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$\Delta^{11,12}$ Double Bond Formation in Tirandamycin Biosynthesis is Atypically Catalyzed by TrdE, a Glycoside Hydrolase Family Enzyme

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Supporting Information

ABSTRACT: The tirandamycins (TAMs) are a small group of *Streptomyces*-derived natural products that target bacterial RNA polymerase. Within the TAM biosynthetic cluster, *trdE* encodes a glycoside hydrolase whose role in TAM biosynthesis has been undefined until now. We report that in vivo *trdE* inactivation leads to accumulation of pre-tirandamycin, the earliest intermediate released from its mixed polyketide/nonribosomal peptide biosynthetic assembly line. In vitro and site-directed mutagenesis studies showed that TrdE, a putative glycoside hydrolase, catalyzes in a highly atypical fashion the installation of the $\Delta^{11,12}$ double bond during TAM biosynthesis.

The tirandamycins (**1**–**9**; Figure 1) are a group of bicyclic ketal and dienoyl tetramic acid natural products isolated

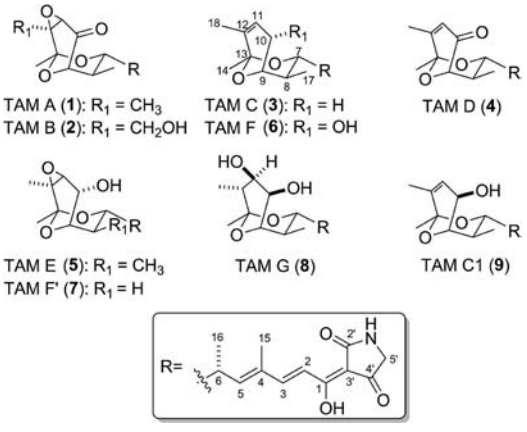

Figure 1. Structures of the tirandamycins.

from a series of terrestrial and marine *Streptomyces* spp. and their mutant strains.[1−3] These compounds are inhibitors of bacterial RNA polymerase and display numerous antimicrobial activities.[4,5] Most recently, TAM B (**2**) was shown to have potent antiparasitic activity by virtue of its ability to selectively inhibit asparaginyl-tRNA synthetase of *Brugia malayi*, one of the causative agents of lymphatic filariasis.[1e] The absolute configuration of TAM A (**1**) was originally determined by X-ray crystallography using the *p*-bromophenacyl ester of tirandamycic acid.[6] The unique structures of the TAMs and their intriguing mechanisms of action have attracted considerable interest from the synthetic community; total synthesis of **1** by DeShong and co-workers dates back to 1985 whereas the total synthesis of **2** was first accomplished in 1991.[7,8] TAM A has been an especially attractive target for synthesis, and its total synthesis has been achieved by several other groups.[9−11]

The TAMs have also attracted significant attention from the biosynthetic community. We[2] and Sherman co-workers[12] have cloned the gene cluster responsible for TAM biosynthesis from marine-derived *Streptomyces* sp. SCSIO 1666 and *Streptomyces* sp. 307-9, respectively. These natural products are assembled by hybrid type-I polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) machineries. We had shown previously that inactivation of TrdI, a cytochrome P450, resulted in accumulation of TAM C (**3**), an early biosynthetic intermediate.[2,12] Moreover, we determined using in vivo and in vitro experiments that TrdL is a novel covalently bonded FAD-dependent dehydrogenase.[3]

Polyketides (PKs) and hybrid PK/nonribosomal peptides (NRPs) belonging to a large group of bioactive secondary metabolites usually contain one or more double bonds. Double bond formation in PKs and PK/NRPs is now widely recognized to be catalyzed by ketoreduction and subsequent dehydration (DH) of the 3-ketoacyl acyl carrier protein (ACP)-tethered intermediate in the PKS biosynthetic assembly line.[13] Occasionally, the double bond is installed by a post-PKS tailoring step. For instance, the $\Delta^{2,3}$ double bond in phoslactomycin biosynthesis has been reported to be installed by PlmT2, a putative NAD-dependent epimerase/dehydratase.[14] We report here another example in which during TAM biosynthesis, the $\Delta^{11,12}$ double bond is installed by TrdE, a glycoside hydrolase, through a post-tailoring dehydration process. As elucidated by TrdE inactivation and site-directed mutagenesis experiments, pre-tirandamycin (**10**) is the earliest intermediate cleaved from the PK/NRPS machinery driving TAM biosynthesis and is the substrate of TrdE.

