

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

Bobby W.K. Lee,[†] He G. Sun,[†] Tianzhu Zang,[†] Byung Ju Kim,[†] Joshua F. Alfaro,[‡] and Zhaohui Sunny Zhou^{*†,‡}

The Barnett Institute and Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115 and Department of Chemistry, Washington State University, Pullman, Washington 99164

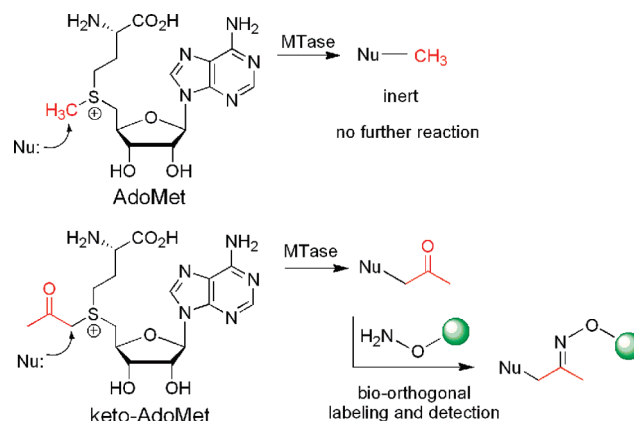
Received October 21, 2009; E-mail: z.zhou@neu.edu

Elucidation of the biochemical activity and specificity of enzymes is an essential step toward understanding their physiological roles. Despite the progress in genomics, structural proteomics, and computational biology, functional annotation of enzymes is often limited to the family level; i.e., the precise substrate specificity for a given enzyme remains largely undefined. These challenges are exemplified by the large and diverse family of *S*-adenosylmethionine (AdoMet or SAM)-dependent methyltransferases (MTases); more than 150 different members have been cataloged by the Enzyme Commission, and the numbers are still rapidly increasing. These enzymes conduct their activities on a host of substrates, including small molecules, DNA, RNA, and proteins.¹ Of the five known MTase structural folds, only the Class IV SPOUT family of RNA MTases and the Class V SET-domain containing family of protein lysine MTases exhibit generally definable targets.^{1a,b} The other classes (I–III) do not correlate to the chemical nature of the substrates which themselves are distinguished by chemical diversity and include amines and guanidines (N), alcohols and carboxylic acids (O), thiols and sulfides (S), alkenes, and even alkanes (C).¹ As such, the structures of many putative MTases have been determined, but their substrates and functions remain elusive.²

Progress in functional analyses of MTases is hampered by the inert properties and small size of the methyl group, making it difficult to directly tag or selectively bind methylation products. The past decade has witnessed the development of chemical approaches for the selective labeling of biomolecules that undergo enzyme-catalyzed group transfer, including phosphorylation, acylation, and glycosylation.³ These approaches typically involve enzymatic introduction of traceable tags using permissive substrate analogues and subsequent recognition of labeled products with bio-orthogonal chemistry.³ For instance, AdoMet analogues substituted with activated alkyl groups in place of the methyl group served as alkyl donors for nucleic acid MTases, and the unnatural groups were transferred in a sequence-specific manner.⁴ When functionalized with an aliphatic amine, the tagged products were visualized by amine reactive fluorescent reporter groups. In a different approach, aziridine containing adenosyl analogues were used as surrogates of AdoMet. To mark the resulting bisubstrate adducts, the purine is substituted with either a fluorescent group or an azide group for subsequent modification by Staudinger ligation.⁵

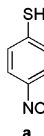
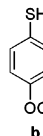
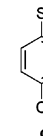
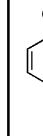
Herein, we report the synthesis and activity of an AdoMet analogue functionalized with a ketone group (keto-AdoMet, Scheme 1), which serves as an attractive probe due to the absence of ketone groups in nucleic acids and proteins and its orthogonal reactivity with hydroxylamines and hydrazides.⁶ The ketone group reacts with these agents rapidly and quantitatively in aqueous solutions, and the resulting oximes and hydrazones are stable; thus, these versatile chemistries are widely used for biological labeling studies.⁶

