Published in final edited form as:

Bioorg Med Chem. 2017 March 15; 25(6): 1990–1996. doi:10.1016/j.bmc.2017.02.028.

In vivo instability of platensimycin and platencin: Synthesis and biological evaluation of urea- and carbamate-platensimycin

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Abstract

Platensimycin (PTM) and platencin (PTN), two natural products and promising drug leads that target bacterial and mammalian fatty acid synthases, are known to have unfavorable pharmacokinetic properties. It is not clear, however, what the metabolic fates of PTM and PTN are and no efforts have been reported to address this key roadblock in the development of these compounds as viable drug options. Here we describe the pharmacokinetics of PTM and PTN, and reveal rapid renal clearance as the primary metabolic liability with three additional sites of chemical liability: (i) amide hydrolysis, (ii) glucuronidation, and (iii) oxidation. We determined that hydrolysis is a viable clearance mechanism in vivo and synthesized two PTM analogues to address in vivo hydrolysis. Urea- and carbamate-PTM analogues showed no detectable hydrolysis in vivo, at the expense of antibacterial activity, with no further improvement in systemic exposure. The antibacterial sulfur-containing analogues PTM D1 and PTM ML14 showed significant decreases in renal clearance. These studies set the stage for continued generation of PTM and PTN analogues in an effort to improve their pharmacokinetics while retaining or improving their biological activities.

Graphical Abstract

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/>.

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Keywords

Platensimycin; Platencin; Natural products; Urea and carbamate analogues; Fatty acid synthase inhibitors; Amide hydrolysis

1. Introduction

Platensimycin (PTM, **1**) and platencin (PTN, **2**) comprise a new class of promising natural product antibiotics.¹ PTM and PTN are potent inhibitors of bacterial fatty acid synthase (FASII) and are effective against a wide range of bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and *Mycobacterium tuberculosis*, while displaying no toxicity.^{2–4} PTM is also a lead compound for the treatment of diabetes and related metabolic disorders as it is a potent and highly selective inhibitor of mammalian fatty acid synthase.^{5,6}

While the intrinsic properties of PTM and PTN provide optimism as a novel class of drug leads, the development of these natural products into practical therapeutics faces many obstacles. In addition to whether FASII is a viable target for antibiotic development, as exogenous fatty acids in circulation may bypass the inhibition of de novo lipogenesis,^{7,8} PTM and PTN displayed unfavorable pharmacokinetic properties as exemplified by the necessity of continuous intravenous (IV) infusion for efficacy in an in vivo mouse model of an *S. aureus* infection.^{2,3} It has been mentioned that continuous delivery was required due to rapid clearance, thereby resulting in decreased systemic exposure.⁹ However, besides the report that PTM preferentially accumulates in the liver,⁵ there was little in the literature detailing the metabolic fate of PTM and PTN prior to the commencement of this study.

PTM and PTN are composed of two distinct moieties, a diterpene-derived lipophilic ketolide and a polar 3-amino-2,4-dihydroxybenzoic acid (ADHBA), connected by an amide linkage.^{10,11} Various analogues of both PTM and PTN have been reported from fermentation of producing strains,^{12–16} biosynthetic investigations,^{17–20} and synthetic methods.^{21–25} These variants have been essential in determining the structure-activity relationships (SAR) of the moieties and functional groups of PTM and PTN. In each of these studies, however, the analogues were only tested for their antibacterial activities. Given the potencies and in vivo efficacies (under continuous delivery conditions) of PTM and PTN, it is unfortunate that none of these compounds, especially the few analogues that retained activity, were tested for improved pharmacokinetics. The major hurdle for advancing PTM and PTN in the clinic is not an improvement in antibacterial activity or fatty acid synthase inhibition, but

rather overcoming the unfavorable pharmacokinetic properties.⁹ Without understanding why PTM and PTN possess unfavorable pharmacokinetics, it is impossible to rationally design analogues to try to address this limitation. Thus, we set out to identify specific reasons why these natural products behave so poorly in vivo.

We found ourselves in an excellent position to contribute towards the design and development of PTM and PTN analogues given our construction of the PTM-PTN dual overproducing strain *Streptomyces platensis* SB12029¹⁷ and its success in revealing new natural products¹⁸ and providing new opportunities for combinatorial biosynthesis.^{19,20} While total synthetic routes of both PTM and PTN are available,^{26,27} titers of PTM from fermentation of SB12029 are >300 mg L⁻¹,^{17,28} with pilot-scale production >1.5 g L⁻¹.²⁹ These high titers significantly reduce the time and resources needed to access the PTM and PTN scaffolds. Chemical modifications can then be performed directly on the natural products, or individually on the two moieties (ADHBA or ketolide) comprising PTM and PTN.

Here we report the metabolic fate of PTM and PTN, revealing (i) high renal clearance and (ii) at least three sites of chemical liability, amide hydrolysis, ketolide oxidation, and benzoate glucuronidation. PTM D1 (**3**) and PTM ML14 (**5**), dimeric analogues of PTM previously isolated,^{18,20} are less prone to renal excretion and are converted into their active monomeric forms **1** and **4**, respectively, in vivo. Amide hydrolysis was seen as a major metabolite detected in in vitro microsome stability experiments and was observed in liver and kidney tissue after in vivo dosing of PTM, with minimal amounts seen in plasma. We designed and synthesized two PTM analogues to address in vivo amide hydrolysis. The amide bond in PTM was replaced with a urea or carbamate functional group, which increased hydrolytic stability in vitro and in vivo. Overall, these studies suggest that carefully designed PTM and PTN analogues may improve the metabolic liabilities and unfavorable pharmacokinetic properties limiting their advancement as antibiotic and antidiabetic drug leads.

