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BRIEF ARTICLE

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Structure-Guided Design of a Methyl Donor Cofactor That Controls a Viral Histone H3 Lysine 27 Methyltransferase Activity

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Supporting Information

ABSTRACT: vSET (a viral SET domain protein) is an attractive polycomb repressive complex 2 (PRC2) surrogate to study the effect of histone H3 lysine 27 (H3K27) methylation on gene transcription, as both catalyze histone H3K27 trimethylation. To control the enzymatic activity of vSET in vivo with an engineered *S*-adenosyl-L-methionine (SAM) analogue as methyl donor cofactor, we have carried out structure-guided design, synthesis, and characterization of orthogonal vSET methyltransferase mutant/SAM analogue pairs using a "bump-and-hole" strategy.

INTRODUCTION

Epigenetic control of gene expression is responsible for fundamental cellular processes such as genetic imprinting, stem cell self-renewal, and differentiation, and it involves many types of chemical modifications on chromatin, including DNA methylation, histone methylation, acetylation, phosphorylation, ubiquitination, and ADP-ribosylation.[1,2] The high complexity of this regulatory system is made possible because different histone modifications function in a context-dependent manner. It is difficult to predict how different modifications work in concert in gene regulation. A means to turn on or off histone modifications on demand would be an invaluable tool.

The polycomb repressive complex 2 (PRC2) methylates histone H3 lysine 27 (H3K27), a mark for gene transcriptional silencing.[3] The PRC2 consists of three components: EZH2, SUZ12, and EED in human.[4,5] EZH2 of 751 amino acids contains the catalytic SET domain lysine methyltransferase activity; its optimal methyltransferase activity requires the other two subunits and other partners. It has proven very challenging to express a functional PRC2 in situ. Notably, our recent studies show that a SET protein (vSET) of 119 amino acids encoded by the *Paramecium bursaria* chlorella virus can specifically methylate H3K27 in eukaryotic cells.[6−8] vSET is active on its own, making it much easier to manipulate than the PRC2 complex.

We envision that vSET activity can be controlled in a variant whose activity is dependent on a chemically engineered *S*-adenosyl-L-methionine (SAM) derivative. The generation of orthogonal vSET/SAM pairing may be accomplished using a "bump-and-hole" strategy, pioneered by Shokat and colleagues in the kinase field.[9] Specifically, one would change a bulky residue in vSET to alanine or glycine and add a substituent to SAM to fill the space created in the protein. Given its function as a common methyl donor cofactor, surprisingly, there are only a few reports on the utilization of SAM analogues by protein methyltransferases. In one study, N^6-substituted *S*-adenosyl-L-homocysteine (SAH) analogues were designed as variant specific RMT1 (a yeast arginine methyltransferase) inhibitors.[10] Other studies reported that SAM analogues, in which the sulfonium methyl group is replaced with a substituent alkynyl group, are useful for identifying enzyme substrates.[11,12] However, no one has yet reported a modified SAM analogue as an effective methyl donor cofactor for a protein methyltransferase. Here, we describe the design and synthesis of several substituted SAM molecules and characterization of their methyl donor capability with vSET H3K27 methylation activity. We succeeded in developing an orthogonal vSET/SAM analogue pair that is very active, thereby demonstrating the feasibility of this strategy for the protein methyltransferases.

