

# ReCYCLE SAM: Catalytic Alkylation Using an S-Adenosylmethionine Regeneration System

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**Abstract:** S-Adenosylmethionine-dependent methyltransferases are versatile tools for specific alkylation reactions of many compounds, such as pharmaceuticals, but their biocatalytic application is severely limited due to the lack of a cofactor regeneration system. Here we report a biomimetic, polyphosphate-based, cyclic cascade for methyltransferases. In addition to the substrate to be methylated, only methionine and polyphosphate have to be added in stoichiometric amounts. The system acts catalytically with respect to the cofactor precursor adenosine in methylation and ethylation reactions of selected substrates, as shown by analysis via HPLC. Further, <sup>1</sup>H and <sup>13</sup>C NMR measurements were performed to unequivocally identify methionine as the methyl group donor and to gain insight into the reactions' selectivity. This system constitutes a vital stage in the development of economical and environmentally friendly applications of methyltransferases.

S-Adenosylmethionine (SAM, AdoMet) is one of the most abundant cofactors in all domains of life.<sup>[1–3]</sup> The “SAM-Empire”<sup>[4]</sup> encompasses the biosynthesis of natural products and complex cofactors, the modification of nucleotides, and drug metabolism, as well as epigenetics and cancer metabolism. Numerous methylations of nucleophilic residues are SAM-driven; moreover, SAM is a donor for radicals, ribosyl moieties, and building blocks derived from the methionine carbon chain. The natural methyl cycle is part of a complex network of different regeneration and salvage pathways. It is also connected to adenosine-5'-triphosphate (ATP) metabolism, as the biosynthesis of SAM is strictly ATP-dependent.<sup>[1,2,5,6]</sup> Given that there is a broad range of selectively methylated pharmaceuticals,<sup>[7]</sup> it is clear that SAM-dependent selective alkylation reactions are highly promising tools for environmentally friendly syntheses of bioactive compounds.<sup>[5]</sup> The impact of methylation on drug molecules has been described as the “magic methyl effect”; in some cases, a boost of potency of up to three orders of magnitude has been observed upon the introduction of a single methyl group.<sup>[7]</sup> Standard methylating agents such as methyl iodide are intrinsically toxic and carcinogenic; consequently, there is a great

demand for selective and environmentally friendly alkylation methods.<sup>[7]</sup> A biocatalytic approach using nature's methylating agent, SAM, could fulfil these needs. In addition to methylation, alkylations using SAM derivatives that are accessible from methionine derivatives would extend the potential of these enzymes as biocatalysts.<sup>[8–13]</sup> The instability of SAM,<sup>[14–16]</sup> the inhibitory nature of its inevitable byproduct S-adenosyl-homocysteine (SAH, AdoHcy),<sup>[17]</sup> and the need for stoichiometric amounts of SAM have so far prohibited the development of up-scaled processes. This highlights the demand for a cofactor regeneration system for SAM-dependent enzymes.<sup>[3,5]</sup> In contrast to other group-delivering cofactors such as nicotinamide adenine dinucleotide (NAD<sup>+</sup>/NADH), the SAM regeneration pathway is not a one-step process that basically reverses the forward reaction. Rather, it is a multistep cyclic pathway. Such pathways are abundant in nature, prominent examples being the Krebs and Calvin cycles. When compared to linear pathways, cyclic pathways offer the advantage of being more efficient in terms of usage of resources and avoidance of byproduct accumulation, as well as having multiple control points and options for variation.<sup>[18]</sup> We, and others, have taken a step towards a SAM regeneration system by integrating the alkylation reaction into a linear three-enzyme cascade (Figure S1).<sup>[12,13,19]</sup> The in situ formation of SAM from ATP and L-methionine is catalyzed by methionine adenosyltransferases (MAT, E.C. 2.5.1.6). SAH is removed by enzymatic cleavage to adenine and S-ribosyl-L-homocysteine catalyzed by methylthioadenosine/SAH nucleosidase (MTAN, E.C. 3.2.2.9). Despite their usefulness for small-scale reactions, these systems are ultimately not genuine cofactor regeneration systems. Rather, they are cofactor supply cascades for which, in addition to methionine, ATP is required stoichiometrically, and S-ribosyl-L-homocysteine and adenine accumulate as byproducts.

