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Brief Communication

Insights into Quinaldic Acid Moiety Formation in Thiostrepton Biosynthesis Facilitating Fluorinated Thiopeptide Generation

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SUMMARY

Thiostrepton (TSR), often referred as to a parent compound in the thiopeptide family, is a bimacrocyclic member that features a quinaldic acid (QA) moiety-containing side ring appended to the characteristic core system. QA biosynthesis requires an unusual ring-expanding conversion, showing a methyl transfer onto and a rearrangement of the indole part of L-tryptophan to give a quinoline ketone. Herein, we report that the process involves the activities of the radical methyltransferase TsrT, aminotransferase TsrA, dehydrogenase TsrE, and cyclase TsrD. TsrU, a stereospecific oxidoreductase, catalyzes the further conversion of the ketone into an enantiomerically pure *S*-alcohol. Elucidation of this chemistry, which is common in the biosynthesis of a number of thiopeptides sharing a QA side ring system, facilitates analog generation, as shown by the achievement of region-specific fluorination of thiostrepton with the improved antibacterial activity.

INTRODUCTION

Thiopeptides, a growing class of sulfur-rich and highly modified polythiazolyl antibiotics, have long been appreciated for their imposing architectures, phenomenal bioactivities, and unusual modes of actions (Bagley et al., 2005; Hegde et al., 2011; Hughes and Moody, 2007; Nicolaou et al., 2009). Many members in this family are active against various drug-resistant bacterial pathogens (Bagley et al., 2005). More recently, certain thiopeptides have been found with the antiproliferative activity in human cancer cells (Bhat et al., 2008; Bowling et al., 2008; Nicolaou et al., 2005), further motivating the interest in new analog development to overcome their physical drawbacks (e.g., poor water solubility) for clinical use. The common paradigm of thiopeptide biosynthesis features a ribosomally synthesized precursor peptide and conserved posttranslational modifications (Ding et al., 2010; Engelhardt et al., 2010; Kelly et al., 2009; Liao et al., 2009; Morris et al., 2009; Wang et al., 2010; Wieland Brown et al., 2009; Young and Walsh, 2011; Yu et al., 2009). Sequence permutation by engineering the precursor peptide therefore proved to be an effective strategy to access the structural diversity in skeleton (Acker et al., 2009; Bowers et al., 2010; Li et al., 2011a and 2011b), for which there is a challenge to the chemical synthesis-based approaches.

In addition to the characteristic framework, many thiopeptides are bimacrocyclic, such as thiostrepton (sometimes called thiostrepton A or A_1. TSR, **1**) and nosiheptide (NOS, **2**) (Bagley et al., 2005), and contain a side ring system whose formation is independent of the precursor peptide (Frenzel et al., 1990; Houck et al., 1988; Mocek et al., 1993; Priestley et al., 1996; Smith et al., 1993; Zhou et al., 1989). The functionalization shares L-tryptophan as a common substrate but can proceed in completely different ways, to afford variable groups as exemplified by the quinaldic acid (QA) moiety of **1** and the indolic acid (IA) moiety of **2** (Figure 1; Figure S1 available online). For IA generation, we have recently characterized NosL in **2** biosynthetic pathway as a radical *S*-adenosylmethionine (SAM) 3-methyl-2-indolic acid (MIA) synthase (Zhang et al., 2011a, 2011b), which alone catalyzes a radical-mediated, unusual fragmentation-recombination to produce MIA. By contrast, QA formation in **1** biosynthesis involves a methylation of the indole and, in particular, a key ring expansion of the methylated indole to a quinoline group, presumably to give 4-acetylquinoline-2-carboxylic acid (**3**) and 4-(1-hydroxyethyl)-quinoline-2-carboxylic acid (**4**) after reduction (Figure 2) (Frenzel et al., 1990; Mocek et al., 1993; Priestley et al., 1996; Smith et al., 1993; Zhou et al., 1989). The process may require multiple enzymes, consistent with **1** biosynthetic gene cluster that harbors the candidates potentially for methylation, desamination, oxidoreduction, and ring opening and recyclization (Kelly et al., 2009; Liao et al., 2009). We focus here on characterization of the roles of **3** and **4** as the intermediates and determination of the specific gene products in QA biosynthesis, including a methyltransferase (TsrF or TsrT), an aminotransferase (TsrA), a dehydrogenase (TsrE), a cyclase (TsrD), and a reductase (TsrU) (*tsrF*, *tsrT*, *tsrA*, *tsrE*, *tsrD*, and *tsrU* in this study, according to Liao et al., 2009, were also named *tsrP*, *tsrM*, *tsrV*, *tsrQ*, *tsrS*, and *tsrN* in Kelly et al., 2009, respectively), toward incorporation of the distinct chemistry into the rational application of biotechnology to develop thiopeptides varying in QA functionalization.

