Function of *lanGT3*, a Glycosyltransferase Gene Involved in Landomycin A Biosynthesis

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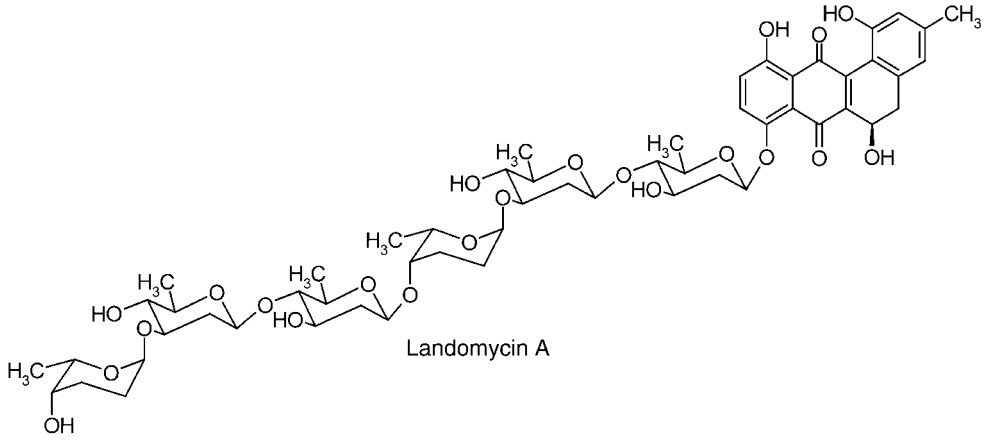
Dedicated to H. G. Floss on the occasion of his 70th birthday

The glycosyltransferase gene lanGT3, *involved in the biosynthesis of the angucyclic antibiotic landomycin A, has been characterised by targeted gene deletion. A* lanGT3 *mutant was shown to produce landomycin E, which consists of a trisaccharide side chain attached to the polyketide moiety. Expression of* lanGT3 *in the mutant restored landomycin A production. Our results indicate that LanGT3 is responsible for the transfer of the fourth sugar during landomycin A biosynthesis.*

Introduction



Significant changes in society and the environment contribute to the emergence of multidrug-resistant bacteria and different cancer tumours. The search for new and more potent compounds has become one of the important tasks of pharmaceutical and medical sciences. Therefore, the carbohydrate-containing metabolites, which show many different biological activities, are of great interest.[1] Molecular biological engineering of glycoside moieties is a promising tool for the rational design of natural products, and a number of new "unnatural" antibiotics have been generated by these attempts.[2,3,4] Surprisingly, little is known about the "extending" glycosyltransferases involved in the biosynthesis of oligosaccharide antibiotics.

Landomycin A, a member of the angucycline group of antibiotics, is produced by *Streptomyces cyanogenus* S136. This compound contains an unusual hexasaccharide side chain consisting of four D-olivose and two L-rhodinose moieties.[5] Landomycin A inhibits [H^3]thymidine uptake in murine smooth muscle and cell-cycle progression[6] and displays strong antitumour activities, in particular against prostate cancer lines.[7] Its unusual activities are due to the oligosaccharide chain, as landomycins with shorter sugar side chains express much weaker antitumour activity.[8] As the hexasaccharide chain is involved in DNA binding of the drug, it is a very interesting and unique structural element. Only four glycosyltransferases (LanGT1, LanGT2, LanGT3 and LanGT4) are responsible for the formation and attachment of the hexasaccharide chain.[9] LanGT1 is an olivosyltransferase, and LanGT4 is a rhodinosyltransferase, as demonstrated by heterologous expression of the responsible genes.[2] However, details of the biosynthesis of the hexasaccharide side chain are so far not well understood. One reason for this was that for a long time no protocol for the genetic engineering of the producer strain *S. cyanogenus* S136 was available, but Fedorenko and co-workers have recently developed a conjugation protocol that can now be used for the introduction of genes into the strain.[10]

Such a targeted gene inactivation experiment has now been successfully applied to elucidate the function of LanGT3 as an olivosyltransferase attaching the fourth sugar of the growing side chain of landomycin A.

