***S*-adenosylhomocysteine as a methyl transfer catalyst in biocatalytic methylation reactions**

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**Abstract.** *S*-adenosylmethionine (SAM)-dependent methyltransferases constitute a large family of enzymes that can catalyze regio-, chemo- and stereospecific methylation of complex natural products. These enzymes could be very useful tools for chemoenzymatic production and diversification natural or artificial compounds. Despite this potential, *in vitro* applications of methyltransferases are limited by their requirement for SAM as a stoichiometric methyl donor. The chemical complexity, the instability, the high cost and the poor atom economy of this reagent prevent preparative *in vitro* methylation reactions from becoming routine protocols in natural product research and viable options for process development. In this report we demonstrate that *C*-, *N*- and *O*-specific methyltransferases can be combined with halide methyltransferases to form enzyme cascades that require only catalytic concentrations of SAM and use methyl iodide as the stoichiometric methyl donor.

Biocatalysis has emerged as a gateway technology for green industry innovations in the production of food, pharmaceuticals and materials.[1](#_ENREF_1) Because of their ability to catalyze difficult reactions under benign conditions, enzymes such as hydrolases, aldolases, transaminases or oxygenases have been recruited successfully to many commercial processes. As the discovery of novel enzyme classes progresses, the biocatalytic toolbox is becoming increasingly comprehensive, suggesting that enzyme-based total synthesis of complex artificial molecules may become a viable alternative to traditional organic synthesis.[1-4](#_ENREF_1)

*S*-Adenosyl methionine (**1**) dependent methyltransferases (SAM-dependent MTs) would be important additions to this toolbox.[5](#_ENREF_5) Methylation is a common reaction in natural product biosynthesis and in signal transduction.[6](#_ENREF_6),[7](#_ENREF_7) Introduction of methyl groups onto small and large biomolecules can significantly change their physiochemical properties.[8](#_ENREF_8) Methylation is also an important synthetic approach to optimize the potency and pharmacokinetic properties of therapeutic compounds.[8](#_ENREF_8) Although electrophilic methyl transfers are conceptionally simple, targeting specific nucleophiles on complex molecules is challenging. Not surprisingly, the development of synthetic methodologies to overcome this problem is an important frontier in organic chemistry.[9-16](#_ENREF_9) The large and rapidly growing number of known enzymes that can methylate a broad variety of molecules with exquisite regio-, chemo- and stereoselectivity, compounded with the maturing abilities to adapt the substrate scope of enzymes by design and selection,[1](#_ENREF_1) provide reasons to believe that biocatalysis may play an important role in overcoming the synthetic challenges of late-stage methylation. Despite this promise, the biocatalytic scope of MTs is limited because these enzymes require SAM as stoichiometric co-substrate.[5](#_ENREF_5),[17](#_ENREF_17) SAM is as a chemically complex, relatively unstable and expensive reagent.

Chemical synthesis has been considered as a possible source for SAM. This approach is complicated by the fact that the last step in this synthesis - methylation of *S*-adenosyl homocysteine (SAH, **2**) - produces mixtures of the active (*S*,*S*)- and the inactive (*R*,*S*)-isomers of SAM.[18](#_ENREF_18) The introduction of chemoenzymatic approaches to produce diastereomerically pure SAM and SAM-derivatives has greatly broadened the scope of enzyme-catalyzed alkylation.[19](#_ENREF_19) Microbial production by fermentation is probably the most efficient source of SAM.[20](#_ENREF_20) Nevertheless, none of these technologies addressed the fundamental problem that SAM-dependent enzymes utilize a 384 Da carrier to transfer a 15 Da methyl group without the possibility of direct recycling.