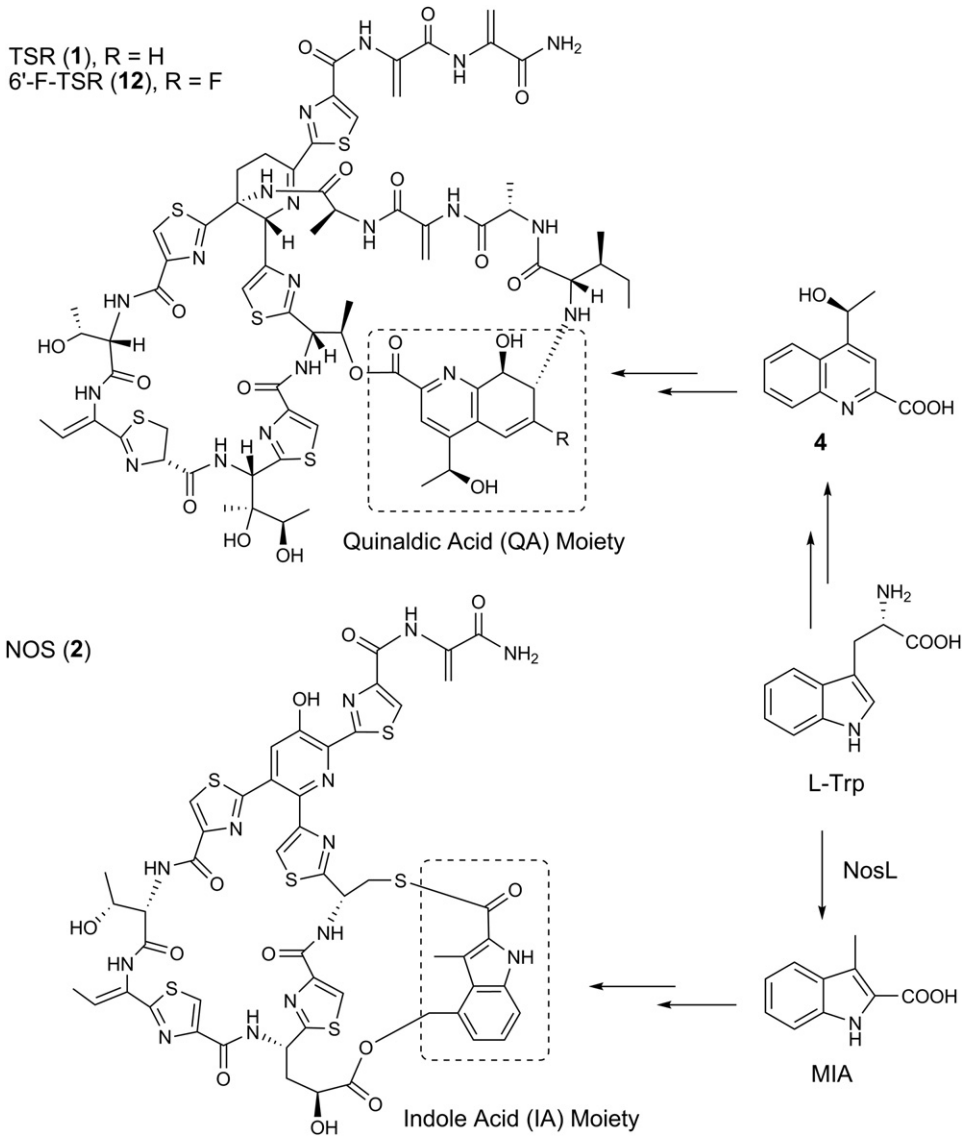

Figure 1. Structures of Bimacrocyclic Thiopeptides TSR (1), 6'-F-TSR (12), and NOS (2) and Their Side Ring System Formation
The dashed rectangles indicate the QA moiety of **1** and the IA moiety of **2**, respectively.
See also Figure S1.

RESULTS AND DISCUSSION

In Vivo Establishment of the Genes Involved in QA Biosynthesis

We began with investigating the relevance of *tsrF*, *tsrT*, *tsrA*, *tsrE*, *tsrD*, and *tsrU* to **1** biosynthesis by gene inactivation in the producing strain *Streptomyces laurentii* ATCC 31255. Each of the mutations was carried out by in-frame deletion, to exclude the polar effects on downstream gene expression. The resulting genotypes have been confirmed by Southern hybridization (Figures S2A–S2F). Upon HPLC-MS analysis (Figure 3A and Figure S2G), inactivation of most of the genes, including *tsrF*, *tsrT*, *tsrA*, *tsrE*, and *tsrU*, which correspond to generation of the resulting mutant strains SL1101, SL1102, SL1103, SL1104, and SL1106, respectively, completely abolished **1** production, whereas the *tsrD*-inactivated strain SL1105 still produced **1**, the yield of which, however, dramatically decreased to 8.7% of that of the wild-type strain (Figure S2G). These results clearly validated the involvement of the genes in **1** biosynthesis.

Structurally, **1** falls into a group of thiopeptides (as the series *a*, *b*, and *c* members) sharing a QA-containing side ring system (Figure S1A) (Bagley et al., 2005). Comparative analysis of the *tsr* gene cluster with those for other thiopeptides, such as monomacrocyclic members and bimacrocyclic members containing a IA moiety, revealed that *tsrF*, *tsrT*, *tsrA*, *tsrE*, *tsrD*, and *tsrU* are specific, because their counterparts were found only in the available siomycin biosynthetic pathway (Figure 2A) (Liao et al.,

