Generation of new sipanmycin analogues by combinatorial biosynthesis and mutasynthesis approaches relying on the substrate flexibility of key enzymes in the biosynthetic pathway.

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Abstract

The appearance of new infectious diseases, the increase of multi-resistant bacteria and the need for more effective chemotherapeutics have oriented the interests of researchers towards the search for metabolites with novel or improved bioactivities. Sipanmycins are disaccharyl glycosylated macrolactams that exert antibiotic and cytotoxic activities. By applying combinatorial biosynthesis and mutasynthesis approaches, we have generated eight new members of the sipanmycin family. The introduction of plasmids harboring genes responsible for the biosynthesis of several deoxysugars into the sipanmycin-producing strain *Streptomyces* sp. CS149 led to the production of six derivatives with altered glycosylation patterns. After structural elucidation of these new metabolites, we conclude that some of these sugars are the result of the combination of the enzymatic machinery coded by the introduced plasmids and the native enzymes of the D-sipanose biosynthetic pathway of the CS149 wild-type strain. In addition, two analogues of the parental compounds with a modified polyketide backbone were generated by a mutasynthesis approach, feeding cultures of a mutant strain defective in sipanmycin biosynthesis with 3-aminopentanoic acid. The generation of new sipanmycin analogues shown in this work relay on the substrate flexibility of key enzymes involved in sipanmycin biosynthesis, in particular the glycosyltransferase pair SipS9/SipS14 and enzymes SipL3, SipL1, SipL7 and SipL2 involved in the incorporation of the polyketide synthase starting unit.

Importance

Combinatorial biosynthesis has proven its usefulness in generating derivatives of already known compounds with novel or improved pharmacological properties. Sipanmycins are a family of glycosylated macrolactams produced by *Streptomyces* sp.

CS149 whose antiproliferative activity is dependent on the sugar moieties attached to the aglycone. In this work, we report the generation of several sipanmycin analogues with different deoxysugars, showing the high degree of flexibility exerted by the glycosyltransferase machinery related to the recognition of diverse nucleotide-activated sugars. In addition, modifications in the macrolactam ring were introduced by mutasynthesis approaches, indicating that the enzymes involved in incorporating the starter unit have a moderate ability to introduce different types of β-amino acids. In conclusion, we have proven the substrate flexibility of key enzymes involved in sipanmycin biosynthesis, specially the glycosyltransferases, which can be exploited in future experiments.

Introduction

Discovery and development of bioactive compounds has become of a great concern in recent years since not only some infectious diseases are emerging, but also several pathogens are becoming resistant to commonly used clinical treatments. Furthermore, there is a need for generating novel compounds to be used in cancer chemotherapy due to the rapid development of resistance to chemotherapeutics, the high toxicity associated with these drugs, their undesirable side-effects, and the demand for novel antitumor chemical entities active against untreatable tumors, with fewer side-effects or with greater therapeutic efficiency (1,2).

Natural products are the most commonly used agents against both animal and human diseases. Among them, the most abundant are those produced by bacteria, specifically from the genus *Streptomyces*. The rate of new drug discovery has been speed down in last decades due to the low success of classical screening programs and the reisolation of already known compounds. Researchers have focused their efforts in searching new bioactive compound producers in poorly studied ecosystems, as marine sediments or associated with other organisms such as ants, wasps or sponges (3-5). In addition, the improvement in sequence data analysis and genetic molecular modification techniques have opened new fields in drug development by (i) the awakening of silent gene clusters leading to the production of bioactive compounds, as for example it was the case of stambomycin, a glycosylated polyketide discovered by the overexpression of a LuxR transcriptional activator in *S. ambofaciens* ATCC 23877 (6); and (ii) combinatorial biosynthesis strategies that have been successfully applied to generate, among others, several paulomycin derivatives by changing the sugar moiety attached to the core structure (7) or different caboxamycins bearing distinct substitutions in the aryl ring (8).

Glycosylated natural compounds with important biological properties have been described and, some of them, as is the case of erythromycin and doxorubicin, have been widely used for antibacterial and anticancer treatments, respectively. Glycosylation has a great influence in the biological activity of compounds. Thus, it can modify drug pharmacokinetics, solubility and transport and also participates in the recognition of the drug molecular target (9, 10). Cytotoxic activity of the polyketide jadomycin depends on the sugar attached to the aglycone (11). Furthermore, mithramycin bioactivity was improved by combinatorial biosynthesis leading to derivatives with different saccharides (12). Thus, glycodiversification could be a valuable tool to obtain improved new analogues of core scaffolds by changing the sugar moieties attached to them.

Sipanmycins are glycosylated 24-membered macrolactams isolated from *Streptomyces* sp. CS149 that have been described as antibacterial and cytotoxic agents (13). Their biosynthesis starts by the incorporation of a 3-aminobutyrate molecule (generated from α-glutamic acid through the action of seven enzymes, SipL1 to SipL7) to the polyketide synthase (PKS) machinery. Then, six units of malonyl-CoA, three units of methylmalonyl-CoA and one isobutylmalonyl-CoA (in sipanmycin A, SIP-A) or 2-(2-methylbutyl)-malonyl-CoA (in sipanmycin B, SIP-B) are condensed by five PKS enzymes to give rise to the complete macrolactam ring skeleton. Finally, two aminodeoxysugars are attached to the aglycone: first, UDP-D-xylosamine (synthetized from *N*-acetyl-glucosamine by SipS1, SipS2 and SipS3) and then TDP-D-sipanose (synthetized by seven enzymes: SipS7, SipS6, SipS10, SipS11, SipS13, SipS8 and SipS5). It was demonstrated by knockout experiments that each glycosylation step requires the coordinated activity of two different glycosyltransferases (GT): SipS4/SipS15 pair attach UDP-D-xylosamine to the aglycone and SipS9/SipS14 pair (with the aid of additional helper protein SipO2) introduce TDP-D-sipanose (14; Fig.

