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Engineering Deoxysugar Biosynthetic Pathways from Antibiotic-Producing Microorganisms: A Tool to Produce Novel Glycosylated Bioactive Compounds

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Summary

A plasmid (pLN2) was generated in which genes involved in the biosynthesis of L-oleandrose in the oleandomycin producer *Streptomyces antibioticus* ATCC11891 were cloned. pLN2 was used to direct the biosynthesis of different deoxysugars by exchanging and/or adding genes from other antibiotic biosynthetic clusters. Transfer of the synthesized deoxysugars to the tetracenomycin C aglycon, 8-demethyl-tetracenomycin C, through the use of the "sugar flexible" glycosyltransferase ElmGT, validated the system. Several pLN2 derivatives were constructed by replacement of the *oleU* 4-ketoreductase gene by different 4-ketoreductase genes. Some of them, such as EryBIV and UrdR, reduced the keto group of the 4-keto intermediates, generating L-olivosyl and D-olivosyl derivatives, respectively. The system was also used to generate an L-rhamnosyl derivative (through a two-gene deletion) and an L-rhodinosyl derivative (through a combination of a gene replacement and a gene addition).

Introduction

Sugars are frequently structural components of natural products. Important antibiotics (erythromycin), antifungals (amphotericin B), antiparasites (avermectins), and anticancer drugs (doxorubicin) possess sugars attached to the aglycon core. These sugar components usually participate in the molecular recognition of the cellular target by the bioactive compound and, therefore, its presence is important, in many cases essential, for the biological activity of many natural products [1]. A great majority of these sugars belong to the 6-deoxyhexoses (6DOHs). These sugars are synthesized from nucleoside diphosphate-activated hexoses (mainly D-glucose) via a 4-keto-6-deoxy intermediate. Two common enzymatic steps leading to the biosynthesis of this intermediate are catalyzed by a dNDP-D-hexose synthase and dNDP-D-hexose-4,6-dehydratase [2, 3]. The different 6DOHs will vary depending on the substituents and/or the stereochemistry at carbon atoms at positions 2, 3, 4, or 5 of the hexose carbon chain, resulting from deoxygenations, transaminations, and/or C, N, or O methylations [2, 3, 4]. D- and L-isomeric forms of many 6DOHs also exist as a result of the action of a 5- or a 3,5-epimerase.

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In recent years, a number of 6DOHs gene clusters have increasingly been characterized, most of them participating in the biosynthesis of different antibiotics produced by actinomycetes [5]. In a few cases, biosynthetic gene clusters for the same 6DOHs have been characterized from different producer organisms. This is the case for the D-desosamine gene cluster that has been characterized from the erythromycin [6, 7], oleandomycin [8, 9], pikromycin [10], and megalomicin [11] producer streptomycetes. Also the D-olivose gene cluster has been cloned from the mithramycin [12, 13], urdamycin [14], and landomycin [15] producers and the L-oleandrose cluster from the oleandomycin [8, 9] and avermectin [16] producers. In spite of the increasing knowledge about 6DOH biosynthesis, the isolation and purification of nucleotide-activated sugars has been hampered because of instability and technological aspects. The early report by Schulman and coworkers on the isolation and purification of dTDP-L-oleandrose from the avermectin producer [17] was not followed by the purification of other NDP-activated sugars from producer organisms.

The deoxysugars are transferred to the corresponding aglycon by glycosyltransferases, which are generally sugar-, aglycon-, and site-specific. In recent years, increasing evidence has suggested some degree of "flexibility" of glycosyltransferases involved in the biosynthesis of secondary metabolites, and there have been some reports of examples in which different deoxysugars have been transferred by a glycosyltransferase to its aglycon [9, 13, 18–26]. One of these glycosyltransferases, the elloramycin glycosyltransferase (ElmGT), has been shown to be especially "flexible" in accepting different L- and D-deoxysugars and also being able to transfer a disaccharide [24]. Recently, several plasmids that direct the biosynthesis of either L-daunosamine [27], L-olivose [9], L-oleandrose [9, 28] or D-desosamine [29] have been reported. These plasmids contain different subsets of genes involved in the biosynthesis of these deoxysugars from several antibiotic-producing organisms.

Here we report the construction of a novel plasmid by using genes from L-oleandrose biosynthesis in the oleandomycin producer *Streptomyces antibioticus* ATCC11891, which can be used as a "plug and play" system for the exchange of selected genes and substitution by homologous genes. It can thus potentially generate a great variety of deoxysugars. We also provide evidence of the formation of the different sugars through the ability of the "sugar flexible" elloramycin glycosyltransferase, ElmGT, to transfer the newly synthesized sugars to the elloramycin aglycon 8-demethyl-tetracenomycin C.

Results and Discussion

Rationale behind the Selected Approach

A great variety of 6DOHs are present in a large number of bioactive natural products [1]. They can be grouped on the basis of their isomer forms at carbon 5 in L- and D-sugars and also according to the deoxygenation pattern of the different carbons [2–4, 30, 31]. We planned to design a plasmid-based sugar-synthesizing system

