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Platensimycin and Platencin: Inspirations for Chemistry, Biology, Enzymology, and Medicine

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Abstract

Natural products have served as the main source of drugs and drug leads, and natural products produced by microorganisms are one of the most prevalent sources of clinical antibiotics. Their unparalleled structural and chemical diversities provide a basis to investigate fundamental biological processes while providing access to a tremendous amount of chemical space. There is a pressing need for novel antibiotics with new mode of actions to combat the growing challenge of multidrug resistant pathogens. This review begins with the pioneering discovery and biological activities of platensimycin (PTM) and platencin (PTN), two antibacterial natural products isolated from *Streptomyces platensis*. The elucidation of their unique biochemical mode of action, structure-activity relationships, and pharmacokinetics is presented to highlight key aspects of their biological activities. It then presents an overview of how microbial genomics has impacted the field of PTM and PTN and revealed paradigm-shifting discoveries in terpenoid biosynthesis, fatty acid metabolism, and antibiotic and antidiabetic therapies. It concludes with a discussion covering the future perspectives of PTM and PTN in regard to natural products discovery, bacterial diterpenoid biosynthesis, and the pharmaceutical promise of PTM and PTN as antibiotics and for the treatment of metabolic disorders. PTM and PTN have inspired new discoveries in chemistry, biology, enzymology, and medicine and will undoubtedly continue to do so.

Graphical Abstract

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1. Introduction

Platensimycin (PTM, **1**) and platencin (PTN, **2**) are unique natural products with a short, but fascinating history, and a promising future (Figure 1A). Traditional paradigms in bacterial natural products, terpenoid biosynthesis, targets for antibiotic and antidiabetic therapies, and fatty acid metabolism have been questioned through the study of PTM and PTN. Natural products, with their diverse chemical structures and wide range of biological activities, have a long history of inspiring other scientific disciplines, and PTM and PTN are no exception. We hope to encompass in this review why PTM and PTN have generated significant worldwide interest and how these two natural products have inspired natural product discovery, chemistry, biology, enzymology, and medicine.

This review covers (i) the discovery of PTM and PTN and determination of its mode of action, structure-activity relationships, and preliminary pharmacokinetic properties; (ii) the impact of microbial genomics on PTM and PTN, including biosynthetic investigations, titer improvement, bioengineering, and self-resistance mechanisms; and (iii) future perspectives of PTM and PTN. Although extensive work has been done regarding the total synthesis of PTM and PTN, as well as chemical modification of the PTM and PTN scaffolds, this review is not intended to cover these synthetic approaches as these topics have been extensively reviewed elsewhere [1–4]. Other reviews on various aspects of PTM and PTN have also appeared [5–7].

2. Discovery of PTM and PTN

2.1. Screening and discovery

Traditional natural product discovery programs have been and continue to be successful, but are increasingly challenged by rediscoveries of known natural products, and new compounds with novel modes of action are being discovered at a painstakingly slow rate [5, 8, 9]. It is known that natural products, particularly antibiotics, produced by actinomycetes are discovered at different frequencies [8, 9]. It has been estimated that it will take virtual or actual screening of more than 10 million random actinomycetes to discover the next clinically relevant antibiotic [8]. Innovations in natural product discovery are clearly needed to meet clinical needs and combat emerging antibiotic resistant pathogens. By targeted discovery of natural products with modes of action that are different than the currently used therapeutic drugs, the success rate of identifying a novel drug or drug lead is substantially increased.

One promising, yet underexploited, target is bacterial type II fatty acid synthesis (FASII) (Figure 1B) [10]. FASII catalyzes the biosynthesis of fatty acids essential for the cell membrane and is a proven antibacterial target based on the clinical success of the FabI inhibitors isoniazid and triclosan [11, 12]. FASII is an attractive target given the differences in fatty acid biosynthesis between bacteria and eukaryotes [10, 13]. Most bacteria, and all plants, use a system of discrete enzymes to synthesize fatty acids (i.e., FASII). Fatty acid biosynthesis in mammals is catalyzed by a dimeric multifunctional protein that contains seven domains (i.e., FASI). The discrete and highly conserved condensing enzymes, including the initiation condensing enzyme FabH and the elongation condensing enzymes FabB/FabF (Figure 1B), are essential components of FASII and potential targets for antibacterial drug discovery.

Researchers at Merck Laboratories developed an innovative antisense differential sensitivity whole-cell two-plate agar diffusion assay for the discovery of FabF/FabH inhibitors from natural products [14]. By expressing antisense mRNA of a desired target, in this case FASII enzymes, the cells are more sensitive to inhibitors of the targeted gene product due to lower levels of the protein. Thus, by comparing the antibacterial activities of microbial crude extracts against wild-type (WT) *Staphylococcus aureus* and *S. aureus* expressing *fabF* (or *fabH*) antisense mRNA, natural products specifically inhibiting FabF (or FabH) were preferentially detected.

Through the systematic screening of 250,000 natural product extracts (~83,000 microbial strains cultivated in three media), several known and new FabF and FabH inhibitors were identified, two of which were PTM and PTN (Figure 1A) [14]. PTM (**1**) was isolated from *Streptomyces platensis* MA7327, a strain from a soil sample collected in South Africa [15]; PTN (**2**) was isolated from *Streptomyces platensis* MA7339, a strain collected in Mallorca, Spain [16]. PTM selectively inhibited *S. aureus* FabF ($IC_{50} = 48$ nM), was a weak inhibitor of *S. aureus* FabH ($IC_{50} = 67$ μ M), and exhibited minimum inhibitory concentration (MIC) values of 0.1–1 μ g mL⁻¹ against various common drug-resistant Gram-positive pathogens (Table 1), including macrolide- and vancomycin-resistant *Enterococci* (VRE), macrolide-, linezolid-, vancomycin- and methicillin-resistant *S. aureus* (MRSA), and *Streptococcus pneumoniae* [15]. PTM was also active against *Mycobacterium tuberculosis* (MIC = 12 μ g mL⁻¹) due to the inhibition of mycolic acid biosynthesis [17]. PTN was a dual inhibitor of FabF ($IC_{50} = 4.6$ μ M) and FabH ($IC_{50} = 9.2$ μ M), and exhibited MIC values of <0.06–4 μ g mL⁻¹ against the Gram-positive pathogens listed above (Table 1) [16]. It is unclear why the IC_{50} values of PTM and PTN, which are different for FabF and FabH, result in similar MIC values; however, the target selectivity of PTM and PTN in these bacteria, or the possibility that the in vitro assay does not accurately represent the biology inside the cell, may account for this phenomenon [15, 16]. As PTM and PTN selectively target enzymes in FASII, they exhibit no cross-resistance to common antibiotic resistant bacteria [15, 16]. Although PTM and PTN are potent bacteriostatics for a wide range of Gram-positive pathogens, they are ineffective against Gram-negative bacteria. Both PTM and PTN show antibacterial activity against the efflux-negative *Escherichia coli* (*tolC*), but not against WT *E. coli*, indicating that TolC-dependent efflux mechanisms limit the effectiveness of PTM and PTN in Gram-negative bacteria [15, 16]. The case of PTM and PTN, antibiotics with novel structures and

