Biosynthesis

DOI: 10.1002/anie.201205414

Cooperation of Two Bifunctional Enzymes in the Biosynthesis and Attachment of Deoxysugars of the Antitumor Antibiotic Mithramycin**

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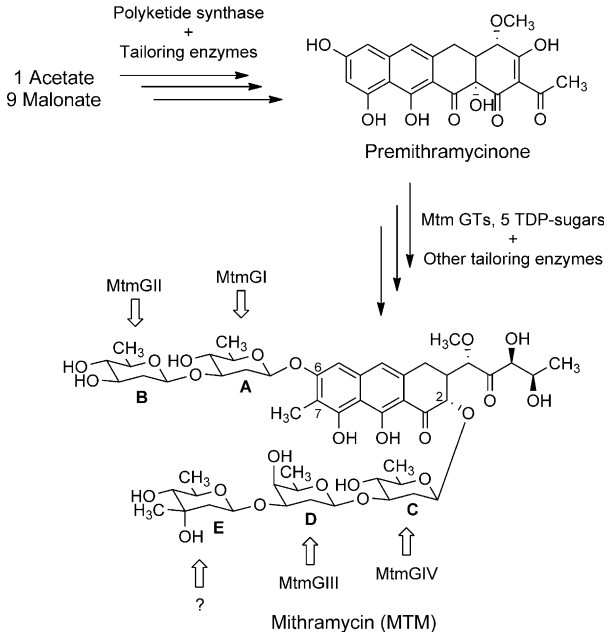
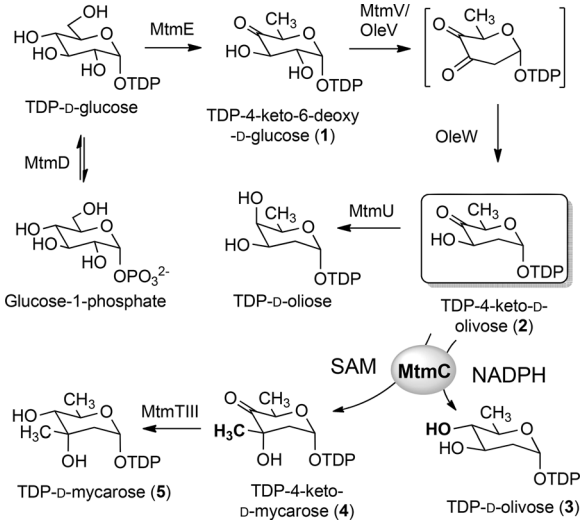
Glycosylation reactions catalyzed by glycosyltransferases (GTs) are important structural modifications that are often necessary for the biological activities of natural products.[1] Studying these is an increasingly important and rigorously pursued research field. Although GTs usually display strict substrate and regiospecificities, some examples were found showing surprising tolerance, which can be exploited in combinatorial biosynthetic approaches.[1b,c]

Mithramycin (MTM), a highly glycosylated aureolic acid anticancer agent, consists of a tricyclic polyketide-derived core with two alkyl side chains and five sugar moieties (three D-olivoses, one D-oliose, and a D-mycarose) attached as disaccharide (sugars A and B) and trisaccharide (sugars C–E) chains at the 6- and 2-positions, respectively (Scheme 1).[2] The sugar chains are important for the ability of MTM to bind and cross-link DNA, thereby blocking replication and transcription;[3] considerable efforts have been made to elucidate the biosynthetic assembly of the MTM sugar chains. Gene inactivation experiments suggested that MtmGIV catalyzes the first glycosylation (attaching sugar C), followed by the transfer of sugar D by MtmGIII.[4] After the assembly of the trisaccharide, MtmGI and MtmGII then transfer the two D-olivoses (sugars A and B) of the disaccharide.[5] However, an obvious GT candidate for the transfer of the D-mycarose moiety (sugar E) of the trisaccharide was missing. Although MtmGIV was suggested for this role,[6] such a dual function of a GT using two different donor and two significantly different acceptor substrates would be unprecedented, and has remained speculation. Furthermore, the biosynthetic pathway leading to D-olivose, the major monosaccharide building block of MTM, remained incomplete until recently, when a reconstitution of the biosynthesis of TDP (thymidine diphosphate)-D-olivose utilizing enzymes of the MTM pathway or functionally equivalent homologues (Scheme 2)[7] revealed that MtmC catalyzes the 4-ketoreduction step.



