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Synthesis and Antibacterial Evaluation of Derivatives of Natural Product Denbinobin

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Abstract

Antibiotic resistant bacteria pose a serious and growing threat to human health. Over the past decade, the number of resistant strains of bacteria has increased while the introduction of new antibiotics has stagnated, exacerbating this health crisis. The discovery and synthesis of novel antibacterial compounds is a crucial step towards inhibiting the rise of drug resistant bacteria. This study represents an initial investigation of the potential antibacterial properties of denbinobin, a substituted phenanthrenequinone commonly extracted from the orchid genus *Dendrobium*. Denbinobin has numerous bioactive properties including anticancer, anti-inflammatory, anti-HIV, and antioxidant effects. A sister compound of denbinobin, denbinobin B, has displayed inhibition against *Staphylococcus aureus,* leading to the identification of denbinobin as target molecule for antibacterial analysis. Stilbene precursor compounds denbinobin were produced via Perkin Condensation chemistry. Investigation into the production of phenanthrene denbinobin analogs via Lewis Acid-mediated oxidative ring closures is ongoing. A cis stilbene analog of denbinobin was found to have some activity against *Staphylococcus aureus* (1 μg/mL<IC_{50}<10 μg/mL) and *Bacillus subtilis* (1 μg/mL<IC_{50}<10 μg/mL) The results of this study may direct the field of novel antibiotic discovery towards a new natural compound of interest.

1. Introduction

1.1. Background: Antibiotic Resistance

Strains of antibiotic resistant bacteria pose a serious and increasing global threat to human health. A report from the Centers for Disease Control states that in the United States alone, at least 35,000 people die from drug resistant bacterial infections each year. Antibiotic resistant bacteria cost the United States an estimated $20 billion in direct healthcare expenses, and an additional $35 billion in lost productivity.[1] If no significant changes are made, global deaths due to antibiotic resistance by 2050 are projected to surpass ten million a year, overtaking current fatalities from all cancers combined. [2]

Many factors have contributed to the rise of drug resistant bacteria. Antibiotics are some of the most commonly prescribed pharmaceuticals, and an estimated 50% of antibiotic prescriptions considered unnecessary or suboptimal.[1] Additionally, antibiotics are often unnecessarily employed to promote the growth of commercial livestock.[1] The increasing prevalence of antibiotic resistant bacteria is directly linked to the global overuse and misuse of antibiotics. Antibiotic compounds only exterminate drug-susceptible bacteria, leaving resistant bacteria free to proliferate.[3] In addition, bacteria with drug resistant mutations may transfer their genetic material through horizontal gene transfer, allowing for the fast spread of antibiotic resistance through commensal populations.[4] Once the majority of a population of bacteria becomes resistant to a certain antibiotic, a new antibiotic with a different mechanism of action must be employed to treat the infection. Accordingly, it is necessary for clinicians to possess a diverse arsenal of antibiotics.

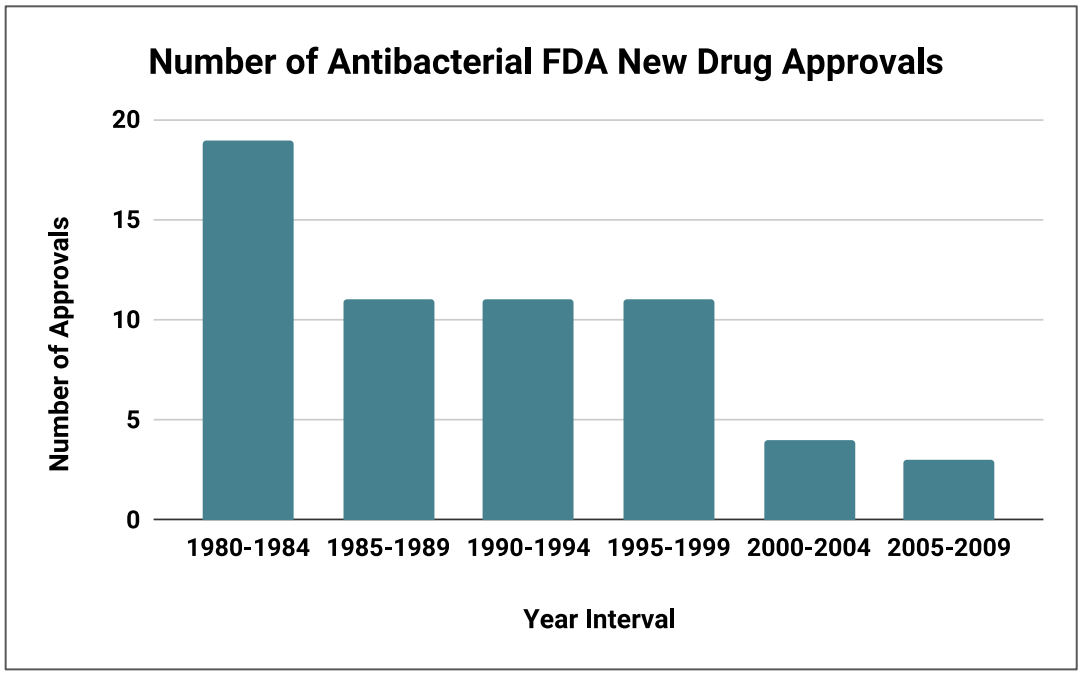


Figure 1: Graph of new antibiotic approvals between 1980 and 2009. Adapted from CDC 2013 report.

