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Characterization of the Terminal Activation Step Catalyzed by Oxygenase CmmOIV of the Chromomycin Biosynthetic Pathway from *Streptomyces griseus*[†]

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Abstract

Inactivation and initial interrogation of key oxygenase CmmOIV of the biosynthetic pathway of chromomycin A₃ in *Streptomyces griseus* ssp. *griseus* revealed that a completely methylated and acetylated prechromomycin is the preferred substrate of this enzyme. This suggests that the three sugar decoration reactions, two *O*-acetylations and an *O*-methylation, which were previously believed to occur as the final steps of chromomycin A₃ biosynthesis, indeed take place prior to the CmmOIV reaction. Upon inactivation of CmmOIV, four new compounds accumulated, from which the fully decorated prechromomycin and its incompletely acetylated precursor along with a diketoprechromomycin-type compound were fully characterized and assayed with CmmOIV.

Chromomycin A₃ **9** (Scheme 1) is a member of the aureolic acid family of antitumor antibiotics that also includes mithramycin (**7**), olivomycin, durhamycin A, UCH9, and chromocyclomycin (1,2). Aureolic acids act on gram-positive bacteria, and inhibit growth and multiplication of several cancer cell lines by interacting in an Mg²⁺-dependent manner with GC-rich regions of the minor groove of DNA (3,4). Durhamycin A is also an inhibitor of HIV Tat transactivation (5). Additionally, chromomycin and mithramycin 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 (6). Chromomycin A₃ and mithramycin can also inhibit Zn²⁺ metalloenzymes including alcohol dehydrogenase (ADH) by binding at the zinc centers and disrupting the quaternary structure of the metalloenzyme complex. This activity makes chromomycin A₃ and mithramycin potential therapeutics for metal dyshomeostasis and neurodegenerative disorders (7).

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SUPPORTING INFORMATION

The insertional inactivation of the gene encoding CmmOIV is depicted, and tables with NMR data for compounds **6**, **11**, and **12** are given. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Chromomycin A₃ **9** 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 as well as two aliphatic side chains at C-3 and C-7 (Scheme 1). The aglycone is formed by condensation of one acetyl-CoA and nine malonyl-CoA units and belongs to the polyketide group of natural products (8-11). In each aureolic acid compound except UCH9 and durhamycin A, the oligosaccharides at C-2 and C-6 are di- and trisaccharide chains, respectively, and contain unique deoxyhexose sugars for each of the aureolic acid compounds. The disaccharide chain of chromomycin A₃ contains 4-*O*-acetyl- β -oliose (sugar A) and 4-*O*-methyl- β -oliose (sugar B), while the trisaccharide chain contains two β -olivoses (sugars C and D), and 4-*O*-acetyl- α -chromose B (sugar E). Four glycosyltransferase enzymes (CmmGI/GII/GIII/GIV) encoded in the chromomycin A₃ gene cluster glycosylate the aglycone precursor premithramycinone starting with the buildup of the trisaccharide chain followed by the generation of the disaccharide (10). Two tailoring enzymes are responsible for the transfer of acetyl and methyl groups to the sugars, CmmA and CmmMIII, respectively (8). Both the sugars and their acetyl groups contribute to the biological activity of chromomycin A₃ (3,4,12-25).

The biosyntheses of mithramycin and chromomycin A₃ progress through common intermediates, up to premithramycin A₁ **3**, and then differ with regard to the assembly of their remaining saccharide patterns (Scheme 1). Consequently, both compounds share a common aglycone but are each glycosylated with a unique pattern of sugars. In addition, the decoration of chromomycin A₃'s sugar moieties with two acetyl groups and one methyl group distinguish the chromomycin A₃ from the mithramycin biosynthesis. To date, the exact timing of these decoration reactions within the chromomycin A₃ biosynthesis remains ambiguous. Previously, we proposed the acetyl and methyl transfers catalyzed by CmmA and CmmMIII, respectively, were the terminal steps in chromomycin A₃ biosynthesis, based on gene inactivation studies and the likelihood for CmmA to be embedded in the cell membrane (8,26). The isolation of compounds from inactivation of CmmA and CmmMIII yielded chromomycin A₃ derivatives with missing acetyl and methyl decorations, and the di-deacetyl-chromomycin A₃ appears to be a substrate for the out-transporters CmrAB (26). On the other hand, mutants in which glycosyltransfer steps affecting sugar units B alone or both A and B, respectively, were inactivated and yielded still tricyclic tetrasaccharidal and tetracyclic trisaccharidal prechromomycins with already *O*-acetylated sugar moieties A and/or E (10). While these contradictory results could be explained by substrate flexibility of the sugar decorating enzymes, a potential substrate flexibility of key oxygenase CmmOIV is an alternative. CmmOIV – in analogy to MtmOIV (27) in the well-studied mithramycin biosynthesis – is proposed to catalyze the oxidative rearrangement of a fully sugar-decorated prechromomycin type compound (**5** or **6**) into a chromomycin type compound (**8** or **9**) (9). A certain substrate flexibility may allow this conversion to occur with or without the *O*-acetyl and *O*-methyl sugar decorations in place, thus leaving it ambiguous whether the *O*-acetylation and/or the *O*-methylation reactions occur prior to or after the CmmOIV reaction (Scheme 1, either **5** $\rightarrow$ **6** $\rightarrow$ **9** or **5** $\rightarrow$ **8** $\rightarrow$ **9**). In this study, we aimed to further clarify these late steps of chromomycin biosynthesis by exploring oxygenase CmmOIV and its substrate specificity.