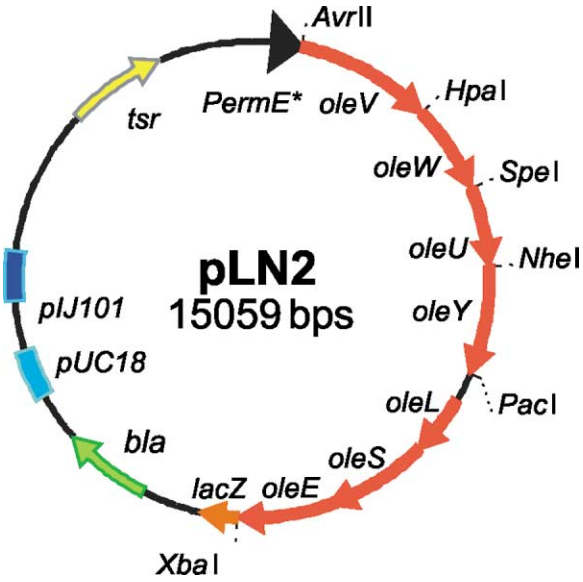

Figure 1. Map of pLN2

Only restriction sites flanking the different *ole* genes that were used for subcloning are indicated. Abbreviations are as follows: P*ermE**, erythromycin resistance promoter; *tsr*, thiostrepton resistance gene; *bla*, β-lactamase gene: pUC18, replication origin of pUC18; pIJ101, replication origin of pIJ101.

("cassette plasmid") that could be a useful genetic tool for endowing different host strains with the capability of synthesizing various 6DOHs. This plasmid would also be useful for assaying potential activities and substrate flexibility of different sugar biosynthetic gene products by replacing selected genes from the plasmid with homologous counterparts. To acheive these goals, we gave the plasmid several specific features. First, the different sugar biosynthetic genes needed to be organized in such a way that they could be easily removed and replaced ("plug and play system") by other related genes; for this reason, in this "cassette plasmid" the different genes had to be cloned with unique and rare restriction sites flanking each gene to facilitate its exchange. Second, we chose a bifunctional replicative plasmid that would make it easy to create the different constructs in *E. coli* and to transfer them into the *Streptomyces* recipient host by protoplast transformation. Finally, the plasmid had to include a promoter region to control the transcription of the genes; we used the erythromycin resistance gene promoter (*ermEp**) from *Saccharopolyspora erythraea*.

Construction of an L-Olivose-Synthesizing Cassette Plasmid and Validation of the Assay System

A plasmid (pLN2) was constructed to serve as starting-base material for the generation of different plasmids capable of directing the biosynthesis of L-2,6-deoxysugars (Figure 1). This plasmid was also designed in such a way that it could be easily converted into a derivative directing the biosynthesis of different L-6-deoxysugars (see below). Seven genes (*oleV, oleW, oleU, oleY, oleL, oleS*, and *oleE*) involved in the biosynthesis of the neutral sugar L-oleandrose (*ole* genes) by *Streptomyces antibioticus,* producer of the 14-membered macrolide oleandomycin [8, 9], were used for the construction of this plasmid (Table 1). Six out of the seven genes (*oleV, oleW, oleU, oleL, oleS*, and *oleE*) code the enzymatic functions that are necessary for the biosynthesis of NDP-L-olivose [9], and the seventh one, *oleY,* codes for a 3-O-methyltransferase responsible for the conversion of L-olivose into L-oleandrose late during oleandomycin biosynthesis, in a step occurring after the transfer of L-olivose to the aglycon [32]. Three of the genes (*oleL, oleS*, and *oleE*) were incorporated to the construct as a single DNA fragment because they code for enzymes catalyzing the three early and common steps in the biosynthesis of all L-6-deoxysugars: activation of glucose-1-phosphate into NDP-D-glucose (*oleS*), dehydration at carbons 4 and 6 (*oleE*), and epimerization at carbons 3 and 5 (*oleL*). The rest of the genes were incorporated into the construct as PCR-amplified DNA fragments flanked by unique restriction sites not frequently found in *Streptomyces* DNA.

Due to the difficulties in detecting NDP-6DOH in living organisms, it was necessary to establish a system that would allow the detection of the plasmid-directed 6DOHs. This system consisted of detecting the formation of the NDP-6DOHs by their transfer to an aglycon and the subsequent formation of the corresponding glycosylated compounds. For this purpose we developed engineered host strains containing the appropriate gly-

Table 1. Genes Used for the Construction of the Different Plasmids

Gene	Microorganism	Antibiotic	Sugar pathway	Proposed function	Reference
oleS	*S. antibioticus*	Oleandomycin	L-oleandrose and D-desosamine	glucose-1-phosphate: TTP thymidylyl transferase	[9]
oleE	*S. antibioticus*	Oleandomycin	L-oleandrose and D-desosamine	glucose 4,6-dehydratase	[9]
oleL	*S. antibioticus*	Oleandomycin	L-oleandrose	3,5-epimerase	[9]
oleV	*S. antibioticus*	Oleandomycin	L-oleandrose	2,3-dehydratase	[9]
oleW	*S. antibioticus*	Oleandomycin	L-oleandrose	3-ketoreductase	[9]
oleU	*S. antibioticus*	Oleandomycin	L-oleandrose	4-ketoreductase	[9]
oleY	*S. antibioticus*	Oleandomycin	L-oleandrose	3-O-methyltransferase	[8]
eryBIV	*Sacc. erythraea*	Erythromycin	L-mycarose	4-ketoreductase	[6, 7]
snogC	*S. nogalater*	Nogalamycin	L-nogalose	4-ketoreductase	AAF01815
urdZ3	*S. fradiae*	Urdamycin A	L-rhodinose	4-ketoreductase	[14]
dnmV	*S. peucetius*	Daunorubicin	L-daunosamine	4-ketoreductase	[32]
tylD	*S. fradiae*	Tylosin	6-deoxy-D-allose	4-ketoreductase	[33, 34]
urdR	*S. fradiae*	Urdamycin A	D-olivose	4-ketoreductase	[14]
urdQ	*S. fradiae*	Urdamycin A	L-rhodinose	3,4-dehydratase	[14]

