The NDP-sugar co-substrate concentration and the enzyme expression level influence the substrate specificity of glycosyltransferases: cloning and characterization of deoxysugar biosynthetic genes of the urdamycin biosynthetic gene cluster

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Background: *Streptomyces fradiae* is the principal producer of urdamycin A. The antibiotic consists of a polyketide-derived aglycone, which is glycosylated with four sugar components, 2× D-olivose (first and last sugar of a C-glycosidically bound trisaccharide chain at the 9-position), and 2× L-rhodinose (in the middle of the trisaccharide chain and at the 12b-position). Limited information is available about both the biosynthesis of D-olivose and L-rhodinose and the influence of the concentration of both sugars on urdamycin biosynthesis.

Results: To further investigate urdamycin biosynthesis, a 5.4 kb section of the urdamycin biosynthetic gene cluster was sequenced. Five new open reading frames (ORFs) (*urdZ3*, *urdQ*, *urdR*, *urdS*, *urdT*) could be identified each one showing significant homology to deoxysugar biosynthetic genes. We inactivated four of these newly allocated ORFs (*urdZ3*, *urdQ*, *urdR*, *urdS*) as well as *urdZ1*, a previously found putative deoxysugar biosynthetic gene. Inactivation of *urdZ3*, *urdQ* and *urdZ1* prevented the mutant strains from producing L-rhodinose resulting in the accumulation of mainly urdamycinone B. Inactivation of *urdR* led to the formation of the novel urdamycin M, which carries a C-glycosidically attached D-rhodinose at the 9-position. The novel urdamycins N and O were detected after overexpression of *urdGT1c* in two different chromosomal *urdGT1c* deletion mutants. The mutants lacking *urdS* and *urdQ* accumulated various known diketopiperazines.

Conclusions: Analysis of deoxysugar biosynthetic genes of the urdamycin biosynthetic gene cluster revealed a widely common biosynthetic pathway leading to D-olivose and L-rhodinose. Several enzymes responsible for specific steps of this pathway could be assigned. The pathway had to be modified compared to earlier suggestions. Two glycosyltransferases normally involved in the C-glycosyltransfer of D-olivose at the 9-position (UrdGT2) and in conversion of 100-2 to urdamycin G (UrdGT1c) show relaxed substrate specificity for their activated deoxysugar co-substrate and their alcohol substrate, respectively. They can transfer activated D-rhodinose (instead of D-olivose) to the 9-position, and attach L-rhodinose to the 4A-position normally occupied by a D-olivose unit, respectively.

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Keywords: Deoxysugar biosynthesis; Glycosyltransferase; D-Olivose; L-Rhodinose; Urdamycin

Received: **26 May 2000**
Revisions requested: **19 July 2000**
Revisions received: **4 August 2000**
Accepted: **31 August 2000**

Published: **19 September 2000**

Chemistry & Biology 2000, **7**:821–831

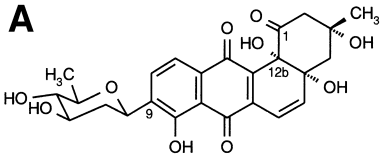
1074-5521/00/$ – see front matter

PII: S1074-5521(00)00029-6

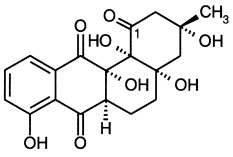
Introduction

Urdamycin A (Figure 1A), produced by *Streptomyces fradiae* Tü2717, is an antibiotic and anticancer agent [1]. It consists of an angucyclic polyketide core and four deoxyhexoses: two L-rhodinoses and two D-olivoses. Urdamycin polyketide formation as well as tailoring reactions and glycosyl-

A



Aquayamycin



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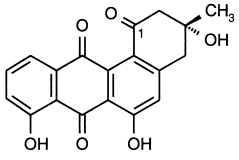

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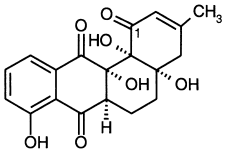
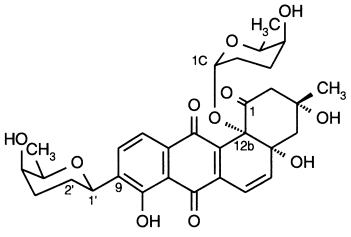
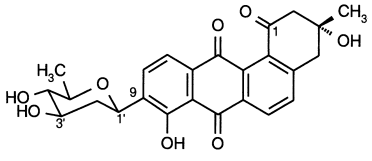


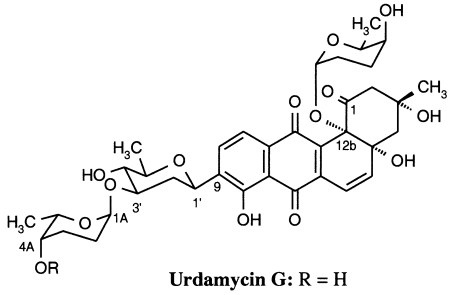
Urdamycin J



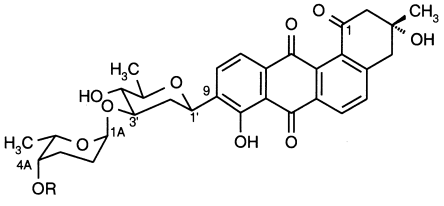
Urdamycin M



Urdamycinone B

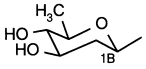


Urdamycin G: R = H



100-1: R = H

Urdamycin A: R =



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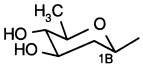
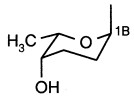
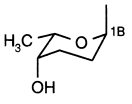


Urdamycin O: R =



Urdamycin N: R =



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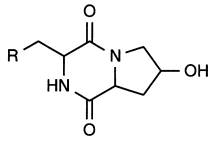

cyclo-(alanyl-4-hydroxyprolyl): R = H

cyclo-(phenylalanyl-4-hydroxyprolyl): R =

cyclo-(leucyl-4-hydroxyprolyl): R =

transfer are genetically well understood. A type II polyketide synthase (PKS) is responsible for decaketide synthesis. After cyclization and reduction, a C-glycosidic D-olivose transfer at position 9 of the polyketide core is performed by the glycosyltransferase UrdGT2 [2,3]. The later glycosylation steps include a L-rhodinose transfer to position C-12b, and an attachment of L-rhodinose and D-olivose to complete the trisaccharide side chain at C-9 [4]. As no urdamycin derivative with variation in the order of the sugar moieties has ever been found, a high specificity of the glycosyltransferases towards the sugar substrates has been concluded [4]. The biosynthetic pathway to NDP-D-olivose and NDP-L-rhodinose in *S. fradiae* has never been explored in detail. Early genetic work on the urdamycin biosynthetic gene cluster revealed the presence of a NDP-glucose synthase gene (*urdG*) and a NDP-glucose-4,6-dehydratase gene (*urdH*). Therefore we expected the early steps of deoxysugar formation to be essentially the same as found for D-desosamine and L-mycarose production in the erythromycin producer [5–7], D-olivose, D-oliose and D-mycarose formation in the mithramycin producer [8] or D-daunosamine synthesis in the daunomycin producer [9], which is the conversion of glucose-1-phosphate to NDP-glucose and further conversion to NDP-4-keto-6-deoxy-D-glucose.

