LanGT2 Catalyzes the First Glycosylation Step during Landomycin A Biosynthesis

Andriy Luzhetskyy,[a] Takaaki Taguchi,[a] Marta Fedoryshyn,[b] Clemens Dürr,[a] Sven-Eric Wohlert,[c] Volodymyr Novikov,[b] and Andreas Bechthold*[a]

The glycosyltransferase LanGT2 is involved in the biosynthesis of the hexasaccharide side chain of the angucyclic antibiotic landomycin A. Its function was elucidated by targeted gene inactivation of lanGT2*. The main metabolite of the obtained mutant was identified as tetrangulol (**4**), the progenitor of the landomycin aglycon (**7**). The lack of the sugar side chain indicates that LanGT2 catalyzes the priming glycosyl transfer in the hexasaccharide biosynthesis: the attachment of a* D*-olivose to O-8 of the polyketide backbone. Heterologous expression of urdGT2 from* S. fradiae *Tü2717 in this mutant resulted in the production of a novel C-glycosylated angucycline (**6**).*

Introduction

A glycosyltransferase (GT) transfers a sugar from an activated sugar donor to an acceptor molecule. GTs are involved in the synthesis and modification of a multitude of secondary metabolites, including some valuable antibiotics and anticancer therapeutics.[1] A recent estimate suggests that over half of the world's drug leads derive directly from the natural product pool, many of which are glycosylated.[2] The application of GTs in a combination of chemoenzymatic and in vivo methods is therefore promising for advancing the diversification of pharmaceutically relevant drugs.

Although carbohydrate-containing metabolites have been known for decades, research devoted to the precise function of GTs is often complicated. In vitro studies on GTs are often limited by the unavailability of the activated sugar donors and by the insolubility of enzymes,[3] whereas in vivo studies cannot be performed in many cases because of the absence of suitable gene-cloning systems in the carbohydrate-producing organisms.[4]

Landomycin A, produced by *Streptomyces cyanogenus* S136, and urdamycin A, produced by *S. fradiae* Tü2717, belong to the angucycline group of antibiotics. They show an interesting correlation between sugar-chain length and biological activity.[5] Landomycin A displays strong antitumor activity, due to its unusual hexasaccharide side chain, made up of two iterative trisaccharide motifs (D-olivose-D-olivose-L-rhodinose, **1**; Scheme 1).[6] Urdamycin A (**3**), with only a trisaccharide side chain, displays much weaker antitumor activity.[5] This correlation is further supported by the decreased activity of landomycin E (**2**), the trisaccharide analogon of landomycin A from *S. globisporus* 1912. The functions of the GTs involved in urdamycin A biosynthesis have been elucidated, and the generation of urdamycins with novel sugar patterns has been described.[7] One important difference between landomycin A and urdamycin A is the way in which the first D-olivose is linked to the polyketide backbone. In the former compound, the hexasaccharide is attached to the aglycon through an O-glycosidic bond,[5] while in the latter, the trisaccharide is connected through an unusual C-glycosidic bond. Such C–C-bound glycosides are of particular interest from the pharmaceutical viewpoint since they are stable towards glycosidase degradation.[1] In an effort to switch the O-glycosidically bound sugar chain of landomycin A to a more stable C-bound one, we first had to identify the GT that catalyzes the attachment of the first D-olivose moiety.

Results

Inactivation of *lanGT2*

Identification of the function of *lanGT2* was performed by targeted gene disruption, achieved by homologous recombination between plasmid pKC-lanGT2-aadA carrying the spectinomycin resistance casette (*aadA*) within *lanGT2* and the chromosomal allele of this gene in *S. cyanogenus* S136. Mutant *S. cyanogenus* ΔlanGT2 was obtained after introduction of *aadA* into the chromosome by a double crossover event (Figure 1). The mutated region in *lanGT2* was confirmed by PCR.