EXPERIMENTAL PROCEDURES

Generation of the *cmmOIV*-minus mutant *S. griseus* C6004

For the generation of a *cmmOIV*-minus mutant, plasmid pABF10 was constructed as follows (Figure S1). A 4.77 kb *Pst*I-*Bsp*QI fragment (blunt-ended) of cosGR60, containing *cmmMI*, *cmmGI*, *cmmOIV*, *cmmWI*, and the 5'-end of *cmmLI*, was subcloned into the *Sma*I site of pIJ2925, generating pABF8. Then, a *Sma*I-*Eco*RV fragment obtained from pEFBA (28), containing an apramycin-resistance cassette, was inserted into the unique *Bsm*I site (blunt-

ended) of pABF8 located within *cmmOIV*, and in the same direction of transcription, generating pABF9. Afterwards, the whole insert was rescued from pABF9 as a *Bgl*II fragment (using these sites from the poly-linker), and subcloned into the *Bam*HI site of pHZ1358, generating pABF10. This construct was introduced by intergeneric conjugation into *S. griseus*, and apramycin-resistant thiostrepton-sensitive transconjugants were selected for further characterization by PCR analysis, using oligonucleotides *cmmOIV*-f (5'-ATGGAGTACGACGTCGTCGTGGC-3') and *cmmOIV*-r (5'-GGAACGTCCTTCTCCGGAAGTGT-3'). PCR was performed in 50 μ L volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 2mM MgSO₄, 0.2 mM of each NTPs, 0.2 mM of the primers, 1.5 U of Taq-polymerase (Roche Diagnostics) and 2 μ L of DNA. The amplification reaction was performed under the following conditions: an initial denaturation of 5 min at 98 °C, 35 cycles of 1 min at 98 °C, 1 min at 50 °C, and 2 min at 72 °C; after the 35 cycles, and extra extension step of 5 min at 72 °C was added.

HPLC analysis and purification of the new compounds

Chromatographic analyses were performed by UPLC or HPLC-MS as previously described (29), except that bi-dimensional chromatograms were extracted at 280 nm. For purification of new compounds produced by mutant C60O4, this strain was inoculated from solid agar plates with R5A solid medium into 500 mL of R5A liquid media in 13 2 L Erlenmeyer flasks. A total of 7.5 L of media was used for cultures, which were incubated at 28 °C for 5 days with shaking at 250 rpm. Culture broth was harvested and extracted three times with ethyl acetate. The extract obtained after evaporation of the solvent was dissolved in 25 mL methanol and the compounds of interest were purified by preparative HPLC with a SunFire C18 column (10 $\times$ 250 mm, Waters), using mixtures of acetonitrile and 0.2% formic acid in water as solvents, at a flow rate of 2.5 mL/min. The solutions obtained were partially evaporated to reduce the concentration of the organic solvent and finally lyophilized. The final yields were 28.0 mg, 2.4 mg, 1.3 mg and 1.0 mg of **6**, **12**, **11**, and **10**, respectively (Figure 1.)

Cloning of the *cmmOIV* Gene in pET28a

From plasmid pABF8, *cmmOIV* was amplified via a two-round cloning procedure. Primers were optimized using IDT's Primer Quest (Coralville, Iowa) upstream and downstream of the start and stop codons of the *cmmOIV* gene. Amplification of this product using Pfu polymerase (Stratagene) yielded a template for a subsequent amplification using Pfu (Stratagene) and primers with *Nde*I and *Eco*RI restriction sites immediately upstream and downstream of the start and stop codons, respectively (Table 3). The blunt-ended amplification product was then ligated into the pCRBluntII-TOPO vector with the TOPO cloning kit (Invitrogen), transformed into TOP10 cells (Invitrogen), and plated on LB with 50 μ g/mL kanamycin sulfate overnight at 37 °C. The *cmmOIV* gene was cut from pCRBluntII-TOPO with *Nde*I/*Eco*RI and ligated into freshly digested pET28a (Novagen), resulting in pET-CmmOIV with an N-terminal His₆-tag, then transformed into XL1-Blue cells. Single colonies were grown in 5mL LB media and plasmid DNA was isolated (Fermentas) and confirmed by sequencing (SeqWright, Houston, TX). Finally, a transformation of pET-CmmOIV into *E. coli* BL21(DE3) cells for protein production was performed, and 50% glycerol was added for storage at -80 °C.

Expression and purification of CmmOIV

Glycerol stocks of pET-CmmOIV in BL21(DE3) cells were grown in fresh LB media in the presence of kanamycin sulfate (50 μ g/mL). 250 mL flasks with 100 mL of LB/Kan were inoculated with 3 mL of CmmOIV preculture and grown at 37 °C/250 rpm and then induced at OD₆₀₀ = 0.5-0.7 with 1 mM IPTG. Cultures were grown overnight at 18 °C/250 rpm. Harvested cultures were spun at 4000 K rpm for 15 minutes at 4 °C. 10 mL of Lysis Buffer

(50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) were added to ~5 g of bacterial cells and re-suspended before lysis by two passes through a French Press. Lysed cells were spun at 18,000 rpm for 45 min and then the supernatant was filtered through a 0.45 µm syringe filter. Purified protein was collected by passage across IMAC purification and P-6 desalting columns (BioRad) using a Profinia purification system (BioRad) and a standard IMAC purification protocol. Approximately 10 mg/L of soluble CmmOIV was produced. Protein concentration was determined by Bradford assay (Sigma).