Scheme 1. Enzyme-Catalyzed Transmethylation from AdoMet, Transfer of Ketone Group from keto-AdoMet, and the Detection of Ketone Containing Products via Selective Derivatization



Keto-AdoMet was synthesized in a single step from *S*-adenosylhomocysteine (AdoHcy) and bromoacetone in a manner analogous to the preparation of other AdoMet analogues.⁴ The activity and specificity of keto-AdoMet were first assessed in vitro using Class I MTases catechol *O*-methyltransferase (COMT, EC 2.1.1.6) from porcine liver and human thiopurine *S*-methyltransferase (TPMT, EC 2.1.1.67). Our results show the ketone group is transferred from keto-AdoMet to the nucleophilic substrates for both MTases in a time and enzyme concentration dependent manner (see Supporting Information (SI) 3 and 4.1). Detailed studies were carried out with TPMT, an enzyme which exhibits substrate promiscuity in the methylation of a broad spectrum of aromatic thiols, including thiopurine drugs.⁷ Despite its role in drug metabolism, the endogenous substrates of TPMT, if any, remain unknown. As summarized in Table 1, the substrate specificity of AdoMet and keto-AdoMet mirror each other. For example, three known substrates (aromatic thiols **a–c**) are alkylated by keto-AdoMet. Conversely, the non-substrate (4-nitrophenol, **d**) is not alkylated by either AdoMet or keto-AdoMet.

Table 1. Specificity of TPMT with AdoMet and keto-AdoMet

Substrate	Kinetic Parameters				
Alkyl Donor		a	b	c	d
AdoMet	K_m : $6.7 \pm 0.7 \mu\text{M}$ k_{cat} : $13.6 \pm 0.4 \text{ min}^{-1}$	known	known	known	non-substrate
keto-AdoMet	K_m : $17.9 \pm 1.5 \mu\text{M}$ k_{cat} : $0.13 \pm 0.01 \text{ min}^{-1}$	yes	yes	yes	no

[†] Northeastern University.

[‡] Washington State University.

Subsequent kinetic analysis using 4-nitrobenzenethiol (**a**) as the alkyl accepting substrate indicates that keto-AdoMet is a respectable substrate of TPMT (Table 1); the calculated $k_{\text{cat}}/K_{\text{m}}$ value ($120 \text{ M}^{-1} \text{ s}^{-1}$) is approximately 300-fold lower than the value measured with AdoMet. Competition assays conducted at varied ratios of AdoMet to keto-AdoMet indicate these substrates bind to the same site on TPMT (see SI 4.3).

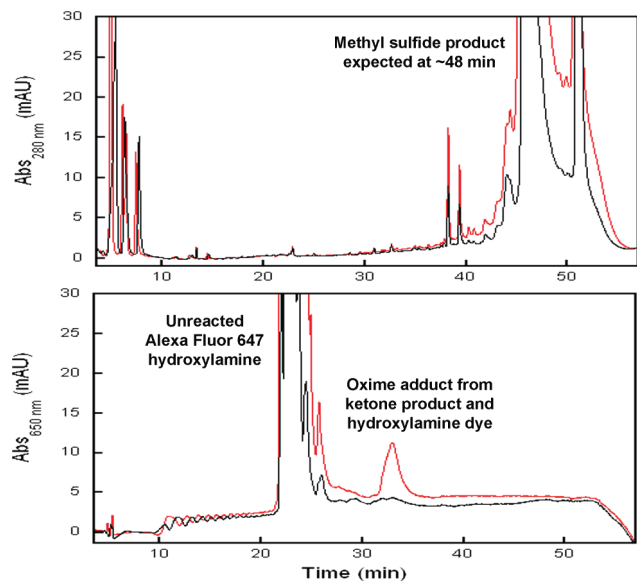


Figure 1. Top: HPLC trace of the erythrocyte lysate reaction containing 4-nitrobenzenethiol in the presence (red) and absence (black) of AdoMet. Bottom: Chromatogram at 650 nm after reactions with (red) and without (black) keto-AdoMet followed by derivatization with Alexa Fluor 647 hydroxylamine.

