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Cooperative Involvement of Glycosyltransferases in the Transfer of Amino Sugars during the Biosynthesis of the Macrolactam Sipanmycin by *Streptomyces* sp. Strain CS149

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ABSTRACT Macrolactams comprise a family of natural compounds with important bioactivities, such as antibiotic, antifungal, and antiproliferative activities. 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 proven to be essential for cytotoxicity. In this work, the gene cluster responsible for the biosynthesis of sipanmycins (*sip* cluster) in *Streptomyces* sp. strain 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 deoxy sugar is addressed. In light of the results obtained by the generation of mutant strains and *in silico* characterization of the *sip* cluster, a biosynthetic pathway for sipanmycins and the two deoxy sugars 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 increased 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, and 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.

KEYWORDS *Streptomyces*, actinomycetes, antibiotics, biosynthesis gene cluster, glycosylation, glycosyltransferases, sugars

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, with actinomycetes being responsible for the production of approximately two-thirds of all natural bioactive
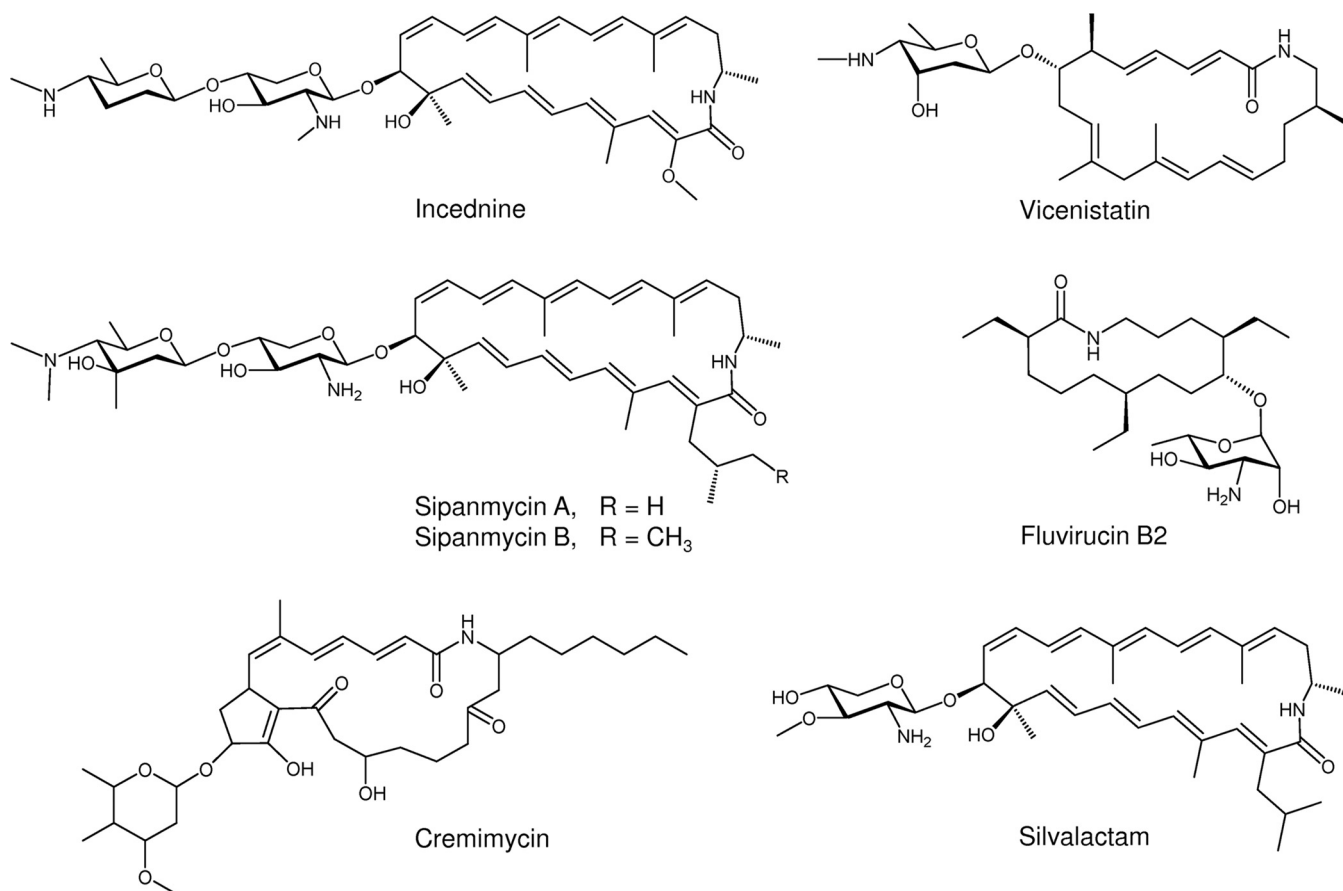
FIG 1 Structures of glycosylated macrolactams.

products known so far (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 with marine or terrestrial macroorganisms, such as sponges, plants, ants, termites, and 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 facilitate 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, mass spectrometry (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 activity (rifamycin) (14), antifungal and antiviral activities (fluvirucins) (15), antiproliferative activity (vicenistatin and leinamycin) (16, 17), and even to overcome the resistance to chemotherapeutic drugs in certain tumor cell lines caused by over-expression of the antiapoptotic oncoprotein Bcl-xL (incednine) (18). Interestingly, many of the macrolactam family members have at least one deoxy sugar moiety attached to the macrocyclic ring, as in the case of sipanmycins, vicenistatin, cremimycin, fluvirucin B2, incednine, and silvalactam (Fig. 1). It has been demonstrated that this deoxy sugar

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 amino sugars 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 sipan-mycins and incednine contain a disaccharide attached to the ring but with different amino sugars.

