# Activation of Primary C-H Bonds in Oxidative Cyclizations of Tambjamines Catalyzed by Rieske Oxygenases TamC and *Pt*TamC

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*Supporting Information Placeholder*

**ABSTRACT:** Tambjamines are complex bipyrrole-containing natural products that possess promising bioactive properties. Although *Pseudoalteromonas citrea* is known to produce both cyclic tambjamine MYP1 and the linear precursor (YP1), the biosynthetic machinery used to catalyze the site-selective oxidative carbocyclization at the unactivated 1° carbon of YP1 has remained unclear. Here, we demonstrate that a three-component Rieske system consisting of an oxygenase (TamC) and two redox partner proteins is responsible for this unprecedented activity on YP1 and a non-native substrate (BE-18591). We also show that a homologous oxidase from *Pseudoalteromonas tunicata* (*Pt*TamC) can function together with the partner proteins from *P. citrea* to process both YP1 and BE-18591. These reactions represent the first Rieske oxygenase-catalyzed activations of C-H bonds at 1° carbons. The use of TamC and *Pt*TamC to generate the new-to-Nature cyclic analogue of BE-18591 illustrates the enormous biocatalytic potential of these Rieske systems to facilitate late-stage oxidative cyclizations at terminal C($sp^3$)-H bonds.

Tambjamines are bipyrrolic alkaloids isolated from marine invertebrates[1, 2] and several bacteria.[3-6] These compounds contain a methoxybipyrrole core (MBC) connected to either substituted/unsubstituted enamine groups or decarboxylated amino acids.[1, 2, 7-10] Tambjamines share a pyrrolylpyrromethene skeleton with tripyrrolic prodiginines and marineosins.[11-13] Each of these classes of natural products exhibits an impressive repertoire of bioactive properties including antitumor,[5, 14, 15] antimicrobial,[5, 16] and antimalarial activities[13, 17] owing to their bipyrrole cores and ability to coordinate metals effectively.[18-20]

To date, four tambjamines have been identified from bacteria: BE-18591 **(1)** from the terrestrial *Streptomyces* sp. BA18591[5, 9] and *Streptomyces albus*,[21] YP1 **(2)** from marine bacterium *Pseudoalteromonas tunicata*,[3] the first reported cyclic tambjamine, MYP1 **(3)** from marine bacterium *Pseudoalteromonas citrea*,[4] and an analogue of MYP1, paucibactin A **(4)** from a freshwater bacterium *Paucibacter aquatile* DH15 (**Figure 1A**).[6] MYP1 is encoded by the *tam* gene clusters in *P. tunicata* and *P. citrea*.[4, 11, 22, 23] MYP1 was first isolated from *P. citrea*, although a recent report suggests that *P. tunicata* also produces this compound.[24] The macrocycle in MYP1 appears to be formed by ligation of C19 in the linear alkyl chain and C5′ of the bipyrrole core of YP1 (**Figure 1D**), this reaction requires the unprecedented enzyme-catalyzed activation of a C-H bond at a 1° carbon. Here, we report our efforts to inspect the *tam* gene cluster and characterize this possible macrocyclization event.

*P. citrea* and *P. tunicata* both encode predicted oxygenases that share moderate sequence identity with RedG, a Rieske oxygenase (RO) that catalyzes the oxidative carbocyclization of undecylprodigiosin to streptorubin B (**5**) in *Streptomyces coelicolor* A3(2) (**Figure SI-1**).[4, 11, 25] ROs are typified by N-terminal Rieske [2Fe-2S] clusters that are coordinated by two His residues and two Cys residues. These oxygenases require redox partner enzymes: a flavin-dependent ferredoxin-reductase (and an additional ferredoxin in some cases) to shuttle electrons from NAD(P)H to the catalytic non-heme iron center via the Rieske center of the oxygenase. The reduced Fe(II) then activates $O_2$ to oxidize the substrate (**Figure SI-2**).[26, 27] Both TamC (*P. citrea*) and *Pt*TamC (the homologous oxidase from *P. tunicata*) contain conserved [2Fe-2S] cluster binding motifs and mononuclear non-heme iron domains characteristic of ROs, including a Asp-to-Glu mutation that facilitates electron transfer between the Rieske and the non-heme catalytic centers in ROs that catalyze oxidative carbocyclizations (**Figure SI-1**).[26] Therefore, we hypothesized TamC and *Pt*TamC are ROs responsible for macrocyclizing YP1 to MYP1 in *P. citrea* and *P. tunicata*.[4, 11, 24]

