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LanV, a Bifunctional Enzyme: Aromatase and Ketoreductase during Landomycin A Biosynthesis

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LanV is involved in the biosynthesis of landomycin A. The exact function of this enzyme was elucidated with combinatorial biosynthesis by using Streptomyces fradiae *mutants that produce urdamycin A. After expression of* lanV *in* S. fradiae ΔurdM*, which is a mutant that accumulates rabelomycin, urdamycinon B and urdamycin B were found to be produced by the strain. This result indicates that LanV is involved in the 6-ketoreduction of the angucycline core, which preceeds a 5,6-dehydration reaction. 9-C-D-Olivosyltetrangulol was also produced by this strain; this demonstrates that LanV catalyses the aromatization of ring A of the angucycline structure. Coexpression of* lanV *and* lanGT2 *in* S. fradiae *AO, a mutant that lacks all four urdamycin glycosyltransferases, resulted in the production of tetrangulol and the glycoside landomycin H, both of which have an aromatic ring A. As glycosylated angucyclines were not observed after expression of* lanGT2 *in the absence of* lanV*, we conclude that LanGT2 needs an aromatized ring A for substrate recognition.*

Introduction

Landomycins and urdamycins are streptomyces-derived angucycline-type anticancer agents.[1,2] Landomycins in particular show powerful anticancer activities, primarily against prostate cancer cell lines.[3] Landomycin A is the major product of *Streptomyces cyanogenus* S136;[4] urdamycin A and urdamycin B are primarily produced by *Streptomyces fradiae* Tü2717[2] (Scheme 1). The biosynthetic pathways that lead to landomycins and urdamycins are similar: the polyketide backbone is thought to be constructed in an identical manner.[5] However, the compounds differ in their sugar linkage, functional groups and oxidation state (Scheme 1). In landomycin A a deoxysugar chain is attached through an *O*-glycosidic linkage to the polyketide core. In urdamycin A, in addition to a single L-rhodinose moiety, a trisaccharide is connected to the polyketide by an unusual C–C bond. It has been shown that four glycosyltransferases are needed for the biosynthesis of the hexasaccharide side chain of landomycin A and for the biosynthesis of urdamycin A.[6–8] In addition, the function of each glycosyltransferase in both pathways has been determined.[7–13] The angucyclic cores of landomycin A and urdamycin B differ in several respects. Importantly, landomycin A contains an aromatic-A ring and two additional hydroxyl groups at position C6 and C11.

While genes and enzymes involved in both hydroxylation steps have been identified,[14,15] the mechanism that leads to the aromatization of ring A during landomycin biosynthesis has not been elucidated. The function of genes involved in landomycin A and urdamycin B biosynthesis has mostly been elucidated by gene-deletion experiments.[7,8,10–12,14–16] Furthermore, combinatorial-biosynthesis experiments have provided information about the exact function of genes only in a relatively small number of cases.[9] In this study we aimed at identifying a putative aromatizing enzyme by expressing landomycin biosynthetic genes in the urdamycin producer. The successful generation of compounds was possible in *S. fradiae* mutants but not in the wild-type strain. This indicates that metabolic flux is a central issue in the production of genetically engineered drugs. Our data suggest that LanV is involved in the removal of the oxygen at position C6 during landomycin A biosynthesis and that it is responsible for the aromatization of ring A of the angucyclic polyketide core. In addition we show that LanGT2, which is involved in the attachment of the first D-olivose moiety during landomycin A biosynthesis, requires the aromatized ring A for substrate recognition.

Results

Expression of landomycin biosynthetic genes in *S. fradiae*

This project was aimed at identifying an aromatizing enzyme in the landomycin pathway that could be used to generate novel compounds in the urdamycin producer, *S. fradiae*. We selected *lanV*, which is located in the landomycin A cluster and can be excised with *Bgl*II to give a 3.7 kb fragment;[17] a gene similar to *lanV* has not been established in the urdamycin B

