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Peptidic transition state analogues as PRMT inhibitors

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A B S T R A C T

Protein arginine *N*-methyltransferases (PRMTs) methylate arginine residues in target proteins using the ubiquitous methyl donor *S*-adenosyl-L-methionine (AdoMet) as a cofactor. PRMTs play important roles in both healthy and disease states and as such inhibition of PRMTs has gained increasing interest. A primary challenge in the development of PRMT inhibitors is achieving specificity for the PRMT of interest as the active sites are highly conserved for all nine members of the PRMT family. Notably, PRMTs show very little redundancy *in vivo* due to their specific sets of protein substrates. However, relatively little is known about the interactions of PRMTs with their protein substrates that drive this substrate specificity. We here describe the extended application of a methodology recently developed in our group for the production of peptide-based transition state mimicking PRMT inhibitors. Using this approach, an adenosine moiety, mimicking that of the AdoMet cofactor, is covalently linked to the guanidine side chain of a target arginine residue contained in a peptidic fragment derived from a PRMT substrate protein. Using this approach, histone H4 tail peptide-based transition state mimics were synthesized wherein the adenosine group was linked to the Arg3 residue. H4R3 is a substrate for multiple PRMTs, including PRMT1 and PRMT6. The inhibition results obtained with these new H4-based transition state mimics show low micromolar IC_{50} values against PRMT1 and PRMT6, indicating that the methodology is applicable to the broader family of PRMTs.

1. Introduction

The methylation of arginines in proteins is performed by the family of protein arginine *N*-methyltransferases (PRMTs). The family consists of nine members which are classified into three categories; type I PRMTs, which form asymmetrically dimethylated arginine (aDMA), type II PRMTs, which form symmetrically dimethylated arginine (sDMA) and the much less common type III PRMTs which only form monomethylated arginine (MMA). Within these categories the members are differentiated by their protein substrate specificity and cellular localizations [1,2]. In common with the majority of small molecule and peptide methyltransferase the PRMTs employ the ubiquitous methyl donor *S*-adenosyl-L-methionine (AdoMet) as a cofactor. In all PRMTs the methylation of the target arginine residue is facilitated by the presence of two conserved glutamate residues that serve to lock the guanidine moiety in close proximity to the AdoMet cofactor's methyl group [3]. These features in PRMT enzymes result in a highly conserved active site configuration (Fig. 1A).

PRMTs are involved in a variety of cellular functions in both healthy and disease states. Cellular functions include the regulation of gene transcription, nuclear transport, DNA repair, protein-protein interactions and RNA processing [4,5]. Upon methylation of histone tails, gene transcription can be activated or repressed, depending on the arginine residue and the type of methylation (aDMA or sDMA) [6]. Notably, most PRMTs are implicated in one or more types of cancer and inhibition of PRMTs has been shown to inhibit cancer cell growth [7]. In addition, several studies have indicated the involvement of PRMTs in cardiovascular disease [8,9], pulmonary disease [10,11] and viral infection [12,13]. Therefore, the development of inhibitors against PRMTs has gained interest over the past decade as also evidenced by recently initiated clinical trials initiated with inhibitors against PRMT5 for the treatment of solid tumours and non-Hodgkin's lymphoma [14].

A major challenge in the development of PRMT inhibitors is how to achieve specificity for a given PRMT considering the highly conserved active site architecture shared among all members of this methyltransferase family. To this end, technologies that can provide new insights into PRMT-substrate interactions can be of great value. We here describe such an approach wherein known PRMT substrate peptides are converted into PRMT specific inhibitors.

