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Isolation, enzyme-bound structure, and activity of platensimycin A_1 from *Streptomyces platensis*

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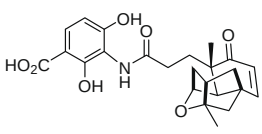
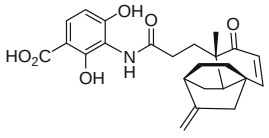
ABSTRACT

Inhibition of fatty acid synthesis is emerging as a valuable target for antibacterial agents. Platensimycin and platencin are novel natural products that were reported recently to inhibit the FabF and FabF/FabH condensing enzymes, respectively, present in the fatty acid biosynthetic pathway. Selective inhibition of these enzymes by platensimycin and platencin accounts for their potent antibiotic activity. We have continued our quest to find additional members of this class of compounds leading to discovery of platensimycin A_1, a hydroxylated congener. We report herein the isolation, structure, antibacterial and enzymatic activities, and co-crystal structure bound to *Escherichia coli* FabF. The lower activity of platensimycin A_1 suggests that substitution at C-14 is detrimental for the activity.

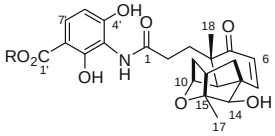
Fatty acid synthesis has been validated as a target for antibacterial agents. Recently platensimycin (**1**) and platencin (**2**) two novel antibiotics were reported from various strains of *Streptomyces platensis*.[1–4] A novel antisense differential sensitivity screening strategy in which *fabH*/*fabF* expression was reduced thereby making these strains hypersensitive to target-based inhibitors was a key factor for the discovery of these compounds.[3–6] Platensimycin is a selective inhibitor of the elongation condensing enzyme FabF of the bacterial fatty acid synthesis pathway in which it interacts with the malonyl binding site of the catalytic triad of the FabF acyl-enzyme intermediate. On the other hand, platencin is a balanced inhibitor of both the initiation condensing enzyme (FabH) and elongation enzyme (FabF). Both of these compounds showed potent in vitro activity against cell-free and whole-cell systems. They exhibited in vivo efficacy in *Staphylococcus aureus*-infected mouse model when dosed by continuous infusion. The poor in vivo activity under conventional administration is attributed to its poor pharmacokinetic properties. This PK problem could be potentially overcome by chemical modification of platensimycin and platencin or by discovery of the new congeners from the fermentation.[7] The present study describes the discovery of four new congeners of platensimycin: platensimycin A_1 (**3a**), methyl ester (**3b**), hydroxyplatensic acid (**4a**), and its methyl ester (**4b**) from the fermentation broth by chemical screening.



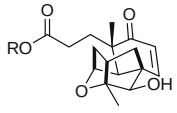
1 (Platensimycin)



2 (Platencin)



3a: R = H (Platensimycin A_1)
3b: R = Me



4a: R = H
4b: R = Me

S. platensis MA7327 was grown in fermentation tanks in the media that were used for the production of platensimycin.[2] The fermentation batches from 3 tanks were combined yielding 43 L of broth.[2] The combined broth containing 50–60 mg/L of platensimycin was acidified by 2 N HCl to pH 3.0 and extracted with MeOH. The filtered broth was chromatographed on reversed-phase Amberchrome resin eluting with 40–100% aqueous MeOH gradient. The fractions eluting with 70–80% MeOH was concentrated and extracted with CH_2Cl_2 at pH 9.0 and pH 2.0. The CH_2Cl_2 extract at pH 2.0 was chromatographed by RP HPLC on Zorbax RX C_8 column eluting with 35% aqueous CH_3CN + 0.1% TFA to give **3a** (4 mg, 0.1 mg/L) as a gum, $[\alpha]_D^{23}$ +3.7 (c 0.27, MeOH), UV (MeOH), λ_{max} 234 (ε 10,078), 247 (sh), 295 (5000) nm; IR (ZnSe) ν_{max} 3287, 2967, 1651, 1535, 1379, 1308, 1202, 1154, 1061, 924, 831 cm^{-1}.

