm correspond to the 3 hydrogens at Carbon 1, Carbon 2, and Carbon 3 of the nonyl chain.
Figure 15. $^1$HNMR of compound 1.

The NMR for 2 is shown in Figure 16. Compound 2 was confirmed because there were a lot more peaks from 1.00 ppm to 2.00 ppm in Figure 16 than Figure 15. Also the peaks in Figure 15 around 5.20 ppm, 6.40 ppm and 6.60 ppm are gone. This shows that the hydrogenation reaction worked and most of the double bonds became saturated. Lastly, the peaks at 7.00 ppm to 7.30 ppm representing the benzene ring and the singlet at 2.25 ppm representing the acetate group was still intact.34

Figure 16. $^1$HNMR of compound 2.
The NMR for 3 is shown in Figure 17. Compound 3 was confirmed mainly because the strong singlet peak shown in Figure 15 and Figure 16 for the acetate group at 2.25 ppm disappeared. Also, the benzene ring is still shown with peaks at 6.8 ppm and 7.0 ppm.  

![Figure 17. ^1H NMR of compound 3.](image-url)
Inhibition Testing Results

Figure 18 shows some of the agar plates results when exposed to the compounds at 0, 12, and 24 hour streaking times. It shows the bacteria growth and the three compounds from 0 hour to 24 hours. It is seen in Figure 18 that 3 did not have colonies on the 0 hour plate and an error may have occurred due to contamination, or it may be that inhibition occurred instantly when the compound solution was added. However, 1, 2, and the repeated test of 3 did show 0 hour plates with colonies and there was significant inhibition over time and in the 12 hour and 24 hour plates.
Trypticase Soy Agar Plates With *S. mutans* Exposed to Compounds

<table>
<thead>
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<th>Time (Hours)</th>
<th>0</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 (Test 1)</strong></td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>2 (Test 1)</strong></td>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>3 (Test 1)</strong></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>3 Repeat (Test 1, 2 &amp; 3)</strong></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 18.** Agar plates exposed with compounds and *S. mutans*.

The values of Colony Forming Units/milliliter versus Time (in hours) are shown in **Figure 19**. The percent decrease from 0 hour to 24 hour is calculated for **1**, **2**, and **3** by the formula \((1 - (24 \text{ hour CFU/mL} \div 0 \text{ hour CFU/mL}))\). This number was multiplied by 100 to get the percentage decrease. Compound **1** showed 99.88% inhibition from 0 hour to 24 hour which started with approximately \(550 \times 10^6\) CFU/mL but decreased down to approximately \(0.667 \times 10^6\) CFU/mL. Compound **2** showed 77.02% inhibition starting...
with approximately 557 x 10^6 CFU/mL at 0 hour down to approximately 128.33 x 10^6 CFU/mL at 24 hour. Lastly, the repeat of compound 3 showed approximately 64.28% inhibition from 0 hour to 24 hour.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>CFU/mL x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>726</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>24</td>
<td>682</td>
</tr>
<tr>
<td>Percent Inhibition after 24 hr.:</td>
<td>99.88%</td>
</tr>
</tbody>
</table>

**Figure 19.** Colony forming units/mL vs time and percent inhibition.

The graph below, **Figure 20**, Inhibition of *S. mutans*, shows all three compounds and control for CFU/mL x 10^6 versus Time (in hours). When the control was added, the amount of CFU/mL stayed pretty similar throughout the 24 hours and it showed a slight exponential growth around 5 to 10 hours. This observation suggests that the control did not affect the inhibition percentage and would not affect the results of the compounds.

Compounds 1 and 2 show a significant decrease in the amount of CFU/mL x 10^6. The amount of colonies started at a higher number and decreased within the first 5 hours for 1 and within the first 12 hours for 2. This observation suggests that 1 and 2 show significant inhibition against *S. mutans* and may be considered as antimicrobial compounds.
Figure 20. Graph of *S. mutans*, colony forming units (CFU/mL x 10^6) vs time (hours).\(^{35}\)

The 3 repeated tests show a decrease but not as much as previously expected. It was hypothesized that the phenol group in 3 would have a great effect in inhibiting *S. mutans* based off of previous studies; however, this research shows inhibition of approximately 64%. It is assumed that there may have been rapid inhibition that instantly inhibited the bacteria as soon as the compound solution was added, or that there was contamination or lack of bacteria in the dilution tube. The first test of 3 did not have any bacteria throughout all the plates from 0 hour to 24 hour and that is why repeated testing was necessary.

