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Engineering Biosynthetic Pathways for Deoxysugars: Branched-Chain Sugar Pathways and Derivatives from the Antitumor Tetracenomycin

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Summary

Sugar biosynthesis cassette genes have been used to construct plasmids directing the biosynthesis of branched-chain deoxysugars: pFL942 (NDP-L-mycarose), pFL947 (NDP-4-deacetyl-L-chromose B), and pFL946/pFL954 (NDP-2,3,4-tridemethyl-L-nogalose). Expression of pFL942 and pFL947 in *S. lividans* 16F4, which harbors genes for elloramycinone biosynthesis and the flexible ElmGT glycosyltransferase of the elloramycin biosynthetic pathway, led to the formation of two compounds: 8-α-L-mycarosyl-elloramycinone and 8-demethyl-8-(4-deacetyl)-α-L-chromosyl-tetracenomycin C, respectively. Expression of pFL946 or pFL954 failed to produce detectable amounts of a novel glycosylated tetracenomycin derivative. Formation of these two compounds represents examples of the sugar cosubstrate flexibility of the ElmGT glycosyltransferase. The use of these cassette plasmids also provided insights into the substrate flexibility of deoxysugar biosynthesis enzymes as the *C*-methyltransferases EryBIII and MtmC, the epimerases OleL and EryBVII, and the 4-ketoreductases EryBIV and OleU.

Introduction

Many bioactive natural products are glycosylated by one or more deoxysugar saccharide chains of variable sugar length. These include important antibiotics (erythromycin), antifungals (amphotericin B), antiparasites (avermectins), and anticancer drugs (doxorubicin). These deoxysugar moieties participate in the molecular recognition of the drug target site and therefore they are usually very important for the biological activity of many natural products [1]. The glycosylation steps usually occur late during biosynthesis by transferring the deoxysugar to the aglycon from an NDP-sugar activated form [2–4]. A great majority of the deoxysugars in natural products belong to the 6-deoxyhexoses family (6DOH), which includes at least 80 different representatives in metabolites from various sources [1–3]. These deoxysugars can be classified as neutral sugars, aminosugars, or branched-chain sugars [2]. Deoxysugar biosynthesis occurs through nucleoside diphosphate-activated hexoses (mainly D-glucose) via a 4-keto-6-deoxy intermediate. The two earliest biosynthetic steps are common to the biosynthesis of all deoxysugars, and it involves the activation of D-glucose into NDP-D-glucose and a further dehydration step generating NDP-4-keto-6-deoxy-D-glucose. These two reactions are catalyzed by an NDP-D-hexose synthase and a NDP-D-hexose-4,6-dehydratase [2, 3]. Further enzymatic reactions (deoxygenations, transaminations, C-, N-, or O-methylations) affecting carbon atoms at positions 2, 3, 4, or 5 in the hexose carbon backbone will introduce different modifications increasing the structural diversity of the family [2, 3]. D- and L-stereoisomeric forms of many 6DOH also exist as a result of the action of a 5- or a 3,5-epimerase.

In the last several years, a number of deoxysugar biosynthetic gene clusters from antibiotic-producing organisms have been described. In a few cases, biosynthetic gene clusters for the same 6DOH have been characterized from different producer organisms. For example, this is the case of the D-desosamine gene cluster, which has been characterized from streptomycetes producing erythromycin A [5, 6], oleandomycin [7, 8], pikromycin [9], and megalomicin [10], and is also true for D-olivose, whose gene cluster has been characterized in the mithramycin [11, 12], chromomycin [13], urdamycin [14], and landomycin [15] producers. The gene cluster for L-oleandrose has been also characterized in the oleandomycin [7, 8] and avermectin [16] producers, and the L-mycarose cluster in producers of erythromycin [5, 6], megalomycin [10], and tylosin [17–20]. In vitro assays for testing the activity of many of the enzymes involved in deoxysugar biosynthesis or glycosyltransfer reactions have been hampered by the unavailability of suitable activated deoxysugars, although in a few cases it was possible to assign putative roles to the different enzymes in deoxysugar biosynthesis by carrying out enzymatic assays to demonstrate the activity of such enzymes and to verify their substrates [19–25]. However, only in the case of L-epivancosamine from the producer of the glycopeptide chloroeremomycin [26] was the complete in vitro reconstitution of the pathway for the biosynthesis of a 6DOH reported. More frequently, functions of enzymes of deoxysugar pathways were assigned on the basis of similarities with related enzymes in databases and through the analysis of the compounds accumulated by mutants affected in selected deoxysugar genes [5, 6, 9, 12–15, 17, 27–30].

An important aspect for the generation of novel glycosylated derivatives is to provide the host strain with the capability of synthesizing various deoxysugars, which then could be potentially transferred by existing glycosyltransferases of the host strain to the aglycon. Several plasmids containing a set of genes involved in 6DOH biosynthesis and capable of directing the bio-

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synthesis toward specific deoxysugars have been already constructed and heterologously expressed: L-daunosamine [31], L-olivose [8], L-oleandrose [8, 32], and D-desosamine [33]. In all these cases, a native DNA fragment was cloned from the chromosome of the producer organism into the appropriate vector. More recently, a "plug and play" plasmid system has been constructed allowing the easy and quick replacement of specific sugar biosynthesis genes by other related genes, thus introducing or removing enzymatic activities and facilitating the generation of novel deoxysugar moieties [34]. This plasmid vector, pLN2, containing genes from the L-oleandrose biosynthesis of the oleandomycin biosynthetic gene cluster from *Streptomyces antibioticus*, has been used to endow the recipient host strain with the ability of synthesizing different deoxysugars [34].

Generation of novel glycosylated compounds requires the availability of glycosyltransferases with some degree of flexibility. In the last several years, increasing evidence suggested the existence of a certain degree of "sugar cosubstrate flexibility" of glycosyltransferases involved in the biosynthesis of secondary metabolites, and some examples have been reported in which various deoxysugars have been transferred to the aglycon [12, 25, 28, 32–40]. One of these glycosyltransferases, the elloramycin glycosyltransferase ElmGT has been shown to be especially "flexible" accepting different L- and D-deoxysugars and being also able to transfer a disaccharide [34, 40–43]. An alternative method for increasing the glycosylation pattern of a compound is the so-called "in vitro glycorandomization," This approach uses an NDP-D-hexose synthase for activating a library of chemically synthesized sugar-1-phosphate precursors that can be now used for glycosylation assays [44–46].

Here we report the use of deoxysugar biosynthesis genes to construct plasmids directing the biosynthesis of different branched-chain deoxysugars and the formation of two tetracenomycin derivatives taking advantage of the high flexibility of the ElmGT glycosyltransferase. The results presented here reveal new insights about 6DOH biosynthetic pathways, including flexibility for the use of several sugar genes and *à la carte* designed pathway deviations in order to obtain the desired final activated branched-chain deoxysugar. In this way, it was also possible to establish some rules about the use of these deoxysugar genes for combinatorial biosynthesis.

Results and Discussion

L-Nogalose (L-NOG) or its 2,3,4-tridemethyl-derivative, L-mycarose (L-MYC) or its 3-*O*-methyl-derivative cladinose, and L-chromose B (L-CHR, also called L-olivomycose) are three deoxysugars occurring in a number of bioactive natural products (Figure 1). They are all branched-chain deoxysugars possessing a *C*-methyl group at C-3 of the hexose carbon backbone. Besides the difference in the functionalization of the OH groups, L-NOG and L-CHR differ in the oxygenation state at C-2, L-NOG being a 6-deoxyhexose and L-CHR a 2,6-dideoxyhexose. L-MYC and L-CHR, both being 2,6-dideoxyhexoses, differ in that the 4-OH group of L-CHR is acetylated and, more importantly, in the stereochemistry at C-3, which is *S*-configurated in L-CHR and *R*-configurated in L-MYC. In the experiments described below, we show the construction of plasmids directing the biosynthesis toward the nonmethylated and nonacetylated derivatives, namely L-MYC, 2,3,4-tri-*O*-demethyl-L-NOG, and 4-O-deacetyl-L-CHR (Figure 1), of these three branched-chain sugars. Assaying their incorporation to an aglycon tested formation of these deoxysugars. To achieve this, we expressed the different "sugar plasmids" into *Streptomyces lividans* 16F4, which harbors cos16F4 [47]. Cosmid 16F4 contains part of the elloramycin gene cluster and it has been shown that its expression leads to the production of the elloramycin aglycon, 8-demethyl-tetracenomycin C (8DMTC) [47] and also contains the *elmGT* glycosyltransferase encoding gene [42].