Unfortunately, as the global use of antibiotics has increased, the discovery of new antibiotics has stagnated. Between 1980 and 2009, the production of new antibacterial compounds dropped significantly due to several factors including the steep price of antibiotic development and the rapid rate at which antibiotics become obsolete.[5] The last novel antibiotic to be approved for use was Bedaquiline, which was released in the United States in 2012 to treat multi-drug resistant *Mycobacterium tuberculosis* infections.[6] However, recourse against many other strains of resistant bacteria—including methicillin-resistant *Staphylococcus aureus* and carbapenem-resistant *Escherichia coli*—is limited to the administration of previously abandoned antibiotics with poor toxicity profiles, such as colistin.[7] To expand the global arsenal of antibiotics, many academic laboratories have begun projects that employ common drug discovery strategies such as natural products research. In the Wolfe Laboratory, we take a two-pronged approach to antibacterial natural products research by both extracting and characterizing unique antibiotics from co-cultures of bacteria, and by undertaking the synthesis and optimization of newly characterized and previously reported antibiotic leads. This study represents an endeavor from the latter focus of the Wolfe Laboratory.

1.2. Background: Denbinobin

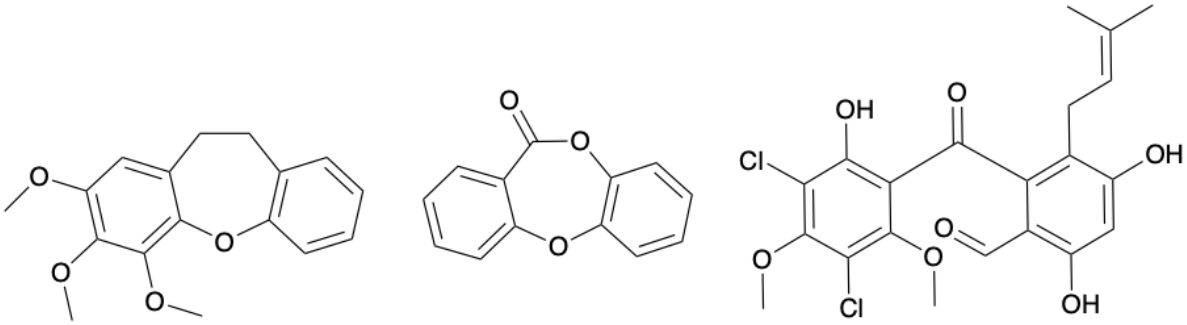


Figure 2: Wolfe Laboratory antibacterial lead compounds empetroxepin (left), depsidone (middle), and pestalone (right).