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Amberchrome fractions eluting with 80–90% MeOH were basified by $NaHCO_3$ to pH 9.0 and were extracted with CH_2Cl_2. The CH_2Cl_2 extract was chromatographed on Sephadex LH20 and eluted with a step gradient of various proportions of hexane–CH_2Cl_2, acetone–CH_2Cl_2, and MeOH. Fractions eluting at 1.5 and 1.6 column volumes were chromatographed by RP HPLC using Zorbax C_8 column eluting with gradient of aqueous CH_3CN + 0.1% TFA affording **4b** (1.3 mg, 0.03 mg/L), and **3b** (35.2 mg, 0.81 mg/L) all as gums. Compound **3b**: $[\alpha]_D^{23}$ +6 (*c* 0.5, MeOH), UV (MeOH) λ_{max} 227 (ε 24,520), 257 (16,690), 298 (5160) nm, IR (ZnSe) ν_{max} 3395, 2957, 1662 (br, strong), 1534, 1439, 1377, 1341, 1263, 1202, 1150, 1076, 1028, 924 cm^{-1}. Compound **4b**: $[\alpha]_D^{23}$ +22 (*c* 0.5, MeOH), UV (MeOH) λ_{max} 223 (ε 4,860) nm, IR (ZnSe) ν_{max} 3426, 2962, 1733, 1667 (br, strong), 1540, 1440, 1378, 1302, 1201, 1178, 1154, 1076, 1030, 988, 925, 830 cm^{-1}. Acid–base treatment of the Amberchrome fractions eluting with 60–70% MeOH followed by extraction with CH_2Cl_2 at pH 2.0 and RP HPLC on Zorbax SB phenyl column and elution with aqueous CH_3CN + 0.1% TFA gradient afforded **4a** (1 mg, 0.023 mg/L) as a colorless powder, Compound **4a**: $[\alpha]_D^{23}$ +18.3 (*c* 0.6, MeOH), UV (MeOH) λ_{max} 221 (ε 4,950) nm, IR (ZnSe) ν_{max} 3347 (br), 2968, 1651 (br, strong), 1202, 1076 cm^{-1}.

Platensimycin A_1 (**3a**) and the methyl ester (**3b**) produced molecular ions at *m/z* 458.1807 and 472.1961, respectively, by HRESIFTMS analysis affording molecular formulae of $C_{24}H_{27}NO_8$ (calcd for M+H, 458.1815) and $C_{25}H_{29}NO_8$ (calcd for M+H, 472.1971). The formulae of **3a** and **3b** indicated that the two compounds differ by a methyl group which was easily attributed to a methyl ester by ^1H and ^{13}C NMR analyses (Table 1). The mass spectrum of **3a** and **3b** showed a common fragment ion at *m/z* 289, due to the cleavage of the amide bond, assigned for the tetracyclic enone portion of the molecule consistent with the presence of extra oxygen in this portion of the molecule. The comparison of the molecular formula of these compounds with that of platensimycin indicated that this set of compounds possessed extra oxygen atom without changing the degree of unsaturation. The UV spectra of the two compounds were similar to the UV spectrum of platensimycin. The ^{13}C NMR spectrum of **3a** was identical to the spectrum of platensimycin (**1**) except for the absence of the downfield methylene resonance $\sim\delta_C$ 52 ppm assigned to C-14 and presence of an oxygenated methine resonance at δ_C 85.6. This carbon correlated to a singlet at δ_H 3.92 in the HMQC spectrum. The methyl ester **3b** showed similar resonances in the ^{13}C (δ_C 85.9) and ^1H (δ_H 3.55) NMR spectrum in addition to corresponding resonances for the methyl ester. The oxygen substitution at C-14 was supported by oxygen-induced downfield shift of α-carbons (C-8 and 15) and upfield shift of β-carbons (C-7, 9, 12, 13, and 17). The singlet resonating at δ_H 3.92 (**3a**) and 3.55 (**3b**) showed HMBC correlations to C-12, C-13, and C-15 confirming the hydroxylation site at C-14. Irradiation of the singlet at δ_H 3.55 showed NOE enhancements of H-7, H-9, and H_3-17 confirming the axial orientation of this proton at C-14. Remaining COSY, TOCSY, and HMBC correlations exhibited by **3a** and methyl ester **3b** were similar to the corresponding correlations observed for platensimycin thus establishing the structure **3a** for platensimycin A_1 which was confirmed by X-ray crystallographic study of the enzyme-bound complex (vide infra).

HRESIFTMS analysis of compound (**4a**) and methyl ester (**4b**) produced parent ions at *m/z* 307.1546 and *m/z* 321.1681 analyzed for a molecular formula $C_{17}H_{22}O_5$ (calcd for M+H, 307.1545) and $C_{18}H_{24}O_5$ (calcd for M+H, 321.1701), respectively. The formulae of the two compounds differ by a methyl group and were supported by their ^{13}C NMR spectra (Table 1). These compounds showed a UV maximum at 221–223 nm different from platensimycin and platensimycin A_1 and similar to platensic acid. The ^{13}C NMR spectrum (Table 1) of **4a** and **4b** showed the presence of 17 and 18 carbon resonances, respectively, consistent with the platensic acid type structure. Comparison of the ^{13}C NMR spectra of these two compounds with platensic acid suggested the shift of one carbon resonance from $\sim\delta_C$ 52 ppm to $\sim\delta_C$ 85.5 which showed HSQC correlation to a methine singlet at $\sim\delta_H$ 4.00 ppm consistent with the hydroxylation at C-14 of platensic acid and the tetracyclic enone acid of platensimycin A_1 (**3a**). All methyl esters are likely isolation artifacts.

