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Synthesis and Antibiotic Evaluation of Simplified Pestalone Analogs

Rhapsody Taylor
Chemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Amanda L. Wolfe

Abstract

The natural product pestalone has been shown to have antibiotic activity against resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). However, pestalone is not readily isolated from its natural source, and its total synthesis has proven to be challenging due to low yielding reactions. Presented is the synthesis of various pestalone analogs. The analogs were synthesized from either substituted benzaldehydes or phthalic anhydride using a Grignard reaction as the key step, with most yields between 65 and 85% for both reactions. Currently, the analogs are being tested in bacterial assays against Gram-positive *S. aureus* and Gram-negative *E. coli*.

1. Introduction

Antibiotics are important to modern medicine because bacteria can cause illnesses which can be harmful or potentially fatal to humans. However, some bacteria are becoming resistant to antibiotics and causing problems for the medical community. Novel antibiotics are therefore necessary to continue to suppress antibiotic-resistant bacterial infections that can quickly become life-threatening to patients.

A bacterium is a single celled microorganism, usually with a simple cell structure and living in colonies with bacteria of the same species. There are many different types of bacteria, and they are broadly classified into two groups based on their cellular structures. Gram-positive bacteria contain only a thick peptidoglycan layer as their cell wall, whereas Gram-negative have an outer membrane in addition to a thinner peptidoglycan layer. This is important because any antibiotic effective against both types of bacteria must either act upon or penetrate the cell wall of the bacteria.[1]

Antibiotics are compounds that can either inhibit the growth of bacteria or cause bacterial cell death. They also have a variety of classes in order to target many strains of bacteria, both Gram-positive and Gram-negative. These classes are determined based on the mechanism of how the drug inhibits the bacteria, and some examples include β-lactams, quinolones, tetracyclines, and gylcopeptides.[2] Most antibiotics act on the peptidoglycan wall of Gram-positive bacteria. However, due to overuse of similar antibiotics within the same class, some bacteria are becoming resistant. Bacteria can become resistant to antibiotics when a genetic mutation occurs within the single bacterium. This change in the genetic sequence can render the medicine ineffective in a number of ways. For example, the mutated bacterium might not let the antibiotic enter the cell, or it removes the antibiotic from the cell before it can reach is biological target. The bacterium could also alter the target site of the antibiotic, or even change the structure of the antibiotic itself. The resistant bacteria then survive and pass their resistance onto other bacteria, resulting in ineffective antibiotics and the uninhibited growth of harmful bacteria.[3] A common type of antibiotic resistant bacteria is methicillin-resistant *Staphylococcus aureas* (MRSA). MRSA is resistant to any antibiotics within the methicillin family, which is commonly used to treat *S. Aureas* infections. MRSA commonly causes skin infections, but can also cause life-threatening bloodstream and surgical site infections.[4]

One way to combat antibiotic resistant bacteria such as MRSA is to create new antibiotics that work using novel mechanisms of action. These new antibiotic compounds can often be found by studying compounds found in known

medicinal plants or bacteria rich soil and water. These compounds, known as natural products, often have more diverse structures as well as novel modes of action against bacteria.[3]

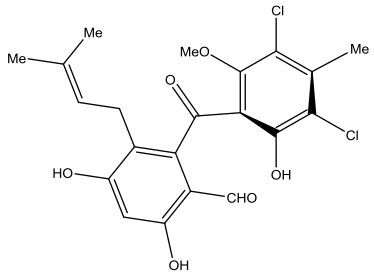


Figure 1. Pestalone, a natural product

In 2001, researchers Cueto et al.[5] discovered that when a brown algae from the Bahamas Islands was co-cultured with a unicellular marine bacterium, a new compound was created. This new compound, known as pestalone (Figure 1), showed significant antibiotic capability against methicillin-resistant *Staphylococcus aureas*, with a Minimum Inhibitory Concentration (MIC) of 37 ng/mL.[5] For comparison, the antibiotic chloramphenicol has been shown to have an MIC against MRSA between 8-16 µg/mL.[6]

