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# Optimization and Characterization of Antibiotic Compounds Produced by Bacteria

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## Abstract

Increasing bacterial resistance to current antibiotic treatments poses a huge threat to the health of the human population. Over the past 20 years, there has been a significant decline in the production of new antibiotics, despite the rapid emergence of drug resistant pathogens. The isolation and extraction of structurally unique antibiotics from bacteria remains largely uninvestigated by current researchers. Natural products, however, are an abundant source of structurally diverse compounds with antibacterial activity that can be used to develop new and potent antibiotics. This research investigates techniques for determining optimal growth media and optimal time of antibiotic production for each bacteria sample from a library of bacteria species, as well as the extraction of antibiotic active compounds from bacteria. In addition, this research also explores the isolation and extraction of the compound pseudopyronine B, from a *Pseudomonas* species found in garden soil in Western North Carolina, and SAR evaluation of C3 and C6 alkyl analogs of the natural product for antibacterial activity against Gram-positive and Gram-negative bacteria. A direct relationship between antibacterial activity and C3/C6 alkyl chain length was observed. For inhibition of Gram-positive bacteria, alkyl chain lengths between 6-8 carbons were found to be the most active ($IC_{50}$ = 0.04-3.8 μg/mL) and short alkyl chain analogs showed modest activity against Gram-negative bacteria ($IC_{50}$ = 222-303 μg/mL).

## 1. Introduction

Due to the widespread misuse and overuse of antibiotics in agriculture and medicine there has been a dramatic increase in the number of antibiotic resistant bacterial infections, especially those caused by the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) over the past two decades, and have been recognized as one of the leading causes of death around the world.[1, 2] Unfortunately, as these infections are continuously rising, antibiotic development has acutely declined. There is a constant need for the development of novel antibiotics with unique structures and mechanisms of action against these pathogens, especially against Gram-negative pathogens that are notoriously difficult to treat. The longer society goes without new antibiotics, the more bacteria will continue developing resistances to current effective drugs thus facilitating the potential for multidrug resistant pathogens to arise. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) has been increasing and was responsible for roughly 18,000 deaths in the United States alone in 2011.[1-4] The demand for the development of broad spectrum antibacterial agents are at an all time high, but only two new and successful classes of antibacterial drugs have been discovered and brought to market in the last thirty years. Natural products, however, have been the foundation of antibiotic development since the ‘‘golden age” of antibiotic discovery in the 1940s due to their unique and often complex structures.[1, 5-8]

Natural products are compounds produced by organisms in nature that often have unique and complex structures, making them challenging to synthesize in a laboratory setting. Bacterial secondary metabolites, in particular, have been and continue to be a rich source of novel compounds with a wide range of biological activity.[1, 5-8] Secondary

metabolites are compounds that are not vital to the survival of the organism and are excreted naturally through biological processes. Some secondary metabolites have shown to have antibiotic properties, making them a target of interest. Secondary metabolites can be produced in one of two ways, either asocially or socially. Asocial production is when the organism readily produces active compounds when in the presence of what is called a target strain, or pathogen. Social production is when the organism cannot produce asocially but production can be induced when in close proximity to another organism, called a modifier strain.[9]

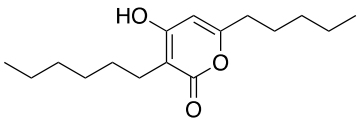
There are two classes of bacteria identified as Gram-positive and Gram-negative bacteria. Efforts have been mostly focused on Gram-positive bacteria treatments, especially MRSA in particular. There are currently a substantial amount for treatments for Gram-positive pathogens, and even some antibiotics that treat resistant strains.[10] Conversely, there are significantly fewer treatments for Gram-negative pathogens, let alone resistant strains. The difficulty of treating Gram-negative bacterial infections has been associated with the presence of an outer lipid membrane surrounding a thin peptidoglycan cell wall, preventing compounds from penetrating the cell membrane. Gram-positive cells lack an outer lipid membrane and have a thick and exposed peptidoglycan cell wall, allowing compounds to enter the cell membrane more easily.[10] The proposed research project will be looking at the activity of different compounds against a panel of pathogens including *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*) as Gram-positive targets and *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) as Gram-negative targets.

Previous work done by the Wolfe research group had been in collaboration with Dr. Sarah Seaton formally in the biology department at University of North Carolina Asheville. The Seaton group collected a library of approximately 400 bacteria samples from water in pitcher plants and soil native to Western North Carolina, as well as from soil of the southwestern United States. The bacteria samples were initially screened for activity using agar diffusion assays and qualitative visualization techniques, which are prone to complications including the appearance of false positives and difficulties in reproducibility. The entire library of bacteria has now been screened against both *S. aureus* and *E. Coli* in a liquid antibiotic assay. All bacteria samples have subsequently been characterized as strong producers (for either *S. aureus, E. coli,* or both), moderate producers (for either *S. aureus, E. coli,* or both), or non-producer through a series of quantitative statistical analysis studies. The Wolfe research group is currently working on extracting, isolating, and characterizing antibiotic active compounds first from the bacteria characterized as strong producers, followed by moderate producers.

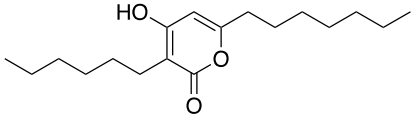
The overall goal of the proposed research project is to isolate new natural occurring antibiotics that contain structures unique to the current antibiotic catalog. It is important to find new compounds with different structures than those of existing antibiotics because if a bacterium is resistant to one drug, it will be resistant to all the drugs with a similar structure. Additionally, it is important to understand under what environmental conditions natural products are secreted. This research explores methods for optimizing growth and antibiotic producing conditions, the extraction and isolation of active compounds from bacteria liquid cultures, and evaluation of the Structure Activity Relationship (SAR) of synthesized analogs of an α-pyrone compound, pseudopyronine B, successfully isolated and characterized from bacteria.

Many microorganisms produce the α-Pyrone class of natural products, including the pseudopyronines, as both biosynthetic precursors and secondary metabolites. α-Pyrones, both natural and synthetic, have been found to possess a wide range of medicinal properties including antibiotic, antifungal, cytotoxic, and antiatherosclerotic activity, making them highly desirable targets for drug development.[11-15] For this reason, their syntheses have been thoroughly explored and are continually being diversified to rapidly access the large number of a-pyrone possessing natural products to allow for further biological evaluation.[16-21]

Figure 1. Pseudopyronines A and B.




pseudopyronine A **(1)**




pseudopyronine B

Pseudopyronine A and B (**Fig. 1**) are produced by phylogentically diverse and geographically distinct bacterial species, including various rhizosphere-associated *Pseudomonas*[22,23] and *Photorhabdus* species[24], marine sponge-associated *Pseudomonas*[25,26], and a marine *Alteromonas*[27], among others. The pseudopyronines were first isolated and structurally elucidated in 2002[22] and 2003[25] and were found to be produced by the action of a PpyS ketosynthase.[28] The pseudopyronines have also been found to have antibacterial[25,28] (against both resistant and nonresistant Gram-positive bacteria), algicidal ($LC_{50}$ = 0.8– 2.0 mg/mL) [27], antimalarial ($IC_{50}$ = 14 mg/mL), and antituberculosis (pseudopyronine B MIC = 2.7 mM) [19,29] properties. The mechanism of action for the pseudopyronines' antimicrobial activity involves selective membrane disruption[25] and inhibition of fatty-acid synthase (FAS) II.[19] Although some evaluation of the antimicrobial activity of pseudopyronines[12-15,19,29] has been performed, no studies to date have evaluated the antibacterial SAR profile of the C3 and C6 alkyl chain lengths, nor have they allowed for the development of pseudopyronine analogs with antibacterial activity against Gram-negative pathogens.[1]

## 2. Methods and Experimental

### 2.1 General Optimization and Culture Techniques

All procedures involving the use of bacteria were done under sterile conditions using sterilized equipment and materials unless otherwise stated. In order to grow bacteria in large-scale liquid culture, the culture media and growth time needed to be optimized. The goal was to find a way to screen all of the bacteria from the collected library and have repeatable quantitative results oppose to qualitative results that have been done in prior literature. A 96-well plate liquid assay technique was developed to screen bacteria in multiple minimal medias to determine which it grows best in. Minimal media is used to provide enough nutrients for the bacteria to proliferate but still inducing a stressful environment were the antibiotic compounds would be released. However, the minimal media the bacteria grow best in may not promote the most antibiotic production. The top two minimal medias are then chosen and subjected to a time trial screen to determine at what point in time is the bacteria producing the most antibiotic active compounds and in what media. Another 96-well plate liquid assay was designed to test the activity of the bacteria in minimal media at incremental points in time. These combined assays give insight into the optimal growing conditions for the bacteria of interest, which the information is then used to precede with large-scale cultures and extractions.