Compounds **3a, 3b, 4a,** and **4b** were first evaluated for inhibition of *S. aureus* fatty acid synthesis in cell-free system using FASII assay.[8] Compound **3a** inhibited the FASII assay with an IC_{50} value of 1.25 μg/mL (2.7 μM) which is 2.5-fold lower than that of platensimycin (IC_{50} 1.13 μM). Platensimycin A_1 inhibited saFabH with an IC_{50} of 219 μM. The other three compounds were inactive at 167 (350–550 μM) and 100 μg/mL (210–325 μM) against FASII and saFabH, respectively.

Table 1
^1H (500 MHz) and ^{13}C (125 MHz) NMR assignments of **3a** (C_5D_5N), **3b** (CD_3OD), **4a** (C_5D_5N), and **4b** (C_5D_5N)

	3a			3b		4a		4b	
#	δ_C	Type	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	175.2	C°		175.7		176.3		174.1	
2	32.3	CH_2	2.73, m	32.0	2.44, m	30.8	2.57, m	30.2	2.49, dd, 10.7, 4.6
			2.85, m		2.29, m		2.70, m		2.41, dd, 9.3, 2.9
3	33.1	CH_2	1.92, m	33.1	2.29, m	32.6	2.07, m	32.1	2.54, m
			2.10, m		1.85, m		2.70, m		1.90, m
4	47.0	C°		47.5		47.0		46.8	
5	203.4	C°		205.2		203.3		203.1	
6	128.1	CH	6.07, d, 10.0	128.3	5.95, d, 9.8	128.1	6.12, d, 10	128.0	6.12, d, 10.2
7	151.9	CH	7.26, d, 10.0	152.7	7.04, d, 9.8	151.8	7.31, d, 10	151.9	7.29, d, 10.2
8	50.0	C°		51.6		51.4		51.3	
9	47.8	CH	2.54, br s	47.9	2.35, br s	47.7	2.64, br s	47.6	2.54, m
10	76.8	CH	4.50, br s	77.7	4.43, br s	76.9	4.57, t, 3.6	76.8	4.47, t, 3.3
11	41.3	CH_2	1.98, m	41.4	2.09, d, 11.7	41.3	1.98, br d, 11.4	41.3	2.03, m
			1.93, d, 11.3		2.02, dd, 11.7, 5.6		2.01, m		1.97, br d, 11.3
12	44.8	CH	2.31, t, 6.5	45.0	2.29, m	44.8	2.3, t, 6.3	44.7	2.33, t, 6.3
13	39.5	CH_2	1.84, d, 11.3	39.5	2.37, m	39.5	1.87, br d, 11.8	39.4	2.69, br dd, 11.7, 6.8
			2.68, m		1.89, m		2.7, dd, 11.8, 6.3		1.87, br d, 11.7
14	85.6	CH	3.92, s	85.9	3.55, s	85.6	4.01, s	85.5	4.00, br s
15	90.6	C°		91.2		90.6		90.6	
17	19.6	CH_3	1.64, s	18.7	1.37, s	19.6	1.66, s	19.6	1.66, s
18	25.47	CH_3	1.24, s	25.5	1.26, s	25.6	1.29, s	25.4	1.24, s
1′	175.2	C°		171.8					
2′	107.6	C°		105.8					
3′	158.8	C°		159.7					
4′	115.8	C°		113.9					
5′	158.3	C°		159.0					
6′	110.4	CH	6.88, d, 8.5	109.8	6.44, d, 9.2				
7′	129.9	CH	8.10, d, 8.5	130.0	7.62, d, 9.2				
NH			10.50, s						
OMe				52.6	3.88, s			51.9	3.59, s

Platensimycin A_1 (**3a**) inhibited growth of wild type *S. aureus* and *Enterococcus faecalis* with MIC value of 16 μg/mL. The other three compounds did not show any activity at 64 μg/mL. None of the compounds showed any activity against *Streptococcus pneumoniae* at 64 μg/mL. Platensimycin exhibited MIC value of 0.5 μg/mL against the same *S. aureus* strain. Platensimycin A_1 (**3a**) showed minimum detection concentration (MDC, minimum concentration of the compound showing differential zone (5 mm) of clearance between antisense plate versus control plate) at <20 μg/mL in the antisense two-plate differential sensitivity assay[6] demonstrating about 500-fold lower activity than platensimycin.[1] The MDC of the other three compounds was >1000 μg/mL. In ecFabF (C163A) direct binding assay **3a** showed binding affinity IC_{50} of 390 nM which is 20-fold lower than that of platensimycin (IC_{50} 20 nM).

