SUPPORTING INFORMATION

Nucleoside-modified AdoMet analogues for differential methyltransferase targeting

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Methods

All chemicals were purchased from Sigma Aldrich, Alfa Aesar or TCI unless otherwise noted. HPLC grade acetonitrile was purchased from VWR. Nucleoside triphosphates and cap analogues were purchased from Jena Bioscience, NEB, TriLink Biotechnologies, Solis BioDyne and Sigma Aldrich. All chemicals were used without further purification.

Synthesis of allyl-D/L-homocysteine (1b)

Allyl-D/L-homocysteine was synthesized as previously reported.[1]

Synthesis of propargyl-L-selenohomocsyteine (*Se*-1c)

First, L-selenohomocsyteine was synthesized in two steps starting from L-homoserine (Activate Scientific) as previously reported by Bothwell *et al.*[2] Under an argon atmosphere L-selenohomocsyteine (119 mg, 0.65 mmol, 1 eq.) was dissolved in 20 mL dry ethanol. Sodium borohydride (124 mg, 3.28 mmol, 5 eq.) was added and the mixture was stirred for 15 min at room temperature. Sodium bicarbonate (150 mg, 1.78 mmol) and propargyl bromide (80 % in toluene, 156 µL, 208 mg, 1.26 mmol, 2 eq.) were added and the mixture stirred for 16 h. The solvent was removed under reduced pressure and the raw product was dissolved in 6 mL ddH_2O with 1 % trifluoroacetic acid. The solution was acidified to pH = 3 using HCl. The solution was centrifuged for 5 min at 21,000 g and purified *via* preparative HPLC. The product was obtained as white crystalline solid (100.4 mg, 0.50 mmol, 77 %)

^{1}H NMR (300 MHz, D_2O): δ / ppm = 3.85 (m, 1H), 3.32 (m, 2H), 2.87 (m, *J* = 7.8 Hz, 2H), 2.67 (t, 1H), 2.27 (m, 2H).

MS (ESI-pos.): calculated mass for $[C_7H_{11}NO_2SeNa]^+$: *m*/*z* = 243.9847, found *m*/*z* = 243.9852 $[M+Na]^+$.

Amino acid sequences of enzymes used in this study

The purification tag is marked in yellow and the substitution site in green. The protein sequence is underlined.

hTGS1

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSEVKKKKNKKKNKKVNG
LPPEIAAVPELAKYWAQRYRLFSRFDDGIKLDREGWFSVTPEKIAEHIAGRVSQSFK
CDVVVDAFCGVGGNTIQFALTGMRVIAIDIDPVKIALARNNAEVYGIADKIEFICGDFL
LLASFLKADVVFLSPPWGGPDYATAETFDIRTMMSPDGFEIFRLSKKITNNIVYFLPR
NADIDQVASLAGPGGQVEIEQNFLNNKLKTITAYFGDLIRRPASET

LuxS

MPSVESFELDHNAVVAPYVRHCGVHKVGTDGVVNKFDIRFCQPNKQAMKPDTIHTL
EHLLAFTIRSHAEKYDHFDIIDISPMGCQTGYYLVVSGEPTSAEIVDLLEDTMKEAVEI
TEIPAANEKQCGQAKLHDLEGAKRLMRFWLSQDKEELLKVFGAAALEHHHHHH

MTAN

MSYYHHHHHHDYDIPTTENLYFQGAMDPEFSMKIGIIGAMEEEVTLLRDKIENRQTIS
LGGCEIYTGQLNGTEVALLKSGIGKVAAALGATLLLEHCKPDVIINTGSAGGLAPTLK
VGDIVVSDEARYHDADVTAFGYEYGQLPGCPAGFKADDKLIAAAEACIAELNLNAVR
GLIVSGDAFINGSVGLAKIRHNFPQAIAVEMEATAIAHVCHNFNVPFVVVRAISDVAD
QQSHLSFDEFLAVAAKQSSLMVESLVQKLAHG

MATIIa I117A

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSELENGQLNGFHEAFIEE
GTFLFTSESVGEGHPDKICDQISDAVLDAHLQQDPDAKVACETVAKTGMILLAGEITS
RAAVDYQKVVREAVKHIGYDDSSKGFDYKTCNVLVALEQQSPDAAQGVHLDRNEE
DIGAGDQGLMFGYATDETEECMPLTIVLAHKLNAKLAELRRNGTLPWLRPDSKTQV
TVQYMQDRGAVLPIRVHTIVISVQHDEEVCLDEMRDALKEKVIKAVVPAKYLDEDTIY
HLQPSGRFVIGGPQGDAGLTGRKIIVDTYGGWGAHGGGAFSGKDYTKVDRSAAYA
ARWVAKSLVKGGLCRRVLVQVSYAIGVSHPLSISIFHYGTSQKSERELLEIVKKNFDL
RPGVIVRDLDLKKPIYQRTAAYGHFGRDSFPWEVPKKLKY

Ecm1

MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGSMEGKKEEIREHYNSIRE
RGRESRQRSKTINIRNANNFIKACLIRLYTKRGDSVLDLGCGKGGDLLKYERAGIGE
YYGVDIAEVSINDARVRARNMKRRFKVFFRAQDSYGRHMDLGKEFDVISSQFSFHY

AFSTSESLDIAQRNIARHLRPGGYFIMTVPSRDVILERYKQGRMSNDFYKIELEKMED
VPMESVREYRFTLLDSVNNCIEYFVDFTRMVDGFKRLGLSLVERKGFIDFYEDEGR
RNPELSKKMGLGCLTREESEVVGIYEVVVFRKLVPESDA

CouO

MGIEPITGSEAEAFHRMGSRAFERYNEFVDLLVGAGIADGQTVVDLCCGSGELEIILT
SRFPSLNLVGVDLSEDMVRIARDYAAEQGKELEFRHGDAQSPAGMEDLLGKADLV
VSRHAFHRLTRLPAGFDTMLRLVKPGGAILNVSFLHLSDFDEPGFRTWVRFLKERP
WDAEMQVAWALAHYYAPRLQDYRDALAQAADETPVSEQRIWVDDQGYGVATVKC
FARRAAALEHHHHHH

ChMAT

MAHHHHHHMDSSRLSGNKSTYTDLQTTSEQFLFSSESVCSGHPDKLCDQISDAILD
ACLEQDPESFVACETCTKTGFIMVFGEITTKANVNYERVVRETVKEIGYDSEEKGLD
YKTMDVIIKLEQQSNQIAGCVHVDKNVEDIGAGDQGMMFGYATNETKELMPLTHVL
ATSITRELDYIRMKGVSSRVGWLRPDGKAQVTVEYNCKHGVLIPKRIHTILVSVQHD
ENIENEEIREFVLENVIKKVCPSDLMDKETRILINPSGRFTIGGPAADAGLTGRKIIVDT
YGGWGAHGGGAFSGKDATKVDRSGAYMARLVAKSIVFSGLCSRCLVQVSYGIGIA
RPLSLYINTFGTAKDGYNDTKLLEIVNKVFDFRPGILIKQLNLKSPIFKKTSSGGHFGR
SEKEFLWEKPIILQ

Tab. S1 Plasmids used in this study.

Vector	Insert	Resistance	Producer
pRSET-A	hTGS1	Ampicillin	Lab plasmid collection[3]
pET-29a(+)	LuxS	Kanamycin	Prof. Zhaohui Zhou, Prof. Birgit Dräger[4]
pProEX HTa	MTAN	Ampicillin	Prof. Zhaohui Zhou, Prof. Birgit Dräger[4]
pRSET-A	MATIIa I117A	Ampicillin	Lab plasmid collection[1]
pRSET-A	Ecm1	Ampicillin	Lab plasmid collection[5]
pET-28a(+)	CouO	Kanamycin	This study
BG1861	ChMAT	Ampicillin	SSGCID

Cloning

The gene *CouO* of *Streptomyces rishiriensis* was codon optimized and synthesized by BioCat GmbH. The obtained sequence was cloned into the expression vector pET-28a(+) upstream of the histidine tag using restriction enzymes FastDigest NcoI and XhoI (Thermo Scientific ™).

Recombinant expression in *E. coli*

For protein production *E. coli* BL21(DE3) cells were transformed with the respective plasmid and cultivated on LB-agar plates supplemented with the respective antibiotics. Single colonies were picked to inoculate an overnight pre-culture. Main cultures were inoculated with 1 % of the pre-culture and induced using isopropyl-*β*-D-1-thiogalactopyranoside (IPTG). Expression conditions for all constructs used in this study are summarized below. After production, cells were centrifuged at 5,000 g for 20 min and pellets were stored at –80 °C for further use.

Tab. S2 Expression conditions of enzymes used in this study.

enzyme	expression host	medium	OD_{600} for induction	IPTG addition	expression conditions
ChMAT	E. coli BL21 (DE3)	LB	0.6-0.8	0.5 mM	16 h at 17 °C
hMAT2a I117A	E. coli BL21 (DE3)	LB	0.6-0.8	0.5 mM	16 h at 17 °C
Ecm1	E. coli BL21-Gold (DE3)	LB	0.6	0.2 mM 2 % EtOH	16 h at 18 °C
hTGS1	E. coli BL21 (DE3)	2YT	0.6	0.32 mM 2 % glucose	16 h at 18 °C
CouO	E. coli BL21 (DE3)	LB	0.8	0.1 mM	16 h at 25 °C
MTAN	E. coli BL21 (DE3)	LB	0.6-0.7	0.2 mM	3 h at 37 °C
LuxS	E. coli BL21 (DE3)	LB	0.6	0.8 mM	6 h at 37 °C

Protein purification using immobilized metal affinity chromatography

Proteins were purified *via* their His-tag by immobilized metal affinity chromatography (IMAC). Cell pellets were resuspended in lysis buffer and PMSF (100 µM final concentration) was added. The suspension was sonicated for 3×3 min at an amplitude of 30 % using a Sonopuls GM3100 (Bandelin, Berlin). The cell lysate was centrifuged at 21,000 g for 30 min (4 °C). The supernatant was filtered through a 0.2 µm filter and loaded into the Superloop of the ÄKTApurifier. A HisTrap FF column (1 mL) was equilibrated in lysis buffer, the sample was injected and proteins were eluted by an increasing gradient of imidazole.

Fractions containing the desired protein were pooled. Concentration and buffer exchange to storage buffer was performed using Amicon Ultra-15 centrifugal units (10 kDa cut-off). Concentrated proteins were flash frozen in liquid nitrogen and stored in aliquots at -80 °C.

Tab. S3 Buffers used for IMAC.

ChMAT
Lysis buffer: 20 mM NaH_2PO_4 pH 7.8, 300 mM NaCl, 10 mM imidazole
Elution buffer: 20 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 500 mM imidazole
Storage buffer: 25 mM Tris HCl pH 8.0, 80 mM KCl, 10 % glycerol
CouO
Lysis buffer:100 mM Tris HCl pH 8.0, 150 mM NaCl, 10 mM imidazole
Elution buffer: 100 mM Tris HCl pH 8.0, 150 mM NaCl, 500 mM imidazole
Storage buffer: 25 mM Tris HCl pH 8.0, 80 mM KCl, 10 % glycerol

HPLC analysis

HPLC was performed on an Agilent 1260 Infinity HPLC equipped with a diode array detector (DAD) (190–640 nm).

For analytical reversed-phase HPLC, a NUCLEODUR® C18 Pyramid (5 µm, 125×4 mm) column from Macherey-Nagel was used. Elution was performed at a flow rate of 1 mL/min in a linear gradient of buffer A (100 mM K_2HPO_4/KH_2PO_4 pH = 6.5) and buffer B (50 % buffer A, 50 % MeCN).

Detection was performed using a diode array detector (DAD) at a wavelength of 225 nm for methionine analogues (**1a, 1b, Se-1c**) and at 260 nm for GpppA (**16**) ATP analogues, AdoMet analogues as well as their degradation products. For the assignment of the cap analogues (**16a-c**, **17a,c**) a detection wavelength of 300 nm was used. Coumarin derivatives were detected at 330 nm.

Evaluation for Figure 3 was performed as follows:

1. Integration of peaks for GpppA, m^7GpppA or p^7GpppA
2. Calculation of conversion (in %) (product/(educt+product)
3. Normalization of conversion obtained with AdoMet analog to conversion obtained with AdoMet.

LC/MS analysis

UHPLC-ESI-TOF-MS analysis was performed using an UltiMate 3000® UHPLC (Thermo Scientific, Waltham, USA) coupled to a maXis II ultra-high resolution QTOF (Bruker, Bremen). Separation was performed using a NUCLEODUR® C18 Pyramid column (125×2 mm, Macherey-Nagel) eluting in a gradient of 20 mM ammonium formate (pH = 3.5, buffer A) and methanol (buffer B) at a flow-rate of 0.6 mL/min.

For LC/MS analysis of AdoMet analogues the respective methionine analogue **1a, 1b,** or **Se-1c** (6 mM) was incubated with an ATP analogue (**2**,**4** or **5,** 2.5 mM) and 100 µM ChMAT in a total volume of 25 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8.0). The reaction was incubated for 3 h at 23 °C. The enzymes were inactivated by the addition of 10 % (v/v) of 1 M $HClO_4$ and centrifuged for 10 min at 21,000 g. The supernatant was directly analyzed *via* LC/MS.

Enzymatic generation of AdoMet analogues

For the enzymatic generation of AdoMet analogues, L-methionine (**1a**, 6 mM) was incubated with ATP analogues (**2**-**8**, 2.5 mM), 25 µM MAT (hMAT2a I117A, ChMAT), 30 µM Ecm1, 5 µM MTAN, 5 µM LuxS and 300 µM GpppA in a total volume of 30 µL.[†1],[6, 7] Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). Samples were taken after 0 h, 1 h, 2 h, 3 h, 4 h and 8 h, stopped by the addition of 10 % (v/v) of 1 M $HClO_4$ and centrifuged for 10 min at 21,000 g. The supernatant was directly analyzed *via* HPLC.

Enzymatic cascade using N^6-modified ATP analogues

For the enzymatic cascade, the respective methionine analogue **1a, 1b** or **Se1-c**, (6 mM) was incubated with an ATP analogue (**4** or **5**, 2.5 mM), 100 µM ChMAT, 30 µM Ecm1, 5 µM MTAN, 5 µM LuxS and GpppA (**16**, 300 µM) in a total volume of 50 µL.

[†1] MTAN and LuxS are added to degrade the coproduct SAH (or SAH analogs) which are known to inhibit MTases.

Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8.0). Samples were taken after 0 h, 1 h, 2 h, 3 h, 4 h and 8 h.

