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# Labeling Substrates of Protein Arginine Methyltransferase with Engineered Enzymes and Matched *S*-Adenosyl-L-methionine Analogues

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Supporting Information

**ABSTRACT:** Elucidating physiological and pathogenic functions of protein methyltransferases (PMTs) relies on knowing their substrate profiles. *S*-adenosyl-L-methionine (SAM) is the sole methyl-donor cofactor of PMTs. Recently, SAM analogues have emerged as novel small-molecule tools to efficiently label PMT substrates. Here we reported the development of a clickable SAM analogue cofactor, 4-propargyloxy-but-2-enyl SAM, and its implementation to label substrates of human protein arginine methyltransferase 1 (PRMT1). In the system, the SAM analogue cofactor, coupled with matched PRMT1 mutants rather than native PRMT1, was shown to label PRMT1 substrates. The transferable 4-propargyloxy-but-2-enyl moiety of the SAM analogue further allowed corresponding modified substrates to be characterized through a subsequent click chemical ligation with an azido-based probe. The SAM analogue, in combination with a rational protein-engineering approach, thus shows potential to label and identify PMT targets in the context of a complex cellular mixture.

Protein arginine methyltransferases (PRMTs) catalyze arginine methylation of a broad range of substrates.[1,2] PRMTs use the cofactor *S*-adenosyl-L-methionine (SAM, **1**, Figure 1) as a methyl donor to modify arginine's guanidino nitrogen in three ways: monomethylation, asymmetric dimethylation (type I PRMTs), and symmetric dimethylation (type II PRMTs).[3] The biological consequences of arginine methylation have been implicated in multiple cellular events, such as signal transduction, transcriptional regulation, mRNA splicing, and protein translocation.[2,4−7] The dysregulation of protein arginine methyltransferases has also been linked to various diseases including cancer.[2] Among 11 known human PRMTs,[2] PRMT1 and PRMT6 are overexpressed in many clinical cancer tissues;[8] PRMT4 is upregulated significantly in breast tumors and hormone-dependent prostate tumors;[9,10] PRMT5 acts as a pro-survival factor by downregulating tumor suppressors ST7 and NM23 in fibroblast cells.[11] Although the importance of the physiological and pathogenic roles of PRMTs is well-known, how PRMTs recognize their substrates in a cellular context remains to be elucidated.[12,13]

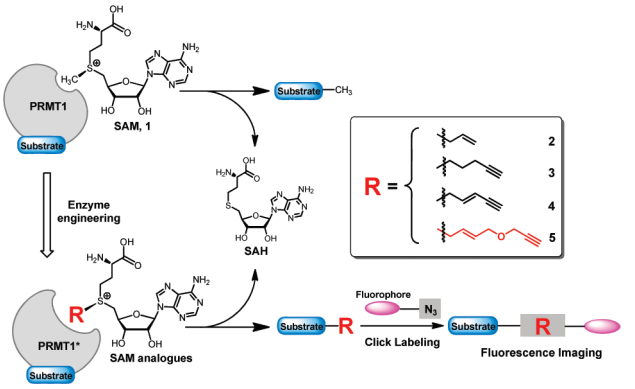


**Figure 1.** SAM analogues combined with a protein-engineering approach to label PRMT1 substrates. The SAM-binding pocket of PRMT1 will be rationally modified to utilize a clickable SAM analogue for substrate labeling. This modification can react with an azido-based probe for further characterization. PRMT1* = PRMT1 mutants.

