Supporting information

Substrate-Dependent Cleavage Site Selection by Unconventional Radical SAM Enzymes in Diphthamide Biosynthesis

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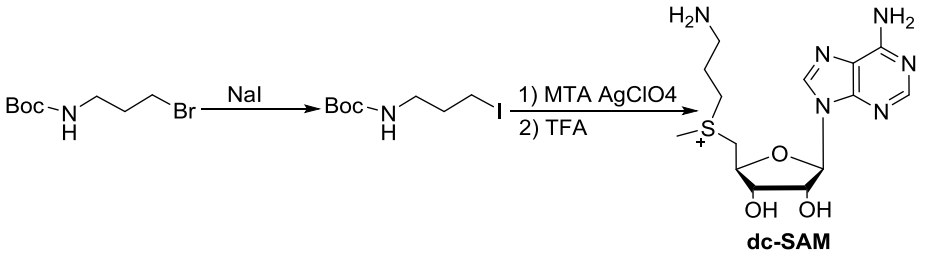
Materials and methods

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. ^{1}H 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 100×4.6 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, and for negative mode were water and acetonitrile. 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 100×4.6 mm column with UV detection at 215 nm and 254 nm. Preparative HPLC purification was carried out using Targa^{TM} Prep C18 10 μm 250×20mm 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 $Na_2S_2O_5$ 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.

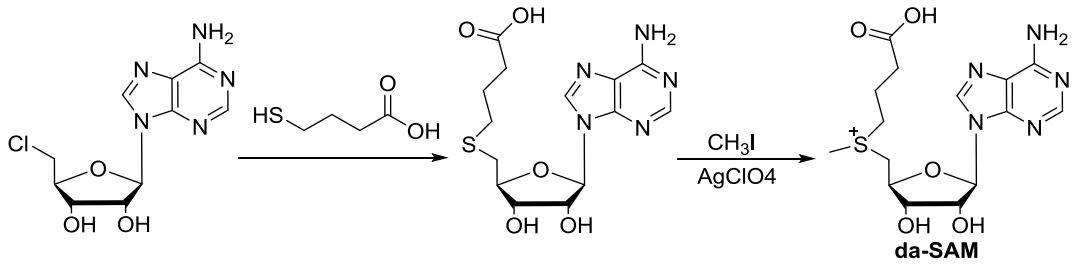


dc-SAM

The synthetic method used for dc-SAM was similar to that reported for

synthesizing other SAM analogues[3] with slight modification. tert-Butoxycarbonyl -amino-3-iodopropane from previous step was dissolved in acetic acid (200 μL) and formic acid (200 μL). 5'-Deoxy-5'-methylthioadenosine (MTA) (15 mg, 50 μmol) was added at 0℃. $AgClO_4$ (35 mg, 169 μmol) was dissolved in 50 μl acetic acid and then added to the former solution at 0 ℃. This mixture was stirred for 20 min at 0℃ 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). ^{1}H 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 $C_{14}H_{23}N_6O_3S$ $[M]^+$ 355.1, obsd. 355.0.

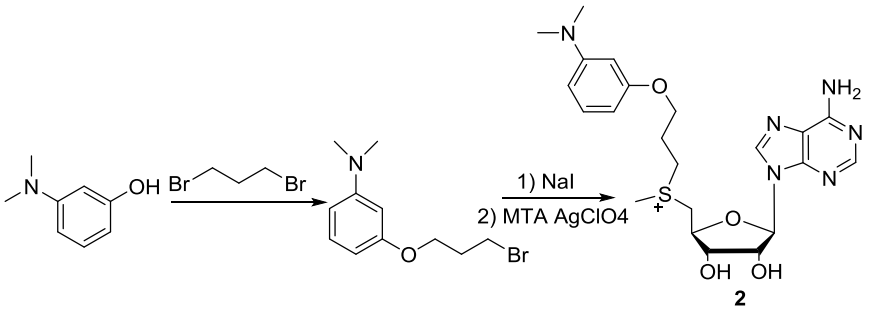
Synthesis of da-SAM. 5'-*S*-(3-Carboxypropyl)-5'-thioadenosine was synthesized from 5'-chloro-5'-deoxyadenosine and 4-mercaptobutyric acid using reported procedure.[4]



5'-*S*-(3-Carboxypropyl)-5'-thioadenosine (20mg, 54μmol) was dissolved in acetic acid (200 μL) and formic acid (200 μL). Methyl iodide (34 μL, 500 μmol) was added at 0℃. $AgClO_4$ (51 mg, 250 μmol) was dissolved in 50 μl of acetic acid and then added to the former solution at 0℃. This mixture was stirred for 20 min at 0℃ 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).^{1}H 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 $C_{15}H_{22}N_5O_5S$ $[M]^+$ 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 × 50 mL). The combined organic phases were dried over anhydrous Na_2SO_4. 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-bromopropoxyl)-*N*, *N*-dimethyl-benzenamine (372 mg, 58% yield) ^{1}H 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).



