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Engineering of *Streptomyces platensis* MA7339 for Overproduction of Platencin and Congeners

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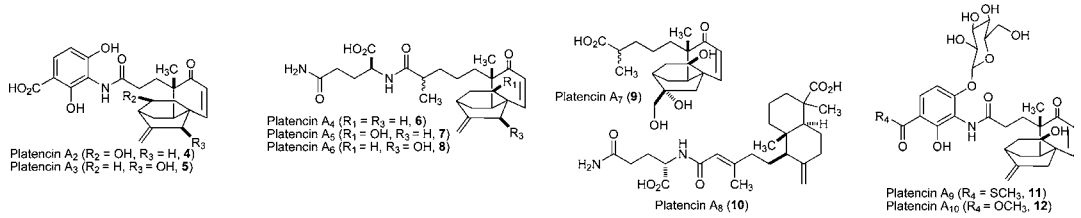
ABSTRACT



Platensimycin (1) and platencin (2) are novel antibiotic leads against multidrug resistant pathogens. The production of 2 in *Streptomyces platensis* MA7339 is under the control of *ptnR1*, a GntR-like transcriptional regulator. Inactivating *ptnR1* afforded *S. platensis* MA7339 mutant strain SB12600 that overproduces 2 at a titer ~100-fold greater than that from the wild-type strain and accumulates platencin A_1 (3) and eight new congeners, platencins A_2−A_9 (4−11). The isolation, structural elucidation, and antibacterial activity of 4−11, in comparison to 1−3, are described.

The recently discovered platensimycin (**1**) and platencin (**2**) represent one of only a few new classes of antibiotics that have been discovered since the early 1960s.[1−4] They potently inhibit the growth of a range of Gram-positive bacteria, including multidrug-resistant *Staphylococcus aureus* and *Enterococcus* sp., making them important antibiotic leads against drug-resistant pathogens. Although both compounds work by inhibiting fatty acid biosynthesis, they have slightly different mechanisms of action: **1** specifically inhibits FabF, whereas **2** synergistically inhibits FabF and FabH subunits of the bacterial type II fatty acid synthase.[1,2] To determine structure−activity relationships (SAR) of **1** and **2**, libraries of analogues featuring the **1** and **2** scaffolds have been generated by organic synthesis.[5−12] Congeners of **1** and **2** have also been isolated from the wild-type producers upon

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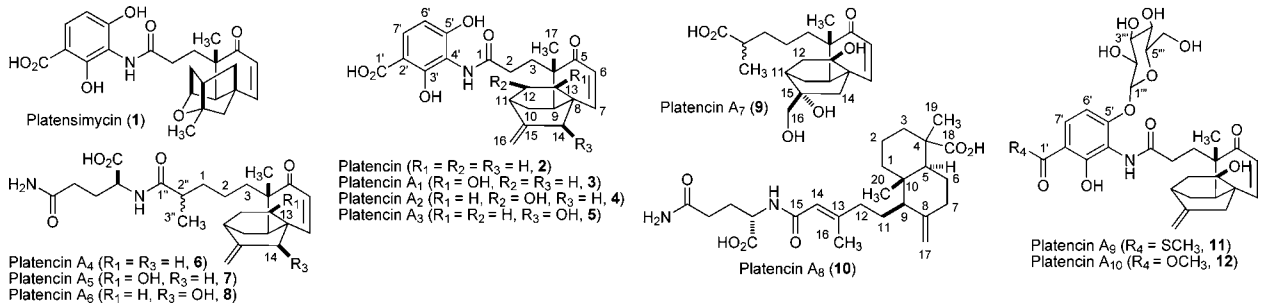

Figure 1. Structures of platensimycin (**1**), platencin (**2**), and the platencin congeners (**3**−**11**) isolated from the engineered *S. platensis* SB12600 strain.

large-scale fermentations.[13−17] Of the analogues reported to date, only platencin A_1 (**3**) and its methyl ester retain the carbon scaffold of **2** (Figure 1).[14]

We recently reported engineered strains from *Streptomyces platensis* MA7327, the wild-type dual producer of **1** and **2**, that are capable of overproducing **1** and **2** with ∼100 fold improved titers.[18] We now report an engineered strain from *S. platensis* MA7339, the wild-type exclusive producer of **2**, that overproduces **2**, **3**, and eight new congeners platencin A_2−A_9 (**4**−**11**) (Figure 1). The isolation, structural elucidation, and antibacterial activity of **4**−**11** in comparison to **1**−**3** are described.