Construction of an L-Mycarose-Synthesizing Plasmid and Generation of L-Mycarosyl-Tetracenomycin C

To generate a plasmid encoding the biosynthesis of L-MYC, we expressed all genes under the control of two erythromycin resistance promoters cloned in divergent directions. Two genes from the mithramycin cluster, *mtmD* (NDP-D-glucose synthase) and *mtmE* (NDP-D-hexose-4,6-dehydratase) [11] were subcloned downstream of one of the promoters and *oleV* (2,3-dehydratase), *oleW* (3-ketoreductase), *eryBIV* (4-ketoreductase), *eryBIII* (C-3-methyltransferase), and *oleL* (3,5-epimerase) genes [5, 6, 8] subcloned under the control of the second divergent promoter. Each gene was flanked by unique restriction sites, which do not frequently cut *Streptomyces* DNA (Figure 2). The final construct, pFL821, contains the same enzymatic functions as the previously described pLNBIV [34], but with a different genetic organization and with the incorporation of the *eryBIII* methyltransferase gene (Figure 2). When pFL821 was expressed in *S. lividans* 16F4, no L-mycarosyl-tetracenomcyin C (LMYC-TCMC) was detected, but it caused the formation of two different compounds. The major one (peak 3 in Figure 3A) showed the same retention time as a pure sample of L-digitoxosyl-tetracenomycin C (LDIG-TCMC) and with m/z ions 459 (corresponding to the 8DMTC aglycon fragment) and 589 (corresponding to LDIG-TCMC). The second minor peak (peak 2 in Figure 3A) showed the same m/z ions as LDIG-TCMC and identical mobility to that of L-olivosyl-tetracenomycin C (LOLV-TCMC). LDIG-TCMC and LOLV-TCMC represented approximately 35% and 13% of all tetracenomycins produced, respectively. These two glycosylated derivatives were also generated in similar proportions when pLNBIV was used [34, 43].

The lack of formation of L-MYC can probably be explained if the *C*-methyltransferase EryBIII acts like the TylC3 C-methyltransferase, namely on an NDP-D-sugar intermediate with an axial hydroxyl group at C-3 [19]. As a consequence of the action of 3-ketoreductase OleW [24, 34], an intermediate not with an axial but with an equatorial 3-OH group is expected when using pFL821. Therefore we concluded that the C-methyltransferase EryBIII was probably unable to act on the

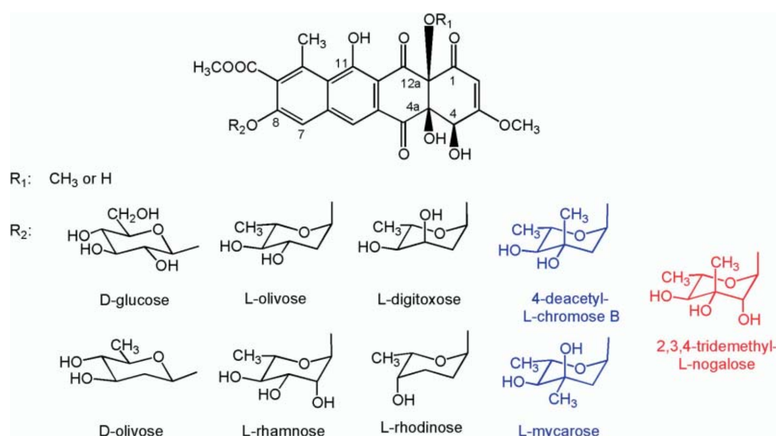

Figure 1. Structures of Glycosylated Derivatives of 8-Demethyl-Tetracenomycin C (R_1=H) and Elloramycinone (R_1=CH_3)

Black sugar moieties: constructed and described in earlier publications (see references in text); blue sugar moieties: newly constructed derivatives with branched deoxysugars (this publication); red sugar moiety: yield too low to be detected.

intermediate produced by the strain expressing pFL821. To generate a sugar intermediate with the appropriate axial hydroxyl group at C-3, we replaced the *oleW* 3-ketoreductase gene in pFL821 by its counterpart *eryBII* [6, 29]. This is a 3-ketoreductase involved in L-MYC biosynthesis, which is supposed to introduce a hydroxyl group at C-3 with axial configuration. Such an enzymatic function has been proved by in vitro assays with the EryBII-homologous reductase TylC1 [20]. Again, after expressing the resultant construct pFL822 (Figure 2) into *S. lividans* 16F4, the same two peaks (peaks 3 and 2 respectively, in Figure 3B), corresponding to LDIG-TCMC (79%) and LOLV-TCMC (12%), were identified. As expected, the production of LDIG-TCMC was higher than when using pFL821 due to the gene combination present in pFL822, since the action of the 3,5-epimerase OleL and the 4-ketoreductase EryBIV on the EryBII product should efficiently render L-digitoxose. The fact that no L-mycarosyl-derivative was produced prompted us eventually to assume that the failure to produce this deoxysugar could be due to OleL, which is a 3,5-epimerase acting on a C-3 nonmethylated intermediate [8]. However, the EryBIII (in pFL822)-mediated C-3 methylation could hamper the epimerization event. Therefore, we replaced the *oleL* gene in pFL822 with the *eryBVII* epimerase gene [5, 6, 29], resulting in pFL942 (Figure 2). Analysis of cultures of *S. lividans* 16F4 harboring pFL942 showed the presence of a new compound (peak 9 in Figure 3C) corresponding to L-mycarosyl-elloramycin (LMYC-ELM), together with LDIG-TCMC (peak 3) and small amounts of LOLV-TCMC (peak 2). HPLC-MS analysis of the corresponding peaks showed m/z values of 617, 589, and 589 respectively, which match the expected masses for these three compounds. In the case of LMYC-ELM, the fragmentation ion corresponding to the aglycon (m/z 473, $M-H^+$) indicates that the aglycon is probably methylated. This suggests that this glycosylated derivative contains elloramycinone as the aglycon and not 8-demethyl-tetracenomycin C (8DMTC), usually found as aglycon of cosmid16F4-mediated glycosylation products. Elloramycinone differs from 8DMTC in that it has a 12a-OCH_3 instead of a 12a-OH group. This 12a-O-methylation can be explained, because cos16F4 contains a gene coding for an *O*-methyltransferase, ElmMIV, which acts during elloramycin biosynthesis usually once 8DMTC has been glycosylated (F.L., unpublished data). Confirmation of the structure of this new compound was achieved after its purification by preparative HPLC and structural elucidation by MS and NMR (see below). The L-DIG-, L-MYC-, and L-OLV-glycosylated derivatives represented approximately 27%, 13%, and 10%, respectively, of the total tetracenomycins produced. The preference of ElmGT for L-DIG rather than L-MYC probably reflects a preference of ElmGT for unmethylated substrates rather than methylated ones, since ElmGT uses NDP-L-rhamnose, an unmethylated deoxysugar, as its natural sugar cosubstrate. The low transfer of L-MYC using this plasmid system does not indicate a low efficiency in the formation of L-MYC. Using pFL942 in another system, we have shown that L-MYC is efficiently transferred by the EryBV glycosyltransferase (which