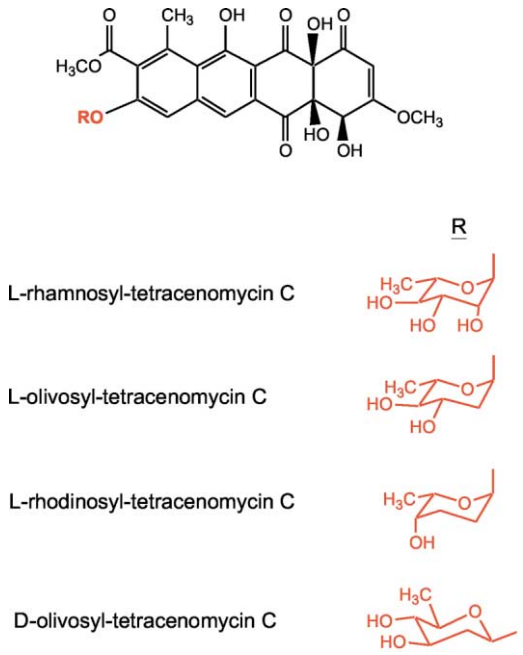

Figure 2. Chemical Structure of 8-Demethyl-Tetracenomycin C and the Different Glycosylated Compounds Generated with the ElmGT Glycosyltransferase and pLN2 Derivatives

cosyltransferase gene. We took advantage of the "sugar flexibility" of the ElmGT glycosyltransferase from the elloramycin pathway, which transfers different sugars to the aglycon 8-demethyl-tetracenomycin C (8DMTC) (Figure 2). Capable of transferring several L-6-deoxysugars (L-olivose, L-rhamnose, L-rhodinose) and D-6-deoxysugars (D-olivose, D-mycarose) into 8DMTC [24, 26], ElmGT has been shown to possess a "flexible" substrate specificity with respect to the sugar. We used two different approaches, providing the aglycon either endogenously or exogenously. In the former, we used *S. albus* 16F4, a recombinant strain harboring cosmid 16F4; this is a cosmid that directs the biosynthesis of 8DMTC and also contains *elmGT* [24, 26]. In the latter, we provided the aglycon by feeding *S. albus* GB16 with 8DMTC; this is a recombinant strain in which *elmGT* is integrated into the chromosome and has its expression under the control of *ermEp* [24].

To assay the functionality of pLN2, we transformed strains *S. albus* 16F4 and GB16 with pLN2. HPLC analysis showed that the new recombinant strain *S. albus* 16F4 (pLN2) produced a new compound that was presumed to be L-olivosyl-tetracenomycin C (LOLV-TCMC) because of its HPLC mobility and absorption spectrum when compared with those of the pure compound used as a standard (Figure 3A). The same compound was detected after biotransformation of *S. albus* GB16 (pLN2) with 8DMTC. MALDI-TOF analysis of this compound showed molecular peaks at m/z 611.0815 and 627.0728 for the sodium and potassium adducts of LOLV-TCMC, respectively. The efficiency of the formation of LOLV-TCMC was much higher with strain 16F4 (64% of all 8DMTC formed was converted into the glycosylated compound) than with strain GB16 (12% conversion). This difference can be explained by the endogenous formation of the aglycon in the former case in contrast to the exogenously added aglycon in the latter.

The conclusion from these experiments is that pLN2 was functional and directs the biosynthesis of L-olivose. Consequently, this plasmid could now be used as a starting point for further studies, including analysis of the function of different sugar biosynthetic enzymes.

Assaying the Function and Substrate Flexibility of Different Sugar 4-Ketoreductase Genes by Single Gene Substitutions

In order to assay the versatility of the pLN2 "cassette plasmid," we replaced *oleU* by other genes that are also reported to code 4-ketoreductases in other antibiotic biosynthetic pathways but that act on different sugar intermediates (Table 1; Figures 4 and 5). In a first step, we chose *eryBIV* and *snogC*, which code 4-ketoreductases involved in the biosynthesis of erythromycin [6, 7] and nogalamycin (accesion number AAF01815). The function of EryBIV was assigned after analysis of products accumulated by a strain with a mutation in its coding gene. EryBIV would catalyze the final 4-keto reduction step in the biosynthesis of L-mycarose [6, 7]. In the case of SnogC, its function in L-nogalose biosynthesis has been proposed based on its similarity to known 4-ketoreductases. Although the three ketoreductases (OleU, EryBIV, and SnogC) act on different intermediates, ketoreduction at C-4 occurs with the same stereospecificity (Figure 4). After replacement of *oleU* by *eryBIV* (plasmid pLNBIV) a new HPLC peak appeared in both host strains (16F4 and GB16), the conversion being higher with 16F4 (20% for 16F4 and 3% for GB16). The compound was also identified as LOLV-TCMC by its HPLC mobility, absorptium spectrum, and m/z values, as shown previously for pLN2. The amounts of LOLV-TCMC formed were lower with pLNBIV than with pLN2. This can be explained by the different natural substrates of the OleU and EryBIV reductases. EryBIV would act on a sugar biosynthetic intermediate (in L-mycarose biosynthesis) with a hydroxyl group in axial configuration and a methyl group in equatorial configuration at C-3, while OleU would act (in L-olivose biosynthesis) on an unmethylated sugar intermediate with a hydroxyl group in equatorial configuration at C-3. The fact that this last intermediate is formed (in the presence of pLNBIV) would explain the difference in yields of LOLV-TCMC.

The fact that *eryBIV* can be used to produce an L-olivose-glycosylated derivative demonstrates a certain degree of flexibility of this ketoreductase because it can act on both C-3-methylated and -unmethylated intermediates with different configurations of the hydroxyl group at C-3.