Within the TAM biosynthetic gene cluster, immediately downstream of the NRPS gene (*trdD*) resides *trdE*, which is

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transcribed in the same direction. Bioinformatics analysis revealed that TrdE (276 amino acids) shows the greatest identity to SlgC1 (76%); the protein is involved in the biosynthesis of the tetramic acid streptolydigin, but its precise function remains unclear.[15] TrdE also shows high identity to a group of glycoside hydrolases that includes BglF (36% identity) from *Nocardiopsis* sp. strain F96[16] and BglIIa (35% identity) from *Cellulosimicrobium cellulans*.[17] Intriguingly, there are no glycosyltransferase genes in the TAM cluster, nor is there any sugar moiety in TAM. Thus, the role of TrdE has been unclear.

To elucidate a possible role for TrdE, we inactivated *trdE* by in-frame replacement of this gene with an apramycin gene cassette using λ-RED recombination technology to yield a Δ*trdE* mutant. The desired mutant was selected on the basis of a kanamycin-sensitive and apramycin-resistant phenotype and was further verified by polymerase chain reaction [Figure S1 in the Supporting Information (SI)]. The validated Δ*trdE* mutant was fermented and the resulting broth extracted with butanone. HPLC analysis of the butanone fermentation extract revealed that the Δ*trdE* mutant accumulated a more polar new product, **10** (Figure 2, trace III). This Δ*trdE* mutant was also fermented

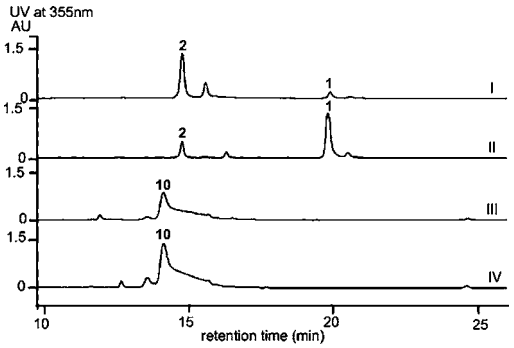


Figure 2. HPLC analyses of fermentation extracts of (I) wild-type strain, (II) wild-type strain with 2% resin added, (III) Δ*trdE* mutant strain, and (IV) Δ*trdE* mutant strain with 2% resin added. Peak identities are denoted with compound numbers.

in the presence of 2% XAD-16 resin to trap the possible early biosynthetic intermediate.[3,18] Subsequent HPLC analysis of the acetone extract of the mycelium and resin revealed that in this case the Δ*trdE* mutant still accumulated **10**, although its yield was improved by ~40% (Figure 2, trace IV). Large-scale fermentation (8 L) of the Δ*trdE* mutant provided quantities of **10** (75 mg) sufficient for structure elucidation.

The molecular formula of **10** was determined by high-resolution electrospray ionization mass spectrometry to be $C_{22}H_{31}NO_6$, which accounts for one more H_2O than in **3**. Complete spectral data including 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC, HMBC, NOESY) NMR spectra of **10** were acquired, thereby allowing rigorous assignment of the ^{1}H and ^{13}C signals (Table S1 in the SI). Comparisons of the ^{1}H and ^{13}C NMR data obtained for **3** and **10** revealed the absence of the $\Delta^{11,12}$ double bond signals in **10**. In turn, the H-18 methyl group of **10** appeared as a doublet at δ 1.10 (J = 7.0 Hz), and H-11 was shifted to δ 3.81 (br s), relative to the same position in **3**, consistent with attachment to an oxygen-bearing carbon in **10**. These data indicated $\Delta^{11,12}$ double bond saturation and the presence of a C-11 hydroxy group in **10**. The H-10α/H-10β/H-11, H-11/H-12, and H-12/H-18 correlation spectroscopy (COSY) correlations as well as the heteronuclear multiple bond correlation (HMBC) correlations from H-10 to C-12 and from H-11 to C-13 substantiated these assignments (Figure 3A,B). Inspection of other NMR data for **10** revealed other structural