Samples containing **4** were inactivated (5 min, 95 °C) and dephosphorylated with FastAP (ThermoFisher Scientific) for 30 min at 23 °C to avoid coelution of **4** with **16b**. Next, the samples were acidified by the addition of 10 % (v/v) of 1 M $HClO_4$, centrifuged for 10 min at 21,000 g and the supernatant was directly analyzed *via* HPLC.

Enzymatic cascade using different MTases

For the enzymatic cascade using different MTases 6 mM L-methionine (**1a**) or propargyl-L-selenohomocysteine (***Se*-1c**) was incubated with 2.5 mM ATP (**2**) or b^6ATP (**5**), 100 µM ChMAT, 30 µM MTase (Ecm1, hTGS1 or CouO), 5 µM MTAN, 5 µM LuxS and 300 µM MTase substrate (GpppA (**16**), m^7GpppA (**16a**) or 4,5,7-trihydroxy-3-phenylcoumarin (**18**)) in a total volume of 20 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). Samples were taken after 0 h, 30 min and 8 h. The reaction was stopped by the addition of 10 % (v/v) of 1M $HClO_4$ and centrifuged for 10 min at 21,000 g. The supernatant was directly analyzed *via* HPLC.

Supplementary Figures

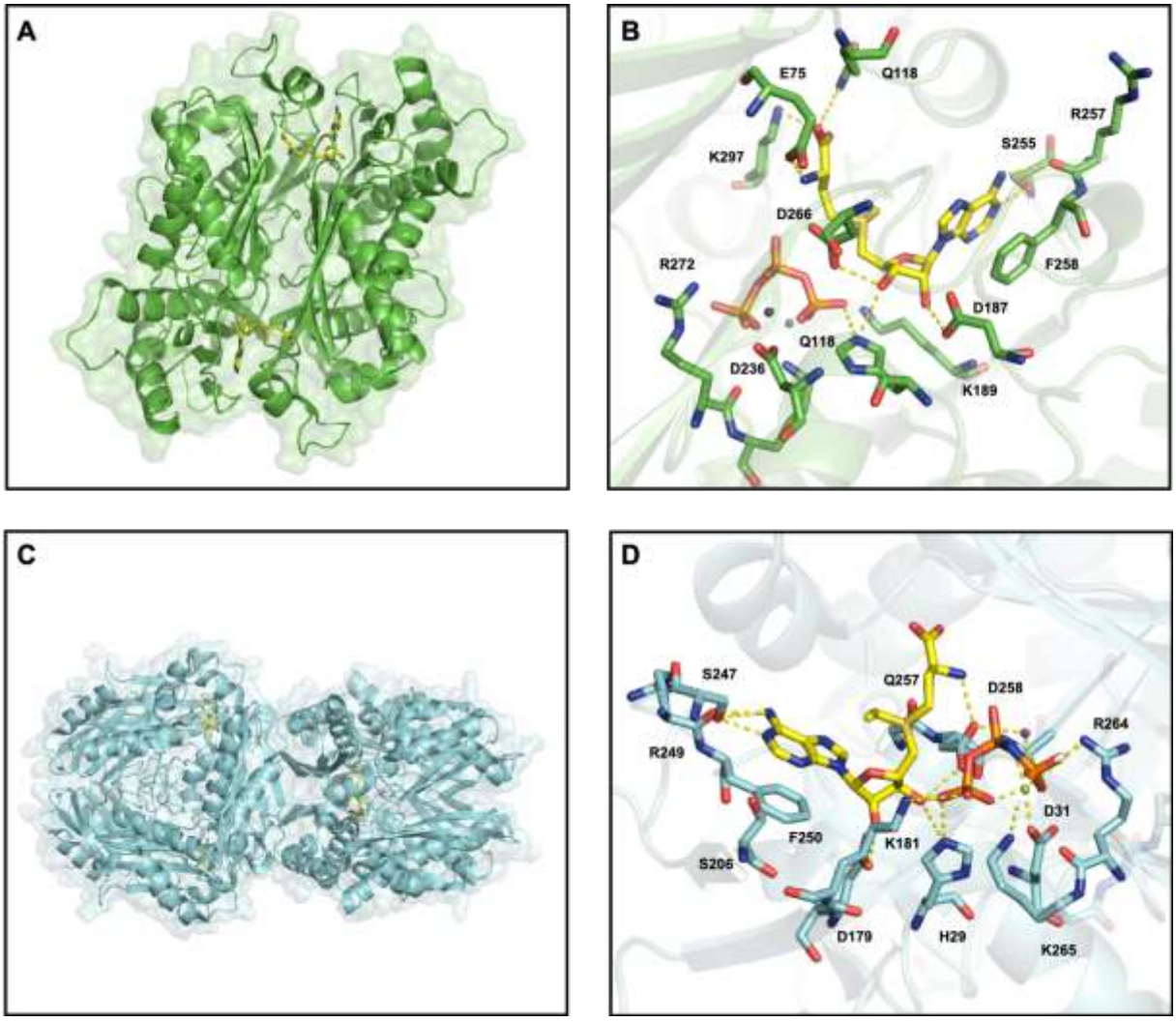

Fig. S1: Crystal structure of ChMAT (PDB: 4ODJ) deposited by the Seattle Structural Genomics Center for Infectious Disease (SSGCID) and of hMAT2a (PDB: 5A1G). A) Homodimer of ChMAT in complex with *S*-adenosylmethionine (SAM and triphosphate (1.6 Å). The SAM molecule indicates the position of the binding sites. B) Active site of ChMAT dimer. Residues that form H-bonds with SAM (yellow) and triphosphate (orange) are shown in sticks, H-bonds are shown as dashed yellow lines and magnesium ions are shown as grey spheres. C) Homotetramer of hMAT2a in complex with S-adenosylethionine (SAE) and (diphosphono)aminophosphonic acid (PPNP) (1.83 Å). The SAE molecule indicates the position of the binding sites. D) Active site of ChMAT dimer. Residues that form H-bonds with SAE (yellow), triphosphate (orange) are shown in sticks, H-bonds are shown as dashed yellow lines, magnesium and potassium ions are shown as green and purple spheres respectively.

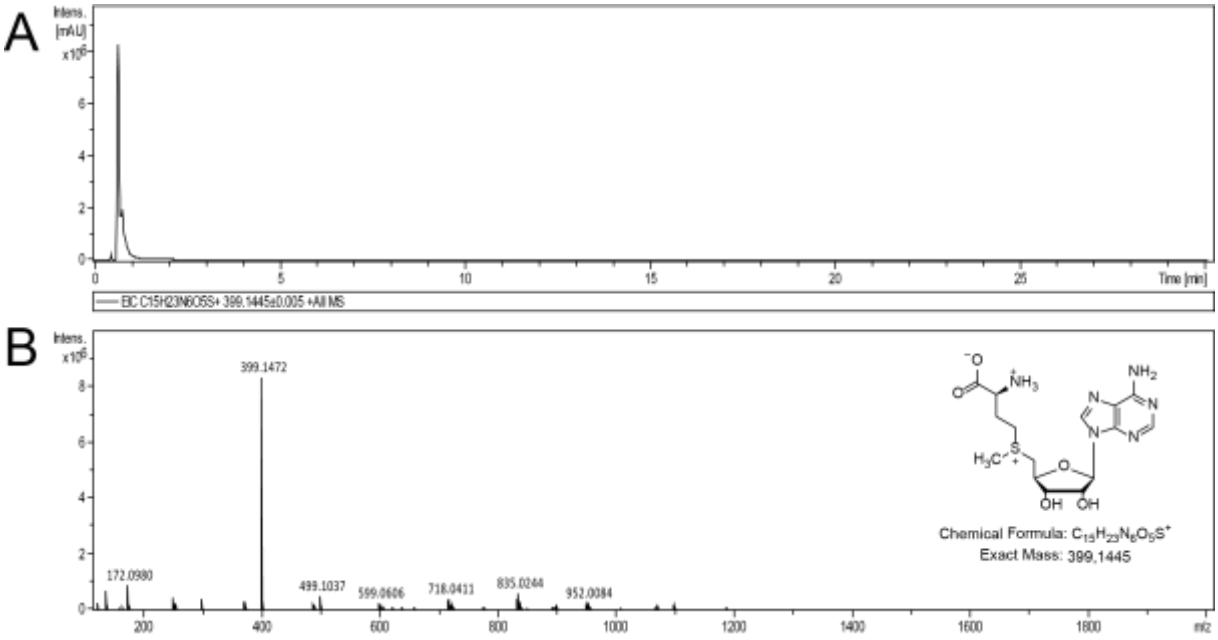

Fig. S2: LC/MS analysis of compound **9a**. Calculated mass for $[M]^+$ = 399.1445 Da, found m/z = 399.1472.

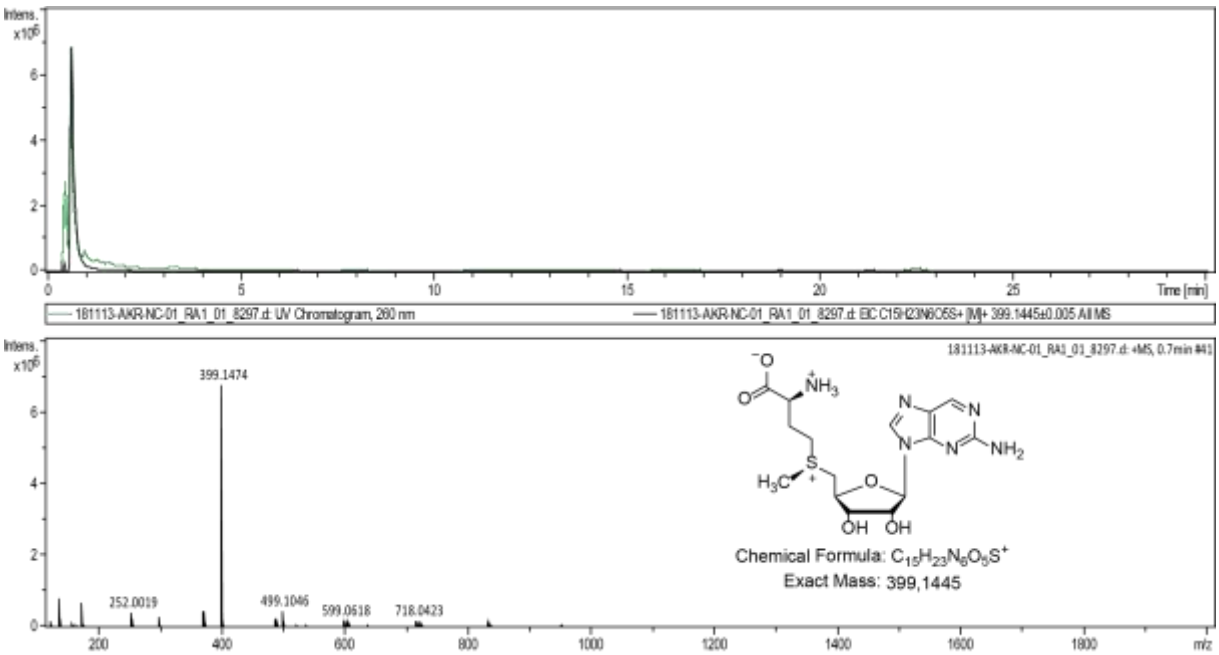

Fig. S3: LC/MS analysis of compound **10a**. Calculated mass for $[M]^+$ = 399.1445 Da, found m/z = 399.1474.

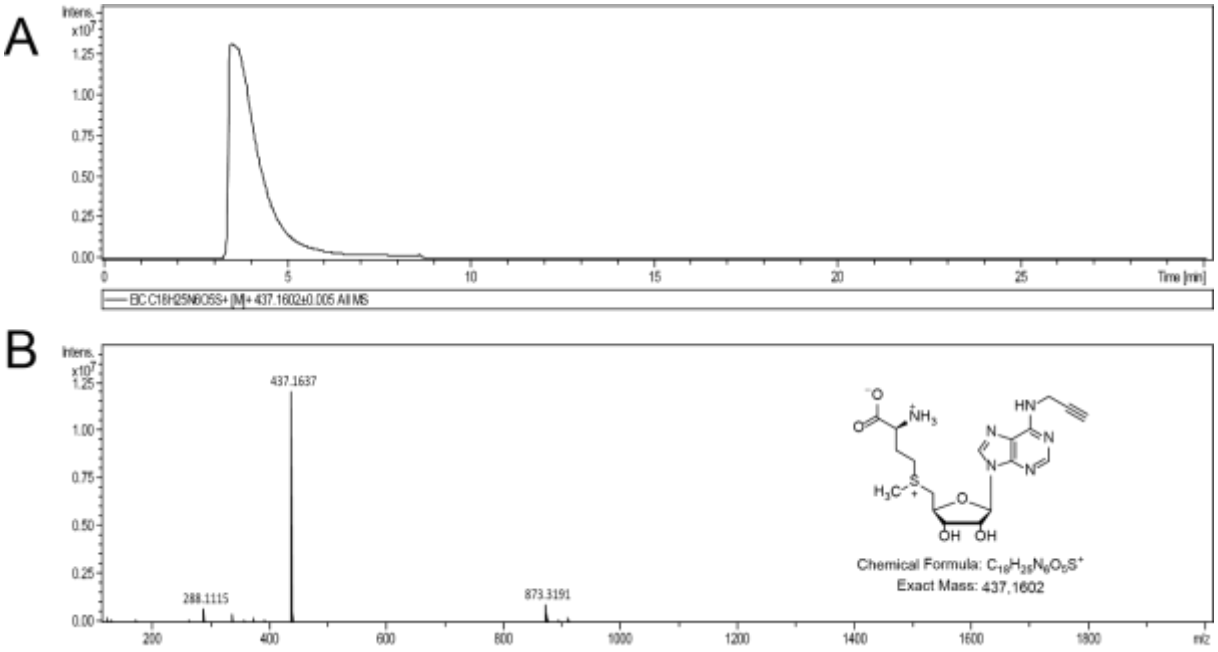

Fig. S4: LC/MS analysis of compound **11a**. Calculated mass for $[M]^+$ = 437.1602 Da, found m/z = 437.1637.

