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Design and synthesis of methionine analogues for the enzymatic synthesis of S-adenosyl-methionine analogues: tools for the analysis of methyltransferase substrate specificity

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DESIGN AND SYNTHESIS OF METHIONINE ANALOGUES FOR THE ENZYMATIC
SYNTHESIS OF *S*-ADENOSYL-METHIONINE ANALOGUES:
TOOLS FOR THE ANALYSIS OF METHYLTRANSFERASE SUBSTRATE SPECIFICITY

A thesis presented
by

Nathaniel Thomas Kenton

to
The Department of Chemistry and Chemical Biology

In partial fulfillment of the requirements for the degree of
Master of Science

in the field of
Chemistry

Northeastern University
Boston, Massachusetts
April 2012

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ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemistry
in the Graduate School of Northeastern University
April 2012

Abstract

Determining enzyme-substrate specificity is tantamount to understanding biology on a molecular level. In medicinal chemistry, for example, this is often the crucial starting point from which a drug discovery project emanates. Understanding specificity can be a markedly difficult task, however, especially in the case of *S*-adenosyl-methionine (AdoMet or SAM)-dependent methyltransferases. Functional analysis of these enzymes is complicated by the fact that the methyl group is only 15 Daltons and unreactive, which, especially in the case of more complex systems, necessitates the use of creative solutions.

The development of AdoMet analogues is one such solution, allowing for substrate labeling with larger and more reactive groups or enzyme-mediated adduct formation. As presented herein, an AdoMet analog that contains an electrophilic “hook” forms a covalent adduct with the nucleophilic methyltransferase substrate. In the case of *S*-adenosyl-vinthionine (AdoVin), the electrophilic group is a vinyl sulfonium, which forms a bi-substrate adduct with its substrate, making detection of substrates facile. I describe herein the enzymatic synthesis of *S*-adenosyl-vinthionine and the chemical synthesis of vinthionine, its corresponding methionine analogue. Furthermore, the method is validated with thiopurine methyltransferase (TPMT).

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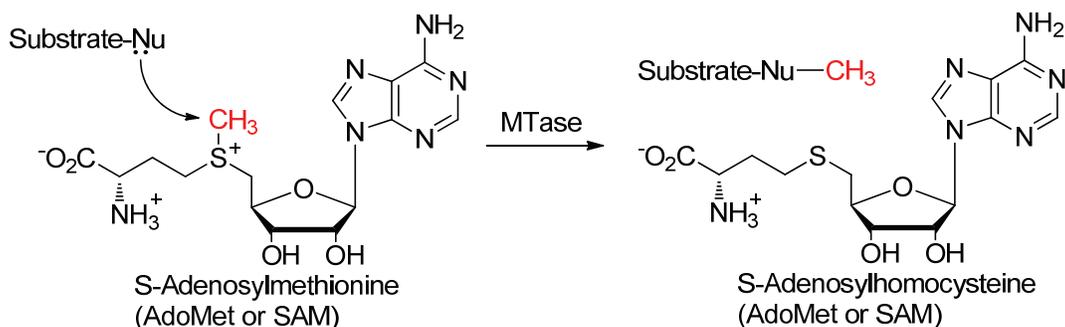
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Chapter 1: Introduction

Determining enzyme-substrate specificity is tantamount to understanding biology on a molecular level. In medicinal chemistry, for example, this is often the crucial starting point from which a drug discovery project emanates. Understanding specificity can be a markedly difficult task, however, because, while other enzymes have extremely specific tasks and only one natural substrate, many enzymes show unexpected activity. This promiscuity is exemplified by the methyltransferase enzymes, which are present in all organisms. In fact, most methyltransferases do not exhibit obvious substrate definition, alkylating all types of biologically relevant functional groups in DNA, RNA, proteins, and natural products [1]; they play important roles in biosynthesis, protein repair, signal transduction, and gene silencing [2]. Methyltransferases are linked to disease as well; for example, methylation of tumor suppression genes has been shown to accelerate tumor growth [3].

Most methyltransferases rely on the molecule *S*-adenosyl-methionine (AdoMet or SAM) as a methyl source. The archetypical methyltransferase reaction is shown in Scheme 1.1. Present in all known organisms [1], AdoMet is demethylated by the methyltransferase to form *S*-adenosyl-homocysteine (AdoHcy or SAH).

Scheme 1.1. An archetypical transmethylation reaction.



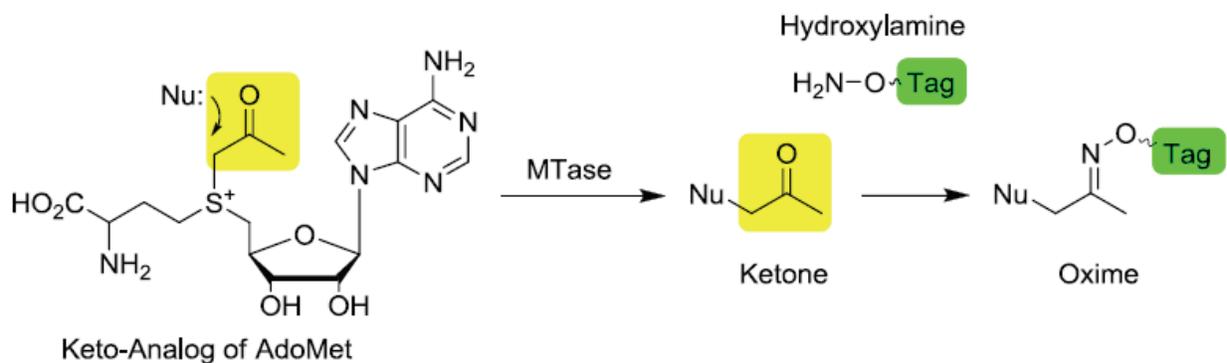
Analysis of methyltransferase function is challenging. The methyl group itself is only 15 Daltons and chemically unreactive, which limits the options for analysis—in particular, that of more complex systems—by typical methods like mass spectrometry and biochemical assays. Analysis of phosphorylation, by contrast, is much easier due to the size and charge of the phosphate group: phosphorylation can be easily detected in biological samples with phosphate-specific antibodies (Western blotting), isoelectric focusing (due to the increased negative charge), or mass spectrometry (due to the mass increase of 80 Da), techniques that are, in many cases, somewhat routine.

Due in part to the fact that homology within a given class of methyltransferase can be down to 10% [2], analysis of substrate specificity can be very challenging—one enzyme may have two substrates whose structures look entirely different, and each substrate may be methylated by other enzymes as well. There are more than 160 methyltransferases cataloged by the Enzyme Commission [1]; there are about 80 protein methyltransferases in humans, and their non-histone targets remain mostly unknown [2].

The use of AdoMet analogues featuring detectable or reactive substitutions for the methyl group can be used to probe enzyme-substrate specificity. One method is to alkylate the substrate

with a traceable R group that replaces the methyl group in AdoMet, which is detectable by HPLC-UV or orthogonal derivatization. We have shown that keto-AdoMet can be used to label several different substrates in red blood cell lysate [1]. Keto-AdoMet is an attractive probe because ketones are not present in most biological samples, allowing for bioorthogonal detection. Thiopurine S-methyltransferase (TPMT, EC 2.1.1.67) catalyzed transfer of an acetyl group from keto-AdoMet to a thiol substrate, which was selectively derivatized with Alexa Fluor 647 hydroxylamine dye and detected at 650 nm, a task otherwise near impossible [1].

Scheme 1.2. Using keto-AdoMet to determine substrate specificity.

