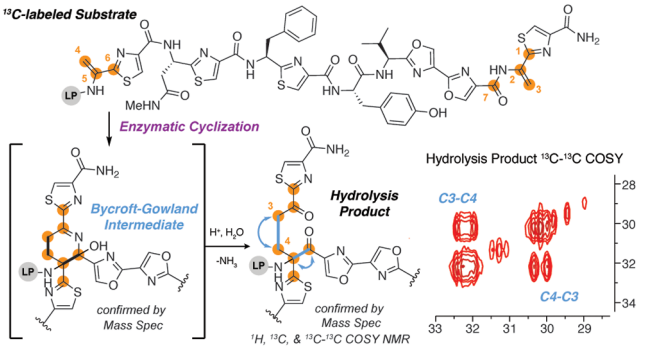
Interception of the Bycroft−Gowland Intermediate in the Enzymatic Macrocyclization of Thiopeptides

Jonathan W. Bogart,[§] Nicholas J. Kramer,[§] Aneta Turlik, Rachel M. Bleich, Daniel S. Catlin, Frank C. Schroeder, Satish K. Nair, R. Thomas Williamson, K. N. Houk, and Albert A. Bowers*

ABSTRACT: Thiopeptides are a broad class of macrocyclic, heavily modified peptide natural products that are unified by the presence of a substituted, nitrogen-containing heterocycle core. Early work indicated that this core might be fashioned from two dehydroalanines by an enzyme-catalyzed aza-[4 + 2] cycloaddition to give a cyclic-hemiaminal intermediate. This common intermediate could then follow a reductive path toward a dehydropiperidine, as in the thiopeptide thiostrepton, or an aromatization path to yield the pyridine groups observed in many other thiopeptides. Although several of the enzymes proposed to perform this cycloaddition have been reconstituted, only pyridine products have been isolated and any hemiaminal intermediates have yet to be observed. Here, we identify the conditions and substrates that decouple the cycloaddition from subsequent steps and allow interception and characterization of this long hypothesized intermediate. Transition state modeling indicates that the key amide−iminol tautomerization is the major hurdle in an otherwise energetically favorable cycloaddition. An anionic model suggests that deprotonation and polarization of this amide bond by TbtD removes this barrier and provides a sufficient driving force for facile (stepwise) cycloaddition. This work provides evidence for a mechanistic link between disparate cyclases in thiopeptide biosynthesis.



INTRODUCTION

Thiopeptides are a class of highly modified, macrocyclic natural products that have garnered significant attention due to their demonstrated therapeutic value and their remarkable biosynthesis.[1−5] Thiopeptides are well known for their antibiotic activity and show impressive potency against multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and penicillin-resistant *Streptococcus pneumoniae*.[2,3] In addition, some thiopeptides have been suggested to use a dual mechanism of action to clear *Mycobacterium tuberculosis* from host macrophages by simultaneously inducing an autophagic response.[6,7] Other thiopeptides hold promise as antimitotic,[8−10] antifungal,[11] antiplasmodial,[12,13] or immunosuppressive[14] therapeutics. Despite this broad therapeutic potential, clinical use of thiopeptides has been hampered by their low aqueous solubility, and major efforts toward thiopeptide synthesis and engineering have been focused on improving this shortcoming.[15−20]

Thiopeptide biosynthesis has proven to be a powerful tool for manipulating their complex structures and creating new analogs.[21−28] Like other ribosomally synthesized and post-translationally modified peptide (RiPP) natural products, thiopeptides are produced through extensive enzymatic modification of a linear precursor peptide.[29,30] Each precursor peptide contains discrete recognition elements within an N-terminal leader sequence that engage and direct tailoring enzymes to modify a C-terminal core sequence. Thiopeptides are distinguished from other RiPPs, however, by the presence of a unique feature: a trisubstituted, nitrogen-containing six-membered ring. These *N*-heterocyclic components can take several forms, such as the dehydropiperidine of thiostrepton or the pyridine rings found in thiomuracins and thiocillins (Figure 1a). TclM, from thiocillin biosynthesis, was the first of a unique set of enzymes shown to form one of these core heterocycles.[31] This work demonstrated that TclM and related cyclases[32,33] act late in thiopeptide biosynthesis to simultaneously form the pyridine core, macrocyclize the modified peptide, and remove the N-terminal leader peptide (LP), thus furnishing the fully mature thiopeptide antibiotic.

Received: May 23, 2020
Published: July 1, 2020

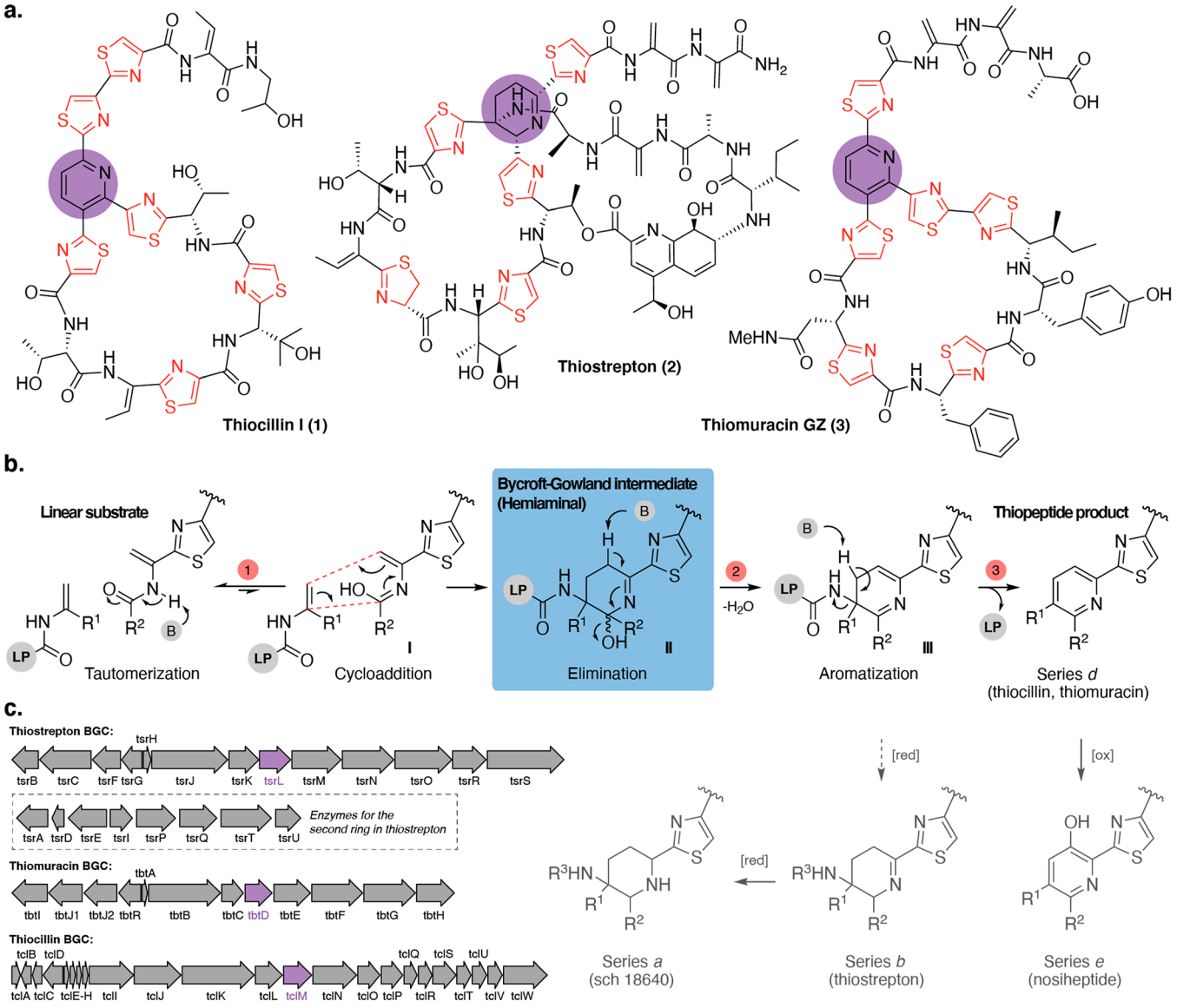


Figure 1. Thiopeptide structures and the proposed enzymatic mechanism of *N*-heterocyclic core installation. (a) Thiopeptides thiocillin and thiomuracin have pyridine cores, while thiostrepton bears a dehydropiperidine core. (b) Proposed mechanism describing the formation of diverse thiopeptide cores. Series *c* is not shown. (c) Biosynthetic gene clusters (BGC) for each thiopeptide are shown. Highlighted in purple are the enzymes that are either putatively responsible for the core formation (tsrL) or have been successfully reconstituted in vitro (tbtD, tclM).

