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The Effects of Triclosan Derivatives against the Growth of *Staphylococcus Aureus*

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Abstract

Triclosan is an antimicrobial commonly used in many different antiseptics and everyday products. Unfortunately, many bacteria are now resistant to triclosan due to innate resistance, mutations in the *fabI* gene, and/or overexpression of certain other genes (*soxS*, *marA*, and an efflux pump encoded by *acrAB*). Therefore, it is essential that drugs be developed to destroy bacteria now resistant to triclosan. In this experiment, four different derivatives of triclosan were tested for antibacterial capabilities under the supervision of Dr. Hubbard at Liberty University. The derivatives were synthesized by Professor McGibbon (professor of organic chemistry at LU). Solutions of 4.0 ug/mL of each derivative were made and then tested against *S. aureus* for inhibition capabilities. Some of the derivatives seemed to have some inhibition capabilities, but none of them were as inhibitive as triclosan itself. Of special note, some of the results seem to indicate that the added benzene ring may inactivate triclosan's antibacterial capabilities while the added chlorines provide at least some inhibition to *S. aureus*. Ultimately, the derivatives of triclosan did not have high inhibition rates at 4.0 ug/mL, but more experiments need to be performed in order to determine their effects at higher concentrations and on different species of bacteria. Hopefully, the data gathered and inferred from this experiment, including several valuable dissolving ratios and laboratory techniques which were discovered, will be implemented into future research on triclosan derivatives that will lead to the discovery of compounds even more inhibitive against bacteria growth than triclosan.

The Effects of Triclosan Derivatives against the Growth of *Staphylococcus Aureus*

Background

*Bacteria and Bacterial Diseases*

Pneumonia, MRSA (Methicillin-Resistant *Staphyloccus aureus*), tetanus, anthrax, plague—these infections and diseases have one major thing in common: they are all caused by bacteria. Bacteria are single-celled organisms that contain peptidoglycan (a unique structural component) in their cell walls. They generally have one of three typical cell shapes: spherical, cylindrical, or spiral. They reproduce via binary fission—the division of one cell into two (usually identical) daughter cells (Nester, Anderson, Roberts, & Nester, 2007).

The human body provides an excellent host for many bacteria types, but not all bacteria are harmful to humans. Internally and externally, the body is covered with bacteria. In fact, one square centimeter of the human back contains approximately 1,000 organisms (including bacteria and fungi) whereas one square centimeter of the human groin or armpit contains more than 10 million microorganisms (Nester et al., 2007). These microorganisms compose what is known as the normal flora of the human body; the normal flora consists of the microorganisms generally growing on healthy persons. The bacteria which compose the normal flora, in many ways, actually help protect the body from infection and disease by stimulating the immune system and preventing other harmful bacteria from growing on or in the body (Nester et al.).

Unfortunately, however, some bacteria are harmful or pathogenic—capable of causing disease. The problem occurs when bacteria have a parasitic relationship with the body—also known as an infection. The infection itself, however, may or may not be

noticeable. If, however, the infection leads to obvious damage to the body or bodily functions, the infection is said to have become an infectious disease (Nester et al., 2007). The diseases mentioned at the beginning of the introduction (pneumonia, tetanus, anthrax, and plague) are all infectious diseases caused by bacteria.

One example of a bacterium which can cause infectious disease is *Staphylococcus aureus*. *S. aureus* is part of the normal flora of bacteria present on the human body. In fact, the bacterium dwells in mucous membranes of most individuals' nostrils at some point during their lives. This bacterium can get transferred to the skin (especially moist skin) of individuals via their hands. Unfortunately, this bacterium is pathogenic and can cause many different types of infections (primarily skin infections). Some of the infections that *S. aureus* causes include folliculitis (infection of a hair follicle), food poisoning, impetigo (skin infection), toxic shock syndrome, wound infections and several other infectious diseases (Nester et al., 2007). Although usually not the cause of fatal infectious disease, *S. aureus* can cause death especially in cases of toxic shock syndrome which is caused by the release of toxins from the bacteria into the host. Toxic shock syndrome usually involves hypotension, fever and the formation of a rash; these symptoms can ultimately cause organ failure and/or lethal shock (McCormick, Yarwood, & Schlievert, 2001). Thus, from this example of *S. aureus*, one can see how even normal flora bacteria can cause severe infectious diseases.

Bacterial diseases have plagued the world for centuries. In Europe in the 14th century, the bubonic plague killed an estimated twenty-five million people— approximately one third to one half of the European population at the time (Thompson and Combee, 1997). In many hospitals in Great Britain in the 19th century, patients would

survive amputations of arms and limbs only to die shortly thereafter due to a disease referred to as "hospitalism" (Bankston, 2005, p. 7-8), diseases apparently caused by bacteria. Primarily due to the plague and "hospitalism," millions have died from bacterial infections over the past several centuries.

*Antibiotics and Antiseptics*

These bacterial diseases would no doubt have continued to plague modern-day societies if it had not been for the discovery and development of antiseptics and antibiotics. Antiseptics are chemicals—mild enough to be used on human skin—that are used to kill or inhibit bacteria, fungi, or viruses (Nester et al., 2007). Joseph Lister is the man credited with the discovery and promotion of the use of antiseptics and antiseptic techniques. After studying the work of Louis Pasteur, Joseph Lister decided to treat a young patient's severe wound with linseed oil and carbolic acid in order to kill the germs that could cause gangrene. The treatment worked. A cure had now been discovered for hospitalism using carbolic acid and linseed oil as an antiseptic to prevent bacterial infections! As a result of his use of this antiseptic, the death rate from major operations plummeted to only 7.1 percent (Bankston, 2005). In addition, Lister helped to establish several important antiseptic techniques involving increased sanitation and cleanliness in hospitals to decrease the chance of bacterial infections. Surgeons operating today wear masks and gloves largely as a result of Lister's establishment of antiseptic techniques (Bankston, 2005).

The discovery of antiseptics did much to prevent bacterial infections. The discovery of antibiotics, however, did much not only to prevent bacterial infections but also to treat and cure them. According to Nester et al. (2007), an antibiotic is "A

compound naturally produced by mold or bacteria that inhibits the growth of or kills

other microorganisms" (p. 496). In 1928, Dr. Alexander Fleming discovered that a certain

mold growing on a petri dish seemed to have certain antibacterial effects. This discovery

soon led to the breakthrough of the antibiotic he named penicillin (Bankston, 2005). After

much experimentation, Penicillin G (a specific version of penicillin) began to be used to

treat many bacterial infections. Penicillin G was the first widely used antibiotic.