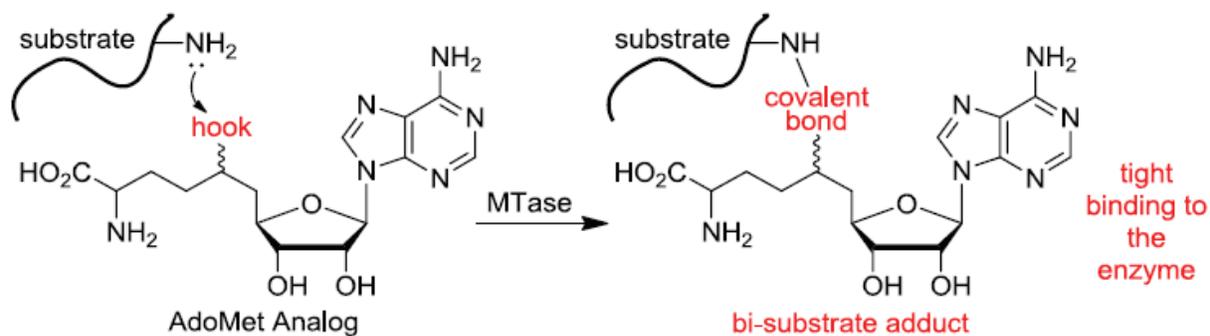


Enzymatic synthesis of AdoMet analogues from methionine analogues

The use of methionine analogues to enzymatically generate AdoMet analogues (rather than direct chemical synthesis of the latter) is advantageous for two reasons: first, they are more chemically assessable, easier to work with, and easier to purify than AdoMet analogues, which are unstable above pH 3; and second, methionine analogues can be used for *in vivo* studies—AdoMet will not penetrate a cellular membrane, nor is it stable in a cellular environment. Methionine analogues can be easily absorbed by cells and are stoichiometrically reacted with ATP to form AdoMet *in situ*, catalyzed by the enzyme methionine *S*-adenosyltransferase (MAT, EC 2.5.1.6).

An alternative to using traceable R groups like ketones is the use of an AdoMet analogue with an electrophilic “hook” that can form a covalent adduct between the electrophilic AdoMet analogue and the nucleophilic substrate. Due to the synergy of binding interactions between the nucleophilic and electrophilic substrates with the methyltransferase, the resulting bisubstrate-adduct is likely to have enhanced binding affinity to the corresponding methyltransferase. Even if the reaction product dissociates from the enzyme, it can be detected by recognition of the adenosyl moiety—which is present on the AdoMet analogue and is rarely observed in proteins—by commercial antibodies. Moreover, our approach can be used to generate highly specific inhibitors to any given methyltransferase.

Scheme 1.3. General enumeration of Mtase-catalyzed bi-substrate adduct formation.

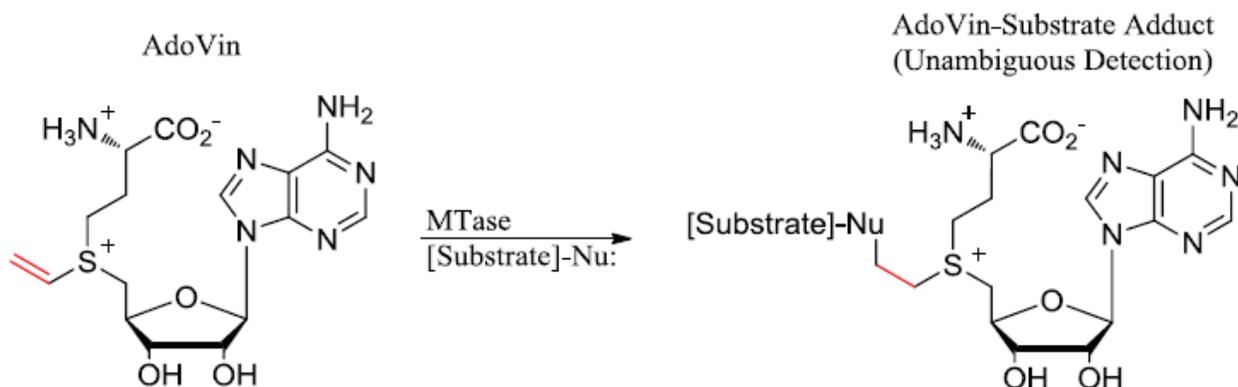


In the following chapter, the chemical synthesis of vinthionine, a methionine analogue with a vinyl group in place of the methyl group, is described, along with the enzymatic synthesis of its corresponding AdoMet analogue, S-Adenosyl-vinthionine (AdoVin), which reacts with a nucleophilic substrate to form a covalent bi-substrate adduct. Furthermore, AdoVin is validated as a probe using thiopurine methyltransferase (TPMT).

Chapter 2: S-Adenosyl-Vinylhomocysteine Synthesis and Formation of Bi-Substrate Adduct

S-Adenosyl-*S*-vinylhomocysteine (AdoVin, depicted in Scheme 2.1) is a particularly attractive candidate for enzyme specificity studies because it employs a vinyl group in lieu of a methyl: sterically, this is the simplest possible alkyl analogue, having only one additional carbon atom. This is advantageous in an experimental setting because the transfer of large R groups on AdoMet analogues is less favorable than methyl transfer. Considering the promiscuity of methyltransferases, it is likely that many will accept AdoVin as a substrate.

Scheme 2.1. Formation of Bisubstrate Adduct between AdoVin and the Nucleophilic Substrate.



Design.

The decision to employ a vinyl sulfonium analogue is not without precedent: we showed that the vinyl sulfonium functional group is a potent proteolytic enzyme inhibitor [4].

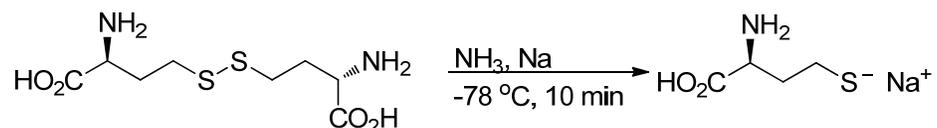
Nucleophiles do not displace the vinyl group from the sulfur atom; instead, the nucleophile attacks the vinyl group, which is more electrophilic, forming a bi-substrate adduct. This adduct is much larger (410 Da) and chromophoric ($\lambda_{\text{max}} = 260 \text{ nm}$), and should result in tight binding.

General Procedures for Synthesis. Reagents of ACS grade were used without further purification unless otherwise noted. Acetylene gas was generated as follows: to 100 mL of 75% aqueous ethanol, several pieces of calcium carbide (~50 g) were added; the flask was then sealed with a rubber septum and connected to the reaction vessel via a 20 cm drying tube with 3-4 cm of Acros 8 mesh Drierite® salt on either end and KOH pellets in the middle. NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer and processed with ACD/NMR Processor Academic Edition, version 12.01. HPLC data were acquired on either Agilent 1100 or 1200 HPLCs; both are equipped with binary pumps, an autosampler, a column cooler, a column heater, and a diode array detector, and used Apollo C18 150 x 4.6 mm, 5 µm columns. Mass spectral data were acquired on a Thermo LCQ Deca Ion trap mass spectrometer attached to an Agilent 1200 HPLC with binary pumps, autosampler, column heater, and diode array detector. UV data were acquired from a Varian Cary 50 Bio UV-Visible Spectrophotometer and analyzed with Scanning Kinetics software. Recombinant histidine-tagged human thiopurine methyltransferase (TPMT) was purified as described [5-6]. Recombinant histidine-tagged human methionine *S*-adenosyltransferase (MAT) was purified from a procedure described previously [5-6].

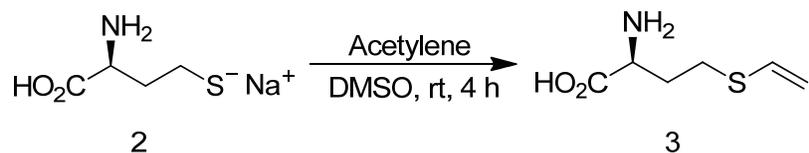
Synthesis of L-Vinthionine.

