Identifying Unknown Substrates of S-Adenosyl-methionine-dependent Methyltransferases via \textit{in situ} Formation of Tight Binding Bisubstrate Adducts

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Dissertation directed by

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Abstract of Dissertation

Identification of enzyme substrates is an essential step toward understanding the physiological functions of enzymes. However, progress in substrate elucidation is hampered by the transient nature of enzyme-substrate interactions. This challenge is exemplified by S-adenosyl-methionine (AdoMet or SAM) dependent methyltransferases (MTases). AdoMet dependent MTases catalyze the methyl-transfer reaction from AdoMet to a wide array of nucleophilic substrates. These nucleophilic substrates, however, are only transiently associated to the enzyme. Inspired by the concept of multisubstrate adduct inhibition, a novel approach of enzyme-catalyzed in situ bisubstrate-adduct formation is presented for capturing substrates of MTases via tight binding MTase-adduct complexes. In our approach, the electrophilic methyl sulfonium in AdoMet is replaced with a vinyl sulfonium in S-adenosyl-vinthionine (AdoVin). AdoVin forms a covalent bisubstrate-adduct with the nucleophilic substrate and the resulting adduct tightly binds the enzyme. Using this strategy, an unknown substrate was readily identified in crude cell lysates, and was found to be a derivative of exogenous substrates.

Another significant achievement is the detection of the complex between the bisubstrate-adduct and enzyme using native mass spectrometry directly from the reaction mixture, without prior purification even using crude cell lysates. The subsequent identification of substrates was achieved by employing various tandem mass spectrometric techniques, such as collision induced dissociation (CID) and surface induced dissociation (SID), in a single run. To the best of our knowledge, this is the first example of MTase substrate identification from cell lysates without any separation prior to mass spectrometric analysis. The approaches outlined here should be generally applicable for identifying substrates of other group transferases or enzymes sharing similar mechanisms.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>¹³C</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>¹⁵N</td>
<td>Nitrogen-15</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>AdoHcy</td>
<td>S-adenosyl homocysteine</td>
</tr>
<tr>
<td>AdoVin</td>
<td>S-adenosyl vinthionine</td>
</tr>
<tr>
<td>AMBA</td>
<td>2-amino-5-mercaptobenzoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>CADD</td>
<td>Computer-aided drug design</td>
</tr>
<tr>
<td>CASS</td>
<td>Computer-aided substrate screening</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol O-methyltransferase</td>
</tr>
<tr>
<td>D</td>
<td>Deuterium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>D (Asp)</td>
<td>Aspartate acid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
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<td>Hey</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>i.d.</td>
<td>Internal Diameter</td>
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<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
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<td>IMS</td>
<td>Ion mobility spectrometry</td>
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<tr>
<td>K (Lys)</td>
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<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
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<td>Kilovolt</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
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<tr>
<td>m/z</td>
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<tr>
<td>M⁻¹cm⁻¹</td>
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</tr>
<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MAT</td>
<td>L-methionine S-adenosyltransferase</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium chloride</td>
</tr>
<tr>
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</tr>
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<td>Minute</td>
</tr>
<tr>
<td>mM</td>
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</tr>
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<td>MS</td>
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</tr>
<tr>
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</tr>
<tr>
<td>MTAN</td>
<td>5'-methylthioadenosine nucleosidase</td>
</tr>
<tr>
<td>MTases</td>
<td>Methyltransferases</td>
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<td>-------------</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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</tr>
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<td>Trifluoroacetic acid</td>
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<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TIC</td>
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</tr>
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<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
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<td>TMT</td>
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<td>Time of flight</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine S-methyltransferase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible spectroscopy</td>
</tr>
<tr>
<td>v</td>
<td>Volume</td>
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<td>XIC</td>
<td>Extracted ion chromatogram</td>
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Chapter 1: Overview of Methylation and Methyltransferases

1.1. Protein post-translational modifications

Protein post-translational modifications (PTMs) are one of the essential routes for protein diversification. PTMs are covalent modifications of proteins, and can be classified based on different classification methods.\textsuperscript{1-2} One classification scheme is based on the modified amino acid side chains of proteins; for examples, modification of lysine by phosphorylation, N-methylation and N-acylation; oxidation to sulfoxide from the methionine; potential modifications on asparagine by deamidation, N-glycosylation, N-ADP-ribosylation and protein splicing; while the potential covalent modifications for aspartic acid (Asp) are phosphorylation and isomerization to isoaspartic acid (isoAsp). In a different classification scheme, PTMs can be sorted into five common covalent additions: phosphorylation,\textsuperscript{3-5} acylation,\textsuperscript{6-8} alkylation,\textsuperscript{8-11} glycosylation,\textsuperscript{2,12} and oxidation.\textsuperscript{2,13}

PTMs are found in numerous classes of proteins and have strong influences with protein structures and biological functions.\textsuperscript{2,14} Thereby, the discovery and characterization of PTMs are required for the elucidation of protein structure modifications and the study of protein-mediated biological functions.\textsuperscript{14-16} However, the biological significance of most of protein side chain modifications has not been established. Moreover, multiple PTMs often occur at multiple sites of proteins, which are crucial to their physiological functions.\textsuperscript{2}

Even the smallest PTMs, by changes in mass, can make big differences to protein structures and functional activities, for example, 0 Da mass change of isomerization between aspartic acid (Asp) and isoaspartic acid (IsoAsp), and 1 Da mass change of deamidation from Asparagine (Asn) to Asp (Scheme 1.1).\textsuperscript{17} Therefore, the detection and analysis of PTMs are important for uncovering the nature of proteins. Two efficient analysis methods for PTMs are mass spectrometry and
antibody based detection. Mass spectrometry, antibody based detection and their combinations with other techniques have been widely applied to the determination of site specific PTMs and quantification.\textsuperscript{18-21} This is exemplified by the detection and quantification of isoAsp in Asp/Asn containing proteins\textsuperscript{22} and glycosylation in protein therapeutics.\textsuperscript{18} With the rapid development of biological chemistry, computational chemistry and analytical chemistry, many PTMs have been discovered, like eliminylation, a PTM of host cells signaling proteins performed by phosphotheronine lyases.\textsuperscript{23}

Scheme 1.1. Deamidation of asparagine and formation of aspartic acid and isoaspartic acid.
1.2. Methylation and *S*-Adenosyl-Methionine Dependent Methyltransferases

Methylation involves a transfer of methyl group from the methyl donor *S*-adenosyl-methionine (AdoMet) to nucleophilic substrates (Scheme 1.2) through an *S*<sub>2</sub> reaction with a linear transition state<sup>24</sup> catalyzed by *S*-Adenosyl-methionine dependent methyltransferases (AdoMet dependent MTases). MTases belong to a large and diverse family, catalyzing over 300 different reactions (EC 2.1.1.1-313), and the number is still increasing. Varied in size, the substrates of MTases range from small molecules to biological macromolecules, such as proteins, mRNA and DNA.<sup>25-26</sup> Atomic targets of methylation include nitrogen (amines and guanidines), oxygen (alcohols and carboxylic acids), sulfur (thiols) and even carbon (alkanes).

Scheme 1.2. AdoMet-dependent MTases catalyze methyl transfer from AdoMet to the nucleophilic substrates.
Currently, MTases have been cataloged into five structurally distinct classes. Only two families of MTases exhibit definable substrate specificity. One is the Class V SET-domain containing family of protein lysine MTases and the other is the Class IV SPOUT family of RNA MTases. While the other classes of MTases do not correlate to any certain chemical natures of their substrates, some of them have considerable overlapping of substrates. As such, the structures of many putative MTases have been determined, but their substrates and functions remain elusive.

Even though all MTases are considered to directly transfer a methyl group to substrates via $\text{S}_\text{N}2$ mechanism with proton transfer throughout the methylation process, a wide variety of mechanisms have been described to activate the nucleophilic substrates, mostly dependent on the polarizability of the atomic targets, i.e. $N$-methylation, $O$-methylation, $S$-methylation and $C$-methylation.

The detection of methylation remains challenging, due to their intrinsic properties: the modification is small relative to overall protein structures (-CH$_3$, 15 Da), has no charge and is chemically inert. Despite its inert chemical properties, methylation plays an essential role in in biosynthesis, signal transduction, protein repair, chromatin regulation and gene silencing. The study of MTase substrates is critical to the full understanding and functional analysis of MTases.
1.3. **Analytical Methods for Methylation**

1.3.1. **Radioactivity Assay**

Incorporation of radioactive [³H] methyl group is commonly used in the study of *in vitro* and *in vivo* DNA methylation, protein methylation, and even methylation of small molecules.²⁸⁻²⁹ To be more precise, a [³H] methyl group is transferred from [³H]-CH₃-AdoMet to nucleophilic substrate via the catalysis of methyltransferases, and then the [³H]-CH₃ containing products can be quantified by liquid scintillation, while the unreacted from [³H] methyl-AdoMet can be easily separated from labeled products using gel filtration,³⁰ filter-binding,³¹ or thin layer chromatography.³²

[¹⁴C] labeling could also be used with higher energy and higher efficiency in liquid scintillation counting, compared to [³H].³³ Compared to radiolabeling by chemical reagents, this “radioenzymatic” methylation is more specific. Taking the advantage of radiolabeling, not only *in vitro* studies but *in vivo* identification of all methyltransferase substrates is underway. However, this method has numerous drawbacks, including radioactive wastes, high cost, highly laborious work, and low throughput. Moreover, the direct connection between the enzymes and the substrates cannot be established using this methods, aside from the complicated operation procedure of radio labeling.

1.3.2. **Mass Spectrometry with Stable Isotope Labeling**

Stable isotope labeling combined with mass spectrometry is an efficient and effective tool for identification and quantification, especially in proteomics screening, because of specifically detectable mass shift but consistent properties, i.e. LC retention time, ionization efficiency and fragmentation patterns.²², ³⁴⁻³⁸ -CD₃ and -¹³CH have been used for the detection of AdoMet or
methylated products. Combined with mass spectrometry, stable isotope labeling was available to determine nanomolar concentrations of AdoMet in complicated environment, such as plasma and even in the cells.\textsuperscript{39} As shown in Scheme 1.3, a rapid screening for \textit{in vivo} methylation products with isotope labeling has been developed by Zhou’s group.\textsuperscript{40} The cells take up isotope labeled methionine through the membrane, and AdoMet synthetase catalyzes the synthesis of AdoMet starting from methionine. Therefore, the isotope -CD$_3$, is introduced into the cells after the methylation catalyzed by AdoMet-dependent MTases in the cells. The 1:1 ratio of CH$_3$-methionine and CD$_3$-methionine is introduced in the media, and the isotopic ratio of the methyl group throughout the transformations will be maintained as a detection signature from the methionine auxotroph strain \textit{E. coli} B834 used in the experiments.

\textit{E. coli} B834 (methionine auxotroph)

Scheme 1.3. \textit{in vivo} methylation workflow, showing uptake of methionine into the cells, enzymatic synthesis of AdoMet, and subsequent methylation.
1.3.3. Design of Traceable S-adenosyl-methionine Analogues

Due to the intrinsic properties of the methyl group, small and chemically inert (-CH$_3$, 15 Da), AdoMet analogues have been developed with traceable tags. The methyl group in AdoMet has been replaced by propargyl, ketone, and other functional groups. After the transfer of these alkyl groups to the nucleophilic substrates, the resulting products can be labeled and then traced by click (azide-alkyne) or oxime (ketone-hydroxylamine) chemistry (Scheme 1.4). Moreover, most AdoMet analogues share similar substrate specificity with AdoMet, which makes the replacement of methyl group remain similar to native biological activities.

Scheme 1.4. MTase catalyzed transfer of a methyl (AdoMet, natural substrate), propargyl or ketone group (substrate surrogates). The traceable products can be detected via click or oxime chemistry.
As shown in Scheme 1.4, an AdoMet analogue with a propargyl group has been shown to be a substrate for protein arginine N-methyltransferases 1 (PRMT1), which catalyzes the methylation of arginine in proteins and peptides. Click chemistry has been applied to label the propargyl-transferred nucleophilic substrates, which then allows fluorescence detection. Similarly, in the methodology utilizing keto-AdoMet, the ketone group was first transferred onto the nucleophilic substrates, and oxime chemistry was applied for the labeling and detection of alkylated substrates. This method was used to discover a previously unidentified, 4-methoxybenzylthiol (an aliphatic thiol), for thiopurine S-methyltransferase (TPMT). Thus, the design of new traceable AdoMet analogues contributes to the identification of substrate specificity of MTases, which is crucial to fully understand their biological activities.