Polyacrylamide gel analysis

Both native and denaturing gels were run to validate the molecular weight and assay for quaternary structure in solution. CmmOIV was run on 10% native gel run in 1X TBE while the 10-16% SDS-PAGE gel (BioRad) was run in 1X Tris-Glycine-SDS buffer.

Gel filtration experiments

A molecular weight standard kit (Sigma) was used to establish a standard curve. Molecular weight standards (Blue Dextran, Bovine Serum Albumin, Cytochrome C, Alcohol Dehydrogenase, β-amylase, Carbonic Anhydrase) were run across a Superose 12 10/300 GL (Amersham Biosciences) column by FPLC (BioRad) and elution times were monitored. Elution and void volumes were calculated from the elution times and a standard curve was plotted from the volumes and molecular weights of these proteins. CmmOIV was also run across the column and an elution time was recorded and used for determination of the molecular weight of the enzyme. Each protein (~200 µL) was run across the column in crystallization buffer (20 mM Tris, pH 7.5, 200 mM NaCl) at a flow rate of 0.5 mL/min.

CmmOIV activity assays

CmmOIV assays were conducted as described previously for MtmOIV (27). In brief, 1 µM enzyme was combined with 250 µM NADPH, 100 µM FAD, 100 mM Tris, pH 8.25 (unless otherwise noted), and 0 – 200 µM substrate in a 120 µL reaction. Reaction turnover was assessed by NADPH oxidation and was monitored by continuous absorbance measurements at λ = 340 nm, T = 30 °C for up to 30 min.

RESULTS

Insertional Inactivation of cmmOIV

Analysis of the chromomycin A₃ gene cluster of *S. griseus* subsp. *griseus* revealed significant similarity to the related mithramycin gene cluster. One of these genes, *cmmOIV*, shows high sequence similarity to *mtmOIV*, which encodes a FAD-dependent Baeyer-Villiger monooxygenase responsible for the oxidative cleavage of the fourth ring of premithramycin B (9). In order to determine its specific role in chromomycin A₃ biosynthesis, inactivation of *cmmOIV* was conducted. A construct in the unstable plasmid pHZ1358 was generated (pABF10), in which the *cmmOIV* gene was interrupted by an apramycin resistance cassette inserted in the direction of transcription of the targeted gene (Figure S1A). This construct was introduced into *S. griseus* by intergeneric conjugation from *E. coli*, transconjugants were selected for resistance to apramycin and then tested for their susceptibility to thiostrepton as a consequence of a double crossover. One mutant (C6004) was selected and characterized by PCR analysis to verify the occurrence of the double recombination event (Figure S1B). Using specific oligonucleotide primers, a 0.57 kb fragment of *cmmOIV* was amplified in the wild type strain, while a 2.17 kb fragment was amplified in the mutant C6004, confirming the replacement.

The mutant was further characterized for production of chromomycin A₃ (or derivatives) by UPLC and HPLC-MS analysis. No chromomycin A₃ was detected in extracts of the mutant,

indicating the involvement of *cmmOIV* in chromomycin A₃ biosynthesis. Analysis by HPLC of cultures of C60O4 showed one major peak and three minor peaks. The peaks were identified as tetracyclic prechromomycin-type compounds based on a hallmark UV absorption spectrum.

Isolation and Structure Elucidation of Novel Compounds Accumulated by the CmmOIV-minus mutant

The new compounds produced by mutant C60O4 were purified by preparative HPLC from cultures grown in liquid R5A media. HRMS analysis of each of the four mutant products yielded molecular weights that allowed preliminary identification of the compounds (Figure 2). The HRMS of **10**, a minor compound of which ~ 1 mg was isolated yielded a mass that indicated a molecular formula of C₅₁H₆₆O₂₃, which corresponds to a prechromomycin-type compound with only four sugars rather than the normal five. The mass difference compared to the fully decorated 4B-*O*-methyl-4A,4E-di-*O*-acetyl-prechromomycin B **6** (see below and Figure 2) indicated that the *O*-methylated sugar B is missing, which in fact is the last sugar attached during chromomycin A₃ biosynthesis. Thus, its structure is likely 4A,4E-di-*O*-acetyl-3A-des-(4B-*O*-methyl-*D*-oliosyl)-prechromomycin B **10**. This minor compound is possibly a shunt product derived from the tetrasaccharidal prechromomycin intermediate of chromomycin biosynthesis.

The HRMS of product **6**, consistent with a molecular formula of C₅₈H₇₈O₂₆, indicates a fully decorated prechromomycin derivative, with all five sugars and both the *O*-methyl and the two *O*-acetyl groups attached at sugars B, A and E respectively. Thus, HRMS putatively identified compound **6** as 4B-*O*-methyl-4A,4E-di-*O*-acetyl-prechromomycin B, and 28.0 mg of **6** was isolated from the major peak of the CmmOIV-minus mutant strain (Figure 1).