Vinthionine is prepared from homocystine and acetylene based on a procedure provided by the Honek Research Group (University of Waterloo, Waterloo, Ontario, personal communication).

Scheme 2.2. Preparation of L-vinthionine.



L-Vinthionine (3, CAS [70858-14-9]). To a 250 mL, three-necked, round-bottom flask over a dry-ice bath, L-homocystine (1.01 g, 7.47 mmol) was added. A dry-ice condenser was attached to the three-necked flask, and the other two necks were sealed with septa. The sealed system was evacuated and then purged with anhydrous nitrogen gas for ten minutes. Anhydrous ammonia was passed through 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 a navy blue color for ten minutes. NH_4Cl was added slowly until the blue color faded, and the solution was allowed to warm to room temperature.



Once all of the ammonia had evaporated from the round-bottom flask, the off-white residue in the round-bottom flask was taken up in 15 mL of anhydrous DMSO and the vessel was placed in a water-ice bath. Acetylene gas was bubbled through the reaction mixture for 4 hours. The

reaction was monitored by TLC using UV absorption and ninhydrin staining. After 4 hours, the reaction was neutralized by addition of 1 N HCl. After 24-48 hours in storage at -20 °C, a precipitate formed. The mother liquor was filtered, leaving behind an off-white solid, which was washed with ethanol and dried. TLC: R_f 0.7 (7:1:1 n-BuOH, H₂O, AcOH). ¹H NMR (D₂O/K₂CO₃, 400 MHz): δ 6.36 (dd, 1H, J = 16.9 Hz, 10.3 Hz), 5.22 (d, 1H, J = 10.3 Hz), 5.14 (d, 1H, J = 16.9 Hz), 3.78 (t, 1H, J = 6.2 Hz), 2.81 (t, 2H, J = 7.3 Hz), 2.13 (m, 2H). ¹³C NMR (D₂O, 100 MHz): δ 174.45, 130.68, 112.70, 53.94, 30.17, 26.42. The NMR data were consistent with the reported values [7].

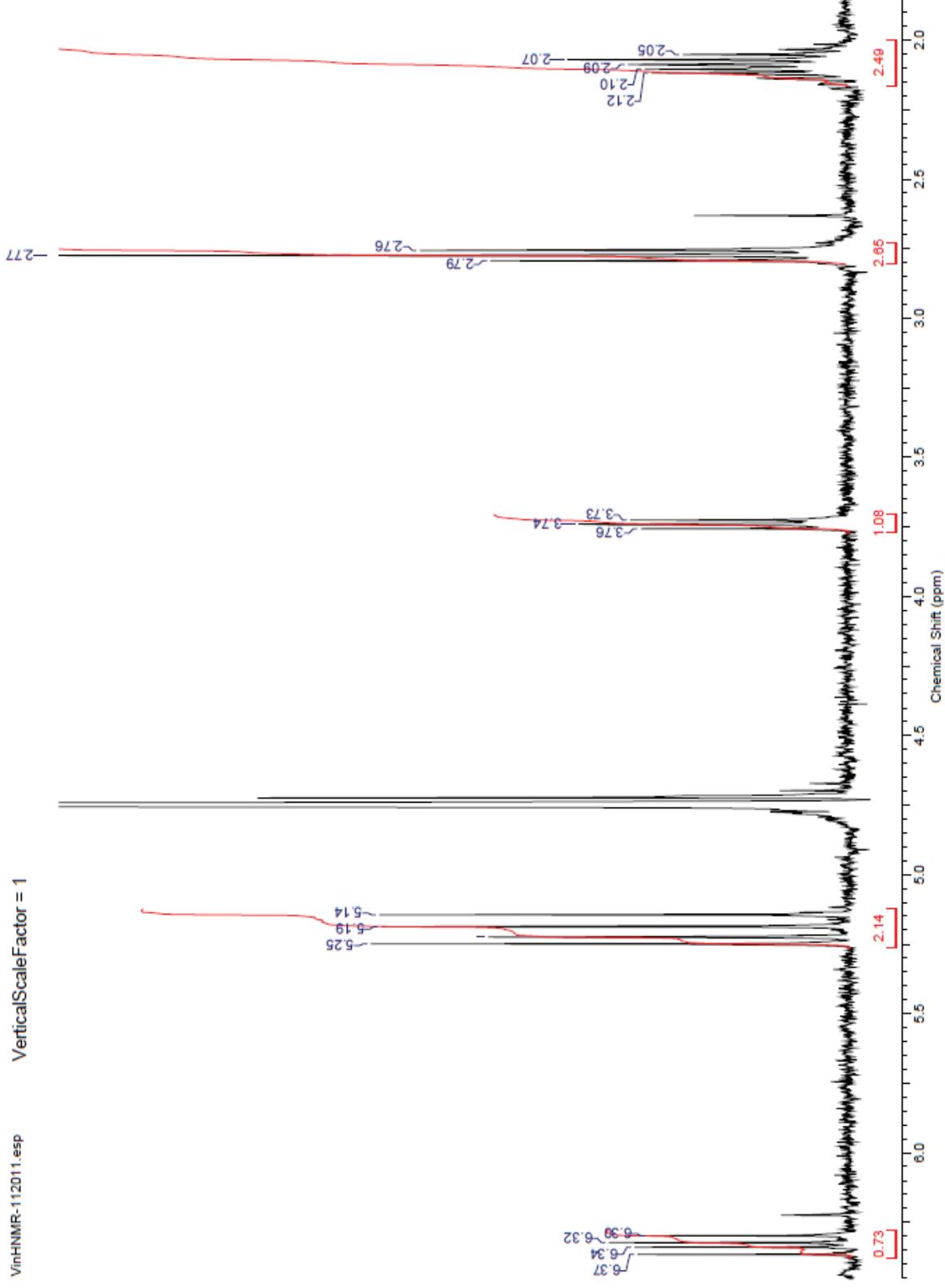


Figure 2.1: $^1\text{H-NMR}$ spectrum of vinthionine (400 MHz, $\text{D}_2\text{O}/\text{K}_2\text{CO}_3$).

