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## Ketopremithramycins and Ketomithramycins, Four New Aureolic Acid-Type Compounds Obtained upon Inactivation of Two Genes Involved in the Biosynthesis of the Deoxysugar Moieties of the Antitumor Drug Mithramycin by *Streptomyces Argillaceus*, Reveal Novel Insights into Post-PKS Tailoring Steps of the Mithramycin Biosynthetic Pathway

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### Abstract

Mithramycin is an aureolic acid-type antimicrobial and antitumor agent produced by *Streptomyces argillaceus*. Modifying post-polyketide synthase (PKS) tailoring enzymes involved in the production of mithramycin is an effective way of gaining further information regarding the late steps of its biosynthetic pathway. In addition, new “unnatural” natural products of the aureolic acid-type class are likely to be produced. The role of two such post-PKS tailoring enzymes, encoded by *mtmC* and *mtmTIII*, was investigated, and four novel aureolic acid class drugs, two premithramycin-type molecules and two mithramycin derivatives, were isolated from mutant strains constructed by insertional gene inactivation of either of these two genes. From data bank comparisons, the corresponding proteins MtmC and MtmTIII were believed to act as a C-methyltransferase involved in the production of the D-mycarose (sugar E) of mithramycin and as a ketoreductase seemingly involved in the biosynthesis of the mithramycin aglycon, respectively. However, gene inactivation and analysis of the accumulated products revealed that both genes encode enzymes participating in the biosynthesis of the D-mycarose building block. Furthermore, the inactivation of MtmC seems to affect the ketoreductase responsible for 4-ketoreduction of sugar C, a D-olivose. Instead of obtaining premithramycin and mithramycin derivatives with a modified E-sugar upon inactivation of *mtmC*, compounds were obtained that completely lack the E-sugar moiety and that possess an unexpected 4-ketosugar moiety instead of the D-olivose at the

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beginning of the lower deoxysaccharide chain. The inactivation of *mtmTIII* led to the accumulation of 4E-ketomithramycin, showing that this ketoreductase is responsible for the 4-ketoreduction of the D-mycarose moiety. The new compounds of the mutant strains, 4A-ketopremithramycin A2, 4A-keto-9-demethylpremithramycin A2, 4C-keto-demycarosylmithramycin, and 4E-ketomithramycin, indicate surprising substrate flexibility of post-PKS enzymes of the mithramycin biosynthetic pathway. Although the glycosyltransferase responsible for the attachment of D-mycarose cannot transfer the unmethylated sugar to the existing lower disaccharide chain, it can transfer the 4-ketoform of sugar E. In addition, the glycosyltransferase MtmGIV, which is responsible for the linkage of sugar C, is also able to transfer an activated 4-ketosugar. The oxygenase MtmOIV, normally responsible for the oxidative cleavage of the tetracyclic premithramycin B into the tricyclic immediate precursor of mithramycin, can act on a substrate analogue with a modified or even incomplete trisaccharide chain. The same is true for glycosyltransferases MtmGI and MtmGII, both of which partake in the formation and attachment of the A–B disaccharide in mithramycin.

## Introduction

Mithramycin (**10**, also known as plicamycin), a natural product of *Streptomyces argillaceus* (ATCC 12956) and other *Streptomyces* spp., along with chromomycin A<sub>3</sub>, olivomycin A, chromocyclomycin, and UCH9, belongs to the discrete group of aureolic acid anticancer antibiotics.<sup>1–19</sup> The aureolic acid antibiotics are effective against a variety of experimental and human tumors.<sup>1–4,20–25</sup> Mithramycin has been used clinically to treat testicular carcinoma,<sup>1,3,4,19</sup> Paget's bone disease, and other bone growth disorders.<sup>26–29</sup> In particular, its hypocalcemic effect has been used to manage hypercalcemia in patients with malignancy-associated bone lesions.<sup>30</sup> Unfortunately, **10** exhibits gastrointestinal, hepatic, renal, and bone marrow toxicity resulting in nausea, vomiting, and bleeding.<sup>1,3,29,31</sup> Therefore, its widespread clinical use is limited, making the identification of improved therapeutic mithramycin analogues an important endeavor.

The biosynthetic pathway of **10** has been studied in our laboratories over the past years.<sup>4,32–44</sup> On the basis of the knowledge gained thus far, alteration of the biosynthetic pathway provides a mechanism through which modified **10** analogues can be produced “naturally”. We use this approach to target mainly post-polyketide synthase (post-PKS) modifying enzymes, which allow modifications to be made in regions of the molecule identified as crucial for the activity of **10**. The mechanism of action of **10** and in particular its DNA binding have been extensively studied. Mithramycin inhibits replication and transcription processes via cross-linking of DNA strands, thereby blocking their template activity for DNA- and RNA-dependent polymerases. It was shown that the deoxysaccharide moieties and the highly functionalized pentyl side chain play major roles<sup>2,7–9,19,23,24,28,45</sup> in the DNA binding of **10**. In particular, the trisaccharide residue is positioned inside of the DNA minor groove with the D-mycarose moiety sitting in its floor and interacting through H-bonds to both DNA strands. This suggests that changes in the trisaccharide segment may affect binding affinity and sequence selectivity of new **10** analogues. Consequently, the aim of our studies described in this article was to modify the D-mycarose moiety found at the end of the trisaccharide chain.

Past biosynthetic studies revealed to a great extent the sequence of events and role of important post-PKS oxygenases and group transferases in the mithramycin biosynthetic pathway. Inactivation of the four glycosyltransferases (GT) and elucidation of the accumulated products of the resulting mutant strains showed that the GTs MtmGI and MtmGII are involved in the assembly and attachment of the diolivoside fragment A–B,<sup>36</sup> while MtmGIII and MtmGIV participate in the assembly of the trisaccharide chain, linking sugar D (a D-oliose) and sugar C (a D-olivose), respectively (see Figures 1 and 2).<sup>41</sup> The studies also revealed that the disaccharide A-B can be attached only after the trisaccharide chain has been completed, suggesting the biosynthetic sequence **1** → **3** → **6** → **8** → **9** (Figures 1 and 2). Currently, no GT has been identified for the linkage of D-mycarose, the last sugar of the trisaccharide chain, and so there seemed to be no way to selectively modify the trisaccharide by inhibiting its attachment or to investigate the substrate specificity of this GT.

However, three methyltransferases (MT), MtmMI, MtmMII, and MtmC, are present in the *mtm* gene cluster. Recent investigations on the role of MtmMI and MtmMII identified them as being involved in the biosynthesis of the aglycon. MtmMI is the 4-O-methyltransferase responsible for the conversion of 4-demethylpremithramycinone into premithramycinone (**1**, Figure 1) prior to the sugar attachments. MtmMII is the C-methyltransferase, which introduces the aromatic methyl group predominantly after completion of the trisaccharide (product **8**), but occasionally also after attachment of the first or second sugar of the trisaccharide chain.<sup>42</sup> The role for the third potential MT in the *mtm* gene cluster (MtmC) seemed to be the addition of the one remaining methyl group in **10**, specifically that of the mycarose moiety. Thus, it was decided to inactivate MtmC with the hope of revealing a mechanism through which the third sugar could be modified or eliminated. During these studies, the role of the ketoreductase MtmTIII came into question. It seemed that MtmTIII, whose corresponding gene *mtmTIII* is clustered with the deoxysugar biosynthetic genes and is located immediately upstream of *mtmC* in the same reading direction, may be responsible for the 4-ketoreduction of sugar C (a D-olivose). To investigate this hypothesis, MtmTIII was also inactivated.

