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Isolation, structure and biological activities of platencin A_2–A_4 from *Streptomyces platensis*

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ABSTRACT

Natural products serve as a great reservoir for chemical diversity and are the greatest source for antibacterial agents. Recent discoveries of platensimycin and platencin as inhibitors of bacterial fatty acid biosynthesis enzymes supplied new chemical scaffolds for potential antibacterial agents to overcome resistant pathogens. Discovery of natural congeners augment chemical modification in understanding of structure–activity relationship (SAR). Chemical and biological screening of the extracts led to isolation of three hydroxylated analogs of platencin. The C-12, C-14 and C-15 hydroxylated analogs showed attenuated activities which provided significant understanding of functional tolerance in the diterpenoid portion of the molecule. A truncated and oxidized C-13 natural congener was isolated which suggested direct intermediacy of *ent*-copalyl diphosphate for the biosynthesis of platensimycins and platencins.

1. Introduction

Bacterial resistance for existing antibiotics continues to grow and is becoming serious health hazard. To combat the infections by MRSA and other drug resistant bacteria it is incumbent upon scientific community to discover new compounds potentially with novel modes of action. Recently we reported the discovery of platensimycin (**1**) and platencin (**2**) from various strains of *Streptomyces platensis*.[1–4] The key to the discovery of these compounds was a novel antisense differential sensitivity screening strategy in which FabH/FabF was sensitized in *Staphylococcus aureus*.[3–6] This strategy led to increased sensitivity for the detection of target based inhibitors and allowed the detection of platensimycin and platencin in crude extracts. Mechanistically, platensimycin selectively inhibited acyl–enzyme intermediate of the elongation condensing enzyme FabF. On the other hand, platencin inhibited both condensing enzymes, FabH and FabF, with almost equal efficiency. Both of these compounds demonstrated potent in vitro activity in both cell-free and whole-cell assay systems. Although the in vitro activity translated very well in in vivo efficacy when dosed by intravenous continuous infusion, the lack of in vivo efficacy under conventional routes of administration was attributed to poor pharmacokinetic (PK) properties.[1–4] Several approaches has been undertaken to improve PK properties and efficacy of these compounds including chemical modification of platensimycin at some of the chemically accessible sites.[7] Discovery of congeners and total synthesis approaches are the alternative strategies which provide opportunities to access compounds not accessible by chemical modifications. Various total synthetic strategies have been reported both for platensimycin[8–22] and platencin.[23,24] Chemical screening of the *S. platensis* extracts have allowed for the discovery of a number of congeners with additional functional groups that provides SAR information which was inaccessible by the chemical methods. These congeners include platensimide A,[25] homoplatensimide A,[26] platensimycin A_1,[27] and platencin A_1.[28]

Continued search for additional congeners and related analogs from a large scale fermentation of *S. platensis* led to the isolation of three new hydroxylated congeners of platencin. These congeners are platencin A_2 (**4**), platencin A_3 (**5**) and platencin A_4 (**6**). Here we describe the isolation, structure elucidation, and biological activity of these compounds.

2. Results and discussion

2.1. Production and isolation

For large scale production of platensimycin, *S. platensis* MA7327 was grown in six stirred tanks on CLA production medium. The production level of platensimycin was monitored by HPLC (Fig. 1). Same media components were used in all six tanks except for the amount of media (see Section 3) which appears to have no effect on the production of platensimycin (**1**). The induction of

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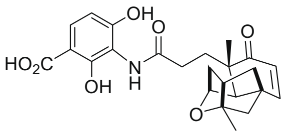
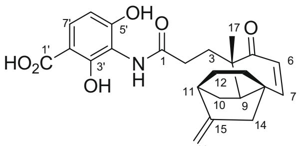


1 (Platensimycin)



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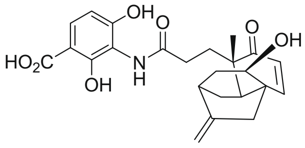

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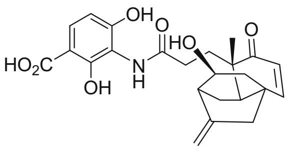

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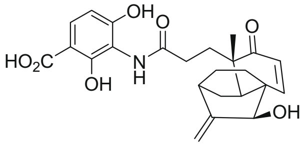
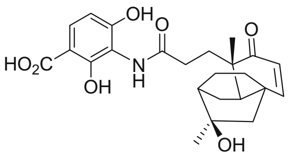
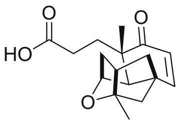


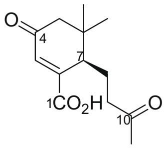
5 (Platencin A_3)



6 (Platencin A_4)



7(Platensic acid)



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platensimycin production appears to be at about 5 days and matured at about 12–13 days when the fermentation was harvested. Five of the six tanks consistently produced 20–30 mg/L of platensimycin at 12–13 days. For an unknown reason, one of the tanks produced 2–3 times of that amount with the peak titer of over 80 mg/L at 12 days.

The harvested broth (3400 L) was acidified to pH 2.8 by addition of 50% aqueous H_2SO_4 and was extracted with isopropyl acetate. The isopropyl acetate extract was concentrated and extracted with 5% aqueous $NaHCO_3$ solution and was directly loaded on to a reversed phase amberchrome column (Fig. 2). After the completion of loading, the column was washed with water until the eluent water showed neutral pH followed by washing with 0.1 M H_2SO_4 which helped in protonation of the carboxylic acid group. The combined wash was used for the isolation of new compounds platencins A_2–A_4 (Fig. 2). The column was eluted with a step gradient of 20–80% aqueous MeOH containing 0.1% TFA followed by washing with MeOH. Platensimycin eluted in fractions eluting with 60–100% MeOH which was concentrated to remove MeOH and extracted with isopropyl acetate. Concentration of the isopropyl acetate extract followed by cooling at 0–4 °C led to the precipitation of platensimycin which was collected and washed with hexanes to yield >90% purity of platensimycin with >30.5% unoptimized isolation yield.

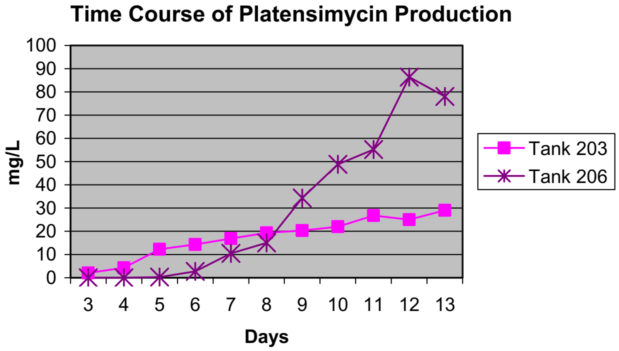


Figure 1. Production time course of platensimycin by *S. platensis* in six stirred tanks. The data from the four tanks were essentially identical to Tank 203 and omitted from the graph for clarity.