Following the discovery of penicillin, Selman Waksman discovered that a certain bacteria

produced an antibiotic called streptomycin. Thus a rapid search for more microorganisms

that produced antibiotics began. In the 1960s, researchers began to change certain

structural components of these early antibiotics to yield even more effective antibiotics—

e.g. ampicillin and methicillin (Nester et al.). Due to these new antibiotic discoveries, the

1960s were an exciting time period for microbiologists. These new antibiotics were truly

wonder drugs in that they could cure most bacterial infections. In fact, it was predicted

that with the discovery of these new antibiotics that one day bacterial infections would be

completely eliminated (Larson, 2007).

Antibiotics and antiseptics together form a class of drugs known as antimicrobials

which inhibit the growth of bacteria via several different mechanisms including

disrupting or interfering with cell wall synthesis, protein synthesis, nucleic acid synthesis,

metabolic pathways, and cell membrane integrity (Nester et al., 2007). For example,

triclosan, the antiseptic on which this study is focusing, is a phenolic compound (a

compound structurally similar to carbolic acid—also known as phenol) used in many

everyday products including toothpaste, soaps, and lotions (Nester et al.); triclosan

inhibits bacterial fatty acid synthesis and thus inhibits bacteria growth by disrupting bacterial cell membrane integrity (Ellison & Champlin, 2007).

*Resistance to Antibacterial Compounds*

It has been said: "Resistance to each new antibiotic…. is inevitable" (Larson, 2007, p. 436). Some bacteria have a natural resistance to certain antibiotics; this type of resistance is called intrinsic or innate resistance. Other bacteria become resistant over time due to mutations or due to obtaining new genetic material (e.g. the R plasmid); this type of resistance is referred to as acquired resistance. As previously mentioned, the discoveries of novel antibiotics led to the hope that bacterial infections would eventually be eliminated. In fact, with the use and overuse of certain antibiotics, the non-resistant strains of certain bacteria have been almost completely eradicated. Unfortunately, what remains are resistant versions of bacteria. For example, at the first use of Penicillin G, only 3% of *S. aureus* were resistant to the drug. Over time, however, the extreme use of Penicillin G has all but eliminated non-resistant strains of *S. aureus* to the point that now 90% of *S. aureus* are resistant to this drug (Nester et al., 2007).

Antimicrobial resistance comes in many shapes and forms. Sometimes bacteria produce enzymes which modify the drug in such a way as to inactivate it and thus inhibit its toxic effects. Another method of resistance occurs when the antimicrobial's target molecule (the molecule to which the antimicrobial binds and inactivates) changes structurally in such a way that the antimicrobial no longer binds to it. Bacteria can also lower the amount of drug uptake by changing the structure of their outer membrane porin proteins (proteins which determine the permeability of the bacteria's membranes to

certain molecules) or by amplifying the expression of their efflux pumps, which in turn pump more of the drug back out of the cell (Nester et al., 2007).

Fortunately, there are quite often multiple antibiotics which can be used to treat resistant forms of bacteria. For example, methicillin is another antibiotic (besides Penicillin G) which can be used to treat *S. aureus* infections, but *S. aureus* is becoming increasingly resistant to methicillin—MRSA (Larson, 2007). Vancomycin is an important antibiotic often used to treat MRSA. Unfortunately, in 1997, a case of *S. aureus* resistant to vancomycin was reported in Japan. Since then, there have been multiple reports of vancomycin-resistant *S. aureus* (Walsh & Howe, 2002). This new resistance to vancomycin is of special concern because vancomycin is often considered "the antibiotic of last resort" (Nester et al., 2007, p. 191). This growing resistance in many different types of bacteria (not just *S. aureus)* threatens to bring modern-day society back to the "preantibiotic era" in regards to treating certain bacterial diseases (Larson, 2007, p. 435).

In addition to being resistant to some antibiotics, some bacteria are no longer inhibited by certain antiseptics. For example, certain streptococci (group of bacteria species) have cellular components innately resistant to triclosan. With this innate resistance and the extreme usage of triclosan in many products, it is speculated that soon there will be multiple strains of bacteria no longer inhibited by triclosan (Campbell & Cronan, 2001). Therefore, it is imperative that new antiseptics are developed to help prevent the growth of bacteria—especially bacteria resistant to certain antiseptics (e.g. triclosan).

With this new threat of an antimicrobial-resistant world, what then can be done to decrease the progression of antimicrobial-resistance in bacteria? Increasing personal

hygiene practices, educating the public about proper use of antimicrobials, vaccinating against certain diseases, limiting the use of antimicrobials in agriculture, lowering the overuse and misuse of antimicrobials and increasing incentives to develop new antimicrobials would all help slow the increase of antimicrobial resistance in bacterial infections. Unfortunately, resistance cannot be totally eliminated. Therefore, it is essential that new antimicrobials be continuously developed for prevention and treatment of antimicrobial-resistant bacterial infections (Larson, 2007). The purpose of this study is to evaluate four new antiseptics derived from triclosan.

<div align="center">Introduction</div>

*Staphylococcus aureus* is a Gram-positive pathogenic bacterium known to cause skin infections (Nester et al., 2007). Experiments have shown, however, that triclosan (IUPAC name: 5-chloro-2-(2,4-dichlorophenoxy)phenol—Menoutis & Parisi, 2006, Triclosan, para. 1)., an antibacterial compound used in many antiseptics and consumer items (Campbell & Cronan, 2001), inhibits the growth of *S. aureus* (see Fig. 1).
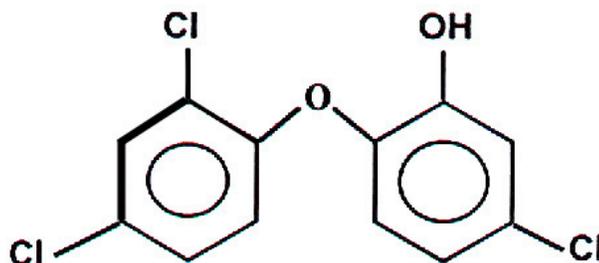


*Figure 1.* Organic Structure of Triclosan (Menoutis & Parisi, 2006, Triclosan, para. 2).

The bacterium *Staphylococcus aureus* was originally chosen for this experiment for two important reasons: *S. aureus* is a pathogenic bacterium; and secondly, it grows rapidly (and thus is easy to culture and use in experiments). During the course of this experiment, however, the value of using *S. aureus* in this experiment increased due to frequently reported MRSA (Methicillin-Resistant *Staphylococcus aureus*) outbreaks in the local and national news. If some of these derivatives could inhibit *S. aureus* growth, it is probable that they could also inhibit MRSA as well.

