Supporting Information

Enzyme-Catalyzed Transfer of a Ketone Group from an *S*-Adenosylmethionine Analog: A Tool for the Functional Analysis of Methyltransferases

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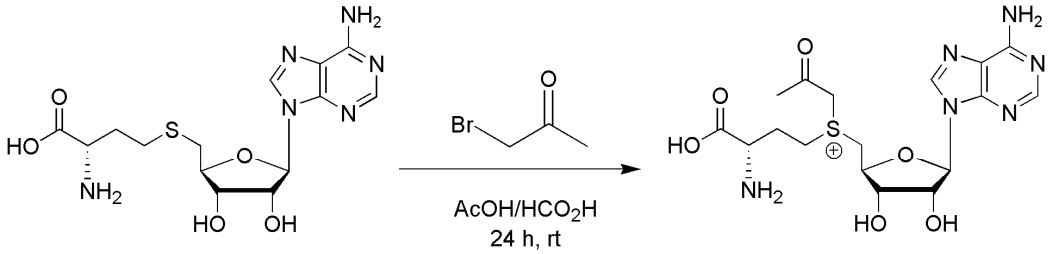
1. General Procedures. Reagents of ACS grade or better were used without further purification unless otherwise noted. Analytical reversed phase chromatography was performed on an Apollo C18 150 x 4.6 mm, 5 μm column and semi-preparative reversed phase chromatography was performed on an Apollo C18 250 x 10 mm, 5 μm column, both from Alltech. HPLC was conducted using Dynamax SD-200 pumps and a Varian ProStar 330 photodiode array detector interfaced with Varian ProStar Chromatography Workstation software version 6.41. UV absorbance measurements were recorded on a Varian Cary 50 Bio UV/vis spectrophotometer. The temperature was controlled using a Varian Cary dual cell peltier accessory temperature module. NMR spectra were obtained on either a Varian Mercury 300 MHz or a Varian INOVA 500 MHz spectrometer. The data were processed and analyzed with VNMR version 6.1C software. Mass spectral data were acquired on a Surveyor HPLC system (Thermo Finnigan, San Jose, CA) coupled to a LCQ ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a PicoView ESI source (New Objective, Woburn, MA) and processed using Xcalibar Data System 2.0 (Thermo Fisher, Waltham, MA). Data analysis was performed and graphics were constructed using the Kaleidagraph software package version 4.01 (Synergy Software, Reading, PA). Alexa Fluor 647 C5-aminooxyacetamide was from Invitrogen (Carlsbad, CA). Recombinant histidine-tagged human thiopurine methyltransferase (TPMT) and *E. coli S*-adenosylhomocysteine nucleosidase (MTAN) were purified as described.[1,2] The concentration of keto-AdoMet was determined using $\varepsilon_{260\ nm}$ = 15,400 M^{-1} cm^{-1} based on the values for AdoMet and AdoHcy.[3-6] Stock solutions of thiol substrates were prepared in dimethyl formamide (DMF) and the final concentration of organics in enzyme reactions did not exceed 5%.

2. Preparation of Keto-AdoMet.

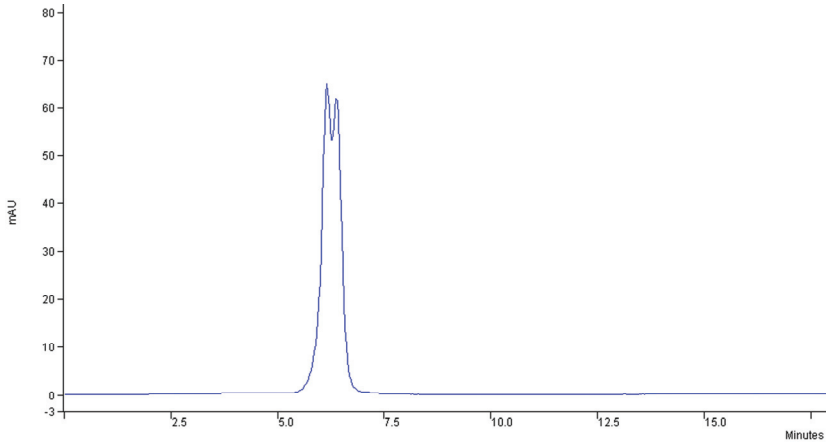
Synthesis of *S*-adenosyl-*S*-(propan-2-one)-L-methionine (keto-AdoMet, 1)



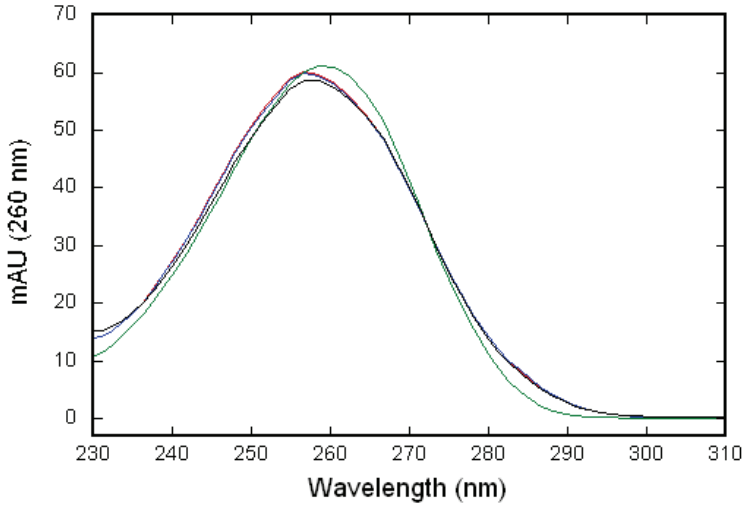
S-adenosylhomocysteine **1**

***S*-Adenosyl-*S*-(propan-2-one)-L-methionine (1).** The procedure is based on the syntheses of AdoMet analogs reported in the literature.[6,7] Bromoacetone (65 μL, 0.73 mmol) was added dropwise to a stirred solution of *S*-adenosylhomocysteine (AdoHcy, 10.5 mg, 0.026 mmol) in 0.5 mL formic acid/acetic acid (1:1) at 0 °C. The solution turned brown immediately and was further stirred in the dark at room temperature for 24 h. HPLC of the reaction mixture indicated ~10% conversion of AdoHcy to the ketone analog. The reaction was quenched by the addition of 1.5 mL water and washed with diethyl ether (3 × 2 mL). The aqueous portion was subjected to lyophilization to yield a white solid which was redissolved in 0.1% aqueous trifluoroacetic acid (TFA) and stored at −80 °C until further use. A sample (~1 mg) of compound **1** was purified by HPLC for characterization. Preparative scale chromatography was performed using 0.1% aqueous TFA at a flow rate of 4 mL/min. The product was collected at 10 min, frozen, and lyophilized to dryness to yield a white solid. ^{1}H-NMR (500 MHz, D_2O): δ 2.07 (s, CH_3), 2.11 (s, CH_3), 3H total; 2.30 (m, 2H, Hβ), 3.63 (m, 2H, Hγ), 3.80 (t, 1H, Hα), 4.01 (m, 2H, H5′), 4.59 (m, 1H, H4'), 4.97 (t, 1H, H2'), 6.12 (q, 1H, H1'), 8.28 (s, 1H, aromatic), 8.30 (s, 1H, aromatic H). The methylene protons between the sulfonium center and the carbonyl were not observed due to exchange with the deuterium oxide solvent. The reported pKa range for these protons is 6.0-8.0.[8,9] ESI-MS m/z (calculated): 441.40 (441.16); [*S*-adenosyl-*S*-(propan-2-one)-L-

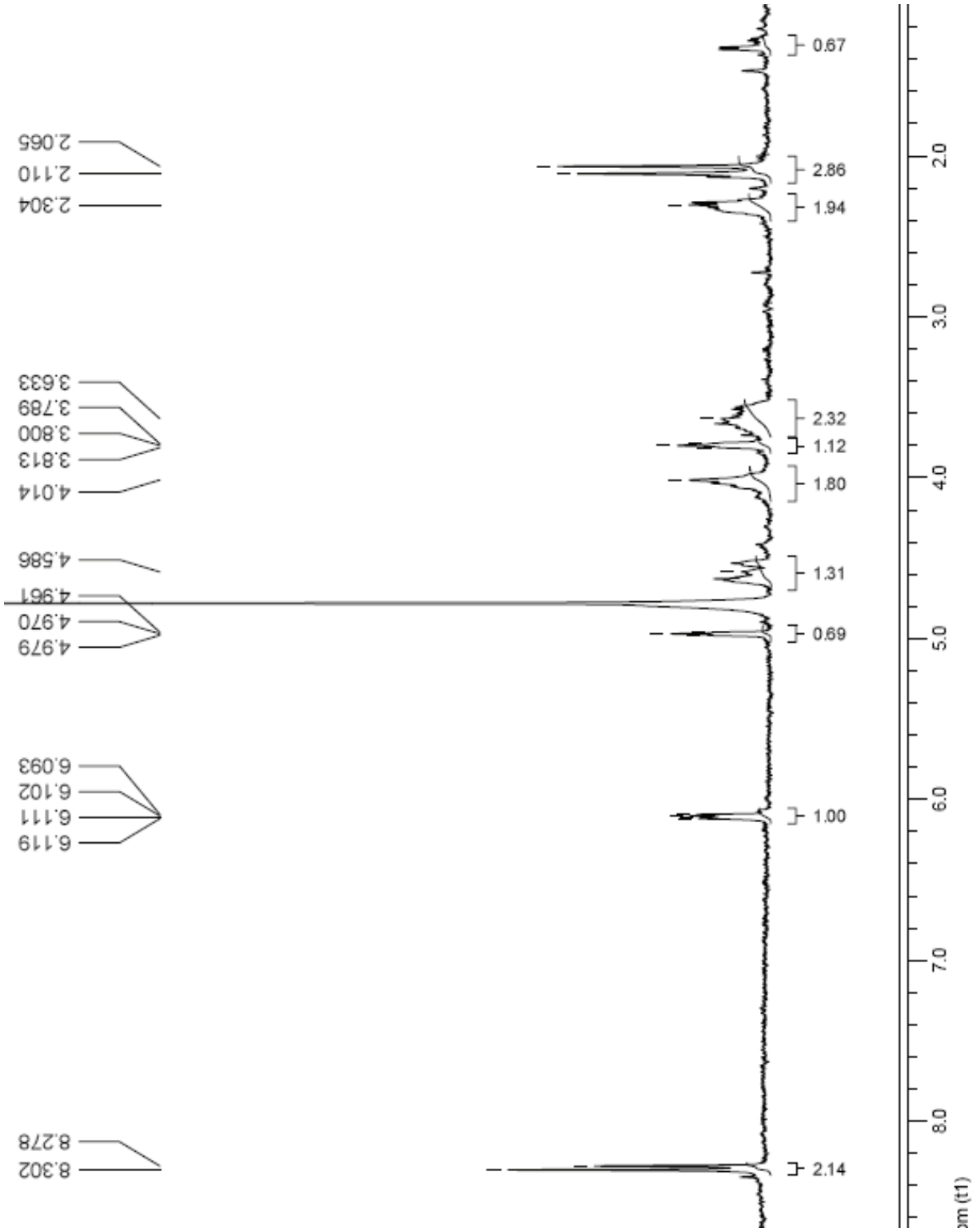
methionine+H]$^+$, 340.53 (340.11); [5'-(2-oxo-propyl)thio-5'-deoxyadenosine+H]$^+$, 250.51 (250.07); [*S*-ribosylhomocysteine+H]$^+$, 136.25 (136.06); [adenine+H]$^+$.