unique mode of action, highlights the propensity for successful discovery of natural products using innovative screening methods.

2.2. Chemical structures

PTM and PTN are hybrid natural products consisting of two distinct moieties connected by an amide bond (Figure 1A) [18, 19]. Both PTM and PTN possess a 3-amino-2,4-dihydroxybenzoic acid (ADHBA) moiety. The differences in the structures of PTM and PTN reside in the aliphatic cages that are linked to ADHBA through a flexible propionamide chain. The aliphatic moieties, or ketolides, of PTM and PTN consist of 17 carbons and contain a cyclohexenone ring, but vary in the remaining portion of the cage moieties [18]. PTM features an unprecedented tetracyclic ketolide with a fused cyclohexyl-cyclopentyl-furan; PTN has a tricyclic unit with an exocyclic methylene [19].

2.3. Mode of action

PTM and PTN inhibit the two classes of decarboxylating condensing enzymes in FASII (Figure 1B). The condensing enzyme β -ketoacyl-acyl carrier protein (ACP) synthase III (FabH) catalyzes chain initiation by Claisen condensation of acetyl-CoA with malonyl-ACP. The β -ketoacyl-ACP synthase I/II (FabB/FabF) elongates the chain through condensation of malonyl-ACP with the growing fatty acyl-ACP [20]. Both FabH and FabB/FabF utilize two substrates in a three step ping-pong reaction mechanism (Figure 2A). Acetyl-CoA in FabH or acyl-ACP in FabB/FabF is covalently transferred to a cysteine in the active site, forming an acyl-enzyme intermediate and releasing reduced CoA or ACP, respectively. Malonyl-ACP, in both enzymes, then binds to the acyl-enzyme intermediate and undergoes decarboxylation. The resulting enolate-ACP nucleophilically attacks the acyl-enzyme intermediate resulting in an elongated acyl-ACP product.

PTM and PTN were shown to bind to the covalent acyl-enzyme intermediate by a direct binding assay with *E. coli* FabF [15]. PTM was observed to bind poorly when incubated directly with WT FabF. However, when incubated with WT FabF and lauroyl-CoA (C_{12}), which mimicked the natural acyl-ACP substrate and created a surrogate intermediate, PTM binding increased 19-fold compared to that of apo FabF. Thus, PTM binds in the malonate binding pocket and is a competitive inhibitor of malonyl-ACP, the second substrate of FabF. This was confirmed by the structural determination of *E. coli* FabF in complex with PTM (Figure 2B) [15]. To mimic the acyl-enzyme intermediate of FabF, the catalytic cysteine was mutated to a glutamine, which positioned the active site residues into an acyl-enzyme-like conformation, and resulted in a 50-fold increase, compared to WT FabF, in binding affinity of PTM to FabF(C163Q). This is exemplified by a significant shift in the side chain of F400 that changes the active site from a 'closed' conformation to an 'open' conformation.

The ADHBA moiety of PTM sits inside the malonate binding pocket of FabF and makes key interactions with active site residues, while the ketolide moiety sits in the mouth of the active site, partially exposed to solvent (Figure 2B and 2C) [15]. The carboxylate of ADHBA ionically interacts with H303 and H340, two of the three residues that comprise the Cys-His-His catalytic triad. The 4-OH group of ADHBA interacts with residues in a side pocket of the active site through a water-mediated hydrogen bond. The amide group hydrogen bonds

to both the side chain of T307 and backbone carbonyl of T270. At the entrance of the binding pocket, the aliphatic cage of PTM acts as a greasy plug, with the oxygens of the ether ring and enone moiety forming hydrogen bonds to the side chain of T270 and the main chain of A309, respectively (Figure 2C).

The differences in the biological selectivities of PTM and PTN must reside in their distinct ketolide moieties (Figure 1A). The ADHBA moieties of PTM and PTN are identical, and make the same active site interactions that are key to inhibiting the fatty acid condensation enzymes. PTN, however, is less active than PTM in FabF, and more active than PTM in FabH [16]. The lack of the ether ring characteristic of PTM likely contributes to this selectivity. PTN is unable to make the hydrogen bond to T270 that PTM makes at the entrance of the active site in FabF (Figure 2C). Conversely, the analogous region in FabH is lined with nonpolar residues, allowing PTN, but not PTM, to inhibit FabH [19].

2.4. Structure-activity relationships

The hybrid structures of PTM and PTN and their selective biological activities encouraged chemists to modify both the ADHBA and ketolide moieties to investigate their structure-activity relationships (SAR). The SAR of PTM and PTN have now been extensively studied through the biological evaluation of both natural and synthetic derivatives (Figure 3 and Table 1). Natural congeners of PTM and PTN, isolated from both WT and overproducing strains, gave a preliminary understanding of the SAR. Platensic acid (**3**), platensimide A (not shown) [21], homoplatensimide A (**13**) [22], and PTM B₁ (**5**), B₂ (not shown) and B₃ (**12**) [23] isolated from *S. platensis* MA7327, exhibited poor antibacterial activities underscoring the necessity of the ADHBA moiety [21]. Several natural glucoside congeners of PTM and PTN (**15–19**) have also been isolated and show no antibacterial activity (Table 1) [24–26], which is consistent with the ADHBA moiety binding in a cavity too small to accommodate an extra sugar moiety.