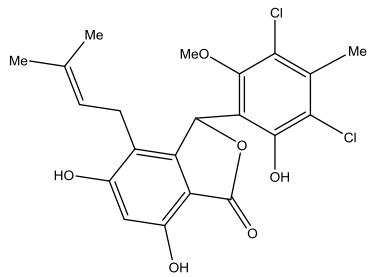


Figure 2. Pestalalactone, an inactive compound

Two years after its discovery, Kaiser et al attempted the synthesis of pestalone analogs. However, the synthesis was much more difficult than anticipated, with multiple synthetic schemes proposed and no completed isolation of pestalone.[2] Later, in 2004, Iijima et al were able to complete the first total synthesis of pestalone.[7] The synthesis of pestalone was again completed in 2010 by researchers Slavov et al.[8] This synthesis was difficult and produced low yields of 16%. Slavov et al. attributed this to the steric hindrance of functional groups as well as torsional strain upon the ring structure. During the project, Slavov et al noted that once synthesized, pestalone easily converted into pestalalactone. To form this compound, the ketone bridge and aldehyde groups of pestalone would cyclize to form a 5 membered ring, creating pestalalactone, shown in Figure 2, which showed no antibiotic properties.[8] In 2013, Augner et al tested the antibiotic and antifungal capabilities of pestalone and pestalachloride A, a derivative of pestalone. Their research showed a MIC of 6.25 µg/mL for pestalone against MRSA, higher than initially reported in 2001.[9]

The objectives of this project were divided into two categories. A simple synthetic scheme was adapted to make similar analogue structures based on pestalone. Then, these analogs were tested individually in bacterial assays. Two simple synthetic schemes were used to create the simplified pestalone derivatives. The first of these schemes is shown in Figure 5, where substituted benzylic ring systems are joined together via a Grignard reaction, then an oxidation reaction creates the ketone bridge. The second of the schemes, shown in Figure 6, also begins with a Grignard reaction, but specifically used phthalic anhydride to create the ketone bridge in a single step along with a carboxylic acid substituent. This carboxylic acid is then methylated in the second step of the scheme. Both the carboxylic acid and the methylated substituents are structurally similar to the aldehyde functional group found on pestalone, but neither will lead to the undesired cyclization reaction. The bacterial assays were ran against *Staphylococcus aureas* and *Bacillus subtilis*, two Gram-positive bacteria, as well as *Escherichia coli* and *Pseudomonas aeruginosa*, two Gram-negative bacteria. This was used to determine if any of the analogues show antibiotic capability similar to that of pestalone.

2. Experimental Methods

2.1 Benzylic Derivative Grignard General Procedure:

Under an inert N_2 atmosphere and in a flame dried round bottom flask at 0 ˚C, the bromobenzene (1 eq), Mg˚ (1 eq), and I_2 (cat) were dissolved in 0.2 M anhydrous tetrahydrofuran (THF). The ice bath was removed and the flask was heated up to 50 °C until the solution exhibited a color change to clear and then black. At this time, the flask was returned to 0 °C (approximately 20 minutes between color change and addition to ensure temperature stability), and the substituted benzaldehyde (0.2 eq) was added. The mixture was then allowed to stir at 23 ˚C for 24 hours. Upon completion, the reaction was quenched with approximately 10 mL of 1N aqueous HCl, and diluted with ethyl acetate. The organic layer was then washed with deionized water and saturated aqueous NaCl. The resultant organic layer was dried over Na_2SO_4, concentrated under reduced pressure, purified via column chromatography (SiO_2, 10 → 30% ethyl acetate/hexane), and characterized.

2.2 Carboxylic Acid Grignard General Procedure:

Under an inert N_2 atmosphere and in a flame dried round bottom flask at 0 ˚C, the bromobenzene (1 eq), Mg˚ (3 eq), and I_2 (cat) were dissolved in 0.9 M anhydrous tetrahydrofuran (THF). The ice bath was removed and the reaction continued at room temperature until the solution exhibited a color change to clear and then black. At this time, the flask was returned to 0 °C. In a separate flask, phthalic anhydride (1 eq) was dissolved in 1.01 M anhydrous tetrahydrofuran (THF). This solution was syringed into the first reaction flask. The mixture was then allowed to stir at 23 ˚C for 24 hours. Upon completion, approximately 10 mL of saturated aqueous $NaHCO_3$ and diluted with ethyl acetate, then washed with approximately 10 mL saturated aqueous NaCl. The aqueous layer was kept and acidified to a pH above 4 using 1N aqueous HCl. This was then reextracted using fresh ethyl acetate, saturated aqueous NaCl, and deionized water. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure, and characterized.