The first enzyme-catalyzed methylation reaction that dispels the stoichiometric requirement of SAM was published only recently.[21](#_ENREF_21) This pioneering work described an *in vitro* reconstituted enzyme cascade that regenerates SAM from SAH mimicking part of the cellular adenosine metabolism. Briefly, after methyl transfer from SAM to product the cascade catalyzes the hydrolysis of SAH to adenosine (**3**, EC 3.3.1.1), phosphorylation of adenosine to ATP (**4**, EC 2.7.1.20, EC:2.7.4.6 or EC:2.7.1.40), and condensation of ATP with methionine to regenerate SAM (EC 2.5.1.6) (Figure 1). The chemical energy required for adenosine phosphorylation was provided by polyphosphates. This system was shown to turnover up to eleven times and marks an important step towards biocatalytic applications of MTs. On the other hand, reaction containing six enzymes and 14 metabolites is inherently difficult to implement, expensive to scale, and laborious to adapt by protein engineering. A related study examined the possibility to use cobalamine-dependent enzymes to catalyze the transfer of methyl groups between different phenol derivatives.[22](#_ENREF_22)

Here we report a much simpler and more versatile SAM recycling system that requires only one regenerating enzyme and uses the off-the-shelf reagent methyl iodide as methyl donor. This system can use SAH as a catalytic alkyl carrier in more than 500 turnovers. We demonstrate that this system can be used for specific methylation of natural products on preparative scale.



**Fig 1: Natural and artificial SAM-cycles.** SAM-dependent methyltransferases (MTs, black) catalyze methyl transfers from SAM (**1**) to substrate forming methylated product and SAH (**2**). In vivo, cyclic SAM regeneration occurs via hydrolysis of SAH (adenosylhomocysteine hydrolase, SAHH) to homocysteine and adenosine (**3**), phosphorylation of **3** to ATP (**4**, adenosine kinase, ADK; polyphosphate kinases PPK2-I and PPK2-II) and ATP-dependent adenylation of methionine (methionine adenosyltransferase, MAT). This system was reconstituted using polyphosphate as chemical energy.[21](#_ENREF_21) The present study demonstrates that halide methyl transferase (HMT, red) can directly remethylate SAH to SAM using methyl iodide as methyl donor. This reaction is driven by the high methyl transfer potential of methyl iodide as chemical energy.

**Results**

**Concept.** The following reasoning led us to construct this system. The sulfonium moiety of SAM is kinetically stable so that alkyl transfers only occur in the confinement of enzyme active sites.[23](#_ENREF_23) The thermodynamic reactivity of SAM, on the other hand, is high enough to make methylation of almost any nucleophile (N, C, O, P, S) favorable (G < 0).[23](#_ENREF_23) The high methyl transfer potential of the sulfonium group of SAM is related to the high acidity of *S-*protonated thioethers (p*K*a = – 5.4).[24](#_ENREF_24) Most nucleophiles that are subject to enzyme-catalyzed methylation form much weaker acids, making methyl transfers from SAM essentially irreversible. In this regard, halide methyl transferases (HMTs) present a notable exception. In fungi, bacteria, marine algae, diatoms, and halophytic plants HMTs are responsible for the production of methyl halides using SAM as the methyl donor and iodide, bromide and chloride as acceptors.[25-28](#_ENREF_25) Hydrogen halides are strong acids (p*K*a,HI – 10; p*K*a,HBr – 8.8; p*K*a,HCl – 6.4;) indicating that methyl transfers from SAM to halides are endergonic. In the cellular context methyl halide production is most likely driven by product removal. Methyl halides evaporate and *S-*adenosyl homocysteine is degraded. However, under proper *in vitro* conditions HMT should be able to transfer methyl groups from methyl iodide to SAH and therefore provide a simple mechanism for SAM regeneration. In the following we describe the demonstration and application of this concept.

**Halide methyl transferase (HMT).** In a first step we produced methyl halide transferase from the acidobacterium *Chloracidobacterium thermophilum* (HMT) and examined whether this enzyme can convert SAH to SAM under physiological conditions. Protocols for production, purification and analysis of this protein are described in the Supplementary Methods (Supplementary Figure 1). Our results confirmed that HMT can readily produce SAM from SAH and methyl iodide under physiological conditions (Supplementary Figures 2 and 3). In a 100 mM phosphate buffer at pH 8, SAH methylation by HMT is characterized by a *k*cat of 0.33 ± 0.03 s-1, a *K*M,MeI of 6.5 ± 1.4 mM and a *K*M,SAH of 0.17 ± 0.04 µM (Supplementary Figure 4).