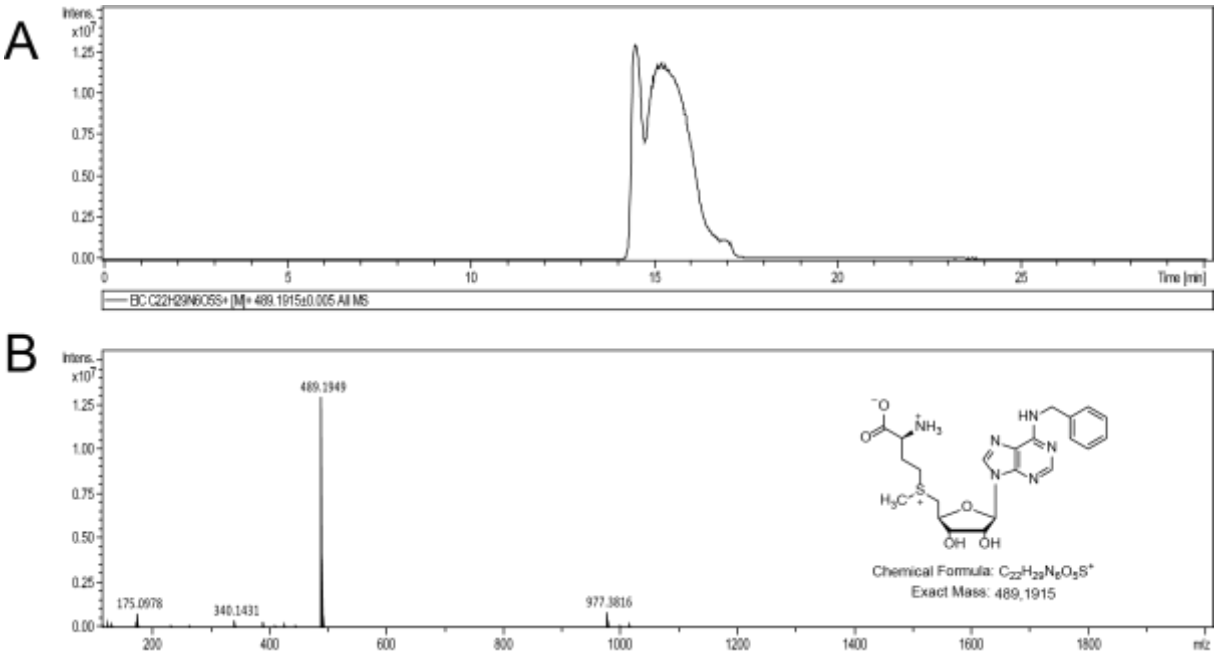

Fig. S5: LC/MS analysis of compound **12a**. Calculated mass for $[M]^+$ = 489.1915 Da, found m/z = 489.1949.

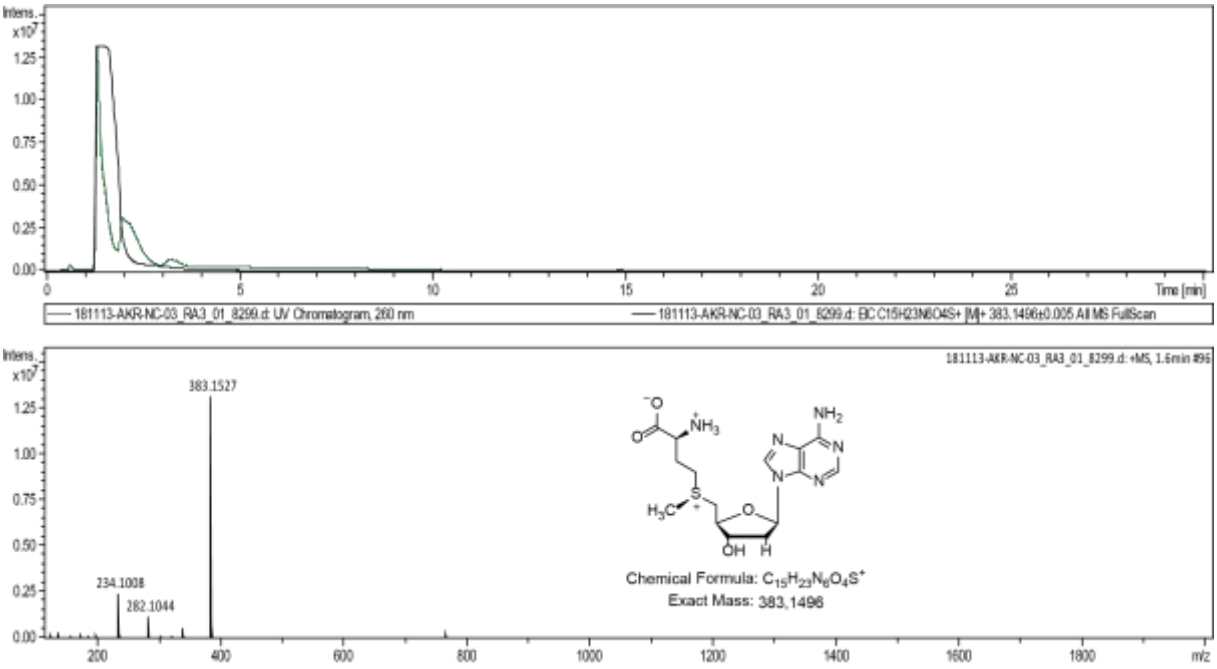

Fig. S6: LC/MS analysis of compound **13a**. Calculated mass for $[M]^+$ = 383.1496 Da, found m/z = 383.1527.

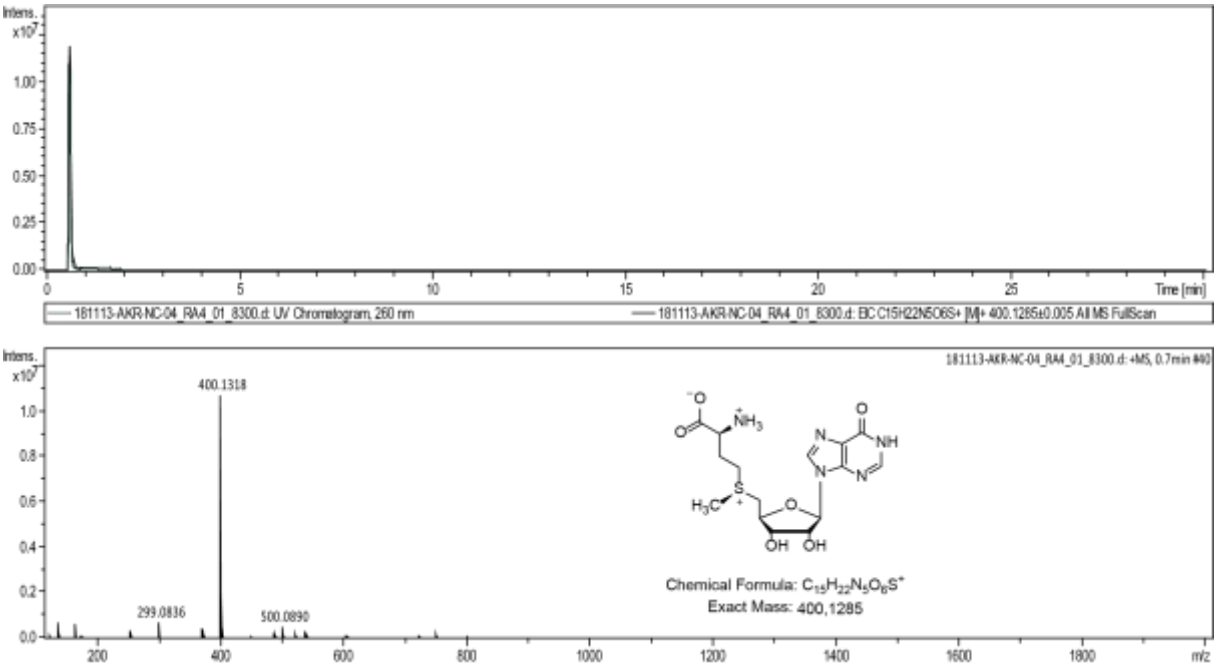

Fig. S7: LC/MS analysis of compound **14a**. Calculated mass for $[M]^+$ = 400.1285 Da, found m/z = 400.1318.

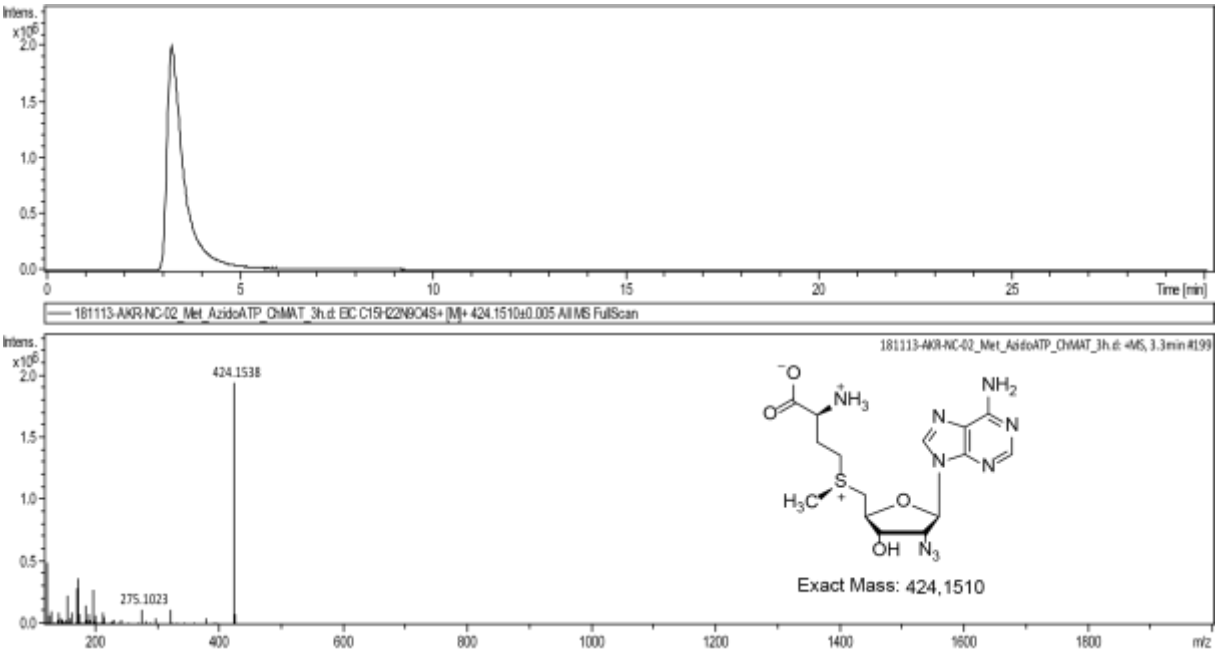

Fig. S8: LC/MS analysis of compound **15a**. Calculated mass for $[M]^+$ = 424.1510 Da, found m/z = 424.1538.

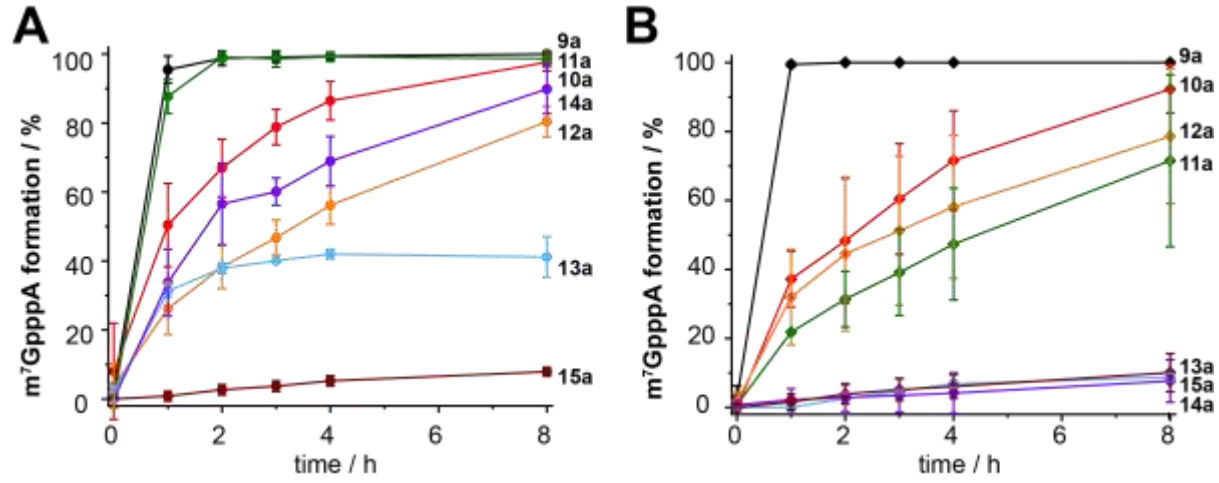

Fig. S9: Data from Figure S3 including error bars.

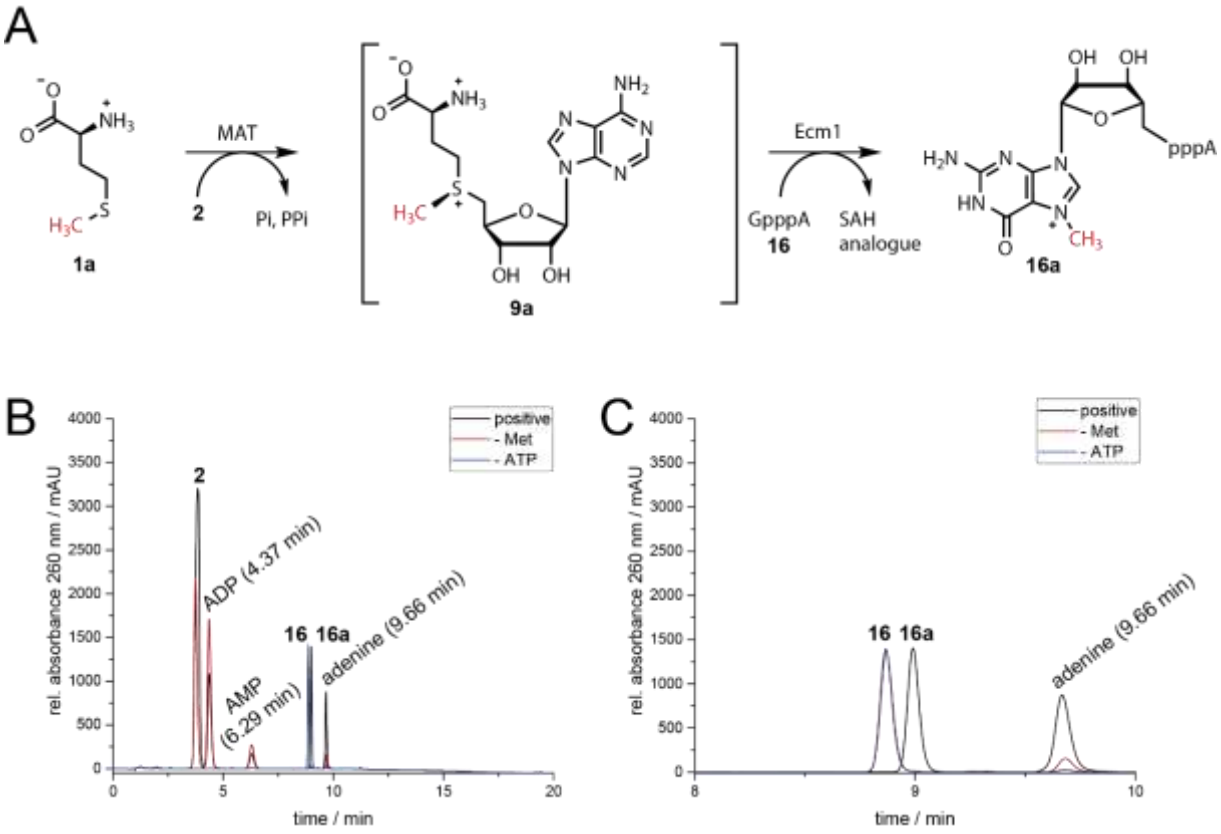

Fig. S10 [A] Controls for enzymatic cascade reaction with ChMAT and Ecm1 (**1a**, **2**, **16** leading to formation of **16a**). [B] Entire chromatogram of the reaction and [C] Enlarged view for relevant compounds. No product formation (**16a**) is observed if methionine or ATP are left out. Conditions: 6 mM L-methionine (**1a**) 2.5 mM ATP (**2**), 25 µM ChMAT, 30 µM Ecm1, 5 µM MTAN, 5 µM LuxS and 300 µM GpppA (**16**) in a total volume of 20 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). Reaction mixture was incubated for 8 h.

