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Research Article

Bacterial Diterpene Synthases Prenylate Small Molecules

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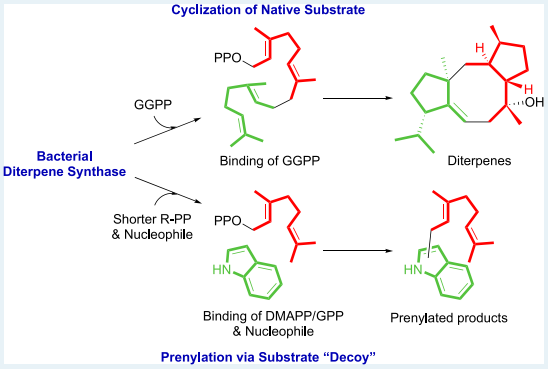
ABSTRACT: The biosynthesis of terpenoid natural products begins with a carbocation-based cyclization or prenylation reaction. While these reactions are mechanistically similar, there are several families of enzymes, namely, terpene synthases and prenyltransferases, that have evolved to specifically catalyze terpene cyclization or prenylation reactions. Here, we report that bacterial diterpene synthases, enzymes that are traditionally considered to be specific for cyclization, are capable of efficiently catalyzing both diterpene cyclization and the prenylation of small molecules. We investigated this unique dual reactivity of terpene synthases through a series of kinetic, biocatalytic, structural, and bioinformatics studies. Overall, this study unveils the ability of terpene synthases to catalyze *C*-, *N*-, *O*-, and *S*-prenylation on small molecules, proposes a substrate decoy mechanism for prenylation by terpene synthases, supports the physiological relevance of terpene synthase-catalyzed prenylation in vivo, and addresses questions regarding the evolution of the prenylation function and its potential role in natural product biosynthesis.



KEYWORDS: *terpene synthase, prenyltransferase, prenylation, substrate decoy, natural product*

INTRODUCTION

Terpenoids are the largest and most structurally diverse family of natural products with over 80,000 known members.[1] They are ubiquitous among all domains of life, are essential constituents of both primary and secondary metabolism, and have a wide range of biological activities. In nature, terpenoids play important ecological roles in membrane stability, photosynthesis, communication among species, and chemical defense mechanisms.[2,3] In medicine, terpenoids have been developed into some of the most successful and important clinically used pharmaceuticals with taxol and artemisinin representing the gold standards of natural product drug development.[4−6]

The chemodiversity of terpenoid natural products arises through an array of biosynthetic mechanisms including prenyltransfers, regio- and stereoselective cyclizations, attachments to a variety of other chemical scaffolds, and additional tailoring reactions.[3,7,8] All terpenoids are constructed from a series of allylic diphosphates that are used as substrates for cyclization reactions or as prenyl donors to alkylate a multitude of chemical scaffolds (i.e., prenylation). These two central biosynthetic steps, cyclization and prenylation, are catalyzed by terpene synthases (TSs) and prenyltransferases (PTs), respectively.

Mechanistically, type I terpene synthases (TSs) and prenyltransferases (PTs) trigger catalysis following the same general strategy: generation of an allylic carbocation via abstraction of the diphosphate moiety.[8−10] Once the carbocation is formed, the fate of the cation is controlled by the enzyme to determine whether a cyclization or prenylation reaction occurs (Figure 1). In cyclization reactions, the terpene chain folds into a conformation allowing an electron-rich olefin direct access to intramolecularly attack the carbocation. The final carbocation quench is achieved by an elimination reaction forming an alkene or a nucleophilic attack, most commonly by a water molecule in the active site. In most of the prenylation reactions, the initial carbocation is directly quenched through a nucleophilic attack of a small molecule or protein. Other TSs, such as the canonical type II TSs and several families of noncanonical TSs, also utilize carbocation chemistry to drive cyclization reactions.[8,9,11]

Three major families of enzymes utilize diphosphate abstraction to catalyze terpene cyclization or prenylation reactions. The most well-known and well-studied are the type I TSs (hereafter referred to as TSs). TSs, which have a characteristic all-α-helical structural fold and two conserved metal-binding motifs, require a trinuclear Mg^{2+} cluster to abstract the diphosphate group.[8,12] Prenylation reactions on small molecules are catalyzed by two different classes of enzymes: UbiA PTs and ABBA PTs (Figure 1). UbiA PTs are membrane-bound enzymes that are responsible for the

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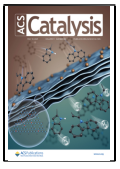

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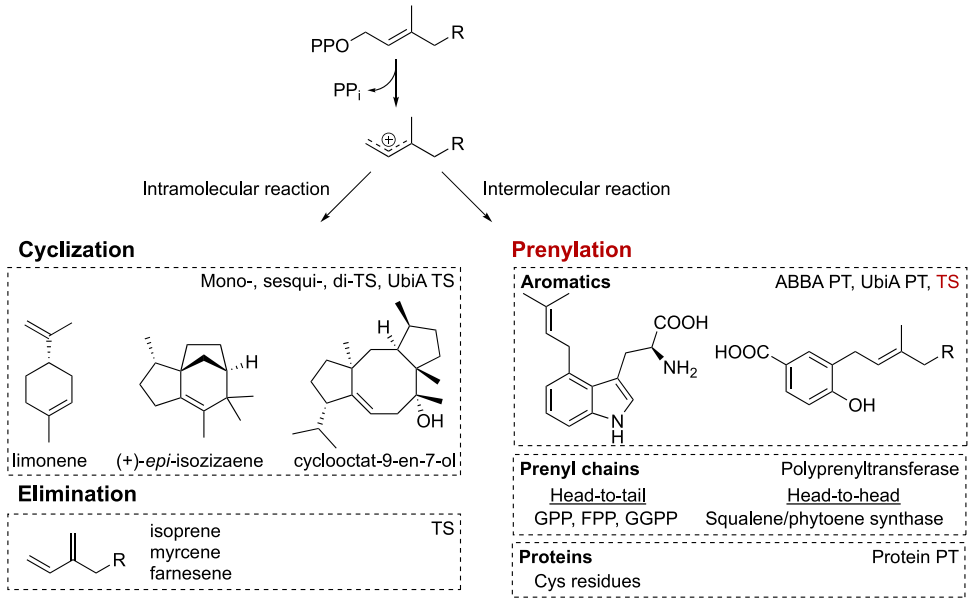