We report herein the identification of the gene cluster for sipanmycin biosynthesis (*sip* cluster) and the functional characterization of genes involved in deoxy sugar biosynthesis. Furthermore, we prove the complex glycosylation steps during sipanmy-cin biosynthesis that include the unexpected cooperation of two different glycosyl-transferases for the attachment of each deoxy sugar, and the need for a P450-like helper protein in the incorporation of the second deoxy sugar. 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 sipanmycin gene cluster, identification and characterization. For the iden-tification of the *sip* cluster in *Streptomyces* sp. 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 version 4) (21). Thirty-one putative BGCs were identified. They included three clusters containing nonribosomal peptide synthetases (NRPSs), three clusters containing polyketide syn-thases (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 lasso peptide, one bacteriocin, one thiopeptide, ectoine, and melanin (see Table S1 in the supplemental material). 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 for the different enzymatic functions that are necessary for the biosynthesis of sipanmycins (Table 1). In addition, amino acid se-quence analysis of this cluster showed similarity to proteins from a *Streptomyces* sp. strain ML694-90F31 cluster involved in incednine (*idn*) biosynthesis (22) (Table 1). Furthermore, three genes of the cluster involved in amino sugar 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. strain Tü 6075, 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 the putative limits of the cluster to be defined. The *sip* cluster comprises approximately 93 kb and 36 open reading frames (ORFs) (Fig. 2 and 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 synthesize: (i) β-amino acid starter unit, (ii) polyketide chain, (iii) branched-chain extender unit, and (iv) deoxy sugar 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* to *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* to *sipP5*) were found within the *sip* cluster, and their gene products showed high similarity to orthologs from *idn*

TABLE 1 Sipanmycin biosynthesis gene cluster in *Streptomyces* sp. CS149

Protein	Proposed function	Protein encoded in *Streptomyces* sp. ML694-90F3, % identity/similarity	Proteins by *Streptomyces* sp. Tü 6075 accession no., % 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, 74/83	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

cluster (ranging from 69 to 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 10 modules within PKS genes in *sip* and *idn* clusters, led us to assume that the order of PKS reactions is the same as that proposed for incednine biosynthesis, starting at SipP1 and ending at SipP5 (Fig. 3). A comparison of the predicted substrate specificities of AT domains revealed that the only
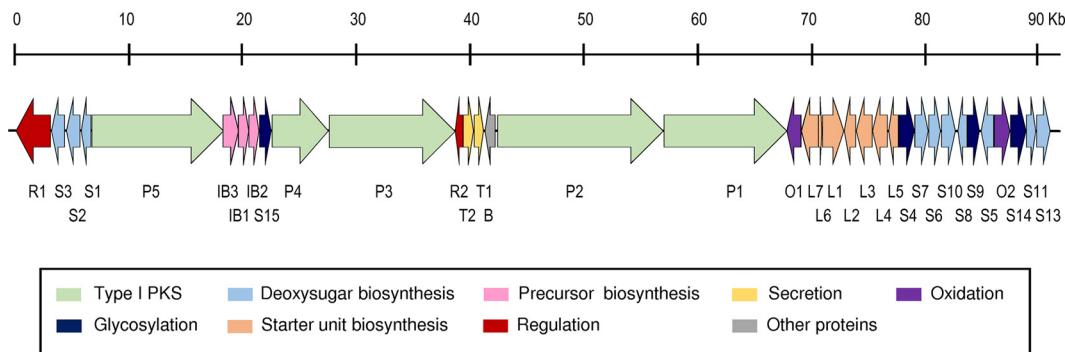

FIG 2 Sipanmycin biosynthesis gene cluster. Gene names of the sipanmycin (*sip*) cluster are indicated beneath its corresponding ORF representation.
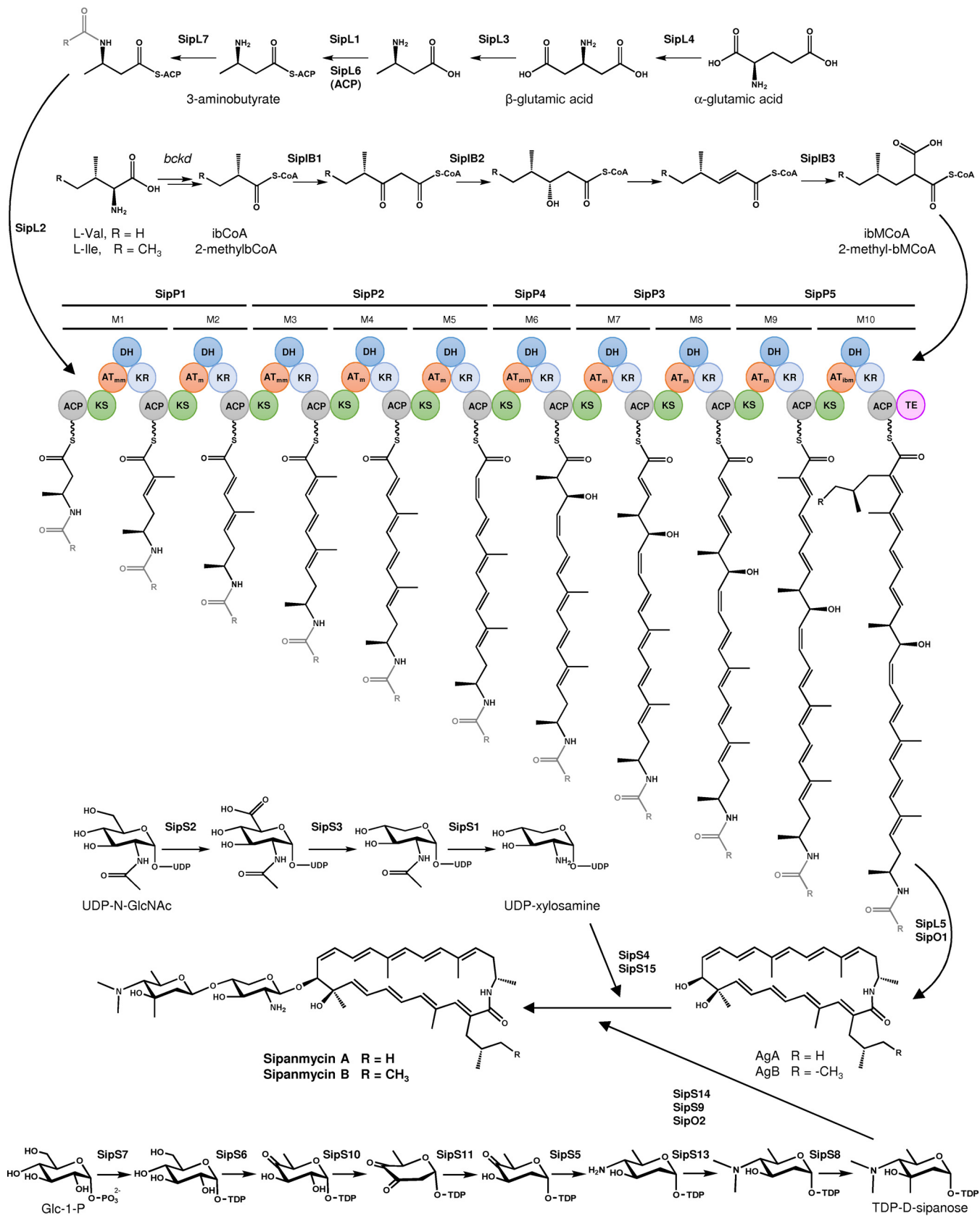
FIG 3 Proposed biosynthesis pathway for sipanmycins.

difference between them in sipanmycins and incednine is AT10 (see Fig. S1 in the supplemental material at https://figshare.com/s/49de24d11120b7b114ba), which recognizes methoxymalonyl-acyl-carrier protein (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 the *sip* cluster revealed the presence of three genes putatively involved in the biosynthesis of this branched-chain extender unit, *siplB1* to *siplB3*, 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. strain HKI0576 (27). For clarity and brevity, aglyca from sipanmycin A and sipanmycin B are referred to here as AgA and AgB, respectively.

