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**Supporting Information**

# Lessons learned in engineering interrupted adenylation domains when attempting to create trifunctional enzymes from three independent monofunctional ones

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**1. Bacterial strains, plasmids, materials, and instruments.** The Sfp protein was expressed and purified as previously reported.[1] The MbtH-like protein (MLP) Ecm8 was expressed and purified as previously described,[2] along with the wild-type (wt) and engineered A domains generated in this study in the *E. coli* BL21 (DE3) ybdz::aac(3)IV strain,[3] a gift from Professor Michael G. Thomas (University of Wisconsin-Madision, USA). Centrifugation for large scale protein purification was done using a Sorvall RC-6 PLUS (Thermo, Ashville, NC, USA). Tabletop centrifugation was done with a Microfuge® 18 Centrifuge (Beckman Coulter, Palo Alto, CA, USA). Chemically competent *E. coli* TOP10 cells were purchased from Invitrogen (Carlsbad, CA, USA) and used for cloning of the expression plasmids in pET28a, which was purchased from Novagen (Gibbstown, NJ, USA). Polymerase chain reactions (PCRs) were carried out using a Robocycler® Gradient 96 (Stratagene, La Jolla, CA, USA) with primers purchased from Sigma-Aldrich (St. Louis, MO, USA). All remaining molecular cloning reagents were purchased from New England BioLabs (NEB, Ipswich, MA, USA). DNA purification of PCR products and plasmids was done using the spin kits QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit, and QIAprep Spin Miniprep Kit, which were purchased from Qiagen (Valencia, CA, USA) and used according to the manufacturer's instructions. Sequencing of the DNA constructs was conducted at Eurofins Scientific (Louisville, KY, USA). The chemical reagents and buffer components for enzymatic assays and protein purifications were purchased from Sigma-Aldrich or VWR (Atlanta, GA, USA) and used without any further purification. The *N*-Me-L-Cys used for HPLC studies was synthesized by Dr. Atefeh Garzan as previously described.[4] The *N*,*O*-diMe-L-Ser standard used for mass spectrometry (MS) and tandem mass spectrometry ($MS^2$) was synthesized by Dr. Nishad Thamban Chandrika as previously described.[5] MS and $MS^2$ were done using an AB SCIEX TripleToF$^{TM}$ 5600 mass spectrometer (SCIEX, Concord, ON, Canada). HPLC was conducted on an Agilent 1260 Infinity equipped with a quaternary pump and 1260 Infinity photo-diode array (Santa Clara, CA, USA) with a Grace Vydac Protein and Peptide C18 column (250 × 4.6 mm) (cat #218TP54). For use in radioactive experiments, [methyl-$^3$H]SAM (*S*-adenosyl-L-methionine) and [$^{32}$P]$PP_i$ were purchased from PerkinElmer (Waltham, MA, USA). Radioactivity was counted using a TriCarb 2900TR Liquid Scintillation Analyzer (PerkinElmer). EcoLumeTM liquid scintillation cocktail was purchased from MP Biomedicals (Santa Ana, CA, USA).

**2. Preparation of p*ecm6(AM$_s$M$_b$A)*-pET28a, p*ecm6(AM$_b$M$_s$A)*-pET28a, p*ecm6(AM$_s$M$_b$AT$_1$)*-pET28a, p*ecm6(AM$_b$M$_s$AT$_1$)*-pET28a, and p*ecm6(T$_1$)*-pET28a constructs.** To construct the p*ecm6(AM$_s$M$_b$A)*-pET28a, p*ecm6(AM$_b$M$_s$A)*-pET28a, p*ecm6(AM$_s$M$_b$AT$_1$)*-pET28a, p*ecm6(AM$_b$M$_s$AT$_1$)*-pET28a, and p*ecm6(T$_1$)*-pET28a overexpression plasmids, the p*ecm6(A$_8$M$_b$A$_9$T$_1$)*-pET28a (originally published as p*ecm6(A$_{1a}$M$_{3S}$A$_{1b}$T$_1$)*-pET28a)[2] was used as a starting point. The p*ecm6(A$_8$M$_b$A$_9$)*-pET28a and p*ecm6(T$_1$)*-pET28a were cloned from p*ecm6(A$_8$M$_b$A$_9$T$_1$)*-pET28a in the following manner. The PCR mixtures contained Phusion High-Fidelity DNA Polymerase (0.5 μL), Phusion GC buffer (5 μL), p*ecm6(A$_8$M$_b$A$_9$T$_1$)*-pET28a template (1 μL), DMSO (1.5 μL), dd$H_2O$ for a final reaction volume of 50 μL, and forward and reverse primers 11 and 12 (0.5 μL each) for *ecm6(A$_8$M$_b$A$_9$)*, or 9 and 10 (0.5 μL each) for *ecm6(T$_1$)* (Table S1). 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 70 °C, and 1 min/1 kb at 72 °C, and a final extension for 10 min at 72 °C. The resulting PCR products were analyzed on a 1% agarose gel and subsequently purified using QIAquick Gel Extraction Kit according to the manufacturer's (Qiagen) protocol. The purified PCR products were then subjected to enzymatic digestion with *Nde*I and *Hin*dIII per the manufacturer's (NEB) instructions. The digested PCR products were then ligated into the *Nde*I and *Hin*dIII sites of linearized pET28a, which resulted in formation of p*ecm6(A$_8$M$_b$A$_9$)*-pET28a and p*ecm6(T$_1$)*-pET28a. From either p*ecm6(A$_8$M$_b$A$_9$)*-pET28a or p*ecm6(A$_8$M$_b$A$_9$T$_1$)*-pET28a, the corresponding p*ecm6(AM$_s$M$_b$A)*-pET28a, p*ecm6(AM$_b$M$_s$A)*-pET28a, p*ecm6(AM$_s$M$_b$AT$_1$)*-pET28a, and p*ecm6(AM$_b$M$_s$AT$_1$)*-pET28a were constructed by removing the M$_b$ *via* enzymatic digestion with *Nhe*I and *Eco*RI restriction enzymes, resulting in a vector backbone of the split Ecm6(A$_1$)/(A$_1$T$_1$) (Fig. S1C) (*Note*: a sequence alignment of Ecm6(AM$_s$M$_b$A) and ColG(AM$_s$M$_b$A) is presented in Fig. 5). After gel extraction of the vector backbone, the purified *M$_s$M$_b$* (fragment "a") or *M$_b$M$_s$* (fragment "b") insert was ligated into the *Nhe*I and *Eco*RI restriction sites (Fig. S1). The *M$_b$M$_s$* and *M$_s$M$_b$* fragments were constructed using overlapping PCR (Fig. S1A,B, Tables S1 and S2). For *M$_s$M$_b$* (fragment "a"), two first round of PCRs were set up that contained the same PCR mixtures and conditions described above except primers 3 and 4 with template KtzH(A$_8$M$_s$A$_9$) (previously published as KtzH(A$_{4a}$M$_H$A$_{4b}$)) or primers 1 and 2 with template TioS(A$_8$M$_b$A$_9$) (previously published as TioS(A$_{3a}$M$_{3S}$A$_{3b}$)) to create *M$_s$* with 3' *M$_b$*-overhang (piece 1) and *M$_b$* with 5' *M$_s$*-overhang (piece 2). In the second round of PCR, pieces 1 and 2 were used as a template with primers 2 and 3 to create fragment "a" (Fig. S1, Tables S1 and S2) in the same reaction mixtures

