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RESEARCH ARTICLE

Exploiting a C-N bond forming cytochrome P450 monooxygenase for C-S bond formation

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Abstract: C-S bond formation reactions are widely distributed in the biosynthesis of biologically active molecules, and thus have received much attention over the past decades. Here, we report intramolecular C-S bond formation by a P450 monooxygenase, TleB, which normally catalyzes a C-N bond forming reaction in indolactam V biosynthesis. Based on the proposed reaction mechanism of TleB, a thiol substituted substrate analogue was synthesized and tested in the enzyme reaction, which afforded the unprecedented sulfur-containing thio-indolactam V, in addition to an unusual indole-fused 6/5/8-tricyclic product whose structure was determined by the crystalline sponge method. Interestingly, the conformational analysis revealed that the SOFA conformation is stable in thio-indolactam V, in sharp contrast to the major TWIST form in indolactam V, thereby resulting in differences in their biological activities.

Introduction

Sulfur is a ubiquitously distributed, essential element in natural biochemical systems, including both primary and secondary metabolisms.[1] Apart from methionine and cysteine amino acids in proteins or peptides, sulfur atoms in natural products are a relatively rare component as compared to carbon, hydrogen, oxygen, and nitrogen atoms. However, there are many examples of sulfur-containing natural products with significant biological activities, such as penicillin, gliotoxin, and yersiniabactin.[2] In the biosyntheses of these sulfur-containing natural products, C-S bond forming reactions are catalyzed by diverse types of enzymes, including cystathionin β-synthase in cysteine biosynthesis, S-adenosylmethionine (SAM) synthetase, and glutathione-S-transferases in primary metabolism, and radical SAM enzymes, heterocyclases, nonheme iron-dependent enzymes, and cytochrome P450 monooxygenases, in secondary metabolism.[3]

Cytochrome P450 monooxygenases catalyze a wide range of oxidative transformations, from the detoxification of xenobiotics to the oxidative tailoring of natural products.[4] In addition to their broad substrate specificity, naturally occurring and engineered P450 enzymes catalyze many different chemical reactions, including hydroxylation, epoxidation, amination, nitration, sulfoxidation, and synthetically important unnatural reactions.[5]

Indolactam V (**1**), isolated from actinomycetes, is an essential pharmacophore of teleocidin's tumor-promoting activity, which involves binding and activating various kinase receptors, including protein kinase C.[6] Because of their potent activities and unique 9-membered lactam structural scaffolds, teleocidin derivatives have attracted keen attention in the areas of medicinal and organic chemistry. Accordingly, numerous total synthesis and structure activity studies of **1** and teleocidin derivatives have been reported.[7] In the biosynthesis of teleocidins, a cytochrome P450 monooxygenase catalyzes the formation of a 9-membered lactam ring to generate **1** from *N*-methyl-L-valyl-L-tryptophanol (NMVT, **2**) as the substrate, through intramolecular C(sp_2)–H bond amination at the C4 position of the indole with the N13-methyl group (Scheme 1A).[8]

We previously reported the mechanistic analysis of TleB and HinD from *Streptomyces blastmyceticus* NBRC 12747, by using N13 substituted substrate analogues and performing an X-ray crystallographic structural analysis.[9] The TleB enzyme reactions with three substrates featuring different functional groups at the 13th position (i.e., hydroxy (**3**), methoxy (**4**), and primary amino (5) groups) revealed that these substrates are converted into the 6/5/6 tricyclic products **6**, **7**, and **8**, respectively, but not the 9-

