C–C bond cleavage in biosynthesis of 4-alkyl-L-proline precursors of lincomycin and anthramycin cannot precede C-methylation

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Zhong et al.[1] confirmed that γ-glutamyltranspeptidase (γ-GTs) homologs are capable of cleaving a C–C bond, which was previously inferred by Jiraskova et al.[2] in 2016 in a study based on gene inactivation experiments. The intriguing C–C bond cleavage catalyzed by LmbA and Ant6 γ-GT homologs from the biosynthesis of lincomycin A and anthramycin, respectively, was conclusively documented by Zhong et al.[1]. However, assignment of 2/3 as the LmbA and Ant6 substrate and 4/5 as the reaction product is questionable for several reasons; most importantly, it contradicts the current state of knowledge of the biosynthesis of 4-alkyl-L-proline derivatives (ALDP or APD used in previous literature; Fig. 1a)[2]. Here, we argue that LmbA/Ant6 γ-GT homologs do not utilize 2/3, but intermediate 9/10, which was previously proposed to be the main native substrate of LmbA[2] and which is biosynthesized from 2/3 by a C-methylation reaction. Consequently, the main LmbA/Ant6 product is not 4/5 but compound 12, which is a subject of isomerization in order to proceed towards the final ALDP of lincomycin A and anthramycin.

Here, we bring evidence that 2/3 is not the main native substrate of LmbA/Ant6 γ-GT homologs, but of LmbW/Ant5 C-methyltransferases. Indeed, we observed in vitro C-methylation of 2/3 by LmbW affording 9/10 and we also detected intermediate 9/10 in the cultivation broth of the ΔlmbA mutant of lincomycin producing strain Streptomyces lincolnensis (Fig. 1b). Even though the conversion of 2/3 into 9/10 by LmbW was only partial, it clearly showed that 2/3 serves as an LmbW/Ant5 substrate. To support that conversion of 2/3 by LmbW is not a side reaction resulting from broader substrate specificity of LmbW and that its main native substrate is indeed 2/3 and not 4/5 as the work by Zhong et al.[1] suggests, we carried out a bioinformatic analysis of LmbW/Ant5. We found out that LmbW/Ant5 and their homologs (SibZ[3], HrmC[4], and Por10[5]) from the biosyntheses of other ALDPs are similar to ALDP-unrelated C-methyltransferases MppJ with known structure[6] and MrsA[7] (26% identity to LmbW according to BLAST for both MppJ and MrsA along the whole sequence; sequence alignment of LmbW and MppJ is available in Supplementary Fig. 1). MppJ

and MrsA methylate phenylpyruvic and 5-guanidino-2-oxopentanoic acids, respectively, i.e., substrates structurally analogous to 2/3 and not 4/5.

Furthermore, methylation of phenylpyruvic acid catalyzed by MppJ is part of the biosynthesis of β-methyl-L-phenylalanine from L-phenylalanine[8]. Instead of direct methylation of L-phenylalanine, the machinery requires to proceed via phenylpyruvic acid, indicating the importance of the α-keto(enol)-carboxylic moiety of phenylpyruvic acid for the MppJ-catalyzed methylation. We propose that the same applies also to LmbW/Ant5 because their substrate 2/3 also contains the α-keto(enol)-carboxylic moiety. Importantly, conversion of the analogous substrates of MppJ and LmbW/Ant5 through a common reaction mechanism is supported by comparison of the active sites of MppJ (based on the protein crystal structure)[6] vs. LmbW (based on a homology model) depicted in Fig. 2. The α-keto(enol)-carboxylic moiety appears to play an important role in fixation of the substrate within the active site not only in the case of MppJ, but also LmbW/Ant5. All these enzymes share the residues important for α-keto(enol)-carboxylic moiety fixation as well as the methylation (four residues depicted in blue in Fig. 2c, d). In contrast to 9/10, intermediate 4/5 (proposed as the LmbA/Ant6 reaction product and thus the LmbW/Ant5 substrate by Zhong et al.[1]) does not possess the α-keto(enol)-carboxylic moiety for the substrate fixation in the active site.

