Thiopeptide Pyridine Synthase TbtD Catalyzes an Intermolecular Formal Aza-Diels−Alder Reaction

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ABSTRACT: Thiopeptide pyridine synthases catalyze a multistep reaction involving a unique and nonspontaneous intramolecular aza-[4 + 2] cycloaddition between two dehydroalanines to forge a trisubstituted pyridine core. We discovered that the *in vitro* activity of pyridine synthases from the thiocillin and thiomuracin pathways are significantly enhanced by general base catalysis and that this broadly expands the enzymes substrate tolerance. Remarkably, TbtD is competent to perform an *inter*-molecular cyclization in addition to its cognate *intra*-molecular reaction, underscoring its versatility as a biocatalyst. These data provide evidence that pyridine synthases use a two-site substrate recognition model to engage and process their substrates.

The Diels−Alder (DA) reaction has been a staple of organic synthesis since its discovery by Otto Diels and Kurt Alder in 1928.[1] Numerous total syntheses have exploited different versions of this typically concerted cycloaddition reaction and related transformations to efficiently construct carbon frameworks and stitch together complex organic scaffolds.[2] In recent years, it has become apparent that similar chemistry is employed widely in the biosynthetic pathways of complex natural products.[3−6] Several enzymes have been found to catalyze DA or DA-like reactions, including PyrI4 in pyroindomycin biosynthesis, SpnF in spinosyn biosynthesis, and LovB in lovastatin biosynthesis, among others.[7−11] In many of these cases, the enzymes work to activate, accelerate, and/or direct the stereochemical outcome of an otherwise spontaneous cycloaddition reaction. As a result, reactions catalyzed by these Diels−Alderases can be highly substrate dependent and their biocatalytic utility is limited.

Pyridine synthases in thiopeptide biosynthesis provide unique examples of what is thought to be an enzyme catalyzed aza-DA. The biosyntheses of thiopeptides are similar to other ribosomally synthesized and post-translationally modified peptides (RiPPs) in that the C-terminal "core" of a precursor peptide is transformed into the mature natural product by a suite of peptide modifying enzymes that are recruited by an N-terminal "leader" peptide.[12,13] Pyridine synthases are responsible for the late stage formation of the class-defining nitrogenous heterocycles at the core of the thiopeptide pharmacophores.[14−16] The key reaction is thought to entail an intramolecular formal [4 + 2] cycloaddition between two dehydroalanines (Dhas) followed by elimination of water and

leader peptide (LP) to yield a trisubstituted pyridine core (Figure 1a).[17,18] In contrast to other DAs, this reaction is nonspontaneous and incurs a significant thermodynamic
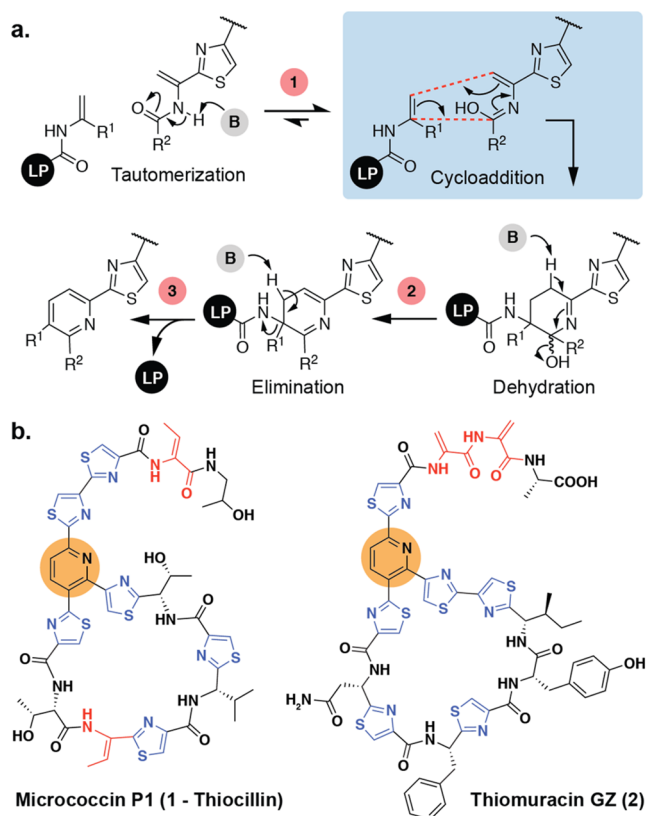
Figure 1. (a) Proposed mechanism of pyridine synthases. Steps potentially influenced by base (1−3) are highlighted. (b) Thiopeptides thiocillin (left) and thiomuracin (right) and their precursor peptide sequences (c, d). Orange regions highlight pyridine synthase recognition sequences used in this work. Core residues in red are converted to Dhas/Dhbs, and residues in blue are converted to thiazoles.