To profile substrates of designated protein methyltransferases (PMTs), some prior approaches relied on recombinant enzymes and radiolabeled SAM with a PMT-knockout proteome or peptide array libraries as substrate candidates.[14−16] Recently, SAM analogues, particularly those carrying transferable chemical reporters (terminal alkynyl, keto, or amino groups), have emerged as novel small-molecule tools to examine methyltransferases and label their substrates.[17−25] For instance, several aziridinium-based SAM analogues have been applied to label the targets of DNA and protein methyltransferases.[20−23] Given the potential product inhibition of aziridinium-based SAM cofactors,[22] double-activated SAM analogues that contain alkenyl/alkynyl/keto-activated sulfonium carbons have been further developed.[17,19,25,26] Recent successes include using (*E*)-pent-2-en-4-ynyl (**4**, Figure 1), propargyl, and keto SAM analogues to label the substrates of MLL4, SETDB1, and catechol *O*-methyltransferase, respectively.[17−19] However, evidence also suggests that SAM analogues may only act as cofactors for certain methyltransferases.[19] For instance, propargyl SAM is a cofactor

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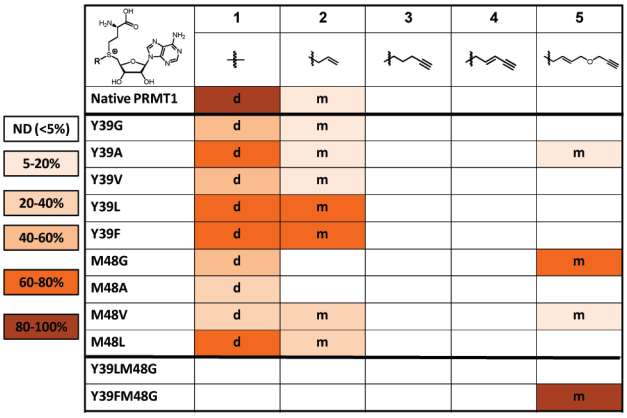


**Figure 2.** Enzymatic activity of PRMT1 and its mutants on RGG peptide substrate with SAM analogues as cofactors (SAM, **1** and SAM analogues **2**−**5**). Letters "m" and "d" refer to mono- and dimodifications. Color boxes code for percentages of modification under our assay conditions; "ND" means nondetectable with the current MS assay. (See Figure S1B, Supporting Information [SI] for the LC-ESI mass spectra.)

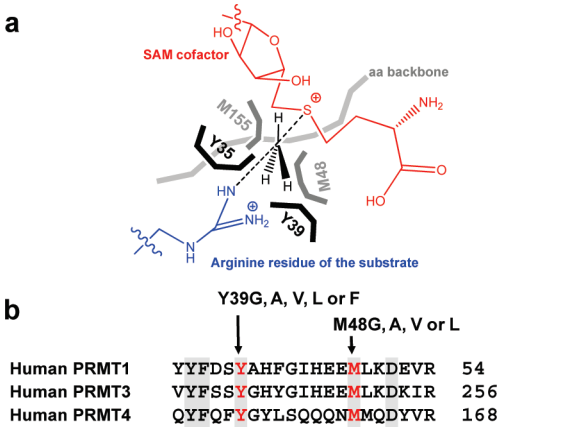


**Figure 3.** (a) Hypothetical transition-state structure of PRMT1-catalyzed methylation. The key contact residues were constructed according to the crystal structures of human PRMT1 (PDB file: 1OR8), human PRMT3 (PDB file: 2FYT) and mouse PRMT4 (PDB file: 3B3F). (b) Sequence alignment of human PRMT1, PRMT3, and PRMT4 with the conserved residues highlighted. Y39 and M48 of human PRMT1 (red) were engineered to adapt bulky SAM analogues.

of SETDB1 but not SET7/9, SMYD2, PRMT4, or PRMT1.[19] This observation thus triggered us to develop SAM analogues that, though may not be active for native PMTs, can be used by engineered PMTs for substrate labeling (Figure 1). This approach is expected to expand the capability of SAM analogues as small-molecule tools to label the targets of designated PMTs.