Triclosan inhibits the growth of *S. aureus* and other bacteria by inhibiting certain enoyl ACP-reductases—essential enzymes involved in bacterial fatty acid synthesis (Ellison & Champlin, 2007). Triclosan forms a tightly-bound, non-covalent ternary complex with co-factor NAD(P)+ and an enoyl-ACP reductase encoded by *fabI* (fatty acid biosynthesis gene I); this tight binding prevents the reductase from participating in fatty acid synthesis—a form of competitive inhibition. The tight ternary complex is achieved due to triclosan's  binding to the reductase's active site—the site where the fatty acid substrate normally binds (see Fig. 2). Triclosan's binding site is located next to NAD(P)+'s nicotinamide ring; thus, triclosan's phenol ring interacts significantly with NAD(P)+'s nicotinamide ring via hydrogen bonding leading to the formation of the complete ternary complex (Escalada, Harwood, Maillard, & Ochs, 2005; Heath, Rubin, Holland, Zhang, Snow, & Rock, 1999). Enonyl ACP-reductase is crucial for acyl chain elongation in fatty acid synthesis in bacteria (Heath et al., 1999). Therefore, by inhibiting this enzyme, triclosan inhibits the production of phospholipids which disturbs the assembly of bacterial membranes which ultimately leads to the inhibition of bacterial metabolism and growth (Ellison & Champlin, 2007; Heath et al., 1999).
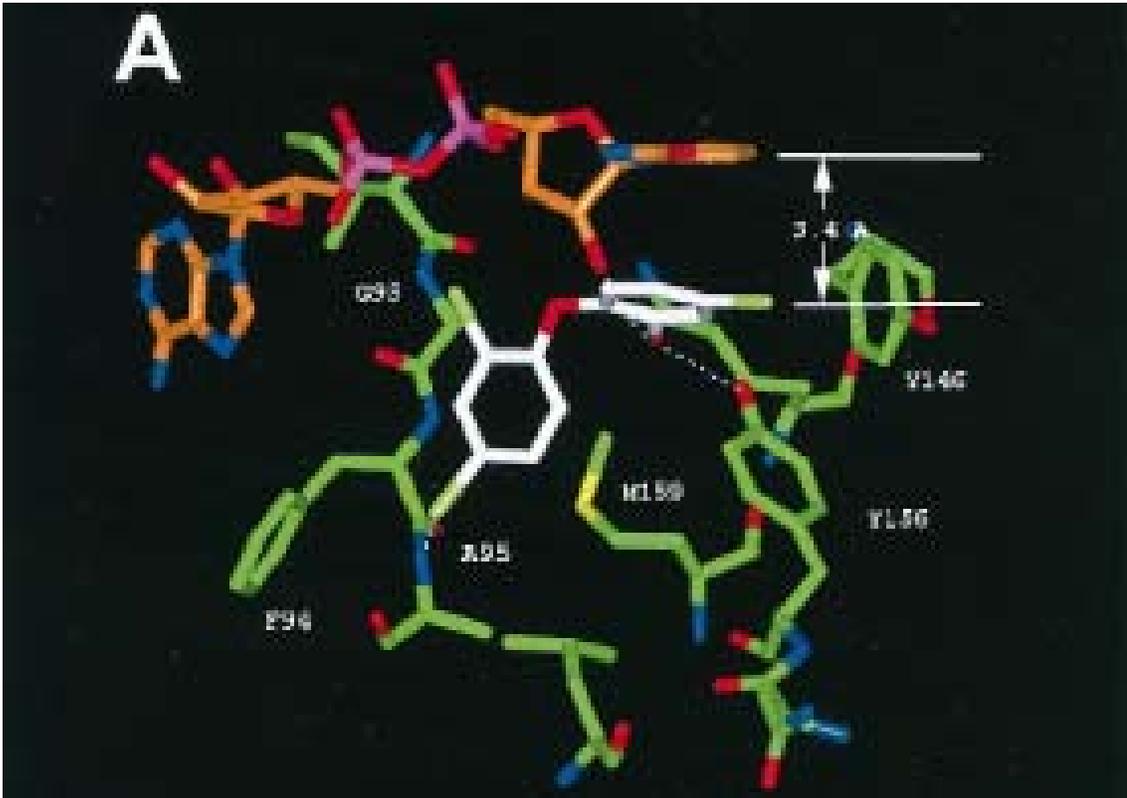
*Figure 2.* "The active site region of the FabI-NAD+-triclosan ternary complex. The hydroxychlorophenyl ring stacks with the nicotinamide ring of the NAD+ with an interplanar distance of 3.4 Å and contacts Tyr-146 and Tyr-156 on the protein. The hydroxyl group of the ligand forms hydrogen bonds with phenol of Tyr-156 and with the 2'-hydroxyl of the NAD+ ribose. The 2,4-dichlorophenyl ring of triclosan sits in a hydrophobic pocket in contact with Met-159. The 4-chloro substituent accepts a hydrogen bond from the amide backbone amide nitgrogen of Ala-95" (Heath et al., 1999, p. 11114, fig. 6).

Unfortunately, recent studies have shown that certain mutations in *fabI* as well as overexpression of *soxS*, *marA*, and an efflux pump encoded by *acrAB* can all contribute to triclosan resistance in bacteria. In fact, due to innate resistance to triclosan in some bacteria and due to the heavy use and overuse of triclosan in everyday products, many bacteria may be resistant to triclosan in the near future (Campbell & Cronan, 2001). Therefore, it is essential that new antimicrobials be developed in order to destroy bacteria that acquire resistance to triclosan.

One possibility in the development of these new antimicrobials would be to synthesize derivatives structurally similar to triclosan. Over the course of the past several years, Professor McGibbon (professor of organic chemistry at Liberty University, Lynchburg, VA) has synthesized several derivatives of triclosan. He added a benzene ring via a dinitrogen double bond para to the alcohol on triclosan. The added benzene ring in turn has anywhere from zero to four chlorines attached to it. Derivative 1 (burnt orange) has chlorines attached to the added benzene ring at the 2,3,5,6 positions going clockwise around the ring (the carbon attached to the nitrogen is in position 1—IUPAC name: 5-chloro-2-(2,4-dichlorophenoxy)-4-(2,3,5,6-tetrachlorophenylazo)phenol —see Table 1 and Fig. 3). Derivative 2 (rusty yellow and clumpy in appearance) has a chlorine at the meta position (position 3) on the added ring (IUPAC name: 5-chloro-2-(2,4-dichlorophenoxy)-4-(3-chlorophenylazo)phenol). Derivative 3 (small brown particles) has just the extra benzene ring attached (no added chlorines—IUPAC name: 5-chloro-2-(2,4-dichlorophenoxy)-4-(phenylazo)phenol). Derivative 4 (clumpy, claylike, dull, yellow particles) has two chlorines attached to the added ring at the meta and para positions (positions 3 and 4—IUPAC name: 5-chloro-2-(2,4-dichlorophenoxy)-4-(3,4-dichlorophenylazo)phenol).