penalty due to the inactivated nature of the reaction subcomponents. Synthetic versions of this cycloaddition are low yielding and require high temperatures.[19]

The *in vivo* manipulation of thiopeptide biosynthetic gene clusters has demonstrated that pyridine synthases are very promiscuous enzymes and can be used to generate a variety of thiopeptide derivatives with different sized macrocycles and pyridine substituents.[20−25] More specifically, enzymatic macrocyclization is independent of any structural preorganization imposed by the core peptide, as neither its length nor rigidity prevent cyclization.[23] Thus, we reasoned that pyridine synthases might be able to catalyze an *intermolecular* reaction between isolated 2π and 4π components. Such a reaction would provide perhaps the most extreme example of substrate promiscuity and support a two-site model of substrate recognition. Therefore, we sought to test whether pyridine synthases TclM or TbtD, from the biosynthetic pathways of thiocillin (**1**) and thiomuracin (**2**), respectively, could catalyze an intermolecular reaction (Figure 1b). Optimization of the enzyme reaction conditions was an important initial step to test this hypothesis because our previous work showed that the enzymes could be slow and several modifications known to be permitted *in vivo* either refused to be cyclized *in vitro* and/or generated byproducts.[26]

We first looked to improve the *in vitro* activity of TclM and TbtD by investigating the influence of pH on the rate of pyridine formation. Several steps of the proposed mechanism could be influenced by pH: (1) tautomerization of the enamide to iminol, (2) elimination of water, and (3) aromatization (Figure 1a). We used our previously described solid-phase strategy to prepare substrates for TclM and TbtD (compounds **3** and **4**, respectively, Figure 2a, b) based on their respective precursor peptides, TclE and TbtA (Figure 1c).[26]

a. TclM substrate (**3**)

ENEALEIMGADhaThzThrAlaThzAlaThzAlaThzDhaThzThz

b. TbtD substrate (**4**)

VESLTAGHGMTEVGADhaThzAsnThzPheThzTyrValThzThzDhaThzDha

c.
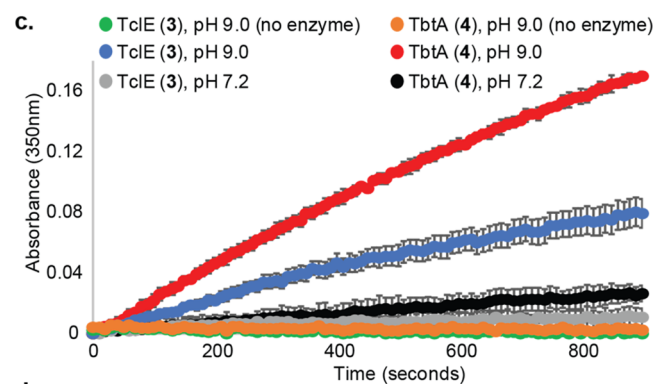
d.

Substrate	pH	k_{cat} (min^{-1})	K_d (μM)
15-res LP TbtA (**4**)	7.2	0.21±0.02	3.2±0.9
15-res LP TbtA (**4**)	8.0	0.94±0.19	3.9±1.1
15-res LP TbtA (**4**)	9.0	1.90±0.08	7.1±2.1

Figure 2. Simplified TclM (a) and TbtD substrates (b). (c) The rate of TclM or TbtD catalyzed pyridine formation is enhanced under basic pH. (d) Impact of pH on $k_{cat.}$ and K_d.