These cyclases have been harnessed as versatile biocatalysts in a chemoenzymatic strategy that mirrors the natural thiopeptide biosynthesis but uses synthetic substrates with minimized LP sequences to probe enzyme promiscuity and access a wide range of analogs.[34] This approach has uncovered the extreme substrate flexibility of these cyclases in vitro. In particular, under optimized conditions, we showed that the thiomuracin cyclase, TbtD, can catalyze an intermolecular transformation between two relatively small fragments of the native substrate.[35] Despite much work on this class of enzymes, their mechanism remains poorly understood.[36,37]

In 1978, Bycroft and Gowland proposed that distinct core motifs, like those of thiocillins and thiostrepton, could be accessed by a single, unifying enzymatic reaction.[38] Specifically, they hypothesized that two dehydroalanines (Dhas) might undergo a formal aza-[4 + 2] cycloaddition to yield a cyclic hemiaminal intermediate which could serve as a key branching point for the different series of thiopeptides. Reductive steps led to a thiostrepton dehydropiperidine core, while elimination and aromatization steps led to a thiocillin or thiomuracin pyridine core (Figure 1b). Importantly, for the proposed cycloaddition, one Dha-adjacent amide would tautomerize to an iminol in order to establish a suitable diene. While amide C−N bonds exhibit substantial double-bond character, there is a steep energetic penalty for complete tautomerization (~11−12 kcal/mol) to the imidic acid, which would presumably preclude a spontaneous cycloaddition.[39−42] Indeed, several efforts to employ synthetically equivalent reactions could only achieve successful aza-cycloadditions at elevated temperatures by forced tautomerization through *O*-alkylation.[43] Nevertheless, a growing body of research has supported the Bycroft−Gowland hypothesis. A combination of genetic and feeding studies confirmed that heterocyclic cores across many families of thiopeptides are fashioned from serine-derived Dhas in the linear peptide precursors.[44−52] Moreover, the in vitro reconstitution of several of these cyclases, including TclM and

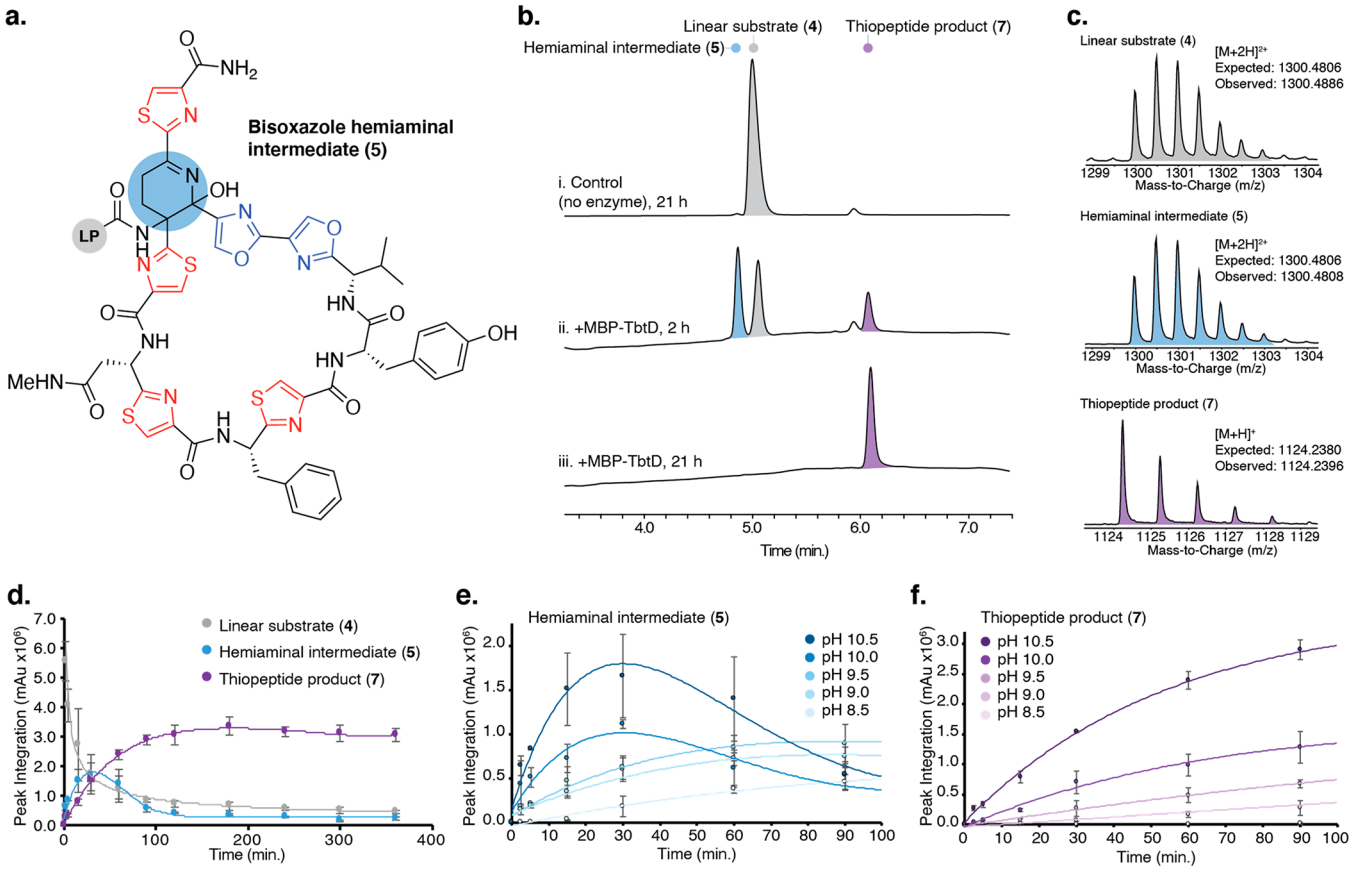


Figure 2. Observation of a Bycroft−Gowland hemiaminal intermediate. (a) Proposed structure of the bisoxazole hemiaminal intermediate observed during the course of the cyclization. (b) RP-HPLC traces (UV at 254 nm) of the MBP-TbtD-catalyzed reaction with compound **4** at 2 and 21 h. (c) MS of linear substrate **4**, hemiaminal intermediate **5**, and thiopeptide product **7** in profile. (d) Time course of the MBP-TbtD-catalyzed reaction at pH 10.5. Area under each peak corresponding to **4**, **5**, and **7** is plotted. Similar time courses were performed across a range of basic pHs, and hemiaminal intermediate (e) and thiopeptide product (f) are plotted. All time points were performed in triplicate with error bars representing the standard deviation.

TbtD, demonstrates that substrates bearing these Dhas can be converted to the pyridine class of thiopeptides in a single enzymatic transformation.[31−35]

Still, a Bycroft−Gowland hemiaminal intermediate has yet to be observed, leaving the open question of the conserved mechanistic intermediate of this transformation. The rapidly expanding genomic data and characterization of numerous thiopeptide biosynthetic gene clusters has firmly established a genetic link between thiopeptides with distinct core motifs; however, the biochemical links between core-forming enzymes of different thiopeptide classes linger.

Herein, we use established chemoenzymatic methods to perform a systematic heterocycle scan of thiomuracin-like thiopeptides. For several analogs, an intermediate is observed during the course of the pyridine synthase-catalyzed reaction. The core structure of an isolated intermediate was confirmed by a combination of LC-MS and 1D and 2D NMR experiments with ^{13}C-labeled material and confirms the presence of a Bycroft−Gowland intermediate. We track the generation and consumption of one particular intermediate under several conditions and demonstrate that the enzymatic cycloaddition step is highly pH dependent. Finally, transition state modeling was used to understand the energetic landscape of the proposed reaction and highlight the differences between the analogs and their parent structure.

