Supporting information

Post-synthetic benzylation of the mRNA 5′ cap via enzymatic cascade reactions

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Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich, Fluorochem, Acros Organics, VWR and Jena Biosciences and were used without further purification unless otherwise stated.

NMR spectra were measured at 299 K on a Bruker Neo 400 spectrometer. The chemical shifts (δ) were reported in ppm relative to deuterated solvents as internal standard (D_2O = 4.79 ppm).

LC-QTOF-MS analysis was performed on a Bruker maXis II ultra-high resolution QTOF coupled to a Thermo Scientific UltiMate 3000® UHPLC. Runs are referenced to high mass standard m/z = 1222.0000 (Agilent, part: G1982-85001).

column	Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 4 mm) from Macherey-Nagel		
Buffer A	7 mM NH_4HCO_2 and 12 mM HCOOH, pH = 3.5		
Buffer B	MeOH		
Flowrate	0.6 mL/min		
Temperature	20 °C		
Gradient	Min	%A	%B
	0	100	0
	5	100	0
	65	0	100
	70	0	100
	75	100	0
	80	0	100
	85	100	0
	90	100	0

LC-QqQ-MS analysis was performed on an Agilent Ultivo using an Agilent Poroshell 120EC-C18, 3×150 mm, 2.7 µm column (Agilent Technologies). Elution was performed at a flow rate of 0.6 mL/min applying a linear gradient for buffer A (10 mM NH_4OAc, pH = 6.0) and buffer B (MeOH).

Preparative HPLC purification of methionine analogues was performed on a Büchi PrepChrom C-700 V5 using a High-Performance Gold C_{18} Aq 150 g reverse-phase column from Teledyne ISCO. Elution was performed at a flow rate of 63 mL/min using a gradient from buffer A (50 mM NH_4OAc, pH = 6.0) to buffer B (MeCN).

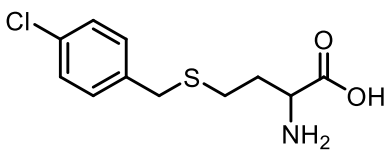
Semi-Preparative HPLC purification of Cap analogues was performed on an Agilent 1260 Infinity HPLC using a Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 10 mm) from Macherey-Nagel. Elution was performed at a flow rate of 5 mL/min using a gradient from buffer A (10 mM NH_4OAc, pH = 6.0) to buffer B (MeCN).

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

	Method A			Method B			Method C		
column	Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 4 mm) from Macherey-Nagel			Agilent Poroshell 120EC-C18, 3×150 mm, 2.7 µm column (Agilent Technologies)			Nucleodur® C18 Pyramid reversed-phase column (5 µm, 125 x 4 mm) from Macherey-Nagel		
Buffer A	100 mM K_2HPO_4/KH_2PO_4 pH = 6.5			10 mM NH_4OAc pH = 6.0			100 mM K_2HPO_4/KH_2PO_4 pH = 6.5		
Buffer B	50 % Buffer A, 50 % MeCN			MeCN			50 % Buffer A, 50 % MeCN		
Flowrate	1 mL/min			0.8 mL/min			1 mL/min		
Temperature	20 °C			40 °C			20 °C		
Gradient	Min	%A	%B	Min	%A	%B	Min	%A	%B
	0	100	0	0	100	0	0	100	0
	5	100	0	3	100	0	5	100	0
	18	40	60	12	70	30	20	0	100
	20	0	100	15	0	100	25	0	100
	25	0	100	17	0	100	28	100	0
	28	100	0	20	100	0	33	100	0
	33	100	0	22	100	0			

Synthesis of D,L-methionine analogues

4-Chlorobenzyl-D,L-homocysteine (**1b**)



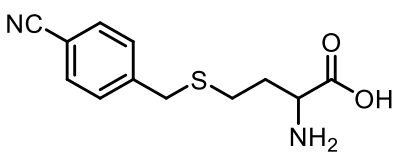
Under argon D,L-homocysteine thiolactone hydrochloride (HCTL, 691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 5 mL freshly prepared NaOH (ddH_2O, degassed, 5 M). The mixture was stirred for 10 min. Sodium bicarbonate (420 mg) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of 4-chlorobenzyl bromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (72.4 mg, 0.28 mmol, 9 %).

HRMS: Calculated for $C_{11}H_{15}ClNO_2S^+$ $[M+H]^+$ = 260.0507 Da, found 260.0517 m/z.

^{1}H NMR (400 MHz, D_2O + 1 % NaOD) δ (ppm) = 7.41 – 7.27 (m, 4H), 3.74 (s, 2H), 3.29 (dd, *J* = 7.6, 5.4 Hz, 1H), 2.56 – 2.44 (m, 2H), 1.99 – 1.68 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, D_2O + 1 % NaOD) δ (ppm) = 183.3, 138.0, 132.9, 131.2, 129.4, 56.1, 39.5, 35.1, 28.0.

4-Cyanobenzyl-D,L-homocysteine (**1c**)



Under argon D,L-homocysteine thiolactone hydrochloride (691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH_2O, degassed, 2 M). The mixture was stirred for 15 min. Sodium bicarbonate (420 mg) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of 4-nitrilbenzyl bromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (141.5 mg, 0.57 mmol, 19 %).

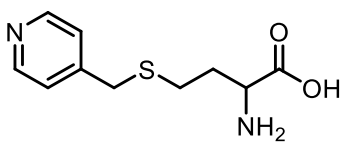
Note: Under basic or acidic conditions the nitrile hydrolysis first to the corresponding carboxamide and finally to the carboxylic acid. The conditions used for NMR (D_2O + 1 % NaOD) lead to visible hydrolysis. In the enzymatic reactions no hydrolysis was detected by LC-MS.

HRMS: Calculated for $C_{12}H_{15}N_2O_2S^+$ $[M+H]^+$ = 251.0849 Da, found 251.0846 m/z.

^{1}H NMR (400 MHz, D_2O + 1 % NaOD) δ (ppm) = 7.80 - 7.67 (m, 2H), 7.55 - 7.43 (m, 2H), 3.82 (s, 2H), 3.33 - 3.25 (m, 1H), 2.58 - 2.44 (m, 2H), 2.00 - 1.66 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, D_2O + 1 % NaOD) δ (ppm) = 182.6, 144.8, 132.8, 129.6, 119.8, 109.4, 55.3, 34.8, 34.4, 27.3.

4-Pyridinylmethyl-D,L-homocysteine (**1d**)



Under argon D,L-homocysteine thiolactone hydrochloride (691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH_2O, degassed, 5 M). The mixture was stirred for 10 min and the thiolactone cleavage was monitored by TLC. Sodium bicarbonate (420 mg, 5 mmol, 1,7 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl.

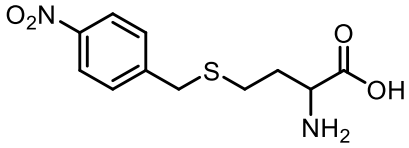
A solution of 4-(bromomethyl)pyridine hydrobromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 40 % B: ACN). The product was obtained as white powder (501.1 mg, 2.21 mmol, 74 %).

HRMS: Calculated for $C_{10}H_{15}N_2O_2S^+$ $[M+H]^+$ = 227.0849 Da, found 227.0857 m/z.

^{1}H NMR (400 MHz, D_2O + 1 % NaOD) δ (ppm) = 8.58 – 8.41 (m, 2H), 7.58 – 7.41 (m, 2H), 3.83 (s, 2H), 3.84 – 3.79 (m, 1H), 2.58 (t, *J* = 7.4 Hz, 2H), 2.26 – 2.02 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, D_2O + 1 % NaOD) δ (ppm) = 174.7, 150.8, 148.9, 125.5, 54.5, 34.6, 30.8, 27.1.

4-nitrobenzyl-D,L-homocysteine (**1e**)



Under argon D,L-homocysteine thiolactone hydrochloride (691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH$_2$O, degassed, 5 M). The mixture was stirred for 10 min and the thiolactone cleavage was monitored by TLC. Sodium bicarbonate (420 mg, 5 mmol, 1,7 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl.

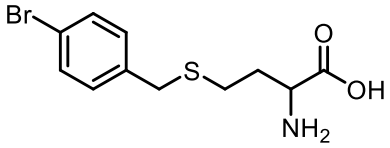
A solution of 4-nitrobenzyl bromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (533.9 mg, 1.98 mmol, 66 %).

HRMS: Calculated for $C_{11}H_{15}N_2O_4S^+$ $[M+H]^+$ = 271.0747 Da, found 271.741 m/z.

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 8.16 (m, 1H), 7.60 (m, 2H), 3.85 (s, 2H), 3.34 – 3.20 (m, 1H), 2.64 – 2.42 (m, 2H), 2.05 – 1.77 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, DMSO) δ (ppm) = 170.4, 147.4, 146.3, 130.1, 123.6, 53.2, 34.0, 31.2, 27.2.

4-Bromobenzyl-D,L-homocysteine (**1f**)



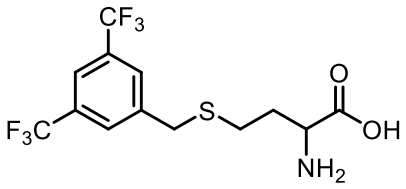
Under argon D,L-homocysteine thiolactone hydrochloride (691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH_2O, degassed, 2 M). The mixture was stirred for 15 min. Sodium bicarbonate (420 mg) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of 4-bromobenzyl bromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (37 mg, 0.12 mmol, 4 %).

HRMS: Calculated for $C_{11}H_{15}BrNO_2S^+$ $[M+H]^+$ = 304.0001 Da, found 304.0016 m/z.

^{1}H NMR (400 MHz, D_2O + 1 % NaOD) δ (ppm) = 7.57 – 7.43 (m, 2H), 7.33 – 7.20 (m, 2H), 3.71 (s, 2H), 3.29 (t, *J* = 6.4 Hz, 1H), 2.54 – 2.42 (m, 2H), 2.00 – 1.68 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, D_2O + 1 % NaOD) δ (ppm) = 182.5, 137.7, 131.6, 130.8, 120.4, 55.4, 34.5, 34.5, 27.4.

3,5-Bis(trifluoromethyl)benzyl-D,L-homocysteine (**1g**)



Under argon D,L-homocysteine thiolactone hydrochloride (691 mg, 4.5 mmol, 1.5 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH_2O, degassed, 5 M). The mixture was stirred for 10 min and the thiolactone cleavage was monitored by TLC. Sodium bicarbonate (420 mg, 5 mmol, 1,7 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of 3,5-bis(trifluoromethyl)benzyl bromide in 1,4-dioxane (6 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature over 4 h and the progress was monitored *via* HPLC. The crude mixture was acidified to pH = 6 using 1 M HCl. The residual solvent was evaporated under reduced pressure. The crude product was dissolved in 10 mL DMSO and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH_4Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (27.4 mg, 76 µmol, 2 %).

HRMS: Calculated for $C_{13}H_{14}F_6NO_2S^+$ $[M+H]^+$ = 362.0644 Da, found 362.0645 m/z.

^{1}H NMR (400 MHz, D_2O + 1 % NaOD) δ (ppm) = 7.98 (s, 3H), 3.92 (m, 2H), 3.29 (dd, J = 7.6, 5.4 Hz, 1H), 2.51 (m, 2H), 1.98 – 1.68 (m, 2H).

^{13}C-{^{1}H}-NMR (101 MHz, D_2O + 1 % NaOD) δ (ppm) = 182.7, 141.3, 131.5, 131.2, 130.8, 130.5, 129.3, 124.8, 122.1, 121.3, 55.2, 34.3, 34.3, 27.3.

Note that the carbon signal for CF_3 groups typically splits to a quartet with intensities of 1:3:3:1 which can be seen for δ (ppm) = 131.5, 131.2, 130.8, 130.5.

^{19}F-{^{1}H}-NMR (377 MHz, D_2O + 1 % NaOD) δ (ppm) = -62.64 (s).

Plasmids, enzyme expression and purification

Plasmids used in this study.

Vector	Insert	Resistance
pET-28a(+)	Ecm1	Kanamycin
pET-28a(+)	PC-MjMAT	Kanamycin
pET26b	NovO	Kanamycin
pProEx	MTAN	Ampicillin
pMRNA-GLuc	GLuc (Φ6.5 promoter)	Ampicillin
pMRNA-qcGLuc	GLuc (Φ2.5 promoter)	Ampicillin
pMRNA-RLuc	RLuc (Φ6.5 promoter)	Ampicillin
pMRNA-FLuc	FLuc (Φ6.5 promoter)	Ampicillin
pMRNA-qcFLuc	FLuc (Φ2.5 promoter)	Ampicillin

Protein purification was performed on ÄKTApurifier using HisTrap™ columns (GE HealthCare) for IMAC and HiLoad™ 16/600 superdex™ 200 pg (GE HealthCare) for SEC.

Ecm1

For Ecm1 production, transformed *E. coli* Tuner (DE3) cells were cultivated in 2YT medium with kanamycin at 37 °C to an OD_{600} of 0.6. Expression was induced with 0.5 mM IPTG at 30 °C for 3 h. The protein was purified by IMAC and SEC using the ÄKTApurifier system. The protein was concentrated and aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

IMAC binding buffer	50 mM Tris-HCl pH = 7.5, 200 mM NaCl, 10 % glycerol, 10 mM imidazole
IMAC elution buffer	50 mM Tris-HCl pH = 7.5, 200 mM NaCl, 10 % glycerol, 500 mM imidazole
SEC/ storage buffer	50 mM Tris-HCl pH = 8, 150 mM NaCl, 1 mM EDTA, 10 % glycerol

PC-MjMAT

For PC-MjMAT (MjMAT L147A/I351A) production, transformed *E. coli* BL21 (DE3) cells were cultivated in LB medium with kanamycin at 37 °C to an OD_{600} of 0.6. Expression was induced with 0.5 mM IPTG at 18 °C for 16 h. The protein was purified by heat purification (incubation at 80 °C for 20 min), IMAC and SEC using the ÄKTApurifier system. The protein was concentrated and aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

IMAC binding buffer	50 mM Tris-HCl pH = 8, 300 mM NaCl
IMAC elution buffer	50 mM Tris-HCl pH = 8, 300 mM NaCl, 500 mM imidazole
SEC/ storage buffer	25 mM Tris-HCl pH = 8, 80 mM KCl, 10 % glycerol

NovO

For NovO production, transformed *E. coli* BL21 (DE3) cells were cultivated in LB medium with kanamycin at 37 °C to an OD_{600} of 0.6. Expression was induced with 0.5 mM IPTG at 18 °C for 16 h. The protein was purified by IMAC using the ÄKTApurifier system. The protein was concentrated and aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

IMAC binding buffer	50 mM Tris-HCl pH = 8, 300 mM NaCl
IMAC elution buffer	50 mM Tris-HCl pH = 8, 300 mM NaCl, 500 mM imidazole
Storage buffer	50 mM Tris-HCl pH = 8, 200 mM NaCl, 10% glycerol

MTAN

For MTAN production, transformed *E. coli* BL21 (DE3) cells were cultivated in LB medium with ampicillin at 37 °C to an OD_{600} of 0.6. Expression was induced with 0.2 mM IPTG at 18 °C for 16 h. The protein was purified by IMAC and SEC using the ÄKTApurifier system. The protein was concentrated and aliquots were flash frozen in liquid nitrogen and stored at -80 °C.

IMAC binding buffer	50 mM sodium phosphate buffer pH = 7.5
IMAC elution buffer	50 mM sodium phosphate buffer pH = 7.5, 250 mM imidazole
SEC/ storage buffer	50 mM sodium phosphate buffer pH = 7.5, 50 mM Hepes and 10% glycerol

MTA assay

Conditions: 5 mM L-methionine (**1a**) or D,L-methionine analogues (**1b**-**g**) are incubated with 5 mM ATP and 100 µM PC-MjMAT in a total volume of 30 µL. 10 µL are used as 0 h sample, 10 µL are incubated at 37°C for 1 h and 10 µL are incubated at 65 °C for 1 h. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). Samples were taken at indicated time points, acidified by the addition of 10 % (v/v) of 1 M $HClO_4$ and centrifuged for 10 min at 21,000 ×g. 3 µL of the supernatant was directly analysed *via* HPLC method A.

Cascade PC-MjMAT/NovO

Conditions: 5 mM L-methionine (**1a**) or D,L-methionine analogues (**1b**-**g**) are incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM NovO, 1 mM 4,5,7,-Trihydroxy-3-phenylcoumarin (THPC, 20 mM stock in DMSO, results in 5 vol% DMSO in the reaction mixture), 10 µM MTAN at 37 °C for 2 h in a total volume of 20 µL. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). Samples were taken at indicated time points, quenched by addition of 3 volumes of ice cold MeCN and centrifuged for 10 min at 21,000 ×g. 12 µL of the supernatant was directly analysed *via* HPLC method C.

Cascade PC-MjMAT/Ecm1

Conditions: 5 mM L-methionine (**1a**) or D,L-methionine analogues (**1b**-**g**) are incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM Ecm1, 500 µM GpppA (**5**), 10 µM MTAN at 37 °C for 2 h in a total volume of 20 µL. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). Samples were taken at indicated time points, acidified by the addition of 10 % (v/v) of 1 M $HClO_4$ and centrifuged for 10 min at 21,000 ×g. 3 µL of the supernatant was directly analysed *via* HPLC method A.

Preparation of modified GpppA and GpppG caps via PC-MjMAT/Ecm1 cascade

To modify GpppA cap, 5 mM of L-methionine (**1a**) or the respective analogue (**1b-g**) was incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM Ecm1 methyltransferase, 10 µM MTAN, and 500 µM GpppA at 37 °C for 2 h. Reaction buffer consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). Samples were taken at indicated time points, acidified by the addition of 10 % (v/v) of 1 M $HClO_4$ and centrifuged for 10 min at 21,000 ×g. 3 µL of the supernatant was directly analysed *via* HPLC method A.

To modify GpppG cap, 5 mM **1b/c** or 8 mM **1d** was incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM Ecm1, 10 µM MTAN, and 2 mM GpppG (**6**) using the same conditions as described above for GpppA modification. The supernatant was directly used to analyse reaction products on HPLC method A.

To purify modified GpppA (**5b-d**) and GpppG (**6b-d**) caps, 200 µL reactions were performed and quenched as described above. Three times 60 µL of each sample was injected into semi-preparative HPLC. The desired products were eluted with a gradient from 10 mM NH_4OAc, pH = 6.5 to acetonitrile. The collected fractions were lyophilized and dissolved in 30 µL nuclease-free water.

Determination of purified **6b-d** concentration

1 µL of purified **6b-d** cap of unknown concentration was mixed with 0.5 mM cytidine (as internal control) and 0.2 U/µL Snake Venom phosphodiesterase (PDE, phosphodiesterase I from Crotalus adamanteus, MP Biomedicals Germany) in 1× snake venom buffer (10×: 110 mM Tris-HCl, 110 mM NaCl, 15 mM $MgCl_2$) in a total volume of 20 µL. 10 µL of sample (control) was immediately taken out, reaction quenched by the addition of 10 % (v/v) of 1 M $HClO_4$, and sample mixed with 1 µL nuclease-free water. Remaining 10 µL of sample was incubated at 37°C for 2 h. Afterwards, into 10 µL reaction, 1 µL FastAP (Thermo Scientific) was added and sample was incubated at 37°C for 1 h. The reaction was stopped by the addition of 10 % (v/v) of 1 M $HClO_4$ and all quenched samples were centrifuged for 10 min at 21,000 × g. 8 µL of the supernatant was analysed by HPLC using method B. The concentration of the 5′ caps was determined based on guanosine peak via external calibration.

Preparation of co-transcriptionally modified mRNAs

DNA template coding for Gaussia luciferase (GLuc), Renilla luciferase (RLuc), and firefly luciferase (FLuc) were prepared for run-off *in vitro* transcription from pMRNA vectors containing the respective sequence with appropriate promoter sequences (T7 Φ2.5 promoter, initiating transcription with A, or Φ6.5 promoter, initiating with G). Plasmid DNA (6 µg) was incubated with 1× FastDigest buffer (Thermo Fisher) and 6 µl of PacI FastDigest enzyme in a total volume of 120 µL for 10 min at 37 °C, followed by inactivation at 65 °C for 10 min. Subsequently, the linear plasmid ends were dephosphorylated by adding 6 µl of FastAP (Thermo Fisher) and incubating at 37 °C for 15 min. Enzyme was inactivated by incubation at 65 °C for 5 min. The products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey–Nagel). The concentration was measured at 260 nm with a Tecan Infinite M1000 PRO instrument. The resulting linear dsDNA was used as template for run-off *in vitro* transcription.

The *in vitro* T7 transcription of RNA with G as first transcribed nucleotide was performed in 1× transcription buffer (40 mM Tris/HCl, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine$(HCl)_3$) using 8 ng/µL corresponding DNA template, 0.5 mM A/C/UTP mix, 0.25 mM GTP, 1 mM ARCA, m^7GpppG or cap analogues **6b-d**, 1.2 U/µL RiboLock RNase Inhibitor (Thermo Scientific), 2 U/µL T7 RNA polymerase (Thermo Scientific) and 0.004 U/µL pyrophosphatase (Thermo Scientific) for 4 h at 37 °C.