Each substrate consisted of a simplified core with a minimal LP fragment that was previously found to be sufficient for complete processing.[14,26] The TclM substrate (**3**) was synthesized with a 10-residue LP, while the TbtD substrate (**4**) had a 15-residue LP (Figure 2a, b). Enzymatic reactions were monitored at neutral and basic pH by measuring the distinctive absorbance of the newly forming, trisubstituted pyridine at 350 nm over time. Reaction rates were calculated based on the standard curve (see Supporting Information (SI)). A significant increase in rate was observed for each enzyme at pH 9.0 (Figure 2c). Specifically for TbtD, a nearly 10-fold increase in $k_{cat.}$ was observed from pH 7.2 to 9.0 (Figures 2d, S1). Although $k_{cat.}$ at pH 9.0 is still generally considered low, it is comparable to other late stage RIPP-modifying enzymes.[27−32]

Next, we assessed whether pH influenced substrate binding. We measured the dissociation constant, K_d, for TbtA bearing the 15-residue LP and MBP-TbtD via fluorescence polarization assay. A nonreactive substrate for TbtD, with alanines instead of Dhas, was synthesized and derivatized with fluorescein isothiocyanate (FITC) at the N-terminus (**5**). K_d's were determined at pH 7.2, 8.0, and 9.0 (Figures 2d, S2). The effects of pH on K_d were minor compared to those on $k_{cat.}$. At pH 7.2, a K_d of 3.2 μM was measured whereas values of 3.9 and 7.1 μM were observed at pH 8.0 and 9.0 respectively. These data suggest that the pH does not significantly influence substrate binding.

Due to the pronounced binding affinity of TbtA's N-terminal 16-residue LP for TbtD,[33] we sought to determine whether this region of the LP might further enhance the reaction rate by allosteric regulation *in trans*. Studies have shown that this region contributes much of the full-length LP binding affinity to TbtD (K_d = 1.3 μM),[33] but is not strictly essential for enzymatic processing.[16,26] The 16-residue N-terminal fragment (**6**) was synthesized by solid-phase peptide synthesis (SPPS), (see SI), and the reaction rate for substrate **4** was measured at pH 7.2 and 9.0 in presence or absence of **6**. No appreciable change in rate was observed (Figure S3). These data suggest that the N-terminal LP fragment does not act as an allosteric modulator. In the context of full length TbtA, this fragment's higher affinity may allow the LP to bind first, thereby increasing the local concentration of the core around the active site to facilitate faster modification.

As an initial test of TbtD's improved substrate promiscuity under the optimized reaction conditions, we assessed its ability to cyclize TbtA LP mutants. Previously, alanine scanning mutagenesis had been used to investigate LP binding to TbtD[16,33] but not substrate processing. We reasoned that similar experiments might identify residues within the LP that are important for catalysis in addition to acting as a metric for promiscuity. A suite of 16, largely alanine-substituted, LP variants were prepared in parallel by SPPS. The LP variants were separately tested at pH 7.2 and 9.0, and LCMS analysis revealed several differences (Table 1, Figure S4a−q). Overall, 12 of the 16 substrates were processed at neutral pH, while 15 were cyclized under basic pH. Interestingly, only the H27A LP variant failed to cyclize under either condition. These data indicate that TbtD exhibits broader substrate promiscuity under basic pH.

With the enhanced activity of pyridine synthases confirmed, we sought to test whether TclM or TbtD could catalyze an *intermolecular* reaction. 2π components for both TclM and TbtD were prepared by SPPS and consisted of the

Table 1. Cyclization Efficiency of TbtA LP Mutants

| TbtA Leader | % Conversion | | TbtA Leader | | |
	pH 7.2	pH 9.0		pH 7.2	pH 9.0
16-res WT	85	96	16-res G28A	83	86
16-res E21Q	74	69	16-res M29A	0	45
16-res E21A	71	65	16-res T30A	74	81
16-res S22A	82	74	16-res E31Q	30	14
16-res L23A	2	51	16-res E31A	65	18
16-res T24A	72	77	16-res V32A	49	76
16-res A25G	80	72	16-res G33A	84	87
16-res G26A	1	47	16-res A34G	68	81
16-res H27A	0	0			