2.3 Carboxylic Acid Methylation General Procedure:

Under an inert N_2 atmosphere and in a flame dried round bottom flask with a reflux condenser, the Carboxyl Acid Grignard Product (1 eq) was added, followed by 0.25 M anhydrous methanol and 5 M concentrated H_2SO_4. This was then heated to reflux using a hot oil bath and allowed to react for 1-2 hours. The solution was neutralized using $NaHCO_3$ and diluted with ethyl acetate. The organic layer was washed with deionized water and saturated aqueous NaCl. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure, purified via column chromatography (SiO_2, 10 → 30% ethyl acetate/hexane), and characterized.

2.4 Pestalone Core Derivative General Procedure:

The Benzylic Derivative Grignard General Procedure was followed, specifically utilizing bromobenzene (1 eq) and 2-bromobenzaldehyde (0.66 eq) in 1.0 M THF.

Under an inert N_2 atmosphere and in a flame dried round bottom flask containing molecular sieves and a stir bar, the synthesized bicyclic alcohol (1 eq) and pyridium chlorochromate (PCC, 2 eq) were dissolved in 0.12 M dichloromethane (DCM). The reaction proceeded at room temperature for 1 hour, then purified via column chromatography (SiO_2 and celite, 20% ethyl acetate/hexane). The resultant ketone was concentrated under reduced pressure and characterized before proceeding.

Under an inert N_2 atmosphere and in a flame dried round bottom flask, the brominated ketone (1 eq) was dissolved in 0.15 M THF. The flask was then submerged in a dry ice/acetone bath at -78˚C. Once the temperature equilibrated, phenyl lithium (1.2 eq) was added via syringe and inert atmosphere. After half an hour, CuCN•2LiBr (0.3 eq), a catalytic copper cyanide complex, as well as allyl bromide (4 eq) were added. The ice bath was removed after roughly 5-15 minutes, and after 15 additional minutes, 5 mL of 1N HCl was added. The organic layer was then washed with deionized water and saturated aqueous NaCl. The resultant organic layer was dried over Na_2SO_4, concentrated under reduced pressure, purified via column chromatography (SiO_2, 5% ethyl acetate/hexane), and characterized.

2.4 Adjusted Pestalone Core Derivative General Procedure:

Under an inert N_2 atmosphere and in a flame dried round bottom flask containing molecular sieves, bromobenzaldehyde (1 eq) and *p*-toluene sulfonic acid (0.3 eq) were added to 0.1 M ethanol. This was allowed to react at 23 ˚C for roughly 12 hours. The organic layer was extracted with ethyl acetate and copious amounts of brine, then dried over Na_2SO_4 and concentrated under reduced pressure.

Under an inert N_2 atmosphere and in a flame dried round bottom flask with a reflux condenser at 0˚C, the protected aldehyde (1 eq), Mg˚ (1.2 eq), and I_2 (cat) were dissolved in 1.6 M THF. The ice bath was removed and, if needed, the flask was heated up to 50 °C until the solution exhibited a color change to clear and then black. At this time, the flask was returned to 0 °C (approximately 20 minutes between color change and addition to ensure temperature stability), and allyl bromide (1.5 eq) was added. The mixture was then allowed to stir at 23 ˚C for 24 hours. Upon completion, the reaction was quenched with approximately 10 mL of 1N aqueous HCl, and diluted with ethyl acetate. The organic layer was then washed with deionized water and saturated aqueous NaCl. The resultant organic layer was dried over Na_2SO_4, concentrated under reduced pressure, purified via column chromatography (SiO_2, 5% ethyl acetate/hexane), and characterized.

