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Insights in the glycosylation steps during biosynthesis of the antitumor anthracycline cosmomycin: characterization of two glycosyltransferase genes

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Abstract

Glycosylation pattern in cosmomycins is a distinctive feature among anthracyclines. These antitumor compounds possess two trisaccharide chains attached at C-7 and C-10, each of them with structural variability, mainly at the distal deoxysugar moieties. We have characterized a 14-kb chromosomal region from *Streptomyces olindensis* containing 13 genes involved in cosmomycin biosynthesis. Two of the genes, *cosG* and *cosK*, coding for glycosyltransferase were inactivated with the generation of five new derivatives. Structural elucidation of these compounds showed altered glycosylation patterns indicating the capability of both glycosyltransferases of transferring deoxysugars to both sides of the aglycone and the flexibility of CosK with respect to the deoxysugar donor. A model is proposed for the glycosylation steps during cosmomycins biosynthesis.

Introduction

Anthracyclines are an important family of natural compounds produced by actinomycetes. They include clinically important compounds used in the treatment of different cancers such as breast, lymphomas, acute leukemias, neuroblastomas, bone, and soft-tissue sarcomas (Brockmann et al. 1969; Hortobagyi 1997; Binaschi et al. 2001). These compounds consist of a linear tetracyclic polyketide backbone of the type 7,8,9,10-tetrahydro-5,12-naphthacenequinone,which derives from the condensation of a propionyl-CoA starter unit (less frequently acetyl-CoA or butyryl-CoA) and nine malonyl-CoA extender units, due to the action of a type II polyketide synthase. A key intermediate in this family of compounds is aklavinone. The level of final hydroxylation on this common structure is quite variable (Arcamone and Cassinelli 1998). This backbone is glycosylated more frequently at 7-position of the nonaromatic ring A, and in some cases at 10-position, or even at both positions by up to five sugar moieties (Arcamone and Cassinelli 1998). These sugars are 6-deoxyhexoses derived biosynthetically from glucose-1-phosphate and the presence of aminosugars is a common feature.

More than 2,000 different anthracyclines have so far been described. However, only few of them are clinically important, most significantly daunorubicin and doxorubicin (and their derivatives epirubicin, idarubicin, valrubicin), but also aclarubicin and carminomycin (Strohl et al. 1997), and some newer derivatives that are in clinical trials (Binaschi et al. 2001). The major limitation for the clinical use of these drugs is their cumulative cardiotoxicity (Platel et al. 1999) and the development of multiple drug resistance (MDR) (Nielsen et al. 1996; Rooverts et al. 1999; Minotti et al. 2004). The exact mechanism of action of anthracyclines remains unclear. However, some effects have been reported as the intercalation-induced double helix distortion of DNA, stabilization of cleavage complex ofDNA:topoisomesase II, and free radical generation, which cause DNA damage, interruption of macromolecular biosynthesis, and apoptosis induction (Muller et al. 1998; Gewirtz 1999; Furlan et al. 2002, 2004).

Sugars play an important role in the biological activity of these drugs (Cipollone et al. 2002). Changes in glycoside composition, number of deoxysugar moieties, or site of attachment to the aglycone could be interesting to obtain derivatives with novel pharmacological properties. For example, epirubicin only differs from doxorubicin in the stereochemistry of the hydroxyl group at 4-position of the deoxysugar moiety, being L-4-epidaunosamine instead of L-daunosamine. This derivative is a better tolerated drug and possesses less cardiotoxicity than daunorubicin (Bonfante et al. 1980).

The cosmomycins (Fig. 1) are an interesting group of compounds because they show one of the most complex glycosylation patterns found in anthracyclines with two trisaccharide chains attached at 7- and 10-positions (Miyamoto et al. 2002). These compounds are produced by *Streptomyces olindensis* originally described as a producer of an anthracycline complex called

retamycin (Gonçalves de Lima et al. 1969). It was initially proposed that retamycin consisted of a γ-rhodomycinone aglycone with a trisaccharide attached at 10-position (Bieber et al. 1989). However, more recently, we have verified that some of the compounds of the retamycin complex have the same structure of some cosmomycins (Furlan et al. 2004). One of the interesting structural features of cosmomycins is the existence of a trisaccharide chain attached at 10-position. This position of the aglycone is not as frequently glycosylated as the 7-position. The isolation of glycosyltransferases able to act on 10-OH could increase the possibilities to generate novel anthracyclines with new patterns of glycosylation by combinatorial biosynthesis.

In this work, we report the cloning and sequencing of a 14-kb DNA fragment from *S. olindensis* encoding 13 genes involved in cosmomycin biosynthesis, two of them coding for glycosyltransferases. These two glycosyltransferases were inactivated by gene replacement, the phenotype of the mutants characterized, the accumulated products purified, and their structures elucidated.