STRUCTURE-GUIDED ENGINEERING OF ORTHOGONAL VSET/SAM COFACTOR PAIR

We focused on chemical modifications of SAM at three positions, the N^6-amino of the adenine ring that is a site used in other studies,[10] and the 2′- or 3′-hydroxyl in the ribose moiety that has yet to be explored. Close inspection of our crystal structure of the vSET/SAH/H3K27me1 peptide complex (Figure 1)[8] reveals that the adenine ring of SAH forms aromatic and hydrophobic interactions with Trp110 and the side chain methyls of Leu13, and the N^6-amino group is hydrogen-bonded to the carboxylate of the C-terminal residue Asn119 and the main chain carbonyl oxygen of His70. This set of interactions is bolstered by a hydrogen bond formed between the side chain amino of Asn119 and the backbone carbonyl oxygen of Leu13. While pointing outward, the 2′-hydroxyl is packed next to Leu116. Further, the phenoxyl of Tyr109 forms two hydrogen bonds, one with the 3′-hydroxyl of SAH and another with the main chain carbonyl of Asp49. Thus, we hypothesized that change of Leu13, Asn119, Tyr109, or Leu116 to Ala or Gly would create a cavity that could accommodate a substituent group from a SAM analogue.

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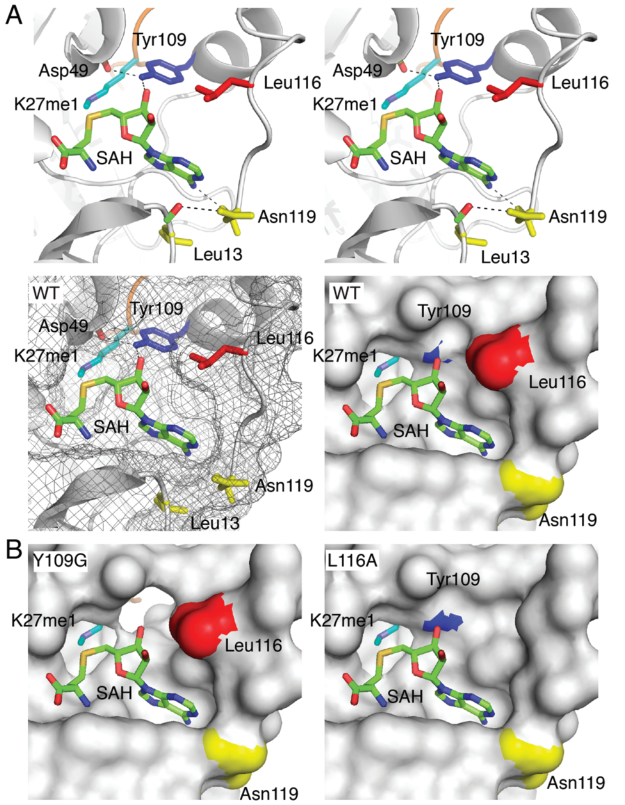
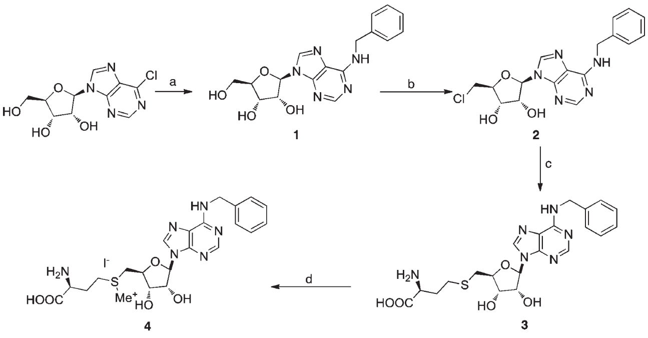


Figure 1. (A) Crystal structure of vSET complex (PDB code 3KMT). SAH and key vSET residues are shown as sticks, and H3 peptide is in orange: (top) stereoview; (bottom) mesh and solid forms of vSET surface. (B) Pymol modeled surfaces of Y109G and L116A variants.

