

Figure S1 | S-Adenosylvinthionine Probe Preparation

S-Adenosylvinthionine (AdoVin) is synthesized enzymatically using S-adenosylmethionine synthetase (MAT, EC 2.5.1.6). Vinthionine (1 mM) is combined with adenosine triphosphate (ATP) (2 mM) in the presence of KCl (10 mM) and MgCl₂ (5 mM) in tris buffered saline (pH 7.9). MAT (25 μM) is added to start the reaction, which is then incubated at 37°C for 4 hours. ATP can be labeled with stable isotopes ¹³C and ¹⁵N at the highlighted positions to produce AdoVin with a +15 Da mass shift.

For the *in vitro* samples, the isotopically labeled probe was reacted with the TNB substrate and the TPMT enzyme to form the [TPMT•TNB-AdoVin] complex. Whole cell samples were prepared using unlabeled AdoVin probe, which, along with the TNB substrate, was added to cell lysate from a TPMT-expressing *E. coli* strain to form the complex.

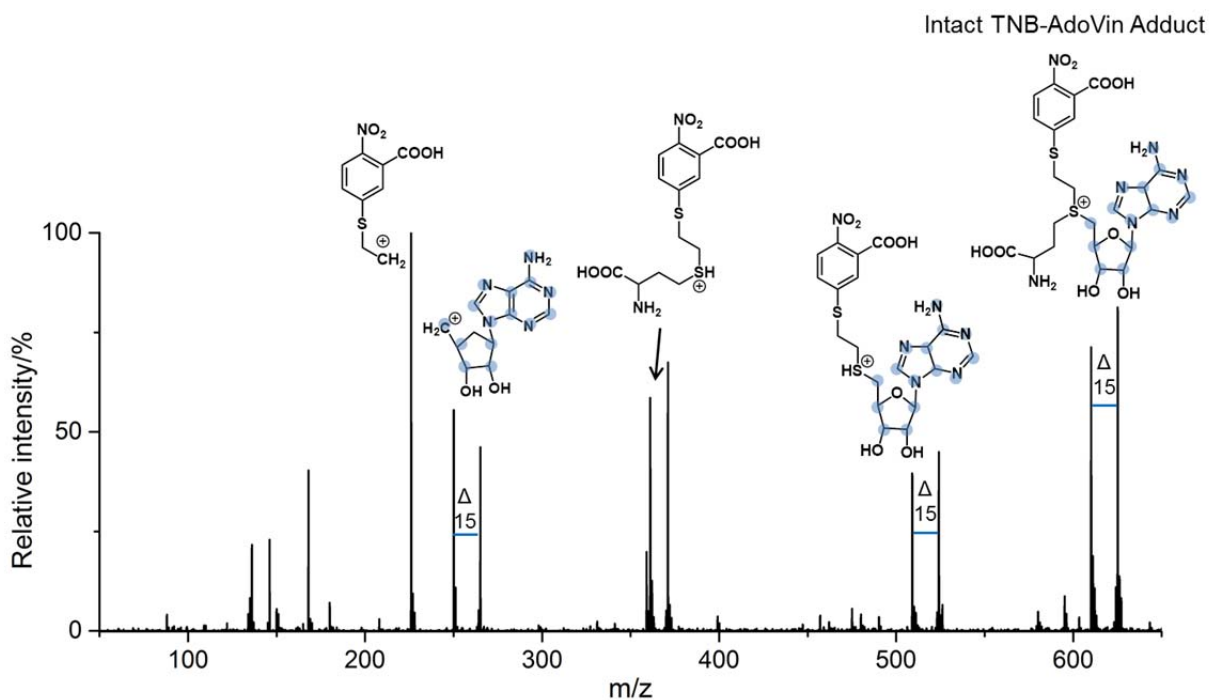


Figure S2 | Zoom in of Collision Induced Dissociation Mass Spectrum of TNB-AdoVin adduct (mixture of heavy and light reagent) (Fig. 3)

CID tandem mass spectrum of precursor ion m/z 3084 ($z=10$) of the [TPMT•TNB-AdoVin] adduct at a collision energy of 1200 eV, which results in dissociation and fragmentation of the bisubstrate adduct (m/z 50 – 650). The TNB-AdoVin adduct is identified intact with the 15 Da stable isotope labeling present in the adenine group (m/z 610, $z=1$; m/z 625, $z=1$). Additional fragments are identified, some containing the adenine isotope flag (indicated by Δ).