In practice, a major limitation for such an approach is that the substrates of multiple enzymes in the same family may be labeled non-specifically, thereby the direct association between a particular substrate-enzyme pair cannot be readily established. This is exacerbated for MTases as this large family of enzymes catalyzes over 300 different reactions with considerable overlap of substrates. For instance, more than fifty protein lysine and nine arginine MTases exist in humans alone.
Another problem for methylation and for all enzyme-catalyzed reactions, is the transient interaction between products and enzymes. The direct connection between the substrates and enzymes is lost due to the immediate dissociation of products from the enzymes. Thus, the traceable products mentioned often cannot be directly linked to a particular MTase (Scheme 1.5). The direct connection between substrates and their corresponding enzymes is very important but challenging for MTases.

Scheme 1.5. Transient interactions between the substrates and products with methyltransferases.
1.4. **Multisubstrate Adduct Inhibitors**

In drug discovery, different methods have been developed to create new inhibitors to achieve satisfactory biological activity. One such method is the multisubstrate adduct inhibitor approach that consists of a covalent link between the substrate analogues, simultaneously present and reacting in the enzyme active site.\(^4^6\) Potentially, the multisubstrate adduct inhibitor approach can be utilized for most enzymes that catalyze the reactions with two or more substrates (including cofactors). A multisubstrate adduct inhibitor contains the key structural features in one molecule, and moreover, enhanced binding affinity to enzymes due to the synergistic effect from the substrates.\(^4^6\)–\(^4^8\) Therefore, the multisubstrate adduct inhibitor approach is an efficient method to obtain structural information about enzymes and to create new inhibitors with potentially high potency. The concept of multisubstrate adduct inhibitor has been successfully applied as a potential tool to identify unknown enzymes and substrates, i.e. the formation of kinase-substrate complex via ATP-based cross-linkers.\(^4^9\)–\(^5^2\)

Many efforts have been made towards design and synthesis of multisubstrate adduct inhibitor for AdoMet-dependent MTases, such as catechol \(O\)-MTase (COMT)\(^2^4\) and protein \(N\)-MTase (PNMT),\(^4^7\) since the mechanism of AdoMet-dependent methylation matches well with the requirement of multisubstrate adduct inhibitor, a \(S_N\)\(^2\) addition reaction with a colinear transition state when the nucleophilic substrate attacks the methyl group in AdoMet (Scheme 1.6).
Scheme 1.6. (a) COMT catalyzed methylation of epinephrine via a SN2 linear transition state; (b) structural design of multisubstrate adduct for COMT, containing the catechol portion, sulfonium ion and homocysteine (left) or adenosyl moiety (right).

Utilizing the concept of multisubstrate adduct inhibitor, the Rajski and Thompson group has performed in situ formation of bisubstrate-adduct catalyzed by protein arginine methyltransferases 1, via an AdoMet analogue with 5’-aziridinyl adenylates,\textsuperscript{53-54} produced from 5’-(diaminobutyric acid)-N-iodoethyl-5’-deoxyadenosine ammonium hydrochloride (AAI) (Scheme 1.7). This bisubstrate analogue was expected to be a potential bisubstrate-adduct inhibitor to PRMT1.\textsuperscript{53} Even though its ability to identify novel PRMT substrates is not clear, the idea of multisubstrate adduct inhibitor brings light to the design of new AdoMet analogues.
1.7. Aziridinium ion formed from the N-mustard (AAI) in situ reacted with arginine catalyzed by PRMT1 to produce bisubstrate-adduct.

1.5. **Bisubstrate-adduct Formation via S-adenosyl-vinthionine**

A conceptually different approach to study methyltransferases is to capture nucleophilic substrates with the corresponding enzyme by forming bisubstrate-adducts, which should bind tightly with the enzyme due to the synergistic binding interactions between the enzyme and moieties in both substrates (Scheme 1.8). This is the premise of multisubstrate adduct inhibitor, a venerable idea championed by Coward and Pegg,\textsuperscript{24, 48} as mentioned before.

Our new approach is demonstrated herein with \textit{S}-adenosyl-vinthionine (AdoVin, Scheme 1.9), in which a vinyl sulfonium replaces the methyl sulfonium in AdoMet. Via an addition reaction to the vinyl sulfonium, AdoVin and the nucleophilic substrate forms a covalent tight-binding adduct.
Scheme 1.8. a) Transient interactions between the substrates and products with methyltransferases; b) “Permanent” interaction (tight binding) between an enzyme and the bisubstrate-adduct formed \textit{in situ}.

Scheme 1.9. AdoMet-dependent MTases catalyze bisubstrate-adduct formation between AdoVin and nucleophilic substrates.
1.6. Conclusions

The detection of methylation remains challenging due to small and chemically inert properties of the methyl group. Most efforts have been made to develop traceable AdoMet analogues, in order to facilitate the functional analysis of AdoMet dependent methyltransferases. However, substrates cannot be linked directly to their corresponding MTases, due to substrate overlap and transience of interactions between MTases and their substrates/products. Using a conceptually distinct approach, we designed a new AdoMet analogue to capture nucleophilic substrates with their corresponding MTases via in situ formation of bisubstrate-adducts.
1.7. References


17. Noguchi, S., Structural changes induced by the deamidation and isomerization of asparagine revealed by the crystal structure of Ustilago sphaerogena ribonuclease U2B. *Biopolymers* 2010, 93 (11), 1003-10.


Chapter 2: Affinity Capturing Substrates via \emph{in situ} Formation of Bisubstrate-Adducts

This chapter is based on a manuscript with the title of “Capturing Unknown Substrates via \emph{in situ} Formation of Tightly Bound Bisubstrate-adducts: \textit{S}-Adenosyl-Vinthionine as a Functional Probe for \textit{S}-Adenosyl-Methionine-Dependent Methyltransferases” with the authors list: Wanlu Qu, Kun Zhang, Michael Pablo, James Glick, Yuan Xiu, Nathaniel Kenton, R. I. Duclos, Jr, and Zhaohui Sunny Zhou

Co-authors’ works in this chapter: Wanlu Qu: experimental design and performance, data analysis, manuscript writing and revision; Kun Zhang, kinetics study; Michael Pablo, AdoVin purification and revision during paper writing; James Glick, idea contributions to mass spectrometric analysis; Yuan Xiu and Nathaniel Kenton, synthesis and characterization of vinthionine; R. I. Duclos, Jr, synthesis and characterization of 2-amino-5-mercaptobenzoic acid; Zhaohui Sunny Zhou, principal investigator.
2.1 Introduction

2.1.1 S-Adenosyl-methionine Synthetase

AdoMet is the second most widely used enzyme substrate, following adenosine 5’-triphosphate (ATP). One of enzymatic ways to synthesize AdoMet is transferring adenosyl group from ATP to sulfur of methionine catalyzed by L-methionine S-adenosyltransferase (S-adenosyl-methionine synthetase, MAT, EC 2.5.1.6), as shown in Scheme 2.1.

Scheme 2.1. MAT catalyzed AdoMet synthesis stoichiometrically from ATP and methionine.

Many analogues of methionine have behaved as possible substrates or inhibitors of MAT, for example, ethionine, a methionine analogue, is the inhibitor to the rat liver MAT. Many efforts have been taken to synthesize more AdoMet analogues (as substrates of MTases), by using the corresponding methionine analogues, even in cellular level.

In my work, MAT was used to catalyze the reaction between vinthionine and ATP, and the subsequent product, AdoVin forms a bisubstrate-adduct with the nucleophilic substrates of MTases. There are two advantages of using methionine analogues to enzymatically synthesize AdoMet analogues: firstly, methionine analogues are more stable than AdoMet analogues during
synthesis and purification;\textsuperscript{5-7} and secondly, methionine analogues are readily absorbed by cellular membranes and thus are accessible for \textit{in vivo} studies, while AdoMet analogues are not.

We used MAT from archaeon \textit{Methanococcus jannaschii}, and the gene encoding MAT was identified as MJ1208, in which the majority of the sequence is highly diverse from those of bacterial and eukaryal MAT proteins. With respect to catalytic activities, archaeon MAT shares most of functional properties with bacterial or eukaryal MAT. For instance, Mg\textsuperscript{2+} and K\textsuperscript{+} are essential to their high catalytic activity. In addition, all the MATs catalyze two-step reactions, that is, AdoMet formation and triply phosphate (PPP\textsubscript{i}) cleavage and release. One significant difference between archaeon MAT and bacterial or eukaryal MAT is that archaeon MAT is quite thermally stable, and its functional properties were evaluated at 70 °C.\textsuperscript{8}

Aside from more stability of vinthionine than AdoVin and accessibility of vinthionine for \textit{in vivo} studies, formation of AdoVin bisubstrate-adduct via enzyme MAT-MTases coupled reactions is advantageous with another reaction: the detection of AdoVin and AdoVin adduct can be facilitated by introduction of stable isotope labeling.

Scheme 2.2. Formation of isotope labeled bisubstrate-adduct via isotope labeled ATP.
As shown in Scheme 2.2, isotope labeled ATP can be used to generate AdoVin with stable isotope labeling catalyzed by MAT, and subsequently, AdoVin bisubstrate-adduct will be isotope labeled due to the catalysis of MTases. Combining with mass spectrometry, isotope labeled AdoVin and AdoVin bisubstrate-adduct will be observed with +15 mass shift, but sharing identical LC retention time, ionization efficiency and fragmentation patterns.

One limitation of mass spectrometry is that unionized substrates or methylation products cannot be detected in mass spectrometry, most commonly with small molecule substrates or products, like catechol, the substrate of catechol O-MTase. It is more accessible to detect AdoVin and its bisubstrate-adduct by mass spectrometry, because they are always positively charged at the LC-MS conditions (mildly acidic).
2.2 Results and Discussions

2.2.1 Enzymatic Synthesis of S-adenosyl-vinthionine

**Synthesis of Vinthionine**

Vinthionine was prepared by organic synthesis (Scheme 2.3), and was characterized by using $^1$H-NMR and $^{13}$C-NMR. Purified vinthionine was dissolved in Milli Q water and subjected to LC-MS for further verification. Figure 2.1 shows that vinthionine was observed as singly charged (m/z 162.0), and the detailed fragmentation of vinthionine under CID was shown in Figure 2.2.

Scheme 2.3. Synthesis of vinthionine (vinyl homocysteine).
Figure 2.1. Mass spectrum of vinthionine (expected m/z 162.05 for vinthionine M+H⁺, observed m/z 162.07; mass difference 0.02 Da).

Figure 2.2. CID MS/MS spectrum of vinthionine from LC- ESI/MS. The +1 precursor ion for MS/MS analysis was m/z 162.07.
Enzymatic Synthesis of S-adenosyl-vinthionine

As shown in Scheme 2.4, AdoVin was synthesized stoichiometrically from ATP and vinthionine catalyzed by L-methionine S-adenosyltransferase (AdoMet synthetase, MAT, EC 2.5.1.6). With similar structure with AdoMet, AdoVin shares similar characteristics with AdoMet. First of all, AdoVin has the same UV absorbance with AdoMet at 260 nm (Figure 2.4), due to the same UV active adenosyl group (ε_{260\text{nm}} = 15,400 \text{ M}^{-1} \text{ cm}^{-1}). Another significant characteristic of AdoVin is that the permanent positive charge from the sulfonium ion makes both AdoMet and AdoVin very hydrophilic, and hardly retained on C18 column, even though AdoVin is a little more hydrophobic than AdoMet replacing methyl group by vinyl group, as Figure 2.3 shown.

Scheme 2.4. Synthesis of S-adenosyl-vinthionine (AdoVin) catalyzed by MAT.
Figure 2.3. Reverse-phase HPLC chromatogram (monitored at 260 nm) of AdoVin synthesis with (top trace) or without (middle trace) MAT.

Figure 2.4. UV/Vis spectra of synthesized AdoVin (blue) and AdoMet (red).
Instead of reverse phase HPLC, strong cation exchange (SCX) is a good method to separate positively charged AdoVin from other species in the reaction mixture at pH 3.0, such as negatively charged ATP and neutral vinthionine. Indeed, AdoVin binds with SCX column more strongly than C18 column, and it retained much longer than ATP and vinthionine. As shown in Figure 2.3 and Figure 2.5, AdoVin was produced only when MAT was provided, indicating that formation of AdoVin is MAT-dependent.

