Dedicated *ent*-Kaurene and *ent*-Atiserene Synthases for Platensimycin and Platencin Biosynthesis

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Supporting Online Materials (SOM)

General experimental procedures

Bacterial strains, plasmids, and media. *Escherichia coli* DH5α (*1*) was used for routine cloning, *E. coli* XL1-Blue MRF and Gigapack III XL (Stratagene, La Jolla, CA) for library construction, *E. coli* BW25113/pIJ790 for λRED-mediated PCR targeting (*2*), and *E. coli* ET12567/pUZ8002 (*3*) was used for intergenic conjugation. Vectors SuperCos1 (Stratagene, La Jolla, CA), pGEM-3zf, and pGEM-5zf (Promega, Madison, WI) were obtained from commercial sources. Platensimycin and platencin producers *S. platensis* MA7327 and MA7339 were kindly provided by Merck Research Laboratories (Rahway, NJ). *E. coli* carrying plasmids were grown in Luria-Bertani (LB) medium and were selected with appropriate antibiotics (*1*). Standard media and protocols were used for *Streptomyces* growth and sporulation (*3*). Media components and all other chemicals were from standard commercial sources.

DNA isolation, manipulation, and sequencing. Plasmid preparation and gel extraction from *E. coli* was carried out with commercial kits (Qiagen, Valencia, CA). Total *S. platensis* DNA isolation was performed according to standard procedures (*3*), as were all restriction endonuclease digestions and ligations (*1*). For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed as described in protocols provided by the manufacturer (Roche Diagnostics, Indianapolis, IN). Automated DNA sequencing and oligonucleotide primer synthesis were performed at the Biotechnology Center, University of Wisconsin-Madison. Long primers for PCR-Targeting were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

General Instrumentation. Unless otherwise noted, PCR experiments were performed with LA Taq polymerase (Takara Bio Inc, Shiga, Japan) and run in an Eppendorf Gradient Thermocycler (Brinkman Instruments, Westbury, NY). Analytical high performance liquid chromatography (HPLC) was performed on a Waters 510 HPLC system with a photodiode array detector (Waters, Milford, MA) equipped with an Apollo C18 column (particle size, 5 µm; 4.6 x 250 mm; Grace Davison Discovery Sciences, Deerfield, IL). Semi-preparative HPLC was performed with an Apollo C18 column (particle size, 5 µm; 10 x 250 mm; Grace Davison Discovery Sciences) on a Varian Liquid Chromatography System (Varian, Walnut Creek, CA) consisting of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector. Unless otherwise noted, HPLC was performed with a 1 mL/min (analytical) or 3 mL/min (semi-prep) flow rate and a 20 min gradient from 15% acetonitrile in H_2O with 0.1% formic acid to 90% acetonitrile in H_2O with 0.1% formic acid. Liquid chromatography-mass spectroscopy (LC-MS) analysis was performed under the same conditions on an Agilent Technologies 1100 Series LC/MSD with DAD (Santa Clara, CA). Column chromatography was performed either on silica gel (230-400 mesh, Natland International Corp, Research Triangle Park, NC) or Sephadex LH-20 (Pharmacia, Kalamazoo, MI). ^{1}H and ^{13}C NMR spectra were recorded at 25 ˚C on a Varian Inova 500 instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei.

Cloning, sequencing, and annotation of the *ptm* and *ptn* gene clusters

The isolation of cosmids carrying the *ptm* cluster from *S. platensis* MA7327 was described previously (*4*). Additional isolated cosmids pBS12003-pBS12006 were used for sequencing and construction of mutants (Table S2). Cosmids harboring the *ptn* cluster were isolated from a library of *S. platensis* MA7339 constructed in SuperCos1following the manufacturer's protocols (Stratagene). The genomic library was screened by colony hybridization with two digoxigenin-labeled PCR products amplified from MA7339 genomic DNA with primers "ptnprobeF" and "ptnprobeR" and "ptmForward" and "ptmReverse" (*4*). Isolated cosmids pBS12614 and pBS12615 were used to sequence the *ptn* gene cluster. Shotgun sequencing was performed by subcloning *Bam*HI or *Eco*RI digest fragments into the same restriction sites of cloning vector pGEM-3zf (Promega, Madison, WI) and sequencing with M13 forward and reverse primers. Contigs were assembled using BioEdit software (*5*), and gaps in the sequence were filled by chromosome walking on overlapping cosmids. To aid in the annotation of each gene cluster, a

codon preference plot was generated using GCG BioComp software (Genetics Computer Group, Madison, WI). Open reading frames were compared to public databases using BLAST analyses (*6*) to identify homologous sequences. Annotated DNA sequences are available from Genbank with the accession numbers FJ655920 and CG398492 for the *ptm* and *ptn* gene clusters, respectively

Construction of mutants by gene replacement

The primers used in the λRED-mediated PCR targeted gene replacements of *orf4*, *ptmO1*, *ptmT1*, *ptmT3*, *ptmB2*, and *orf5* with the *aac(3)IV-oriT* resistance cassette from pIJ773 by standard protocols (*2*) are summarized in Table S3. The resulting modified cosmids were introduced to *S. platensis* MA7327 by intergenic conjugation using *E. coli* ET12567/pUZ8002 as the donor stran (*3, 7*). Briefly, *E. coli* ET12567/pUZ8002 cells harboring the modified cosmids were grown to OD_{600} 0.6 in LB supplemented with 20 mM $MgCl_2$ at 37 ˚C. During this incubation, ~10^8 *S. platensis* MA7327 spores were heat shocked in modified TSB medium (30 g/L tryptic soy broth, 100 g/L sucrose, 4 g/L glycine) at 50˚C for 10 min and incubated at 28 ˚C. The donor *E. coli* and recipient *S. platensis* cells were then mixed and plated on IWL4 medium (37 g/L ISP4, 0.5 g/L yeast extract, 1 g/L tryptone) supplemented with 20 mM $MgCl_2$ (*8*). The plates were incubated for 16 hr at 30˚C before being overlaid with 1 mL H_2O containing final concentrations of 25 μg/mL nalidixic acid to select against *E. coli* and 50 μg/mL apramycin to select for exconjugants. Plates were incubated for ~6 days at 30 ˚C and the resultant colonies were replica plated to find apramycin resistant and kanamycin sensitive clones that had undergone homologous recombination on each side of the locus of gene replacement.

Determination of the gene cluster boundaries

To determine the upstream cluster boundary of the *ptm* cluster in *S. platensis* MA7327, *orf4* and *ptmO1* were independently knocked out using the PCR-targeted cosmids, pBS12007 and pBS12008, respectively, to generate Δ*orf4* mutant SB12003 and Δ*ptmO1* mutant SB12004. The genotype of apramycin-resistant, kanamycin-sensitive exconjugates was verified by Southern analysis (Figure S2A and S2B). Fermentation of the mutant strains and wild-type *S. platensis* MA7327 under standard PTM production conditions (*4*) revealed PTM production by SB12003 but not SB12004. These data suggest a role for PtmO1 but not Orf4 in PTM biosynthesis and therfore that the upstream boundary is located between these genes.

The downstream boundary of the *ptm* cluster is predicted to be located between *ptmO9* and *orf5*. PtmP4, just inside of this boundary is predicted to be an efflux pump, thereby conferring PTM/PTN resistance by exporting the antibiotics, based on its homology to known enzymes. As the ferredoxin-encoding *ptmO9* appears to be cotranscribed with *ptmP4*, the most likely boundary location is between *ptmO9* and the glycosyltransferase, *orf5*. Attempts to knockout *orf5* using the modified cosmid, pBS12009, yielded a single-crossover mutant, SB12005 (Figure S3). Following two rounds of growth in antibiotic free medium, 10/60 colonies isolated had reverted back to wildtype, while 0/60 resolved to the desired knockout mutation. These results suggest that *orf5* is essential for viability, and thus support the hypothesis that it is not involved in PTM biosynthesis.

By analogy, the boundaries for the *ptn* cluster from *S. platensis* MA7339 were assigned from *ptnO1* to *ptnO9*. The proposed *ptn* cluster boundary locations were validated by the production of PTN in selected heterologous hosts bearing the construct pBS12631 that harbor the intact *ptn* cluster.