Scheme 1[a]

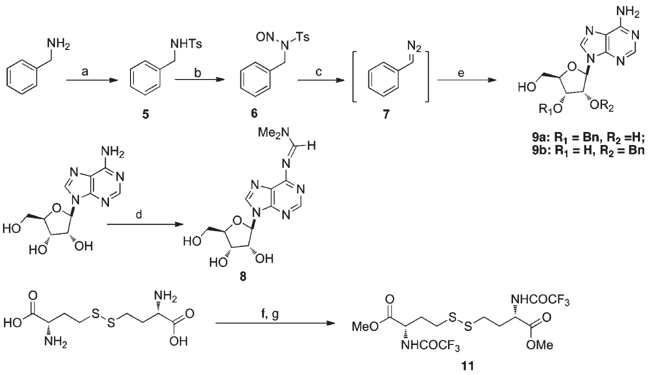


[a] Reagents and conditions: (a) benzylamine, EtOH, 70 °C, 16 h; (b) $SOCl_2$, Py, CH_3CN, 0 → 25 °C, 6 h; (c) L-homocysteine thiolactone/HCl, NaOH, KI, 100 °C, O/N; (d) MeI, HCOOH, 25 °C, 4 days.

SYNTHESIS OF SUBSTITUTED SAM ANALOGUES

We first synthesized (±)-N^6-benzyl-*S*-adenosyl-L-methionine **4** in a four-step synthetic route (Scheme 1). The reaction started with condensation of 6-chloropurine riboside with benzylamine in ethanol[10] followed by replacement of the 5′-hydroxyl of **1** with chloride and subsequent coupling with L-homocysteine thiolactone and methylation of the sulfur, affording the product of **4** with an overall yield of 6%. The intermediates and final product were confirmed by mass spectrometry and NMR spectra.

Scheme 2[a]



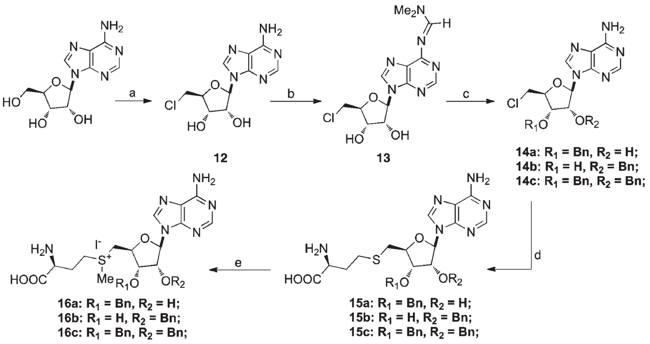
[a] Reagents and conditions: (a) tosyl chloride, Py, 25 °C, 1 h; (b) $NaNO_2$, CH_3COOH/Ac_2O = 1:4, 0 → 25 °C, overnight; (c) NaOMe, MeOH/Et_2O = 1:6, reflux, 30 min; (d) *N*,*N*-dimethylformamide dimethyl acetal, DMF, 25 °C, overnight; (e) (i) $SnCl_2 \cdot 2H_2O$, **8**, 1,2-dimethoxyethane, 25 °C, overnight; (ii) NH_4OH/MeOH, 25 °C, 5 h; (f) $SOCl_2$, MeOH, 25 °C, 16 h; (g) $(CF_3CO)_2O$, 60 °C, 1.5 h.

For the synthesis of 2′- and/or 3′-benzyl SAM, we generated adenosine derivatives with dialkyl disulfides under Hata's condition.[13,14] Our initial attempt at direct benzylation of unprotected adenosine with phenyldiazomethane **7**,[15] prepared in three steps from benzylamine in 44% yield (Scheme 2), resulted in a very low yield (<10%).[16] We found that protection of the amino at the 4-position of adenosine with *N*,*N*-dimethylformamide dimethyl acetal improved the yield to 55%,[17] producing the two regioisomers **9a** and **9b** (Scheme 2) that were separated by HPLC in a ratio of 3:2. The regiochemistry assignment of **9a** and **9b** was made by comparison of the ^1H NMR spectra with those reported previously.[18] The disulfide moiety *N*,*N*-bis(trifluoroacetyl)-L-homocysteine dimethyl ester **11**[17] was obtained in 59% yield after two steps from L-homocystine (Scheme 2).