To overcome these limitations and to exploit the advantages of a cyclic pathway, we sought to establish a biocatalytic system where no cofactor has to be added in stoichiometric quantity. Potentially, this could be achieved using a biomimetic SAM cycle. In nature, the building blocks for a new SAM molecule, ATP and L-methionine, are regenerated in separate pathways from the SAH cleavage products adenosine and L-homocysteine, respectively. As methionine is produced in large amounts as feedstock,<sup>[20]</sup> the stoichiometric addition of methionine is economically viable. The phosphorylation of adenosine to provide ATP is carried out by the cell's primary metabolism, and can be catalyzed by a broad range of enzymes; however, not all of these enzymes are optimal for in vitro applications as they are membrane-bound or require complex and expensive cofactors.<sup>[21]</sup> Although many ATP regeneration systems starting from ADP have been described,<sup>[21]</sup> only a few in vitro systems starting from adenosine or AMP have been developed. Of these, the family-2 polyphosphate kinases (PPK2, E.C. 2.7.4.1)<sup>[22,23]</sup> are particularly

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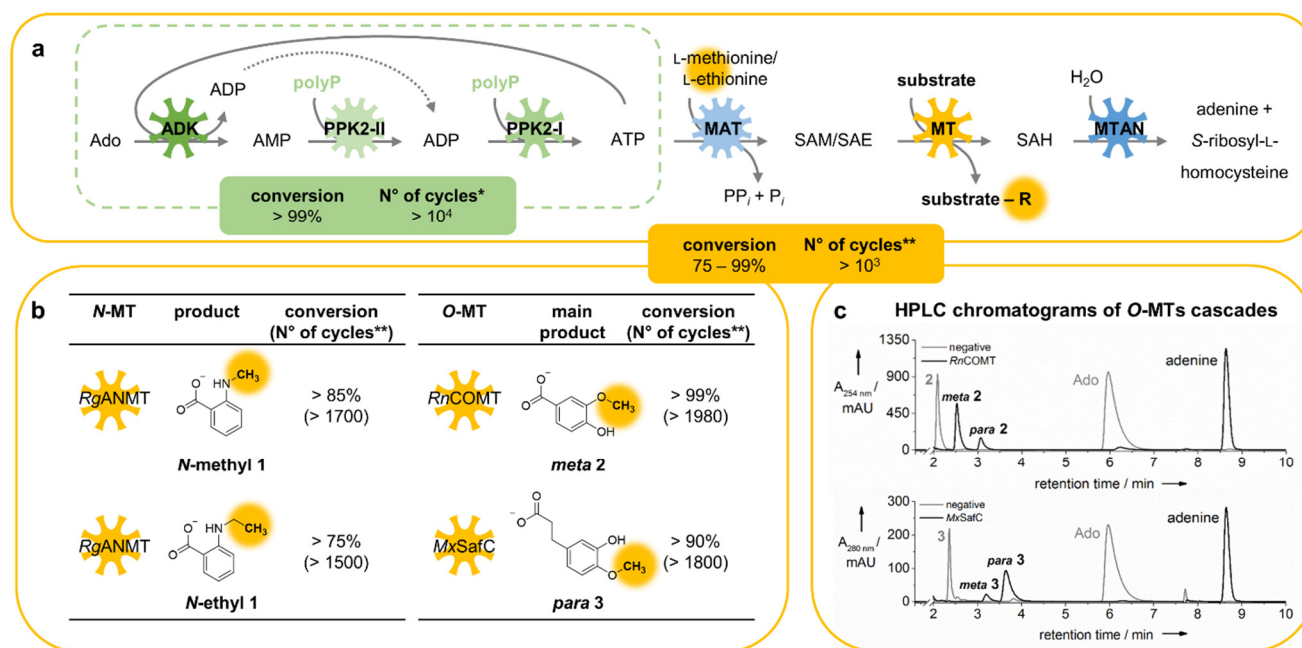
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suitable: the phosphate donor, polyphosphate (polyP), is available in large amounts and is sufficiently stable in aqueous solution.<sup>[24]</sup> Further, depending on the PPK2 enzyme, both AMP and ADP can be phosphorylated. Although the initial phosphorylation of adenosine is exclusively carried out by adenosine kinase (ADK, E.C. 2.7.1.20), the ATP used as phosphate donor can be regenerated using PPK2 and polyP. The enzymes from *Acinetobacter johnsonii* (PPK2-II, A<sub>J</sub>PPK)<sup>[23]</sup> and *Sinorhizobium meliloti* (PPK2-I, S<sub>m</sub>PPK)<sup>[22]</sup> were used for AMP and ADP phosphorylation, respectively (Figure 1a, Figure S4). When adenosine was incubated with polyP, ADK from *Saccharomyces cerevisiae*, PPK2-II and PPK2-I, a catalytic amount of AMP was added to start ATP production. The nucleoside was completely converted into a mixture of nucleotides (AMP/ADP/ATP) in a 1:1:2.6 ratio (Figure S4). Moreover, full conversion of adenosine was reached starting from an AMP/adenosine molar ratio as low as 1:10000. These results highlight the potential of PPK2s for use in large-scale ATP regeneration systems and are an attractive alternative to conventional ATP regeneration systems such as acetyl phosphate/ acetate kinase or phosphoenolpyruvate/ pyruvate kinase.<sup>[21,24]</sup>