Inactivation of *ptmB2* in *S. platensis* MA7327

A modified cosmid, pBS12010, was produced by λRED-mediated PCR targeting pBS12003 with the primers Δ*ptmB2*Forward and Δ*ptmB2*Reverse (Table S3) to replace *ptmB2* with the apramycin resistance cassette. Introduction of pBS12010 to *S. platensis* MA7327 afforded the *ptmB2* mutant strain, *S. platensis* SB12006, whose genotype was confirmed by Southern analysis (Figure S4). Phenotypic analysis of PTM/PTN production by SB12006 followed the same method described previously for other strains (*4*). To determine the identity of new compounds eluting at 13.85 min and 18.45 min, respectively (Figure 3D), production cultures were scaled-up to a total of 4 L (10 x 400-mL cultures in 2-L baffled shake flasks). Extraction and purification of the newly produced compounds was performed as follows: Amberlite XAD-16 resin was separated from culture broth by centrifugation and washed with distilled H_2O prior to extraction with 4 x 250 mL of acetone. Fractions were combined and solvent removed under reduced pressure to afford a crude extract. Column chromatography over silica gel with increasing concentrations of methanol in chloroform afforded semi-pure fractions of the two compounds. Further purification by column chromatography over LH-20 resin with methanol as a mobile phase and finally semi-preparative, reverse-phase HPLC using identical conditions to those described previously (*9*) yielded the pure materials. The identity of the compound eluting at 13.85 min was confirmed as platensic acid (*10, 11*), while that of the compound eluting at 18.45 min was confirmed as platencinic acid (*12*), both of which have been characterized previously; the mass data and ^{1}H NMR and ^{13}C NMR spectra (Figures S5-S8) from the current study were identical to those reported in the literature. Chemical complementation of SB12006 for PTM and PTN production was achieved by adding a filter-sterilized aqueous solution of 3,4-aminohydroxybenzoic acid to the autoclaved production medium at a final concentration of 0.65 mM.

Inactivation of *ptmT1* and *ptmT3* in *S. platensis* MA7327

Following the same λRED-mediated PCR targeted gene replacement protocols (*2*), modified cosmids, pBS12011 and pBS12012, were produced by PCR-targeting pBS12003 with primer pairs Δ*ptmT1*Forward-Δ*ptmT1*Reverse (Table S3) and Δ*ptmT3*Forward-Δ*ptmT3*Reverse (Table S3) to replace *ptmT1* and *ptmT3*, respectively, with the apramycin resistance cassette. Introducing pBS12011 and pBS12012 to *S. platensis* MA7327 by intergenic conjugation afforded mutant strains *S. platensis* SB12007 and *S. platensis* SB12008, respectively, whose genotypes were confirmed by Southern analysis (Figure S9). Phenotypic analyses comparing crude extracts generated from SB12007 and SB12008 cultures in standard PTM/PTN production conditions (*4*) to wild-type MA7327 crude extracts as well as to authentic standards of PTM and PTN identified SB12007 as a PTM-only producing strain and SB12008 as a PTN-only producing strain.

Expression of *ptn* cluster in *S. lividans* K4-114 for PTN production

Cosmid pBS12615 was determined to contain the entire *ptn* gene cluster by direct sequencing. For heterologous expression constructs, *ptnR1* was replaced with the apramycin resistance cassette to make pBS12616 using λRED-mediated PCR targeting (*2*) and the primers 34lambdaF and 34lambdaR (Table S3) (*4, 9*). This gene replacement was converted to a markerless gene deletion by introducing the modified cosmid to *E. coli* DH5α/BT340 via electroporation, and incubating overnight at 42 ^{o}C to induce expression of FLP recombinase, resulting in the loss of *aac(IV)3* cassette and the generation of an 81 bp in-frame scar in the new cosmid pBS12617 (*2*). The SuperCos backbone was modified for integration into the chromosome of various *Streptomyces* via the following protocol. A 270-bp fragment corresponding to the 3' end of β-lactamase gene from SuperCos was amplified with primers 3'AmpF and 3'AmpR (Table S3) and cloned into the *Xba*I-*Bam*HI sites of pSET152 (*7*). The resulting plasmid, pBS12618, was linearized via a *Bam*HI-*Eco*RI double digest and used in place of PCR product for the PCR targeting of pBS12617. Recombination across the 270 bp “3'-Amp” region on one side and the conserved origin of replication (rep^{pUC}) on the other side resulted in the replacement of the SuperCos1

backbone with the pSET152 backbone to afford the heterologous expression construct, pBS12618. The final construct was verified by PCR and sequencing of the modified sites and introduced to *S. lividans* K4-114 species by *E. coli – Streptomyces* conjugation (*3, 7*) to generate heterologous producer *S. lividans* SB12606. PTN production by SB12606 was verified by HPLC and LC-MS analyses with comparison to an authentic standard. Fermentations done in triplicate yielded an average titer of 1.20 ± 0.54 mg/L, as determined by the absorbance at 240 nm.

Identification of the "PTM cassette" and its possible role in the evolution of the *ptm* and *ptn* gene clusters

The "PTM cassette" is a four-gene contig comprised of *ptm04*, *ptmT3*, *ptmO5*, and *ptmR3* located between the duplicated gene, *ptmO3/ptmO6* (99.9% identical over 1 kb). This cassette is replaced with just a single copy of *ptnO6* in the *ptn* gene cluster. Because the Sanger sequencing method used during sequencing and assembly of the gene clusters typically yields reads of ~800 bp, it was not possible to obtain direct sequence evidence for the absence of this cassette in the *ptn* gene cluster. Instead, a PCR strategy was devised to support the current organization of the *ptn* gene clusters. Primers "CassetteCheckF" and "CassetteCheckR" (Table S3) were used to amplify across *ptnT2-ptnT4* in the *ptn* gene cluster. If the PTM cassette is missing, as expected, the primers are expected to produce a 1.4 kb product. Indeed, the expected product was produced, supporting the reported gene sequences and organization.

The presence of the 1-kb duplicated sequence on either side of the PTM cassette affords a possible mechanism to explain how the *ptm* and *ptn* gene clusters could have evolved. In one scenario, a duplication of *ptmO3* in a predecessor of the *ptm* gene cluster created the 1-kb flanking repeats still apparent in the *ptm* gene cluster. Homologous recombination between these two regions would result in the precise excision of the PTM cassette, and only one copy of the duplicated region would remain (Figure S10). This matches the genotype seen in the *ptn* gene cluster. In a second scenario, the *ptn* gene cluster could have predated the *ptm* gene cluster. Recombination across *ptnO6* between the *ptn* gene cluster and a small circular plasmid bearing the genes *ptmO3*, *ptmO4*, *ptmT3*, and *ptmR3* would result in the incorpation of the PTM cassette while at the same time generating the 1-kb flanking repeats (Figure S10).

Expression of the PTM cassette in *S. platensis* MA7339 for PTM production

Creating an expression construct for the entire PTM cassette required a PCR approach to introduce convenient restriction cleavage sites on either end of the cassette. As the entire cassette was not efficiently amplified as a single product, smaller fragments were individually amplified and cloned together to eventually yield the pSET152 derivative, pBS12603, containing *ptmO3-ptmR3* under the expression of the *ErmE** promoter. This construct was introduced to *S. platensis* MA7339 following the same protocol as described above for mutagenesis experiments.

Fermentation and HPLC analysis of strains containing the PTM cassette revealed PTM production at approximately 25 mg/L. PTN was still produced in trace amounts and was readily identified by LC-MS. The high level of PTM production compared to PTN is attributed to the high level expression of the PTM cassette by the *ErmE** promoter. The high titer of PTM permitted isolation by normal phase column chromatography and structure confirmation by ^{1}H NMR spectroscopy (Figure S11).