Figure 1. Biosynthesis of terpenoids. Prenyl diphosphates undergo diphosphate abstraction to form a carbocation intermediate in the active site of terpene-related enzymes. These reactive intermediates are then used for both intramolecular reactions (e.g., cyclization) and intermolecular reactions (e.g., prenylation). In this study, TSs are shown to catalyze the prenylation of small molecules.

prenylation of aromatic, and typically charged, substrates (e.g., *p*-hydroxybenzoate in ubiquinone biosynthesis).[13] Despite significant sequence disparity between TSs and UbiA PTs, they share high structural homology, have two Asp-rich metal-binding motifs (albeit different from those found in TSs), and require divalent cations for activity.[8,13] ABBA PTs, which are also known as aromatic PTs, are completely unique in sequence and structure, and not all ABBA PTs require divalent cations for catalysis.[8,14] While TSs and ABBA PTs are solely known for their cyclization and prenylation abilities, respectively, there are recent examples of UbiA cyclases (Figure 1).[11,15] Given their structural and functional similarities to TSs, it was not entirely surprising that the members of the UbiA family of enzymes were able to catalyze cyclization or prenylation reactions. At the outset of this study, TSs were not generally considered to be able to catalyze direct prenylation reactions, although there are a few known examples in natural product biosynthesis (see the discussion section) and there are proposals that a single TS may catalyze both cyclization and prenylation.[3,16] However, during manuscript preparation, a few fungal and plant sesqui- and di-TSs were reported to primarily prenylate indole.[17]

Here, we report our discovery that a previously uncharacterized di-TS is capable of efficiently catalyzing both diterpene cyclization and the prenylation of small molecules. We tested other functionally characterized mono-, sesqui-, and di-TSs for this cryptic prenylation ability and found that di-TSs were the most efficient PTs and capable of accepting a variety of prenyl donors and acceptors. The selected and tested di-TSs showed different levels of catalytic efficiencies and regioselectivities when administered different prenyl acceptors. We investigated this unique dual reactivity of TSs through a series of studies including kinetic experiments, structural examination and ligand docking, and sequence and phylogenetic analysis of TSs and PTs.

RESULTS AND DISCUSSION

Discovery of a Diterpene Synthase that Catalyzes Both Cyclization and Prenylation. In an effort to discover new terpene skeletons produced by bacteria, we routinely test TSs (Supporting InformationTables S1−S3) against a panel of prenyl diphosphates [i.e., geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP)]. HPLC analysis of our initial test reactions with TS29, a presumed di-TS from *Streptomyces* sp. CB02400[18] with 51.8% sequence identity to spata-13,17-diene synthase (Figure S1),[19] and GPP revealed two products, geraniol (**1**) and an unknown compound **2** (Figure 2A). Product **2** was obtained from a large-scale enzyme reaction, and its structure was determined using NMR spectroscopy and LC−MS (Supporting Results, Table S4, and Figures S2−S6). We were surprised to find that **2** was *S*-geranyldithiothreitol (**2**), a C_{10} prenylation adduct of dithiothreitol (DTT) (Figure 2A). DTT is a common additive used to stabilize enzymes and has been used in reactions containing TSs with no reports of being prenylated.[20−22] The addition of EDTA to the reaction mixture precluded formation of both **1** and **2**, supporting that the formation of **1** and prenylation of DTT is enzyme-catalyzed and requires Mg^{2+}-dependent diphosphate abstraction; in the absence of DTT, TS29 only produces **1** (Figure 2A). No significant differences were observed when the enzyme reaction was performed under mildly acidic or alkaline conditions (Figure 2A). DTT was not prenylated with FPP (data not shown) or GGPP (Figure 2B) in our test reactions with TS29. Incubations of TS29 with GGPP revealed two known products, cneorubin Y (**3**) and spata-13,17-diene (**4**) (Figure 2B, Supporting Results, and Figures S7−S17). It is worth noting that TS29 produced **3**:**4** in a ratio of 1.4:1, while spata-13,17-diene synthase produced **3**:**4** in a ratio of 1:7 with another minor product, prenylkelsoene, also being present.[19] Based on these findings, we hypothesized that TSs use their natural prenyl diphosphates for cyclization reactions and can use shorter prenyl diphosphates for prenylation reactions, provided that a nucleophile is available and able to bind in the active site (Figure 2C).

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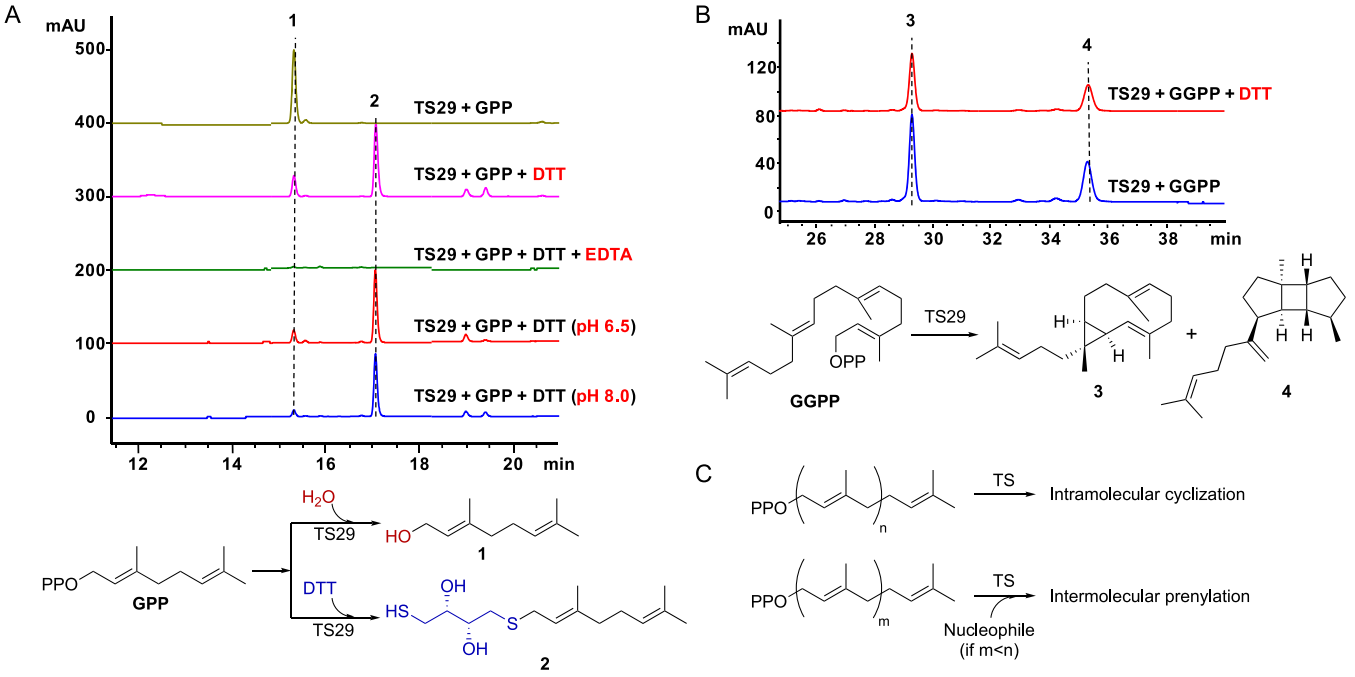

