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Total Synthesis and Biological Investigation of (−)-Promysalin

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Supporting Information

ABSTRACT: Compounds that specifically target pathogenic bacteria are greatly needed, and identifying the method by which they act would provide new avenues of treatment. Herein we report the concise, high-yielding total synthesis (eight steps, 35% yield) of promysalin, a natural product that displays antivirulence phenotypes against pathogenic bacteria. Guided by bioinformatics, four diastereomers were synthesized, and the relative and absolute stereochemistries were confirmed by spectral and biological analysis. Finally, we show for the first time that promysalin displays two antivirulence 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 a profound impact on our understanding of life, most notably in the human body. There has been a call to provide small-molecule probes that could be used to deconvolute such systems by specifically targeting pathogenic 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 be of value not only to farming but also to human health (i.e., oral, gastrointestinal) and other commercial interests.

A well-studied example of a multispecies community is the root system of plants, generally termed the rhizosphere microbiome.[3] The predominant players in this arena are the *Pseudomonads*, which comprise 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 co-workers isolated a novel metabolite, promysalin (**1**), from *Pseudomonas putida* (*PP*) RW10S1, which resides in the rhizosphere of rice plants (Figure 1).[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 shows 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* in clinical settings[9] (cystic fibrosis, immunocompromised patients) and in agriculture, promysalin could serve as an attractive alternative to current therapies. The

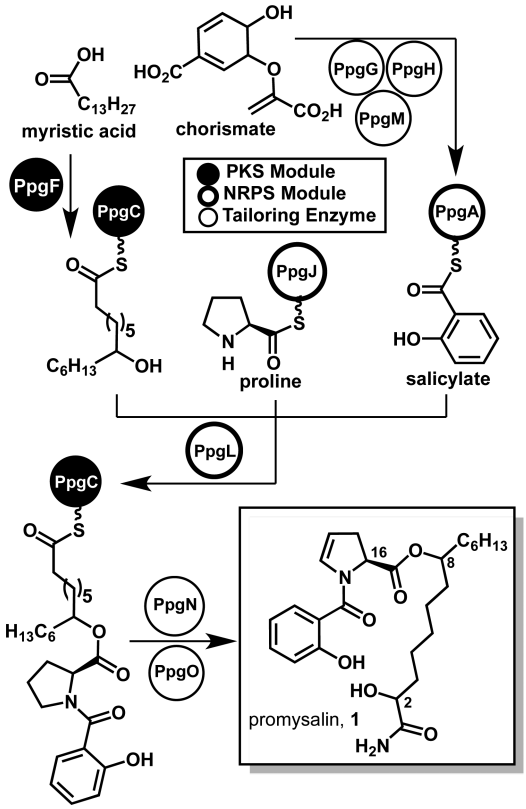

Figure 1. Proposed biosynthesis of promysalin. Polyketide synthase (PKS), NRPS, and tailoring enzymes (Rieske iron−sulfur cluster, asparagine synthase, chorismate synthase) are depicted.

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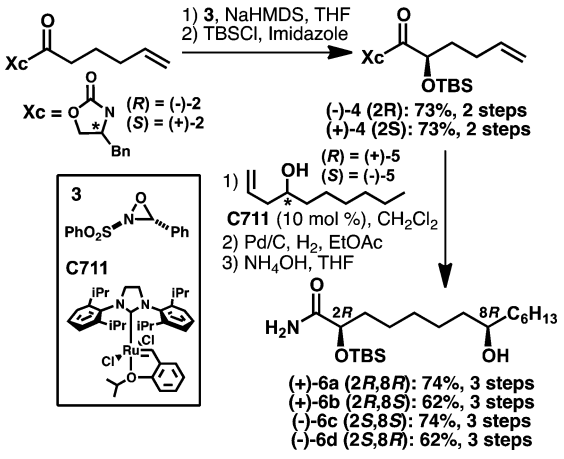
unique bioactivity, unknown mode of action, and structural ambiguity are what prompted the synthesis reported herein.

Before initiating our synthetic investigation, we sought to reannotate the biosynthetic gene cluster using AntiSMASH (Figure 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 nonribosomal peptide synthetase (NRPS) module containing both adenylation (A) and thiolation domains but lacking a condensation domain, reminiscent of *sylC* found in *Pseudomonas syringae*.[11] Upon closer inspection, we were unable to identify any putative epimerase or thioesterase domains contained in either the characterized gene cluster or the flanking regions. Bioinformatic investigation of the *ppgJ* A domain revealed the Stachelhaus code,[12] DVQFVAHV, corresponding to the selective activation of L-proline as previously hypothesized by De Mot.[8] This exercise led us to the conclusion that the absolute configuration of the C16 stereocenter should be assigned as (*S*). On the basis of these results, 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 the construction of the four diastereomers of the myristic acid fragment (Scheme 1). We