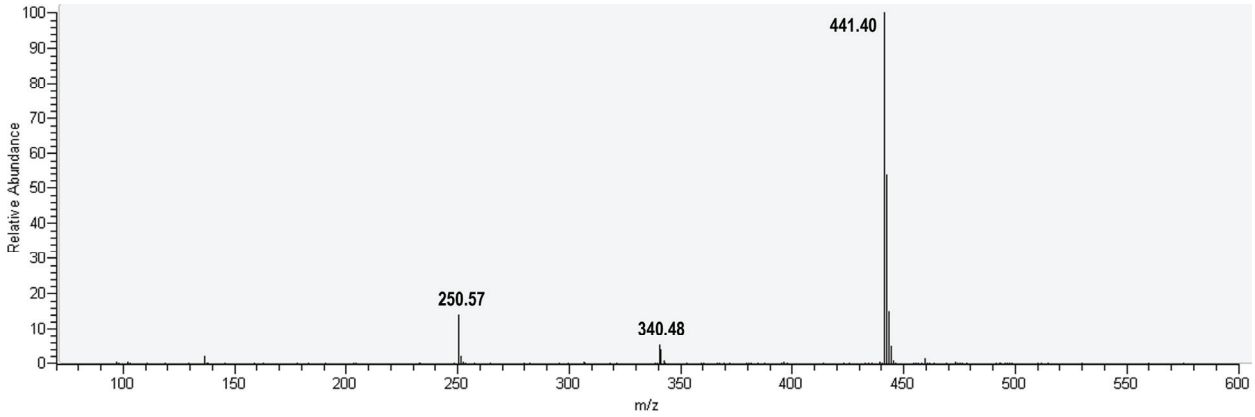
Supplemental Figure 2.1. Analytical HPLC chromatogram (260 nm) of purified keto-AdoMet. The compound was eluted with 0.1 % aqueous TFA at a flow rate of 1 mL/min. Under these conditions, the mixture of *R*- and *S*-sulfonium epimers generated by the chemical synthesis of keto-AdoMet was not fully resolved.



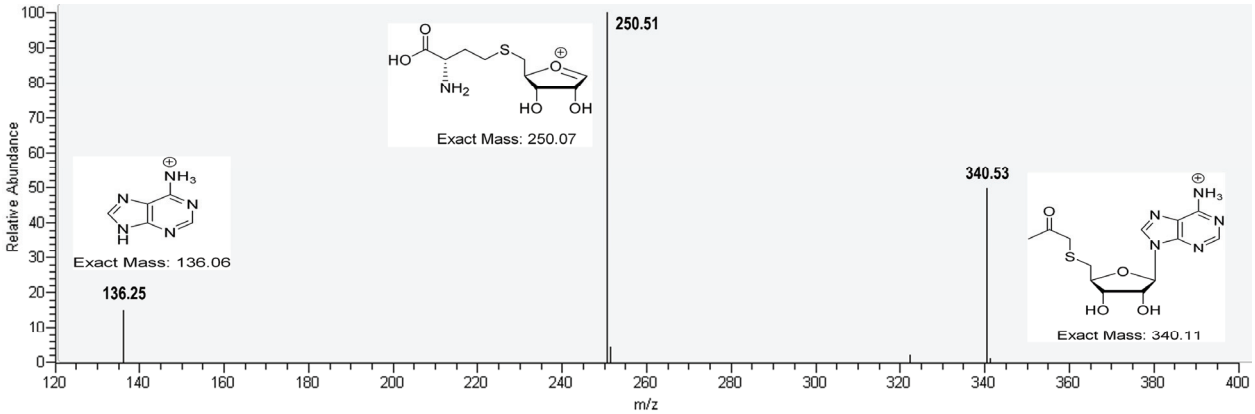
Supplemental Figure 2.2 UV/vis absorbance spectra of purified keto-AdoMet. The spectra of the isomers (Supplemental Figure 2.1.) eluting at 6.2 (blue) and 6.4 min (red). The absorbance maximum for keto-AdoMet is 257 nm. For comparison, the UV/vis absorbance spectrum of AdoMet (green) and AdoHcy (black) is shown.



Supplemental Figure 2.3. ^{1}H-NMR spectrum of keto-AdoMet (500 MHz, D_2O).



Supplemental Figure 2.4. Mass spectrum and fragmentation of keto-AdoMet (expected m/z 441.16). Purified keto-AdoMet was dissolved in 0.1 % aqueous formic acid and subjected to ESI-LC-MS.

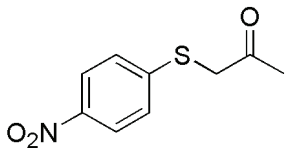


Supplemental Figure 2.5. ESI-MS/MS of keto-AdoMet. The precursor ion of the MS^2 spectrum was m/z 441.40.

Preparation of Product Standards.

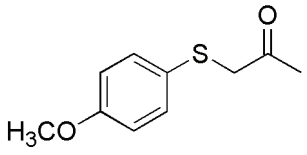
General Procedure for the Synthesis of Standards

Triethylamine (0.07 mL, 0.5 mmol) was added to a solution of thiol (0.32 mmol) in 2 mL tetrahydrofuran (THF) and after stirring for 10 min at room temperature, chloroacetone (40 μL, 0.48 mmol) was added dropwise. The reaction was allowed to proceed for 4 h before the mixture was quenched by the addition of saturated aqueous ammonium chloride (2 mL). The solution was extracted with ethyl acetate (3 x 10 mL) and the organic layer was dried over magnesium sulfate. The product was purified by silica gel column chromatography (0-40% ethyl acetate/hexanes).



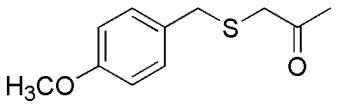
2

1-[(4-Nitrophenyl)thio]acetone (2). [CAS: 25784-85-4] Yellow solid; yield: 80% ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.32 (s, 3H), 3.82 (s, 2H), 7.35 (d, J = 9.6 Hz, 2H), 8.13 (d, J = 9.6 Hz, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 28.06, 42.60, 124.00, 126.56, 145.13, 145.44, 201.90.



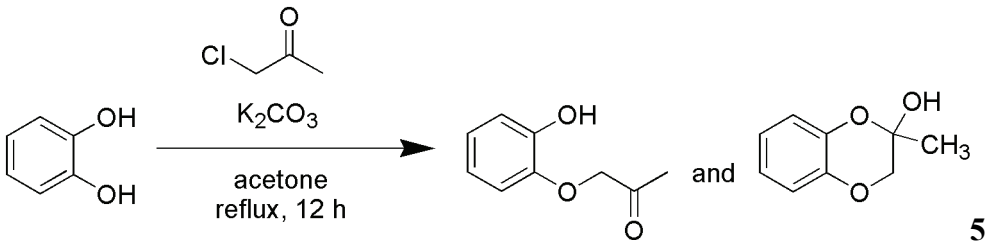
3

1-[(4-Methoxyphenyl)thio]acetone (3). [CAS: 25784-84-3] White solid; yield: 90% ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.25 (s, 3H), 3.54 (s, 2H), 3.78 (s, 3H), 6.83 (d, J = 8.7 Hz, 2H), 7.34 (d, J = 8.7 Hz, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 28.18, 46.68, 55.45, 114.98, 124.72, 133.77, 159.72, 203.71.



4

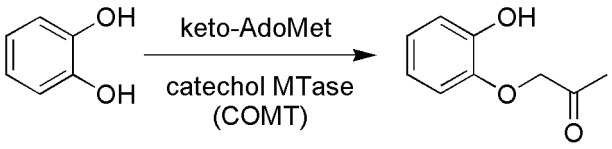
1-[(4-Methoxybenzyl)thio]acetone (4). [CAS: 675576-47-3] White solid; yield: 92% ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 2.24 (s, 3H), 3.10 (s, 2H), 3.64 (s, 2H), 3.79 (s, 3H), 6.84 (d, J = 8.7 Hz, 2H), 7.21 (d, J = 8.7 Hz, 2H). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 28.14, 35.53, 40.83, 55.39, 114.07, 129.25, 130.42, 158 .94, 203.92.



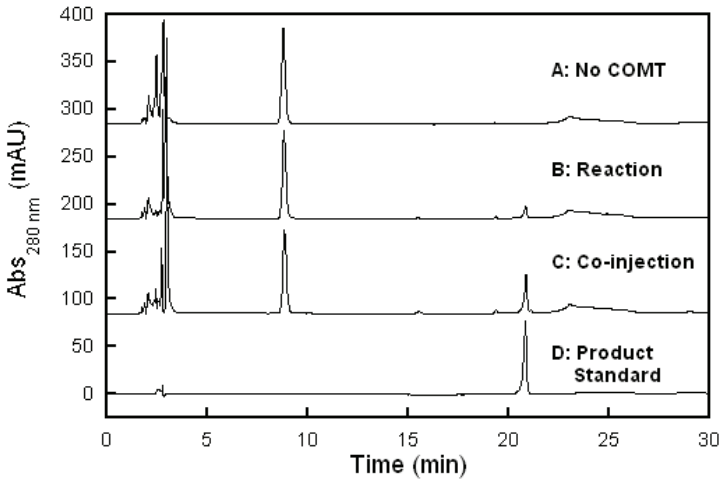
5

1-(2-Hydroxyphenoxy)acetone (5). [CAS: 5740-96-5]. Compound **5** was prepared as described.[10] Chloroacetone (1.67 g, 1.4 mL, 18 mmol) was added dropwise to a stirred solution of catechol (2.2 g, 20 mmol) and potassium carbonate (3.3 g, 20 mmol) in 20 ml acetone. The mixture was refluxed overnight, cooled and then evaporated to dryness. The resulting solids were separated between dichloromethane and water. The aqueous portion was further extracted with dichloromethane and the combined organics were washed with water and dried over anhydrous Na_2SO_4. The organics were evaporated and the crude material was subjected to silica gel flash column chromatography (40% ethyl acetate/hexanes) to provide the product as a white solid (1.96 g, yield: 59%). ESI-MS m/z (calculated): 167.45 (167.07). ^{1}H-NMR ($CDCl_3$, 500 MHz): open form (< 5%) δ 2.10 (s, 3H, CH3), 4.65 (s, 2H), 6.71-6.92 (m, 4H, aromatic); closed form δ 1.59 (s, 3H, CH3), 3.43 (s, 1H, OH), 3.84 (d, 2H, J = 11.0 Hz), 4.09 (d, 1H, J = 11.1 Hz), 6.83-7.08 (m, 4H, aromatic). ^{13}C-NMR ($CDCl_3$, 75 MHz): δ 23.70, 70.29, 93.58, 117.38, 118.06, 121.96, 122.80, 141.70, 142.21.

