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Adenylation and *S*-Methylation of Cysteine by the Bifunctional Enzyme TioN in Thiocoraline Biosynthesis

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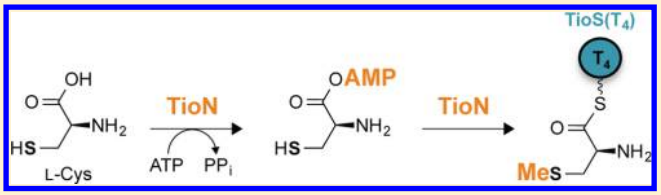
Supporting Information

ABSTRACT: The antitumor agent thiocoraline is a nonribosomally biosynthesized bisintercalator natural product, which contains in its peptidic backbone two *S*-methylated L-cysteine residues. *S*-Methylation occurs very rarely in nature, and is observed extremely rarely in nonribosomal peptide scaffolds. We have proposed that during thiocoraline biosynthesis, TioN, a stand-alone adenylation domain interrupted by the *S*-adenosyl-L-methionine binding region of a methyltransferase enzyme, is capable of performing two functions: the adenylation and *S*-methylation of L-cysteine. Herein, by preparation of knockouts of TioN and its MbtH-like protein partner TioT, we confirmed their role in thiocoraline biosynthesis. We also co-expressed recombinant TioN and TioT and biochemically investigated three potential pathways involving activation, methylation, and loading of L-cysteine onto the TioN partner thiolation domain, TioS(T_4). The valuable insights gained into the pathway(s) followed for the production of *S*-Me-L-Cys-*S*-TioS(T_4) will serve as a guide for the development of novel engineered interrupted adenylation enzymes for combinatorial biosynthesis.



INTRODUCTION

Thiocoraline is a nonribosomal peptide (NRP) bisintercalator[1,2] natural product that contains unique structural features: (i) two 3-hydroxyquinaldic acid (3HQA) moieties required for binding of the molecule with high affinity to the minor groove of DNA,[3] (ii) two rare and intriguing *S*-methylated L-cysteine (*S*-Me-L-Cys) moieties, (iii) an intramolecular disulfide linkage, and (iv) a thiodepsipeptide backbone (Figure 1A). We previously thoroughly investigated the 3HQA formation. By demonstrating the broad substrate profile of the tryptophan 2,3-dioxygenase TioF,[4] by proving the role of the type II thioesterase TioQ in thiocoraline production,[5] and by confirming the involvement of TioK, an adenylation−thiolation (A-T) didomain involved in the conversion of L-Trp into L-Trp-adenosine monophosphate (AMP) as the first committed step during 3HQA formation,[6] we revised the original proposed pathway[7] and unambiguously established the correct pathway for the formation of 3HQA.

To our knowledge, the *S*-Me-L-Cys residue is only found in three known NRP scaffolds, which are all DNA bisintercalators: the quinomycins, which include echinomycin (quinomycin A),[8] SW-163D,[9] and thiocoraline, the subject of the current study. In the quinomycins and in SW-163D, there is only one *S*-Me-L-Cys engaged in an S−C bond as part of a thioacetal bridge, which creates asymmetry in these bicyclic compounds. In contrast, in thiocoraline there are two *S*-Me-L-Cys moieties in the peptidic skeleton, and the central disulfide bond is intact, preserving the two-fold symmetry. The chemistry of the rearrangement of the disulfide bond into the thioacetal linkage during echinomycin biosynthesis had for a long time been postulated, but never confirmed. This year, by structural studies, the *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase Ecm18 was shown to perform this conversion in two stages: the methylation of one of the sulfur atoms of the disulfide bond of triostin A,[10] followed by a rearrangement to form the thioacetal bridge of echinomycin.[11] However, no homologues of Ecm18 are found in the thiocoraline biosynthetic gene cluster (Figure 1B).

