Supporting Information

Capturing Unknown Substrates via *in situ* Formation of Tightly Bound Bisubstrate Adducts: *S*-Adenosyl-Vinthionine as a Functional Probe for AdoMet-Dependent Methyltransferases

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1. General Procedures

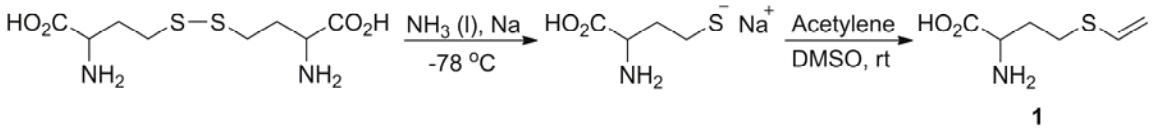
Reagents of ACS grade or better were obtained from either Sigma or Fisher unless otherwise noted. Recombinant histidine-tagged archaeon *Methanococcus jannaschii* L-methionine *S*-adenosyltransferase (*S*-adenosyl-methionine synthetase, MAT, EC 2.5.1.6), histidine-tagged human thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.67) and histidine-tagged *E. coli* 5'-methylthioadenosine/S-adenosyl-homocysteine nucleosidase (MTAN, EC 3.2.2.9) were purified as reported.[1-3]

High performance liquid chromatography (HPLC) was done on an Agilent 1100 HPLC with Dynamax SD-200 pumps and a Varian ProStar 330 photodiode array detector interfaced with the Varian ProStar Chromatography Workstation software version 6.41. Graphics were constructed using Kaleidagraph (4.1, Synergy Software, Reading, PA).

Prior to mass spectrometry, all samples were first buffer exchanged into 50 mM ammonium bicarbonate (pH 8.0); then aqueous trichloroacetic acid (TCA, 5 g TCA in 3.5 mL Milli-Q water) was used to precipitate the proteins for 10 min at room temperature (sample to aqueous TCA stock solution, 4:1, v:v). The protein precipitation was then spun down and the supernatants were collected for the subsequent analysis. LCQ ion trap mass spectrometer was used in-line with an HPLC system (Agilent 1200) for the characterization of AdoVin and AdoVin adducts. The spray voltage was 2.3 kV and the capillary temperature was 285 °C. Selected-ion monitoring chromatograms (SIM) were collected using Triple quadrupole mass spectrometer in-line with an HPLC system (Agilent 1200). Data were processed using the Xcalibar Data System 2.0 (Thermo Fisher, Waltham, MA).

2. Synthesis of S-Adenosyl-Vinthionine (AdoVin)

2.1. Synthesis of Vinthionine



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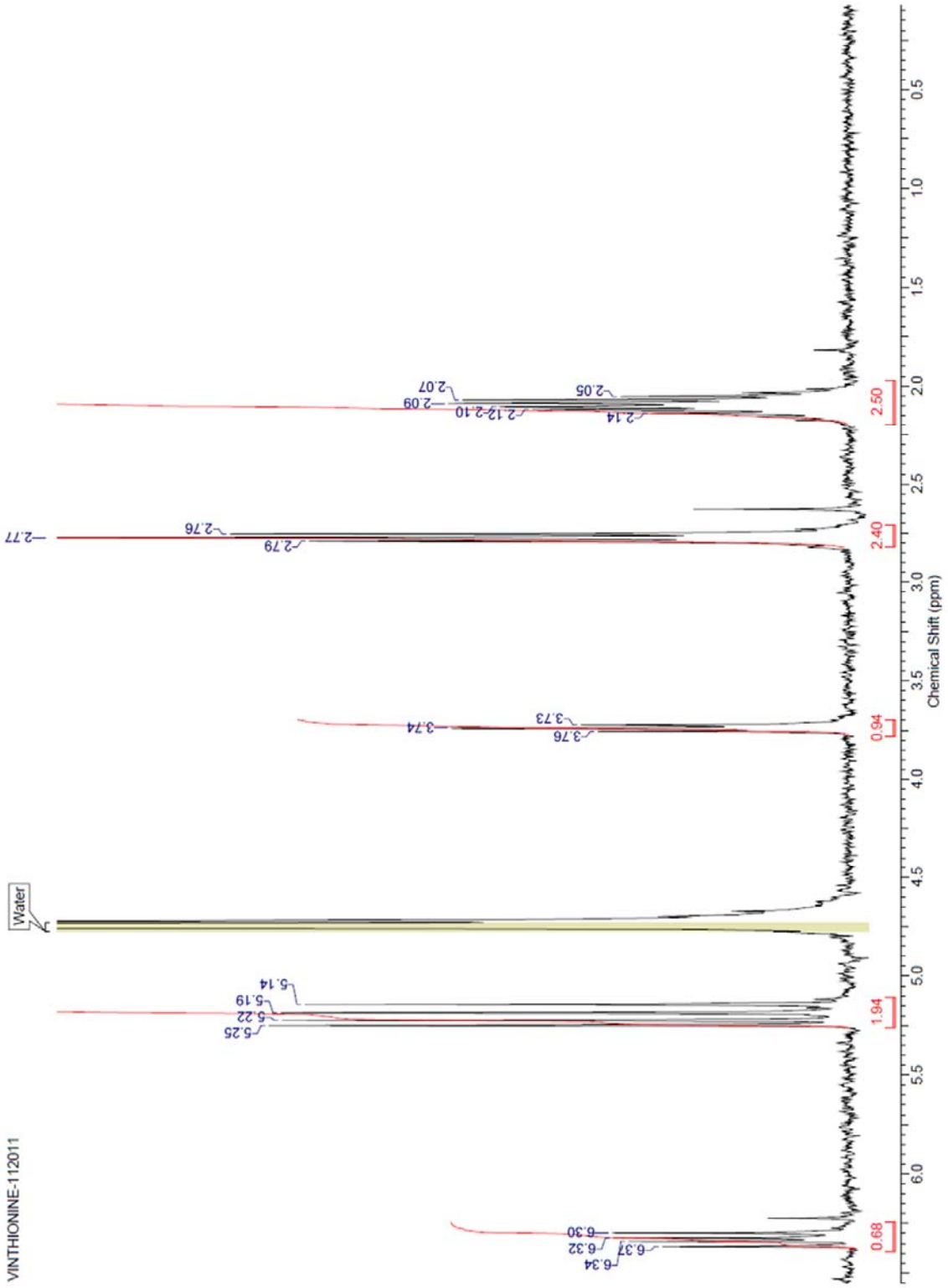
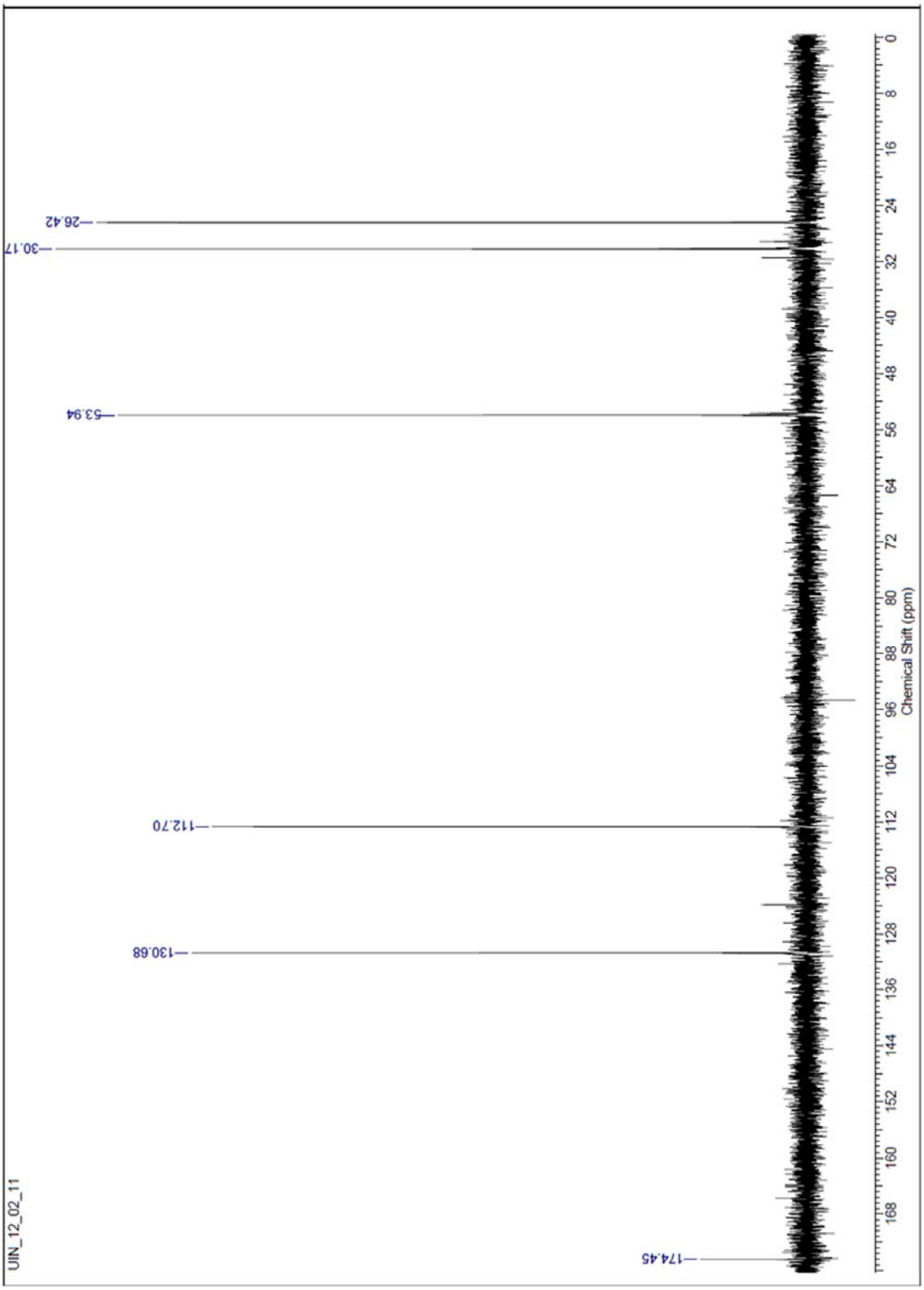
Supporting Scheme 2.1.1. Synthesis of vinthionine (vinyl homocysteine**, 1**).

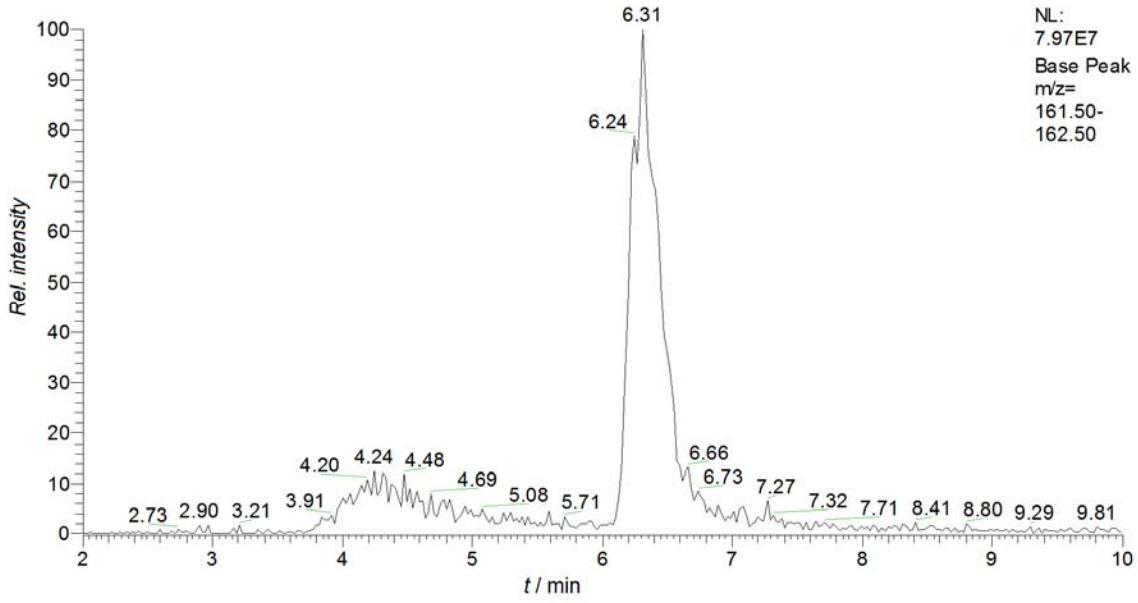
Vinthionine (1). [CAS: 83768-87-0] As reported, to a 250 mL, three-necked, round-bottom flask over a dry-ice/ethanol bath, DL-homocystine (1.01 g, 7.47 mmol) was added.[4] The sealed system was evacuated and then purged with anhydrous nitrogen several times. Anhydrous ammonia was then condensed into the round-bottom flask until the volume of liquid ammonia was approximately 50 mL. Small pieces of sodium metal were added slowly with stirring until the solution remained blue for ten minutes. Ammonium chloride was added slowly until the blue color faded, and the solution was allowed to warm to room temperature. After all ammonia was evaporated, the flask was cooled to 0°C, and 15 mL of anhydrous DMSO was added to dissolve the residue. Dried acetylene gas (passing through a calcium chloride column) was then bubbled though the solution for 4 hours, during which the flask was allowed to return room temperature. The syrup was then neutralized to pH near 7 with 1N HCl. The mixture was cooled to -20°C overnight to allow crystallization before filtering and washing with cold ethanol. The light-yellow powder was dried under vacuum. The product was used in the following procedures without further purification. ^{1}H-NMR (D_2O/K_2CO_3, 400 MHz): δ 6.33 (dd, 1H, J = 16.9 Hz, 10.3 Hz), 5.24 (d, 1H, J = 10.3 Hz), 5.16 (d, 1H, J = 16.9 Hz), 3.74 (t, 1H, J = 6.2 Hz), 2.77 (t, 2H, J = 7.3 Hz), 2.10 (m, 2H); ^{13}C-NMR (D_2O, 100 MHz): δ 174.45, 130.68, 112.70, 53.94, 30.17, 26.42.



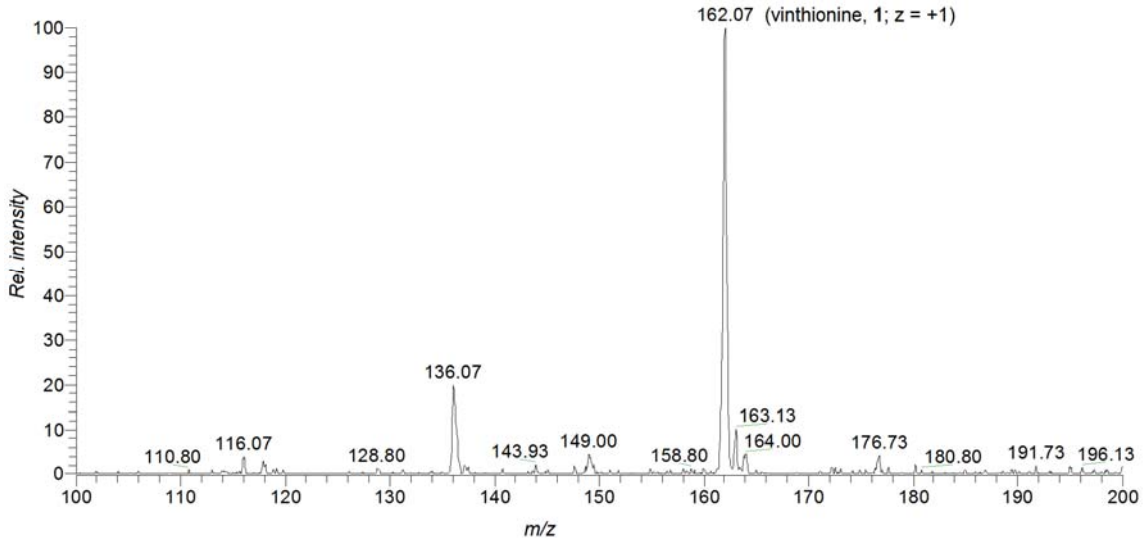
Supporting Figure 2.1.1. ^{1}H-NMR spectrum of vinthionine (400 MHz, D_2O).



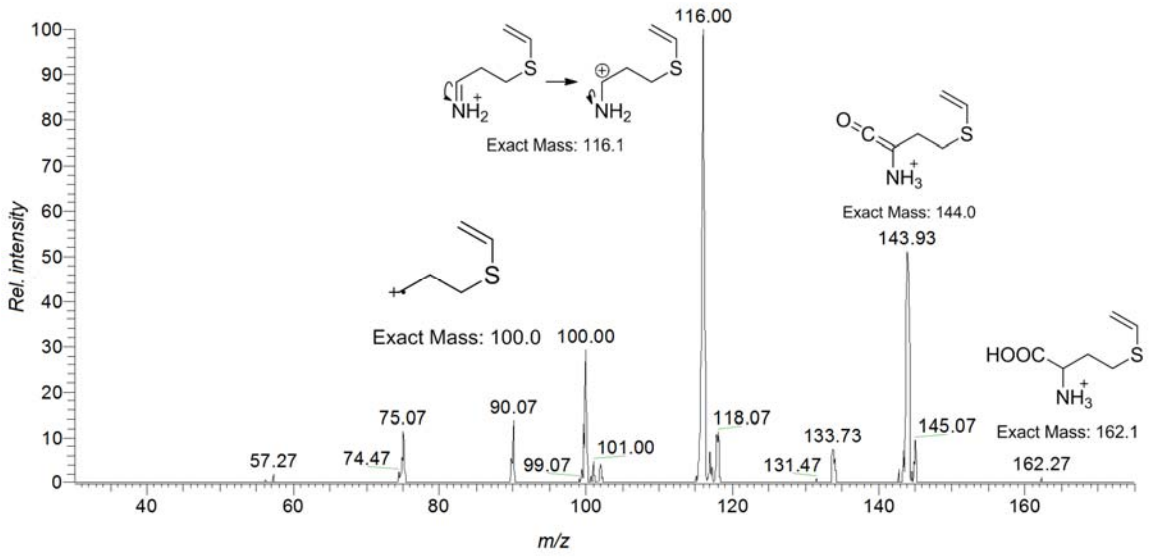
Supporting Figure 2.1.2. ^{13}C-NMRspectrum of vinthionine (100 MHz, D_2O).



Supporting Figure 2.1.3. Extracted ion chromatogram of m/z 162.0 (calculated for vinthionine).

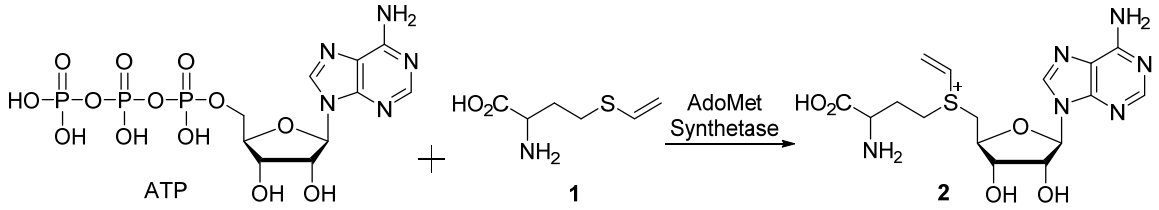


Supporting Figure 2.1.4. Mass spectrum of vinthionine (M^+; expected m/z 162.05, observed m/z 162.07; mass difference 0.02 Da). Purified vinthionine was dissolved in Milli-Q water and subjected to ESI-LC-MS.



Supporting Figure 2.1.5. CID MS/MS spectrum of precursor ion m/z 162.07 (M^+ of vinthionine, expected m/z 162.05) from LC-ESI/MS.

2.2. Synthesis of S-Adenosyl-Vinthionine (AdoVin)

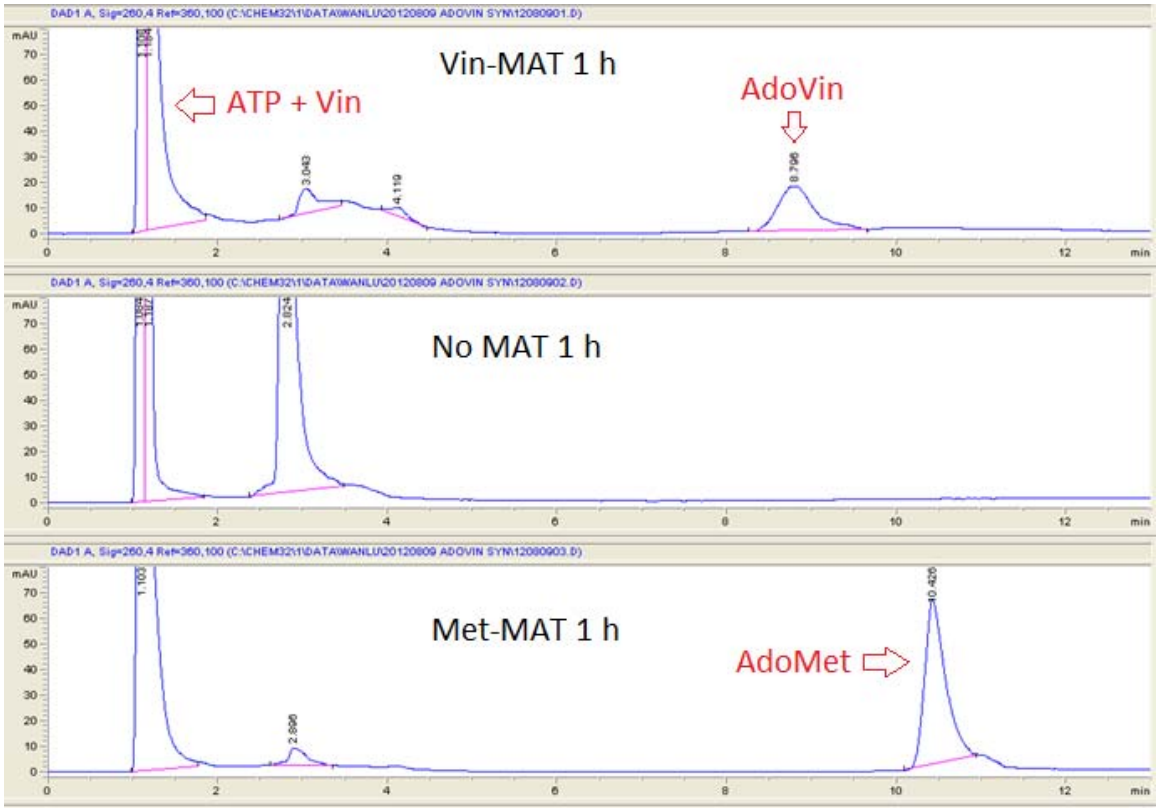


Supporting Scheme 2.2.1. Synthesis of *S*-adenosyl-vinthionine (AdoVin, **2**) catalyzed by methionine *S*-adenosyl transferase (MAT, EC 2.5.1.6).