Materials and methods

Bacterial strains, culture conditions, and plasmids

S. olindensis DAUFPE 5622 was obtained from "Departamento de Antibióticos da Universidade Federal de Pernambuco", Brazil. *Escherichia coli* DH10B (Invitrogene) was used for routine subcloning. *E. coli* SURE (Stratagene) was used for the construction of a cosmid library of *S. olindensis* chromosomal DNA. *E. coli* ET12567 (pUB307) (Flett et al. 1997) was used as donor strain for conjugation with *S. olindensis*. *Bacillus subtilis* 1012 (kindly provided by Dr.W. Schumann, Department of Genetics, University of Bayreuth, Germany) was used as a test microorganism in antibiotic activity assays. *S. olindensis* was cultivated on A medium (Fernández et al. 1998) for sporulation and in liquid R5M medium (Furlan et al. 2004) for mycelium propagation and antibiotic production (30 °C, 200 rpm). For protoplasts regeneration, it was grown on R5 solid medium (Kieser et al. 2000). *E. coli* strains were grown in LB or TSB media at 37 °C. Disrupted mutants of *S. olindensis* were grown in the presence of 25 μg apramycin ml-1, and the complemented mutants were grown in the presence of 50 μg thiostrepton ml-1 (solid medium) or 5 μg thiostrepton ml-1 (liquid medium). Antibiotic concentrations for *E. coli* were 100 μg ampicillin ml-1 or 30 μg chloramphenicol ml-1. Plasmids used in this work were pUC18 (Invitrogene), pBCSK+ (Stratagene), Supercos 1 (Stratagene), pEM4 (Quirós et al. 1998), pHZ1358 (Sun et al. 2002), pFL7APA (Lombó et al. 1997), and pIJ2921 (Kieser et al. 2000).

DNA manipulation

Plasmid DNA preparation, restriction endonucleases digestion, Klenow treatment, alkaline phosphatase treatment, and ligation were performed according to standard procedures for *E. coli* (Sambrook et al. 1989). Streptomyces protoplasts, PEG-mediated transformation, chromosomal DNA isolation, and *E. coli*/*Streptomyces*-conjugation were made as described in (Kieser et al. 2000).

Construction and screening of a genomic library

A genomic library of *S. olindensis* DAUFPE 5622 was generated in Supercos1 as described (Redenbach et al. 1998). Two different probes were used for the screening of the cosmid library: (1) a PKS probe, a 0.6-kb DNA fragment containing part of the type II polyketide synthase amplified by polymerase chain reaction (PCR) from *S. olindensis* chromosomal DNA using oligoprimers previously described by Metsa-Ketela et al. (1999) and (2) a deoxysugar probe, consisting of a *Pst*I–*Bgl*II DNA fragment contaning the *mtmDE* genes involved in deoxysugar

biosynthesis (Lombó et al. 1997). Colony hybridization was performed with Alkphos/CDP Star direct labeling and detection system (GE Health Care).

DNA sequencing and analysis

Sequencing was performed on pUC18 or pBCSK+ templates, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 or ABI Prism 3100 (Applied Biosystems) systems. The sequencing method was based in the dideoxynucleotide chain termination method (Sanger et al. 1977). The assembly of the 14,080-bp DNA sequence was carried out by using the Phred-Phrap-Consed package of The University of Washington, and the ORFs were determined with the FramePlot program (Ishikawa and Hotta 1999) and GCUA (http://www.gcua.de) tools. Cosmid p5E11 was partially digested with *Sau*3AI to maximize DNA fragments ranging from 2 to 4 kb and these fragments were cloned into a *Bam*HI-digested pBCSK+; thus, generating the sublibraries of the cosmid, including pT19, pT2, and pU12 (see below).

Generation of mutants

For generation of mutants in genes cosB, cosG, and cosK, three different constructs were made as follows.

For *cosB* mutation, an *Eco*RI–*Bam*HI apramycin resistance cassette was blunt-ended with Klenow fragment and inserted at the unique blunt-ended *Bam*HI site of *cosB* in plasmid clone pT19. pT19 is a pBCSK+ derivative which contains a 2.5-kb *Sau*3AI fragment from cosmid p5E11. The resulting 4.2 kb insert was recovered as an *Eco*RI–*Xba*I fragment, blunt-ended with Klenow and cloned into the Klenow-treated *Bam*HI site of pHZ1358, generating pT1N3.

For *cosG* mutation, the apramycin resistance cassette was inserted into the unique *Nru*I site (blunt-ended) of pT2 and located within *cos*G. The resulting insert was rescued as a *Pvu*II fragment (sites present in pBCSK+) and subcloned into pHZ1358 as described above, resulting in pT2N3.

For *cosK* mutation, pU12 (a pBCSK+ derivative which contains a 3.3-kb Sau3AI fragment from cosmid p5E11) was digested with *Eco*RI–*Xba*I (using these sites from the polylinker) and the insert was subcloned into the same sites of pIJ2921, generating pT3N1. The blunt-ended *Eco*RI–*Bam*HI apramycin resistance cassette was introduced at the blunt-ended NcoI site of pT3N1 located within *cosK*. The insert containing the disrupted gene was rescued as a *Bgl*II fragment and cloned into a *Bam*HI-digested pHZ1358, thus generating pT3N3.

All these constructs (pT1N3, pT2N3, and pT3N3) were introduced into *E. coli* ET12567 (pUB307) by transformation and the resultant strains were used to conjugate *S. olindensis*. Transconjugants were selected and the occurrence of the double crossover in the apramycin-resistant thiostrepton-sensitive exconjugants was verified by Southern hybridization.

Complementation of mutant strains

With the aim to complement the *cosG* and *cosK* glycosyltransferase mutants, two PCR cassettes containing these genes were amplified and cloned in pEM4, downstream from the constitutive *Streptomyces* promotor PermE. The following primers carrying *Eco*RI or *Bam*HI sites (underlined) were designed to facilitate the cloning in the right orientation for each of the gene cassettes:

1. cosG1: GGATCCCATCACCAGGGGAGGAGCAC
2. cosG2: GAATTCCACCAGGATGTTCACGGTTC
3. Cosk1019er1: TTAAATGAATTCCGAGGTAGGGGGGTACGG

4. CosK2404bh1: AAATTAGGATCCATCGCATACGGGTCGT

The resulting plasmids pEM4G (containing *cosG*) and pEM4K (containing *cosK*) were used for transforming protoplasts of the mutants in the respective genes. Transformants were selected in R5M liquid medium in the presence of 5 μg thiostrepton per milliliter and production of cosmomycins was analyzed as described below.