4-Keto-6-deoxy-D-glucose has been discussed to be the key intermediate in the biosynthesis of many different deoxysugars [10]. In strains producing different deoxysugars, independent pathways from 4-keto-6-deoxyglucose to each sugar have been found [11]. In this work we cloned, sequenced and characterized further deoxysugar biosynthetic genes of the urdamycin cluster, and investigated whether the substrate specificity of glycosyltransferases can be modified.

Results

Cloning and sequencing of a 5.4 kb section of the urdamycin cluster

A 5.4 kb section of the urdamycin biosynthetic gene cluster located downstream of *urdH* was sequenced (Figure 2). Computer-based sequence analysis showed that it harbors five new open reading frames (ORFs) (*urdZ3*, *urdQ*, *urdR*, *urdS*, and partially *urdT*). Throughout this region we found an average G/C content of 68.7% which is typical for *Streptomyces* DNA, and third codon positions were significantly biased towards G and C. Upstream of all genes putative ribosome binding sites similar to the consensus sequence published by Strohl [12] were detected.

Database comparison of UrdZ3, a 346 amino acid (aa) protein, revealed close similarity to proteins of different origin which act as (or presumably are) NDP-hexose 4-ketoreductases, highest homology is found to LanZ3 (accession number: AF080235), a NDP-hexose 4-ketoreductase homolog from the landomycin producer *Streptomyces cyanogenus* S 136 (42% identical aa) [13]. The deduced protein of *urdQ* has 434 aa (molecular weight 48.081 kDa) and shows highest similarity to LanQ (AF080235), a NDP-hexose 3,4-dehydratase homolog from *S. cyanogenus* S 136 (85% identical aa) [13], to GraOrf23 (AJ011500), a putative dTDP-4-keto-6-deoxyglucose 3-dehydratase of the granaticin producer *S. violaceoruber* Tü22 (70% identical aa) [14], and to a NDP-hexose 3-dehydratase from *Amycolatopsis mediterranei* [15] (71% identical aa). *urdR* encodes a 247 aa protein with 26.837 kDa molecular weight. It most resembles LanR, a NDP-hexose 4-ketoreductase homolog from *S. cyanogenus* (69% identical aa) [13], and an unnamed protein of *Actinobacillus actinomycetemcomitans* (27% identical aa) [16]. UrdR, like the other proteins, contains the NAD^+ cofactor binding motif YxxxKxxxD/E which is conserved in numerous putative 4-ketoreductases. The *urdS* reading frame should yield a 469 aa protein of 52.112 kDa molecular weight. It closely resembles proteins identified as NDP-hexose 2,3-dehydratases in various species. Highest homology (75% identical aa) is found to LanS [13], a NDP-hexose 2,3-dehydratase homolog of *S. cyanogenus*, to a putative 2,3-dehydratase (ORF23) of the chloroeremomycin producer *Amycolatopsis orientalis* (68% identical aa) [17] and to AveB-VI, a TDP-4-keto-6-deoxy-L-hexose 2,3-dehydratase of *S. avermitilis* (64% identical aa) [18]. Downstream of *urdS* we found an incomplete ORF named *urdT*. Homology of the deduced 269 amino acids gene product suggests that *urdT* encodes an oxidoreductase, since throughout the cloned and sequenced part significant similarity is found to the product of *lanT*, an oxidoreductase homolog of *S. cyanogenus* (58% identical aa) [13], to an oxidoreductase from *A. mediterranei* (53% identical aa) [15], and to GraOrf26 of *S. violaceoruber* [14].

Inactivation of deoxysugar biosynthetic genes

For inactivation of *urdQ*, *urdR*, *urdS*, *urdZ1* and *urdZ3*, plasmids were constructed allowing the replacement of the wild-type (wt) gene by a mutated allele.

A mutant lacking *urdS* does not accumulate any urdamycin derivative but accumulates cyclo-(alanyl-4-hydroxyprolyl)

To determine the function of *urdS*, mutants were gener-

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Figure 1. (A) Selected structures of compounds produced by *S. fradiae* Tü2717 and mutants generated during this study. **(B)** Structures of diketopiperazines produced by mutants generated during this study.