Several natural PTM and PTN analogues with hydroxylated ketolide moieties (**20–28**) are biologically active, but show decreased MIC values compared to PTM and PTN (Table 1) [25, 27–30]. This is exemplified by PTM A₁ (**20**), which possesses an axial hydroxyl at C-14 [27]. PTM A₁ inhibited FASII with IC₅₀ (2.7 μ M) and MIC (16 μ g mL⁻¹) values that are 2.5-fold and 32-fold lower than those of PTM, respectively. A crystal structure of *E. coli* FabF with PTM A₁ revealed that the hydroxyl group causes a shift of the ketolide moiety in the binding site and disrupts key polar interactions [27].

We recently discovered two new sulfur-containing congeners of PTM from an engineered dual PTM-PTN overproducing strain *S. platensis* SB12029 (see Section 3.3) (Figure 3) [31]. We first identified the sulfur-containing pseudodimer PTM D1 (**29**), a likely nonenzymatic product due to a heteroatom Michael addition of a sulfur atom attacking the enone, which lead to the identification of its presumptive precursor PTM S1, a thioacid analogue of PTM (**11**). While PTM S1 was intrinsically unstable and prevented us from directly determining its antibacterial activity, PTM D1 (MIC = 0.5 μ g mL⁻¹) was found to retain the strong antibacterial activity of PTM (Table 1) [31]. It is still unclear how the pseudodimer exerts its activity.

The total syntheses of PTM and PTN, reviewed elsewhere [1–4], made it possible to perform a systematic SAR study of these hybrid natural products. Following an initial study [32] where two series of analogues with varying degrees of complexity were synthetically constructed, several groups have continued to synthesize PTM and PTN variants [18, 33–40]. One series possessed constant aromatic moieties and variable ketolides (**30–50**); the other series had constant ketolides and variable aromatic moieties (**51–59**) (Figure 3). Confirming the SAR of the natural congeners, structural modifications on the ADHBA moiety had a significant impact on antibacterial activity, while the polycyclic cage is tolerant to certain modifications without drastically impacting biological activity (Figure 3). Deletion of any, or all, of the functional groups on ADHBA completely abolished activity (**51–53**) [32]. Similarly, 2- or 4-methoxy groups (**54** and **55**) or addition of large halogens onto ADHBA negatively impacts activity (**56–59**) [33, 34].

Several analogues have shown that the conformation of the cyclohexenone ring plays an important role in binding. Dihydro-PTM (**36**) [18] phenyldihydro-PTM (**38**) and cyclopropyldihydro-PTM (**39**) [35] had cyclohexane rings that were in twist chair conformations, as opposed to the boat conformation in PTM, which disrupted proper binding, namely the orientation of the C-5 ketone for favorable hydrogen bonding (Figure 2C). Retaining the cyclohexenone ring, while making minor modifications to the rest of the ketolide, typically resulted in retention of activity. Carbaplatensimycin (**31**) and adamantaplatensimycin (**32**) [32, 36, 37] and the endocyclic alkene analogue iso-PTN (**30**) [34] inhibited bacterial growth on a comparable level with that of PTM and PTN (Table 1). 7-Phenyl-PTM (**34**) and 11-methyl-7-phenyl-PTM (**35**) were the first analogues that were more potent than PTM (Table 1) [38]. Even the dialkylamino analogues (+)-myrtemycin (**48**), (–)-myrtemycin (**49**), and myrtamycin (**50**), the most dissimilar analogues of PTM, showed only 8–32-fold decreases in MICs (Table 1) [39]. However, minor modifications, e.g., changing the carbon connectivity of only one atom as seen in iso-PTM (**47**) [40] can also dramatically decrease potency (Table 1), suggesting that the SAR of both PTM and PTN is still not completely understood. The current SAR model, which is an essentiality of the ADHBA moiety and modest variability of the ketolide (Figure 3), still has room for improvement. The amide linker has not yet been optimized; however, analogues of PTM with modified linkers (but also with modified ADHBA moieties) have been constructed using mutasynthesis (**60–63**, see Section 3.4.3.) and set the stage for future studies involving the amide linker of PTM and PTN [41]. It is also unknown how other modifications, such as fluorination of the ADHBA moiety or expanding ketolide diversity, will affect biological activity and selectivity.

The natural and synthetic analogues mentioned above and shown in Figure 3 and Table 1 are meant to showcase the current understanding of the SAR of PTM and PTN, as well as to highlight any analogues that approach the biological activities of PTM and PTN. There are numerous other analogues that were isolated or synthesized but not discussed in this review (see selected reviews [1–4] and articles [18, 32–40]).

2.5. Pharmacokinetic properties

PTM and PTN have been prevented from entering clinical trials as antibiotics due to their poor pharmacokinetics. PTM and PTN showed efficacy in an in vivo mouse model of an *S. aureus* infection with no toxicity after administration for up to 17 days ($IC_{50} > 1 \text{ mg mL}^{-1}$ in a HeLa cytotoxicity assay) [15, 16]. However, efficacy was only achieved when the antibiotics were administered by continuous intravenous (IV) infusion. It is thought that continuous delivery was necessary due to rapid clearance, thereby resulting in low concentrations of the natural products in plasma, which are vital for successful antibiotics [6]. While much work has been done to understand the SAR of PTM and PTN, and improve its biological activity (see Section 2.4), no reports are available of analogues addressing this vital roadblock.