Substitution of *oleU* by *snogC* (generating pLNS) did not generate any glycosylated compound after biotransformation with the aglycon 8DMTC. Because the expected compounds would contain either an L-olivose or an L-rhamnose moiety and these sugars have been shown to be transferred by ElmGT, the absence of any glycosylated compound indicates that the SnogC ketoreductase did not act on the 4-keto intermediates that could have been synthesized. The conclusion was that SnogC has a narrower sugar-substrate specificity.

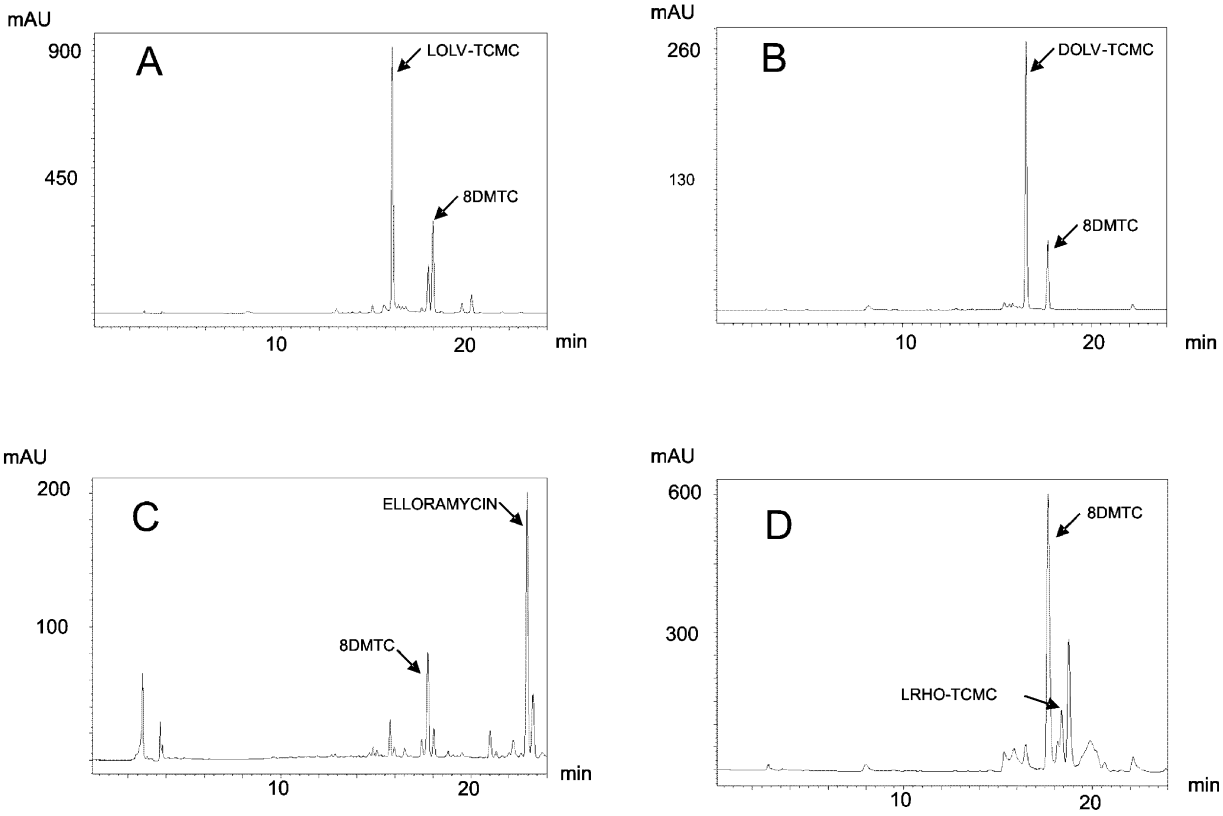

Figure 3. HPLC Analysis of Cultures of Different Recombinant Strains Harboring pLN2 Derivatives and Producing 8DMTC Glycosylated Compounds

(A) Strain harboring pLN2.
(B) Strain harboring pLNR.
(C) Strain harboring pLN2Δ.
(D) Strain harboring pLNRHO.

We also assayed two other 4-ketoreductases, DnmV and UrdZ3, which have different steroespecificity than OleU and also act on different sugar intermediates (Figures 4 and 5). DnmV is a 4-ketoreductase involved in the biosynthesis of the aminosugar L-daunosamine in *S. peucetius* [33]. The UrdZ3 4-ketoreductase participates in the biosynthesis of the 2,3,6-trideoxysugar L-rhodinose, one of the sugars forming part of the angucycline urdamycin A [14]. Provided that these two reductases could recognize the pLN2-directed 4-keto intermediate and because of the different stereoespecifity at C-4 with respect to OleU, the formation of 2-deoxy-L-fucose (L-oliose) instead of L-olivose (formed with the participation of OleU) should be expected when *oleU* is replaced by either *dnmV* (plasmid pLNV) or *urdZ3* (plasmid pLNZ3). Neither of the strains 16F4 or GB16 harboring either pLNV or pLNZ3 produced any new compound, suggesting that either L-oliose was not generated or, if it was, that it could not be recognized and transferred by the ElmGT glycosyltransferase.