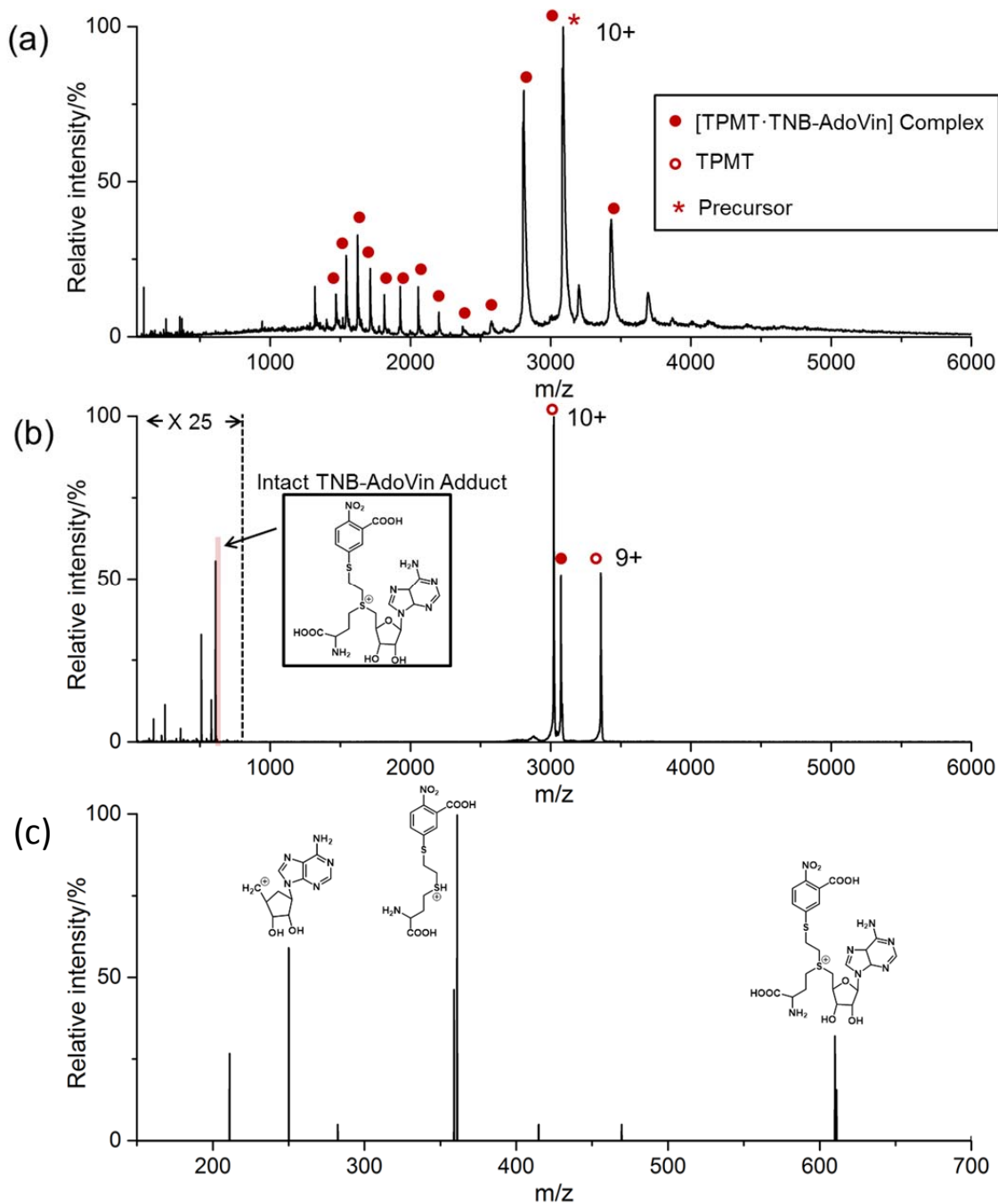


Figure S3 | Quasi MS³ of unlabeled TNB-AdoVin adduct

(a) Mass spectrum of *in vitro* [TPMT•TNB-AdoVin] complex (*) at m/z 3089 ($z=10$).

(b) Tandem mass spectrum after CID at a collision energy of 500 eV of [TPMT•TNB-AdoVin] complex (precursor ion m/z 3089), resulting in apo-TPMT singly charged TNB-AdoVin adduct (highlighted) at m/z 610.

(c) Quasi MS³ spectrum of CID at a collision energy of 20 eV of TNB-AdoVin adduct (precursor ion m/z 610, $z=1$), resulting in various adduct fragments.

