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Se-Adenosyl-L-selenomethionine Cofactor Analogue as a Reporter of Protein Methylation

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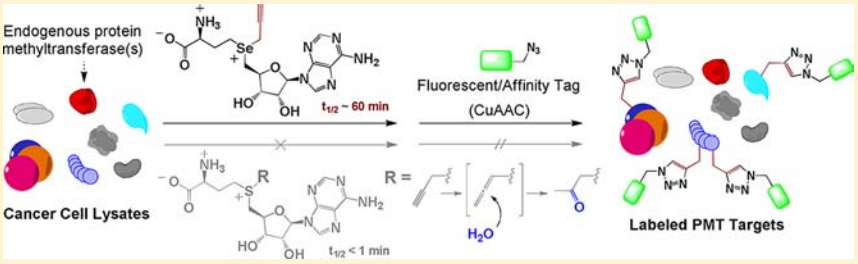
Supporting Information



ABSTRACT: Posttranslational methylation by *S*-adenosyl-L-methionine(SAM)-dependent methyltransferases plays essential roles in modulating protein function in both normal and disease states. As such, there is a growing need to develop chemical reporters to examine the physiological and pathological roles of protein methyltransferases. Several sterically bulky SAM analogues have previously been used to label substrates of specific protein methyltransferases. However, broad application of these compounds has been limited by their general incompatibility with native enzymes. Here we report a SAM surrogate, ProSeAM (propargylic *Se*-adenosyl-L-selenomethionine), as a reporter of methyltransferases. ProSeAM can be processed by multiple protein methyltransferases for substrate labeling. In contrast, sulfur-based propargylic SAM undergoes rapid decomposition at physiological pH, likely via an allene intermediate. In conjunction with fluorescent/affinity-based azide probes, copper-catalyzed azide−alkyne cycloaddition chemistry, in-gel fluorescence visualization and proteomic analysis, we further demonstrated ProSeAM's utility to profile substrates of endogenous methyltransferases in diverse cellular contexts. These results thus feature ProSeAM as a convenient probe to study the activities of endogenous protein methyltransferases.

INTRODUCTION

The enzymatic methylation of proteins following their translation is an important means of modulating their function and activity.[1−3] These modifications are typically carried out by *S*-adenosyl-L-methionine(SAM)-dependent methyltransferases, which transfer the sulfonium methyl group of SAM to a variety of amino acid residues, including acidic glutamyl groups,[3] neutral cysteine,[2] and basic lysine/arginine/histidine.[1] The downstream effects of these methylation events have been implicated in numerous biological processes, such as epigenetic regulation through modification of lysine and arginine side chains of histones,[4] bacterial chemotaxis through the methylation of glutamic acid residues of bacterial chemoreceptors,[3] and disruption of NF-κB signal transduction through the modification of the zinc-coordinating cysteine of human TAB2.[2] SAM-dependent methyltransferases are also widely recognized for their roles in nucleic acid and metabolite methylation.[5] Because of the broad impact of SAM-dependent enzymes, significant efforts have been made to develop tools for the elucidation of their substrates and associated biological effects.[6−10]

To characterize posttranslational modifications (PTMs) in biological systems, one powerful approach is to use alkyne or azide-containing PTM chemical reporters coupled with detection/pull-down tags, via copper-catalyzed azide−alkyne cycloaddition (CuAAC) chemistry, to visualize or enrich PTM targets for further analysis.[11] This and similar approaches have been reported for the examination of several PTM events such as glycosylation,[12] acetylation,[13] lipidation,[14−16] poly(ADP-ribosyl)ation[17] and AMPylation.[18,19] Given the importance of posttranslational methylation, these prior examples have inspired us and others to develop comparable approaches for protein methylation (Figure 1).[6,7,9,20] To date, several bulky SAM derivatives containing sulfonium-β-sp^2 alkyne/azido clickable functionality have been reported.[6−9] However,

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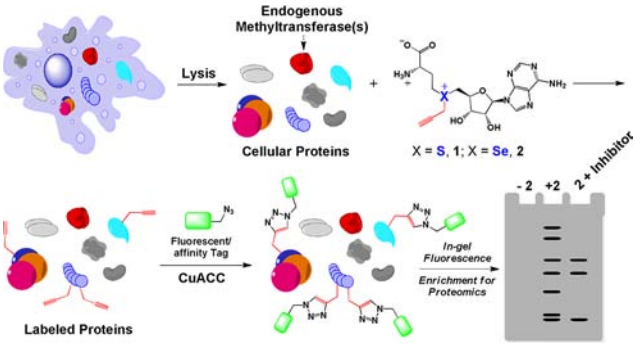

Figure 1. SAM analogue cofactors as chemical reporters of protein methylation. Endogenous methyltransferases can utilize certain SAM analogues to label cellular proteins. The resultant modified proteins can carry a terminal-alkyne moiety, which can be coupled with CuAAC for further characterization.

application of these molecules has been limited by their general incompatibility with native enzymes, and thus requires the engineering of specific enzymes to achieve efficient substrate labeling.[6,7,10,20]

While developing SAM analogues to probe methyltransferases, our attention was caught by an apparent discrepancy concerning propargylic SAM **1** (Figures 1 and 2). This

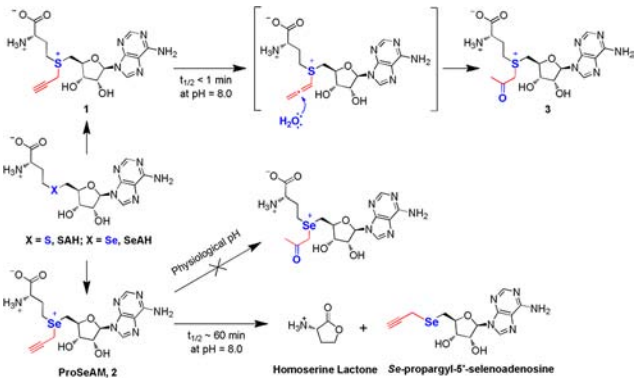

Figure 2. Preparation and stability of propargyl SAM **1** and ProSeAM **2** cofactor analogues.

compound was expected to be of particular value as a probe of methyltransferases, because it contains the smallest transferable chemical handle for CuAAC chemistry and thus should be more compatible with native methyltransferases. Indeed, **1** was reported as a SAM surrogate for both small-molecule methyltransferases (NovO and CouO)[21] and the lysine methyltransferase SETDB1.[8] However, the same compound has also been reported to be unstable at physiological pH.[6,9] Intrigued by these findings, we explored the nature of this instability and circumvented it by developing a structurally similar, but significantly more stable propargylic *Se*-adenosyl-L-selenomethionine (ProSeAM **2**, Figures 1 and 2). Here we demonstrate that ProSeAM is a suitable cofactor for multiple native protein methyltransferases and its ability to label and identify PMT substrates in various cellular contexts (Figure 1).