Moreover, the methylation of 4/5 would have to proceed through a different mechanism than reactions catalyzed by MppJ and MrsA, which would be inconsistent with the high conservation of the key catalytic residues within the active sites of MppJ and LmbW/Ant5. Based on the above-mentioned arguments, we claim that 2/3 is first C-methylated by LmbW/Ant5 and the reaction product 9/10 is utilized as a substrate of LmbA/Ant6 γ-GT homologs. However, 2/3 can serve as a minor substrate of LmbA if the C-methylation step is omitted and lincomycin B[9], a side product of lincomycin A biosynthesis, is formed. Similarly, 2/3 undergoes C–C bond cleavage if the C-methyltransferase is not encoded within the biosynthetic gene cluster, which applies to the biosynthesis of e.g., tomaymycin[10,11] and

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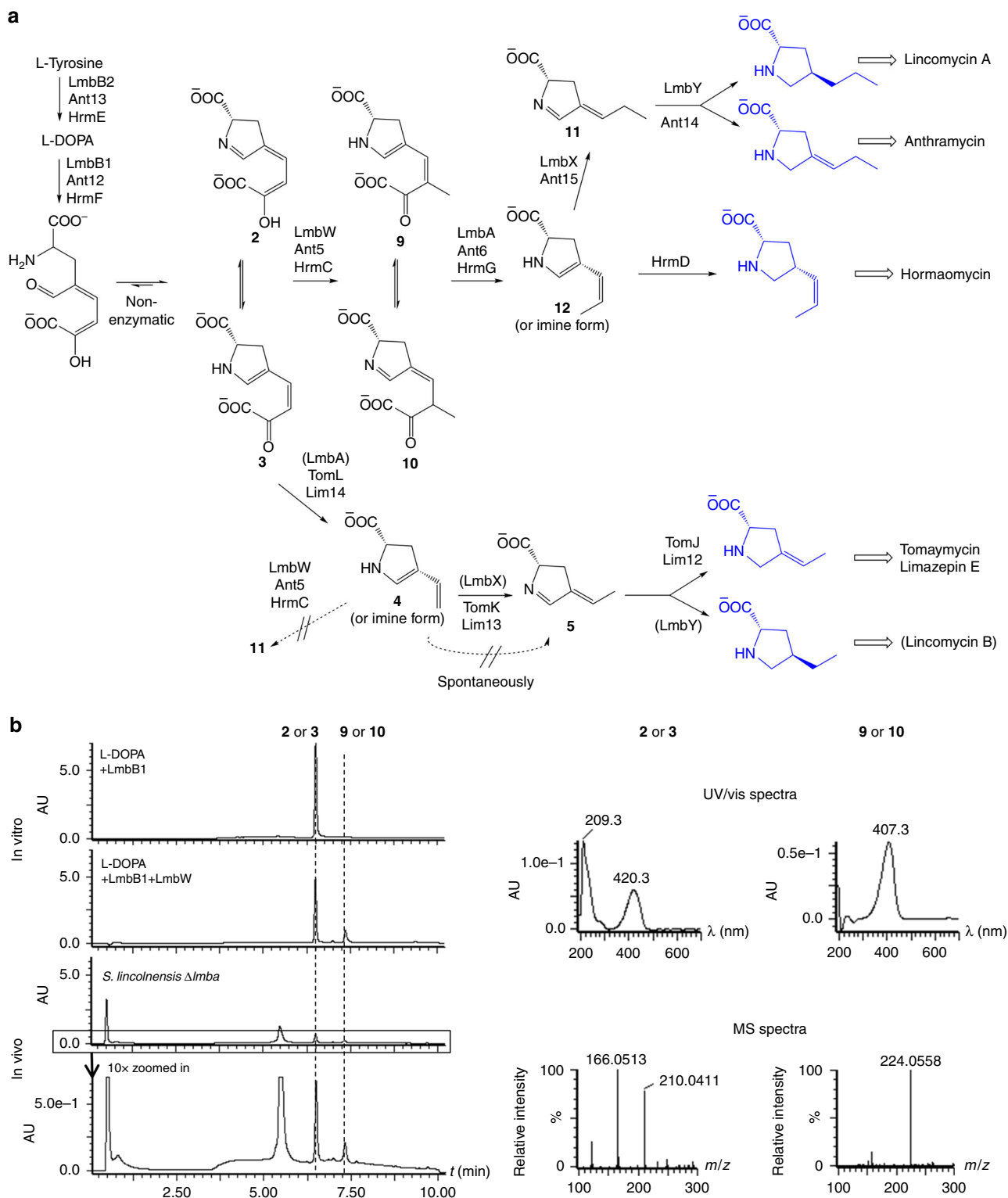
Fig. 1 Biosynthetic steps catalyzed by LmbA/Ant6 and LmbW/Ant5 in the context of ALDP pathway. **a** Scheme of ALDP biosynthetic pathway (adopted from Jiraskova et al.[2] and modified according to Kamenik et al.[10]); Dotted arrows indicate steps proposed by Zhong et al.[1], brackets indicate a side-pathway, final ALDP precursors highlighted in blue are incorporated into the secondary metabolites. **b** In vitro (experiments from Jiraskova et al.[2] re-examined using a more suitable chromatographic method) and in vivo (new experiments) C-methylation of **2/3** by LmbW; Chromatographic conditions: UPLC BEH Amide 1.7 μm, 2.1 × 50 mm column (Waters, USA), mobile phase: A-acetonitrile and B-50 mM ammonium acetate pH8:acetonitrile 1:1 (v/v), elution: 99% A for 2.5 min followed by a linear decrease from 99 to 1% A in 10 min, UV/VIS chromatograms extracted at 405 nm, MS spectra were recorded using an electrospray ionization technique in a negative mode
