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Research Paper

Identification of a sugar flexible glycosyltransferase from *Streptomyces olivaceus*, the producer of the antitumor polyketide elloramycin

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Abstract

Background: Elloramycin is an anthracycline-like antitumor drug related to tetracenomycin C which is produced by *Streptomyces olivaceus* Tü2353. Structurally is a tetracyclic aromatic polyketide derived from the condensation of 10 acetate units. Its chromophoric aglycon is glycosylated with a permethylated L-rhamnose moiety at the C-8 hydroxy group. Only limited information is available about the genes involved in the biosynthesis of elloramycin. From a library of chromosomal DNA from *S. olivaceus*, a cosmid (16F4) was isolated that contains part of the elloramycin gene cluster and when expressed in *Streptomyces lividans* resulted in the production of a non-glycosylated intermediate in elloramycin biosynthesis, 8-demethyl-tetracenomycin C (8-DMTC).

Results: The expression of cosmid 16F4 in several producers of glycosylated antibiotics has been shown to produce tetracenomycin derivatives containing different 6-deoxysugars. Different experimental approaches showed that the glycosyltransferase gene involved in these glycosylation events was located in 16F4. Using degenerated oligoprimers derived from conserved amino acid sequences in glycosyltransferases, the gene encoding this sugar flexible glycosyltransferase (*elmGT*) has been identified. After expression of *elmGT* in *Streptomyces albus* under the control of the erythromycin resistance promoter, *ermEp*, it was shown that *elmG* can transfer different monosaccharides (both L- and D-sugars) and a disaccharide to 8-DMTC. Formation of a diolivosyl derivative in the mithramycin producer *Streptomyces argillaceus* was found to require the cooperative action of two mithramycin glycosyltransferases (MtmGI and MtmGII) responsible for the formation of the diolivosyl disaccharide, which is then transferred by ElmGT to 8-DMTC.

Conclusions: The ElmGT glycosyltransferase from *S. olivaceus* Tü2353 can transfer different sugars into the aglycon 8-DMTC. In addition to its natural sugar substrate L-rhamnose, ElmGT can transfer several L- and D-sugars and also a diolivosyl disaccharide into the aglycon 8-DMTC. ElmGT is an example of sugar flexible glycosyltransferase and can represent an important tool for combinatorial biosynthesis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Polyketide; Anticancer; Deoxyhexose; Anthracycline; Mithramycin

1. Introduction

A number of bioactive compounds contain sugars attached to their aglycons. Usually, glycosylation occurs as late steps in biosynthesis and the addition of the respective sugars is important and, in many cases, essential for biological activity of the compounds. Most of these sugars belong to the large family of the 6-deoxyhexoses (6-DOH) which form part of a great number of natural products and at least 80 different 6-DOH have been identified in different metabolites [1–3]. In the last years, a number of deoxysugar biosynthetic pathways from antibiotic-producing organisms are increasingly being reported [3], providing the information needed to design new gene combinations directing the biosynthesis of different 6-DOHs. In

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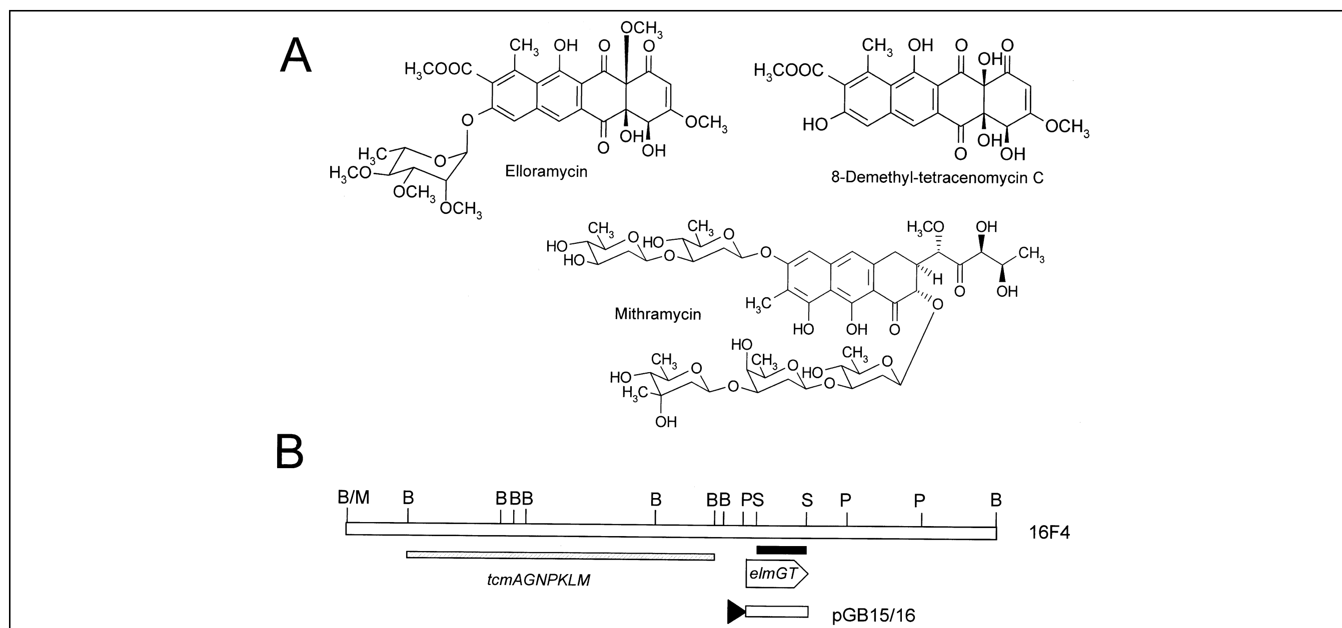

Fig. 1. (A) Structures of elloramycin, 8-DMTC and mithramycin. (B) Schematic representation of the restriction map of cosmid 16F4: the shadowed bar indicates the region that has been shown to hybridize with the *tcmAGNPKLM* genes of the tetracenomycin C gene cluster [17]. The black bar shows the location of the fragment hybridizing against the 350 bp PCR probe. The black triangle represents the *ermEp*. B, *Bam*HI; M, *Mbo*I; P, *Pst*I; S, *Sal*I.

addition, several glycosyltransferase genes have been cloned and characterized [4–13], supplying the necessary tools for the potential generation of new glycosylated bioactive compounds.