The water and 0.1 M H_2SO_4 wash from amberchrome column was combined and extracted with isopropyl acetate and the organic layer was concentrated to dryness and chromatographed on Sephadex LH20 (Fig. 2). The column was eluted with a step gradient of hexanes–CH_2Cl_2, CH_2Cl_2–acetone, and MeOH. Trituration and RPHPLC of an early eluting fraction yielded platensic acid (**7**).[25] Other early eluting fractions were chromatographed by RPHPLC to yield compound **8** (11.3 mg, 0.003 mg/L). A portion of the late eluting fractions containing platencins A_2–A_4 (**4**–**6**) were chromatographed on reversed phase HPLC to afford pure platencin A_2 (**4**) (21.1 mg, 0.081 mg/L), (**5**) A_3 (2.9 mg, 0.011 mg/L), and (**6**) A_4 (3.1 mg, 0.012 mg/L) as amorphous powders.

2.1.1. Platencin A_2 (4)

HRESIFTMS analysis of **4** showed a protonated molecular ion at *m/z* 442.1857 (M+H) affording a molecular formula of $C_{24}H_{27}NO_7$ which was isomeric with platensimycin. The ^{13}C NMR spectrum showed the presence of 24 resonances and supported the molecular formula. The UV spectrum was very similar to platensimycin and platencin spectrum indicating the presence of identical chromophore. The IR spectrum showed the presence of the absorption bands for hydroxy group, conjugated ketone, carboxylic acid and aromatic systems. Comparison of ^1H and ^{13}C NMR spectra of **4** with the corresponding spectra of platensimycin and platencin indicated that **4** possessed platencin type structure. The key features were the presence of resonances for exocyclic methylene group and only one methyl group. Since the molecular formula was isomeric to platensimycin which would suggest that compound **4** contained extra oxygen atom than platencin. The ESIMS spectrum showed the presence of a fragment ion at *m/z* 273 due to cleavage of the amide bond suggesting that the extra oxygen was located in the tricyclic portion of the molecule and not in the benzoic acid portion. Comparison of the ^{13}C NMR spectrum of **4** with the corresponding spectrum of platencin revealed the absence of one of the methylene resonances and the presence of a methine resonance (δ_C 68.8). The methine carbon showed HSQC correlation to a proton resonating as a doublet of doublet of doublets (J = 9.0, 4.0, 3.0 Hz) at δ_H 4.25. The methine proton showed COSY correlation to a methine triplet δ_H 2.63 (H-11) and to a methylene group (H_2-13). H-13 did not show any other couplings except for a weak W-coupling to one of the methylene protons (δ_H 2.02) at C-14. These data indicated that the oxygen was located at C-12 in the form of a hydroxy group. This assignment was further confirmed by a HMBC correlation of H-13 to the methine carbon at C-12. The hydroxy substitution at C-12 caused downfield shifts of C-11 and C-13 by 8.5 and 10.9 ppm, respectively. The remainder of the spectra and correlations were similar to that of platencin. The configuration at C-12 was determined by NOE difference measurements. Irradiation of H-12 showed enhancements of H-11, H-13 (δ_H 2.42) and H-23 (δ_H 1.39) indicating that the proton was located on the back side of the bicyclo ring system thus placing the hydroxy group in the front and in *R* configuration (Fig. 3a). This configuration would be consistent with the cytochrome P450 oxidation of platencin post biosynthesis from the less hindered face of the molecule. Thus structure **4** was assigned to platencin A_2 as 12*R*-hydroxyplatencin.