Analysis of cosmomycin production

For analysis of cosmomycins production, 5 ml samples of cultures were centrifuged and the supernatants were applied to solid-phase extraction cartridges (Sep-Pak Vac; C-18 50 mg;Waters). The adsorbed compounds were eluted with 1 ml methanol and concentrated under vacuum. After resuspension of the residue in a small volume of methanol, 10 μl samples were analyzed by high performance liquid chromatography mass spectrometry (HPLC-MS), using a reverse phase column (Symmetry C18, 2.1×150 mm, Waters) and acetonitrile and a mixture of 0,1 % trifluoroacetic acid in water as solvents. During the first 4 min, the concentration of the mobile phase was maintained at 10 % acetonitrile (isocratic step). Then, a linear gradient from 10 to 100 % acetonitrile was established. The flow rate was 0.25 ml/min. Spectral detection and characterization of the different compounds were carried out by using a photodiode array detector and the software Millennium (Waters). Chromatograms were extracted at 488 nm. Mass analyses in positive mode were done by electrospray ionization in the positive mode, with a capillary voltage of 3 kV and cone voltage of 20 V.

Purification of compounds

For purification, each mutant strain was incubated during 5 days at 30 °C in Erlenmeyer flasks (250 ml with 50 ml R5M medium each). The supernatant was filtered and applied to a solid-phase extraction cartridge (SepPak Vac 35 cc, Waters). The retained material was eluted with methanol and water with a linear gradient from 0 to 100 % methanol in 1 h at 10 ml/min. Fractions were taken every 5 min and analyzed by high performance liquid chromatography (HPLC). The desired products from the corresponding fractions were purified by preparative HPLC in a μBondapak C18 radial compression cartridge (PrepPak cartridge, 25×100 mm, Waters). The purified material was diluted fourfold with water, applied to a solid-phase extraction column (SepPak Vac C18, Waters), washed with water, eluted with methanol, and dried in vacuo.

Structure determination

All structures (1–5) found upon the described inactivation experiments were elucidated using mass spectrometry and NMR spectroscopy. The data were compared to cosmomycin D and to published data of various related compounds, e.g., the obelmycins (Johdo et al. 1991c, 1997), alldimycins (Johdo et al. 1991b,e), alpharubicin I (Koch et al. 1982), yellamycins (Johdo at al. 1991d), β-rhodomycinones I and II (Ihn et al. 1982, 1985), the CG-anthracyclins (Matsuzawa et al. 1980; Oki et al. 1980; Yoshimoto et al. 1984), and some patented rhodomycin group antibiotics (Oki et al. 1981). While compounds 1 and 4 were identified as the previously described β-rhodomycins I (4) and II (1), the others (2, 3 and 5) appear to be new (Supplementary material, Tables S1 and S2). The molecular formulae of all five compounds were verified by mass spectrometry. Compounds 1–4 are variants with regard to the substitution pattern with phenolic hydroxy groups (1-, 6-, and 11-positions) and with respect to the substitution of L-rhodosamine moieties (in 7- and/or 10-positions). In contrast, compound 5 is a truly novel anthracycline, in which a different sugar, namely, L-rhodinose is directly attached at both 7- and 10-positions of its aglycone β-rhodomycinone. Its structural formula was confirmed by HRMS (positive-FAB): calc for C32H37O12: 613.2285 (MH+); found: 613.2278. The ^{1}H-NMR data clearly show the lack of the *N*-methyl groups typical for rhodosamine, which is normally attached at these positions and are in agreement with the

trideoxysugar rhodinose. All the shown stereochemical features of compounds 1–5 were deduced by biosynthetic logic.

Results

Isolation and characterization of glycosyltransferase genes involved in cosmomycin biosynthesis

A cosmid library of *S. olindensis* DAUFPE 5622 genomic DNA was screened by in situ colony hybridization using two different probes: a PKS probe and a deoxysugar probe (see "Materials and methods"). Two positive hybridizing cosmid clones, named p3E9 and p5E11, were isolated and specific hybridization against both probes was verified by Southern analysis. Restriction digestions showed that both cosmids had the same band pattern and cosmid p5E11 was chosen for further experiments. A region of 14,080 bp encomprising the hybridization signals for both probes was sequenced and 13 complete and one incomplete ORFs were identified (Fig. 2). The accession number for this sequence is DQ280500. All ORFs showed the high G+C content and the GC bias in the third position which is characteristic of *Streptomyces* genes. Analysis of the deduced products of the different ORFs showed clear similarities with anthracycline biosynthetic proteins from different organisms. The functions for the deduced products are shown in Table 1.

The sequenced region contains two genes that code for the minimal polyketide synthase, *cosB* (ketoacyl synthase α) and *cosC* (ketoacyl synthase β), and are responsible for the biosynthesis of the 20-carbon anthracyclic backbone. The gene coding for the acyl carrier protein (ACP) is not present in the sequenced region. It is not located just downstream of the ketoacyl synthases as usually happens in most aromatic polyketide clusters; a similar situation is found in the case of the daunorubicin cluster in *S. peucetius* (Lomovskaya et al. 1999). Two other genes, *cosE* and *cosF*, code proteins involved in the generation of the propionyl-CoA starter unit; a similar situation occurs in the daunorubicin pathway, where those two functions are represented by DpsC and DpsD (Rajgarhia et al. 2001; Bao et al. 1999). According to these authors, DpsC, the extra ketoacyl synthase, would be a "fidelity factor" helping to maintain the propionyl-CoA specificity for the starter unit and DpsD would be the putative propionyl-CoA:ACP acyltransferase. We propose a similar role for CosE and CosF, respectively.