Once the growth conditions are optimized, the bacteria was grown on a large-scale ranging anywhere from 1-12 L. The bacteria were grown for the ideal amount of time as determined by the time trial assay. After the allotted time, the cultures were centrifuged and the supernatant was collected and extracted from by organic solvents. Crude extracts are then tested for activity against either *S. aureus* or *E. coli*, depending on the activity results from the bacteria's preliminary screen, in either a 96-well plate assay or a solid agar diffusion assay. If crude extract retained activity, then it was purified via normal phase column chromatography. Purified fractions are tested for activity against its respective pathogen. Activity of fraction indicates the target compound and is amplified by repeating this process. When a substantial amount of target compound is isolated, characterization techniques are used to identify the compound's structure.

### 2.2 Minimal Media Screening Assay

To determine the optimal growth media for antibiotic production, bacteria were screened in a minimal media (0.35 M $K_2HPO_4$; 0.22 M $KH_2PO_4$; 0.08 M $(NH_4)_2SO_4$; 200 mM $MgSO_4$) supplemented with either sodium acetate (12.5 mM), sodium citrate (12.5 mM), D-glucose (12.5 mM), or sodium succinate (25 mM). The bacteria were initially cultured in 10% tryptic soy broth (TSB) media for 24 h. The liquid cultures of bacteria were pipetted into a 96-well plate (10 μL) with each minimal media (140 μL) in different columns. The plate is put into the BioTek plate reader that continuously measured the absorbance at 590 nm over the course of 72 h. After 72 h, the contents of the wells were pipetted and transferred into a 96-well filter plate and centrifuged at 2000 g for 5 minutes at 23 °C. The supernatant was collected in a new plate and tested for activity against *S. aureus*. Full strength tryptic soy broth (FS TSB; 100 μL) and *S. aureus* (10 μL) were added to the 96-well plate and incubated at 37 °C for 18 h. After 18 h, the absorbances are read at 590 nm using the BioTek plate reader. The wells with the lowest absorbance indicate the optimal media for antibiotic production and the top two medias for each bacterium are subjected to a time trial assay (Table 1).

## 2.3 Time Trial Assay

To determine the point in time where the bacteria were producing the most antibiotic compound in the minimal media, time trial assays were done. The bacteria were inoculated in the top two minimal medias of interest from the minimal media assay and put in the rotatory shaker. After 24 h, 48 h, 72 h, and 96 h, 1 mL samples were removed and filtered through a 0.22 μm filter. The samples were concentrated then re-dissolved in FS TSB (50 μL) and loaded into a 96-well plate along with FS TSB (130-220 μL) and *S. aureus* (20-30 μL). The plate was incubated at 37 °C for up to 18 h. After 18 h, the absorbance was read at 590 nm using the BioTek plate reader. The optimal minimal media and duration of growth for each bacterium are determined (Table 1).

## 2.4 Extraction of SS 616

SS 616 was characterized to be a strong producer against both Gram-positive *S. aureus* and Gram-negative *E. coli* in preliminary screenings. The optimal growth and antibiotic producing conditions for SS 616 were determined to be minimal media supplemented with sodium succinate (25 mM) and grown for 72 h. Sequential-scale ups of liquid culture were done by inoculating one colony of bacteria in 10 mL of 10% TSB and grown in the rotatory shaker for 24 h. This was then transferred into 300 mL of 25 mM sodium succinate and grown in the rotatory shaker for 24 h. This was then transferred into 3 L of 25 mM sodium succinate and grown in the rotatory shaker for 72 h. Large-scale cultures ranged from 9-12 L following the sequential scale up. After 72 h in the rotatory shaker, the large-scale cultures were centrifuged at 4000 rpm for 12 minutes at 23 °C. The supernatant was collected and extracted with hexane (1x) and ethyl acetate (2x). The organic extracts were combined, washed with saturated aqueous NaCl, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The crude extract has yet to be tested for activity and has not been purified.

## 2.5 Extraction and isolation of an unknown antibiotic compound from CPSSIV

CPSSIV, a *Chromobacterium* species, was characterized to be a strong producer against Gram-positive *S. aureus* and a non-producer against Gram-negative *E. coli* in preliminary screenings. The optimal growth and antibiotic producing conditions for CPSSIV were determined to be minimal media supplemented with sodium succinate (25 mM) and grown for 72 h. Sequential-scale ups of liquid culture were done by inoculating one colony of bacteria in 10 mL of 10% TSB and grown in the rotatory shaker for 24 h. This was then transferred into 300 mL of 25 mM sodium succinate and grown in the rotatory shaker for 24 h. This was then transferred into 3 L of 25 mM sodium succinate and grown in the rotatory shaker for 72 h.

For small-scale liquid cultures, 3-5 mL of minimal media was inoculated with one colony of bacteria and grown in the rotatory shaker for 48-72 h. Large-scale cultures ranged from 3-12 L following the sequential scale up. After 72 h in the rotatory shaker, the large-scale cultures were centrifuged at 4000 rpm for 12 minutes at 23 °C. The supernatant was collected and extracted with hexane (1x) and ethyl acetate (2x). The organic extracts were combined, washed with saturated aqueous NaCl, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The crude extract was purified using gradient column chromatography ($SiO_2$, 1 × 8 cm, 50% ethyl acetate/hexane gradient elution) and concentrated under reduced pressure. The separated fractions were tested via an agar diffusion assay against *S. aureus* and the antibiotic active fraction was isolated (Rf = 0.38). It has yet to be characterized, for antibiotic activity has been inconsistent.

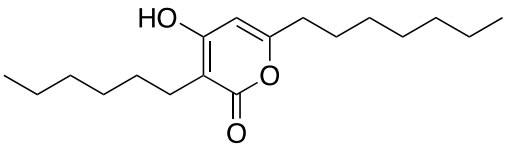
## 2.6 Extraction and isolation of pseudopyronine B from RG/RF B10[1]

RG/RF B10 was characterized to be a strong producer against Gram-positive *S. aureus* and a non-producer against Gram-negative *E. coli* in preliminary screenings. To determine optimal growth and antibiotic producing conditions for RG/RF B10, *Pseudomonas* species B10 was cultured in a minimal media supplemented with either sodium acetate (12.5 mM), sodium citrate (12.5 mM), D-glucose (12.5 mM), or sodium succinate (25 mM). The optical density and antibiotic production via an agar diffusion assay against *S. aureus* of each minimal media were measured at 2h, 12h, 24h, 36h, 48h, and 72h. At 24 h $OD_{600}$ for each carbon source were: sodium acetate (0.359), sodium citrate (0.676), D-glucose (0.071), and sodium succinate (0.267) and zones of inhibition were: sodium acetate (10 mm), sodium citrate (12 mm), D-glucose (14 mm), and sodium succinate (15 mm).