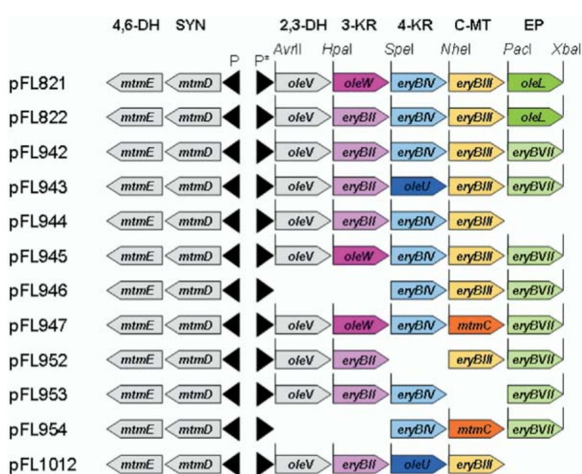

Figure 2. Genetic Organization of Deoxysugar Biosynthesis Gene Cassettes in the Different Plasmids

Genes in gray were not replaced in the different plasmids. Colored genes show those that were replaced in the different plasmids. Abbreviations: SYN, NDP-D-glucose synthase; 4,6-DH, 4,6-dehydratase; 2,3-DH, 2,3-dehydratase; 3-KR, 3-ketoreductase; 4-KR, 4-ketoreductase; C-MT, C-methyltransferase; EP, epimerase; and P, erythromycin resistance promoter.

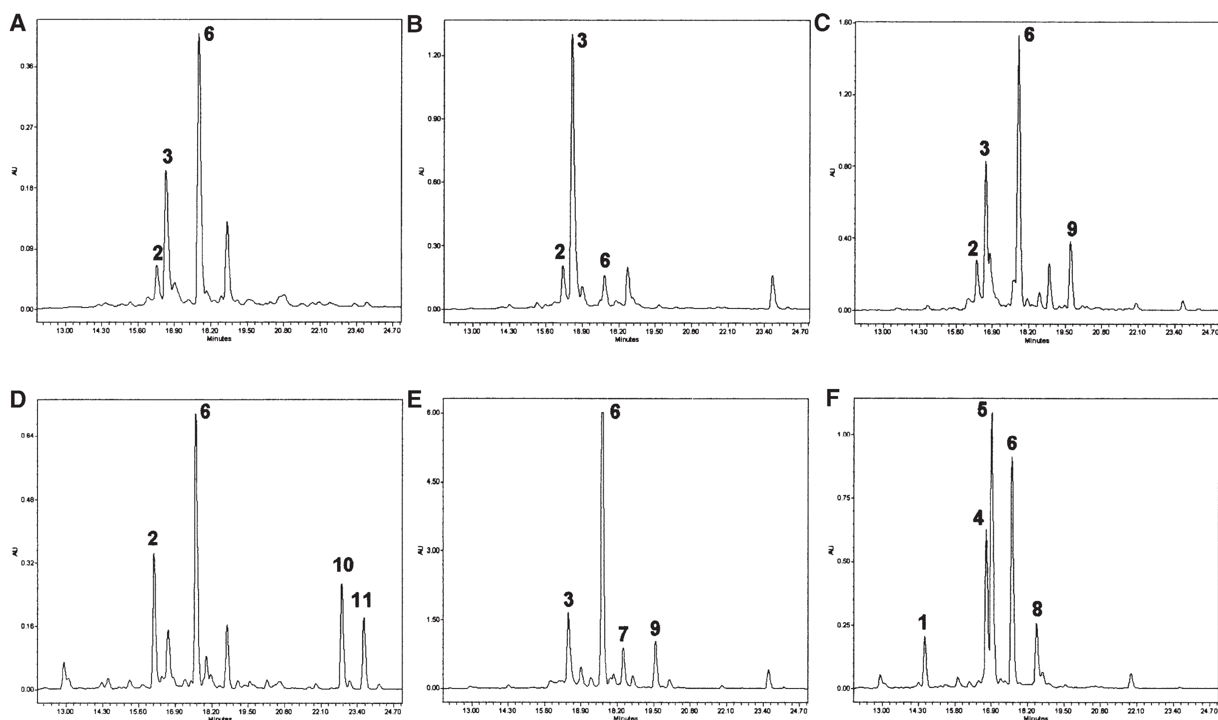

Figure 3. HPLC Analyses of Cultures of *S. lividans* 16F4

HPLC analyses of cultures of *S. lividans* 16F4 harboring (A) pFL821, (B) pFL822, (C) pFL942, (D) pFL943, (E) pFL945, and (F) pFL947. Peaks corresponding to the different tetracenomycins/elloramycins are indicated as follows: D-glucosyl-tetracenomycin C (1); L-olivosyl-tetracenomycin C (2); L-digitoxosyl-tetracenomycin C (3); 4-deacetyl-L-chromosyl-tetracenomycin C (4); D-olivosyl-tetracenomycin C (5); 8-demethyl-tetracenomycin C (6); L-digitoxosyl-elloramycin (7); 4-deacetyl-L-chromosyl-elloramycin (8); L-mycarosyl-elloramycin (9); elloramycin (10); 3′,4′-demethoxy-elloramycin (11).

normally transfers L-mycarose) with the conversion of approximately 65% of erythronolide B into L-mycarosyl-erythronolide B (F.L., unpublished data).

To discard the possibility that genes present in the *S. lividans* 16F4 host strain were participating in the biosynthesis of L-MYC, several control experiments were run using various derivatives of pFL942, in which *eryBIV* (pFL952), *eryBVII* (pFL944), *eryBIII* (pFL953), or *eryBII* + *oleV* (pFL946) were independently deleted (Figure 2). In all four cases, no L-MYC-containing elloramycin derivative was detected (data not shown). These experiments clearly excluded the participation of host-mediated enzymatic activities in the formation of L-MYC.

Once we got a plasmid (pFL942) able to direct the biosynthesis of L-mycarose, we wondered if we could generate L-mycarose using another combination of genes. Thus, we analyzed the possibility of using *oleU* for this purpose. OleU is a 4-ketoreductase involved in L-olivose biosynthesis [8], which renders the final C-4 hydroxy group into the equatorial position, thus establishing the same stereochemistry as EryBIV does. It has been shown that OleU possesses some substrate flexibility [40]. Therefore, we replaced *eryBIV* in pFL942 by *oleU* (generating pFL943) (Figure 2). Upon transformation of pFL943 into *S. lividans* 16F4, no LMYC-ELM was formed. However, three different peaks were observed. One of them corresponded to LOLV-TCMC (peak 2 in Figure 3D) with approximately 23% conversion of the aglycon. The other two peaks (peaks 10 and 11 in Figure 3D) showed retention times and masses identical to elloramycin (peak 10) and 3′,4′-demethyl-elloramycin (peak 11). Both compounds posses L-rhamnose attached to the elloramycinone aglycon and they only differ in the degree of methylation of the sugar, elloramycin being permethylated and 3′,4′-demethyl-elloramycin lacking the methyl groups at positions 3 and 4 of the L-rhamnose moiety. Therefore, in total, the L-rhamnosyl derivatives account for 31% of all tetracenomycins.

The above experiments designed to generate LMYC-TCMC have allowed us to draw up some conclusions about the specificity and flexibility of some of the genes (and their products) used for these constructs (Figure 4). On one hand, the EryBIV 4-ketoreductase possesses a certain relaxed specificity, since it is able to reduce either C-3 methylated or unmethylated intermediates, the latter ones with different stereochemistry at C-3. However, its counterpart OleU showed lower substrate flexibility, since it is unable to act on C-3 methylated intermediates. It can also be concluded that for generating C-3 methylated sugar derivatives, the EryBVII epimerase is more appropriate than the OleL epimerase, since OleL cannot epimerize C-5 once C-3 is already methylated.