3. Impact of microbial genomics on PTM and PTN

3.1. Early biosynthetic studies

The hybrid skeletons of PTM and PTN, consisting of unique 17-carbon polycyclic enones and an ADHBA moiety (Figure 1A), suggested two biosynthetic pathways converge to form the natural products. Stable-isotope precursor incorporation experiments revealed the biosynthetic origins of both PTM [42] and PTN [43]. Both sodium acetate and sodium pyruvate were successfully incorporated into the ADHBA moieties of PTM and PTN. The labeling pattern of the enriched ADHBA moiety indicated a 4 + 3 carbon unit biosynthesis using a tricarboxylic acid (TCA) cycle intermediate and phosphoenolpyruvate, respectively. The ketolide moieties of PTM and PTN were both found to incorporate pyruvate, but not acetate or mevalonate, indicating that their biogenesis is derived from the non-mevalonate, or methylerythritol phosphate (MEP) [44], terpenoid pathway [42, 43]. Subsequent isolation of homoplantensimide A (**13**) (Figure 3), a congener possessing twenty carbons, from *S. platensis* MA7327 supported the hypothesis that the 17-carbon ketolide scaffold of PTM is derived from a diterpenoid precursor [22]. Based on their polycyclic skeletons, the ketolides of PTM and PTN were proposed to originate from *ent*-kaurene and *ent*-atiserene intermediates, respectively [19].

3.2. The *ptm* and *ptn* gene clusters and a unified biosynthetic model

Inspired by the inherent processing of two distinct diterpene scaffolds into the final natural products of PTM and PTN, we set out to find the biosynthetic gene clusters of PTM and PTN. Utilizing designed primers based on conserved regions of AHBA synthases (e.g., grixazone biosynthesis [45]), we identified a single gene, later named *ptmB2*, encoding AHBA synthase in *S. platensis* MA7327 [46]. On the assumption that *ptmB2* was present within a larger cluster of genes responsible for encoding PTM biosynthesis, as many microbial biosynthetic pathways are, we cloned and sequenced the *ptm* gene cluster from *S. platensis* MA7327 (Figure 4A) [47]. Similarly, we located, cloned, and sequenced the *ptn* gene cluster from *S. platensis* MA7339 (Figure 4B) [47]. The *ptm* and *ptn* biosynthetic gene clusters are highly homologous in genetic organization and sequence. The only major difference between the two clusters is the absence of a five gene operon, seen in the middle of the *ptm* cluster and named the PTM cassette, in the *ptn* cluster. The similarities between the two gene clusters, with the exception of the PTM cassette, supported our earlier

discovery that *S. platensis* MA7327 was in fact a dual PTM-PTN producer [46]. We thus hypothesized that the PTM cassette must control the partitioning between PTM and PTN biosynthesis. This was confirmed by inserting the PTM cassette into *S. platensis* MA7339, the PTN-only producer, resulting in the dual PTM-PTN producer *S. platensis* SB12604 [47].

It was, however, still unclear how the two diterpene scaffolds were biosynthesized. Only one *ent*-kaurene synthase of bacterial origin was known [48] and no *ent*-atiserene synthases, of either prokaryotic or eukaryotic origin, had ever been reported. *ent*-Atiserene had been isolated as a minor metabolite from a mutant of *ent*-kaurene synthase from rice (*Oryza sativa*) [49], suggesting that one diterpene synthase may be responsible for creating both diterpene scaffolds. To address this question, we first deleted *ptmT3*, a bioinformatically predicted *ent*-kaurene synthase, in *S. platensis* MA7327, which afforded a PTN-specific producer [47]. We then searched for an *ent*-atiserene specific synthase and targeted *ptmT1*, which was originally annotated as an aromatic prenyltransferase. Deletion of *ptmT1* in *S. platensis* MA7327 yielded a PTM-specific producer [47]. Thus, we discovered the first *ent*-atiserene synthase and established that two dedicated diterpene synthases govern the divergence in the biosynthesis of PTM and PTN.

A unified pathway for PTM and PTN biosynthesis was proposed based on the results described above, as well as bioinformatics analysis of the two gene clusters (Figure 4) [47]. (i) The ADHBA moiety is constructed from aspartate 4-semialdehyde (ASA) and dihydroxyacetone phosphate (DHAP) (Figure 4C). In a process similar to that seen in grixazone biosynthesis in *Streptomyces griseus* [45], AHBA is biosynthesized by PtmB1/PtnB1 and PtmB2/PtnB2. AHBA is then likely hydroxylated by PtmB3/PtnB3, a flavin-dependent hydroxylase, to afford ADHBA [47]. (ii) In a separate pathway, the terpenoid building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are formed via enzymes in the MEP pathway (Figure 4D). IPP and DMAPP are then condensed into the 20-carbon linear GGPP, the universal precursor for all known diterpenoids, by the geranylgeranyl diphosphate (GGPP) synthase PtmT4/PtnT4. (iii) GGPP is first cyclized into *ent*-copalyl diphosphate (CPP) synthase by PtmT2/PtnT2 [50]; *ent*-CPP is the most advanced intermediate shared in the biosynthesis of both PTM and PTN (Figure 4D). Then, dedicated diterpene synthases, *ent*-kaurene synthase PtmT3 and *ent*-atiserene synthase PtmT1/PtnT1, split *ent*-CPP into the initial 20-carbon ketolide scaffolds for PTM and PTN, respectively (Figure 4D) [47]. (iv) A group of enzymes, then processes the diterpene scaffolds into the corresponding ketolide acids by enzymes either specific for PTM (to furnish the ether linkage) or flexible enough to accommodate both scaffolds (general ketolide processing) (Figure 4D). (v) In the final step, the ketolides are coupled to ADHBA by the amide synthase PtmC/PtnC affording PTM and PTN (Figure 4D) [41].

Equipped with the *ptm* and *ptn* gene clusters and a preliminary hypothesis how these natural products are biosynthesized, we sought to discover alternative PTM and PTN producers with improved phenotypes, e.g., higher production titers or enhanced genetic amenability (see Sections 3.3 and 3.4), for future biosynthetic studies. We developed a high-throughput strain prioritization method for diterpene discovery [51]. There are four putative diterpene-associated genes in the *ptm* gene cluster: *ptmT4*, *ptmT2*, *ptmT1*, and *ptmT3* (Figure 4A) [47]. By targeting these four genes, we identified six new, alternative dual PTM-PTN

producers from a collection of 1911 strains within the Actinomycetales strain collection at The Scripps Research Institute [51]. These six strains, although genetically very similar to *S. platensis* MA7327, were morphologically different from each other and *S. platensis* MA7327, i.e., they sporulated well and were genetically amenable, leading to the use of one of these strains as a model system for PTM and PTN biosynthesis and engineering [52].