RESULTS AND DISCUSSION

Degradation of Propargylic SAM 1 and Development of a More Stable Surrogate ProSeAM 2. Several previous efforts had shown that **1** underwent rapid decomposition at physiological pH.[6,9] Further MS analysis in the current work indicated that the half-life of **1** is shorter than 1 min at pH 8.0 (Figure 2 and Supporting Information Figure S2). The major decomposition product from the parent compound was featured by its +18 Da molecular ion peak, which we surmised arose from the hydration ($+H_2O$) of **1** via a putative allene intermediate (Figure 2 and Supporting Information Figure S2). The formation of **3** was confirmed upon comparing the HPLC-purified decomposition product with independently synthesized **3** (NMR and MS data in Supporting Information).[22]

Because of the inherent instability of **1**, we envisioned a strategy of stabilizing the alkyne handle by replacing the former's sulfonium with selenonium (ProSeAM **2**). A related molecule, *Se*-adenosyl-L-selenomethionine (SeAM), has been reported to be a SAM surrogate for methyltransferases.[23,24] In certain enzymatic reactions, SeAM is a better methyl donor than SAM, likely because of the former's weaker Se−C bond and thus higher reactivity toward S_N2-type methylation.[24] Given its chemical properties, ProSeAM's selenonium functionality is also expected to reduce the acidity of the protons on its adjacent sp^3 carbons,[25] and thus suppress decomposition toward the allene intermediate. Here, ProSeAM was synthesized in a fashion similar to other SAM analogues through alkylation of *Se*-adenosyl-L-homoselenocysteine[23] using propargyl bromide under acidic conditions (Figure 2).[6,7,20] The resolved HPLC peaks between the selenonium *R*- and *S*-epimer also afforded diasteriomerically pure ProSeAM, which was characterized and used in all subsequent experiments (Supporting Information Figure S1).

The stability of ProSeAM was examined via HPLC and LCMS under the same conditions used to analyze **1**. The propargyl moiety of ProSeAM **2** is significantly more stable than the sulfonium counterpart (Figure 2 and Supporting Information Figures S1 and S2). Further analysis revealed that the predominant decomposition pathway of **2** is through the intramolecular lactonization of the 2-aminobutyrate moiety to afford the byproducts homoserine lactone and *Se*-propargyl-5′-selenoadenosine (Figure 2 and Supporting Information Figure S2). A similar decomposition pathway was also reported for SAM and SeAM.[25] Overall, the half-life of ProSeAM was shown to be approximately 60-fold longer than that of **1** (∼ 60 min versus <1 min in Tris HCl buffer at pH 8.0, Supporting Information Figure S2). Despite the anticipated high electrophilicity at α-carbon of the selenium propargyl moiety, ProSeAM was found to be compatible with free-thiol-containing reagents such as β-mercaptoethanol (<10% decrease of half-life, Supporting Information Figure S3). Here the half-life of **2** has fallen within the time range used for efficient substrate labeling with SAM or bulkier sulfonium SAM analogues as cofactors.[6,7,9,20]

Compatibility of ProSeAM with Native Methyltransferases. The enhanced stability of ProSeAM **2** enabled us to examine the ability of native protein lysine/arginine methyltransferases (PKMT/PRMT) to utilize **2** as a SAM surrogate. MALDI-TOF mass spectrometry was applied to monitor the abilities of a panel of PKMTs/PRMTs (GLP1, SET7/9, SET8, G9a, SUV39H2, PRMT1 and PRMT3) to modify peptide substrates in the presence of ProSeAM (Figure 3a,b). Reactions with SAM were carried out in parallel as positive controls (Supporting Information Figures S4−S10). ProSeAM was shown to be active toward three PKMTs (GLP1, G9a, and SUV39H2) for efficient substrate labeling (alkylation of N-terminal histone H3 peptide, Figure 3a, Supporting Information

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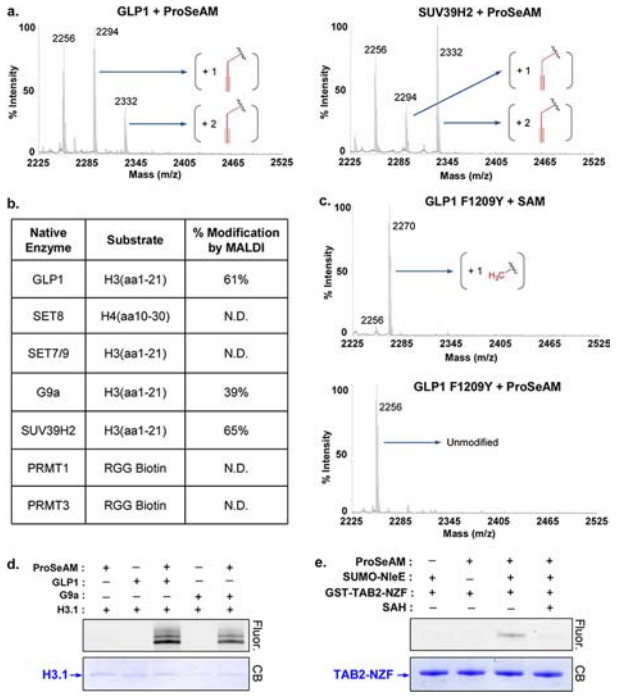

Native Enzyme	Substrate	% Modification by MALDI
GLP1	H3(aa1-21)	61%
SET8	H4(aa10-30)	N.D.
SET7/9	H3(aa1-21)	N.D.
G9a	H3(aa1-21)	39%
SUV39H2	H3(aa1-21)	65%
PRMT1	RGG Biotin	N.D.
PRMT3	RGG Biotin	N.D.