Figure 2.5. Strong cation exchange (SCX) HPLC chromatogram (monitored at 260 nm) showing the MAT-catalyzed synthesis of AdoVin and AdoMet; a) MAT-catalyzed AdoVin synthesis; b) MAT-catalyzed AdoMet synthesis.
**Characterization of Enzymatic Synthesis of AdoVin by MS**

Since all the compounds containing adenine group have the maximum absorbance at 260 nm in the reaction mixture, we need further verification of the newly found peak as AdoVin. Due to the instability of AdoVin, similar with AdoMet, purification of AdoVin is not practical, thus NMR is not the appropriate tool for the characterization. Instead, LC-MS was used to separate and then verify the synthesized AdoVin in the reaction mixture. Linear ion trap mass spectrometer was used to detect AdoVin synthesized from MAT catalyzed reactions. In order to maintain the cleanness of MS, volatile buffer salts NH$_4$HCO$_3$ was used for AdoVin synthesis at pH 8.0. MAT was precipitated by using 100% trichloroacetic acid (TCA) prior to the injection into LC-MS system. AdoVin was observed as m/z 411.07 with its single positive charge (Figure 2.6-2.7). CID MS/MS spectrum matches the theoretical prediction of AdoVin fragmentation, and adenosyl fragment is the most abundant species (Figure 2.8).

![Figure 2.6](image.png)

**Figure 2.6.** Extracted ion chromatogram of m/z 411.0 (expected for AdoVin).
Figure 2.7. Mass spectrum of MAT-catalyzed AdoVin synthesis (expected m/z 411.10 for AdoVin M+, observed m/z 411.07; mass difference 0.03 Da).

Figure 2.8. CID MS/MS spectrum of AdoVin from LC-ESI/MS. The +1 precursor ion for MS/MS analysis was m/z 411.07.
2.2.2 \textit{in situ} formation of AdoVin Adduct Catalyzed by Methyltransferases

Formation and Characterization of AdoVin-TNB Adduct

In order to test the activity of AdoVin, thiopurine methyltransferase (TPMT, EC 2.1.1.67) was selected for two reasons: first, TPMT has a broad substrate specificity to aromatic thiols,\textsuperscript{10-13} which we anticipated would show high activity to the vinyl group of AdoVin, based on Michael addition.\textsuperscript{14} Second, some of the aromatic thiol substrates and their methyl products or alkyl products have strong UV absorbance above 260 nm, therefore, the expected adduct between AdoVin and the aromatic thiols would have a signature UV absorbance.

Reduced Ellman’s reagent, 5-thio-2-nitrobenzoic acid (TNB) was used as the substrate to test the activity of AdoVin catalyzed by TPMT.\textsuperscript{15} The maximum UV absorbance of TNB is at 325 nm at pH 2-3, while the UV absorbance of methyl-TNB shifts to 350 nm.\textsuperscript{16} Our theoretical prediction of the UV spectrum for AdoVin-TNB is maximal at both 260 nm and the range 325-350 nm (Scheme 2.5).
Scheme 2.5. TPMT catalyzed methylation of TNB (top) and formation of bisubstrate-adduct between AdoVin and TNB (bottom).

In our experiments, AdoVin was synthesized from vinthionine and ATP catalyzed by MAT first, and then an AdoVin-TNB adduct was formed in situ, catalyzed by TPMT. A negative control without MAT (AdoVin) was included in the investigation. As Figure 2.9 shows, there was a peak after 1 hour incubation in the reaction mixture, while not in the negative control. Moreover, the UV absorbance of the new peak matches the expected combination of both the UV active chromophores, adenosyl group (260 nm)\(^9\) and alkyl-TNB (330 nm),\(^{16}\) and the ratio between 260 nm and 330 nm are very close to the estimated ratio of AdoVin-TNB adduct (Figure 2.10, Table 2.1). Therefore, the new peak was estimated as the AdoVin-TNB adduct.
Figure 2.9. HPLC chromatogram (325 nm) of AdoVin-TNB reaction catalyzed by MAT-TPMT (top), and AdoVin-TNB reaction mixture without MAT (bottom).
Figure 2.10. UV/Vis absorbance spectra of adenosine (red), methylated TNB (blue) and AdoVin-TNB adduct (black).
Table 2.1. Calculation of extinction coefficient of the AdoVin-TNB Adduct.

<table>
<thead>
<tr>
<th>Extinction Coefficient (ε, M⁻¹ cm⁻¹)</th>
<th>ε 260 nm</th>
<th>ε 350 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>15,400⁹</td>
<td>0</td>
</tr>
<tr>
<td>Methyl-TNB</td>
<td>5,254¹⁶</td>
<td>10,835¹⁶</td>
</tr>
<tr>
<td>AdoVin-TNB Adduct, Calculated</td>
<td>20,654</td>
<td>10,835</td>
</tr>
<tr>
<td>Calculated Ratio (260 nm: 350 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For the AdoVin-TNB Adduct</td>
<td>20,514/10,835 = 1.91</td>
<td></td>
</tr>
<tr>
<td>Observed Ratio (260 nm: 350 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For the AdoVin-TNB Adduct</td>
<td>392/198 = 1.98</td>
<td></td>
</tr>
</tbody>
</table>

To further verify the enzymatically synthesized AdoVin-TNB adduct, LC-MS was used. A species was observed with single positive charge (610.0 m/z). AdoVin-TNB was only observed in reaction mixture, not in the negative control “No TPMT” in which the region around 7.1 min (where the adduct eluted) was enlarged 50 times (Figure 2.11 and 2.12). This observation indicated that AdoVin-TNB adduct formation is TPMT dependent, and the subsequent CID MS/MS fragmentation of m/z 610.27 matches the theoretical prediction of AdoVin-TNB adduct (Figure 2.13).
Figure 2.11. Extracted ion chromatogram of m/z 411.0 (expected AdoVin), and 610.0 (expected AdoVin-TNB adduct) in AdoVin-TNB reaction (top), and AdoVin-TNB reaction without TPMT (bottom).
Figure 2.12. Mass spectrum of AdoVin-TNB adduct (expected m/z 610.14 for AdoVin-TNB adduct $M^+$, observed 610.27; mass difference 0.13 Da).

Figure 2.13. CID MS/MS spectrum of AdoVin-TNB adduct from LC-ESI/MS. The +1 precursor ion for MS/MS analysis was m/z 610.07.
2.2.3 Substrate Specificity toward AdoVin

When designing enzyme substrate analogues, the first priority is for enzyme specificity, that is, whether AdoMet analogues have the similar substrate specificity as AdoMet. To this end, we studied substrate specificity towards AdoVin by testing other known substrates of TPMT, and the structures of the thiol substrates are included in Table 2.2. Using HPLC-UV and LC-MS, we observed that all the tested known substrates reacted with AdoVin to form bisubstrate-adducts catalyzed by TPMT. For example, bisubstrate-adducts between AdoVin and 2-Bromothiophenol and 3-Bromothiophenol were observed in the MAT-TPMT coupled reactions, but not in the negative control (Figure 2.14 and Figure 2.15, c and d in Table 2.2). In the meanwhile, no adduct was observed between AdoVin and the non-substrate toward AdoMet, 4-nitrophenol (g in Table 2.2), indicating that 4-nitrophenol did not show activity to AdoVin, either (Figure 2.16). These observations indicate that the substrate specificity of TPMT toward AdoVin and AdoMet mirrors each other.

Table 2.2. Similar substrate specificity toward AdoVin and AdoMet

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Alkyl donor</th>
<th>AdoMet</th>
<th>AdoVin</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>HO₂C -</td>
<td>known</td>
<td>yes</td>
</tr>
<tr>
<td>b</td>
<td>NO₂</td>
<td>known</td>
<td>yes</td>
</tr>
<tr>
<td>c</td>
<td>SH</td>
<td>known</td>
<td>yes</td>
</tr>
<tr>
<td>d</td>
<td>Br</td>
<td>known</td>
<td>yes</td>
</tr>
<tr>
<td>e</td>
<td>NO₂</td>
<td>known</td>
<td>yes</td>
</tr>
<tr>
<td>f</td>
<td>HO₂C -</td>
<td>unknown</td>
<td>yes</td>
</tr>
<tr>
<td>g</td>
<td>NH₂</td>
<td>non-substrate</td>
<td>no</td>
</tr>
</tbody>
</table>
Figure 2.14. Selected ion chromatography of m/z 599.0 (theoretical AdoVin-BrPhSH adduct, m/z 599.07) in AdoVin-2-Bromothiophenol (2BrPhSH, c) and AdoVin-3-Bromothiophenol (3BrPhSH, d) reactions. The adducts between AdoVin and 2BrPhSH and 3BrPhSH were observed at 13.5 and 14.3 min, respectively.
Figure 2.15. Extracted ion chromatogram of m/z 599.0 (theoretical AdoVin-BrPhSH adduct, m/z 599.07) in AdoVin-2BrPhSH reaction (top) and negative control “No 2BrPhSH” (bottom).
Figure 2.16. HPLC chromatogram (325 nm) of \textit{in vitro} AdoVin-Nitrophenol (g) reactions (top) and negative control at 3 hours incubation (bottom). No adduct was not observed, indicating that no reaction occurred between AdoVin and 4-nitrophenol (non-substrate of TPMT).
2.2.4 Tight Binding between Bisubstrate-Adduct and Methyltransferases

Taking the concept of bisubstrate-adduct inhibition into consideration, a synergistic interaction between the bisubstrate-adduct and MTases is expected. To investigate the binding strength of the AdoVin adduct for methyltransferases, ultrafiltration was performed to separate TPMT and other large proteins from small molecules (free ligands) in the reaction mixture by molecular weight (MW of TPMT is 30,343; MWCO of filters is 10,000). Immobilized metal ion affinity chromatography (IMAC) was also applied to isolate TPMT from other components, since our recombinant TPMT contains a hexa-histidine tag.

After isolation of TPMT using both methods, TPMT and other large proteins were precipitated using 100% TCA, and the supernatant was injected into LC-MS. Under both ultrafiltration and IMAC conditions, AdoVin-TNB adduct was observed only in the enzyme complex (Table 2.3 and Figure 2.17), indicating significantly strong binding between AdoVin-TNB adduct and TPMT. While other small molecules were only observed in free ligands fraction, but not in TPMT complex fraction. This suggests markedly weak binding or no interaction between other small molecules and TPMT, including the non-natural substrates, vinthionine and AdoVin (Table 2.3 and Figure 2.18). The adducts between AdoVin and other thiol substrates of TPMT, for example, AdoVin-2-Bromothiophenol and AdoVin-3-Bromothiophenol, were also tested for the tight binding event (Figure 2.19-2.20). All the AdoVin-thiols adducts were observed tightly bound with TPMT, suggesting that the tight binding event is the common property of AdoVin adducts.
Table 2.3. Main components observed in the fractions of isolated TPMT and free ligands

<table>
<thead>
<tr>
<th>Isolated TPMT fraction</th>
<th>Free ligands fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdoVin-thiol bisubstrate-adduct</td>
<td>Vinthionine</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td></td>
<td>TNB</td>
</tr>
<tr>
<td></td>
<td>AdoVin</td>
</tr>
</tbody>
</table>

Figure 2.17. HPLC chromatogram of isolated TPMT fraction (top) and free ligands fraction (bottom) with UV detection at 325 nm.
Figure 2.18. Extracted ion chromatogram of m/z 162.07 (theoretical vinhionine, m/z 162.05) and 610.14 (theoretical AdoVin-TNB adduct, m/z 610.27) in isolated TPMT fraction (top) and free ligands fraction (bottom).
Figure 2.19. Selected ion chromatogram of m/z 599.0 (theoretical AdoVin-BrPhSH adduct, m/z 599.07) for the binding assay between AdoVin-BrPhSH adduct and TPMT. The intensity of peaks between 13 and 14 min in “free ligands b)” fraction is over 50 fold less than that of AdoVin-2BrPhSH and AdoVin-3BrPhSH adducts in “TPMT complex a)”, indicating that the no AdoVin-BrPhSH adduct was detected in “free ligands b)”.
Figure 2.20. CID MS/MS spectra of AdoVin-2BrPhSH adduct (top) and AdoVin-3BrPhSH adduct (bottom) from LC- ESI/MS. The +1 precursor ions for MS/MS analysis were m/z 598.91 and 598.97, respectively.
2.2.5 Kinetics of Methyltransferases-catalyzed AdoVin Adduct Formation

To further investigate the mechanism of TPMT catalyzed AdoVin bisubstrate-adduct formation, kinetic studies of TPMT catalyzed AdoVin-TNB adduct formation were performed, and the concentration change of TNB with time were monitored at 411 nm at pH 8.0. As shown in Figure 2.21, AdoVin-TNB adduct formation is both time-dependent (first-order kinetics) and enzyme concentration dependent. This is consistent with the first order reaction mechanism, involving a rapid initial binding of the thiol substrate and AdoVin with TPMT and then a subsequent addition reaction between the thiol substrate and AdoVin. Better still, only a single turnover formation of the bisubstrate-adduct was observed (Figure 2.22), suggesting that the resulting adduct binds much more tightly than either substrate, and thus inhibits the enzyme from further turnover. This observation is also consistent with the enhanced binding affinity by the bisubstrate-adduct compared to the individual substrates.