Figure 2. Discovery of di-TS-catalyzed prenylation. (A) TS29 prenylation activity with GPP and DTT. (B) TS29 cyclization activity with GGPP. (C) Hypothesis proposing that TSs catalyze prenylation when incubated with prenyl diphosphates that are shorter than their native substrates. Enzyme assays were analyzed by HPLC at 210 nm.

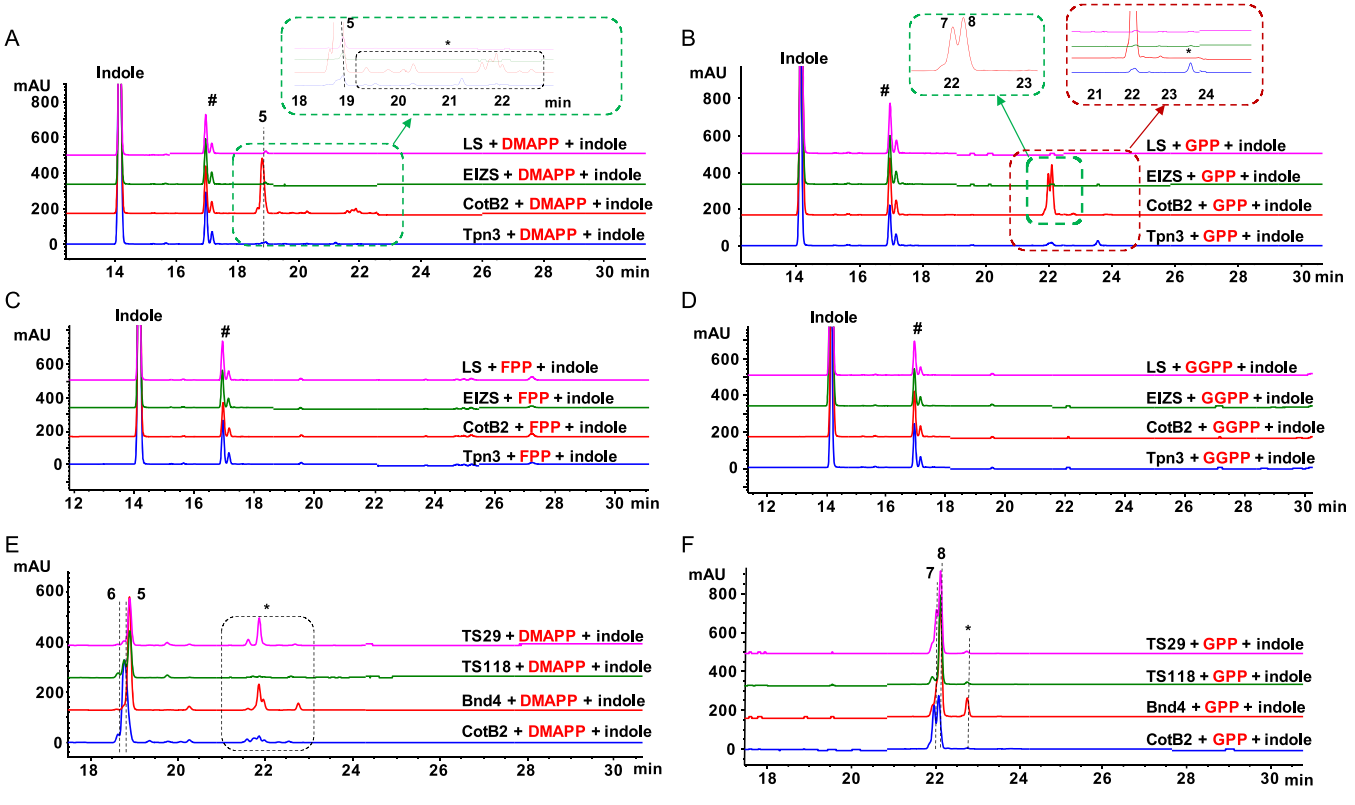

Figure 3. Analysis of prenylation activities with selected mono-, sesqui-, and di-TSs. Four TSs were incubated with indole and prenyl diphosphates (A–D, C_5–C_{20}). Four di-TSs incubated with indole and (E) DMAPP or (F) GPP. Enzyme assays were analyzed by HPLC at 280 nm. Enzyme products labeled with numbers were structurally characterized (see Figure 4B); enzyme products labeled with asterisks (*) were uncharacterized; peaks labeled with hash marks (#) denote impurities in the indole sample.