and conditions as above. For $M_bM_s$ (fragment "b"), two first rounds of PCRs were set up that contained the same PCR mixtures and conditions described above except primers 7 and 8 with template KtzH($A_8M_sA_9$) or primers 5 and 6 with template TioS($A_8M_bA_9$) to create $M_b$ with 3' $M_s$-overhang (piece 3) and $M_s$ with 5' $M_b$-overhang (piece 4) (Fig. S1 and Tables S1 and S2). In the second round of PCR, pieces 3 and 4 were used as a template with primers 5 and 8 to create fragment "b" (Fig. S1A) in the same reaction mixtures and conditions as above. Fragments "a" and "b" were digested with *Nhe*I and *Eco*RI restriction enzymes and subsequently ligated into the linearized A/AT domain backbone pET28a in the *Nhe*I and *Eco*RI restriction sites to create p*ecm6(AM$_s$M$_b$A)*-pET28a, p*ecm6(AM$_b$M$_s$A)*-pET28a, p*ecm6(AM$_s$M$_b$AT$_1$)*-pET28a, and p*ecm6(AM$_b$M$_s$AT$_1$)*-pET28a.

**Table S1:** Primers used in this study.

| Primer # herein | SGT primer # | Primer name | Primer sequence | 5' or 3' primer | Restriction site[a] |
|---|---|---|---|---|---|
| 1 | 1541 | *tioS(M$_b$)9bp ovrhg of ktzH(M$_s$)F* | CCGGCCCCGACGGTGGAGAGCGTGGTCGCC | 5' | N/A |
| 2* | 914 | *tioS(M$_b$)R* | GGTGCCgaattcGGCCACCGGGAGGTAGG | 3' | *Eco*RI |
| 3* | 796 | *ktzH(M$_s$)F* | GCCGACgctagcACGGCGGCCCCCGCGGCG | 5' | *Nhe*I |
| 4 | 1542 | *ktzH(M$_s$)9bp ovrhg of tioS(M$_b$)R* | CTCCACCGTCGGGGCCGGCATCGGGGCCAG | 3' | N/A |
| 5* | 913 | *tioS(M$_b$)F* | CCCGATgctagcACGGTGGAGAGCGTGGTC | 5' | *Nhe*I |
| 6 | 1546 | *tioS(M$_b$)9bp ovrhg of ktzH(M$_s$)R* | GGCCGCCGTGGCCACCGGGAGGTAGGTGCC | 3' | N/A |
| 7 | 1545 | *ktzH(M$_s$)9bp ovrhg of tioS(M$_b$)F* | CCGGTGGCCACGGCGGCCCCCGCGGCGACC | 5' | N/A |
| 8* | 797 | *ktzH(M$_s$)R* | GCTGTTgaattcCGGGGCCGGCATCGGGGC | 3' | *Eco*RI |
| 9 | 1576 | *ecm6(T$_1$)F* | GACCGGcatatgCTGCCCGAGCCGGAGATCAC | 5' | *Nde*I |
| 10* | 903 | *ecm6(T$_1$)R* | GGCGTGaagcttTTACATGGGGGGCAGCGGCAG CAG | 3' | *Hin*dIII |
| 11* | 902 | *ecm6(A$_8$M$_b$A$_9$)F* | CTCGCGcatatgTTCACCACGTTCCTGCGGAGG | 5' | *Nde*I |
| 12 | 1354 | *ecm6(A$_8$M$_b$A$_9$)R* | GGCCTGaagcttTTACTCCGGCTCGGGCAGCCCG | 3' | *Hin*dIII |

[a] The introduced restriction sites are in lowercase in each relevant primer. N/A = not applicable. All final constructs encode a NHis$_6$-tagged protein. * These primers were previously reported in Ref [2] and are presented here to make it easier for the readers.

