Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
This journal is © The Royal Society of Chemistry 2019

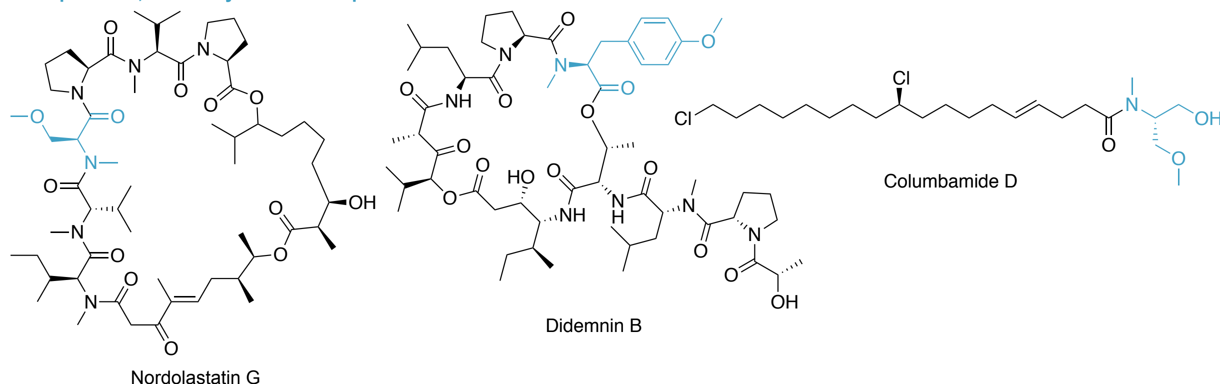
Supporting Information

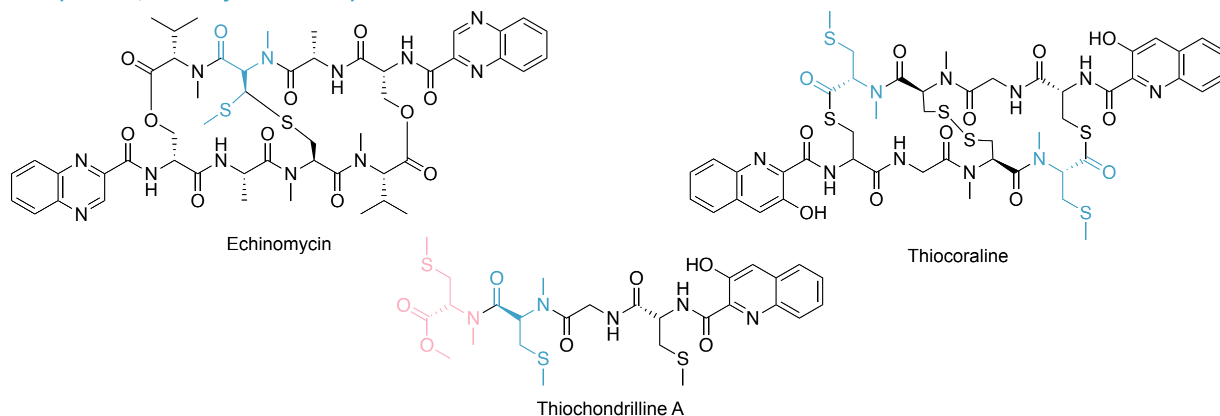
Probing the limits of interrupted adenylation domains by engineering a trifunctional enzyme capable of adenylation, *N*-, and *S*-methylation

Taylor A. Lundy,[a] Shogo Mori,[a] and Sylvie Garneau-Tsodikova[a,*]
[a]University of Kentucky, Department of Pharmaceutical Sciences, College of Pharmacy, Lexington, KY 40536-0596, USA. *sylviegtsodikova@uky.edu

Table of Contents **Pages**

Fig. S1: Examples of structures of natural products containing dimethylated amino acids S2
1. Bacterial strains, plasmids, materials, and instruments S2-3
Fig. S2: NRPS assembly-line for thiocoraline biosynthesis S3
Fig. S3: Sequence alignment used to determine insertion points of M_{3S} in TioN($A_aM_NA_b$) S4
2. Preparation of p*tioN(AM$_N$AM$_{3S}$A)*-pET28a and p*tioN(AM$_N$AM$_{3S}$A)D818A*-pET28a overexpression constructs S4-5
Table S1: Primers used in this study S5
Fig. S4: Cloning strategy for the preparation of p*tioN(AM$_N$AM$_{3S}$A)*-pET28a S6
3. Preparation of p*tioN(A$_a$M$_N$A$_b$)D167A*-pET28a overexpression construct S6
4. Co-overexpression and co-purification of TioN(AM$_N$AM$_{3S}$A) and TioN(AM$_N$AM$_{3S}$A)D818A with MLP TioT S6-7
5. Substrate specificity and determination of kinetic parameters of TioN(AM$_N$AM$_{3S}$A) and TioN(AM$_N$AM$_{3S}$A)D818A by ATP-[^{32}P]PP$_i$ exchange assays S8-9
Fig. S5: A domain activity of TioN($A_aM_NA_b$)D167A S8
Fig. S6: Time course for adenylation activity of wt TioN($A_aM_NA_b$) and TioN($A_aM_NA_b$)D167A with *N*,*S*-diMe-L-Cys S9
Fig. S7: Michaelis-Menten kinetic plots of TioN(AM$_N$AM$_{3S}$A) and TioN(AM$_N$AM$_{3S}$A)D818A S9
6. Characterization of methylation (M) domain activity by TioN(AM$_N$AM$_{3S}$A) and TioN(AM$_N$AM$_{3S}$A)D818A by TCA precipitation assays S10-11
Fig. S8: M domain (in)activity of TioN($A_aM_NA_b$)D167A S10
7. Evaluation of methylated products liberated from the T domain by potassium hydroxide (KOH) cleavage and Quadrupole time-of-flight mass spectrometry (Q-TOF MS) S11-12
Fig. S9: Mass spectra of amino acid standards and *N*,*S*-diMe-L-Cys from enzymatic reaction S13
Fig. S10: Tandem mass spectra (MS^2) of *N*,*S*-diMe-L-Cys standard and generated enzymatically S14
8. References S14





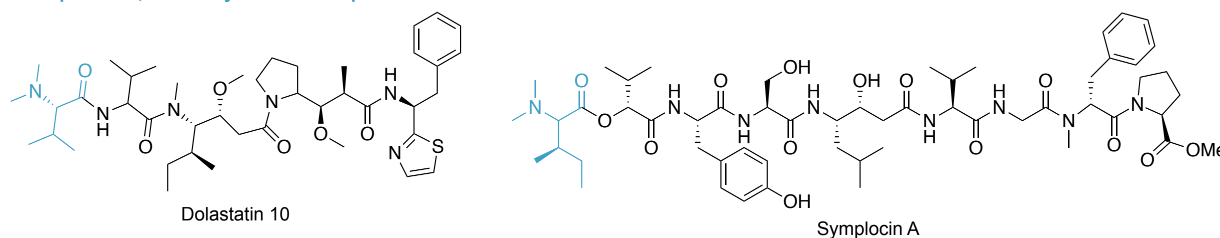


Fig. S1: Examples of structures of natural products containing dimethylated (blue) and trimethylated (pink) amino acids.

