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## Surprising production of a new urdamycin derivative by *S. fradiae* $\Delta$ urdQ/R

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### Abstract

A strain (*S. fradiae*  $\Delta$ urdQ/R) with mutations in *urdQ* and *urdR* encoding a dTDP-hexose-3,4-dehydratase and a dTDP-hexose-4-ketoreductase, respectively, produces a new urdamycin analogue (urdamycin X) with changes in the polyketide structure. The structure of urdamycin X has been elucidated by NMR spectroscopy. Urdamycin X was not detectable, even in small amounts, in either *S. fradiae*  $\Delta$ urdQ, in *S. fradiae*  $\Delta$ urdR or in *S. fradiae* A0, a mutant lacking all glycosyltransferase genes. Complementation of *S. fradiae*  $\Delta$ urdQ/R restored urdamycin A production indicating that the mutations did not cause any polar effect.

### Keywords

*Streptomyces fradiae*; Secondary metabolism; Urdamycins; Deoxysugar biosynthetic enzymes

## 1. Introduction

Urdamycin A (Fig. 1), produced by *Streptomyces fradiae* Tü2717 (*S. fradiae* Tü2717), is an angucycline antibiotic with some anticancer activity (Drautz et al., 1986). It has been used as model substance for biosynthetic studies focusing especially on the biosynthesis of the sugar side chain (Hoffmeister et al., 2000; Trefzer et al., 2000). In addition, mutants of *S. fradiae* Tü2717 have been used as hosts for the production of hybrid natural products obtained by combinatorial biosynthesis (Trefzer et al., 2001; Luzhetskyy et al., 2005).

Combinatorial biosynthesis has been described as an efficient implement for drug discovery and development, once the enzymology of the pathway being manipulated and the physiology of the organisms used for production is perfectly understood (Floss, 2006; Reeves, 2003). The enzymology for natural product biosynthesis ranges from single catalytic steps to multistep assemblies. Examples for single step reactions are glycosylation, hydroxylation and methylation, examples for multistep assemblies are polyketide synthases or non-ribosomal peptide synthetases (Reeves, 2003). Inactivation of a gene encoding a multifunctional protein very often leads to a non producing strain while inactivation of a gene responsible for a single step reaction very often leads to simple structural changes and these changes are often

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predictable. For example, the inactivation of a sugar biosynthetic gene generates a mutant producing a novel compound with changes in the glycan structure, and the inactivation of a gene involved in modifying the polyketide backbone results in a mutant producing a compound with changes in the aglycon structure (Reeves, 2003; Rix et al., 2002).

In this article, surprisingly and for the first time, we have observed that a mutant with modified sugar biosynthetic genes produces a new compound with changes in the polyketide structure.

## 2. Materials and methods

### 2.1. Baterial strains, plasmids and culture conditions

*S. fradiae* Tü2717 (Drautz et al., 1986), *S. fradiae*  $\Delta$ urdR (Hoffmeister et al., 2000) and all mutants were grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1mM CaCl<sub>2</sub>, pH adjusted to 7.2 (HA medium) at 28 °C. DNA manipulation was carried out using *Escherichia coli* XL-1 Blue MRF' (Stratagene) as host strain. Plasmids pBluecript SK- was from Stratagene. Plasmid pKC1132 and pKC1218ermE were kind gifts from Eli Lilly and Company. For gene expression (complementation experiments) plasmid pUWL201 (Trefzer et al., 2000) and pIJ2925 (Kieser et al., 2000) were used. The construction of the gene inactivation plasmid pKurdQpm has been described (Hoffmeister et al., 2000). Plasmids pLR234 $\Delta$ 7 and pUC18U containing *oleL* and *oleU* (Aguirrezabalaga et al., 2000; Lombo et al., 2004; Salas and Mendez, 2005) were a kind gift of Prof. Dr. J.A. Salas, Oviedo, Spain. *E. coli* was grown on Luria-Bertani (LB) agar or liquid medium containing the appropriate antibiotic. For urdamycin production *S. fradiae* and all mutants were grown in NL111V medium as described (Trefzer et al., 2000).