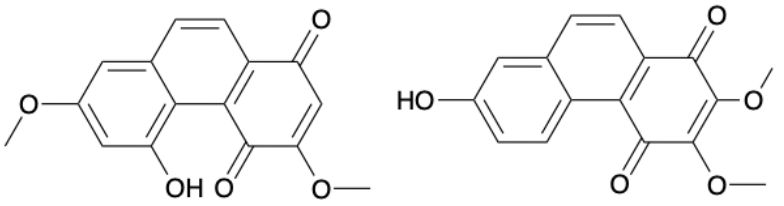
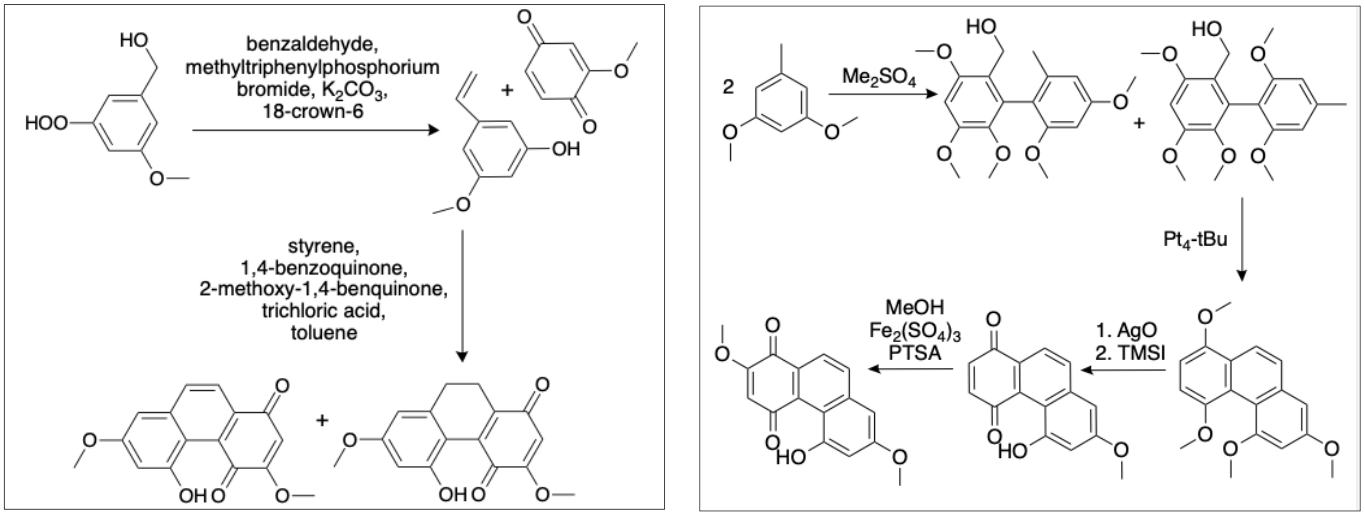
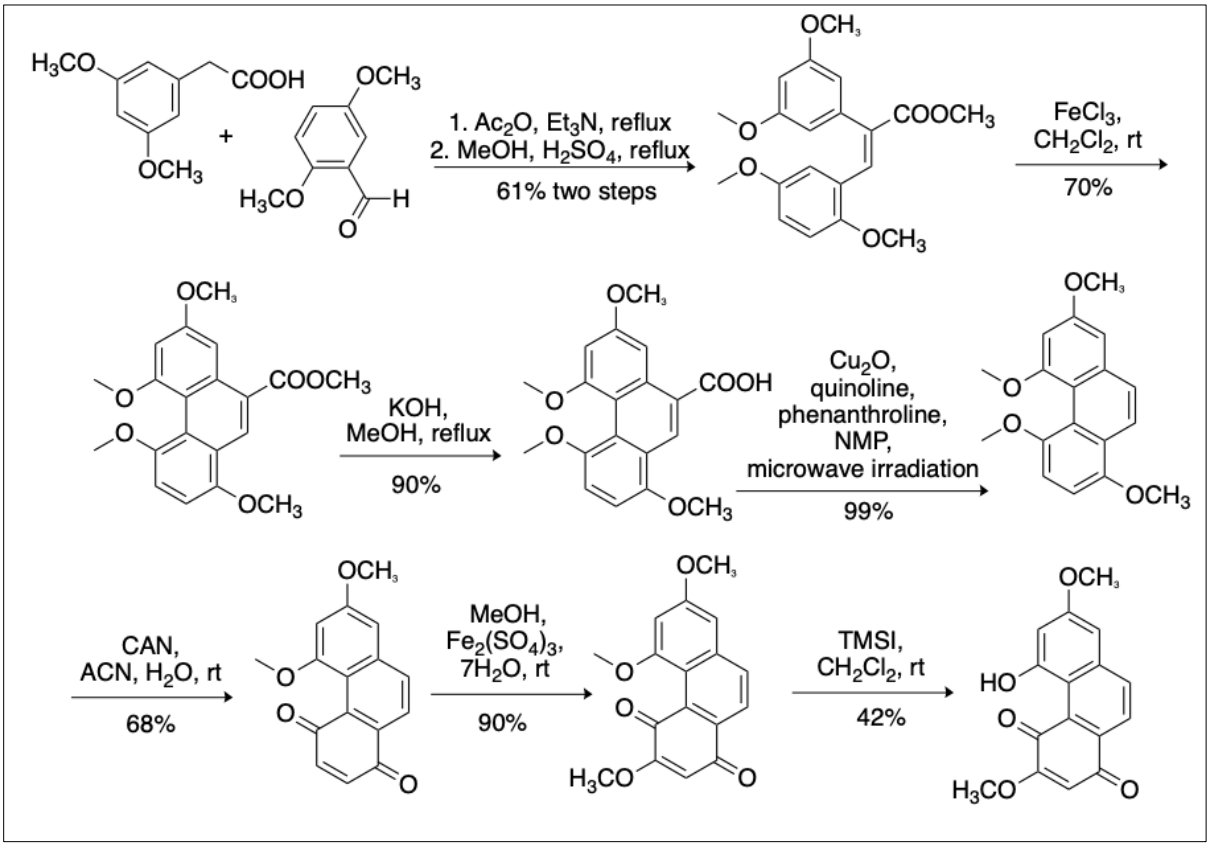


Figure 3: Structures of denbinobin (Compound **1**, left) and denbinobin B (Compound **2**, right)

The Wolfe Laboratory has engaged in the synthesis and antibacterial evaluation of numerous bicyclic and tricyclic natural products, including depsidone, empetroxepin A and B, and pestalone (Figure 2). This study concerns a tricyclic natural product known as denbinobin (Figure 3). Denbinobin is a phenanthrenequinone which has been extracted from multiple species of *Dendrobium* orchids, including *Dendrobium nobile*, *Dendrobium wardianum warner*, and *Dendrobium moniliforme*.[8-10] For hundreds of years, Chinese medicine practitioners have utilized *Dendrobium* stems containing bioactive phenanthrenes.[11] Contemporarily, denbinobin has shown medicinal promise. *In vitro*, denbinobin displays potent anti-inflammatory effects which may one day be used to treat a variety of maladies, including septic shock, arthritis, and asthma.[12] Denbinobin has also been demonstrated to inhibit a kinase involved in the second stage of HIV-1 viral replication.[13] Most notably, denbinobin has been shown to inhibit the growth and mobility of multiple human cancer cell lines, including breast cancer, pancreatic cancer, colon cancer, leukemia, and glioblastoma multiforme.[14-18] In addition, a related analog of denbinobin, called denbinobin B, has produced some inhibitory affects against *S. aureus* (16.5mm at 20mg/mL after 24 hours).[19] Despite the numerous studies on denbinobin's medicinal potential and denbinobin B's antibacterial properties, there are no current studies which report screening denbinobin itself for antibacterial activity.



Scheme 1: Diels-Alder synthesis of denbinobin (left) and Scheme 2: P4-tBu catalyzed synthesis of denbinobin (right).



