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Synthesis and Evaluation of Amino Acid Ester Substituted Pseudopyronine Derivatives

Stephanie Holz
Chemistry and Biochemistry
University of North Carolina at Asheville
One University Heights
Asheville, North Carolina 28804 USA
Research Advisor: Dr. Amanda Wolfe

Abstract

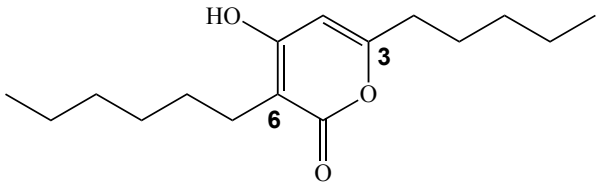
As antibiotic resistance continues to be an increasing problem worldwide, the synthesis and evaluation of novel antibiotic compounds is becoming necessary. Natural products have become a promising source of exploration, as many of these compounds have not been used clinically, and therefore bacterial pathogens have no associated resistance mechanisms. Pseudopyronines A and B of the α-pyrone class have been isolated and seen to exhibit both antibacterial and anticancer activities. Previous research by Fields et al. has demonstrated that the α-pyrone core has potential inhibitory effects on efflux pumps, which are commonly overexpressed in many Gram-negative bacteria. Additionally, Rath et al. determined that the addition of bulky amino acid substituents to natural compounds also increases inhibition of NorA efflux activity in Gram-positive bacteria. Finally, Bouthillette et al. determined through SAR study of pseudopyronine that specific chain alterations allow for specificity in antibacterial activity against Gram-negative and -positive bacteria. Based on this prior work, this research aims to synthesize short chain pseudopyronine derivatives in which previous methods were used to substitute the hydroxyl group of the α-pyrone core using various L-amino acid methyl esters. Based on these methods being mostly ineffective, the site of amino acid addition has been changed to a carbonyl present on the alkyl chain at the C6 position of the α-pyrone core. These synthesized compounds were tested biologically using a cell death assay in determination of whether they stand alone as antibiotic compounds themselves. Further testing will be required to discern if these compounds are more suited as an adjuvant therapy.

1. Introduction

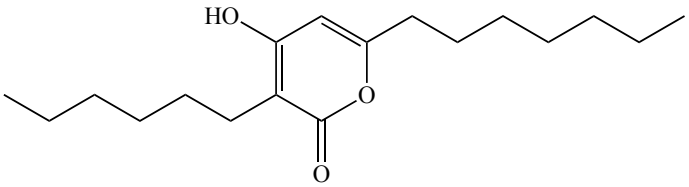
Antibiotic resistance is an ongoing public health crisis caused by the misuse and overuse of antibiotics, and it affects a multitude of people worldwide.[1] In 2019, the Center of Disease Control (CDC) estimated that at least 2.8 million people were infected with multidrug resistant bacteria in the United States, and greater than 35,000 people died as a result of those infections.[2] Additionally, the CDC estimates that multidrug resistant infections will result in more than 10 million deaths by the year 2050 if current trends continue, establishing a demand for the development of antibiotic compounds which utilize unique mechanisms of action.[2] According to the World Health Organization (WHO), antimicrobial resistance occurs when bacteria and other pathogens undergo a genetic mutation and therefore no longer respond to antibiotics or other drugs that were once effective in treatment of the associated infection.[3] These mutations can take many forms, including modification of the antibiotic target, or even complete bypass of the drugs target system.[4] Moreover, bacteria can undergo genetic mutations such as horizontal gene transfer, which refers to the obtaining of foreign genes by bacteria from surrounding organisms in the environment.[5]

Due to these mechanisms, multidrug resistant (MDR) bacteria are often harder, or can be nearly impossible to treat, requiring extended hospital stays, or even toxic alternative regimens to treat infections.[2] These treatments can become expensive, making the necessary care to treat these infections inaccessible to many people. Additionally, it is important to recognize the rate at which antibiotic resistant bacteria proliferate, as this has made it difficult for modern medicine to keep up with. Moreover, there is an important distinction to be made between the two major classes of bacteria: Gram-positive and -negative bacteria. Due to structural differences, these two classes often require treatments which employ different mechanisms of action. More specifically, Gram-negative bacteria have an additional outer membrane consisting of a negatively charged lipopolysaccharide layer, making these bacteria harder to penetrate and treat.[6] Additionally many Gram-negative bacteria overexpress efflux pumps, which further reduce antibiotic accumulation within the cell. Efflux pumps are a class of transmembrane proteins that rapidly remove both endogenous molecules and exogenous toxins, such as antibiotics.[7] Many antibiotics meant to treat bacterial infections are substrates of these efflux pumps, leading to their disposal out of the cell. Efflux pump inhibition is thought to be a promising approach to help combat the growing problem of antibiotic resistance, as it can reestablish the efficacy of ineffective drugs that lost potency. Connectively, it is predicted that the antibacterial potency can be achieved at lower concentrations of the antibiotic.[7]

As previously noted, there are two distinct types of bacteria, Gram-positive and -negative. Just as these bacteria differ in their membrane composition, they typically also express different efflux proteins. More specifically, NorA and Rv2686c–2687c–2688c pumps are expressed in Gram-positive bacteria, while AcrAB-TolC efflux pumps are expressed in Gram-negative bacteria.[7,8] Due to the structural cell wall complexity mentioned previously associated with Gram-negative bacteria, in tandem with the overexpression of efflux pumps, accumulation of antibiotics within the cell is rather difficult. This makes AcrAB-TolC pumps a necessary target for novel antibiotic compounds.



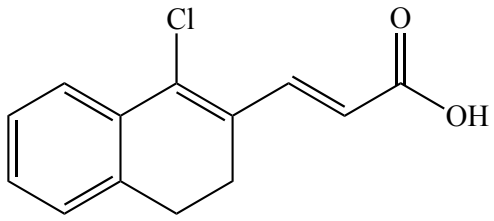
Pseudopyronine A



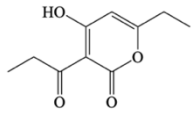
Pseudopyronine B

Moreover, natural products are being rapidly explored to develop these antibiotics, as many naturally occurring compounds have not been used clinically, and therefore do not have any associated resistance. One previously studied class of compounds, the α-pyrones, are naturally occurring products of many microorganisms as both biosynthetic precursors and secondary metabolites which have exhibited moderate antibacterial activity upon their isolation.[9] Of this class, pseudopyronines A and B (Figure 1) have been studied in the Wolfe research laboratory for their variety of medicinal effects upon alterations to chain lengths at both carbons 3 and 6 of the ring system. The results discerned that chain lengths of six to seven carbons increase activity against specifically Gram-positive bacteria, whereas shorter chain lengths exhibited moderate activity against Gram-negative bacteria, such as *Escherichia coli*.[9] Based on this finding and a focus on Gram-negative bacteria, derivatizing pseudopyronine with short alkyl chain lengths at C3 and C6 may be a promising approach in treating multidrug resistant *Escherichia coli* and *Pseudomonas aeruginosa,* two of the most common strains of MDR Gram-negative bacteria.