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 DOI: 10.1002/cbic.200400123

Results and Discussion

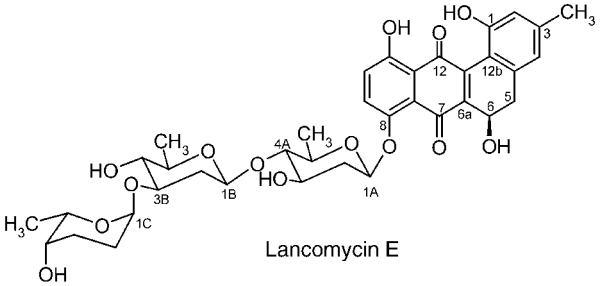
Inactivation of the lanGT3 gene

Identification of the function of LanGT3 was performed by targeted gene disruption, which was achieved by homologous recombination between plasmid pOJΔGT3 (Experimental Section) carrying the deleted *lanGT3* gene and the chromosomal allele of this gene in *S. cyanogenus* S136. *LanGT3* disruption was achieved after a second crossover introducing the deleted *lanGT3* into the chromosome. The mutated region in *lanGT3* was confirmed by Southern hybridisation and sequencing analysis.

For complementation, plasmid pKCGT3—consisting of *lanGT3* ligated behind the ermE promotor—was introduced into the mutant strain by intergeneric conjugation. As landomycin A production was restored, we were able to rule out any upstream or downstream polar effects.

Chromatographic separation of metabolites produced by S. cyanogenus ΔlanGT3

No landomycin A could be detected in extracts of the *lanGT3* mutant. A new landomycin derivative, with a UV/Vis spectrum identical to that of landomycin A, was produced instead. HPLC-MS investigations revealed the molecular mass of the new compound to be $m/z = 711$ amu. The mass difference with landomycin A (374 amu) exactly matches the mass of the trisaccharide L-rhodinose-D-olivose-D-olivose. From this result we concluded the new metabolite to be landomycin E, bearing half of the hexasaccharide chain of landomycin A.



Lancomycin E

Structure elucidation

The *S. cyanogenus* ΔlanGT3 mutant accumulates only one major product, which was identified as the known compound landomycin E by TLC, HPLC-MS and NMR spectroscopy. The NMR data (^1H, ^{13}C; see Table 1) revealed that this compound consists of 37 carbons and of 44 protons; this is consistent with the molecular formula of landomycin E ($C_{37}H_{44}O_{14}$, $M_W = 712.75\ g\,mol^{-1}$). Relative to the spectra of landomycin A, the signals of three sugar moieties were missing, while the remaining signals indicated one rhodinose and two olivose moieties. The TLC, HPLC, UV, NMR and MS data were identical with those of the previously described landomycin E.[8]

Table 1. *^1H and ^{13}C NMR data for landomycin E.*[a]

Position	δ ^1H [ppm]	Multiplicity (*J* [Hz])	δ ^{13}C [ppm]
1			156.3
2	6.62	s	117.8
3			142.9
3-CH_3	2.30	s	21.4
4	6.71	s	123.1
4a			139.3
5 α	2.85	dd (16.5, 3.5)	37.7
β	2.99	dd (16.5, 2)	
6	5.14	m	60.2
6a			145.1
7			182.4
7a			120.3
8			151.2
9	7.49	d (9.5)	131.4
10	7.22	d (9.5)	126
11			158.3
11a			116.8
12			191.1
12a			140.5
12b			116.2
1A	5.13	dd (10, 1.5)	99.9
2A a	1.81	ddd (12, 12, 10)	39.1
e	2.57	ddd (12, 5, 1.5)	
3A	3.60–3.69	m	70.0
4A	3.11	dd (9, 9)	88.7
5A	3.39–3.47	m	71.6
5A-CH_3	1.28	d (6)	18.4
1B	4.64	dd (9.5, 1.5)	101.8
2B a	1.39–1.49	m	37.4
e	2.35	ddd (12, 5, 1.5)	
3B	3.60–3.69	m	78.0
4B	3.07	dd (9, 9)	76.0
5B	3.39–3.47	m	73.2
5B-CH_3	1.35	d (6)	18.1
1C	4.95	br s	96.2
2C a	1.39–1.49	m	24.8
e	2.00–2.15	m	
3C a	1.66	m	26.4
e	2.00–2.15	m	
4C	3.55	br s	67.3
5C	4.19	dq (7, 1.5)	68.0
5C-CH_3	1.143	d (6.5)	17.6