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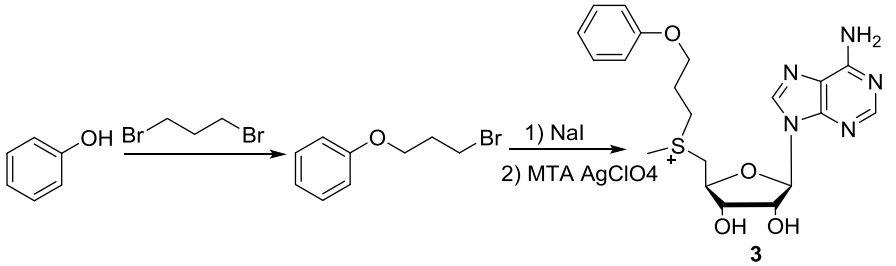
Compound **2** (3:2 mixture of two stereoisomers) was synthesized with 3-(3-bromopropoxy)-*N*,*N*-dimethyl-benzenamine according to the same procedure for the synthesis of da-SAM. ^{1}H NMR (400 MHz, D_2O) δ 8.20 (t, s, 1H), 8.18, 8.13 (d, d, 1H), 7.27 (t, *J* = 8.3 Hz, 0.4H), 7.18 (t, *J* = 8.3 Hz, 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 $C_{22}H_{31}N_6O_4S$ $[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. ^{1}H NMR (400 MHz, D_2O) 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 $C_{20}H_{26}N_5O_4S$ $[M]^+$432.2, obsd. 432.0.



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Synthesis of $^{13}C_{Methyl}$-dc-SAM. 5'-*S*-(3-aminopropyl)-5'-thioadenosine (10 mg, 29μmol) was dissolved in formic acid (200 μL). ^{13}C-Methyl iodide (5.3 mg, 348 μmol) was added at 0℃. $AgClO_4$ (6.1 mg, 290 μmol) was dissolved in 50 μl of acetic acid and then added to the former solution at 0 ℃. This mixture was stirred for 20 min at 0 ℃ 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 H_2O and loaded onto preparative HPLC for purification. Freeze drying of the HPLC fractions yielded $^{13}C_{Methyl}$-dc-SAM as a white powder (8 mg, 78% yield, 1:1 mixture of two stereoisomers). ^{1}H NMR (400 MHz, D_2O) 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 $C_{13}{}^{13}CH_{23}N_6O_3S$ $[M]^+$356.2, obsd. 356.1.

Expression and purification of *Ph*Dph2 and Dph1-Dph2. *Ph*Dph2 was overexpressed in *E. coli* BL21(DE3) with pRARE2 and purified as previously described[5] but with the following modification. The cells were cooled down to 18 ℃ before induced at OD_{600} of 0.6 with 0.1 mM isopropyl-β-D- thiogalactopyranoside (IPTG). Cells were incubated in a shaker at 18 ℃ and 200 rpm for 20 h before harvested. Dph1-Dph2 was expressed and purified as previously reported[6].

Analyzing the enzymatic reactions of *Ph*Dph2 and SAM analogues with high-performance liquid chromatography (HPLC). The reactions were set up under anaerobic condition. The full reaction contained 200 μM *Ph*Dph2, 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 *Ph*Dph2, 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 ℃ 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 ℃ 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 ℃ 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 μL for each time point in an anaerobic chamber at room temperature. The reactions were transferred to EPR tubes and frozen in liquid N_2 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 $^{13}C_{Methyl}$-dc-SAM was used.

Sample preparation for ^{13}C-NMR to detect reaction product. A complete reaction (400 μM *Ph*Dph2, 10 mM dithionite, and 5 mM $^{13}C_{Methyl}$-dc-SAM in 60 μL 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 ℃. D_2O (300 μL) 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 D_2O-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 *Ph*Dph2 with $^{13}C_{Methyl}$-dc-SAM for ^{13}C-NMR was treated with KF and 6-bromoacetyl-2-dimethylaminonaphthalene (Badan) following the reported procedure[7]. 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 $^{13}C_{Methyl}$-dc-SAM for the detection of ^{13}C-methanethiol. Control reactions without dithionite were set up with both substrates. After incubation, ethyl ether (300 μl) 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 μL). The mixture was treated with KF and Badan as in the detection of methylated DTT.