3.3. Titer improvement

One common obstacle in natural product discovery and development is low production titer resulting in inadequate supply of the desired natural product or related congeners. Much progress has been made to circumvent the supply issues of PTM, PTN, and their natural congeners since the discovery of these natural products. Initially, production titers of PTM and PTN ranged from 1 to 4 mg L⁻¹ [15, 16], with later studies reporting up to 52 mg L⁻¹ for PTM [30]. However, many of the natural congeners were isolated from fermentations as large as 3400 L at titers as low as 3 µg L⁻¹ [24, 28, 29]. Semi-defined and chemically defined media for PTM production in *S. platensis* MA7327 have also been reported [53, 54]. Although the titers of PTM under these conditions are less than those in previous reports, they support PTM and PTN production in simple, defined, and soluble medium.

In contrast to the empirical and fermentation optimization methods, we exploited the natural genetic regulatory mechanism of PTM and PTN biosynthesis to rationally improve the titers of both the natural products and their congeners [55]. Deletion of *ptmR1*, a GntR-like transcriptional regulator, in *S. platensis* MA7327 yielded dual PTM-PTN overproducing strains with titers up to 323 mg L⁻¹, over 100-fold greater than that of the WT strains [46]. Similarly, deletion of the pathway-specific negative regulator *ptnR1* in *S. platensis* MA7339 afforded a PTN-only overproducing strain, again with a 100-fold improvement in PTN titer [25]. These overproduction strains also accumulated congeners not detectable in the WT strains [25, 26]. The same strategy was used to construct dual PTM-PTN overproducing strains that were genetically amenable (see Section 3.4) [51, 52]. Exploiting a combination of pathway engineering and fermentation optimization, pilot scale production of PTM was developed. Manipulation of the medium and optimization of the fermentation procedures of one of the *ptmR1* strains, *S. platensis* SB12026, afforded PTM production titers of 1.56 g L⁻¹ [56].

3.4. Engineering

In addition to manipulating the PTM and PTN machinery for titer improvement (see Section 3.3), we have used genetic engineering to (i) develop a heterologous production system for PTN, (ii) study the biosynthesis of bacterial diterpenoids and discover novel enzymes and biochemistries, and (iii) construct a model system for biotechnology applications.

3.4.1. Heterologous production of PTN—Early technical difficulties when working with the original native PTM and PTN producers, which were later addressed by discovering alternative producing strains with improved genetic traits (see Section 3.2) [51] inspired us to develop a heterologous production system. A heterologous production system would also set the stage for future synthetic biology applications for the PTM and PTN class of natural products. By mobilizing the entire *ptn* gene cluster into five model *Streptomyces* hosts, PTN

production was achieved in *Streptomyces lividans* K4–114 [57]. Corresponding with increased production in the *S. platensis* *ptnR1* overproducing strain [25], PTN production in *S. lividans* was only achievable in an expression system containing a *ptn* gene cluster with its *ptnR1* gene inactivated. Although the PTN titer was modest (1–2 mg L⁻¹), five novel PTN congeners were isolated revealing new insights in PTN biosynthesis (see Section 3.4.2.).

3.4.2. Fundamental biosynthesis of PTM and PTN—Impressed by the complexity of the *ptm* and *ptn* biosynthetic gene clusters, two distinct metabolic pathways caught in the action of evolution, we set out to understand the fundamental biosynthetic pathways of PTM and PTN. Through comparative bioinformatics of the two gene clusters, a unified model was proposed (see Section 3.2 and Figure 4) [47]. This model has been further supported through the elucidation of individual biosynthetic steps within the overall pathway. These advancements were achieved through engineering the PTM biosynthetic pathway, accumulation of intermediates and congeners from the engineered and heterologous producing strains, and in vitro biochemical enzyme characterizations.

The convergent nature of PTM and PTN biosynthesis was confirmed by exploring the roles of key enzymes involved in the biosynthesis of ADHBA (Figure 4C), the ketolide moieties, and the coupling of these two moieties (Figure 4D) [41]. Three different phenotypes were observed when the three “pathways” were individually disrupted. (i) Inactivation of *ptmC* abolished PTM and PTN, but not ADHBA or ketolide, production, (ii) inactivation of *ptmB1* or *ptmB2* abolished ADHBA, but not ketolide, production; and (iii) inactivation of *ptmT2* abolished ketolide, but not ADHBA, production. These phenotypes supported the proposal that two distinct moieties, ADHBA and the diterpene-derived ketolides, are biosynthesized separately, and coupled together by the amide synthase PtmC (Figure 4).

Diterpene production and processing begin with the cyclization of the universal diterpenoid precursor GGPP (Figure 4D). In vitro and structural characterization of PtmT2 confirmed its role as an *ent*-CPP synthase [50]. As mentioned above (see Section 3.2), the *ptmT3* and *ptmT1* genes encode two dedicated diterpene synthases that control the divergence of PTM and PTN biosynthesis, respectively [47]. *ent*-CPP, the final shared intermediate in both PTM and PTN biosynthesis, is cyclized into (16*R*)-*ent*-kauran-16-ol by PtmT3 [58] and *ent*-atiserene by PtmT1 [47]. PTM-specific enzymes, such as the cytochrome P450 monooxygenase PtmO5, then catalyze PTM-specific biosynthetic reactions. PtmO5 was found to catalyze ether formation, from a 16*R*-hydroxy intermediate, in the ketolide of PTM. The remaining enzymes complete the processing of both the PTM and PTN diterpene scaffolds into their respective ketolide moieties. These enzymes must process intrinsic substrate flexibility given their acceptance of two different diterpene scaffolds. For example, PtmO4, a long-chain acyl-CoA dehydrogenase, was found to catalyze β -oxidation for both scaffolds of PTM and PTN (Figure 4D) [52].

