

Supporting information

Substrate-Dependent Cleavage Site Selection by Unconventional Radical *S*-Adenosylmethionine Enzymes in Diphthamide Biosynthesis

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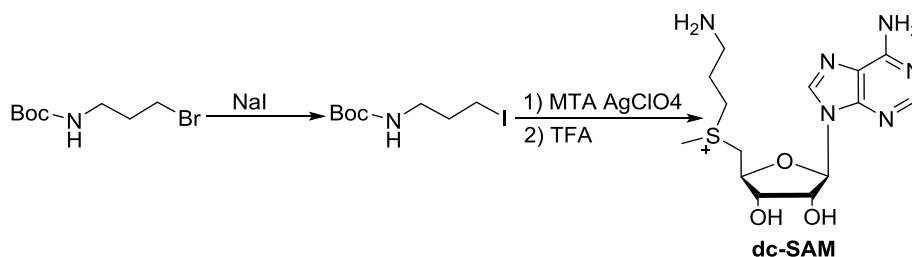
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A. Reagents and Methods

General methods. The reagents for organic synthesis were purchased from Sigma-Aldrich. Badan (6-Bromoacetyl-2-Dimethylaminonaphthalene) was purchased from Thermo Fisher Scientific Inc. ^1H and ^{13}C NMR were performed on INOVA 400 and 600 MHz spectrometers. LCMS was carried out on a SHIMADZU HPLC and Thermo LCQ FLEET mass spectrometer with a Kinetex 2.6 μm XB-C18 100 \times 4.6 mm column monitoring at 215 and 254 nm. Solvents used in LCMS for positive mode were water with 0.1% acetic acid and acetonitrile with 0.1% acetic acid. Mobile phases used for analytic and preparative HPLC were water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). Analytic HPLC analysis was performed using Kinetex 2.6 μm XB-C18 100 \times 4.6 mm column with UV detection at 215 nm and 254 nm. Preparative HPLC purification was carried out using TargaTM Prep C18 10 μm 250 \times 20mm reverse phase column with UV detection at 215 nm and 254 nm. Protein purification, enzymatic reactions were performed in an anaerobic chamber (Coy Laboratory Products). X band EPR spectra were recorded at National Biomedical Center for Advanced ESR Technology (ACERT) on a Bruker ElexSys E500 EPR spectrometer at a frequency of 9.25 GHz in quartz tubes with internal diameters of 4 mm. EPR measurements at 12 K and 35K were carried out using an ESR910 liquid-helium cryostat (Oxford Instruments). The spectrometer settings were as follows: modulation frequency, 100 kHz; modulation amplitude, 8 G; microwave power, 0.63 mW. The field sweeps were calibrated with a BRUKER ER 035 Gauss meter and the microwave frequency was monitored with a frequency counter. Data acquisition and manipulation were performed with Xepr software. ENDOR spectra were collected on a spectrometer with a helium immersion dewar previously reported.^{1,2} ENDOR measurements were done at 2 K.

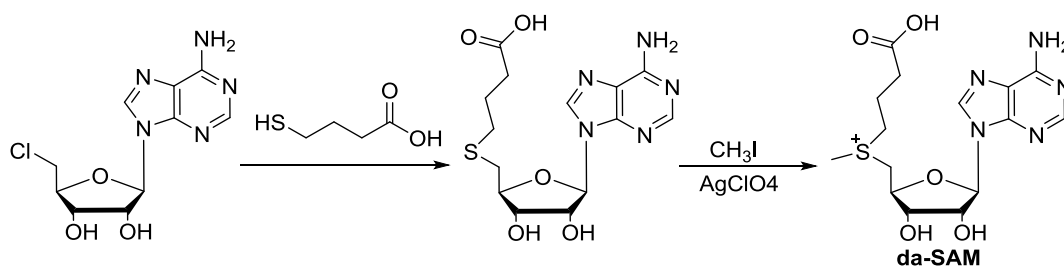
Synthesis of dc-SAM. tert-Butoxycarbonylamino-3-bromopropane (100mg, 0.42mmol) was dissolved in acetone (1.5 mL). Sodium iodide (75 mg, 0.5 mmol) in 0.5 mL of acetone was added to the reaction. The mixture was stirred overnight. White precipitate was filtered and the filtrate was evaporated under vacuum. The residue was dissolved in 10 mL chloroform, washed with saturated $\text{Na}_2\text{S}_2\text{O}_5$ and NaCl solution sequentially. The organic layer was dried over anhydrous Na_2SO_4 and the solvents were evaporated under vacuum. The crude product was used directly without further purification.



The synthetic method used for dc-SAM was similar to that reported for synthesizing other SAM analogues³ with slight modification. tert-Butoxycarbonyl

-amino-3-iodopropane from previous step was dissolved in acetic acid (200 μ L) and formic acid (200 μ L). 5'-Deoxy-5'-methylthioadenosine (MTA) (15 mg, 50 μ mol) was added at 0 °C. AgClO_4 (35 mg, 169 μ mol) was dissolved in 50 μ L acetic acid and then added to the former solution at 0 °C. This mixture was stirred for 20 min at 0 °C and then for 5 h at room temperature. After diluted with 1 mL of cold water, the reaction was filtered to remove the precipitated AgI. The filtrate was dissolved in 1 mL of trifluoroacetic acid and stirred for 30 min at room temperature. The mixture was evaporated under vacuum. The residue was dissolved in 2 mL of H_2O and loaded onto preparative HPLC for purification. Freeze drying of the HPLC fractions yielded dc-SAM as a white powder (3.8 mg, 22% yield, 1:1 mixture of stereoisomers). ^1H NMR (600 MHz, D_2O) δ 8.27 (s, 1H), 8.26 (s, 1H), 6.02 (d, 1H), 4.77 – 4.69 (m, 1H), 4.50 – 4.36 (m, 2H), 3.87 – 3.72 (m, 2H), 3.42 – 3.34 (m, 1H), 3.33 – 3.25 (m, 1H), 2.95 (dt, J = 18.9, 7.7 Hz, 3H), 2.83 (s, s, 3H), 2.05 (p, J = 7.6 Hz, 2H). LCMS (ESI) calcd. for $\text{C}_{14}\text{H}_{23}\text{N}_6\text{O}_3\text{S}$ $[\text{M}]^+$ 355.1, obsd. 355.0.

