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Processing 2-Methyl-L-Tryptophan through Tandem Transamination and Selective Oxygenation Initiates Indole Ring Expansion in the Biosynthesis of Thiostrepton

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Supporting Information

ABSTRACT: Thiostrepton (TSR), an archetypal member of the family of ribosomally synthesized and post-translationally modified thiopeptide antibiotics, possesses a biologically important quinaldic acid (QA) moiety within the side-ring system of its characteristic thiopeptide framework. QA is derived from an independent L-Trp residue; however, its associated transformation process remains poorly understood. We here report that during the formation of QA, the key expansion of an indole to a quinoline relies on the activities of the pyridoxal-5′-phosphate-dependent protein TsrA and the flavoprotein TsrE. These proteins act in tandem to process the precursor 2-methyl- L-Trp through reversible transamination and selective oxygenation, thereby initiating a highly reactive rearrangement in which selective C2−N1 bond cleavage via hydrolysis for indole ring-opening is closely coupled with C2′−N1 bond formation via condensation for recyclization and ring expansion in the production of a quinoline ketone intermediate. This indole ring-expansion mechanism is unusual, and represents a new strategy found in nature for L-Trp-based functionalization.

Thiopeptide antibiotics are a class of sulfur-rich, ribosomally synthesized and post-translationally modified peptides (RiPPs) that possess a wide variety of biological properties.[1] These antibiotics share an unusual macrocyclic core that contains a six-membered heterocycle central to multiple azoles and dehydroamino acids (Figure 1). As with many RiPPs,[2] the biosynthesis of thiopeptides starts with the conversion of a peptide precursor composed of an N-terminal leader sequence and a C-terminal core sequence. A myriad of post-translational modifications (PTMs) occur solely on the latter, exemplifying how nature develops structural complexity from a Ser/Thr and Cys-rich sequence.[3]

The establishment of a characteristic thiopeptide framework requires a series of common PTMs in the formation of azoles, dehydroamino acids and a central heterocyclic domain.[4] In addition to sequence permutation of precursor peptides, the

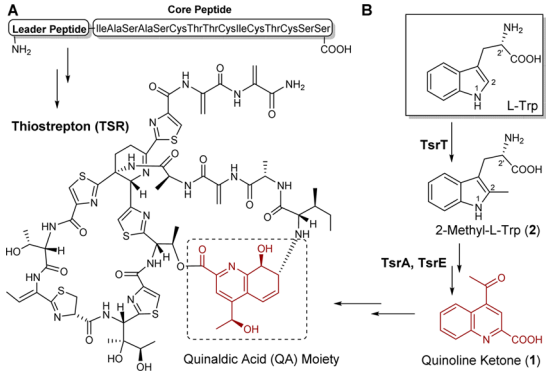

Figure 1. Bicyclic thiopeptide TSR, its ribosomally synthesized precursor peptide and related biosynthetic pathway. (A) Structures of TSR, in which the QA (ochre) moiety is indicated in a dashed rectangle. (B) Specific PTMs that involve related enzymatic activities for processing the precursor L-Trp to achieve QA (via quinoline ketone intermediate **1**) within the side-ring system.

constitution of the thiopeptide family, which includes over 100 different entities, depends on a number of specific PTMs for individualized treatment,[5] as exemplified by the biosynthesis of the bicyclic members thiostrepton (TSR, Figure 1A) and nosiheptide (NOS). Specifically, TSR bears a quinaldic acid (QA) moiety within a 27-membered large side-ring system, whereas in NOS, an indolic acid (IA) moiety is directly attached to the macrocyclic core, forming a 19-membered compact side-ring system. Benefiting from these moieties, which expand the chemical spaces available for biological functions, bicyclic thiopeptides appear to be unique regarding their anti-infectious actions.[6]

Intriguingly, both the QA moiety of TSR and the IA moiety of NOS originate from a common substrate, L-Trp (Figure 1B).[7] The formation of IA relies primarily on the activity of NosL, an *S*-adenosylmethionine (SAM)-dependent radical protein.[8] This protein catalyzes a complex arrangement of the carbon side

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chain of L-Trp, i.e., the removal of the C2′−N unit and a shift of the carboxylate group onto the indole ring, and produces 3-methyl-2-indolic acid for further incorporation. In contrast, the process through which QA is formed remains poorly understood. This process results in more complex changes in the structure of L-Trp, including ring expansion by cleavage of the C2−N1 bond for indole ring opening and the connection of C2′ of the carbon side chain to N1 for recyclization.[7b] Focusing on this key transformation, we here dissect an unusual mechanism for indole ring expansion and demonstrate that processing the precursor 2-methyl-L-Trp through tandem transamination and selective oxygenation triggers an intramolecular rearrangement during the formation of a quinoline ketone intermediate.

