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Cloning and Sequencing of the Biosynthetic Gene Cluster for Saquayamycin Z and Galtamycin B and the Elucidation of the Assembly of Their Saccharide Chains

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The Gram-positive bacterium, *Micromonospora* sp. Tü6368 produces the angucyclic antibiotic saquayamycin Z and the tetracenequinone galtamycin B. The structural similarity of both compounds suggests a common biosynthetic pathway. The entire biosynthetic gene cluster (*saq* gene cluster) was cloned and characterized. DNA sequence analysis of a 36.7 kb region revealed the presence of 31 genes that are probably involved in saquayamycin Z and galtamycin B formation. Heterologous expression experiments and targeted gene inactivations were carried out to specifically manipulate the saquayamycin Z and galtamycin B pathways; this demonstrated unambiguously that both compounds are derived from the same cluster. The inactivation of glycosyltransferase genes led to the production of novel saquayamycin and galtamycin derivatives, provided information on the assembly of the sugar chains, and showed that tetracenequinones are formed from angucyclines.

Introduction

Actinomycetes are Gram-positive bacteria that have been isolated from soil as well as from marine habitats. Representatives of this genus are a particular rich source of bioactive natural products, including valuable antibiotics, antitumor agents, immunosuppressants, and enzyme inhibitors; further, many of these compounds are glycosylated.[1] Recently, a new actinomycete strain, *Micromonospora* sp. Tü6368, has been successfully cultivated and shown to produce two novel glycosylated compounds: saquayamycin Z and galtamycin B. Both metabolites show cytostatic effects against human tumor cell lines, and saquayamycin Z additionally exhibits antibiotic activity against Gram-positive bacteria.[2] Besides saquayamycin Z and galtamycin B, the strain *M.* sp. Tü6368 produces the monoglycosylated tetracenequinone galtamycinone and several nonglycosylated metabolites, among these 3-deoxyrabelomycin and retimycin.

Saquayamycin Z and galtamycin B are structurally related compounds that both possess a similar polyketide aglycone, which is an angucycline in the case of saquayamycin Z and a tetracenequinone in the case of galtamycin B (Figure 1). Common to both substances is a tetrasacchaide side chain that is attached via an unusual C–C linkage to C9 of the respective polyketide. In contrast to galtamycin B, saquayamycin Z incorporates an additional sugar chain: a pentasaccharide that is attached to 3-O of the aglycon. Saquayamycin Z carries nine deoxy sugars in total and is therefore the largest angucycline that has been reported to date.[3]

The biosynthesis of the pentasaccharide chain especially raised our interest because it might involve glycosyltransferases that have new functions. First, the attachment of the sugar at the 3-O position of the saquayamycin aglycones requires a glycosyltransferase with new substrate specificity because no other aglycones that have been reported so far carry a saccharide chain at this position. Second, the pentasaccharide chain consists of three L-rhodinoses that alternate with two D-oliveses. The structure of the aglycones suggested that the biosyn-

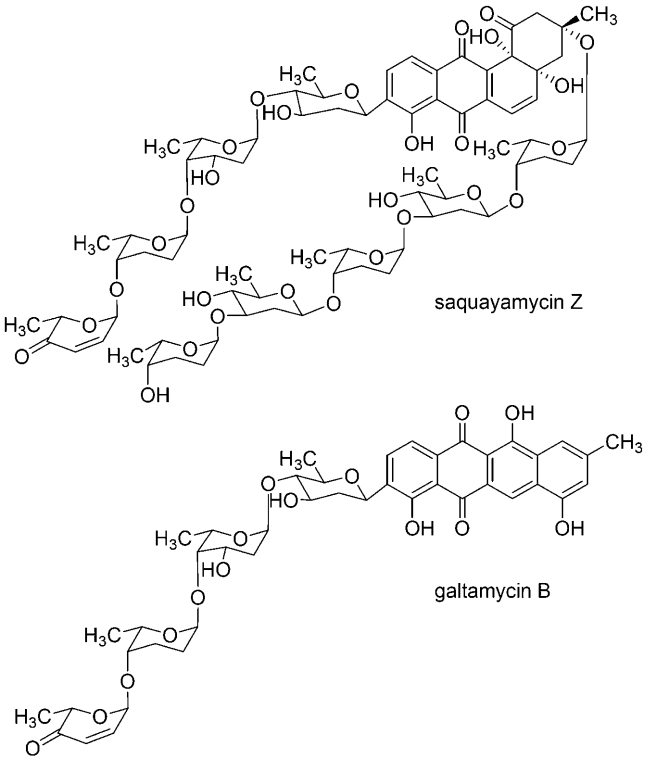

Figure 1. Chemical structures of saquayamycin Z and galtamycin B.

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thetic pathway would contain additional glycosyltransferases of flexible substrate specificity as shown for the glycosyltransferases involved in the biosynthesis of landomycin A.[4]

Here, we report the cloning, sequencing, and functional analysis of the saquayamycin Z and galtamycin B biosynthetic gene cluster in *M.* sp. Tü6368. In an effort to examine details of saquayamycin Z and galtamycin B biosynthesis, knockout mutants were generated in five glycosyltransferase genes. Structure elucidation of novel derivatives that accumulated in the mutant strains, together with the annotation analysis of the cluster sequence led to a detailed proposal for the biosynthesis, especially for the assembly of the sugar chains.

Results and Discussion

Cloning and identification of the saquayamycin Z biosynthetic gene cluster

The chemical structures of the saquayamycin Z and galtamycin B aglycones indicated that a type II polyketide synthase (PKS II) is involved in the biosynthesis of both compounds. The so-called "minimal PKS" consists of two ketosynthase units (KSα and KSβ) and an acyl carrier protein (ACP). Additional PKS subunits, including ketoreductases, cyclases, and aromatases define the folding pattern of the intermediate. In addition to the PKS II, the deoxy sugars attached to both aglycones suggest that a 4,6-deydratase operates during biosynthesis.[5,6] Therefore, a genomic cosmid library of *M.* sp. Tü6368 in *E. coli* was screened with both a probe of the ketosynthase α gene of PKS II and an NDP-glucose-4,6-dehydratase gene probe. Five cosmids were identified by these initial screenings and were subjected subsequently to a second screening approach. A PCR was carried out with primers that were directed against conserved gene sequences of a cyclase that is probably involved in the formation of the fourth ring. This resulted in three positively hybridizing clones. The DNA of these cosmids was analyzed subsequently by restriction mapping, which revealed that they contained overlapping DNA fragments. These fragments were subcloned and partially sequenced. Based on restriction and sequence analysis, two cosmids, 20 and 23, were chosen for complete sequencing.

A contiguous region of 36.7 kbp could be assembled according to the sequencing results. The 34 kpb that was assigned to the *saq* gene cluster was flanked upstream by a 1.1 kpb region, and downstream by a 1.6 kbp region. The average G+C content of 72.7% is well in line with the reference value for *Actinomyces* sp. DNA.[7] Annotation analysis revealed 31 open reading frames (ORF), of which 30 could be assigned a function in the formation of saquayamycin Z (Table 1). The genetic organization of the biosynthetic gene cluster is shown in Figure 2.

Sequence analysis of Genes/enzymes putatively involved in aglycone formation and modification

Three ORFs (*saqA* (**6**), *saqB* (**7**), and *saqC* (**8**)) located at the 5′-end of the cluster are homologous to a set of genes derived from *Streptomyces* species, and exhibit 67 to 82% identity to the gene products on the amino acid level. SaqA and SaqB resemble the subunits α and β of a minimal ketoacylsynthase, and SaqC is the acyl carrier protein homologue; together this constitutes the expected minimal PKS. Two deduced proteins of the *saq* cluster, SaqF (**5**) and SaqL (**10**), show similarity to cyclases involved in angucycline formation. SaqF shares 76% identical amino acids with LndF from the landomycin E producer *S. globisporus*.[8] SaqL is 70% identical with SimA5, which is derived from *S. antibioticus* Tü6040.[9] The cluster also harbors (keto)reductase genes (*saqD* (**9**), *saqN* (**12**), *saqO* (**13**)) and genes encoding oxygenases (*saqE* (**4**), *saqM* (**11**)) that are probably involved in the biosynthesis of the saquayamycin aglycone.