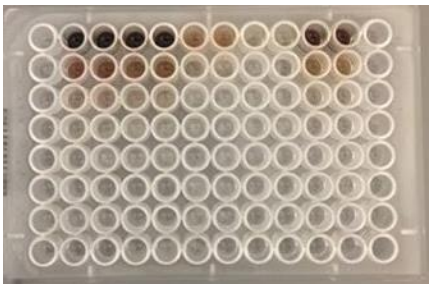


Figure 3. Example 96-well assay master plate

2.5 Bacterial Assay General Procedure:

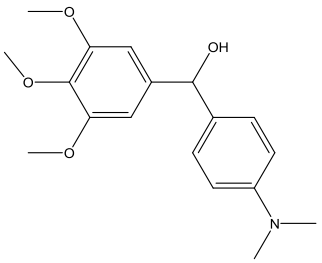
Firstly, a master plate of the tested compounds was created, such as depicted in Figure 3. Using a 96-well assay plate, the first row was charged with a known antibiotic (either penicillin G or chloramphenicol) in column 1, duplicates of 5 synthesized compounds in columns 2-11, and a negative control of pure dimethyl sulfoxide (DMSO) in column 12. Each row was then diluted with DMSO to be tenfold less concentrated than the row above it, creating a dilution range between 0.005 and 50,000 µg/mL (exception of Plate 1, which was twice as concentrated, 0.01 µg/mL at the most dilute).

Overnight bacteria cultures of *S. aureas*, *B. subtilis*, *E. coli*, and *P. aeruginosa* were prepared in 10% tryptic soy broth (TSB) and incubated for 24 hours at 37˚C. In a separate 96-well assay plate, each well was filled with 89 µL of 10% TSB, followed by 10 µL of the overnight bacteria culture (one bacteria per plate). To each corresponding well, 1µL from the master plate was also added. For the negative control, at least two wells were left completely empty, two wells with only broth and DMSO, and two with broth, bacteria, and DMSO. The plate was then incubated 24 hours at 37˚C.

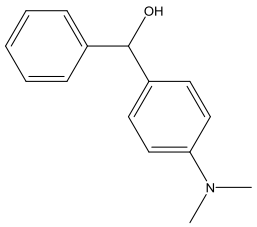
Following incubation, the data from the plate was collected via BioTek plate reader. This scanned each well of the plate using visible light, and measured the absorbance on the other side. This was then plotted onto a chart with higher

absorbance charted in a darker color and with a larger absorbance value. Higher absorbance of the well correlated to more dense bacterial growth, and thus less antibiotic activity of the compound at that concentration.

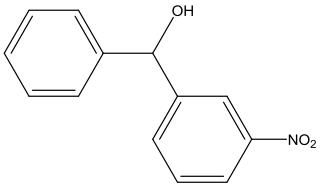
2.6 Synthesized Derivatives:



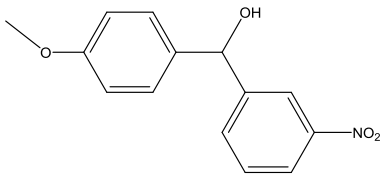
(4-(dimethylamino)phenyl)(3,4,5-trimethoxyphenyl)methanol (7.1). Benzylic Derivative Grignard General Procedure. 3,4,5-trimethoxy-1-bromobenzene (1.0 g), magnesium turnings (0.87 g), THF (30 mL), 4-(dimethylamino) benzaldehyde (0.50 g) was added, stirred at room temperature for 24 hours. Column chromatography (2x, 250 mL, SiO_2, 20% EtOAc/hexanes solvent system). Green crystals. 82% yield.



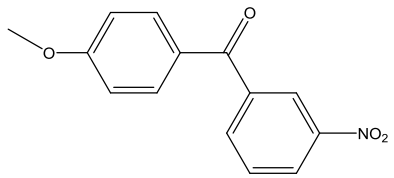
(4-(dimethylamino)phenyl)(phenyl)methanol (7.2). Benzylic Derivative Grignard General Procedure. Bromobenzene (4.0 mL), magnesium turnings (0.8 g), THF (30 mL), 4-(dimethylamino) benzaldehyde (1.01 g) was added, stirred at room temperature for 24 hours. Column chromatography (500 mL, SiO_2, 20% EtOAc/hexanes solvent system). Pale yellow solid. 64% yield.