3. Catechol *O*-Methyltransferase (COMT) Activity Assays.

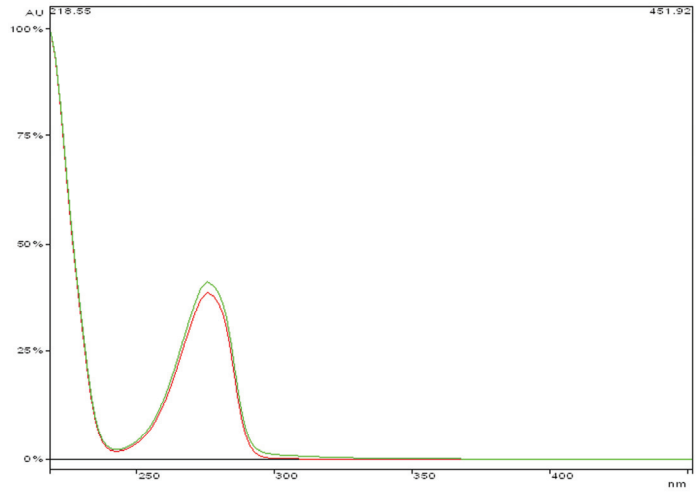


Catechol *O*-methyltransferase (COMT) transfer of the ketone moiety from keto-AdoMet to catechol was monitored by HPLC. The assays contained 200 mM potassium phosphate (pH 8.0), 2 mM tris(2-carboxyethyl)phosphine (TCEP), 2 mM $MgCl_2$, 1 μM MTAN, 500 μM catechol and 400 μM keto-AdoMet. The reactions were initiated with 5 μM catechol *O*-methyltransferase from porcine liver (EC 2.1.1.6, MP Biomedicals, catalog number 153880) and incubated at 37 °C. Aliquots were removed (typically at 4, 8, 12, or 24 h) and analyzed by HPLC (280 nm) using the following conditions: 10% acetonitrile for 10 min followed by a linear increase to 50% acetonitrile over 10 min, then a hold at 50% acetonitrile for 3 min, and finally a return to 10% acetonitrile over 2 min. The flow rate was 1 mL/min. Peaks corresponding to the alkylated products were confirmed by co-injection with synthetic standard.



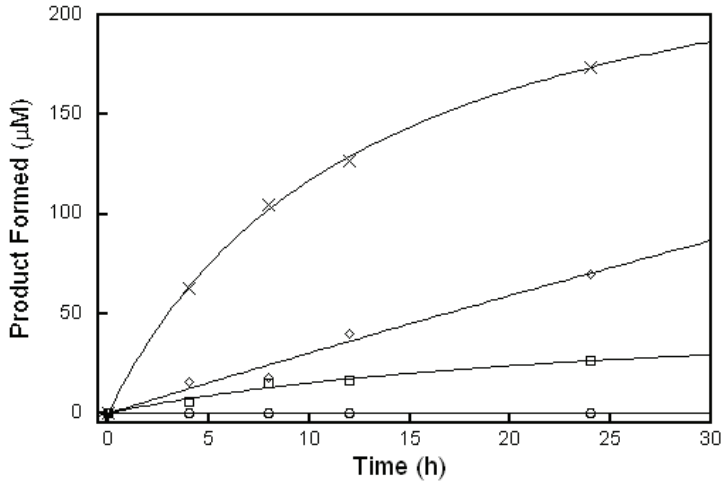
A

Supplemental Figure 3.1. COMT-catalyzed transfer of the ketone moiety from keto-AdoMet to catechol. A) A solution containing 500 μM catechol (elutes at 8.9 min) and 400 μM keto-AdoMet without COMT after 12 h. B) The same solution as (A) at 12 h except with 20 units of COMT. The alkylated product elutes at 20.8 min. C) Co-injection of the COMT reaction product with synthetic 1-(2-hydroxyphenoxy)acetone (**5**). D) Synthetic 1-(2-hydroxyphenoxy)acetone (**5**) standard (20 nmoles).



Supplemental Figure 3.2. UV/vis absorbance spectra of the reaction product eluting at 20.8 min shown in Supplemental Figure 3.1, trace B (red) and a synthetic standard of 1-(2-hydroxyphenoxy)acetone (**5**) (green).

COMT Activity Assay: Time-Course and Enzyme Concentration Dependence. The transfer of the ketone group to catechol by COMT was monitored by HPLC. The assay solutions contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 2 mM $MgCl_2$, 1 μM MTAN, 400 μM keto-AdoMet, 2 mM catechol, and variable COMT concentrations (0, 2, 5, and 10 μM). Reactions were initiated with COMT and incubated at 37 °C. Aliquots (10 μL) were removed at the specified time points and analyzed directly by HPLC.



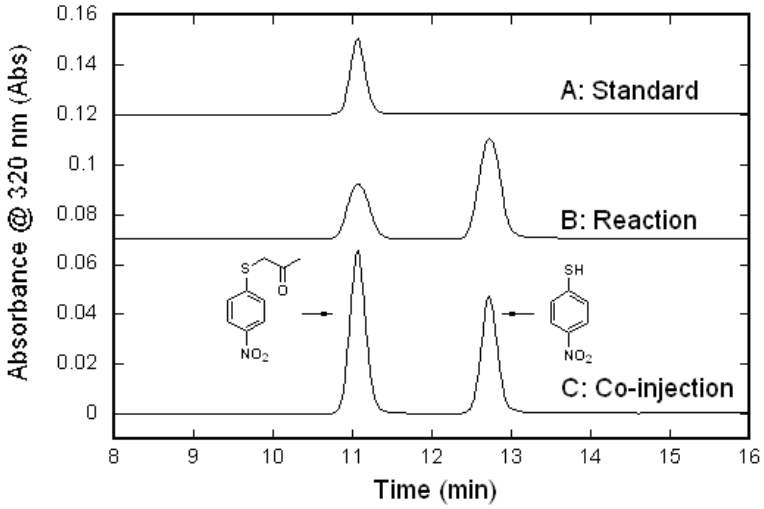
Supplemental Figure 3.3. The time and COMT concentration-dependent formation of 1-(2-hydroxyphenoxy)acetone (**5**). The enzyme concentrations were 0 μM (○), 2 μM (□), 5 μM (◊), and 10 μM (×).

4. Thiopurine *S*-Methyltransferase (TPMT).

4.1 Substrate Specificity.

Thiopurine *S*-Methyltransferase UV-vis Assays. TPMT catalyzed transfer of the ketone group from **1** to 4-nitrobenzenethiol was monitored continuously following the disappearance of the thiol substrate at 411 nm as described.[2] The assay solution contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN, 50 μM 4-nitrobenzenethiol, and 200 μM keto-

AdoMet at 37 °C. The reaction was initiated with 3 μM TPMT or no enzyme as control. To confirm the transfer of the ketone group, a parallel reaction was analyzed by HPLC. At specific time points (typically 0, 30, 60 and 120 min), aliquots of 20 μL were removed from the reaction and analyzed directly by HPLC with monitoring at 320 nm. The chromatography was performed using 0.1% aqueous TFA (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 1 mL/min. The gradient program was initiated with 40% acetonitrile for 5 min followed by a linear increase to 60% acetonitrile over 15 min, then a hold at 60% acetonitrile for 5 min, and finally a return to 40% acetonitrile over 5 min.

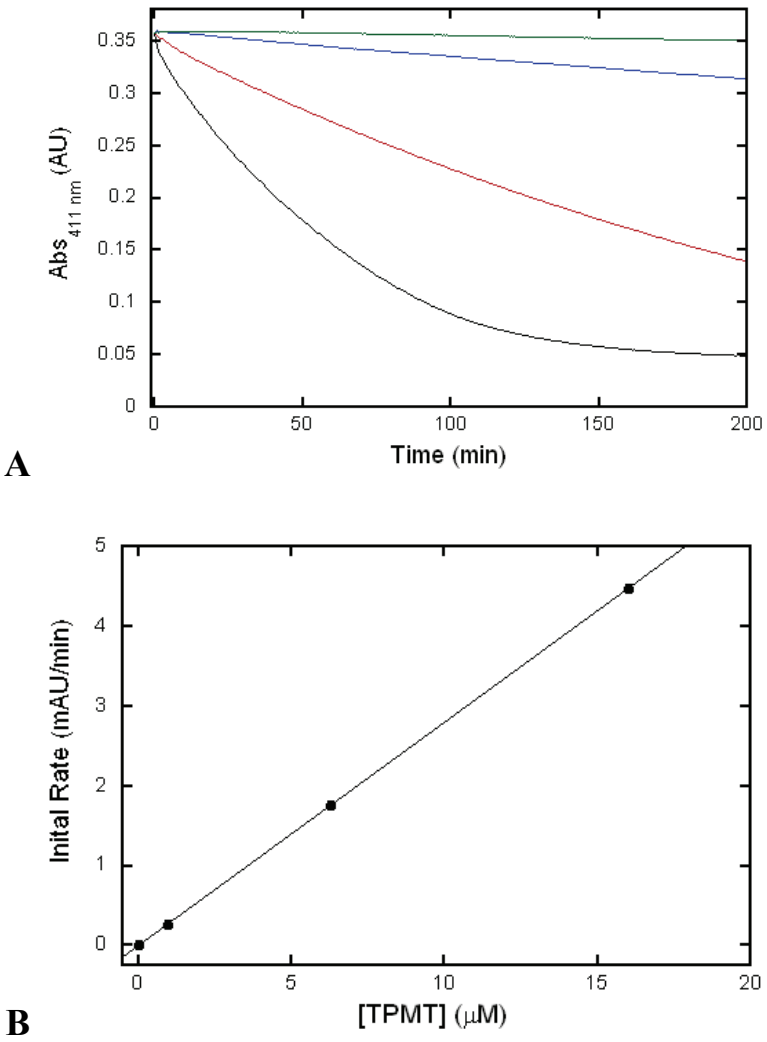


Supplemental Figure 4.1.1. Representative chromatogram showing the transfer of the ketone moiety to 4-nitrobenzenethiol. A) Synthetic **2** eluting at 11.1 min. B) TPMT catalyzed reaction containing 4-nitrobenzenethiol (eluting at 12.7 min) and keto-AdoMet. C) Co-injection of the reaction product with synthetic **2**.

HPLC Activity Assays. HPLC was performed to monitor the transfer of the ketone or the methyl group to compounds other than 4-nitrobenzenethiol. The assays contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN and either 200 μM keto-AdoMet or

100 μM AdoMet. Final concentrations of the testing compounds were 50 μM, except for reactions containing 4-methoxybenzylthiol and 4-nitrophenol which were conducted at 500 μM. For keto-AdoMet activity assays, the reactions were initiated with 3 μM TPMT, except for 4-methoxybenzylthiol and 4-nitrophenol reactions which were initiated with 10 μM enzyme. For assays containing AdoMet, 16 nM TPMT was added to initiate reactions. In all cases, the reactions were carried out at 37 °C and aliquots of 20 μL (typically at 0, 30, 60 and 120 min) were removed and analyzed by HPLC with monitoring at 280 nm. The gradient program described above for 4-nitrobenzenethiol was used for all analyses except for 4-methoxybenzylthiol assays which were analyzed using the following conditions: 50% acetonitrile for 5 min followed by a linear increase to 70% acetonitrile over 15 min, then a hold at 70% acetonitrile for 3 min, and finally a return to 40% acetonitrile over 2 min. Peaks corresponding to the methylated or alkylated products were confirmed by co-injection with authentic or synthetic standards.

Time-Course and Enzyme Concentration Dependence Assays. The transfer of the ketone moiety to 4-nitrobenzenethiol by TPMT was monitored continuously at 411 nm. The assay solutions contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 40 μM keto-AdoMet, 30 μM 4-nitrobenzenethiol, 1 μM MTAN, and variable TPMT concentrations (0, 1, 6, and 16 μM). Reactions were initiated with enzyme and incubated at 37 °C.

