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Novel Avilamycin Derivatives with Improved Polarity Generated by Targeted Gene Disruption

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Summary

The oligosaccharide antibiotics avilamycin A and C are produced by *Streptomyces viridochromogenes* Tü57. Both consist of a heptasaccharide chain, which is attached to a polyketide-derived dichloroisoeverninic acid moiety. They show excellent antibiotic activity against Gram-positive bacteria. Both molecules are modified by O-methylation at different positions, which contributes to poor water solubility and difficulties in galenical drug development. In order to generate novel avilamycin derivatives with improved polarity and improved pharmacokinetic properties, we generated a series of mutants with one, two, or three mutated methyltransferase genes. Based on the structure of the novel avilamycin derivatives, the exact function of three methyltransferases, AviG2, AviG5, and AviG6, involved in avilamycin biosynthesis could be assigned.

Introduction

Antibiotic resistance is increasing dramatically among both Gram-positive and Gram-negative bacteria [1]. The continuous evolution of new resistance mechanisms results in a permanent need for the development of novel drugs. Avilamycin, a natural product of *Streptomyces viridochromogenes* Tü57 (*S. viridochromogenes* Tü57), along with curamycins, everninomycins, and flambamycins, belongs to the orthosomycin class of antibiotics [2]. Structural features of the avilamycins are a terminal dichloroisoeverninic acid moiety (residue A in Figure 1) and a heptasaccharide side chain consisting of D-olivose (residues B and C), 2-deoxy-D-evalose (residue D), 4-O-methyl-D-fucose (residue E), 2,6-di-O-methyl-D-mannose (residue F), L-lyxose (residue G), and eurekanate (residue H). Avilamycin A and avilamycin C are the main products of *S. viridochromogenes* Tü57. The structures of these compounds differ in one residue at C56 of the molecule (avilamycin A: $COCH_3$; avilamycin C: $CH(OH)CH_3$). Avilamycin and evernimicin, the most prominent examples of the orthosomycins, show excellent activity against a broad range of Gram-positive pathogenic bacteria including glycopeptide-resistant *enterococci*, methicillin-resistant *staphylococci*, and penicillin-resistant *streptococci* [3–6] and are therefore interesting candidates for the generation of new therapeutics. Evernimicin (Ziracin), which is structurally very similar to avilamycin, already passed through clinical studies phase II and phase III. Due to a mismatch of activity to side effects and due to impurities in the drug, its further development as a drug was stopped by Schering-Plough in May 2000 [7, 8]. Both avilamycin and evernimicin were shown to inhibit protein biosynthesis by binding exclusively to the 50S ribosomal subunit of bacterial ribosomes [9, 10]. Recently we reported that methylation of G2535 and U2479 in domain V of the 23S rRNA confers resistance to avilamycin by preventing the antibiotic from binding to the ribosome [11]. This was in accordance with results obtained by footprinting avilamycin on *Escherichia coli* ribosomal subunits [12]. Based on these data, it is suggested that avilamycin interacts with the ribosomal A-site and interferes with initiation factor IF2 and tRNA binding. No crossresistance to other antibiotics has been described. Avilamycin and other orthosomycins show only poor solubility in water, which makes their therapeutical use difficult. Therefore, it is of interest to investigate whether new, structurally modified avilamycin derivatives may be able to overcome the limitations of the known compounds. Avilamycin A contains at least five methyl groups, presumably transferred by methyltransferases, which contribute to its lipophilic character. We have already functionally identified two of these methyltransferases, AviG1, which is a C-methyltransferase and is involved in evalose biosynthesis [13], and AviG4, which is an O-methyltransferase and is responsible for methylation of the phenolic hydroxyl group in the dichloroisoeverninic acid moiety [14]. While an *aviG1*-defective mutant did not produce any avilamycin derivatives, the *aviG4* mutant (*S. viridochromogenes* GW4) produced novel derivatives. The major compounds were named gavibamycin A1 and A3. Sequence analysis of the avilamycin biosynthetic gene cluster [14] led to the identification of three additional putative methyltransferase genes, i.e., *aviG2*, *aviG5*, and *aviG6* (Figure 2). In this study, we report the functional identification of *aviG2*, *aviG5*, and *aviG6* by means of targeted gene inactivation, the generation of double and triple mutants, and the production of new antibiotically active secondary metabolites. This work sets the stage for the genetic engineering of further chemically inaccessible, modified avilamycin derivatives and the generation of novel potent antibiotics.

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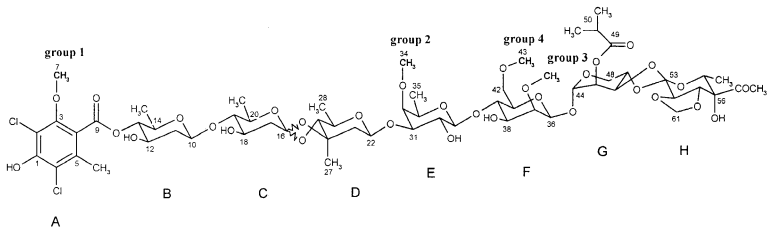

Figure 1. Structure of Avilamycin A

Methyl groups, investigated during these studies, are shown in bold and are denoted as "groups" 1–4.