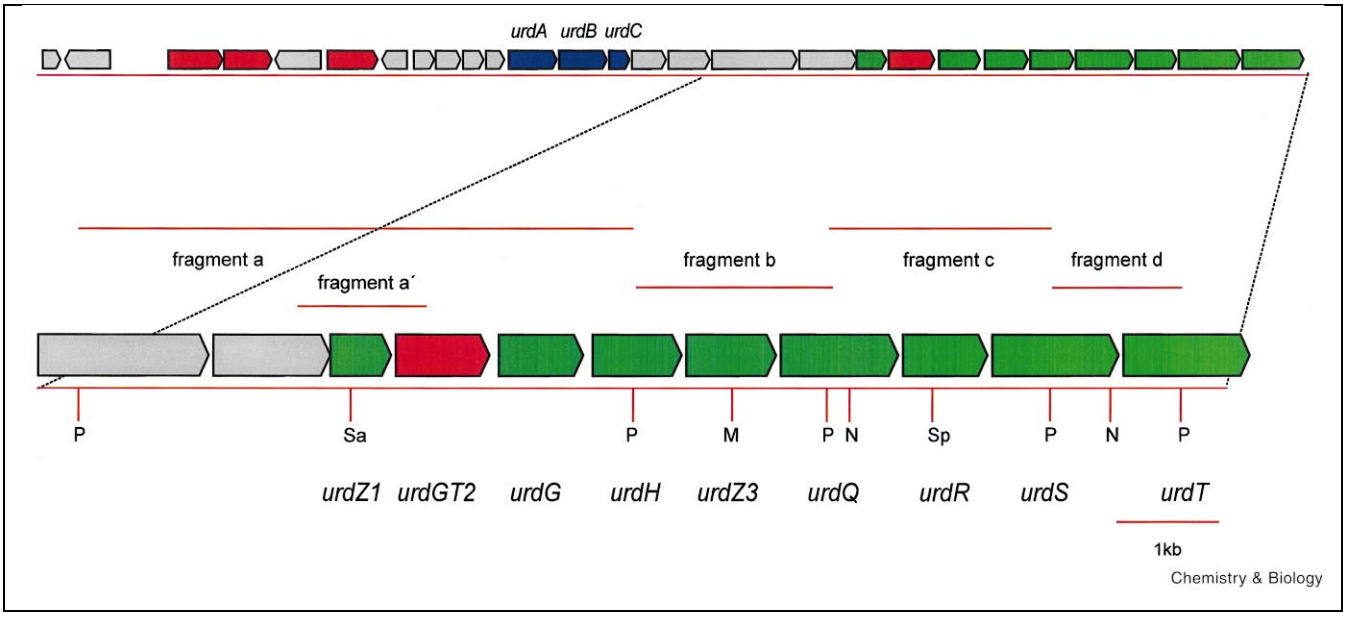

Figure 2. Genetic organization of the urdamycin biosynthetic gene cluster. Genes are indicated by arrows oriented in the direction of transcription. Genes encoding glycosyltransferases are red, PKS genes are blue. Genes encoding enzymes involved in deoxysugar biosynthesis are green. Fragments a–d have been used in constructing plasmids for gene disruption and gene expression experiments. (P, *Pst*I; Sa, *Sal*I; M, *Mro*I; N, *Nco*I; Sp, *Spl*I).

ated using plasmid pKurdSpm which contains a frameshift mutation within *urdS* due to the alteration of a *Nco*I restriction site. After *S. fradiae* protoplasts were transformed with plasmid pKurdSpm, apramycin-resistant colonies were obtained. The transformation efficiency was about 100 colonies per µg DNA. Numerous colonies were grown on plates containing no apramycin to select for loss of resistance. Eight apramycin-sensitive colonies were obtained suggesting that they were the consequence of a double cross-over. Mutant urdSpm was further examined. PCR fragments, obtained from urdSpm using primers S-1 and S-2, could be digested by *Pst*I and *Dra*III, but not by *Nco*I, whereas PCR fragments obtained from the wt strain could be digested by all three enzymes. Analysis of the culture supernatant of urdSpm showed that it did not accumulate any urdamycin derivative. A compound accumulated instead was determined to be cyclo-(alanyl-4-hydroxyprolyl) (Figure 1B).

Mutants lacking either *urdQ*, *urdZ1*, or *urdZ3* predominantly accumulate urdamycinone B

To inactivate *urdQ*, *urdZ1*, and *urdZ3*, gene inactivation plasmids pKurdQifD and pKurdQpm (inactivation of *urdQ*), pKC-urdZ1d (inactivation of *urdZ1*) and pKC-urdZ3d (inactivation of *urdZ3*) were constructed. *S. fradiae* Tü2717 protoplasts were transformed with these plasmids. About 80 apramycin-resistant colonies per µg DNA for each *urdQ* inactivation construct and approximately 150 per µg DNA for pKC-urdZ1d and pKC-urdZ3d were obtained. Some of each were further cultivated screening for loss of apramycin resistance. Mutants urdQifD, urdQpm (both lacking *urdQ*), BF-3-1 (lacking *urdZ1*), and Z3-148 (lacking *urdZ3*) were chosen for further investigation. The size of PCR fragments obtained from urdQifD with primers Q-1 and Q-2 was 0.9 kb while the wt strain gave a PCR fragment of the expected lower mobility (1.23 kb). To confirm the presence of the deletion within *urdQ* Southern blot analysis was performed as follows. When *Pst*I-digested chromosomal DNA prepared from urdQifD was probed with a 2.3 kb *Pst*I fragment (fragment c in Figure 2) a 1.97 kb fragment was detected while the expected 2.3 kb fragment could be identified in the *S. fradiae* wt strain. There was no hybridization signal when an internal 0.7 kb *Sac*I fragment of pKC1132 was used. These results confirmed the presence of the expected deletion. PCR fragments obtained from mutants urdQpm (primers Q-1 and Q-2), BF-3-1 (primers Z1-1 and Z1-2) and Z3-148 (primers Z3-1 and Z3-2) were analyzed by restriction analysis. Fragments could not be restricted by *Nco*I (urdQpm), *Sal*I (BF-3-1) and *Mro*I (Z3-148) which were the endonucleases employed for frameshifting. In contrast, PCR products amplified with wt DNA as template were digestible in each case. Secondary metabolites of all mutants were predominantly urdamycinone B (Figure 1A). Aquayamycin (Figure 1A) was also detectable in mutant Z3-148, and mutant urdQpm also accumulated cyclo-(leucyl-4-hydroxyprolyl) and cyclo-(phenylalanyl-4-hydroxyprolyl) (Figure 1B).

A mutant lacking *urdR* accumulates the new urdamycin M

A chromosomal mutant of *urdR* was created by transforming *S. fradiae* Tü2717 with plasmid pKCRN resulting in approximately 200 apramycin-resistant colonies per μg DNA. After screening for double cross-over the apramycin-sensitive strain RN-435 was obtained. PCR fragments obtained from RN-435 using primers R-1 and R-2 could not be digested by *Sph*I, while PCR fragments obtained from the wt strain were restrictable by this enzyme. The analysis of culture supernatants of mutant RN-435 showed that one new compound, urdamycin M (Figure 1A), along with rabelomycin, urdamycins I and J (Figure 1A) and several urdamycin derivatives not yet identified, were produced instead of urdamycin A.

Complementation of urdSpm, urdQifD, urdQpm, BF-3-1, Z3-148, and RN-435

To determine clearly that the mutation event only affected the desired genes and no other genes, *urdQ*, *urdR*, *urdS*, *urdZ1*, and *urdZ3* were cloned into pUWL201 [4] or pEM4 [20] and introduced by protoplast transformation into the corresponding mutants. In each case urdamycin A production was restored. Thus, we could rule out any upstream or downstream effects.

