Function of glycosyltransferase genes involved in urdamycin A biosynthesis

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Background: Urdamycin A, the principle product of *Streptomyces fradiae* Tü2717, is an angucycline-type antibiotic. The polyketide-derived aglycone moiety is glycosylated at two positions, but only limited information is available about glycosyltransferases involved in urdamycin biosynthesis.

Results: To determine the function of three glycosyltransferase genes in the urdamycin biosynthetic gene cluster, we have carried out gene inactivation and expression experiments. Inactivation of *urdGT1a* resulted in the predominant accumulation of urdamycin B. A mutant lacking *urdGT1b* and *urdGT1c* mainly produced compound 100-2. When *urdGT1c* was expressed in the *urdGT1b*/*urdGT1c* double mutant, urdamycin G and urdamycin A were detected. The mutant lacking all three genes mainly accumulated aquayamycin and urdamycinone B. Expression of *urdGT1c* in the triple mutant led to the formation of compound 100-1, whereas expression of *urdGT1a* resulted in the formation of compound 100-2. Co-expression of *urdGT1b* and *urdGT1c* resulted in the production of 12b-derhodinosyl-urdamycin A, and co-expression of *urdGT1a*, *urdGT1b* and *urdGT1c* resulted in the formation of urdamycin A.

Conclusions: Analysis of glycosyltransferase genes of the urdamycin biosynthetic gene cluster led to an unambiguous assignment of each glycosyltransferase to a certain biosynthetic saccharide attachment step.

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Introduction

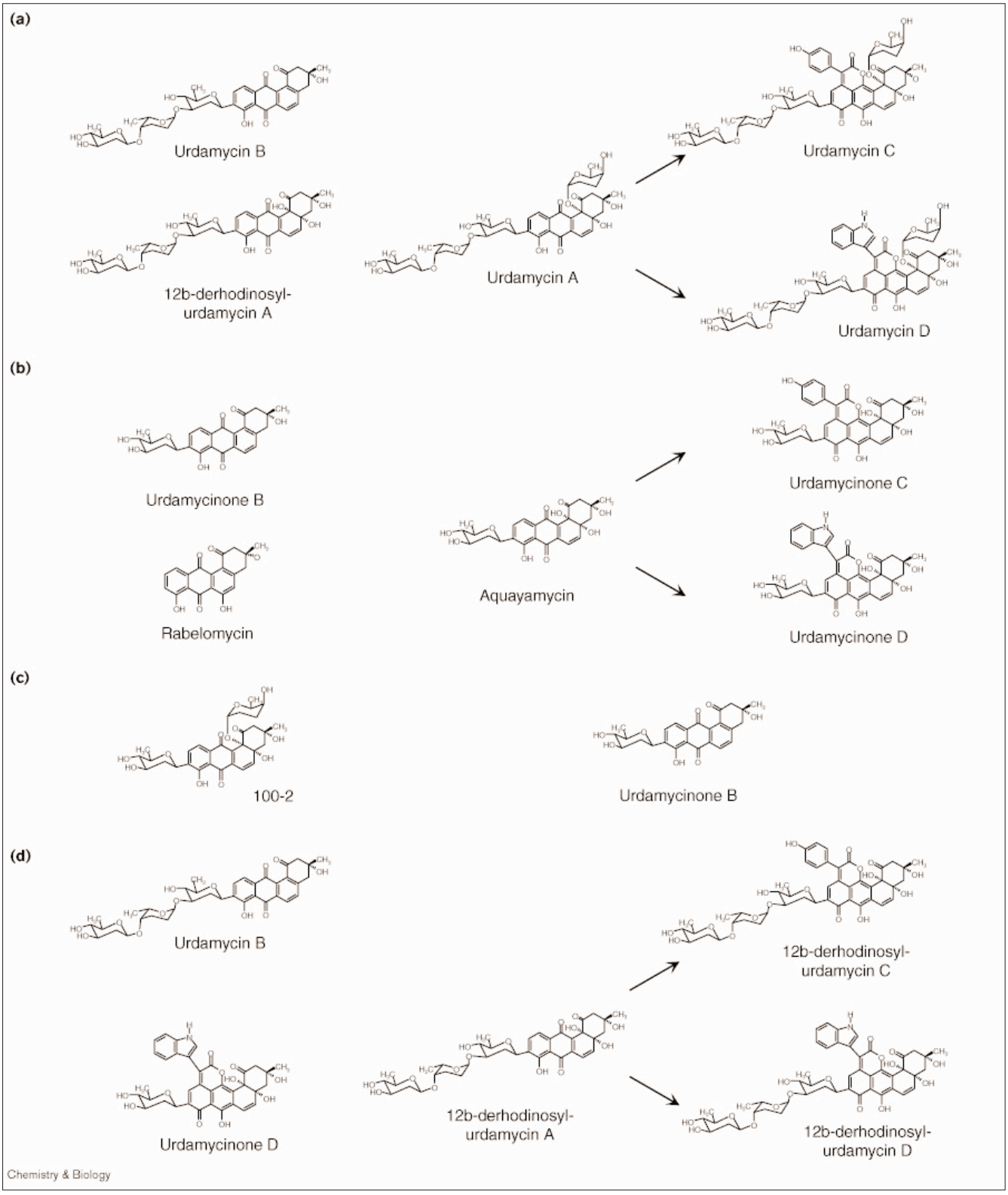
Recently, many research groups have started to manipulate polyketide biosynthetic pathways, attempting to produce hybrid or novel antibiotics. Work from a number of laboratories has shown that it is possible to combine individual polyketide synthase (PKS) domains that govern synthesis of different aromatic polyketides [1–5]. An important source of diversity in natural products is the range of sugars attached to specific positions of an aglycone core. These sugar components have almost invariably been found to be involved in the molecular recognition between the antibiotic and its cellular target [6,7]. Urdamycin A (Figure 1), an angucycline-type antibiotic and anticancer agent [8], is the principle product of *Streptomyces fradiae* Tü2717. It consists of aquayamycin, which contains a C-glycosidically linked D-olivose, and three additional O-glycosidically linked deoxysugars: two L-rhodinoses and another D-olivose. Other metabolites produced by *S. fradiae* Tü2717 are 12b-derhodinosyl-urdamycin A, urdamycin B, urdamycin C and urdamycin D [8]. Genes encoding the PKS involved in the formation of urdamycin and genes involved in tailoring reactions have been cloned and sequenced [9–13]. Inactivation of one gene, *urdGT2*, created a mutant that produced three new urdamycins (urdamycin I, urdamycin J and urdamycin K), all of which lack the C-glycosidically linked saccharide sidechain. This result indicates that UrdGT2 catalyzes the earliest glycosyltransfer step in the urdamycin biosynthesis (the C-glycosyltransfer of one dNDP-olivose moiety [12,13]). As the next step to analyse the formation and attachment of the other three deoxysugar moieties, we cloned and sequenced more of the urdamycin biosynthetic cluster. Three genes encoding glycosyltransferases were detected. Here we report their sequences and the results of gene inactivation and expression experiments, which have allowed us to assign the individual functions of each glycosyltransferase of the urdamycin cluster.