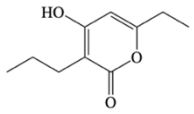
As shown in another study performed by Rath et al, the addition of bulky amino acid substituents to a naturally occurring compound has been seen to function as effective efflux pump inhibitors (Figure 2). Specifically, of the amino acid substituents biologically evaluated, tryptophan, tyrosine, and proline were found to have the highest inhibition of NorA efflux in *Staphylococcus aureus* (SA), a Gram-positive bacterium. The percent efflux inhibition associated with these three analogs were 83.87%, 85.24%, and 80.33%.[7] Additionally, research conducted by Fields et al. further supported the potential application of pseudopyronine as a co-dosed adjuvant therapy.[10] As seen in Figure 3, this research demonstrated that when penicillin G was administered with compound 1, the antibiotic recovered a significant amount (greater than 256-fold) of its antibacterial activity against *Pseudomonas aeruginosa*. This redeemed activity is likely associated with the ability of pseudopyronine to effectively inhibit the efflux pumps within the inner membrane of *P. aeruginosa*. The research proved the inhibition of efflux pumps by adjuvant therapy by first assessing the pseudopyronine compounds as purely an antibiotic, in which there was no inhibition of *Pseudomonas aeruginosa,* supporting that these compounds work strictly as adjuvants. This finding was also supported using an efflux inhibition assay, which ruled out other mechanisms of action such as cell wall synthesis disruption and inhibition of biofilm formation.[10] Therefore, based on the findings of Bouthillette et al., Fields et al. and Rath et al., altering a short chain pseudopyronine core with amino acid esters may be an advantageous approach to a novel mechanism of action to treat multidrug resistant Gram-negative bacteria, highlighting the objective of this research (Figure 4).



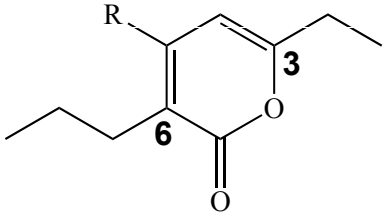
	P. aeruginosa + Adjuvant (50 µM)		
	-	1	2
Antibiotic			
Amoxicillin	2	2(1)	2(1)
Erythromycin	0.5	0.5(1)	0.5(1)
Penicillin G	>128	0.5(>256)	128(>1)
Vancomycin	1	1(1)	1(1)



1



2



R= Ala, Val, Trp, etc.

2. Experimental

All solvents and reagents were purchased without further purification prior to use in reactions. Anhydrous solvents specifically were obtained from an inert solvent system. All water and air sensitive reactions were carried out under argon gas in a flame dried flask. Characterization was done by 1H-NMR; the spectra were taken on a Varian Inova NMR. LCMS was also used in determining if synthesis of derivatives were successful. This was done using a shimadzu single quadrupole LCMS-2020.

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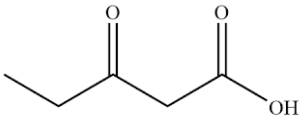
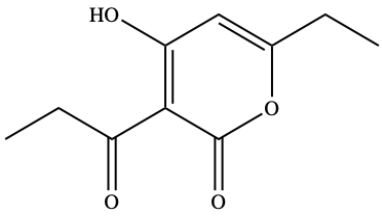


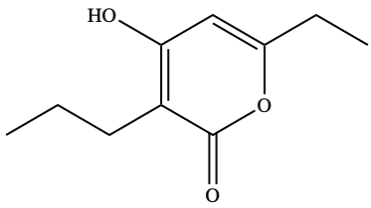
2.1 3-oxopentanoic acid (1)

Commercial grade methyl-3-oxopentanoate (10.00 g, 86.1 mmol) was dissolved in a 1 to 1 ratio of tetrahydrofuran (THF): H2O (200 mL; 0.38 M each) at room temperature. While the solvent was stirred, lithium hydroxide was slowly added to the flask (3.69 g, 154.1 mmol); the reaction ran for approximately 24 hours. Upon completion, the reaction was quenched to a pH of approximately 1 using 1 N aqueous hydrochloric acid (HCl), and then concentrated under reduced pressure. The compound was then extracted using 300 mL of ethyl acetate (3x). The organic layers were then washed with 500 mL of deionized water, and dried using sodium sulfate. The drying agent was removed via filtration, and the compound was then concentrated under reduced pressure. The product was obtained and further used without additional purification. **Yield:** 30%



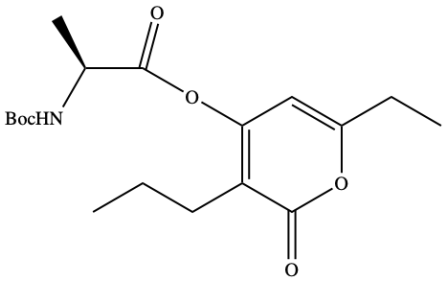
2.2 6-ethyl-4-hydroxy-3-propionyl-2H-pyran-2-one (2)

A solution of **1** (2.66 g, 22.9 mmol) in anhydrous THF (127 mL; 0.18 M) was stirred in a flask under inert atmospheric conditions at room temperature. Subsequently, carbonyldiimidazole (CDI) (5.05 g, 31 mmol; 1.36 eq) was added to the solution and allowed to stir for 24 hours. The reaction was acidified to a pH of 1 using 1N HCl (aqueous), followed by distillation of the THF. Upon this, a white-brown solid crashed out of the solution and was isolated via vacuum filtration. H-NMR spectroscopy confirmed this to be pure product.
Yield: 59% **^{1}H NMR:** ($CDCl_3$, 400 MHz) 5.90 (s, 1H), 3.06 (q, 2H) 2.50 (q, 2H) 1.21 (t, 3H), 1.11 (t, 3H).



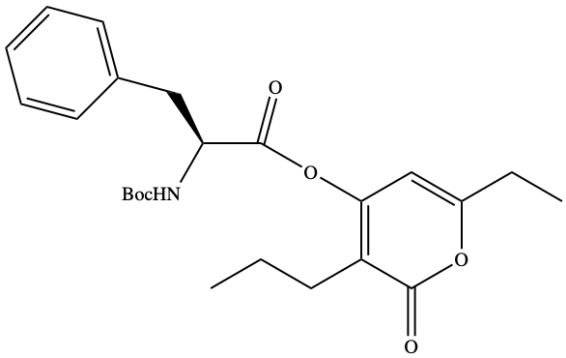
2.3 6-ethyl-4-hydroxy-3-propyl-2H-pyran-2-one (3)

To a solution of anhydrous THF (1.4 mL) and 1N HCl (2.62 mL), **2** (0.5 g, 2.5 mmol; 2.5 eq) was added under inert atmospheric conditions at room temperature. Subsequently, sodium cyanoborohydride (0.4 g, 6.4 mmol) was slowly added to the stirring solution. Upon completion of the reaction, the solvent was distilled, and the compound was dissolved in dichloromethane (DCM). This was then extracted twice with water, and the organic layer was dried using sodium sulfate. The product was then concentrated under reduced pressure, followed by purification using column chromatography by a solvent system of 40% ethyl acetate/ 60% hexanes. This was confirmed to be a pure product by H-NMR spectroscopy.
Yield: 16% **^{1}H NMR:** ($CDCl_3$, 400 MHz) 6.26 (s, 1H), 2.53 (m, 4H), 1.53 (m, 2H), 1.20 (t, 3H), 0.94 (t, 3H).



