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Combinatorial Biosynthesis of Antitumor Deoxysugar Pathways in *Streptomyces griseus*: Reconstitution of "Unnatural Natural Gene Clusters" for the Biosynthesis of Four 2,6-D-Dideoxyhexoses†

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Combinatorial biosynthesis was applied to *Streptomyces* deoxysugar biosynthesis genes in order to reconstitute "unnatural natural gene clusters" for the biosynthesis of four D-deoxysugars (D-olivose, D-oliose, D-digitoxose, and D-boivinose). Expression of these gene clusters in *Streptomyces albus* 16F4 was used to prove the functionality of the designed clusters through the generation of glycosylated tetracenomycins. Three glycosylated tetracenomycins were generated and characterized, two of which (D-digitoxosyl-tetracenomycin C and D-boivinosyl-tetracenocmycin C) were novel compounds. The constructed gene clusters may be used to increase the capabilities of microorganisms to synthesize new deoxysugars and therefore to produce new glycosylated bioactive compounds.

Microorganisms, mainly actinomycetes, produce a huge variety of glycosylated compounds possessing biological activity, such as antibiotics or antitumors. In the last few years, remarkable efforts have been made to modify the sugar patterns of bioactive compounds by using combinatorial biosynthesis approaches (22, 38, 39). This requires the use of sugar donor flexible glycosyltransferases and the ability to provide microorganisms with the capability to synthesize new sugars. However, the extension of these studies is limited by the small variety of nucleotide diphosphate (NDP) sugars that can be produced by a single strain.

The saccharide moieties of bioactive glycosylated compounds are mainly deoxysugars. This is an important class of carbohydrates formed from common monosaccharides by replacement of one or more hydroxyl groups with hydrogen. Among them, 2,6-dideoxyhexoses (2,6-DOHs) are found ubiquitously in nature, but they are especially abundant in antibiotic or antitumor compounds, in which they play an important role in biological activity, participating in the interaction with the cell targets. Most 2,6-DOHs derive from glucose-1-phosphate, which is first activated to dTDP (TDP)-D-glucose and then undergoes a 4,6 dehydration to give rise to the common biosynthesis intermediate TDP-4-keto-6-deoxy-D-glucose (19, 40). Next, C-2 deoxygenation takes place, involving a dehydration step followed by a reduction reaction. Two different products can arise from this C-2 deoxygenation process, depending on the type of ketoreductase involved. When a TylC1-like ketoreductase is involved, TDP-2,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose is formed (7), whereas if a Gra-Orf26-like protein participates, the product will be TDP-4-keto-2,6-dideoxy-D-glucose (9). These two products differ only in the configuration at the C-3 hydroxyl group, which is axial in the former and equatorial in the latter. By further C-4 ketoreduction or C-3,5 epimerization and C-4 ketoreduction steps of these two biosynthesis intermediates, the different D- and L-2,6-DOHs are generated (14, 29, 32).

Four possible 2,6-D-DOHs exist, differing in the configuration of the hydroxyl groups at C-3 and C-4 (Fig. 1): D-olivose (also known as D-canarose or D-chromose C), D-oliose, D-digitoxose, and D-boivinose. Of these, D-olivose is the most commonly found in bioactive compounds. However, D-oliose, and especially D-digitoxose and D-boivinose, are quite unusual among bioactive compounds produced by microorganisms. Genes involved in the biosynthesis of D-olivose and D-oliose have been identified from different antibiotic producer microorganisms (9, 13, 15, 16, 20, 24, 40, 42, 43). Very recently, a gene cluster for the biosynthesis of D-digitoxose was also identified (5). However, no gene cluster involved in the biosynthesis of D-boivinose has been identified so far. Here, we report the use of combinatorial biosynthesis to reconstitute "unnatural natural gene clusters" for the biosynthesis of 2,6-D-DOH, which can be used to provide *Streptomyces* species with the capability to synthesize these four 2,6-D-DOHs. These DOH gene clusters were expressed in a bifunctional (*Escherichia coli-Streptomyces*) multicopy plasmid and were tested for the generation of glycosylated compounds using the sugar flexible glycosyltransferase ElmGT from the elloramycin cluster. Three glycosylated tetracenomycin derivatives were generated, two of which were new.

MATERIALS AND METHODS

Microorganisms, culture conditions, and vectors. *Streptomyces griseus* subsp. *griseus* ATCC 13273 (a chromomycin producer) was used as the source of DNA.

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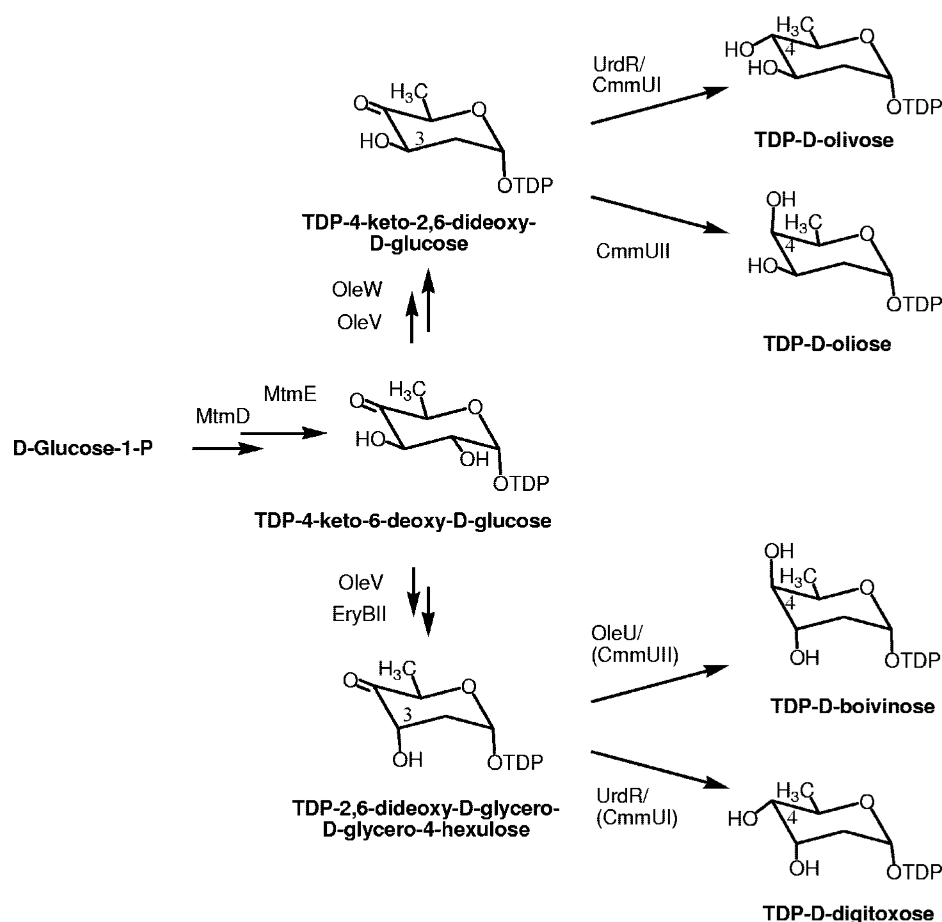