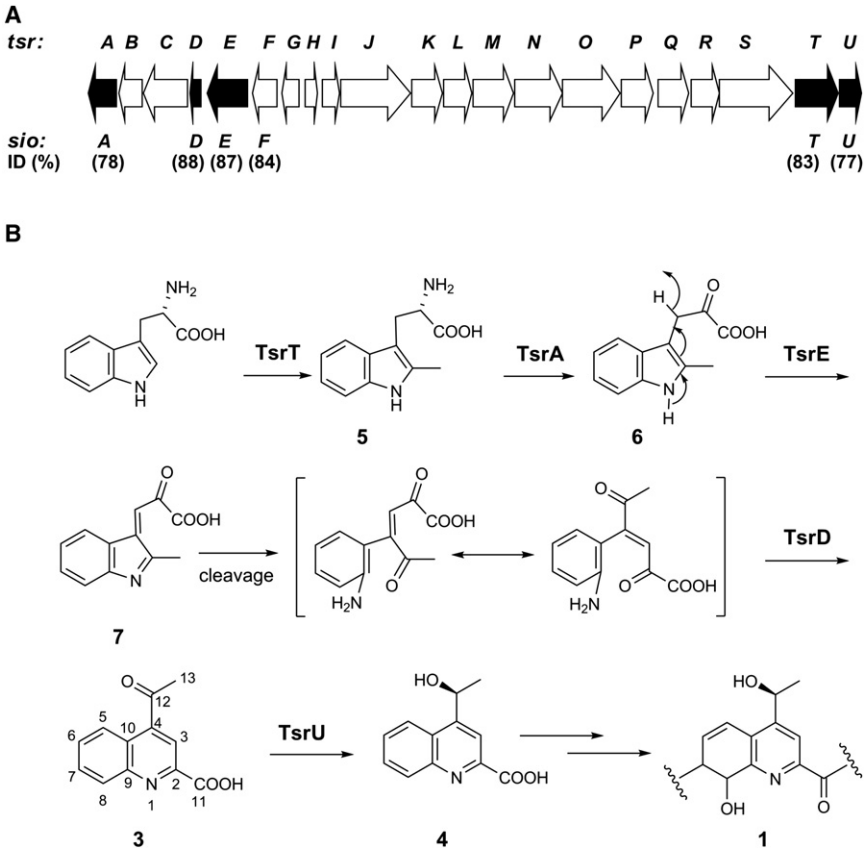

Figure 2. Gene Cluster and Biosynthetic Pathway for QA Formation

(A) Organization of the TSR biosynthetic genes. The genes *tsrA*, *tsrD*, *tsrE*, *tsrT*, and *tsrU* (along with their counterparts *sioA*, *sioD*, *sioE*, *sioT*, and *sioU* in siomycin biosynthesis), labeled in black color, are relevant to QA biosynthesis. ID, sequence identity.

(B) Functional assignment of the corresponding enzymes TsrA, TsrD, TsrE, TsrT, and TsrU in the biostransformation of L-tryptophan into QA.

2009). To confirm the functional identity, we selected the **1**-nonproducing strain SL1106 (by inactivating *tsrU* that was confirmed to be involved in QA formation below) for complementation by in *trans* expression of the native gene *tsrU* (to give SL1107) and heterologous gene *sioU* (to give SL1108), respectively. Indeed, the production of **1** was restored in both the recombinant strains SL1107 (Figure 3B, IV) and SL1108 (Figure 3C, IV). The gene interchangeability supported the generality of QA formation in the biosyntheses of these series thiopeptides.

To experimentally determine their participation, we synthesized ketone **3**, which was then fed to the corresponding mutant strains to examine **1** production. Supplementation of **3** into SL1101 (*tsrF* mutation) failed to produce **1** (Figure 3B, II), therefore excluding the role of *tsrF* in QA biosynthesis. In contrast, feeding of **3** to SL1102 (*tsrT* mutation), SL1103 (*tsrA* mutation), SL1104 (*tsrE* mutation), and SL1106 (*tsrD* mutation), respectively, restored **1** production to a yield comparable to that of the wild-type strain (Figure 3B, III and Figure S2G, I–III), unambiguously confirming that they are essential for QA formation. Thus, the synthesis of **3** from L-tryptophan can proceed with the following enzymatic reactions (Figure 2B): (1) TsrT, a radical SAM/methylcobalamin-dependent methyltransferase, may catalyze C-2 methylation to give **5**; (2) TsrA, a pyridoxal phosphate (PLP)-dependent aminotransferase, is responsible for desamination of **5** to provide the α-keto acid **6**; (3) TsrE, a putative dehydrogenase, may convert the enamine indole of **6** into the imine of **7** via a dehydrogenation; and (4) TsrD, a polyketide cyclase-like protein, could be involved in hydrolysis of the imine indole ring and subsequent addition of the resulting amino group onto α-keto to afford **3**. In an imine indole form, **7** is unstable and may spontaneously undergo a ring opening/recyclization to give **3**, consistent with the fact that the *tsrD* mutant strain produced a trace of **1** (Figure S2G, III). Together with the recent validation of the aminotransferase activity of TsrA by using 3-indolylpyruvic acid as the amino acceptor (Kelly et al., 2009), these findings provided insights into the proposal with respect to **3** as an intermediate (Priestley et al., 1996). On the other hand, the feeding results provided the evidence to differentiate the two methyltransferases TsrT and TsrF that are distinct in mechanism. TsrT likely participates in C-2 methylation of L-tryptophan via an uncovered radical-mediated way, in line with previous labeling study showing an unusual methyl transfer from SAM with a net retention of stereochemistry (Zhou et al., 1989). TsrF, belonging to a large family of SAM-dependent proteins known to catalyze the nucleophilic attack of the substrates to the methyl of SAM by a S_N2 displacement, may be responsible for the newly identified C-terminal methylation of the precursor peptide that is cryptic in **1** biosynthesis (Liao and Liu, 2011). The fact that the *tsrF* mutant strain SL1101 lost the ability to produce **1** implied that this C-terminal methylation is critical for downstream peptide modifications to generate a **1**-like, methyl ester intermediate, which undergoes a deesterification-amidation process for maturation to eventually afford **1**.