## Results

The deduced amino acid sequence of MtmC closely resembles various methyltransferases, most of which are involved in deoxysugar biosynthesis. The highest identity was found with SnoG from the nogalamycin pathway in *S. nogalater* (62.2%),<sup>54</sup> DnrX from the daunorubicin pathway in *S. peucetius* (37%),<sup>55</sup> TyICIII from the tylosin pathway in *S. fradiae* (32.4%),<sup>56,57</sup> and EryBIII from the erythromycin pathway in *Sacc. erythraea* (31%).<sup>58</sup> TyICIII and EryBIII have been proposed to be *S*-adenosylmethionine-dependent methyltransferases involved in C-3-methylation during the biosynthesis of L-mycarose. On the basis of these similarities, we predicted that *mtmC* may encode a C-3-methyltransferase involved in the biosynthesis of the D-mycarose moiety of mithramycin. To prove this hypothesis, *mtmC* was inactivated by gene replacement.<sup>44</sup> An apramycin resistance cassette was inserted within the gene in both the same and the reverse orientations with respect to the transcriptional direction of *mtmC* (M7C1 and M7C1R). As expected, HPLC analysis of cultures of both mutants showed that they did not produce **10**. Instead, two identical

compounds accumulated in both strains, while a third compound was detected only in M7C1.

The compounds were purified by preparative HPLC, and their structures were determined by NMR and mass spectroscopy. The major compound of both mutant strains exhibits all of the typical NMR signals of a tetracyclic premithramycin-type aglycon including the 9-methyl group, but only has two sugar moieties present. Unexpectedly, one of these sugars contains a hydrated keto group in the 4-position. This is strongly supported by the NMR data (Tables 1 and 2), which reveal a quaternary carbon in the  $^{13}\text{C}$  NMR spectrum ( $\delta_{\text{C}}$  90.6, no signal in the HSQC spectrum) and no proton in the 4-position ( $^1\text{H}$  NMR). The H,H-COSY, HSQC, and HMBC data (Table 3) allow the unambiguous assignment of all of the carbons and protons of this 4-ketosugar and also, through the observation of the  $^3J_{\text{C-H}}$  coupling between C-12a and 1-H of the sugar in the HMBC spectrum, indicate its linkage to the 12a-position of the aglycon. The H,H coupling constants, along with the other  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, prove the other sugar moiety to be an olivose. The presence of only small couplings to the 4B-H indicating the axial position of the 4-OH group is most important. The olivose is linked with its C-1 to the oxygen in the 3-position of the ketosugar, as implied by the  $^3J_{\text{C-H}}$  couplings between its 1-H and C-3 of the ketosugar as well as between its C-1 and 3-H of the ketosugar moiety visible in the HMBC spectrum (Table 3). The negative FAB mass spectrum shows a molecular ion ( $\text{M}^-$  703, 100%, HR calculated for  $\text{C}_{34}\text{H}_{39}\text{O}_{16}$ , 703.2238; found, 703.2206), which corresponds to a mass difference of 16 amu from premithramycin A2 (9-methyl-6)<sup>36</sup> and thus supports the structure with the hydrated keto group. Therefore, the main compound is 4A-ketopremithramycin A2 (**11**, Figure 3).

Compared to this compound, the minor compound found in both mutant strains differs only in that it is missing the aromatic 9-methyl group. Instead, 9-H can be observed in the  $^1\text{H}$  NMR spectrum at  $\delta$  6.45. The chemical shifts and coupling constants of all other  $^1\text{H}$  NMR signals are almost identical with the major compound allowing us to suggest 4A-keto-9-demethylpremithramycin A2 (**12**) as the structure for this minor compound.

The third compound, which is only observed as a product of the M7C1 mutant, exhibits the typical UV light (366 nm)-induced yellow fluorescence of the tricyclic mithramycin chromophore. Indeed, the NMR data reveal a compound that possesses the tricyclic mithramycinone skeleton, but only four deoxysugar building blocks. Comparison with the mithramycin NMR data<sup>11,37,39</sup> indicates that the A-B disaccharide attached at C-6 is unchanged, while the lower chain attached at C-2 is identical to the disaccharide containing the hydrated 4-ketosugar found in **11** and **12**. Thus, 4C-keto-demycarosylmithramycin (**13**) was deduced as the structure of this third compound. Long-range couplings observed in the HMBC spectrum (Table 3) as well as the negative FAB mass spectrum ( $\text{M}^-$  956, 100%) confirm these conclusions.

Interestingly, all three novel compounds discovered by inactivation of *mtmC* are chemically in a keto/hydrate equilibrium. The keto form of the olivose attached at C-12a and C-2, respectively, is the minor form (10–20%), visible in the  $^1\text{H}$  NMR spectrum (shifted 1-, 3-, and 5-positions, data not shown). The keto form is not detectable in the  $^{13}\text{C}$  NMR. Changing

the solvent from acetone to DMSO increases the amount of the keto form (data not shown), which becomes dominant after being dissolved in DMSO for 72 h.

While the absence of the D-mycarose moiety in all three new compounds is in agreement with the proposed function for MtmC as the C-3-methyltransferase necessary for the formation of the D-mycarose unit (see Discussion), the appearance of a hydrated keto group at the 4-position of the first sugar of the lower saccharide chain (normally a D-olivose, as in **10**) was unexpected and intriguing. This suggested that in both mutants another enzymatic function, a sugar 4-ketoreductase, was also affected. Our initial interpretation was that the inactivation of *mtmC* was causing a polar effect on genes transcribed in the same direction and following *mtmC*.<sup>34,36,38–42</sup> Since a potential ketoreductase gene, *mtmTIII*, is located adjacent to *mtmC* and immediately upstream in the same reading direction, this gene was consequently thought to be most likely affected.

To further clarify this situation, two experiments were carried out: (i) complementation experiments with *mtmC* cloned in a multicopy plasmid using the M7C1 and M7C1R mutants as transformation hosts, and (ii) inactivation of *mtmTIII*. In the first experiment, overexpression of *mtmC* restored the production of mithramycin in both the M7C1R and the M7C1 mutants.<sup>44</sup> The anticipated 4C-ketomithramycin was not produced. Thus, it is unlikely that the presence of the 4-keto group of sugar A in **11** and **12** and sugar C in **13** is caused by a polar effect. For the second experiment, the *mtmTIII*-minus mutant was constructed by inserting an apramycin resistance cassette within the *mtmTIII* gene (Figure 4). This mutant (M7T3) yielded only one compound, a mithramycin derivative, which was subsequently identified as 4E-ketomithramycin (**14**). The negative APCI-MS reveals an (M–H)<sup>–</sup> peak at *m/z* 1081.3 (31%), which is 2 amu smaller than the corresponding peak for mithramycin (**10**) and in agreement with a calculated molecular formula of C<sub>52</sub>H<sub>74</sub>O<sub>24</sub>, which lacks two protons as compared to the mithramycin molecular formula. While most of the NMR signals of this novel compound **14** are very similar to those of mithramycin,<sup>11,37,39</sup> significant differences can be seen for the signals of sugar E, which is a 4-keto-D-mycarose in **14** and D-mycarose in **10**. Most obvious, the signal for C-4E ( $\delta$ 76.1 in **10**) is shifted downfield to  $\delta$ 206.0 in **14**, typical for a keto group. This keto group signal shows <sup>3</sup>J<sub>C–H</sub> couplings to the 3E- and 5E-methyl group protons (see Table 3), which proves its location at the 4-position. Other significant shift differences for C-1E, C-3E, and C-5E as well as for the protons attached to these carbons were also observed, all in agreement with a 4E-carbonyl group. In contrast to the keto group in the 4C-position mentioned in context with structure **13**, the 4E-keto group in **14** clearly appears as a carbonyl, not as a hydrate (keto:hydrate  $\approx$  95:5), as the <sup>13</sup>C NMR spectrum shows no signal in the  $\delta$ 90 region.