Scheme 1. Overview of the biosynthesis and structure of MTM. Sugars A, B, and C: D-olivose; sugar D: D-oliose; sugar E: D-mycarose.



Scheme 2. Biosynthetic pathway of the MTM sugars. Note the dual role of MtmC as *C*-methyltransferase and 4-ketoreductase.

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[**] This work was supported by the NIH grant CA 091901 to J.R.
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205414.

Previously, MtmC was only recognized as SAM (*S*-adenosylmethionine)-dependent *C*-methyltranferase involved in the biosynthesis of the branched sugar D-mycarose (sugar E).[6] In addition to MtmC, MtmTIII acts as a unique 4-ketoreductase of the MtmC product in the biosynthesis of the D-mycarose building block (Scheme 2).[6]

To determine the exact functions of MtmGIV, MtmC, and MtmTIII in the assembly of the trisaccharide chain, we now reconstituted the pathway of D-mycarose in vitro. MtmC was overproduced in *Escherichia coli* as described previously,[7] purified to near homogeneity (see Supporting Information, Figure S1), and tested for its *C*-methyltransferase activity by using TDP-4-keto-D-olivose (**2**; Scheme 2) and SAM as substrates. Substrate **2** was prepared from TDP-4-keto-6-deoxy-D-glucose (**1**) by using the enzyme pair OleV (an MtmV homologue from the oleandomycin pathway)/OleW,[8] together with an NADPH regeneration system consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G6PD).[7]

Substrate **2** disappeared when MtmC was incubated with pure **2** and SAM, yet no unique TDP-sugar product was detectable (Supporting Information, Figure S2). In contrast, replacement of pure **2** by crude preparations (OleV/OleW reactions for in situ generation of **2**) did reveal a new product that co-eluted with TDP-D-olivose (**3**; Figure 1, trace b). The

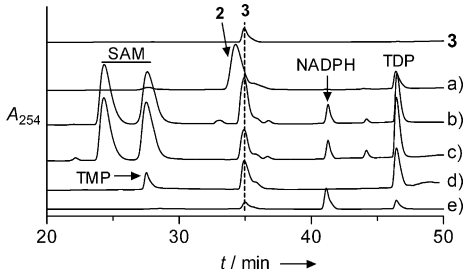


Figure 1. HPLC traces of sugar reactions catalyzed by MtmC. a) Crude **2** without MtmC; b) crude **2** + MtmC + SAM + NADPH; c) adding MtmTIII and NADPH into (b); d) adding $NaBH_4$ into (b); e) MtmC + pure **2** + NADPH. A_{254}: absorbance at 254 nm.

new product could not be reduced, neither by MtmTIII nor chemically with $NaBH_4$ (Figure 1, traces c and d), excluding a ketosugar as a possibility. The product was confirmed to be **3** by ^{1}H NMR spectroscopy (see the Supporting Information), and its generation can be explained by the 4-ketoreductase activity of MtmC. This reduction also occurred when MtmC was incubated with pure **2** and NADPH (Figure 1, trace e). Although we were unable to detect a methylated MtmC product starting with pure **2** and SAM, the disappearance of **2** suggested that a reaction had occurred, but leading to an unstable product. Possibly, the axial 3-OH group generated concomitantly with the 3-methylation reaction can attack the proximal phosphate to cleave the phosphodiester bond, thereby removing the thymidine chromophore (Supporting Information, Figure S2), which is analogous to a mechanism we and others identified recently.[9]

