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Structural insight into MtmC, a bifunctional ketoreductase-methyltransferase involved in the assembly of the mithramycin trisaccharide chain

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Abstract

More and more post-PKS tailoring enzymes are recognized to be multifunctional and co-dependent on other tailoring enzymes. One of the recently discovered intriguing examples is MtmC, a bifunctional TDP-4-keto-D-olivose ketoreductase-methyltransferase, which – in co-dependence with glycosyltransferase MtmGIV – is a key contributor to the biosynthesis of the critical trisaccharide chain of the antitumor antibiotic mithramycin (MTM), produced by *Streptomyces argillaceus*. We report crystal structures of three binary complexes of MtmC with its methylation co-substrate SAM, its co-product SAH, and a nucleotide TDP as well as crystal structures of two ternary complexes, MtmC-SAH-TDP-4-keto-D-olivose and MtmC-SAM-TDP, in the range of 2.2–2.7 Å in resolution. The structures reveal general and sugar-specific recognition and catalytic structural features of MtmC. Depending on the catalytic function that is carried out by MtmC, it must bind either NADPH or SAM in the same co-factor binding pocket. A tyrosine residue (Tyr79) appears as a lid covering the sugar moiety of the substrate during the methyl transfer reaction. This residue swings out of the active site by about 180° in the absence of the substrate. This unique conformational change likely serves to release the methylated product and, possibly, to open up the active site for binding the bulkier co-substrate NADPH prior to the reduction reaction.

Keywords

crystal structure; conformational change; bifunctional enzyme; natural product; sugar biosynthesis

INTRODUCTION

Mithramycin **1** (MTM; Fig. 1) is a member of the aureolic acid family of antitumor antibiotics that also includes the chromomycins, olivomycin, durhamycin A, UCH9, and chromocyclomycin.^{1–4} Aureolic acids act on gram positive bacteria, and inhibit growth and multiplication of several cancer cell lines through dysregulation of transcription, by

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interacting in an Mg^{2+} -dependent manner with the minor groove of GC-rich regions of DNA.^{5, 6} Durhamycin A is also an inhibitor of HIV Tat transactivation.⁴ Additionally, chromomycin and MTM are strong inducers of erythroid differentiation in K562 cells and potent inhibitors of neuronal apoptosis, making these compounds candidates for therapeutics of haematological diseases and neurological disorders, respectively.⁷ MTM was recently discovered as the only drug out of 50,000 screened compounds to potently antagonize the abnormal oncogenic transcription factor EWS-FLI1 in Ewing sarcoma, a poorly treatable bone and soft tissue cancer that affects mostly children and young adults.⁸ MTM is currently in clinical trials to treat this devastating cancer (National Cancer Institute, clinical trial NCT01610570). MTM was also identified as a potent down-regulator of ABCG2 efflux pumps,⁹ whose overexpression is responsible for resistance to lung cancer chemotherapeutics. MTM recently entered clinical trials to treat cancers of the respiratory tract, including esophageal and lung cancers (National Cancer Institute, clinical trial NCT01624090).

MTM and all other aureolic acid compounds (except chromocyclomycin) contain a tricyclic chromophore with two oligosaccharide chains attached via *O* glycosidic bonds at position C-2 and C-6 of the aglycone, respectively, as well as two aliphatic side chains at C-3 and C-7 positions, respectively (Figure 1). The aglycone is formed by a type-2 polyketide synthase (PKS) from one acetyl-CoA and nine malonyl-CoA units.¹⁰⁻¹³ In each aureolic acid compound, except durhamycin A and UCH9, the oligosaccharides at C-2 and C-6 are di- and trisaccharide chains, respectively, and they contain unique deoxyhexose sugars. The trisaccharide chain of MTM is a key structural part that appears to be evolutionary optimized for binding DNA in a minor groove of GC-rich regions. This chain contains a *D*-olivose (sugar C), a *D*-oliose (sugar D) and a *D*-mycarose (sugar E). Although this chain contains three sugar units, its biosynthesis requires only two glycosyltransferases, namely MtmGIV and MtmGIII. MtmGIV initiates the glycosylation cascade by transferring a *D*-olivose moiety to the aglycone precursor premithramycinone, and finishes the trisaccharide chain by transferring a *D*-mycarose unit (Fig. 2).¹⁴ MtmGIII acts by transferring the middle sugar, *D*-oliose.^{15, 16} Both of the MtmGIV catalyzed steps are supported by MtmC, which – in situ – generates either TDP-*D*-olivose by reduction or TDP-*D*-4 keto mycarose by methylation from the same precursor, TDP-4-keto-*D*-olivose (TDP-KOL).¹⁷ Furthermore, MtmTIII is required for the reduction of 4-keto-*D*-mycarose to *D*-mycarose, either prior or immediately after the glycosyltransfer step.¹⁴ Therefore, activities of MtmC, MtmGIV and MtmTIII need to be coordinated. How these enzymes cooperate remains one of the most intriguing mysteries of the MTM biosynthetic pathway. Here we present the first piece of the puzzle of the multi-faceted biosynthesis of MTM's trisaccharide chain, the crystal structure of the bi-functional ketoreductase-methyltransferase MtmC.