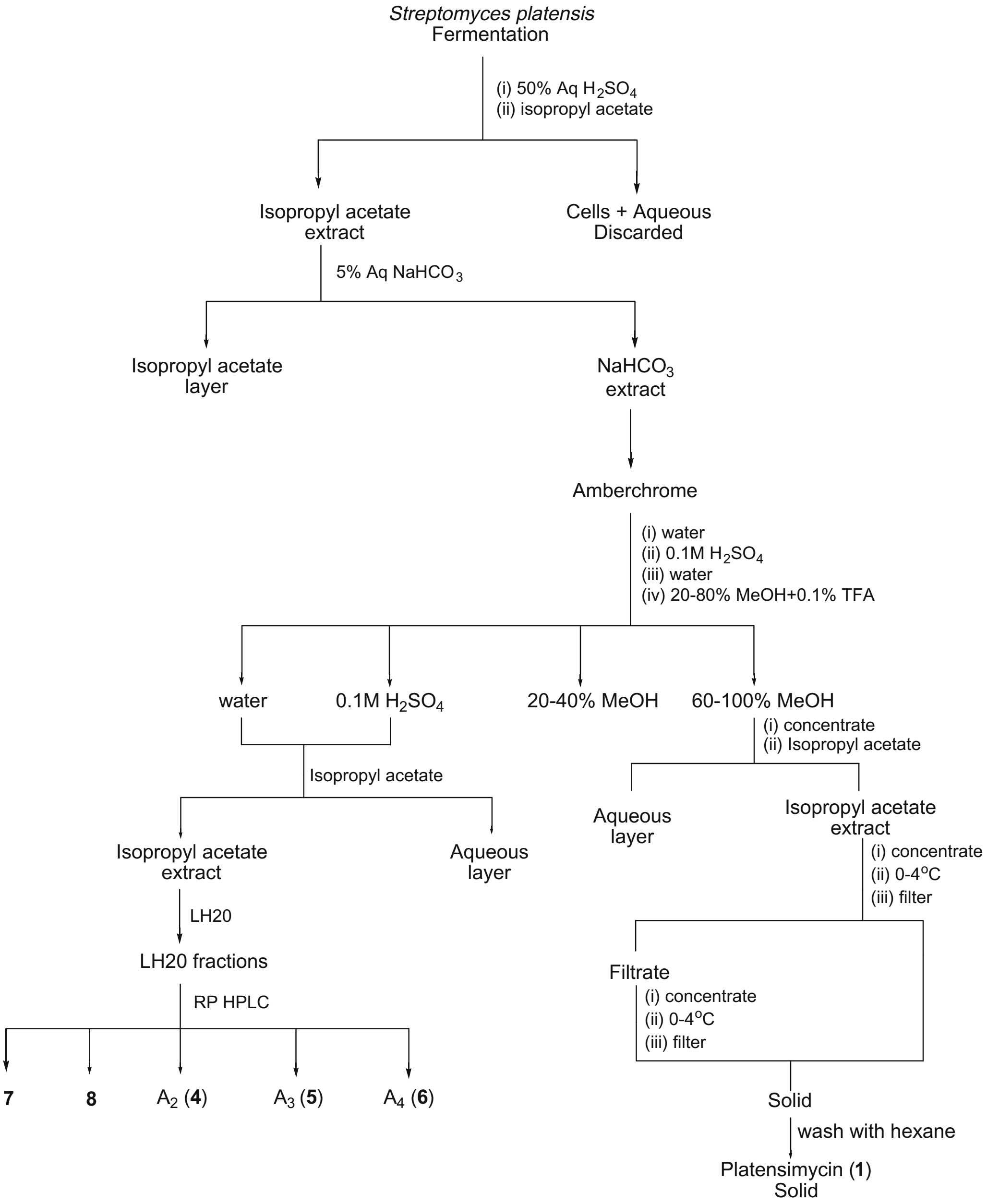


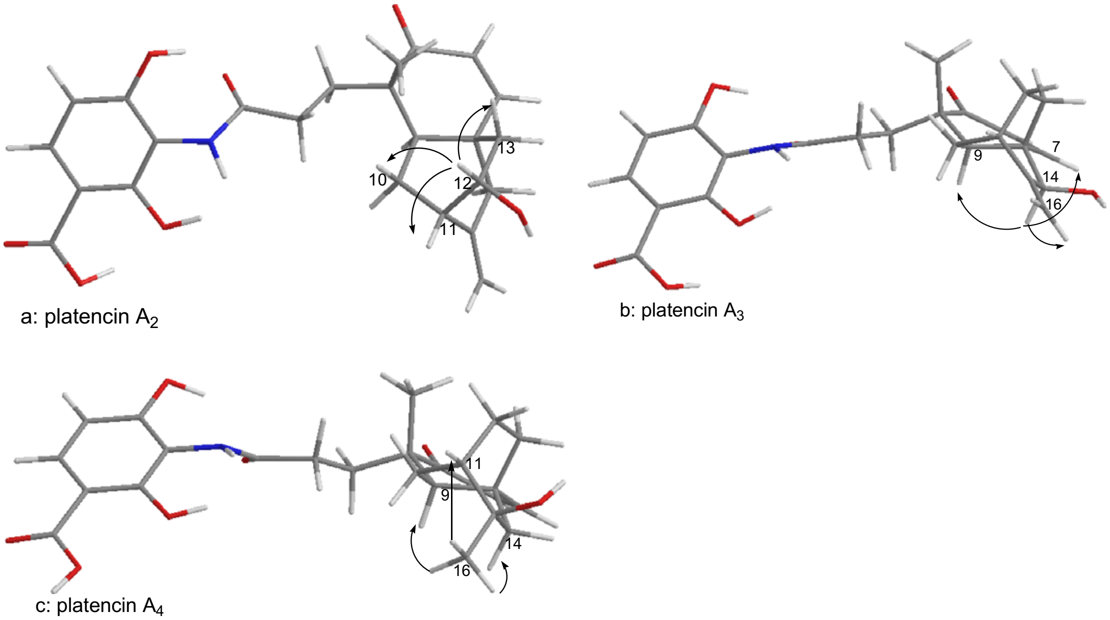
Figure 2. Isolation scheme of platensimycin (**1**) and platencin A_2–A_4 (**4**–**6**).

2.1.2. Platencin A_3 (5)

Mass spectral analysis of **5** produced a molecular formula of $C_{24}H_{27}NO_7$ which was isomeric to platencin A_2 (**4**). The UV and IR spectrum suggested the presence of similar chromophore and functional groups. The mass spectral fragmentation produced *m/z* 273 ion consistent with the hydroxylation in the tricyclic enone part of the molecule. The comparison of ^{13}C and ^{1}H NMR spectra showed similarity to platencin A_2 except for the presence of a singlet of a methine proton resonating at δ_H 3.97. This singlet showed HSQC correlation with a carbon at δ_C 76.3. The presence of a singlet methine suggested that the hydroxylation was at C-14 which was confirmed by the HMBC correlations of H_2-16 and H-9 to C-14. The hydroxy substitution at C-14 caused downfield shift to two adjacent carbons, C-8 and C-15, by 4.4 and 5.4 ppm, respectively, and an upfield shift to C-13 by 7.8 ppm confirming the location of the substitution. Irradiation of H-14 showed NOE enhancements to H-7, H-9 and H-16 (δ_H 5.28) indicating that it was pseudo axially oriented in a boat conformation and placing the hydroxy group on the top face and in a *S* configuration at C-14 (Fig. 3b). Thus structure **5** was established for platencin A_3 as 14*S*-hydroxyplatencin.

2.1.3. Platencin A_4 (6)

HR mass spectral analysis of platencin A_4 (**6**) provided a molecular formula of $C_{24}H_{29}NO_7$ which contained an additional molecule of hydrogen compared to platencins A_2 and A_3. The UV spectrum was similar to other two compounds and IR spectrum displayed the absorption bands for hydroxy, acid, enone and aromatic groups like other compounds. The ^{13}C NMR spectrum of **6** displayed the presence of 24 resonances like other platencins. Examination of ^{1}H and ^{13}C NMR spectrum of **6** indicated the absence of the exocyclic methylene group and the presence of an additional methyl group resonating as a singlet at δ_H 1.57 which correlated with δ_C 25.3 in HSQC. The ESIMS spectrum showed a fragment ion at *m/z* 275, due to the cleavage of the amide bond, indicating the addition of a molecule of water compared to the corresponding unit of



a: platencin A$_2$

b: platencin A$_3$

c: platencin A$_4$

Figure 3. ChemDraw 3D model showing NOE difference enhancements of (a) platencin A$_2$ (**4**), (b) platencin A$_3$ (**5**) and (c) platencin A$_4$ (**6**).