Scheme 3: Seven-step denbinobin synthesis with yields from Lee. et al.

A number of synthetic pathways have been developed to produce denbinobin. In 2001 Krohn et al. published a method for the synthesis of denbinobin which utilized Diels-Alder chemistry (Scheme 1).[20] The following year, Kraus and Zhang outlined an alternative method of denbinobin synthesis in which phenanthrene formation was catalyzed by basic P4-*t*Bu (Scheme 2).[21] Recently, Lee et al. described a seven-step synthetic pathway to denbinobin featuring a $FeCl_3$ mediated oxidative ring closure.[8] Lee et al.'s pathway is unique in that it contains several stilbene and phenanthrene intermediate compounds. As both stilbenes and phenanthrenes are well known bioactive molecular scaffolds, the precursor compounds produced by the Lee et al. synthesis may also display some antibacterial properties.[22,23] By leveraging Lee et al.'s synthesis, the antibiotic potential of denbinobin and denbinobin related compounds will be efficiently assessed. The results of this study may guide the field of novel antibiotic discovery towards a new natural compound of interest

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2. Experimental

2.1 Synthesis

2.1.1 general methods

All reagents including starting compounds **3** and **4** were purchased commercially and used without further purification with the exception of 2-methyl-4-chlorophenoxyacetic acid (mCPBA). To purify mCPBA, the solid reagent was washed with a phosphate buffer and dried via vacuum filtration. Anhydrous solvents were either purchased or dried. Purification by means of standard silica gel (SiO_2) gradient chromatography occurred using a solvent system comprised of hexane and ethyl acetate, with mobile phase solutions being 5%, 10%, 15%, 20%, 30% or 100% ethyl acetate depending on the compound being isolated. A Varian Oxford 400 was used to obtain all ^{1}H and ^{13}C nuclear magnetic resonance spectra which are reported in parts per million with tetramethylsilane as an internal standard (see supplemental information). Abbreviations for reagents are as follows:

Dichloromethane (DCM), N-Methyl-2-pyrrolidone (NMP), *N*,*N*′-Dicyclohexylcarbodiimide (DCC), Tetrahydrofuran (THF), 4-Dimethylaminopyridine (DMAP), Dimethylforamide (DMF), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI),

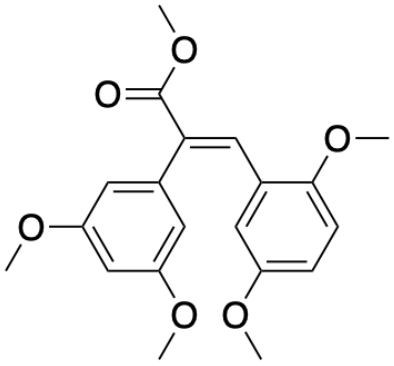


Figure 5: Intermediate compound **5**

2.1.2 compound 5

A mixture of 3,5-dimeoxyphenylacetic acid (**3**) (2.74 g, 14.0 mmol), 2,5-dimethoxybenzaldehyde (**4**) (2.32 g, 14.0 mmol), acetic anhydride (4.61 mL, 3.04 M), and trimethylamine (2.30 mL, 6.09 M) was warmed to reflux with stirring under an Ar atmosphere for 26 hours. The mixture was cooled, diluted with H_2O (50 mL), and extracted (DCM, 3 × 60 mL). The organic layers were combined, dried over anhydrous Na_2SO_4, and dried via rotary evaporation. The crude solid was then dissolved in 41mL CH_3OH (0.34 M) and concentrated H_2SO_4 (2.72 mL) was added dropwise. The mixture was warmed to reflux with stirring under an Ar atmosphere for 26 hours and 25 minutes. The mixture was cooled, diluted with H_2O (50 mL), and extracted (DCM, 3 × 60 mL). The organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude material was purified via flash chromatography (SiO_2, 15% ethyl acetate/hexane) to afford compound 5 (2.87g, 57% yield). ^{1}H-NMR ($CDCl_3$, 400 MHz) 3.33 (3H, s,), 3.72 (6H, s), 3.81 (3H, s), 3.83 (3H, s), 6.39 (1H, s), 6.41 (1H, s), 6.41 (1H, s), 6.42 (1H, d), 6.76 (1H, d), 6.77 (1H, s), 8.16 (1H, s). ^{13}C-NMR ($CDCl_3$, 100 MHz) 52.38 (1C), 55.16 (1C), 55.37 (2C), 56.13 (1C), 100.05 (1C), 107.70 (2C), 111.78 (1C), 114.05 (1C), 117.55 (1C), 123.53 (1C), 131.89 (1C), 134.92 (1C), 138.08 (1C), 152.57 (1C), 152.78 (1C), 160.93 (2C), 168.17 (1C).

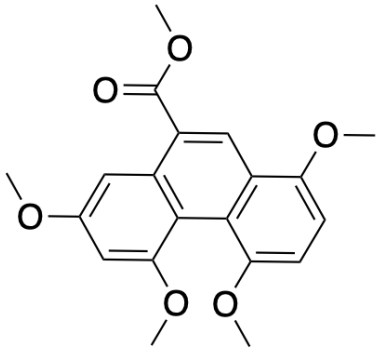


Figure 6: Intermediate compound **6**.