2. Results and discussion

2.1. Evaluation of the metabolic properties of PTM and PTN

Studies were designed to evaluate the overall pharmacokinetics (PK) of PTM and PTN. The PK studies were augmented with in vitro experiments where metabolites of PTM or its analogues were identified. Further in vivo evaluation examined both depletion of the parent compounds and appearance of the specific metabolites.

2.1.1. Pharmacokinetic properties of PTM, PTN, and PTM analogues—Mouse PK of both PTM and PTN were evaluated in male C57Bl/6 mice (Table 1). Mice were dosed at 3 mg/kg (mpk) intravenously or 10 mpk orally with PTM (**1**) or PTN (**2**). Both PTM and PTN exhibited poor pharmacokinetics with high clearance and low oral bioavailability. PTM concentration rapidly decreased from 20 μ M five minutes after dosing to 10 nM at the two hour time point. From 2–8 h, the rate of PTM depletion greatly reduced with a calculated elimination half-life of 3.5 h. This was likely due to slow reabsorption of PTM that had distributed into tissue. Tissue distribution studies supported this hypothesis, with similar

results observed for PTN. After a 60 min IV dose of 10 mpk, PTM was detected in plasma, liver, lung, kidney, muscle, and adipose tissue (Fig. 2). The tissue-to-plasma ratios were 6.2×, 0.5×, 0.5×, 0.1×, and 0.1×, respectively. This matches with a previous report that PTM primarily accumulates in the liver.⁵ No PTM was detected in the brain. A parallel artificial membrane permeability assay (PAMPA, BD Biosciences) using PTM revealed an apparent permeability (P_{app}) of $1.3 \times 10^{-7} \text{ cm s}^{-1}$, corresponding to poor permeability and likely accounting for the lack of exposure to the brain. Similarly, PTN was detected in plasma, liver (3.8× plasma level), kidney (1.4×), lung (0.4×), and muscle (0.1×), but not in adipose tissue or the brain. Most significantly, PTM was found in extremely high concentrations (3790× plasma level) in urine, indicating that the low levels of PTM and PTN in vivo are the result of rapid renal clearance. During the preparation of this manuscript, PTM was shown to preferentially accumulate in the liver of monkeys, with high concentrations in urine and bile.⁶

Mice were also dosed with the sulfur-containing analogues PTM D1 (**3**) and PTM ML14 (**5**), which were previously isolated from PTM-PTN overproducing strains and retain the antibacterial activity of PTM.^{18,20} We proposed that these larger molecular weight analogues would be poorer candidates for renal elimination and may achieve more sustained plasma concentrations. It was clear that these “dimeric” analogues reverted back to their monomeric forms given the presence of **1** and **4** when **3** and **5** were dosed, respectively (Table 1). PTM D1 (**3**) has a single thioester and both the intact **3** and **1**, formed by the hydrolysis or retro-heteroatom Michael addition of **3**, were detected in plasma (3.3× plasma level of **1**), liver (0.6× plasma level), and kidney (0.2×), (**5** was not tested for tissue distribution). Like PTM, **3** and its metabolic product **1** were present in urine, but at dramatically decreased levels compared to that of PTM when PTM was directly dosed (<1%) (Fig. 2).

2.1.2. Hepatic microsome stability assay revealing three sites of chemical liability for PTM and PTN—As PTM and PTN both accumulate primarily in liver, and studies have focused on the phenotypes observed due to the inhibition of fatty acid synthesis by PTM in mammalian hepatocytes, we tested PTM for metabolism in hepatic microsomes. PTM was relatively stable with a calculated $Cl_{in\ vitro}$ of $13.6 \mu\text{L min}^{-1} \text{ mg}^{-1}$. Hydrolysis of the amide bond in PTM was observed in microsomal incubations initiated with the P450 cofactor NADPH, as well as in control incubations with no NADPH added. PTM clearance was low, approximately 4-fold reduced in the no-NADPH control samples where the disappearance of PTM corresponded to the stoichiometric generation of a hydrolyzed acid metabolite (**9** or **1h**). Hydrolysis of PTM was considered a possible metabolic liability because other tissues besides the liver contain enzymes capable of hydrolyzing amides. Two other metabolites were observed in microsomal incubations, corresponding to hydroxylation and glucuronidation of the carboxylic acid of PTM (Fig. 3). However, standards were not available and their relative concentrations were therefore not determined.

2.2. Design and synthesis of urea- and carbamate-PTM analogues

A crystal structure of PTM bound with its target FabF (a C163Q variant)² and synthetic SAR studies²³ exposed a simple guide to the biological activity of the PTM pharmacophore. There are several key drug-protein interactions, with the benzoate moiety sitting deep within

the binding pocket, that account for the binding of PTM to FabF (Fig. 4). The hydrophobic diterpene-derived ketolide forms two hydrogen bonds with T270 and A309. However, structural modifications of the ketolide is, in some cases, tolerated without severe effects on its biological activity.²³ PTN, the most obvious example of this, showcases that nature has already developed a way to modify this moiety, resulting in both retention of biological activity and a change in target selectivity.³ In contrast, the benzoate moiety possesses a strict SAR, with most structural modifications severely impacting biological activity.²³ This is due to the strong ionic interaction between the carboxylate of PTM and H303 and H340, along with the benzoic acid ring π -stacking with F400 (Fig. 4). Finally, the amide bond of the linker forms hydrogen bonds with T270 and T307. The amide bond of PTM and PTN, was previously modified using mutasynthesis, however, the ADHBA moiety was also modified.¹⁹ Therefore, it remains unclear how modifying this functional group, including the length of the linker, joining the two moieties together will alter its biological activity.