**Construction of a methylation cascade with SAM regeneration.** In a second step we combined HMT with EgtD, a histidine-specific MT from *Mycobacterium smegmatis* (Figure 2).[29](#_ENREF_29) This enzyme-catalyzes three methyl transfers from SAM to histidine via the intermediates N-monomethyl histidine and N-dimethyl histidine to produce the final product N-trimethyl histidine (**5**, TMH). In a mechanistic study we showed that the three EgtD-catalyzed methyl transfers follow a highly cooperative process with limited accumulation of the mono- and dimethylated intermediates.[30](#_ENREF_30) TMH is a precursor for ergothioneine biosynthesis in bacteria and fungi.[31-33](#_ENREF_31) We assembled a reaction containing HMT, EgtD, SAH and methyl iodide to test whether continuous methylation of SAH by HMT could sustain EgtD-catalyzed TMH production (Figure 2a). Time-dependent product formation was monitored by HPLC (Figure 2b). In a reaction containing 1 mM histidine, 6 mM methyl iodide, 50 *μ*M SAH, 10 *μ*M of EgtD and 10 *μ*M of HMTthermo in a 100 mM phosphate buffer at pH 8, we observed TMH formation at an initial rate of (1.1 ± 0.1) x 10-2 µM/s (Figure 2c). After two hours this rate decreased significantly, and after 23 hours we determined that about 15 % of histidine was converted to TMH (Table 1, entry 1). This yield, divided by the concentration of added SAH indicated that every SAH molecule was remethylated by HMT nine times. Control reactions lacking either of the two enzymes produced no detectable TMH (Figure 2b). Importantly, the use of SAH as a catalytic methyl carrier is rather unnatural. In cellular SAM-cycles SAH does not act as a catalyst, because it is degraded, rather than remethylated (Figure 1).

**Hydrolysis of SAH limits productivity of methylation cascade.** Closer inspection of the HPLC traces showed that SAH was hydrolyzed to adenine (**6**) and ribosyl homocysteine (**7**) at an initial rate of (7 ± 1) x 10-3 µM/s (Figure 2d, Supplementary Figure 5). This rate is significantly faster than expected based on the rate for spontaneous depurination of SAH (*k* = 10-5 s-1),[34](#_ENREF_34) suggesting that SAH degradation maybe due to SAH nucleosidase (EC 3.2.2.9) contaminations in the HMT and EgtD preparations. Indeed, HMT and EgtD contained about 10-5 μmol/s SAH nucleosidase activity per mg protein.

Further purification of HMT and EgtD by gel-filtration reduced the initial rate of SAH hydrolysis, by 2-fold ((4 ± 0.3) x 10-3 µM/s) corroborating the suspicion that the SAH instability is due to contamination (Figure 2F, Supplementary Figure 6). In the presence of re-purified enzymes the half-life of SAH/SAM was significantly increased, allowing the cascade to remethylate SAH up to 30-fold and converting 51 % histidine to TMH (Figure 2e, Table 1, entry 2). Nevertheless, SAH degradation still limited turnover and yield.



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**Fig. 2. HMT/EgtD methyl transfer cascade**. **a**: Schematic representation of the HMT/EgtD cascade. **b:** HPLC analysis monitoring HMT/EgtD-mediated production of TMH in a reaction containing 1 mM histidine, 6 mM methyl iodide, 50 *μ*M SAH, 10 *μ*M HMT, 10 *μ*M EgtD 100 mM phosphate buffer at 25 °C. **c** & **d:** Time-dependent substrate-consumption/product formation by HMT and EgtD purified from *E. coli* BL21 (DE3) over Ni(II)-NTA agarose; **e** & **f:** HMT/EgtD purified over Ni(II)-NTA agarose and by size-exclusion chromatography; **g** & **h:** HMT/EgtD purified from a SAH nucleosidase-deficient *E. coli* strain (*E. coli* Δmtn (DE3)) over Ni(II)-NTA agarose. Error bars represent standard deviations from averages of three independent measurements.



**Fig. 3. Activity of SAH nucleosidase.** SAH nucleosidase *E. coli* (EC 3.2.2.9) catalyzes the irreversible cleavage of the glycosidic bond in SAH to adenine (**6**) and *S-*ribosylhomocysteine (**7**).

