Electronic Supplementary Information

A bicyclic *S*-adenosylmethionine regeneration system applicable with different nucleosides or nucleotides as cofactor building blocks

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Experimental

Materials

Substrates and reference standards were purchased in the highest purity available from Sigma-Aldrich (ATP, ADP, AMP, adenosine, IDP, IMP, inosine, hypoxanthine, CTP, CDP, CMP, cytidine, cytosine, SAM, SAH, MTA, L-methionine, L-ethionine, D/L-SMM, *N*-ethylanthranilic acid, and 3,4-dihydroxybenzoic acid), Acros Organics (isovanillic acid and sodium polyphosphate), Alfa Aesar (*N*-methylanthranilic acid, vanillic acid), AppliChem (adenine), Th. Geyer (ITP) and Fluka (anthranilic acid). Ingredients for buffers and cultivation media were purchased from Carl Roth.

Enzymes used in the regeneration systems (cloning, expression, and purification)

The cloning, expression, and purification of the majority of enzymes used in the regeneration system have been described in previous publications.[1–3] Only modifications and newly added enzymes are described here. An overview of all enzymes and their UniProt accession numbers are listed in Table S3, as well as their DNA and protein sequences below.

The 975 bp gene coding for the L-homocysteine *S*-methyltransferase from *Saccharomyces cerevisiae* (*Sc*HSMT) was amplified by PCR from genomic DNA of baker's yeast (primers used: *Sc*HSMT-NdeI-fwd 5′-TATATACATATGAAGCGCATTCCAATCAAAG-3′ and *Sc*HSMT-HindIII-rv 5′-TATATAAGCTTAGGAGTATTTATCTACAGCTGATGC-3′) and cloned into pET28a(+) using T4 DNA ligase (New England Biolabs GmbH, Frankfurt am Main, Germany). The 1224 bp gene coding for the betaine-L-homocysteine *S*-methyltransferase from *Rattus norvegicus* (*Rn*BHMT) was purchased as synthetic DNA strings from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA), amplified by PCR (primers used: *Rn*BHMT-NdeI-fwd 5′-CGCGCGGCAGCCATATGGCACCGATTGCAGGT-3′ and *Rn*BHMT-HindIII-rv 5′-GTGCGGCCGCAAGCTTAAGCTTACTGTGCGCTCTTAAATTT-3′) and cloned into pET28a(+) using In-Fusion Cloning (Takara Bio Europe, Saint-Germain-en-Laye, France). Restriction sites were included in the primers to leave the option for using restriction enzyme cloning.

The enzymes used in the regular SAM regeneration system were produced in *E. coli* BL21-Gold(DE3) (Agilent, Santa Clara, CA, USA). To eliminate any nucleosidase contamination, *E. coli* Δ*mtn* (DE3) cells were used, which were kindly provided by C. Liao and F. Seebeck (University of Basel, Switzerland).[4]

The expression of *Tk*MAT (*Thermococcus kodakarensis* methionine adenosyltransferase) was improved by the addition of the RIPL plasmid (Agilent, Santa Clara, CA, USA) to the *E. coli* strains, as well as using 2xYT medium for expression. *Rn*BHMT was also expressed in 2xYT medium. As the expression of *Mm*SAHH (*Mus musculus* SAH hydrolase) was decreased in the nucleosidase-deficient strain, 2xYT medium was used for expression.

Seed cultures (5 mL) were grown in LB medium with the corresponding antibiotics at 37 °C overnight. The main culture (400 mL plus added seed culture) was grown in LB medium, if not otherwise stated, supplemented with the needed antibiotics, at 37 °C. When the OD_{600} was higher than 0.5, the expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 0.25 mM, for *Rn*BHMT 0.20 mM) and the cultures were shaken at 160 rpm for at least 18 h at 20 °C. The cells were harvested by centrifugation and lysed by sonication [Branson Sonifier 250, Emerson, St. Louis, MO, USA (duty cycle 50%,

intensity 50%, 5 x 30 s with 30 s breaks)] in lysis buffer [40 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% (*w*/*v*) glycerol].

After centrifugation the crude lysate was either directly used for assays or the soluble proteins were purified by nickel-NTA affinity chromatography (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and desalted using a PD-10 column (GE Healthcare Life Sciences, Little Chalfont, UK). The storage buffer of the enzymes was the same as the lysis buffer. Protein concentration was determined using the absorbance at 280 nm, the molecular weight (including the His_6-tag), and the protein extinction coefficient (calculated with ExPASy ProtParam tool[5]) with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Purified protein samples were visualised by SDS-PAGE (Fig. S2).[6]

HPLC analysis

All substrates, products, and intermediates in the regeneration system that are commercially available have been used as authentic reference standards and for calibration curves.

All assays were analysed with an Agilent 1100 Series HPLC using an ISAspher SCX 100-5 column (250 mm x 4.6 mm, 5 µm, ISERA GmbH, Düren, Germany). The HPLC method for the substrates and products has been described previously.[2] The injection volume was set to 5 µL for assays with **12** and to 10 µL for assays with **18**. All retention times and calibration curves are listed in Table S4.

Buffer screening for SAM regeneration system

The standard conditions for the assays were 50 mM buffer, 20 mM $MgCl_2$, 50 mM KCl, and 20 mM polyP (calculated as single phosphate residues). The MT substrate **12** was used at 2 mM and the cofactor building block AMP was supplied at 40 µM, while L-methionine was added in excess at 3 mM. The concentrations of the enzymes were 1 µM for *Sc*ADK, *Aj*PPK2, and *Sm*PPK2 and 10 µM for *Ec*MAT, *Rn*COMT, and *Mm*SAHH. Different buffer systems with various pHs (potassium phosphate pH 7.5, 8.0; HEPES-NaOH pH 7.5, 8.0; Tris-HCl, pH 7.0, 7.5, 8.0, 8.5, 9.0) were tested for overall activity of the regeneration system looking at the *Rn*COMT conversion of 3,4-dihydroxybenzoic acid (**12**) to its methylated products vanillic acid (**13**) and isovanillic acid (**14**). A master mix of all necessary enzymes was prepared and added to the assay to start the reaction. Negative controls were prepared without any addition of enzymes. The samples were incubated at 37 °C at 300 rpm in a final volume of 200 µL and the reaction stopped by the addition of 10% (*v*/*v*) perchloric acid [final concentration 2.5% (*v*/*v*)] after 20 h. The samples were immediately frozen in liquid nitrogen and stored at −20 °C. Prior to HPLC analysis, the samples were re-thawed, centrifuged for 30 min at 18213 × g at 4 °C, and 80 µL of the supernatant was transferred to an HPLC vial with inlet.

Assays for cyclic cofactor regeneration system

SAM regeneration

The standard conditions for the assays were 50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 50 mM KCl, and 20 mM polyP (calculated as single phosphate residues). The substrate of the MTs, **12** or **18,** was used at 2 mM and the cofactor building block AMP was varied between 0 and 40 µM, resulting between 50 and 200 maximum turnover numbers possible in the reaction set-up. L-Methionine was added in excess at 3 mM. The concentrations of the enzymes were 1 µM for *Sc*ADK, *Aj*PPK2, and *Sm*PPK2 and 10 µM for *Ec*MAT, *Rn*COMT or *Rg*ANMT, and *Mm*SAHH. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

SAM and L-methionine regeneration with 5 mM MT substrate

Enzyme concentrations and set-ups were the same as for all other assays. 5 mM MT substrate **12** were used with either an excess of 7.5 mM L-methionine or 7.5 mM *S*-methyl-D/L-methionine (D/L-SMM) and 40 µM L-methionine. AMP was varied between 10 and 100 µM. The samples were incubated and stopped described above. After centrifugation, the samples were diluted 1:1 with water and 80 µL of the dilution was transferred to a HPLC vial with inlet.

SAE regeneration

The same assay set-up was used as described for the SAM regeneration only the alkyl group donor was swapped to L-ethionine and *Tk*MAT was used. Anthranilic acid (**18**) was used as substrate with *Rg*ANMT as MT. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

S^{7dz}AM regeneration

The same assay set-up was used as described for the SAM regeneration only the cofactor building block was exchanged. 7-Deazadenosine (**7b**, tubercidin) was used as starting material, as a nucleoside monophosphate was not available, AMP (1 µM) was added to start the reaction. Both *Ec*MAT and *Tk*MAT were tested with the modified cofactor building block. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

SAM and L-methionine regeneration

The same assay set-up was used as described for the SAM regeneration, except that L-methionine was reduced to 40 µM or not added at all. Either D/L-SMM or betaine were added at 3 mM in excess and an additional enzyme for L-methionine regeneration, *Sc*HSMT or *Rn*BHMT, was added at 10 µM. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

SIM and SCM regeneration

The same assay set-up was used as described for the SAM regeneration only the cofactor building block was exchanged. Either IMP or CMP was added at 40 µM. As *Ec*MAT did not accept ITP and CTP as substrates, *Tk*MAT was used in all assays containing cofactor analogues. Assays featuring L-methionine regeneration included 40 µM L-methionine, 3 mM D/L-SMM and 10 µM *Sc*HSMT. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

SAM regeneration with crude lysates

The assays were set up in the same way as the ones featuring purified enzymes. The final concentration of crude lysates was 10% (*v*/*v*) for *Ec*MAT, *Rn*COMT, *Mm*SAHH, and *Sc*HSMT, and 1% (*v*/*v*) for *Sc*ADK, *Aj*PPK2, and *Sm*PPK2. Negative controls were set up with crude lysate of empty pET28a(+) vector [final concentration 33% (*v*/*v*)] cultivated in the same way as the other cultures. The samples were incubated, stopped, and prepared for HPLC analysis as described above.

Assays for extended three-enzyme cascade starting from L-homocysteine

The standard conditions for the assays were 50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, and 50 mM KCl. The starting material was 2 mM L-homocysteine, 2.5 mM betaine or D/L-SMM, 2.5 mM ATP, and 2 mM MT substrate **12** and the enzymes added were *Rn*BHMT or *Sc*HSMT, *Ec*MAT, *Rn*COMT, and *Ec*MTAN at 10 µM each. The samples were incubated for 20 h, stopped, and prepared for HPLC analysis as described above.

Control assays for side reactions

SAH as methyl acceptor

To check if SAH can directly be methylated by *Sc*HSMT (or *Rn*BHMT), 10 µM of the enzyme was incubated with 1 mM SAH and 2 mM D/L-SMM (or 2 mM betaine), in the optimised buffer system. The samples were incubated for 5.5 h, stopped, and prepared for HPLC analysis as described above.

12 as methyl acceptor

To check if **12** can directly be methylated by *Sc*HSMT (or *Rn*BHMT), 10 µM of the enzyme was incubated with 2 mM **12,** and 2 mM D/L-SMM (or 2 mM betaine) in the optimised buffer system. The samples were incubated for 20 h, stopped, and prepared for HPLC analysis as described above.

Methionine alternatives as substrate for *Ec*MAT

To check if *Ec*MAT accepts either D/L-SMM (or betaine as an L-methionine alternative, the reaction was coupled to *Rn*COMT with **12** as substrate for easier detection. 10 µM of each enzyme were added to 3 mM ATP and either 3 mM L-methionine (positive control), 3 mM D/L-SMM or 3 mM betaine with 2 mM **12** as MT substrate, in the optimised buffer system. The samples were incubated for 20 h, stopped, and prepared for HPLC analysis as described above.

Methyl donors directly used by *Rn*COMT

It was checked if *Rn*COMT accepts either D/L-SMM or betaine directly as a methyl donor. 10 µM of the enzyme was added to either 3 mM SAM (positive control), 3 mM D/L-SMM or 3 mM betaine with 2 mM **12** as MT substrate, in the optimised buffer system. The samples were incubated for 20 h, stopped, and prepared for HPLC analysis as described above.

Statistical analysis

As MT conversion was detected without the addition of cofactor building block, a statistical analysis was conducted to check if the conversion with modified cofactor building blocks was significant. An unpaired t-test was used.

DNA sequences

>*Aj*PPK2 (codon-optimised for *E. coli*)

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TAA

>*Ec*MAT

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>*Ec*MTAN

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GTGCAGAAACTTGCACATGGCTAA

>*Mm*SAHH (codon-optimised for *E. coli*)

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>*Rg*ANMT (codon-optimised for *E. coli*)

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>*Rn*BHMT (codon-optimised for *E. coli*)

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>*Rn*COMT (codon-optimised for *E. coli*)

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>*Sc*ADK

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>*Sc*HSMT

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>*Sm*PPK2 (codon-optimised for *E. coli*)

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TAA

>*Tk*MAT

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Protein sequences

His$_6$-tags are underlined.