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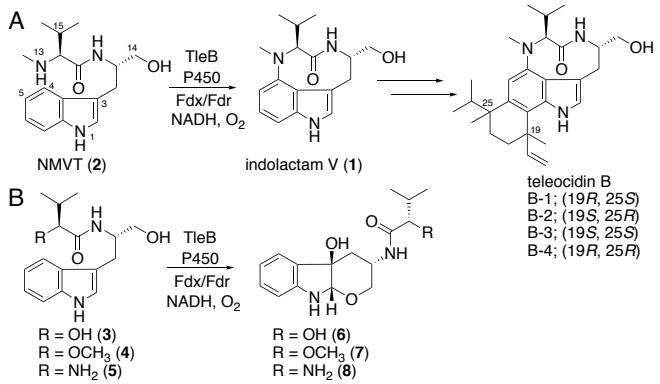
membered indolactam skeleton. (Scheme 1B). Together with the in vitro results, further X-ray structural analyses of TleB and its homologous enzyme HinD suggested that the stability of the radical on the heteroatoms and the H-R (R = NCH_3, NH, or O) bond dissociation energy (BDE) at the 13th position are important for the formation of the 9-membered indolactam.

To further test this hypothesis and generate new derivatives for future drug development, here we investigated the enzymatic synthesis of sulfur-containing indolactam derivatives by utilizing the promiscuous TleB and the 13-SH substituted substrate **9**, which has a lower BDE as compared to those of H-N and H-O.



Scheme 1. TleB catalyzed enzyme reactions. (A) Biosynthetic pathway of teleocidins. TleB is involved in the formation of indolactam V through a direct C-N bond formation reaction. (B) Enzyme reactions of TleB with substrate analogues.

Results and Discussion

Synthesized **9** (0.5 mM) was incubated with the purified TleB, in the presence of the P450 redox partners CamA and CamB from *Pseudomonas putida*, 10 mM NADH, 0.5 mM TCEP hydrochloride, and 10 μg catalase. The liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) analysis revealed that the enzyme generated a mixture of two products, **10** and **11**, possessing the same parent ion peaks with *m/z* 303 $[M-H]^-$, indicating the loss of two hydrogen atoms from the substrate (Figures 1A and S1A). The UV spectra of these products were significantly different, suggesting that **10** and **11** formed different scaffolds (Figure S1B). The ^{1}H-, ^{13}C-NMR analyses of **10** showed the elimination of the indole C4 proton. Most importantly, the HMBC correlation of H-12/C-4 clearly indicated the formation of a direct C-S bond between S13 and the indole C4, which was further supported by the downfield-shifted C-12 position (**10**: 66.1 ppm, **9**: 49.9 ppm) (Figure S2A and Table S1). Thus, the structure of **10** was determined to be a sulfur-containing indolactam-like (named thio-indolactam) skeleton (Figure 1B). The structure of **10** was also confirmed by the single crystal X-ray crystallography, and its absolute stereochemistry was established as (9*S*, 12*S*), based on the Flack parameter [0.002(3)] (Figures 2A and S3). These results clearly demonstrated that the BDE is important for the cyclization reaction, and TleB can form an indole C4-heteroatom bond with not only N-methylamine but also a thiol.

The high-temperature molecular dynamics (MD) calculation of **1** derivatives suggested that the 9-membered lactam moiety forms 10 possible types of conformations, although only two conformations derived from the cis-lactam amide (TWIST) and the trans-lactam amide (SOFA) are stable in **1**.[10] Indeed, the NMR analysis revealed that **1** exists as the mixture of TWIST and SOFA, and the TWIST conformation is preferred (Figures 3 and S4).[10] Distinct from the case of **1**, the series of NMR analyses of **10**, including NOESY correlations and coupling constants, suggested that **10** exists as only the SOFA conformer (Figure S2B). The crystal structure of **10** was also obtained as the SOFA conformation (Figure S3), which is compatible with the NMR result. The difference in the conformations between **1** and **10** probably results from the 13-methyl group and/or the different bond lengths between the C-S and C-N bonds.

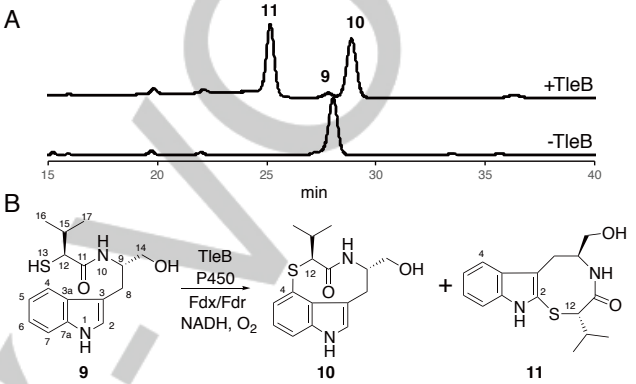


Figure 1. TleB enzyme reaction with 13-thiol substituted **9**. (A) HPLC profiles of the enzyme reaction. (B) Structures of the enzymatic products of TleB from **9**. The chromatograms were monitored at 280 nm.