Detection of ^{14}C-methyl labeled SAM decarboxylase using $^{14}C_{methyl}$-dc-SAM. $^{14}C_{methyl}$-dc-SAM was prepared using $^{14}C_{methyl}$-SAM and SAM decarboxylase. $^{14}C_{methyl}$-SAM (80 μM; 55 mCi/mmol, American Radiolabeled Chemicals Inc.) was incubated with SAM decarboxylase [8](80 μM) in 1 M Tris-HCl at pH 8.0. The reaction mixture was incubated at 65 ℃ for 15 min. The conversion of $^{14}C_{methyl}$-SAM to $^{14}C_{methyl}$-dc-SAM was confirmed by LC-MS using a parallel experiment with unlabeled SAM. The reactions containing 30 μM *Ph*Dph2, 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 $^{14}C_{methyl}$-dc-SAM mixture or $^{14}C_{methyl}$-SAM (60uM) was injected using needles into each reaction vial to start the reaction. The reaction mixtures were vortexed briefly to mix and incubated at 65 ℃ 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 ℃ for 1 hr.

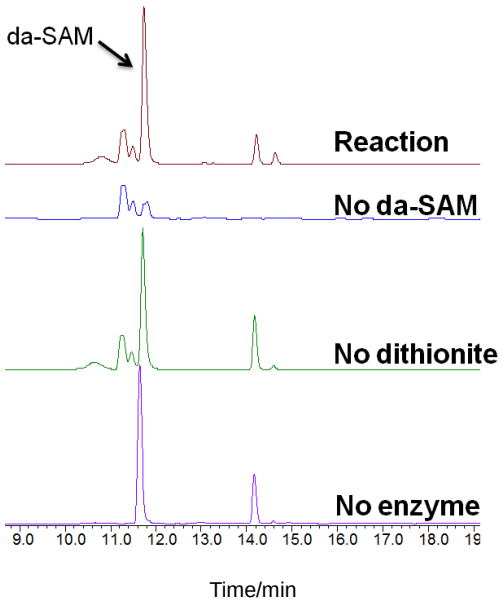

Figure S1. Reaction of *Ph*Dph2 with da-SAM.

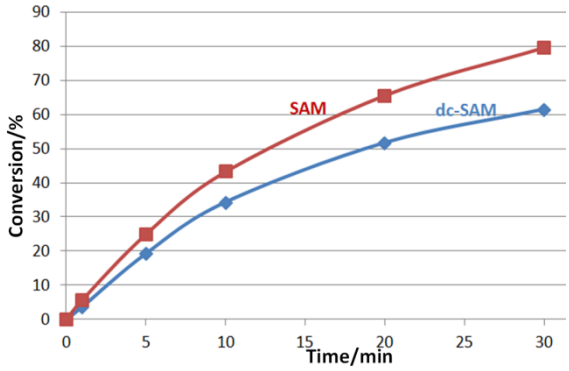

Figure S2 Reaction rate of *Ph*Dph2 with SAM and dc-SAM.

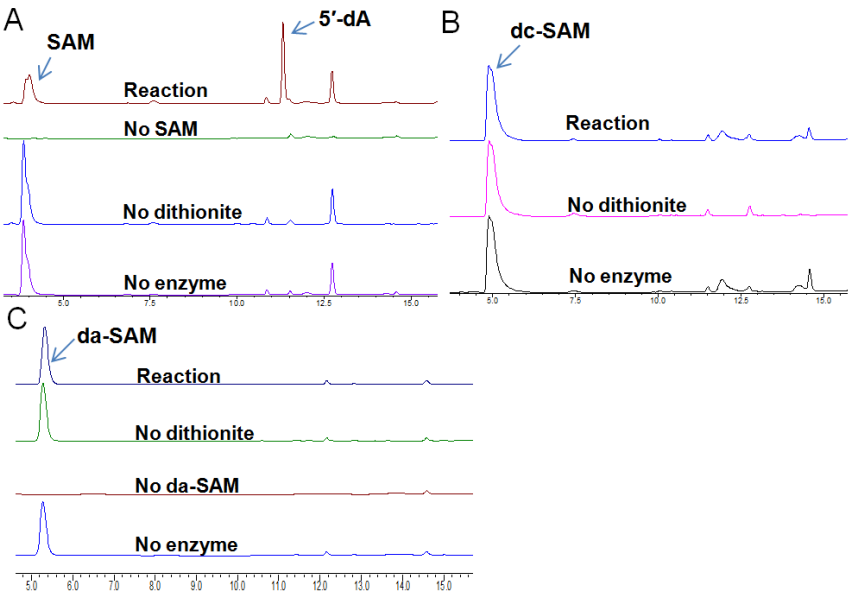

Figure S3. Activity of BtrN with SAM, dc-SAM and da-SAM.