**Table S2**. Overlapping PCR for $M_sM_b$ and $M_bM_s$ fragments.

| PCR round 1 | Template(s) | Primer set | Product |
|---|---|---|---|
| | KtzH($A_8M_sA_9$) | 3 & 4 | $M_s$ with 3' $M_b$-overhang |
| | KtzH($A_8M_sA_9$) | 7 & 8 | $M_s$ with 5' $M_b$-overhang |
| | TioS($A_8M_bA_9$) | 1 & 2 | $M_b$ with 5' $M_s$-overhang |
| | TioS($A_8M_bA_9$) | 5 & 6 | $M_b$ with 3' $M_s$-overhang |
| PCR round 2 | $M_s$ with 3' $M_b$ overhang and $M_b$ with 5' $M_s$ overhang | 2 & 3 | $M_sM_b$ fragment "a" |
| | $M_b$ with 3' $M_s$ overhang and $M_s$ with 5' $M_b$ overhang | 5 & 8 | $M_bM_s$ fragment "b" |

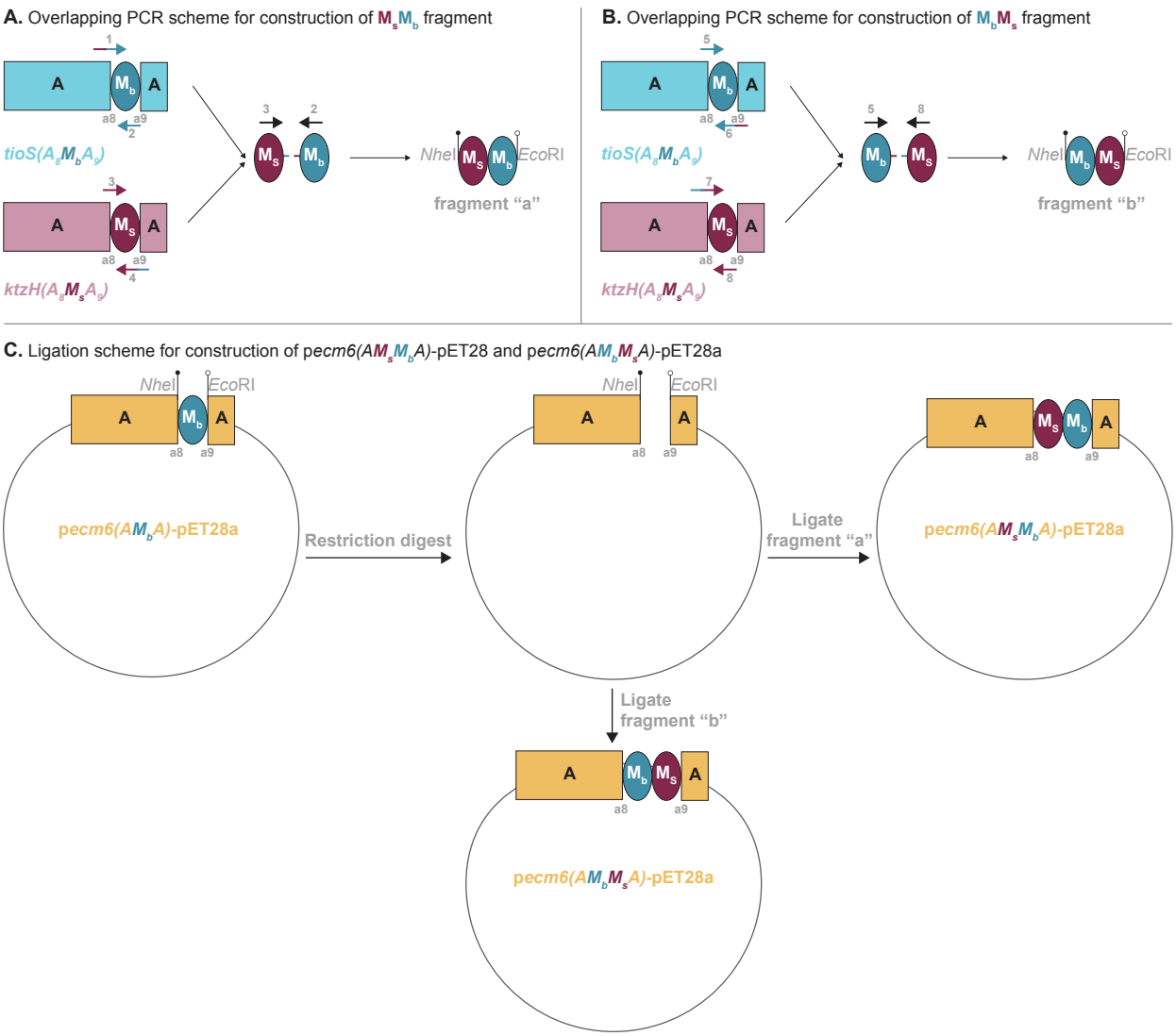


**Fig. S1.** Cloning strategy for preparation of p*ecm6(AMsMbA)*-pET28a and p*ecm6(AMbMsA)*-pET28a. *Note*: The same strategy was used to construct p*ecm6(AMsMbAT1)*-pET28a and p*ecm6(AMbMsAT1)*-pET28a from the p*ecm6(A8MbA9T1)*-pET28a plasmid, which was originally described in Ref [2].