Figure 3. Screening native methyltransferases with ProSeAM as a cofactor. (a) MALDI mass spectra of *in vitro* assays of GLP1 and SUV39H2 with ProSeAM as an active cofactor. See Supporting Information Figures S4–S10 for other PMTs. (b) Summary of enzymes, peptides and %modification MALDI MS of the screening. (c) MALDI mass spectra of *in vitro* assays of GLP1 F1209Y mutant compatibility with SAM and ProSeAM. (d) In-gel fluorescence validation of G9a and GLP1 activity on H3.1 with ProSeAM **2** as a cofactor. (e) NleE-mediated labeling of the NZF domain of TAB2 using **2** as a cofactor. SAH, a pan-methyltransferase inhibitor; Fluor, in-gel fluorescence; CB, Coomassie staining as loading control; N.D., Not Detected.

Figures S4–S6). ProSeAM can also be processed twice to generate the dipropargylated peptide products (Figure 3a, Supporting Information S4 and S6), consistent with processive character of GLP1 and SUV39H2.[26,27] Here no modification was observed in no-enzyme/cofactor controls or with the unstable SAM analogue **1** (Supporting Information Figures S4–S10). In addition, only the propargylated products but no product via nucleophilic attack at C6 of ProSeAM's ribose or Cδ of ProSeAM's *Se*-homocysteine was detected for the active PMTs (see the expanded MS regions in Figure 3a, Supporting Information Figures S4 and S6). These observations collectively indicate that ProSeAM mainly serves as an enzymatic propargylation cofactor rather than a nonspecific alkylation reagent.

To evaluate the catalysis efficiency of ProSeAM, we analyzed the steady-state kinetics of a representative PMT, GLP1. Upon measuring the initial rates of GLP1-catalyzed propargylation by ProSeAM, the apparent k_{cat} of 0.375 min^{-1} and K_m of 45.4 μM were obtained. These kinetic parameters are only different from k_{cat} of 1.97 min^{-1} and K_m of 3.1 of native SAM by 5- to 15-fold (Supporting Information Figure S11) (the apparent k_{cat} and K_m of ProSeAM are sufficient for target labeling as demonstrated later). In contrast, ProSeAM was shown to be inert for native SET7/9, SET8, PRMT1 and PRMT3 under our MALDI-based assay conditions (Supporting Information Figures S7–S10). These results argue that ProSeAM can be utilized by a subset of PKMTs for substrate propargylation.

Recognition Mechanism of ProSeAM 2 by PKMTs. Given that the 3 characterized ProSeAM-utilizing enzymes are protein lysine methyltransferases (PKMTs), which generally contain SET(suppressor of variegation, enhancer of zeste and Trithorax) domains,[5] we investigated the possible nature of this selectivity. Here we noticed that the activities of PKMTs toward ProSeAM correlate with their product specificity: ProSeAM-active lysine di/trimethyltransferases, which contain relatively vacant product-binding sites such as G9a, GLP1 and SUV39H2 versus ProSeAM-inert lysine monomethyltransferases, which contain less vacant product-binding sites such as SET7/9 and SET8. To further examine the correlation, we generated the F1209Y mutation of GLP1, which was previously demonstrated to be a di/tri-to-mono 'switch' mutation in the family of PKMTs.[28] As anticipated, this mutation shifts the product specificity of GLP1 from a lysine di/trimethyltransferase to a predominantly lysine monomethyltransferase (Figure 2c). More importantly, the F1209Y switch mutation is inert toward ProSeAM under our assay conditions (Figure 2c). The loss-of-function experiment therefore suggests that the ProSeAM-active PKMTs may utilize their pre-existing methyllysine-binding sites to accommodate the cofactor. This putative explanation remains to be further validated with the mutants of other PKMTs

Enzymatic Labeling of Full-length Protein Substrates with ProSeAM as a Cofactor. The cofactor activity of ProSeAM as a SAM surrogate for GLP1 and G9a was further confirmed by in-gel fluorescence, with full-length histone H3.1 as the substrate and an azido fluorescent dye (Az-Rho)[15] as a probe (H3.1 labeling was visualized after reacting with Az-Rho via CuAAC, Figure 3d). The multiple labeled bands of H3.1 revealed by the in-gel fluorescence (Figure 3d) correlate well with the different degree of alkylation (~0.7 kDa increase for each modification due to Az-Rho conjugation). In addition, MS/MS analysis of GLP1-modified full-length histone H3.1 confirmed the transfer of ProSeAM's propargyl group to lysine 9, the same site when native SAM is used as a cofactor (Supporting Information Figure S12).

To further extend the utility of ProSeAM **2** as a chemical reporter of protein methyltransferases beyond PKMTs, we examined ProSeAM as a potential cofactor of a newly discovered bacterial cysteine methyltransferase NleE.[2] This family of methyltransferases has been implicated as bacterial virulence factors through methylation of the zinc-coordinating cysteine residues of the NZF domain of human TAB2, disrupting NF-κB signaling.[2] Using the in-gel fluorescence method described above, we observed that ProSeAM can also be recognized by NleE to label the NZF domain of TAB2 (Figure 3e). No significant labeling was detected either in the absence of NleE or in the presence of *S*-adenosyl-L-homocysteine (SAH), a pan-selective inhibitor of methyltransferases, indicating that labeling is enzyme-dependent.

Fluorescent Labeling of Protein in Whole Cell Extract Using ProSeAM as a Cofactor. To demonstrate the potential utility of ProSeAM in more complex biochemical systems, HEK293T cell lysates were labeled using ProSeAM. For this purpose, HEK293T cells were lysed after the treatment with adenosine-2′,3′-dialdehyde (Adox), a SAH hydrolase inhibitor that was reported to generate hypomethylated proteomes via the accumulation of SAH, the byproduct of SAM-dependent methylation, and the resultant product inhibition.[29] The lysates containing hypomethylated proteomes were then treated with ProSeAM and visualized using the in-gel fluorescence method

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described above. Labeled proteins were readily visible in the samples treated, even with 1 μM ProSeAM (Supporting Information Figure S13). In contrast, the samples treated with the unstable SAM analogue **1** showed barely detectable labeling, even at the high concentration of 1 mM (Supporting Information Figure S13). Furthermore, the labeling efficiency of ProSeAM is proportional to ProSeAM concentration and can be suppressed by treatment with the pan-methyltransferase inhibitor SAH or by heat inactivation (100 °C for 10 min, Supporting Information Figures S13, S14). The SAH/heat-sensitive labeling (comparing the treatment with and without SAH/heat, Supporting Information Figure S13, S14) is consistent with the involvement of endogenous methyltransferases in the labeling process.