Results

Cloning and sequencing of *urdGT1a*, *urdGT1b*, *urdGT1c*, and other genes in the urdamycin biosynthetic gene cluster

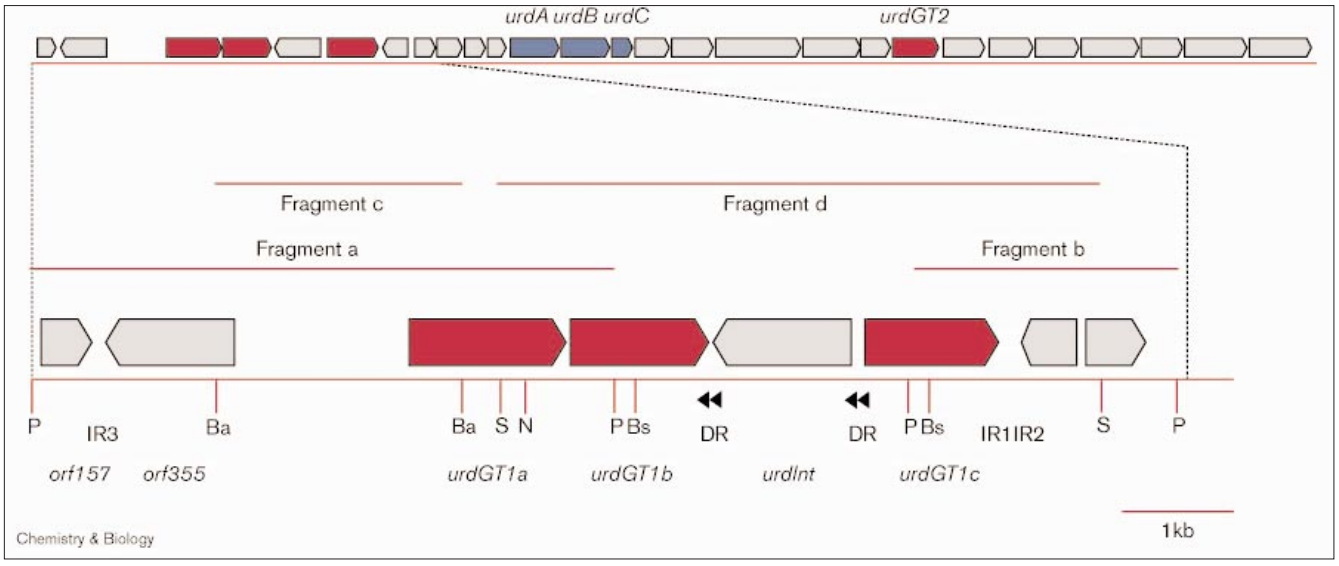
Previously, it was shown that cosmid purd8 carries the PKS genes of the urdamycin biosynthetic cluster [10]. An 8.5 kb DNA fragment of purd8 was sequenced. In total, six open reading frames (*orf157*, *orf355*, *urdGT1a*, *urdGT1b*, *urdInt* and *urdGT1c*) with characteristics of *Streptomyces* genes (overall G+C content of 67% and a high bias towards G

Figure 1



Structures of aromatic polyketides produced by **(a)** wild-type *S. fradiae* Tü2717 and by **(b)** the Ax mutant, **(c)** the 16-14 mutant and **(d)** the D331-9 mutant of *S. fradiae* Tü2717 generated in this study.

Figure 2



Genetic organization of the urdamycin biosynthetic gene cluster. Genes are indicated by arrows oriented in the direction of transcription. Genes encoding glycosyltransferases are red and PKS genes are blue.

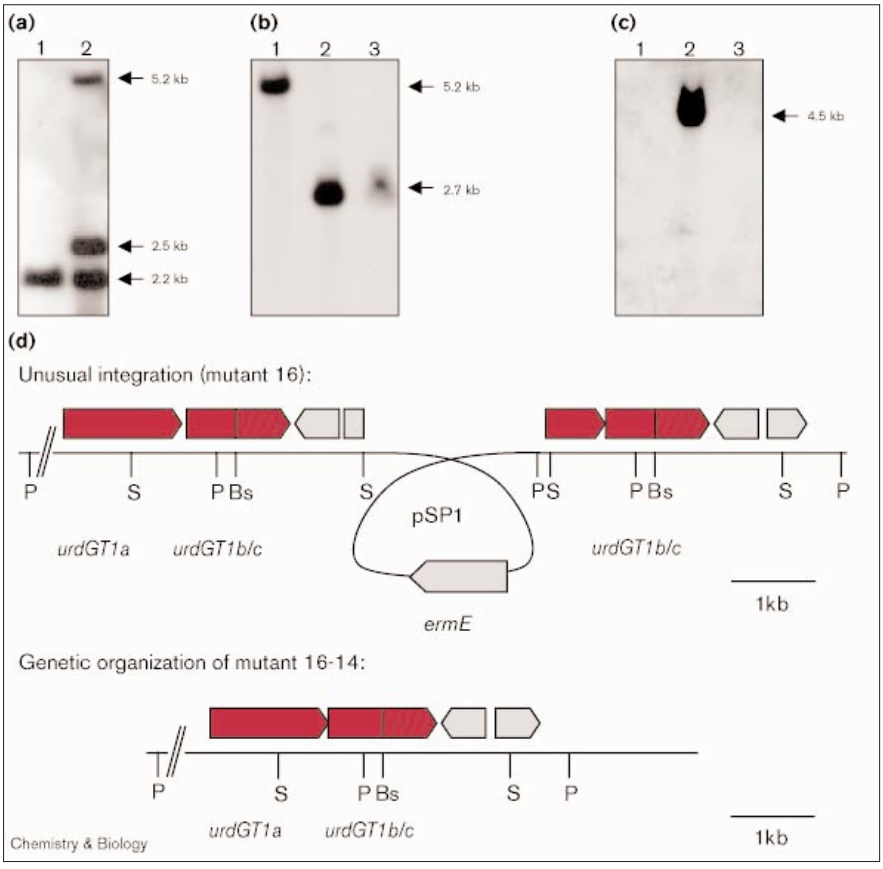
Fragments a–d have been used in constructing plasmids for gene-disruption experiments. (Ba, *Bam*HI; P, *Pst*I; S, *Sac*I; N, *Nco*I; Bs, *Bsm*I; DR, direct repeat; IR, inverted repeat.)