In an attempt to understand the molecular basis for why the presence of the hydroxy group in the tetracyclic enone portion of the molecule significantly affected the activity of platensimycin A_1, we determined the structures of the *Escherichia coli* FabF (C163A) protein in complex with platensimycin and platensimycin A_1. The protein expression, purification, and crystallization were precisely as described for the ecFabF(C163Q) variant previously reported.[1] The structures were determined by molecular replacement using the structure of the apo enzyme (PDB ID 2GFW) at resolutions of 2.40 Å for the platensimycin structure and 1.90 Å for the platensimycin A_1 structure. Both structures showed clear and unambiguous electron density for the bound inhibitor which enabled a direct comparison of the two structures.

The structures of ecFabF(C163A) in complex with platensimycin (**1**) (PDB code 3HN) and the hydroxy congener of platensimycin (**3a**) (PDB code 3IAP) are extremely similar and superpose with a cα rmsd of 0.175 Å. Despite this high degree of similarity, there are small but significant differences in the positioning of the two inhibitors with respect to the active site of the enzyme (Fig. 1). The hydroxylated tetracyclic enone is shifted and rotated in the binding site compared to platensimycin (Fig. 1A), which results in the lengthening of the ether oxygen hydrogen bond to Thr270 from 2.62 Å in the platensimycin structure to 2.89 Å in the platensimycin A_1 structure. The relative shift of A_1 also moves the position of the carbonyl oxygen on the opposite side of the tetracyclic enone by about 0.5 Å, which, in turn decreases the length of the interaction between the carbonyl oxygen and the backbone amide nitrogen of Ala309 from 3.09 Å to 2.85 Å. Small torsional rotations in the linker joining the tetracyclic enone to the benzoic acid moiety result in the **3a** benzoic acid ring being shifted slightly in the pocket away from catalytic histidine 340 (Fig. 1B). The hydrogen bond/salt-bridge distance increases from 2.79 to 2.83 Å, which is close to the estimated experimental coordinate error making this movement's significance ambiguous.

Despite solid evidence that the hydroxylated platensimycin variant is shifted in the binding site in a manner that changes key polar interactions between the inhibitor and the protein, it is unclear how the presence of the hydroxyl group causes the shift in binding modes to occur. The hydroxyl group is situated on the edge of the tetracyclic enone ring system that is fully-exposed to bulk solvent, and therefore no distinct cause and effect relationship can be made regarding the hydroxyl and binding modes. Unappreciated effects on charge distribution, solvation/desolvation energies, and other kinetic and/or thermodynamic parameters may contribute to the weaker binding affinity of the hydroxylated variant. Future studies could be designed to address these questions more fully.

In summary, we have described the isolation, structure, and activities of four congeners of platensimycin with hydroxylation of the aliphatic tetracyclic enone carboxylic acid unit which provided key insight of functional group tolerance for the activity.

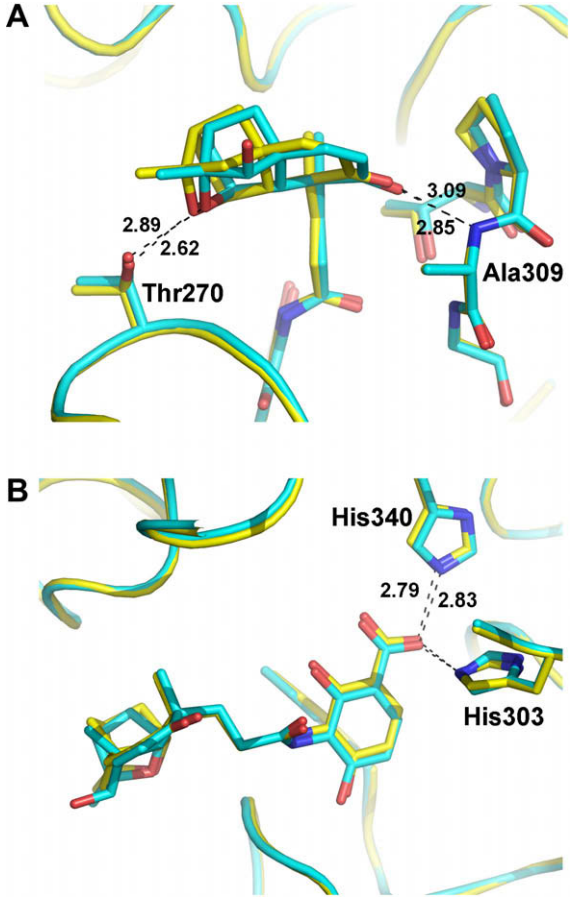

Figure 1. Interactions between platensimycin (yellow) and platensimycin A1 (cyan). (A) Ketolide interactions. (B) Benzoic acid interactions. Residues and distances (Å) discussed in the text are labeled.

The hydroxylation is likely a result of oxidation by P450 enzymes post terpenoid cyclization events.[9]

Acknowledgments

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