We hoped to circumvent this instability of the methylated sugar by using MtmGIV-coupled reactions, and therefore attempted to overproduce MtmGIV in *E. coli*, which led to a soluble but inactive protein. We also failed to obtain functional MtmGIV from *S. lividans* TK64, but succeeded by overexpressing the *mtmGIV* gene in the *S. argillaceus* Δ*mtmGIV* mutant strain M3G4. This mutant strain produces premithramycinone (**6**, Schemes 1 and 3),[4] and complementation with *mtmGIV* completely restored the MTM production, suggesting that functional MtmGIV was produced (Supporting Information, Figure S3). Using this complemented strain, MtmGIV was purified to near homogeneity (Supporting Information, Figure S4).

First, we wanted to demonstrate the glycosyltransferase activity of MtmGIV by monitoring the transfer of D-olivose (sugar C) onto **6** (the first glycosylation event; Scheme 3). Incubations of MtmGIV with **6** and pure **3** resulted in the production of a new peak (Figure 2, trace a) with a $[M-H]^-$ ion at m/z 543.1 (Supporting Information, Figure S5), and HRMS (Supporting Information, Table S1) and ^{1}H NMR spectra (Supporting Information, Figure S6) that are consistent with structure of premithramycin A1 (**7**), thus confirming the enzyme activity and the role of MtmGIV in initiating the trisaccharide assembly. As expected, the control proteins prepared from M3G4 cells harboring only the empty vector failed to glycosylate **6**. By monitoring this glycosylation, MtmGIV was found to optimally react at pH 8 (Supporting Information, Figure S7). Interestingly, MtmGIV was also able to transfer multiple D-olivose units iteratively onto **6**, resulting in diolivosyl- and triolivosylpremithramycinone (**10** and **11**; Scheme 3) when excess amounts of **3** were present (Figure 2, trace b). The identity of **10** and **11** was confirmed by HRMS (Supporting Information, Table S1).