As the *S*-Me-L-Cys is of rare occurrence in NRPs, we are interested in gaining a greater understanding of its formation and its incorporation into the growing peptide chain during thiocoraline production. It was originally suggested that TioX, a protein with sequence homology to the type I glyoxalase superfamily, would be responsible for *S*-methylation of L-Cys during thiocoraline biosynthesis.[7] By gene deletion, biochemical, and structural studies, we previously demonstrated that TioX is not responsible for *S*-methylation of L-Cys, and instead is involved in thiocoraline resistance or secretion.[12]

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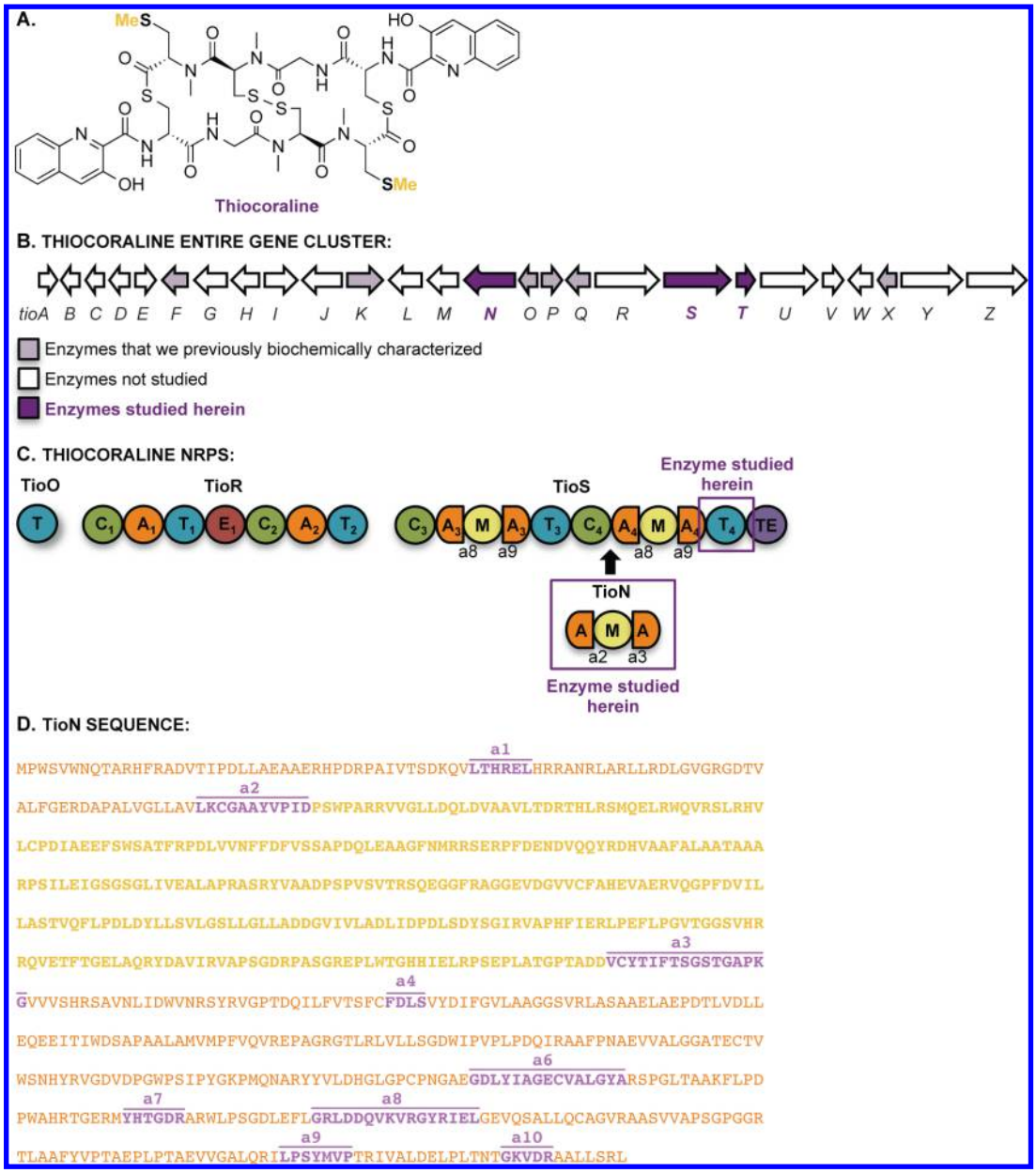

Figure 1. (A) Thiocoraline structure. (B) Thiocoraline gene cluster. (C) Structural organization of the thiocoraline NRPS. Abbreviations used: A = adenylation, C = condensation, T = thiolation, E = epimerization, M = methylation, TE = thioesterase. (D) Amino acid residue sequence of the bifunctional enzyme TioN, an A domain interrupted between a2 and a3 by the SAM-binding domain of a methyltransfease. The TioN A and M domains are depicted in orange and yellow, respectively. The core signature sequences (a1−a10) are depicted in purple.