MATERIALS AND METHODS

Protein expression, and purification and site-directed mutagenesis of MtmC

The original annotated sequence of the *mtmC* gene from *Streptomyces argillaceus* contained inaccuracies; the corrected sequence was deposited into GenBank under submission numbers GUSub26197 and GUSub26196 for the amino acid residue and nucleotide

sequences, respectively. MtmC protein was expressed following a previously reported protocol.¹⁷ The fractions containing MtmC were pooled and dialysed against 20 mM Tris pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol and 10% glycerol. For biochemical assays, the enzyme was concentrated to 14 mg/mL, flash-frozen and stored at -80°C . For crystallization, the purified protein was further passed through a size-exclusion Sephacryl S-200 column (GE Healthcare) equilibrated in 40 mM Tris-HCl pH 8.0 (pH adjusted at room temperature), 0.1 M NaCl and 2 mM β -mercaptoethanol and the fractions containing the protein were pooled and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore) to 12 mg/ml.

MtmC Tyr79Phe and MtmC Tyr79Ala mutants were generated by site-directed mutagenesis with the QuikChange kit (Stratagene), following the manufacturer's protocol. Incorporation of the desired mutation into each plasmid was confirmed by DNA sequencing at the University of Kentucky DNA Sequencing Core. Mutant proteins were expressed and purified analogously to the wild-type enzyme.

Crystallization, data collection and crystal structure determination

The initial crystallization condition was found by a sparse incomplete factorial screen (Hampton Research Crystal Screen), by vapor diffusion in hanging drops at 21°C . At the optimized conditions, the drops contained 1 OL of MtmC with 1 mM ligand (SAM, SAH, TDP, TDP-4-keto D-olivose or their mixtures, as specified) and 1 OL of the reservoir solution (0.1 M MES pH 5.5, 0.2 M ammonium acetate, 16% PEG 4000). The crystals were gradually transferred into the cryoprotectant buffer (0.1 M MES pH 5.5, 0.2 M ammonium acetate, 16% PEG 4000, 20% glycerol) and rapidly frozen in liquid nitrogen.

X-ray diffraction data were collected at 100 K at beamlines 21ID-G (for MtmC-SAM-TDP crystals) and 22ID (for the other crystals) of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL). The data were processed with HKL2000.¹⁸ The structure of MtmC-SAM-TDP complex was determined by molecular replacement with MOLREP¹⁹ with the structure of methyltransferase TcaB9 (PDB accession code 4E2X)²⁰ as a search model. The structure was then iteratively rebuilt and refined with COOT²¹ and REFMAC,²² respectively. The ligands in this and other structures were modeled without uncertainty into strong omit $F_o - F_c$ electron density. The structure of MtmC-SAM-TDP served as a starting point for rebuilding and refinement of all other structures of MtmC. The data collection and refinement statistics are given in Table 1. Crystal structures listed in Table 1 were deposited in the Protein Data Bank under accession codes 1RV9 (complex with SAH), 4RVD (complex with SAM), 4RVF (complex with TDP), 4RVG (complex with SAM and TDP) and 4RVH (complex with SAH and TDP-4-keto D-olivose).