The *in vitro* T7 transcription of RNA with an A as first transcribed nucleotide was performed in 1× transcription buffer using 8 ng/µL corresponding DNA template, 0.5 mM A/C/UTP mix, 0.25 mM GTP, 1 mM m^7GpppA or cap analogues **5b-d**, 1.2 U/µL RiboLock RNase Inhibitor, 2 U/µL T7 RNA polymerase and 0.004 U/µL pyrophosphatase for 4 h at 37 °C.

After *in vitro* transcription. DNA template was digested by incubation with 0.08 U/µL DNase I (Thermo Scientific) for 1 h at 37 °C followed by purification using the RNA Clean & Concentrator™-5 Kit (Zymo Research). To digest non-capped RNAs, up to 5 µg of purified RNA was incubated with 10 U of the RNA 5′-polyphosphatase (Epicentre) in 1× polyphosphatase reaction buffer for 1 h at 37 °C, followed by 1 h incubation at 37 °C after addition of 5 mM $MgCl_2$ and 0.5 U of the 5′–3′ exoribonuclease XRN1 (New England Biolabs). Afterwards, mRNAs were purified using the RNA Clean & Concentrator™-5 Kit. Concentration of mRNA was determined via absorbance at

260 nm with a Tecan Infinite M1000 PRO instrument. The quality of mRNA was assessed via dPAGE and mRNAs were stored at –20 °C.

Preparation of post-transcriptionally modified mRNAs via PC-MjMAT/Ecm1 cascade

The enzymatic mRNA modification at the *N*7 position of the terminal guanosine of 0.2 µM 5′-GpppG-mRNA was performed in the presence of 1 µM Ecm1, 2.5 µM MTAN, 2.5 µM PC-MjMAT, 50 µM **1a-d**, 50 µM ATP, 1.2 U/µL RiboLock, and 0.5× CutSmart buffer (25 mM KOAc, 10 mM Tris-acetate, 2.5 mM $Mg(OAc)_2$, 50 µg/ml BSA, pH = 7.9). After incubation for 3 h at 37 °C, the mRNAs were extracted via phenol/chloroform and precipitated with EtOH. Concentration of RNA was determined via absorbance at 260 nm. Then, the integrity of modified mRNAs was analysed by 7.5 % dPAGE. mRNA was stored at –20 °C until further use.

Quantification of post-transcriptional mRNA modification efficiency

To quantify the reaction efficiency of mRNA modification via PC-MjMAT/Ecm1 cascade, 400 ng RNA was incubated with 0.02 U/µL nuclease P1 in 1 × nuclease P1 reaction buffer (10× reaction buffer: 200 mM NH_4OAc, 1 mM $ZnCl_2$, pH = 5.3 adjusted with glacial acetic acid) in 17 µL reaction volume at 50 °C for 1 h. Nucleosides were dephosphorylated by addition of 1/10th volume of 10× fast alkaline phosphatase (FastAP) buffer and 1 U FastAP (Thermo Scientific) followed by incubation at 37 °C for 1 h. Proteins were precipitated by addition of $HClO_4$ as described above and the supernatant analysed by LC-QqQ-MS.

LC-QqQ-MS analysis and quantification of **6** and **6a-d** were performed on an Agilent 1260 Infinity II HPLC system with an Agilent Poroshell 120EC-C18, 3×150 mm, 2.7 µm column (Agilent Technologies) coupled to an Agilent Ultivo triple-quad mass spectrometer. Chromatographic separation was performed in a linear gradient from 100 % 50 mM NH_4OAc (0–1 min) to 60 % acetonitrile (7 min) and 100 % MeCN (8 min) at a flow rate of 0.8 mL/min at 40 °C. Samples were ionized via electrospray ionization in positive mode with the following parameters: gas temperature: 250 °C, gas flow: 7.0 L/min, nebulizer 40 psi, sheath gas temperature 375 °C, sheath gas flow 12 L/min. MRM mode was used with the fragmentor voltage and collision energy optimized for each nucleoside (Fig. S51B).

For quantification, standard curves were prepared using standard mixes of **6** and **6a-d** in different concentrations. Standard curves were then created and used for calculation of the relative abundance of different nucleosides using the software Agilent Mass Hunter Quantitative Analysis (for QqQ).

Transfection of HeLa and HEK-NF-κB cells with mRNA

For transfection, the HeLa cells (Merck) were cultured in MEM Eagle's media (PAN) supplemented with L-glutamine (2 mM, PAN), fetal calf serum (FCS, 10 %, PAN), non-essential amino acids (1 %, PAN) and penicillin and streptomycin (1 %, PAN). One day before transfection, the cells were seeded in a 96-well plate (30.000 cells/well). At the day of transfection, the media was replaced by transfection medium (medium without antibiotics). The transfection was performed by lipofection using Lipofectamine™ MessengerMAX™ (Invitrogen). 100 ng mRNA in 6.25 µL Opti-MEM was mixed with 0.15 µL Lipofectamine™ MessengerMAX™ in 6.25 µL Opti-MEM. The mixture was added to the well with cells. The cells were incubated with the transfection reagents under standard conditions (5 % CO_2, 37 °C) for 4.5 h. After incubation, transfection medium was replaced to medium with antibiotics.

HEK-NF-κB cells (from TRON in Mainz, Germany) were cultured in Dulbecco´s Modified Eagle´s medium (DMEM) supplemented with FCS (10 %, PAN), HEPES (1 %), L-glutamine (2 mM), non-essential amino acids (1 %, PAN) and sodium pyruvate (1 %). For selection the antibiotics blasticidin (10 µg/mL), zeocin (100 µg/mL) and geneticin (G418) (250 µg/mL) were added to the culture of the HEK-NF-κB-Null, HEK-NF-κB-TLR7 and HEK-NF-κB-TLR8 cell lines. The HEK-NF-κB-TLR3 cell line was cultured in the absence of G418. One day before transfection 40.000 cells/well were seeded in a 96-well plate in a medium without antibiotics. Transfection was performed as described above without medium exchange.

Luminescence assays *in cellulo*

Translational output of differently capped GLuc mRNAs in HeLa cells was determined at 24 h post-transfection. As GLuc is a secreting protein, the supernatant was collected from each well and the luminescence assay was performed with the Gaussia-Juice Luciferase Assay-Kit (pjk). The reagents were prepared as suggested by the manufacturer. 5 µL of supernatant (in triplicates) was transferred to a white 96-well plate, then 50 µL of the freshly prepared mix of reconstruction buffer and

coelenterazine was injected to the well with an acquisition time of 3000 ms and luminescence was measured using the Tecan Infinite© M1000 PRO plate reader. As negative control, untransfected cells were used. The luminescence signal from this untransfected control was regarded as background. The ARCA-capped mRNA was used as a reference.

Luminescence of differently capped firefly luciferase (FLuc) and Renilla luciferase (RLuc) mRNAs was measured at 24 h post-transfection. The cells were washed with warm PBS and incubated with 60 µL 1× lysis buffer (pjk) for 3 minutes. Cells were detached by pipetting, transferred to another tube, and incubated at 37 °C (450 U/min) for 10 min. 20 µL of each sample was transferred to a 96-well plate (in triplicates) and 50 µL of the freshly prepared Firefly or Renilla (Gaussia) reaction mixture was injected to the wells in case of FLuc or RLuc, respectively with an acquisition time of 3000 ms. Luminescence was measured using the Tecan Infinite© M1000 PRO plate reader. As negative control, untransfected cells were used. The luminescence signal from this untransfected control was regarded as background. The m^7GpppG-capped mRNA or ARCA-mRNA was used as a reference.

The dual-luminescence assay was performed for simultaneous determination of translational output and immunogenicity. HEK-NF-κB cells lines (HEK-NF-κB-Null, HEK-NF-κB-TLR3, HEK-NF-κB-TLR7 and HEK-NF-κB-TLR8) were transfected with differently capped Renilla luciferase mRNA as described above. The cell lines overexpress NF-κB driven firefly luciferase. The readout of FLuc luminescence gives information on immunogenicity while RLuc on protein production. At 20 h post-transfection the medium was removed and cells were washed with 100 µL warm PBS. The cells were detached from wells by pipetting with 40 µL cold PBS. For luminescence measurement, Gaussia-Juice Luciferase Assay Kit (pjk) and the Beetle-Juice Luciferase assay Firefly Kit (pjk) were used for RLuc and FLuc detection respectively. The reagents were prepared according to the manufacturer´s instructions. The cell-suspension was mixed with 40 µL of 2× Lysis Juice and incubated for 10 min at 37° C (450 U/min). Then, 20 µL of the cell lysate was transferred to a 96-well plate (in triplicates) and 50 µL of the freshly prepared Firefly reaction mixture was injected to the well with an acquisition time of 3000 ms and the FLuc signal was determined. Later, 50 µL of the freshly prepared Renilla (Gaussia) reaction mixture was injected to the same well with an acquisition time of 3000 ms, for determination of the RLuc signal.

m^7GpppG-capped mRNA or ARCA-mRNA was used as reference. As negative control, untransfected cells were used. The luminescence signal from this untransfected control was regarded as background.

In vitro luminescence assay

To investigate mRNA translational output *in vitro*, the Retic Lysate IVT™ kit (Invitrogen), a eukaryotic cell-free protein expression system, was used. The experiments were performed in a total volume of 15 µL and contained 10 ng ARCA RLuc-mRNA, 40 ng of the differently capped FLuc-mRNAs or 10 ng ARCA-FLuc and 40 ng of differently capped RLuc-mRNAs, 50 µM L-methionine, 0.8 U/µL Ribolock and 150 mM potassium acetate. Samples were mixed with 8.5 µL of the Reticulocyte lysate and incubated for 90 min at 30 °C. The translational outputs of RLuc and FLuc mRNAs were assessed in a dual luciferase assay using Gaussia-Juice Luciferase Assay Kit (pjk) and the Beetle-Juice Luciferase assay Firefly Kit (pjk), respectively. 2 µL of the translation mix (in triplicates) was transferred to a white 96-well plate and 50 µL of the freshly prepared Firefly reaction mixture was injected to the well with an acquisition time of 3000 ms and the FLuc signal was determined. Later, 50 µL of the freshly prepared Renilla (Gaussia) reaction mixture was injected to the same well with an acquisition time of 3000 ms, for determination of the RLuc signal. m^7GpppG-capped mRNA or ARCA-mRNA was used as reference.

Supplementary figures

Preparative HPLC purification of **1b**-**g**

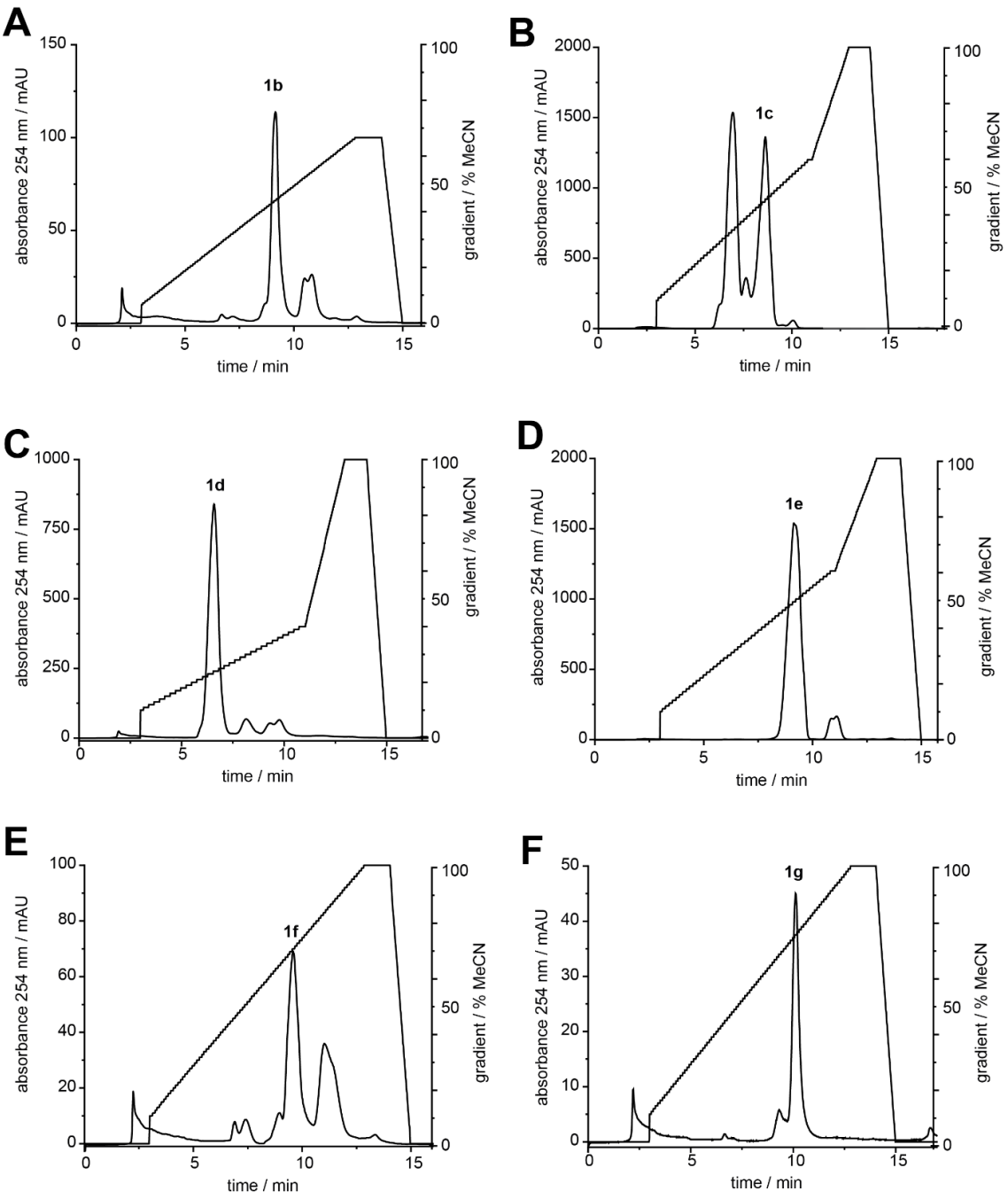


Figure S1: Chromatograms of the preparative HPLC purification of methionine analogues. A)-F) Preparative HPLC purification (BÜCHI C-700) of methionine analogues **1b**-**g** as indicated. The y-axis (left) indicates the absorbance at 254 nm of the chromatogram, while the y-axis (right) shows the % MeCN of the gradient.

LC-QTOF-MS analysis of methionine analogues **1b**-**g**

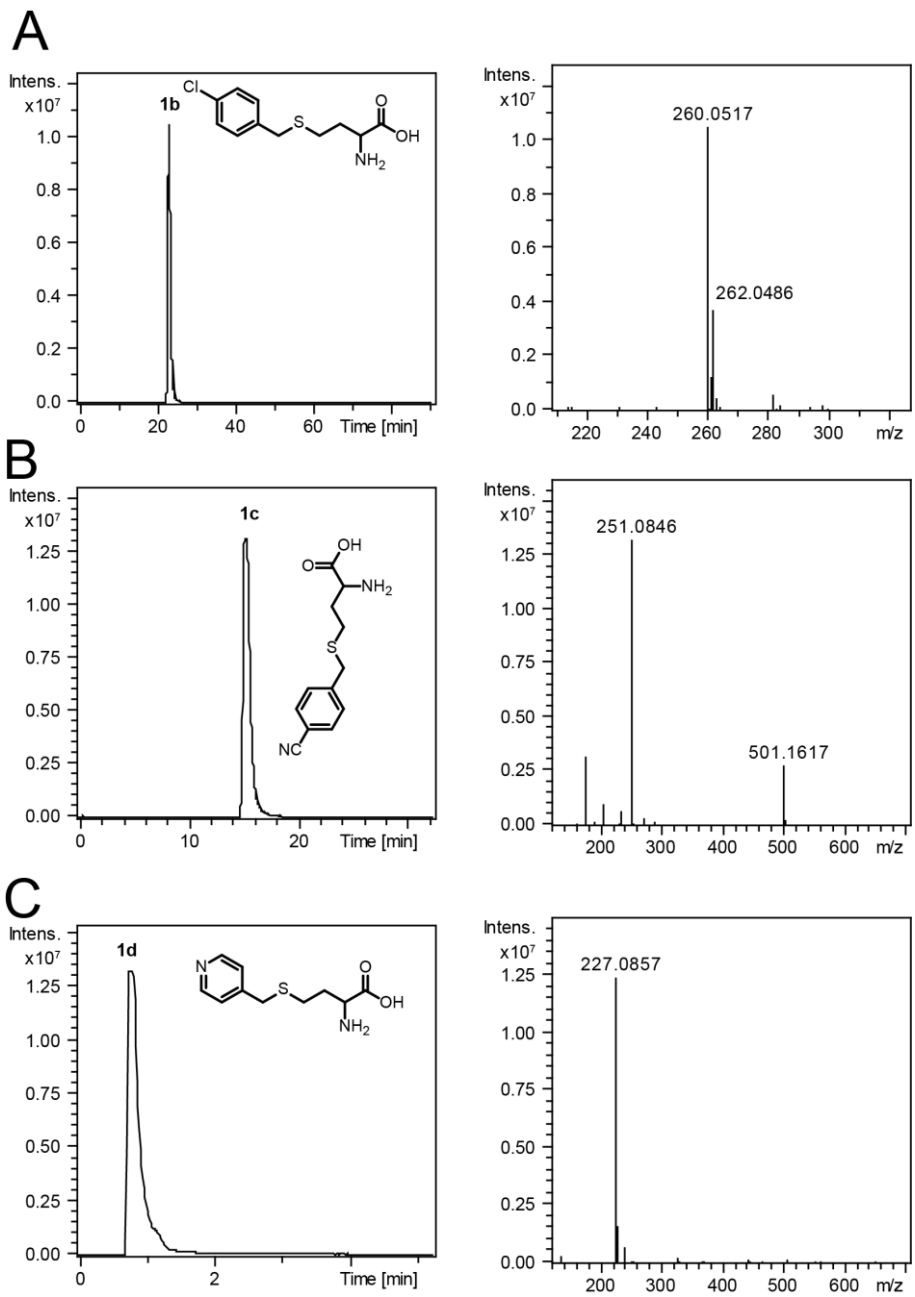


Figure S2: LC-QTOF-MS analysis of **1b**-**d**. Shown is the EIC in the left panel and the MS analysis of the main peak on the right panel. A) 4-Chlorobenzyl-D,L-homocysteine (**1b**). Retention time: 23.5 min. Calculated for $C_{11}H_{15}ClNO_2S^+$ $[M+H]^+$ = 260.0507, found m/z = 260.0517. Note the typical isotope pattern expected for chlorine. B) 4-Cyanobenzyl-D,L-homocysteine (**1c**). Retention time: 15.2 min. Calculated for $C_{12}H_{15}N_2O_2S^+$ $[M+H]^+$ = 251.0849, found m/z = 251.0846. C) 4-Pyridinylmethyl-D,L-homocysteine (**1d**). Retention time: 0.8 min. Calculated for $C_{10}H_{15}N_2O_2S^+$ $[M+H]^+$ = 227.0849, found m/z = 227.0857.