Table 1. *Derivative Numbers Assigned to Various Triclosan Derivatives.*

| Derivative number | Description of actual Triclosan Derivative |
|---|---|
| Derivative 1 | T with benzene ring attached with chlorines at positions 2,3,5,6 on the ring (position 1 is the location of the attachment bond to Triclosan) |
| Derivative 2 | T with benzene ring attached with chlorine meta to attachment bond |
| Derivative 3 | T with benzene ring attached |
| Derivative 4 | T with benzene ring attached with 2 chlorines meta and para to attachment bond |
| Compound 5 | Triclosan (standard of comparison) |
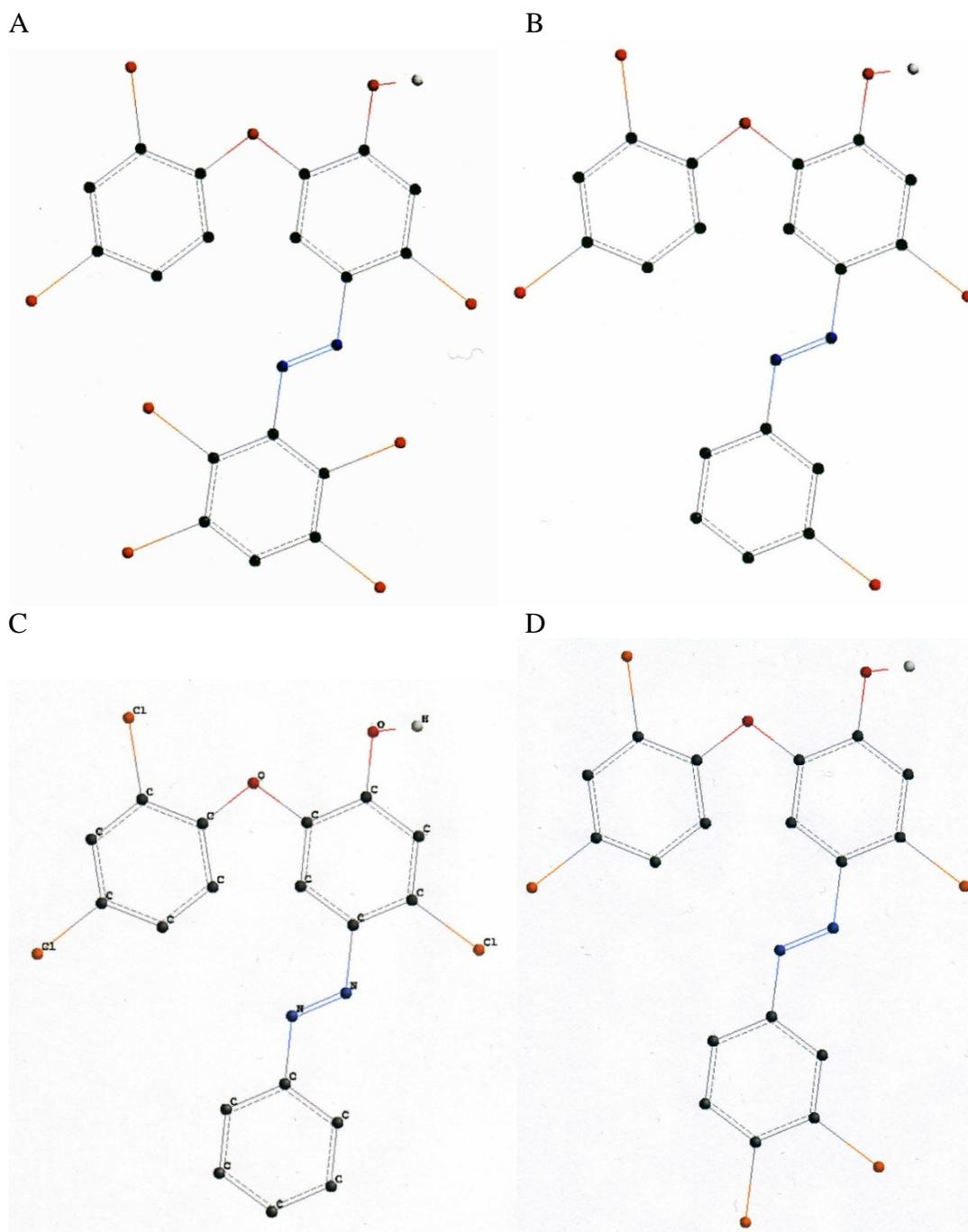
*Figure 3.* Organic Structures of the Four Triclosan Derivatives. In all of the derivatives, the added benzene ring is added via a dinitrogen double bond (-N=N-) to the 2nd ring of triclosan para to the alcohol group. A) Derivative 1: The added ring has four chlorines attached in positions 2,3,5,6 (ortho and meta positions). B) Derivative 2: The added ring has one chlorine attached in the meta (3rd) position. C) Derivative 3: The added ring has no chlorines attached. (Since this is the base structure of all the derivatives, the various atoms are labeled in this figure.) D) Derivative 4: The added ring has two chlorines attached in the meta and para (3rd and 4th) positions.

A search of the relevant literature has revealed no tests on these triclosan derivatives to date. Thus, one can easily see the potential importance of testing these derivatives in this experiment especially considering the threat of growing bacterial resistance against triclosan. If derivatives more inhibitive against bacteria than triclosan could be synthesized, they could possibly be used against triclosan-resistant bacteria. Therefore, four of Professor McGibbon's derivatives (designated Derivatives 1-4) were tested under the supervision of Dr. Hubbard (professor of microbiology at Liberty University) for inhibitive capabilities against *S. aureus*.

## Materials and Methods

Before any experiments were performed on triclosan and its derivatives, a sample of *Staphylococcus aureus* was obtained from Dr. Hubbard. The *S. aureus* was streaked for isolation on a Trypticase Soy Agar (TSA; Becton-Dickinson & Co., Sparks, MD) plate (incubated at 37°C for approximately 1 day). A colony on the plate was also gram-stained to ensure that the bacteria were indeed the gram-positive *S. aureus*. Two colonies were then transferred into two test tubes of Trypticase Soy Broth (TSB—approximately 5mL each; Becton-Dickinson & Co.) to provide stock solutions for the duration of the experiment. These colonies were incubated at 37°C for 1 day before being stored at 4°C for the remainder of the experiment. Approximately halfway through the experiment, the bacteria were re-isolated and two new broths of the bacteria were prepared and stored in a similar fashion to be used for the remainder of the experiment.

Throughout this experiment, a modification of the Kirby-Bauer disc diffusion test (Nester et al., 2007) was performed multiple times. In these tests, 0.1mL of *S. aureus* was taken from the stock broth and spread onto a TSA plate. The 7mm paper discs were then

soaked in their relative solutions for 5 sec. before being placed onto the TSA plate. The plate was then incubated at 37°C for approximately 24 hours, and the zones of inhibition around each disc were then measured.

A stock supply of triclosan and each derivative were obtained from Professor McGibbon. He had synthesized each of the derivatives from triclosan in an organic chemistry laboratory at Liberty University. The derivatives were designated Derivative 1, 2, 3, and 4 (see Table 1, p. 13).