Methyltransferase activity assays—The product of the 3-methylation reaction by wild-type MtmC, TDP-4-keto D-mycarose , was unobservable likely because the axial 3-OH group generated concomitantly with 3-methylation attacked the proximal phosphate to cleave the phosphodiester bond of TDP, resulting in the loss of UV absorbance.¹⁷ For this reason, we monitored formation of the co-product SAH. Kinetic assays were carried out for the wild-type and mutant MtmC (Tyr79Phe and Tyr79Ala) by discontinuous HPLC (Waters

600 system, consisting of a controller, a Waters 996 photodiode array detector, and a Delta 600 pump). A 100 L reaction mixture contained 25 mM Hepes pH 7.5, 50 mM NaCl, 1 mM EDTA, 90 M TDP KOL, 2 mM SAM, and 5 M MtmC. The reactions were initiated by the addition of enzyme and incubated at 22 °C, to minimize the non-enzymatic degradation of SAM. 50 µL aliquots were quenched at 60 and 300 min (in the range where SAH concentration increased linearly over time) with 5 µL of 1.5 g/mL trichloroacetic acid (13.6% w/v final concentration) and then incubated on ice for 10 min. After centrifugation at 13,000 rpm for 3 min, 50 µL of the supernatant was passed through a Phenomenex Kinetex™ 5 µm EVO C18 100 Å column (250 × 4.6 mm) and eluted isocratically in 10 mM ammonium formate with 5% methanol (pH = 3.0) at 0.8 mL/min. The area under the chromatogram absorbance peak at 260 nm corresponding to SAH was measured. The rate of conversion of SAM to SAH for the wild-type enzyme and the two mutants was calculated from the 60 and 300 min data points.

RESULTS

Homologs of MtmC—A BLAST search²³ for MtmC homologs yielded a number of mostly putative bacterial Class I family SAM-dependent sugar C-methyltransferases dominated by those from *Streptomyces*. MtmC homologs whose function has been demonstrated or postulated are listed in Table 2 together with the respective sugar moiety and the final natural product, whose biosynthesis they are involved in. The multiple sequence alignment of MtmC and these homologs are given in Supplementary Fig. 1. The only homolog of MtmC that has been rigorously characterized both functionally and structurally, by Holden and co-workers, is C-3'-methyltransferase TcaB9 from *Micromonospora chalybeata*, from the biosynthetic pathway for D-tetronitrose in tetrocarcin A.^{20, 24} Structural information on MtmC would not only help elucidate its intriguing catalytic mechanism, but also reveal divergent structural features of this family of C-methyltransferases responsible for recognition of specific sugar substrates.

Overview of crystal structures of MtmC with its substrates and co-substrates

—We determined crystal structures of three binary complexes: MtmC-SAM, MtmC-SAH and MtmC-TDP and two ternary complexes: MtmC-SAH-TDP-4-keto-D-oligose and MtmC-SAH-TDP (Fig. 3). In all the structures, the ligands were very clearly resolved in the strong omit $F_o - F_c$ electron density map (Fig. 3). The apo-MtmC did not form crystals suitable for a diffraction experiment, likely due to mobility of protein regions involved in substrate or co-substrate binding. The overall structure of MtmC is similar to that of TcaB9.²⁴ MtmC is a monomer with a tri-partite fold. Its N-terminal domain (residues 1-68) consists of a β -sheet and extensive regions lacking secondary structure. As in TcaB9, these loop regions contain a tightly bound Zn^{2+} ion coordinated tetrahedrally to four conserved cysteines (Cys13, Cys16, Cys56 and Cys59; Fig. 3 and Supplementary Fig. 1). The central domain (residues 69-288) and the C terminal domain (residues 289-423) are structurally similar, with a Rossmann-type fold forming a SAM and a TDP binding site, respectively. Except for one region of the central domain, whose residues interact both with SAM/SAH and the sugar moiety of the TDP-sugar substrate (residues 76 to 84; described in detail in the next section), all structures of MtmC are similar to each other.

MtmC-SAM and MtmC-SAH complexes—In the crystal structures of MtmC-SAM and MtmC-SAH complexes, the co-factor and its product are bound in the conserved binding pocket formed by residues of the central domain, similarly to the bound SAH in the crystal structure of TcaB9²⁴ (Fig. 3A-D). The protein conformations in the complexes with SAM and SAH are nearly identical (Figs 3C, D). A striking difference in the substrate binding pocket between MtmC-SAM/SAH and TcaB9 structures is in the conformation of a loop region between residues 76 and 84, which we will call the sensor loop (Fig. 3). The backbone of the sensor loop in MtmC switches its conformation based on whether the substrate or the co-substrate occupies their respective binding pockets. In contrast, the conformation of this region in TcaB9 does not differ significantly among any of its binary and ternary complexes with the co-substrate, substrate or their products.^{20, 24} The conformations of the sensor loop of MtmC when only SAM/SAH or only TDP is bound, allow relatively facile access to the active site from outside. In contrast, when both SAM and TDP are bound this loop adopts a conformation that is more closed onto the active site. Finally when both SAH and TDP-4'-keto-olivose are bound, the sensor loop fits in the most closed state, sterically blocking the dissociation of the ligands from it.