To eliminate this problem completely we produced HMT and EgtD in a SAH nucleosidase-deficient strain of *E. coli* (KEIO collection, strain JW0155-1).[35](#_ENREF_35) To make this strain compatible with gene expression from a pET28a vector, we deleted the genomic kanamycin-resistant cassette, and introduced a T7 RNA polymerase gene by lysogenesis (see Supplementary Methods). The resulting strain (*E. coli* Δmtn (DE3)) produced HMT and EgtD with a similar efficiency as BL21(DE3) cells. HMT purified from this strain contained less than 10-6 μmol/s SAH nucleosidase activity per mg protein (Supplementary Figure 7). The methylation cascade assembled from these proteins showed less than 5 % SAH degradation after 10 h at 25°C corresponding to a depurination rate of *k*SAH\_hydrolysis = (2.0 ± 0.1) x 10-5 s-1 (Figure 2h, Supplementary Figure 8), consistent with the published rate for the uncatalyzed reaction.[34](#_ENREF_34) Instead, the reaction produced TMH at an initial rate of (4.8 ± 0.3) x 10-2 uM/s and converted 94% of substrate, corresponding to 56 SAH regeneration cycles (Figure 2g, Table 1, entry 3). The concentration of the reaction intermediate N-dimethyl histidine increased in the first 20 minutes to 18 uM and then declined to less than 1 uM after 48 h (Supplementary Figure 9). Doubling the substrate concentration also doubled the yield and the number of SAH regeneration cycles (Table 1, entry 4). Increasing the concentration of EgtD by two-fold increased substrate conversion to 99 % (Table 1, entry 5). Interestingly, reduction of the SAH/SAM concentration by 2.5-fold did not significantly reduce productivity, and consequently doubled the number of catalytic cycles per SAH (Table 1, entry 6). The reactions in entries 1 – 6 all contained a six-fold higher concentration of methyl iodide than histidine, corresponding to a two-fold excess because each histidine is methylated three times. Reactions with no or little excess of methyl iodide also resulted in more than 90 % of conversion (entries 7 – 9). Increases of substrate concentration increased the SAH turnover number to almost 500 but at the same time reduced conversion, possibly due to a combination of effects such as product inhibition and limited supply of methyl iodide (Table 1, entries 10 - 12). Finally, we could also demonstrate that a 200 ml HMT/EgtD reaction containing 4 mM histidine and 18 mM methyl iodide (1.5 eq.) produced 140 mg TMH (88 %, isolated product).

**Kinetic analysis of the HMT/EgtD cascade.** The following quantitative considerations provide clues as to why the HMT/EgtD cascade reaction (entry 5) works so efficiently. HMT is characterized by a remarkably high substrate affinity (*K*M,SAH = 0.17 µM) (Supplementary Figure 4). The steady state concentrations of SAH and SAM were determined to be 2 and 48 µM (Figure 2h). Hence, most available SAH is bound to HMT. This is a crucial feature because many SAM-dependent enzymes are subject to product inhibition by SAH.[30](#_ENREF_30) *In vitro* MT assays usually include SAH nucleosidase to avoid product inhibition.[30](#_ENREF_30) *In vivo* the activities of MTs are believed to be modulated by the SAH concentration and the SAM/SAH ratio.[36](#_ENREF_36) SAH concentrations are typically maintained at low- or sub-micromolar concentrations by SAH nucleosidases or SAH hydrolases.[37](#_ENREF_37) The high SAH affinity of HMT combined with the high methyl transfer potential of methyl iodide provide a simple way to establish a similarly high steady state SAM/SAH ratio as observed in living cells.

**Occurrence and effects of unspecific methylation.** We also examined whether uncatalyzed methyl transfers to solvent or to other reaction components reduce productivity. Measured rates of methyl iodide hydrolysis in neutral aqueous solutions at 55°C (*k*hydrolysis = 3 x 10*-6*)[38](#_ENREF_38) or at 85°C (*k*hydrolysis = 1 x 10-4)[39](#_ENREF_39) indicate that this reaction is exceedingly slow at room temperature. Indeed, a solution containing 6 mM methyl iodide and 1 mM SAH in 100 mM phosphate buffer at pH 8 produced only 160 µM methanol, 100 µM methyl phosphate, 185 µM dimethyl phosphate after 48 h of incubation at 20°C (Supplementary Figure 10). Non-enzymatic methylation of SAH or histidine was not observed.

