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Characterization of *TrdL* as a 10-Hydroxy Dehydrogenase and Generation of New Analogues from a Tirandamycin Biosynthetic Pathway

Xuhua Mo,[†,‡] Hongbo Huang,[†] Junying Ma,[†] Zhongwen Wang,[†] Bo Wang,[†] Si Zhang,[†] Changsheng Zhang,[†] and Jianhua Ju*[,†]

CAS Key Laboratory of Marine Bio-resources Sustainable Utilization, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, P.R. China, and Graduate University of Chinese Academy of Sciences, 19 Yuquan Road, Beijing, 110039, P.R. China

jju@scsio.ac.cn

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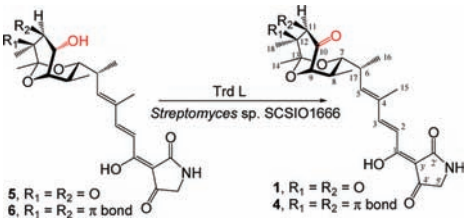
ABSTRACT



***TrdL*, encoding a flavin-dependent oxidoreductase in the tirandamycin gene cluster, was inactivated to afford a Δ*trdL* mutant, the fermentation of which yielded a new intermediate, tirandamycin E (5), and an additional early intermediate, tirandamycin F (6), if XAD-16 resin was introduced. *TrdL* was overexpressed in *E. coli*, and the protein was shown to efficiently catalyze the transformations from 5 to tirandamycin A (1) and from 6 to tirandamycin D (4), demonstrating its function as a 10-hydroxy dehydrogenase.**

The tirandamycins belong to a small group of tetramic acid natural products featured with a 2,4-pyrrolidinedione ring system.[1] Compounds containing a tetramic acid structural unit have been demonstrated to exhibit extensive biological activities such as antibacterial, antiviral, antiulcerative, cytotoxicity, mycotoxicity, and antitumor.[1,2]

In 1971, tirandamycin A (**1**) was first isolated from the terrestrial bacterium *Streptomyces tirandis*, and subsequently its absolute stereochemistry was determined by X-ray diffraction of a *p*-bromophenacyl ester of tirandamycic acid in 1973.[3,4] **1** and its other congeners tirandamycins B (**2**), C (**3**), and D (**4**) were later reisolated or discovered from *Streptomyces flaveolus*,[5] marine-derived *Streptomyces* sp. 307−9,[6] and *Streptomyces* sp. SCSIO 1666 originating from South China Sea marine sediment in our laboratory.[7] We have reported that by addition of XAD-16 resin into the *Streptomyces* sp. SCSIO 1666 fermentation, the yield of **1** was dramatically improved 250-fold, suggesting that **2** serves as the final oxidized product in the tirandamycin biosynthesis.[7]

Tirandamycins are bacterial RNA polymerase (RNAP) inhibitors, and the mode of action is to inhibit both chain initiation and chain elongation during the transcriptional process.[8] In addition to the dienoyltetramic acid moiety,

[†] South China Sea Institute of Oceanology.
[‡] Graduate University of Chinese Academy of Sciences.

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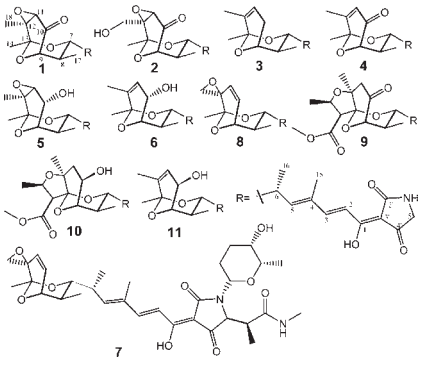

Figure 1. Representatives of tetramic acid compounds containing a bicyclic keto structure: tirandamycin A (**1**), tirandamycin B (**2**), tirandamycin C (**3**), tirandamycin D (**4**), tirandamycin E (**5**), tirandamycin F (**6**), streptolidigin (**7**), tirandaligin (**8**), BU-2313B (**9**), nocamycin II (**10**), and tirandamycin C2 (**11**).