Having established the adenosine to ATP regeneration cascade, it was then linked to the three-enzyme methylation cascade developed in our laboratory (Figure 1).<sup>[19]</sup> The modular construction of the system allows the use of different enzymes

tailored to a particular application. To evaluate the broad applicability, two regiocomplementary Mg<sup>2+</sup>-dependent *O*-methyltransferases (*O*-MTs) and one *N*-methyltransferase (*N*-MT) were chosen as model enzymes. While the *O*-MTs RnCOMT (catechol-*O*-MT from *Rattus norvegicus*)<sup>[25]</sup> and MxSafC (from *Myxococcus xanthus*)<sup>[26]</sup> were each used for the methylation of different catechol substrates, the *N*-MT RgANMT (anthranilate *N*-MT from *Ruta graveolens* L.)<sup>[27]</sup> was tested with anthranilic acid (**1**) using both L-methionine and L-ethionine as alkyl donor substrates (Figure S2). MAT from *Escherichia coli* was employed for methylation reactions, whereas MAT from the archaeon *Thermococcus kodakarensis* was used for ethylation, as archaeal MATs have favorable kinetic parameters for ethionine.<sup>[28,29]</sup> Overall, the setup provides an extended SAM supply cascade using adenosine as a less expensive and more stable SAM precursor than ATP. The extended supply cascade allowed us i) to adapt buffer conditions for all enzymes and to test the enzymes under cascade conditions, before making the link to the complete cycle, and ii) to test the ATP regeneration linked to the SAM production step. In the first instance, an HPLC assay was used to monitor and quantify the conversion of substrate into methylated product. The MT-catalyzed reaction resulted in conversions of 75–100%; this corresponds to more than 1500 regeneration cycles for ATP in all cases [total turnover number (TTN) calculated as the amount of MT product produced divided by the catalytic amount of AMP; Figure 1, Table S3].



**Figure 1.** Linear SAM supply cascade starting from adenosine. (a) Cascade setup consisting of ATP production from adenosine using polyP as sole phosphate donor (green dashed box) and SAM supply/methylation/SAH degradation cascade (see also Figure S1). Enzymes: ADK – adenosine kinase from *Saccharomyces cerevisiae*, PPK2-II – polyphosphate kinase from *Acinetobacter johnsonii*, PPK2-I – polyphosphate kinase from *Sinorhizobium meliloti*, MAT – methionine adenosyltransferase from *Escherichia coli* (or *Thermococcus kodakarensis*), MT – selected methyltransferases (for details see main text), MTAN – methylthioadenosine/S-adenosylhomocysteine nucleosidase from *E. coli*. (b) Results from HPLC assays for selected MTs and substrates (see also Figures S5–S7 and Table S3). (c) Typical HPLC chromatograms. SAM: R = methyl; SAE: R = ethyl; Ado: adenosine. \*TTN calculated as the amount of product (AMP, ADP, ATP) divided by the amount of AMP used to start the reaction. \*\*TTN calculated as the amount of MT product produced divided by the catalytic amount of AMP. All reactions were prepared and analyzed in triplicates.