**Table 1.** **Optimization of the HMT/EgtD methylation cascade.**a

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Entry | Histidine (mM) | CH3I (mM) | Equivalents of CH3I  | SAH (*μ*M) | HMT (*μ*M) | EgtD (*μ*M) | Conversion (%) | No of Cycle |
| 1b | 1 | 6 | 2 | 50 | 10 | 10 | 15 | 9 |
| 2c | 1 | 6 | 2 | 50 | 10 | 10 | 51 | 31 |
| 3 | 1 | 6  | 2 | 50  | 10 | 10 | 94 | 56 |
| 4 | 2 | 12 | 2 | 50  | 10 | 10 | 92 | 110 |
| 5 | 2 | 12 | 2 | 50 | 10 | 20 | 99 | 120 |
| 6 | 2 | 12 | 2 | 20 | 10 | 20 | 97 | 290 |
| 7 | 2 | 6 | 1 | 20 | 10 | 20 | 93 | 280 |
| 8 | 2 | 8 | 1.3 | 20 | 10 | 20 | 94 | 280 |
| 9 | 2 | 10 | 1.7 | 20 | 10 | 20 | 94 | 282 |
| 10 | 4 | 20 | 1.3 | 20 | 10 | 20 | 72 | 440 |
| 11 | 6 | 20 | 1.1 | 20 | 10 | 20 | 55 | 500 |
| 12 | 8  | 20 | 0.8 | 20 | 10 | 20 | 48 | 580 |

a) All reactions contained 100 mM phosphate buffer, pH 8.0 and were incubated at 25°C b) Proteins were produced in BL21 (DE3) cells and purified over Ni(II)-NTA agarose. c) Proteins were produced in BL21 (DE3) cells and purified over Ni(II)-NTA and by size exclusion chromatography. Proteins used for the remaining reactions (entries 3 – 9) were purified from *E. coli* Δmtn (DE3) over Ni(II)-NTA agarose. Conversion was determined by HPLC (Supplementary Figures 13). Numbers of cycle were determined as (3 x [product])/[SAH]t=0. Reaction conditions are described in the methods section.

Methyl iodide could also poison the reaction by methylating HMT or EgtD. To examine this possibility, we compared the catalytic activities of HMT and EgtD after incubation with 0 or 5 mM methyl iodide for 5 or 48 h at 25°C in phosphate buffer (Supplementary Figures 11 & 12). As a result, we found no significant methyl iodide-dependent inactivation. Analysis by High-Resolution Electrospray Mass Spectrometry (HR-ESI-MS) of the entire proteins showed that HMT is methylated three times (Supplementary Table 1). We did not detect any methylated EgtD species. Tryptic digestion followed by Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS) showed that only cysteine-containing fragments were alkylated. In contrast to the unmethylated control, the methylated fragments no longer reacted with iodoacetamide, confirming that the methyl groups are attached to the side chain of cysteine residues. The same LC-HRMS characterization of alternative MTs (IMT, PMT, SgvM and NovO, see below) supported the general notion that under the conditions used for *in vitro* catalysis methyl iodide exclusively alkylates cysteine residues (Supplementary Table 1). Since MTs do not usually contain cysteines as essential catalytic residues, we anticipate that methyl iodide sensitive MTs could be cured by mutating specific cysteine residues to alanine, serine or valine. In conclusion, the slow rate of unspecific methyl iodide consumption, and the limited effect of methyl iodide on enzyme activity reveal the HMT/EgtD cascade as a robust and efficient system for methyl transfer from methyl iodide to histidine.

**Application of other MTs.** The Braunschweig Enzyme Database (BRENDA) lists more than 300 SAM-dependent MTs (EC 2.1.1.-) most of which transfer methyl groups to *O*-, *N*- or *C*-nucleophiles. In principle, HMT-mediated cofactor regeneration could be applicable to any SAM-dependent MT that is producible, active and stable under *in vitro* conditions. To provide a few test cases for this proposition we examined the productivity of the following natural product methyl transferases in the context of the SAM regeneration cascade.