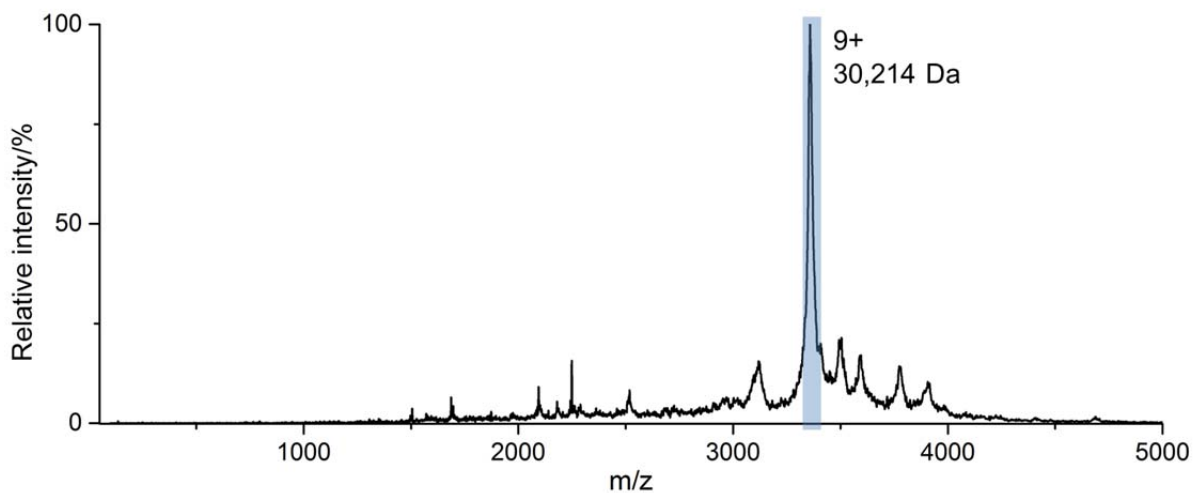


Figure S4 | Mass Spectrum of Apo-TPMT

CID at a collision energy of 1350 eV of precursor ion m/z 3407 ($z=9$, highlighted in blue) dissociates salt adducts and results in TPMT (m/z 3358, $z=9$) with a mass of 30,214 Da. The theoretical mass given the enzyme sequence is 30,343 Da, suggesting N-terminal methionine removal.