CosX shows similarity to several anthrone oxygenases from anthracycline biosynthetic routes such as AknX from the aclacinomycin cluster in *S. galilaeus*, which converts aklanonic acid anthrone into aklanonic acid (Chung et al. 2002). Therefore, *cosX* is involved in the generation of the quinone ring present in cosmomycin.

The products of four additional genes (*cosM*, *cosL*, *cosT*, and *cosH*) are related to NDP-deoxysugar biosynthesis enzymes. Cosmomycins possess two trisaccharide chains linked at 7- and 10-positions of ring D. In cosmomycin D, these chains contain the same pattern of deoxysugars: L-rhodosamine, 2-deoxy-L-fucose, and L-rhodinose. In contrast, in cosmomycin C, both chains differ in the second deoxysugar of the trisaccharide: 2-deoxy-L-fucose in the chain linked at 7-position and L-rhodinose in the chain linked at 10-position (Furlan et al. 2004). CosH shows strong similarity with NDP-hexose 4,6-dehydratases, which are enzymes catalyzing the second enzymatic step during deoxysugars biosynthesis. All cosmomycin deoxysugars are L-sugars, and CosL is the 3,5-epimerase responsible for conversion of the corresponding D-intermediate to the L-form. CosM is highly similar to several 4-ketoreductases, which usually carry out the last enzymatic step during deoxysugar biosynthesis. Aclacinomycins and cosmomycins share the presence of two deoxysugars, L-rhodosamine and 2-deoxy-L-fucose. CosM shows high similarity (53 % identical aminoacids) with AclM, a 4-ketoreductase involved in aclacinomycin biosynthesis (Räty et al. 2002). Therefore, *cosM* is involved in the final ketoreduction step during biosynthesis of either L-rhodosamine or 2-deoxy-

L-fucose. Finally, CosT is very similar to DnrQ, EryCII, DesVIII, and other proteins showing similarities to cytochromes P450, but lacking the conserved cysteine residue that acts as the heme iron-binding site. All these proteins have been implicated in the biosynthesis of several amino deoxysugars like L-daunosamine in the daunorubicin pathway (Otten et al. 1995), D-desosamine in the erythromycin pathway (Summers et al. 1997), and D-desosamine in the pikromycin pathway (Chen et al. 2001). More recently, it has been shown that some of these proteins such as DesVIII and AknT are necessary for the glycosylation step collaborating with the glycosyltransferase (Borisova et al. 2004; Lu et al. 2004). By analogy, CosT is necessary for L-rhodosamine transfer to the cosmomycin aglycone by the corresponding glycosyltransferase.

Two glycosyltransferase genes, *cosG* and *cosK*, were identified based on sequence comparisons. CosG shows similarities with several glycosyltransferases which transfer aminodeoxysugars like RhoG from the rhodomycin pathway in *S. violaceus* (Miyamoto et al. 2002), DnrS from the daunorubicin pathway in *S. peucetius* (Otten et al. 1995), AclS from the aclacinomycin pathway in *S. galilaeus* (Chung et al. 2002), and DesVII from the pikromycin pathway in *S. venezuelae* (Xue et al. 1998). Based on this, CosG is the glycosyltransferase responsible for L-rhososamine transfer to the 7- or 10-position (or both) in the cosmomycin aglycone. The other glycosyltransferase CosK shows similarities with AknK, which is responsible for the addition of 2-deoxy-L-fucose during aclacinomycin biosynthesis in *S. galilaeus* (Räty et al. 2002) and, therefore, CosK is the responsible for the incorporation of the second deoxysugar (2-deoxy-L-fucose) during cosmomycin D biosynthesis.

The last two deduced products from the sequenced region correspond to an ATP-binding subunit of an avidinbiotin- complex transporter, CosI, which is involved in cosmomycin secretion and resistance and a putative transcriptional regulator of the PadR family, CosS. The gene *cosI* contains a TTA codon at position 107, codon that has been proposed to have a regulatory role on secondary metabolism and development gene expression (Lawlor et al. 1987). *S. coelicolor* mutants affected in *bldA*, which codes for a leucine-tRNA specific for codon UUA, are defective in antibiotic production and development of aerial hyphae and spores.

Generation of different glycosyltransferases mutants and isolation of new compounds

To get a definitive proof of the involvement of the sequenced region in cosmomycin biosynthesis, a mutant was generated by gene replacement in which an internal fragment of *cosB* (ketoacyl synthase α) was replaced by an apramycin resistance cassette. To do this, pT1N3 was constructed and introduced into *S. olindensis* by intergeneric conjugation from *E. coli* ET12567 (pUB307). Transconjugants in which a double crossover occurred (apramycin-resistant and thiostreptone-sensitive mutants) were confirmed by Southern analysis. The mutant *S. olindensis* Δ*cosB* did not produced any cosmomycin or biosynthetic intermediate as determined by HPLC (data not shown) therefore demonstrating the involvement of the mutated gene in cosmomycin biosynthesis.