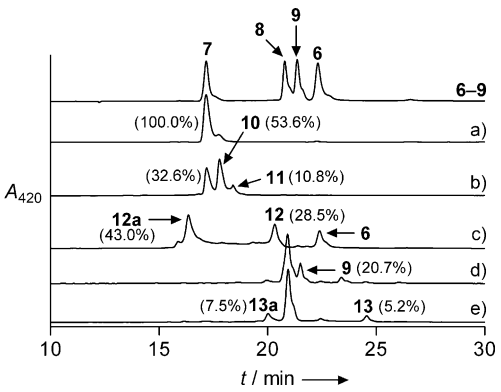
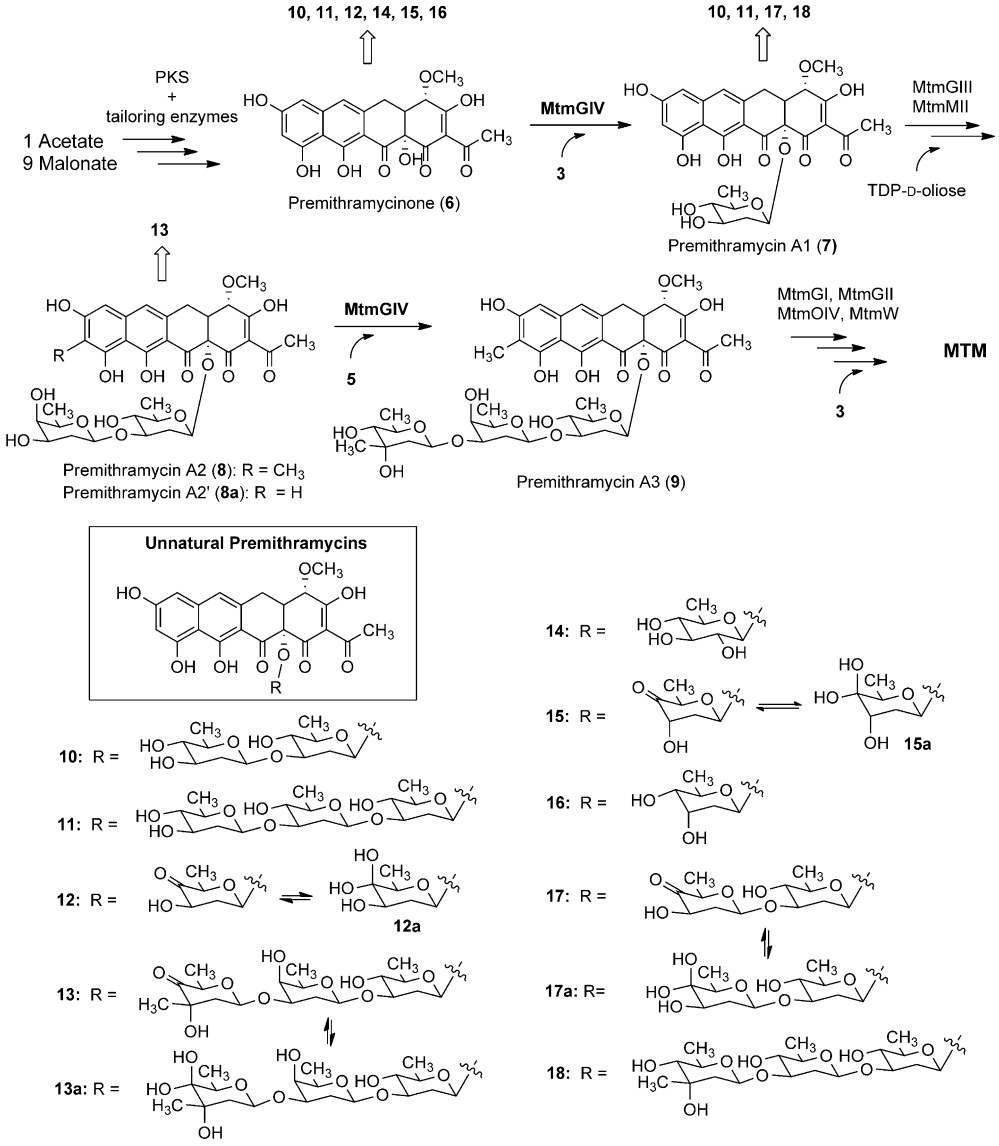


Figure 2. HPLC traces of MtmGIV-catalyzed glycosylations using **6** (a–c) or **8** (d,e) as the acceptor substrate. a) MtmGIV + **6** + **3**; b) MtmGIV + **6** + excess amount of **3** (0.8 mM); c) MtmGIV + **6** + **2**; d) MtmGIV + **8** + in situ generated **5**; e) MtmGIV + **8** + **4** generated in situ. The conversion rate [%] is shown in parentheses. A_{420}: absorbance at 420 nm.

As some earlier isolated MTM derivatives contained ketosugars in C and E positions,[6] we wanted to confirm that MtmGIV can also transfer ketoolivose (**2**). Thus, the activity of MtmGIV was tested with **6** and **2**. Two new peaks were observed using LC-MS, with retention times $t_{ret} = 20.3$ and 16.3 min (Figure 2, trace c). Corresponding $[M-H]^-$ ions



Scheme 3. Proposed pathway for the assembly of the MTM trisaccharide chain and structures of non-naturally glycosylated premithramycins.