Elloramycin is an anthracycline-like antitumor drug produced by *Streptomyces olivaceus* Tü2353 [14]. It is active against Gram-positive bacteria and also exhibits antitumor activity [15]. Structurally, elloramycin belongs to the large and important family of the aromatic polyketides [16]. Its aglycon closely resembles tetracenomycin C, but has an additional C-12a-*O*-methyl group and, in contrast to tetracenomycin C, is glycosylated with a permethylated L-rhamnose residue at the C-8 hydroxyl group (Fig. 1A). Not too much information exists about genes involved in elloramycin biosynthesis. Using a cosmid library of chromosomal DNA from *S. olivaceus*, several overlapping cosmid clones were isolated by probing this cosmid library with two polyketide synthase genes of the tetracenomycin cluster (*tcmK* and *tcmL*) [17]. Two positive cosmid clones (16F4 and 4F5) were characterized in some detail. Different tetracenomycin C genes were used as probes to detect the presence of homologous elloramycin genes in these cosmids (Fig. 1B). The conclusion was that basically the sequential order of the *tcm* and *elm* genes appeared to be very similar [17]. Expression of 16F4 in *Streptomyces lividans* TK64 resulted in the production of several putative intermediates in elloramycin biosynthesis, 8-demethyl-tetracenomycin C (8-DMTC; Fig. 1A), a non-glycosylated intermediate, being the major compound. It was therefore assumed that 16F4 should contain all genes necessary for the biosynthesis of the polyketide moiety of elloramycin and should probably lack some of the genes required for L-rhamnose biosynthesis or its transfer to the aglycon.

Interestingly, 16F4 has been shown to be a good genetical tool for the generation of novel compounds by combinatorial biosynthesis. Novel hybrid compounds were obtained after introducing 16F4 into different producer strains. These were hybrid compounds between urdamycin and elloramycin [18], mithramycin and elloramycin [19] and oleandomycin and elloramycin [20]. All these compounds have a common feature: they share the aglycon (8-DMTC encoded by 16F4) and contain different sugar moieties synthesized by the host strain. Here we report experimental evidence demonstrating the presence of a sugar flexible glycosyltransferase in 16F4, its cloning and expression and evidence of the sugar flexible capability of this enzyme.

2. Results and discussion

2.1. *cos16F4 contains a gene encoding a glycosyltransferase responsible for the formation of glycosylated tetracenomycins*

The formation of different glycosylated tetracenomycins after expression of 16F4 into several producer organisms raised a question about the genetic location of the gene encoding the glycosyltransferase responsible for sugar transfer into the aglycon: would it be coded by 16F4 or would it be a glycosyltransferase present in the transformation host? To discern between these two possibilities we

carried out several experiments using several hosts with different genetic backgrounds (Table 1). When 16F4 was transformed into the mithramycin producer, *Streptomyces argillaceus*, four compounds were produced by the recombinant strain: the elloramycin aglycon 8-DMTC and three glycosylated derivatives. Two of them contained a D-olivose or a D-mycarose moiety attached to the aglycon, 8-demethyl-8-β-D-olivosyl-tetracenomycin C (DOLV-TCM) and 8-demethyl-8-β-D-mycarosyl-tetracenomycin C (DMYC-TCM). The third one contained a diolivosyl disaccharide attached to the aglycon, 8-demethyl-8-β-D-diolivosyl-3′-1″-D-β-tetracenomycin C (DDIOLV-TCM). The purification and structural elucidation of these compounds was previously carried out [19] and from that moment it was possible to identify these compounds in the different recombinant strains by comparison of their high performance liquid chromatography (HPLC) mobility and absorption spectra with pure samples.

To verify if a glycosyltransferase from the host strain was responsible for the sugar transfer, *S. argillaceus* wild type strain was transformed with pWHM1026Δ*tcmO*. This plasmid contains the entire tetracenomycin C gene cluster with the exception of the *tcmO* gene that codes for a 8-*O*-methyltransferase and therefore directs the production of 8-DMTC. The resultant recombinant strain produced 8-DMTC but none of the glycosylated compounds were formed. Since in this strain the aglycon was produced and the sugars to be transferred (D-olivose and D-mycarose) were synthesized by the host strain, the absence of glycosylated tetracenomycins was a clear indication that the glycosyltransferase was absent from this recombinant strain and therefore from the *S. argillaceus* host genome. A similar result and conclusion was obtained by feeding *S. argillaceus* wild type strain with 8-DMTC since no glycosylated tetracenomycin derivatives were generated. These results strongly suggested that the glycosyltransferase should be coded by 16F4.

Another strong indication that 16F4 harbors the glycosyltransferase gene came from experiments in which 16F4 was used to transform the *S. argillaceus* M3ΔMG mutant. This is a mithramycin non-producing mutant in which a DNA fragment has been deleted by gene replacement eliminating a region containing four mithramycin glycosyltransferases (*mtmGI*, *mtmGII*, *mtmGIII* and *mtmGIV*) and two methyltransferases (*mtmMI* and *mtmMII*) from the chromosome [21]. When 16F4 was transformed into the M3ΔMG mutant, three of the four compounds were produced (8-DMTC, DOLV-TCM and DMYC-TCM), DDIOLV-TCM not being detected. This demonstrates that generation of the monoglycosylated derivatives is not dependent upon the activity of any mithramycin glycosyltransferase (since none of them are present in this mutant) but it requires the presence of a glycosyltransferase gene in 16F4. Interestingly, DDIOLV-TCM was only formed in *S. argillaceus* wild type strain but not in this mutant (see below for further experiments and discussion).