However, treatment of **9a** or **9b** with **11** under Hata's condition, in the presence of tri-*n*-butylphosphine in pyridine did not give the desired product. The coupling reaction did not proceed in several solvent conditions tested (Supporting Information Table 1). We noticed that the reaction of free adenosine with **11** under the same conditions gave the desired product in 46% yield after 5 days (Supporting Information, Table 1, entry 3). The ^1H NMR spectrum of the product matches well with that reported previously.[19] Thus, we reasoned that introduction of the bulky benzyl substituent near the coupling site likely interferes with the reaction, and dialkyldisulfides are generally much less reactive than diaryldisulfides in the phosphine-mediated coupling reaction, which may also contribute to the failure of the reaction.[14,19]

We next introduced a good leaving group at the 5′-position to install the alkylthio substituent (Scheme 3). Selective conversion of adenosine to 5′-chloro derivative **12** was performed with thionyl chloride in acetonitrile,[20] followed by protection of the amino group with *N*,*N*-dimethylformamide dimethyl acetal.[17] Subsequent benzylation of **13** with phenyldiazomethane **7** afforded two regioisomers **14a** and **14b** in a ratio of 3:2. Their regiochemistry assignment was done by comparison of the ^1H NMR spectra with those obtained from chlorination of **9a** and **9b**. In addition, the dibenzylated adenosine analogue **14c** was synthesized by increasing the amount of phenyldiazomethane in the reaction (Scheme 3).

Scheme 3[a]



[a] Reagents and conditions: (a) $SOCl_2$, Py, CH_3CN, 0 → 25 °C, 6 h; (b) *N*,*N*-dimethylformamide dimethyl acetal, DMF, 25 °C, overnight; (c) (i) $SnCl_2 \cdot 2H_2O$, 7, 1,2-dimethoxyethane, 25 °C, overnight; (ii) NH_4OH/MeOH, 25 °C, 5 h; (d) L-homocysteine thiolactone hydrochloride, NaOH, KI, 100 °C, O/N; (e) MeI, HCOOH, 25 °C, 4 days.

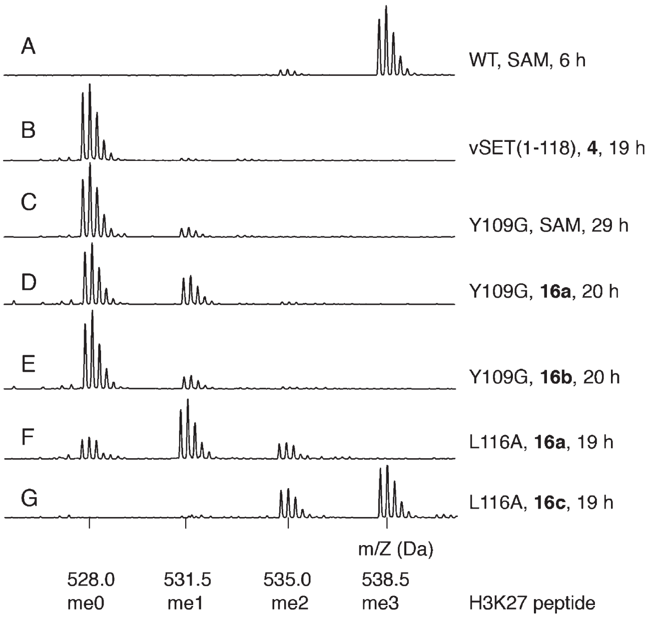


Figure 2. Mass spectrometry data showing the activity of different enzyme/cofactor pairs.

As expected (Scheme 3), coupling of **14a**, **14b**, or **14c** with L-homocysteine thiolactone proceeded successfully and led to **15a**, **15b**, and **15c**, respectively.[21] Because of a resulting sulfonium chiral center,[22] a diastereomeric mixture of (±)-3′-benzyl-*S*-adenosyl-L-methionine **16a**, (±)-2′-benzyl-*S*-adenosyl-L-methionine **16b**, and (±)-2′,3′-dibenzyl-*S*-adenosyl-L-methionine **16c** was synthesized by methylating the sulfur atoms of **15a**, **15b**, and **15c** with excess amount of methyl iodide in formic acid in the dark for 4 days. The final products of the SAM derivatives **16a**−**c** were obtained as diastereomeric mixtures.