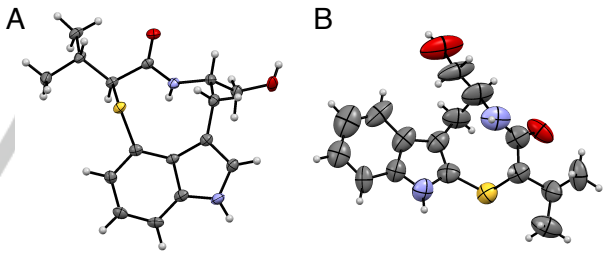


Figure 2. Crystal structures of **10** and **11**. (A) ORTEP drawing with 50% probability for **10**, which was observed by the single crystal X-ray crystallography. (B) ORTEP drawing (30% probability) of **11**, which was observed as a part of the **11**/crystalline sponge complex.

To further verify the stable conformation of **10**, we examined the conformational distribution of **10** by DFT calculations (Tables S2 and S3, Figures 3 and S5-S9). Based solely on the calculated thermal free energies, while the SOFA conformation is dominant (72.3% distribution), the r-trans-FOLD conformation can also exist (21.7%). Among the conformations predicted by the MD calculations by Kawai et al.,[10] the "r"-conformations were classified into group B based on the dihedral angle of C5-C4-S-C12 (Figure 4).[10b] The molecule is folded toward the reverse side of the indole ring, as compared to the SOFA and TWIST conformations. However, the chemically synthesized **1** and the natural teleocidins reportedly do not exist as r-conformations. Furthermore, the metadynamics calculation of 10 revealed that the SOFA and r-trans-FOLD conformations are not interconvertible (Figure 4). These results suggest that **10** is fixed

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in the SOFA conformation when the C-S bond is enzymatically formed inside the TleB active site pocket, and it is not converted into other conformations because of the high energy barrier at the ambient temperature.

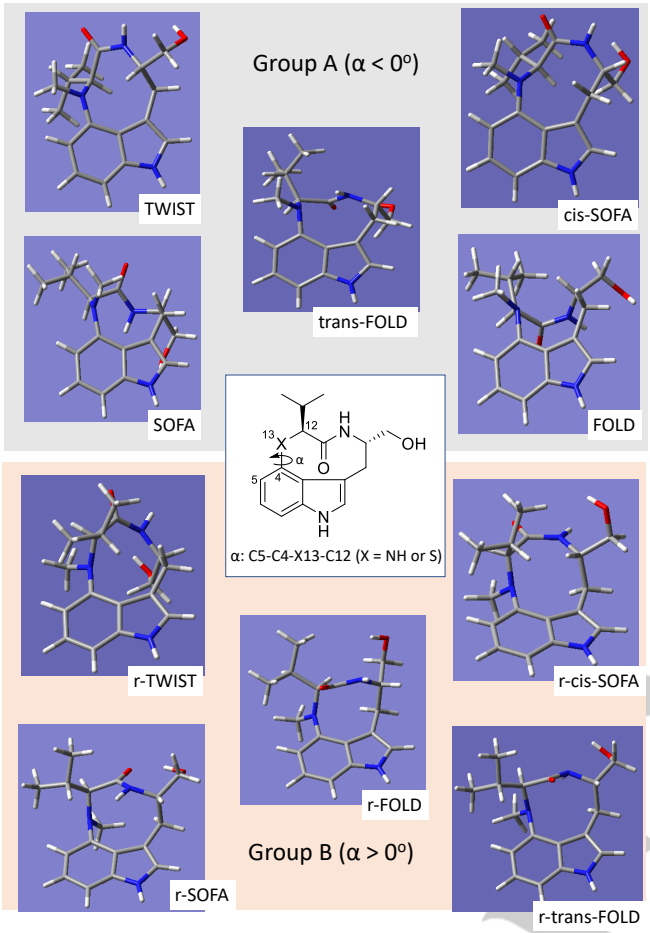

Figure 3. Possible conformations of indolactam analogue proposed by Kawai *et. al.* Based on the dihedral angle of C5-C4-X13-C12 (α, X = heteroatom), 10 conformations were divided into two groups (Group A: α < 0o/ Group B: α > 0o).