HRMS analysis of **11** indicated a molecular formula of C₅₃H₆₈O₂₄, consistent with a diketoprechromomycin B structure. The structure of **11** matches earlier discussions that concluded that CmmGIV, the glycosyltransferase responsible for the transfers of both sugars A and E, actually transfers 4-ketosugars, which are reduced after the attachment, presumably by CmmTIII (10). Thus, compound **11** might indeed be an (unstable) intermediate of the chromomycin A₃ biosynthetic pathway.

Product **12** was separated as a very minor 'contamination' from the main product peak (2.4 mg). The HRMS of **12**, consistent with a molecular formula of C₅₆H₇₆O₂₅, indicates a monoacetylated, methylated prechromomycin B product, and was putatively identified as 4B-*O*-methyl-4E-*O*-acetyl prechromomycin B **12**. Similar to compounds **6** and **11**, **12** is possibly an intermediate of chromomycin A₃ biosynthesis, an immediate precursor of **6**. This is consistent with earlier conclusions in that the 4E-*O*-acetyltransfer occurs prior to the 4A-*O*-acetyltransfer (8).

For NMR analysis, we could isolate only compounds **6**, **11** and **12** in sufficient quantities.

4B-*O*-methyl-4A,4E-di-*O*-acetyl-prechromomycin B, **6**

Compound **6** was obtained as a UV absorbing yellow solid of medium polarity. The physicochemical properties are summarized in Table 1. The molecular weight was determined by ESI MS: one *quasi*-molecular ion peak in negative ESI MS mode, respectively, confirmed the molecular weight of **6** as 1190 g/mol, the (–)-HRESI MS delivered the molecular formula C₅₈H₇₈O₂₆. The UV data and ¹H NMR spectrum indicated similarity with premithramycin B **4** (30) with respect to the aglycone moiety and with chromomycin A₃ **9** (9) with respect to the sugars (Table S1).

The NMR data comparison revealed that the polyketide-derived aglycone moiety of **6** is identical to the one of premithramycin B, while its sugar pattern is the same as found in chromomycin A₃. Comparing the aglycone data to chromomycin A₃ showed that the oxymethines in chromomycin A₃ at δ 4.96 (2-H, d, J = 12 Hz) and 4.85 (4'-H, dq, J = 6.5, 3 Hz) were missing and replaced with two quaternary carbons (δ 78.0 and 201.3) of C-12a and C-1' in **6**. Furthermore, the doublet methyl at δ 1.60 (C-5', d, J = 6.5 Hz) of chromomycin A₃ was replaced by the singlet at δ 2.72 (C-2') indicating its attachment at an sp^2 -carbon in compound **6**. From the HMBC and ^1H - ^1H COSY correlations of **6** (Figure S2), the final structure was deduced and named 4B-*O*-methyl-4A,4E-*O*-diacetyl-prechromomycin B **6**. The absolute stereochemistry of **6** was deduced based on NOESY experiments (Figure S3), coupling constants as well as in comparison with the related chromomycin A₃ (9) and premithramycin B (30). The coupling constants of the anomeric protons in sugars A, C and D (J = 9.6~10.1 Hz), indicating its axial orientation, as in chromomycin A₃, and the anomeric protons of the remaining two sugars B and E (J = 2.9~3.2 Hz) indicated their equatorial orientations. Thus, the sugar residues were linked as in chromomycin A₃.

4C,4E-diketoprechromomycin B, **11**

Compound **11** was isolated as yellow solid and shows similar physicochemical properties as **6** and **12** (Table 1). The molecular formula C₅₃H₆₈O₂₄ determined by HRESI MS (m/z = 1087.4028 [M-H]⁻) differs from **6** by C₅H₁₀O₂. Comparison of the ^1H and ^{13}C NMR spectra of **6** and **11** (Table S2) revealed that the signals of the two acetyl groups and of the sugar-B methoxy signal in compound **6** were missing in the spectra of **11**. The remaining 4H-difference was due to the oxidation at positions of 4C and 4E of the sugar residues, also observable in the NMR spectra (Table S2), which lack the 4-H signals of sugars C and E. Note that the carbonyl signals could not be observed in the ^{13}C NMR spectrum of **11** due to the small quantities isolable. In summary, compound **11** was identified as 4C,4E-diketoprechromomycin B.

4B-*O*-methyl-4E-*O*-acetyl-prechromomycin B, **12**

In the same way, **12** was obtained as an UV absorbing yellow solid, slightly more polar than **6** (Table 1). The molecular weight was determined by the negative mode ESI MS as 1148 g/mol, the (–)-HRESI MS delivered the molecular formula C₅₆H₇₆O₂₅ with 42 amu less than **6**, indicating a loss of one of the two acetyl groups. The ^1H NMR spectrum of **12** was very similar to that of **6**, the sole difference was the missing of the singlet methyl at δ 2.09 (4A-OAc) found in **12**. Thus, the 4-*O*-acetyl- β -D-oliose (sugar A) of **12** was replaced by β -D-oliose, and the structure was identified as 4B-*O*-methyl-4E-*O*-acetyl-prechromomycin B.