RESULTS

Unnatural TbtD Substrates Lead to the Accumulation of Intermediates. As part of our ongoing efforts to improve the solubility of thiopeptides, we designed a small series of variants that would systematically exchange the native thiazoles for oxazoles using our established chemoenzymatic strategy (Table S1). These variants were of particular interest because the oxazoles are predicted to be significantly more hydrophilic than the isosteric thiazoles[53] (Table S1) but had proven challenging to install by purely biosynthetic means.[26] We prepared eight linear variants based on the features of thiomuracins and related thiopeptides (Figure S1). In five of these variants each monothiazole was individually substituted with a monooxazole; in a sixth variant, the bisthiazole was replaced with a bisoxazole; in another variant, the four thiazoles directly surrounding the pyridine core were simultaneously replaced; in the final variant, all of the thiazoles were replaced with oxazoles (Table S1). These substrates were synthesized with a minimized, 15-residue LP sequence based on the natural precursor peptide as we had previously shown this portion to be necessary and sufficient to allow processing by MBP-TbtD.[34] We recently reported that similar unnatural substrates are processed poorly at neutral pH. However, we discovered that the rate of TbtD-mediated pyridine formation is significantly accelerated at elevated pH, which allowed much more efficient cyclization for these variants.[35] We therefore

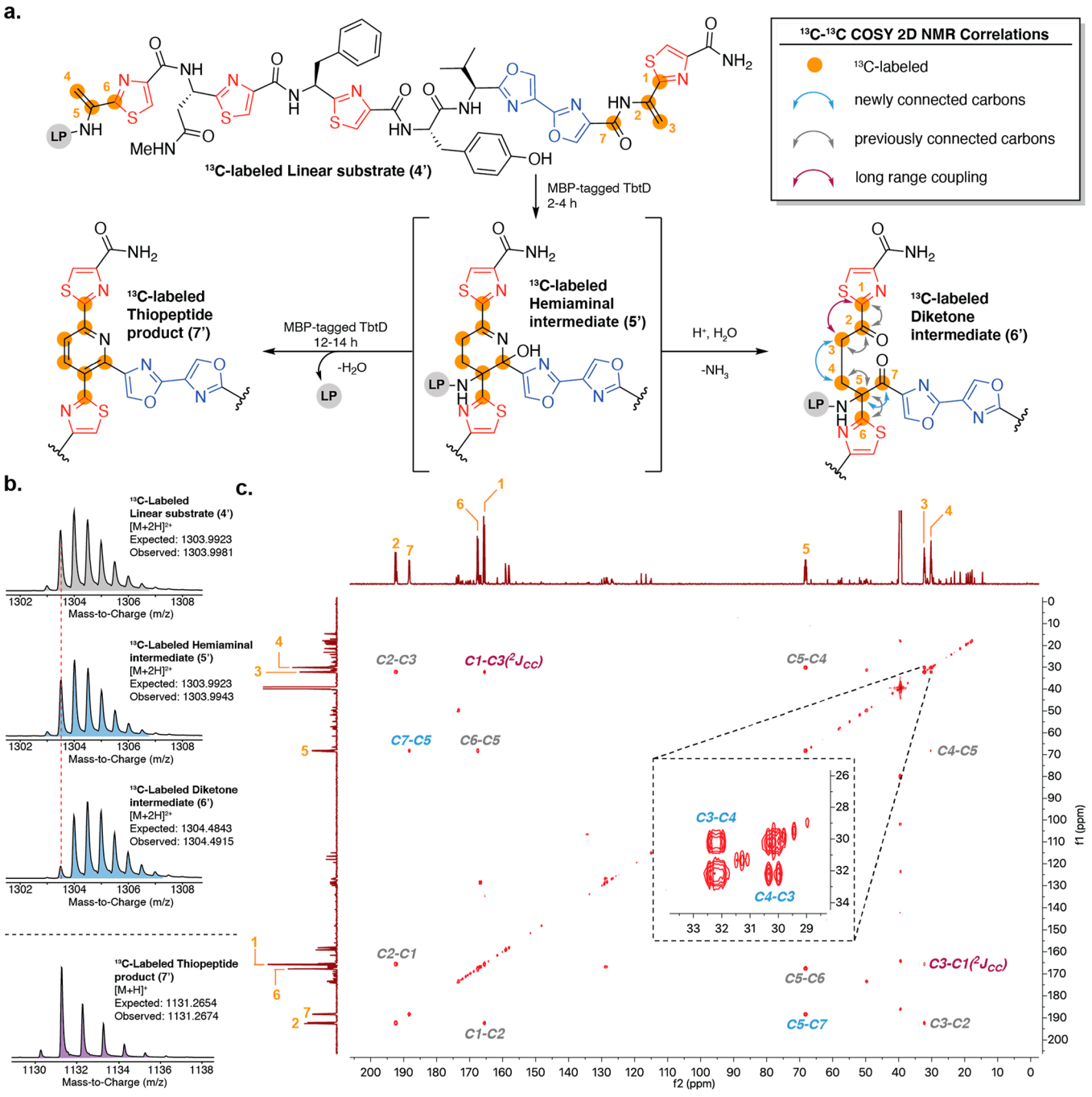


Figure 3. $^{13}C-^{13}C$ COSY confirms the diketone intermediate structure. (a) Reaction scheme of the MBP-TbtD-catalyzed cyclization of the ^{13}C-labeled bisoxazole linear substrate **4′**. Short exposures of **4′** to MBP-TbtD allow the buildup of hemiaminal **5′**. Introducing an acidic aqueous solution to the reaction quickly hydrolyzes **5′** to the diketone intermediate **6′**. Prolonged incubation of **4′** with MBP-TbtD yields the thiopeptide product **7′** as expected. (b) Profile MS of **4′** and **5′** generated in situ, the isolated diketone **6′**, and the thiopeptide product **7′**. (c) $^{13}C-^{13}C$ COSY (DMSO-d_6) of **6′**. C−C bonds formed in the formal cycloaddition are highlighted in blue.

tested our suite of oxazole-containing variants at pH 9.0. Gratifyingly, after overnight incubation, many of these variants underwent complete cyclization to the expected thiopeptide product (Figure S2). However, a few variants exhibited minimal to moderate conversion, even after extended incubation. Curiously, in each case, new, more polar peaks could be observed in the UV traces. LC-MS analysis indicated that in all cases the new peaks were associated with mass to charge ratios (m/z) identical to those of their respective starting materials (Figures 2b, 2c, and S2).

We chose to focus on a single polyoxazole substrate **4** to investigate further. This substrate contained the fewest changes from the parent thiopeptide but shows formation of this new species after relatively short, 2 h, enzymatic assays. A time course of the reaction clearly shows that the new species grows in and then is consumed cleanly, providing strong evidence that this species is an intermediate on route to the final pyridine product (Figures 2b, 2d–f, S2, and S3). We further monitored the progress of the reaction across a range of basic pHs by RP-HPLC (Figure 2e, 2f, and S3). At pH 8.5 or below