We previously demonstrated that, in the TSR-producing *Streptomyces laurentii* strain, the formation of the QA moiety involves a ring-expanded intermediate, quinoline ketone **1** (Figure 1B).[9] The genes *tsrT*, *tsrA* and *tsrE* are related to the biogenesis of **1** because the inactivation of each gene completely abolished the production of TSR, which was then restored by feeding **1** into the corresponding mutant strain. Among these genes, the only one that has been functionally characterized thus far is *tsrT*, which encodes a SAM-dependent radical protein for the 2-methylation of L-Trp to produce 2-methyl-L-Trp (**2**) (Figure 1B).[10] To determine whether the remaining genes code for the transformation of **2**, *tsrA* and *tsrE* were coexpressed heterologously in *Escherichia coli*, where an excess of 2-methyl-DL-Trp ($[M + H]^+$ *m/z*: calcd. 219.1134 for $C_{12}H_{15}N_2O_2$, found 219.1134), a racemate synthesized in this study (Supplementary Results), was supplemented. The production of quinoline ketone **1** was observed; however, it was completely abolished by omitting either *tsrA* or *tsrE* (Figure S1). The necessity of *tsrA* and *tsrE* was further confirmed using the recombinant *E. coli* cell homogenate to transform **2** because **1** was produced (Figure S1). Both TsrA and TsrE were then purified from *E. coli* to assay their specific activities *in vitro* (Figure S2).

The recombinant TsrA protein appeared colorless and exhibited activity only in the presence of exogenous pyridoxal-5′-phosphate (PLP) (Figure 2). Consistent with previous findings,[11] this protein catalyzed the transformation of 2-methyl-L-Trp (**2**) using indolylpyruvate (IPA) as an ammonia acceptor, yielding 2-methyl-indolylpyruvate (**3**, $[M - H]^-$ *m/z*: calcd. 216.0661 for $C_{12}H_{10}NO_3$, found 216.0662) and the coproduct L-Trp (Figure 2).Therefore, TsrA is a characteristic PLP-dependent aminotransferase. Intriguingly, the ammonia acceptor is changeable, as the replacement of IPA with other α-keto acids varying in α-substitution, e.g., phenylpyruvate (aromatic) or α-ketoglutarate (linear), still produced **3** with a different coproduct, e.g., L-Phe or L-Glu, respectively (Figure 2), indicating that TsrA is flexible with respect to its substrate. Indeed, TsrA tolerates L-Trp and L-Phe and catalyzed reversible conversions between L-Trp and indolylpyruvate and between L-Phe and phenylpyruvate when each of these α-keto acids served as the ammonia acceptor for the other transamination reaction (Figure S3). TsrA functions in a stereoselective manner, because none of the tested D-amino acids, including D-Trp and D-Phe, can be transformed into the corresponding α-keto acids.

Bioinformatics suggests that TsrE is a flavin-dependent protein and is homologous to various acyl-CoA dehydrogenases that catalyze acyl chain desaturation accompanied by the reduction of the flavin cofactor from its oxidized form (e.g., flavin adenine dinucleotide, FAD) to reduced form (e.g., $FADH_2$).[12] Accordingly, it was previously proposed that TsrE follows TsrA activity to dehydrogenate indole enamine **3** in a

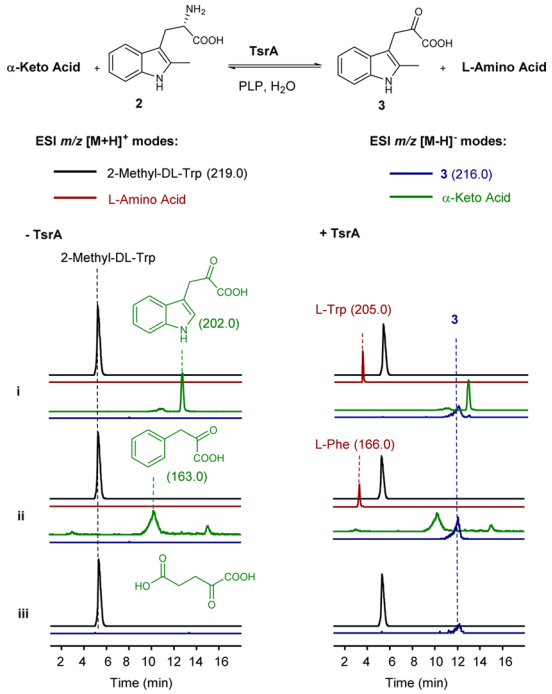

Figure 2. *In vitro* transamination of **2** to **3** (top) in the absence (lower left) and presence (lower right) of TsrA, using IPA (i), phenylpyruvate (ii) or α-ketoglutarate (iii) as an ammonia acceptor.