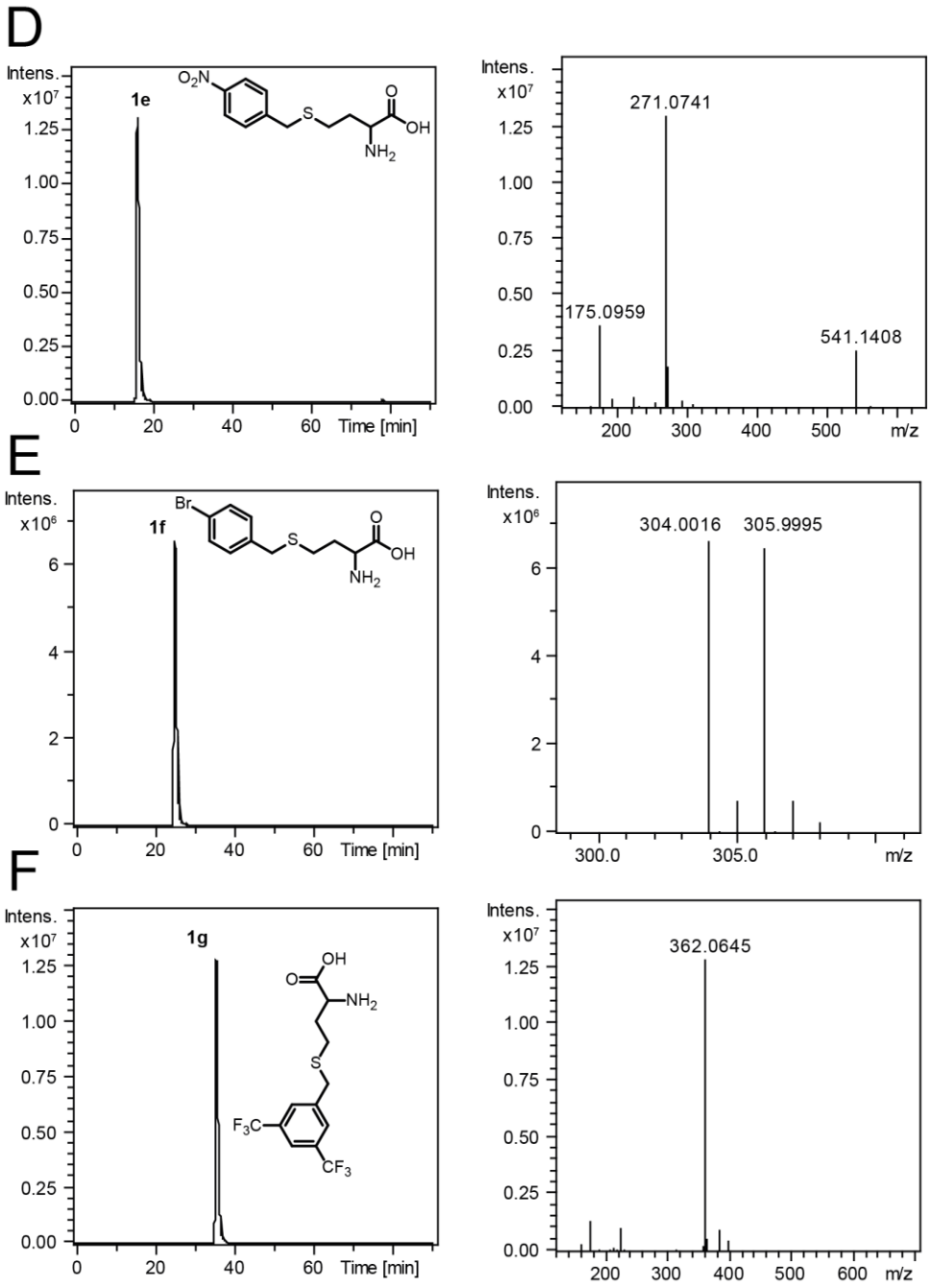


Figure S3: LC-QTOF-MS analysis of **1e**-**g**. Shown is the EIC in the left panel and the MS analysis of the main peak on the right panel. D) 4-Nitrobenzyl-DL-homocysteine (**1e**) retention time: 16.6 min. Calculated for $C_{11}H_{15}N_2O_4S^+$ $[M+H]^+$ = 271.0747, found m/z = 271.741. E) 4-Bromobenzyl-DL-homocysteine (**1f**) retention time: 25.5 min. Calculated for $C_{11}H_{15}BrNO_2S^+$ $[M+H]^+$ = 304.0001, found m/z = 304.0016. Note the typical isotope pattern expected for bromine. F) 3,5-Bis(trifluoromethyl)benzyl-DL-homocysteine (**1g**) retention time: 35.9 min. Calculated for $C_{13}H_{14}F_6NO_2S^+$ $[M+H]^+$ = 362.0644, found m/z = 362.0645.

NMR spectra of methionine analogues **1b**-**g**

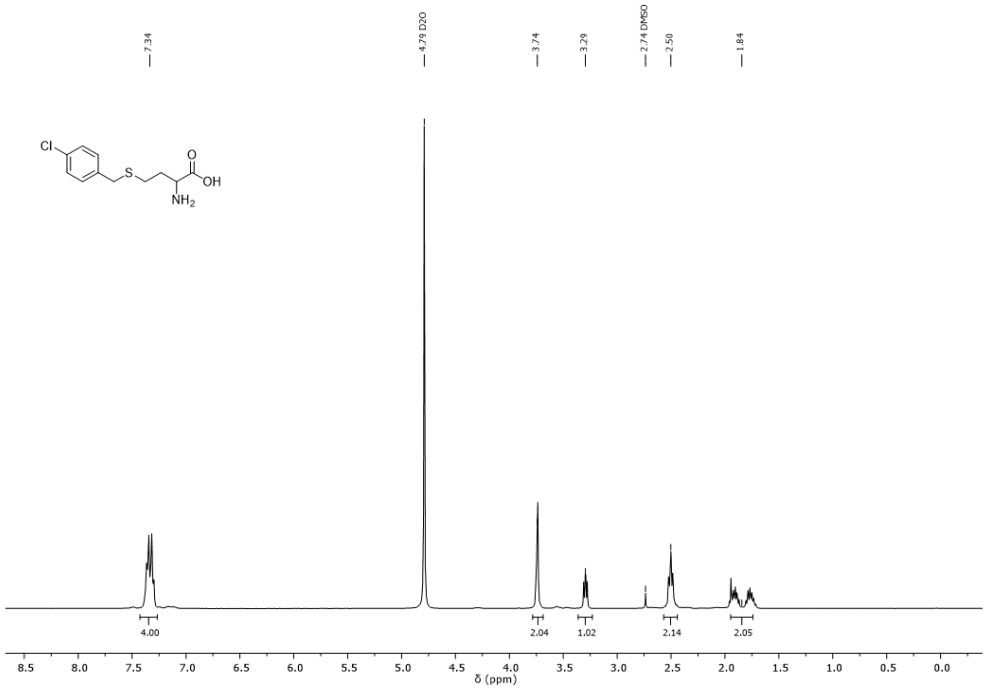


Figure S4: ^{1}H NMR spectrum of 4-chlorobenzyl-D,L-homocysteine (**1b**).

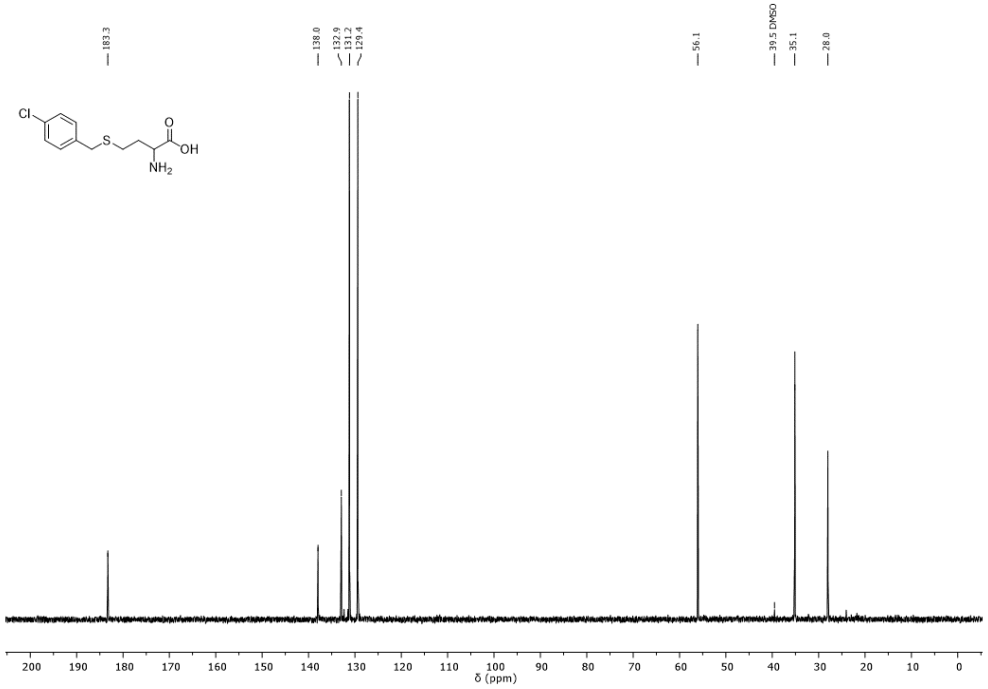


Figure S5: ^{13}C-{^{1}H}-NMR spectrum of 4-chlorobenzyl-D,L-homocysteine (**1b**).

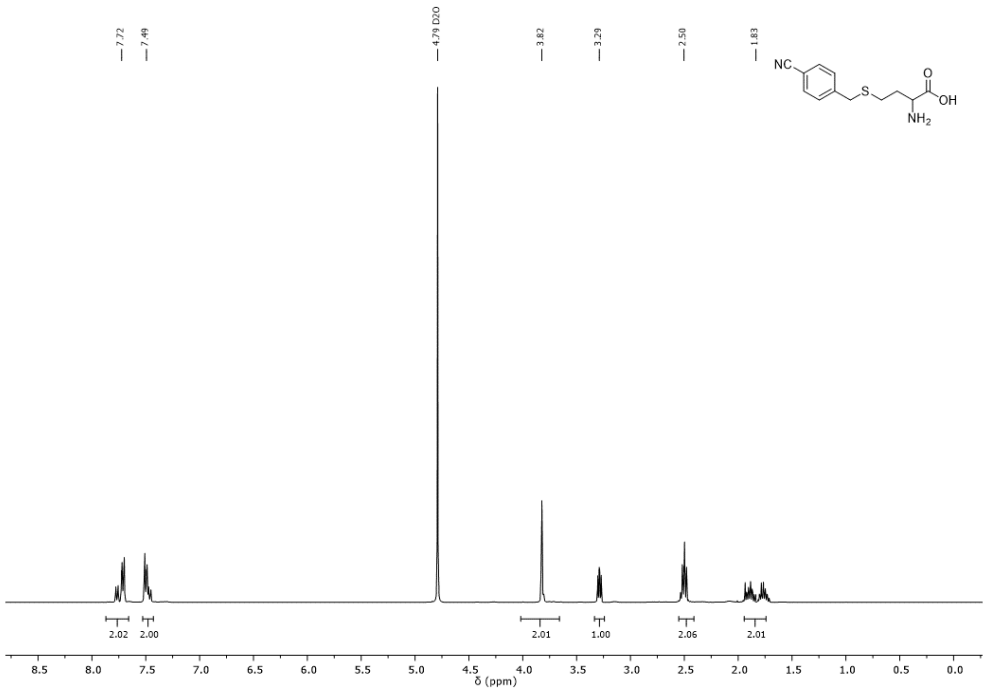


Figure S6: ^{1}H NMR spectrum of 4-cyanobenzyl-D,L-homocysteine (**1c**).

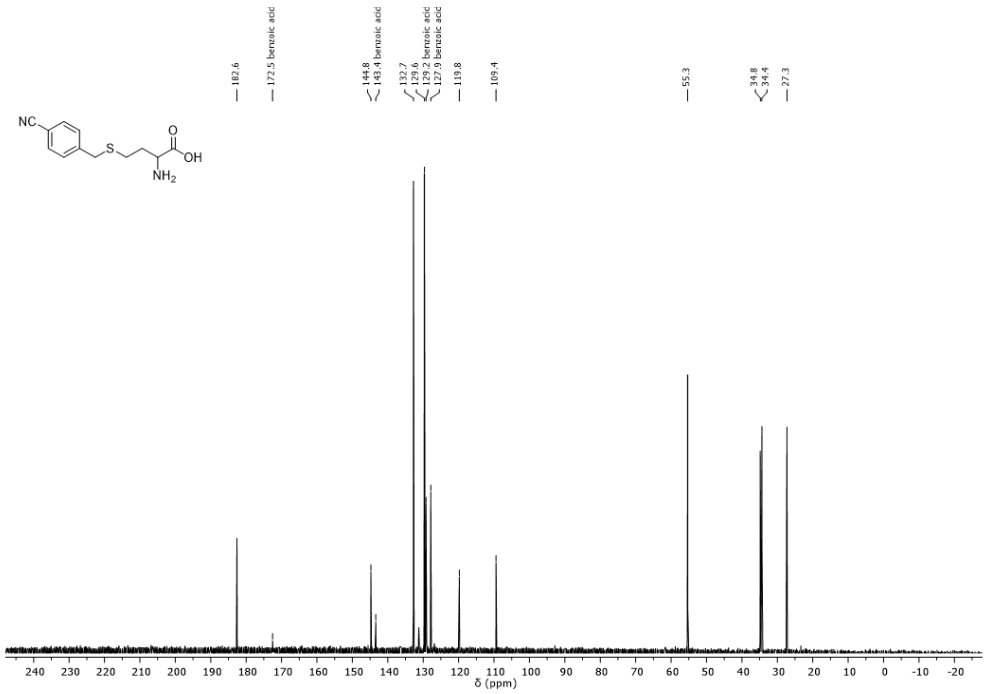


Figure S7: ^{13}C-{^{1}H}-NMR spectrum of 4-cyanobenzyl-D,L-homocysteine (**1c**).

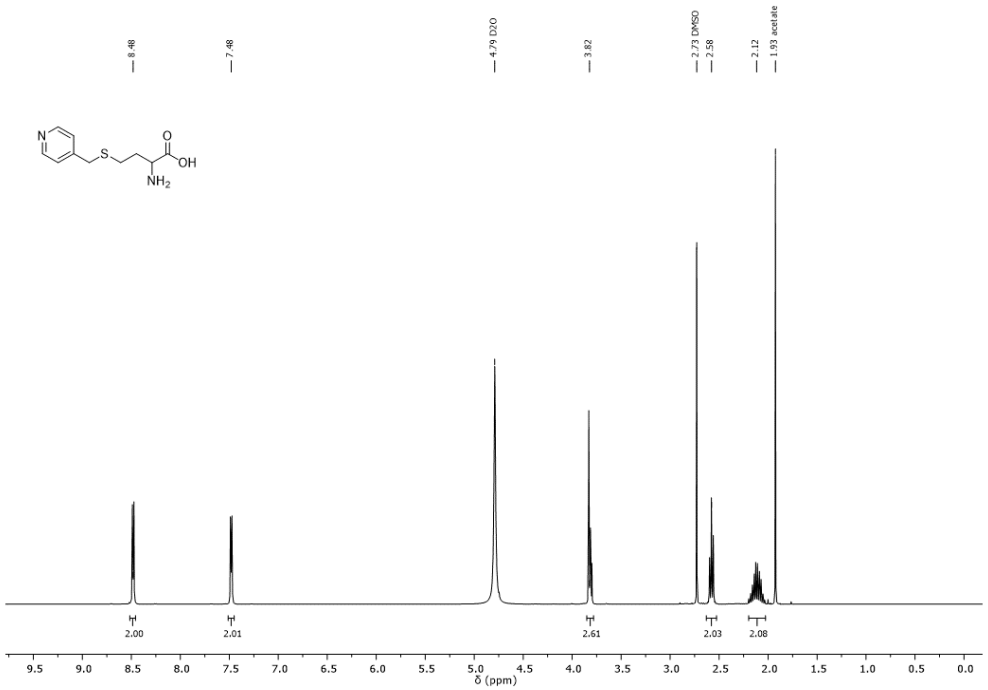


Figure S8: ^{1}H NMR spectrum of 4-pyridinylmethyl-D,L-homocysteine (**1d**).

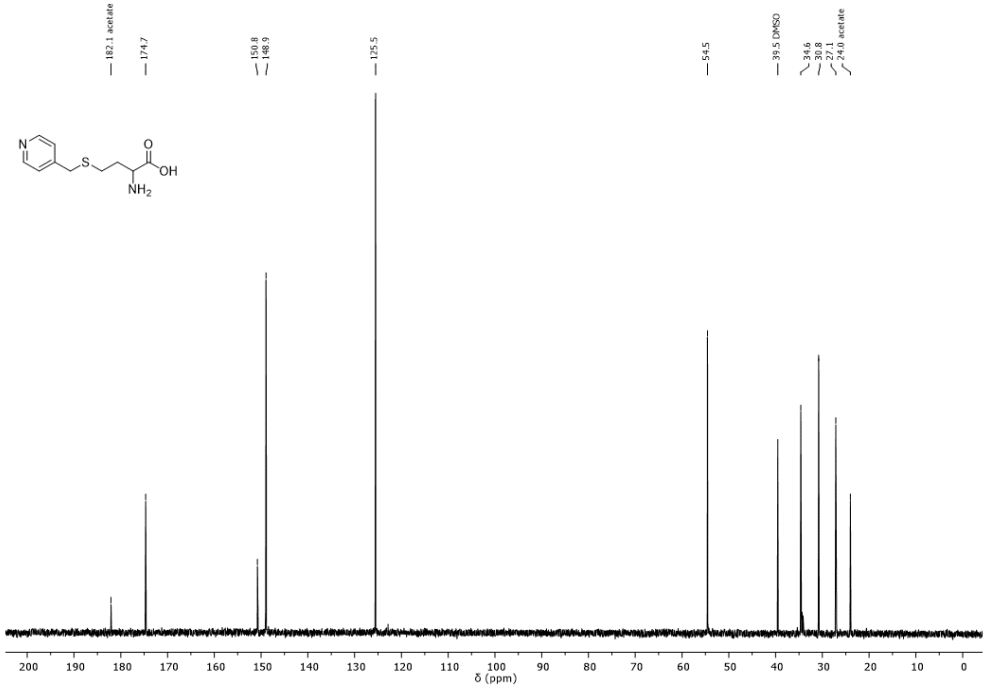


Figure S9: ^{13}C-{^{1}H}-NMR spectrum of 4-pyridinylmethyl-D,L-homocysteine (**1d**).

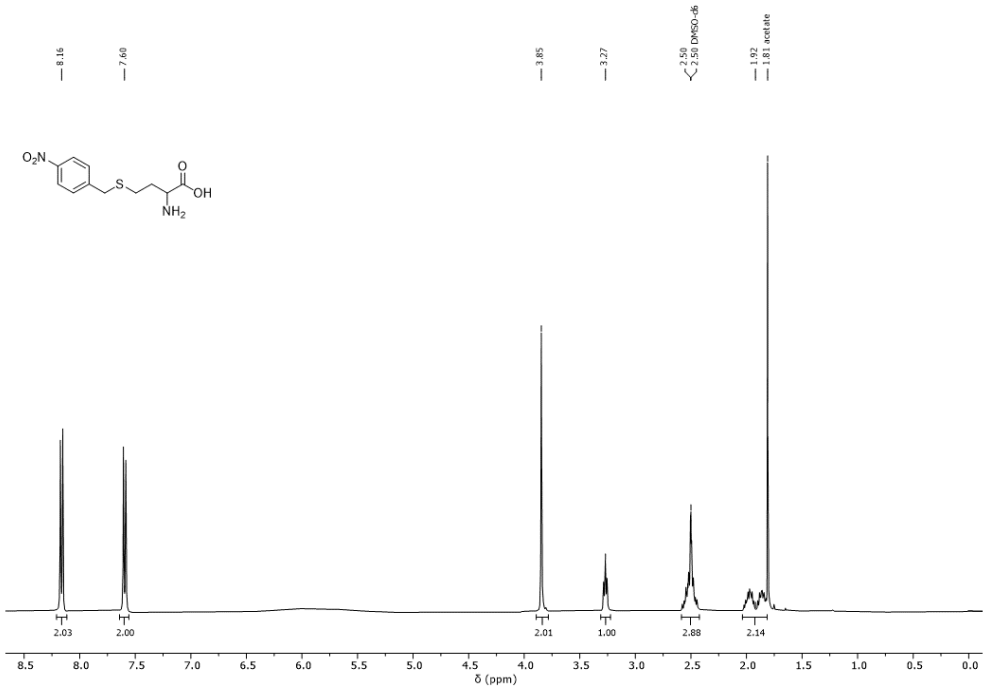


Figure S10: ^{1}H NMR spectrum of 4-nitrobenzyl-D,L-homocysteine (**1e**).

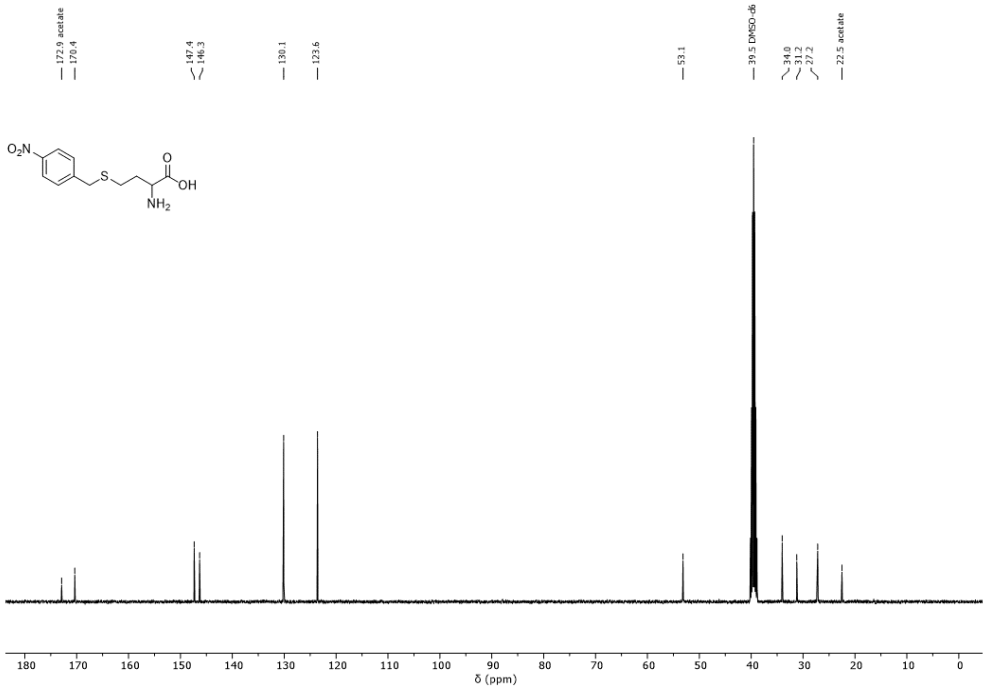


Figure S11: ^{13}C-{^{1}H}-NMR spectrum of 4-nitrobenzyl-D,L-homocysteine (**1e**).

