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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 multi-drug 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 titer ~100-fold greater than that from the wild-type strain and accumulates platencin A₁ (3) and eight new congeners platencin A₂–A₉ (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 1960's.^{1–4} They potently inhibit the growth of a range of Gram-

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Supporting Information Available Experimental procedures, MS and ¹H and ¹³C NMR data of 4–12. This material is available free of charge via the Internet at <http://pubs.acs.org>.

positive bacteria, including multi-drug 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 analogs featuring the **1** and **2** scaffold have been generated by organic synthesis.^{5–12} Congeners of **1** and **2** have also been isolated from the wild-type producers upon large-scale fermentations.^{13–17} Of the analogs reported to date, only platencin A₁ (**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 A₂–A₉ (**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 homolog 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 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).

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 multiple steps of chromatography, affording **2** and nine additional metabolites (**3**–**11**) (Figure 1) (see Supporting Information).

Compound **2** was confirmed as platencin by comparison with an authentic standard.^{2,4,18} Compound **3** was identified as platencin A₁ by spectroscopic comparisons with literature, including a NOESY correlation between H-13 and H₃-17 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 analog 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 downfield-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 H₃-17 determined the relative stereochemistry of the hydroxy group at C-12; **4** can be assigned as 12(*S*)-hydroxyplatencin (i.e. platencin A₂) 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 H₂-16 and with H-7. Thus, like **4**, **5** was similarly assigned as 14(*S*)-hydroxyplatencin (i.e., platencin A₃) 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 $CH_3-CHR-CH_2R'$ 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 A₄ 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 A₅ and A₆ 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 HMBC correlations between H₂-16 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 A₇ with a 15(*S*)-hydroxy group.

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*-copaly]-glutamine (i.e., platencin A₈). 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 CH₃-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 analogs, hence the final assignment of **11** as platencin A₉.

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 Supplementary Information). LC-ESI-MS analysis of **12** gave an M⁺ ion at *m/z* 612.2, corresponding to 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 methylester. ¹H NMR data finally confirmed the assignment of **12** as platencin A₁₀, showing the expected downfield shift of the CH₃-S methyl at δ_H 2.50 in **11** to δ_H 3.91 for the CH₃-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* MA7327¹⁸ 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) illustrates the utility and effectiveness of such an approach for lead production and optimization.

The new congeners reported here add to the list of **1** and **2** analogs generated thus far for SAR studies and constitute the majority of analogs 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 non-polar terpene moiety can strengthen binding, ¹ and the previously described regioisomer **3** inhibits bacterial fatty acid synthase in a cell free assay with an IC₅₀ 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 B₄¹⁵, 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 B₄ similarly showed no antibacterial activity.¹⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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.

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.

Figure 3.
Key COSY and HMBC correlations for platencin A₈ (**10**) (A) and NOESY correlations for the bicyclic moiety of **10** (R = the side chain) (B).

Estimated titers of platencin (2) and congeners (3–11) produced by *S. platensis* MA7339 wild-type and SB12600 strains (mg/L).^a

Table 1

strain	2	3	4	5	6	7	8	9	10	11
MA7339 ^b	0.05 ± 0.04	nd	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

nd, not detected;

^asee Supporting Information;

^bin PTNM medium;

^cin SLY medium;

^disolated yield.

Table 2

Antibacterial activity of **3–11** in comparison to **1–3** on two selected *S. aureus* strains as measured by MIC ($\mu\text{g/mL}$)^a

<i>S. aureus</i> 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 $\mu\text{g/mL}$;

^b Methicillin resistant;

^c Methicillin sensitive.