Expression of *urdGT1a*, *urdGT1b*, *urdGT1c* and *urdGT2* in mutant Ax and mutant 16-14

Expression of *urdGT1c* in mutant Ax, in which three glycosyltransferase genes had been deleted before, resulted in the formation of 12b-derhodinosyl-urdamycin G as the main product [4]. This experiment allowed the unambiguous functional assignment of UrdGT1c as a glycosyltransferase catalyzing the transfer of NDP-L-rhodinose to the first sugar of the trisaccharide side chain during urdamycin A biosynthesis. Further in-depth analysis of this strain, in which *urdGT1c* was overexpressed, revealed a new, previously unidentified compound. We now determined the structure of this new urdamycin derivative, urdamycin N (Figure 1A), which is not detectable in the wt strain. Urdamycin N contains an additional L-rhodinose moiety attached to the second sugar of the side chain of compound 100-1, an intermediate of the branch pathway leading to urdamycin B [4,19]. Thus, urdamycin N is a urdamycin B analog with a L-rhodinose instead of a D-olivose as terminal sugar of the C-glycosidically bound trisaccharide chain. In contrast, Ax mutants complemented with either *urdGT1a*, *urdGT1b* or *urdGT2* did not produce any other urdamycin derivative than the one described by Trefzer et al. [4].

To rule out any incidental effects on urdamycin biosynthesis we also reinvestigated the urdamycin spectrum after expression of *urdGT1c* in mutant 16-14 [4], which is defective for both *urdGT1b* and *urdGT1c* but carries instead a chromosomal hybrid thereof. Analysis of the culture supernatant revealed the presence of a hitherto unknown urdamycin derivative, urdamycin O. It arises from an attachment of another L-rhodinose to the second sugar of the C-glycosidically linked side chain of urdamycin G (Figure 1A), and thus is an urdamycin A analog with a terminal α-L-rhodinose instead of a β-D-olivose in the trisaccharide chain.

Discussion

The biosynthesis of the angucycline antibiotic urdamycin A is a multi-step process in which the elaboration of the polyketide chain is followed by a number of steps including specific hydroxylation and glycosylation. Single sugar residues of urdamycin A are D-olivose and L-rhodinose. Previous work demonstrated that *urdGT1a*, *urdGT1b*, *urdGT1c*, and *urdGT2* encode glycosyltransferases each responsible for a certain biosynthetic saccharide attachment step during urdamycin biosynthesis. Our work now was aimed at identifying and elucidating the function of genes involved in the biosynthesis of NDP-D-olivose and NDP-L-rhodinose. Analysis of a 5.4 kb fragment identified several putative deoxysugar biosynthetic genes. Based on the sequencing data the biosynthetic pathways from glucose-1-phosphate to NDP-D-olivose and NDP-L-rhodinose together should comprise eight steps. This would include NDP-D-glucose synthesis [21] (UrdG), a 4,6-dehydratation [22] (UrdH), a 2,3-dehydratation (UrdS) and a reduction (UrdT) [23] to yield NDP-4-keto-2,6-dideoxy-D-glucose. The remaining four steps would be a 4-ketoreduction (UrdR) to prepare NDP-D-olivose and a pyridoxamine-catalyzed dehydration [24] (UrdQ) followed by a 5-epimerization (UrdZ1) and a 4-ketoreduction (UrdZ3) to prepare NDP-L-rhodinose (Figure 3). Targeted inactivation of *urdQ*, *urdR*, *urdS*, *urdZ1*, and *urdZ3* was accomplished during this study. Analysis of compounds produced by the mutants provided unequivocal evidence that *urdQ*, *urdZ1*, and *urdZ3* are involved in NDP-L-rhodinose biosynthesis, since strains lacking such genes accumulated urdamycinone B (Figure 1A), as expected if they are specifically defective in the synthesis of NDP-L-rhodinose. However, further biochemical studies are necessary to confirm our functional assignment of *urdQ*, *urdZ1*, and *urdZ3*.

urdR has been shown to be the gene specifically required for NDP-D-olivose formation, as the *urdR* mutant accumulates urdamycin M. This novel urdamycin exclusively contains rhodinose moieties and lacks any D-olivose. Therefore it must be a single-step process to convert the central intermediate NDP-4-keto-2,6-dideoxy-D-glucose to D-olivose. The D-rhodinose moiety in urdamycin M also reveals that the 3-deoxygenation occurs prior to the 5-epimerization (D → L switch), as was recently suggested for landomycin biosynthesis [25]. Interestingly, the 4-ketoreductase UrdZ3 seems to be able to reduce NDP-D-cinerulose as well as NDP-L-cinerulose (Figure 3). Anyway, UrdZ3 might act specifically on the D-sugar but then reduction by UrdZ3 must occur prior to epimerization by UrdZ1. Deletion of *urdS* was accomplished by introducing a frame-

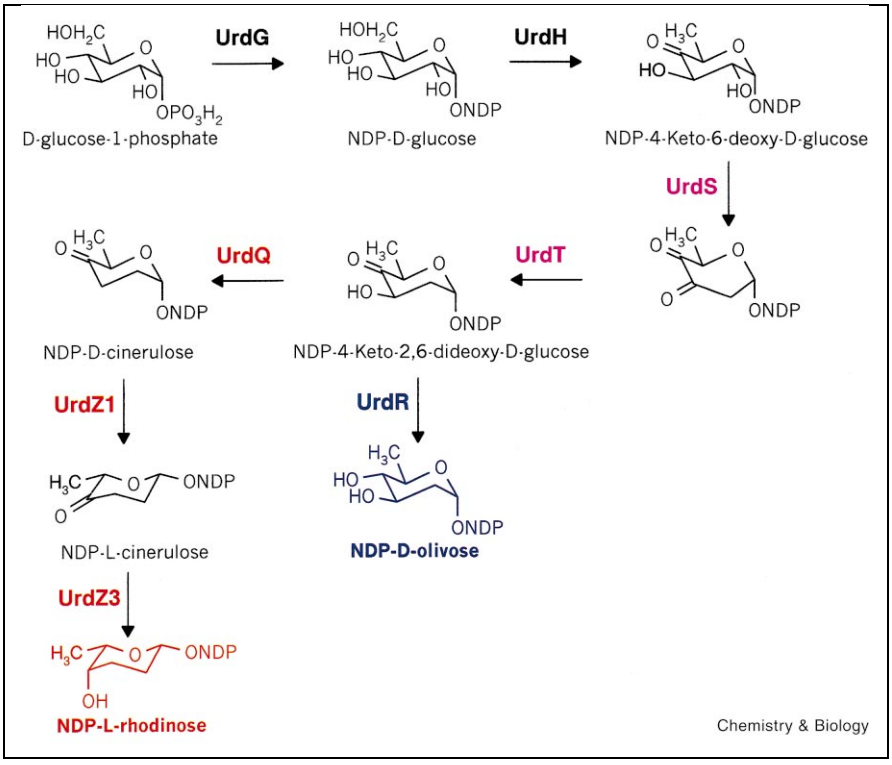

Figure 3. Biosynthetic pathway to NDP-D-olivose and NDP-L-rhodinose in *S. fradiae* Tü2717.