To identify such cofactors, a panel of SAM analogues was synthesized (Figure 1). These compounds include allyl SAM **2** and (*E*)-pent-2-en-4-ynyl SAM **4**, which were identified first as active cofactors of DNA adenine-N6 methyltransferase M.*Taq*I and human MLL4, respectively.[17,26] Using similar strategies,[20,26] 4-pentynyl SAM **3** and 4-propargyloxy-but-2-enyl SAM **5** (Pob-SAM) were also prepared (Figure 1 and SI). The latter two SAM analogues, as well as **4**, are featured by a terminal alkynyl group, which can serve as a clickable reporter of azido-based probes.[27−29] Among these synthetic SAM derivatives, **2** and **4** have a sulfonium-$\beta$ vinyl moiety, which favors $S_N2$-like transition states of methyltransferases,[17,26] and **3** contains an ethylene linker. Pob-SAM **5** mimics **2** except that it contains an additional methylene-glycol linker. Propargyl SAM was reported to be active for certain methyltransferases.[19,30] However, its rapid decomposition, as noticed by others and confirmed by us (data not shown),[17] prevented us from examining propargyl SAM further.

PRMT1 is the predominant type I human PRMT and accounts for 80% of total human PRMT activity.[31] Here human PRMT1 was chosen as a model enzyme to examine activities of the SAM analogues as cofactors. SAM, SAM derivatives **3** and **5**, and previously reported active cofactors **2** and **4**,[17,26] were first tested against native PRMT1. After incubating these compounds with PRMT1 and its RGG peptide substrate,[12,13,32] the products were analyzed by HPLC−MS to trace desirable modifications. Although native SAM and **2** can be utilized by native PRMT1, none of the clickable SAM analogues (**3**−**5**) showed detectable activity toward native PRMT1 and RGG substrate (Figures 2 and S1).

Given the undetectable activity of the clickable SAM analogues **3**−**5** on the RGG substrate with native PRMT1, the feasibility of the SAM analogues to be accommodated by engineered PRMT1 was then explored (Figure 3). Similar approaches have been applied to other enzymes, particularly kinases, for target labeling.[33] Analysis of SAM-binding pockets of PRMT1 and other PRMTs (Figure 3a) reveals several conserved residues (Y35, F36, Y39, M48, and D51 of PRMT1).[32] Since Y39 and M48 of PRMT1 are in close proximity to SAM's sulfonium methyl group, as a proof-of-principle approach, the two residues were replaced with less-sterically hindered amino acids (Y39G, A, V, L, F and M48G, A, V, L, Figure 3b), a strategy expected to expand PMT SAM-binding pockets.[34] After screening the mutants, native SAM was shown to be active toward the panel of PRMT1 single mutants. In contrast, analogue **2** and **5** were shown to be active toward Y39F, Y39L mutants and the M48G mutant, respectively (Figures 2 and S1). To further boost the selectivity of SAM analogues, two double mutants were generated by combining the most active single mutants (Figure 2). Although SAM and SAM analogues **2**−**4** are inert to the double mutants, Pob-SAM **5** exhibited excellent activity toward the PRMT1 Y39FM48G mutant (Figure 2 and S1).

To further examine the catalytic efficiency of the PRMT1 Y39FM48G mutant on Pob-SAM **5**, the apparent $k_{cat}$ and $K_{m,Pob\text{-}SAM}$ of the enzyme−cofactor pair were measured (SI and Figure S5). Although the $K_{m,Pob\text{-}SAM}$ of 63 $\mu$M for the PRMT1 mutant is 3-fold higher than $K_{m,\ SAM}$ (21 $\mu$M) of native PRMT1 and the $k_{cat}/K_{m,Pob\text{-}SAM}$ ($1.4 \times 10^3$ $M^{-1}$ $min^{-1}$) is around 20-fold lower than that of native PRMT1 and SAM ($2.6 \times 10^4$ $M^{-1}$ $min^{-1}$),[35] the engineered enzyme−cofactor pair has gained sufficient activities to label PRMT1 targets (see results below).

