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Biosynthesis of Chuangxinmycin Featuring a Deubiquitinase-like Sulfurtransferase

Xingwang Zhang, Xiaokun Xu, Cai You, Chaofan Yang, Jiawei Guo, Moli Sang, Ce Geng, Fangyuan Cheng, Lei Du, Yuemao Shen, Sheng Wang, Haidong Lan, Fan Yang, Yuezhong Li, Ya-Jie Tang, Youming Zhang, Xiaoying Bian, Shengying Li,* and Wei Zhang**

Abstract: *The knowledge on sulfur incorporation mechanism involved in sulfur-containing molecule biosynthesis remains limited. Chuangxinmycin is a sulfur-containing antibiotic with a unique thiopyrano[4,3,2-cd]indole (TPI) skeleton and selective inhibitory activity against bacterial tryptophanyl-tRNA synthetase. Despite the previously reported biosynthetic gene clusters and the recent functional characterization of a P450 enzyme responsible for C–S bond formation, the enzymatic mechanism for sulfur incorporation remains unknown. Here, we resolve this central biosynthetic problem by in vitro biochemical characterization of the key enzymes and reconstitute the TPI skeleton in a one-pot enzymatic reaction. We reveal that the JAMM/MPN$^+$ protein Cxm3 functions as a deubiquitinase-like sulfurtransferase to catalyze a non-classical sulfur-transfer reaction by interacting with the ubiquitin-like sulfur carrier protein Cxm4GG. This finding adds a new mechanism for sulfurtransferase in nature.*

Sulfur-containing molecules play important physiological and ecological functions in nature.[1] Although numerous natural sulfur-containing compounds have been discovered,[2] the biogenetic mechanisms for sulfur incorporation remain poorly understood despite some recent progresses.[3] In some sulfur-containing molecule biosynthesis, a sulfur carrier protein (SCP) usually acts as the direct sulfur donor after its conserved *C*-terminal diglycine tail (GG-COO$^-$) is activated to the thiocarboxylate form (GG-COS$^-$).[4] A sulfurtransferase is required to catalyze the sulfur-transfer from the activated SCP-GG-COS$^-$ to the acceptor-substrate by forming a ternary sulfurtransferase·aceptor–substrate·SCP complex (Figure 1 A).[5] Such enzyme-mediated sulfur-transfer reactions are essential for the biosynthesis of sulfur-containing molecules.[5] The JAB1/MPN/MOV34 (JAMM/MPN$^+$) proteins, a superfamily of metallopeptidases ubiquitously distributed in eukaryotes, archaea and bacteria,[6] have been found in bacteria to serve as SCP peptidases to cleave the *C*-terminal peptide, thereby exposing the GG-tail for the subsequent sulfurization (Figure 1 A).[5b,7] In eukaryotic and archaeal organisms, the JAMM/MPN$^+$ proteins generally act as deubiquitinases (DUBs) by recognizing and cleaving the ubiquitin (UB) motif, which is evolutionarily related to and structurally similar with SCP, of a ubiquitinated protein (Figure 1 B).[6,8]

The sulfur-containing antibiotic chuangxinmycin (**1**; Figure 1 C), originally isolated from *Actinoplanes tsinanensis* in 1970s, is a potent and selective inhibitor of bacterial tryptophanyl-tRNA synthetase (TrpRS).[9] Although its clinical application has been suspended due to its narrow antimicrobial spectrum, **1** still draws continuing attention on drug development since there has not been any clinical antibiotic targeting bacterial TrpRS.[10] With respect to the biogenesis of **1**, the biochemical basis for construction of the unique TPI scaffold is of special interest for further understanding the sulfur metabolism during natural product biosynthesis. Previously, the biosynthetic gene cluster and the putative biosynthetic pathway of **1** have been independently reported by our and other laboratories (Figure S1).[11] In this study, we elucidated and reconstituted the assembling pathway of TPI from the starting material L-tryptophan (**2**), with a focus on the sulfur incorporation process (Figure 1 C).