2. Design and synthesis of PRMT transition state analogues

2.1. Design of the transition state analogues

In an attempt to obtain general PRMT inhibitors, we previously synthesized a series of small molecule bisubstrate compounds with a guanidine group attached to the adenosine unit with different linker lengths [15]. For these compounds, we observed surprising selectivity among the PRMTs tested. Building on those results, we hypothesized that enhanced specificity could be achieved by linking the adenosine

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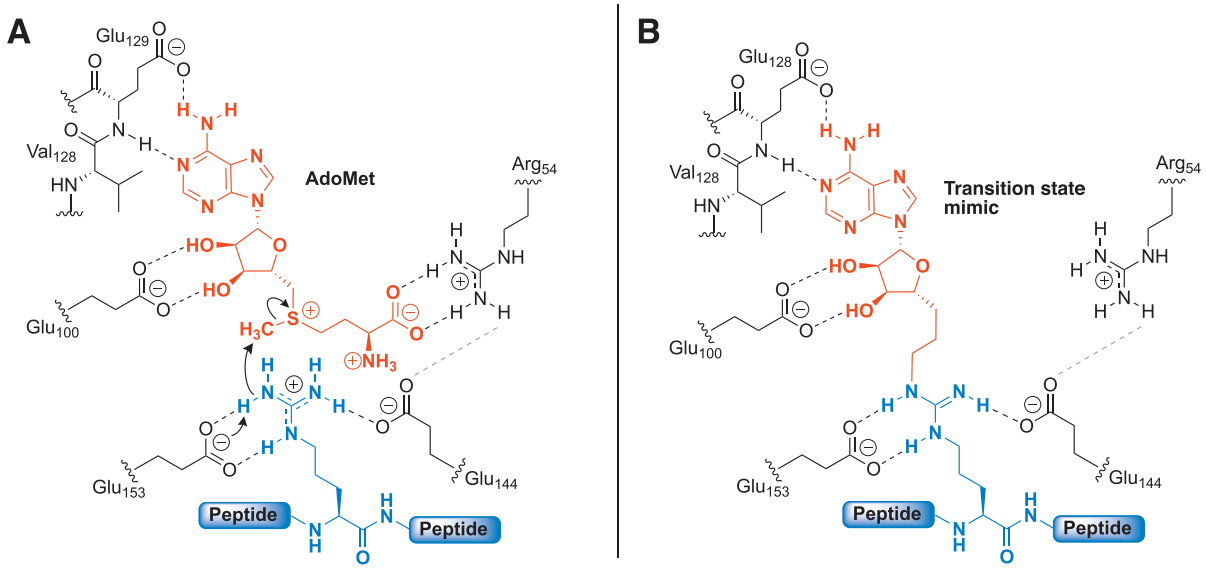


Fig. 1. A) Schematic representation of the conserved residues in the PRMT active site interacting with the AdoMet cofactor (in red) and the guanidine of the target arginine residue (in blue) (PRMT1 numbering). B) The design of the transition state mimics is based on the covalent linkage of the adenosine group (as shown in red) to the arginine sidechain in a peptide (as shown in blue). This approach leads to binding interactions with the conserved active site residues of both the AdoMet cofactor and peptide substrate binding pockets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unit to the target arginine residue of a given PRMT substrate peptide (Fig. 1B). The adenosine-peptide conjugates mimic the transition state of the first methylation step, making the approach applicable to all three types of PRMTs. In addition, by binding in the protein substrate binding pocket, more information could be gathered about the binding interactions of the PRMTs with their (specific) protein substrates.

This approach was initially validated on coactivator-associated arginine methyltransferase 1 (CARM1 also known as PRMT4) [16]. Transition state analogues were prepared based on a peptide sequence of its known substrate PABP1. Biochemical evaluation showed nanomolar inhibition against CARM1 with up to 300-fold selectivity over PRMT1. Subsequent co-crystallization experiments quickly led to high resolution crystal structures of CARM1 bound to the transition state analogue revealing the interactions in both the cofactor's and peptide binding sites. The fact that the crystals of the complex were readily obtained was attributed to the stabilizing effect the transition state mimics have on the enzyme by binding in the different substrate binding pockets simultaneously.

