

Iteratively Acting Glycosyltransferases Involved in the Hexasaccharide Biosynthesis of Landomycin A

Brief Communication

Andriy Luzhetskyy,^{1,3} Marta Fedoryshyn,^{2,3}
Clemens Dürr,¹ Takaaki Taguchi,¹
Volodymyr Novikov,² and Andreas Bechthold^{1,*}

¹Albert-Ludwigs-Universität
Institut für Pharmazeutische Wissenschaften
Stefan-Meier-Strasse 19
79104 Freiburg
Germany

²Institut of Chemistry and Chemical Technologies
National University
Lvivs'ka Politechnika
St. Jury sq. 3/4
Lviv 79000
Ukraine

Summary

Detailed studies on the biosynthesis of the hexasaccharide side chain of landomycin A, produced by *S. cyanogenus* S136, revealed the function of each glycosyltransferase gene of the biosynthetic gene cluster. Analyses of generated mutants as well as feeding experiments allowed us to determine that LanGT2 and LanGT3 catalyze the attachment of one sugar, whereas LanGT1 and LanGT4 attach two sugars during landomycin A biosynthesis. The generation of a *lanZ2* deletion mutant provided evidence that LanZ2 is controlling the elongation of the saccharide side chain.

Introduction

Sugar modifications of many antibiotics play essential roles in maintaining the biological activities of these compounds [1]. It has been clearly demonstrated that anthracyclines, glycopeptide antibiotics, aminocoumarines, and macrolides require sugar moieties for their full biological activities [2, 3]. A small but growing number of natural products with promising antibacterial activities mainly consists of sugar chains. Examples for such oligosaccharide antibiotics are the orthosomycins avilamycin A and evernimicin, the huge polysaccharide saccharomycin, and the angucyclines urdamycin A and landomycin A [3–5].

A fundamental challenge in the field of oligosaccharide antibiotics is to identify the exact functions of glycosyltransferases that are involved in the biosynthesis of the glycan structures. Biosynthetic gene clusters of a few oligosaccharide antibiotics have been cloned and sequenced [6–8]. But only the biosynthesis of the urdamycin sugar side chain has been investigated in detail, and it was shown that one glycosyltransferase is needed for the attachment of each individual sugar [6].

In this study, we are presenting data on the biosynthesis of landomycin A, a compound containing a hexa-

saccharide portion that is produced by *Streptomyces cyanogenus* S136 [9]. Landomycin A possesses strong antitumor activities, in particular against prostate cancer cell lines [10]. It inhibits thymidine uptake in murine smooth-muscle cells and cell-cycle progression. Its unusual activities depend on the long oligosaccharide chain because landomycins with shorter sugar chains, such as landomycin E (Figure 1) produced by *S. globisporus*, have much weaker antitumor activity [11].

The sugar portion consists of four D-olivose and two L-rhodinose moieties (Figure 1). Its formation is extremely puzzling because only four glycosyltransferases (LanGT1, LanGT2, LanGT3, and LanGT4) are available for the six consecutive elongations steps toward the hexasaccharide [12].

By heterologous expression and gene inactivation, we could recently demonstrate that LanGT2 is the priming glycosyltransferase connecting D-olivose to the landomycin aglycon [13]. We could also show that LanGT1 is an olivosyltransferase catalyzing the attachment of the second sugar and that LanGT4 is a rhodinosyltransferase attaching the third sugar during landomycin A biosynthesis [13, 14]. Furthermore, a mutant lacking *lanGT3* was accumulating landomycin E, a truncated landomycin derivative with a sugar side chain comprising the first three sugars only (Figure 1). This indicates that LanGT3 is the olivosyltransferase responsible for the transfer of the fourth sugar moiety [15]. However, despite the elucidation of the sugar substrate specificities of LanGT1, LanGT2, LanGT3, and LanGT4, it remains elusive as to which GTs account for the fifth and sixth chain-elongation step.

In this paper, we report results that answer the riddle of the “six sugars, four glycosyltransferases” paradox: it is shown by feeding experiments that LanGT1 and LanGT4 are iteratively acting enzymes both catalyzing the attachment of two sugar moieties during landomycin A maturation. We also present data on LanZ2, which apparently controls the smooth course of the biosynthesis of the hexasaccharide side chain.