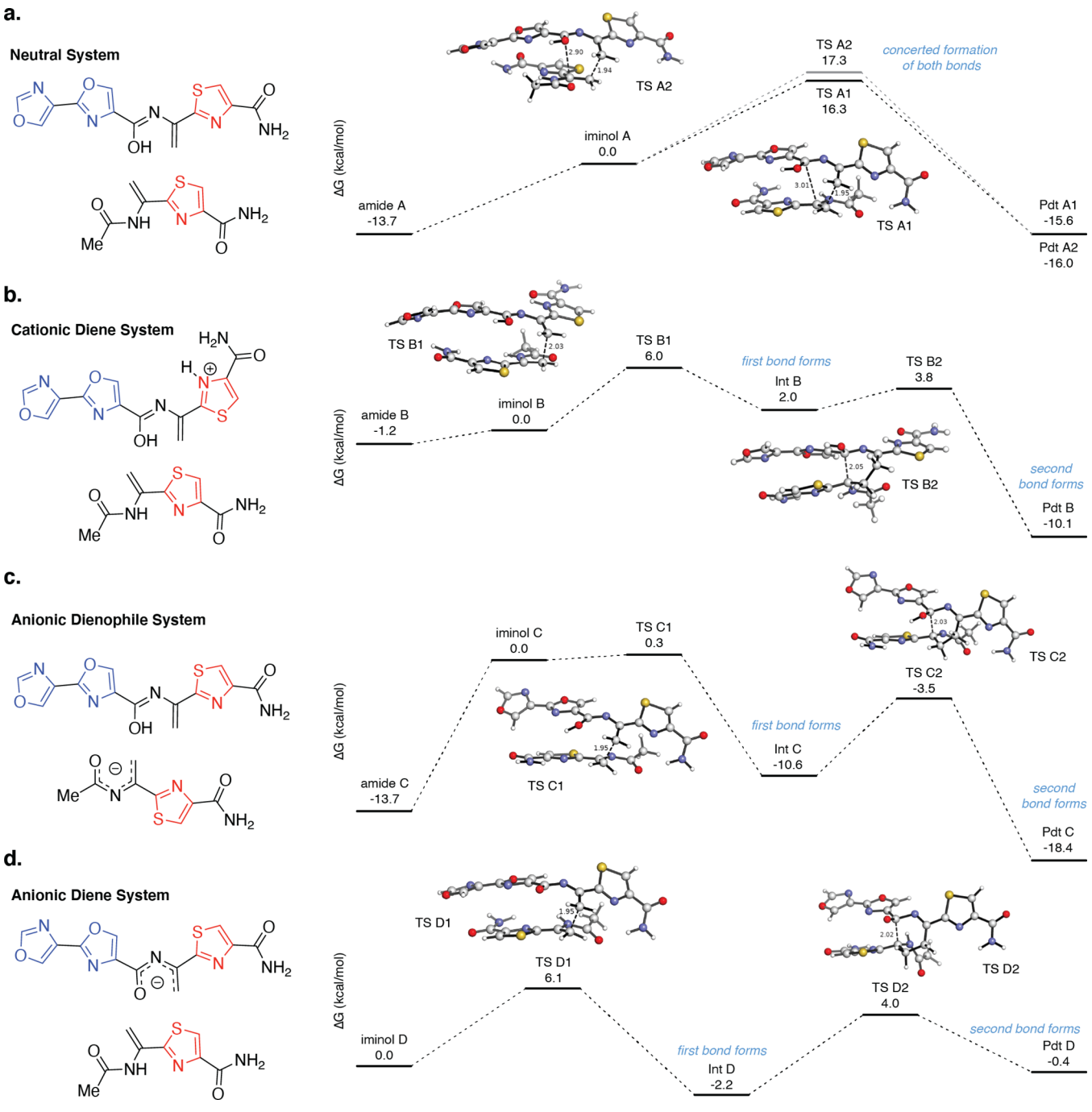


Figure 4. Possible reaction mechanisms for the aza-[4 + 2] cycloaddition. Neutral (a), cationic (b), and anionic (e, d) manifolds are investigated. (a) Cycloaddition may proceed via a concerted manner under a neutral reaction system; however, a high-energy amide−iminol tautomerization barrier is expected. This energy barrier may be overcome in some cationic (b) or anionic (d) systems to facilitate a stepwise cycloaddition mechanism.

the reaction is sluggish, but as pH is increased a substantial increase in the generation and consumption of the putative intermediate is observed, with the majority of the material being converted to the mature thiopeptide after about 90 min at pH 10.5 (Figures 2d−f and S3). Notably, no other intermediates along the proposed mechanism were observed by LC-MS analysis. We hypothesized that this compound might be a Bycroft−Gowland hemiaminal intermediate (Figure 2a).

^{13}C−^{13}C COSY Confirms Formation of a Bycroft−Gowland Intermediate. We sought to confirm the identity of this intermediate by NMR. Given the complexity of the substrate (due to a large core and a 15-residue leader peptide), we reasoned that an equivalent substrate, site-selectively labeled at carbons that are incorporated into the central nitrogen-containing ring, should significantly simplify spectra and allow unambiguous structural determination (Figure 3). This strategy would allow facile acquisition of ^{1}H, ^{13}C, and importantly, ^{13}C−^{13}C-correlation spectra to confirm the new connectivity of the proposed heterocyclic hemiaminal intermediate. Isotopically labeled building blocks were synthesized, and the linear substrate, incorporating seven ^{13}C-labeled

carbons, was assembled by solid-phase peptide synthesis (SPPS) as described previously (Figure 3a, see SI). A large-scale cyclization was performed, and multimilligram quantities of the intermediate were isolated by RP-HPLC. Following purification, LC-MS analysis revealed the isolated compound exhibited a 1 Da increase in m/z compared to the intermediate observed during the course of the reaction (Figure 3b). Given the propensity of secondary ketimines, like the one in the anticipated hemiaminal intermediate, to undergo rapid hydrolysis to ketones in acidic, aqueous environments,[52,54−58] we reasoned the observed mass shift was likely a consequence of the loss of the imine nitrogen during RP-HPLC purification and proposed the structure of the isolated intermediate as a cyclic diketone (Figure 3a). Importantly, the two key carbon−carbon bonds expected to form during the anticipated cycloaddition would still be preserved in the diketone species.

Comparing ^{1}H and ^{13}C NMR spectra of the isolated intermediate to the linear substrate reveals several differences. First, disappearance of the four Dha proton signals from the linear substrate can clearly be discerned in the ^{1}H NMR of the intermediate (Figure S4). As a result of the site-specific labeling, the ^{13}C NMR spectrum of the intermediate provides conclusive evidence that these Dhas had been conjoined. The quartets at ∼134 ppm and doublets at ∼106 ppm (representing the ^{13}C-labeled alpha and beta carbons of the Dhas, respectively) that were present in the linear substrate spectrum are absent from the ^{13}C NMR spectrum of the intermediate (Figure S5). These have been replaced by three additional peaks much farther upfield: (i) a multiplet at 68.8 ppm, (ii) a triplet of doublets at 32.6 ppm, and (iii) a triplet at 30.6 ppm (Figure S5). These chemical shifts and ^{13}C−^{13}C splitting patterns are in good agreement with those previously observed for the single tertiary carbon and two methylene carbons in the dehydropiperidines of thiostrepton and related compounds.[59−61] As expected, the two thiazole carbon shifts at 166 and 168 ppm are mostly unchanged. The two remaining ^{13}C chemical shifts at 193 and 182 ppm clearly indicate the diketone structure. Indeed, calculated NMR shifts for every labeled carbon in the diketone are in close agreement with those experimentally observed (Figure S6).

In an effort to better establish the carbon−carbon bond connectivity and definitively show the newly formed bonds from the cycloaddition, we collected 2D ^{13}C−^{13}C COSY NMR spectra of the intermediate. The ^{13}C−^{13}C COSY's easy interpretation and ability to explicitly map carbon−carbon connectivity within a single experiment make it a useful tool for structure determination. As expected, unambiguous signals in the isolated intermediate (**6′**) spectra indicate connectivity across C1−C2−C3 and C4−C5−C6 (Figure 3c, gray). Most notably, two distinct cross peaks indicated the formation of two new carbon−carbon bonds between C3−C4 and C5−C7 (Figure 3c, blue). Importantly, these two connections are precisely those expected to form during the aza-[4 + 2] cycloaddition. Finally, an unusually large long-range $^{2}J_{CC}$ coupling (about 15 Hz) between carbons 1 and 3 was observed (Figure 3c, purple). Such large two bond ^{13}C−^{13}C couplings are a characteristic feature of carbons that are left and right adjacent to a ketone and have been observed in similar NMRs of complex natural products.[62,63] Collectively, 1D and 2D NMRs, LC-MS, and established reactivity of (ket)imines conclusively establish the structure of the diketone species (**6′**) and its origin from hydrolysis of the hemiaminal intermediate (**5′**).

Transition State Modeling Informs the Energetic Landscape of the Cycloaddition. Density functional theory (DFT) calculations were performed to evaluate various mechanisms for the formation of the central heterocycle in a formal [4 + 2] cycloaddition and to examine potential impacts of oxazoles and pH on this reaction. A simplified set of substrates (closely resembling those that undergo intermolecular cyclizations) were used for the calculations (Figure 4). All optimizations were performed with M06-2X-D3/6-31G(d), and single-point calculations of the optimized structures were performed with M06-2X-D3/6-311+G(d,p).

We first evaluated the possibility for this aza-[4 + 2] cycloaddition under neutral conditions (no charged species, Figures 4a and S6). Starting from the iminol intermediate (iminol A), a concerted asynchronous [4 + 2] cycloaddition leads to an exo adduct (Pdt A1) with a transition state barrier of 16.3 kcal/mol (TS A1, Figure 4a). The formation of the exo adduct is favored over the endo adduct by 1.0 kcal/mol. This preference is in agreement with the natural stereochemistry observed in the dehydropiperidine core of thiostrepton and others.[59,64] Despite the low barrier for the [4 + 2] cycloaddition, the difference in energy between iminol A and amide A (13.7 kcal/mol) leads to a high overall barrier of 30.0 kcal/mol. This suggests that the [4 + 2] reaction cannot occur without the aid of the enzyme, presumably through forced tautomerization by acidic or basic residues within the active site. A similar pattern was observed in the system with a bisthiazole instead of the bisoxazole, with transition state barriers of 18.3 and 18.5 kcal/mol for formation of the exo and endo products, respectively (see SI). For the bisthiazole substrate, the difference in energy between the iminol and the amide structure is 13.2 kcal/mol. The "anti" bis-azole ring conformation is preferred for both the bisoxazole and the bisthiazole systems, although this preference is stronger for the bisthiazoles (3.2 vs 0.4 kcal/mol for the bisthiazole vs bisoxazole of the diene component). In addition, many important intra- and intermolecular hydrogen-bonding interactions are present between the two fragments.