### 2.2. General genetic manipulation

Standard molecular biology procedures were performed as described (Kieser et al., 2000). Isolation of *E. coli* DNA and DNA restriction, were performed by the protocols of the manufactures of kits, enzymes, and reagents (Amersham, Pharmacia, Boehringer, Mannheim, Promega, Stratagene).

### 2.3. Generation of *S. fradiae* $\Delta$ urdQ/R, a mutant lacking UrdQ and UrdR

Intergeneric conjugation between *E. coli* and *S. fradiae*  $\Delta$ urdR was performed as described earlier (Luzhetskyy et al., 2006) using the plasmid pKurdQpm. After conjugation apramycin resistant colonies were obtained. Numerous colonies were grown on plates containing no apramycin to select for loss of resistance. Six apramycin-sensitive colonies were obtained suggesting that they were the consequence of a double crossover. One mutant named *S. fradiae*  $\Delta$ urdQ/R was further examined. PCR fragments obtained from *S. fradiae*  $\Delta$ urdQ/R using primers Q1: 5'-GGAACCACCGAATTCTGGCCGT-CC-3' and Q2: 5'-CTAGCCACAAGCTTCGACGAACTCCTT-GAT-3' were analyzed by restriction analysis. Fragments could not be restricted by *NcoI* which was the endonuclease employed for frame shifting. In contrast, PCR fragments amplified with the wt-DNA as template were digestible.

### 2.4. Construction of gene expression (complementation) plasmids

The construction of gene complementation plasmids containing either *urdQ* or *urdR* has been described (Hoffmeister et al., 2000). For the construction of pKColeUL *oleL* was amplified by PCR using pLR234 $\Delta$ 7 as template. *SacI* restriction sites were introduced 5' to the ribosome binding site and 3' to the termination codon using primers oleLF: 5'-TTGAATGGATCCGAGCTCCAG-3' and oleLR: 5'-CGGCCGGAGCTCGGGTGCCGG-3'. The fragment containing *oleL* was cloned into the *SacI* site of pIJ2925 (Kieser et al., 2000) to yield plasmid pIJoleL. Then plasmid pUC18U was restricted by *HindIII*-*XbaI*. A 1.0 kb

fragment containing *oleU* was ligated into pIJoleL previously restricted by the same enzymes to yield pIJoleUL. A 2,0 kb *Bgl*II fragment of pIJoleUL containing *oleU* and *oleL* was ligated behind the *ermE* promoter of pKC1218ermE to generate pKColeUL. For the construction of pKClanQR a 3.0 kb *Bam*HI fragment from cosmid H2-26 (Westrich et al., 1999), containing *lanQ* and *lanR*, was ligated behind the *ermE* promoter into the *Bgl*II site of pKC1218ermE.

## 2.5. Analysis of secondary metabolites produced by *S. fradiae* Tü2717 and by the mutants

Strains were cultured in production medium for 4 days at 28 °C in a rotary shaker (180 rpm) as described (Drautz et al., 1986). Samples (0.9 ml) were extracted with an equal volume of ethyl acetate. The solvent of the organic phase was removed and the residue was dissolved in 30 µl methanol. This solution was used for HPLC–UV–MS analyses. HPLC–UV–MS analysis was performed on an Agilent 1100 series LC/MS liquid chromatograph equipped with a photodiode array detector. For mass detection electrospray ionization (ESI) with detection in the positive and negative modes were used. The LC-system was equipped with a Hewlett Packard ZORBAX SB C-18 column (5 mm particle size, 4.6 mm × 150 mm), maintained at 35 °C. The gradient profile was: Solvent A: 0.5% acetic acid in H<sub>2</sub>O, Solvent B: 0.5% acetic acid in CH<sub>3</sub>CN. 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<sup>-1</sup>.

## 2.6. Isolation of urdamycin X

Cultures of *S. fradiae*  $\Delta$ *urdQ/R* were extracted with an equal volume of ethyl acetate. After evaporation of the solvent the dried extract was redissolved in methanol.

Urdamycin X was isolated from the extract using several chromatographic steps: (i) RP18 silica gel column (15 cm × 3 cm), eluted with water and acetonitrile (67:33), (ii) HPLC with gradient mixture of water (with 0.1% formic acid) and acetonitrile (standard gradient). A total of 23.8 mg of urdamycin X has been purified, and characterized by mass, <sup>1</sup>H NMR, <sup>13</sup>C NMR including 2D NMR (Table 1).