>*Aj*PPK2

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>*Ec*MAT

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>*Ec*MTAN

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>*Mm*SAHH

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RY

>*Rg*ANMT

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NLHIMEFFK

>*Rn*BHMT

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>*Rn*COMT

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>*Sc*ADK

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>*Sc*HSMT

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>*Sm*PPK2

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EAAIGEVDEKILGSGPGFLR

>*Tk*MAT

MGSS<u>HHHHHH</u>SSGLVPRGSHMAGKVRNIVVEELVRTPVEMQKVELVERKGIGHPDSIADGIAEAVSRALSREYVK
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State of the art

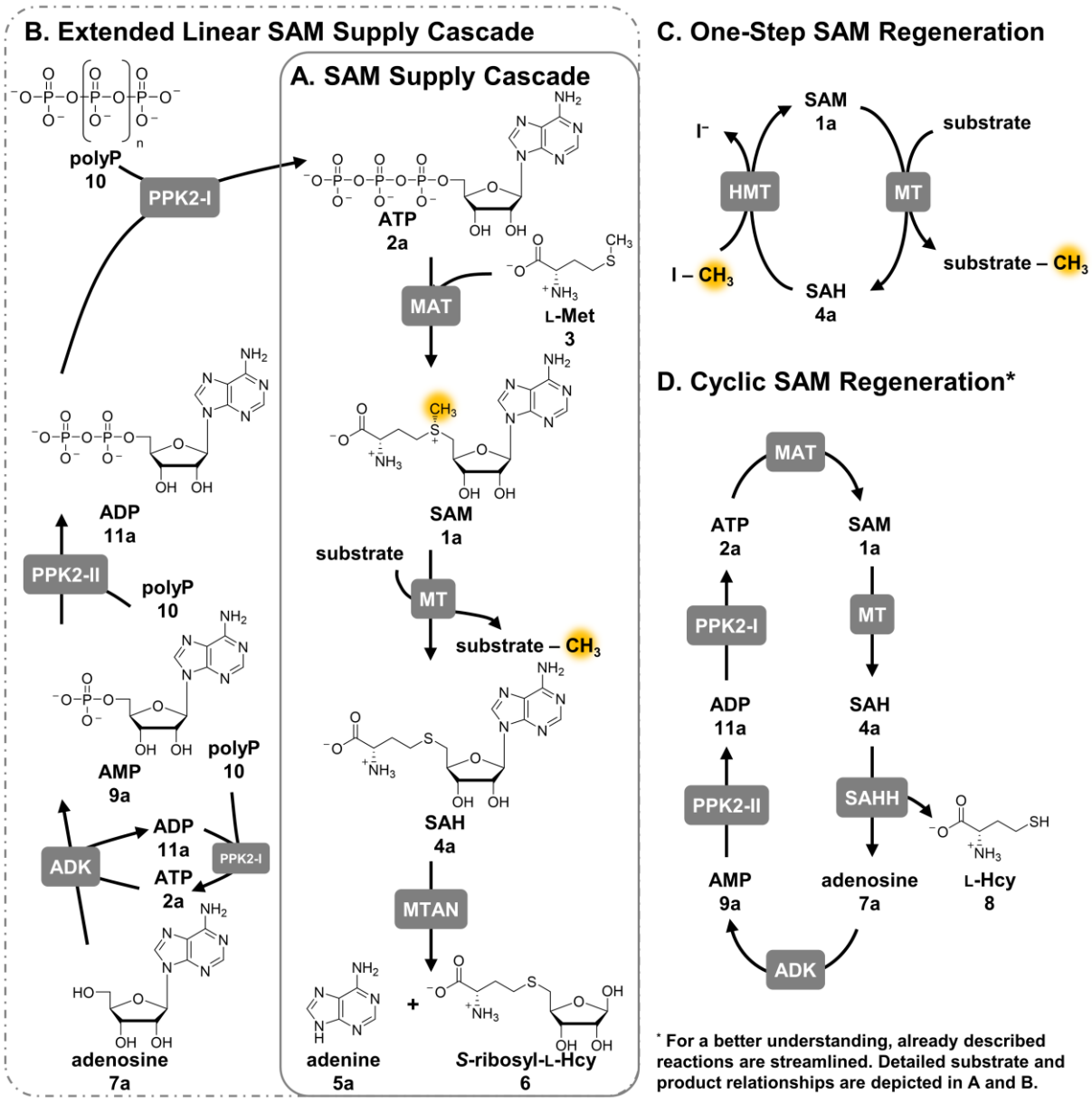


Fig. S1. Previously published systems for enzymatic SAM supply or SAM regeneration. A. Three-enzyme SAM supply and SAH degrading cascade employing MAT, MT, and MTAN; B. ATP regeneration coupled to SAM supply using six enzymes starting from adenosine (extended supply cascade); C. SAM regeneration using two enzymes directly methylating SAH back to SAM by HMT with methyl iodide; D. Cyclic SAM regeneration system using six enzymes with AMP as starting cofactor building block. MAT, methionine adenosyltransferase; MT, methyltransferase; MTAN, MTA/SAH nucleosidase; ADK, adenosine kinase; PPK2-I/II, polyphosphate kinases; SAHH, SAH hydrolase; HMT, halide methyltransferase.

Table S1. Overview of existing SAM supply and regeneration systems and examples for MT and MT substrate used.

System	Co-factor	Buffer and salts	Substrates	Enzymes	Mol% of MT	Incu-bation	Con-ver-sion [%][a]	TTN[b]
Supply (3 enzymes)[2]	SAM	100 mM Hepes, pH 8.0, 20 mM $MgCl_2$, 200 mM KCl	5 mM ATP, 5 mM L-Met, 5 mM **12**	*Tk*MAT (11 µM), *Rn*COMT (4 µM), *Ec*MTAN (1 µM)	0.08	1.5 h, 37 °C	50	n.a.[c]
Supply (6 enzymes)[1]	SAM (ATP)	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-Met, 3 mM adenosine, 1 µM AMP, 2 mM **12**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Ec*MAT (11 µM), *Rn*COMT (19 µM), *Ec*MTAN (3.8 µM)	0.93	20 h, 37 °C	>99	n.a.[c] (>1980)
Supply (6 enzymes)[1]	SAM (ATP)	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-Met, 3 mM adenosine, 1 µM AMP, 2 mM **18**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Ec*MAT (11 µM), *Rg*ANMT (12 µM), *Ec*MTAN (3.8 µM)	0.59	20 h, 37 °C	>85	n.a.[c] (>1700)
Supply (6 enzymes)[1]	SAE (ATP)	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-ethionine, 3 mM adenosine, 1 µM AMP, 2 mM **18**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Tk*MAT (11 µM), *Rg*ANMT (12 µM), *Ec*MTAN (3.8 µM)	0.59	20 h, 37 °C	>75	n.a.[c] (>1500)
Regeneration (2 enzymes)[4]	SAM	100 mM sodium phosphate, pH 8.0	20 µM SAH, 4 mM methyl iodide, 2 mM α-ketovaleric acid	SgvM (20 µM), HMT (10 µM)	1.00 (SgvM), 0.50 (HMT)	72 h, 25 °C	>95	100
Regeneration (2 enzymes)[4]	SAM	100 mM sodium phosphate, pH 8.0	20 µM SAH, 20 mM methyl iodide, 8 mM histidine	EgtD (20 µM), HMT (10 µM)	1.00 (EgtD), 0.50 (HMT)	48 h, 25 °C	48	580

Regeneration (2 enzymes)[7]	SAE	50 mM chloride-free sodium phosphate, pH 7.5	100 µM SAH, 80 mM ethyl iodide, 10 mM luteolin	IeOMT T133M-Y326L (292 µM), *At*HMT-V140T (364 µM)	2.92 (IeOMT T133M-Y326L), 3.64 (HMT)	72 h, 25 °C	41	41
Regeneration (2 enzymes)[7]	SAA	50 mM chloride-free sodium phosphate, pH 7.5	100 µM SAH, 80 mM allyl iodide, 10 mM 3,4-dihydroxy-benzalde-hyde	human COMT (292 µM), *At*HMT-V140T (291 µM)	2.92 (IeOMT), 2.91 (HMT)	24 h, 25 °C	48	48
Regeneration (6 enzymes)[1]	SAM	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-Met, 40 µM AMP, 2 mM **12**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Ec*MAT (11 µM), *Rn*COMT (19 µM), *Mm*SAHH (10 µM)	0.93	20 h, 37 °C	20	10
Regeneration (6 enzymes)[1]	SAM	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-Met, 40 µM AMP, 2 mM **18**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Ec*MAT (11 µM), *Rg*ANMT (12 µM), *Mm*SAHH (10 µM)	0.59	20 h, 37 °C	22	11
Regeneration (6 enzymes)[1]	SAE	50 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 50 mM KCl	20 mM polyP, 3 mM L-ethionine, 40 µM AMP, 2 mM **18**	*Sc*ADK (1.3 µM), *Aj*PPK2 (0.9 µM), *Sm*PPK2 (1.4 µM), *Ec*MAT (11 µM), *Rg*ANMT (12 µM), *Mm*SAHH (10 µM)	0.59	20 h, 37 °C	19	10

[a] Conversion based on MT reaction. [b] The number of cycles was calculated based on the ratio of alkylated product to catalytic amount of AMP. [c] n.a. – not applicable; the cofactor was not regenerated, but supplied in the reaction, and thus no TTN was calculated.

Table S2. Selected studies using (co)substrate analogues with enzymes from the SAM regeneration system.

Enzyme class	Organism/ enzyme name	Accepted substrates
MAT	*Cryptosporidium hominis*	Natural (hypoxanthine) and non-natural nucleobase analogues with allyl or propargyl group (medium conv.)[8] Selenium analogues of L-methionine with other modifications (medium conv.)[8]
	E. coli	L-Ethionine (low conv.)[9,10] Selenium analogues of L-methionine (high conv.)[10] and longer alkyl, allyl, alkenyl, alkinyl groups (very low conv.)[10] Analogues of L-methionine with longer alkyl, allyl, alkenyl, alkinyl groups (very low conv.)[10]
	*Homo sapiens (*MAT 2A)	L-Ethionine (high conv.)[10] Selenium analogues of L-methionine (high conv.)[10] and longer alkyl, allyl, alkenyl, alkinyl groups (low to medium conv.)[10] Analogues of L-methionine with longer alkyl, allyl, alkenyl, alkinyl groups (low to medium conv.)[10] Analogue of L-methionine with modified carboxylate moieties (low to high conv.)[11,12]
	Methanocaldococcus jannaschii	L-Ethionine (high conv.)[9,10] Natural (cytosine, guanine, hypoxanthine, uracil) and non-natural nucleobase analogues (high conv.)[9] Selenium analogues of L-methionine (high conv.)[10] and longer alkyl, allyl, alkenyl, alkinyl groups (low to high conv.)[10] Analogues of L-methionine with longer alkyl, allyl, alkenyl, alkinyl groups (low to medium conv.)[10]
MT	CouO/ *Streptomyces rishiriensis*	Non-natural nucleobase SAM analogue (medium conv.)[8] Selenium analogue with propargyl group (high conv.)[8]
	DnrK/ *Streptomyces peucetius*	SAM analogue with modified carboxylate moiety[11]
	Ecm1/ *Encephalitozoon cuniculi*	SAM analogues with photo-cross-linking moieties (high conv.)[13] Non-natural nucleobase SAM analogue (high conv.)[8] Selenium analogue with propargyl group (high conv.)[8]
	hTGS1/ *Homo sapiens*	Non-natural nucleobase SAM analogue (medium conv.)[8] Selenium analogue with propargyl group (low conv.)[8]
	M.TaqI/ *Thermus aquaticus*, MHhaI/ *Haemophilus parahaemolyticus*, M.BcnIB/ *Bacillus centrosporus*	Alkyl SAM analogues (low to high conv.)[14]
	NovO/ *Streptomyces niveus*	Alkyl SAM analogues and SAM analogues with modified carboxylate moiety (low to high conv.)[15]

Enzyme class	Organism/ enzyme name	Accepted substrates
	RapM/ *Streptomyces hygroscopicus*	Ethyl and allyl SAM analogues[16]
SAHH	*Alcaligenes faecalis*	Nucleobase analogues of adenosine (low to high conv.)[17]
	Methanocaldococcus jannaschii	Inosine analogues of SAH and adenosine (high conv.)[18]
ADK	*Mycobacterium tuberculosis*	Ribose analogues of adenosine (low to high conv.)[19]
	Saccharomyces cerevisiae	Natural nucleobase (cytosine, guanine, hypoxanthine, uracil) and ribose analogues of adenosine (low to medium conv.)[20]
	Trypanosoma brucei	Natural nucleotide analogues (CTP, GTP, UTP) and non-natural adenosine analogues[21]
PPK2	*Francisella tularensis*	Guanine analogues[22]
	Arthrobacter (Paenarthrobacter) aurescens	Guanine analogues[23]
	Ruegeria pomeroyi	Natural nucleobase (cytosine, guanine, uracil) analogues[24]
	Sinorhizobium meliloti	Guanine analogues[25]

Results and discussion

Starting point for optimisation

Table S3. Enzymes used in this study and their UniProt accession numbers (gene and protein sequences are given above).

Enzyme	UniProt accession number
*Aj*PPK2	Q83XD3 (Q83XD3_ACIJO)
*Ec*MAT	P0A817 (METK_ECOLI)
*Ec*MTAN	P0AF12 (MTNN_ECOLI)
*Mm*SAHH	P50247 (SAHH_MOUSE)
*Rg*ANMT	A9X7L0 (ANMT_RUTGR)
*Rn*BHMT	O09171 (BHMT1_RAT)
*Rn*COMT	P22734 (COMT_RAT)
*Sc*ADK	P47143 (ADK_YEAST)
*Sc*HSMT	Q08985 (SAM4_YEAST)
*Sm*PPK2	Q92SA6 (Q92SA6_RHIME)
*Tk*MAT	Q5JF22 (METK_THEKO)

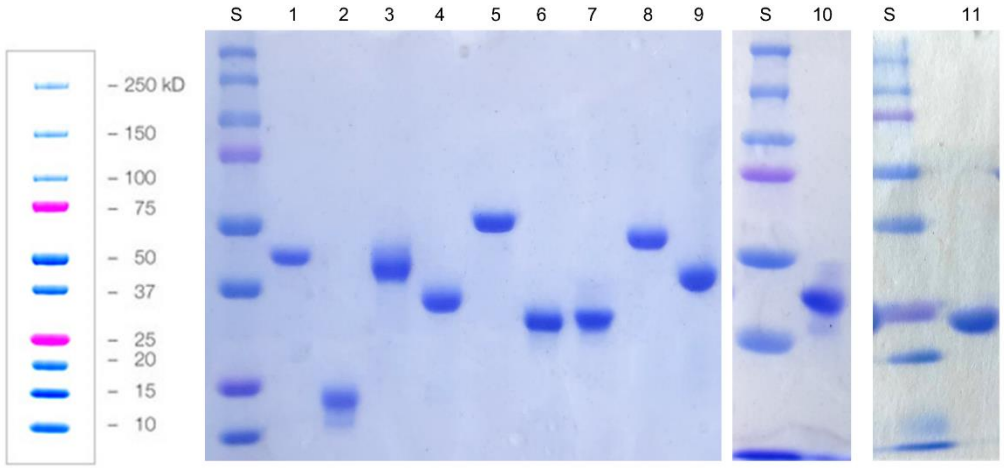


Fig. S2. SDS-PAGE analysis of purified proteins. For each protein 2 µg was applied; S – standard, 1 – *Ec*MAT (44 kDa), 2 – *Rn*COMT (27 kDa), 3 – *Mm*SAHH (50 kDa), 4 – *Sc*ADK (37 kDa), 5 – *Aj*PPK2 (58 kDa), 6 – *Sm*PPK2 (37 kDa), 7 – *Sc*HSMT (39 kDa), 8 – *Tk*MAT (47 kDa), 9 – *Rg*ANMT (42 kDa), 10 – *Rn*BHMT (47 kDa), 11 – *Ec*MTAN (27 kDa), the calculated molecular weight always includes the His_6-tag. Standard taken from https://www.bio-rad.com/en-de/sku/1610374-precision-plus-protein-dual-color-standards-500-ul?ID=1610374.