1. Bacterial strains, plasmids, materials, and instruments. The TioS(T_3)[1] and Sfp[2] proteins were expressed and purified as previously reported. For the purpose of constructing the expression plasmids, chemically competent *E. coli* TOP10 cells were purchased from Invitrogen (Carlsbad, CA, USA). For expression of the di-interrupted A domains and the accompanying MbtH-like protein (MLP) TioT, the *E. coli* BL21 (DE3)ybdZ::aac(3)IV strain[3] was used and was originally obtained as a gift from Professor Michael G. Thomas (University of Wisconsin-Madison, USA). The expression vector used for the construction of the di-interrupted A domains in the current study were originally purchased from Novagen (Gibbstown, NJ, USA). DNA primers were purchased from Sigma-Aldrich (St. Louis, MO, USA). All remaining reagents used for cloning were from New England BioLabs (NEB, Ipswich, MA, USA). DNA

sequencing was performed at Eurofins Scientific (Louisville, KY, USA). All chemical reagents and buffer components used for protein purification and enzymatic assays were used without any further purification and were from Sigma-Aldrich or VWR (Atlanta, GA, USA), except for *N*-Me-L-Cys and *N*,*S*-diMe-L-Cys whose synthesis was previously described.[1] A Sorvall RC-6 PLUS (Thermo, Ashville, NC, USA) was used for large scale protein purification. A Microfuge® 18 Centrifuge (Beckman Coulter, Palo Alto, CA, USA) was used for table top centrifugation. PCRs were conducted on a Robocycler® Gradient 96 (Stratagene, La Jolla, CA, USA). The radioactive reagents used in the enzymatic assays, [methyl-^{3}H]SAM (*S*-adenosyl-L-methionine) and [^{32}P]PP_i were from PerkinElmer (Waltham, MA, USA), and EcoLume™ liquid scintillation cocktail was from MP Biomedicals (Santa Ana, CA, USA). Radioactivity was counted by using a TriCarb 2900TR Liquid Scintillation Analyzer (PerkinElmer).

Note: Explanation of naming of engineered plasmids. The wild-type (wt) proteins (Fig. S2) used for cloning of the novel di-interrupted adenylation (A) domains were: (i) TioN($A_aM_NA_b$) where the methylation (M_N) domain was naturally found to be inserted between two A domain fragments (a and b) between the a2 and a3 recognition motifs,[4] and (ii) TioS($A_{3a}M_{3S}A_{3b}$) from the 3rd module in the thiocoraline biosynthetic assembly-line where the M_{3S} domain was naturally found to be inserted between two A domain fragments (3a and 3b) between the a8 and a9 recognition motifs. In order to simplify the naming of the di-interrupted A domains that we constructed in this study, we omitted the subscript letter indicating each part of the A domain and used TioN($AM_NAM_{3S}A$) where the M_N is located between a2-a3 and M_{3S} between a8-a9.

