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Isolation and Structure of Platencin: A FabH and FabF Dual Inhibitor with Potent Broad-Spectrum Antibiotic Activity

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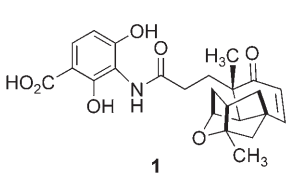
The discovery of antibiotics for the treatment of bacterial infections has been one of the most important medical achievements of the past 60 years.[1] However, the emergence of bacterial resistance to current antibiotics is a major challenge faced by the medical community. The emergence of resistance is inevitable, and hence the continued discovery of novel chemotype antibiotics with novel modes of action is critical to overcome drug-resistant organisms.[2] Several drug-discovery strategies have emerged, including incremental improvements to existing antibiotics by chemical manipulation and the search for novel drug targets on the basis of genomic approaches. An alternative strategy is to study the biochemical pathways or processes inhibited by known antibiotics and look for as yet unexploited targets within a pathway. A major benefit of employing such an approach is that these pathways are validated targets for drug intervention. The fatty acid synthesis is one such pathway. The targets we chose to study were the fatty acid biosynthesis condensing enzymes FabH and FabF, which are conserved and essential for bacterial viability.[3–6] The FabF target has been the subject of studies for over 20 years, and two classes of inhibitors have been reported:[7–12] cerulenin[13] and thiolactomycin[14–16] and its analogues. Cerulenin is a covalent modifier and a selective inhibitor of FabF, whereas thiolactomycin is a dual inhibitor of FabH and FabF. However, both exhibit poor antibacterial activity (*Staphylococcus aureus* MIC (minimum inhibitory concentration) 64 $\mu g\,mL^{-1}$).[17] Potent inhibitors of these enzymes are expected to allow the development of antibiotics with no cross-resistance to existing drugs.

We employed a differential-sensitivity method in which each target was differentially expressed by using antisense methodology under control of a xylose-inducible promoter.[18] In this method, the strain expressing fabF/fabH antisense RNA exhibits hypersensitivity to FabH- and/or FabF-specific inhibitors relative to a control strain.[18] This allowed us to develop a target-based whole-cell high-throughput-screening assay for the discovery of FabH and FabF inhibitors.[19,20]

Recent screening of natural product extracts by employing this differential-sensitivity whole-cell two-plate agar-diffusion assay[19] led us to discover platensimycin (**1**) as a



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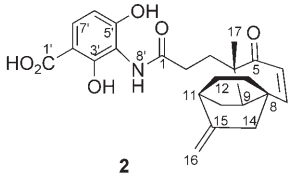

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selective inhibitor of *Staphylococcus aureus* FabF (IC_{50} = 48 nM) over FabH (IC_{50} = 67 μM).[21] Continued screening led to the identification of another extract of a new strain of *Streptomyces platensis* MA7339, which was isolated from a soil sample collected in Spain and showed activity in the FabH/FabF assay.

Bioassay-guided fractionation of the extract led to the isolation of platencin (**2**), which is a potent and dual inhibitor of FabH and FabF. Platencin consists of a tetracyclic unit (the right side of the molecule as drawn, different from platensimycin) connected to 3-amino-2,4-dihydroxybenzoic acid (common with platensimycin) through a two-carbon chain and an amide function. Platencin exhibits broad-spectrum antibacterial activity against all key pathogens harboring resistance to current antibiotics. It exhibits MIC values $\leq$ 0.06–4 $\mu g\,mL^{-1}$ ($\leq$ 0.14–9.4 μM) against key Gram-positive pathogens such as *S. aureus*, MRSA, macrolide, linezolid-resistant *S. aureus*, vancomycin intermediate *S. aureus*, vancomycin-resistant enterococci, and *Streptococcus pneumoniae*). Furthermore, it inhibits *S. aureus* fatty acid synthesis (IC_{50} = 0.45 μM), SaFabH (IC_{50} = 9.2 μM), and SaFabF (IC_{50} = 4.6 μM) and shows 3 log reduction of *S. aureus* colony forming units in an in vivo mouse model compared to untreated control.[22] Herein we describe the isolation, structure elucidation, and additional biological activity of platencin as well as a modeling-based rationale for the differences in activity between platencin and platensimycin.