and C at the third codon position) and a plausible ribosome-binding site were identified (Figure 2). *Orf157* encodes a hypothetical 17.125 kDa protein of 157 amino acids (aa), which has highest homology to StgU of *Streptomyces anulatus* [14]. *Orf355* appears to encode a 355 aa protein (33.622 kDa), of which an 82 aa portion shows 36% identity to SC5C7.06 of *Streptomyces coelicolor* A3 [15], a putatively secreted protein. There is a 34 bp sequence nearly identical to the rp3 actinophage attachment site between *orf355* and *urdGT1a* [16]. The *urdGT1a* gene is 1281 base pairs, and a TTG triplet is thought to be the initiation codon. The deduced protein is 426 aa and has a molecular weight of 46.747 kDa. UrdGT1a is most similar (42% identical at the amino acid level) to LanGT4 of the landomycin biosynthetic gene cluster from *Streptomyces cyanogenus* [17], and also resembles EryCIII (38% identical at the amino acid level), a glycosyltransferase devoted to D-desosamine attachment in erythromycin biosynthesis [18,19], DnrH (38% identical at the amino acid level), which is involved in daunorubicin biosynthesis [20], and OleG2 (38% identical at the amino acid level) [21] of the oleandomycin gene cluster. The *urdGT1b* gene encodes a 388 aa protein (41.036 kDa), and its initiation codon appears to be located 17 bases downstream of *urdGT1a*. UrdGT1b is closely related to the glycosyltransferases LanGT1 and LanGT3 from *S. cyanogenus* (49% and 45% identical, respectively) [17], OleG2 (32% identical at the amino acid level) [21] and the mithramycin glycosyltransferase MtmGII (32% identical at the amino acid level) [22]. The *urdGT1c* gene encodes a 391 aa protein (40.883 kDa). UrdGT1c and UrdGT1b share strikingly high homology (89% identical at the amino acid level). The deduced amino acid sequence of *urdInt* was very similar to TnpA, a transposase of *Rhodococcus rhodochrous* [23] (69% identical at the amino acid level), and to an IS116-element transposase of *Streptomyces clavuligerus* [24] (62% identical at the amino acid level). *UrdInt* is flanked by two direct repeats (DR1 and DR2). Several obvious inverted repeat sequences were also identified. IR1 and IR2 are located in the intergenic region downstream of *urdGT1c* and IR3 is located between *orf157* and *orf355* (Figure 2). Within a 150 bp region located upstream of *urdGT1a*, a motif that strongly resembles streptomycete promoter consensus sequences was found [25].

A mutant lacking all three glycosyltransferase genes predominantly accumulates urdamycinone B and aquayamycin

Apramycin-resistant colonies were obtained after transforming *S. fradiae* with plasmid pKC-12-B2. Integration of pKC-12-B2 into the chromosome was confirmed by Southern analysis. To allow for the second recombination event, integrants were screened for apramycin sensitivity. The chromosomal mutation in mutant Ax, which was sensitive to apramycin, was analyzed by Southern hybridization. A 5.2 kb *Sac*I fragment (fragment d in Figure 2) was used to probe *Pst*I-digested chromosomal DNA. Analysis of wild-type *S. fradiae* showed the expected 5.2, 2.6 and 2.4 kb fragments after hybridization. When chromosomal DNA from mutant Ax was treated similarly, one 2.4 kb fragment was detected indicating that the 4 kb *Bam*HI–*Pst*I fragment had been removed (Figure 3a).

Figure 3



Southern hybridization of genomic *S. fradiae* DNA. **(a)** Genomic DNA of the Ax mutant (lane 1) and wild-type *S. fradiae* Tü2717 (lane 2) was digested with *Pst*I and probed with fragment d (Figure 2). **(b,c)** Genomic DNA of wild-type *S. fradiae* Tü2717 (lane 1), mutant 16 (lane 2) and mutant 16-14 (lane 3) was digested with *Sac*I and probed with fragment d (Figure 2) (b) and the *ermE* gene (c). **(d)** Genomic organization of mutant 16 obtained by integration of pBsmSP3 into *S. fradiae* Tü2717 by an unusual integration event and mutant 16–14 (generated from mutant 16 by a double cross-over event). P, *Pst*I; S, *Sac*I; Bs, *Bsm*I.

Polymerase chain reaction (PCR) amplification with chromosomal DNA of mutant Ax as template and primers 1F and 4R (which are located directly upstream of *urdGT1a* and 500 bp downstrean of *urdGT1c*, respectively) produced a 1.56 kb fragment, confirming the expected deletion. The Ax mutant did not produce urdamycin, but instead produced urdamycinone B and aquayamycin (major products) and urdamycinone C, urdamycinone D and rabelomycin (minor products; Figure 1 and Table 1).

A mutant lacking UrdGT1b and UrdGT1c but containing a hybrid enzyme UrdGT1b/c accumulates the urdamycin intermediate 100-2 and minor amounts of urdamycinone B

After *S. fradiae* protoplasts were transformed with plasmid pBsmSP3, erythromycin-resistant colonies were obtained. Chromosomal DNA of several colonies was analyzed by Southern blot using a 5.2 kb *Sac*I DNA fragment (fragment d in Figure 2) as a probe. Integration of pBsmSP3 by a single cross-over event should give hybridization signals at 5.2 kb and 2.7 kb after *Sac*I digestion (Figure 3b,c). These hybridization patterns were obtained in several cases. Chromosomal DNA of one mutant (mutant 16) gave only one hybridization signal at 2.7 kb, however. The use of a 1.7 kb *Sma*I fragment containing the erythromycin-resistance gene as a probe resulted in one hybridization signal at 4.5 kb after *Sac*I digestion. This hybridization pattern can be explained by the unusual integration event given in Figure 3d. To confirm our results, *Pst*I-restricted chromosomal DNA of *S. fradiae* and mutant 16 were subjected to Southern analysis again using fragment d (Figure 2) as a probe. Hybridization patterns (*S. fradiae*, 5.2 kb, 2.6 kb and 2.4 kb; mutant 16, 5.5 kb, 5.2 kb, 2.4 kb and 1.6 kb) were consistent with the proposed integration event (data not shown). Mutant 16-14 was generated from mutant 16 after screening for loss of resistance to erythromycin. Southern analysis of 16-14 confirmed the presence of the expected deletion: there was no hybridization signal using the 1.7 kb *Sma*I fragment (*ermE*) as a probe, and only one signal at 2.7 kb when the 5.2 kb *Sac*I DNA fragment was used as a probe. The analysis of culture supernatants of mutant 16-14 showed that compound 100-2 and minor amounts of urdamycinone B [26] (Figure 1 and Table 1) were produced instead of urdamycin A.