Table S4. HPLC retention times and calibration curves (n.d. – not determined).

Substance	Retention time [min]	Calibration curve	R^2
ATP (**2a**)	1.7	n.d.	n.d.
ADP (**11a**)	1.9	n.d.	n.d.
AMP (**9a**)	2.0	n.d.	n.d.
Adenosine (**7a**)	7.8	n.d.	n.d.
SAM (**1a**)	1.6	n.d.	n.d.
SAH (**4a**)	8.1	n.d.	n.d.
MTA	8.4	n.d.	n.d.
Adenine (**5a**)	9.2	n.d.	n.d.
ITP (**2c**)	1.3	n.d.	n.d.
IDP (**11c**)	1.3	n.d.	n.d.
IMP (**9c**)	1.5	n.d.	n.d.
Inosine (**7c**)	2.8	n.d.	n.d.
Hypoxanthine (**5c**)	3.2	n.d.	n.d.
CTP (**2d**)	1.3	n.d.	n.d.
CDP (**11d**)	1.5	n.d.	n.d.
CMP (**9d**)	2.0	n.d.	n.d.
Cytidine (**7d**)	7.8	n.d.	n.d.
Cytosine (**5d**)	10.0	n.d.	n.d.
7-Deazadenosine (Tubercidin, **7b**)	12.3	n.d.	n.d.
3,4-Dihydroxybenzoic acid (**12**)	2.3	324.7 x	>0.99
Vanillic acid (**13**)	3.3	371.4 x	>0.99
Isovanillic acid (**14**)	4.5	465.4 x	>0.99
Anthranilic acid (**18**)	4.0	91.1 x	>0.99
N-Methylanthranilic acid (**19**)	7.5	151.4 x	>0.99
N-Ethylanthranilic acid	7.9	163.9 x	>0.99

Quantitative data for MT conversion and TTNs

For all regeneration systems, the MAT, MT, MT substrate, and cofactor building block used are given, as well as if the L-methionine regeneration system was in place. The regeneration systems start from the monophosphorylated nucleotide in catalytic amount and for methylation 2 mM MT substrate was supplied, if not otherwise stated. Conversions are based on the MT reaction; conversions and total turnover numbers (TTN) are given as mean values ± standard deviation. All reactions were performed and analysed in triplicates. TTNs were calculated as

the amount of MT product formed divided by the catalytic amount of AMP added to the reaction. The maximum possible TTNs for 2 mM MT substrate and 40, 20, or 10 µM cofactor building block are consequently 50, 100, or 200 TTNs, respectively. Increasing the MT substrate to 5 mM and adding 100, 40, 20, or 10 µM cofactor building block correspond to turnovers of at most 50, 125, 250, or 500, respectively.

Table S5. Results from SAM regeneration systems using enzymes produced in *E. coli* BL21-Gold(DE3) strain with AMP as cofactor building block and different L-methionine regeneration systems.

MAT	MT	Substrate	L-Methionine Regeneration	AMP [µM]	Conv. [%]	N° of cycles
*Ec*MAT	*Rn*COMT	**12**	no	40	92 ± 2.2	46 ± 1.1
				20	68 ± 1.0	68 ± 1.0
				10	47 ± 0.7	95 ± 1.5
				0	9.9 ± 0.3	n.a.
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	40	>99 ± 0.0	50 ± 0.0
				20	>99 ± 0.0	100 ± 0.0
				10	>99 ± 0.0	200 ± 0.0
				0	25 ± 1.4	n.a.
			*Sc*HSMT 3 mM D/L-SMM	40	>99 ± 0.0	50 ± 0.0
				20	>99 ± 0.0	100 ± 0.0
				10	82 ± 2.8	164 ± 5.7
			*Rn*BHMT 3 mM betaine 40 µM L-Met	40	16 ± 0.9	8 ± 0.5
				20	13 ± 0.3	13 ± 0.3
				10	9 ± 0.4	17 ± 0.9
*Ec*MAT	*Rg*ANMT	**18**	no	40	59 ± 3.1	30 ± 1.5
				20	39 ± 1.8	39 ± 1.8
				10	27 ± 1.4	59 ± 2.9
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	40	81 ± 0.6	41 ± 0.3
				20	53 ± 3.3	53 ± 3.3
				10	38 ± 1.6	75 ± 3.1

n.a.; not applicable, as the number of cycles is calculated from the cofactor building block added to the system.

Table S6. Results from cofactor regeneration systems using enzymes produced in *E. coli* BL21-Gold(DE3) strain with AMP analogues as cofactor building block and use of *Sc*HSMT for L-methionine regeneration.

MAT	MT	Substrate	L-Methionine Regeneration	NMP [µM]	Conv. [%]	N° of cycles
*Ec*MAT	*Rn*COMT	**12**	no	AMP [1]	43 ± 1.5	22 ± 0.8
*Tk*MAT				^{7dz}ADO [40]	23 ± 0.4	11 ± 0.2
*Tk*MAT	*Rn*COMT	**12**	no	AMP [40]	65 ± 1.3	32 ± 0.6
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met		83 ± 0.1	42 ± 0.0
			no	CMP [40]	20 ± 0.5 (p < 0.0001)*	10 ± 0.3
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met		38 ± 1.9 (p = 0.0007)*	19 ± 1.0
			no	IMP [40]	23 ± 1.7 (p = 0.0057)*	11 ± 0.8
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met		33 ± 2.8 (p = 0.0115)*	16 ± 1.4

*compared to the conv. without cofactor building block added (Table S5)

Table S7. Results from SAM regeneration systems using enzymes produced in *E. coli* Δ*mtn* (DE3) strain with AMP as cofactor building block and L-methionine regeneration systems.

MAT	MT	Substrate	L-Methionine Regeneration	AMP [µM]	Conv. [%]	N° of cycles
*Ec*MAT	*Rn*COMT	**12**	no	40	99 ± 0.3	50 ± 0.1
				20	85 ± 0.1	85 ± 0.1
				10	54 ± 1.1	108 ± 2.3
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	40	>99 ± 0.0	50.0 ± 0.0
				20	99 ± 1.6	99 ± 1.6
				10	64 ± 0.2	129 ± 0.4
*Ec*MAT	*Rg*ANMT	**18**	no	40	61 ± 5.8	31 ± 2.9
				20	46 ± 0.5	46 ± 0.5
				10	35 ± 6.0	74 ± 12.0
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	40	75 ± 2.4	37 ± 1.2
				20	78 ± 6.6	78 ± 6.6
				10	61 ± 4.8	123 ± 9.5

Table S8. Results from SAE regeneration systems using enzymes produced in *E. coli* BL21-Gold(DE3) strain or *E. coli* Δ*mtn* (DE3) strain with AMP as cofactor building block.

MAT	MT	Substrate	AMP [µM]	*E. coli* strain	Conv. [%]	N° of cycles
*Tk*MAT	*Rg*ANMT	**18**	40	BL21-Gold(DE3)	20 ± 1.6	10 ± 0.8
				Δmtn (DE3)	23 ± 0.1	11 ± 0.0

Table S9. Results from SAM regeneration systems using enzymes produced in *E. coli* BL21-Gold(DE3) strain with AMP as cofactor building block and 5 mM MT substrate.

MAT	MT	Substrate	L-Methionine Regeneration	AMP [µM]	Conv. [%]	N° of cycles
*Ec*MAT	*Rn*COMT	**12**	no	100	63 ± 1.0	32 ± 0.5
				40	37 ± 0.3	46 ± 0.4
				15	23 ± 0.7	76 ± 2.2
				10	18 ± 0.6	90 ± 2.8
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	100	72 ± 1.4	36 ± 0.7
				40	47 ± 3.2	58 ± 4.0
				15	33 ± 1.1	110 ± 3.6
				10	27 ± 0.1	136 ± 0.7

Table S10. Results from SAM regeneration systems using crude lysates produced in *E. coli* BL21-Gold(DE3) strain with AMP as cofactor building block based on the decrease of substrate **12**.

MAT	MT	Substrate	L-Methionine Regeneration	AMP [µM]	Conv. [%]	N° of cycles
*Ec*MAT	*Rn*COMT	**12**	no	40	45 ± 2.1	22 ± 1.1
			*Sc*HSMT 3 mM D/L-SMM 40 µM L-Met	40	88 ± 0.5	44 ± 0.3

Buffer screening

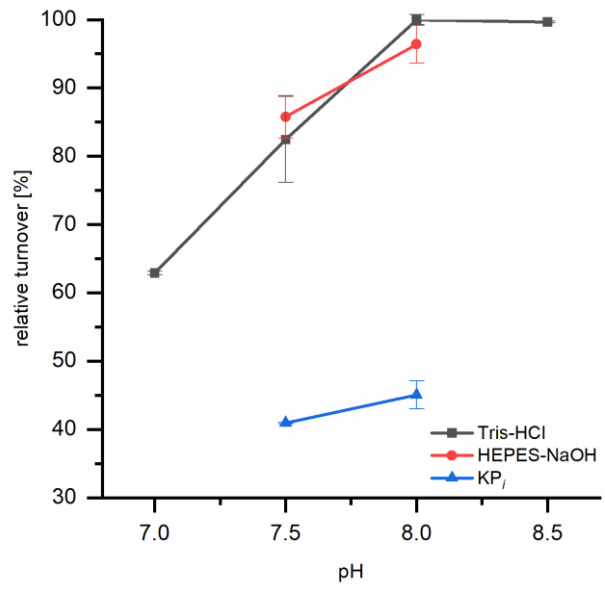


Fig. S3. Impact of buffer salt and pH on MT conversion. Tris-HCl pH 8.0, was the optimal buffer choice. The substrate was oxidised at Tris-HCl pH 9.0, and was not included in the data set.

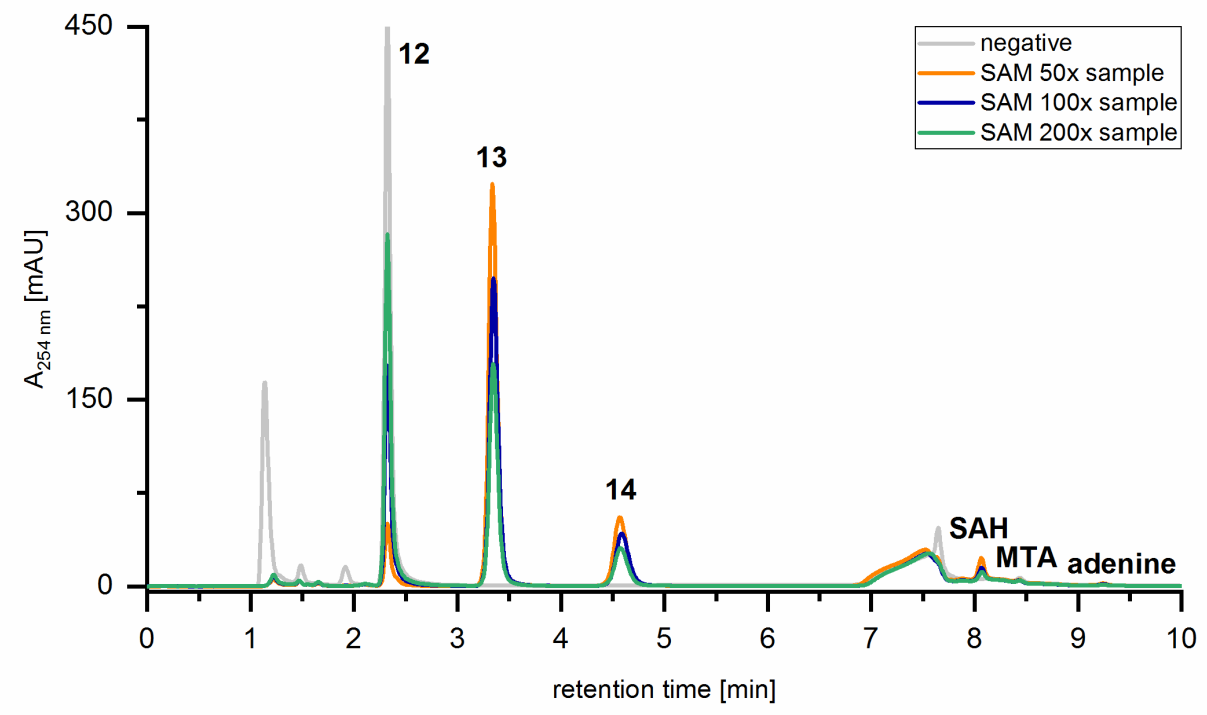


Fig. S4. Typical HPLC chromatograms of the SAM regeneration system with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