The final and definitive evidence demonstrating that the glycosyltransferase is present in 16F4 and not in the host strains came from another experiment in which 16F4 was transformed into *S. lividans* harboring pFLOS. This is a plasmid that contains several genes of the mithramycin gene cluster (*mtmD*, *mtmE*, *mtmC*, *mtmU*, *mtmV*, *mtmT3* and *mtmW*) most of which were supposed to be involved in sugar biosynthesis. The recombinant strain *S. lividans* FLOS/16F4 directed the production of two of the glycosylated compounds, but not DDIOLV-TCM. Since no mithramycin glycosyltransferases are present in this host strain, this experiment was a clear and definitive proof that the glycosyltransferase is encoded by 16F4. The conclusion of all these experiments was that 16F4 must contain a gene encoding a glycosyltransferase that could transfer individually D-olivose and D-mycarose or a diolivosyl disaccharide to the 8-DMTC aglycon, providing this disaccharide is previously synthesized by the mithramycin glycosyltransferases (see below).

2.2. *Cloning of the elmGT glycosyltransferase gene*

To clone the elloramycin glycosyltransferase gene from 16F4 we initially used a hybridization approach. The

Table 1
Biosynthesis of glycosylated tetracenomycins by different recombinant strains

Host	Plasmid/cosmid constructs	Products			
		8-DMTC	DOLV-TCM	DMYC-TCM	DDIOLV-TCM
S. argillaceus wt	16F4	+	+	+	+
S. argillaceus wt	pWHM1026Δ*tcmO*	+	–	–	–
S. argillaceus M3ΔMG	16F4	+	+	+	–
S. lividans TK21	16F4	+	–	–	–
S. lividans TK21	16F4+pFLOS	+	+	+	–
S. argillaceus M3G1	16F4	+	+	+	–
S. argillaceus M3G2	16F4	+	+	+	–
S. argillaceus M3ΔMG	pGB15+pEPG1	+	+	+	–
S. argillaceus M3ΔMG	pGB15+pEPG2	+	+	+	–
S. argillaceus M3ΔMG	pGB15+pEPG12	+	+	+	+

8-DMTC, 8-demethyl-tetracenomycin C; DOLV-TCM, 8-demethyl-8-β-D-olivosyl-tetracenomycin C; DMYC-TCM, 8-demethyl-8-β-D-mycarosyl-tetracenomycin C; DDIOLV-TCM, 8-demethyl-8-β-D-diolivosyl-3′-1″-D-β-tetracenomycin C.

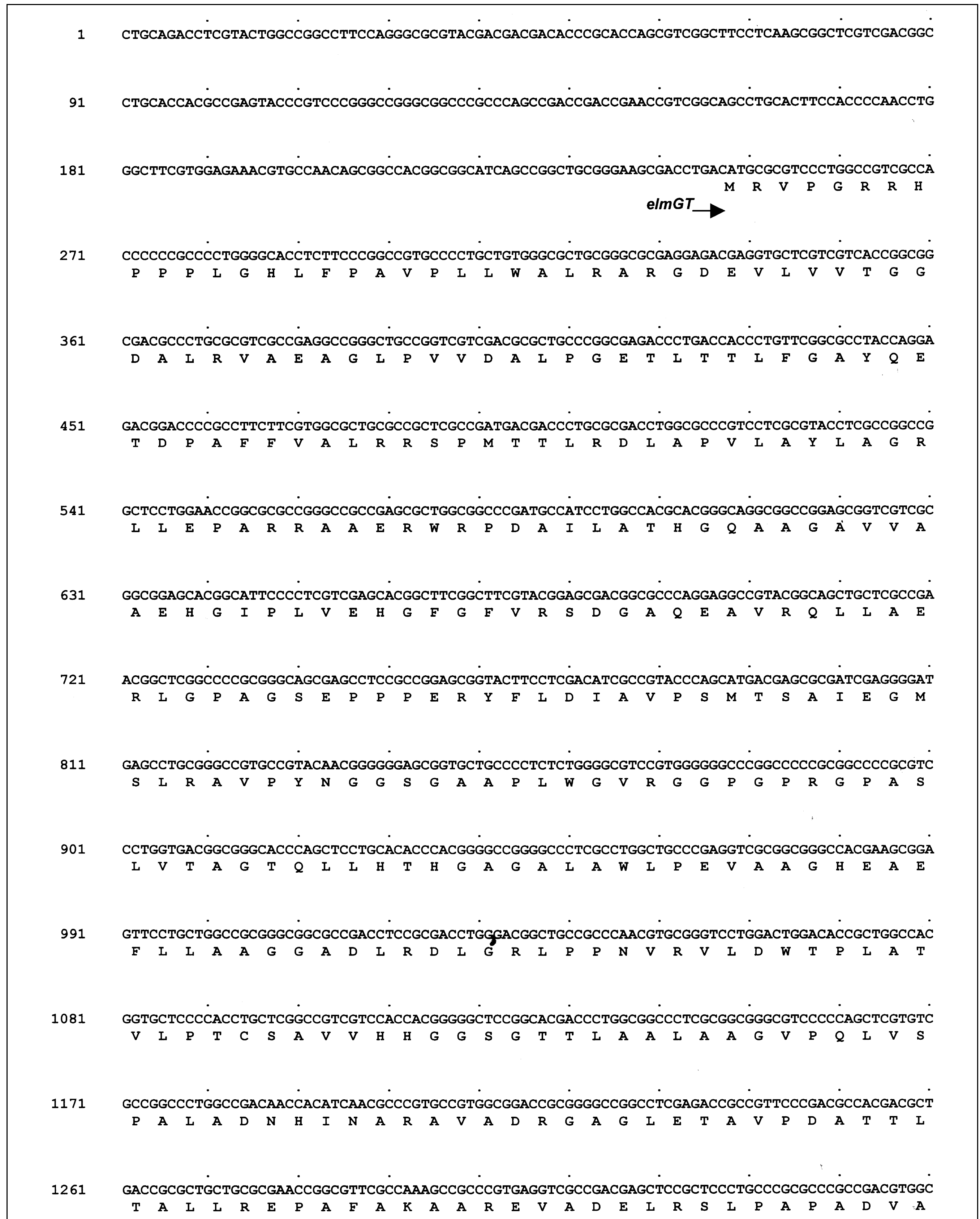

Fig. 2. Nucleotide sequence of the 1850 bp DNA region sequenced containing elmGT. The complete nucleotide sequence of the fragment is shown with the deduced amino acid sequence of elmGT in a single letter code. The sequence has been deposited in GenBank under the accession number AJ300305.