S1, https://figshare.com/s/87fcece083b744468230). The disaccharide attached to the sipanmycin aglycone by its unique GT machinery has been proved to be essential for their antibacterial and cytotoxic activities. Similarly, the presence or absence of a hydroxy group at C-10 position of the aglycone determines the biological activity of this class of compounds (14). Thus, both the sugar moiety and the aglycone could be promising modification targets to obtain new analogues with improved pharmacological properties.

In this work, we have explored the versatility of some enzymes involved in sipanmycin biosynthesis to obtain novel derivatives. We tested the substrate flexibility of the GT pairs SipS4/SipS15 and SipS9/SipS14 that naturally transfer UDP-D-xylosamine and TDP-D-sipanose respectively, to the sipanmycin aglycone (14). In addition, we have substituted the natural β-amino acid unit that serves as a starter in the biosynthesis of the macrolactam ring. By using combinatorial biosynthesis and mutasynthesis approaches, we have generated novel sipanmycin analogues and tested their biological activities.

Results

Sipanmycin glycosylated derivatives obtained by combinatorial biosynthesis

Plasmids pLNRT, pFL844T and pLNBIVT (directing the biosynthesis of D-olivose, L-amicetose and L-digitoxose, respectively; Fig. S2, https://figshare.com/s/87fcece083b744468230) (15) were introduced into wild-type (wt) *Streptomyces* sp. CS149 strain by intergeneric conjugation (*E. coli-Streptomyces*). Clones harboring these sugar biosynthesis plasmids were grown on R5A liquid medium, samples extracted with ethyl acetate at 3, 5 and 7 days and analyzed by UPLC and HPLC-MS.

Comparative analysis of production profiles between CS149 wt and CS149+pLNBIVT strains showed the production of compounds SIP-A and SIP-B in both of them and two extra peaks in the latter strain sharing UV absorption spectra with sipanmycins (Fig. 1A,B), but differing in their $[M+H]^+$ ions, being *m/z* 753 for compound **1** and *m/z* 767 for compound **2**. These compounds were purified by HPLC and their chemical structure was determined by high-resolution mass spectrometry (HRMS) and NMR spectroscopy (Figs. S3-S14 and Table S1 for **1**, Figs. S15-S21 and Table S2 for **2**: https://figshare.com/s/87fcece083b744468230). Structural elucidation of compounds **1** and **2** identified them as α-L-digitoxyl-(1→4')-3'-*O*-demethylsilvalactam (**1**), which we designated as sipanmycin A1 (SIP-A1) and its congener (**2**) carrying an extra carbon in the aliphatic substituent at C-2, sipanmycin B1 (SIP-B1), following the same trivial nomenclature we originally employed for the wild-type sipanmycins A and B (SIP-A and SIP-B). SIP-A1 and SIP-B1 thus correspond to analogues of compounds SIP-A and SIP-B respectively, in which the second sugar moiety, β-D-sipanose, has been replaced by α-L-digitoxose (Fig. 1B).

Similarly, CS149+pLNRT strain produced compounds SIP-A and SIP-B but also two extra compounds (**3** and **4**, Fig. 1A,C) with $[M+H]^+$ ions at *m/z* 753 and *m/z* 767. Compounds **3** and **4** were purified by HPLC and their structures determined by HRMS and NMR (Figs. S22-S29 and Table S3 for **3**, Figs. S30-40 and Table S4 for **4**: https://figshare.com/s/87fcece083b744468230). Structural elucidation of compounds **3** and **4** identified them as β-D-olivosyl-(1→4')-3'-*O*-demethylsilvalactam (**3**), which we designated as sipanmycin A2 (SIP-A2) and β-D-olivomycosyl-(1→4')-3'-*O*-demethylsilvalactam (**4**), sipanmycin A2b (SIP-A2b), respectively. Both compounds shared the aglycone and the first deoxysugar with compound SIP-A, but differed in the second one, being β-D-olivose for SipA2 or β-D-olivomycose for SIP-A2b (Fig. 1C).

On the other hand, in culture extracts of CS149+pFL844T strain, compounds SIP-A and SIP-B were not observed but two novel peaks (corresponding to compounds **5** and **6**) showing the characteristic absorption spectra of sipanmycins, with only slightly different UPLC retention times from those expected for compounds SIP-A and SIP-B (Fig. 1A,D) and $[M+H]^+$ ions at *m/z* 764 and *m/z* 778 were identified. After purification and analysis of their HRMS and NMR data their structures were elucidated as β-D-forosaminyl-(1→4')-3'-*O*-demethylsilvalactam (**5**), which we propose to name sipanmycin A3, and its congener (**6**) carrying an extra carbon in the aliphatic substituent at C-2 which we designated as sipanmycin B3 (SIP-B3). Compared with wild-type sipanmycins A and B, in SIP-A3 and SIP-B3 the terminal sugar has been substituted by β-D-forosamine (which corresponds to β-3-demethyl,3-deoxy-D-sipanose) (Figs. S41-S52 and Table S5 for **5**, Figs. S53-S61 and Table S6 for **6**: https://figshare.com/s/87fcece083b744468230; Fig. 1D).

All the novel sipanmycin analogues so far described have altered sugars in the second (terminal) position of the disaccharide chain. In order to evaluate the possibility to obtain new sipanmycin analogues containing alternative sugars replacing the first sugar moiety, D-xylosamine, a mutant defective in the biosynthesis of UDP-xylosamine (149ΔXyl) was generated by the replacement of *sipS1*, *sipS2* and *sipS3* genes (encoding putative *N*-acetylglucosaminyl deacetylase, UDP-glucose-6-dehydrogenase and nucleoside-diphosphate sugar epimerase, respectively) by an apramycin resistance cassette (Fig. S62, https://figshare.com/s/87fcece083b744468230). Culture extracts of 149ΔXyl mutant strain showed neither production of compounds SIP-A and SIP-B nor even the aglyca of each compound. However, no novel glycosylated sipanmycin derivatives were produced by 149ΔXyl strain harboring plasmids pLNBIVT, pLNRT or pFL844T.