shift mutation into the gene resulting in the formation of a fusion protein UrdS–UrdT. Surprisingly, no urdamycin derivative was detectable in this mutant urdSpm. Similar to the urdQifD and urdQpm mutants urdSpm accumulates diketopiperazines, which are normally not detected in the wt strain. In total, the structures of three different diketopiperazines were determined: cyclo-(phenylalanyl-4-hydroxyprolyl), cyclo-(leucyl-4-hydroxyprolyl), and cyclo-(alanyl-4-hydroxyprolyl). They have been described before as natural products isolated from a *Jaspidae* sponge [26], from rabbit [27], or as a product made by combinatorial chemistry [28]. The accumulation of these cyclic dipeptides in the urdamycin mutants might represent an example for the onset of another biosynthetic pathway if the biosynthesis of the main product is blocked. At this stage, we cannot explain why mutant urdSpm does not produce any non-glycosylated urdamycin derivative. A similar result has been observed by Fish and Cundliffe [29]: the targeted disruption of *tylM2*, a glycosyltransferase from the tylosin producer *S. fradiae* abolished the biosynthesis of the aglycone tylactone because of an intricate regulatory feedback mechanism in the producer strain.

The accumulation of urdamycin M in the *urdR* mutant not only indicates the putative function of *urdR*, but also shows that UrdGT2, which was identified to catalyze the C-glycosylation of NDP-D-olivose in the wt strain, now accepts NDP-D-rhodinose as the sugar co-substrate. This differs from the normal sugar co-substrate in that it has a deoxygenated 3-position and an inverted stereochemistry at the 4-position. As not even traces of urdamycin M can be found in the wt strain, we postulate that the function of UrdGT2 is controlled by several parameters. Beside kinetic parameters (K_M, V_{max}) some kind of channeling of the sugar substrates towards the glycosyltransferase and the formation of enzyme complexes by the deoxysugar forming enzymes and the glycosyltransferases might help to explain the accumulation of the new urdamycin derivatives.

Recently, we described the generation of glycosyltransferase mutants (mutant Ax, mutant 16-14) of the urdamycin producer, in which three (Ax) or two (16-14) of the glycosyltransferase encoding genes had been deleted [4]. These mutants were then used as hosts for expressing single glycosyltransferase genes of the cluster, which had been deleted before. The experiment supported the assignment of each glycosyltransferase of the urdamycin cluster to a certain biosynthetic saccharide attachment step. We now reinvestigated various extracts of the Ax mutant, which was complemented with either *urdGT1a*, *urdGT1b* or *urdGT1c*, and extracts of 16-14 complemented with *urdGT1c*. While we could not find new urdamycin derivatives in extracts of mutant Ax complemented with either *urdGT1a* or *urdGT1b*, a new derivative (urdamycin N) was detected in the Ax mutant complemented with *urdGT1c*. Analo-

gously, the novel urdamycin O was found to be produced in mutant 16-14 complemented with *urdGT1c*. Both new urdamycins contain a second L-rhodinose unit in the trisaccharide side chain. These compounds with the unusual trisaccharide chain were previously observed neither in the wt strain, nor in the Ax mutant or mutant 16-14. Thus, UrdGT1c must be responsible for the linkage of the terminal L-rhodinose moiety. Here it attaches its NDP-sugar co-substrate to a foreign alcohol substrate at a position which in the normal biosynthetic pathway is reserved for a D-olivose moiety linked through UrdGT1b. The result of this experiment again supports our assumption that the specificity of a glycosyltransferase might be regulated by a well-defined control system in the cell. Here, overexpression of the glycosyltransferase gene *urdGT1c* leads to a relaxed specificity regarding its alcohol substrate.

Relaxed specificity for both sugar co-substrates and alcohol substrates (often just the aglycones) has been described already for various glycosyltransferases [30–32]. Recently, OleG2, a glycosyltransferase from *S. antibioticus*, which transfers L-oleandrose in the wt strain, was shown to be able to transfer L-rhamnose if expressed in a different strain [32]. TylM2, a glycosyltransferase from *S. fradiae*, was integrated into the chromosome of a triple mutant of *Saccharopolyspora erythraea*. The resulting strain, fed with tylactone, produced new hybrid antibiotics [33].

Despite all these results it is still difficult to predict the specificity of a given glycosyltransferase and it will need more study to understand the parameters controlling the function of these enzymes.

Significance

1. **NDP-4-keto-2,6-dideoxy-D-glucose seems to be the central intermediate in the biosynthesis of NDP-D-olivose and NDP-L-rhodinose during urdamycin A biosynthesis.**
2. **The specificity of two glycosyltransferases (UrdGT2, UrdGT1c) of urdamycin biosynthesis is influenced by the available concentration of the NDP-sugar co-substrate as well as the expression level of the glycosyltransferase. While an over-concentration of the activated sugar co-substrate can lead to a somewhat relaxed sugar co-substrate specificity an overexpressed glycosyltransferase can overcome restrictions for its alcohol substrate.**

Materials and methods

Bacterial strains, plasmids and culture conditions

For standard purposes, *S. fradiae* Tü2717 [1] was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1 mM $CaCl_2$, 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. Before transforming *S. fradiae* Tü2717, plasmids were propagated in *E. coli* ET 12567 (*dam*$^-$, *dcm*$^-$, *hsd*S, Cm^R) [34] to obtain unmethylated DNA. *E. coli* strains were routinely grown on Luria–Bertani (LB) agar or liquid medium containing the appropriate antibiotic. pBluescript SK− was from Stratagene and pLitmus 28 was from New England Biolabs. Plasmid pKC1132, conferring apramycin resistance, was a kind gift of Eli Lilly and Company. For gene expression experiments, plasmid pUWL201 [4] and plasmid pEM4 [20] both conferring thiostrepton and ampicillin resistance were used.