While MYP1 resembles cyclized prodiginine analogues like streptorubin B and deschlororoseophilin (**Figures 1A, 1B**),[12, 26, 28] the macrocyclization in MYP1 connects C19 of the linear alkyl chain in YP1 with the C5′ position of the bipyrrole suggesting C-C bond formation at the terminal methyl group of the alkyl chain (**Figure 1D**). In contrast, the late-stage oxidative carbocyclizations with prodiginine analogues likely involve significantly more stable pyrollic or methylene radicals, although carbocationic intermediates have not been ruled out (**Figure SI-3**).[26] The TamC-catalyzed macrocyclization of YP1 would likely require formation of a far less stable radical/cation following abstraction of a hydrogen/hydride from the 1° carbon at the terminal methyl group of the alkylamine tail.[4] To date, the only other known cyclic tambjamine, paucibactin A, is cyclized at a 2º carbon and bears similar ring topology with most cyclic prodiginines. To probe the macrocyclization reaction of tambjamine YP1 to MYP1, we have reconstituted the activity of TamC and redox co-factors in *E. coli*.

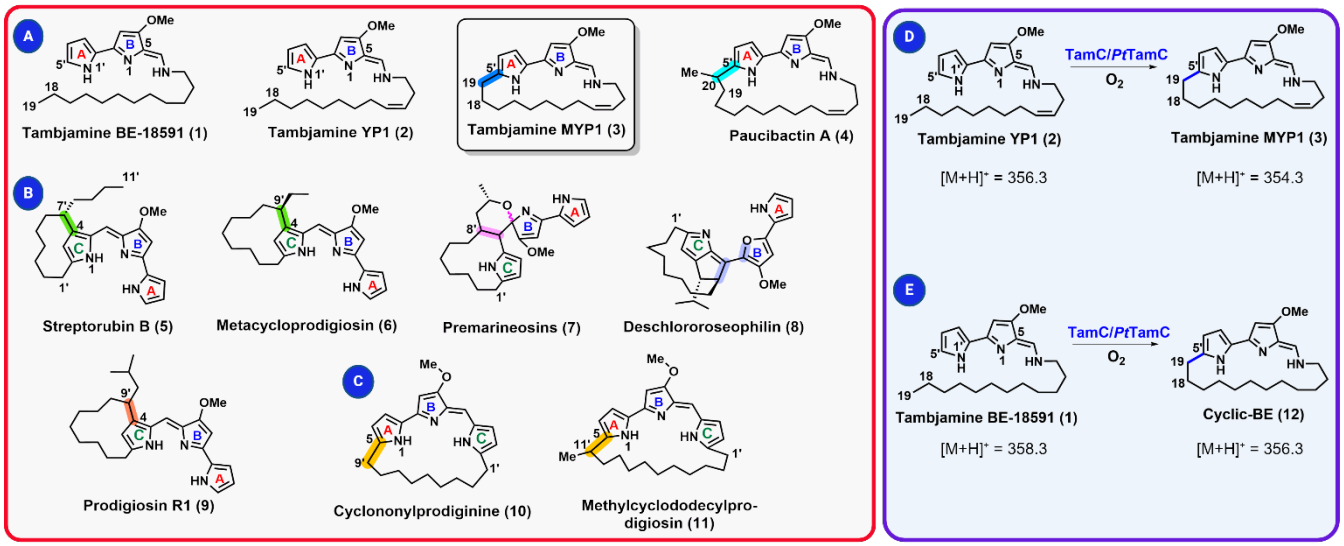


**Figure 1**. **(A)** Bacterial tambjamines include the only known cyclic variants: MYP1 (**3**) and paucibactin A (**4**). **(B)** Cyclic prodiginines with macrocyclization via pyrrole ring C. **(C)** Cyclononylprodiginine (**10**) and methylcyclododecylprodiginine (**11**)[29-31] exhibit connectivities similar to MYP1, but the biosynthesis of these cyclic compounds remains unknown. **(D)** Macrocyclization of YP1 (**2**) to MYP1 (**3**) putatively catalyzed by TamC/*Pt*TamC. **(E)** Macrocyclization of non-native substrate BE-18591 (**1**) to cyclic-BE (**12**) putatively catalyzed by TamC/*Pt*TamC.

To avoid issues of air sensitivity associated with ROs, we first evaluated the role of TamC when heterologously expressed in whole cells of *E. coli*. Since the redox partners were not known, we first attempted to exploit *E. coli's* endogenous electron transport proteins and reducing intracellular environment to reconstitute the activity of the RO enzymes.[32, 33] Following synthesis and purification of BE-18591, a saturated analogue of YP1 (**Figure SI-4**) that has been used previously to assay activity of *tam* biosynthetic enzymes, this compound was fed to *E. coli* Lemo21(DE3) cells overexpressing TamC or *Pt*TamC. No cyclic analogue of BE-18591 (cyclic-BE; **Figure 1E**) could be detected from these experiments (**Figure SI-5**). This suggested that BE-18591 is not accepted by either TamC and/or that more specific Rieske redox partners are required for TamC.