The *aadA* cassette from pHP45omega is flanked with transcriptional and translational terminators of phage T4. The insertion into *lanGT2* caused a polar effect on two genes: *lanX* and *lanGT1*, both located downstream of the integration site. *S. cyanogenus* ΔlanGT2 was therefore complemented with pUWLoriT-lanGT1-lanX. No difference in product formation be-

[a] *Dr. A. Luzhetskyy, Dr. T. Taguchi, C. Dürr, Prof. Dr. A. Bechthold*
Albert-Ludwigs-Universität, Institut für Pharmazeutische Wissenschaften
Stefan-Meier-Straße 19, 79104 Freiburg (Germany)
Fax: (+49) 761-203-8383
E-mail: andreas.bechthold@pharmazie.uni-freiburg.de

[b] *M. Fedoryshyn, Prof. Dr. V. Novikov*
Institute of Chemistry and Chemical Technologies
National University "Lvivs'ka Politechnika"
St. Jury Square 3–4, Lviv 79000 (Ukraine)

[c] *Dr. S.-E. Wohlert*
Combinature Biopharm AG
Robert-Roessle-Straße 10, 13125 Berlin (Germany)

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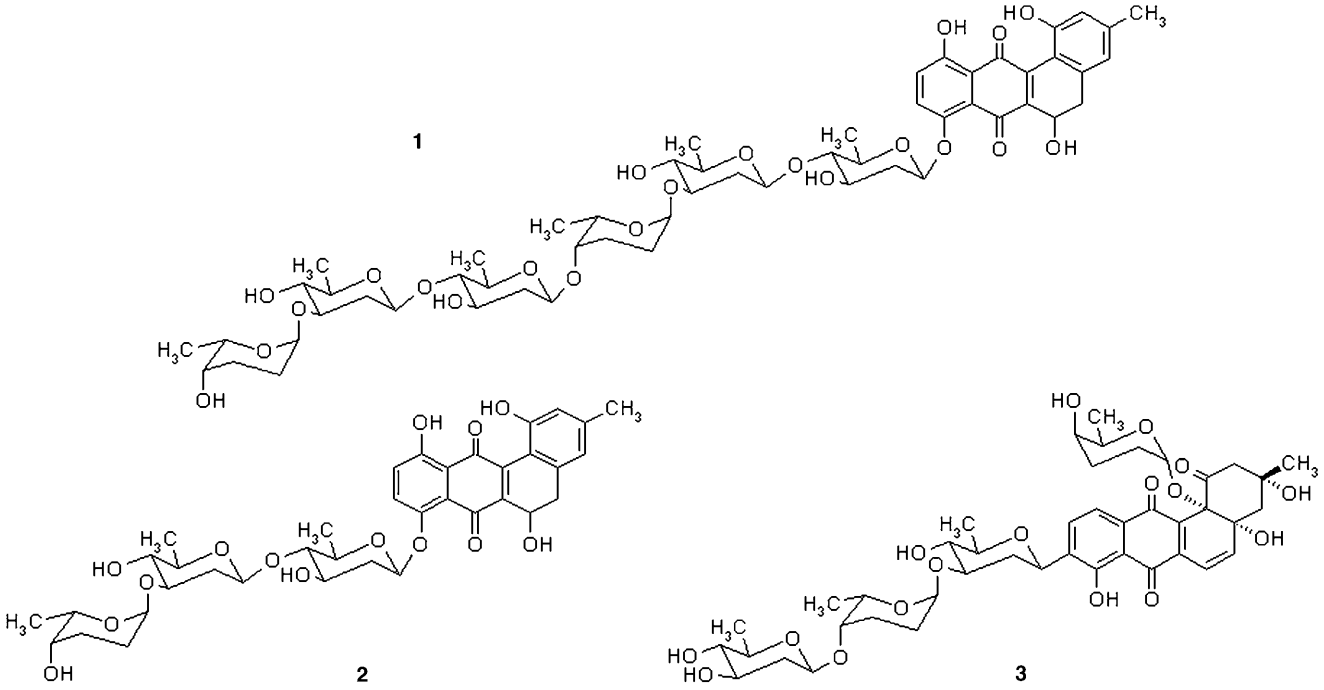

Scheme 1. Chemical structures of landomycin A (**1**, *S. cyanogenus* S136), landomycin E (**2**, *S. globisporus* 1912), and urdamycin A (**3**, *S. fradiae* Tü2717).