The genes responsible for the biosynthesis of the two amino sugars attached to the sipanmycin aglyca are grouped into two separate subclusters within the *sip* cluster. The first one consisted of three genes (*sipS1* to *sipS3*) that might be involved in the biosynthesis of UDP-xylosamine (Fig. 3).

The second subcluster (*sip6*, *sip7*, *sip8*, *sip10*, *sip11*, and *sip13*) might be involved in the biosynthesis of a *N,N*-dimethyl derivative of a monosaccharide which formally corresponds to a 3,5-diepimer of lemonose (the amino sugar contained in lemonomycin) (28, 29) and which we propose to trivially name D-sipanose (Fig. 3). For the transfer of the amino sugars to the aglyca, four glycosyltransferase (GT) genes are present in the cluster (*sipS4*, *sipS9*, *sipS14*, and *sipS15*) that are described below (Table 1 and Fig. 2).

Two gene products, SipO1 and SipO2, showed similarity to cytochrome P-450 monooxygenases (Table 1). A thorough analysis of their sequences revealed that SipO1 might act as a P450-monooxygenase catalyzing the C-10 hydroxylation of sipanmycin aglyca. In contrast, SipO2 lacks the Cys residue at the active site responsible for the coordination of the heme iron; thus, it could not act as a monooxygenase (Fig. S2, https://figshare.com/s/49de24d11120b7b114ba). These kinds of enzymes have been described as GT helper proteins with a chaperone-like function. These genes are normally located directly upstream of the corresponding GT gene, as is the case of *sipO2*. Several examples of GT auxiliary protein/GT systems have been described, including DesVIII/DesVII (methymycin/pikromycin) (30), TylM3/TylM2 (tylosin) (31), EryCII/EryCIII (erythromycin) (32), and AknT/AknS (aclacinomycin) (33). Thus, we proposed a similar role for SipO2 in sipanmycin biosynthesis (see below).

Apart from structural genes, two genes with a high degree of similarity to transcriptional regulators were found in the *sip* cluster (Table 1 and Fig. 2). Sequence analysis showed that *sipR1* could code for a LuxR family transcriptional regulator (usually described as activators of secondary metabolite pathways) and *sipR2* could code for a putative TetR family transcriptional repressor. In *trans* overexpression of *sipR1* (149esipR1 strain) enhanced sipanmycin production during the first 3 days of cultivation in R5A medium (Fig. S3), supporting the role of SipR1 as an activator. After day 4, sipanmycin accumulation decreased in the wild-type and 149esipR1 strains, with 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 sipanmycin export, and *sipB* may code for a type II thioesterase, often related to the removal of aberrant extender units loaded onto PKSs (34) (Table 1 and Fig. 2).

Involvement of four glycosyltransferases in the transfer of two amino sugars. As mentioned above, sipanmycins contain two amino sugars 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 amino sugar 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 ultrahigh-performance liquid chromatography (UPLC). Inactivation
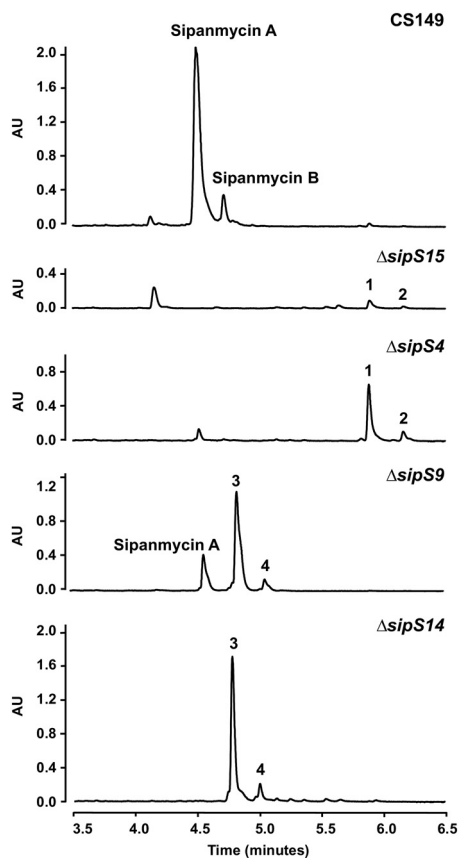
FIG 4 Effect of glycosyltransferase knockout experiments on sipanmycin biosynthesis. UPLC analysis of ethyl acetate extracts of mutants in the four GTs involved in the attachment of the two deoxy sugars to the sipanmycin aglycon. Peak 1, AgA; peak 2, AgB; peak 3, AgA+S1; peak 4, AgB+S1.