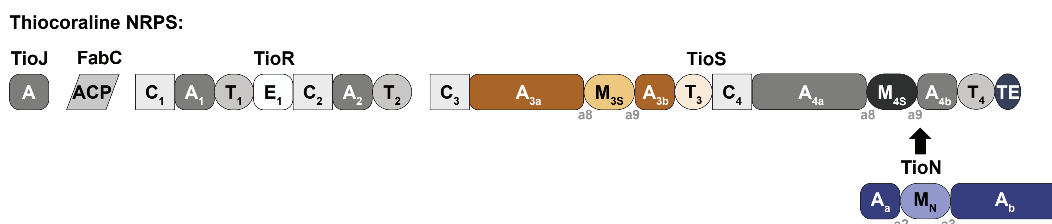


Fig. S2: NRPS biosynthetic assembly-line for the production of thiocoraline. The wild-type (wt) proteins, TioS($A_{3a}M_{3S}A_{3b}$), TioS(T_3), and TioN($A_aM_NA_b$) used in this study are highlighted in brown, peach, and blue, respectively. Domain abbreviations: A = adenylation; ACP = acyl carrier protein; C = condensation; E = epimerization; M = methylation; T = thiolation; TE = thioesterase. *Note*: The following enzymes presented above have been previously studied: TioJ/FabC,[5] TioS($A_{3a}M_{3S}A_{3b}T_3$),[1] TioS($A_{4a}M_{4S}A_{4b}T_4$),[6] and TioN($A_aM_NA_b$).[7]

```
TioS(A4aM4SA4b)     1 ............DAPIDAVEILNRDDLAALERWTGRARGTDRVVGTIPERFAAVVAEQPEAVALVAADGEESWTYGELDRWAN
TioN(AΔMNAM3SA)     1 ...........................MPWSVWNQTARHFRADV.TIPDLLAEAAERHPDRPAIVT.SDKQVLTHRELHRRAN
Ecm6(A1aM3SA1b)     1 FTTFLRRALADPRAPVGTVTALHPCEQRALLTEYAGPTVPTPPV.TLTELLDRQAHATPDAVALLW.EGSPT.TYRELAERSG
                                                                                              a1

TioS(A4aM4SA4b)    72 RIAHHLHARGVGRQHRVALVMERSPLLVAAVLGTLKAGACYVPVEPTWPRARIDLVLADLDPALVIDER.............
TioN(AΔMNAM3SA)    55 RLARLLRDLGVGRGDTVALFGERDAPALVGLLAVLKCGAAYVPIDPSWPARRVVGLLDQLDVAAVLTDRTHLRSMQELRWQRE
Ecm6(A1aM3SA1b)    81 RLARLLTERGAGPERCVAVAVPRSPELVVALIAVLRAGAAYLPVDPDYPAARVAFMLADARPALLLTAKD...TADRLPPTDV
                                                       a2

TioS(A4aM4SA4b)   141 ..LAEEDLTGYPTRPLDTADVGGEHLAYLMYTSGSTGTPKGVEVSHRNVLSLA..LDPCWADADHQRVLVHAPPTFDASTYEM
TioN(AΔMNAM3SA)   138 PLWTGHHIELRPSEPLATGP.TADDVCYTIFTSGSTGAPKGVVVSHRSAVNLIDWVNRSYRVGPTDQILFVTSFCFDLSVYDI
Ecm6(A1aM3SA1b)   161 PVLLVDDVPPAEGPVAPAAH.GVDHPAYMIYTSGSTGRPKGVVVTHAAIVNRLLWMQDRFRLDGTDRVLQKTSASFDVSVWEF
                                                    a3                                     a4

TioS(A4aM4SA4b)   220 WVPLLHGGAAVVAPPGKL.DAARLATLIAERGVTAL.WLPAGLF...DLITQHHPKSFVQVREVWAGGDVLSPAAVRRLVRDD
TioN(AΔMNAM3SA)   220 FGVLAAGGSVRLASAAELAEPDTLVDLLEQEEITIWDSAPAALAMVMPFVQVREPAGRGTLRLVLLSGDWIPVPLPDQIRAAF
Ecm6(A1aM3SA1b)   243 FWPLITGATLVIARPDGHRDPDYLAELIRRAGVTTAHFVPSMLA...EFVTDGAAAACTGLRRVVCSGEALPAELAARFHRTF

TioS(A4aM4SA4b)   298 GTLTVVNGYGPTETTTFAARYRMSAPARCKDPLPIGEPMAGSRLYALDDRLRQVPQGVIGELYVGGDGVARGYANHPPLTSER
TioN(AΔMNAM3SA)   303 PNAEVVALGGATECTVWSNHYRVGDVDPGWPSIPYGKPMQNARYYVLDHGLGPCPNGAEGDLYIAGECVALGYARSPGLTAAK
Ecm6(A1aM3SA1b)   323 .GVPLHNLYGPTEAAV...DVTAWEYRPGARTVPIGTPIWNTALYVLDSRLRPLPPGVHGDLYIAGAGLARGYHDRPGLTAER
                          a5                                                    a6

TioS(A4aM4SA4b)   381 FVADPFGR.PGERMYRTGDLVRWNHDGQLEFLGRVDEQVKIRGFRVEPGEIRAALRKRDGVAQAVVVPRTDRLGER..RLVAY
TioN(AΔMNAM3SA)   386 FLPDPWAHRTGERMYHTGDRARWLPSGDLEFLGRLDDQVKVRGYRIELGEVQSALLQCAGVRAASVVAPSGPGGRT...LAAF
Ecm6(A1aM3SA1b)   402 FVACPFGE.PGRRMYRTGDLARWNAQGELEFAGRADHQVKIRGFRVEPQEIEDTLTGHPAVLRAAVVARPGRGADAAAQLVAY
                              a7                    a8

TioS(A4aM4SA4b)   461 VVPEVPAGADED...STEHVEKWRAIYDSMYDETEADATEIGNDFTGWKSSYTRDNIPLSEMRRWRDSVVEEVRGLRARRILE
TioN(AΔMNAM3SA)   466 YVP.TARSTVESVVAADEQVREWQEIYDQGY..LEVTDQDFGDDFNLWVSSYTGEPIPVGQMREWQDAAVDRILSFTPRRVLE
Ecm6(A1aM3SA1b)   484 IVPVTARSTVESVVAADEQVREWQEIYDQGY..LEVTDQDFGDDFNLWVSSYTGEPIPVGQMREWQDAAVDRILSFTPRRVLE

TioS(A4aM4SA4b)   541 IGVGSGLLLGPLAPEAEAYWGTDFSLPVIERLEVQVGTDPCLKEKVSLRCQHADVADGLPVKYFDTVILNSVVQYFPDAAYLS
TioN(AΔMNAM3SA)   546 VGAGTGLLLARVAGSVEAYWATDFSEPVIERLGRQV.TEAGWAERVRLLCRRADDLDGIP.RIFDTVVLNSVVQYFPNERYLE
Ecm6(A1aM3SA1b)   565 VGAGTGLLLARVAGSVEAYWATDFSEPVIERLGRQV.TEAGWAERVRLLCRRADDLDGIP.RIFDTVVLNSVVQYFPNERYLE

TioS(A4aM4SA4b)   624 RVLDVALDRLAPGGRILVGDVRNYGTLREFLTAVHHAQHPQDSASAVRAAVERAVLAEKELVIDPDFFTEWARTRPDVVAVDI
TioN(AΔMNAM3SA)   627 QVLDGVWAMLEPGGRLVLGDIRRARSLRAFQVAVQQAKHGNLPPAQLRSAVEQGLLLEKELVIDPEWFQRWAE.RAGAAGVDV
Ecm6(A1aM3SA1b)   646 QVLDGVWAMLEPGGRLVLGDIRRARSLRAFQVAVQQAKHGNLPPAQLRSAVEQGLLLEKELVIDPEWFQRWAE.RAGAAGVDV

TioS(A4aM4SA4b)   707 RLKPGADQNELTRHRYEVILHKQ.PSQPLRLADVRTANWGSEVPDLSGLETALARHGGRL.RLARIPNARLVSE...AVQCGV
TioN(AΔMNAM3SA)   709 RLKEGAFQNELTRHRYEIVVHKPGTTEAGRPYAVDTVPRLEWNGDLDGLAERIRTLGGPVVRIAGIPNARVAQEVAAARDLGL
Ecm6(A1aM3SA1b)   728 RLKEGAFQNELTRHRYEIVVHKPGTTEAGRPYAVDTVPRLEWNGDLDGLAERIRTLGGPVVRIAGIPNARVAQEVAAARDLGL

TioS(A4aM4SA4b)   785 PTNVGGT..PLDPHELASWGGQRGYSVHCTWSAEAPGWFEAVIIPVDSGHCR..DGVYRPVGPRPRQLVNLPAAARRVSRLPS
TioN(AΔMNAM3SA)   792 EESEPPTSVPVDPHEMATWAARQGWSIALTWSGSAVDQFEAVLFTDATTEHRALSGTYLPVAEF.............EPLPTA
Ecm6(A1aM3SA1b)   811 EESEPPTSVPVDPHEMATWAARQGWSIALTWSGSAVDQFEAVLFTDATTEHRALSGTYLPVAEFGGSRPAGADWDLHAGVDLA

TioS(A4aM4SA4b)   864 WLREELAAELPEHLVPGDIVVMERLPLTTNGKIDHSRLPEVEQADREYGSPRTPLEAELAGLFAEVLGVDRVGIEDDFFDCGG
TioN(AΔMNAM3SA)   862 EVVGALQRILPSYMVPTRIVALDELPLTNTGKVDRAALLSRL.........................................
Ecm6(A1aM3SA1b)   894 ELRGFVAARLPAHLVPAAFVALDRLPMTANGKLDRAGLPEPEITRQAHRPPATREESLLAAAFAEVLGLSDIGVDDDFFALGG
                               a9                a10

TioS(A4aM4SA4b)   947 SSLQVIRLIWRIRAELGFDIPVRTIFQHPTVAEVAEHLAAGREDVEFDDPFSVVLP......
TioN(AΔMNAM3SA)       ..............................................................
Ecm6(A1aM3SA1b)   977 DSIRAIQVVARARAG.GLALSPRTVFDCRTVAALAQAASRTDAPAALAEPEGGGVGLLPLPP
```

Fig. S3: Alignment of TioS($A_{4a}M_{4S}A_{4b}$),[6] TioN($A\Delta M_N AM_{3S}A$) (current study), and Ecm6($A_{1a}M_{3S}A_{1b}$)[8] generated by using Multalin[9] and showing the insertion site of TioS(M_{3S}) (orange) of the two engineered interrupted A domains compared to the M domain insertion naturally found in TioS($A_{4a}M_{4S}A_{4b}$) (blue). The ten conserved motifs of A domains are underlined in red. The cut sites generated by the sequential ligation explained in Fig. S3 are in yellow.

2. Preparation of p*tioN($AM_NAM_{3S}A$)*-pET28a and p*tioN($AM_NAM_{3S}A$)D818A*-pET28a overexpression constructs. The constructs used for heterologous co-overexpression of di-interrupted A domains designed based on the sequence alignment presented in Fig. S3, with the MLP TioT, were generated by using the primers presented in Table S1, in a series of PCRs and ligations outlined in Fig. S4 similar to that reported in ref [8]. Initially, two PCRs were done with p*tioN($A_aM_NA_b$)*-pET28a[7] as a template with primers 1 and 2 (fragment a) and primers 5 and 6 (fragment b) and one PCR with p*tioS($A_{3a}M_{3S}A_{3b}$)*-pET28a[1] as the template and primers 3 and 4 (fragment c). The PCR conditions were as follows: initial denaturation for 30 s at 98 °C, 30 cycles of 30 s at 98 °C, 30 s at 62 °C (59 °C or 60 °C for fragments a, b, and c, respectively), and 1 min/kb at 72 °C, and a final extension for 10 min at 72 °C. The PCR products were analyzed on a 1% agarose gel and purified by using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified PCR products were then subjected to enzymatic digestion with *Nde*I and *Nhe*I (fragment a), *Eco*RI and *Hin*dIII (fragment b), or *Nhe*I and *Eco*RI (fragment c) following manufacturer's (NEB) instructions. The digested PCR products were purified by using a QIAquick PCR purification kit (Qiagen) for use in subsequent ligation reactions. Fragment a was ligated into pET28a (digested with *Nde*I and *Nhe*I) using T4

DNA ligase (NEB) to generate p*tioN*(AM_NA)-pET28a, which was transformed into chemically competent *E. coli* TOP10 cells. The resulting p*tioN*(AM_NA)-pET28a from positive transformants, as confirmed by double digest and colony PCR, was purified by using the QIAprep spin miniprep kit (Qiagen), digested with *Nhe*I and *Eco*RI and ligated with fragment c as described above. The ligation was transformed, screened, confirmed as detailed above. The resulting p*tioN*(AM_NAM_{3S})-pET28a was then purified by using the QIAprep spin miniprep kit (Qiagen), digested with *Eco*RI and *Hin*dIII, ligated with fragment b and the process repeated to yield the final p*tioN*($AM_NAM_{3S}A$)-pET28a expression vector. The purified plasmid was subject to DNA sequencing and comparison to the section of the wt enzymes TioN($A_aM_NA_b$) and TioS($A_{3a}M_{3S}A_{3b}$) from *Micromonospora* sp. ML1 (protein accession number CAJ34370 and CAJ34375, respectively) used to generate these chimeras.

