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Evaluation of Amino Acid Methyl Ester Substituted Pseudopyronine Derivatives

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Abstract

As strains of drug resistant bacteria become more common, it is becoming increasingly necessary to synthesize novel compounds to overcome known resistance mechanisms. Natural products remain a robust source of novel bioactive molecules. Pseudopyronines, first isolated from *Pseudomonas* bacteria in 2002, are members of the α-pyrone class of antibiotics and have shown significant promise as natural product antibiotics. This work derivatizes the α-pyrone core of short chain pseudopyronine analogs to probe the effects of L-amino acid methyl esters on antibiotic activity against *Escherichia coli*. The 4' and 5' carbons of the α-pyrone core will be substituted with various L-amino acid esters as these positions have previously been found to contribute heavily to biological activity. This is based on previous research which showed that the addition of bulky amino acid groups inhibited AcrAB-TolC efflux pumps, the "garbage chutes" of the bacteria which are commonly overexpressed in many Gram-negative bacteria. This opens the potential for the synthesized molecules to be dosed adjuvantly with a known antibiotic. Each analog was tested for antibiotic activity in a cell death assay against Gram-negative and Gram-positive bacteria, as well as assessed for efflux inhibition potential via an AcrAB-TolC efflux pump assay.

1. Introduction

Since the early 1970s, the rate at which bacteria have become resistant to currently available antibiotics has drastically increased.[1] This phenomenon was first observed as early as the 1940s when sulfonamides, a class of antibiotics developed in the 1930s, began to lose effectiveness. Since then, the problem has grown exponentially, with modern bacteria developing some resistance to a drug within one year of clinical implementation.[2] Bacterial resistance to an antibiotic occurs when a population is exposed to a drug allowing only those that have naturally occurring resistance due to genetic mutations to remain and proliferate after treatment. These mutations can take many forms such as a modification of the antibiotic target, the synthesis of drug inactivating enzymes, or a complete bypass of a drugs target system.[3] The population that survives is thus immune to the effects of that antibiotic. Similarly, resistant bacteria are also able to transfer their resistance to other organisms via horizontal gene transfer (Figure 1).[3] These factors render known antibiotics obsolete.

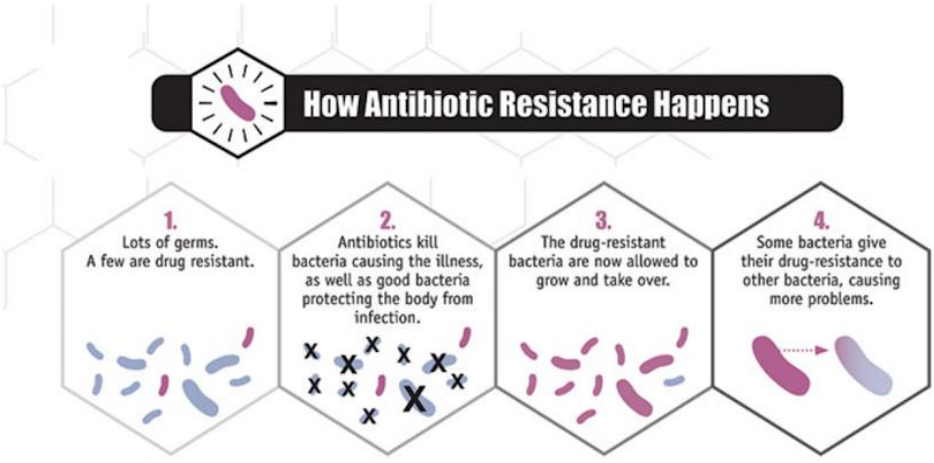


Figure 1. Mechanism of Antibiotic Resistance [3]

The World Health Organization (WHO) estimates that by 2050, 10 million lives and a total of $100 trillion (USD) will be lost annually if an effective method of controlling antibiotic resistance is not reached. The most urgent threats to public health are from infections caused by *Clostridium difficile* (*C. diff.)* and multidrug-resistant *Staphylococcus aureus* (MRSA), with over 29,000 and 20,000 annual deaths respectively.[3,4] Similarly, Gram-negative bacteria, such as *Escherichia coli*, are much more difficult to treat due to their lipopolysaccharide layer. However, regardless of bacterial species, this antibiotic resistance is further exacerbated by the presence of efflux pumps.

Efflux pumps are a category of transmembrane proteins which work to remove both endogenous metabolites, as well as exogenous toxins, such as antibiotics, from the cell. Although efflux pumps are over expressed in almost 44% of antibiotic resistant infections, a broad spectrum efflux pump inhibitor is not plausible due to the pump diversity within a single cell as well as the lack of similarities between Gram stains, e.g. NorA and Rv2686c–2687c–2688c efflux pumps only expressing themselves in Gram-positive and mycobacterium respectively.[11] However, when compounding the penetration problems associated with lipopolysaccharide layers with the overexpression of their AcrAB-TolC efflux pumps, Gram-negative bacteria become a necessary target.