As we sought to address the hydrolytic instability of PTM and PTN, while retaining biological function, we focused on replacing the susceptible amide bond with a more stable functional group without modifying other positions in the molecule. Given our capacity to produce PTM in excellent yields by fermentation,^{13–18,28,29} we envisioned that both the urea and carbamate analogues could be easily accessed through a coupling reaction between a ketolide moiety and ADHBA or trihydroxybenzoic acid, respectively. Urea functional groups are much more stable to hydrolysis than amides³⁰ and several urea- and carbamate-containing compounds are clinically-relevant drugs or drug leads.^{31,32}

2.2.1. Production and hydrolysis of PTM—Our synthesis commenced with PTM (**1**), which was easily isolated from the PTM-PTN dual overproducing strain *S. platensis* SB12029.¹⁷ Approximately 1.2 g of PTM was isolated from a 6 L fermentation of *S. platensis* SB12029. Previously, only one method was reported for hydrolysis of PTM, a sequential two-step sodium nitrite and lithium hydroxide treatment giving platensic acid (**9**) at a yield of 60%.²² During the preparation of this manuscript, an two-step LiOH-mediated sulfuric acid hydrolysis in ethanol was reported with an overall yield of 85%.²⁵ In this work, 6 N HCl was added to **1** and refluxed in an oil bath for 1–2 h, resulting in direct hydrolysis of **1** to yield **9** with a yield of 76%.

2.2.2. Synthesis of urea- and carbamate-PTM—With the ketolide acid of PTM (**9**) in hand, we planned to synthesize the urea- and carbamate-PTM analogues using the Curtius rearrangement.³³ Ketolide **9** was readily transformed into platensic azide using diphenylphosphoryl azide (DPPA).^{34,35} As platensic azide is unstable, a single flash silica gel column was used to obtain >85% pure platensic azide, which was used directly in the next step. For the synthesis of urea-PTM methyl ester (**6**), 3-amino-2,4-dihydroxybenzoic acid methyl ester³⁶ (**11**) and platensic azide were added together and heated to 90 °C in toluene for 12 h. Although the overall yield from **9** was only 11%, 16 mg of **6** was produced, enough for preliminary biological assays and demonstrating the feasibility of replacing the natural amide with a urea linkage by Curtius rearrangement. The characteristic signals of δ_{H} 8.38 (1H, s, NH-1') and 8.30 (1H, br s, NH-2') in the ¹H NMR spectrum and δ_{C} 159.3 (C-1, s) in the ¹³C NMR spectrum supported **6** as a urea derivative (Figs. S1 and S2). The linkage

of the urea group of **6** was further supported by HMBC correlations of $\text{NH}-1'$ with C-20 (δ_{C} 155.0) and C-22 (δ_{C} 156.7) (Fig. S3).

No urea-PTM (**7**) was detected by LC-MS when platensis azide was incubated with ADHBA (**12**) using the method described above. A suspension of **12** in toluene suggested poor solubility. To increase the solubility of **12** in the reaction mixture, DMF was added after platensis azide was fully converted into the Curtius reaction product, platensis isocyanate (**10**). An overall yield of 35% of **7** was produced, the ^1H and ^{13}C NMR of which was very similar to those of **6**, except the expected lack of the methyl signal and a downfield shift of the C-18 signal in the ^{13}C NMR spectrum (Figs. S5 and S6).

Carbamate-PTM (**8**) was synthesized with an overall yield of 10% using a similar method for that of **7** by adding 2,3,4-trihydroxybenzoic acid (**13**) and DMF. The ^{13}C NMR chemical shifts of C-20 (δ_{C} 156.1), C-21 (δ_{C} 126.4), and C-22 (δ_{C} 156.2) in **8** (Figs. S7 and S8) were very similar to those of the corresponding positions (δ_{C} 155.7, 116.6, and 155.8) in **7**, with the C-21 chemical shift of **8** expectantly downfield of the C-21 of **7** due to the stronger electronegativity of the oxygen. Thus, **8** was determined to possess a carbamate group coupled to the *meta*-OH of **13**.

2.3. Urea- and carbamate-PTM lost their antibiotic activities

Compounds **6–8** were tested for antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 9431 and minimum inhibitory concentrations (MICs) were determined using the broth dilution method.³⁷ Both **7** and **8** were inactive (MICs >64 mg mL^{-1}) against both strains, while **6** showed no activity against *S. aureus* (>64 mg mL^{-1}) and weak activity (64 mg mL^{-1}) against *M. luteus*. PTM, used as a positive control, had MICs of 0.5 mg mL^{-1} against both strains. Given that the only structural difference between PTM (**1**), urea-PTM (**7**), and carbamate-PTM (**8**) is the urea or carbamate functional group, thereby adding one additional atom to the linker region, the loss of antibacterial activity suggests that the four atom (three carbons and one nitrogen) linker region is necessary for proper orientation of both the ADHBA and ketolide moieties while binding to FabF.