Novel sipanmycin derivatives generated by mutasynthesis

In an attempt to modify the sipanmycin aglycone by substituting the natural starter unit (β-glutamic acid) by other β-amino acids, a *Streptomyces* sp. CS149 non-producing sipanmycin mutant was generated by the replacement of *sipL4* (encoding a lysine 2,3-aminomutase) by the apramycin resistant cassette using plasmid pUH149ΔLAM (Fig. S63, https://figshare.com/s/87fcece083b744468230). Mutant obtained (149ΔLAM) was cultivated in R5A liquid medium and the production of sipanmycins was checked by UPLC analysis. Neither SIP-A nor SIP-B could be detected in this mutant strain (Fig. 2A,B). Production of sipanmycins was restored when 10 mM β-glutamic acid was added to a 24-hour old R5A cultures of 149ΔLAM mutant strain (Fig. 2C), thus proving the essential role of SipL4 enzyme in the biosynthesis of β-glutamic acid, the proposed starter unit in the biosynthesis of sipanmycins (14).

Twenty-four-hours R5A cultures of 149ΔLAM were independently fed with 10 mM of DL-β-leucine, DL-β-phenylalanine, β-alanine or racemic 3-amino-pentanoic acid (3-APA) and samples were extracted with ethyl acetate at 24 and 48 hours post-feeding. Analysis of UPLC chromatograms showed that, only in cultures fed with 3-APA two different peaks with the characteristic UV absorption spectra of sipanmycins were produced (Fig. 2D). Compounds **7** (*m/z* 808 $[M+H]^+$) and **8** (*m/z* 822 $[M+H]^+$) were purified and analyzed by HRMS and NMR allowing their structural elucidation (Figs. S64-S72 and Table S7 for **7**, Figs. S73-S77 and Table S8 for **8**: https://figshare.com/s/87fcece083b744468230). Compound **7**, 28-methylsipanmycin A, which we propose to name sipanmycin C (SIP-C), and its congener (**8**) carrying an extra carbon in the aliphatic substituent at C-2, which we propose to name sipanmycin D (SIP-D), correspond respectively to SIP-A and SIP-B analogues in which the starter unit

3-aminobutanoic acid (derived from β-glutamic acid) has been replaced by 3-amino-pentanoic acid (Fig. 2E).

Antibacterial and *in vitro* cytotoxicity of compounds

The novel sipanmycin analogues showed neither antibacterial activity against Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* nor antifungal activity against *Candida albicans*, as previously reported for the parental compounds (14), but they exerted moderate antibacterial activity against the Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus* (Table 1). In particular, some of them (SIP-A2b, SIP-A3, SIP-B3 and SIP-C) showed lower MIC values against *Staphylococcus aureus* than those of the corresponding parental compounds. *In vitro* cytotoxicity assays were also carried out against several tumor cell lines. Results pointed out that the strongest activity was exerted by the parental compound SIP-A (Table 2), with values of IC_{50} ranging between 96 nM and 1.72 μM, depending on the cell line tested. All the derivatives in which the D-sipanose has been replaced by another sugar presented higher IC_{50} values, suggesting that the second sugar moiety plays an essential role in the bioactivity of sipanmycins. For most of the cell lines tested, SIP-B and SIP-C showed a similar IC_{50} value compared to SIP-A, indicating that minor changes in the structure of the aglycone have a minor effect over sipanmycin cytotoxicity.

Discussion

Searching for new bioactive compounds to fight against multi-resistant bacteria, emerging infectious diseases or more effective chemotherapeutics has become a great challenge in the last years. Two of the strategies that are being employed to achieve this

goal are the isolation of drug producers from unexploited ecosystems and the modification of already known bioactive metabolites. Glycosylated compounds have proven to be one of the most important types of metabolites regarding their use in clinical treatments, as it is the case of erythromycin (antibiotic), amphothericin B (antifungal) and doxorubicin (chemotherapy) (9). In this sense, glycodiversification is a powerful tool directed to expand the structural diversity of glycosylated metabolites but it requires the presence of a GT capable of recognizing several aglyca and sugars as substrates. Combinatorial biosynthesis approaches have been successfully used to generate new derivatives of already known metabolites of diverse chemical structures (16,17). In the case of glycosylated compounds, this strategy led to the generation of novel analogues of mithramycin (12), paulomycin (7) or jadomycin (11) just to mention a few examples. Thus, we focused our work in the determination of the promiscuity of the GTs involved in sipanmycin glycosylation as well as the enzymes responsible of the introduction of the β-amino acid that serves as a starter unit of the PKS machinery and the possibility of generating novel derivatives by combinatorial biosynthesis and mutasynthesis.

Plasmids that encode the biosynthesis of L-digitoxose, L-amicetose, and D-olivose (pLNBIVT, pFL844T and pLNRT, respectively) (15) were introduced by intergeneric conjugation into *Streptomyces* sp. CS149 wild-type strain. Applying this combinatorial biosynthetic approach, six novel sipanmycin derivatives were obtained (Fig.1). SIP-A1 and SIP-B1 were the result of the interchange of the β-D-sipanose residue (the native terminal deoxyaminosugar in sipanmycins A and B) by an α-L-digitoxose residue, the sugar synthetized by pLNBIVT (Fig. 3). Similarly, when pLNRT was introduced into CS149 wild-type strain, the β-D-sipanose moiety was replaced by a β-D-olivose (SIP-A2; Fig. 3). In this case, we could not detect the sipanmycin analog of SIP-B containing