Platencin (**2**) was isolated (1 $mg\,L^{-1}$) as colorless amorphous powder from the fermentation broth of *Streptomyces platensis* MA7339 by a two-step isolation method using amberchrome and reversed-phase HPLC chromatography.

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It displayed absorption bands at 226 and 296 nm in the UV/Vis spectrum and gave rise to bands for hydroxy (3300 cm^{-1}), enone (1713 cm^{-1}), and benzoic acid (1657 cm^{-1}) groups in the IR spectrum. High-resolution ESI FTMS analysis was consistent with the molecular formula $C_{24}H_{27}NO_6$, which was corroborated by the ^{13}C NMR spectrum in C_5D_5N (Table 1).

Table 1: ^1H and ^{13}C NMR assignments of platencin (**2**) in C_5D_5N.

#	δ_C	Type	δ_H	mult (*J* [Hz])	HMBC[a] (C→H)
1	175.2	C°			3-H, NH
2	32.2	CH_2	2.70	m	3-H
			2.68	m	
3	31.7	CH_2	1.93	ddd (14.4, 10.8, 5.4)	17-H_3
			2.53	ddd (14.4, 10.8, 5.4)	
4	48.1	C°			9-, 10-, 17-H_3
5	204.2	C°			7-, 9-, 17-H_3
6	126.8	CH	5.92	d (10)	
7	154.9	CH	6.36	d (10)	
8	36.7	C°			6-, 7-, 9-, 10-, 12-H
9	40.4	CH	2.02	t (9.6)	3-, 7-, 10-, 11-, 13-, 17-H_3
10	27.1	CH_2	1.60	m	9-H
			1.36	ddd (12.0, 9.6, 1.2)	
11	36.6	CH	2.26	m	9-, 11-, 12-, 16-H
12	26.5	CH_2	1.78	m	10-, 11-, 13-H
			1.56	m	
13	28.5	CH_2	1.30	m	9-, 12-H
14	44.9	CH_2	1.95	d (16.0) (equatorial)	7-, 11-, 13-, 16-H
			2.19	d (16.0) (axial)	
15	149.8	C°			10-, 11-, 12-, 14-H
16	107.8	CH_2	4.71	br s	14-H
			4.87	br s	
17	21.5	CH_3	1.09	s	3-, 9-H
1′	175.2	C°			7′-H
2′	107.6	C°			6′-H
3′	158.8	C°			7′-H, NH
4′	115.8	C°			6′-H, NH
5′	158.3	C°			6′-, 7′-H, NH
6′	110.4	CH	6.88	d (8.4)	6′-H
7′	129.8	CH	8.12	d (8.4)	
8′-NH			10.5	s	

The ^1H (600 MHz) and ^{13}C NMR (125 MHz) spectroscopic analysis of **2** in C_5D_5N in combination with a DEPT spectrum provided evidence for the presence of one angular methyl, seven methylene (including one vinyl), six methine (including four sp^2-hybridized carbon centers), and 10 quaternary carbon atoms (including an enone carbonyl group, two carboxy- and/or amide-type carbonyl groups, and two sp^3-hybridized carbon atoms). The $^1H,^1H$-COSY correlations led to four contiguous fragments consisting of two methylene groups (C2–C3), a *cis* alkene (C6–C7), the C9–C13 fragment, and an *ortho*-coupled (C6′–C7′) aromatic moiety. The two- and three-bond HMBC correlation of the angular methyl group to C3, C4, and C5 and of the olefinic methine protons 6-H to C4 and C8 and 7-H to C9 allowed the elucidation of the carbon chain C2 to C9. The three-bond HMBC correlation of the angular methyl group 17-H_3 to C9 permitted the construction of the six-membered enone-containing ring. The structure of the more-complex unprecedented tetracyclic unit of the molecule was elucidated by extensive application of HMBC correlations (Table 1). Three-bond HMBC correlations of 7-H to C14, of 9-H to C11, C13, and C14, of 16-H_2 to C11 and C14, and of 14-H_2 to C8, C9, C15 and C16 allowed the unambiguous identification of the tetracyclic unit with a vinyl substituent at C15.