were m/z 541.1 and 559.1 (Supporting Information, Figure S5), respectively, which is consistent with a molecular formula $C_{27}H_{26}O_{12}$ of 4-keto-D-olivosyl-premithramycinone (**12**; Scheme 3) and its hydrated form $C_{27}H_{28}O_{13}$ (**12a**), which was corroborated by HRMS (Supporting Information, Table S1). Thus, MtmGIV was able to transfer both the 4-keto and the reduced sugar to the acceptor substrate **6**, confirming for the first time in vitro such a sugar donor substrate flexibility of MtmGIV. To probe whether the 4-ketoreduction of sugar C in MTM biosynthesis occurs before or following the MtmGIV catalysis, kinetic studies were carried out. The results showed a complete conversion of **3** into product within 0.5 h, while the transfer of **2** required at least 8 h (Supporting Information, Figure S8), suggesting the reduced sugar **3** to be the preferred sugar donor for MtmGIV, which was further confirmed by single-substrate kinetic experiments. Both substrates followed typical Michaelis–Menten kinetic patterns with $K_M = (0.25 \pm 0.04)$ mM and $k_{cat} = (2.2 \pm 0.1)\ min^{-1}$ with respect to **3** and $K_M = (0.21 \pm 0.04)$ mM and $k_{cat} = (0.11 \pm 0.02)\ min^{-1}$ with respect to **2** (Supporting Information, Figure S8).

After confirming the function of MtmGIV in transferring D-olivose onto **6**, we next tested whether a mixture of MtmC, MtmTIII, and MtmGIV could produce D-mycarose in situ from **2** and transfer it to premithramycin A2 (**8**; Scheme 3). When MtmC, MtmTIII, and MtmGIV were incubated with **2**, acceptor substrate **8**, SAM, and NADPH, the HPLC analysis revealed a new peak that co-eluted with premithramycin A3 (**9**; Figure 2, trace d). The LC-MS yielded an $[M-H]^-$ ion at m/z 831.3 (Supporting Information, Figure S5) consistent with the molecular formula $C_{41}H_{52}O_{18}$ of **9**, which was further corroborated by HRMS (Supporting Information, Table S1) and ^{1}H NMR (Supporting Information, Figure S9). These data confirm that MtmGIV indeed catalyzes two glycosylation events in MTM biosynthesis by attaching two structurally distinct sugars onto two significantly different acceptor molecules. Moreover, MtmGIV seemed to closely cooperate with MtmC in its glycosylation events, triggering us to further investigate MtmC. The *C*-methyltransferase activity of MtmC was further tested by omitting MtmTIII and NADPH from the reaction mix that now consisted of MtmC, MtmGIV, **2**, **8**, and SAM. This enzyme mixture yielded two new peaks with $t_{ret.} = 24.3$ and 19.8 min (Figure 2, trace e). LC-MS analysis of these new peaks revealed $[M-H]^-$ ions at m/z 829.3 and 847.4 (Supporting Information, Figure S5), respectively, consistent with the molecular formula $C_{41}H_{50}O_{18}$ of 4-keto-D-mycarosyl-premithramycin A2 (**13**; Scheme 3) and its hydrated form $C_{41}H_{52}O_{19}$ (**13a**), which was further confirmed by HRMS (Supporting Information, Table S1). These results clearly confirmed the transfer of a methyl group onto **2** to afford keto-D-mycarose. The same glycosylation reactions occur when premithramycin A2′ (**8a**, Scheme 3) was used as the acceptor substrate (Supporting Information, Figure S10). Indirectly, these results also confirmed the function of

MtmTIII in vitro, which had previously been assigned as a 4-ketoreductase for the D-mycarose biosynthesis based on an *mtmTIII* inactivation experiment,[6] which led to the accumulation of 4E-ketomithramycin.