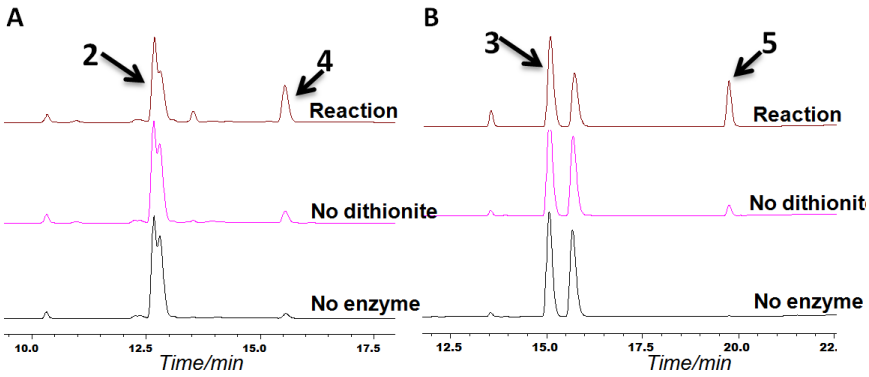

Figure S4. Reaction of *Ph*Dph2 with SAM analogues **2** and **3**.

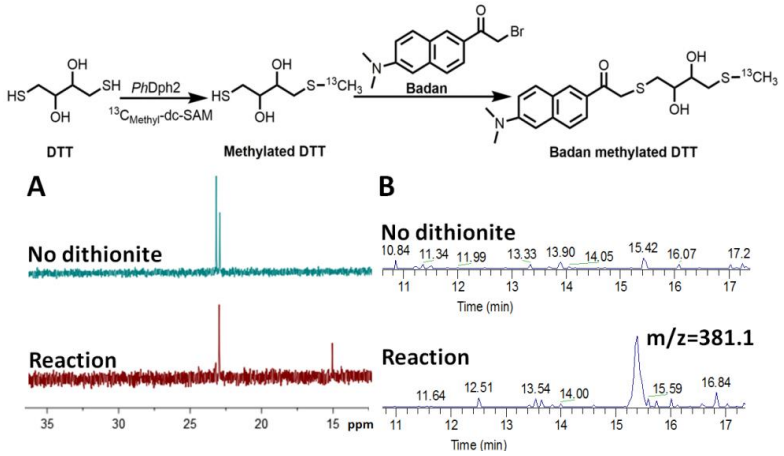

Figure S5. *Ph*Dph2 transfers ^{13}C-methyl group to DTT from $^{13}C_{methyl}$-dc-SAM. A) ^{13}C NMR showing that a new peak formed in the reaction, but not in a control without dithionite. B) Detection of Badan methylated DTT by LC–MS. The mass spectroscopy (MS) traces are shown for the reaction and control.

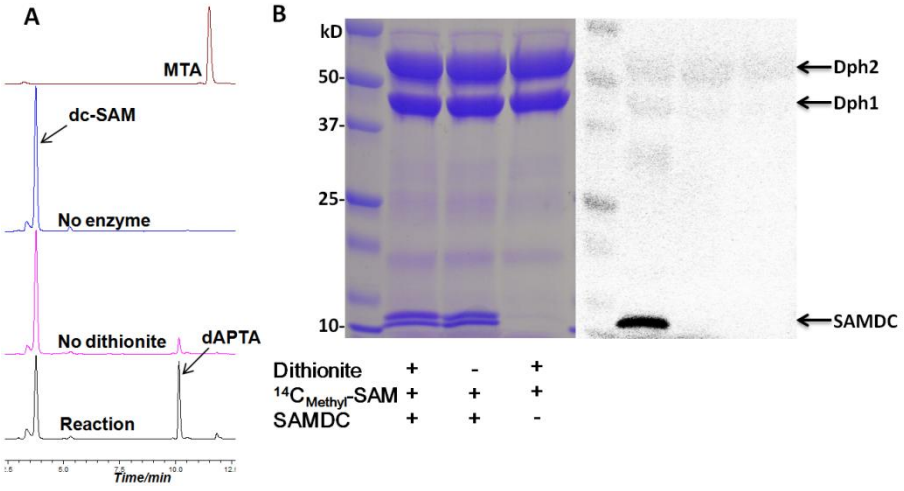

Figure S6. Dph1-Dph2 catalyzes the methyl cleavate reaction with dc-SAM, similar to *Ph*Dph2. A) HPLC analysis showing dAPTA formation catalyzed by Dph1-Dph2. B) Dph1-Dph2 transfers ^{14}C-methyl group to SAM decarboxylase (SAMDC) from $^{14}C_{methyl}$-dcSAM. The left panel shows the Coomassie blue-stained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