To establish that both methylation domains were functional, a point mutant of the added TioS(M_{3S}) M domain was utilized. We previously demonstrated that mutating aspartate 599 to alanine (D599A) abolished methylation activity in the wt TioS($A_{3a}M_{3S}A_{3b}$).[8] In the engineered TioN($AM_NAM_{3S}A$) that corresponding mutation is D818A. Therefore, we utilized the single overlap extension (SOE) method and previously reported mutation primers 7 and 8 to first create D818A overhang fragments then used those fragments as a template to generate the full length *tioN*($AM_NAM_{3S}A$)*D818A* PCR product. Round one of PCR reactions used p*tioN*($AM_NAM_{3S}A$)-pET28a as the template with primers 1 and 8 (fragment *tioN*($AM_NAM_{3S}A$)*D818A-overhang-fwd*) and primers 6 and 7 (fragment *tioN*($AM_NAM_{3S}A$)*D818A-overhang-rev*) as described above with the exception that the annealing temperature was set to 68 °C. The resulting PCR products were analyzed on a 1% agarose gel and purified as above. The purified products were used in equal amounts (1 μL of each) as templates in a subsequent round two PCR with the reaction conditions described in round one using primers 1 and 6. The resulting PCR product was purified and digested with *Nde*I and *Hin*dIIII, and ligated into pET28a as described above to yield the final p*tioN*($AM_NAM_{3S}A$)*D818A*-pET28a construct. The point mutation was confirmed by DNA sequencing.

Table S1: Primers used in this study.

Primer #	Primer name	Primer sequence[a]	5' or 3' primer	Restriction site
1[b]	*tioN*(AM_NA)*-fwd*	TCTCCGcatatgCCCTGGAGCGTATGGAACCAG	5'	*Nde*I
2	*tioN*(AM_NA)*-rev*	GGGAAGgctagcGGCCGTCGGAACGTAGAAGG	3'	*Nhe*I
3[c]	*tioS*(M_{3S})*-fwd*	CCCGATgctagcACGGTGGAGAGCGTGGTC	5'	*Nhe*I
4[c]	*tioS*(M_{3S})*-rev*	GGTGCCgaattcGGCCACCGGGAGGTAGG	3'	*Eco*RI
5	*tioN(A)-fwd*	GTTCGGgaattcGAGCCGCTTCCCACGGCCGAG	5'	*Eco*RI
6	*tioN(A)-rev*	CCACAGaagcttTCAGAGCCGGCTGAGCAGCG	3'	*Eco*RI
7[c]	*tioN*($AM_NAM_{3S}A$)*D818A-fwd*	GTACTGGGCGACC***gcc***TTCTCGGAGCCGG	5'	N/A
8[c]	*tioN*($AM_NAM_{3S}A$)*D818A-rev*	CCGGCTCCGAGAA***ggc***GGTCGCCCAGTAC	3'	N/A
9	*tioN*($A_aM_NA_b$)*D167A-fwd*	GGTGAACTTCTTC***gcc***TTCGTGTCGAGCGC	5'	N/A
10	*tioN*($A_aM_NA_b$)*D167A-rev*	GCGCTCGACACGAA***ggc***GAAGAAGTTCACC	3'	N/A

[a] The introduced restriction sites are in lowercase in each relevant primer. The codon mutated is in bold italic lowercase. All constructs encode an $NHis_6$-tagged protein.
[b] Primer 1 was previously used in ref [7].
[c] Primers 3, 4, 7, and 8 were previously used in ref [8] and were labeled primers 7, 8, 11, and 12, respectively, in that previous study.

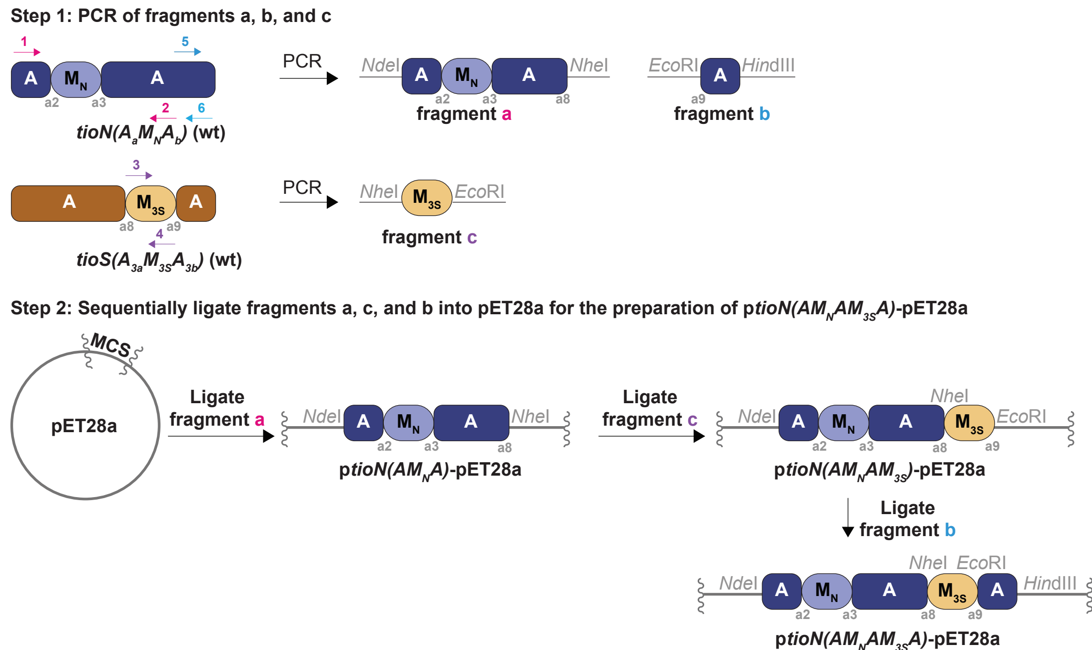


Fig. S4: Cloning strategy employed for the construction of the p*tioN(AM$_N$AM$_{3S}$A)*-pET28a overexpression construct.

3. Preparation of p*tioN(A$_a$M$_N$A$_b$)D167A*-pET28a overexpression construct. For activation and loading of *N*,*S*-diMe-L-Cys, TioN(A$_a$M$_N$A$_b$)D167A, which M$_N$ domain was inactivated, was used. The point mutation was achieved by using the SOE method by using pairs of primers 1 and 10 as well as 6 and 9 to first create the D167A overhang fragments, which were then used as templates to generate the full length *tioN(A$_a$M$_N$A$_b$)D167A* PCR product. Round one of PCR reactions used p*tioN(A$_a$M$_N$A$_b$)*-pET28a[7] as the template with primers 1 and 10 to generate fragment *tioN(A$_a$M$_N$A$_b$)D167A-overhang-fwd*, and primers 6 and 9 to generate fragment *tioN(A$_a$M$_N$A$_b$)D167A-overhang-rev* as described above in section 2 with the exception that the annealing temperature was set to 72 °C. The resulting PCR products were analyzed on a 1% agarose gel and purified as above. The purified products were used (1 µL of each) as templates in a subsequent round two PCR with the reaction conditions described in round one using primers 1 and 6. The resulting PCR product was purified and digested with *Nde*I and *Hin*dIIII, and ligated into pET28a as described above to yield the final p*tioN(A$_a$M$_N$A$_b$)D167A*-pET28a construct. The point mutation was confirmed by DNA sequencing.

