Cooperative involvement of glycosyltransferases in the transfer of aminosugars in the biosynthesis of the macrolactam sipanmycin by *Streptomyces* sp. CS149

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Running Head: Glycosylation in sipanmycin biosynthesis

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Abstract

Macrolactams comprise a family of natural compounds with important bioactivities such as antibiotic, antifungal or antiproliferative. Sipanmycins A and B are two novel members of this family with two sugar moieties attached to the aglycon. In the related macrolactam vicenistatin, the sugar moiety has been proved essential for cytotoxicity. In this work, the gene cluster responsible for the biosynthesis of sipanmycins (*sip* cluster) in *Streptomyces* sp. CS149 is described and the steps involved in the glycosylation of the final compounds unraveled. Also, the cooperation of two different glycosyltransferases in each glycosylation step is demonstrated. Additionally, the essential role of SipO2 as an auxiliary protein in the incorporation of the second deoxysugar is addressed. In light of the results obtained by generation of mutant strains and *in silico* characterization of the *sip* cluster, a biosynthetic pathway for sipanmycins and the two deoxysugars attached is proposed. Finally, the importance of the hydroxyl group at C-10 of the macrolactam ring and the sugar moieties for cytotoxicity and antibiotic activity of sipanmycins is shown.

Importance

The rapid emergence of infectious diseases and multiresistant pathogens has risen the necessity for new bioactive compounds, thus novel strategies have to be developed to find them. Actinomycetes isolated in symbiosis with insects have attracted attention in recent years as producers of metabolites with important bioactivities. Sipanmycins are glycosylated macrolactams produced by *Streptomyces* sp. CS149, isolated from leaf-cutting ants, that show potent cytotoxic activity. Here, we characterize the *sip* cluster and propose a biosynthetic pathway for sipanmycins. As far as we know, it is the first time that the cooperation between two different glycosyltransferases is demonstrated to be strictly necessary for the incorporation of the same sugar. Also, a third protein with homology to P450 monooxygenases, SipO2, is

shown to be essential in the second glycosylation step forming a complex with the glycosyltransferase pair SipS9-SipS14.

Introduction

Searching for novel bioactive natural products is a great challenge for researchers in order to find novel antibiotics to combat emergent infectious diseases and multiresistant pathogens or novel chemotherapeutic drugs in the field of cancer. Microorganisms produce a large number of natural products, actinomycetes being responsible for the production of approximately two thirds of all natural bioactive products so far known (1). However, due to the low hit rate for novel compounds obtained using classical screening approaches and the frequent rediscovery of already known compounds (2) novel strategies must be developed. Some of these approaches involve the isolation of bioactive compound microbial producers from unexplored environments, paying special attention to microorganisms associated to marine or terrestrial macroorganisms such as sponges, plants, ants, termites or wasps (3-6), but also to mammals (7), including human beings (8). Furthermore, the development of DNA sequencing technologies and the increasing number of genomes sequenced facilitates the use of other strategies such as transcriptome analysis by RNA sequencing (9), mining of microbial genomes or metagenomes in searching specific gene homologs (10) or new biosynthetic gene clusters (BGCs) (11), and the activation of low expressed or silent clusters (12).

We have recently applied a targeted screening in the search for the presence of glycosylated bioactive natural products in a collection of *Streptomyces* strains isolated from leaf-cutting ants (13). By combining PCR-based screening, MS dereplication, and generation of mutants, we identified two novel macrolactams, sipanmycins A and B (Fig. 1). Macrolactams are a growing family of macrocyclic polyketides that have attracted attention since they show potent bioactivities such as antibacterial (rifamycin) (14), antifungal and antiviral (fluvirucins) (15), antiproliferative (vicenistatin and leinamycin) (16,17), and even to overcome the resistance to chemotherapeutic drugs in certain tumor cell lines caused by overexpression of the anti-apoptotic oncoprotein Bcl-xL (incednine) (18). Interestingly, many of the macrolactam family

members have at least one deoxysugar moiety attached to the macrocyclic ring, as it is the case of sipanmycins, vicenistatin, cremimycin, fluvirucin B2, incednine and silvalactam (Fig. 1). It has been demonstrated that this deoxysugar is essential for the bioactivity of vicenistatin (19). Thus, the study of the glycosylation steps during glycosylated macrolactam biosynthesis could be a great opportunity to obtain different derivatives with improved biological properties.

Sipanmycins A and B belong to the family of 24-membered macrolactams (Fig. 1) that include incednine (18) and silvalactam (20). The macrolactam rings of sipanmycin A and silvalactam are identical but they differ in the number of aminosugars attached. In contrast, sipanmycins and incednine possess very similar macrolactam rings only differing in the side chain substituent at C-2:an isobutyl chain or 2-methylbutyl (in sipanmycins A and B, respectively) and a methoxy group (in incednine). Both sipanmycins and incednine contains a disaccharide attached to the ring but with different aminosugars.

We report herein the identification of the gene cluster for sipanmycins biosynthesis (*sip* cluster) and the functional characterization of genes involved in deoxysugar biosynthesis. Furthermore, we prove the complex glycosylation steps during sipanmycins biosynthesis that include the unexpected cooperation of two different glycosyltransferases for the attachment of each deoxysugar, and the need for a P450-like helper protein in the incorporation of the second deoxysugar. We also describe the isolation and structural elucidation of several novel macrolactam derivatives and test their antibiotic activity and cytotoxicity against several tumor cell lines.

Results

The sipanmycins gene cluster: identification and characterization

For the identification of the *sip* cluster in strain CS149, the genome of this strain was sequenced and searched for the presence of putative BGCs using the bioinformatic tool "*antibiotics and Secondary Metabolite Analysis Shell*" (antiSMASH v.4) (21). Thirty-one putative BGCs were identified. They included three clusters containing NRPSs, three clusters containing PKSs (one type I and two type III), seven clusters comprising hybrid PKS-NRPS and five clusters coding for enzymes involved in the biosynthesis of terpenes. In addition, antiSMASH analysis revealed the presence of other putative BGCs related to the biosynthesis of two lantipeptides, two butyrolactones, two siderophores, one oligosaccharide, one lassopeptide, one bacteriocin, one thiopeptide, ectoine and melanine (Supplemental material, Table S1). Cluster 20, classified as belonging to the PKS type I-oligosaccharide class, appeared as a clear candidate to encode the *sip* cluster since it contained all genes coding the different enzymatic functions that are necessary for the biosynthesis of sipanmycins (Table 1). In addition, amino acid sequence analysis of this cluster showed similarity to proteins from a *Streptomyces* sp. ML694-90F31 cluster involved in incednine (*idn*) biosynthesis (22) (Table 1). Furthermore, three genes of the cluster involved in aminosugar biosynthesis (*sipS6*, *sipS7* and *sipS10*) contained DNA sequences that were previously used to generate sipanmycin nonproducing mutants in this strain (13). In addition, a cluster very similar to the *sip* cluster is also present in *Streptomyces* sp. Tü6075 with percentages of protein identity higher than 70% (Table 1). Comparison of the genetic organization between cluster 20 in CS149 and the *idn* cluster allowed defining the putative limits of the cluster. The *sip* cluster comprises approximately 93 kb and 36 open reading frames (ORFs) (Fig. 2; Table 1). Genes coding for structural enzymes in the *sip* cluster can be divided into four different groups depending on

which part of the final molecule they synthetize: (i) β-amino acid starter unit; (ii) polyketide chain; (iii) branched-chain extender unit; and (iv) deoxysugar moieties.