2.3.1. Amide hydrolysis as a viable clearance mechanism—With at least three metabolic liabilities associated with using PTM and PTN in an in vivo system, we sought to determine if any of these liabilities were observable in tissue and plasma. PTM and PTN were hydrolyzed in vivo, with detectable concentrations in plasma, liver, kidney, and urine (Table 2). No hydrolyzed metabolites of PTM (**9**) or PTN were detected in lung, muscle, adipose, or brain tissue. Qualitative amounts of oxidized and glucuronidated metabolites of PTM and its hydrolysis product were also detected in liver and kidney (only oxidation seen), and urine. When the amide of PTM was replaced with urea (**7**) or carbamate (**8**) functionalities, hydrolysis was eliminated in vivo. Levels of the parent compounds **7** and **8** were comparable to that of PTM, but no hydrolyzed products of **7** or **8**, **7h** or **8h**, in analogy to **1h** (or **9**), respectively, were observed in any tissue or urine (Table 2). Overall, these observations support hydrolysis of the amide bond, as well as oxidation and glucuronidation, as viable clearance mechanisms of PTM and PTN.

3. Conclusions

The history of drug discovery is full of success stories where a lead compound suffers initially from poor pharmacokinetics but extensive SAR and analogue generation lead to the development of a clinically useful drug. The epothilones are such an example. These macrolide natural products initially suffered from inactivation via hydrolysis, but the macrolactam analogues showed significantly higher stability.³⁸ Success stories such as this highlight the wisdom of investigating the metabolic fate of PTM and PTN and developing analogues that address current drug metabolism and pharmacokinetic concerns.

PTM and PTN have been colloquially reported as having unfavorable pharmacokinetics. Although much work has been done to isolate natural congeners, perform SAR experiments, and synthesize analogues of these natural products, there has been no reported effort to address the poor pharmacokinetic properties. We set out to uncover the causes of these poor properties and to design and test PTM analogues to address these issues.

The need for continuous infusion of PTM and PTN for in vivo efficacy is undoubtedly due to its rapid renal clearance, resulting in low levels of drug in plasma. Thus, PTM and PTN need to overcome their propensity for rapid clearance, and thus low levels of systemic exposure, to be effective as clinical antibiotics. We successfully eliminated PTM hydrolysis as a minor clearance pathway by designing analogues with stable urea or carbamate linkages. Showing additional promise were the sulfur-containing “dimers” **3** and **5**, which exhibited significantly reduced renal clearance. The dimers readily reverted back to their monomeric forms; however, the plasma concentration of the monomer was not higher than when PTM was directly dosed. While **3** and **5** are not the solution to solving the puzzle of the poor pharmacokinetics of PTM, they demonstrate that analogues can be generated with reduced renal clearance and support the design, isolation, or synthesis of biologically active analogues of PTM and PTN to improve the pharmacokinetic properties. In addition, the presence of the monomers of **3** and **5** in vivo suggests that PTM and PTN analogues designed as prodrugs are another feasible approach to improving these drug leads.

As a lead compound for the treatment of diabetes, liver steatosis, and other metabolic disorders,^{3,6} enrichment of PTM in liver may be desirable; rapid degradation of PTM, however, is not. We uncovered three sites of liability for PTM and PTN: (i) hydrolysis of the amide bond (ii) glucuronidation of the ADHBA moiety, and (iii) oxidation of the hydrophobic ketolide moiety. Improvement in the stability for each of these pathways becomes more important after the renal clearance issue has been successfully addressed. By designing analogues targeting the amide linkage of PTM, a functional group not previously modified in SAR studies, we sought to improve the hydrolytic stability of PTM, and possibly the pharmacokinetic properties as well, while maintaining the inherent biological activity. By replacing the amide with urea or carbamate functional groups, we dramatically improved the hydrolytic stability of PTM, although at the cost of antibacterial activity. We are optimistic that additional studies will lead to potent and stable analogues. For example, synthetic or semi-synthetic approaches to construct a urea- or carbamate-linker that retains the typical four atom length may result in nonhydrolyzable analogues that retain biological activity.

Although the development of PTM and PTN as clinically relevant pharmaceutical options faces major obstacles, it would be unwise to abandon the study of these natural products. As researchers see promise in scouring the literature and databases for orphaned antibiotics, with a hope to resurrect them as viable drug candidates,³⁹ promising, yet undeveloped, current drug leads, such as PTM and PTN, should remain in the pipeline.

4. Experimental section

4.1. Chemistry

4.1.1. General chemical experimental procedures—All chemicals were purchased from standard commercial sources. All ^1H , ^{13}C , and 2D NMR (HSQC, ^1H – ^1H COSY, HMBC) spectra were collected using a Bruker Avance III Ultrashield 700 at 700 MHz for ^1H and 175 MHz for ^{13}C nuclei. MPLC separation was conducted on a Biotage Isolera One equipped with a Biotage SNAP Cartridge HP-Sil column (60 g). Preparative HPLC was carried out on an Agilent 1260 Infinity LC equipped with an Agilent Eclipse XDB-C18 column (250 mm $\times$ 21.2 mm, 7 μm). LC-MS was performed on an Agilent 1260 Infinity LC coupled to a 6230 TOF (HRESI) equipped with an Agilent Poroshell 120 EC-C18 column (50 mm $\times$ 4.6 mm, 2.7 μm). Optical rotations were measured using an AUTOPOL IV automatic polarimeter (Rudolph Research Analytical). UV spectra were obtained with a NanoDrop 2000C spectrophotometer (Thermo Scientific). IR spectra were collected with a Spectrum One FT-IR spectrometer (PerkinElmer).