Diterpene Synthases Are Most Effective at Catalyzing Prenylation. To test our hypothesis that TSs are capable of catalyzing prenylation reactions with shorter prenyl diphosphates, we first examined the prenylation ability of four different families of type I TSs. We selected three characterized mono-, sesqui-, and di-TSs, namely, limonene synthase (LS),[23]

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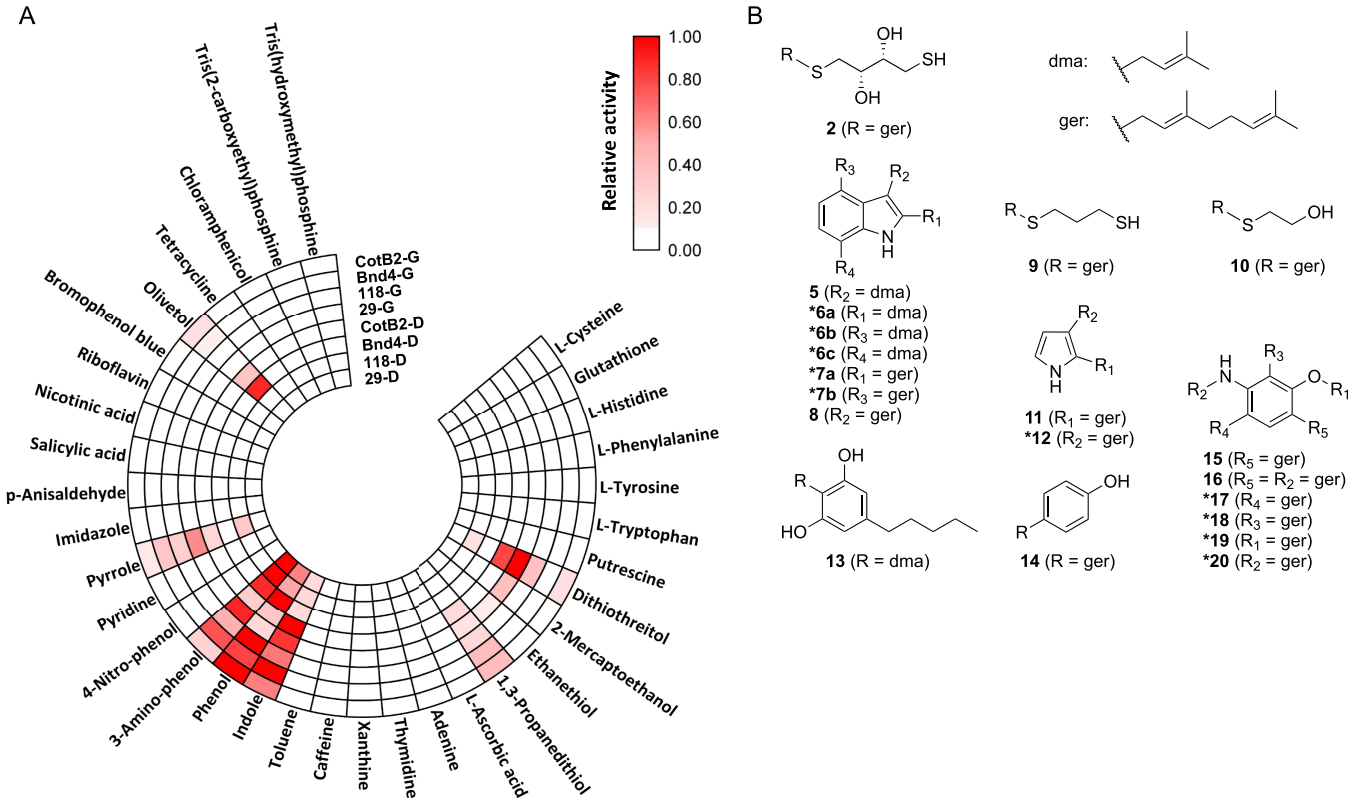

Figure 4. Substrate scope of di-TS prenylation reactions. (A) Heat map of relative activities for selected nucleophiles. (B) Structures of prenylated enzyme products isolated in this study. Compounds labeled with asterisks (*) indicate that structures were proposed based on limited spectroscopic data (see the Supporting Results).

epi-isozizaene synthase (EIZS),[24] and cyclooctat-9-en-7-ol synthase (CotB2),[25] respectively, that act directly on the acyclic prenyl diphosphates; we also selected terpentetriene synthase (Tpn3), a type I di-TS that catalyzes the diphosphate elimination of terpentedienyl diphosphate, a clerodienyl-type bicyclic substrate (Figure S1).[26] To facilitate detection of enzyme prenylation, we used indole as the prenyl acceptor. Incubation of each enzyme with indole and a C_5−C_{20} prenyl donor (Figure 3A−D) revealed that other TSs can indeed prenylate indole when dimethylallyl diphosphate (DMAPP) or GPP is present. CotB2 was the most efficient enzyme at catalyzing prenylation, accepting both DMAPP and GPP and producing several prenylation products. Tpn3 also produced several hemi- and monoprenylation products but at much lower levels. Both LS and EIZS showed minor prenylation activity with DMAPP but produced very little to no detectable product with GPP. No prenylation activity was seen for any enzyme when incubated with FPP or GGPP. Four polyprenyltransferases, namely, GPP synthase, FPP synthase, GGPP synthase, and geranylfarnesyl diphosphate (GFPP) synthase, were also tested but did not show prenylation activity with indole and DMAPP or GPP (Figure S18).

The realization that CotB2 was much more efficient at catalyzing prenylation than the other TSs tested led us to test additional type I di-TSs that accept GGPP as their native substrate. TS29, CotB2, the recently reported benditerpe-2,6,15-triene synthase Bnd4,[27] and an additional unpublished di-TS from our lab, TS118, were tested for prenylation activity with indole and DMAPP or GPP (Figure 3E,F). All four di-TSs effectively catalyzed prenylation. Interestingly, each enzyme showed different product profiles, suggesting that each enzyme not only has a different level of prenylation activity but also likely preferred regioselectivities. Isolation and spectroscopic characterization of major products **5**−**8** revealed that the major products were indoles with single prenyl units at various carbon positions of the indole ring (Figure 3, Supporting Results, and Figures S19−S31). These initial experiments led us to conclude that (i) prenylation activity of TSs is likely a widespread characteristic among TSs, (ii) TSs that accept larger substrates (e.g., $\geq C_{20}$) are more efficient at catalyzing prenylation than their counterparts that use shorter substrates, and (iii) the active sites of TSs must control the regioselectivity of prenylation.