Results and Discussion

Sequence Analysis of *aviG2, aviG5*, and *aviG6*

Besides *aviG1* and *aviG4*, three further putative methyltransferase genes, *aviG2, aviG5*, and *aviG6*, could be detected in the avilamycin biosynthetic gene cluster [13, 14]. The predicted gene product of *aviG2* comprises 241 amino acids and shows only low sequence similarity to putative methyltransferases in the database (e.g., 31% identity to a putative methyltransferase of *Deinococcus radiodurans*) [15]. The protein coded by *aviG5* consists of 239 amino acids and is similar to a methyltransferase in *Bacillus cereus* ATCC 14579 (29% identical amino acids). Database comparison with the deduced product of *aviG6* (240 amino acids) revealed similarity to a putative methyltransferase from *S. avermitilis* [16] (33% identical amino acids). Characteristic sequence regions, which are usually found in *S*-adenosylmethionine-dependent methyltransferases [17], could be detected. Conserved motif I (V/I/L)(L/V)(**D/E**)(V/I)**G**(G/C)**G**(T/P)**G**, motif II (P/G)(Q/T)(F/Y/A)**D**A(I/V/Y)(F/I)(C/V/L), and motif III LL(R/K)P**GG**(R/I/L)(L/I)(L/F/I/V)(I/L) are present in AviG2, motif I and motif II are present in AviG5, and motif I is present in AviG6.

Generation of *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, *S. viridochromogenes* GW4-GW6, *S. viridochromogenes* GW4-GW2-GW5, *S. viridochromogenes* GW2, *S. viridochromogenes* GW5, and *S. viridochromogenes* GW6 and Complementation Experiments

For the generation of methyltransferase mutants (double gene replacement mutants), gene inactivation experiments were carried out. Plasmids pMIKG2, pMIKG5, and pSP1-aviG6m were constructed as described in the Experimental Procedures, allowing the replacement of the wild-type gene by a mutated allele in *S. viridochromogenes* GW4. For the generation of the triple mutant *S. viridochromogenes* GW4-GW2-GW5 defective in three methyltransferase genes, plasmid pMIKG5 was used as inactivation plasmid and *S. viridochromogenes* GW4-GW2 as host strain (Figure 3). The deletions within the methyltransferase genes were confirmed by PCR. PCR fragments obtained from the double crossover mutants using primers G2R and G2F, G5R and G5F, and G6R and G6F, respectively, could not be digested by *Sph*I, *Xho*I, and *Aat*II, respectively, whereas the PCR fragments obtained from *S. viridochromogenes* GW4 could be digested by the enzymes. In all cases, Southern hybridization confirmed these results (data not shown).

S. viridochromogenes GW2, *S. viridochromogenes* GW5, and *S. viridochromogenes* GW6 were obtained after transformation of *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, and *S. viridochromogenes* GW4-GW6 with pSET-G4erm containing *aviG4* behind the *ermE** promoter [14]. To determine clearly that the mutation event affected only the desired genes and not other genes, *aviG2*, *aviG5*, and *aviG6* were ligated behind the *ermE** promoter, cloned into the integration plasmid pSET152, and introduced by protoplast transformation into the corresponding mutants. Gavibamycin A1 and A3 (derivatives of mutant *S. viridochromogenes* GW4) production was restored in each case. The amount of gavibamycin A1 and A3 produced by the complemented mutants was similar to the amount produced by *S. viridochromogenes* GW4.

Isolation and Identification of Novel Avilamycin Derivatives Produced by the Defective Mutants

S. viridochromogenes Tü57, the *aviG4* mutant *S. viridochromogenes* GW4, the double gene replacement mutants *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, and *S. viridochromogenes* GW4-GW6, the triple gene replacement mutant *S. viridochromogenes* GW4-GW2-GW5, as well as *S. viridochromogenes* GW2, *S. viridochromogenes* GW5, and *S. viridochromogenes* GW6 were grown under the conditions described in the Experimental Procedures. Extracts from culture supernatants were analyzed by TLC, HPLC-UV, and HPLC/ESI-MS. Avilamycin A ($[M+Na]^+$: 1425) and avilamycin C ($[M+Na]^+$: 1427) were detected in extracts of *S. viridochromogenes* Tü57 as sodium adduct ions ($[M+23]^+$). The observed distributions of the isotopic patterns of the pseudomolecular ions were in accordance with calculated values. With the assignment of molecular masses, avilamycin A was produced as main compound. The molecular masses of the pseudomolecular ions detected as the respective sodium adduct ions of two major compounds produced by *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, and *S. viridochromogenes* GW4-GW6 were $[M+Na]^+ = 1397$ and $[M+Na]^+ = 1399$, indicating the loss of two methyl groups in comparison to the wild-type avilamycin A and C. *S. viridochromogenes* GW4-GW2-GW5 gave masses of $[M+Na]^+ = 1383$ and $[M+Na]^+ = 1385$, which is in accordance with avilamycin A and C derivatives missing three methyl groups. *S. viridochromogenes* GW2, *S. viridochromogenes* GW4, *S. viridochromogenes* GW5, and *S. viridochromogenes* GW6 gave masses of $[M+Na]^+ = 1411$ and $[M+Na]^+ = 1413$, which are in accordance with avilamycin A and C missing one methyl group.