4. Co-overexpression and co-purification of TioN(AM$_N$AM$_{3S}$A), TioN(AM$_N$AM$_{3S}$A)D818A, and TioN(A$_a$M$_N$A$_b$)D167A with the MLP TioT. p*tioN(AM$_N$AM$_{3S}$A)*-pET28a, p*tioN(AM$_N$AM$_{3S}$A)D818A*-pET28a, and p*tioN(A$_a$M$_N$A$_b$)D167A*-pET28a were co-overexpressed and co-purified with the wt TioN(A$_a$M$_N$A$_b$)'s MLP partner TioT, previously shown to be necessary for successful heterologous expression of active protein.[7] p*tioN(AM$_N$AM$_{3S}$A)*-pET28, p*tioN(AM$_N$AM$_{3S}$A)D818A*-pET28a, and p*tioN(A$_a$M$_N$A$_b$)D167A*-pET28a were individually transformed into chemically competent *E. coli* strain

BL21 (DE3)ybdz::aac(3)IV containing the previously reported p*tioT*-pACYCDuet-1.[7] The transformation mixture was then plated on Luria-Bertani (LB) agar containing 50 µg/mL of kanamycin and 35 µg/mL of chloramphenicol for selection of positive transformants harboring both expression plasmids, and incubated overnight at 37 °C. Colonies from the transformation were used to inoculate 7×2 mL of LB medium supplemented with antibiotics. The 2-mL cultures were incubated for several hours at 37 °C to reach an OD_{600} of ~1.4, at which point the cultures were combined into one tube and mixed for homogeneity. From the homogenous culture, 2 mL was inoculated into 1 L of LB medium (6 L total) containing both antibiotics and 10 mM $MgCl_2$. The 6×1 L cultures were then incubated at 37 °C with shaking at 200 rpm until an OD_{600} of 0.2-0.3. From there, the temperature was reduced to 16 °C and the cells were incubated with shaking at 200 rpm until the culture reached an OD_{600} of 0.5-0.8, at which point 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added and incubation continued at 16 °C for an additional 16-18 h. To collect the cells, the cultures were centrifuged at 5,000 rpm for 10 min at 4 °C. The resulting cell pellet was then resuspended in ~30 mL of lysis buffer (25 mM Tris-HCl, 400 mM NaCl, and 10% glycerol at pH 8.0 adjusted at room temperature (rt)) until homogenous consistency and lysed on ice by sonication (4 cycles of 2 min alternating with 2 s "on", 10 s "off"). The lysate was then divided equally into 8 centrifuge tubes and tubes were filled to the top with lysis buffer (~40 mL). The cell debris was cleared from the lysate by centrifugation at 16,000 rpm for 35-45 min at 4 °C. The supernatant was incubated with 1 mL/L of culture of washed Ni^{II}-NTA agarose resin (Qiagen) at 4 °C for 2 h with gentle mixing. The resin/lysate was passed through a column and the flow-through was collected and run once more. The resin was then washed with 10×10 mL of wash buffer (25 mM Tris-HCl, 400 mM NaCl, 40 mM imidazole, and 10% glycerol at pH 8.0 adjusted at rt). After washing, the (di-)interrupted A domains and TioT were co-eluted with 3×5 mL of elution buffer (25 mM Tris-HCl, 400 mM NaCl, 500 mM imidazole, and 10% glycerol at pH 8.0 adjusted at rt). After analysis by SDS-PAGE, the fractions that contained the proteins of interest were dialyzed by using 3,500 MWCO SnakeSkin® dialysis tubing (Thermo Scientific, Rockford, IL, USA) in 3×2 L of dialysis buffer (40 mM Tris-HCl, 200 mM NaCl, 2 mM β-mercaptoethanol, and 10% glycerol at pH 8.0 adjusted at rt) at 4 °C for 18-20 h with a minimum of 3 h between dialysis buffer changes. The resulting solution was concentrated by using Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Billerica, MA, USA) with a 3K membrane, and the concentration was spectrophotometrically determined by calculated extinction coefficient at 280 nm (178,390 $M^{-1}cm^{-1}$ for TioN($AM_NAM_{3S}A$) and TioN($AM_NAM_{3S}A$)D818A, and 98,530 $M^{-1}cm^{-1}$ for TioN($A_aM_NA_b$)D167A) (http://protcalc.sourceforge.net/cgi-bin/protcalc). The production yields for TioN($AM_NAM_{3S}A$), TioN($AM_NAM_{3S}A$)D818A, and TioN($A_aM_NA_b$)D167A were 2.3, 2.2, and 3.4 mg/L of culture, respectively.

5. Substrate specificity and determination of kinetic parameters of TioN($AM_NAM_{3S}A$), TioN($AM_NAM_{3S}A$)D818A, and TioN($A_aM_NA_b$)D167A by ATP-[^{32}P]PP_i exchange assays. For each di-interrupted A domain, different amino acids and amino acid derivatives were evaluated to determine substrate specificity using the following reactions, done in duplicate (Fig. 2). Each 100-μL reaction contained 75 mM Tris-HCl (pH 7.5 adjusted at rt), 5 mM tris(2-carboxyethyl)phosphine (TCEP), 10 mM $MgCl_2$, 5 mM ATP, 1 mM $Na_4P_2O_7$ spiked with ~500,000-800,000 cpm of [^{32}P]PP_i, 5 mM amino acid substrate, and 1 μM of enzyme. Reactions were initiated by the addition of the substrate and incubated for a total of 2 h at rt. To quench the reactions, 500 μL of a quenching solution (1.6% (*w*/*v*) activated charcoal, 4.5% (*w*/*v*) $Na_4P_2O_7$, and 3.5% (*v*/*v*) perchloric acid in H_2O) was added. The quenched reactions were centrifuged at 14,000 rpm for 7 min at rt to pellet the charcoal. The supernatant was discarded and the pelleted charcoal was resuspended in 500 μL of a wash solution (4.5% (*w*/*v*) $Na_4P_2O_7$ and 3.5% (*v*/*v*) perchloric acid in H_2O) and pelleted again by centrifugation as above. The supernatant was discarded and the process repeated once more. After the supernatant was again discarded, the charcoal pellet was resuspended in 500 μL of water and transferred to 5 mL of scintillation cocktail. The radioactivity was then counted with a liquid scintillation analyzer. The A domain activity of TioN($A_aM_NA_b$)D167A was compared with that of wt TioN($A_aM_NA_b$) for L-Cys, *N*-Me-L-Cys, *S*-Me-L-Cys, and *N*,*S*-diMe-L-Cys by using the assay described above. These data are presented in Fig. S5. For *N*,*S*-diMe-L-Cys, the time course assay was performed to test if TioN($A_aM_NA_b$)D167A can be used to activate it. In this assay, the reactions were performed with 2.35 μM of enzyme, quenched at 0, 0.5, 1, 2, 4, 8, 12 h, and processed as mentioned above. These data are presented in Fig. S6.