Following up on our promising initial results with transition state analogues designed for CARM1, we here describe the application of a similar approach for generating transition state mimics as inhibitors of PRMT1. PRMT1 is the most abundant PRMT and it is estimated that 85% of all methylated arginine residues in the proteome are methylated by PRMT1 [4,17]. PRMT1 preferentially methylates the RGG motif in target proteins and is found primarily in the cytoplasm. Alternative splicing variants show different activities, substrate specificity and cellular localizations [18]. PRMT1 substrates are diverse and include histones (H2AR3 and H4R3), splicing factors, DNA damage proteins, RNA-binding proteins, transcription factors, viral proteins and signalling proteins [2,7]. As a player in human disease, PRMT1 is overexpressed in different types of cancer [19] and is further involved in pulmonary disease [20] and cardiovascular disease [21,22]. A well-known substrate of PRMT1 is Arg3 on the histone H4 tail (H4R3), which is often used as a marker in studies concerning the role of PRMT1 [7,23]. The design of the compounds here described focuses on H4R3. As this residue is also a substrate of several other PRMTs (at least *in vitro*) we additionally included PRMT6 in the biochemical evaluation of the new H4R3 based transition state analogues. In contrast with PRMT1, PRMT6 is exclusively found in the nucleus [1]. Similar to PRMT1, it preferentially methylates the RGG motif and known substrates include histone tails (H2AR3, H2AR29, H3R2, H3R8, H3R42 and H4R3), chromatin proteins, DNA-binding proteins and viral proteins [2,24]. PRMT6 is overexpressed in bladder, lung and prostate cancer and associated with pulmonary disorders [7,11,25]. Interestingly, PRMT6 is reportedly downregulated in melanoma and reduces HIV-1 production and viral replication [26–28].

We here describe methodology for the synthesis of PRMT1 inhibiting transition state mimics through the covalent linkage of the adenosine moiety to PRMT1 target peptides. Using chemistry developed in our group [29], the adenosine moiety can be conveniently linked to the arginine side chain of any target peptide, making the methodology widely applicable to the entire family of PRMTs.

The design of the H4R3-based transition state analogues here described is based on the N-terminal 7 or 8 amino acids of the H4 tail peptide. Because the target arginine is close to the N-terminus, the N-terminal serine residue was evaluated as both the free amine as well as in its acetylated form to investigate the effect of the N-terminus on the inhibitory activity of the modified peptides. The sequences designed around H4R3 were selected on the basis of the kinetic data on methylation of the histone H4 tail by PRMT1 as reported by Thompson and coworkers [30]. The results of their study revealed that the best catalytic efficiency was achieved with a sequence of histone 4 covering the first 21 residues ($H4_{1–21}$). A slight reduction in turnover was found for two mid-sequence truncations of $H4_{1–21}$ where either residues 11–13 or 9–15 were eliminated. Conversely, sequences $H4_{1–13}$ and $H4_{1–15}$ showed a greatly reduced methylation rate. These findings suggest that binding of the H4 tail is driven by two contributing parts: one part that interacts at the methylation site (residues 1–8) and one part that binds at a more distal binding pocket containing several negatively charged residues. Without the positive residues ($H4_{16–21}$) to bind in this negatively charged region of the enzyme, 'linker residues' 9–15 seem to hinder more than attribute to the methylation process. Therefore, sequences $H4_{1–7}$ and $H4_{1–8}$ were selected with a C-terminal amide and both a free or acetylated N-terminus. The rationale for examining the $H4_{1–7}$ peptide in addition to the $H4_{1–8}$ peptide was to elucidate the contribution of the Lys8 residue in binding.