4.1.2. Production and isolation of PTM and PTN—As previously described,¹⁷ fresh spores of *S. platensis* SB12029 were inoculated into BactoTM tryptic soy broth (BD) and shaken at 250 rpm and 28 $^{\circ}\text{C}$ for 48 h. Twenty milliliters of the resultant seed culture were used to inoculate 500 mL of PTM medium supplemented with 15 g of Amberlite XAD-16 resin (Sigma-Aldrich). After shaking at 250 rpm for 7 d, 6 L (500 mL $\times$ 12 flasks) of the fermentation culture were harvested by centrifugation to obtain the resin, which was subsequently washed three times with H_2O and extracted three times with ca. 600 mL of methanol. Methanol was removed by rotary evaporation and the resulting oil was adsorbed onto C18 reverse phase resin (Biotage) and fractionated using MPLC with an elution system of methanol- H_2O (5–100%). The fractions containing PTM were combined and further purified by preparative HPLC using a 30 min solvent gradient elution system of 10–70% CH_3CN in H_2O containing 0.1% formic acid at a flow rate of 17 mL min^{-1} to obtain 1.2 g of PTM (**1**).

4.1.3. Hydrolysis of PTM

Platensic acid (9): A stirred solution of **1** (136 mg, 0.308 mmol) in 6 N HCl (5.0 mL) was refluxed for 1.5 h. The reaction mixture was then cooled to room temperature and quenched with 3 mL of 6 N NaOH and saturated NaHCO_3 solution. The resultant mixture was extracted with EtOAc three times, dried over Na_2SO_4 , concentrated in vacuo, and purified by preparative HPLC using a 30 min gradient elution system of 30–100% CH_3CN in H_2O containing 0.1% formic acid at a flow rate of 17 mL min^{-1} to afford **9** (68 mg, 76%) as a white solid. ^1H NMR (700 MHz, pyridine- d_5) δ_{H} : 6.41 (1H, d, J = 9.8 Hz, H-7), 6.00 (1H, d, J = 9.8 Hz, H-6), 4.57 (1H, br t, J = 3.0 Hz, H-10), 2.69 (1H, m, H-3a), 2.67 (1H, m, H-2a),

2.59 (1H, m, H-2b), 2.56 (1H, s, H-9), 2.23 (1H, t, $J = 6.3$ Hz, H-12), 2.04 (1H, m, H-3b), 1.96 (1H, m, H-11a), 1.87 (1H, br d, $J = 11.2$ Hz, H-12b), 1.85 (1H, dd, $J = 11.2, 3.5$ Hz, H-14a), 1.81 (1H, dd, $J = 10.5, 3.5$ Hz, H-13a), 1.60 (1H, ddt, $J = 11.2, 7.0, 1.4$ Hz, H-13b), 1.51 (1H, br d, $J = 10.5$ Hz, H-14b), 1.42 (3H, s, Me-16), 1.19 (3H, s, Me-17); ^{13}C NMR (175 MHz, pyridine- d_5) δ_{C} : 203.6 (C-5, s), 176.1 (C-1, s), 154.3 (C-7, d), 127.7 (C-6, d), 87.2 (C-15, s), 76.9 (C-10, d), 55.3 (C-14, t), 47.0 (C-4, s), 46.8 (C-8, s), 46.5 (C-9, d), 45.4 (C-12, d), 43.4 (C-13, t), 41.2 (C-11, t), 32.1 (C-3, t), 30.7 (C-2, t), 25.0 (C-17, q), 23.6 (C-16, q). HRESIMS affording the $[\text{M} + \text{H}]^+$ ion at m/z 291.1587 (calcd $[\text{M} + \text{H}]^+$ for $\text{C}_{17}\text{H}_{22}\text{O}_4$ at 291.1591).

4.1.4. PTM analogues 6–8—CAUTION! While we had no issues, any synthetic experiments performed involving azides should be handled with appropriate precautions against exothermic decomposition.