General genetic manipulation, DNA sequencing/sequence analysis and PCR

Standard molecular biology procedures were performed as described [35]. Isolation of *E. coli* plasmid DNA, DNA restriction, DNA modification such as filling-in sticky ends, and Southern hybridization were performed following the protocols of the manufacturers of kits, enzymes and reagents (Amersham Pharmacia, Boehringer Mannheim, Promega, Stratagene). *Streptomyces* protoplast formation, transformation, and protoplast regeneration were performed as described [36].

Nucleotide sequences were determined by the dideoxy chain termination method using automatic laser fluorescence sequencers (Molecular Dynamics Vistra 725 or Perkin Elmer ABI). Sequencing reactions were done using a thermosequenase cycle sequencing kit with 7-deaza-dGTP (Amersham) and standard primers (M13 universal and reverse, T3, T7). Computer-aided sequence analysis was done with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), database searches were performed with the BLAST 2.0 program [37] on the server of the National Center for Biotechnology Information, Bethesda, MD, USA. PCR was carried out using a Perkin Elmer GeneAmp 2400 thermal cycler. The conditions were as described [4] using oligonucleotide primers listed in Table 1.

Construction of gene inactivation plasmids

urdQ: A unique *Nco*I restriction site inside the gene *urdQ*, which is located at bases 287–292 on a 2.3 *Pst*I fragment (fragment c in Figure 2) was chosen for targeted inactivation by shifting the reading frame. After *Nco*I restriction, treatment with the Klenow fragment of *E. coli* DNA polymerase I and religation, the intended alteration was checked by DNA sequencing. The manipulated 2.3 kb *Pst*I insert was then ligated into the same site of pKC1132 to yield inactivation construct pKurdQpm. For the construction of pKurdQifD, which would allow the introduction of an in-frame deletion into *urdQ*, two fragments (urdQA and urdQB), containing DNA that encodes aa 33–171 (urdQA) and aa 288–430 (urdQB) of UrdQ, were amplified by PCR. Primers for the amplification of urdQA were Q-1 and Q-3 and primers for the amplification of urdQB were Q-4 and Q-2. After restriction of fragment urdQA with *Eco*RI and *Bam*HI, and restriction of fragment urdQB with *Bgl*II and *Hin*dIII both fragments were ligated into pKC1132 digested with *Eco*RI and *Hin*dIII to create pKurdQifD.

Table 1
Oligonucleotide primers used in PCR analyses of the different urdamycin mutants of *S. fradiae* Tü2717 and for PCR amplification of DNA fragments.

Name of primer	Oligonucleotide sequence
S-1	5′-TGACCGGATCCACCCAGCCCCTCT-3′
S-2	5′-GTGAGCCGTCCGTCACCGCCCAAC-3′
Q-1	5′-GGAACCACCGAATTCTGGCCGTCC-3′
Q-2	5′-CTAGCCACAAGCTTCGACGAACTCCTTGAT-3′
Q-3	5′-GGCCATCTCGGATCCCTCGAACGG-3′
Q-4	5′-CGGTTACAGATCTAAGGCCACCGACATACAG-3′
Z1-1	5′-ACTGCACACGGTACCGGGCTGGAA-3′
Z1-2	5′-GCCGACGATGAATTCGGGACGCCA-3′
Z3-1	5′-GAAGAGCATCGAGCTCGGCGGGGACAG-3′
Z3-2	5′-ACCTCTTCCTGCAGCAGTTCCTTGCGG-3′
R-1	5′-ATGTGTCACTGCAGCACAGAGATCAGG
R-2	5′-TTCAGAAAGCATCAGAATTCTCCCGTG-3′

urdR: The unique *Spl*I site inside the *urdR* gene, which is located on a 2.3 kb *Pst*I fragment (fragment c in Figure 2), was altered by *Spl*I restriction and subsequent treatment as described for *urdQ*. Sequencing of several plasmids revealed correct alteration (CGTACGTACG) along one plasmid carrying an irregularly filled *Spl*I site (CGTGTACG). Screening for double cross-over events by PCR requires the loss of the restriction site used for introducing the frameshift. Correct fill-in alters the frame but recreates the *Spl*I site making impossible a PCR-based strategy for a double cross-over recognition. Therefore the plasmid with the irregularly altered *urdR* gene was chosen for further work. Its 2.3 kb *Pst*I insert was then ligated into the *Pst*I site of pKC1132 to create inactivation plasmid pKCRN. For its integration into the chromosome the inactivation construct provides 1.25 kb (upstream of the manipulated *Spl*I site) and 1.05 kb (downstream) flanking homologous DNA.

urdS: A 1.3 kb *Pst*I fragment (fragment d in Figure 2) was restricted with *Nco*I, treated with Klenow fragment and religated resulting in pKurdSpm. For cross-over events there are 0.8 kb upstream and 0.5 kb downstream of the altered *Nco*I site available. The intended alteration was checked by DNA sequencing. pKurdSpm carries a frameshift mutation which would create a fusion protein UrdS–UrdT containing 464 aa of UrdS.

urdZ1: A 1.4 kb section of fragment a (Figure 2) including the entire reading frame of urdZ1 was amplified by PCR (fragment a′). Primers were Z1-1 and Z1-2 which introduced restriction sites for *Bam*HI and *Eco*RI. This PCR fragment a′ was cloned into pLitmus 28 to give pLurdZ1. *urdZ1* contains a unique *Sal*I restriction site which is located 0.7 kb from either end of fragment a′. It was used for shifting the reading frame by *Sal*I restriction and subsequent end-filling using T4 DNA polymerase and religation. DNA sequencing confirmed correct fill-in. The

Table 2
^{1}H NMR data of the new urdamycins (δ in ppm relative to TMS (J in Hz)).