The gene cluster encoding **2** production in *S. platensis* MA7339 contains *ptnR1*, a closely related homologue to the pathway specific repressor *ptmR1* characterized from the **1** and **2** dual producer *S. platensis* MA7327; inactivation of *ptmR1* previously afforded recombinant strains that overproduce **1** and **2**.[18] We similarly inactivated *ptnR1*, using REDIRECT Technology,[19] in *S. platensis* MA7339 to afford the desired mutant strain SB12600, the genotype of which was confirmed by PCR and Southern analyses (see the Supporting Information).

To determine the effect of *ptnR1* inactivation on **2** production, SB12600 was fermented in two different production media with the wild-type strain as a control. In the standard production medium (PTNM),[18] the titer of **2** increased from ∼0.05 mg/L in the wild-type to ∼22 mg/L in SB12600. In addition, HPLC analysis of the crude extract from SB12600 revealed many other metabolites with similar UV−vis spectra to **2** (Figure 2, panel i, and Table 1). In the second medium (SLY) that was previously used for **1** and **2** overproduction by the *S. platensis* MA7327 dual producers,[18] no improvement in **2** titer was seen; however, the additional metabolites were produced in increased titers (Figure 2, panel ii, and Table 1) (see Supporting Information).

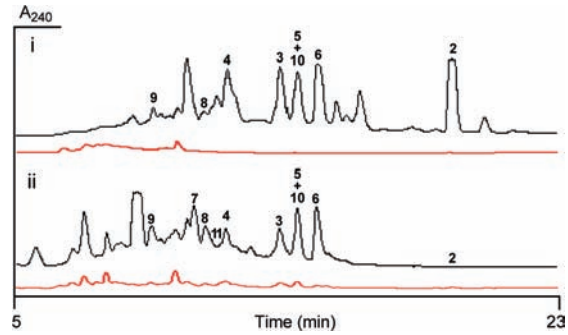

Figure 2. Metabolite profiles of *S. platensis* MA7339 wild-type (red) and SB12600 (black) strains fermented in PTNM (i) or SLY (ii) medium upon HPLC analyses of the crude extracts.

To isolate the overproduced metabolites, SB12600 was cultured in both PTNM (7.2 L) and SLY (4.4 L) media, and the crude extracts made from the combined fermentation culture were subjected to mutiple steps of chromatography, affording **2** and nine additional metabolites (**3**−**11**) (Figure 1) (see the Supporting Information).

Compound **2** was confirmed as platencin by comparison with an authentic standard.[2,4,18] Compound **3** was identified as platencin A_1 by spectroscopic comparisons with literature, including a NOESY correlation between H-13 and H_3-17 that verified the stereochemistry of the hydroxyl group at C-13.[14]

Compounds **4** and **5** share the same molecular formula of $C_{24}H_{27}NO_7$ as established by HiResMALDI-FTMS that yielded $[M + Na]^+$ ions at *m/z* 464.16586 and 464.16605, respectively (calcd for $C_{24}H_{27}NO_7Na$, 464.16797). Each was predicted to be a hydroxylated analogue of **2** based on the 16 mass increase and the initial ^1H NMR spectra confirming the presence of an exomethylene characteristic of **2**. Similar to compound **3**, the loss of a methylene carbon signal and the appearance of unique oxygenated methine signals in compounds **4** and **5** suggested hydroxylation of the terpene moiety. The hydroxyl group in **4** was assigned to C-12 on the basis of a clear gCOSY correlation between the down-