of either *sipS4* or *sipS15* completely abolished the 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 compound 1 and m/z 506 $[M+H]^+$ for compound 2, which matched the expected masses for AgA and AgB, respectively. The accumulation of compounds 1 and 2 was considerably higher in the Δ*sipS4* mutant than in the Δ*sipS15* mutant. On the other hand, the inactivation of *sipS14* also blocked the production of sipanmycins A and B, while inactivation of *sipS9* abolished the 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 compound 3 and m/z 637 $[M+H]^+$ for compound 4, both consistent with those expected for AgA and AgB, with just the first amino sugar of sipanmycins (D-xylosamine) attached, respectively (referred to here for brevity as AgA+S1 and AgB+S1, respectively) (Fig. 5). Compound 3 is nearly identical to silva-lactam (20), only lacking methylation at 3′-OH in the amino sugar unit; thus, it can be named 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 (Fig. S4), thus con-firming that there were no polar effects upon amikacin resistance (Am^r) cassette insertions. Major compounds (peaks 1 and 3) were purified by preparative high-performance liquid chromatography (HPLC) and their chemical structures confirmed by MS and nuclear magnetic resonance (NMR) (Fig. S5 to S16 and Table S2 for compound 1 and Fig. S17 to S24 and Table S3 for compound 3, https://figshare.com/s/49de24d11120b7b114ba).
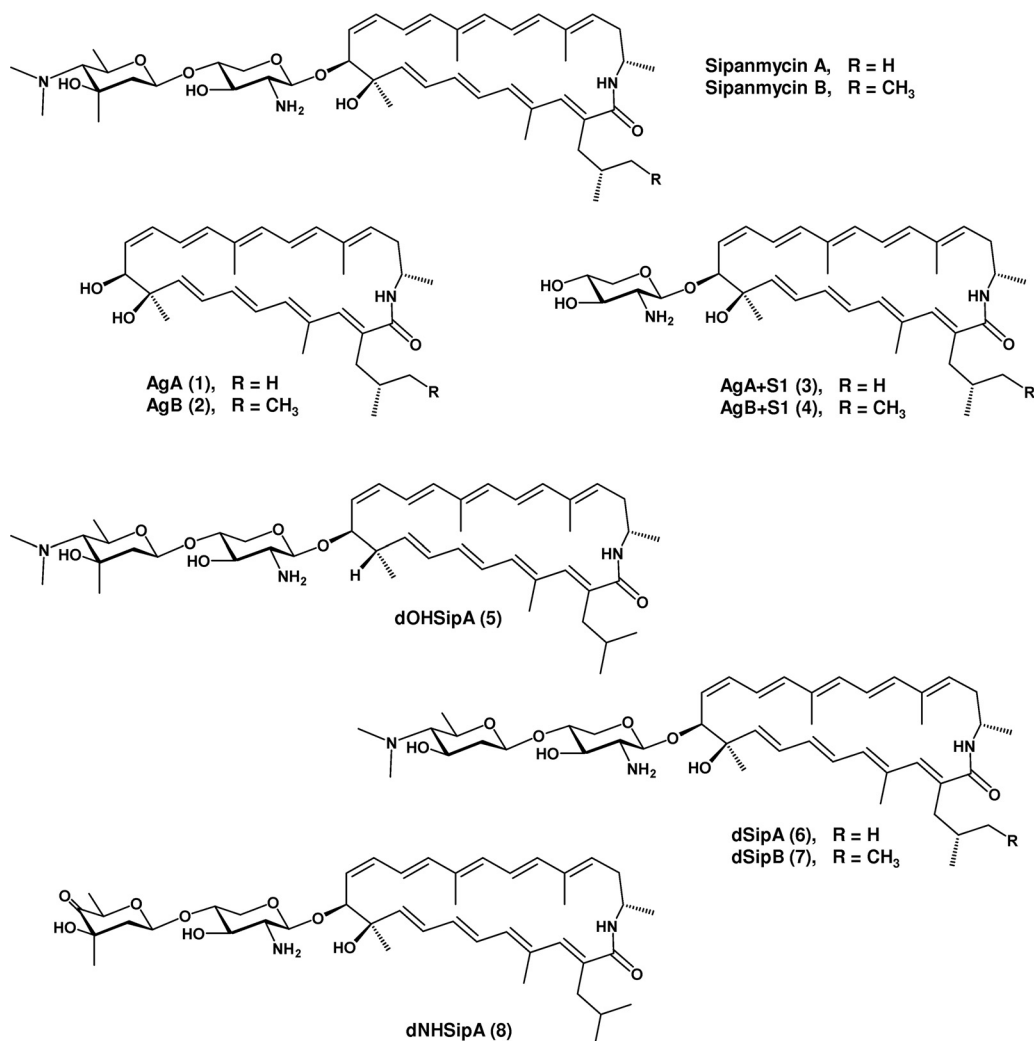
FIG 5 Structures of sipanmycin derivatives obtained during this work.

The conclusion of these experiments is that the four GTs in the *sip* cluster are required for the transfer of the two amino sugars, with two GTs, SipS15 and SipS4, working together in the transfer of D-xylosamine, and the other two GTs, SipS9 and SipS14, working together 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 the Δ*sipO2* mutant revealed results similar to those obtained by the inactivation of GTs *sipS9* and *sipS14*, an 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 amino sugar in sipanmycin biosynthesis. In cultures of the Δ*sipO1* mutant, we could not detect sipanmycins but rather the accumulation of a new compound (compound 5) with the characteristic UV absorption spectrum of sipanmycins and a mass of *m/z* 778 [M+H]$^+$ that matched that expected for sipanmycin A lacking the hydroxyl group at C-10 (10-deoxysipanmycin A [dOHSipA]; Fig. 6). This was confirmed after purification of the compound and elucidation of its chemical structure by high-resolution mass spectrometry (HRMS) and NMR (Fig. 5 and S25 to S35 and Table S4). Genetic complementation of each P450 monooxygenase-coding genes restored the wild-type phenotype in both mutant strains (Fig. S4).
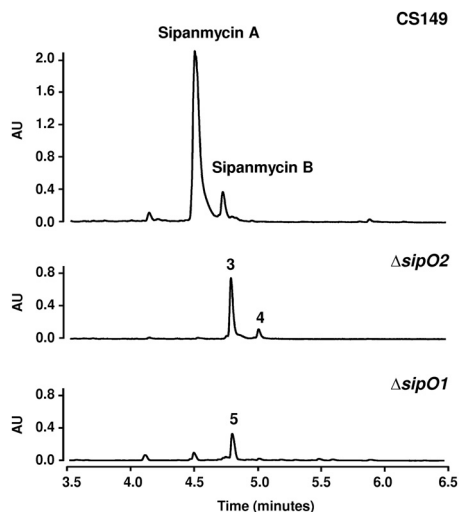
FIG 6 Effect of P450 cytochrome knockout experiments on sipanmycin biosynthesis. UPLC analysis of ethyl acetate extracts of mutants in *sipO2* and *sipO1* genes. Peak 3, AgA+S1; peak 4, AgB+S1; peak 5, dOHSipA.