Urea-PTM methyl ester (6): To a solution of **9** (87 mg, 0.3 mmol) in toluene (3 mL), Et_3N (280 μL , 2.0 mmol) and diphenylphosphoryl azide (372 mg, 1.35 mmol) were added at room temperature under argon. After stirring for 3 h at room temperature, the solution was directly concentrated in vacuo and purified by flash silica gel chromatography (2:1 *n*-hexanes/ EtOAc) to obtain partially pure (>85%) platensic azide (80 mg). Platensic azide was used in the next step without additional purification. Platensic azide (80 mg) was dissolved in toluene (2 mL), and 3-amino-2,4-dihydroxybenzoic acid methyl ester³⁵ (**11**) (69 mg, 0.377 mmol) was added under argon at room temperature. The reaction mixture was heated to 90 °C and stirred for 12 h. After concentration in vacuo, the crude product was purified by preparative HPLC using a 30 min gradient elution system of 30–100% CH_3CN in H_2O containing 0.1% formic acid at a flow rate of 17 mL min^{-1} to afford **6** (16 mg, 11% overall yield from **9**) as a white solid. $[\alpha]_{\text{D}}^{23} -49.2$ (c 0.12, CH_3OH); UV (CH_3OH) λ_{max} (log ϵ) 240 (5.59) nm; IR ν_{max} 3364, 2955, 1661, 1595, 1560, 1438, 1340, 1260, 1212, 1148, 831, 788 cm^{-1} ; ^1H NMR (700 MHz, pyridine- d_5) δ_{H} : 8.38 (1H, s, NH-1'), 8.30 (1H, br s, NH-2'), 7.57 (1H, d, $J = 8.4$ Hz, H-24), 6.79 (1H, d, $J = 8.4$ Hz, H-23), 6.40 (1H, d, $J = 9.8$ Hz, H-7), 5.99 (1H, d, $J = 9.8$ Hz, H-6), 4.70 (1H, s, H-10), 3.73 (3H, s, OMe-25), 3.68 (1H, m, H-2a), 3.32 (1H, m, H-2b), 2.59 (1H, s, H-9), 2.37 (1H, ddd, $J = 14.0, 10.5, 5.6$ Hz, H-3a), 2.22 (1H, t, $J = 6.3$ Hz, H-12), 1.96 (1H, m, H-11a), 1.87 (1H, ddd, $J = 14.0, 10.5, 5.6$ Hz, H-3b), 1.86 (1H, br d, $J = 11.2$ Hz, H-11b), 1.82 (1H, dd, $J = 11.9, 3.5$ Hz, H-13a), 1.79 (1H, dd, $J = 11.2, 3.5$ Hz, H-14a), 1.58 (1H, dd, $J = 11.2, 7.0$ Hz, H-13b), 1.49 (1H, br d, $J = 11.2$ Hz, H-14b), 1.42 (3H, s, CH_3 -16), 1.14 (3H, s, CH_3 -17); ^{13}C NMR (175 MHz, pyridine- d_5) δ_{C} : 203.6 (C-5, s), 171.3 (C-18, s), 159.3 (C-1, s), 156.7 (C-22, s), 155.0 (C-20, s), 154.4 (C-7, d), 127.4 (C-6, d), 126.6 (C-24, d), 116.7 (C-21, s), 111.6 (C-23, d), 104.9 (C-19, s), 87.2 (C-15, s), 77.0 (C-10, d), 55.3 (C-14, t), 52.4 (C-25, q), 46.9 (C-9, d), 46.5 (C-4, s), 46.5 (C-8, s), 45.4 (C-12, d), 43.4 (C-13, t), 41.2 (C-11, t), 37.2 (C-2, t), 36.8 (C-3, t), 24.9 (C-17, q), 23.6 (C-16, q). HRESIMS affording the $[\text{M} + \text{H}]^+$ ion at m/z 471.2128 (calcd $[\text{M} + \text{H}]^+$ for $\text{C}_{25}\text{H}_{30}\text{N}_2\text{O}_7$ at 471.2126).

Urea-PTM (7): Platensic azide was prepared as described above. Platensic azide (42.5 mg) was dissolved in toluene (1 mL) and stirred at 90 °C for 2 h until all of the starting material had disappeared. After the solution was cooled to room temperature, ADHBA⁴⁰ (**12**) (60

mg, 0.355 mmol) and 125 μ L of DMF were added and stirred for an additional 3.5 h. Direct evaporation of the solvent gave a crude product which was subjected to preparative HPLC using a 30 min gradient elution system of 30–80% CH₃CN in H₂O containing 0.1% formic acid at a flow rate of 17 mL min⁻¹ to afford a mixture of **7** and an unknown byproduct. The mixture was redissolved in CH₂Cl₂ and washed three times using 2 N HCl to remove the unknown byproduct. The desired product was repurified by preparative HPLC using a 30 min gradient elution system of 40–100% CH₃OH in H₂O containing 0.1% formic acid at a flow rate of 17 mL min⁻¹ to obtain **7** (25 mg, 35% overall yield from **9**) as a white solid.

$[\alpha]_D^{23}$ -30.7 (c 0.15, CH₃OH); UV (CH₃OH) $\lambda_{\max}$ (log ϵ) 297 (4.53), 224 (5.68) nm; IR $\nu_{\max}$ 2960, 1772, 1655, 1595, 1389, 1021, 953, 829 cm⁻¹; ¹H NMR (700 MHz, pyridine-*d*₅) δ_H : 8.39 (1H, s, NH-1'), 8.34 (1H, br s, NH-2'), 7.98 (1H, d, *J* = 8.4 Hz, H-24), 6.88 (1H, d, *J* = 8.4 Hz, H-23), 6.40 (1H, d, *J* = 9.8 Hz, H-7), 5.99 (1H, d, *J* = 9.8 Hz, H-6), 4.71 (1H, s, H-10), 3.71 (1H, m, H-2a), 3.33 (1H, m, H-2b), 2.60 (1H, s, H-9), 2.38 (1H, ddd, *J* = 14.0, 11.2, 5.6 Hz, H-3a), 2.21 (1H, t, *J* = 6.3 Hz, H-12), 1.96 (1H, m, H-11a), 1.89 (1H, ddd, *J* = 14.0, 11.2, 5.6 Hz, H-3b), 1.86 (1H, br d, *J* = 11.2 Hz, H-11b), 1.82 (1H, dd, *J* = 11.9, 3.5 Hz, H-13a), 1.80 (1H, dd, *J* = 11.2, 3.5 Hz, H-14a), 1.58 (1H, dd, *J* = 11.2, 7.0 Hz, H-13b), 1.49 (1H, br d, *J* = 11.2 Hz, H-14b), 1.42 (3H, s, CH₃-16), 1.14 (3H, s, CH₃-17); ¹³C NMR (175 MHz, pyridine-*d*₅) δ_C : 203.6 (C-5, s), 175.2 (C-18, s), 159.4 (C-1, s), 155.8 (C-22, s), 155.7 (C-20, s), 154.4 (C-7, d), 127.4 (C-6, d), 127.3 (C-24, d), 116.6 (C-21, s), 111.0 (C-23, d), 106.8 (C-19, s), 87.2 (C-15, s), 77.0 (C-10, d), 55.3 (C-14, t), 46.9 (C-9, d), 46.5 (C-4, s), 46.5 (C-8, s), 45.4 (C-12, d), 43.4 (C-13, t), 41.2 (C-11, t), 37.2 (C-2, t), 36.8 (C-3, t), 24.9 (C-17, q), 23.6 (C-16, q). HRESIMS affording the [M + H]⁺ ion at *m/z* 457.1971 (calcd [M + H]⁺ for C₂₄H₂₈N₂O₇ at 457.1969).