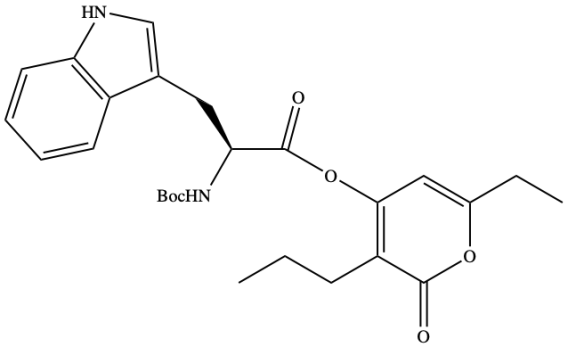
2.4 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl(*tert*butoxycarbonyl)-*L*-alaninate (4)

To a stirring solution of anhydrous dimethylformamide (DMF; 7.8 mL) and **3** (100 mg, 0.55 mmol) under inert atmospheric conditions at room temperature, 4-dimethylaminopyridine (DMAP) (0.02 g, 0.16 mmol) was added to the mixture. Subsequently, N-Boc-L-alanine (0.209 g, 1.1 mmol) and dicyclohexylcarbodiimide (DCC) (0.3 g, 1.5 mmol) were added. The reaction was allowed to run for approximately 18 hours. A pipette filtration was done to filter a white solid from the reaction, which was then washed using DMF. The liquid from the reaction was dissolved in ethyl acetate and extracted first using 150 mL of 1M HCl. The organic layer was then extracted with sodium bicarbonate, followed by water, and lastly brine. The organic layer was then dried using sodium sulfate and the solvent was distilled. The crude product was purified via column chromatography by a solvent system of 8% ethyl acetate/ 92% hexane. The polarity of the solvent system eventually moved up to about 20% ethyl acetate.
Yield:0%



2.5 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl(*tert*butoxycarbonyl)-*L*-phenylalaninate (5)

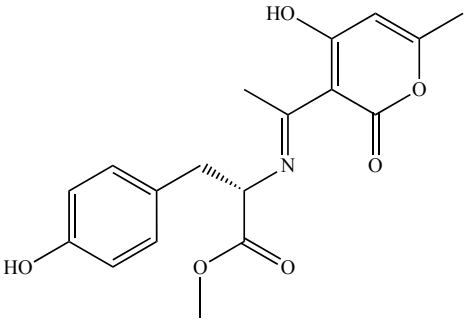
To a stirring solution of anhydrous THF (4 mL) and **3** (50 mg; .274 mmol) under inert atmospheric conditions at room temperature, 4-dimethylaminopyridine (0.01 g, 0.08 mmol) was added to the mixture. Subsequently, N-Boc-L-phenylalanine (0.159 g, 0.60 mmol) and dicyclohexylcarbodiimide (0.115 g, 0.56 mmol) were added. The reaction ran for approximately 24 hours before 70 mL of ethyl acetate was added to the reaction. Upon this, the compound was extracted first using 1M HCl, followed by sodium bicarbonate, water, and brine. The organic layer was dried using sodium sulfate and the compound was then concentrated under reduced pressure. The crude product was purified via column chromatography by a solvent system of 8% ethyl acetate/ 92% hexane. The polarity of the solvent system eventually moved up to about 15% ethyl acetate at fraction 35.
Yield:0%



2.6 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl(*tert*butoxycarbonyl)-*L*-tryptophanate (6)

To a stirring solution of anhydrous DCM (1.5 mL), **3** (50 mg; .274 mmol) and N-Boc-L-tryptophan (80 mg; 0.26 mmol) were added under inert atmospheric conditions at room temperature. To a separate small vial, another 1.5 mL of anhydrous DCM was added, followed by dicyclohexylcarbodiimide (DCC) (60 mg; 0.29 mmol). Next, 4-dimethylaminopyridine (1.7 mg; 0.014 mmol) was added to the solution containing **3.** This solution was then stirred in an ice bath until it reached approximately 0 °C. Once the flask was cooled, the DCC solution was then added dropwise to the flask over a five-minute period. The reaction ran for approximately 1 hour before the progress of the reaction was checked via H-NMR. Upon this, the mixture was separated via column chromatography by a solvent system of 30% ethyl acetate/ 70% hexane.

Yield: 0%

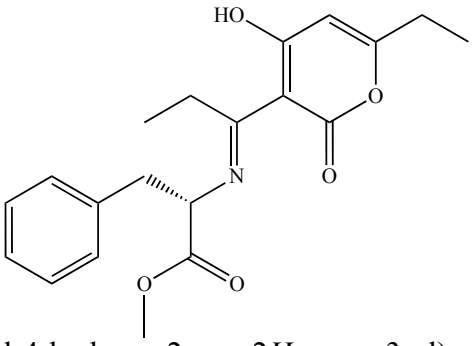


2.7 methyl (*S*, *E*)-2-((1-(4-hydroxy-6-methyl-2-oxo-2*H*-pyran-3-yl)ethylidene)amino)-3-(4-hydroxyphenyl)propanoate (7)

To a stirring solution of methanol (MeOH) (1.3 mL), 3-acetyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (Figure 6) (20 mg, 0.12 mmol) and methyl ester *L*-tyrosine (23 mg, 0.12 mmol) were added. As this was stirring, N,N-Diisopropylethylamine (DIPEA) (76 mg, 0.60 mmol) was then added. This stirred under inert atmospheric conditions at room temperature for approximately 24 hours. Then, sodium borohydride was added (8.9 mg, 0.24 mmol); this was then allowed to stir for approximately 30 minutes before the reaction was quenched using water. The solution was then extracted with ethyl acetate (3x) and the organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure. The product was then purified via column chromatography using a solvent system of 30% ethyl acetate/ 70% hexanes.

