Communication

Total Synthesis and Biological Investigation of (-)-Promysalin

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Total Synthesis and Biological Investigation of (-)-Promysalin

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Supporting Information Placeholder

ABSTRACT: Compounds that specifically target pathogenic bacteria are in great need and identifying the method by which they act would provide new avenues of treatment. Herein we report the concise, high-yielding total synthesis (8 steps, 35% yield) of promysalin, a natural product that displays anti-virulence phenotypes against pathogenic bacteria. Guided by bioinformatics, four diastereomers were synthesized and the relative and absolute stereochemistry was confirmed by spectral and biological analysis. Finally, we show for the first time, that promysalin displays two anti-virulence phenotypes: the dispersion of mature biofilms and the inhibition of pyoverdine production hinting at a unique pathogenic-specific mechanism of action.

Advances in culture-independent genome sequencing coupled with computational analysis methods have revolutionized research into microbiomes – the entirety of the microbial community in a given system.[1] At the same time, the burgeoning field of microbial ecology has shed light on the inherent complexity of interrelationships in such ecosystems.[2] Studies investigating the coexistence of organisms, ranging from mutualistic to parasitic, have had profound impact on our understanding of life, most notably in the human body. There has been a call for providing small molecule probes that could be used to deconvolute such systems by targeting pathogen-specific bacteria. For example, a narrow-spectrum antibiotic that can specifically target pathovars without disrupting the remaining population would be of interest in agriculture. If successful, these compounds would not only be of value to farming but also to human health (i.e. oral, gastrointestinal) and other commercial interests.

A well-studied example of a multi-species community is the root system of plants, generally termed the rhizosphere microbiome.[3] The predominant players in this arena are the *Pseudomonads*, which are comprised of both commensal and pathogenic species competing for vital resources, either ensuring or jeopardizing the health of the host. The competitors produce an array of secondary metabolites with unique bioactivities evolutionarily designed to promote survival. Functions include siderophores,[4] virulence factors,[5] biosurfactants,[6] and antibiotics.[7] Such species-specific compounds represent attractive targets for agricultural and medicinal needs and may shed light on novel virulence targets for future drug design.

In 2011, De Mot and coworkers isolated a novel metabolite, promysalin (**1**), from *Pseudomonas putida* (*PP*) RW10S1 which resides in the rhizosphere of rice plants.[8] The natural product showed unique species-specific bioactivity, most notably against *Pseudomonas aeruginosa* (*PA*), inhibiting growth at low micromolar concentrations. Promysalin selectively inhibits certain *PA* strains and other Gram-negative bacteria, but showed no activity against their Gram-positive counterparts. In contrast, the compound was also shown to promote swarming of the producing organism, hinting at two discrete modes of action. The original report characterized the biosynthetic gene cluster and proposed a biosynthesis via annotation and the characterization of shunt products. The authors elucidated the structure of promysalin through spectroscopic methods; however, no absolute or relative stereochemical assignments were made. Considering the significance of *PA* clinical settings[9] (cystic fibrosis, immunocompromised patients) and in agriculture, promysalin could serve as an attractive alternative to current therapies. The unique bioactivity, unknown mode of action, and structural ambiguity are what prompted the synthesis reported herein.

Before initiating our synthetic investigation we sought to reannotate the biosynthetic gene cluster using AntiSMASH (Scheme 1).[10] We postulated that this computational work would aid in determining the absolute stereochemistry of dehydroproline thus limiting the synthesis to one enantiomeric series. This study confirmed that *ppgJ* encodes for a truncated Non-Ribosomal Peptide Synthetase (NRPS) module containing both an adenylation (A) and thiolation domain but lacking a condensation domain, reminiscent of *sylC* found in *P. syringae*.[11] Upon closer inspection, we were unable to identify any putative epimerase or thioesterase domains contained in either the characterized gene cluster or in

the flanking regions. Bioinformatic investigation of the *ppgJ* A-domain revealed the