Initial Characterization of CmmOIV

CmmOIV is homologous to the well-studied MtmOIV oxygenase from the mithramycin biosynthetic pathway (27,31,32). Both enzymes are FAD- and NADPH-dependent and require molecular oxygen. Crystallography studies of MtmOIV showed the enzyme acts as a dimer with both FAD binding sites filled with an FAD moiety, although the biochemical studies showed a 2:1 ratio of protein:FAD (27). Like MtmOIV, CmmOIV catalyzes the insertion of an oxygen atom into the fourth ring of a so far not clearly identified prechromomycin B-type compound, which eventually converts a tetracyclic substrate into a tricyclic product. This breakage of the fourth ring catalyzed by CmmOIV is a key reaction, since it goes hand-in-hand with the establishment of the anti-tumor activity found for chromomycin.

The molecular weight of CmmOIV was calculated to be 54211.9844 g/mol. Figure 3A shows purified CmmOIV, with the His₆-tag removed running at approximately 55 kD on an 10-16% SDS-PAGE gradient gel (BioRad). In all further experiments, the His₆-tag was left

uncleaved and a separate molecular weight calculation indicated a molecular weight of 56012.9023 g/mol for the His₆-tagged enzyme. A dimer of the His₆-tagged protein was calculated to be 112,026 g/mol. To verify that CmmOIV, like MtmOIV, also acts as a dimer, purified CmmOIV was run under native conditions on a 10% native PAGE gel, and the protein ran at a molecular weight of approximately 120 kD, indicative of the dimer (Figure 3B). To confirm that CmmOIV is in fact a dimer, we also ran the protein across a gel filtration column under native conditions (Figures 3C, 3D), using a series of five proteins as molecular weight standards (see Experimental Section) and blue dextran to estimate the void volume. Gel filtration experiments indicated a molecular weight of 118,111 g/mol further confirming the dimeric nature of CmmOIV.

CmmOIV Reaction Optimization

Prior to characterization of CmmOIV, we first needed to optimize the reaction conditions used to assay enzymatic activity. The CmmOIV reaction was first tested in the presence or absence of 100 μ M FAD (data not shown). At pH 8.5 and 100 μ M substrate, approximately 30% of the amplitude was lost without addition of FAD to the reaction mixture (data not shown). This result indicates that the FAD binding is non-covalent and the FAD binding sites on CmmOIV are not completely filled prior to reaction initiation. Further experiments measuring the FAD concentration following enzyme purification resulted in an only 1:2–1:4 FAD:CmmOIV ratio. Incubation of CmmOIV with FAD on ice increased the ratio of FAD:CmmOIV, but not consistently 1:1. These data determined that supplementing each reaction with 100 μ M FAD eliminated any artificial variations in enzyme activity stemming from FAD concentration. The pH of the CmmOIV reaction with **6** as the substrate was optimized using 100 mM Tris buffers with varying pH (Figure 4). The initial rate of these reactions was measured and a maximum was reached at pH 8.25. Also, NADH and NADPH were both evaluated as co-substrates for the CmmOIV reaction by determining initial rates in the presence of each co-substrate. Like MtmOIV, CmmOIV reaction rates were greatly reduced in the presence of NADH compared to NADPH, and overall reaction amplitudes decreased up to 30% (data not shown). Additionally, the K_M **6** with NADH as the co-substrate decreased more than 10 fold compared to the K_M **6** in the presence of NADPH, confirming that NADPH is the preferred co-substrate (Table 2).

CmmOIV Steady-State Kinetics

To identify the natural substrate of CmmOIV and to delineate the sequence of the terminal reactions of the chromomycin A₃ biosynthetic pathway, we determined the steady-state kinetic parameters of the CmmOIV enzyme with three of the isolated compounds available from the CmmOIV-mutant strain: **6**, **11**, and **12**. Surprisingly, CmmOIV enzyme efficiency decreased when reacted with **12** compared to when reacted with **6** and further decreased efficiency when reacted with **11** (Figure 5, Table 2). Although compounds **6** and **12** only vary by a single acetyl group existing in sugar A of **6**, the change in rate and amplitude of the reaction is significant. It appears that both acetyl groups are required for full enzymatic activity of CmmOIV and that the loss of one acetyl group negatively affects the turnover rate. The diketone substrate **11** lost nearly all CmmOIV activity compared to **6** suggesting either that the keto groups play an inhibitory role or that the lack of decoration at the sugar residues prevent substrate binding or CmmOIV turnover.

DISCUSSION

In summary, none of the compounds accumulated by the CmmOIV-minus mutant were identified as the expected substrate, prechromomycin B **5** (Scheme 1). This was surprising as the MtmOIV minus mutant strain from the sister mithramycin biosynthetic pathway yielded predominantly premithramycin B **4** along with minor amounts of 3A-deolivosyl-

premithramycin B (30). The fact that **5** does not accumulate upon CmmOIV inactivation indicates that the *O*-acetylation and *O*-methylation reactions occur fast, possibly to stabilize an otherwise unstable intermediate. Premithramycin B **4** was clearly identified as the MtmOIV substrate, and 3A-deoliviosyl-premithramycin B as its immediate biosynthetic precursor. Ignoring the minor products **10** and **11** of the CmmOIV inactivation, which were only found in miniscule amounts, the accumulation of fully functionalized **6** as the major compound accompanied by minor amounts of the monoacetylated compound **12** suggests **6** to be the CmmOIV substrate and **12** to be its immediate precursor, to match perfectly the findings of the chromomycin pathway with those established for the mithramycin biosynthetic pathway. Investigations with purified CmmOIV were designed to further substantiate this possible revision of the late chromomycin A₃ biosynthetic pathway. The previously favored CmmOIV substrate, prechromomycin B **5**, was never isolated and apparently does not accumulate upon CmmOIV inactivation. Thus, although previous work suggested a terminal role for the tailoring enzymes, CmmA and CmmMIII (8), our findings here now suggest that it is more likely that CmmOIV naturally acts on a fully *O*-acetyl- and *O*-methyl functionalized substrate.