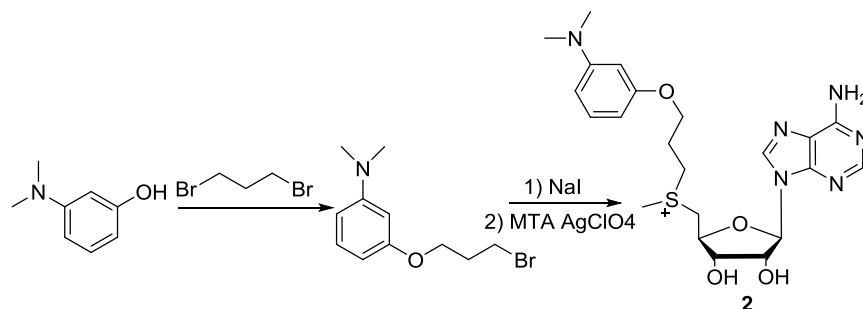
Synthesis of da-SAM. 5'-S-(3-Carboxypropyl)-5'-thioadenosine was synthesized from 5'-chloro-5'-deoxyadenosine and 4-mercaptoputyric acid using reported procedure.⁴



5'-S-(3-Carboxypropyl)-5'-thioadenosine (20mg, 54 μ mol) was dissolved in acetic acid (200 μ L) and formic acid (200 μ L). Methyl iodide (34 μ L, 500 μ mol) was added at 0 °C. AgClO_4 (51 mg, 250 μ mol) was dissolved in 50 μ L of acetic acid and then added to the former solution at 0 °C. This mixture was stirred for 20 min at 0 °C and then for 2 h at room temperature. After diluted with 1 mL of cold water, the reaction was filtered to remove the precipitated AgI. The filtrate was evaporated under vacuum. The residue was dissolved in 2 mL of H_2O and loaded onto preparative HPLC for purification. Freeze drying of the HPLC fractions yielded da-SAM as a white powder (14 mg, 70% yield, 3:2 mixture of stereoisomers). ^1H NMR (400 MHz, D_2O) δ 8.27 (s, 2H), 5.98 (t, 1H), 4.73 (m, 1H), 4.47 – 4.38 (m, 2H), 3.83 – 3.64 (m, 2H), 3.32 – 3.11 (m, 2H), 2.77, 2.75 (s,s, 3H), 2.29 – 2.23 (m, 2H), 1.97 – 1.66 (m, 2H). LCMS (ESI) calcd. for $\text{C}_{15}\text{H}_{22}\text{N}_5\text{O}_5\text{S}$ $[\text{M}]^+$ 384.1, obsd. 383.9.

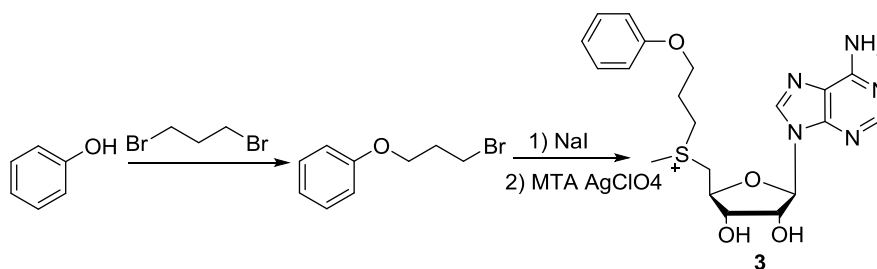
Synthesis of compound 2. 3-(Dimethylamino)-phenol (343 mg, 2.5 mmol) and 1,3-dibromopropane (1.27 mL, 12.5 mmol) were added to 10 mL of acetonitrile. Then potassium carbonate (520 mg, 3.75 mmol) and potassium iodide (20 mg, 5%) were added to the reaction. The mixture was stirred at room temperature for 24 hr and evaporated under vacuum. The residue was dissolved in 50 mL of ethyl acetate and washed with 50 mL of water. The aqueous phase was extracted with ethyl acetate (3 \times

50 mL). The combined organic phases were dried over anhydrous Na₂SO₄. The solvent was evaporated in vacuum and the crude product was purified by silica gel column chromatography (hexane:ethyl acetate 20:1) to give 3-(3-bromopropoxy)-*N,N*-dimethyl-benzenamine (372 mg, 58% yield) ¹H NMR (400 MHz, Acetonitrile-*d*₃) δ 7.13 – 7.06 (m, 1H), 6.37 (ddd, *J* = 8.3, 2.2, 1.0 Hz, 1H), 6.29 – 6.25 (m, 2H), 4.08 (t, *J* = 5.9 Hz, 2H), 3.64 (t, *J* = 6.6 Hz, 2H), 2.90 (s, 6H), 2.29 – 2.22 (m, 2H).