## Discussion

The results of the above-described experiments clearly point out that MtmC is the 3-C-methyltransferase necessary for the biosynthesis of the D-mycarose moiety of mithramycin. This is supported by its sequence similarity to TylC3 (TylCIII) and EryBIII, other proteins that act as methyltransferases involved in L-mycarose building block biosyntheses. Particular proof came from the inactivation of the *mtmC* gene leading to the accumulation of compounds lacking the D-mycarose moiety of the trisaccharide chain. It seems that the

glycosyltransferase responsible for the attachment of D-mycarose to the preceding D-oliose moiety, possibly MtmGIV (see below), is unable to recognize an unmethylated sugar donor substrate for this particular glycosyl transfer step and, therefore, seems to be inhibited when the methyl group is missing in the sugar cosubstrate.

The accumulation of products showing an unreduced C-4 carbonyl group at the first sugar moiety of the lower disaccharide chain was also proven to be the consequence of the inactivation of *mtmC*, since mithramycin biosynthesis was restored in the corresponding mutants by expressing in trans the *mtmC* gene. Since a polar effect on the neighbor gene *mtmTIII* could be ruled out, this result suggests that inactivation of *mtmC* is affecting a ketoreductase other than MtmTIII. Possible candidates are MtmTI or MtmTII, whose functions within the biosynthetic pathway of **10** have yet to be determined. Type-II PKSs are likely to be assembled in a highly structured multifunctional array resulting in a 3-dimensional structure, which might be crucial to assist folding of the growing polyketide chain as well as for the activity of certain biosynthetic enzymes. Similar unpredicted results as found here led already to the conclusion that a complex association of many proteins is sometimes necessary in type-II PKS systems and that removal of one component can have unpredictable indirect effects on the behavior of the remaining activities.<sup>59</sup> Thus, the modification of the spatial structure of the protein cluster by inactivation of *mtmC* may in this case be the underlying cause by which the ketoreductase responsible for the 4-reduction of the first sugar building block of the trisaccharide chain, although expressed, is rendered inactive.

Inactivation of MtmTIII showed that this enzyme is responsible for the 4-ketoreduction step of the mycarose building block biosynthesis, considering the product isolated from the MtmTIII mutant (**14**) contains a 4-ketomycarose instead of a mycarose unit at the end of the trisaccharide chain. That MtmTIII is responsible for a ketoreduction in a sugar building block biosynthesis seemed at first somewhat surprising, since the deduced amino acid sequence of MtmTIII resembles mostly ketoreductases involved in polyketide aglycon biosyntheses. The closest resemblance was found for DnrE and DauE,<sup>40</sup> which catalyze an early step of the biosynthesis of anthracyclines, namely the reduction of the tetracyclic aklaviketone to aklavinone. Only weak resemblance could be found with ketoreductases involved in deoxysugar biosyntheses. A possible explanation for this is that the reduction of the 4-keto group of this sugar moiety happens *after* its attachment. Thus, MtmTIII has to act on a large molecule, composed of a tetracyclic polyketide-derived aglycon (like aklaviketone) and an attached trisaccharide, instead of on a single NDP-deoxysugar. Transfer of biosynthetic intermediates of deoxysugars that are still unreduced at their 4-position is possible; however, it is very unusual and rarely found in antibiotic biosynthetic pathways. For example, in a recent publication, Liu and co-workers generated new methymycin/pikromycin analogues bearing modified sugars, revealing a highly relaxed sugar cosubstrate specificity of the glycosyltransferase DesVII. Despite its unusual substrate flexibility, DesVII failed to transfer 4-ketosugars.<sup>60</sup> In contrast, an EryBIV mutant from the erythromycin producer, *Saccharopolyspora erythraea*, has been previously reported to transfer a 4-keto-L-mycarose unit to the erythronolide B aglycon.<sup>61</sup> Also, the GT responsible for the glycosyltransfer step, through which the D-mycarose moiety is linked

during the biosynthesis of **10**, as well as the GT, which attaches the first sugar unit of the trisaccharide chain, are capable of transferring 4-ketosugars, as was shown in products **11–13** and **14**, respectively. Since such a capability is rarely found among GTs, it seems reasonable to assume that a single GT may be responsible for both of these transfer steps and that, as suggested by the results presented above, the corresponding sugars are reduced after attachment as ketosugars. A dual role for one of the *mtm* GTs is also necessary because of the presence of five sugar moieties, but only four GT encoding genes in the *mtm* gene cluster. MtmGIV has previously been shown to be responsible for the attachment of the first sugar of the trisaccharide chain, a D-olivose, and thus is the best candidate for such a double role. As with all GTs, MtmGIV is believed to have two binding sites, one for the alcohol substrate or the acceptor substrate (here **1**) and a second for the sugar cosubstrate, the donor substrate. It seems that MtmGIV may have a unique sugar binding site, which recognizes and selectively attaches two different sugars, a 4-ketoolivose as the first sugar and a 4-ketomycarose as the last sugar of the trisaccharide chain, respectively. The change of the alcohol acceptor substrate, here in particular the absence or presence of a D-olivose-D-oliose disaccharide chain, might also influence the sugar donor binding site, thereby changing its specificity and thus accounting for the differentiation between NDP-ketoolivose and NDP-ketomycarose as sugar cosubstrates.