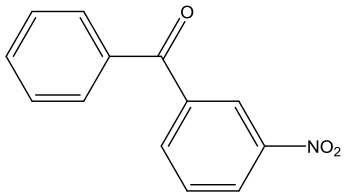
(3-nitrophenyl)(phenyl)methanol (7.3). Benzylic Derivative Grignard General Procedure. Bromobenzene (3.5 mL), magnesium turnings (0.81 g), THF (30 mL), 3-nitrobenzaldehyde (1.00 g). Solution bubbled violently upon addition of aldehyde. Column chromatography (500 mL, SiO_2, 10% EtOAc/hexanes solvent system). 27% yield.



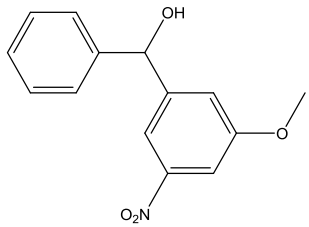
(4-methoxyphenyl)(3-nitrophenyl)methanol (7.4). Benzylic Derivative Grignard General Procedure. Bromoanisole (4 mL), magnesium turnings (0.80 g), THF (30 mL), 3-nitrobenzaldehyde (1.04 g, 6.884 mmol). Solution bubbled violently upon addition of aldehyde. Dark brown with wet clay consistency. This crude compound was carried on to the next reaction.



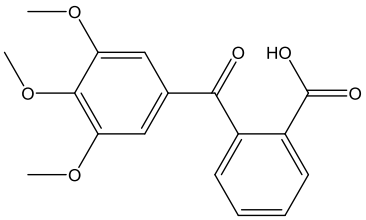
(4-methoxyphenyl)(3-nitrophenyl)methanone (7.5). In an inert flask containing molecular sieves, 2 equivalents of pyridium chlorochromate (PCC, 1.7 g) was partially dissolved in dichloromethane (30 mL). In a separate inert flask, the bicyclic alcohol product, Compound **7.4** (1.0 g, crude) was dissolved in THF (8 mL) and added to the first flask via syringe. The reaction ran at room temperature for 24 hours. The solution was then filtered via fritted funnel and DCM to remove excess PCC. The solution was concentrated under reduced pressure, and characterized. 77% yield.



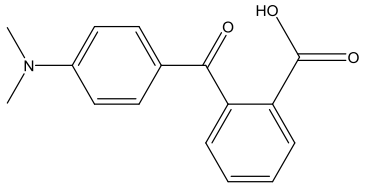
(3-nitrophenyl)(phenyl)methanone (7.6) In an inert flask with a reflux condenser, 1 equivalent of Compound **7.3** (0.2 g) was dissolved in 0.14 M of ethyl acetate (6 mL). Then, 3 equivalents of iodoxybenzoic acid (IBX, 0.74 g) was added. The flask was heated to 80°C for 2.5 hours. Note that the reflux condenser used was slightly cracked, so a small amount of water was added to the reaction. The reaction was removed from heat and filtered via fritted funnel with ethyl acetate. This was then dried over Na_2SO_4, concentrated under reduced pressure, purified via column chromatography (250 mL, SiO_2, 10% EtOAc/hexanes solvent), and characterized. 9.4% yield.



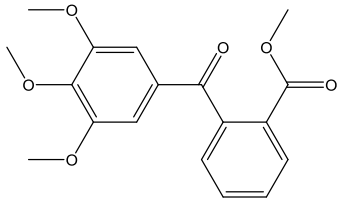
(3-methoxy-5-nitrophenyl)(phenyl)methanol (7.7) Benzylic Derivative Grignard General Procedure. Bromobenzene (3 mL), magnesium turnings (0.68 g), THF (30 mL), 4-methoxy-3-nitrobenzaldehyde (1.00 g). Column chromatography (500 mL, SiO_2, 10% EtOAc/hexanes solvent system). 63% yield.



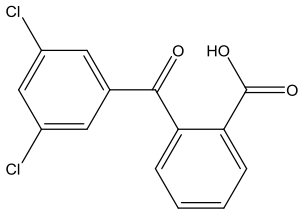
2-(3,4,5-trimethoxybenzoyl)benzoic acid (7.8) Carboxylic Acid Grignard General Procedure. 5-bromo-1,2,3-trimethoxybenzene (1.01 g), magnesium turnings (0.30 g), THF (4.5 mL, 4 mL), phthalic anhydride (0.60 g). Yellow/orange solid. 71% yield.