Identification and role of aminotransferases and methyltransferases in amino sugar biosynthesis. Biosynthesis of the second amino sugar requires the involvement of two methyltransferases and an aminotransferase. BLAST analysis of the *sip* cluster pointed to *sipS8* and *sipS5* as potential candidates as *N*- and *C*-methyltransferase genes, respectively, and to *sipS13* as the aminotransferase gene. To clarify their role, we generated replacement mutants in each of these genes, analyzed the corresponding mutants, and isolated and determined the structures of the accumulated compounds. UPLC analysis of the Δ*sipS5* mutant in comparison to the wild-type strain showed the absence of peaks corresponding to sipanmycins and the appearance of three new peaks (Fig. 7). Compounds in these peaks showed the characteristic UV absorption spectrum of sipanmycins and masses of m/z 781 [M+H]$^+$ for compound 6, m/z 795 [M+H]$^+$ for compound 7, and m/z 623 [M+H]$^+$ for compound 3. Structural elucidation of compounds 6 and 7 identified them as 3"-demethyl-sipanmycins A and B (dSipA and dSipB), respectively, both lacking the methyl group at position C-3 in the second amino sugar, which in this case corresponds to *N,N*-dimethyl-D-pyrrolosamine, the enantiomer of the amino sugar found as L- form in the antitumor antibiotics lomaiviticin A and B (35) (Fig. 5 and S36 to S46 and Table S5 for compound 6 and Fig. S47 to S56 and Table S6 for compound 7). The third peak (compound 3) was the smallest one and corresponds to AgA+S1, confirming the role of SipS5 in the biosynthesis of the second amino sugar (Fig. 7).

A comparative profile analysis of the Δ*sipS8* mutant versus the wild-type strain showed the accumulation of two already-described compounds (compounds 3 and 4; Fig. 7), thus indicating that the inactivation of this *N*-methyltransferase gives rise to a sugar that is not recognized and transferred by the joint action of SipS14, SipS9, and SipO2.

Analysis of ethyl acetate extracts from the Δ*sipS13* 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). In a comparison of retention time and mass (m/z 637 [M+H]$^+$), one of them was identified as compound 4, which is previously described in other mutants in this study. The other two peaks (labeled 8 and 8′ in Fig. 7) contained compounds that did not show clear pseudomolecular ion mass spectra, making a structural proposal impossible. After isolating compound 8, it was observed that its analytical chromatographic reanalysis 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 con-
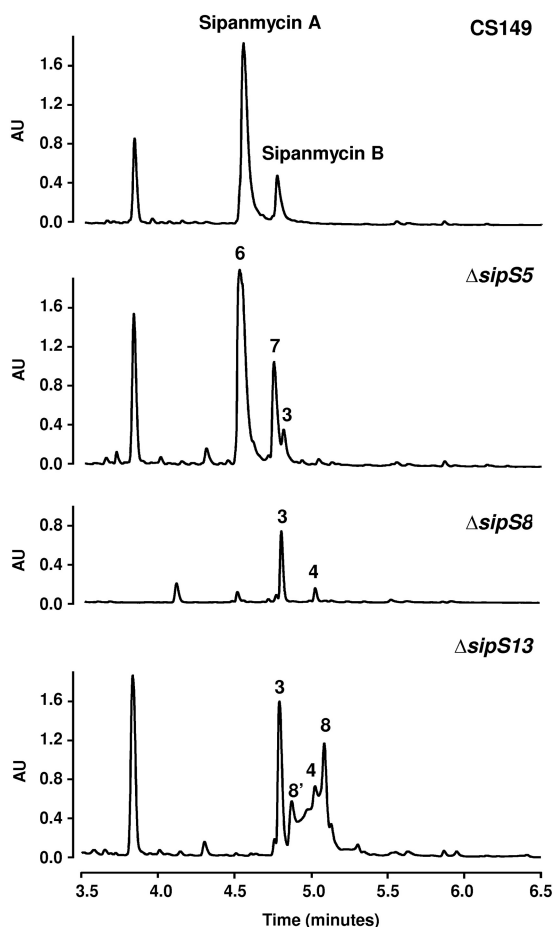
FIG 7 Effect of methyltransferases and aminotransferase knockout experiments on sipanmycin production. Chromatograms correspond to mutants in *sipS5*, *sipS8*, and *sipS13* genes. Peak 3, AgA+S1; peak 4, AgB+S1; peak 6, dSipA; peak 7, dSipB; peak 8, dNHSipA; 8', dNHSipA ketal derivative.

ditions. Analysis of HRMS and NMR data allowed the identification of compound 8 (*m/z* 765 [M+H]$^+$) as a sipanmycin A derivative with the dimethylated amino group at C-4 of the second amino sugar replaced by a ketone, rendering the monosaccharide 4-keto-β-D-olivomycose. Based on the structure of compound 8, it could be proposed that compound 8' (*m/z* 783 [M+H]$^+$) was an artifact that originated under acidic chromatographic conditions in which the ketone of compound 8 was hydrated rendering the monosaccharide 4-hydroxy-β-D-olivomycose. Compound 8 is proposed to be named 4"-deamino-4"-oxosipanmycin A (dNHSipA), and compound 8' corresponded to its ketal derivative (Fig. 5 and S57 to S68 and Table S7). Taking into account the accumulation of compound 3 and the production phenotype of the Δ*sipS13* mutant, it is probably the case that in the absence of amination at C-4, recognition of the second amino sugar by GTs SipS14 and SipS9 would be much less efficient.

The Δ*sipS8*, Δ*sipS5*, and Δ*sipS13* mutants recovered the sipanmycin production after genetic complementation (Fig. S4).

***In vitro* cytotoxicity and antibiotic activity analyses of sipanmycins and derivatives.** Sipanmycins exerted potent cytotoxicity against several tumor cell lines (Table 2), with sipanmycin A being the one with strongest activity, showing 50% inhibitory concentrations (IC$_{50}$s) 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* (Table S8). The conclusions are that the deoxy sugars attached to the aglycon and the

TABLE 2 *In vitro* cytotoxicities of sipanmycins and derivatives

| Sipanmycin or derivative | Cytotoxicity (IC$_{50}$ [μM]) by cell type (source) | | | | | |
	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 (compound 1)	≫10	≫10	≫10	2.549	≫10	≫10
AgB (compound 2)	≫10	>10	>10	0.698	≫10	>10
AgA+S1 (compound 3)	0.91	0.647	0.431	1.16	2.17	0.827
dOHSipA (compound 5)	1.614	1.75	0.873	1.089	2.68	1.2
dSipA (compound 6)	0.163	0.147	0.147	0.468	0.181	0.085
dSipB (compound 7)	0.293	0.211	0.213	1.129	0.493	0.241

hydroxyl group at C-10 are essential for both the antiproliferative and antibiotic activities of this kind of compounds.