MtmC-SAH-TDP-4-keto- α -olivose, MtmC-SAM-TDP and MtmC-TDP structures—

The ternary complex MtmC-SAH-TDP-4-keto- α -olivose represents a mimic of the reaction intermediate prior to the catalytic methyl transfer. Here, we observe a number of interactions that stabilize the bound substrate TDP-4-keto- α -olivose (Fig. 4). These interactions are similar to those observed in the ternary complex TcaB9-SAH-TDP-3-amino 2,3,6-trideoxy-4-keto- α -glucose, except His178 of MtmC is replaced by Asn 177 in TcaB9, both making a van der Waals contact between their respective C β -methylene group and the 5-methyl group of the sugar. The environment of the 3-OH group of the sugar is highly hydrophilic, with the side chains of Glu225 and His182 engaged in hydrogen bonds with this hydroxyl group. Tyr77 is in the sensor loop, but its conformation does not change significantly in different complexes, and its hydroxyl group appears to form a weak hydrogen bond with the 3-OH of the sugar (with the O-O distance of 3.35 Å). His226 forms a hydrogen bond with the 4-keto group of the olivose, consistent with its potential role as the catalytic acid, demonstrated for its counterpart in TcaB9, His225.²⁰ The 3-OH group of the sugar points towards the sulfur atom of SAH, indicating that the methylation occurs with inversion of stereochemistry at this position, in agreement with the biosynthetic pathway and the enzymatic properties of MtmC.¹⁷ In contrast, the counterpart 3-amino-group of TDP-3-amino 2,3,6-trideoxy-4-keto- α -glucose points away from the SAH in the complex with TcaB9, indicating that methylation by this enzyme occurs without inversion at this stereocenter.²⁴

The sensor loop and especially Tyr79 exhibit a progression of conformational states in complexes MtmC-SAM/SAH, MtmC-TDP, MtmC-SAM-TDP and MtmC-SAH-TDP-4-keto- α -olivose, as follows. In the MtmC-SAM and MtmC SAH complexes (Fig. 5A; MtmC-SAH is nearly identical), Tyr79 points out of the active site. When only TDP is bound (Fig. 5B), the sensor loop remains essentially unchanged, except for Tyr79, whose side chain is rotated by 130° where it does not interact with the TDP phosphates (Fig. 5B). In the MtmC-TDP complex, the only complex not containing SAM or SAH, the sensor loop exhibits

partial disorder, whereas it is well ordered in the MtmC-SAM and MtmC-SAH complexes, indicating that this loop undergoes an order-disorder transition upon dissociation of SAH. This is consistent with Tyr77, Tyr79 and Thr81 in this loop forming a part of the co-factor binding interface in the ternary complexes (Figs. 4 and 5C, D). In the ternary complex MtmC-SAM-TDP (Fig. 5C), the backbone of the sensor loop around Tyr79 is moved towards the active site, with Tyr79 pointing out of the active center. In this conformation, Tyr79 does not interact with either SAM or TDP, apparently because the previous conformation would place the aromatic ring of Tyr in the unfavorable charged environment of the TDP phosphate groups SAM. In all the above complexes, waters fill empty co-substrate or substrate binding pockets. Specifically, in complexes MtmC-SAM/SAH and MtmC-TDP, a water molecule is invariably found in place of the 3-OH group of the sugar (Fig. 5A-C). In complex MtmC-SAH-TDP-4-keto-D-olivose (Fig. 5D), the sensor loop stays in the same backbone conformation, and Tyr79 is in a different rotamer state (different from that in the MtmC-SAM complex by 180°), capping the substrate by the steric contact between the phenol ring of Tyr79 and the nonpolar sugar-β-phosphate junction as well as a making a hydrogen bond between the hydroxyl group of Tyr79 and the α-phosphate. In addition, in this conformation Tyr79 interacts sterically with SAH. This conformation of the sensor loop and Tyr79 is similar to that observed in all structures of TcaB9, with and without the substrates, co-substrates or products. Therefore, unlike TcaB9, MtmC exhibits conformational plasticity of the sensor loop and its residues, especially Tyr79.