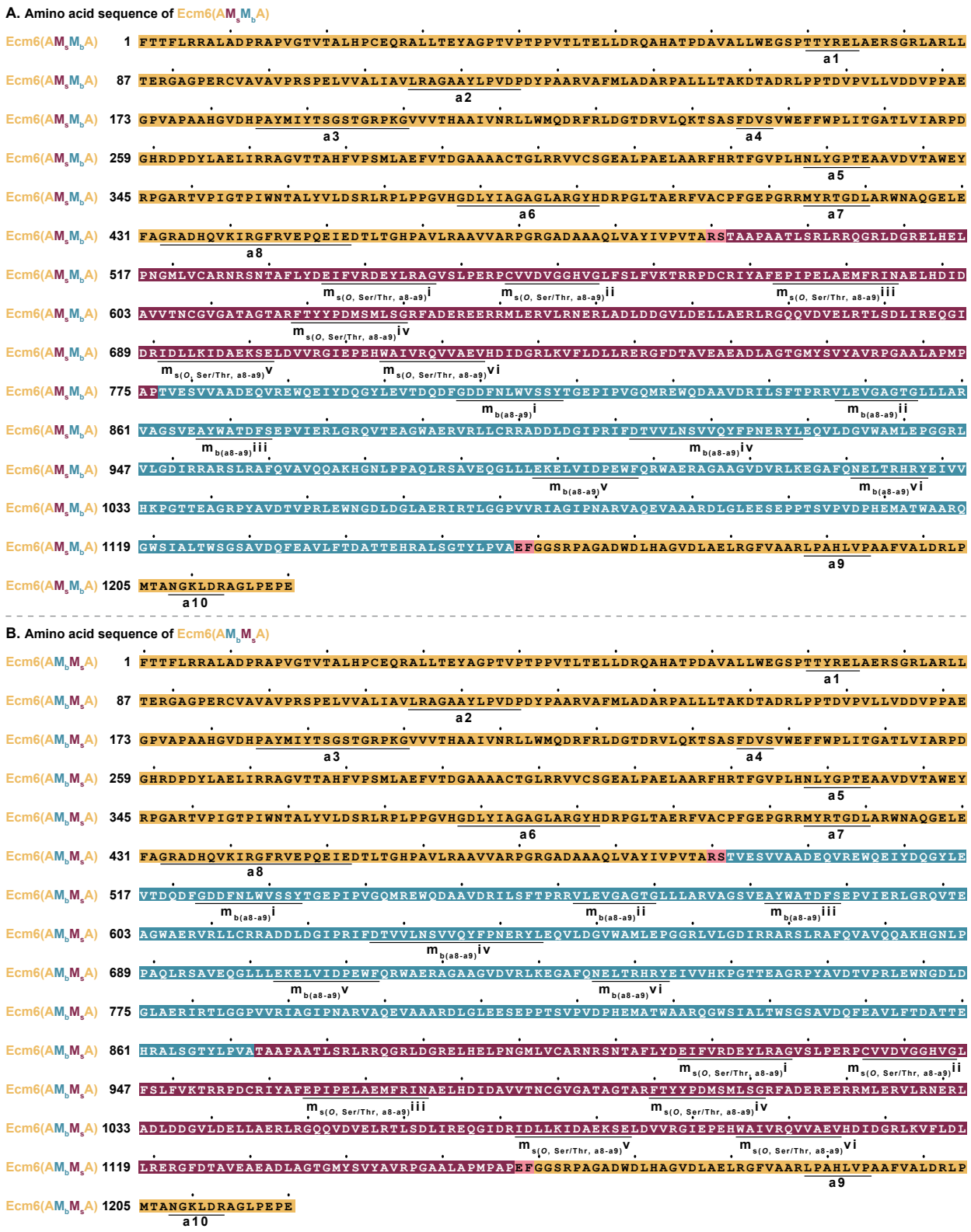


**Fig. S2.** Amino acid sequences of the **A.** Ecm6(AM$_s$M$_b$A) and **B.** Ecm6(AM$_b$M$_s$A) engineered for this study. The A domain is highlighted in dark yellow, the M$_s$ in burgundy, and the M$_b$ in teal. The amino acid residues added as a result of the addition of the restriction sites used to generate the engineered constructs are highlighted in pink. The

conserved A domain motifs are underlined and labeled a1-a10. The conserved $M_s$ domain motifs are underlined and labeled $m_{s(O,\ Ser/Thr,\ a8\text{-}a9)}$i-vi. The $M_b$ domain conserved motifs are underlined and labeled $m_{b(a8\text{-}a9)}$i-vi.

**3. Overexpression/co-overexpression and purification/co-purification of proteins used in this study.** The p*ecm6(AM$_s$M$_b$A)*-pET28a, p*ecm6(AM$_b$M$_s$A)*-pET28a, p*ecm6(AM$_s$M$_b$AT$_1$)*-pET28a, and p*ecm6(AM$_b$M$_s$AT$_1$)*-pET28a constructs were transformed into *E. coli* BL21 (DE3) ybdzD::aac(3)IV, which contained the MLP partner in a pACYCDuet-1 vector as we previously reported,[6] p*ecm8*-pACYCDuet-1 for co-overexpression and co-purification. The construct p*ecm6(T$_1$)*-pET28a was transformed into *E. coli* BL21 (DE3) cells for overexpression and purification. The transformation mixture was plated onto Luria-Bertani (LB) agar and incubated overnight at 37 °C. The LB agar contained 50 μg/mL of kanamycin and 35 μg/mL of chloramphenicol for the MLP/A domain transformations and just 50 μg/mL of kanamycin for the T domain transformation. All the interrupted A domains and the T domain were co-overexpressed/overexpressed and co-purified/purified following the same protocol; the only differences were the antibiotics and the addition of 10 mM $MgCl_2$ during interrupted A domain overexpression. Transformants were used to inoculate 3×2 mL of LB medium with the corresponding antibiotics. These 2-mL cultures were then incubated at 37 °C until they reached an $OD_{600}$ of ~1.4. The cultures were then combined in a single tube to yield a homogenous seed culture (6-mL total) from which 2-mL was then used to inoculate 3×1 L LB medium supplemented with the appropriate antibiotics (*Note*: for each protein, a total of 6 L was purified). The 1 L cultures were incubated at 37 °C with shaking at 200 rpm until an $OD_{600}$ of 0.2-0.3 was reached, at which point, the temperature was decreased to 16 °C and the cells were incubated with shaking at 200 rpm until an $OD_{600}$ of 0.5-0.8 was reached. Overexpression of the proteins was then initiated with the addition of 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and continued incubation at 16 °C for an additional 16-18 h. The cells were collected by centrifugation at 5,000 rpm for 10 min at 4 °C. The supernatant was decanted, and the cell pellet was resuspended until homogenous 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)). The cells were then lysed on ice by sonication (4 cycles of 2 min alternating with 2 s "on" and 10 s "off"). After sonication, the cell lysate was divided into 4 centrifuge tubes, and additional lysis buffer was added (~40 mL each). The cell lysate was cleared of cellular debris by centrifugation at 16,000 rpm at 4 °C for 35-45 min. The cleared lysate was then incubated with ~1.5 mL of washed $Ni^{II}$-NTA agarose resin (Qiagen) at 4 °C with gentle