Compound **2** (3:2 mixture of two stereoisomers) was synthesized with 3-(3-bromopropoxy)-*N,N*-dimethyl-benzenamine according to the same procedure for the synthesis of da-SAM. ¹H NMR (400 MHz, D₂O) δ 8.20 (t, s, 1H), 8.18, 8.13 (d, d, 1H), 7.27 (t, *J* = 8.3 Hz, 0.4H), 7.18 (t, *J* = 8.3 Hz, 0.6H), 7.01 – 6.90 (m, 1H), 6.82 (s, 0.4 H), 6.73 (d, *J* = 8.5 Hz, 0.4H), 6.65 (s, 0.6H), 6.54 (d, *J* = 8.4 Hz, 0.6H), 5.94 (m, 1H), 4.92 (t, *J* = 5.4 Hz, 0.6H), 4.72 (t, *J* = 4.6 Hz, 0.4H), 4.43 (m, 2H), 4.02 – 3.73 (m, 3H), 3.64 – 3.26 (m, 3H), 3.08 (s, 3H), 3.05 (s, 3H), 2.83 (s, s, 3H), 2.14 (m, 2H). LCMS (ESI) calcd. for C₂₂H₃₁N₆O₄S [M]⁺475.2, obsd. 474.9.

Synthesis of compound 3. Compound 3 (3:2 mixture of two stereoisomers) was synthesized starting from phenol according to the same procedure used for the synthesis of compound 2. ¹H NMR (400 MHz, D₂O) δ 8.23 (d, 1H), 8.11 (d, 1H), 7.16 – 7.06 (m, 1H), 7.04 – 6.96 (m, 1H), 6.87 – 6.75 (m, 1H), 6.59 (dt, *J* = 8.0, 1.1 Hz, 1H), 6.47 – 6.37 (m, 1H), 5.94 (d, 1H), 4.87 (t, *J* = 5.3 Hz, 0.6H), 4.73 – 4.68 (t, *J* = 5.3 Hz, 0.4H), 4.49 – 4.39 (m, 2H), 3.99 – 3.71 (m, 3H), 3.62 (dd, *J* = 13.4, 2.4 Hz, 1H), 3.52 – 3.24 (m, 2H), 2.83 (s, s, 3H), 2.19 – 2.01 (m, 2H). LCMS (ESI) calcd. for C₂₀H₂₆N₅O₄S [M]⁺432.2, obsd. 432.0.



Synthesis of ¹³C_{Methyl}-dc-SAM. 5'-*S*-(3-aminopropyl)-5'-thioadenosine (10 mg, 29 μmol) was dissolved in formic acid (200 μL). ¹³C-Methyl iodide (5.3 mg, 348 μmol) was added at 0 °C. AgClO₄ (6.1 mg, 290 μmol) was dissolved in 50 μL of acetic acid and then added to the former solution at 0 °C. This mixture was stirred for 20 min at 0 °C and then for 5 h at room temperature. After diluted with 1 mL of cold water, the reaction was filtered to remove the precipitated AgI. The filtrate was evaporated under

vacuum. The residue was dissolved in 2 mL of H₂O and loaded onto preparative HPLC for purification. Freeze drying of the HPLC fractions yielded ¹³C_{Methyl}-dc-SAM as a white powder (8 mg, 78% yield, 1:1 mixture of two stereoisomers). ¹H NMR (400 MHz, D₂O) δ 8.28 (d, 2H), 6.00 (d, 1H), 4.71 (ddd, *J* = 10.0, 5.4, 3.9 Hz, 1H), 4.52 – 4.34 (m, 2H), 3.77 (q, *J* = 3.6 Hz, 2H), 3.42 – 3.19 (m, 2H), 3.00 – 2.60 (d, d, *J* = 144 Hz, 3H), 2.96 (m, 2H), 2.03 (p, *J* = 7.8 Hz, 2H). LCMS (ESI) calcd. for C₁₃¹³CH₂₃N₆O₃S [M]⁺ 356.2, obsd. 356.1.

Expression and purification of *PhDph2* and *Dph1-Dph2*. *PhDph2* was overexpressed in *E. coli* BL21(DE3) with pRARE2 and purified as previously described⁵ but with the following modification. The cells were cooled down to 18 °C before induced at OD₆₀₀ of 0.6 with 0.1 mM isopropyl-β-D- thiogalactopyranoside (IPTG). Cells were incubated in a shaker at 18 °C and 200 rpm for 20 h before harvested. *Dph1-Dph2* was expressed and purified as previously reported⁶.

Analyzing the enzymatic reactions of *PhDph2* and SAM analogues with high-performance liquid chromatography (HPLC). The reactions were set up under anaerobic condition. The full reaction contained 200 μM *PhDph2*, 10 mM dithionite, 2 mM SAM analogues, 150 mM NaCl, 10 mM DTT in 200 mM Tris-HCl buffer at pH 7.4 in a final volume of 10 μL. The control samples (without *PhDph2*, dithionite, or SAM analogues) were set up similarly by replacing the corresponding component with equal volume of water respectively. The reactions were incubated at 65 °C for 30 min and quenched by adding 50 μL of 10 % TFA in water. Protein was precipitated by centrifugation and the supernatant was analyzed using HPLC with a linear gradient of 0 to 40 % buffer B over 15 min at a flow rate of 0.5 mL/min.

The time dependent experiments were set up as described above. A total volume of 60 μL reaction was set up, 10 μL aliquot of which was quenched with 50 μL of 10% TFA at different time points: 1 min, 5 min, 10 min, 20min and 30 min. Then the protein was spun down and the supernatant was analyzed using HPLC.