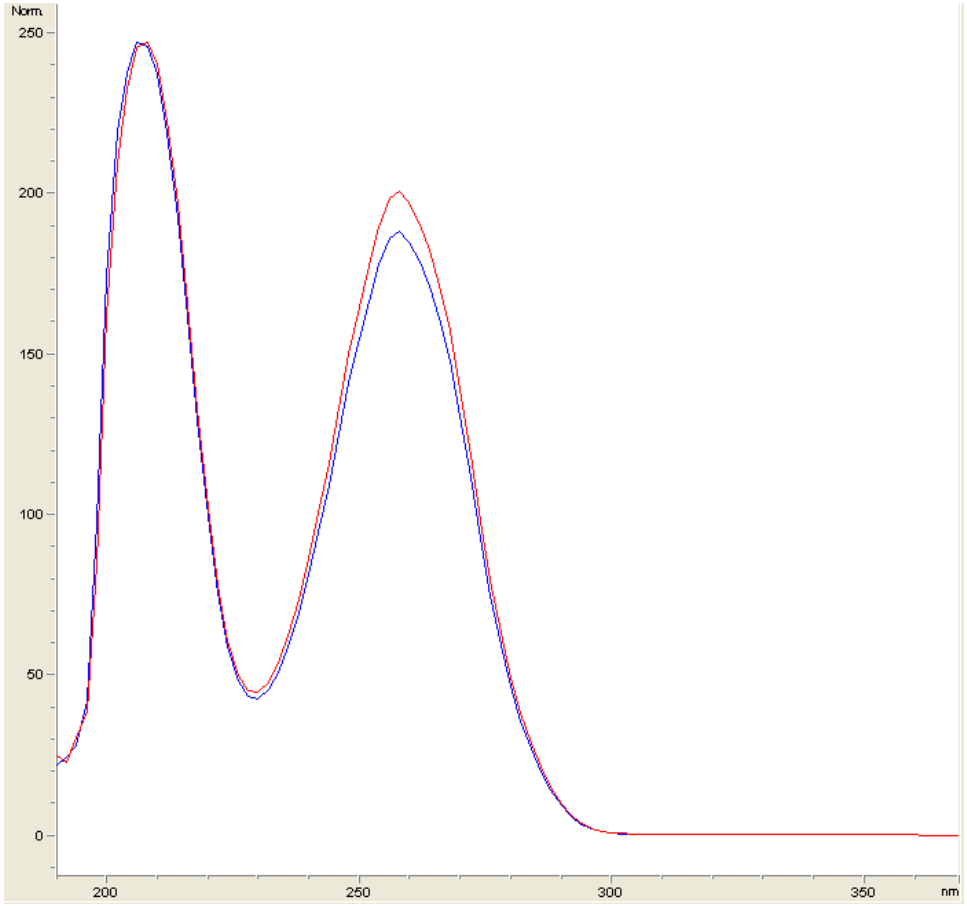
***S*-Adenosyl-Vinthionine (2).** [83768-89-2] The reaction contained 50 mM potassium phosphate (pH 8.0), 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP and 500 μM vinthionine, and was initiated with 50 μM MAT and incubated at 37 °C. The same solutions without MAT was performed as the negative control "No MAT". The same reaction containing methionine instead of vinthionine was performed as the positive control "AdoMet synthesis". About 150 μM AdoVin was synthesized after 2 hours incubation. The concentration of AdoVin was determined using $\varepsilon_{260\ nm}$ = 15,400 M^{-1} cm^{-1} based on the value for AdoMet.[6]

Separation of AdoVin using Strong Cation Exchange HPLC

Aliquots of reaction mixture (10 μL) were analyzed by HPLC and monitored by UV absorption at 260 nm. The chromatography was performed on a strong cation exchange (SCX) column (ProPac SCX-10 analytical, 4×250 mm) using 20% acetonitrile and 0.07% NH_4OH in water (mobile phase A, pH 3) and 50 mM $(NH_4)_2SO_4$, 20% acetonitrile and 0.07% NH_4OH in water (mobile phase B, pH 3) at a flow rate of 1 mL/min. The gradient program was initiated with 0% mobile phase B, followed by a linear increase to 50% mobile phase B over 20 min, then a return to 0% mobile phase B over 1 min, and finally a hold at 0% mobile phase B over 9 min.



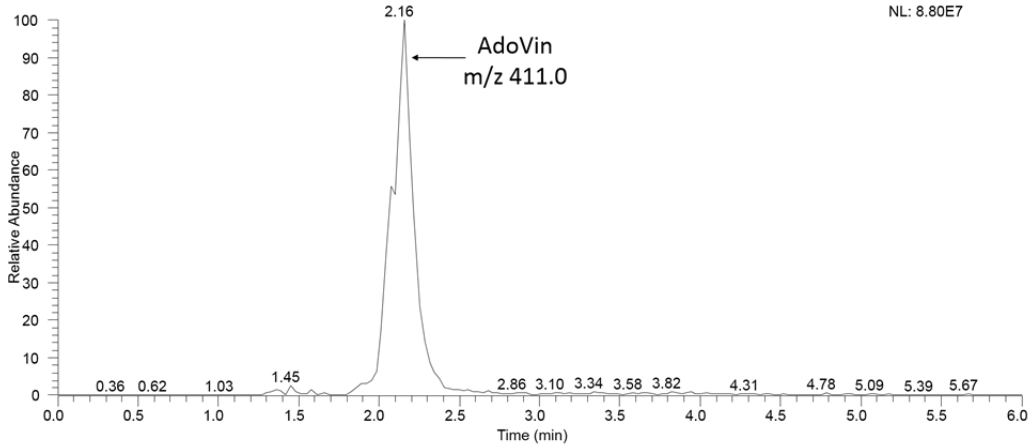
Supporting Figure 2.2.1. Strong cation exchange HPLC chromatograms (260 nm) of MAT-catalyzed AdoVin synthesis (top), reaction mixture without MAT (middle) and MAT-catalyzed AdoMet synthesis (bottom).



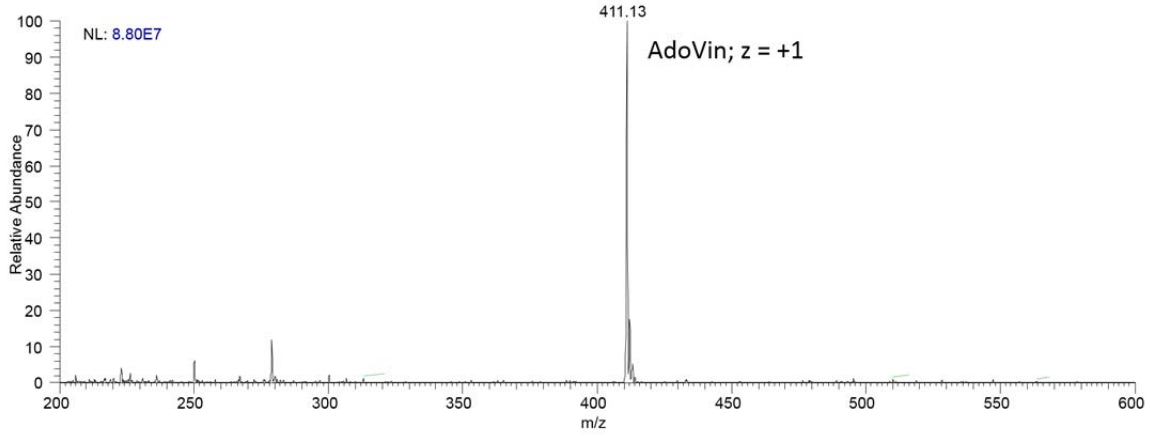
Supporting Figure 2.2.2. UV/vis absorbance spectra of AdoVin (blue) and AdoMet (red).

HPLC–UV-MS Assays of Enzymatic Synthesis of AdoVin

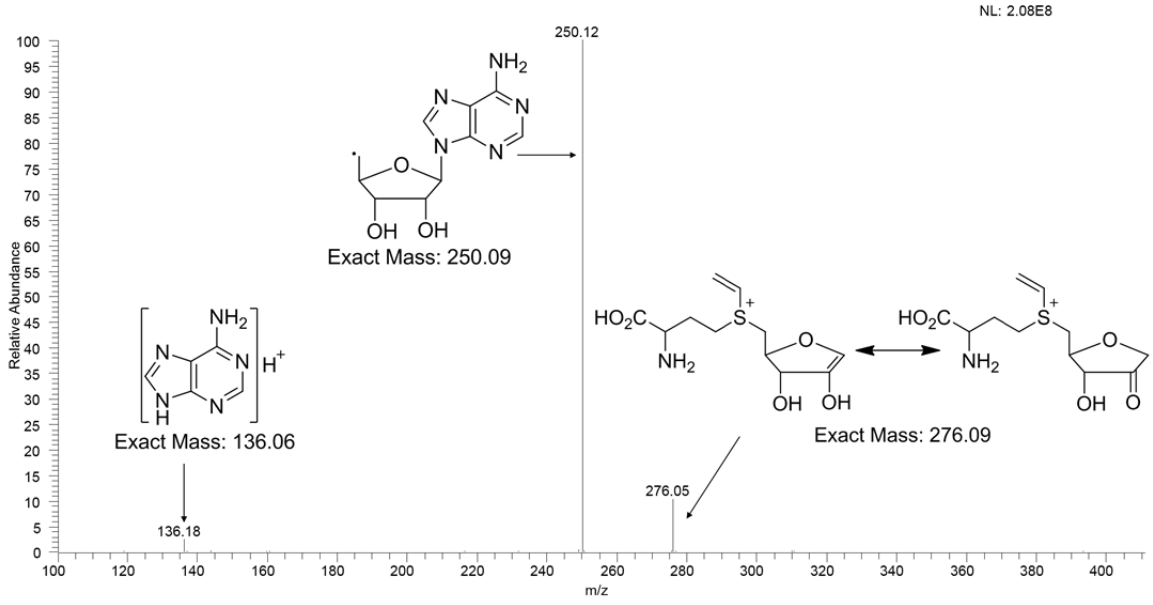
Aliquots of reaction mixture (10 μL) were analyzed by HPLC and monitored at 260 nm. The separation of AdoVin was carried out on a reverse-phase column (Apollo, C_{18}, 5μ, 4.6 mm x 150 mm), using 0.1% aqueous formic acid (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 1 mL/min. The gradient program was initiated with 2% mobile phase B, followed by a linear increase to 10% mobile phase B over 8 min, then a return to 2% mobile phase B over 1 min, and finally a hold at 2% mobile phase B over 5 min.



Supporting Figure 2.2.3. Extracted ion chromatogram of m/z 411.0 for AdoVin (calculated m/z 411.1).

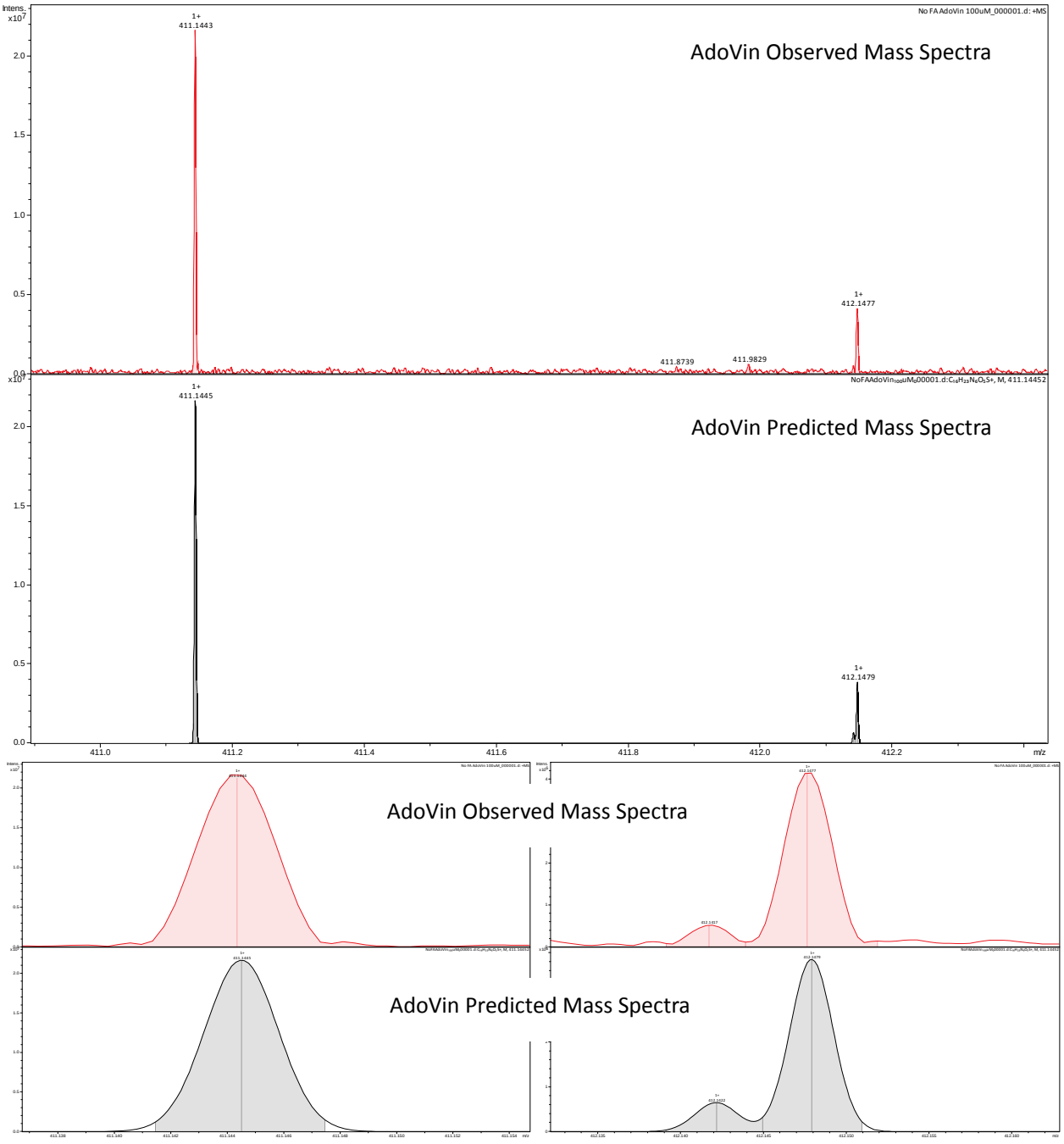


Supporting Figure 2.2.4. Mass spectrum of AdoVin (M^+; expected m/z 411.10, observed m/z 411.13; mass difference 0.03 Da).

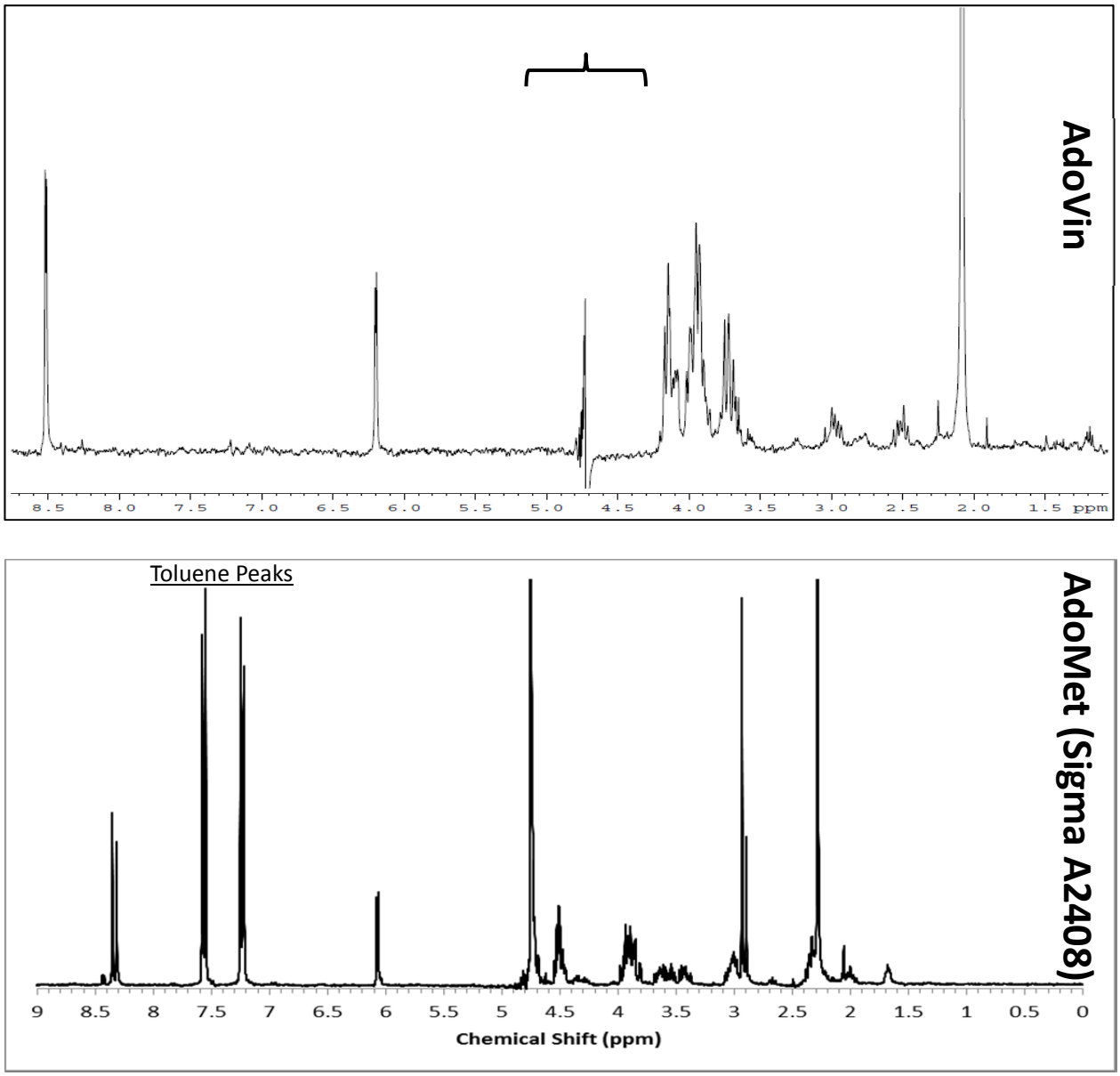


Supporting Figure 2.2.5. CID MS/MS spectrum of precursor ion m/z 411.07 (M^+ of AdoVin, expected m/z 411.10).

AdoVin was prepared enzymatically as previously described. Proteins were precipitated by adding in 10% trichloro acetic acid and pelleting the proteins by centrifugation. High-resolution mass spectrometry was performed on the protein depleted AdoVin reaction mixture using a Bruker Solarix XR ESI-FT-ICR. A 1:1 water/acetonitrile solution contain 100 μM AdoVin was electrosprayed at a flow rate of 2 μL/min. Acquisition of the spectrum was performed using ftms Control in the Compass 1.5 software and the expected mass spectra was simulated using DataAnlysis (v4.2).



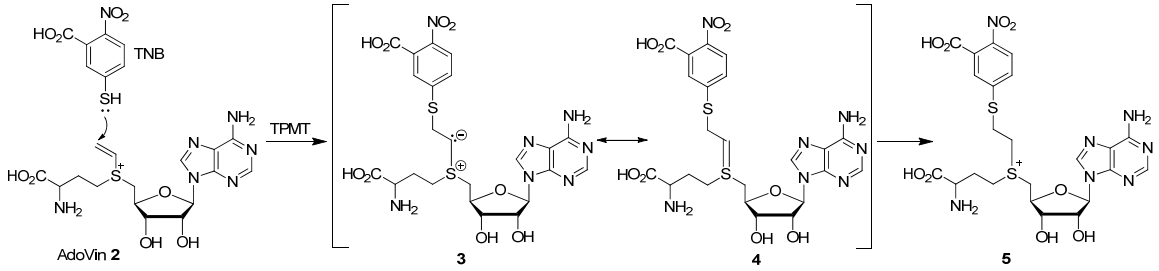
Supporting Figure 2.2.6. (Top) High-resolution mass spectrum of AdoVin; monoisotopic m/z 411.1444 (M+ of AdoVin expected m/z 411.1445; observed error -0.24 ppm). Spectrum is an average of 24 scans. (Middle) Predicted mass spectrum of AdoVin. (Bottom, left) Zoom-in of monoisotopic peak. (Bottom, right) Zoom-in of the +1 Da peak that shows fine isotopic structure.



Supporting Figure 2.2.7. (Top) ^{1}H-NMR spectrum of AdoVin in H_2O/D_2O. The spectra was collected on a Bruker Ultrashield 400 WB Plus and analyzed with Bruker Topspin (v2.1). Water suppression was achieved through excitation sculpting.[7] The bracket shows the region of signal suppression. (Bottom) ^{1}H-NMR spectrum of AdoMet-toluenesulfonate salt (Sigma A2408). The spectra agrees with published data.[8,9] The peaks due to the toluenesulfonate are marked.