D-olivose (the expected SIP-B3 congener) probably due to its low production titer. Surprisingly, we have also observed the production of sipanmycin derivatives in which the second sugar moiety was not replaced by the sugar encoded by the plasmid introduced into the wild-type strain, but rather by a modified or non-expected sugar. Presumably, the biosynthetic machinery of the native CS149 D-sipanose (14) was interfering in the biosynthesis of the deoxysugar encoded in the introduced plasmid rendering a different than expected terminal deoxysugar in the isolated products or alternatively, the biosynthetic machinery related to the introduced deoxysugar-encoding plasmid was interfering in the biosynthesis of the native CS149 D-sipanose rendering an unexpected terminal deoxyaminosugar in the isolated products. In the context of the first scenario, TDP-4-keto-2,6-dideoxy-D-glucose (also known as TDP-4-keto-D-olivose), which is a common intermediate derived from both native *sip* genes or *ole* genes from the different introduced plasmids, might be methylated at C-3 position by the C-methyltransferase SipS5, the enzyme responsible of the 3-methylation of D-sipanose (14), and then the 4-keto group might be reduced by UrdR (from the introduced pLNRT plasmid encoding D-olivose biosynthesis) to generate the D-olivomycose present in SIP-A2b (Fig. 3). Conversely, in line with the second scenario, the aminodeoxysugar D-forosamine present in SIP-A3 and SIP-B3 could be biosynthetized by the coordinated action of genes involved in native CS149 D-sipanose biosynthesis with the extra participation of a gene from pFL844T (plasmid encoding L-amicetose biosynthesis). The reductase UrdQ from L-amicetose biosynthetic pathway would accept the common intermediate TDP-4-keto-2,6-dideoxy-D-glucose, catalyzing its C-3 deoxygenation to render TDP-4-keto-2,3,6-trideoxy-D-glucose which, by the action of aminotransferase SipS13, would be converted into TDP-4-amino-2,3,6-trideoxy-D-glucose that later would be dimethylated by SipS8 (as in D-sipanose

biosynthesis; Fig. 3) rendering the final TDP-D-forosamine. In conclusion, the GT pair SipS9/SipS14 involved in the second glycosylation step of sipanmycins, which act as an inverting GT, could accept, as activated TDP glycosides, different D-configured sugars, including neutral and aminodeoxysugars, and also deoxysugars from the L series such as L-digitoxose thus rendering sipanmycin analogues where the terminal sugar residue has a β-D or α-L absolute configuration according to Klyne's rule (18). Very interestingly, the SipS9/SipS14 pair is capable of transferring D-forosamine (as found in the new analogues SIP-A3 and SIP-B3), the same monosaccharide natively transferred by the spinosyn forosaminyltransferase SpnP (member of the GT-1 family) involved in the last glycosylating step in the biosynthetic pathway of spinosyns (19). Thus, it is logical to propose SipS9 and/or SipS14 as homologues of SpnP. We had already indicated (14) that SipS14 (but not SipS9) contain the 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-X4-G) already described for the GT SpnP (20). Fortunately, the structural studies carried out with SpnP (20) have identified the key residues for the glycosyl transfer such as the basic residue (H13) involved in deprotonation of the acceptor hydroxyl in the aglycon, and also the key negatively charged residues (D356 and E357) which interact with the positively charged tertiary amine at C-4 of the aminodeoxysugar forosamine. Likewise key residues forming hydrophobic interactions with the thymine moiety (L254, Y315, and L318) or the asparagine/threonine pair (N230 and T335) where the N230 amino group forms a hydrogen bond with the 3'-OH of the TDP unit (this asparagine/threonine pair sterically excludes UDP by clashing with its 2′-OH) have been identified together with key residues interacting with the pyrophosphate moiety via backbone NH groups (T335, T336) or side chain contacts (S11, S12, H331, S333, T335 and T336) (20). Alignment of the complete sequences of the structurally characterized GT SpnP with both SipS14

and SipS9 (and their homologs in incednine biosynthesis IdnS14 and IdnS9, involved in the transfer of *N*-demethyl-D-forosamine) (Fig.S78: https://figshare.com/s/87fcece083b744468230) revealed that the key basic residue H13 and the key residues involved in C-4-deoxyaminosugar recognition, D356 and E357, are present in SipS14 (and IndS14) but not in SipS9 (or IndS9) confirming this last protein as a mere auxiliary protein not having native GT activity for transferring C-4 aminodeoxysugars in agreement with our previous results where residual production of sipanmycin A was only observed in the Δ*sipS*9 mutant but not in the Δ*sipS*14 (14). Likewise, some of the SpnP key residues involved in the interaction with the TDP moiety were only observed in SipS14 (and IndS14) but not in SipS9 (or IndS9), whose classification as GT is thus doubtful (21). Overall, SpnP and SipS14 (and IdnS14) must be structurally similar GTs, belonging to the GT-1 family, which natively transfer C-4 aminodeoxysugars. The flexibility of SipS14 for transferring neutral and C-4 aminodeoxysugars of both L- and D- series described in this work not surprisingly parallels the flexibility already reported for its homolog SpnP which showed the ability to transfer L-mycarose (22) and L-olivose (19) instead of the native D-forosamine in biotransformation experiments with engineered strains of *Saccharopolyspora erythraea* leading to the generation of new spinosyn analogues. In a similar manner, the substrate-flexible glycosyltransferase DesVII (which also requires an auxiliary protein DesVIII), responsible for the transfer of the D-desosamine unit (a C-3 aminodeoxysugar) in the glycosylation step involved in narbomycin and macrolide antibiotic YC-13 biosynthesis, have shown its capacity to transfer also a variety of L- and D- neutral sugars instead of the native aminosugar (D-desosamine) in combinatorial biosynthesis experiments leading to new narbomycin analogues (23) and new antibiotic YC-13 analogues (24). Other flexible GTs worth mentioning include StaG, one of the GTs

involved in the indolocarbazole staurosporine biosynthesis, which is able to transfer several NDP-activated sugars (including L, D, neutral and aminated sugars) in heterologous expression experiments (25) or AraGT, the GT involved in aranciamycin biosynthesis, which accepts different nucleotide-activated neutral sugars of both L and D series (such as D-amicetose, L-rhodinose, L-rhamnose and L-axenose) in heterologous expression experiments (26).