Recently, we established that an adenylation (A) domain interrupted by a part of a methyltransferase (M) enzyme in the KtzH module of the kutznerides nonribosomal peptide synthestase (NRPS) assembly line is responsible for both activation and *O*-methylation of L-Ser during kutznerides biosynthesis.[13] Upon close inspection of the thiocoraline biosynthetic gene cluster, we identified TioN, also an A domain interrupted by a part of an M enzyme, as a potential candidate for *S*-Me-L-Cys formation (Figure 1C). A domains interrupted by *O*-methyltransferase,[13] *N*-methyltransferase,[14−20] oxidase,[21−23] monooxygenase,[22,23] and ketoreductase,[24−26] are found in the NRPS machinery of a variety of natural products, but very few have been investigated. In almost all of these cases, the interruption of the A domain is found between the a8 and a9 core signature sequences (A domains contain 10 core signature sequences, "a1−a10"[27]). Interestingly, in TioN, the only identified interrupted A domain that could perform the unique *S*-methylation of L-Cys, the interruption by the M domain occurs between the core signature sequences a2 and a3 (Figure 1D). Additionally, TioN is unique in that it is the only known stand-alone interrupted A domain. All others are found within NRPS modules.

There are three possible pathways involving TioN and TioS(T_4) that could lead to the formation, in three steps, of an *S*-Me-L-Cys covalently tethered to the phosphopantetheinyl (Ppant) arm of the TioN partner T domain, TioS(T_4) (Figure 2). In this study, we report our efforts toward delineating this *S*-Me-L-Cys-*S*-TioS(T_4) formation in thiocoraline biosynthesis and propose the most likely pathways.

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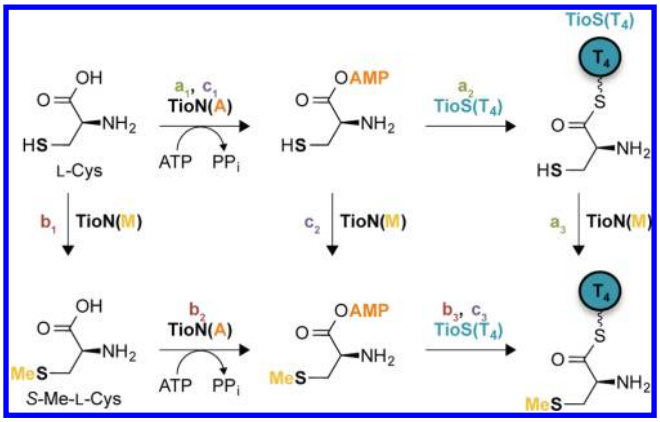

Figure 2. Three potential pathways (a–c, with three steps each) for the formation of *S*-Me-L-Cys-*S*-TioS(T_4) by TioN. All steps involving TioN require the presence of the MbtH-like protein partner TioT.

RESULTS AND DISCUSSION

Dependence of Thiocoraline Production on TioN and TioT Proteins. As TioN was originally proposed to be inactive,[7] in order to test its involvement in thiocoraline production, we prepared a *tioN* knockout in the thiocoraline-overproducing strain *Streptomyces albus*-pFL1049, *S. albus*-pFL1049-Δ*tioN* (Figure S1). By HPLC and mass spectrometry (MS), we confirmed that this Δ*tioN* strain does not produce thiocoraline (Figure S3), providing a proof that TioN is directly involved in the biosynthesis of this bisintercalator.

Having established the importance of TioN in thiocoraline production, we set out to purify this enzyme for biochemical studies. We successfully cloned, heterologously overexpressed, and purified to homogeneity the TioN protein. Unfortunately, although expressed and purified in large quantities, TioN was completely devoid of adenylation and methylation activities.

We previously demonstrated the importance of the MbtH-like protein TioT for production and activity of another enzyme in the thiocoraline gene cluster, the A-T didomain TioK.[6] MbtH-like proteins from other NRPS assembly lines have also been reported to play an important role in solubility and activity of A domains.[28−32] MbtH-like proteins have been suggested to serve a variety of functions during NRP biosynthesis,[33] as allosteric regulators of adenylating enzymes,[34] folding chaperones,[35,36] and enzymes being integral components of NRPS machineries.[30] To test whether TioT is an essential enzyme in the thiocoraline biosynthetic pathway or only an accessory protein, we generated a *tioT* knockout in *S. albus*-pFL1049, *S. albus*-pFL1049-Δ*tioT* (Figure S2). By HPLC and MS, we established that this strain did not produce thiocoraline (Figure S4), demonstrating the essentiality of TioT for thiocoraline biosynthesis and identifying TioT as an integral component of the *tio* NRPS complex.