The chemical structure of sipanmycins suggests that their biosynthesis should start with the incorporation of a β-amino acid to the PKS assembly line. *In silico* analysis of the *sip* cluster showed the presence of seven genes (*sipL1-sipL7*) putatively involved in the biosynthesis of this starter unit, which are conserved in other BGCs involved in the biosynthesis of related macrolactams such as incednine, vicenistatin, ML-449, cremimycin and hitachimycin (22-26). Five genes coding for type I PKSs (*sipP1-sipP5*) were found within the *sip* cluster and their gene products showed high similarity to orthologs from *idn* cluster (ranging between 69 and 74%; Table 1). The fact that the chemical structure of the sipanmycin A aglycon is identical to the incednine one (except for the last unit introduced by the PKS), together with the same distribution of the ten modules within PKS genes in *sip* and *idn* clusters let us to assume the order of PKS reactions is the same as those proposed for incednine biosynthesis, starting at SipP1 and ending at SipP5 (Fig. 3). Comparison of the predicted substrate specificity of AT domains revealed that the only difference between them in sipanmycins and incednine is AT10 (Supplemental material, Fig. S1), which recognizes methoxymalonyl-ACP in incednine biosynthesis or, based on the chemical structure, isobutylmalonyl-CoA (ibMCoA) or 2-(2-methylbutyl)-malonyl-CoA (2-methylbMCoA) in sipanmycin A/B biosynthesis, respectively.

As mentioned above, the last unit introduced by module 10 of SipP5 may be ibMCoA or 2-methylbMCoA. Bioinformatic analysis of *sip* cluster revealed the presence of three genes putatively involved in the biosynthesis of this branched-chain extender unit, *sipIB1-sipIB3*, which showed a high degree of similarity to *divS, divT and divR* respectively, involved in the biosynthesis of divergolides and germicidins in the mangrove endophyte *Streptomyces* sp. HKI0576 (27). For clarity and brevity, aglyca from sipanmycin A and sipanmycin B will be referred as AgA and AgB, respectively.

153 The genes responsible for the biosynthesis of the two aminosugars attached to the sipanmycin
154 aglyca are grouped into two separate subclusters within the *sip* cluster. The first one consisted
155 of three genes (*sipS1*-*sipS3*) that might be involved in the biosynthesis of UDP-xylosamine
156 (Fig. 3).
157 The second subcluster (*sip6, sip7, sip8, sip10, sip11* and *sip13*) might be involved in the
158 biosynthesis of a *N*,*N*-dimethyl derivative of a monosaccharide which formally corresponds to
159 a 3,5 diepimer of lemonose (the aminosugar contained in lemonomycin) (28,29) and which we
160 propose to trivially name as D-sipanose (Fig. 3). For the transfer of the aminosugars to the
161 aglyca, four glycosyltransferase (GT) genes are present in the cluster (*sipS4, sipS9, sipS14* and
162 *sipS15)* that will be described below (Table 1; Fig. 2).
163 Two gene products, SipO1 and SipO2, showed similarity to cytochrome P-450
164 monooxygenases (Table 1). Thorough analysis of their sequences revealed that SipO1 might
165 act as a P450-monooxygenase catalyzing the C-10 hydroxylation of sipanmycin aglyca. In
166 contrast, SipO2 lacks the Cys residue at the active site responsible for the coordination of the
167 heme iron, thus it could not act as a monooxygenase (Supplemental material, Fig. S2). This
168 kind of enzymes have been described as GT helper proteins with a chaperone-like function.
169 These genes are normally located directly upstream of the corresponding GT gene, as is the
170 case of *sipO2*. Several examples of GT auxiliary protein/GT systems have been described,
171 including DesVIII/DesVII (methymycin/pikromycin) (30), TylM3/TylM2 (tylosin) (31),
172 EryCII/EryCIII (erythromycin) (32) and AknT/AknS (aclacinomycin) (33). Thus, we proposed
173 a similar role for SipO2 in sipanmycins biosynthesis (see below).
174 Apart from structural genes, two genes with high degree of similarity to transcriptional
175 regulators were found in the *sip* cluster (Table 1; Fig. 2). Sequence analysis showed that *sipR1*
176 would code for a LuxR-family transcriptional regulator (usually described as activators of
177 secondary metabolite pathways) and *sipR2* could code for a putative TetR-family

transcriptional repressor. *In trans* overexpression of *sipR1* (149esipR1 strain) enhanced sipanmycins production during the first three days of cultivation in R5A medium (Supplemental material, Fig. S3), supporting the role of SipR1 as activator. After day 4, sipanmycin accumulation decreased in the wild type and 149esipR1 strains, this reduction being faster in 149esipR1 probably due to the higher depletion of precursor supply in this culture.

Finally, two putative ABC transporters, SipT1 and SipT2, would participate in sipanmycins export and *sipB* may code for a type II thioesterase, often related to removal of aberrant extender units loaded onto PKSs (34) (Table 1; Fig. 2).

Involvement of four glycosyltransferases in the transfer of two aminosugars

As mentioned above, sipanmycins contain two aminosugars in their structures but four GT encoding genes are present in the cluster (*sipS4*, *sipS9*, *sipS14* and *sipS15*). To get further insight on the role and possible involvement of these GTs in aminosugar transfer, we individually inactivated the four genes by gene replacement through the insertion of the apramycin resistance cassette *aac(3)IV* within each gene. The resultant strains were grown on R5A liquid medium and ethyl acetate extracts of cultures of each mutant analyzed by UPLC. Inactivation of either *sipS4* or *sipS15* completely abolished biosynthesis of sipanmycins and instead two new peaks were detected by UPLC (Fig. 4). These peaks showed the characteristic absorption spectrum of sipanmycins and ions in their mass spectra at *m/z* = 492 $[M+H]^+$ for **1** and *m/z* = 506 $[M+H]^+$ for **2** that matched the expected masses for AgA and AgB, respectively. Accumulation of **1** and **2** was considerably higher in mutant *ΔsipS4* than in *ΔsipS15*. On the other hand, inactivation of *sipS14* also blocked production of sipanmycins A and B while inactivation of *sipS9* abolished production of sipanmycin B but trace amounts of sipanmycin A could be detected (Fig. 4). Both mutants showed the presence of two new peaks with the

characteristic absorption spectrum of sipanmycins and ions at *m/z* = 623 $[M+H]^+$ for **3** and *m/z* = 637 $[M+H]^+$ for **4**, both consistent with those expected for AgA and AgB with just the first aminosugar of sipanmycins (D-xylosamine) attached, respectively (referred for brevity as AgA+S1 and AgB+S1) (Fig. 5). Compound **3** is nearly identical to silvalactam (20) just lacking methylation at 3'-OH in the aminosugar unit and thus it can be named as 3'-*O*-demethylsilvalactam. All four mutants recovered the capability to produce sipanmycins A and B upon complementation with the corresponding native gene expressed under the control of *ermE*p* in an integrative plasmid (Supplemental material, Fig. S4), thus confirming that there were no polar effects upon Am^R cassette insertions. Major compounds (peaks **1** and **3)** were purified by preparative HPLC and their chemical structures confirmed by MS and NMR (Supplemental material: Figs. S5-S16, Table S2 (**1**) and Figs. S17-S24, Table S3 (**3**)).