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Table 1. Estimated Titers of Platencin (**2**) and Congeners (**3**−**11**) Produced by *S. platensis* MA7339 Wild-Type and SB12600 Strains (mg/L)[a]

strain	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
MA7339[b]	0.05 ± 0.04	nd[e]	nd	nd	nd	nd	nd	nd	nd	nd
SB12600[b]	22 ± 3.0	15 ± 4.7	14 ± 3.2	12 ± 3.6	42 ± 4.4	nd	2.0 ± 0.37	4.5 ± 0.75	6.3 ± 3.8	nd
MA7339[c]	0.26 ± 0.10	1.5 ± 0.45	2.8 ± 2.1	1.8 ± 0.90	0.90 ± 0.22	2.7 ± 2.0	1.8 ± 1.5	1.8 ± 0.72	2.3 ± 0.43	nd
SB12600[c]	0.24 ± 0.06	9.8 ± 3.1	7.4 ± 2.6	16 ± 2.5	35 ± 3.2	8.5 ± 6.6	22 ± 6.6	22 ± 8.8	21 ± 2.1	~0.25[d]

[a] See the Supporting Information. [b] In PTNM medium. [c] In SLY medium. [d] Isolated yield. [e] nd = not detected.

field-shifted H-12 and H-11. C-10 could be excluded as there is a gCOSY correlation between its two protons with both H-9 and H-11. A NOESY correlation between H-12 and H_3-17 determined the relative stereochemistry of the hydroxy group at C-12; **4** can be assigned as 12(*S*)-hydroxyplatencin (i.e., platencin A_2) if the absolute stereochemistry is assumed to be the same as in **2**.[4] The hydroxy group on **5** could be placed on C-14 by the presence of an oxygenated methine proton (H-14) with a singlet peak at δ_H 3.97. This was corroborated by HMBC correlations of C-14 with H_2-16 and with H-7. Thus, like **4**, **5** was similarly assigned as 14(*S*)-hydroxyplatencin (i.e., platencin A_3) by a clear NOESY correlation between H-14 and H-9.

HiResMALDI-FTMS analysis of **6** gave an $[M + Na]^+$ ion at *m*/*z* 467.24902, consistent with a molecular formula of $C_{25}H_{36}N_2O_5$ (calcd for $C_{25}H_{36}N_2O_5Na$, 467.25164). The UV−vis spectrum of **6** contained a single peak at 229 nm indicating an enone moiety but lacked the smaller 300 nm peak characteristic of the benzoic acid moiety common to **2** and other congeners. ^{13}C NMR analysis confirmed the presence of 25 carbons, and combining gHSQC and ^{13}C NMR, **6** was shown to contain all of the carbon signals expected from the terpenoid moiety of **2** with the addition of one methyl, three methylenes, two methines, and two carbonyl carbons. ^1H NMR along with gCOSY, TOCSY, gHSQC, and gHMBC data suggested the presence of glutamine, similar to homoplatensimide.[16] This accounted for all extra carbon signals except for one methylene, one methine, and one methyl carbon. gCOSY spectra clearly established the three carbons as a CH_3CHRCH_2R' unit. The chemical shifts of the methine carbon and its proton were indicative of a position α to a carbonyl group. gHMBC showed connectivity between the amide carbon (C-1″) and all of the protons in the new three-carbon fragment. gCOSY correlations between the exocyclic methylene carbons C-2/C-3 and C-2/C-1 confirmed that the extra carbons were located in the flexible linker between the amide and the cyclic region, affording the final assignment of **6** as platencin A_4 with the relative stereochemistry of the methyl group at C-2″ undetermined.

Solving the chemical structure of **6** greatly facilitated the structural elucidation of compounds **7** and **8**. HiResMALDI-FTMS analysis of **7** and **8** yielded $[M + Na]^+$ ions at *m*/*z* 483.24382 and 483.24395, respectively, and the 16 mass increase suggested that they are hydroxylated-**6** isomers with the same molecular formula of $C_{25}H_{36}N_2O_6$ (calcd for $C_{25}H_{36}N_2O_6Na$, 483.24656). Using the same correlations that were used to determine the regiochemistry and stereochemistry of the hydroxy groups in **3**−**5**, **7** and **8** were assigned as platencin A_5 and A_6 with a 13(*R*)- and 14(*S*)-hydroxyl group, respectively.