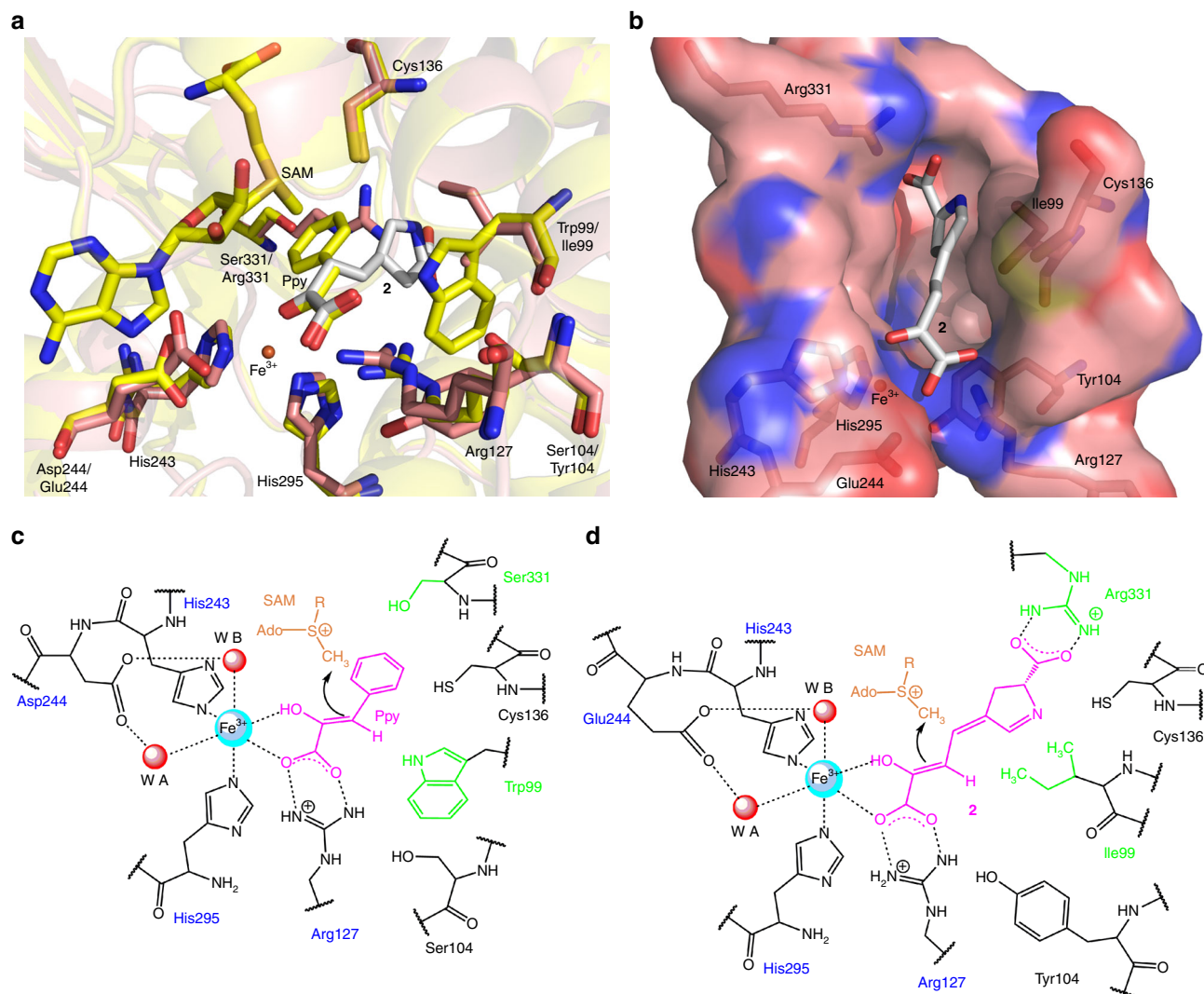
Fig. 2 Comparison of the active sites and proposed reaction mechanism of MppJ and LmbW. **a** Comparison of active sites of MppJ (in yellow, crystal structure PDB ID: 4KIC [https://www.rcsb.org/structure/4KIC] with the substrates phenylenolpyruvate (Ppy) and S-adenosyl methionine (SAM)—adopted[6]) and LmbW (a homology model built using the MppJ structure and the SWISS-MODEL server[15]); LmbW is in pink; substrate **2** is in white. The positions of compound **2**, Fe^{3+}, and SAM in the model were determined by superimposing the model on the 4KIC template in PyMOL[16] and adjusting the position of **2** based on the position of the α-keto(enol)-carboxylic moiety of Ppy bound to MppJ. **b** Arrangement of the putative substrate binding pocket with **2** in the homology model of LmbW. **c** Schematic active site and a proposed mechanism of action of MppJ[6], modified according to panel **a**. **d** Schematic active site and proposed mechanism of action of LmbW. Panels **c** and **d**: abbreviations of residues reflecting the common α-keto(enol)-carboxylic moiety of Ppy and **2** and the common proposed mechanism are in blue; abbreviations of residues differing in MppJ vs. LmbW, reflecting the uncommon moieties of Ppy vs. **2** (aromatic ring of Ppy vs. heterocyclic carboxylic moiety of **2**), are in green. Residue numbering corresponds to MppJ