The conclusion of these experiments is that the four GTs in the *sip* cluster are required for the transfer of the two aminosugars: two GTs, SipS15 and SipS4, working together in the transfer of D-xylosamine and the other two GTs, SipS9 and SipS14, in the transfer of D-sipanose.

Role of P450-like genes in sipanmycin biosynthesis

As mentioned above, two Sip proteins showed similarity to P450 monooxygenases but only one seemed to be a real monooxygenase. To investigate the possible role of both proteins, we inactivated *sipO1* and *sipO2* by gene replacement. Analysis of cultures of mutant *ΔsipO2* revealed similar results to those obtained by the inactivation of GTs *sipS9* and *sipS14*: accumulation of AgA+S1 and AgB+S1 (compounds **3** and **4**, respectively) (Fig. 6). These results suggest that SipO2 is an auxiliary protein of GTs SipS9 and SipS14 during the incorporation of the second aminosugar in sipanmycins biosynthesis. In cultures of mutant *ΔsipO1* we could not detect sipanmycins, but rather the accumulation of a new compound (**5**) with the characteristic UV absorption spectrum of sipanmycins and a mass of *m/z* = 778

228 $[M+H]^+$ that match the expected for sipanmycin A lacking the hydroxyl group at C-10 (10-
229 deoxysipanmycin A, dOHSipA; Fig. 6). This was confirmed after purification of the compound
230 and elucidation of its chemical structure by HRMS and NMR (Fig. 5; Supplemental material,
231 Figs. S25-S35, Table S4). Genetic complementation of each P450 monooxygenase coding
232 genes restored the wild-type phenotype in both mutant strains (Supplemental material, Fig. S4).
233
234 **Identification and role of aminotransferases and methyltransferases in aminosugar**
235 **biosynthesis**
236 Biosynthesis of the second aminosugar requires the involvement of two methyltransferases and
237 an aminotransferase. Blast analysis of the *sip* cluster pointed out to *sipS8* and *sipS5* as potential
238 candidates as *N*- and *C*-methyltransferase genes respectively, and *sipS13* as the
239 aminotransferase gene. To clarify their role, we generated replacement mutants in each of these
240 genes, analyzed the corresponding mutants and isolated and determined the structures of the
241 accumulated compounds. UPLC analysis of *ΔsipS5* mutant in comparison to the wild type
242 strain showed the absence of peaks corresponding to sipanmycins and the appearance of three
243 new peaks (Fig. 7). Compounds in these peaks showed the characteristic UV absorption
244 spectrum of sipanmycins and masses of *m/z* = 781 $[M+H]^+$ for **6**, *m/z* = 795 $[M+H]^+$ for **7** and
245 *m/z* = 623 $[M+H]^+$ for peak **3**. Structural elucidation of compounds **6** and **7** identified them as
246 3''-demethyl-sipanmycins A and B (dSipA and dSipB) respectively, both lacking the *C*-methyl
247 group at position C-3'' in the second aminosugar, which in this case corresponds to *N*,*N*-
248 dimethyl-D-pyrrolosamine, the enantiomer of the aminosugar found as L- form in the antitumor
249 antibiotics lomaiviticins A and B (35) (Fig. 5; Supplemental material: Figs. S36-S46, Table S5
250 (**6**) and Figs. S47-S56, Table S6 (**7**)). The third peak (compound **3**) was the smallest one and
251 correspond to AgA+S1, confirming the role of SipS5 in the biosynthesis of the second
252 aminosugar (Fig. 7).

Comparative profile analysis of *ΔsipS8* mutant *vs.* the wild type strain showed the accumulation of two already described compounds (**3** and **4**; Fig. 7), thus indicating that inactivation of this *N*-methyltransferase give rises to a sugar that it is not recognized and transferred by the joint action of SipS14, SipS9 and SipO2.

Analysis of ethyl acetate extracts from *ΔsipS13* mutant showed the presence of a main peak (compound **3**), and several small peaks absent in the wild-type strain that share absorption UV spectra with sipanmycins (Fig. 7). By comparison of retention time and mass (*m/z* = 637 $[M+H]^+$) one of them was identified as compound **4**, previously described in other mutants along this study. The other two peaks (labeled **8** and **8'** in Fig. 7) contained compounds that did not show clear pseudomolecular ions mass spectra, making impossible a structural proposal. After isolating compound **8**, it was observed that its analytical chromatographic re-analysis surprisingly rendered two peaks (**8** and **8'**) rather than the expected single peak. This immediately pointed out a probable interconversion of both compounds under the acidic chromatographic conditions. Analysis of HRMS and NMR data allowed to identify compound **8** (*m/z* = 765 $[M+H]^+$) as a sipanmycin A derivative with the dimethylated amino group at C-4'' of the second aminosugar replaced by a ketone, rendering the monosaccharide 4-keto-β-D-olivomycose. Based on the structure of **8** it could be proposed that compound **8'** (*m/z* = 783 $[M+H]^+$) was an artifact originated under acidic chromatographic conditions in which the ketone of compound **8** was hydrated rendering the monosaccharide 4-hydroxy-β-D-olivomycose. Compounds **8** is proposed to be named as 4''-deamino-4''-oxosipanmycin A (dNHSipA), and compound **8'** corresponded to its ketal derivative (Fig. 5; Supplemental material, Figs. S57-S68, Table S7). Taking into account the accumulation of **3** and the production phenotype of *ΔsipS13* mutant, it is probably that, in the absence of amination at C-4, recognition of the second aminosugar by GTs SipS14 and SipS9 would be much less efficient.

Mutants *ΔsipS8, ΔsipS5* and *ΔsipS13* recovered the sipanmycin production after genetic complementation (Supplemental material, Fig. S4).

In vitro cytotoxicity and antibiotic activity analysis of sipanmycins and derivatives

Sipanmycins exerted potent cytotoxicity against several tumor cell lines (Table 2), sipanmycin A being the one with strongest activity, showing IC_{50} values ranging from 0.095 to 0.796 µM, depending on the cell line tested. This family of compounds neither exhibited antifungal activity against *Candida albicans*, nor antibacterial activity against *Escherichia coli*. In contrast, they were strong antibiotics against Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus* (Supplemental material, Table S8). The conclusions are that the deoxysugars attached to the aglycon and the hydroxyl group at C-10 are essential for both the antiproliferative and antibiotic activity of this kind of compounds.

Discussion

It has been previously described that the deoxysugar moieties attached to the aglyca from several bioactive compounds play a major role in their activities, usually participating in the interaction between the compound and its cellular target. Thus, we focused our attention in those genes involved in aminosugar biosynthesis and its transfer to the sipanmycin aglyca.

