

SAM-III aptamer enables detection of enzymatic SAM analogue generation

Jonas Schöning^{a,b}, Aileen Tekath^b, Nicolas V. Cornelissen^b, Arne Hoffmann^{a,b}, Andrea Rentmeister^{a,b}

^a Institute of Chemical Epigenetics, Ludwig-Maximilians-Universität München, Germany

^b Institute of Biochemistry, University of Münster, Germany.

^a Department of Chemistry, Ludwig-Maximilians-University Munich, Butenandtstr. 5-13, Haus F, D-81377 Munich, Germany.

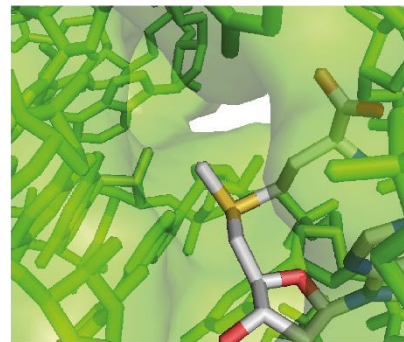
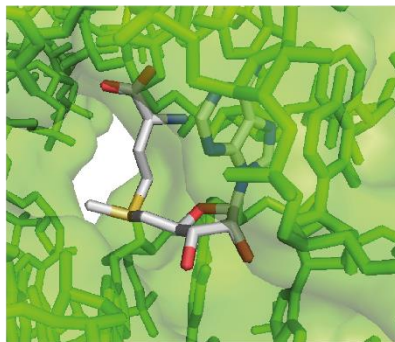
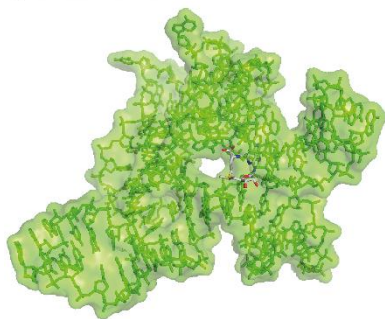
^b Institute of Biochemistry, University of Münster, Corrensstr. 36, D-48149 Muenster, Germany.

Table S1: Comparison of interactions between SAM and nucleotides of the SAM-riboswitches and SAM/SAH riboswitch.

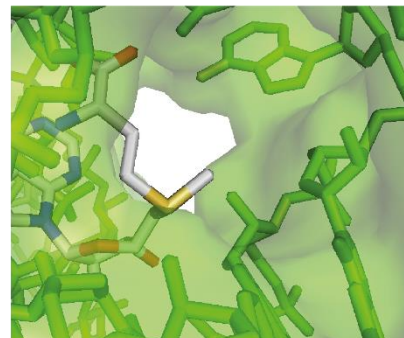
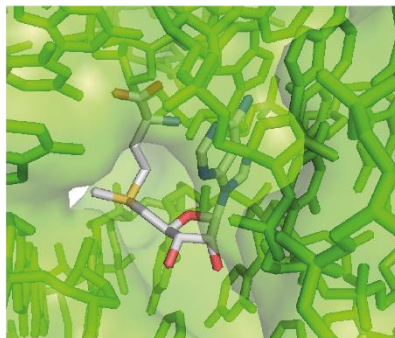
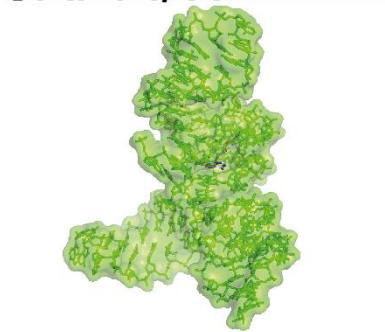
Riboswitch PDB-code	SAM conformation	SAM element	Nucleic acid interaction
SAM-I 3GX5	<i>syn</i>	adenine N7 adenine N6 adenine N6 adenine N1 methionine NH ₃ methionine NH ₃ methionine COOH methionine COOH methionine COOH sulfonium sulfonium	U57 N3 U57 O6 A45 N3 A45 2'OH G58 N3 G58 2'OH G58 N2 G11 N2 G11 N1 U7 O2 U88 O2
SAM-II 2QWY	<i>anti</i>	adenine N7 adenine N6 methionine NH ₃ methionine COOH sulfonium sulfonium	U44 N3 U44 O2 A47 N1 A47 N6 U11 O4 U21 O4
SAM-III 3E5C	<i>syn</i>	adenine N7 adenine N6 adenine N6 adenine N1 ribose 2'OH ribose 3'OH sulfonium sulfonium	A38 N6 G7 N3 G7 2'OH G7 N2 G47 N7 G47 phosphate O U37 O4 G36 2'OH
SAM-IV 6UET	<i>syn</i>	adenine N7 adenine N6 adenine N6 adenine N1 methionine NH ₃ methionine NH ₃ methionine COOH methionine COOH methionine COOH sulfonium sulfonium	U63 N3 U63 O6 A42 N3 A42 2'OH G64 N3 G64 2'OH G64 N2 G7 N2 G7 N1 U3 O2 C23 U2
SAM-I/IV 4L81	<i>syn</i>	adenine N7 adenine N6 adenine N6 adenine N1 methionine NH ₃ methionine NH ₃ methionine COOH methionine COOH methionine COOH sulfonium	U46 N3 U46 O6 A25 N3 A25 2'OH G42 N3 G42 2'OH G42 N2 G8 N2 G8 N1 U4 O2
SAM-V 6FZ0	<i>anti</i>	adenine N7 adenine N6 methionine NH ₃ methionine COOH sulfonium	U44 N3 U44 O2 A50 N1 A50 N6 U9 O4

		sulfonium	U20 O4
SAM-VI 6LAS	<i>anti</i>	adenine N7 adenine N6 adenine N6 adenine N1 methionine NH ₃ ribose 2`OH sulfonium sulfonium	U8 N3 U8 O2 A37 N1 A36 N6 U8 O4 G38 phosphate O U6 O4 U6 O4
SAM/SAH 6YMM	<i>anti</i>	adenine N7 adenine N6 adenine N1 ribose 2`OH methionine NH ₃	U23 N3 U23 O2 U44 2`OH G46 N7 U23 O4