FIG. 1. Proposed pathways for the biosynthesis of TDP-2,6-dideoxy-D-hexoses, showing the enzymes catalyzing the indicated biosynthetic steps. In parentheses are enzymes with similar functions that were unable to carry out the corresponding reaction here.

pFL942 and pFL943 (21) and pLN2 and pLNR (30) were used as sources of sugar genes. *Streptomyces lividans* 16F4 (6) was used as the host for gene expression. Growth was carried out on trypticase soy broth (Oxoid) or R5A medium (10) for product isolation. For sporulation, agar plates containing A medium (10) were used, and cultures were grown 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 trypticase soy broth medium. pCRBlunt (Invitrogen) and pUC18 were used as vectors for subcloning experiments and DNA sequencing. pWHM3 (41) was used for expressing genes in *Streptomyces*. 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 was used.

DNA manipulation, PCR amplification, and sequencing. Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed according to standard procedures for *Streptomyces* (17) and for *E. coli* (33). Specific oligoprimers were used to amplify by PCR *cmmUI* (NMUI-U, 5′-AAAAACTAGTGTGTCTGAG

TABLE 1. Plasmid constructs generated in this work

Plasmid	Genes	Characteristics
pMP3*	*mtmE*, *mtmD*, *oleV*, *oleW*, *urdR*, *oleY*	Derived from pLNR (30) and pFL942 (21)
pMP1*UI	*mtmE*, *mtmD*, *oleV*, *oleW*, *cmmUI*, *oleY*	pMP3* was digested with SpeI and NheI, and the *urdR* gene was replaced by the *cmmUI* gene, flanked by the same restriction sites.
pMP1*UII	*mtmE*, *mtmD*, *oleV*, *oleW*, *cmmUII*, *oleY*	pMP3* was digested with SpeI and NheI, and the *urdR* gene was replaced by the *cmmUII* gene, flanked by the same restriction sites.
pMP3*BII	*mtmE*, *mtmD*, *oleV*, *eryBII*, *urdR*, *oleY*	pMP3* was digested with HpaI and SpeI, and the *oleW* gene was replaced by the *eryBII* gene, flanked by the same restriction sites.
pMP1*UIBII	*mtmE*, *mtmD*, *oleV*, *eryBII*, *cmmUI*, *oleY*	pMP1*UI was digested with HpaI and SpeI, and the *oleW* gene was replaced by the *eryBII* gene, flanked by the same restriction sites.
pMP1*UIIBII	*mtmE*, *mtmD*, *oleV*, *eryBII*, *cmmUII*, *oleY*	pMP3*BII was digested with SpeI and NheI, and the *urdR* gene was replaced by the *cmmUII* gene, flanked by the same restriction sites.
pMP1*BII	*mtmE*, *mtmD*, *oleV*, *eryBII*, *oleU*, *oleY*	pMP1*UIIBII was digested with SpeI and NheI, and the *cmmUII* gene was replaced by the *oleU* gene, flanked by the same restriction sites.
pMP1*BIIΔU	*mtmE*, *mtmD*, *oleV*, *eryBII*, *oleY*	pMP1*BII was digested with SpeI and NheI and religated to delete the *oleU* gene.