Diterpene Synthases Have a Broad, but Selective, Substrate Scope for Prenylation. To investigate the substrate scope of TS-catalyzed prenylation reactions and to probe if this activity may have some physiological relevance, we tested CotB2, TS29, Bnd4, and TS118 for prenylation activity with 34 potential substrates. These 34 compounds included amino acids, reductants, amines, nucleobases, aromatic compounds, heterocyclic aromatics, vitamins, and antibiotics (Figure 4A). In addition to **2** and **5**−**8**, we isolated 12 additional enzymatic products (**9**−**20**) and determined the structures of **9**−**11** and **13**−**16**; due to minor amounts of **12** and **17**−**20**, we proposed their structures based on limited NMR experiments (Figure 4B, Supporting Results, Tables S4−S6, and Figures S32−S64). Overall, *C*-, *N*-, *O*-, and *S*-prenylation products were found. Among the tested compounds, thiols and small aromatic compounds were the most efficiently transformed; charged compounds were not accepted, even those (e.g., Cys or Trp) with similar nucleophilic groups to compounds that were previously

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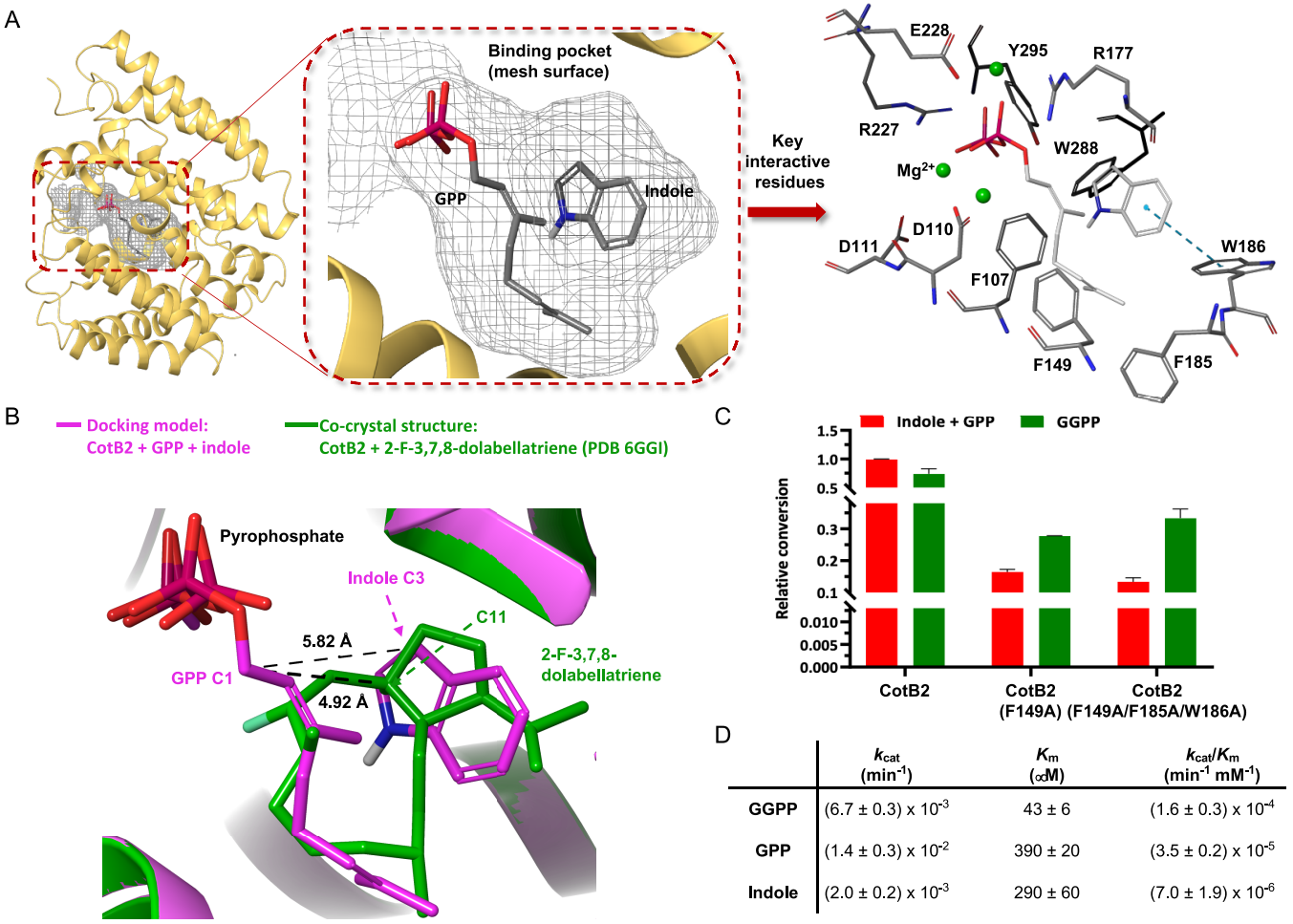

D

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} mM^{-1})
GGPP	(6.7 ± 0.3) x 10^{-3}	43 ± 6	(1.6 ± 0.3) x 10^{-4}
GPP	(1.4 ± 0.3) x 10^{-2}	390 ± 20	(3.5 ± 0.2) x 10^{-5}
Indole	(2.0 ± 0.2) x 10^{-3}	290 ± 60	(7.0 ± 1.9) x 10^{-6}

Figure 5. Structural and kinetic analysis of the prenylation activity of CotB2. (A) Docking model of GPP and indole in CotB2 showing overview and zoomed-in perspectives. PDB 6GGI was used as the CotB2 model. (B) Overlay showing the GPP and indole docking model with the crystal structure of CotB2 in complex with 2-*F*-3,7,8-dolabellatriene. (C) Relative cyclization (GGPP) and prenylation (GPP + indole) activities of native CotB2 and mutants. (D) Kinetic parameters of CotB2 cyclization (GGPP) and prenylation (GPP + indole).

prenylated (e.g., DTT or indole) (Figure 4A). It is noteworthy that 4-nitrophenol was not accepted, while phenol and 3-aminophenol were good prenyl acceptors, suggesting that substrates with strong electron-withdrawing substituents can prevent prenylation. Likewise, pyrrole was easily prenylated, whereas pyridine and imidazole were not. During these studies, we reasoned that olivetol, a neutral phenol, may be easily prenylated by TSs and produce cannabigerol, the decarboxylated version of the cannabinoid central precursor cannabigerolic acid (CBGA); in *Cannabis sativa*, geranylation naturally occurs on olivetolic acid and is catalyzed by cannabigerolic acid synthase.[28,29] While olivetol was not prenylated with GPP, 4-dimethylallylolivetol (**13**), which was found in studies with the ABBA PT SCO7190,[30] was formed.