Inositol 4-MT from *Mesembryanthemum crystallinum* (IMT, EC 2.1.1.129) converts inositol (**8**) to ononitol (**9**, Table 2, entry 3).[40](#_ENREF_40) O-Methyl inositols are plant osmoprotectants, that also have antidiabetic, anti-cancer or anti-inflammatory effects in humans.[41](#_ENREF_41) Inositol MTs reflect the synthetic prowess of enzyme-mediated methylation in that they afford regiospecific alkylation of one out of six nearly equivalent hydroxyl groups. Chemical approaches for the same transformation include laborious multistep syntheses.[42-44](#_ENREF_42)

Putrescine *N*-MT (PMT, EC2.1.1.53) catalyzes the methylation of the diamine putrescine (**10**) to form the first specific intermediate on the biosynthetic path to nicotine, tropane, and nortropane alkaloids (Table, entry 4).[45](#_ENREF_45) Transformation of primary amines to secondary amines by chemical synthesis is often hampered by the higher nucleophilicity of the secondary amine which results in overalkylation. Hence, secondary methyl amines are usually synthesized through indirect routes. In contrast, the PMT/HMT cascade can use methyl iodide to produce *N,N′*-dimethylputrescine (**11**) in a direct and selective reaction.

*C*-Methylating enzymes are notable for their abilities of asymmetric alkylation of the -carbon of -keto acids (Table 2, entry 5).[46-48](#_ENREF_46) This activity is of particular interest, since direct asymmetric alkylation  to carbonyl groups is still a challenging objective for synthetic methods.[17](#_ENREF_17) As a test enzyme we examined the MT SgvM from *Streptomyces griseoviridis*, which can methylate the C3 position of -ketovaleric acid (**12**), among other substrates.[46](#_ENREF_46) Another class of C-methylating enzymes is represented by the enzyme NovO,[49](#_ENREF_49) which can methylate the sp2-hybridized carbon on aromatic compounds such as 2,7-dihydroxynaphthalene (**14**, entry 6).[50](#_ENREF_50)

These four enzymes (Table 2) were purified from *E. coli* Δmtn (DE3) cells and assayed following similar protocols as described for EgtD. The methylated products emerging from the corresponding HMT/MT cascades were identified and quantified by NMR and ESI-MS (Supplementary Figures 14 - 25). The reactions containing PMT or SgvM converted 2 mM of **10** or **12** almost completely, using 20 µM of SAH in 100 cycles. Determination of the enantiomeric excess (*ee*) of the product as a function of time suggests that SgvM-catalyzed methyl transfer likely occurs with complete stereoselectivity, but that the product is prone to uncatalyzed racemization at a rate of approximately (3 ± 0.6) x 10-7 s-1 (Supplementary Figure 23). For example, after five hours the *ee* was 98 % but declined to 91 % after 48 h.

The enzymes IMT and NovO converted almost half of their substrates **8** and **14** respectively. Two more additions of IMT and NovO to the running reactions drove substrate conversion to near completion (>90 % conversion, Table 2), suggesting that enzyme stability is the limiting factor in these examples. Finally, as a demonstration that HMT/MT cascades could be used for isotope labeling we produced deuterated TMH using deuterated methyl iodide (entry 2) using the same conditions as described for production of TMH (entry 1). Again, this process afforded almost quantitative conversion of histidine to product (Supplementary Figures 26 - 31).



**Scheme 1**

**Table 2.** **Conversion of cascade methyl transfer combining different type of MTs and HMT**.a

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| entry | enzyme | substrate/product | Conversion [%] | Number of cycles |
| 1 | EgtD | Histidine/**5** | 97 | 290 |
| 2 | EgtD | Histidine/*d9*-**5** | 96 | 290 |
| 3 | IMT | **8**/**9** | 43 (> 90) | 43 |
| 4 | PMT | **10**/**11** | 87 | 87 |
| 5 | SgvM | **12**/**13** | > 95 | 100 |
| 6 | NovO | **14**/**15** | 35 (>90) | 35 |

aConversion was determined by 1H NMR (Supplementary Figures 14, 16, 19, 24) or HPLC (Supplementary Figures 13 and 26). Numbers of cycle were determined as [product]/[SAH]t=0. Reaction conditions are described in the methods section.