For installation of the adenosine unit a fully saturated three-carbon spacer was used to link to the arginine side-chain. This linker was found

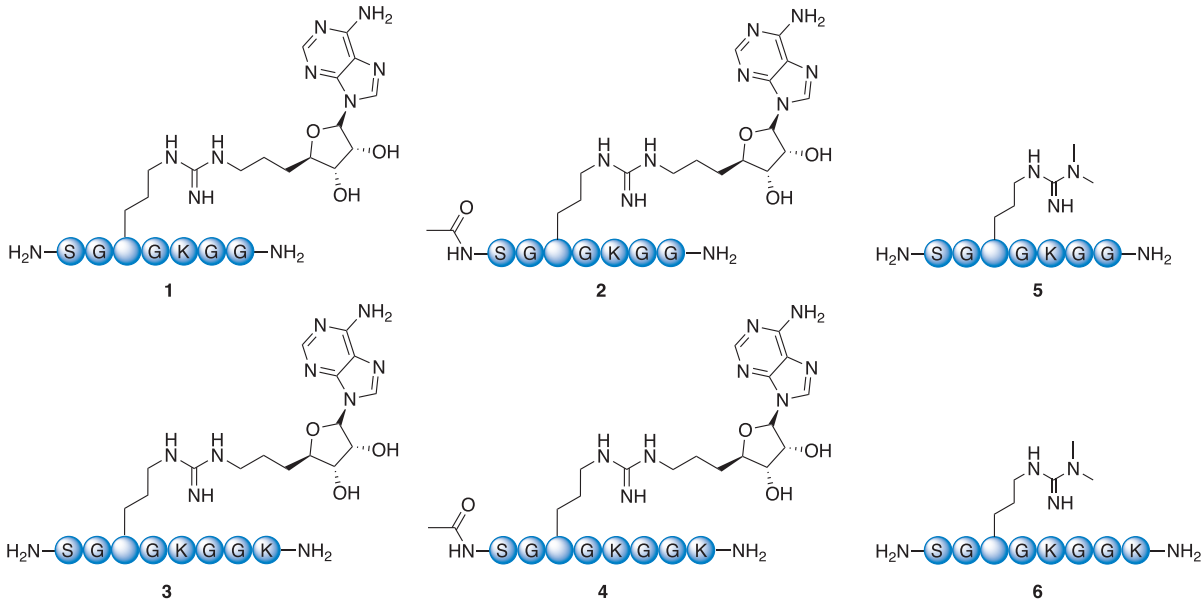


Fig. 2. Schematic representations of the structures of compounds **1–6**. Compounds **1**, **2** and **5** are based on residues 1–7 of the histone H4 tail and compounds **3**, **4** and **6** are based on residues 1–8 of the histone H4 tail.

to be most effective among the small molecule bisubstrate inhibitors tested against PRMT1 and PRMT6 in previous work [15]. As a control we also prepared the corresponding asymmetrically dimethylated arginine containing peptides of sequences $H4_{1-7}$ (**5**) and $H4_{1-8}$ (**6**). The structures of peptides **1–6** are presented in Fig. 2.

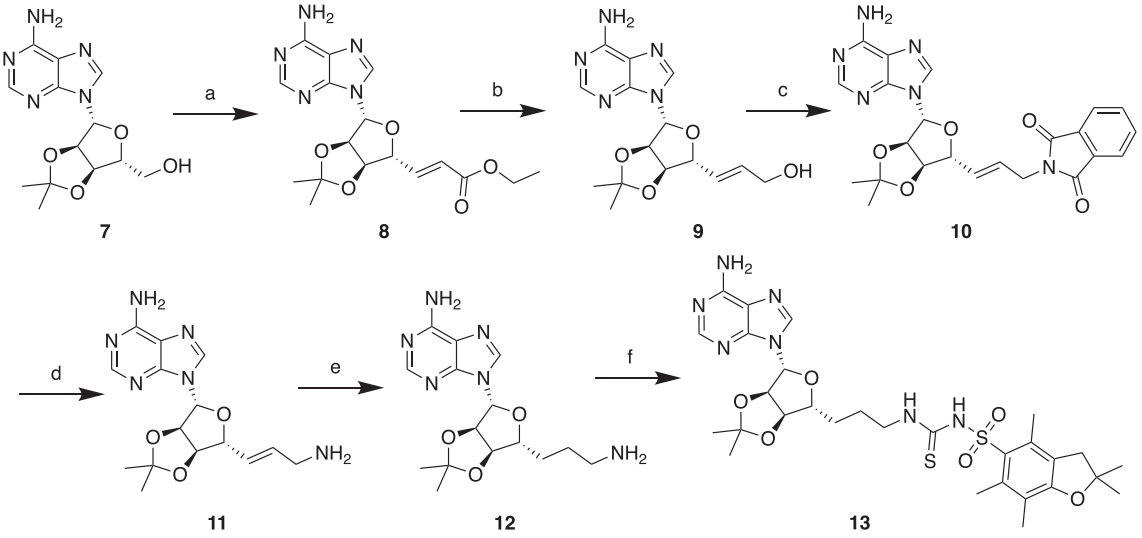
2.2. Synthesis of transition state analogues

