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Cryptic and Stereospecific Hydroxylation, Oxidation, and Reduction in Platensimycin and Platencin Biosynthesis

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Abstract

Platensimycin (PTM) and platencin (PTN) are highly functionalized bacterial diterpenoids of *ent*-kauranol and *ent*-atiserene biosynthetic origin. C7 oxidation in the B-ring plays a key biosynthetic role in generating structural complexity known for *ent*-kaurane and *ent*-atisane derived diterpenoids. While all three oxidation patterns, α -hydroxyl, β -hydroxyl, and ketone, at C7 are seen in both the *ent*-kaurane and *ent*-atisane derived diterpenoids, their biosynthetic origins remain largely unknown. We previously established that PTM and PTN are produced by a single biosynthetic machinery, featuring cryptic C7 oxidations at the B-rings that transform the *ent*-kauranol and *ent*-atiserene derived precursors into the characteristic PTM and PTN scaffolds. Here, we report a three-enzyme cascade affording C7 α -hydroxylation in PTM and PTN biosynthesis. Combining *in vitro* and *in vivo* studies, we show that PtmO3 and PtmO6 are two functionally redundant α -ketoglutarate-dependent dioxygenases that generate a cryptic C7 β -hydroxyl on each of the *ent*-kauranol and *ent*-atiserene scaffolds and PtmO8 and PtmO1, a pair of NAD⁺/NADPH-dependent dehydrogenases, subsequently work in concert to invert the C7 β -hydroxyl to α -hydroxyl via a C7 ketone intermediate. PtmO3 and PtmO6 represent the first dedicated C7 β -hydroxylases characterized to date, and, together with PtmO8 and PtmO1, provide

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and methods. Strains, plasmids, and primers used in this study (Tables S1–S3); summary of NMR data for compounds **6**, **7**, **10**, and **11** (Tables S4 and S5); chemical synthesis of **13** and **14** (Schemes S1 and S2); C7 β -hydroxylation as an essential early biosynthetic step in the B-ring construction of gibberellins bio-synthesis (Figure S1); structures and proposed biosynthetic pathway of PTM, PTN, thioPTM, and thioPTN (Figure S2); Southern analysis of mutants SB12045–SB12049 (Figures S3–S5, S28 and S29); Selected 1D and 2D NMR spectra of compounds **5–12** (Figures S6–S9, S11–S18, S20–S25 and S30–S35); SDS-PAGE analysis and size exclusion chromatography of PtmO3, PtmO6, PtmO8, and PtmO1 (Figures S10 and S36); Steady-state kinetics of PtmO3, PtmO6, PtmO8, and PtmO1 (Figures S19 and S37); Homology modeling and docking of substrates in PtmO6 (Figure S26); A proposed mechanism of stereoselective hydroxylation of PtmO6 (Figure S27); Homology models of PtmO8 and PtmO1 (Figure S38); A plausible biosynthetic pathway to the enone in PTM and PTN biosynthesis (Figure S39). X-ray crystallographic data for compounds **5**, **6**, and **8** (CIF)

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an account for the biosynthetic origins of all three C7 oxidation patterns that may shed light on other B-ring modifications in bacterial, plant, and fungal diterpenoid biosynthesis. Given their unprecedented activities in C7 oxidations, PtmO3, PtmO6, PtmO8, and PtmO1 enrich the growing toolbox of novel enzymes that could be exploited as biocatalysts to rapidly access complex diterpenoid natural products.