Unfortunately, no sipanmycin derivatives carrying differences in the first aminosugar were found, even when plasmids harboring sugar biosynthetic genes were introduced into a mutant strain in which the three genes responsible of UDP-xylosamine were replaced by the apramycin resistance cassette. A reasonable explanation would be the inability of the GT pair SipS4/SipS15 to recognize a deoxysugar activated by TDP instead of UDP. A few examples of GTs that can recognize and transfer sugars activated with different NDPs have been described though. This is the case of VinC, the GT responsible of the attachment of TDP-vicenisamine to vicenistatin *in vivo* but that is also able to transfer UDP and ADP activated vicenisamine *in vitro* (27).

It has been demonstrated that the nature of the starter unit could determine important characteristics of the polyketide (28). In the case of the macrolactams the starter unit is a β-amino acid that is recognized by an adenylation enzyme, ligated to a standalone acyl carrier protein (ACP) and finally transferred to the PKS machinery (29). We explored the possibility of generating sipanmycin derivatives by substituting the 3-aminobutanoic acid (derived from β-glutamic acid) used by the PKS as a starter unit. Feeding experiments of 149ΔLAM cultures with β-Leu, β-Phe or β-Ala did not retrieve any sipanmycin analogue production, but compounds SIP-C and SIP-D were generated by adding 3-APA. These results are in accordance to those obtained by Cieslak and co-workers in 2017 (30). *In vitro* studies of the substrate specificity of IdnL1 (the

adenylation enzyme involved in the recognition of 3-aminobutanoic acid as the starter unit for the biosynthesis of incednine) showed its preference for short-chain 3-amino fatty acids such as 3-aminobutanoic acid or 3-APA. In contrast, IdnL1 exhibits weak activity against β-Ala, medium-chain fatty acids or the aromatic β-amino acid β-Phe. Thus, the failure in obtaining sipanmycin derivatives with β-Leu, β-Ala or β-Phe could be due to the high specificity of the adenylation enzyme SipL1. Amino acid sequence comparison between SipL1 and IdnL1 pointed out their high similarity (76.4%) which might explain the *in vivo* behavior observed in sipanmycin biosynthesis. Indeed, amino acids of the substrate-binding pocket showed that SipL1 could be classified into short-chain fatty acid recognition type of adenylation enzymes (group that includes IndL1) (29).

In conclusion, with the generation of eight novel sipanmycin analogues we have shown the substrate flexibility of some of the enzymes involved in the biosynthesis of these compounds, in particular those participating in the incorporation of the polyketide backbone starter unit and the glycosyltransferases involved in the attachment of D-sipanose, which could recognize different deoxysugars and aminodeoxysugars. The biosynthetic flexibility of those enzymes contrasts with the apparent stiffness of the biological activity showed by the novel compounds generated, which in general terms exert a lower cytotoxic or antibiotic activity compared to their parental compounds. The only exception to that observation corresponds to derivatives SIP-A2b, SIP-A3, SIP-B3 and SIP-C that were more active against *Staphylococcus aureus* than the parent compounds SIP-A or SIP-B.

Material and methods

Bacterial strains and culture conditions

Sipanmycin producer, *Streptomyces* sp. CS149 (13), was used as host strain during this work. For metabolite production, 30 ml of tryptic soy broth (TSB) were inoculated with spores and grown at 30ºC and 250 rpm for 24 h. This seed culture was used to inoculate 50 ml of R5A medium (31) to a final optical density at 600 nm of 0.2. Medium A (MA; 32) was used for sporulation; and mannitol soy medium + 20mM $MgCl_2$ (MS; 33) for intergeneric conjugation. *Escherichia coli* DH5α (34) was used for routine cloning procedures and *E. coli* ET12567/pUB307 (35,36) for intergeneric conjugation; both strains were grown on 2 x TY medium. Culture media were supplemented with antibiotics when needed: apramycin (100 µg/mL for *E. coli*, 25 µg/mL for *Streptomyces*), thiostreptone (25 µg/mL), kanamycin (25 µg/mL), tetracycline (10 µg/mL), chloramphenicol (25 µg/mL), and/or nalidixic acid (50 µg/mL).

DNA manipulation and plasmids used in this work

DNA manipulations were performed according to standard procedures for *E. coli* (37) and *Streptomyces* (38). All PCR amplifications were carried out with the high-fidelity polymerase Herculase II Fusion (Agilent Technologies) following the manufacturer instructions.

Plasmids pLNRT, pLNBIVT and pFL844T (15) were used for combinatorial biosynthesis (Fig. S2, https://figshare.com/s/87fcece083b744468230). pEM4T (39) was used as an empty vector for comparative purposes.

pUH149ΔXyl was generated to eliminate the genes involved in UDP-xylosamine biosynthesis (*sipS3*, *sipS2* and *sipS1*) in *Streptomyces* sp. CS149. Upstream and

downstream flanking regions were amplified by PCR (oligonucleotide pairs 149dXyl.5F (5'-TAT GAA TTC AAC TGG ACA TCG TCG CTG A -3') /149dXyl.5R (5'-TAT AAG CTT GCA CGC TCG ACG AGA TCA T-3') and 149dXyl.3F (5'-TAT CAT ATG GCG CAA CCA CTA TCA GGA GT-3')/149dXyl.3R (5'- TAT TCT AGA GCC AGG ACC ATC TTC ATC AC-3'); and cloned into pUO9090 (40). After digestion with SpeI, the replacement cassette was cloned into pHZ1358 (41) (Fig. S62, https://figshare.com/s/87fcece083b744468230).

pUH149ΔLAM was constructed in order to obtain a non-producing sipanmycin strain (149ΔLAM) by the replacement of the *sipL4* gene (coding for a lysine 2,3-aminomutase). Similarly as described above, flanking regions were amplified by PCR using oligonucleotides pairs 149dLAM64.5F (5'-TAT GAA TTC AGA CTG TAG ATG TGC GTG CG-3')/149dLAM64.5R (5'- TAT AAG CTT CCA CCT CGT CCA TGT GC TG -3') and 149dLAM64.3F (5'- TAT CAT ATG TGG ATG GAC CAT CTG GAG CT -3') /149dLAM64.3R (5'- TAT TCT AGA GTC CGG GTA CAC GTA GAA GC -3'), respectively. Amplicons were cloned into pUO9090 (40) at both sides of the apramycin resistance gene. The resulting plasmid was digested with SpeI and the replacement cassette was cloned into pHZ1358 (41) XbaI site to obtain the final conjugative vector for knockout experiments (Fig. S63, https://figshare.com/s/87fcece083b744468230).