One further and interesting aspect coming out from the experiments with pFL943 is that the formation of

Figure 4. Proposed Pathways for the Biosynthesis of the Different Deoxysugars Directed by the Plasmids Described in This Work

Pathways producing branched-chain deoxysugars are colored. Genes unable to carry out the indicated steps are shown in brackets. Plasmids leading to the formation of the different deoxysugars are indicated below the name of the corresponding deoxysugar. LNOG, 2,3,4-tridemethyl-L-nogalose; LOLV, L-olivose; LRHA, L-rhamnose; LDIG, L-digitoxose; LMYC, L-mycarose; and L-CHR, 4-deacetyl-L-chromose B.

LRHA-ELM and LOLV-TCMC requires two different epimerization events: 3,5-epimerization in the case of LRHA-ELM and 5-epimerization in the case of LOLV-TCMC. There are several possibilities to explain the formation of these two different sugars in the presence of a unique epimerase, EryBVII (which is supposed to catalyze a 5-epimerization in L-MYC biosynthesis in *Saccharopolyspora erythraea*). First, it is possible that another epimerase in the host strain exists that catalyzes the 3,5-epimerization. This possibility could be ruled out, since when we used pFL1012 (Figure 2), a derivative of pFL943 lacking *eryBVII*, neither LRHA-TCMC nor LOLV-TCMC was produced (data not shown). A second possibility would be that EryBVII could act both as a 5- (in the formation of LOLV-TCMC) and as a 3,5-epimerase (in the formation of LRHA-ELM) depending on the substrate intermediates. A similar situation has been demonstrated for the EvsA epimerase, which is involved in the biosynthesis of the 4-epi-L-vancosamine moiety decorating chloroeremomycin. It has been shown by in vitro enzymatic assays [26, 48] that EvsA can act both as a 5- or a 3,5-epimerase when using different substrates. A final possibility would be that, after the 5-epimerization event, both C-5 and C-3,5 epimers could coexist due to a C-3 to C-4 tautomerism equilibrium, one of them being a better substrate for the OleU 4-ketoreductase. We have recently proposed this mechanism to explain the formation of LDIG-TCMC by pLNBIV [43].

Construction of a 4-Deacetyl-L-Chromose B (4-Deacetyl-L-Olivomycose)-Synthesizing Plasmid and Generation of 8-Demethyl-8-(4-Deacetyl-L-Chromosyl B)-Tetracenomycin C

L-chromose B, L-CHR (also designated as L-olivomycose, Figure 1) is a neutral and branched-chain sugar present in the aureolic acid group antitumor drugs olivomycin and chromomycin [13, 49, 50]. We were interested in constructing a plasmid directing the biosynthesis of 4-deacetyl-L-CHR and in testing if ElmGT was also able to transfer this sugar. As a first step and, since 4-deacetyl-L-CHR only differs from L-MYC in the stereochemistry at C-3, we replaced in pFL942 *eryBII* with *oleW*. This was based on the assumption that the product of the latter gene could generate an intermediate with the appropriate stereochemistry at C-3 as a substrate for the *C*-methyltransferase EryBIII, thus generating 4-deacetyl-L-CHR. Expression of this construct (pFL945; Figure 2) in *S. lividans* 16F4 gave rise to the formation of LDIG-TCMC (peak 3 in Figure 3E; 34% conversion) and LMYC-ELM (peak 9 in Figure 3E; 8% conversion) but no 4-deacetyl-LCHR-TCMC was detected. Formation of LMYC-ELM probably implies that an intermediate with an axial C-3 hydroxyl group (normal substrate for EryBIII) is being generated via a C-3 to C-4 tautomerization on the product of OleW 2,3-ketoreductase. The lack of formation of 4-deacetyl-LCHR-TCMC with pFL945 prompted us to replace then the *eryBIII* C-methyltransferase gene with the *mtmC* C-methyltransferase. The rationale behind this exchange was that, after the 2-deoxygenation steps catalyzed by OleV and OleW, intermediate NDP-4-keto-2,6-dideoxy-D-glucose should be formed, the natural substrate of *C*-methyltransferase MtmC during D-mycarose biosynthesis in the mithramycin pathway [12]. Upon introduction of the resultant plasmid (pFL947; Figure 2) into *S. lividans* 16F4, three major peaks (peaks 4, 5, and 8 in Figure 3F) were detected by HPLC-MS, whose m/z values (603, 589, and 617) were consistent with the formation of 4-deacetyl-LCHR-TCMC, D-olivosyl-tetracenomycin C (DOLV-TCMC) and 4-deacetyl-LCHR-ELM. Confirmation of the structure of 4-deacetyl-LCHR-

TCMC was achieved after its purification by preparative HPLC and subsequent elucidation of its structure by NMR (see below). The 4-deacetyl-L-CHR- (both derivatives taken together) and D-OLV-derivatives represented approximately 32% and 37%, respectively, of the total tetracenomycins produced.

Taking together all experiments shown above, we can draw several conclusions on the functional activity and substrates of some sugar biosynthesis enzymes (Figure 4).

MtmC: It has been proposed that the *mtmC* gene codes for a C-3 methyltransferase in D-mycarose biosynthesis [12]. Formation of 4-deacetyl-L-CHR-containing tetracenomycin derivatives by the recombinant strain harboring pFL947 confirms this role for MtmC, acting on NDP-4-keto-2,6-dideoxyglucose, which presumably is its natural substrate.

EryBIII: The EryBIII C-methyltransferase probably acts on a D-sugar intermediate with a C-3 hydroxyl group in axial configuration, as it was demonstrated for its homologous TylC3 in L-MYC biosynthesis in the tylosin pathway [19]. If this was not the case, and EryBIII could act on an L-intermediate, L-MYC would have been produced using pFL821, since the action of OleW and OleL renders an appropriate L-intermediate. Thus, since no L-MYC was synthesized with pFL821, the possibility of EryBIII acting on an L-intermediate can be ruled out. Moreover, EryBIII cannot convert an NDP-2,6-deoxy-D-sugar substrate if it possesses an equatorial hydroxyl group at C-3 such as that produced by OleW. This conclusion follows from the result that no LCHR-TCMC is generated using pFL945, which contains *eryBIII*, however, LCHR-TCMC is generated when using pFL947, which contains *mtmC* instead.

EryBVII: The fact that EryBIII acts on a D-sugar intermediate indicates that EryBVII causes C-5 epimerization acting on a 3-C-methylated substrate during L-MYC biosynthesis in the erythromycin pathway. Furthermore, EryBVII acts regardless of how C-3 is configurated, i.e., on both a substrate with an equatorial methyl group or a substrate with an axial methyl group, leading to LCHR-TCMC when using pFL947 (Figure 4, blue) or to LMYC-ELM using pFL942 (Figure 4, red).

Approach to Construct a Plasmid that Could Synthesize 2,3,4-Tridemethyl-L-Nogalose

Several attempts were made to construct a plasmid directing 2,3,4-tridemethyl-L-NOG biosynthesis. First, we took advantage of the previously constructed pFL946, in which the *oleV* and *eryBII* genes were deleted (Figure 2). This deletion was supposed to eliminate the deoxygenation step affecting C-2 and therefore potentially rendering 2,3,4-tridemethyl-L-NOG instead of L-MYC. Using this construct, no formation of 2,3,4-tridemethyl-L-nogalosyl-tetracenomycin C was observed when analyzed by HPLC-MS. A second approach was done by replacing *eryBIII* with *mtmC* in pFL946 (Figure 2). Both genes code for *C*-methyltransferases: *mtmC* involved in the biosynthesis of a D-sugar and *eryBIII* in the biosynthesis of an L-sugar, and acting on intermediates with different stereochemistry at C-3. We assumed, when designing this construct, that the possible substrate intermediate would be more appropriate for the activity of MtmC than that of EryBIII. However, when the corresponding construct (pFL954) was introduced into *S. lividans* 16F4, no LNOG-TCMC was found but only D-glucosyl-tetracenomycin C (DGLU-TCMC). This sugar has been previously shown to be transferred by ElmGT when using other gene constructs [43]. These results suggest that either the C-methyltransferases or the 4-ketoreductase (or both), are unable to act on the intermediates formed or, if the deoxysugar is formed, the possibility cannot be discarded that it could not be substrate for the ElmGT glycosyltransferase.