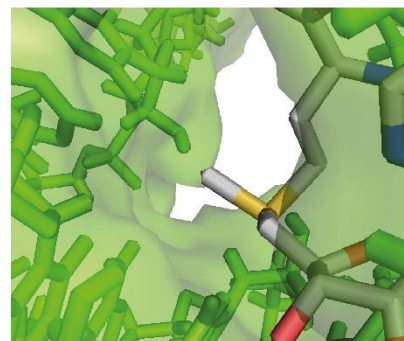
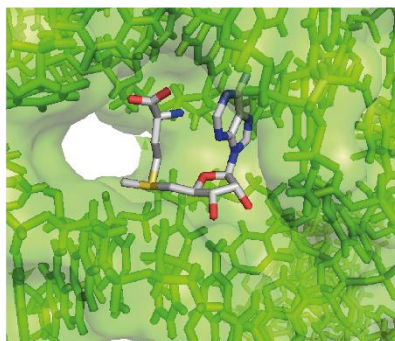
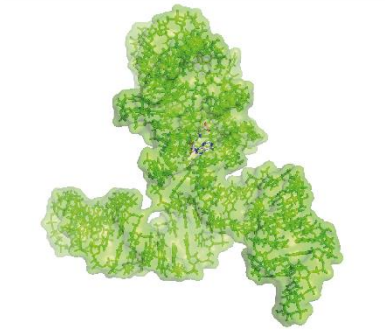
SAM-I



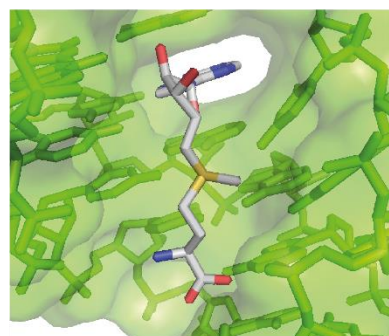
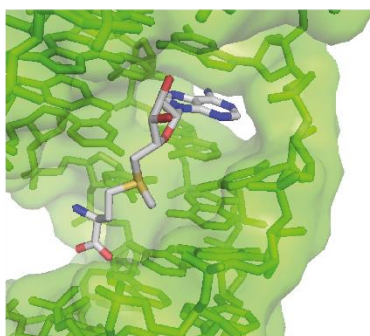
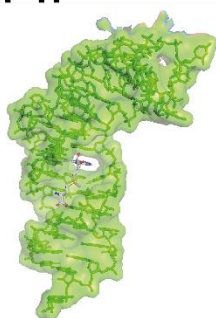
SAM-I/IV



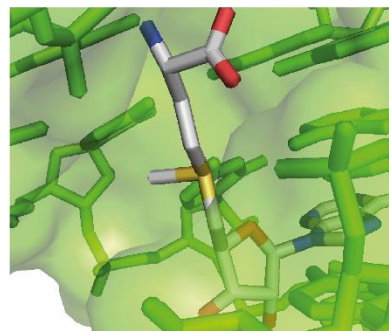
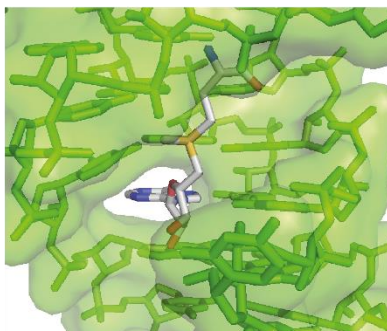
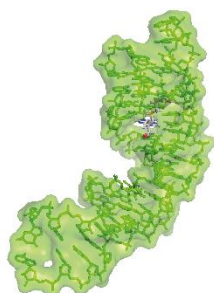
SAM-IV



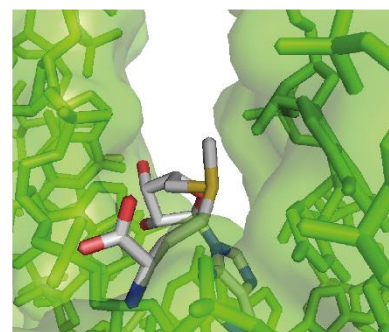
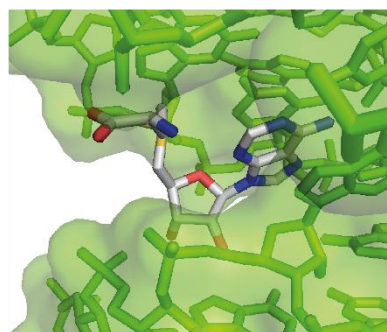
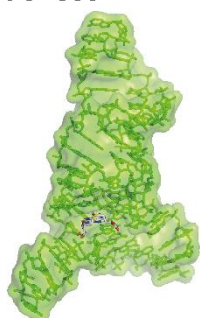
SAM-II



SAM-V



SAM-III



SAM-VI

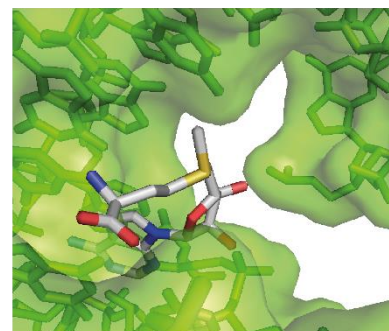
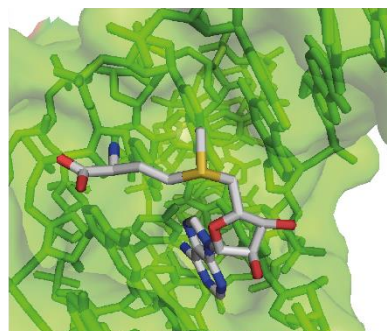
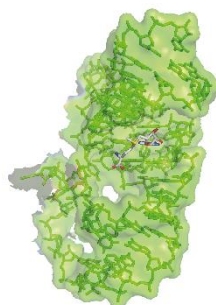


Figure S1: Comparison of the position of SAM in crystal structures of different SAM-riboswitch classes. The PDB files indicated in Table S2 were used. The SAM-III riboswitch offers a wide binding-pocket and the methyl group of SAM points out of the binding pocket, suggesting that alterations of the moiety may be tolerated.