Graphical Abstract

INTRODUCTION

Terpenoids play essential physiological roles and are found in all living organisms, comprising the largest, most structurally diverse family of natural products with over 70,000 known compounds (<http://dnp.chemnetbase.com>). Diterpenoids, bio-synthetically originating from the C₂₀ precursor geranylgeranyl diphosphate, are well represented with ~18,000 members. For *ent*-kaurane and *ent*-atisane derived diterpenoids, C7 oxidation in the B-ring plays a key biosynthetic role in generating structural complexity via new ring formation, ring cleavage, and ring contraction (Figure 1A).^{1–4} Examples of diterpenoid natural products likely resulting from these modifications include plant hormone gibberellins (GAs), the plant-derived anticancer agent oridonin, maoecrystal V, and the diterpenoid alkaloid spiramine C (Figure 1A).^{1,5–7} All three oxidation patterns, α -hydroxyl, β -hydroxyl, and ketone, are seen at C7 in both the *ent*-kaurane and *ent*-atisane derived diterpenoids.^{1–4} C7 β -hydroxylation is known as an essential early biosynthetic step in the B-ring construction of the gibberellin skeleton. This committed step in GA biosynthesis is catalyzed by an *ent*-kaurenoic acid oxidase (KAO), as exemplified by CYP88A, P450–1, and CYP114 from plants, plant-associated fungi, and bacteria, respectively (Figure S1).^{8–11} However, they are not dedicated C7 hydroxylases; they continue to catalyze B-ring rearrangement to generate GA12-aldehyde (Figure S1). Furthermore, it remains unknown how Nature biosynthetically generates an α -hydroxyl or ketone at C7 in either *ent*-kaurane or *ent*-atisane derived diterpenoids.

Platensimycin (**1**, PTM) and platencin (**2**, PTN) are two highly functionalized bacterial diterpenoid natural products of *ent*-kauranol and *ent*-atiserene biosynthetic origin (Figure 1B).¹² They are potent inhibitors of bacterial and mammalian fatty acid synthases: PTN dually inhibits the chain-elongation condensing enzyme FabF/FabB and the chain-initiation condensing enzyme FabH while PTM selectively inhibits FabF/FabB.^{13,14} The chemical

structures of PTM and PTN are comprised of two distinct moieties, a 3-amino-2,4-dihydroxybenzoic acid (ADHBA) and an aliphatic ketolide derived from the diterpene scaffolds *ent*-kauranol and *ent*-atiserene, respectively, linked by an amide (Figure 1B). Within the ketolide moieties of PTM and PTN, the enone functional group is one of the key features as, in binding assays, the C6/C7 double bond keeps the B-ring in the boat conformation and positions the carbonyl group to interact with Ala309 of FabF.¹⁵ Saturation of the double bond resulted in a >10-fold loss of activity against FabF by antisense assay.¹⁵

We previously cloned and sequenced the *ptm* gene cluster from three PTM–PTN dual producers, *S. platensis* MA7327, CB00739, and CB00765, together with the *ptn* gene cluster from a PTN-exclusive producer, *S. platensis* MA7339.^{16,17} The *ptm* and *ptn* gene clusters are highly homologous in genetic organization and sequence, with the major difference being the inclusion of a 5.4-kb insertion, designated the PTM cassette, in the middle of the *ptm* gene cluster (Figure 2AB). Inactivation of the pathway-specific negative regulator *ptmR1* in one of these dual producers, CB00739, afforded the genetically-amenable PTM–PTN overproducer SB12029, which has been developed into a model strain to study and engineer PTM and PTN biosynthesis.¹⁸ In our previous biosynthetic studies of PTM and PTN, the C6/C7 double bond in the B-ring was proposed to be derived from dehydration of a C7 α -hydroxylated intermediate (Figures 1B and S2). Several C7 α -hydroxylated intermediates, either with the *ent*-kauranol or *ent*-atiserene skeletons, were identified as major products in multiple recombinant strains. The first intermediate bearing a C7 α -hydroxyl functional group, platencin SL4, was discovered by heterologous expression of the *ptn* biosynthetic gene cluster in a model host, *Streptomyces lividans* K4–114 (Figure S2C).¹⁹ Later, *ent*-kauranol derived intermediates with a C7 α -hydroxyl as major products, as well as their *ent*-atiserene derivative counterparts, were produced in *ptmO5*, *ptmA1*, and *ptmA2* mutants (Figure S2).^{20,21} However, the biosynthetic origin of this α -hydroxyl at the C7 position remains unknown, and thus the stage is set to explore novel chemistry and enzymology in B-ring modifications of *ent*-kauranol and *ent*-atiserene derived diterpenoids.