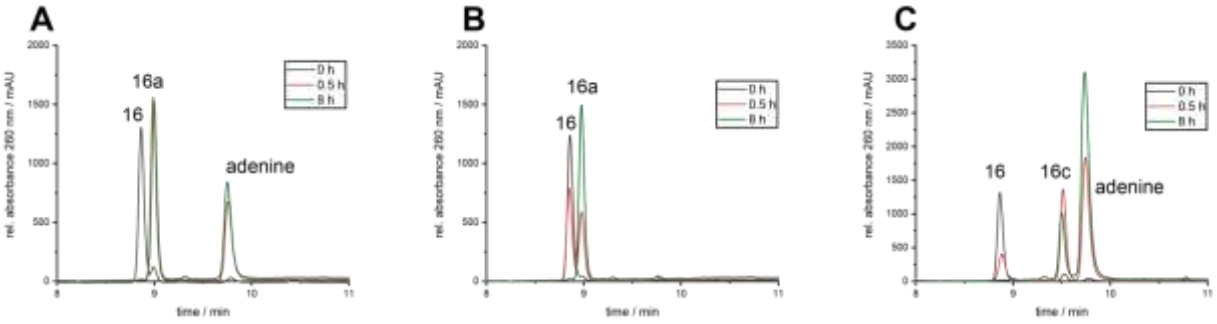

Fig. S11: HPLC analysis of cascade reactions. AdoMet analogue formation starting from different methionine analogues (6 mM) and ATP analogues (2.5 mM) catalyzed by ChMAT and subsequent alkylation reactions catalyzed by Ecm1. Reaction mixture contained 100 µM ChMAT, 30 µM Ecm1, 5 µM MTAN, 5 µM LuxS and 300 µM GpppA (**16**) in a total volume of 20 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). [A] L-methionine (**1a**) and ATP (**2**) [B] L-methionine (**1a**) and b^6ATP (**5**) [C] propargyl-L-selenohomocysteine (***Se*-1c**) and ATP (**2**). Samples were taken after 0 h and 0.5 h and 8 h. Note that adenine is only formed in [A] and [C].

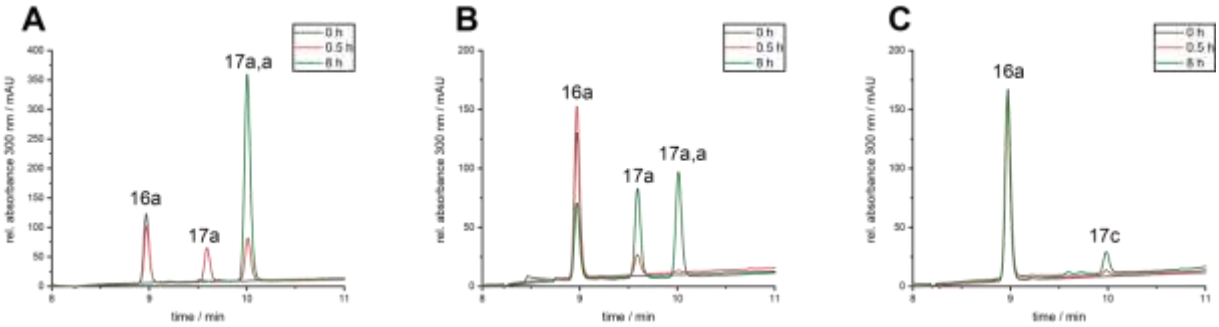

Fig. S12: HPLC analysis of cascade reactions. AdoMet analogue formation starting from different methionine analogues (6 mM) and ATP analogues (2.5 mM) catalyzed by ChMAT and subsequent alkylation reactions catalyzed by hTGS1. Reaction mixture contained 100 µM ChMAT, 30 µM hTGS1, 5 µM MTAN, 5 µM LuxS and 300 µM m^7GpppA (**16a**) in a total volume of 20 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). [A] L-methionine (**1a**) and ATP (**2**) [B] L-methionine (**1a**) and b^6ATP (**5**) [C] propargyl-L-selenohomocysteine (***Se*-1c**) and ATP (**2**). Samples were taken after 0 h and 0.5 h and 8 h. Note that a single methyl group can be attached at the N^2 position of **16a** to form **17a**. Subsequently a second methylation occurs at the same *N* atom to form **17a,a**. For the propargylation reaction only the single alkylated product **17c** is observed.

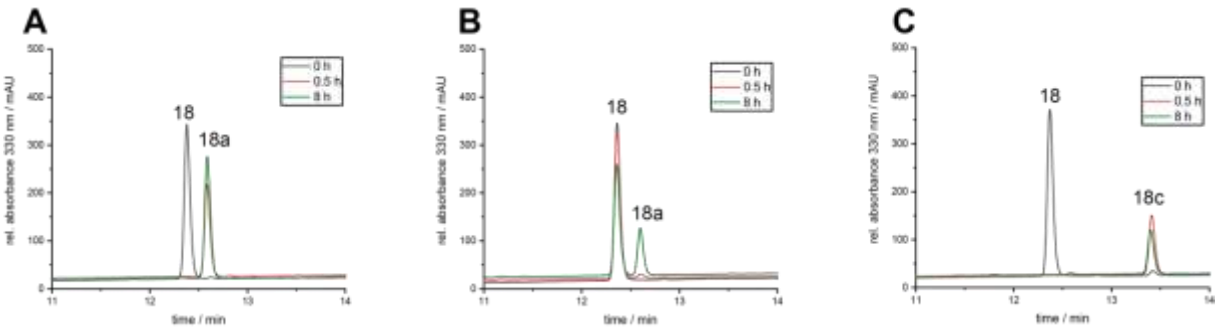

Fig. S13: HPLC analysis of cascade reactions. AdoMet analogue formation starting from different methionine analogues (6 mM) and ATP analogues (2.5 mM) catalyzed by ChMAT and subsequent alkylation reactions catalyzed by CouO. Reaction mixture contained 100 µM ChMAT, 30 µM CouO, 5 µM MTAN, 5 µM LuxS and 300 µM THPC (**18**) in a total volume of 20 µL. Reaction buffer consisted of 5 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM KCl (pH = 8). [A] L-methionine (**1a**) and ATP (**2**) [B] L-methionine (**1a**) and b^6ATP (**5**) [C] propargyl-L-selenohomocysteine (***Se*-1c**) and ATP (**2**). Samples were taken after 0 h and 0.5 h and 8 h.

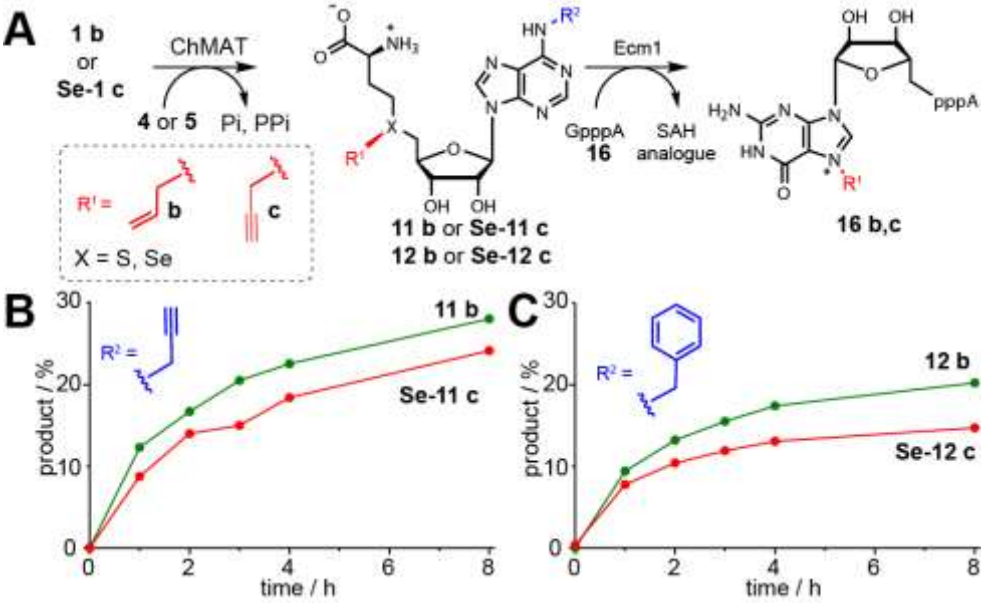

Fig. S14: Methionine analogues (**1b**, **Se-1c**) can be reacted with N^6-modified-ATP. Double modified AdoMet analogues (**11a,b**, **Se-11c** or **12a,b, Se-12c**) are formed and are converted by Ecm1. A) Reaction scheme. B)-C) Cascade reactions with indicated methionine analogues and N^6-propargyl-ATP (**4**) (B) or N^6-benzyl-ATP (**5**) (C). Conditions: 6 mM **1b** or **Se-1c**, 2.5 mM **4** or **5,** 4 mol% ChMAT (rel. to **4** or **5**), 300 µM **16**, 10 mol% Ecm1 (rel. to **16**)**.**

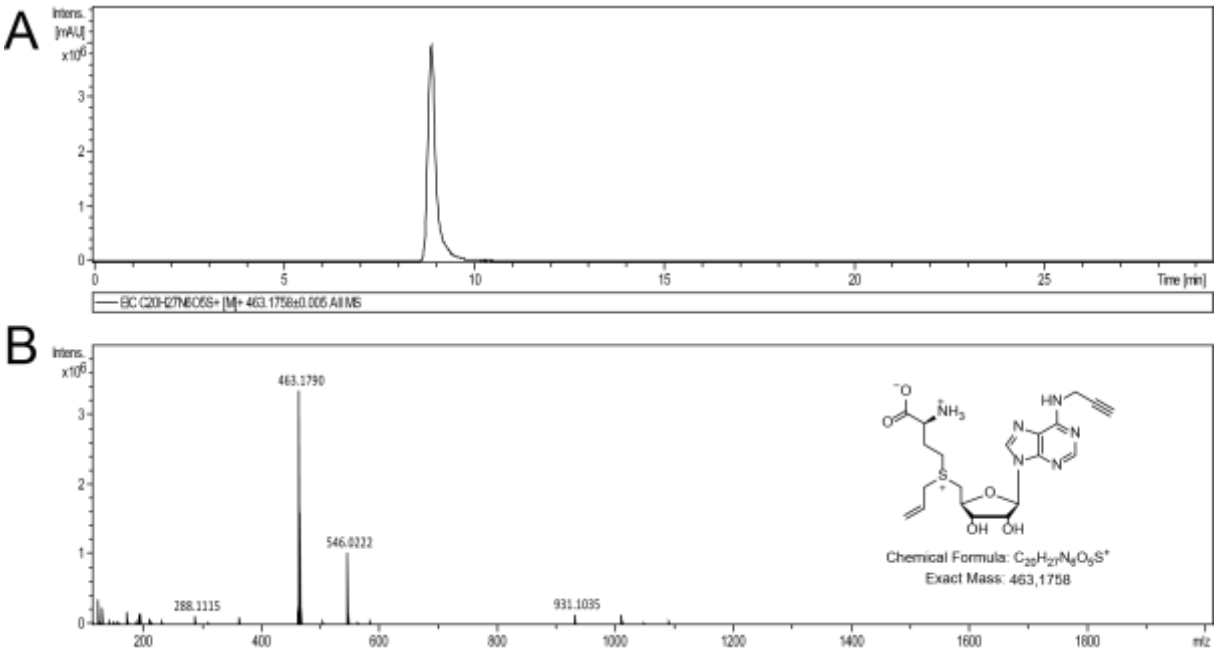

Fig. S15: LC/MS analysis of compound **11b**. Calculated mass for $[M]^+$ = 463.1748 Da, found m/z = 463.1790.