Genes/enzymes putatively involved in the biosynthesis and transfer of deoxy sugars

In saquayamycin Z four different deoxy sugars are attached to the aglycone: L-rhodinose, D-olivose, L-2-deoxyfucose and the ketosugar L-aculose. Fifteen genes are predicted to be involved in the formation and transfer of these deoxyhexoses.

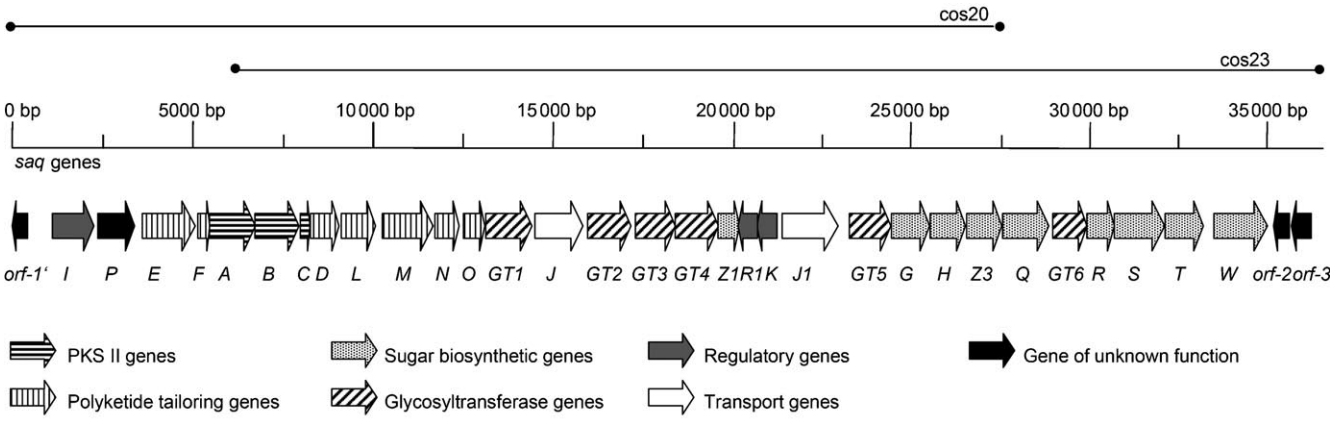


Figure 2. Organization of the biosynthetic gene cluster of saquayamycin Z and galtamycin B.

Table 1. Deduced functions of ORFs located within the saquayamycin Z and galtamycin B gene clusters of *Micromonospora* sp. Tü6368.

ORF (running number)	aa[a]	Most similar protein (aa identity [%])	Accession No.	Proposed function
orf-1′ (**1**)	>142[b]	Strop_0581; *Salinispora tropica* CNB-440 (54)	YP_001157439	unknown function
saqI (**2**)	382	KasT; *S. kasugaensis* (49)	BAC53615	transcriptional regulator
saqP (**3**)	299	PgaK; 250; *S.* sp. PGA64 (48)	AAK57521	unknown function
saqE (**4**)	490	CabE; *S.* sp. H022 (71)	2A2A	hydroxylase
saqF (**5**)	109	LndF; *S. globisporus* (76)	AAU04837	cyclase
saqA (**6**)	422	PgaA; *S.* sp. PGA64 (82)	AAK57525	ketosynthase
saqB (**7**)	407	LanB; *S. cyanogenus* (71)	AAD13537	chain length factor
saqC (**8**)	90	ORF3; *S. griseus* (67)	CAA54860	acyl carrier protein
saqD (**9**)	254	SimA6; *S. antibioticus* (77)	AAK06787	ketoreductase
saqL (**10**)	314	SimA5; *S. antibioticus* (70)	AAK06788	cyclase
saqM (**11**)	491	Sim7; *S. antibioticus* (57)	AAL15585	monooxygenase
saqN (**12**)	253	LanV; *S. cyanogenus* (65)	AAD13552	ketoreductase
saqO (**13**)	194	UrdO; *S. fradiae* (67)	AAF00220	reductase
saqGT1 (**14**)	424	UrdGT1a; *S. fradiae* (53)	AAF00214	glycosyltransferase
saqJ (**15**)	469	PgaJ; *S.* sp. PGA64 (52)	AAK57531	transporter
saqGT2 (**16**)	443	UrdGT1a; *S. fradiae* (56)	AAF00214	glycosyltransferase
saqGT3 (**17**)	404	LanGT1; *S. cyanogenu* (57)	AAD13555	glycosyltransferase
saqGT4 (**18**)	396	UrdGT1c; *S. fradiae* (58)	AAF00217	glycosyltransferase
saqZ1 (**19**)	199	LanZ1; *S. cyanogenus* S136 (69)	AAD13558	NDP-hexose 3,5-epimerase
saqR1 (**20**)	162	*S. violaceoruber* Tü22 (63)	CAA09641	transcriptional activator
saqK (**21**)	207	*S. avermitilis* MA-4680 (55)	NP_825878	transcriptional regulator
saqJ1 (**22**)	518	UrdJ; *S. fradiae* (58)	AAF00219	transporter
saqGT5 (**23**)	383	UrdGT2; *S. fradiae* (65)	2P6P_A	*C*-glycosyltransferase
saqG (**24**)	353	LanG; *S. cyanogenus* S136 (69)	AAD13545	NDP-hexose synthetase
saqH (**25**)	326	Med-ORF17; *S.* sp. AM-7161 (78)	BAC79030	NDP-glucose 4,6-dehydratase
saqZ3 (**26**)	322	UrdZ3; *S. fradiae* (47)	AAF72549	NDP-hexose 4-ketoreduktase
saqQ (**27**)	436	UrdQ; *S. fradiae* (83)	AAF72550	NDP-hexose 3,4-dehydratase
saqGT6 (**28**)	416	SnogZ; *S. nogalater* (46)	CAB59003	glycosyltransferase
saqR (**29**)	251	LanR; *S. cyanogenus* S136 (70)	AAD13548	4-ketoreductase
saqS (**30**)	467	LanS; *S. cyanogenus* S136 (73)	AAD13549	NDP-hexose 2,3-dehydratase
saqT (**31**)	336	LanT; *S. cyanogenus* S136 (66)	AAD13550	oxidoreductase
saqW (**32**)	498	AknOx; *S. galilaeus* ATCC 31615 (57)	ABI15166	oxidoreductase
orf-2 (**33**)	151	AclJ; *S. galilaeus* (51)	BAB72053	unknown function
orf-3 (**34**)	197	Strop_0020; *Salinispora tropica* CNB-440 (38)	YP_001156883	DSBA-oxidoreductase

[a] Amino acid; [b] incomplete ORF.

The conversion of glucose-1-phosphate to dNDP-4-keto-2,6-dideoxy-D-glucose, the common intermediate of all four sugars, involves NDP-D-glucose synthesis (SaqG; **24**), a 4,6-dehydration (SaqH; **25**), a 2,3-dehydration (SaqS; **30**), and a 3-ketoreduction (SaqT; **31**). At this stage, the biosynthetic pathways to the various deoxy sugars branch out. The trideoxyhexose NDP-L-rhodinose is formed by subsequent 3-deoxygenation (SaqQ; **27**), 5-epimerization (SaqZ1; **19**), and 4-ketoreduction (SaqZ3; **26**). The biosynthesis of NDP-D-olivose is accomplished by a 4-ketoreduction that is catalyzed by SaqR (**29**). The final steps toward NDP-L-2-deoxyfucose are 5-epimerization (SaqZ1; **19**) and 4-ketoreduction (SaqZ3; **26**). Finally, six glycosyltransferases (SaqGT1–SaqGT6; **14**, **16**–**18**, **23**, **28**) catalyze the attachment of all nine deoxy sugars to the aglycone. The ketosugar L-aculose is probably generated from L-rhodinose via oxidoreduction (SaqW; **32**).[10]

Genes/enzymes putatively involved in the regulation and self-resistance and genes/enzymes of unknown function

Three genes, *saqI* (**2**), *saqR1* (**20**), and *saqK* (**21**), could be identified in the biosynthetic gene cluster and are probably involved in the regulation of the saquayamycin Z biosynthesis. Located at the 5′-end of the cluster is *saqI*, which encodes a protein with similarity to transcriptional activators of the StrR family, whereas *saqR1* and *saqK* are the only genes transcribed on the antisense strand, which is also typical of regulatory genes.[11] SaqR1 shows similarities to several regulators of the MerR family, and SaqK belongs to the TetR family of regulators.