NMR Analysis of Avilamycin A and Derivatives

In order to determine which methyl groups of avilamycin A and avilamycin C, respectively, were affected in our

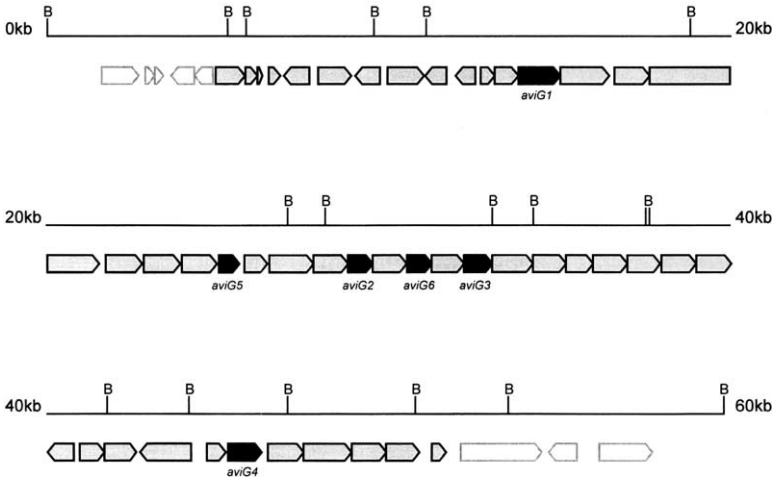

Figure 2. Genetic Organization of the Avilamycin Biosynthetic Gene Cluster

Genes belonging to the cluster are indicated as dark arrows. Methyltransferase genes are shown as black arrows.

mutants, it was necessary to complete the partial previous assignment [18, 19] of the ^{1}H and ^{13}C resonances of avilamycin A (see Supplemental Data). Samples were available both partially ^{13}C-labeled and unlabeled. The partially labeled products were prepared by feeding the bacteria with ^{13}C-labeled L-methionine. This resulted in ^{13}C-labeling of the carbons of the methoxy group in the dichloroisoeverninic acid moiety (group 1), of the methoxy group at position C4 of the fucose moiety (group 2), of the methoxy group at position C2 of the mannose moiety (group 3), of the methoxy group at position C6 of the mannose moiety (group 4), as well as the methylgroup at position C3 of the evalose moiety and the methylene group of the eurekanate (Figure 1). The NMR analysis was complicated due to severe overlapping of signals. For instance seven of the ten hydrogen signals of the mannose moiety have resonances within ~0.3 ppm. Furthermore, the labeled samples were available only in relatively low quantities (concentration of *S. viridochromogenes* GW4-GW5 sample about 3 mmol/liter; concentration of wild-type avilamycin A sample about 45 mmol/liter).

The assignment of the four methyl groups of interest of the wild-type avilamycin A is shown in the HMQC spectrum in Figure 4A. The assignments of groups 1 and 4 were straightforward and are consistent with the literature data. The partially overlapping resonances of groups 2 and 3 could be unequivocally assigned based on HMBC data.

The HMQC spectra of the derivatives (only avilamycin A analogs) produced by the mutants are shown in Figures 4B–4E. As expected, in the spectrum of samples obtained from *S. viridochromogenes* GW4, the signal of group 1 is missing (Figure 4B). In samples obtained from *S. viridochromogenes* GW4-GW2, this group is also absent, as well as group 4 (see Figure 4C). Samples obtained from *S. viridochromogenes* GW4-GW5 lack group 1 and group 2 (Figure 4D). Due to the partial overlap of the signal of groups 2 and 3 in Figure 4A, an unequivocal identification of the methoxy signal at 3.43 ppm was not possible based on chemical shift information only. However, the chemical shift of the signal suggests the presence of group 3. This assumption is further supported by the fact that the chemical shifts of all spins in the vicinity of group 2 in the fucose moiety in samples of *S. viridochromogenes* GW4-GW5 are significantly changed compared to the wild-type avilamycin, indicating the absence of group 2. The signals of the mannose moiety and of the C1 position of the fucose moiety were unchanged. The presence of group 3 is affirmed by the observation of key NOEs, indicating the close spatial proximity of the methoxy group to protons in the lyxose- and mannose moieties (Figure 5). For the *S. viridochromogenes* GW4-GW6 sample (Figure 4E), an identification of the methoxy signal at 3.43 ppm was also not possible based on chemical shift information alone. However, the HMBC spectrum showed clear cross-peaks, which allowed the unequivocal assignment of

