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Characterization of an Orphan Diterpenoid Biosynthetic Operon from Salinispora arenicola

Abstract

While more commonly associated with plants than microbes, diterpenoid natural products have been reported to have profound effects in marine microbe–microbe interactions. Intriguingly, the genome of the marine bacterium *Salinispora arenicola* CNS-205 contains a putative diterpenoid biosynthetic operon, *terp1*. Here recombinant expression studies are reported, indicating that this three-gene operon leads to the production of isopimara-8,15-dien-19-ol (**4**). Although **4** is not observed in pure cultures of *S. arenicola*, it is plausible that the *terp1* operon is only expressed under certain physiologically relevant conditions such as in the presence of other marine organisms.

Keywords

Salinispora arenicola, marine, terp1

Disciplines

Biochemistry, Biophysics, and Structural Biology | Marine Biology | Natural Products Chemistry and Pharmacognosy

Comments

Rights

Authors

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Note

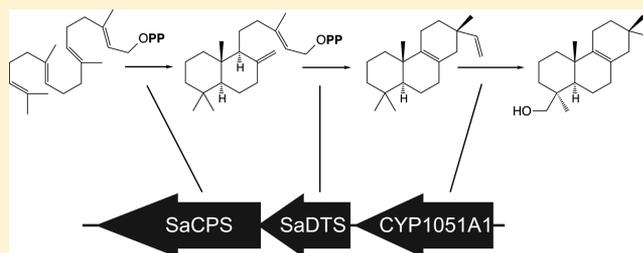# Characterization of an Orphan Diterpenoid Biosynthetic Operon from *Salinispora arenicola*

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Ⓢ *Supporting Information*

ABSTRACT: While more commonly associated with plants than microbes, diterpenoid natural products have been reported to have profound effects in marine microbe–microbe interactions. Intriguingly, the genome of the marine bacterium *Salinispora arenicola* CNS-205 contains a putative diterpenoid biosynthetic operon, *terp1*. Here recombinant expression studies are reported, indicating that this three-gene operon leads to the production of isopimara-8,15-dien-19-ol (**4**). Although **4** is not observed in pure cultures of *S. arenicola*, it is plausible that the *terp1* operon is only expressed under certain physiologically relevant conditions such as in the presence of other marine organisms.

The production of terpenoids is most commonly associated with plants, which have clearly expanded their ability to produce this class of natural products. For example, terpene synthases form moderate sized gene families, with more than 10 such enzymatic genes found in each of the known vascular plant genome sequences.[1] By contrast, there appear to be just over 100 terpene synthases among the more than 1000 sequenced bacterial genomes, suggesting the relative scarcity of terpene synthases and, hence, terpenoid production, among commonly studied bacterial genera.[2]

This relative paucity of bacterial terpenoid production is also illustrated by the labdane-related diterpenoids, a large super-family of ~7000 known natural products whose biosynthesis is characterized by the initiating reaction. Specifically, acid–base catalyzed bicyclization of the general diterpenoid precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **1**), which is mediated by class II diterpene cyclases that generally form the eponymous labdadienyl/copalyl diphosphate (CPP). This is typically followed by an additional cyclization and/or rearrange-ment reaction initiated by ionization of the allylic diphosphate catalyzed by a class I diterpene synthase.[3] All vascular plants have at least one class II diterpene cyclase in order to produce the requisite gibberellin phytohormones, and many plant species have multiple such enzymes.[4] However, less than 100 class II diterpene cyclases are present in the known bacterial genomes[5] and less than 10 have been characterized.[6–12]

Nevertheless, there are a handful of bacteria that produce labdane-related diterpenoids of significant interest. The relevant class II diterpene cyclases have been identified for a number of these bacterial natural products, including such enzymes involved in the production of gibberellin phytohormones by plant symbiotic rhizobia and phytopathogens,[9,11,12] the potential antibacterial and antidiabetic compounds platencin and platensimycin by *Streptomyces platensis*,[10] and an immuno-modulatory factor by *Mycobacterium tuberculosis*.[8] Intriguingly, it has been reported that an epiphytic marine bacterium produces a labdane-related diterpenoid that acts at subpico-molar levels to promote the aggregation of marine green macroalgae (e.g., sea lettuce).[13]

With the advent of next generation sequencing, there has been a tremendous increase in the availability of microbial genome sequences,[14] which has generally revealed that there are many more putative natural product biosynthetic gene clusters than known metabolites.[15] A variety of approaches have been taken toward identifying the compounds resulting from such orphan operons.[16] Here is reported the use of recombinant expression to characterize a bioinformatics-predicted labdane-related diterpenoid biosynthetic operon from the marine bacterium *Salinispora arenicola* CNS-205.