References

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Table S1. Strains and plasmids used for this study

Strains	**Description**	**Reference**
E. coli DH5α	*E. coli* host for cloning	Commercial
E. coli XL1-Blue MRF	*E. coli* host for cosmid library	Commercial
E. coli BW25113/pIJ790	*E. coli* host for PCR targeting	(*2*)
E. coli ET12567/pUZ8002	*E. coli* host for conjugation	(*3*)
S. platensis MA7327	Wildtype PTM/PTN producer	(*13,14*)
S. platensis MA7339	Wildtype PTN producer	(*15,16*)
S. platensis SB12003	MA7327 Δ*orf4*::*aac(IV)3*	This study
S. platensis SB12004	MA7327 Δ*ptmO1*::*aac(IV)3*	This study
S. platensis SB12005	MA7327 pBS12009	This study
S. platensis SB12006	MA7327 Δ*ptmB2*::*aac(IV)3*	This study
S. platensis SB12007	MA7327 Δ*ptmT1*::*aac(IV)3*	This study
S. platensis SB12008	MA7327 Δ*ptmT3*::*aac(IV)3*	This study
S. platensis SB12604	MA7339 harboring pBS12603	This study
S. platensis SB12605	MA7339 harboring pSET152	This study
S. lividans K4-114	*S. lividans* with RED cluster deletion	(*17*)
S. lividans SB12606	K4-114 harboring pBS12619	This study
Plasmids		
SuperCos1	Construction of MA7327 cosmid library	Commercial
pGEM-3zf	Used to subclone *ptm* and *ptn* gene cluster fragments from cosmids	Commercial
pGEM-5-zf	Used to subclone *ptm* and *ptn* gene cluster fragments from cosmids	Commercial
pGEM-Tez	Used for PCR cloning during PTM cassette experiment	Commercial
pIJ773	Contains apramycin resistance cassette (*aac(IV)3*)	Gust 2003
pSET152	*E. coli – Streptomyces* shuttle vector	(*7*)
pBS12001	Cosmid harboring partial *ptm* gene cluster	(*4*)
pBS12003	Cosmid harboring partial *ptm* gene cluster	This study
pBS12004	Cosmid harboring partial *ptm* gene cluster	This study
pBS12005	Cosmid harboring partial *ptm* gene cluster	This study
pBS12006	Cosmid harboring partial *ptm* gene cluster	This study
pBS12007	pBS12006 Δ*orf4*::*aac(IV)3*, generated by PCR targeting with primers Δ*orf4*Forward and Δ*orf4*Reverse	This study
pBS12008	pBS12006 Δ*ptmO1*::*aac(IV)3*, generated by PCR targeting with primers Δ*ptmO1*Forward and Δ*ptmO1*Reverse	This study
pBS12009	pBS12001 Δ*orf5*::*aac(IV)3*, generated by PCR targeting with primers Δ*orf5*Forward and Δ*orf5*Reverse	This study
pBS12010	pBS12003 Δ*ptmB2*::*aac(IV)3*, generated by PCR targeting with primers Δ*ptmB2*Forward and Δ*ptmB2*Reverse	This study
pBS12011	pBS12003 Δ*ptmT1*::*aac(IV)3*, generated by PCR targeting with primers Δ*ptmT1*Forward and Δ*ptmT1*Reverse	This study
pBS12012	pBS12003 Δ*ptmT3*::*aac(IV)3*, generated by PCR targeting with primers Δ*ptmT3*Forward and Δ*ptmT3*Reverse	This study
pBS12603	pSET152 derivative harboring the PTM cassette under control of promoter *ErmE**;	This study
pBS12614	Cosmid harboring *ptn* gene cluster	This study
pBS12615	Cosmid harboring *ptn* gene cluster	This study
pBS12616	pBS12615 Δ*ptmR1*::*aac(IV)3*, generated by PCR targeting with primers 34lambdaF and 34lambdaR	This study
pBS12617	pBS12616 Δ*aac(IV)3*, generated by FLP-mediated excision of disruption cassette; contains 81 bp scar	This study
pBS12618	pSET152::3'Amp	This study
pBS12619	pBS12617 with SuperCos1 backbone replaced by pSET152 backbone	This study

Table S2. Primers used for this study

Primer Name	Nucleotide Sequence (5'-3')
ptnprobeF	GCCGTCTCGTCGTGCTG
ptnprobeR	CTGGACCACCACATCCGG
ptmForward	CCAAGATCCCGCTGGAGATC
ptmReverse	GGCAGAACTCGTTGATCGGG
M13 Forward	GTAAAACGACGGCCAG
M13 Reverse	CAGGAAACAGCTATGAC
Δ*orf4*Forward	ACACCCGCACGTTCCTTTGCAGATTGGAGGCGGACAGTGATTCCGGGGATCCGTCGACC
Δ*orf4*Reverse	CATGTCGACGTACTCCTGACCGGAGAGCACCGCGATCTCTGTAGGCTGGAGCTGCTTC
Δ*ptmO1*Forward	CGCCCCCGTACCGCACGCGAAAGGGCACACCACTCCATGATTCCGGGGATCCGTCGACC
Δ*ptmO1*Reverse	GTCGTCGGCCAGCGCCACCACGGCCCGGCCGATGTCCTGTGTAGGCTGGAGCTGCTTC
Δ*ptmT1*Forward	AGACCTGAGAATGACGGTGACCGAGTGTCCAGCATGACGATTCCGGGGATCCGTCGACC
Δ*ptmT1*Reverse	CTCCCGCGAGATGCTCCGCAGCAGTTTGATCTTGTCCACTGTAGGCTGGAGCTGCTTC
Δ*ptmT3*Forward	GGGGCTGACATCGGCGACCGTGAGGAAGCAGGAATGAACATTCCGGGGATCCGTCGACC
Δ*ptmT3*Reverse	CGCGGCGGCGGAGGCCGGGGCGCTGCGGTGCAGCTCGTCTGTAGGCTGGAGCTGCTTC
Δ*ptmB2*Forward	GTCGACCTTGCCTCACCATATTTGTGGAGAAATAACATGATTCCGGGGATCCGTCGACC
Δ*ptmB2*Reverse	GGGCTTGAGCTCGGTGCTGTTGAGCACGGCGCCACCGGGTGTAGGCTGGAGCTGCTTC
Δ*orf5*Forward	CGCGCAGTGGAGCCATTGATGACCAAGTCGTACGCAGCCATTCCGGGGATCCGTCGACC
Δ*orf5*Reverse	TCACCGCTCCACCAGCGTGAACAGGCTCTCCCAGCGGCCTGTAGGCTGGAGCTGCTTC
34lambdaF	AAGGGACCCCAGAAGCGAAACGGGGCGGCACTTCGTGTGATTCGGGGGATCCGTCGACC
34lambdaR	ATGGGCCTCGGCCTCGTCGGCCTCGCCCGCCTGAATCAGTTGAGGCTGGAGCTGCTTC
3'AmpF	CCGAGTTGCTCTAGACCGGCGTCAACACGG
3'AmpR	AGAGTATGAGGATCCAACATTTCCGTGTCGCC
CassetteCheckF	CCGCGAGGTCGTCGTG
CassetteCheckR	CCACATCAGCAGGCCGTAG

Figure S1. Structures of PTM, PTN, and biosynthetically related natural products. (A) PTM and PTN with their common 3-amino-2,5-dihydroxybenzoic acid (ADHBA) moiety shown in red and the *ent*-kaurene-derived diterpenoid moiety for PTM and *ent*-atiserene-derived diterpenoid moiety shown in blue. Wavy lines denote bonds broken during biosynthesis en route to PTM and PTN. (B) While the ADHBA moiety has not been seen in other known natural products, natural products featuring 3-amino-5-hydroxybenzoic acid (AHBA) moiety whose biosynthetic origins are known, and the biosynthetic origin of AHBA has been studied and traced to aspartate 4-semialdehyde and dihydroxyacetone phosphate as exemplified by grixazone (*18*), murayaanthraquinone (*19*), and exfoliazone (*20*). (C) Pierisformoside G (*21*) and gibberellin A3 (*22*) of *ent*-kaurene biosynthetic origin. (D) Spiramilactone (*23*) and serofendic acid (*24*) of *ent*-atiserene origin.