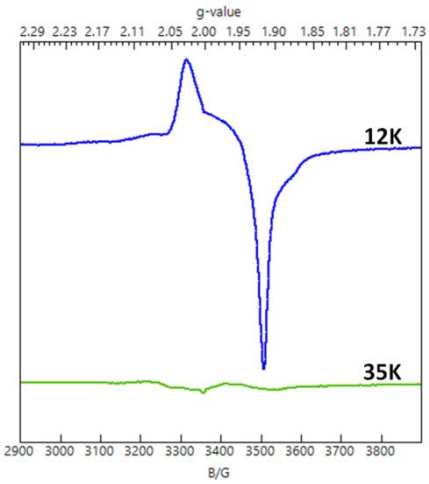

Figure S7. Temperature dependency of the intermediate formed with Dph1-Dph2 and dc-SAM.

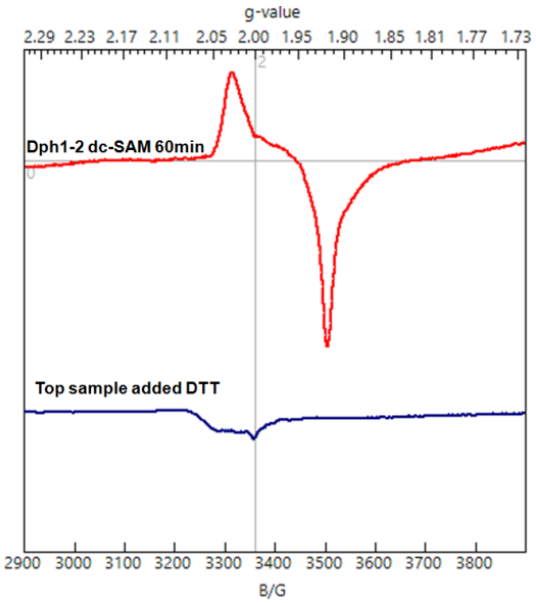

Figure S8. X-band EPR at 12K showing that DTT can react with the EPR-active intermediate formed with Dph1-Dph2 and dc-SAM.

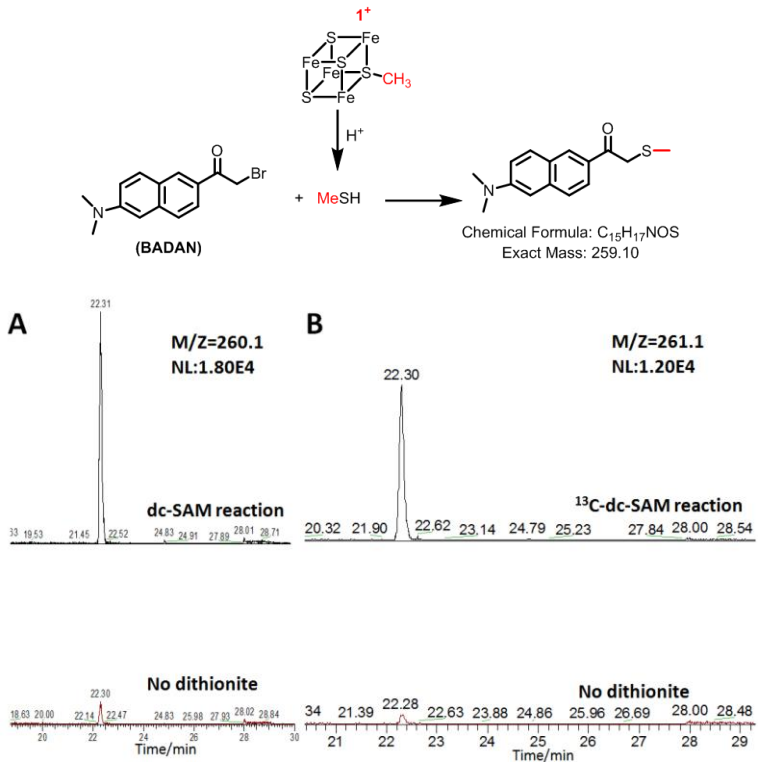

Figure S9. The Badan-derivatization reaction for the detection of methanethiol (A) and ^{13}C-methanethiol (B) generated with dc-SAM and $^{13}C_{methyl}$-dc-SAM as substrate, respectively.

