# Tirandamycin biosynthesis is mediated by co-dependent oxidative enzymes

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Elucidation of natural product biosynthetic pathways provides important insights into the assembly of potent bioactive molecules, and expands access to unique enzymes able to selectively modify complex substrates. Here, we show full reconstitution, *in vitro*, of an unusual multi-step oxidative cascade for post-assembly-line tailoring of tirandamycin antibiotics. This pathway involves a remarkably versatile and iterative cytochrome P450 monooxygenase (Taml) and a flavin adenine dinucleotide-dependent oxidase (TamL), which act co-dependently through the repeated exchange of substrates. Taml hydroxylates tirandamycin C (TirC) to generate tirandamycin E (TirE), a previously unidentified tirandamycin intermediate. TirE is subsequently oxidized by TamL, giving rise to the ketone of tirandamycin D (TirD), after which a unique exchange back to Taml enables successive epoxidation and hydroxylation to afford, respectively, the final products tirandamycin A (TirA) and tirandamycin B (TirB). Ligand-free, substrate- and product-bound crystal structures of bicovalently flavinylated TamL oxidase reveal a likely mechanism for the C10 oxidation of TirE.

he antibiotic tirandamycin is one of several dienoyl tetramic acid natural products containing an intriguing and often heavily tailored bicyclic ketal moiety. Recently, we have expanded the family of known tirandamycins to four compounds, tirandamycins A-D (TirA-D, 1-4), which differ in the extent of oxidative tailoring of the bicyclic ketal (Fig. 1); this has been found to be a key determinant of potency against vancomycin-resistant Enterococcus faecalis (VRE)<sup>1</sup>. Similar modifications are apparent in the ketone of nocamycin (5), and the epoxide of tirandalydigin (6) and streptolydigin (7), but TirB is unique in that it contains a ketone (C10), epoxide (C11/C12) and an additional hydroxyl (C18) group (Fig. 1)<sup>2</sup>. Our interest in the formation and tailoring of this pharmacophore prompted a search for the gene cluster encoding tirandamycin biosynthesis in Streptomyces sp. 307-9, resulting in identification of a hybrid polyketide synthase (PKS)/non-ribosomal peptide synthetase (NRPS) system<sup>3</sup>. Flanking the PKS-NRPS genes were coding regions for two predicted oxidative tailoring enzymes: a cytochrome P450 homologue TamI, which we regarded as a likely candidate for installation of the epoxide and C18 hydroxyl group, and a flavoprotein homologue TamL, which we proposed to be involved in the formation of the C10 ketone. Disruption of tamI led to exclusive accumulation of TirC (ref. 3), which is devoid of oxidative modifications, suggesting that TamI is responsible for the first step in the tirandamycin tailoring pathway. This finding motivated our interest in establishing the precise role of TamI and other oxidative enzymes involved in the introduction of the C10 keto, C11/C12 epoxide and C18 hydroxyl functional groups through in vitro analysis and reconstitution of the TirC  $\rightarrow$  TirB enzymatic system. Finally, the troika of ligand-free, substrate and product-bound X-ray structures of TamL provide compelling evidence for the mechanism of C10 keto-group installation.

#### Results

We began by examining the reactions catalysed by TamI, a predicted cytochrome P450 monooxygenase (Supplementary Fig. S1), on each of the intermediates that were previously isolated from fermentation

broths of *Streptomyces* sp. 307-9 (ref. 1). The N-terminal His<sub>8</sub>-tagged recombinant TamI was heterologously overexpressed and purified from *Escherichia coli* (Supplementary Fig. S2) to yield an orange-red enzyme solution that was characterized by ultraviolet-visible spectroscopy using standard techniques<sup>4</sup>. The enzyme solution displayed an absorbance peak at 417 nm, with a 448 nm peak arising after reduction with sodium dithionite and bubbling of the solution with CO (Fig. 2a).