We propose that prenylation by TSs is mediated through a combination of active site architecture, where binding and orientation of the prenyl acceptor are controlled, and substituent electronic effects that direct the site of alkylation. While we only isolated the major compounds for each enzyme reaction, it is interesting to note that only one diprenylated product was isolated, and no reverse-prenylated products were detected. This suggests that the active site size and shape preclude multiple prenylation reactions as well as nucleophilic attack at C3 of the prenyl diphosphate; however, it is possible that some of the minor compounds are reverse prenylation products.

TSs, due to their inherent prenylation activity, may make an excellent starting scaffold for theoretical studies coupled with rationale mutagenesis or directed evolution to develop a series of catalytic tools for prenylation reactions, as has been previously done with ABBA PTs.[31−33] The substrate binding pockets of TSs are already optimized to bind various lengths of prenyl diphosphates, and their accommodation of a shorter prenyl donor and a putative hydrophobic prenyl acceptor is now shown to be possible. Logic should dictate that prenylation reactions with longer prenyl donors or larger nucleophiles would also be possible in TSs that inherently accept longer (>C_{20}) prenyl diphosphates for cyclization reactions. At the very least, it is clear that future TS cyclization reactions should not include potential competing nucleophiles such as DTT or 2-mercaptoethanol; tris(2-carboxyethyl)-phosphine (TCEP), due to its inactivity in our assay, may be a good alternative if reducing agents are required.

Docking Studies and Mutational Analysis Support the Substrate Decoy Mechanism for Prenylation by TSs. The ability of TSs to prenylate small aromatic and thiol molecules drove us to ask what is the relationship between cyclization and prenylation activities of di-TSs. We selected CotB2 as a model di-TS as it and its reaction mechanism have

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been extensively characterized by structural characterization, mutagenesis, and theoretical calculations.[34] In the CotB2-GGSPP complex structure (PDB ID 5GUE), GGSPP, an *S*-thiolodiphosphate analogue of GGPP, is bound in a twisted conformation with the diphosphate group coordinated to the Mg^{2+} ions, and the alkyl chain folded into a conformation that provides regio- and stereoselective cyclization to proceed.[35] We performed docking experiments using the structure of CotB2 in its closed conformation with three co-crystallized Mg^{2+} ions (PDB ID 6GGI)[20] and the prenylation reaction ligands GPP and indole. In the model, both substrates fit well into the GGPP binding pocket with the diphosphate of GPP located near the three Mg^{2+} ions and indole positioned deep in the hydrophobic binding pocket with W186 providing a key $\pi-\pi$ interaction (Figure 5A). By overlaying our docked GPP/indole model with that of CotB2 in complex with 2-fluoro-3,7,8-dolabellatriene (F-Dola) and the cleaved-off diphosphate, it is evident that our model of prenylation mimics the active site during cyclization (Figure 5B). Specifically, GPP binds in a similar manner to that of GGPP with its diphosphate moiety coordinated to the trinuclear metal center and the alkyl chain positioned over C-1−C-7 of F-Dola; indole is positioned over C-10−C-15 of F-Dola. The distance between C-1 of GPP and C-3 of indole is ∼5.8 Å, indicating that a minor conformational or positional change may be required for nucleophilic attack. Comparison of our CotB2-GPP-indole model and the crystal structure of FgGS in complex with inorganic pyrophosphate and imidazole, the latter of which was previously suggested to represent the indole binding site,[17] revealed that imidazole is located very near to the pyrrole ring of indole (Figure S65). As would be expected with two proteins sharing only 22% sequence identity over 48% coverage, not all active site residues are conserved between CotB2 and FgGS. This comparison supports that the prenyl acceptor binding site is similarly located in CotB2 and FgGS, although the exact binding mode is dependent on the shape of the active site cavity and its surrounding residues.

We constructed a small set of CotB2 mutants in an effort to determine active site residues that were important for prenylation activity. Three mutants, namely, D110A/D111A/D113A, N220A/S224A/E228A, and D110A/D111A/D113A/N220A/S224A/E228A, were made to assess the expected importance of the DDxxD and NSE motifs. Unfortunately, these mutants were insoluble and precluded activity assays; however, the inhibition of both cyclization and prenylation by the addition of EDTA conclusively supports that Mg^{2+} ions are required for prenylation (Figure 2A). Two other mutants, F149A and the triple mutant F149A/F185A/W186A, were constructed to determine the importance of the aromaticity of the binding pocket while intentionally creating a larger pocket for an extended prenyl donor (i.e., FPP or GGPP). The relative activities of both mutants decreased for both their cyclization reaction with GGPP and prenylation reaction with GPP and indole (Figure 5C). These studies suggested that both cyclization and prenylation are likely controlled by the same residues within the catalytic domain. Thus, the hydrophobic pocket, which consists of mostly aromatic residues as in most of the TSs, not only serves to shape the prenyl chain of GGPP for cyclization but can also recruit small molecules to trap the reactive carbocation, i.e., prenylation reactions. Overall, our CotB2 prenylation model indicates a substrate decoy-like catalytic mechanism[36] where GPP is a small and relatively unreactive substrate, at least in the framework of a di-TS cyclization reaction, and indole functions as a substrate decoy and carbocation trap. Based on the alkyl chain of GGPP and the hydrophobic pocket, it is unsurprising that small, uncharged, and mostly aromatic compounds would be good prenyl acceptors in a TS prenylation reaction.

Enzyme Kinetic Experiments Support the Possibility of In Vivo Activity. An important question of the prenylation activity of TSs is whether or not this occurs in vivo or is just an artifact of in vitro enzyme reactions. To address this, we first tested whether pH influenced the cyclization and prenylation reactions of CotB2. There was not a significant difference between the optimal pH values for cyclization (7.0) and prenylation (7.5) (Figure S66). This supports that both cyclization and prenylation can occur in physiological pH and is in contrast to the prenylation activity switch seen for AaTPS when prenylation only occurred at pH values greater than 7.[17] Next, we determined the kinetic parameters of CotB2 under steady-state conditions using a nonlinear fit of initial velocities versus [GGPP] (Figure S67). The values of k_{cat} and K_m were determined to be $(6.7 \pm 0.3) \times 10^{-3}$ s^{-1} and 43 ± 6 μM ($k_{cat}/K_m = 1.6 \times 10^{-4}$ s^{-1} μM^{-1}), respectively. We then determined the kinetic parameters of GPP and indole under nonvaried substrate saturating conditions revealing their K_m values to be ∼9-fold (390 ± 20 μM) and ∼7-fold (290 ± 60 μM) higher and their k_{cat}/K_m values ∼4-fold (3.5×10^{-5} s^{-1} μM^{-1}) and ∼23-fold (7.0×10^{-6} s^{-1} μM^{-1}) lower, respectively, than those of GGPP. These values matched reasonably well with the values calculated for prenylation by AaTPS.[17] While these values support that CotB2 is more efficient at catalyzing diterpene cyclization, the relatively small (<25-fold) differences in kinetic parameters support that prenylation by CotB2, and other TSs, is possible in vivo. As potential small molecule nucleophiles including indole, polyketides, and thiols are present in cells, it is reasonable to consider that prenylation by TSs may at least occur to a minor extent in native hosts.