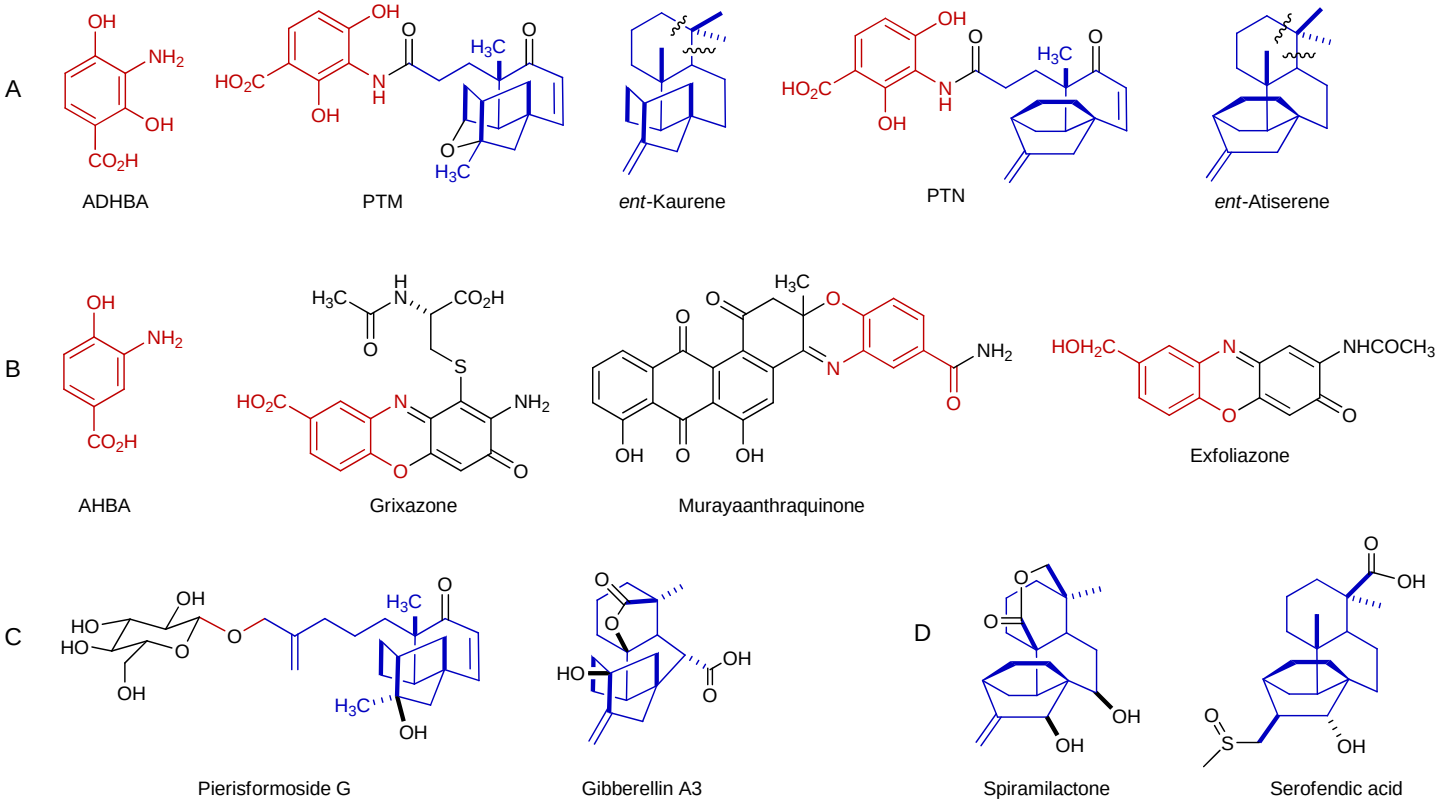
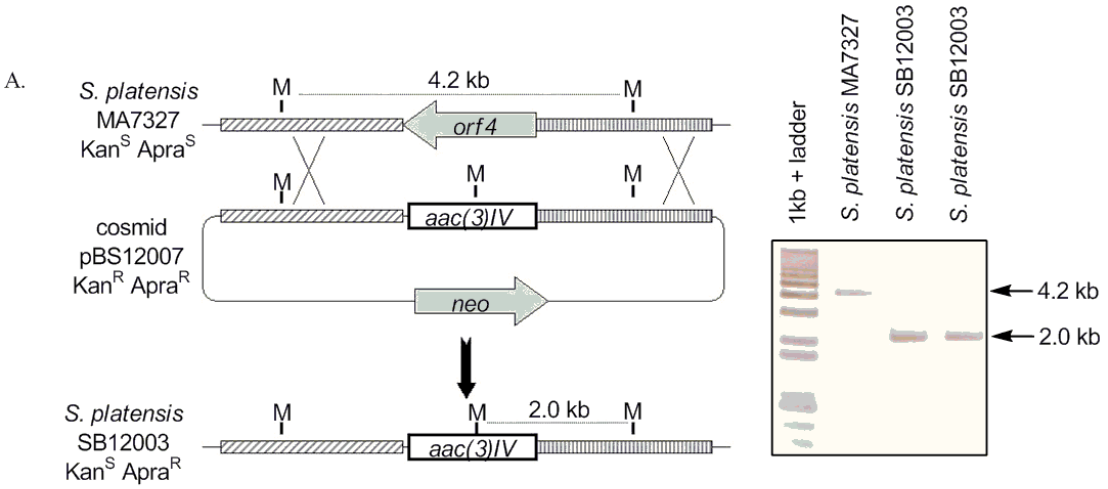
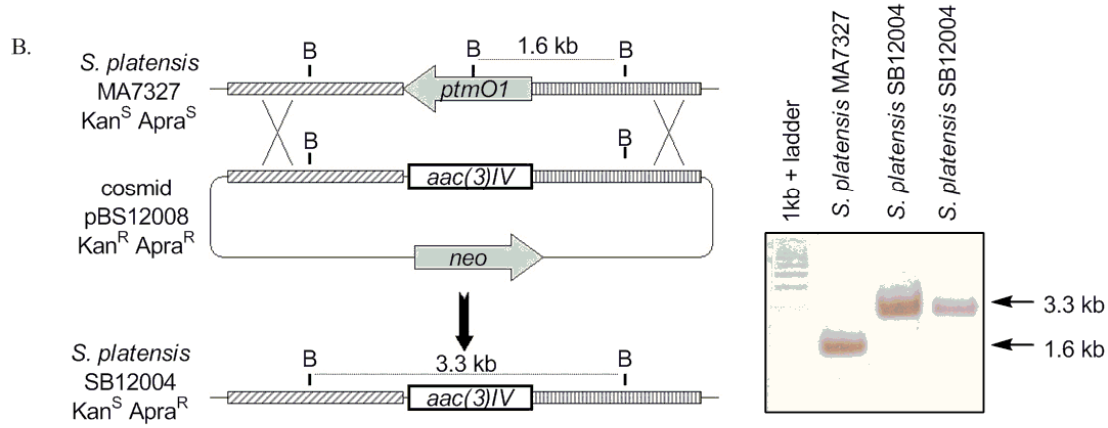


Figure S2. Determination of the upstream boundary of the *ptm* cluster in *S. platensis* MA7327. Genetic maps depict *orf4* (A) and *ptmO1* (B) gene replacements to generate *S. platensis* SB12003 and SB12004, respectively, which are confirmed by Southern analysis. Phenotypic analysis of resulting strains (C) show that HPLC chromatograms with UV detection at 240 nm and UV-vis spectra (insets) for authentic PTM (i), crude extract from SB12003 fermenation (ii), and crude extract from SB12004 fermentation (iii). These data suggest that *ptmO1* is essential for PTM production while *orf4* is not, hence establishing the upstream boundary of the *ptm* cluster. Kan^R, kanamycin resistant; $Apra^R$, apramycin resistant; M, *Mlu*I cleavage site; B, *Bmr*I cleavage site; *aac(3)IV*, apramycin resistance marker; *neo*, kanamycin resistance marker.





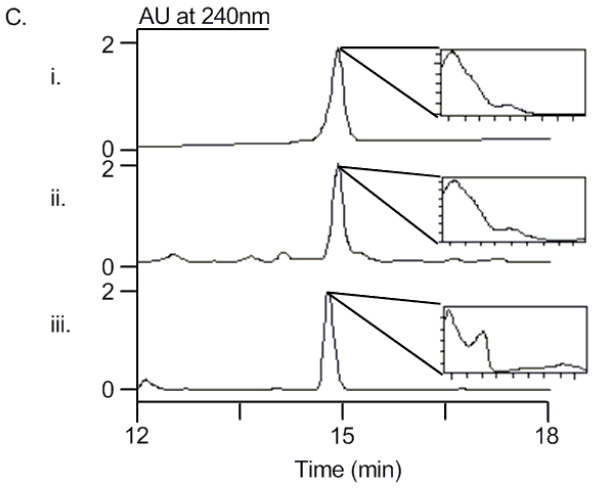


Figure S3. Determination of the downstream boundary of the *ptm* cluster in *S. platensis* MA7327. Genetic map depicts *orf5* gene replacement strategy to generate *S. platensis* SB12005 (A), which is confirmed by PCR analysis (B). Putative exconjugants were screened by PCR with primers Δ*orf5*Forward and Δ*orf5*Reverse (Table 3, represented by half arrows) to amplify a ~1.3 kb fragment of *aac(3)IV*. PCR was performed using DNA isolated from SB12005 (lane 1) and MA7327 (lane 2). Size of fragment was verified by comparison to 1 kb Plus DNA Ladder (lane 3). Kan^R, kanamycin resistant; $Apra^R$, apramycin resistant; *aac(3)IV*, apramycin resistance marker; *neo*, kanamycin resistance marker.