2.2.1. Synthesis of the building block

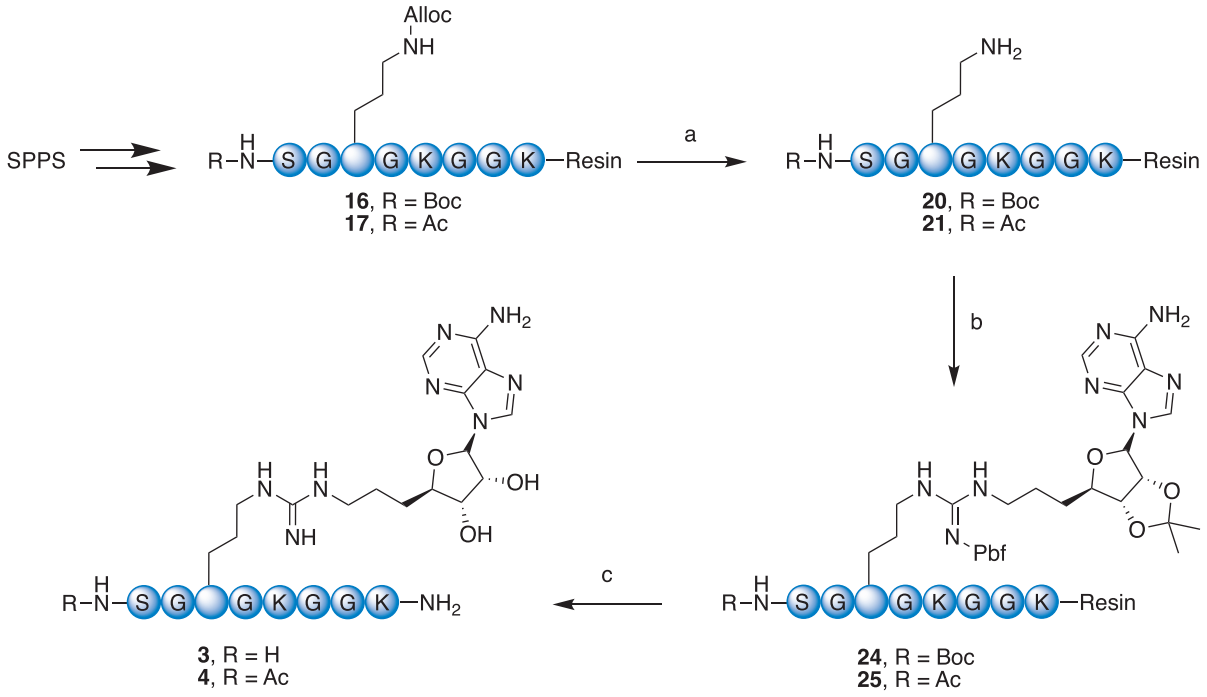
Installation of the adenosine moiety at the target arginine residue in the peptides prepared required access to a specific thiourea building block (compound **13**, Scheme 1), The preparation of this thiourea begins from commercially available 2,3-*O*-isopropylidine adenosine alcohol **7** as we previously reported [31]. Briefly, **7** is first transformed into unsaturated ethyl ester **8** in a one-pot oxidation and Wittig reaction. Subsequently, the ester is reduced to alcohol **9** using diisobutylaluminium hydride (DiBAL-H). The alcohol is converted to phthalimide-protected amine **11** via a Mitsunobu reaction with phthalimide and subsequent deprotection using methylamine resulting in amine **12**. The amine is then reacted with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl isothiocyanate (Pbf-NCS) [29] to form the Pbf-protected thiourea building block **13**.