Before triclosan or its derivatives' solutions were prepared, 0%, 5%, 8%, 10%, 10.75%, 13%, 20%, and 25% acetone dilutions were all prepared; using a modification of the Kirby-Bauer disc diffusion test, each of these acetone dilutions were tested against *S. aureus* growth. Next, 20ug/mL, 10ug/mL, 5ug/mL, 4ug/mL, 2ug/mL, and 1ug/mL triclosan solutions were all prepared by dissolving various amounts of triclosan in 20% acetone solutions. These various concentrations of triclosan were then tested against the growth of *S. aureus* once again using a modification of the Kirby-Bauer disc diffusion test.

Next, 4ug/mL solutions of triclosan and the various triclosan derivatives were prepared. In preparing the triclosan solution, 1.0mg of triclosan was dissolved in a 100mL 50% acetone/50% deionized water solution. The solution was lightly heated and intensely stirred for 15 minutes. Another 150mL of deionized water was added approximately 10 minutes into the stirring to yield a 20% acetone solution. Each of the derivatives was prepared in a similar fashion to yield a 4ug/mL 20% acetone solution of each compound. These solutions were sealed and stored at room temperature for the duration of the experiment.

Finally, several Kirby-Bauer disc diffusion tests were performed testing triclosan and its derivatives against *S. aureus* growth. In the first of these tests, triclosan and Derivatives 1-2 were tested against the bacteria growth. In the second experiment, two Kirby-Bauer disc diffusion tests were performed testing triclosan and all four derivatives against *S. aureus* growth. In the final experiment, five Kirby-Bauer disc diffusion tests were performed testing triclosan and all four derivatives against the bacteria growth.

## Results

In the experiment with the various acetone/water solutions, the 0%, 5%, 8%, 10%, 10.75%, 13%, and 20% acetone solutions did not exhibit resistance/inhibition to *S. aureus*. The 25% acetone solution did inhibit the growth of *S. aureus*; it produced a 9mm (diameter) zone of inhibition.

The results of the experiment with the various concentrations of triclosan on the two TSA plates are included in the table and graph below. Table 2 lists the zones of inhibition of each triclosan concentration on each TSA plate. Figure 4 plots the average zones of inhibition of the various concentrations of triclosan from both plates (in 20% acetone solution).

Table 2. *Inhibition Zones of Various Concentrations of Triclosan.*

| Inhibitor (compound and concentration) | Plate 1: Inhibition zone (mm) | Plate 2: Inhibition zone (mm) |
|---|---|---|
| Water | 0 | 0 |
| 20% acetone | 0 | 0 |
| Triclosan (1ug/mL) | 0 | 1 |
| Triclosan (2ug/mL) | 0 | 7 |
| Triclosan (4ug/mL) | 7 | 14 |
| Triclosan (5ug/mL) | 12 | 14 |
| Triclosan (10ug/mL) | 13 | 12 |
| Triclosan (20ug/mL) | 13 | 18 |
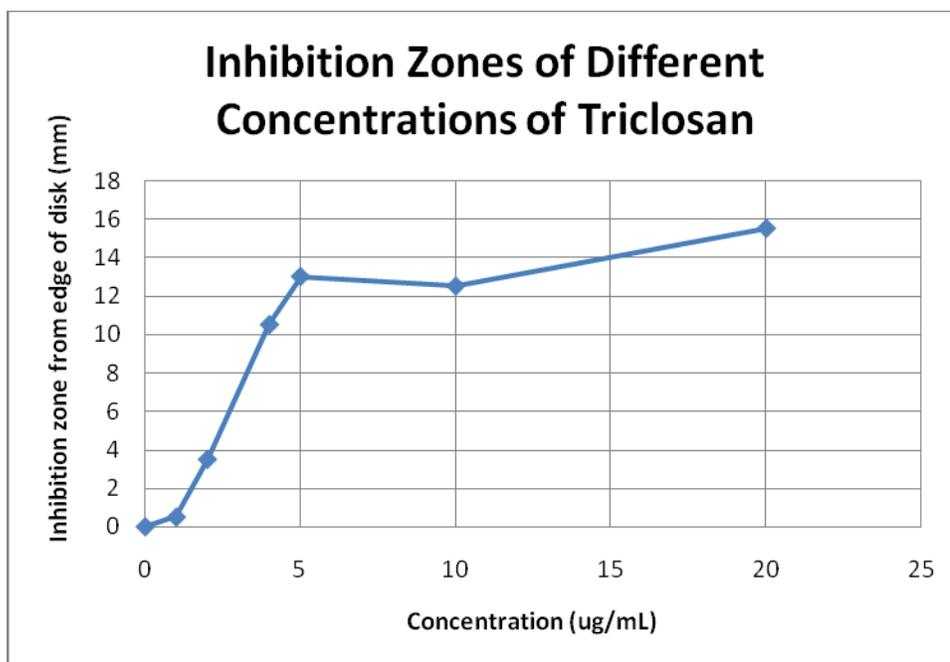


*Figure 4.* Inhibition Zones of the Different Concentrations of Triclosan in 20% Acetone Solutions. Generally, the zones of inhibition increased in diameter as the concentration of the triclosan solution increased; at a concentration of 5ug/mL, the increase in the zones of inhibition began to level off.

The results of the early experiment with Derivatives 1-2 and triclosan are seen in

Table 3. Triclosan produced a zone of inhibition with a 20mm diameter. Derivatives 1

and 2 produced 8mm zones of inhibition (measured at approximately 22 hours). At 43

hours, Derivative 2's zone of inhibition had shrunk to 7mm (see Table 3) while

triclosan's and Derivative 1's zones of inhibition had remained the same.

Table 3. *Inhibition Zones of Derivative 1, Derivative 2, and Triclosan. D1 & D2 did not display nearly as much inhibition as triclosan did, and D2's zone of inhibition actually shrunk over time.*

| Derivative | Inhibition Zones at 22 hours (mm) | Inhibition Zones at 43 hours (mm) |
|---|---|---|
| Derivative 1 | 8 | 8 |
| Derivative 2 | 8 | 7 |
| Triclosan | 20 | 20 |

In the first experiment with all four derivatives and triclosan, Derivative 1

produced zones of inhibition with 8 mm and 7 mm diameters on Plates 1 and 2

respectively (see Table 4). Derivative 2 produced zones of inhibition with 7 mm

diameters on both plates. Derivative 3 had no zone of inhibition on Plate 1 and a 7mm

zone of inhibition on Plate 2. Derivative 4 produced 8mm and 7mm zones of inhibition

on Plates 1 and 2 respectively. Finally, Derivative 5 (triclosan) produced 26mm and

19mm zones of inhibition on Plates 1 and 2 respectively.