Here we also note that labeling of many proteins but not all was diminished in the SAH/heat-treated samples, likely due to slight nonspecific labeling. In addition, given that SAH's IC_{50} values can vary by more than 2 orders of magnitude among protein methyltransferases,[10,30] some SAH-insensitive labeling might be due to certain protein methyltransferases that cannot be inhibited by 500 μM SAH used under the current conditions. However, the background labeling does not prevent the identification of PMT-labeled novel targets (See the latter section of new target identification with ProSeAM). Collectively, these data suggest that ProSeAM is suitable to exploit endogenous methyltransferases for substrate labeling in a complex biochemical setting.

Weinhold and colleagues recently showed that ProSeAM was active not only toward G9a but also Set7/9 and PRMT1,[31] which is different from our observation that only G9a but not the other two PMTs are active toward ProSeAM. We reasoned that these differences may be due to the sensitive antibody-coupled horseradish peroxidase assay used under Weinhold's conditions, in contrast to the MS-based and in-gel fluorescence assays presented here. The former is expected to capture the signals of extremely weak modifications, while the latter, though less sensitive, better reflects the efficiency of ProSeAM-involved target labeling and thus merits for subsequent target identification as will be detailed later. Here we could not rule out other possibilities to cause the discrepancy such as the different constructs of Set7/9 and PRMT1 used in the two sets of experiments.

ProSeAM-Mediated Target Labeling across Cancer Cell Lines. To further explore ProSeAM's general utility as a chemical reporter of protein methylation beyond HEK293T context, we compared the labeling pattern in the lysates of 8 common cancer cell lines (Figure 4, Supporting Information Figure S15). Close examination of these samples revealed that, although many labeled proteins are shared among all the cell lines and can be suppressed by SAH treatment (e.g., proteins ***g***, ***h*** in Figure 4), a subset of labeling is cell-type-specific. For example, protein ***c*** appeared to be strongly labeled in MCF7, HT-29, RKO, A549, and H1299 cells, but not in MDA-MB-231, Jurkat, or U937 cells (Figure 4). Such cell-type-specific labeling patterns were also observed for proteins ***a***, ***b***, ***c***, ***d*** and ***f*** (Figure 4). Interestingly, the labeling patterns also varied between cancer cell lines of the same tissue type (e.g., protein ***e*** in RKO, but not in HT-29 of colon cancer, Figure 4). Here we reasoned that, since certain protein methyltransferases and their targets can be distinctly expressed and regulated in different cancer cell lines, the labeling patterns upon the treatment of ProSeAM may reflect such heterogeneity. For instance, MCF7, MDA-MB-231, RKO, H1299 cells contain an elevated gene

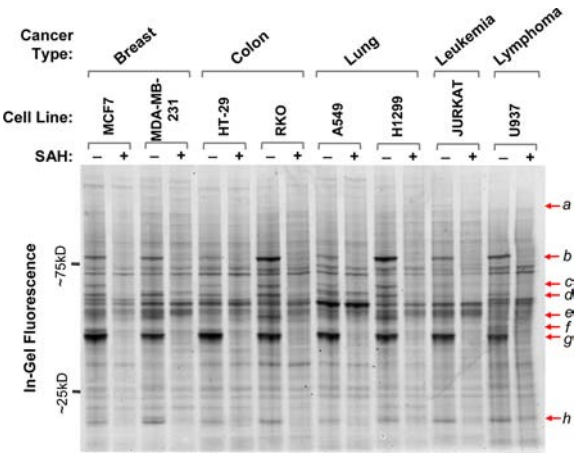


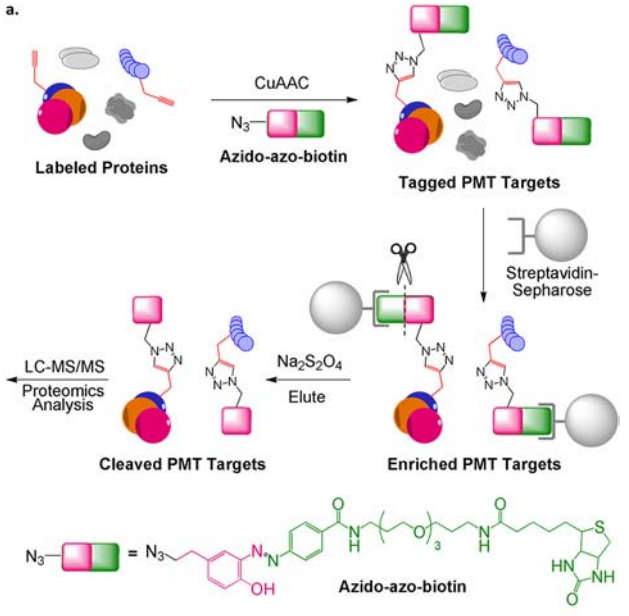
Figure 4. Protein labeling with ProSeAM as a chemical reporter of endogenous methyltransferases. In-gel fluorescence of ProSeAM-labeled proteins is presented across cancer cell lines. Cells were treated with Adox during growth to generate hypomethylated proteomes for more efficient target labeling. Cell lysates were treated with **2** (with or without 500 μM SAH) prior to Az-Rho conjugation and in-gel visualization. See Supporting Information Figure S15 for loading control.

copy number of GLP1 or G9a (Supporting Information Figure S16),[32] which may correlate to the distinct labeling of protein ***e*** (Figure 4,). A lack of labeling for protein ***c*** in HT-29 and Jurkat cells (Figure 4) may reflect the unaltered gene copy number of GLP1, G9a, SUV39H1 and SETDB1 in the both cell lines (Supporting Information Figure S16).[32] Since cancer is a heterogeneous disease and has been linked to aberrant protein methylation,[33] we are intrigued by the potential of ProSeAM as a chemical reporter to explore such heterogeneity.