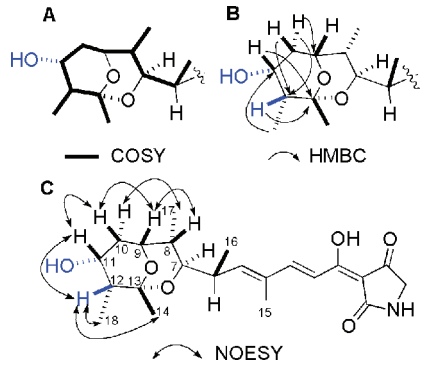
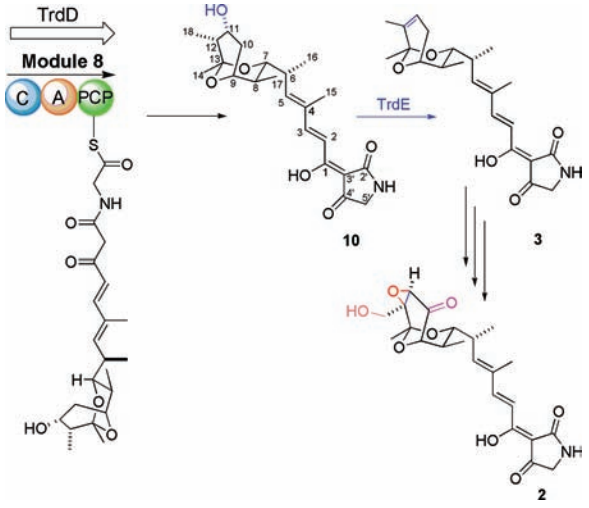


Figure 3. Selected key COSY, HMBC, and NOE correlations for **10**.

elements identical to those of **3**. The stereochemistry at C-11 and C-12 was determined to be 11*R*,12*R* on the basis of nuclear Overhauser effect (NOE) correlations between H-8 and H-9, H-9 and H-10β, H-10α and H-17, H-10β and H-11β, H-11β and H-12β, and H-12β and H-14 (Figure 3C). Consequently, the structure of **10** was found to be that shown in Scheme 1.

Scheme 1. Role of TrdE in the Biosynthetic Pathway of TAM B (2)



The isolation of **10** from the Δ*trdE* mutant indicated that TrdE is a post-PKS-NRPS tailoring enzyme that catalyzes dehydration of **10** to install the $\Delta^{11,12}$ double bond. To obtain further confirmation of its function in vitro, we cloned the *trdE* gene from cosmid 19G12 into the *Nde*I and *Bam*HI sites of a pET28a(+) vector. TrdE was overexpressed as an N-terminal His_6-tagged soluble protein and purified to homogeneity by Ni-affinity chromatography. The activity of TrdE at various pH values was investigated. We found that (i) TrdE efficiently catalyzed the transformation of **10** into **3** over the pH range from 4.0 to 8.0, (ii) the activity of TrdE decreased gradually at pH >8.0, and (iii) a small fraction of nonenzymatic transformation was detected in the negative control (Figure S2). Hence, the nonenzymatic transformation of **10** into **3** was investigated more rigorously at distinct pH values (4.0, 6.0, 7.4, and 10.0). At a reaction time of 12 h, the corresponding yields of the elimination product were 9.8, 0.4, 0.7, and 0.9%.

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Nonenzymatic dehydration was clearly enhanced at low pH (pH < 4) (Figure S3). A typical in vitro assay of TrdE was conducted in the presence of 50 mM Tris-Cl, 300 μM substrate **10**, and 0.5 μM TrdE at pH 7.4. HPLC data acquired at different time points revealed that TrdE efficiently catalyzed the transformation of **10** into **3** (Figure 4).

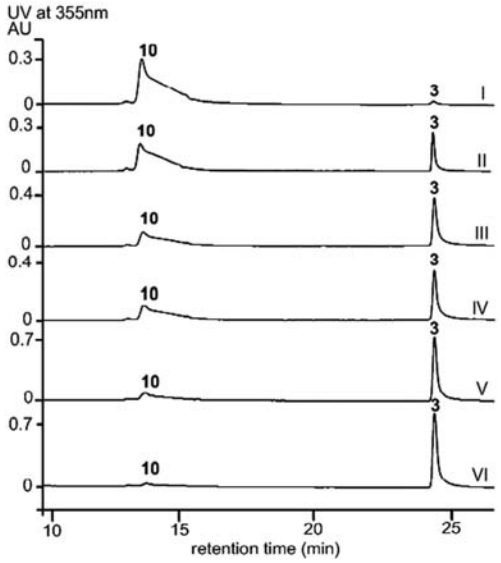

Figure 4. TrdE time trials involving HPLC analyses of TrdE-containing reactions in which **10** and TrdE were incubated for (II) 2 min, (III) 5 min, (IV) 10 min, (V) 30 min, and (VI) 1 h. Trace I is a negative control (1 h) involving denatured TrdE. The results validate the role of TrdE in the dehydration of **10**.