mixing for 2 h. After incubation, the resin was separated from the lysate by passing the resin/lysate mixture through a column and collecting the flow-through. To ensure maximum collection of resin bound protein, the flow-through was passed through the column a second time. The $Ni^{II}$-NTA agarose resin was then washed with 10×10 mL wash buffer (25 mM Tris-HCl, 400 mM NaCl, 40 mM imidazole, and 10% glycerol at pH 8.0 adjusted at rt). The bound proteins were then 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). Samples collected were then analyzed with SDS-PAGE, and fractions that contained the proteins were combined and transferred to a 3,500 molecular weight-cut off (MWCO) SnakeSkin® dialysis tubing (Thermo Scientific) and dialyzed at 4 °C against 3×2 L dialysis buffer (40 mM Tris-HCl, 200 mM NaCl, 2 mM β-mercaptoethanol, and 10% glycerol at pH 8.0 adjusted at rt) for 18-20 h with a minimum of 3 h between dialysis buffer changes. The proteins were concentrated with Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Burlington, MA, USA) with a 3 K MWCO membrane. For the interrupted A domains, the concentration was spectrophotometrically determined by calculated extinction coefficient at 280 nm, 158,190 $M^{-1}cm^{-1}$ for Ecm6($AM_sM_bA$), Ecm6($AM_bM_sA$), Ecm6($AM_sM_bAT_1$), and Ecm6($AM_bM_sAT_1$) (*Note*: The T domain does not absorb UV light at 280 nm). The concentration of Ecm6($T_1$) was calculated using a Bradford assay with the Bovine Serum Albumin (BSA) standard. The production yields for Ecm6($AM_sM_bA$), Ecm6($AM_bM_sA$), Ecm6($AM_sM_bAT_1$), Ecm6($AM_bM_sAT_1$), and Ecm6($T_1$) were 5 mg/L of culture, 2.4 mg/L of culture, 1.2 mg/L of culture, 2.3 mg/L of culture, and 7.5 mg/L of culture, respectively. SDS-PAGE gels of the purified proteins are presented in Fig. S3.

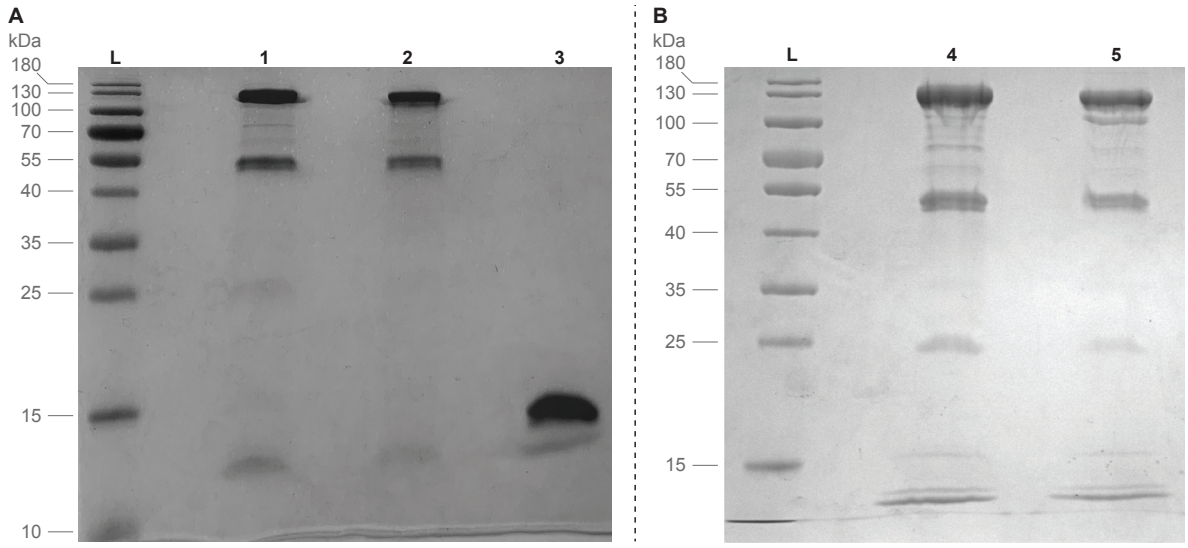


**Fig. S3.** SDS-PAGE gels of **A.** purified Ecm6($AM_sM_bA$) (136,097 Da; lane 1), Ecm6($AM_bM_sA$) (136,097 Da; lane 2), and Ecm6($T_1$) (13,365 Da; lane 3), and **B.** purified Ecm6($AM_sM_bAT_1$) (146,584 Da; lane 4) and Ecm6($AM_bM_sAT_1$) (146,584 Da; lane 5). L indicates the pre-stained protein ladder from Thermo Scientific. 6 μg of each of the proteins were loaded on the gels.

**4. Substrate specificity and determination of kinetic parameters of the engineered interrupted A domains by ATP-[$^{32}$P]$PP_i$ exchange assays.** The substrate profiles of Ecm6($AM_sM_bA$) and Ecm6($AM_bM_sA$) were evaluated with the 20 natural L-amino acids as well as *N*-Me-L-Ser and *O*-Me-D,L-Ser by using ATP-[$^{32}$P]$PP_i$ exchange assays (Fig. 2) (*Note*: The previously published[2] substrate profile for the wt uninterrupted Ecm6($A_1T_1$) is presented in Fig. S4 for comparison purposes). Each reaction (100 μL), containing Tris-HCl (75 mM, pH 7.5 adjusted at rt), Tris(2-carboxyethyl)phosphine (TCEP) (5 mM), $MgCl_2$ (10 mM), ATP (5 mM), $Na_4P_2O_7$ (1 mM) with up to 600,000 cpm of [$^{32}$P]$PP_i$, amino acid substrate (5 mM or 10 mM for *O*-Me-D,L-Ser), and enzyme (2.5 μM), was initiated by the addition of the substrate. After incubation of the reactions at rt for 2 h, the reactions were quenched by the addition of a quenching solution (500 μL; 1.6% (*w*/*v*) activated charcoal, 4.5% (*w*/*v*) $Na_4P_2O_7$, and 3.5% (*v*/*v*) perchloric acid in $H_2O$). The charcoal was pelleted by centrifugation (14,000 rpm, 7 min, rt) and washed twice with a wash solution (500 μL; 4.5% (*w*/*v*) $Na_4P_2O_7$, and 3.5% (*v*/*v*) perchloric acid in $H_2O$). The washed charcoal pellet was resuspended in $H_2O$ (500 μL) and mixed with scintillation cocktail (5 mL) in a scintillation vial. The samples were transferred into a liquid scintillation analyzer to measure radioactivity. Having established that L-Ser remained the preferred substrate for the engineered Ecm6($AM_sM_bA$) and Ecm6($AM_bM_sA$) (Fig. 2), this amino acid was used to compare