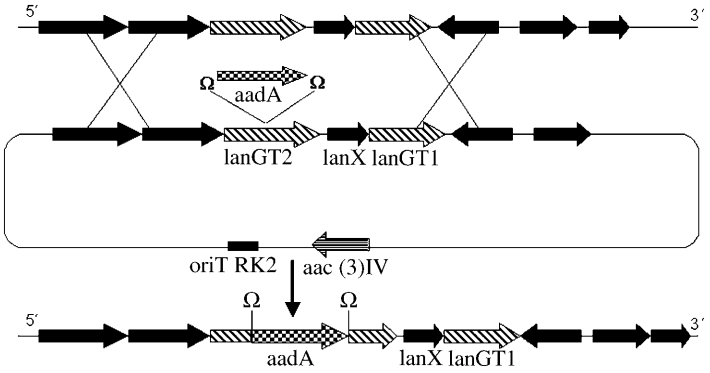

Figure 1. Generation of *S. cyanogenus* ΔlanGT mutant by aadA casette integration into the *lan* cluster of the *S. cyangenus* chromosome.

tween *S. cyanogenus* ΔlanGT2 and *S. cyanogenus* ΔlanGT2 containing pUWLoriT-lanGT1-lanX was observed.

Tetrangulol is the main product of *S. cyanogenus* ΔlanGT2

No landomycins could be detected in extracts of the *S. cyanogenus* ΔlanGT2 mutant. Instead, this mutant was accumulating one major product, not produced by the wild-type strain, at $t_R = 25$ min (Figure 2). This new derivative showed a UV-visible spectrum different from those of landomycins. The compound was extracted from a 5 L fermentation and purified by silica gel column chromatography and subsequent recrystallization. High-resolution ESI-MS measurements indicated its composition to be $C_{19}H_{12}O_4$ (observed: 304.0736, calculated: 304.0739). One- and two-dimensional NMR analyses unambiguously identified the derivative as tetrangulol (Scheme 2; **4**), the progenitor of the landomycin aglycon lacking the hydroxyl groups at C-6 and C-11 and the hexasaccharide side chain.

Function of LanGT1

After expression of *lanGT2*, *lanX*, and *lanGT1* (plasmids pSET-lanGT2 and pUWLoriT-lanGT1-lanX) in *S. cyanogenus* ΔlanGT2 the wild-type landomycin production was restored. HPLC-MS analysis of the mutant complemented with only pSET-lanGT2 (carrying *lanGT2* ligated behind the ermE promotor) identified a monoolivosylated landomycinone (**5**) as the main compound. This result indicates that LanGT1, which is under polar effect in the mutant, is involved in the transfer of the second olivosyl residue during the hexasaccharide biosynthesis.

Heterologous expression of *urdGT2* in *S. cyanogenus* ΔlanGT2 results in the formation of 9-*C*-D-olivosyltetrangulol

The C-GT UrdGT2, responsible for the attachment of the first D-olivose moiety during urdamycin A biosynthesis, shows wide substrate flexibility.[11] The expression of this gene in *S. cyanogenus* ΔlanGT2 under the strong ermE promotor resulted in the accumulation of a new compound at $t_R = 25.1$ min with a UV/visible spectrum typical of tetrangulol. HPLC-MS analysis indicated a molecular weight of $m/z = 434$ for the new derivative, consistent with the attachment of an olivose to **4**. The new variant (57 mg) was extracted from a 6 L fermentation and purified. One- and two-dimensional NMR analyses showed