AcrAB-TolC efflux pumps, like all efflux pumps, are membrane spanning proteins which work to remove both endogenous metabolites, as well as exogenous toxins, such as antibiotics, from the cell. AcrAB-TolC is composed of the outer membrane protein TolC, the periplasmic membrane fusion protein AcrA, and the inner membrane transporter AcrB, which cycles through three different conformational states during the drug export process (Figure 2).[12]

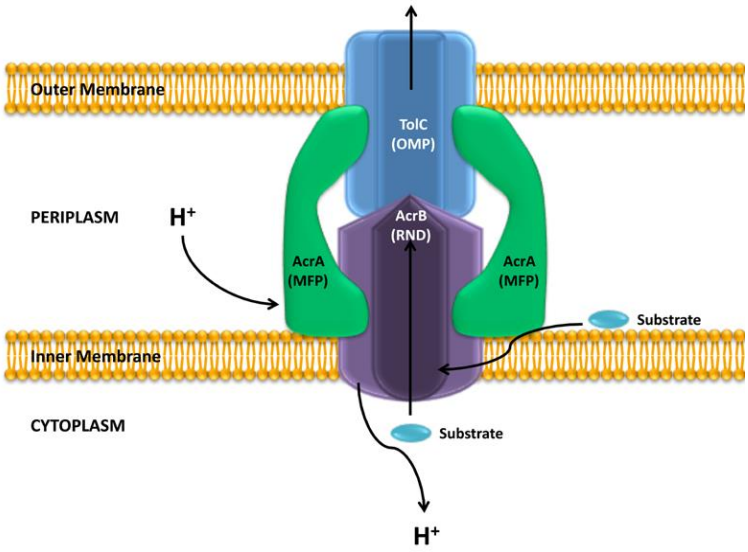


Figure 2. AcrAB-TolC Efflux Pumps

Because antibiotic resistance has stemmed from the abuse of current antibiotics, there is a push to embrace naturally occurring antimicrobial molecules in order to treat mutated infections, as there is little to no resistance present due to lack of clinical use. These compounds can be isolated from plants, adapted from indigenous medicine, or taken directly from bacterial isolates.[4] These techniques have been used in the discovery of compounds such as artemisinin, lysolipin, and, most notably, penicillin.[16] Various *Pseudomonas* species have been shown to produce bioactive α-pyrone compounds, which when isolated, exhibit moderate activity. These molecules have been deemed pseudopyronines A and B.

Pseudopyronines A and B (Figure 3) were discovered by Bauer et al. (2002) and Wyeth et al. (2003) by isolating compounds from *Pseudomonas fluorescens* and *Pseudomonas* sp. PB22.5 respectively.[5] These compounds were found to have antibacterial and anticancer properties (pseudopyronine B being more potent in both respects than pseudopyronine A), with some reports of insecticidal potential.[5,6] This is likely due to its mechanism of action - cell wall disruption and fatty acid synthase (FAS) II inhibition.[7] Due to the relatively short time since their discovery, and their limited bioactivity, pseudopyronines have yet to be fully adopted by the medical community. Thus it falls to medicinal chemists to optimize these compounds to better suit clinical applications.

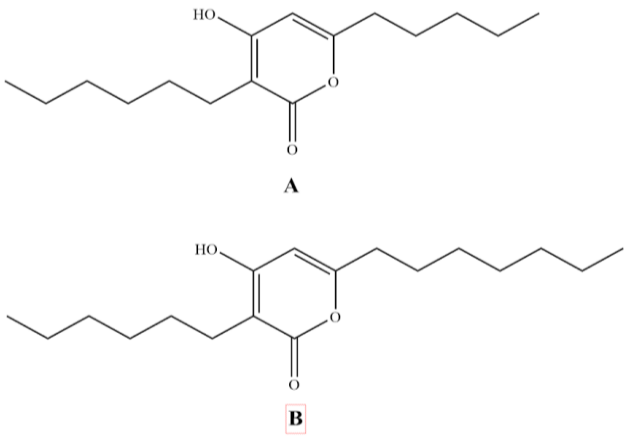


Figure 3. Pseudopyronines A and B Respectivley

Prior research conducted in the Wolfe research laboratory worked to synthesize derivatives of the pseudopyronines in order to determine how the alkyl side chain length of C3 and C6 affected the bioactivity of the compound. Sixteen analogs were produced by Bouthillette et al., while maintaining the α-pyrone core, and tested against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa*. The results indicated that shorter alkyl chain lengths correlated to greater activity against *E. coli*, while longer chain lengths were more active against *B. subtilis* and *S. aureus* (IC_{50} 0.1 μg/mL).[8] Furthermore, the Wolfe research laboratory discovered that increasing polarity of the compound, via the addition of electronegative groups to the C4 position, increased inhibition against *S. aureus*. A continuation of Bouthilliette's study conducted by Chappel, using a short-chain pseudopyronine B derivative, found that the C4 position contained optimization potential according to electronegativity[9]. The addition of electronegative motifs increased inhibition of both Gram-positive and Gram-negative bacteria.[10]

Furthermore, research conducted by Fields *et al.* showed the potential for pseudopyronine as an adjuvant therapy. It was shown that, when dosed with pseudopyronine, penicillin G recovered a significant portion of its antibiotic activity against *Pseudomonas aeruginosa*.[16] This increase in activity is attributed to pseudopyronines ability to interact with, and effectively block, the inner membrane protein of *P. aeruginosa* efflux pumps. This prevents the antibiotic from being shuttled out and allows it to interact with its intended target. This sort of efflux pump inhibition was also studied by Rath *et al.* who found that efflux pump inhibitors could be optimized with the addition of various L-amino acid ester substituents. It was determined that with the addition of L-amino acid esters, particularly one that are more sterically bulky such as Phe or Tyr, AcrAB-TolC efflux pumps in *Escherichia coli* could be obstructed.[11]

Building upon previous conclusions, this research seeks to substitute a short chain derivative of pseudopyronine, known to have moderate activity against *E. coli*. with L-amino acid esters in order to increase affinity for AcrAB-TolC efflux pumps. This will take place on the C4 and C5 positions of the α-pyrone ring, as these positions have been shown to be vital to biological activity (Figure 4***)***.[9]