Menéndez et al. previously showed that the acetyl groups of chromomycin A₃ contribute to its antitumor properties (8,26), and it was shown by Chakrabarti et al. that the *O*-acetyl groups help to stabilize the DNA-interaction of the chromomycin-Mg²⁺-complex (22). Our work presented here, the inactivation and initial interrogation of CmmOIV, revealed the fully functionalized substrate **6** to be the major accumulated product of the CmmOIV-minus mutant strain, followed by minor amounts of the monoacetylated substrate **12**, the diketo compound **11** and the four sugar compound **10**. Fully characterizing the steady state kinetics of these accumulated compounds with CmmOIV clearly indicates that a completely acetylated and methylated prechromomycin **6** is preferred by this critical bottle-neck oxygenase over its non-decorated or incompletely acetylated precursors **11** and **12**, respectively. This in turn would suggest that the sugar decoration (*O*-acetylation and *O*-methylation) reactions, previously believed to occur at the very end of the chromomycin A₃ biosynthesis, indeed take place prior to the CmmOIV reaction. Thus, the biosynthetic sequence **5** → **6** → **9** is more likely than the previously suggested sequence **5** → **8** → **9** (see Scheme 1). Crystallization of CmmOIV (currently under investigation) will hopefully shed further light into this question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

ADH	alcohol dehydrogenase
HIV	human immunodeficiency virus
PCR	polymerase chain reaction
UPLC	ultra-high performance liquid chromatography

HPLC	high performance liquid chromatography
DMSO	dimethylsulfoxide
LB medium	Luria-Bertani medium
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

REFERENCES

- Skarbek, JD.; Speedie, MK. Antitumor antibiotics of the aureolic acid group: chromomycin A3, mithramycin A, and olivomycin A. In: Aszalos, A., editor. Antitumor compounds of natural origin: chemistry and biochemistry. CRC Press; Boca Raton, Florida: 1981. p. 191-235.
- Lombó F, Menendez N, Salas JA, Méndez C. The aureolic acid family of antitumor compounds: structure, mode of action, biosynthesis, and novel derivatives. Appl. Microbiol. Biotechnol 2006;73:1–14. [PubMed: 17013601]
- Majee S, Sen R, Guha S, Bhattacharyya D, Dasgupta D. Differential Interactions of the Mg²⁺ Complexes of Chromomycin A3 and Mithramycin with Poly(dG-dC)-Poly(dC-dG) and Poly(dG)·Poly(dC). Biochemistry 1997;36:2291–2299. [PubMed: 9047331]
- Silva DJ, Goodnow R Jr, Kahne D. The sugars in chromomycin A3 stabilize the Mg(2+)-dimer complex. Biochemistry 1993;32:463–471. [PubMed: 8422355]
- Jayasuriya H, Lingham RB, Graham P, Quamina D, Herranz L, Genilloud O, Gagliardi M, Danzeisen R, Tomassini JE, Zink DL, Guan ZQ, Singh SB. Durhamycin A, a potent inhibitor of HIV Tat transactivation. J. Nat. Prod 2002;65:1091–1095. [PubMed: 12193009]
- Bianchi N, Osti F, Rutigliano C, Corradini FG, Borsetti E, Tomassetti M, Mischiati C, Feriotto G, Gambari R. The DNA-binding drugs mithramycin and chromomycin are powerful inducers of erythroid differentiation of human K562 cells. Brit. J. Haematol 1999;104:258–265. [PubMed: 10050705]
- Devi PG, Chakraborty PK, Dasgupta D. Inhibition of a Zn(II)-containing enzyme, alcohol dehydrogenase, by anticancer antibiotics, mithramycin and chromomycin A3. J. Biol. Inorg. Chem 2009;14:347–359. [PubMed: 19034537]
- Menéndez N, Nur-e-Alam M, Braña AF, Rohr J, Salas JA, Méndez C. Tailoring modification of deoxysugars during biosynthesis of the antitumour drug chromomycin A by Streptomyces griseus ssp. griseus. Mol. Microbiol 2004;53:903–915. [PubMed: 15255901]
- Menéndez N, Nur-e-Alam M, Braña AF, Rohr J, Salas JA, Méndez C. Biosynthesis of the antitumor chromomycin A3 in Streptomyces griseus: analysis of the gene cluster and rational design of novel chromomycin analogs. Chem. Biol 2004;11:21–32. [PubMed: 15112992]
- Menéndez N, Nur-e-Alam M, Fischer C, Braña AF, Salas JA, Rohr J, Méndez C. Deoxysugar Transfer during Chromomycin A3 Biosynthesis in Streptomyces griseus subsp. griseus: New Derivatives with Antitumor Activity. Appl. Environ. Microbiol 2006;72:167–177. [PubMed: 16391039]
- Montanari A, Rosazza JP. Biogenesis of chromomycin A3 by Streptomyces griseus. J. Antibiot 1990;43:883–889. [PubMed: 2117602]