Methyltransferase activity of MtmC mutants Tyr79Ala and Tyr79Phe—To probe the functional importance of Tyr79, we measured the rate of SAH formation upon methyl transfer. We found that Tyr79Ala and Tyr79Phe mutants of MtmC catalyzed the methyl transfer ~7-8 times less efficiently than the wild-type MtmC (Fig. 6). This demonstrated the functional importance of the hydroxyl group of the Tyr79 in positioning the substrate for catalysis, consistent with its interaction with the α-phosphate of the TDP moiety shown by the structural analysis.

DISCUSSION

An intriguing enzyme complex MtmC/MtmGIV plays a key role in the biosynthesis of the important trisaccharide chain of the anticancer drug mithramycin. As a stepping stone towards elucidation of how these two enzymes cooperate, we determine the protein crystal structure of ketoreductase/C-methyltransferase MtmC. We obtained crystal structures of MtmC with its biologically relevant ligands, including that of the ternary complex of MtmC with bound substrate 4-keto-D-olivose and SAH. Because the TDP-sugar binding pocket appears to be highly conserved in this family of methyltransferases, and the only other access to the sugar moiety (without a major protein conformational change) is via the SAM-binding channel, MtmC must use the same channel to bind either NADPH or SAM, depending on the catalytic function. The conformationally versatile sensor loop and, specifically, Tyr79 likely play an important role in active site dehydration and the control of substrate and co-substrate binding and product release. Our biochemical and structural data explain the universal conservation of this Tyr among MtmC and its homologs (Supplementary Fig. 1): the hydrogen bond between the OH of Tyr79 and the TDP moiety is

critically important for the catalytic turnover, since the Tyr79Phe mutant is unable to support methylation *in vitro*. Moreover, Tyr79 is likely needed to ensure discrimination of the sugar-TDP from TDP or other abundant ligands, via its ability to establish a bi-dentate interaction with both the sugar and the TDP moieties of the substrate, while also making a contact with SAM. Indeed, Tyr79 is seen in the conformation ensuring such interaction only in the ternary complex MtmC-SAH-TDP-4-keto-D-olivose, but not in complex MtmC-SAM-TDP. Because the active site pocket appears too constricted for binding of the larger co-factor, NADPH, when Tyr79 is in the “in” conformation, we propose that MtmC has uniquely evolved to accommodate NADPH by swinging Tyr79 out of the active site to make room for NADPH binding. In support of this idea, the TcaB9, which is a monofunctional methyltransferase, the homologous Tyr78 does not appear to undergo this conformational change. We modeled NADPH bound to MtmC in this Tyr79 “out” conformation (Fig. 7); the nicotinic acid moiety of NADPH in this model is in place of the phenol ring of Tyr79 in the “in” conformation, with the ribose rings of SAM and SAH occupying the same site. Because NADPH is larger than SAM, the adenine base of NADPH in the model is more solvent-exposed, as expected, since the binding site has evolved to bind mainly SAM in this family of enzymes. Indeed, as we observed only partial density for NADPH in the crystals grown or soaked with NADPH or NADPH and the substrate (insufficient for building its structure in the active site of MtmC), NADPH is likely only loosely bound.