Feeding experiments

All reagents were purchased from Sigma-Aldrich: β-Glutamic acid (Cat. Reference G1763-50MG), DL-β-Leucine (Cat. Reference 17988-1G-F), DL-β-Phenylalanine (Cat. Reference 159492-5G), β-Alanine (Cat. Reference 146064-25G) and racemic 3-amino-pentanoic acid (Cat. Reference BBO000720-1G). Aqueous solutions of these reagents

were added to 24-hour cultures of 149ΔLAM strain in R5A medium to a final concentration of 10 mM. Samples were extracted at 24 and 48 hours post-feeding to check the compound production.

Extraction, analysis by UPLC and HPLC-MS, and isolation of compounds by semipreparative HPLC

Whole cultures (1 mL) of selected strains were extracted with one volume of ethyl acetate at different times and analyzed by UPLC and HPLC-MS as described previously (13). Two-liter cultures of CS149 mutant strains in R5A medium were used to purify sipanmycin derivatives by semipreparative HPLC, as previously described (13).

Structural elucidation of novel compounds

Compounds **1**-**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. The structural elucidation of each compound was carried out by detailed analysis of 1D and 2D NMR spectra further assisted by comparison with the spectroscopic data reported for incednine (42), silvalactam (43), 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). Absolute configuration proposals were supported by biosynthetic arguments. A detailed description of the structural elucidation of each compound is presented in the supporting information alongside the corresponding spectral data (https://figshare.com/s/87fcece083b744468230).

452

453 **LC-DAD-ESI-TOF and NMR analyses**

454 HRMS and UV-vis spectra spectra were obtained by LC-DAD-ESI-TOF analyses
455 performed using an Agilent 1200RR HPLC equipped with a SB-C8 column (2.1 × 30
456 mm, Zorbax) coupled to a Bruker maXis Spectrometer. Chromatographic and ionization
457 conditions were identical to those we employed for our dereplication routines (44),
458 which are also the same previously employed for sipanmycins A and B (13) and the first
459 series of derivatives we obtained while studying their biosynthesis (14).
460 NMR spectra were recorded in CD_3OD at 24ºC on a Bruker AVANCE III-500 MHz
461 (500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively) equipped with a 1.7 mm TCI
462 MicroCryoProbe^{TM}, using the residual solvent signal as internal reference.
463

464 ***In vitro* cytotoxicity and antibiotic activity assays**

465 Cytotoxic activity of compounds was tested against the following human tumor cell
466 lines: colon adenocarcinoma (HT29), non-small cell lung cancer (A549), breast
467 adenocarcinoma (MDA-MB-231), promyelocytic leukemia (HL-60) and pancreatic
468 cancer (CAPAN-1). Mouse embryonic fibroblast cell line NIH/3T3 was used as control
469 to evaluate cytotoxicity against non-malignant cells. These analyses were carried out as
470 described before (14).
471 Antibiotic activity tests (minimal inhibitory concentration, MIC) were performed in 96-
472 wells microtiter plates. Fresh cultures of each microorganism (*Micrococcus luteus*,
473 *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and
474 *Candida albicans*) were used as seed cultures to inoculate the plates, with the
475 appropriate compound concentration, to a final OD=0.1 and total volume of 150µl per
476 well. Plates were incubated overnight at 37ºC (30ºC for *C. albicans*).