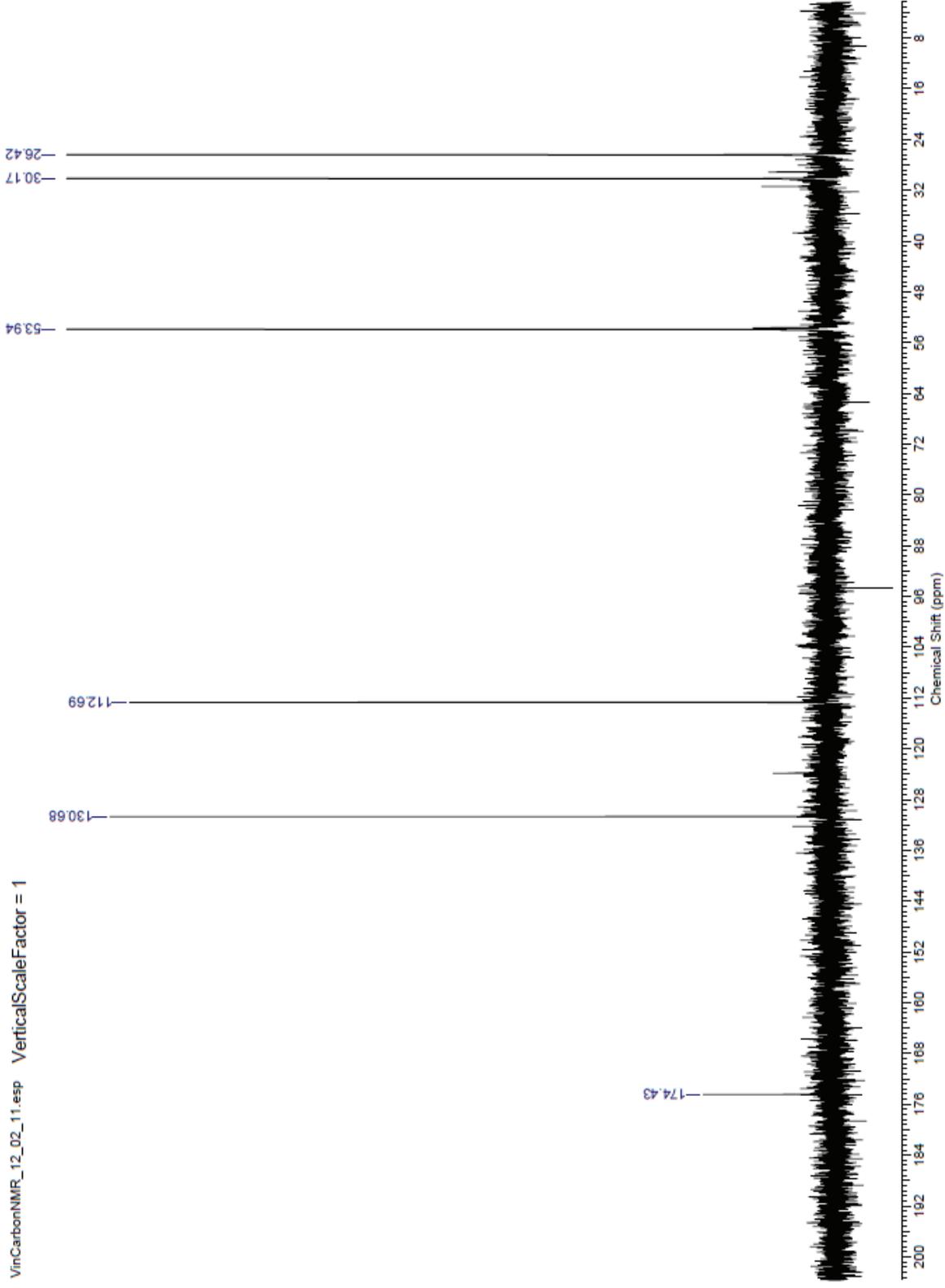


Figure 2.2: ^{13}C -NMR spectrum of vinthionine (400 MHz, $\text{D}_2\text{O}/\text{K}_2\text{CO}_3$).

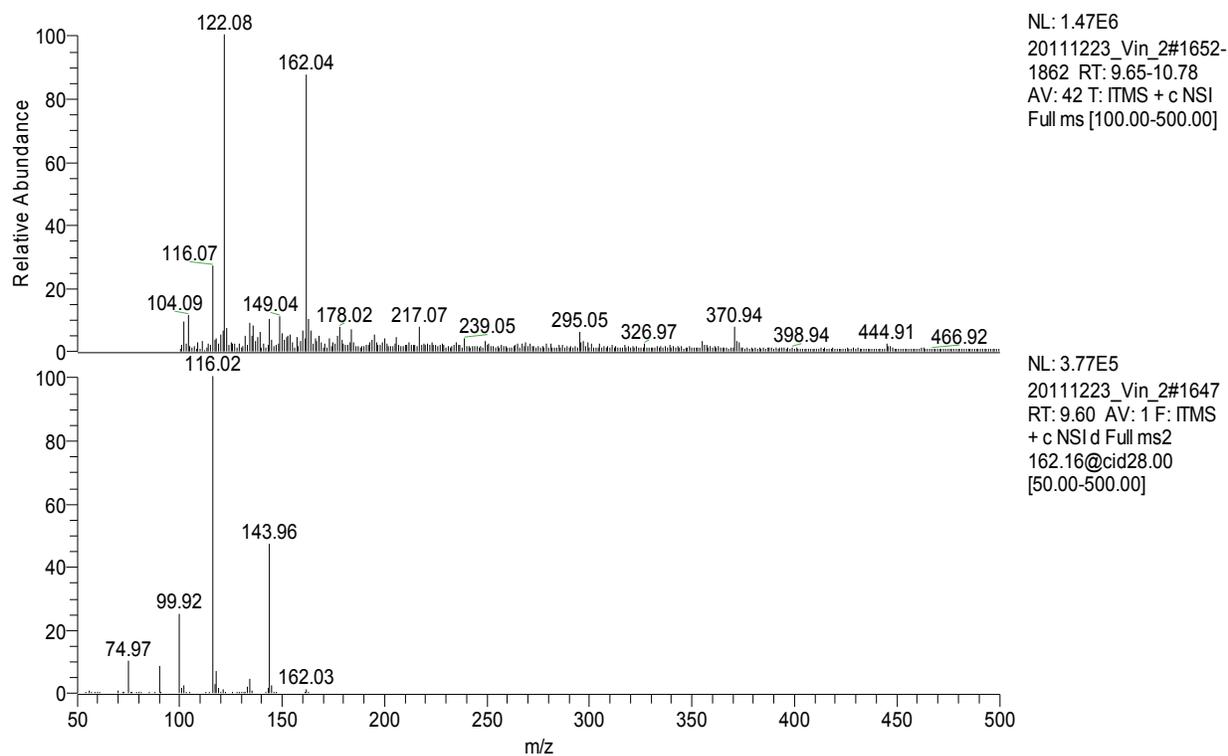


Figure 2.3: Mass spectrum (top) and MS² spectrum (bottom) of vinthionine. The parent ion is 162.04. The exact mass was calculated to be 161.22 m/z. The peaks at 143.96 m/z, 116.02 m/z, and 99.92 m/z may result from consecutive loss of H₂O, CO, and NH₂, respectively.

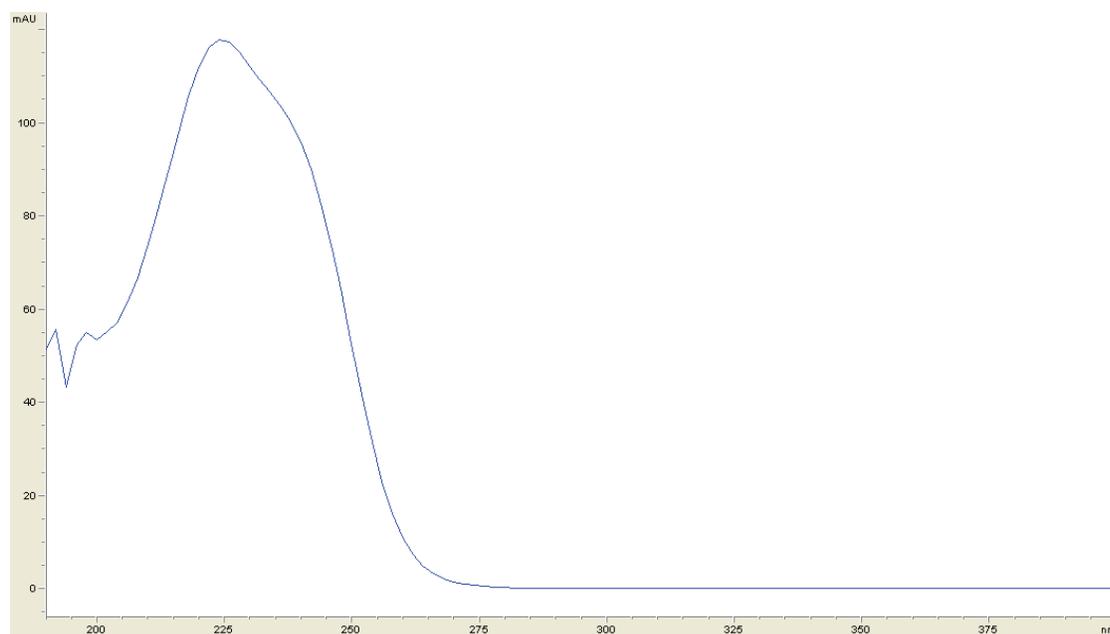
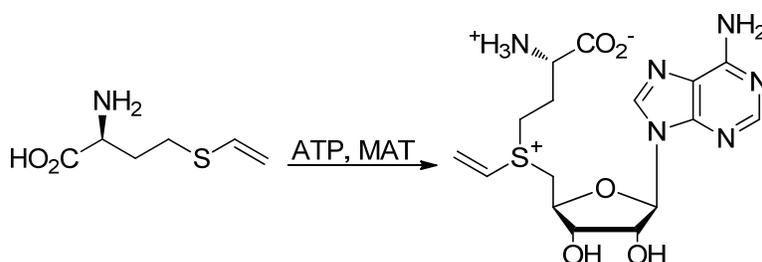


Figure 2.4: UV spectrum of vinthionine. In accordance with a previous report [7], the maximum absorbance was found to be 224 nm.