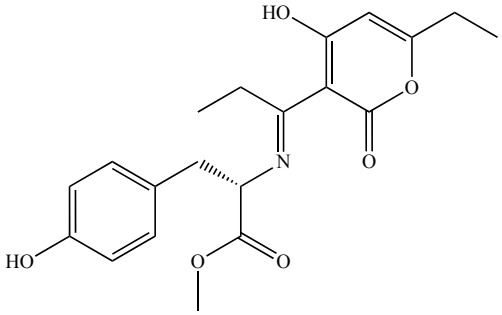
Yield: 14% **^{1}H NMR:** ($CDCl_3$, 400 MHz) 2.18 (s, 3H), 2.34 (s, 3H), 3.04 (m, 1H), 3.25 (m, 2H), 3.80 (s, 3H), 5.12 (s, 1H), 6.75, J=6.8 (d, 2H), 7.04, J=2.4 (d, 2H).

IR (film) ν_{max} 1001, 1106, 1237, 1330, 1363, 1394, 1467, 1517, 1560, 1580, 1654, 1701, 1747, 2853, 2921, 2954 cm^{-1}



2.8 methyl (*S*, *E*)-2-((1-(6-ethyl-4-hydroxy-2-oxo-2*H*-pyran-3-yl)propylidene)amino)-3-phenylpropanoate (8)

To a stirring solution of methanol (MeOH) (1.7 mL), **2** (30 mg, 0.15 mmol) and methyl ester *L*-phenylalanine (39 mg, 0.18 mmol) were added. As this was stirring, N, N-Diisopropylethylamine (DIPEA) (98 mg, 0.76 mmol) was then added. This stirred under inert atmospheric conditions at room temperature for approximately 24 hours. Then, sodium borohydride was added (12 mg, 0.31 mmol); this was then allowed to stir for approximately 30 minutes before the reaction was quenched using water. The solution was then extracted with ethyl acetate (3x) and the organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure. The product was then purified via column chromatography using a solvent system of 22% ethyl acetate/ 78% hexanes.
Yield: 8% **^{1}H NMR:** ($CDCl_3$, 400 MHz) 0.94, J=7.6 (t, 3H), 1.17, J=6.8 (t, 3H), 2.37 (m, 2H), 2.47 (m, 2H), 2.80 (m, 1H), 2.84 (m, 2H), 5.82 (s, 3H), 6.73, J=8.4 (d, 2H), 7.02, J=8.8 (d, 2H)
IR (film) ν_{max} 1119, 1236, 1326, 1414, 1458, 1509, 1588, 1654, 1675, 2855, 2923, 2961 cm^{-1}



2.9 methyl (*S*, *E*)-2-((1-(6-ethyl-4-hydroxy-2-oxo-2*H*-pyran-3-yl)propylidene)amino)-3-(4-hydroxyphenyl)propanoate (9)

To a stirring solution of methanol (MeOH) (1.7 mL), **2** (30 mg, 0.15 mmol) and methyl ester *L*- tyrosine (36 mg, 0.18 mmol) were added. As this was stirring, N, N-Diisopropylethylamine (DIPEA) (98 mg, 0.76 mmol) was then added. This stirred under inert atmospheric conditions at room temperature for approximately 24 hours. Then, sodium borohydride was added (12 mg, 0.31 mmol); this was then allowed to stir for approximately 30 minutes before the reaction was quenched using water. The solution was then extracted with ethyl acetate (3x) and the organic layers were combined, dried over Na_2SO_4, and concentrated under reduced pressure. The product was then purified via column chromatography using a solvent system of 22% ethyl acetate/ 78% hexanes.
Yield: 5% **^{1}H NMR:** ($CDCl_3$, 400 MHz) 0.88, J=2.4 (t, 3H), 1.16, J=7.6 (t, 3H), 2.37 (m, 2H), 2.42 (m, 2H), 3.07 (m, 1H), 3.32 (m, 2H), 3.79 (s, 3H), 5.71 (s, 1H), 7.18 (m, 3H), 7.28 (m, 2H)
IR (film) ν_{max} 1330, 1376, 1453, 1487, 1573, 1658, 1708, 1746, 2855, 2923, 2961 cm^{-1}

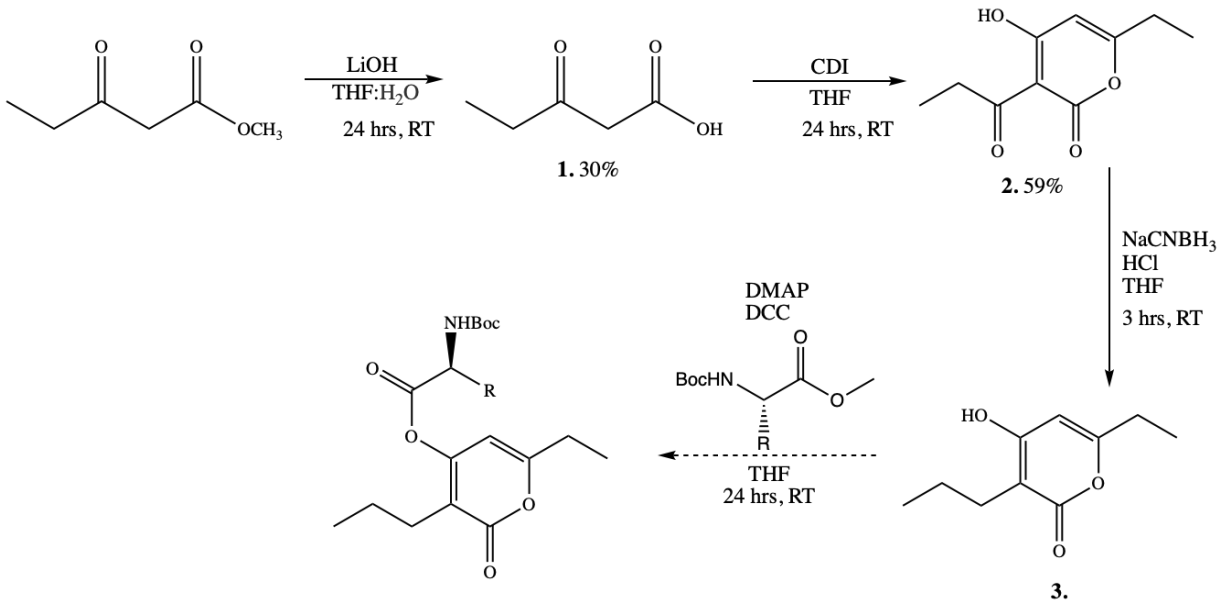
2.10 Biological Testing

2.10.1 Cell Death Assay

Approximately 10 mg of compounds **7**, **8**, and **9** were measured out into vials, with their exact weights recorded. The compounds were diluted using biological grade DMSO to make 25.6 mg/mL solutions. One µL of the dissolved products was added to a 96 well plate in triplicate. The compounds were further diluted with 89 µL of Full-Strength Tryptic Soy Broth (FS TSB) and 10 µL of FS TSB overnight cultures containing either S. aureus or E. coli. DMSO-only columns were used as a negative control. The plate was shaken for 24 hours at approximately 36 degrees Celsius. The antibacterial activity of each compound was then assessed using a Biotek plate reader (OD 590) after the 24-hour incubation. Lower absorbance values would be seen for compounds with higher antibacterial properties. Compounds **7**, **8**, and **9** all showed minimum inhibitory concentrations greater than 256 µg/mL, indicating these compounds do not contain antibacterial properties themselves. Typically, a MIC in the nanomolar range is indicative that a compound is likely a good antibiotic candidate, therefore further supporting that these are not potent antibiotics.