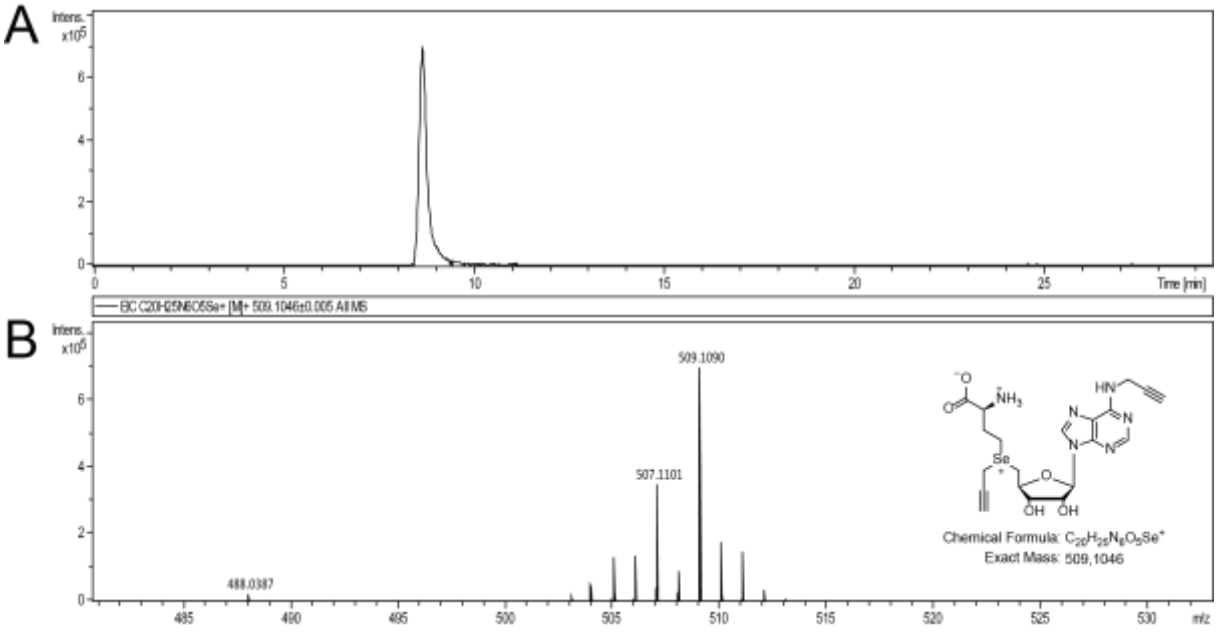

Fig. S16: LC/MS analysis of compound ***Se*-11c**. Calculated mass for $[M]^+$ = 509.1046 Da, found m/z = 509.1090.

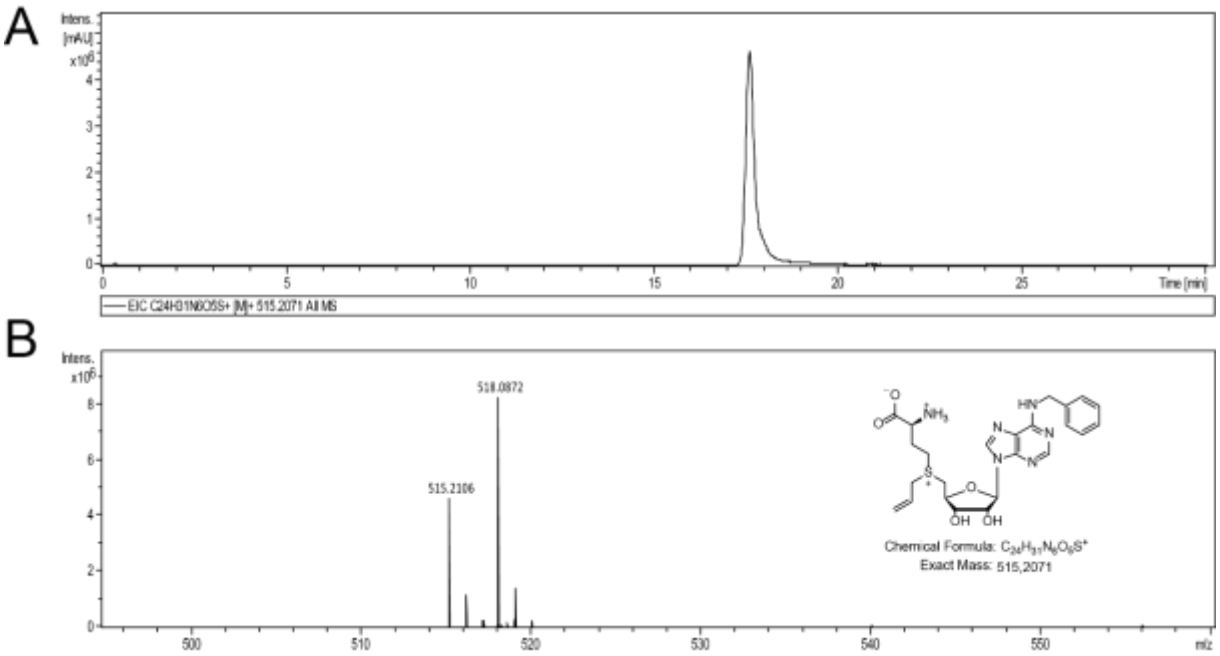

Fig. S17: LC/MS analysis of compound **12b**. Calculated mass for $[M]^+$ = 515.2071 Da, found m/z = 515.2106. Note that the m/z = 518.0872 corresponds to the mass of N^6-benzyl-ADP with a calculated mass of $[M+H]^+$ = 518.0836 that coelutes.

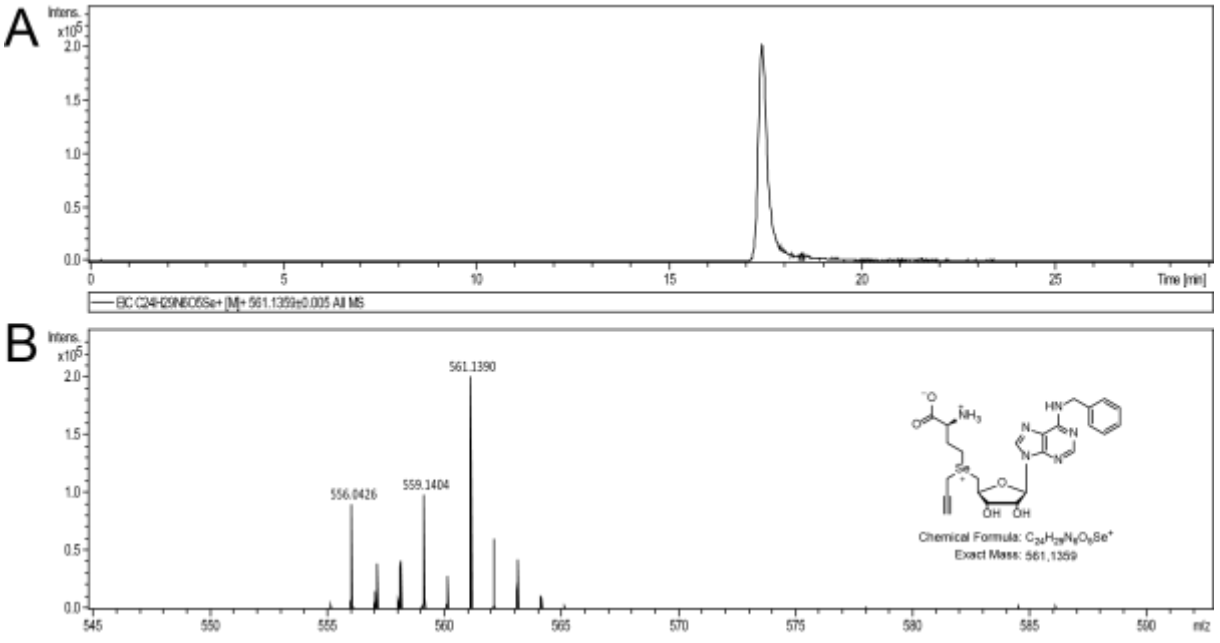

Fig. S18: LC/MS analysis of compound ***Se*-12c**. Calculated mass for $[M]^+$ = 561.1359 Da, found m/z = 561.1390.

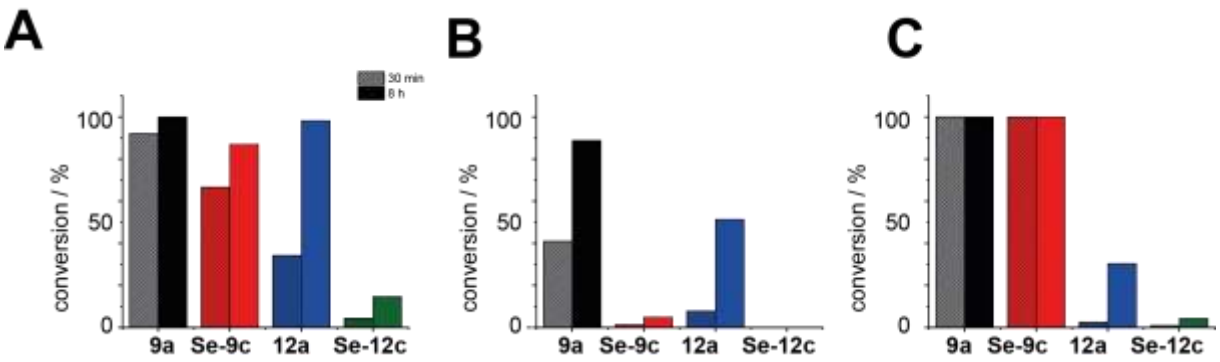

Fig. S19: Differential MTase targeting by AdoMet analogues bearing no modification, a modification at the sulfur/selenium atom, at the nucleoside or both. Absolute conversions for Ecm1 (A), hTGS1 (B) or CouO (C), respectively. Conditions: 6 mM **1a** or ***Se*-1c**, 2.5 mM **2** or **5**, 4 mol% ChMAT (rel. to **2** or **5**), 300 µM **16, 16a**, or **18**, 10 mol% MTase (rel. to **16**, **16a**, or **18**). Reaction mixture is incubated at 23 °C for 30 min (striped bars) or 8h.

Additional supplementary data for figures shown in main text

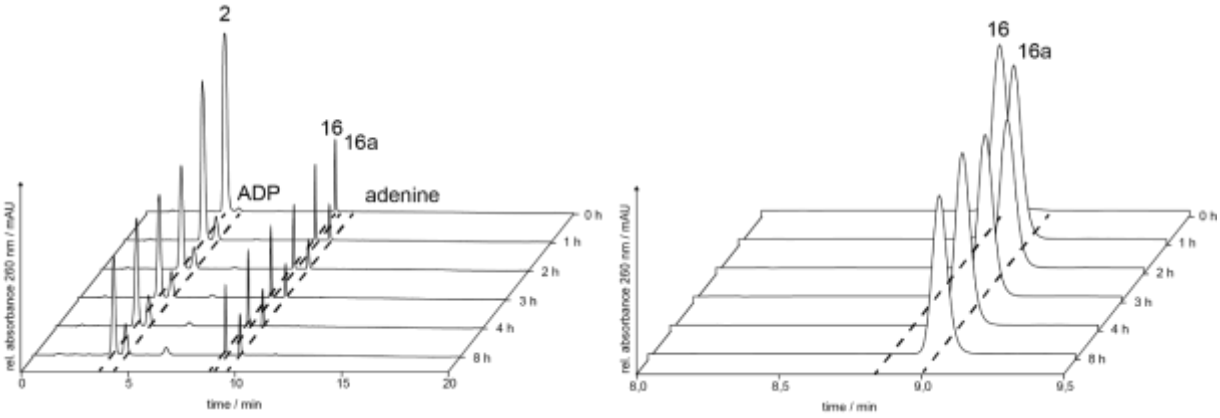

Fig. S20: HPLC analysis of the enzymatic cascade using methionine (**1a**), ATP (**2**, 3.66 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min)

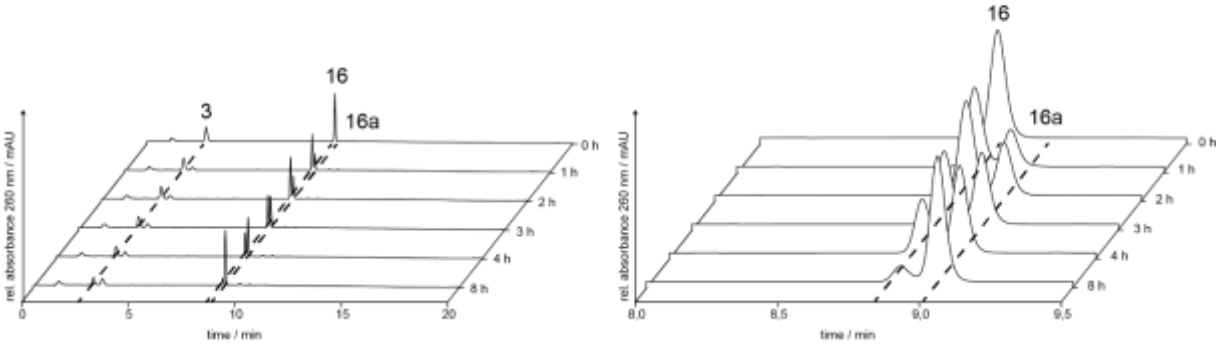

Fig. S21: HPLC analysis of the enzymatic cascade using methionine (**1a**), 2-APTP (**3**, 2.79 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min).

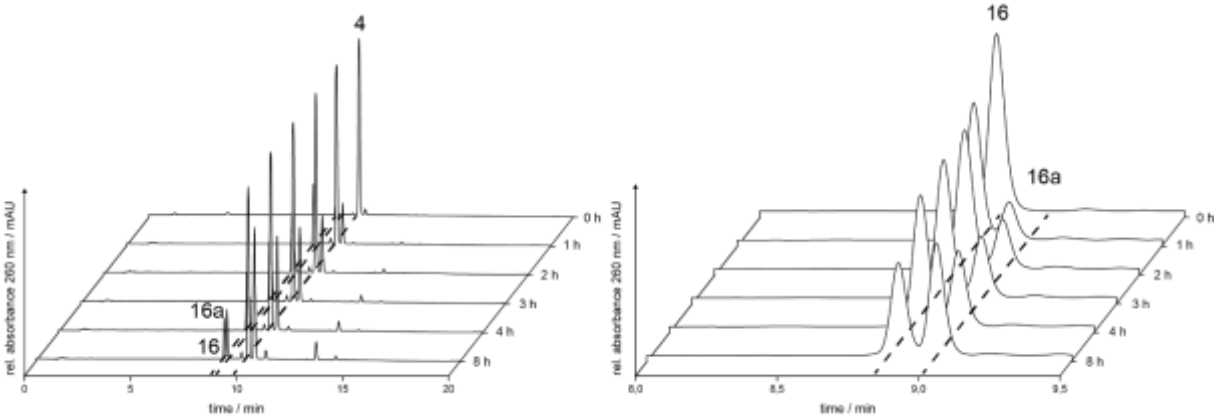

Fig. S22: HPLC analysis of the enzymatic cascade using methionine (**1a**), p^{6}ATP (**4**, 9.91 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min).