For the data processing, the concentration change of TNB was fitted to the first order equation: \( y = m1 + m2 \cdot e^{(-m3 \cdot x)} \), and the calculated “m3” is the “turnover number”, \( k_{cat} \), for TPMT catalyzed AdoVin-TNB, and the half time, \( t_{1/2} \) is thus calculated by using equation \( t_{1/2} = \frac{0.693}{k_{cat}} \). The fitting parameters were shown in Table 2.4.
Figure 2.21. Absorbance change of TNB at 411 nm of AdoVin-TNB reactions catalyzed by TPMT under various concentrations
Figure 2.2. The overall concentration change of TNB at 411 nm at different concentration of TPMT, indicating a single turnover of the reaction.

\[ y = 0.9241x; \quad R^2 = 0.9971 \]
Table 2.4. Fitting parameters of first order kinetics

<table>
<thead>
<tr>
<th></th>
<th>5 µM TPMT</th>
<th>10 µM TPMT</th>
<th>20 µM TPMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitting equation</td>
<td>( y = m_1 + m_2 e^{-m_3 x} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m_1</td>
<td>-0.0602</td>
<td>-0.111</td>
<td>-0.212</td>
</tr>
<tr>
<td>m_2</td>
<td>0.0635</td>
<td>0.113</td>
<td>0.220</td>
</tr>
<tr>
<td>m_3</td>
<td>0.490</td>
<td>0.298</td>
<td>0.201</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>0.847</td>
<td>0.997</td>
<td>0.998</td>
</tr>
<tr>
<td>( t_{1/2} ) (min) = ( 0.693/ k_{cat} )</td>
<td>1.4</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>average of ( t_{1/2} ) (min)</td>
<td></td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>standard deviation ( \sigma ) of ( t_{1/2} ) (min)</td>
<td></td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>( k_{cat} ) (min(^{-1})) = m_3</td>
<td>0.490</td>
<td>0.298</td>
<td>0.201</td>
</tr>
<tr>
<td>average of ( k_{cat} ) (min(^{-1}))</td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>standard deviation ( \sigma ) of ( k_{cat} ) (min(^{-1}))</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
</tbody>
</table>
2.2.6 Discovery and Identification of Unknown Substrates of Methyltransferases

2.2.6.1 Discovery of Unknown Adduct

One of our goals is to identify unknown substrates of MTases; in particular, no endogenous substrate for TPMT has been reported. To this end, AdoVin was incubated with the crude cell lysate of the \textit{E. coli} strains that expressed recombinant human TPMT, but no AdoVin adduct was detected (Figure 2.23 and 2.26), which was not unexpected as no aromatic thiol metabolites have been reported for \textit{E. coli}.

As a positive control, TNB was added to the \textit{E. coli} crude cell lysate, and the corresponding adduct was detected only in the enzyme complex (see Figure 2.23 and 2.24), indicating tight binding under physiological conditions as well. Furthermore, Figure 2.24 illustrates the significant enrichment of the adducts from very complex cellular mixtures, thereby making it possible to readily identify the unknown substrate.

Interestingly and unexpectedly, besides the AdoVin-TNB adduct (m/z 610.0), another adduct (m/z 580.0 in Figure 2.23 and 2.26) was detected in the TPMT complex, but only when TNB was added to cell lysate, suggesting this unknown peak was derived from TNB. Isotope labeled AdoVin (+15 Da shift) was introduced to verify the unknown peak. Based on the mass shift (+15 Da) and its UV-Vis spectrum (Figure 2.25), it could be inferred that the unknown contains both AdoVin (260 nm) and thiol moieties (330 nm). Isotope labeling was used to verify the unknown adduct. Furthermore, based on the MSMS fragmentation (Figure 2.27) and the mass change between the unknown adduct and AdoVin-TNB adduct (-30 Da), we postulated that the nitro-group was reduced to an amine, with the knowledge of the existence of at least four nitroreductases with broad specificities in \textit{E. coli}.\textsuperscript{17-22} Further verification will be discussed in the next section.
Figure 2.23. Selected ion chromatogram of AdoVin adducts (m/z 610.0 and 580.0) in isolated TPMT fractions from crude cell lysates of ex vivo AdoVin-TNB and negative control (without exogenous thiols).
Figure 2.24. HPLC chromatogram (260 nm) of *ex vivo* AdoVin-TNB reaction at 1 hour (top), isolated TPMT fraction (middle), and free ligands fraction (bottom), showing the adducts tightly bound with TPMT complex in *ex vivo* AdoVin-TNB reactions.
Figure 2.25. UV-Vis spectra of AdoVin-TNB adduct (black) and unknown adduct (red).

Figure 2.26. Extracted ion chromatogram (XIC) of m/z 610.0 and 580.0 in TPMT fraction of ex vivo AdoVin-TNB (top) and ex vivo “No thiols” negative control (bottom), indicating that unknown adduct m/z 580.0 was found, and also no AdoVin adduct was observed without exogenous thiols.
Figure 2.27. CID MS/MS spectra of AdoVin-TNB adduct, regular and isotope labeled unknown adduct from LC-ESI/MS. The +1 precursor ions for MS/MS analysis were m/z 610.01, 580.08 and 595.08, respectively.
2.2.6.2 Elucidation and Confirmation of Unknown Adduct

**Synthesis of 2-Amino-5-Mercaptobenzoic Acid**

We hypothesized that the nitro group of the AdoVin-TNB adduct was reduced to amine group, and postulated the unknown thiol to be 2-amino-5-mercaptobenzoic acid (Scheme 2.6). In order to confirm this hypothesis, authentic 2-amino-5-mercaptobenzoic acid (AMBA) was synthesized, and the reaction between AMBA and AdoVin was investigated. First, the disulfide form, 5,5’-dithio-bis-(2-aminobenzoic acid), was synthesized from the disulfide form of TNB (5,5’-dithio-bis-(2-aminobenzoic acid), DTNB), and then AMBA was synthesized from its disulfide form by the reduction of tris(2-carboxyethyl)phosphine (TCEP).

![Scheme 2.6. Synthesis of 2-amino-5-mercaptobenzoic acid.](image-url)
**in vitro** Bisubstrate-adduct Formation between AdoVin and AMBA

Next, the reaction between authentic AMBA and AdoVin was performed under the catalysis of TPMT (Scheme 2.7). It was expected that AMBA would react with AdoVin to form a bisubstrate-adduct. The co-elution of the *in vitro* formed AdoVin-AMBA adduct and the *ex vivo* unknown adduct indicates that they are the same compound (Figure 2.28), which was also verified by overlying their UV spectra (Figure 2.29) and their mass spectra with isotope labeled AdoVin-AMBA adduct (Figure 2.30). The CID MSMS fragmentation of unknown adduct was then assigned as AdoVin-AMBA adduct, sharing similar fragmentation with AdoVin-TNB adduct (Figure 2.31).

Scheme 2.7. *in vitro* bisubstrate-adduct formation between AdoVin and AMBA catalyzed by thiopurine S-methyltransferase.
Figure 2.28. HPLC chromatogram (350 nm) of authentic AdoVin-AMBA adduct (top), co-injection of ex vivo AdoVin-TNB reaction and authentic AdoVin-AMBA adduct (middle), and ex vivo AdoVin-TNB reaction (bottom).
Figure 2.29. UV spectra of \textit{ex vivo} AdoVin-AMBA adduct (black) and authentic AdoVin-AMBA adduct standard (red).

Figure 2.30. Extracted ion chromatogram of m/z 580.08 (AdoVin-AMBA adduct) and 595.06 (isotope labeled) in isolated TPMT fraction (top) and free ligands fraction (bottom).
Figure 2.31. CID MS/MS spectra of AdoVin-TNB adduct, regular and isotope labeled 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.
2.2.6.3 Confirmation of 2-Amino-5-Mercaptobenzoic Acid as TPMT Substrate

It is worth noting that this amino thiol (AMBA) had not been reported as a substrate of TPMT, so next, methylation of AMBA was performed to characterize AMBA as the substrate of TPMT (Scheme 2.8). A new peak was observed eluting after AMBA in C18 column, and only occurred when TPMT was used (Figure 2.32). Together with its UV spectrum (Figure 2.33), the new peak was confirmed as methylation product of AMBA. Thereby, AMBA was confirmed as a substrate of methylation. Altogether, this serendipitous finding underscores the utility of our approach in directly identifying enzyme substrates, even unknowns.

Scheme 2.8. Proposed methylation of 2-amino-5-mercaptopbenzoic acid catalyzed by thiopurine methyltransferase.
Figure 2.32. HPLC chromatogram (350 nm) of TPMT catalyzed methylation of 2-amino-5-mercaptobenzoic acid (top), negative control for AMBA methylation without TPMT (middle), and co-injection of AMBA methylation and authentic AMBA standard (bottom).
Figure 2.3. UV spectra of methylated AMBA (black) and AMBA (red). The spectra were normalized at 260 nm (AMBA) and 270 nm (Me-AMBA).
2.2.7 No Modification of Enzymes by AdoVin

One of the general concerns of AdoMet analogues is whether the analogue modifies enzymes. MALDI-TOF/TOF was performed to monitor the potential background adduct formation between AdoVin and proteins. As shown in Figure 2.34 and 2.35, no modification was detected on either TPMT or MAT. While the vinyl sulfonium group is intrinsically reactive, extensive solvation in aqueous solution renders low reactivity of highly charged sulfoniums. For instance, no background reaction between aromatic thiols and AdoVin was observed in the absence of TPMT, and moreover, even under ex vivo conditions where many metabolites exist, no adducts were detected unless exogenous thiols were provided.
Figure 2.34. MALDI mass spectra of native MAT (top) and treatment with AdoVin (bottom). The molecular weight of native MAT is 48019.0. Under the instrument error range, no mass shift (+411.1 Da) was observed, indicating that no modification with AdoVin was observed.
Figure 2.35. MALDI mass spectra of native TPMT (top) and treatment with AdoVin (bottom). The molecular weight of native TPMT is 30343.6. Under the instrument error range, no mass shift (+411.1 Da) was observed, indicating that no modification with AdoVin was observed.
2.3 Conclusions

In sum, our strategy can indeed capture and identify enzyme substrates, even unknowns. Conversely, if a substrate or methylation product is known, the corresponding enzyme can be identified as well. Applications in whole cells and organisms can also be envisioned, as AdoVin can be synthesized \textit{in vivo} when vinthionine is supplemented. Moreover, the formation of bisubstrate-adducts may also have broad utility in facile generation of strong specific inhibitors and structural elucidation of substrate-enzyme interactions. Lastly, our approach can be applied to many enzymes in other classes, particularly those catalyzing group transfers.
2.4 Experimental Section

2.4.1 General Procedures

All chemicals with reagent purity or above were purchased from Sigma and Fisher. A 100 mM Adenosine-$^{13}$C$_{10}$,$^{15}$N$_5$ 5’-triphosphate sodium salt solution (+15 ATP) in 5 mM Tris buffer was purchased from Sigma (catalog No. 645702). Immobilized metal ion affinity chromatography was performed on HisTrap HP columns (GE Healthcare Life Sciences, catalog No. 17-52447-01), and the HisPur Ni-NTA Spin Column with 0.2 mL resin bed (ThermoScientific Pierce, catalog No. 12393730), respectively. Ultrafiltration was carried out using filters with 30,000 molecular weight cut off (MWCO). The filters were purchased from Fisher Scientific (EMD Millipore Amicon Ultra 0.5 mL, catalog No. UFC50VL96).

UV absorbances were measured on a Cary 50 Bio UV/VIS spectrophotometer. HPLC chromatograms were acquired 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. Mass spectral data were acquired on a Thermo LCQ Deca XP Ion trap mass spectrometer in lined with an Agilent 1200 HPLC, and processed by using Xcalibar Data System 2.0.7 (Thermo Fisher Scientific Inc. Waltham, MA). Graphics were constructed by using the Kaleidagraph software package 4.1 (Synergy Software, Reading, PA).
2.4.2 Expression and Purification of Enzymes

**Recombinant Thiopurine S-Methyltransferase (TPMT)**

Recombinant human TPMT was prepared as described previously. The human thiopurine S-methyltransferase gene was ligated into pET28a vector (Novagen, Madison, WI) between NdeI and EcoRI sites. The amino acid sequence of the N-terminal histidine tag prior to the original starting methionine is MGSSHHHHHH SSGLVPRGS. The recombinant plasmid, TPMT7A, was transformed into *Escherichia coli* strain BL21 (DE3) (Novagen) for protein expression. A starter culture of 10 mL in Luria-Bertani broth (LB) with 50 μg/mL kanamycin was initiated with a single transformed colony and grown overnight at 37 °C in a shaker (50 rpm) to reach an OD600 reading around 0.6. The BL21 (DE3)/TPMT7A cells were then transferred to 1 L LB broth with 50 μg/mL kanamycin and grown aerobically at 37 °C in a shaker (50 rpm) to an OD600 reading of 0.6. Then, isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression. Cells were harvested 8 h post induction by centrifugation (4500 g, 4 °C, and 20 minutes) and resuspended in a cold solution containing 20 mM potassium phosphate and 0.5 M NaCl at pH 8.0. Cells were lysed via sonication on ice, and the cell debris and unbroken cells were removed by centrifugation (8000 g, 4 °C, and 60 minutes).