Heterologous Co-expression and Purification of TioN and TioT Proteins. To evaluate the proposed bifunctional role (adenylation and methylation) of the discrete interrupted A domain TioN in the generation of *S*-Me-L-Cys-*S*-TioS(T_4), we cloned the *tioN* and *tioT* genes in pET28a and pACYCDuet1, respectively. We then co-expressed TioN and TioT, and purified them in their soluble and active form (as determined during the establishment of the substrate specificity profile presented in the next section) by Ni^{2+}-affinity chromatography (Figure S5). All biochemical experiments presented in this study were performed using the TioN co-expressed with TioT. A previous study by Thomas and co-workers showed that MbtH-like proteins are needed in stoichiometric amounts to form a complex with their partner A domains.[29]

We originally did not expect TioT to be required for activity of TioN, as TioN could be purified without this MbtH-like protein partner. However, we have now established that, in contrast to TioK and KtzH, where the respective MbtH-like partner proteins TioT[6] and KtzJ[13] are required for both production and activity of the enzymes, TioT is essential for the enzymatic activity of TioN, but not for the expression of TioN in its soluble form.

Substrate Specificity and Kinetic Characterization of the A Domain Portion of TioN. There are three possible pathways, designated by a_1–a_3, b_1–b_3, and c_1–c_3 in Figure 2, by which *S*-Me-L-Cys-*S*-TioS(T_4) could be generated. To establish if pathways a and c are favored over b, we first determined the substrate specificity profile of the A domain portion of TioN by ATP-[^{32}P]PP_i exchange assays (Figure 3A). These data show

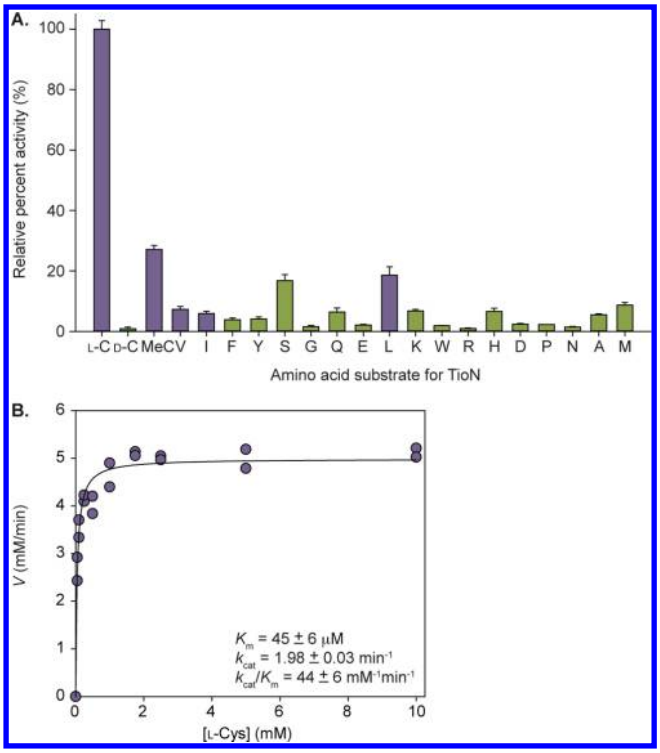

Figure 3. (A) Relative substrate specificity of the A domain portion of TioN co-expressed with TioT (2.5 μM), as determined by ATP-[^{32}P]PP_i exchange assays. The data are obtained in 2-h end-point assays. The substrates for which kinetics parameters were determined (Table 1) are designated by the purple bars. (B) Michaelis–Menten parameters of the TioN-catalyzed adenylation of amino acids. The natural TioN substrate, L-Cys, was selected as a representative example. Standard errors as depicted by the duplicate on the graph ranged from 0.04 to 0.25 mM/min.

that L-Cys is the natural substrate for the enzyme, whereas *S*-Me-L-Cys, L-Val, L-Ile, and L-Leu are poorer substrates of TioN. These data strongly suggest that pathway b is unlikely to generate *S*-Me-L-Cys-*S*-TioS(T_4) *in vivo*.