The decision to study PTM and PTN biosynthesis in overproducing strains and heterologous hosts has been extremely rewarding. This is due to accumulation of a plethora of potential intermediates and congeners during fermentation. Oxidation at C-19 of the diterpene scaffold, in a similar fashion to gibberellin biosynthesis [59], was implied by the isolation of

oxidized congeners (either alcohol or carboxylic acids) [52, 57]. This oxidation likely occurs early (i.e., before A-ring cleavage) in biosynthesis. The resultant C-19 carboxylic acid is presumably activated into its CoA thioester. CoA activation is a reasonable presumption given the late-stage β -oxidation cycle to afford the 17-carbon ketolide. Another oxidation reaction, C-7 hydroxylation, is supported by the isolation of several congeners and also appears to occur early, although the exact timing of this hydroxylation is unknown [52, 57, 58]. After a possible retro-aldol cleavage of the A-ring, a complete β -oxidation cycle, including dehydrogenation (PtmO4) [52] hydration, oxidation, and thiolitic loss of the propyl moiety, yields platensicyl- or platencinyl-CoA. All together, the unified model of PTM and PTN biosynthesis is outlined in Figure 4.

3.4.3. Biotechnology applications—With an excellent dual PTM-PTN overproducing and genetically amenable strain available, we sought to utilize this model system for biotechnological applications. First, we exploited the inherently flexible coupling enzyme PtmC to generate a mutasynthetic library of PTM and PTN analogues with varied benzoic acid moieties [41]. By inactivating the ADHBA biosynthetic genes *ptmB1* or *ptmB2* (Figure 4C), we abolished ADHBA production in the dual PTM-PTN overproducing strain SB12029. These recombinant strains, however, were still able to produce platensic (**3**) and platencinic acid, and PTM and PTN if ADHBA was added exogenously. Precursor-directed feeding experiments in the *ptmB1* strain SB12032 with 34 aryl variants afforded more than 30 PTM and PTN analogues (**60–63** are selected examples). Although none of the generated analogues possessed antibacterial activity, this study paves the way to future modifications of the ADHBA moiety that may result in improved biological activities and/or pharmacological properties.

In a similar fashion, we expect that the *ptmB1* strain SB12032 will be used to produce the difficult to synthesize ketolides for semi-synthetic experiments. For example, *S. platensis* SB12032 produces platensic acid at an initial, unoptimized titer of $\sim 150 \text{ mg L}^{-1}$ [41]. Facile access to the diterpene-derived scaffolds of PTM or PTN would greatly simplify efforts to synthetically modify the ketolide or link the ketolide to other desired moieties.

3.5. Self-resistance

PTM and PTN exhibit no cross-resistance to common antibiotic resistant bacteria [15, 16]. This is due to the fact that they selectively target enzymes in FASII, an attractive, yet rarely used, target for antibiotics [10]. Upon determination of the mode of action of PTM and PTN, there were two observations regarding the possible mechanism of resistance. First, the nature of the interactions between PTM and PTN and its target, i.e., targeting the acyl-enzyme intermediate, suggests that resistance arising through mutation of the target protein may be unlikely [15]. Second, simultaneously targeting two or more essential enzymes, as PTN does, instead of one, greatly reduces the likelihood of acquired resistance through mutation of the target protein [16, 60]. While this sounds promising for potential clinical use of PTM and PTN, there is an unavoidable and highly efficient resistant system that must be considered: the producing organism's self-resistant mechanism. Development of antibiotic resistance in pathogenic bacteria can be attributed to horizontal gene transfer of the resistant elements from nonpathogenic bacteria, with one potential source being the producing

organism itself. Clinical resistance to vancomycin and aminoglycosides are important examples of this model [61–63].

Using microbial genomics, we identified two mechanisms for PTM and PTN resistance in the *S. platensis* producers (Figure 1B) [64]. The *ptmP3/ptnP3* gene, found within the *ptm* and *ptn* biosynthetic gene clusters, respectively, was identified as the primary self-resistant element. PtmP3/PtnP3 is a FabF homologue and expression of *ptmP3* in *Streptomyces albus*, a strain sensitive to PTM and PTN, conferred resistance to both PTM and PTN. Mutagenesis of the catalytic cysteine (C162A, C162L, or C162Q) abolished its ability to confer resistance and supported the notion that PtmP3/PtnP3 confers resistance through target replacement, as opposed to antibiotic sequestration [64]. Deletion of *ptmP3* in *S. platensis* resulted in a mutant that retained PTM resistance, but was still susceptible to PTN, suggesting a secondary form of PTM resistance. The second self-resistant mechanism found in *S. platensis* was target modification of native FabF [64]. Expression of *fabF* from *S. platensis* in *S. albus* conferred resistance to PTM, while expression of *fabH* from *S. platensis* in *S. albus* did not alter the resistance phenotype. As PTN inhibits both FabF and FabH, yet the native FabH was unable to confer resistance to PTN, we suspected that PtmP3/PtnP3 is capable of replacing both native FabF and FabH enzymes. Successful deletion of *fabF* or *fabH* within the housekeeping FASII locus, an experiment that is not possible if the essential genes *fabF* or *fabH* are not functionally replaced by another gene, supports that PtmP3/PtnP3 may in fact be a dual-acting enzyme of FASII [64]. Although the resistance elements and overall mechanisms of resistance have been revealed, detailed biochemical, i.e., structural and enzymological, studies would further advance our understanding of these complicated mechanisms.

In addition to horizontal gene transfer of the natural resistance elements from the antibiotic-producing organism, clinically relevant pathogens may acquire resistance through evolution. Laboratory-evolved resistance to PTM and PTN has now been seen in *S. aureus*; however, resistance was not acquired by mutation in *fabF*, as expected. Growth of *S. aureus* on agar plates containing antibiotic at 4× the MIC value, resulted in colonies with mutations in *fabH*, instead of *fabF* [65]. It was established that the FabH mutants were catalytically defective, which likely resulted in control of the substrate concentrations of FabF. Specifically, the concentration of acyl-ACP is lowered, limiting the amount of acyl-enzyme intermediate available for inhibition, while the concentration of malonyl-ACP is increased, increasing competition for the acyl-enzyme intermediate binding site. The MICs of PTM and PTN increased >64× compared with the WT; however, the growth phenotype of each mutant was impaired and fatty-acid dependent. Overall, these findings challenge the current paradigm of fatty acid biosynthesis and should be considered while developing inhibitors of fatty acid condensing enzymes.