12. Aich P, Dasgupta D. Role of Mg^{++} in the mithramycin-DNA interaction: evidence for two types of mithramycin- Mg^{++} complex. *Biochem. Biophys. Res. Commun* 1990;173:689–696. [PubMed: 2148084]
13. Majee S, Dasgupta D, Chakrabarti A. Interaction of the DNA-binding antitumor antibiotics, chromomycin and mithramycin with erythroid spectrin. *Eur. J. Biochem* 1999;260:619–626. [PubMed: 10102989]
14. Mir MA, Majee S, Das S, Dasgupta D. Association of chromatin with anticancer antibiotics, mithramycin and chromomycin A3. *Bioorg. Med. Chem* 2003;11:2791–2801. [PubMed: 12788353]
15. Silva DJ, Kahne DE. Studies of the 2:1 Chromomycin A₃- Mg^{2+} Complex in Methanol: Role of the Carbohydrates in Complex Formation. *J. Am. Chem. Soc* 1993;115:7962–7970.
16. Mir MA, Dasgupta D. Association of the Anticancer Antibiotic Chromomycin A3 with the Nucleosome: Role of Core Histone Tail Domains in the Binding Process. *Biochemistry* 2001;40:11578–11585. [PubMed: 11560508]
17. Keniry MA, Banville DL, Simmonds PM, Shafer RH. Nuclear magnetic resonance comparison of the binding sites of mithramycin and chromomycin on the self-complementary oligonucleotide d(ACCCGGGT)₂. Evidence that the saccharide chains have a role in sequence specificity. *J. Mol. Biol* 1993;231:753–767. [PubMed: 8515449]
18. Keniry MA, Owen EA, Shafer RH. The three-dimensional structure of the 4:1 mithramycin:d(ACCCGGGT)₂ complex: evidence for an interaction between the E saccharides. *Biopolymers* 2000;54:104–114. [PubMed: 10861371]
19. Hou MH, Robinson H, Gao YG, Wang AH. Crystal structure of the $[Mg^{2+}-(\text{chromomycin A}_3)_2]$ -d(TTGGCCAA)₂ complex reveals GGCC binding specificity of the drug dimer chelated by a metal ion. *Nucleic Acids Res* 2004;32:2214–2222. [PubMed: 15107489]
20. Hou MH, Lu WJ, Lin HY, Yuann JM. Studies of sequence-specific DNA binding, DNA cleavage, and topoisomerase I inhibition by the dimeric chromomycin A3 complexed with Fe(II). *Biochemistry* 2008;47:5493–5502. [PubMed: 18426223]
21. Gao X, Patel DJ. Solution structure of the chromomycin-DNA complex. *Biochemistry* 1989;28:751–762. [PubMed: 2713341]
22. Chakrabarti S, Bhattacharyya B, Dasgupta D. Interaction of Mithramycin and Chromomycin A₃ with d(TAGCTAGCTA)₂: Role of Sugars in Antibiotic-DNA Recognition. *J. Phys. Chem. B* 2002;106:6947–6953.
23. Chakrabarti S, Bhattacharyya D, Dasgupta D. Structural basis of DNA recognition by anticancer antibiotics, chromomycin A(3), and mithramycin: roles of minor groove width and ligand flexibility. *Biopolymers* 2001;56:85–95. [PubMed: 11592055]
24. Chakrabarti S, Dasgupta D. Interactions of chromomycin A3 and mithramycin with the sequence d(TAGCTAGCTA)₂. *Indian J. Biochem. & Biophys* 2001;38:64–70. [PubMed: 11563333]
25. Chakrabarti S, Mir MA, Dasgupta D. Differential interactions of antitumor antibiotics chromomycin A(3) and mithramycin with d(TATGCATA)₂ in presence of Mg^{2+} . *Biopolymers* 2001;62:131–140. [PubMed: 11343281]
26. Menéndez N, Brana AF, Salas JA, Mendez C. Involvement of a chromomycin ABC transporter system in secretion of a deacetylated precursor during chromomycin biosynthesis. *Microbiology* 2007;153:3061–3070. [PubMed: 17768249]
27. Beam MP, Bosserman MA, Noinaj N, Wehenkel M, Rohr J. Crystal structure of Baeyer-Villiger monooxygenase MtmOIV, the key enzyme of the mithramycin biosynthetic pathway. *Biochemistry* 2009;48:4476–4487. [PubMed: 19364090]
28. Fernandez-Lozano MJ, Remsing LL, Quiros LM, Braña AF, Fernandez E, Sanchez C, Méndez C, Rohr J, Salas JA. Characterization of two polyketide methyltransferases involved in the biosynthesis of the antitumor drug mithramycin by *Streptomyces argillaceus*. *J. Biol. Chem* 2000;275:3065–3074. [PubMed: 10652287]
29. Olano C, Gómez C, Pérez M, Palomino M, Pineda-Lucena A, Carbajo RJ, Braña AF, Méndez C, Salas JA. Deciphering biosynthesis of the RNA polymerase inhibitor streptolydigin and generation of glycosylated derivatives. *Chem. Biol* 2009;16:1031–1044. [PubMed: 19875077]