Structural Elucidation of the Compounds

Two of the compounds mentioned above were chosen for structural elucidation: LMYC-ELM and LCHR-TCMC, since they were compounds not previously described. Yields of these compounds in the fermentation experiments were 4 and 6 mg/l, respectively.

LMYC-ELM was identified from its ^{1}H and ^{13}C NMR spectra in comparison to elloramycinone. The ^{1}H NMR spectrum (300 MHz, in d_6-acetone) of LMYC-ELM shows all signals of elloramycinone, except the 8-OH signal. The only significantly shifted signal of the aglycon moiety is 7-H (δ 7.78 versus δ 7.43 in elloramycinone) because of the nearby attached sugar moiety. The ^{1}H NMR signals of the sugar moiety consist of three spin systems observable in the H,H-COSY spectrum, one stretching from the anomeric 1′-H (δ 6.00) to 2′-H_2 (δ 2.14 and δ 2.26), the second from 6′-H_3 (δ 1.20) over 5′-H (δ 3.81) to 4′-H (δ 3.09), the third being the 3′-CH_3 singlet (δ 1.28). This is consistent with a sugar moiety containing a 3-methyl branch. The stereochemistry of the sugar moiety follows from the coupling pattern observed for each sugar proton. For instance, the large diaxial coupling of 10 Hz between 5′-H and 4′-H indicates that both the 5′-CH_3 group as well as 4-OH are in equatorial position, and the pattern of 1′-H (d, J = 3.5 Hz) is in agreement with its equatorial position and therefore proves an α-glycosidically linked L-sugar, considering that ElmGT (like most streptomycete GTs) follows a mechanism inverting the anomeric configuration of the NDP-sugar-cosubstrate, thus belongs to the GT-1 family and is in agreement with Klyne's rule [51]. The *R*-configuration of C-3′ was confirmed by 2D-NOESY cross-couplings of 3′-CH_3 with 4′-H, 2′-H_a, and 6′-H_3, which is only possible if 3′-CH_3 is in an equatorial position, while no NOE-coupling could be observed between 5′-H and 3′-CH_3 as one would have expected, if C-3′ were *S*-configurated (with 3′-CH_3 being in an axial position). An NOE effect observed between 7-H and 1′-H also confirms the regiochemistry of the glycosidic linkage. These data prove that the sugar moiety of LMYC-ELM is indeed 8-O-α-glycosidically linked L-mycarose. The ^{13}C NMR data are also in agreement with this conclusion. All the ^{1}H and ^{13}C NMR data of LMYC-ELM are listed in Table 1.

LCHR-TCMC was also identified from its HPLC-MS, ^{1}H and ^{13}C NMR data in comparison with tetracenomycin C, elloramycinone, and known 8-glycoslyated tetracenomycins. From the above-mentioned HPLC-MS data, it was evident that LCHR-TCMC contains

Table 1. ^{1}H and ^{13}C NMR Data of LMYC-ELM and LCHR-TCMC in d_6-Acetone

Position	LMYC-ELM		LCHR-TCMC	
	^{1}H (300 MHz), multiplicity (*J* in Hz)	^{13}C (100 MHz)	^{1}H (300 MHz), multiplicity (*J* in Hz)	^{13}C (100 MHz)
1	—	189.0	—	190.8
2	5.49 s	101.1	5.63 s	99.3
3	—	173.0	—	176.1
3-OCH_3	3.78 s	55.1	3.84 s	56.7
4	4.88 s	69.1	5.06 s	69.9
4-OH	4.98 s[a]	—	5.01 br s[b]	—
4a	—	76.3	—	79.2
4a-OH	5.31 s[a]	—	5.14 br s[b]	—
5	—	193.3	—	194.2
5a	—	140.5	—	140.5
6	7.99 s	120.5	7.99 s	120.6
6a	—	131.0	—	130.0
7	7.55 s	111.1	7.75 s	111.4
8	—	155.2	—	155.2
9	—	128.4	—	128.4
9-CO	—	168.3	—	n.o.[d]
9-OCH_3	3.96 s	52.4	3.96 s	52.2
10	—	136.5	—	137.7
10-CH_3	2.86 s	20.4	2.84 s	20.3
10a	—	120.5	—	121.1
11/11-OH	14.03 s[c]	167.7	14.00 s[c]	167.6
11a	—	110.0	—	110.9
12	—	n.o.[d]	—	197.3
12a/12a-OH	—	85.0	10.43 s[c]	81.5
12a-OCH_3	3.59 s	56.4	—	—
1′	6.0 br d (3.5)	96.1	5.97 br d (3.5)	96.6
2′/2′-H_a	2.14 dd (15, 3.5)	40.9	2.14 dd (15, 3.5)	43.1
2′-H_e	2.26 dd (15, 1)	—	2.19 dd (15, 1)	—
3′/3′-OH	3.31 br s[a]	66.5[e]	5.78 br s[b]	69.0[f]
3′-CH_3	1.28 s	25.7	1.43 s	21.8
4′	3.09 d (9.5)	70.1[e]	3.31 d (9.5)	70.1[f]
4′-OH	3.21 br s[a]	—	4.20 br s[b]	—
5′	3.81 dq (9.5, 6)	66.5[e]	3.64 dq (9.5, 6)	69.9[f]
6′/6′-H_3	1.20 d (6)	17.7	1.22 d (6)	17.9

δ in ppm relative to TMS.
[a] Exchangeable with D_2O, assignments interchangeable.
[b] Exchangeable with D_2O, assignments interchangeable.
[c] Not observed.
[d] Exchangeable by D_2O.
[e] Assignments interchangeable.
[f] Assignments interchangeable.

8-demethyltetracenomycin C, not elloramycinone, as its aglycon moiety (note that tetracenomycin C (TCMC) and elloramycinone are structural isomers, which only differ in the position of one of their OCH_3 groups, which is at 12a-position in elloramycinone instead of in 8-position in TCMC). This was confirmed by the ^{1}H NMR spectrum, in which no 12a-OCH_3 group, but a 12a-OH group (δ 10.43) was found along with all other signals representing this aglycon (see Table 1). The ^{1}H NMR signals of the sugar moiety are in agreement with the expected α-L-chromose B (α-L-olivomycose); like in LMYC-ELM (above), the H,H-COSY spectrum shows three spin systems, (1) 1′-H (δ 5.97) to 2′-H_2 (δ 2.14 and 2.19), (2) 6′-H_3 (δ 1.22) over 5′-H (δ 3.64) to 4′-H (δ 3.31), and (3) the 3-CH_3 singlet (δ 1.43). A large diaxial coupling between 4′-H and 5′-H of 9.5 Hz indicates these two protons to be in an axial position of a $_4C^1$-conformation of an L-configured chair. The small coupling (3.5 Hz) between the anomeric 1′-H and 2′-H_a indicates its α-glycosidic bondage, considering ElmGT belonging to the GT-1 family of glycosyltransferases following Klyne's rule [51]. The *S*-configuration of C-3′ (= axial CH_3-residue) was already indicated by a small $^{4}J_{H\text{-}H}$ coupling between 2′-H_a and 3′-CH_3 observable in the H,H-COSY spectrum, but is unambiguously proven by the 2D-NOESY spectrum, which shows a strong coupling between 5′-H and 3′-CH_3. The 2D-NOESY spectrum also shows a coupling between 7-H and 1′-H, confirming the linkage position of the sugar moiety at 8-O. The ^{13}C NMR data are also in agreement with these conclusions. All the ^{1}H and ^{13}C NMR data of LCHR-TCMC are also listed in Table 1.