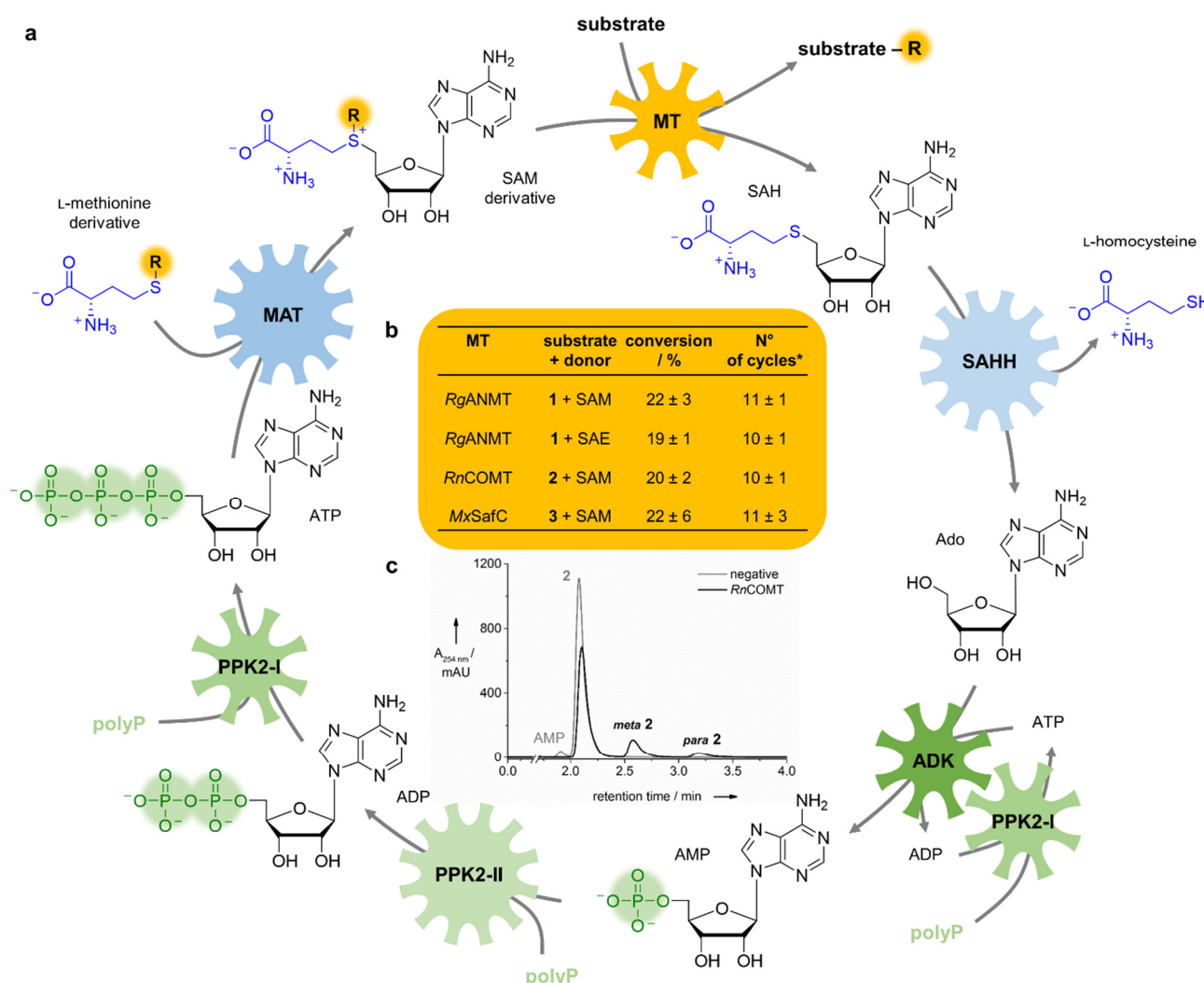
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In order to verify that the transferred methyl group truly originates from L-methionine, additional assays were performed with  $^{13}\text{C}$ -labeled methionine ( $^{13}\text{CH}_3$ -L-methionine) and analyzed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy. Anthranilic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), and dihydrocaffeic acid (**3**) were used as control substrates for *RgANMT*, *RnCOMT*, and *MxSafC*, respectively. The  $^{13}\text{C}$  NMR signal(s) detected in the assay samples corresponded to the methyl group signals of authentic reference compounds. Further, the NMR experiments (Figures S8–S10, Table S3) also confirmed the regioselectivity of the *O*-MTs as initially determined by HPLC.