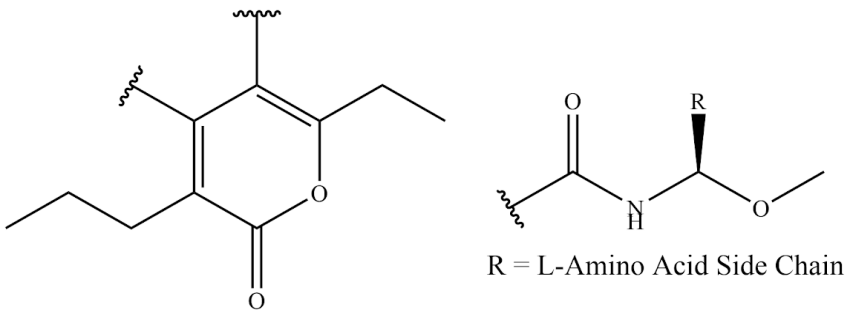


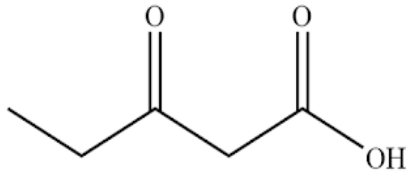
Figure 4. Proposed Amino Acid Substituted Pseudopyronine Analogs

In summation, pseudopyronine derivatives have shown the potential to assist in the epidemic of antibiotic resistance. Multiple studies have concluded that the drug can be optimized in order to enhance its innate ability as an efflux pump inhibitor, and thus, increase the biological activity of other antibacterial molecules. This research attempts to utilize amino acid ester substitution in order to determine what modifications are important in the functionality of the *α*-pyrone. This experimentation, when combined with cell death, and efflux pump, assay data,will assist in verifying this hypothesis.

2. Methodology

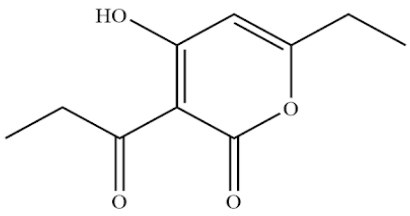
All solvents and reagents were purchased as described and used without further purification. Anhydrous solvents were obtained via an inert solvent system. Water is considered to be deionized unless otherwise stated. All water and air sensitive reactions were carried out under argon gas in a flame dried flask. Evaporation and concentration in vacuum was performed at 50 °C. ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Varian Inova NMR with an Oxford Instruments 400MHz superconducting magnet. Chemical shifts were given in ppm with patterns being expressed as singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m). IR and LCMS spectra were taken using a Thermo

Scientific Nicolet iS10 and Shimadzu LCMS-2020 respectively. LCMS data is expressed as *m/z* with *m* representing the ionized molecule. (EtoAc: ethyl acetate, Hex: hexane, DCM: dichloromethane, THF: tetrahydrofuran, MeOH: methanol, Et_2O: diethyl ether, EtOH: ethanol)



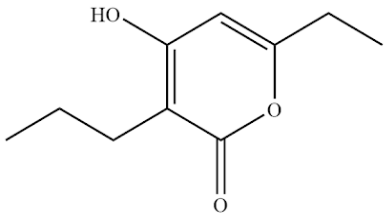
2.1 3-oxopentanoic acid (1)

Commercially available methyl-3-oxopentanoate (5.00g) was dissolved in a 1 to 1 ratio CH_3OH:H_2O (101 mL; 0.40 M, each) at 23 ºC. $NaOCH_3$ (30% w/v in CH_3OH) (2.78mL; 1.45 eq) was added dropwise and the reaction was left to stir for 18 hrs. Once completed reaction was concentrated under reduced pressure the acidified to pH<4 using 1N HCl (aq). The compound was then extracted using 500mL EtOAc (x3). The organic layers were subsequently washed with 750mL H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. Resulting 3-oxopentanoic acid was then further utilized without purification. **Yield:** 86% **^{1}H-NMR:** ($CDCl_3$, 400 MHz) 13.826 (s, 1H) 3.411 (s, 2H) 2.438 (q, 2H) 1.066 (t, 3H) **^{13}C-NMR:** ($CDCl_3$, 100 MHz) 206, 173, 51, 31, 8 **IR:** ν_{max} 3307, 3011, 2863, 2935, 2926, 2864, 2853, 1717, 1711, 1662, 1464, 1457, 1442, 1420, 1270, 1106, 918, 727 cm^{-1}



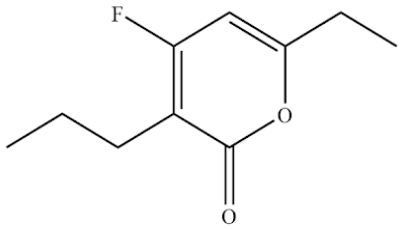
2.2 6-ethyl-4-hydroxy-3-propionyl-2H-pyran-2-one (2)

A solution of **1** (1.00g) in anhydrous THF (47.8mL; 0.18 M) was added to a flask under an inert atmosphere at 23 ºC. To this, CDI (1.89g; 1.36 eq) was added and the reaction was allowed to stir for 24 hrs. Upon completion, the reaction was concentrated under reduced pressure then acidified to pH<4 with 1N HCl (aq). The compound was extracted with 500mL of EtOAc, and the organic layers washed with 250mL of H_2O, before being dried over Na_2SO_4 and concentrated under reduced pressure. Crude mixture was then purified by recrystallization in CH_3OH to afford the desired 6-ethyl-4-hydroxy-3-propionyl-2H-pyran-2-one. **Yield:** 63% **^{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)**.^{13}C NMR:** ($CDCl_3$, 100 MHz) 208.4, 181.2, 173.6, 161.2, 99.9, 99.6, 35.4, 27.6, 10.5, 7.8. **IR:** ν_{max} 2975, 2940, 2882, 1732, 1638, 1558, 1441, 1391, 1283, 1219, 1152, 1074, 1014, 955, 913, 826 cm^{-1}



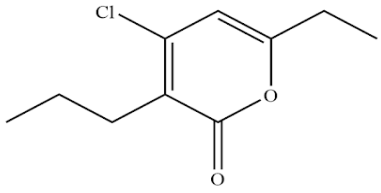
2.3 6-ethyl-4-hydroxy-3-propyl-2H-pyran-2-one (3)