Application of ProSeAM as a Reporter for Proteome-wide Identification of PMT Substrates. Encouraged by the ability to label known methylation targets *in vitro* and the apparent labeling of methytransferase targets in cell lysate, we then advanced ProSeAM's utility by coupling it with mass spectrometry-based proteomic analysis to identify protein targets. Here HEK293T lysates were treated with ProSeAM in the presence or absence of SAH inhibition. The putative propargylated proteins were conjugated to the cleavable azido-azo-biotin affinity tag[34] via CuAAC and enriched over streptavidin beads (Figure 5a). Labeled proteins were then liberated from the streptavidin beads using sodium dithionite, separated by SDS-PAGE and then subject to trypsin digestion and LCMS-MS analysis (Figure 5a).[34]

Analysis of the two data sets revealed that a total of 297 targets are identified in lysates treated with ProSeAM but not those treated with the pan-methyltransferase inhibitor SAH (Supporting Information Table S2).[35−39] The presence of SAH in cell lysates prevented the pull-down of these targets, suggesting that these proteins are putative substrates of endogenous protein methyltransferases, whose activities can be inhibited by SAH. Although some proteins such as Histone H3, H1.2, and DNMT1[37,39,40] were reported previously as the substrates of PMTs such as G9a, GLP1, or SUV39H2 and can be found in the list of the 297 targets (Figure 5b, Supporting Information Table S2), many proteins revealed here are linked to endogenous activities of PMTs for the first time. This observation suggests that PMTs may act on diverse unknown substrates in complex cellular contexts. Here we were not able

a.



b.

Accession #	Description	Score	Ref
IPI00215884.4	Serine/arginine-rich splicing factor 1	6.0	35
IPI00217465.5	Histone H1.2	6.2	39
IPI00018534.4	Histone H2B type 1-L	7.9	36
IPI00395674.1	Small nuclear ribonucleoprotein-associated proteins B and B'	13.3	35
IPI00020194.1	TATA-binding protein-associated factor 2N	16.2	35
IPI00216493.1	Heterogeneous nuclear ribonucleoprotein H3	16.4	38
IPI00455457.4	Histone H3	18.2	36
IPI00798375.2	Probable ATP-dependent RNA helicase DDX5	20.2	35
IPI00220919.1	DNMT1	38.3	37
IPI00418313.3	Interleukin enhancer-binding factor 3	48.4	35

Figure 5. Pulldown and identification of reported methylation targets in HEK293T lysate treated with ProSeAM. (a) A cleavable azido-azo-biotin[34] was used to pulldown and enrich labeled proteins from HEK293T lysate prior to LC–MS/MS analysis. (b) A short list of previously reported methylation targets that were detected in HEK293T lysate treated with ProSeAM, but not in lysates pretreated with the general methyltransferase product inhibitor, SAH.

to pull down all the known targets of specific enzymes such as G9a, GLP1, or SUV39H2.[37,39,40] However, this may be due to endogenous expression levels of the targets or the corresponding PMTs. Increasing the levels of these proteins, either through transient expression or by selecting more relevant cell lines, is expected to address these issues. The robust pull down of propargylated proteins in cellular contexts therefore presents ProSeAM as a suitable probe for endogenous protein methylation and provides a foundation for future work aimed at expanding its utility to various disease-relevant cell types.

CONCLUSION

Determining that **1** rapidly decomposes to compound **3** prompted us to develop the more stable SAM surrogate, ProSeAM, which contains the smallest chemical handle for CuAAC chemistry. The well-resolved HPLC profiles of ProSeAM's selenonium-*R*/*S* epimers permitted the access to the diastereomerically pure ProSeAM to explore its application. Here ProSeAM demonstrated compatibility toward a set of PKMTs (G9a, GLP1, and SUV39H2) and the newly discovered cysteine methyltransferase NleE. Given that DNA methyltransferases (M.TaqI, M.HhaI and M.BcnIB),[41] RNA methyltransferase (Trm1),[42] protein methyltransferases (SETDB1, MLL4 and Dim-5),[8,9] and small-molecule methyltransferases-(NovO and CouO)[21] can act on the SAM analogues that are structurally bulky or similar to ProSeAM **2**, we envision that more native methyltransferases can recognize ProSeAM as a SAM surrogate (see Notes). Consistent with its *in vitro* activity, ProSeAM appears to be processed by endogenous protein methyltransferases in various cellular contexts. Furthermore, the resultant cell-type-specific labeling may relate to the methylation events respective to pathological states and thus can be the focus of future research.

Several alkyne/azido-containing SAM analogues have been described previously.[6,7,9,20] These SAM surrogates are generally too bulky for native protein methyltransferases and thus demand engineered enzymes to achieve efficient substrate labeling.[6,20] ProSeAM is distinguishable by its small and stable propargylic moiety and its ability to be utilized by multiple native protein methyltransferases. This feature presents ProSeAM as a chemical reporter to probe the activities of endogenous protein methyltransferases. Here we have demonstrated this utility through the pull-down and identification of a series of protein targets in HEK293T cells. The same approach could be readily applied to primary tumor samples, for which knocking in engineered protein methyltransferases can be challenging. Since the replacement of sulfonium with selenonium makes SAM a better methyl donor,[24] the sulfonium-to-selenium-replacement approach can also be implemented to previously reported SAM analogues. These SAM derivatives, combined with native or engineered protein methyltransferases, may be expected to be more effective for substrate labeling. Given that various other families of methyltransferases can utilize SAM analogues that are structurally similar to ProSeAM (see Notes and ref 31 and 45), this SAM surrogate is expected to serve as a chemical reporter to interrogate biological methylation in a broader context.

EXPERIMENTAL SECTION

Synthesis of Propargyl SAM 1 and ProSeAM 2. SAH (Aldrich Chemical; 10 mg, 26 μmol, 1 equiv) or SeAH (15 mg, 34.8 μmol, 1 equiv; synthesized according to the method of Skupin)[23] was dissolved into a mixture of 1:1 formic acid and acetic acid (1 mL) and cooled on ice. Propargyl bromide (50 equiv, 80% v/v in toluene) was then slowly added. After addition $AgClO_4$ (5.4 mg, 1 equiv) in 0.5 mL of 1:1 formic and acetic acid mixture, the reaction was continued at ambient temperature (22 °C) for another 8 h then quenched with 5 mL of distilled water containing 0.1% TFA (v/v). The aqueous phase was washed three times with diethyl ether (3 × 5 mL), centrifuged to remove the precipitate, and then passed through a Nalgene 0.2 μM syringe filter. Compound **1** or ProSeAM **2** was purified by preparative reversed-phase HPLC (XBridge Prep C18 5 μm OBD 19 × 150 mm) at a flow rate of 10 mL/min with acetonitrile in 0.1% aqueous trifluoroacetic acid (linear gradients from 0% to 10% in 20 min and then to 70% in 5 min). The sulfonium-*R*/*S* diastereomers of **1** could not be resolved under the present HPLC conditions. A mixture of epimers was thus collected and used in subsequent reactions. In contrast, the selenonium-*R*/*S* diastereomers of ProSeAM **2** were separable by XBridge Prep C18 reverse phase HPLC column (Supporting Information Figure S1). The active epimer of ProSeAM **2** (the compound with a longer retention time, Supporting Information Figure S1) was collected and concentrated by