Dph1-Dph2 catalyzed reactions were set up and treated similarly except that the reactions were incubated at 30 °C for 1 hr.

Analyzing reaction product from *BtrN* with HPLC. The reactions were set up under anaerobic conditions. The full reaction contained 20 μM *BtrN*, 10 mM dithionite, 100 μM SAM or SAM analogues, in 25 mM HEPES buffer at pH 7.5 in a final volume of 30 μL. The control samples (without *BtrN*, dithionite, or SAM analogues) were set up similarly by replacing the corresponding component with equal volume of water respectively. The reactions were incubated at 37 °C for 30 min and quenched by adding 30 μL of 10 % TFA in water. Protein was precipitated by centrifugation and the supernatant was applied to HPLC and resolved with a linear gradient of 0 to 40 % buffer B over 15 min at a flow rate of 0.5 mL/min.

Sample preparation for EPR and ENDOR experiments. The time dependent EPR experiments (360 μM *Dph1-Dph2*, 1 mM dithionite, and 4 mM dc-SAM in 200

mM Tris-HCl with 150 mM sodium chloride, pH 7.4) were set up with the volume of 60 μ L for each time point in an anaerobic chamber at room temperature. The reactions were transferred to EPR tubes and frozen in liquid N₂ at different time points: 5 min, 30 min, and 60 min. Then the tubes were sealed in the anaerobic chamber and taken out for EPR analysis. The procedure of sample preparation for ENDOR experiments was similar to that for EPR, except ¹³C_{Methyl}-dc-SAM was used.

Sample preparation for ¹³C-NMR to detect reaction product. A complete reaction (400 μ M *PhDph2*, 10 mM dithionite, and 5 mM ¹³C_{Methyl}-dc-SAM in 60 μ L of 200 mM Tris-HCl with 1 mM DTT and 150 mM sodium chloride, pH 7.4) and controls (without dithionite) were set up in an anaerobic chamber. The samples were incubated for 30 min at 65 °C. D₂O (300 μ L) was added to each sample and the solution was transferred to a Millipore Microcon YM-10 filter unit. The filtrate was transferred to a Shigemi D₂O-matched NMR tube and directly used for NMR measurements.

Detection of methylated DTT using Badan. The following steps were all performed in an anaerobic chamber. A portion of enzymatic reaction of *PhDph2* with ¹³C_{Methyl}-dc-SAM for ¹³C-NMR was treated with KF and 6-bromoacetyl-2-dimethylaminonaphthalene (Badan) following the reported procedure⁷. The reaction was extracted with dichloromethane. The organic layer was evaporated and re-dissolved in acetonitrile before analysis by LC-MS.

Detection of Methanethiol using Badan. The 60 min time point EPR sample was used to detect methanethiol generated from the decomposition of the intermediate. A similar reaction was set up with ¹³C_{Methyl}-dc-SAM for the detection of ¹³C-methanethiol. Control reactions without dithionite were set up with both substrates. After incubation, ethyl ether (300 μ L) was added to the reaction, followed by the addition of 6 N HCl to a final concentration of 1 M. The HCl was added directly in the aqueous phase. The tubes were sealed and sat for 10 min followed by gently mixing. The ether phase was transferred to a tube with 1 M Tris-HCl pH 8.0 (50 μ L). The mixture was treated with KF and Badan as in the detection of methylated DTT.

Detection of ¹⁴C-methyl labeled SAM decarboxylase using ¹⁴C_{methyl}-dc-SAM. ¹⁴C_{methyl}-dc-SAM was prepared using ¹⁴C_{methyl}-SAM and SAM decarboxylase. ¹⁴C_{methyl}-SAM (80 μ M; 55 mCi/mmol, American Radiolabeled Chemicals Inc.) was incubated with SAM decarboxylase⁸ (80 μ M) in 1 M Tris-HCl at pH 8.0. The reaction mixture was incubated at 65 °C for 15 min. The conversion of ¹⁴C_{methyl}-SAM to ¹⁴C_{methyl}-dc-SAM was confirmed by LC-MS using a parallel experiment with unlabeled SAM. The reactions containing 30 μ M *PhDph2*, 10 mM dithionite in 150 mM NaCl and 200 mM Tris-HCl at pH 7.4 were set up in an anaerobic chamber. The reaction vials were sealed with rubber stoppers before being taken out of the anaerobic chamber. The prepared ¹⁴C_{methyl}-dc-SAM mixture or ¹⁴C_{methyl}-SAM (60 μ M)

was injected using needles into each reaction vial to start the reaction. The reaction mixtures were vortexed briefly to mix and incubated at 65 °C for 30 min. The reactions were stopped by adding protein loading dye and subsequently heating at 95 °C for 5 min, followed by 4-20% SDS–polyacrylamide gradient gel electrophoresis. The dried gel was exposed to a PhosphorImaging screen (GE Healthcare Life Science) and the radioactivity was detected using a Typhoon FLA 7000 (GE Healthcare Life Science).

Dph1-Dph2 catalyzed labeling experiments were set up and treated similarly except that the reactions were incubated at 30 °C for 1 hr.