The prenylation of indole by AaTPS in *Escherichia coli* (*E. coli*), a heterologous host, supports that TS-catalyzed prenylation can indeed occur in vivo,[17] although whether it occurs in its native host is unknown. It was postulated that the in vivo prenylation activity of TSs in *E. coli* was a chemoprotective function that regulated the concentration of prenyl diphosphates below toxic thresholds.[17] A similar phenomenon was seen when the mevalonate pathway was first engineered into *E. coli* to increase isoprenoid production: the accumulation of IPP resulted in cell toxicity, while the addition of a TS prevented toxicity, likely by channeling IPP to a nontoxic terpenoid.[37,38] These isoprenoid pathways in *E. coli* created, by design, an unnaturally high level of isoprenoids. However, in most of the natural systems, IPP and DMAPP concentrations are not high enough to be toxic, and polyprenyl synthases and TSs would also be present to push equilibrium away from toxic levels of IPP. Therefore, we propose that in addition to any putative chemoprotective effect, the innate prenylation activity of TSs may ultimately result in the prenylation of small molecules (i.e., meroterpenoid biosynthesis) in their native hosts.

Phylogenetic Analysis Suggests High Complexity in the Sequence and Function of Cyclization or Prenylation Enzymes. In an effort to understand the evolutionary history of prenylation versus terpene cyclization in bacterial enzymes, we generated a phylogenetic tree of TSs, ABBA PTs, and the UbiA family of PTs and TSs from actinobacteria. Using the Enzyme Function Initiative Enzyme Similarity Tool

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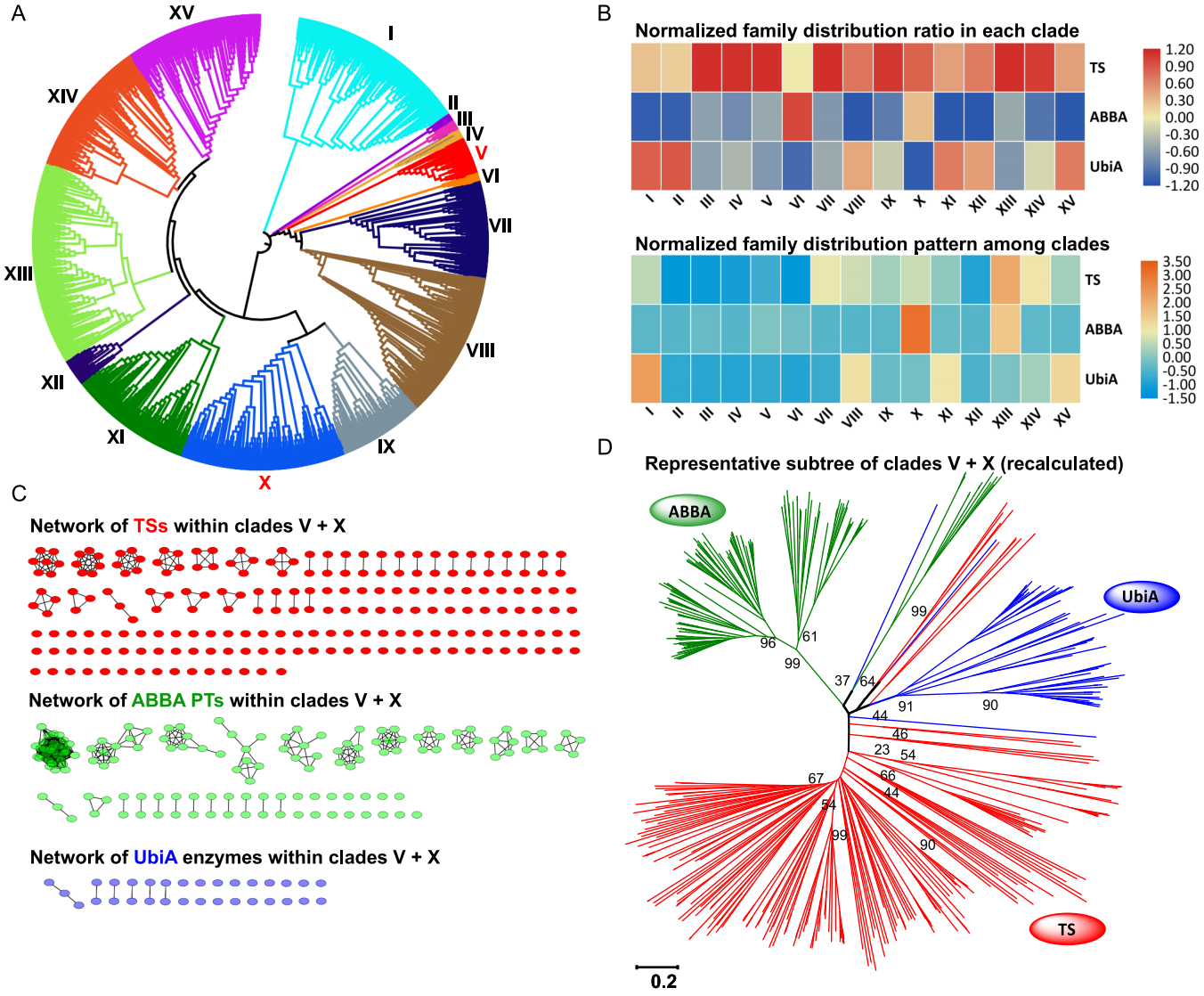

Figure 6. Phylogenetic and sequence analysis of bacterial TS, ABBA PT, and UbiA families of enzymes. (A) Uncorrected neighbor joining phylogenetic tree of selected TS, ABBA, and UbiA enzymes from actinobacteria. (B) Distribution pattern of TS, ABBA, and UbiA enzymes in each clade (top heat map) and among clades (bottom heat map). (C) Sequence similarity networks of TS, ABBA PT, and UbiA enzymes within clades V and X. For a full SSN, see Figure S68. (D) Recalculated neighbor joining phylogenetic tree showing only clades V and X.