Furthermore, plating was not conducted before the addition of 1, 2, and 3. This was not completed because it was assumed that the compounds would take time to inhibit the bacteria and we were specifically looking for a decrease in the amount of colonies. This decrease was shown in all compounds, but unlike 1 and 2, 3 started with significantly fewer amount of colonies. Even though the bacterium was viable and inhibition occurred over time with 3, we do not know if it inhibited the bacteria instantly.
at 0 hour. In future research, plating of the dilution should be completed before and after the addition of the compounds to see if inhibition occurs rapidly for 3.
Summary

*Streptococci mutans* is the leading cause of dental decay, which is the most common infection in humans. There is a need for efficient and affordable dental care materials that inhibit *S. mutans*.

Previous research was analyzed to find structural molecular characteristics that have inhibited *S. mutans*. This led to the discussions that long hydrophobic carbon chains are able to infiltrate bacterial membranes while hydrophilic phenol groups can interact with the phospholipid membranes. Both saturated and unsaturated hydrophobic carbon chains with hydrophilic components were able to inhibit *S. mutans* with reasonable Minimum Inhibitory Concentrations. With these characteristics, lipophilicity measurements close to a log P of 6 were desired for compounds 1, 2 and 3. Furthermore, cholesterol is known to interact with the phospholipid membrane and the length of the molecule, 16 Angstroms, was considered a reasonable guideline for the lengths of our compounds. The compounds 1, 2, and 3 were characterized by Proton Nuclear Magnetic Resonance Spectroscopy and with the characteristics for modeling our compounds, this led to the initial hypothesis that compound 1, 2 and 3 would inhibit *S. mutans*.

Testing was conducted by counting the Colony Forming Units/mL x 10^6 versus Time in hours with trypsinase soy agar and the *S. mutan* bacteria ATCC 25175. Plating and counting were conducted every 4 hours from 0 hour to 24 hours, and inhibition was calculated by the percentage of decrease from the 0 hour to the 24 hour.
Compound 1, 2 and 3 showed a decrease in the Colony Forming Units/mL x 10^6 in a 24 hour period. Compound 1 had the most inhibition with approximately 99% decrease, while Compound 2 was approximately 77% and Compound 3 repeated approximately 64%. All three compounds showed a significant decrease in the amount of CFU/ml x 10^6 with very small amounts of the compounds used. The compounds in this research were able to inhibit the oral bacteria strain, *Streptococci mutans*. 
Conclusions

Three compounds were able to inhibit *Streptococci mutans* by approximately 99% inhibition with 1, approximately 77% with 2, and approximately 64% with 3. Based off of previous studies, high percentages of inhibition were expected for the compounds since they included hydrophobic and hydrophilic characters. Compound 3 was hypothesized that it would inhibit the strain the most; however, 1, which included double bonds in the hydrophobic carbon chain, inhibited *S. mutans* by approximately 99% in this research. Compound 3 may have had rapid inhibition since the first plating at 0 hour showed a significantly lower amount of colonies, but that cannot be proven until repeated testing has a plating before and after the addition of the compound in order to see if inhibition occurs right away. However, these results are significant since all three compounds inhibited the strain over time by more than 50% and shows that these compounds may be considered as antimicrobial agents against *S. mutans*. 
Recommendations

This particular thesis research has been a starting point and further studies are highly recommended. An important recommendation is repeated testing of the current research. However, they should be conducted in order to synthesize more amounts of the compounds, have various sources for the Streptococci mutans bacterial strain, and there should be numerous plating tests with varying times and mediums to view the changes in CFU/mL. Furthermore, testing should include plating the dilution before and after the addition of the compound at 0 hour in order to see if rapid inhibition occurs. Overall, the tests should be done in order to see if the percentages of inhibition are consistent with this research.

Another recommendation is to find the Minimum Inhibitory Concentrations for the three compounds. This could be done by lowering the compounds concentrations until inhibition stops occurring. With the appropriate instruments that can weigh extremely small amounts of the compounds, this process could be completed.

Lastly, the compounds should be researched and prepared for in vivo testing. Before testing, toxicity issues and membrane interactions of these compounds with the bacteria should be studied and addressed for conditions in the mouth. Then, in vivo testing should eventually occur to see if these compounds could be placed into dental hygiene materials and to see if there are side effects or symptoms to these compounds.
1. Loesche, W. J. Role of Streptococcus mutans in Human Dental Decay. Microbiological Reviews. 1986, 50, 371
   (accessed May 27, 2013).
29. Kyler, K. S. Ref. KSK-IUP-II-87. 2, 87