Two deduced proteins are probably involved in conferring saquayamycin resistance to the producing strain. SaqJ (**15**) and SaqJ1 (**22**) resemble transport proteins that are likely responsible for transport of the secondary metabolites across the membrane.

The product of *saqP* (**3**) shows homology to various proteins of unknown function located within other angucycline biosynthetic gene clusters.

Characterization of genes of the *saq* cluster

To confirm the involvement of the *saq* gene cluster in saquayamycin Z and galtamycin B biosynthesis and to investigate the sugar chain formation, we sought the rational design of novel derivatives by targeted gene inactivation. A promising candi-

date was *saqGT5*, which encodes a protein that is homologous to UrdGT2, a *C*-glycosyltransferase that is involved in urdamycin A production. Inactivation of *urdGT2* has successfully been applied to generate the novel nonglycosylated urdamycins I, J, and K.[12]

To gain insight into the assembly of the deoxy sugar chains, putative glycosyltransferase genes were selected for heterologous expression (*saqGT2*) and inactivation (*saqGT1*, *saqGT2*, *saqGT3*, and *saqGT6*). Therefore, a protocol for DNA transfer from *E. coli* via conjugation had to be developed. Crucial aspects of the conjugation protocol for the strain were the ratio of *E. coli* and *M.* sp. Tü6368 cells, the choice of medium, and the time that was used for the conjugation process. The mutations were achieved either by a shift of the reading frame (*saqGT1*, *saqGT5*) or by replacing the gene of interest by the spectinomycin-resistance cassette *aadA* (*saqGT2*, *saqGT3*, *saqGT6*). Inactivation plasmids were introduced into the chromosome of the wild type by homologous recombination. The mutant strains of *saqGT1* and *saqGT5* were also complemented in trans with a full-length copy of the respective gene under control of the constitutive promoter, *ermE**. This restored wild-type production in both cases.

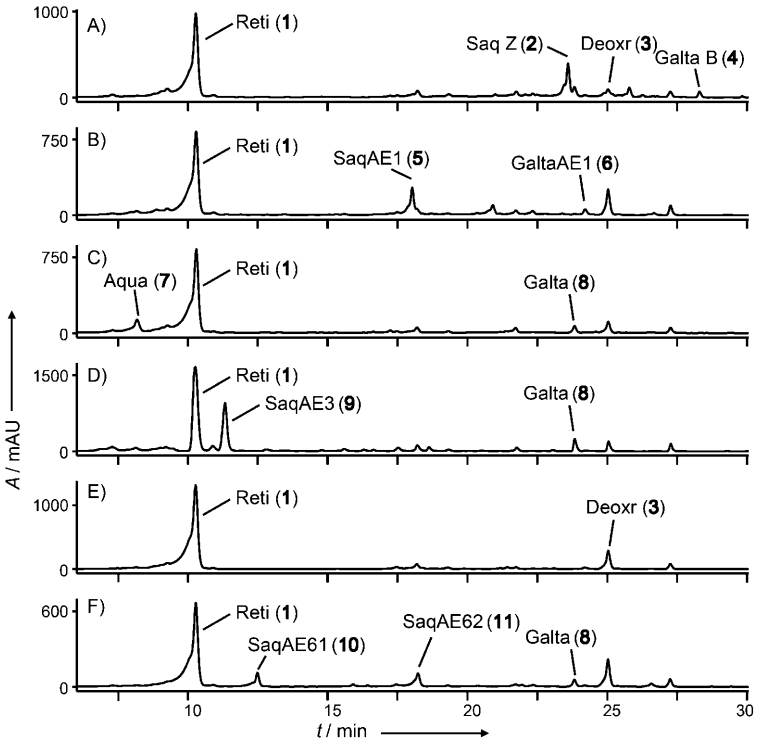


Figure 3. HPLC/ESI-MS analysis of crude extracts of *Micromonospora* sp. Tü6368. A) Wild type, B) Δ*saqGT1*, C) Δ*saqGT2*, D) Δ*saqGT3*, E) Δ*saqGT5* and F) Δ*saqGT6*; Reti: retimycin.

Inactivation of *saqGT5* and expression of glycosyltransferases in the *saqGT5* deletion mutant

The production profile of the mutant strain *M.* sp. Tü6368 Δ*saqGT5* was analyzed by HPLC/electrospray ionization mass spectrometry (ESI-MS) and compared to that of the wild type. The mutant was neither able to produce saquayamycin Z nor the tetracenequinone galtamycin B or any other tetracenequinone. Instead, 3-deoxyrabelomycin (**3**, Figure 3 E), a nonglycosylated angucyclic derivative which has also been detected in the wild type,[3] accumulated. This result demonstrates that SaqGT5 is a D-olivosyltransferase that forms a C–C glycosidic linkage. Furthermore, it proves that both saquayamycin Z and galtamycin B biosynthesis are encoded by the same gene cluster.

In a combinatorial biosynthetic approach, we expressed in the *saqGT5* mutant the olivosyltransferase genes *urdGT2*, simB7, and *lanGT2*, which are all involved in the biosynthesis of different angucyclines (Figure 4). UrdGT2 is derived from the urdamycin A producer *Streptomyces fradiae* Tü2717.[12] SimB7 is a C-glycosyltransferase that is involved in simocyclinone biosynthesis,[9] and LanGT2 forms an O-glycosidic linkage during the landomycin A biosynthesis.[13] As expected, UrdGT2 was able to restore wild-type production of saquayamycin Z and galtamycin B. Surprisingly, expression of *simB7* only led to the production of the monoglycosylated galtamycinone and did not restore wild-type production, which had been

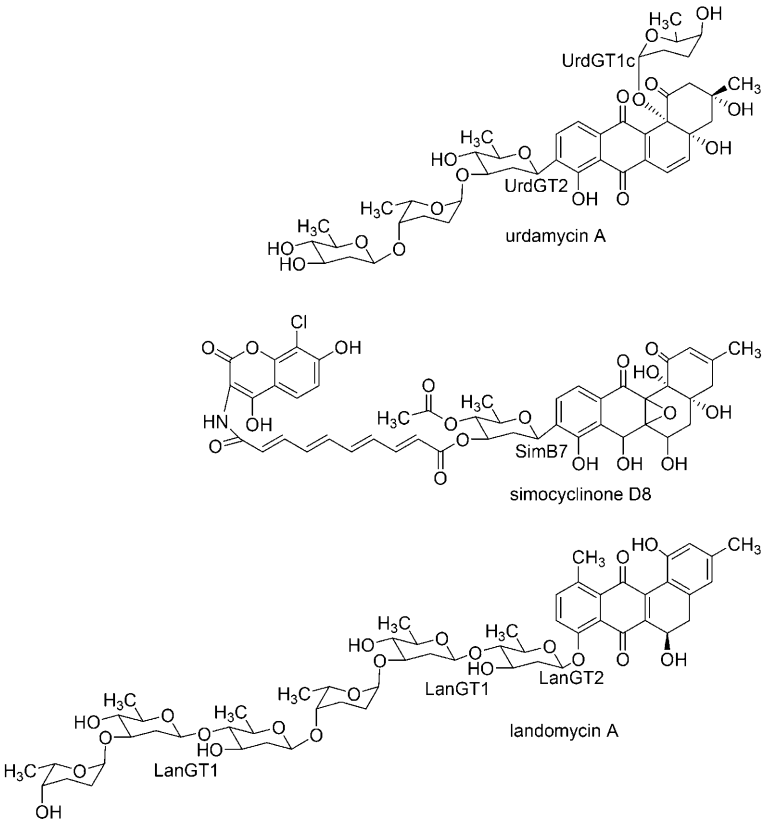


Figure 4. Chemical structures of urdamycin A, simocyclinone D8 and landomycin A. Only glycosyltransferases mentioned in this manuscript are shown.

expected. Expression of the *O*-glycosyltransferase *lanGT2* was expected to lead to *O*-glycosylated derivatives. However, it did not have any effect on the production profile of the *saqGT5* mutant (data not shown).