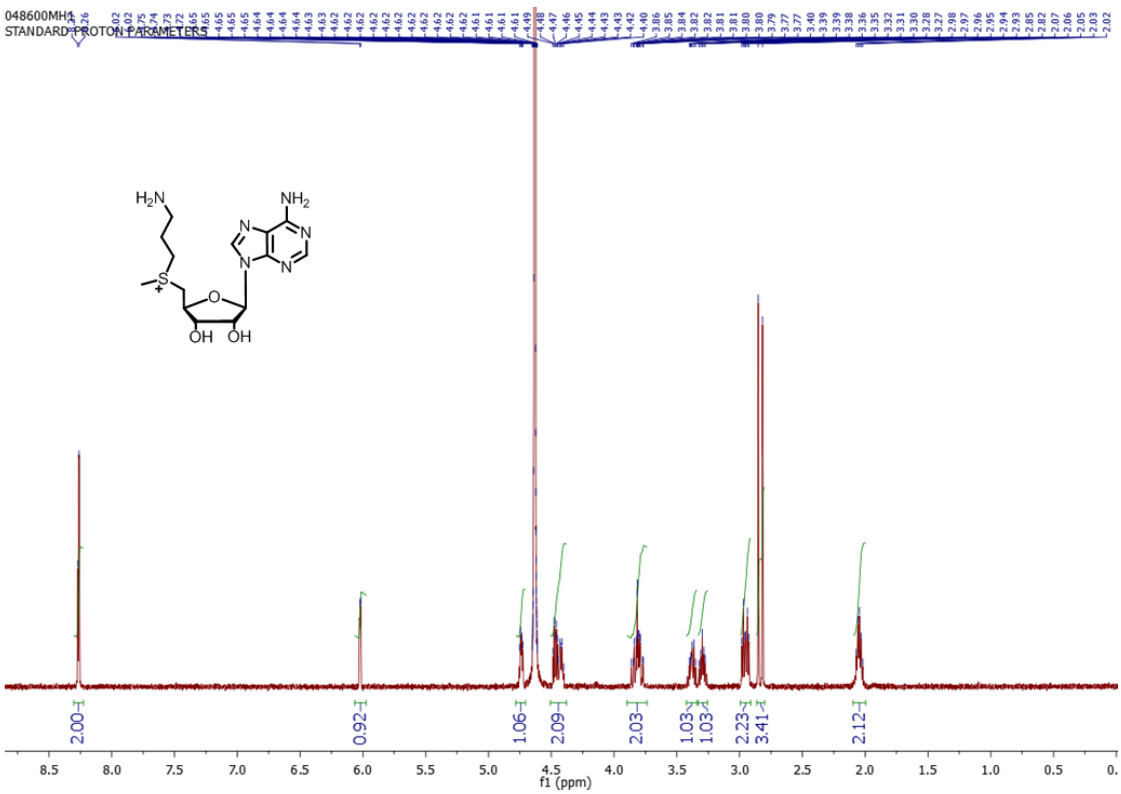

Figure S10. ^{1}H-NMR spectrum of dc-SAM

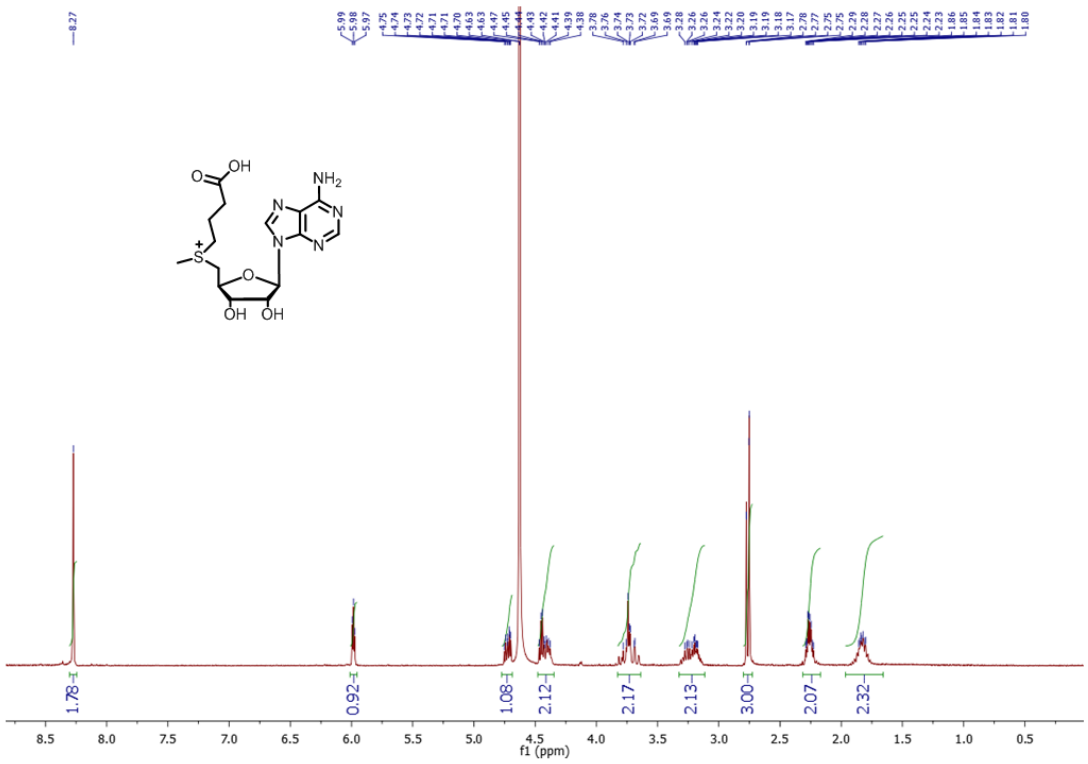

Figure S11. ^{1}H-NMR spectrum of da-SAM