In Vitro Characterization of TsrU as a Stereospecific Oxidoreductase

For further conversion of ketone **3** into alcohol **4**, we then explored the in vitro reaction catalyzed by the reductase TsrU. First, TsrU was overproduced in a 6-His-tagged form and purified from *Escherichia coli* B12(DE3) to homogeneity (Figure S3A). With saturated NADPH, effective conversion of **3** to a distinct product occurred in the presence of active TsrU, in contrast to the negative control using the inactivated enzyme as the catalyst (Figure 4A, III and IV). This compound was determined as **4** on the basis of the same retention time of HPLC, UV-vis absorptions and HR-MS data with the synthetic standard. The steady-state

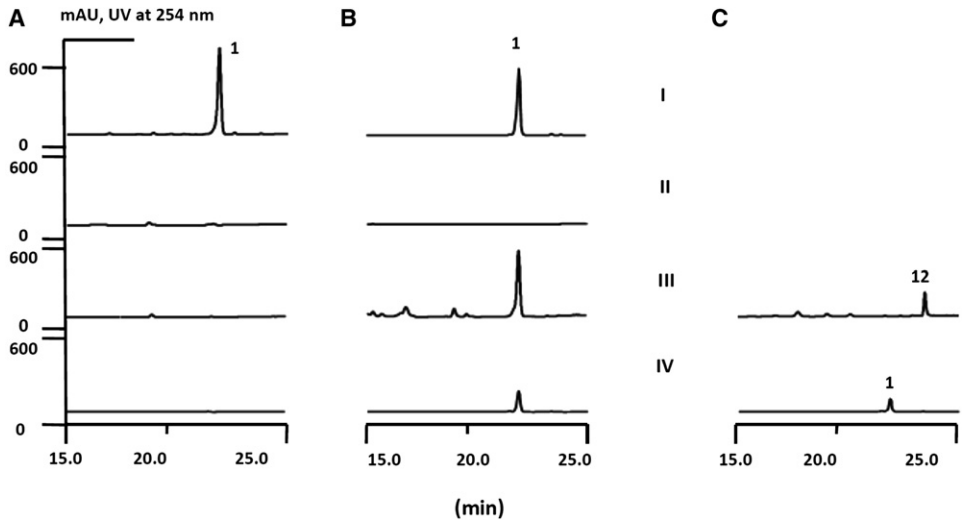

Figure 3. Product Examination in *S. laurentii*

(A) Abolishment of TSR production, with authentic TSR as the control (I), as exemplified by inactivating *tsrF* (SL1101, II), *tsrT* (SL1102, III), and *tsrU* (SL1106, IV), respectively.

(B) Restoration of TSR production, with the wild-type strain as the control (I), as exemplified by feeding of compound **3** individually into the mutants SL1101 (II) and SL1102 (III), and in SL1107 (a SL1106 derivative carrying *tsrU*, IV).