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1351   CGCCCGTCTGCACACCGCGTTCGGCCTCCCGACCACTCAAGGAGACGCATGACCACCCCTTCACCCACGCCACCGCTCGCGGCCGCCGAG
        A  R  L  H  T  A  F  G  L  P  T  T  Q  G  D  A  *

1441   GTGGCCGCCGCCGCGGGACTCCAGCAGCGGACCTGCTCGCGGTGCTCGACCGGGTCGGCCTGGAACCCGCCGTCGCGTTCCTCGTCCATG

1531   ACCTCACGGCCCGCTGCGACACCCCCGACAACCCGGACGCGGCCCGGATCGGCCTCGCCGTCGAGCACAGCGGACACCGGACCGAGCGGG

1621   TCCTCGCCGTGCGCAAGGGCGAGCCCGTCCGGGTGGACGAGAAGACGGCGGGTCCGCCTCCCGTACGTCTGACCTTCGACCTCGCCGACC

1711   TGGTCCGTGGCGTGTACGGACCGCCGCCCGGTCCCGGAGCCGGTCTGTTCCGGGTCGAACGCGACGACGCCTGGTTCGTCAAGAACGCCG

1801   ACGACGCCGAGCCCTTCCGGATCTTCGAGTCGTACATGCGCGCCGTCGAC    1850
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Fig. 2 (*continued*).

mtmGI glycosyltransferase gene from the mithramycin cluster was used as a probe against *Bam*HI-digested cosmid 16F4 DNA; however, no clear hybridization signal was observed in spite of using different stringency conditions. We therefore used another approach by using polymerase chain reaction (PCR) and two oligoprimers derived from consensus amino acid sequences obtained after comparison of known glycosyltransferases from antibiotic-producing microorganisms (Fig. 3A). Using this pair of oligoprimers and 16F4 DNA as template, a 350 bp PCR fragment was amplified. Preliminary partial sequence of this fragment and comparison of its deduced amino acid sequence with proteins in databases showed that it could code for an internal region of a glycosyltransferase. This PCR fragment was then used as probe to locate the glycosyltransferase gene within 16F4 and found to hybridize within a 9.5 kb *Bam*HI fragment located at the right-hand-side of 16F4 (Fig. 1B). Further subcloning and Southern analysis reduced the hybridizing region to a minimal region of 1.5 kb *Sal*I fragment (Fig. 1B). A region of 1.85 kb was sequenced and the nucleotide sequence (Fig. 2) analyzed for the presence of potential coding regions using the CODONPREFERENCE program [22]. The presence of an open reading frame (ORF) was observed (named *elmGT*) that started at an ATG codon and ended at a TGA codon. This ORF comprised 1152 nucleotides and coded for a polypeptide of 384 amino acids and had an estimated M_r of 39 674 Da. This ORF showed the characteristic high GC content of *Streptomyces* genes and the bias in the third codon position characteristic of the genes of this genus. Comparison of the deduced product of *elmGT* with proteins in databases, showed clear similarities with different glycosyltransferases. The highest scores were with SnogD, a glycosyltransferase from *Streptomyces nogalater* involved in nogalamycin biosynthesis (39.5% identity) [23], UrdGT1b and UrdGT1c, glycosyltransferases from *Streptomyces fradiae* involved in urdamycin biosynthesis (39 and 38% identity, respectively) [13], IroB a glycosyltransferase from *Salmonella typhi* (34% identity) [23], OleG2 a glycosyltransferase from *Streptomyces antibioticus* involved in oleandomycin biosynthesis (34% identity) [8], MtmGII a glycosyltransferase from *S. argillaceus* involved in mithramycin biosynthesis (34% identity) [7] and LanGT2 a glycosyltransferase from *Streptomyces cyanogenus* involved in landomycin biosynthesis (30% identity) [11]. A comparative dendrogram showing similarities between the ElmGT and other antibiotic glycosyltransferases is shown in Fig. 3B.

2.3. Confirmation of the ElmGT sugar flexibility

Once the elloramycin glycosyltransferase gene was identified, it was expressed in different hosts for in vivo assay of its capability to transfer several dTDP-sugars into the elloramycin aglycon. The *elmGT* gene was subcloned under the control of the erythromycin resistance promoter *ermEp* in the multicopy plasmid vector pIAGO (generating pGB15) and then together with the promoter in the integrative plasmid vector pKC796 (generating pGB16). After transforming protoplasts of *Streptomyces albus* with pGB16, clones in which the plasmid was integrated into the chromosome through the *att* integration site were identified by resistance to apramycin and verified by Southern analysis. The resultant recombinant strain (GB16) was then transformed independently with several plasmid constructs that direct the biosynthesis of different dTDP-L-sugars (Table 2). These constructs (pRHAM, pOLE and pOLV) contain different combinations of oleandrose biosynthetic genes from *S. antibioticus*, an oleandomycin producer, and direct the biosynthesis of dTDP-L-