Isosteric adenosine analogues are not well tolerated by each cycle enzyme

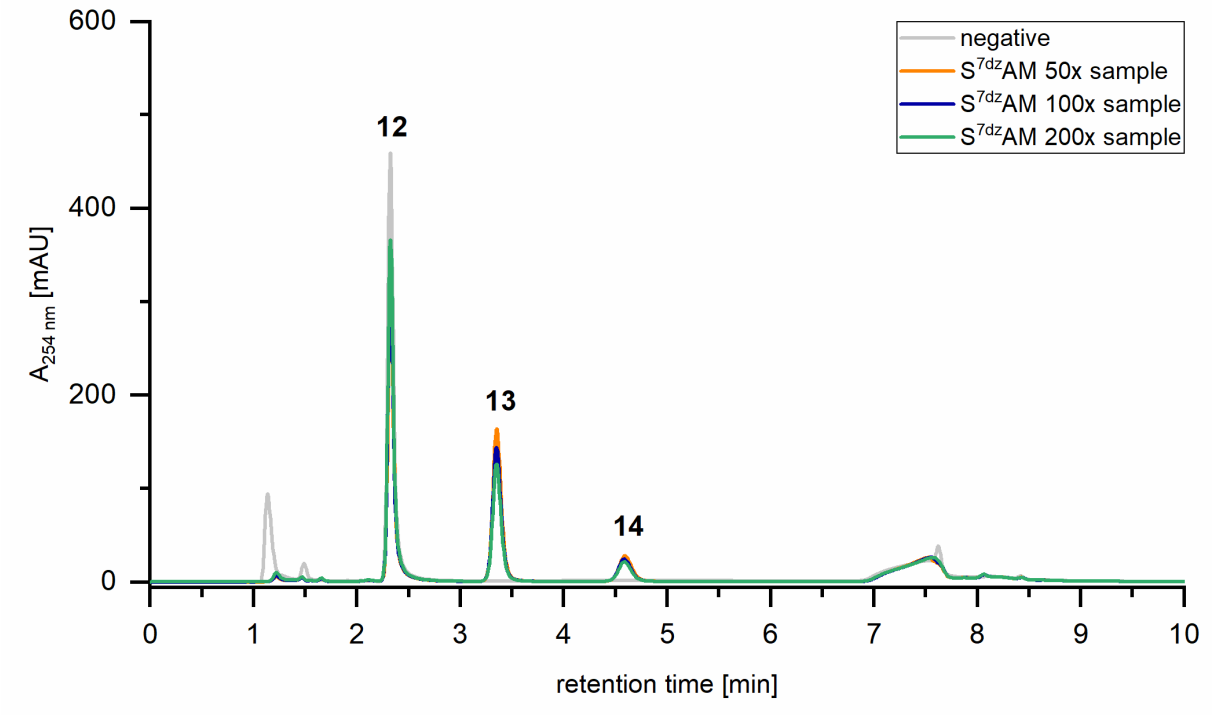


Fig. S5. Typical HPLC chromatograms of the $S^{7dz}AM$ regeneration system with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine, 20 mM polyP (calculated as single phosphate residues), 1 µM AMP, tubercidin (orange – 40 µM, blue – 20 µM, green – 10 µM).

Co-purified MTAN is not the only reason for adenine accumulation

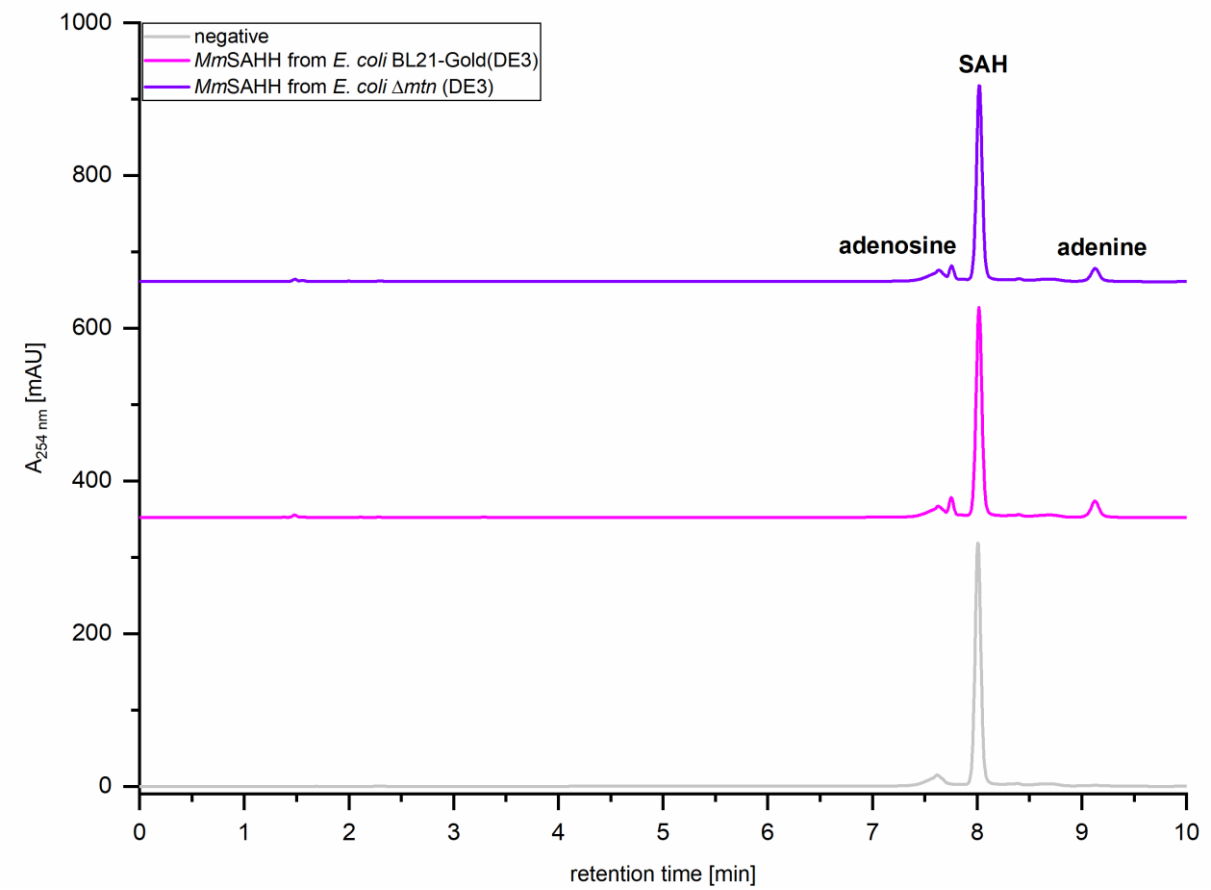


Fig. S6. HPLC chromatograms of the hydrolysis of SAH catalysed by either *Mm*SAHH purified from the *E. coli* BL21-Gold(DE3) (pink) or *E. coli* Δ*mtn* (DE3) strain (purple) after 20 h. The SAH hydrolysis reaction that is included in the regeneration system is not the preferred *in vitro* reaction of the enzyme and adenine is formed as a side product.

Options for the removal of L-homocysteine

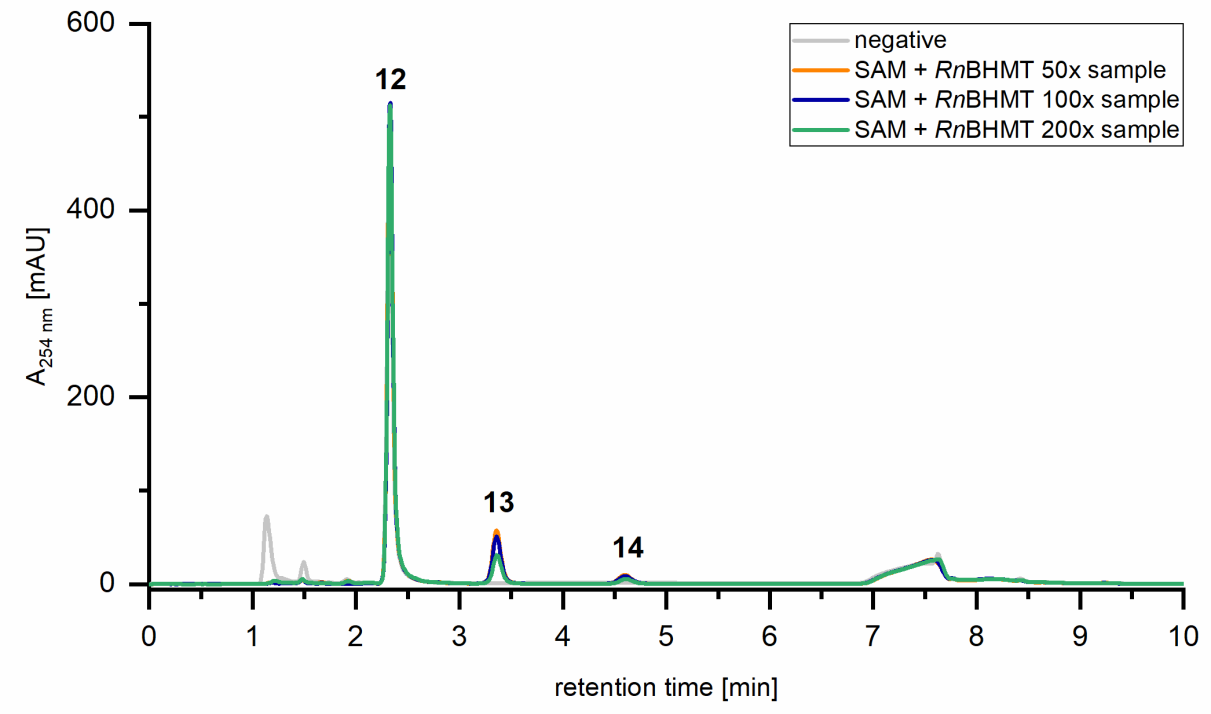
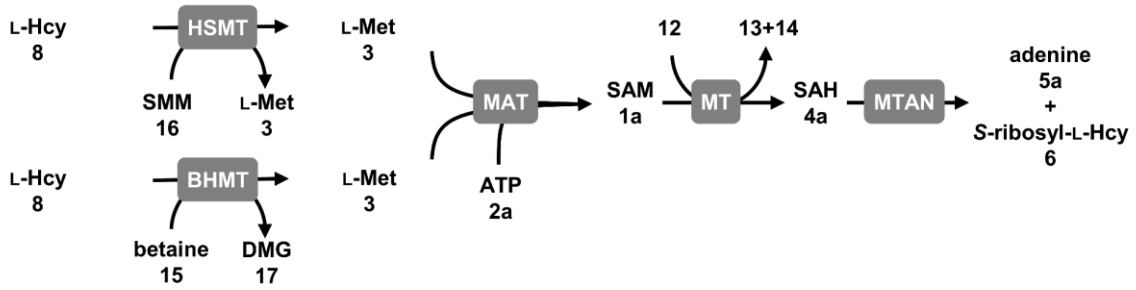


Fig. S7. Typical HPLC chromatograms of the SAM regeneration system with *Rn*COMT and additional L-methionine regeneration using *Rn*BHMT. Starting material: 2 mM MT substrate **12**, 3 mM betaine, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

A.



B.

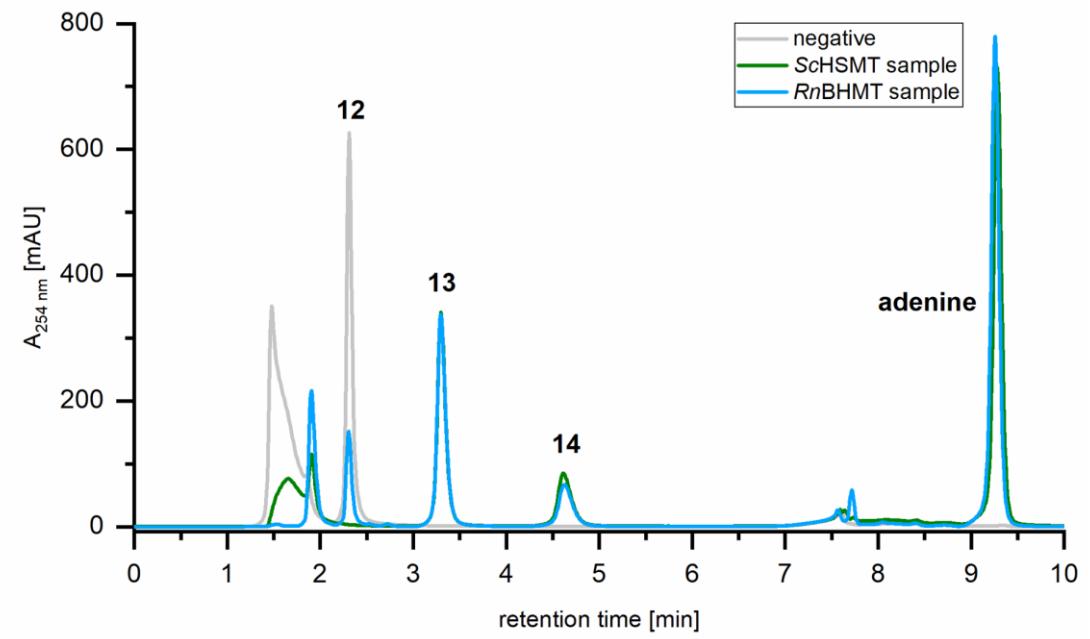


Fig. S8. A. Three-enzyme cascade (MAT–MT–MTAN) coupled to either HSMT or BHMT starting from L-homocysteine; B. HPLC chromatograms of the three-enzyme cascade (*Ec*MAT, *Rn*COMT, *Ec*MTAN) coupled to either *Sc*HSMT or *Rn*BHMT starting from L-homocysteine. Starting material: 2 mM MT substrate **12**, 2 mM L-homocysteine, 3 mM D/L-SMM or betaine, 3 mM ATP.

The NMR spectra were recorded on a DRX 400 spectrometer (Bruker, Billerica, MA, USA) operating at 400.1 MHz (for ^{1}H NMR). Spectra were analysed with TopSpin 3.6.2 (Bruker, Billerica, MA, USA). All NMR measurements were performed once at 25 °C.