The *sip* cluster contains four genes encoding GTs while only two aminosugars form part of the sipanmycins structure. There are reports in the literature of BGCs containing a lower number of GT genes with respect to the number of sugars attached to the aglycon (36-39), and it has been claimed that some GTs can act twice by incorporating the same sugar at different positions (38), incorporating different sugars (40,41) or acting cooperatively to achieve dual O- and C-glycosyltransferase activity (39). However, reports on BGCs containing more GT genes than sugars in the molecule are scarce, PM100117/PM100118 (42) and oleandomycin (43) are two

examples of them. In the PM100117/PM100118 BGC there is no clear role for a fourth GT in the biosynthesis pathway that otherwise involves the incorporation of three deoxysugars (44). In the case of oleandomycin, which contains two deoxysugars in its structure, the third GT acts as an inactivation mechanism (43). In addition to these examples, the BGC for incednine has also been reported to possess more GT genes than aminosugars in the molecule and. the authors proposed that two of the GT genes could be inactive since they lack internal protein sequences, which are conserved in active GTs, such as the essential histidine responsible for the deprotonation of the hydroxyl group of the glycosyl acceptor molecules (22). However, no experimental data were provided supporting the lack of functionality of these GTs. In contrast, for the incorporation of the two aminosugars into the sipanmycins aglycon, we have demonstrated that the participation of the four GTs is required. They work together in pairs (Sip4 and Sip15 for the transfer of the first aminosugar and Sip9 and Sip14 for the second one). Apparently, none of them can replace the activity of any of the others since independent inactivation of each of the four GT genes caused the disappearance of sipanmycins in culture broths (the exception is mutant *ΔsipS9* in which the presence of a small amount of sipanmycin A is still can be detected by LC/MS analysis).

Interestingly, the joint cooperation of Sip9 and Sip14 is not sufficient for the incorporation of the second aminosugar since there is a requirement for the presence of a P450 helper protein coded by *sipO2*. The *in vitro* activity of the GT EryCIII has been shown to be enhanced by the addition of its auxiliary P450-like EryCII (45) and it has been proposed that this auxiliary protein has the function to stabilize the fold and quaternary structure of its partner GT (32). Thus, the catalytic complex of GT-auxiliary cytochrome P450 adopted a tetrameric complex formed by two homodimers, one of the GT and the other of the auxiliary P450. In the case of the sipanmycin GTs pair SipS9/SipS14 in combination with auxiliary SipO2; this picture might be slightly different. Since both GTs are required for the attachment of the second aminosugar

and strictly require the assistance of SipO2, the structure of the complex might involve a GT heterodimer (SipS9/SipS14) in combination with the auxiliary enzyme. Interestingly, an important structural difference between these two GTs exists: SipS9 that share high similarity to IndS9, which was proposed by Takahishi et al (22) as inactive, is 99 amino acids shorter than SipS14. Structural elucidation and domain swapping experiments have shown that GTs contain two distinctive domains for substrate binding, an N-terminal domain recognizing the aglycon and a C-terminal domain involved in NDP-deoxysugar binding (46-48). The main differences between SipS9 and SipS14 are located at the N-terminal region where the aglycon recognition domain might reside. According to these differences, one member of the GT heterodimer (SipS14) could be in charge of recognizing the sipanmycins aglycon while the other member (SipS9), or both in cooperation, could be involved in aminosugar binding. Another fact that should be taken into account is that in this N-terminal region resides a putative motif involved in GT-auxiliary protein interaction (H-X-R-X_5-D-X_5-R-$X_{12–20}$-D-P-X_3-W-L-$X_{12–18}$-E-X_4-G) described for EryCIII and SpnP GTs (49). This motif is present in SipS14 but absent in SipS9 (Supplemental material, Fig. S68) indicating that auxiliary protein SipO2 could interact only with one of the partners of the GT pair giving to SipS9 a secondary role in this glycosylation step as indicated by the residual sipanmycin A production in *ΔsipS9* mutant (Fig. 4).

A similar scenario may occur in the first glycosylation step with the difference that GT pair SipS15/SipS4 is not dependent on the auxiliary protein SipO2 as xylosamine is attached to AgA and AgB in *ΔsipO2* mutant (**3** and **4**, respectively). In a similar way to the GT pair SipS9/SipS14, the N-terminal region of SipS15 is shorter than the SipS4 one, lacking the aglycon recognition domain and the motif involved in the auxiliary protein interaction (Supplemental material, Fig. S68). This last motif is present in SipS4 but with a lower level of conservation than in other GTs previously described, or even in SipS14, which could explain the independency from SipO2 of this GT pair.

Xylosamine, transferred from UDP-xylosamine, is the first aminosugar directly attached to the aglycon and present in both sipanmycins and incednine, but the second aminosugar is different. Three genes present in the *sip* cluster would be responsible for the aforementioned differences. The *sip5* gene would participate in the C-methylation step at C-3 of this monosaccharide as was confirmed by the isolation of dSipA and dSipB (**6** and **7**, respectively), both lacking this methyl group. Furthermore, sipanmycin A derivative with a ketone in C-4'' of the second deoxysugar, instead of a dimethylated amino group (**8**), has been isolated from cultures of the *ΔsipS13* mutant indicating the aminotransferase activity of SipS13. Incorporation of the unmethylated aminosugar (in **6** and **7**) or neutral deoxysugar (in **8**) shows a certain degree of flexibility of the complex SipS9/SipS14/SipO2. Such flexibility regarding the sugar donor has been reported for other GTs such as ElmGT (biosynthesis of elloramycin) (50,51) or UrdGT2 (biosynthesis of urdamycin) (52). In contrast, the lack of the dimethyl group at the amino group at C-4 restricts this flexibility since a mutant in which the *sip8 N*-dimethyltransferase gene was inactivated accumulates biosynthetic intermediates in which only the first aminosugar was incorporated (**3** and **4**).

Material and methods

Bacterial strains and culture conditions

Streptomyces sp. CS149, sipanmycins producer, has been previously reported (13). MA medium (53) was used for sporulation. For metabolite production, strains were added to flasks containing 30 mL TSB medium and grown at 30ºC and 250 rpm. After 24h, this seed culture was used to inoculate 50mL of R5A medium (36) to a final $O.D_{600nm}$ = 0.2. For intergeneric conjugation, MS medium (54) was used. *Escherichia coli* strains were grown in 2×TY medium supplemented with the appropriate antibiotic. All bacterial strains used in this work are listed in Table 3. Culture media were supplemented with antibiotics when needed: apramycin (100

µg/mL for *E. coli*, 25 µg/mL for *Streptomyces*), hygromycin (200 µg/mL), kanamycin (25 µg/mL), tetracycline (10 µg/mL), chloramphenicol (25 µg/mL), and/or nalidixic acid (50 µg/mL).

DNA manipulation and vectors

DNA manipulations were performed according to standard procedures for *E. coli* (63) and *Streptomyces* (64). All amplifications were carried out with the high-fidelity polymerase Herculase II Fusion (Agilent Technologies) and PCR conditions were: initial denaturation at 98°C, 2 min.; 30 cycles of 98°C, 30 s, 58°C, 30 s and 72°C, X s (1Kb / 30s) and a final extension at 72°C, 3 min. All plasmids used in this work are summarized in Table 3. pUKHyg was constructed by cloning the PstI-HindIII fragment containing the hygromycin resistance cassette from pLHyg (65) into pUK21 (60).