Carbamate-PTM (8): Platensic azide was prepared as described above. Platensic azide (44.0 mg) was dissolved in toluene (1 mL) and stirred at 90 °C for 2 h until all of the starting material had disappeared. After the solution was cooled to room temperature, 2,3,4-trihydroxybenzoic acid (**13**) (33 mg, 0.194 mmol, Sigma-Aldrich) and 60 μ L of DMF were added and stirred for an additional 24 h. Direct evaporation of the solvent gave a crude product which was subjected to preparative HPLC using a 30 min gradient elution system of 5–60% CH₃CN in H₂O containing 0.1% formic acid at a flow rate of 17 mL min⁻¹ to afford **8** (7.9 mg, 10% overall yield from **9**) as a white solid. $[\alpha]_D^{23}$ -24.3 (c 0.12, CH₃OH); UV (CH₃OH) $\lambda_{\max}$ (log ϵ) 291 (4.78), 244 (5.41), 214 (5.66) nm; IR $\nu_{\max}$ 3190, 2963, 1732, 1662, 1457, 1316, 1253, 1053, 1016, 953, 831, 798 cm⁻¹; ¹H NMR (700 MHz, DMSO-*d*₆) δ_H : 10.42 (1H, s, COOH), 7.64 (1H, t, *J* = 5.6 Hz, NH-2'), 7.48 (1H, d, *J* = 8.4 Hz, H-24), 6.65 (1H, d, *J* = 9.8 Hz, H-7), 6.44 (1H, d, *J* = 8.4 Hz, H-23), 5.82 (1H, d, *J* = 9.8 Hz, H-6), 4.43 (1H, s, H-10), 3.06 (1H, m, H-2a), 2.75 (1H, m, H-2b), 2.34 (1H, t, *J* = 6.3 Hz, H-12), 2.24 (1H, br s, H-9), 1.97 (1H, overlapped, H-3a), 1.96 (1H, br d, *J* = 11.2 Hz, H-11a), 1.93 (1H, dd, *J* = 11.9, 3.5 Hz, H-13a), 1.92 (1H, m, H-11b), 1.77 (1H, dd, *J* = 11.9, 7.0 Hz, H-13b), 1.68 (2H, m, H-14), 1.64 (1H, overlapped, H-3b), 1.34 (3H, s, CH₃-16), 1.12 (3H, s, CH₃-17); ¹³C NMR (175 MHz, DMSO-*d*₆) δ_C : 202.8 (C-5, s), 172.0 (C-18, s), 156.2 (C-22, s), 156.1 (C-20, s), 154.4 (C-7, d), 153.5 (C-1, s), 127.2 (C-24, d), 126.4 (C-21, s), 126.2 (C-6, d), 107.4 (C-23, d), 105.1 (C-19, s), 86.3 (C-15, s), 75.6 (C-10, d), 54.1 (C-14, t), 45.9 (C-9, d), 45.6 (C-8, s), 45.3 (C-4, s), 44.2 (C-12, d), 42.3 (C-13, t), 40.1 (C-11, t), 36.4 (C-2,

t), 35.0 (C-3, t), 24.1 (C-17, q), 22.9 (C-16, q). HRESIMS affording the $[M + H]^+$ ion at m/z 458.1807 (calcd $[M + H]^+$ for $C_{24}H_{27}NO_8$ at 458.1809).

4.2. Biological Evaluations

4.2.1. Antibacterial assay—The antibacterial activities of compounds **6–8** were tested against *S. aureus* ATCC 25923 and *M. luteus* ATCC 9431. MIC values were determined using a 96-well plate format with Müller-Hinton (MH) broth.³⁷ Each strain was grown in MH broth to an $OD_{600} = 0.5$, diluted 100× with MH broth, and 98 μ L of the resultant cell culture was pipetted into each well. Two microliters of each compound, serially diluted in DMSO, were added to each well, resulting in final concentrations of 0.125–64 μ g mL^{-1} . PTM (**1**) and DMSO were used as positive and negative controls, respectively. The MIC values were determined after incubation at 37 °C for 18 h. Each MIC determination was performed in duplicate.

4.2.2. Hepatic microsome stability assay—Microsome stability was evaluated by incubating 1 μ M compound with 1 mg mL^{-1} hepatic microsomes (mouse) in 100 mM potassium phosphate buffer (pH 7.4) at 37 °C with continuous shaking. Reactions were performed in the absence or presence of 1 mM NADPH. The final incubation volumes were 200 μ L and 20 μ L aliquots were removed at six time points: 0, 5, 10, 20, 40 and 60 min. Each aliquot was added to 150 μ L of CH_3CN to quench the reaction and precipitate the protein. Resultant samples were centrifuged through a 0.45 μ m filter plate (Millipore Solventer low binding hydrophilic plates) and analyzed by LC-MS/MS. The data were log-transformed and reported as half-lives (min).