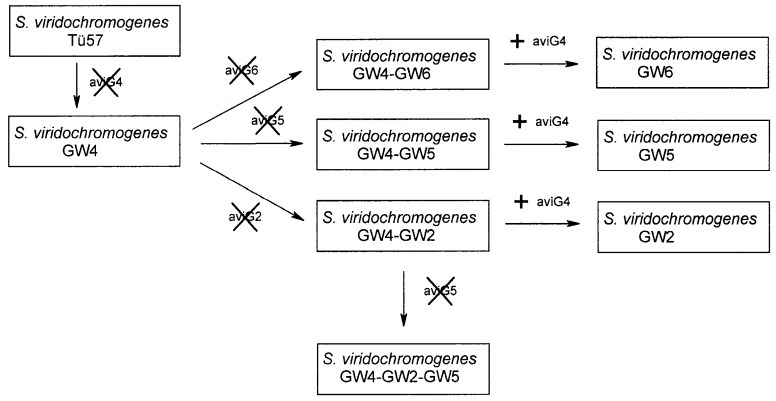

Figure 3. Methyltransferase Mutants Generated in This Study

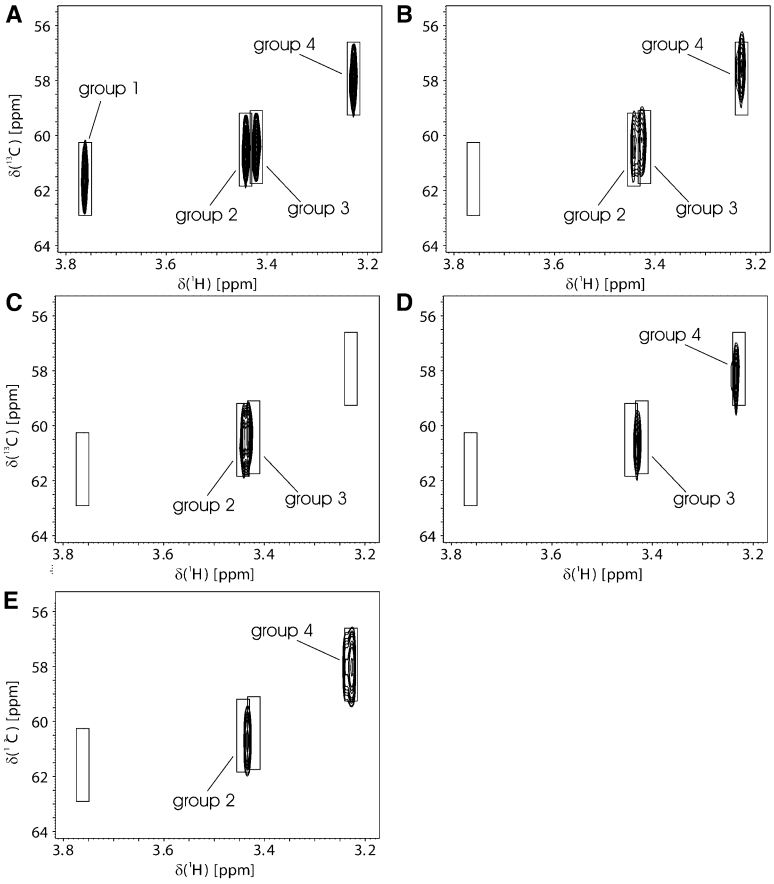

Figure 4. Sections of the HMQC Spectra

Sections of the HMQC spectra of (A) wild-type avilamycin A and of derivatives produced by the mutants *S. viridochromogenes* (B) GW4, (C) GW4-GW2, (D) GW4-GW5, and (E) GW4-GW6. The contour level was chosen such that only the methoxy group signals are visible. The boxes indicate the reference positions of methoxy groups 1–4 of Figure 4A.

the remaining signal to group 2, indicating that group 3 is missing in extracts of this mutant. From these results, it is deduced that AviG2 is responsible for methylation of the hydroxyl group at position 6 of the D-mannose moiety, AviG5 for methylation of the hydroxyl group at position 4 of the D-fucose moiety, and AviG6 for methylation of the hydroxyl group at position 2 of the D-mannose moiety. In consideration of the results of HPLC/ESI-MS analysis, the structure of the avilamycin C analogs could also be assigned. New derivatives were named gavibamycins (Figure 6).

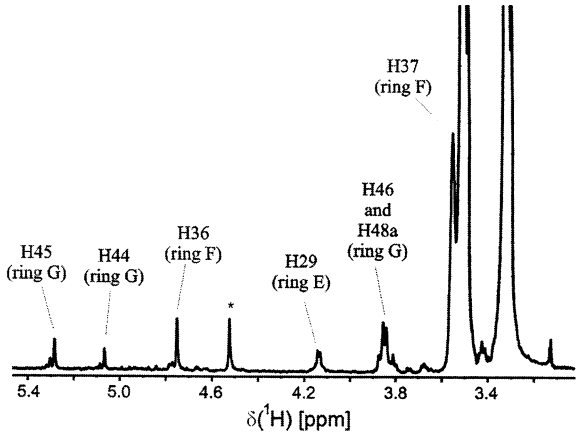

Figure 5. 1D Slice of a ^{13}C-Filtered NOESY Spectrum of the Sample Derived from *S. viridochromogenes* GW4-GW5 Showing the NOE Contacts of the Methoxy Group at 3.43 ppm

The NOEs show the spatial proximity of protons in the lyxose and mannose moieties to the methoxy group supporting the assignment of this signal to group 3. The asterisk marks the NOE to the proton of the OH group at C38 in ring F.