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

The AdoMet analog is synthesized from vinthionine and ATP catalyzed by recombinant methionine S-adenosyl-transferase (MAT). Formation of S-adenosyl-vinthionine is MAT-dependent and is confirmed by liquid chromatography, ultraviolet spectroscopy and mass spectrometry.

Scheme 2.3. Enzymatic synthesis of AdoVin.



The reaction conditions for the MAT-catalyzed formation of AdoVin are as follows: 20 mM NH₄HCO₃ (pH 8.0), 25 mM KCl, 10 mM MgCl₂, 5 mM ATP, 1.05 μM vinthionine. The reaction was initiated with 114 μM MAT and incubated at 37 °C. Aliquots were removed after 2 hours of incubation and analyzed by LC-UV-MS (ion trap MS) using the following conditions: linear increase from 2% to 10% acetonitrile with 0.1% TFA over 8 minutes, followed by a return to 2% acetonitrile with 0.1% TFA over 2 minutes. The flow rate was 1 mL/min.

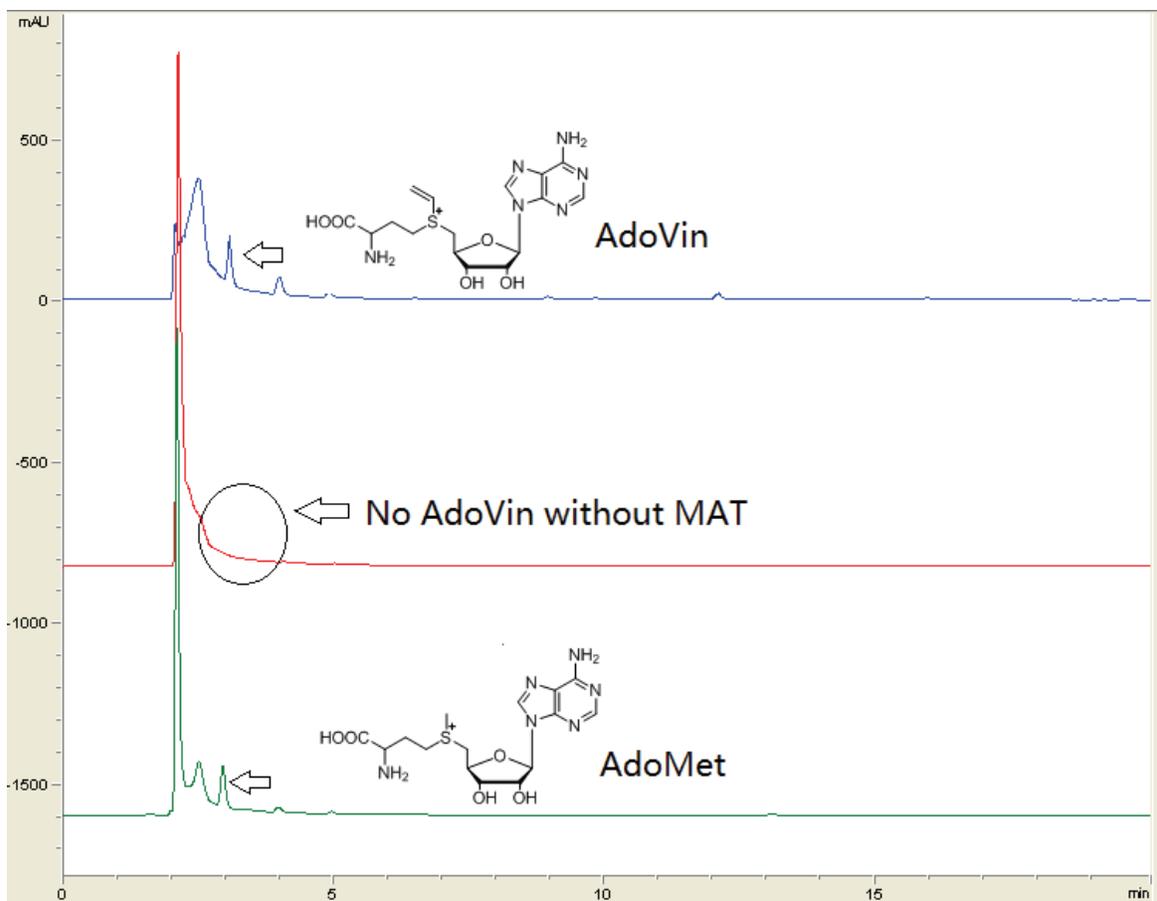


Figure 2.5: The formation of AdoVin is MAT-dependent (top and center), shown here at 260 nm. AdoMet is shown for comparison.