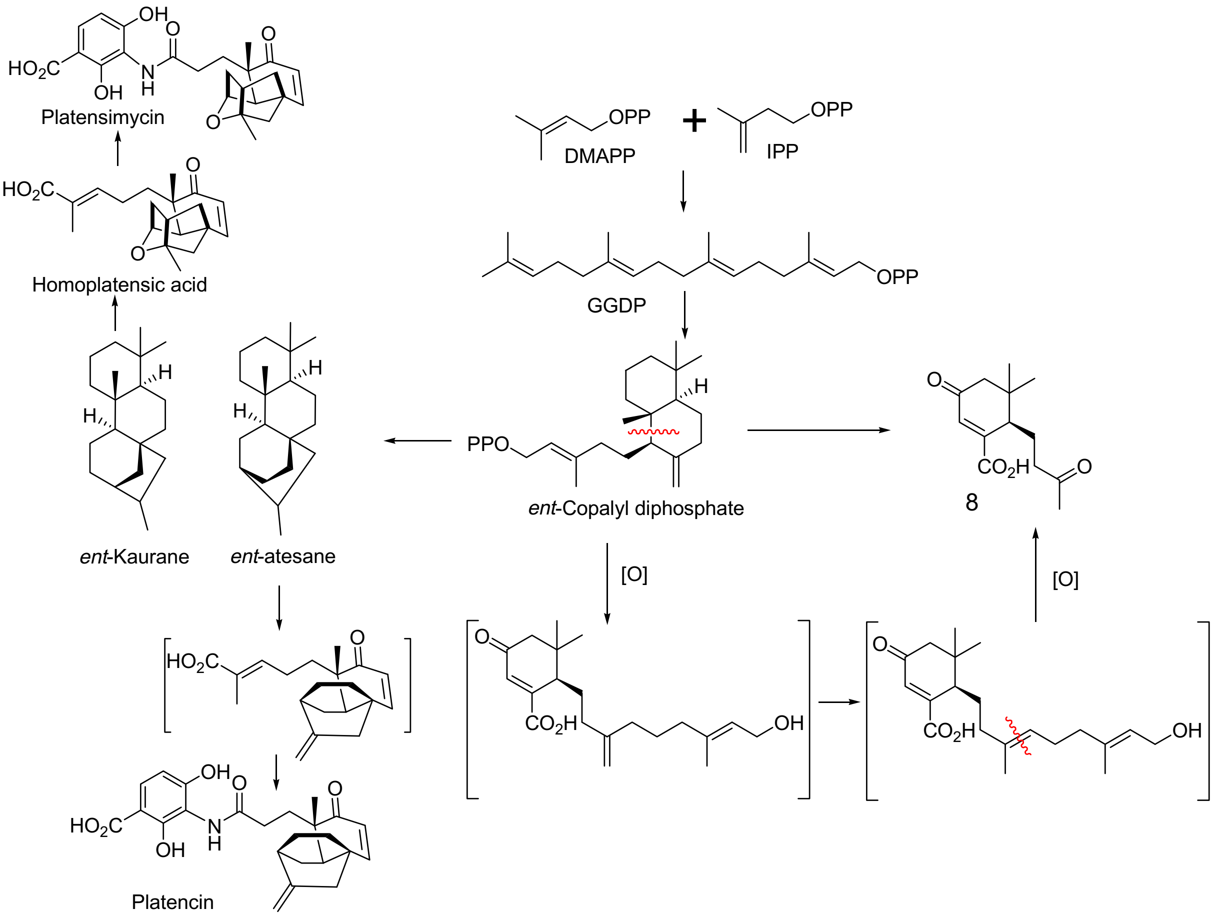
platencin. The methyl group δ_H 1.57 (H$_3$-16) exhibited strong HMBC correlations to C-15 (δ_C 79.1), C-14 (δ_C 58.2), and C-11 (δ_C 49.0) confirming the hydroxylation at C-15. The hydroxy substitution at C-15 led to downfield shifts of C-8, C-11, C-12 and C-14 by 10, 12.4, 12.3, and 13.3 ppm, respectively, and an upfield shift of C-13 by 9.8 ppm due to a change in the ring strain. Irradiation of H$_3$-16 afforded NOE enhancements of H-11, H-9 and one of the two methylene protons of H-14 (δ_H 1.83, d) suggesting through space proximity and pseudo axial orientation of the methyl group and thus establishing *R* configuration for C-15 and assigning structure **6** for platencin A$_4$ (Fig. 3c).

2.1.4. Compound (8)

The mass spectral analysis gave a molecular formula $C_{13}H_{18}O_4$ which was confirmed by the ^{13}C NMR spectral analysis which showed the presence of 13 resonances including three methyl groups, three methylenes, an aliphatic methine, an olefinic methine, an enone carbonyl, an acyclic saturated ketone and a carboxyl type carbon. The gCOSY together with TOCSY correlations showed a –CH–CH$_2$CH$_2$– and an isolated CH$_2$ fragments which were assembled to compound **8** by HMBC correlations. The olefinic methine singlet (δ_H 7.11) showed strong HMBC correlations to C-1 (δ_C 170.9), C-2 (δ_C 155.5), C-5 (δ_C 47.8), and C-7 (δ_C 44.5); H-5 to C-3 (δ_C 131.3), C-4 (δ_C 200.7), C-6 (37.1), C-7 (δ_C 44.5) and both methyl groups (δ_C 28.7 and δ_C 27.9); H-7 to C-1, C-2, C-3, C-5, C-6, C-8, C-9 and both methyl groups, and HMBC correlations of H-8, H-9 and acetyl methyl established the structure **8** as 7-(3-butanonyl)-6,6-dimethyl-2-cyclohex-4-none-2-carboxylic acid. The absolute configuration was rationalized based on its potential origin from *ent*-copalyl diphosphate and thus assigned the same as in *ent*-copalyl diphosphate and *ent*-kaurene (C-5) and establishes biosynthesis relationship of these diterpenoids to platensimycin (**1**)[29] and platencin (**2**) (Scheme 1).[30] Platensimycin is formed by a number of oxidative cleavages leading to homoplatensic acid[26] and platensic acid (**7**).[25] Platencin is likely biosynthesized from a similar but isomeric C-20 diterpenoid acid and a putative C-17 platencinic acid), respectively.