Both in vivo and in vitro experiments validated that TrdE catalyzes the post-tailoring dehydration responsible for the formation of the $\Delta^{11,12}$ double bond (Scheme 1) *en route* to **2**. Sequence alignments revealed that TrdE contains the catalytic motif EXDXXE characteristic of glycoside hydrolase family 16 (Figure S4).[16,17,19,20] Consequently, we measured the glycoside hydrolase activity of TrdE in reactions containing 2.5 μM TrdE, 1% (w/v) laminarin (a canonical substrate), and 50 mM sodium acetate (pH 4.5) or 50 mM Tris-Cl (pH 7.4). TLC analyses of reactions lasting for up to 24 h (Figure S5) revealed the complete absence of glycoside hydrolase activity. These data support the conclusion that despite its primary structure, TrdE does not behave like a glycoside hydrolase.

The importance of the conserved residues E145, D147, and E150 for glycoside hydrolases is well-known and has been validated by X-ray crystallography and mutagenesis experiments.[19−22] To probe whether the glycoside hydrolase catalytic motif $E_{145}XD_{147}XXE_{150}$ is involved in the biochemical activity of TrdE, site-directed mutagenesis of these amino acid residues was carried out. In addition, TrdE was found to contain four His residues in its C-terminal half. Since the His residue in the PKS DH domain is well-known for the catalytic chemistry of dehydration,[13b,23] the four His residues in TrdE were also mutated. Consequently, seven mutant TrdE proteins (E145L, D147A, E150L, H130F, H164F, H169F, and H193F) were created using standard site-directed mutagenesis methods and subsequently overproduced in *Escherichia coli* BL21 (DE3) and purified to homogeneity (Figure S6). The ability of these site-directed mutant proteins to convert **10** into **3** was assessed in vitro over the course of 1 h at 30 °C; the reaction mixtures contained 50 mM Tris-Cl, 300 μM substrate **10**, and 0.5 μM TrdE mutant. A reaction containing wild-type TrdE served as a positive control, and **10** in reaction buffer without TrdE or related variants served as a negative control. HPLC analysis of each reaction revealed that H130F retained biochemical activity on par with wild-type TrdE and that D147A retained ~60% of the biochemical activity of wild-type TrdE (Figure 5, traces I–III). None of the other site-directed TrdE mutants showed apparent conversion of **10** into **3** (Figure 5, traces IV–IX).

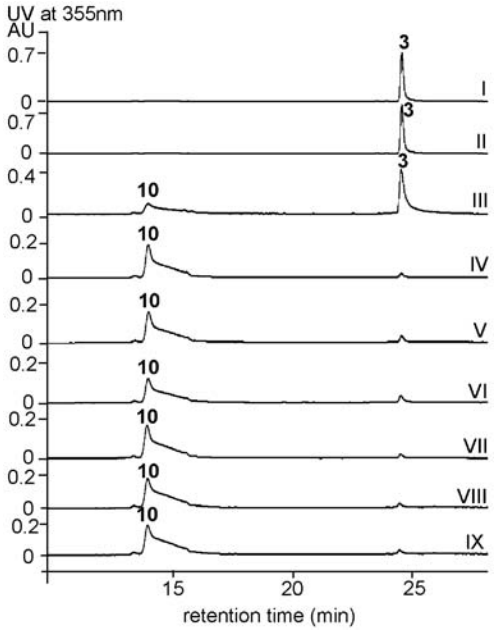

Figure 5. TrdE site-directed mutagenesis. HPLC analyses of incubations of **10** with (I) wild-type TrdE (positive control), (II) H130F, (III) D147A, (IV) E145L, (V) E150L, (VI) H164F, (VII) H169F, (VIII) H193F, and (IX) **10** in Tris-Cl buffer (negative control) are shown.