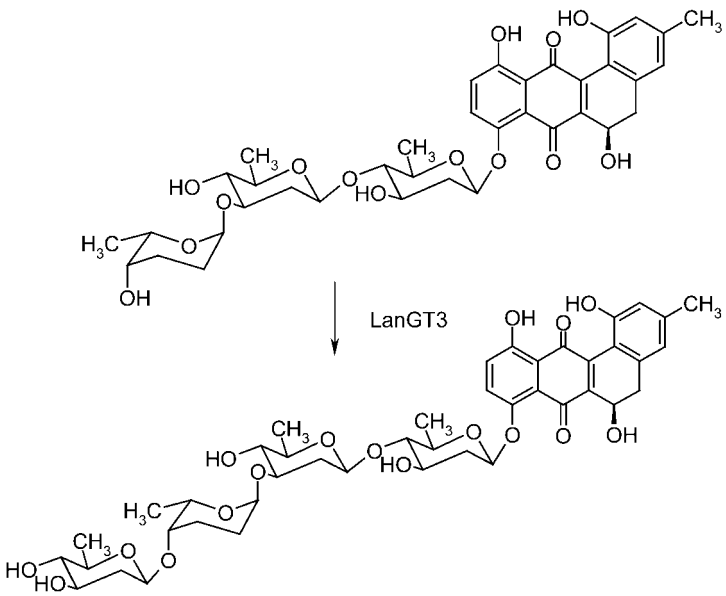
[a] ^1H NMR data were recorded at 400 MHz in CD_3OD, ^{13}C NMR data at 75 MHz in [D_6]acetone.

General implications

The unique landomycin A hexasaccharide is a deoxyoligosaccharide containing four 2,6-dideoxy-glycosydic linkages and two 2,3,6-trideoxy-glycosidic linkages. This is the longest glycan so far reported in the family of angucycline and anthracycline antibiotics.[1] The chemical synthesis of the landomycin A hexasaccharide chain has been reported by several groups, indicating the great importance of this side chain.[11,12] This process is very laborious, yields are low, and the stereochemical control is poor,[12] so knowledge about the biosynthesis of this unique hexasaccharide is of particular interest. Recently, we reported that four glycosyltransferases—LanGT1, LanGT2, LanGT3 and LanGT4—are necessary to compose the hexasaccharide chain during landomycin A biosynthesis.[9] From se-

quence similarities, LanGT2 is believed to be responsible for the attachment of the first sugar to the polyketide moiety. LanGT1 was shown to be a D-olivosyltransferase catalysing the attachment of the sugar to the 4-OH group of a D-olivose already attached to a polyketide-derived aglycon.[2,13] LanGT4 was shown to be a rhodinosyltransferase, exhibiting broad substrate specificity.[13,15] In this study we now report that LanGT3 is involved in the attachment of the fourth sugar of the hexasaccharide chain. In contrast to LanGT1 and LanGT4, this enzyme seems to be much more specific, as we were not able to synthesize novel urdamycin derivatives when expressing *lanGT3* in different *S. fradiae* mutants.

The exact role of each glycosyltransferase in landomycin A biosynthesis still remains to be elucidated, but it is now clear that LanGT3 is a D-olivosyltransferase that introduces the fourth sugar of the hexasaccharide side chain (Scheme 1). The broad substrate specificity of LanGT1 and LanGT4 indicates that both enzymes work iteratively, both twice on the growing chain. In contrast LanGT3 shows very narrow substrate specificity and acts only once during landomycin A biosynthesis.