Given the unusual substrate flexibility of MtmGIV in MTM biosynthesis, we wanted to more closely examine its substrate specificity by using alternative acceptors and TDP–sugar donors. The attachment of sugar C to **6** was first examined by using a panel of TDP-activated sugars (Supporting Information, Figure S11). A new peak with $t_{ret.} = 16.2$ min appeared when TDP-D-quinovose was used as donor (Figure 3, trace a), with an $[M-H]^-$ ion at m/z 558.9 (Supporting Information, Figure S5), consistent with the molecular formula $C_{27}H_{28}O_{13}$ of D-quinovosylpremithramycinone (**14**), which was further supported by HRMS (Supporting Information, Table S1). Notably, two TDP-sugars with axial 3-OH groups were also prepared, TDP-4-keto-D-digitoxose and TDP-D-digitoxose (Supporting Information, Figure S11). The former sugar was generated in situ by incubating **1** with OleV and EryBII (an earlier identified 3-ketoreductase from the erythromycin pathway).[10] LC-MS analysis revealed two new peaks eluted at 20.4 and 16.6 min, respectively (Figure 3, trace b), with $[M-H]^-$ ions at m/z 541.0 and 559.0 (Supporting Information, Figure S5) that are consistent with the molecular formula $C_{27}H_{26}O_{12}$ of 4-keto-D-digitoxosyl-premithramycinone (**15**) and its hydrated form $C_{27}H_{28}O_{13}$ (**15a**; Supporting Information, Table S1). The latter sugar was generated by utilizing the same conditions as above but including MtmTIII and NADPH. LC-MS revealed a new peak with $t_{ret.} = 17.2$ min (Figure 3, trace c) with an $[M-H]^-$ ion at m/z 543.2 (Supporting Information, Figure S5), consistent with the molecular formula $C_{27}H_{28}O_{12}$ of D-digitoxosyl-premithramycinone (**16**). The identities of **15** and **16** were confirmed by HRMS (Supporting Information, Table S1). In total, our results suggested that MtmGIV has an unusually relaxed substrate specificity towards the stereochemical configuration at the 3-position of sugar donors, but rigidly controls that of the 4-position and prefers 2,6-deoxygenated D-sugars.

The same panel of TDP-activated sugars was tested for the second glycosyltransfer reaction catalyzed by MtmGIV, for which we checked the transfer to both the potential disaccharidal acceptor substrates **8** and **8a**. Here, MtmGIV was unable to use any other TDP-sugars besides **4** and **5** (Supporting Information, Table S2). Most surprising was the fact that MtmGIV failed to recognize TDP-D-digitoxose, although MTM analogues with D-digitoxose as the E sugar have been reported.[2b,11]

Following up on our observation that MtmGIV can catalyze multiple, sequential transfers of D-olivose to generate mono-, di- and triglycosylated MTMs onto the acceptor substrate **6**, we also checked whether MtmGIV would tolerate monosaccharide **7** (Scheme 3) as an acceptor substrate. In the presence of **3** and **7**, MtmGIV rapidly generated compounds **10** and **11** (Figure 4 trace a; Supporting Information, Figure S12). Incubation of MtmGIV with **7** and the ketosugar **2** also yielded two new products with $t_{ret.} = 20.5$ min and 16.9 min (Figure 4, trace b). LC-MS revealed two $[M-H]^-$ ions at m/z 671.2 and 689.1 (Supporting Information, Figure S5), respectively, as confirmed by HRMS (Supporting Information, Table S1), which were consistent with the molecular formula $C_{33}H_{36}O_{15}$ of 4-keto-D-olivosyl-premithramycin A1 (**17**, m/z 672.2) and its hydrated form $C_{33}H_{38}O_{16}$ (**17a**, m/z 690.2). Next, we tested the ability of MtmGIV to transfer D-mycarose onto **7** by using **5** generated in situ. Instead of the expected D-mycarosyl-premithramycin A1 (m/z 702.2), LC-MS revealed two peaks with $t_{ret.} = 17.8$ and 20.7 min (Figure 4, trace c) with $[M-H]^-$ ions at m/z 673.2 and 817.2, respectively (Supporting Information, Figure S5). The former was consistent with a disaccharide product containing an additional D-olivose that is formed by MtmC-catalyzed reduction of the ketosugar **2** instead of a D-mycarose formed by MtmC-catalyzed methylation. Indeed, the same product was obtained when MtmTIII and SAM were omitted from the enzyme mixture (Figure 4, trace d). The latter peak, analyzed by HRMS (Supporting Information, Table S1), was consistent with the molecular formula of $C_{40}H_{50}O_{18}$ for the trisaccharidal compound **18** generated by sequential transfer of D-olivose and D-mycarose. These results clearly indicated that MtmGIV itself is able to assemble trisaccharide-containing products, and that MtmC can pro-