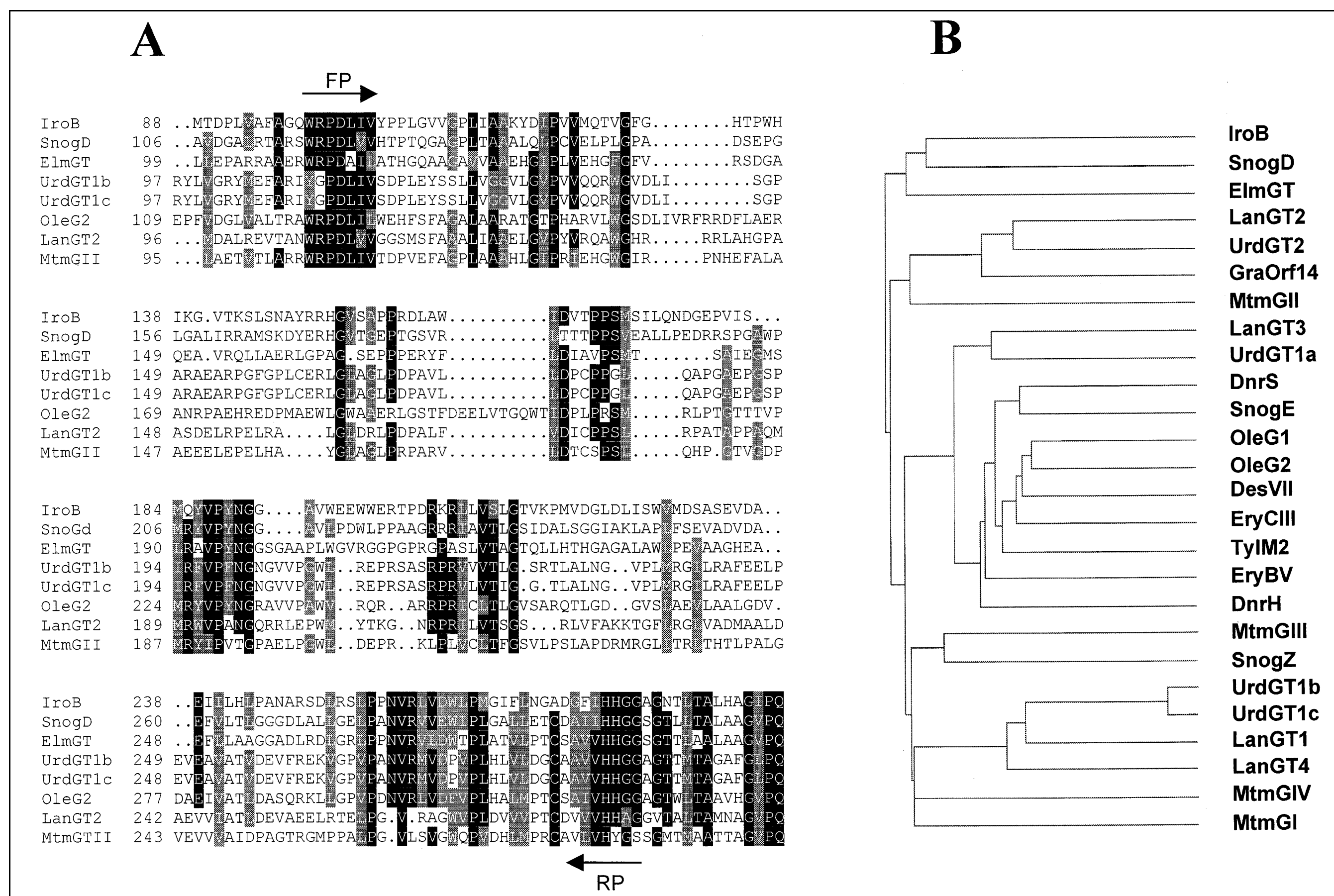

Fig. 3. (A) Alignment of the deduced amino acid sequences of different glycosyltransferases around the conserved regions chosen for designing the two oligoprimers for PCR amplification. FP and RP indicate the amino acid regions selected for the forward and reverse primer, respectively. IroB, glycosyltransferase from *S. typhi* [24]; SnogD, glycosyltransferase from *S. nogalater* [23]; UrdGT1b and UrdGT1c, glycosyltransferases from *S. fradiae* [3]; OleG2, glycosyltransferase from *S. antibioticus* [8]; LanGT2, glycosyltransferase from *S. cyanogenus* [11]; MtmGII, glycosyltransferase from *S. argillaceus* [7]. (B) Dendrogram showing the similarities among different glycosyltransferases: all glycosyltransferases shown are involved in antibiotic biosynthesis in producer organisms, with the exception of IroB.

Table 2
Biotransformation experiments to assay in vivo the activity of the ElmG glycosyltransferase in *S. albus* using different constructs directing the biosynthesis of several 6-deoxysugars

Construct	Genes	Sugar synthesized	Compound generated
pRHAM	▶ oleL oleS oleE oleU	L-rhamnose	RHA-TCM
pOLV	oleW oleV oleL oleS oleE oleU	L-olivose	OLV-TCM
pOLE	oleW oleV oleL oleS oleE oleU ▶ oleY	L-oleandrose	OLV-TCM

The black triangle indicates the position and orientation of the erythromycin resistance promoter.