## 2.7. Structure elucidation of urdamycin X: high resolution NMR and ESI-MS measurement

All NMR assignments have been confirmed through combining <sup>1</sup>H NMR, <sup>13</sup>C NMR with 2D NMR spectra, including H, H-COSY, HSQC and HMBC. The stereochemistry at the chiral centers was determined through a NOESY spectrum, where a strong coupling has been found between 3-OH, 4a-OH, 12b-OH. The 3-CH<sub>3</sub> group (C-14) showed coupling with the axial protons attached to C-2, C-4, C-5 and C-6.

High resolution ESI-MS was measured using a Micromass QTOF2 mass spectrometer.

## 3. Results

Urdamycin A (Fig. 1) served as a model compound for the investigation of the formation of a saccharide side chain during the past 10 years (Hoffmeister et al., 2000; Trefzer et al., 2000; Rix et al., 2003; Luzhetskyy et al., 2005). Functions of all glycosyltransferases as well as most enzymes involved in deoxy-sugars formation have been elucidated (Fig. 1) (Trefzer et al., 2000; Hoffmeister et al., 2000). The biosynthesis of D-olivose and L-rhodinose starts from dTDP-D-glucose. dTDP-4-keto-2,6-dideoxy-D-glucose was shown to be the central intermediate in the biosynthesis of dTDP-D-olivose and dTDP-L-rhodinose during urdamycin A biosynthesis. Inactivation of *urdR* involved in D-olivose biosynthesis led to the formation of urdamycin M (Fig. 2, Table 2), which carries a C-glycosidically attached D-rhodinose at the 9-position and inactivation of *urdQ* prevented the mutant strain from producing L-rhodinose resulting in the accumulation of urdamycinone B (Fig. 2, Table 2).

In this study, a strain (*S. fradiae*  $\Delta$ urd*Q/R*) with mutations in *urdQ* and *urdR* encoding a dTDP-hexose-3,4-dehydratase and a dTDP-hexose-4-ketoreductase, respectively (Fig. 1) was generated. Analysis of the culture supernatant of *S. fradiae*  $\Delta$ urd*Q/R* showed that one major compound was produced by the strain (Fig. 3) and its structure was elucidated using NMR spectroscopy (Table 1) and mass spectrometry.

The unusual hemiketal-lactone ring A followed from the  $^{13}\text{C}$  NMR data ( $\delta$  86.7 for C-12b,  $\delta$  175.8 for C-1) and indicative HMBC-couplings, e.g.  $^3J_{\text{C-H}}$  between 5-H<sub>a</sub> and C-12b, and between 4-H<sub>a</sub> and C-12b), along with the HR mass of 374.1002 confirming the molecular formula of C<sub>19</sub>H<sub>18</sub>O<sub>8</sub>. The stereochemistry of the three centres at C-3, C-4a, and C-12b followed from observed NOESY couplings between the three OH groups located at these positions along with the given stereochemistry at C-3 from the biosynthetic process, while the 3-methyl group protons couple with the axial protons at 2-, 4-, 5- and 6-position. In addition to this new compound, named urdamycin X (Fig. 2), the mutant strain was also producing small amounts of urdamycinone B (Fig. 2, Table 2), detected by mass spectrometry.

Complementation of the double mutant with either *urdQ* or *urdR* resulted in a complete disappearing of urdamycin X; instead urdamycin M (after complementation with *urdQ*) and aquayamycin and urdamycinone B (after complementation with *urdR*) (Figs. 2 and 3, Table 2) were accumulated in both strains. Complementation with *lanQ* (encoding a dTDP-hexose-3,4-dehydratase) and *lanR* (encoding a dTDP-hexose-4-ketoreductase), two genes from the landomycin biosynthetic gene cluster from *S. cyanogenus* S136 resulted in urdamycin A and urdamycin B production (Figs. 1 and 3, Table 2).

Expression of *oleL* and *oleU*, genes encoding a dTDP-4-keto-6-deoxyglucose 3,5-epimerase and a dTDP-hexose-4-ketoreductase, respectively, in *S. fradiae*  $\Delta$ urd*Q/R* again resulted in disappearing of urdamycin X. Now urdamycin I and urdamycin J (Figs. 2 and 3, Table 2) were accumulated in the strain.