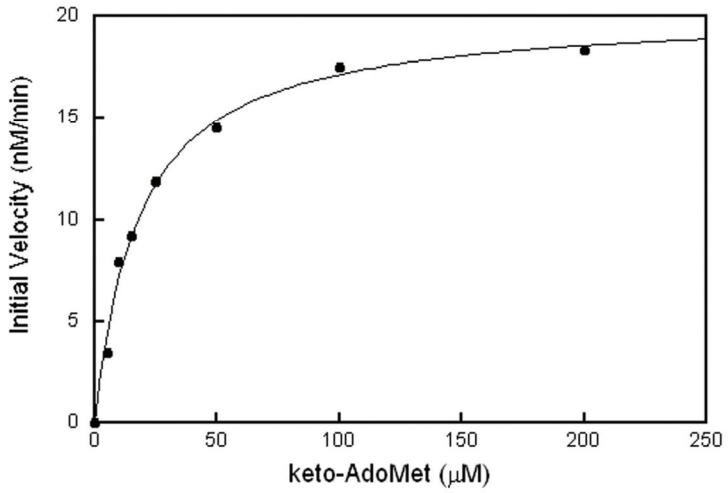


Supplemental Figure 4.1.2. A) The time and TPMT concentration-dependent transfer of the ketone group to 4-nitrobenzenethiol. The TPMT concentrations were 0 μM (green), 1 μM (blue), 6 μM (red), and 16 μM (black). B) Linear dependence of the initial rates of activity on TPMT concentration.

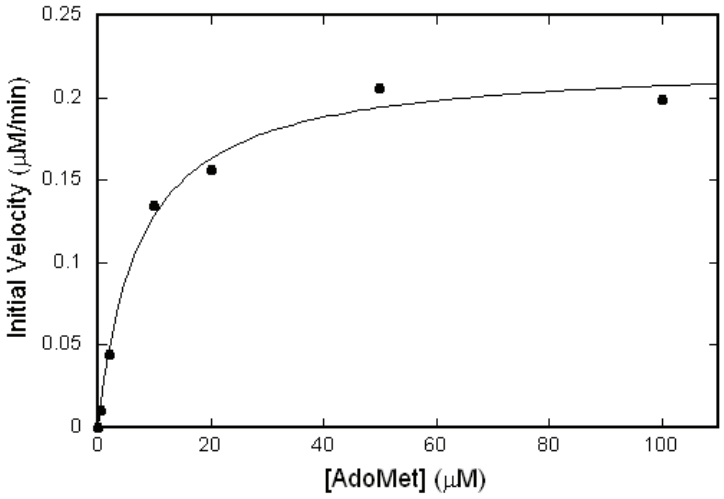
4.2. Steady State Kinetics of Keto-AdoMet and AdoMet.

Alkyl Donor. Steady-state kinetic parameters for keto-AdoMet and AdoMet following the transfer of the ketone or the methyl group to 4-nitrobenzenethiol were determined using the UV/vis method described above. For kinetic analysis of keto-AdoMet, the assays contained 200

mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN and variable concentrations (0–200 μM) of keto-AdoMet. This mixture was incubated at 37 °C for 5 min prior to the addition of 60 μM 4-nitrobenzenethiol and 0.16 μM TPMT (final concentrations). Parallel reactions containing no enzyme were carried out and the background rate was subtracted from the enzyme catalyzed reaction. For kinetic analysis of AdoMet, the assays solutions were the same except with variable concentrations (0-100 μM) of AdoMet as alkyl donor. The reaction was initiated with 0.16 μM TPMT and incubated at 37 °C with monitoring at 411 nm.



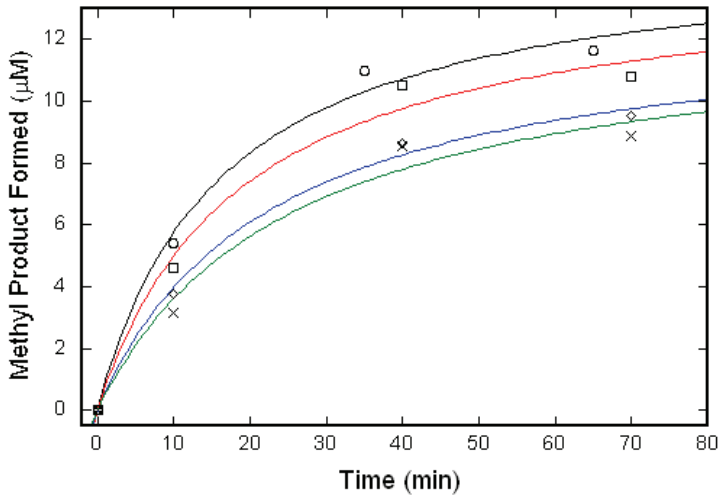
Supplemental Figure 4.2.1. Michaelis-Menten kinetics for the TPMT-catalyzed transfer of the ketone group to 4-nitrobenzenethiol. The data were fit to the Michaelis-Menten equation and the kinetic parameters K_M and k_{cat} were 17.9 ± 1.5 μM and 0.13 ± 0.01 min^{-1}, respectively. The data points represent the average of at least three experiments performed in duplicate.



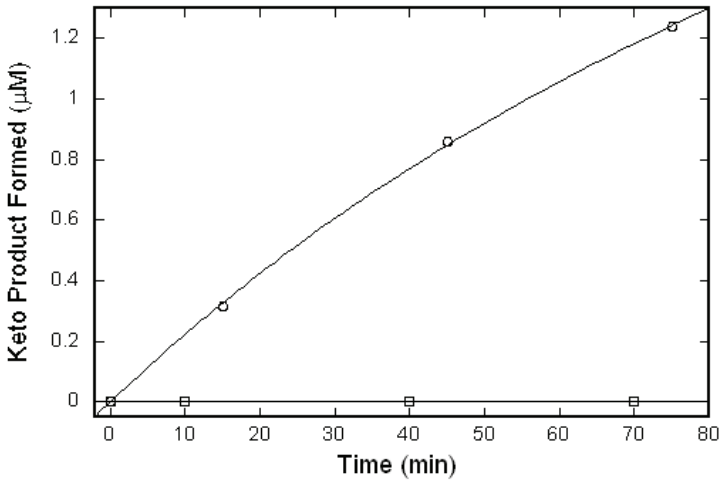
Supplemental Figure 4.2.2. Steady-state kinetic analysis for the TPMT catalyzed methylation of 4-nitrobenzenethiol. The data were fit to the Michaelis-Menten equation and the kinetic parameters K_M and k_{cat} were 6.7 ± 0.7 μM and 13.6 ± 0.4 min^{-1}, respectively. The data points represent the average of at least three experiments performed in duplicate.

4.3. Keto-AdoMet and AdoMet Competition Assays.

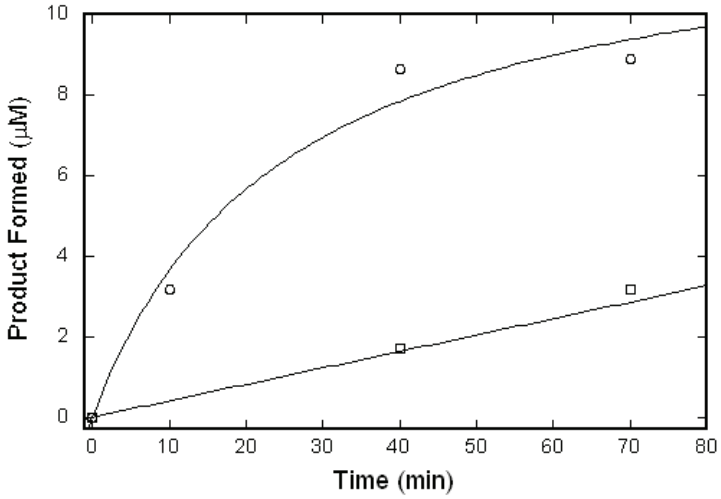
Alkyl Donor Competition Assays. The competition between AdoMet and keto-AdoMet for the TPMT catalyzed reaction was monitored by HPLC. The assay solutions contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 2 μM MTAN, 60 μM 4-nitrobenzenethiol, 20 μM AdoMet, and variable concentrations (20, 100 or 200 μM) of keto-AdoMet. Reactions were initiated by the addition of 0.16 μM TPMT followed by incubation at 37 °C. Timed aliquots were removed and analyzed by HPLC. Quantification of the methyl and ketone products formed was determined using standard curves generated from authentic standards.



Supplemental Figure 4.3.1. Competition assay between AdoMet and keto-AdoMet for the TPMT catalyzed reaction. The formation of methyl product in a reaction containing AdoMet (20 μM) is inhibited by the presence of keto-AdoMet (0 μM, ○; 20 μM, □; 100 μM, ◊; 200 μM, ×).



Supplemental Figure 4.3.2. Competition assay between AdoMet and keto-AdoMet for the TPMT catalyzed reaction. The formation of **2** in a reaction containing keto-AdoMet (20 μM) is inhibited by the presence of AdoMet (0 μM, ○; 20 μM, □).



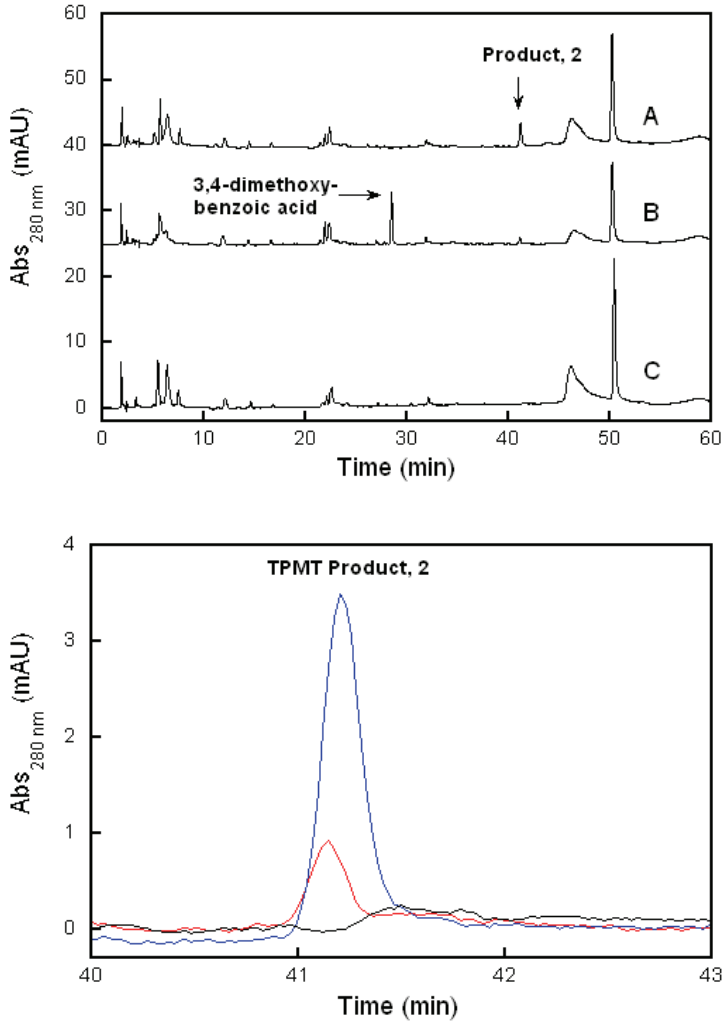
Supplemental Figure 4.3.3. Product distribution of 4-nitrophenyl methyl sulfide (○) and **2** (□) in a reaction containing 0.16 μM TPMT, 20 μM AdoMet and 200 μM keto-AdoMet.