The results of this experiment on Derivatives 1-4 and triclosan (labeled 5) are

summarized by Table 4 and Figures 5-9 below. Figure 9 is a comparison of the average

inhibition zones of Derivatives 1-4 and triclosan (Compound 5). The results clearly show

that none of the derivatives are nearly as inhibitive against the growth of *S. aureus* as

triclosan is.

Table 4. *Inhibition Zones of Various Triclosan Derivatives. None of the derivatives were as inhibitive against* S. aureus *as triclosan. Although most of the derivatives showed some inhibition, on plate 1, D3 showed no inhibition at all against* S. aureus *growth.*

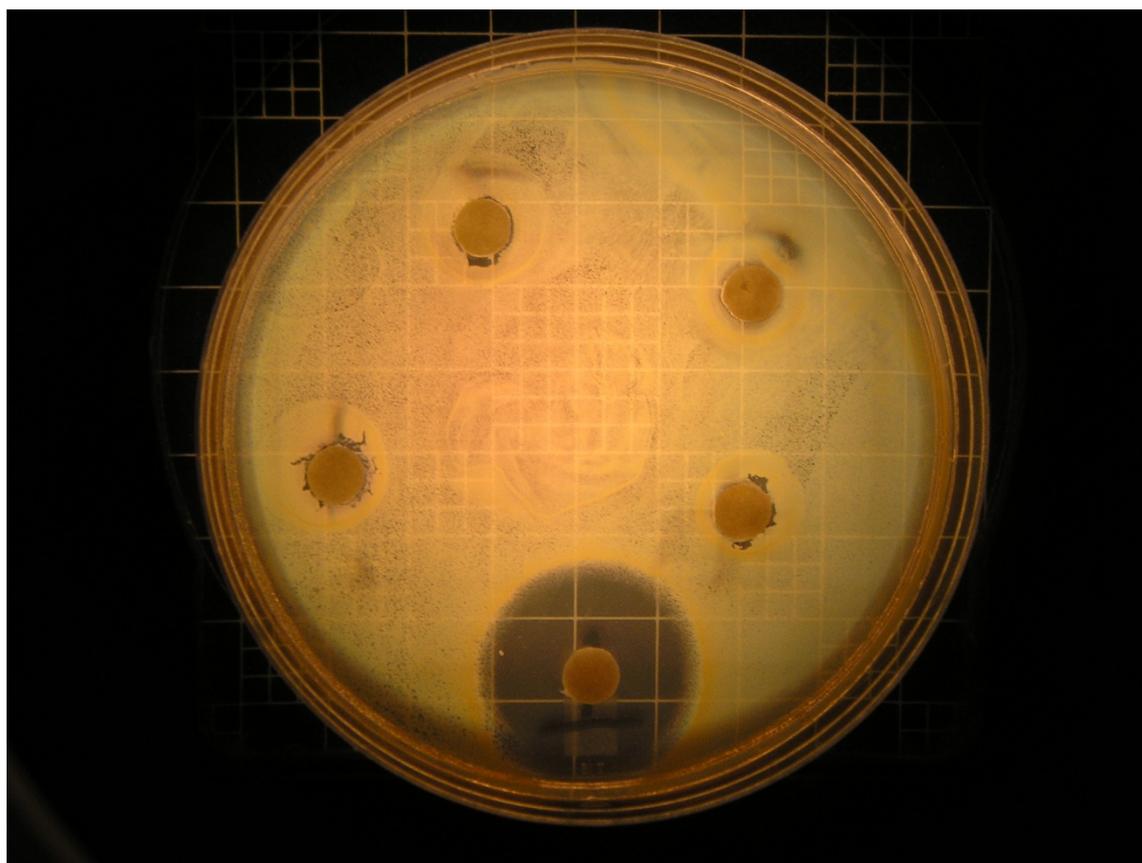| Derivative | Plate 1: Inhibition Zone (mm) | Plate 2: Inhibition Zone (mm) |
|---|---|---|
| Derivative 1 | 8 | 7 |
| Derivative 2 | 7 | 7 |
| Derivative 3 | 0 | 7 |
| Derivative 4 | 8 | 7 |
| Triclosan (5) | 26 | 19 |