To a stirred solution of **2** (1.00g) in anhydrous THF (28.3mL; 0.18 M) 2 M HCl (aq) (26.8mL; 0.19 M) was added and allowed to stir at 23 ºC. $NaCNBH_3$ (0.78g; 2.5 eq) was then added and the mixture was allowed to react for 3 hrs. Upon completion, the reaction was diluted with 250mL CH_2Cl_2 and washed with 200mL H_2O (x3). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. Flash chromatography (SiO_2, 25-40% EtOAc/Hex mobile phase) afforded 6-ethyl-4-hydroxy-3-propyl-2H-pyran-2-one. **Yield:** 77% . **^{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). **^{13}C NMR:** ($CDCl_3$, 100 MHz) 168.9, 168.1, 164.8, 103.4, 100.5, 26.9, 25.1, 21.5, 14.2, 11.1. **IR:** ν_{max} 3099, 2962, 2931, 2874, 2658, 1663, 1575, 1463, 1438, 1408, 1303, 1267, 1245, 1170, 1126, 1092, 1012, 987, 939, 908, 840, 733 cm^{-1}



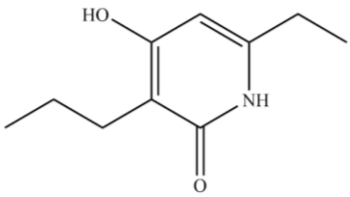
2.4 6-ethyl-4-fluoro-3-propyl-2H-pyran-2-one (4)

Product **3** (0.5g) was reacted with NaF (0.23g; 2 eq) in THF (30.5mL; 0.18 M). A catalytic amount of H_2SO_4 (aq) was added and reaction was allowed to stir at 23 ºC overnight. Once complete, the reaction was diluted with 200mL of EtOAc and washed with 400mL of H_2O. Organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. Crude mixture was then purified via flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) to afford 6-ethyl-4-fluoro-3-propyl-2H-pyran-2-one. **Yield:** 89% **^{1}H-NMR:** (CDCl3-dl 400MHz) 5.938 (s, 1H) 2.528 (t, 2H) 2.4873 (t 2H) 1.647 (t, 2H) 1.310 (t, 2H) 0.893 (t, 3H) **^{13}C-NMR:** ($CDCl_3$, 100 MHz) 165, 162, 157, 102, 101, 30, 22, 20, 14, 12 **IR:** ν_{max} 3029, 2918, 2852, 1735, 1731, 1680, 1603, 1457, 1387, 1101, 844. 815 cm^{-1}



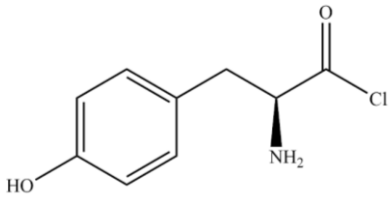
2.5 4-chloro-6-ethyl-3-propyl-2H-pyran-2-one (5)

$POCl_3$ (7.68mL; 10 eq) was added to **3** (1.5g) and allowed to stir at reflux for 4 hrs, after which the reaction was extracted with 300mL of Et_2O, washed with saturated 500mL of $NaHCO_3$, and condensed under reduced pressure. Product was isolated by recrystallization in CH_3OH to yield 4-chloro-6-ethyl-3-propyl-2H-pyran-2-one. **Yield:** 74% **^{1}H-NMR:** (CDCl3-dl 400MHz) 5.928 (s, 1H) 2.508 (t, 2H) 2.488 (t 2H) 1.657 (t, 2H) 1.321 (t, 2H) 0.895 (t, 3H) **^{13}C-NMR:** ($CDCl_3$, 100 MHz) 165, 159, 147, 129, 105, 30, 22, 20, 14, 12 **IR:** ν_{max} 3029, 2918, 2852, 1735, 1731, 1680, 1603, 1457, 1387, 1218, 859, 819 709 cm^{-1}



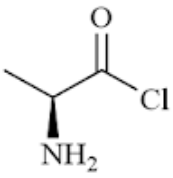
2.6 6-ethyl-4-hydroxy-3-propylpyridin-2(1H)-one (6)

Concentrated NH_3 (aq) (0.69mL; 13 eq) was combined with product **3** (0.5g) and allowed to reflux for 4 hrs. Once complete, reaction was extracted with 150mL of EtOAC and 150mL of H_2O then washed with 500mL of saturated NaCl (aq). The organic layers were combined and dried over Na_2SO_4 before condensed under reduced pressure. The crude mixture was purified via flash chromatography (SiO_2, 25% EtOAc/Hex mobile phase) to isolate 6-ethyl-4-hydroxy-3-propylpyridin-2(1H)-one. **Yield: 94% ^{1}H-NMR:** (CDCl3-dl 400 MHz) 5.928 (s, 1H) 7.200 (s, 1H) 2.508 (t, 2H) 2.488 (t 2H) 1.657 (t, 2H) 1.321 (t, 2H) 0.895 (t, 3H) **^{13}C-NMR:** ($CDCl_3$, 100 MHz) 167, 157, 155, 111, 102, 27, 22, 22, 13, 11 **IR:** ν_{max} 3300, 3164, 3032, 2929, 2867, 1680, 1651, 1599, 1457, 1372, 1339, 1252, 1054, 823 cm^{-1}



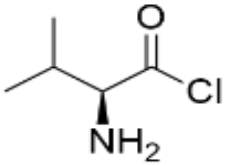
2.7 L-tyrosinoyl chloride (7.a)