To test the hypothesis that TamI installs the C11/C12 epoxide and C18 hydroxyl, the recombinant enzyme was incubated with the putative substrate TirA in reaction buffer containing spinach ferredoxin/ferredoxin-NADP<sup>+</sup> reductase as heterologous redox partners, and NADPH. Liquid chromatography-mass spectrometry (LC-MS) analysis of the reaction supernatants revealed a small but reproducible conversion of TirA to TirB due to hydroxylation at C18. Under the same reaction conditions but with TirD as the substrate, we observed complete conversion predominantly to TirA but with a minor amount of TirB (Fig. 3a). Together, these experiments demonstrate that the penultimate and final steps of tirandamycin biosynthesis are a C11/C12 epoxidation and a C18 hydroxylation, respectively.

We next investigated the possible role of TamI in the formation of the C10 ketone by incubating TirC with recombinant P450 enzyme, and observed the formation of a very small amount of TirA (Fig. 3a), as well as a new species that had not been previously identified from fermentation broths. The new compound, tirandamycin E (TirE, **8**), had a mass and polarity consistent with a single hydroxylation of TirC. To characterize TirE by NMR, we required several milligrams of product prepared enzymatically, which was impractical using the existing reaction procedure with spinach ferredoxin/ferredoxin-NADP<sup>+</sup> reductase. To overcome this limitation, we used a recently developed strategy to generate a self-sufficient biosynthetic P450 reaction system by fusing TamI to the RhFRED reductase domain from *Rhodococcus* sp. (ref. 5), thus obviating the need for costly exogenous redox partners

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**Figure 1** | **Tetramic acid natural products bearing a bicyclic ketal moiety** (red) with varying degrees of oxidative modification. Tirandamycin E is described within this study; all other compounds have been reported previously<sup>1,2</sup>. Nocamycin I and Bu-2313B are synonymous.

(Fig. 2b, Supplementary Fig. S2). Using this system we conducted a preparative scale conversion of TirC to TirE, purified the product, and assigned the structure by NMR analysis (Supplementary Table S1, Figs S3–S9), which confirmed the presence of a hydroxyl group at C10 of the bicyclic ketal moiety. The stereochemistry was assigned based on the observation of through-space deshielding between the C10 hydroxyl and the H7 and H17 protons, and by comparison of the nuclear Overhauser enhancement spectroscopy (NOESY) data for TirC and TirE, which confirmed the disappearance of the pro-*S* proton at C10.

These results demonstrate that one major activity of TamI is to catalyse hydroxylation at C10, but the low levels of TirA formation indicated that TamI is capable of further reaction to generate the corresponding ketone. It is likely that formation of this keto group by TamI proceeds through sequential hydroxylations at C10 to form a geminal-diol that exists (in equilibrium) predominantly in the ketone form of TirD (Fig. 3b). This route resembles that used by P450s for keto-group formation in doxorubicin biosynthesis<sup>6</sup>, or to install a carboxylate at a primary carbon atom as in the biosynthesis of artemisinic acid7. However, because this activity for TamI was low, we reasoned that another oxidative enzyme in the pathway might catalyse the predominant route to TirD. We therefore examined the ability of TamL, a predicted flavin-dependent oxidoreductase, to catalyse this reaction. Alignments revealed a sequence motif for the bicovalent attachment of flavin adenine dinucleotide (FAD) to invariant histidine and cysteine residues (Supplementary Fig. S10)<sup>8,9</sup>. This suggested that TamL may use a mode of cofactor binding similar to those of glucooligosaccharide oxidase  $(GOOX)^{10}$ , aclacinomycin oxidoreductase  $(AknOx)^{11}$ , glycopeptide hexose oxidase  $(Dbv29)^{12}$ , hexose oxidase  $(HOX)^{12,13}$  and berberine bridge enzyme  $(BBE)^{9,14,15}$ , all of which belong to the *p*-cresol methylhydroxylase scaffold family<sup>16</sup>. These proteins show high sequence similarity in the flavin binding site, whereas few residues are conserved in their substrate binding domains owing to the structural diversity of their substrates (Supplementary Fig. S10).