(C) Production of 6'-F-TSR (**12**) by feeding of compound **10** into SL1102 (III) and of TSR in SL1108 (a SL1106 derivative carrying *sioU*, IV).

See also Figure S2 and Table S1.

kinetic parameters were measured at the optimal pH of 7.0 (Figures S3B and S3C, I), showing the conversion with a K_m value of 670 ± 50 μM for **3**, a K_{cat} value of 202.8 ± 6.5 min^{-1}, and a K_{cat}/K_m value of 0.3. Second, the TsrU-catalyzed reverse reaction was accordingly examined with **4** as the substrate under the condition where NADPH was replaced with $NADP^+$, showing a visible production of **3** (Figure 4A, V); however, the yield (by a ~15.6% conversion of **4** with a K_m value of 90.0 ± 8.9 μM for **4**, a K_{cat} value of 17.4 ± 0.5 min^{-1}, and a K_{cat}/K_m value of 0.2; Figure S3C, II) were much lower than that of the forward reaction. These findings demonstrated that TsrU acts as a $NADPH/NADP^+$-dependent ketone-alcohol oxidoreductase on the conversion, the equilibrium of which apparently favors alcohol **4** rather than ketone **3** as the product (Figure 4B). Finally, the TsrU-catalyzed forward reaction was scaled up for product **4** isolation. This product, displaying a specific value of optical rotation at $[\alpha]_D^{32} = -60.32$ (c = 0.1, CH_3OH), is a pure enantiomer, showing a absolute configuration as *S* (same as that in **1**) of the C-12 hydroxylethyl group upon NMR analysis of the Mosher ester derivatives **9a** and **9b** from the **4**-methylated product **8** (Figure S3D and Supplemental Experimental Procedures). Consequently, these results confirmed that TsrU catalyzes a stereospecific ketoreduction by converting ketone **3** into the enantiomerically pure *S*-alcohol **4** in QA formation.

TsrU belongs to a large, functionally diverse family of short chain dehydrogenases/reductases (SDR) (Kavanagh et al., 2008), which share a conserved a motif TGXXXG characteristic for NADP(H)-binding and a sequence YXXXK to provide the active site (Figure S3E), and afford a hydroxyl group via the transfer of a 4-pro-*S* hydride from nicotinamide to ketone and relay of a proton involving the conserved residue Tyr. To test the substrate tolerance of TsrU for region-specific fluorination of QA, we synthesized the compound **10**, by substitution of a fluorine atom at C-6 of **3** (Figure 4B). Intriguingly, TsrU catalyzed the C-12 ketoreduction more effectively to generate the product **11** (Figures 4A, VI and VII and 4B), with a comparable K_m value of 440 ± 80 μM for **10**, an improved K_{cat} value of 651.9 ± 29.0 min^{-1}, and a K_{cat}/K_m value of 1.5 that is 4-fold higher than that for **3** (Figure S3C, III). A likely explanation is that the increase of the electrophilicity at C-12 facilitates the nucleophilic attack of the hydride from nicotinamide, by introducing fluorine, similar to hydrogen in size to minimize the structural change, as a potent electron-withdrawing group.

Generation of a Fluorinated Thiostrepton

The fact that TsrU tolerates **10** thus promoted us to feed it into the **1**-nonproducing, *tsrT* mutant strain SL1102, to determine whether the resulting alcohol **11** can be further incorporated into the TSR biosynthetic machinery for fluorinated thiopeptide generation. A product was clearly observed (Figure 3C, III), showing a yield approximately 20% of that for **1** production in the wild-type strain. This compound was purified and eventually characterized to be **12** (Figure 1), a thiopeptide containing a fluorine atom at C'-6 of the QA ring, on the basis of HR-ESI-MS, ^{19}F NMR, and 1D and 2D NMR analyses (Figure 4C and Figure S3F). Remarkably, the 6'-fluoro-substitution has a positive effect on the antibacterial activity, because the bioassays against the test strain *Bacillus subtilis* displayed a minimum inhibitory concentration (MIC) at 0.005 μg/ml for **12**, 3-fold higher than that of **1** (0.020 μg/ml).