The purification procedure was based on the protocol from the manufacturer. A 5 mL HiTrap nickel-chelate column (GE Healthcare) was preconditioned with 25 mL MilliQ water, 5 mL 0.1 M Nickel Sulfate (filtered by 0.2 μm membrane), 25 mL MilliQ water, 30 mL binding buffer (20 mM potassium phosphate, 0.5 M NaCl and 5 mM imidazole, pH 8.0, filtered by 0.2 μm membrane). After column conditioning, the supernatants from TPMT lysate were loaded onto the column, equilibrated with 50 mL binding buffer, and washed with 25 mL washing buffer (20 mM potassium phosphate, 0.5 M NaCl and 50 mM imidazole, pH 8.0, filtered by 0.2 μm membrane).
The TPMT protein was eluted with 25 mL elution buffer (20 mM potassium phosphate, 0.5 M NaCl and 500 mM imidazole, pH 8.0, filtered by 0.2 µm membrane). Stripping buffer (25 mL, 20 mM potassium phosphate, 0.5 M NaCl and 500 mM EDTA, pH 8.0, filtered by 0.2 µm membrane) was used to strip the remaining proteins on the column. The protein solution was dialyzed in 50 mM potassium phosphate at pH 8.0 containing 2 mM DTT. The purified TPMT solutions were mixed with glycerol (10%, v/v) and stored at -80 °C.

Protein purity was analyzed by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) and visualized by Coomassie blue staining. The molecular weight of TPMT is calculated as 34401.3 Da, and the concentration of TPMT is determined by UV absorbance at 280 nm, using the estimated extinction coefficient $\varepsilon_{280\text{ nm}} = 39,420 \text{ M}^{-1}\text{cm}^{-1}$.

Recombinant Methionine S-Adenosyltransferase (MAT)

The MJ1208 gene was amplified from *M. jannaschii* genomic DNA by polymerase chain reaction using the primers MJ1208FWD (GCATATGAGAACATAATTGTATAAAAATATTAG) and MJ1208REV (GGATCTTTAGAATGTAGTTACTTTTCC). The NdeI and BamHI sites introduced into the gene through the primers were used to clone the DNA into the pET-19b vector (Novagen Inc.). *E. coli* BL21 (DE3) cells were transformed with plasmid (pMJ1208-1) for protein expression. Ampicillin (100 mg/ml) was used as the antibiotic for MAT expression. The detailed expression and purification procedure is similarly described in TPMT section. The molecular weight of MAT is calculated as 50427.7, and the concentration of MAT is determined by UV absorbance at 280 nm using the estimated extinction coefficient $\varepsilon_{280\text{ nm}} = 24,870 \text{ M}^{-1}\text{cm}^{-1}$. 
Recombinant *E. coli* 5'-methylthioadenosine/S-adenosyl-homocysteine nucleosidase

An EcoRI/NotI fragment from p5Xmtan (Cornell & Riscoe, 1998) containing the complete *E. coli* MTA/AdoHcy nucleosidase gene (accession No. U24438) was ligated into EcoRI/NotI-digested pPROEX HTa expression vector (Gibco BRL) and transformed into *E. coli* strain TOP10F'. The expressed enzyme contains a 31-residue N-terminal tag consisting of a six-histidine tag, a spacer sequence and an rTEV protease cleavage site prior to the native initiating methionine of the nucleosidase. Ampicillin (100 mg/ml) was used as the antibiotic for 5'-methylthioadenosine/S-adenosyl-homocysteine nucleosidase (MTAN) expression. The detailed expression and purification procedure is similarly described in TPMT section. The molecular weight of MTAN is calculated as 28157.0 Da, and the concentration of MTAN is determined by UV absorbance at 280 nm using the estimated extinction coefficient $\varepsilon_{280\text{ nm}} = 11,920 \text{ M}^{-1}\text{ cm}^{-1}$.

2.4.3 Protein Precipitation

100% (w/v) trichloroacetic acid (TCA) was prepared by dissolving 5 g TCA into 3.5 mL Milli Q water. The TCA stock solution was stored at room temperature, protected from light. To precipitate proteins in the reaction mixtures, 1 volume TCA stock solution was added to 4 volumes of protein containing reaction mixtures, and incubated 10 minutes at 4 °C. The protein precipitation was then spun down at 14K rpm for 5 minutes. The supernatant was saved for further analysis.
2.4.4 Preparation of Vinthionine

Vinthionine. [CAS: 83768-87-0] Vinthionine was prepared as reported.\textsuperscript{25} 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. 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 as evaporated, the flask was cooled to 0 °C, and 15 mL of anhydrous DMSO was added to dissolve the residue. Dried acetylene gas 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 1 N 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. \textsuperscript{1}H NMR (D\textsubscript{2}O/K\textsubscript{2}CO\textsubscript{3}, 400 MHz): \(\delta\) 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); \textsuperscript{13}C NMR (D\textsubscript{2}O, 100 MHz): \(\delta\) 174.45, 130.68, 112.70, 53.94, 30.17, 26.42.
Figure 2.36. $^1$H-NMR spectrum of vinthionine (400 MHz, D$_2$O).
Figure 2.37. $^{13}$C-NMR spectrum of vinthionine (100 MHz, D$_2$O).
2.4.5 Enzymatic Synthesis of AdoVin

*S-Adenosyl-Vinthionine. [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 ε$_{260}$ nm = 15,400 M$^{-1}$ cm$^{-1}$ based on the value for AdoMet.$^9$

2.4.6 in vitro Formation of AdoVin Adduct in MAT-TPMT Coupled Reactions

AdoVin-TNB Adduct Formation

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, “No MAT” and “No TPMT.” The concentration of TNB was determined using ε 411 nm = 13,600 M$^{-1}$ cm$^{-1}$.26

AdoVin-Thiophenol Adduct Formation

The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM MgCl$_2$, 1 mM ATP, 900 μM vinthionine, 510 μM thiophenol, 2 mM TCEP, 120 μM TPMT, and 1.75 μM MTAN. The reaction was initiated with 300 μM MAT and incubated at 37 °C. HPLC-UV and LC-
MS were used to monitor the reactions. The same solutions without MAT or TPMT were performed as negative controls, “No MAT” and “No TPMT.”

**AdoVin-Bromothiophenol Adduct Formation**

The reaction contained 50 mM potassium phosphate, pH 8.0, 10 mM KCl, 5 mM MgCl₂, 2 mM ATP, 900 μM vinthionine, 410 μM 2-Bromothiophenol (2BrPhSH) or 3-Bromothiophenol (3BrPhSH), 2 mM TCEP, 120 μM TPMT, and 1.75 μM MTAN. The reaction was initiated with 300 μM MAT and incubated at 37 °C. The same reactions without MAT, TPMT and 2BrPhSH were performed as negative controls, “No MAT”, “No TPMT” and “No 2BrPhSH,” and the negative controls contained both 2-Bromothiophenol and 3-Bromothiophenol.

**AdoVin-4-Nitrothiophenol Adduct Formation**

The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM MgCl₂, 1 mM ATP, 450 μM vinthionine, 400 μM 4-nitrothiophenol, 2 mM TCEP, 1.75 μM MTAN, and 74 μM TPMT. The reaction was initiated with 160 μM MAT and incubated at 37 °C. The same reactions without MAT or TPMT were conducted as negative controls. The concentration of 4-nitrothiophenol was determined using ε 411 nm = 13,650 M⁻¹ cm⁻¹.¹⁵

**AdoVin-4-Nitrophenol Adduct Formation**

The reaction contained 50 mM potassium phosphate, pH 8.0, 10 mM KCl, 5 mM MgCl₂, 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 MAT or TPMT were conducted as negative controls, “No MAT” and “No TPMT.” The reaction was initiated with 182 μM MAT and incubated at 37 °C.
2.4.7 Binding Assay

The binding assay was conducted at 4 °C using the HisPur Ni-NTA Spin Column (0.2 mL resin bed, Thermo scientific). Storage buffer of column was removed by centrifuging 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 reaction samples were mixed with an equal volume of equilibration buffer and then loaded onto the columns. The column was then mixed 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 eluted with 3 x 200 μL elution buffer (50 mM potassium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0).

Ultrafiltration was carried out using filters with 30,000 MWCO (0.5 mL). The reaction sample (100 μL) was loaded to the membrane containing vials. Ammonium bicarbonate, pH 8.0 (400 μL 50 mM) was added to the retentate, and the diluted free ligands were filtered out again by 12,000 g for 5 min. The buffer exchange procedure was continued until the UV absorbance of the flow through was 0. The retentate was removed for further analysis, as well as free ligands fraction by the first filtration.

The protein containing fractions from both methods were monitored by measuring the UV absorbance at 280 nm, and injected into HPLC after protein acidic precipitation.
2.4.8 *ex vivo* AdoVin Adduct Formation Catalyzed by TPMT

**Preparation of *ex vivo* AdoVin-TNB reactions**

The concentration of TPMT in TPMT lysate was estimated as 336 μM by the TPMT band on SDS-PAGE gel. The TPMT lysate was prepared as described in general procedure without the purification. The reaction (200 μL) contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 10 mM ATP, 1 mM vinthionine, 489 μM TNB, 2 mM TCEP, 3.5 μM MTAN and 100 μL TPMT cell lysate. The reaction was initiated with 295 μM MAT and incubated at 37 °C.

To do the binding assay between *ex vivo* formed AdoVin-TNB adduct and TPMT, the *ex vivo* TNB reaction sample was loaded onto the HisPur Ni-NTA Spin Column, and isolation of TPMT fraction from TPMT lysates was described previously.

**Preparation of isotope labeling AdoVin-TNB adduct**

The reaction contained 50 mM potassium phosphate, pH 8.0, 5 mM KCl, 2.5 mM MgCl₂, 1 mM Adenosine-¹³C₁₀,¹⁵N₂-5′-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.
2.4.9 Elucidation and Confirmation of Unknown Adduct

Synthesis of 5,5'-dithio-bis-(2-aminobenzoic acid)

As reported,\textsuperscript{27-28} an 8 mL solution of 7.5 M ammonium hydroxide was degassed by bubbling N\textsubscript{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 (5,5-dithio-bis-(2-nitrobenzoic acid), TNB, 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\textsubscript{f} = 0.08 in 25:75 i-PrOH/CHCl\textsubscript{3}; m.p. 200°C; \textsuperscript{1}H-NMR (CDCl\textsubscript{3}/DMSO-d\textsubscript{6} 95:5) \( \delta \) 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)\superscript{+} 337.0 [(M+H)+, calcd for C\textsubscript{14}H\textsubscript{13}N\textsubscript{2}O\textsubscript{4}S\textsubscript{2}: 337.0] and 319.0 [(M-OH)+, calcd for acylium ion C\textsubscript{14}H\textsubscript{11}N\textsubscript{2}O\textsubscript{3}S\textsubscript{2}: 319.0].
**in vitro Bisubstrate-Adduct Formation between AdoVin and AMBA**

The reaction contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 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 adduct peak in AdoVin-AMBA reaction, the *in vitro* reaction mixture was co-injected with *ex vivo* AdoVin-TNB reaction mixture. The *ex vivo* AdoVin-TNB reaction sample was prepared as described previously.

### 2.4.10 Confirmation of 2-Amino-5-Mercaptobenzoic Acid as TPMT Substrate

The assays 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 13.2 μM TPMT and incubated at 37 °C. 10 μL reaction samples (at 0, 15, 30, 60 and 120 min) were analyzed by HPLC. The eluting profiles were monitored by UV absorbance at both 260 and 350 nm.

### 2.4.11 Modification of MAT and TPMT by AdoVin

AdoVin was enzymatically synthesized in 50 mM ammonium bicarbonate, pH 8.0, MAT was filtered out by using filters with 30,000 Da molecular weight cut off (MWCO). The AdoVin solution was injected into HPLC with UV detector, and the concentration of AdoVin was determined by UV absorbance at 260 nm.

The assay solution contained 50 mM ammonium bicarbonate (pH 8.0), 71.32 μM AdoVin was incubated with 41.2 μM MAT and 42 μM TPMT, respectively. Samples of MAT and TPMT
without treatment with AdoVin were prepared as control. All the samples were incubated at 37 °C for 18 h and analyzed by MALDI-TOF/TOF.

2.4.12 Kinetics Analysis of TPMT-Catalyzed AdoVin-TNB Adduct Formation

The reaction contained 200 mM Tris buffer, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 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).