Scheme 1. *Function of lanGT3 during landomycin A hexasaccharide chain biosynthesis.*

Experimental Section

General genetic manipulation: Standard molecular biology procedures were performed as described.[14] Isolation of *E. coli* DNA, DNA restriction, DNA modification (such as filling sticky ends) and Southern hybridisation were performed by the protocols of the manufactures of the 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.[10] A frozen mycelial culture of *S. cyanogenus* (1 mL) was diluted in TS broth[14] (9 mL) and was agitated at 28 °C and 180 rpm for 16 h. A proportion of this seed culture (2 mL) was transferred into fresh TS broth (18 mL). The culture was again agitated at 28 °C and 180 rpm for 16 h. The mycelium was recovered by centrifugation, washed once in fresh TS broth and resuspended in TS broth (2 mL; recipient culture). The *E. coli* donor ET12567 (pUB307) was grown at 37 °C for 16–18 h in LB medium plus apramycin (25 $\mu g\,mL^{-1}$) and kanamycin (25 $\mu g\,mL^{-1}$). Cells were pelleted, washed once in LB medium and resuspended in TS broth (1 mL; donor culture). Equal volumes of donor and recipient cultures were combined, and samples (300 μL) were plated on oatmeal medium [oat (30 $g\,L^{-1}$), agar (18 $g\,L^{-1}$), pH 7.2]. Plates were incubated at 28 °C for 14–16 h and then covered with water (1 mL) containing nalidixic acid (1 mg) and apramycin (0.5 mg) for selection of exconjugants. Incubation at 28 °C was continued for 7–9 days until exconjugants appeared.

Construction of gene inactivation and complementation plasmids: The 2.2 kb *BamHI-SacI* fragment from cosmid H2–26 containing *lanGT3* gene was ligated into the same sites of pUC19 to yield plasmid pUClanGT3. A unique *NotI* restriction site inside the gene *lanGT3* was chosen for targeted inactivation by shifting the reading frame. After *Not*I restriction, treatment with the Klenow fragment of *E. coli* DNA polymerase I and religation, the intended alteration was checked by DNA sequencing. The 2.2 kb fragment was inserted into the *BamHI-EcoRI* sites of pOJ260 mel to yield the inactivation construct pOJlanGT3.

A plasmid for the complementation of the *lanGT3* mutant was generated by ligation of a 2.2 kb *Bam*HI-*Eco*RI fragment from pUClanGT3 to the expression vector pKC1218ermE.

Landomycin E production and purification: The *S. cyanogenus* ΔlanGT3 mutant strain was 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 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 $CHCl_3$ and purified by column chromatography on silica gel (column 2.5×25 cm, $CHCl_3$/MeOH 96:4). Yield of pure landomycin E: 25 $mg\,L^{-1}$.

HPLC-MS: HPLC/MS was performed on a Waters Alliance 2695 system, equipped with a Waters 2996 photodiode array detector and a Micromass ZQ 2000 mass spectrometer with an APCI probe (solvent A: 0.1 % formic acid in H_2O; solvent B: acetonitrile; flow rate = 0.5 $mL\,min^{-1}$; 0–6 min 75 % A and 25 % B to 100 % B, linear gradient, 6–7 min 100 % B, 7–7.5 min 100 % B to 75 % A and 25 % B [linear gradient], 7.5–10 min 75 % A and 25 % B). We used a Waters Symmetry C-18 column (4.6×50 mm, particle size 5 μm). The column temperature was 23 °C and the UV detection wavelength was 451 nm. Under these conditions landomycin E was detected at a retention time of 4.79 min, showing the typical UV spectrum of the landomycins and a mass of 711 in the negative mode APCI ($[M-H]^-$, 52 %; 337, 100 %, landomycinone, M-olivose-olivoserhodinose).

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), grant Bechthold 5–1 to A.B.

Keywords: biosynthesis · glycosylation · glycosyltransferases · landomycin · olivose

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Received: April 28, 2004