[*] X. Zhang, X. Xu, C. You, C. Yang, J. Guo, M. Sang, F. Cheng, L. Du, Y. Li, Y.-J. Tang, Y. Zhang, X. Bian, Prof. Dr. S. Li, Prof. W. Zhang
State Key Laboratory of Microbial Technology, Shandong University
Qingdao, Shandong, 266237 (China)
E-mail: bianxiaoying@sdu.edu.cn
lishengying@sdu.edu.cn
zhang_wei@sdu.edu.cn

X. Zhang, Prof. Dr. S. Li
Laboratory for Marine Biology and Biotechnology
Qingdao National Laboratory for Marine Science and Technology
Qingdao, Shandong 266237 (China)

C. Geng
Shandong Provincial Key Laboratory of Synthetic Biology
CAS Key Laboratory of Biofuels
Qingdao Institute of Bioenergy and Bioprocess Technology
Chinese Academy of Sciences, Qingdao, Shandong, 266101 (China)

Y. Shen
Key Laboratory of Chemical Biology (Ministry of Education)
School of Pharmaceutical Sciences, Shandong University
Jinan, Shandong, 250012 (China)

S. Wang, H. Lan
Tencent AI Lab, Shenzhen, Guangdong, 518063 (China)

F. Yang
Research Center for Marine Drugs
State Key Laboratory of Oncogenes and Related Genes
Department of Pharmacy, Renji Hospital, School of Medicine
Shanghai Jiao Tong University, Shanghai, 200127 (China)

Y. Zhang, X. Bian
Helmholtz International Lab for Anti-Infectives
Shandong University-Helmholtz Institute of Biotechnology
Shandong University, Qingdao, Shandong 266237 (China)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.202107745.