DISCUSSION

It has been previously described that the deoxy sugar 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 on those genes involved in amino sugar biosynthesis and its transfer to the sipanmycin aglyca.

The *sip* cluster contains four genes encoding GTs, while only two amino sugars form part of the sipanmycin 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 activities (39). However, reports on BGCs containing more GT genes than sugars in the molecule are scarce, with PM100117/PM100118 (42) and oleandomycin (43) being 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 deoxy sugars (44). In the case of oleandomycin, which contains two deoxy sugars 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 amino sugars 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 amino sugars into the sipanmycin 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 amino sugar and Sip9 and Sip14 for the second amino sugar). 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 the Δ*sipS9* mutant, in which the presence of a small amount of sipanmycin A 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 amino sugar since there is a requirement for the presence of a P450 helper protein encoded 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 of stabilizing 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 GT pair SipS9/SipS14 in combination with auxiliary SipO2, this picture might be slightly different. Since both GTs are required for the

TABLE 3 Strains and plasmids used in this work

Strain or plasmid	Genotype/characteristics	Use	Reference(s) or source
Strains			
Escherichia coli			
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Cloning	63
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	64, 65
Streptomyces			
CS149	Wild type		1
Δ*sipS15* mutant	Δ*sipS15*∷*aac(3)-IV*		This work
Δ*sipS4* mutant	Δ*sipS4*∷*aac(3)-IV*		This work
Δ*sipS9* mutant	Δ*sipS9*∷*aac(3)-IV*		This work
Δ*sipS14* mutant	Δ*sipS14*∷*aac(3)-IV*		This work
Δ*sipS8* mutant	Δ*sipS8*∷*aac(3)-IV*		This work
Δ*sipS5* mutant	Δ*sipS5*∷*aac(3)-IV*		This work
Δ*sipS13* mutant	Δ*sipS13*∷*aac(3)-IV*		This work
Δ*sipO1* mutant	Δ*sipO1*∷*hyg*		This work
Δ*sipO2* mutant	Δ*sipO2*∷*hyg*		This work
149esipR1	*ermE*p-sipR1*		This work
Plasmids			
pSETec	Integrating vector, *aac(3)-IV oriT$_{RK2}$ intΦC31 attPΦC31 ermE*p*	Gene overexpression	66
pSETeTc	Integrating vector, *aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31 ermE*p*	Gene overexpression	66
pLHyg	*lacZ bla hyg*	Source of *hyg* gene	57
pUK21	*lacZ km*	Backbone for pUKHyg construction	58
pUKHyg	*lacZ km hyg*	Replacement cassette construction	This work
pUO9090	*aac(3)-IV km*	Replacement cassette construction	67
pHZ1358	*bla tsr oriT$_{RK2}$*	Knockout mutant generation	68
pUO149Δ*sipS15*	Carries up- and downstream flanking regions of *sipS15* at both sides of *aac(3)-IV km*	Source of *sipS15* replacement cassette	This work
pUH149Δ*sipS15*	Carries *sipS15* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS15* replacement	This work
pSETT149esipS15	Carries *sipS15* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS15* complementation	This work
pUO149Δ*sipS4*	Carries up- and downstream flanking regions of *sipS4* at both sides of *aac(3)-IV km*	Source of *sipS4* replacement cassette	This work
pUH149Δ*sipS4*	Carries *sipS4* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS4* replacement	This work
pSETT149esipS4	Carries *sipS4* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS4* complementation	This work
pUO149Δ*sipS9*	Carries up- and downstream flanking regions of *sipS9* at both sides of *aac(3)-IV km*	Source of *sipS9* replacement cassette	This work
pUH149Δ*sipS9*	Carries *sipS9* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS9* replacement	This work
pSETT149esipS9	Carries *sipS9* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS9* complementation	This work
pUO149Δ*sipS14*	Carries up- and downstream flanking regions of *sipS14* at both sides of *aac(3)-IV km*	Source of *sipS14* replacement cassette	This work
pUH149Δ*sipS14*	Carries *sipS14* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS14* replacement	This work
pSETT149esipS14	Carries *sipS14* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS14* complementation	This work
pUO149Δ*sipS8*	Carries up- and downstream flanking regions of *sipS8* at both sides of *aac(3)-IV km*	Source of *sipS8* replacement cassette	This work
pUH149Δ*sipS8*	Carries *sipS8* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS8* replacement	This work
pSETT149esipS8	Carries *sipS8* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS8* complementation	This work
pUO149Δ*sipS5*	Carries up- and downstream flanking regions of *sipS5* at both sides of *aac(3)-IV km*	Source of *sipS5* replacement cassette	This work
pUH149Δ*sipS5*	Carries *sipS5* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS5* replacement	This work
pSETT149esipS5	Carries *sipS5* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS5* complementation	This work
pUO149Δ*sipS13*	Carries up- and downstream flanking regions of *sipS13* at both sides of *aac(3)-IV km*	Source of *sipS13* replacement cassette	This work
pUH149Δ*sipS13*	Carries *sipS13* replacement cassette, *aac(3)-IV bla tsr oriT$_{RK2}$*	*sipS13* replacement	This work
pSETT149esipS13	Carries *sipS13* under control of *ermE*p aac(3)-IV tsr oriT$_{RK2}$ intΦC31 attPΦC31*	*sipS13* complementation	This work

(Continued on next page)

TABLE 3 (Continued)

Strain or plasmid	Genotype/characteristics	Use	Reference(s) or source
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 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 control of *ermE*p aac(3)-IV oriT$_{RK2}$ intΦC31 attPΦC31*	*sipO2* complementation	This work
pSET149eLuxR	Carries *sipR1* under control of *ermE*p aac(3)-IV oriT$_{RK2}$ intΦC31 attPΦC31*	*sipR1* overexpression	This work

attachment of the second amino sugar 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, in that SipS9 (homolog of IndS9 and 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 nucleotidyl-diphosphate (NDP)-deoxy sugar 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 sipanmycin aglycon, while the other member (SipS9), or both in cooperation, could be involved in amino sugar 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 (Fig. S68, https://figshare.com/s/49de24d11120b7b114ba), indicating that the auxiliary protein SipO2 could interact only with one of the partners of the GT pair, giving SipS9 a secondary role in this glycosylation step, as indicated by the residual sipanmycin A production in the Δ*sipS9* mutant (Fig. 4).