2.2. Biological activity

Platencin A$_2$–A$_4$ (**4**–**6**) were first evaluated for their ability to inhibit *S. aureus* fatty acid synthesis in cell-free system measuring FASII assay.[31] Platencin A$_2$, A$_3$ and A$_4$ exhibited IC$_{50}$ values of 6.3 (14.3 μM), 7.1 (16.1 μM) and 25.1 (56.7 μM) μg/mL, respectively (Table 2). In this assay platensimycin (**1**) and platencin (**2**) exhibited IC$_{50}$ values of 0.5 (1.1 μM) and 12 (28.2 μM) μg/mL, respectively. The hydroxylated compounds A$_2$ (**4**) and A$_3$ (**5**) are about twofold more active in the FASII assay than platencin but the A$_4$ (**6**) is about twofold less active. These differences in the enzyme inhibitory activity could be rationalized by modeling studies (Fig. 4) using the X-ray co-crystal structures of platencin (**2**) and platencin A$_1$ (**3**) with *Escherichia coli* FabF (C163A).[28] The modeled structures of platencin A$_2$ (**4**) and A$_3$ (**5**) with *Ec*FabF (C163A) show C-12 (**4**, Fig. 4a) and C-14 (**5**, Fig. 4b) hydroxy groups are 2.6 Å and 4.1 Å away from Arg206 indicating hydrogen bond interactions and hence better enzymatic activities compared to platencin, which lacks these interactions. On the other hand hydration of the olefin at C-15 of platencin A$_4$ (**6**) pushes the methyl group in the hydrophilic region (Fig. 4c) near Ser271 and Thr270 providing a negative hydrophilic–hydrophobic interaction leading to slight loss of enzyme inhibitory activity. However the activity in the cell-free system did not translate in the cellular assays perhaps due to poor cell penetration because of increased polarity of the terpenoid unit. In the most sensitive antisense two-plate differential sensitivity cellular assay[5] platencin A$_3$ (**5**) was most active and showed MDC of 20 μg/mL which was 100-fold less active than platencin (MDC of 0.2 μg/mL). MDC is defined by minimum concentration of the compound showing differential zone of clearance between antisense plates compared to control plate. Platencin A$_2$ (**4**) and A$_4$ (**6**) was 500-fold less active than platencin and fivefold less active than A$_3$ (**5**). The discrepancy between IC$_{50}$ obtained from cell-free FASII assay and MDC from cellular assay suggested that hydroxylation in the terpenoid part of platencin has a negative impact on bacterial uptake. Since these compounds showed poor activity in the antisense assay they were not expected to show strong activities against wild type *S. aureus* and other bacterial strains. Indeed that was the case when they were tested for their activities against such strains. Again platencin A$_3$ which showed better activity in the antisense assay showed the best activity. It inhibited growth of *S. aureus* with an MIC of 16 μg/mL, *Enterococcus faecalis* (MIC 64 μg/mL) and *Bacillus subtilis* (MIC 64 μg/mL). While it did not inhibit the growth of wild type *E. coli* (MIC >64 μg/mL) it showed inhibition of envelope disrupted (envA negative) *E. coli* with an MIC of



Scheme 1. Plausible biogenesis of platencin.

16 μg/mL. The other two compounds (**4** and **6**) only showed some activity against envA-*E. coli* (MIC 32 μg/mL). These compounds did not show any toxicity against HeLa cells and did not show activity against *Candida albicans* (MIC ≫64 μg/mL) suggesting selectivity for bacterial inhibition. In general, the polar groups in the terpenoid portion of the molecule are detrimental for the antibacterial activity though two of the compounds retained the cell-free enzymatic activity. While hydroxy groups do play negative role in the activity it appears some substitution locations are more tolerant than others. For example, hydroxylation at C-14 has less detrimental impact than C-12 and C-15. Whether poor cellular activity is due to poor penetration or enhanced efflux is not understood.

In summary, we have described here the large scale and efficient isolation of platensimycin and three new hydroxylated congeners of platencin. We have demonstrated that hydroxylation in the terpenoid part of the molecule has negative impact on the activity of these compounds. However these compounds could serve additional lead structures for further modifications particularly using hydroxy group as an additional handle.

3. Experimental

3.1. General methods

Optical rotations were measured with a Perkin–Elmer 241 polarimeter. UV spectra were recorded on a Beckman DU-70 spectrophotometer. IR spectra were recorded with a Perkin–Elmer Spectrum One FT-IR spectrophotometer. All NMR spectra were recorded with a Varian Unity 500 (^{1}H, 500 MHz, ^{13}C, 125 MHz) spectrometer. Residual protons in deuterated solvents were used as internal reference (DMSO-d_6 δ_H 2.50, δ_C 39.51; C_5D_5N δ_H 8.74, δ_C 150.35). ^{1}H–^{1}H COSY, DEPT, TOCSY, HMQC and HMBC spectra were measured using standard Varian pulse sequences. ESIMS data were recorded on an Agilent 1100 MSD with ESI ionization. HRESIFTMS was acquired on a Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe. Three scan events were used. The ion trap was scanned from 150–2000 first in negative ion mode and then in positive ion mode. The FT was scanned from 200–2000 in the positive ion mode only. In all cases the SID was set to 18 V to try to reduce multiple ion clusters. Instrument resolution is set to 100,000 at *m/z* 400. No internal calibration is required the instrument is calibrated once a week and checked daily to assure accuracy. An Agilent HP1100 instrument was used for analytical HPLC.

3.2. Fermentation condition for production of compounds: 625 L tank productions

A 1.5 mL frozen suspension of *S. platensis* MA7327 (ATCC PTA-5316) was inoculated in 50 mL ATCC-2 seed medium [consist of (g/L) Difco starch (20), dextrose (10), NZ amine type E (5), Difco beef extract (3), Difco Bacto peptone (5), Difco yeast extract (5), calcium carbonate (1)] (3% inoculum) in a 250 mL baffled Erlenmeyer flask and incubated at 28 °C at 220 rpm agitation on a shaker for 48 h. The second stage seed was developed by aseptically transferring of 10 mL broth from the stage-one seed into four 2 L non-baffled Erlenmeyer flasks, containing 500 mL of ATCC-2 medium each (2% inoc), and cultivated for 33 h at 180 rpm shaker speed, at 28 °C. Pooled contents from all four flasks (2000 mL) were transferred into 275 L tank containing 180 L of ISM-3 seed medium