2.1.3 compound 6

The first attempt to produce compound **6** began with the addition of anhydrous $AlCl_3$ (1.20 g, 9.00mmol) to a solution of compound **5** (2.00 g, 5.58 mmol) dissolved in dry DCM (60 mL, 0.09 M). The mixture was left with stirring at room temperature under argon atmosphere for 50 minutes. The reaction was then quenched with CH_3OH (100 mL, 0.06 M), and dried via rotory

evaporation. The crude mixture was dissolved in water (50 mL) and extracted (DCM, 3 × 50 mL) The organic layers were combined, dried over anhydrous Na_2SO_4, and dried via rotary evaporation. The reaction was then purified via flash chromatography (SiO_2, 20% ethyl acetate/hexane). No reaction was observed, and compound **6** was not obtained. The starting material, compound **5**, was recovered in good yield as a pale yellow solid (1.68 g, 84%). A second attempt at producing compound **6** began with the addition of anhydrous $FeCl_3$ (0.58 g, 3.58 mmol), to a solution of compound **5** (0.50 g, 1.40 mmol) dissolved in dry DCM (30mL, 0.12 M). The mixture was left stirring at room temperature under argon atmosphere for 90 minutes. The reaction was then quenched with CH_3OH (25mL, 0.06 M), and dried via rotory evaporation. The crude mixture was dissolved in water (30 mL) and extracted (DCM, 3 × 40 mL) The organic layers were combined, dried over anhydrous Na_2SO_4, and dried via rotary evaporation. The reaction was then purified via flash chromatography (SiO_2, 15% ethyl acetate/hexane). No reaction was observed, and compound **6** was not obtained. A third attempt to produce compound **6** proceeded via the addition of purified mCPBA (0.24g, 1.39 mmol) and anhydrous $FeCl_3$ (0.58 g, 3.58 mmol) to a solution of compound **5** (0.50g, 1.40 mmol) dissolved in dry DCM (30 mL, 0.12 M). The mixture was left stirring under argon atmosphere at room temperature for 2 hours and 25 minutes. The reaction was then quenched with CH_3OH (25mL, 0.06 M), and dried via rotary evaporation. The crude mixture was dissolved in water (10 mL) and extracted (DCM, 3 × 20 mL) The organic layers were combined, dried over anhydrous Na_2SO_4, and dried via rotary evaporation. The reaction was then purified via flash chromatography (SiO_2, 7% ethyl acetate/hexane). Initial ^{1}H-NMR results suggested that compound **6** had been produced and eluted from the column in a mixture with 3-chlorobenzoic acid, the byproduct of mCPBA. The reaction was purified a second time via flash chromatography (SiO_2, 10% ethyl acetate/hexane), and the product again eluted in a mixture with 3-chlorobenzoic acid. A mixture of hexanes, ethyl acetate, and DCM (65% hexanes/30% ethyl acetate/5% DCM) was then added to the solid mixture of compound **6** and 3-chlorobenzoic, and the product was obtained as a red crystalline solid via vacuum filtration (4.40 mg, 2% yield). ^{1}H-NMR ($CDCl_3$, 400 MHz) 3.94 (3H, s), 3.98 (6H, s), 4.00 (6H, s), 6.75 (1H, s), 6.89 (1H, d), 7.09 (1H, d), 8.03 (1H, s), 8.85 (1H, s). ^{13}C-NMR ($CDCl_3$, 100 MHz) 50.57 (1C), 52.12 (1C), 55.50 (1C), 56.14 (1C), 56.30 (1C), 100.07 (1C), 107.71 (1C), 111.80 (1C), 114.05 (1C), 117.57 (1C), 134.93 (1C), 138.10 (1C), 152.58 (1C), 160.94 (1C), 168.20 (1C).

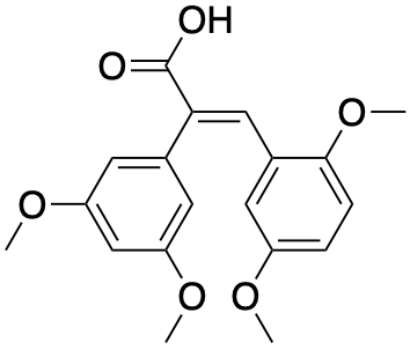


Figure 7: Intermediate compound **7**

2.1.4 compound 7

A mixture of compound **5** (1.5 g, 4.25 mmol), CH_3OH (12.12 mL), and 1M KOH (12.12 mL) was heated to reflux with stirring under an Ar atmosphere for 7 hours. The mixture was then quenched with 37% HCl at 0 °C and filtered via vacuum filtration. The resulting solid was dried under reduced pressure to afford compound **7** as a pale yellow solid (1.02 g, 68% yield). ^{1}H-NMR ($CDCl_3$, 400MHz) 3.33 (3H, s), 3.73 (6H, s), 3.84 (3H, s), 6.42 (1H, s), 6.43 (1H, s), 6.43 (1H, s), 6.45 (1H, d), 6.79 (1H, d), 6.79 (1H, s), 8.30 (1H, s). ^{13}C-NMR ($CDCl_3$, 100MHz) 55.15 (1C), 55.39 (2C), 56.16 (1C), 100.33 (1C), 107.64 (2C), 111.90 (1C), 114.00 (1C), 118.19 (1C), 123.24 (1C), 130.86 (1C), 136.82 (1C), 137.57 (1C), 152.57 (1C), 153.01 (1C), 161.01 (2C), 172.79 (1C).