*Figure 5.* Inhibition Zones of Various Triclosan Derivatives (Plate 1). Triclosan is pictured at the bottom, and the derivatives are pictured in order (D1-D4) clockwise around the plate starting at triclosan. Notice the lack of inhibition in Derivative 3.
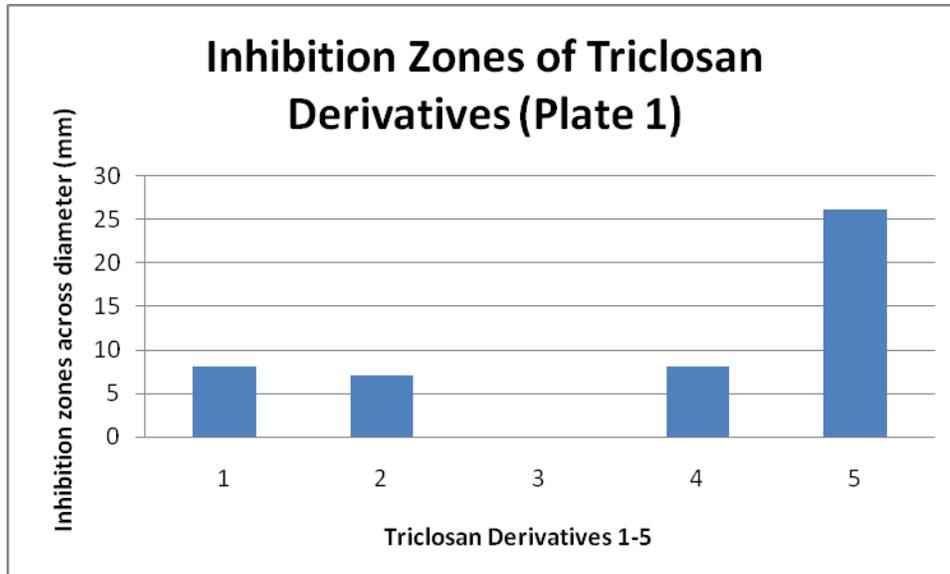
*Figure 6.* Comparison of the Inhibition Zones of Triclosan Derivatives (Plate 1). As noted in Table 3, D3 showed no inhibition against *S. aureus* growth.
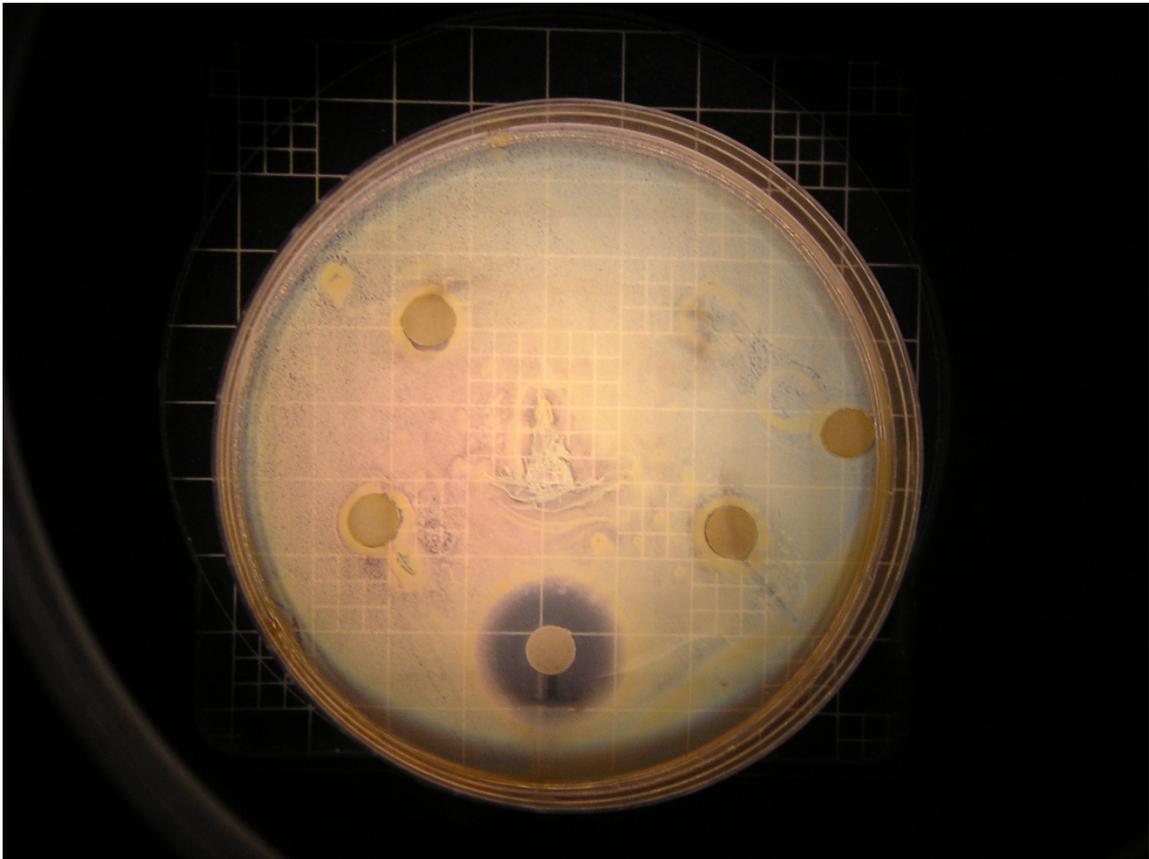


*Figure 7.* Inhibition Zones of Various Triclosan Derivatives (Plate 2). Triclosan is pictured at the bottom; the derivatives are pictured in order (D1-4) clockwise around the plate starting at triclosan.
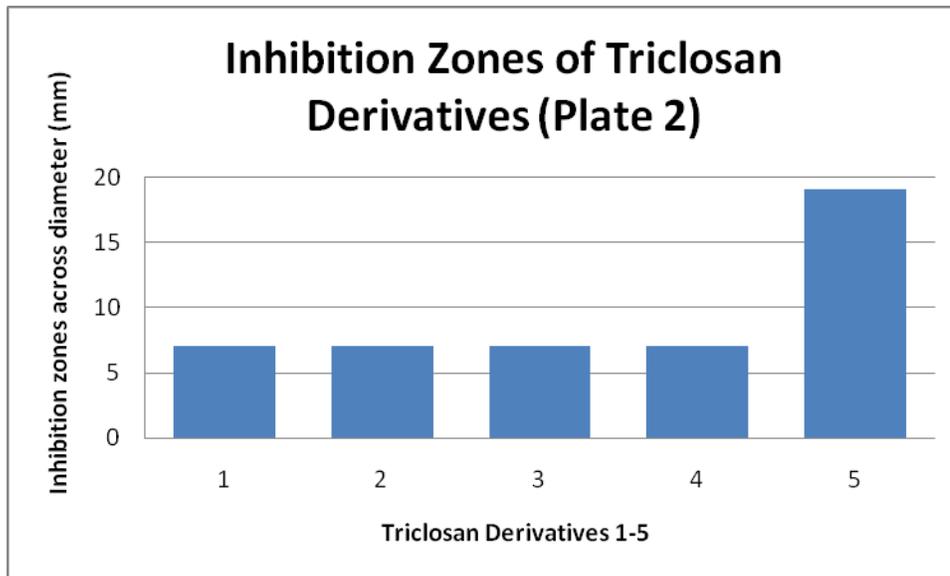
*Figure 8.* Comparison of the Inhibition Zones of Triclosan Derivatives (Plate 2). On this plate, D3 did show some inhibition against *S. aureus*.
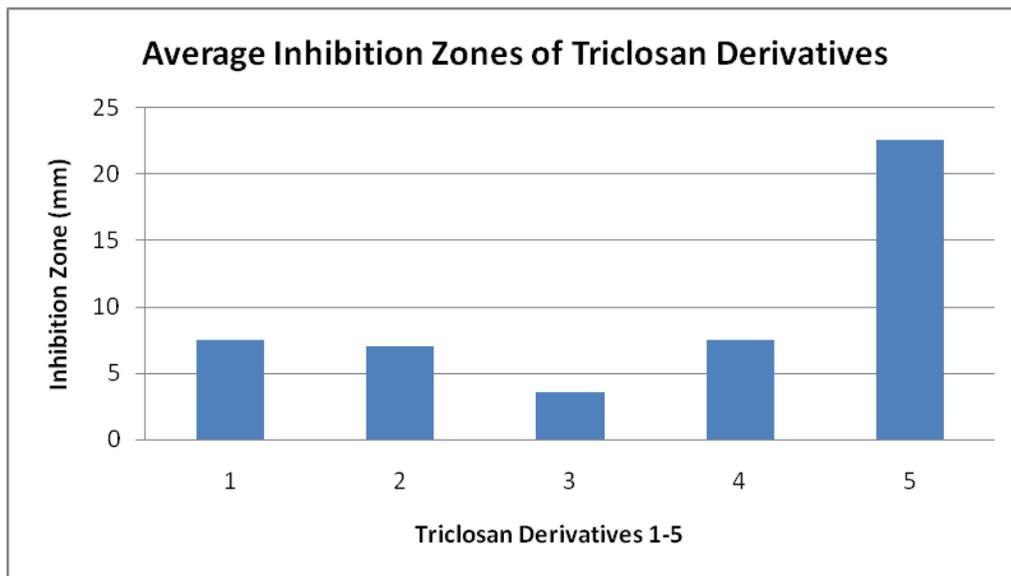


*Figure 9.* Comparison of the Average Inhibition Zones of Triclosan Derivatives. This graph takes the average of the zones of inhibition of triclosan and each of the derivatives from the two TSA plates.