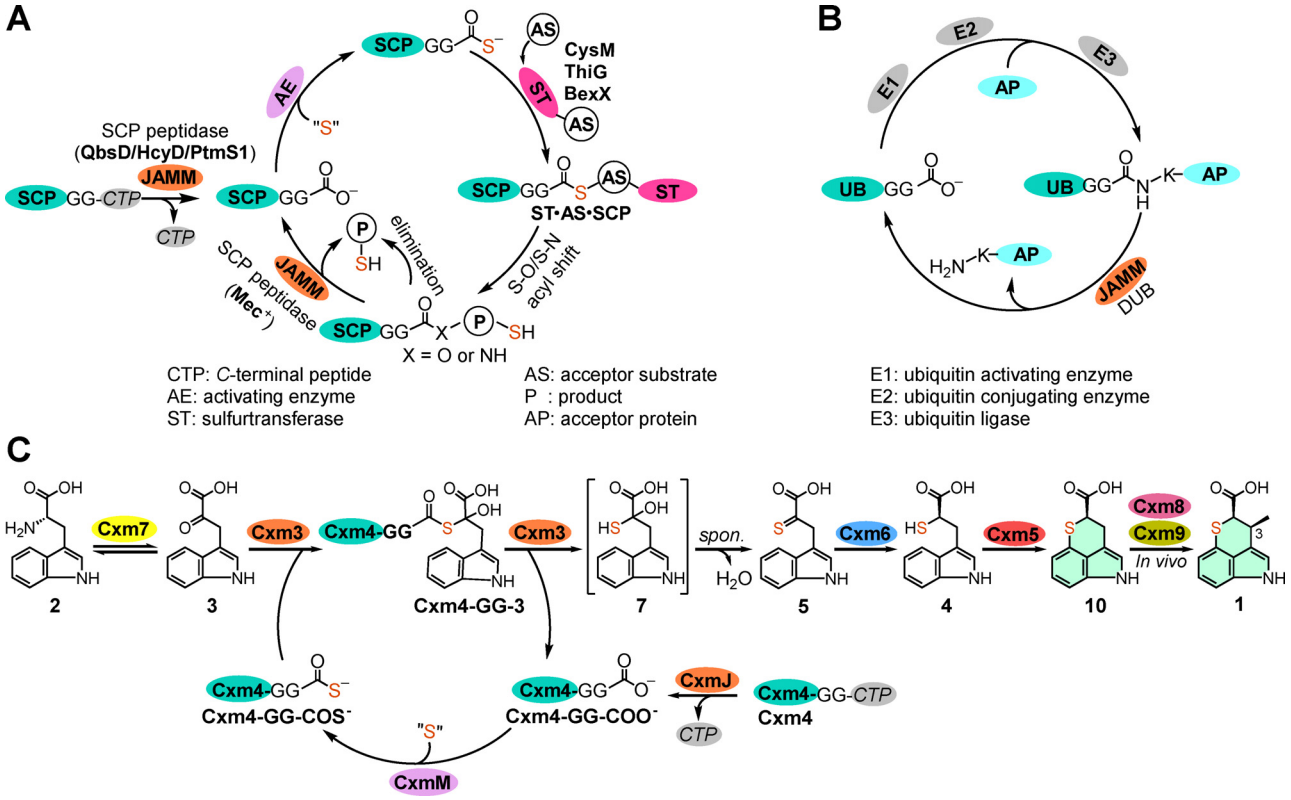

Figure 1. A) The typical SCP-involved sulfur-transfer cycle during SM biosynthesis. B) The ubiquitination/deubiquitination process. C) The biosynthetic pathway of chuangxinmycin (**1**). The TPI skeleton is highlighted in green.

Cxm4 was predicted to be an SCP with 10 additional amino acids after the functional GG moiety (Figure S2). We originally hypothesized that the decapeptide might be cleaved by the predicted JAMM/MPN$^+$ protein Cxm3 to expose the functional GG-tail (Figure 1 A).[5b,7a,b] However, the co-incubation of Cxm3 and Cxm4 (Figures S3 and S4) did not give any detectable Cxm4-GG-COO$^-$ (Figure S5). Whole genome scanning of *A. tsinanensis* located another putative JAMM/MPN$^+$ family SCP peptidase encoding gene *cxmJ* (named after JAMM/MPN$^+$) outside the *cxm* cluster (Figure S6). The reaction of CxmJ and Cxm4 revealed a new protein band with lower molecular mass by SDS-PAGE analysis (Figure S7), which was confirmed as Cxm4GG-COO$^-$ by electro-spray ionization-mass spectrometry (ESI-MS) analysis (Figure 2 A, traces i, ii). The gene *cxmM* (named after MoeZ, an identified SCP activating enzyme) responsible for Cxm4GG-COO$^-$ activation was revealed by using *moeZ* as a probe (Figure S1A).[5d] As expected, the purified CxmM (Figure S4) was able to convert Cxm4GG-COO$^-$ into Cxm4GG-COS$^-$ via the intermediate Cxm4GG-CO-AMP (Figures 2 A and S8).[5d,7a,b] Thus, CxmJ and CxmM co-mediate the transformation from the intact Cxm4 to Cxm4GG-COS$^-$.

The fact that inactivation of the JAMM/MPN$^+$ protein Cxm3 completely abolished the production of **1** and related sulfur-containing intermediates strongly suggests the essential role of Cxm3 in the sulfur-incorporation process.[11a] Indole-3-pyruvic acid (**3**) was previously proposed as the substrate of Cxm3.[11a] Thus, we co-incubated Cxm3, **3**, together with Cxm4GG-COO$^-$ and CxmM in the presence of ATP, $MgCl_2$, $Na_2S_2O_3$ and the thiol protectant dithiothreitol (DTT). As a result, **3** was completely converted into three products **4**, **5** and **6** within 4 h (Figure 2 B, trace ii), while such a conversion did not occur in absence of Cxm3 (Figure S9). The structure of **4** was determined as the α-SH substituted analogue of **3**, while **5** being its α-thione derivative by high-resolution (HR) ESI-MS analyses (Figure S10) and comparisons with synthesized standards (Figures 2 B, traces i, ii, and 2D).[12] With regard to biogenesis of **3**, we confirmed that the PLP-dependent aminotransferase Cxm7 is responsible for the conversion of **2** to **3** (Figure 2 C), which is consistent with the previous prediction.[11a]

Compound **6** was identified as an en route intermediate between **3** and **4**/**5** as evidenced by the time-course study (Figure S11). However, the instability of **6** prevented it from purification and nuclear magnetic resonance (NMR) structure determination. Nonetheless, HR-ESI-MS (Figure S10) and HR-ESI-MS/MS (Figure S12) analysis together with derivatization with the thiol-trapping reagent monobromobimane (Figures S13 and S14),[5d,13] supported **6** to be a DTT adduct of **4** with two free thiol groups (Figure 2 D).[14] We reasoned that **6** might be a DTT-trapped product, likely derived from the real but unstable product **7** (or **5**, the dehydration product of **7**), during the Cxm3 catalyzed sulfur-transfer reaction (Figure 2 D and Figure S15). Of note, when DTT was omitted from the above reaction, **4**, **5** and **6** were all undetectable (Figure S9). With regard to the transformation from **6** to **4**/**5**, the DTT moiety in **6** could be spontaneously cleaved to yield **4** with a mechanism similar to the DTT-