Because cosmomycins possess a complex glycosylation pattern, we decided to carry out gene inactivation experiments in two genes of the cluster coding for the glycosyltransferases identified, *cosK* and *cosG*. Construct pT3N3 (in which *cosK* was inactivated) was introduced into *S. olindensis* and transconjugants, in which the replacement of the wild type allele by the in vitro mutated one took place, were verified by Southern analysis. After grown in liquid production medium R5M, ethyl acetate extracts were analyzed by HPLC-MS (Supplementary material, Fig. S1a). Four peaks showing the characteristic absorptium spectra of anthracyclines were found. None of them presenting the retention time of any of the previously known cosmomycins: two major peaks with retention times of 13.1 (compound 1) and 16.3 min (compound 4) and two minor peaks eluting at 13.6 (compound 2) and 15.7 min (compound 3)

(Supplementary material, Fig. S1a). These four compounds were purified by preparative HPLC and their structures (Fig. 3a) elucidated by NMR and MS. The two major compounds (1 and 4) contained a cosmomycin-like aglycone, β-rhodomycinone. Compound 4 contains only one L-rhodosamine moiety attached at 7-position, thus corresponding to rhodomycin B (β-rhodomycin I) (Brockmann et al. 1969; Ihn et al. 1982), while compound 1 contains this aminosugar attached to both, the 7- and 10-positions, matching the structure of rhodomycin A (β-rhodomycin II) (Brockmann et al. 1969; Ihn et al. 1985). This probably implies that L-rhodosamine is firstly attached at 7-position and then at 10-position. The two minor compounds (compounds 2 and 3) possess an aglycone slightly different from that of cosmomycin. They lack the hydroxyl group(s) at 11-position (in both compounds) and at 6-position (in compound 2). In addition, both compounds contain an extra hydroxyl group at 1-position. Compound 2 shows two L-rhodosamine residues linked at 7- and 10-position, while compound 3 only possesses an L-rhodosamine residue in 10-position. Because all these compounds contain L-rhodosamine attached to the aglycone, the conclusion is that CosK is involved in the transfer of the second sugar of the saccharide chains.

To generate a mutant in the second glycosyltransferase gene, *cosG*, construct pT2N3 (in which *cosG* was inactivated) was introduced into *S. olindensis* by conjugation. After selection and verification by Southern analysis of transconjugants in which the gene replacement took place, they were grown in liquid production medium R5M and ethyl acetate extracts were analyzed by HPLC-MS. A single cosmomycin-like UV-spectra peak, compound 5, not corresponding to any of the known cosmomycins, was observed (Supplementary material, Fig. S1b). Analysis of the structure of the purified compound showed the characteristic cosmomycin aglycone but having L-rhodinose instead of L-rhodosamine residues attached at 7- and 10-positions (Fig. 3b). The absence of L-rhodosamine in this compound strengthens that CosG is the glycosyltransferase involved in the transfer of this sugar.

To discard any polar effect on the generation of both mutants, they were complemented in trans by individually expressing *cosK* and *cosG* genes in the corresponding mutants. In both cases, normal levels of cosmomycins production were recovered (data not shown).

Discussion

One of the main challenges for drug discovery is searching for compounds with enhanced pharmacological properties. In the case of anthracyclines, sugar residues are crucial for biological activity due to their role in the interaction with the DNA minor groove (Quigley et al. 1980; Wang et al. 1987). Therefore, modifications in the glycosylation pattern could potentially render derivatives with improved properties. The isolation and characterization of genes involved in the biosynthesis of cosmomycins, a group of anthracyclines with a characteristic glycosylation pattern, opens up new possibilities for combinatorial biosynthesis in anthracyclines.

On the basis of similarities with proteins in databases, it was expected that CosG would be the glycosyltransferase responsible for the incorporation of L-rhodosamine. Because L-rhodosamines are directly attached to the aglycone at 7- and 10-positions (ring D), it was anticipated that a mutant in *cosG* would accumulate the cosmomycin aglycone. Surprisingly, this mutant accumulated a compound that contains two L-rhodinose moieties directly attached to the oxygen in 7- and 10-positions. In cosmomycins, L-rhodinose is present as the second and/or third positions of the trisaccharide chains. Two conclusions can be drawn from the analysis of this mutant: (1) CosG is the glycosyltransferase responsible for the incorporation of L-rhodosamine and (2) in the absence of CosG, another not yet identified glycosyltransferase is able of transferring L-rhodinose directly to the aglycone. It is interesting that these glycosyltransfer steps occur exactly at the same aglycone positions (7 and 10), where the

saccharide chains always start, normally with L-rhodosamine. Because this occurs despite the inactivation of glycosyltransferase CosG, it can be assumed that CosG is normally assisted by another DesVIII-like enzyme, possibly CosT, that is important to help to direct the first sugars to the wanted positions of the acceptor substrate (in this study, aglycone).

Based on the compounds isolated from the mutant in the glycosyltransferase CosK, it was deduced that this transferase is responsible for the addition of the second deoxysugar to the trisaccharide chains, which is 2-deoxy-L-fucose or L-rhodinose. CosK shows similarity with AknK, which has been shown to be responsible for the incorporation of 2-deoxy-L-fucose (the second deoxysugar) during aclacinomycin biosynthesis (Lu et al. 2004). Two of the compounds accumulated from the Δ*cosK* mutant contain aglycones with an incomplete or modified hydroxylation pattern, which probably represent shunt products. Formation of these compounds indicates that either glycosyltransferase CosG (responsible for direct glycosylation of the aglycone) or some hydroxylases have some substrate flexibility. The former is more likely because glycosylation events usually occur at the very end of biosynthetic sequences, that feeding anthracyclinones with various hydroxylation patterns to blocked mutants of anthracycline producers resulted in glycosylated anthracyclines (Oki et al. 1980; Matsuzawa et al. 1980; Yoshimoto et al. 1984). The combination in the different compounds of unusual glycosylations with alternative hydroxylation patterns increases the structural diversity of cosmomycins.