In the final experiment with the 5 TSA plates, the results were observed to confirm

the results of the main experiment (See Fig. 5-9). The results, however, were not physically

measured.

Discussion

*Introductory Summary of Findings*

The results indicate that, as tested in this experiment, none of the derivatives were as inhibitive as triclosan against the growth of *S. aureus* (The reason that no results were physically measured in the final experiment was that the results appeared to be virtually identical to the results of the previous experiments and clearly indicated that the derivatives were not as inhibitive as triclosan). Fortunately, several observations and laboratory techniques were discovered and developed during the experimental procedures. It is hoped that these observations and techniques will aid in future research on triclosan derivatives.

*Inhibition by Triclosan Is Greater than All Derivatives*

As tested in this experiment, none of the derivatives were as inhibitive as triclosan. In fact, none of the derivatives produced zones of inhibition even half the average diameter of triclosan's zone of inhibition, and in one experiment, Derivative 3 showed no inhibition at all against the bacteria growth (see Fig. 6, p. 21).

One possible explanation for the derivatives' small zones of inhibition would be that the derivatives may not dissolve (nor stay dissolved) as well as triclosan. The derivatives, structurally, are less polar than triclosan due to the addition of the extra benzene ring. Therefore, it would be more difficult to dissolve them in the primarily polar 20% acetone solution (80% deionized water). It is possible that when the discs soaked in the derivative solutions were placed onto the TSA plate that the derivatives did not stay completely dissolved; therefore, as the acetone/water solution diffused outward onto the agar, the derivatives could have crystallized onto the disc and not diffused out with the

solution, therefore producing only small zones of inhibition against *S. aureus*. Although this proposition is only a hypothesis, certain observations during the experiment seem to support it. For example, the derivatives proved more difficult to dissolve than triclosan, a fact which indicates that the derivatives would most likely come out of solution more easily than triclosan. Two simple ways to test this hypothesis would be to perform a minimum inhibitory concentration (MIC) test (Nester et al., 2007) or to test for a bacteria that can tolerate higher levels of acetone (30-50% solutions) and to repeat this Kirby-Bauer disc diffusion test using compounds dissolved in these more concentrated acetone solutions—the higher acetone concentration should ensure the derivatives stay dissolved. Another way to test this hypothesis would be to dissolve the derivatives in the 20% acetone solution, then to make an agar directly from this solution, and finally to test if *S. aureus* is capable of growing on this agar containing the derivative.

Assuming that it was not the derivatives' insolubility that caused their small zones of inhibition, there was another observation that may explain the derivatives' apparent lack of significant inhibition against *S. aureus*. Figures 6 and 9 show that the benzene ring added to triclosan by itself (no chlorines attached to the ring—Derivative 3) seems to inactivate triclosan's inhibitive capabilities toward *S. aureus*. But as more chlorines are attached to the added ring, the inhibition seems to be slightly increased as compared to just the added ring by itself (see Fig. 5-9, p. 20-22). Therefore, a possible future experiment would be to synthesize triclosan derivatives that consist of chlorines added to one (or both) of the two benzene rings of triclosan itself instead of adding another benzene ring (with attached chlorines) to triclosan. These compounds may prove to be more inhibitive than the derivatives tested in this experiment.

Based on these observations and hypotheses, recommendations for future research would therefore include more tests performed with Derivatives 1-4 not only against *S. aureus* (using 4ug/mL and higher concentrations as well) but also against other pathogenic bacteria. In addition, compounds should be synthesized based on the description above (with chlorines added to the triclosan benzene rings) and tested for antibacterial capabilities. Finally, to determine the inhibitory capabilities of all of these derivatives, other antimicrobial tests (see above paragraphs), including a minimum inhibitory concentration (MIC) test, should be performed.

*Methodological Benefits of Study*

Despite the negative results of this experiment, several beneficial observations and techniques developed during the course of the experiment could aid in future efforts to develop/discover more effective antiseptic compounds. Well over half the time devoted to experimental methods was spent primarily on developing effective procedures. These procedures were used to provide stock solutions and to determine ratios and concentrations to use during the main experiment. The observations and discovered techniques which have been established from this study will prove extremely helpful in future research on triclosan.

Because triclosan is insoluble in a purely aqueous solution, it was determined that a partial acetone solution would be used to dissolve triclosan and its derivatives. Therefore, the original experiment testing various concentrations of acetone against *S. aureus* growth was performed to determine the maximum concentration of acetone that could be used to dissolve the compounds. Since 25% acetone concentration showed a complete inhibition against bacteria growth and the 20% acetone concentration showed

no inhibition against bacteria growth, it was determined to use a 20% acetone solution for dissolving triclosan and its derivatives.

To determine the concentration of triclosan and its derivatives to use for the comparison experiment, the experiment testing the various concentrations of triclosan (1ug/mL-20ug/mL) was performed. Figure 4 clearly indicates that increasing the concentration of triclosan increases the zones of inhibition significantly up until a concentration of 5ug/mL is reached; at this point, the zones of inhibition begin to level off even if the triclosan concentration is doubled or quadrupled. Therefore, it was determined that 4ug/mL would be the best concentration to use for a comparison study between triclosan and its derivatives.

Despite using a low concentration for the experiment, dissolving the derivatives still proved extremely difficult. Therefore, at the recommendation of Dr. Hubbard, the compounds were originally dissolved in a 50% acetone solution by stirring on low heat, and more deionized water was added approximately 10 minutes into stirring to bring the acetone concentration down to 20% and the compound concentration down to 4ug/mL. This method proved efficient in dissolving the compounds and should be used in future efforts to dissolve triclosan and/or its derivatives.

## Conclusion

In conclusion, although the tests performed in this study indicate that none of the derivatives were nearly as inhibitive as triclosan itself, several valuable insights were gained. First of all, it was discovered that a 4ug/mL 20% acetone solution of each compound was the best concentration for dissolving triclosan and its derivatives and performing comparison experiments. Secondly, dissolving the compounds into a 50%

acetone solution (stirring on low heat) and diluting to a 20% acetone solution (while still stirring on low heat) proved far more effective than dissolving the compounds straight into a 20% acetone solution. Finally, although none of these derivatives seem to hold much promise as components of future antiseptics, there is hope that other derivatives will be synthesized by adding chlorines directly to triclosan (as described in the Discussion section) and that these derivatives will be subsequently tested for antibacterial capabilities. All three of these observations could prove extremely useful in future experiments with triclosan derivatives. With time and effort, antibacterial compounds even more inhibitive than triclosan may be discovered to aid in the battle against pathogenic bacteria.

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