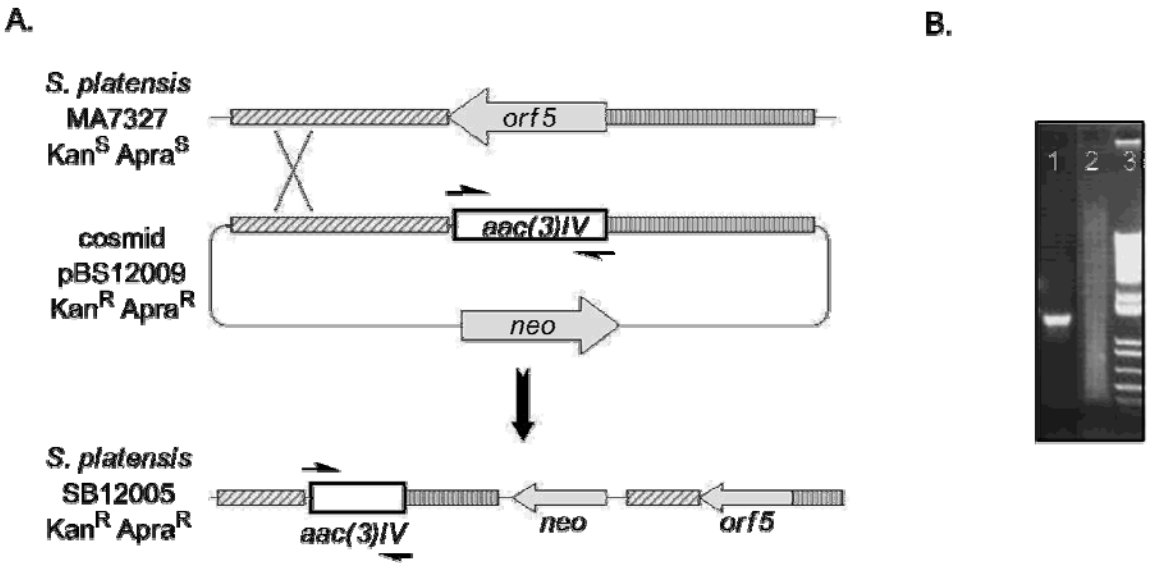


Figure S4. Genotype confirmation of *S. platensis* SB12006. Genetic map depicts *ptmB2* gene replacement to generate *S. platensis* SB12006, which is confirmed by Southern analysis. Kan^S, kanamycin sensitive; $Apra^S$, apramycin sensitive; Kan^R, kanamycin resistant; $Apra^R$, apramycin resistant; N, *Not*I cleavage site; *aac(3)IV*, apramycin resistance marker; *neo*, kanamycin resistance marker.

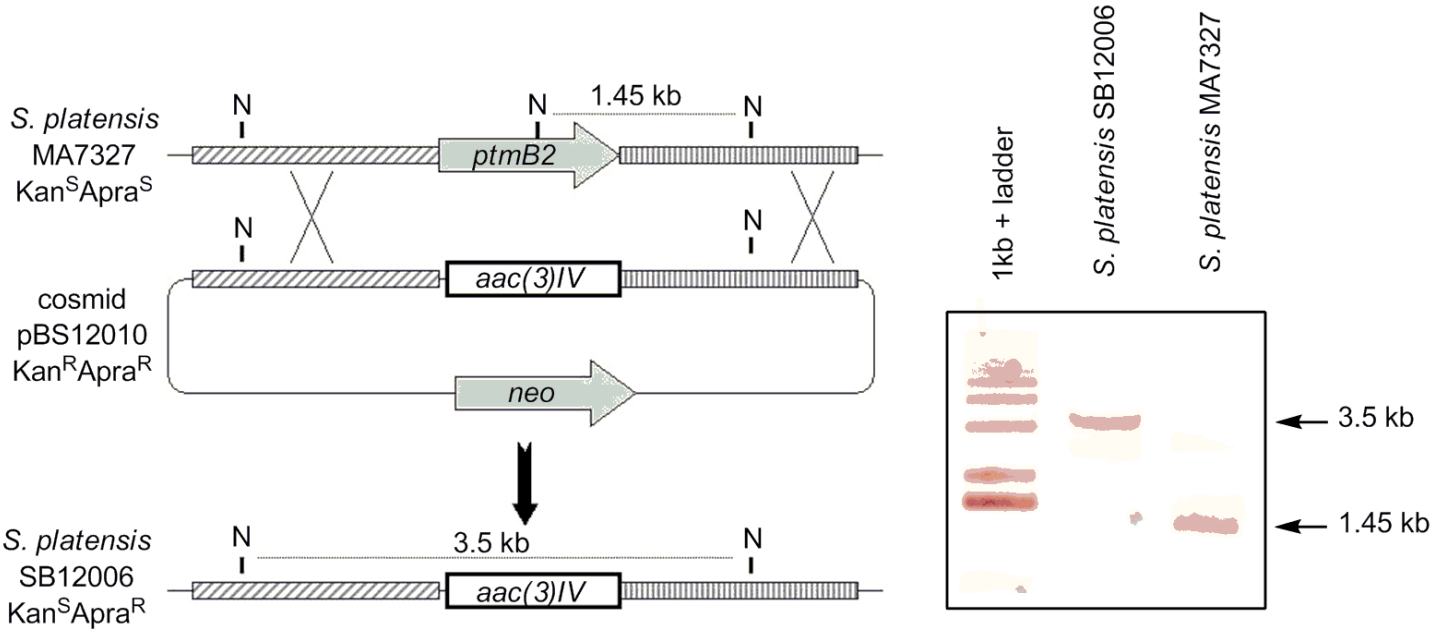


Figure S5. ^{1}H NMR of platensic acid in $CDCl_3$, isolated from *S. platensis* SB12006

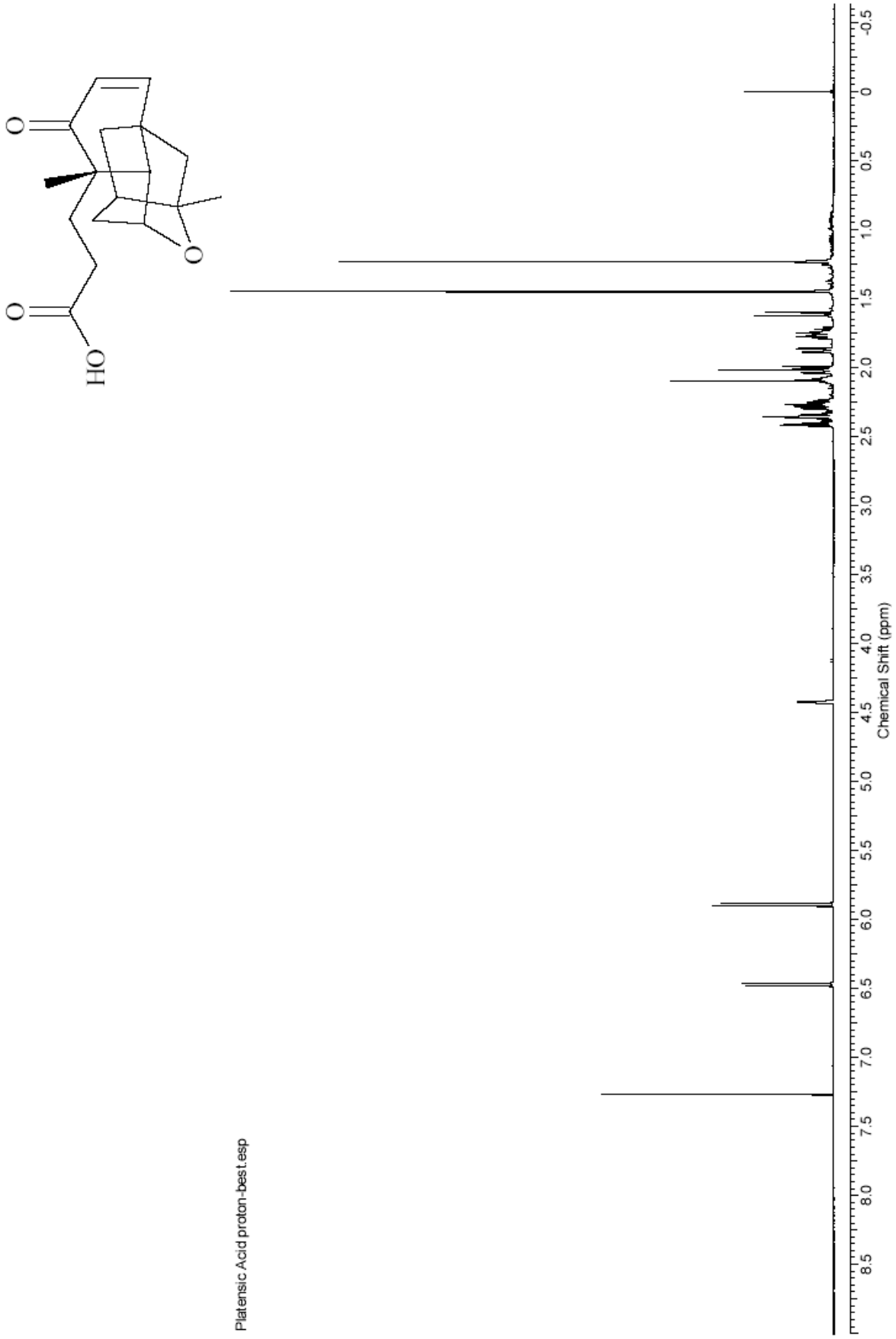


Figure S6. ^{13}C NMR of platensic acid in $CDCl_3$, isolated from *S. platensis* SB12006