AdoVin-TNB adduct formation was initiated with TPMT at various concentrations, and incubated at 37 °C. The spectral changes were monitored continuously between 260-610 nm for 240 min. Three scanning cycles were set up with different scan time intervals: 1 min for the first 60 min, then 5 min between 60 and 120 min incubation, and finally increased to 30 min until 240 min. Kaleidagraph software package 4.1 (Synergy Software, Reading, PA) was used to analyze the absorbance change at 411 nm.

2.4.13 HPLC Analysis of AdoVin and AdoVin reactions

Reverse Phase HPLC Separation of AdoVin

Aliquots of reaction mixture (10 μL) were removed and analyzed by HPLC, and elution profiles were monitored by UV absorption at 260 nm. The separation of AdoVin was carried out on a reverse-phase column (Apollo, C₁₈, 5μ, 4.6 mm x 150 mm), using 0.1% aqueous trifluoroacetic acid (TCA) (mobile phase A) and 0.1% TCA 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.

**Strong Cation Exchange HPLC Separation of AdoVin**

Aliquots of reaction mixture (10 μL) were removed and analyzed by HPLC, and elution
profiles were 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% NH₄OH in water (mobile phase A, pH 3) and 50 mM (NH₄)₂SO₄, 20% Acetonitrile and 0.07% NH₄OH 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.

**Reverse Phase HPLC Separation of AdoVin Adducts**

All the samples were injected and analyzed by HPLC, elution profiles were monitored by
UV absorption at 260 nm and 350 nm, based on the absorbance for AdoMet and alkyl TNB. The
chromatography was performed on a reversed-phase column (Apollo, C₁₈, 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.
Reverse Phase HPLC Separation of methylated AMBA

Aliquots (10 μL) of reactions and negative controls were injected and analyzed by HPLC, elution profiles were monitored by UV absorption at 260 nm and 350 nm, based on the absorbance for AdoMet and alkyl TNB. The chromatography was performed on a reversed-phase column (Apollo, C18, 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. Same chromatography gradient program was used in separation of AdoVin adducts.

2.4.14 Mass spectrometry

All the analysis samples were exchanged into 50 mM ammonium bicarbonate, pH 8.0. TCA (100% v/v) was used to precipitate the proteins in the samples, and the supernatants were saved for further analysis by mass spectrometry. Reaction and negative control samples (15 μL of each sample) were taken for HPLC-MS analysis. Free ligands fraction after ion metal affinity chromatography (50 μL) were taken for HPLC-MS analysis.

LCQ Ion Trap mass spectrometer was used in-line with HPLC system (Agilent 1100) for the identification of AdoVin and AdoVin adducts, respectively. The chromatography was performed on a reversed-phase column (Apollo, C18, 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, 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. LCQ was operated with a full scan and data-independent MSMS scan of the top ten most abundant ions. The spray voltage was 2.3 kV, and the capillary temperature was 285 °C. Data were processed using the Xcalibur Data System 2.0 (Thermo Fisher, Waltham, MA).

2.4.15 MALDI-TOF/TOF Analysis

Protein samples was diluted to 50 µM using a solution of acetonitrile/water/TFA (v:v:v:, 50:50:0.5). Protein samples (0.5 µL) were spotted on a clean standard 192 well stainless steel MALDI sample plate followed by the addition of sinapinic acid matrix solution (0.5 µL, 10 mg/mL in a mixture of acetonitrile/water/TFA, v:v:v:, 50:50:0.5). The resulting mixtures were air-dried and analyzed using an AB 5800 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems Framingham, MA). MS spectra were acquired in linear positive mode. Lysozyme ([M + H]+: 14,308 Da) and BSA ([M + H]+: 66,464 Da) were used for external calibration. Software Data Explorer 4.6 was used to analyze the data.
2.5 References


Chapter 3: Identification of Substrates by Native Mass Spectrometry

This chapter is based on a manuscript with the title of “Identifying Substrates of Thiopurine Methyltransferase via Enzyme-Ligand Complex Using Native Mass Spectrometry” with the author list: Jing Yan, Wanlu Qu, Kalli Catcott, Vicki H. Wysocki, and Zhaohui Sunny Zhou

Co-authors’ works in this chapter: Jing Yan and Wanlu Qu contributed equally; Jing Yan, mass spectrometric analysis; Wanlu Qu, experimental design and performance on AdoVin reactions; Kalli Catcott, cell lysates preparation, manuscript writing and idea contributions; Vicki H. Wysocki, idea contributions; Zhaohui Sunny Zhou, principal investigator.
3.1 Introduction

3.1.1 Native Mass Spectrometry

Understanding proteins and conformation of protein networks is of high importance. However, the characterization of proteins and native protein networks, including protein-protein interactions and protein-small molecule interactions, remains challenging, despite the routine determination of protein primary sequence. Recently, the application of soft ionization methods in mass spectrometry (native MS) allows the efficient transfer of intact large proteins into the gas phase, making it possible to characterize intact proteins with higher-order structure and detect native protein networks.¹

Native protein mass spectrometry was termed by Heck and van den Heuvel as a powerful technique to characterize proteins in their native functional form, and analyze large intact protein complexes, even non-covalent complexes.²⁻⁴ Currently, native MS with the soft ionization method, nano-ESI, is applicable for analyzing protein complexes with masses up to 18 MDa.³ Furthermore, the development of tandem MS followed by native MS has broadened the utility of MS in the characterization of protein structures and protein complexes, especially those with unknowns.
3.1.2 Tandem Mass Spectrometry and Fragmentation in Tandem MS

Tandem mass spectrometry, also known as MS/MS or MS², is a common technique for generating fragments that can contribute to the structural analysis of precursor ions. There are mainly three steps in tandem mass spectrometry: 1) selection of precursor ions from the first stage mass analyzer; 2) fragmentation of selected precursor ions; 3) analysis of the fragmentation ions by a second stage mass analyzer. Multiple fragmentation can be performed as MSⁿ.⁵ Additionally, the precursor ions can be selected and isolated by both data-dependent and precursor ion targeting modes.

There are two commonly-used fragmentation techniques in tandem mass spectrometry: collision-induced dissociation (CID, also known as collisionally activated dissociation) and electron transfer dissociation (ETD). In our work, another newly developed fragmentation technique has been applied, that is, surface induced dissociation (SID).

CID involves collision of the selected precursor ions with inert gas ions, i.e., helium or nitrogen, resulting in a vibrational excitation in the precursor ions. Part of the kinetic energy of the precursor ions can be converted into internal energy that generates corresponding product ions. CID is commonly applied to break the amide bonds of peptide backbone.⁶ The limitation of CID is exemplified in the detection of protein posttranslational modifications (PTMs), because most modifications bonds are weaker than peptide bonds, and thus are cleaved first under CID fragmentation.⁷

ETD is a newly developed fragmentation technique that transfers electrons to positively charged proteins or peptides, which turns proteins or peptides into radicals, thus generating fragmentation ions.⁶ Unlike CID, ETD cleaves Cα-N bonds along the peptide backbones, while
leaving the side chains and their modifications intact. Therefore, ETD is widely applicable in the discovery and localization of PTMs.\textsuperscript{5}

In CID, the activation occurs with internal energy via many low-energy collisions with inert gas atoms. Differently, in SID, the fragmentation is caused by the collision of precursor ions with a surface under high vacuum in a much shorter time.\textsuperscript{8} Therefore, SID is more effective and efficient for detecting protein folding/unfolding transition states than CID, in which significant structural rearrangement may occur and lead to unfolded monomers. Currently, the combination of CID and SID is applied to investigate the structures and the dissociation pathways of protein complexes.

### 3.1.3 Ion Mobility Spectrometry with Mass Spectrometry

Ion mobility spectrometry (IMS)\textsuperscript{9} is used to separate ions according to their mobilities in gas phase,\textsuperscript{10-11} and it has been highlighted as a potential tool to elucidate the structures of proteins.\textsuperscript{12} The application of a unique gas-phase electrophoretic mobility molecular analyzer (GEMMA) has extended the measurement of large protein complexes up to the megadalton range.\textsuperscript{13-16}

Separately, mass spectrometry has been applied in many fields, such as proteomics, nanomaterials, and medicinal screening, because of its advantages: easy operation, high sensitivity, high throughput, and high resolution. Ion mobility separates ions based on their size and shape, and MS identifies the ions by their mass-to-charge ratio (m/z). Currently, the combination of ion mobility spectrometry and mass spectrometry is an analytical technique that is widely applied on structural biology with more sensitivity and greater peak capacity than mass spectrometry.\textsuperscript{17-19}
3.1.4 Identification of Enzyme Substrates

Identification of substrates of enzymes is the essential step to fully understand the functional activities of enzymes. Previous efforts to identify the substrate specificity of enzymes have included broad substrate screening, computer-aided substrate screening and substrate-trapping screening.

High-throughput Screening

High-throughput screening on a broad range of substrates has been developed to identify substrates of enzymes, in which the substrate binding domains are not conserved.\textsuperscript{20-21} For example, Gcn5-related N-acetyltransferases (GNATs), which catalyze the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to a substrate.\textsuperscript{22} Even though the structure elucidation for GNATs has been established, the identification of its substrates remains challenging, due to the flexible substrate binding domains and a wide variety of substrate specificity. This method is a potential way to identify substrate specificity of enzymes, but cannot be applied to enzymes with unknown structures.

Computer-Aided Substrate Screening (CASS)

Computer-Aided Drug Design (CADD) has been developed rapidly in the design of protein inhibitors.\textsuperscript{23} Based on the similar mechanism between protein-inhibitor binding and enzyme-substrate interaction, a computer-aided substrate screening (CASS) using restricted molecular docking was designed.\textsuperscript{24-25} Structure-based CASS is a reliable and highly accurate method for identifying the substrate specificity of enzymes. Compared to experimental screening, CASS is less laborious, less time consuming and less costly. However, the limitation of this method is that molecular docking may not be appropriate for prediction of the substrate-enzyme interaction, since
the substrate-enzyme binding may undergo energetically unfavorable intermediate that cannot be predicted by molecular docking.\textsuperscript{26} Also, the method is only applicable to enzymes with known structures.

**Substrate-Trapping System**

Substrate-trapping system relies on the protein mutants of its active sites. Due to the active site mutations, the binding affinity between protein and its substrates is retained, or even enhanced, but their catalytic activity is reduced. Therefore, stable enzyme-substrate intermediates can be formed in the catalytically inactive sites of enzymes.\textsuperscript{27-28} This method enables the isolation and identification of both in vitro and in vivo enzyme substrates.\textsuperscript{27,29} However, the application of this strategy is restricted to the abundance of substrates\textsuperscript{30} and conserved enzymes structures.\textsuperscript{28,30}

### 3.1.5 Identification of Enzyme Substrates via Enzyme-Substrate Complex

In our approach, AdoVin is an efficient “substrate-trapper” to identify substrates of MTases, independent of the structures of enzymes. Previously, the TPMT-adduct complex was initially isolated from the reaction mixture and then injected into HPLC and LC-MS for analysis after protein acidic precipitation. Herein, we developed a new method to identify substrates of enzymes via enzyme-substrate complex without any isolation of enzymes. In our method, native MS was used to separate proteins and protein complexes by their mass to charge ratio (m/z), followed by IMS, which separates proteins and protein complexes based on their size and shape.\textsuperscript{17} Subsequently, both CID and SID were used to dissociate the binding substrates from enzyme-substrate complex, and thus the released substrates could be identified by the tandem MS fragmentation (Scheme 3.1). Moreover, the binding affinity between enzymes and substrate was
investigated under various collision energies of CID and SID. Using this method, TPMT complex with AdoVin adduct was observed even in crude *E. coli* cell lysates. Altogether, our method provides, for the first time, the observation of MTase-substrate binding complex in native MS.

Scheme 3.1. Detection of enzyme-ligand complex by using native mass spectrometry and subsequent structural elucidation by tandem mass spectrometry (CID or SID).
3.2 Results and Discussions

3.2.1 TPMT in Gas Phase

Two charge state distributions of TPMT were observed in gas phase, folded and unfolded. As shown in Figure 3.1, TPMT with charge states from +9 to +13 was folded with salts attached, so these peaks are broad, while TPMT with larger charge state, +14 to +16, was unfolded with fewer salt attached, providing narrow peaks.

The structure of TPMT is not stable in gas phase. Two major peaks were observed at each charge state (Figure 3.1). One was calculated to be 30212.6, 131.0 less than the theoretical mass of TPMT (30343.6). The other one was 30596.0, 253.0 more than theoretical mass.