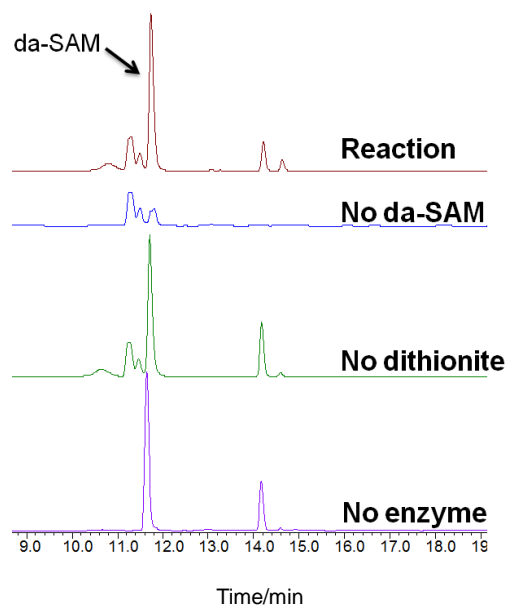


Figure S1. Reaction of *PhDph2* with da-SAM.

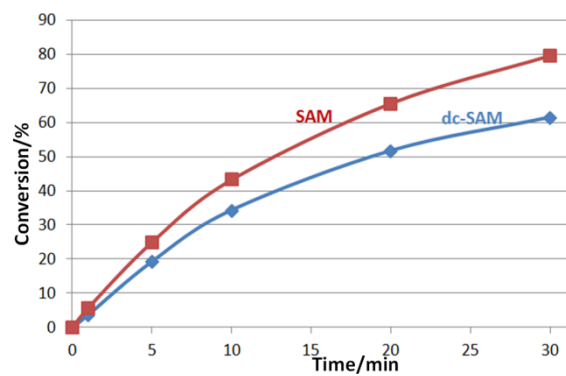


Figure S2 Reaction rate of *PhDph2* with SAM and dc-SAM.

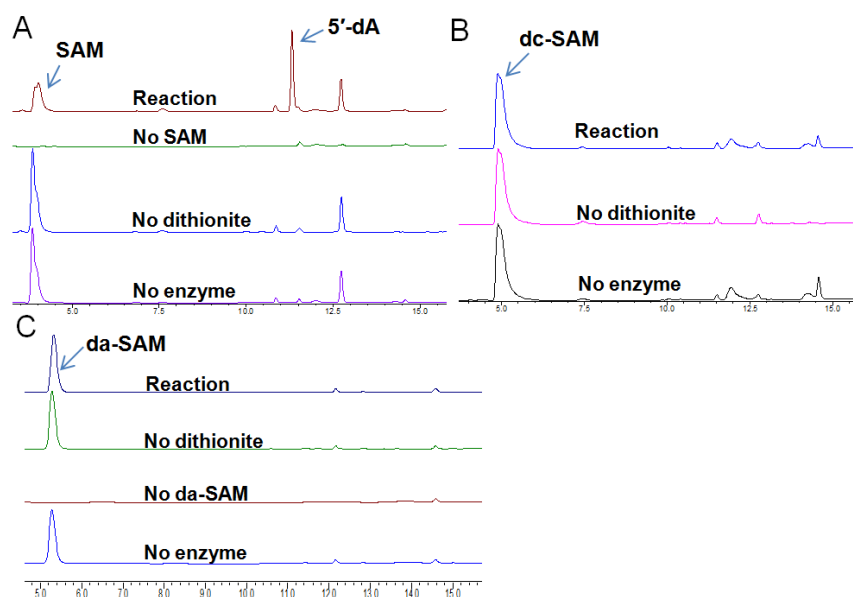


Figure S3. Activity of BtrN with SAM, dc-SAM and da-SAM.

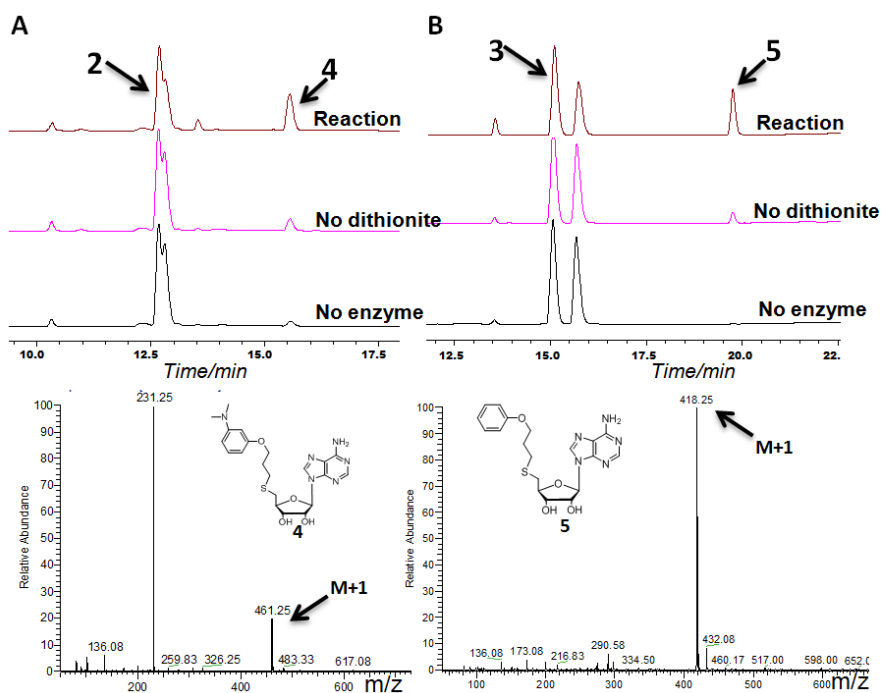


Figure S4. Reaction of PhDph2 with SAM analogues 2 and 3 with ESI-MS of the products 4 and 5