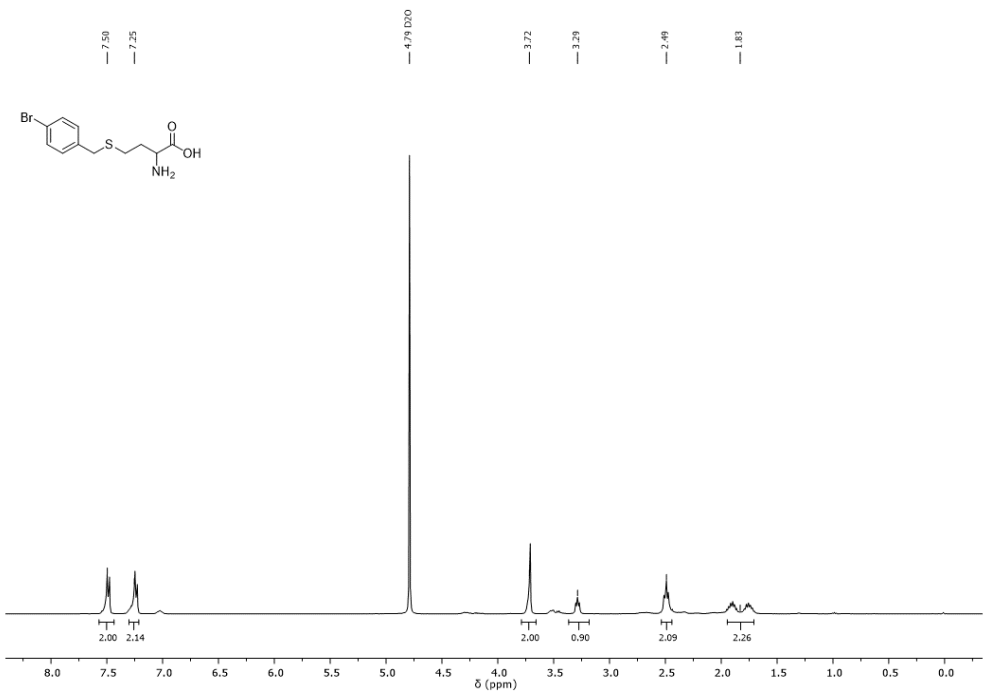


Figure S12: ^{1}H NMR spectrum of 4-bromobenzyl-D,L-homocysteine (**1f**).

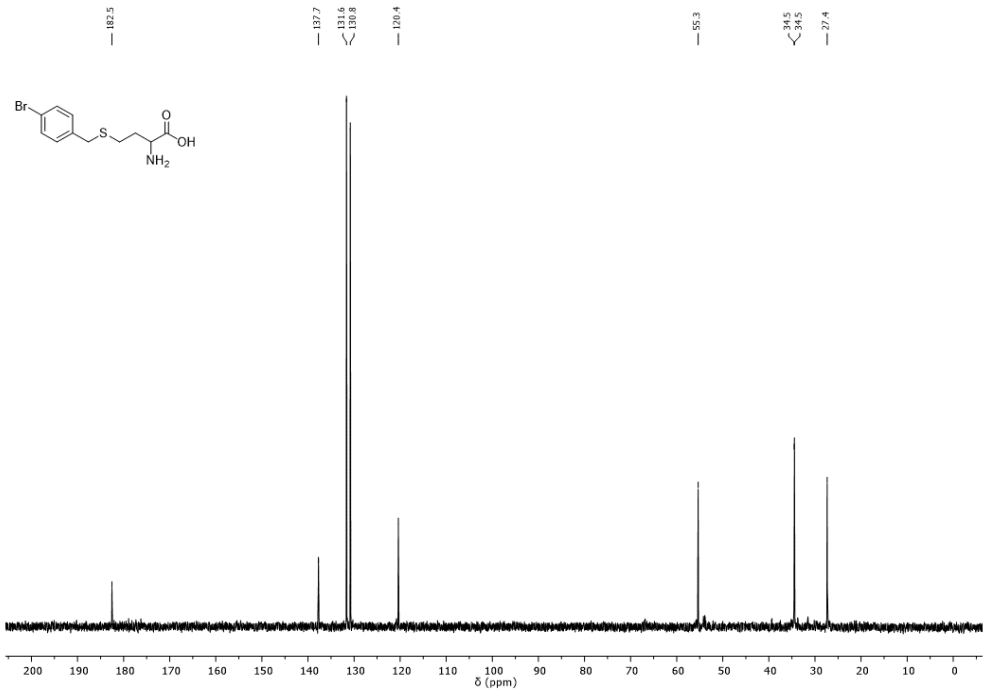


Figure S13: ^{13}C-{^{1}H}-NMR spectrum of 4-bromobenzyl-D,L-homocysteine (**1f**).

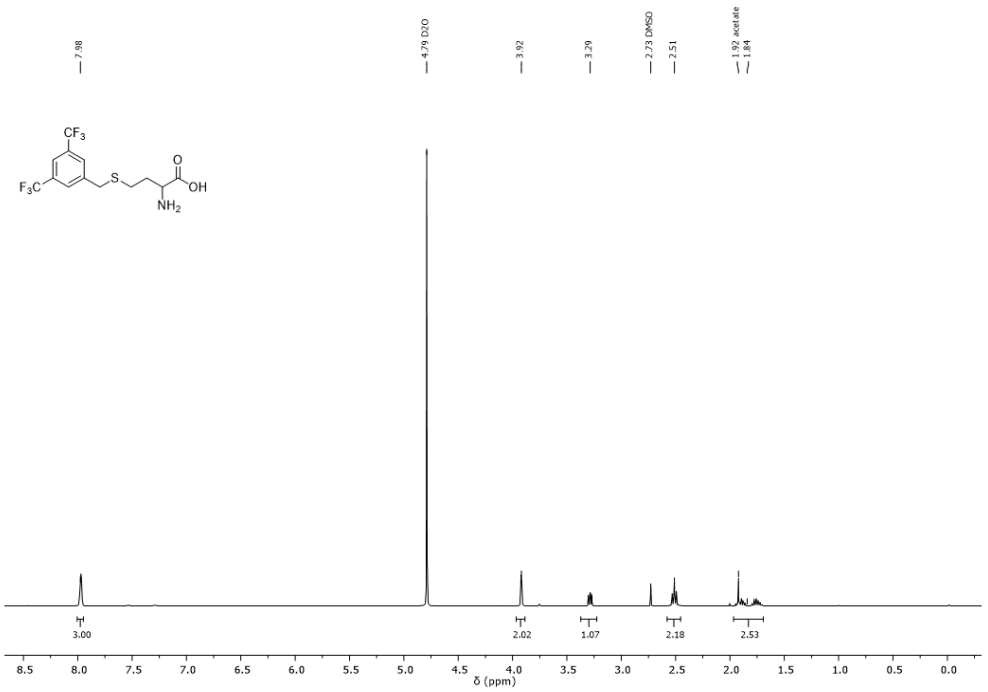


Figure S14: ^{1}H NMR spectrum of 3,5-bis(trifluoromethyl)benzyl-D,L-homocysteine (**1g**).

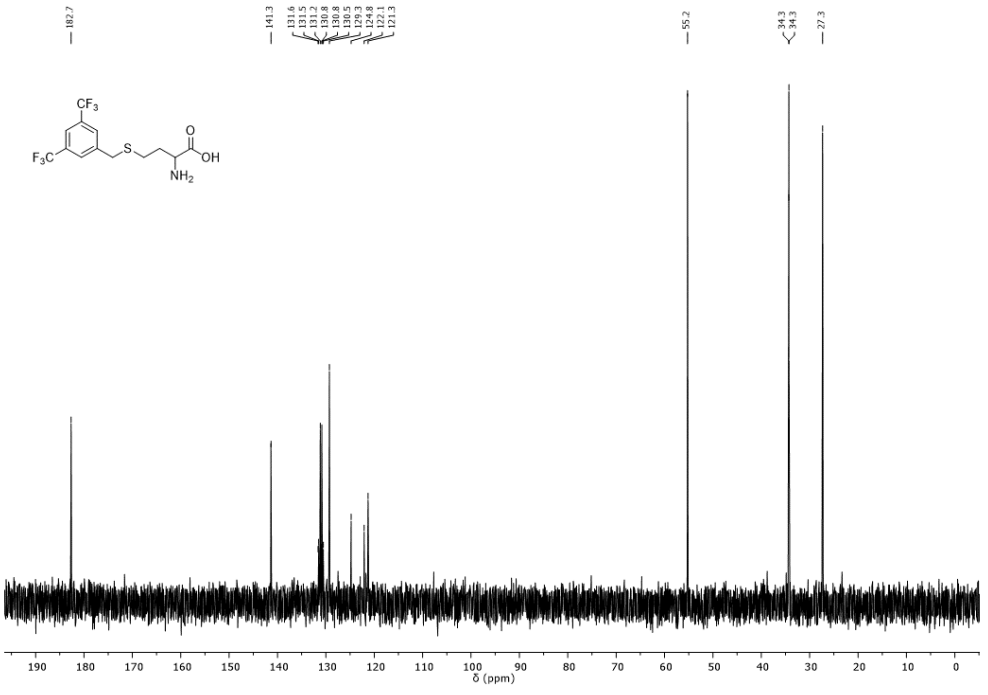


Figure S15: ^{13}C-{^{1}H}-NMR spectrum of 3,5-bis(trifluoromethyl)benzyl-D,L-homocysteine (**1g**).

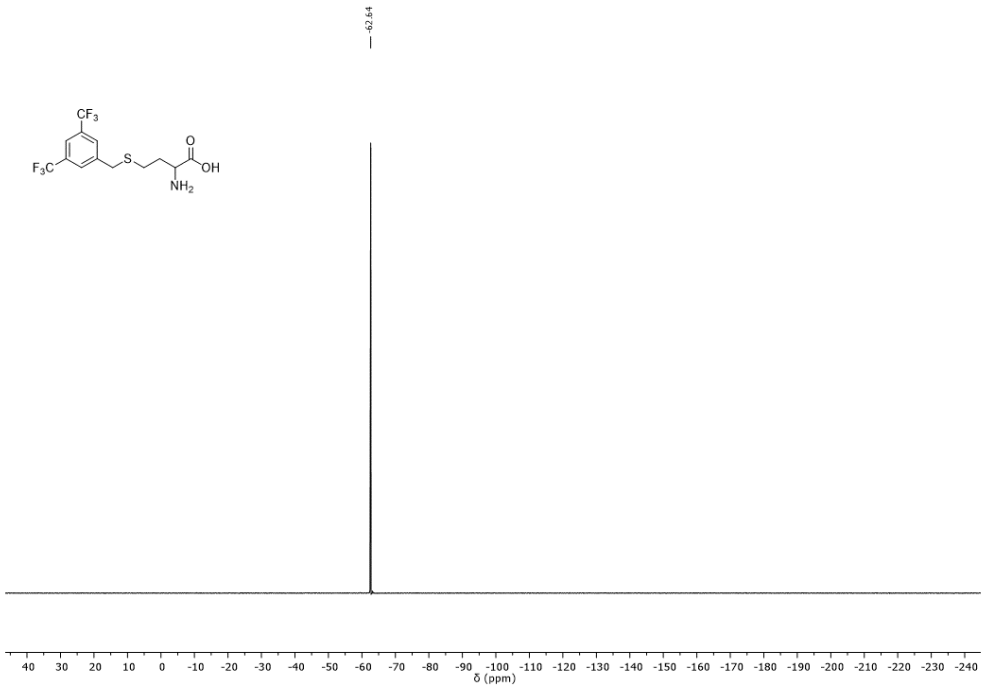


Figure S16: ^{19}F-{^{1}H}-NMR spectrum of 3,5-bis(trifluoromethyl)benzyl-D,L-homocysteine (**1g**).

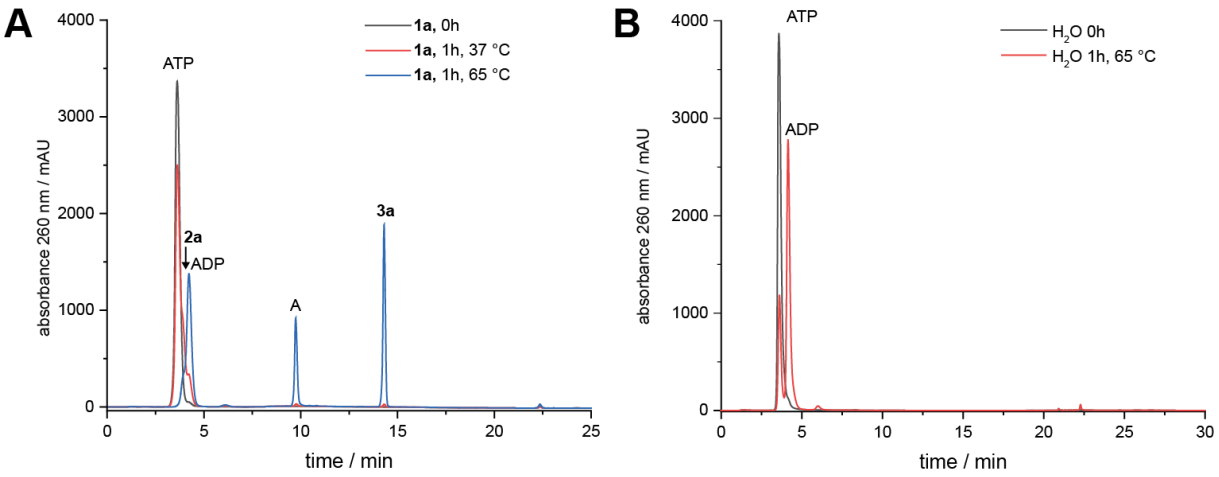


Figure S17: Representative HPLC analysis of MTA assays for control samples as shown in Fig. 2 (main text). A) MTA assay of methionine **1a**. Adenine (A) is formed during the assay. B) Negative control (amino acid solution was replaced by ddH_2O).

LC-QTOF-MS analysis of AdoMet analogues **2b**-**g**

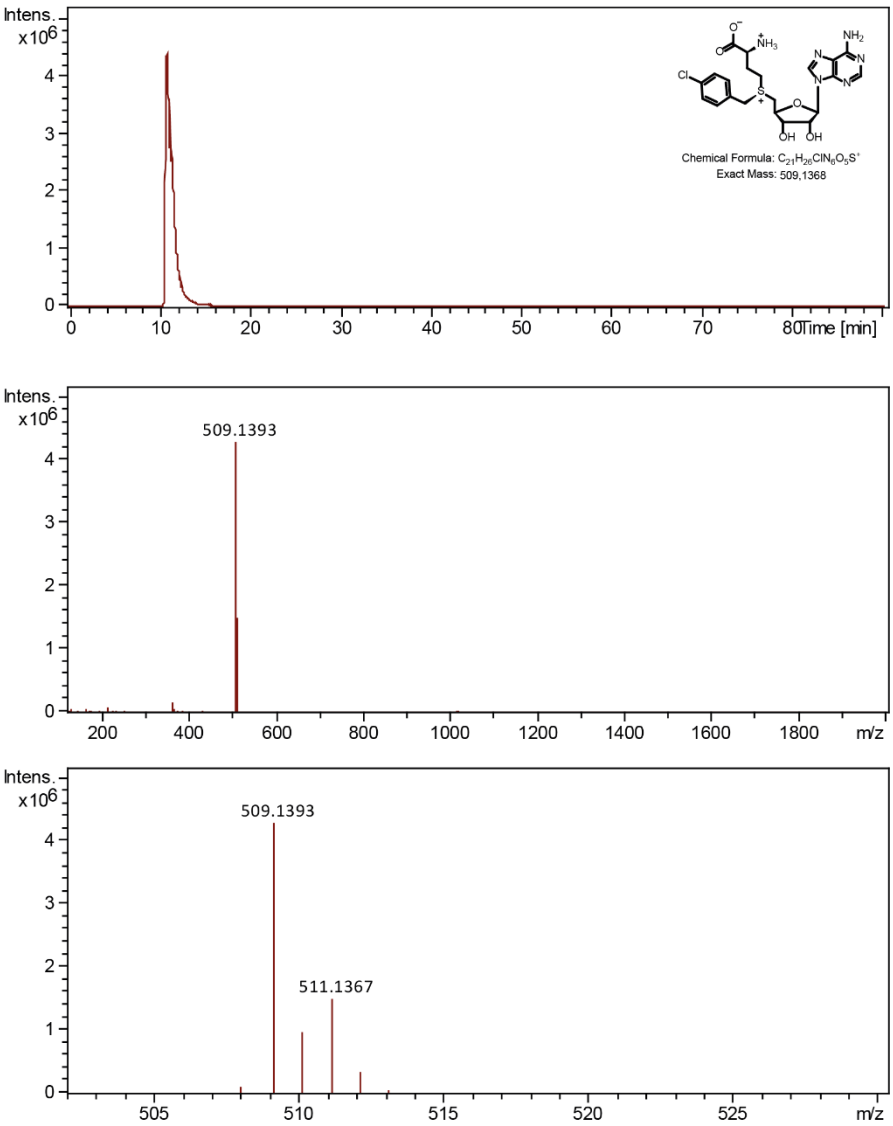


Figure S18: LC-QTOF-MS analysis of compound **2b**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the main peak. Calculated mass for $C_{21}H_{26}ClN_6O_5S^+$ $[M]^+$ = 509.1368 Da, found m/z = 509.1393. Note the typical isotope pattern for chlorine.

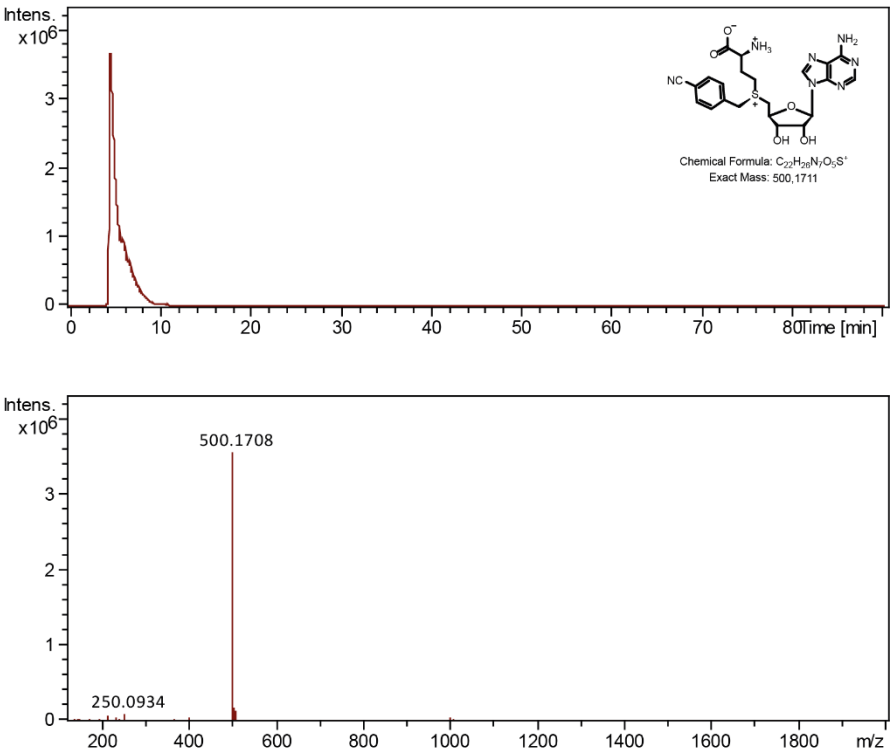


Figure S19: LC-QTOF-MS analysis of compound **2c**. Upper panel shows the EIC and lower panel shows the mass spectrum at the main peak. Calculated mass for $C_{22}H_{26}N_7O_5S^+$ $[M]^+$ = 500.1711 Da, found m/z = 500.1708.