3. *in vitro* Formation and Characterization of AdoVin Adducts

3.1. Formation and Characterization of AdoVin-TNB Adduct



Supporting Scheme 3.1.1. Formation of bisubstrate-adduct between AdoVin and TNB catalyzed by TPMT. Either the ylide (**3**, or its tautomer form, **4**) or the sulfonium ion after protonation (**5**) may be the form bound to the enzyme.

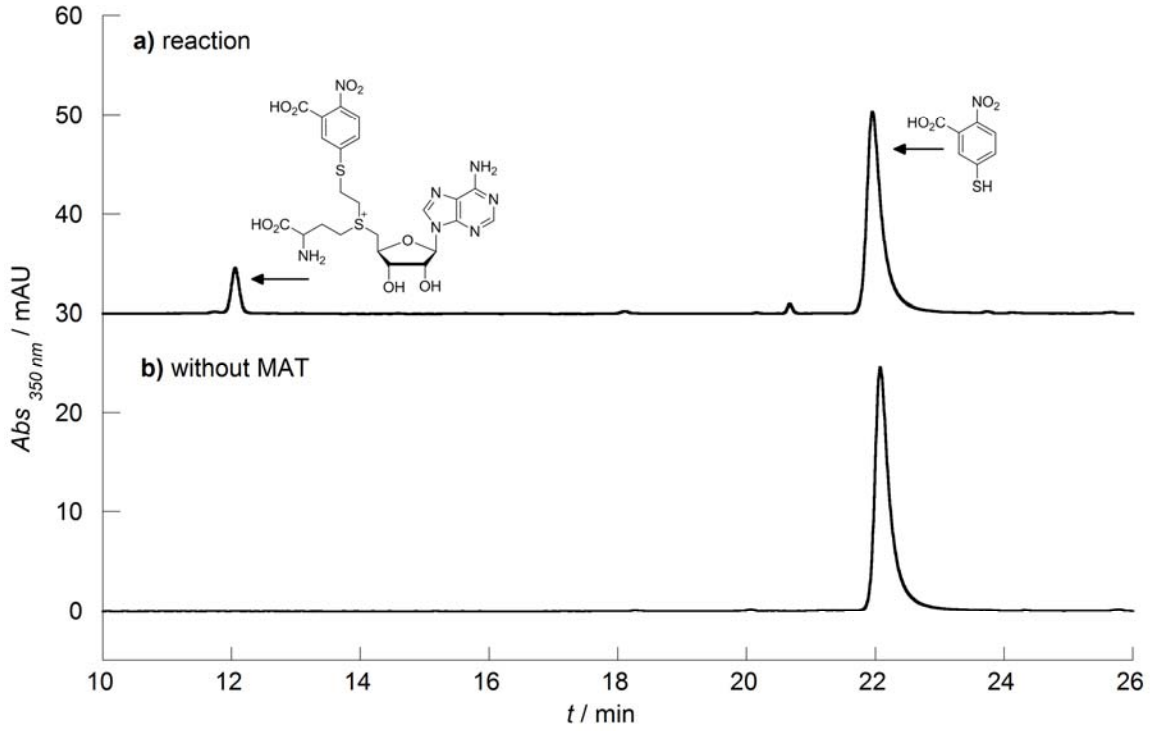
AdoVin-TNB Adduct (5). The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP, 600 μM vinthionine, 480 μM 2-nitro-5-mercaptobenzoic acid (TNB), 2 mM TCEP, 83 μM TPMT and 1.75 μM MTAN. The reaction was initiated with 120 μM MAT and incubated at 37 °C. The same solutions without MAT or TPMT were performed as negative controls. The concentration of TNB was determined using ε 411 nm = 13,600 M^{-1} cm^{-1}.[5]

Isotope Labeling of the AdoVin-TNB Adduct

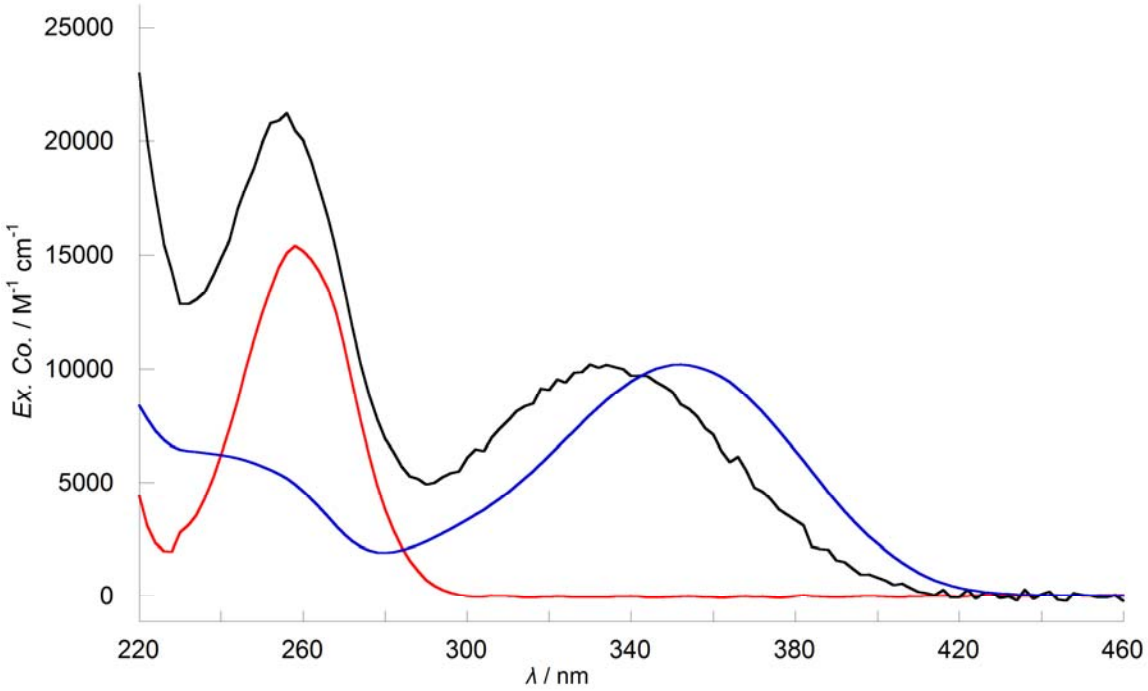
The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM adenosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate sodium salt solution (isotope labeled ATP, Sigma-Aldrich, catalog No. 645702), 600 μM vinthionine, 489 μM TNB, 2 mM TCEP, 1.75 μM MTAN, and 70 μM TPMT. The reaction was initiated with 120 μM MAT and incubated at 37 °C.

HPLC-UV-MS Assays of Formation of Bisubstrate-Adduct between AdoVin and TNB

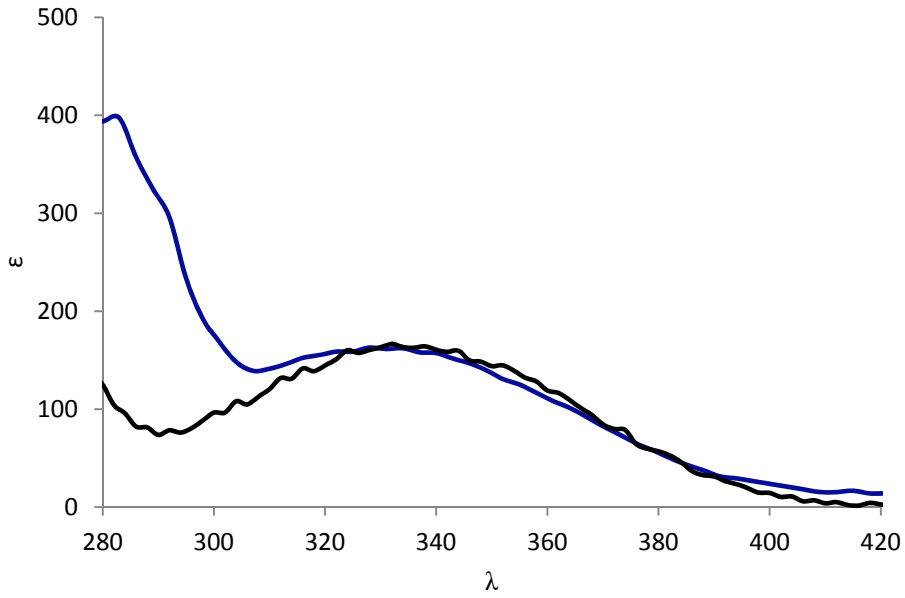
Reaction and negative control (10 μL) were analyzed by HPLC-MS using a reversed-phase column (Apollo, C_{18}, 5μ, 4.6 mm x 150 mm) with 0.1% aqueous formic acid (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at a flow rate of 1 mL/min. The gradient was initiated with 2% mobile phase B, and increased to 10% mobile phase B over 8 min, then increased to 30% mobile phase B over 12 min, then increased to 70% mobile phase B over 15 min, followed by an increase to 90 % mobile phase B over 5 min, then a return to 2% mobile phase B over 1 min, and finally a hold at 2% mobile phase B over 5 min.



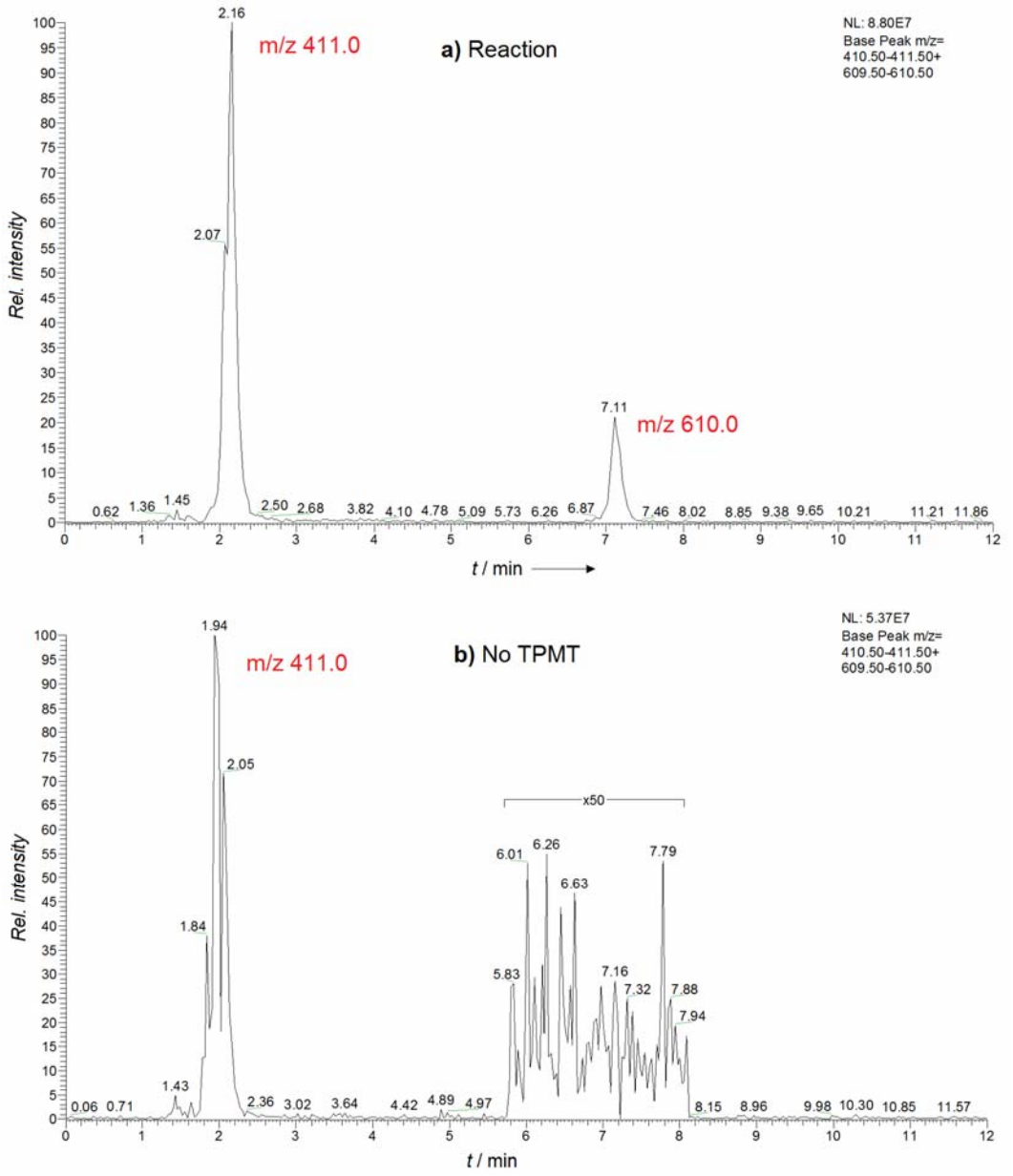
Supporting Figure 3.1.1. HPLC chromatograms (350 nm) of AdoVin-TNB reaction (a) and reaction without MAT (i.e., no AdoVin) (b).



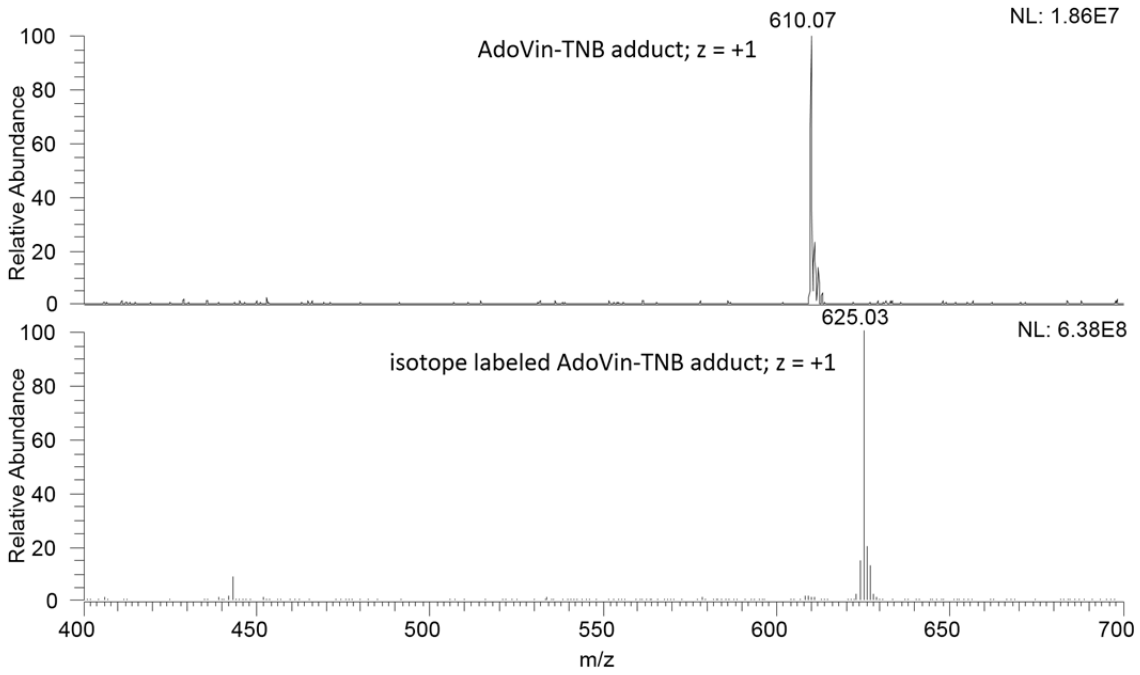
Supporting Figure 3.1.2. UV/vis absorbance spectra of adenosine (red), methylated TNB (blue) and AdoVin-TNB adduct (black). The spectra show that the adduct contains both the chromophores from adenosyl group and alkyl-TNB. The extinction coefficient of the adduct at 350 nm was based on that for methyl-TNB as previously described.[5]



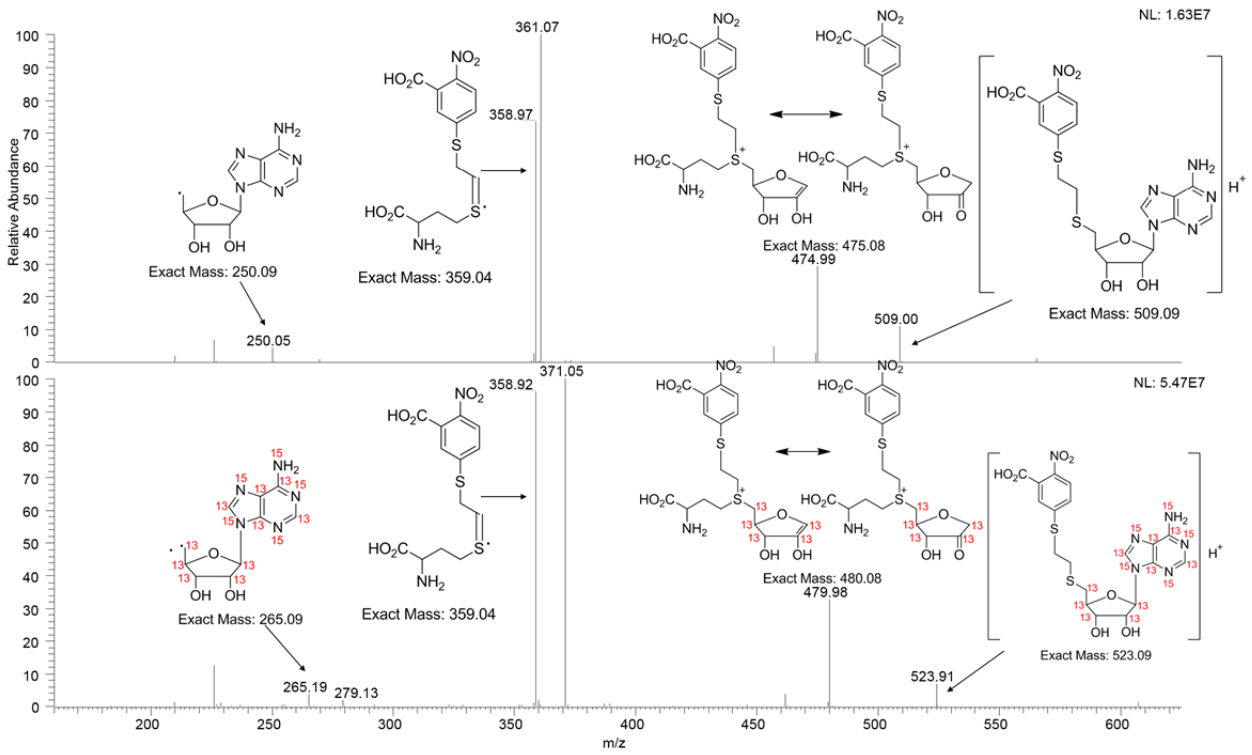
Supporting Figure 3.1.3. UV/vis absorbance spectra AdoVin-TNB adduct (black) and AdoVin-TNB adduct bound to TPMT (blue). Differences in the spectra are due to TPMT absorbance.



Supporting Figure 3.1.4. Extracted ion chromatograms of m/z 411.0 for AdoVin, and 610.0 for the AdoVin-TNB adduct. (a) Reaction of AdoVin and TNB in the presence of TPMT; (b) negative control: AdoVin and TNB but without TPMT. The region around 7.1 min (where the adduct eluted) was enlarged 50 times, showing that no adduct was observed under these conditions.



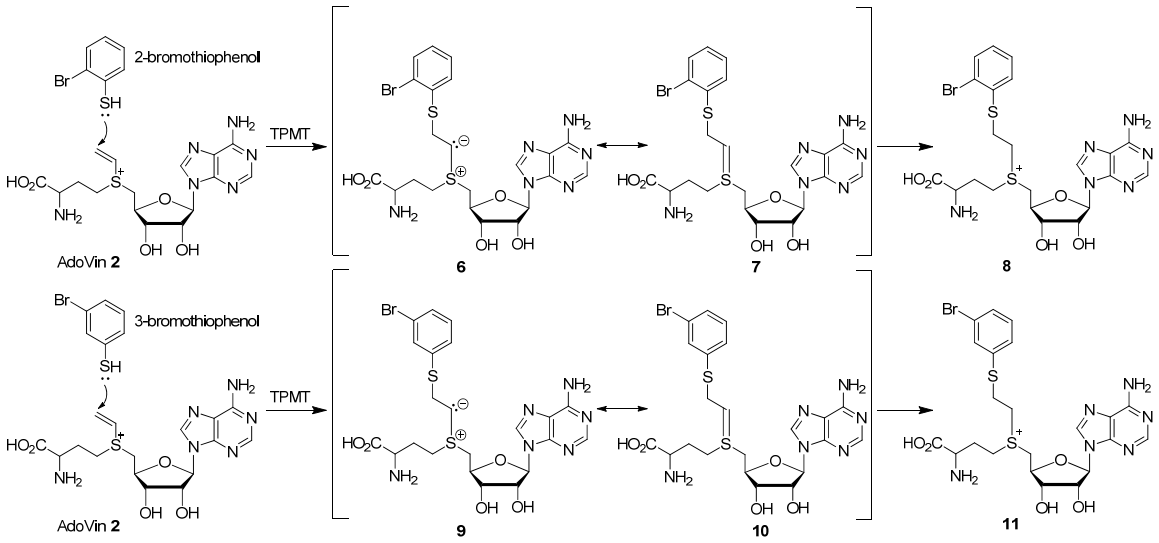
Supporting Figure 3.1.5. Mass spectra of TPMT-catalyzed AdoVin-TNB adduct and its isotope labeled form (M^+ of AdoVin-TNB adduct and its isotope labeled form: calculated m/z 610.14 and 625.14; observed m/z 610.07 and 625.03; mass difference 0.13 Da and 0.11 Da, respectively).