lyophilization overnight. Into the resultant concentrated solution, a small amount of TFA was added to adjust its pH to ~2. Stock solutions of ProSeAM **2** were stored at −80 °C before use. The concentrations of the stock solutions of **1** and ProSeAM **2** were determined by their UV absorption (ε_{260} = 15 400 L·mol^{-1}·cm^{-1}) and gave the final isolation yields of 15% for ProSeAM **2** (the active epimer) and 40% for **1** (the mixture of two epimers), respectively. ^{1}H NMR and ESI-MS of **1** ($C_{17}H_{24}N_6O_5S^+$) match the reported value.[8] ^{1}H NMR for ProSeAM **2** (500 MHz, D_2O + 0.1% TFA-*d*): δ = 2.34 (m, 2H), 3.15 (s, 1H), 3.52 (m, 2H), 3.86 (m, 1H), 3.9 (ddd, 2H; J_a =34.3 Hz, J_b = 12.8 Hz, J_c = 3.2 Hz), 4.14 (m, 2H), 4.49 (m, 1H), 4.56 (t, 1H, J = 6.13 Hz), 4.75 (m, 1H), 6.09 (d,1H, J = 3.85 Hz), 8.37−8.40 (m, 2H, arom.); HPLC retention time for ProSeAM = 7.5 min; ESI-MS for ProSeAM (m/z): 471.11 [M]$^+$, 369.99 [5′-(propargyl)-selenoadenosine + H]$^+$, 249.91 [5′-deoxyadenosine]$^+$, 135.73 [adenine + H]$^+$. HRMS for ProSeAM: 471.0871 calculated and 471.0880 observed for $C_{17}H_{24}N_6O_5Se$.

Analysis of Stability of Compound 1 and ProSeAM 2 by Analytical HPLC and LCMS. See Supporting Information for details. Briefly, ProSeAM and **1** in 0.1% aqueous TFA (pH ~ 2.0) are stable for at least 8 h at ambient temperature (conditions of synthesis and HPLC) and for 3 months at −80 °C (storage conditions). To characterize the stability of these compounds, stock solutions of either **1** or ProSeAM were prepared in 50 mM Tris-HCl (pH = 8.0). Aliquots were removed and analyzed by analytic HLPC or LCMS at various time intervals. Rapid decomposition of **1** was featured by the loss of the expected MS of 423.38 of **1** and the accumulation of a new hydrated species with the MS of 441.58 (423.38 + 18). The HPLC profile (Supporting Information Figure S2), ^{1}H NMR and ESI-MS of the decomposition product match those of Compound **3**, which was synthesized by an independent method.[1] The half-life of **1** was estimated to be <1 min on the basis of the time-dependent analytic HLPC and LCMS analysis (Supporting Information Figure S2). In contrast, no significant loss of ProSeAM through the formation of the +18 adduct was detected within 60 min. In addition, ProSeAM primarily decomposes to *Se*-propargyl-5′-selenoadenosine as revealed by HLPC and LCMS analysis (Supporting Information Figure S2).

Synthesis and Characterization of Compound 3, the Decomposition Product of 1. Compound **1** (6 mg, 0.014 mmol, synthesized as described above) was dissolved in 5 mL of 50 mM Tris-HCl (pH = 8.0) buffer in 15 mL Falcon tube and rotated end-over-end for 20 min at ambient temperature (22 °C). ESI-MS analysis confirmed the complete disappearance of the starting material **1** and the sole appearance of a product, whose MS and fragment pattern are consistent with those of **3** (**1** + H_2O, Supporting Information Figure S2). The reaction mixture was then acidified to pH 4.0 by dropwise addition of hydrochloric acid. The crude decomposition product of **3** ($C_{17}H_{26}N_6O_6S^+$) was purified by preparative reversed-phase HPLC eluting at a flow rate of 10 mL/mins with acetonitrile in 0.01% aqueous trifluoroacetic acid (linear gradients to 10% in 15 min and then to 70% in 5 min). A single peak of **3** was collected and lyophilized to dryness. The dried product was dissolved in water containing 0.01% TFA (v/v) and stored at −80 °C prior to analysis. The concentration of **3** was determined by UV absorption with ε_{260} = 15 400 L·mol^{-1}·cm^{-1} with the isolation yield of 57% (3.5 mg, 0.008 mmol). ^{1}H NMR (600 MHz, D_2O): δ 2.1 (s, 1.5H), 2.13 (s, 1.5 H), 2.26−2.19 (m, 2H), 3.6−3.47 (m, 2H), 3.74 (t, 0.5H, J = 6.6 Hz), 3.77 (t, 0.5H, J = 6.6 Hz), 3.97−3.86 (m, 2H), 4.41 (q, 0.5H, J = 6 Hz), 4.48−4.45 (m, 0.5H), 4.53 (t, 0.5H, J = 5.9 Hz), 4.56 (t, 0.5H, J = 6 Hz), 4.74−4.72 (m, 1H), 6.07 (q, 1H, J = 3.84 Hz), 8.33 (s, 0.5), 8.34 (s, 0.5H), 8.35 (s, 1H). The data match those of the authentic compound **3** synthesized via an independent method.[22] Here the total 16 protons rather than 18 protons were detected. Missing the two methylene protons adjacent to the sulfonium center is likely due to a rapid deuterium exchange, which has been described in a previously reported synthesis.[22] HPLC retention time = 5 min; ESI-MS (m/z): 441.07[M]$^+$, 339.96 [5′-(2-oxopropyl)thio-5′-deoxyaden-osine + H]$^+$, 250.0 [5′-deoxyadenosine]$^+$, 135.82 [adenine + H]$^+$.