3. Results and Discussion

Only three amino acid derivatives have been attempted to be synthesized using the synthetic route illustrated in Figure 5. However, upon attempted purification and characterization by NMR spectroscopy and mass spectrometry, none of these analogs were successfully synthesized. These coupling reactions were attempted using 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), and a Boc-protected amino acid.



Both mass spectrometry and H-NMR spectroscopy reflect that the reagents and compound **3** are likely not reacting, as starting material (**3**) is predominantly what is found upon analysis. Based on a redirected focus in method development, the alanine derivative has been a primary focus, as the goal was to optimize reaction conditions to then apply them to other amino acid coupling reactions to compound **3.** With the issues faced with the original coupling method, a synthesis method which utilizes thionyl chloride was attempted, as this was a successful method used by a previous researcher in the Wolfe Laboratory.[11]

The first two reaction attempts using thionyl chloride were crudely analyzed using H-NMR and mass spectrometry, in which starting material was mostly found. Due to this, it was hypothesized that due to resonance destabilization, the hydroxyl group of compound 3 was less nucleophilic than desired, ultimately leading to the use of potassium carbonate, as seen in the last two attempts as shown in Table 1. This was done with the expectation that the base would deprotonate the hydroxyl group at the C1 position of the α-pyrone core, making it theoretically more nucleophilic and therefore more reactive. However, the analysis of these attempts suggested that no analog was synthesized. The last two attempts using this method were also slightly modified, so that the reagents were split up and able to stir separately before being combined to stir. More specifically, the first attempt using potassium

carbonate allowed the base and starting material to stir in half of the solvent, while the amino acid and thionyl chloride were allowed to stir separately. Each solution stirred for approximately 30 minutes before the solution containing the stating material was added to the solution containing the amino acid. Since the results of this reaction concluded unsuccessful, it was hypothesized that reversing the addition may encourage reactivity. This is because a lot of base was left over in the vial during the transfer of the starting material-containing solution and may have not been able to fully react with the starting material. Therefore, the fourth attempt entailed the addition of the amino acid solution to the starting material and base, however, this also proved unsuccessful. Analysis using H-NMR and mass spectrometry support this conclusion, as starting material was mostly present.

Table 1. Attempts utilizing thionyl chloride to successfully synthesize amino-acid ester pseudopyronine derivatives.

Trial	Amino Acid	Solvent (anhydrous)	Reagents	Starting material (g)	Temperature	Time	Yield	# of attempts
1	ALA	DCM	2 eq $SOCl_2$	0.05	RT	24 hrs	-	1
2	ALA	DCM; 1 drop DMF	2 eq $SOCl_2$	0.05	RT	24 hrs	-	1
3	ALA	DCM; 1 drop DMF	2 eq $SOCl_2$, 2 eq K_2CO_3	0.05	RT	24 hrs	-	2

Upon coming to these realizations, another method was attempted which used triflic anhydride (Table 2), which was predicted to be more reactive than the thionyl chloride or DCC used previously. However, resonance destabilization of the starting material was still expected, therefore pyridine was used. This was used instead of potassium carbonate as previous reactions utilizing triflic anhydride had commonly utilized pyridine. Upon running this reaction, crude analysis using mass spectrometry proposed that the desired product was present (354 m/z).

Table 2. Attempt utilizing triflic anhydride to successfully synthesize amino-acid ester pseudopyronine derivatives.

Trial	Amino Acid	Solvent (anhydrous)	Reagents	Starting material (g)	Temperature	Time	Yield	# of attempts
1	ALA	DCM	2 eq Tf2O, 2 eq pyridine	0.05	RT	24 hrs	-	1

However, upon purification, several fractions still showed this mass via mass spectrometry. Furthermore, none of the compounds separated on the column show any promise of successful synthesis when using H-NMR. Therefore, it is hypothesized that the product was synthesized, however, in very trace amounts. Characterization via mass spectrometry also showed a peak which indicated that the α-pyrone core had been oxidized (370 m/z). Due to this, the results of this reaction need to be further explored. Therefore, two more reactions were run using ALW 038, as seen in Table 3. This was done based on a hypothesis that the carbonyl coming off the alkyl chain at the C6 position may offer stabilization to the hydroxyl group, due to intramolecular hydrogen bonding between these substituents (Figure 6). This was thought to promote reactivity of the starting material as a nucleophile; however, these reactions show no successful product formation upon analysis after attempted purification.

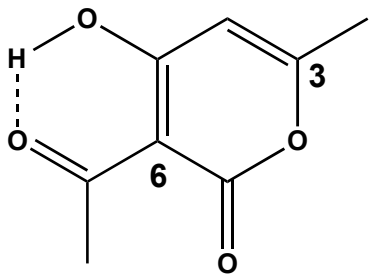


Table 3. Recent reaction attempts utilizing ALW 038.

Trial	Amino	Solvent	Reagents	Starting	Temperature	Time	Yield	# of

	Acid	(anhydrous)		material (g)				attempts
1	ALA	DCM	2 eq Tf_2O, 2 eq pyridine	0.05	RT	24 hrs	-	1
2	ALA	DCM	2 eq $SOCl_2$, 2 eq K_2CO_3	0.05	RT	24 hrs	-	1

Due to the lack of product being formed despite multiple attempts, the project switched gears, attempting to make the starting material the electrophile rather than a nucleophile. More specifically, the carbonyl present at the C6 position of the α-pyrone core would undergo nucleophilic attack by the amine present within the amino acid backbone. This was attempted 3 times, once using ALW 038 (Figure 6) and the other two times using compound **2**. ALW 038 was reacted with *L*-tyrosine methyl ester, whereas one of the reactions which used compound **2** was run with *L*-tyrosine methyl ester and the other with *L*-phenylalanine methyl ester. All these reactions were proven successful upon their purification, producing compounds **7**, **8**, and **9**; this was confirmed via H-NMR and mass spectrometry. However, due to the reaction yields being low, the NMR samples were not very concentrated. Therefore, ^{13}C-NMR was not completed for these compounds, and some peaks are hard to detect using ^{1}H-NMR, as seen in the supporting information.