Here, we report an unusual three-step biosynthetic strategy, rather than one-step direct stereoselective α -hydroxylation, to install the C7 α -hydroxyl group in PTM and PTN biosynthesis by: (i) a pair of functionally-redundant α -ketoglutarate-dependent dioxygenases, PtmO3 and PtmO6, hydroxylates C7 generating a β -hydroxyl; (ii) a NAD⁺-dependent dehydrogenase, PtmO8, oxidizes the β -hydroxyl forming a C7 ketone; and (iii) a NADPH-dependent dehydrogenase, PtmO1, reduces the ketone finally affording a C7 α -hydroxyl. Kinetic studies, *in vitro* assays with substrate analogues, and homology modeling reveal insights into the stereoselective hydroxylation by PtmO3 and PtmO6 and their ability to process varying *ent*-kauranol and *ent*-atiserene scaffolds. PtmO3 and PtmO6 represent the first dedicated C7 β -hydroxylases characterized to date, and, together with PtmO8 and PtmO1, provide an account for the biosynthetic origins of all three C7 oxidation patterns that may shed light on other B-ring modifications in bacterial, plant, and fungal diterpenoid biosynthesis.

RESULTS AND DISCUSSION

Highly Homologous α -Ketoglutarate-Dependent Dioxygenases in the *ptm* Gene Cluster.

α -Ketoglutarate-dependent (Fe/ α KG) dioxygenases and cytochrome P450 monooxygenases are well-known candidates to catalyze C–H functionalization, thereby introducing oxygen functionalities into various hydrocarbon scaffolds, serving as excellent candidates for C7 oxidations as proposed for PTM and PTN biosynthesis (Figure 1B). The PTM cassette contains five open reading frames (*ptmO3*, *ptmO4*, *ptmT3*, *ptmO5*, and *ptmR3*, Figure 2A), three of which (*ptmO4*, *ptmT3*, and *ptmO5*) were experimentally characterized in our previous studies: PtmO4 as a long-chain acyl-CoA dehydrogenase necessary for late-stage β -oxidation of both PTM and PTN scaffolds;¹⁸ PtmT3 as a (16*R*)-*ent*-kauran-16-ol synthase, the first divergent step in the biosynthesis of PTM and PTN;²¹ and PtmO5 as a cytochrome P450 monooxygenase that catalyzes C11 hydroxylation of the *ent*-kaurane scaffold to initiate ether formation in PTM biosynthesis.^{21,22} PtmR3, a putative regulator, is unlikely to play a catalytic role in the biosynthesis of PTM and PTN.¹⁷ The last uncharacterized gene, located at the 5'-end of the PTM cassette, is *ptmO3*, encoding an Fe/ α KG dioxygenase. Although the enzymes involved in the PTM cassette were initially proposed to function solely in PTM biosynthesis,¹⁷ it is notable that *ptmO6*, which shares 99% sequence identity with *ptmO3*, is positioned as the first gene downstream of the 3'-end of the PTM cassette (Figure 2A). The concurrence of the highly identical *ptmO3* and *ptmO6* genes in the *ptm* gene cluster is likely a result from a gene duplication event (*vide infra*). Additionally, a homologue of *ptmO6*, *ptnO6*, is discovered in the *ptn* gene cluster (Figure 2A),¹⁷ suggesting a functional role of PtmO6/PtnO6 in the bio-synthesis of PTM and PTN. Taken together, we reasoned that PtmO3 and PtmO6 might be functionally redundant and unlikely to be PTM-specific.

In vivo Characterization of PtmO3 and PtmO6 Reveals Their Functional Redundancy.

To verify the function of PtmO3 and PtmO6 in PTM and PTN biosynthesis, we individually inactivated *ptmO3* and *ptmO6* in the PTM-PTN dual over-producer *S. platensis* SB12029,^{18,23} yielding SB12045 and SB12046, respectively. The genotypes of both mutants were confirmed by Southern analysis (Figures S3 and S4). Under the fermentation conditions previously developed for PTM and PTN production,¹⁸ both mutants were able to produce PTM (**1**) and PTN (**2**), as well as their thioacid analogues, thioPTM (**3**) and thioPTN (**4**),^{24,25} in comparable titers, thus confirming their functional redundancy (Figure 2C). We then constructed a *ptmO3*/*ptmO6* double mutant SB12047 and confirmed its genotype by Southern analysis (Figure S5). Under the same fermentation condition, SB12047 lost production of PTM, PTN, thioPTM, and thioPTN, but accumulated the known compound (**5**), (11*S*,16*S*)-*ent*-kauran-11,16-epoxy-19-oic acid (Figures 2CD, S6 and S7).^{18,26}

If **5**, a putative PTM intermediate, is indeed the substrate of PtmO3 and PtmO6, its counterpart in PTN biosynthesis, *ent*-atise-16-en-19-oic acid (**9**), should also accumulate in the *ptmO3*/*ptmO6* double mutant SB12047. However, due to the low production yield and undetectable nature of **9** by HRESIMS, we were unable to detect **9** from SB12047 fermentation initially, hence preventing us from its isolation. We therefore synthesized **9**

from (–)-steviol (Scheme S1).²⁷ Using chemically synthesized **9** as a standard, a larger scale (6-L) fermentation of SB12047 led to the isolation of 25 mg of **9**. The ¹H and ¹³C NMR spectra of **9** were identical to those reported in the literature (Figures 2D, S8 and S9).