Given the distinct reactivity of Pob-SAM **5**, we further explored the mechanism that Pob-SAM **5**, rather than **3** or **4**, is favored as a cofactor by the PRMT1 Y39FM48G mutant. Our competition results showed that **3** and **4** inhibit the reaction of PRMT1 Y39FM48G mutant and Pob-SAM **5** with an $IC_{50}$ of 4−6 $\mu$M (see Figure S5 and SI). This observation suggests that **3** and **4** indeed bind to the PRMT1 mutant but fail to be processed further. Since Pob-SAM **5** differs from **4** only by a methylene-glycol linker, this moiety must render some steric or electronic effects to promote the chemical conversion of Pob-SAM **5** by the engineered PRMT1. Although follow-up studies are needed to further elucidate the origin of the distinct reactivity of PRMT1


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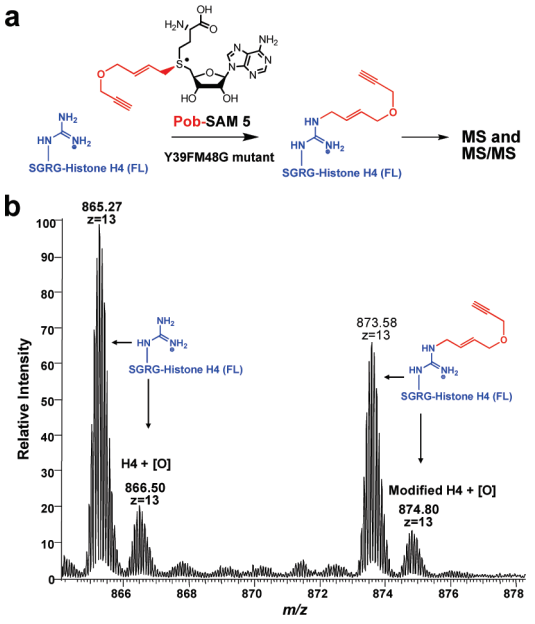


**Figure 4.** (a) Schematic representation of the reaction of PRMT1 Y39FM48G mutant, Pob-SAM cofactor **5**, and human histone H4 substrate. (b) ESI-MS spectrum of the modified H4.

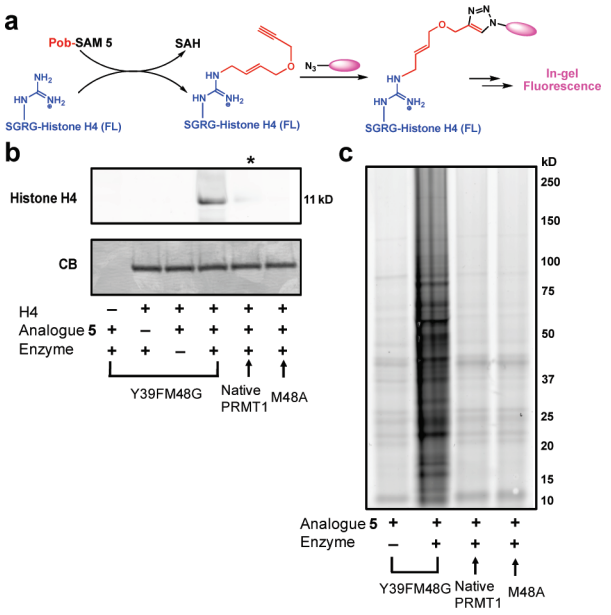


**Figure 5.** (a) H4 modification by PRMT1 Y39FM48G mutant and Pob-SAM **5**, followed by click ligation and in-gel fluorescence analysis. (b) In-gel fluorescence of 4-propargyloxy-but-2-enylated histone H4. Recombinant human histone H4 was modified as described (Figure 5a and SI). The strong in-gel fluorescence was detected for the sample treated with Y39FM48G mutant (lane 4), but barely detectable for native PRMT1 (lane 5) and undetectable for other controls (H4-negative, cofactor-negative, enzyme-negative, and the dead M48A mutant). Coomassie blue loading controls (CB) are shown in the bottom panel. *This weak labeling might be due to slightly residual activity of native PRMT1 on Pob-SAM **5** only when pure H4 was used as a substrate. No such labeling has been observed for the RGG peptide (Figure 2) or the hypomethylated cell lysate (lane 2 versus lane 3 in Figure 5c). (c) In-gel fluorescence of the hypomethylated HEK293T cell extraction treated with Pob-SAM **5** followed by an azido-rhodamine probe. See Figure S6 for CB control.