4.4. ex vivo Labeling of Substrate.

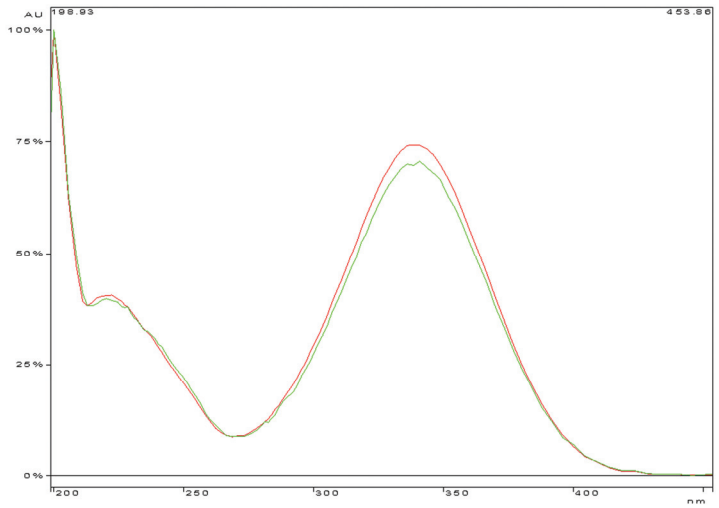
Preparation of Red Blood Cell Lysate. Human whole blood in sodium citrate was obtained from Lampire Biological Laboratories (Pipersville, PA) and red blood cell lysate was prepared following described protocols.[11,12] Erythrocytes were separated by centrifugation (1200 × *g*, 5 min) and the supernatant containing plasma was discarded. The pellet was washed twice gently with 0.9 % NaCl followed by lysis of the erythrocytes (200 μL) by the addition of 800 μL ice-cold water. The lysate was centrifuged (5000 × *g*, 10 min), and the supernatant was collected and stored at −80 °C until further use. TPMT protein is stable for over four weeks in our hands and as previously reported.[11,13,14]

Red Blood Cell Lysate Assay and Specific Inhibition of TPMT Activity. The assay solution with keto-AdoMet contained 200 mM potassium phosphate (pH 8.0), 5 mM TCEP, 1 μM MTAN, 400 μM keto-AdoMet, and 100 μM 4-nitrobenzenethiol in a total volume of 100 μL.

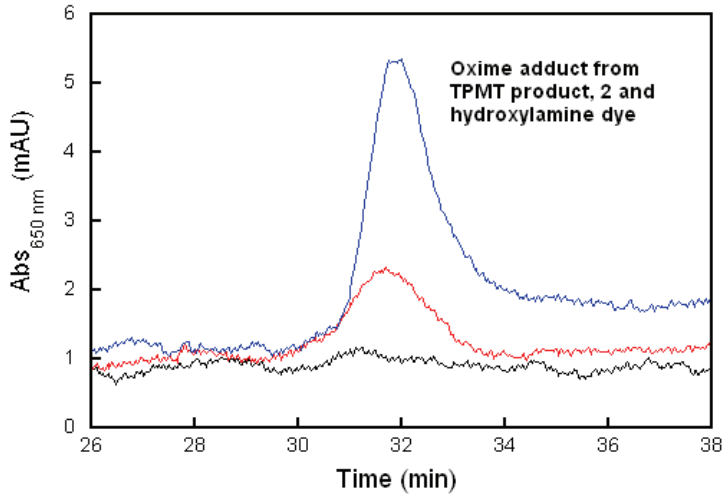
Reactions without keto-AdoMet and a reaction containing 100 μM 3,4-dimethoxybenzoic acid (TPMT inhibitor, reported K_i 10 μM, ref. 7b in the main text) and without MTAN (enzyme hydrolyzes AdoHcy, a feedback inhibitor of TPMT) were carried out in parallel. The reactions were initiated by the addition of 200 μL of the prepared lysate and incubated at 37 °C for 12 h. After incubation, 0.5 M trichloroacetic acid (50 μL) was added and the mixture was vortexed (1 min) to precipitate proteins. An aliquot of the supernatant (15 μL) was derivatized in a reaction containing 200 mM MES (pH 5.5) and 50 μM Alexa Fluor 647 C5-aminooxyacetamide. The labeling step was allowed to proceed in the dark for 2 h at room temperature and then subjected to analytical HPLC with monitoring at 280 nm and 650 nm under the following conditions: 1% acetonitrile for 5 min followed by a linear increase to 50% acetonitrile over 40 min, then a hold at 50% acetonitrile for 5 min, and finally a return to 1% acetonitrile over 5 min. The flow rate was 1 mL/min.



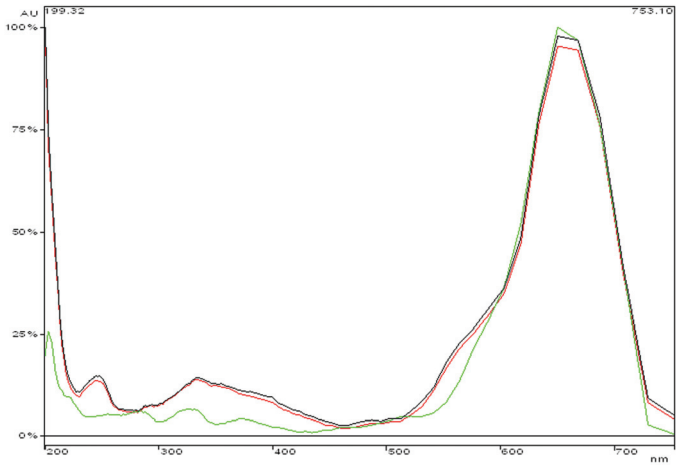
Supplemental Figure 4.4.1. top. HPLC analysis (280 nm) of the RBC lysate reactions after treatment with fluorophore. A) RBC reaction without inhibitor. B) Reaction performed in the presence of 100 μM 3,4-dimethoxybenzoic acid (elutes at 28.8 min) and without MTAN. C) A trace of the reaction mixture performed without keto-AdoMet. Bottom. Overlay trace showing the production of **2** in lysate reactions containing no keto-AdoMet (black), in the presence of inhibitor (red), and in the absence of inhibitor (blue). Based on the peak area, the formation of **2** is inhibited by 70% in the presence of 100 μM 3,4-dimethoxybenzoic acid.



Supplemental Figure 4.4.2. UV/vis absorbance spectra of the products eluting at 42 min as shown in Supplemental Figure 4.4.1 (green, ketone product generated in the lysate assay containing keto-AdoMet; red, compound **2**).

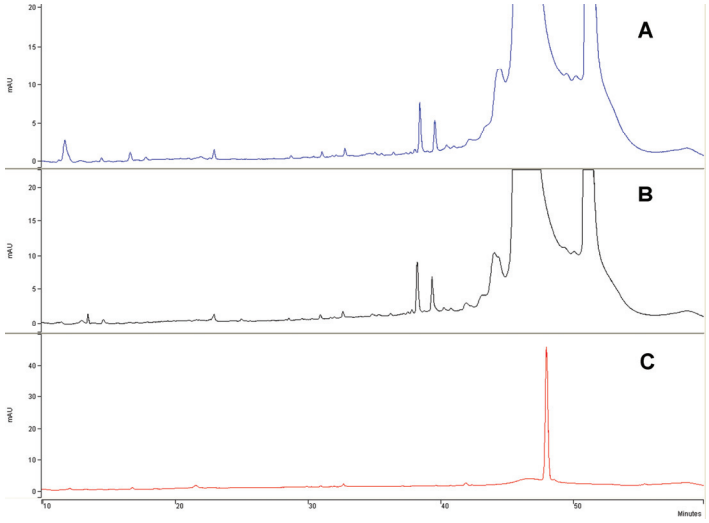


Supplemental Figure 4.4.3. HPLC analysis (650 nm) of lysate reactions after treatment with hydroxylamine fluorophore. Overlay of the traces from a reaction conducted without keto-AdoMet (black), a reaction in the presence of 100 μM 3,4-dimethoxybenzoic acid and without MTAN (red), and a reaction without inhibitors and with MTAN (blue).

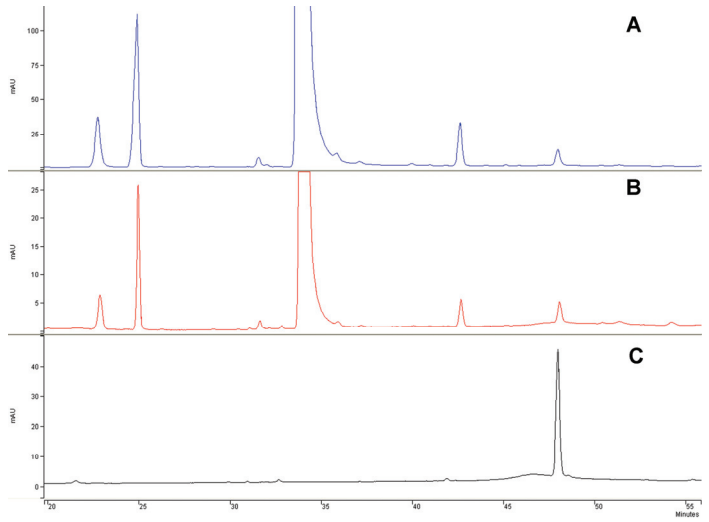


Supplemental Figure 4.4.4. UV/vis absorbance spectra of Alexa Fluor 647 hydroxylamine (green), the product generated from a coupling reaction between **2** and Alexa Fluor 647 hydroxylamine (black), and the product eluting ~33 min from the RBC lysate containing 4-nitrobenzenethiol and keto-AdoMet after treatment with Alexa Fluor 647 hydroxylamine (red).

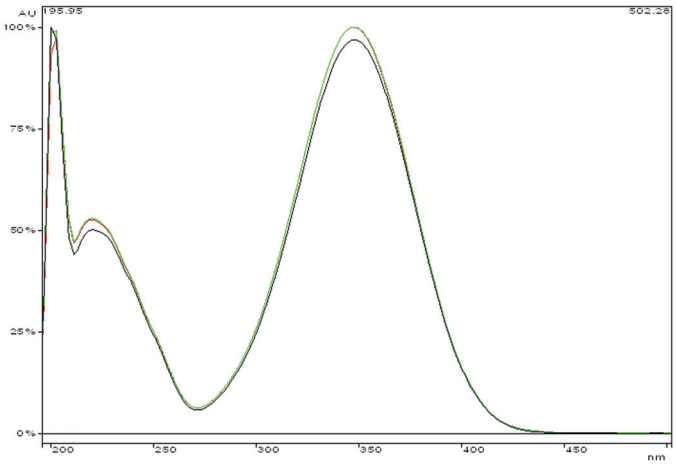
Cell Lysate Assay with AdoMet. The assay solution contained 200 mM potassium phosphate (pH 8.0), 5 mM TCEP, 1 μM MTAN, 200 μM AdoMet or no AdoMet, and 100 μM 4-nitrobenzenethiol in a total volume of 30 μL. The reaction was initiated by the addition of 70 μL lysate and incubated at 37 °C for 2 h. After incubation, 0.5 M trichloroacetic acid (15 μL) was added and the mixture was vortexed (1 min) to precipitate proteins. The resulting supernatant was analyzed directly by HPLC using the method described above. To confirm formation of the methyl sulfide product, water (200 μL) was added to the supernatant followed by liquid extraction with ethyl acetate (4 x 200 μL), concentration of the organics to ~25 μL under a stream of nitrogen and then analysis by HPLC using the method described above.