Isolation of Gavibamycin Derivatives for Biological Testing

For biological studies, avilamycin A and the corresponding gavibamycin derivatives were isolated from *S. viridochromogenes* Tü57 and from each mutant respectively after cultivation of 6–12 liters medium. A total of 1–2 mg/l avilamycin A could be isolated from extracts of *S. viridochromogenes* Tü57. A total of 1–2 mg/l gavibamycin derivatives corresponding to avilamycin A could be isolated from *S. viridochromogenes* GW4, *S. viridochromogenes* GW2, and *S. viridochromogenes* GW6. *S. viridochromogenes* GW5, *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, and *S. viridochromogenes* GW4-GW6 gave around 0.1–0.3 mg/l. The decrease in productivity was not due to a decreased efflux as determined by cell lysis, but was mainly due to the instability of the gavibamycin derivatives. Degradation to nonactive compound was observed in each case (data not shown).The purity of each sample was >90% as determined by HPLC-UV- and HPLC/ESI-MS analysis. *S. viridochromogenes* GW4-GW2-GW5 produced less than 0.1 mg/l. As the gaviba-

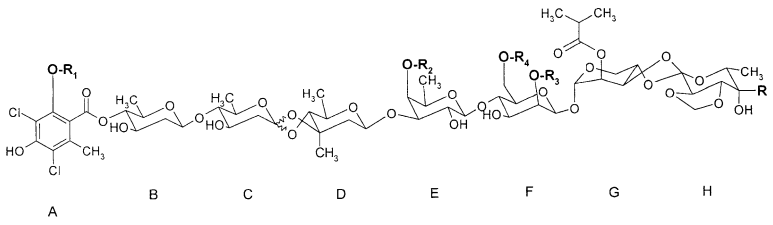

Figure 6. Structure of Compounds Generated during This Study

strain/mutant	R	R_1	R_2	R_3	R_4	main compound
wild-type	$COCH_3$	CH_3	CH_3	CH_3	CH_3	avilamycin A
wild-type	$CH(OH)CH_3$	CH_3	CH_3	CH_3	CH_3	avilamycin C
GW2	$COCH_3$	CH_3	CH_3	CH_3	H	gavibamycin J1
GW2	$CH(OH)CH_3$	CH_3	CH_3	CH_3	H	gavibamycin J3
GW4	$COCH_3$	H	CH_3	CH_3	CH_3	gavibamycin A1
GW4	$CH(OH)CH_3$	H	CH_3	CH_3	CH_3	gavibamycin A3
GW5	$COCH_3$	CH_3	H	CH_3	CH_3	gavibamycin K1
GW5	$CH(OH)CH_3$	CH_3	H	CH_3	CH_3	gavibamycin K3
GW6	$COCH_3$	CH_3	CH_3	H	CH_3	gavibamycin L1
GW6	$CH(OH)CH_3$	CH_3	CH_3	H	CH_3	gavibamycin L3
GW4-GW5	$COCH_3$	H	H	CH_3	CH_3	gavibamycin E1
GW4-GW5	$CH(OH)CH_3$	H	H	CH_3	CH_3	gavibamycin E3
GW4-GW2	$COCH_3$	H	CH_3	CH_3	H	gavibamycin C1
GW4-GW2	$CH(OH)CH_3$	H	CH_3	CH_3	H	gavibamycin C3
GW4-GW6	$COCH_3$	H	CH_3	H	CH_3	gavibamycin I1
GW4-GW6	$CH(OH)CH_3$	H	CH_3	H	CH_3	gavibamycin I3
GW4-GW2-GW5	$COCH_3$	H	H	CH_3	H	gavibamycin H1
GW4-GW2-GW5	$CH(OH)CH_3$	H	H	CH_3	H	gavibamycin H3

mycin derivatives of this mutant (gavibamycin H1 and H3) were very unstable, we could not isolate enough material for biological studies.

Physicochemical and Biological Properties of Gavibamycin Derivatives

The logP coefficient describes the distribution of a compound between n-octanol and water and is used to compare hydrophilic properties of pharmaceutical drugs. The logP coefficient was determined for each derivative. As shown in Table 1, the loss of one or two methyl groups influences the polarity of the novel gavibamycin derivatives and increases the water solubility of unmethylated derivatives in general. The loss of the methyl group of the 4-O-methyl-D-fucose moiety has the strongest influence on the polarity. The logP value for gavibamycin E1, the major product of *S. viridochromogenes* GW4-GW5, is 1 unit lower than the coefficient found for avilamycin A, indicating a 10-fold higher water solubility (Figure 6).

Extracts of our mutants were pretested against *Bacillus subtilis* using the agar diffusion test. Antibiotic activity could be detected in each case. Furthermore, all gavibamycin derivatives of *S. viridochromogenes* GW4-GW2, GW4-GW5, GW4-GW6, GW4, GW2, GW5, GW6, and wild-type avilamycin A were tested against a panel of pathogenic Gram-positive organisms, including two vancomycin-resistant strains, using the microdilution assay. The antibacterial activity of each derivative against the clinical isolates is presented in Table 2. All isolates were susceptible to avilamycin A (MIC range, <0.25 to 4 μg/ml). Activity of avilamycin A was good against *Streptococcus* and *Enterococcus* strains and good to moderate against *Staphylococcus* strains. All gavibamycin derivatives were less active against *Enterococcus* strains, slightly less active against *Staphylococcus* strains and *Streptococcus pyogenes*, and as active as avilamycin A against *Streptococcus pneumoniae*.