The *ortho*-coupled aromatic protons 6′-H and 7′-H showed three-bond HMBC correlations to C2′ and C4′ and to C3′ and C5′, respectively. Hydroxy substituents were assigned to C3′ and C5′ because of their downfield shifts. The N8′-H produced HMBC correlations to C3′, C4′, and C5′, thus allowing the placement of phenol groups at C3′ and C5′ and supporting the presence of the amino benzoic acid moiety as found in platensimycin, whose structure was confirmed by X-ray crystallographic analysis of a 6-bromo derivative.[23] Finally the HMBC correlation of N8′-H and 3-H_2 to C1 allowed us to conclude where the two pieces were joined.

The relative configuration of the tetracyclic unit was elucidated by a NOESY experiment. The 17-H_3 axial methyl group produced strong NOESY correlations to the methylene groups at C12 and C13, which allowed us to establish that the methyl and 12-H_2 and 13-H_2 were on the same side of the molecule (Figure 1). Whereas this *Streptomyces platensis* strain only produces platencin, another strain—MA7374—produces both platencin and platensimycin (1:5 ratio), thus suggesting that both compounds likely have the same absolute configuration. The biogenesis of platensimycin could be envisaged from *ent*-

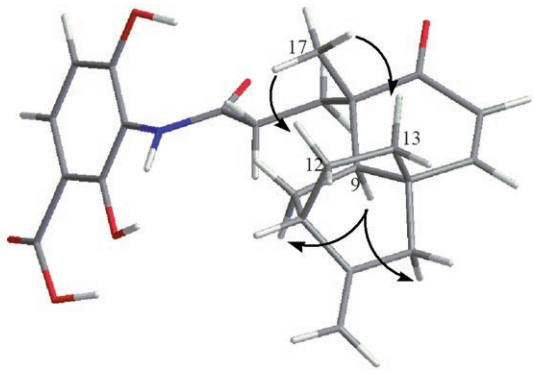
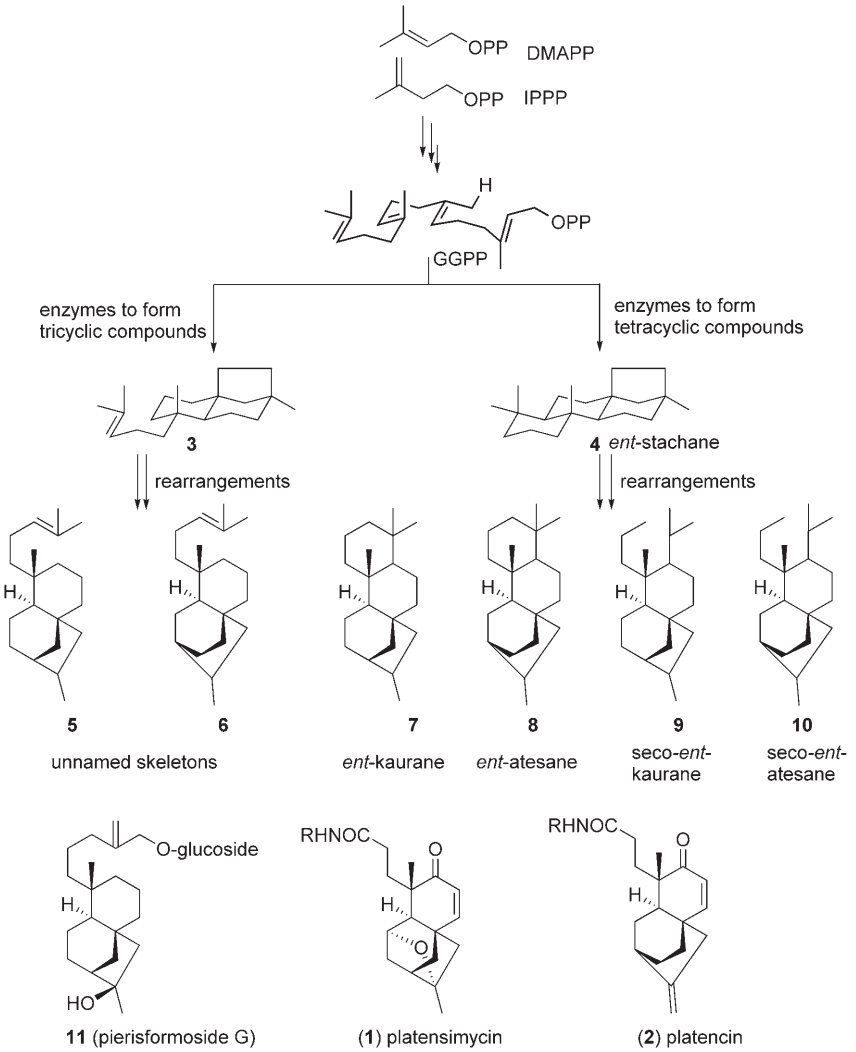