similar manner, producing an indole imine, which is reactive and may readily undergo hydrolysis to break the N1−C2 bond and initiate the ring expansion process (Figure 3, Route 1).[9] However, in the presence of FAD, the addition of purified TsrE protein to the TsrA-containing reaction mixture did not result in further transformation of **3** (Figure 4A).

We thus reconsidered the nature of TsrE catalysis. In addition to FAD-based dehydrogenation, O_2 and $FADH_2$-dependent oxygenation or halogenation is a direct alternative for achieving indole ring activation and expansion (Figure 3, Routes 2, 3 and 4).[12] Consequently, Fre,[13] a flavin reductase, was purified from *E. coli* (Figure S2) and complemented to the above reaction mixture to recycle $FADH_2$ from oxidized FAD with dihydronicotinamide adenine dinucleotide phosphate (NADPH) *in situ*. In the presence of Fre, FAD and saturated NADPH, TsrE drove TsrA-mediated reversible transamination forward by effectively converting **3**, completed the consumption of the precursor 2-methyl-L-Trp (**2**) in the racemate and produced the quinoline ketone intermediate **1** in a time-dependent manner (Figure 4A). This transformation failed to occur under strictly anaerobic conditions, supporting the notion that O_2 is indispensable. TsrE could not transform indolylpyruvate or **2**, indicating that indole ring expansion follows the activities of 2-methylation and transamination. In addition, omitting halide ions from the reaction buffer had little effect on the production of **1**. More likely, this protein is a flavin-dependent oxygenase essential for an intramolecular rearrangement.

Careful analysis of the TsrA and TsrE-involving transformation that proceeded at 30 °C revealed a minor compound ($[M + H]^+$ *m/z*: calcd. 206.0812 for $C_{11}H_{12}NO_3$, found 206.0817) (Figure 4A), which displays an ultraviolet absorption spectrum different from those associated with **2**, **3** and **1** (Figure S4). According to the established molecular formula and the hypothesis that TsrE catalysis is essentially an oxygenation

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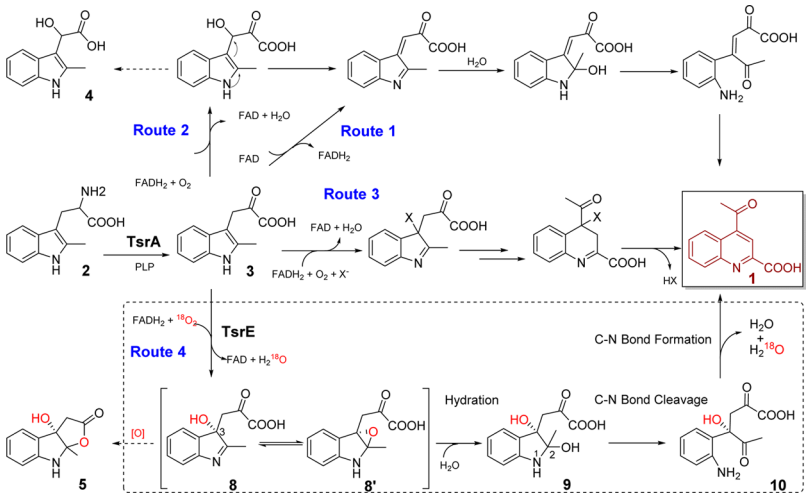

Figure 3. Potential routes of indole ring expansion toward the formation of quinoline ketone **1** (ochre, shown in the solid rectangle). The TsrE-initiated ring-expansion process is indicated in the dashed rectangle, where the C3 stereochemistry of related chemicals is proposed based on the assignment of the stereochemistry of **7**. The atom ^{18}O is indicated in red when using $^{18}O_2$.