Supporting Figure 3.1.6. CID MS/MS spectra of precursor ion m/z 610.07 (M^+ of AdoVin-TNB adduct, expected m/z 610.14), and 625.03 (M^+ of isotope labeled AdoVin-TNB adduct, expected m/z 625.14).

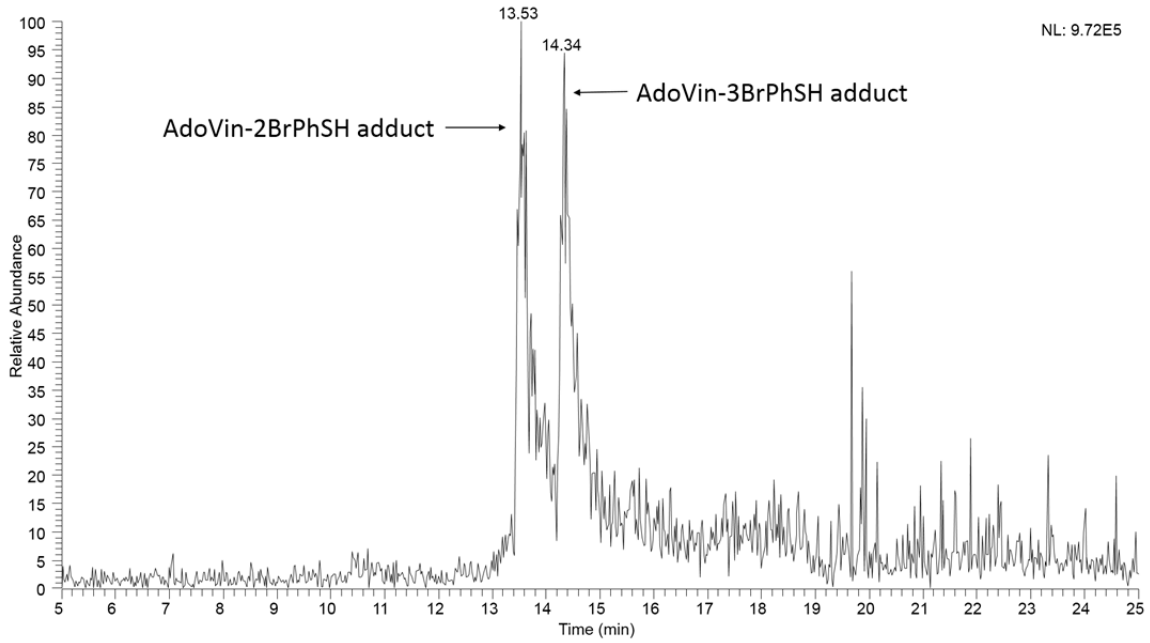
3.2. Substrate Specificity Towards AdoVin

2-Bromothiophenol and 3-Bromothiophenol

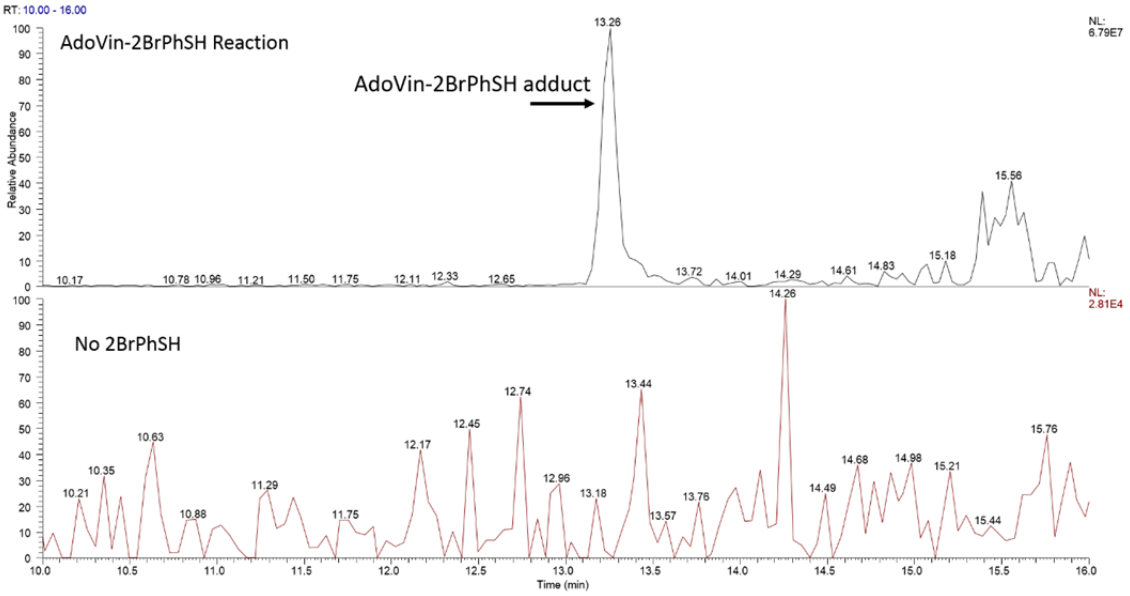


Supporting Scheme 3.2.1. Formation of bisubstrate-adduct between AdoVin and 2-bromothiophenol (top) or 3-bromothiophenol (bottom) catalyzed by TPMT. Either the ylides (**6** and **9**, or their tautomer form, **7** and **10**) or the sulfonium ions after protonation (**8** and **11**) may be the form bound to the enzyme.

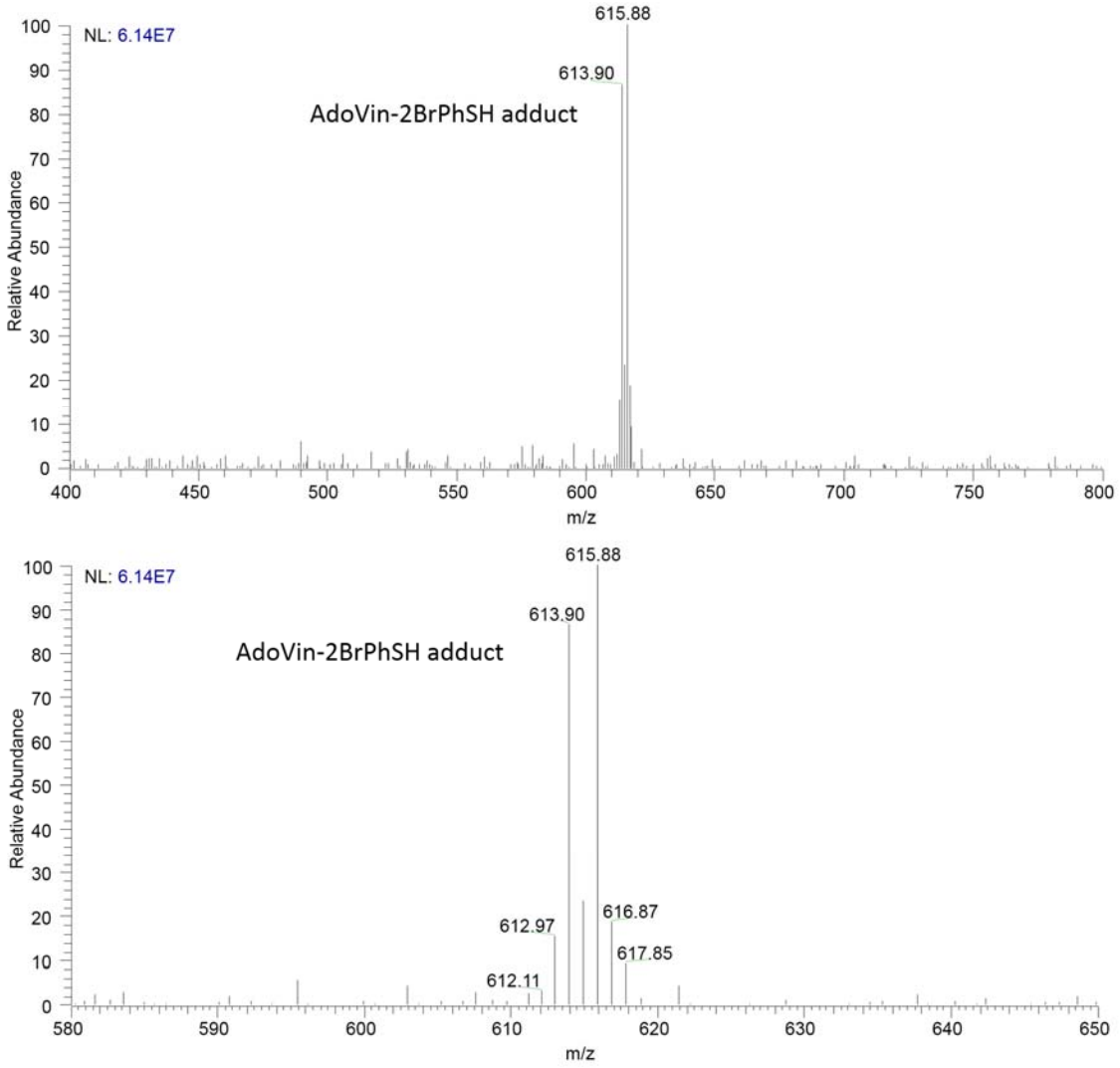
AdoVin-2-Bromothiophenol Adduct (8) and AdoVin-3-Bromothiophenol (11) Adduct. The reaction contained 200 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 1 mM vinthionine, 410 μM 2-bromothiophenol (2BrPhSH) or 3-bromothiophenol (3BrPhSH), 2 mM TCEP, 70 μM TPMT, 3.5 μM MTAN and the reaction was initiated with 100 μM MAT and incubated at 37 °C.



Supporting Figure 3.2.1. Selected-ion monitoring chromatogram of m/z 599.0 for the AdoVin-2-bromothiophenol (2BrPhSH) adduct (13.5 min) and AdoVin-3-bromothiophenol (3BrPhSH) adducts (14.3 min).



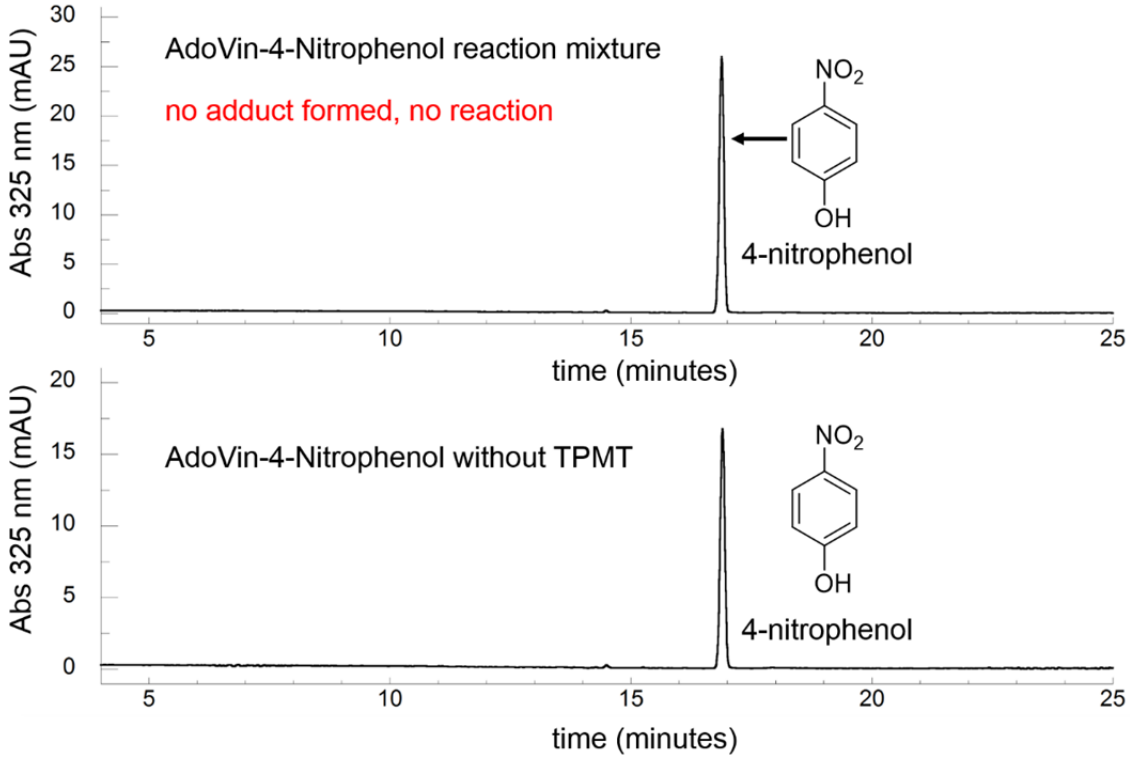
Supporting Figure 3.2.2. Extracted ion chromatograms of m/z 614.1 and 616.1 for heavy isotope labeled AdoVin-2BrPhSH adduct (calculated m/z 614.07 and 616.07) in the complete reaction (top) and negative control without 2BrPhSH (bottom).



Supporting Figure 3.2.3. Mass spectrum (top) and zoom-in mass spectrum (bottom) of isotope labeled AdoVin-2BrPhSH (M^+; expected m/z 614.07 observed m/z 613.97, mass difference 0.1 Da; expected m/z 616.07 observed m/z 615.88, mass difference 0.19 Da).

No Formation of the AdoVin-4-Nitrophenol Adduct.

The reaction contained 50 mM potassium phosphate, pH 8.0, 10 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 900 µM vinthionine, 1.0 mM 4-nitrophenol, 2 mM TCEP, 1.75 µM MTAN, and 74 µM TPMT. The same reactions without TPMT were conducted as negative control. The reaction was initiated with 182 µM MAT and incubated at 37 °C.



Supporting Figure 3.2.4. HPLC chromatograms (325 nm) of the mixture containing AdoVin and 4-nitrophenol in the presence (top) and absence (bottom) of TPMT for 3 hours. Neither depletion of 4-nitrophenol nor formation of any new species was observed, indicating that no reaction occurred between AdoVin and 4-nitrophenol (non-substrate of TPMT).

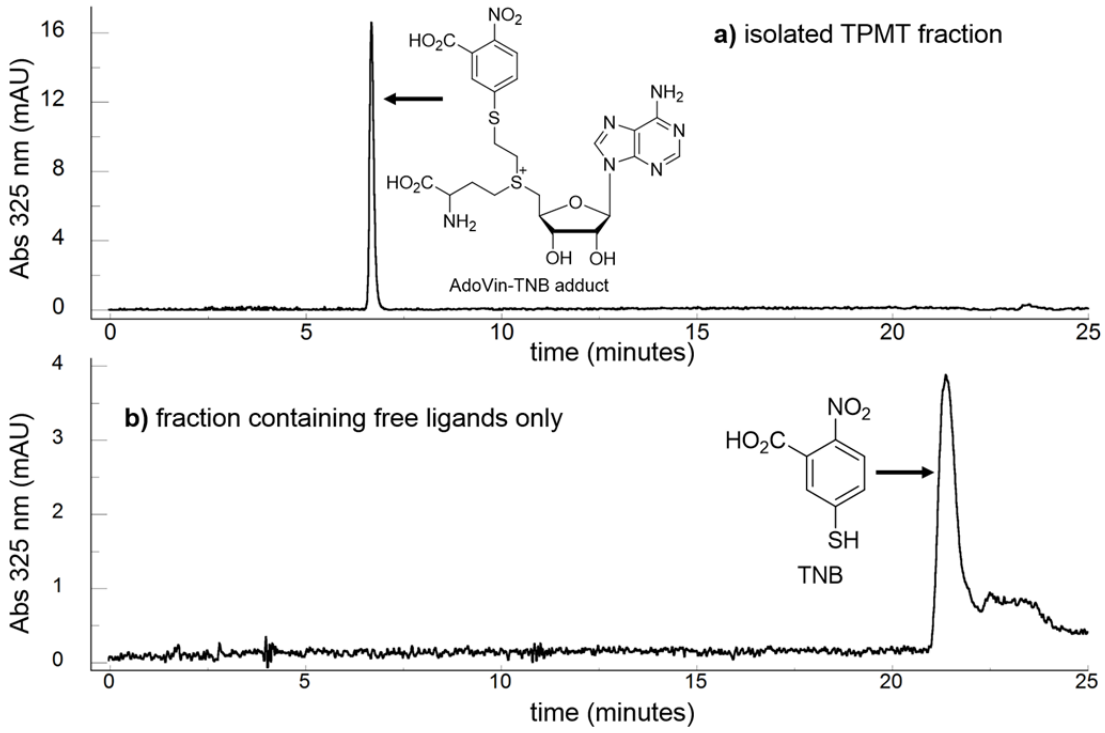
3.3. Tight Binding of Bisubstrate Adduct with Methyltransferase

The formation of adduct and HPLC-UV-MS analysis were performed as described above. Two binding assays were performed as detailed next.

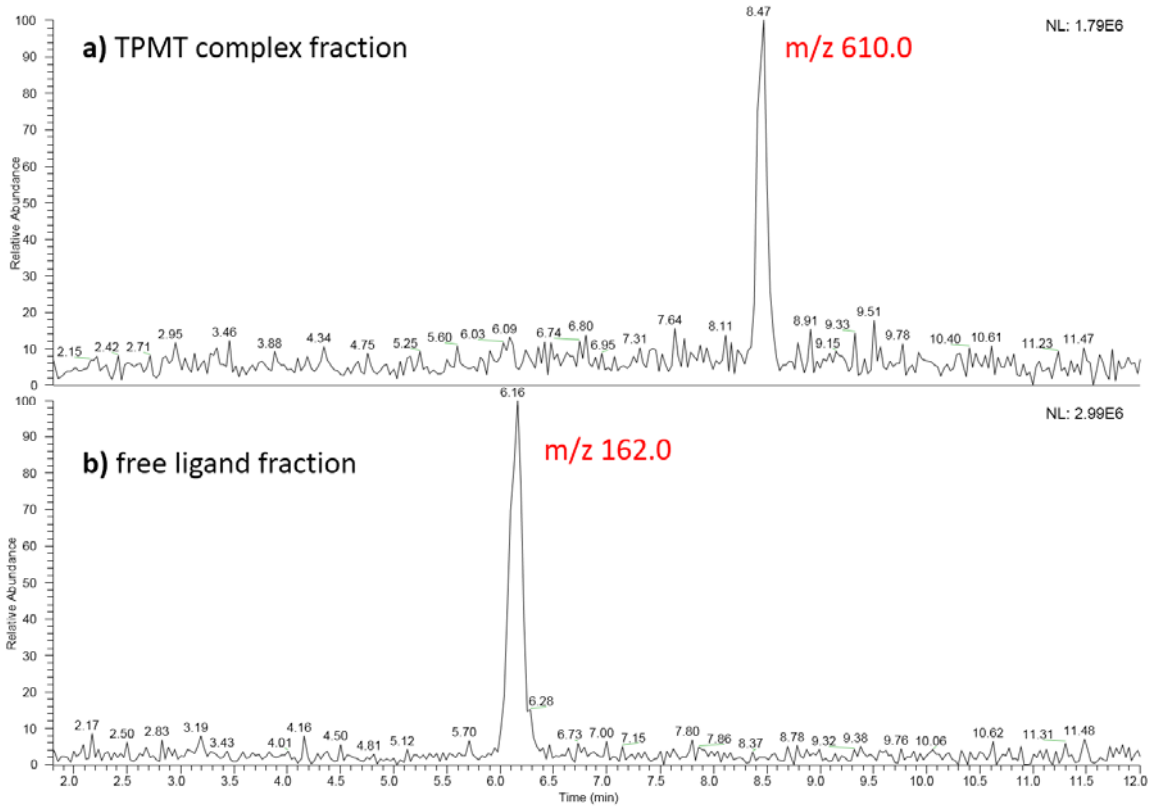
In the first assay, histidine-tagged TPMT was separated from other components via immobilized metal ion affinity chromatography (IMAC) at 4 °C using the HisPur Ni-NTA spin column (0.2 mL resin bed, ThermoScientific Pierce, catalog No. 12393730). Storage buffer for the resins was removed by centrifuging the column at 700 g for 2 min, and the column was equilibrated with 400 μL equilibration buffer (20 mM potassium phosphate, 300 mM NaCl and 10 mM imidazole, pH 8.0). The reactions were mixed with an equal volume of equilibration buffer and then loaded onto the columns. Each column was then turned on an end-over-end mixer for 30 min at 4 °C. Then 3 x 400 μL washing buffer (50 mM potassium phosphate, 300 mM NaCl, and 25 mM imidazole, pH 8.0) was used to wash the resin. TPMT and TPMT-adduct complex was eluted with 3 x 200 μL elution buffer (50 mM potassium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0). The elutes were analyzed separately.

The second binding assay was based on ultrafiltration using filters with 30,000 MWCO (0.5 mL, EMD Millipore Amicon Ultra 0.5 mL, catalog No. UFC50VL96). An aliquot of the reaction (100 μL) was loaded to the filter, washed with ammonium bicarbonate (400 μL, 50 mM, pH 8.0) at 12,000 g for 5 min, and the process continued until the UV absorbance of the filtrate was less than 0.01 at 260 nm. The final retentate and all filtrates were analyzed.