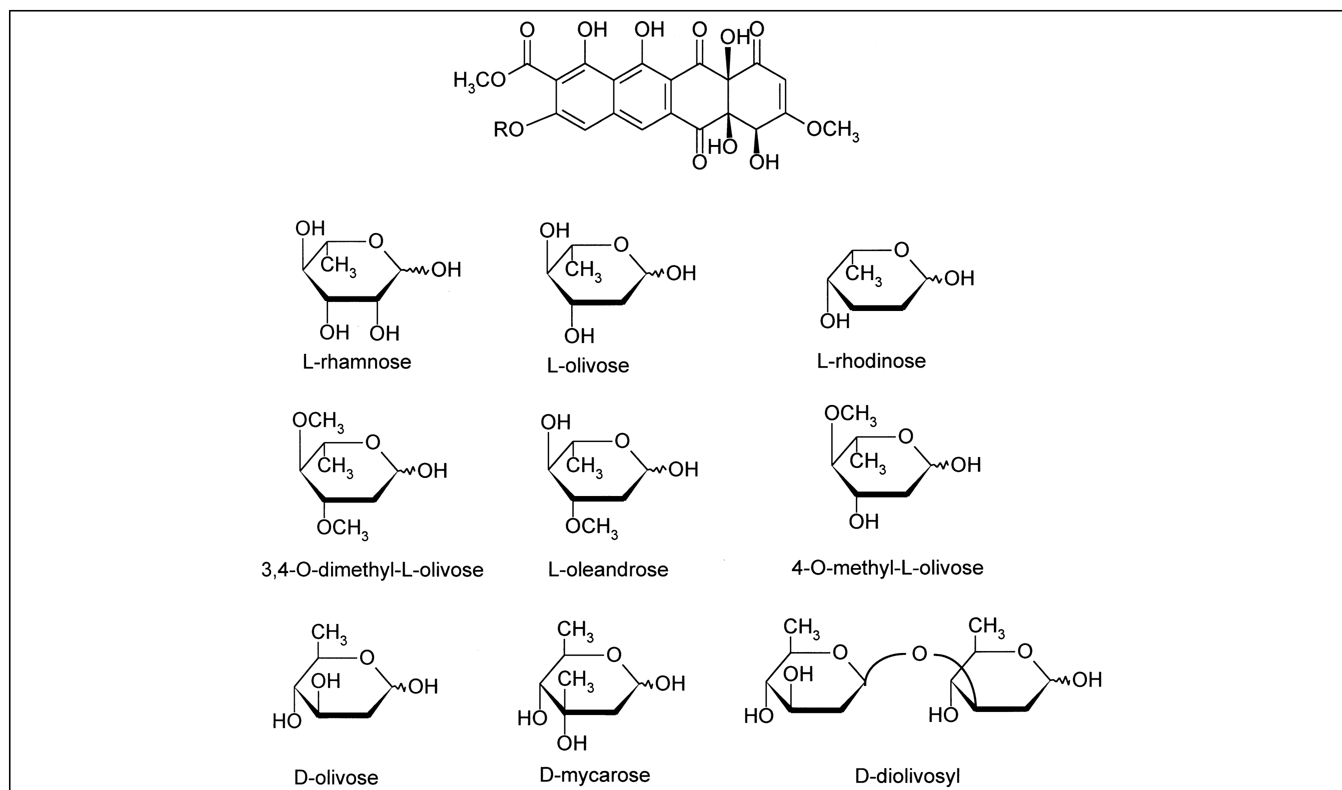

Fig. 4. Structures of 8-DMTC and 6-DOH (substituted at R) forming part of novel glycosylated tetracenomycin derivatives generated with the participation of the ElmGT glycosyltransferase.

rhamnose, dTDP-L-olivose and dTDP-L-oleandrose, respectively [20,25]. Transformants were selected for resistance to thiostrepton. The double recombinant strains harboring the *elmGT* gene integrated into the chromosome and the replicative plasmids directing the biosynthesis of different 6-DOH were used for biotransformation experiments. They were incubated for 24 h in liquid medium and then 8-DMTC was added (100 μg/ml final concentration). After 2 days of further incubation, the bioconversion of 8-DMTC to glycosylated derivatives was monitored (thin layer chromatography (TLC) and HPLC). In the presence of pRHAM, bioconversion of 8-DMTC was a very efficient process. Since it was previously shown [20] that pRHAM directs the biosynthesis of dTDP-L-rhamnose, it was assumed that the bioconverted product was L-rhamnosyl-tetracenomycin C. The product was purified and nuclear magnetic resonance analysis confirmed this presumption. Strains harboring either pOLV or pOLE gave a good bioconversion of 8-DMTC into the same compound, L-olivosyl-tetracenomycin C (LOLV-TCM), as it was confirmed by running the bioconversion products in parallel with pure LOLV-TCM as standard. L-Olivose has been proposed to be an intermediate in L-oleandrose biosynthesis in *S. antibioticus* [25]. These experiments confirm that ElmGT can recognize L-2,6-deoxysugars (i.e. L-olivose) in addition to its natural sugar substrate, an L-6-deoxysugar like L-rhamnose. The *elmGT* gene was also expressed (in pGB15) in the *S. argillaceus* mutant M3ΔMG in order to verify its capability to transfer D-sugars. Transformants were selected by their resistance to thiostrepton and used in biotransformation experiments with 8-DMTC. Formation of the monoglycosylated tetracenomycin derivatives (DOLV-TCM and DMYC-TCM) was observed but the presence of the diglycosylated derivative (DDIOLV-TCM) was not detected, suggesting the involvement of some mithramycin glycosyltransferases in the formation of the disaccharide derivative.

2.4. *Formation of DDIOLV-TCM requires the cooperation of two mithramycin glycosyltransferases and the elloramycin glycosyltransferase, ElmGT*

As shown above, the diglycosylated tetracenomycin DDIOLV-TCMC was not produced in *S. argillaceus* mutant M3ΔMG in which all the mithramycin glycosyltransferases were absent. This cannot be explained by the absence of an intracellular pool of dTDP-D-olivose in this mutant since the monoglycosylated derivative DOLV-TCMC was still produced. This raised an interesting question about how the diolivosyl disaccharide was formed and transferred. From the above shown results, it can be concluded that ElmGT plus some mithramycin glycosyltransferases are required for the biosynthesis of DDIOLV-TCM. Mithramycin contains a diolivosyl disaccharide attached to the C-6 position of the mithramycinone aglycon. It has been proposed that during mithramycin biosynthesis