Alternatively, protonation of one of the exocyclic thiazoles has been previously proposed as a potentially relevant enzymatic mechanism (Figure 4b).[65] When the thiazole of the diene is protonated, the formal [4 + 2] cycloaddition becomes a stepwise inverse-electron-demand hetero-Diels−Alder process with a discrete intermediate present between the two C−C bond formation steps. Protonation of the thiazole on the diene fragment leads to activation of the diene component toward nucleophilic addition by lowering its LUMO and decreasing the barrier toward the initial C−C bond formation (6.0 kcal/mol). In addition, the conversion of amide B to iminol B is much more favorable. Only a small 1.2 kcal/mol energy difference is observed, due to the presence of an additional favorable hydrogen-bonding interaction between the carbonyl of the iminol tautomer and the protonated thiazole. This potentially acid-catalyzed mechanism, while favorable, stands in contrast to the rate enhancement observed under basic conditions.

Under basic conditions, deprotonation of the enamide N−H in either the diene or the dienophile might precede the cycloaddition step (Figure 4c and 4d). We investigated these two potential anionic manifolds. In both cases, this led to identification of stepwise bond-forming mechanisms. In the first, deprotonation of the dienophile amide C increases the nucleophilicity of the dienophile fragment by raising the

HOMO of the alkene and facilitates an inverse-electron-demand hetero-Diels–Alder cycloaddition (Figure 4c). Both C–C bond formation steps are strongly exergonic with energy differences of 10.6 and 18.4 kcal/mol compared to the iminol for the formation of the first and second bonds, respectively. The barrier of amide–iminol tautomerization remained comparable to the neutral condition manifold. In the second, negatively charged diene system, the initial C–C bond formation is also facile, with a TS energy barrier of only 6.1 kcal/mol (TS D1, Figure 4d). The intermediate (Int D) and product (Pdt D) are both lower in energy than the starting structure with energies of −2.2 and −0.4 kcal/mol, respectively. Importantly, deprotonation of this amide N–H yields a resonance-stabilized anion and effectively removes the barrier to tautomerization, thereby lowering the overall activation energy required for cycloaddition (iminol D). This last mechanism provides a potentially efficient solution to the problem of amide bond tautomerization that is in agreement with our kinetic data and might reasonably be exploited by this group of enzymes.

Lastly, we sought to understand the potential effects of the bisoxazole in slowing the rate of elimination after formation of the initial [4 + 2] adduct (see SI). This is supported by computational studies that show that formation of a cation after removal of water is more stable in the structure containing the bisthiazole moiety by 1.7 kcal/mol. If this factor were operating in the transition state for dehydration, the bisthiazole would be dehydrated about 16 times faster than the bisoxazole at room temperature.

DISCUSSION

Cumulatively, these data confirm the intermediacy of a cyclic hemiaminal in the biosynthesis of thiopeptide heterocyclic cores and substantiates the long-standing Bycroft–Gowland hypothesis of an enzyme-catalyzed formal aza-[4 + 2] cycloaddition. The fact that the intermediate accumulates without spontaneously undergoing conversion to the pyridine suggests that the enzyme is required for the dehydration and elimination steps (Figure 2). It is unclear what catalytic base(s) in the enzyme's active site might contribute to these steps, especially since regions of the enzyme thought to be involved in catalysis are still largely hypotheses based on limited mutational analyses and homology modeling.[37] Still, some coordination is likely needed to drive dehydration and elimination toward the aromatic pyridine products, and the absence of this key residue(s) may help to keep the thiostrepton core at this important intermediate, en route to the dehydropiperidine. Isolation of the diketone suggests yet another need for the enzyme in protecting against this alternative hydrolytic pathway.

We previously reported that pyridine formation is pH dependent, but we could not attribute this rate enhancement with any step in the proposed mechanism.[35] Monitoring the generation of the hemiaminal intermediate across pH clearly indicates that cycloaddition is strongly influenced (Figure 2). These data align well with our computation work. Evidence in the literature reporting the high energetic barrier of amide–iminol tautomerization was reaffirmed for our more thiopeptide-specific system with transition state modeling. These data imply that any increase in the rate of cycloaddition would be a consequence of an increased rate in tautomerization, which may be more easily accomplished in a more basic environment. While the amide pK_a of our specific substrate is unknown, electronically similar anilides are much more acidic than typical amides: 18.8 (DMSO) for benzanilide or 21.5 (DMSO) for acetanilide as compared to 25.5 (DMSO) for acetamide (15.1 in water).[66,67] Moreover, an anionic cycloaddition route, in which the negatively charged diene is produced, removes this high-energy barrier and facilitates cycloaddition (Figure 4). Therefore, a pH-dependent mechanism that deprotonates and stabilizes an anionic diene would support the observed rate enhancement at elevated pHs. Indeed, several basic residues have already been identified in TbtD that strongly influence pyridine formation and are likely candidates to perform this type of chemistry.[32,37] The subtle differences in transition states calculations between the bisthiazole and the bisoxazole substrate suggest the tautomerization-based rate enhancement may also describe reactions with more natural substrates in which the intermediates are not observed.

The spectroscopic data indicate that a Bycroft–Gowland intermediate is formed as a single stereoisomer, as only one corresponding peak in the LC-MS data and one sharp set of ^{13}C NMR signals were detected (Figure S5). This observed diastereoselectivity and the preference for the exo adduct from our calculations is consistent with the exclusively observed exo stereochemistry in the dehydropiperidine series compounds. Although we assumed the same stereochemistry for the rest of our calculations (ionic manifolds), it is possible that TbtD or other members of the growing cadre of cryptic thiopeptide cyclases may be less stereoselective. Indeed, recent work with the paraherquamides and related prenylated indole alkaloids has shown that Diels–Alderases are used elsewhere in nature for chiral resolution of full natural product enantiomers let alone diastereomers.[68] It remains to be seen whether the stereochemistry among the thiopeptide family of cyclases is substrate or enzyme controlled or a combination of the two. Given the meager preference for exo over endo (0.2 kcal/mol) in our bisthiazole calculations, perhaps stereoselectivity may even be manipulated by specific substrates or reaction conditions.

Lastly, these results also inform on the potential for incorporation of more hydrophilic heterocycles into the relatively hydrophobic scaffolds of thiopeptides (Figure S2). The chemoenzymatic approach used here provides unimpaired access to the oxazole substrates, where mutasynthesis had previously been found to produce complex mixtures of differently processed compounds.[26] This work shows that the oxazole replacements are tolerated under the optimized conditions. Still, these oxazole substrates undergo cyclization much more slowly than the parent thiazole-containing counterparts, in particular in the second step dehydration/aromatization. The reason for diminished turnover is not clear at present. Our calculations suggest that the oxazole/thiazole substitution has a minimal electronic impact on the reactivity of the diene component and that the transition states for both oxazole and thiazole substitutions are comparable. For the cycloaddition, it may be that a larger conformational effect is responsible and that the oxazole substrates do not sit in the active site in quite the same manner as the thiazole substrates and thus do not access a potential catalytic base as efficiently for ready turnover to the dehydration product. Regardless, these data demonstrate that the oxazole substrates can be made to react after prolonged incubation with the enzymes, opening up the possibility for incorporation of these groups into new thiopeptide analogs.

CONCLUSIONS

Pyridine synthases catalyze a complicated reaction that simultaneously macrocyclizes a linear substrate, removes the leader peptide, and forms the hallmark trisubstituted pyridine core that defines this genre of natural products. Although the mechanism of catalysis is currently unknown, the prospect of the enzyme catalyzing a unique hetero-Diels–Alder has garnered much investigation. Here, we have described how the use of an established chemoenzymatic platform allowed the investigation of otherwise challenging linear substrates and led to the discovery of a cryptic intermediate along the reaction pathway. The discovery of this intermediate provides major evidence to support that the mechanism goes through a formal [4 + 2] cycloaddition step and further substantiates the 42-year-old hypothesis originally proposed by Bycroft and Gowland. These data suggest a mechanistic link between disparate thiopeptide cyclases is probable. Discovery of this intermediate now separates the cycloaddition from the rest of the reaction steps and may allow more thorough investigation of one of the most exciting steps in this enzymatic transformation. This intermediate could also prove to be synthetically useful, as it could allow access to thiostrepton-like piperidine cores or "new-to-nature" thiopeptide scaffolds. Collectively, this work represents a major advancement in the understanding of RIPP enzymology and once again expands the utility of pyridine synthases to access further reaches of complex natural product-inspired chemical space.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c05639.