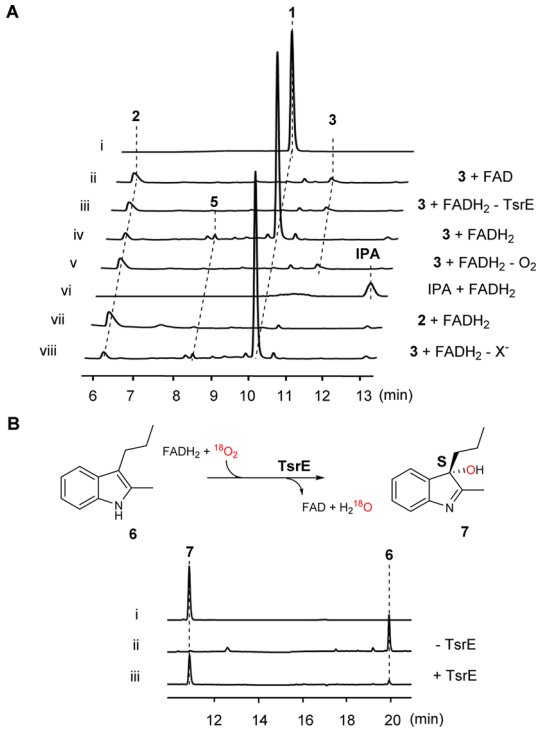

Figure 4. Characterization of the role of TsrE in the formation of **1**. (A) *In vitro* assays of TsrE activity. Substrate **3** was prepared *in situ* using TsrA-catalyzed transamination reaction in which the ammonia acceptor is phenylpyruvate. $FADH_2$ was produced by reducing FAD with NADPH in the presence of Fre. i, authentic **1**; ii, transforming **3** with cofactor FAD; iii, iv and v, transforming **3** with cofactor $FADH_2$ in the absence (negative control) and presence of TsrE (aerobically and anaerobically), respectively; vi and vii, transforming IPA and **2**; and viii, transforming **3** in the absence of halide ions. (B) TsrE-catalyzed selective hydroxylation of **6** to **7** (top, the atom ^{18}O is indicated in red) upon HPLC analysis (bottom). i, authentic **7**; and ii and iii, transforming **6** in the absence (negative control) and presence of TsrE.

process, a shunt product was proposed to arise from the decarboxylation of an uncharacterized oxygenated intermediate during the formation of **1**. Two related 2-methyl-indole derivatives, α-hydroxyl carboxylate **4** from Route 2 and lactone **5** from Route 4 (Figure 3), were then synthesized and served as standards for structural determination (Supplementary Results). High-performance liquid chromatography with mass spectrometric detection (HPLC-MS) indicated that this product differs from **4**. Instead, it is identical to **5**, leading to a hypothesis that TsrE-catalyzed oxygenation directly targets the pyrrole part of the indole ring. Furthermore, TsrE was found to hydroxylate 2-methyl-3-propyl-indole (**6**, a synthetic mimic of **3**, $[M + H]^+$ *m/z*: calcd. 174.1277 for $C_{12}H_{16}N$, found 174.1278) at C3 and trigger a double bond shift within the pyrrole ring, yielding an imine product, **7** ($[M + H]^+$ *m/z*: calcd. 190.1226 for $C_{12}H_{16}NO$, found 190.1226) (Figure 4B). Chiral separation of **7** on HPLC and subsequent electronic circular dichroism (ECD) calculation validated the stereoselectivity of TsrE (Figures S6 and S7), which produced a (3*S*)-isomer with an enantiomeric excess (*ee*) value of up to 96%. Using $^{18}O_2$ in the TsrE-catalyzed hydroxylation of **6** led to the production of single ^{18}O-labeled **7** ($[M + H]^+$ *m/z*: calcd. 192.1269 for $C_{12}H_{16}N^{18}O$, found 192.1265). Consequently, these observations provide strong evidence that TsrE can catalyze the selective oxygenation of **3** and generate (*S*)-3-(3-hydroxy-2-methyl-3H-indol-3-yl)-pyruvic acid (**8**) (Figure 3), which exists in an imine form and appears to be too unstable to be detected at 30 °C.

By lowering the incubation temperature, we slowed the transformation of the precursor **2** that involves both TsrA and TsrE activities. Remarkably, the reaction proceeding at 4 °C did accumulate **8** ($[M - H]^-$ *m/z*: calcd. 232.0615 for $C_{12}H_{10}NO_4$, found 232.0612), which results from 3-hydroxylation (or **8′** from 2,3-epoxidation, an oxygenation alternative that cannot be completely excluded at this time because the facile tautomerization between **8** and **8′** could occur in solution), as judged by careful HPLC-high resolution (HR)-MS and MS/MS analyses (Figure S8). At this temperature, using $^{18}O_2$ in the reaction mixture produced single ^{18}O-labeled **8** (or **8′**, $[M - H]^-$ *m/z*: calcd. 234.0658 for $C_{12}H_{10}NO_3{}^{18}O$, found 234.0659), consistent with the notion that TsrE-mediated indole ring expansion is initiated by a cryptic selective oxygenation of **3** rather than dehydrogenation (Route 1) or halogenation (Route 3). These analyses revealed a set of related derivatives, further supporting the following conversion process toward the formation of **1** through Route 4 (Figures 3 and S8). Intermediate **8** (or **8′**), which possesses a highly reactive pyrrole imine ring, could be