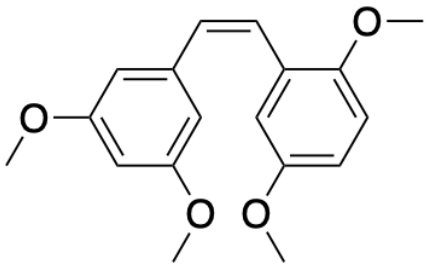


Figure 8: Intermediate compound **8**

2.1.5 compound 8

A mixture of compound **7** (0.50 g, 1.45 mmol), Cu_2O (0.01 g, 0.07 mmol), 1,10-phenanthroline (0.03g, 0.17 mmol), quinoline (0.5 mL, 2.9 M) was dissolved in NMP (1mL, 1.45M). The reaction was left stirring under reflux conditions and Ar atmosphere for 22 hours and 35 minutes. The mixture was then quenched with 37% HCl at 0 °C and filtered via vacuum filtration, and purified via flash chromatography (SiO_2, 15, 20, 30, and 100% ethyl acetate/hexane). No compound **8** was obtained. Compound **7** was recovered in trace amounts only.

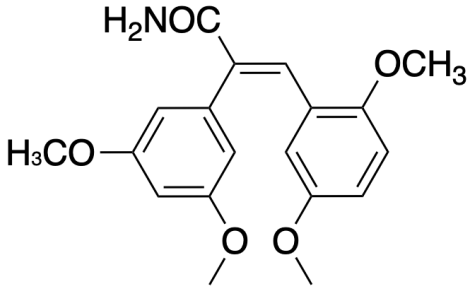


Figure 9: Intermediate compound **9**

2.1.6 compound 9

A mixture of compound **7** (0.010 g, 0.03 mmol), DCC (0.07 g, 0.03 mmol), and NH_4Cl (0.015 g, 0.03 mmol), was dissolved in dry dry THF (3 mL, 0.01 M). The reaction was left stirring under reflux conditions and Ar atmosphere for 51.5 hours. The mixture was then diluted with Et_2O, quenched with water and saturated aqueous $NaHCO_3$, then washed with Et_2O (3 x 30 mL). The organic layers were combined, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude material was purified via flash chromatography (SiO_2, 15, 30, 50, 80 and 100% ethyl acetate/hexane) Compound **9** was not obtained. Trace amounts of Compound **7** were recovered.

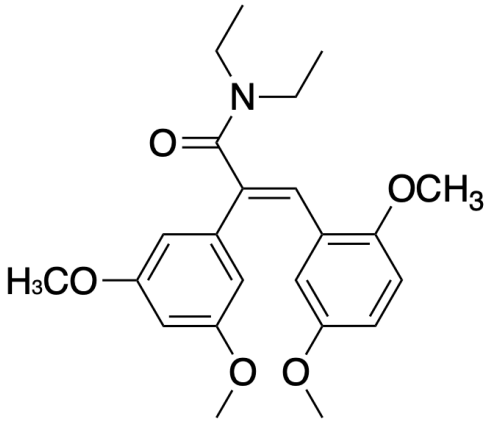


Figure 10: Intermediate compound **10**

2.1.7 compound 10

A mixture of compound **7** (0.015 g, 0.04 mmol), DMAP (90.0 µmol), and Et_2N (0.20 mL, 0.2 mmol) was added to DMF (10 mL, 0.004 M) and left to stir for 10 minutes at 0 °C. EDCI (0.084 g, 0.04 mmol) was then added slowly, and the mixture was allowed to come to room temperature overnight. After 19 hours the reaction was stopped, quenched with water, and washed with DCM (6 x 30 mL). The organic layers were combined and dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude material was purified via flash chromatography (SiO_2, 25, 50, 80 and 100% ethyl acetate/hexane, then 100% methanol). The presence of compound **10** has not yet been confirmed by spectroscopy.

2.2 Antibacterial Assays

2.2.1 sterile conditions

All surfaces and gloves were sterilized with ethanol. Pipette tips, 96 well plates, and petri dishes were sterilized via autoclave. Sterile procedures were conducted under a propane torch flame. The lids of all sterile containers were flamed before replacement. The lip of the jar containing autoclaved full strength tryptic soy broth (FSTSB) was flamed before use. All contaminated waste was treated with a 10% bleach solution for fifteen minutes before disposal in dedicated biohazard accumulation containers.