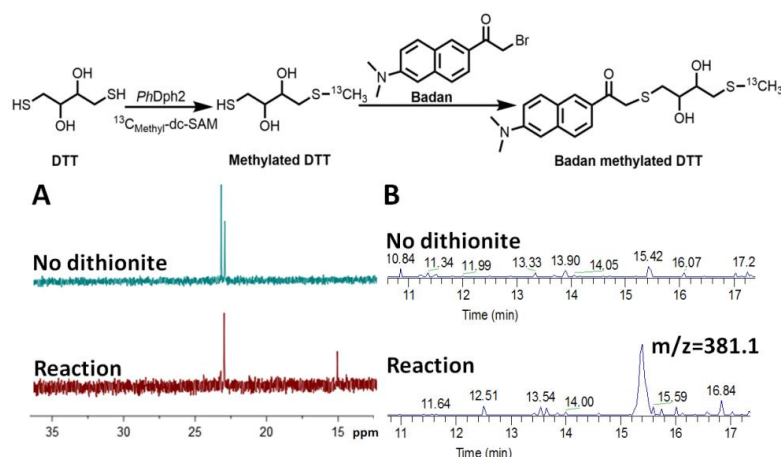


Figure S5. *PhDph2* transfers ^{13}C -methyl group to DTT from $^{13}\text{C}_{\text{methyl}}\text{-dc-SAM}$. A) ^{13}C NMR showing that a new peak formed in the reaction, but not in a control without dithionite. B) Detection of Badan methylated DTT by LC-MS. The mass spectroscopy (MS) traces are shown for the reaction and control.

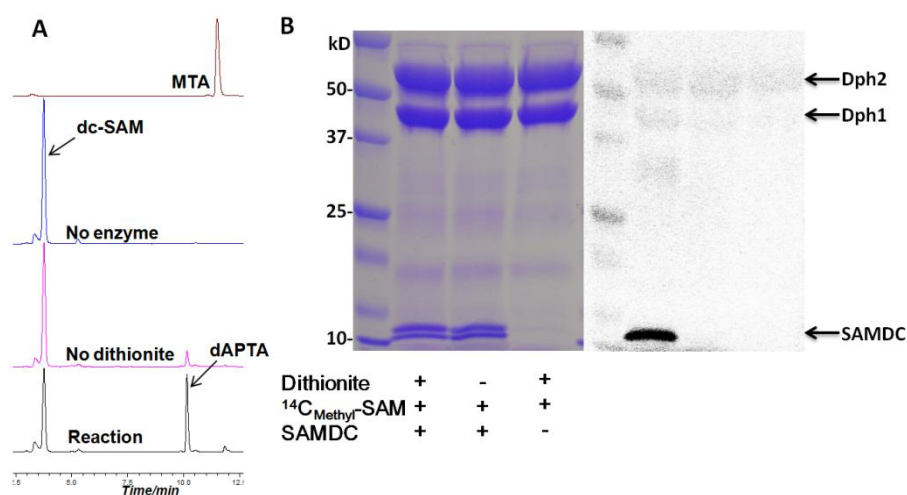


Figure S6. *Dph1-Dph2* catalyzes the methyl cleavage reaction with dc-SAM, similar to *PhDph2*. A) HPLC analysis showing dAPTA formation catalyzed by *Dph1-Dph2*. B) *Dph1-Dph2* transfers ^{14}C -methyl group to SAM decarboxylase (SAMDC) from $^{14}\text{C}_{\text{methyl}}\text{-dcSAM}$. The left panel shows the Coomassie blue-stained gel; the right panel shows the autoradiography.

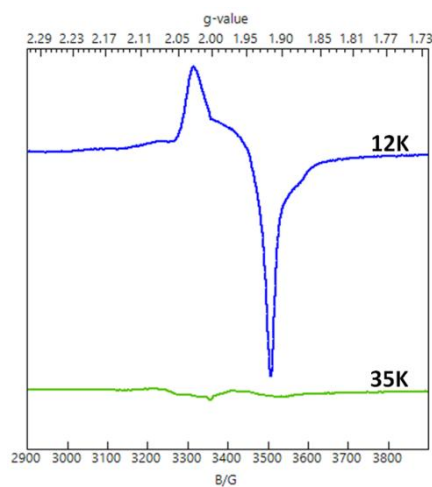


Figure S7. Temperature dependency of the intermediate formed with Dph1-Dph2 and dc-SAM.

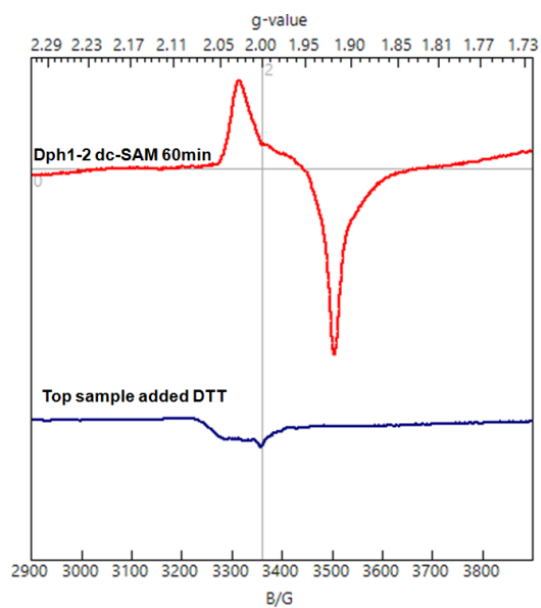


Figure S8. X-band EPR at 12K showing that DTT can react with the EPR-active intermediate formed with Dph1-Dph2 and dc-SAM.