Based on the structures of the compounds isolated in this study, we propose a feasible model for glycosylation during cosmomycin D biosynthesis (Fig. 4). The first event would be the attachment of two L-rhodosamines to the β-rhodomycinone aglycone. Probably, this occurs in two steps, first attachment at position 7- then at 10-position, based on the following facts. First, in mutant Δ*cosK*, the monoglycosylated derivative containing L-rhodosamine moiety connected to the 7-position was a major compound while that with the sugar attached to the 10-position was a minor product. Second, the only monoglycosylated compound isolated from cultures of *S. violaceus*, another cosmomycin producer, also contained L-rhodosamine in 7-position (Johdo et al. 1991a). It is interesting to note that the glycosyltransferase CosG responsible for these glycosylation steps is probably able of incorporating both L-rhodosamines. These glycosylations probably will require the participation of the cytochrome P450-like protein CosT. The formed rhodomycin A intermediate (compound 1 in Fig. 4) would be the substrate for CosK, probably adding 2-deoxy-L-fucose at both saccharide chains. Accordingly to the structures of the cosmomycins isolated from the wild type, CosK must have some flexibility regarding its sugar donor substrate because cosmomycin C contains L-rhodinose instead of 2-deoxy-L-fucose. As a last glycosylation step, a not yet identified glycosyltransferase would be responsible for the incorporation of the last deoxysugar residue, L-rhodinose, to both saccharide chains, generating cosmomycin D. It is important to notice that in the absence of CosG, the action of a flexible glycosyltransferase incorporates two residues of L-rhodinose directly to the aglycone, generating compound 5. The identity of this glycosyltransferase remains unknown: it could be a third glycosyltransferase in this cluster not yet identified or, alternatively, CosK could play this role. As discussed above, CosT, apparently a chaperone that normally supports the L-rhodosamine-transfer by CosG, seems also to support this unidentified glycosyltransferase and seems to play an essential role to direct the sugars to the 7- and 10-positions of the aglycones. In context with glycosyltransfer events of anthracycline biosyntheses, it was recently shown by in vitro studies with the aclacinomycin AknK glycosyltransferase that this enzyme is able to transfer two consecutive sugars of the aclacinomycin trisaccharide chain (Lu et al. 2004).

The results shown in this paper indicate that the glycosyltransferases CosG and CosK are able to glycosylate at both sides of ring D of the cosmomycin aglycone. CosK has been shown to be a flexible glycosyltransferase regarding the sugar donor substrate, leading to the formation

of cosmomycins with modified glycosylation patterns. This fact is responsible for the diversity found in the trisaccharide chains of this type of anthracyclines. This opens the way for the use of these glycosyltransferases for the generation of new anthracycline derivatives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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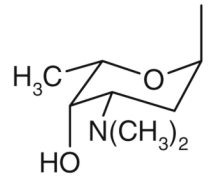
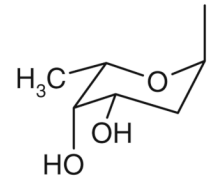
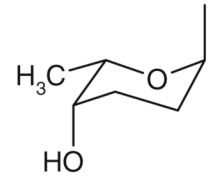


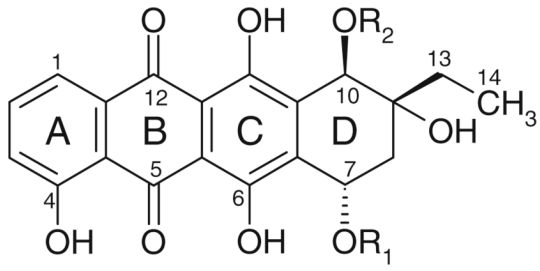
L-**rhodosamine**
(L-**rhm**)



2-deoxy-L-**fucose**
(L-**dfuc**)



L-**rhodinose**
(L-**rho**)



	R_1	R_2
Cosmomycin D	L-**rhm**-L-**dfuc**-L-**rho**	L-**rhm**-L-**dfuc**-L-**rho**
Cosmomycin C	L-**rhm**-L-**dfuc**-L-**rho**	L-**rhm**-L-**rho**-L-**dfuc**
Cytorhodin N	L-**rhm**-L-**rho**-L-**dfuc**	L-**rhm**-L-**dfuc**-L-**rho**

Fig. 1.
Chemical structures of cosmomycins produced by *Streptomyces olindensis*

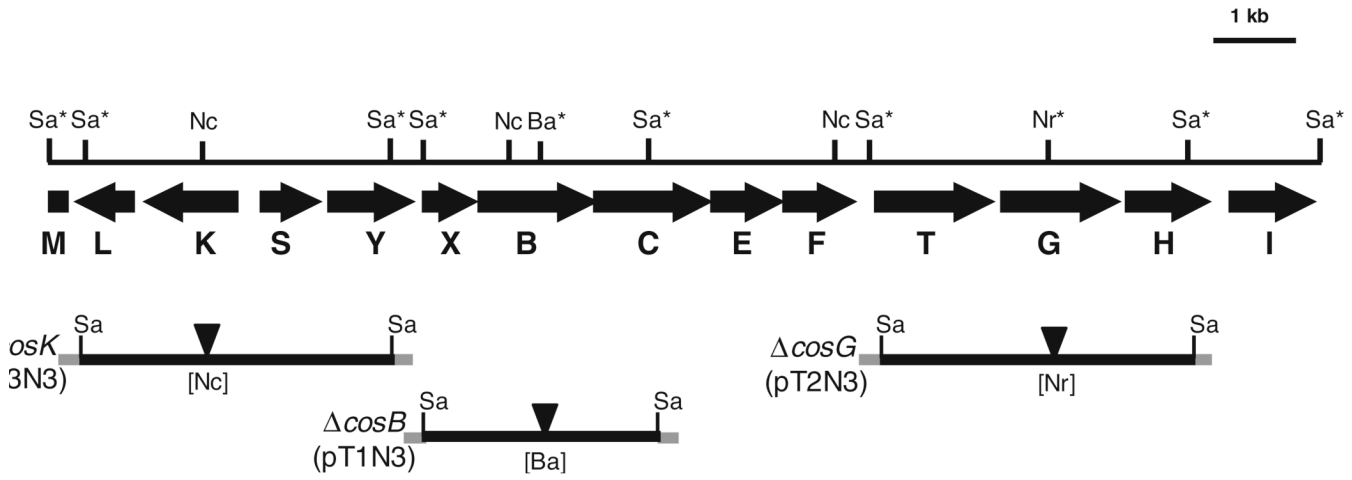


Fig. 2.