Results

Feeding Experiments Revealed LanGT1 and LanGT4 to Be Iteratively Acting Glycosyltransferases of the Landomycin A Pathway

If one considers that the hexasaccharide side chain of landomycin A is built up by six consecutive glycosyltransfers, it is impossible to identify an iteratively acting GT solely by inactivation of the respective gene because the chain elongation would always be stopped prior to its first glycosyltransfer. No information of a potential second glycosyltransfer would be provided. Feeding experiments with landomycin derivatives with varying chain length were performed as an alternative strategy to overcome this limitation. Landomycin E, containing a trisaccharide side chain, was fed to the wild-type (wt) strain and to a *lanGT1* and *lanGT4* mutant

*Correspondence: andreas.bechthold@pharmazie.uni-freiburg.de

³These authors contributed equally to this work.

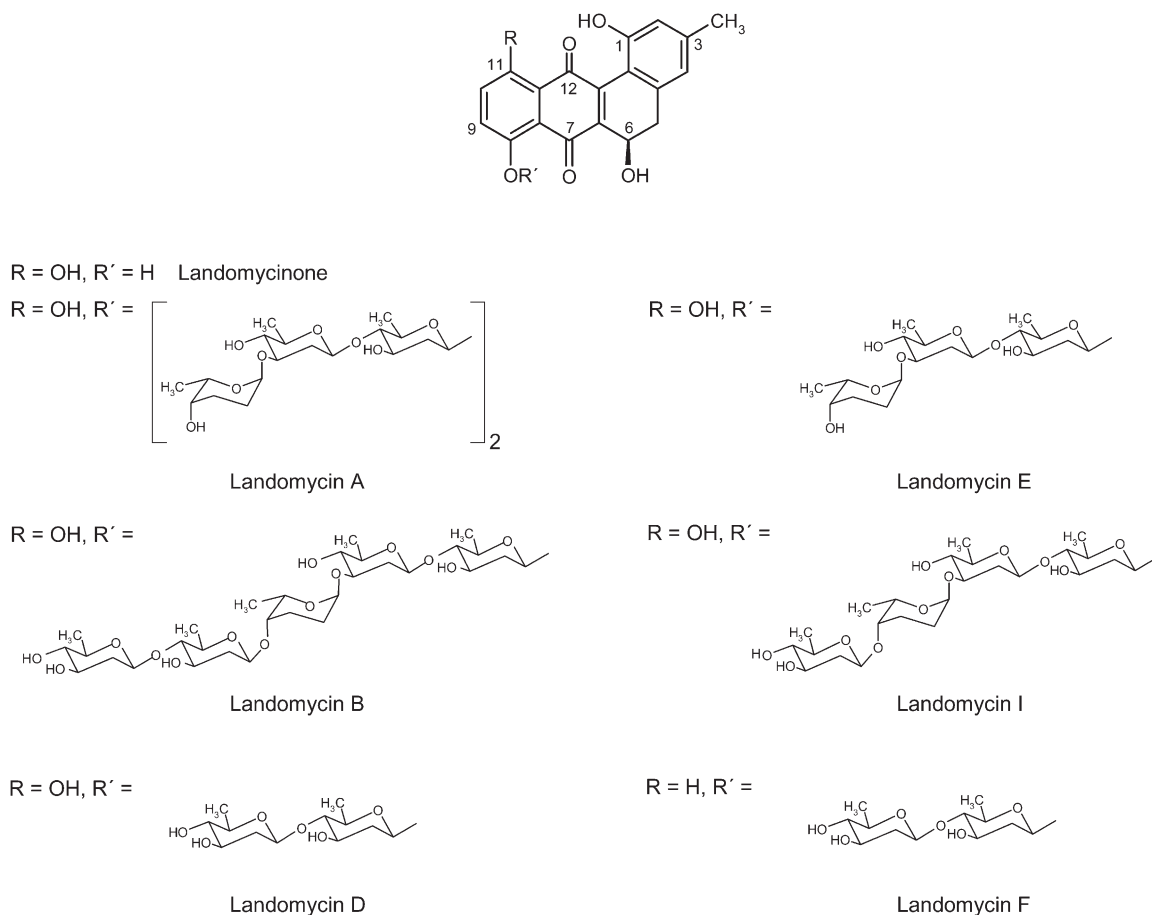


Figure 1. Structures of Different Landomycin Derivatives

of *S. cyanogenus*. The chain elongation of fed landomycin derivatives was monitored by HPLC-MS. Feeding of landomycin E to the wt strain led to its conversion to landomycin A (data not shown). This demonstrates that *S. cyanogenus* S136 is able to convert truncated landomycin derivatives to the full-length final product. However, feeding of landomycin E to the *lanGT1* mutant resulted in the accumulation of landomycin I, the tetrasaccharide congener. This indicates that the fifth sugar transfer cannot take place in the absence of LanGT1. Hence, we conclude that LanGT1 catalyzes also the fifth glycosyltransfer additionally to the second as previously shown (Figure 2A). After feeding of landomycin E to the *lanGT4* mutant, landomycin B containing five sugars was accumulated. This shows that the final glycosyltransfer depends on the presence of LanGT4. From this result, we can conclude that LanGT4 is also responsible for the terminal L-rhodosyltransfer (Figure 2B). This data clearly documents that both GTs LanGT1 and LanGT4 are iteratively acting during the hexasaccharide formation of landomycin biosynthesis.

