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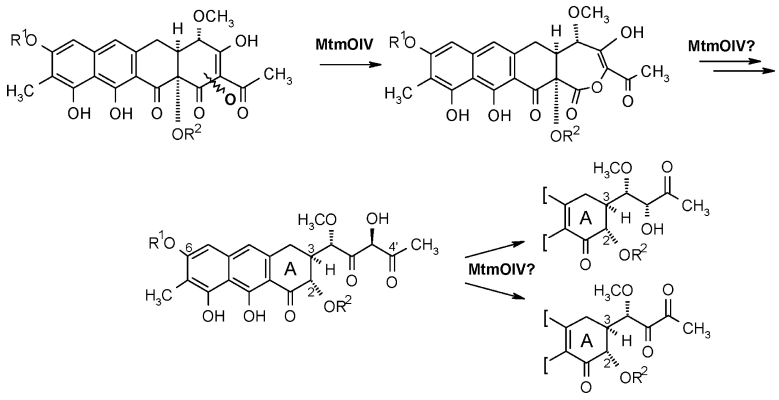

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Characterization of Kinetics and Products of the Baeyer−Villiger Oxygenase MtmOIV, The Key Enzyme of the Biosynthetic Pathway toward the Natural Product Anticancer Drug Mithramycin from *Streptomyces argillaceus*

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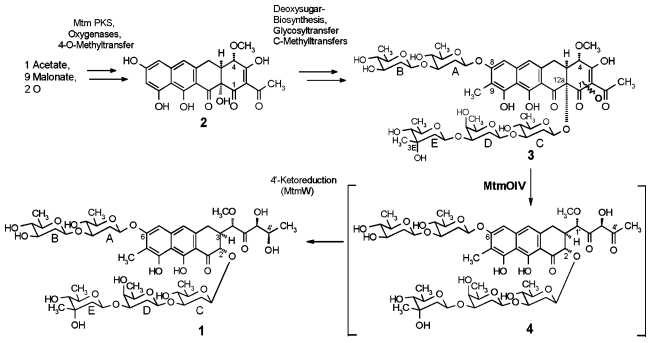
Mithramycin (**1**), a clinically used aureolic acid-type anticancer drug and calcium-lowering agent produced by *Streptomyces argillaceus* as well as various other streptomycetes, consists of a polyketide-derived tricyclic core with a pentyl side chain attached in 3-position and five deoxysugars linked as trisaccharide and disaccharide chains in 2- and 6-positions, respectively. Its biosynthesis proceeds through the condensation of multiple acyl-CoA units catalyzed by a type II polyketide synthase.[1a] The initial condensation phase leads to the formation of the tetracyclic intermediate premithramycinone (**2**), which is subsequently glycosylated and C-methylated, resulting in premithramycin B (**3**).[1b] The final steps in mithramycin biosynthesis, catalyzed by MtmOIV and MtmW, respectively, are an oxidative cleavage of the fourth ring of **3**, followed by decarboxylation and reduction of the 4′-keto group (Scheme 1).[1c,d] Oxygenase MtmOIV plays a key role in mithramycin biosynthesis and was suggested to be responsible for a series of reactions leading to the oxidative C−C bond cleavage and following decarboxylation, which essentially establishes the characteristic molecular frame found in all aureolic acid drugs and converts biologically completely inactive **3** into active molecules.[1e,f] From incorporation experiments with isotope-labeled precursors, it was suggested that this key oxidative cleavage reaction proceeds through a Baeyer−Villiger oxidation mechanism, but a retro-aldol mechanism could not be ruled out as an alternative[1a,c] (see also Supporting Information).

To further elucidate this key step of mithramycin biosynthesis, we overexpressed MtmOIV, isolated this enzyme as N-terminally His_6-tagged protein, and monitored the conversion of its substrate premithramycin B (**3**) by HPLC/MS. This way we not only established the steady state kinetics of the pH-optimized reaction of MtmOIV but also isolated and physicochemically characterized various reaction intermediates, products, and shunt products leading to the new and much more detailed reaction sequence for the mithramycin formation shown in Scheme 2.