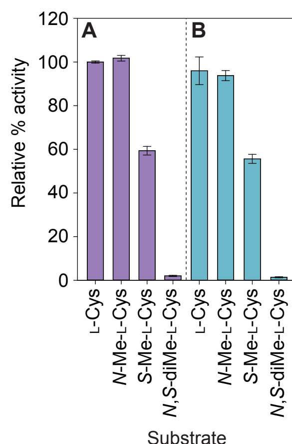


Fig. S5: Substrate profile of **A.** wt TioN($A_aM_NA_b$) (purple bars) and **B.** TioN($A_aM_NA_b$)D167A (turquoise bars) with L-Cys, *N*-Me-L-Cys, *S*-Me-L-Cys, and *N*,*S*-diMe-L-Cys. *Note*: All % activity values are relative to the % activity of wt TioN($A_aM_NA_b$) with L-Cys.

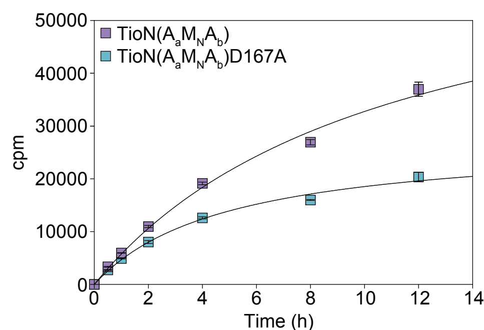


Fig. S6: Time course of adenylation activity for wt TioN($A_aM_NA_b$) (purple squares) and TioN($A_aM_NA_b$)D167A (turquoise squares) with *N*,*S*-diMe-L-Cys.

The kinetic parameters (k_{cat} and K_m) for the adenylation of L-Cys, *N*-Me-L-Cys, and *S*-Me-L-Cys by TioN($AM_NAM_{3S}A$) and TioN($AM_NAM_{3S}A$)D818A were determined. The reaction conditions, done in duplicate, were the same as described above for the substrate profile with the exception that the concentration of the substrate varied (0, 0.05, 0.1, 0.25, 0.5, 1, 1.75, 2.5, 5, 10, and 15 mM) and the reactions were incubated for 12 min (determined to be within the linear range of the enzyme based on a time course experiment) at rt before being quenched. After quenching, the reactions were washed and counted as described for the substrate profile. The Michaelis-Menten kinetic plots are presented in Fig. S7 and the kinetic parameters summarized in Table 1.

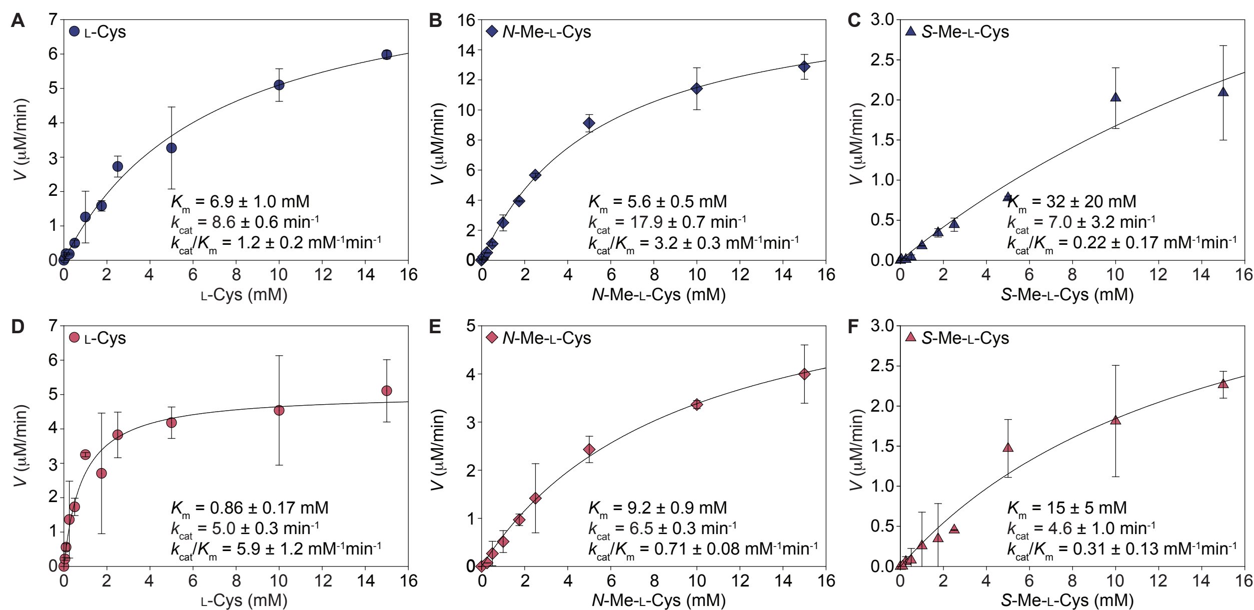


Fig. S7: Michaelis-Menten kinetic plots of TioN($AM_NAM_{3S}A$)-catalyzed adenylation of **A.** L-Cys, **B.** *N*-Me-L-Cys, and **C.** *S*-Me-L-Cys, as well as those for TioN($AM_NAM_{3S}A$)D818A-catalyzed adenylation of **D.** L-Cys, **E.** *N*-Me-L-Cys, and **F.** *S*-Me-L-Cys.

6. Characterization of methylation (M) domain activity of TioN($AM_NAM_{3S}A$), TioN($AM_NAM_{3S}A$)D818A, and TioN($A_aM_NA_b$)D167A by trichloroacetic acid (TCA) precipitation assays. To investigate the methylation activity of both M domains in the di-interrupted A domains, TCA precipitation assays were performed by using selected substrates with differing methylation sites available or blocked. These assays were conducted with both TioN($AM_NAM_{3S}A$) and TioN($AM_NAM_{3S}A$)D818A, as the M domains have differing methylation capabilities. The time courses were initiated by the addition of a SAM mixture (0.5 mM SAM (spiked with ~1,000,000 cpm of [methyl-^{3}H]SAM)) and quenched at specific time points post-initiation. For L-Cys and *N*-Me-L-Cys, the reactions were started after a 2 h pre-incubation period to allow time for the apo to holo conversion of TioS(T_3), adenylation, and loading of the substrate. The pre-incubated mixture (22.5 μL/reaction) for L-Cys and *N*-Me-L-Cys contained 83.33 mM Tris-HCl (pH 7.5 adjusted at rt), 11.11 mM $MgCl_2$, 1.11 mM TCEP, 0.55 mM coenzyme A (CoA), 0.55 μM Sfp, 55.5 μM TioS(T_3), 1.39 mM ATP, 2.22 μM TioN($AM_NAM_{3S}A$) or TioN($AM_NAM_{3S}A$)D818A, and 1.39 mM substrate. 2.5 μL/reaction of the SAM mixture was added to start the reaction. The final reaction (25 μL) contained 75 mM Tris-HCl (pH 7.5 adjusted at rt), 10 mM $MgCl_2$, 1 mM TCEP, 0.5 mM CoA, 0.5 μM Sfp, 50 μM TioS(T_3), 1.25 mM ATP, 2 μM TioN($AM_NAM_{3S}A$) or TioN($AM_NAM_{3S}A$)D818A, and 1.25 mM substrate. At 0, 3, 6, 15, 30, 60, and 90 min, 25-μL aliquots were quenched with 100 μL of 10% TCA to precipitate the protein. The precipitated protein was then pelleted by centrifugation at 14,000 rpm for 7 min at rt. The supernatant was discarded and the pellet washed with 100 μL of 10% TCA and centrifuged again. The supernatant was again discarded and the protein pellet washed once more. The pellet was dissolved in 100 μL of 88% formic acid and added to 5 mL of scintillation cocktail and counted by using a liquid scintillation analyzer. These data are presented in Fig. 3A,B. To test if TioN($A_aM_NA_b$)D167A had lost its methylating activity, the assay described above was performed by using L-Cys as a substrate and TioN($A_aM_NA_b$)D167A and wt TioN($A_aM_NA_b$) as enzymes. These data are presented in Fig. S8.