PtmO3 and PtmO6 are Dedicated C7 β-Hydroxylases.

PtmO3 and O6 were overproduced as soluble proteins in *E. coli* (Figure S11A). Size exclusion chromatography suggested that these proteins exist as homodimers in solution (Figure S11B). Using boiled PtmO3 as a control, incubation of native PtmO3 with **5**, αKG, Fe²⁺, and ascorbic acid and subsequent analysis by LC-MS revealed the disappearance of **5** and a concomitant appearance of a new peak (**6**) (Figure 3AB). Surprisingly, although this new peak possessed an identical molecular weight (*m/z* at 333.2089 for the [M – H][–] ion) with that of **8**, their elution times were different (8.24 min of **6** vs. 8.80 min of **8**, Figure 3), indicating that **6** was an isomer of **8**. Analysis of their 1D and 2D NMR spectra revealed that the only difference between **6** and **8** was the stereochemistry of a hydroxyl group at C7; the β configuration at **6** was supported by the ROESY correlations of H7 with H14a, H14b, and H15b (Figure 4A and S11–S15). We further obtained the X-ray crystal structures of both **6** and **8**, unambiguously establishing their chemical structures with different C7 hydroxyl stereochemistries (Figure 4B). PtmO3 was also reactive with the *ent*-atiserene diterpenoid **9** to form a C7 β-hydroxyl product, **10** (Figures 3AB, 4A and S16–S18). When PtmO6 was incubated with either **5** or **9** under the same conditions as PtmO3, the same products, **6** or **10**, respectively, were observed, again confirming that both enzymes are dedicated C7 β-hydroxylases (Figure 3AB). Thus, the unexpected ability of PtmO3/PtmO6 to catalyze the production of both C7 β-hydroxylation products **6** and **10** from **5** and **9**, respectively, was verified.

Kinetic Studies Suggest the *ent*-Atiserene Scaffold is Favored.

Our *in vivo* studies of the *ptmO3/ptmO6* mutant SB12047 and our recent *in vitro* study of PtmO5 support the timing of the PtmO3/PtmO6 hydroxylation as occurring immediately after the introduction of the C19 carboxylate and the ether ring formation in the *ent*-kauranol scaffold.²² Therefore, C7 β-hydroxylation represents the initial step in the unified biosynthetic pathway en route to PTM and PTN, providing an outstanding opportunity to use PtmO3/PtmO6 to explore this unified biosynthesis proposal.¹⁷

PtmO3 and PtmO6 showed similar *k*_{cat} and *K*_m values with either **5** or **9** that further supported their functional redundancy in the biosynthesis of PTM and PTN (Table 1 and Figure S19). Although a unified biosynthetic pathway was proposed for PTM and PTN biosynthesis, an ~8-fold catalytic efficiency variance was observed for **5** versus **9** when using either PtmO3 or PtmO6. In our previous *in vitro* study of PtmA2, a unique acyl-CoA ligase lacking adenylation functionality, the catalytic efficiencies were much more similar (only a ~1.4-fold change) with the *ent*-kauranol and *ent*-atiserene acyl-adenylated indicated that (i) due to the inherent chemical structural distinctions between the *ent*-kauranol and *ent*-atiserene scaffolds, not all tailoring steps are equally efficient with both PTM and PTN intermediates, and (ii) the enzymes may be more efficient with the *ent*-atiserene intermediates.

Enzyme Assays with Substrate Analogues and Molecular Modeling Provide Initial Insights into Stereoselective Hydroxylation by PtmO3 and PtmO6.