the activity of these back-to-back interrupted A domains to their counterparts with the additional C-terminal naturally attached T domain, Ecm6($AM_sM_bAT_1$) and Ecm6($AM_bM_sAT_1$), using the exact same conditions as above (Fig. 3).

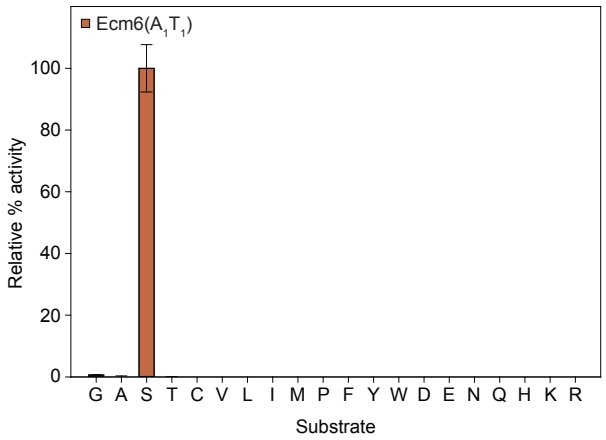


**Fig. S4.** Substrate profile of the wt uninterrupted Ecm6($A_1T_1$) presented here *for comparison purposes only* and was originally reported in Ref [2].

The kinetic parameters of Ecm6($AM_sM_bA$) and Ecm6($AM_bM_sA$) were measured by using the reaction rate with varied concentrations of L-Ser (0, 0.05, 0.1, 0.25, 0.5, 1, 1.75, 2.5, 5, 10, and 15 mM). The reaction conditions were the same as described above with the exception of the incubation time, which was 12 min (in the linear range of the reaction). After incubation, the reactions were processed as described above. All radiometric assays described in this section were performed in duplicate. These Michaelis-Menten plots are presented in Fig. S5 and summarized in Table 1. Since the purified enzymes contained some degradation product (Fig. S3), the concentrations of Ecm6($AM_sM_bA$) and Ecm6($AM_bM_sA$) may not accurately reflect the amount of active protein. Therefore, only $V_{max}$ could be confidently measured instead of $k_{cat}$; likewise, $V_{max}/K_m$ was calculated instead of $k_{cat}/K_m$. In these parameters, $\mu M_{(E)}$ and $mM_{(S)}$ represent concentrations of enzyme and substrate, respectively.

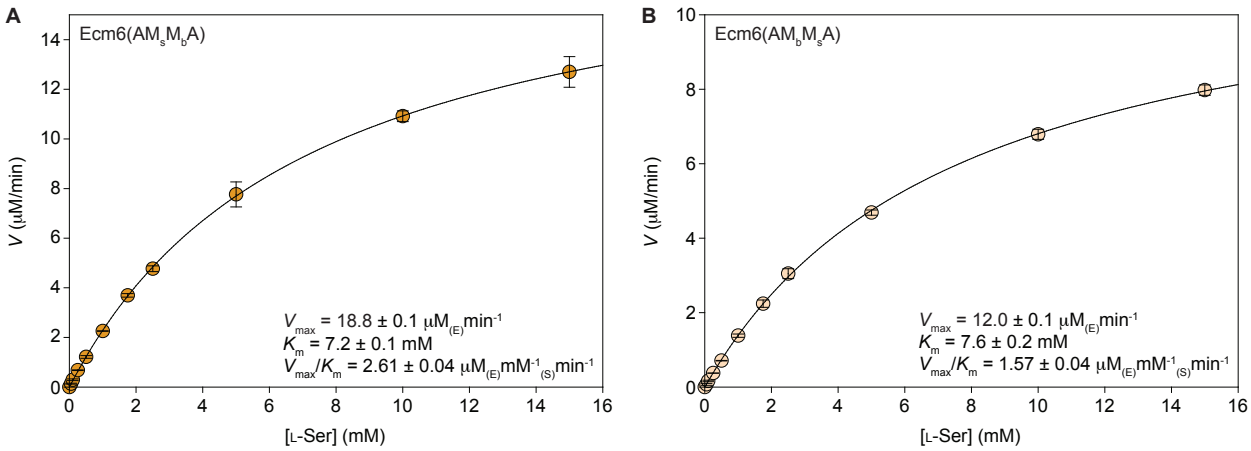


**Fig. S5.** Michaelis-Menten kinetic plots for the adenylation of L-Ser by **A.** Ecm6($AM_sM_bA$) and **B.** Ecm6($AM_bM_sA$).

## 5. Attempts at methylation of L-Ser by (i) trichloroacetic acid (TCA) precipitation, (ii) high performance liquid chromatography (HPLC), as well as (iii) mass spectrometry (MS) and tandem mass spectrometry ($MS^2$).