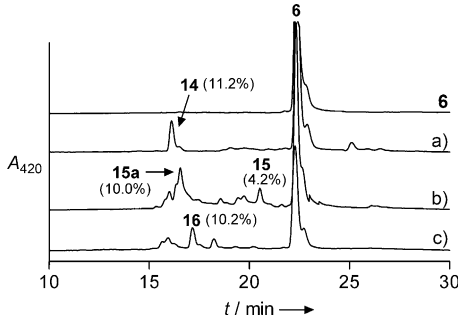


Figure 3. HPLC traces of substrate flexibility studies of MtmGIV using **6** as the acceptor substrate. a) MtmGIV + **6** + TDP-D-quinovose; b) MtmGIV + **6** + TDP-4-keto-D-digitoxose generated in situ; c) MtmGIV + **6** + TDP-D-digitoxose generated in situ.

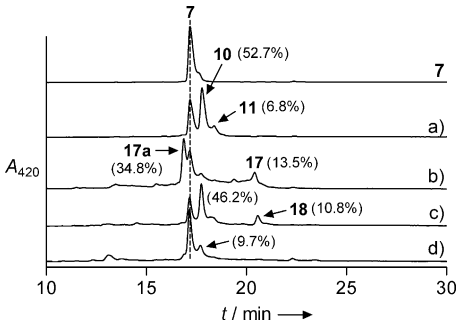


Figure 4. HPLC traces of substrate flexibility studies of MtmGIV using **7** as the acceptor substrate. a) MtmGIV + **7** + **3**; b) MtmGIV + **7** + **2**; c) MtmGIV + **7** + **3** and **5** generated in situ by adding MtmC, MtmTIII, SAM, and NADPH into (b); d) MtmGIV + **7** + **3** generated in situ by adding only MtmC and NADPH into (b).

vide parallel formation of both D-olivose and D-mycarose, from **2** through its 4-ketoreductase and *C*-methyltransferase activities, respectively.

In conclusion, we have functionally assigned two enzymes that closely cooperate for the assembly of the MTM trisaccharide chain; both have a dual role. MtmGIV is responsible for two distinct sugar transfers, leading to sugars C and E in MTM. It closely cooperates with MtmC, which was shown to use a single substrate **2** yet remarkably catalyzes either a reduction or a methyl transfer reaction using the appropriate co-substrate (NADPH or SAM) depending on which of the two MtmGIV actions is supported. This way MtmC generates the two building blocks of MTM's trisaccharide chain, TDP-D-olivose and TDP-D-mycarose, respectively, which serve as sugar donors for MtmGIV. Although a few iteratively acting GTs, such as AveBI, AknK, LanGT1, and LanGT4 had been reported,[12] they all utilized only one sugar donor substrate. To our knowledge, MtmGIV is the first characterized GT able to recognize two distinct acceptor and two distinct donor substrates in a given metabolite pathway. The discovery of these dual functional enzymes explains two previously missing activities that are essential for MTM biosynthesis and that were not readily predicted from the bioinformatic analysis of the gene products. Taken together, the results have allowed us to propose a more complete biosynthetic pathway for MTM, and also provide the impetus for generating new glycosylated MTMs by exploiting the substrate flexibility of MtmGIV. Apart from the mentioned GTs, also iteratively acting ketosynthases or cyclases/aromatases, and most recently a reductase/methyltransferase were identified previously. Such multifunctional enzymes are likely to be just the tip of the iceberg and will continue to be discovered in secondary metabolism. Only a few of these have been previously studied biochemically.[9a,13]

Received: July 9, 2012
Published online: September 20, 2012

Keywords: bifunctional enzymes · glycosyltransferase · 4-ketoreductase · *c*-methyltransferase · mithramycin

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