Supplemental Figure 4.4.5. HPLC analysis (280 nm) of the RBC lysate assay containing 100 μM 4-nitrobenzenethiol in the absence (A) and presence (B) of 200 μM AdoMet after 2 h reaction time. C) Standard of 4-nitrophenyl methyl sulfide (10 nmoles) eluting at 48 min. Detection of 4-nitrophenyl methyl sulfide is masked by the overlapping heme peaks between 44-54 min.



Supplemental Figure 4.4.6. HPLC analysis (280 nm) of the organic extracts from the RBC lysate assay containing 4-nitrobenzenethiol and AdoMet. A) Organic extract of the assay solution. B) Co-injection of the reaction with 4-nitrophenyl methyl sulfide. C) Standard of 4-nitrophenyl methyl sulfide standard eluting at 48 min.

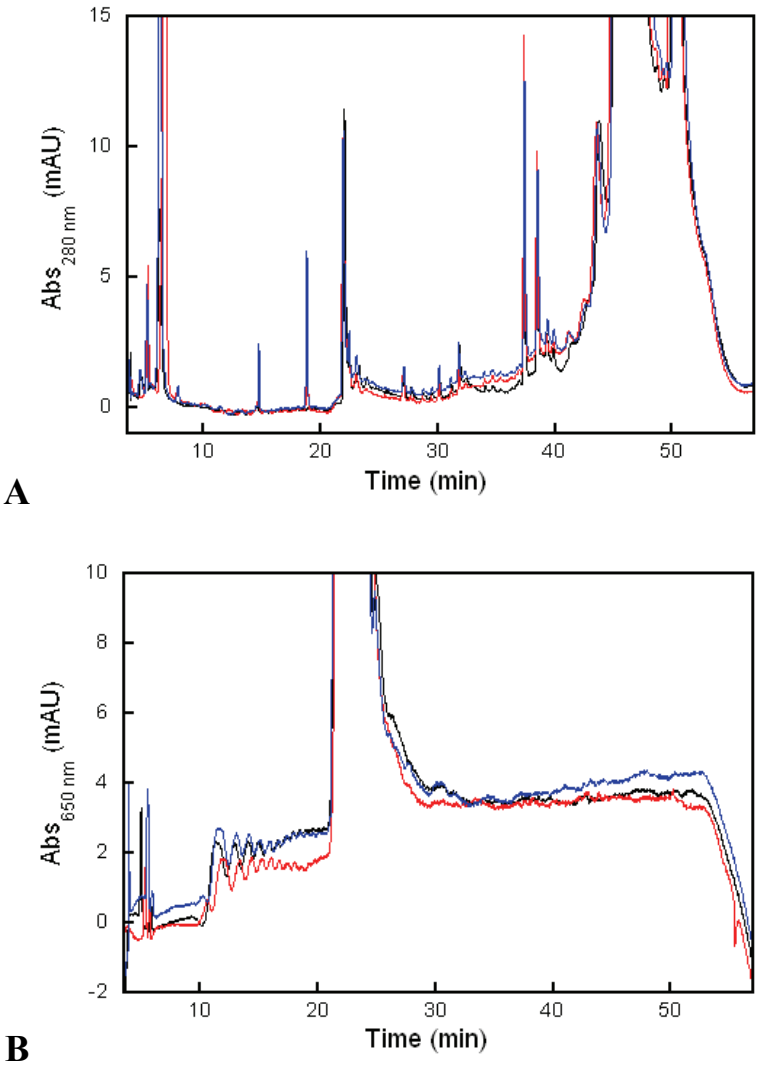


Supplemental Figure 4.4.7. UV/vis absorbance spectra of the products eluting at 48 min as shown in Supplemental Figure 4.4.6 (black, organic extract; red, co-injection with 4-nitrophenyl methyl sulfide; green, standard of 4-nitrophenyl methyl sulfide).

5. Identification of Unknown Substrates of TPMT.

5.1. ex vivo Assay for Endogenous Substrates of TPMT.

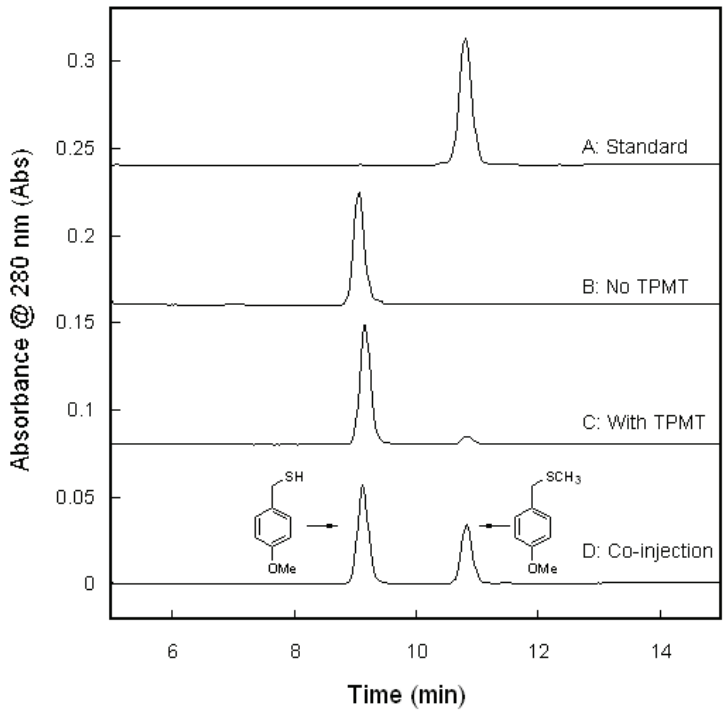
Detection of Putative Endogenous TPMT Substrates in Red Blood Cell Lysate. An assay containing 200 mM potassium phosphate (pH 8.0), 5 mM TCEP, 1 μM MTAN, and 400 μM keto-AdoMet was prepared to a total volume of 30 μL. Solutions containing the above components without keto-AdoMet, and an assay solution supplemented with recombinant TPMT (10 μM) was carried out in parallel. The reactions were initiated by the addition of 70 μL cell lysate and incubated at 37 °C for 8 hours. After 8 hours, an additional equivalent of keto-AdoMet was added and the reactions were further incubated overnight. After incubation, 0.5 M trichloroacetic acid (15 μL) was added and the mixture was vortexed (1 min) to precipitate proteins. An aliquot of the supernatant (15 μL) was derivatized in a reaction containing 200 mM MES (pH 5.5) and 100 μM Alexa Fluor 647 C5-aminooxyacetamide. The analysis was carried out by HPLC using the conditions described above.



Supplemental Figure 5.1.1. Detection of putative endogenous TPMT substrates in red blood cell lysate. A) Chromatograms (280 nm) of RBC lysate reactions after treatment with fluorophore. The traces represent reactions performed in the absence of keto-AdoMet (black), with keto-AdoMet (red) and an assay containing both keto-AdoMet and purified recombinant TPMT (10 μM, blue). B) The above traces monitored at 650 nm.

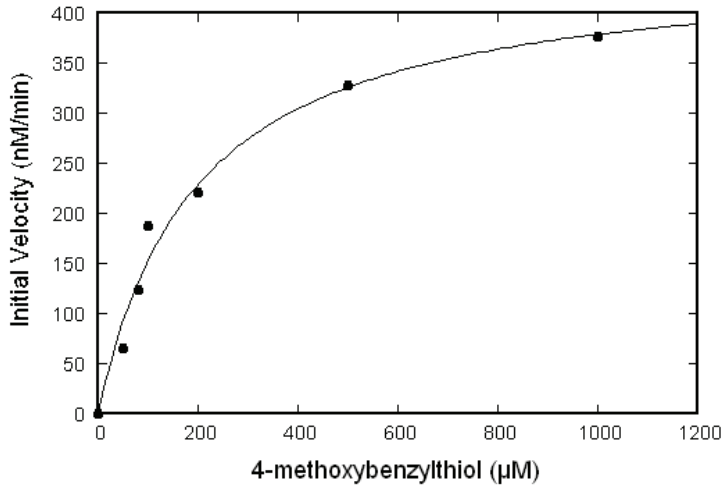
5.2. Characterization of 4-Methoxybenzylthiol as a Substrate of TPMT.

HPLC Activity Assays. The assays contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN, 500 μM 4-methoxybenzylthiol and 100 μM AdoMet. The reactions were initiated with 10 μM TPMT. The reactions were carried out at 37 °C and aliquots of 20 μL (typically at 0, 30, 60 and 120 min) were removed and analyzed by HPLC with monitoring at 280 nm. The following gradient was used: 50% acetonitrile for 5 min followed by a linear increase to 70% acetonitrile over 15 min, then a hold at 70% acetonitrile for 3 min, and finally a return to 40% acetonitrile over 2 min. Peaks corresponding to the methylated products were confirmed by co-injection with synthetic standard.



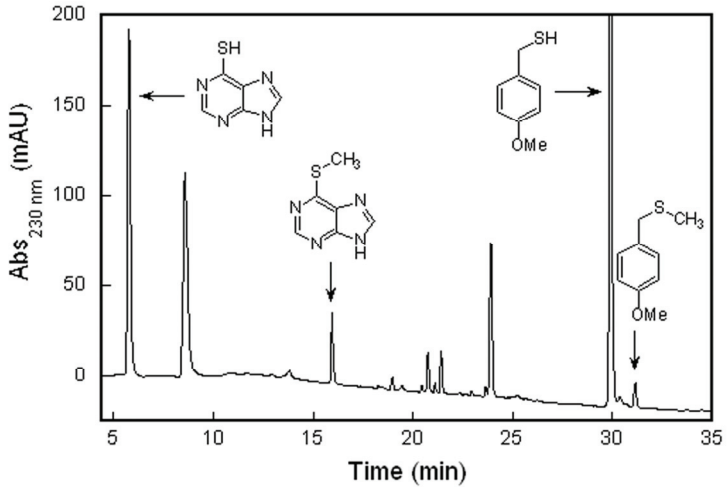
Supplemental Figure 5.2.1. The TPMT catalyzed methylation of 4-methoxybenzylthiol. A) 4-Methoxybenzyl methyl sulfide standard eluting at 10.8 min. B) A solution containing 500 μM 4-methoxybenzylthiol (eluting at 9.2 min) and 100 μM AdoMet without TPMT after 90 min. C) The same solution as (B) at 90 min except with 0.16 μM TPMT. D) Co-injection of the TPMT reaction product with synthetic 4-methoxybenzyl methyl sulfide.

Steady State Kinetics of 4-Methoxybenzylthiol. Kinetic parameters for the TPMT catalyzed methylation of 4-methoxybenzylthiol were measured using the HPLC assay described above. The assays contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN, 200 μM AdoMet and variable 4-methoxybenzylthiol (0–1000 μM). The reactions were initiated by the addition of 0.16 μM TPMT followed by incubation at 37 °C. Timed aliquots were removed and analyzed by HPLC. The amount of product formed was determined using an HPLC standard curve generated for 4-methoxybenzyl methyl sulfide.

