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 gene inactivation experiments. The intriguing C

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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. and modified according to Kamenik et al.); Dotted arrows indicate steps proposed by Zhong et al., 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. 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 x 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.
limazepine E\textsuperscript{12} with a two-carbon side-chain ALDP (Fig. 1a). Therefore, Zhong et al.\textsuperscript{1} elucidated the unusual C–C bond cleavage function of LmbA/Ant\textsubscript{6}, but using other than the main native substrate.

Furthermore, Zhong et al.\textsuperscript{1} claim that 4, which they propose to be the product of 2/3 cleavage by LmbA/Ant\textsubscript{6}, 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 Lmb\textsubscript{X}/Ant\textsubscript{15}. 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 Lmb\textsubscript{X}\textsuperscript{4}, and (2) the production profile of the \textit{ΔlmbX} and \textit{ΔlmbXΔlmbW} mutants of lincomycin producing strain \textit{S. lincolnensis}\textsuperscript{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.\textsuperscript{1} from enzymatic reaction of 2/3 with LmbA/Ant\textsubscript{6} are not sufficient for unambiguous structural elucidation of this.

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**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 \textit{S}-adenosyl methionine (SAM)—adopted\textsuperscript{6}) and LmbW (a homology model built using the MppJ structure and the SWISS-MODEL server\textsuperscript{15}; LmbW is in pink; substrate 2 is in white. The positions of compound 2, Fe\textsuperscript{3+}, and SAM in the model were determined by superimposing the model on the 4KIC template in PyMOL\textsuperscript{16} and adjusting the position of 2 based on the position of the \(\alpha\)-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\textsuperscript{6}, modified according to panel a. d Schematic active site and proposed mechanism of action of LmbW. Panels a and c: abbreviations of residues differing in MppJ vs. LmbW, re.
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$_4$OAc, which together with a relatively low quality of the overlap of the terminal methyl group signal by the signal of enzymatically and by chemical synthesis is complicated by partial 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. 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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