4. Conclusion

Compounds **7**, **8**, and **9** were further evaluated upon their isolation using biological testing. Specifically, each of these compounds were tested in a cell death assay against both *Staphylococcus aureus* (*S.aureus*) and *Escherichia coli* (*E.coli*). These two bacteria were specifically used as they are some of the most common of Gram-positive and -negative bacteria. Due to this research focusing entirely on short alkyl chain pseudopyronine molecules, the results of the cell death assay can potentially be attributed to the same findings found by Fields et al. It was discerned by their research that short chain pseudopyronine molecules exhibit moderate activity against Gram-negative bacteria as an adjuvant therapy specifically. All three of these compounds exhibited no activity (MIC: > 256 μg/mL) on their own, therefore aligning with the previously mentioned results. Therefore, the next step taken would be to assess these compounds as an adjuvant therapy using an adjuvant assay. This would further assess these findings and determine if these compounds do act as an adjuvant therapy against Gram-negative and -positive bacteria. These compounds will also likely be tested against an efflux knockout strain to determine if they exhibit efflux inhibition properties.

Since the new synthesis pathway has proven successful, it is expected that other derivatives using any commercially available L- amino acid could be synthesized. This allows for a wide variety of substituents to test biologically in determining which side chains or functional groups possess the best inhibitory activity. With this being said, future work done on this project may include creating a library of these molecules with a variety of *L*-amino acids. These could also be further assessed using cell death and adjuvant assays. Furthermore, all the synthesized compounds could be tested against an efflux knock-out bacterial strain to determine if this is a potential mechanism of action for these compounds, as exhibited by other previous work.

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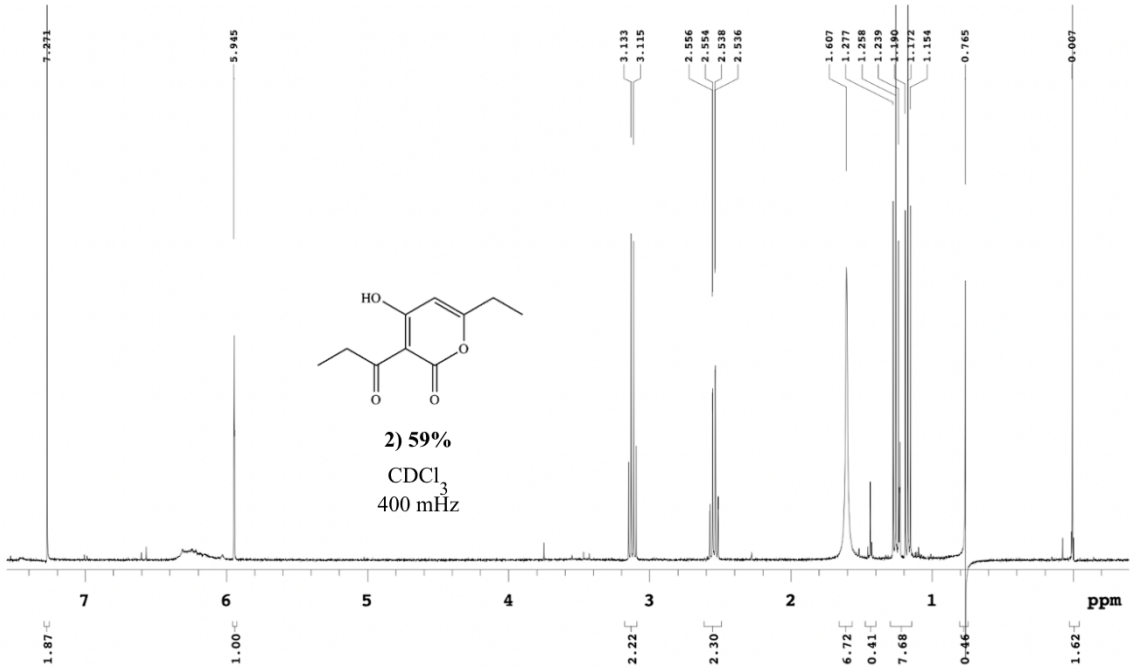
Supporting Information



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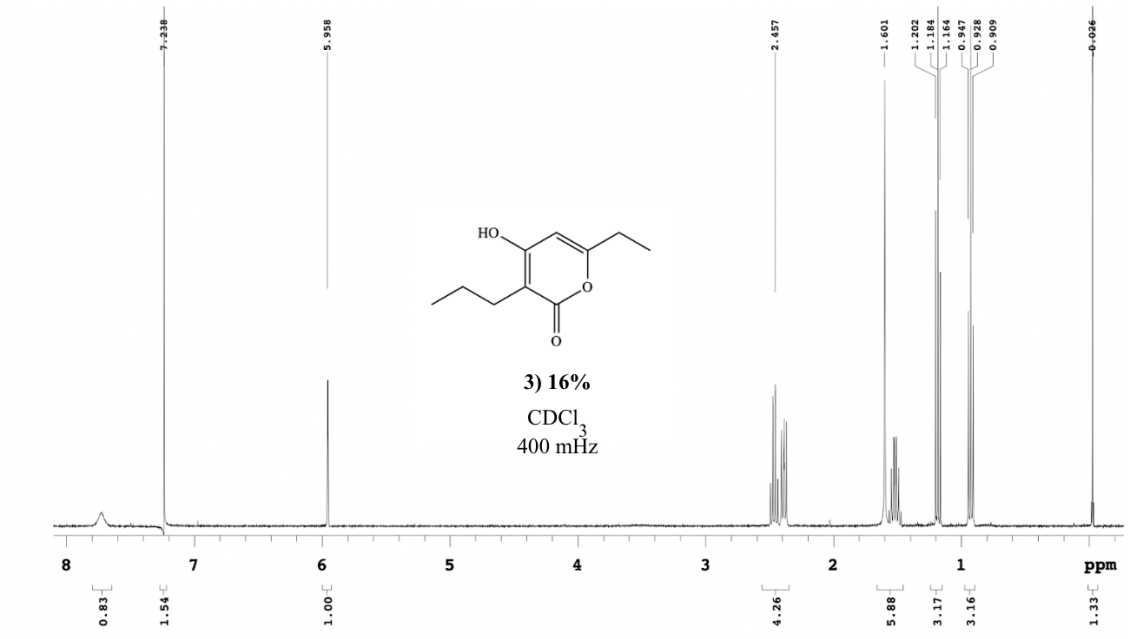