2.2.2. Peptide synthesis

In preparing the peptides, the target arginine in the sequence was replaced by an Alloc-protected ornithine residue, which allows for orthogonal deprotection and modification of the required residue. As a representative example Scheme 2 illustrates the synthetic route used in preparing transition state mimics **3** and **4**. To begin, the Alloc-protected ornithine peptides were synthesized manually following standard Fmoc solid phase peptide synthesis (SPPS) protocols using Rink Amide tentagel S resin. The peptide couplings were performed in *N*,*N*-dimethylformamide (DMF) at ambient temperature for 1 h using standard Fmoc-protected amino acids with (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activator and di-isopropylethylamine (DiPEA) as base. Deprotection of the Fmoc



Scheme 1. Synthesis of Pbf-protected thiourea building block **13**. Reagents and conditions: (a) IBX, Ph_3P = $CHCO_2Et$, DMSO, 79%; (b) DIBAL-H, hexane, DCM, 78%; (c) phthalimide, PPh_3, diethyldiazocarboxylate, THF, 94%; (d) $MeNH_2$, EtOH, 94%; (e) 10% Pd/C, H_2 (g), EtOH, 98%; (f) Pbf-NCS, Et_3N, DCM, 83%.



Scheme 2. On-resin modification procedure for the synthesis of transition state analogues **1**–**4**, presented for H4 1–8 peptides **3** and **4**. The H4 1–7 peptides **1** and **2** were prepared following the same route. Reagents and conditions: a) $Pd(PPh_3)_4$, phenylsilane, DCM, Ar (g), dark, rt, 1 h; b) **13**, EDCI, DCM, N_2 (g), rt, 90 min; c) TFA/TIPS/H_2O (95:2.5:2.5), rt, 1 h.

protecting group was done with 20% piperidine in DMF. Each step was checked by means of a Kaiser test [32,33] to ensure completion of the reaction. After coupling and Fmoc deprotection of the last residue the resin was split and one half treated with di-*tert*-butyldicarbonate (Boc_2O) to generate resin bound intermediate **16** while the other half was acetylated with acetic anhydride to yield intermediate **17**. Resin bound **16** and **17** were subsequently converted to the corresponding adenosine-linked arginine peptides (see Section 2.2.3 below).

The asymmetrically dimethylated control peptides **5** and **6** were synthesized using a microwave-assisted peptide synthesizer using standard Fmoc-protected amino acids, *N,N′*-diisopropylcarbodiimide (DIC) as the activator and ethyl cyano(hydroxyimino)acetate (Oxyma) as base. Deprotection of the Fmoc protecting group was done with 20% piperidine in DMF at 90 °C. Only for the Fmoc-aDMA(Pbf)-OH building block different conditions were used as the amino acid did not appear to be compatible with the high temperatures used in the microwave assisted coupling. Therefore, the aDMA residue was coupled at room temperature using two equivalents of the amino acid and left to react overnight.

2.2.3. On-resin installation of the adenosine unit

An on-resin modification procedure was used to install the adenosine unit in the transition state mimic peptides prepared. To this end the Alloc-group in resin-bound peptides **16** and **17** was removed from the ornithine side-chain using tetrakis-(triphenylphosphine)-palladium ($Pd(PPh_3)_4$) as catalyst and phenylsilane as a scavenger to yield intermediates **20** and **21**. The reaction is performed in dichloromethane (DCM) under an argon atmosphere and protected from light. Pbf-protected thiourea **13** was subsequently coupled to the free amine of the ornithine side chain by activation with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) in DMF to yield fully protected and resin bound adenosine linked peptides **22** and **23**.