Commercially available L-tyrosine methyl ester (2.00g) was added to a 1 to 1 solution of EtOH:H_2O (29.96mL; 0.38M each) and NaOH (0.62g; 2eq) and allowed to stir at 60 ^oC overnight. The next day, the reaction was acidified to pH<2 using 1N HCl (aq), extracted with 100mL of Et_2O and washed with 250mL of saturated NaCl (aq),. Product was dried over Na_2SO_4 and condensed under reduced pressure before being diluted with 200mL of DCM. To this flask, three drops of $SOCl_2$ were added and the reaction was allowed to stir, at reflux, for 2 hrs. Once complete, the mixture was washed with 500mL of saturated sodium bicarbonate followed by 300mL of H_2O before being condensed under reduced pressure to isolate L-tyrosinoyl chloride. No further purification required. **Yield:** 93**% ^{1}H-NMR:** (CDCl3-dl 400 MHz) 9.062 (s, 1H) 8.864 (s, 2H) 6.969 (t, 2H) 6.682 (d, 2H) 3.870 (t, 1H) 3.326 (m, 2H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 174, 155, 130, 129, 116, 74, 41 **IR:** ν_{max} 3369, 3281, 3270, 3043, 2930, 2896, 2849, 1614, 1603, 1500, 1482, 1464, 1372, 1339, 1259, 1208, 1182, 1112, 1101, 1042, 1013, 984, 822, 804, 709, 691 cm^{-1}



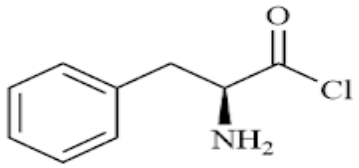
2.8 L-alaninol chloride (7.b)

Commercially available L-alanine methyl ester (1.00g) was added to a 1 to 1 solution of EtOH:H_2O (18.96mL; 0.38M each) andNaOH (0.572g; 2eq) and allowed to stir at 60 ^oC overnight. The next day, the reaction was acidified to pH<2 using 1N HCl (aq), extracted with 100mL of Et_2O and washed with 250mL of saturated NaCl (aq),. Product was dried over Na_2SO_4 and condensed under reduced pressure before being diluted with 200mL of DCM. To this flask, $SOCl_2$ (1.56mL; 3eq) was added and the reaction was allowed to stir, at reflux, for 2 hrs. Once complete, the mixture was washed with 500mL of saturated sodium bicarbonate followed by 300mL of H_2O before being condensed under reduced pressure to isolate L-alaninol chloride. No further purification required. **Yield:** 60**%** **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.763 (s, 2H) 3.612 (q, 1H) 1.231 (d, 3H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 173.6, 68.2, 20.6 **IR:** ν_{max} 3366, 3289, 2970, 2889, 2874, 1625, 1456, 1376, 1339, 1101, 1042, 804, 724 cm^{-1}



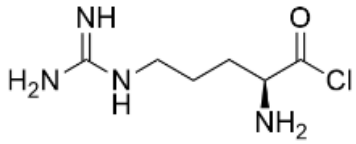
2.9 L-valinoyl chloride (7.c)

Commercially available L-valine methyl ester (1.00g) was added to a 1 to 1 solution of EtOH:H_2O (20.06mL; 0.38M each) andNaOH (0.61g; 2eq) and allowed to stir at 60 ^oC overnight. The next day, the reaction was acidified to pH<2 using 1N HCl (aq), extracted with 100mL of Et_2O and washed with 250mL of saturated NaCl (aq),. Product was dried over Na_2SO_4 and condensed under reduced pressure before being diluted with 200mL of DCM. To this flask, $SOCl_2$ (1.83mL; 3eq) was added and the reaction was allowed to stir, at reflux, for 2 hrs. Once complete, the mixture was washed with 500mL of saturated sodium bicarbonate followed by 300mL of H_2O before being condensed under reduced pressure to isolate L-alaninol chloride. No further purification required. **Yield:** 76**%** **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.764 (s, 2H) 3.294 (d, 1H) 1.976 (m, 1H) 0.958 (d, 6H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 173.6, 78.4, 33.1, 18.1 **IR:** ν_{max} 3366, 3292, 2959, 2893, 2867, 1610, 1460, 1379, 1332, 1149, 1116, 1039, 804, 713 cm^{-1}



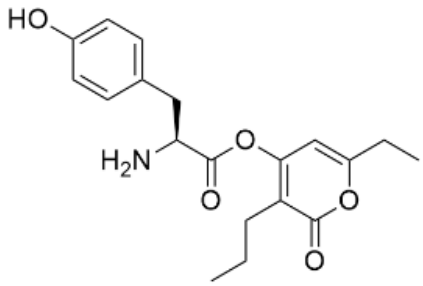
2.10 L-phenylalaninol chloride (7.d)

Commercially available L-phenylalanine methyl ester (1.00g) was added to a 1 to 1 solution of EtOH:H_2O (14.7mL; 0.38M each) and NaOH (0.45g; 2eq) and allowed to stir at 60 ^oC overnight. The next day, the reaction was acidified to pH<2 using 1N HCl (aq), extracted with 100mL of Et_2O and washed with 250mL of saturated NaCl (aq),. Product was dried over Na_2SO_4 and condensed under reduced pressure before being diluted with 200mL of DCM. To this flask, $SOCl_2$ (1.26mL; 3eq) was added and the reaction was allowed to stir, at reflux, for 2 hrs. Once complete, the mixture was washed with 500mL of saturated sodium bicarbonate followed by 300mL of H_2O before being condensed under reduced pressure to isolate L-phenylalaninol chloride. No further purification required. **Yield:** 74**%** **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.863 (s, 2H) 7.166 (m, 5H) 3.873 (t, 1H) 3.282 (m, 2H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 174, 136, 129, 128, 126, 74, 41 **IR:** ν_{max} 3362, 3274, 3044, 2934, 2893, 2838, 1611, 1577, 1504, 1464, 1449, 1339, 1237, 1178, 1152, 1108, 1075, 1039, 991, 881, 801, 749, 713, 698 cm^{-1}