We overexpressed and purified recombinant  $His_8$ -tagged TamL in *E. coli* (Supplementary Fig. S2) to yield a deep yellow protein solution with fluorescence excitation/emission and ultravioletvisible absorption spectra consistent with a flavoprotein (Fig. 2c,d)<sup>8,17</sup>. The presence of a covalently bound cofactor was supported by boiling the enzyme solution and observing that the yellow colour was retained with denatured protein, and released cofactor was absent from the supernatant (determined by LC-MS). Acid hydrolysis treatment of the protein released adenosine monophosphate (AMP), which confirmed FAD (and not flavin



**Figure 2 | Spectral analysis of tirandamycin tailoring enzymes. a**, Ultraviolet-vis absorption spectrum for purified Taml P450 enzyme in oxidized form (solid line), sodium dithionite-reduced form (dotted line) and CO-reduced form (dashed line). Insets: CO-bound reduced difference spectra. **b**, Ultraviolet-vis absorption spectrum for purified Taml-RhFRED fusion. **c**, Fluorescence excitation and emission spectra of purified TamL flavoprotein. **d**, Ultraviolet-vis absorption spectrum for purified TamL. Insets: Ultraviolet-vis absorption spectra of authentic FAD and FMN cofactors.

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**Figure 3 | Elucidation of individual steps in the tirandamycin oxidative cascade. a**, *In vitro* reconstitution of Taml- and TamL-mediated oxidation steps, and metabolite profiles of *Streptomyces* sp. 307-9 wild-type and mutant strains. Peaks were identified by comparison to authentic standards and MS detection of anticipated species. Relative zoom of the bottom three traces: wild-type ×4.1, *Ataml* ×1, *AtamL* ×17.5. **b**, Complete oxidative cascade scheme; the predominant Taml P450 reactions are highlighted in red, with the intervening TamL oxidation reaction highlighted in yellow; dashed arrows represent minor activity towards the TamL-independent route. The R group is shown in Fig. 1. Taml mediates the initial hydroxylation of TirC at C10, to form TirE, after which TamL oxidizes TirE to the ketone. Subsequent epoxidation and hydroxylation, both catalysed by Taml, complete the cascade.

mononucleotide (FMN)) as the cofactor (Supplementary Fig. S11)<sup>18</sup>, and MS analysis of the intact protein corroborated the presence of a single FAD per enzyme molecule (Fig. S12).

X-ray structure analysis of TamL revealed that FAD is attached covalently to the enzyme by bonds between the C6 atom of the isoalloxazine ring and the sulfur atom of invariant cysteine C122, as well as the  $8\alpha$ -methyl group to the N1 atom of H62, resulting

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in 6-S-cysteinyl,  $8\alpha$ -N1-histidyl FAD (Fig. 4a). The spacious substrate binding cleft of TamL is open to the bulk solvent and is built largely of a seven-stranded antiparallel  $\beta$ -sheet flanked by five  $\alpha$ -helices (Fig. 4b). The mouth of the cleft is surrounded by residues 323–336 (pink in Fig. 4b), which are the most variable of the closest TamL homologues from both sequence and structural perspectives (Supplementary Fig. S10). This region participates in substrate binding and mediates the formation of tightly associated dimers in the crystal, as observed for other members of the *p*-cresol methylhydroxylase superfamily<sup>16</sup> (Fig. 4c). The 1,680 Å<sup>2</sup> dimerization interface in the substrate-free TamL is also stabilized by two solvated Mg<sup>2+</sup> cations, bound to D124 of each protein monomer. Five other Mg<sup>2+</sup> coordinates are occupied by water molecules (Fig. 4a) making multiple hydrogen-bonding interactions with the protein.