Genetic organization of the sequenced DNA region and constructs used for the generation of the different mutants. The *black triangle* indicates the apramycin resistance cassette. Restriction sites indicated with an *asterisk* are not unique sites. *Sa*Sau3AI; *Nc*Nco I; *Ba*Bam HI; *Nr*Nru I

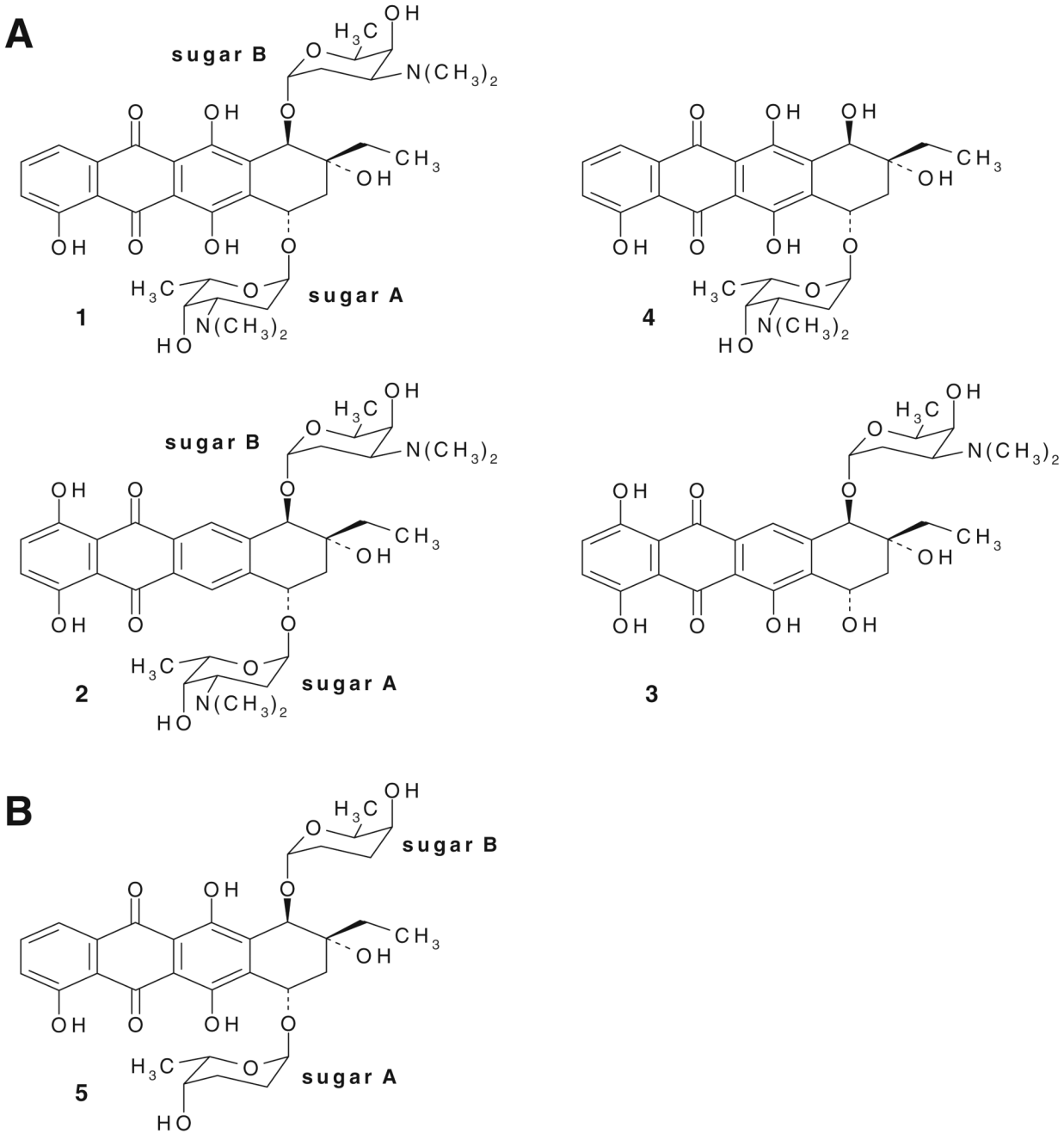


Fig. 3.
Chemical structures of compounds isolated from *Streptomyces olindensis* Δ*cosK* (**a**) and *S. olindensis* Δ*cosG* (**b**)