## 4. Discussion

The modifications observed in the polyketide-derived urdamycin X, particularly the hemiketal-lactone and the saturated 5,6 bond, were very surprising. We can rule out any polar effects caused by the gene inactivation experiments as complementation of our mutants restored activity. Urdamycin X was not detectable, even in small amounts, in either *S. fradiae*  $\Delta$ urd*Q*, in *S. fradiae*  $\Delta$ urd*R* or in *S. fradiae* A0, a mutant lacking all glycosyltransferase genes. Major compounds produced by *S. fradiae* A0 were urdamycin I and J (Fig. 2).

An explanation for the unusual production of urdamycin X might be that the biosynthesis of urdamycin A takes place in a complex. If two enzymes of the complex are not active or cannot interact with other enzymes, biosynthetic intermediates are not transferred correctly along the biosynthetic machinery. In our case it might influence the activities of two oxygenases UrdE and/or UrdM involved in urdamycin A biosynthesis.

There are reports in the literature indicating that polyketide biosynthesis takes place in a complex. The recent structural study by Stroud, Khosla and coworkers, in which the authors were able to 'catch a type II PKS in action', was a breakthrough. They provided the direct proof for a KS heterodimer in showing that KS and CLF (or KS $\beta$ ) have evolved highly complementary contacts. In addition, they deduced from the structure that a protein cleft keeps the nascent polyketide chain extended. In this fashion the reactive ketide groups are separated and probably contact the KS-CLF mainly as enols (Keatinge-Clay et al., 2004). This study well complements the previously available structures of type II PKS components, mainly an X-ray structure of a priming KS (KAS III homolog) from the R1128 pathway (Pan et al., 2002), and

NMR solution structures of ACPs from the actinorhodin (Crump et al., 1997), oxytetracycline (Findlow et al., 2003), and frenolicin (Li et al., 2003) PKSs.

Interestingly a very similar compound to urdamycin X, urdamycin L (Fig. 2) has been detected in an *urdM* mutant of *S. fradiae* as a minor compound (Rix et al., 2003). Urdamycin X differs from urdamycin L in possessing an almost saturated ring B (additional OH groups at the angular 4a- and 12b-positions, and a saturated 5,6-bond), while ring B in urdamycin L is a phenolic arene ring with an OH group in 6-position. However, both compounds are related by having an unusual  $\epsilon$ -lactone as ring A. It was discussed that urdamycin L accumulates in the cell as a consequence of a Baeyer-Villiger-oxygenation process in context with the 12b-oxygenation. Our finding now might indicate that the 12b-oxygenation catalyzed by UrdM is even more complex than suggested, and leads here to an over-oxidized 12-carbon.

When *oleU* and *oleL*, two genes involved in dTDP-L-oleandrose biosynthesis in *S. antibioticus* were expressed in *S. fradiae*  $\Delta$ urdQ/R, urdamycin I and J were produced by the strain but not urdamycin X. OleL (dTDP-4-keto-2,6-dideoxy-D-glucose-3,5-epimerase) and OleU (dTDP-4-deoxy-4-keto-L-oliose-4-ketoreductase) are known to convert dTDP-4-keto-2,6-dideoxy-D-glucose to dTDP-L-olivose. dTDP-L-olivose is obviously not accepted as substrate by UrdGT2 but interestingly, OleL and OleU are influencing product formation.

*S. fradiae*  $\Delta$ urdQ/R was also producing small amounts of urdamycinone B. We believe that an unspecific ketoreductase is converting dTDP-4-keto-2,6-dideoxyglucose to dTDP-D-olivose which is the substrate for glycosyltransferase UrdGT2 that form the C-glycosidic link. However, the major sugar component accumulating in the mutant strain is presumably dTDP-4-keto-2,6-dideoxy-D-glucose (4, Scheme 2), which appears to be unsuited as a substrate of UrdGT2. Therefore urdamycin X remains not glycosylated.