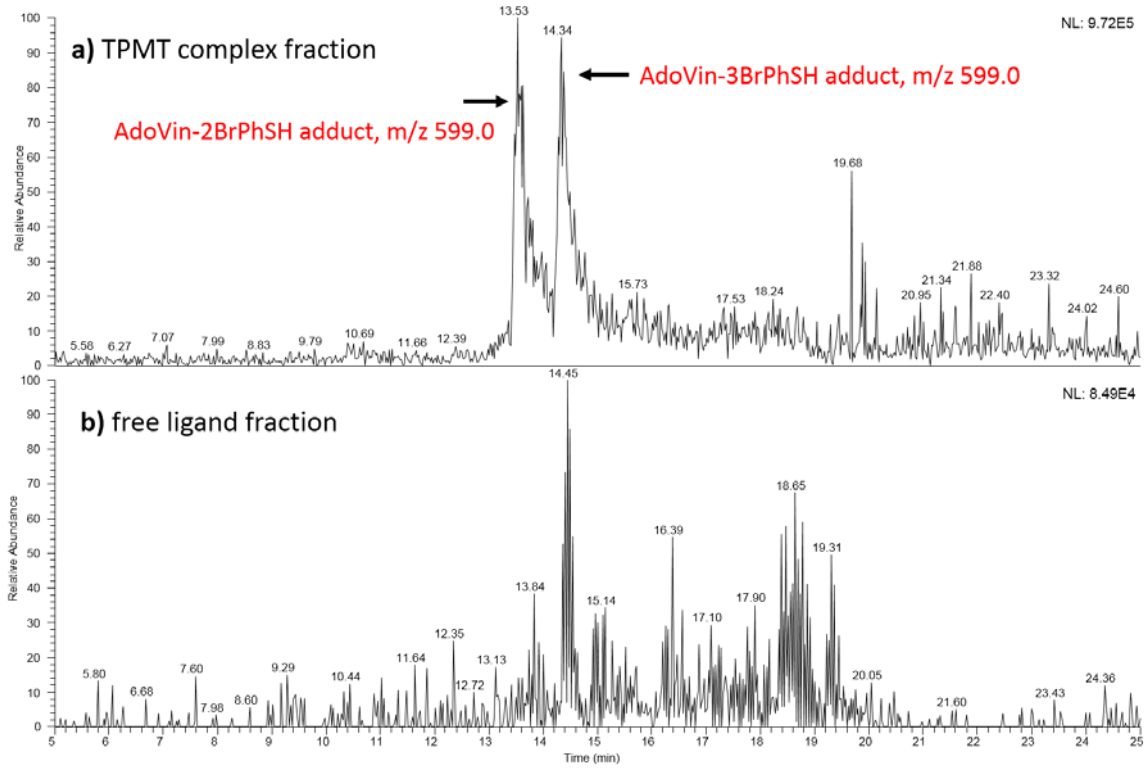
For both assays, the TPMT concentrations were determined by UV absorbance at 280 nm; and the proteins were precipitated by TCA before samples were injected for HPLC as described above.



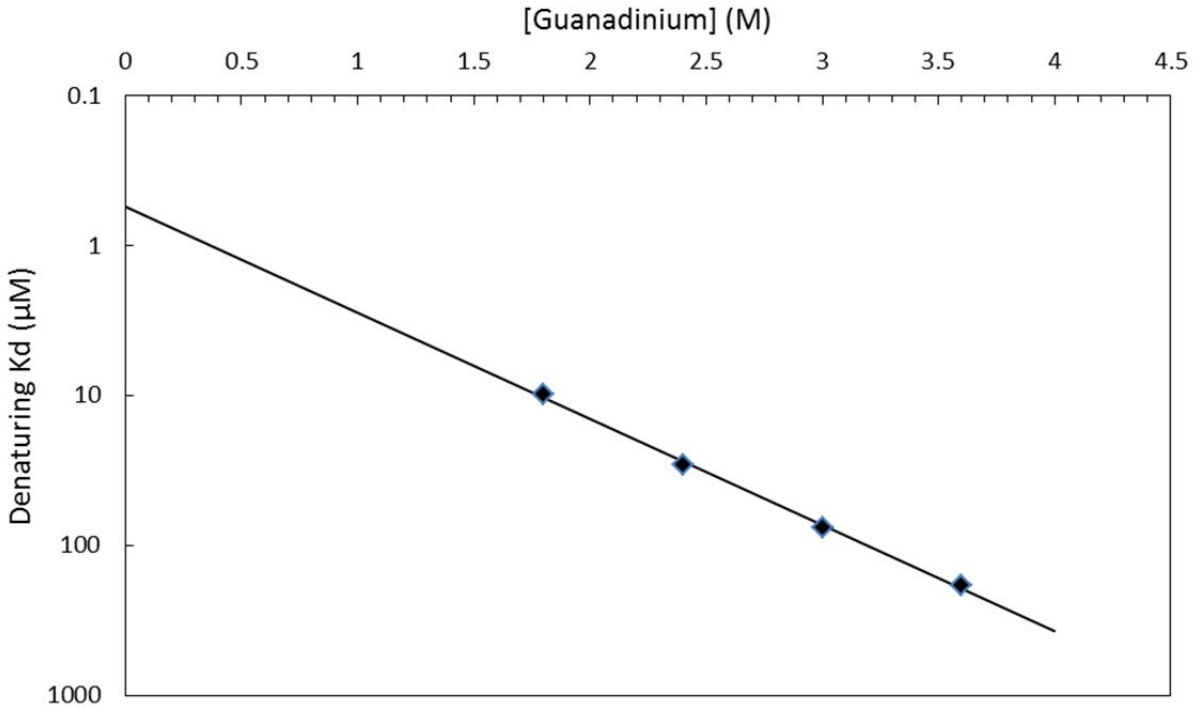
Supporting Figure 3.3.1. HPLC chromatograms (325 nm) of the TPMT complex and free ligand fraction from binding assay via immobilized metal ion affinity chromatography (IMAC). AdoVin-TNB adducts were observed in TPMT complex, but not in free ligand fraction, indicating that AdoVin-TNB adduct was tightly binding with TPMT, but not TNB.



Supporting Figure 3.3.2. Extracted ion chromatograms of m/z 162.0 for vinthionine and m/z 610.0 for the AdoVin-TNB adduct. (a) The TPMT complex was isolated by ultrafiltration using 30,000 MWCO filters. (b) The free ligands fraction was from the first filtrate.

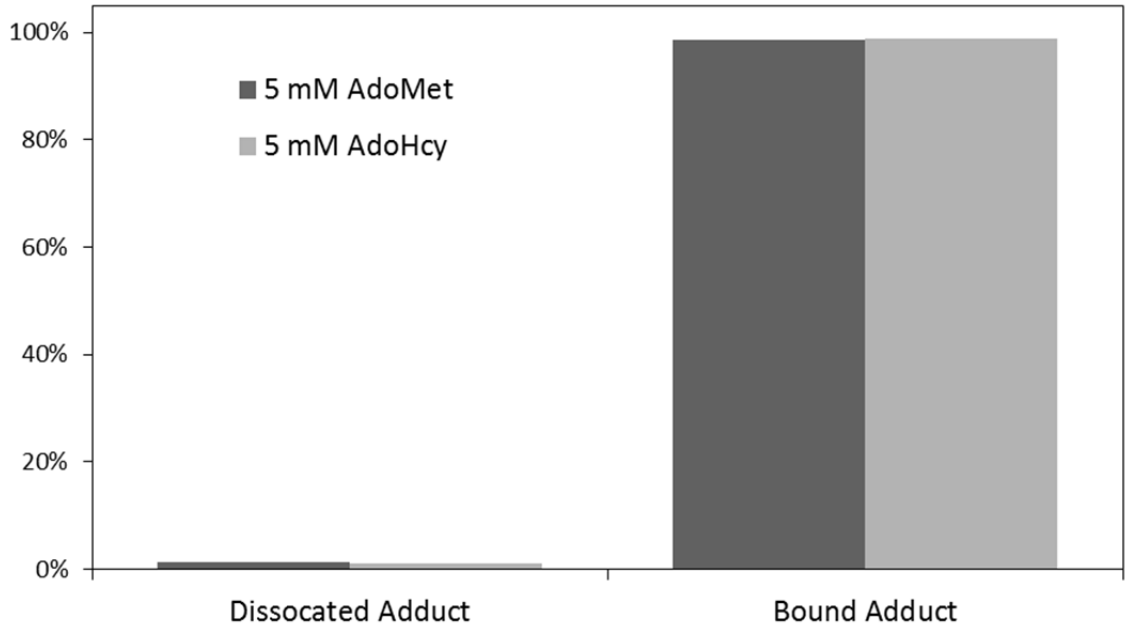


Supporting Figure 3.3.3. Selected-ion monitoring chromatograms of m/z 599.0 for the AdoVin-BrPhSH adducts of AdoVin adduct formation with 2-bromothiophennol or 3-bromothiophenol. (a) The TPMT complex was isolated by ultrafiltration using 30,000 MWCO filters. (b) The free ligands fraction was from the first filtrate.



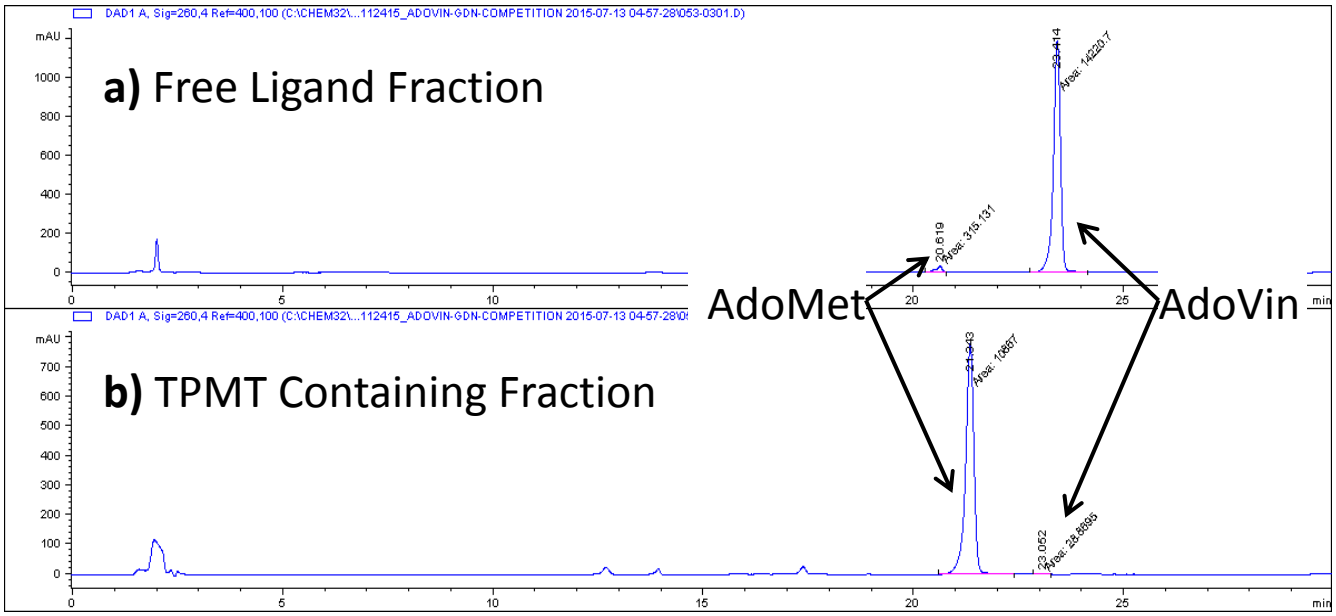
Supporting Figure 3.3.4. TPMT-AdoVin-TNB Adduct complex was prepared as described above. The complex was then exposed to increasing concentrations of guanidine hydrochloride. Small molecules were then separated from the protein fraction by using centrifugal ultrafiltration with a 10 kDa MWCO. The percent dissociation was monitored by strong cation exchange (SCX) HPLC. The extrapolated *Kd* for the TPMT-Adduct complex was calculated to be 0.55 µM. The actual *Kd* is expected to be considerably smaller than this.

As seen in Supporting Figure 3.3.5, based on AdoMet and AdoHcy competition, the *Kd* can be estimated in the nanomolar range.



Supporting Figure 3.3.5. TPMT-AdoVin-TNB-Adduct complex was prepared as described above. The complex was then incubated with a 370-fold molar excess of AdoHcy or AdoMet. The small molecules were then separated from the protein fraction by using centrifugal ultrafiltration with a 10 kDa MWCO. The protein bound fraction was denatured using trichloroacetic acid. The resulting small molecule fractions were analyzed by SCX HPLC. Even with high concentrations of competing ligands, more than 99% of the adduct remains bound to TPMT.

Based on *Kd* values for AdoHcy (0.75 μM), the *Kd* for the AdoVin-TNB adduct can be estimated at <1 nM.[6]



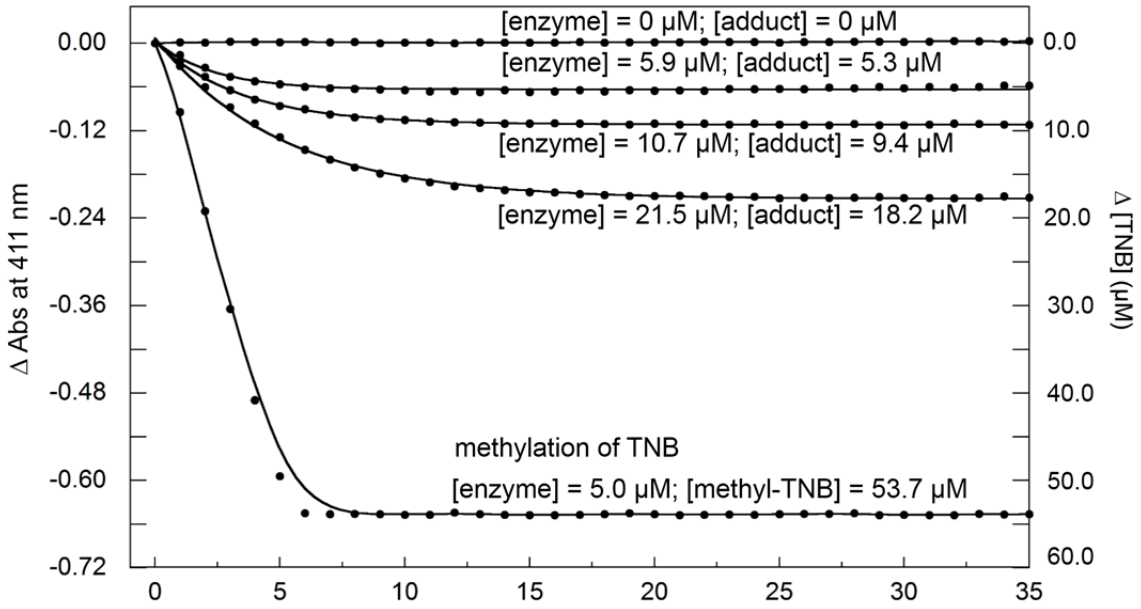
Supporting Figure 3.3.6. TPMT was incubated with AdoMet and AdoVin. Free ligands were separated from the mixture by ultrafiltration with a 10 kDa MWCO. Protein was removed from the remaining fraction by denaturation with trichloroacetic acid; the resulting small molecule fraction (b) along with the free ligand fraction (a) were analyzed by SCX HPLC. AdoMet binds TPMT much more strongly than AdoVin and is enriched in the protein fraction by a factor of 300.

3.4 First-Order Kinetics and Single-Turnover of Adduct Formation

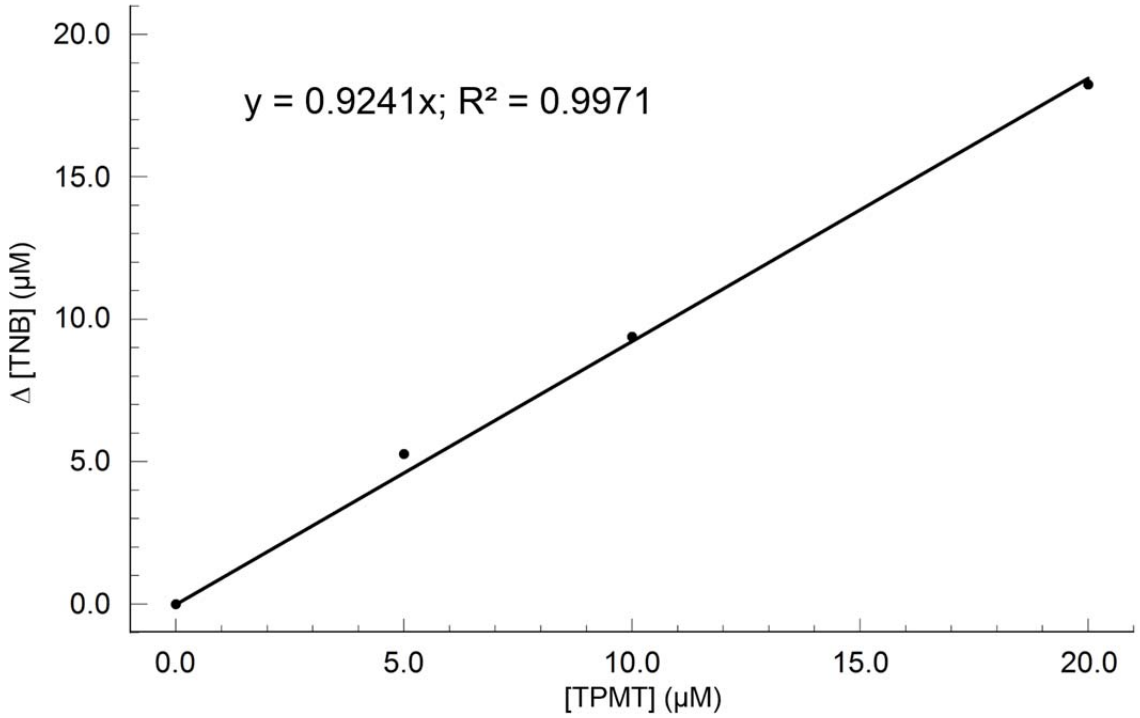
The reaction contained 200 mM Tris buffer, pH 8.0, 50 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 1 mM vinthionine, 56.8 μM TNB, 2 mM TCEP, 3.5 μM MTAN, 100 μM MAT and variable TPMT concentrations (0, 5.9, 10.7 and 21.5 μM), with expected saturation of the enzyme. Reactions were initiated with TPMT at various concentrations and incubated at 37 °C. The spectral changes were monitored continuously (1-minute interval) between 260-610 nm for 240 min on a Cary 50 Bio UV/VIS spectrophotometer.

Kaleidagraph software package 4.1 (Synergy Software, Reading, PA) was used to analyze the absorbance change at 411 nm. For data processing, changes in the concentration of TNB was fitted to the first order equation: y = m1 + m2*e^(-m3x), while "m3" is the "first-order rate constant" (*k)* for the formation of the AdoVin-TNB adduct catalyzed by TPMT. As shown in Figure 3 in the main text, pseudo-first-order kinetics were observed; and the first-order rate constant k_{app} was 0.33 ± 0.12 min^{-1} and the half-life was 2.4 ± 0.8 min. These parameters are comparable with these for the transmethylation reactions with AdoMet (k_{cat} was 13.6 ± 0.4 min^{-1}).[10]

Furthermore, Supporting Figure 3.2.3 demonstrated stoichiometric correlation between the concentrations of the TPMT enzyme and the changes in the concentrations of the TNB substrate, indicating that the adduct binds much tightly than each substrates and thus inhibits the enzyme from further turnover. It is worth noting that multiple turnovers (over 10) were observed by transmethylation between TNB and AdoMet under the same conditions (Supporting Figure 3.2.2).



Supporting Figure 3.4.1. Changes of absorbance at 411 nm for the methylation of TNB catalyzed by 5.0 μM TPMT (bottom), and the formation of adduct between AdoVin and TNB, catalyzed by 0, 5.9, 10.7, and 21.5 μM TPMT.



Supporting Figure 3.4.2. Stoichiometric correlation between the concentrations of the TPMT enzyme and the changes in the concentrations of TNB, indicating that the adduct binds much tightly than each substrate and thus inhibits the enzyme from further turnover.

4. Discovery and Elucidation of an Unknown Adduct from *ex vivo* Reactions

4.1. Discovery and Characterization of the Unknown Adduct

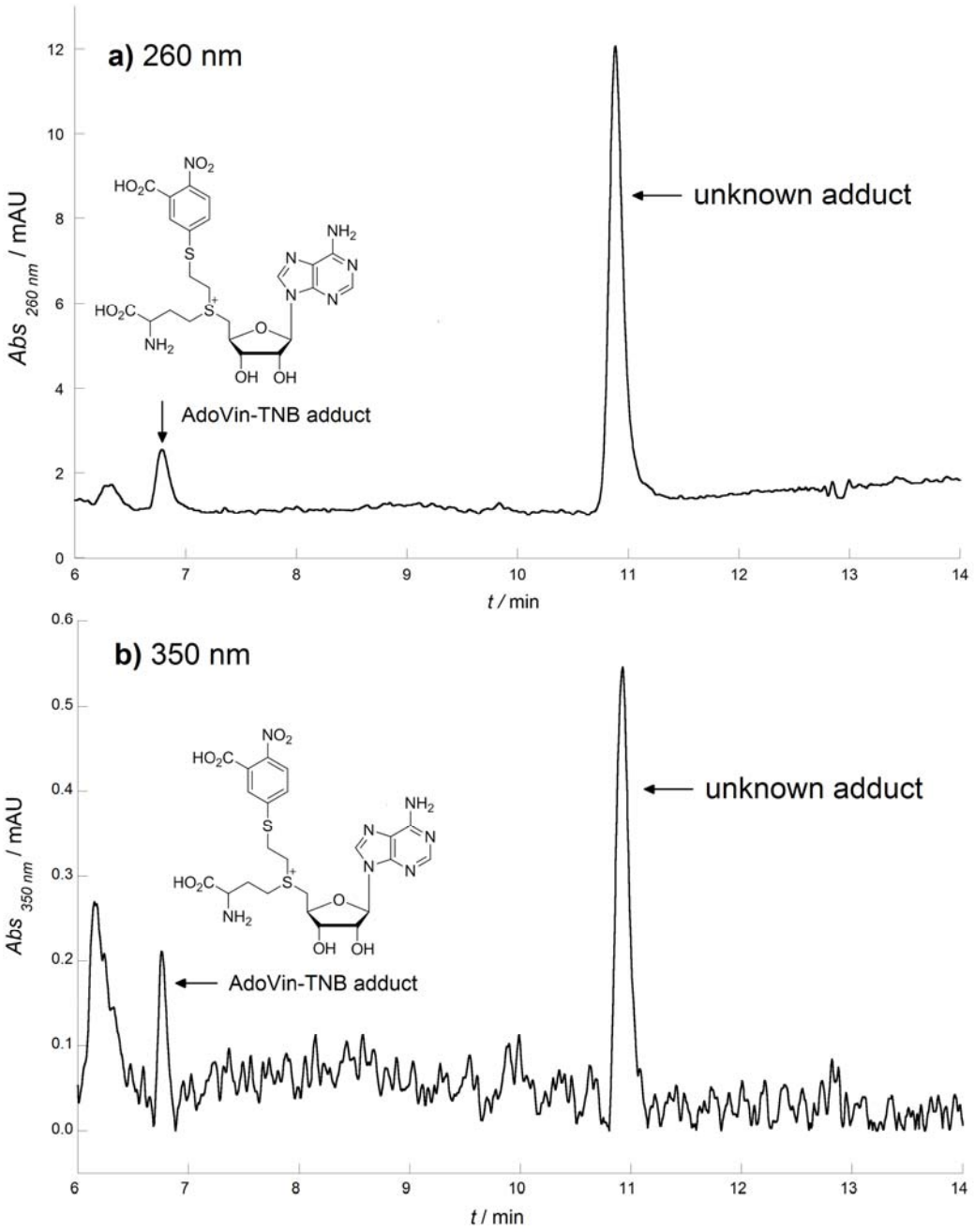
The cell lysate was prepared as described in general procedure without the purification step. The concentration of TPMT in cell lysate was estimated as 336 μM by the density of the TPMT band on SDS-PAGE gel. The reaction (200 μL) contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 1 mM vinthionine, 489 μM TNB, 2 mM TCEP, 3.5 μM MTAN and 100 μL cell lysate. The reaction was initiated with 295 μM MAT and incubated at 37 °C.