The structures of MtmC and TcaB9 in complexes with their respective substrates together with the sequence alignment (Table 2 and Supplementary Fig. 1) allow one to analyze potential sugar recognition and regiospecificity rules among close homologs of MtmC. Interestingly, some of the homologs function as 5-methyltransferases, methylating a position symmetrical with respect to a 180° rotation around the axis going through positions 1-4 of the sugar ring. Such rotation is needed to expose the 5-position for methylation and, consequently, the ring oxygen of the sugar will then be located in place of the 2-methylene group in the structure of MtmC-SAH-TDP-KOL. These 5-C methyltransferases are CloU,²⁵ CouU^{26, 27} and NovU²⁸ from the clorobiocin, coumermycin A₁ and novobiocin biosynthetic pathways, respectively, and a more divergent homolog TiaS2²⁹ from the tiacumicin B pathway. The 2-methylene in the MtmC and TcaB9 structures in complexes with their respective substrates are dehydrated and are not in close proximity to potential hydrogen bond donors. The side chain of Asp34 that points towards this sugar position is too far to interact with it and, instead, Asp34 interacts with a potential catalytic residue Glu225. Interestingly, in CloU, CouU and NovU Asp34 is replaced by a Gly, creating a void. A coupled residue difference in these three 5-C-methyltransferases is a replacement of Pro78 in the sensor loop by an Arg. These two changes may either expose this position, now occupied by a ring oxygen, to solvent, or through a potential conformational change of the backbone due to the Pro78 substitution, provide the Arg as a hydrogen bond donor for the ring oxygen. TiaS2 appears to be a special case, where Asp34 is not replaced and Pro78 is replaced by a Val. However, TiaS2 is also the only exception in that in place of Tyr223 it contains a His, which has a potential for formation of strong hydrogen bonds. In even more divergent 3-C-methyltransferases EryBIII³⁰ TylCIII³¹ and AviG1,³² a Gly is in place of both Asp34 and Pro78, but a Cys in place of Tyr223 may preserve the overall dehydrated environment of the methylene at the 2-position. These last three examples are more

speculative, since the sensor loop sequence in these homologs is very different from that of MtmC and is very Gly- and Ser-rich; therefore, its conformation may not be well predicted by the structures of MtmC and TcaB9. Finally, a much smaller Cys in place of Tyr223 in EryBIII is able to accommodate a bulky methoxy group that is also installed at 3-position, which would otherwise clash with a Tyr.

While both the sugar donor substrate generation and transfer are catalyzed by MtmC, MtmTIII and MtmGIV for the first and the third sugar of the trisaccharide chain, the overall biosynthetic sequence of the trisaccharide chain biosynthesis necessitates one MtmC-independent step in between, namely the transfer of α -oliose, the second sugar of the chain, by MtmGIII. Thus, many intriguing questions remain; for example, how is the product of the first MtmC/MtmGIV reaction passed onto MtmGIII, and then back to MtmC/MtmGIV? Are three or all four of these enzymes somehow assembled into a multi-enzyme complex? We hope that complementary ongoing structural studies of MtmGIV and MtmTIII will shed light onto these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MTM	mithramycin
MT	methyl transferase
PCR	polymerase chain reaction
HPLC	high performance liquid chromatography
DMSO	dimethylsulfoxide
LB	Lysogeny broth
IPTG	isopropyl- β -D-1-thiogalactopyranoside
IMAC	Immobilized metal ion affinity chromatography
Tris	tris(hydroxymethyl)aminomethane
TBE	Tris/Borate/EDTA
MS	mass spectrometry
HRMS	high resolution mass spectrometry
NMR	nuclear magnetic resonance

ESI-MS	electrospray ionization mass spectrometry
HRESI-MS	high resolution electrospray ionization mass spectrometry
TDP	thymidine diphosphate
TDP-KOL	TDP-4-keto-D-olivose
SAM	S-adenosyl-methionine
SAH	S-adenosyl homocysteine
NADPH	nicotinamide adenine dinucleotide phosphate
GDD	5,5- <i>gem</i> -dimethyl deoxysugar

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Figure 1.
Chemical structures of MTM and other representative aureolic acid drugs.

Figure 2.

The biosynthetic route for generation of the trisaccharide chain of MTM, highlighting the central role of MtmC and MtmGIV in the sugar elaboration the chain assembly.

Figure 3.

A cartoon representation of crystal structures of MtmC with its biologically relevant ligands. The N-, central and C-terminal domains are shown in yellow, blue and green, respectively. The ligands are shown as sticks, SAM and SAH in pink and the substrate and TDP in orange. The sensor loop (residues 76-84) is shown in black. The Zn²⁺ ion is a red sphere. A. The structure of MtmC-SAH-TDP-4-keto-olivose (MtmC-SAH-TDP-KOL). The zoom-in views of the active sites of MtmC-SAM-TDP, MtmC-SAM, MtmC-SAH and MtmC-TDP complexes are shown in panels B, C, D and E, respectively.

Figure 4. The active site of the MtmC-SAH-TDP KOL complex. The ligands and the residues interacting with the sugar moiety of the substrate are shown as sticks and colored according to the protein region to which they belong, as in Fig. 1.