DNA sequencing and analysis

The *Streptomyces* sp. CS149 chromosome was sequenced at the Department of Biochemistry, University of Cambridge (Cambridge, UK) using Illumina MiSeq Sequencing technology. De novo assemblies were achieved using default parameters in Newbler assembler software version 2.9. Annotation was performed using the PGAAP pipeline (https://submit.ncbi.nlm.nih.gov/subs/wgs) (66). Database searching and sequence analysis were carried out with the bioinformatic tool antiSMASH (21).

Plasmid construction for gene replacement

The same steps were carried out to construct *sipO1* and *sipO2* gene replacement plasmids. Briefly, upstream and downstream flanking regions of the genes of interest were amplified by PCR using genomic DNA from CS149 as template and oligonucleotides indicated in Table 4.

These amplicons were gel purified and cloned into pUKHyg (using restriction enzyme sites added to the oligonucleotide sequences) at both sides of the hygromycin resistance gene. The resulting plasmids were digested with SpeI and the gene replacement cassette was cloned into compatible XbaI site of pHZ1358 resulting in the final conjugative plasmids used for knockout experiments. For the rest of the genes, the same strategy was applied but flanking regions were cloned into pUO9090 (instead of pUKHyg) at both sides of the apramycin resistance gene. Plasmids were introduced into their respective strains by intergeneric conjugation. Mutant strains were confirmed by PCR using oligonucleotides listed in Table 5.

Plasmid construction for genetic complementation of CS149 mutant strains

The complete ORF of each gene was amplified by PCR using oligonucleotides listed in Table 6, and the whole sequences were verified by sequencing. Amplicons were gel purified, digested with restriction enzymes indicated in Table 6 and cloned into pSETec (*sipO1* and *sipO2*) or pSETeTc (rest of genes), thus each gene was constitutively expressed under the control of *ermE** promoter (*ermE*p*).

Plasmid construction for *sipR1* overexpression

sipR1 was amplified by PCR using oligonucleotides 149-LuxR.5 (5´-TATGGATCCCGGGGGTAGGTGTACATGAG-3´) and 149-LuxR.3 (5´-TATGAATTCATCGGCTACGAAGTGCTCTG-3´). The resulting 3.042 pb fragment was gel purified, digested with BamHI/EcoRI and cloned into pSETec, leading to final plasmid pSET149eLuxR.

Extraction, analysis and isolation of metabolites by UPLC and HPLC-MS

Whole cultures (1 mL) of selected strains were extracted with one volume of ethyl acetate at three different times and analyzed by UPLC as described previously (13).

Compounds were isolated from two-liters cultures of CS149 mutant strains in R5A medium as previously described (13) but using a different mobile phase during semipreparative HPLC: MQ water + 0.05% TFA / ACN (ranging between 40 and 45% ACN depending on compound).

Structural elucidation of new compounds

Compounds **1**, **3**, **5**, **6**, **7**, **8** (and the artifact **8'**) were analyzed by LC-DAD-ESI-TOF to determine their UV-vis (DAD) spectra and their molecular formula based on the experimental accurate masses and the corresponding isotopic distribution. HRMS based dereplication against our in-house library (67) and the Dictionary of Natural Products Version 26:2 (68) was carried out to ensure their novelty. The structural elucidation of these compounds was carried out by detailed analysis of 1D and 2D NMR spectra further assisted by comparison with the spectroscopic data reported for incednine (18), silvalactam (20), and specially sipanmycins A and B (13) Relative configurations were determined by coupling constants and NOE analyses, assisted by comparison with the NMR data of sipanmycins A and B (13).

LC-DAD-ESI-TOF and NMR analyses

HRMS and UV-vis spectra spectra were obtained by LC-DAD-ESI-TOF analyses performed using an Agilent 1200RR HPLC equipped with a SB-C8 column (2.1 × 30 mm, Zorbax) coupled to a Bruker maXis Spectrometer. Chromatographic and ionization conditions were identical to those previously employed for sipanmycins A and B (13).

NMR spectra were recorded in CD_3OD at 24 $^{\circ}C$ on a Bruker AVANCE III-500 MHz (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI MicroCryoProbe™, using the residual solvent signal as internal reference.

In vitro cytotoxicity and antibiotic activity assays

Cytotoxic activity of compounds was tested against the following human tumor cell lines: colon adenocarcinoma (HT29), non-small cell lung cancer (A549), breast adenocarcinoma (MDA-MB-231), promyelocytic leukemia (HL-60) and pancreatic cancer (CAPAN-1). Mouse embryonic fibroblast cell line NIH/3T3 was used as control to evaluate cytotoxicity against non-malignant cells. Cells were previously grown for a week on DMEM-10%FBS medium, then aliquoted to 5000 cells per well in 96-well plates using the Cell counting kit-8-(96992) (Sigma-Aldrich) and grown for an extra 24 h. Compounds were dissolved in DMEM medium. After the incubation, 10 μL of compound (in diverse concentrations) were added to each well and incubated for another 48 h. Lastly, 10 μL of CCK-8 reagent (Sigma-Aldrich) were added, left to develop for 2 h in the incubator, and measured at 450 nm using an Elisa Bio-tek ELx 800 (BioTek).

Antibiotic activity tests (minimal inhibitory concentration, MIC) were performed in 96-wells microtiter plates. Fresh cultures of each microorganism were used as seed cultures to inoculate the plates, with the appropriate compound concentration, to a final OD=0.1 and total volume of 150μl per well. Plates were incubated overnight at 37ºC (30ºC for *C. albicans*).

Accession numbers

The Whole Genome Shotgun project of *Streptomyces* sp. CS149 has been deposited at DDBJ/ENA/GenBank under the accession PVZY00000000. The version described in this paper is version PVZY01000000.

The nucleotide sequence of the *sip* cluster was deposited in the European Nucleotide Archive (accession number: LT986736 [http://www.ebi.ac.uk/ena/data/view/LT986736]) and at

475 Minimum Information about a Biosynthetic Gene Cluster (MIBiG) repository (69) under the
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484 **SUPPLEMENTAL MATERIAL**
485 Supplemental material for this article may be found at Figshare through the link
486 https://figshare.com/s/49de24d11120b7b114ba
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Figure legends

Figure 1. Glycosylated macrolactams.

Figure 2. Sipanmycin biosynthesis gene cluster.

Figure 3. Proposed biosynthesis pathway for sipanmycins.

Figure 4. Effect of glycosyltransferase knockout experiments on sipanmycin biosynthesis. UPLC analysis of ethyl acetate extracts of mutants in the four GTs involved in the attachment of the two deoxysugars to the sipanmycin aglycon. **1**: AgA; **2**: AgB; **3**: AgA+S1 and **4**: AgB+S1.

Figure 5. Effect of P450 cytochrome knockout experiments on sipanmycin biosynthesis. UPLC analysis of ethyl acetate extracts of mutants in *sipO2* and *sipO1* genes. **3**: AgA+S1; **4**: AgB+S1 and **5**: dOHSipA.