Production of Spinach/SAM aptamers

The sequence of the Spinach/SAM aptamer and its amplification conditions were based on literature.¹ The aptasensor sequence of Jaffrey et al. was amplified with newly designed primers to introduce the T7-promotor sequence (see. Tab. S1). Planning of amplification and cloning was performed with the *Sci Ed Software* Clone Manager 9 tool. Annealing temperatures were calculated with the *Thermo Fisher T_m* calculator.

PCR-amplification

Briefly, a mixture of dNTPs (200 μ M each), HF-buffer (1x), fwd./rev. primer (each 0.5 μ M), single stranded template DNA (200 ng), and Phusion Polymerase (1 U) were mixed and ddH₂O was added to a volume of 50 μ L. For amplification, the mixture was first denatured (30 s at 98 °C), then amplified in 30 cycles (denaturation: 98 °C, 10 s; annealing 57 °C, 10 s; elongation: 72 °C, 15 s) and finally elongated (72 °C, 10 min). The crude PCR-product was purified via the *Machery-Nagel*® kit. Success of the amplification was validated by agarose gel electrophoresis (Fig. S2).

T7-transcription

The T7-transcription mix consisted of the hybridized aptaswitch template after PCR-amplification (400 ng per 100 μ L mixture), CARO buffer 3x (1:3 diluted; 120 mM TRIS, 6 mM spermidine, 0.03 % Triton-X-100, 4.5 (w/v) % PEG6000, 15 mM DTT), 2.5 mM NTP-mix, 15 mM Mg(OAc)₂, T7 RNA polymerase (1 mg/mL, recombinant, non-commercial), 0.25 U pyrophosphatase and 1 μ L of Ribolock RNase inhibitor. Transcription volumes varied between 100 μ L and 800 μ L. The mixture was incubated for 4 h at 37 °C. Afterwards 5 μ L of DNase I per 100 μ L transcription mix were added and the remaining DNA was digested for another 2 h at 37 °C. Purification of the RNA was performed with the RNA Clean & Concentrator-5 kit by *Zymo Research*. Success of the T7-transcription was analyzed by dPAGE (Fig. S2).

Table S2: Sequences of the Spinach aptaswitch sensor used in this work. Aptaswitches include the SAM-binding aptamer domain (red), a transducer (magenta) and the fluorophore-binding aptamer domain (green). The primers include restriction sites for *NdeI* (bright green) and *XbaI* (yellow). Furthermore, the forward primers include the T7-promoter sequence (blue). Overlap with the aptamer sequences either on the 5'-end or 3'-end is shown in brown. Non-coloured bases function as spacers to ensure complete amplification.

ssDNA name	Sequence (5' -> 3')
Spinach aptamer core	GACGCGACTGAATGAAATGGTGAAGGACGGGTCCA CCGAAAGGATGGCGGAAACGCCAGATGCCTTGTAAACCGAAAGGGG TTGTTGAGTAGAGTGTGAGCTCCGTAAGTGTGCGCTC
Spinach fwd. primer	GAGCAGCCATATGTAATACGACTCACTATAGGGGAGACGCAACTGAA GACGCGACTGAATGAAATG
Spinach rev. primer	CGCTGCTCTAGAGACGCG
Spinach aptasensor PCR-product	GAGACGCAACTGAAGACGCGACTGAATGAAATGGTGA AGGACGGGTCCACCGAAAGGATGGCGGAAACGCCAGA TGCCTTGTAAACCGAAAGGGG TTGTTGAGTAGAGTGTGA GCTCCGTAAGTGTGCGTCTCTAGAGCAGCG
Spinach aptasensor RNA	GGGAGACGCAACUGAAGACGCGACUGAAUGAAAUGGUGAAG GACGGGUCCACCGAAAGGAUGGCGGAAACGCCAGAUG CCUNNKAACCGAAAGGGGUUGUUGAGUAGAGUGUGAGCUC CGUAACUAGUCGCGUCUCUAGAGCAGCG

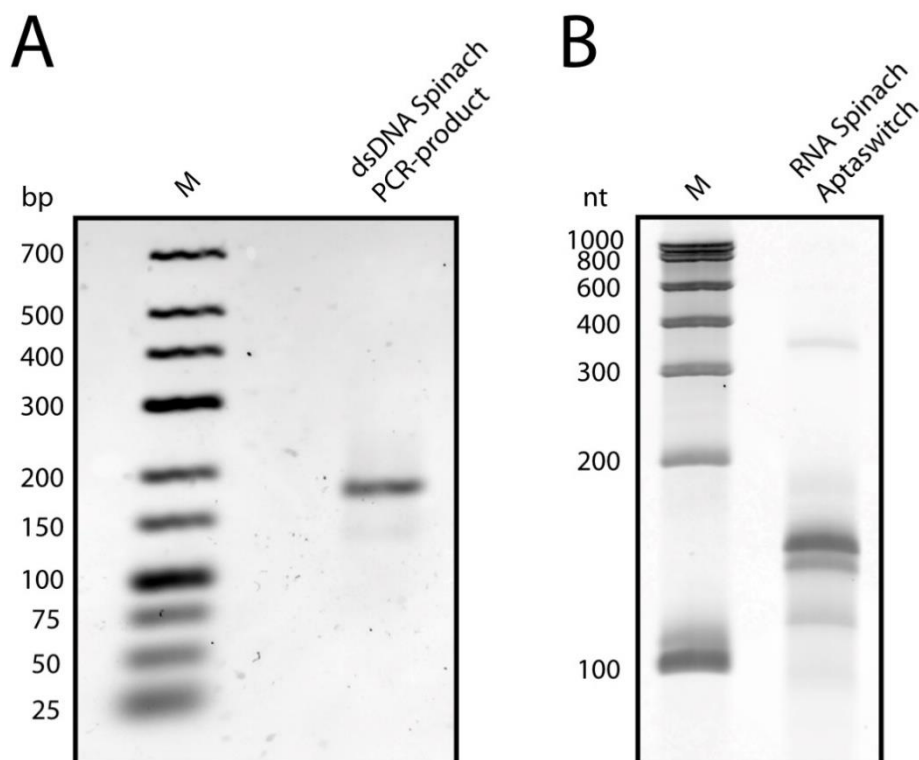


Figure S2: Gel analysis of Spinach/SAM aptasensor **A)** dsDNA (176 bp) after PCR amplification (3 %-agarose mini-gel, 1 h, 100 V, 200 ng DNA) and **B)** the Spinach/SAM aptasensor RNA (146 nt) after IVT (10 %-TBE mini-gel, 12 W, 30 min, 100 ng RNA).