4.2.3. Single dose pharmacokinetic evaluation—Compounds **1** and **2** were dosed at 3 mpk by IV into the tail vein or 10 mpk by oral gavage in 8–10-week-old C57Bl/6 mice ($n = 3$). Each drug was formulated to a concentration of 0.6 mg mL^{-1} in 10:10:80 DMSO/Tween80/water. Compounds **3** and **5** were dosed at 10 mpk by IV at 1.0 mg mL^{-1} . Plasma samples were collected at approximately 5 min, 15 min, and 0.5, 1, 2, 4, 6, and 8 h. Each sample was mixed with 6× v/v CH_3CN to precipitate the protein and filtered through a 0.2 μ m filter prior to analysis by LC-MS/MS. All procedures were approved by the Scripps Florida IACUC and Scripps vivarium is fully AAALAC accredited.

4.2.4. Plasma and tissue concentrations of PTM, PTN, PTM derivatives, and their degradation products—PTM (**1**), PTN (**2**), PTM D1 (**3**), urea-PTM (**7**) and carbamate-PTM (**8**) were dosed to 8–10-week-old C57Bl/6 mice ($n = 3$) at 10 mpk by IV. Each drug was formulated to a concentration of 2 mg mL^{-1} solution in 10:10:80 DMSO/Tween80/water. For **1** and **2**, after 1 h, blood, liver, lung, kidney, muscle, adipose tissue, brain, and urine were collected. For **3**, **7**, and **8**, only plasma, liver, kidney, and urine were collected. Plasma was generated using standard centrifugation techniques, and the plasma and tissues were frozen at -80 °C. Plasma and tissues were mixed with CH_3CN (1:5 v/v or 1:5 w/v, respectively) and tissues were subsequently disrupted with brief pulses of a probe tip sonicator on ice. Samples were analyzed for drug or metabolite by LC-MS/MS. Drug levels were determined against standards made in plasma and tissue levels against standards made in the appropriate blank tissue matrix.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported in part by the NIH Grant GM114353. JDR is supported in part by an Arnold O. Beckman Postdoctoral Fellowship. We thank Zhengren Xu for insightful discussions on urea- and carbamate-PTM synthesis. This is manuscript #29457 from The Scripps Research Institute.

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Figure 1.
Structures of PTM, PTN, and the PTM analogues generated and/or used in this study.

Figure 2.

Tissue distribution of PTM (1), PTN (2), and PTM D1 (3) after 1 hr of a 10 mpk IV dose (n = 3). The panel graph displays concentrations of compound from 0–4 µM (lower panel) and 0–2000 µM (upper panel). PTM (1) was found when PTM D1 (3) was dosed (i.e., 1 from 3). PTN concentration in urine was not determined. Error bars represented standard error of the mean.

Figure 3.
Three sites of liabilities for PTM.

Figure 4.

PTM bound in the active site pocket of *E. coli* FabF(C163Q). The interactions with active site residues that are responsible for the structure-activity relationships of PTM are shown (PDB entry 2GFX). PTM is shown in green, protein residues are shown in magenta, and the active site cavity is shaded in gray.

Scheme 1.

Synthesis of **6** from PTM (**1**). Reagents and conditions: a) 6 N HCl, reflux, 1.5 h, 76%; b) DPPA, Et₃N, toluene, RT, 3 h; c) **11**, toluene, 90 °C, 12 h, 11% overall yield from **9**. DPPA = diphenylphosphoryl azide.

Scheme 2.

Synthesis of **7** and **8** from platensic acid (**6**). Reagents and conditions: a) DPPA, Et₃N, toluene, RT, 3 h; b) toluene, 90 °C, 2 h; c) **12**, DMF, RT, 3.5 h, 35% overall yield from **6**; d) **13**, DMF, 40 °C, 24 h, 10% overall yield from **9**. DMF = dimethylformamide.

Table 1In vivo pharmacokinetic data in mice^a

Cmpd	<i>T</i> _{1/2} (h)	<i>C</i> _{max} (μM)	AUC ₀₋₈ (μM·h)	Cl _{obs} (mL min ⁻¹ kg ⁻¹)	<i>V</i> _d (L kg ⁻¹)	%F, po
1-IV^b	3.5	20	10	16	0.90	-
1-PO^c	3.2	2.1	1.5	-	-	4.4
2-IV^b	0.22	11	6.8	23	0.46	-
2-PO^c	1.3	4.0	2.3	-	-	10
3-IV^d	1.1	38	18	11	0.33	-
1 from 3	1.9	15	6.3	62	2.5	-
5-IV^d	0.76	150	71	3.0	0.08	-
4 from 5	1.8	2.3	2.5	170	15	-

^aUnless noted, data were the mean of n = 3 mice.^bIV, 3 mpk.^cPO, 10 mpk; only one determination.^dIV, 10 mpk.

Table 2Hydrolytic stability of PTM and urea- and carbamate-PTM^a

Tissue	1	1h (9) ^b	7	7h ^b	8	8h ^b
Plasma	1.0 ^c	0.1	1.4	n.d. ^d	2.1	n.d.
Liver	1.0	0.04	0.5	n.d.	5.0	n.d.
Kidney	1.0	0.2	2.2	n.d.	2.2	n.d.
Urine	1.0	0.0005	1.2	n.d.	1.0	n.d.

^a Data were the mean of n = 2 or 3 mice.^b h, hydrolyzed metabolite detected (**1h** or **9**) or predicted (**7h** or **8h**).^c All concentrations were normalized to that of **1**.^d n.d., not detected.