To further confirm this hypothesis, we determined the Michaelis–Menten kinetic parameters (K_m and k_{cat}) in substrate-dependent AMP derivatization assays by TioN for L-Cys, *S*-Me-L-Cys, L-Val, L-Ile, and L-Leu (Table 1 and Figure 3B). All other amino acids tested were also poor substrates of

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Table 1. Steady-State Kinetic Parameters for AMP Derivatization of Different Substrates by TioN

substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
L-Cys	45 ± 6	1.98 ± 0.03	44 ± 6
S-Me-L-Cys	16 168 ± 1167	1.23 ± 0.05	0.076 ± 0.006
L-Val	71 ± 11	0.215 ± 0.006	3.05 ± 0.52
L-Ile	11 ± 5	0.156 ± 0.004	14 ± 7
L-Leu	55 ± 6	0.213 ± 0.004	3.87 ± 0.45

TioN (Figure 3A) and determination of kinetic parameters, although attempted, were not successful for these amino acids. TioN displayed the highest catalytic efficiency with the natural substrate L-Cys (k_{cat}/K_m = 44 ± 6 mM^{-1} min^{-1}), followed by L-Ile (k_{cat}/K_m = 14 ± 7 mM^{-1} min^{-1}), L-Leu (k_{cat}/K_m = 3.87 ± 0.45 mM^{-1} min^{-1}), and L-Val (k_{cat}/K_m = 3.05 ± 0.52 mM^{-1} min^{-1}). Interestingly, unlike KtzH($A_4MA_4T_4$) (k_{cat}/K_m = 0.77 ± 0.07 mM^{-1} min^{-1} for the activation of L-Ser),[13] where the interruption of the A domain results in a greatly diminished catalytic efficiency of the enzyme when compared to that of uninterrupted A domains, for TioN, the catalytic efficiency for L-Cys activation (k_{cat}/K_m = 44 ± 6 mM^{-1} min^{-1}) is similar to that of the uninterrupted A domain TioK involved in the formation of L-Trp-AMP (k_{cat}/K_m = 49 ± 8 mM^{-1} min^{-1}).[6] Because the catalytic efficiency of TioN with *S*-Me-L-Cys (k_{cat}/K_m = 0.076 ± 0.006 mM^{-1} min^{-1}) is much lower than with L-Cys (k_{cat}/K_m = 44 ± 6 mM^{-1} min^{-1}), pathway b can likely be ruled out.

Methyltransferase Activity of TioN and Loading of Activated Amino Acids onto TioS(T_4). To compare the covalent attachment of L-Cys-AMP (steps a_1−a_2 in Figure 2) to that of *S*-Me-L-Cys-AMP (steps c_1−c_3) onto the Ppant arm of holo TioS(T_4), we used [^{35}S]L-Cys as our starting material in two parallel time course trichloroacetic acid (TCA) precipitation assays (Figure 4A). By using TioN, we first generated [^{35}S]L-Cys-AMP (steps a_1 and c_1). We then either (i) used this [^{35}S]L-Cys-AMP directly in combination with holo TioS(T_4) thiolation domain to monitor its loading onto the enzyme (step a_2, green squares in Figure 4A), or (ii) treated it with SAM for 2 h (step c_2) prior to monitoring the loading of the resulting *S*-Me-[^{35}S]L-Cys-AMP onto the holo TioS(T_4) (step c_3, orange circles in Figure 4A). These assays show that both L-Cys-AMP and *S*-Me-L-Cys-AMP can be loaded onto TioS(T_4). However, one could argue that, in these assays there was no guarantee that the methylation of [^{35}S]L-Cys-AMP had taken place and that in both sets of reactions, only the loading of the unmethylated [^{35}S]L-Cys-AMP was observed.