The experiments described here also raise important issues regarding the flexibility of other enzymes involved in the biosynthesis of **10**. For example, MtmGIII, which normally attaches D-oliose to the (already 4-reduced!) D-olivose of premithramycin A1 (**3**), shows a unique flexibility toward its acceptor substrate by attaching D-oliose also to a 4-ketosugar. To our knowledge, this is the first example in a deoxysaccharide biosynthesis in which a GT was able to link a deoxysugar to another genetically modified deoxysugar that includes a keto group neighboring the linkage position. The hydrate formation, which changes 4-keto-D-olivose to 4-hydroxy-D-olivose, may be indeed helpful for this process, since 4-hydroxy-olivose is stereoelectronically more similar to olivose than the keto form. In addition, compound **13** is the first case in which MtmGI and MtmGII have been shown to attach the disaccharide chain even though the trisaccharide chain is incomplete. It is also worth pointing out that oxygenase MtmOIV was capable of oxidatively opening the fourth ring of the **13** precursor, although this lacks the mycarose moiety and has the (hydrated) keto function in the 4C-position as compared to the natural substrate premithramycin B (**9**). Furthermore, MtmOIV was able to process the precursor of 4E-ketomithramycin (**14**) despite its keto function in the 4E-position. Along with the previously published **10** derivatives, 7-demethylmithramycin and premithramycin A4, **13** and **14** are further examples demonstrating the relatively high degree of substrate flexibility of MtmOIV. This is an important finding, since MtmOIV plays a key role in the generation of novel mithramycin derivatives by combinatorial biosynthesis due to its ability to convert biologically inactive tetracyclic premithramycins into active tricyclic mithramycins.<sup>39</sup>

In conclusion, the work described here on the two neighboring genes *mtmC* and *mtmTIII* reveals that the corresponding enzymes, MtmC and MtmTIII, are responsible for the 3-methyltransfer and the 4-ketoreduction, respectively, occurring during the generation of the D-mycarose moiety of **10**. Glycosyltransferase MtmGIV presumably displays a double role,

likely being responsible for the linkage of both the first and the third sugar of the trisaccharide chain. These sugars appear to be attached as 4-ketosugars, and the 4-ketoreduction happens after the glycosyltransfer step. Finally, four new compounds, two of which are potentially active **10** analogues, were created as a result of the inactivation experiments. All these results also provide important additional information regarding the substrate specificity of several of the post-PKS enzymes involved in mithramycin biosynthesis.

## Experimental Section

### Microorganisms, Culture Conditions, and Vectors

*Streptomyces argillaceus* ATCC 12956, a mithramycin producer, was used as the source of chromosomal DNA. For sporulation it was grown for 7 days at 30 °C on plates containing A medium.<sup>36</sup> For protoplast regeneration, the organism was grown on R5 solid medium,<sup>46</sup> and R5A medium was used as the liquid medium for production.<sup>36</sup> *Escherichia coli* XL1-Blue<sup>47</sup> was used as the host for subcloning and was grown at 37 °C in TSB medium (tryptic soy broth, Oxoid). When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 5 or 50 µg/mL of thiostrepton for liquid or solid cultures, respectively, 100 µg/mL of ampicillin, 25 µg/mL of apramycin, or 20 µg/mL of tobramycin. Plasmid pBSKT<sup>38</sup> was the vector used for insertional inactivation experiments.

### DNA Manipulation

Plasmid DNA preparations, restriction endonuclease digestions, alkaline phosphatase treatments, ligations, and other DNA manipulations were performed according to standard procedures for *E. coli*<sup>48</sup> and for *Streptomyces*.<sup>46</sup>

### Insertional Inactivation of *mtmC*

A 5.5 kb *Pst*I fragment containing the *mtmC* gene was subcloned as a *Xba*I–*Hind*III fragment (using these sites from the polylinker) into the same sites of pBSKT, generating pM7C0. A *Bam*HI–*Bgl*III fragment from pUO9090, containing an apramycin resistance cassette, was then subcloned in both orientations into the unique *Bam*HI site in pM7C0 located within the *mtmC* gene, generating two different constructs: pM7C1, with the resistance gene oriented in the same direction of transcription as *mtmC*, and pM7C1R, with the resistance gene inserted in the opposite direction. These constructs were used to transform protoplasts of the wild-type strain *S. argillaceus* ATCC 12956. Transformants were selected on the basis of resistance to 25 µg/mL of apramycin. To select disruptants in which the wild-type region of the chromosome was replaced by the in vitro mutated one through a double crossover, transformant colonies were grown in TSB medium without antibiotics. After 72 h, protoplasts were obtained and plated onto a regeneration medium. Growing colonies were tested for resistance to apramycin and sensitivity to thiostrepton (50 µg/mL). The replacement was also confirmed by Southern analysis.

### Complementation of the *mtmC*-Minus Mutants

The 2 kb *Sna*BI–*Pml*I fragment from pLP7 was subcloned into the *Sma*I site of pUK21, generating PAGC0. Then the *Spe*I fragment using the sites from the flanking polylinker was



subcloned into the *Xba*I site of pIAGO, in the correct orientation and downstream of the erythromycin resistance promoter, generating the multicopy plasmid pAGC1. Protoplasts of mutants M7C1 and M7C1R were transformed with pAGC1.

### Insertional Inactivation of *mtmTIII*

A 4.5 kb *Bam*HI fragment containing the *mtmTIII* gene was subcloned into the *Bam*HI site of pBSKT. In this construct, an apramycin resistance cassette was subcloned as an *Eco*RI–*Eco*RV (this site blunt-ended) fragment into the unique *Pml*I site which is located within the *mtmTIII* coding region. This construct (pJG140) was digested with *Eco*RI, and a 1.7 kb *Eco*RI fragment was inserted to produce pJG141, in which the right-hand side of the insert was enlarged with additional chromosomal DNA from that region to facilitate the occurrence of a crossover. This final construct (pJG141) was used to transform protoplasts of the wild-type strain *S. argillaceus* ATCC 12956. Transformants in which the replacement of the wild-type region of the chromosome was replaced by the in vitro mutated one through a double crossover at both sides of the apramycin cassette were recognized by resistance to apramycin and sensitivity to thiostrepton (Figures 4 and 5). The replacement was also verified by Southern analysis.

### Isolation, Purification, and HPLC of the New Compounds

Detection of mithramycin-related compounds was performed on a reversed phase column (Symmetry C18, 4.6 × 250 mm, Waters), with acetonitrile and water supplemented with 0.1% trifluoroacetic acid as solvents (linear gradient from 10 to 100% acetonitrile in 30 min, at a flow rate of 1 mL/min). Detection and spectral characterization of peaks were accomplished with a photodiode array detector and Millennium software (Waters), extracting bidimensional chromatograms at 280 nm. To isolate the new compounds, spores of the mutants were initially grown in TSB medium during 24 h at 30 °C and 250 rpm. This seed culture was used to inoculate (at 2.5%, v/v) eight 2 L Erlenmeyer flasks containing 400 mL of R5A medium. After incubation for 4 days at the above conditions, the cultures were centrifuged, filtered, and extracted as described.<sup>36</sup> Mithramycin-related compounds (identified according to their spectral characteristics) were purified by preparative HPLC on a  $\mu$ Bondapak C18 radial compression cartridge (PrepPak Cartridge, 25 × 100 mm, Waters). Short gradients using 0.1% trifluoroacetic acid in water and either methanol or acetonitrile, at 10 mL/min, were optimized for resolution of individual peaks. The purified material collected in each case was diluted 4-fold with water, applied to a solid-phase extraction column (Lichrolut RP-18, Merck), washed with water to eliminate trifluoroacetic acid, eluted with methanol, and dried in vacuo. Yields: **11**, 12 mg/L; **12**, 10 mg/L; **13**, 3 mg/L; **14**, 12 mg/L (for comparison reasons; the production of **10** by the wild-type strain of *S. argillaceus* is 10–12 mg/L).