Figure 6. Effect of methyltransferases and aminotransferase knockout experiments on sipanmycin production. Chromatograms corresponding to mutants in *sipS5*, *sipS8* and *sipS13* genes. **3**: AgA+S1; **4**: AgB+S1; **6**: dSipA; **7**: dSipB; **8**: dNHSipA and **8'**: dNHSipA ketal derivative.

765 **Tables**

766 **Table 1**. Sipanmycin biosynthesis gene cluster in *Streptomyces* sp. CS149 [a].

Protein	Proposed function	*Streptomyces* sp. ML694-90F3 (% identity/similarity)	*Streptomyces* sp. Tü 6075 (% identity/similarity)
SipR1	Transcriptional regulator (LuxR family)	IdnR1 54 / 67	WP_075268275 97 / 98
SipS3	Nucleoside-diphosphate sugar epimerase	IdnS3 76 / 84	WP_075263857 99 / 99
SipS2	UDP-glucose 6-dehydrogenase	IdnS2 72 / 80	WP_075263856 99 / 99
SipS1	N-acetylglucosaminyl deacetylase	IdnS1 65 / 78	WP_075263855 99 / 99
SipP5	Type I PKS	IdnP5 64 / 74	WP_075263854 98 / 98
SipIB3	Crotonyl-CoA carboxylase/reductase	-	WP_075263853 99 / 99
SipIB1	3-oxoacyl-ACP synthase	-	WP_075263852 99 / 99
SipIB2	3-hydroxybutyryl-CoA dehydrogenase	-	WP_075268274 99 / 98
SipS15	Glycosyltransferase	IdnS15 40 / 50	WP_075263851 96 / 97
SipP4	Type I PKS	IdnP4 62 / 70	WP_075263850 97 / 97
SipP3	Type I PKS	IdnP3 65 / 73	WP_075263849 95 / 96
SipR2	Transcriptional regulator (TetR family)	IdnR2 58 / 73	WP_075263848 99 / 100
SipT2	ABC transporter	IdnT2 73 / 83	WP_075263847 99 / 99
SipT1	ABC transporter	IdnT1 68 / 83	-
SipB	Thioesterase	IdnB 58 / 72	WP_075263846 98 / 98
SipP2	Type I PKS	IdnP2 64 / 72	WP_079181603 70 / 75
SipP1	Type I PKS	IdnP1 60 / 69	WP_079181603 89 / 90
SipO1	Cytochrome P450	IdnO1 82 / 89	WP_075263845 99 / 99
SipL7	AMP-dependent synthetase	IdnL7 64 / 73	WP_075263844 94 / 95
SipL6	Acyl carrier protein	IdnL6 58 / 70	WP_075263843 96 / 97
SipL1	AMP-dependent synthetase	IdnL1 70 / 81	WP_075263842 98 / 98
SipL2	Malonyl-CoA-ACP transacylase	IdnL2 69 / 77	WP_079181433 94 / 94
SipL3	Aminotransferase	IdnL3 74 / 80	WP_075268271 98 / 98
SipL4	Lysine 2,3-aminomutase	IdnL4 75 / 86	WP_075263841 99 / 99
SipL5	L-proline amide hydrolase	IdnL5 75 / 84	WP_075268270 99 / 99
SipS4	Glycosyltransferase	IdnS4 77 / 83	WP_079181602 99 / 99
SipS7	Glucose-1-phosphate thymidylyltransferase	IdnS7 63 / 77	WP_075263839 99 / 99
SipS6	dTDP-glucose 4,6-dehydratase	IdnS6 66 / 74	WP_075263838 97 / 98
SipS10	NDP-hexose 2,3-dehydratase	IdnS10 51 / 61	WP_075263837 98 / 98
SipS8	N-methyltransferase	IdnS8 58 / 71	-
SipS9	Glycosyltransferase	IdnS9 47 / 57	WP_075263836 98 / 98
SipS5	C-methyltransferase	-	WP_075263835 99 / 99
SipO2	P450-derived glycosyltransferase activator	IdnO2 47 /57	WP_075263834 95 / 95
SipS14	Glycosyltransferase	IdnS14 60 / 76	WP_075263833 99 / 99
SipS11	NDP-hexose-3-ketoreductase	IdnS11 68 / 75	WP_079181432 96 / 98
SipS13	dTDP-4-dehydro-6-deoxyglucose aminotransferase	IdnS13 74 / 83	WP_075263832 99 / 98

767 [a] Comparison against proteins encoded in the incednine (*Streptomyces* sp. ML694-90F31) biosynthesis gene cluster. In addition,
768 comparison against proteins in *Streptomyces* sp. Tü 6075 is shown.

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770 **Table 2.** *In vitro* cytotoxicity of sipanmycins and derivatives (IC_{50} , µM).

	3T3 (fibroblasts)	A549 (lung)	HT29 (colon)	HL60 (leukemia)	CAPAN-1 (pancreas)	MDAMB231 (breast)
Sipanmycin A	0.302	0.117	0.189	0.796	0.178	0.095
Sipanmycin B	0.411	0.118	0.264	1.52	0.747	0.188
AgA (1)	>>10	>>10	>>10	2.549	>>10	>>10
AgB (2)	>>10	>10	>10	0.698	>>10	>10
AgA+S1 (3)	0.91	0.647	0.431	1.16	2.17	0.827
dOHSipA (5)	1.614	1.75	0.873	1.089	2.68	1.2
dSipA (6)	0.163	0.147	0.147	0.468	0.181	0.085
dSipB (7)	0.293	0.211	0.213	1.129	0.493	0.241

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773 **Table 3.** Strains and plasmids used in this work.