**Conclusions**. In this report we demonstrated that HMT can be combined with *O*-, *N*- or *C*-specific MTs to engineer efficient reaction cascades that afford preparative and nearly quantitative methylation of complex molecules. Fueled with the off-the-shelf reagent methyl iodide and catalytic concentrations of SAM/SAH these cascades can exploit the exquisite regio-, chemo-, and stereoselectivity of the large family of naturally evolved SAM-dependent MTs for *in vitro* methylation reactions. Because of its simplicity this technology is easy to implement and is ready to use as a tool for natural product research and for small scale chemo-enzymatic syntheses. The protocols described here may also serve as a starting point to examine the applicability of HMT/MT cascades to large scale reactions. A major weakness of the HMT/MT technology in its current form is the dependence on methyl iodide. Even though methyl iodide is a natural product,[28](#_ENREF_28),[51](#_ENREF_51) and one of the standard reagents for methylation in organic synthesis, this volatile electrophile poses a significant safety hazard particularly when handled in large quantities. Therefore, another important future goal in our research is the development of SAM-dependent methyl transfer cascades that use less hazardous methyl donors.

**Methods**

**General procedures.** All enzymes were produced by recombinant strains of *Escherichia coli* transformed with pET28a expression vectors. Cells were grown in LB medium supplemented with 50 mg/L kanamycin and 35 mg/L chloramphenicol. Gene-expression was induced by addition of (Isopropyl β-D-1-thiogalactopyranoside) IPTG. Protein were purified over Ni(II)-NTA agarose following standard protocols. Purified proteins were characterized SDS\_PAGE (Supplementary Figure 1), and mass spectroscopy (Supplementary Table 1). Protein concentrations were determined by UV-Vis absorption using calculated extinction coefficients (280).[52](#_ENREF_52) Methylated products (*d9*-**5**, **9**, **11**, **13** and **15**) were characterized by HR-ESI-MS and NMR (Supplementary Figures 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 27, 28, 29, 30).

**Analytical HMT\_EgtD cascade reactions**. 0.5 ml reactions containing different concentrations of histidine, SAH and EgtD were incubated in 100 mM sodium phosphate buffer, pH 8.0 at 25 °C (Table 1). 30 *μ*L reaction aliquots were quenched by addition of 15 *μ*L of 1 M phosphoric acid. Time-dependent product formation was monitored by HPLC using a cation exchange column (Phenomenex Luna® 5 µm SCX 100 Å) and a diode array detector. Reaction products were identified by comparison with authentic samples (**1**, **2**, **5**, **6**, methanol, methyl phosphate Supplementary Figures 2, 3, 6, 10). The cascade reactions listed in Table 2 contained similar compositions of enzymes and substrates (see supplementary information).

**Preparative HMT-EgtD cascade reaction**. A 200 ml reaction containing 4 mM L-histidine (124 mg), 12 mM CH3I (1 eq.), 20 *µ*M SAH, 10 *µ*M HMT and 20 *µ*M EgtD in 100 mM sodium phosphate buffer (pH 8.0) was incubated at 21 °C for two days. After addition of 170 mg CH3I (0.5 eq), reaction was incubated for three more days. Subsequently, residual CH3I was extracted with ethyl acetate. 800 ml of ethanol was added to aqueous solution to precipitate the phosphate salts. The volume of the filtrate was reduced by evaporated under vacuum. The resulting aqueous solution was passed through an anion-exchange column (2 x 15 cm, Amberjet® 4200 in hydroxide form). The immobilized product was eluted from this column with milli-Q water. Fractions containing product were collected and freeze-dried to afford TMH (**5**) (140 mg, yield 88%).

**Data availability**

The primary data that support the plots within this paper and other findings of this study are available from the corresponding author upon reasonable request.

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**Competing interests:** The authors have submitted a patent application (European Patent EP18193563) protecting the methodology described in this manuscript.

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