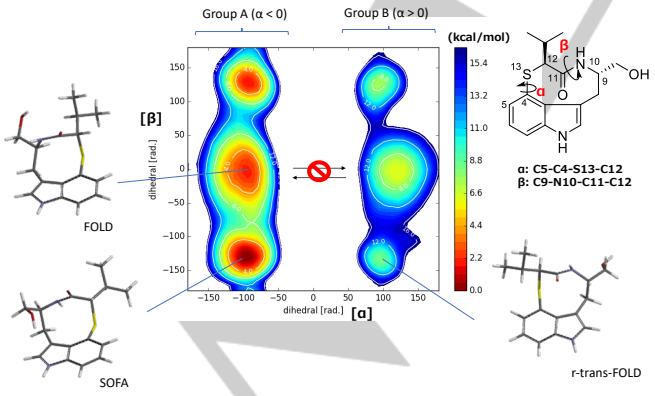

Figure 4. Metadynamics simulation of **10** in methanol at 300 K. The conformational states between group A and group B (divided based on the dihedral angle of C5-C4-S-C12) are not interchangeable due to the high energy barrier.

A structural model of TleB with a substrate analogue also supports this result (Figure S10). We previously obtained two different conformations of substrates in the active site of TleB and its homologue HinD.[9] The first binding mode should represent the initial conformation for the abstraction of the N1-proton, and we proposed that **2** undergoes re-orientation after the N1-hydrogen abstraction in the active site to the second conformation. In the second binding mode, the N13 (or S13) attacks the C4 atom from the *si*-face to generate the natural conformers. Thus, the enzyme strictly regulates the conformation of the substrate in the active site, indicating that we can rule out the possibility of group B formation (e.g., r-trans-FOLD, Figure 4) in nature.

The previous structure-activity studies with conformationally restricted **1** analogues demonstrated that the TWIST conformation is mainly responsible for the tumor promoting activity of **1**.[11] Furthermore, the TWIST-restricted analogue of **1** binds both the Ca^{2+}-dependent and Ca^{2+}-independent PKC isozymes, while the SOFA-restricted analogue selectively binds to the Ca^{2+}-independent PKC isozymes.[12] Considering the significant conformational difference, the biological function of **10** could be distinct from those of other indolactams. Indeed, the biological activity test of **10** for 39 cancer cell line revealed that **10** has moderate cytotoxicity against several cell lines (GI50 = ~27 μM), and thus the molecular targets of **10** are likely different from those of **1** (Table S4, Figures S11 and S12).

On the other hand, the ^{13}C-, ^{1}H-NMR, and 2D-NMR spectra of **11** and the (*S*)-MTPA ester of **11** (Table S1 and Figure S13) clearly showed the retention of four protons on the C4-C7 of the indole, the elimination of H-2, and the remarkable downfield C-12 chemical shift, as compared to **9** (Table S1). Considering these observations, we speculated that **11** possesses an unusual indole-fused 6/5/8 tricyclic ring system, with a C-S bond between C-2 and S-13. However, direct evidence for the C-S bond between C-2 and S13 could not be obtained from the NMR spectra. Therefore, the structure of **11** was determined by the crystalline sponge method, which enables the crystallization-free X-ray diffraction analysis.[13] In this study, a crystal of the porous complex $[(ZnCl_2)_3(tpt)_2 \cdot x(\text{cyclopentylmethylether})]_n$ [tpt = 2,4,6-tris(4-pyridyl)-1,3,5-triazine][14] was incubated with 2.5 μg of **11**. Since **11** entered the host crystal as guest molecules and aligned neatly, the X-ray analysis of the guest-absorbed crystal enabled the structural determination of **11** (Figures 2B and S14). Moreover, its absolute stereochemistry was established as (9*S*, 12*S*), based on the Flack parameter [0.060(12)]. Interestingly, the indole ring of **11** was not β-hydroxylated, as observed in the C-O bond forming **6**, **7**, and **8** (Figure 1).[9]