In the linear cascade, at least four out of the six reactions (ADK, MAT, MT, MTAN) are irreversible. The presence of multiple irreversible steps, caused either by the enzyme's mechanism or by effectively removing byproducts, is even more important in a cyclic system, as these drive the cycle in the desired direction and maintain it. After the successful implementation of the linear cascade, we hypothesized that swapping MTAN for SAH

hydrolase (SAHH, E.C. 3.3.1.1) would turn the supply cascade into a true regeneration cycle (Figure 2).

SAHH produces adenosine from SAH and so provides the starting material for a new cycle. SAHH is not frequently used in *in vitro* cascades as it is relatively unstable and has only moderate activity. Additionally, the fact that the reaction's equilibrium lies on the SAH side makes it unsuitable for use in a linear setup as it would be unable to drive the reaction to completion. However, in the cyclic cascade envisioned in Figure 2 this should be irrelevant, as ADK would subsequently irreversibly convert adenosine into AMP. We considered SAHs from several organisms, with respect to stability and activity. SAHH from *Mus musculus*<sup>[30]</sup> had the most favorable overall properties and was therefore used in the cascade. Ultimately, the cyclic regeneration cascade contained six enzymes (MAT, MT, SAHH, ADK, PPK2-II, PPK2-I), stoichiometric amounts of L-methionine and MT substrate, and an excess of polyP. Finally, a catalytic amount of AMP (1:50 relative to the MT substrate) was added as cofactor, and the reactions were monitored by HPLC.



**Figure 2.** SAM regeneration cycle. (a) The biocatalytic alkylation with cofactor regeneration is powered by polyP and uses L-methionine (or a derivative) as alkyl donor. Methionine (or ethionine), polyP, and the corresponding MT substrate were added in a stoichiometric amount, along with a catalytic amount of AMP as cofactor precursor. (b) Results from HPLC assays for selected MTs. (c) Typical HPLC chromatogram. See also Figures S11 and S12, and Table S4. For enzyme abbreviations, see Figure 1 and main text; SAHH – SAH hydrolase from *Mus musculus*. \*TTN calculated as the amount of MT product produced divided by the amount of cofactor building block. All reactions were prepared and analyzed in triplicates; the values given refer to the arithmetic mean ± the standard deviation.



Gratifyingly, the reactions produced significantly more methylated product than the initial amount of cofactor precursor. This clearly indicates that the cycle is operating catalytically with regard to ATP. However, the linear cascade, which uses MTAN instead of SAHH, provided significantly higher turnover numbers than the cyclic cascade. Presumably, the bottleneck in the system is the SAH degradation step, as SAHH is the only enzyme that could not be evaluated in the extended supply cascade. It is notable that also adenine accumulated in the cyclic reaction. As it cannot be regenerated to ATP with the current setup, adenine effectively forms a dead end. The adenine detected could occur for multiple reasons, among them acid-catalyzed depurination of ATP or other derivatives during sample workup, the nonenzymatic degradation of either SAM or SAH or a side activity of SAHH or another enzyme common to all assays. Incubation of SAM as well as SAH under assay conditions resulted in the described SAM decay products SAH and adenine,<sup>[14,15,31]</sup> while SAH seemed to be stable for 24 h. Incubation with *Mm*SAHH shows incomplete degradation to adenine (compared to the reaction with *Ec*MTAN), which points towards a side activity of *Mm*SAHH (Figure S13). The detailed analysis of the cascades' kinetics is carried out in ongoing experiments, this includes potential improvements to the cyclic cascade, e.g., the use of engineered SAHH variants with improved stability, activity and selectivity towards the desired reaction, and the removal of homocysteine from the system, e.g., by remethylation to methionine. In addition, the utilization of SAM derivatives that are less prone to depurination<sup>[32]</sup> are a promising alternative.