limazepine E[12] with a two-carbon side-chain ALDP (Fig. 1a). Therefore, Zhong et al.[1] elucidated the unusual C–C bond cleavage function of LmbA/Ant6, but using other than the main native substrate.

Furthermore, Zhong et al.[1] claim that **4**, which they propose to be the product of **2**/**3** cleavage by LmbA/Ant6, is prone to spontaneous isomerization into **5** (Fig. 1a). They observed this isomerization during their unsuccessful attempt to synthesize **4**. However, **4** was previously synthesized by Saha et al.[13], it was structurally characterized by nuclear magnetic resonance (NMR) and used for enzymatic assays, but its spontaneous isomerization into **5** was not reported. Specifically, Saha et al.[13] conducted a two-step deprotection of an analogous compound (methyl ester was used instead of *tert*-butyl ester) using LiOH for methyl ester hydrolysis and trifluoroacetic acid for Boc deprotection, affording **4**, not **5**. Therefore, we consider the formation of **5** during

deprotection of **4'** observed by Zhong et al.[1] to be caused by the used deprotecting method. Importantly, spontaneous isomerization of **4** into **5** would be also inconsistent with the function of putative isomerases LmbX/Ant15. They were assigned for enzymatic isomerization of **4** into **5** based on (1) the comparison of the hormaomycin structure and its biosynthetic gene cluster, which does not encode a homolog of LmbX[4], and (2) the production profile of the Δ*lmbX* and Δ*lmbX*Δ*lmbW* mutants of lincomycin producing strain *S. lincolnensis*[2]. These data show that if the enzymatic isomerization step of **4** into **5** is not involved in the ALDP biosynthesis, **4** or its analog **12** with a three-carbon side-chain is after reduction of its endocyclic double bond incorporated into the final secondary metabolite.

In addition, analytical chemistry data for **5** obtained by Zhong et al.[1] from enzymatic reaction of **2**/**3** with LmbA/Ant6 are not sufficient for unambiguous structural elucidation of this

compound. Comparison of ^{1}H NMR spectra of **5** obtained enzymatically and by chemical synthesis is complicated by partial overlap of the terminal methyl group signal by the signal of NH_4OAc, which together with a relatively low quality of the spectrum complicates easy identification in the case of the enzymatic product. Without analogous comparison of at least ^{13}C NMR spectra of **5** obtained from both sources, it is difficult to see their virtual identity. The expansion present in the spectrum of **5** from enzymatic reaction looks like an expansion from a different spectrum. Moreover, the signal at 2.00 ppm (expansion in spectrum a) should be a doublet, similarly as in the spectrum b. Another misleading point is also the chemical name of **5** in page 39 of Supplementary Information, in which its name corresponds to the structure of **4**.

In summary, considering also our arguments, work of Zhong et al.[1] represents a crucial missing proof of the ALDP biosynthetic pathway puzzle, i.e., the role of γ-GT homologs in the cleavage of oxalate from **2/3** (for compounds with a two-carbon side-chain ALDP) or its methylated derivative **9/10** (for compounds with a three-carbon side-chain ALDP including lincomycin A and anthramycin). The subsequent step in anthramycin and lincomycin A biosynthesis presumably involves isomerization catalyzed by LmbX/Ant15 so that the pathway proceeds towards the final ALDP intermediate.[14]

Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information file and from the corresponding author on request.

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Author contributions

J.J. and Z.K. designed the experiments; R.G. and S.K. built the homology model of LmbW; L.S. and V.R. performed the experiments; R.G., S.K., and Z.K. wrote the text; J.J. revised the text.

Additional information

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