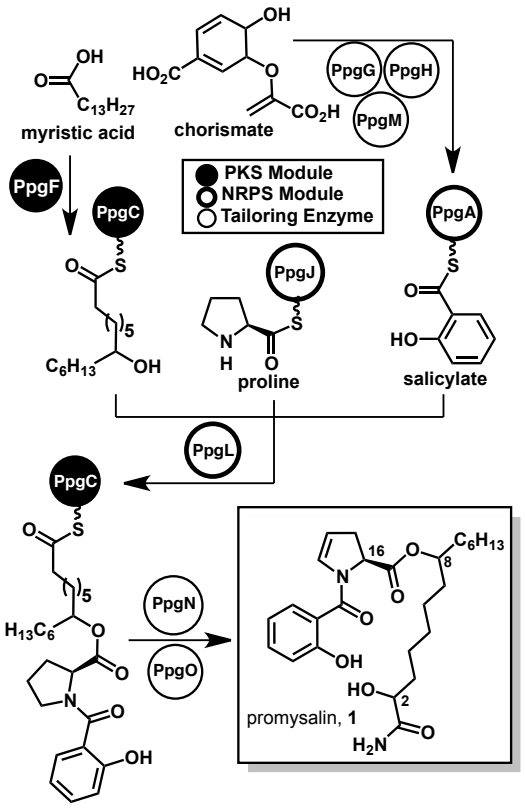
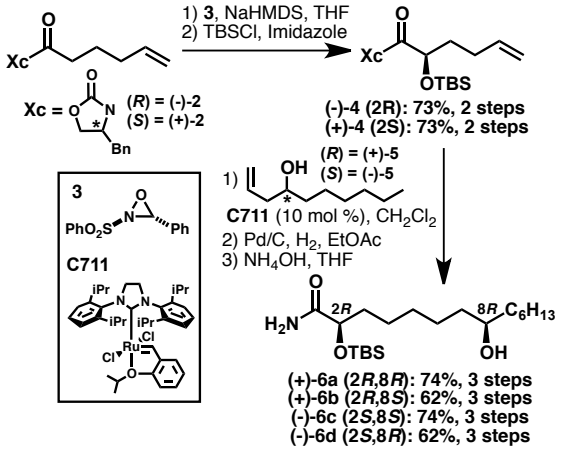


Figure 1. Proposed biosynthesis of promysalin. Polyketide synthase (PKS), NRPS, and Tailoring enzymes (Rieske Iron-Sulfur cluster, asparagine synthase, chorismate synthase) are depicted.

Stachelhaus code,[12] DVQFVAHV, corresponding to the selective activation of L-proline as previously hypothesized by De Mot. This exercise led us to the conclusion that the absolute configuration of the C16-stereocenter should be assigned as (*S*). Taken together, we reevaluated the proposed biosynthesis of promysalin, which is depicted in Figure 1. With this information in hand, we began our campaign to synthesize the four diastereomers generated from the two unresolved stereocenters (C2 and C8).

Our synthetic efforts began with construction of the four diastereomers of the myristic acid fragment (Scheme 1). We envisioned utilizing a convergent route wherein cross metathesis followed by hydrogenation would be used to forge the complete aliphatic chain providing a succinct route to all four L-proline diastereomers. Beginning with the known compound (-)-**2** available in one step from 5-hexenoic acid and the phenylalanine-derived Evans' oxazolidinone,[13] diastereoselective oxidation using the Davis oxaziradine followed by silyl-protection furnished compound (-)-**4** in good yield. Cross metathesis with known, enantiomerically pure homoallylic alcohol (+)-**5** or (-)-**6** in the presence of catalyst **C711**,[14] subsequent hydrogenation, and ammonolysis provides diastereomers **6a/b**. Analogously, the enantiomeric series of compounds was synthesized starting with (+)-**2** providing **6c/d**. This route provides concise access to all four diastereomers in enantiomerically pure form (45-54% over five steps).