CID was then used to remove the salts attached to TPMT (+9 TPMT). Interestingly, after targeting the broad +9 peak as a precursor ion in CID, the salts were dissociated from the +9 TPMT peak and covalent bond cleavage was observed on parts of them (Figure 3.2). The mass of protein was then calculated as 30214.0, according to the remaining +9 precursor ions. This observation indicated that the TPMT sequence is one methionine less than theoretical.
Figure 3.1. Mass spectrum of TPMT (top) and zoomed in mass spectrum of TPMT (bottom).
Figure 3.2. Tandem mass spectrum of +9 precursor ions under CID.
3.2.2 Detection of Enzyme-Substrate Complex

As described in Chapter Two, AdoVin reacted in situ with TNB in the presence of thiopurine methyltransferase (TPMT), and the resulting bisubstrate-adduct tightly bound with TPMT. In order to develop a method to identify substrate of enzyme via enzyme-substrate complex, native MS was used to monitor the interaction between AdoVin adduct and TPMT in gas phase.

In these experiments, isotope labeled AdoVin-TNB was used to introduce an isotope doublet pattern, and the ratio between regular and isotopic AdoVin-TNB adduct was 1:1. Both TPMT and TPMT with AdoVin-TNB adduct were observed as different charge states (Figure 3.3). Using the most abundant peaks “+10 TPMT and +10 TPMT-ligand”, the deconvoluted masses of TPMT was calculated as 30214.1, and the TPMT-ligand was calculated as 30832.1. The difference between TPMT and TPMT-ligand is 618.0, which exactly matches the mass average between AdoVin-TNB (610.0) and isotope labeled AdoVin-TNB adduct (625.0). This result indicates that AdoVin-TNB was formed, and the TPMT complex with AdoVin-TNB adduct can be observed in the native MS. The two adduct forms were also clearly observed in the zoomed in MS spectrum (Figure 3.3). The deconvoluted masses of the peaks are labeled, 30846.1 and 30831.6, respectively. Compared to the mass of TPMT (30214.1), the mass difference (Δm) between the species and TPMT are 609.8 and 624.3, close to the theoretical mass of AdoVin-TNB adduct (610.0) and its isotopic form (625.0), indicating that AdoVin-TNB adduct is also bound to TPMT in the gas phase and the isotope shift (15 Da) can be distinguished. Therefore, isotope labeled AdoVin adduct can be applied to the verification of unknown enzyme-ligand complex in the future.
Figure 3.3. Mass spectrum of reaction mixture of TPMT catalyzed AdoVin-TNB adduct formation (top), and zoomed in mass spectrum of +10 charged TPMT complex with AdoVin-TNB adduct. Isotopic AdoVin-TNB adduct was used for verification (bottom).
Next, to investigate the fragmentation of TPMT-ligand complex, various collision energies were tested for both CID and SID. Apoenzyme TPMT, TPMT-AdoVin-TNB complex, and dissociated AdoVin-TNB adduct were all observed. More AdoVin-TNB adduct dissociated from TPMT with higher collision energies in both CID and SID (Figure 3.4 and 3.5). Moreover, higher collision energies cause more fragmentation of AdoVin-TNB adduct (Figure 3.4 and 3.5), which is consistent with the structure of AdoVin-TNB determined in chapter two. It worth noting that the isotope labeling introduced isotopic doublet fragmentations, and this method could be extended to enzyme-unknown ligand system, in which the fragmentations is the main evidence to identify unknown ligand, and even better with the introduction of isotopic patterns.

In theory, CID could generate more fragmentation than SID at equivalent energies for enzyme-ligand complexes, due to different ion dissociation mechanism (discussed in the introduction), however, for TPMT-AdoVin-TNB complex, no significant difference in fragmentation was observed between CID and SID (Figure 3.4 and 3.5). This observation is explainable, since TPMT-AdoVin-TNB complex is not a real three component complex. Instead, AdoVin and TNB was covalently bound and dissociated as one part in both CID and SID.
Figure 3.4. Tandem mass spectra of +10 charged TPMT-ligand complex in AdoVin-TNB reaction mixture under different CID energy (top). Zoomed in tandem mass spectra of +10 charged TPMT-ligand complex in AdoVin-TNB reaction mixture under various CID energy (bottom).
Figure 3.5. Tandem mass spectra of +10 charged TPMT-ligand complex in AdoVin-TNB reaction mixture under different SID energy (top). Zoomed in tandem mass spectra of +10 charged TPMT-ligand complex in AdoVin-TNB reaction mixture under various SID energy (bottom).
3.2.3 Detection of Enzyme-Substrate Complex in Crude Biological Samples

In order to test the ability of native MS to detect enzyme-ligand complex in more complicated cellular samples, over-expressed TPMT lysates (~50% total protein by weight) were used to generate AdoVin-TNB adducts in situ with exogenous MAT. TPMT-ligand complexes were observed (Figure 3.6) and isolated for both CID and SID fragmentation. Similar to the results of in vitro samples, apoenzyme TPMT, TPMT-AdoVin-AMBA complex and AdoVin-AMBA adduct were observed under both CID and SID fragmentation (Figure 3.7). However, the significant difference is that AdoVin-AMBA adduct (2-amino-5-mercaptobenzoic acid, AMBA) was the major adduct, rather than AdoVin-TNB adduct. This is likely due to the reduction of TNB by any of four nitroreductases present in E. coli, as discussed in chapter two.

Figure 3.6. Mass spectrum of ex vivo reaction mixture of TPMT catalyzed AdoVin-TNB adduct formation.
Figure 3.7. Tandem mass spectra of +10 charged TPMT-ligand complex in *ex vivo* AdoVin-TNB reaction by CID (500 eV) and SID (500 eV).

The TPMT complex with AdoVin-AMBA adduct was readily detected in *ex vivo* level with the existence of over 50% TPMT. In typical biological system, most enzymes do not exist at such high concentrations. Therefore, the possibility to detect enzyme-ligand complex in more complicated samples needs to be explored. Different from previous experiments, TPMT was expressed without any further induction (in the studies described above, *E. coli* cells were induced with IPTG to express TPMT), and the cells were then lysed as the source of TPMT in AdoVin-TNB reactions without further purification.
We were happy to see, as shown in Figure 3.8, TPMT-adduct complex was observed in native MS with lower content of TPMT (under 5% of total protein by weight). AdoVin-AMBA adduct was also the major adduct, and readily identified under both CID and SID fragmentation (Figure 3.9). Compared to the previous methods, no purification of TPMT was needed in this convenient and streamlined method. Moreover, native MS provides a more sensitive detection for TPMT-adduct complex and identification of AdoVin adduct. For instance, only 1 pmole TPMT-adduct complex was needed, and the detection limit is much lower than that of previous detection method, in which at least 10 pmole of AdoVin adduct was required.

![Mass spectrum](image)

Figure 3.8. Mass spectrum of *ex vivo* reaction mixture of TPMT catalyzed AdoVin-TNB adduct formation in TPMT lysate from non-induced *E. coli* cells. AdoVin was enzymatically synthesized by MAT, and AdoVin-TNB adduct was formed *in situ*.
Figure 3.9. Tandem mass spectrum of +10 charged TPMT-ligand complex in *ex vivo* AdoVin-TNB reaction with non-induced TPMT lysate by CID (500 eV).
More importantly, prior to MS analysis, all the samples were buffer exchanged by ultrafiltration, and the small molecules under 30,000 Da were removed by ultrafiltration, and therefore the fact that TPMT-AdoVin complex was not observed in the retentate suggests no modification on TPMT by AdoVin, consistent with the results reported in Chapter 2.

Using the example of tight binding AdoVin adduct with TPMT, this method has been proven as an efficient way to detect enzyme-ligand complex by native MS and identify the ligands by subsequent tandem MS. It is important to mention that TPMT complex with AdoMet, the natural methyl donor of MTases, and AdoHcy, a natural product inhibitor to MTases, were also observed using Native MS (Figure 3.10 and 3.13). Subsequently, both TPMT-AdoMet and TPMT-AdoHcy complexes were detectable in low CID (300 eV) (Figure 3.10-3.13). In contrast to the AdoVin adducts, with low energy SID (500 eV) and high energy CID (1000 eV), AdoMet and AdoHcy (Kd = 0.75 µM)\textsuperscript{31} were readily dissociated from TPMT (Figure 3.12 and 3.14), due to their weaker binding to TPMT than AdoVin-TNB or AdoVin-AMBA adduct. These results further indicate that this method could be used to discover enzyme substrates and identify those substrates with tandem MS fragmentation (Figure 3.12 and 3.15).

Overall, the tight binding event of AdoVin-TNB with TPMT was determined by using native MS and the tandem MS with CID and SID, without any separation of TPMT from the reaction mixture. Moreover, the observation of TPMT-AdoMet and TPMT-AdoHcy complex also supports that this method is efficient and sufficient for enzyme-ligand screening, and drug candidate development.
Figure 3.10. Mass spectra of TPMT-AdoMet complex.
Figure 3.11. Tandem mass spectrum of +10 peak (TPMT-AdoMet) dissociated by low energy CID. The calculated mass of TPMT-AdoMet complex is 30955.8 Da, and the calculated mass of TPMT is 30557.9 Da. The difference is 397.9, close to the mass of AdoMet (399.0 Da).
Figure 3.12. Tandem mass spectra of +10 peak (TPMT-AdoMet) for high energy CID and low energy SID.
Figure 3.13. Mass spectra of \textit{in vitro} AdoVin-TNB reaction (top) and \textit{in vitro} AdoVin-TNB reaction without MTAN (bottom). 5'-methylthioadenosine/\textit{S}-adenosyl-homocysteine nucleosidase (MTAN) is used to hydrolyze AdoHcy produced in methylation reactions.
Figure 3.14. Tandem mass spectra of +10 peak (TPMT-AdoHcy) under various CID energies.
Figure 3.15. Tandem mass spectra of AdoHcy under various CID energies.
3.2.4 Ion Mobility and Mobiligram

Ion mobility cell was assembled after native MS and CID/SID dissociation. The conformation information and drifting time of enzymes and enzyme-ligand complexes can be collected. Enzymes and enzyme-ligand complexes can be separated by two dimension separation: mass to charge in mass spectrometry and drift time (size and shape) in ion mobility. Therefore, in these experiments, ion mobility was also used to monitor the conformation change of TPMT when binding with AdoVin-TNB and AdoVin-AMBA adduct.

In a mobiligram, the higher intensity of peaks gives a lighter color. In general, the folded proteins have narrower drift time than their unfolded forms, and the lower charge state of the precursor helps to maintain the folded structure of TPMT. Precursors with lower charge states require higher collision energy for fragmentation. As shown in Figure 3.16, high charge states of TPMT and TPMT-AdoVin-TNB complex (+9 and +10) were unfolded, while TPMT and TPMT-AdoVin-TNB complex with lower charge states (lower than +9) are more folded. Due to the unfolded structure of TPMT-AdoVin-TNB complex, AdoVin-TNB adduct was able to dissociate from TPMT under low dissociation energy (300 eV), as discussed in previous section.
Next, we investigated the different roles of CID and SID on protein conformation change by ion mobility. Compared to CID fragmentation, SID produces more folded (narrower drift time range) TPMT fragments (Figure 3.17 and 3.18). This observation supports that SID is more advantageous to detect the transition state of protein structure change than CID.

Although, ion mobility did not provide as much information as native mass spectrometry with our goal to identify substrates of enzyme, we still believe that the native mass spectrometry combined with ion mobility spectrometry can be a powerful tool for exploring structural changes of unknown enzyme-ligand complexes.
Figure 3.17. Mobiligram of the fragmentation of +10 TPMT-AdoVin-TNB complex under CID (500 eV).
Figure 3.18. Mobiligram of the fragmentation of +10 TPMT-AdoVin-TNB complex under SID (500 eV).
3.2.5 Detection of Potential Modification on MTases by AdoVin

AdoVin has been tested with other types of MTases, including catechol O-MTase (COMT),\textsuperscript{32-33} protein L-isoaspartate O-MTases (PIMT),\textsuperscript{34-35} protein arginine N-MTase 1 (PRMT1),\textsuperscript{36-37} protein N-terminal MTases (NMT).\textsuperscript{38} However, it is very disappointing that none of the expected AdoVin bisubstrate-adducts were observed under the catalysis of MTases, nor the degradation products of AdoVin adducts. Despite the possibility in organic chemistry that AdoVin (vinyl sulfonium ion) may only show activity to –SH group even under the efficient enzyme catalysis, these results can be connected to the general concern of AdoMet analogue as discussed previously: is there any modification of these MTases by AdoVin that inhibit further bisubstrate-adduct formation? Therefore, potential AdoVin modification of these MTases can be studied into two directions in the future: 1) non-covalent inhibition by AdoVin; 2) covalent modification on MTases by AdoVin, since AdoVin may react with the cysteine side chains exposed in the active sites of enzymes.