Sodium citrate was chosen and used for all large-scale cultures. For small-scale liquid cultures, 3-5 mL of minimal media was inoculated with one colony of bacteria and grown in the rotatory shaker for 24-48 h. Large-scale liquid cultures were grown in 4-6 L of sodium citrate inoculated with one colony of bacteria. After 48-72 h in the rotatory shaker the large-scale cultures were centrifuged at 5000 rpm for 12 minutes at 23 °C. The supernatant was collected and extracted with ethyl acetate (3x). The organic extracts were combined, washed with saturated aqueous NaCl, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The crude extract was purified using gradient column chromatography ($SiO_2$, 1 × 8 cm, 20% ethyl acetate/hexane gradient elution) and concentrated under reduced pressure. The separated fractions were tested via an agar diffusion assay against *S. aureus* and the antibiotic active fraction was isolated.[1]

## 2.7 Identification of the antibiotic pseudopyronine B[1]

The structure of the compound being produced by RG/RF B10 was assessed through $^{1}$H-NMR, $^{13}$C-NMR, DEPT, IR, LC-MSMS, and HRMS analysis, and subsequently confirmed with prior published spectroscopic data.[22,25-26] It was determined to be the known antibiotic pseudopyronine B. Scale-up and extraction of the culture supernatant allowed for the isolation of pseudopyronine B (2–5.5 mg/L) as a white solid.[1]




pseudopyronine B **(2)**

6-heptyl-3-hexyl-4-hydroxy-2*H*-pyran-2-one (Pseudopyronine B, 2). $^{1}$H NMR ($CD_3OD$, 400 MHz) δ 6.16 (s, 1H), 2.44 (m, 4H), 1.63 (m, 2H), 1.50 (m, 2H), 1.38-1.26 (m, 14H), 0.88 (m, 6H). $^{13}$C NMR ($CD_3OD$, 100 MHz) δ 169.0, 167.9, 165.3, 104.0, 101.2, 34.4, 33.1, 33.0, 30.4, 30.2, 30.1, 29.1, 28.1, 24.0, 23.9, 23.8, 14.6 (2C). IR (film) $\nu_{max}$ 2955, 2922, 2872, 2851, 1655, 1627, 1551, 1465, 1435, 1409, 1294, 1257, 1233, 1099, 999 cm$^{-1}$. DEPT $^{13}$C NMR ($CD_3OD$, 100 MHz) δ 101.2 (CH), 34.4 ($CH_2$), 33.1 ($CH_2$), 33.0 ($CH_2$), 30.4 ($CH_2$), 30.2 ($CH_2$), 30.1 ($CH_2$), 29.1 ($CH_2$), 28.1 ($CH_2$), 24.0 ($CH_2$), 23.9 ($CH_2$), 23.8 ($CH_2$), 14.6 (2C, $CH_3$). HRMS *m/z* 295.2264 ($M+H^+$, $C_{18}H_{30}O_3$ requires 295.2273).

## 2.8 Bacterial isolation and phylogenetic identification[1]

Isolate B10 was cultured from a sample of rhizosphere garden soil collected in the Rhoades Garden on the campus of the University of North Carolina Asheville. Soil was aseptically collected, subjected to a series of ten-fold dilutions, and spread plated on dilute Tryptic Soy Agar (dTSA; 3g Difco Tryptic Soy Broth per liter; solidified with 1.5% Bacto agar). After incubation at 25 °C, colonies were streaked purified 3X on fresh dTSA plates, and screened for antibiotic production. Isolates of interest, including B10, were phylogenetically identified via sequencing of 16S rDNA, as described below. DNA was liberated using the 5Prime ArchivePure Genomic DNA Isolation Kit, and used as the template in a PCR reaction to amplify the bacterial 16S rRNA gene using universal primers, 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT). Each PCR sample contained the following; Taq polymerase (10 units), 25 µL 1X OneTaq PCR Buffer, 200 µM dNTPs, 0.1 µM forward and reverse primers, and 100 ng of template DNA. Thermocycling conditions were as follows: initial denaturation (95 °C for 3 min); 30 cycles of 95 °C for 30 sec, 50°C for 30 sec, 72 °C for 1 min 30 sec; followed by a final extension at 72 °C for 5 min. PCR products were analyzed by size separation on a 1% agarose gel, and products corresponding to the appropriate size (approximately 1460 bp) were purified using the QiaQuick PCR Purification Kit (Qiagen), and sequenced using 27F and 1492R primers. DNA sequencing was performed by GeneWiz (Cambridge, MA), and rDNA sequences were then compared to publicly available 16S sequences available through National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases.[1]

## 2.9 Initial screen for antibacterial activity[1]

*Pseudomonas* sp. B10 was initially screened for production of antibiotic compounds with activity against a Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) target, using a standard agar overlay zone of inhibition assay. Briefly, dTSA plates were topped with a soft agar overlay (TSA solidified with 0.6% agar) containing approximately 5 x $10^7$ target cells per mL. Plates were incubated at 25 °C for 24-48 hours, and antibacterial activity was apparent as a zone of inhibition of the target.[1]

## 2.10 PCR-based discovery of the pseudopyronine synthase gene[1]

To confirm the presence of the PpyS gene in *Pseudomonas* sp. B10, MAFFT (Multiple Alignment using Fast Fourier Transform) was used to generate a multiple sequence alignment of the PpyS gene and its flanking regions from strains: *Pseudomonas putida* BW11M1, *Pseudomonas mosselii* SJ10, *Pseudomonas* sp. CCOS191, and *Pseudomonas putida* 1A00316. Utilizing areas of conserved sequence in the alignment and the PriFi tool, degenerate primer pairs were designed based on the orthologous sequences. PCR samples were prepared, as above, except that 250 mM primers were used, and thermocycling conditions were as follows: 95 °C for 3 min; 6 cycles of 95 °C for 30 sec, 40 °C for 30 sec, 72 °C for 1 min 30 sec; 20 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min 30 sec; and final extension at 72 °C for 5 min. Products of the correct size were gel-excised and purified using the Qiaquick Gel purification kit (Qiagen), followed by DNA sequencing (GeneWiz).[1]

| Primers | Sequence |
|---|---|
| PpyS_FOR_1<br>PpyS_REV_1 | GCAGAAAGCCGGTCAGGGCGATCT<br>GCTGTTGATGACGTCCAT |
| PpyS_FOR_2<br>PpyS_REV_2 | ATGGACGTCATCAACAGC<br>CCGGTGCMGGTGCTGTYCAGCGC |
| PpyS_FOR_3<br>PpyS_REV_3 | GCGGCGCTGGCCCAGGCCAATATCAGCAAGGCCG<br>CCCCGAGGTGGCCCAGCGCCTGGGCG |
| PpyS_FOR_4<br>PpyS_REV_4 | CGGCCGGCAGAAAGCCGGTCAGGGCGATCTGCAGC<br>CGTCCATGGCCGTCACCCAGCCGTTGCAGGCGTCG |

## 2.11 Nucleotide Accession Number[1]

The nucleotide sequence of the *Pseudomonas* sp. B10 PpyS, pseudopyronine synthase, gene is deposited in GenBank under accession number KY491008.[1]

## 2.12 General Procedure A: Synthesis of β-ketoesters[1]

To a flame dried round bottom flask under inert atmosphere, Meldrum's acid (1 eq) and anhydrous $CH_2Cl_2$ (0.2 M) were added. The solution was then cooled to 0 °C in an ice bath, and anhydrous pyridine (2 eq) was added. Finally, the acid chloride (1.1 eq) was added dropwise, and the reaction mixture was allowed to stir at 0 °C for 1 h before being allowed to warm to 23 °C to react for an additional 2 h. Upon completion, the reaction was poured into a separatory funnel and washed with 1 N aqueous HCl followed by $H_2O$. The organic layer was then dried over $Na_2SO_4$ and concentrated under reduced pressure. The crude residue was then dissolved in anhydrous $CH_3OH$ and warmed to reflux for 2-6 h. Upon completion solvent was removed under reduced pressure and the crude β-ketoester was carried on without further purification.[1]