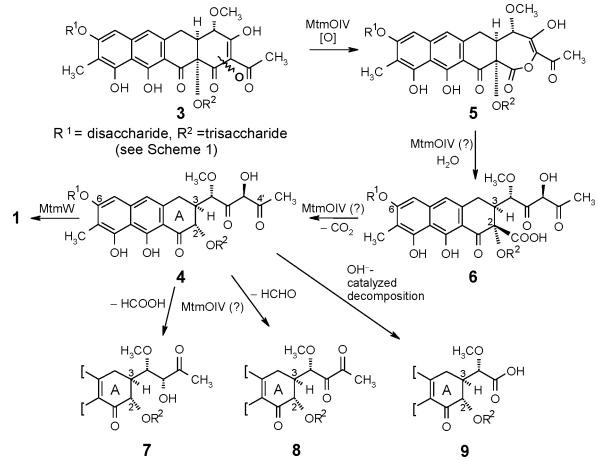
All intermediates (**4**−**6**) and the MtmOIV product mithramycin SDK (**8**) are new mithramycin analogues, while the shunt product demycarosyl-SK (not shown) as well as the product mithramycin SK (**7**) were previously described as products of the MtmW$^-$ mutant.[1d] The lactone intermediate, premithramycin B-lactone (**5**), ultimately proves the Baeyer−Villiger mechanism of the MtmOIV reaction. All immediate products, mithramycins DK (**4**), SK (**7**), and SDK (**8**), are highly active anticancer agents, with SK and especially SDK having significantly improved therapeutic indices over the parent drug **1**.[1e,g] While it is possible that MtmOIV partakes in the rearrangement reactions toward **7** and **8** (via β-shift),[1e] mithramycin SA (**9**)[1e] is most likely a decomposition product caused by a base-catalyzed retro-aldol reaction of the highly unstable β-diketone **4** (for mechanisms, see Supporting Information).

The overexpression of MtmOIV was achieved by inserting the *mtmOIV* gene into the pRSETB overexpression vector that contains

Scheme 1. The Key Oxidative Cleavage Reaction Catalyzed by Oxygenase MtmOIV within the Mithramycin Biosynthetic Pathway



Scheme 2. The Reaction of Premithramycin B with Isolated MtmOIV is Initiated by a Baeyer−Villiger Reaction (**5** formation) and Proceeds on to the Four Products, Mithramycins DK (**4**), SK (**7**), SDK (**8**), and SA (**9**)[a]



[a] At 4 h and at pH 8.25, the following composition was found: **3**, 15%; **4**, 20%; **5**, 15%; **6**, 5%; **7**, 10%; **8**, 10%; **9**, 25% (see also Figure 1).

the IPTG-inducible T7 promoter and provides an N-terminal His_6-tag that facilitated its production in *E. coli* BL21 (DF3)plys*S* and purification to homogeneity using Ni-affinity chromatography, respectively, and allowed a 3 mg/L production for MtmOIV. The optimal pH to study the enzyme reaction with its substrate premithramycin B (**3**) was found to be 8.25, where the **3** conversion increases significantly, and the most products/intermediates were produced at maximum range (Figure 1; for details regarding the enzyme purification and reaction, see Supporting Information).

With the purified, stable enzyme in hand, we studied the conversion of premithramycin B at pH 8.25 to determine the kinetic parameters and repeated the procedure using 10 mM quantities of the enzyme over a 4 h time period to allow the isolation of sufficient

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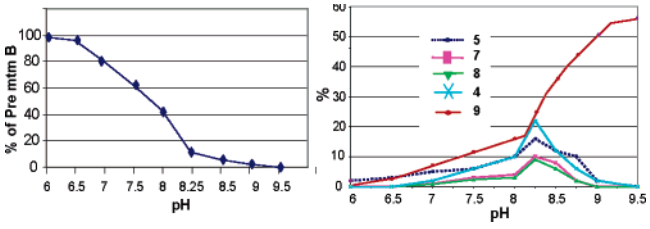

Figure 1. The pH dependence of the MtmOIV reaction (data shown are after 4 h reaction time). Optimal pH to study the reaction was 8.25. At lower pHs, the reaction slows down significantly, and at higher pHs, decomposition product **9** becomes increasingly dominant, being the only "product" at pH 9.5. The numbers refer to the structures shown in Scheme 2.