corresponding minimal leader sequences with a C-terminal Dha-thiazole. The TclM 4π component was synthesized via SPPS following the same synthetic strategy that was used to prepare the intramolecular substrates. The TbtD 4π component was synthesized in solution from thiazole building blocks following standard peptide coupling procedures (see SI). In either case, each 4π component was designed to present the amide bond and Dha proposed to be involved in the cycloaddition plus two-to-three modified residues flanking either side. Incubation of each set of substrates with their respective pyridine synthases at pH 7.2 for 21 h yielded only trace amounts of product by LCMS. At pH 9.0, we again observed only trace amounts of product in reactions with TclM (Table S1). However, TbtD cleanly consumes both components and produces a new UV peak with characteristic absorbance at 350 nm and an $[m/z]$ consistent with a fully formed pyridine product (Figures 3, S5a). The intermolecular reaction was scaled up, and the identity of the product was confirmed by ^{1}H and ^{13}C NMR. TbtD is capable of recognizing the 2π and 4π reaction partners separately, without
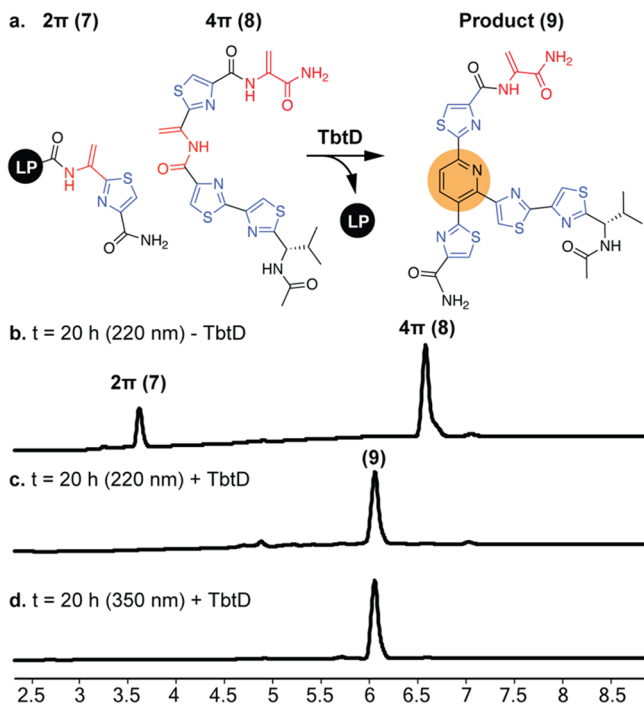
Figure 3. (a) TbtD-catalyzed intermolecular reaction. (b–d) UV traces of 2π and 4π substrates after 20 h in the presence (c and d) and absence (b) of TbtD.

the need for tethering between them, suggesting that an exceptionally broad array of macrocycles could likely be prepared by this enzyme.

In order to further define the limits of the TbtD-catalyzed intermolecular reaction, we prepared a small library of 2π and 4π substrates. With respect to the 2π component, previous LP truncation experiments and the above LP variants have defined the important residues in the LP so we focused further variation on the C-terminal side on the Dha. The thiazole (Thz) was changed to an oxazole (Oxz, Figure 4a, entry 2) or
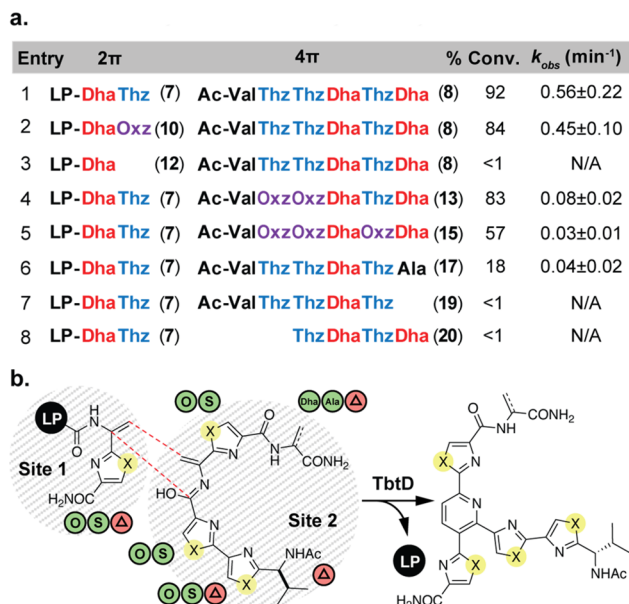
Entry	2π	4π	% Conv.	k_{obs} (min^{-1})
1	LP-DhaThz (7)	Ac-ValThzThzDhaThzDha (8)	92	0.56±0.22
2	LP-DhaOxz (10)	Ac-ValThzThzDhaThzDha (8)	84	0.45±0.10
3	LP-Dha (12)	Ac-ValThzThzDhaThzDha (8)	<1	N/A
4	LP-DhaThz (7)	Ac-ValOxzOxzDhaThzDha (13)	83	0.08±0.02
5	LP-DhaThz (7)	Ac-ValOxzOxzDhaOxzDha (15)	57	0.03±0.01
6	LP-DhaThz (7)	Ac-ValThzThzDhaThzAla (17)	18	0.04±0.02
7	LP-DhaThz (7)	Ac-ValThzThzDhaThz (19)	<1	N/A
8	LP-DhaThz (7)	ThzDhaThzDha (20)	<1	N/A