this disaccharide is not formed by a sequential addition of the two D-olivose moieties but rather a diolivosyl disaccharide is firstly formed and then transferred [7]. The *mtmGI* and *mtmGII* gene products would be responsible for this synthesis and incorporation, although it is not yet clear which one was responsible for the formation of the disaccharide and its transfer [7]. Therefore, we thought that the biosynthesis of DDIOLV-TCM should require either one or both MtmGI and MtmGII glycosyltransferases for the formation of the diolivosyl disaccharide and ElmGT to transfer the disaccharide. To test this hypothesis, 16F4 was used to transform protoplasts of two *S. argillaceus* mutants (M3G1 and M3G2 mutants), in which either *mtmGI* or *mtmGII* genes were independently inactivated, respectively [7]. Analysis of the products synthesized by these recombinant strains showed that only the monoglycosylated tetracenomycins were produced, suggesting that the presence of both mithramycin glycosyltransferases was required for the formation of DDIOLV-TCM. Further confirmation was obtained when *S. argillaceus* M3ΔMG containing pGB15 (and therefore expressing *elmGT*) was independently transformed with pEM4A (control), pEPG1 (expressing *mtmGI*), pEPG2 (expressing *mtmGII*) or pEPG12 (expressing both *mtmGI* and *mtmGII*). Selected clones from these transformations were then incubated in the presence of 8-DMTC. Analysis of the biotransformation products after 2 days of incubation showed that no DDIOLV-TCM formation was observed with clones individually expressing *mtmGI* or *mtmGII* but DDIOLV-TCM was detected in the culture supernatant of clones that simultaneously expressed both mithramycin glycosyltransferases. From these results three main conclusions can be drawn: (i) addition of the diolivosyl disaccharide does not occur by sequential incorporation of the two sugars but rather by the direct transfer of the disaccharide; (ii) the MtmGI and MtmGII glycosyltransferases are responsible for the formation of the diolivosyl disaccharide and (iii) the disaccharide is then transferred by ElmGT to 8-DMTC.

2.5. *Sugar flexible glycosyltransferases for generating new bioactive compounds*

For the generation of novel glycosylated compounds by combinatorial biosynthesis, a certain degree of substrate flexibility of the glycosyltransferases is required. Recent studies by different laboratories have shown that some glycosyltransferases from different antibiotic biosynthetic pathways have relaxed substrate specificity. Glycosyltransferases involved in sugar transfer during the biosynthesis of glycopeptide antibiotics have been shown to transfer sugars to different, but structurally related, heptapeptides [26]. In addition, some macrolide glycosyltransferases can transfer different sugars into an aglycon [10,25,27–29]. The ElmGT we reported here adds a new and important example of sugar flexibility to antibiotic glycosyltransferases. ElmGT apparently shows a broader flexibility for the sugar substrate than any other glycosyltransferase from antibiotic pathways so far described. ElmGT is capable of transferring to its natural substrate aglycon (8-DMTC) different L-DOHs as its natural sugar substrate, L-rhamnose (6-DOH), L-olivose (2,6-DOH) and L-rhodinose (2,3,6-DOH) (Fig. 4). In addition, it can also transfer some D-sugars as D-olivose and D-mycarose (2,6-DOHs) and it can even transfer a D-olivose disaccharide. Interestingly, for the biosynthesis of this disaccharide the joint action of two mithramycin glycosyltransferases (MtmGI and MtmGII) is required. As far as we know this is the first report on antibiotic biosynthetic pathways in which a collaborative action of two glycosylating enzymes for the formation of a disaccharide has been stated.

3. Significance

The generation of novel bioactive compounds with an altered glycosylation pattern requires substrate flexibility of the glycosyltransferases. The rhamnosyl glycosyltransferase ElmGT from *S. olivaceus*, and other flexible glycosyltransferases, constitute powerful tools for producing novel hybrid glycoside antibiotics through the incorporation of different sugar moieties into the corresponding aglycons. This will require an understanding of which amino acid residues/regions are involved in the recognition of the cofactor (dTDP-activated 6-DOH) and the substrate (aglycon) to act on specific amino acid residues in order to improve sugar flexibility.

4. Materials and methods

4.1. *Bacterial strains, culture conditions and vectors*

S. olivaceus Tü2353, the elloramycin producer, was used as the source of chromosomal DNA. The following wild type *Streptomyces* strains were used as transformation hosts: *S. albus G*, *S. lividans* TK21 and *S. argillaceus* ATCC 12956. In addition, several mithramycin non-producing mutants were also used as transformation hosts: *S. argillaceus* M3ΔMG [21] and *S. argillaceus* M3G1 and M3G2 [7]. Growth was carried out on trypticase soya broth (TSB; Oxoid) or R5A medium [7]. For sporulation, growth was for 7 days at 30°C on agar plates containing A medium [7]. Protoplast formation and transformation were carried out using standard procedures [30] with some minor modifications for *S. argillaceus* [31]. *Escherichia coli* XL1-Blue [32] was used as a host for subcloning and was grown at 37°C in TSB medium (Oxoid). The bifunctional (*Streptomyces–E. coli*) replicative plasmid vectors pIAGO [25], pEM4A (this paper) and the integrative vector pKC796 [33] were used for gene expression in *Streptomyces*. pEM4A is a pEM4 derivative [34] containing the apramycin resistance cassette subcloned as a blunt-ended *Hin*dIII–*Bam*HI fragment from pEFBA [35] into the unique *Eco*RV site located

within the thiostrepton resistance gene. Consequently, pEM4A confers resistance to apramycin and not to thiostrepton. When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 5 or 50 μg/ml thiostrepton for liquid or solid cultures, respectively, 100 μg/ml for ampicillin, 2.5 or 25 μg/ml apramycin for liquid or solid cultures, respectively, or 20 μg/ml tobramycin.

4.2. *DNA manipulation and sequencing*

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations and other DNA manipulations were performed according to standard procedures for *E. coli* [36] and for *Streptomyces* [30]. Sequencing was performed on double-stranded DNA templates in pUC18 by using the dideoxynucleotide chain-termination method [37] 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) using an ALF-express automatic DNA sequencer (Pharmacia). Computer-aided data base searching and sequence analyses were carried out using the University of Wisconsin Genetics Computer Group programs package (UWGCG) [22] and the BLAST program [38].