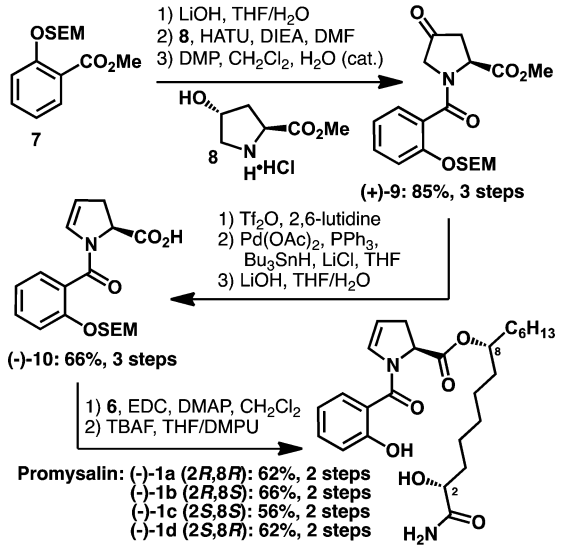
Scheme 1. Synthesis of Aliphatic Precursors 6a–d



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 oxaziridine[14] followed by silyl protection furnished compound (−)-**4** in good yield. Cross-metathesis with the known enantiomerically pure homoallylic alcohol (+)-**5** or (−)-**5** in the presence of catalyst **C711**,[15] subsequent hydrogenation, and ammonolysis provided diastereomers (+)-**6a** and (+)-**6b**, respectively. Analogously, the enantiomeric series of compounds (−)-**6c** and (−)-**6d** were synthesized starting with (+)-**2**. This route provides concise access to all four diastereomers in enantiomerically pure form (45–54% yield over five steps).

The synthesis of the proline–salicylate fragment commenced with ester hydrolysis of (2-(trimethylsilyl)ethoxy)methyl (SEM)-protected methyl salicylate, amidation with *trans*-4-L-hydroxyproline methyl ester, and Dess–Martin oxidation to provide (+)-**9** (Scheme 2). At this stage, we sought to develop a

Scheme 2. Synthesis of Promysalin Diastereomers 1a–d



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 to furnish the corresponding enamine with the desired regiochemistry found in the natural product.[16] 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 nontrivial. Most literature methods for 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 1 M TBAF in THF with DMPU as a cosolvent cleanly removed both silyl protecting groups in a single operation.[17] Our method of SEM deprotection provides a straightforward alternative to previously published precedent, as it is performed at ambient temperature using commercially available TBAF/THF solution and short reaction times (30–60 min).

With the four diastereomers in hand, we set out to unequivocally define the relative and absolute stereochemistries through both NMR spectral comparison and biological assays. Upon careful examination of the chemical shift differences in the ^{1}H and ^{13}C NMR spectra of compounds **1a–d**, we identified distinct features that were used to assess the correct configurations at C2 and C8. As shown in Figure 2, the spectral data of compound **1a** best correlate to those of the isolated material compared with the other diastereomers. Key chemical shifts of protons located on C3, C7, C9, and C19 strongly

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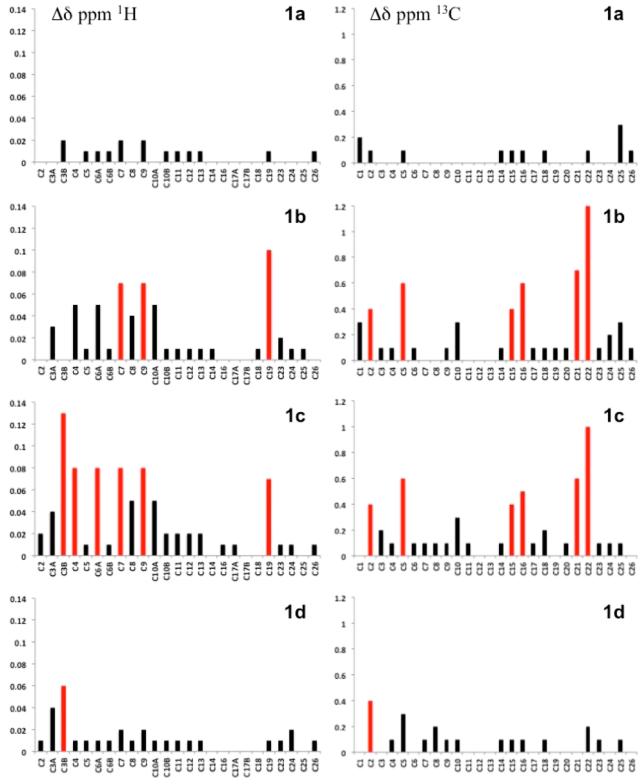
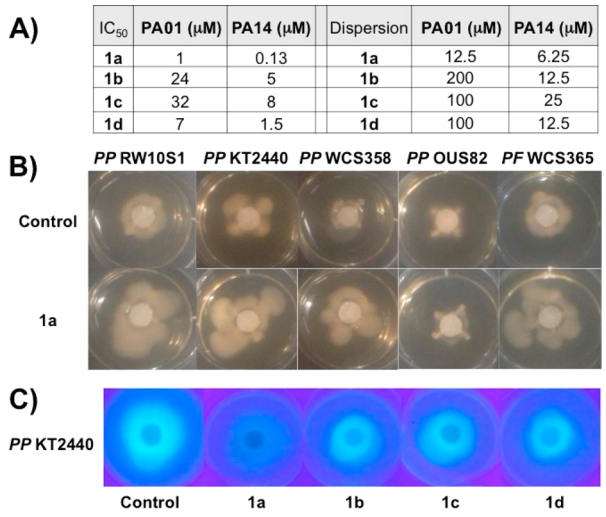