Two other 4-ketoreductases, TylD and UrdR, were tested (Figures 4 and 5). Both act on different D-6-deoxysugar intermediates but have the same stereoespecificity at C-4. TylD catalyzes a 4-keto reduction as the final step in the formation of 6-deoxy-D-allose, one of the three 6DOHs present in the macrolide antibiotic tylosin [34, 35]. Strains 16F4 and GB16 harboring *tylD* (plasmid pLNT) did not produce any glycosylated product. UrdR is a 4-ketoreductase involved in the biosynthesis of D-olivose in urdamycin A biosynthesis by *S. fradiae* [14]. In contrast to that of *tylD*, replacement of *oleU* by *urdR* led to the formation of a new glycosylated derivative (Figure 3B) with m/z values of 611.1532 and 627.1467 for the sodium and potassium adducts; these values are equivalent to those obtained for LOLV-TCMC (see above). However, this compound showed an HPLC retention different from that of LOLV-TCMC but had the same absorption spectrum, suggesting its identity with the D-isomer form, D-olivosyl-tetracenomycin C (DOLV-TCMC). This was confirmed by comparison of its HPLC mobility with that of pure DOLV-TCMC, used as a standard. Formation of DOLV-TCMC occurred with a high efficiency (79%) in the case of strain 16F4. It is worth to mentioning that this D-sugar was formed in the presence of the OleL 3,5-epimerase. To explain the formation of D-olivose, one must assume that NDP-4-keto-2,6-dideoxy-D-glucose is a common intermediate in the biosynthesis of L- and D-olivose. In the case of L-olivose biosynthesis (directed by pLN2), this 4-keto intermediate would first be a substrate for the OleL 3,5-epimerase,

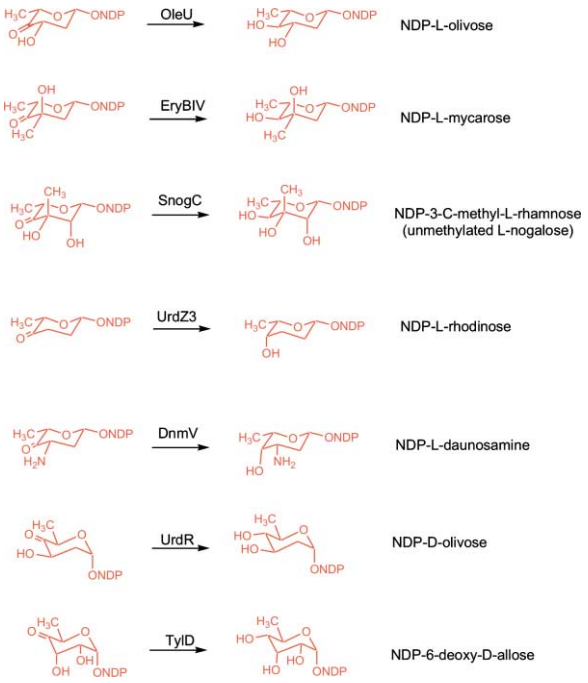

Figure 4. Enzymatic Steps Catalyzed by the Different 4-Ketoreductases Used in this Study

and the OleU reductase would reduce the resulting 4-keto product (Figure 6). However, when *oleU* was replaced by *urdR* (plasmid pLNR), the NDP-4-keto-2,6-dideoxy-D-glucose would be diverted by the UrdR ketoreductase, generating D-olivose (Figure 6).

Designing a Construct Directing the Biosynthesis of the 6DOH L-Rhamnose by Two-Gene Deletion

L-Rhamnose and L-olivose are two 6DOHs that only differ in the oxygenation state at C-2; L-rhamnose contains a hydroxyl group, and L-olivose does not. Because pLN2 was designed in such a way that one can easily delete the genes involved in the 2-deoxygenation step (*oleV* and *oleW*), we were able to create a derivative of pLN2 by deleting these two genes. The resultant construct (plasmid pLN2Δ) should direct the biosynthesis of the 6DOH L-rhamnose. These two genes were removed by digestion of pLN2 with AvrII and SpeI and further religation (these enzymes generate compatible cohesive ends). The final construct (pLN2Δ) was introduced into strains 16F4 and GB16, and the formation of glycosylated derivatives was analyzed by HPLC and MALDI-TOF. 8DMTC was efficiently converted into glycosylated compounds (Figure 3C). In the case of strain 16F4, 8DMTC was converted into elloramycin because, once L-rhamnose is transferred by ElmGT, the three O-methyltransferases present in cos16F4 methylate the free hydroxyl groups of L-rhamnose [36]. MALDI-TOF analysis of the glycosylated compound formed by strain GB16 showed molecular peaks at m/z values of 627.0641 and 643.0388 for the sodium and potassium adducts, which is consistent with the formation of LRHA-TCMC. In the case of strain 16F4, MALDI-TOF analysis in the negative mode showed a molecular peak at an m/z value of 659.0380, corresponding to elloramycin. The identity of these compounds was also verified by comparison with pure standards. Three conclusions can be drawn from this experiment: (1) pLN2Δ contains all genes required for the biosynthesis of L-rhamnose; (2) the OleU 4-ketoreductase that naturally has as a substrate a 2,6-dideoxy sugar intermediate in the biosynthesis of L-olivose can also reduce a 6-deoxy intermediate, and (3) although the OleL epimerase usually acts on 2,6-dideoxy intermediates (see the above results for pLNR), it can also recognize and epimerize a 6-deoxy intermediate.

We also attempted to construct another plasmid that directs the biosynthesis of L-rhamnose. The two genes (*oleV* and *oleW*) involved in the 2-deoxygenation step were deleted from pLNBIV, a construct that has been also shown to direct the biosynthesis of L-olivose (see above). The resultant construct (pLNBIVΔ) was introduced in *S. albus* 16F4 and *S. albus* GB16 and assayed for the potential formation of 8DMTC glycosylated derivatives. In both cases, no glycosylated derivatives were formed. This result indicates that, in contrast to OleU, which was able to reduce a 6-deoxy intermediate (see above), EryBIV did not recognize that intermediate. This suggests that EryBIV probably requires a C-2-deoxygenated sugar intermediate for its action.