A similar scenario may occur in the first glycosylation step, with the difference that the GT pair SipS15/SipS4 is not dependent on the auxiliary protein SipO2, as xylosamine is attached to AgA and AgB in the Δ*sipO2* mutant (compounds 3 and 4, respectively). In a way similar 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 (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 independence from SipO2 of this GT pair.

Xylosamine, transferred from UDP-xylosamine, is the first amino sugar directly attached to the aglycon and present in both sipanmycins and incednine, but the second amino sugar 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 (compounds 6 and 7, respectively), both lacking this methyl group. Furthermore, a sipanmycin A derivative with a ketone in C-4″ of the second deoxy sugar, instead of a dimethylated amino group (compound 8), has been isolated from cultures of the Δ*sipS13* mutant, indicating the aminotransferase activity of SipS13. The incorporation of the unmethylated amino sugar (in compounds 6 and 7) or neutral deoxy sugar (in compound 8) shows a certain degree of flexibility of the SipS9/SipS14/SipO2 complex. Such flexibility regarding the sugar donor has been reported for other GTs, such as ElmGT (for the biosynthesis of elloramycin) (50, 51) and UrdGT2 (for the biosynthesis of urdamycin) (52). In contrast, the lack of the dimethyl group at the amino group

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 bp)
149dGT47.5R	TATAAGCTTACCAGGCTGTGCAGTCGT	HindIII	
149dGT47.3F	TATGGATCCGGAACTGCGGGAGGAGAT	BamHI	*sipS15* replacement (3′ flanking region) (1,069 bp)
149dGT47.3R	TATTCTAGACGCTTGATGACCTTCACCTG	XbaI	
149dGT66.5F	TATGAATTCTGAGCTCGGACAGCACATAG	EcoRI	*sipS4* replacement (5′ flanking region) (1,357 bp)
149dGT66.5R	TATAAGCTTCGGAAACGACATCAACATGA	HindIII	
149dGT66.3F	TATCATATGCGATCTCGTCCCCGAACT	NdeI	*sipS4* replacement (3′ flanking region) (1,124 bp)
149dGT66.3R	TATTCTAGAGATCTGGCTGGTGTCGATG	XbaI	
149dGT71.5F	TATGAATTCCCACCACGTCAACGTCCAG	EcoRI	*sipS9* replacement (5′ flanking region) (1,101 bp)
149dGT71.5R	TATAAGCTTGAGGACGGCACGGTCAGC	HindIII	
149dGT71.3F	TATCATATGGAGGTCGTGCCGCTGCTG	NdeI	*sipS9* replacement (3′ flanking region) (1,127 bp)
149dGT71.3R	TATTCTAGACGATCTGTCGCTCCTGTACG	XbaI	
149dGT74.5F	TATGAATTCATGCGAGGGAATAAGTGACG	EcoRI	*sipS14* replacement (5′ flanking region) (1,408 bp)
149dGT74.5R	TATAAGCTTGCGAAGACCGTGAACAGAAC	HindIII	
149dGT74.3F	TATGGATCCGTGACCAAGGACAGCGAGAG	BamHI	*sipS14* replacement (3′ flanking region) (1,118 bp)
149dGT74.3R	TATTCTAGACCTGTCTGATCCGTTCCAGT	XbaI	
149-DORF70.5F	TATGAATTCGAACATCCCGATCGTCTCC	EcoRI	*sipS8* replacement (5′ flanking region) (1,420 bp)
149-DORF70.5R	TATAAGCTTTCTGCTCATTCTCGATGACG	HindIII	
149-DORF70.3F	TATCATATGGCGTCTGTGACCGGAGCA	NdeI	*sipS8* replacement (3′ flanking region) (1,377 bp)
149-DORF70.3R	TATTCTAGAGTGATGCGCACCGAGGAC	XbaI	
149-DORF72.5F	TATGAATTCTCCCAACCGGTTTCTCAA	EcoRI	*sipS5* replacement (5′ flanking region) (1,527 bp)
149-DORF72.5R	TATAAGCTTCTCGGCATGCAGAAATGG	HindIII	
149-DORF72.3F	TATCATATGAGGCTGTCCGAGGCGTAGT	NdeI	*sipS5* replacement (3′ flanking region) (1,369 bp)
149-DORF72.3R	TATTCTAGAGCGATCAGGTCGTGAATTTG	XbaI	
149dAT76.5F	TATGAATTCAGTTGCAGCAGGAGATCCAC	EcoRI	*sipS13* replacement (5′ flanking region) (1,125 bp)
149dAT76.5R	TATAAGCTTCGAGGATGGCGAGTTCAG	HindIII	
149dAT76.3F	TATGGATCCGGAGTGACGCTCGACCAG	BamHI	*sipS13* replacement (3′ flanking region) (1,419 bp)
149dAT76.3R	TATTCTAGATTCAACTCGATGCACGACAC	XbaI	
149dP450_58.5F	TATTCTAGATCGAAGTTCCGCATGCTCAT	XbaI	*sipO1* replacement (5′ flanking region) (2,028 bp)
149dP450_58.5R	TATGGATCCCCTCCATGTCGAACGGGTAG	BamHI	
149dP450_58.3F	TATAAGCTTATTCAGTGGAAGTTCGGCGT	HindII	*sipO1* replacement (3′ flanking region) (4,050 bp)
149dP450_58.3R	TATAGATCTGGTATGGGGCGTGGTTTGTA	BglII	
149dP450_73.5F	TATTCTAGAGTCCGGTTCCTTCCCTACG	XbaI	*sipO2* replacement (5′ flanking region) (2,022 bp)
149dP450_73.5R	TATCATATGAGCCTCGGGTCGATCTGATA	NdeI	
149dP450_73.3F	TATAAGCTTGCTGCTTGAGAAACCGGTTG	HindIII	*sipO2* replacement (3′ flanking region) (2,203 bp)
149dP450_73.3R	TATAGATCTCGATGATCTCTCGACGCTGA	BglII	

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 amino sugar was incorporated (compounds 3 and 4).