## Acknowledgments

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## Abbreviations

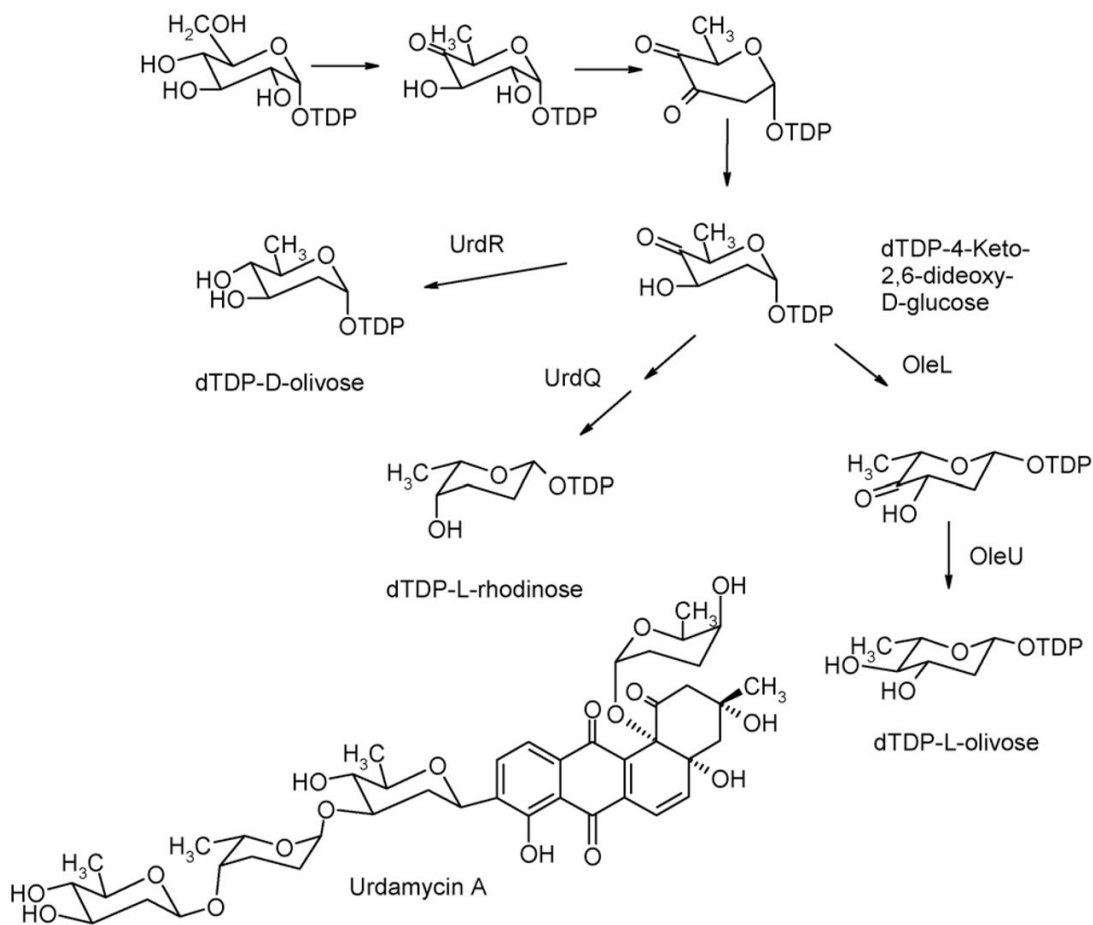
PCR	polymerase chain reaction
dTDP	deoxythymidine phosphate
NMR	nuclear magnetic resonance
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
PMP	pyridoxamine 5'-phosphate