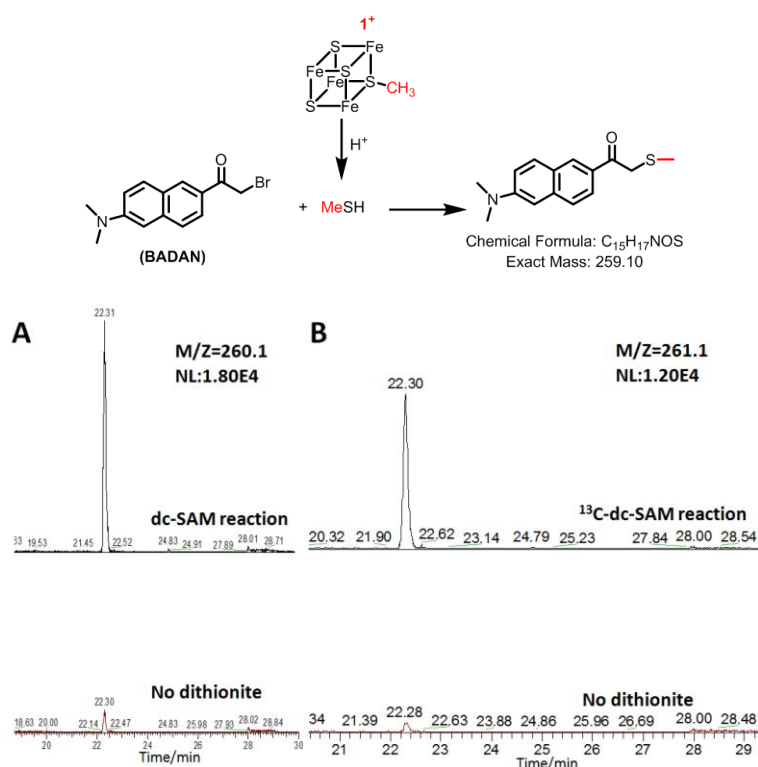


Figure S9. The Badan-derivatization reaction for the detection of methanethiol (A) and ¹³C-methanethiol (B) generated with dc-SAM and ¹³C_{methyl}-dc-SAM as substrate, respectively.