Strain	Genotype / characteristics	Use	Reference
Escherichia coli			
DH5α	*fhuA2, Δ(argF-lacZ)U169, phoA, glnV44, Θ80, Δ(lacZ)M15, gyrA96, recA1, relA1, endA1, thi-1, hsdR17*	Cloning	55
ET12567/pUB307	*dam13::Tn9, dcm6, hsdM, hsdR, recF143, zjj201::Tn10, galK2, galT22, ara14, lacY1, xyl5, leuB6, thi1, tonA31, rpsL136, hisG4, tsx78, mtli, glnV44, F-* , carries plasmid pUB307	Intergeneric conjugation	56, 57
Streptomyces			
CS149	wild-type		1
ΔsipS15	*ΔsipS15:aac(3)IV*		This work
ΔsipS4	*ΔsipS4:aac(3)IV*		This work
ΔsipS9	*ΔsipS9:aac(3)IV*		This work
ΔsipS14	*ΔsipS14:aac(3)IV*		This work
ΔsipS8	*ΔsipS8:aac(3)IV*		This work
ΔsipS5	*ΔsipS5:aac(3)IV*		This work
ΔsipS13	*ΔsipS13:aac(3)IV*		This work
ΔsipO1	*ΔsipO1:hyg*		This work
ΔsipO2	*ΔsipO2:hyg*		This work
149esipR1	*ermE*p-sipR1*		This work
Plasmids			
pSETec	integrating vector, *aac(3)IV*, $oriT_{RK2}$, *intΦC31*, *attPΦC31*, *ermE*p*	gene overexpression	58
pSETeTc	integrating vector, *aac(3)IV*, *tsr*, $oriT_{RK2}$, *intΦC31*, *attPΦC31*, *ermE*p*	gene overexpression	58
pLHyg	*lacZ, bla, hyg*	source of *hyg* gene	59
pUK21	*lacZ, km*	backbone for pUKHyg construction	60
pUKHyg	*lacZ, km, hyg*	replacement cassette construction	This work
pUO9090	*aac(3)IV, km*	replacement cassette construction	61
pHZ1358	*bla, tsr,*$oriT_{RK2}$	knockout mutant generation	62
pUO149ΔsipS15	carries up- and downstream flanking regions of *sipS15* at both sides of *aac(3)IV, km*	source of *sipS15* replacement cassette	This work
pUH149ΔsipS15	carries *sipS15* replacement cassette, *aac(3)IV, bla, tsr,*$oriT_{RK2}$	*sipS15* replacement	This work
pSETT149esipS15	carries *sipS15* under the control of *ermE*p*, *aac(3)IV, tsr,* $oriT_{RK2}$, *intΦC31, attPΦC31*	*sipS15* complementation	This work
pUO149ΔsipS4	carries up- and downstream flanking regions of *sipS4* at both sides of *aac(3)IV, km*	source of *sipS4* replacement cassette	This work
pUH149ΔsipS4	carries *sipS4* replacement cassette, *aac(3)IV, bla, tsr,*$oriT_{RK2}$	*sipS4* replacement	This work

pSETT149esipS4	carries *sipS4* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS4* complementation	This work
pUO149ΔsipS9	carries up- and downstream flanking regions of *sipS9* at both sides of *aac(3)IV, km*	source of *sipS9* replacement cassette	This work
pUH149ΔsipS9	carries *sipS9* replacement cassette, *aac(3)IV, bla, tsr,oriT$_{RK2}$*	*sipS9* replacement	This work
pSETT149esipS9	carries *sipS9* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS9* complementation	This work
pUO149ΔsipS14	carries up- and downstream flanking regions of *sipS14* at both sides of *aac(3)IV, km*	source of *sipS14* replacement cassette	This work
pUH149ΔsipS14	carries *sipS14* replacement cassette, *aac(3)IV, bla, tsr,oriT$_{RK2}$*	*sipS14* replacement	This work
pSETT149esipS14	carries *sipS14* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS14* complementation	This work
pUO149ΔsipS8	carries up- and downstream flanking regions of *sipS8* at both sides of *aac(3)IV, km*	source of *sipS8* replacement cassette	This work
pUH149ΔsipS8	carries *sipS8* replacement cassette, *aac(3)IV, bla, tsr,oriT$_{RK2}$*	*sipS8* replacement	This work
pSETT149esipS8	carries *sipS8* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS8* complementation	This work
pUO149ΔsipS5	carries up- and downstream flanking regions of *sipS5* at both sides of *aac(3)IV, km*	source of *sipS5* replacement cassette	This work
pUH149ΔsipS5	carries *sipS5* replacement cassette, *aac(3)IV, bla, tsr,oriT$_{RK2}$*	*sipS5* replacement	This work
pSETT149esipS5	carries *sipS5* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS5* complementation	This work
pUO149ΔsipS13	carries up- and downstream flanking regions of *sipS13* at both sides of *aac(3)IV, km*	source of *sipS13* replacement cassette	This work
pUH149ΔsipS13	carries *sipS13* replacement cassette, *aac(3)IV, bla, tsr,oriT$_{RK2}$*	*sipS13* replacement	This work
pSETT149esipS13	carries *sipS13* under the control of *ermE*p*, *aac(3)IV, tsr, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipS13* complementation	This work
pUK149ΔsipO1	carries up- and downstream flanking regions of *sipO1* at both sides of *hyg, km*	source of *sipO1* replacement cassette	This work
pHH149ΔsipO1	carries *sipO1* replacement cassette, *hyg, bla, tsr,oriT$_{RK2}$*	*sipO1* replacement	This work
pSET149esipO1	carries *sipO1* under the control of *ermE*p*, *aac(3)IV, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipO1* complementation	This work
pUK149ΔsipO2	carries up- and downstream flanking regions of *sipO2* at both sides of *hyg, km*	source of *sipO2* replacement cassette	This work
pHH149ΔsipO2	carries *sipO2* replacement cassette, *hyg, bla, tsr,oriT$_{RK2}$*	*sipO2* replacement	This work
pSET149esipO2	carries *sipO2* under the control of *ermE*p*, *aac(3)IV, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipO2* complementation	This work
pSET149eLuxR	carries *sipR1* under the control of *ermE*p*, *aac(3)IV, oriT$_{RK2}$, intΦC31, attPΦC31*	*sipR1* overexpression	This work

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777 **Table 4.** Oligonucleotides used for gene replacement in *Streptomyces* sp. CS149.