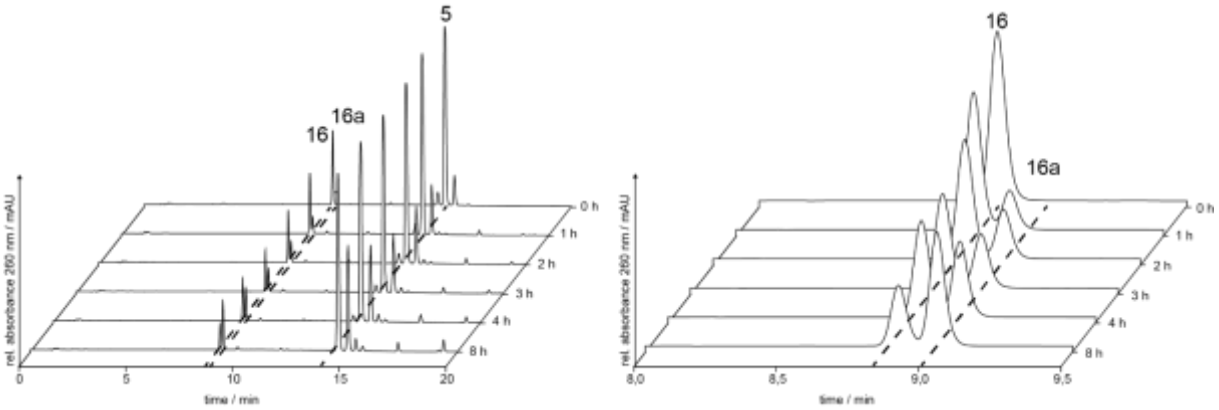

Fig. S23: HPLC analysis of the enzymatic cascade using methionine (**1a**), b^{6}ATP (**5**, 14.10 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min).

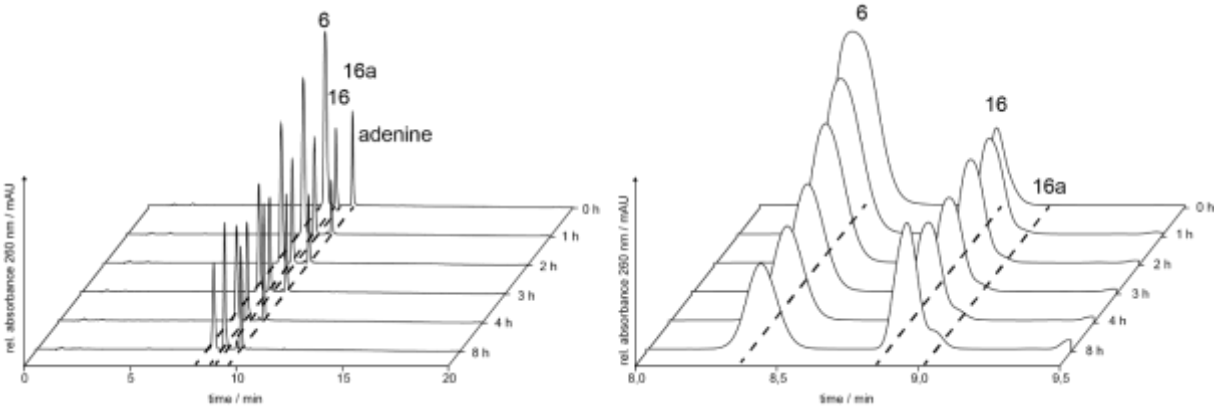

Fig. S24: HPLC analysis of the enzymatic cascade using methionine (**1a**), dATP (**6**, 8.33 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min).

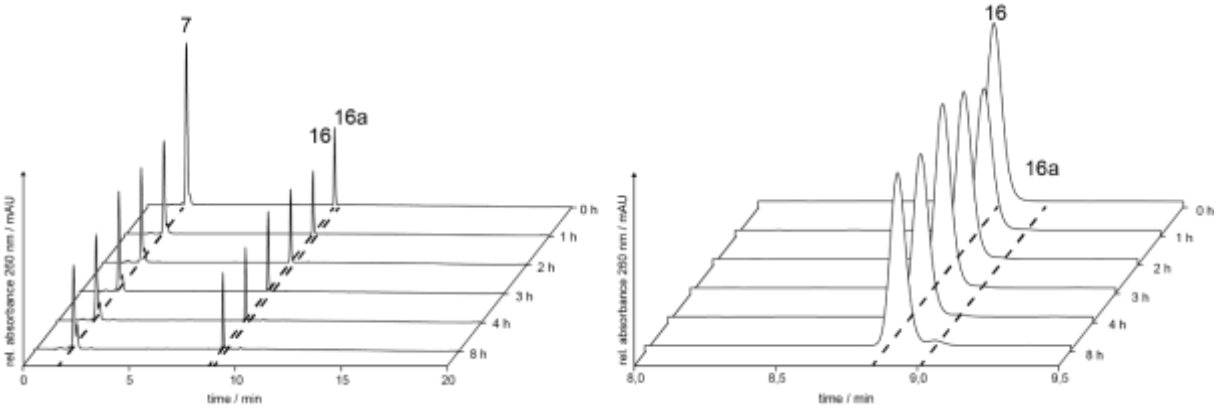

Fig. S25: HPLC analysis of the enzymatic cascade using methionine (**1a**), ITP (**7**, 1.84 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min).

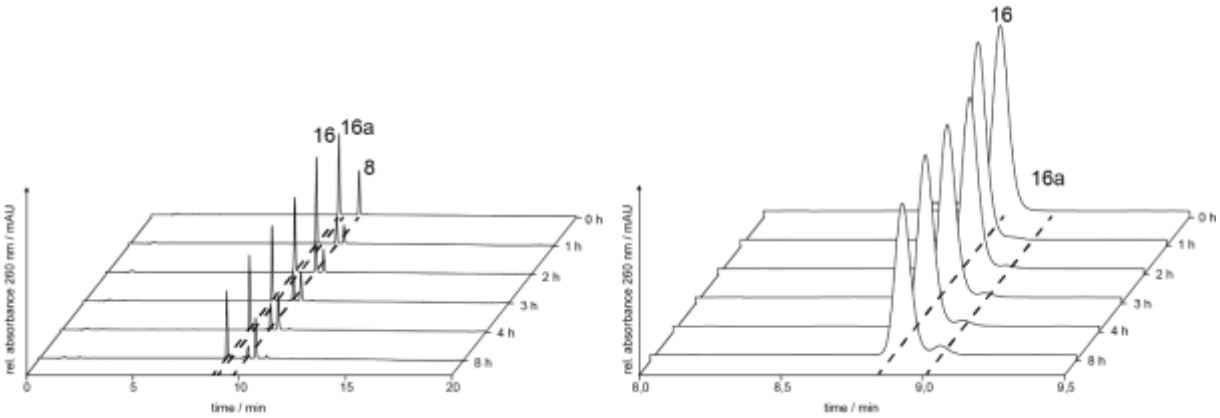

Fig. S26: HPLC analysis of the enzymatic cascade using methionine (**1a**), 2´-azido-2´-dATP (**8**, 9.77 min), MAT2a I117A, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^7GpppA (**16a**, 9.02 min).

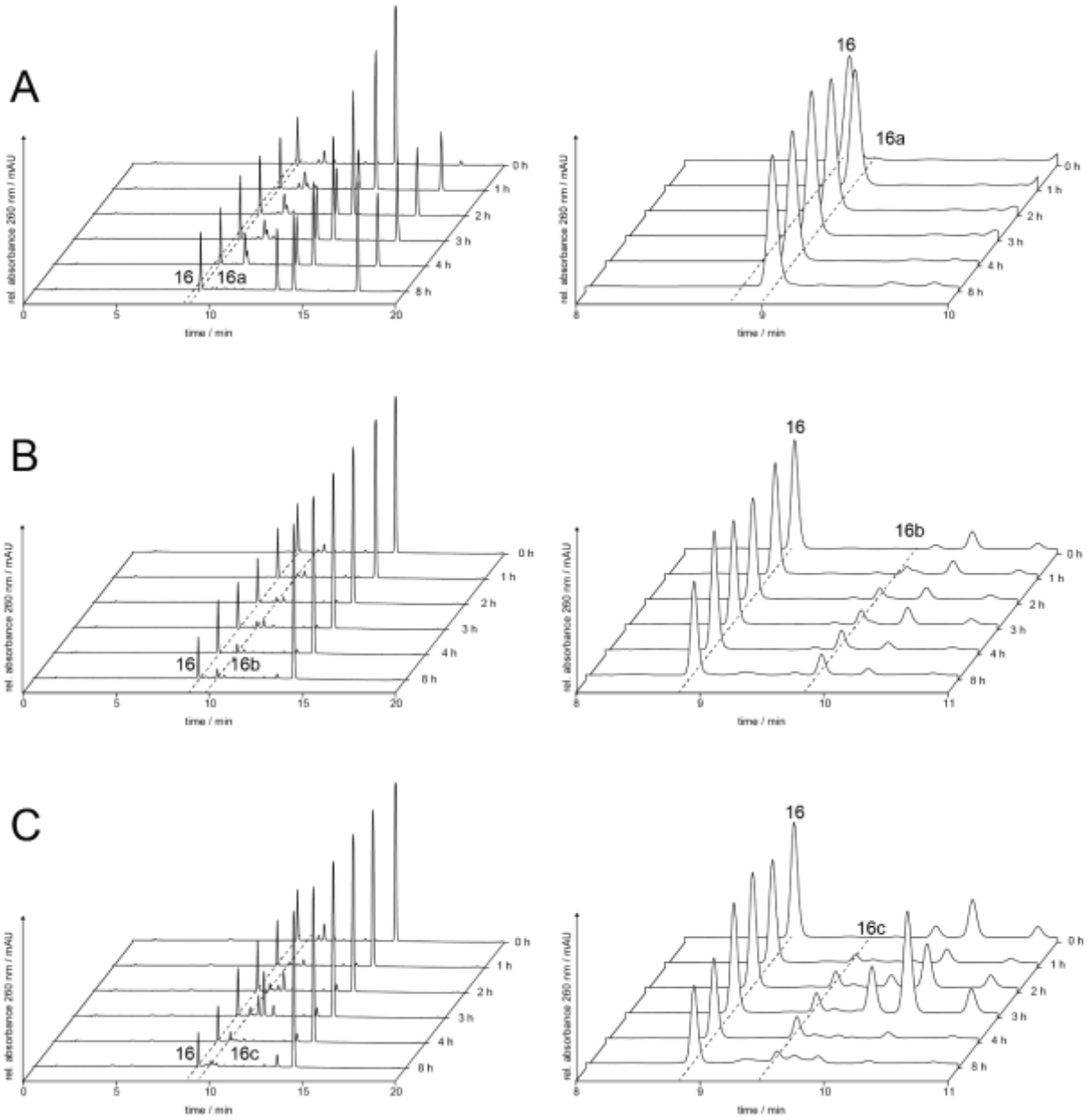

Fig. S27: HPLC analysis of the enzymatic cascade. [A] Methionine (**1a**), p^{6}ATP (**4**, 9.88 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min). [B] Allyl-D/L-homocysteine (**1b**), p^{6}ATP (**4**, 9.88 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to a^{7}GpppA (**16b**, 9.85 min). [C] Propargyl-L-selenohomocysteine (***Se*-1c**), p^{6}ATP (**4**, 9.88 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to p^{7}GpppA (**16c**, 9.50 min). Note that the reaction mixture was dephosphorylated with FastAP® to avoid coelution of **16b** with **4** as described above.

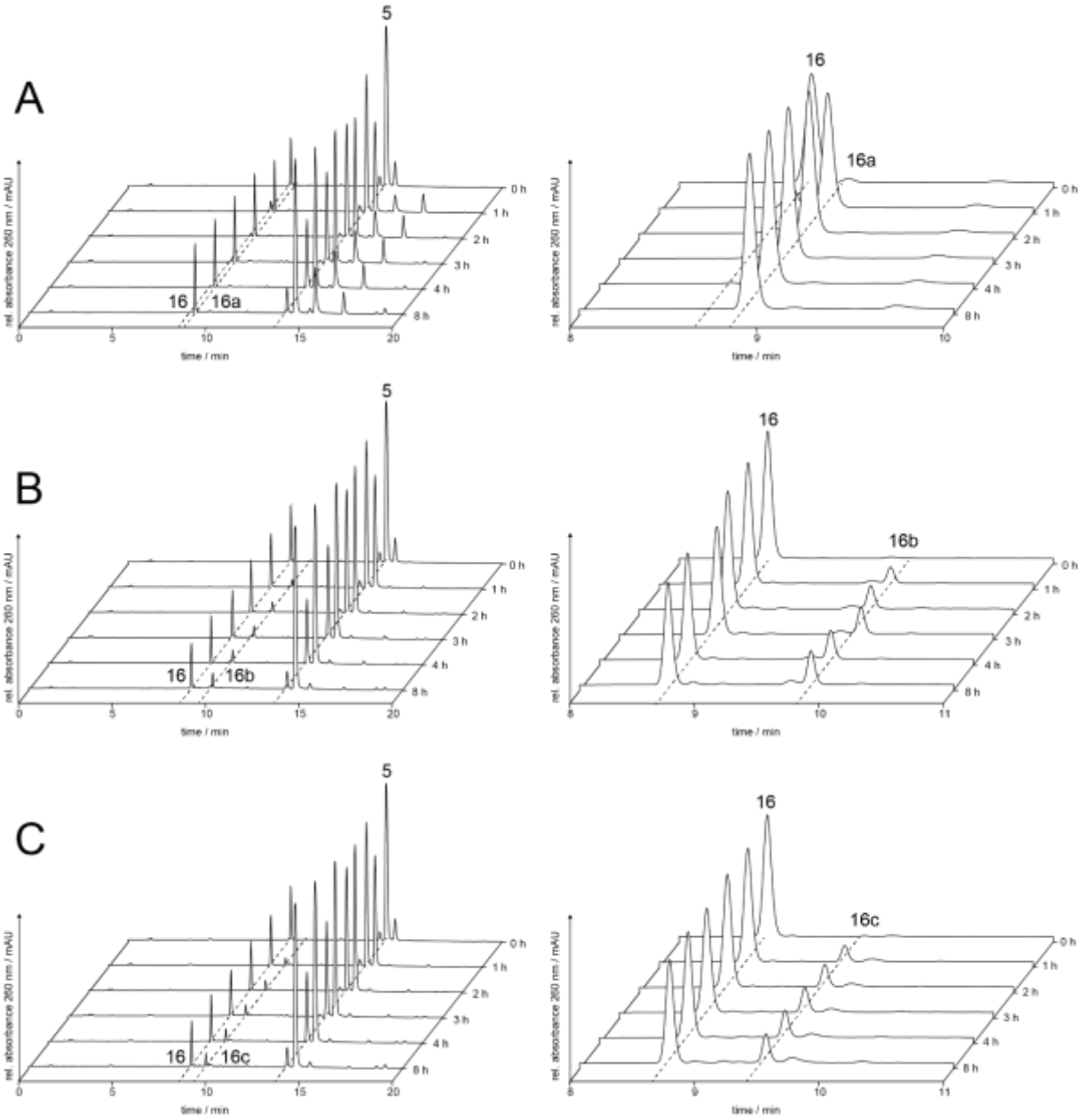

Fig. S28: HPLC analysis of the enzymatic cascade. [A] Methionine (**1a**), b^{6}ATP (**5**, 14.10 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to m^{7}GpppA (**16a**, 9.02 min). [B] Allyl-D/L-homocysteine (**1b**), b^{6}ATP (**5**, 14.10 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to a^{7}GpppA (**16b**, 9.85 min). [C] Propargyl-L-selenohomocysteine (***Se*-1c**), b^{6}ATP (**5**, 14.10 min), ChMAT, and Ecm1. The substrate GpppA (**16**, 8.84 min) is converted to p^{7}GpppA (**16c**, 9.50 min).