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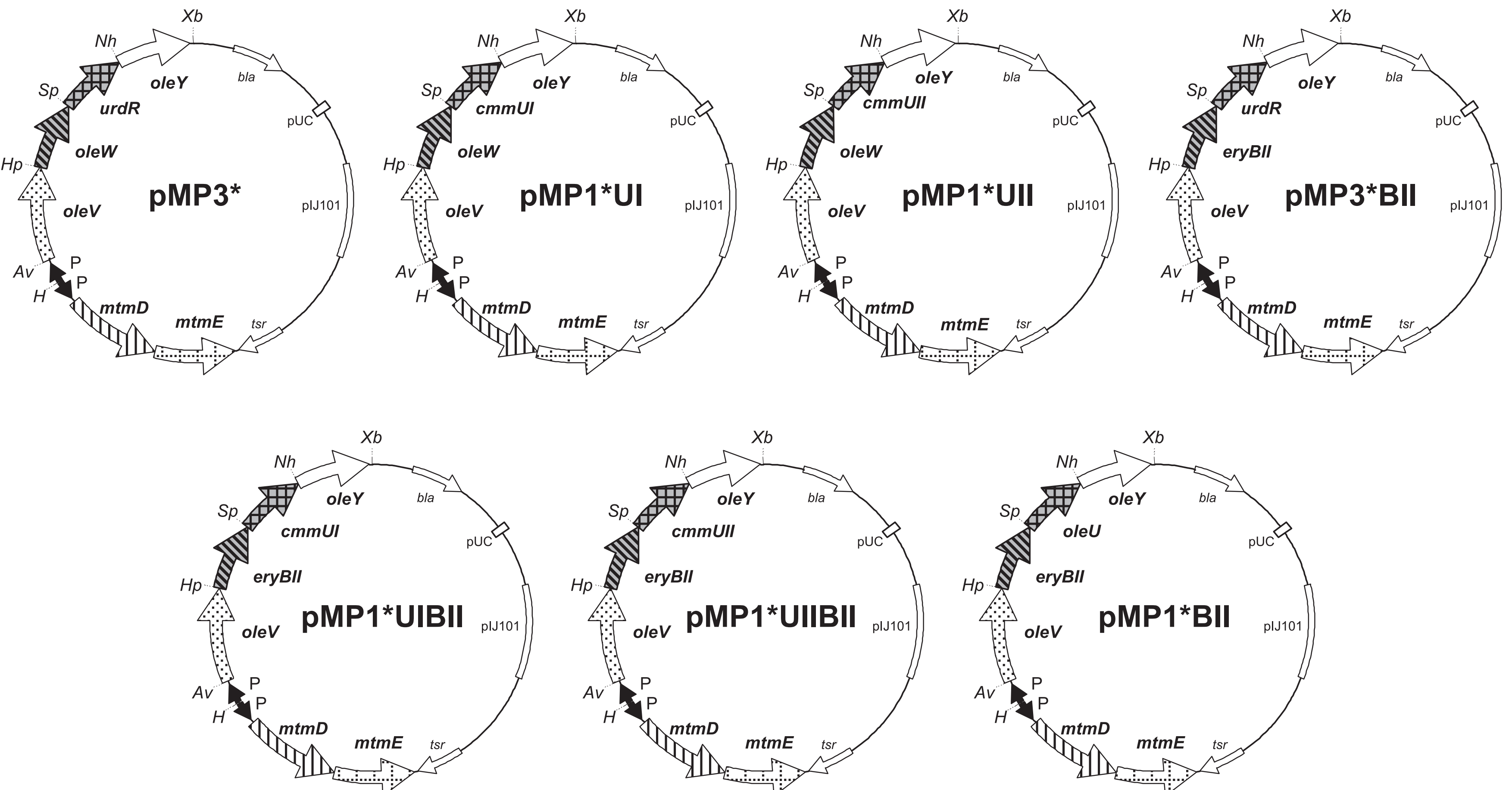

FIG. 2. Genetic organizations of plasmids for the biosynthesis of TDP-2,6-dideoxy-D-hexoses: TDP-D-olivose (pMP3* and pMP1*UI), TDP-D-oliose (pMP1*UII), TDP-D-digitoxose (pMP3*BII and pMP1*UIBII), and TDP-D-boivinose (pMP1*UIIBII and pMP1*BII). Abbreviations: P, erythromycin resistance promoter; *bla*, β-lactamase gene; *tsr*, thiostrepton resistance gene; *Av*, AvrII; *H*, HindIII; *Hp*, HpaI; *Nh*, NheI; *Sp*, SpeI; *Xb*, XbaI.

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TABLE 2. ^{1}H and ^{13}C NMR data for DDIG-TCMC in pyridine-d_5 at 300 MHz

Carbon	^{1}H NMR δ (*J* in Hz)	^{13}C NMR (δ)	Multiplicity
1		192.72	C
2	6.01 br s	101.37	CH
3		175.54	C
3-OCH_3	3.56 (s)	57.06	OCH_3
4	5.77 br s	71.74	CH
4a		87.55	C
5		195.71	C
5a		141.03	C
6	8.18 (s)	121.39	CH
6a		130.34	C
7	7.70 (s)	112.01	CH
8		156.08	C
9		130.08	C
9-C=O		168.59	C
9-CH_3CO	4.01	53.15	CH_3
10		138.54	C
10-CH_3	2.89 (s)	21.71	CH_3
10a		121.96	C
11		167.34	C
11-OH	14.03[a] br (s)		
11a		110.67	C
12		199.70	C
12a		86.01	C
1′	6.27 (dd, *J* = 9, 3 Hz)	97.37	CH
2′a	2.24 (ddd, *J* = 13, 9, 3 Hz)	39.08	CH_2
2′e	2.60 (ddd, *J* = 13, 3, 3 Hz)		
3′	4.56 (ddd, *J* = 3, 3, 3 Hz)	72.20	CH
4′	3.77 (dd, *J* = 9, 3 Hz)	74.15	CH
5′	4.62 (dd, *J* = 9, 6 Hz)	68.42	CH
6′	1.65 (d, *J* = 6)	19.70	CH_3

[a] Observable in d_6-acetone.

TABLE 3. ^{1}H and ^{13}C NMR data for DBOV-TCMC in pyridine-d_5 at 300 MHz

Carbon	^{1}H NMR δ (*J* in Hz)	^{13}C NMR (δ)	Multiplicity
1		192.76	C
2	6.02 br s	101.38	CH
3		175.57	C
3-OCH_3	3.56 (s)	57.07	OCH_3
4	5.79 br s	71.77	CH
4a		87.59	C
5		195.73	C
5a		141.07	C
6	8.20 (s)	121.39	CH
6a		130.30	C
7	7.74 (s)	112.02	CH
8		156.12	C
9		130.09	C
9-C=O		168.63	C
9-CH_3CO	3.94 (s)	53.10	CH_3
10		138.52	C
10-CH_3	2.91 (s)	21.72	CH_3
10a		121.94	C
11		167.37	C
11-OH	14.03[a] br (s)		
11a		110.65	C
12		199.68	C
12a		86.02	C
1′	6.28 (dd, *J* = 9, 3 Hz)	98.08	CH
2′a	2.80 (ddd, *J* = 13, 9, 3 Hz)	35.29	CH_2
2′e	2.38 (ddd, *J* = 13, 3, 3 Hz)		
3′	4.74 (ddd, *J* = 3, 3, 3 Hz)	71.93	CH
4′	4.00 (dd, *J* = 3.0, 1.5)	71.77	CH
5′	4.85 (dq, *J* = 6.0, 1.5)	69.94	CH
6′	1.66 (d, *J* = 6.0)	18.03	CH_3

[a] Observable in d_6-acetone.