Based on recent efforts to identify redox partner genes of ROs,[34, 35] we searched for sequences with existing annotations based on conserved domains for 'ferredoxin' and 'ferredoxin-reductase' in the genomes of *P. citrea* and *P. tunicata*. Additionally, we performed a BLASTP[36] search against these genomes to look for unannotated sequences with similar functionalities. Accordingly, a ferredoxin and a ferredoxin-reductase were identified from *P. citrea* as potential redox partners of TamC, whereas two putative ferredoxins and a ferredoxin-reductase were identified in the *P. tunicata* genome as potential partners for *Pt*TamC (**Table SI-1**). We initially focused on assessing the activity of TamC by using the pETDuet platform to co-express TamC with the ferredoxin (*Pc*Fdx) and ferredoxin-reductase (*Pc*Red) from *P. citrea* in *E. coli* Lemo21(DE3) cells (**Figure SI-6**). When cells expressing these three enzymes were fed BE-18591 and incubated for 3 hours, an $[M+H]^+$ ion of m/z = 356.3 was identified in extracts, consistent with the formation of cyclic-BE (**Figure 2A**). The fragmentation pattern obtained by MS/MS further supported the assignment of this ion as a cyclic tambjamine (**Figure SI-7**).

Interestingly, a minor peak with the same m/z was also observed in these extracts, suggesting that two isomers were being formed by the TamC-catalyzed reaction of BE-18591. The fragmentation pattern of this minor peak was consistent with that of a linear tambjamine (**13; Figure 2A**) with an unsaturation present within the alkyl chain. While we hypothesize that this corresponds to a terminal alkene (**Figure 2A and Figure SI-7**), attempts to isolate sufficient quantities of this material for structural characterization by NMR have been challenging due to low yields of the linear analogue. Nonetheless, the formation of two previously unreported tambjamines by TamC suggests some plasticity in the substrate scope of these enzymes and may hint at opportunities to employ engineered ROs enzymes as useful biocatalysts.

Encouraged by the observed cyclization of BE-18591 by TamC and its redox partners, we sought to test the activity of the TamC Rieske system on the presumed native substrate: YP1. We synthesized YP1 by converting the alcohol (*Z*)-dodec-3-en-1-ol to (*Z*)-dodec-3-en-1-amine and condensing this amine with MBC (**Scheme SI-1, Figure SI-8**).[37-39] When *E. coli* cells expressing all three components of the Rieske system were fed YP1, two product ion peaks with $[M+H]^+$ ions of m/z = 354.3 (the expected mass of MYP1) were observed (**Figure 2B**). Both peaks showed fragmentation patterns consistent with cyclic tambjamines (**Figures SI-9**).