Separation and Analysis of TPMT and Other Components

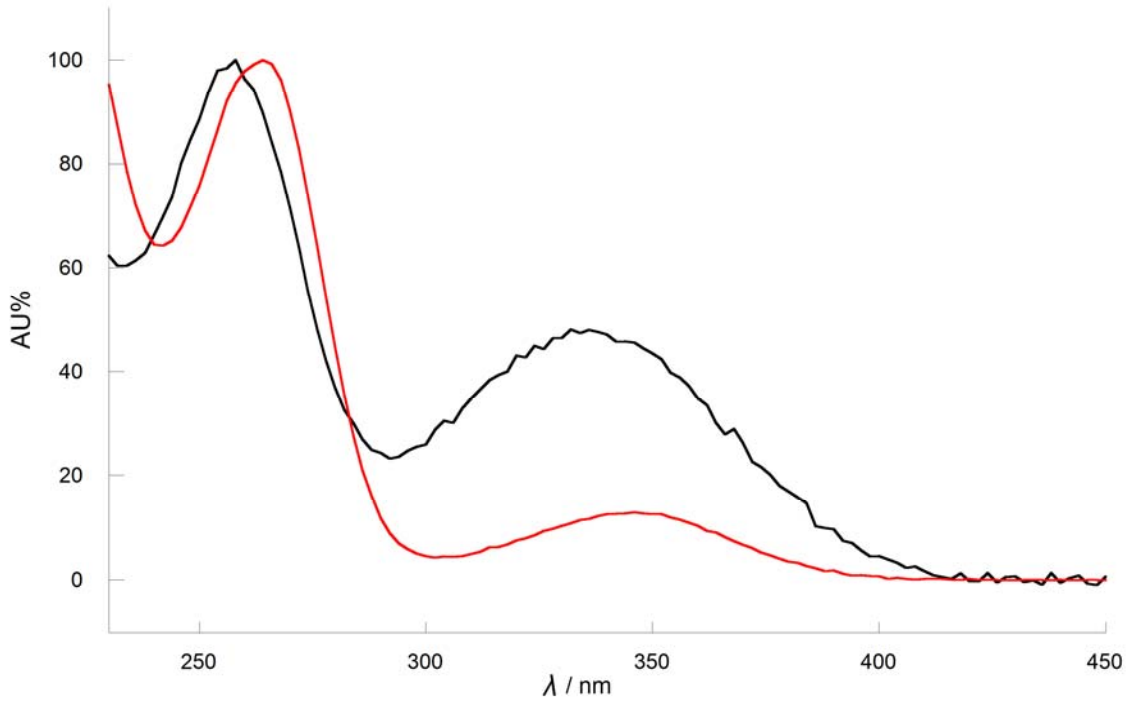
The *ex vivo* TNB reaction was loaded onto the HisPur Ni-NTA spin column. The isolation of TPMT fraction from TPMT lysates was described previously. Both the reaction mixtures and TPMT fraction eluting from the HisPur Ni-NTA Spin Column, after TCA protein precipitation, were injected analyzed by HPLC-UV-Vis and LC-MS.

Isotope Labeling of the AdoVin-TNB Adduct

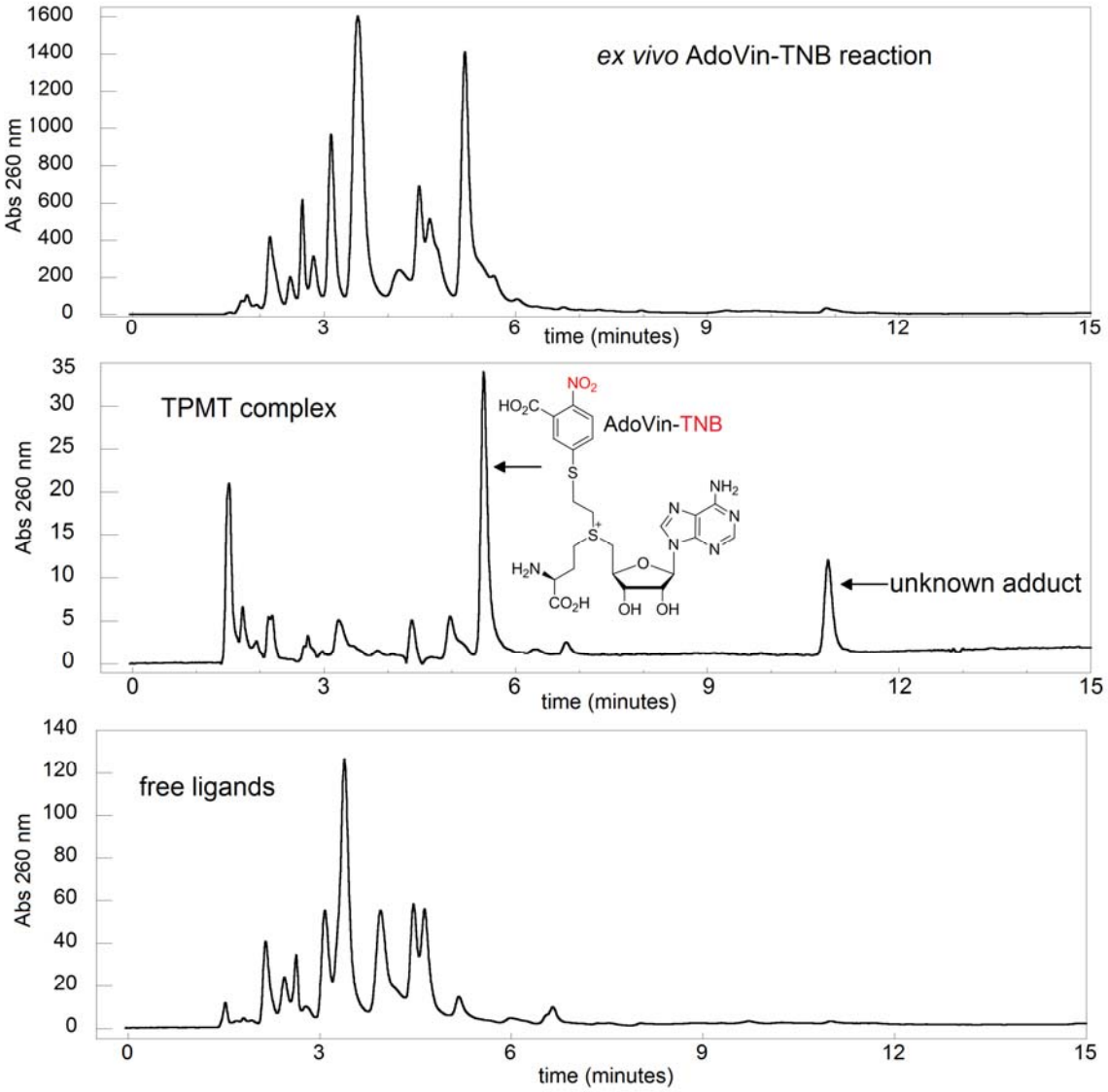
The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM adenosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate sodium salt solution (isotope labeled ATP, Sigma-Aldrich, catalog No. 645702), 600 μM vinthionine, 489 μM TNB, 2 mM TCEP, 1.75 μM MTAN, and 70 μM TPMT. The reaction was initiated with 120 μM MAT and incubated at 37 °C. The heavy isotope labeled samples were analyzed the same way as for those without labeling as described above.



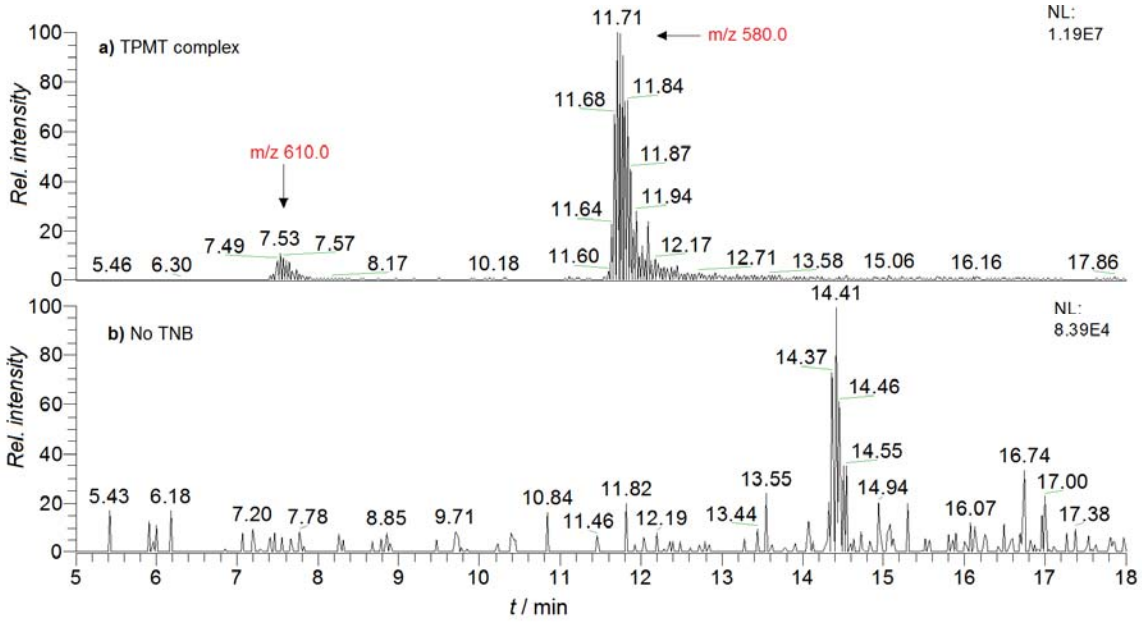
Supporting Figure 4.1.1. HPLC chromatograms (260 and 350 nm) of the TPMT complex isolated by immobilized metal ion affinity chromatography, showing the adducts tightly bound with TPMT complex in the *ex vivo* reactions. It is worth noting that an unknown adduct, also tightly bound to TPMT, was detected in additional to the AdoVin-TNB adduct.



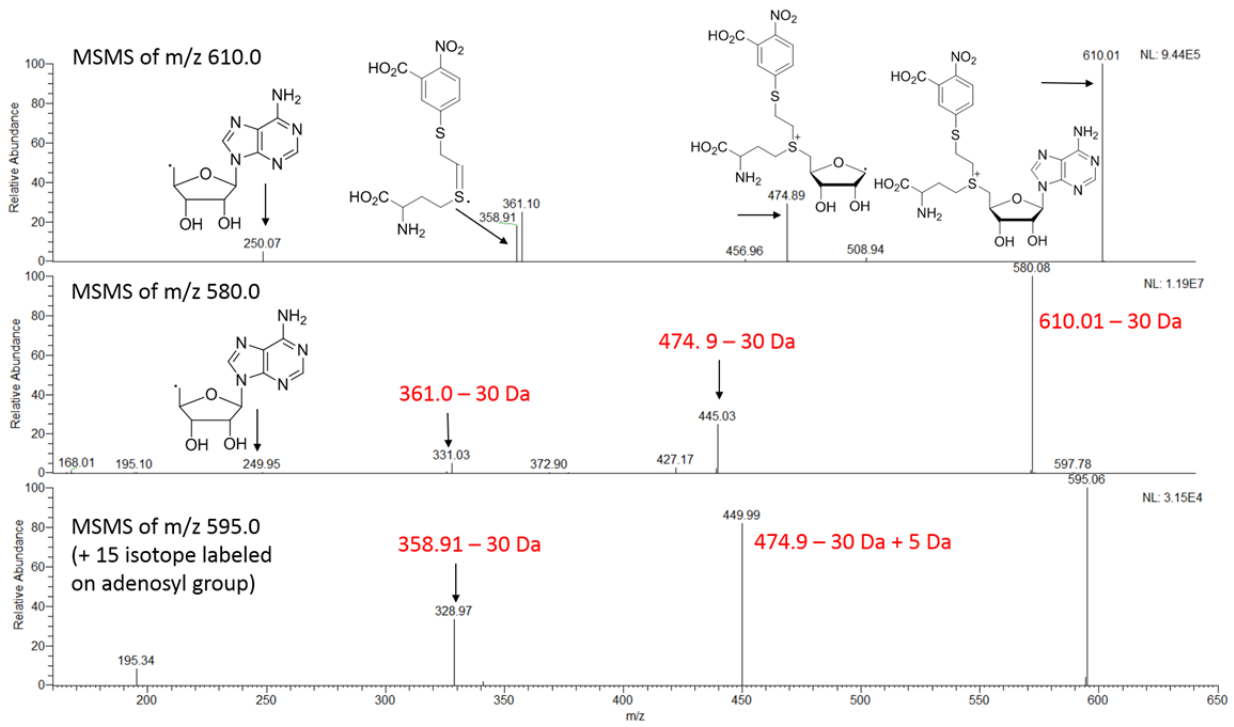
Supporting Figure 4.1.2. UV/vis absorbance spectra of AdoVin-TNB adduct (black) and unknown adduct (red). The spectra were normalized to 260 nm.



Supporting Figure 4.1.3. HPLC chromatograms (260 nm) of *ex vivo* AdoVin-TNB reaction (top), isolated TPMT complex (middle), and free ligands fraction (bottom), showing the adducts tightly bound with TPMT complex but absent in the free ligand fraction. The TPMT complex was isolated by immobilized metal ion affinity chromatography.

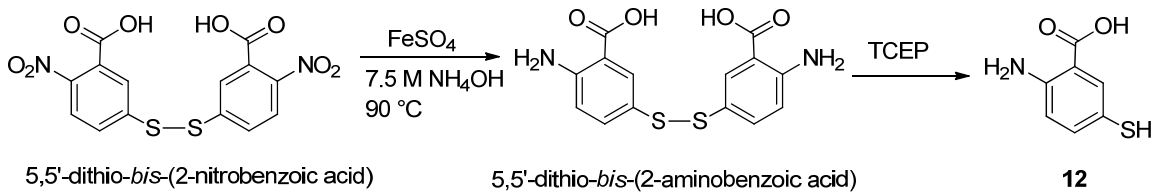


Supporting Figure 4.1.4. Selected-ion monitoring chromatograms of m/z 610.0 for the AdoVin-TNB adduct and 580.0 for the unknown adduct of the isolated TPMT complex (top) and negative control without TNB (bottom).



Supporting Figure 4.1.5. CID MS/MS spectra of the AdoVin-TNB adduct, natural and isotope labeled unknown adduct from LC-ESI/MS. The +1 precursor ions were m/z 610.0, 580.0 and 595.0, respectively.

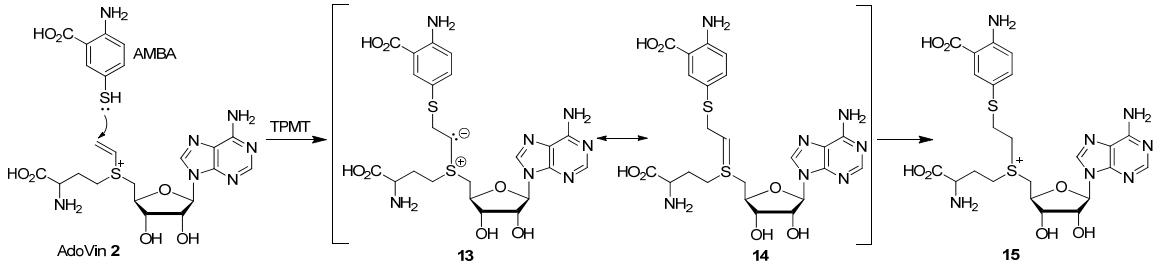
4.2. Elucidation and Confirmation of Unknown Adduct



Scheme 4.2.1. Syntheses of 2-amino-5-mercaptobenzoic acid

5,5'-Dithio-bis-(2-aminobenzoic acid). [857602-17-6] As reported,[11,12] an 8 mL solution of 7.5 M ammonium hydroxide was degassed by bubbling N_2 through the magnetically stirred solution and was heated to 85-90°C with an oil bath at 100°C. Ferrous sulfate heptahydrate (1.63 g, 5.86 mmol) was added to the basic solution while heating, stirring, and maintaining a nitrogen atmosphere. Ellman's reagent (DTNB, 5,5-dithio-*bis*-(2-nitrobenzoic acid), Alfa Aesar A14331, 0.116 g, 0.293 mmol) in 1.5 mL of 7.5 M ammonium hydroxide was added dropwise to the reaction mixture. After two hours, the oil bath was removed and the reaction mixture was allowed to cool to room temperature. Decolorizing carbon (100 mg) was added to the black reaction mixture, which was then filtered through filter paper. The yellow-orange filtrate was collected and cooled to 5°C. The filtrate was acidified with concentrated phosphoric acid to pH 3. The aqueous mixture was extracted with 2-propanol/chloroform (25:75, 4 x 15 mL). The organic extracts were combined and back-washed with water (2 x 30 mL). The organic phase was dried over sodium sulfate, the solvent removed by rotary evaporation, and stored under vacuum overnight to give 5,5'-dithio-*bis*-(2-aminobenzoic acid) as a yellow powder that was homogeneous by TLC R_f = 0.08 in 25:75 *i*-PrOH/$CHCl_3$; m.p. 200°C; ^{1}H-NMR ($CDCl_3$/DMSO-d_6 95:5) δ 7.88 (d, J = 2.1 Hz, 2H), 7.22 (dd, J = 8.6, 2.1 Hz, 2H), 6.65 (d, J = 8.6 Hz, 2H). LC-MS ES-TOF $(m/z)^+$ 337.0 [$(M+H)^+$, calcd for $C_{14}H_{13}N_2O_4S_2$: 337.0] and 319.0 [$(M-OH)^+$, calcd for acylium ion $C_{14}H_{11}N_2O_3S_2$: 319.0]. To the aqueous solution of 5,5'-dithio-*bis*-(2-aminobenzoic acid), TCEP was added to give 2-amino-5-mercaptobenzoic acid (AMBA) (**12**) [42901-73-5].