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readily hydrated to generate **9** ($[M - H]^-$ *m/z*: calcd. 250.0721 for $C_{12}H_{12}NO_5$, found 250.0721; and calcd. 252.0763 for $C_{12}H_{12}NO_4{}^{18}O$, found 252.0757), an extremely unstable 2,3-dihydroxylated pyrroline intermediate. Breaking the N1−C2 bond of **9** would result in intermediate **10** ($[M - H]^-$ *m/z*: calcd. 250.0721 for $C_{12}H_{12}NO_5$, found *m/z* 250.0720; and calcd. 252.0763 for $C_{12}H_{12}NO_4{}^{18}O$, found 252.0766). Ring expansion relies on the condensation occurring between the released amino group and the α-keto group of **10** to produce a recyclized intermediate, which would undergo dehydration/aromatization to form quinoline ketone **1**. Alternatively, intermediate **8** could undergo the oxidative decarboxylation of its carbon side chain (particularly in the presence of O_2 and $FADH_2$, which react to form the oxidizing adduct FAD-4a-OOH), and the subsequent nucleophilic attack of the newly generated carboxylate group onto C2 would furnish an indole lactone to produce shunt product **5** (Figure S9). The production of double ^{18}O-labeled **5** ($[M - H]^-$ *m/z*: calcd. 208.0751 for $C_{11}H_{12}NO^{18}O_2$, found 208.0749) in the presence of $^{18}O_2$ supported this conversion. The oxidative decarboxylation of the carbon side chain appears to compete with the process of indole ring expansion, and the yield of **5** evidently increased with the decrease in incubation temperature (Figure S5).

In conclusion, we demonstrate that the formation of quinoline ketone intermediate **1** in TSR biosynthesis involves the tandem activities of TsrA and TsrE for processing the precursor 2-methyl-L-Trp (**2**) through reversible transamination and selective oxygenation. TsrA, a flexible PLP-dependent aminotransferase, activates C2′ of the carbon side chain through transamination to generate α-keto acid **3**. TsrE, a flavin-dependent protein, selectively oxygenates **3** to produce a highly reactive indole imine, which then undergoes a rearrangement process through intermediates **9** and **10** for ring expansion. More likely, this unstable intermediate is C3(*S*)-hydroxylated **8** according to data presented here and a similar intermediate resulting from unusual FAD-dependent protein activity, which initiates indoloterpenoid cyclization through a selective C3-hydroxylation of indole in xiamycin biosynthesis.[14] The subsequent steps in the biosynthetic pathway of TSR include a selective ketone reduction to generate a (12*S*)-quinoline alcohol,[9] which could then be activated, epoxidated and appended onto the core peptide part of the precursor peptide prior to the formation of the thiopeptide framework.[15] The closure of the side-ring system follows the removal of the leader peptide sequence, and both are post-thiopeptide PTMs that depend on the dual activity of TsrI, an unusual α/β-hydrolase fold protein.[16]

Indole ring expansion was previously observed during the biosynthesis of quinoxaline and quinolone-containing natural products.[17] In that process, the 2,3-dioxygenation of an L-Trp precursor cleaves the C2−C3 bond within the indole ring, followed by deformylation and transamination to release both the amino and α-keto groups between which condensation occurs to form an expanded ring. In contrast, in the biosynthesis of TSR, quinoline formation is initiated by a selective hydroxylation of a 2-substituted L-Trp derivative and subsequent hydrolysis to break the indole ring (by cleaving the N1−C2 bond), and ring reconstruction (by forming the N1−C2′ bond) immediately couples with ring destruction. Notably, the formation of cinchona alkaloids, such as quinine, an antimalarial agent that has been widely used in the clinic for nearly two centuries, also requires N1−C2 bond cleavage and transformation of a 2-substituted L-Trp derivative into a quinoline product.[18] Determining whether these cinchona alkaloids share a similar biochemical mechanism with TSR-type thiopeptides for indole ring expansion would be extremely interesting.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b05337.

Experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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