The removal of the methyl group of orsellinic acid drastically affected the activity against *Streptococcus pyogenes* E12449/98. In contrast, activity against *Enterococcus faecalis* ATCC19212, *Enterococcus faecalis* H10513/99, and *Enterococcus faecium* Vanco-H8914/00 was influenced by the polarity of the compounds and was not dependent on a special methyl group of the molecule.

These results, biological as well as physicochemical,

Table 1. The Logarithm of the Partition Coefficient between N-Octanol and Water of Compounds Generated during This Study

	Avilamycin	Gavibamycin						
	A	A1	L1	J1	I1	K1	C1	E1
Strain	Wild-Type	GW4	GW6	GW2	GW4-GW6	GW5	GW4-GW2	GW4-GW5
logP	4.24	4.00	3.94	3.70	3.67	3.49	3.46	3.22

The logarithm of the partition coefficient between n-octanol and water (logP) was determined by HPLC [41].

Table 2. Minimum Inhibitory Concentration of Avilamycin Derivatives Determined by Microdilution According to NCCLS Guidelines

	Avi. A	Vancomycin	Gavi. J1	Gavi. A1	Gavi. K1	Gavi. L1	Gavi. C1	Gavi. E1	Gavi. I1
Staphylococcus aureus ATCC 25923	2	1	4	8	4	2	4	8	8
MRSA RV 5/98	4	<0.5	2	4	4	2	4	8	8
Staphylococcus epidermidis DSM 1798	2	1	4	4	4	2	4	8	8
Streptococcus pyogenes E12449/98	<0.25	<0.25	<0.25	2	0.5	<0.25	1	2	1
Streptococcus pneumoniae E2919/94	<0.25	<0.25	<0.25	<0.5	<0.25	<0.25	<0.5	<0.5	<0.5
Enterococcus faecalis ATCC 19212	0.5	<0.25	4	2	8	4	16	16	16
Enterococcus faecalis H10513/99	0.5	>128	4	1	4	4	8	8	8
Enterococcus faecium Vanco-H8914/00	2	>128	32	8	32	16	64	32	32

Avi., avilamycin; gavi., gavibamycin.

indicate that our strategy of gene inactivation and generation of double and triple mutants to get novel, antibiotically active avilamycin derivatives in a nonchemical synthetic way is a promising method and a step toward the development of new antibiotics in order to overcome problems in the treatment of infections caused by multiresistant pathogens.

The reduced activity of the novel derivatives might be explained by a decreased permeability of the gavibamycin derivatives through the cell membrane. The use of gavibamycin derivatives together with β-lactame antibiotics could be an interesting strategy to help to overcome this effect. Further preclinical experiments have to be performed to evaluate the use of gavibamycin derivatives as new agents for the treatment of serious infections caused by pathogen microorganisms.

Significance

The avilamycin biosynthetic genes *aviG2*, *aviG5*, and *aviG6* encode methyltransferases methylating different sites of the avilamycin molecule. Our data indicate that AviG2, AviG5, and AviG6 act at a late step during avilamycin biosynthesis. Partially nonmethylated substrates are accepted by these methyltransferases, demonstrating that a strict order for methylation is not required. The majority of clinically useful antibiotics interact with only a few sites in the large ribosomal subunit, confined primarily to the peptidyl transferase center and entrance to the nascent peptide exit tunnel. In contrast, orthosomycins like avilamycin and evernimicin bind to a novel site in the ribosome, which is not used by any other therapeutically important drug. Clearly, this study has provided significant insights into the biosynthesis of avilamycin A and allowed the generation of novel, potentially valuable antibiotics.

Experimental Procedures

Bacterial Strains, Plasmids, and Culture Conditions

S. viridochromogenes Tü57 [20] and all mutants were 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 37°C). For avilamycin production, *S. viridochromogenes* Tü57 and all mutants were grown at 28°C in NL19+ medium containing 2% D-mannitol, 2% soy flour, and 20 mM L-valine (adjusted to pH 7.5). For improved production, SG medium containing 2% D-glucose, 1% soy peptone, 0.1% $CaCO_3$, 20 mM L-Valin, and 1 ml of 0.1% $CoCL_2$ solution was used. Both media were adjusted to pH 7.2. DNA manipulation was carried out using *Escherichia coli* (*E. coli*) XL-1 Blue MRF′ (Stratagene) as the host strain. Before transforming *S. viridochromogenes* strains, plasmids were propagated in *E. coli* ET 12567 (*dam*$^-$, *dcm*$^-$, *hsd*S, Cm^R) [21] to obtain unmethylated DNA. *E. coli* strains were grown on Luria-Bertani (LB) agar or liquid medium containing the appropriate antibiotic. pBluescript SK− (pBSK−) and pBC-SK− were from Stratagene. Plasmids pUC19 and pSP1 [22], conferring erythromycin resistance, were a kind gift of Dr. S. Pelzer, Tübingen, and pSET152 [23], conferring apramycin resistance, was obtained from Eli Lilly & Co. (Indianapolis, IN). The construction of pMun2 [24] and pSET-1cerm [25] has been described.