Combining Gene Replacement and Gene Addition to Design a Construct that Directs the Biosynthesis of L-Rhodinose

L-olivose and L-rhodinose are 6DOHs that differ in the hydroxyl groups at C-3 and C-4; L-olivose is a 2,6-dideoxysugar with an equatorial hydroxyl group at C-4, and L-rhodinose is a 2,3,6-trideoxysugar with an axial hydroxyl at C-4. To design a plasmid that directs the biosynthesis of L-rhodinose, we used pLN2 as starting construct to first replace the ketoreductase *oleU* by another ketoreductase able to direct the reduction step with different stereochemistry. This was already achieved in the previous construct, pLNZ3 (see above). Next, it was necessary to add a second gene coding for a 3,4-dehydratase. For this purpose we chose *urdQ* from the urdamycin biosynthetic cluster [14]. The final construct (pLNRHO) was used to transform protoplasts of the recipient host strains 16F4 and GB16. A new compound was formed (Figure 3D). MALDI-TOF analysis in the negative mode showed an m/z value of 571.0760, which is consistent with the formation of L-rhodinosyl-tetracenomycin C (LRHO-TCMC). HPLC analysis also showed this compound to have mobility identical to that of pure LRHO-TCMC, which was used as a standard.

Discussion

Sugars contribute to the structural biodiversity of natural products. Moreover, biological activity of many of these compounds is dependent on the sugar moieties, which constitute an important part of the molecule in its interaction with the drug target site. Changing the sugar moieties in natural products might be an important contribution to the generation of novel derivatives with potentially new pharmacological properties. Some studies have reported plasmids that direct the biosynthesis of

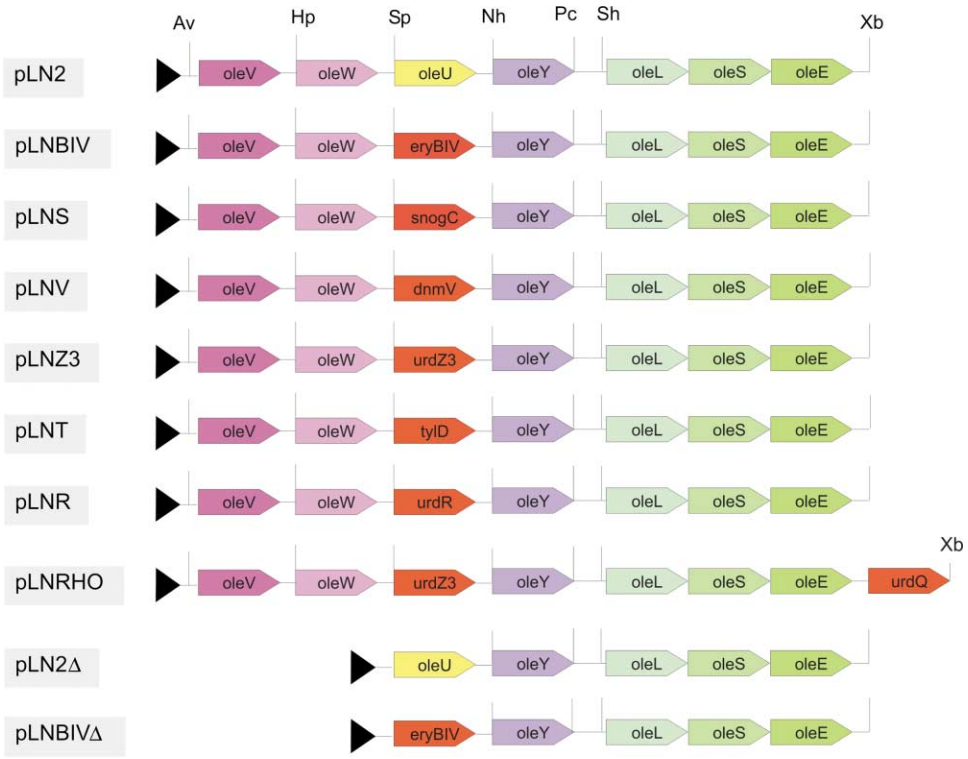

Figure 5. Organization of *ole* Genes in pLN2 and Plasmid-Borne Constructs Derived from pLN2

Black triangles indicate the erythromycin resistance promoter. Red arrows indicate genes substituting *ole* genes in the different constructs. Abbreviations are as follows: Av, AvrII; Hp, HpaI; Nh, NheI; Pc, PacI; Sp, SpeI; Sh, SphI; Xb, XbaI.

different 6DOHs [9, 27–29]. These plasmids were generated through subcloning DNA regions from the chromosome of different antibiotic-producing streptomycetes into appropriate plasmid vectors. The plasmid construct reported in this work (pLN2) confers to the recipient host strains the capability of synthesizing L-olivose in its NDP-activated form. Furthermore, it possesses several important technological advantages over the others. First, some of the genes are flanked by restriction sites for enzymes that do not frequently recognize streptomycetes DNA, so they can be easily replaced by homologous genes. This allows an assay of the specific function of selected genes (and their products) and makes it possible to test the flexibility of the sugar biosynthetic enzymes in recognizing different biosynthetic intermediates. In this work, we have tested the "flexibility" of different 4-ketoreductases and found that some of them possess a certain degree of flexibility with respect to the sugar substrate. An example from the erythromycin pathway is the EryBIV ketoreductase, which has been shown to reduce the 4-keto group at C-4 and to react with different stereochemistry on both C-3-methylated and -unmethylated intermediates, independently of the stereochemistry of the hydroxy group at C-3. In some cases, a single gene exchange was sufficient to generate a different sugar. This was the case for the substitution of *urdR* for *oleU,* both coding 4-ketoreductases. Interestingly, the starting construct (pLN2) directs the biosynthesis of an L-6-deoxysugar, and through this substitution, a D-6-deoxysugar was formed. An explanation to this fact would be that the NDP-4-keto-2,6-dideoxy-D-glucose intermediate is a common biosynthetic intermediate to both L- and D-olivose.