Fluorescence measurements

Fluorescence measurements were performed on a *TECAN* Infinite M1000 PRO® (*Tecan Group Ltd.*, Männedorf, CH) with a *Greiner* 384 well microplate, PS, flat-bottom, round well (*Greiner Bio-One International GmbH*, Kersmünster, AT). The settings for measurements were 100 flashes, manual z-position 21709 μm , manual gain 200 with $\lambda_{Ex} = 460 \text{ nm}/\lambda_{Em} = 504 \text{ nm}$. For compounds **11b** and **16b** the measurements were performed on a *TECAN* Spark® (*Tecan Group Ltd.*, Männedorf, CH) with a *Greiner* 384 well microplate, PS, flat-bottom, square well (*Greiner Bio-One International GmbH*, Kersmünster, AT). The settings for measurements were 100 flashes, manual z-position 21709 μm , manual gain 180 with $\lambda_{Ex} = 460 \text{ nm}/\lambda_{Em} = 504 \text{ nm}$.

Binding affinity measurements for SAM analogues

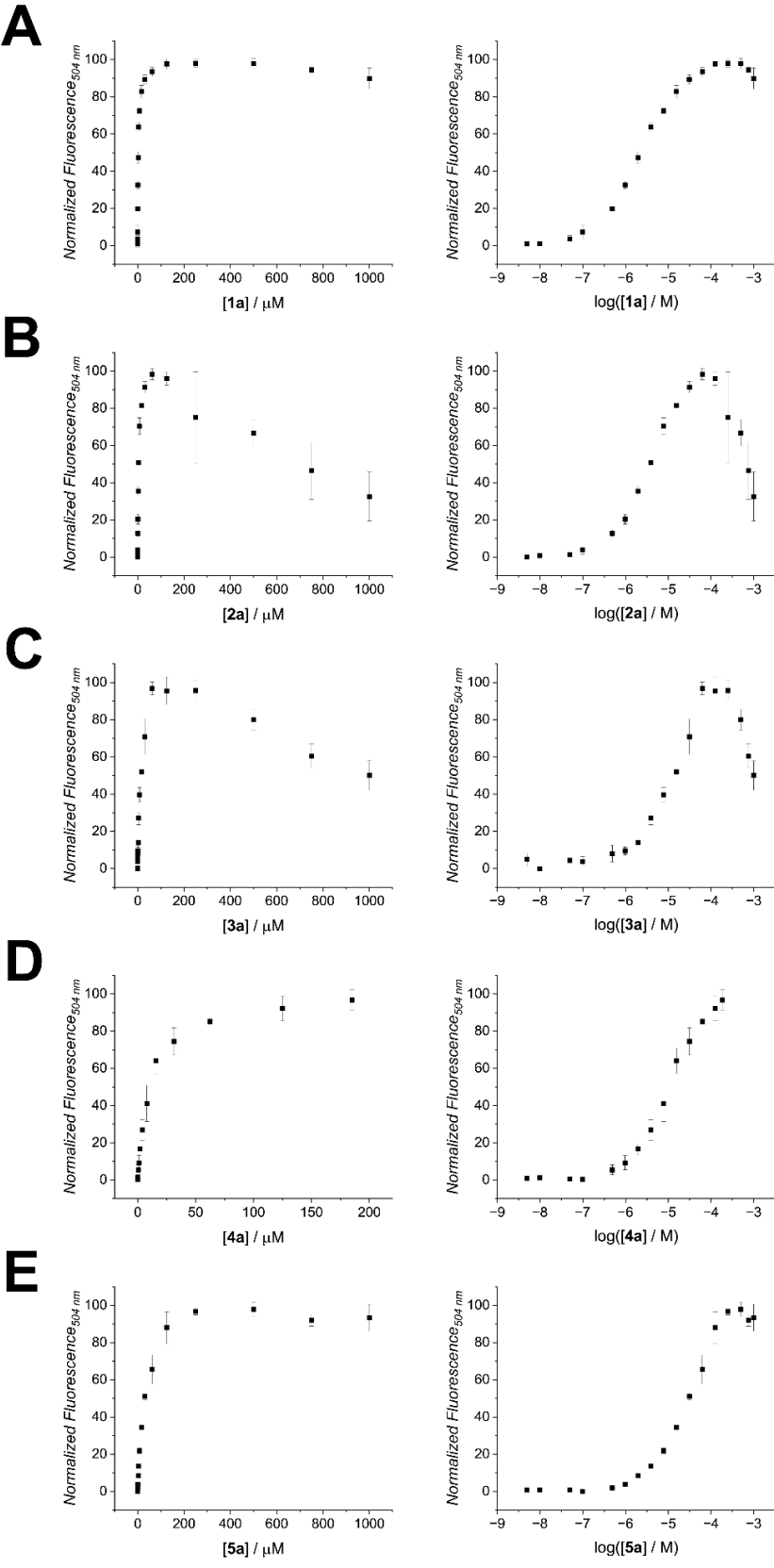


Figure S3: Measurement of fluorescence signal dependence on SAM(-analogue) concentration measured with wavelengths $\lambda_{Ex} = 460 \text{ nm}$ and $\lambda_{Em} = 504 \text{ nm}$. The assay contains $3 \mu\text{M}$ aptasensor and $120 \mu\text{M}$ DFHBI. The plot shows the mean values and standard deviation of three independent experiments ($n = 3$) with four technical replicates for each data point.

Corresponding EC_{50} -values were determined by fitting with dose-response curves with the logarithmic normalized fluorescence. The EC_{50} -values are **A)** for **1a** $EC_{50} = 2.26 \mu\text{M} \pm 0.10 \mu\text{M}$, **B)** for **2a** $EC_{50} = 4.1 \mu\text{M} \pm 0.3 \mu\text{M}$, **C)** for **3a** $EC_{50} = 11.6 \mu\text{M} \pm 0.9 \mu\text{M}$, **D)** for **4a** $EC_{50} = 11.0 \mu\text{M} \pm 0.9 \mu\text{M}$, **E)** for **5a** $EC_{50} = 39.5 \mu\text{M} \pm 3.0 \mu\text{M}$.