Protein Expression and Purification. The expression and purification of protein lysine methyltransferases and protein arginine methyltransferases was described previously.[7,27,43,44] For the bacterial cysteine methyltransferase NleE, the His×6-SUMO-NleE plasmid was obtained from Dr. Feng Shao (National Institute of Biological Science; Beijing, China) and expressed in BL-21 (DE) cells according to manufacturer's instruction with the exception that 10 μM $ZnSO_4$ was included in the growth medium and 0.2 mM IPTG was used for induction at OD_{600} = 0.70 and 22 °C for overnight. His×6-SUMO-NleE was purified with Ni-NTA agarose resin (Qiagen), followed by overnight dialysis against 50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 10% glycerol. Protein concentrations were determined by a Bradford assay kit (Bio-Rad) with BSA as standards. All enzymes were stored at −80 °C and thawed on ice prior to use.

Cell Culture Materials and Procedures. All cell lines were cultured according to ATCC protocols. A list of cell lines and corresponding culture conditions are provided in the Supporting Information.

MALDI−MS Screening for the Compatibility of ProSeAM with Native Methyltransferases. In general, all reactions (50 μL) were carried out for 2 h at ambient temperature (22 °C). Both biotinylated peptides and nonbiotynylated peptides were used as substrates. Nonbiotinylated peptide products were purified over Sep-Pak (Waters) C18 cartridges according to manufacturer's protocol, while biotinylated peptides were enriched by Streptavidin-Sepharose (GE Healthcare) beads after enzymatic reaction. These samples were then subjected to MALDI mass spectroscopy as described previously.[6] Assay conditions for individual enzymes are described below.

For G9a and GLP1, the reaction contained 2 μM enzyme, 25 μM histone H3(a.a. 1−21) peptide and 100 μM SAM, **1** or ProSeAM in 50 mM Tris-HCl (pH = 8.0). The reaction of SUV39H2 contained 2 μM enzyme, 25 μM histone H3(a.a. 1−21) peptide and 100 μM SAM, **1** or ProSeAM in 50 mM Tris-HCl (pH = 8.0), 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 2.5 mM $MgCl_2$. The reaction of SET8 contained 5 μM enzyme, 25 μM histone H4 (a.a. 10−30) peptide and 100 μM SAM, **1** or ProSeAM in 50 mM HEPES (pH = 8.0), 0.005% Tween 20 and 0.0005% BSA. The reaction of SET7/9 contained 1 μM enzyme, 25 μM histone H3(a.a. 1−21) peptide and 100 μM SAM, **1** or ProSeAM in 50 mM HEPES (pH = 8.0), 0.005% Tween 20 and 0.0005% BSA. The reaction of PRMT1 contained 2 μM enzyme, 100 μM RGG-biotin peptide and 100 μM SAM, **1** or ProSeAM in 50 mM Tris-HCl (pH = 8.0). The reaction of PRMT3 contained 1 μM enzyme, 100 μM RGG-biotin peptide and 100 μM SAM, **1** or ProSeAM in 200 mM HEPES (pH = 8.0).

Labeling of Full-Length Histone H3.1 and GST-TAB2-NZF. For the G9a/GLP1-catalyzed labeling of full-length histone H3.1 with ProSeAM (Figure 3d), 2 μM G9a or GLP1 was incubated with 25 μM recombinant human histone H3.1 (New England BioLabs) and 50 μM ProSeAM for 1 h at 25 °C in 50 mM Tris-HCl (pH = 8.0). For NleE-catalyzed labeling of GST-TAB2-NZF with ProSeAM (Figure 3e), 3 μM His×6-SUMO-NleE was incubated with 50 μM GST-TAB2-NZF and 100 μM ProSeAM for 30 min in the buffer containing 50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 5 mM DTT and 0.1% NP-40. Alternatively, 3 μM His×6-SUMO-NleE was preincubated with 500 μM SAH for 15 min prior to addition of ProSeAM. Upon the completion of the reaction, the crude samples were precipitated with 1:2:3 $CHCl_3$/H_2O/MeOH and subjected to CuAAC chemistry as described below.

MS/MS Analysis of ProSeAM-Modified Histone H3.1. Histone H3.1 (25 μM; New England Biolabs) in 40 μL of 50 mM Tris HCl pH 8.0 was incubated overnight at ambient temperature (22 °C) in the presence of 2 μM GLP1 and 50 μM ProSeAM. Samples were then separated by SDS-PAGE. The resultant histone band was extracted from the gel, treated with propionic anhydride and then subject to trypsin digestion and LCMS analysis as described previously.[6]

Labeling Cell Lysates with ProSeAM. Whole cell lysates were made fresh from frozen cell pellets prepared as described in the Supporting Information. Cells were suspended in buffer containing 50 mM Tris-HCl (pH = 8.0), 50 mM KCl, 10% glycerol, 1 mM TCEP, 0.005% Tween 20 and 1× Roche protease inhibitor cocktail and incubated on ice for 20 min. The suspended cells were then lysed via

sonication (Misonix Ultrasonic Liquid Processor with a single 15 min pulse at 65% amplitude) and centrifuged at 15 000*g* for 30 min. The supernatants were isolated and their protein concentrations were measured by a Bradford assay kit (Bio-Rad) with BSA as standards.

For each 50 μL labeling reaction, 100 μg of total lysate protein was incubated with the indicated concentrations of ProSeAM or **1**. Here 0.1 μM 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) was also added into the samples to degrade SAH and thus release the potential inhibition of SAH in lysates or from the byproduct (SAH or SeAH) of methylation reaction.[27,44] MTAN treatment was omitted when the samples were treated with SAH to inhibit endogenous methyltransferases. SAH treated samples were preincubated for 15 min with 500 μM SAH, 2 μM adenosine-2′,3′-dialdehyde (an irreversible inhibitor of SAH hydrolase to block SAH degradation)[27,44] and 2 μM methylthio-DADMe-ImmA (an inhibitor of *S*-methyl-5′-thioadenosine phosphorylase to block SAH degradation, a generous gift from the Schramm lab at Albert Einstein College of Medicine).[27] After the 2-h treatment of ProSeAM or **1**, the samples were precipitated and washed with 1:2:3 $CHCl_3$/H_2O/MeOH and subject to CuAAC chemistry as described below.