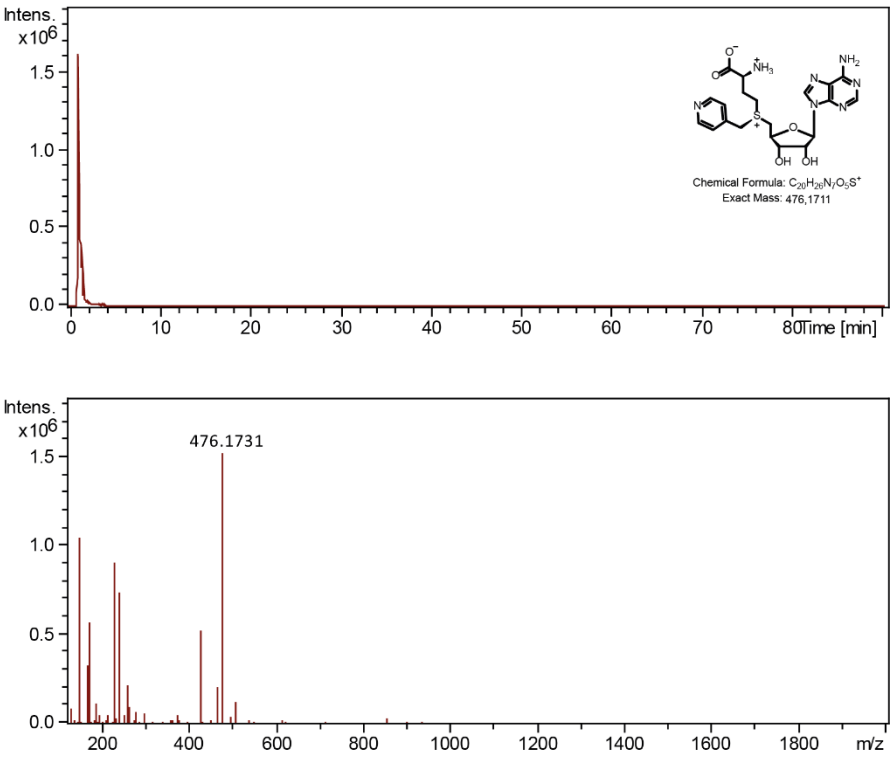


Figure S20: LC-QTOF-MS analysis of compound **2d**. Upper panel shows the EIC and lower panel shows the mass spectrum at the main peak. Calculated mass for $C_{20}H_{26}N_7O_5S^+$ $[M]^+$ = 476.1711 Da, found m/z = 476.1731.

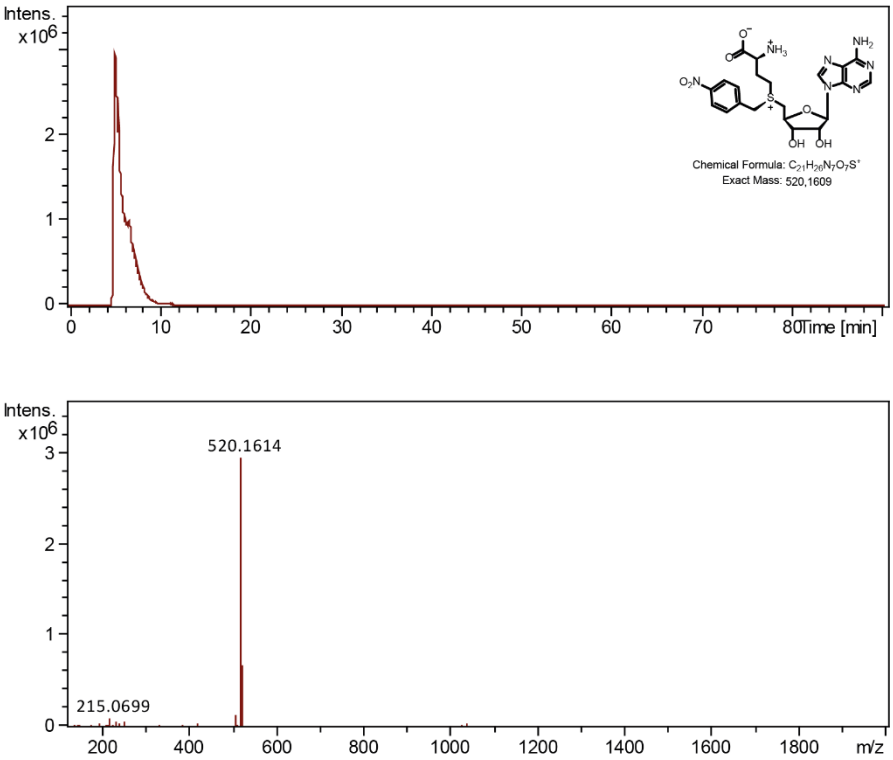


Figure S21: LC-QTOF-MS analysis of compound **2e**. Upper panel shows the EIC and lower panel shows the mass spectrum at the main peak. Calculated mass for $C_{21}H_{26}N_7O_7S^+$ $[M]^+$ = 520.1609 Da, found m/z = 520.1614.

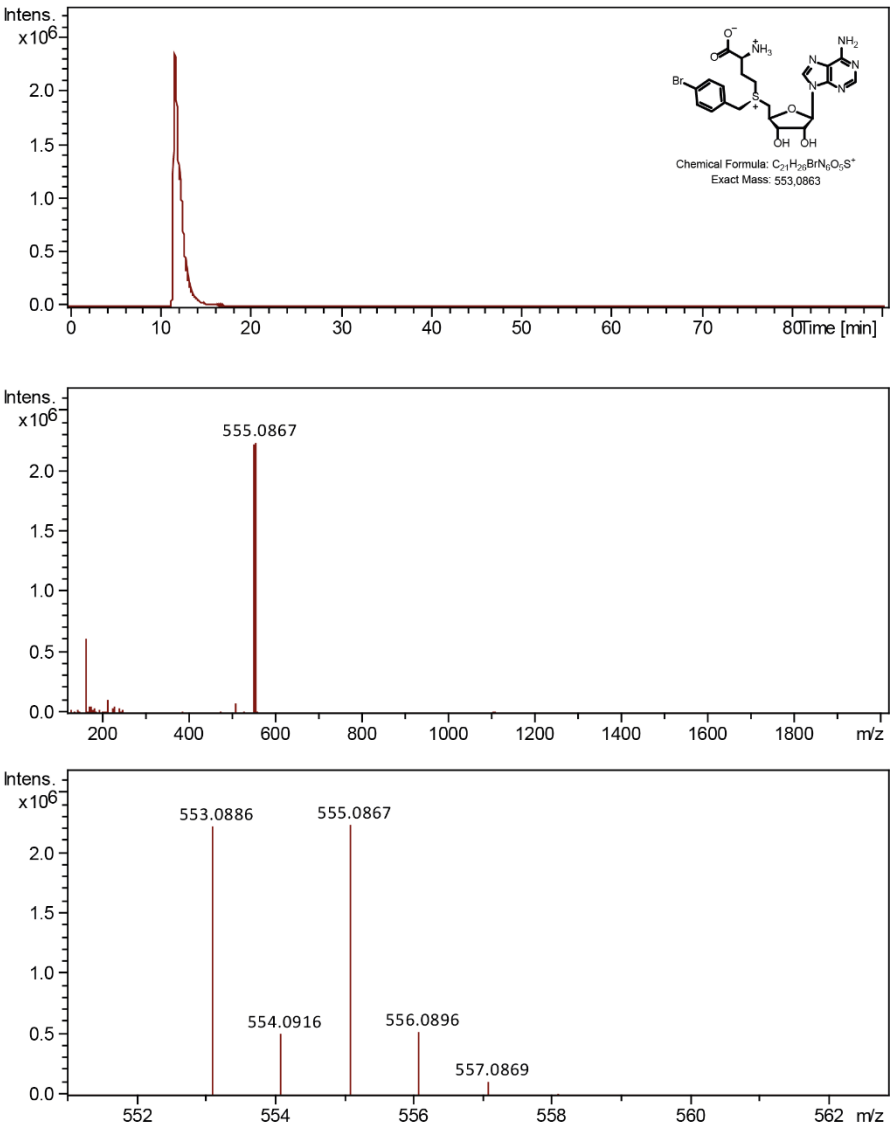


Figure S22: LC-QTOF-MS analysis of compound **2f**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the main peak. Calculated mass for $C_{21}H_{26}BrN_6O_5S^+$ $[M]^+$ = 553.0863 Da, found m/z = 553.0886. Note the typical isotope pattern for bromine.

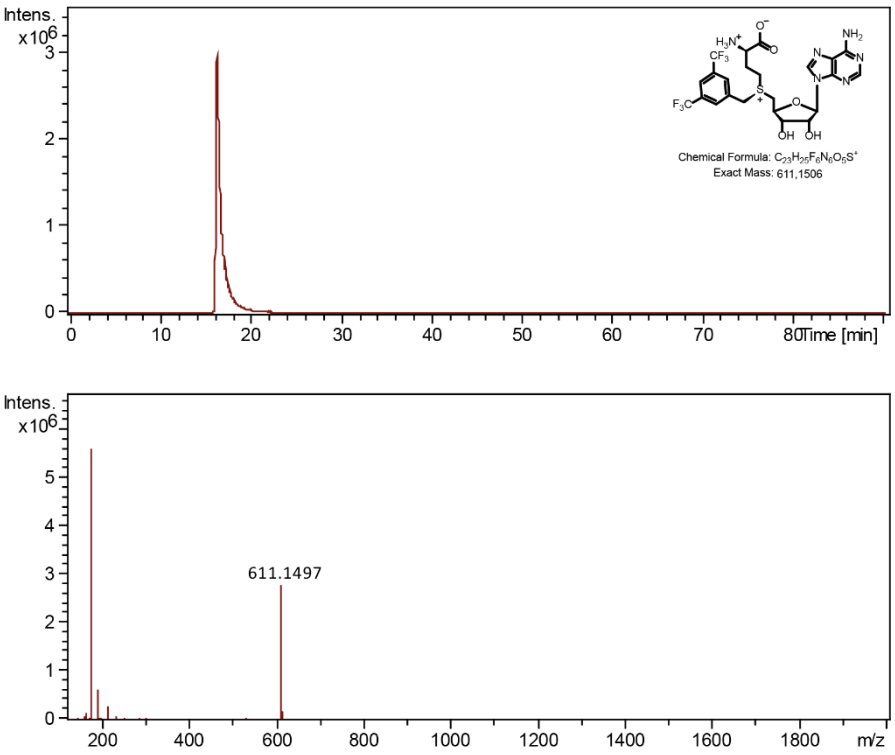


Figure S23: LC-QTOF-MS analysis of compound **2g**. Upper panel shows the EIC and lower panel shows the mass spectrum at the main peak. Calculated mass for $C_{23}H_{25}F_6N_6O_5S^+$ $[M]^+$ = 611.1506 Da, found m/z = 611.1497.

Cascade PC-MjMAT/NovO

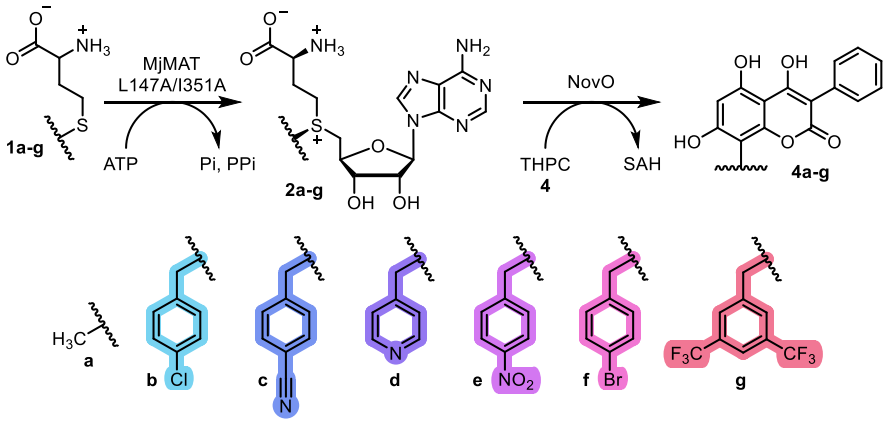


Figure S24: PC-MjMAT/NovO cascade used to convert 4,5,7-trihydroxy-3-phenylcoumarin (THPC, **4**) to derivatives benzylated at 8-position (**4a**-**g**). 5 mM **1a-g** are incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM NovO, 10 µM MTAN, 1 mM **4** (stock solution in DMSO resulting in 5 vol.-% DMSO in the reaction mixture) at 37 °C for 2 h in a total volume of 40 µL. Samples were quenched by addition of three volumes of ice cold MeCN. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4).

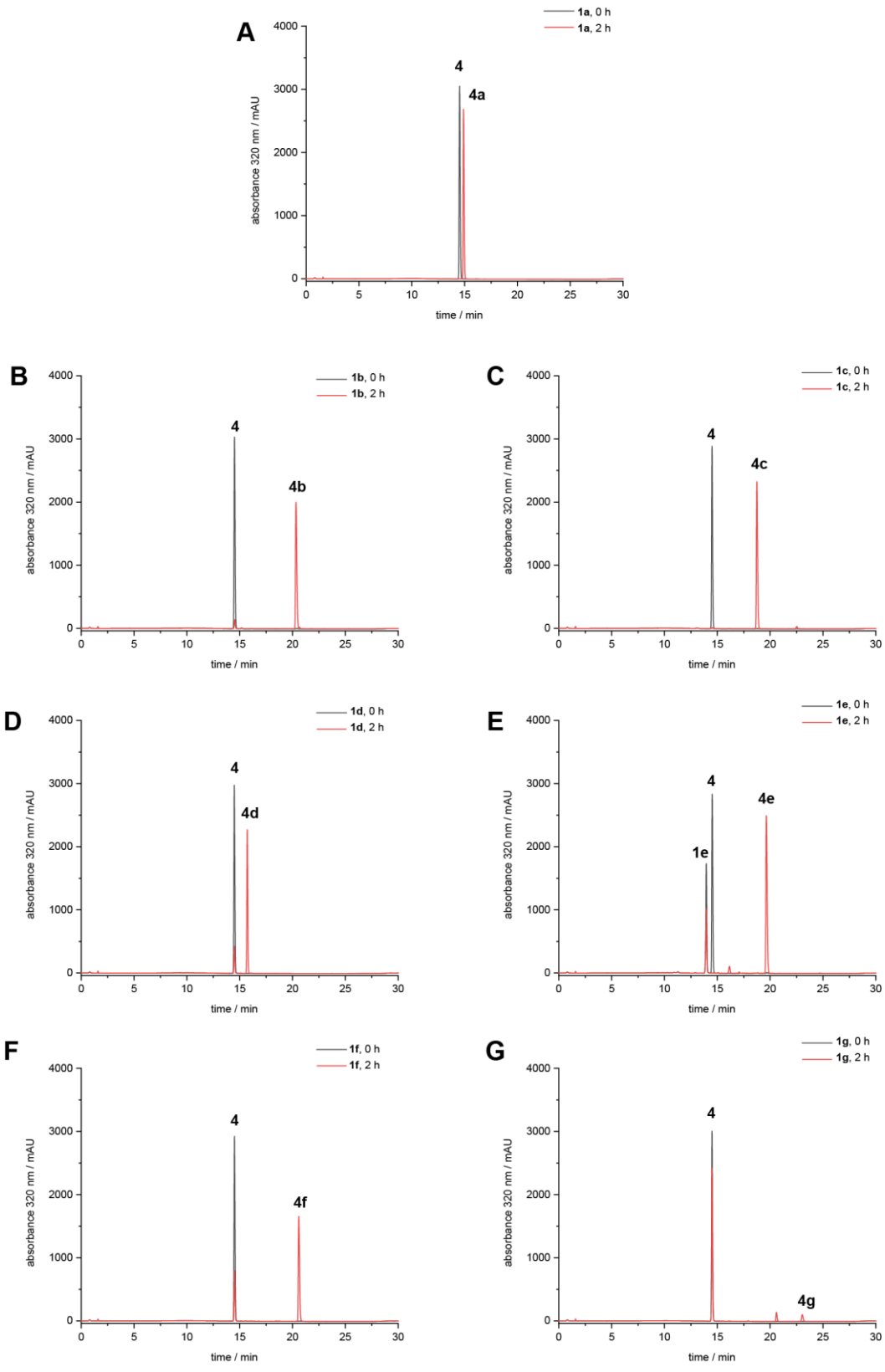


Figure S25: Representative HPLC analyses of PC-MjMAT/NovO cascades as shown in Figure 3B (main text). Here, the traces are grouped by the methionine analogue used and the additional 0 h time point is shown. A)-G) PC-MjMAT/NovO cascades of **4** with **1a**-**g**. Absorbance at 320 nm is shown. HPLC method C was used.

LC-QTOF-MS analysis of THPC analogues **4a**-**g**

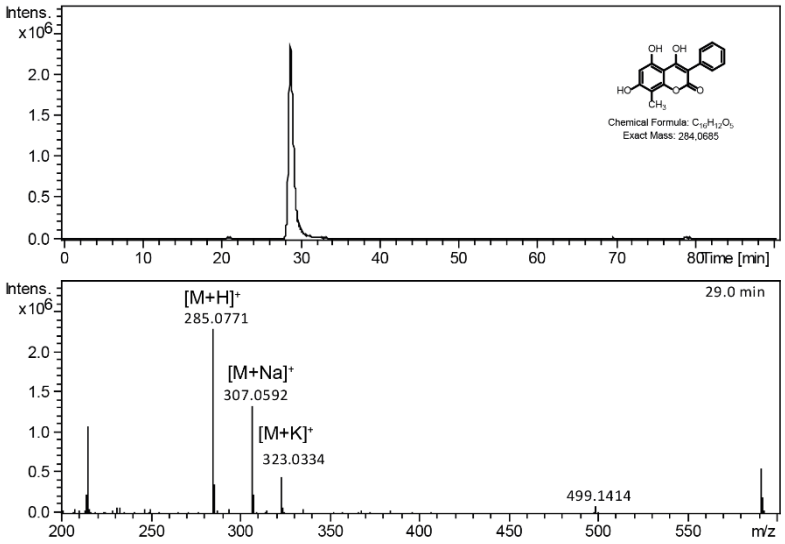


Figure S26: LC-QTOF-MS analysis of compound **4a**. Upper panel shows the EIC and lower panel shows the mass spectrum at indicated retention time. Calculated mass for $C_{16}H_{13}O_5^+$ $[M+H]^+$ = 285.0758 Da, found m/z = 285.0771.

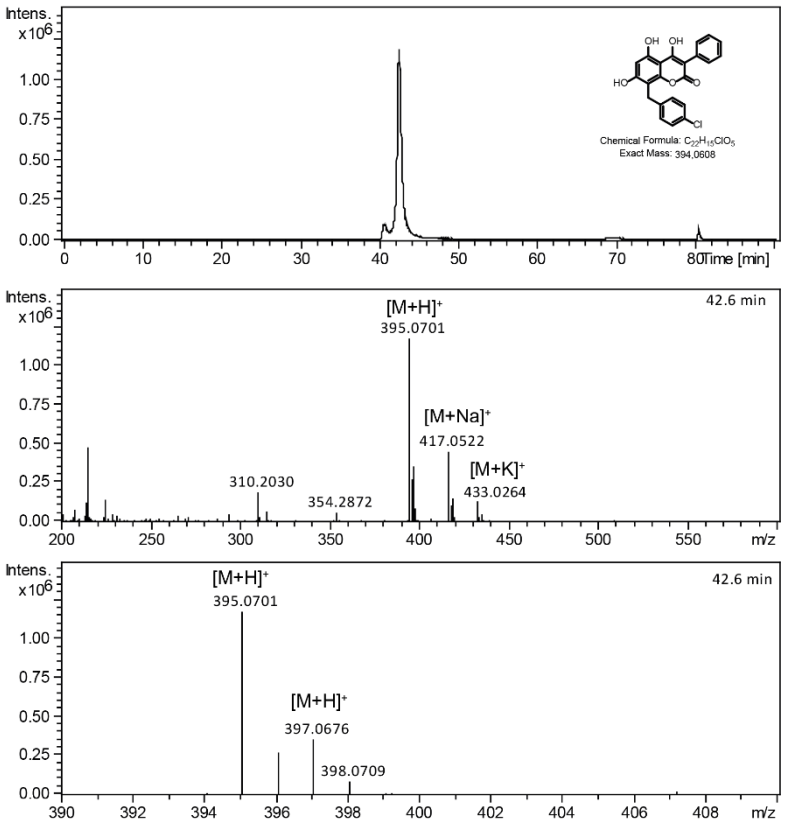


Figure S27: LC-QTOF-MS analysis of compound **4b**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the indicated retention time. Calculated mass for $C_{22}H_{16}ClO_5^+$ $[M+H]^+$ = 395.0681 Da, found m/z = 395.0701. Note the typical isotope pattern for chlorine.

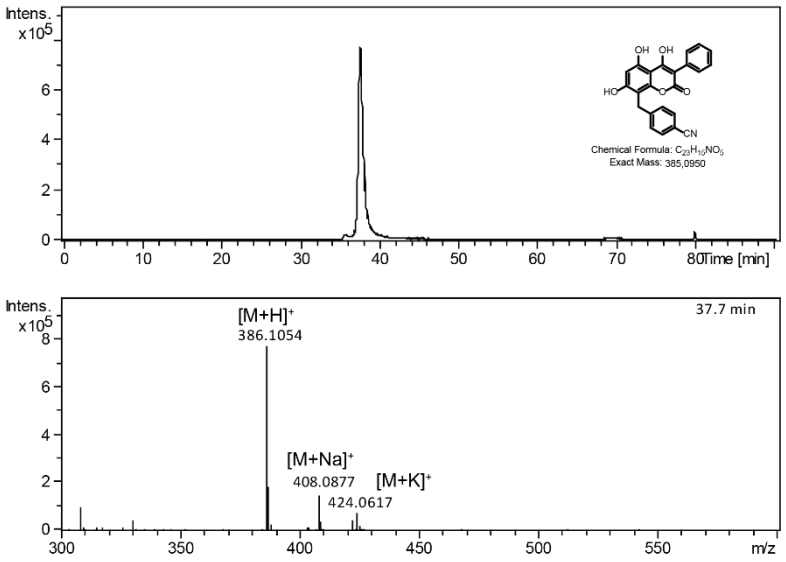


Figure S28: LC-QTOF-MS analysis of compound **4c**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{23}H_{16}NO_5^+$ $[M+H]^+$ = 386.1022 Da, found m/z = 386.1054.