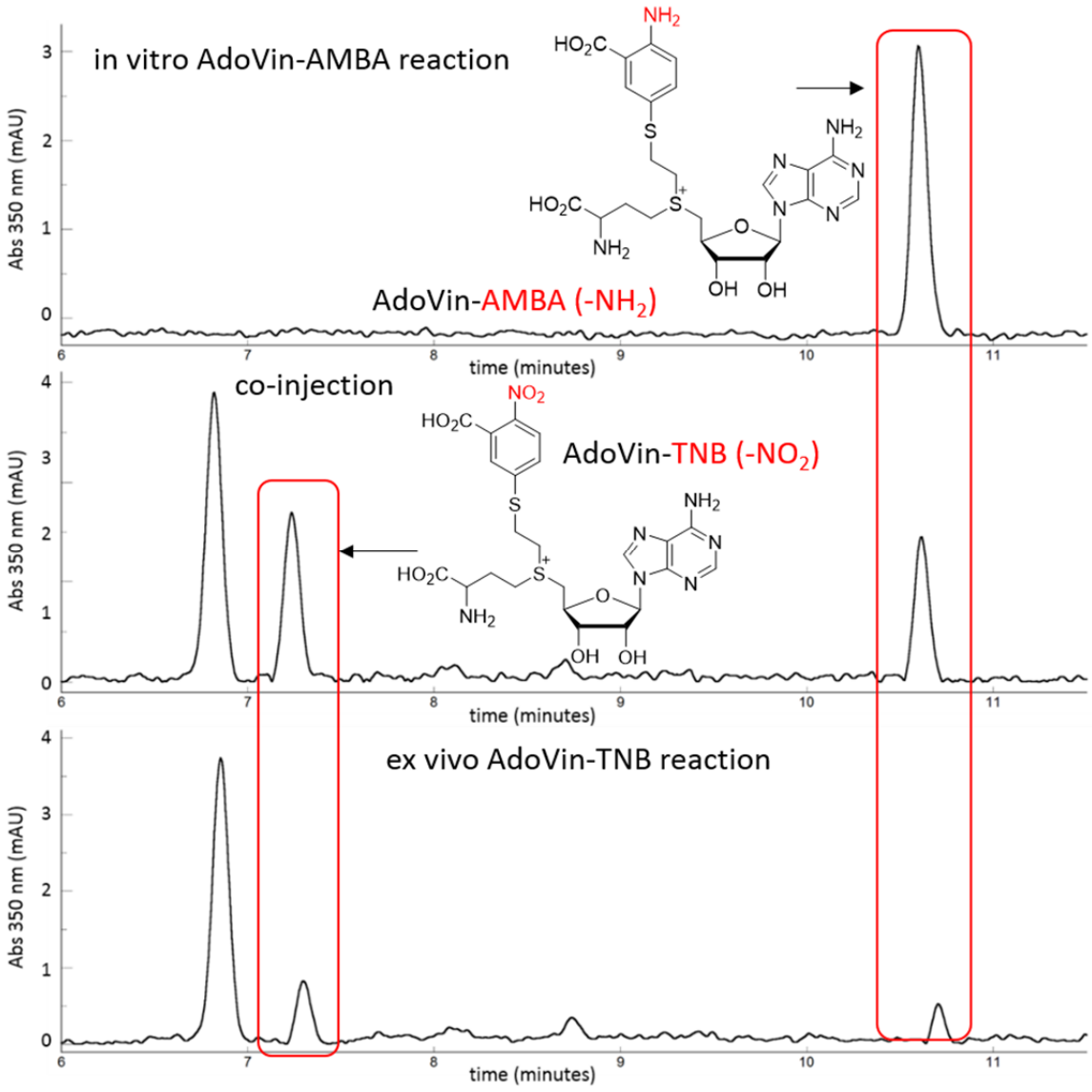
in vitro Bisubstrate Adduct Formation between AdoVin and AMBA



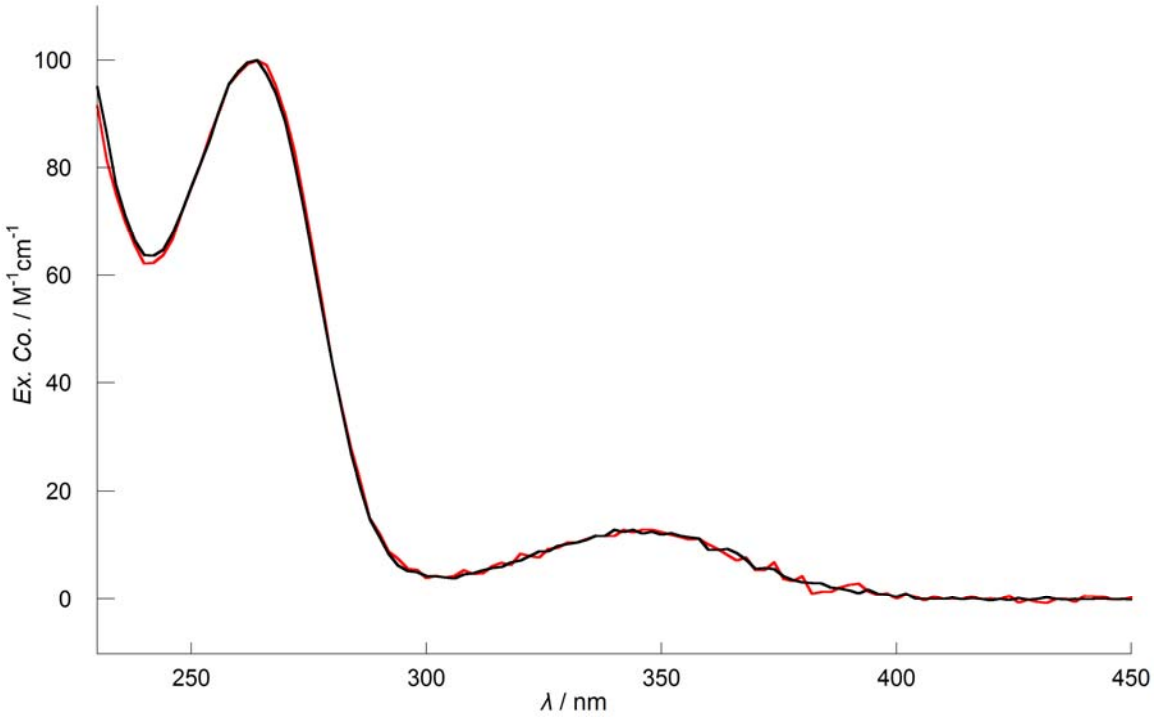
Supporting Scheme 4.2.1. Formation of bisubstrate adduct between AdoVin and AMBA catalyzed by TPMT. Either the ylide (**13**, or their tautomer form, **14**) or the sulfonium ions after protonation (**15**) may be the form bound to the enzyme.

in vitro Formation of AdoVin-AMBA adduct (15)

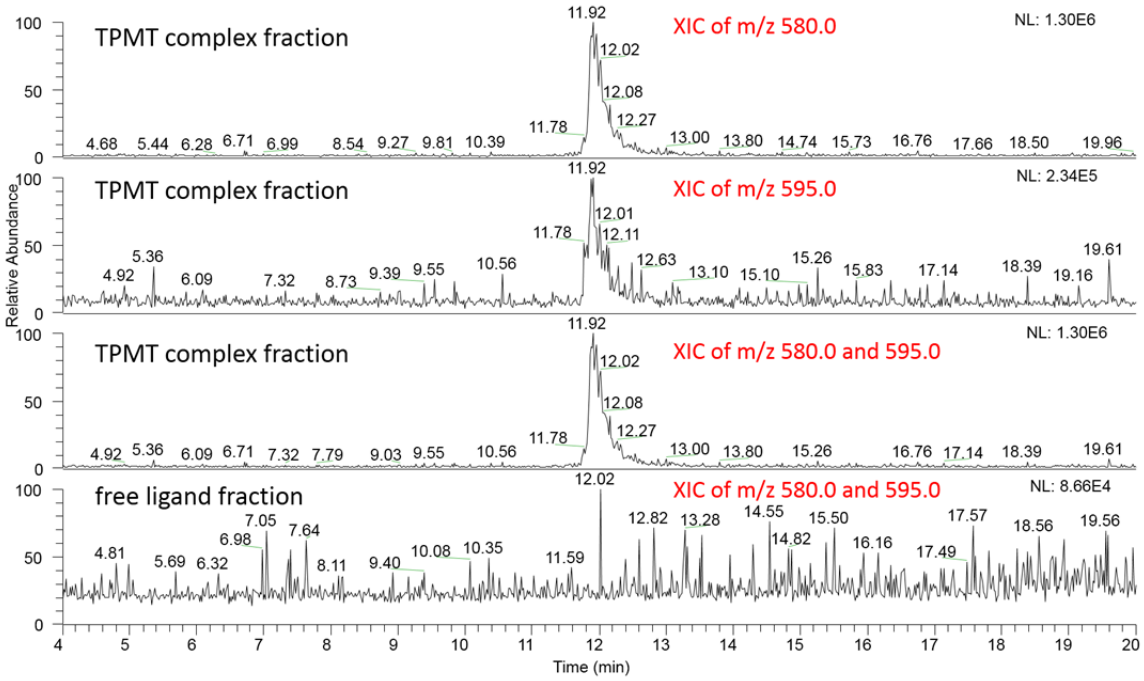
The reaction contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 1 mM vinthionine, 500 μM AMBA, 2 mM TCEP, 3.5 μM MTAN and 100 μM TPMT. The reaction was initiated with 200 μM MAT and incubated at 37 °C. To verify the adducts, the *in vitro* reaction was co-injected with *ex vivo* AdoVin-TNB reaction.



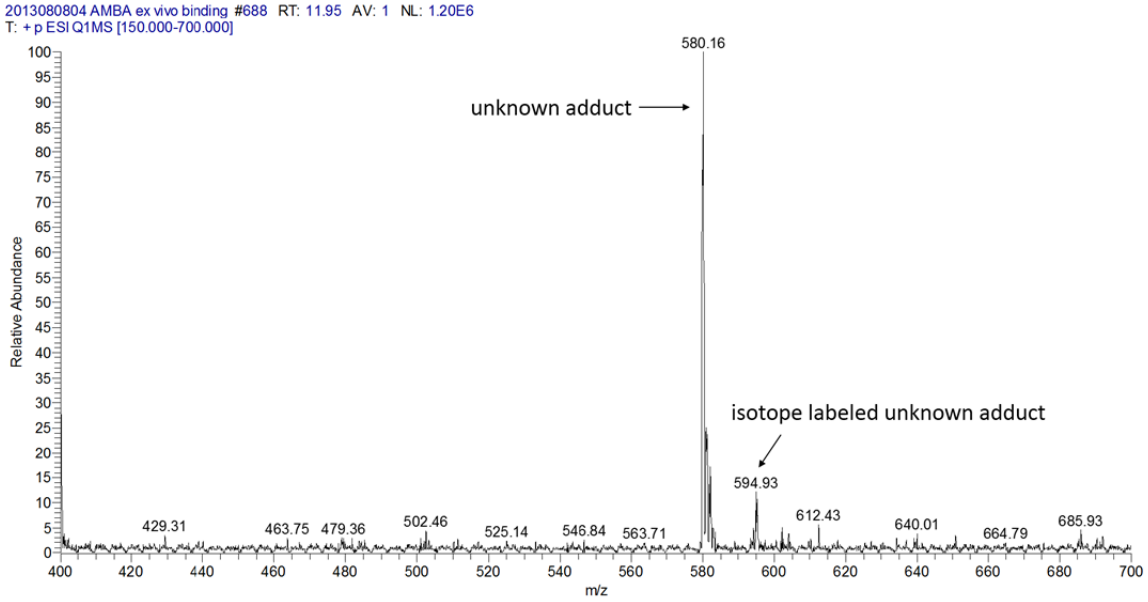
Supporting Figure 4.2.1. HPLC chromatograms (350 nm) of authentic AdoVin-AMBA adduct (top), *ex vivo* AdoVin-TNB reaction (bottom) and co-injection (middle).



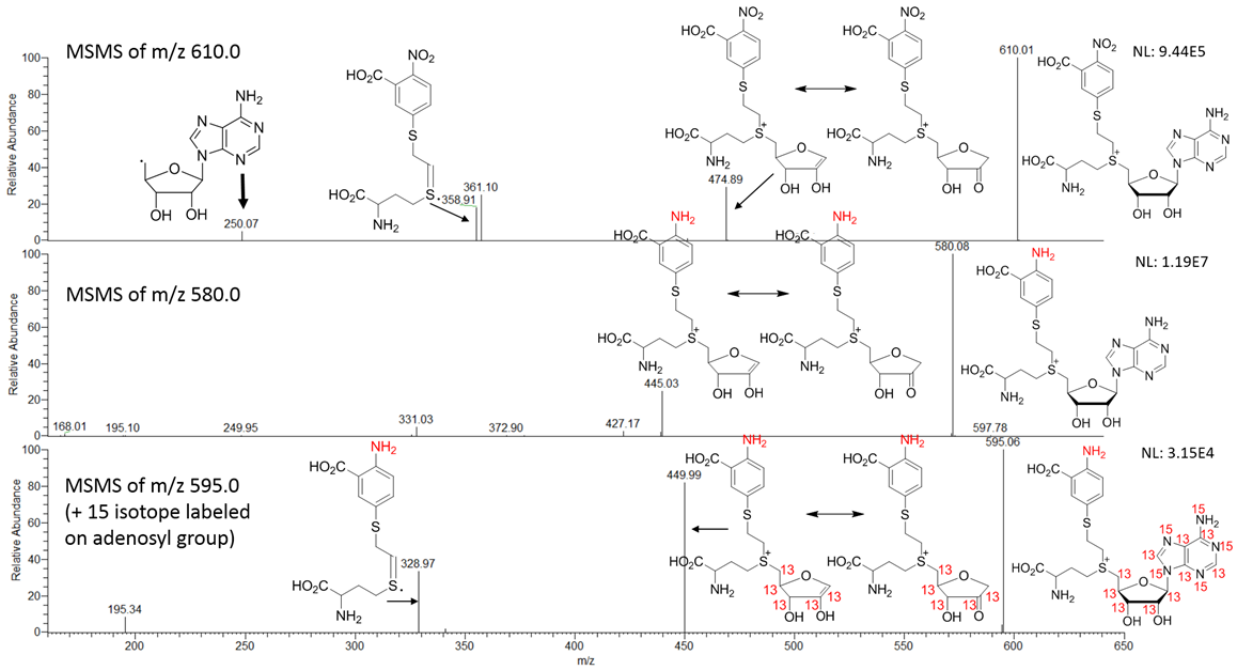
Supporting Figure 4.2.2. UV/vis absorbance spectra of the unknown adduct from the *ex vivo* reaction (black) and authentic AdoVin-AMBA adduct standard from the *in vitro* reaction (red).



Supporting Figure 4.2.3. Extracted ion chromatograms of m/z 580.0 (unknown adduct, i.e., AdoVin-AMBA) and 595.0 (isotope-labeled unknown adduct) in the isolated TPMT complex and the free ligand fraction. The coelution of these two species indicated that the adducts were derived from AdoVin (natural and isotope labeled).

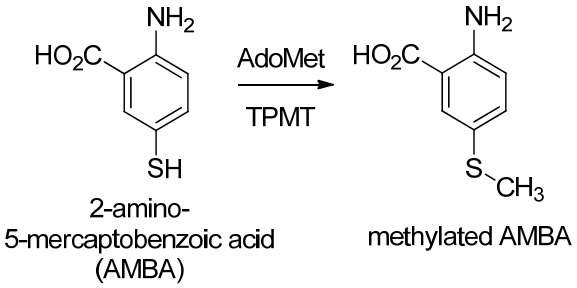


Supporting Figure 4.2.4. Mass spectrum of the unknown adduct (M^+; expected m/z 580.16 observed m/z 580.16; mass difference 0 Da) and isotope labeled unknown adduct (M^+; expected m/z 595.16 observed m/z 595.93; mass difference 0.77 Da).



Supporting Figure 4.2.5. CID MS/MS spectra of the AdoVin-TNB adduct (top), natural (middle) and isotope labeled (bottom) AdoVin-AMBA from LC-ESI/MS. The +1 precursor ion for MS/MS analysis was m/z 610.01, 580.08 and 595.06, respectively.

4.3. Confirmation of 2-Amino-5-mercaptobenzoic acid as a Methylation Substrate

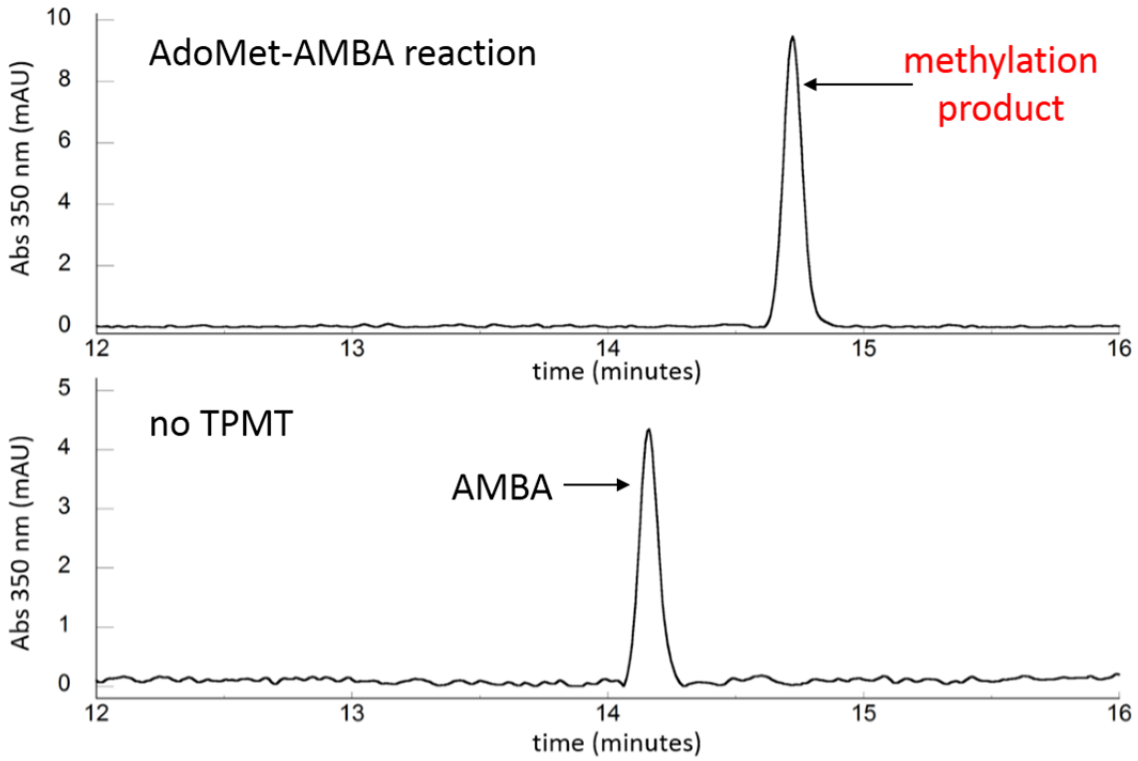


Supporting Scheme 4.3.1. Methylation of 2-amino-5-mercaptobenzoic acid (AMBA) catalyzed by thiopurine methyltransferases (TPMT).

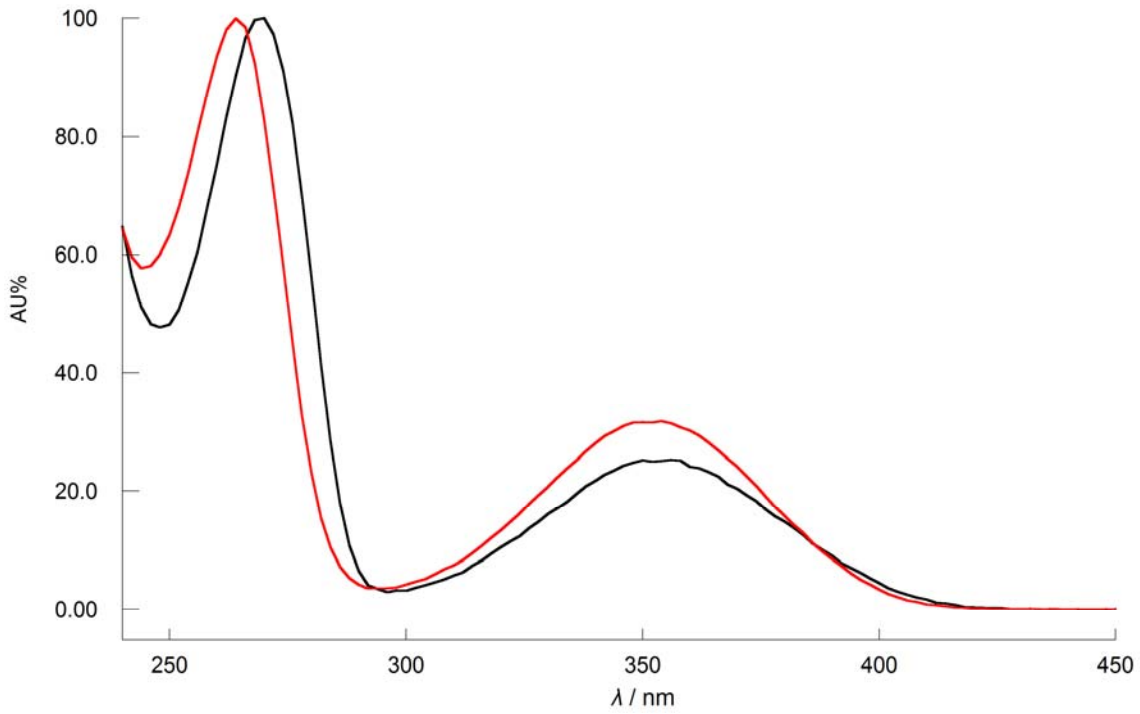
HPLC Activity Assays of Methylation of 2-Amino-5-Mercaptobenzoic Acid

The reactions contained 50 mM potassium phosphate, pH 8.0, 1 mM AdoMet, 1 mM AMBA, 2 mM TCEP, and 3.5 μM MTAN. The reaction was initiated with 13 μM TPMT and incubated at 37 °C. Aliquots of the reactions (10 μL each at 0, 15, 30, 60 and 120 min) were analyzed by HPLC monitored at both 260 and 350 nm.

The chromatography was performed on a reversed-phase column (Apollo, C_{18}, 5μ, 4.6 mm x 150 mm) using 0.1% aqueous TFA (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B) at a flow rate of 1 mL/min. The gradient program was initiated with 2% mobile phase B, and increased to 10% mobile phase B over 8 min, then increased to 30% mobile phase B over 12 min, then increased to 70% mobile phase B over 15 min, followed by an increase to 90 % mobile phase B over 5 min, then a return to 2% mobile phase B over 1 min, and finally a hold at 2% mobile phase B over 5 min.



Supporting Figure 4.3.1. HPLC chromatograms (350 nm) of TPMT-catalyzed methylation of 2-amino-5-mercaptobenzoic acid (AMBA) (top) and negative control without TPMT (bottom). Complete methylation of AMBA was observed and accounted for over 75 turnovers during 120 min incubation (the concentration of AMBA and TPMT were 1 mM and 13 μM, respectively).