The molecular formula of compound **9** was deduced as $C_{20}H_{30}O_6$ based on the HiResMALDI-FTMS spectrum that gave an $[M + Na]^+$ ion at *m*/*z* 389.19346 (calcd for $C_{20}H_{30}O_6Na$, 389.19339). The ^1H and ^{13}C NMR spectra showed similar signals to those produced by the terpenoid moiety of **7**, with the notable differences being the loss of signals representing the C-15/C-16 vinyl group. Combination of gHSQC and ^{13}C NMR analyses revealed new signals for an oxygenated methine and an oxygenated methylene, suggesting the presence of two hydroxy groups on C-15/C-16. This was confirmed by gHMBC correlations between H_2-16 and C-15, C-11, and C-14. A NOESY correlation between H-16 and H-12 determined the stereochemistry of C-15, allowing the final assignment of **9** as platencin A_7 with a 15(*S*)-hydroxy group.

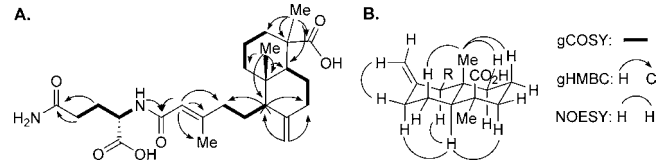

Figure 3. Key COSY and HMBC correlations for platencin A_8 (**10**) (A) and NOESY correlations for the bicyclic moiety of **10** (R = the side chain) (B).

The molecular formula of $C_{25}H_{38}N_2O_6$ for compound **10** was established on the basis of HiResMALDI-FTMS analysis that afforded an $[M + Na]^+$ ion at *m*/*z* 485.26221 (calcd for $C_{25}H_{38}N_2O_6Na$ 485.25856). ^1H and ^{13}C NMR spectra of **10** readily revealed a glutamine moiety similar to **6**−**8**, subtraction of which results in a predicted terpene moiety with a molecular formula of $C_{20}H_{29}O_3$, bearing one less degree of unsaturation compared to **6**−**8**. A significant difference between the spectra from **6**−**8** and that of **10** is the presence of the carbon signal at δ_C 181.5 indicating a carboxyl functional group. On the basis of extensive gHMBC and gCOSY correlations (Figure 3A), **10** was established as *N*-[15,18-dicarboxy-*ent*-copalyl]glutamine

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(i.e., platencin A_8). NOESY correlations (Figure 3B) allowed the assignment of the relative stereochemistry around most of the ring system, although the lack of any correlations to the C-19 methyl protons prevented us from determining the C-4 stereochemistry.

HiResMALDI-FTMS analysis of compound **11** yielded an $[M + Na]^+$ ion at *m/z* 656.21335, predicting a molecular formula of $C_{31}H_{39}NO_{11}S$ (calcd for $C_{31}H_{39}NO_{11}SNa$, 656.21361). The presence of a sulfur atom was supported by the isotopic distribution of the parent ion. The UV−vis spectrum of **11** displayed an altered absorbance pattern in the 300 nm range compared with other **2** congeners, with a peak at 280 nm and a pronounced shoulder at 320 nm, signifying changes in the benzoic acid moiety. The ^1H and ^{13}C NMR spectra were identical to corresponding spectra of **3**, except for the presence of seven additional carbons, one methyl signal and six oxygenated resonances, and associated protons. The presence of intact **3** was confirmed by gCOSY, gHSQC, and gHMBC. Additionally, the ^1H and ^{13}C NMR data suggested a hexose moiety, which was confirmed by a 1D-TOCSY experiment and verified to be a glucoside by gHSQC, gHMBC, and TOCSY correlations. The anomeric proton H-1‴ (δ_H 5.07) showed a gHMBC correlation to C-5‴, consistent with a pyran sugar moiety. The large coupling constants of all protons in the sugar moiety revealed equatorial placement of all hydroxy groups also suggestive of a glucoside. The gHMBC correlations of H-1‴ with C-5′ (δ_C 158.1) established the glycosidic linkage at C-5′. This was further supported by the NOESY correlations between H-6′ and H-1‴, which also showed correlations to H-3‴ and H-5‴, confirming the location of the β-glucoside. The sugar is suspected to be the β-D-glucoside as this is the most prevalent glucoside enantiomer in nature. Lastly, gHMBC correlations between the CH_3−S methyl protons (δ_H 2.50) and the carbonyl carbon C-1′ (δ_C 196.6) and H-7′ (δ_H 7.88) with C-1′ (δ_C 196.6) indicated the presence of a methyl thioester, in place of the free acid found in **2** and related analogues, hence the final assignment of **11** as platencin A_9.