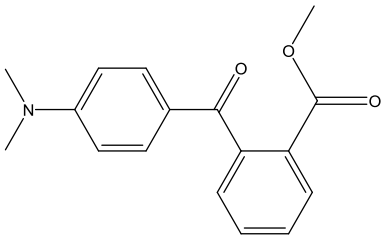
2-(4-(dimethylamino)benzoyl)benzoic acid (7.9) Carboxylic Acid Grignard General Procedure. 4-bromo-N,N-dimethylaniline (1.01 g), magnesium turnings (0.37 g), THF (5.5 mL, 5 mL), phthalic anhydride (0.75 g). Green solid, highly insoluble. 73% yield.



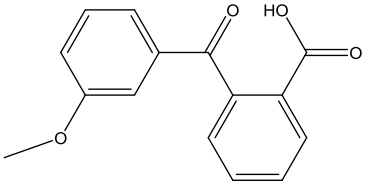
methyl 2-(3,4,5-trimethoxybenzoyl)benzoate (7.10) Carboxylic Acid Methylation General Procedure. Compound **7.8** (0.18 g), MeOH (2.5 mL), H_2SO_4 (0.13 mL). Column chromatography (250 mL, SiO_2, 10-20% EtOAc/hexanes solvent system) 12% yield.



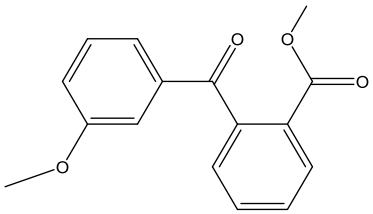
2-(3,5-dichlorobenzoyl)benzoic acid (7.11) Carboxylic Acid Grignard General Procedure. 1-bromo-3,5-dichlorobenzene (1.00 g), magnesium turnings (0.32 g), THF (5 mL, 4.5 mL), phthalic anhydride (0.67 g, small amount spilled while adding to the flask). 66% yield.



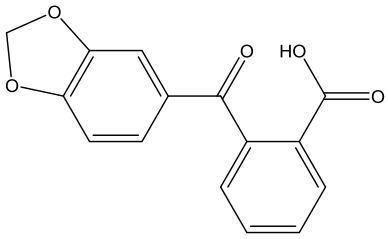
methyl 2-(4-(dimethylamino)benzoyl)benzoate (7.12) Carboxylic Acid Methylation General Procedure. Compound **7.9** (0.21 g), MeOH (3 mL), H_2SO_4 (0.15 mL). Column chromatography (250 mL, SiO_2, 10-50% EtOAc/hexanes solvent system). 65% yield.



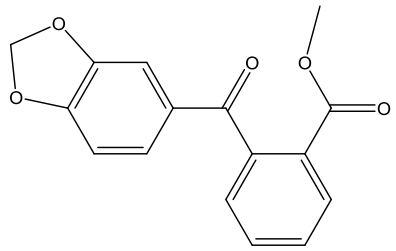
2-(3-methoxybenzoyl)benzoic acid (7.13) Carboxylic Acid Grignard General Procedure. Bromoanisol (0.67 mL), magnesium turnings (0.40 g), THF (6 mL, 5.5 mL), phthalic anhydride (0.80 g). Pale yellow powder. 93% yield.



methyl 2-(3-methoxybenzoyl)benzoate (7.14) Carboxylic Acid Methylation General Procedure. Compound **7.13** (0.51 g), MeOH (8 mL), H_2SO_4 (0.4 mL). Column chromatography (250 mL, SiO_2, 10% EtOAc/hexanes solvent system). Accidentally contaminated with salicylaldehyde, recolumned with same conditions. 40% yield.



2-(benzo[d][1,3]dioxole-5-carbonyl)benzoic acid (7.15) Carboxylic Acid Grignard General Procedure. Bromo-3,4-methylendioxy-benzol (0.6 mL), magnesium turnings (0.36 g), THF (5.5 mL, 5 mL), phthalic anhydride (0.74 g). 82% yield.



methyl 2-(benzo[d][1,3]dioxole-5-carbonyl)benzoate (7.16) Carboxylic Acid Methylation General Procedure. Compound **7.15** (0.50 g), MeOH (7.5 mL), H_2SO_4 (0.38 mL). Column chromatography (250 mL, SiO_2, 10% EtOAc/hexanes solvent system). 38% yield.