Inactivation of *saqGT2*

The HPLC/ESI-MS analysis of the mutant strain *M.* sp. Tü6368 Δ*saqGT2* revealed galtamycinone and one new compound (**7**, Figure 3C), which exhibited the characteristic UV/Vis spectrum of a saquayamycin derivative. The corresponding molecular mass was determined as *m/z* 485 $[M-H]^-$. This metabolite was identified as aquayamycin (Scheme 1) by comparison to an authentic standard. Aquayamycin is a mono-glycosylated angucycline, the structure of which has been known since the 1970s.[14] This suggested that SaqGT2 transfers the first sugar of the pentasaccharide.

Inactivation of *saqGT3*

Besides galtamycinone (**8**, Figure 3D), which also accumulates in the wild type, HPLC/ESI-MS analysis of extracts of the mutant *M.* sp. Tü6368 Δ*saqGT3* identified one novel compound, now referred to as SaqAE3 (**9**, Figure 3D), with a molecular ion of *m/z* 599 $[M-H]^-$. SaqAE3 showed the characteristic UV/Vis spectrum of a saquayamycin. To elucidate its structure, SaqAE3 was purified from a scaled-up fermentation and subjected to 1D (^{1}H, ^{13}C, NOE-1D) and 2D (^{1}H,^{1}H COSY, HSQC, HMBC) NMR spectroscopy. The resulting spectra of the orange powder were compared to the assignments of saquayamycin Z as published by Ströch and co-workers.[3] The ^{13}C NMR spectroscopic data of the aglycone were in full agreement with those of saquayamycin Z. In contrast to saquayamycin Z, however, only signals for two of the nine deoxy sugars were present in the NMR spectra of SaqAE3. These two sugars were the β-D-olivose of the aglycone (1′H ($\delta_H = 4.9$) to C 9 ($\delta_C = 138.3$)) and the α-L-rhodinose, which is connected to 3-O of the aglycone (1D H ($\delta_H = 5.25$) to C3 ($\delta_C = 82.4$)). The position of both sugars is the same as in saquayamycins A, B and Z.[3] The structure of the product SaqAE3 (Scheme 1) showed that *saqGT3* encodes the glycosyltransferase that transfers D-olivose to 4A-O of the L-rhodinose. Interestingly, the biosynthesis of the tetrasaccharide chain at C4′ seems to depend on the activity of SaqGT3 because no saquayamycin derivative with an intact tetrasaccharide chain was detected in the *saqGT3* mutant.

Inactivation of *saqGT6*

Analysis of extracts of the *saqGT6* deletion mutant by HPLC/ESI-MS revealed galtamycinone and two new peaks, SaqAE61 and SaqAE62, which exhibited UV/Vis spectra that were typical of saquayamycin (**10** and **11**, Figure 3F). The corresponding molecular masses were determined as *m/z* 729 $[M-H]^-$ for SaqAE61 and *m/z* 1087 $[M-H]^-$ for SaqAE62. The molecular mass of SaqAE62 reflected a mass difference of 354 Da compared to saquayamycin Z; this is consistent with the loss of the three deoxy sugars, L-2-deoxyfucose, L-rhodinose, and L-aculose of the tetrasaccharide chain. The mass difference of 358 Da compared to SaqAE62 suggests that SaqAE61 lacks another three deoxy sugars (two L-rhodinoses and one D-olivose) and is therefore a precursor of SaqAE62. Based on the production of galtamycinone, SaqAE61, and SaqAE62, which all contain the first sugar of the tetrasaccharide, we concluded that SaqGT6 attaches the second deoxy sugar of the tetrasaccharide chain, an L-2-deoxyfucose.

Inactivation of *saqGT1*

The HPLC/ESI-MS analysis of the mutant *M.* sp. Tü6368 Δ*saqGT1* showed that both saquayamycin Z and galtamycin B were absent in the mutant. Instead, two novel compounds, named SaqAE1 and GaltaAE1, which exhibited the typical UV/Vis spectrum of saquayamycin and galtamycin, respectively, were detected (**5** and **6**, Figure 3B). The corresponding molecular ions of *m/z* 1218 $[M-H]^-$ for SaqAE1 and 579 $[M-H]^-$ for GaltaAE1 reflected a mass difference of 224 Da in both cases in reference to saquayamycin Z and galtamycin B, respectively. This was consistent with the loss of the last two deoxy sugars of the tetrasaccharide chain, L-rhodinose and L-aculose, and shows that SaqGT1 transfers L-rhodinose onto the growing tetrasaccharide side-chain.