The genome sequence of the CNS-205 strain of the widely distributed marine actinobacterium *S. arenicola* was previously reported, with bioinformatic analysis indicating the presence of 30 putative natural product biosynthetic gene clusters.[17] Included among these was *terp1*, annotated as producing an unidentified diterpene. This small operon contains genes encoding a putative class II diterpene cyclase (Sare_1288) and class I (di)terpene synthase (Sare_1287) as well as a cytochrome P450 (CYP) mono-oxygenase (Sare_1286) that has been assigned as CYP1051A1 (Figure 1). Thus, the *terp1*

Figure 1. Schematic of the *terp1* diterpenoid biosynthetic operon from *S. arenicola* CNS-205.

operon is predicted to encode for the production of a labdane-related diterpenoid, although this appears to be unique to the originally sequenced CNS-205 strain,[17] as similar sequences do not seem to be present in the other 36 strains whose genome sequences have recently been reported.[18]

The putative class II diterpene cyclase is most closely related to two previously characterized *ent*-CPP synthases (CPSs) from *Streptomyces* species of terrestrial actinobacteria (42−45% amino acid sequence identity)[7,10] and is referred to henceforth as SaCPS. While class II diterpene cyclases almost invariably contain a DxDD motif that cooperatively acts as the catalytic acid,[19] SaCPS contains a Thr in place of the last Asp (Supporting Information (SI), Figure S1). Such a variant DxDT motif has been previously noted in the active class II diterpene cyclase from *M. tuberculosis*,[8] suggesting that SaCPS might be functional.

Similarly, the putative class I diterpene synthase is most closely related to a previously characterized *ent*-pimaradiene synthase from *Streptomyces* sp. KO-3988 (~30% amino acid sequence identity)[20] and is referred to henceforth as SaDTS. Although class I terpene synthases almost invariably contain a DDxD motif involved in binding the requisite divalent magnesium ion cofactors,[21] SaDTS does not contain a corresponding sequence. Amino acid sequence alignments indicate that the corresponding sequence in SaDTS is EDWQVD$_{83-88}$ instead (SI, Figure S2). While the *ent*-kaurene synthase from *S. platensis* also does not have the canonical DDxD motif at this position, sequence conforming to the DDxD motif can be found nearby,[10] which is not true in SaDTS. On the other hand, SaDTS does contain the NDxxSxxxE motif also involved in binding the requisite divalent magnesium ion co-factors,[21] leaving open the possibility that SaDTS may be active as well.

Characterization of the bioinformatics-predicted labdane-related diterpenoid product of this operon was undertaken with a previously developed modular metabolic engineering system.[22] This enabled recombinant expression of SaCPS with a GGPP synthase (GGPS) in *Escherichia coli*, which led to the production of CPP, observed as the dephosphorylated copalol by GC-MS analysis of hexane extracts of the induced culture (Figure 2A). To determine the absolute configuration of this CPS, SaCPS was co-expressed with GGPS and selective class I diterpene synthases, much as previously described for investigation of other class II diterpene cyclases. Briefly, SaCPS and the GGPS were co-expressed with either the *ent*-kaurene synthase from *Arabidopsis thaliana* (AtKS) that selectively reacts with *ent*-CPP,[9] or a mutant of the abietadiene synthase from *Abies grandis* (AgAS) that no longer exhibits class II activity and only reacts with normal CPP (AgAS:D404A).[23] No *ent*-kaurene was observed upon co-expression of SaCPS (and GGPS) with AtKS, while the same products made by wild-type AgAS were readily observed upon co-expression with AgAS:D440A (SI, Figure S3). Thus, SaCPS makes normal (5*S*,9*S*,10*S*) CPP (**2**).

Co-expression of SaDTS with GGPS and SaCPS led to production of an unknown diterpene, observed by GC-MS analysis of hexane extracts of the induced culture. To determine the structure of this compound, SaDTS was co-expressed with GGPS and a plant CPS in *E. coli* (Figure 2B), which produces larger quantities of **2** (i.e., than SaCPS), and the culture volume was increased to 2 L. This enabled isolation of ~2 mg for NMR analysis (SI, Figures S4−S6 and Table S1), which indicated that this was a (iso)pimara-8,15-diene, with resolution of the configuration at C-13 derived from comparison to previously reported NMR chemical shift data,[24,25] which led to assignment of the SaDTS product as isopimara-8,15-diene (**3**).