### Structure Elucidation of 4A-Ketopremithramycin A2, 4A-Keto-9-demethylpremithramycin A2, 4C-Keto-demycarosylmithramycin, and 4E-Ketomithramycin

The structures of new premithramycin and mithramycin derivatives were elucidated by NMR and mass spectroscopy. The positive fast atom bombardment (FAB) and high-resolution (HR-FAB) mass spectra were acquired at the University of South Carolina,

Department of Biochemistry and Chemistry facilities in Columbia, SC, using a VG70SQ double focusing magnetic sector MS instrument. The atmospheric pressure chemical ionization mass spectra (APCI-MS) were acquired at the Medical University of South Carolina, Regional Mass Spectroscopy Center, using a Finnigan MAT LCQ. All the NMR data were performed in *d*<sub>6</sub>-acetone/D<sub>2</sub>O (95:5), either on a Varian Inova 400 or a Bruker DMX 500 instrument. The NMR data are listed in Tables 1 and 2; the important long-range C–H couplings observed in the HMBC spectra are shown in Table 3. All NMR assignments are based on DEPT, H,H-COSY, HSQC, TOCSY, and HMBC spectra,<sup>37,49–53</sup> allowing an unambiguous assignment of all NMR signals.

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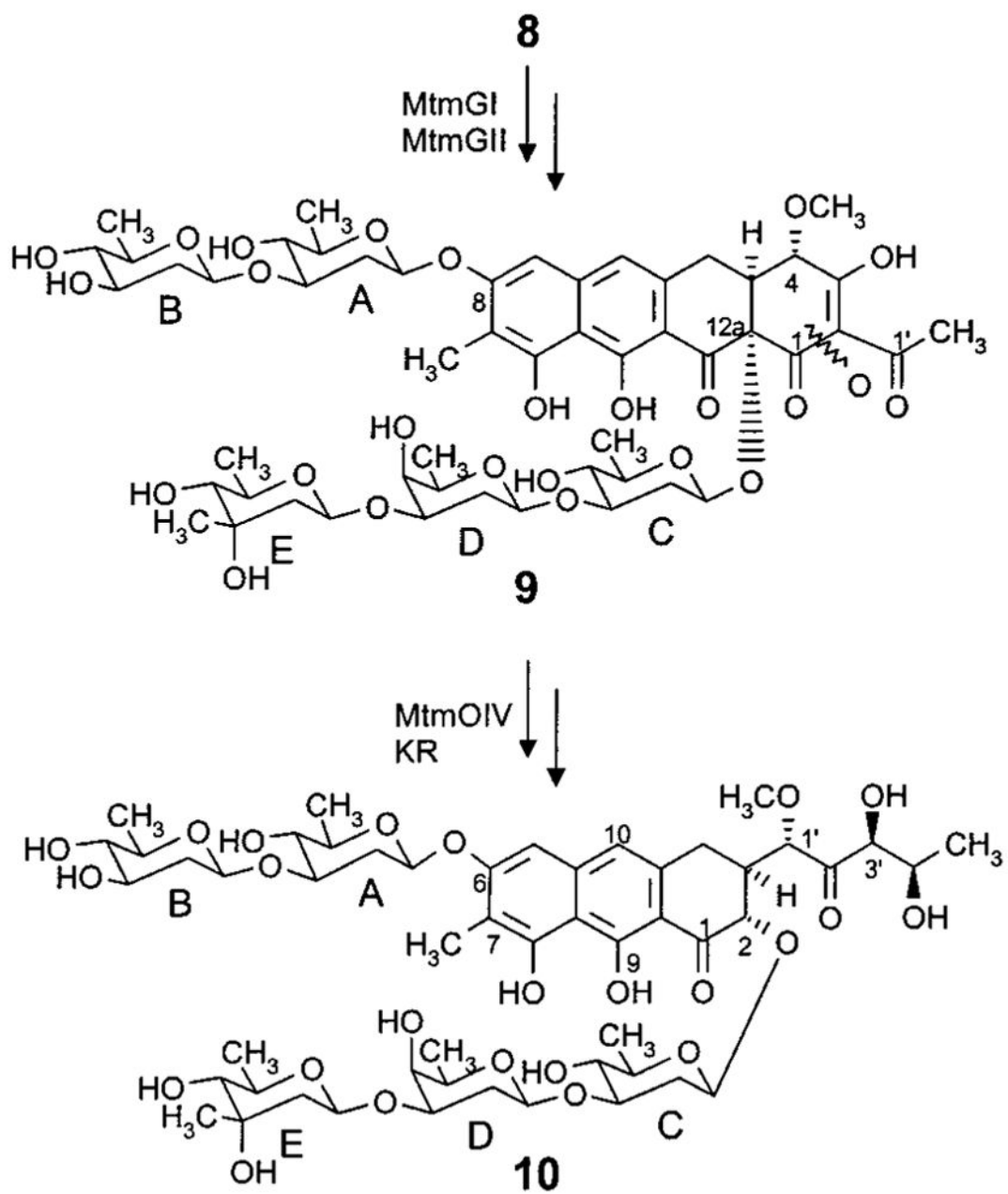
## References

1. Skarbek, J.D.; Speedie, M.K. Antitumor Antibiotics of the Aureolic Acid Group: Chromomycin A<sub>3</sub>, Mithramycin A, and Olivomycin A. In: Aszalos, A., editor. *Antitumor Compounds of Natural Origin*. Vol. 1. CRC Press; Boca Raton, FL: 1981. p. 191-235.
2. Van Dyke MW, Dervan PB. *Biochemistry*. 1983; 22:2373–2377. [PubMed: 6222762]
3. Remers, W.A. *The Chemistry of Antitumor Antibiotics*. Vol. 1. Wiley-Inter-science; New York: 1979. p. 133-175.
4. Rohr J, Méndez C, Salas JA. *Bioorg Chem*. 1999; 27:41–54.
5. Katahira R, Katahira M, Yamashita Y, Ogawa H, Kyogoku Y, Yoshida M. *Nucleic Acids Res*. 1998; 26:744–755. [PubMed: 9443966]
6. Katahira R, Uosaki Y, Ogawa H, Yamashita Y, Nakano H, Yoshida M. *J Antibiot*. 1998; 51:267–274. [PubMed: 9589061]
7. Sastry M, Patel DJ. *Biochemistry*. 1993; 32:6588–6604. [PubMed: 8329387]
8. Sastry M, Fiala R, Patel DJ. *J Mol Biol*. 1995; 251:674–689. [PubMed: 7666419]
9. Rama Krishna N, Miller DM, Sakai TT. *J Antibiot*. 1990; 43:1543–1552. [PubMed: 2177461]
10. Thiem J, Schneider G, Sinnwell V. *Liebigs Ann Chem*. 1986:814–824.
11. Thiem J, Meyer B. *Tetrahedron*. 1981; 37:551–558.
12. Roush WR, Lin XF. *J Am Chem Soc*. 1995; 117:2236–2250.
13. Thiem J, Schneider G. *Angew Chem*. 1983; 95:54–55.
14. Thiem J, Meyer B. *J Chem Soc, Perkin Trans 2*. 1979:1331–1336.
15. Thiem J, Schöttner B. *Angew Chem*. 1987; 99:591–592.
16. Roush WR, Lin XF. *Tetrahedron Lett*. 1993; 34:6829–6832.
17. Franck RW, Marzabadi CH. *J Org Chem*. 1998; 63:2197–2208.
18. Roush WR, Hartz RA, Gustin DJ. *J Am Chem Soc*. 1999; 121:1990–1991.
19. Majee S, Dasgupta D, Chakrabarti A. *Eur J Biochem*. 1999; 260:619–626. [PubMed: 10102989]
20. Jones DE Jr, Cui DM, Miller DM. *Oncogene*. 1995; 10:2323–2330. [PubMed: 7784080]
21. Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. *J Clin Invest*. 1991; 88:1613–1621. [PubMed: 1834700]
22. Ray R, Thomas S, Miller DM. *Am J Med*. 1990; 300:203–208.
23. Ray R, Snyder RC, Thomas S, Koller CA, Miller DM. *J Clin Invest*. 1989; 83:2003–2007. [PubMed: 2542379]
24. Snyder RC, Ray R, Blume S, Miller DM. *Biochemistry*. 1991; 30:4290–4297. [PubMed: 1827033]