3. Results

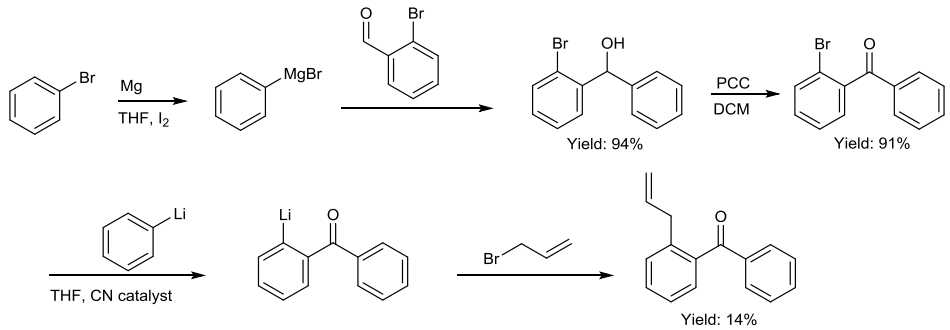


Figure 4. Pestalone core derivative scheme

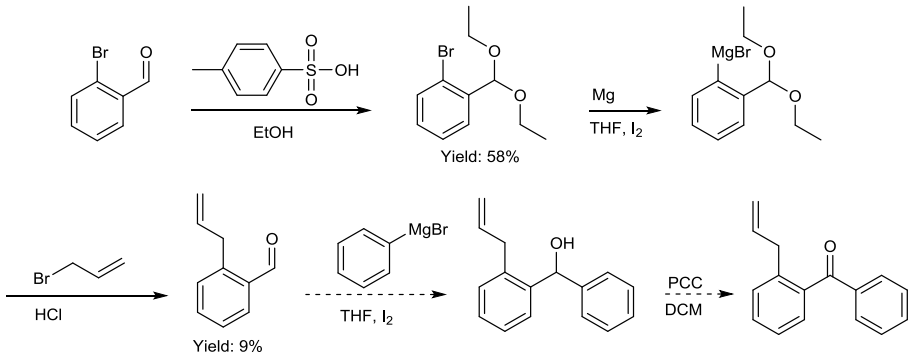


Figure 5. Adjusted pestalone core derivative scheme

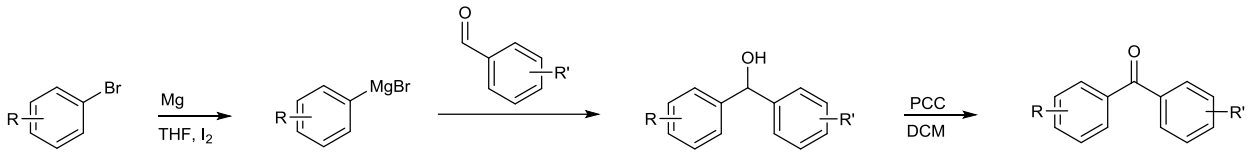


Figure 6. Benzylic derivative scheme of pestalone analogs

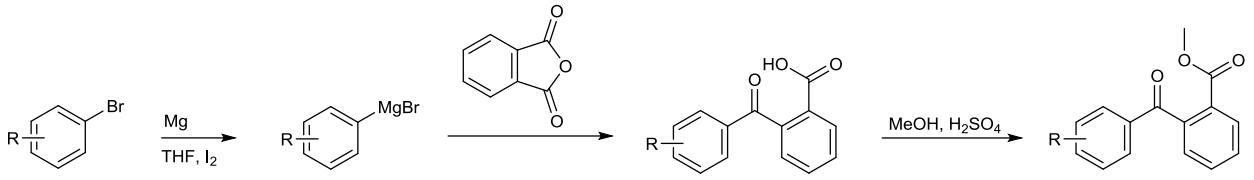


Figure 7. Carboxylic acid derivative scheme of pestalone analogs

This research project was began using the synthetic scheme in Figure 4 to create bicyclic ring systems connected by a ketone bridge. This reaction scheme was successful in producing the desired core structure, but with a very low yield and using harsh reaction conditions. This was due to the addition of the allyl chain via Grignard reaction being more difficult than originally anticipated, so an additional modified synthesis scheme was required. This modified reaction scheme is shown in Figure 5, and attaches the allyl chain prior to joining the two ring systems. Current work focuses on the addition of the allyl chain, which has been observed, though still at low yields, and the joining of the ring systems has not yet been attempted.