## References

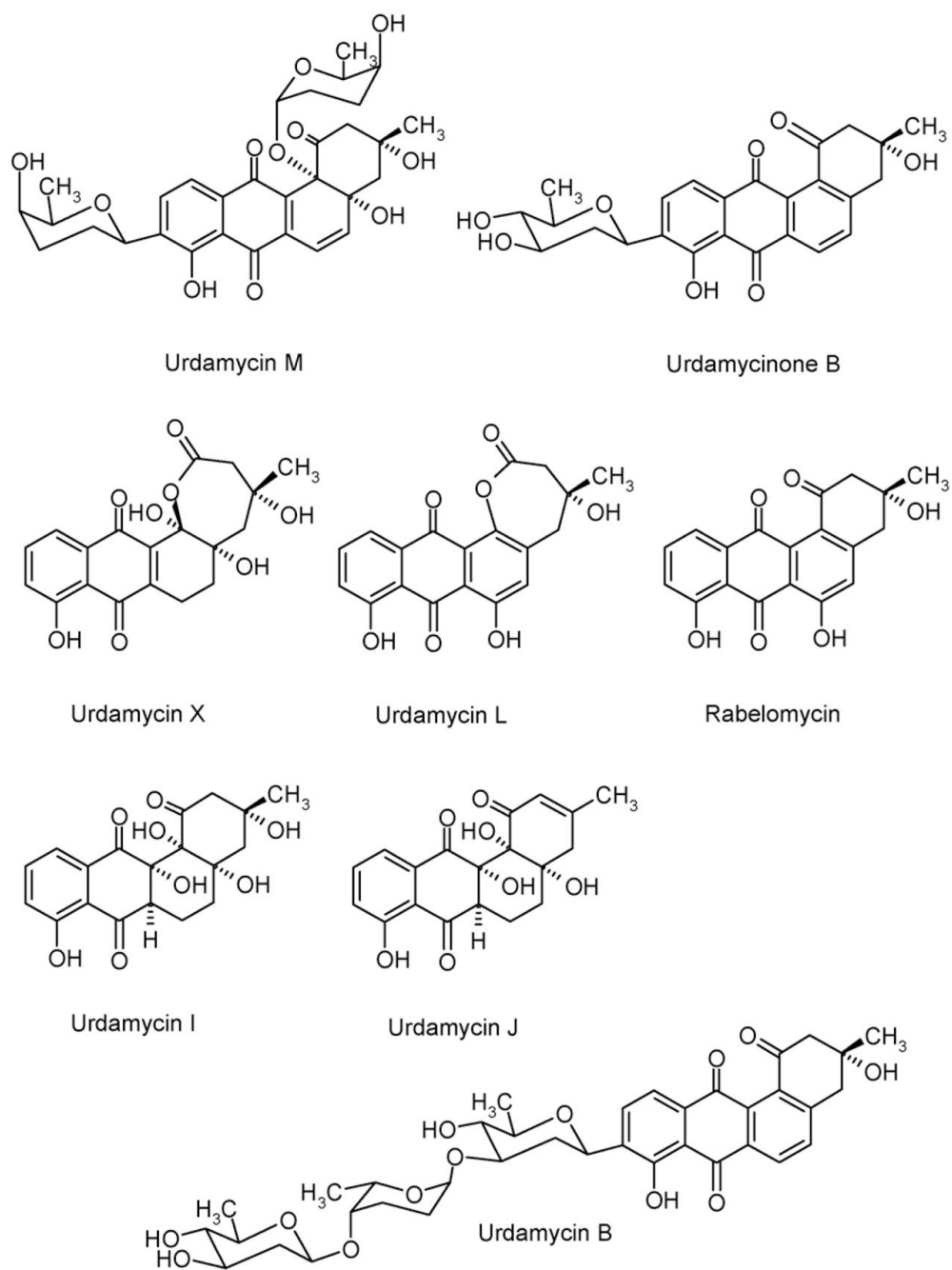
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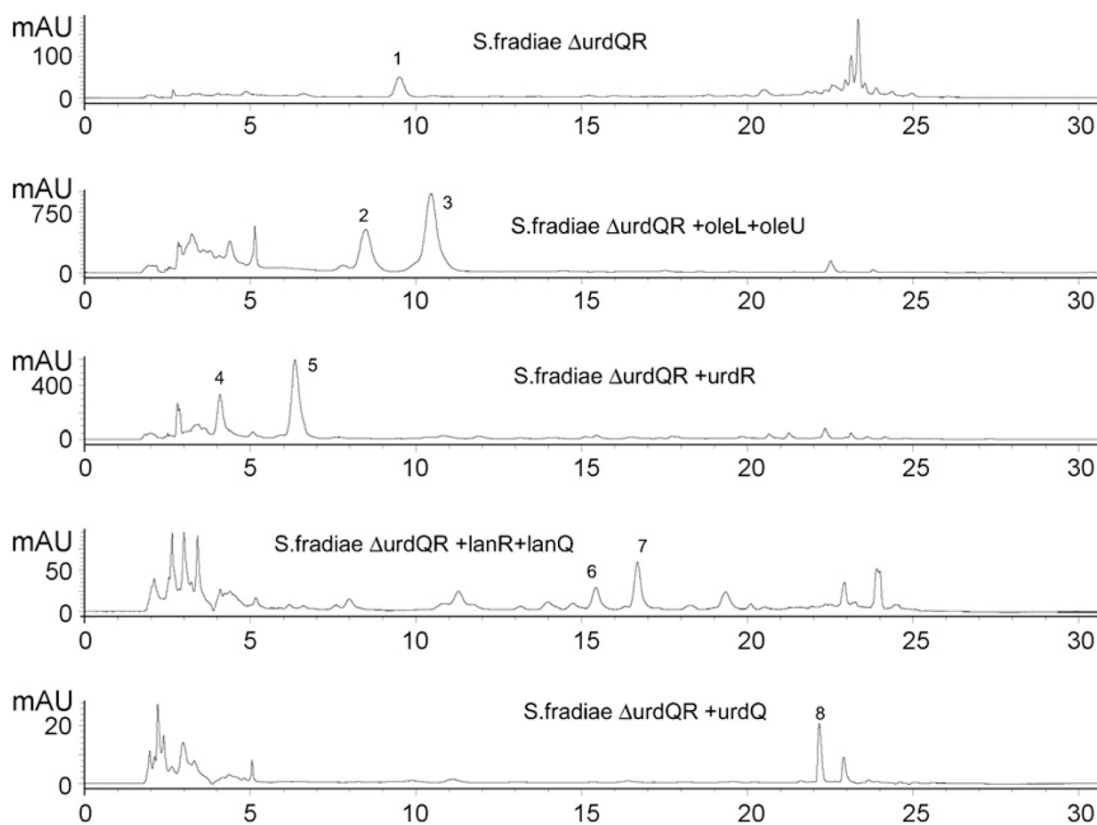
**Fig. 1.**