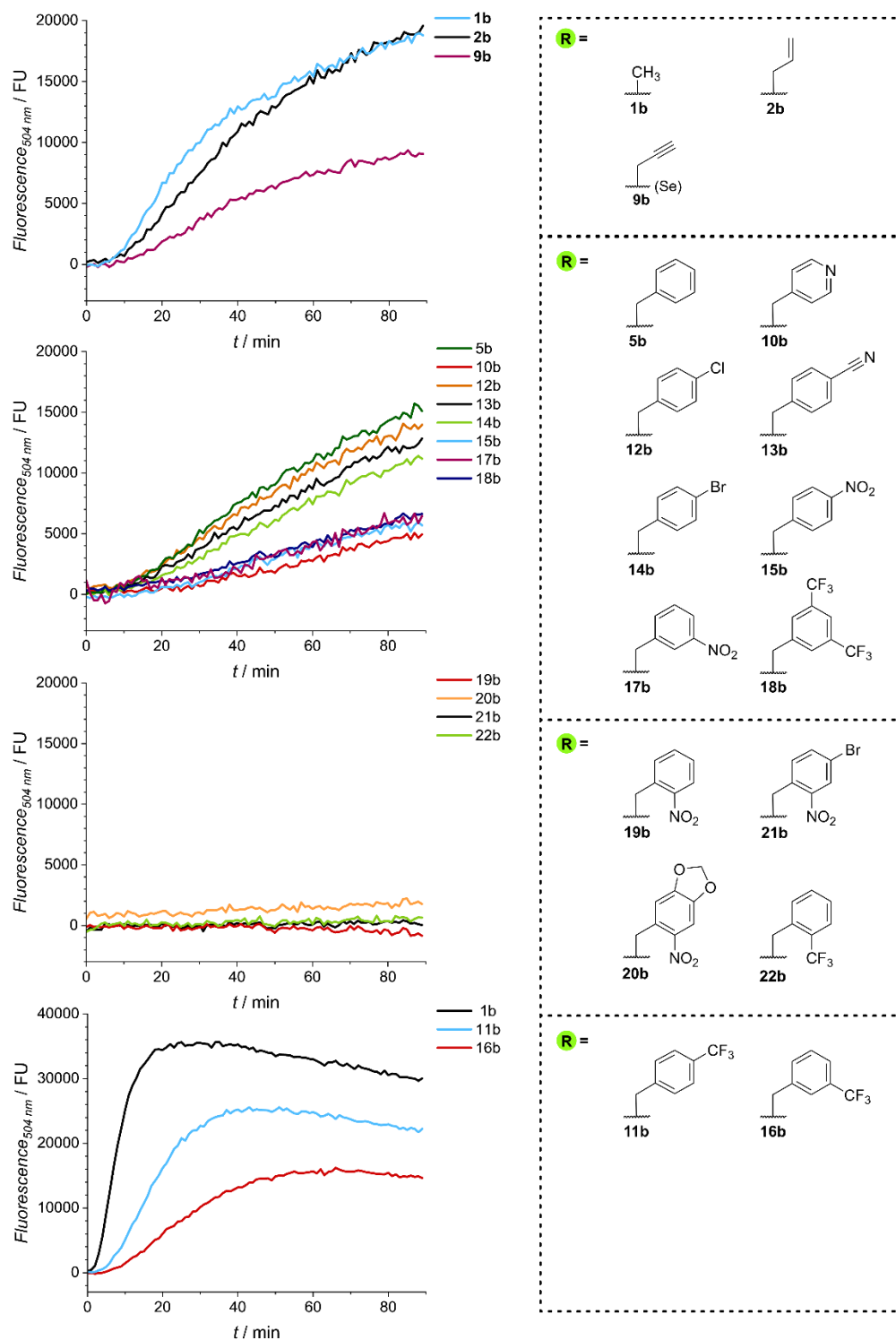


Figure S4: Screening of SAM analogue acceptance of the SAM aptasensor by PC-MjMAT chemoenzymatic synthesis starting with methionine analogues. The increase in fluorescence upon SAM analogue formation is monitored over time. The assay contains 1 mM *L*-methionine analogue, 1 mM ATP, 100 μM PC-MjMAT, 120 μM DFHBI, 3 μM aptasensor and

MAT/aptasensor buffer (50 mM HEPES, 100 mM KCl, 10 mM MgCl₂, pH = 7.4). The fluorescence signal is detected with the TECAN infinite 1000pro with datapoints after 1 min during incubation at 25 °C. The control signal was subtracted from the raw data to exclude analogue-independent signal increase by fluorophore binding. As **15b** is a fluorophore, the control signal here included the methionine analogue but no PC-MjMAT to subtract background fluorescence. The assay was performed n = 3 times with similar results.

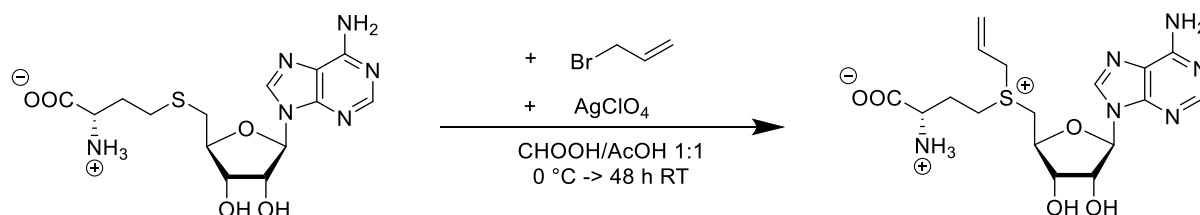
PC-MjMAT purification

The protein PC-MjMAT was produced and purified as described by Peters *et al.*^{2,3}

SAM analogue synthesis

The SAM analogue **3a** was synthesized as described before.⁴ Likewise, the SAM analogue **4a** was synthesized as described before.⁵ SAM analogues **3a-5a** were purified by a preparative *PrepChrom C-700 Purification system* (Büchi, Flawil, CH) with a *Nucleodur C18 Pyramid* (10 µm, 250 x 21 mm) column (*Macherey-Nagel GmbH*, Düren, GER) and a flow rate of 18 mL/min. A gradient of buffer A (10 mM NH₄OAc, pH = 6.5) and buffer B (50 % buffer A, 50 % ACN) was used. Concentrations of the analogues were determined by UV absorption analysis with $\epsilon_{260} = 15.400 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$.