tirandamycins possess another intriguing structure element, the bicyclic ketal skeleton, which could also be found in other antimicrobial antibiotics such as streptolydigin (**7**), tirandaligin (**8**), BU-2313B (**9**), and nocamycin II (**10**)[1] (Figure 1). Recently, cocrystal complexes of **7** with *E. coli* and *Thermus thermophilus* RNAP have unveiled the structural basis of inhibition of bacterial RNA polymerase by binding the site adjacent to an RNAP active site and stabilizing the inactive conformation.[9] The unique structural features and mode of action of this class of compounds have aroused the attention of synthetic chemists. Total synthesis of **1**, **2**, **7** and efforts toward **8** have been reported.[10,11]

The biosynthetic gene cluster of tirandamycins has been cloned and sequenced independently from *Streptomyces* sp. 307-9[12] and SCSIO1666 in our laboratory.[13] The tirandamycin gene cluster in *Streptomyces* sp. SCSIO1666 consists of 15 genes which encode three type I PKSs (TrdAI, AII, AIII), one NRPS (TrdD), one phosphopantetheinyl transferase (TrdM), one Type II thioesterase (TrdB), one FAD-dependent oxidoreductase (TrdL), one cytochrome P450 monooxygenase (TrdI), three proteins related to regulation (TrdHJK), and four proteins with unknown function (TrdCEFG).[13] We have reported that TrdH acts as a positive regulator and TrdK acts as a negative regulator and inactivation of *trdI* led to accumulation of **3** and a new trace product tirandamycin C2 (**11**).[13] The goal of this study was to characterize the function of the *trdL* gene in the tirandamycin postmodification process. Herein

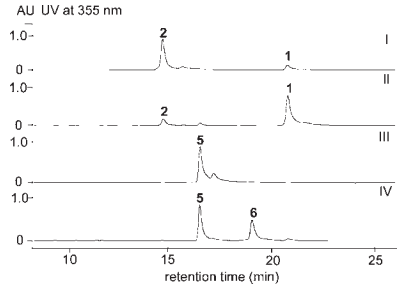

Figure 2. HPLC analyses of fermentation extracts of: (I) wild type, (II) wild type supplemented with 2% resin, (III) Δ*trdL* mutant strain, and (IV) Δ*trdL* mutant strain supplemented with 2% resin. Numbers above the peaks repesent the compounds. See Figure 1 for structures of **1**, **2**, **5**, and **6**.

we report (i) gene inactivation of *trdL* leading to accumulation of a key biosynthetic intermediate tirandamycin E (**5**); (ii) fermentation of the Δ*trdL* mutant with adsorbent resin addition resulting in accumulation of an additional new intermediate, tirandamycin F (**6**); (iii) *in vitro* biochemical characterization of TrdL; and (iv) a proposed post-tailoring biosynthetic pathway based on the intermediates produced by the *trdL* gene disruption mutant.

Bioinformatics analysis suggested that *trdL* encodes a putative flavin-dependent oxidoreductase and shares 59% identity with StfE, an uncharacterized putative dehydrogenase in the gene cluster of anthracycline antibiotic steffimycin.[14] Analysis of the conserved domains of the *trdL* product reveals that it possesses a Rossmann fold dinucleotide-binding motif (GXXGXXXG)[15] responsible for binding the adenosine moiety of FAD and a berberine-bridge enzyme (BBE) motif[16] for covalent attachment of FAD in the N-terminus (see Figure S1 in the Supporting Information, SI). To probe the exact role of *trdL*, it was inactivated by in frame replacement with an apramycin resistance gene cassette using a PCR-targeting strategy according to the established methods.[13] The double-crossover mutant was selected by apramycin^{R}kanamycin^S phenotype and then verified by PCR (see SI).