2.2.2 bacteria culture

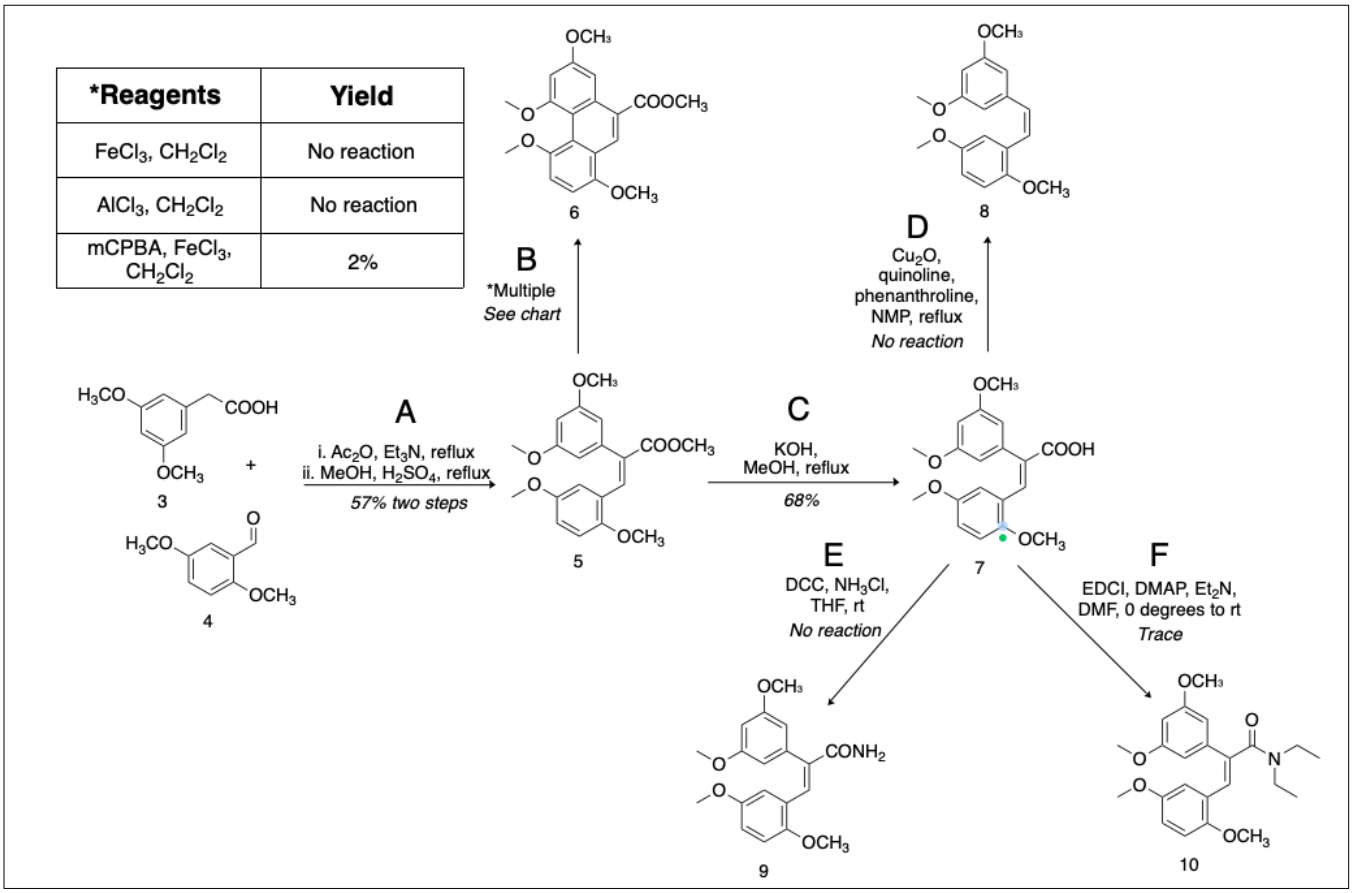
Under sterile conditions, 4 mL of FSTSB were added to two separate test tubes, one for *Staphylococcus aureus* and one for *Escherichia coli*. A single colony of each bacteria was placed into each respective test tube. Both test tubes were incubated with shaking at 37 ºC for 20 hours.

2.2.3 96-well plate bioassay

Compound **7b** was tested for antibacterial activity *in vitro* via standard broth microdilution assay technique. Compound **7b** was diluted to 100 mg/mL in DMSO. A 100 mg/mL dilution of Chloramphenicol was also prepared as a positive control. A master plate was prepared by micropipetting 9uL of DMSO into each well except for the top row. Into the first and second wells of the top row, 10 µL of the positive control were micropipetted. Into the third and fourth wells of the top row, 10µL of compound **7b** were micropipetted. Into the fifth and sixth wells of the top row, 10µL of DMSO were micropipetted. In order to create a ten-fold dilution down each column of the master plate, 1 µL of liquid from each well except the bottom row was pipetted and mixed into the well directly below it. Bacteria specific test plates were then created under sterile conditions. To create the test plates, 89µL of FSTSB were pipetted into each well of a new plate. Next, 10 µL of the overnight culture of desired bacteria were pipetted into each well. A 1µL sample from each well of the master plate was then pipetted into each

corresponding well of the test plate. Test plates were incubated with shaking for 20 hours at 37 ºC, read by a BioTek multimode plate-reader at 590nm, and analyzed using Gen5 2.09 software.

3. Results and Discussion



Scheme 4: Synthesis of precursor denbinobin analogs with table of reagents and yields for reaction B.

3.1 Synthetic Strategy and Outcomes

To begin the synthesis of denbinobin, compound **5** was obtained by the Perkin Condensation of compounds **3** and **4** in moderate yield (57%). To produce compound **6,** a $FeCl_3$ mediated oxidative ring closure of **5** was then attempted at room temperature under inert atmosphere as described by Lee et al. However, no product was obtained from this reaction. The substitution of $FeCl_3$ with $AlCl_3$, a stronger Lewis acid, also failed to produce compound **6**. The exclusive use of $FeCl_3$ at room temperature to afford methoxy substituted phenanthrenes from stilbenes was first reported by Wang et al. in 2008.[24] The following year, Wang et al. published a study detailing the use of the oxidizing peracid *m*CPBA with catalytic $FeCl_3$ to achieve the same reactions in good yield.[25] Accordingly, *m*CPBA was added to reaction **B**, and the presence of compound **6** was confirmed in the crude mixture by ^{1}H-NMR spectroscopy. During the reaction workup, compound **6** proved difficult to separate from solid inorganics via filtration as it is poorly soluble in DCM, diethyl ether, water, hexane, and ethyl acetate. The poor solubility of compound **6** also hindered separation from polar organic byproducts via flash chromatography. The aggregation of these purification challenges likely resulted in the low final yield of compound **6** (2%). Further investigation into solvent systems and purification methods suitable for compound **6** may improve the results of reaction **B**.