Figure 2. Comparative (left) ^{1}H and (right) ^{13}C NMR spectra depicting the absolute $\Delta\delta$ of compounds **1a–d** relative to the natural product. Red lines indicate $\Delta\delta$ values of >0.06 (^{1}H NMR) and >0.4 (^{13}C NMR). For numerical comparison, see Table S1 in the Supporting Information.

indicate that (2*R*,8*R*,16*S*) is the proper relative stereochemical assignment for promysalin. Unfortunately, with neither a reported optical rotation nor an authentic sample available, we were unable to determine the absolute configuration unequivocally. 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 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 activities of >100 bacterial strains through halo diffusion assays with cotreatment of the producing organism, noting qualitative inhibition.[8] More specifically, they quantified the IC_{50} value for *PA14*, providing a strain with which we could directly compare. In accordance with their findings, compound (−)-**1a** possessed the most potent biological activity of the four compounds, with IC_{50} values of 125 nM against *PA14* (1.8 μM reported) and 1 μM against *PAO1* (not reported). Compounds **1b–d** were each ∼10–60 times less effective against both strains (Figure 3A left). On the basis of these results, we propose that the absolute stereochemistry of promysalin be assigned as (2*R*,8*R*,16*S*), as 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 *Pseudomonas fluorescens* (*PF*)). Curiously, the



IC_{50}	PA01 (μM)	PA14 (μM)	Dispersion	PA01 (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) 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 h. (C) Pyoverdine production by *PP* KT2440 upon treatment with control (DMSO) and compounds **1a–d**, visualized with UV light.

strain *PP* OUS82, which was originally isolated from oil-contaminated soil and is known for its ability to cannibalize hydrocarbons, was unaffected.[18] *PP* OUS82 has been previously shown to contain enzymes capable of degrading salicylate; therefore, it is possible that the bacteria consumed promysalin before it was 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.[19] We postulated that **1a** could act 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 in the Supporting Information). It is well-established that amphipathic molecules, such as rhamnolipids, can disperse and/or eradicate mature biofilms.[20] We hypothesized that if compounds **1a–d** act 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 h and then dosed each trial with varying concentrations of compounds **1a–d**. All of the diastereomers dispersed *PA* biofilms at 100 μM; however, compound (−)-**1a** again showed the most potent biological activity, dispersing biofilms at both 6.25 and 12.5 μM (Figure 3A right and Figure S4). These results suggest that promysalin acts on a specific target.

Finally, during the course of these studies we serendipitously observed that **1a** inhibits fluorescence in *PP* KT2440 compared with 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.[21] Furthermore, it has been shown that pyoverdine-deficient mutants of *P. syringae* pv *tabaci* 6605 exhibit reduced virulence in host tobacco infection.[22] Recent reports have shown that strains deficient in pyoverdine have increased swarming and biosurfactant phenotypes,[23] in accordance with observations reported herein. Taken together, these results

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suggest that promysalin either directly or indirectly affects pyoverdine biosynthesis and/or transport in this strain.

In conclusion, we have reported 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 steps from known compound **7** in 31−37% overall yield. This culminated in compound (−)-**1a**, which is identical to the isolated material as determined by ^{1}H NMR, ^{13}C NMR, and HRMS analyses and proposed to be the structure of promysalin. Furthermore, biological investigations support that the synthesized enantiomer is that of the natural product. Finally, we have demonstrated 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 to act 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 acts directly on pyoverdine production or through a pyoverdine-signaling pathway. The route presented herein allows the preparation of gram quantities of the natural product and analogues 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. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04767.

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Notes

The authors declare no competing financial interest.

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