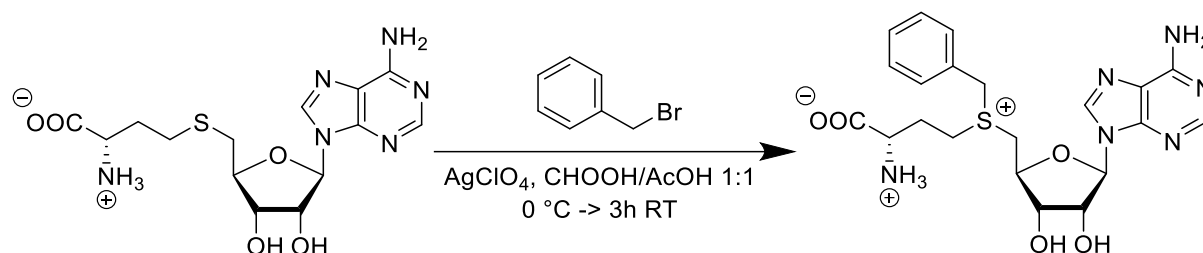
Synthesis of Allyl-SAM (5'-[(R/S)(3S)-3-Amino-3-carboxypropyl]-vinylsulfonio]-5'-deoxyadenosine) (2a)



Allyl bromide (34.1 µL, 394 µmol, 29 eq.) was dissolved in 1:1 formic acid/acetic acid (1.5 mL) and *S*-adenosyl-*L*-homocysteine (5.2 mg, 13.5 µM, 1 eq.) and silver perchlorate (2.7 mg, 13.0 µmol, 0.96 eq.) were added at 0 °C. Progress of the reaction was monitored by HPLC-UV/vis. The reaction is stirred for 48 h at RT and stopped by dilution with water (3 mL). Organic byproducts were separated by extraction with diethyl ether (15 mL). The aqueous phase was lyophilized and dissolved in water (1.5 mL) with 0.01 % TFA. Purification was performed via semi-preparative HPLC with a gradient of buffer A (water/0.01 % TFA) and buffer B (50 % A, 50 % ACN). Compound **2a** was obtained after lyophilization and dissolved as a stock solution in a racemic *R/S*-mixture (0.04 mL, 61.77 mM, 48 %).

High-res. MS (ESI-pos): Calculated mass of [C₁₇H₂₅N₆O₅S₁]⁺ = 425.16017; found: 425.16010

Synthesis of Benzyl-SAM (5'-[(R/S)-[(3S)-3-Amino-3-carboxypropyl]-benzylsulfonio]-5'-deoxyadenosine) (5a**)**



Benzyl bromide (93 μL , 780 μmol , 24 eq.) was dissolved in 1:1 formic acid/acetic acid (1.5 mL) and S-adenosyl-L-homocysteine (12.4 mg, 32.2 μmol , 1 eq.) and silver perchlorate (2.7 mg, 13.0 μM , 0.5 eq.) were added at 0 °C. Progress of the reaction was monitored by LC-MS with a $\text{NH}_4\text{OAc}/\text{ACN}$ gradient and further silver perchlorate was added to enhance the reaction. The reaction stirred for 3 h at RT and was stopped by dilution with water (3 mL). Organic byproducts were separated by extraction with diethyl ether (15 mL). The aqueous phase was lyophilized and dissolved in water (2 mL) with 0.01 % TFA. Purification was performed as described above. Compound **5a** was obtained after lyophilization as a racemic *R/S*-mixture and dissolved as a stock solution (70 μL , 112 mM, 24 %). Success of the reaction was monitored by LC-MS (Fig. S6).

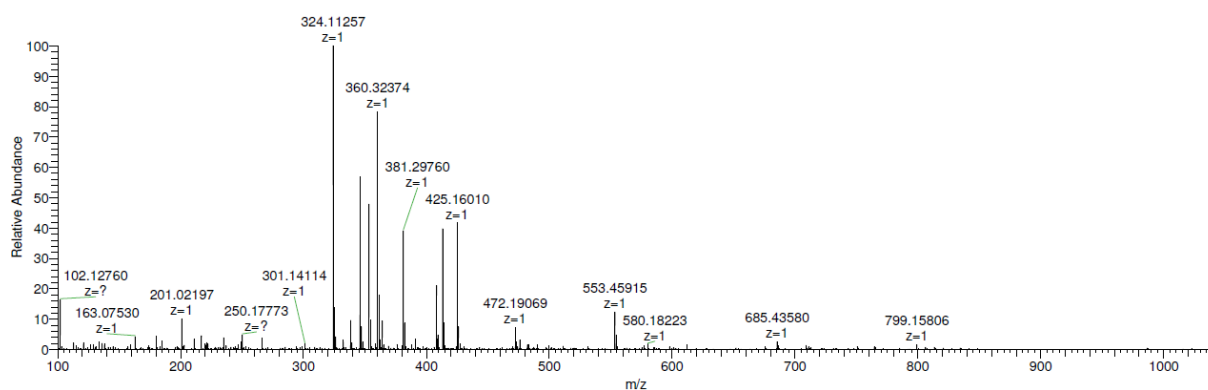


Figure S5: Mass spectrum of compound **2a**. Calculated mass of $[\text{C}_{17}\text{H}_{25}\text{N}_6\text{O}_5\text{S}]^+ = 425.16017$ $[\text{M}+\text{Na}]^+$; found: 425.16017.