Antitumor Activity

The antitumor activity of the glycosylated tetracenomycins was tested against three tumor cell lines. Compilation of the average GI_{50} values showed that similar to the original compound elloramycin, all the com-

pounds had very low antitumor activity, being inactive at concentrations below 10^{-5} M. The exception was LMYC-ELM, which showed antitumor activity above a concentration 10^{-6} M. This indicates that substitution of the original sugar residue by L-MYC improves the antitumor activity of these compounds. Probably the presence and orientation of the methyl group at C3 of L-MYC is related to this improvement in the antitumor activity of LMYC-ELM.

Significance

Glycosylation of natural products is usually very important for their biological activity. Therefore, altering the sugar profile may improve the bioactivity or pharmacological properties of the parent molecules. We have, to our knowledge, successfully constructed for the first time plasmids directing the biosyntheses of branched NDP-deoxysugars. Such sugars play key roles for drug-target interactions of many biologically active natural products, such as the macrolide antibiotics erythromycin A, tylosin, and pikromycin, or the aureolic acid anticancer drugs olivomycin, mithramycin, and chromomycin. These plasmids allow for the pathway-engineered drug design of novel derivatives of glycosylated natural products, if glycosyltransferases are present, which show flexibility toward their NDP-sugar donor substrate. The proof of concept was given here using the well established, NDP-sugar-cosubstrate-flexible glycosyltransferase ElmGT to engineer novel glycosylated tetracenomycin derivatives.

Experimental Procedures

Microorganisms, Culture Conditions, and Vectors

Streptomyces antibioticus ATCC11891 (oleandomycin producer), *Streptomyces argillaceus* ATCC12956 (mithramycin producer), and *Saccharopolyspora erythraea* NRRL2338 (erythromycin producer) were used as sources of DNA. cosAR7 [11], pLN2, and pLNBIV [34] were used as sources of sugar DNA cassettes. *Streptomyces lividans* 16F4 [42] was used as host for gene expression. Growth was carried out on trypticase soy broth (TSB; Oxoid) or R5A medium [52] for product isolation. For sporulation, we used agar plates containing medium A [52] for 7 days at 30°C. *Escherichia coli* DH10B (Invitrogen) was used as a host for subcloning and it was grown at 37°C in TSB medium. pCRBlunt (Invitrogen), pUC18, and pIAGO [8] were used as vectors for subcloning experiments and DNA sequencing. When antibiotic selection of transformants was needed, 50 μg/ml of thiostrepton, 25 μg/ml of apramycin, 50 μg/ml of kanamycin, 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 *Streptomyces* [53] and for *E. coli* [54]. Sequencing was performed by using the dideoxynucleotide chain-terminator method [55] and the Thermo Sequenase Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences). Both DNA strands were sequenced with primers supplied in the kits or with internal oligoprimers (18-mer) using an ALF-express automatic DNA sequencer (Amersham Biosciences). Computer-assisted database searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package [56] and the BLAST program [57].

PCR Amplification

Several genes were amplified by PCR using the following oligoprimers.

oleL: FL-oleL-up, 5′-AAAA**TTAATTAA**AATTCGGCAGAATCGGGATCG-3′; FL-oleL-rp, 5′-AAAA**TCTAGA**TCACGGGCCGGTCCCACGC-3′.
eryBII: FL-eryB2-up, 5′-AAAA**GTTAAC**GCCCGCAGGAAGGAGAGAACC-3′; FL-eryB2-rp, 5′-AAAA**ACTAGT**CTTTCTCGGTTCCTCTTGTGC-3′.
eryBVII: FL-eryB7-up, 5′-GG**TTAATTAA**CTGCGCGGAAGGTGAACCGG-3′; FL-eryB7-rp, 5′-AA**TCTAGA**ACCAAGGCCACCGAGGTCGC-3′.
mtmC: FL-mtmC-up, 5′-AAAA**GCTAGC**GCGCGGCGCCTTCCCCTGTGACC-3′; FL-mtmC-rp, 5′-**TTAATTAA**GTTCGTCGTCCGTGCCACGGTCACTCC-3′.

PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 1.25 U of Platinum-Pfx DNA Polymerase (Invitrogen) in a total reaction volume of 50 μl containing 1 mM $MgSO_4$, 0.3 mM of each dNTP, 1× Pfx-buffer, and in some cases PCRx Enhancer Solution. The polymerization reactions were performed in a thermocycler (PT-100, MJ Research). General conditions for PCR amplification were as follows: 2 min at 94°C; 30 cycles composed of 30 s at 94°C; 1 min at the primer annealing temperature and 80 s at 68°C; 5 min at 68°C; and 15 min at 4°C. Primer annealing temperatures for the different genes were 53°C, 56°C, 73°C, and 60°C for *oleL*, *eryBII*, *eryBVII*, and *mtmC*, respectively. The PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), subcloned into pCRBlunt, and sequenced.

Plasmid Constructs

pFL0DE

A 2.8 kb PstI-SphI fragment from cosAR7, containing the *mtmD* (NDP-glucose synthase) and *mtmE* (NDP-4,6-glucose dehydratase) genes [11] was subcloned into pUC18 and then rescued as an XbaI (using this site from the polylinker)-SphI fragment, and subcloned into the same sites of pIAGO, downstream of the *ermE* promoter.

pFL821

For constructing this plasmid, pLNBIV was chosen as the starting construct. It was first digested with NheI and PacI and the *eryBIII* gene flanked by the same restriction sites replaced the released fragment. Then, the PacI-XbaI fragment, containing *oleL*, *oleS*, and *oleE* genes, was replaced by the *oleL* gene flanked by the same restriction sites, generating plasmid pFL820. In parallel, pFL0DE was digested with XbaI, and after filling ends with Klenow polymerase, religated to eliminate the XbaI site. Then, the 3.1 kb EcoRI-SphI fragment blunt-ended with the Klenow fragment and containing the *mtmD* and *mtmE* genes under the control of the *ermEp*, was subcloned into the unique blunt-ended HindIII site of pFL820, generating pFL821. In this final construct, *oleV*, *oleW*, *eryBIV*, *eryBIII*, and *oleL* are under the control of the *ermE**p, and the *mtmD* and *mtmE* genes are divergently transcribed from an *ermEp*.

Several other constructs were made derived from plasmid pFL821, as shown in Figure 2 (for details, see Table S1 in Supplemental Data).

Production Conditions and Chromatographic Techniques

Spores of *S. lividans* 16F4 containing the different constructs were grown in R5A medium according to conditions previously described [34]. HPLC analyses were performed as previously described [34].

Mass Spectra and NMR Analysis

HPLC-MS analysis of the glycosylated compounds was carried out using chromatographic equipment coupled to a ZQ4000 mass spectrometer (Waters-Micromass), using electrospray ionization in the positive mode, with a capillary voltage of 3 kV and a cone voltage of 20 V. Chromatographic conditions were as previously described [34], except for the column size (2.1 × 150 mm) and flow rate (0.25 ml/min). A SymmetryPrep C_{18} column (7.8 × 300 mm, Waters) was used for compound purification, with acetonitrile and

0.1% trifluoroacetic acid as solvents. The new compound LCHR-TCM was eluted in isocratic mode with 32.5% acetonitrile, while 40% acetonitrile was used for LMYC-ELM. Standards with pure compounds were used for L-olivosyl, L-digitoxosyl-, permethylated L-rhamnosyl-, D-olivosyl-, and D-glucosyl-8-demethyl-tetracenomycin C and -elloramycinone comparisons.

NMR spectra were recorded on Varian Inova 300 and Varian Inova 400 NMR spectrometers at magnetic field strengths of 7.05 T and 9.40 T, respectively. The δ values were adjusted on the solvent peaks (d_6-acetone, δ 2.05 and δ 29.9 ppm for ^1H and ^{13}C NMR, respectively), and standard conditions were used for the 2D-NMR spectra.