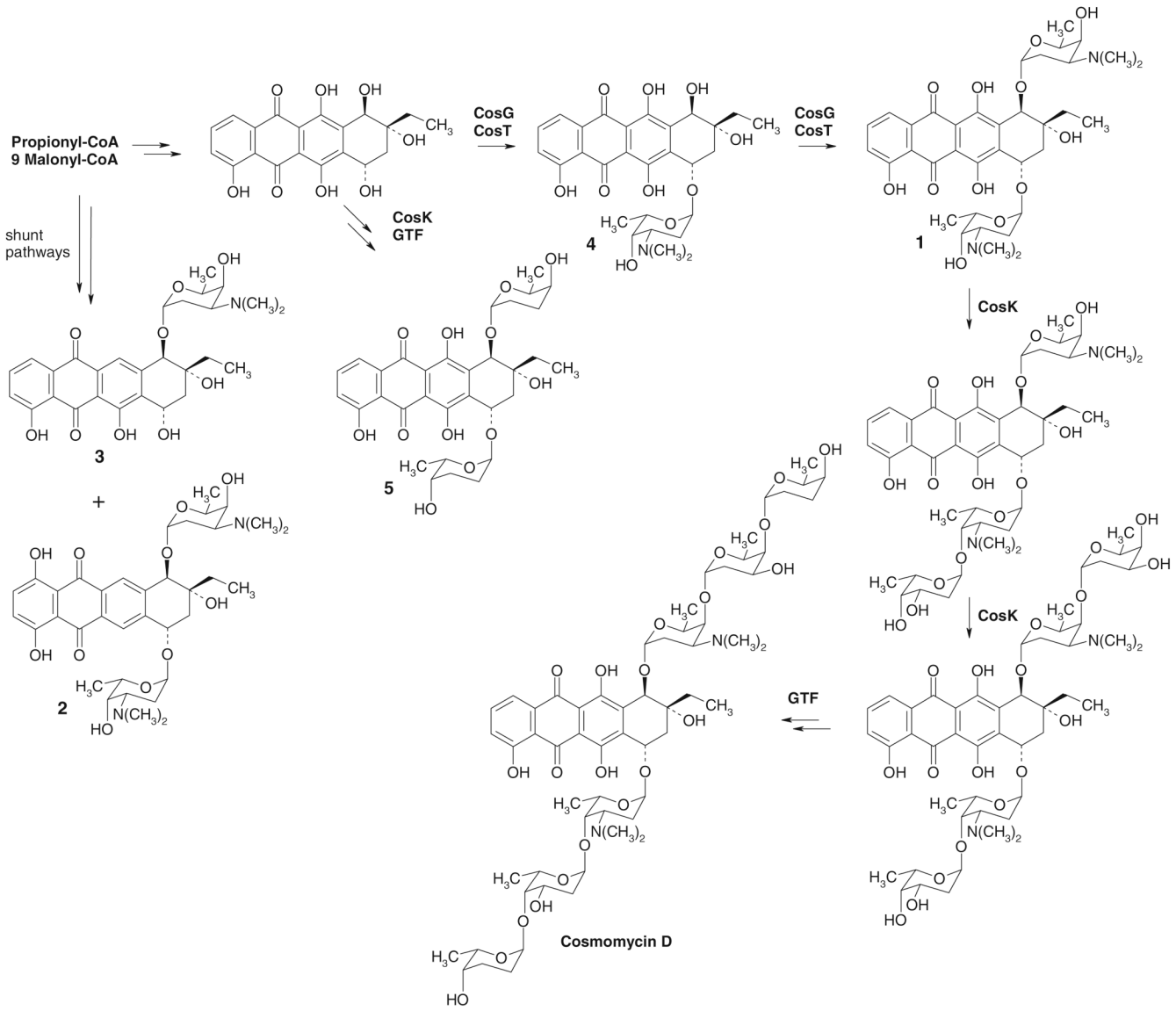


Fig. 4.
Proposed pathway for glycosylation events in cosmomycin D biosynthesis

Table 1

Deduced functions for genes in the cosmomycin gene cluster

Gene	Amino acids	Closest similar protein (% identity/similarity) accession no.	Proposed function
cosM	121[a]	dTDP-4-keto-6-deoxyhexose reductase AclM from *Streptomyces galilaeus* (53/61 %) BAB72039	NDP-hexose 4-ketoreductase
cosL	208	3,5-Epimerase from *Streptomyces* sp. TP-A0274 (66/77 %) BAC55217	NDP-hexose 3,5-epimerase
cosK	430	Glycosyltransferase AknK from *Streptomyces galilaeus* (64/79 %) AAF70102	Glycosyltransferase
cosS	187	AclS from *Streptomyces galilaeus* (54/68 %) BAB72058	PadR-family regulator
cosY	325	Ocd ornithine cyclodeaminase *Azoarcus* sp. EbN1 from (28/42 %) YP_159383	Unknown
cosX	117	Anthrone oxygenase AknX from *Streptomyces galilaeus* (55/65 %) AAF70105	Monooxygenase
cosB	422	Ketosynthase α AknB from *Streptomyces galilaeus* (73/83 %) BAB72045	Ketosynthase α
cosC	414	Ketosynthase β AknC from *Streptomyces galilaeus* (65/77 %) BAB72046	Ketosynthase β
cosE	344	Ketoacyl-(ACP) synthase AknE2 from *Streptomyces galilaeus* (64/74 %) BAB72048	Ketoacylsynthase for starter unit
cosF	349	Acyltransferase AknF from *Streptomyces galilaeus* (66/73 %) BAB72049	Acyltransferase for starter unit
cosT	416	RhoF from *Streptomyces violaceus* (84/88) BAB87834	L-rhodosamine transfer
cosG	426	Glycosyltransferase RhoG from *Streptomyces violaceus* (96/98 %) BAB87835	Glycosyltransferase
cosH	327	4,6-Dehydratase RhoH from *Streptomyces violaceus* (95/96 %) BAB87836	NDP-hexose 4,6-dehydratase
cosI	329	RhoI from *Streptomyces violaceus* (94/97 %) BAB87837	ABC transporter (ATP-binding subunit)

[a] Incomplete ORF