Figure 1. ChemDraw 3D model of platencin showing key NOESY correlations.

kaurane (**7**), seco-*ent*-kaurane (**9**) or unnamed diterpenoid skeleton **5** (A-ring uncyclized, e.g., pierisformoside G (**11**))[24,25] and that of platencin from *ent*-atesane (**8**), seco-*ent*-atesane (10),[26] or unnamed diterpenoid skeleton **6**, which originate from isoprenoid precursors dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPPP) via geranyl geranyl pyrophosphate (GGPP) and tricyclic intermediate **3** or tetracyclic intermediate **4** (Scheme 1). The



Scheme 1. Plausible biogenesis of platensimycin (**1**) and platencin (**2**) from isoprenoid precursors.

biogenesis of platencin could potentially also be envisioned from platensimycin (see Figure S1-2 in the Supporting Information). Precursor feeding experiments could define the precise biosynthesis route of these compounds, and such experiments are in progress.

Although the cellular and in vivo activity of platencin and platensimycin are similar, their cell-free enzymatic activity differ significantly.[22] Platencin inhibits both SaFabF and SaFabH with similar potency ($IC_{50} = 4.6\ \mu M$ and $9.2\ \mu M$, respectively), whereas platensimycin inhibits SaFabF very potently ($IC_{50} = 0.29\ \mu M$) but SaFabH only weakly ($IC_{50} = 247\ \mu M$), as measured in FabH/FabF PAGE gel elongation assays.[22] These data suggest that unlike platensimycin, which is a selective inhibitor of FabF (>800-fold selectivity), platencin is a balanced dual FabH/FabF inhibitor and its overall inhibition of bacterial growth results from the inhibition of both enzymes. This difference in the FabF activity of the two compounds was confirmed in a direct binding assay using [^{3}H]dihydroplatensimycin and ecFabF (wild-type),[21] for which an approximately sixfold difference in affinity is observed (platencin: $IC_{50} = 113$ nM; platensimycin: $IC_{50} = 19$ nM). The differences in the binding affinities of the two compounds are consistent with the observed interaction differences in the docking studies with ecFabF and ecFabH (Figure 2).