Overall, the Grignard reactions used to produce compounds **7.1**-**7.16** were successful in synthesizing desired products, although yields varied widely. Each Grignard reaction produced a highly viscous by-product following acidification. This was usually removed by filtering the solution through cotton prior to extraction, and did not present further complications but could have contributed to loss of product. The products of the Benzylic Ring Grignard Reactions also required column chromatography to separate the desired product from starting material and by-products. This was often difficult as the column was ran in a very low polarity solvent system and still resulted in very close spotting via thin layer chromatography (TLC).

Compound **7.4** was confirmed as one compound via TLC, and proton NMR was inconclusive, but seemed to suggest that the desired product was synthesized. Part of the difficulty is that **7.4** was isolated as a very thick solid, similar in consistency to wet clay, and was highly insoluble in most solvent systems, including dichloromethane (DCM). This made it very difficult to accurately mass, as well as for analysis or synthesis.

The oxidation reactions used to produce compounds **7.5** and **7.6** were successful in synthesizing desired products. However, the solutions following the reaction required filtration through fritted funnel to remove excess starting material, and required multiple rinses with DCM to separate desired product from waste material. The yield for compound **7.5** was calculated based on an assumption of 100% yield for the starting material. This is probably inaccurate as the compound used was crude and yield was not calculated for the compound.

The bacterial assay results seem to indicate antibiotic activity against Gram-positive bacteria *S. aureus* and *B. subtilis* for Compounds **7.8**, **7.9**, **7.11**, **7.13**, and **7.15**. All of these compounds are carboxylic acid derivatives prior to methylation. Additional bacterial assays against *E. coli* and *P. aeruginosa* seem to indicate antibiotic activity for Compound **7.2**. As the other analogs with alcohol bridges did not show activity, the amine group off of the benzylic ring could be contributing to its antibiotic activity, although more testing on this and similar compounds is necessary. The negative controls of empty wells and wells with just tryptic soy broth were used to remove any absorbance value from the actual plate itself, as well as the absorbance of the medium with no bacteria present. Despite this, the bacterial assay results were not clear enough to assign any minimum inhibitory concentrations and additional testing of these compounds is needed. Additionally, the core structure was not synthesized in high enough quantity to test antibiotic activity.

4. Conclusion

The general synthetic schemes used in this project was successful in creating analogs of pestalone. The carboxylic acid derivative scheme was found to produce higher yields of the desired pestalone derivatives than the benzylic ring derivative scheme. This is partially due to the extraction technique for purification, which is both simpler and faster than column chromatography. However, both of these methods are much more efficient than the original and modified core derivative schemes, both in terms of time and yields of desired product. Although the original core derivative scheme was able to produce the desired product, it was in very low yield and is not feasible to produce analogs, especially in large quantities. The feasibility of the adjusted core derivative scheme has yet to be determined, as only the first two steps have been completed thus far, but shows promise of synthesizing the desired product. The oxidation and methylation reactions were not completed on all of the Grignard products. However, the bacterial assay results seem to indicate these steps are not necessary for antibiotic activity.

The results of the bacterial assays completed thus far seem to indicate that antibiotic activity is favored among derivatives with carboxylic acid substituents. However, results from the assays were not clear enough to assess any minimum inhibitory concentrations for the synthesized derivatives. This project is on-going, and additional analogs will be created, potentially adding additional functional groups to derivatives with carboxylic acid side chains to increase antibiotic activity. More testing against *Staphylococcus aureus* and *Escherichia coli,* as well as other Gram-positive and Gram-negative bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa* will be ran to fully assess the antibiotic capabilities of the synthesized molecules.

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