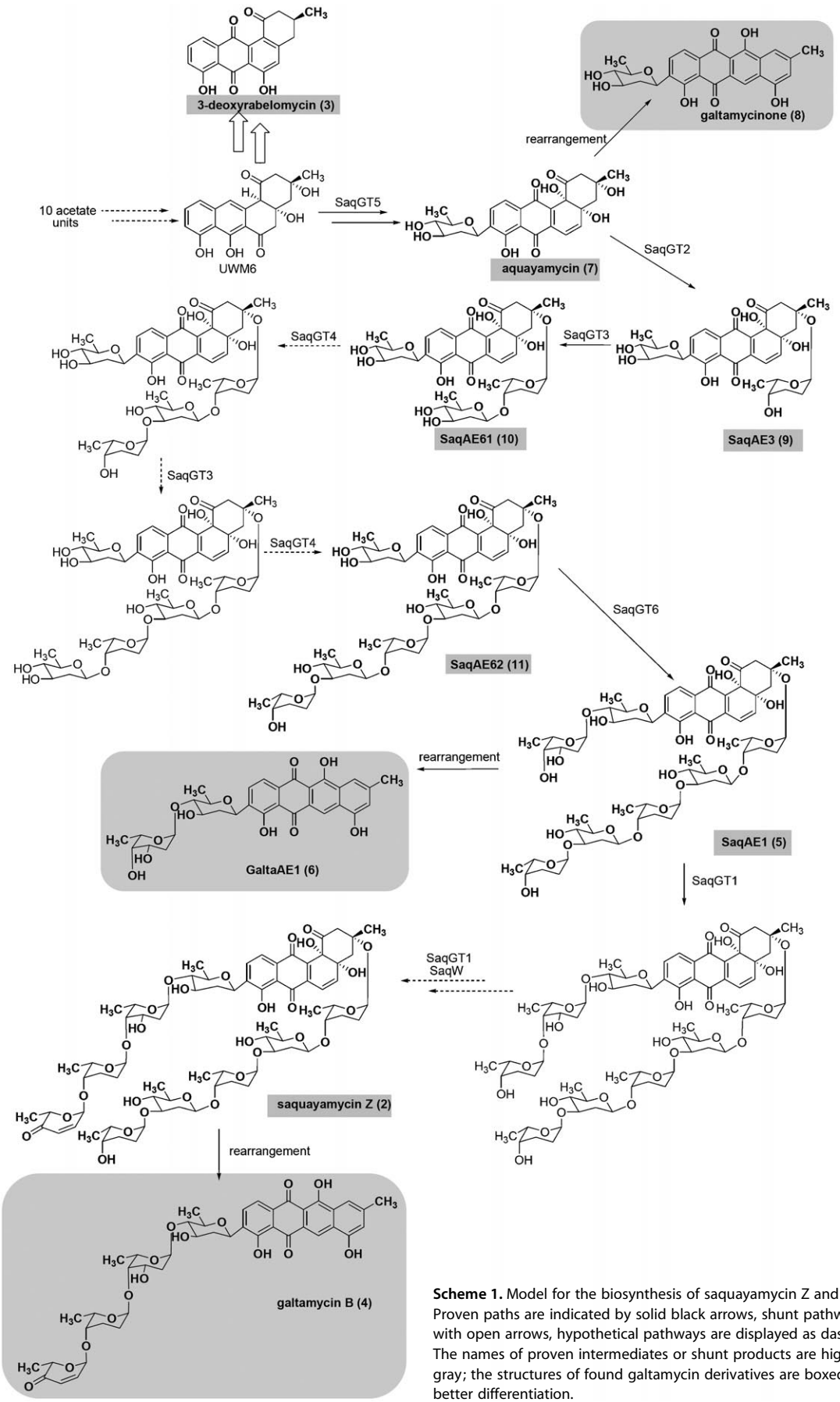
Heterologous expression of *saqGT2*

To confirm the function of SaqGT2, the gene *saqGT2* was heterologously expressed in *Streptomyces fradiae* Ax, an aquayamycin-producing mutant (Scheme 1) that contains only one glycosyltransferase (UrdGT2).[15] Because of an observed different codon usage of *Streptomyces* and *Micromonospora* species, two rare codons were substituted by PCR to optimize translation. In contrast to the original expression of the unmodified gene of *saqGT2*, which did not lead to any new product, expression of the modified gene led to a new compound with the characteristic UV/Vis spectrum of a saquayamycin. The substance exhibited the same molecular ion peak (*m/z* 599 $[M-H]^-$) and the same retention time as SaqAE3; this indicates that SaqGT2 is indeed the first glycosyltransferase that is involved in the assembly of the pentasaccharide chain that connects α-L-rhodinose to 3-O of the aglycone.

Model for saquayamycin Z and galtamycin B biosynthesis

The functional study of the *saq* gene cluster combined with the results from the inactivation and expression experiments allows a proposal for the biosynthesis of both compounds, with a detailed insight into the assembly of the sugar chains, as presented in Scheme 1. The pathway is comprised of three main parts: 1) formation and modification of the polyketide aglycone, 2) biosynthesis of the four deoxysugars D-olivose, L-rhodinose, L-2-deoxyfucose, and L-aculose and 3) attachment of sugars by glycosyltransferases.

The assembly of the aglycone begins with the biosynthesis of the decaketide, which is catalyzed by the enzymes SaqA, SaqB, and SaqC that form the type II minimal polyketide syn-



Scheme 1. Model for the biosynthesis of saquayamycin Z and galtamycin B. Proven paths are indicated by solid black arrows, shunt pathways are shown with open arrows, hypothetical pathways are displayed as dashed arrows. The names of proven intermediates or shunt products are highlighted in gray; the structures of found galtamycin derivatives are boxed in gray for a better differentiation.

thase.[16] The decaketide chain is modified by the ketoreductases SaqD and SaqN, the cyclases SaqF and SaqL, and the reductase SaqO. The oxygenases SaqE and SaqM introduce a keto group and a hydroxyl group to the aglycone, respectively, resulting in the angucyclic aglycone.

All our results indicate that the identified gene cluster is responsible for the biosynthesis of both saquayamycin Z and galtamycin B, because in various mutants, the production of both compounds was affected. Because no galtamycin derivative was detectable in the *saqGT5* mutant, we conclude that the tetracenequinone galtamycin B is derived from the angucyclic saquayamycin Z, most probably through a rearrangement. This might be catalyzed by a so-far unknown mechanism.

The accumulation of 3-deoxyrabelomycin in the *saqGT5* mutant indicates that SaqGT5 is a *C*-glycosyltransferase, which transfers β-D-olivose to *C*9 of the angucyclic aglycone. Interestingly, the production of saquayamycin Z and galtamycin B could be restored by expressing either *saqGT5* or *urdGT2* in this mutant. Expression of *simB7* only led to the accumulation of galtamycinone, which might indicate that SimB7 is not able to interact with other glycosyltransferases in a putative enzyme complex.

SaqGT2 is responsible for the second enzymatic glycosylation step, the attachment of L-rhodinose at 3-O of the aglycone. This was shown by the accumulation of aquayamycin and galtamycinone in the *saqGT2* mutant and by the production of SaqAE3 by *S. fradiae* Ax x *saqGT2*.

SaqAE3 was the major compound that was produced by the *saqGT3* mutant; this indicates that SaqGT3 is the olivosyltransferase that attaches the second sugar of the pentasaccharide. SaqGT3 shows the highest similarity to LanGT1, an iteratively acting olivosyltransferase that is involved in landomycin A biosynthesis (Figure 4).[4] Therefore we suggest that SaqGT3 is also acting iteratively and is responsible for the attachment of the second and the fourth sugar (both D-olivoses) during the biosynthesis of the pentasaccharide chain.

The transfer of the third and the fifth sugar of the pentasaccharide, both L-rhodinoses, is probably accomplished by SaqGT4, which is similar to the rhodinosyltransferase UrdGT1c (Figure 4) that is involved in urdamycin A biosynthesis.[15] Unfortunately, we did not succeed in generating a mutant of *saqGT4*, although several experiments have been performed. Interestingly, in the cluster, *saqGT4* overlaps with *saqGT3*, which itself overlaps with *saqGT2*. Because both SaqGT2 and SaqGT3 act during the biosynthesis of the pentasaccharide, this might be an additional hint that SaqGT4 is also involved in the assembly of this chain.

The production of SaqAE62 and galtamycinone in the *saqGT6* deletion mutant identified SaqGT6 as the glycosyltransferase that transfers an L-2-deoxyfucose onto the 4-*O* of the D-olivose; this establishes a 4→1 linkage between those two sugars. Interestingly, most L-deoxysugars that are components of saccharide chains of natural products (e.g., mithramycin, chromomycin A_3, landomycin, and urdamycin A) are connected via 3→1 linkages to D-olivose.[17–20] Therefore, SaqGT6, which differs from other angucycline glycosyltransferases and shows less than 46% identity on amino acid level is a unique enzyme with special regioselectivity. Together with the results from the *saqGT2* and *saqGT3* mutants that accumulated derivatives that contained only the first sugar of the tetrasaccharide, we conclude that the enzymatic activity of SaqGT6 depends on the presence of the pentasaccharide chain at 3-*O*.

SaqGT1 transfers the third saccharide, an L-rhodinose, onto the growing tetrasaccharide chain. This was demonstrated by the formation of the biosynthetic intermediates SaqAE1 and GaltaAE1 that were produced by the *saqGT1* mutant.

SaqW is similar to the oxidoreductase AknOx, which converts L-rhodinose via cinerulose A to L-aculose in the biosynthesis of the anthracycline aclacinomycin.[10] We propose that SaqGT1 attaches the third and the fourth sugars (both L-rhodinoses) in a similar fashion to the tandem addition of two L-2-deoxyfucoses to rhodosaminyl aklavinone catalyzed by AknK.[21] We suggest that SaqW is involved in the subsequent conversion of the last sugar to an L-aculose moiety. Further studies on what controls the level of iteration will be performed, similar to the studies carried out with glycosyltransferases involved in landomycin biosynthesis.[22]

Interestingly, the mono-glycosylated galtamycinone instead of the expected fully glycosylated galtamycin B accumulated in the *saqGT2* and *saqGT3* mutants. This suggests that glycosyltransferases that act on the tetrasaccharide chain are only acting on the angucycline and not on the tetracenequinone.

Conclusions

We have cloned and sequenced the common biosynthetic gene cluster of the largest known angucycline, saquayamycin Z, and the tetracenequinone galtamycin B. The formation of the tetracenequinone galtamycin B results from a rearrangement reaction from angucyclines. The function of five glycosyltransferases has been elucidated; this led to a detailed insight into the assembly of the sugar chains. The biosynthesis of saquayamycin Z probably involves three iteratively acting glycosyltransferases. SaqGT2 was identified as a glycosyltransferase with a new position of transfer. This sets the stage for future studies on the function and specificity of glycosyltransferases

Experimental Section

Bacterial strains, growth conditions, media and vectors: For standard purposes, *M.* sp. Tü6368[2] and its mutant strains and *S. fradiae* Tü2717 Ax[15] were grown on tryptone soy broth (TS broth),[23] which was prepared as solid or liquid medium, at 28 °C. For saquayamycin Z and galtamycin B production, NL MMM[2] liquid medium (glucose (1%), soluble starch (2%), yeast extract (0.5%), Bacto casitone (0.5%) and $CaCO_3$ (0.1%) in tap water; the pH was adjusted to 7.6 prior to sterilization) with Amberlite® XAD-16 (4%) was used. For production of SaqAE3 by *S fradiae* Ax x *saqGT2*, E1 liquid medium (starch (20%), glucose (20%), yeast extract (2.5%), pharmamedia (3%), $CaCO_3$ (1%), NaCl (1%), $K_2HPO_4 \cdot 3H_2O$ (1%), $MgSO_4 \cdot 7H_2O$ (1%) in tap water, the pH was adjusted to 7.2 prior to sterilization) was used. For genomic DNA isolation, *M.* sp. Tü6368 was grown for 24–48 h in TSB^+ liquid medium (tryptic soy broth (3%), glycine (0.4%), sucrose (10%) in tap water). DNA manipulation was carried out in *E. coli* XL1-Blue (Stratagene).