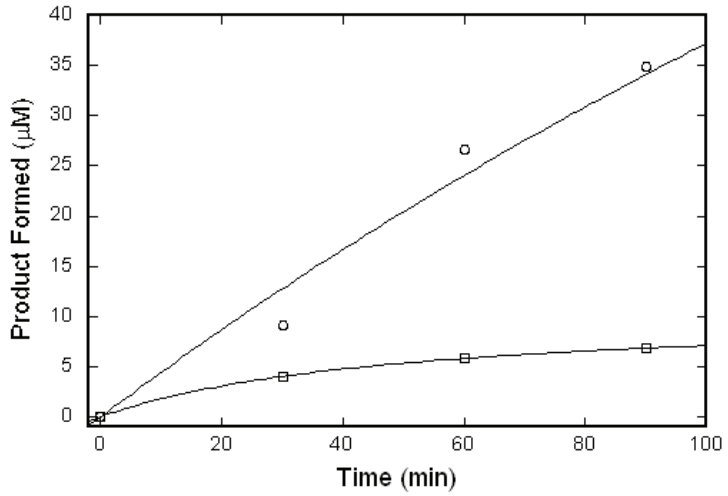


Supplemental Figure 5.2.2. Steady-state kinetic analysis for the TPMT catalyzed methylation of 4-methoxybenzylthiol. The data were fit to the Michaelis-Menten equation and the kinetic parameters K_M and k_{cat} were 195 ± 35 μM and 2.9 ± 0.2 min^{-1}, respectively. The data points represent the average of at least three experiments performed in duplicate.

Competition Assays of 4-Methoxybenzylthiol and 6-Thiopurine. The competition between 6-thiopurine and 4-methoxybenzylthiol for turnover by TPMT was monitored by HPLC. The assays contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 1 μM MTAN, 200 μM AdoMet, 200 μM 6-thiopurine, and 200 μM 4-methoxybenzylthiol. The reaction was initiated by the addition of 0.16 μM TPMT followed by incubation at 37 °C. Timed aliquots were removed and analyzed by HPLC according to the following conditions: 4% acetonitrile for 6 min followed by a linear increase to 80% acetonitrile over 29 min, then a hold at 80% acetonitrile for 5 min, and finally a return to 4% acetonitrile over 5 min. Quantification of the methyl products formed was determined using standard curves generated from authentic standards.



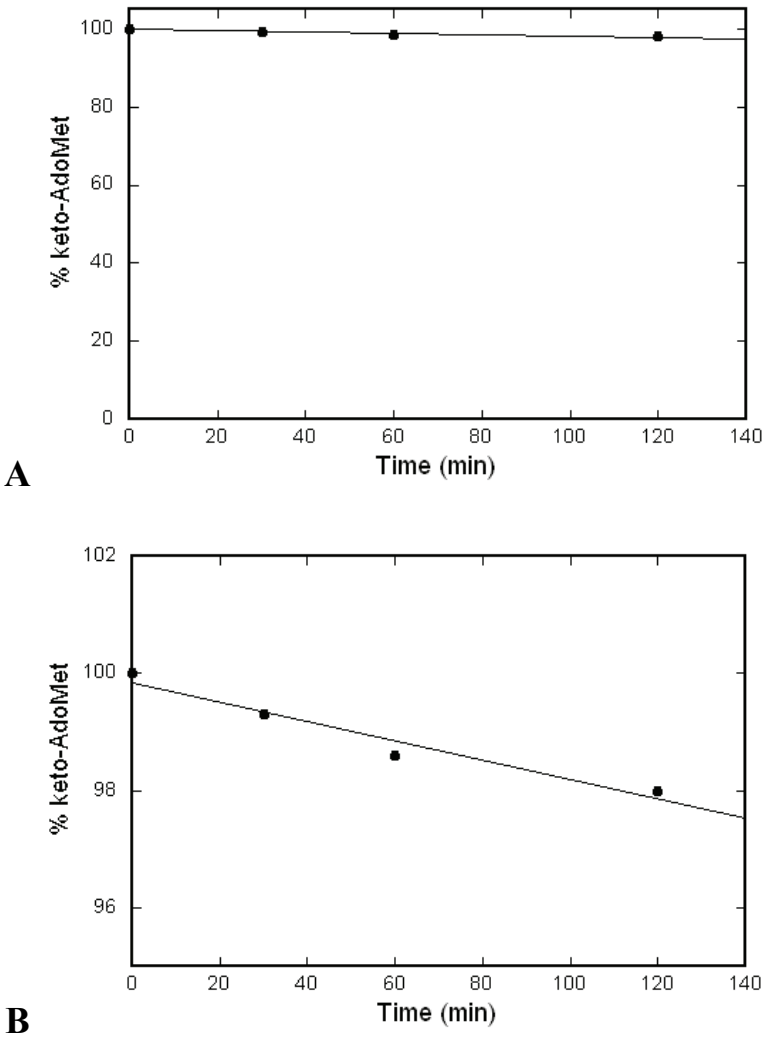
Supplemental Figure 5.2.3. HPLC chromatogram of a competition assay between 6-thiopurine (200 μM) and 4-methoxybenzylthiol (200 μM) in the presence of 200 μM AdoMet. Under these conditions, the corresponding products were formed at a ratio of ~ 4 to 1 (6-methylthiopurine to 4-methoxybenzyl methyl sulfide).



Supplemental Figure 5.2.4. TPMT competition assay between 6-thiopurine (200 μM) and 4-methoxybenzylthiol (200 μM) and the formation of 6-(methylthio)purine (○) and 4-methoxybenzyl methyl sulfide (□).

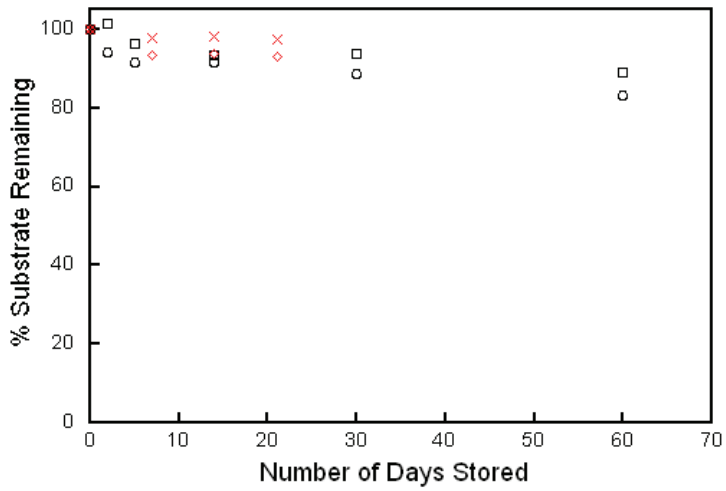
6. Stability and Reactivity of Keto-AdoMet.

Keto-AdoMet Stability. The stability of keto-AdoMet under the enzyme assay conditions were monitored by HPLC. A solution containing 200 mM potassium phosphate (pH 8.0), 2 mM TCEP and 100 μM keto-AdoMet was incubated at 37 °C. Aliquots were removed at 0, 30, 60, and 120 min and subjected to analytical HPLC using the keto-AdoMet purification conditions described above. The traces indicate that adenine and AdoHcy are among the degradation products of keto-AdoMet. The observation of these products suggests keto-AdoMet degrades in a manner similar to the reported pathways of AdoMet degradation.[15,16]



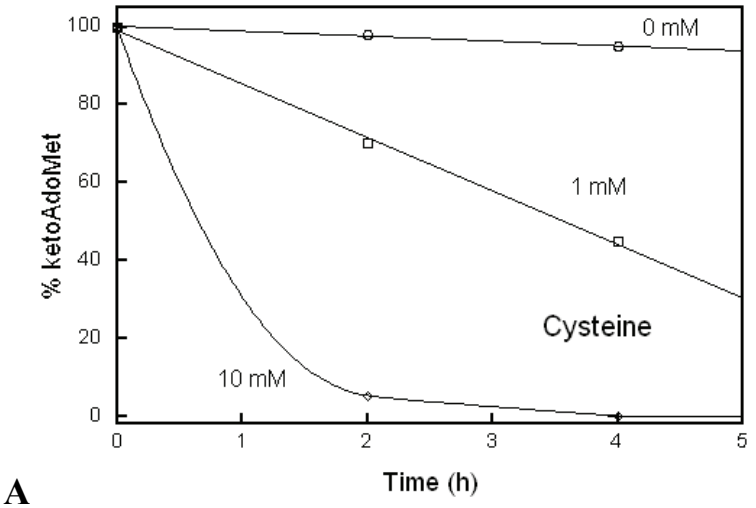
Supplemental Figure 6.1. A) Stability of keto-AdoMet under the assay conditions. B) Expanded view of the data indicates keto-AdoMet does not undergo significant degradation during the time course of the assay and its composition remains greater than 98 % after 2 h incubation at 37 °C.

Long-term Storage Stability. To monitor the long-term storage stability of keto-AdoMet, aliquots of purified compound were stored in 0.1% TFA at both −20 °C and −80 °C and analyzed by HPLC at intervals over a two-month period. For comparison, AdoMet was prepared, stored and analyzed in the same manner.

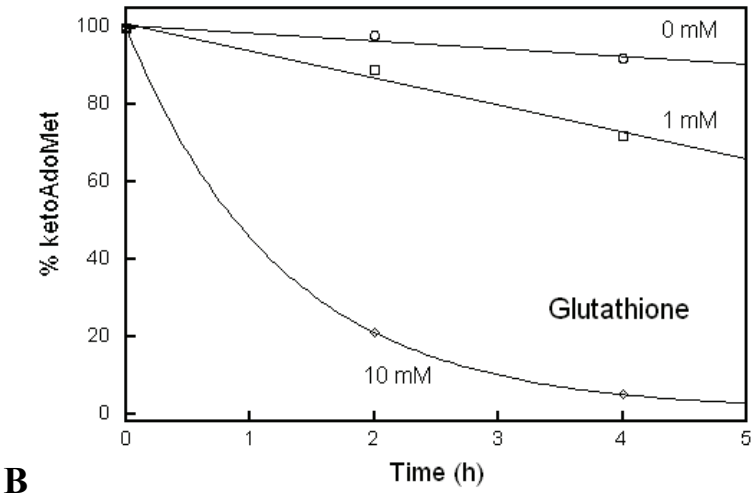


Supplemental Figure 6.2. Storage stability of keto-AdoMet and AdoMet. Keto-AdoMet (0.1% aqueous TFA, final pH 2.0) was stored at both –20 °C (○) and –80 °C (□) and tested at the indicated time points. Under either condition, keto-AdoMet is stable and remains greater than 80% pure after two months storage. The analog behaves similarly to AdoMet stored at –20 °C (◊) and –80 °C (×).

Keto-AdoMet Stability in the Presence of Free Thiols. The stability of keto-AdoMet under the assay conditions and in the presence of free thiol was measured. A solution containing 200 mM potassium phosphate (pH 8.0), 5 mM TCEP, 100 μM keto-AdoMet and either L-cysteine or glutathione (1 or 10 mM) was incubated at 37 °C. A control assay containing no thiol was prepared and carried out in parallel to monitor keto-AdoMet in the absence of thiols. Aliquots were removed at 0, 2, and 4 h and analyzed using the HPLC conditions described above for the purification of keto-AdoMet.