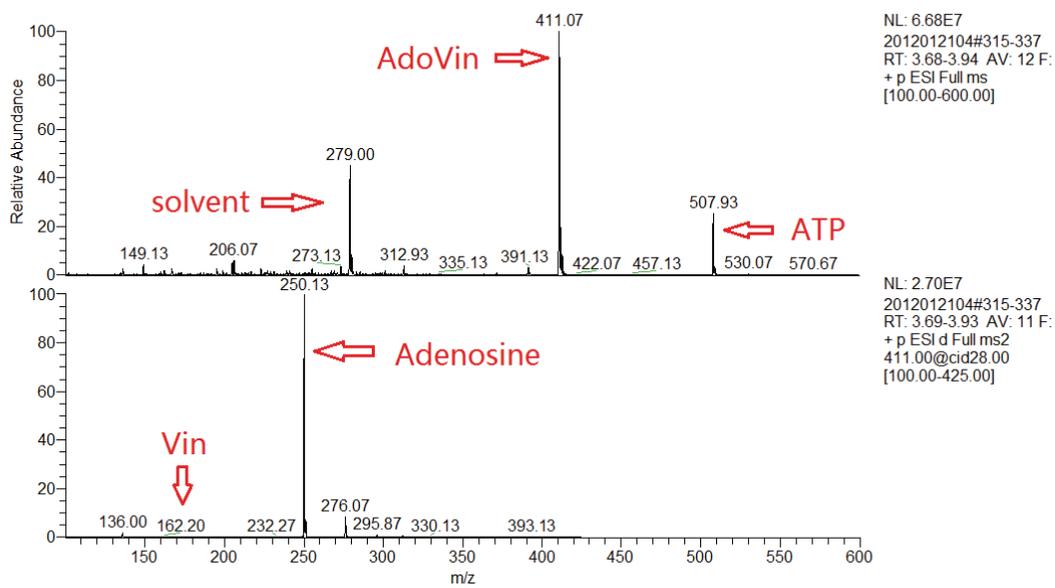
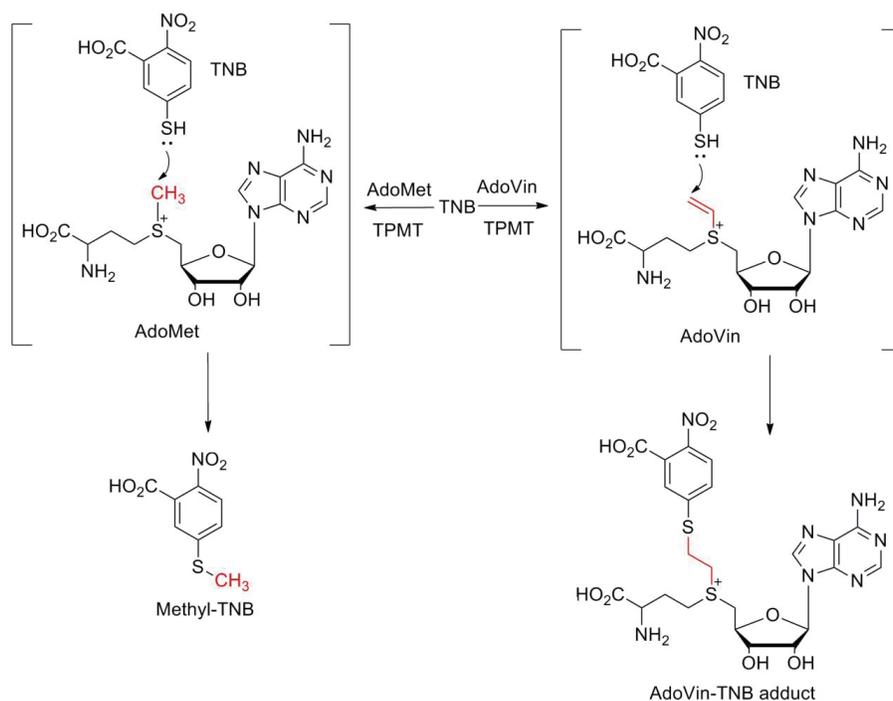


Figure 2.6: Mass spectrum (top) and MS² spectrum (bottom) of AdoVin. The parent ion is at 411.07 m/z (calculated 410.5 m/z).

Formation of Bi-Substrate Adduct by Thiopurine Methyltransferase

The feasibility of *S*-adenosyl-vinithionine as a bi-substrate precursor was assessed using recombinant human thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.67) and the substrate 3-carboxy-4-nitro-benzenethiol (TNB). Much work remains to be done on TPMT (it is unknown whether it has any endogenous substrates, and it is implicated in the methylation of thiopurine drugs), so this experiment can be conducted in biological systems to further elucidate TPMT's substrate specificity. The AdoVin-TNB adduct was observed, and the results show that the reaction occurs only with both enzymes (Figure 2.7) and in a time-dependent (Figure 2.8) manner.

Scheme 2.4. TPMT-catalyzed formation of AdoVin-TNB adduct.



The reaction mixture contained the following: 50 mM NH₄HCO₃ (pH 8.0), 10 mM KCl, 4 mM MgCl₂, 2 mM ATP, 600 μM vinthionine, 480 μM TNB, 83 μM TPMT. The reaction was initiated with 120 μM MAT and incubated at 37 °C. An adduct concentration of 6.54 μM was detected after 1 hour incubation. 10 μL samples were injected and analyzed by HPLC (260 nm and 350 nm) after 1 hour, 3 hours, and 6 hours. The chromatography was performed 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 as follows: 2% mobile phase B for 5 minutes, followed by a linear increase to 60% mobile phase B over 22 minutes, then a return to 2% mobile phase B over 1 minute, and finally a hold at 2% mobile phase B over 3 minutes. Formation of the AdoVin-TNB adduct was detected after 1 hour. 20 μL reaction samples after 1 hour of incubation were analyzed with LC-UV-MS (Ion Trap MS).

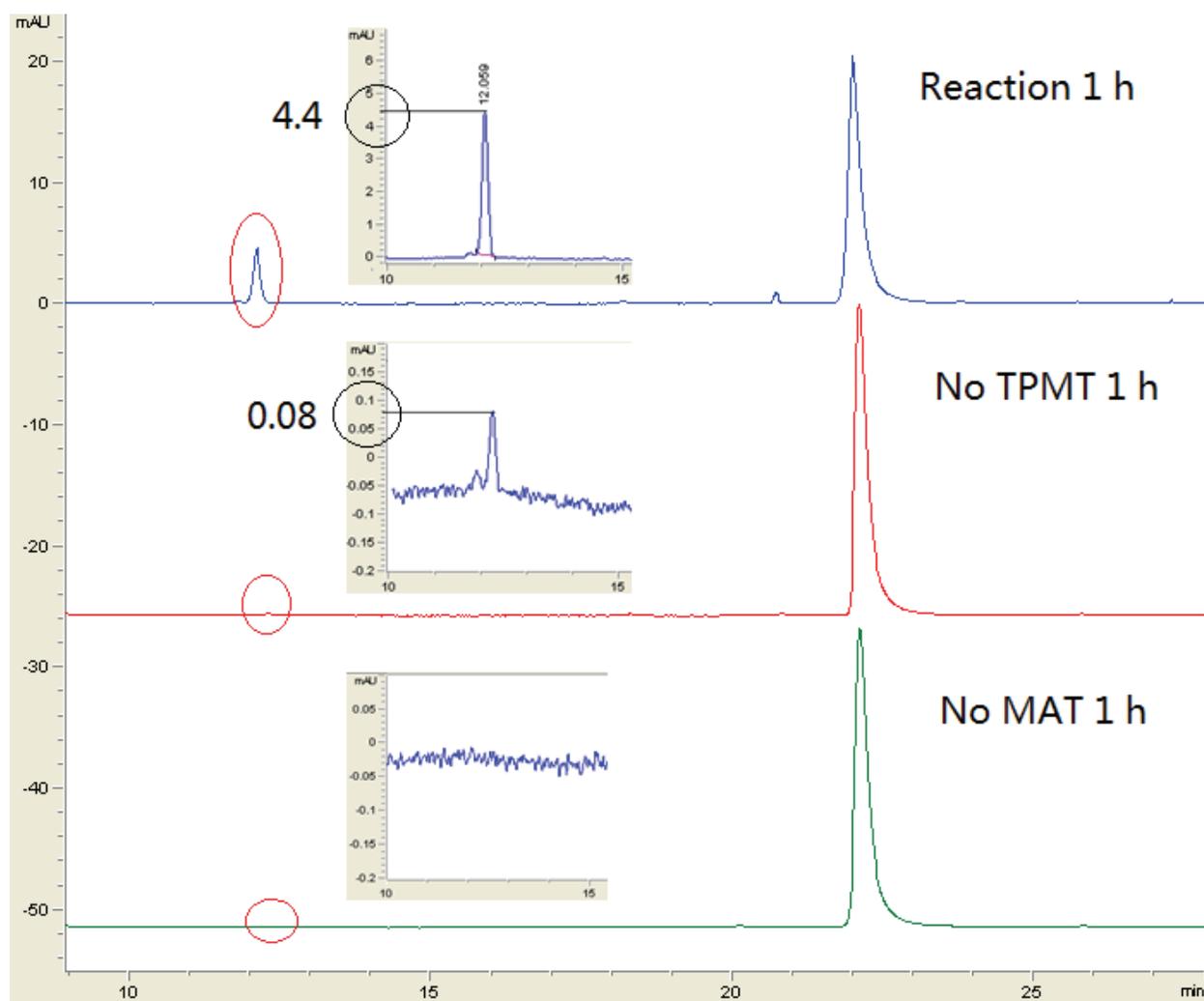


Figure 2.7: HPLC chromatogram of formation of the AdoVin-TNB adduct (top), and negative controls with no TPMT (center) and no MAT (bottom).