Y39FM48G mutant on Pob-SAM **5**, the successful identification of the matched cofactor−enzyme pair, as well as its robust activity, presents a suitable system for substrate labeling.

After confirming the activity of Pob-SAM **5** on PRMT1 RGG peptide substrate, its activity on other substrates was examined. Using the full-length histone H4 and histone octamer as substrates, Pob-SAM **5** was shown to be utilized efficiently by the PRMT1 Y39FM48G mutant with the desirable 4-propargyloxy-but-2-enylation delivered to histone H4 arginine 3 (H4R3), the reported H4 methylation site of native PRMT1,[12,13,35,36] but not H2A, H2B, H3, and other arginines on H4 (Figures 4 and S2, MS and MS/MS data). A competition assay was also performed by incubating H4 and the PRMT1 mutant with both Pob-SAM **5** and SAM. Consistent with the RGG substrate (Figures 2 and Figure S1), histone H4 was solely modified by Pob-SAM **5** but not SAM (4-propargyloxy-but-2-enylation versus no methylation, Figure S3). The lack of the activity of PRMT1 Y39FM48G mutant on native SAM was also confirmed by using the histone H4 (1−21) peptide as a substrate (Figure S4). In contrast, native PRMT1 and its Y39F, Y39L, M48G mutants can process native SAM and H4 to various extents (Figure S4). The bioorthogonal character of Pob-SAM **5** and PRMT1 Y39FM48G mutant thus makes the cofactor−enzyme pair suitable for substrate labeling even in the presence of native SAM.

Given the clickable feature of the terminal-alkyne of Pob-SAM **5**, we further examined whether the alkynyl functionality can be implemented for substrate characterization in combination with Cu(I)-catalyzed click chemical ligation. Full-length histone H4 was treated with Pob-SAM **5** in the presence of the PRMT1 Y39FM48G mutant as well as enzyme- and cofactor-negative controls. The products were then reacted with an azido-rhodamine fluorescent probe, followed by gel electrophoresis separation and in-gel fluorescence visualization. A specific fluorescent band was detected only for the H4 treated with Pob-SAM **5** and the PRMT1 Y39FM48G mutant (Figure 5b). In contrast, the control experiments in the absence of Pob-SAM **5** or the active PRMT1 mutant did not exhibit significant labeling (Figure 5b). To further prove that Pob-SAM **5** and the matched PRMT1 mutant are efficient to label potential PRMT1 targets in the context of complex cellular proteome, the hypomethylated lysate of HEK293T cells were used as protein substrates and treated with the mutant−cofactor pair. The resultant modified proteome was then subject to azido-rhodamine labeling. In comparison with the enzyme-negative control, multiple new protein bands can be visualized readily through in-gel fluorescence (lane 2 versus lane 1 in Figure 5c). The substrate-labeling activity of Pob-SAM **5** is highly specific for the PRMT1 Y39FM48G mutant because neither native PRMT1 nor a dead PRMT1 mutant exhibited such a labeling pattern. Although the newly labeled protein bands remain to be characterized by MS and validated through vigorous *in vitro* and *in vivo* assays using native PRMT1 and SAM, the current finding has demonstrated the potential to implement Pob-SAM in combination with engineered methyltransferases to identify PRMT1 targets.

Here Pob-SAM **5** was successfully identified as a clickable SAM analogue cofactor that can be utilized by engineered human PRMT1 but not by native PRMT1 for substrate labeling. It was reported previously that adenine-N6-derivatized SAM analogues can serve as bioorthogonal cofactors of engineered Rmt1 (a yeast PRMT). Since the adenine-derivatized SAM analogue cofactors