Synthesis of the proline-salicylate fragment commenced



with the ester hydrolysis of the SEM-protected methyl *trans*-4-L-hydroxyproline methyl ester and Dess-Martin oxidation to

Scheme 1. Synthesis of Aliphatic Precursors 6a-d

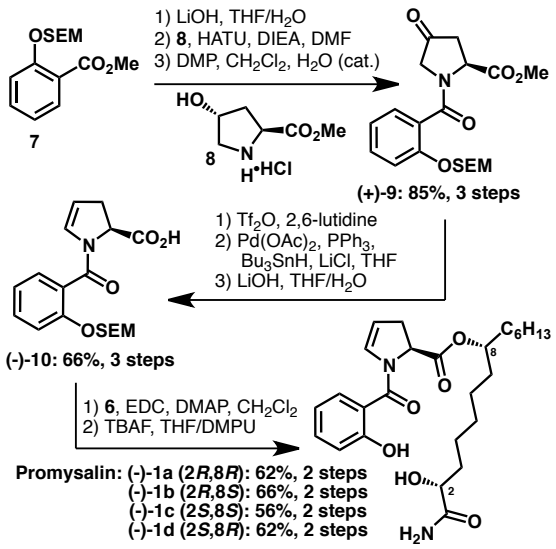
provide (+)-**9** (Scheme 2). At this stage, we sought to develop a method for the regioselective dehydration of (+)-**9** to give the delicate enamine functionality. To this end, we treated the ketone with triflic anhydride and 2,6-lutidine to provide the desired enol-triflate which was cleanly reduced using a modified Stille reaction furnishing the corresponding enamine with the desired regiochemistry found in the natural product.[15] Base hydrolysis of the methyl ester ultimately led to the key coupling fragment (-)-**10** in six steps and an overall yield of 56%.

EDC-mediated esterification of alcohols **6a-d** with (-)-**10** proceeded smoothly to give all four diastereomers of fully protected promysalin. As is the case in many total syntheses, the final global deprotection proved to be non-trivial. Most literature methods of SEM-deprotection call for either Brønsted or Lewis acidic conditions or fluoride (TBAF or TASF) at elevated temperatures. Unfortunately, the substrate was unstable to both prolonged heat and/or acid, providing only trace amounts of the desired product. Undeterred, we sought milder deprotection conditions. After much experimentation we found that 1M TBAF in THF with DMPU as a co-solvent cleanly removed both silyl protecting groups in a single operation.[16] Our method of SEM-deprotection provides a straightforward alternative to previous published precedent as it is performed at ambient temperature, using commercially available TBAF/THF solution, and with short reaction times (30-60 min).

With the four diastereomers in hand we set out to unequivocally define the relative and absolute stereochemistry through both NMR spectral comparison and biological assays. Upon careful examination of the chemical shift difference in the ^{1}H and ^{13}C NMR spectra of compounds **1a-d**, we identified distinct features that were used to assess the cor-

rect configurations at C2 and C8. As shown in Figure 2, the spectral data of compound **1a** best correlates to that of the isolated material when compared to the other diastereomers. Key chemical shifts of protons located on C3, C7, C9, and C19 strongly indicate that (2*R*,8*R*,16*S*) is the proper relative stereochemical assignment for promysalin. Unfortunately, without a reported optical rotation nor authentic sample available we were unable to unequivocally determine the absolute configuration. We postulated that only the correct enantiomer would elicit the biological responses reported previously and, therefore, we sought to recapitulate both the inhibitory activity with

Scheme 2. Synthesis of Promysalin Diastereomers 1a-d.



compounds **1a-d** versus *PAO1* and *PA14* and the swarming activity identified in the producing strain.

In De Mot's initial report, they surveyed the biological activity of >100 bacterial strains through halo diffusion assays with co-treatment of the producing organism noting qualitative inhibition.[8] More specifically, they quantified the IC_{50} value for *PA14* providing a strain by which we could directly compare. In accordance with their findings, compound (-)-**1a** possessed the most potent biological activity of the four compounds with an IC_{50} of 125 nM against *PA14* (1.8 μM reported) and 1 μM for *PAO1* (not reported). Compounds **1b-d** were each ~10-60 times less effective against both strains (Figure 3A, left). Taken in sum, we propose that the absolute stereochemistry of promysalin be assigned as (2*R*,8*R*,16*S*) and depicted by structure (-)-**1a** (Scheme 2).