In the substrate-bound form (Fig. 4b),  $Mg^{2+}$  was found chelated by two adjacent carbonyl groups of the tirandamycin tetramic acid moiety and its 3-acyl substituent, with the four other coordination sites occupied by the water molecules mediating contacts with the side chain of D330 (Fig. 4d). Location of the metal centre in both structures seems to exclude its direct involvement in catalysis. This was consistent with the observation that pre-incubation of TamL with ethylenediaminetetraacetic acid (EDTA) does not change its enzymatic activity. However,  $Mg^{2+}$  may play a stabilizing role in the monomer or dimer structure, facilitate substrate binding, or assist in covalent flavinylation through hydrogen bonding between the  $Mg^{2+}$  aqueous shell and the FAD attachment site at H62 (Fig. 4a).

Biochemical analysis of recombinant TamL demonstrated complete conversion of purified TirE to TirD, thus confirming its role in oxidizing the C10 hydroxyl of its presumed natural substrate to a ketone (Fig. 3). Furthermore, when reactions containing TamI P450 and TirC were amended with TamL, we observed nearly complete conversion to TirA (bearing the C11/C12 epoxide) with no residual TirE detected, indicating that oxidation of TirE by TamL affords the C10 ketone more effectively than TamI alone. These *in vitro* results are supported by our previous *in vivo* gene disruption experiments<sup>3</sup>, and are entirely consistent with initiation of the oxidative cascade by TamI. To confirm the role of TamL *in vivo*, we created a  $\Delta tamL$  mutant and observed accumulation of the TirE biosynthetic intermediate from this strain, demonstrating that TamI alone is unable to catalyse conversion to TirD *in vivo* (Fig. 3a).

To better understand the interplay of TamI and TamL in this oxidative cascade, we obtained the kinetic parameters for each reaction (Table 1). Consistent with the above-described *in vitro* qualitative results (Fig. 3a), TamI was most efficient in catalysing the initial hydroxylation of TirC and epoxidation of TirD ( $k_{cat}/K_m = 19.3$ and 3.6  $\mu$ M<sup>-1</sup> min<sup>-1</sup>, respectively), in contrast to the inefficient final hydroxylation of TirA ( $k_{cat}/K_m = 5.8 \times 10^{-4} \mu$ M<sup>-1</sup> min<sup>-1</sup>), which resulted from both a substantially lower  $k_{cat}$  and higher  $K_m$ . Moreover, TamL-mediated oxidation of TirE to the corresponding ketone was very rapid ( $k_{cat} = 4.08 \times 10^3 \min^{-1}$ ), highlighting the significance of this enzyme in overcoming a biochemical challenge that is poorly met by TamI alone.

To establish the mechanism of catalysis, we determined the X-ray structures of TamL alone (PDB ID code 2Y08) and of the TamL-tirandamycin complex (PDB ID codes 2Y3R, 2Y4G and 2Y3S). Given the high catalytic activity of TamL, the product, TirD, rather than the substrate, TirE, would be expected to occupy the active site in the crystals. However, the active-site electron density map for the 2Y4G and 2Y3S structures unambiguously matched TirE, which was finally fitted in both monomers in the asymmetric unit in these structures. In contrast, TirD better satisfied the electron density in monomers B and D in the 2Y3R structure and, therefore, TirE was fitted in chains A and C, and TirD in chains B and D in 2Y3R (Fig. 4d). Collectively, the structures revealed that tirandamycins are bound in the active site, with the bicyclic ketal moiety



**Figure 4 | Ligand-free and substrate/product-bound TamL. a**, Catalytic and Mg<sup>2+</sup>-binding sites in the substrate-free TamL with covalently bound FAD (yellow sticks) (PDB ID 2Y08). Residues from the same monomer are in cyan, and from the symmetry-related monomer in pink. **b**, Tirandamycin (blue sticks) in the active site of TamL (PDB ID 2Y3R). Residues 323-336 at the mouth of the substrate binding cleft are in pink. **c**, Ribbon representation of TamL dimer formed by the green and pink monomers related by non-crystallographic symmetry (PDB ID 2Y08). Mg<sup>2+</sup> atoms (spheres of matching colours) stabilize the dimerization interface. **d**, Interactions between the C10 site of oxidation in TirE and the N5 locus in FAD are highlighted in a magenta dashed line defining an angle with the N5/N10 flavin atoms of 110°. Installation of the keto group in TirD flattens the ketal ring, pulling C10 away from the N5 atom by 0.3 Å (PDB ID 2Y3R). **e**, Superimposition of the amino-acid residues in the tirandamycin-bound TamL (cyan sticks) on substrate-free TamL (grey sticks). **f**, Mechanism of dehydrogenation at C10 in TirE. In panels **a-e**, O atoms are in red, N in blue, S in dark yellow, Mg<sup>2+</sup> in green. Electron density 2F<sub>o</sub>-F<sub>c</sub> map (grey mesh) is contoured at 1.5*o*. Distances are in Å.