Antitumor Tests

The antitumor activity of the compounds was tested against tumor cell lines of breast (MDA-MB-231), NSCL (A549), and colon (HT-29). Quantitative measurement of cell growth and viability was carried out by using a colorimetric type of assay, using sulforhodamine reaction [58].

Supplemental Data

A table with the nucleotide sequences of the primers used for PCR amplification of the different genes and a table with a list of plasmid constructs are available online at http://www.chembiol.com/cgi/content/full/11/12/1709/DC1/.

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References

1. Weymouth-Wilson, A.C. (1997). The role of carbohydrates in biologically active natural products. Nat. Prod. Rep. *14*, 99–110.
2. Liu, H.-W., and Thorson, J.S. (1994). Pathways and mechanisms in the biogenesis of novel deoxysugars by bacteria. Annu. Rev. Microbiol. *48*, 223–256.
3. Piepersberg, W. (1994). Pathway engineering in secondary metabolite-producing actinomycetes. Crit. Rev. Biotechnol. *14*, 251–285.
4. Trefzer, A., Salas, J.A., and Bechthold, A. (1999). Genes and enzymes of deoxysugar biosyntheses. Nat. Prod. Rep. *16*, 283–299.
5. Gaisser, S., Böhm, G., Cortés, J., and Leadlay, P.F. (1997). Analysis of seven genes from the *eryAI-eryK* region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. Mol. Gen. Genet. *256*, 239–251.
6. Summers, R.G., Donadio, S., Staver, M.J., Wendt-Pienkowski, E., Hutchinson, C.R., and Katz, L. (1997). Sequencing and mutagenesis of genes from the erythromycin biosynthetic gene cluster of *Saccharopolyspora erythraea* that are involved in L-mycarose and D-desosamine production. Microbiol. *143*, 3251–3262.
7. Olano, C., Rodriguez, A.M., Michel, J.M., Méndez, C., Raynal, M.C., and Salas, J.A. (1998). Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis that contains two glycosyltransferases responsible for glycosylation of the macrolactone ring. Mol. Gen. Genet. *259*, 299–308.
8. Aguirrezabalaga, I., Olano, C., Allende, N., Rodriguez, L., Braña, A.F., Méndez, C., and Salas, J.A. (2000). Identification and expression of genes involved in biosynthesis of L-oleandrose and its intermediate L-olivose in the oleandomycin producer *Streptomyces antibioticus*. Antimicrob. Agents Chemother. *44*, 1266–1275.
9. Xue, Y., Zhao, L., Liu, H.-W., and Sherman, D.H. (1998). A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: architecture of metabolic diversity. Proc. Natl. Acad. Sci. USA *95*, 12111–12116.
10. Volchegursky, Y., Hu, Z., Katz, L., and McDaniel, R. (2000). Biosynthesis of the anti-parasitic agent megalomicin: transformation of erythromycin to megalomicin in *Saccharopolyspora erythraea*. Mol. Microbiol. *37*, 752–762.
11. Lombó, F., Siems, K., Braña, A.F., Méndez, C., Bindseil, K., and Salas, J.A. (1997). Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in earliest steps of sugar biosynthesis of the antitumor polyketide mithramycin. J. Bacteriol. *179*, 3354–3357.
12. González, A., Remsing, L.L., Lombó, F., Fernández, M.J., Prado, L., Braña, A.F., Kunzel, E., Rohr, J., Méndez, C., and Salas, J.A. (2001). The *mtmVUC* genes of the mithramycin gene cluster in *Streptomyces argillaceus* are involved in the biosynthesis of the sugar moieties. Mol. Gen. Genet. *264*, 827–835.
13. Menéndez, N., Nur-e-Alam, M., Braña, A.F., Rohr, J., Salas, J.A., and Méndez, C. (2004). Biosynthesis of the antitumor chromomycin A_3 in *Streptomyces griseus*: analysis of the gene cluster and rational design of novel chromomycin analogs. Chem. Biol. *11*, 21–32.
14. Hoffmeister, D., Ichinose, K., Domann, S., Faust, B., Trefzer, A., Drager, G., Kirschning, A., Fischer, C., Kunzel, E., Bearden, D., et al. (2000). The NDP-sugar co-substrate concentration and the enzyme expression level influence the substrate specificity of glycosyltransferases: cloning and characterization of deoxysugar biosynthetic genes of the urdamycin biosynthetic gene cluster. Chem. Biol. *7*, 821–831.
15. Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, D.A., and Bechthold, A. (1999). Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin biosynthesis. FEMS Microbiol. Lett. *15*, 381–387.
16. Ikeda, H., Nonomiya, T., Usami, M., Ohta, T., and Omura, S. (1999). Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. Proc. Natl. Acad. Sci. USA *96*, 9509–9514.
17. Merson-Davies, L.A., and Cundliffe, E. (1994). Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. Mol. Microbiol. *13*, 349–355.
18. Bate, N., Butler, A.R., Smith, I.P., and Cundliffe, E. (2000). The mycarose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. Microbiology *146*, 139–146.
19. Chen, H., Zhao, Z., Hallis, T.M., Guo, Z., and Liu, H.-W. (2001). Insights into the branched-chain formation of mycarose: methylation catalyzed by an *S*-adenosylmethionine-dependent methyltransferase. Angew. Chem. Int. Ed. Engl. *40*, 607–610.
20. Chen, H., Agnihotri, G., Guo, Z., Que, N.L.S., Chen, X.H., and Liu, H.-W. (1999). Biosynthesis of mycarose: isolation and characterization of enzymes involved in the C-2 deoxygenation. J. Am. Chem. Soc. *121*, 8124–8125.
21. Chang, C.-W., Zhao, L., Yamase, H., and Liu, H.-W. (2000). DesVI: a new member of the sugar N,N-dimethyltransferase family involved in the biosynthesis of desosamine. Angew. Chem. Int. Ed. Engl. *39*, 2160–2163.
22. Chen, H., Guo, Z., and Liu, H.-W. (2000). Expression, purification and characterization of TylM1, a N,N-dimethyltransferase involved in the biosynthesis of mycaminose. J. Am. Chem. Soc. *120*, 9951–9952.
23. Chen, H., Yamase, H., Murakami, K., Chang, C.-W., Zhao, L., Zhao, Z., and Liu, H.-W. (2001). Expression, purification and characterization of two N,N-dimethyltransferases, TylM1 and DesVI, involved in the biosynthesis of mycaminose and desosamine. Biochemistry *41*, 9165–9183.
24. Draeger, G., Park, S.H., and Floss, H.G. (1999). Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin. J. Am. Chem. Soc. *121*, 2611–2612.