When stored in methanol, **11** readily degraded to a new compound (**12**), which migrated faster than **11** during C18 HPLC and displayed a distinct UV−vis spectrum (see the Supporting Information). LC-ESI-MS analysis of **12** gave an M^- ion at *m/z* 612.2, corresponding to a 16 mass decrease compared to **11**. Additionally, **12** no longer produced the sulfur-specific isotope distribution, suggesting that the thioester bond was cleaved by solvolysis in methanol to afford the corresponding methyl ester. ^1H NMR data finally confirmed the assignment of **12** as platencin A_{10}, showing the expected downfield shift of the CH_3-S methyl at δ_H 2.50 in **11** to δ_H 3.91 for the CH_3-O methyl in **12**.

Rational metabolic engineering for titer improvement of *Streptomyces* secondary metabolites is an attractive means of enabling the production and characterization of new natural products.[20] Here, we applied knowledge gained from studying the regulation of **1** and **2** dual production in *S. platensis* MA7327[18] to quickly generate improved strains from the **2**-producing *S. platensis* MA7339. The improved titers of **2**−**11** in the engineered *S. platensis* SB12600 strain relative to the wild-type MA7339 (Table 1) illustrate the utility and effectiveness of such an approach for lead production and optimization.

Table 2. Antibacterial Activity of **3**−**11** in Comparison to **1**−**3** on Two *S. aureus* Strains As Measured by MIC (μg/mL)[a]

S. aureus strain	**1**	**2**	**3**	**4**	**5**
ATCC33591[b]	0.1	0.1	16	>64	8
ATCC25923[c]	0.36	2.6	64	>64	32

[a] Congeners **6**−**11** all possessed MIC values >64 μg/mL. [b] Methicillin resistant. [c] Methicillin sensitive.

The new congeners reported here add to the list of **1** and **2** analogues generated thus far for SAR studies and constitute the majority of analogues possessing the **2**-scaffold. The newly isolated **2** congeners (**4**−**11**) were therefore assayed for antibacterial activities against selected *S. aureus* strains, in comparison to **1**−**3** (Table 2). Similar to the naturally isolated amino acylated **1**-like diterpenes,[13,16] **6**−**10** did not show any bioactivity confirming the importance of benzoate-to-FabH/F active site binding interactions.[5] Congeners **4** and **5** were more intriguing, as the chemical structures and binding interactions of **1** and **2** to FabF suggest that a hydrogen-bond acceptor in the otherwise nonpolar terpene moiety can strengthen binding,[1] and the previously described regioisomer **3** inhibits bacterial fatty acid synthase in a cell free assay with an IC_{50} of 7.12 μg/mL.[14] Indeed, **5** displayed the best activity of all congeners tested, with a MIC of 8 μg/mL against the methicillin-resistant *S. aureus* strain. Although significantly less active than **2**, the MIC for **5** is similar to that of the clinical drug Linezolid.[2] Compound **11** is a highly modified congener of **2**, with a C-13 hydroxylation, an O-5′ glucosidation, and a methyl thioester on C-1′. Although the first two modifications have been seen previously in **3**[14] and platencimycin B_4,[15] respectively, this is the first reported example of a sulfur-containing member of the platensimycin/platencin family of compounds. Compound **11** lacked activity due most likely to its glycosylation, as platensimycin B_4 similarly showed no antibacterial activity.[15]

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Supporting Information Available: Experimental procedures, MS ^1H and ^{13}C NMR data of **4**−**12**, and full citations of references 1−4 and 9. This material is available free of charge via the Internet at http://pubs.acs.org.

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