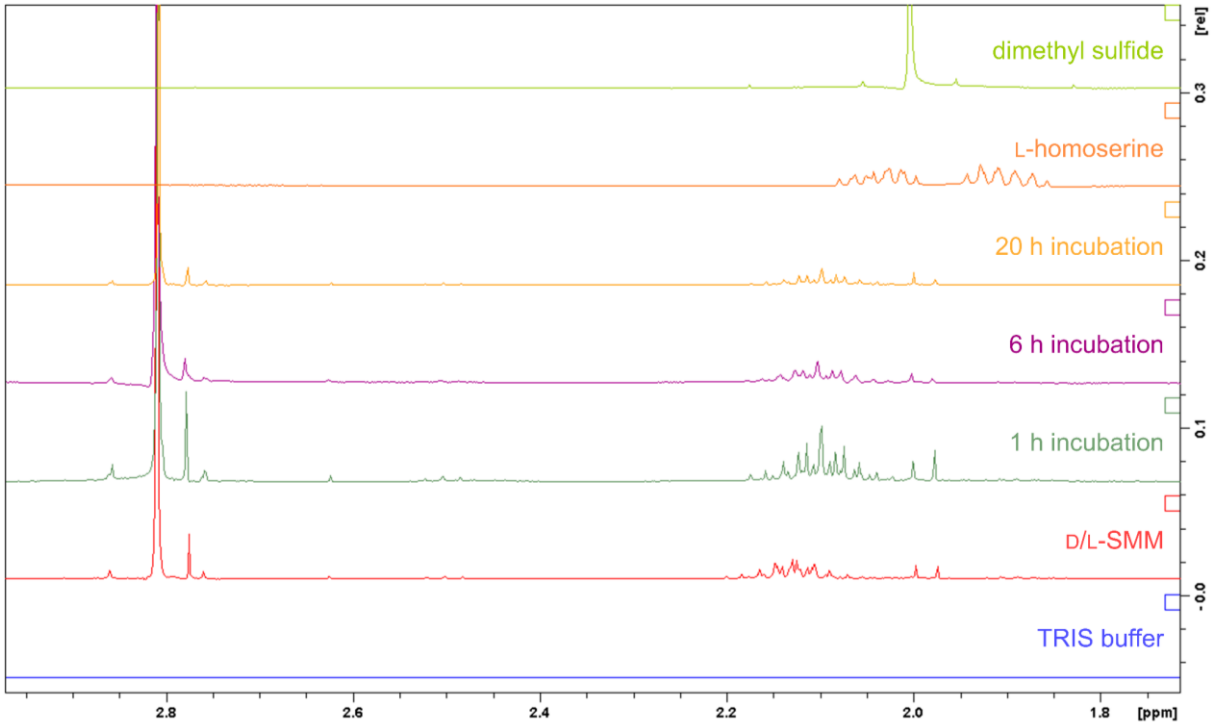


Fig. S9. ^{1}H NMR spectra of the possible degradation products of D/L-SMM, dimethyl sulfide (light green) and homoserine (orange), compared with D/L-SMM (red) incubated for 1 h (dark green), 6 h (purple) and 20 h (yellow) in buffer (blue) at 37 °C under assay conditions in 5% D_2O. No degradation products were found.

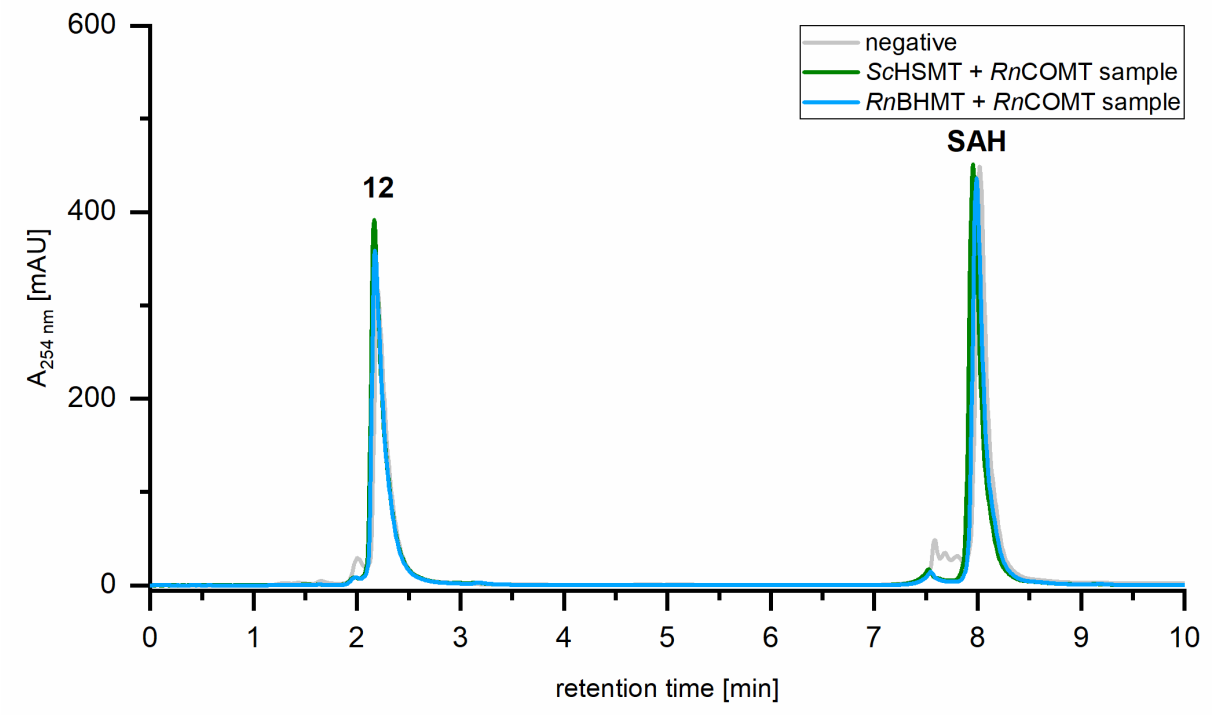


Fig. S10. HPLC chromatograms of *Sc*HSMT or *Rn*BHMT incubated with *Rn*COMT and SAH and MT substrate **12**. Starting material: 2 mM MT substrate **12**, 1 mM SAH, 2 mM D/L-SMM or 2 mM betaine.

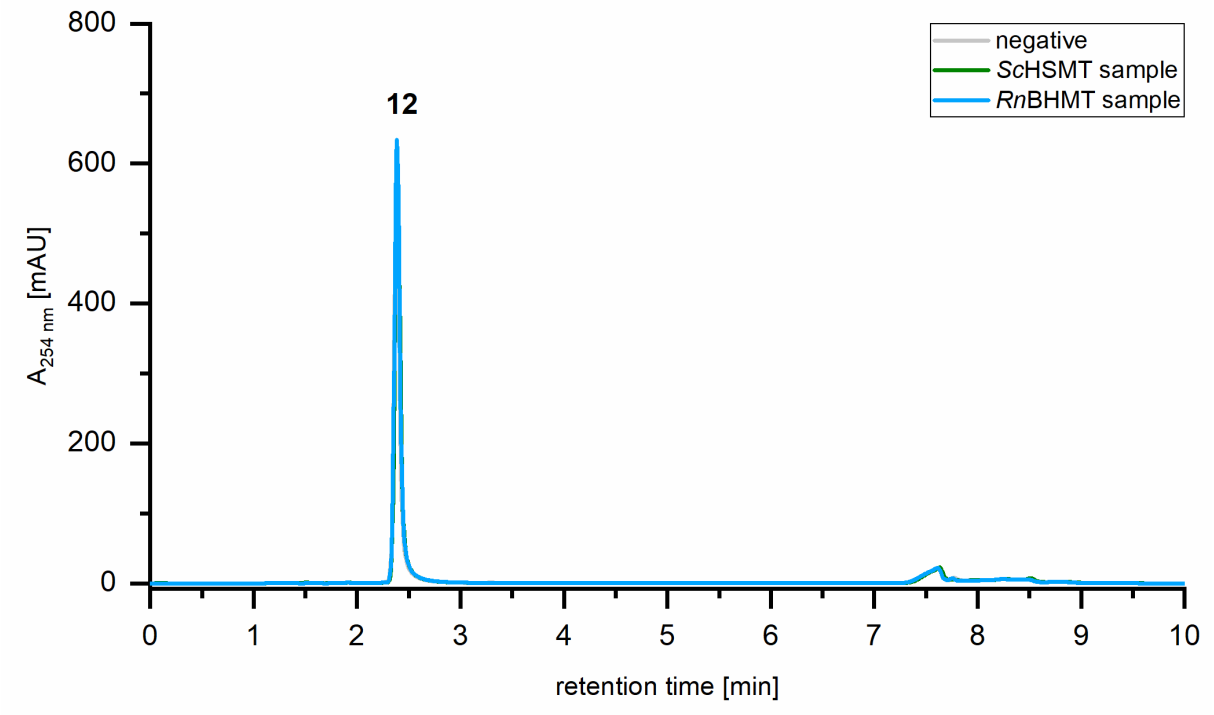


Fig. S11. HPLC chromatograms of *Sc*HSMT or *Rn*BHMT incubated with **12**. Starting material: 2 mM MT substrate **12**, 3 mM D/L-SMM or 3 mM betaine.

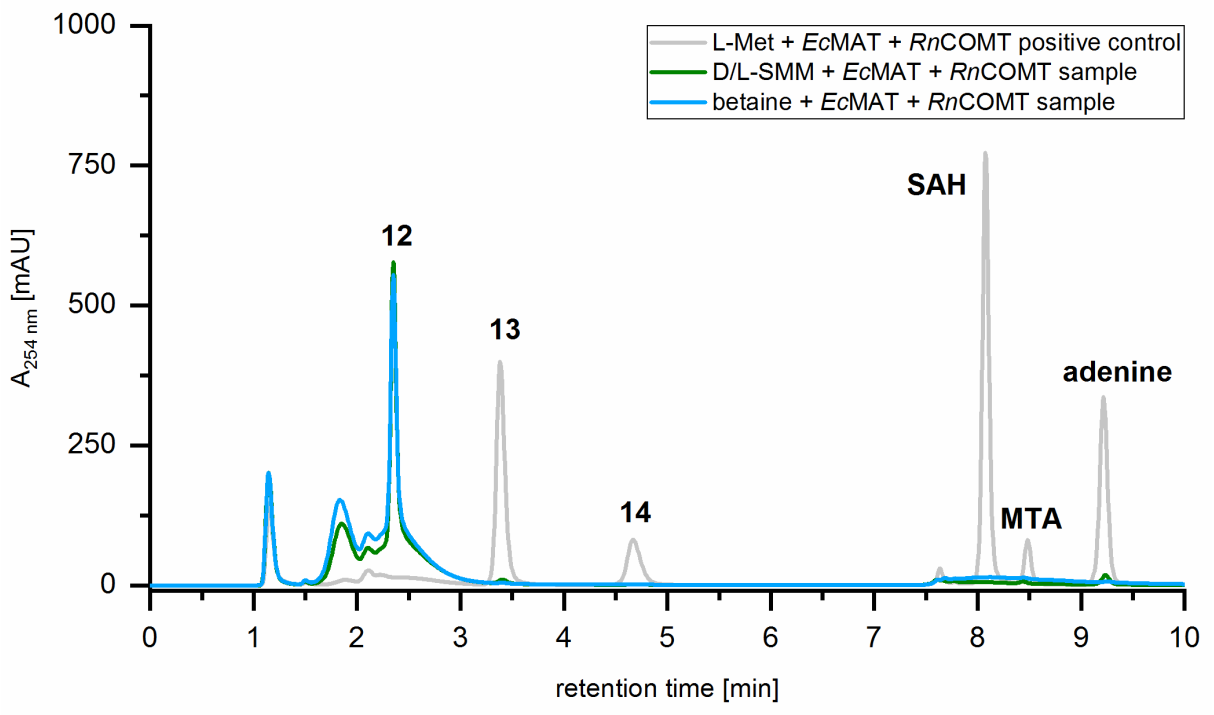


Fig. S12. HPLC chromatograms of *Ec*MAT and *Rn*COMT incubated with L-methionine or some analogues and ATP. Starting material: 2 mM MT substrate **12**, 3 mM ATP, 3 mM L-methionine, 3 mM D/L-SMM or 3 mM betaine.

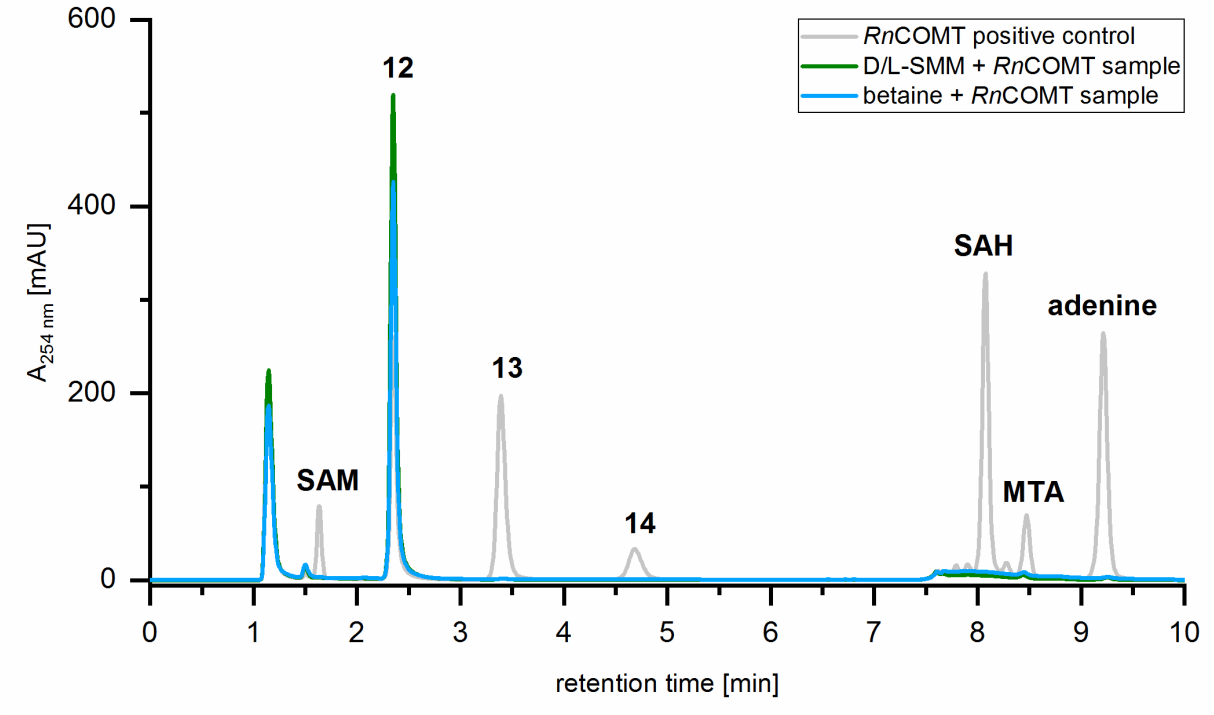


Fig. S13. HPLC chromatograms of *Rn*COMT incubated with SAM, D/L-SMM or betaine as possible methyl donors. Starting material: 2 mM MT substrate **12**, 3 mM SAM, 3 mM D/L-SMM or 3 mM betaine. A small peak for **13** is detected in the D/L-SMM sample because of the L-methionine impurity.