facing FAD and the tetramic acid moiety extending towards the mouth of the substrate binding cleft (Fig. 4b). The ketal ring positioned for oxidation runs parallel to the plane of the isoalloxazine ring, with the C10 hydrogen atom pointing directly at the N5 of FAD (distance, 2.7 Å) (Fig. 4d,e). The geometry of this disposition is consistent with the hydride ion transfer proposed for many flavin-dependent oxidases<sup>19,20</sup>. This mechanism requires concomitant abstraction of the proton from the C10–OH group, which is probably achieved through interactions with the invariant Y447 activated by the highly conserved Y136 residue, as proposed for TamL homologue Dbv29 (ref. 21; Fig. 4e,f).

The TamL-TirD/TirE co-crystal structure provides considerable mechanistic insights into the TirE  $\rightarrow$  TirD oxidation process. In addition to the repositioning of the 323–336 loop running across the opening of the substrate binding cleft (highlighted pink in Fig. 4b), binding of the substrate causes

Table 1   Kinetic parameters for the reaction of					
TamI-RhFRED and TamL with tirandamycin intermediates.					
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Enzyme (substrate)	κ <sub>m</sub> (μΜ)	k <sub>cat</sub> (min ')	k <sub>cat</sub> /K <sub>m</sub> (μM <sup>-1</sup> min <sup>-1</sup> )
Taml-RhFRED (TirA)	189.7 <u>+</u> 25.1	0.11±0.01	0.00058
Taml-RhFRED (TirC)	2.1 <u>+</u> 0.3	40.5 <u>+</u> 0.8	19.3
Taml-RhFRED (TirD)	23.2 <u>+</u> 3.7	83.8 <u>+</u> 3.4	3.6
TamL (TirE)	174.1 <u>+</u> 37.0	4,082.7 <u>+</u> 375.7	23.4

hydride abstraction and subsequent re-oxidation of cofactor by molecular oxygen through the stabilization of certain electronic forms of FAD. One major event clearly observed in the 2Y3R structure includes flipping of the main chain carbonyl group of A121 towards the isoalloxazine ring, which ideally positions the carbonyl oxygen hydrogen-bond acceptor (within 2.9 Å) to transiently stabilize the reduced rather than oxidized state of the cofactor (Fig. 4e). Assuming that the inversion of the A121 carbonyl group happens each time the hydride ion is transferred to the N5 locus of FAD, it should occur with high precision to maintain the fast rate of the reaction. We propose that the covalent attachment of FAD to the nearby C122 may serve to ensure the precision of this interaction.

conformational changes near FAD that may facilitate both

In addition, rotation of the Y444 side chain brings its OH group within the hydrogen-bonding distance (2.5 Å) of the O2 locus of FAD (Fig. 4e), which may increase the oxidative power of the cofactor<sup>22</sup>. Other tyrosine residues Y64, Y136, Y323 and Y447, and the main chain at G134–G137, undergo adjustments to accommodate the incoming substrate. As a result, the OH group of Y447 that apparently serves as a base to abstract a proton from C10–OH ends up between the C10–OH (2.3 Å) and Y136–OH (2.5 Å) groups (Fig. 4e,f). The presumed requirement for conformational changes to accommodate substrate binding and catalysis may explain the surprising presence of substrate in the TamL active site. A tenfold molar excess of TirE over the enzyme could

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subsequently displace TirD in the active site whose FAD cofactor is stalled in its reduced form in the crystal.