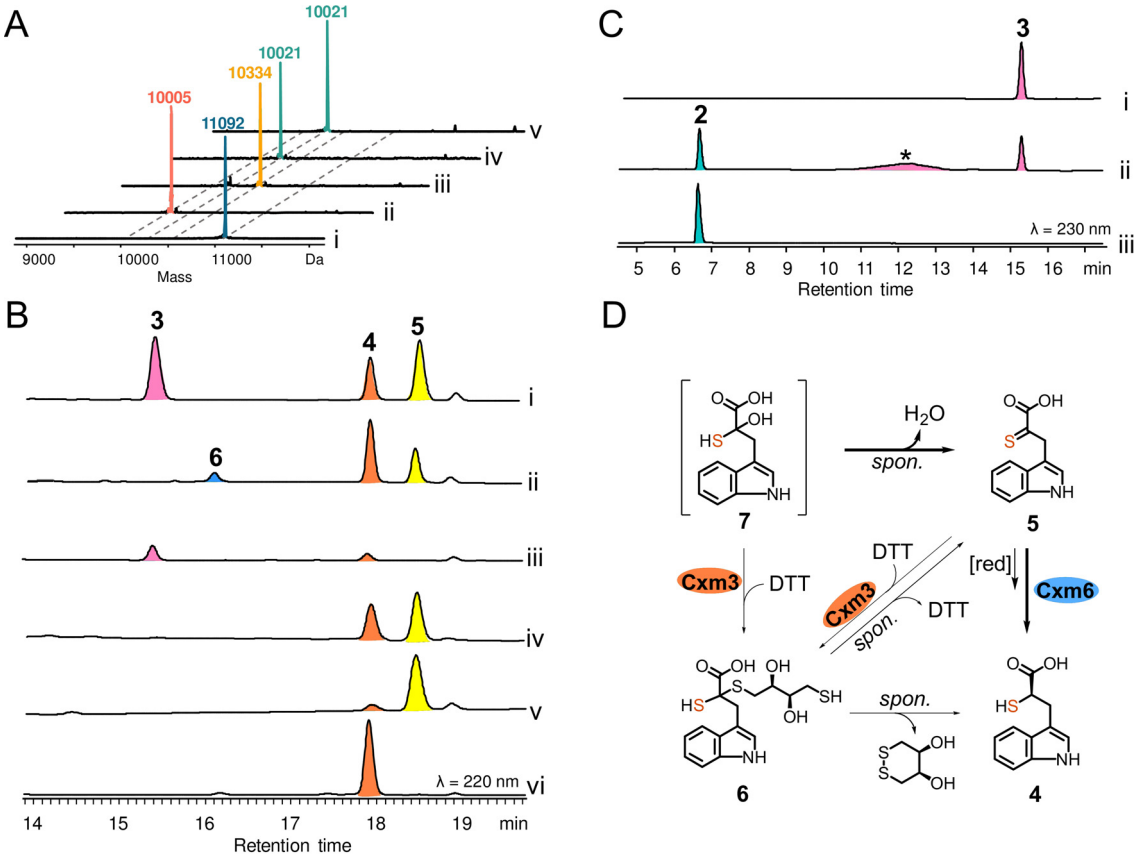

Figure 2. A) Deconvoluted ESI-MS analyses of the different forms of Cxm4. (i) The original Cxm4 (*calc.* 11092.5 Da); (ii) Cxm4GG-COO$^-$ (*calc.* 10005.4 Da) after the cleavage of decapeptide by CxmJ; (iii) Cxm4GG-CO-AMP (*calc.* 10334.5 Da) resulted from the reaction of Cxm4GG-COO$^-$ with CxmM in the presence of ATP and $MgCl_2$; (iv) Cxm4GG-COS$^-$ (*calc.* 10021.4) generated by the addition of $Na_2S_2O_3$ into the reaction of (iii); (v) No Cxm4GG-COS·**3** adduct (*calc.* 10223.2 Da) was detected when the in situ generated Cxm4GG-COS$^-$ (*calc.* 10021.4) was incubated with **3**. B) HPLC analysis of the sulfur-incorporation reaction and the Cxm6-mediated reductive reactions. (i) Mixed standards of **3**, **4** and **5** in the presence of DTT; (ii) The incubation of **3** with Cxm3, Cxm4GG-COO$^-$ and CxmM in the presence of ATP, $MgCl_2$, $Na_2S_2O_3$ and DTT for 4 h; (iii) The reaction of (ii) using TCEP as a reductant in place of DTT; (iv) The incubation of **5** with Cxm6 in the presence of NADPH and DTT for 1 h; (v) The reaction of (iv) with Cxm6 omitted; (vi) The incubation of **3** with Cxm3, Cxm4GG-COO$^-$, CxmM and Cxm6 in the presence of ATP, NADPH, $MgCl_2$, $Na_2S_2O_3$ and DTT for 1 h. C) HPLC analysis of the Cxm7 mediated reactions. (i) Authentic standard of **3**; (ii) Reaction of **2** with Cxm7 in the presence of PLP and sodium pyruvate (*: the decomposed benzopyrrole products of **3** resulted from spontaneous C–C bond cleavage); (iii) negative control of (ii) with boiled Cxm7. D) The transformation network of **4**/**5**/**6**/**7**.

mediated disulfide bond cleavage (Figure S15).[15] Alternatively, the lone pair electrons of α-SH in **6** could attack the electrophilic α-carbon to cleave the DTT fragment, giving rise to **5**. Interestingly, when DTT was replaced by the non-thiol reductant tris-(2-carboxyethyl)-phosphine (TCEP), only a small amount of **4** was also detected (Figure 2B, trace iii). Moreover, **5** could be slowly reduced to **4** by TCEP (Figure S16). Taken together, we proposed the transformation network of **4**/**5**/**6**/**7** (Figure 2D).