A mutant lacking UrdGT1a predominantly accumulates urdamycin B and 12b-derhodinosyl-urdamycin A

To determine the function of UrdGT1a, mutants were generated using plasmid pKC33 containing a frame-shift

Table 1

Mutants, glycosyltransferase gene constructs and resulting metabolites.

Mutant	Expressed glycosyltransferase(s)	Main products produced by the recombinant strain
Wild type	–	Urdamycin A, urdamycin B, 12b-derhodinosyl-urdamycin A, urdamycin C, urdamycin D
Ax	–	Aquayamycin, urdamycinone B, urdamycinone C, urdamycinone D, rabelomycin
16-14	–	100-2, urdamycinone B
D331-9	–	urdamycin B, 12b-derhodinosyl-urdamycin A, 12b-derhodinosyl-urdamycin C, 12b-derhodinosyl-urdamycin D, urdamycinone D
Ax	urdGT1a	100-2, aquayamycine, urdamycinone B
Ax	urdGT1b	Aquayamycin, urdamycinone B, urdamycinone C, urdamycinone D, rabelomycin
Ax	urdGT1c	12b-derhodinosyl-urdamycin G, aquayamycin, 12b-derhodinosyl-4A-deolivosyl-urdamycin C, 12b-derhodinosyl-4A-deolivosyl-urdamycin D, 100-1
Ax	urdGT1a + urdGT1c	Urdamycin G, 100-1
Ax	urdGT1b + urdGT1c	12b-derhodinosyl-urdamycin A, aquayamycin, urdamycin B
Ax	urdGT1a + urdGT1b	100-2, aquayamycin, urdamycinone B
Ax	urdGT1a + urdGT1b + urdGT1c	Urdamycin A, 12b-derhodinosyl-urdamycin A, 100-2
D331-9	urdGT1a	Urdamycin A, urdamycin B, urdamycin C, 12b-derhodinosyl-urdamycin C
16-14	urdGT1b	100-2, urdamycinone B
16-14	urdGT1c	Urdamycin A, urdamycin G, 100-2, urdamycin C, 12b-derhodinosyl-urdamycin C, urdamycin B, 100-1

mutation within *urdGT1a*. After transforming *S. fradiae* protoplasts with pKC33, several apramycin-resistant colonies were obtained. Two of these colonies were grown on plates containing no apramycin to select for loss of the resistance gene. Apramycin-sensitive colonies were obtained, suggesting that they were the consequence of a double crossover. Mutant D331-9 was examined further. PCR fragments obtained from mutant D331-9 could be digested by *Bam*HI but not by *Nco*I, whereas PCR fragments obtained from wild-type DNA could be digested by both enzymes. Results of the PCR analysis were confirmed by Southern blot (data not shown). Analysis of culture supernatants of D331-9 showed that urdamycin B and 12b-derhodinosyl-urdamycin A were the predominant products, instead of urdamycin. Further compounds, detected in small amounts, were 12b-derhodinosyl-urdamycin C, 12b-derhodinosyl-urdamycin D, and urdamycinone D [27,28] (Figure 1 and Table 1).

Complementation of D331-9 with *urdGT1a*

To determine clearly that the mutation event only affected *urdGT1a* and no other genes, *urdGT1a* was subcloned into pUWL201 and introduced by transformation into D331-9. In eight of ten transformants urdamycin A production was restored, indicating that only *urdGT1a* was affected by the mutation.

Expression of glycosyltransferase genes in the Ax mutant

In order to verify (*urdGT1a*) and specify (*urdGT1b* and *urdGT1c*) the function of the glycosyltransferase genes we expressed these genes singly and in various combinations. The genes *urdGT1a*, *urdGT1b* and *urdGT1c* were amplified by PCR and cloned into the vector pMunII. They were then cloned as single genes or in combination into the expression vector pUWL201 (the expression of genes in this vector is controlled by the erythromycin-resistance promoter (*ermE**; [29]; Table 1). Constructs were used to transform the Ax mutant. Urdamycinone B was converted to 100-1 and aquayamycin to 12b-derhodinosyl-urdamycin G and forward into the corresponding C- and D-analogs after the expression of *urdGT1c*, and compound 100-2 was identified when *urdGT1a* was expressed. No conversion of urdamycinone B and aquayamycin was observed after the expression of *urdGT1b* alone. Urdamycin G and minor amounts of 100-1 were produced after coexpression of *urdGT1a* and *urdGT1c;* 12b-derhodinosyl-urdamycin A and urdamycin B were produced after expression of *urdGT1b* and *urdGT1c*; and urdamycin A production was restored after coexpression of *urdGT1a*, *urdGT1b*, and *urdGT1c* (Figure 4 and Table 1).

Expression of *urdGT1c* in the 16-14 mutant, which accumulates compound 100-2

The deletion in mutant 16-14 was leading to the formation of a hybrid glycosyltransferase gene. The product of this hybrid gene contains 238 aa of UrdGT1b (amino-terminal sequence) and 153 aa of UrdGT1c (carboxy-terminal sequence). The genes *urdGT1c* and *urdGT1b* were expressed separately in mutant 16–14. (Table 1). Transformants containing *urdGT1c* accumulated urdamycin G and urdamycin A (Figure 4), indicating that the hybrid gene was still active.