AGGTGTCATG-3′; NMUI-R, 5′-AAAAGCTAGCTCAGGCGGAGGCCTCGG-3′) and *cmmUII* (NMUII-U, 5′-AAAAACTAGTGCATAGGAGCCACACCATG-3′; NMUII-R, 5′-AAAAGCTAGCTCAAGCCGGACTCGAAGG-3′). The PCR conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 1.25 units of Platinum-*Pfx* DNA Polymerase (Invitrogene) in a total reaction volume of 50 μl containing 1 mM $MgSO_4$, 0.3 mM of each deoxynucleoside triphosphate, 1× *Pfx* buffer, and, in some cases, PCRx Enhancer Solution. The polymerization reactions were performed in a thermocycler (PT-100; MJ Research). The general conditions for PCR amplification were as follows: 2 min at 94°C; 30 cycles composed of 15 s at 94°C, 30 s at 55°C, and 1 min at 68°C; 5 min at 68°C; and 15 min at 4°C. The PCR products were purified with GFX PCR DNA and a Gel Band Purification Kit (Amersham Biosciences), subcloned into pCRBlunt, and sequenced. Sequencing was performed using the dideoxynucleotide chain terminator method (34) 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 (Pharmacia). Computer-assisted database searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group program package (8) and the BLAST program (4).

Plasmid constructs. For the construction of pMP3*, first the SphI-XbaI polylinker fragment of pUC18 was used to replace the SphI-XbaI fragment in pLNR (30) containing the *oleL*, *oleS*, and *oleE* genes. Then, from the resultant construct, the 4.2-kb AvrII-XbaI fragment containing the *oleV*, *oleW*, *urdR*, and *oleY* genes was used to replace the AvrII-XbaI fragment in pFL942 (21), generating pMP3*. In this final construct, *oleV*, *oleW*, *urdR*, and *oleY* are under the control of *ermE**p*, and the *mtmD* and *mtmE* genes are divergently transcribed from *ermEp*.

Several other constructs were made using pMP3* as the starting plasmid, as shown in Table 1 and Fig. 2.

Production conditions and chromatographic techniques. Spores of *S. lividans* 16F4 containing the different constructs were grown in R5A medium under conditions previously described (30). High-performance liquid chromatography (HPLC) analyses were performed as previously described (30). Pure compounds were used as standards for D-olivosyl-tetracenomycin C and 8-demethyl-tetracenomycin C comparisons. They were purified from cultures of *S. lividans* strains 16F4 and 16F4(pLNR) (6, 30).

For the isolation of new glycosylated tetracenomycins, *S. lividans* strains 16F4(pMP3*BII) and 16F4(pMP1*BII) were cultured for 7 days in 2-liter Erlenmeyer flasks with R5A medium, as previously described (27). In each case, 3.2 liters of culture was centrifuged, filtered, and extracted (10). The compounds were purified by preparative HPLC, using a μBondapak C_{18} radial compression cartridge (PrepPak Cartridge; 25 by 100 mm; Waters). An isocratic elution with a mixture of acetonitrile and 0.1% trifluoroacetic acid in water (35:65) was used in both cases. The peaks corresponding to the desired compounds were collected, diluted fourfold with water, applied to a reverse-phase extraction cartridge, washed with water to eliminate trifluoroacetic acid, and finally eluted with methanol. After lyophilization, 7.9 mg of D-digitoxosyl-tetracenomycin C (DDIG-TCMC) and 25.7 mg of D-boivinosyl-tetracenomycin C (DBOV-TCMC) were obtained. HPLC-mass spectrometry (MS) analysis was carried out as previously described (21).

NMR analysis. The nuclear magnetic resonance (NMR) data (Tables 2 and 3) were recorded on a Varian Mercury 300 NMR spectrometer at a magnetic field strength of 7.05 T.

RESULTS

In vivo reconstitution of gene clusters for the biosynthesis of TDP-D-olivose and TDP-D-oliose. D-Olivose and D-oliose (Fig. 1) are both 2,6-D-DOHs with an equatorial C-3 hydroxyl group, but they differ in the configuration of the hydroxyl group at C-4: equatorial in the former and axial in the latter. D-Olivose is present in several antibiotic and antitumor compounds, such as mithramycin, chromomycin, urdamycin, and granaticin. D-Oliose is more unusual, but it is also found in mithramycin and, as a methylated or an acetylated derivative, in chromomycin

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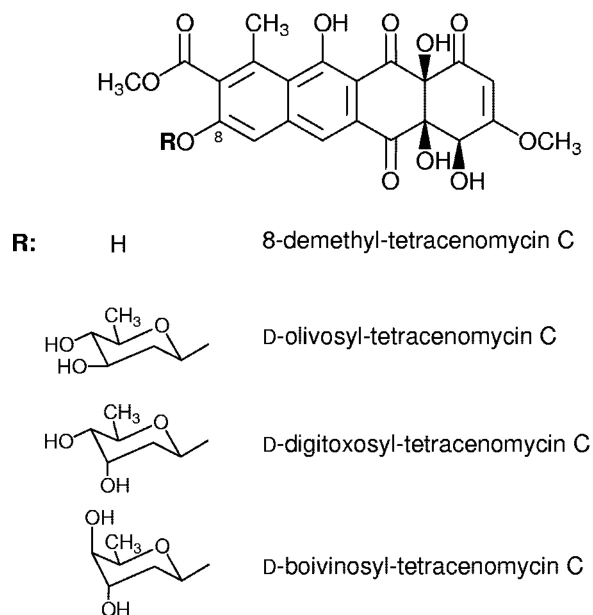

FIG. 3. Chemical structures of glycosylated tetracenomycin derivatives.