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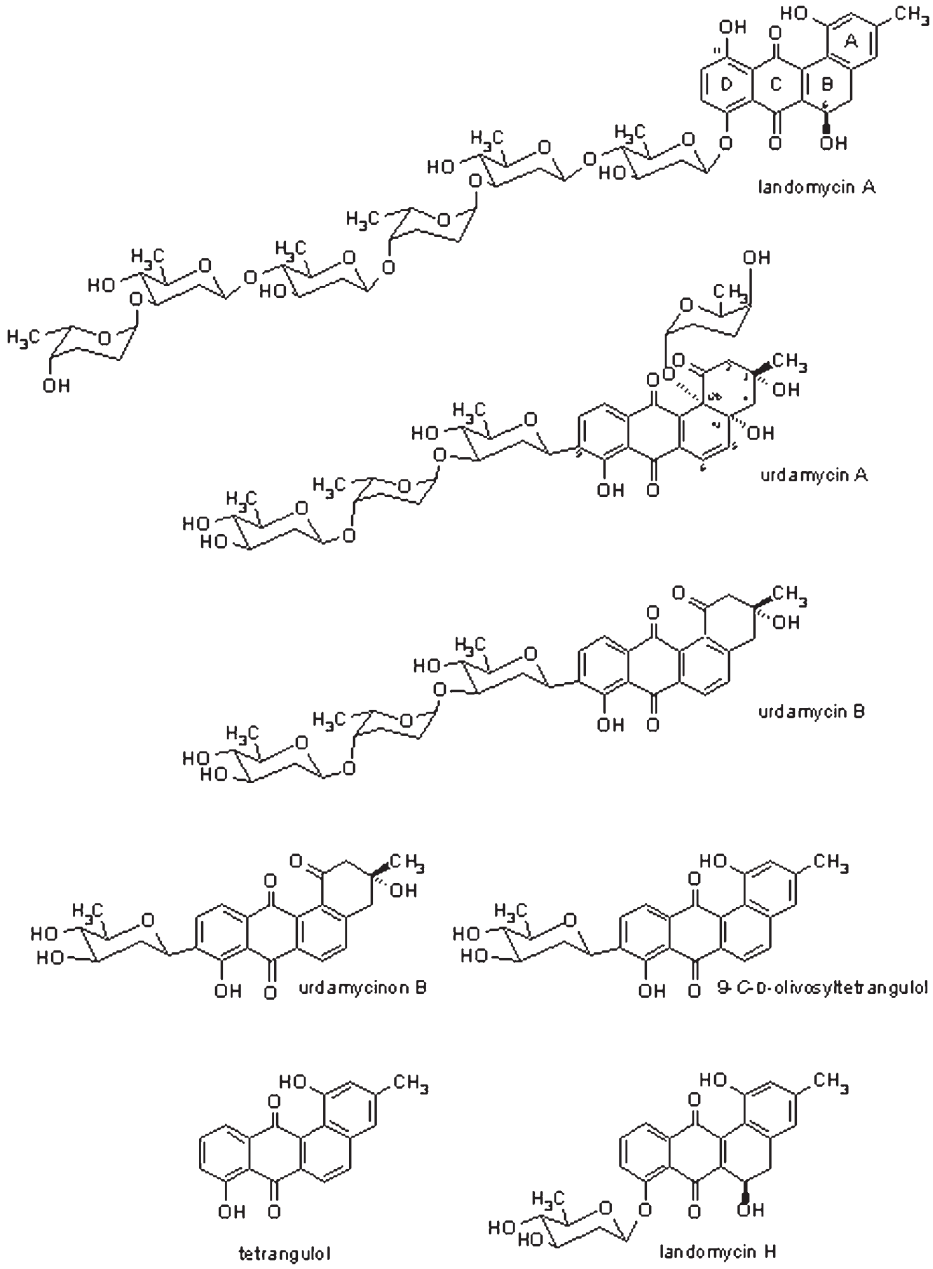

Scheme 1. Chemical structures of landomycin A, urdamycin A, urdamycin B, urdamycinon B, 9-*C*-D-olivosyltetrangulol, tetrangulol and landomycin H.

biosynthetic gene cluster. The 3.7 kb *Bgl*II fragment also contains *lanGT2* which encodes the first-acting glycosyltransferase in landomycin A biosynthesis. The fragment was engineered to be under the control of the *ermE* promoter in plasmid pUWL201oriT, and the product pU3705 was generated. After the expression, no new compound was detected, only those produced by the exoconjugates in comparison to the control cells, *S. fradiae*–pUWL201oriT. Therefore, experiments were repeated with *S. fradiae* AO[9] a mutant that lacks all four glycosyltransferase genes of the urdamycin pathway, and *S. fradiae ΔurdM*[16] a mutant that lacks the oxygenase gene *urdM*. Both mutants accumulate polyketide derived products (*S. fradiae* AO: urdamycin I, urdamycin J and rabelomycin;[8] *S. fradiae ΔurdM*: rabelomycin[16]). The expression of pU3705 in these mutants led to the formation of compounds previously not produced, as indicated below.