We then measured the circular dichroism (CD) spectra of TrdE and its seven mutants. The CD spectrum of wild-type TrdE exhibited an apparent negative Cotton effect at 225 nm as a sharp peak (panel I in Figure S7). This band clearly shifted to 210 nm as a broad peak in the TrdE mutants E145L, H164F, H169F, and H193F (panels IV–VII in Figure S7), indicating a change in protein conformation that accounts for the decreased bioactivity. These results also imply that the residues E145, H164, H169, and H193 are important for the peptidic scaffold of TrdE and indispensible for its enzymatic activity.

Interestingly, the CD spectra of the H130F, E150L and D147A mutants (panels III, II, and VIII in Figure S7) were similar to that obtained for wild-type TrdE. Finally, we acquired steady-state kinetic parameters for TrdE and these three mutants enzymes. The parameters for TrdE were acquired using 75 nM TrdE, 50 mM Tris-Cl (pH 7.4), and concentrations of **10** ranging from 37.5 to 800 μM. The results are shown in Table 1 and Figure S8a–d. Among the TrdE mutants, E150L showed distinct K_m and k_{cat} values, suggesting its direct role in catalytic activity. D147A retained biochemical activity but lost ~50% of its k_{cat}. It is now clear that TrdE possesses characteristics typical of the glycoside hydrolase class.

The mechanism by which TrdE catalyzes the dehydration of **10** while also clearly possessing structural characteristics consistent with glycoside hydrolase activity is likely enabled

Table 1. Steady-State Kinetic Parameters for TrdE and Mutants

enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
TrdE	144.3 ± 9.5	22.3 ± 0.5	0.15
H130F	144.1 ± 19.8	31.7 ± 1.6	0.22
D147A	139.7 ± 26.3	11.4 ± 0.8	0.082
E150L	247.3 ± 31.2	0.98 ± 0.04	0.0040

by the fact that in the TAMs, the C-13 ketal carbon resembles the anomeric carbon of a glycosidic linkage. This structural feature of the TAMs suggests that transient loss of the TAM bicyclic ketal system may lead to acidification at C-12 in such a way as to facilitate dehydrative installation of the C-11−C-12 double bond (Figure S9). Though one can envision multiple variations on the general theme (Figure S9), such a mechanism would be analogous to the middle step of the dehydration process catalyzed by dTDP-D-glucose-4,6-dehydratase (RmlB) involved in L-rhamnose biosynthesis.[24] Such a mechanistic rationale, as opposed to a direct elimination on the intact TAM bicyclic ketal (I in Figure S9), draws support from the established mechanistic pathway of RmlB, which entails initial ketone formation at C-4 prior to C-5 deprotonation to install the C-5−C-6 double bond. Moreover, transient conversion of the TAM C-13 ketal to either an oxonium or keto species (II−IV in Figure S9) is supported by the aforementioned acid-accelerated nonenzymatic reaction.

In summary, in vivo inactivation of TrdE, a glycoside hydrolase, resulted in accumulation of pre-tirandamycin (**10**), the earliest intermediate released from the TAM biosynthetic assembly line leading to TAM C (**3**), an intermediate previously identified through inactivation of TrdI, a cytochrome P450 enzyme. In vitro overproduction and biochemical assays demonstrated that TrdE installs the cis-$\Delta^{11,12}$ double bond through a post-tailoring dehydration process leading to the conversion of **10** into **3**. Site-directed mutagenesis of TrdE revealed that the conserved residue E150 is indispensable for the catalytic activity and that residues E145, H164, and H193, which are well-known to be conserved among the glycoside hydrolases, are required for enzymatic activity. Thus, TrdE was identified as the enzyme responsible for formation of the $\Delta^{11,12}$ double bond in TAM biosynthesis. This function, combined with the role of TrdE as a putative glycoside hydrolase, suggests a novel, heretofore unprecedented mechanism for polyketide double bond formation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental section, 1D and 2D NMR spectral data for compound **10**, gene replacement data for *trdE*, and in vitro biochemical data for TrdE. This material is available free of charge via the Internet at http://pubs.acs.org.http://pubs.acs.org/page/jacsat/submission/authors.html.

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Notes

The authors declare no competing financial interest.

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