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Tables and Figures

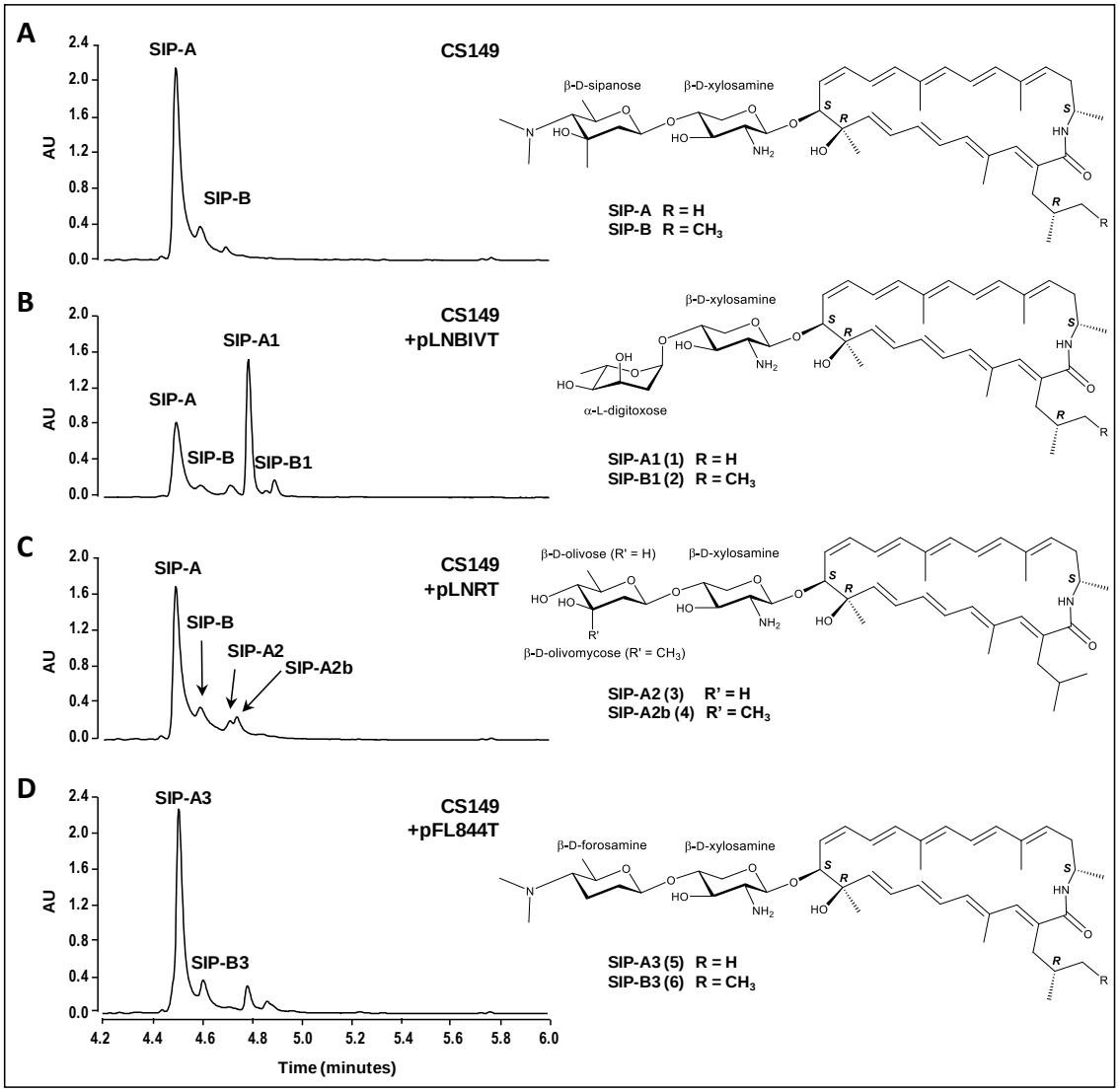
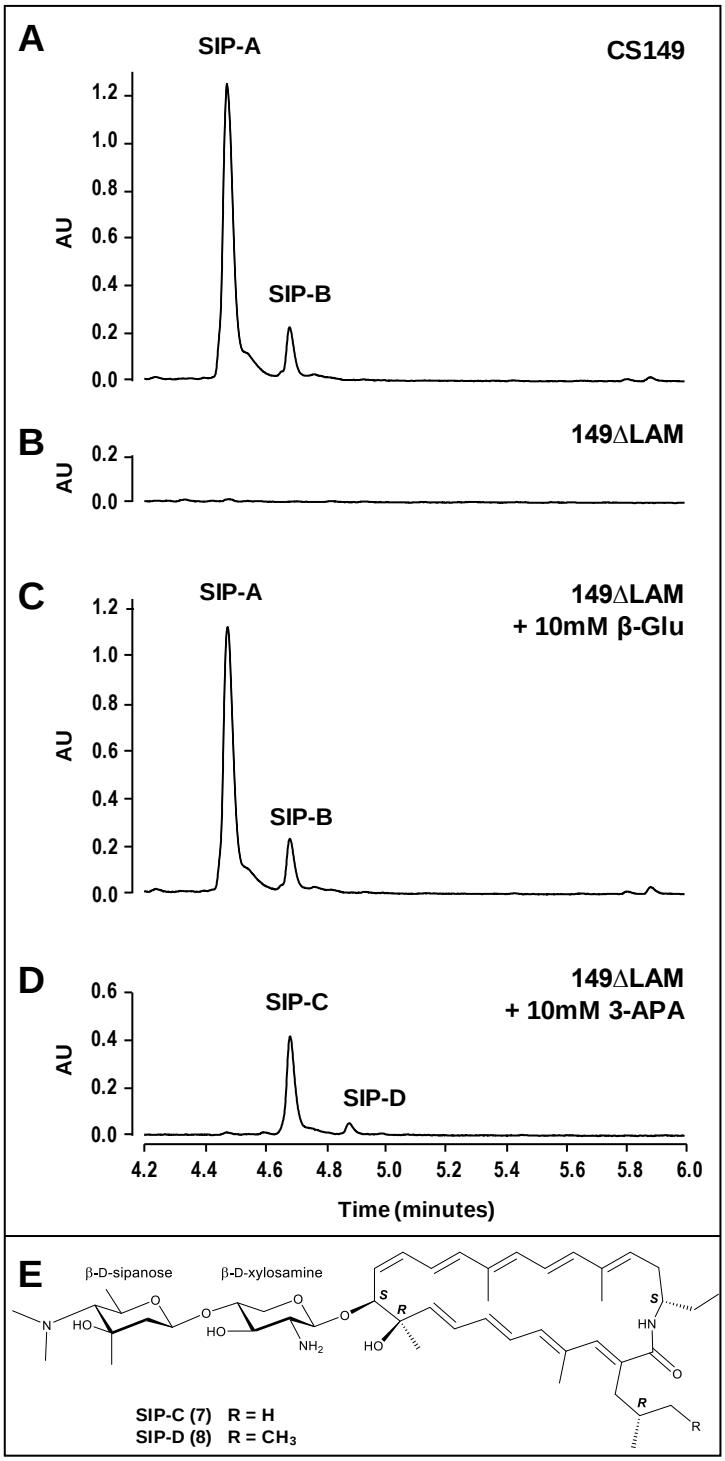
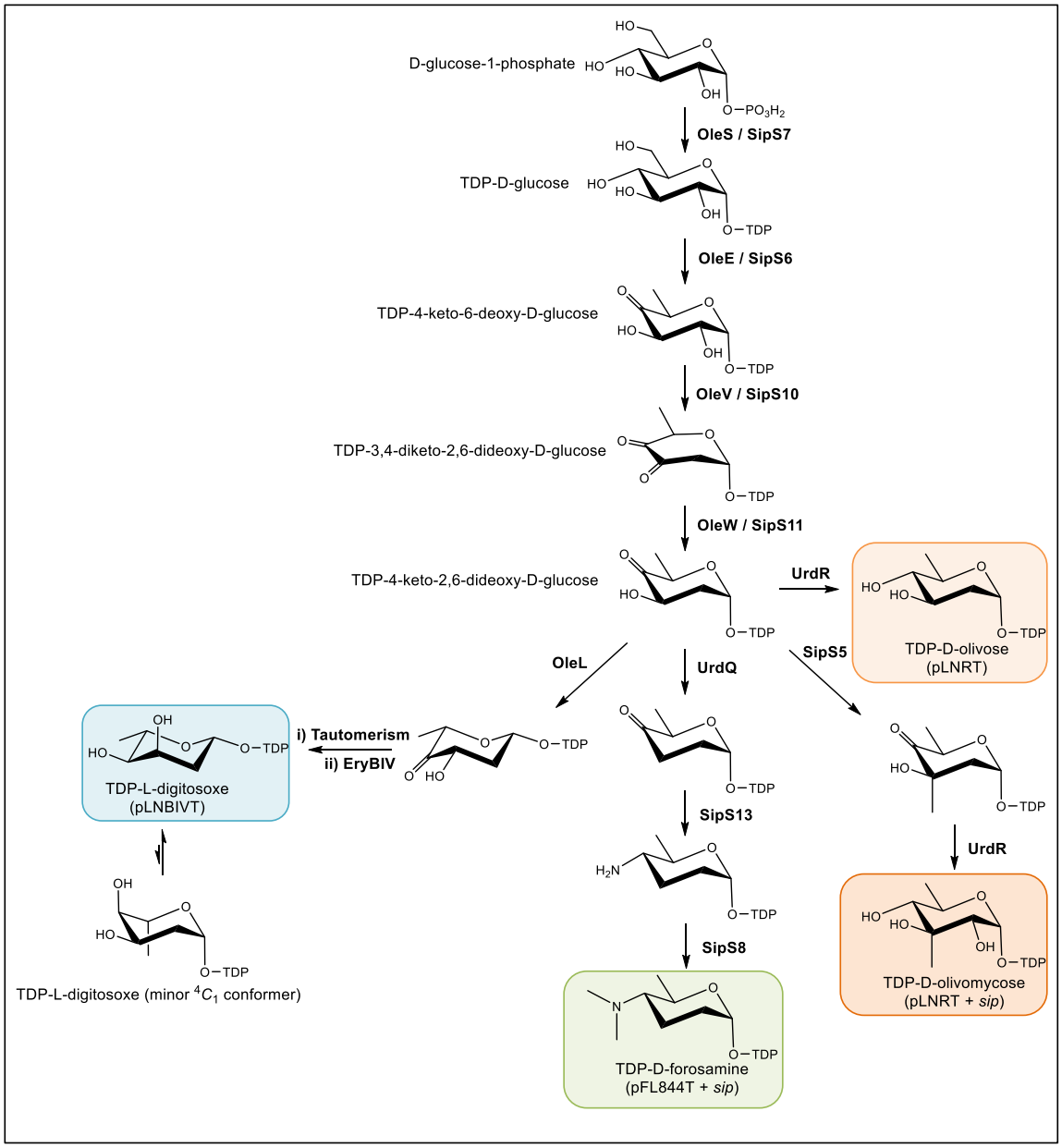