The Δ*trdL* mutant and wild type strain as a control were fermented, extracted with butanone, and analyzed by HPLC-UV (Figure 2, III, I). Since the addition of adsorbent resin has been proved efficient in capturing earlier biosynthetic intermediates or derivatives,[17,7] we also fermented the Δ*trdL* mutant and the wild type strain with the addition of 2% XAD-16 resin. After fermentation, the culture broth was centrifuged; the mycelium and resin were extracted with MeOH and then analyzed by HPLC-UV (Figure 2, IV, II). The results revealed that the Δ*trdL* mutant accumulated a new tirandamycin analog **5**, and one more analog **6** if the resin was added during fermentation, both

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compounds exhibiting similar characteristic UV absorptions to those of **1** and **2**. In order to solve the two structures, an 8-L scale fermentation of the Δ*trdL* mutant was conducted and led to the purification of **5** and **6** (see SI).

Compound **5** has a molecular formula of $C_{22}H_{29}NO_7$ as determined by HR-ESI-MS, two mass units smaller than that of **1**. A full set of 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC, HMBC and NOESY) NMR spectra of **5** was acquired, which allowed us to completely assign its ^{1}H and ^{13}C signals (Table S1, SI). In comparison with that of **1**, the C-10 keto group signal in **5** was missing, and in turn, an oxygen-bearing carbon signal at δ 67.7 (C-10) was present, indicating **5** had a hydroxyl group substituted at the C-10 position. Consistently, the ^{1}H NMR spectrum and splitting patterns of **5** were similar to those of **1**, except for the appearance of one oxygen-bearing H-10 signal at δ 4.21 (d, J = 7.0 Hz), and the splitting pattern for H-9 changed to a triplet at δ 3.93 (t, J = 6.0 Hz). The presence of the C-10 hydroxy group in **5** was further confirmed by COSY correlations between H-9/H-10/H-11 and HMBC correlations from H-10 to C-8, C-9, C-11, and C-12 and from H-9 to C-7, C-8, C-10, and C-11. Inspection of other 1D and 2D NMR data confirmed **5** had other substructures identical to those of **1**. Since the absolute stereochemistry of **1** was determined,[4] to solve the absolute configuration of the C-10 hydroxy group in **5**, conformation analysis using the Chem3D MM2 energy minimization model was conducted. The model suggested that when the C-10 hydroxy group was in the α position, the C-10 OH and C-17 methyl group were in 1,3-syn disposition, whereas when the C-10 hydroxy group was in the β position, the distance between C-10 OH and C-17 methyl group was much greater. The former scenario was consistent with the observed dramatic downfield shift of H-17 by 0.32 ppm when compared with those of **1**. Hence, the hydroxy group at C-10 in **5** was determined to be in an α configuration (10*R*), which was supported by NOESY correlations between H-8/H-9/H-10 and H-10/H-11/H-18.

Compound **6** has a molecular formula of $C_{22}H_{29}NO_6$ as determined by HR-EI-MS, 16 mass units greater than that of **3**. A full set of 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC, HMBC, and NOESY) NMR spectra data of **6** was also acquired, which led to full assignments of its ^{1}H and ^{13}C signals (Table S1, SI). Careful analyses of the ^{1}H and ^{13}C NMR data of **6** and comparison with those of **3** revealed that **6** also has a hydroxy group substituted at C-10 (δ 69.3) rather than a keto group in **3**, which was supported by the COSY correlations between H-9/H-10/H-11 and HMBC correlations from H-10 to C-8, C-9, C-11, and C-12. As in **5**, the C-10α hydroxy configuration in **6** was suggested by the dramatic downfield shift of H-17 by 0.27 ppm when compared with that of **3**. Hence, **6** is the C-10 epimer of tirandamycin C2 (**11**) that has been isolated in trace amounts from the Δ*trdI* mutant.[13] Comparisons of the

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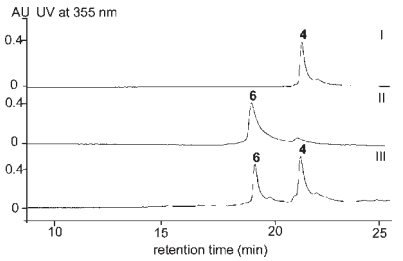

Figure 3. HPLC detection of the stability of **6**: (I) **4** standard, (II) **6** standard, and (III) a methanol solution of **6** stored at room temperature for 60 days. See Figure 1 for structures of **6** and **4**.