A second utility of this plasmid is the incorporation of new enzymatic functions through the insertion of additional genes. This is facilitated by the presence of several unique restriction sites in these plasmids. This is exemplified by the generation of pLNRHO in which, in addition to exchange of the 4-ketoreductase gene, a new gene function was incorporated (a 3,4-dehydratase) in order to direct the biosynthesis of L-rhodinose. An additional advantage of pLN2 is the simplicity of converting 2,6-deoxysugars into 6-deoxysugars by a single two-gene deletion. This utility was proven by the generation of pLN2Δ from pLN2 and the consequent biosynthesis of L-rhamnose instead of L-olivose.

These plasmids are also important tools for producing novel glycosylated compounds within recipient hosts. In this work, we have taken advantage of the sugar flexibility of the ElmGT glycosyltransferase to produce several glycosylated tetracenomycin derivatives containing L-rhamnose, L-olivose, L-rhodinose, and D-olivose. These compounds were previously reported to be produced by antibiotic-producing microorganisms containing a cosmid clone conducting the biosynthesis of 8DMTC and having the endogenous capability of synthesizing these sugars [24, 26, 37]. Here we show a novel approach that can be used to produce these compounds in antibiotic-producing and antibiotic-nonproducing microorganisms through the incorporation of specific constructs directing sugar biosynthesis. Although in the present work these constructs have been

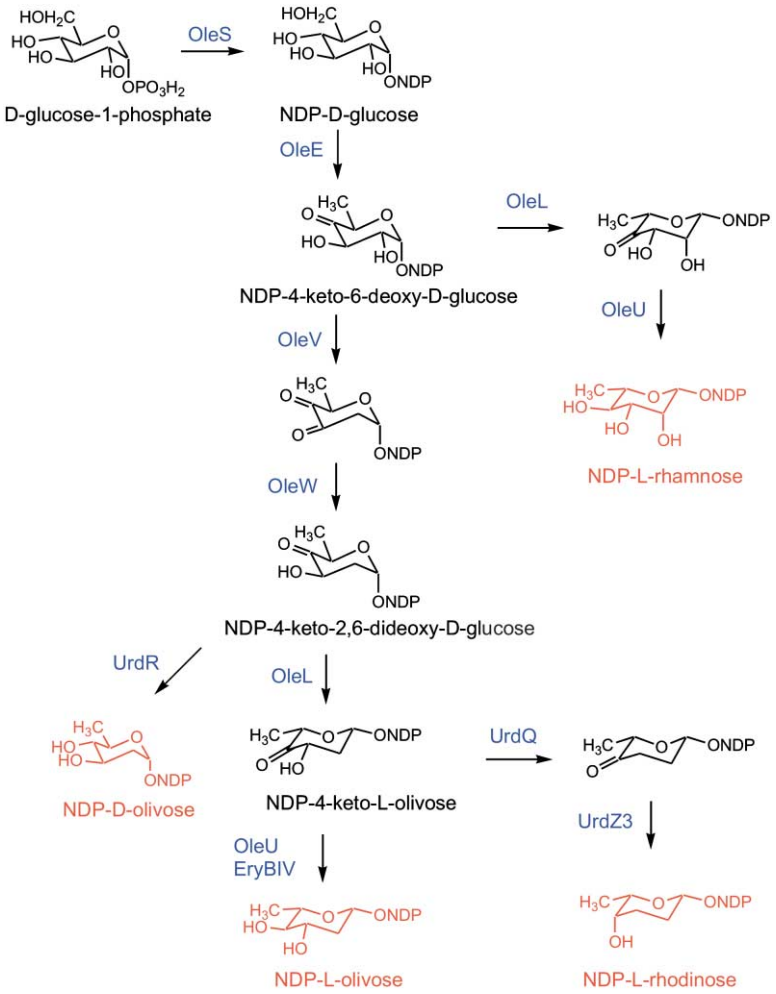

Figure 6. Proposed Pathways for the Biosynthesis of the Different NDP-Activated Deoxysugars Generated in this Work

used to produce glycosylated tetracenomycin derivatives, the potential of pLN2 derivatives is not restricted to this system. Recent experiments in our laboratory have shown that combining pLN2 derivatives with the EryBV L-mycarosyl glycosyltransferase from the erythromycin pathway can lead to the production of different monoglycosylated macrolides (N.A., unpublished data).

Significance

Increasing evidence suggests the existence of a relative sugar substrate flexibility in antibiotic biosynthesis glycosyltransferases. pLN2 (and derivatives) can serve to engineer antibiotic-producing strains to endow them with the capability of synthesizing different 6DOHs. These plasmids can also be introduced in nonproducing strains to be used as biotransformation recipient hosts to generate novel glycosylated derivatives. The use of these plasmids will facilitate the assay of substrate flexibility of glycosyltransferases.