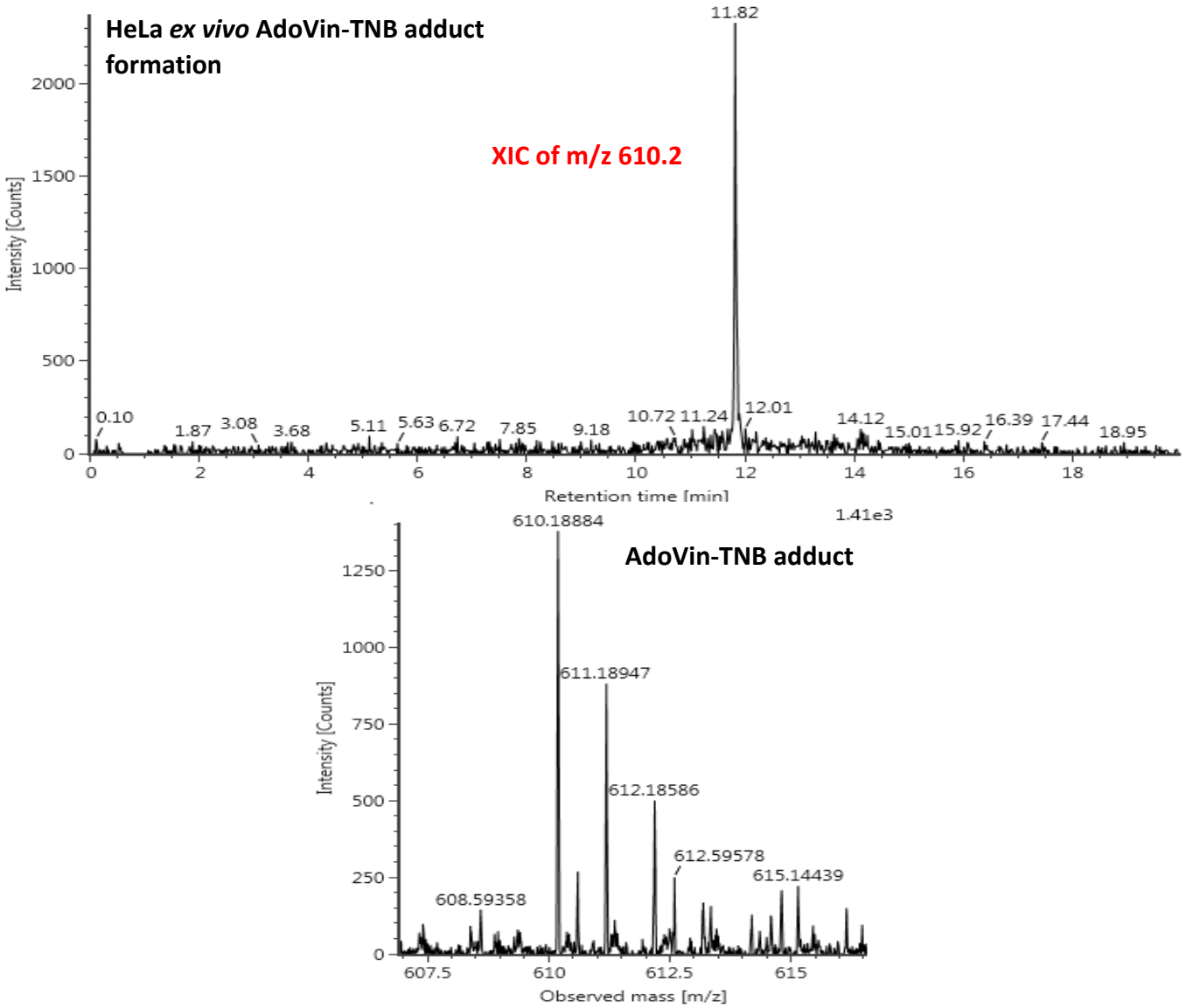


Supporting Figure 4.3.2. UV/vis absorbance spectra of methylated AMBA (black) and AMBA (red). The spectra were normalized at 260 nm (AMBA) and 270 nm (Me-AMBA).

Ex vivo adduct formation in HeLa lysate

In 10% HeLa cell lysate (AbNova L013V2), 1 mM vinthionine, 4 mM ATP, 2.5 mM $MgCl_2$, 5 mM KCl, 200 μM MAT, 0.4 mM TNB, 100 μM TPMT, and 2 mM TCEP were combined in pH 8 50 mM Tris and incubated at 37°C for 6 hours. The protein portion of the reaction was then precipitated with 10% trichloroacetic acid. The remaining small molecule fraction was then run on LC-MS.

LC-MS data was obtained using an H-Class Acquity UPLC system coupled to a Xevo G2-S Q-ToF mass spectrometer (Waters Corp, Milford, MA). Liquid chromatography was performed on a BEH-C18, 2.1 mm x 150 mm column, with pore size of 1.7 μm (Waters Corp, Milford, MA). Mobile phase A consisted of 0.1% formic acid (v/v) in HPLC grade water and mobile phase B consisted of 0.1% formic acid (v/v) in HPLC grade acetonitrile with a flow rate at 0.61 mL/min. A gradient was applied by starting at 2% mobile phase B increasing to 30% mobile phase B over 6.8 min, then increasing to 100% mobile phase B over 2.7 min, holding at 100% mobile phase B for 3.4 min, and finally decreasing to 2% mobile phase B over 1 min and holding there for 6.1 min. After liquid chromatography, samples were introduced via an electrospray ion source in-line with the Xevo G2-S Q-ToF. Data were processed manually using Waters UNIFI 1.7.1 software.

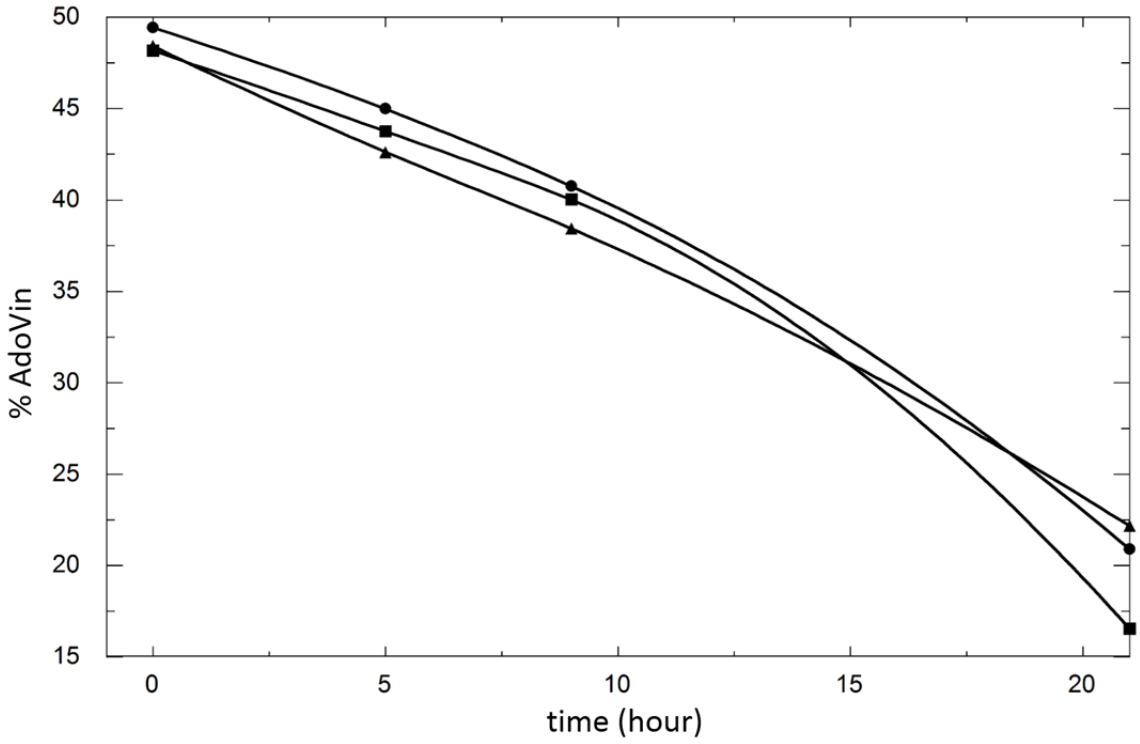


Supporting Figure 4.4. Extracted ion chromatogram (top) of m/z 610.2 (AdoVin-TNB adduct) in the small molecule fraction of the TPMT catalyzed reaction in HeLa lysate. The mass spectra (bottom) of the AdoVin-TNB adduct from this reaction.

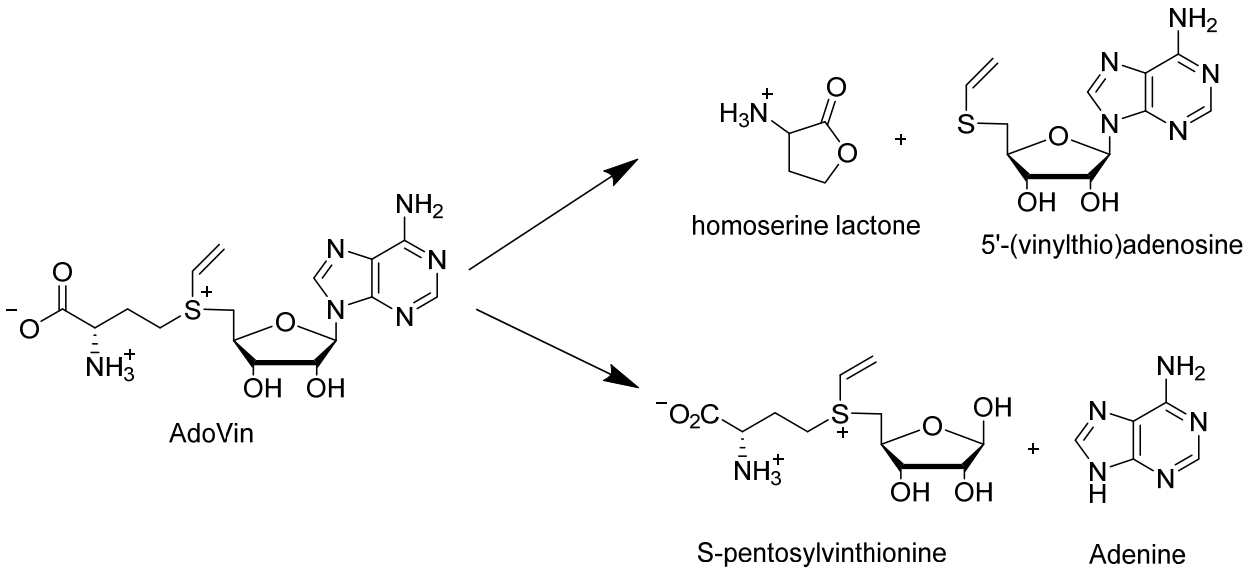
5. Stability and Reactivity of AdoVin

AdoVin Stability in the Presence of Free Thiol

AdoVin was synthesized in 50 mM ammonium bicarbonate, pH 8.0, as described above. The MAT protein was removed by ultracentrifugation using filters with 30,000 Da molecular weight cut off (MWCO). The solutions containing 50 μM AdoVin with and without DTT (1 mM or 10 mM) was incubated at 37 °C. Aliquots of the reactions (10 μL each at 0, 5, 9 and 21 h) were analyzed by strong cation exchange HPLC monitored at 260 nm as described above.



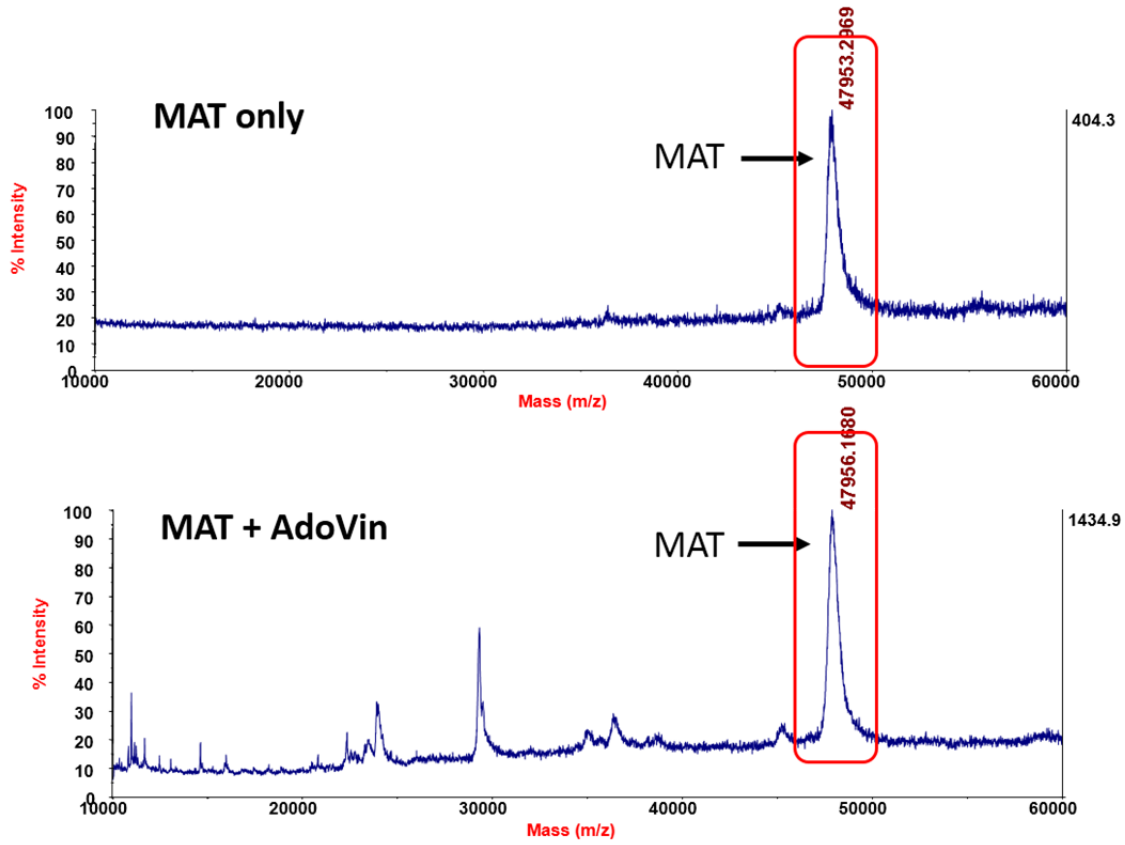
Supporting Figure 5.1.1. Stability of AdoVin in the absence (■) and presence of 1 mM (▲) or 10 mM (●) of DTT.



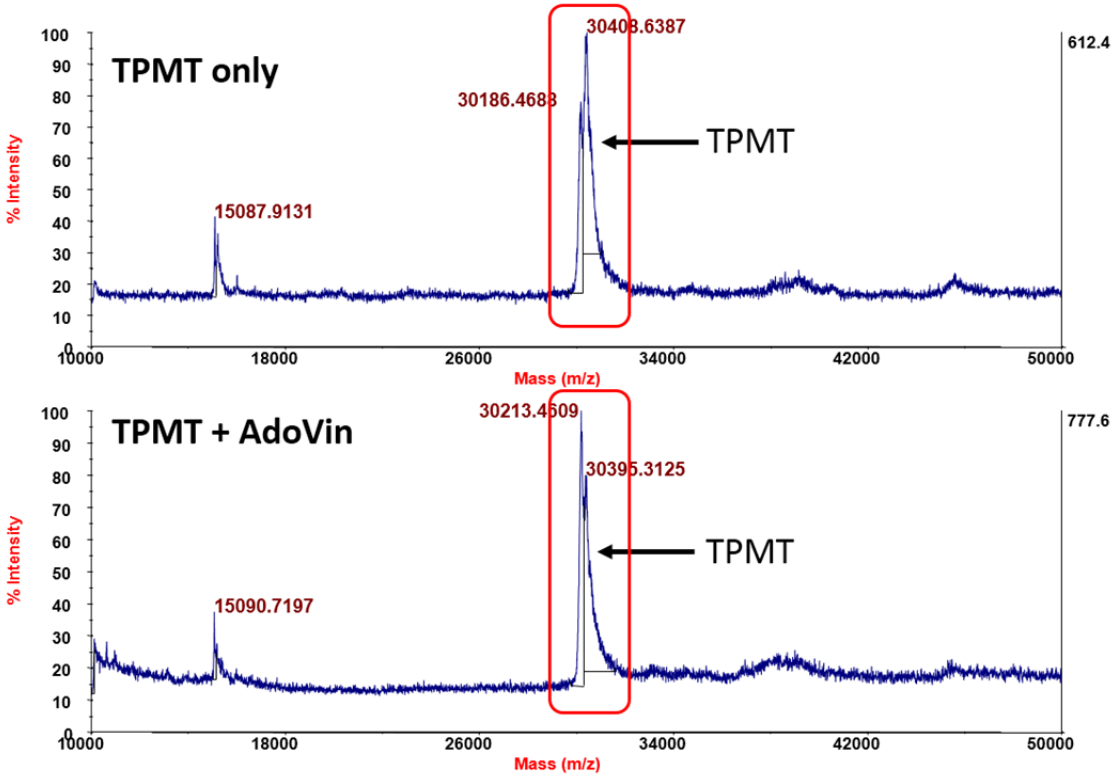
Supporting Figure 5.1.2. Proposed degradation pathways for AdoVin. Top: AdoVin undergoes intramolecular nucleophilic attack to form homoserine lactone and 5'-(vinylthio)adenosine. Bottom: AdoVin in hydrolyzed to form *S*-pentosylvinthionine and adenine.

No Modification of Proteins by AdoVin

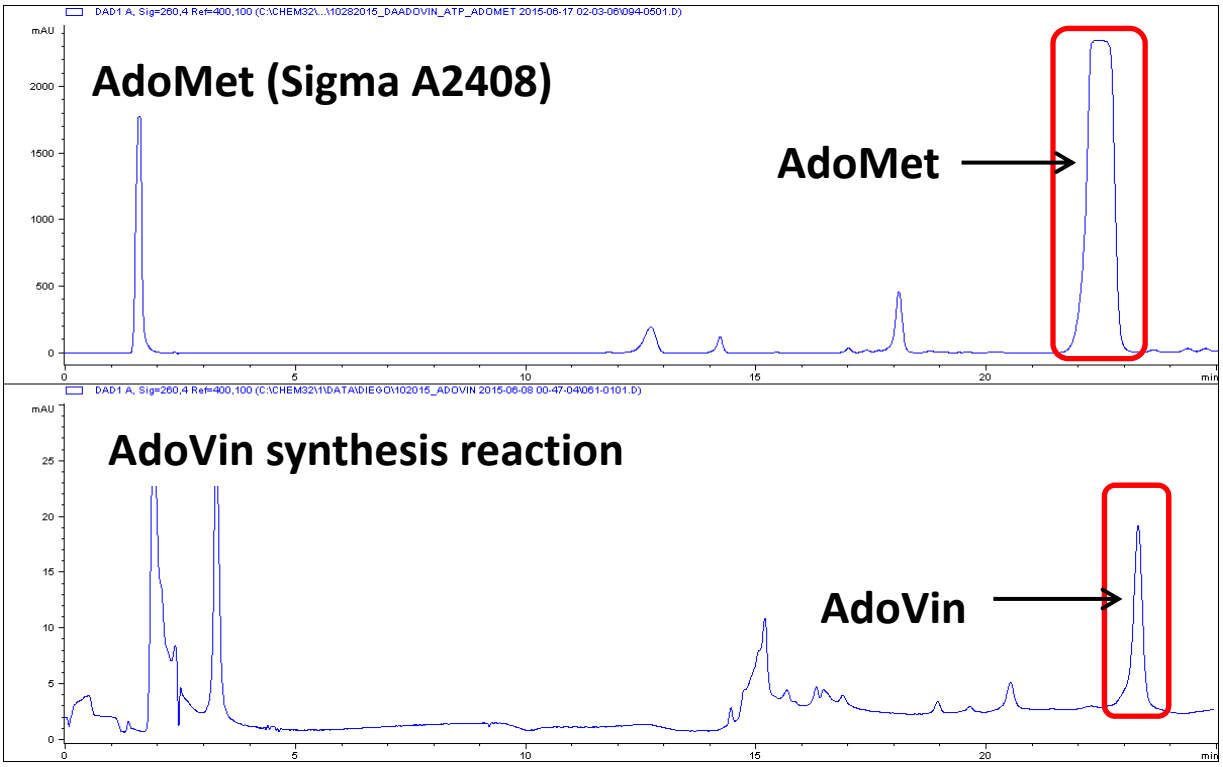
In 50 mM ammonium bicarbonate (pH 8.0), 71 μM AdoVin was incubated with 41 μM MAT and 42 μM TPMT, respectively. Reactions of MAT and TPMT without AdoVin were prepared as control. All the samples were incubated at 37 °C for 18 h and analyzed by MALDI-TOF.



Supporting Figure 5.2. MALDI mass spectra of MAT native (top) and treated with AdoVin (bottom). The theoretical molecular weight of native MAT is 48019.0. Within the resolution of the instrument, no significant mass shift (e.g., 411.1 Da for AdoVin) was observed, indicating that no modification by AdoVin.



Supporting Figure 5.3. MALDI mass spectra of TPMT native (top) and treated with AdoVin (bottom). The theoretical molecular weight of native TPMT is 30343.6 with the N-terminal methionine and 30212.6 without the N-terminal methionine. Within the resolution of the instrument, no significant mass shift (e.g., 411.1 Da for AdoVin) was observed, indicating that no modification by AdoVin.



Supporting Figure 5.4. AdoVin was prepared enzymatically as previously described then proteins in the reaction were removed through TCA precipitation. The small molecule portion was then separated by strong cation exchange (SCX). AdoVin is sufficiently resolved for purification by this method.

The chromatography was performed on a strong cation exchange column (PolyLC PolySulfoethyl A 5µ, 4.6 mm x 100 mm) using 10 mM KH_2PO_4, pH 2.9 and 20% acetonitrile (mobile phase A) and 500 mM KCl, 10 mM KH_2PO_4, pH 2.9 and 20% acetonitrile (mobile phase B) at a flow rate of 0.5 mL/min. The gradient program was initiated with 5 min at 0% mobile phase B, and increased to 100% mobile phase B over 15 min, then held at 100% mobile phase B for 5 min, then decreased to 0% mobile phase B over 1 min, and finally a hold at 0% mobile phase B for 4 more min.

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