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On the Acceptor Substrate of C-Glycosyltransferase UrdGT2: Three Prejadomycin C-Glycosides from an Engineered Mutant of *Streptomyces globisporus* 1912 $\Delta IndE(urdGT2)^{**}$

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The landomycins **1–4** produced by *Streptomyces cyanogenus* S-136 and *S. globisporus* 1912 are angucycline antibiotics with a strong activity against various cancer cell lines, in particular against prostate cancer cell lines.[1–6] The landomycins are closely related to the urdamycins (e.g., urdamycin A (**5**)).[7–9] Both possess a polyketide-derived angucyclinone core and sugar moieties consisting of D-olivose and L-rhodinose building blocks. Major structural differences were found in the assembly of the sugar moieties and in the oxygenation pattern of the polyketide core moiety.[1,4,5,10–12]

landomycins: **urdamycin A (5)**

The availability of the biosynthetic gene clusters of urdamycins and landomycins[7,13–17] made it possible to track down the genes encoding the biosynthetic enzymes responsible for the unique structural features of the two types of compounds, which are also responsible for the significant variations in the biological activities of these related anti-cancer drugs.[18] The overall structural and biosynthetic similarity between these two closely related sets of antibiotics allowed several successful combinatorial, biosynthetic gene-combination experiments, leading to new hybrid molecules.[5,6,15,19–24]

The most striking structural difference is that the trisaccharide chain found in the urdamycins is C-glycosidically linked at C9, while the oligosaccharide chains found in the different

landomycins are O-glycosidically linked at the 8-position. The enzymes responsible for the first glycosyl transfer step for these chains, the glycosyl transferases (GT) UrdGT2 and LanGT2/LndGT2, are closely related. LndGT2 shows 53% amino acid identity (68% similarity) with UrdGT2. However, the acceptor substrates of these related GTs appear quite different, not only because one is a C-GT and the other an O-GT, but also with regard to the timing of the first GT step within the urdamycin and landomycin biosyntheses. In landomycin biosynthesis, all but one oxygenation step in the aglycon formation appear to occur prior to the first GT step,[5] while for the urdamycin biosynthesis the sequence of biosynthetic events (oxygen attachment at 12- and 12b-positions by UrdE and UrdM, respectively, before or after the C-glycosylation step) remained the subject of debate, and the nature of the acceptor substrate of the important C-glycosyltransferase UrdGT2 was still ambiguous.[8,11]

Here we describe our attempts to further investigate UrdGT2, to identify its acceptor substrate, and to possibly generate C-glycosidic landomycin derivatives through heterologous expression of the corresponding gene *urdGT2*. Our work has showed that the C-glycosylation in the urdamycin biosynthesis occurs prior to the two aglycon oxygenations through oxygenases UrdE and UrdM, and that the early intermediate UWM6 serves as the acceptor substrate for UrdGT2.

Regarding the oxygenases of the landomycin biosynthesis, it was found that oxygenase Lan/LndZ5 is responsible for the 11-hydroxylation,[5] and Lan/LndM2 for the 6-hydroxylation, [12] while Lan/LndE most likely catalyzes the 12-oxygenation (quinone formation), which was assumed to be the first oxygenation step in the landomycin biosynthesis. All oxygenations steps except the 11-hydroxylation occur before Lan/LndGT2 attaches the first sugar moiety. Thus we first wanted to confirm these conclusions regarding the oxygenation sequence in landomycin biosynthesis by inactivation of *lndE*, leading to the early block mutant *S. globisporus* Δ *lndE*. This mutant of the landomycin E producer accumulates prejadomycin (2,3-dehydro-UWM6; **6**), an early intermediate of various angucyclines and angucycline-derived compounds that was first discovered in the context of the jadomycin biosynthesis; more recently it was also found in blocked mutants of the gilvocarcin V and the oviedomycin producers. The *S. globisporus* Δ *lndE* was obtained by directed disruption of the *lndE* gene within the chromosome of *S. globisporus* Smu622 by insertion of the hygromycin resistance cassette. The accumulation of **6** clearly supports the conclusion that LndE is the first acting oxygenase of the landomycin pathway. Moreover, the mutant *S. globisporus* Δ *lndE* appeared to be an ideal host for a heterologous expression of *urdGT2*, the gene encoding C-GT in urdamycin biosynthesis, since it provided prejadomycin (**6**), a compound almost identical to UWM6 (**10**), which was recently discussed as a possible acceptor substrate of UrdGT2,[19] as well as NDP-activated D-olivose, its sugar donor substrate. Moreover, the construction of an *S. globisporus* Δ *lndE* (*urdGT2*) mutant not only promised to provide information about the acceptor substrate of UrdGT2 but also would allow us to further test the promiscuity of the other *lnd/lan* glycosyltransferases, two of which (LndGT1 and LndGT4) were present in the construct along with their required NDP-activated sugar donor substrates. Finding GTs that can elongate a sugar moiety into di-, tri-, or longer saccharide chains regardless of where and how the first sugar is attached, is important for future saccharide constructs by combinatorial biosynthesis. While one such experiment was already successfully completed with LanGT1, which is closely related to LndGT1,[19] LndGT4/LanGT4 were never tested on unnatural acceptor substrates.