SIGNIFICANCE

We have uncovered a potentially common pathway for forming the QA moiety present in the series *a*, *b*, and *c*

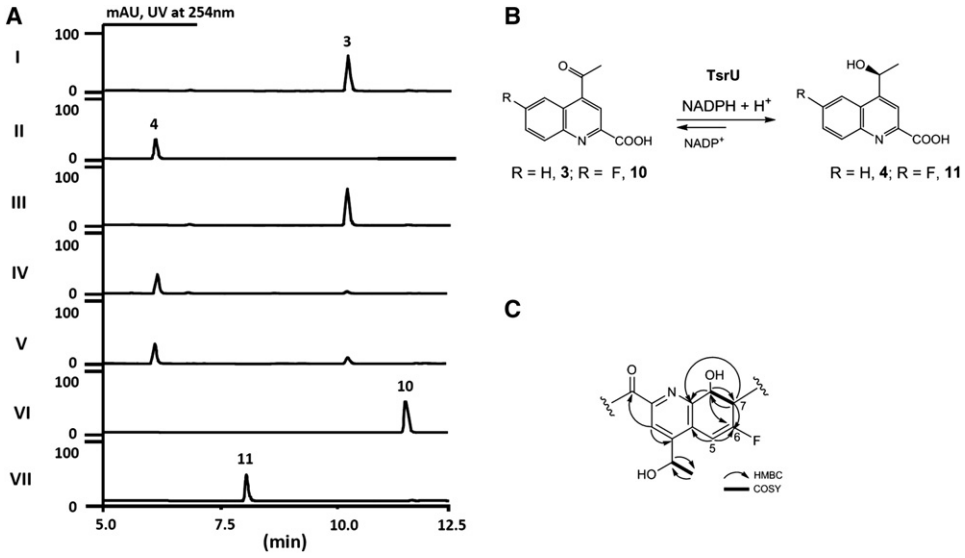

Figure 4. Characterization of TsrU

(A) HPLC analysis for product examination with authentic **3** (I), **4** (II), and **10** (VI) as the controls. The forward conversion of **3** to **4** in the presence of TsrU inactivated by heating (III) and of active TsrU (IV), respectively; the reverse conversion of **4** to **3** in the presence of active TsrU (V); and the transformation of the C-6 fluorinated ketone **10** (VI) to the corresponding alcohol **11** by TsrU (VII).

(B) TsrU-catalyzed stereospecific oxidoreduction.

(C) Selected HMBC and 1H-1H COSY correlations of the QA moiety of **12**.

See also Figure S3 and Table S1.

thiopeptides, which features a methyl transfer onto and a rearrangement of the indole part of L-tryptophan. It involves multiple reactions catalyzed by a set of specific enzymes to reach the ring-expanded quinoline ketone, including the SAM radical protein TsrT, rather than the typically SAM-dependent protein TsrF, for C-2 methylation. TsrU, a stereospecific oxidoreductase, catalyzes the further conversion of the ketone to the enantiomerically pure *S*-alcohol. TsrU tolerates the fluorinated analog as the substrate, allowing for production of a region-specifically fluorinated thiopeptide that showed improved antibacterial activity. This application, together with that for 5′-fluoro-NOS production, enabled by elucidating the distinct chemistry to process L-tryptophan, provides an effective strategy for structural diversity on the side ring systems of the bimacrocyclic thiopeptides.

EXPERIMENTAL PROCEDURES

All details about the Experimental Procedures and materials used in this study are given in accompanying Supplemental Information. They can be found at the following URL: doi:10.1016/j.chembiol.2012.02.008.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, two tables, Supplemental Experimental Procedures, and can be found with this article online at doi:10.1016/j.chembiol.2012.02.008.

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