A_3. A gene cluster was constructed for the biosynthesis of TDP-D-olivose in plasmid pWHM3, generating pMP3* (Fig. 2). This construct contained all the gene functions required to synthesize TDP-D-olivose expressed under the control of two divergent erythromycin resistance promoters, one controlling the expression of two genes from the mithramycin gene cluster, *mtmD* (TDP-D-glucose synthase) and *mtmE* (TDP–D-hexose-4,6-dehydratase) (20), and the other controlling a set of four genes, three of them from the oleandomycin gene cluster, *oleV* (2,3-dehydratase), *oleW* (a 3-ketoreductase similar to Gra-orf26), and *oleY* (*O*-methyltransferase) (3), and a 4-ketoreductase gene (*urdR*) from the urdamycin gene cluster (15). To confirm that this gene cluster was able to direct the biosynthesis of TDP-D-olivose, pMP3* was introduced into *Streptomyces lividans* 16F4. This strain harbors cos16F4, a cosmid containing part of the elloramycin biosynthetic gene cluster from *Streptomyces olivaceus* Tü2353, and its expression in streptomycete hosts generates 8-demethyl-tetracenomycin C (8DMTC) (44) (Fig. 3). This cosmid also contains a gene for the sugar flexible glycosyltransferase ElmGT (6). Upon incubation of *S. lividans* 16F4(pMP3*) and analysis of cultures by HPLC-MS, in addition to the corresponding aglycone 8DMTC, a new peak (representing approximately 35% of all tetracenomycins) was detected (Fig. 4B). This peak was absent in a control experiment using *S. lividans* 16F4(pWHM3) (Fig. 4A). The new compound showed the same retention time as a pure sample of D-olivosyl-tetracenomycin C (DOLV-TCMC) and *m/z* values of 459 (corresponding to the 8DMTC aglycone fragment) and 589 (corresponding to DOLV-TCMC). The formation of this compound confirmed that pMP3* was directing the biosynthesis of TDP-D-olivose.

The biosynthesis of TDP-D-oliose differs from that of TDP-D-olivose only in the final ketoreduction step, which requires a 4-ketoreductase rendering a hydroxyl group at C-4 in axial configuration. To construct a gene cluster for the biosynthesis of TDP-D-oliose, it was necessary to replace the *urdR* gene (coding for a 4-ketoreductase) with another one able of generating the hydroxyl group in axial configuration. Chromomycin A_3 contains both D-olivose and modified D-oliose moieties. In the chromomycin cluster, there are two putative 4-ketoreductase genes that could be involved in the biosynthesis of either D-olivose or D-oliose (24). These two genes, *cmmUI* and *cmmUII*, were independently used to replace *urdR* in pMP3*, generating pMP1*UI and pMP1*UII, respectively (Fig. 2). Upon introduction of pMP1*UI into *S. lividans* 16F4, most 8DMTC was converted into DOLV-TCMC (Fig. 4C). The formation of this compound confirmed that CmmUI is the 4-ketoreductase involved in D-olivose biosynthesis in the chromomycin pathway and pointed to CmmUII as the candidate for the 4-ketoreductase in D-oliose biosynthesis. However, when pMP1*UII was introduced into *S. lividans* 16F4, no oliosyl-tetracenomycin or glycosylated tetracenomycin was observed (data not shown). In order to prove the functionality of pMP1*UII, i.e., its capability to direct the biosynthesis of D-oliose, we expressed the plasmid in *Streptomyces argillaceus* M7U1, a mithramycin nonproducer mutant in which D-oliose biosynthesis is affected (13). Complementation of this mutant was achieved, indicating that D-oliose biosynthesis was recovered and therefore that genes in pMP1*UII were functional (data not shown).

In vivo reconstitution of gene clusters for the biosynthesis of TDP-D-digitoxose and TDP-D-boivinose and generation of novel glycosylated derivatives of tetracenomycin C. D-Digitoxose and D-boivinose are both 2,6-D-DOHs with an axial C-3 hydroxyl group, but they differ in the configuration of the hydroxyl group at C-4: equatorial in the former and axial in the latter (Fig. 1). D-Digitoxose is well known as a constituent of plant cardiac and other steroidal glycosides (1, 2). D-Boivinose is an unusual DOH. As far as we know, it has been described only as a component of two flavone C-glycosides from *Zea mays* with glycation-inhibitory activities (37). As a constituent of antibiotic or antitumour compounds, D-digitoxose is present in the apoptosis inducer ammocidin produced by *Saccharothrix* sp. (25) and in the antibacterial saccharomicin produced by *Saccharotrix espanaensis* (18, 35). Very recently, a gene cluster for the biosynthesis of TDP-D-digitoxose was identified (5). However, no gene cluster for the biosynthesis of TDP-D-boivinose has been identified in antibiotic-producing microorganisms. We anticipated that a pathway for the biosynthesis of 2,6-D-DOH with a hydroxyl group at C-3 in axial configuration would require the same enzymatic steps needed to synthesize D-olivose and D-oliose but involving a TylC1-like ketoreductase to render the sugar intermediate TDP-2,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (Fig. 1). Consequently, we decided to test this hypothesis.