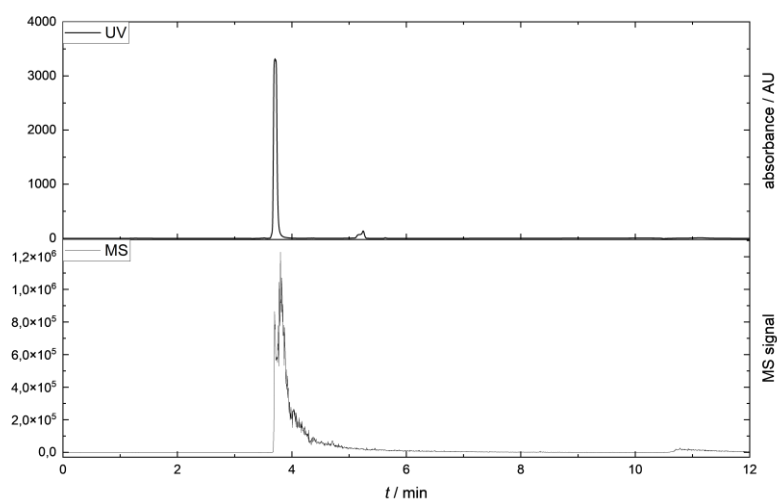
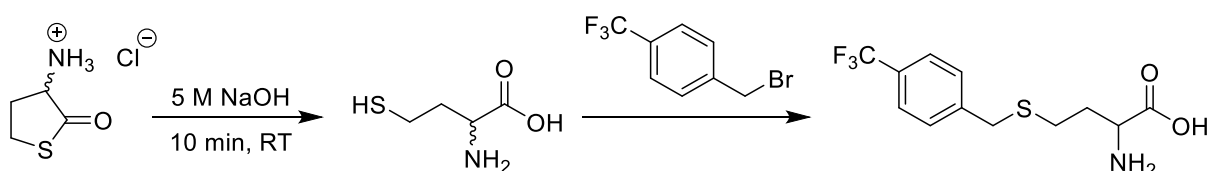


Figure S6: LC analysis of **5a**. Upper panel: HPLC chromatogram with detection at 260 nm. Lower panel: Extracted Ion Count (+EIC) at $m/z = 425 \pm 1$.

Methionine analogue Synthesis

Methionine analogues were synthesized based on the procedure described by Peters *et al.*² Methionine analogues **10b**, **12-15b** and **18b** have been previously synthesized by Cornelissen *et al.*⁶ Analogues **2b** and **5b** were synthesized as described by Hoffmann *et al.*⁷ The analogues **19b-21b** were synthesized as described by Peters *et al.*²

Synthesis of *S*-(4-(trifluoromethyl)benzyl)homocysteine (**11b**)



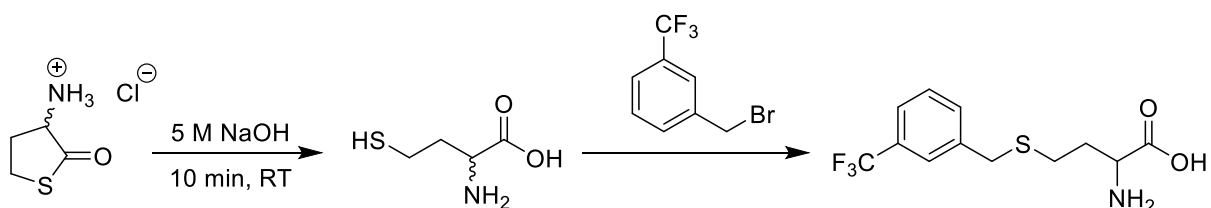
Homocysteine thiolactone (346 mg, 2.25 mmol, 1.50 eq.) was dissolved in 5 mL freshly prepared NaOH (ddH₂O, degassed, 5 M) under argon. The mixture was stirred for 10 min. Sodium bicarbonate (210 mg, 1.25 mmol, 1.20 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of *meta*-trifluoromethylbenzyl bromide (229 μ L, 1.5 mmol, 1 eq.) in dry 1,4-dioxane (3 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature for 4 h and the progress was monitored *via* TLC. The crude mixture was acidified to pH = 6 using 1 M HCl and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH₄Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (168 mg, 0.57 mmol, 38 %)

¹H-NMR (500 MHz, D₂O): δ = 7.71 (*d*, *J* = 8.1 Hz, 2H, H-C(8/10)), 7.56 (*d*, *J* = 8.0 Hz, 2H, H-C(7/11)), 3.87 (*s*, 2H, H₂-C(5)), 3.29 (*dd*, *J* = 7.4, 5.5 Hz, 1H, H-C(2)), 2.51 (*ddd*, *J* = 8.2, 6.8, 1.4 Hz, 2H, H₂-C(4)), 1.99-1.67 (*m*, 2H, H₂-C(3)) ppm.

¹³C-NMR (126 MHz, D₂O): δ = 182.67 C(1), 129.32 C(7/11), 125.53 C(12), 55.26 C(2), 34.48 C(5), 34.23 C(3), 27.02 C(4) ppm.

High-res. MS (ESI-pos): Calculated mass of [C₁₂H₁₅NF₃O₂S]⁺ = 294.0776 = [M+H]⁺; found: 294.0777

Synthesis of *S*-(3-(trifluoromethyl)benzyl)homocysteine (**16b**)



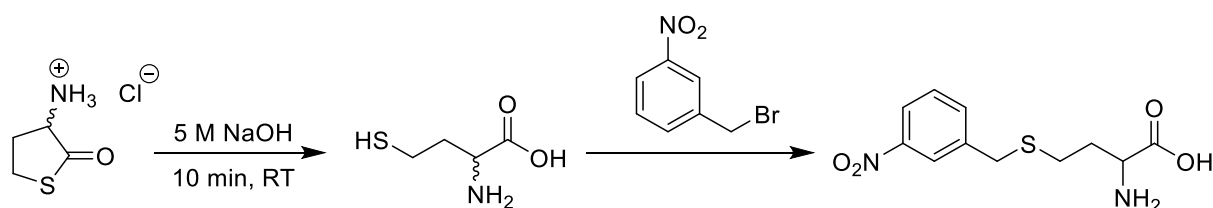
Homocysteine thiolactone (691 mg, 4.50 mmol, 1.50 eq.) was dissolved in 10 mL freshly prepared NaOH (ddH₂O, degassed, 5 M) under argon. The mixture was stirred for 10 min. Sodium bicarbonate (420 mg, 2.5 mmol, 1.20 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of *para*-trifluoromethylbenzyl bromide (458 μ L, 3.00 mmol, 1 eq.) in dry 1,4-dioxane (3 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature for 4 h and the progress was monitored *via* TLC. The crude mixture was acidified to pH = 6 using 1 M HCl and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH₄Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (141 mg, 0.48 mmol, 16 %)

¹H-NMR (500 MHz, *D*₂O): δ = 7.75 (s, 1H, H-C(11)), 7.64 (t, 1H, H-C(8)), 7.63 (s, 1H, H-C(7)) 7.56 (t, *J* = 7.7 Hz, 1H, H-C(9)), 3.88 (s, 2H, 2H-C(5)), 3.29 (dd, *J* = 7.5, 5.5 Hz, 1H, H-C(2)), 2.52 (ddd, *J* = 8.5, 6.7, 2.0 Hz, 2H, H2-C(4)), 1.97-1.71 (m, 2H, H2-C(3)) ppm.