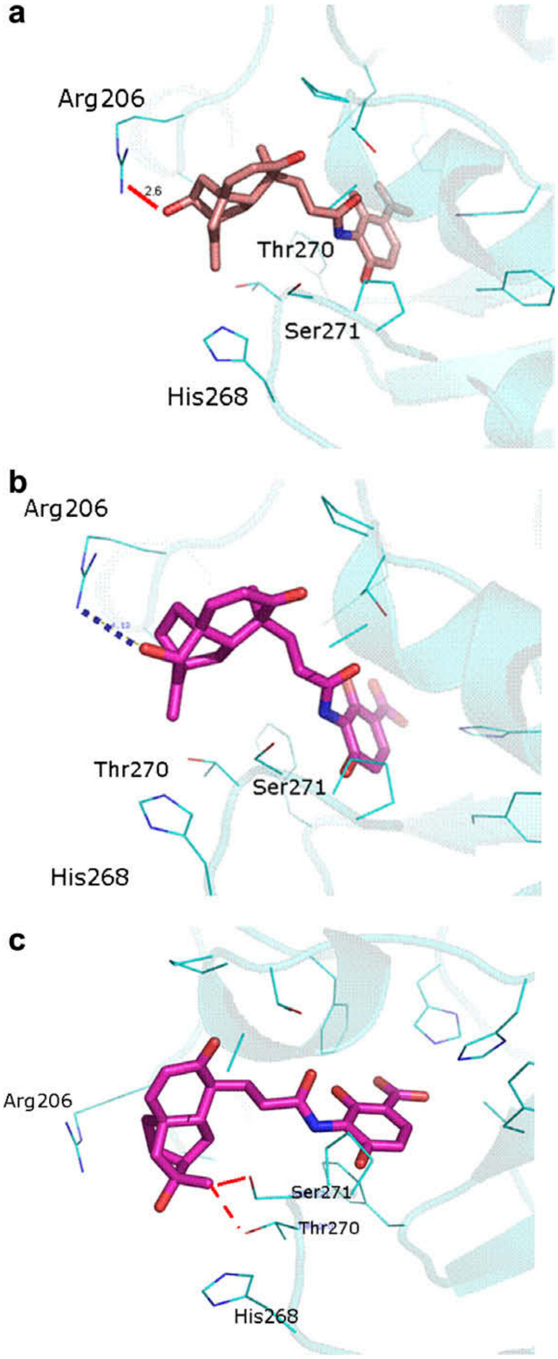


Figure 4. Modeled structures of platencin A_2 (**4**, a), platencin A_3 (**5**, b) and platencin A_4 (**6**, c) with *E. coli* FabF (C163A).

[consist of in g/L: yeast extract 14, maltoline ME 9.3, cerelose 27.5, ferric chloride monohydrate 0.47, polypropylene glycol (P2000) 1.9 mL/L] and incubated for 48 h (temperature 30 °C, pressure 0.7 kg/cm^2, airflow 100 slpm, agitation at 100 rpm). A 25 L aliquot of this culture was transferred to each of the six 800 L tanks containing 600 L of 'CLA' production medium (4% inoc) in three set of media proportions. First set of two tanks contained following medium components in g/L: yellow corn meal (10), amberex pH (1.25), lactose (10) and polypropylene glycol (P2000, 2 mL/L). The all media components were doubled and quadrupled in next set of two tanks each except for antifoaming agent P2000 which was maintained at 2 mL/L. The production fermenters were harvested after 13 days. The operating condition for the production fermenters was: pressure 0.7 kg/cm^2, airflow 150 slpm, agitation 10–160 rpm, dissolved oxygen 50%, temperature 30 °C. The batch with different production levels of platensimycin was harvested after 13 days affording 3400 L of broth.

3.3. HPLC quantification of platensimycin in fermentation broths

Measured volume of broth samples were acidified with 2 N HCl to pH 2.0 and extracted with two volumes of methyl ethyl ketone (MEK) by shaking for 0.5 h and centrifuged on a table top centrifuge for 20 min. Two milliliters of MEK extract was taken up from top from each sample and were concentrated to dryness and re-dissolved in 100 μL THF. Ten microliters of each sample was injected to an optimized RP HPLC (Zorbax RX C_8, 4.6 × 250 mm, 15 min gradient of 10–90% aqueous CH_3CN + 0.1%TFA, 1 mL/min, quantification at 230 nm). Platensimycin eluted at ~10 min.

3.4. Isolation of compounds

Six fermenters (3400 L, titer 16–78 mg/L, total 121 g) were harvested and adjusted to pH 2.8 by addition of 50% H_2SO_4 and extracted with equal volume of isopropyl acetate by agitating in a tank and phases were separated. The isopropyl acetate extract was concentrated to a volume of 260 L and extracted with 75 L of 5% aqueous sodium bicarbonate. The layers were separated and the isopropyl acetate layer was extracted a second time with 56 L of 5% sodium bicarbonate. The bicarbonate extracts were combined, centrifuged and charged to a 40 L amberchrome 161C column and washed with six column volumes (CV) of water until the pH of the eluent reached to 7.5 followed by washing with one CV of 0.1 M H_2SO_4 and three CV of water to neutral pH. The column was eluted with a step gradient using one CV each of 20%, 40%, 60%, 80% MeOH/0.1% aqueous TFA. This elution was followed by three CV of 100% MeOH. Two 20 L fractions were collected for each elution step. Fractions 5–11 contained platensimycin (**1**). These pooled fractions were concentrated to remove most of the methanol and extracted with 8 L of isopropyl acetate. The isopropyl acetate layer was separated and concentrated to a volume of ~700 mL and stored in a cold room at 0–4 °C leading to precipitation of platensimycin which was collected by filtration to give 20 g. The filtrate was concentrated to 100 mL and cooled again and filtered. The combined solid was washed with hexanes to remove non polar material and thus affording 37 g (30.5% recovery) of platensimycin with >90% purity.

The first six CV water and 0.1 M H_2SO_4 amberchrome column wash was extracted with 18 L of isopropyl acetate. The organic extract was concentrated under reduced pressure to dryness giving 26 g solid which was dissolved in a minimum volume of dichloromethane and chromatographed on Sephadex LH-20 (4 L column) and eluted with a step gradient (1:4, hexanes/CH_2Cl_2 one CV, 1:4, acetone/CH_2Cl_2 one CV, 3:2, acetone/CH_2Cl_2 two CV; 50 mL/min; finally wash with MeOH after unpacking). 320 LH-20 fractions were collected and analyzed by TLC plate (Silica Gel 60 F254; 5, 10%, 15%, 20%, 25% and 30% MeOH in CH_2Cl_2 as eluent). The fractions were combined into 39 fractions, which were further analyzed by analytical HPLC (Zorbax C_8, 4.6 × 250 mm, 10 min linear gradient of 30–100% aqueous CH_3CN + 0.05% TFA). This analysis allowed for further combination of fractions leading to 16 fractions. LH-20 pooled fraction 7 (elution between 2.0 and 2.2 CV's) was concentrated to dryness and re-dissolved in MeOH. The supernatant was separated, concentrated and purified by preparative reversed phase HPLC to give platensic acid (**7**), as an amorphous powder.