D-Olivose differs from D-digitoxose only in the stereochemistry at C-3 (Fig. 1). To construct a gene cluster for the biosynthesis of TDP-D-digitoxose, we used as starting plasmids pMP3* and pMP1*UI, which direct the biosynthesis of D-olivose. We replaced in these plasmids the *oleW* 3-ketoreductase gene with the TylC1-homologous 3-ketoreductase gene *eryBII* (12, 36), generating pMP3*BII and pMP1*UIBII, respectively (Fig. 2). After expressing pMP1*UIBII in *S. lividans* 16F4, only a peak corresponding to 8DMTC was observed (data not shown). However, when pMP3*BII was used, ap-

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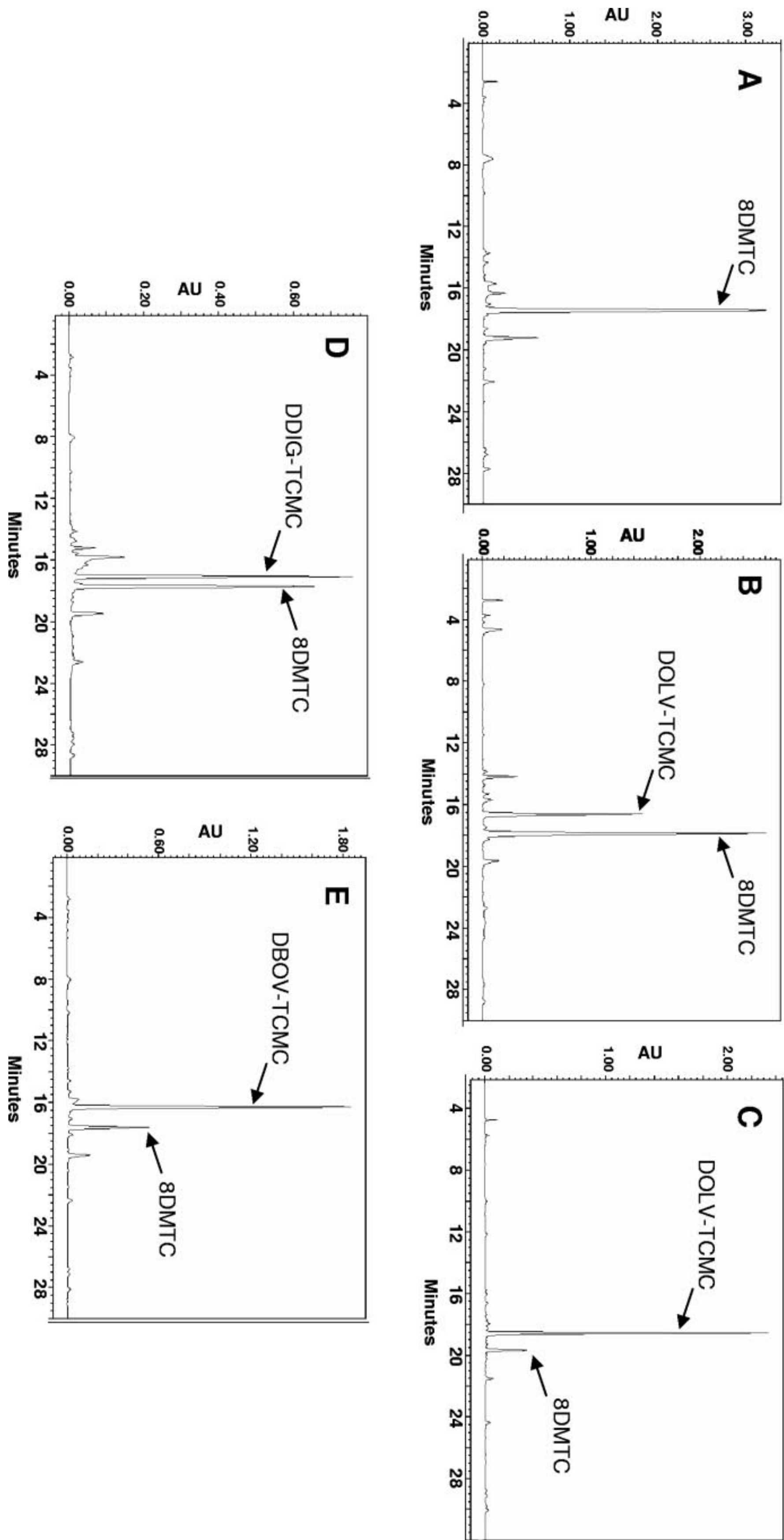

FIG. 4. HPLC analyses of cultures of *S. lividans* 16F4 harboring (A) pWHM3, (B) pMP3*, (C) pMP1*UI, (D) pMP3*BII, and (E) pMP1*BII. Peaks corresponding to the different compounds are indicated.

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proximately 55% of the aglycone 8DMTC was converted into a novel compound eluting at 17.0 min (Fig. 4D). The compound in this peak showed an *m/z* value in positive mode of 589 (with a fragmentation ion of *m/z* 459, corresponding to 8DMTC). This compound was purified, and NMR analysis (see below) confirmed that it corresponded to the novel compound DDIG-TCMC (Fig. 3). The formation of this compound confirmed that pMP3*BII was directing the biosynthesis of TDP-D-digitoxose. These results also indicated that, as has been reported (15, 28), the UrdR 4-ketoreductase shows a certain degree of substrate flexibility and can be used to generate 2,3,6-trideoxyhexoses and 2,6-dideoxyhexoses with different stereochemistries at C-3. On the other hand, the 4-ketoreductase CmmUI shows low substrate flexibility, since it is not able to act on a substrate differing from its natural one in the stereochemistry at C-3.