(EFI-EST) webtool,[39] we first generated a sequence similarity network (SSN) of TS (IPR008949), ABBA (IPR033964), and UbiA (IPR000537) enzymes with representative nodes containing proteins with >50% sequence identities. We then collected 3209 sequences, one from each node and 916 randomly selected singletons, for phylogenetic analysis (Figure 6A). We manually assigned 15 clades (I–XV) and quickly realized that the three families of enzymes were not exclusively separated and were distributed with varying ratios (Figure 6B). To further exemplify the distribution within clades, clades V (majority of TSs) and X (majority of ABBA PTs) were combined into an SSN and a subtree (Figure 6C,D). In the phylogenetic subtree, the majority of enzymes fall into subgroups containing the three enzyme families, but some UbiA proteins and TSs share clade roots with ABBA PTs and UbiA proteins, respectively. This analysis may suggest that there is a possible evolutionary relationship between TSs, ABBA PTs, and UbiA proteins. Accurate prediction of the natural functionality of these proteins, i.e., cyclization or prenylation, would be beneficial, but more functional and evolutionary analysis is needed to define how their functions are controlled.

TSs with Prenylation Activity May Have Important Implications in Natural Product Biosynthesis. Meroterpenoids are hybrid natural products partially derived from terpenoid precursors and are commonly the result of direct prenylation reactions by either ABBA or UbiA PTs.[3,40] However, there are a few examples of meroterpenoids that do not have "canonical" PTs associated with their biosynthesis (Figure S69). (i) 1-Tuberculosinyladenosine (1-TbAd), an unusual terpene nucleoside found in *Mycobacterium tuberculosis*, is constructed by two enzymes. First, the type II di-TS Rv3377c cyclizes GGPP into the bicyclic tuberculosinyl diphosphate.[41] Then, the TS-like PT Rv3378c catalyzes prenylation of adenosine at N-1 with tuberculosinyl diphosphate.[42] Interestingly, Rv3378c was initially published as a TS that catalyzed the diphosphate elimination of tuberculosinyl diphosphate.[43] (ii) In xiamycin biosynthesis, farnesylation of indole at C-3 is proposed to be catalyzed by the polyprenyl synthase XiaM/P (two BGCs were independ-

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ently discovered and named).[44,45] (iii) In moenomycin biosynthesis, two unusual PTs act to build the unusual C_{25} moenocinol moiety. First, MoeO5, a member of the geranylgeranylglycerolphosphate synthase family with a triose-phosphate isomerase (TIM)-barrel fold, farnesylates 3-phosphoglycerate,[46,47] then, several steps downstream, the TS MoeN5 catalyzes an unusual head-to-middle geranylation reaction that proceeds with multiple rearrangement steps to yield the moenocinol moiety.[48] Very recently, a new family of PTs that appears to have repurposed the TS structural fold was discovered in marine algae.[49] These examples inspire us to consider that some enzymes annotated as TSs may instead be functional PTs that are responsible for the biosynthesis of known or new meroterpenoids. The family of pyrrole meroterpenoids, which includes pyrrolostatin, glaciapyrroles, nitropyrrolins, and heronapyrroles,[3] is one possible candidate. Although ABBA PTs are likely candidates for the prenylation of pyrroles, no biosynthetic gene clusters (BGCs) have been identified, and widespread testing of ABBA PTs from producing strains revealed no activity with pyrroles.[50]

If TSs use prenylation for natural product biosynthesis, a major question for future biosynthesis and genome mining studies is how to differentiate between moonlighting activity and a PT-only (or PT-dominant) TS. This is already a challenge for the study of UbiA proteins where there are examples of both prenylation and cyclization.[15,51,52] In some cases, UbiA-like TSs are encoded near type II di-TSs,[53] thereby providing a hint of its function. In *Streptomyces*, there are many examples of genes encoding putative TSs clustered near those of PKS-related genes, even within the same predicted operon (Figures S70 and S71), suggesting that prenylation may be a natural function.

Finally, if both TSs and UbiA enzymes can catalyze both cyclization and prenylation reactions, what about ABBA PTs, can they also catalyze cyclization reactions? Preliminary docking studies with NphB (PDB ID 6GGI) and GGPP suggest that the cavity is too narrow for the prenyl tail to wrap around to approach C-1 or C-3 (Figure S72). However, further studies on ABBA PTs, in addition to whether UbiA cyclases can also catalyze prenylation reactions, are needed.

CONCLUSIONS

Type I TS cyclization and prenylation reactions are mechanistically similar, and both begin with diphosphate abstraction. In the intramolecular cyclization reaction, the tail of the substrate interacts with the resulting carbocation. Prenylation, an intermolecular reaction, can be considered a carbocation quench where the proximity of a small molecule nucleophile to the carbocation prevents cyclization or water quench. Given the similarities in carbocation generation and the hydrophobicity of the substrate binding pocket, it is not surprising that TSs can also catalyze prenylation reactions under certain conditions. While there are several pieces of evidence that support that TS-catalyzed prenylation may be physiologically relevant, additional studies are needed to conclude that this cryptic function is more than just an in vitro or heterologous expression artifact.

METHODS

See the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c01113.

Supporting methods, results, and references; strains, plasmids, and primers used in this study (Tables S1−S3); summary of NMR data for compounds **2**, **9**, **11**, **12**, **15**, and **16** (Tables S4−S6); SDS-PAGE analysis of purified proteins (Figure S1); NMR and EIMS spectra of **2**−**20** (Figures S2−S17, S19−S31, and S33−S64); HPLC traces of enzyme reactions (Figures S18 and S32); docking models (Figures S65 and S72); kinetic and pH characterization of enzyme reactions (Figures S66 and S67); bioinformatics analysis (Figures S68, S70, and S71); biosynthesis of natural products using noncanonical PTs (Figure S69) (PDF)

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B.X. and J.D.R. conceived the project and designed the experiments; B.X., Z.L., T.A.A., and M.A.E. performed the experiments; B.X. and J.D.R. analyzed the results; B.X. and J.D.R. wrote the manuscript with input from all co-authors.

Notes

The authors declare no competing financial interest.

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