^{1}H NMR data of **6** and **11** revealed that the chemical shift of H-10 in **6** appears at 4.65 ppm, whereas in **11** it appears at 3.83 ppm. The downfield shift of H-10 in **6** could be readily interpreted by the 1,3 diaxial effect due to H-10β and the oxygen atom between C-9 and C-13 in a chair form of the six-membered ring. Thus, the 10α (10*S*) configuration in **6** was established.

Interestingly and unexpectedly, we found that **6** was relatively unstable at room temperature. Purified **6** was stored in methanol-d_4 at room temperature in an NMR tube for 2 months, and then it was analyzed by HPLC. An additional peak was observed in this methanol solution (Figure 3, III). This newly appearing compound was isolated and identified as **4** by MS, ^{1}H, and ^{13}C NMR spectral data. This suggests that **6** could be gradually converted to **4** in a methanol solution by O_2 oxidation. Under the same conditions, **5** remains unchanged upon HPLC analysis, indicating **5** was stable. One way to interpret this phenomenon is that the α position of the C-11/C-12 double bond in **6** readily suffered from air oxidation.

Based on the structures of **5** and **6**, we determined that TrdL is responsible for the dehydrogenation of the C-10 hydroxy group to form the keto group. To testify its function *in vitro*, the *trdL* gene was amplified from a cosmid DNA and cloned into a pET-28a(+) vector for overexpression as a soluble N-terminal His_6-tagged protein in *E. coli* BL21 (DE3). The recombinant protein was obtained at a final concentration of 4 mg/mL by using Ni-NTA affinity chromatography (see SI). Purified TrdL was yellow, indicating that it probably binds the cofactor FAD. This was confirmed by UV−vis spectrum determination of TrdL for characteristic FAD absorption (SI, Figure S4). TrdL was denatured by heating for 15 min, resulting in a colorless supernatant and yellow precipitate. No FAD was detected in the denatured supernatant by measuring UV−vis absorption spectrum and through HPLC analysis (SI, Figure S4), demonstrating that FAD was covalently attached to the protein. The activity of TrdL was then tested *in vitro* (see SI). The enzymatic reaction was conducted by mixing **5** individually or **6** individually as a substrate in 50 mM Tris-HCl and a catalytic amount of TrdL. At the same time, the control experiments were also set. Upon incubation at 30 °C for 1 h, **5** was almost all converted to **1** (Figure 4, II), and **6** was almost completely

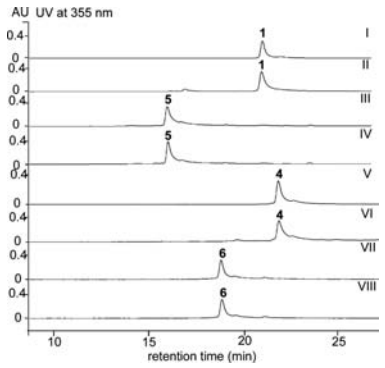

Figure 4. HPLC analyses of products of TrdL assays: (I) tirandamycin A (**1**) standard, (II) incubation of **5** with TrdL, (III) incubation of **5** with no TrdL, (IV) incubation of **5** with boiled TrdL, (V) tirandamycin D (**4**) standard, (VI) incubation of **6** with TrdL, (VII) incubation of **6** with no TrdL, (VIII) incubation of **6** with boiled TrdL. See Figure 1 for structures of **1** and **4**–**6**.