In contrast to the activity observed in *PA*, promysalin has been shown to increase both swarming and biofilm formation in *PP* RW10S1. We were curious as to whether the compound would elicit a swarming response in multiple strains of *PP* or solely in the producing organism. As can be seen in Figure 3B, compound **1a** clearly promotes swarming in the native producing organism and three other species (two strains of *PP* and one of *P. fluorescens* (*PF*)). Curiously, the strain *PP* OUS82, which was originally isolated from oil-contaminated soil and is known for its ability to cannibalize hydrocarbons, was unaffected.[17] *PP* OUS82 has been previously shown to contain enzymes capable of degrading salicylate; therefore, it is possible that the bacteria is consuming promysalin before it is able to elicit a biological response. Nevertheless, these results indicate that (-)-**1a** is responsible for a swarming phenotype in a broad range of closely related organisms.

The main mode by which bacteria swarm is through biosurfactant production.[18] We postulated that **1a** could be acting either directly as a biosurfactant or as a trigger for biosurfactant production. In order to differentiate the former from the latter, we performed a simple surface tension assay. We found that all four diastereomers displayed similar biosurfactant-properties at equimolar concentrations (Figure S1). It is well established that amphipathic molecules, like rhamnolipids, can

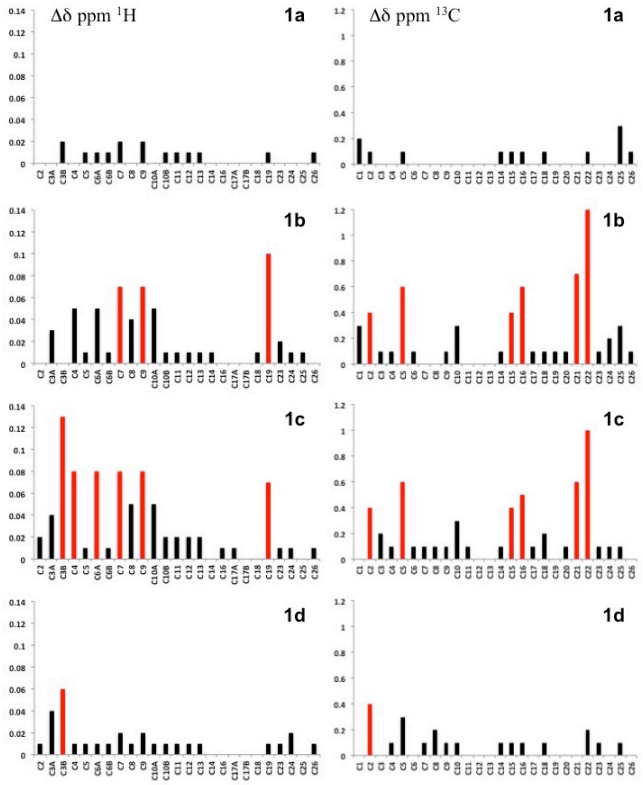


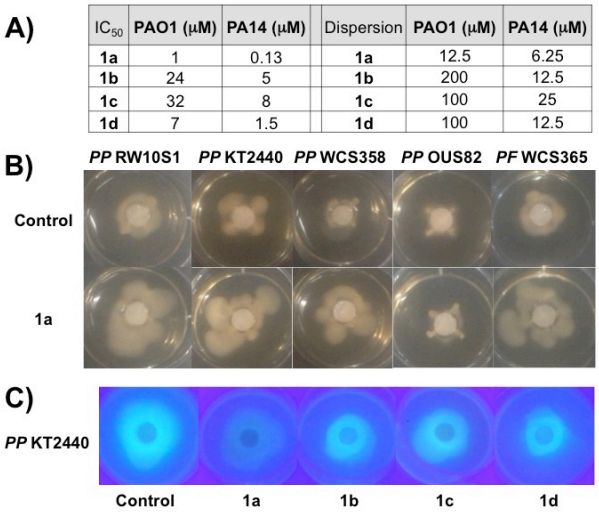
Figure 2. Comparative ^{1}H NMR (left) and ^{13}C NMR (right) spectra depicting the absolute Δδppm of compounds **1a-d** when compared to the natural product. Red lines indicate Δδppm values of >0.06 (^{1}H NMR) and >0.4 (^{13}C NMR). For numerical comparison see Table S1.