The reconstituted sulfur incorporation indicated that Cxm3 functions as a sulfurtransferase to mediate the sulfur-transfer from Cxm4GG-COS$^-$ to **3**. To rationalize the catalytic mechanism of Cxm3, we first examined if Cxm4GG-COO$^-$ and **3** could bind with Cxm3 to form a ternary complex by isothermal titration calorimetry (ITC) experiments. The dissociation constant (K_D) value between Cxm4GG-COO$^-$ and Cxm3 was determined to be 7.0 ± 1.2 μM (Figure S17A). Since **3** fast decomposed in aqueous solution (Figure S17B),[16] we elected to titrate Cxm3 with indole-3-lactic acid (**8**), a stable α-OH analog of **3**. The K_D value was determined to be 2.0 ± 0.8 μM (Figure S17C). These results suggested that Cxm3, Cxm4GG-COS$^-$ and **3** could form a ternary complex for the sulfur-transfer reaction. However, unlike the previously reported sulfurtransferases that form a transient covalent enzyme·substrate adduct during catalysis, such as CysM, BexX and ThiG (Figure 1A),[5] no covalent enzyme·substrate adduct (Cxm3·**3**) could be detected by the established $NaBH_4$ treatment (Figure S18),[17] suggesting an atypical sulfur-transfer mechanism for Cxm3.

At the protein level, Cxm3 is divergent from all known JAMM/MPN$^+$ family proteases (Figure S19) with an atypical motif ($Rx_{80}HxHx_{23}D$ versus the typical core $Ex_{40\text{-}60}HxHx_{10}D$) (Figure S20).[8d] Using the artificial intelligence (AI) dependent de novo protein folding system tFold,[18] the modelled Cxm3 structure showed considerable structural homology with the core fold of the JAMM/MPN$^+$ family DUBs or DUB-like proteins, including the metalloprotease domain