Expression of pU3705 in *S. fradiae Δ urdM* and *S. fradiae* AO results in the production of angucycline derivatives that are not produced by the mutant strain

Expression of pU3705, which encodes both *lanV* and *lanGT2*, in *S. fradiae ΔurdM* resulted in the production of compounds that were not found in the mutant strain that contained the control plasmid, pUWL201oriT. These compounds were also produced by expression of a plasmid that only contained *lanV* (pUlanV). Products were identified by TLC, HPLC-MS (Figure 1) and NMR spectroscopy to be compounds 9-*C*-D-olivosyltetrangulol,[11] urdamycin B and urdamycinon B[2,8] (Scheme 1).

Plasmid pU3705 was also expressed in *S. fradiae* AO. Extracts of the strain were analysed by TLC and HPLC-MS and compared to those of *S. fradiae* AO–pUWl201oriT.

Two minor peaks were detected with *S. fradiae* AO–pU3705 extracts which did not accumulate in the control strain. Retention time, UV and mass spectrum of each compound indicated the formation of tetrangulol[11] and landomycin H[14] (Scheme 1, Figure 2).

Discussion

The aim of this study was to identify a gene from the landomycin biosynthetic-gene cluster that encoded a landomycin-ring A 6-aromatase. The accumulation of 9-*C*-D-olivosyltetrangulol in *S. fradiae ΔurdM*, which contained *lanV*, clearly demonstrated that LanV is the responsible aromatase.

The accumulation of two further compounds, urdamycin B and urdamycinon B, indicated that LanV is also involved in the ketoreduction step at position C6 of the polyketide core followed by a dehydration reaction. The enzyme complements an unidentified second function for UrdM—the other function being hydroxylation at position C12b during urdamycin A bio-

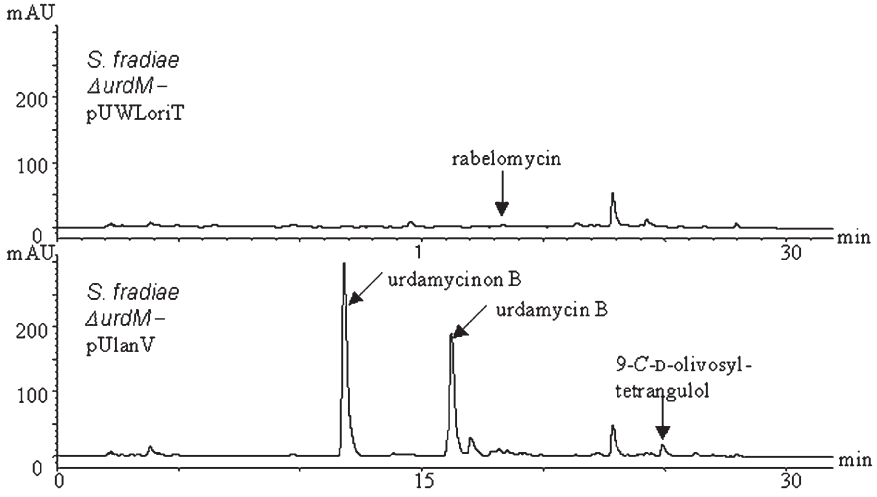

Figure 1. HPLC chromatograms of extracts obtained from *S. fradiae ΔurdM*–pUWLoriT and *S. fradiae ΔurdM*–pUlanV ($\lambda = 254$ nm).