Comparing these two product peaks with extracts from the native producers of MYP1 (*P. citrea* and *P. tunicata*) showed that the major peak from the whole cell *E. coli* assay had the same retention time as MYP1 obtained from *P. citrea*. On the other hand, the retention time of MYP1 obtained from *P. tunicata* extracts was found to match the minor peak obtained from the *E. coli* assay (**Figure 3**). Both prodiginines and tambjamines have been reported to exist as separable rotamers due to restricted rotation about the methylene/imine bond (**Figure 4**), and these different conformational states will have implications for biological activity based on the differential preferences for anion transport and/or cation binding.[40-43]

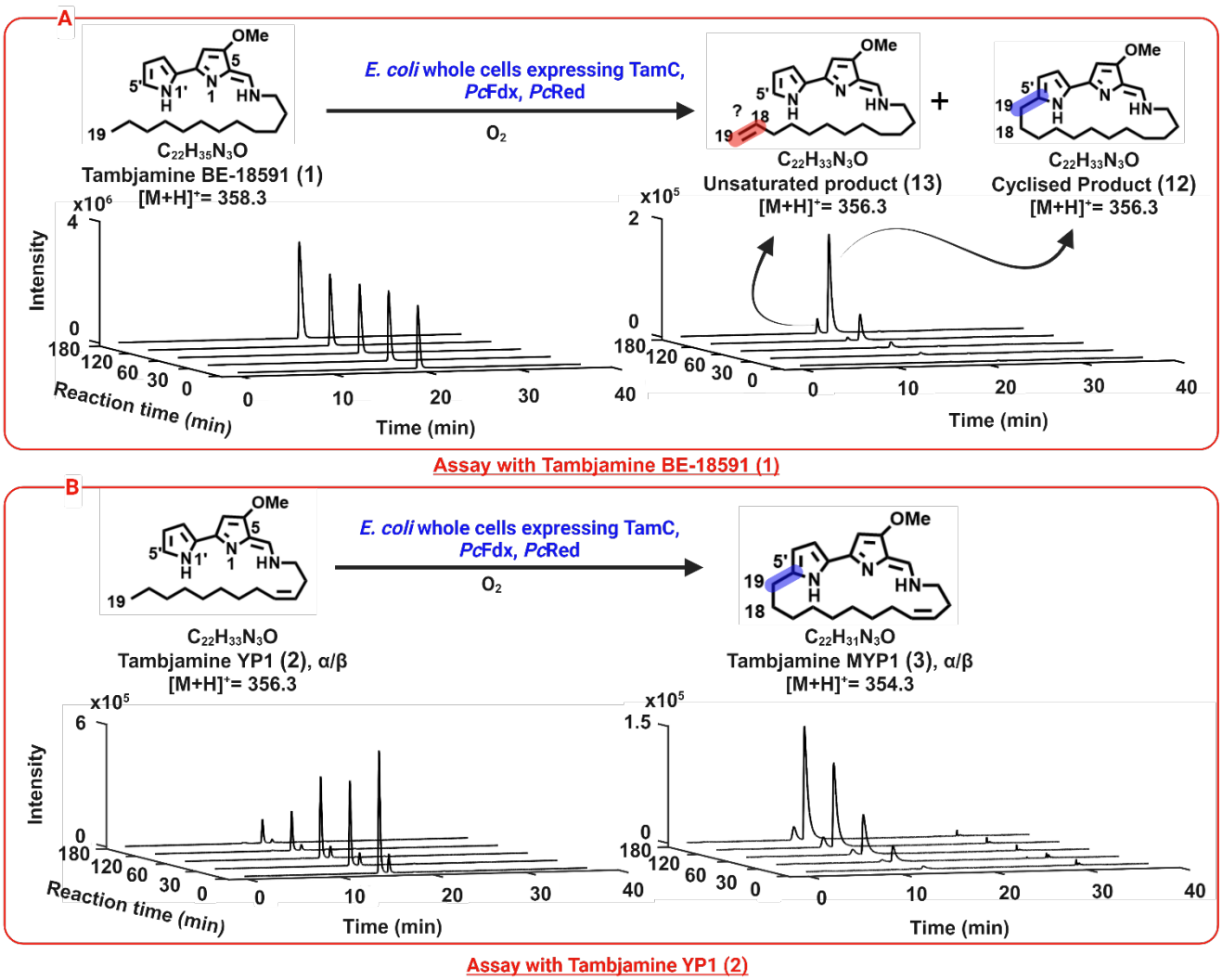


**Figure 2**. **(A)** Chromatograms demonstrating that TamC converts BE-18591 (**1**) into a cyclic tambjamine (major) and a linear one (minor). **(B)** Chromatograms demonstrating that TamC converts YP1 (**2**) (present as α (*E*) and β (*Z*) rotamers)[40] into two new peaks with the expected mass of MYP1 (m/z = 354.3). Fragmentation patterns suggest both peaks are cyclic tambjamines.

To evaluate the possibility that the peaks produced in the *E. coli* assay correspond to different rotamers of MYP1, we isolated the tambjamine produced by *P. tunicata* to elucidate the structure. The chromatogram taken immediately after isolation of the compound indicated that it had a different retention time to the MYP1 of *P. citrea* **(Figures 3A, 3B, and 3C)**, while NMR spectra **(Table SI-2)** were nearly indistinguishable from the MYP1 isolated from *P. citrea*.[4] After a month of storage at -20 ºC, the retention time of the isolated *P. tunicata* tambjamine shifted to match the MYP1 peak from *P. citrea* (**Figures 3A, 3C, and 3D**). We interpret this as evidence that the isolated tambjamines are interconvertible rotamers of MYP1. Consequently, we postulate that the whole cell *E. coli* assay and the native producers both make MYP1: the whole-cell assay results in both rotamers being produced, whereas *P. tunicata* and *P. citrea* each produce a single rotamer. Since tambjamines are involved in the chemical defense of their native producers,[42, 44] *P. citrea* and *P. tunicata* may each produce a single rotamer in response to different environmental stressors, unlike the production of both rotamers observed in *E. coli* under assay conditions. To the best of our knowledge, this represents the first demonstration of homologous enzymes following homologous pathways producing different rotamers of the same complex natural product.