To construct a gene cluster for the biosynthesis of TDP-D-boivinose, it was necessary to incorporate, in addition to *eryBII*, a gene coding for a 4-ketoreductase that would render a hydroxyl group in axial configuration. We therefore replaced in pMP3*BII the 4-ketoreductase *urdR* gene (which renders hydroxyl groups in equatorial configuration) with the *cmmUII* gene. The resultant plasmid pMP1*UIIBII was expressed in *S. lividans* 16F4, but no glycosylated tetracenomycin was detected (data not shown). This suggested that, similarly to CmmUI, the CmmUII ketoreductase was unable to act on the substrate TDP-2,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose, which has stereochemistry at C-3 opposite to that of its normal substrate. Alternatively, the possibility exists that ElmGT is unable to transfer D-boivinose. To clarify this situation, we decided to use a different 4-ketoreductase, OleU, which is involved in the biosynthesis of L-oleandrose in the oleandomycin pathway (3). A first indication that OleU could be suitable for the biosynthesis of D-boivinose arose from the analysis of cultures of *S. lividans* 16F4 expressing pFL1012 (21). This plasmid derives from pFL943, which has been shown to direct the biosynthesis of TDP-L-olivose and TDP-L-rhamnose. pFL1012 contains the same gene functions as pFL943, but it lacks the *oleL* 3,5-epimerase gene, which prevents it from directing the biosynthesis of L-DOH (21). Notably, cultures of *S. lividans* 16F4(pFL1012) produced a glycosylated tetracenomycin with the same mass as DOLV-TCMC or DDIG-TCMC but showing a different retention time. This result prompted us to think that this compound could correspond to DBOV-TCMC. Consequently, we used the *oleU* 4-ketoreductase to replace *cmmUII* in pMP1*UIIBII. Upon transforming *S. lividans* 16F4 with the resultant construct, pMP1*BII (Fig. 2), a major peak (representing 76% of all tetracenomycins) was detected (Fig. 4E), eluting at 16.2 min and showing an *m/z* value of 589 (with a fragmentation ion of *m/z* 459, corresponding to 8DMTC). This mass was in accordance with the presence of a 2,6-DOH attached to 8DMTC. The compound from this peak was purified, and NMR analysis (see below) revealed that it corresponded to the novel compound DBOV-TCMC (Fig. 3). The formation of this compound confirmed that pMP1*BII was directing the biosynthesis of TDP-D-boivinose. To eliminate the possibility that a 4-ketoreductase gene from the *S. lividans* 16F4 host strain was participating in the biosynthesis of D-boivinose, a control experiment was run using pMP1*BIIΔU, a derivative of pMP1*BII in which *oleU* was deleted. Using this construct, no DBOV-TCMC was detected, confirming that OleU was the 4-ketoreductase involved in the formation of this boivinosyl derivative. This result also indicated that OleU shows a certain degree of flexibility, since, although it normally reduces a 2,6-L-DOH intermediate, it can also act on a 2,6-D-DOH substrate.

Structure elucidation of D-digitoxosyl-tetracenomycin C and D-boivinosyl-tetracenomycin C. The two novel tetracenomycins were characterized by liquid chromatography-MS (see below) and NMR spectroscopy in comparison with various previously described 8-position glycosylated tetracenomycins and elloramycins (11, 31, 44).

The positive atmospheric pressure chemical ionization (APCI) mass spectrum of DDIG-TCMC showed two major mass fragments, at *m/z* 589 (M^+) and at *m/z* 459 (M^+-sugar). While the former confirms the deduced molecular formula of $C_{28}H_{28}O_{14}$, the latter is consistent with the fragment obtained from the aglycon after cleavage of a 2,6-DOH unit. This pattern of M^+ and M-sugar fragmentations is typical of the glycosylated tetracenomycins and elloramycins. The ^{1}H NMR data (Table 2) revealed a β-glycosidically bound D-sugar (deduced from the large coupling of 1′-H of 9 Hz), as well as a sugar in $^4C_1$ conformation typical of D-sugars (from the overall H-H coupling pattern observed in the sugar unit, particularly the two axial protons in the 1″ and 5′ positions). The observed stereochemistry at the 3′ position (a multiple resulting from three small $^3J_{H\text{-}H}$ couplings with neighboring protons reveals an axially attached OH group) and in the 4′ position (dd, $J = 9$, 3 Hz, revealing an equatorially attached OH group) is consistent with digitoxose stereochemistry. This suggests the structure of 8-demethyl-8-β-D-digitoxosyltetracenomycin C for DDIG-TCMC, which was fully confirmed by the ^{13}C NMR data (Table 2).

The positive APCI mass spectrum of DBOV-TCMC gave the same molecular formula ($C_{28}H_{28}O_{14}$) and major fragmentation (M^+ at *m/z* 589; M-sugar at *m/z* 459) found for DDIG-TCMC, also consistent with a tetracenomycin derivative with a 2,6-dideoxysugar attached in the 8 position (for the APCI mass spectrum and the major fragmentation in the APCI mass spectrum, see the supplemental material). The ^{1}H NMR spectrum (Table 3) revealed the difference from DDIG-TCMC. While we also observed a β-glycosidically bound D-sugar in $^4C_1$ conformation, the signals of 3′-H (δ 4.74, ddd, $J = 3.0$, 3.0, 3.0 Hz) and 4′-H (δ 4.00, dq, $J = 6.0$, 1.5 Hz) of the sugar moiety clearly show that the OH groups in these two positions are attached axially in both cases, which gives rise to a boivinose stereochemistry. Therefore, the structure of 8-demethyl-8-β-D-boivinosyl-tetracenomycin C could be deduced for DBOV-TCMC. This was fully confirmed by the ^{13}C NMR data (Table 3).