A

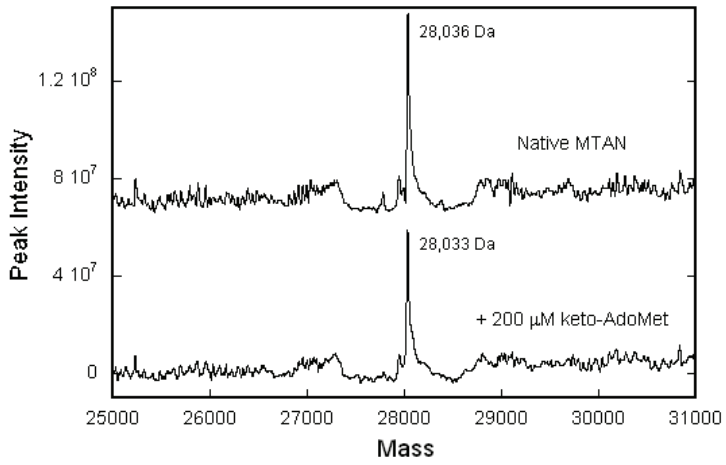


B

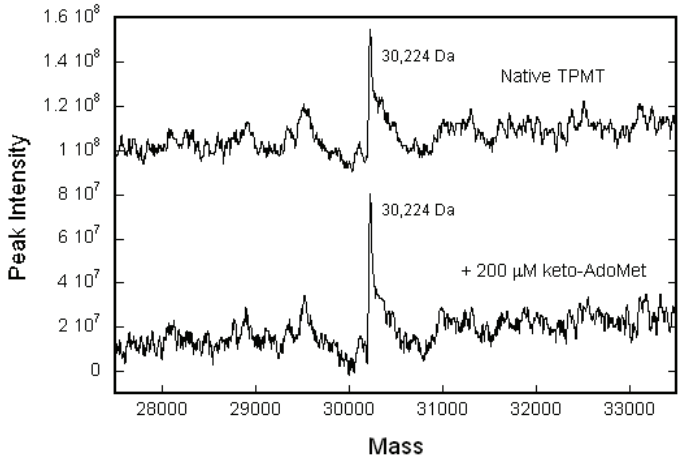
Supplemental Figure 6.3. A) Stability of keto-AdoMet under assay conditions in the absence (○) and presence of 1 mM (□) or 10 mM (◊) of cysteine. B) Stability of keto-AdoMet under assay conditions in the absence (○) and presence of 1 mM (□) or 10 mM (◊) of glutathione. The percentage of keto-AdoMet remaining was calculated as a fraction of the keto-AdoMet present in the solution at time zero.

Modification of MTAN and TPMT by Keto-AdoMet. Mass spectrometry was performed to monitor the potential background transfer of the ketone moiety to proteins. The assay solution

contained 200 mM potassium phosphate (pH 8.0), 2 mM TCEP, 200 μM keto-AdoMet and either TPMT or MTAN (1 μM). Samples of TPMT and MTAN without treatment with keto-AdoMet were prepared as control. The mixtures were incubated at 37 °C for 2 h and analyzed by LC-MS. The proteins were loaded onto a self-packed reversed phase column (75 μm i.d. × 10 cm, Magic C4 resin, 5 μm particle size, 300 Å pore size, Michrom Bioresources, Auburn, CA) and eluted at 200 nL/min. The chromatography was run using 0.1% aqueous formic acid (mobile phase A) and acetonitrile (mobile phase B). The run was initiated at 1% mobile phase B and increased to 30% mobile phase B over 5 min, then increased to 55% mobile phase B over 25 min, and finally increased to 90% mobile phase B over 5 min. The ion transfer tube of the linear ion trap was held at 250 °C and the ion spray voltage was set at 2.3 kV.



Supplemental Figure 6.4. Deconvoluted mass spectra of native MTAN and MTAN after treatment with 200 μM keto-AdoMet indicate the protein is not modified by the analog. Expected averaged mass for MTAN is 28,026 Da.

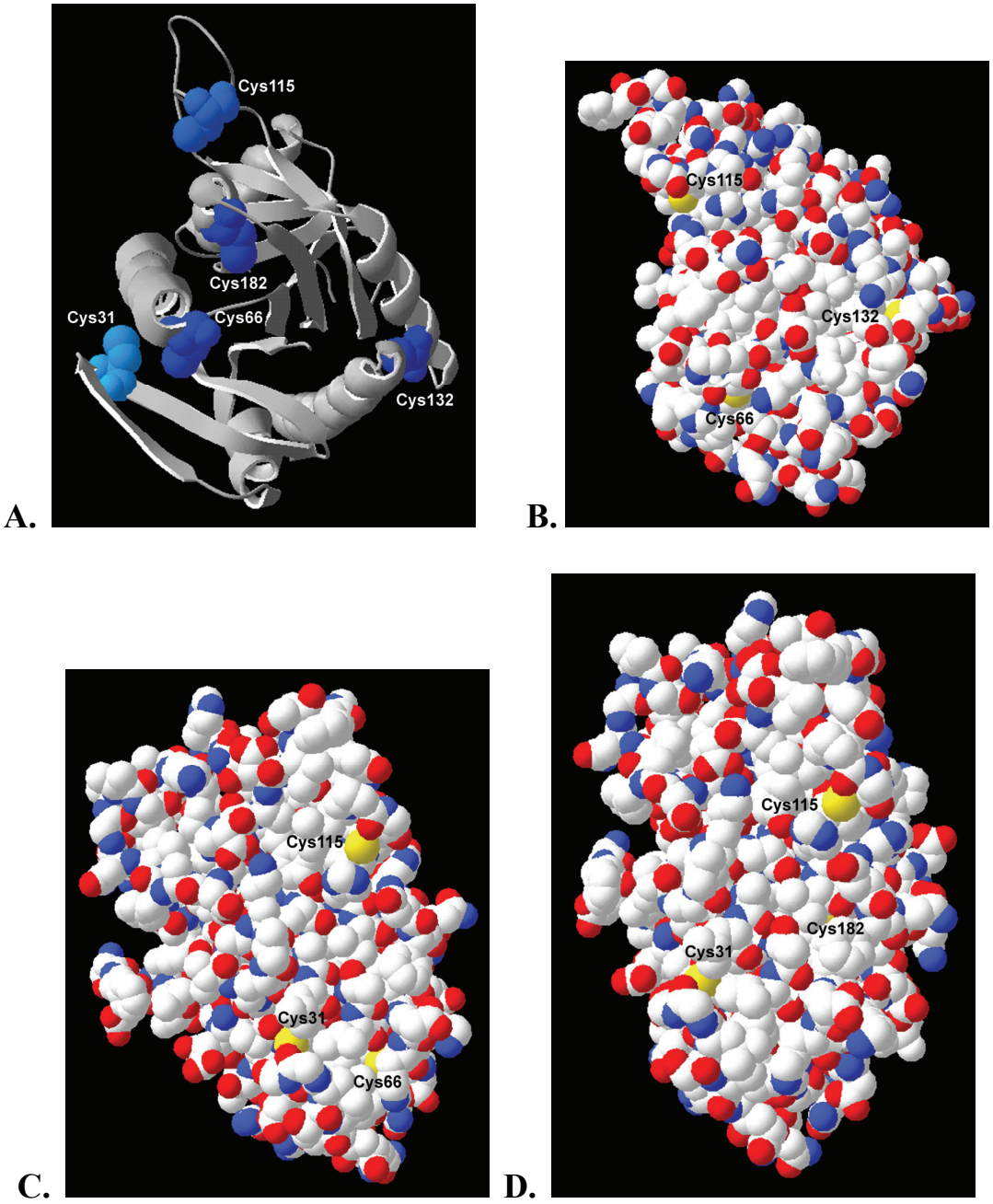


Supplemental Figure 6.5. Deconvoluted mass spectra of native TPMT and TPMT after treatment with 200 µM keto-AdoMet indicate the protein is not modified by the analog. Expected averaged mass for TPMT is 30,212 Da.

Solvent Accessibility of Cysteine Residues in MTAN and TPMT

Supplemental Table 1. Percentage Solvent Accessibility of MTAN Cysteine Residues Predicted using DeepView/ Swiss-PdbViewer 3.7 and PDB Code 1JYS.

Cysteine Residue	% Solvent Accessibility
31	11.4
66	4.1
115	9.1
132	0
182	0

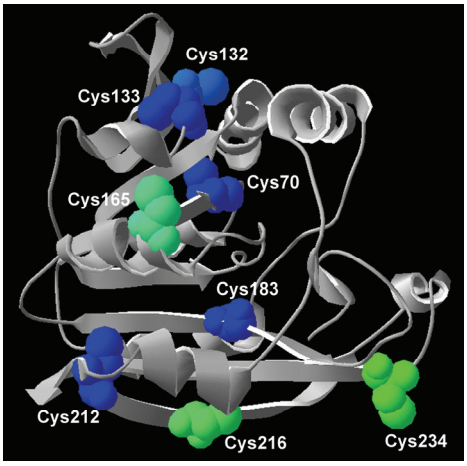


Supplemental Figure 6.6. Images of MTAN illustrating the location and degree of solvent accessibility of cysteine residues in the MTAN structure (A), and surface exposure of cysteine thiols on the protein (B-D).

Supplemental Table 2. Percentage Solvent Accessibility of TPMT Cysteine Residues Predicted using DeepView/ Swiss-PdbViewer 3.7 and PDB Code 2BZG.

Cysteine Residue	% Solvent Accessibility
70	0.5
132	7.3
133	0.5
165	27.9
183	1.8
212	1.8
216	31.1
234	33.3

A.



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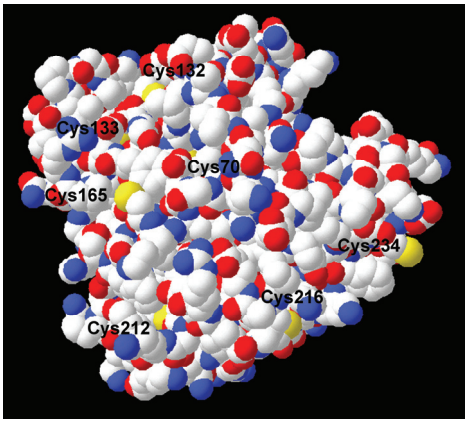
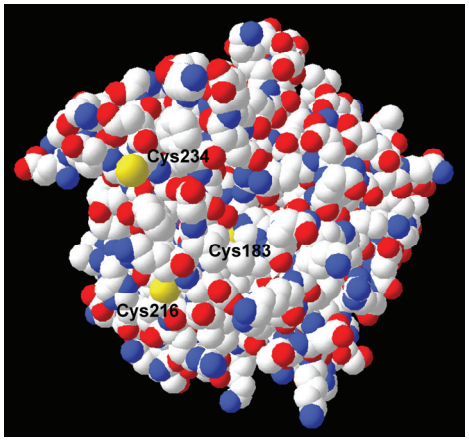


C.



Supplemental Figure 6.7. Images of TPMT illustrating the location and degree of solvent accessibility of cysteine residues in the TPMT structure (A), and surface exposure of cysteine thiols on the protein (B-C).

Acknowledgements. The authors are indebted to Drs. Roger Kautz for his expertise with NMR, Richard Weinshilboum for providing TPMT cDNA, Ken Cornell and Michael Riscoe for providing AdoHcy nucleosidase expression strain.

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