Figure 4. Substrate scope of the intermolecular reaction. (a) Consistent with intramolecular substrate 4, a 15-residue LP was used for all 2π components. Compound numbers for 2π and 4πs components are listed next to each sequence. See SI for calculations regarding percent conversion and k_{obs}. (b) Green circles represent permissible changes (S for thiazoles, O for oxazoles). Removing residues with red triangles prevents cyclization.

removed entirely (Figure 4a, entry 3). The 4π substrates were again synthesized via solution-phase chemistry, and Dhas were installed either through alkylation−elimination chemistry of cysteine thiols or via mesylation−elimination of serine alcohols (see SI). As with the 2π substrates, thiazoles were iteratively changed to oxazoles and residues flanking the necessary Dha were removed to isolate the crucial components of the 4π substrate. For each new reaction, a percent conversion was calculated based on LCMS analysis and the rate of cyclization was measured by change in absorbance at 350 nm over time relative to a standard curve of purified product (Figures 4a, S5a−h, S6). The results illustrate several key requirements for intermolecular substrates (summarized in Figure 4): (1) the C-terminal heterocycle must be present in the 2π component (Figure 4a, entry 3), but a thiazole can be exchanged for an oxazole without significantly perturbing the rate or conversion (Figure 4a, entry 2); (2) TbtD is sensitive to changes to both the N- and C-termini of the 4π component (comparing entry 1 to 6, 7, and 8 in Figure 4a); (3) the bisazole motif is critical for cyclization; truncation to a single thiazole results in no product (Figure 4a, entry 8); (4) removal of the C-terminal Dha results in no product—this Dha can be replaced with an alanine, albeit at significant cost to the overall rate (Figure 4a, entries 6

and 7); (5) in all 4π substrates, exchanging thiazoles for oxazoles is tolerated but there is a decrease in efficiency in each case (comparing entry 1 to 2, 4, and 5 in Figure 4a). Ultimately, all permissible reactions could be forced to full conversion with higher enzyme loadings, allowing isolation and structural confirmation of products by ^{1}H and ^{13}C NMR. These data demonstrate TbtD is tolerant of major changes to nearly every residue outside of the two Dhas undergoing pyridine ring formation.

In conclusion, we found that the thiomuracin pyridine synthase TbtD is capable of catalyzing an *inter*molecular pyridine formation in addition to its cognate *intra*molecular reaction. The intermolecular reaction proved possible only under rate-enhancing alkaline conditions, which allow an approximate 10-fold increase in enzyme turnover ($k_{cat.}$) while minimally affecting substrate binding (K_d). The thiocillin pyridine synthase, TclM, observed similar base-catalyzed rate enhancement but could not perform an intermolecular reaction, suggesting that different families of pyridine synthases may have distinct substrate requirements. The intermolecular chemistry of TbtD provides evidence that pyridine synthases use a two-site model to engage and process their precursor peptides; this reaction is a strong indicator of this enzyme family's broad promiscuity, suggesting that their use as biocatalysts could be extended beyond natural thiopeptide scaffolds, to allow larger ring sizes, incorporation of non-natural functionality, or even novel linear substrates. The intermolecular reaction may be of use in elucidating the mechanism of these enzymes and may provide a barometer for evaluating new pyridine synthases for biotechnology applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b11852.

Experimental details, synthetic schemes, figures (PDF)

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Notes
The authors declare no competing financial interest.

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