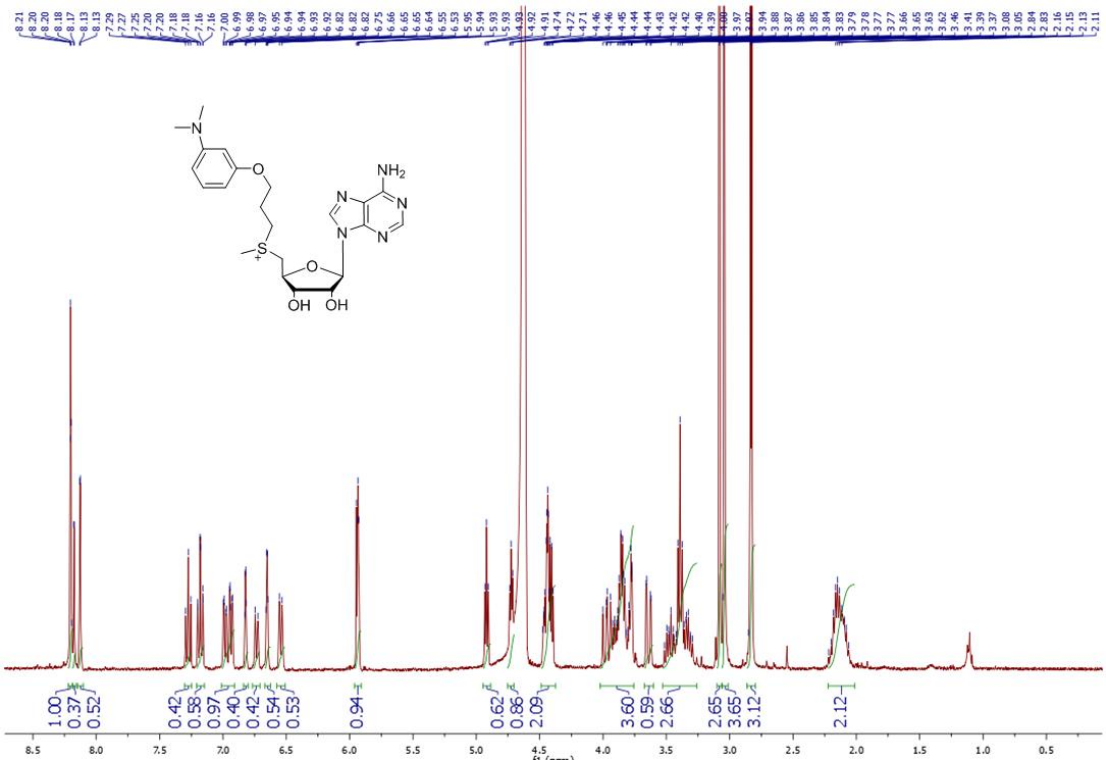

Figure S12. ^{1}H-NMR spectrum of SAM analogue 2

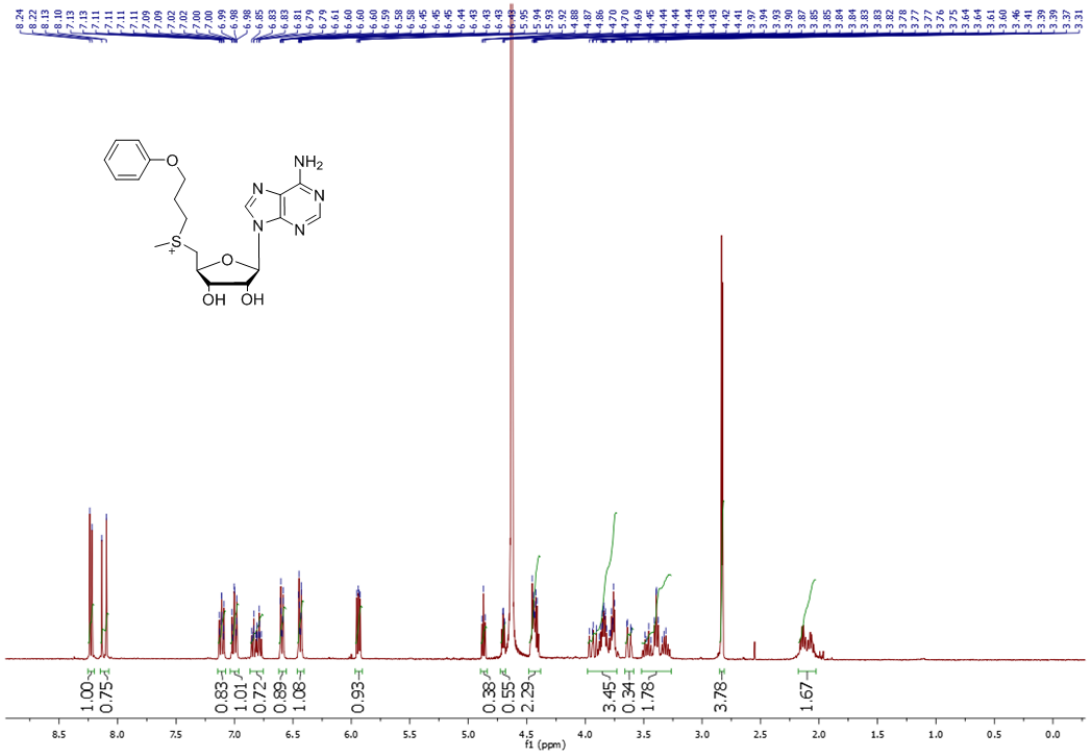

Figure S13. ^{1}H-NMR spectrum of SAM analogue 3.