General information; synthesis of building blocks; solid-phase peptide synthesis; protein expression and purification; general information for intramolecular cyclization assays with TbtD; summary of cyclizations with variant substrates; thiopeptides with 29-membered macrocycles; cyclization assays with thiazole-to-oxazole substitutions; time courses of **4** at pHs 8.5–10.5; synthesis and isolation of ^{13}C-labeled intermediate **6′** and thiopeptide **7′**) stacked ^{13}C-labeled ^{1}H NMRs; stacked ^{13}C-labeled ^{13}C NMRs; calculated NMR shifts for two potential intermediates; NMR calculations; DFT calculations for a possible aza-[4+2] cycloaddition mechanism; DFT calculations of the aza-[4+2] reaction; peptide characterization; NMR spectra (PDF)

AUTHOR INFORMATION

Corresponding Author

Albert A. Bowers − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy and Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States;* orcid.org/0000-0001-8214-7484; Email: abower2@email.unc.edu

Authors

Jonathan W. Bogart − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Nicholas J. Kramer − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Aneta Turlik − *Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States*

Rachel M. Bleich − *Division of Chemical Biology and Medicinal Chemistry, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Daniel S. Catlin − *Department of Biochemistry, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States*

Frank C. Schroeder − *Boyce Thompson Institute and Department of Chemistry and Chemical Biology, Ithaca, New York 14853, United States;* orcid.org/0000-0002-4420-0237

Satish K. Nair − *Department of Biochemistry and Center for Biophysics and Computational Biology, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, United States;* orcid.org/0000-0003-1790-1334

R. Thomas Williamson − *Department of Chemistry and Biochemistry, University of North Carolina Wilmington, Wilmington, North Carolina 28403, United States*

K. N. Houk − *Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States;* orcid.org/0000-0002-8387-5261

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.0c05639

Author Contributions

[§]J.W.B. and N.J.K. contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank C. Neumann and B. Li for informative discussions and useful feedback. J.W.B. is a recipient of an AFPE predoctoral fellowship, a GSK predoctoral fellowship, and an Eshelman Institute of Innovation (EII) Award. This work was supported by the EII award (J.W.B.), by NIH Grants GM125005 (A.A.B.) and GM131347 (S.K.N.) and NSF grant CHE-1806581 (K.N.H.).

REFERENCES

(1) Bagley, M. C.; Dale, J. W.; Merritt, E. A.; Xiong, X. Thiopeptide Antibiotics. *Chem. Rev.* **2005**, *105* (2), 685–714.

(2) Just-Baringo, X.; Albericio, F.; Álvarez, M. Thiopeptide Antibiotics: Retrospective and Recent Advances. *Mar. Drugs* **2014**, *12* (1), 317–351.

(3) Just-Baringo, X.; Albericio, F.; Álvarez, M. Thiopeptide Engineering: a Multidisciplinary Effort Towards Future Drugs. *Angew. Chem., Int. Ed.* **2014**, *53* (26), 6602–6616.

(4) Shen, X.; Mustafa, M.; Chen, Y.; Cao, Y.; Gao, J. Natural Thiopeptides as a Privileged Scaffold for Drug Discovery and Therapeutic Development. *Med. Chem. Res.* **2019**, *28* (8), 1063–1098.

(5) Schwalen, C. J.; Hudson, G. A.; Kille, B.; Mitchell, D. A. Bioinformatic Expansion and Discovery of Thiopeptide Antibiotics. *J. Am. Chem. Soc.* **2018**, *140* (30), 9494–9501.

(6) Zheng, Q.; Wang, Q.; Wang, S.; Wu, J.; Gao, Q.; Liu, W. Thiopeptide Antibiotics Exhibit a Dual Mode of Action Against