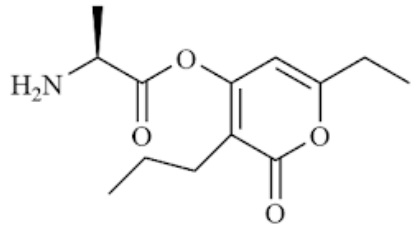
2.11 L-argininoyl chloride (7.e)

Commercially available L-arginine methyl ester (1.00g) was added to a 1 to 1 solution of EtOH:H_2O (13.9mL; 0.38M each) and NaOH (1.11g; 2eq) and allowed to stir at 60 ^oC overnight. The next day, the reaction was acidified to pH<2 using 1N HCl (aq), extracted with 100mL of Et_2O and washed with 250mL of saturated NaCl (aq),. Product was dried over Na_2SO_4 and condensed under reduced pressure before being diluted with 200mL of DCM. To this flask, $SOCl_2$ (3.02mL; 3eq) was added and the reaction was allowed to stir, at reflux, for 2 hrs. Once complete, the mixture was washed with 500mL of saturated sodium bicarbonate followed by 300mL of H_2O before being condensed under reduced pressure to isolate L-phenylalaninol chloride. No further purification required. **Yield:** 68**%** **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.763 (s, 2H) 7.825 (s, 1H) 6.634 (s, 2H) 3.410 (m, 1H) 2.275 (s, 1H) 1.783 (q, 2H) 1.493 (m, 2H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 173.6, 158.6, 71.4, 41.6, 31.2, 23.8 **IR:** ν_{max} 3369, 3289, 2922, 2882, 2856, 1625, 1460, 1325, 1101, 1039, 804, 716 cm^{-1}



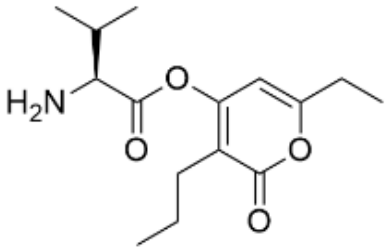
2.12 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl L-tyrosinate (8.a)

Compound **3** (1.00g) was dissolved in anhydrous DCM (22.04mL; 0.21M) and left to cool in an ice bath. Once the reaction reached 0 ºC Et_3N (0.83mL; 2eq) and DMAP (0.42g; 1.20eq) were added to the flask, followed by compound **7.a** (0.57g; 1.2eq). The reaction was then taken off ice and allowed to rise to room temperature over the course of 16 hrs. Once complete, the DCM was removed under reduced pressure and the resulting solid was redissolved in EtoAc. The solution was then washed with 25mL of 1N HCl, 25mL of saturated sodium bicarbonate, and 250mL of brine in succession before being dried over sodium sulfate and condensed under reduced pressure. The resulting product was then purified using flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) in order to isolate the desired product **Yield:** 39**% ^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.712 (s, 2H) 6.958 (t, 2H) 6.673 (d, 2H) 6.217 (s, 1H) 4.156 (t, 1H) 3.423 (m, 1H) 3.178 (m, 1H) 2.413 (t, 2H) 2.001 (q, 2H) 1.362 (m, 2H) 1.026 (m, 6H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 168.1, 166.6, 165.2, 158.3, 155.6, 130.1, 129.6, 115.3, 110.8, 99.7, 55.4, 37.6, 30.0, 22.2, 21.7, 14.1, 10.9 **IR:** ν_{max} 3370, 3301, 3265, 3021, 2964, 2922, 2880, 2859, 1674, 1623, 1602, 1466, 1373, 1367, 1331, 1217, 1118, 1045, 814 cm^{-1}



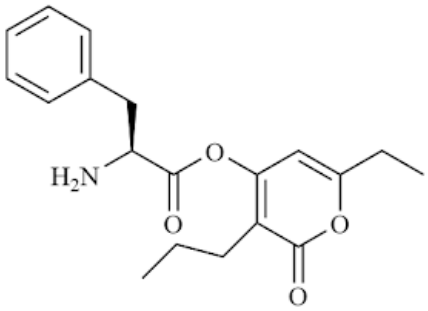
2.13 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl L-alaninate (8.b)

Compound **3** (1.00g) was dissolved in anhydrous DCM (26.09mL; 0.21M) and left to cool in an ice bath. Once the reaction reached 0 ºC Et_3N (1.53mL; 2eq) and DMAP (0.802g; 1.20eq) were added to the flask, followed by compound **7.b** (0.917g; 1.2eq). The reaction was then taken off ice and allowed to rise to room temperature over the course of 16 hrs. Once complete, the DCM was removed under reduced pressure and the resulting solid was redissolved in EtoAc. The solution was then washed with 25mL of 1N HCl, 25mL of saturated sodium bicarbonate, and 250mL of brine in succession before being dried over sodium sulfate and condensed under reduced pressure. The resulting product was then purified using flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) in order to isolate the desired product. **Yield:** 45% **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.762 (s, 2H) 6.227 (s, 1H) 3.574 (q, 1H) 2.231 (t, 2H) 2.038 (q, 2H) 1.375 (m, 2H) 1.284 (d, 3H) 1.017 (m, 6H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 168, 167,165, 110, 99, 49, 31, 22, 18, 14, 11 **IR:** ν_{max} 3355, 3285, 3021, 2948, 2882, 2863, 1732, 1676, 1618, 1460, 1372, 1339, 1229, 1119, 1031, 855, 790 cm^{-1}



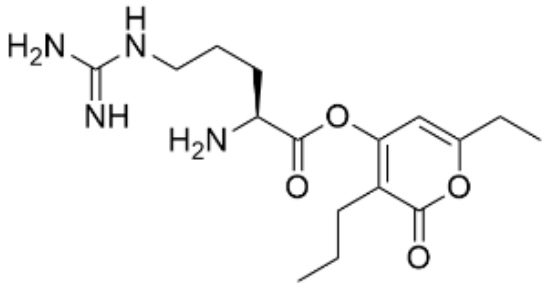
2.14 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl L-valinate (8.c)