Name	Sequence (5´--- 3´)	Restriction site	Use
149dGT47.5F	TATGAATTCCGAACTCGTCCTGCTCGT	EcoRI	*sipS15* replacement (5' flanking region) (1.067 pb)
149dGT47.5R	TATAAGCTTACCAGGCTGTGCAGTCGT	HindIII	
149dGT47.3F	TATGGATCCGGAACTGCGGGAGGAGAT	BamHI	*sipS15* replacement (3' flanking region) (1.069 pb)
149dGT47.3R	TATTCTAGACGCTTGATGACCTTCACCTG	XbaI	
149dGT66.5F	TATGAATTCTGAGCTCGGACAGCACATAG	EcoRI	*sipS4* replacement (5' flanking region) (1.357 pb)
149dGT66.5R	TATAAGCTTCGGAAACGACATCAACATGA	HindIII	
149dGT66.3F	TATCATATGCGATCTCGTCCCCGAACT	NdeI	*sipS4* replacement (3' flanking region) (1.124 pb)
149dGT66.3R	TATTCTAGAGATCTGGCTGGTGTCGATG	XbaI	
149dGT71.5F	TATGAATTCCCACCACGTCAACGTCCAG	EcoRI	*sipS9* replacement (5' flanking region) (1.101 pb)
149dGT71.5R	TATAAGCTTGAGGACGGCACGGTCAGC	HindIII	
149dGT71.3F	TATCATATGGAGGTCGTGCCGCTGCTG	NdeI	*sipS9* replacement (3' flanking region) (1.127 pb)
149dGT71.3R	TATTCTAGACGATCTGTCGCTCCTGTACG	XbaI	
149dGT74.5F	TATGAATTCATGCGAGGGAATAAGTGACG	EcoRI	*sipS14* replacement (5' flanking region) (1.408 pb)
149dGT74.5R	TATAAGCTTGCGAAGACCGTGAACAGAAC	HindIII	
149dGT74.3F	TATGGATCCGTGACCAAGGACAGCGAGAG	BamHI	*sipS14* replacement (3' flanking region) (1.118 pb)
149dGT74.3R	TATTCTAGACCTGTCTGATCCGTTCCAGT	XbaI	
149-DORF70.5F	TATGAATTCGAACATCCCGATCGTCTCC	EcoRI	*sipS8* replacement (5' flanking region) (1.420 pb)
149-DORF70.5R	TATAAGCTTTCTGCTCATTCTCGATGACG	HindIII	
149-DORF70.3F	TATCATATGGCGTCTGTGACCGGAGCA	NdeI	*sipS8* replacement (3' flanking region) (1.377 pb)
149-DORF70.3R	TATTCTAGAGTGATGCGCACCGAGGAC	XbaI	
149-DORF72.5F	TATGAATTCTCCCAACCGGTTTCTCAA	EcoRI	*sipS5* replacement (5' flanking region) (1.527 pb)
149-DORF72.5R	TATAAGCTTCTCGGCATGCAGAAATGG	HindIII	
149-DORF72.3F	TATCATATGAGGCTGTCCGAGGCGTAGT	NdeI	*sipS5* replacement (3' flanking region) (1.369 pb)
149-DORF72.3R	TATTCTAGAGCGATCAGGTCGTGAATTTG	XbaI	
149dAT76.5F	TATGAATTCAGTTGCAGCAGGAGATCCAC	EcoRI	*sipS13* replacement (5' flanking region) (1.125 pb)
149dAT76.5R	TATAAGCTTCGAGGATGGCGAGTTCAG	HindIII	
149dAT76.3F	TATGGATCCGGAGTGACGCTCGACCAG	BamHI	*sipS13* replacement (3' flanking region) (1.419 pb)
149dAT76.3R	TATTCTAGATTCAACTCGATGCACGACAC	XbaI	
149dP450_58.5F	TATTCTAGATCGAAGTTCCGCATGCTCAT	XbaI	*sipO1* replacement (5' flanking region) (2.028 pb)
149dP450_58.5R	TATGGATCCCCTCCATGTCGAACGGGTAG	BamHI	
149dP450_58.3F	TATAAGCTTATTCAGTGGAAGTTCGGCGT	HindII	*sipO1* replacement (3' flanking region) (4.050 pb)
149dP450_58.3R	TATAGATCTGGTATGGGGCGTGGTTTGTA	BglII	
149dP450_73.5F	TATTCTAGAGTCCGGTTCCTTCCCTACG	XbaI	*sipO2* replacement (5' flanking region) (2.022 pb)
149dP450_73.5R	TATCATATGAGCCTCGGGTCGATCTGATA	NdeI	
149dP450_73.3F	TATAAGCTTGCTGCTTGAGAAACCGGTTG	HindIII	*sipO2* replacement (3' flanking region) (2.203 pb)
149dP450_73.3R	TATAGATCTCGATGATCTCTCGACGCTGA	BglII	

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781 **Table 5.** Oligonucleotides used for PCR confirmation of *Streptomyces* sp. CS149 mutants.

Name	Sequence (5´--- 3´)	Gene	Amplicon size in wild-type strain (bp)	Amplicon size in mutant strain (bp)
comp149dGT47.5	TACGCGGAGTTCAAGGAACC	*sipS15*	1.223	1.739
comp149dGT47.3b	CCGTTCACGAGAGGTCTGTG			
comp149dGT66.5	CAGGGGTCGGACGGTATTTC	*sipS4*	1.297	2.004
comp149dGT66.3	AGCGGTTCCTCCTGGTAGAT			
comp149dGT71.5	TCTTCCGGCGTTGTACTGTC	*sipS9*	1.262	1.769
comp149dGT71.3	GAACATCCCGATCGTCTCCG			
comp149dGT74.5	GCTGCTTGAGAAACCGGTTG	*sipS14*	1.474	1.684
comp149dGT74.3	GGTCCGCCTTCTCCTTGTC			
comp149dMT70.5	GACAGTACAACGCCGGAAGA	*sipS8*	1.266	2.037
comp149dMT70.3	GTGCACCCCGGAGAACTAC			
comp149dMT72.5	TGGGTGCGTCACTTATTCCC	*sipS5*	1.655	1.922
comp149dMT72.3	CAGCTGATCCTCACGGACG			
comp149dAT76.5	TGACGGCACATCTGACCTTC	*sipS13*	1.983	2.410
comp149dAT76.3	CTGGAGTCGCTGAACTTCGT			
comp149dP450_58.5	CCGAAGCACTACCACCATCT	*sipO1*	1.738	2.141
comp149dP450_58.3	AGCAGTACGCCTCGGTGAT			
comp149dP450_73.5	ATGCGAGGGAATAAGTGACG	*sipO2*	1.408	1.691
comp149dP450_73.3	GCGAAGACCGTGAACAGAAC			
ermE	TAGCTTGCGAGTGTCCGT	*sipR1*	-	
149-LuxR.3	TATGAATTCATCGGCTACGAAGTGCTCTG			

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785 **Table 6.** Oligonucleotides used for genetic complementation of *Streptomcyes* sp. CS149
786 mutants.

Name	Sequence (5´--- 3´)	Restriction site	Purpose
149eGT47.F	TATGGATCCCCGATGTACACCCGATTCCC	BamHI	*sipS15* expression (1.060 pb)
149eGT47.R	TATGAATTCGAGGTCTGTGTCGGTCGTG	EcoRI	
149eGT66.F	TATGGATCCCAGGGGTCGGACGGTATTTC	BamHI	*sipS4* expression (1.511 pb)
149eGT66.R	TATGAATTCGAGGAAAACCAGGCGGACAT	EcoRI	
149eGT71.F	TATTCTAGATCTTCCGGCGTTGTACTGTC	XbaI	*sipS9* expression (1.138 pb)
149eGT71.R	TATGAATTCACGATGGTGTTCCCGCTG	EcoRI	
149eGT74.F	TATGGATCCGCTGCTTGAGAAACCGGTTG	BamHI	*sipS14* expression (1.469 pb)
149eGT74.R	TATGAATTCGCCTTCTCCTTGTCGCGG	EcoRI	
149eMT70.F	TATGGATCCCTCACGCTTGGGATGATCGT	BamHI	*sipS8* expression (750 pb)
149eMT70.R	TATGAATTCGTGCTCCGGTCACAGACG	EcoRI	
149eMT72.F	TATTCTAGACTGGCGGGATTTCGTTTGGA	XbaI	*sipS5* expression (1.272 pb)
149eMT72.R	TATGAATTCCTCGGACTACGCCTCGGA	EcoRI	
149eAT76.F	TATGGATCCCCATGGGGCCTGAACTCG	BamHI	*sipS13* expression (1.202 pb)
149eAT76.R	TATGAATTCCTTCCCGTCTCACGCCAG	EcoRI	
149eP450_58.F	TATGGATCCCCACGATGAGCACGGAGAAG	BamHI	*sipO1* expression (1.203 pb)
149eP450_58.R	TATGAATTCCCTACCAGGTGACACGCAG	EcoRI	
149eP450_73.F	TATGGATCCGCCGGATCATGACATGACCA	BamHI	*sipO2* expression (1.344 pb)
149eP450_73.R	TATGAATTCTCCCAACCGGTTTCTCAAGC	EcoRI	

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