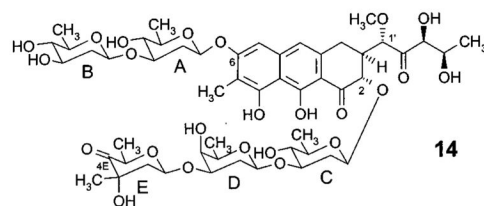
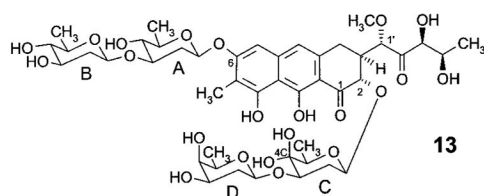
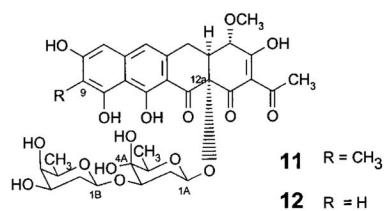
25. Kennedy BJ, Yarbro JW, Kickert V, Sandberg-Wollheim M. *Cancer Res.* 1968; 28:91–97. [PubMed: 5635376]
26. Reddy SV, Menaa C, Singer FR, Demulder A, Roodman GD. *J Bone Miner Res.* 1999; 14:3–8. [PubMed: 10510206]
27. Cortes EP, Holland JF, Moskowitz R, Depoli E. *Cancer Res.* 1972; 32:74–76. [PubMed: 4257415]
28. Hall TJ, Schaeublin M, Chambers TJ. *Biochem Biophys Res Commun.* 1993; 195:1245–1253. [PubMed: 8216256]
29. Elias EG, Evans JT. *J Bone Jt Surg.* 1972; 54A:1730–1736.
30. Robins P, Jowsey J. *J Lab Clin Med.* 1973; 82:576–586. [PubMed: 4271121]
31. Hardmann, JG.; Limbird, LE.; Molinoff, PB.; Ruddon, RW.; Goodman Gilman, A., editors. Goodman & Gilman's *The Pharmacological Basis of Therapeutics.* McGraw-Hill; New York, St. Louis: 1996. p. 1267–1268.
32. Lombó F, Blanco G, Fernández E, Méndez C, Salas JA. *Gene.* 1996; 172:87–91. [PubMed: 8654997]
33. Fernández E, Lombó F, Méndez C, Salas JA. *Mol Gen Genet.* 1996; 251:692–698. [PubMed: 8757400]
34. Lombó F, Siems K, Braña AF, Méndez C, Bindseil K, Salas JA. *J Bacteriol.* 1997; 179:3354–3357. [PubMed: 9150235]
35. Rohr J, Weissbach U, Beninga C, Kunzel E, Siems K, Bindseil KU, Lombó F, Prado L, Braña AF, Méndez C, Salas JA. *Chem Commun.* 1998:437–438.
36. Fernández E, Weissbach U, Sánchez Reillo C, Braña AF, Méndez C, Rohr J, Salas JA. *J Bacteriol.* 1998; 180:4929–4937. [PubMed: 9733697]
37. Wohler SE, Kunzel E, Machinek R, Méndez C, Salas JA, Rohr J. *J Nat Prod.* 1999; 62:119–121. [PubMed: 9917296]
38. Lombó F, Braña AF, Méndez C, Salas JA. *J Bacteriol.* 1999; 181:642–647. [PubMed: 9882681]
39. Prado L, Fernández E, Weissbach U, Blanco G, Quirós LM, Braña AF, Méndez C, Rohr J, Salas JA. *Chem Biol.* 1999; 6:19–30. [PubMed: 9889148]
40. Prado L, Lombó F, Braña AF, Méndez C, Rohr J, Salas JA. *Mol Gen Genet.* 1999; 261:216–225. [PubMed: 10102355]
41. Blanco G, Fernández E, Fernández MJ, Braña AF, Weissbach U, Kunzel E, Rohr J, Méndez C, Salas JA. *Mol Gen Genet.* 2000; 262:991–1000. [PubMed: 10660060]
42. Fernández Lozano MJ, Remsing LL, Quiros LM, Braña AF, Fernández E, Sánchez C, Méndez C, Rohr J, Salas JA. *J Biol Chem.* 2000; 275:3065–3074. [PubMed: 10652287]
43. Lombó F, Kunzel E, Prado L, Braña AF, Bindseil KU, Frevert J, Bearden D, Méndez C, Salas JA, Rohr J. *Angew Chem, Int Ed.* 2000; 112:808–811.
44. González A, Remsing LL, Lombó F, Fernández MJ, Prado L, Braña AF, Kunzel E, Rohr J, Méndez C, Salas JA. *Mol Gen Genet.* 2001; 263:827–835. [PubMed: 11254130]
45. Aich P, Dasgupta D. *Biochemistry.* 1995; 34:1376–1385. [PubMed: 7827085]
46. Hopwood, DA.; Bibb, MJ.; Chater, KF.; Kieser, T.; Bruton, CJ.; Kieser, HM.; Lydiate, DJ.; Smith, CP.; Ward, JM.; Schrepf, H. *Genetic Manipulation of Streptomyces. A Laboratory Manual.* The John Innes Foundation; Norwich, U.K: 1985.
47. Bullock WO, Fernández JM, Short JN. *BioTechniques.* 1987; 5:376.
48. Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning. A Laboratory Manual.* Vol. 1–3. Cold Spring Harbor Laboratory Press; Plainview, NY: 1989.
49. Norwood TJ, Boyd J, Heritage JE, Soffe N, Campbell ID. *J Magn Reson.* 1990; 87:488–501.
50. Bax A, Summers MF. *J Am Chem Soc.* 1986; 108:2093–2094.
51. Norwood TJ, Boyd J, Heritage JE, Soffe N, Campbell ID. *J Magn Reson.* 1990; 87:488–501.
52. Bax A, Ikura M, Kay LE, Torchia DA, Tschudin R. *J Magn Reson.* 1990; 86:304–318.
53. Sklenar V, Poggio M, Leppik R, Staudek V. *J Magn Reson, Ser A.* 1993; 102:241–245.
54. Torkkell S, Ylihonko K, Hakala J, Skurnik M, Mäntsälä P. *Mol Gen Genet.* 1997; 256:203–209. [PubMed: 9349712]