The formation of **11** should proceed via a 3β-hydroxy-indolenine (or 2,3-epoxide) intermediate, as in the case of the formation of the 6/5/6 tricyclic products **6**, **7**, and **8**. Thus, TleB catalyzes both the direct C-S bond formation and the C-S bond formation through the oxidation of the indole ring. However, interestingly, when we used the **2**-analogues **3**, **4**, and **5** as substrates, the C2-O13 or C2-N13 bond forming **11** type products were not obtained.[9] The product formation would be controlled by the nucleophilicity of the sulfur atom, which is higher than those of the hydroxyl and amine groups. For **11** production, the indole N1 hydrogen is firstly abstracted by a ferryl-oxo species (compound I) to generate a radical on the indole ring. It is then translocated onto the C3 position and hydroxylated with a ferryl-hydroxo species (compound II) to form the 3β-hydroxy-indolenine

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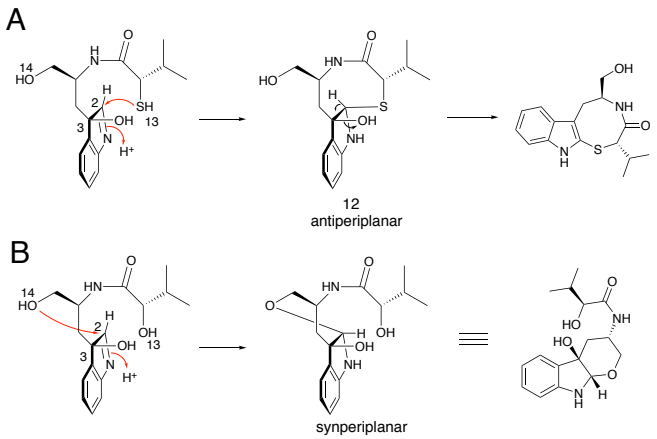
(or 2,3-epoxide) intermediate. The intramolecular attack of S13 to the indole C2 generates the cyclized intermediate **12**.



Scheme 2. Reaction mechanism of formation of (A) 6/5/8 from **9** and (B) 6/5/6 ring structures from **3**.

It is notable that the cyclization reaction is followed by the spontaneous re-aromatization of the indole ring through H_2O elimination, while the dehydration is not observed in the formation of the 6/5/6 tricyclic compounds, in which the 3β-hydroxyl group is retained in the structures. The spontaneous H_2O elimination would be contributed by the antiperiplanar conformations of the C2-H and C3-OH bonds (Scheme 2A). The S13 group attacks the indole C-2 from the *re*-face to produce the 2*R*, 3*R* cyclic intermediate **12**. The elimination of the hydroxyl group readily proceeds to stabilize the indole ring by restoration of the aromaticity. This is different in the 6/5/6 tricyclic compounds, in which the C2-H and C3-OH bond conformations should be synperiplanar, because the 14-OH in **3**, **4**, and **5** can attack only from the *si*-face of the indole C2 (Scheme 2B).

Since our attempt at obtaining complexed structure of TleB with **9** failed, the enzyme reactions of TleB T286A, F287A, and Q387A mutants, residues that interact with substrate **2** in the crystal structure[9], were performed to explore the interaction of **9** in the active site (Figure S15). As a result, the F287A mutant determined to be inactive as in the case when **2** was used as a substrate, suggesting the importance of the π-π interaction and radical-π interaction with the indole ring of substrate. However, mutation of T286 and Q387, which interact with amide-carbonyl and 14-OH of **2** respectively, to alanine, resulted in mutants T286A and Q387A maintained the significant activity toward **9** while these mutants dramatically decreased the activity (~20%) toward **2**. [9] These results suggested that loss of the methyl group and the large atomic radius of sulfur atom changed the initial binding mode in the active site. Because of the difference of the binding mode, the second conformational change of the radical intermediate and hydroxylation of the C3 indole ring would be competed. Furthermore, when the 3β-hydroxy-indolenine is generated, C-S bond formation between S13 and C2 is preferred due to the high nucleophilicity of sulfur atom to produce 6/5/8 tricyclic **11** but not 6/5/6 tricyclic product.