Nonetheless, even with the wild-type enzymes and substrates, conversions of up to 25% were achieved (Figure 2). This corresponds to more than a 10-fold regeneration of SAM and was observed for all tested methylation and ethylation reactions.

In summary, we have designed and developed a genuine in vitro SAM regeneration system. The cyclic cascade represents a breakthrough towards a generally applicable cofactor regeneration system for SAM-dependent alkylation reactions. It provides an ideal starting point for further optimization, with improved efficiency of the SAH degradation step being a possible first step. Consistent results were obtained for all model enzymes, highlighting the fact that this platform could be used for C-, O-, N-, and S-MTs, namely all SAM-dependent MTs producing SAH as byproduct. This brings the use of MTs as biocatalysts for regioselective, and asymmetric methylation<sup>[33]</sup> a step closer to application. The system offers many advantages typical for in vitro biocatalytic systems using isolated enzymes, for example usefulness for the production of fine chemicals, where a precise control of the reaction is necessary, or the possibility to introduce selective isotopic labels. An added bonus is that many SAM-dependent MTs can also use a variety of methionine analogues.<sup>[8,9]</sup> Our results with ethionine show that it should be feasible to develop an in vitro platform for more general alkylation reactions, this will be followed-up in further studies.

## Experimental Section

Experimental Details are given in the Supporting Information.