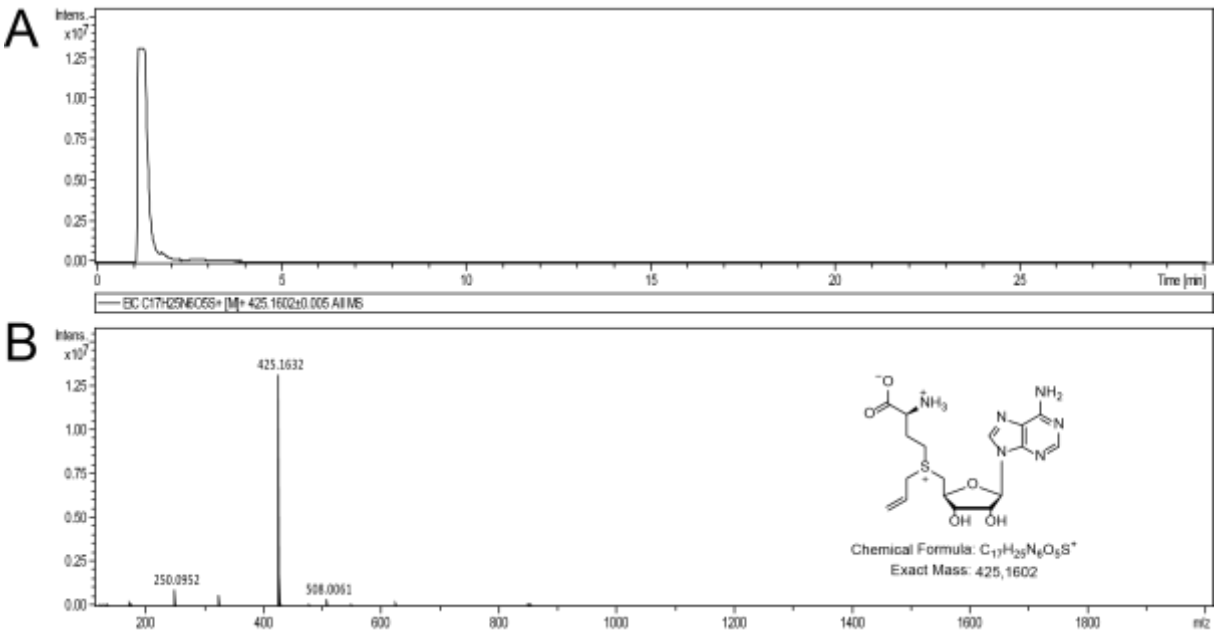

Fig. S29: LC/MS analysis of compound **9b**. Calculated mass for $[M]^+$ = 425.1602 Da, found m/z = 425.1632.

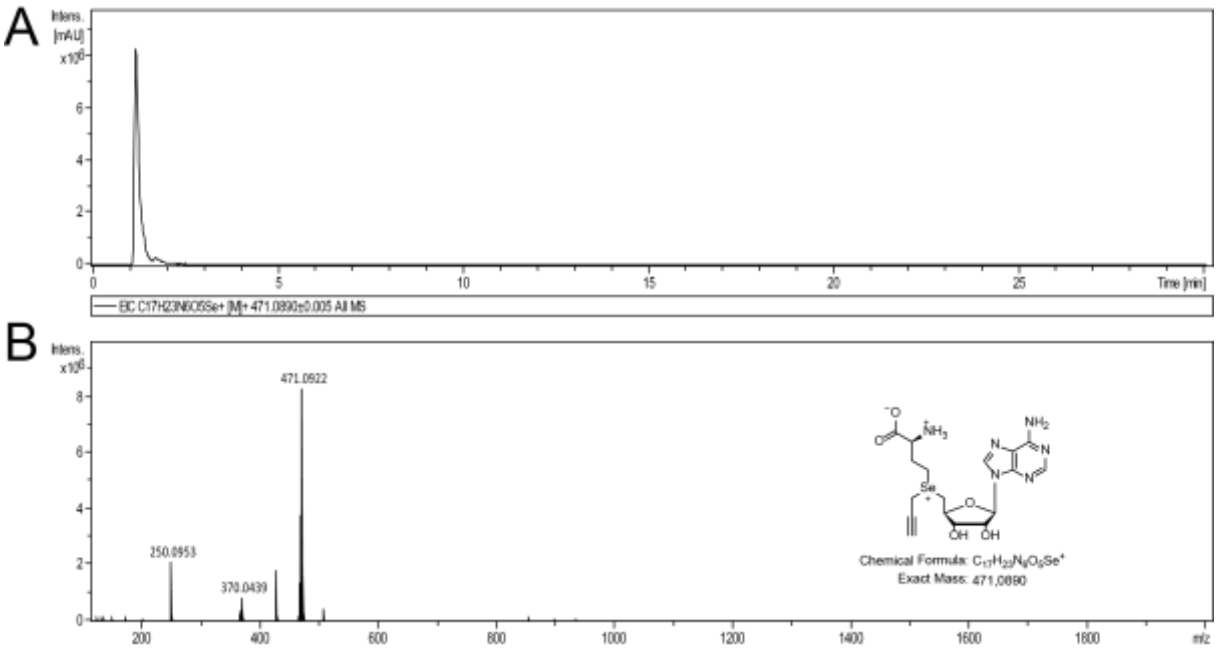

Fig. S30: LC/MS analysis of compound ***Se*-9c**. Calculated mass for $[M]^+$ = 471.0890 Da, found m/z = 471.0922.

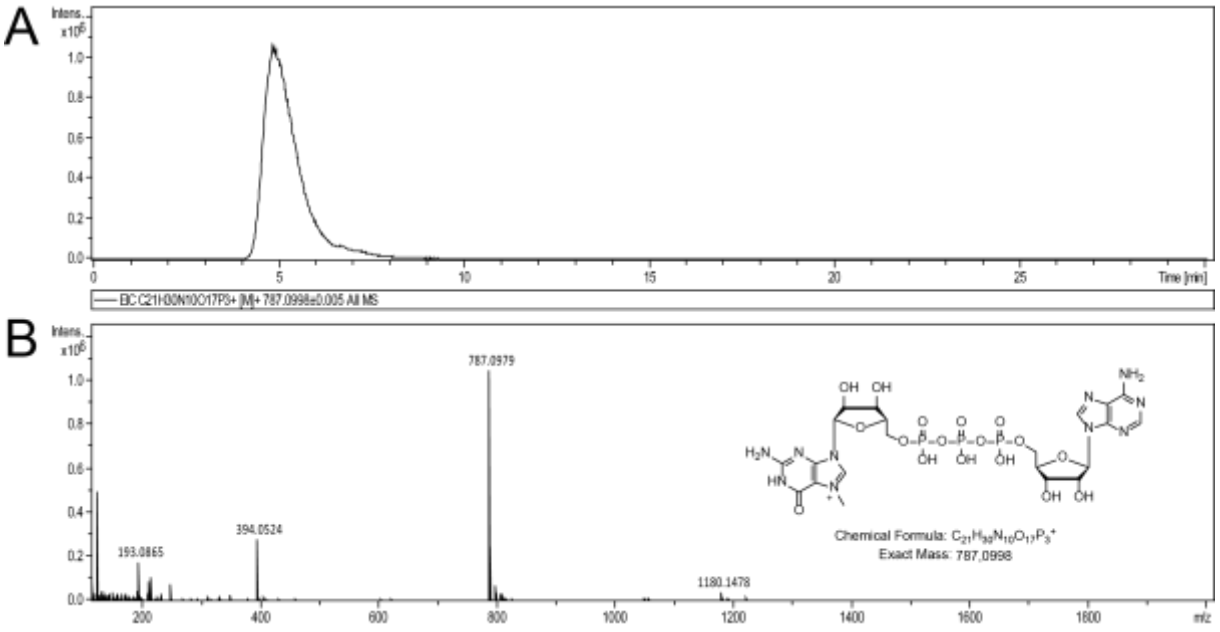

Fig. S31: LC/MS analysis of compound **16a**. Calculated mass for $[M]^+$ = 787.0998 Da, found m/z = 787.0979.

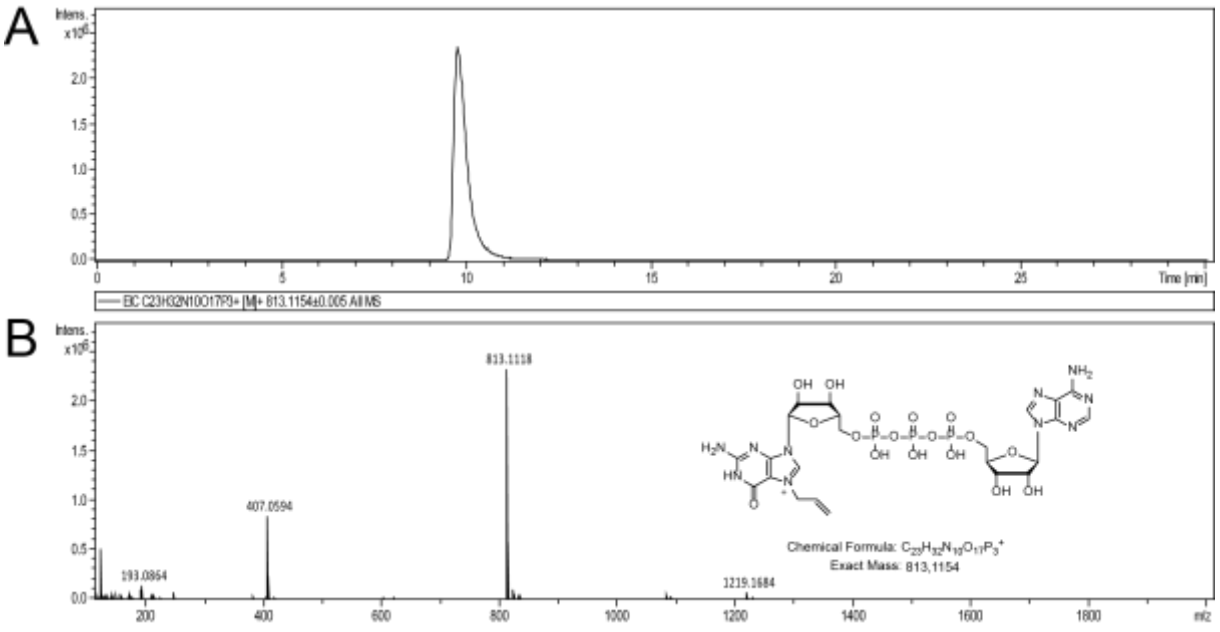

Fig. S32: LC/MS analysis of compound **16b**. Calculated mass for $[M]^+$ = 813.1154 Da, found m/z = 813.1118.

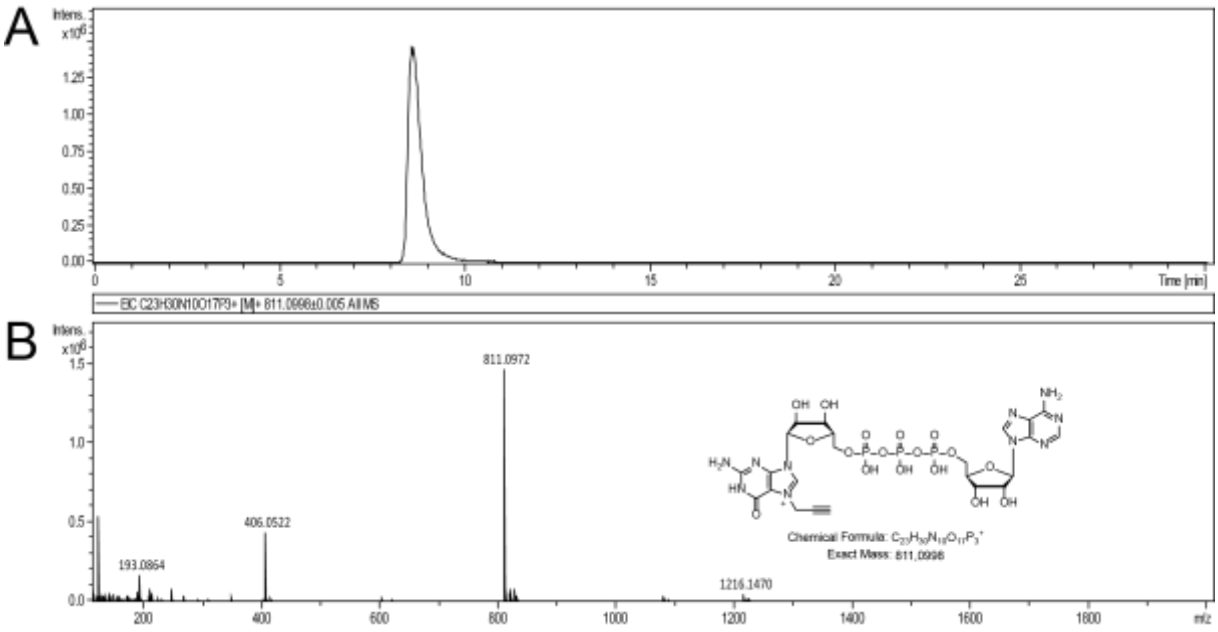

Fig. S33: LC/MS analysis of compound **16c**. Calculated mass for $[M]^+$ = 811.0998 Da, found m/z = 811.0972.