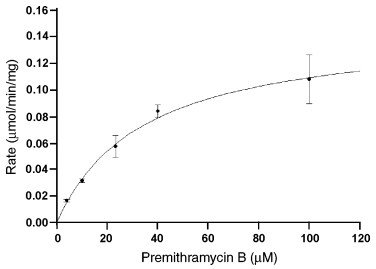

Figure 2. The Michaelis−Menten curve of NADPH depletion (UV, 340 nm) mediated by the reaction of MtmOIV with premithramycin B.

amounts of the observed intermediates and products for structure elucidation. All compounds shown in Scheme 2, except for **6**, were isolated in multiple milligram amounts and were fully characterized by NMR, IR, UV spectroscopy, and HR mass spectrometry (see Supporting Information). Mithramycin DKA (**6**) could only be isolated in submilligram amounts and, therefore, was only characterized by HR-MS and UV data.

The kinetic data for the reaction of premithramycin B with MtmOIV at pH 8.25 were obtained by monitoring the oxidation of cofactor NADPH at 340 nm and 30 °C (in the presence of FAD and O_2). The kinetic constants were calculated using the program SigmaPlot 8.0 with an enzyme kinetics module. The nonlinear regression analysis resulted in a Michaelis−Menten curve with values of $K_m = 34(\pm 12)$ μM, $v_{max} = 147(\pm 22)$ nmol min^{-1} mg protein^{-1} (Figure 2).

Baeyer−Villiger monooxygenases (BVMOs) were established as valuable tools for organic synthesis as well as postulated as key enzymes in various biosynthetic oxidative rearrangement sequences.[2,3] An involvement of a Baeyer−Villiger or similar reaction was postulated for the biosyntheses of many microbial polyketides,[3a−h] a marine natural product,[3i] an insect metabolite,[3j] and certain plant alkaloids.[3h] Protein sequence alignment studies have implicated an aspartate residue located five amino acids upstream of the dinucleotide-binding motif (the $\beta\alpha\beta$- or Rossmann-fold GxGxxG) close to the N-terminus with type I (FAD- and NADPH-dependent) BVMOs.[2d] This particular aspartate residue is present in MtmOIV, upstream of its N-terminal Rossmann-fold (residues 19−24), and the enzyme also contains a second Rossmann-fold motif at residues 162−168, which also was postulated to be characteristic for type I BVMOs.[2f] However, in contrast to Baeyer−Villiger reaction activities observed with bacterial enzymes that were treated with artificial cyclic or aliphatic ketone substrates,[2] which may not reflect their true biological action because the natural substrates are usually unknown, MtmOIV is to our knowledge the first enzyme identified as Baeyer−Villigerase that performs this reaction in its true biological context. In Scheme 2, it is suggested that MtmOIV may catalyze a series of reactions following the Baeyer−Villiger rearrangement leading to lactone **5**, such as the hydrolysis to **6**, the decarboxylation to **4**, etc. However, some of these reactions, particularly the rearrangements to **7** and **8**, may also occur spontaneously. From the pH-dependence studies, it can be safely concluded that the retro-aldol cleavage from **4** to **9** is a nonenzymatic decomposition reaction.

Previously reported data of Rodriguez et al. for the MtmOIV kinetics state a K_m value of 269 μM and a v_{max} of 12.1 nmol min^{-1} mg protein^{-1} based on NADPH depletion, and a K_m of 23.3 μM based on the depletion of premithramycin B.[1f] However, these data were obtained using a conventionally purified, unstable enzyme at pH 9.5.[4]

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Supporting Information Available: Enzyme overexpression, purification, and reaction protocol; physicochemical data for compounds **4**−**6**; protein sequence of MtmOIV. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) The authors reported a compound with a MW of 1026 g/mol as the sole product of the reaction, for which a wrong (and impossible) structure was suggested, supported only by low-resolution MS. The correct structure of this product is **9** (1026 g/mol; see Scheme 2 and Figure 1).

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