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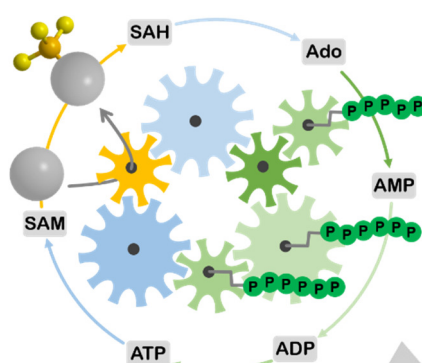
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- [1] M. Fontecave, M. Atta, E. Mulliez, *Trends Biochem. Sci.* **2004**, *29*, 243–249.
- [2] W. A. Loenen, *Biochem. Soc. Trans.* **2006**, *34*, 330–333.
- [3] M. Richter, *Nat. Prod. Rep.* **2013**, *30*, 1324–1345.
- [4] G. L. Cantoni, in *Biochem. -Adenosylmethionine Relat. Compd.*, Palgrave Macmillan UK, **1982**, pp. 3–10.
- [5] A.-W. Struck, M. L. Thompson, L. S. Wong, J. Micklefield, *ChemBioChem.* **2012**, *13*, 2642–2655.
- [6] J. B. Broderick, B. R. Duffus, K. S. Duschene, E. M. Shepard, *Chem. Rev.* **2014**, *114*, 4229–4317.
- [7] H. Schönherr, T. Cernak, *Angew. Chem. Int. Ed.* **2013**, *52*, 12256–12267; *Angew. Chem.* **2013**, *125*, 12480–12492.
- [8] J. Zhang, Y. G. Zheng, *ACS Chem. Biol.* **2016**, *11*, 583–597.
- [9] T. D. Huber, B. R. Johnson, J. Zhang, J. S. Thorson, *Curr. Opin. Biotechnol.* **2016**, *42*, 189–197.
- [10] H. Stecher, M. Teng, B. J. Ueberbacher, P. Remler, H. Schwab, H. Griengl, M. Gruber-Khadjawi, *Angew. Chem. Int. Ed.* **2009**, *48*, 9546–9548; *Angew. Chem.* **2009**, *121*, 9710–9712.
- [11] S. Singh, J. Zhang, T. D. Huber, M. Sunkara, K. Hurley, R. D. Goff, G. Wang, W. Zhang, C. Liu, J. Rohr, S. G. Van Lanen, A. J. Morris, J. S. Thorson, *Angew. Chem. Int. Ed.* **2014**, *53*, 3965–3969; *Angew. Chem.* **2014**, *126*, 4046–4050.
- [12] B. J. C. Law, A.-W. Struck, M. R. Bennett, B. Wilkinson, J. Micklefield, *Chem. Sci.* **2015**, *6*, 2885–2892.
- [13] F. Muttach, A. Rentmeister, *Angew. Chem. Int. Ed.* **2016**, *55*, 1917–1920; *Angew. Chem.* **2016**, *128*, 1951–1954.
- [14] J. L. Hoffman, *Biochemistry* **1986**, *25*, 4444–4449.
- [15] L. W. Parks, F. Schlenk, *J. Biol. Chem.* **1958**, *230*, 295–305.
- [16] J. K. Coward, E. P. Slisz, *J. Med. Chem.* **1973**, *16*, 460–463.
- [17] H. K. Chenault, E. S. Simon, G. M. Whitesides, *Biotechnol. Genet. Eng. Rev.* **1988**, *6*, 221–270.
- [18] J. E. Baldwin, H. Krebs, *Nature* **1981**, *291*, 381–382.
- [19] J. Siegrist, S. Aschwanden, S. Mordhorst, L. Thöny-Meyer, M. Richter, J. N. Andexer, *ChemBioChem.* **2015**, *16*, 2576–2579.
- [20] T. Wilke, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 9893–9914.
- [21] J. N. Andexer, M. Richter, *ChemBioChem.* **2015**, *16*, 380–386.
- [22] B. Nocek, S. Kochinyan, M. Proudfoot, G. Brown, E. Evdokimova, J. Osipiuk, A. M. Edwards, A. Savchenko, A. Joachimiak, A. F. Yakunin, *Proc. Nat. Acad. Sci. U.S.A.* **2008**, *105*, 17730–17735.
- [23] C. F. Bonting, G. J. Kortstee, A. J. Zehnder, *J. Bacteriol.* **1991**, *173*, 6484–6488.
- [24] L. Butler, *Biotechnol. Bioeng.* **1977**, *19*, 591–593.
- [25] J. Vidgren, L. A. Svensson, A. Liljas, *Nature* **1994**, *368*, 354–358.
- [26] J. T. Nelson, J. Lee, J. W. Sims, E. W. Schmidt, *Appl. Env. Microbiol.* **2007**, *73*, 3575–3580.
- [27] B. Rohde, J. Hans, S. Martens, A. Baumert, P. Hunziker, U. Matern, *Plant J.* **2008**, *53*, 541–553.
- [28] Z. J. Lu, G. D. Markham, *J. Biol. Chem.* **2002**, *277*, 16624–16631.
- [29] F. Wang, S. Singh, J. Zhang, T. D. Huber, K. E. Helmich, M. Sunkara, K. A. Hurley, R. D. Goff, C. A. Bingman, A. J. Morris, J. S. Thorson, G. N. Phillips, *FEBS J.* **2014**, *281*, 4224–4239.
- [30] A. Ichikawa, S. Sato, K. Tomita, *J. Biochem.* **1985**, *97*, 189–197.
- [31] L. Laurino, D. S. Tawfik, *Angew. Chem. Int. Ed.* **2017**, *56*, 343–345; *Angew. Chem.* **2017**, *129*, 349–351.
- [32] T. D. Huber, F. Wang, S. Singh, B. R. Johnson, J. Zhang, M. Sunkara, S. G. Van Lanen, A. J. Morris, G. N. Phillips, J. S. Thorson, *ACS Chem. Biol.* **2016**, *11*, 2484–2491.
- [33] C. Sommer-Kamann, A. Fries, S. Mordhorst, J. N. Andexer, M. Müller, *Angew. Chem. Int. Ed.* **2017**, *56*, DOI: 10.1002/anie.201609375R1.

## COMMUNICATION

**SAM-go-round:** Biocatalytic alkylation is an emerging field in biotechnology. In addition to the discovery and engineering of highly efficient and selective methyltransferases, the development of an efficient regeneration system for the cofactor S-adenosylmethionine is a prerequisite for their economic application. Here we present a first draft of an in vitro biomimetic SAM regeneration cycle using different methyltransferases as a model system.



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