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still maintain the sulfonium methyl moiety of SAM, these SAM derivatives can only be used as methyl donors. The chemically inert methylation is less ready to be probed than clickable modifications, since the latter can be further coupled with Cu(I)-catalyzed chemical ligation for target identification (e.g., Pob-SAM **5**). A clickable aziridinium-based SAM analogue was also reported to modify PRMT1 substrates.[22] In that case, the cofactor−substrate adduct inhibits PRMT1 and thus prevents multiple turnover.[22] The present work successfully circumvents these limitations by developing Pob-SAM **5**, which carries a transferable, clickable functionality and meanwhile can be processed enzymatically. The combined features are useful to label and identify PRMT1 substrates from cellular proteome through the enzymatic installation of a clickable reporter, followed by a click ligation to suitable analytic tags. More importantly, accessing Pob-SAM **5** for engineered PRMT1 can be a starting point to engineer other structurally related PRMTs[1−3] to utilize the same cofactor. Another potential application of Pob-SAM **5** is to evolve bioorthogonal cofactor−enzyme pairs and apply them to dissect the substrates of designated PMTs, such as PRMT1, in the context of closely related PMTs.

Unveiling the substrates of PRMTs is of great importance in understanding their biological functions.[1−3] Here we developed a SAM analogue cofactor in combination with a rational protein-engineering approach to label PRMT1 substrates. The clickable feature of the cofactor further facilitates subsequent substrate characterization. In combination with Cu-catalyzed click chemistry and MS analysis, this system is expected to have the ability to identify PMT substrates from complex cellular mixtures. The new SAM analogue cofactor therefore expands our chemical tools to identify novel PMT targets.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Experimental procedures, synthetic methods, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ACKNOWLEDGMENT