Figure 2 shows the platensimycin and platencin docked into the active sites of ecFabH[27] and ecFabF (C163Q).[21] In this figure, platensimycin is shown in green and platencin in cyan. The carboxylic acid group of both compounds interacts with Asn 274 and His 244 of FabH while this group interacts with His 303 and His 310 of FabF. Platensimycin and platencin interact with the enzymes similarly, except for the ketolide groups posed at the entrance of the active sites. In FabF, the ether oxygen atom of the pentacyclic ring of platensimycin makes a weak hydrogen-bond contact with Thr 270. This hydrogen-bond interaction is absent in platencin, which can be a possible explanation for why platencin is less active than platensimycin in FabF. However, there are two unfavorable interactions between platensimycin and FabH that may cost the binding: the polar ether oxygen atom of platensimycin is surrounded by nonpolar residues such as Ile 155, Ile 156, and Trp 32, and the nonpolar methyl group points directly toward the polar residue Arg 151. In contrast, the vinyl group of the tetracyclic ring in platencin interacts with the same nonpolar residues Ile 155, Ile 156, and Trp 32, thus allowing platencin to keep its activity in FabH.

In summary, platencin is a novel and potent broad-spectrum Gram-positive antibiotic that exerts its activity by a novel mode of action by dual inhibition of FabH and FabF. The dual inhibition should provide an advantage over single-enzyme inhibition for lower resistance potential. The novel architecture, unique mode of action, in vivo efficacy, and biological profile provide a great opportunity for platencin to be used as a lead compound for the development of a valuable class of antibiotics.

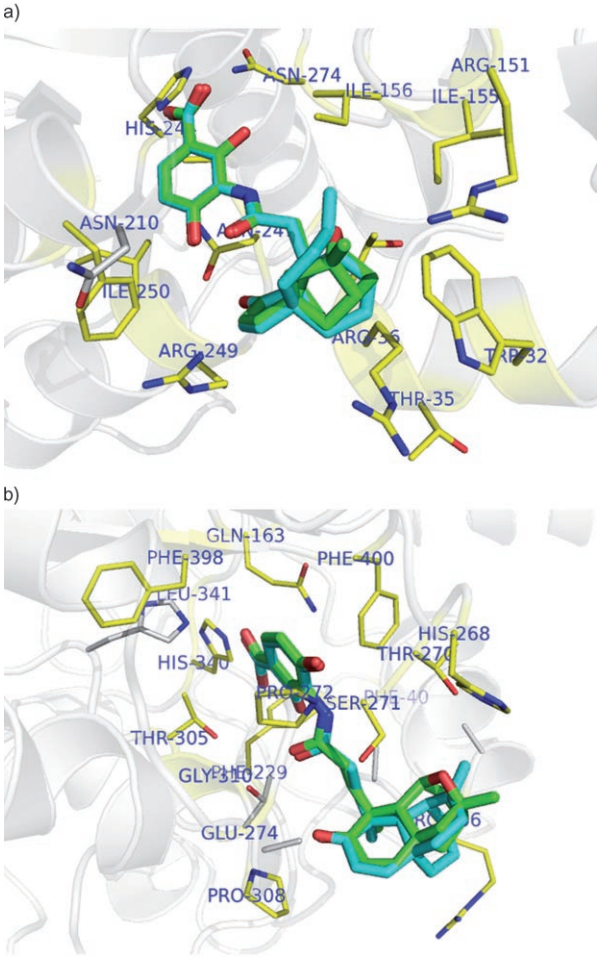


Figure 2. *E. coli* condensing enzyme active sites of a) FabH and b) FabF with docked platensimycin (green) and platencin (cyan).

Experimental Section

Producing organism: The producing strain was obtained from a soil sample collected in Mallorca, Balearic Islands, Spain. The strain was isolated after pretreatment of the soil with chloramines T (1% *w*/*v*) and plating on humic acid based agar supplemented with nalidixic acid (20 μg mL^{-1}). The morphology, fatty acid composition, and 16S rDNA sequence indicated that the organism was consistent with a *Streptomyces* sp. Complete 16S rDNA analysis by using the maximum parsimony method showed a close relationship with *S. platensis* ATCC 13865 and *S. platensis* MA7327 and MA7331 (both producers of platensimycin), highly supported by a 97% bootstrapping value, thus suggesting that this is another strain of *S. platensis* (strain number MA7339).