prejadomycin

(2,3-Dehydro-UWM6; **6**):
R = H

prejadomycin C-glycosides

The *S. globisporus* $\Delta lndE$ (*urdGT2*) recombinant strain was constructed by introduction of plasmid pUWLurdGT2 harboring the *urdGT2* gene, which was controlled by the *PerME* promoter through intergeneric conjugation into the $\Delta lndE$ mutant. The presence of the plasmid was proven by physical isolation, transfer into *E. coli* XL1-blue MRF, restriction enzyme mapping, Southern blotting with *urdGT2* amplified from the urdamycin producer *S. fradiae* Tü2717, and sequencing of the PCR product obtained from the plasmid with suitable primers for *urdGT2*. The resulting construct *S. globisporus* $\Delta lndE$ (*urdGT2*) was cultivated in 4 L of a soya-glucose medium, and the accumulated natural products were isolated. Three novel compounds could be isolated and their structures were elucidated using NMR spectroscopy and mass spectrometry. The mass and UV spectra revealed immediately the chromophore of the poly-ketide moiety to be prejadomycin.[25–27] Typical for this chromophore are the absorption maxima at $\lambda=405$ nm and 266 nm. Furthermore, the mass spectra data reveal also the presence of three, two, and one deoxysugar moiety, respectively, because of characteristic fragmentations ($[M-115] = M$ -rhodnose for the monosaccharide, $[M-115-130] = M$ -rhodnose-olivose for the disaccharide, and $[M-115-130-130] = M$ -rhodnose-olivose-olivose for the trisaccharide). Comparison of the NMR data with those of prejadomycin and the known landomycins[5] indicated the structures to be 9-C- β -D-olivosylprejadomycin (**7**, 3 mg), 9-C- β -D-olivosyl-1,4- β -D-olivosylprejadomycin (**8**, 9 mg), and 9-C- α -L-rhodosyl-1,3- β -D-olivosyl-1,4- β -D-olivosylprejadomycin (**9**, 4.5 mg). The conformation and connectivity of the sugar units (to each other and to the polyketide core) was confirmed from 2D NMR experiments (NOESY and CIGAR-HMBC).[28,29] For instance, the anomeric 1A-H of the C-glycosidically linked D-olivose unit shows NOE couplings with 10-H, 2A-H_e, 3A-H, and 5A-H; and $^3J_{C,H}$ couplings can be observed in the CIGAR-HMBC between 1A-H and C8, C10 and C3A along with a weaker $^2J_{C,H}$ coupling with C9 (see the Supporting Information). This clearly determines this sugar to be in the 4C_1 conformation typical for D-sugars, attached directly at C9, and, based on the large coupling constant between 1A-H and 2A-H_a of $^3J_{H,H}=10$ Hz, containing a β -glycosidic linkage.