Table 2. Oligonucleotides used in this study.

Primer	Sequence (5′→3′)	Restriction site
SaqF	ATGCACAGCACTCTAGATCGTCG	—
SaqR	CCGGGTCGTAGGCCTCGAGAACGG	—
saqGT2aadF	*ATGACGGCGACGATCGCCGCCACCGGGCTGACCCCCGTC***ACTAGT**CCGTATTTGCAGTACCAGCG[a,b]	SpeI
saqGT2aadR	*ATGAGTACGCGCATGTGCTCTCCTGTCCGTGAGGTGATCG***ACTAGT**TGTAGGCTGGAGCTGCTTC[a,b]	SpeI
saqGT3aadF	*ATGGCGCACTCGGCGGGGTTGAGCGCGGTCAGCATCGCC***ACTAGT**CCGTATTTGCAGTACCAGCG[a,b]	SpeI
saqGT3aadR	*AGGCGGCCTCCCTCGTCTCGGCGGCGCCGGGGCGGGCCAG***ACTAGT**TGTAGGCTGGAGCTGCTTC[a,b]	SpeI
saqGT6aad-F	*CTCGTCGATCCGCGAGTTCGTGAAGGCCCGTAGCTGATG***ACTAGT**CCGTATTTGCAGTACCAGCG[a,b]	SpeI
saqGT6aad-R	*CGCGGCGGATGCGGCGGCCGCGAGGGCGCCGGATGGTCA***ACTAGT**TGTAGGCTGGAGCTGCTTC[a,b]	SpeI
SaqGT-f-Mun	ACGGCCGC**CAATTG**CATGCTC[a]	MunI
SaqGT-f-Xba	CGCCACGA**TCTAGA**CGGCGACC[a]	XbaI
saqGT5_3 EcoRI	CGCGGTTC**GAATTC**GGGCAC[a]	EcoRI
saqGT5_3 XbaI	CCGGACAGG**TCTAGA**GCCTTC[a]	XbaI
saqGT2-F-EcoRI	GCGCGATC**GAATTC**CTCGTC[a]	EcoRI
saqGT2-R-XbaI	CGAAGTGCG**TCTAGA**CCGG[a]	XbaI
saqGT2_codonF	AGCAACGGAGGTACGGACTTGCGGGTCTTGTTCGTGACC*TTC*CCC[c]	—
saqGT2_codonR	GCGTGGACACCGGGGTGGCCATCA*GGA*[c]	—
thioF	*ATGACTGAGTTGGACACCATCGCAAATCCGTCCGATCCC*GCCGTATTTGCAGTACCAGCG[b]	—
thioR	*TTATCGGGTGGCCGCGAGATTCCTGTCGATCCTTCT*CGTGCTGTAGGCTGGAGCTGCTTC[b]	—

[a] Artificially introduced restriction sites are in bold. [b] The at-first nonhybridizing part is italicized. [c] Exchanged codons are italicized and underlined.

E. coli DH5α (Invitrogen) was used for construction of the *M.* sp. Tü6368 genomic cosmid library. For intergeneric conjugation between *E. coli* and *M.* sp. Tü6368 or *Streptomyces fradiae* Tü2717 Ax, the methylase deficient *E. coli* ET12567 with plasmid pUZ8002 was used as a donor strain. *E. coli* strains were grown on LB agar or liquid medium that contained the appropriate antibiotic for selection as described.[24] Vector pBluescript SK(−) (pBSK-) was from Stratagene; Litmus28, pET3d,[25] and pUC19[26] were from New England Biolabs (Frankfurt, Germany); and pKC1132, which carried the apramycin-resistance gene that was used for gene disruption, was from Eli Lilly and Company (Indianapolis, IN, USA).[27] pSET-1cerm[28] was used for the generation of complementation plasmids. The vector pUWL-oriT-*aac*(3)IV was constructed during this study. It is derived from pUWL-oriT;[29] the thiostreptone-resistance gene was replaced by the apramycin-resistance gene by using the Red/ET® recombineering method with primers thioF and thioR (Table 2).

General genetic manipulation and PCR: Standard molecular biology procedures were performed as described previously.[24] Isolation of plasmid DNA from *E. coli* and DNA restriction/ligation were performed by following the protocols of the manufacturers of the kits, enzymes, and reagents, Qiagen, Promega, and Roche Diagnostics. PCR reactions were performed with a Gene Amp® PCR System 9700 (Applied Biosystems) by using Pfu polymerase (Promega) for complementation and expression experiments and Taq or GoTaq polymerase (Promega) to verify mutants. Primers were purchased from Operon Biotechnologies, Inc. (Cologne, Germany). Oligonucleotide primers that were used are listed in Table 2.

Construction and screening of a *M.* sp. Tü6368 cosmid library: A cosmid library was prepared by using cosmid pOJ436.[27] For preparation of DNA, the mycelium was embedded in agarose. Cell disruption, partial digestion of the genomic DNA, and separation of DNA fragments were performed as described previously.[30] A total of 2300 cosmid clones were screened with two strain-specific probes, which were both obtained by PCR: a type II polyketide synthase probe and an dNDP-D-glucose-4,6-dehydratase probe,[31] by following standard nonradioactive hybridization procedures with digoxigenin (DIG)-labeled DNA probes. Five cosmids were subjected to PCR screening with primers SaqF and SaqR (Table 2), which were directed against conserved sequences of cyclases.

DNA sequencing and computer-assisted sequence analysis: Nucleotide sequences were determined at 4base lab GmbH (Reutlingen, Germany) and at GATC Biotech AG (Konstanz, Germany) by using either standard primers (M13 universal and reverse, T3 and T7) or customized, internal primers. Computer-assisted sequence analysis was done with the Clone Manager software (Clone Manager 7, version 7.11). Database comparison was performed with the BLAST search tools on the server of the National Center for Biotechnology Information, National Library of Medicine, NIH (http://www.ncbi.nlm.nih.gov/).[32] The sequence that is reported here has been deposited in the *GenBank* database (http://www.ncbi.nlm.nih.gov/Genbank) under the accession number FJ670504.

Intergeneric conjugation between *E. coli* and *Micromonospora* sp. Tü6368: Plasmids were transferred to *M.* sp. Tü6368 by intergeneric conjugation between *E. coli* and *M.* sp. Tü6368. A frozen mycelial culture of *M.* sp. Tü6368 (1 mL) was diluted in TS broth and was agitated at 28 °C and 180 rpm for 24 h. A proportion of this seed culture was transferred into fresh TS broth. The culture was again agitated at 28 °C and 180 rpm for 24 h. The mycelium was recovered by centrifugation, washed once in fresh TS broth, and resuspended in TS broth (recipient culture). The *E. coli* donor ET 12567 (pUZ8002) was grown at 37 °C for 16–18 h on LB agar plus apramycin (50 μg mL^{-1}) and kanamycin (30 μg mL^{-1}). These donor cells were detached from the plate with a loop and resuspended in the recipient culture. Samples of this combination (300 μL) were plated on MS medium (20 g L^{-1} D-mannitol, 20 g L^{-1} soy flour, 18 g L^{-1} agar, pH 7.2). Plates were incubated at 28 °C for 10–12 h and then covered with water (1 mL) that contained phosphomycin and apramycin to a final concentration of 400 μg mL^{-1} and 25 μg mL^{-1}, respectively, for selection of exconjugants. Incubation at 28 °C was continued for 7–10 days until exconjugants appeared.