Proton	Urdamycin M[a]	Urdamycin N[b]	Urdamycin O
2-Ha	ca. 3.00 (complex)	3.32 d (17)	2.85 (obsc. by water)
2-He	2.61 d, br (13)	3.20 dd (17; 2)	2.52 dd (13; 2)
3-CH_3	1.22 s	1.46 s	1.13 s
4-Ha	2.33 d, br (15)	3.06 d (15)	ca. 2.00 (complex)
4-He	ca. 2.2 m	2.87 dd[c] (15; 2)	2.30 dd (15; 1)
5-H	6.95 d (10)	7.58 d (8)	6.48 d (10)
6-H	6.55 d (10)	7.95 d (8)	6.87 d (10)
10-H	8.06 d (8)	8.28 d (8)	7.95 d (8)
11-H	7.69 d (8)	7.74 d (8)	7.61 d (8)
1′-H	4.92 d, br (11)	4.92 dd (11; 1)	4.90 dd (11; 1)
2′-Ha	ca. 1.1–1.8 (complex)	1.1–1.8 (complex)	ca. 1.3 (complex)
2′-He	ca. 1.1–1.8 (complex)	2.72 ddd (13; 5; 1)	2.70 ddd (13; 5; 1)
3′-H	ca. 2.1 (complex)	4.00 ddd (12; 9; 4)	3.98 ddd (9; 9; 5)
4′-H	3.42 s, br	3.37 dd (9; 9)	3.36 dd (9; 9)
5′-H	3.85 q, br (6.5)	3.58 dq (9; 6)	3.60 dq (9, 6)
6′-H_3	1.33 d (6.5)	1.37 d (6)	1.39 d (6)
1A-H	–	5.02 d (2)	4.98 d (1)
2A-Ha	–	1.1–1.8 (complex)	ca. 2.0 (complex)
2A-He	–	1.1–1.8 (complex)	ca. 1.3 (complex)
3A-Ha	–	1.1–1.8 (complex)	ca. 1.6 (complex)
3A-He	–	1.1–1.8 (complex)	ca. 2.0 (complex)
4A-H	–	3.50 s, br	3.52 s, br
5A-H	–	4.23 q, br (6.5)	4.21 q, br (6.5)
6A-H_3	–	1.15 d (6.5)	1.15 d (6.5)
1B-H	–	5.17 d (2)	5.16 d (1)
2B-Ha	–	1.1–1.8 (complex)	1.2–2.0 (complex)
2B-He	–	1.1–1.8 (complex)	1.2–2.0 (complex)
3B-Ha	–	1.1–1.8 (complex)	1.2–2.0 (complex)
3B-He	–	1.1–1.8 (complex)	1.2–2.0 (complex)
4B-H	–	3.53 s, br	3.53 s, br
5B-H	–	4.34 q, br (6.5)	4.33 q, br (6.5)
6B-H_3	–	1.09 d (6.5)	1.08 d (6.5)
1C-H	5.36 d (2)	–	5.30 d (2)
2C-Ha	1.2–2.0 (complex)	–	1.2–2.0 (complex)
2C-He	1.2–2.0 (complex)	–	1.2–2.0 (complex)
3C-Ha	1.2–2.0 (complex)	–	1.2–2.0 (complex)
3C-He	1.2–2.0 (complex)	–	1.2–2.0 (complex)
4C-H	3.65 s, br	–	3.32 s, br
5C-H	3.79 q, br (6.5)	–	3.63 q, br (6.5)
6C-H_3	0.58 d (6.5)	–	0.49 d (6.5)
OH signals	3.36; 3.62; 4.45; 5.32; 12.4	–[d]	3.33; 3.47; 3.55; 4.38; 5.32; 12.40

[a]500 MHz, d_6-acetone.
[b]400 MHz, d_6-acetone, assignment of the rhodinose signals (sugars A and B) is based on comparison with urdamycin B and urdamycin O, and may be interchangeable.
[c]Obscured by water, visible after addition of more D_2O; br = broad signal.
[d]Not observed due to some D_2O in the sample.

altered insert of pLurdZ1 was then excised by *Bam*HI and *Eco*RI restriction and inserted into pKC1132 to create the final inactivation plasmid pKC-urdZ1d.

urdZ3: The unique *Mro*I site inside the *urdZ3* gene, which is located at bases 1114–1119 on a 2010 bp *Pst*I fragment (fragment b in Figure 2) was removed by restriction with this enzyme, end-blunting with T4 DNA polymerase and religation therefore shifting the *urdZ3* reading frame. Correct fill-in was checked by DNA sequencing. After religation the 2.0 kb *Pst*I insert was ligated to the *Pst*I site of pKC1132 to yield inactivation plasmid pKCZ3.

Construction of gene complementation plasmids

urdQ, urdS, and urdZ1: A *Xba*I (*Bam*HI) restriction site 5′ to the ribosome binding site and an *Eco*RI restriction site 3′ to the termination codon were introduced by PCR into the genes *urdQ* and *urdS* (*urdZ1*) using PCR. Fragments were cloned into the *Xba*I (*Bam*HI) and *Eco*RI site of pEM4.

urdR and urdZ3: The 2.0 kb *Pst*I fragment (fragment b in Figure 2) was cloned into the *Pst*I site of pUWL201 to yield complementation construct pUWL-Z3. Correct insert orientation was verified by DNA restriction. Using a 2.3 kb *Pst*I fragment (fragment c in Figure 2), the plasmid for complementation of the *urdR* mutant (RN-435) was constructed identically as described for *urdZ3*.

Analysis of urdamycin A, its intermediates and new compounds

For urdamycin production *S. fradiae* Tü2717 and all mutants mentioned were grown in NL111V medium as described [4].

Cultures were extracted with an equal volume of ethyl acetate. After evaporation of the solvent the dried extracts were redissolved in methanol. TLC analysis was carried out on silica gel plates (silica gel 60 F254, Merck) with methylene chloride/methanol/ethyl acetate (8:1:1, v/v) as solvent. Purification of crude extracts was carried out on Sephadex LH-20 columns with methanol as solvent. Fractions containing new urdamycins were subjected to preparative HPLC as described [4].

Analytical HPLC-UV was performed on a Tosoh SC-8020 liquid chromatograph with a photodiode array detector and a Tosoh TSK-gel ODS-80TM (150 × 4.6 mm) column maintained at 40°C. The detection wavelength range was 250–500 nm. For elution the following gradient profile was used: solvent A: 0.5% AcOH in H_2O, solvent B 0.5% AcOH in CH_3CN, non-linear gradient, 0–95% B within 30 min at a flow rate of 0.8 ml/min. LC/MS was performed on a Thermoquest LCQ equipped with Hewlett Packard HP1000 series LC under the identical conditions described for HPLC, by atmospheric pressure chemical ionization (APCI) and detection in the positive and negative mode. Urdamycin A and its intermediates were identified by HPLC and mass spectrometry.

Production cultures of RN-435 yielded approximately 2 mg/l urdamycin M, cultures of mutants Ax and 16-14, complemented with *urdGT1c*, about 1.5 mg/l urdamycin N and urdamycin O, respectively.