## 2.13 General Procedure B: Synthesis of β-ketoacids[1]

The β-ketoester (1 eq) was dissolved in a 1:1 mixture of $CH_3OH$:$H_2O$ (0.38 M each) at 23 °C. A 30% w/v solution of $NaOCH_3$ in $CH_3OH$ (1.3 eq) was then added dropwise, and the reaction mixture was stirred for 18 h. Upon completion reaction was concentrated under reduced pressure, and the reaction mixture was acidified to pH < 4 with 1 N aqueous HCl. The aqueous suspension was then extracted with $CH_2Cl_2$ (3x). The organic layers were combined, washed with $H_2O$, dried over $Na_2SO_4$, and concentrated under reduced pressure, affording the desired β-ketoacids, which were carried on without further purification.[1]

## 2.14 General Procedure C: Synthesis of β-ketoacids[1]

The β-ketoester (1 eq) was dissolved in a 1:1 mixture of THF:$H_2O$ (0.1 M). LiOH (2 eq) was added and the reaction mixture was stirred at 23 °C for 24 h. Upon completion, the reaction mixture was concentrated under reduced pressure and acidified to pH < 4 with the addition of 1 N aqueous HCl. The aqueous layer was then extracted with EtOAc (3x). The organic extracts were combined, dried over $Na_2SO_4$, and concentrated under reduced pressure, affording the desired β-ketoacids, which were carried on without further purification.[1]

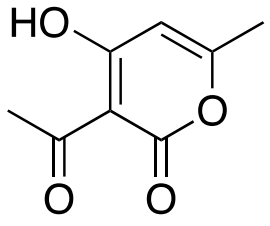
## 2.15 General Procedure D: Synthesis of 3-keto-4-hydroxypyran-2-ones[1]

To a stirred solution of the β-ketoacid (1 eq) in anhydrous THF (0.18 M) under an inert atmosphere at 23 °C was added 1,1'-carbonyldiimidazole (CDI, 1.36 eq). The reaction mixture was allowed to stir for 24 h. Upon completion, the reaction mixture was concentrated under reduced pressure, acidified to pH < 4 with the addition of 1 N aqueous HCl and extracted with EtOAc. The organic layer was then washed with $H_2O$, dried over $Na_2SO_4$, and concentrated under reduced pressure. Flash chromatography ($SiO_2$, 3 × 20 cm, 0−15% EtOAc/hexanes gradient elution) and/or recrystallization in $CH_3OH$ provided the desired 3-keto-4-hydroxypyran-2-ones β-ketoacids.[1]

## 2.16 General Procedure E. Synthesis of 3,5-alkyl-4-hydroxypyran-2-ones[1]

To a stirred solution of the 3-keto-4-hydroxypyran-2-one (1 eq) in THF (0.18 M) at 23 °C was added 2 M HCl (0.19 M). Then $NaCNBH_3$ (2.5 eq) was added, and the reaction mixture was allowed to stir for 3 h. Upon completion, the reaction mixture was diluted with $CH_2Cl_2$ and washed with $H_2O$ (3x). The organic layer was then dried over $Na_2SO_4$, and concentrated under reduced pressure. Flash chromatography ($SiO_2$, 2 × 10 cm, 15−40% EtOAc/hexanes gradient elution) provided the desired 3,5-alkyl-4-hydroxypyran-2-ones β-ketoacids.[1]

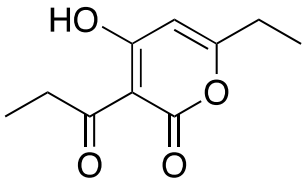
## 2.17 Synthesized Analogs[1]




**23**

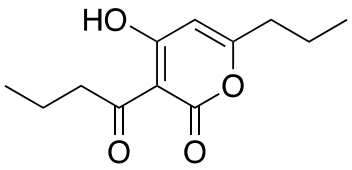
3-acetyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (23). General Procedure C then D starting from commercially available methyl 3-oxobutanoate (5.0 g, 43.0 mmol). Flash chromatography ($SiO_2$, 3 × 20 cm, 15−20% EtOAc/hexanes gradient elution) afforded 23 (316 mg, 15% over two steps) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 5.90 (s, 1H), 2.60 (s, 3H), 2.23 (s, *3*H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 205.3, 181.1, 169.2, 161.2, 101.5, 99.9, 30.2, 20.8. IR (film) $\nu_{max}$

3094, 1712, 1634, 1614, 1552, 1450, 1371, 1349, 1254, 1029, 995, 965 $cm^{-1}$. HRMS *m/z* 169.0497 ($M+H^+$, $C_8H_8O_4$ requires 169.0501)[1]



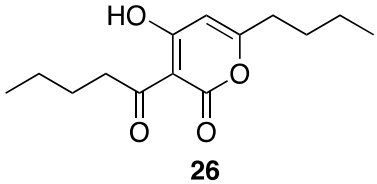
**24**

6-ethyl-4-hydroxy-3-propionyl-2*H*-pyran-2-one (24). General Procedure B then D starting from commercially available methyl 3-oxopentanoate (1.0 g, 7.6 mmol). Flash chromatography ($SiO_2$, 3 × 20 cm, 15−20% EtOAc/hexanes gradient elution) afforded 24 (396 mg, 55% over two steps) as a white solid. $^1H$ NMR ($CDCl_3$, 400 MHz) δ 5.90 (s, 1H), 3.06 (q, *J* = 7.2, 2H), 2.50 (q, *J* = 6.8, 2H) 1.21 (t, *J* = 5.2, 3H), 1.11 (t, *J* = 7.2, 3H).$^{13}C$ NMR ($CDCl_3$, 100 MHz) δ 208.4, 181.2, 173.6, 161.2, 99.9, 99.6, 35.4, 27.6, 10.5, 7.8. IR (film) $\nu_{max}$ 2975, 2940, 2882, 1732, 1638, 1558, 1441, 1391, 1283, 1219, 1152, 1074, 1014, 955, 913, 826 $cm^{-1}$. HRMS *m/z* 197.0807 ($M+H^+$, $C_{10}H_{12}O_4$ requires 197.0814)[1]



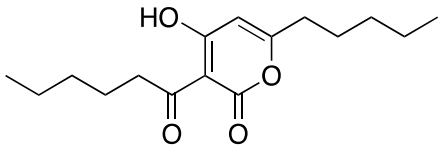
**25**

3-butyryl-4-hydroxy-6-propyl-2*H*-pyran-2-one (25). General Procedure A then B then D starting from commercially available butyryl chloride (4.05 g, 38.0 mmol). Flash chromatography (SiO2, 3 × 20 cm, 10% EtOAc/hexanes gradient elution) afforded 25 (134 mg, 3% over 4 steps) as a yellow oil. 1H NMR (CDCl3, 400 MHz) δ 5.92 (s, 1H), 3.06 (t, *J* = 7.2 Hz, 2H), 2.47 (t, *J* = 7.6 Hz, 2H), 1.703 (m, 4H), 0.99 (m, 6H).13C NMR (CDCl3, 100 MHz) δ 208.0, 181.4, 172.5, 161.3, 101.0, 99.9, 43.7, 36.3, 19.9, 17.5, 13.9, 13.6. IR (film) νmax 2964, 2935, 2874, 1732, 1639, 1610, 1558, 1451, 1260, 1212, 1156, 1091, 1039, 996, 940, 894 cm-1. HRMS *m/z* 225.1118 (M+H+, C12H16O4 requires 225.1127)[1]