General Genetic Manipulation, PCR, and Sequence Analysis

Standard molecular biology procedures were performed as described [26]. Isolation of *E. coli* plasmid DNA, DNA restriction, DNA modification, and Southern hybridization were performed following the protocols of the manufacturers of kits, enzymes, and reagents (Amersham Biosciences, Roche Diagnostics, Promega, Stratagene). Streptomyces protoplast formation, transformation, and protoplast regeneration were performed as described [27]. PCR was carried out using a Perkin Elmer GeneAmp 2400 thermal cycler. Oligonucleotide primers used were G2F (5′-CCGCCAATTGTGGCCCACGA-3′), G2R (5′-CTCGGTCTAGAGCCGTCCCA-3′), G5F (5′-CCTCAATTGGCCC CGCCGAA-3′), G5R (5′-GCGCAGATCTTCGTCGGTGA-3′), G6F (5′-GGACGAATTCCTGGCCGACG-3′), and G6R (5′-AGCGTCTAGATG GTGTCGTGCATG-3′) (restriction sites are underlined). Reaction conditions involved 35 cycles of denaturating at 95°C for 1:30 min, annealing for 1:00 min and extending at 72°C for 1:45 min. Annealing temperatures used were 56°C in the case of *aviG2*, 62°C in the case of *aviG5*, and 66°C in the case of *aviG6*. 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 [28] on the server of the National Center for Biotechnology Information, Bethesda, MD.

Construction of Gene Inactivation Plasmids

aviG2

The *aviG2* gene, located on a 1.8 kb EcoRI/BamHI fragment, was ligated into pBC SK−. After SphI restriction, treatment with the Klenow fragment of *E. coli* DNA polymerase I, and religation, the intended alteration was confirmed by DNA sequencing. The mutated 1.8 kb fragment was cloned in pSP1 to generate plasmid pMIKG2.

aviG5

A 3.2 kb EcoRI/BamHI fragment containing *aviG5* was ligated into pBC SK− to create plasmid pE624. After restriction with EcoRI and

XbaI, the insert was transferred to plasmid pSP1 to generate pSP1-E624/7. A unique XhoI restriction site was then used for modification in a similar way as described for *aviG2*. DNA sequencing showed that treatment with T4 DNA polymerase led to a 50 bp deletion in *aviG5*. The resulting plasmid pMIKG5 was used for further experiments.

aviG6

An EcoRI and an XbaI restriction site were introduced upstream and downstream to *aviG6* using primers G6F and G6R. After restriction with EcoRI and XbaI, a 1 kb fragment containing *aviG6* was ligated into plasmid pBC SK− to generate plasmid pBC-aviG6. A unique AatII restriction site was altered by AatII restriction and subsequent treatment as described for *aviG2*. Sequencing revealed the correct alteration. The 1 kb fragment was cloned into pSP1 to generate plasmid pSP1-aviG6m.

Plasmids pMIKG2, pMIKG5, and pSP1-aviG6m were used to generate methyltransferase mutants as described [14]. As host, we used the *aviG4* gene replacement mutant *S. viridochromogenes* GW4 [14].

Construction of Complementation Plasmids

For the generation of plasmids used to complement the methyltransferase mutants, *aviG2, aviG5*, and *aviG6* were amplified by PCR. Suitable restriction sites (*aviG2*, MunI and XbaI; *aviG5*, MunI and BglII; *aviG6*, EcoRI and XbaI) were introduced upstream and downstream of each gene using oligonucleotide primers G2F and G2R, G5F and G5R, and G6F and G6R, respectively. The PCR product of *aviG5* was restricted with MunI and BglII and ligated into plasmid pMun2 to create plasmid pMun2-aviG5. Plasmid pSET-1cerm was digested with MunI and XbaI to remove *urdGT1c*, and the fragment containing *aviG5* was cloned behind the *ermE** promoter in place of *urdGT1c* after restriction digestion of pMun2-aviG5 using MunI and XbaI. The resulting complementation plasmid was named pSET-G5. The PCR product of *aviG2* was restricted with MunI and XbaI and ligated behind *aviG5* into the EcoRI and XbaI sites of plasmid pMun2-aviG5 to form plasmid pMun2-aviG5G2. To generate the complementation plasmid pSET-G5G2 the MunI/XbaI fragment of pMun2-aviG5G2 containing *aviG5* and *aviG2* was cloned behind the *ermE** promoter in place of *urdGT1c* in plasmid pSET-1cerm. To generate the complementation plasmid pSET-G2, the PCR product obtained was digested by MunI and XbaI and was ligated behind the *ermE** promoter in place of *urdGT1c* in plasmid pSET-1cerm. For construction of the integration plasmid pSET-G6, the PCR product of *aviG6* was restricted using EcoRI and XbaI and cloned into the MunI and XbaI sites of pSET-1cerm after removal of *urdGT1c*.

Feeding of *S. viridochromogenes* Tü57 and All Methyltransferase-Defective Mutants with ^{13}C-Labeled L-Methionine

The strains were grown at 28°C in NL19+ medium (5 × 100 ml) as described above. After 24 hr and 36 hr, 10 mg ^{13}C-labeled L-methionine was added to the culture. After 72 hr of cultivation, secondary metabolites were isolated as described in the following section and analyzed by NMR.