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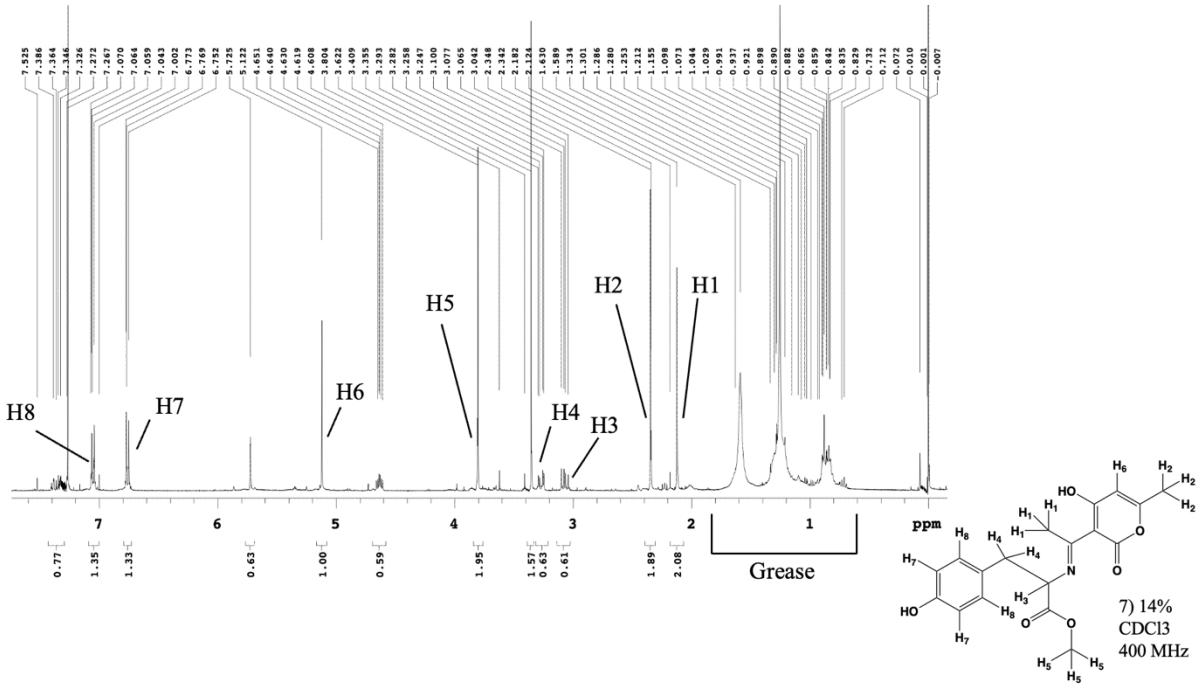
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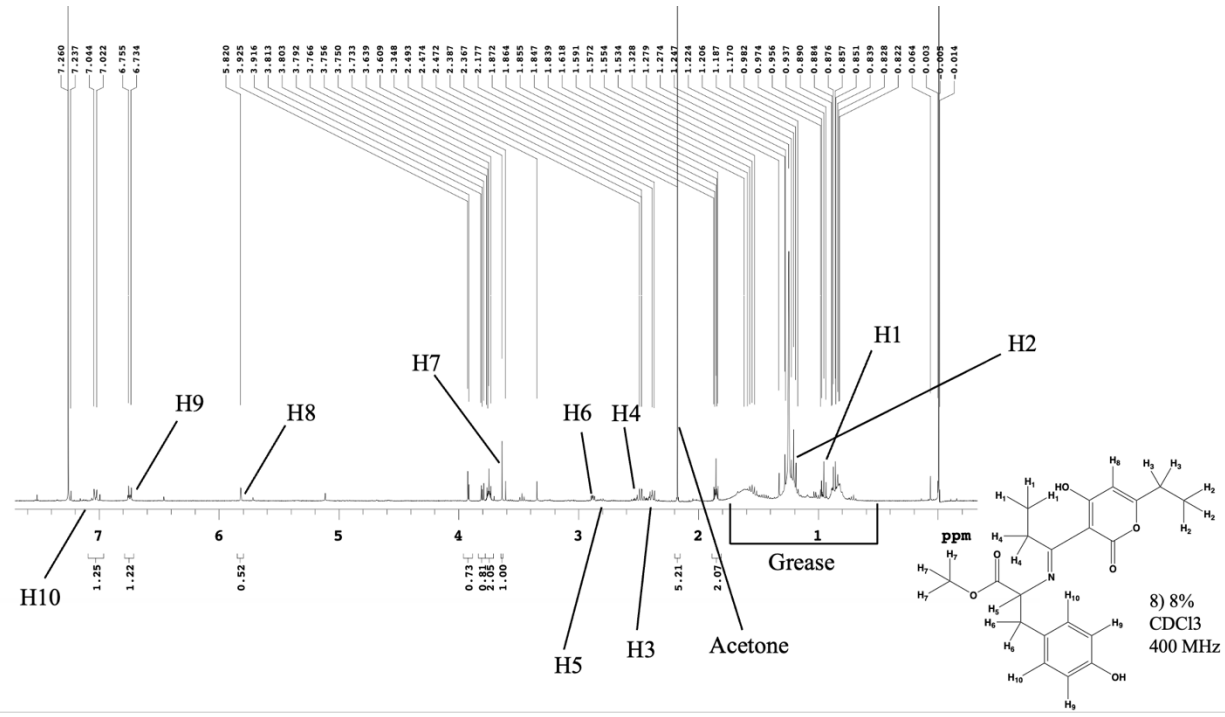
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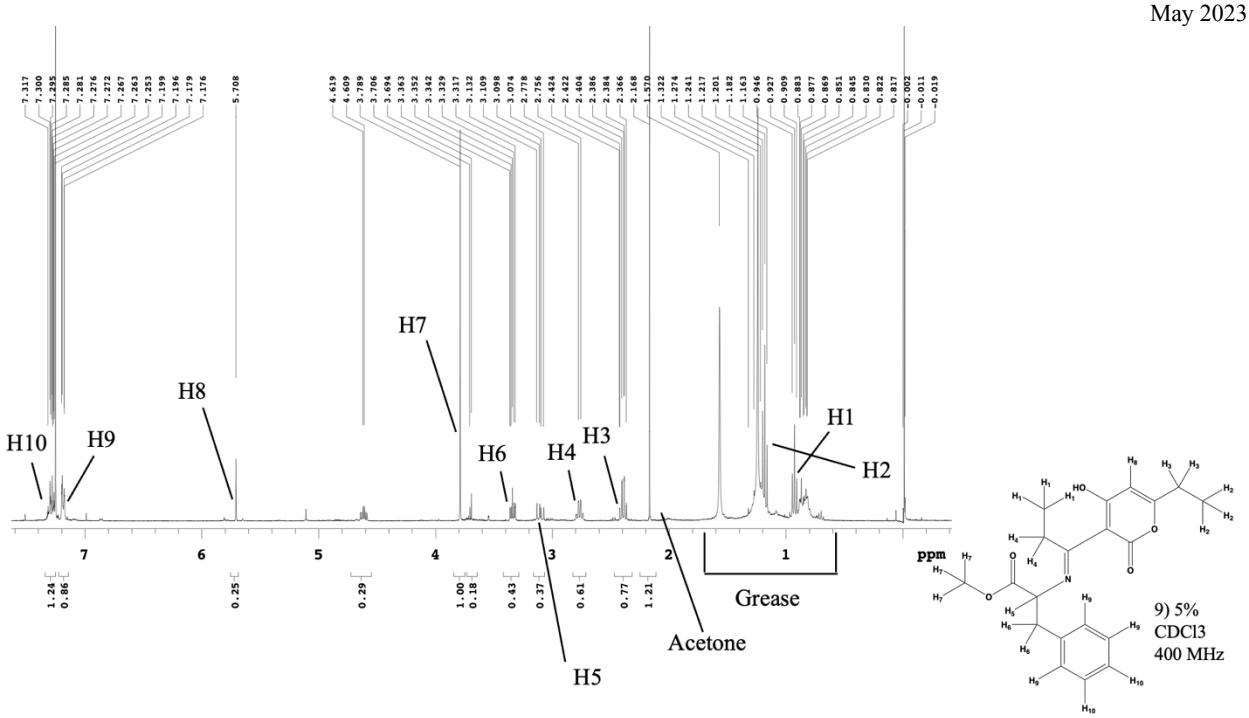
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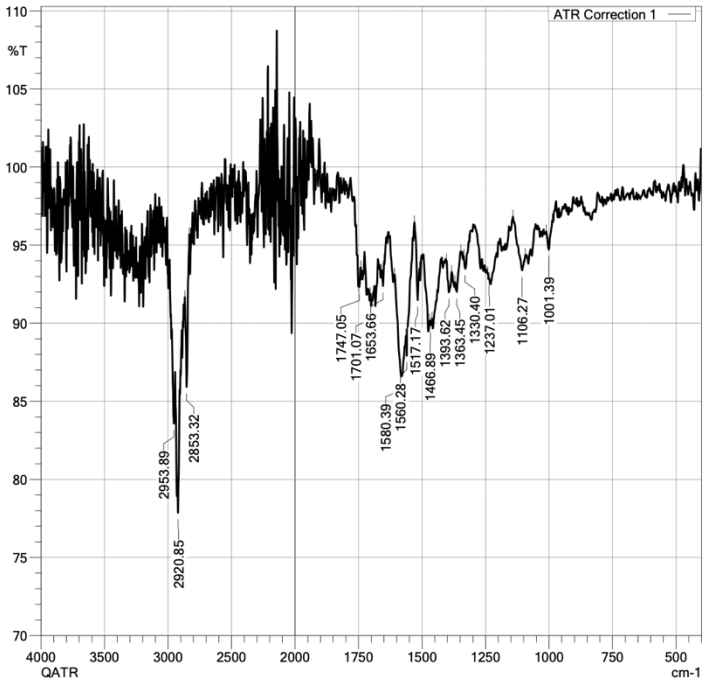
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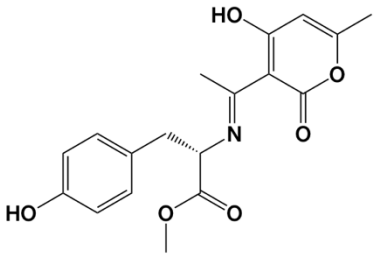