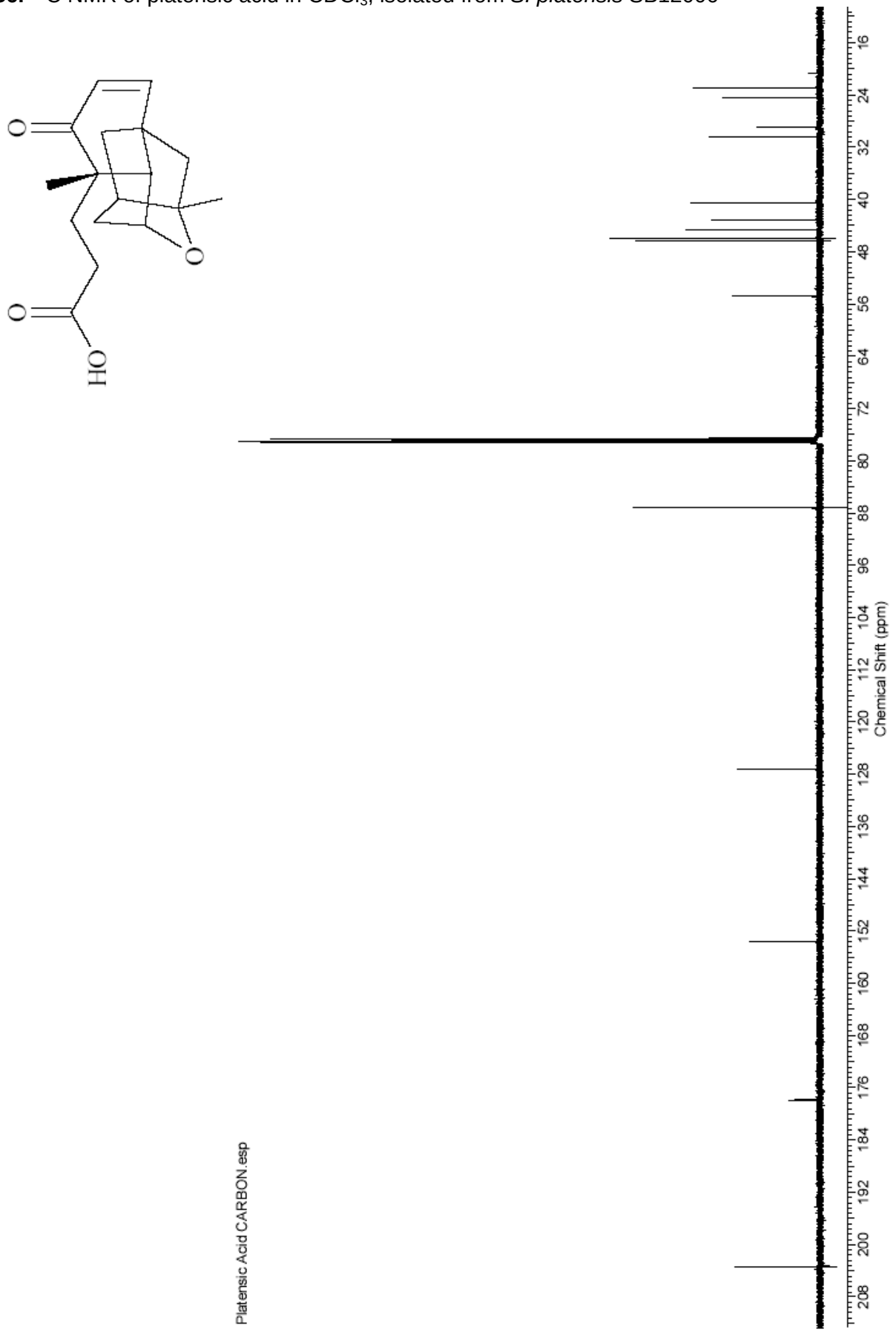


Figure S7. ^{1}H NMR of platencinic acid in $CDCl_3$, isolated from *S. platensis* SB12006.

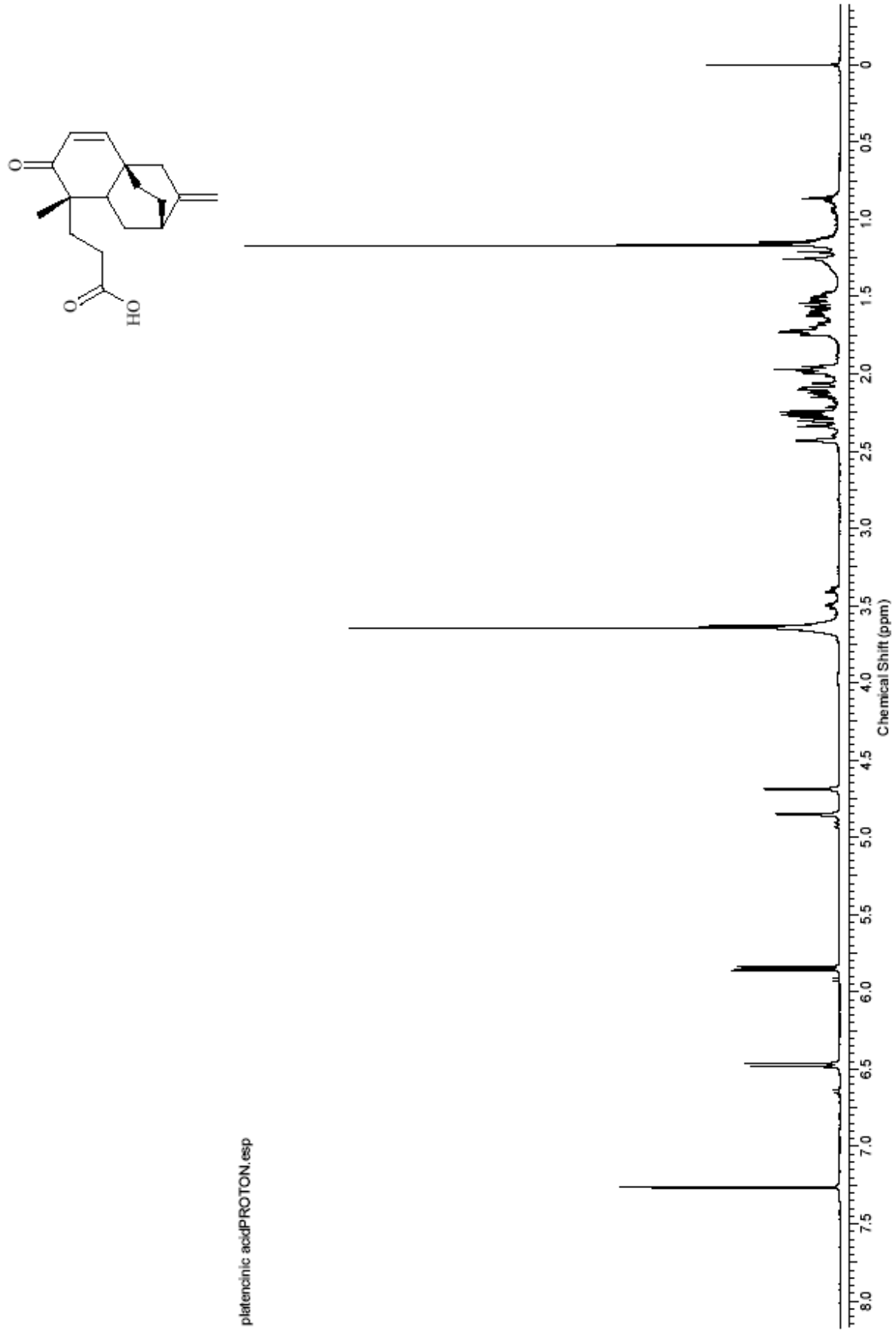


Figure S8. ^{13}C NMR of platencinic acid in $CDCl_3$, isolated from *S. platensis* SB12006