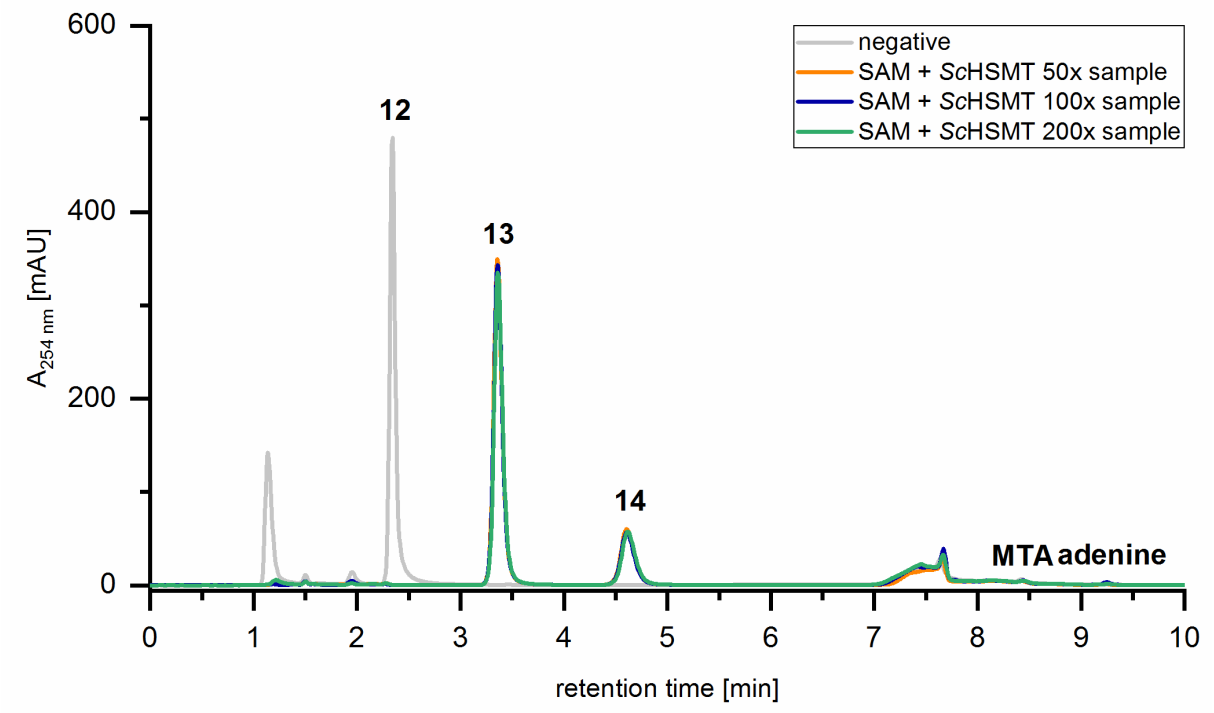


Fig. S14. Typical HPLC chromatograms of the SAM regeneration system with *Rn*COMT and additional L-methionine regeneration using *Sc*HSMT. Starting material: 2 mM MT substrate **12**, 3 mM D/L-SMM, 40 µM L-methionine, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

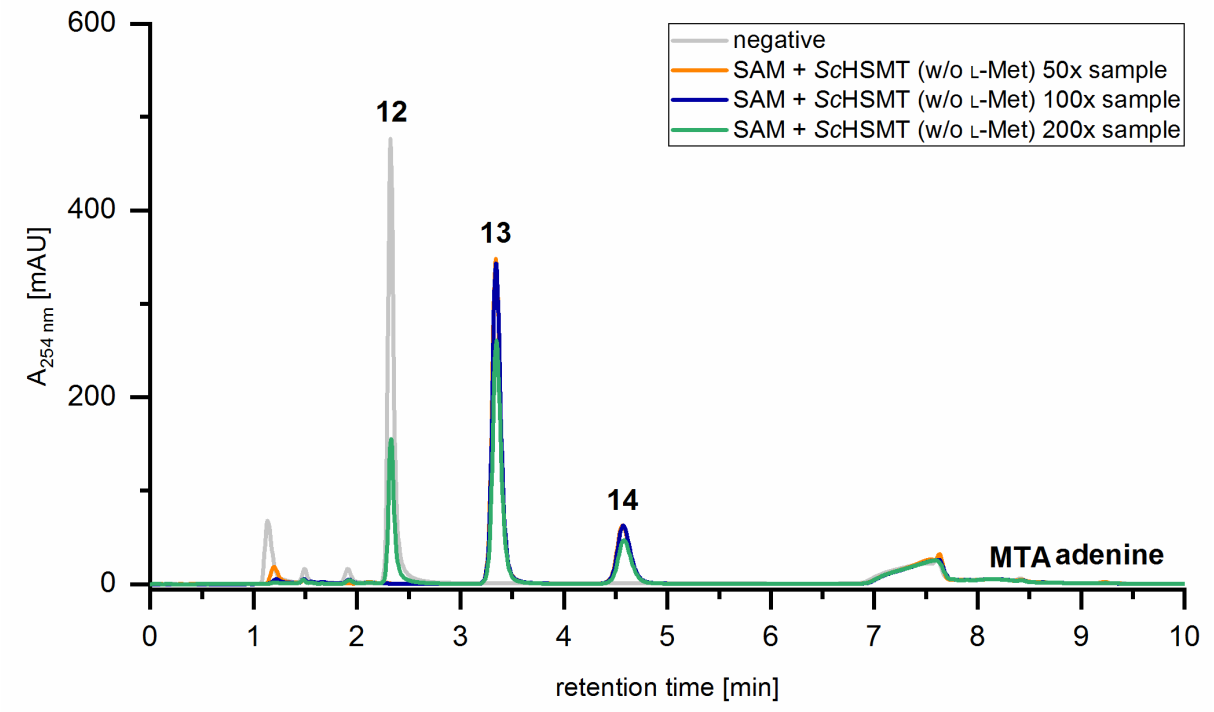


Fig. S15. Typical HPLC chromatograms of the SAM regeneration system with *Rn*COMT and additional L-methionine regeneration using *Sc*HSMT. Starting material: 2 mM MT substrate **12**, 3 mM D/L-SMM, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

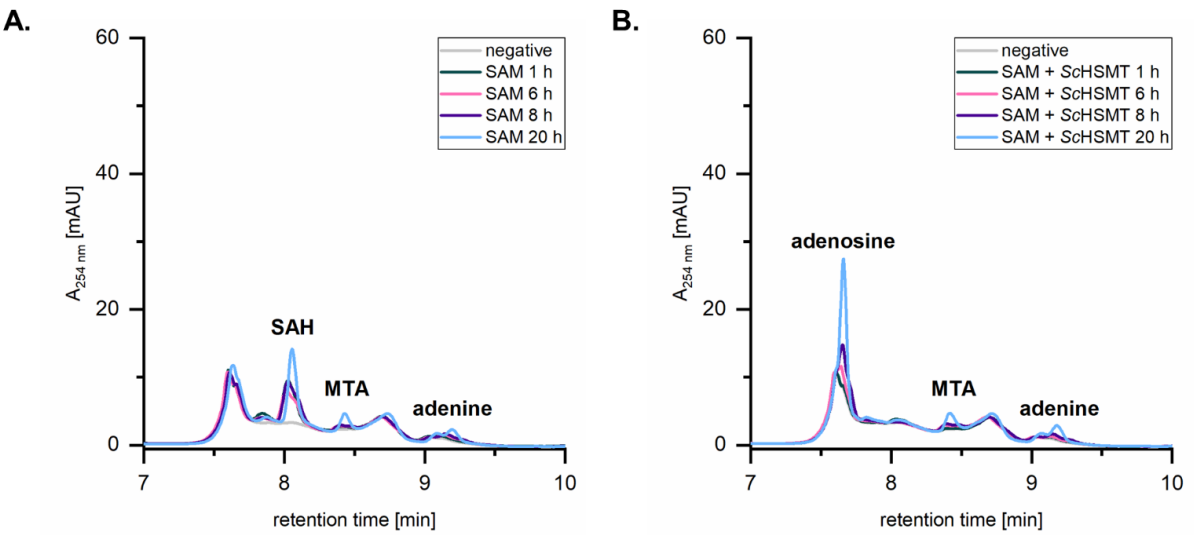


Fig. S16. Time course of MT conversion with A. regular SAM regeneration system and B. with the addition of L-methionine regeneration system using *Sc*HSMT with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine (A) or 3 mM D/L-SMM and 40 µM L-methionine (addition of *Sc*HSMT, B), 20 mM polyP (calculated as single phosphate residues), 40 µM AMP. The same data as in Fig. S21 is shown.

L-Methionine was identified as an impurity in the *S*-methyl-D/L-methionine authentic standard (Fig. S17) when being compared to the L-methionine authentic standard (Fig. S18) using ^{1}H NMR spectroscopy. The manufacturer gives the purity of the chemical at >99.0%.

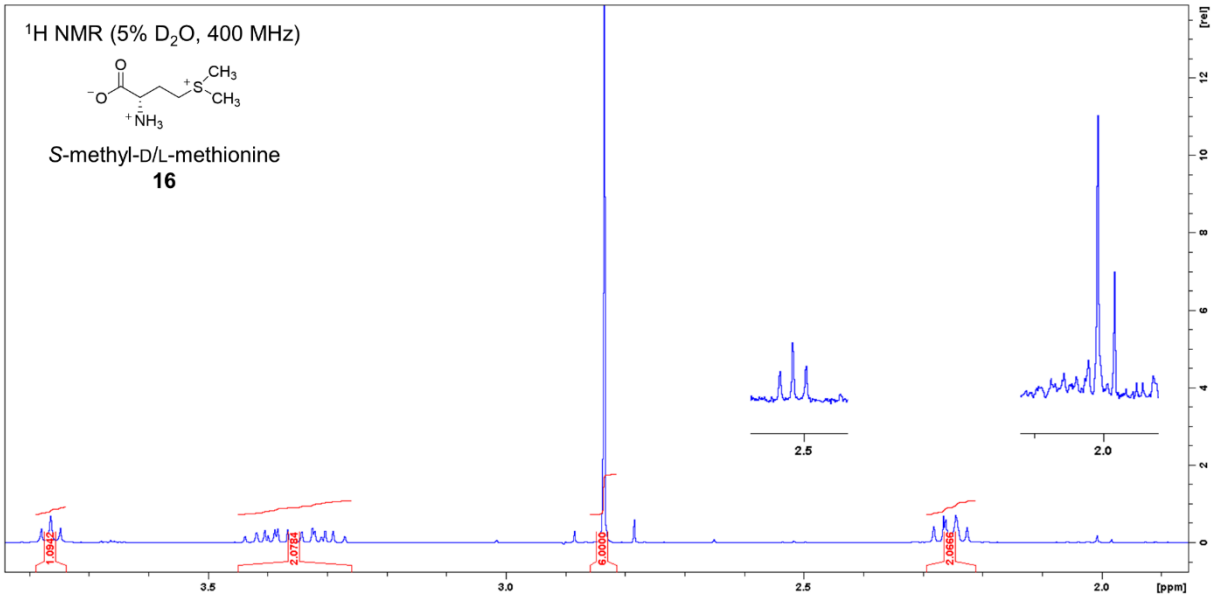


Fig. S17. ^{1}H NMR spectrum of the authentic reference of *S*-methyl-D/L-methionine (**16**) showing the L-methionine impurity in 5% D_2O.

S-Methyl-D/L-methionine (**16**):
^{1}H NMR (5% D_2O, 25 °C, 400 MHz): δ = 3.76 ppm (t, *J* = 6.8 Hz, C_α-H), 3.36 ppm (m, C_γ-H_2), 2.84 ppm (s, CH_3-S), 2.25 ppm (q, *J* = 8.3 Hz, C_β-H_2).