The differences in the chemical structures of **5** and **9** are the connectivity of the C and D rings and the presence of an ether bridge in **5** (Figure 1B). Given that the C19 carboxylate is the only polar functional group in the A/B ring system in both **5** and **9**, this functional group was proposed to play a key role in positioning the substrates in a catalytically-competent conformation. To evaluate this hypothesis, the methyl ester of **5** (**13**) and a C4 gem-dimethyl analogue (**14**) were chemically synthesized as substrate analogues (Scheme S2 and Figures S20–S25). As expected, even when high concentrations of PtmO6 were used, neither **13** nor **14** were converted into any new products (Table 1), indicating that the C19 carboxylate is indispensable for PtmO6-catalyzed C7 hydroxylation.

A homology model of PtmO6 was subsequently generated using I-TASSER.²⁸ The model provided an overall structure of intermediates, PTM ML16 and PTN ML3, respectively (Figure S2).²⁰ Taken together, these observations PtmO6 with a distorted double-stranded β -helix (also known as jelly-roll) fold that is typically seen in members of the Fe/ α KG dioxygenase (Figure S26A).²⁹ The conserved triad of His93, Asp95, and His240 clearly bind to Fe^{2+} and Thr120, Arg251, and Arg255 bind to α KG (Figure S26BE). Each substrate, **5** and **9**, was docked independently into the active site along with Fe^{2+} and α KG (Figure S26CD). Upon docking, hydrogen bonding with the C19 carboxylate group acted as an anchor for the substrate. These interactions would position the H β of C7 to react with the high-valent $\text{Fe}^{\text{IV}}=\text{O}$ intermediate, which is able to abstract this hydrogen and generate a radical. This species would then rebound to the incipient $\text{Fe}^{\text{III}}\text{-OH}$ to form the C7 β -hydroxyls observed in **6** and **10** (Figure S27). In addition, because this step is under enzymatic control, the active site would not accommodate the rotation of this radical species for a C7 α -hydroxylation. These modeling and docking results provide an initial insight into the stereoselective hydroxylation of PtmO3 and PtmO6, which may be significant for future efforts to engineer these dioxygenases as biocatalysts or explore their utilities for natural product structural diversity via synthetic biology.

PtmO8 and PtmO1 are Dehydrogenases and Involved in C7 Epimerization.

The unexpected β -stereoselective hydroxylation by PtmO3 and PtmO6 indicated that at least one additional enzyme is required to epimerize the C7 hydroxyl to account for the isolated α -hydroxylated PTM and PTN intermediates. In the *ptm* gene cluster, no obvious epimerase-encoding gene was detected. However, there are two distinct and uncharacterized dehydrogenase-encoding genes: *ptmO1* and *ptmO8*. Both *ptmO1* and *ptmO8*, and their homologues *ptnO1* and *ptnO8* in the *ptn* gene cluster,¹⁷ encode enzymes belonging to the short-chain dehydrogenases/reductases (SDR) superfamily. Typically, SDRs are NAD(H)- or NADP(H)-dependent oxidoreductases that reversibly transfer hydrides between substrates and cofactors.³⁰ Therefore, a pair of SDRs could serve as an excellent candidate to epimerize the C7 hydroxyl stereochemistry through a ketone intermediate, such as those seen with 7 α - and 7 β -hydroxysteroid dehydrogenases in the biosynthesis of ursodeoxycholic acid.^{31,32}

To confirm the activities of PtmO8 and PtmO1, *ptmO8* and *ptmO1* were individually inactivated to afford the recombinant strains SB12048 and SB12049, respectively, whose genotypes were confirmed by Southern analysis (Figures S28 and S29). Both SB12048 and SB12049 lost production of PTM, PTN, thioPTM, and thioPTN (Figure 2C). The PtmO3 and PtmO6 enzymatic products **6** and **10** were clearly observed in the crude extract of SB12048 (Figure 2C), supporting that PtmO8 is a dehydrogenase responsible for oxidizing the C7 β -hydroxyl into a ketone. Several new metabolites were also detected from the crude extract of SB12049 (*ptmO1*) (Figure 2C). The new metabolites, **7** and **10**, were isolated for structural elucidation. In the ^{13}C NMR spectra, signals of δC 212.2 for **7** and δC 214.6 for **11** were observed clearly, supporting a ketone group in their chemical structures (Figures S30–S35). Taken together, these *in vivo* results suggested PtmO8 and PtmO1 as a pair of dehydrogenases, working in concert, to invert the stereochemistry of the C7 hydroxyl from β to α through a ketone intermediate (**7** or **11**).