Figure 1. Novel sipanmycin analogues obtained by combinatorial biosynthesis. UPLC chromatograms at 320 nm of culture extracts of *Streptomyces* sp. CS149 wild-type **(A)**, CS149+pLNBIVT **(B)**, CS149+pLNRT **(C)** and CS149+pFL844T **(D)**. Molecular structures of the corresponding derivatives are included next to each chromatogram. As expected, a negative control strain harboring the empty vector pEM4T did not show any difference in its production profile compared to the wild-type strain (data not shown).



658

659 **Figure 2. Generation of sipanmycins C and D by mutasynthesis experiments.**
660 UPLC chromatograms at 320 nm of culture extracts of CS149 wild-type strain (A),
661 149ΔLAM mutant strain (B), and 149ΔLAM strain fed with β-glutamic acid (C) or with
662 3-amino-pentanoic acid (D). Chemical structures of SIP-C and SIP-D are shown (E).



663
664

665 **Figure 3. Proposed biosynthetic pathway of the new sugars attached to sipanmycin**
666 **derivatives obtained during this work.** Sip enzymes involved are shown in bold
667 letters. Squares mark the sugars in the final molecule of SIP-A1/SIP-B1 (blue), SIP-A2
668 (light orange), SIP-A2b (dark orange) and SIP-A3/SIP-B3 (green).

669 **Table 1.** Antibiotic activity of sipanmycins and derivatives.

MIC (μg/mL)	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*
SIP-A	3.22 ±1.87	10.58±6.55	>100.	>100.	>100
SIP-B	3.63±2.19	12.15±6.22	>100.	>100	>100
SIP-A1 (1)	10.66±7.06	16.31±9.39	>100	>100	>100
SIP-B1 (2)	19.17±3.56	26.84±8.60	>100	>100	>100
SIP-A2 (3)	9.41±2.34	22.59±3.76	>100	>100	>100
SIP-A2b (4)	4.79±0.25	8.87±0.71	>100	>100	>100
SIP-A3 (5)	4.45±0.55	6.68±2.06	>100	>100	>100
SIP-B3 (6)	7.29±3.43	9.72±1.21	>100	>100	>100
SIP-C (7)	4.71±0.58	6.06±2.85	>100	>100	>100

670

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

IC_{50} (µM)	3T3 (fibroblasts)	A549 (lung)	HT29 (colon)	HL60 (leukemia)	CAPAN-1 (pancreas)	MDAMB231 (breast)
SIP-A	0.134	0.096	0.201	1.72	0.189	0.181
SIP-B	0.103	0.096	0.185	2.54	0.206	0.213
SIP-A1 (1)	0.410	1.15	0.783	1.52	1.99	1.26
SIP-B1 (2)	0.436	0.430	0.718	2.16	2.10	0.466
SIP-A2 (3)	0.618	0.529	0.647	1.26	4.03	0.633
SIP-A2b (4)	0.448	1.28	0.436	0.686	2.81	2.05
SIP-A3(5)	0.184	0.229	0.410	3.39	0.676	0.448
SIP-B3(6)	0.187	0.233	0.364	2.13	0.959	0.469
SIP-C (7)	0.124	0.144	0.146	3.91	0.289	0.194

672