Discussion

Functions of UrdGT1a, UrdGT1b and UrdGT1c

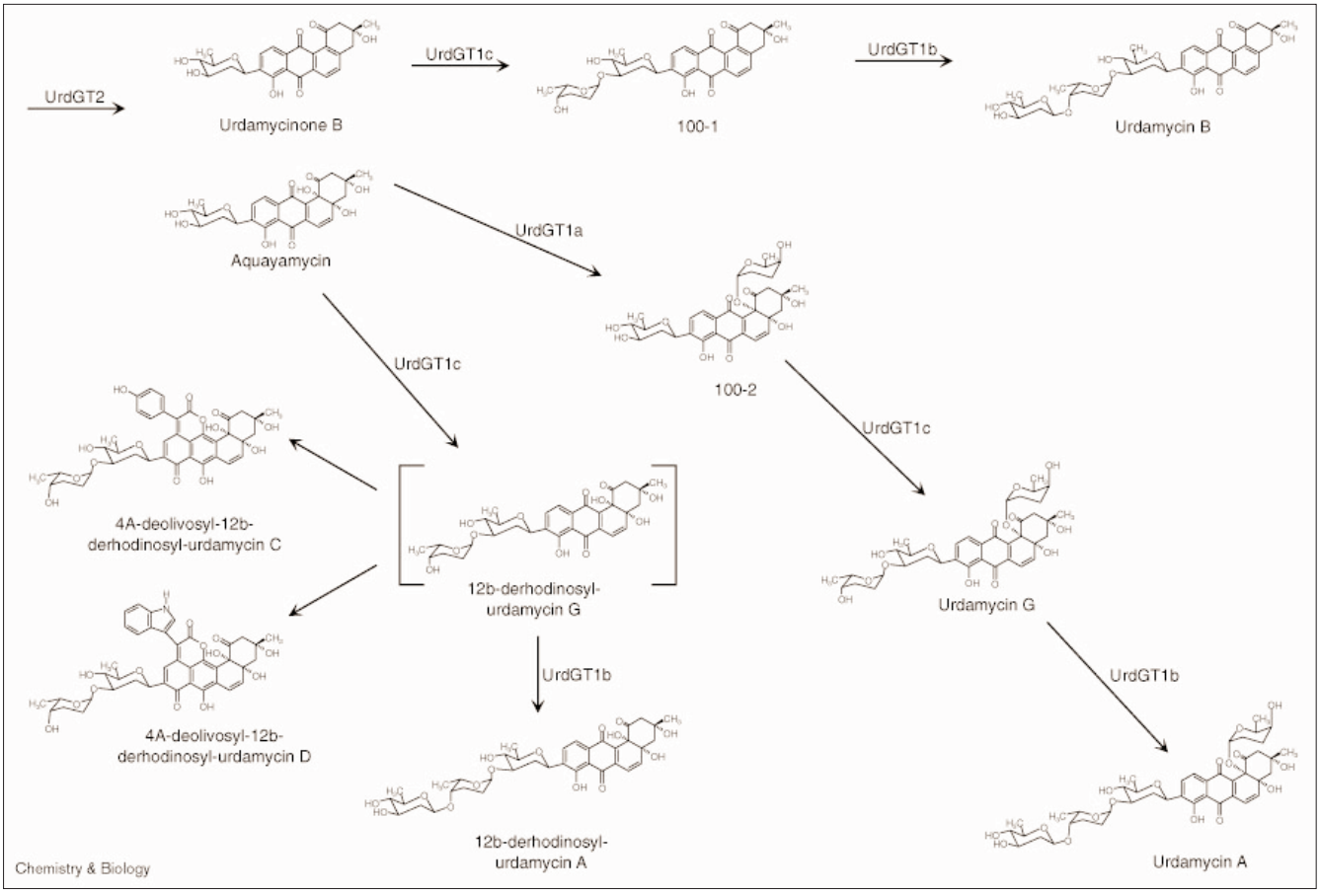
The *urd* PKS genes were originally cloned by homology with tetracenomycin PKS gene probes. Deletion of the PKS genes from the chromosome abolished urdamycin

production, providing evidence that the cloned genes were indeed those for the urdamycin PKS [9]. These genes and genes encoding a ketoreductase, a cyclase and an oxygenase were sequenced. Finding genes apparently involved in deoxysugar biosynthesis and C-glycosylation was consistent with this assignment [12]. It has been shown that urdamycin I, urdamycin J and urdamycin K were produced by a UrdGT2-mutant, and as these angucyclines do not contain the C-glycosidic moiety it was concluded that UrdGT2 must catalyze the C-glycosyl transfer of an activated D-olivose, which is the first glycosyltransfer step in urdamycin A biosynthesis [12,26].

Our work was aimed at identifying and elucidating the function of all glycosyltransferase genes involved in urdamycin biosynthesis. Analysis of an 8.5 kb DNA fragment identified three putative glycosyltransferase genes. A mutant in which all three had been deleted accumulated urdamycinone B and aquayamycin. The *urdGT1a*⁻ mutant predominantly accumulated urdamycin B, which does not contain the L-rhodinose moiety at position 12b, indicating that UrdGT1a is responsible for the transfer of this 12b-linked L-rhodinose. In the biosynthetic sequence leading to urdamycin A, this is the glycosylation of aquayamycin to 100-2, the second glycosyltransfer step [26]. Comparison of the deduced amino acid sequence of *urdGT1b* and *urdGT1c* showed that these two glycosyltransferases are extremely similar to each other—the amino acid sequences differ in only 33 amino acids (of which 23 are located in the first 200 amino-terminal residues). A mutant lacking both genes but containing a hybrid gene consisting of 715 nucleotides from *urdGT1b* and 458 nucleotides from *urdGT1c* accumulated intermediate 100-2, indicating that both UrdGT1b and UrdGT1c are involved in the formation of the trisaccharide chain.

In order to distinguish between the functions of *urdGT1b* and *urdGT1c* both genes were expressed separately in the Ax mutant. Aquayamycin was converted to 12b-derhodinosyl-urdamycin G [30] and forward into the corresponding C- and D-analogs (in which the enlarged chromophore moieties can be described as 4-hydroxyphenyl-δ-lactone and as indolyl-δ-lactone, respectively) after the expression of *urdGT1c*, and no conversion was observed after the

Figure 4



Structure of aromatic polyketides produced after expression of glycosyltransferase genes in the Ax mutant.

expression of *urdGT1b*. Coexpression of *urdGT1b* and *urdGT1c* in the same mutant resulted in the formation of 12b-derhodinosyl-urdamycin A and urdamycin B [30] (Figure 4 and Table 1). These experiments allowed the unambiguous functional assignment of the products of the *urdGT1b* and *urdGT1c* genes. UrdGT1c catalyzes the transfer of dNDP-L-rhodinose to the first sugar of the trisaccharide sidechain, that is the formation of urdamycin G from 100-2, which is the third glycosylation step of the urdamycin A biosynthesis, and UrdGT1b adds the terminal sugar (dNDP-D-olivose) to complete the biosynthesis (Figure 4). UrdGT1b and UrdGT1c have relaxed substrate specificity, because they can act on molecules with or without L-rhodinose at position 12b.

The functions of all three glycosyltransferase genes was confirmed by coexpressing *urdGT1a* and *urdGT1c*, or *urdGT1a*, *urdGT1b* and *urdGT1c* in the Ax mutant resulting in the formation of urdamycin G or urdamycin A, respectively.

We were also interested in investigating whether the product of the hybrid glycosyltransferase gene in the 16-14 mutant was enzymatically active. After expression of *urdGT1c* in mutant 16-14 formation of urdamycin G and urdamycin A was observed. The formation of urdamycin A indicates that the hybrid glycosyltransferase, which differs from UrdGT1b in six amino acids, is enzymatically active and catalyzes the same reaction as UrdGT1b. As UrdGT1b and UrdGT1c are very similar in their amino acid sequences, but transfer different dNDP-deoxysugars to different alcohol substrates, they should be useful in exploring the substrate-binding sites for the activated sugar and the corresponding alcohol substrates. Our plan in this context is to construct further hybrid glycosyltransferase genes and test their catalytic activities.