***In vitro* Study Confirms that PtmO8 and PtmO1 are Sufficient for Reversing C7 Hydroxyl Stereochemistry.**

Both PtmO8 and PtmO1 were overproduced as soluble proteins in *E. coli* (Figure S36A) and existed as homotetramers in solution upon size exclusion chromatography (Figure S36B). When PtmO8 was incubated with **6** or **10** in the presence of NAD^+ , a new peak was detected, consistent with the retention times and molecular formulas of the authentic standards for **7** or **11** (Figure 3C). The catalytic efficiency of **10** is ~ 3 -fold higher than **6** (Table 1 and Figure S37AB). PtmO8 was also able to accept NADP^+ as an electron acceptor (Figure 3C), albeit with a much lower catalytic efficiency (~ 120 -fold) than NAD^+ (Table 1 and Figure S37CD), which suggests that NAD^+ is much more likely to be the native electron acceptor *in vivo*.

Intermediates **7** and **11** were individually incubated with NADPH and PtmO1. As expected, the products had identical retention times and molecular formulas with the authentic standard of **8** or **12** (Figures 3D),²⁰ confirming PtmO1-catalyzed reduction of the C7 ketone of **7** and **11** by NADPH. The catalytic efficiency of **11** is equal to that of **7**, despite a ~ 10 -fold difference in K_m values (Table 1). While no substrate inhibition was detected for PtmO8, high concentrations ($K_i = 43$ – $101\ \mu\text{M}$ and 10 – 35 -fold greater than K_m) of **7** or **11** inhibited product formation in PtmO1 (Table 1 and Figure S37EF). Finally, under the same conditions, when NADH was used as cofactor, no obvious product was observed even after increasing the concentration of PtmO1 to $10\ \mu\text{M}$ (only 0.1 – $0.5\ \mu\text{M}$ used for NADPH reactions) (Figure 3D), suggesting that PtmO1 selectively uses NADPH as a cofactor *in vivo*.

The homology models of PtmO8 and PtmO1 were constructed using I-TASSER.²⁸ Despite PtmO8 and PtmO1 only sharing 36% sequence identity, their modeled 3D structures show highly similar folds (Figure S38). This result, as well as their tetrameric nature, are consistent with those reported for the SDR superfamily.³³ The native cofactors for each, NAD^+ (PtmO1) and NADPH (PtmO8), were docked into their conserved binding sites. The expected charged residues (Asp37 in PtmO8; Arg41 in PtmO1) were located near where the

phosphate of NADPH would be bound, providing evidence to support the cofactor selectivities of the two enzymes.³³

In theory, one would expect Nature to evolve the most efficient way to produce a small molecule, and yet, the *ptm* and *ptn* gene clusters show a counterintuitive approach: using a three-enzyme cascade to install the C7 α -hydroxyl in PTM and PTN biosynthesis, when one would suffice. This inefficiency has already been described in the PTM and PTN pathways with PtmA1 and PtmA2 performing the activity typically catalyzed by a single canonical CoA ligase.²⁰ Additionally, from a chemical diversity point of view, this modular enzymatic cascade is an ideal system to produce myriad secondary metabolites. This system may also be present in other species because all three oxidation patterns, α -hydroxyl, β -hydroxyl, and ketone, are seen at C7 in the *ent*-kaurane and *ent*-atisane derived diterpenoids.^{1–4} Thus, our findings may provide an explanation as to why ~1,000 *ent*-kaurane derived diterpenoids with distinct B-ring modifications have discovered from a single *Isodon* genus.^{2,3} Although the possibility that a C7 α -hydroxylase exists in other unknown diterpenoid biosynthetic pathways cannot be ruled out, no C7 α -hydroxylase is present in the biosynthesis of PTM and PTN. This is supported by the fact that (i) no C7 α -hydroxylated intermediates **8** or **12** were detected in any of the *ptmO3*/*ptmO6*, *ptmO8*, or *ptmO1* mutants; (ii) heterologous expression of the *ptn* biosynthetic gene cluster in a model host, *S. lividans* K4–114, successfully produced PTN along with a C7 α -hydroxylated congener, platencin SL4 (Figure S2C);¹⁹ and (iii) a one-pot reaction using a combination of PtmO3, PtmO8, and PtmO1 with **5** and **9** as substrates efficiently generated **8** and **12**, respectively, with minimal accumulation of either the C7 β -hydroxyl or C7 ketone intermediates (Figure 3E).