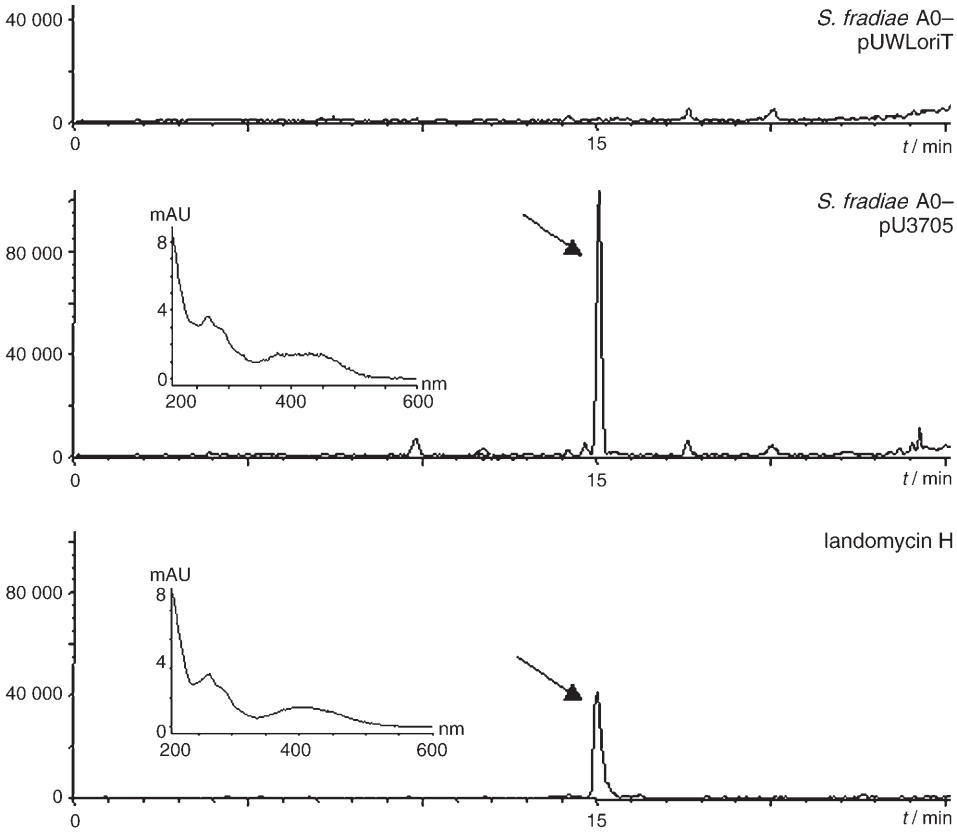

Figure 2. Ion chromatograms (*m/z* 451 amu (negative mode)) of extracts obtained from *S. fradiae* A0–pUWLoriT and *S. fradiae* A0–pU3705. The retention time and UV spectrum of the compound produced by *S. fradiae* A0–pU3705 are identical to those of landomycin H. The landomycin H peaks are indicated with arrows.

synthesis.[16,18] The fact that *S. fradiae ΔurdM* does not produce glycosylated rabelomycin or any other glycosylated secondary metabolites indicates that the OH-group at position C6 prevents the activity of UrdGT2. Therefore, the glycosyltransferase from the urdamycin pathway catalyses the formation of the *C*-glycosidic linkage at C9. Careful comparison of LanV and UrdM showed that LanV and the C-terminal part of UrdM (amino acids 413–669) share a 59% amino-acids identity. Both enzymes belong to the short-chain dehydrogenase/reductase (SDR) family that contains the catalytic triad Ser-Tyr-Lys. One member of this family is 4HNR from *Magnaphorte grisea* which reduces tetrahydroxynaphthalene to scytalone.[19] Surprisingly, LanV is not similar to JadF—a monooxygenase that is involved in the aromatization of ring A during jadomycin B biosynthesis.[20]

The single expression of *lanGT2* in *S. fradiae* A0 did not result in the production of glycosylated angucyclines.[9] The coexpression of *lanGT2* and *lanV* in *S. fradiae* AO led to the accumulation of small amounts of tetrangulol and landomycin H. The latter indicates that LanGT2 requires an aromatized ring A for substrate recognition. The combination of different gene clusters has been shown to result in the formation of novel natural products. This study shows that the success of this method is somewhat limited when using wild-type strains. The reason might stem from metabolic flux that is strongly controlled during the biosynthesis of a given compound and prevents the accumulation of intermediates in the cells.