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## ■ REFERENCES

(1) Bedford, M. T.; Richard, S. *Mol. Cell* **2005**, *18*, 263.
(2) Bedford, M. T.; Clarke, S. G. *Mol. Cell* **2009**, *33*, 1.
(3) Bedford, M. T. *J. Cell Sci.* **2007**, *120*, 4243.
(4) Aletta, J. M.; Cimato, T. R.; Ettinger, M. J. *Trends Biochem. Sci.* **1998**, *23*, 89.
(5) Xu, W.; Chen, H.; Du, K.; Asahara, H.; Tini, M.; Emerson, B. M.; Montminy, M.; Evans, R. M. *Science* **2001**, *294*, 2507.
(6) Gary, J. D.; Clarke, S. *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *61*, 65.
(7) Nichols, R. C.; Wang, X. W.; Tang, J.; Hamilton, B. J.; High, F. A.; Herschman, H. R.; Rigby, W. F. *Exp. Cell Res.* **2000**, *256*, 522.
(8) Yoshimatsu, M.; Toyokawa, G.; Hayami, S.; Unoki, M.; Tsunoda, T.; Field, H. I.; Kelly, J. D.; Neal, D. E.; Maehara, Y.; Ponder, B. A.; Nakamura, Y.; Hamamoto, R. *Int. J. Cancer* **2011**, *128*, 562.
(9) Kim, Y. R.; Lee, B. K.; Park, R. Y.; Nguyen, N. T.; Bae, J. A.; Kwon, D. D.; Jung, C. *BMC Cancer* **2010**, *10*, 197.
(10) Frietze, S.; Lupien, M.; Silver, P. A.; Brown, M. *Cancer Res.* **2008**, *68*, 301.
(11) Pal, S.; Vishwanath, S. N.; Erdjument-Bromage, H.; Tempst, P.; Sif, S. *Mol. Cell. Biol.* **2004**, *24*, 9630.
(12) Fronz, K.; Otto, S.; Kolbel, K.; Kuhn, U.; Friedrich, H.; Schierhorn, A.; Beck-Sickinger, A. G.; Ostareck-Lederer, A.; Wahle, E. *J. Biol. Chem.* **2008**, *283*, 20408.
(13) Wooderchak, W. L.; Zang, T. Z.; Zhou, Z. S.; Acuna, M.; Tahara, S. M.; Hevel, J. M. *Biochemistry* **2008**, *47*, 9456.
(14) Rathert, P.; Dhayalan, A.; Ma, H. M.; Jeltsch, A. *Mol. BioSyst.* **2008**, *4*, 1186.
(15) Rathert, P.; Zhang, X.; Freund, C.; Cheng, X. D.; Jeltsch, A. *Chem. Biol.* **2008**, *15*, 5.
(16) Rathert, P.; Dhayalan, A.; Murakami, M.; Zhang, X.; Tamas, R.; Jurkowska, R.; Komatsu, Y.; Shinkai, Y.; Cheng, X. D.; Jeltsch, A. *Nat. Chem. Biol.* **2008**, *4*, 344.
(17) Peters, W.; Willnow, S.; Duisken, M.; Kleine, H.; Macherey, T.; Duncan, K. E.; Litchfield, D. W.; Luscher, B.; Weinhold, E. *Angew. Chem., Int. Ed.* **2010**, *49*, 5170.
(18) Lee, B. W.; Sun, H. G.; Zang, T.; Kim, B. J.; Alfaro, J. F.; Zhou, Z. S. *J. Am. Chem. Soc.* **2010**, *132*, 3642.
(19) Binda, O.; Boyce, M.; Rush, J. S.; Palaniappan, K. K.; Bertozzi, C. R.; Gozani, O. *ChemBioChem* **2010**, 330.
(20) Dalhoff, C.; Lukinavicius, G.; Klimasauakas, S.; Weinhold, E. *Nat. Protoc.* **2006**, *1*, 1879.
(21) Lukinavicius, G.; Lapiene, V.; Stasevskij, Z.; Dalhoff, C.; Weinhold, E.; Klimasauskas, S. *J. Am. Chem. Soc.* **2007**, *129*, 2758.
(22) Osborne, T.; Roska, R. L.; Rajski, S. R.; Thompson, P. R. *J. Am. Chem. Soc.* **2008**, *130*, 4574.
(23) Pljevaljcic, G.; Pignot, M.; Weinhold, E. *J. Am. Chem. Soc.* **2003**, *125*, 3486.
(24) Weller, R. L.; Rajski, S. R. *ChemBioChem* **2006**, *7*, 243.
(25) Motorin, Y.; Burhenne, J.; Teimer, R.; Koynov, K.; Willnow, S.; Weinhold, E.; Helm, M. *Nucleic Acids Res.* **2010**, 1943.
(26) Dalhoff, C.; Lukinavicius, G.; Klimasauskas, S.; Weinhold, E. *Nat. Chem. Biol.* **2006**, *2*, 31.
(27) Grammel, M.; Zhang, M. Z. M.; Hang, H. C. *Angew. Chem., Int. Ed.* **2010**, *49*, 5970.
(28) Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13.
(29) Raghavan, A. S.; Hang, H. C. *Drug Discovery Today* **2009**, *14*, 178.
(30) Stecher, H.; Tengg, M.; Ueberbacher, B. J.; Remler, P.; Schwab, H.; Griengl, H.; Gruber-Khadjawi, M. *Angew. Chem., Int. Ed.* **2009**, *48*, 9546.
(31) Tang, J.; Frankel, A.; Cook, R. J.; Kim, S.; Paik, W. K.; Williams, K. R.; Clarke, S.; Herschman, H. R. *J. Biol. Chem.* **2000**, *275*, 7723.
(32) Zhang, X.; Cheng, X. *Structure* **2003**, *11*, 509.
(33) Bishop, A. C.; Buzko, O.; Shokat, K. M. *Trends Cell Biol.* **2001**, *11*, 167.
(34) Klimasauskas, S.; Weinhold, E. *Trends Biotechnol.* **2007**, *25*, 99.
(35) Obianyo, O.; Osborne, T. C.; Thompson, P. R. *Biochemistry* **2008**, *47*, 10420.
(36) Osborne, T. C.; Obianyo, O.; Zhang, X.; Cheng, X.; Thompson, P. R. *Biochemistry* **2007**, *46*, 13370.
(37) Lin, Q.; Jiang, F. Y.; Schultz, P. G.; Gray, N. S. *J. Am. Chem. Soc.* **2001**, *123*, 11608.