Chemical analysis of urdamycinone B and other known urdamycins

Urdamycinone B and all other known urdamycins were identical in TLC, HPLC-UV/Vis, and HPLC-MS to those described earlier [1,3,4].

Structure determination of the new urdamycins M, N and O

All three new compounds were identified mainly by NMR spectroscopy, the structures were also supported by mass spectrometry.

Urdamycin M: The ^1H and ^{13}C NMR data indicate two rhodinose moieties as the sole sugar components of this new urdamycin derivative. Typical are the 4-H (broad singlets, δ 3.42 and δ 3.65) and 5-H (broad quartets, δ 3.79 and δ 3.85) signals, which are both due to the axial 4-OH position in rhodinose. The four carbon triplet signals around δ 25 in the ^{13}C NMR spectrum (see Tables 2 and 3) are significant for the deoxygenated 2- and 3-positions. That one rhodinose unit is attached

Table 3
^{13}C NMR data of urdamycin M (125.7 MHz) and O (100.6 MHz) in d_6-acetone.

Carbon	Urdamycin M	Urdamycin O	Carbon	Urdamycin M	Urdamycin O
1	202.6 s	202.5 s	4′	67.3 d	79.8 d
2	54.8 t	54.8 t	5′	77.2 d	77.0 d
3	76.2 s	76.2 s	6′	18.3 q	19.8 q
4	44.3 t	44.0 t	1A	–	92.2 d
4a	82.7 s	81.8 s	2A	–	23.5 t[a]
5	146.6 d	146.1 d	3A	–	26.3 t[a]
6	117.4 d	117.2 d	4A	–	76.1 d[b]
6a	138.1 s	137.7 s	5A	–	74.2 d[b]
7	192.0 s	190.2 s	6A	–	17.9 q
7a	114.9 s	115.0 s	1B	–	98.1 d
8	158.0 s	157.8 s	2B	–	23.3 t[a]
9	140.1 s	138.8 s	3B	–	26.6 t[a]
10	134.8 d	134.1 d	4B	–	67.0 d[b]
11	120.1 d	120.1 d	5B	–	68.1 d[b]
11a	131.7 s	132.0 s	6B	–	14.5 q
12	190.0 s	183.8 s	1C	95.2 d	95.0 d
12a	141.1 s	141.6 s	2C	23.4 t[a]	23.4 t[a]
12b	85.5 s	82.9 s	3C	26.4 t[a]	26.6 t[a]
13	30.6 q	30.0 q	4C	67.0 d	68.3 d[b]
1′	74.3 d	72.0 d	5C	67.9 d	67.2 d[b]
2′	24.0 t[a]	36.9 t	6C	16.9 q	17.0 q
3′	26.9 t[a]	68.0 d			

Assignments and multiplicities from HMBC and HSQC spectra.
[a]Assignments interchangeable.
[b]Assignments interchangeable.

at the 12b-position can be deduced from the significantly downfield shifted 6-H_3 signal (δ 0.58) due to its location in the anisotropy cone of the quinone chromophore. The 12b attachment is normal for an L-rhodinose moiety, which here is also indicated by a small coupling constant of the 1C-H signal (δ 5.36). In contrast, the 1′-H signal (δ 4.92) appears as a broad doublet, with a large coupling constant of 11 Hz, which clearly indicates a β-glycosidically bound sugar, and also a D-sugar, since both types of deoxysugars of the urdamycins, D-olivose and L-rhodinose, are biosynthesized widely within the same pathway. This determines that the activating NDP group is normally in the axial position leading to β-attachment. It is only in the equatorial position (for an α-attachment) after the 5-epimerase step, which performs the D to L switch. The chemical shift of C-1′ (δ 74.3) clearly indicates the C-glycosidic linkage. Urdamycin M has a molecular formula $C_{31}H_{36}O_{11}$ and a MW of 584.64, which has been confirmed by APCI-MS (retention time: 18.08 min).

Urdamycin N: The ^{1}H NMR data indicate two rhodinose (e.g., 4A-H and 4B-H appear as broad singlets, 5A-H and 5B-H as broad quartets) and one olivose moiety along with an urdamycin B-type chromophore, which follows from the two aromatic AB systems (δ 7.58 and 7.95 and δ 7.74 and 8.28, respectively). The D-olivose moiety is C-glycosidically linked, as usually. The NMR data of the deoxysugar trisaccharide are identical to the corresponding moiety in urdamycin O (see Table 2). The molecular formula of urdamycin N, $C_{37}H_{44}O_{12}$, MW 680.76, is confirmed by the pos.-FAB MS of 681 (MH^+).

Urdamycin O: ^{1}H and ^{13}C NMR data (Tables 2 and 3) indicate three L-rhodinose moieties. 4A-H, 4B-H and 4C-H appear as broad singlets (δ 3.52, 3.53, 3.32), 5A-H, 5B-H and 5C-H as broad quartets (δ 4.21, 4.33, 3.63), respectively, and 1A-H, 1B-H and 1C-H (δ 4.98, 5.16, 5.30) show a small coupling constant, which proves their α-glycosidic linkage. The corresponding C-1 carbons all show an O-glycosidic bondage (see Table 3). A fourth sugar unit is the β-C-glycosidically connected D-olivose, typical for many aquayamycin-type angucyclines. The sugar linkages are also proven by the HMBC data, which show $^3J_{C-H}$ couplings between 1′-H and C-9, 1A-H and C-3′, 3′-H and C-1A, 1B-H and C-4A, 4A-H and C-1B, and 1C-H and C-12b. The molecular formula of urdamycin O, $C_{43}H_{56}O_{16}$, MW 828.92, is confirmed by the pos.-FAB MS showing the largest signals at 829 (MH^+).

Diketopiperazines

The diketopiperazines cyclo-(phenylalanyl-4-hydroxy-prolyl), cyclo-(leucyl-4-hydroxy-prolyl), and cyclo-(alanyl-4-hydroxy-prolyl) were identified by their ^{1}H and ^{13}C NMR spectra, which were identical to those reported in the literature.

Accession number

The sequence reported here has been deposited in the GenBank data base under the accession numbers AF164961 and AF269227.

Acknowledgements

This work was supported by a grant of the European Union (BIO-CT96-0068) to A.B. and J.R. Further support came from a grant of the Deutsche Forschungsgemeinschaft (SFB323) to A.B., and from grants of the Medical University of South Carolina Institutional Research Funds of 1999–00 as well as the South Carolina Commission of Higher Education and the US Department of Defense to J.R.

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