Analysis of New Derivatives of Avilamycin A and C

TLC Analysis

S. viridochromogenes Tü57 and mutants *S. viridochromogenes* GW4, *S. viridochromogenes* GW4-GW2, *S. viridochromogenes* GW4-GW5, *S. viridochromogenes* GW4-GW2-GW5, and *S. viridochromogenes* GW4-GW6 were incubated for 3 days. Cultures were filtered, and the filtrate was applied to a solid-phase extraction cartridge (SepPak C_{18}, Waters Associates). The cartridge was eluted using a gradient from 10% to 100% methanol in water. Avilamycin derivatives containing fractions were extracted by ethyl acetate. After evaporation of the organic solvent, avilamycin derivatives were redissolved in methanol and analyzed by TLC on silica gel plates (silica gel 60 F_{254}, Merck) with methylene chloride/methanol (9:1, v/v) as solvent. Avilamycin derivatives could be detected after treatment with anisaldehyde/H_2SO_4.

HPLC-UV Analysis

Detection of avilamycin and avilamycin-related compounds was performed on a reversed-phase column (ProC18, 3 μm, 50 × 4 mm, Kronlab GmbH, Sinsheim), with acetonitrile and water supplemented with 0.5% acetic acid as solvents (nonlinear gradient from 20% to 100% acetonitrile in 20 min, at a flow rate of 1.2 ml/min). The detection wavelength was 220 nm. Detection and spectral characterization of peaks were accomplished with a photodiode array detector and Millennium software (Waters Associates; Eschborn).

HPLC/ESI-MS Analysis

HPLC/ESI-MS was performed on an Agilent 1100 Series System with an electrospray chamber and a quadrupole detector. HPLC analysis was carried out on a Zorbax SB-C18 5 μm, 4.6 × 150 mm column with a Zorbax SB-C18 5 μm, 4.6 × 12.5 mm precolumn (Agilent). A nonlinear gradient from 20% to 70% acetonitrile in 0.5% acetic acid over 22 min at a flow rate of 0.5 ml/min was used. The column temperature was 23°C, and the UV detection wavelengths were 254 and 300 nm. The MSD chamber settings were as following: drying gas flow 12 liter/min, nebulize pressure 50 psig, drying gas temperature 300°C. The samples were analyzed in positive and negative scan mode with a mass range of 700–1500 Da.

NMR Analysis

NMR spectra were recorded in DMSO-d_6 at 295 K on Bruker DMX 600 and 750 MHz spectrometers. The following experiments were initially acquired at 600 MHz. ^{1}H-1D, ^{13}C-1D, COSY [29], TOCSY [30, 31], NOESY [32], HMQC [33] (HSQC [34] for *S. viridochromogenes* GW4-GW6), and HMBC [35]. To solve remaining problems, additional spectra were measured for the wild-type sample and the samples of *S. viridochromogenes* GW4-GW5 and *S. viridochromogenes* GW4-GW6 at a 750 MHz spectrometer: HMQC-COSY [36], HSQC-TOCSY [37], and ^{13}C-filtered-NOESY [38] (mixing time: 200 ms). Both spectrometers are part of the Bavarian NMR Center in Garching. Spectra were assigned using the program SPARKY 3 [39].

Isolation of Novel Gavibamycin Derivatives for the Determination of Physicochemical and Biological Properties

Strains were grown in SG medium for 3–4 days. Cultures were filtered, and the filtrate was extracted twice by ethyl acetate. The mycelium was broken with acetone and filtered again. After evaporation of acetone, it was also extracted by ethyl acetate and evaporated to dryness. Crude extracts were combined and applied to a solid-phase extraction cartridge (SepPak C_{18}, Waters Associates). The cartridge was eluted with 50% and 80% methanol. The 80% fraction contained about 90% of the avilamycin derivatives. The evaporated and lyophilisized fractions were redissolved in acetonitril and water. Further isolation was performed on an Agilent 1100 system using a semipreparative column (Zorbax SB-C18, 9.4 μm × 150 mm; 5 μm). For elution, the following gradient profile was used: solvent A: 5 mM ammoniumacetate in H_2O; solvent B: acetonitrile, nonlinear gradient, 30%–50% B within 20 min at a flow rate of 3.5 ml/min. A mass-based fraction collector was used to isolate the avilamycin derivatives. The final isolation step was performed using a gelpermeation column (PLgel 5 μm; 100 Å; 300 × 7.5 μm; MW < 4000). As solvent, acetonitril at a flow rate of 1 ml/ml was used. Again, a mass-based fraction collector was used to isolate the avilamycin derivatives.

Biological and Physicochemical Properties

The antimicrobial activity of the new derivatives was determined by the agar plate diffusion assay [40] using *Bacillus subtilis* as the test strain. Susceptibility of *Staphylococci*, *Streptococci*, and *Enterococci* to different derivatives was determined by the microdilution test according to NCCLS guidelines. The logarithm of the partition coefficient between n-octanol and water was determined by HPLC [41].

Supplemental Data

Chemical shifts δ(^{1}H) of nonexchanging protons of avilamycin A and δ(^{13}C) are given as supplemental information at http://www.chembiol.com/cgi/content/full/11/10/1403/DC1.

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Accession Numbers

The sequence reported here has been deposited in the GenBank database under the accession number AF333038.