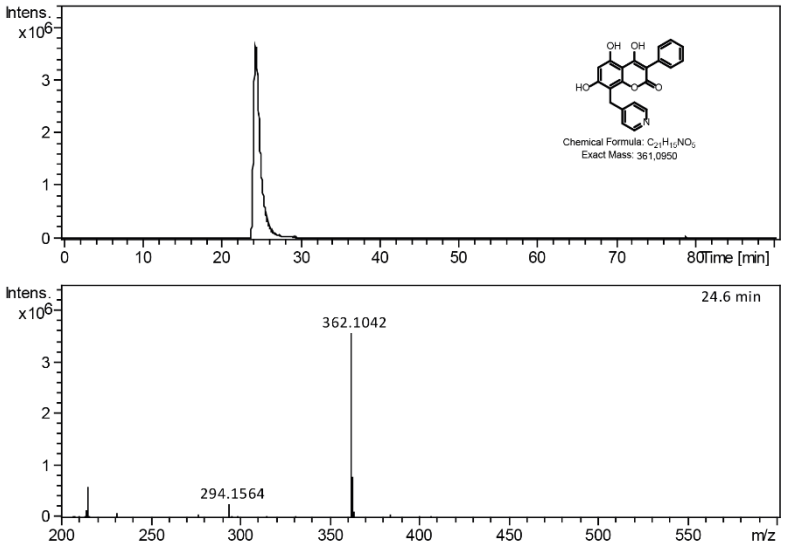


Figure S29: LC-QTOF-MS analysis of compound **4d**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{21}H_{16}NO_5^+$ $[M+H]^+$ = 362.1023 Da, found m/z = 362.1042.

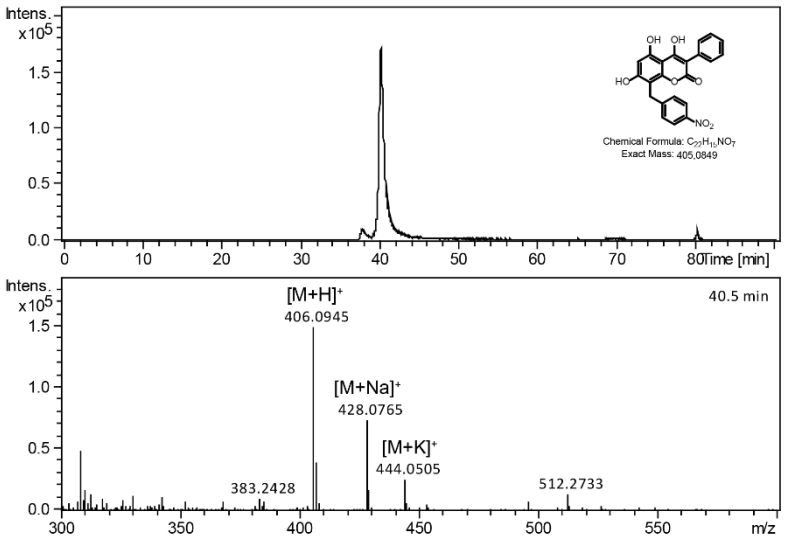


Figure S30: LC-QTOF-MS analysis of compound **4e**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{22}H_{16}NO_7^+$ $[M+H]^+$ = 406.0921 Da, found m/z = 406.0945.

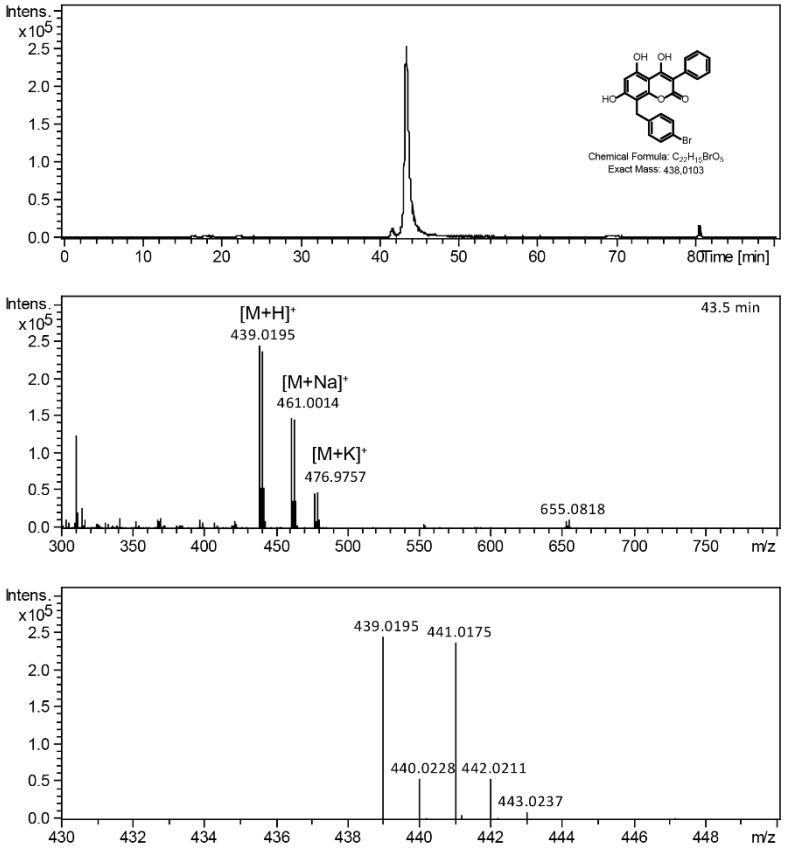


Figure S31: LC-QTOF-MS analysis of compound **4f**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the indicated retention time. Calculated mass for $C_{22}H_{16}BrO_5^+$ $[M+H]^+$ = 439.0176 Da, found m/z = 439.0195.

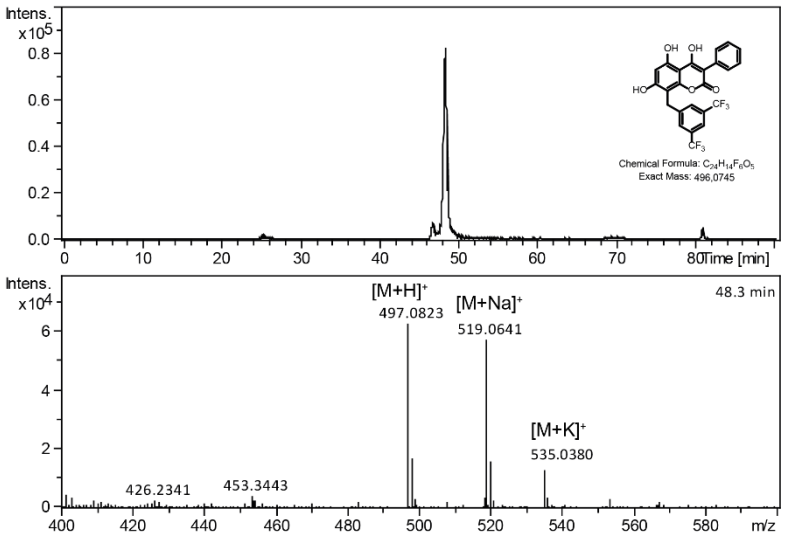


Figure S32: LC-QTOF-MS analysis of compound **4g**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{24}H_{15}F_6O_5^+$ $[M+H]^+$ = 497.0818 Da, found m/z = 497.0832.

Cascade PC-MjMAT/Ecm1

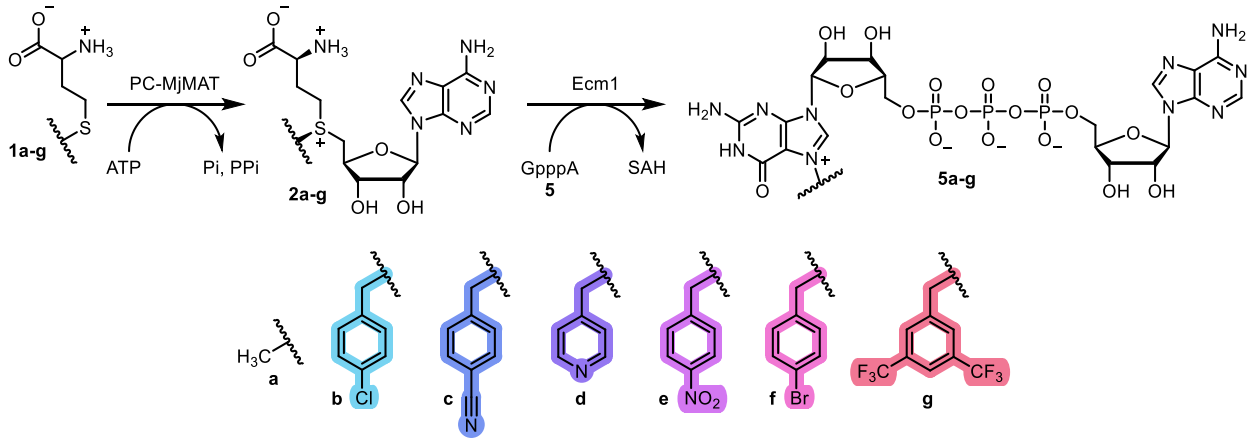


Figure S33: Conversion of GpppA (**5**) to derivatives benzylated at *N*7-position of guanosine (**5a-g**) via PC-MjMAT/Ecm1 cascade. 5 mM **1a**-**g** are incubated with 5 mM ATP, 100 µM PC-MjMAT, 50 µM Ecm1, 10 µM MTAN, 500 µM **5** at 37 °C for 2 h in a total volume of 20 µL. Samples were quenched by addition of 10 % (v/v) $HClO_4$ (1M) and analysed via HPLC. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4).

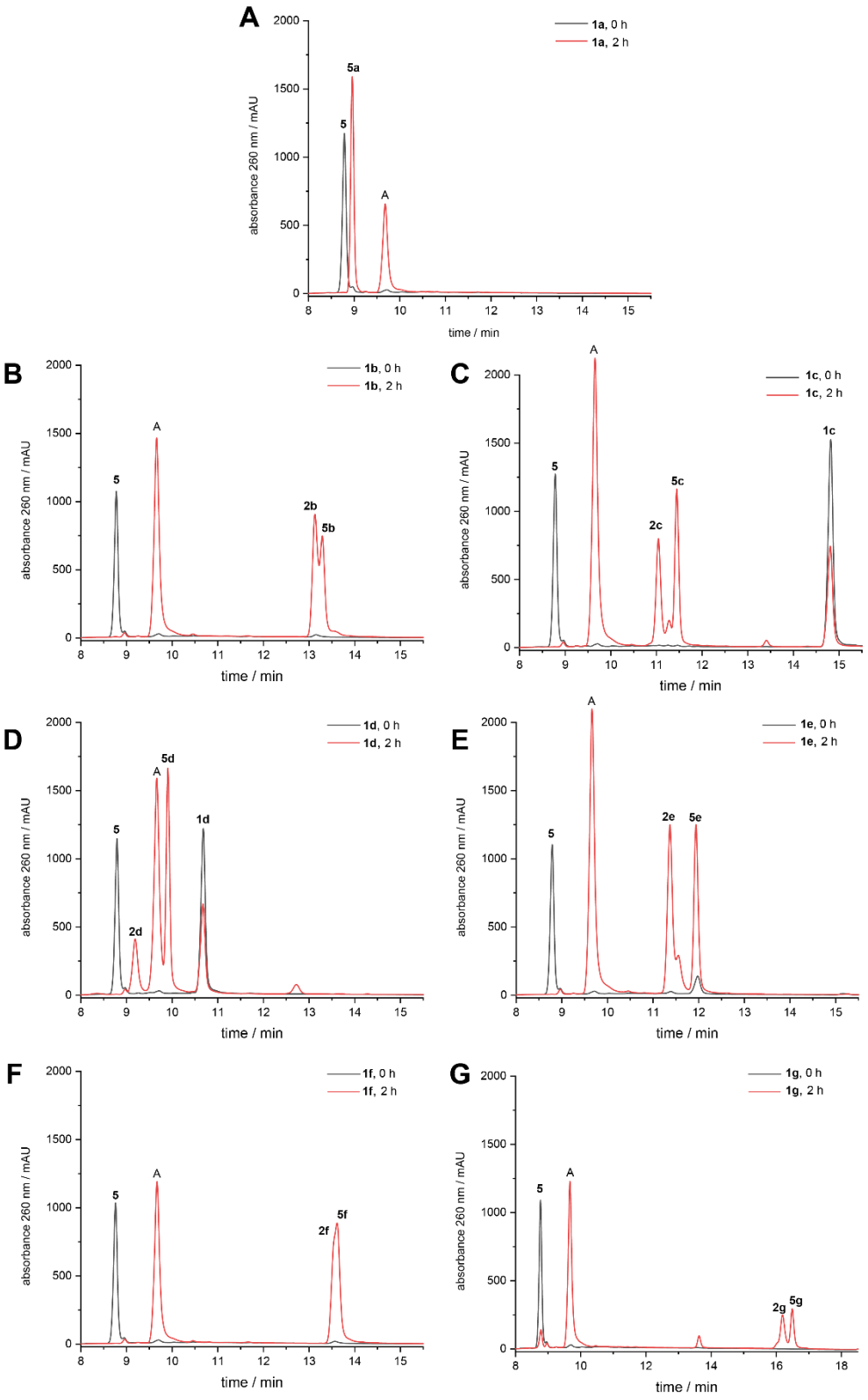


Figure S34: Representative HPLC analyses of PC-MjMAT/Ecm1 cascades as shown in Figure 3E (main text). Here, the traces are grouped by the methionine analogue used and the additional 0 h time point is shown. A)-G) PC-MjMAT/Ecm1 cascades of **5** with **1a**-**g**. Adenine (A) is formed during the assay. Absorbance at 260 nm is shown. HPLC method A was used.

LC-QTOF-MS analysis of GpppA analogues **5a**-**g**

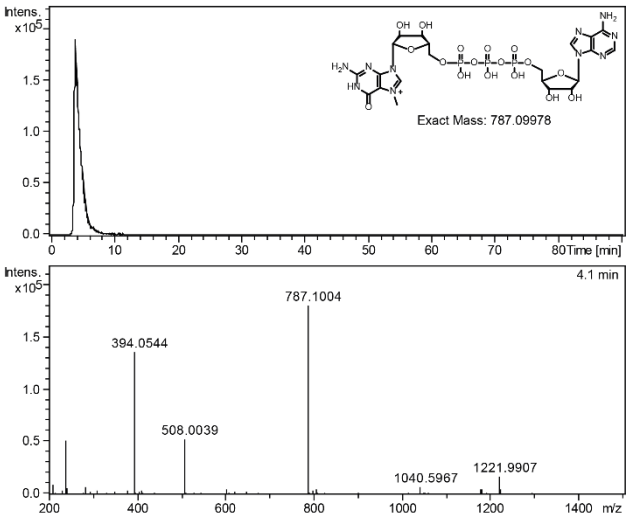


Figure S35: LC-QTOF-MS analysis of compound **5a**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{21}H_{30}N_{10}O_{17}P_3^+$ $[M]^+$ = 787.0998 Da, found m/z = 787.1004.

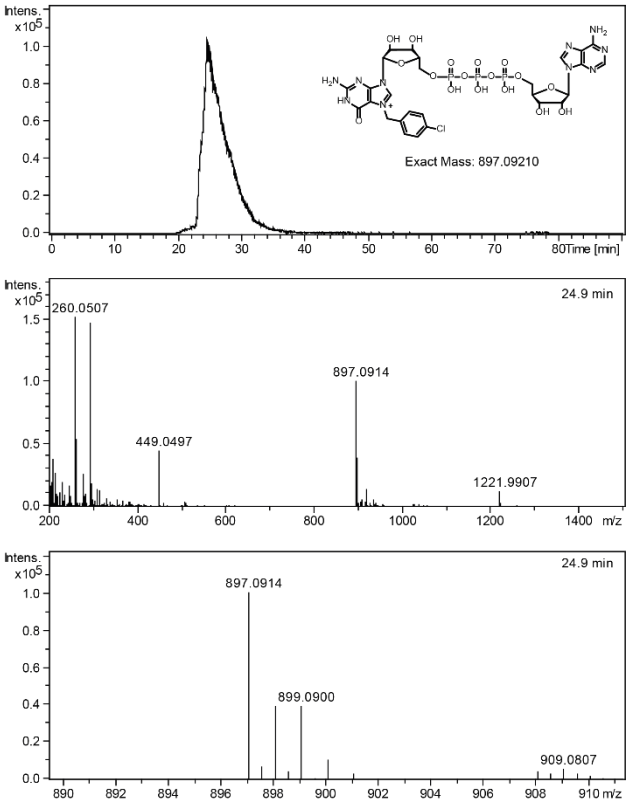


Figure S36: LC-QTOF-MS analysis of compound **5b**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}ClN_{10}O_{17}P_3^+$ $[M]^+$ = 897.0921 Da, found m/z = 897.0914. Note the typical isotope pattern for chlorine.

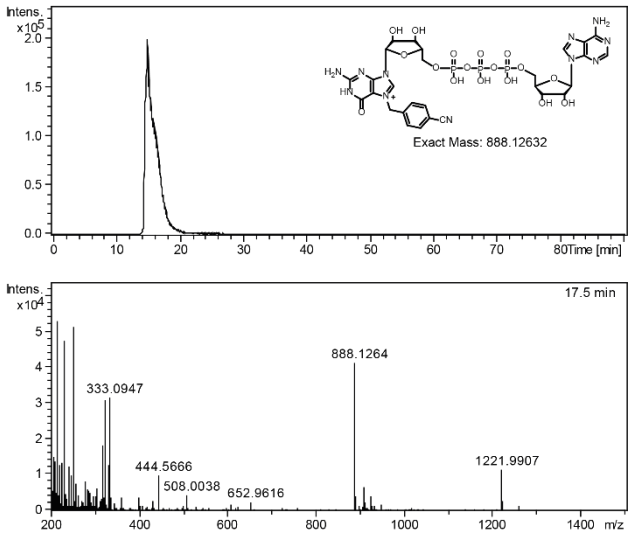


Figure S37: LC-QTOF-MS analysis of compound **5c**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{28}H_{33}N_{11}O_{17}P_3^+$ $[M]^+$ = 888.1263 Da, found m/z = 888.1264.

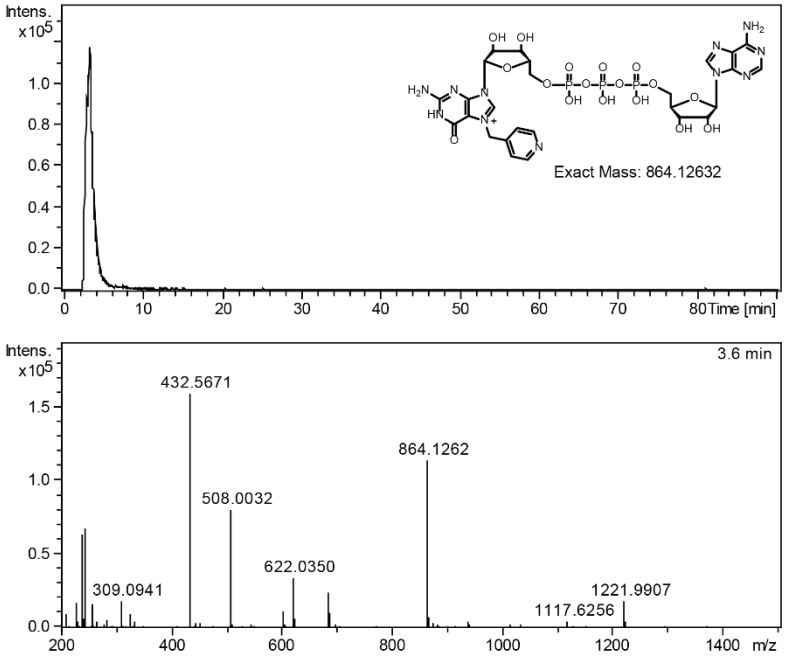


Figure S38: LC-QTOF-MS analysis of compound **5d**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{26}H_{33}N_{11}O_{17}P_3^+$ $[M]^+$ = 864.1263 Da, found m/z = 864.1262.