Labeling of the substrate using the analogue was demonstrated *ex vivo* with human erythrocyte lysate which contains TPMT.⁸ HPLC analysis of the reaction containing AdoMet and 4-nitrobenzenethiol indicates the methyl sulfide product is obscured by the broad peaks eluting between 44 and 54 min resulting from heme and other metabolites (Figure 1, top), even though a fairly large amount of the product (1.1 nmol) was loaded onto the column. This observation further underscores the challenges inherent in the identification of unknown products; under standard analytical conditions, reaction products may not be detectable against the background of cellular components. In comparison, the TPMT product was readily detected using keto-AdoMet as an alkyl donor and derivatization with Alexa Fluor 647 hydroxylamine (Figure 1, bottom). At 650 nm, the complex background associated with the erythrocyte lysate, including the strong absorptions from heme, is absent. Other than the excess hydroxylamine dye, the oxime product is the single prominent peak visible at this wavelength and due to the large extinction coefficient at 650 nm ($203\,000 \text{ M}^{-1} \text{ cm}^{-1}$), 52 pmol of the ketone product (1/20th of the methylation reaction) were sufficient to provide a strong signal.⁹

One of the goals is to identify unknown substrates of MTases. Toward this end, we attempted to identify endogenous substrates of TPMT. Labeled products were not observed from red blood cell lysate treated with keto-AdoMet (see SI 5.1). Since active TPMT was present, endogenous thiol substrates might already be converted into their methylated forms. Another possibility is that no endogenous substrates exist. It has been reported that human subjects with little TPMT activity due to genetic background display no observable phenotype except when undergoing thiopurine treatment.^{7c} Unexpectedly, we found that an aliphatic thiol (4-methoxybenzylthiol) was labeled by keto-AdoMet. Subsequently, TPMT-catalyzed methylation by AdoMet was confirmed. Since

benzylthiols were not previously considered as substrates of TPMT, this aliphatic thiol was intended as a negative control. This serendipitous finding highlights the utility of keto-AdoMet to identify undescribed MTase activities.

A general concern regarding substrate analogues, in particular those with intrinsically reactive aziridines or sulfoniums, is whether the analogue modifies and inactivates enzymes.^{4,5} Using mass spectrometry, no modification was detected for either TPMT or AdoHcy nucleosidase, even in the presence of excess keto-AdoMet. More importantly, the activities of these enzymes were not affected. An additional advantage of keto-AdoMet as a labeling reagent is that multiple turnovers were observed with various substrates (e.g., at least 14 for 4-nitrobenzenethiol). We further report that the analogue is stable for over 2 months when stored under mildly acidic conditions and at either -20 or -80 °C.

In summary, keto-AdoMet, a readily accessible analogue of AdoMet, expands the toolbox available to interrogate the biochemical functions of MTases. When coupled with specific MTases, the analogue may further serve as a site-specific labeling reagent for bioconjugation and structural diversification.^{4,5}

Acknowledgment. This work was supported by the Herman Frasch Foundation (541-HF02 to Z.S.Z.), NIH (1R01AI058146 to Z.S.Z. and 1F31GM073559 to J.F.A.), and the American Heart Association (09PRE2300071 to T.Z.) We thank the reviewers for their constructive comments. This is contribution number 943 from the Barnett Institute.

Supporting Information Available: Full experimental details, synthesis, and characterization of keto-AdoMet. This material is free of charge via the Internet at <http://pubs.acs.org>.

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JA908995P