Experimental Procedures

Microorganisms, Culture Conditions, and Vectors

Streptomyces antibioticus ATCC11891 (oleandomycin producer), *Streptomyces fradiae* ATCC19609 (tylosin producer), *Streptomyces peucetius* ATCC29050 (daunorubicin producer), *Streptomyces nogalater* NRRL3035 (nogalamycin producer), *Streptomyces fradiae* Tü2717 (urdamycin producer), and *Saccharopolyspora erythraea* NRRL2338 (erythromycin producer) were used as sources of DNA. *Streptomyces albus* GB16 [24] and *S. albus* 16F4 (this work) were used as hosts for gene expression and for biotransformation experiments. *Streptomyces argillaceus* 16F4 [24] was used for obtaining 8-demethyl-tetracenomycin C (8DMTC). Growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium [38]. For sporulation, growth was for 7 days at 30°C on agar plates containing A medium [38]. *Escherichia coli* XL1-Blue [39] was used as a host for subcloning and was grown at 37°C in TSB medium. pLITMUS29 (Biolabs) and pUC18 were used as vectors for subcloning experiments and DNA sequencing. pWHM3 [40] and pEM4 [41] were used for expression in *Streptomyces*. When antibiotic selection of transformants was needed, 25 μg/ml of thiostrepton, 25 μg/ml of apramycin, or 100 μg/ml of ampicillin were used.

DNA Manipulation and Sequencing

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were according to standard procedures for *E. coli* [42] and for *Streptomyces* [43]. Sequencing was performed via the dideoxynucleotide chain termination method [44] and the Cy5 AutoCycle Sequencing Kit (Pharmacia Biotech). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (17-mer) via an ALF-express automatic DNA sequencer (Pharmacia). Computer-assisted database searching and sequence analyses were carried out with the University of Wisconsin Genetics Computer Group programs package [45] and the BLASTP program [46].

PCR Amplification of the Genes

Several genes were amplified by PCR with specific oligoprimers. These primers were designed to create HindIII and XbaI sites at the 5′ and 3′ ends, respectively, of all genes to facilitate subcloning. Moreover, each pair of oligoprimers contained two other restriction sites that were specific for each gene, which allowed gene exchange. PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmols of each primer and 2 units of Vent DNA Polymerase (New England Biolabs) in a total reaction volume of 50 μl containing 2 mM of each dNTP, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, and 10% DMSO (Merck). This reaction mix was overlayed with 50 μl of mineral oil (Sigma), and the polimerization reactions were performed in a thermocycler (MinyCycler, MJ Research). The PCR products were purified, subcloned into pUC18, and sequenced. Oligonucleotide sequences are given in the Supplemental Material.

Plasmid Constructs

The cassette plasmid pLN2 was constructed as follows. The oleandomycin sugar genes, *oleV*, *oleW*, *oleU*, and *oleY*, were independently amplified with the corresponding primers. Then, all the genes were sequentially cloned in pUC18 to generate pUC18VWUY. To do this, we used the 5′-specific restriction site and the XbaI site (located at 3′ end of each gene) to subclone each gene into intermediate plasmid constructs that had been digested with the same restriction enzymes. Then, the AvrII-XbaI fragment containing the four genes was subcloned downstream of the erythromycin resistance promoter of pEM4, generating pLN. The *oleL*, *oleS*, and *oleE* genes were then subcloned from pLR234Δ7 [22] into the XbaI site of pLN as a SpeI-XbaI fragment (with these sites used from the polylinker), generating pLN1. In parallel, the *oleV* PCR fragment was subcloned as an AvrII-XbaI fragment into the same sites of pLITMUS29 and rescued as a SpeI (this site was used from the polylinker)-XbaI fragment for subcloning into the pEM4 XbaI site, downstream of the erythromycin resistance promoter, generating pEM4V. Then, the HindIII-HpaI fragment of pLN1 was replaced by the HindIII-HpaI fragment containing *oleV* from pEM4V, generating pLN1b. Finally the pLN1b HindIII-XbaI fragment, which contains all oleandrose genes under the erythromycin resistance promoter, was subcloned into the same sites of pWHM3, generating pLN2.

pLN2 derivatives in which *oleU* was replaced by different 4-ketoreductase genes were generated by digestion of pLN2 with SpeI and NheI. The released fragment was then replaced by the different genes (*urdR*, *snogC*, *tylD*, *eryBIV*, *urdZ3*, and *dnmV*) after PCR amplification with specific primers and digestion of the PCR fragments with the same restriction enzymes. The generated constructs were named pLNR, pLNS, pLNT, pLNBIV, pLNZ3, and pLNV, respectively.

pLN2Δ and pLNBIVΔ were generated by digestion of pLN2 and pLNBIV, respectively, with AvrII and SpeI and further religation. This digestion eliminates *oleV* and *oleW*.

pLNRHO was constructed by insertion into the XbaI site of pLNZ3 a XbaI fragment containing *urdQ* generated by PCR amplification.

Biotransformation and Chromatographic Techniques

Spores of *S. albus* GB16 containing the different constructs were grown in the presence of 8-demethyl-tetracenomycin C according to conditions previously described [24]. TLC and HPLC analyses were performed as previously described [47].

Mass Spectra Analysis

Analysis of the glycosylated compounds was carried out via MALDI-TOF mass spectra analysis on a Voyager-DE STR. The samples were dissolved in a solution of acetonitrile/0.1% trifluoroacetic acid in water (1:1) and were mixed with the α-cyano-4-hydroxycinnamic acid matrix in different serial dilutions. Spectra analyses were carried out on a reflector mode with detection of positive ionization.

Supplemental Material

Oligonucleotide sequences are available as supplemental material. Please write to chembiol@cell.com for a pdf.

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