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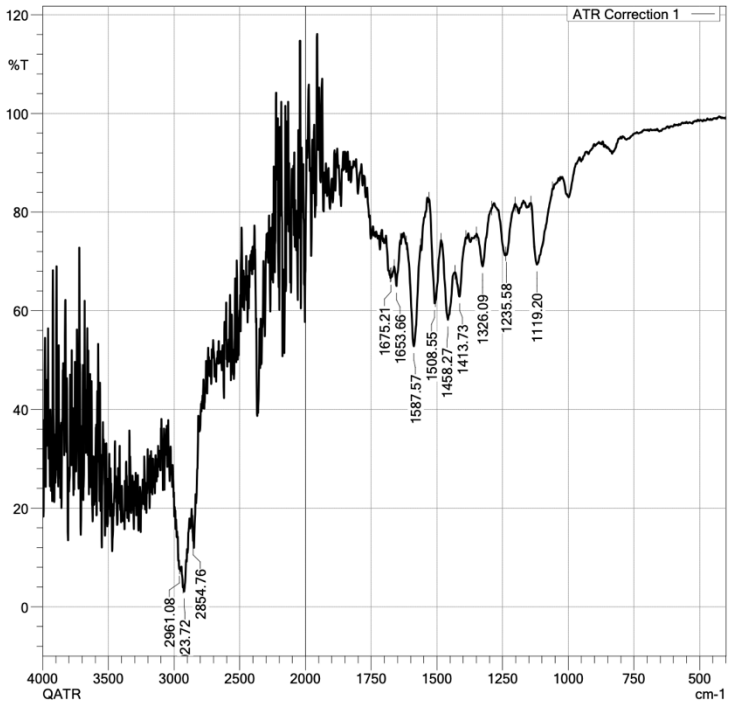
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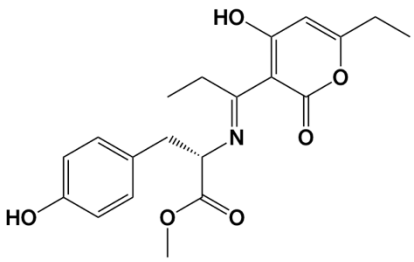
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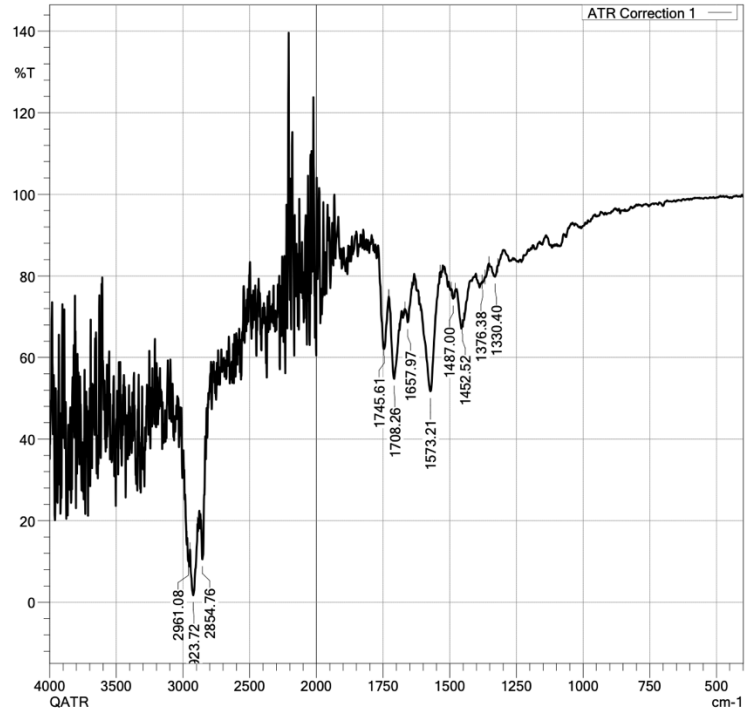
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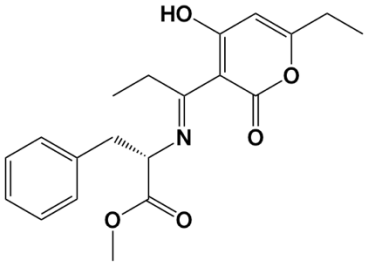
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