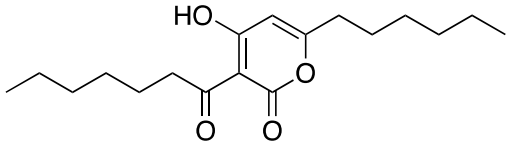
**26**

6-butyl-4-hydroxy-3-pentanoyl-2*H*-pyran-2-one (26). General Procedure A then B then D starting from commercially available pentanoyl chloride (4.59 g, 38.0 mmol). Flash chromatography ($SiO_2$, 3 × 20 cm, 10% EtOAc/hexanes gradient elution) afforded 26 (408 mg, 9% over 4 steps) as an off white solid. $^1H$ NMR ($CDCl_3$, 400 MHz) δ 5.92 (s, 1H), 3.07 (t, *J* = 7.2 Hz, 2H), 2.49 (t, *J* = 7.6 Hz, 2H), 2.35 (t, *J* = 7.2 Hz, 2H), 1.64 (m, 3H), 1.41 (m, 3H), 0.94 (m, 6H).$^{13}C$ NMR ($CDCl_3$, 100 MHz) δ 208.4, 181.5, 172.7, 161.3, 100.9, 99.8, 41.6, 34.2, 28.6, 26.2, 22.6, 22.3, 14.1, 13.9. IR (film) $\nu_{max}$ 2959, 2932, 2871, 1724, 1636, 1606, 1557, 1450, 1396, 1227, 1154, 1027, 990, 933, 847 $cm^{-1}$. HRMS *m/z* 253.1431 ($M+H^+$, $C_{14}H_{20}O_4$ requires 253.1440)[1]



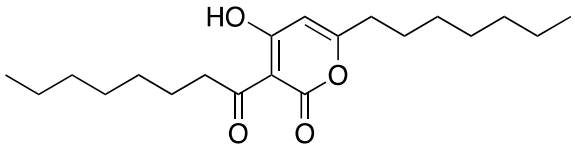
**27**

3-hexanoyl-4-hydroxy-6-pentyl-2*H*-pyran-2-one (27). General Procedure A then B then D starting from commercially available hexanoyl chloride (8.2 g, 60.9 mmol). Flash chromatography ($SiO_2$, 3 × 20 cm, 10% EtOAc/hexanes gradient elution) afforded 27 (1.38 g, 25% over 4 steps) as an off white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 5.89 (s, 1H) 3.02 (t, *J* = 8.0 Hz, 2H), 2.45 (t, *J* = 7.6 Hz, 2H), 1.64 (m, 4H), 1.31 (m, 8H), 0.87 (m, 6H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 208.1, 181.2, 172.7, 161.7, 100.9, 99.7, 41.8, 34.4, 31.5, 31.2, 26.2, 23.8, 22.6, 22.4 14.1, 14.0. IR (film) $\nu_{max}$ 2960, 2934, 2871, 1722, 1634, 1602, 1563, 1448, 1339, 1251, 1221, 1192, 1156, 1013, 982, 946, 855 cm$^{-1}$. HRMS *m/z* 281.1743 (M+H$^+$, $C_{16}H_{24}O_4$ requires 281.1753)[1]



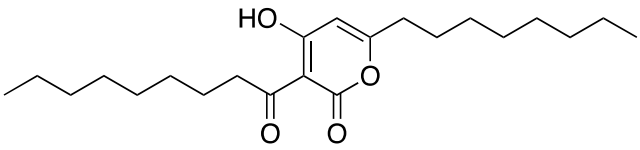
**28**

3-heptanoyl-6-hexyl-4-hydroxy-2*H*-pyran-2-one (28). General Procedure A then B then D starting from commercially available heptanoyl chloride (9.06 g, 60.9 mmol). Flash chromatography ($SiO_2$, 6 × 20 cm, 2-10% EtOAc/hexanes gradient elution) and recrystallization from $CH_3OH$ afforded 28 (2.442 g, 29% over 4 steps) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 5.92 (s, 1H) 3.07 (t, *J* = 7.6 Hz, 2H), 2.48 (t, *J* = 7.6 Hz, 2H), 1.66 (m, 4H), 1.39-1.27 (m, 12H), 0.88 (m, 6H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 208.2, 181.4, 172.7, 161.3, 100.9, 99.8, 41.9, 34.5, 31.8, 31.6, 29.1, 28.8, 26.5, 24.1, 22.7, 22.6, 14.2 (2C). IR (film) $\nu_{max}$ 2954, 2928, 2867, 1730, 1636, 1613, 1564, 1447, 1398, 1242, 1028, 1007, 985, 842 cm$^{-1}$. HRMS *m/z* 309.2063 (M+H$^+$, $C_{18}H_{28}O_4$ requires 309.2066)[1]



**29**

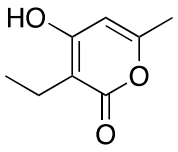
6-heptyl-4-hydroxy-3-octanoyl-2*H*-pyran-2-one (29). General Procedure A then B then D starting from commercially available octanoyl chloride (9.91 g, 60.9 mmol). Flash chromatography ($SiO_2$, 6 × 20 cm, 2-10% EtOAc/hexanes gradient elution) and recrystallization from $CH_3OH$ afforded 29 (2.522 g, 32% over 4 steps) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 5.92 (s, 1H) 3.06 (t, *J* = 7.2 Hz, 2H), 2.48 (t, *J* = 7.6 Hz, 2H), 1.67 (m, 4H), 1.37-1.27 (m, 16H), 0.89 (m, 6H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 208.2, 181.4, 172.7, 161.3, 100.9, 99.8, 41.9, 34.5, 31.9, 31.8, 29.4, 29.3, 29.1 (2C), 26.6, 24.1, 22.8 (2C), 14.3, 14.2. IR (film) $\nu_{max}$ 2953, 2924, 2851, 1723, 1636, 1610, 1565, 1448, 1234, 1000, 940, 910, 847, 729 cm$^{-1}$. HRMS *m/z* 337.2375 (M+H$^+$, $C_{20}H_{32}O_4$ requires 337.2379)[1]



**30**

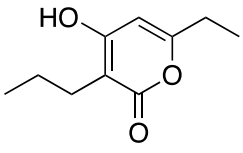
4-hydroxy-3-nonanoyl-6-octyl-2*H*-pyran-2-one (30). General Procedure A then B then D starting from commercially available nonanoyl chloride (10.7 g, 60.9 mmol). Flash chromatography ($SiO_2$, 6 × 20 cm, 2% EtOAc/hexanes gradient elution) and recrystallization from $CH_3OH$ afforded 30 (419 mg, 4% over 4 steps) as a white solid. $^1$H NMR ($CDCl_3$,

400 MHz) δ 5.92 (s, 1H), 3.08 (t, *J* = 5.6, 2H), 2.49 (t, *J* = 7.6, 2H), 1.71-1.62 (m, 4H), 1.37-1.28 (m, 20H), 0.91-0.87 (m, 6H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 298.3, 181.5, 172.8, 161.4, 100.9, 99.8, 41.9, 34.5, 32.1, 31.9, 29.6, 29.5, 29.4 (2C), 29.3, 29.1, 26.6, 24.2, 22.9, 22.8, 14.3 (2C). IR (film) $\nu_{max}$ 2953, 2923, 2852, 11731, 1636, 1605, 1567, 1450, 1396, 1232, 1019, 991, 935 cm$^{-1}$. HRMS *m/z* 365.2686 (M+H$^+$, $C_{22}H_{36}O_4$ requires 365.2686)[1]