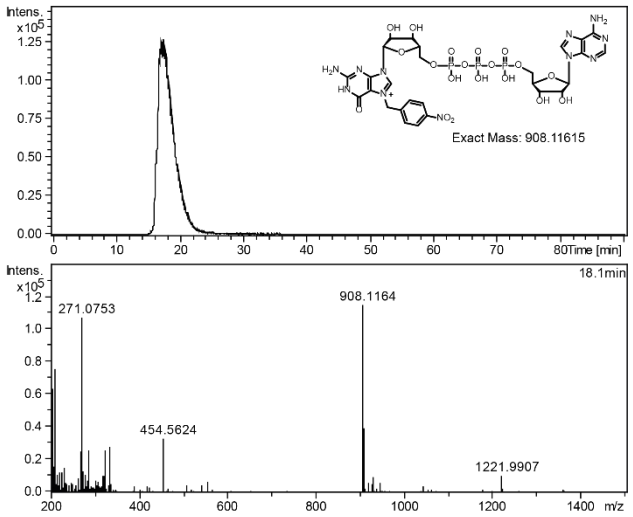


Figure S39: LC-QTOF-MS analysis of compound **5e**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}N_{11}O_{19}P_3^+$ $[M]^+$ = 908.1162 Da, found m/z = 908.1164.

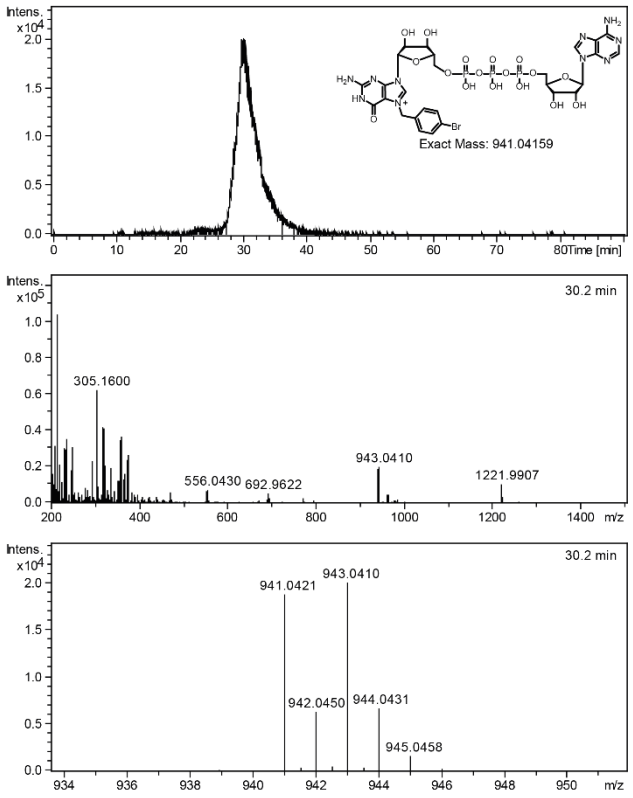


Figure S40: LC-QTOF-MS analysis of compound **5f**. Upper panel shows the EIC, middle and lower panel show the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}BrN_{10}O_{17}P_3^+$ $[M]^+$ = 941.0416 Da, found m/z = 941.0421. Note the typical isotope pattern for bromine.

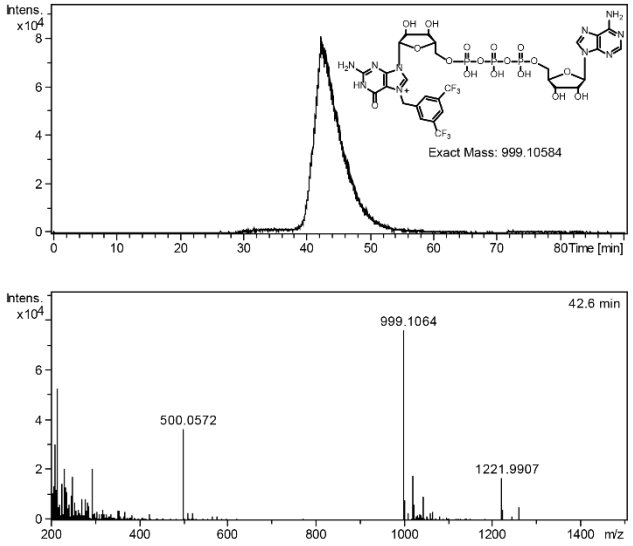


Figure S41: LC-QTOF-MS analysis of compound **5g**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{29}H_{32}F_6N_{10}O_{17}P_3^+$ $[M]^+$ = 999.1058 Da, found m/z = 999.1064.

Luminescence assays

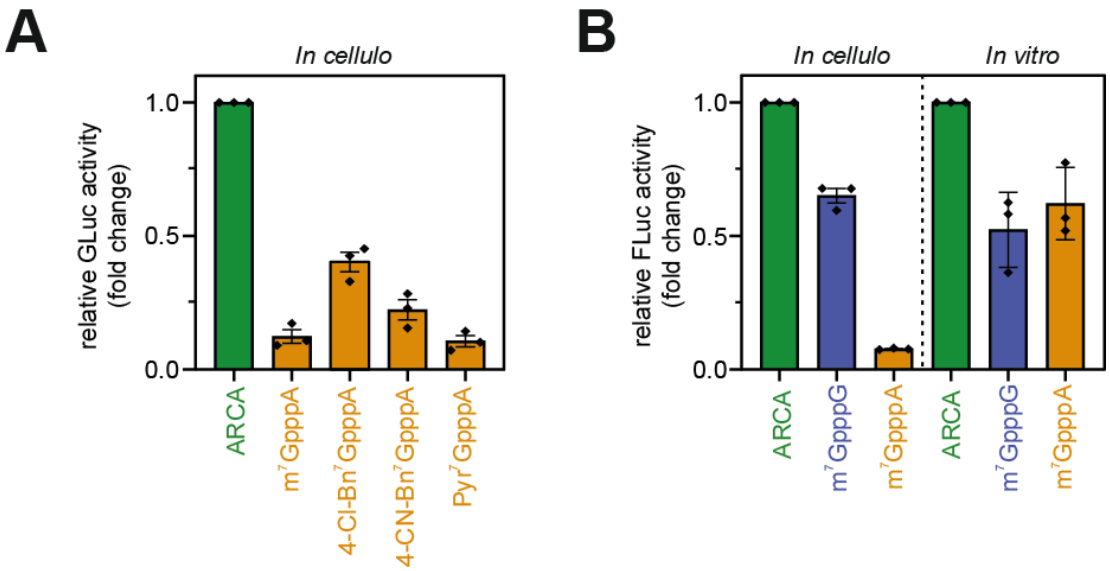


Figure S42: Translational output of indicated luciferase-encoding mRNAs *in vitro* and/or *in cellulo*. A) The influence of *N*7G-modified dinucleotide 5′ cap analogues on the translational properties of GLuc mRNAs in HeLa cells. The resulting data are normalized to ARCA-capped mRNA. The average of n = 3 independent experiments and standard error of the mean is shown. B) The comparison of translational output of m^7GpppG and m^7GpppA capped mRNAs encoding firefly luciferase *in cellulo* and *in*

vitro. Protein production *in cellulo* was determined at 24 h post transfection of HeLa cells with indicated mRNAs. The resulting data are normalized to ARCA-capped mRNA. The average of n=3 independent experiments and the standard error of the mean are shown. FLuc activity in a eukaryotic cell-free expression system was determined using dual luciferase assay. The results are expressed as the ratio of firefly to *Renilla* luciferase activity (FLuc/RLuc) and normalized to ARCA-FLuc. The data represent the average of n = 3 independent assays and standard deviation.

HPLC traces of GpppG modification via PC-MjMAT/Ecm1 cascade

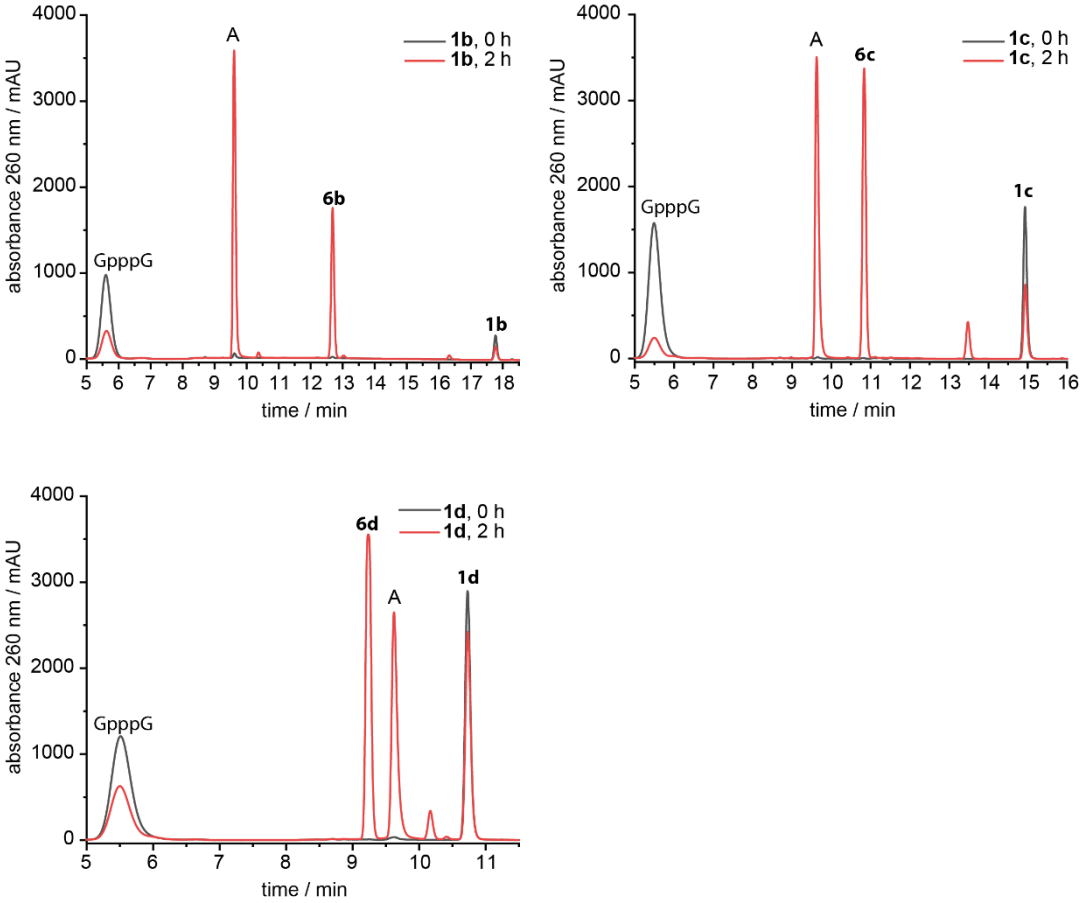


Figure S43: HPLC traces of reaction products of the mRNA 5′-cap GpppG (**6**) conversion to derivatives alkylated at *N*7-position of guanosine (**6b-d**) via enzymatic PC-MjMAT/Ecm1 cascade. Conditions: 5 mM **1b/c** or 8 mM **1d**, 5 mM ATP, 100 µM PC-MjMAT, 50 µM Ecm1, 10 µM MTAN, 2 mM **6** at 37 °C for 2 h in a total volume of 20 µL (analytical scale) or 600 µL (semi-preparative scale). Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). Adenine (A) is formed during the assay.

LC-QTOF-MS analysis of GpppG analogues **6a**-**g**

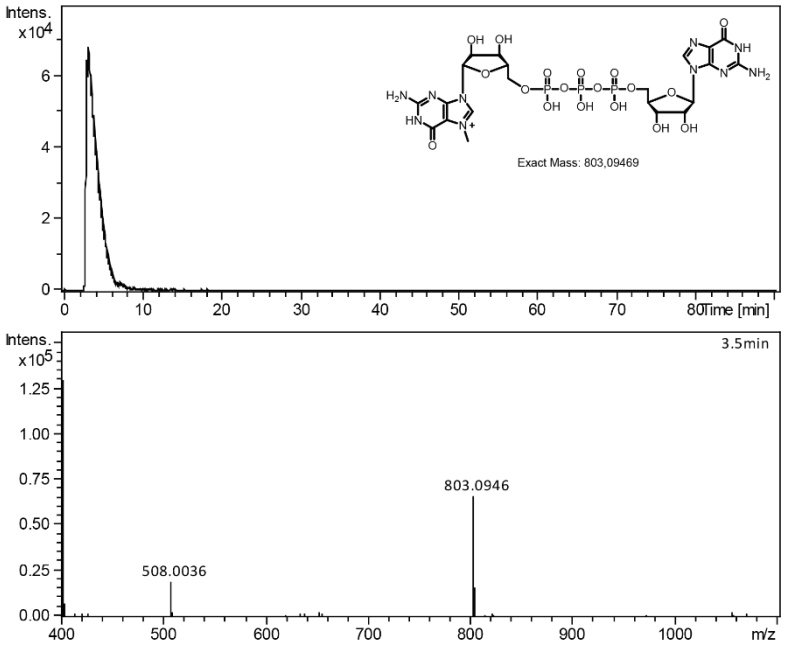


Figure S44: LC-QTOF-MS analysis of compound **6a**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{21}H_{30}N_{10}O_{18}P_3^+$ $[M]^+$ = 803.0947 Da, found m/z = 803.0946.

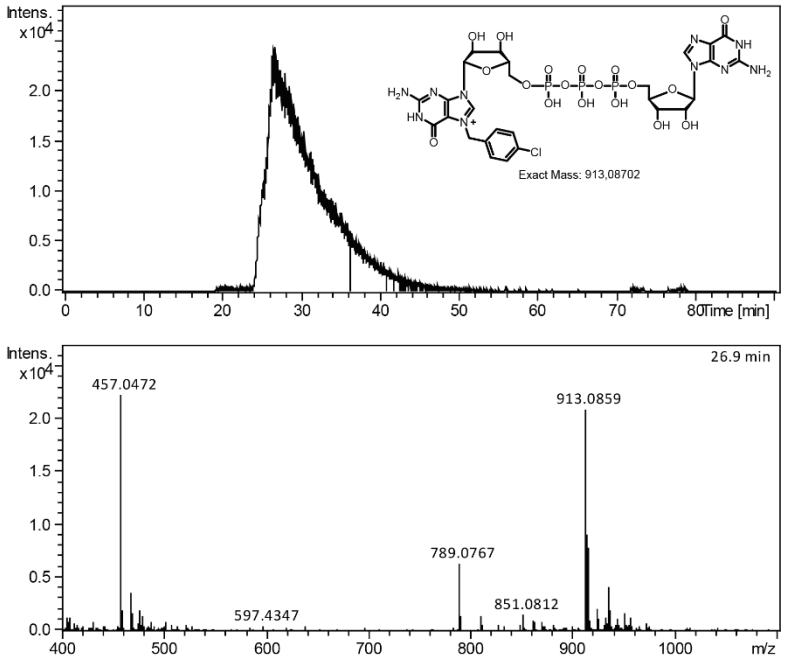


Figure S45: LC-QTOF-MS analysis of compound **6b**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}ClN_{10}O_{18}P_3^+$ $[M]^+$ = 913.0870 Da, found m/z = 913.0859.

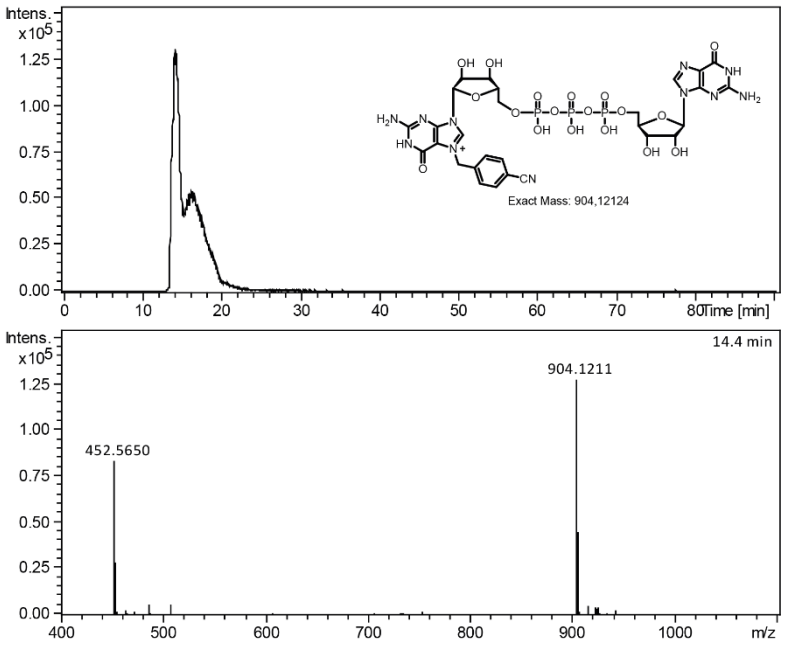


Figure S46: LC-QTOF-MS analysis of compound **6c**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{28}H_{33}N_{11}O_{18}P_3^+$ $[M]^+$ = 904.1212 Da, found m/z = 904.1211.

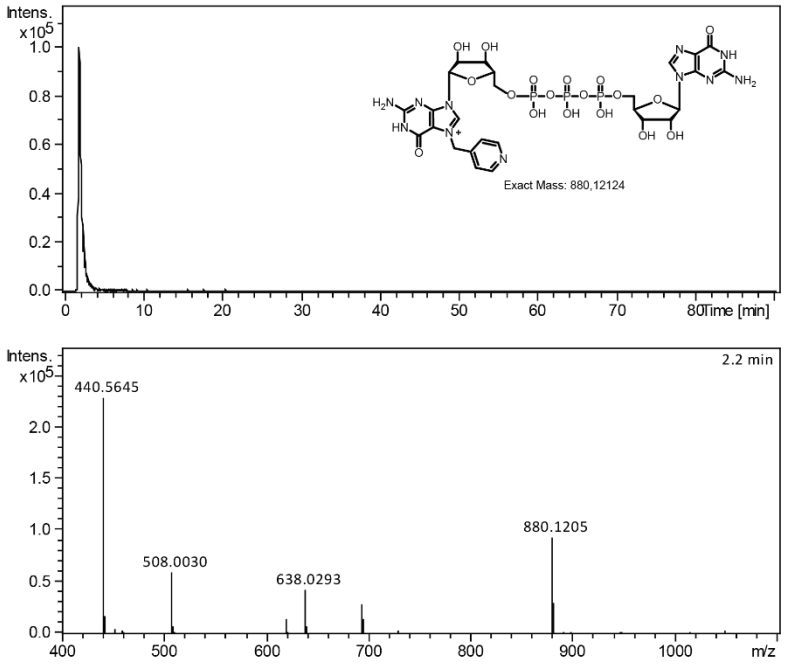


Figure S47: LC-QTOF-MS analysis of compound **6d**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{26}H_{33}N_{11}O_{18}P_3^+$ $[M]^+$ = 880.1212 Da, found m/z = 880.1205.

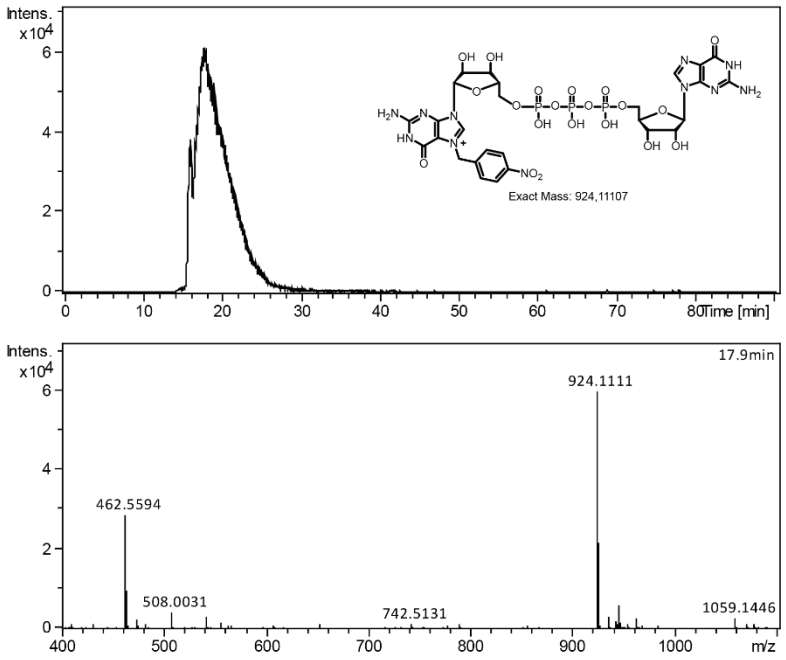


Figure S48: LC-QTOF-MS analysis of compound **6e**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}N_{11}O_{20}P_3^+$ $[M]^+$ = 924.1111 Da, found m/z = 924.1111.