DISCUSSION

Many bioactive compounds produced by actinomycetes are glycosylated, and in many cases, the saccharide moieties are essential for bioactivity. Therefore, efforts to modify the glycosylation pattern of a certain compound could have practical implications, since they could lead to the generation of a more active compound or one with improved pharmacological characteristics. This type of modification can be approached by combinatorial biosynthesis, providing that flexible glycosyltransferases exist, together with the possibility of using differ-

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ent nucleotide DOHs. In this context, the isolation of natural gene clusters and the reconstitution of "unnatural gene clusters" for the biosynthesis of DOHs have allowed their use for the generation of new glycosylated compounds (23, 26, 38). In this study, we have reconstituted "unnatural natural gene clusters" for the biosynthesis of four different activated 2,6-D-DOHs, namely, TDP-D-olivose, TDP-D-oliose, TDP-D-digitoxose, and TDP-D-boivinose. To achieve this, we combined, in a rational way, the required genes involved in sugar biosynthesis from different antibiotic and antitumor biosynthetic gene clusters: the antitumor agents mithramycin, chromomycin, and urdamycin and the antibiotics oleandomycin and erythromycin. Since the biosynthesis of 2,6-D-DOH proceeds through three common initial steps, all the gene clusters constructed contain three genes, which code for the enzymatic activities involved in those steps: the glucose synthase gene *mtmD* and the 4,6-dehydratase gene *mtmE* from the mithramycin cluster (20) and the 3,4-dehydratase gene *oleV* from the oleandomycin cluster (3), whose enzymatic activities had already been proven in vitro (20) or in vivo (30). In addition, gene clusters for the biosynthesis of TDP-D-olivose and TDP-D-oliose include the 3-ketoreductase gene *oleW* from the oleandomycin gene cluster, which had been shown to be involved in L-olivose biosynthesis reducing the keto group at C-3 with the formation of a hydroxyl group in equatorial configuration (3, 30). On the other hand, gene clusters for the biosynthesis of TDP-D-digitoxose and TDP-D-boivinose contain the *eryBII* C-3 ketoreductase from the erythromycin gene cluster, involved in L-mycarose biosynthesis (12, 36). Previous experiments have suggested that EryBII was able to introduce hydroxyl groups at C-3 in axial configuration (21). The fact that pMP3*BII and pMP1*BII were able to direct the biosynthesis of TDP-D-digitoxose and TDP-D-boivinose, respectively, confirms that function for EryBII and also indicates that this process takes place on a nucleotide sugar intermediate of D configuration.

All 2,6-D-DOH gene clusters contain a 4-ketoreductase gene. Thus, those for the biosynthesis of TDP-D-olivose and TDP-D-digitoxose (with hydroxyl groups at C-4 in equatorial configuration) include the 4-ketoreductase gene *urdR* from the urdamycin cluster, which has been shown to code for a 4-ketoreductase in D-olivose biosynthesis (15, 30). A gene cluster (present in pMP1*UI) for the biosynthesis of TDP-D-olivose was also constructed using a different 4-ketoreductase gene, *cmmUI*, a gene from the chromomycin gene cluster, which was proposed to code for a 4-ketoreductase involved in sugar biosynthesis (24). The fact that pMP1*UI directed the biosynthesis of D-olivose demonstrates that CmmUI is the 4-ketoreductase for D-olivose biosynthesis in the chromomycin pathway. On the other hand, in the gene cluster for the biosynthesis of TDP-D-boivinose, we included the *oleU* gene from the oleandomycin gene cluster, which has been shown to be involved in the biosynthesis of L-olivose (3). The fact that OleU can also be used for the biosynthesis of D-boivinose indicates that OleU shows some substrate flexibility, since although its natural substrate is a nucleotide sugar intermediate in L configuration, the results obtained demonstrate that OleU is also able to act on D-DOH biosynthesis intermediates. Finally, the gene cluster for the biosynthesis of TDP-D-oliose (present in pMP1*UII) includes the 4-ketoreductase *cmmUII* from the chromomycin gene cluster.

Validation of the functionality of the described gene clusters was achieved by expressing them in *Streptomyces albus* 16F4 and the subsequent formation of the corresponding glycosylated tetracenomycin. However, when plasmid pMP1*UII was used, no glycosylated tetracenomycin was produced. Two possible hypotheses exist to explain this result: (i) CmmUII is not the 4-ketoreductase for TDP-D-oliose and therefore pMP1*UII is not able to direct the biosynthesis of this deoxysugar, and consequently no glycosylated tetracenomycin is formed, or (ii) even though the biosynthesis of TDP-D-oliose is directed by pMP1*UII, the possibility exists that this DOH was not recognized by the ElmGT glycosyltransferase. We favor the second explanation, since pMP1*UII directs the biosynthesis of D-oliose, as it was able to complement the non-mithramycin-producing mutant *S. argilllaceus* M7U1, in which D-oliose biosynthesis is affected. Moreover, previous experiments also suggested that the glycosyltransferase ElmGT was not able to recognize TDP-D-oliose as a substrate. Thus, it has been shown that by expressing cosmid 16F4 in the mithramycin producer *S. argillaceus*, no D-oliosyl-tetracenomycin C was obtained, despite the fact that this microorganism synthesizes TDP-D-oliose (44).

In this work, two novel glycosylated tetracenomycins were generated, DDIG-TCMC and DBOV-TCMC. The formation of these two new compounds extends the NDP-sugar donor substrate profile usable by the ElmGT glycosyltransferase by two. ElmGT has been previously shown to be able to transfer nine different sugars (23).

Gene clusters for the biosynthesis of TDP-D-boivinose in antibiotic producers had not been described or isolated previously. The reconstituted gene clusters described here for the biosynthesis of this DOH, and also those for TDP-D-digitoxose, TDP-D-olivose, and TDP-D-oliose, will be very useful for the generation of new glycosylated derivatives of bioactive compounds by providing host strains with the capability of synthesizing these D-DOHs, which then could be potentially transferred by existing glycosyltransferases of the host to an aglycone. In addition, the information obtained from the reconstituted gene clusters for the biosynthesis of TDP-D-boivinose could help to predict which gene functions should be present in a natural gene cluster for this deoxysugar.

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