Protected peptides **24** and **25** were then deprotected and cleaved from the resin using a standard cleavage cocktail of trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O in a ratio of 95:2.5:2.5. Precipitation in methyl *tert*-butyl ether (MTBE)/hexane (1:1) yielded crude peptides **3** and **4**, which were purified by preparative HPLC. The purity (> 95%) and identity of the final compounds were confirmed by analytical HPLC and high-resolution mass spectrometry (HRMS).

3. Biochemical evaluation

Methyltransferase inhibition assays were performed using commercially available chemiluminescent assay kits for PRMT1 and PRMT6 (BPS Bioscience, San Diego, CA, USA). The inhibition reactions were performed in duplicate at room temperature for 1 h using 96-well plates precoated with histone $H4_{1–24}$ peptides as the substrate in a total volume of 50 µl containing proprietary assay buffer, 20 µM AdoMet, enzyme: PRMT1 (10 ng per reaction) and PRMT6 (200 ng per reaction) and inhibitors with concentration ranges of 0.0128–200 µM in water. Positive controls were performed by addition of pure water instead of inhibitor solution. Blank and substrate controls were conducted in the absence of enzyme and AdoMet, respectively. After incubation for 1 h at room temperature, the wells were washed and blocked, primary antibody was added to each well and incubated for an additional 1 h. After washing and blocking, a secondary HRP-labelled antibody was added and incubated for another 30 min. After a final washing and blocking step, the HRP-substrate mixture was added to the wells and the luminescence was measured immediately using a Tecan spark plate reader. All the measurements were performed in duplicate and the luminescence data analysed using GraphPad Prism 7.

Blank data was subtracted from the luminescence data and the results were subsequently normalized with the highest value in the concentration range defined as 100% inhibition. The percentage of inhibition activity was plotted as a function of inhibitor concentration and fit using non-linear regression analysis of the sigmoidal dose-response curve generated using the normalized data and a variable slope following equation:

$$Y = \frac{100}{(1 + 10^{((\log IC_{50} - X) \times \text{Hill Slope})})}$$

where Y = percent activity, X = the logarithmic concentration of the

Table 1
Inhibitory activity of compounds **1–6** against PRMT1 and PRMT6.

Compound	Sequence	IC_{50} values (μM)[a]	
		PRMT 1	PRMT 6
1	H_2N-SGR*GKGG-$CONH_2$	2.51 ± 0.24	4.82 ± 0.96
2	AcNH-SGR*GKGG-$CONH_2$	5.36 ± 0.52	10.98 ± 2.25
3	H_2N-SGR*GKGGK-$CONH_2$	4.66 ± 0.65	2.26 ± 0.72
4	AcNH-SGR*GKGGK-$CONH_2$	1.33 ± 0.25	1.22 ± 0.34
5[b]	H_2N-SGR(aDMA)GKGG-$CONH_2$	> 50	> 50
6[b]	H_2N-SGR(aDMA)GKGGK-$CONH_2$	> 50	> 50

[a] IC_{50} values from duplicate data obtained from 7 concentrations ± standard deviations. (see SI Appendix, IC_{50} curves). The R* indicates the position where the adenosine group is incorporated.

[b] In compounds **5** and **6**, the central arginine residue is present in asymmetrically dimethylated form.

compound, Hillslope = slope factor or Hill coefficient. The IC_{50} value was determined by the half maximal inhibitory concentration. The standard deviations were reported using the symmetrical CI function. Table 1 provides an overview of the IC_{50} values thus obtained for peptides **1–6** with the corresponding IC_{50} curves provided in the accompanying supporting information (see Fig. S1).