#### Discussion

Our in vitro characterization of the complete tirandamycin oxidative cascade has revealed a remarkably versatile TamI P450 enzyme that catalyses at least two hydroxylations and one epoxidation at three distinct sites, including a primary allylic C-H bond (C18), a secondary allylic C-H bond (C10) and an olefin (C11/C12), which to our knowledge is the first reported example of a bacterial biosynthetic P450 with such versatile activity. In comparison, non-haem irondependent oxygenases are well known for such catalytic versatility<sup>23,24</sup>, which is thought to be the result of a relatively flexible coordination geometry that might be difficult to achieve through the haem-based coordination of a cytochrome P450. The TamI/TamL reactions occur in a precisely defined order with no detected promiscuity during the individual steps (Fig. 3b). Following the initial oxidation of TirC by TamI, TamL must oxidize the C10 hydroxyl group to the ketone of TirD, which is the exclusively observed substrate for sequential epoxidation to TirA and hydroxylation to TirB. The repeated exchange of substrates between these two enzymes creates a unique tailoring pathway in which a P450 catalyses multiple oxidations co-dependently with a second biosynthetic enzyme, in contrast to numerous examples of multi-step P450 reactions in which no intervening step by an alternative enzyme is required<sup>7,25-27</sup>. This intriguing enzymatic interplay parallels that observed in clavaminate biosynthesis, in which a non-haem iron oxidase catalyses three non-consecutive reactions separated by the action of a separate hydrolase enzyme<sup>23</sup>. Perhaps the recruitment of TamL to the evolving secondary metabolic pathway was prompted by the inability of TamI to efficiently transform TirE to TirD. Oxidative modification in the tirandamycin pathway contributes significantly to antibiotic potency<sup>1</sup>, which might have provided evolutionary pressure for the TamI/TamL series of tailoring steps. It appears that this process is facilitated in TamL through bicovalent attachment of FAD, which has been shown to increase redox potential in related enzymes GOOX<sup>28</sup> and BBE<sup>29</sup> to the remarkably high values of  $\sim$ 130 mV. This cofactor configuration accelerates flavin reduction during turnover to the point where its reoxidation by molecular oxygen becomes the rate-limiting step<sup>29</sup>. It remains to be determined if the nocamycin pathway uses a TamL homologue for oxidation of the C10 hydroxyl of nocamycin II (9) to the ketone of nocamycin, or simply proceeds through P450-mediated geminal-diol formation and equilibration identified as a minor aspect of the TirE  $\rightarrow$  TirD conversion (Fig. 3b).

Conservation of the catalytically essential tyrosine residues in TamL suggests its close mechanistic similarities with GOOX, AknOx and Dbv29 (refs 9,14,15; Supplementary Fig. S10). In TamL, four tyrosine residues are situated in the vicinity of the N5 nitrogen atom of FAD (Fig. 4e): Y136 and Y447 are invariant between TamL, GOOX, AknOx and Dbv29, whereas Y444 is absent from AknOx, and Y64 is present only in TamL and GOOX (Supplementary Fig. S10). Thus, TamL-mediated oxidation is likely to proceed via proton abstraction from the C10 hydroxyl group by Tyr447 and concomitant hydride ion transfer from the C10 carbon atom to the N5 nitrogen atom of FAD (Fig. 4f). Tyr136 may participate in the proton transfer network to initiate H<sup>+</sup> abstraction by Y447. The reduced flavin is probably reoxidized by molecular oxygen, with formation of hydrogen peroxide. The oxidative half of the reaction in the flavin-containing oxidases is believed to proceed by a two-step mechanism via transient formation of the red anionic semiquinone<sup>20,29</sup>. Although it has not been confirmed experimentally for TamL, H130 would be a candidate residue to stabilize the negative charge should it accumulate on the N1-C2 = O2 locus in FAD (Fig. 4f). The exact catalytic mechanism and the actual roles of the catalytically important residues require further analysis, which is currently ongoing in our laboratories.