Conjugation of Azide-Containing Fluorecent Probe via Cu(I)-Catalyzed Azide-Alkynyl (CuAAC) Chemistry. After treating full-length histone H3.1 (20 μL reaction mixture of 25 μM H3.1), GST-TAB2-NZF (100 μL reaction mixture of 50 μM GST-TAB2-NZF) or whole cell lysates (100 μg proteins) with ProSeAM or **1** as described above, the samples were precipitated with a mixture containing 600 μL of methanol, 200 μL of chloroform and 400 μL of water, and centrifuged at 15 000*g* for 10 min. The aqueous phase was discarded and the pellets in the organic phase were washed three times with 1000 μL of methanol. The supernatant was decanted and the protein pellets were air-dried for 1 h. The sample was then redissolved in 20 μL of solution containing 50 mM TEA, pH 7.4, 150 mM NaCl and 4% SDS. This mixture was subjected to CuAAC chemistry by adding 100 μM Az-Rho,[15] 2 mM tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), 1 mM $CuSO_4$ and 100 μM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) for 1 h at ambient temperature (22 °C) and in the absence of light. After the CuAAC reaction, the samples were precipitated with 1000 μL of 1:2:3 $CHCl_3$/H_2O/MeOH mixture as described above. The aqueous phase was discarded and the organic phase was washed three times with 1000 μL of methanol. The dried samples were redissolved in 1× loading buffer and heated for 10 min at 100 °C, followed by SDS-PAGE separation (Criterion Precast gel, 12% or 18% Tris-HCl, Bio-Rad). The fluorescent bands were visualized by in-gel fluorescence using an Amersham Biosciences Typhoon 9400 fluorescent scanner (excitation at 532 nm, 580 nm filter and 30 nm band-pass). After the in-gel fluorescence, Coomassie Blue staining was carried out as loading control (results in Figure 4, Supporting Information Figures S13−S15)

Affinity Pull-Down of HEK293T Lysate Treated with ProSeAM. ProSeAM (a final concentration =25 μM) was added to a total of 10 mg of protein from HEK293T lysate in 5 mL of 50 mM Tris HCl, pH 8.0, 50 mM KCl, 10% glycerol, 1 mM TCEP, 0.005% Tween20 and 1× Roche protease inhibitor cocktail. Noninhibitor treated sample buffer also contained 100 nM MTAN to release potential SAH inhibition. By contrast, the sample treated with SAH inhibitor contained 0.5 mM SAH, 2 μM adenosine-2′,3′-dialdehyde and 2 μM methylthio-DADMe-ImmA.

After 1 h reaction time, the samples were precipitated with 1:2:3 $CHCl_3$/H_2O/MeOH (42.5 mL), centrifuged at 3000*g* for 45 min and the resultant pellets were washed twice with 50 mL of MeOH. The protein pellets were then suspended in (4.45 mL) 50 mM TEA, pH 7.4, 150 mM NaCl, 4% (w/v) SDS buffer, into which was added 550 μL of CuAAC reaction cocktail containing 100 μL of 5 mM azido-azo-biotin[34] (100 μM final concentration), 100 μL of 50 mM TCEP (1 mM final concentration), 250 μL of TBTA (100 μM final concentration), and 100 μL of 50 mM $CuSO_4$ (1 mM final concentration). After a 1.5 h incubation period at ambient temperature (22 °C), the proteins were precipitated again by addition of 45 mL of MeOH at −80 °C, centrifuged at 3000*g* and washed twice with 45 mL of MeOH. Protein was dried and then suspended in 1 mL of 50 mM TEA, pH 7.4, 150 mM NaCl, 10 mM EDTA, 4% (w/v) SDS buffer, and diluted through addition of 2 mL of 50 mM TEA, pH 7.4, 150 mM NaCl, and 1% (w/v) Brij97. Into these mixtures was added 100 μL of Streptavidin-Sepharose beads (GE Healthcare) prewashed 3 times with PBS. The samples were incubated for 1 h with agitation, centrifuged at 3000*g* and 4 °C for 2 min, and then washed three times with 10 mL of PBS containing 0.2% SDS, followed by two additional washes with 10 mL of 250 mM ammonium bicarbonate. After removing supernatant and adding 500 μL of 8 M Urea, 25 μL of 200 mM TCEP, and 25 μL of 400 mM iodoacetamide, the protein samples were then incubated for 40 min in the absence of light. The samples were then washed twice with 10 mL of 250 mM ammonium bicarbonate. The bead-immobilized proteins were then liberated by two consecutive 30-min incubations with 250 μL of 1% SDS, 250 mM ammonium bicarbonate, and 25 mM sodium dithionite. The released proteins were then collected and concentrated using Amicon Ultra 3K centrifugal filter units (Millipore), lyophilized and stored at −80 °C prior to proteomics analysis.

Proteomics Analysis of the Pulldown Samples from HEK293T Cell Lysate. Pulldown samples were separated by SDS-PAGE, extracted, and trypsinized as described previously.[7] The digestion product was then analyzed by LC−MS/MS via separation on a 60 min gradient elution at a flow rate of 0.30 μL/min with the UltiMate 3000 RSLCnano System (Thermo Scientific), which was directly interfaced with the Thermo Q Exactive benchtop mass spectrometer. The analytical column was a homemade fused silica capillary column (75 μm i.d., 150 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 Å, 5 μm,; Varian, Lexington, MA). Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The LTQ-Orbitrap mass spectrophotometer was operated in the data-dependent Acquisition mode using the Xcalibur 2.2.0 software with a single full-scan mass spectrum in the Orbitrap followed by 8 MS/MS scans in the quadrupole collision cell using higher energy collision dissociation (HCD).

The MS/MS data was analyzed using Thermo Proteome discoverer 1.2.0 software against the ipi.HUMAN.v3.82 database with the following search parameters: 10 ppm peptide mass tolerance, 0.8 Da ms/ms tolerance, and two missed cleavages allowed. In addition, modification of carbamidomethylation on Cys, oxidation of Met, deamidated Asn and Gln, and $C_{11}H_{12}N_4O$ (ProSeAM + cleaved azido-azo-biotin linker) or $C_{22}H_{24}N_8O_2$ (2× ProSeAM + cleaved azido-azo-biotin linker) were also used to search the database. A decoy database search was added with the criteria of FDR at 0.01. Peptide filtering criteria were the following: 2, 2.75, and 3 for singly charged, doubly charged, and triply or higher charged ions, respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional procedures, characterization of compounds, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

The authors note that, while preparing this manuscript, the Weinhhold lab also reported the synthesis and characterization of **2** and its activity toward several methyltransferases.[31,45]

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