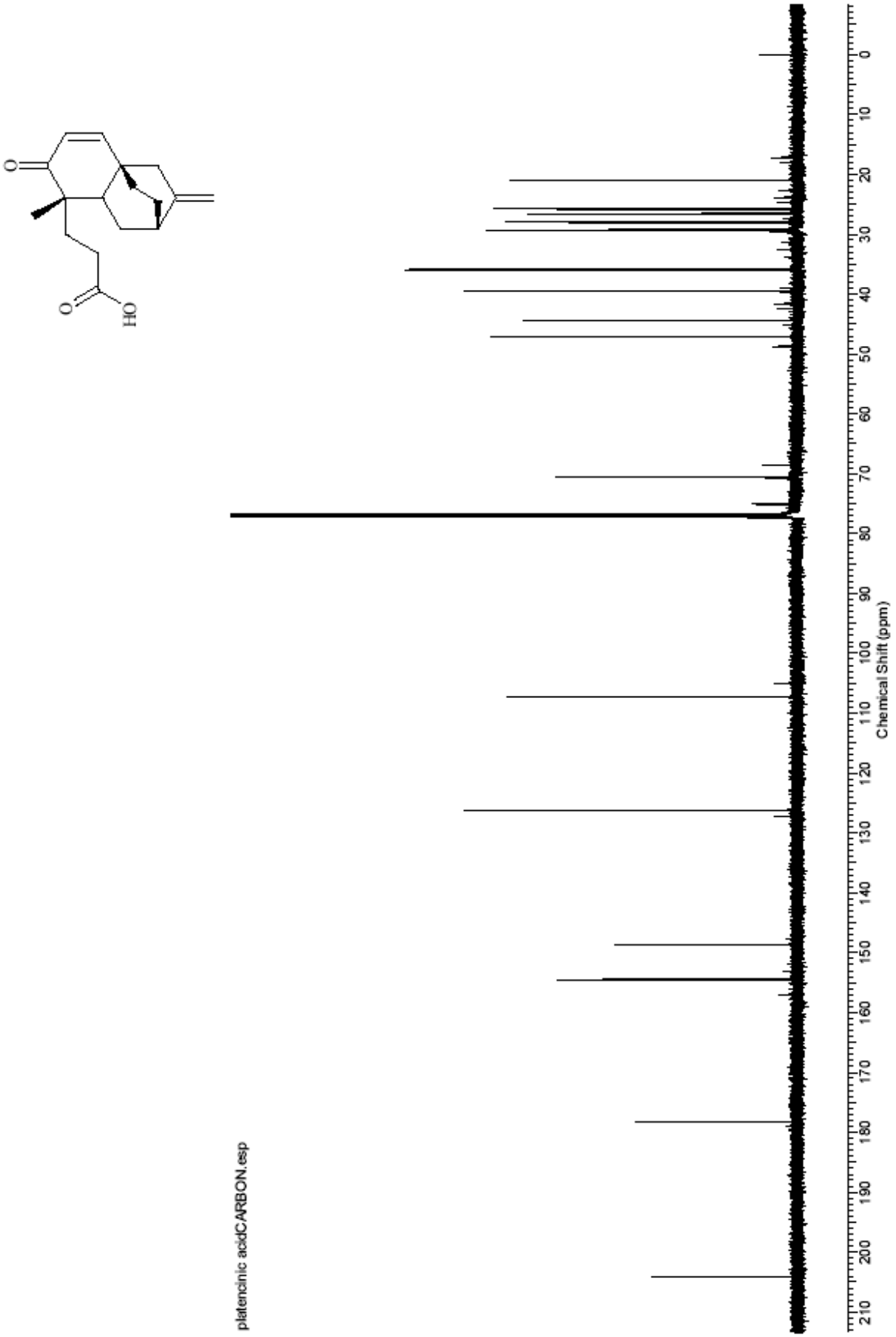


Figure S9. Genotype confirmations of *S. platensis* SB12007 and SB12008. Genetic maps depict *ptmT1* (A) and *ptmT3* (B) gene replacements to generate *S. platensis* SB12007 and SB12008, respectively, which are confirmed by Southern analysis. Kan^S, kanamycin sensitive; Apra^S, apramycin sensitive; Kan^R, kanamycin resistant; Apra^R, apramycin resistant; E, *Eco*R1 cleavage site; M, *Mlu*I cleavage site; *aac(3)IV*, apramycin resistance marker; *neo*, kanamycin resistance marker.

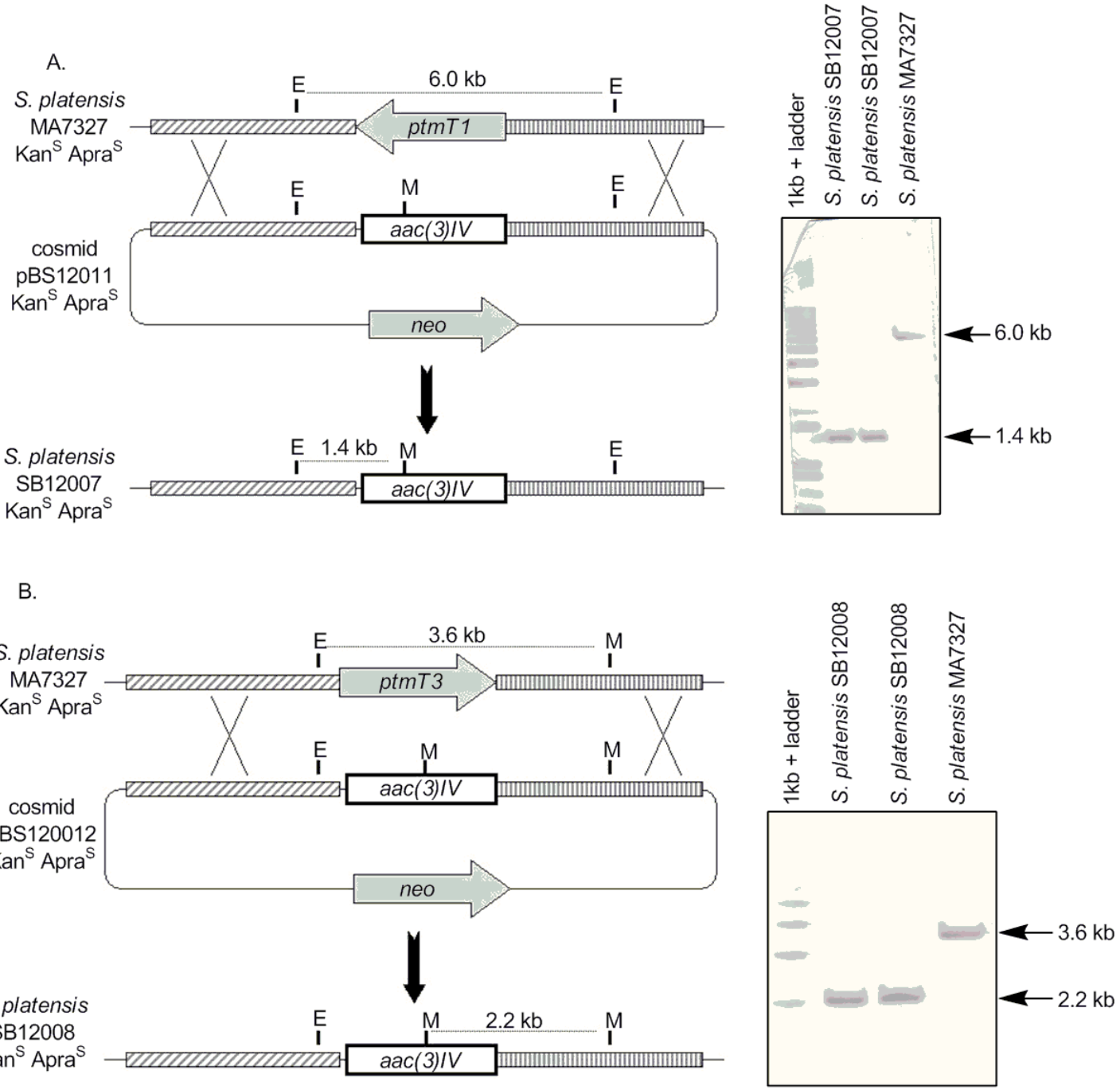


Figure S10. Possible routes of *ptm* and *ptn* gene cluster evolution. In one scenario (top to bottom), a gene duplication in an ancestor of the *ptm* gene cluster produced the nearly identical 1 kb regions (blue) flanking genes of the PTM cassette (green). Homologous recombination between these two nearly identical regions would result in excision of the PTM cassette to afford the *ptn* gene cluster. In the alternative scenario (bottom to top), the PTM cassette present on a plasmid could have been introduced to the *ptn* gene cluster via homologous recombination across *ptnO6* (blue), resulting in the *ptm* gene cluster.