To enable coexpression of multiple genes in our system, a special cloning vector (pMunII) was created. This vector facilitates the construction of gene sets in a convenient way, taking advantage of the compatibility of *Mun*I and *Eco*RI restriction enzymes. Coexpression of several gene combinations in the Ax mutant was achieved using pMunII as the cloning vector.

Urdamycin A was produced when all three glycosyltransferase genes were expressed, indicating that *urdInt*, located between *urdGT1b* and *urdGT1c*, is not required for urdamycin biosynthesis (this gene is also absent in the Ax mutant). *UrdInt*, in contrast to IS116, is flanked by two direct repeats (TGTCA and GAAGA), indicating that the insertion of *urdInt* caused target site duplication. The deduced amino acids DxxDT (in which x denotes any amino acid) found in UrdInt strongly resemble the motif DxxDA present in IS110, IS116 and IS900, and also in reverse transcriptases of retroviral origin. Site-directed mutagenesis demonstrated that the DxxD motif is functionally important. We cannot conclude that transposition of *urdInt* was based on a RNA intermediate, however [24]. The function of genes located upstream of *urdGT1a* remains unknown as the deduced amino acid sequences of both *orf157* and *orf355* do not resemble any essential genes involved in any antibiotic biosynthesis.

Significance

The assembly of the saccharide portions of urdamycin A is controlled by four glycosyltransferases. The function of each glycosyltransferase gene was determined by gene inactivation and expression experiments. Some of the glycosyltransferases have flexibility in their substrate specificity, making them potentially valuable tools for combinatorial biosynthesis of novel polyketides. UrdGT1b and UrdGT1c transfer different NDP-sugars to different alcohol substrates. The fact that these two glycosyltransferases are highly homologous (89% identical at the amino acid level) opens up the possibility for exploring the substrate and co-substrate binding site through constructing UrdGT1b/1c hybrid glycosyltransferases.

Material and methods

Bacterial strains, plasmids, and culture conditions

S. fradiae Tü2717 [7], obtained from the Department of Microbiology, University of Tübingen, was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1 mM $CaCl_2$, pH 7.2 (HA medium) at 28°C. *E. coli* XL1 Blue MRF′ from Stratagene, La Jolla, CA, was used as host for plasmid propagation. Plasmids were passed through *E. coli* ET12567 (*dam*$^-$, *dcm*$^-$, *hsds*$^-$, Cm$^+$) [31] to generate unmethylated DNA before their use to transform *S. fradiae*. *E. coli* strains were grown under standard conditions. Plasmid pBluescript SK$^-$ (pSK$^-$) was obtained from Stratagene, plasmid pSP1 [32] was provided by S. Pelzer, Department of Microbiology, University of Tübingen, pDS101 [33] was from D. Schwarz, Department of Microbiology, University of Tübingen, and pKC1132 [34] was from Eli Lilly and Company, Indianapolis, IN. pUWL201 was a gift from U. Wehmeier and Professor Piepersberg, Department of Chemical Microbiology, University of Wuppertal.

General genetic manipulation

Routine molecular methods were performed as described previously [35]. Protoplast formation, transformation, and regeneration of protoplasts for *S. fradiae* were performed following standard procedures [36]. Digestion of DNA with restriction endonucleases, and southern hybridization were carried out according to the manufacturer's directions (Amersham Pharmacia Biotech, Freiburg, Germany; Boehringer–Mannheim, Mannheim, Germany).

DNA sequencing and computer assisted sequence analysis

DNA was sequenced by the dideoxynucleotide chain-termination method with thermo sequenase. Sequencing reactions were performed on an automated sequencer (Vistra 725) from Molecular Dynamics (Krefeld, Germany). The GenBank accession number for the sequence is AF164961. DNA sequences were analyzed using the DNASIS software package (version 2, 1995; Hitachi Software Engineering, San Bruno, CA). Blast X analysis was run with the GenBank CDC translations + PDB + SwissProt + Spupdate + PIR, release 2.0.

Generation of gene-inactivation plasmids

Generation of pKC-12-B2. A 5.2 kb *Pst*I (fragment a in Figure 2) and a 2.4 kb *Pst*I fragment (fragment b in Figure 2) were ligated into

pBluescript SK$^-$ to create p33 and p12, respectively. A 2.1 kb *Bam*HI fragment (fragment c in Figure 2) of p33 was ligated into the *Bam*HI site of the polylinker of p12 to generate p12-B2. A 4.4 kb *Hind*III–*Xba*I fragment of p12-B2 was cloned into the corresponding sites of pKC1132 generating pKC-12-B2. Fragments b and c (Figure 2) are adjacent in this plasmid.

Generation of pBsmSP3. Plasmid pS5, containing a 5.2 kb *Sac*I fragment (fragment d in Figure 2) was restriction enzyme digested with *Bsm*I to delete a 2630 bp fragment. After religation the resulting 2.6 kb *Sac*I fragment was subcloned into pDS101 to create pBsm8-11. To generate pBsmSP3 a 2.6 kb *Eco*RI–*Xba*I fragment of pBsm8-11 containing the entire SacI fragment was ligated into pSP1. Consequently, pBsmSP3 contains fragment d of Figure 2 containing a 2630 bp *Bsm*I deletion.

Generation of pKC33. Plasmid p33 (containing fragment a in Figure 2) was restricted with *Nco*I, treated with Klenow fragment and religated resulting in p33*. Plasmid p33* carries a frame-shift mutation after the amino-terminal 326 amino acids of UrdGT1a. A 5.2 kb *Pst*I fragment containing the frame-shift mutation was ligated into pKC1132 to create pKC33.

DNA amplification by PCR

Identification of mutants D331-9 and Ax. Oligonucleotide primers were purchased from Applied Biosystems, Weiterstadt. PCR amplification was performed using a GeneAmp PCR System 2400 (Applied Biosystems, Weiterstadt). The amplification of *urdGT1a* in the wild-type strain and in mutant D331-9 was performed using primer 1F: 5′-TGCGTGTAAGCTTTTCGATCATGCCGGCAA-3′ and primer 1F2: 5′-ACGCCAACGACACCAACGGCGTGAAAT-3′. For detecting mutant Ax, primers 1F (see above) and 4R: 5′-CCATTCTCATCAGC-GAATTCCAGGATCTGG-3′ were used. PCR conditions were similar to those described previously [37].