Biosynthetic pathway to dTDP-D-olivose, dTDP-L-rhodinose as sugar components of urdamycin A. A pathway to dTDP-L-olivose catalyzed by OleL and OleU is also shown.



**Fig. 2.** Structures of urdamycin M, urdamycinone B, urdamycin X, urdamycin L, rabelomycin, urdamycin I, urdamycin J and urdamycin B.

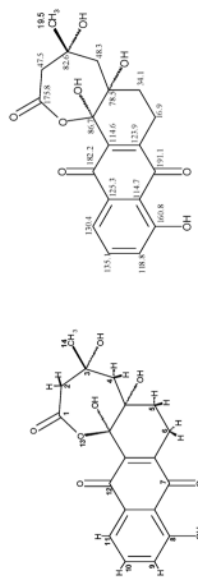




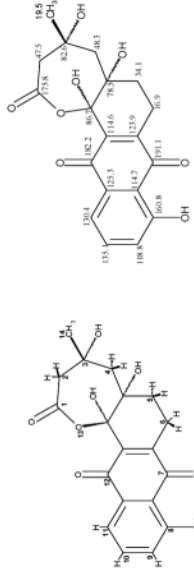
**Fig. 3.** HPLC chromatograms of crude extract from *S. fradiae*  $\Delta$ urd $Q/R$  and from *S. fradiae*  $\Delta$ urd $Q/R$  expressing different genes. First lane: detection of urdamycin X (1) in *S. fradiae*  $\Delta$ urd $Q/R$ ; second lane: detection of urdamycin I (2) and urdamycin J (3) in *S. fradiae*  $\Delta$ urd $Q/R$  expressing *oleL* and *oleU*; third lane: detection of aquayamycin (4) and urdamycinone B (5) in *S. fradiae*  $\Delta$ urd $Q/R$  expressing *urdR*; fourth lane: detection of urdamycin A (6) and urdamycin B (7) in *S. fradiae*  $\Delta$ urd $Q/R$  expressing *lanR* and *lanQ*; fifth lane: detection of urdamycin M (8) in *S. fradiae*  $\Delta$ urd $Q/R$  expressing *urdQ*. Compounds were identified by UV- and MS-spectroscopy (structures of compounds are shown in Figs. 1 and 2).