Experimental Section

Bacterial strains, plasmids and culture conditions: DNA manipulation was performed in *E. coli* XL1 Blue MRF (Stratagene, La Jolla, CA, USA). Plasmids were then passed through *E. coli* ET12567 (*dam*$^-$, *dcm*$^-$, *hsdM*$^-$, *cmr*)[21] to generate unmethylated DNA for transformation. *E. coli* strains were grown under standard conditions.[22] Plasmid pUWL201[23] was a gift from U. Wehmeier and W. Piepersberg (Department of Chemical Microbiology, University of Wuppertal, Germany). A blunt-end fragment containing oriT was cloned into the *Ssp*I site of pUWL201 to yield pUWLoriT. Routine molecular methods were performed as described.[24] Protoplast formation, transformation and regeneration of protoplasts for *S. fradiae* were performed by using standard procedures.[24] The generation of *S. fradiae ΔurdM* and *S. fradiae* AO has been described.[12] NL111 medium (10% malt extract, 2% meat extract Lab Lemco (Oxoid), 1% $CaCO_3$, pH 7.2) was used for pro-

duction. Thiostrepton (50 μg mL^{-1}) was added to the cultures to maintain the expression plasmid. Digestion with restriction endonucleases (Promega) and isolation of plasmid DNA (Wizard Plus, Promega) were carried out according to the manufacturer's instructions.

Construction of expression plasmids: The 3.7 kb *Bgl*II fragment from cosmid H2-26[17] that contained *lanV* and *lanGT2* was ligated into the *Bam*HI site of pBluescript SK$^-$ (Stratagene). The right orientation of the fragment was checked by restriction mapping. The *Hin*dIII–*Spe*I fragment was cloned into pUWL201oriT to generate pU3705. In order to generate the expression plasmid pUlanV, *lanV* was amplified by PCR by using cosmid H2-26 as template. Suitable restriction sites (*Mun*I and *Bgl*II) were introduced up- and downstream of *lanV* by using oligonucleotide primers lanVMfe (5′-CAT GTC CAATTG CGG TTC GCT GAA AGT CAA TTC C-3′) and lanVBglII (5′-CTT CGG AGATCT GCT GCA TCC GGC GCT CAG C-3′); restriction sites are underlined. The PCR product was digested with *Mun*I and *Bgl*II and ligated into plasmid pMUN2[9] to create pMlanU. In order to produce the expression plasmid pUlanV, pMlanV was digested with *Hind*III and *Xba*I and ligated into UWL201oriT.

Analysis of secondary metabolites from *S. fradiae Δ urdM* transformants: Transformants were cultured in production medium for 4 days at 28 °C in a rotary shaker (180 rpm) as described.[2] Samples (0.9 mL) were extracted with an equal volume of ethyl acetate. The solvent was removed from the organic phase and the remaining residue was dissolved in methanol (30 μL). This solution was used for TLC and HPLC-MS analyses.

Analysis of secondary metabolites by TLC and HPLC-MS: TLC was performed on silica-gel plates (Merck) with CH_2Cl_2/CH_3OH (8.5:1.5, *v/v*) as solvent. HPLC-UV/HPLC-MS analysis was carried out on an Agilent 1100 series LC/MS system by ESI in the positive and negative modes. The LC system was equipped with a Hewlett–Packard ZORBAX SB C18 column (5 μm particle size, 4.6×12.5 mm) and a ZORBAX Eclipse XDB-C8 (5 μm particle size, 4.6×150 mm) and was maintained at 30 °C. A nonlinear gradient over 30 min at a flow rate of 0.7 mL min^{-1} was conducted with acetic acid (0.5 %) in H_2O (solvent A) and CH_3CN (solvent B). The gradient started with 20 % and ended with 95 % CH_3CN; the detection wavelength was 254 nm. 9-*C*-D-Olivosyltetrangulol,[11] tetrangulol[11] and landomycin H[15] were used as references.

Purification of urdamycinon B and urdamycin B: *S. fradiae ΔurdM* were transformed with pU3705 and grown in NL111 medium with thiostrepton (50 μg mL^{-1}) for 4 days on a rotary shaker (180 rpm). The culture broth was extracted twice with an equal volume of ethylacetate. The extract was subjected to repeated RP18 gel-column chromatography in methanol/water (65:35–100:0). Finally the products were extracted with ethylacetate and evaporation of the solvent gave a yellow powder. High-resolution mass spectroscopy and one- and two-dimensional NMR spectroscopy showed the existence of urdamycinon B and urdamycin B.[25]

High resolution ESI-MS and NMR measurements: High resolution ESI-MS was measured by using a Micromass QTOF2 mass spectrometer. NMR measurements were obtained with a Bruker AMX 500 spectrometer.

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