In order to investigate the potential antibacterial properties of stilbene denbinobin precursor molecules, compound **7b** was produced via a classic saponification of compound **5** in good yield (68%). A decarboxylation reaction utilizing Cu_2O was then attempted on compound **7** to produce compound **8**. However, no product was obtained. The failure of reaction **D** may be

attributed in part to impurities in the quinoline, which had turned dark brown due to light exposure. Furthermore, Lee et al. described reaction **D** as occurring by microwave irradiation—a method unavailable for this study. Although reflux conditions run for an increased time should have afforded similar results for reaction **D**, a more comprehensive reaction condition screen may be needed to produce compound **8** in-house. To produce compound **9**, a DCC assisted nucleophilic amine addition was attempted, but no product was obtained. To form compound **10,** a different carbodiimide, EDCI, was utilized with catalytic DMAP. Initial ^{1}H-NMR spectra suggested the presence of compound 10 in the crude mixture, but further purification and analysis is needed to confirm the results of reaction **F**.

3.2 Antibacterial Activity

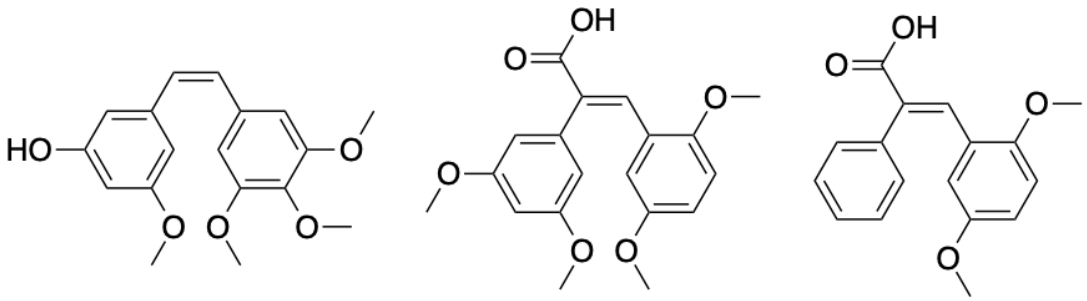


Figure 4: Cis Stilbenes with antibacterial properties: combretastain A-4 (left), compound **7** (middle), and (E)-3-(2,5-dimethoxyphenyl)-2-phenylacrylate (right).

Most pathogenic bacteria fall into two categories: Gram-positive species, which feature a thick peptidoglycan cell wall, and Gram-negative species, which feature a unique lipopolysaccharide outer membrane.[26] To establish spectrum of potential activity, compound **7** was screened for activity against two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, and two Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, via microdilution cell death assays. After 17 hours of incubation, **7** displayed no antibacterial activity against *E. coli* or *P. aeruginosa*. However, the compound demonstrated notable inhibition of *S. aureus* (1 μg/mL<IC_{50}<10 μg/mL) and *B. subtilis* (1 μg/mL<IC_{50}<10 μg/mL), indicating that the activity of the compound is likely limited to Gram-positive bacteria. Additional assay data is required to determine precise IC_{50} values for compound **7**.

Outside of the Wolfe Laboratory, several other methoxy substituted stilbenes have been identified as potential antibacterial compounds of interest (Figure 4). One such compound, combretastain A-4, has demonstrated inhibitory effects against multiple strains of gram positive and gram negative bacteria.[27] In addition, a remarkably similar cis stilbene as compound **7**, (E)-3-(2,5-dimethoxyphenyl)-2-phenylacrylate, has also demonstrated broad spectrum antibiotic properties.[28] Interestingly, our discovery of compound **7** as an inhibitor of exclusively Gram-positive bacteria suggests that variation in the position and number of aromatic methoxy groups on the stilbene scaffold is sufficient to modulate the spectrum of activity of this class of small molecules. Further research into the mechanism of action of compound **7** and additional structure-activity relationship analysis is needed to provide a complete picture of the stilbene's antibiotic potential.

4. Conclusion

Denbinobin is a known bioactive substituted phenanthrenequinone with medicinal promise. In this study, stilbene and phenanthrene denbinobin precursor analogs were produced in moderate yield. One denbinobin related analog, compound **7**, was demonstrated to inhibit two Gram-positive bacteria, *S. aureus* and *B. subtilis* (1 μg/mL<IC_{50}<10 μg/mL). Continued investigation into the antibacterial potential of substituted stilbene derivatives of **7** may help guide the field of towards a novel class of antibiotics.

5. Acknowledgements

The author thanks Dr. Amanda Wolfe, the Wolfe Research Team, the North Carolina Space Grant Program, and the University of North Carolina Asheville Chemistry Department. Additional thanks to the University of North Carolina Asheville Honors Program for their support of undergraduate research.

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