CHARACTERIZATION OF VSET H3K27 METHYLATION WITH THE SAM ANALOGUES

We first tested the idea of adding a bulky group to the N^6-position of the adenine ring of SAM. To generate necessary extra space, Leu13 was substituted with Ala or the C-terminal Asn119 was removed (Figure 1A). Almost no activity was detected after an overnight reaction for both vSET variants in the presence of N^6-benzyl-SAM **4** (Figure 2B,A; Table 1). Similar results were obtained when N^6-methyl-SAM or N^6-isopropyl-SAM was used (data not shown). Note that the previously reported yeast RMT1-E117G/N^6-benzyl-SAM pair yielded only 0.2% arginine methyltransferase activity compared to the WT-RMT1/SAM pair.[10] Given that the adenine ring forms a network of hydrogen bonds with vSET residues Leu13, His70, and Asn119, our results combined with those from the RMT1-E117G/N^6-benzyl-SAM pair argue that the N^6-position of the adenine is not suitable for generating SAM derivatives that would retain significant methylation activity with a paired vSET variant.

Table 1. vSET/Cofactor Pairs with No Activity

vSET	cofactor
L13A	**4**, N^6-MSAM, N^6-IPSAM
vSET (aa 1−118)	**4**, N^6-MSAM, N^6-IPSAM
Y109H	**19**, SAM
Y109D	**19**, SAM
Y109N	**19**, SAM
WT	**19**

Table 2. k_{cat} and K_m of the vSET/Cofactor Pairs

vSET	cofactor	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} M^{-1})
WT	SAM	3.9	126	30952
WT	**16a**	0.14	86	1628
WT	**16c**	0.37	173	2139
WT	**16b**	0.13	86	1512
Y109G	SAM	0.00048	62	7.7
Y109G	**16a**	0.00049	2800	0.18
Y109G	**16c**	0.0024	917	2.6
L116A	SAM	7.4	60	123333
L116A	**16a**	0.15	137	1095
L116A	**16c**	1.3	72	18056
L116A	**16b**	0.12	128	938

We next characterized Tyr109 variants with SAM or its analogues. Mass spectrometry analysis shows that Y109G has very little activity in methylating H3K27 with SAM compared to the wild-type vSET (Figure 2A,C). The same is true with Y109A (data not shown). We then tested the activity of Tyr109 variants in the presence of **16a** or **16b**. The Y109G/**16a** pair was much less active than wild-type/SAM pair as judged by the amount of methylation signal generated in the mass spectrometry study (Figure 2D). However, it was slightly more active than Y109G/SAM and Y109G/**16b** pairs (Figure 2E). The activity of Y109A was lower in the presence of SAM analogues than Y109G (data not shown). Therefore, our further studies were carried out with the Y109G variant. To better compare different enzyme/cofactor pairs, we performed Michaelis−Menten enzyme kinetic study to obtain k_{cat} and K_m (Table 2, Supporting Information Figure 1).

When SAM is used, change of Tyr109 to Gly causes a 8125-fold drop in k_{cat} but little change to K_m (Table 2). It suggests that the substitution at Tyr109 is most likely to directly interfere with its possible function in substrate lysine binding. For Y109G, changing the cofactor from SAM to **16a** results in a small increase of k_{cat} but a much higher K_m. The same cofactor change for the wild-type vSET causes a 28-fold decrease of catalytic efficiency but surprisingly a 1.5-fold decrease of K_m. The decrease in K_m could be explained by the contribution of fortuitous interaction between the benzyl group and some protein residues. The extremely high K_m for Y109G/**16a** might be associated with the entropic penalty of fixing the benzyl group at a specific orientation for its insertion into the artificial cavity. Thus, we postulated that a second benzyl group at the 2′-position might restrict the conformation of 3′-benzyl, securing its insertion into the cavity. An additional 2′-benzyl group in SAM (i.e., 2′,3′-dibenzyl-SAM **16c**) brings little change of the k_{cat}/K_m to the wild-type vSET with an increase in k_{cat} and K_m. But a very different scenario was observed for Y109G: a slight increase of k_{cat} but a much-improved K_m (Table 2).