Inactivation of *lanZ2* Results in the Production of Novel Landomycin Derivatives

The identification of the function of LanZ2, a protein resembling dNDP-hexose synthases from different clusters [8], was aimed by targeted gene disruption. This was achieved by homologous recombination between

plasmid pKC1132lanZ2 (Experimental Procedures) carrying the deleted *lanZ2* gene and the chromosomal allele of this gene in *S. cyanogenus* S136. The mutated region in *lanZ2* was confirmed by PCR and sequencing analysis. For complementation, plasmid pKCermE-GT3Z2Z3 consisting of *lanZ2* ligated behind the *ermE* promoter was introduced into the mutant strain by intergeneric conjugation. This led to the complete reconstitution of landomycin A production.

The HPLC-MS analyses of the *lanZ2* mutant revealed the production of a different spectrum of landomycin derivatives compared to the wt strain. The wt strain was producing landomycin A (44%), landomycin B (30%), and landomycin D (26%). The *lanZ2* mutant produced landomycin A (5.4%), landomycin B (7%), and landomycin D (4%) and, in addition, landomycin F (23.4%) and landomycin E (30%). The latter have both never been described as products of the wt strain. Furthermore, two new landomycin derivatives with unknown structure (30.2%) were produced. From these results, we have to conclude that LanZ2 impacts the formation of the sugar side chain.

Discussion

The hexasaccharide portion of landomycin A is the longest sugar side chain so far detected among angucycline and anthracycline antibiotics. In our work, we