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptomyces* sp. CS149, a sipanmycin producer, has been previously reported (13). Medium A (MA) (53) was used for sporulation. For metabolite production, strains were added to flasks containing 30 ml tryptic soy broth (TSB) medium and grown at 30°C and 250 rpm. After 24 h, this seed culture was used to inoculate 50 ml of R5A medium (36) to a final optical density at 600 nm (OD_{600}) of 0.2. For intergeneric conjugation, mannitol soy medium (MS) (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, as follows: apramycin, 100 μg/ml for *E. coli* and 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* (55) and *Streptomyces* spp. (56). All amplifications were carried out with the Herculase II Fusion high-fidelity polymerase (Agilent Technologies), and PCR conditions were initial denaturation at 98°C for 2 min, 30 cycles of 98°C for 30 s, 58°C for 30 s, and 72°C for *x* s (1 kb/30 s), and a final extension at 72°C for 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 (57) into pUK21 (58).

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 the Newbler assembler software version 2.9. Annotation was performed using the PGAAP pipeline (https://submit.ncbi.nlm.nih .gov/subs/genome/) (59). Database searching and sequence analysis were carried out with the bioinformatic tool antiSMASH (21).

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

Name	Sequence (5′–3′)	Gene	Amplicon size (bp) in:	
			Wild-type strain	Mutant strain
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			

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 the 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 a 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 the 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 the *ermE** promoter (*ermE*p*).

TABLE 6 Oligonucleotides used for genetic complementation of *Streptomyces* sp. CS149 mutants

Name	Sequence (5′–3′)	Restriction site	Gene expression (size [bp])
149eGT47.F	TATGGATCCCCGATGTACACCCGATTCCC	BamHI	*sipS15* (1,060)
149eGT47.R	TATGAATTCGAGGTCTGTGTCGGTCGTG	EcoRI	
149eGT66.F	TATGGATCCCAGGGGTCGGACGGTATTTC	BamHI	*sipS4* (1,511)
149eGT66.R	TATGAATTCGAGGAAAACCAGGCGGACAT	EcoRI	
149eGT71.F	TATTCTAGATCTTCCGGCGTTGTACTGTC	XbaI	*sipS9* (1,138)
149eGT71.R	TATGAATTCACGATGGTGTTCCCGCTG	EcoRI	
149eGT74.F	TATGGATCCGCTGCTTGAGAAACCGGTTG	BamHI	*sipS14* (1,469)
149eGT74.R	TATGAATTCGCCTTCTCCTTGTCGCGG	EcoRI	
149eMT70.F	TATGGATCCCTCACGCTTGGGATGATCGT	BamHI	*sipS8* (750)
149eMT70.R	TATGAATTCGTGCTCCGGTCACAGACG	EcoRI	
149eMT72.F	TATTCTAGACTGGCGGGATTTCGTTTGGA	XbaI	*sipS5* (1,272)
149eMT72.R	TATGAATTCCTCGGACTACGCCTCGGA	EcoRI	
149eAT76.F	TATGGATCCCCATGGGGCCTGAACTCG	BamHI	*sipS13* (1,202)
149eAT76.R	TATGAATTCCTTCCCGTCTCACGCCAG	EcoRI	
149eP450_58.F	TATGGATCCCCACGATGAGCACGGAGAAG	BamHI	*sipO1* (1,203)
149eP450_58.R	TATGAATTCCCTACCAGGTGACACGCAG	EcoRI	
149eP450_73.F	TATGGATCCGCCGGATCATGACATGACCA	BamHI	*sipO2* (1,344)
149eP450_73.R	TATGAATTCTCCCAACCGGTTTCTCAAGC	EcoRI	

Plasmid construction for *sipR1* overexpression. *sipR1* was amplified by PCR using the oligonucleotides 149-LuxR.5 (5′-TATGGATCCCGGGGGTAGGTGTACATGAG-3′) and 149-LuxR.3 (5′-TATGAATTCATCG GCTACGAAGTGCTCTG-3′). The resulting 3,042-bp fragment was gel purified, digested with BamHI/EcoRI, and cloned into pSETec, leading to the 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-liter cultures of CS149 mutant strains in R5A medium, as previously described (13), but using a different mobile phase during semipreparative HPLC with Milli-Q (MQ) water plus 0.05% trifluoroacetic acid-acetonitrile (TFA-can; ranging from 40 to 45% ACN, depending on the compound).

Structural elucidation of new compounds. Compounds 1, 3, 5, 6, 7, and 8 (and the artifact 8′) were analyzed by liquid chromatography-diode array detector-electrospray ionization-time of flight (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 (60) and the Dictionary of natural products version 26:2 (61) was carried out to ensure their novelty. The structural elucidation of these compounds was carried out by detailed analysis of one-dimensional (1D) and two-dimensional (2D) NMR spectra, further assisted by comparison with the spectroscopic data reported for incednine (18), silvalactam (20), and especially sipanmycins A and B (13). Relative configurations were determined by coupling constants and nuclear Overhauser effect (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 were obtained by LC-DAD-ESI-TOF analyses performed using an Agilent 1200RR HPLC equipped with a SB-C$_8$ column (2.1 by 30 mm; Zorbax) coupled to a Bruker maXis spectrometer. The chromatographic and ionization conditions were identical to those previously employed for sipanmycins A and B (13).

NMR spectra were recorded in deuterated methanol (CD$_3$OD) at 24°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 an internal reference.

***In vitro* cytotoxicity and antibiotic activity assays.** The cytotoxic activities of compounds were 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) cells. The mouse embryonic fibroblast cell line NIH/3T3 was used as a control to evaluate cytotoxicity against nonmalignant cells. Cells were previously grown for a week on Dulbecco's modified Eagle medium (DMEM)–10% fetal bovine serum (FBS) medium, aliquoted to 5,000 cells per well in 96-well plates using the cell counting kit CCK-8 (catalog no. 96992; Sigma-Aldrich), and grown for an extra 24 h. The compounds were dissolved in DMEM. After the incubation, 10 μl of compound (in diverse concentrations) was added to each well and incubated for another 48 h. Last, 10 μl of CCK-8 reagent (Sigma-Aldrich) was added, left to develop for 2 h in the incubator, and measured at 450 nm using a Bio-Tek ELx 800 enzyme-linked immunosorbent assay (ELISA) kit (BioTek).

Antibiotic activity tests (in 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 of 0.1 and total volume of 150 μl per well. Plates were incubated overnight at 37°C (30°C for *C. albicans*).

Accession number(s). The whole-genome shotgun project of *Streptomyces* sp. CS149 has been deposited at DDBJ/ENA/GenBank under the accession number PVZY00000000. The version described in this paper is version PVZY01000000. The nucleotide sequence of the *sip* cluster was deposited in the European Nucleotide Archive under accession number LT986736 and at the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) repository (62) under the accession number BGC0001452.

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