4.3. *PCR amplification*

Degenerated oligoprimers were designed to try to locate the presence of the *elmGT* glycosyltransferase gene in cosmid 16F4. They were derived from conserved amino acid sequences present in a number of different antibiotic glycosyltransferases (see Fig. 3A). These oligoprimers were used to attempt PCR amplification using as template cosmid 16F4 DNA or subclones derived from it. The two oligoprimers were: forward primer (5′) CG (C,G) T (A,T) CGT (C,G) CC (C,G) T (A,T) CAACGG (3′) and reverse primer (5′) GT (C,G) CC (C,G) (C,G) C (C,G) CC (C,G) (C,G) CGTGGTG (3′). The PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 2 U of Taq DNA polymerase (Boehringer) in a total reaction volume of 100 μl containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 2.5% DMSO (Merck) and 10% glycerin. This reaction mix was overlayed with 50 μl of mineral oil (Sigma) and the polymerization reaction was performed in a thermocycler (MinyCycler, MJ Research) under the following conditions: an initial denaturation of 5 min at 96°C; 35 cycles of 90 s at 95°C, 30 s at 60°C and 90 s at 72°C; after the 35 cycles, an extra extension step of 10 min at 72°C was added, followed by cooling at 4°C at the end of this program. The PCR product was used as DNA probe for Southern hybridization.

4.4. *Gene expression*

For the expression of *elmGT* in *Streptomyces*, the gene was amplified by PCR. The following oligoprimers were used: (5′) ATCGTCTAGAGGCTGCGGGAAGCGACCTGACATG (3′) for the 5′-end of the gene and (5′) ATCGAAGCTTGTCGCAGCGGGCCGTGAGGTCA (3′) for the 3′-end of the gene (restriction sites for *Xba*I and *Hin*dIII sites were included in the oligoprimers, respectively, to facilitate subcloning and are underlined). PCR reaction conditions were as follows: 100 ng of template DNA was mixed with 30 pmol of each primer and 2 U of Vent DNA Polymerase (New England Biolabs) in a total reaction volume of 50 μl containing 2 mM of each dNTP and 10% DMSO (Merck). This reaction mix was overlayed with 50 μl of mineral oil (Sigma) and the polymerization reaction was performed in a thermocycler (MinyCycler, MJ Research) under the following conditions: an initial denaturation of 3 min at 98°C; 30 cycles of 30 s at 95°C, 60 s at 68°C and 90 s at 72°C; after the 30 cycles, an extra extension step of 5 min at 72°C was added, followed by cooling at 4°C at the end of this program. The PCR product was digested with *Xba*I and *Hin*dIII and subcloned into the same sites of pUC18. The fragment was then rescued as an *Xba*I–*Hin*dIII DNA fragment and subcloned into the same sites of pIAGO, immediately downstream of the erythromycin resistance promoter (*ermE*p), generating pGB15. From this construct, a 1.4 kb *Bgl*II fragment, containing both the *ermE*p and *elmGT* gene, was rescued and subcloned into the unique *Bam*HI site of pKC796, generating pGB16.

For expression of the different mithramycin glycosyltransferases, the following constructs were made: (i) *mtmGI*: a 2.5 kb *Eco*RI–*Hin*dIII fragment from pIAGOG1 [12], that contained the *mtmGI* gene under the control of the *ermEp*, was subcloned in the same sites of pEM4A, generating pEPG1. (ii) *mtmGII*: a 2.3 kb *Eco*RI–*Hin*dIII fragment from pIAGOG2 [12], that contained the *mtmGII* gene under the control of the *ermEp* promoter, was subcloned in the same sites of pEM4A, generating pEPG2. (iii) *mtmGI*+*mtmGII*: a 5.1 kb *Hin*dIII–*Xba*I fragment from pM12G12 [E. Fernández, unpublished results], that contained the methyltransferase genes *mtmMI* and *mtmMII* [13] and the glycosyltransferase genes *mtmGI* and *mtmGII* [12], was subcloned into the same sites of pEM4A, generating pEPG12. In this construct both glycosyltransferase genes are under the control of their own promoters.

4.5. *Preparation of 8-DMTC*

Spores of strain *S. argillaceus* 16F4 were inoculated in TSB medium supplemented with 2.5 μg/ml apramycin. After 24 h of incubation at 30°C and 250 rpm, this culture was used to inoculate (at 2.5%, v/v) eight 2 l Erlenmeyer flasks containing 400 ml of R5A medium [7] also containing apramycin. After further incubation for 5 days under the above conditions, the cultures were centrifuged, filtered and extracted as previously described [7]. 8-DMTC was subsequently purified by reverse-phase preparative HPLC. The mobile phase used for elution in isocratic conditions was a mixture of acetonitrile and 0.1% trifluoroacetic acid in water (30:70). The peak corresponding to 8-DMTC was collected, dried in vacuo and weighted, resulting in 638 mg of pure compound as determined by analytical HPLC.

4.6. *Biotransformation*

For biotransformation experiments, spores of the appropriate

S. albus recombinant strains were used to inoculate 5 ml of TSB liquid medium (containing the selection antibiotic) for 24 h at 30°C and 250 rpm. Then, 100 μl of this culture was used to inoculate 5 ml R5A liquid medium. After 24 h of incubation, 8-DMTC was added at 100 μg/ml final concentration and the cultures further incubated. At 24 h intervals, 900 μl samples were removed and mixed with 300 μl ethyl acetate. After centrifugation to separate the aqueous and organic phases, the organic phase was removed, evaporated under vacuo and the residue resuspended in a small volume of methanol.

4.7. TLC and HPLC analyses

For TLC analysis, 1–2 μl samples were applied onto pre-coated silica gel 60 F_{254} plates and chromatography developed using dichloromethane/methanol (90/10, v/v) as a solvent. Detection was carried out by UV light absorption. HPLC analyses of ethyl acetate extracts were performed as previously described [35].

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