55. Lomovskaya N, Doi-Katayama Y, Filippini S, Nastro C, Fonstein L, Gallo M, Colombo AL, Hutchinson CR. *J Bacteriol.* 1998; 180:2379–2386. [PubMed: 9573189]
56. Bate N, Butler AR, Smith IP, Cundliffe E. *Microbiology.* 2000; 146:139–146. [PubMed: 10658660]
57. Chen H, Zhao Z, Hallis TM, Guo Z, Liu H-w. *Angew Chem Int Ed.* in press.
58. Gaisser S, Böhm GA, Doumith M, Raynal M-C, Dhillon N, Cortés J, Leadlay PF. *Mol Gen Genet.* 1998; 258:78–88. [PubMed: 9613575]
59. Staunton J, Weissman KJ. *Nat Prod Rep.* 2001; 18:380–416. [PubMed: 11548049]
60. Yamase H, Zhao L, Liu H-w. *J Am Chem Soc.* 2001; 123 in press.
61. Salah-Bey K, Doumith M, Michel JM, Haydock S, Cortes J, Leadlay PF, Raynal MC. *Mol Gen Genet.* 1998; 257:542–553. [PubMed: 9563840]

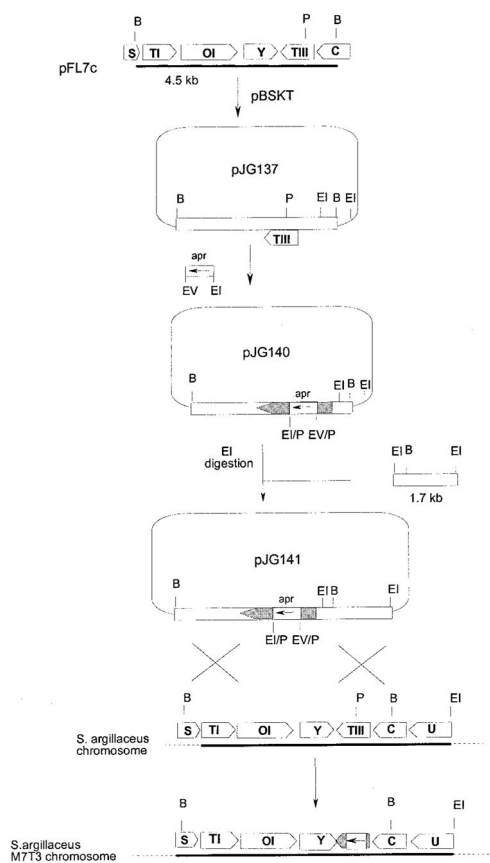




**Figure 2.** Late biosynthetic steps of the mithramycin (**10**) biosynthetic pathway, including the glycosyltransfer steps, the oxidative cleavage of the fourth ring, and some biosynthetic steps of the deoxysugar units.



**Figure 3.** New structures resulting from the *mtmC*- and *mtmTIII*-inactivation experiments: 4A-ketopremithramycin A2 (**11**), 4A-keto-9-demethylpremithramycin A2 (**12**), 4C-keto-demycarosylmithramycin (**13**), and 4E-ketomithramycin (**14**).



**Figure 4.** Scheme representing the insertional inactivation of *mtmTIII* and the replacement in the chromosome of the wild-type *mtmTIII* allele by the in vitro mutated one. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; P, *Pml*I; apr, apramycin resistance gene.





Table 1

<sup>1</sup>H NMR Data of 4A-Ketopremithramycin A2 (**11**), 4A-Keto-9-demethylpremithramycin A2 (**12**), 4C-Keto-demycarosylmithramycin (**13**), and 4E-Ketomithramycin (**14**) in Acetone-*d*<sub>6</sub> at 400 MHz ( $\delta$  in ppm Relative to Internal TMS)<sup>a</sup>

	$\delta$ multiplicity (J/Hz)			
	<b>11</b>	<b>12</b>	<b>13</b>	<b>14<sup>c</sup></b>
2-H			4.70 d (12)	
3-H			2.78 br t (12)	4.80 d (12)
4-H	4.24 s br	4.31 s br	a: 2.92 dd (16) <sup>b</sup> e: 2.60 dd (16,3)	2.82 br t (12) a: 3.00 complex
4-OCH <sub>3</sub>	3.6 s	3.61 d (1)		e: 2.68 dd (16,3)
4a-H	3.13 ddd (11,4,3)	3.13 ddd (11,4,3)		
5-H	a: 3.95 (16,4,1) e: 3.05 (16,3)	3.96 d br (16) 3.07 obsc	6.81 s	6.86 s
6-H	6.97 d (1)	7.03 s br		
7-H	6.75 s	6.69 s		
7-CH <sub>3</sub>			2.09 s	2.15 s
9-H		6.45 s		
9-CH <sub>3</sub>	2.19 s			
10-H			6.85 s	6.84 s
1'			4.88 d (1.5)	4.88 d (2)
1'-OCH <sub>3</sub>			3.40 s	3.44 s
2'	2.65 s	2.64 s		
3'			4.24 d (3) <sup>b</sup>	4.30 d (3) <sup>b</sup>
4'			4.23 dq (6.5,3) <sup>b</sup>	4.30 dq (6,3) <sup>b</sup>
5'			1.22 d (6.5)	1.27 d (6)
1A	4.94 dd (10,2)	4.95 dd (10,2)	5.34 dd (10,2)	5.33 dd (10,2)
2A <sub>a</sub>	1.86 ddd (12,12,10)	1.85 ddd (12,12,10)	1.80 ddd (12,12,10)	1.85 ddd (12,12,10)
2A <sub>e</sub>	2.45 m br	2.44 ddd (12,5,2)	2.43 ddd (12,5,2) <sup>b</sup>	2.40 ddd (12,5,2) <sup>b</sup>
3A	3.70 dd (12,5) <sup>b</sup>	3.71 dd (12,5) <sup>b</sup>	3.81 <sup>b</sup>	3.70 <sup>b</sup>
4A			3.06 dd (9,9)	3.06 dd (9,9)
5A	3.45 q (6)	3.44 q (6) <sup>b</sup>	3.52 dq (9,6)	3.51 dq (9,6)
5A-CH <sub>3</sub>	1.25 d (6)	1.25 d (6)	1.26 d (6)	1.30 d (6)
1B	4.68 dd (10,2)	4.69 dd (10,2)	4.74 dd (10,2)	4.72 dd (10,2)
2B	1.70 ddd (12,12,10) 1.90 ddd (12,5,2)	1.73 ddd (12,12,10) 1.92 ddd (12,5,2)	a: 1.55 ddd (12,12,10) e: 2.19 ddd (12,5,2)	1.58 ddd (12,12,10) 2.20 ddd (12,5,2)
3B	3.82 ddd (12,5,3)	3.83 m <sup>b</sup>	3.6 ddd (12,9,5)	3.60 ddd (12,9,5)
4B	3.52 s br	3.50 s br	2.99 dd (9,9)	3.01 dd (9,9)
5B	3.70 q (6.5)	3.70 q (6.5)	3.40 dq (9,6)	3.41 dq (9,6)
5B-CH <sub>3</sub>	1.25 d (6.5)	1.25 d (6.5)	1.26 d (6)	1.30 d (6)
1C			5.09 dd (10,2)	5.14 dd (10,2)

	$\delta$ multiplicity (J/Hz)			
	11	12	13	14 <sup>c</sup>
2C <sub>a</sub>			1.88 ddd (12,12,10)	1.62 ddd (12,12,10)
2C <sub>e</sub>			2.42 ddd (12,5,2) <sup>b</sup>	2.55 ddd (12,5,2)
3C			3.84 dd (12,5)	3.70 complex
4C				3.02 dd (9,9)
5C			3.40 q (6)	3.32 dq (9,6)
5C-CH <sub>3</sub>			1.26 d (6)	1.30 d (6)
1D			4.74 dd (10,2)	4.7 dd (10,2)
2D <sub>a</sub>			1.80 ddd (12,12,10)	1.76 ddd (12,12,10)
2D <sub>e</sub>			1.98 ddd (12,5,2)	1.93 ddd (12,5,2)
3D			3.81 ddd (12,5,3) <sup>b</sup>	3.82 complex
4D			3.56 s br	3.54 s br
5D			3.70 q br (6.5)	3.74 q (6)
5D-CH <sub>3</sub>			1.26 d (6.5)	1.24 d (6)

<sup>a</sup> br = broad; obsc = obscured by solvent or water; OH signals not visible due to 5% water in the samples.