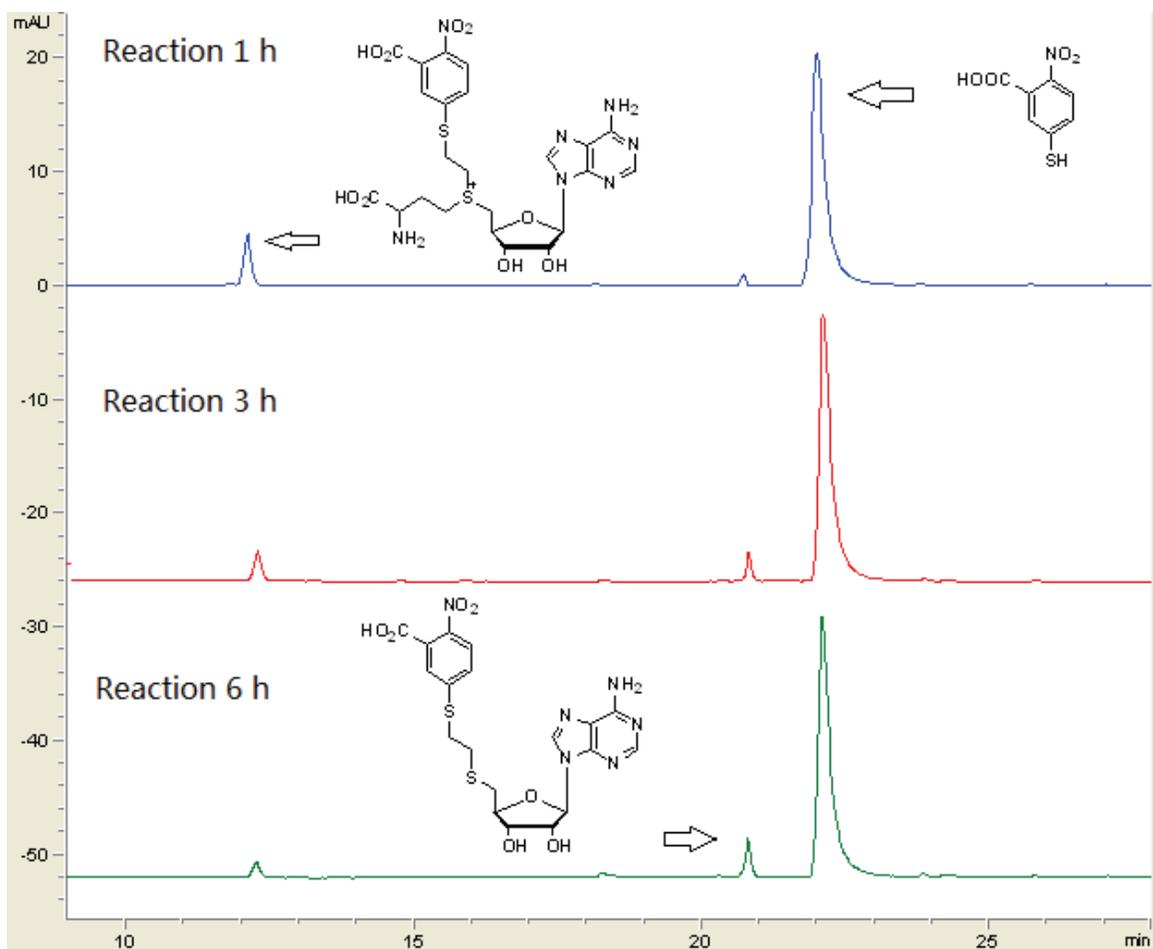


Figure 2.8: HPLC chromatogram of time-dependent formation of the AdoVin-TNB adduct after 1 hour (top), 3 hours (center) and 6 hours (bottom).

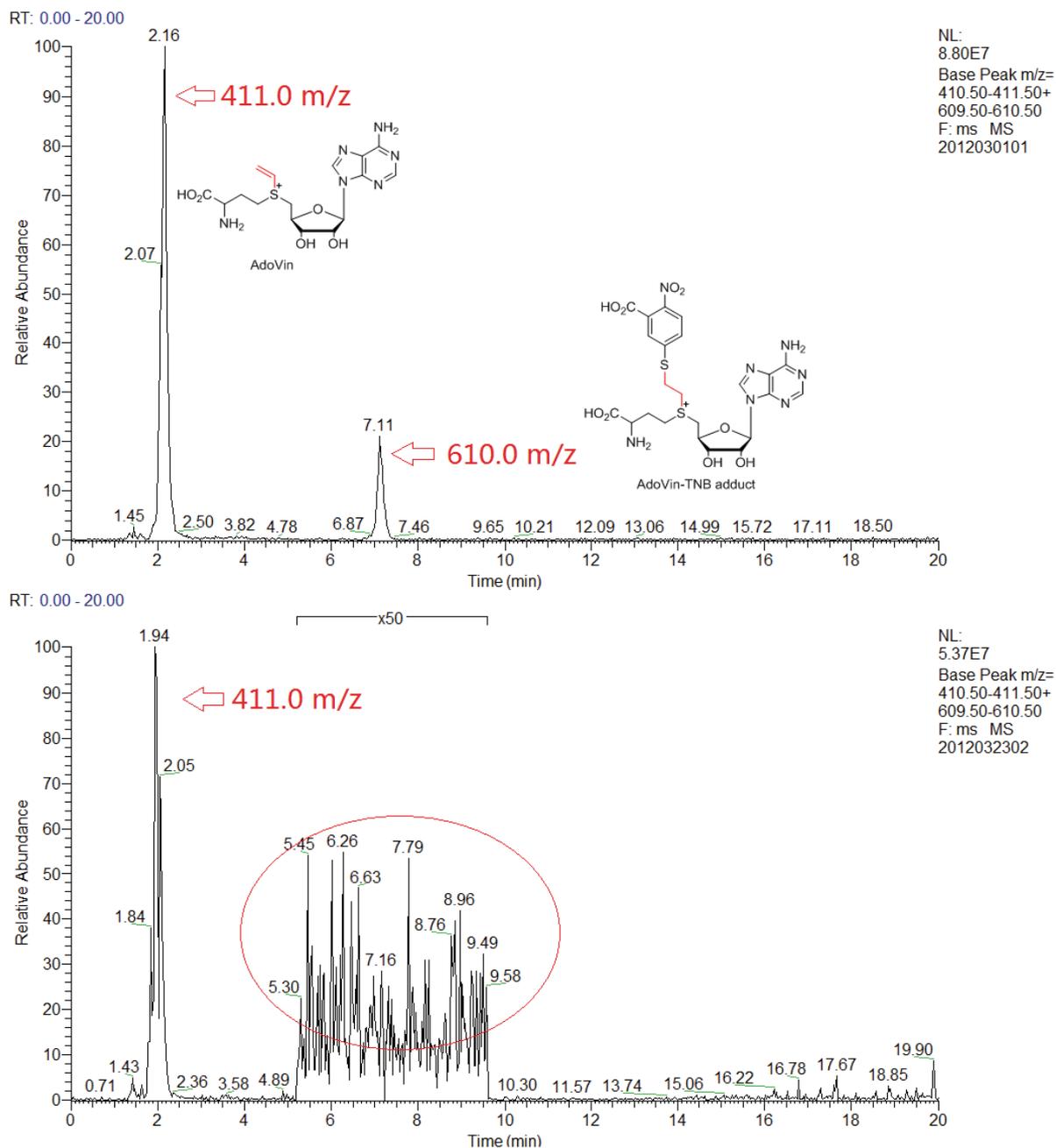


Figure 2.9: HPLC chromatogram of the AdoVin-TNB reaction with (top) and without (bottom) TPMT. AdoVin (calculated 410.5 m/z) and the AdoVin-TNB adduct (calculated 609.6 m/z) are clearly visible at top, but only AdoVin is visible at bottom.