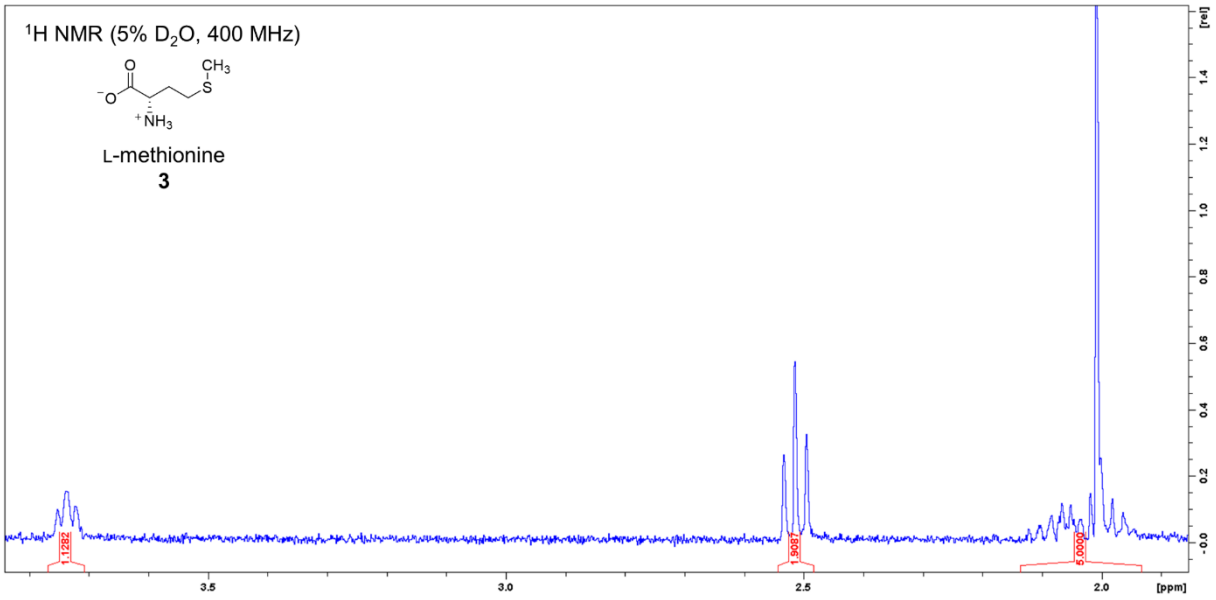


Fig. S18. ^{1}H NMR spectrum of the authentic reference of L-methionine (**3**) in 5% D_2O.

L-Methionine (**3**):
^{1}H NMR (5% D_2O, 25 °C, 400 MHz): δ = 3.74 ppm (t, *J* = 8.0 Hz, C_α-H), 2.51 ppm (t, *J* = 8.0 Hz, C_γ-H_2), 2.04 ppm (m, CH_3-S, C_β-H_2).

N-Methylation also improved by optimised system with L-methionine regeneration

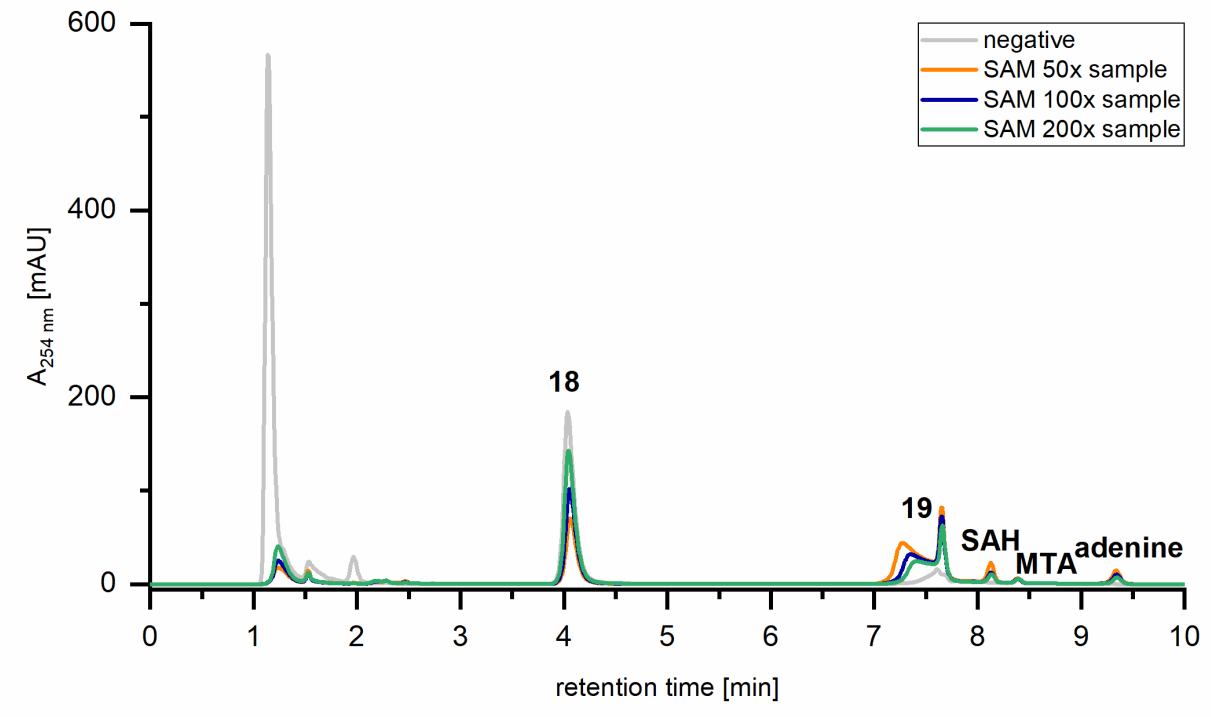


Fig. S19. Typical HPLC chromatograms of the SAM regeneration system with *Rg*ANMT. Starting material: 2 mM MT substrate **18**, 3 mM L-methionine, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

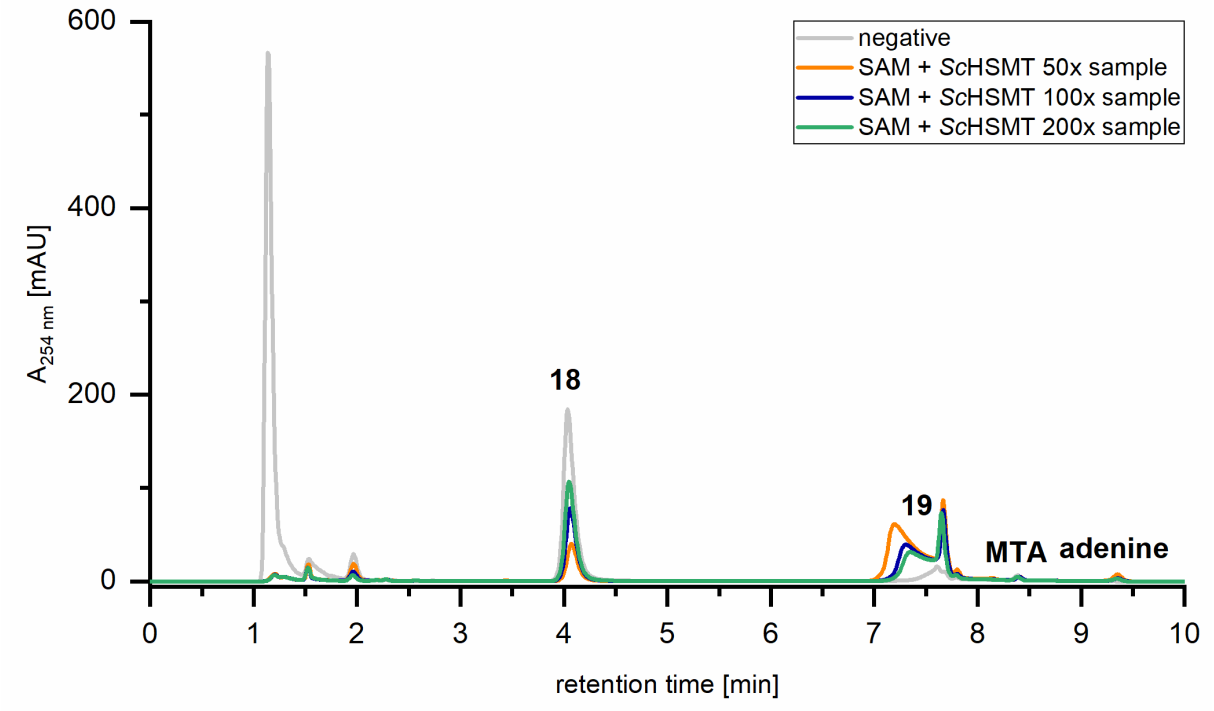


Fig. S20. Typical HPLC chromatograms of the SAM regeneration system with *Rg*ANMT and additional L-methionine regeneration using *Sc*HSMT. Starting material: 2 mM MT substrate **18**, 3 mM D/L-SMM, 40 µM L-methionine, 20 mM polyP (calculated as single phosphate residues), AMP (orange – 40 µM, blue – 20 µM, green – 10 µM).

Cyclic SAM regeneration with L-methionine regeneration is more efficient than without

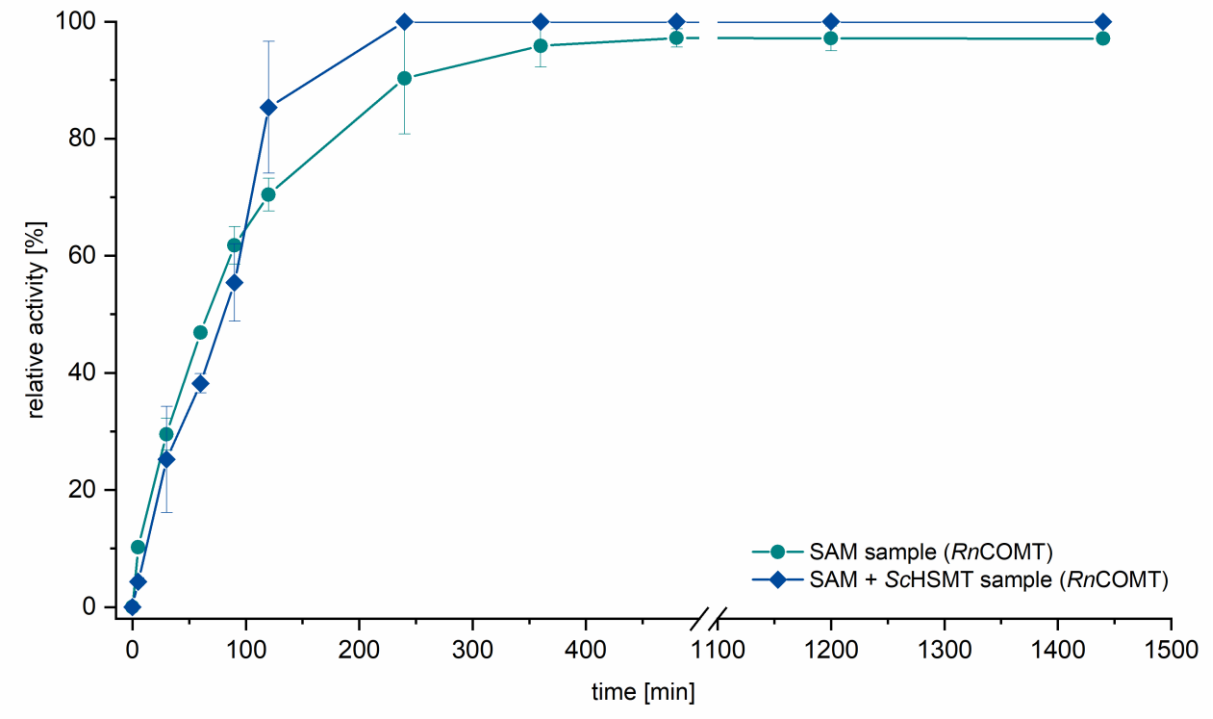


Fig. S21. Time course of MT conversion with regular SAM regeneration system (green) and with the addition of L-methionine regeneration system using *Sc*HSMT (blue) with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine (green) or 3 mM D/L-SMM and 40 µM L-methionine (addition of *Sc*HSMT, blue), 20 mM polyP (calculated as single phosphate residues), 40 µM AMP.

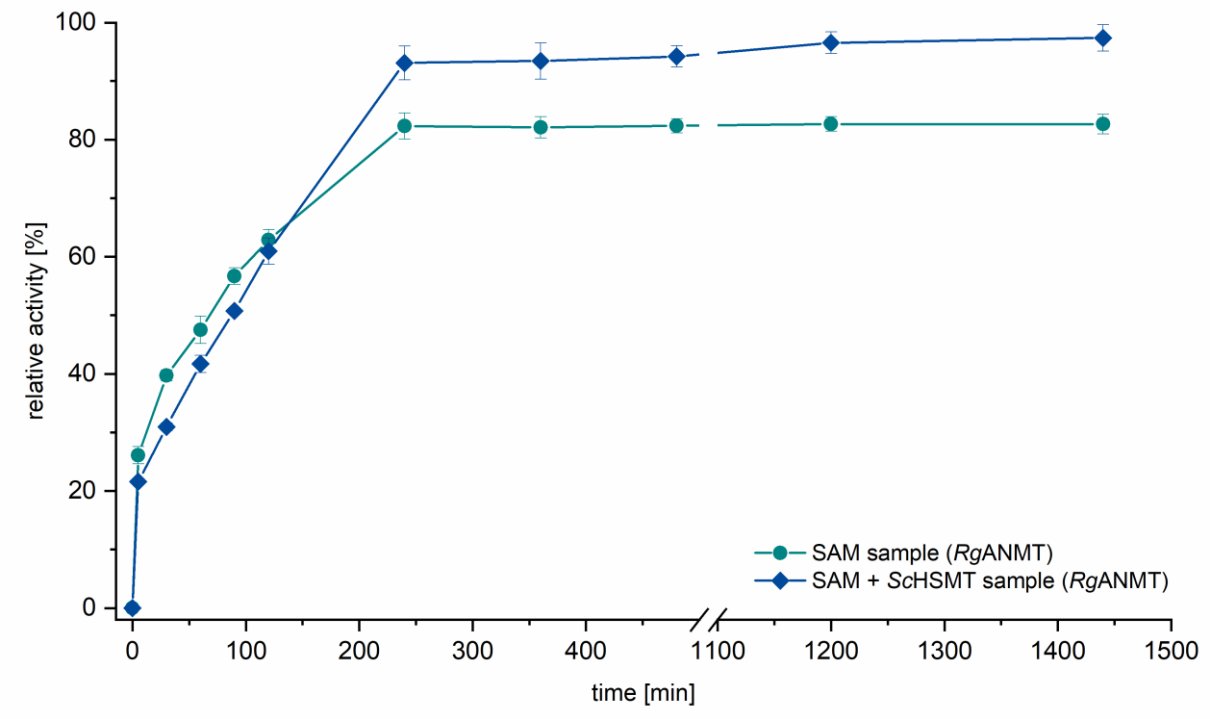


Fig. S22. Time course of MT conversion with regular SAM regeneration system (green) and with the addition of L-methionine regeneration system using *Sc*HSMT (blue) with *Rg*ANMT. Starting material: 2 mM MT substrate **18**, 3 mM L-methionine (green) or 3 mM D/L-SMM and 40 µM L-methionine (addition of *Sc*HSMT, blue), 20 mM polyP (calculated as single phosphate residues), 40 µM AMP.