To functionally characterize CYP1051A1, it was necessary to account for the fact that CYPs require the input of electrons, generally obtained from NADPH, and in the case of bacterial CYPs typically provided by a ferredoxin (Fdx) that has been reduced by a ferredoxin reductase (FdR).[26] Accordingly, CYP1051A1 was co-expressed with an Fdx and FdR from *S. arenicola* (Sare_4141 and Sare_0646, respectively) in a strain of
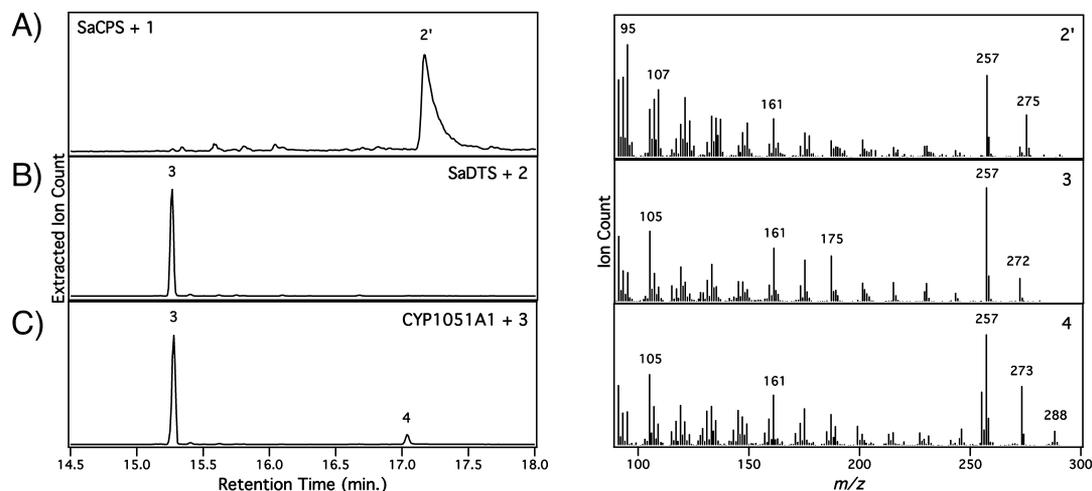
Figure 2. GC-MS extracted ion (*m/z* = 257) chromatograms and associated mass spectra for (A) production of CPP (**2**), detected as dephosphorylated copalol (**2′**; retention time, RT = 17.16 min), from expression of SaCPS in a strain of *E. coli* engineered to make GGPP (**1**) by SaCPS. (B) Isopimara-8,15-diene (**3**; RT = 15.27 min) from expression of SaDTS in a strain engineered to make **2**. (C) Isopimara-8,15-dien-19-ol (**4**; RT = 17.04 min) from co-expression of CYP1051A1 with an Fdx and Fdr in a strain engineered to make **3**.

Scheme 1. Labdane-Related Diterpenoid Biosynthetic Pathway Encoded by _S. arenicola_ CNS-205 _terp1_ Operon
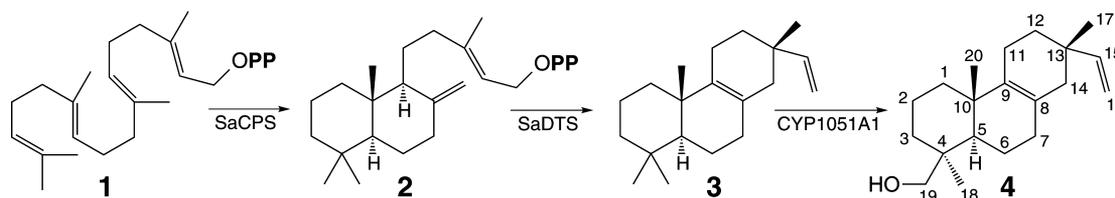
E. coli also engineered to produce **3**. The resulting recombinant strain produced a hydroxylated derivative of **3**, observed by GC-MS analysis of an organic solvent extract (Figure 2C). Attempts to scale up production of the CYP1051A1 product were not fruitful, with a net yield of only ~100 μg from 5 L of culture. Fortuitously, it was discovered that CYP99A3 from rice[27] catalyzed the same reaction (SI, Figure S7) and was more amendable to scale-up. Thus, CYP99A3 was used to produce enough compound (~1 mg), which was mixed with the ~0.1 mg isolated from CYP1051A1 for NMR analysis (SI, Figures S4, S8, and S9 and Table S2). Only a single peak was found in the alcohol region of the ¹³C spectra (SI, Figure S9), consistent with equivalence of the CYP99A3 and CYP1051A1 products, which the collected data indicated was isopimara-8,15-dien-19-ol (**4**). The configuration at C-4 was suggested by the observation of an NOE signal between the secondary alcohol hydrogens on C-19 and the methyl hydrogens of C-20, whose configuration was already known (vide supra) and verified by comparison to previously reported NMR chemical shift data.[28] Thus, the *S. arenicola terp1* operon presumably can lead to the production of isopimara-8,15-dien-19-ol (Scheme 1).