Intracellular Pathogens by Affecting Both Host and Microbe. *Chem. Biol.* **2015**, *22* (8), 1002−1007.
(7) Bird, K. E.; Xander, C.; Murcia, S.; Schmalstig, A. A.; Wang, X.; Emanuele, M. J.; Braunstein, M.; Bowers, A. A. Thiopeptides Induce Proteasome-Independent Activation of Cellular Mitophagy. *ACS Chem. Biol.* **2020**. DOI: 10.1021/acschembio.0c00364
(8) Hegde, N. S.; Sanders, D. A.; Rodriguez, R.; Balasubramanian, S. The Transcription Factor FOXM1 Is a Cellular Target of the Natural Product Thiostrepton. *Nat. Chem.* **2011**, *3* (9), 725−731.
(9) Bhat, U. G.; Halasi, M.; Gartel, A. L. Thiazole Antibiotics Target FoxM1 and Induce Apoptosis in Human Cancer Cells. *PLoS One* **2009**, *4* (5), No. e5592.
(10) Iniyan, A. M.; Sudarman, E.; Wink, J.; Kannan, R. R.; Vincent, S. G. P. Ala-Geninthiocin, a New Broad Spectrum Thiopeptide Antibiotic, Produced by a Marine Streptomyces Sp. ICN19. *J. Antibiot.* **2019**, *72* (2), 99−105.
(11) Mizuhara, N.; Kuroda, M.; Ogita, A.; Tanaka, T.; Usuki, Y.; Fujita, K.-I. Antifungal Thiopeptide Cyclothiazomycin B1 Exhibits Growth Inhibition Accompanying Morphological Changes via Binding to Fungal Cell Wall Chitin. *Bioorg. Med. Chem.* **2011**, *19* (18), 5300−5310.
(12) Gaillard, T.; Madamet, M.; Tsombeng, F. F.; Dormoi, J.; Pradines, B. Antibiotics in Malaria Therapy: Which Antibiotics Except Tetracyclines and Macrolides May Be Used Against Malaria? *Malar. J.* **2016**, *15* (1), 1980.
(13) Schoof, S.; Pradel, G.; Aminake, M. N.; Ellinger, B.; Baumann, S.; Potowski, M.; Najajreh, Y.; Kirschner, M.; Arndt, H.-D. Antiplasmodial Thiostrepton Derivatives: Proteasome Inhibitors with a Dual Mode of Action. *Angew. Chem., Int. Ed.* **2010**, *49* (19), 3317−3321.
(14) Ueno, M.; Furukawa, S.; Abe, F.; Ushioda, M.; Fujine, K.; Johki, S.; Hatori, H.; Ueda, H. Suppressive Effect of Antibiotic Siomycin on Antibody Production. *J. Antibiot.* **2004**, *57* (9), 590−596.
(15) Leeds, J. A.; LaMarche, M. J.; Brewer, J. T.; Bushell, S. M.; Deng, G.; Dewhurst, J. M.; Dzink-Fox, J.; Gangl, E.; Jain, A.; Lee, L.; Lilly, M.; Manni, K.; Mullin, S.; Neckermann, G.; Osborne, C.; Palestrant, D.; Patane, M. A.; Raimondi, A.; Ranjitkar, S.; Rann, E. M.; Sachdeva, M.; Shao, J.; Tiamfook, S.; Whitehead, L.; Yu, D. In Vitro and in Vivo Activities of Novel, Semisynthetic Thiopeptide Inhibitors of Bacterial Elongation Factor Tu. *Antimicrob. Agents Chemother.* **2011**, *55* (11), 5277−5283.
(16) LaMarche, M. J.; Leeds, J. A.; Dzink-Fox, J.; Gunderson, K.; Krastel, P.; Memmert, K.; Patane, M. A.; Rann, E. M.; Schmitt, E.; Tiamfook, S.; Wang, B. 4-Aminothiazolyl Analogues of GE2270 a: Antibacterial Lead Finding. *J. Med. Chem.* **2011**, *54* (7), 2517−2521.
(17) LaMarche, M. J.; Leeds, J. A.; Amaral, K.; Brewer, J. T.; Bushell, S. M.; Dewhurst, J. M.; Dzink-Fox, J.; Gangl, E.; Goldovitz, J.; Jain, A.; Mullin, S.; Neckermann, G.; Osborne, C.; Palestrant, D.; Patane, M. A.; Rann, E. M.; Sachdeva, M.; Shao, J.; Tiamfook, S.; Whitehead, L.; Yu, D. Antibacterial Optimization of 4-Aminothiazolyl Analogues of the Natural Product GE2270 a: Identification of the Cycloalkylcarboxylic Acids. *J. Med. Chem.* **2011**, *54* (23), 8099−8109.
(18) LaMarche, M. J.; Leeds, J. A.; Dzink-Fox, J.; Gangl, E.; Krastel, P.; Neckermann, G.; Palestrant, D.; Patane, M. A.; Rann, E. M.; Tiamfook, S.; Yu, D. Antibiotic Optimization and Chemical Structure Stabilization of Thiomuracin A. *J. Med. Chem.* **2012**, *55* (15), 6934−6941.
(19) LaMarche, M. J.; Leeds, J. A.; Amaral, A.; Brewer, J. T.; Bushell, S. M.; Deng, G.; Dewhurst, J. M.; Ding, J.; Dzink-Fox, J.; Gamber, G.; Jain, A.; Lee, K.; Lee, L.; Lister, T.; McKenney, D.; Mullin, S.; Osborne, C.; Palestrant, D.; Patane, M. A.; Rann, E. M.; Sachdeva, M.; Shao, J.; Tiamfook, S.; Trzasko, A.; Whitehead, L.; Yifru, A.; Yu, D.; Yan, W.; Zhu, Q. Discovery of LFF571: an Investigational Agent for Clostridium Difficile Infection. *J. Med. Chem.* **2012**, *55* (5), 2376−2387.
(20) LaMarche, M. J.; Leeds, J. A.; Brewer, J.; Dean, K.; Ding, J.; Dzink-Fox, J.; Gamber, G.; Jain, A.; Kerrigan, R.; Krastel, P.; Lee, K.; Lombardo, F.; McKenney, D.; Neckermann, G.; Osborne, C.; Palestrant, D.; Patane, M. A.; Rann, E. M.; Robinson, Z.; Schmitt, E.; Stams, T.; Tiamfook, S.; Yu, D.; Whitehead, L. Antibacterial and Solubility Optimization of Thiomuracin A. *J. Med. Chem.* **2016**, *59* (14), 6920−6928.
(21) Acker, M. G.; Bowers, A. A.; Walsh, C. T. Generation of Thiocillin Variants by Prepeptide Gene Replacement and in Vivo Processing by Bacillus Cereus. *J. Am. Chem. Soc.* **2009**, *131* (48), 17563−17565.
(22) Young, T. S.; Dorrestein, P. C.; Walsh, C. T. Codon Randomization for Rapid Exploration of Chemical Space in Thiopeptide Antibiotic Variants. *Chem. Biol.* **2012**, *19* (12), 1600−1610.
(23) Bowers, A. A.; Acker, M. G.; Young, T. S.; Walsh, C. T. Generation of Thiocillin Ring Size Variants by Prepeptide Gene Replacement and in Vivo Processing by Bacillus Cereus. *J. Am. Chem. Soc.* **2012**, *134* (25), 10313−10316.
(24) Luo, X.; Zambaldo, C.; Liu, T.; Zhang, Y.; Xuan, W.; Wang, C.; Reed, S. A.; Yang, P.-Y.; Wang, R. E.; Javahishvili, T.; Schultz, P. G.; Young, T. S. Recombinant Thiopeptides Containing Noncanonical Amino Acids. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (13), 3615−3620.
(25) Tran, H. L.; Lexa, K. W.; Julien, O.; Young, T. S.; Walsh, C. T.; Jacobson, M. P.; Wells, J. A. Structure-Activity Relationship and Molecular Mechanics Reveal the Importance of Ring Entropy in the Biosynthesis and Activity of a Natural Product. *J. Am. Chem. Soc.* **2017**, *139* (7), 2541−2544.
(26) Bowers, A. A.; Acker, M. G.; Koglin, A.; Walsh, C. T. Manipulation of Thiocillin Variants by Prepeptide Gene Replacement: Structure, Conformation, and Activity of Heterocycle Substitution Mutants. *J. Am. Chem. Soc.* **2010**, *132* (21), 7519−7527.
(27) Wang, S.; Zheng, Q.; Wang, J.; Zhao, Z.; Li, Q.; Yu, Y.; Wang, R.; Liu, W. Target-Oriented Design and Biosynthesis of Thiostrepton-Derived Thiopeptide Antibiotics with Improved Pharmaceutical Properties. *Org. Chem. Front.* **2015**, *2* (2), 106−109.
(28) Wang, J.; Lin, Z.; Bai, X.; Tao, J.; Liu, W. Optimal Design of Thiostrepton-Derived Thiopeptide Antibiotics and Their Potential Application Against Oral Pathogens. *Org. Chem. Front.* **2019**, *6* (8), 1194−1199.
(29) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K.-D.; Fischbach, M. A.; Garavelli, J. S.; Goransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Muller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Rebuffat, S.; Ross, R. P.; Sahl, H.-G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Sussmuth, R. D.; Tagg, J. R.; Tang, G.-L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. Ribosomally Synthesized and Post-Translationally Modified Peptide Natural Products: Overview and Recommendations for a Universal Nomenclature. *Nat. Prod. Rep.* **2013**, *30* (1), 108−160.
(30) Hudson, G. A.; Mitchell, D. A. RiPP Antibiotics: Biosynthesis and Engineering Potential. *Curr. Opin. Microbiol.* **2018**, *45*, 61−69.
(31) Wever, W. J.; Bogart, J. W.; Baccile, J. A.; Chan, A. N.; Schroeder, F. C.; Bowers, A. A. Chemoenzymatic Synthesis of Thiazolyl Peptide Natural Products Featuring an Enzyme-Catalyzed Formal [4 + 2] Cycloaddition. *J. Am. Chem. Soc.* **2015**, *137* (10), 3494−3497.
(32) Hudson, G. A.; Zhang, Z.; Tietz, J. I.; Mitchell, D. A.; van der Donk, W. A. In Vitro Biosynthesis of the Core Scaffold of the Thiopeptide Thiomuracin. *J. Am. Chem. Soc.* **2015**, *137* (51), 16012−16015.
(33) Fleming, S. R.; Bartges, T. E.; Vinogradov, A. A.; Kirkpatrick, C. L.; Goto, Y.; Suga, H.; Hicks, L. M.; Bowers, A. A. Flexizyme-Enabled