Generation of gene expression constructs. A *Mun*I restriction site 5′ to the ribosome binding site and a *Bgl*II restriction site 3′ to the termination codon were introduced into the *S. fradiae* glycosyltransferase genes (*urdGT1a*, *urdGT1b*, and *urdGT1c*), using PCR. Templates for the PCR were p33 (for *urdGT1a*) and pS5 (for *urdGT1b* and *urdGT1c*). The synthetic oligonucleotides used for the amplification of *urdGT1a* were primer urdGT1aF, 5′-CTCCAGCAATTGGAGCAGTC-CATATGCGTG-3′ and primer urdGT1aR, 5′-ACACGCATAGATC-TACTCCCTGCGTCA-3′; for the amplification of *urdGT1b* primer urdGT1bF, 5′-CCGCCGCAATTGCAGGGAGTAGTCATATGCG-3′ and primer urdGT1bR, 5′-CTCCAACCGCCGTAGATCTGACATCA-3′; and for the amplification of *urdGT1c* primer urdGT1cF, 5′-CGCC-CAACAATTGACGGAGACCATATGCGA-3′ and primer urdGT1cR, 5′-CAGACGTACGAGATCTAGGGCCGGCTA-3′. PCR fragments were cloned into the *Mun*I and *Bgl*II site of pMunII to create pMun2urdGT1a, pMun2urdGT1b, and pMun2urdGT1c, respectively. Plasmid pMunII had been derived from pBluescript SK$^-$ by removing the *Sma*I, *Pst*I, *Eco*RI and *Eco*RV restriction enzyme sites between the *Bam*HI and *Hind*III site of the polylinker and introducing *Eco*RI, *Bgl*II, *Nde*I, *Nsi*I, *Nco*I and *Mun*I sites instead. For expression, fragments were cloned into the *Hind*III and *Xba*I site of pUWL201 to create the corresponding plasmids pUWLurdGT1a, pUWLurdGT1b and pUWLurdGT1c. For the coexpression of *urdGT1b* and *urdGT1c* pMun2urdGT1c was restricted with *Mun*I and *Xba*I and the fragment containing *urdGT1c* was ligated into pMun2urdGT1b restricted with *Eco*RI and *Xba*I generating pMun2urdGT1b-1c. After *Hind*III and *Xba*I restriction both genes were cloned into pUWL201 to create pUWL1b-1c. The constructions of pUWL1a-1b, pUWL1a-1c, and pUWL1a-1b-1c were performed in a similar way.

Production and purification of urdamycin compounds

All described mutants were grown on agar dishes as well as in liquid medium to produce their metabolites. The medium for the agar dishes consisted of 20 g/l soybean meal and 20 g/l glucose dissolved in 1 l of tap water. The pH was adjusted to 7.00. The mutants were grown for 4 days at 30°C. Medium for liquid cultures (NL111v) consisted of 0.2 g/l Lab-Lemco powder (Oxoid, Hampshire, England), 100 g/l malt extract and 10 g/l $Ca(CO_3)_2$. To isolate the urdamycins, agar medium was chopped and extracted 5× with an ethyl acetate:acetone (1:1) mixture, and liquid medium was extracted 3× with ethyl acetate. The organic layer was concentrated to dryness. The obtained crude extract was separated by HPLC (RP-18 silica gel column, 250 × 10 mm, flow rate 6 ml/min, using an H_2O/acetonitrile/MeOH gradient: 3 min 70:25:5, within 8 min to 60:33.5:6.5, further 10 min at this relation). Final purification was performed by Sephadex LH-20 chromatography (column, 100 × 2.5 cm, MeOH).

Chemical analysis of urdamycin intermediates

The ^{1}H nuclear magnetic resonance (NMR) data of the novel urdamycin C and D derivatives, recorded at 400 MHz in d_6-acetone, are as follows: 12b-derhodinosylurdamycin C ^{1}H NMR (400 MHz, d_6-acetone): d 1.00–2.2 m (2′-H_a), 1.10–1.50 m (2B-H), 1.13 d (J = 6 Hz, 3H, 5A-CH_3), 1.21 s (3H, 3-CH_3), 1.21 d (J = 6 Hz, 5B-CH_3), 1.24 d (J = 6 Hz, 3H, 5′-CH_3), 1.25–1.7 m (2A-H, 3A-H), 1.9–2.2 m (2A-H, 3A-H and 2B-H, partly obscured by solvent), 2.0 (partly obscured by solvent, 4-H_e), 2.1 (partly obscured by solvent, 4-H_a), 2.2 ddd (J = 13, 5 and 2 Hz, 1H, 2′-H_e), 2.55 dd (J = 13 and 2 Hz, 1H, 2-H_e), 2.8 (partly obscured by water, 4′-H), 2.9 (partly obscured by water, 2-H_a), 3.08 dq (partly obscured by water, 1H, 5B-H), 3.10 dd (partly obscured by water, 1H, 4B-H), 3.45 dq (J = 9 and 6 Hz, 1H, 5′-H), 3.52 m (1H, 3B-H), 3.55 s (broad, 1H, 4A-H), 3.76 ddd (J = 12, 8.5 and 5 Hz, 1H, 3′-H), 4.19 dq (J = 6 and 2 Hz, 1H, 5A-H), 4.58 d (J = 11 and 2 Hz, 1H, 1B-H), 4.75 dd (J = 10 and 2 Hz, 1H, 1′-H), 4.98 s (broad, 1H, 1A-H), 6.15 d (J = 10 Hz, 1H, 5-H), 7.05 d (J = 10 Hz, 1H, 6-H), 7.06 d (J = 8 Hz, 2H, 6′-H and 8′-H), 7.4 d (J = 8 Hz, 2H, 5′-H and 9′-H), 7.88 s (1H, 10-H) ppm.

12b-derhodinosyl-4A-deolivosylurdamycin C ^{1}H NMR (400 MHz, d_6-acetone): d 1.00–2.2 m (2′-H_a), 1.15 d (J = 6 Hz, 3H, 5A-CH_3), 1.20 s (3H, 3-CH_3), 1.24 d (J = 6 Hz, 3H, 5′-CH_3), 1.25–1.7 m (2A-H, 3A-H), 1.9–2.2 m (2A-H, 3A-H, partly obscured by solvent), 2.00 (partly obscured by solvent, 4-H_e), 2.08 (partly obscured by solvent, 4-H_a), 2.2 ddd (J = 13, 5 and 1.5 Hz, 1H, 2′-H_e), 2.56 dd (J = 13 and 2 Hz, 1H, 2-H_e), 2.8 (partly obscured by water, 4′-H), 2.9 (partly obscured by water, 2-H_a), 3.46 dq (J = 9 and 6 Hz, 1H, 5′-H), 3.55 s (broad, 1H, 4A-H), 3.79 ddd (J = 12, 9 and 5 Hz, 1H, 3′-H), 4.18 dq (J = 6 and 2 Hz, 1H, 5A-H), 4.75 dd (J = 10 and 1.5 Hz, 1H, 1′-H), 4.98 s (broad, 1H, 1A-H), 6.15 d (J = 10 Hz, 1H, 5-H), 7.05 d (J = 10 Hz, 1H, 6-H), 7.06 d (J = 8 Hz, 2H, 6′-H and 8′-H), 7.4 d (J = 8 Hz, 2H, 5′-H and 9′-H), 7.88 s (1H, 10-H) ppm.