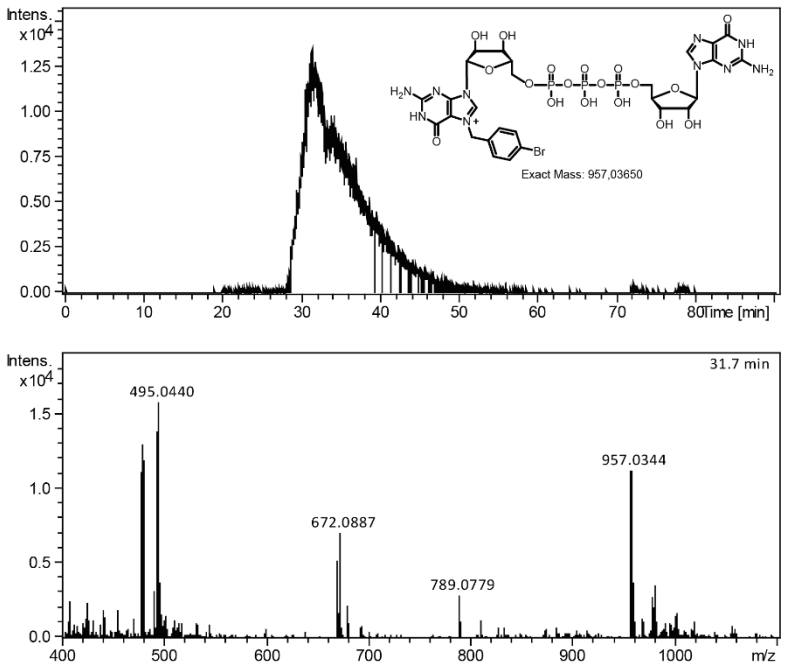


Figure S49: LC-QTOF-MS analysis of compound **6f**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{27}H_{33}BrN_{10}O_{18}P_3^+$ $[M]^+$ = 957.0365 Da, found m/z = 957.0344.

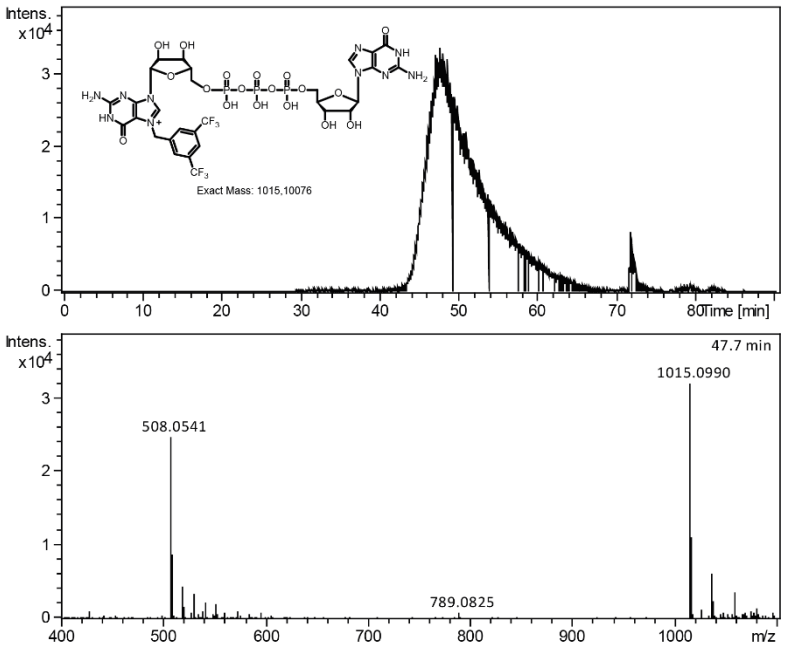
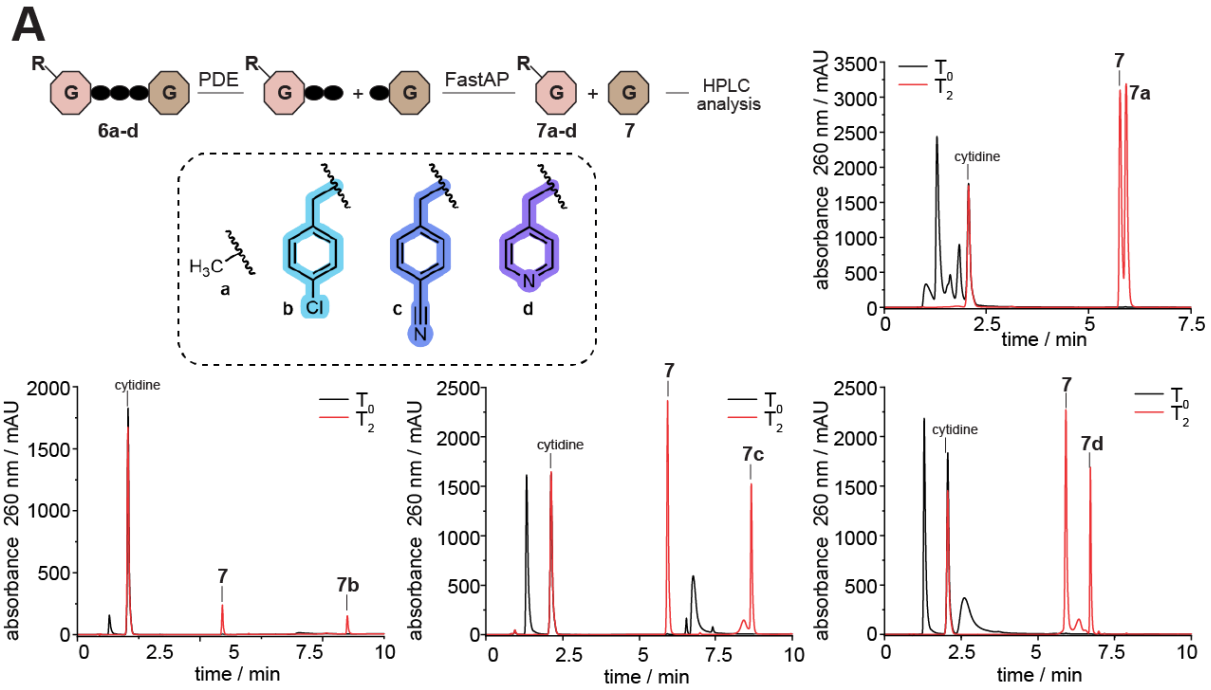


Figure S50: LC-QTOF-MS analysis of compound **6g**. Upper panel shows the EIC and lower panel shows the mass spectrum at the indicated retention time. Calculated mass for $C_{29}H_{32}F_6N_{10}O_{18}P_3^+$ $[M]^+$ = 1015.1008 Da, found m/z = 1015.0990.

Determination of **6b-d** concentration

A



B

Compound (quantifier/qualifier)	Mode	Precursor ion (m/z)	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)	CAV (V)	Retention time (min)
GpppG (quant.)	pos.	789.1	151.8	150	57	9	3.4
GpppG (qual.)	pos.	789.1	248.4	150	21	9	3.4
m^{7}GpppG (quant.)	pos.	803.1	248.1	135	21	9	4.3
m^{7}GpppG (qual.)	pos.	803.1	165.7	135	53	9	4.3
4-Cl-Bn^{7}GpppG (quant.)	pos.	913.1	151.7	135	61	9	6.4
4-Cl-Bn^{7}GpppG (qual.)	pos.	913.1	165.7	135	53	9	6.4
4-CN-Bn^{7}GpppG (quant.)	pos.	904.1	267.2	164	44	9	5.3
4-CN-Bn^{7}GpppG (qual.)	pos.	904.1	248.1	164	25	9	5.3
Pyr^{7}GpppG (quant.)	pos.	880.1	248.1	166	21	9	4.5
Pyr^{7}GpppG (qual.)	pos.	880.1	151.8	166	71	9	4.5

C

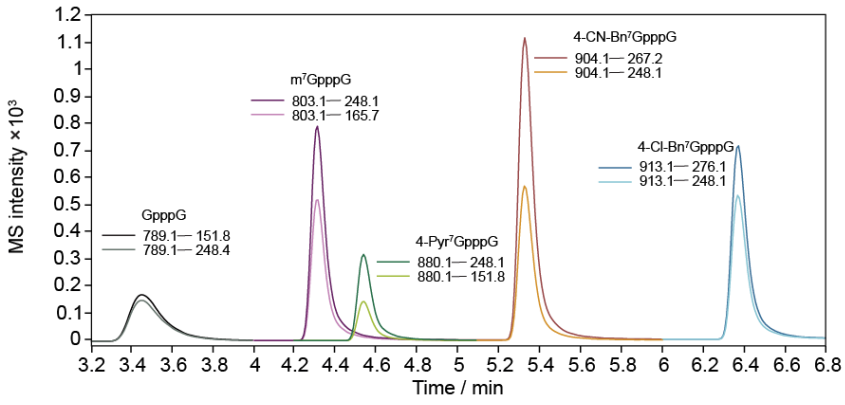


Figure S51: Detection of *N*7-benzylated GpppG caps on LC-QqQ-MS. A) Determination of concentration of purified **6b-d** caps. **6b-d** were digested into nucleosides using Snake Venom phosphodiesterase (PDE) and dephosphorylated using FastAP. Digested caps were analysed on HPLC. Cytidine was added to each sample before reaction as internal standard. The concentration of **6b-d** was

determined based on amount of guanosine (**7**) using external calibration of guanosine. B) Mass spectrometer settings used for **6b-d** analysis (CAV: cell acceleration voltage). Settings for quantifiers (quant.) and qualifiers (qual.) are shown. C) LC-QqQ analysis of cap standards in dMRM mode. Shown are the MS intensities of **6b-d**. MRM-transitions of the respective quantifier and qualifier are shown.

Preparation of co-transcriptionally modified mRNAs

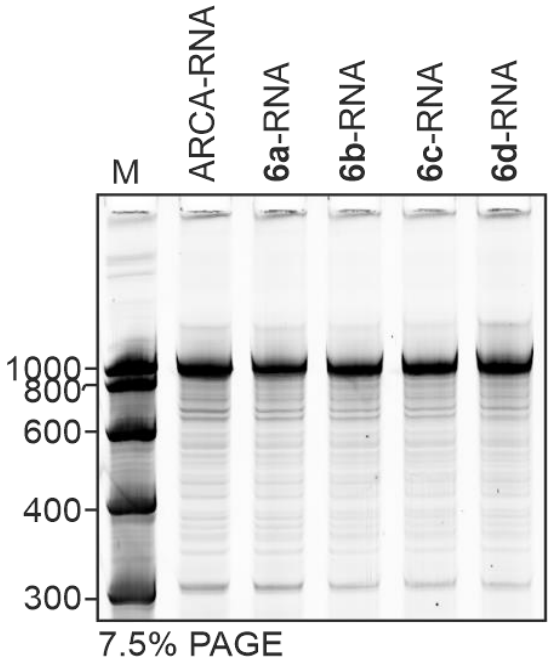


Figure S52: Analysis of co-transcriptionally modified mRNA integrity. M – RiboRuler High Range RNA Ladder (Thermo Scientific).

Correct cap incorporation assay

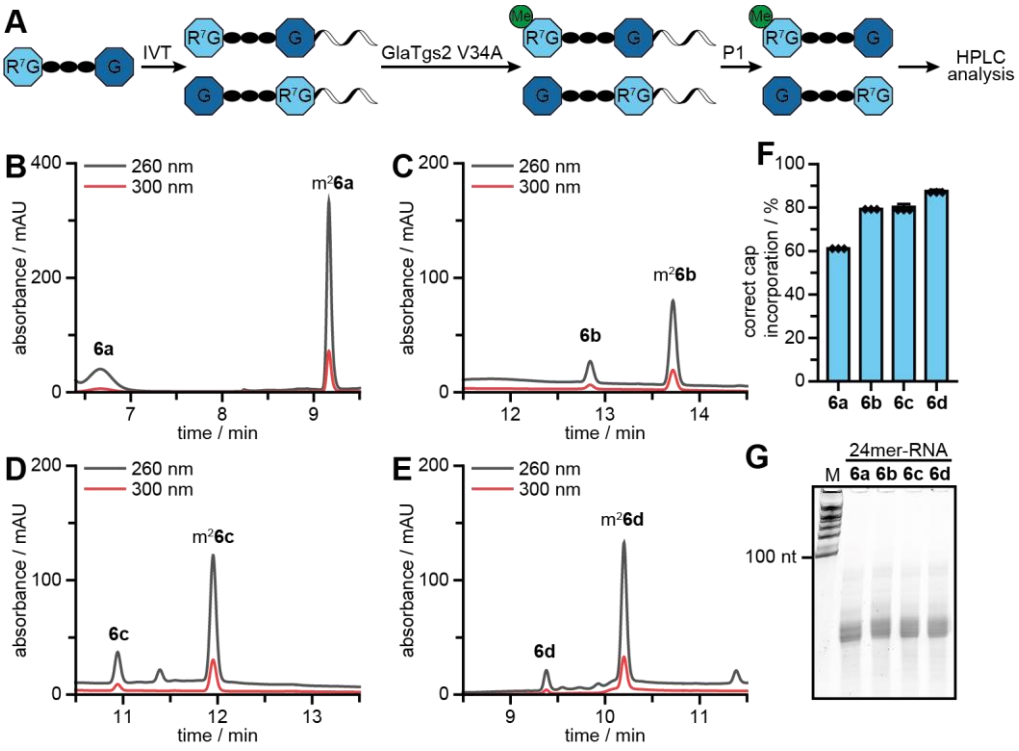


Fig. S53: Extension of main text Fig. 6 A) Scheme of HPLC-based 5′ cap orientation assay. B-E) Representative HPLC analysis of the correct cap incorporation assay in co-transcriptionally modified RNA using **6a**-**d**, respectively. F) Amount of correct cap incorporation, where the *N*7-modified Guanosine is the terminal nucleoside, found for **6a**-**d** (n = 3). G) Integrity of RNA 24mer used in 5’ cap orientation assay, analysed by PAA gel (15 % TBE).

24-mer RNA was produced similar to previous reports.[1]

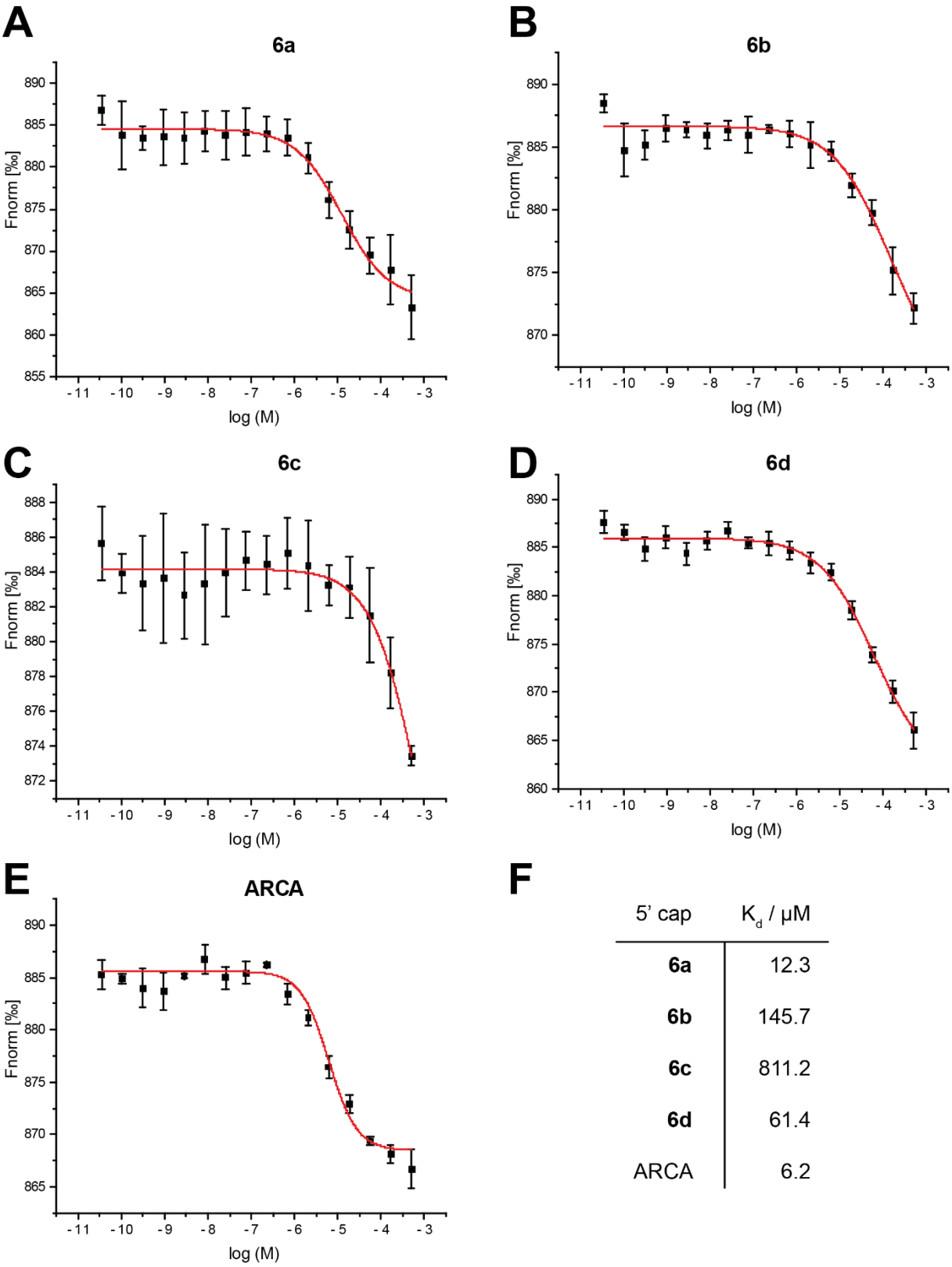
In short, the *in vitro* T7 transcription of 24-mer RNA with G as first transcribed nucleotide (bold) was performed in 1× transcription buffer (40 mM Tris/HCl, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine$(HCl)_3$) using 0.5 µM corresponding DNA template, 1 mM A/C/UTP mix, 1,6 mM cap analogues **6a**-**d**, 0.8 U/µL RiboLock RNase Inhibitor (Thermo Scientific), 2.5 mg/mL T7 RNA polymerase (produced in-house) and 0.004 U/µL pyrophosphatase (Thermo Scientific) for 4 h at 37 °C.

- **fwd:** 5'–GAAATTAATACGACTCACTATA**G**GAGCCAGCCTACGAGCCTGAGCC–3'
- **rev:** 5'–GGCTCAGGCTCGTAGGCTGGCTCCTATAGTGAGTCGTATTAATTTC–3'

DNA template was digested with DNase I (12 µL/100µL) for 1 h at 37 °C. The RNA was then purified using RNA Clean & Concentrator Kit (Zymo Research, Epigenetics) following the manufacturer´s protocol.

For the correct cap incorporation assay 3 µg of IVT produced 24-mer RNA were incubated with 5 mM SAM, 50 µM GlaTgs2 V34A, 10 µM MTAN for 2 h at 30 °C. Reaction buffer (1x) consisted of 50 mM HEPES, 10 mM $MgCl_2$, 5 mM KCl (pH = 7.4). The mixture was purified by phenol-chloroform extraction and resuspended in ddH_2O.

The RNA was digested with 0.4 µL nuclease P1 from *Penicillium citrinum* (Sigma Aldrich, N8630, Stock: 0.33 u/µL 20 mM NH_4OAc, pH = 5.5) in P1 Buffer (1x): 20 mM NH_4OAc, 100 µM $ZnCl_2$, pH = 5.3. The resulting mixture was analysed by HPLC method A.



F

5' cap	K_d / µM
6a	12.3
6b	145.7
6c	811.2
6d	61.4
ARCA	6.2

Figure S54. Binding curve of cap analogues A)-D) **6a**-**d** and E) ARCA are given. F) Calculated K_d for **6a**-**d** and ARCA. Data and error bars show mean values and SD of three independent experiments.

Microscale thermophoresis (MST)

MST measurements were performed on a Monolith NT.115 series instrument (NanoTemper), operated by NTControl v2.2.1. Data processing was carried out in MO.Affinity Analysis v2.1.3. Monolith™ Series Protein Labelling Kit RED-NHS 2nd Generation (Cat# MO-L011) was used for Cy5 labelling of recombinant eIF4E, according to the manufacturer's protocol. The labelled protein was flash frozen in liquid nitrogen and stored in ultra-low temperature freezer until further use. The labelled protein was stored in pH = 7.2 MST buffer containing 50 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, and 0.5 % Tween-20. DTT and Tween-20 were freshly added to the buffer before use and the complete MST buffer could be stored up to several days. Ligand serial dilutions (3-fold) were performed in MST buffer. For each ligand, the same concentration range was used with the highest concentration as 500 µM. Then, the ligand solutions were mixed (further diluted 2-fold) with an equal volume of the labelled protein (~75 nM). These protein-ligand mixtures were filled into Monolith™ Series Premium Capillaries (Cat# MO-K025). For all MST data presented in this study, 20% MST power and 13% LED power (excitation, 625 nm; emission, 680 nm) were used. Thermophoresis measurements were performed with the following settings: fluorescence before, 5 s; MST on, 30 s; fluorescence after, 5 s; and delay, 25 s. MST data were normalized to baseline differences and the data required for binding curve was generated by using the "Thermophoresis with T Jump" advanced setting. Nonlinear regression for curve fitting was performed in OriginPro 2023, using the Sigmoidal dose-response parameter.

[1] M. van Dülmen, N. Muthmann and A. Rentmeister, *Angew. Chem. Int. Ed.* **2021**, *60*, 13280-13286.