Benchtop Biosynthesis of Thiopeptides. *J. Am. Chem. Soc.* **2019**, *141* (2), 758−762.
(34) Wever, W. J.; Bogart, J. W.; Bowers, A. A. Identification of Pyridine Synthase Recognition Sequences Allows a Modular Solid-Phase Route to Thiopeptide Variants. *J. Am. Chem. Soc.* **2016**, *138* (41), 13461−13464.
(35) Bogart, J. W.; Bowers, A. A. Thiopeptide Pyridine Synthase TbtD Catalyzes an Intermolecular Formal Aza-Diels-Alder Reaction. *J. Am. Chem. Soc.* **2019**, *141* (5), 1842−1846.
(36) Zhang, Z.; Hudson, G. A.; Mahanta, N.; Tietz, J. I.; van der Donk, W. A.; Mitchell, D. A. Biosynthetic Timing and Substrate Specificity for the Thiopeptide Thiomuracin. *J. Am. Chem. Soc.* **2016**, *138* (48), 15511−15514.
(37) Cogan, D. P.; Hudson, G. A.; Zhang, Z.; Pogorelov, T. V.; van der Donk, W. A.; Mitchell, D. A.; Nair, S. K. Structural Insights Into Enzymatic [4 + 2] Aza-Cycloaddition in Thiopeptide Antibiotic Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (49), 12928−12933.
(38) Bycroft, B. W.; Gowland, M. S. The Structures of the Highly Modified Peptide Antibiotics Micrococcin P1 and P2. *J. Chem. Soc., Chem. Commun.* **1978**, *6*, 256−258.
(39) Perrin, C. L.; Lollo, C. P.; Johnston, E. R. NMR Site-to-Site Rate Constants and the Mechanisms of Acid-Catalyzed Proton Exchange in Secondary Amides. *J. Am. Chem. Soc.* **1984**, *106* (10), 2749−2753.
(40) Perrin, C. L. Symmetries of Hydrogen Bonds in Solution. *Science* **1994**, *266* (5191), 1665−1668.
(41) Li, Q.-G.; Xue, Y.; Yan, G.-S. Water-Assisted Enol-to-Keto Tautomerism of a Simple Peptide Model: a Computational Investigation. *J. Mol. Struct.: THEOCHEM* **2008**, *868* (1−3), 55−64.
(42) Schlegel, H. B.; Gund, P.; Fluder, E. M. Tautomerization of Formamide, 2-Pyridone, and 4-Pyridone: an Ab Initio Study. *J. Am. Chem. Soc.* **1982**, *104* (20), 5347−5351.
(43) Moody, C. J.; Hughes, R. A.; Thompson, S. P.; Alcaraz, L. Biosynthesis Inspired Diels-Alder Route to Pyridines: Synthesis of the 2,3-Dithiazolylpyridine Core of the Thiopeptide Antibiotics. *Chem. Commun.* **2002**, *225* (16), 1760−1761.
(44) Bycroft, B. W. Structural Relationships in Microbial Peptides. *Nature* **1969**, *224* (5219), 595−597.
(45) Pearce, C. J.; Rinehart, K. L. Berninamycin Biosynthesis. 1. Origin of the Dehydroalanine Residues †. *J. Am. Chem. Soc.* **1979**, *101* (17), 5069−5070.
(46) Houck, D. R.; Chen, L. C.; Keller, P. J.; Beale, J. M.; Floss, H. G. Biosynthesis of the Modified Peptide Antibiotic Nosiheptide in Streptomyces Actuosus. *J. Am. Chem. Soc.* **1988**, *110* (17), 5800−5806.
(47) Zhou, P.; O'Hagan, D.; Mocek, U.; Zeng, Z.; Yuen, L. D.; Frenzel, T.; Unkefer, C. J.; Beale, J. M.; Floss, H. G. Biosynthesis of the Antibiotic Thiostrepton. Methylation of Tryptophan in the Formation of the Quinaldic Acid Moiety by Transfer of the Methionine Methyl Group with Net Retention of Configuration. *J. Am. Chem. Soc.* **1989**, *111* (18), 7274−7276.
(48) Floss, H. G.; Beale, J. M. Biosynthetic Studies on Antibiotics. *Angew. Chem., Int. Ed. Engl.* **1989**, *28* (2), 146−177.
(49) Mocek, U.; Zeng, Z.; O'Hagan, D.; Zhou, P.; Fan, L. D. G.; Beale, J. M.; Floss, H. G. Biosynthesis of the Modified Peptide Antibiotic Thiostrepton in Streptomyces Azureus and Streptomyces Laurentii. *J. Am. Chem. Soc.* **1993**, *115* (18), 7992−8001.
(50) Mocek, U.; Knaggs, A. R.; Tsuchiya, R.; Nguyen, T.; Beale, J. M.; Floss, H. G. Biosynthesis of the Modified Peptide Antibiotic Nosiheptide in Streptomyces Actuosus. *J. Am. Chem. Soc.* **1993**, *115* (17), 7557−7568.
(51) Lau, R. C. M.; Rinehart, K. L. Biosynthesis of Berninamycin: Incorporation of 13C-Labeled Amino Acids. *J. Am. Chem. Soc.* **1995**, *117* (29), 7606−7610.
(52) Bowers, A. A.; Walsh, C. T.; Acker, M. G. Genetic Interception and Structural Characterization of Thiopeptide Cyclization Precursors From Bacillus Cereus. *J. Am. Chem. Soc.* **2010**, *132* (35), 12182−12184.
(53) Horner, K. E.; Karadakov, P. B. Shielding in and Around Oxazole, Imidazole, and Thiazole: How Does the Second Heteroatom Affect Aromaticity and Bonding? *J. Org. Chem.* **2015**, *80* (14), 7150−7157.
(54) Misra, P.; Mishra, B. K.; Behera, G. B. Hydrolysis of Schiff Bases, 1: Kinetics and Mechanism of Spontaneous, Acid, and Base Hydrolysis of N-(2/4-Hydroxybenzylidene)-2-Aminobenzothiazoles. *Int. J. Chem. Kinet.* **1991**, *23* (7), 639−654.
(55) Dash, A. C.; Dash, B.; Praharaj, S. Hydrolysis of Imines: Kinetics and Mechanism of Spontaneous Acid-, Base-, and Metal Ion-Induced Hydrolysis of N-Salicylidene-2-Aminothiazole. *J. Chem. Soc., Dalton Trans.* **1981**, *10*, 2063−2069.
(56) Hine, J.; Craig, J. C., Jr; Underwood, J. G., II; Via, F. A. Kinetics and Mechanism of the Hydrolysis of N-Isobutylidenemethylamine in Aqueous Solution. *J. Am. Chem. Soc.* **1970**, *92* (17), 5194−5199.
(57) Cordes, E. H.; Jencks, W. P. On the Mechanism of Schiff Base Formation and Hydrolysis. *J. Am. Chem. Soc.* **1962**, *84* (5), 832−837.
(58) Culbertson, J. B. Factors Affecting the Rate of Hydrolysis of Ketimines. *J. Am. Chem. Soc.* **1951**, *73* (10), 4818−4823.
(59) Tori, K.; Tokura, K.; Yoshimura, Y.; Terui, Y.; Okabe, K.; Otsuka, H.; Matsushita, K. Structures of Siomycin-B and -C and Thiostrepton-B Determined by NMR Spectroscopy and Carbon-13 Signal Assignments of Siomycins, Thiostreptons, and thiopeptin-B. *J. Antibiot.* **1981**, *34* (1), 124−129.
(60) Nicolaou, K. C.; Safina, B. S.; Zak, M.; Lee, S. H.; Nevalainen, M.; Bella, M.; Estrada, A. A.; Funke, C.; Zécri, F. J.; Bulat, S. Total Synthesis of Thiostrepton. Retrosynthetic Analysis and Construction of Key Building Blocks. *J. Am. Chem. Soc.* **2005**, *127* (31), 11159−11175.
(61) Ichikawa, H.; Bashiri, G.; Kelly, W. L. Biosynthesis of the Thiopeptins and Identification of an F420H2-Dependent Dehydropiperidine Reductase. *J. Am. Chem. Soc.* **2018**, *140* (34), 10749−10756.
(62) Hansen, P. E. Long range ^{13}C-^{13}C Coupling Constants. a Review. *Org. Magn. Reson.* **1978**, *11* (5), 215−233.
(63) Francke, W.; Schröder, F.; Walter, F.; Sinnwell, V.; Baumann, H.; Kaib, M. New Alkaloids From Ants: Identification and Synthesis of (3R,5S,9R)-3-Butyl-5-(1-Oxopropyl)Indolizidine and (3R,5R,9R)-3-Butyl-5-(1-Oxopropyl)Indolizidine, Constituents of the Poison Gland Secretion in Myrmicaria Eumenoides (Hymenoptera, Formicidae). *Eur. J. Org. Chem.* **1995**, *1995* (6), 965−977.
(64) Anderson, B.; Hodgkin, D. C.; Viswamitra, M. A. The Structure of Thiostrepton. *Nature* **1970**, *225* (5229), 233−235.
(65) Kelly, W. L.; Pan, L.; Li, C. Thiostrepton Biosynthesis: Prototype for a New Family of Bacteriocins. *J. Am. Chem. Soc.* **2009**, *131* (12), 4327−4334.
(66) Bordwell, F. G.; Algrim, D. J.; Harrelson, J. A. The Relative Ease of Removing a Proton, a Hydrogen Atom, or an Electron from Carboxamides versus Thiocarboxamides. *J. Am. Chem. Soc.* **1988**, *110* (17), 5903−5904.
(67) Bordwell, F. G.; Ji, G.-Z. Effects of Structural Changes on Acidities and Homolytic Bond Dissociation Energies of the Hydrogen-Nitrogen Bonds in Amidines, Carboxamides, and Thiocarboxamides. *J. Am. Chem. Soc.* **1991**, *113* (22), 8398−8401.
(68) Dan, Q.; Newmister, S. A.; Klas, K. R.; Fraley, A. E.; McAfoos, T. J.; Somoza, A. D.; Sunderhaus, J. D.; Ye, Y.; Shende, V. V.; Yu, F.; Sanders, J. N.; Brown, W. C.; Zhao, L.; Paton, R. S.; Houk, K. N.; Smith, J. L.; Sherman, D. H.; Williams, R. M. Fungal Indole Alkaloid Biogenesis Through Evolution of a Bifunctional Reductase/Diels-Alderase. *Nat. Chem.* **2019**, *11*, 972−980.