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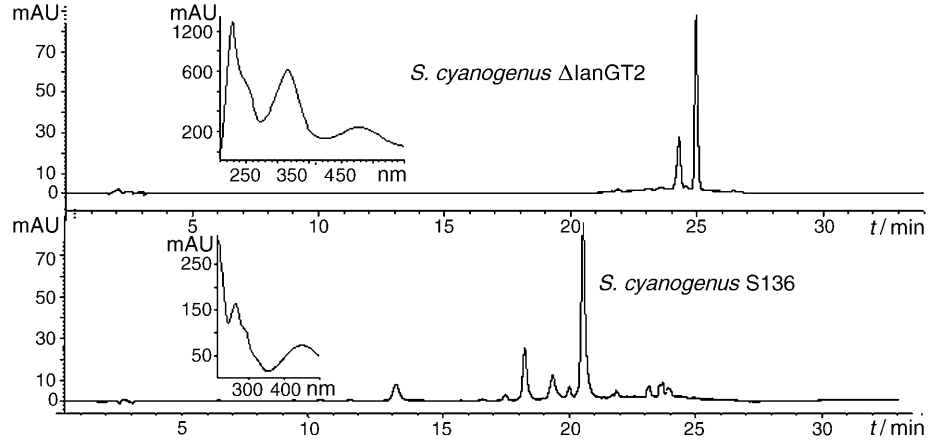
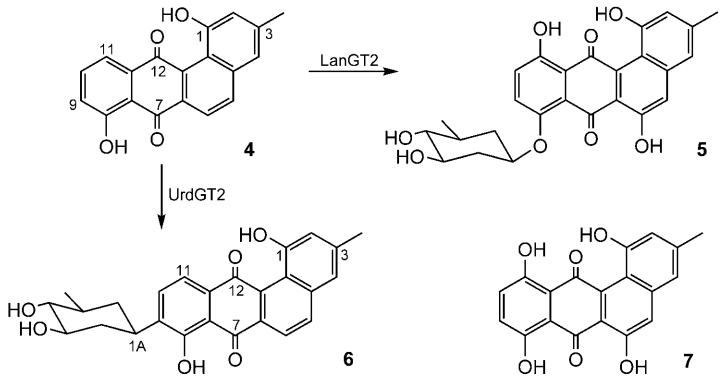


Figure 2. HPLC analysis of extracts of *S. cyanogenus* S136 and *S. cyanogenus* ΔlanGT2, a *lanGT2* defective mutant strain unable to produce landomycin A, instead accumulating tetrangulol.



Scheme 2. Function of LanGT2 during hexasaccharide biosynthesis of landomycin A. Generation of a new tetrangulol derivative (**6**) after heterologous expression of *urdGT2*. Tetrangulol (**4**), 8-O-olivosyllandomycinone (**5**), landomycinone (**7**).

the presence of tetrangulol and olivose moieties. In the ^{1}H NMR the signal corresponding to the 8-hydroxyl group of tetrangulol was still detectable, while the signal of the proton at C-9 was missing. Additionally, the HMBC correlations confirmed that the olivose moiety was attached to C-9 of tetrangulol by C-glycosylation (Table 1). With consideration of the original function of UrdGT2,[7] the new metabolite was identified as 9-*C*-D-olivosyltetrangulol (**6**).

Discussion

Sugar moieties of glycosylated antibiotics and anticancer drugs are often crucial for biological activity as well as for pharmacokinetic properties. The chemical synthesis of such sugar ligands is exceedingly difficult to carry out and therefore impractical to establish on a large scale.[6] This makes glycosyltransferases useful tools for chemoenzymatic and in vivo approaches to the development of complex glycosylated natural products.[12] In recent years around 30 GT genes have been isolated from antibiotic-producing organisms.[12,13,15] The remarkable substrate flexibility displayed by some of these GTs could be exploited for the generation of novel glycosylated compounds.[7]