Fraction 9 (167.5 mg) was chromatographed by reversed-phase HPLC (Zorbax C_8, 21.2 × 250 mm, 10 mL/min, 60 min linear

Table 1

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignment of platencin A_2 (**4**), A_3 (**5**) and A_4 (**6**) in C_5D_5N

#	4				5			6		
	δ_C	Type	δ_H, mult, *J* in Hz	HMBC (H→C)	δ_C	δ_H, mult, *J* in Hz	HMBC (H→C)	δ_C	δ_H, mult, *J* in Hz	HMBC (H→C)
1	175.3	C^{o}			175.3			175.2		
2	32.3	CH_2	2.75, m	C-1, 3, 4	32.3	2.75, m	C-1, 3	32.7	2.65, m	–
									2.73, m	C-1, 3
3	32.0	CH_2	2.53, m	–	31.9	2.57, m	–	34.0	2.64, m	
			1.92, m	C-1, 2, 4, 17		1.95, m	C-1, 2, 4, 17		2.14, m	
4	48.2	C^{o}			48.1			49.7		
5	204.3	C^{o}			204.1			204.4		
6	127.0	CH	5.93, d, 10.1	C-4, 8	127.2	6.09, d, 10.0	C-4, 8	127.4	5.97, d, 9.9	C-4, 8
7	154.6	CH	6.44, d, 10.1	C-5, 9, 14	154.0	7.22, d, 10.1	C-5, 9	158.6	6.74, d, 9.9	C-5, 8, 9, 14
8	37.8	C^{o}			41.1			46.7		
9	39.5	CH	2.05, m	C-3, 4, 10, 13, 14, 17	38.6	1.93, m	C-3, 4, 8, 10, 14, 17	43.4	2.03, br d, 7.7	C-3, 4, 8, 10, 13, 14, 17
10	26.3	CH_2	1.68, m	–	28.3	1.60, m	–	27.0	1.61, m	
			1.39, dd, 12.9, 10.0	C-4, 9, 12, 15		1.46, br t, 10.8	C-4, 12, 15		1.43, m	
11	45.1	CH	2.63, t, 4.2		36.7	2.40, br s		49.0	2.20, m	C-9, 15
12	68.8	CH	4.25, ddd, 9.0, 4.0, 3.0		26.7	1.84, m		38.8	2.41, dd, 11.2, 4.7	–
						1.58, m			1.72, d, 11.2	C-14, 15
13	39.4	CH_2	2.42, ddd, 14.2, 9.1, 3.2	–	20.7	2.26, m		18.7	1.59, m	
			1.64, br d, 15.0	C-9, 12, 14		1.71, br dt, 4.8, 12.5				
14	45.2	CH_2	2.53, br d, 13.9		76.3	3.97, br s	C-13, 15	58.2	2.23, d, 14.6	C-7, 8, 9, 15, 16
			2.02, m						1.83, d, 14.6	
15	146.6	C^{o}			155.2			79.1		
16	111.3	CH_2	5.03, br s	C-14	111.6	5.28, br s	C-11, 14	25.3	1.57, s	C-11, 14, 15
			4.93, br s	C-14		5.15, br s	C-11, 14			
17	21.8	CH_3	1.08, s	C-3, 4, 5, 9	22.1	1.18, s	C-3, 4, 5, 9	24.4	1.18, s	C-3, 4, 5, 9
1′	175.2	C^{o}			175.2			175.0		
2′	107.7	C^{o}			107.8			107.7		
3′	159.0	C^{o}			158.8			159.5		
4′	115.8	C^{o}			115.8			115.8		
5′	158.7	C^{o}			158.3			158.9		
6′	110.5	CH	6.86, d, 8.8	C-2′, 4′, 5′	110.4	6.91, d, 8.8	C-2′, 4′, 5′	110.4	6.89, d, 8.8	C-2′, 4′, 5′
7′	130.1	CH	8.09, d, 8.8	C-1′, 3′, 5′	129.9	8.15, d, 8.8	C-1′, 3′, 5′	129.9	8.15, d, 8.8	C-1′, 3′, 5′
8′		NH	10.53, s	C-1, 5′		10.52, s	C-1, 5′		10.53, s	C-1, 5′

Table 2
Biological activities of platencin (**2**), platencin A_2 (**4**), platencin A_3 (**5**) and platencin A_4 (**6**)

Assay	2	4	5	6
saFASII (IC_{50}, μg/mL)	12	6.3	7.1	25.1
saFabF2 (MDC, μg/mL)	0.2	100	20	100
MIC (μg/mL)[a,b]				
S. aureus Smith	0.5	>64	16	>64
S. aureus Lin^s (50% serum)	8.0	>64	>64	>64
E. faecalis CL8516	2	>64	64	>64
B. subtilis MB964	1	>64	64	>64
Haemophilus influenzae MSD2261	64	>64	64	>64
E. coli (wt) MB2884	>64	>64	>64	>64
E. coli (env-) MB5746	2	32	16	32
C. albicans	>64	>64	>64	>64
Hela MTT (IC_{50})	86	>100	>100	>100

[a] All strains were tested in cation adjusted Mueller Hinton Broth (CAMHB) medium under National Committee for Clinical laboratory Standards (NCCLS) guidelines.

[b] MIC (minimum inhibitory concentration).

gradient of 20–25% aqueous CH_3CN + 0.05%TFA). The compound eluting at 37 min was lyophilized to give **8** (11.3 mg). LC–MS analysis of fraction 13 suggested that this fraction contained compounds that were not isolated earlier. A 100 mg aliquot of the fraction 13 (1.3 g) was chromatographed on reversed phase HPLC (Zorbax C_8, 21.2 × 250 mm, 10 mL/min, 30% aqueous CH_3CN + 0.05% TFA isocratic, detection at 215 nm). Fractions eluting at 20, 24 and 39 min were lyophilized to afford platencin A_2 (**4**) (21.1 mg, 0.081 mg/L), platencin A_4 (**6**) (3.1 mg, 0.012 mg/L) and platencin A_3 (**5**) (2.9 mg, 0.011 mg/L), respectively, all as amorphous powders.