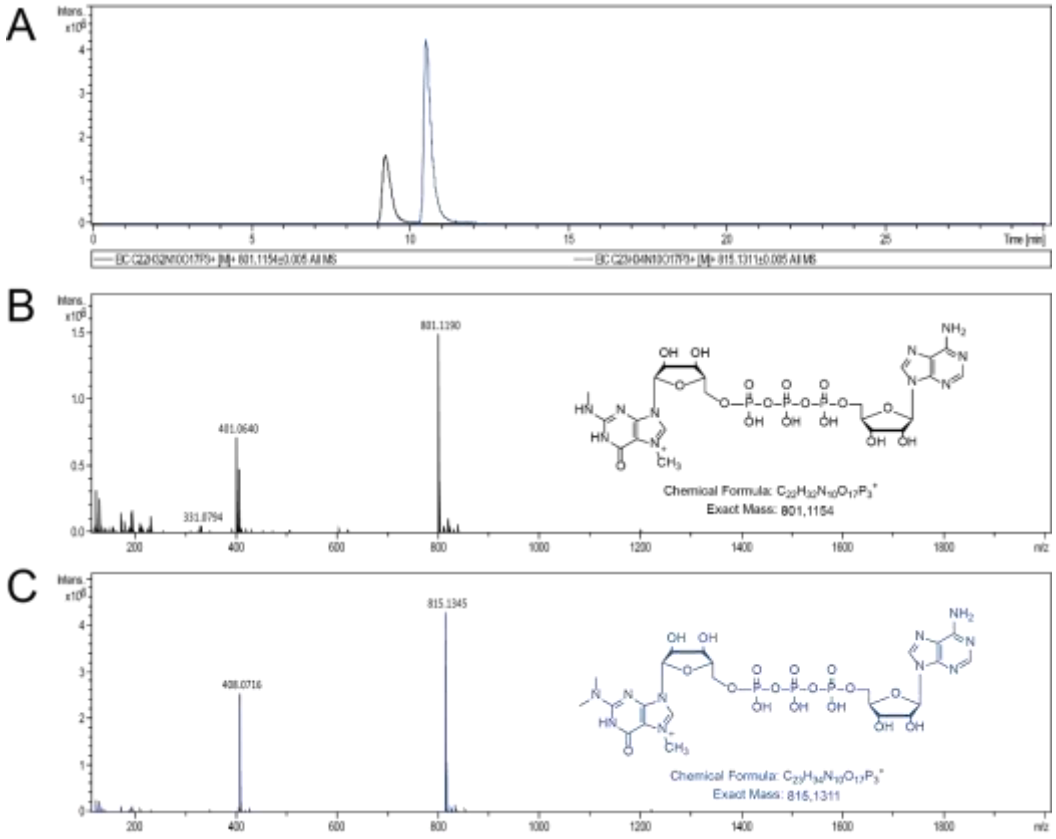

Fig. S34: LC/MS analysis of compounds **17a** and **17a,a**. [A] EIC of **17a** and **17a,a**. [B] Calculated mass for **17a** $[M]^+$ = 801.1154 Da, found m/z = 801.1190. [C] Calculated mass for **17a,a** $[M]^+$ = 815.1311 Da, found m/z = 815.1345.

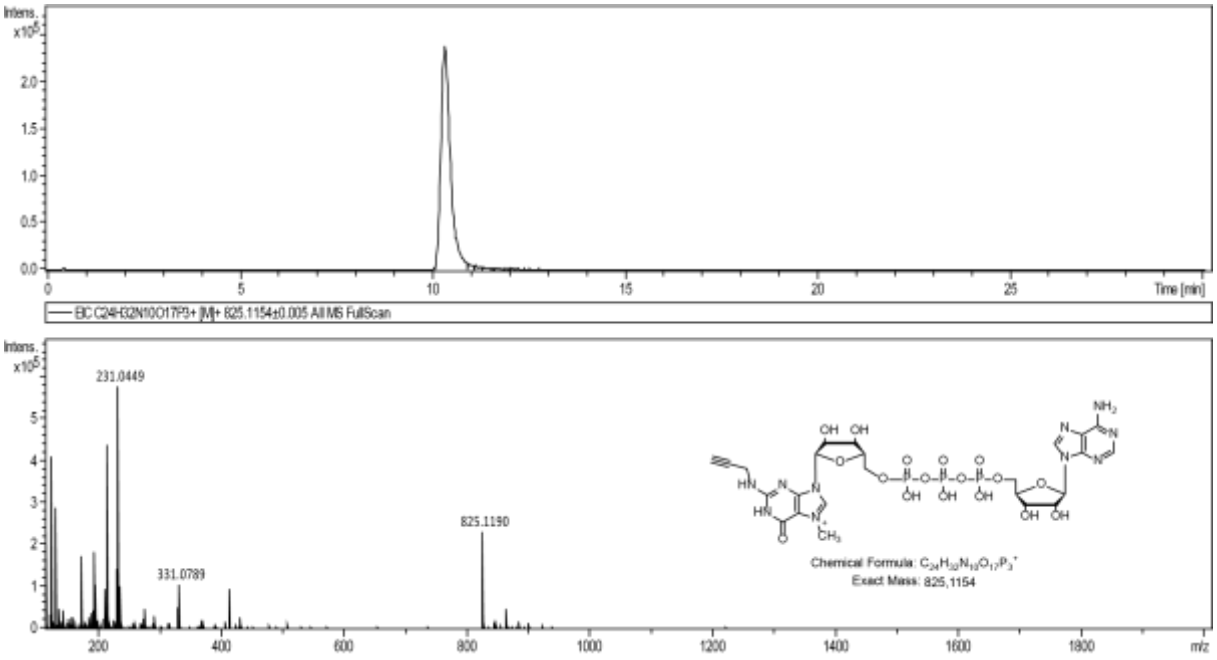

Fig. S35: LC/MS analysis of compound **17c**. [A] EIC of **17c**. [B] Calculated mass for **17c** $[M]^+$ = 825.1154 Da, found m/z = 825.1190.

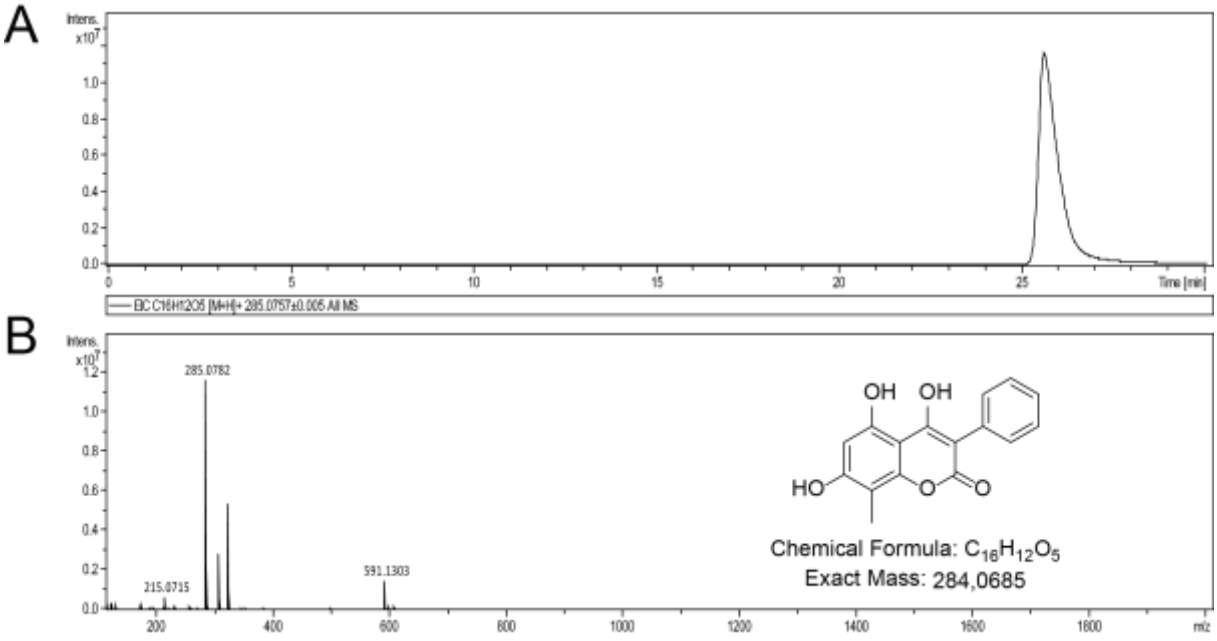

Fig. S36: LC/MS analysis of compound **18a**. Calculated mass for $[M+H]^+$ = 285.0757 Da, found m/z = 285.0782.

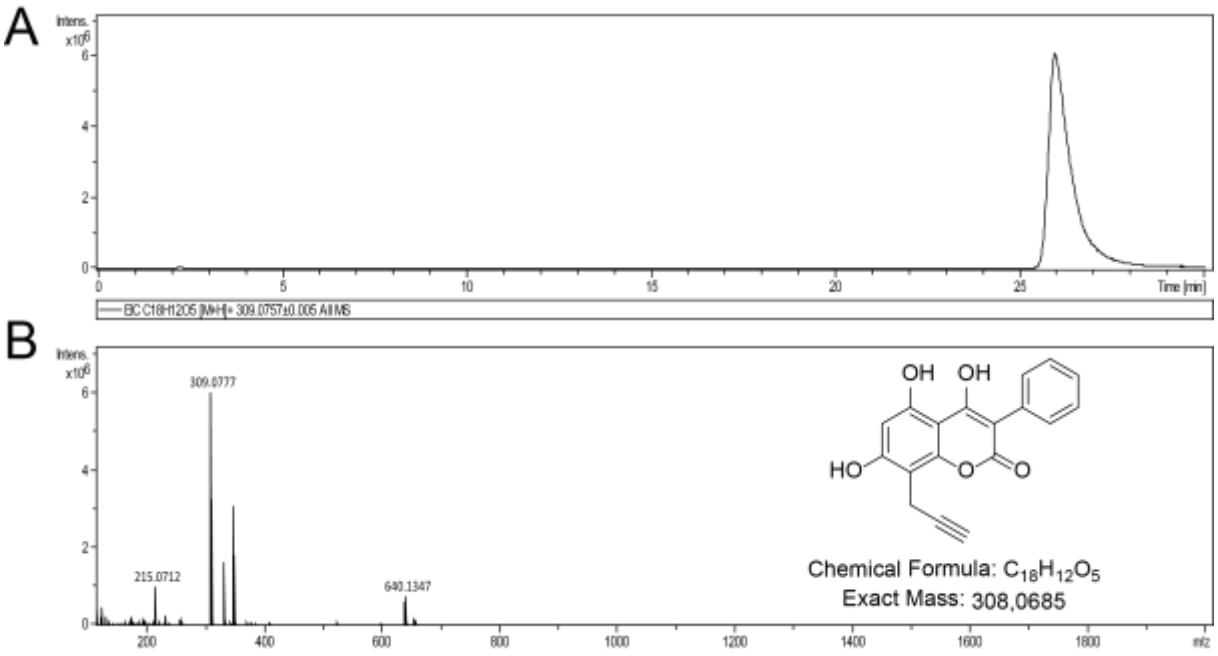

Fig. S37: LC/MS analysis of compound **18c**. [A] EIC of **18c**. [B] Calculated mass for **18c** $[M+H]^+$ = 309.0757 Da, found m/z = 309.0777.

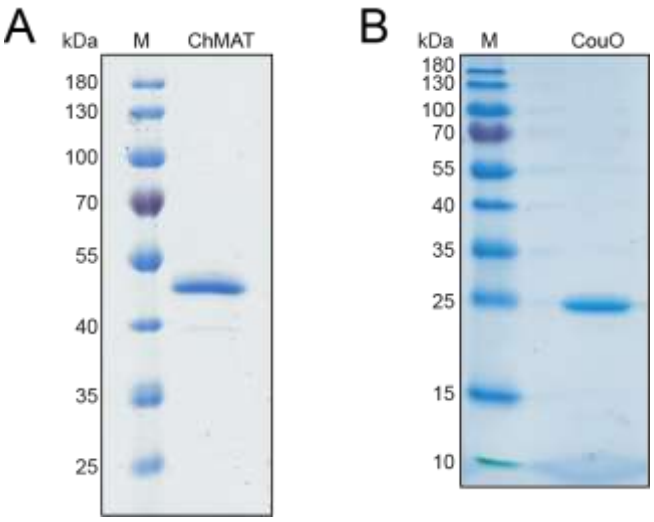

Fig. S38: SDS-PAGE of IMAC purified enzymes. [A] ChMAT (45.9 kDa) and [B] CouO (26.6 kDa).

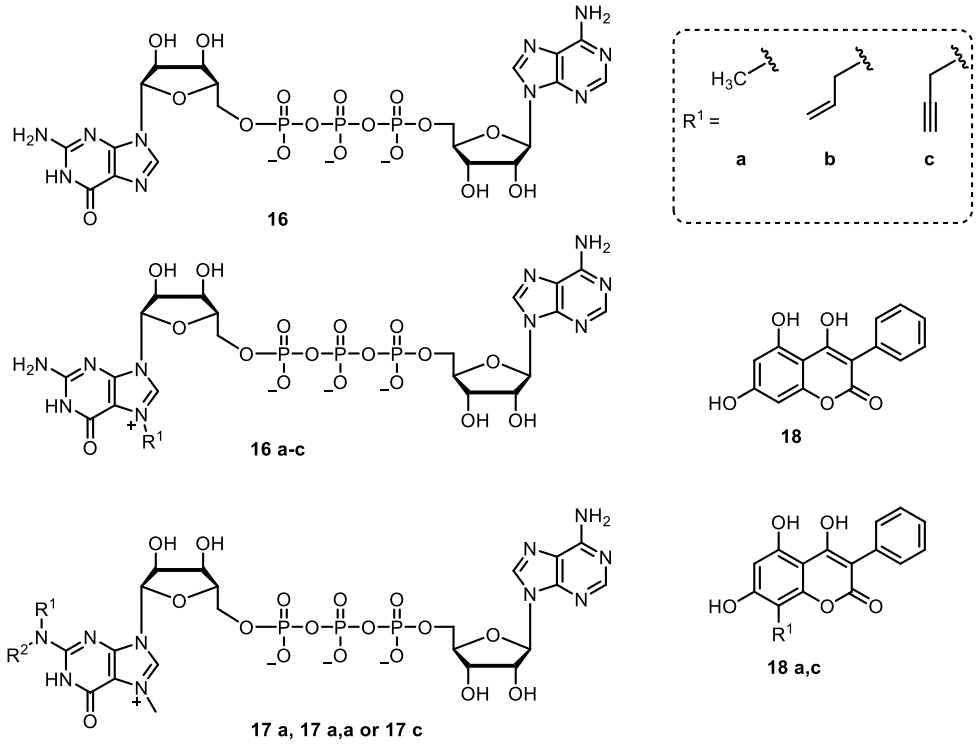

Fig. S39: Methyltransferase substrates used and products produced in this study. mRNA cap and analogues (GpppA **16**, **16a-c** and **17a,c**), 4,5,7-trihydroxy-3-phenylco umarin and analogues (**18a,c**). **17a,a** refers to the $m^{2,2,7}$GpppA.

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