Based on our recent structure-activity relationship analysis of the tirandamycins<sup>1</sup>, the potency of the early intermediate TirC (minimum inhibitory concentration (MIC), 110 µM) is far below that of TirA (MIC, 2.25 µM). This makes the above-described oxidative tailoring steps biologically significant, assuming that the observed antimicrobial (for example, anti-VRE) activity reflects potency against relevant natural targets of the tirandamycins (the molecular target of TirA and TirB is RNA polymerase<sup>2</sup>). Our in vitro enzymatic characterization and analysis of the fermentation broth metabolite profiles all suggest that this biosynthetic pathway is tuned towards the production of TirA, the most potent of the tirandamycin antibiotics. The in vitro conversion of TirC to TirA (passing through TirE and TirD) proceeds efficiently, and the early intermediates TirC and TirD are only observed from wildtype culture fermentations if an adsorbent resin is included in the broth to sequester these compounds during production<sup>1</sup>. Thus, the TirA metabolite that accumulates predominantly in the culture media is the most potent antibiotic. Conversion to the most highly modified, but less potent TirB (MIC,  $100 \mu$ M) occurs with low efficiency in vitro, and TirB only accumulates appreciably in fermentation broths using extended culture times. These observations suggest that the TamI P450 has evolved to efficiently accommodate TirC and TirD, resulting in a substantial increase in potency of the product, but then stalls on the final hydroxylation that converts TirA to the less bioactive TirB.

Note added in proof: During review, two papers were published relating to the topics in this Article: Mo, X. et al. Biochem. Biophys. Res. Commun. **406**, 341–347 (2011); Mo, X. et al. Org. Lett. **13**, 2212–2215 (2011).

#### Methods

General experimental procedures. NMR spectra were acquired on Varian INOVA 400 MHz and Varian INOVA 600 MHz spectrometers at the Center for Chemical Genomics, University of Michigan. NMR spectra were processed using MestReNova software. High-resolution electrospray ionization mass spectrometry (ESI-MS) was performed at the University of Michigan core facility in the Department of Chemistry using a Waters Micromass AutoSpec Ultima. Reversed-phase high-performance liquid chromatography (RP-HPLC) purification was performed using Waters XBridge 5  $\mu$ m C18 columns and a solvent system of acetonitrile and H<sub>2</sub>O supplemented with 0.1% trifluoroacetic acid (TFA). LC-MS analysis was performed on a Shimadzu 2010 EV ESI spectrometer using an XBridge C18 3.5  $\mu$ m, 50 mm column with the same solvent system supplemented with 0.1% formic acid. DNA sequencing was performed at the University of Michigan DNA Sequencing Core Facility using the dideoxy chain-termination method.

Crystallization and structure determination. Crystallization of TamL was sensitive to the presence of Mg<sup>2+</sup>. Substrate-free crystals obtained from 15% PEG4000, 0.2 M MgCl<sub>2</sub> and 0.1 M Tris-HCl, pH 7.0 belong to the space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> with cell dimensions a = 64.4, b = 129.6, c = 134.6. Two molecules in the asymmetric unit related by the twofold non-crystallographic symmetry form a dimer with the ~1,680 Å<sup>2</sup> interface. Tirandamycin-bound crystals were obtained in three distinct forms from similar conditions, all featuring the same dimeric structure packed in the crystals in different ways (Supplementary Table S2). Diffraction data were collected at 100-110 K at the Beamline 8.3.1, Advanced Light Source at Lawrence Berkeley National Laboratory. Consistent with the high redox potential of the bicovalently attached FAD is bleaching of the brilliant yellow colour of the oxidized flavin, which was observed during the first few seconds of data collection, suggesting conversion into the fully reduced, virtually colourless, hydroquinoid form by the flux of the X-ray electrons. Despite that observation, fully oxidized FAD was fitted into the electron density during structure refinement. In none of the structures did the isoalloxazine ring deviate from exact planarity. Data were processed using ELVES<sup>30</sup> to resolutions ranging from  $\sim$ 1.7 to 2.0 Å, depending on the structure. Molecular replacement and refinement routines were carried out using the CCP4 software suite<sup>31</sup> with the hexose oxidase atomic coordinates (PDB ID 2WDX) as an initial search model. Data collection and refinement statistics are shown in Supplementary Table S2.