Table 1

NMR spectral data; NMR analysis of urdamycin X



Position	<sup>1</sup> H NMR (multiplicity)	<sup>13</sup> C NMR	HMBC	COSY	NOESY
1	-	175.8	-	-	-
2 <sub>ax</sub>	2.30 (d, 13.5)	47.5	1, 3, 4	2 <sub>eq</sub> , 4 <sub>ax</sub> , 4 <sub>eq</sub> , 2 <sub>ax</sub> , 4 <sub>ax</sub> , 4 <sub>eq</sub>	
2 <sub>eq</sub>	1.95 (dd, 13.5, 3.6)				
3	-	82.6	-	-	
3-OH	4.05 (br s)	-	-	-	4a-OH, 12b-OH
4 <sub>ax</sub>	2.22 (d, 10.2)	48.3	2, 3, 4a, 12b, 14	2 <sub>eq</sub> , 4 <sub>ax</sub> , 2 <sub>eq</sub> , 2 <sub>ax</sub> , 4 <sub>eq</sub>	
4 <sub>eq</sub>	1.99 (dd, 10.2, 3.6)				
4a	-	78.5	-	-	
4a-OH	4.76 (br s)	-	-	-	
5 <sub>ax</sub>	1.75 (ddd, 10.5, 10.2, 6.3)	34.1	4, 4a, 6, 12b	5 <sub>eq</sub> , 6 <sub>ax</sub> , 6 <sub>eq</sub>	
5 <sub>eq</sub>	2.16 (ddd, 10.5, 10.5, 4.8)			5 <sub>ax</sub> , 6 <sub>ax</sub> , 6 <sub>eq</sub>	
6 <sub>ax</sub>	2.84 (ddd, 10.2, 9.9, 4.8)	16.9	4a, 5, 6a, 7	5 <sub>eq</sub> , 5 <sub>ax</sub> , 6 <sub>eq</sub>	
6 <sub>eq</sub>	2.64 (ddd, 10.5, 9.9, 6.3)			5 <sub>ax</sub> , 6 <sub>ax</sub> , 5 <sub>eq</sub>	
6a	-	123.9	-	-	
7	-	191.1	-	-	
7a	-	114.7	-	-	
8	-	160.8	-	-	
8-OH	12.52 (br s)	-	-	-	
9	7.57 (dd, 7.8, 1.2)	125.3	7, 7a, 9, 10, 11a	10, 11	
10	7.63 (t, 7.8)	135.1	9, 11, 11a	9, 11	
11	7.26, (dd, 7.8, 1.2)	118.8	9, 10, 11a, 12	9, 10	
11a	-	130.4	-	-	



Position	<sup>1</sup> H NMR (multiplicity)	<sup>13</sup> C NMR	HMBC	COSY	NOESY
12	-	182.2	-	-	-
12a	-	114.6	-	-	-
12b	-	86.7	-	-	-
12b-OH	9.62 (br s)	-	-	-	-
14	1.48, (s)	19.5	2, 3, 4	-	5 <sub>BAX</sub> , 4 <sub>BAX</sub> , 2 <sub>BAX</sub> , 6 <sub>BAX</sub>

**Table 2**

Mutated genes, gene constructs and resulting metabolites

<b>Mutated gene</b>	<b>Expressed gene</b>	<b>Main product produced by the recombinant strain</b>
<i>urdR</i>	–	Urdamycin M
<i>urdQ</i>	–	Urdamycinone B
<i>urdR</i> and <i>urdQ</i>	–	Urdamycin X
<i>urdR</i> and <i>urdQ</i>	<i>urdQ</i>	Urdamycin M
<i>urdR</i> and <i>urdQ</i>	<i>urdR</i>	Urdamycinone B
<i>urdR</i> and <i>urdQ</i>	<i>lanQ</i> and <i>lanR</i>	Urdamycin A and urdamycin B
<i>urdR</i> and <i>urdQ</i>	<i>oleL</i> and <i>oleU</i>	Urdamycin I and urdamycin J
<i>urdGT1a</i> , <i>urdGT1b</i> , <i>urdGT1c</i> and <i>urdGT2</i>	–	Urdamycin I and urdamycin J
<i>urdM</i>	–	Rabelomycin and urdamycin L