disperse and/or eradicate mature biofilms.[19] We hypothesized that if compounds **1a-d** acted solely as biosurfactants then all would possess equipotent dispersant activity. To test this proposal, we grew mature biofilms of both *PA14* and *PAO1* for 24 hours and then dosed each trial with varying concentrations of compounds **1a-d**. All diastereomers dispersed *PA* biofilms at 100 μM; however, compound (-)-**1a** again showed the most potent biological activity, dispersing both biofilms at 6.25 and 12.5 μM, respectively (Figure 3A,

right and Figure S4). These results suggest that promysalin is acting on a specific target.

Finally, during the course of these studies we serendipitously observed that **1a** inhibited fluorescence in *PP* KT2440 when compared to either the control or compounds **1b-d** (Figure 3C). Pyoverdine is a siderophore produced by a wide-range of *Pseudomonads* and is responsible for their fluorescent properties.[20] Furthermore, it has been shown that pyoverdine-deficient mutants of *P. syringae* pv. *tabaci* 6605 exhibited reduced virulence in host tobacco infection.[21] Recent reports have shown that strains deficient in pyoverdine have increased swarming and biosurfactant phenotypes,[22] in accordance with observations reported herein. Taken together, this suggests that promysalin is either directly or indirectly affecting pyoverdine biosynthesis and/or transport in this strain.

In conclusion, we report a concise, stereocontrolled synthesis of the four diastereomers of the L-proline series of the natural product promysalin guided by bioinformatics. The compounds were synthesized in a longest linear sequence of eight



IC_{50}	PAO1 (μM)	PA14 (μM)	Dispersion	PAO1 (μM)	PA14 (μM)
1a	1	0.13	**1a**	12.5	6.25
1b	24	5	**1b**	200	12.5
1c	32	8	**1c**	100	25
1d	7	1.5	**1d**	100	12.5

Figure 3. A) The concentrations of compounds **1a-d** at which 50% of growth is inhibited (left) and visual effects of dispersion are observed (right) against PAO1 and PA14. **B)** Swarming assays, performed on 1% agar and visualized after 24 hrs. **C**) Visualization of pyoverdine production by *PP* KT2440 when treated with control (DMSO) and compounds **1a-d** with UV light.

steps from known compound **7** in 31-37% overall yield. This culminated in compound (-)-**1a**, identical by ^{1}H NMR, ^{13}C NMR, and HRMS to that of the isolated material, which we propose to be the structure of promysalin. Furthermore, biological investigations support that the synthesized enantiomer is that of the natural product. Finally, we demonstrate, for the first time, that promysalin disperses established biofilms and inhibits pyoverdine production, two pathogenic phenotypes, which may hint at the role the compound plays in the rhizosphere. The potential of promysalin acting specifically on pyoverdine-related processes is enticing as it could provide a novel method to combat virulence both in agricultural and human health. Current work in our laboratory is focused on deciphering the target triggering *PA* biofilm dispersion and whether the molecule is acting directly on pyoverdine production or through a pyoverdine-signaling pathway. The route presented herein allows for the preparation of gram-quantities of the natural product and analogs to better understand the specific target of promysalin, all of which will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, NMR spectra, and supporting figures. This material is available free of charge on the ACS Publications website at http://pubs.acs.org..

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Notes

The authors declare no competing financial interest.

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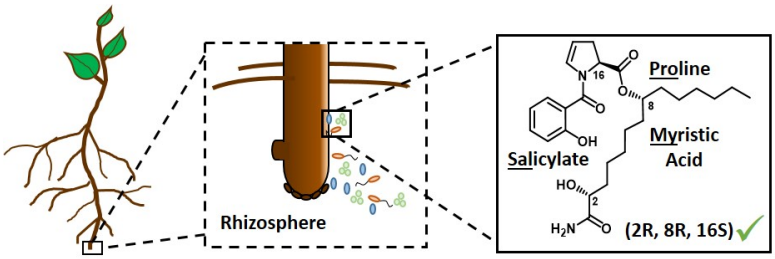

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