3.4.1. Platencin A_2 (4)

$[\alpha]_D^{23}$ −20 (*c*, 0.5, MeOH), UV (MeOH) λ_{max} 228 (ε 20,568), 240 (sh), 296 (3369) nm, FTIR (ZnSe) 3280, 2930, 1649 (br, strong), 1534, 1378, 1347, 1308, 1245, 1186, 1135, 1057, 1017, 894, 826, 797 cm^{-1}, HRESIFTMS (*m/z*) 442.1857 (calcd for $C_{24}H_{27}NO_7$+H: 442.1866), for ^1H and ^{13}C NMR see Table 1.

3.4.2. Platencin A_3 (5)

$[\alpha]_D^{23}$ −4.0 (c 0.5, MeOH), UV (MeOH) λ_{max} 226 (ε 16,837), 240 (sh), 296 (2663) nm, FTIR (ZnSe) 3300, 2935, 2870, 1657, 1536, 1379, 1310, 1247, 1203, 1141, 1094, 1057, 832, 799 cm^{-1}, HRESIFTMS (*m/z*) 442.1863 (calcd for $C_{24}H_{27}NO_7$+H: 442.1866), for ^1H and ^{13}C NMR see Table 1.

3.4.3. Platencin A_4 (6)

$[\alpha]_D^{23}$ −28.0 (*c* 0.5, MeOH), UV (MeOH) λ_{max} 228 (ε 17,675), 240 (sh), 296 (2800) nm, FTIR (ZnSe) 3285, 2934, 1646 (br, strong), 1534, 1442, 1347, 1308, 1249, 1200, 1140, 1120, 1088, 1057, 829, 796 cm^{-1}, HRESIFTMS (*m/z*) 444.2013 (calcd for $C_{24}H_{29}NO_7$+H: 444.2022), for ^1H and ^{13}C NMR see Table 1.

3.4.4. Compound 8

$[\alpha]_D^{23}$ +28.0 (c 0.5, CH_3OH), UV (MeOH) λ_{max} 238 (ε 5474) nm, FTIR (ZnSe) 2962, 1699, 1674, 1416, 1368, 1231, 1167, 1125, 912 cm^{-1}, ESIMS (*m/z*) 261 [M+H], 239 [M+H], 237 [M−H]; HRESIFTMS (*m/z*) 239.1270 (calcd for $C_{13}H_{18}O_4$+H, 239.1278), for ^1H NMR 500 MHz (C_5D_5N) δ 8.98 (1H, br s, COOH), 7.11 (1H, s, H-3), 2.93 (1H, dd, *J* = 8.5, 4.5 Hz, H-7), 2.73 (1H, ddd, *J* = 16, 9, 6.5 Hz, H-9), 2.66 (1H, d, *J* = 16 Hz, H-5), 2.65 (1H, m, H-9), 2.34 (1H, m, H-8), 2.21 (1H, d, *J* = 16 Hz, H-5), 2.07 (3H, s, H_3-11), 1.72 (1H, m, H-8), 1.05 (3H, s, H_3-12*), 1.00 (3H, s, H_3-13*); ^{13}C NMR 125 MHz (C_5D_5N) δ 207.8 (C-10), 200.7 (C-4), 170.9 (C-1), 155.5 (C-2), 131.3 (C-3), 47.8 (C-5), 44.5 (C-7), 42.4 (C-9), 37.1 (C-6), 30.3 (C-11), 28.7 (C-12*), 27.9 (C-13*), 26.1 (C-8); HMBC (H→C) H-3→C-1, 2, 5, 7; H-5→C-3, 4, 6, 7, C-12/13; H-7→C-1, 2, 3, 5, 6, 8, 9, 12/13; H-8→C-2, 6, 7, 9, 10; H-9→C-8,10; H_3-11→C-9, 10; H_3-12→C-5, 6, 7, 13; H_3-13→C-5, 6, 7, 12.

3.5. $FabF_{2P}$ assay

S. aureus cells (RN450) carrying plasmid S1–1941 bearing antisense to FabF (*fabF* AS-RNA strain) or vector (control strain) were inoculated from a frozen vial source into a tube containing 3 mL of Miller's LB Broth (Invitrogen) plus 34 μg/mL of chloramphenicol. Tubes were incubated at 37 °C at 220 rpm for 18–20 h. and kept at room temperature until use. Miller's LB broth was supplemented with 1.2% Select agar (Invitrogen), 0.2% glucose, 15 μg/mL chloramphenicol and 50 mM of xylose (only for the antisense strain). The OD_{600} of the culture was measured and 1:1000 of OD 3.0 inoculated. Next, 100 mL were poured into each NUNC plate and well-caster templates placed into the agar and the agar was allowed to solidify. Then, 20 μL of test samples were added to the wells and the plates incubated at 37 °C for 18 h and zones of inhibition measured.[5]MDC (minimum detection concentration) values were determined by twofold serial dilution.

3.6. FASII assay

The assay was performed in a phospholipid coated 96-well flash plate. Briefly, 1.26 μg of the partially purified protein from *S. aureus* containing all the required fatty acid synthesis enzymes was preincubated with a serial dilution of natural products at room temperature for 20 min in 50 μL of buffer containing 100 mM sodium phosphate (pH 7.0), 5 mM EDTA, 1 mM NADPH, 1 mM NADH, 150 μM DTT, 5 mM β-mercaptoethanol, 20 μM *n*-octanoyl-CoA (or lauroyl-CoA), 4% Me_2SO, and 5 μM ACP. The reaction was initiated by addition of 10 μL of water-diluted [2-^{14}C]-malonyl-CoA, giving a final concentration of 4 μM malonyl-CoA with total counts of about 20,000 cpm. The reaction was incubated at 37 °C for 90 min. The reaction was terminated by adding 100 μL of 14% perchloric acid. The plates were sealed and incubated at room temperature overnight and counted for 5 min using Packard TopCount NXT scintillation counter. Further details of the assay are described in Ref 31.

3.7. Antibiotic assay (MIC)

The MIC (minimum inhibitory concentration) against each of the strains was determined by twofold dilution as previously described and conducted under National Committee for Clinical laboratory Standards (NCCLS)—now called the Clinical Laboratory Standards Institute—guidelines.[5,31] Cells were inoculated at 10^5 colony-forming units/mL followed by incubation at 37 °C with a twofold serial dilution of compounds in the growth medium for 20 h. MIC is defined as the lowest concentration of antibiotic inhibiting visible growth.

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Supplementary data

Supplementary data (^1H and ^{13}C NMR data of compounds platencin A_2 (**4**), A_3 (**5**) and A_4 (**6**)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.030.

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