(i) *TCA precipitation assays*. TCA precipitation assays were set up using the same protocol for both Ecm6($AM_sM_bA$) and Ecm6($AM_bM_sA$) using a time course initiated by the addition of a SAM mixture (0.5 mM SAM spiked with ~1,000,000 cpm of [methyl-$^3$H]SAM). An initial pre-incubation reaction was set up for 2 h to allow for priming of the T domain (conversion from apo to holo along with activation and loading of the amino acid substrate). The pre-incubation reaction (22.5 μL/reaction) 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), 1.39 mM L-Ser (the substrate was omitted for the negative controls), 1.11 μM Sfp, 111 μM Ecm6($T_1$), 1.39 mM ATP, and 27.8 μM Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$). To initiate the time course, 2.5 μL/reaction of the SAM mixture was added for a final reaction (25 μL/reaction) of 75 mM Tris-HCl (pH 7.5 adjusted at rt), 10 mM $MgCl_2$, 1 mM TCEP, 0.5 mM CoA, 1.25 mM L-Ser, 1 μM Sfp, 100 μM Ecm6($T_1$), 1.25 mM ATP, and 25 μM Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$). At 0, 5, 15, 30, 60, and 120 min, 25-μL aliquots were added to 100 μL of 12.5% TCA to quench the reaction and precipitate the protein. The precipitated protein was pelleted by centrifugation at 14,000 rpm for 7 min at rt, the supernatant was carefully removed, and the pellet was washed with 100 μL 12.5% TCA. The samples were centrifuged 14,000 rpm for 7 min at rt, and the wash was repeated. After the second wash, the samples were centrifuged a third time (14,000 rpm for 7 min at rt), and the supernatant was removed. The protein pellet was then dissolved in 100 μL of 88% formic acid. The sample was then transferred to 5 mL

of scintillation cocktail in a scintillation vial, and the radioactivity was measured as above in section 4. For the methylation assays supplemented with wt Ecm6($A_1$), the reaction was carried out by the same method except 2.7 μM of Ecm6($A_1$) was added to the pre-incubation reaction (2.5 μM in the final reaction) and incubated overnight. At 0, 30, 60, and 120 min time points, the reactions were quenched and processed as above.

(ii) *HPLC assays*. Reactions for HPLC analysis of T domain loaded by Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$) were conducted in the following manner, modified from previous publications from our laboratory.[7] The final reaction volume was 50 μL total that consisted of 75 mM Tris-HCl (pH 7.5 adjusted at rt), 10 mM $MgCl_2$, 1 mM TCEP, 0.25 mM CoA, 3 mM L-Ser (or L-Cys or *N*-Me-L-Cys), 2 μM Sfp, 15 μM Ecm6($T_1$), 5 mM ATP, and 5 μM Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$). The apo reaction contained no CoA or interrupted A domain. The holo reactions contained no interrupted A domain. For the methylation reactions, a final concentration of 0.5 mM of SAM was added to the reaction. The reactions were incubated overnight at rt and centrifuged for 10 min at 13,000 rpm at rt prior to transferring to HPLC vials. The samples (10 μL) were injected for each reaction to monitor the modified T domains on HPLC at λ = 215 nm. The reactions that used TioN to activate and load L-Cys and *N*-Me-L-Cys as standards were set up the same way, except TioN was used instead of Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$) and L-Cys or *N*-Me-L-Cys was used instead of L-Ser. TioN is known to be able to load both L-Cys and *N*-Me-L-Cys onto Ecm6($T_1$).[2] The HPLC protocol used is presented in Table S3. These data are presented in Fig. 4. To present the HPLC traces (Fig. 4) we used Sfp as an internal standard and adjusted the retention times so that all the Sfp peaks were at the same retention time. For Fig. 4A, reaction 3 Sfp was set as the standard (retention time = 16.0521 min), so 0.0111 and 0.0063 were added to the entire set of x data for reactions 2 and 3, respectively so that the retention time of Sfp was increased from 16.041 min to 16.0521min and 16.0458 min to 16.0521 min, respectively. The same adjustment was done to the data set in Fig. 4B. The Sfp peak in reaction 9 was set as the standard (retention time = 16.0508) so 0.0229, 0.0517, 0.0246, 0.0415, and 0.0369 were subtracted from reactions 8, 7, 6, 5, and 4, respectively, so that the retention times were all decreased from 16.0738 min, 16.1025 min, 16.0754 min, 16.0923 min, and 16.0877 min to 16.0508 min, respectively.

**Table S3**. Specifications of HPLC protocol.

| Time | %A ($H_2O$ with 0.1% TFA) | %B (Acetonitrile) | Flow rate (mL/min) |
|---|---|---|---|
| 5 | 80 | 20 | 1.00 |
| 20 | 40 | 60 | 1.00 |
| 25 | 40 | 60 | 1.00 |
| 30 | 5 | 95 | 1.00 |
| 35 | 5 | 95 | 1.00 |
| 36 | 80 | 20 | 1.00 |
| 41 | 80 | 20 | 1.00 |

(iii) *Mass spectrometry (MS) and tandem mass spectrometry ($MS^2$) assays*. Reactions for MS and $MS^2$ analyses were conducted on enzymatic extracts. The enzymatic reactions were conducted in accordance with previous publications.[5, 8] The reactions (484.38 µL), which contained 77.4 mM Tris-HCl (pH 7.5 adjusted at rt), 10.3 mM $MgCl_2$, 1.29 mM L-Ser, 1.03 mM TCEP, 0.52 mM CoA, 1.03 µM Sfp, 103 µM Ecm6($T_1$), 5.16 mM ATP, and 25.8 µM Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$), were incubated for 2 h at rt. Methylation was initiated by the addition of 15.62 µL 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-Ser, 1 mM TCEP, 0.5 mM CoA, 1 µM Sfp, 100 µM Ecm6($T_1$), 5 mM ATP, 1 mM SAM, and 25 µM Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$)). A negative control was set up that contained all the same components, except no Ecm6($AM_sM_bA$) or Ecm6($AM_bM_sA$) was added. After the addition of SAM, the reactions were incubated an additional 1 h and then quenched with 2 mL of ice-cold MeOH and incubated at -20 °C for 2 h to precipitate the proteins. For each reaction, the mixture was then centrifuged at 13,000 rpm for 10 min at rt, and the supernatant was removed and discarded. The protein pellet was washed with 1 mL of ice-cold 80% MeOH, which was centrifuged as before, and the supernatant was removed. This process was repeated once more. After the second wash, the protein was centrifuged as above, and the supernatant was discarded. The protein was air dried for 5 min. The product bound to the T domain was cleaved with KOH (500 µL of 0.1 N KOH) by fully dissolving the pellet and incubation at 63 °C for 30 min. The reaction was then neutralized by the addition of 500 µL of 0.1 N HCl. The reaction was then extracted 4× with 1 mL of EtOAc in the following manner: 1 mL EtOAc was added, the mixture was vortexed for 1 min and centrifuged for 5 min at rt, 13,000 rpm. The organic layer was transferred to a pre-weighed vial. During the first extraction, an emulsion/protein layer formed, which was broken and removed (after transferring the organic layer to the glass vial) by filtering with glass wool plug in the neck of a Pasteur pipet. The combined 4 extractions were dried under reduced pressure by using a rotary evaporator and overnight vacuum desiccation. The samples