We have focused our research on the GTs from the urdamycin and landomycin gene clusters. The exact functions of GTs involved in urdamycin biosynthesis have been reported.[5,6,11] In the biosynthesis of the trisaccharide ligand of urdamycin A each glycosylation step is catalyzed by one GT.[5] In contrast, the hexasaccharide side chain in landomycin A is catalyzed by only four GTs.[10] Recently, the functions of LanGT3 and LanGT4 have been described.[9,14] In this study, the priming GT LanGT2 was identified through targeted gene inactivation. In addition, we were able to show that LanGT1 is responsible for the attachment of the second D-olivose moiety. Interestingly, inactivation of the *lanGT2* gene resulted in the accumulation of tetrangulol, and not landomycinone (Scheme 2), tetrangulol lacking landomycinone's two hydroxyl groups at C-6 and C-11. It has been shown by feeding experiments that the O atoms at positions 6 and 11 derive from molecular oxygen.[15] The specific oxygenases catalyzing these reactions, LanM and LanZ4/Z5, were recently identified.[14] As both hydroxyl groups are absent in tetrangulol the oxygenation steps apparently occur after the attachment of the first D-olivose moiety by LanGT2. Remarkably, the oxygen at C-6 is originally introduced in the form of an acetate building block during the polyketide assembly, subsequently removed by deoxygenation, and reintroduced again by an oxygenase.[15]

This unusual, energy-consuming pathway can be explained by the narrow substrate specificity of LanGT2, which accepts only tetrangulol, and not landomycinone, as acceptor substrate. One might assume that the hydroxyl groups at C-6 and C-11, situated at positions that are chemically equivalent to the acceptor hydroxyl group at C-8, would interfere in the proper binding to the active site of LanGT2.

The heterologous expression of the *urdGT2* gene in *S. cyanogenus* ΔlanGT2 resulted in the accumulation of C-glycosylated tetrangulol (Scheme 2, **6**) showing once more the wide substrate flexibility of this extraordinary enzyme. Interestingly, the C-glycosylated compound still lacks hydroxyl groups at both C-6 and C-11. Evidently, both oxygenases LanM and LanZ4/Z5 fail to accept the C-glycosylated tetrangulol; this indicates narrow substrate specificities for both oxygenases.

Combining the present findings with results reported earlier[5,9,14] we can now clearly say how the assembly of the first four sugars of the hexasaccharide biosynthesis takes place: LanGT2 catalyzes the attachment of the first D-olivose to O-8 of the polyketide moiety, followed by the transfer of a second D-olivose to 3-OH of the first olivose by LanGT1. LanGT4 links

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Table 1. NMR data for 9-C-D-olivosyltetrangulol (in [D_6]DMSO: 500 MHz for ^{1}H, 125 MHz for ^{13}C).

Position	^{13}C δ [ppm]	δ [ppm]	^{1}H intensity	multiplicity	J [Hz]	Carbon(s) correlated in HMBC spectrum
1	154.9	10.86	OH	s		C-1, 4, 12b, 3-Me
2	117.2	6.92	1H	brs		
3	141.2					
3-Me	21.0	2.39	3H	s		C-2, 3, 4
4	119.7	7.27	1H	brs		C-1, 2, 4a, 5, 12b, 3-Me
4a	138.3					
5	135.3	8.05	1H	dd	8.5, 3.0	C-4, 4a, 6a, 12b
6	121.2	8.12	1H	dd	8.5, 3.0	C-4a, 7, 12a
6a	133.2					
7	187.7					
7a	114.1					
8	156.7	12.42	OH	s		C-7a, 8, 9
9	137.0					
10	133.0	7.79	1H	d	8.0	C-8, 11, 11a, A1
11	119.4	7.6	1H	d	8.0	C-7a, 8, 9, 11a, 12
11a	134.0					
12	186.5					
12a	133.9					
12b	118.5					
A1	70.6	4.75	1H	brd	12.0	C-8, 9, 10, A2, A3, A5
A2	40.1	ax 1.29	1H	q-like	12.0	C-9, A1, A3
		eq 2.26	1H	ddd	12.0, 4.5, 1.5	C-A3, A4
A3	71.7	3.54	1H	m		C-A4
A4	77.0	2.99	1H	t	8.5	C-A2, A5, A5 -Me
A5	76.1	3.36	1H	dq	8.5, 6.0	C-A3, A4
A5-Me	18.4	1.27	3H	d	6.0	C-A1, A3, A4, A5