4. Future perspectives of PTM and PTN in chemistry, biology, and medicine

4.1. Natural product discovery

Novel natural products with novel targets or mode of actions are challenging to discover because of high frequencies of rediscovery of known natural products and low frequencies of new natural products in actinomycetes, the workhorse producers of antibiotics, and a lack

of strategic visions [8]. Innovations in natural product discovery are needed to address each of these obstacles. The high-throughput antisense differential sensitivity assay that was created and used for the discovery of FabF/FabH inhibitors is an example of such an innovation.

Targeted discovery of natural products with desired biological activities may yield new chemical scaffolds. It is well known that families of similar scaffolds typically interact with targets in similar ways. For example, aminoglycosides (e.g., streptomycin) and macrolides (e.g., erythromycin) both bind to the ribosome and prevent translocation of the peptidyl-tRNA; however, aminoglycosides bind to the 30S ribosomal subunit and macrolides bind to the 50S ribosomal subunit [66]. By choosing underrepresented targets, e.g., fatty acid biosynthesis, natural products with novel skeletons are more likely to be found. Accordingly, PTM and PTN, inhibitors of FabF and FabH, have unique structures consisting of highly processed diterpene scaffolds linked to an aminobenzoic acid. This type of hybrid structure had never been seen before and supports the hypothesis that new targets lead to new structures. Given the propensity of Nature to perform combinatorial biosynthetic chemistry, it is reasonable to assume that there are additional PTM- and PTN-like structures waiting to be discovered.

Natural products with novel structures are understandably exciting discoveries as fresh scaffolds may present new opportunities to investigate other biologies. While PTM and PTN were discovered through a screen targeting inhibitors of FASII, their privileged scaffolds may lead to alternative biological targets. There are many examples, such as rapamycin [67], of natural products being considered as a drug or drug lead for one disease, before ultimately becoming a useful therapeutic for a different affliction. The discovery of PTM and PTN, their novel structures, novel mode of actions, and privileged scaffolds should inspire future natural product chemists to continue natural product discovery efforts.

4.2. Exploration of diterpenoids in bacteria

Terpenoids are one of the most fascinating, structurally and chemically diverse, and abundant classes of natural products. There are over 68,000 known terpenoids, over 18,700 of which are diterpenoids (dnp.chemnetbase.com). Most diterpenoids are produced by plants and fungi; contrarily, diterpenoids of bacterial origin are rare [68]. Before the discovery of PTM and PTN, the few known bacterial diterpenoids were simple carbon scaffolds with only minor modifications, such as hydroxylations. PTM and PTN, as well as the phenalinolactones and brasiliacardins [69, 70], have shown that highly processed diterpenoids are present in bacteria, the discovery of which, however, has been sluggish.

Given the rarity of bacterial diterpenoids, understanding the biosynthesis of the PTM and PTN family of natural products should inspire continued exploration of diterpenoids of bacterial origin. In only a few short years of study, there have been several immense contributions to the knowledge of the biosynthesis of diterpenoids. First, an *ent*-atiserene synthase, a diterpene synthase not yet found in any kingdom of life, was identified [47]. As PtmT1/PtnT1 was not bioinformatically annotated as a diterpene synthase, it represents a new subclass of terpene synthases and can now be used as a model for understanding, and identifying, new terpene synthases. The other diterpene synthase in PTM and PTN

obtain a basic understanding of whether fatty acid biosynthesis is a feasible antibacterial target.

PTM has also emerged as a prospective drug lead for the treatment of diabetes and other related metabolic disorders. Somewhat ironically, PTM is a potent inhibitor of mammalian fatty acid synthase and selectively inhibits de novo lipogenesis and fatty acid oxidation, but not sterol biosynthesis, in hepatocytes [84]. PTM, when administered orally, preferentially concentrates in the liver and leads to improved liver steatosis and insulin sensitivity in *db/+* mice that are fed with a high fructose diet, and lowered glucose levels in *db/db* mice [84]. These results, while only preliminary in nature, are a proof of concept for the treatment of diabetes, liver steatosis, and other metabolic diseases using PTM, PTN, or other derivatives.

5. Conclusion

PTM and PTN have drawn worldwide attention since their discovery a decade ago due to their novel skeletons and intriguing inhibition of FASII. From an innovative natural products screening method to novel enzymes catalyzing unprecedented transformations to native antibiotic resistance mechanisms, PTM and PTN have inspired innovations and discoveries in synthetic chemistry, enzymology, biology, and medicine. Understandably, there was overwhelming enthusiasm regarding the antibiotic potential of PTM and PTN after its initial discovery. While the development of these two natural products has slowed, primarily due to their poor pharmacokinetic properties, significant progress is still being made through the use of microbial genomics. In addition, the recent revelation that PTM is a drug lead for the treatment of diabetes mellitus, suggests that the privileged scaffolds of PTM and PTN may be used to interrogate other biologies. There is no doubt that continued study of this unique class of natural products will continue to yield conceptual breakthroughs in several scientific disciplines.

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Figure 1.

Structures of PTM and PTN, inhibition of fatty acid synthase, and self-resistance in native producers. (A) Structures of PTM and PTN featuring diterpene-derived ketolides linked to 3-amino-2,4-dihydroxybenzoic acid moieties. IC_{50} values of FabH and FabF *S. aureus* (Sa) and human and rat FAS. (B) Bacterial fatty acid biosynthesis catalyzed by FASII. PTM selectively inhibits FabF/B and PTN dually inhibits FabH and FabB/FabF. The two complementary mechanisms of resistance in *S. platensis* by target replacement (i.e., FabH and FabF by PtmP3) and target modification (i.e., native PTM-insensitive FabF).

Figure 2.