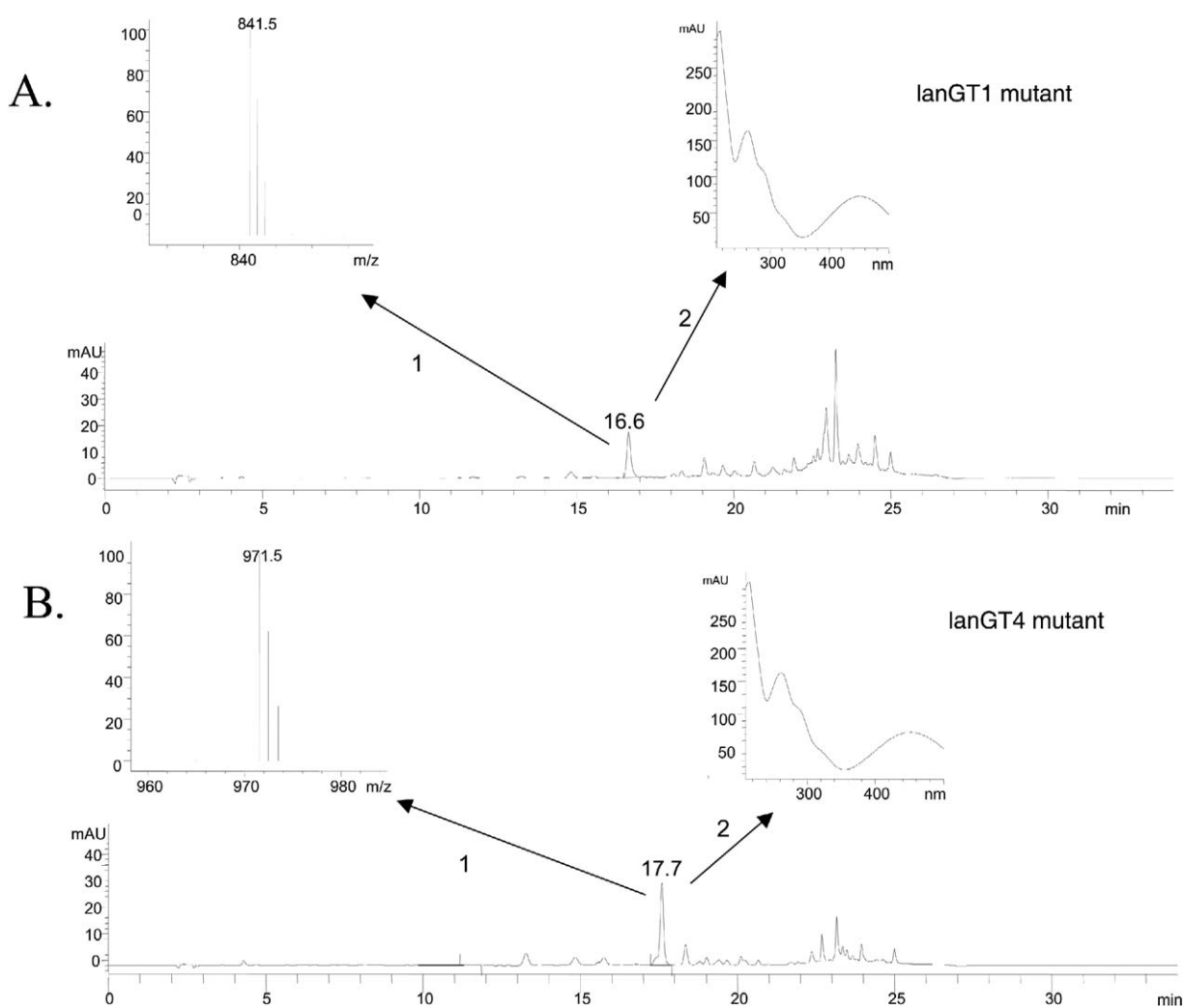


Figure 2. HPLC-MS Chromatograms of Extracts after Feeding of Landomycin E to the *lanGT1* and *lanGT4* Mutants

HPLC-MS chromatograms of extracts after feeding of landomycin E to the *lanGT1* mutant (A) and to the *lanGT4* mutant (B). 1, negative ion chromatograms; 2, UV-VIS spectra.

are now presenting data that clearly shows that two GTs involved in landomycin A biosynthesis are acting twice during the assembly of the sugar side chain (Figure 3).

The function of several GTs involved in antibiotic bio-

synthesis (e.g., erythromycin, urdamycin A, mithramycin) has been described [6, 16]. In all cases, the number of sugars matches the number of available GTs. There are few examples of GTs capable of working repeatedly on a growing sugar chain either by overex-

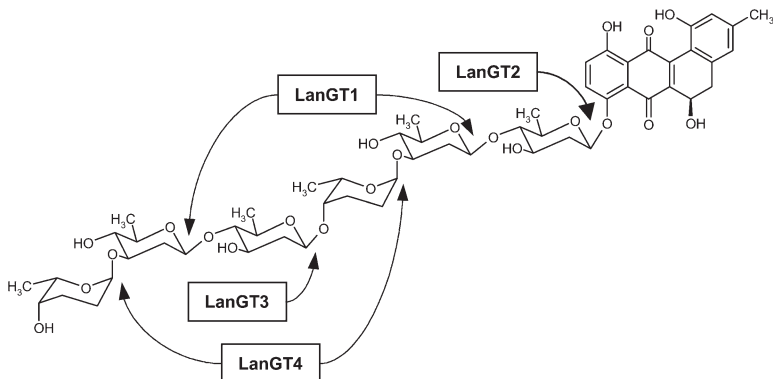


Figure 3. Function of Glycosyltransferases Involved in the Biosynthesis of Landomycin A

pression [17] or by in vitro studies [18]. But to date, no GT has been shown to act twice during natural product formation. Thus, LanGT1 and LanGT4 are the first examples of such iteratively acting GTs. It is likely that there are many further natural-product GTs that belong to this new class of iteratively acting GTs. As an example, avilamycin A consists of a heptasaccharide chain, but only four putative GT genes have been detected in the avilamycin biosynthetic gene cluster. This suggests repeated action also for some of the avilamycin GTs. A big challenge in the future for the generation of novel oligosaccharide antibiotics is to understand the structural features that allow the unique flexibility of iteratively acting GTs.

In this study, we recognized another enzyme to be involved in the process of the hexasaccharide biosynthesis of landomycin A. A *lanZ2* mutant of *S. cyanogenus* S136 produced all main landomycin derivatives, but the production was strongly impaired.