The conversion rate of TleB for **2** was 52.5 ± 6.4%, while that for **9** was 52.2 ± 1.0%. The k_{cat}/K_M value of TleB for **9** (K_M = 63.8 ± 16.7 μM, k_{cat}/K_M = 14.2 $min^{-1}mM^{-1}$) is comparable to that for **2** (K_M = 44.6 ± 9.7 μM, k_{cat}/K_M = 20.9 $min^{-1}mM^{-1}$) (Table S5). Notably, TleB efficiently accepted **9** to produce the thio-indolactam V **10** and the 6/5/8 tricyclic compound **11** with nearly the same efficiency, as compared to the original substrate **2**.

Precedent for C-S bond formation using heme-containing enzymes are observed in the biosynthesis of camalexin, spirobrassinin, cyclobrassinin, thienodolin, and ustiloxin. These C-S bonds are proposed to be constructed through epoxidation or desaturation of compounds.[3] For example, in cyclobrassinin[15] and thienodolin[16] cases, the P450s are proposed to catalyze the epoxidation at the C2-C3 position, and the newly generated 6- or 5-membered ring is also spontaneously dehydrated to rearomatize the indole ring, suggesting that these P450s also strictly control the stereochemistry of the epoxidation and cyclization reactions as in the case of TleB. Other examples of enzymatic C-S bond formation with similar mechanism to production of **10** are the engineered P411/P450 enzymes.[17] The Arnold group recently described a powerful biocatalytic platform based on engineered cytochrome P411/P450 enzymes that enables facile access to a broad array of structurally diverse α-thio-γ-lactones through enzyme catalyzed C-S bond construction. In this mechanism, the iron-carbene intermediate is firstly generated, which is followed by the radical-coupling between a thiyl radical and a free alkyl radical or a heme-bound alkyl species. Thus, heme-containing enzymes catalyze C-S bond formation via either of oxidation-mediated or radical-mediated mechanism. On the other hand, the generation of **10** and **11** suggested that TleB can catalyze both C-S bond formation reactions. Furthermore, the formation of C-S bond through radical-coupling was observed both in naturally occurring TleB and engineered heme-containing enzymes, suggesting that the radical mechanism could be one of the common C-S bond formation reaction in nature.

Conclusion

We demonstrated here the enzymatic generation of two unnatural products from a thiol-containing substrate through different types of C-S bond formation reactions. These results not only support our previously proposed mechanism of the TleB reaction but also shed light on future possibilities of creating new skeletons with various biological activities by using P450 enzymes as biocatalysts.

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Keywords: Cytochrom P450, teleocidin, biosynthesis, enzyme reaction, DFT calculation

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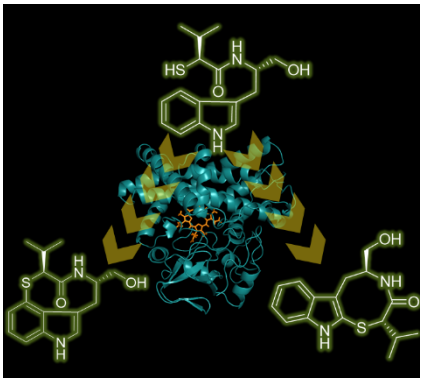

Cytochrome P450 monooxygenases catalyze a wide range of oxidative transformations. TleB in the biosynthesis of teleocidin catalyzes C-S bond formation through both of oxidation-mediated and radical-mediated mechanisms to generate sulfur-containing indolactam and precedent 6/5/8 tricyclic ring compound. These results may provide insight for the production of new unnatural compound by using enzymes as biocatalysts.