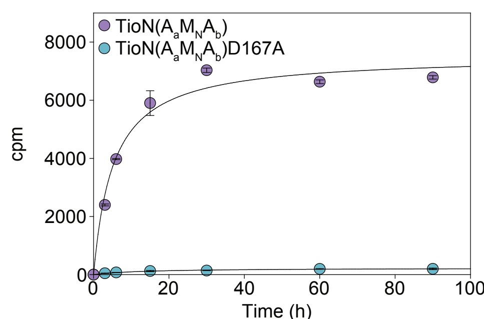


Fig. S8: Time course methylation activity of wt TioN($A_aM_NA_b$) (purple circles) and TioN($A_aM_NA_b$)D167A (turquoise circles) with L-Cys.

For *S*-Me-L-Cys and *N*,*S*-diMe-L-Cys, the pre-incubation was done overnight using a methylation deficient mutant of TioN($A_aM_NA_b$), TioN($A_aM_NA_b$)D167A. Two equal doses of TioN($A_aM_NA_b$)D167A were used to yield a final concentration of 2 μM in 25 μL, one initially when the overnight pre-incubation was started and one again 1 h before starting the reaction. The pre-incubated mixture (21.3 μL) for *S*-Me-L-Cys and *N*,*S*-diMe-L-Cys contained 88.03 mM Tris-HCl (pH 7.5 adjusted at rt), 11.73 mM $MgCl_2$, 1.17 mM TCEP, 0.59 mM CoA, 0.59 μM Sfp, 58.69 μM TioS(T_3), 1.47 mM ATP, 2.35 μM TioN($A_aM_NA_b$)D167A, and 1.46 mM substrate. After pre-incubation, TioN($AM_NAM_{3S}A$) or TioN($AM_NAM_{3S}A$)D818A was added and the reactions were subsequently initiated by the addition of the SAM mixture as above. The final reaction (25 μL) contained 75 mM Tris-HCl (pH 7.5 adjusted at rt), 10 mM $MgCl_2$, 1 mM TCEP, 0.5 mM CoA, 0.5 μM Sfp, 50 μM TioS(T_3), 1.25 mM ATP, 2 μM TioN($A_aM_NA_b$)D167A, 2 μM TioN($AM_NAM_{3S}A$) or 5 μM TioN($AM_NAM_{3S}A$)D818A, and 1.25 mM substrate. At 0, 3, 6, 12, 24, 36, and 48 h, 25-μL aliquots were quenched, washed, and counted as described above. These data are presented in Fig. 3C.

7. Evaluation of methylated products liberated from the T domain by potassium hydroxide (KOH) cleavage and evaluated by quadrupole time-of-flight mass spectrometry (Q-TOF MS). To further confirm the formation of *N*,*S*-diMe-L-Cys, mass spectrometry (MS) and tandem mass spectrometry (MS^2) experiments were performed. We carried out the enzymatic reaction followed by cleavage of the product formed from the T domain and extraction with EtOAc. We analyzed the resulting extract from the enzymatic reaction and chemical standards by using Q-TOF MS to detect the mass of the product and compare fragmentation patterns between the extract and the standards. Doing so, we confirmed the formation of *N*,*S*-diMe-L-Cys by TioN($AM_NAM_{3S}A$). The reaction mixture (484.38 μL) containing 77.4 mM Tris-HCl (pH 7.5 adjusted at rt), 10.3 mM $MgCl_2$, 1.29 mM L-Cys, 1.03 mM TCEP, 0.52 mM CoA, 1.03 μM Sfp, 72.25 μM TioS(T_3), 5.16 mM ATP, and 5.16 μM TioN($AM_NAM_{3S}A$) was incubated at rt for 2 h. The methylation reaction was initiated by the addition of 15.6 μL of SAM to bring the final reaction volume to 500 μL (75 mM Tris-HCl (pH 7.5 adjusted at rt), 10 mM $MgCl_2$, 1 mM L-Cys, 1 mM TCEP, 0.5 mM CoA, 1 μM Sfp, 70 μM TioS(T_3), 5 mM ATP, 1 mM SAM, and 5 μM TioN($AM_NAM_{3S}A$)) and allowed to react for 30 min. The reaction was quenched by the addition of 2 mL of ice-cold MeOH. The mixture was incubated at -20 °C for 2 h to allow the protein to precipitate. The precipitated protein mixture was then equally divided into two microtubes and centrifuged at 13,000 rpm for 10 min at rt. The supernatant was removed and the protein pellet was washed by the addition of 1 mL of ice-cold 80% MeOH. The mixture was centrifuged as above and the supernatant was removed and the protein pellet was washed once more. After centrifugation, the supernatant was removed as much as possible and the tubes were inverted to air dry. To cleave the amino acid from the precipitated protein, 500 μL of 0.1 N KOH was used to fully dissolve the protein pellet in one microtube, then the solution was transferred to the second microtube and

used to dissolve that protein pellet as well so that all the precipitated protein from the methylation reaction was dissolved in 500 μL KOH. The mixture was then incubated in a 63 °C hot water bath for 30 min. After incubation, the reaction was neutralized with 500 μL of 0.1 N HCl. The pH was then adjusted to ~2-3. The resulting 1 mL mixture was extracted 4 times with equal volume of EtOAc in the following manner: 1 mL EtOAc was added, the mixture was vortexed for 1 min and then centrifuged (13,000 rpm for 5 min at rt). The top layer was transferred to a pre-weighed glass vial and the process repeated. The EtOAc was dried under reduced pressure by using a rotary evaporator and subsequent vacuum desiccation overnight. The sample was then dissolved in HPLC grade MeOH to a concentration of 5 mg/mL and subsequently used to make 500 μL of 100 ng/μL for direct sample injection on AB SCIEX TripleTOF™ 5600 (SCIEX, Concord, ON, Canada). The pure chemical standards were also prepared as 500 μL of 100 ng/μL from purchased or synthesized compounds. The software used to analyze the data were Analyst®TF and PeakView. The MS and MS^2 data are presented in Figs. S9 and S10, respectively.