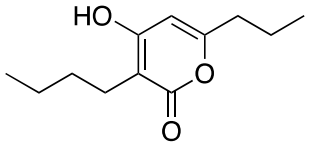
**31**

3-ethyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (31). General Procedure E starting from 23 (99.2 mg, 0.59 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 30−50% EtOAc/hexanes gradient elution) afforded 31 (36 mg, 40%) as a white solid. $^1$H NMR ($CD_3CN$, 400 MHz) δ 5.89 (s, 1H), 2.33 (q, *J* = 7.6 Hz, 2H), 2.14 (s, 3H), 0.99 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR ($CD_3CN$, 100 MHz) δ 166.5, 165.3, 161.6, 104.9, 100.9, 20.1, 17.5, 13.2. IR (film) $\nu_{max}$ 3062, 2973, 2874, 2646, 1666, 1627, 1568, 1406, 1277 cm$^{-1}$. HRMS *m/z* 155.0702 (M+H$^+$, $C_8H_{10}O_3$ requires 155.0630)[1]



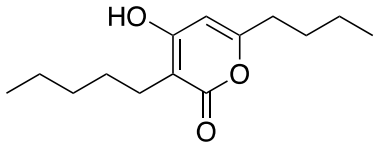
**32**

6-ethyl-4-hydroxy-3-propyl-2*H*-pyran-2-one (32). General Procedure E starting from 24 (100 mg, 0.51 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 20% EtOAc/hexanes gradient elution) afforded 32 (62 mg, 67%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 6.26 (s, 1H), 2.53-2.43 (m, 4H), 1.53 (m, 2H), 1.20 (t, *J* = 7.6 Hz, 3H), 0.94 (t, *J* = 7.6 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.9, 168.1, 164.8, 103.4, 100.5, 26.9, 25.1, 21.5, 14.2, 11.1. IR (film) $\nu_{max}$ 3099, 2962, 2931, 2874, 2658, 1663, 1575, 1463, 1438, 1408, 1303, 1267, 1245, 1170, 1126, 1092, 1012, 987, 939, 908, 840, 733 cm$^{-1}$. HRMS *m/z* 183.1015 (M+H$^+$, $C_{10}H_{14}O_3$ requires 183.1021)[1]



**33**

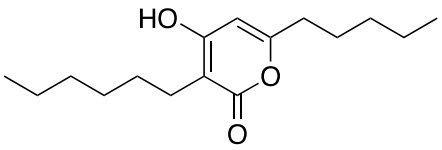
3-butyl-4-hydroxy-6-propyl-2*H*-pyran-2-one (33). General Procedure E starting from 25 (57 mg, 0.25 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 20% EtOAc/hexanes gradient elution) afforded 33 (29 mg, 56%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 9.3 (br, 1H), 6.13 (s, 1H), 2.44 (m, 4H), 1.67 (m, 3H), 1.49 (m, 2H), 1.37 (m, 2H), 0.96 (t, *J* = 7.2 Hz, 3H), 0.92 (t, *J* = 7.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.2, 166.8, 163.6, 103.6, 100.9, 35.6, 30.4, 23.1, 22.9, 20.4, 14.2, 13.7. IR (film) $\nu_{max}$ 3083, 2959, 2930, 2670, 1632, 1573, 1435, 1410, 1289, 1218, 1172 cm$^{-1}$. HRMS *m/z* 211.1327 (M+H$^+$, $C_{12}H_{18}O_3$ requires 211.1334)[1]



**34**

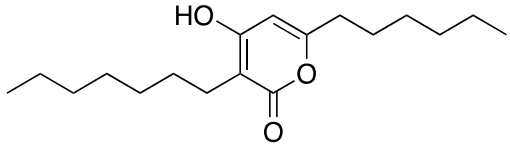
6-butyl-4-hydroxy-3-pentyl-2*H*-pyran-2-one (34). General Procedure E starting from 26 (48 mg, 0.19 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 20% EtOAc/hexanes gradient elution) afforded 34 (21 mg, 47%) as a white

solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 9.9 (br, 1H) 6.18 (s, 1H), 2.45 (t, $J$ = 7.6 Hz, 4H), 1.61 (m, 2H), 1.51, (m, 2H), 1.40-1.30 (m, 6H), 0.93-0.86 (m, 6H).$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.5, 167.2, 163.8, 103.6, 101.0, 33.4, 32.0, 29.1, 27.9, 23.2, 22.8, 22.3, 14.3, 13.9. IR (film) $\nu_{max}$ 2956, 2929, 2873, 2676, 1634, 1574, 1435, 1410, 1304, 1273, 1243, 1130 cm$^{-1}$. HRMS *m/z* 239.1640 (M+H$^+$, $C_{14}H_{12}O_3$ requires 239.1647)[1]



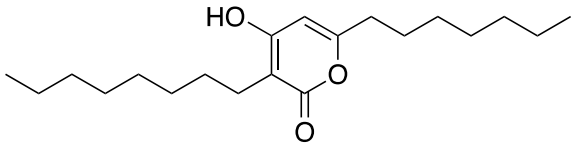

pseudopyronine A **(1)**

3-hexyl-4-hydroxy-6-pentyl-2*H*-pyran-2-one (Pseudopyronine A, 1). General Procedure E starting from 27 (300 mg, 1.07 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 20% EtOAc/hexanes gradient elution) afforded 1 (105 mg, 37%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 10.6 (br, 1H) 6.24 (s, 1H), 2.47-2.42 (m, 4H), 1.63 (m, 2H), 1.50 (m, 2H), 1.36-1.26 (m, 10H), 0.91-0.85 (m, 6H).$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.9, 167.9, 163.8, 103.6, 101.3, 33.7, 32.0, 31.3, 29.6, 28.2, 26.7, 23.3, 22.9, 22.5, 14.3, 14.1. IR (film) $\nu_{max}$ 2995, 2927, 2872, 2858, 2654, 1663, 1633, 1568, 1435, 1409, 1303, 1257, 1231, 1131, 993 cm$^{-1}$. HRMS *m/z* 267.1950 (M+H$^+$, $C_{16}H_{26}O_3$ requires 267.1960)[1]



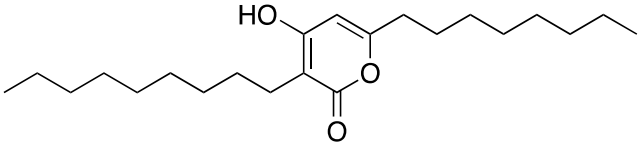

**35**

3-heptyl-6-hexyl-4-hydroxy-2*H*-pyran-2-one (35). General Procedure E starting from 28 (500 mg, 1.62 mmol). Flash chromatography ($SiO_2$, 3 × 10 cm, 10-20% EtOAc/hexanes gradient elution) afforded 35 (303 mg, 64%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 10.6 (br, 1H) 6.26 (s, 1H), 2.46-2.42 (m, 4H), 1.62 (m, 2H), 1.50 (m, 2H), 1.38-1.20 (m, 14H), 0.89-0.84 (m, 6H).$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.9, 167.9, 163.8, 103.6, 101.3, 33.7, 32.1, 31.7, 29.9, 29.5, 28.9, 29.4, 26.9, 23.3, 22.9, 22.7, 14.3, 14.2. IR (film) $\nu_{max}$ 2957, 2924, 2850, 2654, 1633, 1569, 1435, 1409, 1302, 1282, 1250, 1180, 1131 cm$^{-1}$. HRMS *m/z* 295.2271 (M+H$^+$, $C_{18}H_{30}O_3$ requires 295.2273)[1]