Aside from providing the catalytic versatility of a three-enzyme cascade, it is surprising that this pathway has evolved to create a C7 α -hydroxyl that is seemingly at odds with the final facilitate *trans*-dehydration to yield the enone in PTM and PTN, the C7 α -hydroxyl, occupying equatorial position within the rigid chair configuration, cannot access a *trans*-conformation (Figure 4); therefore, we speculate that the dehydrogenation may act in a similar fashion as seen with the *cis*-dehydration mechanism catalyzed by the type I dehydroquinase in the shikimate pathway (Figure S39A).³⁴ As such, it may be that the C7 β -hydroxyl proves disadvantageous for one or more downstream steps, e.g. a C7 β -hydroxyl may hinder the later C5 hydroxylation that was proposed to initiate A-ring cleavage via a retro-aldol reaction,¹⁹ in PTM and PTN biosynthesis (Figure S39B).

CONCLUSIONS

PTM and PTN are two highly functionalized bacterial diterpenoid natural products of *ent*-kauranol and *ent*-atiserene biosynthetic origin. Study on the biosynthesis of PTM and PTN continues to unveil novel chemistry and enzymology in diterpenoid biosynthesis. In this study, we have disclosed a three-step biosynthetic strategy to install the C7 α -hydroxyl in PTM and PTN biosynthesis. Two highly homologous and functionally-redundant Fe/ α KG dioxygenases, PtmO3 and PtmO6, generate a cryptic C7 β -hydroxyl. A pair of NAD⁺/NADPH-dependent dehydrogenases, PtmO8 and PtmO1, sequentially oxidizes the β -hydroxyl to a C7 ketone and reduces the ketone to yield the C7 α -hydroxyl, respectively. This three-step strategy accounts for the biosynthetic origins of all three C7 oxidation

patterns known for *ent*-kaurane and *ent*-atisane derived diterpenoids and may provide a model system to study unanswered questions regarding other B-ring modifications in bacterial, plant, and fungal diterpenoid biosynthesis. PtmO3 and PtmO6 are dedicated C7 hydroxylases and, to the best of our knowledge, the utilization of an Fe/αKG dioxygenase for the C7 hydroxylation is unprecedented in *ent*-kaurane and *ent*-atisane derived diterpenoids and is distinct from reported P450s in GA biosynthesis.¹ Notably, to date, there are no inexpensive organic synthetic methods for C7 C-H bond of installing the C6/C7 double bond into PTM and PTN by dehydration. Whereas a C7 β-hydroxyl would functionalize the *ent*-kaurane and *ent*-atisane scaffolds; however, PtmO3 and PtmO6 have desirable traits to serve as biocatalysts, including inexpensive cofactors (i.e., αKG), potential substrate promiscuity (i.e., both *ent*-kauranol and *ent*-atiserene scaffolds), readily available (i.e., well overproduced in soluble forms in *E. coli*), and high regio- and stereoselectivity (i.e., C7 and β-hydroxylation).³⁵ Finally, future studies are needed to explore how and why this C7 α-hydroxyl, rather β-hydroxyl, is significant for the downstream tailoring steps of PTM and PTN biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Selected *ent*-kaurane and *ent*-atisane derived diterpenoids with B-ring modifications. (A) Selected *ent*-kaurane and *ent*-atisane derived diterpenoids exemplify the importance of C7 oxidations in generating structural complexity. (B) Biosynthesis of the C6/C7 double bond in the B-ring of PTM and PTN is proposed to occur by dehydration of a C7 α -hydroxyl.

Figure 2.