Construction of gene inactivation constructs: For the generation of chromosomal mutants of the saquayamycin Z and galtamycin B

producer *M.* sp. Tü6368 by homologous recombination, the gene disruption plasmids were constructed as described below.

To inactivate *saqGT1*, a 6.2 kb BamHI fragment of cosmid 20 was ligated into the same sites of pBSK- to yield plasmid B2062. A unique NcoI restriction site inside the gene *saqGT1* was chosen for targeted inactivation by shifting the reading frame. After NcoI restriction, treatment with the T4 DNA polymerase and re-ligation, the intended alteration was confirmed by DNA sequencing. The internal fragment was inserted into the BamHI–HindIII sites of pKC1132 to yield the inactivation construct pKC-Δ*saqGT1*.

The deletion of the three genes *saqGT2*, *saqGT3*, and *saqGT6* resulted from the replacement of each gene by the *aadA* cassette of vector pIJ778[33] by using the Red/ET recombineering method. For deletion of *saqGT2* and *saqGT3*, a 9 kb NcoI fragment from cosmid 23 was cloned into Litmus28. Both genes were disrupted by Red/ET recombineering with the corresponding primers saqGT2aadF and saqGT2aadR for *saqGT2* inactivation and saqGT3aadF and saqGT3aadR for *saqGT3* inactivation. These primers were used to introduce SpeI sites to both sites of the cassette. Hence, the NcoI fragments that contained the deleted sequence of *saqGT2* and *saqGT3*, respectively, were cloned to pET3d (pET-Δ*saqGT2-aadA* and pET-Δ*saGT3-aadA*). In both cases the incorporated spectinomycin cassette was removed by SpeI restriction and re-ligation to avoid polar effects. The fragments were then cloned (HindIII and XbaI) into pKC1132 to yield pKC-Δ*saqGT2* and pKC-Δ*saqGT3*. For deletion of *saqGT6* a 5.8 kb KpnI fragment from cosmid 23 was cloned into pUC19. The fragment was then cloned after EcoRI and XbaI restriction into pKC1132. The primers saqGT6-aadF and saqGT6-aadR were used to replace *saqGT6* by *aadA*. The primers were used to introduce SpeI sites to both sites of the cassette; to avoid polar effects the cassette was removed by SpeI restriction, which led to the plasmid pKC-Δ*saqGT6*.

The inactivation construct of *saqGT5* was generated by ligation of a 5.7 kb NcoI fragment of cosmid 23 into the corresponding sites of Litmus28 and successive SphI restriction, treatment with T4 DNA polymerase, and re-ligation. The shifted reading frame was confirmed by sequencing. The mutated 5.7 kb fragment was cloned (EcoRI and XbaI) into pKC1132 to generate pKC-Δ*saqGT5*.

Generation of chromosomal mutant strains of *Micromonospora* sp. Tü6368: For the generation of all deletion mutants, single crossover mutants were screened for loss of vector-resistance as a consequence of a double crossover event. Deletions within the genes were confirmed by PCR and/or Southern hybridization.

Construction of complementation and expression plasmids: For the generation of plasmids that were used to complement the mutant strains Δ*saqGT1* and Δ*saqGT5*, *saqGT1* and *saqGT5* were amplified by PCR by using Pfu polymerase. Suitable restriction sites (for *saqGT1*, MunI and XbaI; for saqGT5, EcoRI and XbaI) were introduced upstream and downstream of each gene by using primers SaqGT-f-Mun/SaqGT-f-Xba and saqGT5_3 EcoRI/saqGT5_3 XbaI (Table 2), respectively. The 1.5 kb PCR product of *saqGT1* was digested with MunI and XbaI and ligated into plasmid pSET-1cerm, which had been digested by the same enzymes to remove *urdGT1c*; this yielded the complementation plasmid pSET–*saqGT1*. To generate the complementation plasmid pSET–*saqGT5*, a 1.2 kb fragment that contained *saqGT5* was amplified. After digestion with EcoRI and XbaI, the fragment was ligated into Litmus28 to create plasmid pLit–*saqGT5*. Plasmid pSET–1cerm was digested with MunI and XbaI to remove *urdGT1c*, and the EcoRI-XbaI fragment from pLit-saqGT5 containing *saqGT5* was fused to *ermE** to generate complementation plasmid pSET–*saqGT5*.

To generate the expression plasmids for *saqGT2*, *saqGT2* was amplified by PCR by using Pfu polymerase and primers saqGT2-F-EcoRI and saqGT2-R-XbaI for the original gene and primers saqGT2_CodonF and saqGT2_CodonR to generate the modified gene (Table 2). The 1.4 kb PCR product of the wild-type gene *saqGT2* was digested with EcoRI and XbaI and ligated into the MunI/XbaI sites of pSET-1cerm to yield the expression plasmid pSET–*saqGT2*. The 1.3 kb PCR product of the modified gene *saqGT2* was ligated into the SmaI site of pBSK-. Hence, the gene was ligated into pUWL-oriT-*aac*(3)IV by using the EcoRI and SpeI sites, to generate the expression plasmid pUWE-saqGT2.

Analysis of saquayamycin Z and galtamycin B production and isolation of new metabolites: Wild type and mutant strains of *M.* sp. Tü6368 were cultured in production medium for seven days at 28 °C in a rotary shaker (180 rpm). Mycelia and Amberlite XAD-16 resin were collected by centrifugation and extracted twice with acetone at room temperature. After removal of the mycelia by centrifugation, the extract was evaporated to reduce the amount of acetone. Finally, this mycelium extract was extracted twice with an equal volume of ethyl acetate. The solvent of the organic phase was removed, and the residue was dissolved in methanol. This solution was used for analysis.

S. fradiae Ax was cultured in production medium for seven days at 28 °C in a rotary shaker (180 rpm). The supernatant was extracted twice with an equal volume of ethyl acetate. The solvent of the organic phase was removed, and the residue was dissolved in methanol. This solution was used for analysis.

Detection of saquayamycin Z, galtamycin B and their intermediates by HPLC-UV/Vis/MS was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) with an electrospray chamber and a quadrupole detector. HPLC analysis was carried out on a Zorbax XDB-C8 column (5 μm, 4.6 by 150 mm) with a Zorbax SB-C8 precolumn (5 μm, 4.6 by 12.5 mm) from Agilent Technologies. A nonlinear gradient from 20% to 95% acetonitrile in 0.5% acetic acid over 30 min at a flow rate of 0.7 mL min^{-1} was used. The column temperature was 30 °C, and the UV detection wavelengths were 254 and 270 nm. The mass-selective detector chamber settings were as follows: drying gas flow rate: 12 L min^{-1}; nebulizing pressure: 35 psi g^{-1}; drying gas temperature: 350 °C. The samples were analyzed in positive and negative-scan modes with a mass range of 250 to 2000 Da.

SaqAE3 was purified by using preparative TLC (silica gel 60 F_{254}, Merck, Darmstadt, Germany; CH_2Cl_2/MeOH 9:1 (*v*/*v*), 0.5% acetic acid). Further purification was performed by using an Agilent 1100 system (see above) equipped with an Agilent® Zorbax SB-C18 column (150×9.6 mm, 5 μm) by using mass-guided fraction collection. An acetonitrile gradient over 24 min ranged from 25 to 95% at a flow rate of 3.0 mL min^{-1}. SaqAE3 (~8 mg) was isolated as an orange powder. For structural elucidation by NMR spectroscopy, SaqAE3 was dissolved in $CDCl_3$.

Structure elucidation of SaqAE3: The chemical structure of SaqAE3 was elucidated with 1D NMR (^1H (400 MHz), ^{13}C (100 MHz), 1D-NOE, 1D-TOCSY)) and 2D NMR (HSQC, HMBC, ^1H-^1H COSY) spectroscopy by using a Bruker Avance DRX400. Chemical shifts are expressed in δ values (ppm) by using the correspondent solvent as internal reference ($CDCl_3$: $\delta_H = 7.26$, s; $\delta_C = 77.0$, t). NMR spectroscopy data are shown in Table 3.