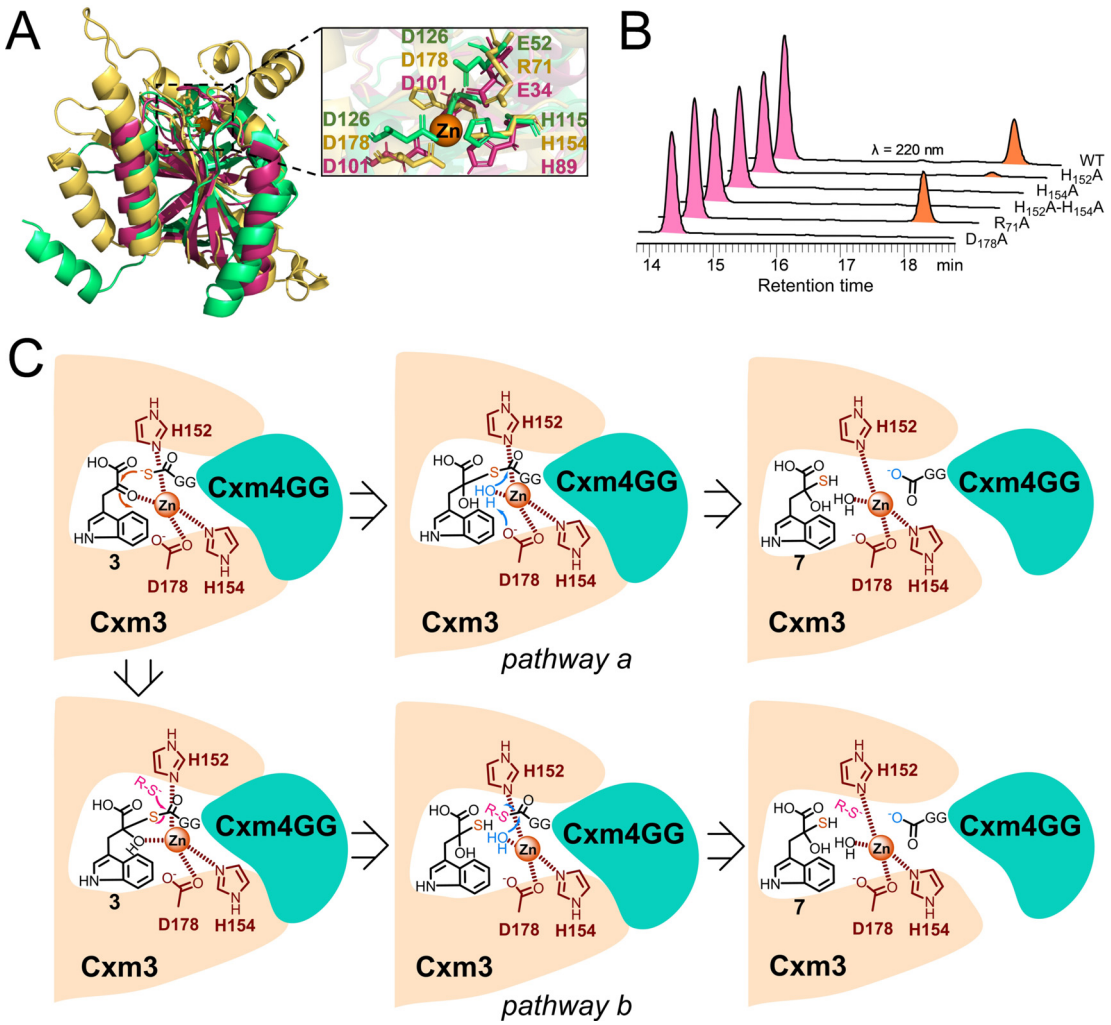

Figure 3. A) Overlay of the modelled Cxm3 (gold) with the Rpn11 domain (green; PDB ID: 5GJQ) and PfJAMM1 (red; PDB ID: 5LDA). The amplified box shows the calculated catalytic groove of Cxm3 comparing with those of Rpn11 and PfJAMM1. B) HPLC analysis of the catalytic activities of Cxm3 mutants. C) The putative mechanisms for the Cxm3-mediated sulfur-transfer reaction.

Rpn11 of human 26S proteasome (PDB: 5GJQ)[19] and PfJAMM1 (PDB: 5LDA) from the archaea *Pyrococcus furiosus*[6b] (Figure 3A). Of note, the conserved H_{152}, H_{154} and D_{178} in Cxm3 was proposed to coordinate a Zn^{2+} ion to form a catalytic groove, which is highly similar to those of Rpn11 and PfJAMM1 (Figure 3A).[6b,8c,20] However, the key glutamate residue for hydrolytic activity in both Rpn11 (E_{52}) and PfJAMM1 (E_{34}) was substituted with an arginine (R_{71}) in the modelled structure of Cxm3 (Figure 3A and S20). To assign its catalytic residues, we performed alanine scanning for the candidate residues of R_{71}, H_{152}, H_{154} and D_{178} (Figure S21A). Specifically, the mutation on the Zn-coordinating residues H_{152}, H_{154} and D_{178} dramatically or completely abolished the sulfur-transfer activity, while the R_{71}A mutant retained the catalytic activity (Figure 3B). Of note, circular dichroism (CD) measurements indicated that the mutations of Cxm3 did not cause any significant conformational changes (Figure S21B).