were dissolved in HPLC grade MeOH and analyzed (100 ng/μL) *via* MS and $MS^2$. The second attempt at improving this reaction and extraction was set up as the first, but with the following changes: First, the reaction incubated overnight after the addition of SAM. Second, after neutralization with HCl, the reaction was acidified to a pH of ~2-3. Third, extraction was done 5× instead of 4, and after the sample cleaned with the glass wool, each subsequent 1-mL EtOAc was filtered through the glass wool plug, then vortexed and centrifuged. These data are presented in Fig. S6D-F).

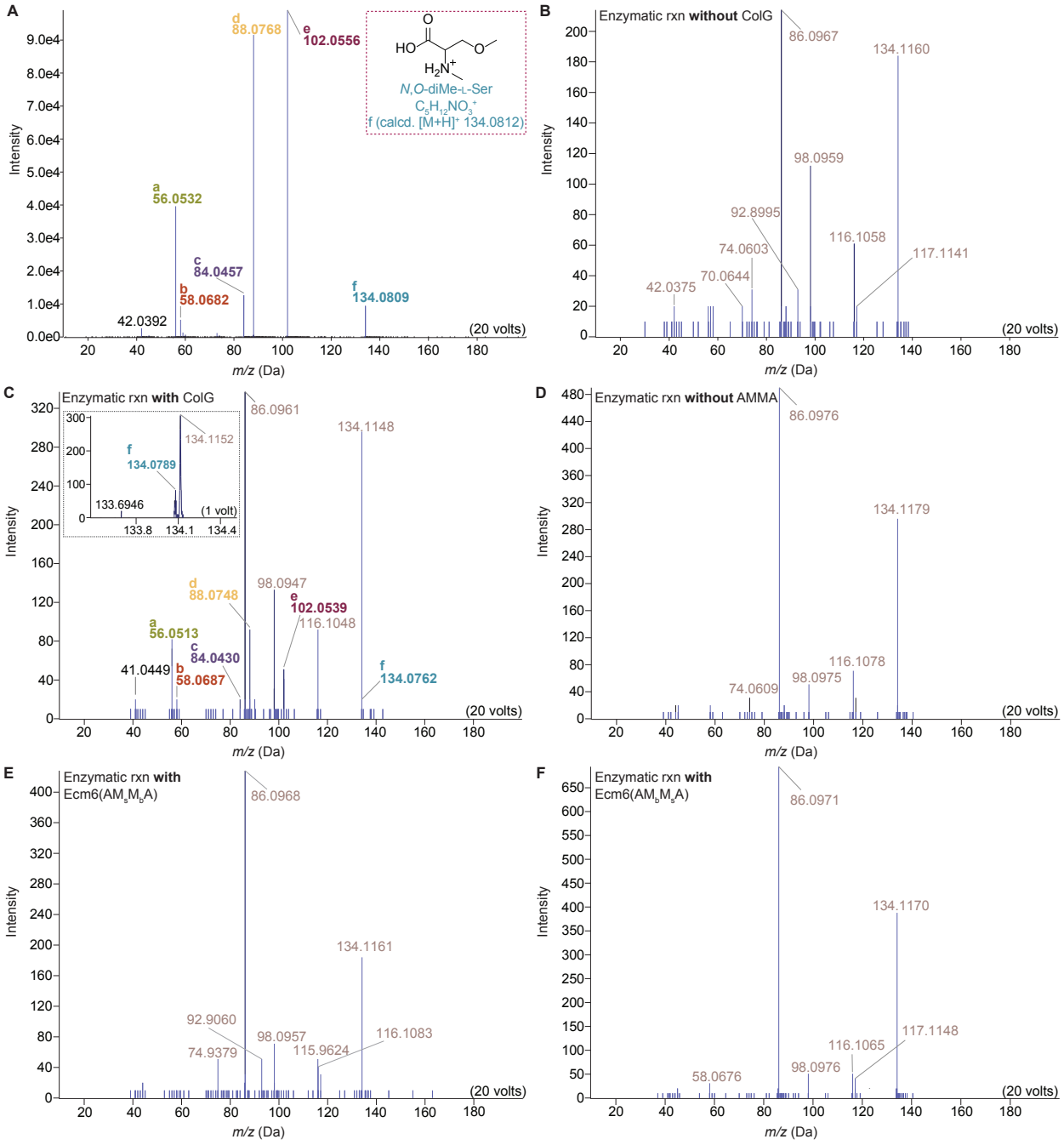


**Fig. S6.** MS$^2$ analysis of enzymatic extracts. Panels A-C were previously published in Ref [5] (*presented here for comparison purposes only*). MS$^2$ of *N*,*O*-diMe-L-Ser data showing the masses obtained from the **A.** synthetic standard, **B.** the enzymatic reaction control without ColG(AM$_s$M$_b$A), and **C.** the enzymatic reaction with ColG(AM$_s$M$_b$A). Panels D-F were generated in this study showing the **D.** reaction without addition of either Ecm6(AM$_s$M$_b$A) or Ecm6(AM$_b$M$_s$A), **E.** enzymatic reaction with Ecm6(AM$_s$M$_b$A), and **F.** enzymatic reaction with Ecm6(AM$_b$M$_s$A).

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