Compound **3** (1.00g) was dissolved in anhydrous DCM (23.47mL; 0.21M) and left to cool in an ice bath. Once the reaction reached 0 ºC Et_3N (1.88mL; 2eq) and DMAP (0.76g; 1.20eq) were added to the flask, followed by compound **7.c** (1.05g; 1.2eq). The reaction was then taken off ice and allowed to rise to room temperature over the course of 16 hrs. Once complete, the DCM was removed under reduced pressure and the resulting solid was redissolved in EtoAc. The solution was then washed with 25mL of 1N HCl, 25mL of saturated sodium bicarbonate, and 250mL of brine in succession before being dried over sodium sulfate and condensed under reduced pressure. The resulting product was then purified using flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) in order to isolate the desired product. **Yield:** 45% **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.764 (s, 2H) 6.216 (s, 1H) 4.227 (d, 1H) 2.405 (m, 3H) 2.080 (q, 2H) 1.357 (m, 2H) 1.102 (d, 3H) 0.978 (m, 9H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 168.3, 166.7, 166.2, 158.7, 111.8, 100.1, 59.9, 30.4, 29.8, 22.1, 18.9, 14.1, 11.0 **IR:** ν_{max} 3361, 3295, 3028, 2961, 2922, 2854, 1674, 1614, 1460, 1452, 1379, 1334, 1139, 1099, 1048, 799 cm^{-1}



2.15 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl L-phenylalaninate (8.d)

Compound **3** (1.00g) was dissolved in anhydrous DCM (18.33mL; 0.21M) and left to cool in an ice bath. Once the reaction reached 0 ºC Et_3N (0.65mL; 2eq) and DMAP (0..38g; 1.20eq) were added to the flask, followed by compound **7.c** (0.86g; 1.2eq). The reaction was then taken off ice and allowed to rise to room temperature over the course of 16 hrs. Once complete, the DCM was removed under reduced pressure and the resulting solid was redissolved in EtoAc. The solution was then washed with 25mL of 1N HCl, 25mL of saturated sodium bicarbonate, and 250mL of brine in succession before being dried over sodium sulfate and condensed under reduced pressure. The resulting product was then purified using flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) in order to isolate the desired product. **Yield:** 33% **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.713 (s, 2H) 7.256 (m, 5H) 6.218 (s, 1H) 4.376 (t, 1H) 2.734 (d, 2H) 2.417 (t, 2H) 2.183 (q, 2H) 1.367 (m, 2H) 1.034 (m, 6H) **^{13}C-NMR: (**$CDCl_3$, 100 MHz) 168, 166, 163, 142, 128, 127, 126, 111, 101, 55, 38, 30, 22, 21, 14, 11 **IR:** ν_{max} 3359, 3296, 3036, 2937, 2900, 2863, 1731, 1673, 1629, 1467, 1383, 1226, 1123, 1042, 999, 877, 808, 694 cm^{-1}



2.16 6-ethyl-2-oxo-3-propyl-2H-pyran-4-yl L-argininate (8.e)

Compound **3** (1.00g) was dissolved in anhydrous DCM (16.83mL; 0.21M) and left to cool in an ice bath. Once the reaction reached 0 ^{o}C Et_3N (0.53mL; 2eq) and DMAP (0.32g; 1.20eq) were added to the flask, followed by compound **7.c** (0.54g; 1.2eq). The reaction was then taken off ice and allowed to rise to room temperature over the course of 16 hrs. Once complete, the DCM was removed under reduced pressure and the resulting solid was redissolved in EtoAc. The solution was then washed with 25mL of 1N HCl, 25mL of saturated sodium bicarbonate, and 250mL of brine in succession before being dried over sodium sulfate and condensed under reduced pressure. The resulting product was then purified using flash chromatography (SiO_2, 40% EtOAc/Hex mobile phase) in order to isolate the desired product. **Yield:** 43% **^{1}H-NMR:** (CDCl3-dl 400 MHz) 8.763 (s, 2H) 7.793 (s, 1H) 6.622 (s, 2H) 6.219 (s, 1H) 3.337 (m, 3H) 2.487 (s, 1H) 2.417 (t, 2H) 2.013 (q, 2H) 1.757 (q, 2H) 1.512 (m, 2H) 1.348 (m, 2H) 1.020 (m, 6H) **^{13}C-NMR:** **(**$CDCl_3$, 100 MHz) 167.9, 166.9, 165.3, 158.8, 158.1, 110.3, 99.8, 53.6, 41.6, 30.0, 28.7, 24.6, 22.4, 20.9, 14.3, 11.5 **IR:** ν_{max} 3367, 3295, 3027, 2952, 2889, 2871, 1725, 1680, 1623, 1463, 1382, 1334, 1223, 1108, 1036, 823 cm^{-1}

3. Bacterial Inhibition Assays

Once synthesized, select compounds were added, in concentrations ranging from 1000 μg/mL to 0.001 μg/mL in a DMSO solution, to a 96 well master plate. Each compound was then tested twice against both *Escherichia coli* and *Staphylococcus aureus* using chloramphenicol and DMSO as a positive and negative control, respectively. The *E. coli* and *S. aureus* were grown in a tryptic soy broth solution and were shaken overnight at 25 ^{o}C. They were then introduced to the analogs and controls and left to shake overnight at 25 ^{o}C. Measured absorbances of each well was obtained with a Synergy HTX plate reader at 590 nm.