4. Results and discussion

The methodology here described provides a flexible and generally applicable approach for the preparation of transition state analogues of PRMTs based on peptidic fragments of their respective protein substrates. By using an orthogonally protected ornithine residue at the position of the target arginine, the peptides can be synthesized by SPPS and modified on the resin. Conveniently, given that the intermediate peptides are manipulated on resin, no intermediate purification steps are necessary and all reagents can simply be washed away. Also of note is the possibility of using this approach to specifically modify a single arginine reside when synthesizing peptides containing multiple arginines.

Key to this methodology is thiourea building block **13** that can be prepared in six steps with good to excellent yields, the details of which are presented in the supplementary information. For groups interested in applying this methodology the building block is also available on request. Also of note, if desired, variants of the thiourea building block can also be prepared with either a two-carbon spacer or an unsaturated three-carbon spacer, as we have previously described [31].

As reported in Table 1, compounds **1–4** display low micromolar inhibition against both PRMT1 and PRMT6. $H4_{1-7}$ analogues **1** and **2** show a 2-fold higher potency against PRMT1 over PRMT6. In addition, the free N-terminus in **1** results in slightly higher inhibition than the acetylated N-terminus in **2**. For $H4_{1-8}$ analogues **3** and **4** the findings are the opposite of those found for compounds **1** and **2**. Compound **3** is slightly more active against PRMT6 over PRMT1, but no significant difference is observed for compound **4** between PRMT1 and 6. Against both enzymes, the acetylated peptide **4** is more active than peptide **3** with a free N-terminus. Notably, no significant inhibition was observed for the asymmetrically dimethylated control peptides **5** and **6** when tested at 50 μM, indicating that the inhibitory activity observed for **1–4** is driven by the incorporation of the adenosine moiety. To gain additional insight into the binding of these transition state analogues to both PRMT1 and PRMT6, structural studies are now underway the results of which will be presented in due course.

As previously reported [31], a key advantage of the methodology here presented is that the transition state analogues can be used to facilitate co-crystallization of PRMTs with mimics of their protein substrates without the need for a cofactor analogue. Obtaining crystal structures of PRMTs bound to their peptidic substrate can give valuable insight into the interactions in the binding site of the peptide substrate, providing information that is crucial for the development of selective small molecule inhibitors. It it known that the co-crystallization of PRMTs with their substrates is often very challenging due to the necessity of including an analogue of the AdoMet cofactor. AdoMet itself cannot be used as this would result in the formation of the (di-)methylated product. Often AdoMet analogues *S*-adenosyl-L-homocysteine (AdoHcy), the product of the methylation reaction, or sinefungin are used to obtain structures of the ternary complex of enzyme, cofactor and substrate. However, the use of AdoHcy in co-crystallization studies of PRMTs is limited for obtaining mechanistical insight as it would only stabilize the PRMT in the conformation it adopts after the methyltransfer takes place. In addition, other AdoMet analogues introduce unnatural interactions into the active site, the effects of which can be difficult to interpret. Furthermore, the crystallization of a ternary complex is often more challenging than for a binary complex of substrate and enzyme. This is evidenced by the limited number of structures of PRMTs bound to their protein substrate. In fact, for only CARM1 and PRMT5 have high resolution crystal structures been reported that show the peptide substrate bound to the enzyme [31,34,35]. In the only structure published to date for PRMT1 bound to a substrate peptide only the target arginine residue was resolved in the active site while in similar studies with PRMT7 only the target arginine-glycine segment of a larger substrate peptide was visible [36,37].

In conclusion, the results of compounds **1–6** show that our peptide transition state analogue methodology is generally applicable throughout the family of PRMTs. Where previous studies focussed on PRMT4, we here demonstrate the applicability of the methodology in generating peptide based bisubstrate inhibitors for PRMT1 and PRMT6 as well. Future efforts will be directed to examining the applicability of peptides **1–4** in co-crystallization studies with PRMT1 and 6 as well as further application of the methodology towards other PRMTs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2019.08.003.

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