To further investigate if *S*-methylation of L-Cys by the M domain portion of TioN occurs before (step c_2) and/or after (step a_3) its loading onto the Ppant arm of TioS(T_4), and to prove that the methylation reaction by the M domain portion of TioN is indeed happening, we used non-radiolabeled L-Cys as the starting material and [methyl-^{3}H]SAM as the methylating agent in two parallel time course TCA precipitation assays (Figure 4B). After conversion of L-Cys to L-Cys-AMP by TioN (steps a_1 and c_1), we either (i) loaded the activated amino acid onto holo TioS(T_4) (step a_2) prior to monitoring the methylation of L-Cys-*S*-TioS(T_4) by using [methyl-^{3}H]SAM and TioN (step a_3, green squares in Figure 4B), or (ii) methylated L-Cys-AMP by using [methyl-^{3}H]SAM and TioN (step c_2) prior to monitoring the loading of the resulting *S*-[^{3}H]Me-L-Cys-AMP onto the holo TioS(T_4) (step c_3, orange circles in Figure 4B). Control experiments in the absence of substrates were performed to eliminate the non-specific methylation of surface cysteine residues. The results of these experiments confirmed the successful *in vitro* methylation of both L-Cys-*S*-TioS(T_4) and L-Cys-AMP by TioN.

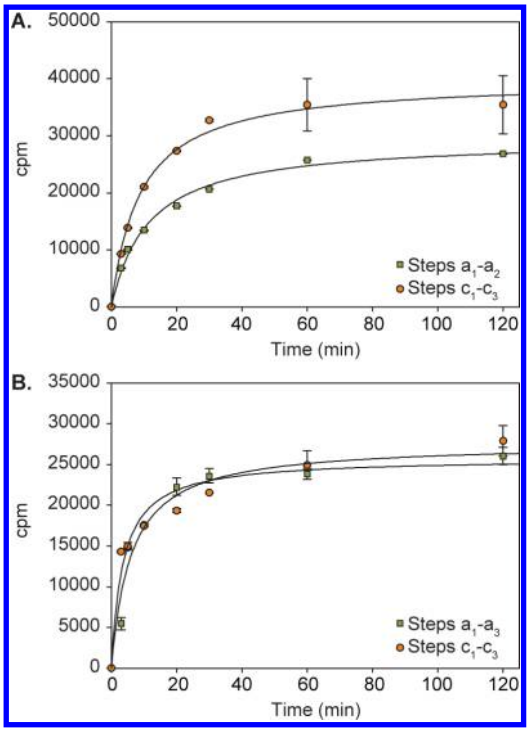

Figure 4. (A) Comparison of the loading of [^{35}S]L-Cys-AMP (steps a_1−a_2 in Figure 2; green squares) to that of *S*-Me-[^{35}S]L-Cys-AMP (c_1−c_3; orange circles) onto TioS(T_4) by TCA precipitation assays using [^{35}S]L-Cys as a starting material. (B) Comparison of the methylation by TioN of L-Cys-*S*-TioS(T_4) (a_1−a_3; green squares) to that of *S*-Me-L-Cys-AMP (c_1−c_3; orange circles) by TCA precipitation assays using [methyl-^{3}H]SAM as the methylating agent.

CONCLUSION

In summary, by testing the *tioN* and *tioT* knockouts of the thiocoraline producer we confirmed the essential role of these genes in thiocoraline biosynthesis. We biochemically characterized the first active stand-alone bifunctional (adenylating and methylating) interrupted A domain TioN in an NRPS complex. By determining the substrate specificity profile of TioN by radioactive adenylation assays, we eliminated pathway b (Figure 2) for the formation of *S*-Me-L-Cys-*S*-TioS(T_4). We confirmed the methylating activity of TioN and demonstrated that both pathways a and c can be achieved *in vitro*. In conjunction with our previous report on the formation of *O*-Me-L-Ser-*S*-KtzH($A_4MA_4T_4$), this work sets the stage for the development of novel interrupted adenylating enzymes by domain swapping experiments. Our laboratory is focused on understanding the significance of the location of the insertion of M enzymes in A domains (between the core signature sequences a2 and a3 versus a8 and a9) toward engineering new bifunctional adenylating−methylating enzymes.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the generation of *tioN* and *tioT* knockout (Δ*tioN* and Δ*tioT*) in *S. albus*-pFL1049 and monitoring of thiocoraline production in these knockout

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strains, preparation of the pTioN-pET28a and pTioT-pACYCDuet overexpression constructs along with the co-expression and purification of TioN and TioT proteins, and ATP-[^{32}P]PP$_i$ exchange as well as TCA precipitation assays with [^{35}S]L-Cys and [methyl-^{3}H]SAM used to investigate the different pathways presented in Figure 2; figures showing the preparation of Δ*tioN* and Δ*tioT*, HPLC chromatograms demonstrating the lack of thiocoraline production in the knockout strains, and a SDS-PAGE gel showing the TioN protein co-purified with TioT. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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