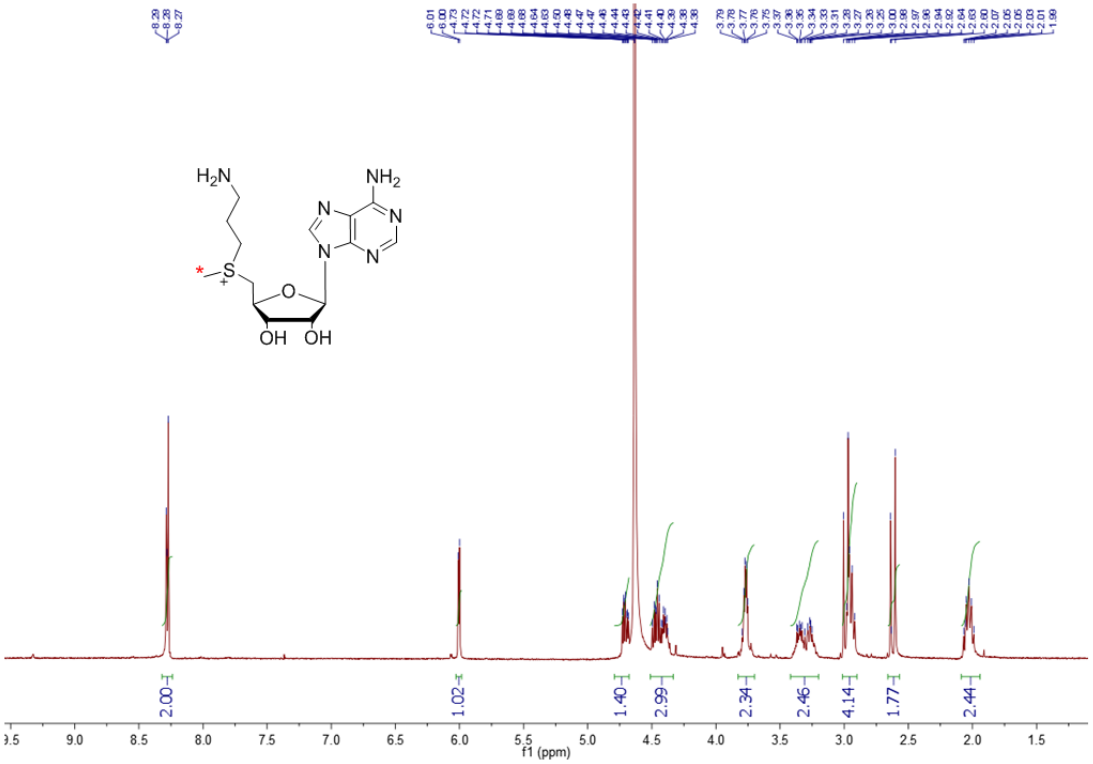

Figure S14. ^{1}H-NMR spectrum of $^{13}C_{Methyl}$-dc-SAM.

References:

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