Compounds **8a-e** were also subject to an adjuvant assay, wherein the compounds were added to a 96 well plate, at a 10mM concentration in DMSO, alongside varying concentrations of either penicillin or erythromycin (0-64 mg/mL) in a DMSO solution. Adjuvant activity was then tested against *E. coli* using pure TSB and DMSO as positive and negative controls, respectively. Tests were performed in the absence of synthesized compounds in order to account for any activity contributed by the antibiotics. The *E. coli* was grown in a tryptic soy broth solution and was shaken overnight at 25 ^{o}C. It was then introduced to the analogs, antibiotics, and controls and left to shake overnight at 25 ^{o}C. Measured absorbances of each well was obtained with a Synergy HTX plate reader at 590

4. Results and Discussion

The complete synthetic scheme for amino acid ester substituted pseudopyronine derivatives is reported below. The parent molecule,a pseudopyronine derivative, can be synthesized in three steps and the amino acid ester in two. To date, only 5 amino acid ester derivatives have been synthesized, Tyr (**8a),** Ala (**8b**), Val (**8c**), Phe (**8d**), and Arg (**8e**), however the benefit of this technique is its versatility and simplicity which allows for the quick production of many analogs. Any commercially available L-amino acid methyl ester can be utilized, without alteration to the procedure, in order to quickly produce analogs. These analogs are produced in moderate yield with the only problem being

solubility of compounds **3** and **7**, which each required sonication for further synthesis. This could potentially be remedied by using DMSO as a solvent in order to increase overall solubility.

As expected, the larger amino acid substituents worked to more effectively inhibit bacterial growth of both *E. coli* and *S. aureus*. Furthermore, it was discovered that hydrophobicity played an integral role in the activity of the compound. This is likely due to the vast amount of hydrophobic residues within efflux pumps which added to the affinity of hydrophobic analogs.

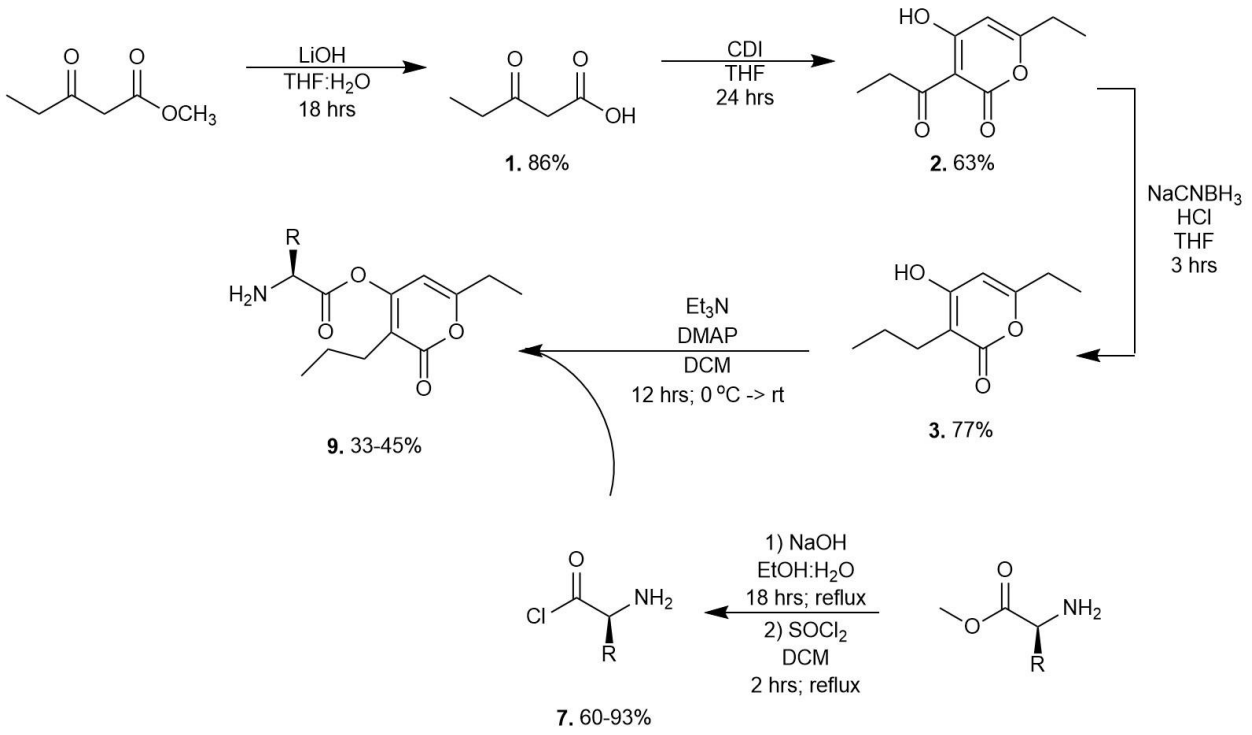


Figure 5. Complete Synthesis of Amino Acid Ester Substituted Pseudopyronine Analogs

Table 1. $1C_{50}$ values of amino acid derivative pseudopyronine analogs against both Gram-positive and Gram-negative bacteria

Analog	*Staphylococcus aureus* IC_{50} (μg/mL)	*Escherichia coli* IC_{50} (μg/mL)
8a	84.5	129.89
8b	21.7	11.9
8c	0.35	0.32
8d	8.9	8.3
8e	0.24	86.6

Furthermore, when dosed adjuvantly to known antibiotics, compounds **8a-b** and **8d-e** showed moderate activity when dosed at a 10mM concentration with erythromycin and compounds **8c-e** showed moderate activity when dosed at a 10mM concentration with penicillin. These findings indicate that the amino acid ester pseudopyronine derivative

holds promise, not just as independent antibiotics, but also as adjuvant therapies in order to prolong the life of current drugs.

Future work will look at substituting the 4' C of the α-pyrone core with all 20 essential L-methyl ester amino acids before doing the same on the 5' C.[8] All of these compounds will be subsequently assessed in a bacterial cell death assay in which it is expected the more massive analogs will show the most activity due to their steric bulk.

5. References

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