We then evaluated whether *Pt*TamC can also catalyze the macrocyclization of BE-18591 and YP1 with the aid of redox partners from *P. citrea* by co-expressing *Pt*TamC with *Pc*Fdx and *Pc*Red in *E. coli*. These experiments revealed *Pt*TamC produces the same two isomeric products from both BE-18591 and YP1 when paired with *Pc*Fdx and *Pc*Red (**Figures SI-10**). These results underscore the importance of choosing redox partner enzymes of closely related ROs for activity of the oxygenases in the Rieske family.[45, 46]

RO enzymes involved in prodiginine oxidative cyclizations are presumed to have evolved to enforce a *cis* conformation that favors cation- and anion-binding in the bioactive cyclic prodiginines.[43, 47] Cyclic tambjamine analogues, likewise, could possess these conformational advantages resulting in increased bioactivity. Therefore, TamC might eventually be leveraged as a biocatalyst to generate other bioactive cyclic tambjamines in a facile manner.

Biocatalysts including cytochrome P450s,[48, 49] non-heme iron α-ketoglutarate-dependent enzymes[50] have been used in the activation of primary C($sp^3$)-H bonds. Other ROs[51, 52] have been successfully used as C-H activating biocatalysts in chemoenzymatic routes towards synthetically challenging molecules. Recently, Liu et al. demonstrated the importance of the substrate access tunnel and the flexible loop in ROs (conserved regions in characterized ROs) in influencing of substrate scope, chemoselectivity, and altered reactivity.[51] The TamC Rieske system represents a unique platform that brings about primary C-H bond activation to form C-C bonds in late-stage macrocyclization reactions offering a multitude of

advantages[53-55] over chemical methods for the synthesis of complex bioactive molecules. Additionally, the formation of a putative terminal alkene by TamC merits further study into the useful exploitation of the enzyme's activity and selectivity as a starting point to engineer enzymes that activate primary carbons.