Figure 5.
The different conformations of the sensor loop (in black sticks) in (A) MtmC-SAM, (B) MtmC-TDP, (C) MtmC-SAM-TDP and (D) MtmC-SAH-TDP-KOL complexes. The water molecule that occupies the site of the 3-OH group of the sugar in its absence is shown as a blue sphere.

Figure 6. Effect of mutations of Tyr79 on the methyltransferase activity of MtmC
Shown are methyltransferase activities of wild-type MtmC and its mutants, Tyr79Phe and Tyr79Ala, relative to that of the wild-type enzyme.

Figure 7.
The model of the MtmC-NADPH-TDP-KOL complex.

Table 1

X-ray diffraction data collection and structure refinement statistics.

<i>Molecular complex</i>	MtmC-SAH-TDP-KOL	MtmC-SAM-TDP	MtmC-SAM	MtmC-SAH	MtmC-TDP
<i>Data collection</i>					
Space group	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22	I4 ₁ 22
Monom./asymm. unit	1	1	1	1	1
Unit cell dimensions					
a, b, c (Å)	135.4, 135.4, 127.3	134.7, 134.7, 127.8	134.8, 134.8, 129.7	134.2, 134.2, 130.0	134.2, 134.2, 132.3
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	50-2.35 (2.39-2.35) ^a	50-2.3 (2.34-2.30)	50-2.2 (2.24-2.20)	50-2.2 (2.24-2.20)	50-2.7 (2.8-2.7)
I/σ	28.2 (2.5)	40.3 (4.7)	39.9 (3.2)	41.8 (3.7)	25.2 (3.3)
Completeness (%)	95.7 (98.9)	99.5 (100)	100 (100)	99.0 (100)	100 (100)
Redundancy	6.6 (6.5)	9.8 (10)	8.1 (8.2)	9.6 (9.8)	9.7 (10.0)
R _{merge}	0.075 (0.61)	0.077 (0.499)	0.064 (0.625)	0.087 (0.627)	0.129 (0.676)
Unique reflections	21204	24771	28993	28774	16056
<i>Structure refinement</i>					
Resolution (Å)	40-2.35	40-2.3	40-2.2	40-2.2	40-2.7
R (%)	20.6	21.2	21.4	20.3	20.9
R _{free} (%)	23.1	23.8	25.7	24.1	24.8
Rmsd from ideal					
bond lengths (Å)	0.005	0.005	0.006	0.006	0.005
bond angles (°)	1.01	1.08	1.13	1.1	0.97
<i>Ramachadran plot^b</i>					
% residues by regions					
most allowed	98.1	97.1	92.2	97.8	90.5
additional allowed	1.9	2.9	7.3	2.2	8.7
generously allowed	0	0	0.6	0	0.3
disallowed	0	0	0	0	0.6

^aValues in parentheses refer to the highest-resolution shell.^bPROCHECK statistics.³³

Table 2

MtmC and its homologs.

Protein	Bacterium	Sugar product	Pathway product	Reference
MtmC	<i>Streptomyces argillaceus</i>	D-mycarose/D-olivose	mithramycin	this study and ¹⁷
SnoG ^a	<i>Streptomyces nogalater</i>	nogalose	nogalamycin	34
CloU	<i>Streptomyces roseochromogenes</i> var. <i>oscitans</i> DS12.976	5,5- <i>gem</i> -dimethyl deoxysugar (GDD)	clorobiocin	25
CouU	<i>Streptomyces rishiriensis</i>	GDD	coumermycin A ₁	26, 27
NovU	<i>Streptomyces spheroides</i> (<i>niveus</i>)	GDD noviose	novobiocin	28
ORF14/SmtA	<i>Amycolatopsis orientalis</i>	vancosamine	chloroeremomycin/vancomycin	35
TcaB9	<i>Micromonospora chalcea</i>	D-tetronitrose	tetrocarcin A	20, 24
TiaS2	<i>Dactylosporangium aurantiacum</i> subsp. <i>hamdegenensis</i> NRRL 18085	modified D-rhamnose	tiacumicin B	29
EryBIII	<i>Saccharopolyspora erythraea</i>	modified L-mycarose	erythromycin A	30
TylCIII	<i>Streptomyces fradiae</i>	L-mycarose	tylosin	31
AviG1	<i>Streptomyces viridochromogenes</i> Tü57	L-mycarose	avilamycin	32

^aThe homologs are presented in the order of their sequence similarity to MtmC, from the most to the least similar homologs.