Inhibition of FabF by PTM. (A) FabF (and FabH) catalyzes the decarboxylating condensation reaction in fatty acid biosynthesis using a three step ping-pong mechanism. (i) The fatty acid chain from acyl-ACP is first transferred to the active site cysteine forming an acyl-enzyme thioester intermediate and releasing free ACP. (ii) Malonyl-ACP then binds to the acyl-enzyme intermediate. (iii) Following decarboxylation of malonyl-ACP, the condensation reaction yields an acyl-ACP product that is two carbons longer than the substrate in (i). FabF is regenerated to its initial reduced state and CO_2 is released. (iv) PTM inhibits FabF by mimicking the malonyl-ACP binding interaction with the acyl-enzyme intermediate. (B) PTM bound *E. coli* FabF (PDB entry 2GFX [15]). The solvent-accessible

surface area of FabF, colored according to electrostatic potential, is shown. PTM is shown as a ball and stick model with green carbon atoms, red oxygen atoms, and blue nitrogen atoms. (C) PTM bound in the malonate-binding pocket of FabF. Residues in the binding pocket that make key interactions (dotted black lines indicating distances of 2.6–3.2 Å) with PTM are shown as magenta sticks. The solvent-accessible surface area of FabF and PTM are shown as described in (B).

Figure 3. Current SAR model of PTM and PTN supported by selected natural, synthetic, and mutasynthetic analogues. The carbons of the ketolide moieties of PTM and PTN are numbered for reference. See Table 1 for a summary of the biological activities (MICs) of each variant shown.

Figure 4.

PTM-PTN dual and PTN exclusive biosynthetic gene clusters and a unified pathway for PTM and PTN biosynthesis. *ptm* and *ptn* biosynthetic gene clusters and biosynthetic model. (A) Genetic organization of the *ptm* gene cluster from *S. platensis* MA7327 and (B) the *ptn* gene cluster from *S. platensis* MA7339. Genes are color-coded corresponding to the predicted function of the encoded proteins in biosynthesis, resistance, and regulation. The “PTM” cassette (shaded in gray), which contains five genes that bestow the *ptm* cluster the ability for dual production of PTM and PTN, is present in the *ptm* cluster and absent in the *ptn* cluster. (C) ADHBA biosynthesis from ASA and DHAP. (D) Diterpene-derived ketolide biosynthesis from IPP and DMAPP, from the MEP pathway. *ent*-CPP is the final common

intermediate in the biosynthesis of PTM and PTN. *ent*-CPP is cyclized by two dedicated diterpene synthases, PtmT3 for PTM and PtmT1/PtnT1 for PTN, and the resultant diterpenes are processed by PTM-specific (e.g., PtmO5) or unspecific (e.g., enzymes for A-ring cleavage or β -oxidation) tailoring enzymes into the penultimate products platensicyl-CoA and platencinyll-CoA. PtmC/PtnC concludes the biosynthetic pathway by coupling ADHBA and the ketolides to afford PTM and PTN.

Table 1

MIC values ($\mu\text{g mL}^{-1}$) of PTM (**1**), PTN (**2**), and selected natural (**3–29**), synthetic (**30–59**), and mutasynthetic variants (**60–63**) against selected Gram-positive pathogens.

Compound	MSSA ^a	MRSA ^b	VRE ^c	Reference(s)
1	0.5	0.5–1	0.1	[15]
2	0.5	1	<0.06	[16]
3	>64	37–58	>58–64	[21, 28, 32]
4	>64	_d	–	[21]
5	>64	–	>64	[27]
6	>64	–	>64	[27]
7	>64	–	>64	[29]
8	>64	–	>64	[30]
9	>64	–	>64	[30, 33]
10	>64	–	–	[23]
11	n.d. ^e	–	–	[31]
12	>64	–	–	[23]
13	>250	–	–	[22]
14	n.a. ^f	n.a.	–	[25]
15	>64	–	–	[24]
16	n.a.	–	–	[26]
17	n.a.	–	–	[26]
18	n.a.	–	–	[26]
19	n.a.	–	–	[25, 26]
20	16	–	16	[27]
21	>64	–	>64	[30]
22	>64	–	>64	[30]
23	>64	–	>64	[30]
24	>64	–	>64	[30]
25	32–64	16	8	[25, 29]
26	>64	>64	–	[25, 28]
27	16–32	8	64	[25, 28]
28	>64	–	>64	[30]
29	0.5	–	–	[31]
30	0.4–25.6	1.6–25.6	>25.6	[34]
31	–	1.1–2.2	1.1–2.2	[32, 36]
32	–	1.3–1.8	1.3–1.8	[32, 37]
33	4	–	–	[35]
34	<0.25–2	<0.25–0.5	<0.5–16	[38]
35	<0.25–0.5	<0.25	<0.5–16	[38]
36	2	–	–	[35]
37	4	–	–	[35]

Compound	MSSA ^a	MRSA ^b	VRE ^c	Reference(s)
38	4	–	–	[35]
39	1	–	–	[35]
40	16	–	–	[35]
41	>64	–	–	[35]
42	–	3.5–4.3	6.5–8.6	[32]
43	>64	–	–	[35]
44	>64	–	–	[35]
45	–	8.0–10	>80	[32]
46	–	17–20	>83	[32]
47	128->128	>128	2–64	[40]
48	–	16	16	[39]
49	–	16	8	[39]
50	–	32	32	[39]
51	–	>85	>85	[32]
52	–	>85	>85	[32]
53	–	>82	>82	[32]
54	n.d.	–	–	[33]
55	n.d.	–	–	[33]
56	n.d.	–	–	[33]
57	n.d.	–	–	[33]
58	n.d.	–	–	[33]
59	>25.6	>25.6	>25.6	[34]
60	n.a.	–	–	[41]
61	n.a.	–	–	[41]
62	n.a.	–	–	[41]
63	n.a.	–	–	[41]

^a methicillin-sensitive *S. aureus*.

^b methicillin-resistant *S. aureus*.

^c vancomycin-resistant *Enterococci*.

^d –, not tested against this pathogen.

^e n.d., not determined (in some cases, compounds were reported as “less than that of PTM” without an MIC value).

^f n.a., no activity without specifying the up limit concentrations tested.