The amino acid sequence encoded by *lanZ2* is similar to dTDP-glucose synthases from different organisms. Because the landomycin biosynthetic gene cluster contains a second putative dTDP-glucose synthase gene (*lanG*), both genes were expressed in *E. coli*, and the corresponding enzymes were purified. Only LanG was able to activate D-glucose-1-phosphate (data not shown). In 1997, the existence of a short sugar-activation pathway in *S. cyanogenus* S136 was shown by incorporation of labeled L-rhodinose [19] into the landomycin molecule. It might be speculated that LanZ2 is involved in this short sugar-activation pathway, maintaining the correct flux of TDP-activated deoxysugar in the cell.

Significance

This is the first description of glycosyltransferases (LanGT1 and LanGT4) involved in natural-product biosynthesis and are working iteratively on a growing oligosaccharide chain. The oligosyltransferase LanGT1 catalyzes the transfer of the second and the fourth sugar, the rhodinosyltransferase LanGT4 attaches the third and the sixth sugar during the biosynthesis of the landomycin A hexasaccharide side chain. Apparently, the putative NDP-hexose synthase LanZ2 is required for the smooth and complete biosynthesis of the hexasaccharide part of landomycin A.

Experimental Procedures

General Genetic Manipulation

Standard molecular biology procedures were performed as described [20]. Isolation of *E. coli* DNA, DNA restriction, DNA modification such as filling-in-sticky ends, and Southern hybridization were performed following the protocols of the manufacturers of kits, enzymes, and reagents (Amersham Pharmacia, Boehringer Mannheim, Promega, Stratagene).

Intergeneric Conjugation between *E. coli* and *S. cyanogenus*

Intergeneric conjugation between *E. coli* and *S. cyanogenus* was performed as described earlier [15].

Construction of Gene Inactivation and Complementation Plasmids

A 4.2 kb *Bam*HI-*Sph*I fragment from cosmid H2-26 [8] containing the *lanZ2* gene was ligated into the same site of pLitmus38 to yield

plasmid pLitZ2. A unique *Sac*I restriction site inside the gene *lanZ2* was chosen for targeted inactivation by shifting the reading frame. After *Sac*I restriction, treatment with the Klenow fragment of *E. coli* DNA polymerase I, and religation, the intended alteration was checked by DNA sequencing. The 4.2 kb fragment containing the mutated *lanZ2* was inserted into the *Eco*RV site of pKC1132 to yield the inactivation construct pKC1132lanZ2. The construction of plasmid pKCermEGT3Z2Z3 used for complementation has been described [14].

Feeding Experiments

Landomycin E (5 mg) was added to a 50 ml liquid culture of *S. cyanogenus* S136, *S. cyanogenus* Δ *lanGT1*, and *S. cyanogenus* Δ *lanGT4*. The production of landomycin derivatives was monitored by HPLC-MS after 48 hr.

Landomycin Production and Purification

Strains were grown in SG medium (soybean meal, 10 g · l⁻¹; glucose, 20 g · l⁻¹; CaCO₃, 2 g · l⁻¹; CoCl₂, 1 mg · l⁻¹ [pH 7.2]) for 2–4 days at 30°C in a rotary shaker at 200 rpm. The culture broth was adjusted to pH 7 and extracted with an equal volume of ethyl acetate. Extracts were dried in vacuo, dissolved in CHCl₃, and purified by column chromatography on silica gel (column 2.5 × 25 cm; CHCl₃, MeOH = 96:4).

HPLC-ESI-MS

HPLC-ESI-MS was performed on an Agilent 1100 series system with an electrospray chamber and a quadrupole detector. The LC-system was equipped with a Hewlett Packard ZORBAX SB C-18 column (5 μm particle size, 4.6 × 150 mm) maintained at 35°C. The gradient profile was: solvent A, 0.5% acetic acid in H₂O; and solvent B, 0.5% acetic acid in CH₃CN. An initial hold for 3 min with 30% B was followed by a step gradient from 30% up to 60% B within 16 min and from 60% up to 95% within 3 min and held at that relation for further 2 min. The solvent flow rate was 0.7 ml · min⁻¹. The UV detection wavelength was 441 nm. Samples were analyzed in a negative scan mode with an m/z range from 200 to 1500.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) grant Bechthold 5-2 to A.B.

Received: March 8, 2005

Revised: April 22, 2005

Accepted: May 6, 2005

Published: July 22, 2005

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