In vivo characterization of C7 α -hydroxylation in PTM and PTN biosynthesis. (A) Genetic organization of the *ptm* gene clusters from the PTM–PTN dual producers *S. platensis* MA7327 and *S. platensis* CB00739. (B) Genetic organization of the *ptn* gene cluster from the PTN-exclusive producer *S. platensis* MA7339. (C) Crude extracts were analyzed by total ion current chromatograms, with SB12029, a PTM–PTN dual overproducer, used as a positive control: (i) SB12029; (ii) SB12045 (*ptmO3*); (iii) SB12046 (*ptmO6*); (iv) SB12047 (*ptmO3/ptmO6*); (v) SB12048 (*ptmO8*); and (vi) SB12049 (*ptmO1*). The accumulated intermediate, *ent*-atiser-16-en-19-oic acid (9), in SB12047 was undetectable by LC-MS. (D) Structures of the biosynthetic intermediates isolated (5–12) and their chemically synthesized structural analogues (13 and 14). Compounds 8 and 12 were previously isolated from the *ptmA1* and *ptmA2* mutants *S. platensis* SB12037 and SB12038, respectively.²⁰ Compounds 13 and 14 were chemically synthesized from 5 (Scheme S2).

Figure 3.

In vitro characterization of C7 oxidations in PTM and PTN biosynthesis. (A) A set of α -ketoglutarate-dependent dioxygenase and dehydrogenase installing the C7 α -hydroxyl in PTM and PTN biosynthesis. (B) Total ion current chromatograms (TICs) of Ptmo3 and Ptmo6 catalyzed reactions with **5** and **9** as substrates, respectively. Substrate **9** was undetectable by LC-MS. (C) TICs of Ptmo8 catalyzed reactions with **6** and **10** as substrates, in the presence of NAD^+ (1 μM Ptmo8) or NADP^+ (4 μM Ptmo8), respectively. (D) TICs of Ptmo1 reactions with **7** and **11** as substrates in the presence of NADH (10 μM Ptmo1) or NADPH (0.1–0.5 μM Ptmo1), respectively. (E) TICs of time-course analysis of the one-pot reaction using a combination of Ptmo3, Ptmo8, and Ptmo1 with **5** and **9** as substrates, respectively.

Figure 4. Stereochemical assignments of the C7 hydroxyl groups in isolated PTM and PTN biosynthetic intermediates. (A) Selected key ROESY correlations of **6** and **10**, confirming the β -hydroxyl at C7. (B) ORTEP drawing of crystal structures of **6** and **8**, confirming the β -hydroxyl and α -hydroxyl at C7 of **6** and **8**, respectively.

Table 1.

Enzyme kinetics of PtmO3, PtmO6, PtmO8, and PtmO1

protein	substrate	K_m (μM)	$V_{\max}$ ($\mu\text{M min}^{-1}$)	k_{cat} (s^{-1})	k_{cat} / K_m ($\text{s}^{-1} \text{M}^{-1}$)	K_i (μM)
PtmO3	5	16.5 ± 3.5	$(4.8 \pm 0.3) \times 10^{-2}$	145 ± 10	8.8×10^6	N.A.
	9	4.19 ± 0.52	$(8.6 \pm 0.2) \times 10^{-2}$	257 ± 7	6.1×10^7	N.A.
	13	N.A.	N.A.	N.A.	N.A.	N.A.
PtmO6	14	N.A.	N.A.	N.A.	N.A.	N.A.
	5	18.9 ± 2.6	$(4.8 \pm 0.2) \times 10^{-2}$	143 ± 6	7.6×10^6	N.A.
	9	3.79 ± 0.40	$(8.1 \pm 0.2) \times 10^{-2}$	244 ± 7	6.5×10^7	N.A.
PtmO8	6^a	29.1 ± 2.4	1.6 ± 0.1	478 ± 11	1.6×10^7	N.A.
	10^a	6.41 ± 0.66	9.5 ± 0.1	285 ± 6	4.5×10^7	N.A.
	NADP ⁺ ^b	34.2 ± 3.6	1.3 ± 0.1	398 ± 11	1.2×10^7	N.A.
PtmO1	NADP ⁺ ^b	$(1.11 \pm 0.11) \times 10^3$	3.7 ± 0.2	110 ± 5	1.0×10^5	N.A.
	7^c	31.9 ± 7.6	2.3 ± 0.3	$(6.90 \pm 0.96) \times 10^3$	2.2×10^8	101 ± 23
	11^c	3.02 ± 0.55	0.66 ± 0.05	996 ± 84	3.3×10^8	42.8 ± 7.8

^aKinetics determined with saturating concentration (500 μM) of cofactor NADP⁺.^bKinetics determined with saturating concentration (100 μM) of **9**.^cKinetics determined with saturating concentration (200 μM) of cofactor NADPH. N.A. not applicable.