converted to **4** (Figure 4, VI), while no conversions were observed in the control experiments (without TrdL or with boiled TrdL; Figure 4, III, IV, VII, and VIII). The structures of enzymatically converted **1** and **4** were confirmed by analyses of their respective MS and ^{1}H NMR data (SI). It is worthwhile to point out that compound **6** remains unchanged after 1 h in the control experiments. The reactions *in vitro* proceed without addition of any other protein to reoxidize the FAD cofactor, because the molecular oxygen could be utilized to reoxidize $FADH_2$ to FAD.[18]

Based on the two biosynthetic intermediates isolated from the △*trdL* mutant, we proposed a postmodification pathway of tirandamycins in *Streptomyces* sp. SCSIO1666 (Figure S5 in SI). In this proposed pathway, **3** is an earlier intermediate; a yet to be identified oxygenase is responsible for the hydroxylation of C-10 position of **3** to yield **6**. **6** could be oxidized to **5** by another unidentified enzyme through C-11/C-12 epoxidation. Both **6** and **5** could be oxidized to **4** and **1** respectively by TrdL. Finally, another unidentified enzyme is responsible for the hydroxylation of the 18-methyl group to form the final product **2**. It should be noted that the three unidentified oxidation steps could be catalyzed by one or more enzymes including TrdI, the P450 cytochrome monooxygenase.

Although numerous flavoenzymes have been reported in nature, most of the proteins were found to bind the flavin cofactor noncovalently. It was reported that there exists a subclass of flavoenzymes in which the FAD cofactor were covalently attached to the protein through a single covalent bond.[19] In recent years, a type of novel flavoenzymes where the cofactor was bicovalently linked to His and Cys residues as an 8α-*N*1-histidyl and 6-*S*-cysteinyl FAD was discovered, such as AknOx from *Streptomyces galilaeus* and gluco-oligosaccharide oxidase (GOOX) from *Acremonium strictum* whose crystal structures had been solved.[20,21] The same type of bicovalent flavinylation was also disclosed through biochemical and mutational investigations of BBE from *Eschscholzia californica*, Chito (chito-oligosaccharide oxidase) from *Fusarium graminearum*, and HOX (hexose oxidase) from *Chondrus crispus*.[22] Our in-depth bioinformatics analysis of TrdL with the aforementioned bicovalent flavinylation proteins as well as TrdL homologues in Genbank related to secondary metabolites revealed (i) the His and Cys dual active site residues (H←55–58 aa→C) were well conserved in TrdL and other proteins; (ii) the Cys active site located three amino acids far away from the other Rossmann fold FAD binding sites (GxxGxxxG); and (iii) the tyrosine residue in the C-terminus of the proteins is also conserved for proton abstraction of the hydroxy group of the substrate[20,21] (see Figure S1 in SI).

On the basis of the above knowledge, we further analyzed the UV–vis spectrum of the native form and unfolded form of TrdL treated with SDS. The native TrdL showed two absorption bands at 350–375 nm (band II) and 450 nm (band I), very similar to the bicovalent flavinylated proteins like BBE and Chito. Band II in TrdL was strongly hypsochromically shifted (Figure S4 in SI) in comparison with that of free FAD, suggesting the 8α-histidyl substitution of FAD.[23] Band II disappeared when TrdL was unfolded by treatment with 0.5% SDS (Figure S4 in SI), which is a signature of 6-*S*-cysteinyl substitution of FAD.[22b,c] Thus, TrdL was also characterized as an 8α-histidyl and 6-*S*-cysteinyl FAD linked protein. To the best of our knowledge, TrdL is the first bacterial alcohol dehydrogenase that has been experimentally characterized as acting on a PKS and mixed PKS/NRPS backbone using a covalently FAD attached protein.

In summary, through *in vivo* and *in vitro* experiments, we have demonstrated that TrdL is a C-10 OH FAD-dependent dehydrogenase, responsible for the C-10 keto formation. **5** And **6** are two key intermediates in the postmodification process, and a hypothetical pathway was proposed based on the discovery of these key intermediates.

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Supporting Information Available. Detailed experimental procedures, NMR data for compounds **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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