Based on the results of AI-dependent structure modelling and mutagenesis analysis, we propose a mechanism for the Cxm3-catalyzed sulfur-transfer reaction (Figure 3C). To initiate the progress, the two co-substrates Cxm4GG-COS^- and **3** bind to Cxm3 in a non-covalent manner to form a ternary complex. In the active site, the Zn^{2+} ion coordinated by H_{152}, H_{154} and D_{178} could further coordinate with the α-carbonyl group of **3** to promote its electrophilicity. This would trigger the nucleophilic attack from the thiocarboxylate of Cxm4GG-COS^- to form the C–S bond, which is supported by the observation that no Cxm4GG-COS·**3** adduct was formed in the absence of Cxm3 (Figure 2A, trace v). Next, a water molecule (that may also be coordinated by the Zn^{2+}) might be deprotonated by D_{178} to facilitate the hydrolytic cleavage of the newly installed thioester bond in the covalent Cxm4GG-COS·**3** adduct, thus yielding **7** and regenerating Cxm4GG-COO^- (Figure 3C, pathway a). Alternatively, after the formation of the Cxm4GG-COS·**3** intermediate via a thioester bond, the Zn^{2+} ion may still coordinate with the α-OH group to prevent the backward reaction. Then, an assistant thiol (R-SH) may be responsible for cleaving the thioester bond in the active site of Cxm3, giving rise to **7** and Cxm4GG-COS-R, which could be further hydrolyzed by Cxm3 to restore Cxm4GG-COO^- and R-SH (Figure 3C, pathway b). To

identify the potential physiological R-SH, we tested a number of natural thiols including coenzyme M, L-cysteine, L-glutathione, and ergothioneine, but none of them could support the Cxm3-mediated sulfur incorporation in vitro (Figure S22).[21] Nonetheless, the possibility that an unknown thiol participates in the physiological reaction in vivo cannot be excluded, especially considering that the abiological thiol DTT can form an artifact **6** during the Cxm3-catalyzed reaction.

Thus, the biosynthesis of **1** likely involves an atypical sulfur-transfer process co-mediated by the deubiquitinase-like sulfurtransferase Cxm3 and the ubiquitin-like SCP Cxm4GG, which is analogous to the deubiquitination process in eukaryote (Figure 1 A). Essentially, the Zn^{2+} ion coordinated by H_{152}, H_{154} and D_{178} in the catalytic groove of Cxm3 plays a central role by coordinating all catalytic elements including the substrate **3**, the catalytic residues of Cxm3 and a water molecule (Figure 3 C). Confirmation of this mechanism requires resolution of the highly challenging co-crystal structure of the Cxm3·**3**·Cxm4GG complex, which is currently ongoing in our laboratories.

Cxm6 was predicted to be a 2-dehydropantoate reductase, which can reduce the α-keto group of substrate.[11] Thus, we surmised that the thioketone **5** might be a substrate of Cxm6. As expected, Cxm6 was capable of reducing **5** to **4** in the presence of NADPH and DTT (Figure 2 B, trace iv). The **5**-to-**4** reduction could also slowly occur non-enzymatically under reducing conditions (Figure 2 B, trace v; Figure S16). Supporting this, Cxm6 was found to be unnecessary (Figure 2 B, traces ii and iii), but beneficial (Figure 2 B, trace vi) for the production of **4.** Next, the catalytic function of cytochrome P450 enzyme Cxm5 was reconstituted in the presence of NADPH as electron donor together with ferredoxin *Sel*Fdx1499 and ferredoxin reductase *Sel*FdR0978 from *Synechococcus elongatus* PCC 7942 as surrogate redox partners (Figure 4 A).[22] As a result, Cxm5 mediated a C-S bond formation reaction by converting **4** into 3-demethylchuangxinmycin (**10**) (Figure 1 C). It should be noted that overlapping in time frame with our work, Hong and coworkers reported the same function of CxnD (a homolog of Cxm5).[23]