**36**

6-heptyl-4-hydroxy-3-octyl-2*H*-pyran-2-one (36). General Procedure E starting from 29 (500 mg, 1.48 mmol). Flash chromatography ($SiO_2$, 3 × 10 cm, 15-20% EtOAc/hexanes gradient elution) afforded 36 (247 mg, 52%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 10.44 (br, 1H) 6.23 (s, 1H), 2.46-2.42 (m, 4H), 1.63 (m, 2H), 1.50 (m, 2H), 1.31-1.26 (m, 18H), 0.89-0.85 (m, 6H).$^{13}$C NMR ($CDCl_3$, 100 MHz) δ168.8, 167.6, 163.8, 103.6, 101.2, 33.8, 32.1, 31.9, 29.9, 29.8, 29.6, 29.2 (2C), 28.3, 27.0, 23.3, 22.9, 22.8, 14.3 (2C). IR (film) $\nu_{max}$ 3087, 2955, 2920, 2872, 2849, 2654, 1662, 1632, 1557, 1467, 1434, 1409, 1295, 1271, 1243 cm$^{-1}$. HRMS *m/z* 323.2584 (M+H$^+$, $C_{20}H_{34}O_3$ requires 323.2586)[1]




**37**

4-hydroxy-3-nonyl-6-octyl-2*H*-pyran-2-one (37). General Procedure E starting from 30 (50 mg, 0.14 mmol). Flash chromatography ($SiO_2$, 2 × 10 cm, 10% EtOAc/hexanes gradient elution) afforded 37 (25 mg, 54%) as a white solid. $^1$H NMR ($CDCl_3$, 400 MHz) δ 10.04 (br, 1H) 6.20 (s, 1H), 2.47-2.42 (m, 4H), 1.63 (m, 2H), 1.50 (m, 2H), 1.29-1.24 (m, 22H), 0.89-0.85 (m, 6H).$^{13}$C NMR ($CDCl_3$, 100 MHz) δ 168.5, 167.3, 163.8, 103.6, 101.0, 33.8, 32.1, 32.0, 29.9 (3C), 29.6, 29.5, 29.4, 29.3, 28.4, 27.0, 22.3, 22.9 (2C), 14.3 (2C). IR (film) $\nu_{max}$ 2954, 2916, 2873, 2849, 2656, 1663, 1630, 1549, 1469, 1432, 1409, 1304, 1284, 1266, 1247, 1182, 1132, 1011, 861 cm$^{-1}$. HRMS *m/z* 351.2896 ($M+H^+$, $C_{22}H_{38}O_3$ requires 351.2899)[1]

## 3. Results and Discussion

### 3.1 Minimal Media and Time Trial Evaluation

The minimal media screens provided the optical density of the bacteria in the different minimal media. The two minimal medias that were evaluated to show the most bacterial density were chosen and subjected to a time trial assay.

Table 1. Optimization of antibiotic producing conditions for each bacteria determined by minimal media and time trial assays.

| Bacteria | Minimal Medias with Highest Absorbances (from media assay) | | Minimal Media with Most Activity (from Time Trial Assay) | Optimal Time of Antibiotic Production (hrs) |
|---|---|---|---|---|
| SS 439 | Glucose | Citrate | Glucose | 96 |
| SS 440 | Glucose | Citrate | Citrate | 96 |
| SS 444 | Glucose | Succinate | Glucose | 72 |
| SS 447 | Glucose | Citrate | Citrate | 96 |
| SS 449 | Glucose | Citrate | Citrate | 96 |
| SS 614 | Glucose | Citrate | Citrate | 96 |
| SS 615 | Glucose | Citrate | Glucose | 72 |
| SS 616 | Glucose | Succinate | Glucose | 72 |
| SS 619 | Glucose | Succinate | Glucose | 96 |
| SS 622 | Glucose | Citrate | Glucose | 96 |
| SS 625 | Glucose | Succinate | Glucose | 72 |
| SS 636 | Glucose | Succinate | Glucose | 72 |
| SS 654 | Glucose | Succinate | Glucose | 72 |
| SS 655 | Citrate | Succinate | Citrate | 96 |
| SS 661 | Glucose | Citrate | Glucose | 72 |
| SS 662 | Glucose | Succinate | Glucose | 96 |
| SS 670 | Glucose | Citrate | Citrate | 72 |
| SS 672 | Glucose | Citrate | Glucose | 96 |
| SS 673 | Glucose | Citrate | Citrate | 96 |
| SS 674 | Glucose | Citrate | Citrate | 72 |
| SS 682 | Glucose | Citrate | Citrate | 72 |
| SS 697 | Glucose | Succinate | Glucose | 72 |
| SS 699 | Glucose | Succinate | Succinate | 96 |
| SS 724 | Glucose | Succinate | Glucose | 96 |
| 827 B | Glucose | Citrate | Glucose | 96 |

### 3.2 Isolation and phylogenetic identification of pseudopyronine B[1]

As part of a larger effort in natural product discovery, various rhizosphere, fresh water, and plant-associated environmental samples were probed for culturable bacteria with antibiotic potential. A fast-growing, aerobic, Gram-negative bacterium, designated B10, was isolated from rhizosphere garden soil at the University of North Carolina Asheville. An initial screen for antibacterial activity revealed that strain B10 inhibited *S. aureus* (Figure 2). PCR

amplification and DNA sequencing of the full-length 16S rRNA gene (V1-V9) allowed identification of strain B10 as a member of the *Pseudomonas putida* group, most closely related to *Pseudomonas mosselii*. Isolate B10 (hereafter designated *Pseudomonas* sp. B10) grows in rich medium (Luria Bertani and Tryptic Soy Broth), and also in a minimal salts medium (0.35 M $K_2HPO_4$; 0.22 M $KH_2PO_4$; 0.08 M $(NH_4)_2SO_4$; 200 mM $MgSO_4$) supplemented with citrate (12.5 mM) as the sole carbon and energy source.[1]

## 3.3 Identification of the antibiotic pseudopyronine B[1]

*Pseudomonas* sp. B10 was cultured on large-scale (6L) in minimal salts medium, with citrate as the sole carbon source, to facilitate extraction and purification of the active antibacterial metabolite(s). Scale-up and extraction of the culture supernatant allowed for the isolation of pseudopyronine B (2-5.5 mg/L) as a white solid whose structure was assessed through $^{1}H$, $^{13}C$, DEPT, IR, LC-MSMS, and HRMS analysis, and subsequently confirmed with prior published spectroscopic data.[11,13-14]

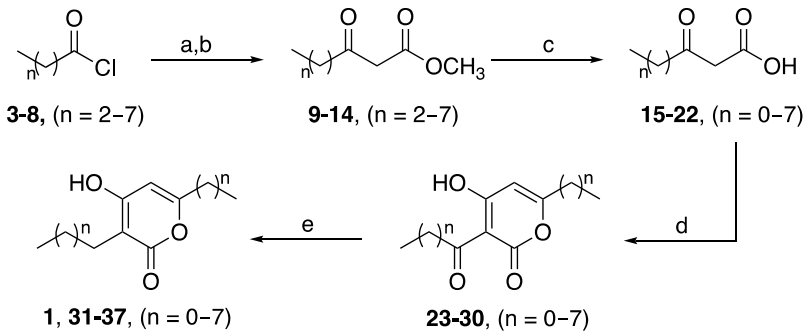
## 3.4 Identification of a PpyS-like pseudopyronine synthase[1]

Several routes for the biosynthesis of α-pyrones have been reported (reviewed recently by Schäberle[30]), and two general pathways exist: 1) formation of the α-pyrone ring by intramolecular cyclization reactions catalyzed by modular polyketide synthase (PKS) pathways or 2) the head-to-head condensation of two independent acyl moieties by freestanding ketosynthase enzymes. Recently, the latter mechanism has been confirmed for the photopyrones produced by *Photorhabdus* bacteria[24] and similarly for the biosynthesis of pseudopyronine A and B by *Pseudomonas putida* BW11M1.[1, 28]