To mimic the hydrogen bond between Tyr109 and the 3′-hydroxyl of SAM, we synthesized another SAM analogue **19**,[23] which bears a carbamoylmethyl substituent at 2′- and 3′-oxygen atoms, and tested its activity with Y109H, Y109D, or Y109N (Supporting Information Scheme 1). We postulated that the amide moiety of the carbamoylmethyl substituent of SAM could form a hydrogen bond with the side chain on the residue replacing Tyr109 in these variants. However, no activity was detected for any of these vSET variants after an overnight incubation and neither was any of these variants active with SAM or the wild-type vSET with **19**. These results emphasize the importance of Tyr109 in substrate binding, and thus, change of Tyr109 to His, Asp, or Asn is detrimental to vSET activity.

Inspection of residues around the ribose moiety of SAH in the crystal structure suggests that changing Leu116 could create a docking site for a benzyl group at the 2′- or 3′-position (Figure 1). Indeed, most of the H3 peptide was converted to mono- or dimethylated species after 19 h of reaction for L116A/**16a** (Figure 2F). The variant was even more active with **16c**, as almost all the H3 peptide was converted to trimethylated species (Figure 2G). Kinetic studies further revealed a k_{cat} of 7.4 $\min^{-1}$ and K_m of 60 μM for L116A/SAM, which notably is even better than the wild-type vSET/SAM pair (Table 2). When **16a** was used, the k_{cat} of L116A was dropped to 0.15 $\min^{-1}$, accompanied by a slight increase of K_m. Importantly, L116A/**16c** was more active than WT/**16c**, with a 3.5-fold higher k_{cat} and a 2.4-fold lower K_m. Comparison of the k_{cat}/K_m of L116A/**16c** with that of L116A/**16a** argues that the presence of the benzyl groups at 2′ and 3′ positions has a synergistic effect on L116A catalytic efficiency when compared to the single benzyl group analogues.

Since the presence of the 3′-benzyl in SAM resulted in a reduction in the enzymatic activity of the wild-type vSET and L116A variant compared to SAM, we investigated whether **16b** would be more potent for L116A. The results show that for the wild-type vSET, the removal of the 3′-benzyl in **16c** causes a small decrease in k_{cat} and K_m with a net result of a 1.4-fold decrease in the k_{cat}/K_m. However, for L116A, a 13-fold decrease of k_{cat} was seen with an overall 19-fold decrease in k_{cat}/K_m. Interestingly, while adding a single benzyl to the 2′- or 3′-position in SAM causes an ~120-fold decrease in k_{cat}/K_m for L116A, these two benzyl groups in **16c** likely act synergistically, leading to only a 7-fold decrease of k_{cat}/K_m.

Histone lysine methylation is a fundamental mechanism for epigenetic control of gene expression in chromatin. Chemical modulators capable of controlling the enzymatic activity of specific lysine methyltransferases are extremely valuable tools. In this study, we illustrated our approach with an H3K27-specific methyltransferase vSET and SAM. We selected the common N^6-position of the adenine ring and the novel 2′- and 3′-hydroxyl groups of the ribose as the modification sites. We synthesized seven SAM analogues and characterized their activity with the wild-type and variant vSET proteins. We succeeded in engineering a paired vSET-L116A/**16c**, which is only slightly less active than vSET-L116A/SAM but more active than the wild-type vSET/**16c**. Given the presence of SAM in cells, further optimization via chemical modifications of the enzyme and the cofactor is needed to generate a paired vSET variant/modified SAM with activity better than that of the variant with SAM. In summary, our study demonstrates the feasibility of developing a methyl donor cofactor that controls a histone lysine methyltransferase, which could be a useful tool to study the effects of histone H3K27 methylation in vivo.