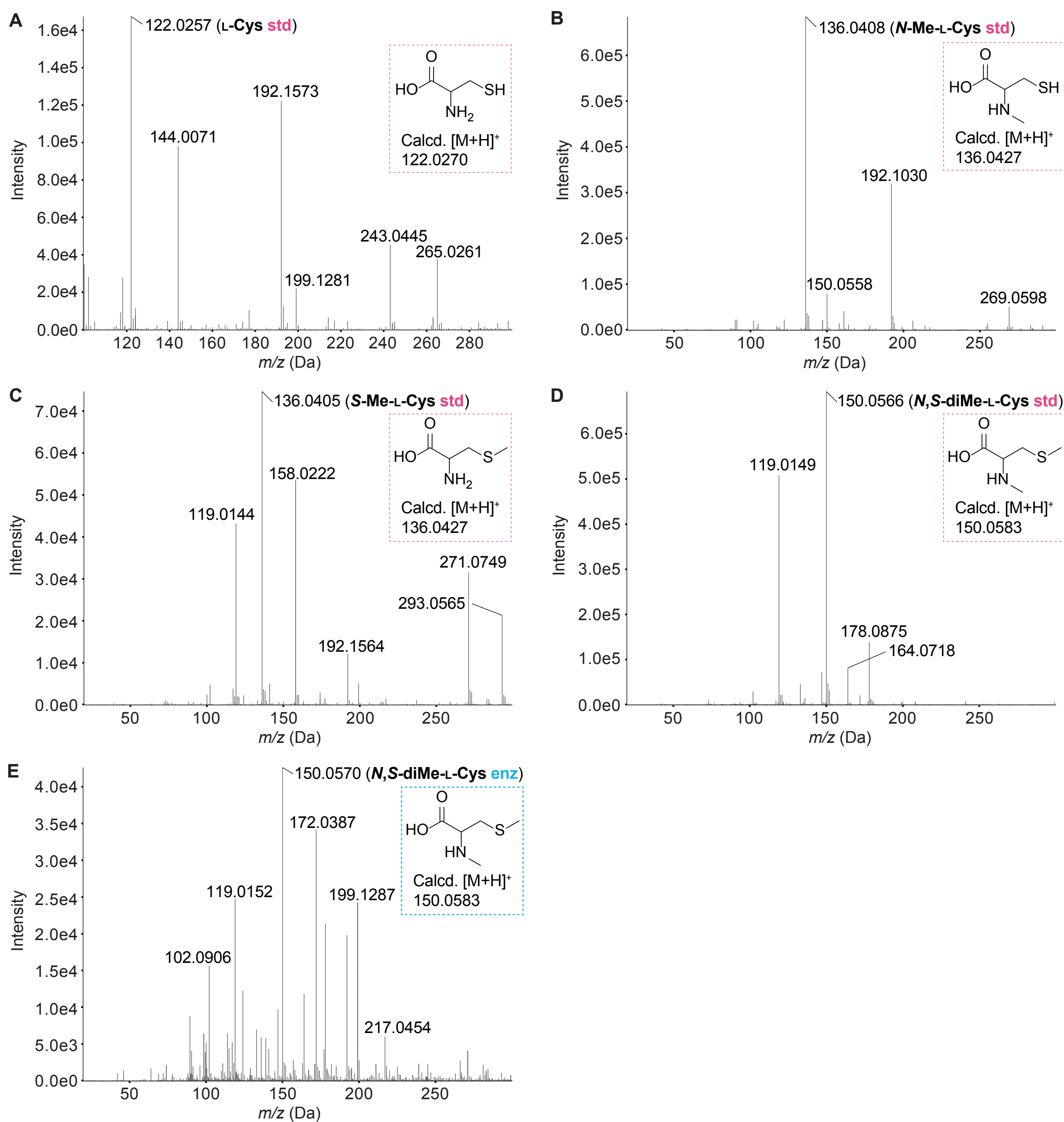


Fig. S9: Mass spectra for the synthetic or commercially available standards: **A.** L-Cys, **B.** *N*-Me-L-Cys, **C.** *S*-Me-L-Cys, and **D.** *N*,*S*-diMe-L-Cys. Panel **E** shows the mass spectrum for the product generated by enzymatic reaction using TioN($AM_NAM_{3S}A$) and TioS(T_3) as shown in Fig. S10. Panel **E** confirms that the product of enzymatic reaction is *N*,*S*-diMe-L-Cys.

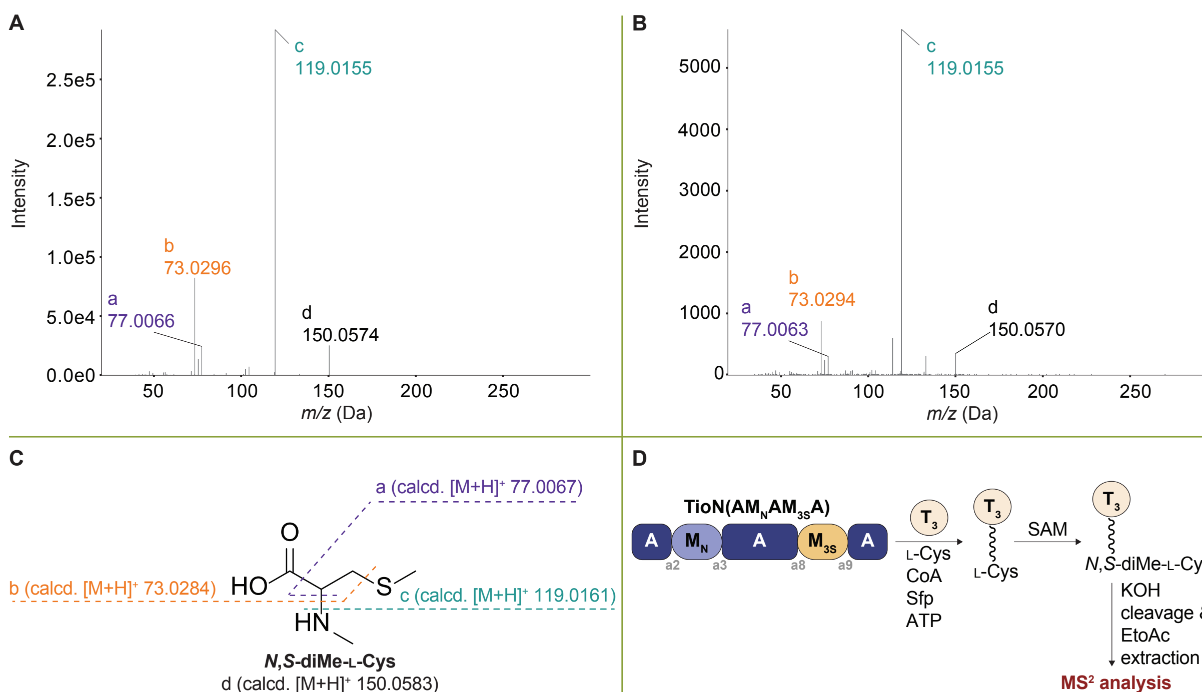


Fig. S10: Tandem mass spectra (MS^2) at 17 volts of **A.** *N*,*S*-diMe-L-Cys synthetic standard and **B.** *N*,*S*-diMe-L-Cys generated by the enzymatic reaction shown in panel **D**. Fragments with *m/z* ratios between 20 to 300 for *N*,*S*-diMe-L-Cys were collected. The fragments of interests in each panel that indicated the position of methylation were labeled both in the spectra and in **C.** the structure of *N*,*S*-diMe-L-Cys with the calculated *m/z* ratios for the fragments shown in panels **A** and **B**.

8. References

1. S. Mori, A. Garzan, O. V. Tsodikov and S. Garneau-Tsodikova, *Biochemistry*, 2017, **56**, 6087-6097.
2. L. E. Quadri, P. H. Weinreb, M. Lei, M. M. Nakano, P. Zuber and C. T. Walsh, *Biochemistry*, 1998, **37**, 1585-1595.
3. E. A. Felnagle, J. J. Barkei, H. Park, A. M. Podevels, M. D. McMahon, D. W. Drott and M. G. Thomas, *Biochemistry*, 2010, **49**, 8815-8817.
4. T. Stachelhaus, H. D. Mootz and M. A. Marahiel, *Chem. Biol.*, 1999, **6**, 493-505.
5. S. Mori, S. K. Shrestha, J. Fernandez, M. Alvarez San Millan, A. Garzan, A. H. Al-Mestarihi, F. Lombo and S. Garneau-Tsodikova, *Biochemistry*, 2017, **56**, 4457-4467.
6. S. Mori, A. H. Pang, T. A. Lundy, A. Garzan, O. V. Tsodikov and S. Garneau-Tsodikova, *Nat. Chem. Biol.*, 2018, **14**, 428-430.
7. A. H. Al-Mestarihi, G. Villamizar, J. Fernandez, O. E. Zolova, F. Lombo and S. Garneau-Tsodikova, *J. Am. Chem. Soc.*, 2014, **136**, 17350-17354.
8. T. A. Lundy, S. Mori and S. Garneau-Tsodikova, *ACS Synth. Biol.*, 2018, **7**, 399-404.
9. F. Corpet, *Nucl. Acids Res.*, 1988, **16**, 10881-10890.