Based upon the established individual biosynthetic steps for TPI skeleton assembly, we sought to reconstitute the biosynthetic pathway from **2** to **10** in vitro. Particularly, when **2** was firstly incubated with an equal concentration (10 μM) of Cxm3, Cxm4, CxmJ, CxmM, Cxm5, Cxm6 and Cxm7 for 6 h in the presence of co-factors**, 2** was mostly consumed, and the intermediate **3** was also efficiently converted into **4**; while **4** was only partially transformed to **10** (Figure 4 B, trace i, ii). When the concentration of Cxm5 and redox partners were doubled, **2** was completely converted to **10** in the one-pot reaction. Thus, the Cxm5-mediated reaction is the rate-limiting step in the in vitro biosynthesis of TPI skeleton (Figure 4 B, trace iii).

The late C3 methylation of **10** was suggested to be mediated by the cooperation of a radical SAM methyltransferase Cxm8 and a functionally unknown protein Cxm9 based on our previous in vivo study (Figure 1 C).[11a] However, it was unsuccessful to reconstitute the in vitro activity of Cxm8/Cxm9 for unknown reasons by using the purified proteins from *E. coli* cells (Figure S4).

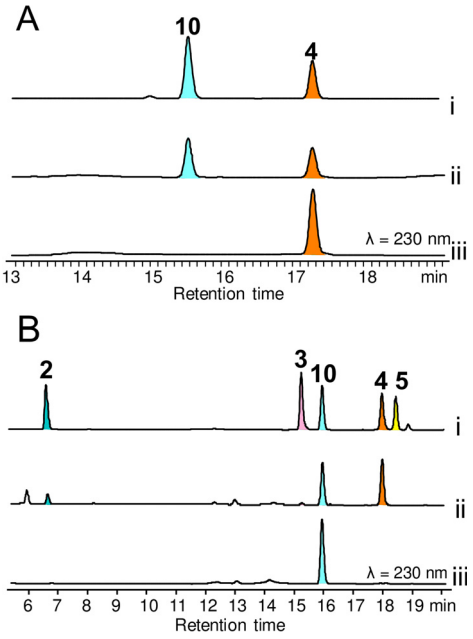

Figure 4. A) HPLC analysis of the Cxm5-catalyzed reaction. (i) Mixed standards of **4** and **10**; (ii) Incubation of **4** with Cxm5 together with the redox partners *Sel*Fdx1499/*Sel*FdR0978 and NADPH led to the formation of **10**; (iii) negative control of (ii). B) HPLC analysis of the in vitro reconstituted conversion from **2** to **10** in one pot. (i) Mixed standards of **2**, **3**, **4**, **5** and **10**; (ii) The one pot reaction from **2** to **10** with an equal concentration of all enzymes; (iii) The concentration of Cxm5 and redox partners were doubled in the reaction of (ii).

In conclusion, we elucidated and reconstituted the biosynthetic pathway of the unique TPI skeleton in **1** by in vitro enzymatic approaches. A novel JAMM/MPN$^+$ superfamily enzyme Cxm3 was characterized as a Zn^{2+}-dependent DUB-like sulfurtransferase. Although the JAMM/MPN$^+$ proteins have been well known as the partners of SCPs or UBs by acting as hydrolases (Figure 1), the identification of Cxm3 as a sulfurtransferase apparently broadens the chemistry of JAMM/MPN$^+$ proteins. Conserved domain and cladogram analysis revealed that Cxm3 and its uncharacterized homologues from various actinomycetes genera form a new clade (V) separated from other subfamilies (clades I–IV) within the proK family (Figure S23). Accordingly, we envision that the co-occurring counterparts of DUB-like Cxm3 and UB-like Cxm4 may have evolved independently for secondary metabolism in prokaryotes, apart from the UB signaling functions in eukaryotes and archaea. Resolution of these biosynthetic mysteries in **1** highlights the intriguing strategies for creating sulfur-containing molecules in nature. Our work also sets the molecular basis for enzymatically generating TPI derivatives as potential therapeutic agents.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: chuangxinmycin · C–S bond biosynthesis · deubiquitinase-like protein · sulfur metabolism · sulfurtransferase

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