an L-rhodinose to the 3-OH group of the second D-olivose, and LanGT3 attaches the fourth sugar, a D-olivose, to 4-OH of the L-rhodinose. The last two steps of glycosyl transfer still remain to be elucidated.

Experimental Section

General genetic manipulation: Standard molecular biology procedures were performed as described.[8] Isolation of *E. coli* DNA, DNA restriction, DNA modification such as filling-in-sticky ends, and Southern hybridization were performed by the protocols of the manufactures 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.[4,9]

Construction of gene inactivation and complementation plasmids: The 9.5 kb *Bam*HI fragment from cosmid H2–26[10] containing the *lanGT2* gene was ligated into the same site of pKC1132[8] to yield plasmid pKC-lanGT2. The resistance gene aadA restricted by *Sma*I was then cloned into the *Sna*BI site of *lanGT2* to yield pKC-lanGT2-aadA. This plasmid was introduced into *S. cyanogenus* S136 by conjugation from *E. coli* ET12567 (pUZ8002). After 10 passages we obtained spectinomycin-resistant, apramycin-sensitive clones indicating that the *aadA* gene had been integrated into *lanGT2*.

A plasmid for the complementation pSET-lanGT2 was generated by ligation of a 1.2 kb *Mun*I-*Bam*HI fragment from pMun-lanGT2[5] to the appropriate sites of integrative vector pSET containing the strong promotor ermE.[7] Genes *lanGTI* and *lanX* were cut as a *Sac*I fragment from H2–26[10] and cloned into the same site of pUC18 (generating plasmid pUC18-LanGT1-lanX). Plasmid pUC18-lanGT1-lanX was digested with *Hind*III *and Eco*RI and the major fragment was ligated to the vector pUWLoriT[7] to give the plasmid pUWLoriT-lanGT1-lanX. To construct pSET-urdGT2 a 1.5 kb *Kpn*I–*Xba*I fragment from pUWL-urdGT2 was ligated into the same sites of integrative vector pSET-urdGT1c.[7]

Tetrangulol and 9-C-D-olivosyltetrangulol production and purification: The *S. cyanogenus* strains were grown in SG medium (soybean meal 10 g L^{-1}, glucose 20 g L^{-1}, $CaCO_3$ 2 g L^{-1}, $CoCl_2$ 1 mg L^{-1}), starting pH 7.2, for 4 days at 30 °C in a rotary shaker (200 rpm). The culture broth was adjusted to pH 7 and extracted with an equal volume of ethyl acetate. The residue from the concentrated extract of *S. cyanogenus* ΔlanGT2 was subjected to repeated silica gel column chromatography in dichloromethane and purified by recrystallization from ethyl acetate to give black needles. The extract from *S. cyanogenus* ΔlanGT expressing urdGT2 was concentrated and subjected to repeated silica gel column chromatography in dichloromethane/ethyl acetate (95:5 to 50:50). Finally, recrystallization from EtOAc gave the green powder.

HPLC-MS: HPLC-MS analysis was performed on an Agilent 1100 series LC/MS system by electrospray ionization (ESI) with detection in the positive and negative modes. 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 H_2O, Solvent B: 0.5 % acetic acid in CH_3CN. 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 that relationship was held for further 2 min. The solvent flow rate was 0.7 mL min^{-1}.

High-resolution ESI-MS and NMR measurements: High-resolution ESI-MS was measured with a Micromass QTOF2 mass spectrometer. NMR measurements were carried out on a Bruker AMX 500 spectrometer.

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