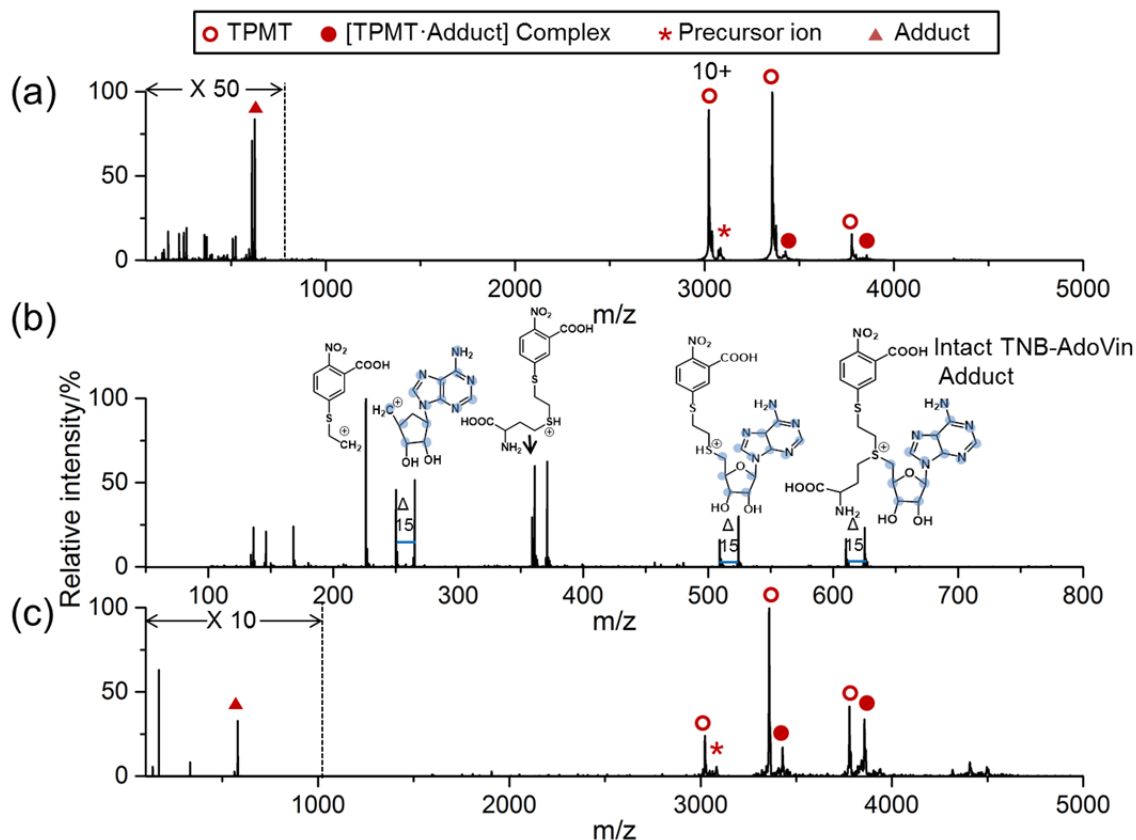


Figure S5 | Surface Induced Dissociation of [TPMT•Adduct] Complex

(a) After initial separation of an *in vitro* sample, surface induced dissociation (SID) tandem mass spectrum of precursor ion m/z 3084 ($z=10$, from Fig. 3a) of the [TPMT•TNB-AdoVin] adduct (star) at a collision energy of 500 eV. After dissociation, apo- (open circles) and adduct-bound (closed circles) enzyme are observed in multiple charge states. The singly-charged TNB-AdoVin adduct (triangle) is observed with the 15 Da pairing due to stable isotope labeling (m/z 610 and 625).

(b) After initial separation of an *in vitro* sample, SID tandem mass spectrum of precursor ion m/z 3084 ($z=10$, from Fig. 3a) of the [TPMT•TNB-AdoVin] adduct at a collision energy of 1000 eV, which results in increased dissociation and fragmentation of the bisubstrate adduct. The TNB-AdoVin adduct is identified intact with the 15 Da stable isotope labeling (m/z 610 and m/z 625). Additional fragments are identified, some containing the adenine isotope flag (indicated by Δ).

(c) After initial separation of an *ex vivo* sample, SID tandem mass spectrum of precursor ion m/z 3080 ($z=10$, from Fig. 3c) of the [TPMT•AMBA-AdoVin] adduct (star) at a collision energy of 500 eV. After dissociation, apo- (open circles) and adduct-bound (closed circles) enzyme are observed in multiple charge states. The singly-charged AMBA-AdoVin adduct (triangle) is observed at m/z 580.