It has been previously reported that at least two species of *Streptomyces* grown in certain media produce the diterpenoid viguiepinol (3α-hydroxy-*ent*-pimara-9(11),15-diene) and derived oxaloterpins, which are very similar to **4**.[29,30] To determine whether *terp1* pathway metabolites were produced by *S. arenicola* CNS-205, this actinomycete was fermented in four different media (SI), and chemical extracts were evaluated by GC-MS with selected ion monitoring for **3** and **4**. None of the evaluated fermentation conditions afforded production of **3** or **4** at levels detectable by GC-MS. Chemical profiles were also compared among wild type *S. arenicola* CNS-205 and PCR-targeted *terp1* gene replacement mutants to facilitate the detection of *terp1* operon-derived metabolites that are modified by additional enzymes in vivo. No differences in GC-MS or LC-MS metabolite profiles were noted for extracts from wild type *S. arenicola* CNS-205, a *sare_1286::Apr^R* mutant lacking the CYP1051A1 cytochrome P450 enzyme and a *sare_1287::Apr^R* mutant lacking the DTS class I (di)terpene synthase enzyme. These data suggest that the *terp1* gene cluster is either inactive in *S. arenicola* CNS-205 under evaluated conditions or that diterpenoid titers are below the limit of detection.

In conclusion, functional characterization of the orphan *terp1* biosynthetic operon from *S. arenicola* is reported here. While the *terp1* operon is only found in strain CNS-205, these results further illuminate the natural products capacity of these widely distributed marine actinobacteria. Although the resulting diterpenoid **4** cannot be found in pure cultures of *S. arenicola* CNS-205 grown in a variety of media, it is possible that **4** serves an ecological function and is only produced by *S. arenicola* CNS-205 under specific environmental conditions (e.g., in the presence of other organisms). Alternatively, it has been previously noted that all other characterized bacterial

diterpenoid biosynthetic operons contain a GGPS as bacteria do not generally produce **1** otherwise,[7,10,12,31−36] and the lack of a GGPS in the *S. arenicola* CNS-205 *terp1* operon may underlie the observed lack of production of **4**. In any case, these results demonstrate the applicability of the utilized modular metabolic engineering system to elucidation of (di)terpenoid biosynthetic operons uncovered by genome mining of actino- and potentially other bacteria as well.

■ EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectroscopic data were recorded on a Bruker Avance 500 spectrometer equipped with cryogenic probe for ¹H and ¹³C measurements (Bruker). GC-MS analyses were carried out using a Varian 3900 GC with Saturn 2100 ion trap mass spectrometer equipped with HP-5 ms capillary column (30 m × 0.25 mm × 0.25 μm) in electron ionization (70 eV) mode (Agilent/Varian). Column chromatography was performed with 80 × 200 mesh silica gel (Fisher Scientific). HPLC was carried out with a ZORBAX Eclipse XDB-C8 column (4.6 mm × 150 mm, 5 μm) on an Agilent 1100 series system equipped with fraction collector and diode array detector. Solvents for chromatographic separations were purchased from Fisher Scientific.

Methods Summary. The genes from *S. arenicola* described herein were cloned from the fosmids used in its genome sequencing project.[17] These were cloned into the Gateway vector system (Invitrogen) to enable their use in the metabolic engineering system, including co-expression with previously characterized enzymes (i.e., to investigate configurations or increase yield). Enzymatic products were analyzed by GC-MS of organic extracts from the relevant recombinant cultures. Where necessary (i.e., for **3** and **4**), these cultures were scaled up to enable production and purification of larger quantities for structural characterization by NMR.

Isopimara-8,15-diene (3). Previously described as a colorless solid;[37,38] ¹H and ¹³C NMR, as well as MS, data largely match literature values,[24,25,39] with the few significant differences supported here by HMBC correlations (SI, Table S1).

Isopimara-8,15-dien-19-ol (4). Previously described as a colorless solid;[38] ¹H and ¹³C NMR data largely match literature values,[28] again with the few significant differences supported here by HMBC correlations (SI, Table S2).

■ ASSOCIATED CONTENT

ⓢ Supporting Information
Detailed description of experimental methods and characterization of the various compounds, along with supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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