12b-derhodinosylurdamycin D ^{1}H NMR (400 MHz, d_6-acetone): d 1.03 d (J = 6 Hz, 3H, 5A-CH_3), 1.10 d (J = 6 Hz, 5B-CH_3), 1.20–1.7 m (2′-H_a), 1.20–1.70 m (2B-H), 1.20 s (3H, 3-CH_3), 1.21 d (J = 6 Hz, 3H, 5′-CH_3), 1.25–1.5 m (2A-H, 3A-H), 1.9–2.2 m (2A-H, 3A-H and 2B-H, partly obscured by solvent), 1.9 (partly obscured by solvent, 4-H_a), 2.2 (partly obscured by solvent, 4-H_e), 2.2 m (1H, 2′-H_e), 2.55 dd (J = 13 and 2 Hz, 1H, 2-H_e), 2.8 (partly obscured by water, 4′-H), 2.8 (partly obscured by water, 2-H_a), 2.8 dd (partly obscured by water, 4B-H), 3.19 dq (partly obscured by water, 1H, 5B-H), 3.38 dq (J = 9 and 6 Hz, 1H, 5′-H), 3.49 m (1H, 3B-H), 3.52 s (broad, 1H, 4A-H), 3.75 ddd (J = 12, 9 and 5 Hz, 1H, 3′-H), 4.18 dq (J = 6 and 2 Hz, 1H, 5A-H), 4.58 d (J = 11 and 2 Hz, 1H, 1B-H), 4.71 dd (J = 10 and 2 Hz, 1H, 1′-H), 4.95 s (broad, 1H, 1A-H), 6.08 d (J = 10 Hz, 1H, 5-H), 7.00 d (J = 10 Hz, 1H, 6-H), 7.16 ddd (J = 8, 8 and 2 Hz, 1H, 9′-H), 7.21 ddd (J = 8, 8 and 2 Hz, 1H, 8′-H), 7.59 dd (J = 8 and 2 Hz, 2H, 7′-H and 10′-H), 8.01 s (1H, 5′-H), 8.18 s (1H, 10-H) ppm.

12b-derhodinosyl-4A-deolivosylurdamycin D ^{1}H NMR (400 MHz, d_6-acetone): d 1.05 d (J = 6 Hz, 3H, 5A-CH_3), 1.20–1.7 m (2′-H_a), 1.20 s (3H, 3-CH_3), 1.20–1.6 m (2A-H, 3A-H), 1.21 d (J = 6 Hz, 3H, 5′-CH_3), 1.9–2.2 m (2A-H, 3A-H, partly obscured by solvent), 2.04 (partly obscured by solvent, 4-H_a), 2.1 (partly obscured by solvent, 4-

H_e), 2.23 m (1H, 2′-H_e), 2.56 dd (J = 13 and 2 Hz, 1H, 2-H_e), 2.8 (partly obscured by water, 4′-H), 2.8 (partly obscured by water, 2-H_a), 3.40 dq (J = 9 and 6 Hz, 1H, 5′-H), 3.52 s (broad, 1H, 4A-H), 3.78 ddd (J = 12, 9 and 5 Hz, 1H, 3′-H), 4.16 dq (J = 6 and 2 Hz, 1H, 5A-H), 4.75 dd (J = 10 and 2 Hz, 1H, 1′-H), 4.98 s (broad, 1H, 1A-H), 6.10 d (J = 10 Hz, 1H, 5-H), 6.98 d (J = 10 Hz, 1H, 6-H), 7.13 ddd (J = 8, 8 and 2 Hz, 1H, 9′-H), 7.22 ddd (J = 8, 8 and 2 Hz, 1H, 8′-H), 7.59 dd (J = 8 and 2 Hz, 2H, 7′-H and 10′-H), 8.01 s (1H, 5′-H), 8.19 s (1H, 10-H) ppm.

The APCI-MS obtained for 12b-derhodinosyl-urdamycin C (*m/z* 862.5, M^+, 100%) and the ESI-MS obtained for 12b-derhodinosyl-urdamycin D (*m/z* 908.3, $M + Na^+$, 100%) confirmed their molecular formulae. The accumulation of the new derivative 12b-derhodinosyl-urdamycin G was only visible in HPLC-UV/Vis, and HPLC-MS, because, during isolation, this compound converted into 12b-derhodinosyl-4A-deolivosyl-urdamycin C and D. The analogous nonenzymatic conversion of urdamycin A into urdamycins C and D has been described previously [28].

The known urdamycins, their derivatives and aglycones mentioned here (i.e. urdamycins A, B, C, D, and G, 12b-derhodinosyl-urdamycin A, urdamycinone B, C and D, 100-1, 100-2, aquayamycin and rabelomycin) were identical in TLC, HPLC-UV/Vis, and HPLC-MS with those described earlier [8,13,26,27,38–41].

HPLC-MS-ELSD analysis was performed by splitting the flow between the MS (220 µl) and the ELSD (780 µl) using the following equipment: HPLC components, 2 × pumps 'Series 200 micro LC Pump'; High-Pressure Micro-Mixer (Perkin-Elmer); Autosampler 'Series 200 Autosampler' (Perkin-Elmer); solvent degasser, ERC-3215; column oven, Jetstream plus (ERC), T = 30°C; chromatographic material, YMC-Pack Pro C-18, 150 x 4.6 mm, 5 µm; mass spectrometer, API 165, Perkin-Elmer-Sciex (single-quadropole); nitrogen generator, Model 75-77, Whatman; software, Masschrom 1.1.1 (PE-Sciex); Evaporative Light-Scattering Detector, Sedex 55 ERC. A linear gradient from 95% A (5 mM ammonium formiat + 0.1% HCOOH) and 5% B (MeOH/MeCN = 50/50 + 5 mM ammonium formiat + 0.1% HCOOH) to 60% A and 40% B in 15 min at 1 ml/min was used. Mass detection: positive and negative spectra were recorded by fast polarity switching during the LC-run (150–1500 amu, step size 0.3 amu).

HPLC-UV was performed on a Hewlett Packard 1090 liquid chromatograph using a Hewlett Packard C-18 column (2.1 × 200 mm) at a flow rate of 0.3 ml/min with UV detection at 200–600 nm. Elution was accomplished with a linear gradient of 5% CH_3CN-95% buffer to 58% CH_3CN-42% buffer over 45 min. The buffer used was 0.1% H_3PO_4 in water.

Accession number

The sequence reported here has been deposited in the GenBank database under the accession number AF164961.

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