Using DNA sequence alignments of syntenic genomic regions from several photopyrone and pseudopyronine producers, degenerate PCR primers were designed (see Supplemental Information) that enabled amplification of the genomic region of Pseudomonas sp. B10 hypothesized to encode the pseudopyronine ketosynthase. Indeed, PCR, followed by DNA sequencing, revealed an open reading frame encoding a PpyS homolog, showing greater than 95% amino acid identity to PpyS of P. putida BW11M1.[1]

## 3.5 Pseudopyronine Analog Synthesis[1]

Following the isolation of pseudopyronine B, a preliminary SAR analysis was conducted on an array of synthetic pseudopyronine analogs. C3/C6 pseudopyronine A-type analogs, where the C6 alkyl chain is one carbon shorter than the C3 alkyl chain, were synthesized as shown in **Scheme 1** via the method described by Copp and co-workers.[19] This synthetic strategy provided rapid access to the desired analogs in a time and cost efficient manner. Alkyl chain lengths ranging from 1–9 carbons were synthesized from either the commercially available acid chlorides (**1**, **33**–**37**, n = 2–7) or the commercially available β-keto esters (**31**–**32**, n = 0,1) in 5 or 4 synthetic steps, respectively. First, the acid chlorides were treated with Meldrum's acid and pyridine in $CH_2Cl_2$ to provide the acyl Meldrum's acids, which were then directly subjected to methanolysis to provide the desired β-keto esters.[31] The β-keto esters could then be carried on crude through the subsequent base mediated hydrolysis and CDI promoted cyclization to provide ketone α-pyrone analogs **23**–**30**. Although moderate to low yields were achieved (4–32% over 4 steps), only a single column chromatography purification or recrystallization step was required for the sequence making this a highly efficient method that could be completed in as little as 3 days. Finally, the ketone α-pyrone analogs were reduced using $NaCNBH_3$ under acidic conditions to provide pseudopyronine A (**1**) and analogs **31**–**37** in reasonable yields after purification. It is worthwhile to note that no α-pyrone to furanone rearrangement described by Carter and co-workers[26] was observed with any of the analogs, pseudopyronine A or pseudopyronine B.[1]

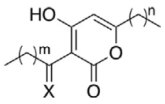



**Scheme 1.** Synthesis of Alkyl Analogs. Reagents and conditions: (a) Meldrum's acid (1 equiv), pyridine (2 equiv), $CH_2Cl_2$, 0–23 °C, 3 h; (b) $CH_3OH$, reflux, 2–6 h; (c) $NaOCH_3$ in $CH_3OH$ (1.3 equiv), $CH_3OH$:$H_2O$ (1:1), 23 °C, 18 h or LiOH (2 equiv), THF: $H_2O$ (1:1), 23 °C, 24 h; (d) CDI (1.36 equiv), THF, 23 °C, 24 h (4–32% over 4 steps); (e) $NaCNBH_3$ (2.5 equiv), 2 M HCl, THF, 23 °C, 2.5 h (37–67%).[1]

## 3.6 Evaluation of antibacterial activity of pseudopyronine A, B, and C3/C6 alkyl analogs[1]

Purified pseudopyronine B (**2**) and all synthesized analogs, including synthetic pseudopyronine A (**1**) and the ketone precursors (**23–30)**, were evaluated for antibacterial activity against a panel of two Gram-positive (*S. aureus* and *B. subtilis*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacteria (Table 2). Pseudopyronine B (**2**) was found to have an $IC_{50}$ of 0.1 mg/mL and 0.3 mg/mL against *S. aureus* and *B. subtilis* respectively and to be inactive against *E. coli* and *P. aeruginosa*, which is consistent with prior reports.[15] For the synthetic C3/C6 analogs, it was found that antibacterial activity is directly related to alkyl chain length at these positions for both the α-pyrones and their ketone precursors. For inhibition of Gram-positive bacteria, activity increased with increasing chain length up to n = 5 (**Scheme 1**) for *S. aureus* with analog **35** being the most active ($IC_{50}$ = 0.04 mg/mL) and with pseudopyronine B (**2**) being the most active against *B. subtilis*. Longer chain lengths (**29**, **30**, **36**, **37**) were found to rapidly lose activity against Gram-positive bacteria. The ketone precursors followed a similar trend against Gram-positive bacteria tested. Interestingly, it was found that the shorter chain lengths (**23**–**26**, **31**, and **32**) displayed modest activity against *E. coli* indicating that perhaps these analogs are employing a different mechanism of action against Gram-negative bacteria compared to Gram-positive. All analogs were found to be inactive against *P. aeruginosa*.[1]

**Table 2.** *In vitro* antibacterial activity[1]




| Compound | $IC_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | *S. aureus* | *E. coli* | *B. subtilis* | *P. aeruginosa* |
| **23** (X = O, n = 0, m = 0) | >1000 | 304 | >1000 | >1000 |
| **24** (X = O, n = 1, m = 1) | 116 | 257 | >1000 | >1000 |
| **25** (X = O, n = 2, m = 2) | 46 | 248 | 1000 | >1000 |
| **26** (X = O, n = 3, m = 3) | 24 | 223 | 227 | >1000 |
| **27** (X = O, n = 4, m = 4) | 3.8 | >1000 | 21 | >1000 |
| **28** (X = O, n = 5, m = 5) | 1.5 | >1000 | 3.5 | >1000 |
| **29** (X = O, n = 6, m = 6) | 48 | >1000 | 27 | >1000 |
| **30** (X = O, n = 7, m = 7) | >1000 | >1000 | >1000 | >1000 |
| **31** (X = $H_2$, n = 0, m = 0) | >1000 | 300 | >1000 | >1000 |
| **32** (X = $H_2$, n = 1, m = 1) | 244 | 278 | 203 | >1000 |
| **33** (X = $H_2$, n = 2, m = 2) | 33 | >1000 | 46 | >1000 |
| **34** (X = $H_2$, n = 3, m = 3) | 4.6 | >1000 | 23 | >1000 |
| **1**, pseudopyronine A (X = $H_2$, n = 4, m = 4) | 2.4 | >1000 | 3.3 | >1000 |
| **2**, pseudopyronine B (X = $H_2$, n = 6, m = 4) | 0.1 | >1000 | 0.3 | >1000 |
| **35** (X = $H_2$, n = 5, m = 5) | 0.04 | >1000 | 0.7 | >1000 |
| **36** (X = $H_2$, n = 6, m = 6) | >1000 | >1000 | 24 | >1000 |
| **37** (X = $H_2$, n = 7, m = 7) | >1000 | >1000 | >1000 | >1000 |

## 4. Conclusion

In summary, we were able to develop techniques to systematically optimize growth media as well as duration of growth that maximizes the amount of antibiotic production that can be used for all of the bacteria in the collected library. Bacteria species SS 616 and CPSSIV are still being worked with to successfully elucidate the antibiotic active compound.

Additionally, we have isolated a *Pseudomonas* species from rhizosphere garden soil in Western North Carolina that produces the known antibiotic pseudopyronine B. In an attempt to improve antibacterial potency of the natural pseudopyronines, a series of C3/C6 alkyl pseudopyronine analogs were synthesized and evaluated for their antibacterial activity against both Gram-positive and Gram-negative target pathogens. We observed a correlation between chain length and antibacterial activity, with 6–7 carbon chains being the most active against Gram-positive bacteria *S. aureus* and *B. subtilis*. Of particular interest, while the natural pseudopyronines show no detectable activity against Gram-negative targets, analogs with short alkyl chain lengths (1–4) exhibit modest activity against *E. coli*. We conclude that the activity seen against *E. coli* (Table 2) is not simply the result of enhanced solubility of the short chain length (1–4) analogs, as the pattern is not mirrored in assays against Gram-positive bacteria. Instead, we suggest that compounds **23**–**26**, **31**, and **32** show the potential to expand the range of activity of this class of antibacterial molecules.[1]

## 5. Acknowledgements

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