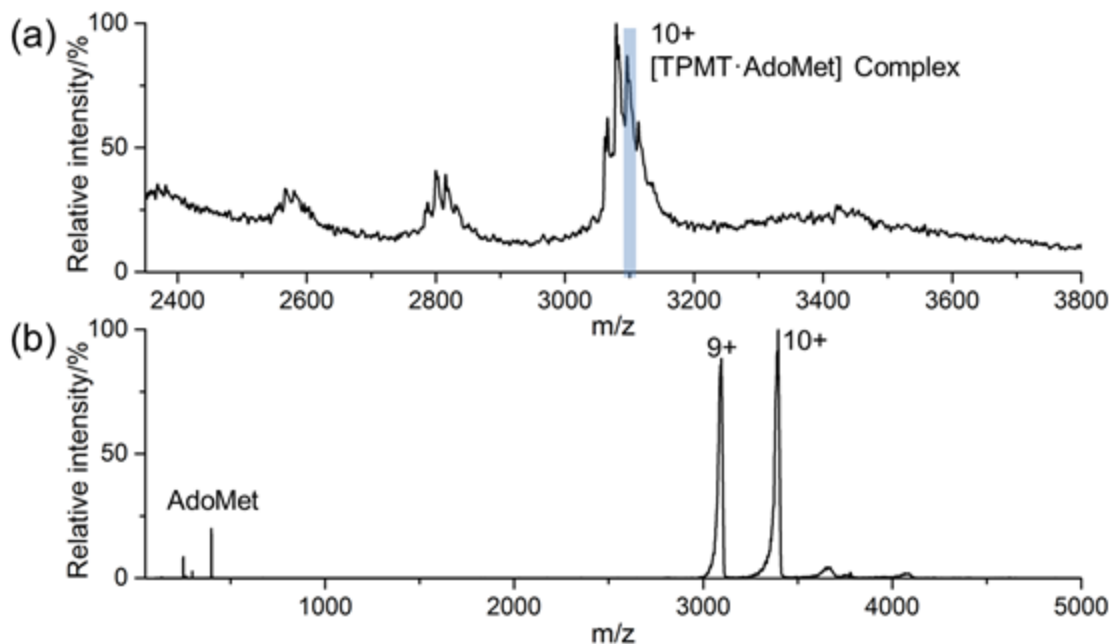


Figure S6 | Mass Spectrum and Tandem Mass Spectrum of [TPMT•AdoMet] Complex

(a) Mass spectrum of [TPMT•AdoMet] complex (highlighted) at m/z 3096 ($z=10$) formed by combining 4 μM TPMT + 40 μM AdoMet *in vitro*.

(b) Collision induced dissociation (CID) tandem mass spectrum of [TPMT•AdoMet] complex from (a) (precursor ion m/z 3096) at a collision energy of 300 eV. Singly charged AdoMet (m/z 399) and TPMT in multiple charge states ($z=9$ and 10) were observed.

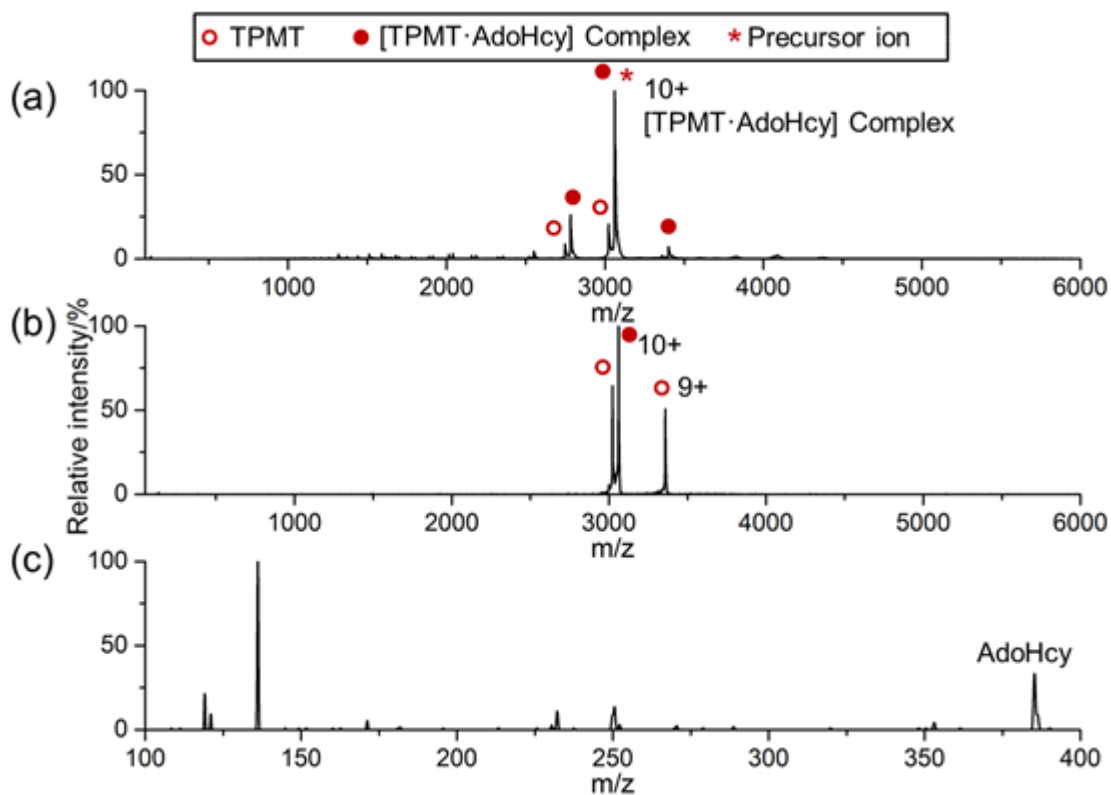


Figure S7 | Mass Spectrum and Tandem Mass Spectrum of [TPMT·AdoHcy] Complex
(a) Mass spectrum of [TPMT·AdoHcy] complex (highlighted) at m/z 3061 ($z=10$).
(b) Tandem mass spectrum after CID at a collision energy of 300 eV of [TPMT·AdoHcy] complex (precursor ion m/z 3061). TPMT in multiple charge states ($z=9$ and 10) and **(c)** singly charged AdoHcy (m/z 385) were observed.