Table 3. ^{1}H NMR (400 MHz, $[D_1]CDCl_3$) and ^{13}C NMR (100 MHz, $[D_1]CDCl_3$) spectroscopy data as well as 2D NMR (HMBC) assignments of SaqAE3 (**9**).

Position	δ_C [ppm]	δ_H [ppm] (m, I, *J* [Hz])[a]	HMBC ($^{1}H \rightarrow ^{13}C$)
1	204.8	–	–
2	50.5	2.55 (m, 1H); 3.2 (m, 1H)	C1, C3, C4, C12b
3	82.4	–	–
4	44.5	1.85 (m, 1H); 2.3 (dd, 1H, 15.2; 2.8)	C2, C4a, C12b
4a	79.8	–	–
4a-OH		4.3 (s, 1H)	C4, C4a, C5
5	145.5	6.4 (d, 1H, 9.8)	C6a, C12b
6	117.4	6.9 (d, 1H, 9.8)	C4a, C6a, C7
6a	138.7	–	–
7	188.2	–	–
7a	113.9	–	–
8	157.9	–	–
8-OH		12.3 (s, 1H)	C7a, C8, C9
9	138.3	–	–
10	133.6	7.9 (d, 1H, 7.8)	C8, C11a, C1′
11	119.7	7.6 (d, 1H, 7.8)	C7a, C9, C10, C12
11a	130.2	–	–
12	182.2	–	–
12a	138.7	–	–
12b	77.4	–	–
13	25.6	1.45 (s, 3H)	C2, C3, C4
1′	71.1	4.9 (d, 1H, 11.1)	C3′, C8, C9, C10
2′	39.5	1.4 (m, 1H); 2.5 (m, 1H)	C8, C1′, C3′, C4′
3′	72.9	3.85 (m, 1H)	C4′
4′	78.0	3.2 (m, 1H)	C3′, C6′
5′	75.9	3.55 (m, 1H)	C1′
6′	18.0	1.4 (d, 3H, 6.3)	C4′, C5′
1A	92.5	5.25 (d, 1H, 3.3)	C3, C3A, C5A
2A	23.8	1.45 (m, 1H); 2.05 (m, 1H)	C1A, C3A
3A	25.5	1.76 (m, 1H); 1.85 (m, 1H)	C2A
4A	66.9	3.7 (s, 1H)	–
5A	67.2	4.2 (q, 1H, 6.7)	C4A, C6A
6A	17.0	1.3 (d, 3H, 6.6)	C3A, C4A, C5A

[a] m: multiplicity; br: broad; I: intensity; *J*: coupling constant.

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[1] J. S. Thorson, T. Vogt, *Carbohydrate-Based Drug Discovery* (Ed.: C.-H. Wong), VCH, Weinheim, **2003**, pp. 685–711.
[2] N. Antal, H.-P. Fiedler, E. Stackebrandt, W. Beil, K. Ströch, A. Zeeck, *J. Antibiot.* **2005**, *58*, 95–102.
[3] K. Ströch, A. Zeeck, N. Antal, H.-P. Fiedler, *J. Antibiot.* **2005**, *58*, 103–110.
[4] A. Luzhetskyy, M. Fedoryshyn, C. Dürr, T. Taguchi, V. Novikov, A. Bechthold, *Chem. Biol.* **2005**, *12*, 725–729.
[5] O. Gabriel, *Adv. Chem. Series* **1973**, *117*, 387–410.
[6] X. He, J. S. Thorson, H. W. Liu, *Biochemistry* **1996**, *35*, 4721–4731.
[7] C. R. Woese, *Microbiol. Rev.* **1987**, *51*, 221–271.
[8] B. Ostash, Y. Rebets, V. Yuskevich, A. Luzhetskyy, V. Tkachenko, V. Fedorenko, *Folia Microbiol.* **2003**, *48*, 484–488.
[9] A. Trefzer, S. Pelzer, J. Schimana, S. Stockert, C. Bihlmaier, H.-P. Fiedler, K. Welzel, A. Vente, A. Bechthold, *Antimicrob. Agents Chemother.* **2002**, *46*, 1174–1182.
[10] I. Alexeev, A. Sultana, P. Mäntsälä, N. Niemi, G. Schneider, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 6170–6175.
[11] K. F. Chater, M. Bibb, *Biotechnology: Products of Secondary Metabolism, Vol. 7* (Eds.: H. Kleinkauf, H. von Dören), VCH, Weinheim **1997**, pp. 57–105.
[12] B. Faust, D. Hoffmeister, G. Weitnauer, L. Westrich, S. Haag, P. Schneider, H. Decker, E. Künzel, J. Rohr, A. Bechthold, *Microbiology* **2000**, *146*, 147–154.
[13] A. Luzhetskyy, T. Taguchi, M. Fedoryshyn, C. Dürr, S. E. Wohlert, V Novikov, A. Bechthold, *ChemBioChem* **2005**, *6*, 1406–1410.
[14] M. Sezaki, S. Kondo, K. Maeda, H. Umezawa, M. Ono, *Tetrahedron* **1970**, *26*, 5171–5190.
[15] A. Trefzer, D. Hoffmeister, E. Künzel, S. Stockert, G. Weitnauer, L. Westrich, U. Rix, J. Fuchser, K. U. Bindseil, J. Rohr, A. Bechthold, *Chem. Biol.* **2000**, *7*, 133–142.
[16] C. Hertweck, A. Luzhetskyy, Y. Rebets, A. Bechthold, *Nat. Prod. Rep.* **2007**, *24*, 162–190.
[17] S. E. Wohlert, E. Künzel, R. Machinek, C. Méndez, J. A. Salas, J. Rohr, *J. Nat. Prod.* **1999**, *62*, 119–121.
[18] N. Menéndez, M. Nur-e-Alam, A. F. Braña, J. Rohr, J. A. Salas, C. Méndez, *Chem. Biol.* **2004**, *11*, 21–32.
[19] T. Henkel, J. Rohr, J. M. Beale, L. Schwenen, *J. Antibiot.* **1990**, *43*, 492–503.
[20] H. Drautz, H. Zähner, J. Rohr, A. Zeeck, *J. Antibiot.* **1986**, *39*, 1657–1669.
[21] W. Lu, C. Leimkuhler, M. Oberthür, D. Kahne, C. T. Walsh, *Biochemistry* **2004**, *43*, 4548–4558.
[22] C. Krauth, M. Fedoryshyn, C. Schleberger, A. Luzhetskyy, A. Bechthold, *Chem. Biol.* **2009**, *16*, 28–35.
[23] T. Kieser, M. J. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, **2000**.
[24] J. Sambrook, D. W. Russell, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, New York, **2001**.
[25] A. H. Rosenberg, B. N. Lade, D. S. Chui, S. W. Lin, J. J. Dunn, F. W. Studier, *Gene* **1987**, *56*, 125–135.
[26] C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **1985**, *33*, 103–119.
[27] M. Bierman, R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, B. E. Schoner, *Gene* **1992**, *116*, 43–49.
[28] D. Hoffmeister, K. Ichinose, A. Bechthold, *Chem. Biol.* **2001**, *8*, 557–567.
[29] A. Luzhetskyy, L. Zhu, M. Gibson, M. Fedoryshyn, C. Dürr, C. Hofmann, D. Hoffmeister, B. Ostash, C. Mattingly, V. Adams, V. Fedorenko, J. Rohr, A. Bechthold, *ChemBioChem* **2005**, *6*, 675–678.
[30] M. Redenbach, H. M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, D. A. Hopwood, *Mol. Microbiol.* **1996**, *21*, 77–96.
[31] A. Luzhetskyy, H. Weiss, A. Chargé, E. Welle, A. Linnenbrink, A. Vente, A. Bechthold, *Appl. Gen. Mol. Biotech.* **2007**, *75*, 1367–1375.
[32] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **1997**, *25*, 3389–3402.
[33] B. Gust, G. L. Challis, K. Fowler, T. Kieser, K. F. Chater, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1541–1546.

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