Regeneration of SAM analogues is possible with nucleobase modifications

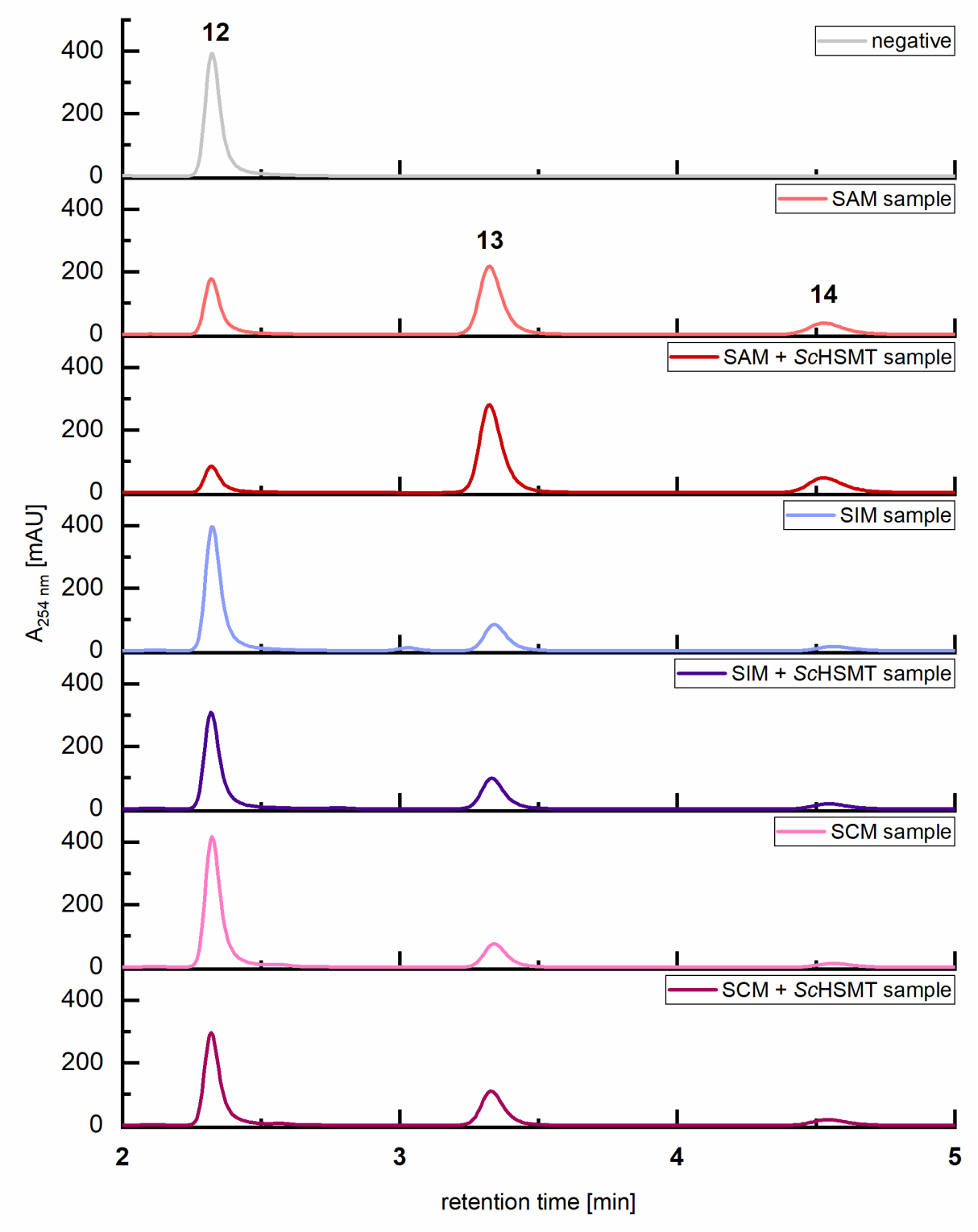


Fig. S23. Typical HPLC chromatograms of the SAM (reds), SIM (purples) and SCM (pinks) regeneration systems with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine (regular regeneration system, light colours) or 3 mM D/L-SMM and 40 µM L-methionine (addition of *Sc*HSMT, dark colours), 20 mM polyP (calculated as single phosphate residues), 40 µM NMP.

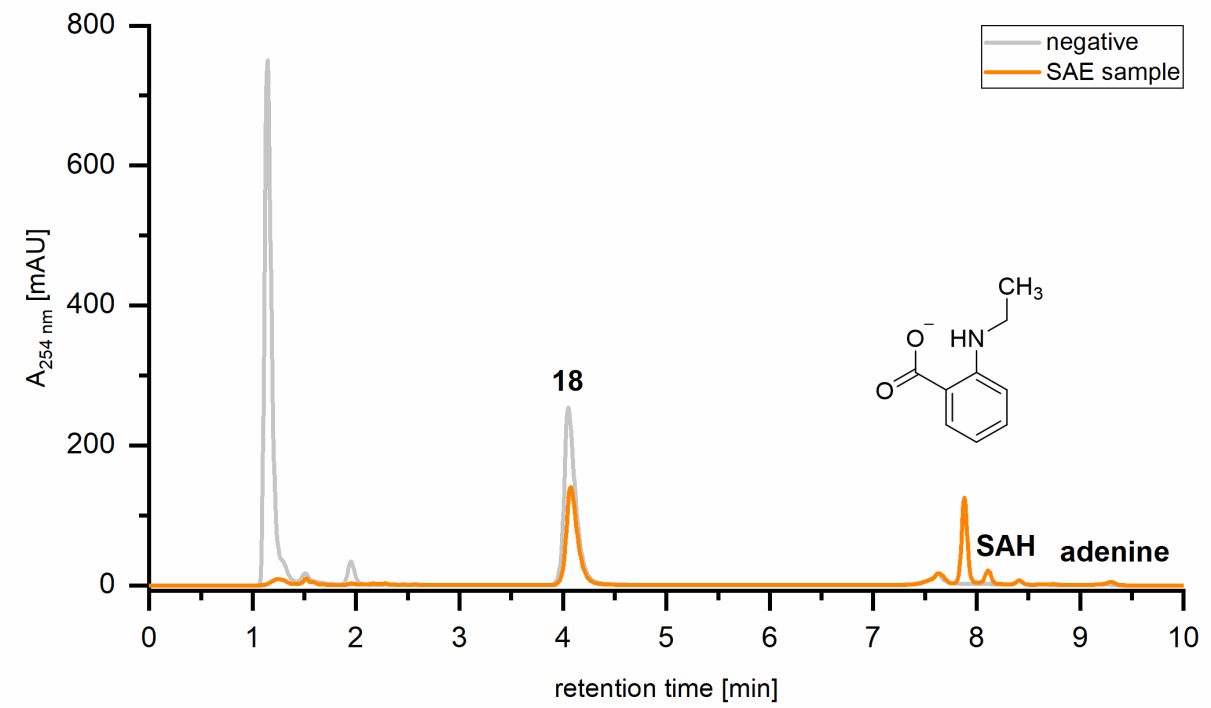


Fig. S24. Typical HPLC chromatograms of the SAE regeneration system with *Rg*ANMT. Starting material: 2 mM MT substrate **18**, 3 mM L-ethionine, 20 mM polyP (calculated as single phosphate residues), 40 µM AMP.

The regeneration system can be used with crude lysates

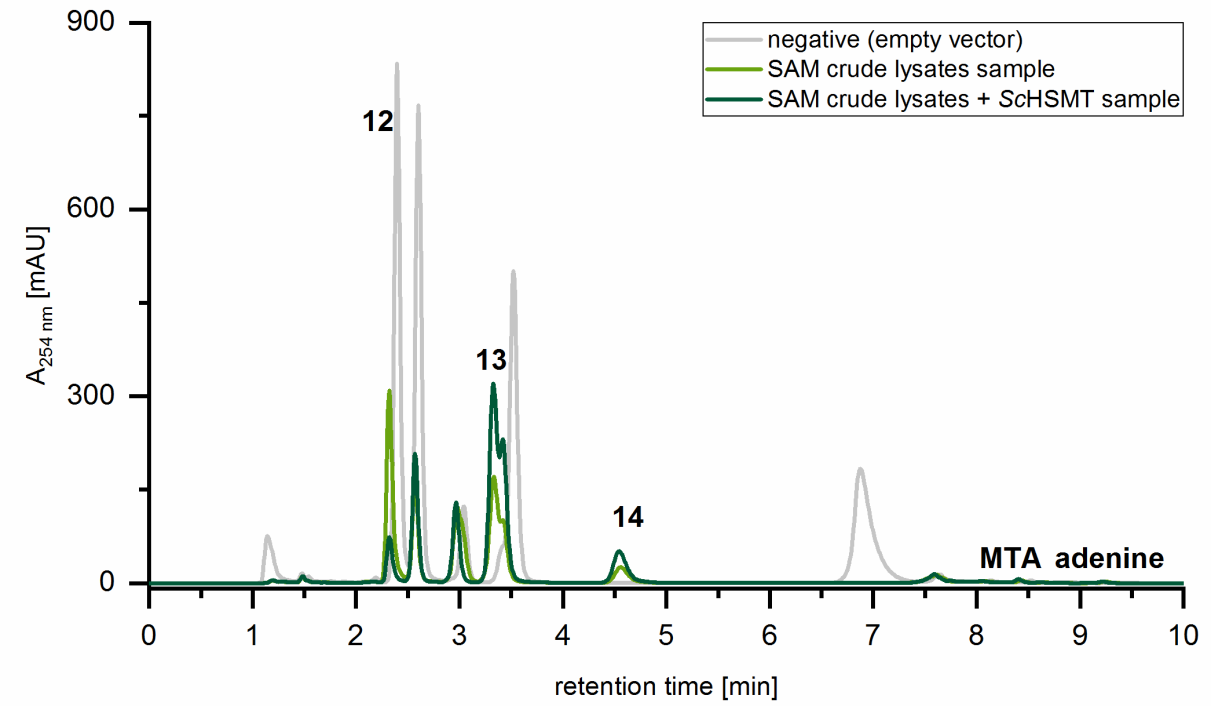


Fig. S25. HPLC chromatograms of the SAM regeneration system using crude lysates with *Rn*COMT. Starting material: 2 mM MT substrate **12**, 3 mM L-methionine (regular regeneration system, green) or 3 mM D/L-SMM and 40 µM L-methionine (addition of *Sc*HSMT, dark green), 20 mM polyP (calculated as single phosphate residues), 40 µM AMP.

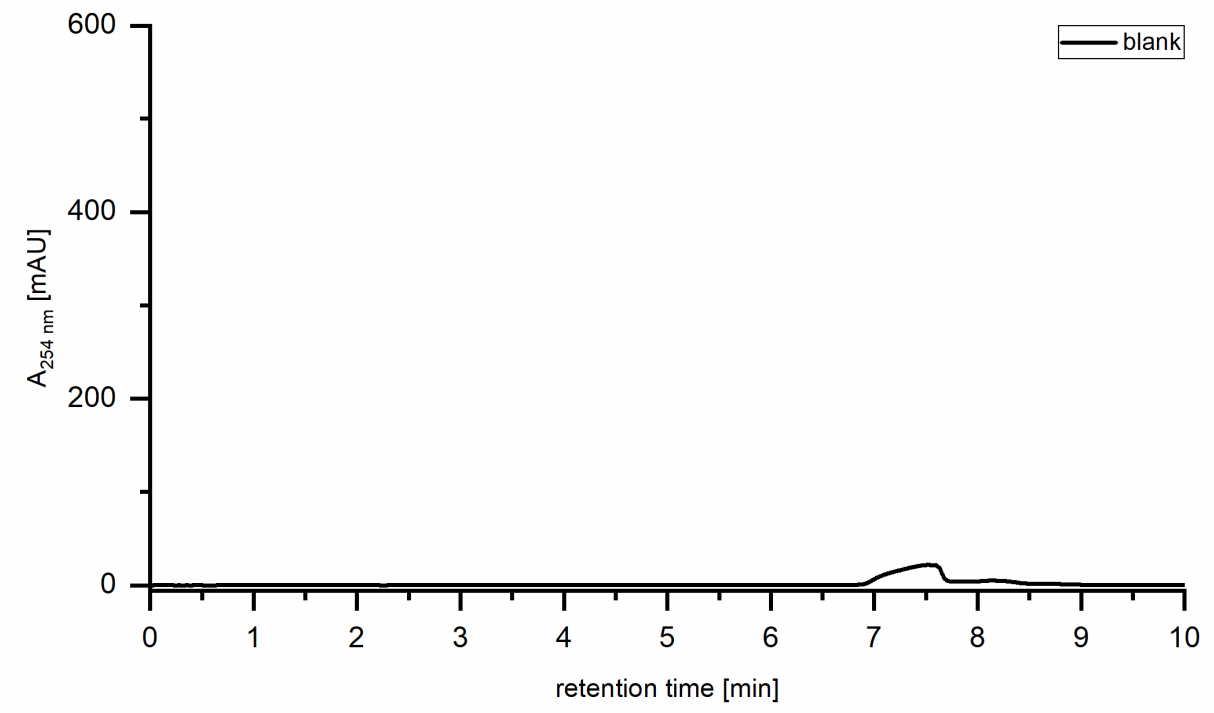


Fig. S26. HPLC chromatogram of a blank sample (injection of water). A peak is always visible between 7 and 8 min.

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