Fermentation of *S. platensis* MA7339: A frozen suspension (1.3 mL) of *S. platensis* MA7339 was inoculated into a 250-mL flask containing aqueous seed medium (50 mL; components: soluble starch (20 g L^{-1}), dextrose (10 g L^{-1}), NZ amine type E (5 g L^{-1}), Difco beef extract (3 g L^{-1}), bacto (Difco) peptone (5 g L^{-1}), Difco yeast extract (5 g L^{-1}), $CaCO_3$ (1 g L^{-1}), at pH 7). The flask was incubated at 28.0 °C with an agitation of 220 rpm for 48 h. The second-stage seed was developed by transferring 3% inoculum of the first-stage seed into a 250-mL shake flask containing seed medium (50 mL). The flask was incubated at 28.0 °C with an agitation of 220 rpm for 24 h. A 5% inoculum of the second-stage seed was transferred into 250-mL shake flasks containing aqueous production medium (30 mL; components: Difco yeast extract (6 g L^{-1}), malt extract (15 g L^{-1}), dextrose (6 g L^{-1}), trace elements (5 mL), 3-(*N*-morpholino)propanesulfonic acid (MOPS; 20 g L^{-1}) at pH 7) and incubated at 32.0 °C with an agitation of 220 rpm for 12 days.

Isolation of platencin: Fermentation broth (2 L) was diluted with acetone (2 L) and shaken on a shaker for 2 h and filtered. The filtrate was concentrated under reduced pressure to remove most of the acetone and charged onto a 75-mL amberchrome (CG161s) column. The column was eluted with a 100-min gradient of 10–100% aqueous methanol at a flow rate of 5 mL min^{-1}. The compound eluted in a broad zone with high methanol content, which upon concentration and lyophilization afforded a semipurified fraction (170 mg). A portion of the fraction (80 mg) was purified by reversed-phase HPLC (Zorbax Rx C_8, 21.4 × 250 mm) with a 37-min gradient of 20–98% aqueous acetonitrile containing trifluoroacetic acid (0.1%) at a flow rate of 12 mL min^{-1} to produce platencin (**2**) (1.1 mg). $[\alpha]_D^{23} = -7.0$ deg cm^3 g^{-1} dm^{-1} ($c = 0.85$ g cm^{-3}, CH_3OH); UV/Vis (CH_3OH): λ_{max} (ε) = 226 (16837), 296 nm (2663); FTIR (ZnSe): $\tilde{\nu} = 3300$, 2935, 1713 (w), 1657, (br, strong), 1536, 1379, 1247, 1141, 1094, 1057, 832, 799 cm^{-1}; HRMS: *m*/*z* calcd for $C_{24}H_{27}NO_6$: 426.1917 $[M+1]^+$; found: 426.1911.

FabF direct-binding assay: The assay was performed as described previously.[21] Briefly, purified His-tagged ecFabF was captured on Cu^{2+}-coated scintillation proximity assay beads in the presence of [^{3}H]dihydroplatensimycin. Direct-binding assays were carried out in potassium phosphate (50 mM; pH 6.5) and NaCl (50 mM) supplemented with bovine serum albumin (0.05% *w*/*v*). To form the acyl intermediate, stock solutions of ecFabF (wild-type) (500 nM) were preincubated on ice for 10 min with excess lauroyl CoA (50 μM) and the activated ecFabF, [^{3}H]dihydroplatensimycin (specific activity 15.8 Ci mol^{-1}) (50 nM each) were combined with Cu^{2+}-coated YtSi SPA beads (200 μg mL^{-1}) (GE Health Care) with defined concentrations of inhibitors in a final volume of 100 μL. Reactions were equilibrated for at least 30 min at room temperature and counted. IC_{50} values were extracted from the data by standard curve-fitting procedures.

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