Based on the success of native mass spectrometry in the identification of TPMT-substrate complex, we believe that native mass spectrometry combined with various tandem MS techniques, such as CID and SID, can be used to identify both non-covalent and covalent modifications on MTases by AdoVin. Additionally, possible confirmation changes of modified MTases can be identified by ion mobility spectrometry.
3.3 Conclusions

In summary, a new workflow was developed for analyzing enzyme-substrate complexes, as well as substrate identification, using native mass spectrometry and ion mobility spectrometry. Tandem MS with both collision induced dissociation (CID) and surface induced dissociation (SID) fragmentation was applied to identify substrates bound to enzymes. Using our approach, TPMT complex with AdoVin bisubstrate-adduct was detectable even in crude *E. coli* cell lysates. The combination of ion mobility and native mass spectrometry is considered as a powerful tool to identify substrates of enzyme and explore structural information of unknown enzyme-ligand complexes.
3.4 Experimental Section

3.4.1 General Procedures

All chemicals with reagent purity or above were purchased from Sigma and Fisher. Ultrafiltration was carried out using filters with 30,000 molecular weight cut off (MWCO). The filters were purchased from Fisher Scientific (EMD Millipore Amicon Ultra 0.5 mL, catalog No. UFC50VL96).

Prior to analysis by MS, all samples were buffer exchanged into 20 mM ammonium acetate (pH 8.0) using at least 10 cycles of concentration and dilution in a centrifuge, ultrafiltration concentrator. Samples were then frozen at -80 °C and thawed immediately prior to analysis. Further sample dilution was done in 20 mM ammonium acetate, pH 8.0. Direct infusion (2 μL) was done for each MS analysis.

3.4.2 HPLC Analysis

All AdoVin adducts were separated by HPLC, elution profiles were monitored by UV absorption at 260 nm 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 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, 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.
3.4.3 Mass Spectrometry

Native mass Spectrometry

The nanoelectrospray experiment was performed on Synapt G2S HDMS (Waters corp., Wilmslow, UK) with a customized surface-induced dissociation (SID) device installed before the ion mobility (IM) cell as previously described. Scheme 3.2 shows the travelling-wave ion guide (TWIG) region in the Waters Synapt G2S HDMS. The arrows indicate the ion trajectory in SID-IM mode. In SID-IM experiments, precursor ions are released in packets from the trap TWIG at the beginning of each separation cycle, and are subsequently dissociated by surface collision. The SID product ions then undergo separation in the ion mobility cell and are transferred into the TOF analyzer for detection. For CID-IM experiments, the potentials on the lenses are adjusted to allow a “fly-through” transmission. The precursor ions are dissociated by collisions with argon gas in the trap TWIG and the product ions are released into the IM cell for mobility separation without surface collision in the SID region. A delay time of 450 μs in the front of IM cell was used to ensure all the ions started the separation at the same time point.

![Scheme 3.2. Workflow of the Q-IM-TOF instrument. The arrow shows the ion trajectory.](image)

Each sample was filled into a glass capillary pulled using a Sutter Instruments P-97 micropipette puller (Novato, CA) and ionized with a nanoelectrospray source at a voltage of 1.2-1.5 kV. The sampling cone voltage was set to 20 V and the source offset voltage was set to 20 V.
Other instrument conditions were $5 \times 10^{-3}$ mbar for the source pressure, 2.0 mL/min gas flow rate to the trap cell, 120 mL/min gas flow to the helium cell and 60 mL/min gas flow to the ion mobility cell.

**Tandem Mass Spectrometry**

Tandem mass spectrometry experiments were performed via dissociation the selected ions with CID and SID. CID experiment was conducted under trap gas flow rate of 4.0 mL/min and SID was conducted under trap gas flow rate of 2.0 mL/min. The acceleration voltage in CID and SID was obtained as described previously.$^{39-40}$ Briefly, a custom SID device was inserted before the ion mobility cell of the instrument. The direct current voltages on the SID lenses can be tuned either to allow a fly-through of the ions for CID experiments or to direct the ions onto the surface for collision. The current instrument setup is able to perform CID or SID experiments after quadrupole m/z selection and separate the CID/SID products in the ion mobility cell. The acceleration voltage in CID is defined by the “Trap CE” setting, which is the potential difference between the direct current offsets of the quadrupole and the trap traveling wave ion guide before the ion mobility cell. The acceleration voltage in SID is defined by the potential difference between the direct current offset of the trap traveling wave ion guide and the surface, which can be adjusted using the “Trap bias” setting. The acceleration voltage multiplied by the charge state of the selected ion provides collision energy in eV.
3.4.4 Preparation of *in vitro* Enzyme-Substrate Complex

**Preparation of *in vitro* Complex between TPMT and AdoVin-TNB Adduct**

The reaction contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 10 mM ATP, 1 mM vinthionine, 500 μM TNB, 2 mM TCEP, 3.5 μM MTAN and 100 μM TPMT. The reaction was initiated with 200 μM MAT and incubated at 37 °C for 3 hours.

**Preparation of *in vitro* Complex between TPMT and AdoVin-AMBA Adduct**

The reaction contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 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 for 3 hours.

3.4.5 Preparation of Enzyme-Substrate Complex in Crude Biological Samples

*E. coli* cell lysates with and without IPTG induction were prepared as described in general procedure without purification. The concentration of TPMT was estimated as 336 μM in induced lysate, and about 4.9 μM in unstimulated lysate.

The reaction (200 μL) contained 50 mM potassium phosphate, pH 8.0, 50 mM KCl, 25 mM MgCl₂, 20 mM ATP, 1 mM vinthionine, 500 μM TNB, 2 mM TCEP, 3.5 μM MTAN and 100 μL TPMT cell lysate. The reaction was initiated with 200 μM MAT and incubated at 37 °C for 3 hours.
3.5 References


Chapter 4: Conclusions and Future Directions

In these two projects, S-adenosyl-vinthionine (AdoVin) serves as a new S-adenosyl-methionine (AdoMet) analogue, which forms a bisubstrate-adduct *in situ* with nucleophilic substrates of methyltransferases. Due to the tight binding between the AdoVin bisubstrate-adduct and TPMT, TPMT and the thiol substrate were enriched and the TPMT-bisubstrate-adduct complexes were characterized by using HPLC-UV and LC-MS. Chapter 2 discussed the progress of the functional activity of AdoVin working with TPMT and its thiol substrates. Moreover, a previously unknown substrate of TPMT, 2-amino-5-mercaptopbenzoic acid (AMBA), has been discovered and identified. More details about future research on the application of AdoVin will be discussed in this chapter.

An additional improvement in this methodology is the ability to observe the adduct-enzyme complex in a crude sample by native mass spectrometry without any purification step. The details have been covered in Chapter 3 and the future directions of substrate discovery and identification will be discussed in this chapter.
4.1. AdoVin Bisubstrate-adduct Formation in Whole Cells

The data reported previously were from either *in vitro* or *ex vivo* studies by ruling out the complexity existing within the living cells. It is possible to expand the utility of AdoVin adducts by employing this technique in whole cells, which may yield new insights into MTases not reflected by the results of *in vitro* or *ex vivo* experiments. There is also potential to use AdoVin as a probe to fish out unknown endogenous substrates of MTases, and even unknown MTases by known substrates. However, a more complicated environment presents more challenges, i.e. specificity of AdoMet synthetase in certain cells, competition between vinthionine and methionine, as well as competition between AdoVin and AdoMet (Scheme 4.1).

![Scheme 4.1. in vivo methylation and bisubstrate-adduct formation with AdoVin starting from methionine and vinthionine, respectively.](image-url)

Scheme 4.1. *in vivo* methylation and bisubstrate-adduct formation with AdoVin starting from methionine and vinthionine, respectively.
Though previous research reported that vinthionine can be utilized by *E. coli* MAT,\(^2\) the specificity of endogenous MAT in *E. coli* strains as well as other cell lines needs to be addressed, because the activity of MAT using vinthionine may not be sufficient for effective labeling. Additionally, vinthionine may not be able to compete with methionine in a cellular context. To address these issues, MAT enzyme can be engineered to obtain desired activities and selectivities, and then expressed in *E. coli* and other cells of interest.\(^3\)

### 4.2. Affinity Capturing Unknown Methyltransferases

Another potentially powerful application of our approach is identifying unknown MTases using the known substrates or methylation products. One target for this approach is human thiol \(S\)-methyltransferase (TMT, EC 2.1.1.9).\(^4\)\(^-\)\(^5\) TMT is a membrane-bound enzyme that catalyzes the \(S\)-methylation of aliphatic sulfhydryl groups and plays a key role in the metabolism of many sulfhydryl drugs, including antihypertensive drug captopril, \(D\)-penicillamine, and \(N\)-acetylcysteine through \(S\)-methylation (Scheme 4.2).\(^6\)\(^-\)\(^7\) However, the pharmacokinetics of each drug could vary depending on the individual TMT isoform, and genetic polymorphism appears to be a determining factor for the variation of activities.\(^8\)\(^-\)\(^9\) There are two reasons to choose human TMT as the target: 1) \(S\)-MTases are the obvious targets for expanding the study of AdoVin activity, given the hypothesis that AdoVin shows good activity towards -SH group (Michael addition); 2) even though the function of human TMT has been studied, its gene and sequence remain undetermined, due to the difficulty of isolating and purifying a membrane-bound enzyme.

AdoVin has shown the activity TPMT, and we believe that AdoVin bisubstrate-adduct may be formed and bind tightly with TMT. The possible persistent interaction between the bisubstrate-
adduct and TMT would make AdoVin an efficient probe to affinity enrich and isolate TMT. Modifying AdoVin or the nucleophilic substrates with affinity tags, such as biotin tag would facilitate this enrichment. Thus, the isolated enzyme may be sequenced by standard peptide mapping and protein mass spectrometry.

Scheme 4.2. Proposed AdoVin bisubstrate-adduct formation catalyzed by TMT and proposed isolation of TMT catalyzed by affinity tag (red) on AdoVin bisubstrate-adduct.
4.3. Substrate Identification for Other MTases using Native Mass Spectrometry via Enhanced Binding without Formation of Covalent Adducts

The elucidation of methyltransferase substrate specificity is hampered by the transient interaction (weak binding) between the nucleophilic substrates and the enzymes, in other words, the binary complex between MTase and substrate as well as the tertiary complex between MTase, substrate, and AdoMet, may not be persistent enough for the detection via Mass Spectrometry. Therefore, tighter binding is required to establish the direction connection between substrates and enzymes. Another method for creating direct connection between the substrates and MTases is to strengthen the binding between nucleophilic substrates and MTases by AdoVin (Scheme 4.3). “Tethering” to AdoVin allows the enhanced interaction between the nucleophilic substrates and MTases to be observed via Mass Spectrometry. This hypothesis expands another possible utility of AdoVin for substrate identification of MTases. Additionally, similar with the “substrate-trapping” system,\textsuperscript{10-11} the enhanced binding between substrate and enzyme is also perfect to investigate the transition state of enzyme catalyzed reactions.

Scheme 4.3. Substrate binding in methylation using AdoMet (top) and proposed binding between nucleophilic substrates and methyltransferases enhanced by AdoVin (bottom).
4.4. Facile Generation of Bisubstrate-adduct for Structural Investigation

It is possible that AdoVin only shows activity to $\text{S}$-MTases via addition reaction with the thiol group (-SH). For other type of MTases, a potentially useful methodology would be to derivatize the nucleophilic groups, such as amine (-NH$_2$) and hydroxyl (-OH), to thiols (-SH). As shown in Scheme 4.4, the modified substrates are expected to react with AdoVin to form tightly binding bisubstrate-adducts catalyzed by their corresponding MTases ($O$-MTases, $N$-MTases or others), for example, though lysine (-NH$_2$) may not be active to AdoVin, its corresponding lysyl thiol analog (-SH) could react with AdoVin and form bisubstrate adduct catalyzed by protein lysine $N$-MTases (PKMTs) (Scheme 4.4 b). Thusly, AdoVin can be used to generate bisubstrate-adduct inhibitors for other type of MTases via the change of various MTases into putative $\text{S}$-MTases.

One utility of the multisubstrate adduct inhibitor approach is to provide essential structural knowledge of enzyme via enzyme co-crystallization. It is expected that co-crystallization of MTases with AdoVin bisubstrate-adducts could elucidate structural information of enzyme-substrate interactions. Additionally, using this methodology, AdoVin could contribute to the generation of specific and strong bisubstrate-adduct inhibitors for many MTases, facilitating the structural investigation of MTases.
Scheme 4.4. (a) Potential generation of bisubstrate-adduct inhibitors by derivatizing other nucleophilic substrates (such as -NH₂ and -OH) to thiol substrates (-SH); (b) modification of –NH₂ group to –SH in lysine for protein lysine N-MTases.
4.5. References