20120210 AdoVin #570 RT: 8.56 AV: 1 NL: 1.52E6
F: +p ESI d Full ms2 610.33@cid28.00 [155.00-625.00]

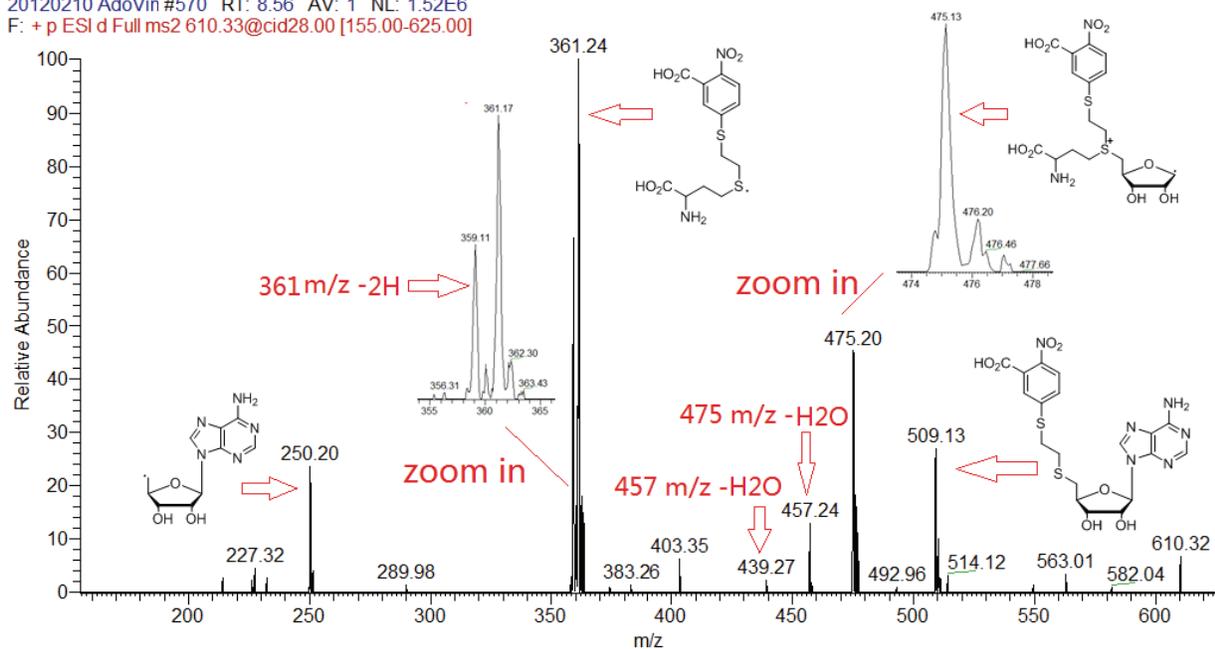


Figure 2.10: MS² spectrum of the AdoVin-TNB adduct. The precursor ion is 610 m/z (calculated 609.6 m/z).

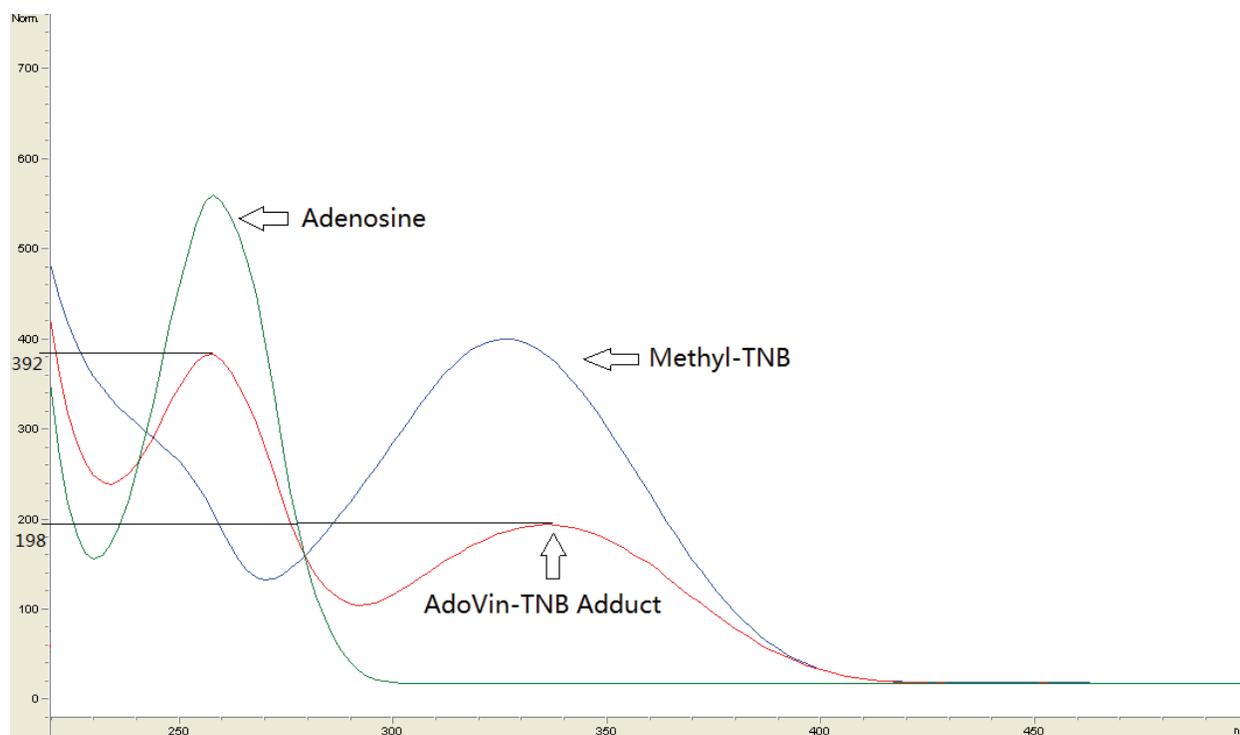


Figure 2.11: UV spectra of the AdoVin-TNB adduct, methyl-TNB and adenosine standards.

Maximum absorbance for the AdoVin-TNB adduct was found to be about 350 nm, and the extinction coefficient at 350 nm was calculated to be $10,835 \text{ M}^{-1}\text{cm}^{-1}$ and confirmed by agreement of the ratio of the extinction coefficients of adenosine and methyl-TNB at 260 nm and 350 nm with the observed intensity on the spectrum.

Chapter 3: Perspective and Future Directions

Identification of methyltransferase substrate specificity is made possible by the use of AdoMet analogues. Our enzymatic synthesis approach, however, circumvents two primary liabilities associated with direct chemical synthesis of AdoMet analogues: their instability at cellular pH and, more importantly, their inability to penetrate a cell membrane. The analogue may prove useful to label specific sites on protein substrates for bioconjugation. There is also, of course, always interest in developing probes for other labeling methods as the shortcomings of our method begin to present themselves.

To our knowledge, this is the first time anyone has used methionine analogues to generate AdoMet analogues for the purposes of labeling substrates. This is an exciting prospect, opening the doors for *in vivo* labeling of methyltransferases, a feat as yet unaccomplished. Demonstration of adduct formation between AdoVin and a single substrate is a small but distinct step toward this end, though the next big step will be probing more complex substrates in biological samples *ex vivo*, such as non-histone proteins and DNA. Having only demonstrated the formation of a single adduct, much work remains to be done, but the outlook is auspicious.

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