EXPERIMENTAL SECTION

Chemical Synthesis. All compounds were synthesized using commercially available starting materials without further purification unless otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 600 MHz NMR spectrometer using the residual signal of the deuterated solvent as internal standard. Chemical shifts (δ) are reported in ppm, coupling constants (J) in hertz. MS (ESI) analysis for new compounds was performed on an Agilent G1969A high-resolution mass spectrometer. All compounds that were tested in the biological assays were analyzed by HPLC and LCMS to confirm the purity, which was ≥95%. *R* and *S* indicate relative configurations.

(±)-2′,3′-Dibenzyl-*S*-adenosylmethionine Iodide (16c). MeI (0.03 mL, 0.5 mmol) was added to a solution of **15c** (22.6 mg, 0.04 mmol) in HCOOH (1 mL). After the addition was completed, the mixture was stirred at room temperature in the dark for 4 days. Isolation of **16c** (6.0 mg, 21.3% yield) was achieved by HPLC on an Agilent Eclipse XDB-C18 9.4 mm × 250 mm column. ^{1}H NMR (600 MHz, D_2O) δ 2.22−2.26 (m, 1H), 2.31−2.35 (m, 1H), 2.83 (s, 3H), 2.95 (s, 2H), 3.45 (t, J = 9.0 Hz, 1H), 3.51−3.56 (m, 2H), 3.60−3.67 (m, 1H), 3.70−3.74 (m, 1H), 3.80 (t, J = 9.0 Hz, 1H), 3.82 (d, J = 12.0 Hz, 1H), 3.88 (d, J = 13.6 Hz, 1H), 4.14−4.23 (m, 2H), 4.34 (d, J = 13.6 Hz, 1H), 4.41 (s, 1H), 4.56−4.57 (m, 1H), 5.89 (d, J = 8.8 Hz, 1H), 6.72−6.78 (m, 1H), 6.86 (t, J = 7.9 Hz, 2H), 6.94 (d, J = 5.6 Hz, 2H), 7.42 (t, J = 5.3 Hz, 1H), 7.46 (t, J = 7.53 Hz, 2H), 7.50 (d, J = 3.9 Hz, 2H), 8.00 (s, 1H), 8.04 (s, 1H); HRMS (ESI) calculated for $C_{29}H_{35}N_6O_5S$ (M)$^+$ 579.2384, found 579.2338.

Cloning, Expression, and Purification of vSET. vSET was cloned into pET-22b and expressed as a nonfusion protein in BL21 (DE3) cells.[8] The protein was purified with a heparin column and then a Superdex 75 column.

Site-Directed Mutagenesis. Variants of vSET were generated with the QuikChange mutagenesis kit (Stratagene). The presence of appropriate amino acid change was confirmed by DNA sequencing. All variants were purified as described for the wild type vSET.

Lysine Methyltransferase Reaction. The activity of a paired vSET/cofactor was measured in a solution containing 1 μM enzyme, 100 μM H3 peptide (residues 13−33), and 1 mM methyl donor cofactor. The reaction buffer consisted of 20 mM Tris at pH 8, 20 mM potassium chloride, and 10 mM magnesium chloride. The reaction was performed at room temperature and stopped at a specified time by the addition of trifluoroacetic acid (to 1%). The products were analyzed by mass spectrometry.

ASSOCIATED CONTENT

Ⓢ Supporting Information. Synthesis procedures, spectroscopic details of the compounds, and enzyme kinetic analysis.

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ABBREVIATIONS USED

PRC2, polycomb repressive complex 2; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine

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