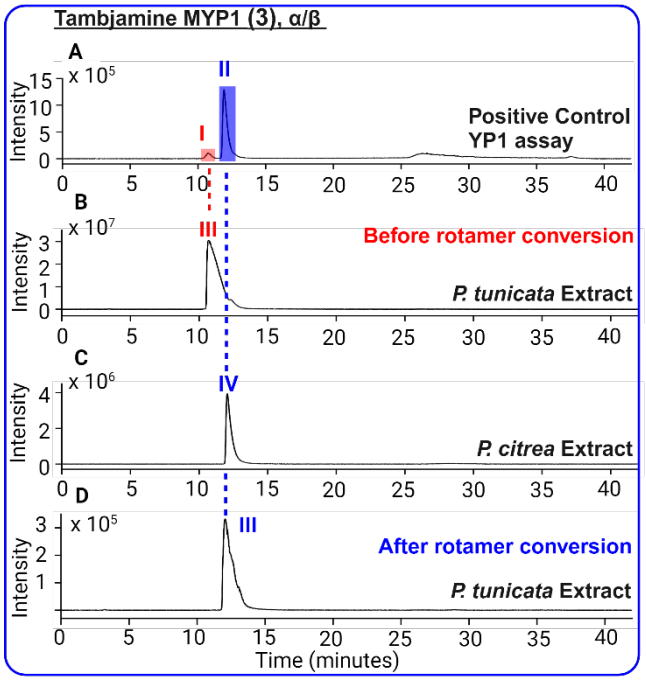


**Figure 3**. Characterization of the products from the YP1 assay. Peaks correspond to an $[M+H]^+$ ion of m/z = 354.3. **(A)** Two MYP1 peaks are formed in the YP1 assay. **(B)** *P. tunicata* extract shows a single MYP1 peak that corresponds to minor peak (I). **(C)** *P. citrea* extract shows one MYP1 peak that corresponds to the major peak (II). **(D)** After four weeks of storage, the *P. tunicata* extract shows one MYP1 peak that corresponds to the major peak (II).

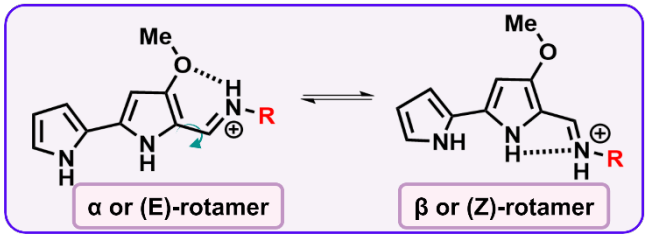


**Figure 4**. The rotamer conformations of general tambjamine structures.

In conclusion, we have functionally reconstituted the multicomponent Rieske oxygenase system, comprising TamC, *Pc*Fdx and *Pc*Red from *P. citrea* in *E. coli*. TamC cyclizes YP1 to generate two different rotamers of MYP1, and converts BE-18591 to two new-to-Nature tambjamines: cyclic-BE and an as-of-yet uncharacterized linear isomer. Collectively, these results represent the first demonstration of a Rieske system capable of activating C-H bonds at 1° carbons in oxidative cyclization reactions. While the initial characterization reported here has demonstrated the role of ROs in the biosynthesis of tambjamine natural products, efforts are underway to characterize the full potential of TamC as a biocatalyst for C-H activation and carbocyclizations.

## ASSOCIATED CONTENT

### Supporting Information

Experimental and synthetic procedures, HRMS spectra, MS/MS analysis, NMR spectra (PDF) are included in the supporting information. The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interests.

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## DEDICATION

This paper is dedicated to our dear colleague Dr. Françoise Sauriol in memory of her outstanding career in NMR and her dedication to the Queen's Department of Chemistry and particularly its students.

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