In summary, the heterologous expression of gene *urdGT2* into the *lndE*-minus mutant of the landomycin E producer *S. globisporus* 1912 yielded three novel prejadomycin analogues that differ in their C-glycosidically bound moieties attached at C9. The sugar residue and oligosaccharide moieties are the same as those previously found in landomycins H (**4**), D (**3**), and E (**1**), but they are attached C-glycosidically at C9 instead of O-glycosidically at C8-O, a

position shift that was expected from using UrdGT2. The glycosyl transfer step occurred on an early angucyclinone intermediate, prejadomycin (**6**), also called 2,3-dehydro-UWM6, which was found to be an intermediate of several pathways. The heterologously expressed C-glycosyl transferase UrdGT2 from the urdamycin biosynthetic pathway, but not its natural competitor, the O-GT LndGT2, which was also present, was able to glycosylate compound **6**, thereby redirecting the attachment of the landomycin sugar chain towards the C9-position. The GTs responsible for the elongation to the landomycin E trisaccharide, LndGT4 and LndGT1, could attach their sugars although the aglycon was structurally quite different and the first sugar unit was C-attached and at different position. Our results strongly suggest that UrdGT2 naturally acts on UWM6 (**10**, Scheme 1) as its acceptor substrate, which differs from prejadomycin (**6**) only by its 3-OH group. Thus the ambiguity is resolved regarding the previously not clearly identified acceptor substrate for UrdGT2, and also the C-GT step in urdamycin biosynthesis is indicated to most likely occur *prior to* the two aglycon oxygenation steps catalyzed by UrdE and UrdM (see Scheme 1).[8,19]

To find out whether prejadomycin (**6**) is an early intermediate of the landomycin pathway or an early intermediate of a shunt pathway branching from the landomycin pathway (Scheme 1), we performed a crossfeeding experiment, in which prejadomycin (**6**, obtained from *Streptomyces globisporus* Δ *lndE*) was fed to an early block mutant of the landomycin biosynthetic pathway (the *lndF* mutant F133).[12] In this mutant the PKS-associated fourth ring cyclase gene *lndF* was inactivated, and consequently it cannot produce any useful polyketides. But the mutant expresses all downstream enzymes necessary for the completion of landomycin E biosynthesis when it is fed with an intermediate containing a completely cyclized polyketide intermediate. Strain *S. globisporus* F133 was cultivated for 24 h, and prejadomycin (**6**) was fed in a single portion (2 mg). HPLC-MS monitoring (every 12 h) showed that significant amounts of landomycin E were produced. This clearly proves that prejadomycin (**6**) is also an intermediate of the landomycin pathway but cannot be glycosylated by LndGT2, since this enzyme acts strictly on a later intermediate, in which two of three oxygenation events have already occurred. Thus, the urdamycin and landomycin pathways differ significantly with regard to their first glycosylation step. For the landomycin biosynthesis, earlier evidence was found that the first glycosylation step, the O-glycosylation at 8-O with D-olivose catalyzed by LndGT2, occurred prior to the 11-hydroxylation, catalyzed by LndZ4/Z5, but after the oxygen atoms had been introduced into both the 12- and 6-positions by LndE and LndM2, respectively. [5] In particular, it was recently shown that the non-glycosylated angucyclinones tetrangomycin (**11**) and rabelomycin (**12**) can be further converted into landomycin E (**1**) by the *lndF* mutant F133, and thus **11** and **12** are biosynthetic intermediates.[12] The results described here confirm this description of a—compared to the urdamycin pathway—later first glycosylation step. The fact that none of the new prejadomycin C-glycosides including monoglycoside **7** could be further modified by the oxygenases that exist in *S. globisporus* Δ *lndE* (urdtGT2), for example, by LndM2 and LndZ4/Z5, shows that these oxygenases require non-glycosylated substrates. The finding that a compound without 3-OH group, namely **6**, serves as an earlier intermediate than two compounds with 3-OH groups, namely **11** and **12**, seems contradictory. However, in studies with the overexpressed oxygenase JadH, an enzyme responsible for the 12-oxygenation in the jadomycin pathway,[25,26] prejadomycin (**6**) was in part directly converted into rabelomycin (**12**), which could be explained only by a rearrangement initiated by a Michael addition of the 4a-OH group at the 3-position.[31] Such a rearrangement was also observed by Hutchinson et al. but not explained.[30] Scheme 1 shows this rearrangement and illustrates the most probable sequences of the urdamycin and landomycin pathways. It also includes the “hybrid” pathway to the new prejadomycin C-glycosides that was initiated by the heterologously expressed UrdGT2.

Supplementary Material

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Scheme 1.

Biosynthetic pathways for landomycin (black) and urdamycin (blue) and the hybrid pathway to the C-glycosylated prejadomycins **7–9** (green). The early urdamycin biosynthetic pathway (blue) proceeds via the hypothetical intermediates **14** and **15**.