25. Zhao, L., Borisova, S., Yeung, S.M., and Liu, H.-W. (2001). Study of C-4 deoxygenation in the biosynthesis of desosamine: evidence implicating a novel mechanism. J. Am. Chem. Soc. *123*, 7909–7910.
26. Chen, H., Thomas, M.G., Hubbard, B.K., Losey, H.C., Walsh, C.T., and Burkart, M.D. (2000). Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. Proc. Natl. Acad. Sci. USA *97*, 11942–11947.
27. Weitnauer, G., Gaisser, S., Kellenberger, L., Leadlay, P.F., and Bechthold, A. (2002). Analysis of a C-methyltransferase gene (*aviG1*) involved in avilamycin biosynthesis in *Streptomyces viridochromogenes* Tü57 and complementation of a *Saccharopolyspora erythraea eryBIII* mutant by *aviG1*. Microbiol. *148*, 373–379.
28. Remsing, L.L., García-Bernardo, J., González, A., Künzel, E., Rix, U., Braña, A.F., Bearden, D.W., Méndez, C., Salas, J.A., and Rohr, J. (2002). Ketopremithramycins and ketomithramycins, four new aureolic acid-type compounds obtained upon inactivation of two genes involved in the biosynthesis of the deoxysugar moieties of the antitumor drug mithramycin by *Streptomyces argillaceus*, reveal novel insights into post-PKS tailoring steps of the mithramycin biosynthetic pathway. J. Am. Chem. Soc. *124*, 1606–1614.
29. Salah-Bey, K., Doumith, M., Michel, J.-M., Haydock, S., Cortés, J., Leadlay, P.F., and Raynal, M.-C. (1998). Targeted gene inactivation for the elucidation of deoxysugar biosynthesis in the erythromycin producer *Saccharopolyspora erythraea*. Mol. Gen. Genet. *257*, 542–553.
30. Méndez, C., and Salas, J.A. (2001). Altering the glycosylation pattern of bioactive compounds. Trends Biotechnol. *19*, 449–456.
31. Olano, C., Lomovskaya, N., Fonstein, L., Roll, J.T., and Hutchinson, C.R. (1999). A two-plasmid system for the glycosylation of polyketide antibiotics: bioconversion of epsilon-rhodomycinone to rhodomycin D. Chem. Biol. *6*, 845–855.
32. Wohlert, S.E., Lomovskaya, N., Kulowski, K., Fonstein, L., Occi, J.L., Gewain, K.M., MacNeil, D.J., and Hutchinson, C.R. (2001). Insights about the biosynthesis of the avermectin deoxysugar L-oleandrose through heterologous expression of *Streptomyces avermitilis* deoxysugar genes in *Streptomyces lividans*. Chem. Biol. *8*, 681–700.
33. Tang, L., and McDaniel, R. (2001). Construction of desosamine containing polyketide libraries using a glycosyltransferase with broad substrate specificity. Chem. Biol. *8*, 547–555.
34. Rodríguez, L., Aguirrezabalaga, I., Allende, N., Braña, A.F., Méndez, C., and Salas, J.A. (2002). Engineering deoxysugar biosynthetic pathways from antibiotic-producing microorganisms. A tool to produce novel glycosylated bioactive compounds. Chem. Biol. *9*, 721–729.
35. Solenberg, P.J., Matsushima, P., Stack, D.R., Wilkie, S.C., Thompson, R.C., and Baltz, R.H. (1997). Production of hybrid glycopeptide antibiotics in vitro and in *Streptomyces toyocaensis*. Chem. Biol. *4*, 195–202.
36. Zhao, L., Ahlert, J., Xue, Y., Thorson, J.S., Sherman, D.H., and Liu, H.-W. (1999). Engineering a methymycin/pikromycin-calicheamicin hybrid: construction of two new macrolides carrying a designed sugar moiety. J. Am. Chem. Soc. *121*, 9881–9882.
37. Borisova, S.A., Zhao, L., Sherman, D.H., and Liu, H.-W. (1999). Biosynthesis of desosamine: construction of a new macrolide carrying a genetically designed sugar moiety. Org. Lett. *15*, 133–136.
38. Doumith, M., Legrand, R., Lang, C., Salas, J.A., and Raynal, M.C. (1999). Interspecies complementation in *Saccharopolyspora erythraea*: elucidation of the function of *oleP1, oleG1* and *oleG2* from the oleandomycin biosynthetic gene cluster of *Streptomyces antibioticus* and generation of new erythromycin derivatives. Mol. Microbiol. *34*, 1039–1048.
39. Gaisser, S., Reather, J., Wirtz, G., Kellenberger, L., Staunton, J., and Leadlay, P.F. (2000). A defined system for hybrid macrolide biosynthesis in *Saccharopolyspora erythraea*. Mol. Microbiol. *36*, 391–401.
40. Rodríguez, L., Oelkers, C., Aguirrezabalaga, I., Braña, A.F., Rohr, J., Méndez, C., and Salas, J.A. (2000). Generation of hybrid elloramycin analogs by combinatorial biosynthesis using genes from anthracycline-type and macrolide biosynthetic pathways. J. Mol. Microbiol. Biotechnol. *2*, 271–276.
41. Wohlert, S.E., Blanco, G., Lombó, F., Fernández, E., Braña, A.F., Reich, S., Udvarnoki, G., Méndez, C., Decker, H., Salas, J.A., et al. (1998). Novel hybrid tetracenomycins through combinatorial biosynthesis using a glycosyltransferase encoded by the *elm*-genes in cosmid 16F4, which shows a broad sugar substrate specificity. J. Am. Chem. Soc. *120*, 10596–10601.
42. Blanco, G., Pérez-Patallo, E., Braña, A.F., Rohr, J., Méndez, C., and Salas, J.A. (2001). Identification of a sugar flexible glycosyltransferase from *Streptomyces olivaceus*, the producer of the antitumor polyketide elloramycin. Chem. Biol. *8*, 253–263.
43. Fischer, C., Rodríguez, L., Patallo, E.P., Lipata, F., Braña, A.F., Méndez, C., Salas, J.A., and Rohr, J. (2002). Digitoxosyltetracenomycin C and glucosyltetracenomycin C, two novel elloramycin analogues obtained by exploring the sugar donor substrate specificity of glycosyltransferase ElmGT. J. Nat. Prod. *65*, 1685–1689.
44. Jiang, J., Biggins, J.B., and Thorson, J.S. (2001). Expanding the pyridine diphosphosugar repertoire: the chemoenzymatic synthesis of amino- and acetaminoglucopyranosyl derivatives. Angew. Chem. Int. Ed. Engl. *40*, 1502–1505.
45. Fu, X., Albermann, C., Jiang, J., Liao, J., Zhang, C., and Thorson, J.S. (2003). Antibiotic optimization via in vitro glycorandomyzation. Nat. Biotechnol. *21*, 1467–1469.
46. Losey, H.C., Jiang, J., Biggins, J.B., Oberthur, M., Ye, X.Y., Dong, S.D., Kahne, D., Thorson, J.S., and Walsh, C.T. (2002). Incorporation of glucose analogs by GtfE and GtfD from the vancomycin biosynthetic pathway to generate variant glycopeptides. Chem. Biol. *9*, 1268–1270.
47. Decker, H., Rohr, J., Motamedi, H., Zähner, H., and Hutchinson, C.R. (1995). Identification of *Streptomyces olivaceus* Tü2353 genes involved in the production of the polyketide elloramycin. Gene *166*, 121–126.
48. Kirkpatrick, P.N., Scaife, W., Hallis, T.M., Liu, H., Spencer, J.B., and Williams, D.H. (2000). Characterization of a sugar epimerase enzyme involved in the biosynthesis of a vancomycin-group antibiotic. Chem. Commun. *166*, 1565–1566.
49. Montanari, A., and Rosazza, J.P. (1990). Biogenesis of chromomycin A3 by *Streptomyces griseus*. J. Antibiot. (Tokyo) *43*, 883–889.
50. Berlin, Y.A., Esipov, S.E., Kolosov, M.N., and Shemyakin, M.M. (1966). The structure of the olivomycin-chromomycin antibiotics. Tetrahedron Lett. *15*, 1643–1647.
51. Rix, U., Fischer, C., Remsing, L.L., and Rohr, J. (2002). Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat. Prod. Rep. *19*, 542–580.
52. Fernández, E., Weissbach, U., Reillo, C.S., Braña, A.F., Méndez, C., Rohr, J., and Salas, J.A. (1998). Identification of two genes from *Streptomyces argillaceus* encoding glycosyltransferases involved in transfer of a disaccharide during biosynthesis of the antitumor drug mithramycin. J. Bacteriol. *180*, 4929–4937.
53. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000). Practical *Streptomyces* Genetics (Norwich, UK: The John Innes Foundation).
54. Sambrook, J., and Russell, D.W. (2001). Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
55. Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.
56. Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. *12*, 387–395.
57. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.
58. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., and Boyd, M.R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. *82*, 1107–1112.