<sup>b</sup> Complex, partially overlapped by other signal(s).

<sup>c</sup> Sugar E signals: 1-E, 5.42 dd (10,2); 2-E<sub>a</sub>, 1.98 dd (14,10); 2-E<sub>e</sub>, 2.30 dd (14,2); 3-E-CH<sub>3</sub>, 1.31 s; 5-E, 4.66 q (6); 5E-CH<sub>3</sub>, 1.30 d (6).

Table 2

<sup>13</sup>C NMR Data of 4A-Ketopremithramycin A2 (11), 4A-Keto-9-demethylpremithramycin A2 (12), 4C-Keto-demycarosylmithramycin (13), and 4E-Ketomithramycin (14) in Acetone-*d*<sub>6</sub> at 125.7 MHz ( $\delta$  in ppm Relative to Internal TMS, Assignments Were Made with the Help of Couplings Determined in the HSQC and HMBC Spectra)<sup>a</sup>

	$\delta$				$\delta$				
	11	12	13	14	11	12	13	14	
1	196.1	n.o.	203.7	204.0	1A	97.9	97.9	96.8	96.9
2	112.6	112.6	77.6	76.8	2A	36.9	36.9	37.2	37.5
3	193.1	n.o.	42.4	42.8	3A	82.0	82.1	80.4	81.4
4	77.6	77.6	27.1	27.3	4A	90.6	91.0	75.2	75.4
4-OCH <sub>3</sub>	61.2	61.2			5A	70.5	70.5	72.3	72.6
4a	42.7	42.5	136.4	136.5	6A	12.7	12.7	17.9	17.9
5	26.5	27.0	101.7	101.7	1B	100.5	100.5	99.4	100.0
5a	134.3	133.0			2B	34.8	34.8	39.7	40.0
6	117.6	117.8	159.6	159.2	3B	68.8	68.9	70.9	71.3
7	102.3	103.0	111.1	111.1	4B	70.3	70.6	77.0	77.5
7-CH <sub>3</sub>			7.97	7.9	5B	71.5	71.5	72.6	72.6
8	161.7	163.0	155.7	156.2	6B	16.4	16.7	17.6	16.5
8a			107.8	108.4	1C			101.3	100.9
9	110.4	102.0	164.0	165.3	2C			36.3	38.0
9a			108.7	108.6	3C			81.4	81.8
9-CH <sub>3</sub>	7.7				4C			91.3	75.7
10	157.1	160.6	117.3	117.2	5C			73.8	72.6
10a	106.8	107.1	138.9	139.0	6C			12.7	17.6
1'	202.9	202.9	81.9	82.1	1D			100.1	100.4
1'-OCH <sub>3</sub>			58.7	58.8	2D			34.4	36.0
2'	27.9	27.3	212.1	211.3	3D			68.7	68.9
3'			79.1	79.2	4D			69.9	70.2
4'			68.3	68.4	5D			71.5	71.1
5'			19.3	19.6	6D			16.4	14.7
					1E				97.5

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	$\delta$				$\delta$			
	11	12	13	14	11	12	13	14
2E				48.0				
3E				69.2				
3E-CH <sub>3</sub>				23.8				
4E				206.0				
5E				71.7				
6E				18.0				

<sup>a</sup> n.o. = not observed.

**Table 3**

Long-Range Couplings Observed in the HMBC Spectra of the New Premithramycin (**11**, **12**) and Mithramycin Derivatives (**13**, **14**) (Most Important Couplings Are Highlighted in Bold)

H-position	C-position			
	11	12	13	14
2			1,3,1',1C	3,1',1C
3				4
4	1,4-OCH <sub>3,4a,5</sub>			
α			4a	4a,10
β			2,3,4a,9a,10	2,4a,9a,10
4-OCH <sub>3</sub>	4	4		
4a				
5			6,7,8,8a,9,9a,10	6,7,10
α	4a,5a			
β				
6	5,6a,7,10a,11a	5,7,11a		
7	6,8,9,10a	6,8,9,10a		
7-CH <sub>3</sub>			5,6,7,8,8a,9a	6,7,8,10a
9		7,8,10,10a		
9-CH <sub>3</sub>	8,9,10			
10			4,5,8a,9a,10a	4,5,8,8a,9,9a,10a
14	2,13	13		
1'			2,3,4,1'-OCH <sub>3,2'</sub>	3,4,1'-OCH <sub>3,2'</sub>
1'-OCH <sub>3</sub>			1',3'	2,1'
3'			2',4',5'	2'
4'			2',5'	
5'			2',3',4'	3',4'
1A	12a		6	6,2A
2A <sub>a</sub>	1A,3A	1A,3A	1A,3A	1A,3A
2A <sub>e</sub>	3A,4A	3A,4A	1A,3A,4A	1A,3A,4A
3A	1B	1B	2A,4A,1B	1B
4A			3A,5A,6A	3A,5A
5A	<b>1A,6A</b>	<b>1A,6A</b>	1A,3A,4A,6A	1A,4A
6A	4A,5A	4A,5A	4A,5A	5A
1B	3A	3A	3A	3A,2B
2B <sub>a</sub>	1B,3B	1B,3B	1B,3B,4B	1B,3B
2B <sub>e</sub>			1B,3B,4B	1B,3B,4B
3B			2B,4B,5B	4B
4B	5B	5B	3B,5B,6B	3B,5B
5B	4B,6B	4B,6B	1B,3B,4B,6B	1B,3B,4B
6B	5B	5B	3B,4B,5B	2B,5B

H-position	C-position			
	11	12	13	14
1C				2,2C
2C <sub>a</sub>			1C,3C	1C,3C
2C <sub>e</sub>			1C,3C,4C	1C,3C,4C
3C			2C,1D	4C,1D
4C				3C,5C
5C			1C,3C,4C,6C	1C,4C
6C			4C,5C	2C,4C,5C
1D			3C	3C
2D <sub>a</sub>			1D,3D	1D,3D
2D <sub>e</sub>			4D	
<b>3D</b>			2D,4D	
4D			2D,3D,5D	2D,3D
5D			1D,4D,6D	3D,4D
6D			4D,5D	
1E				2E
2E <sub>a</sub>				1E
2E <sub>e</sub>				1E,4E
3E-CH <sub>3</sub>				1E,2E,3E,4E
5E				1E,4E
6E				4E,5E