30. Prado L, Fernandez E, Weissbach U, Blanco G, Quiros LM, Brana AF, Méndez C, Rohr J, Salas JA. Oxidative cleavage of premithramycin B is one of the last steps in the biosynthesis of the antitumor drug mithramycin. *Chem. Biol* 1999;6:19–30. [PubMed: 9889148]
31. Rodriguez D, Quiros LM, Braña AF, Salas JA. Purification and characterization of a monooxygenase involved in the biosynthetic pathway of the antitumor drug mithramycin. *J. Bacteriol* 2003;185:3962–3965. [PubMed: 12813091]
32. Gibson M, Nur-e-Alam M, Lipata F, Oliveira MA, Rohr J. Characterization of Kinetics and Products of the Baeyer-Villiger Oxygenase MtmOIV, The Key Enzyme of the Biosynthetic Pathway toward the Natural Product Anticancer Drug Mithramycin from *Streptomyces argillaceus*. *J. Am. Chem. Soc* 2005;127:17594–17595. [PubMed: 16351075]

Scheme 1.

Biosynthetic pathways towards mithramycin in *Streptomyces argillaceus* and chromomycin A₃ in *S. griseus* ssp. *griseus*. For the chromomycin A₃ specific pathway exists an ambiguity whether the sugar unit decorating enzymes CmmMIII and CmmA act before (**5** → **6** → **9**) or after (**5** → **8** → **9**) the oxidative breakage of the fourth ring through CmmOIV.

Figure 1.
HPLC chromatogram traces of wild type (A) and mutant strains (B) measured at 420 nm.

Figure 2.
Suggested chemical structures of the four isolated products of the CmmOIV-minus mutant, C60O4.

Figure 3.

CmmOIV molecular weight under denaturing and native conditions. (a) CmmOIV runs at 56 kDa on standard SDS-PAGE. (b) On a native gel CmmOIV shifts to 120 kDa indicating CmmOIV to be dimeric in solution. (c) Gel filtration column using five proteins as standards (filled squares, ■) shows CmmOIV (filled circles, ●) with an E_v/E_0 ratio of 1.49 where E_v is the elution volume and E_0 is the void volume measured by elution of blue dextran. This ratio yields a molecular weight of 118,111 Da confirming that CmmOIV is dimeric in solution. (d) FPLC chromatogram traces of protein standards and CmmOIV measured at 280 nm. Blue dextran (red), cytochrome C and β -amylase (blue), carbonic anhydrase and alcohol dehydrogenase (green), CmmOIV (orange), and albumin (black).

Figure 4.

pH-Optimization of the CmmOIV reaction. (a) Comparison of NADPH oxidation, measured by decreased absorbance at 340 nm, from CmmOIV turnover of **6** in 0.1 mM Tris-HCl buffer with pH of 7.25 (blue), 7.5 (red), 7.75 (green), 8.0 (purple), 8.25 (orange), 8.5 (cyan). Each line represents an average of three measured spectra. The blank (black) represents NADPH oxidation in the absence of enzyme. (b) Initial rates of NADPH oxidation measured from the turnover of **6** by CmmOIV clearly indicate maximal turnover at pH = 8.25.

Figure 5. CmmOIV reactions fitted with Michaelis-Menton equation. Initial rates (μmol/min) of NADPH oxidation from CmmOIV turnover of substrates. (A) **6**; (B) **12**; and (C) **11**.

Table 1

Physicochemical characterization of the four isolated compounds from the CmmOIV-minus mutant strain, C60O4.

	10	6	11	12
Appearance	Yellow solid	Yellow solid	Yellow solid	Yellow solid
R _f (CH ₂ Cl ₂ /5%MeOH)	-	0.34	0.36	0.32
(-)-ESI MS:	1045 [M-H] ⁻	1189 [M-H] ⁻	1087 [M-H] ⁻	1147 [M-H] ⁻
(+)-ESI MS:	-	1213 [M+Na] ⁺	1111 [M+Na] ⁺	1171 [M+Na] ⁺
(-)-HRESI MS:	1045.3926 [M-H] ⁻	1189.4708 [M-H] ⁻	1087.4040 [M-H] ⁻	1147.4613 [M-H] ⁻
Calcd.	1045.3922 for C ₅₁ H ₆₅ O ₂₃	1189.4708 for C ₅₈ H ₇₇ O ₂₆	1087.4028 for C ₅₃ H ₆₇ O ₂₄	1147.4603 for C ₅₆ H ₇₅ O ₂₅

Table 2

Steady state kinetic parameters of CmmOIV with various substrates.

Substrate	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM s^{-1}]
<i>NADPH</i>			
6	5.74 ± 0.57	0.29 ± 0.01	50.5
12	30.21 ± 3.38	0.25 ± 0.01	8.3
11	<i>n.d.</i>	0.025 ± 0.004	<i>n.d.</i>
<i>NADH</i>			
6	79.58 ± 25.27	0.29 ± 0.03	3.64

n.d. not determined.

Table 3Oligonucleotide primers for *cmmOIV* cloning.

Primer Name	Sequence
<i>cmmOIV</i> _reverse	5'-TTCGAGACCTTCACGGACGTGTTT-3'
<i>cmmOIV</i> _forward	5'-TGGACATGTTCTGGTCCCGA-3'
<i>cmmOIV</i> _NdeI	5'-AATTATCATATGGAGTACGACGTCG-3'
<i>cmmOIV</i> _EcoRI	5'-AATGAATTCTACCTCCCGTCCTGG-3'