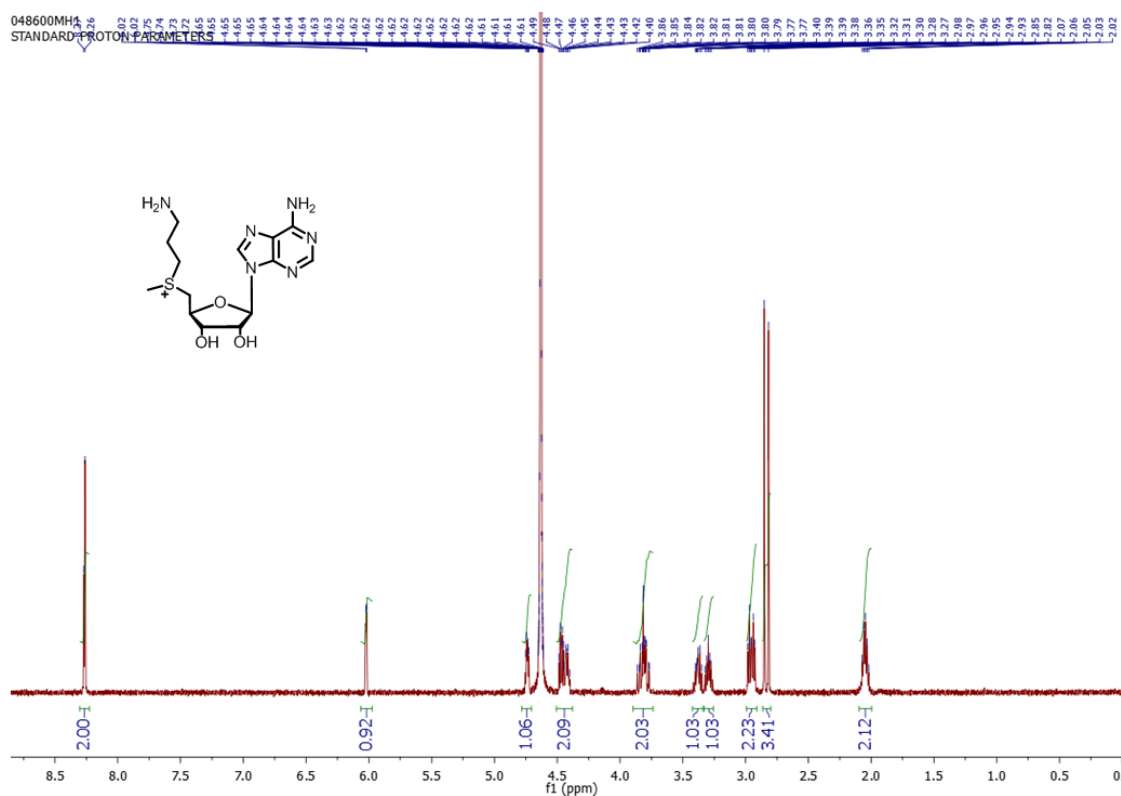


Figure S10. ¹H-NMR spectrum of dc-SAM

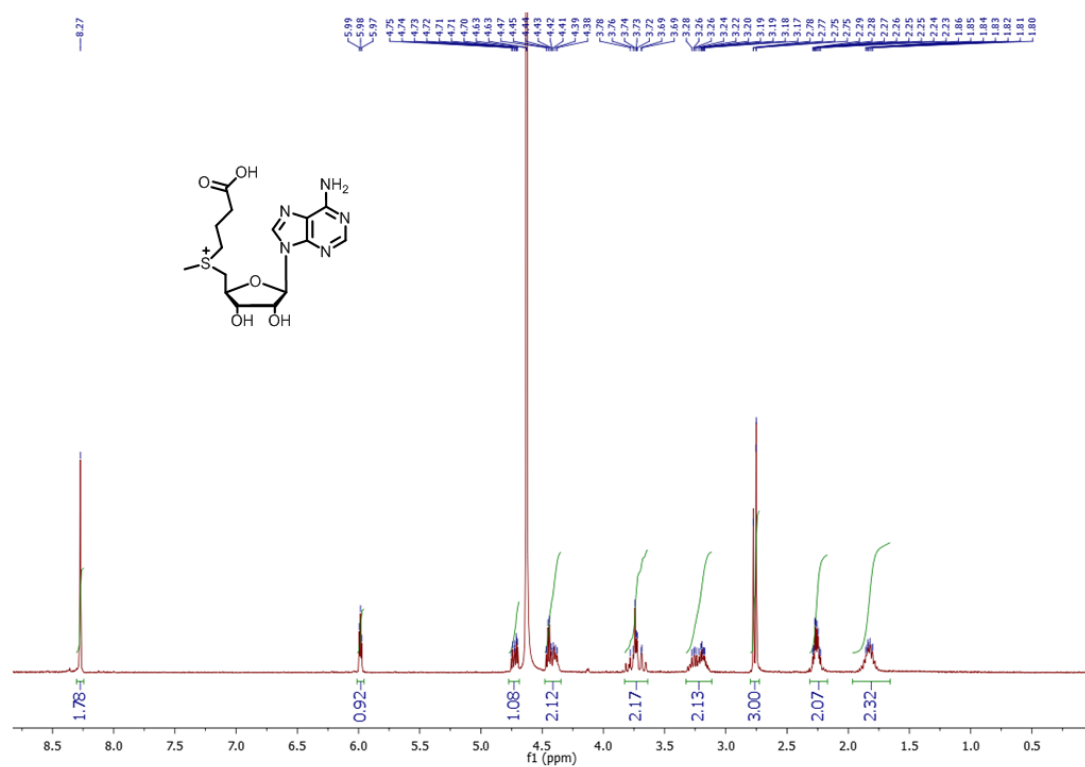


Figure S11. ¹H-NMR spectrum of da-SAM

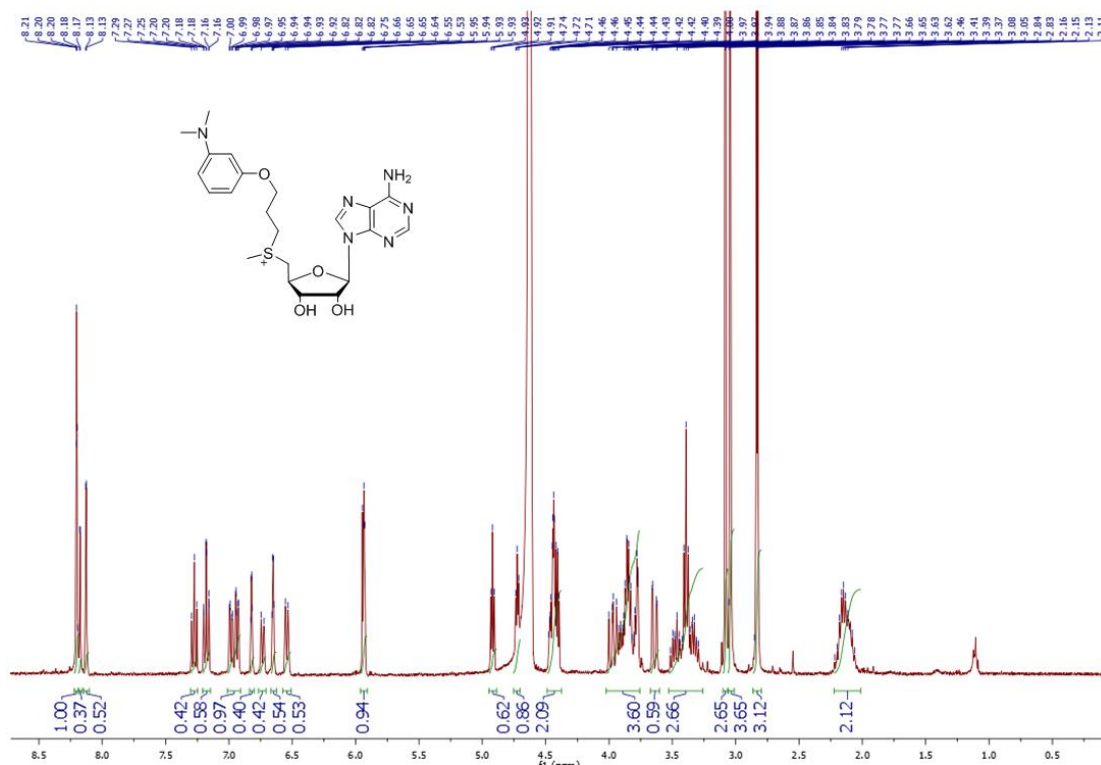


Figure S12. ¹H-NMR spectrum of SAM analogue 2

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