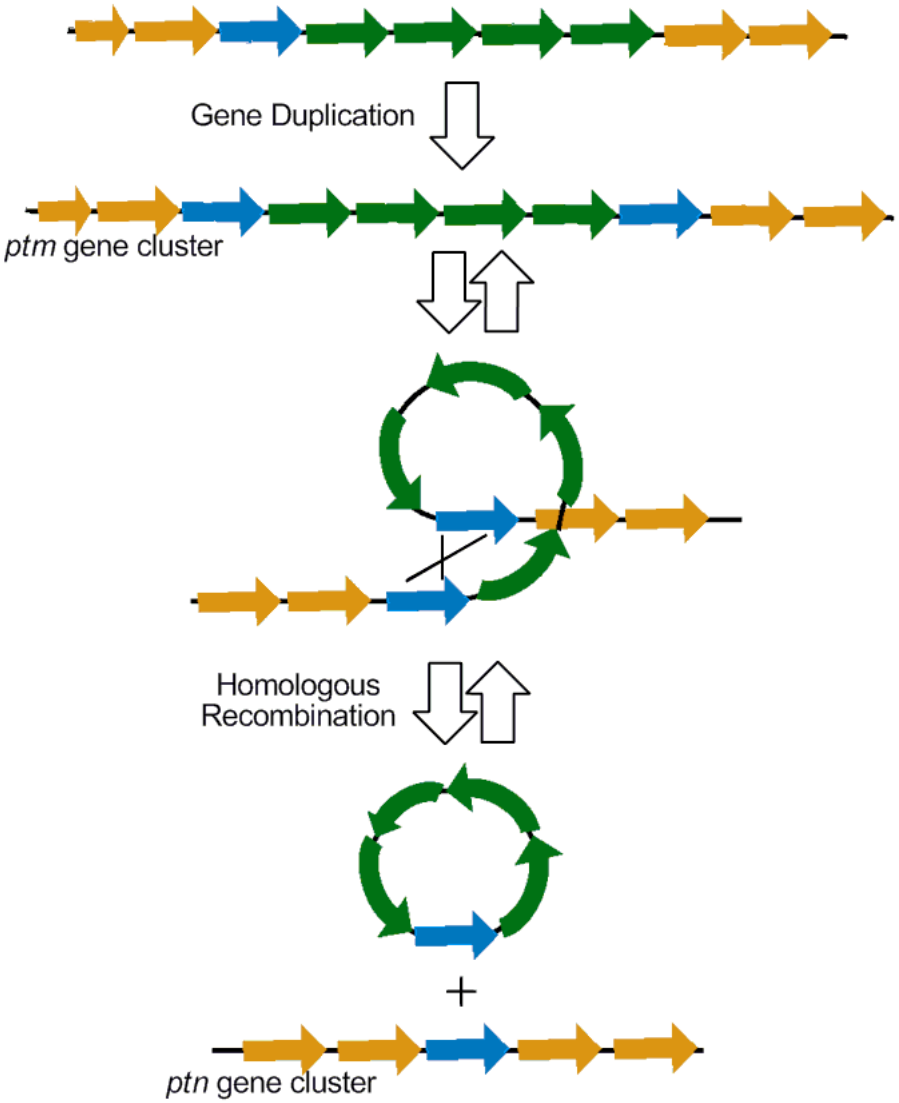


Figure S11. ^{1}H NMR of PTM isolated from *S. platensis* SB12604 in DMSO-d_6

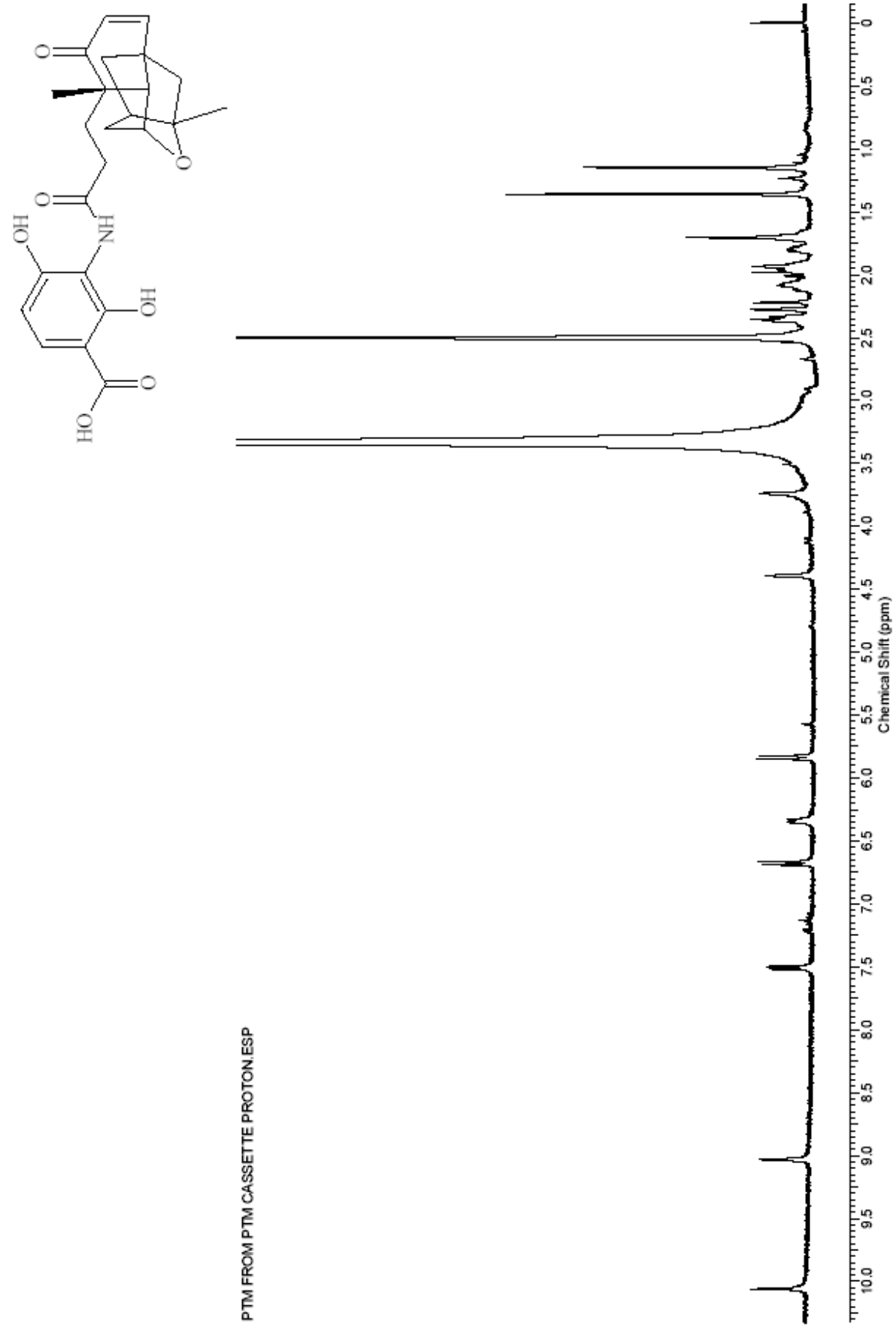


Figure S12. Partial alignment of 16S rDNA sequences of *S. platensis* MA7327 and MA7339. The sole differences between the partial (1410 bp) 16S rDNA sequences of MA7327 and MA7339 lie in a stem-loop of the variable γ-region typically used to distinguish between species in streptomycetes (*25*).

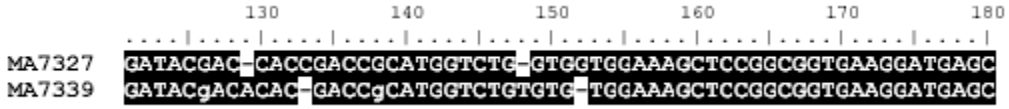


Figure S13. HPLC Analysis of SB12604-SB12606. HPLC chromatograms display compounds with UV absorbance at 240 nm from 12 to 21 minutes including (i) authentic PTN (diamonds), (ii) authentic PTM (circles), and crude extracts generated from (iii) wildtype *S. platensis* MA7339, (iv) heterologous production strain *S. lividans* SB12606, (v) SB12604, a derivative of MA7339 harboring the PTM cassette, and (vi) SB12505, a derivative of MA7339 harboring the empty vector pSET152.