**Enzyme assays and preparation of substrates.** Isolation of TirA, TirB, TirC and TirD have been described previously<sup>1</sup>. TirE was obtained by preparative-scale

enzymatic conversion in 40 × 500 µl parallel reactions (overnight, 30 °C), each containing ~10 µM purified TamI-RhFRED, 0.15 mg TirC, 1 mM NADPH and 5 µM glucose-6-phosphate and 2 Unit/ml glucose-6-phosphate dehydrogenase for NADPH regeneration. The reaction mixtures were combined and extracted by 3 × 40 ml CH<sub>2</sub>Cl<sub>2</sub>, after which the extract was dried, redissolved in 2 ml methanol, and purified by RP-HPLC to yield TirE. The structural assignment of TirE was based on analysis of <sup>1</sup>H, <sup>13</sup>C, correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC) and NOESY data sets. The complete NMR assignment is available in the Supplementary Information. HRMS (*m*/*z*): [M–H]<sup>-</sup> calcd. for C<sub>22</sub>H<sub>29</sub>NO<sub>6</sub>, 402.1922; found, 402.1914.

The *in vitro* enzymatic conversions of tirandamycins were performed in a total volume of 100 µl of conversion buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, 10% glycerol) containing 2 µM TamL (or 1 µM TamI partnered by 3.5 µM spinach ferredoxin, 0.1 Unit/ml spinach ferredoxin-NADP<sup>+</sup> reductase and 500 µM NADPH) and 200 µM substrate. As negative controls, corresponding enzymes were omitted. After incubation at 30 °C for 2 h, the reactions were quenched by extraction, using  $2 \times 200$  µl of CHCl<sub>3</sub>. The resulting organic extract was dried, redissolved in 120 µl of methanol, and analysed by LC-MS using 354 nm UV detection and positive/negative ion MS detection.

Kinetic assays. For TamI kinetics, the standard assays contained TamI-RhFRED (3.2  $\mu M$  for TirA assay, 25 nM for TirC assay and 50 nM for TirD assay) and various concentrations of substrates in 190 µl of P450 desalting buffer (50 mM NaH2PO4, pH 7.3, 1 mM EDTA, 0.2 mM dithioerythritol, 10% glycerol). After a pre-incubation at 30 °C for 5 min, the reactions were initiated by adding 10 µl of 50 mM NADPH. For TamL kinetics, the standard assays contained 4 nM of TamL in 198 µl of storage buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.3, 20% glycerol). After pre-incubation at 30 °C for 5 min, the reactions were initiated by adding 2 µl of TirE (in DMSO) at various concentrations. For both assays, reaction termination and analysis were as follows: three aliquots (50 µl) were taken at three time points within the linear range to thoroughly mix with 100 µl of methanol, and the proteins were pelleted by centrifugation at 13,000g, 4 °C for 15 min. The supernatant was subject to HPLC analysis to monitor substrate consumption within the linear range, thereby deducing the initial velocity of the reactions. All measurements were performed in duplicate, and velocities determined under different substrate concentrations were fit into the Michaelis-Menten equation to calculate the kinetic parameters.

Data deposition note. The atomic coordinates and structure factors (PDBID codes 2Y08, 2Y3R, 2Y4G and 2Y3S) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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#### Author contributions

All authors designed experiments and analysed data. J.C., S.L., S.G., Y.A. and D.B. performed the experiments. J.C., S.L., L.P. and D.S. wrote the manuscript. J.C. and S.L. contributed equally to this study.

#### Additional information

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