¹³C-NMR (126 MHz, *D*₂O): δ = 182.68 C(1), 139.69 C(6), 132.59 C(7), 129.29 C(9), 125.47 C(12), 125.22 C(11), 123.90 C(8), 55.24 C(2), 34.53 C(3), 34.25 C(5), 27.09 C(4) ppm.

High-res. MS (ESI-pos): Calculated mass of [C₁₂H₁₅NF₃O₂S]⁺ = 294.0776 = [M+H]⁺; found: 294.0777

Synthesis of *S*-(3-nitrobenzyl)homocysteine (**17b**)



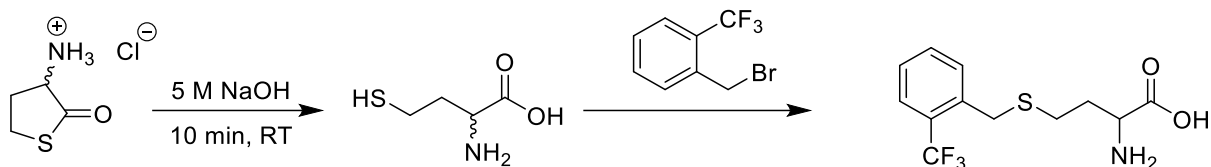
Homocysteine thiolactone (691 mg, 4.5 mmol, 1.49 eq.) was dissolved in 5 mL freshly prepared NaOH (ddH₂O, degassed, 5 M) under argon. The mixture was stirred for 10 min. Sodium bicarbonate (458 mg, 5.45 mmol, 1,7.9 eq.) was added and the mixture was acidified to pH = 9 using conc. HCl. A solution of *meta*-nitrobenzyl bromide (655 mg, 3.03 mmol, 1 eq.) in dry 1,4-dioxane (3 mL) was prepared under argon and slowly added to the amino acid solution. The reaction was stirred at room temperature for 4 h and the progress was monitored *via* TLC. The crude mixture was acidified to pH = 6 using 1 M HCl and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH₄Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (60.4 mg, 0.22 mmol, 7 %)

¹H-NMR (500 MHz, DMSO-*d*₆): δ = 8.20 (t, *J* = 2.0 Hz, 1H, H-C(11)), 8.10 (ddd, *J* = 8.2, 2.4, 1.0 Hz, 1H, H-C(9)), 7.79 (ddt, *J* = 6.5, 5.3, 1.4 Hz, 1H (H-C(7))), 7.61 (t, *J* = 7.9 Hz, 1H, H-C(8)), 3.88 (s, 2H, H2-C(5)), 3.27 (ddd, *J* = 11.0, 6.3, 3.2 Hz, 1H, H-C(2)), 2.45 (m, 2H, H2-C(4)), 2.06-1.78 (m, 2H, H2-C(3)) ppm.

¹³C-NMR (126 MHz, DMSO-*d*₆): δ = 169.76 C(1), 147.82 C(10), 141.48 C(6), 135.61 C(7), 129.89 C(8), 123.31 C(11), 121.77 C(9), 53.00 C(1), 33.91 C(5), 30.95 C(3), 27.19 C(4) ppm.

High-res. MS (ESI-pos): Calculated mass of [C₁₁H₁₅N₂O₄]⁺ = 271.07470 = [M+H]⁺; found: 271.07465; Calculated mass of [C₁₁H₁₄N₂O₄Na]⁺ = 293.05665 = [M+Na]⁺; found: 293.05665.

Synthesis of *S*-(2-(trifluoromethyl)benzyl)homocysteine (**22b**)



Homocysteine thiolactone (697 mg, 4.54 mmol, 1.48 eq.) was dissolved in 5 mL freshly prepared NaOH (ddH₂O, degassed, 5 M) under argon. The mixture was stirred for 10 min. 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 2-trifluoromethylbenzyl bromide (732 mg, 3.06 mmol, 1 eq.) in dry 1,4-dioxane (3 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* TLC. The crude mixture was acidified to pH = 6 using 1 M HCl and separated via flash chromatography (column: HP-C18-Aq. 100 g, gradient: 100 % A: NH₄Ac 50 mM to 60 % B: ACN). The product was obtained as white powder (294 mg, 1.00 mmol, 33 %)

¹H-NMR (500 MHz, D₂O): δ = 7.55 (*d*, 1H, H-C(10)), 7.43 (*t*, 1H, H-C(8)), 7.39 (*t*, *J* = 7.6 Hz, 1H, H-C(7)), 7.28 (*t*, *J* = 7.6 Hz, 1H, H-C(9)), 3.78 (*d*, *J* = 1.2 Hz, 2H, H₂-C(5)), 3.13 (*dd*, *J* = 7.4, 5.5 Hz, 1H, H-C(2)), 2.52-2.39 (*m*, 2H, H₂-C(4)), 1.86-1.52 (*m*, 2H, 2H-C(3)) ppm.

¹³C-NMR (126 MHz, D₂O): δ = 182.51 C(1), 136.65 C(6), 132.40 C(8), 131.65 C(7), 127.50 C(9), 127.23 C(11), 126.41 C(10), 126.36 (*q*, C(12)), 55.26 C(2), 34.43 C(3), 32.12 C(5), 28.13 C(4) ppm.

High-res. MS (ESI-pos): Calculated mass of [C₁₂H₁₄N₁O₁S₁F₃Na]⁺ = 316.05896 = [M+Na]⁺; found: 316.05893.

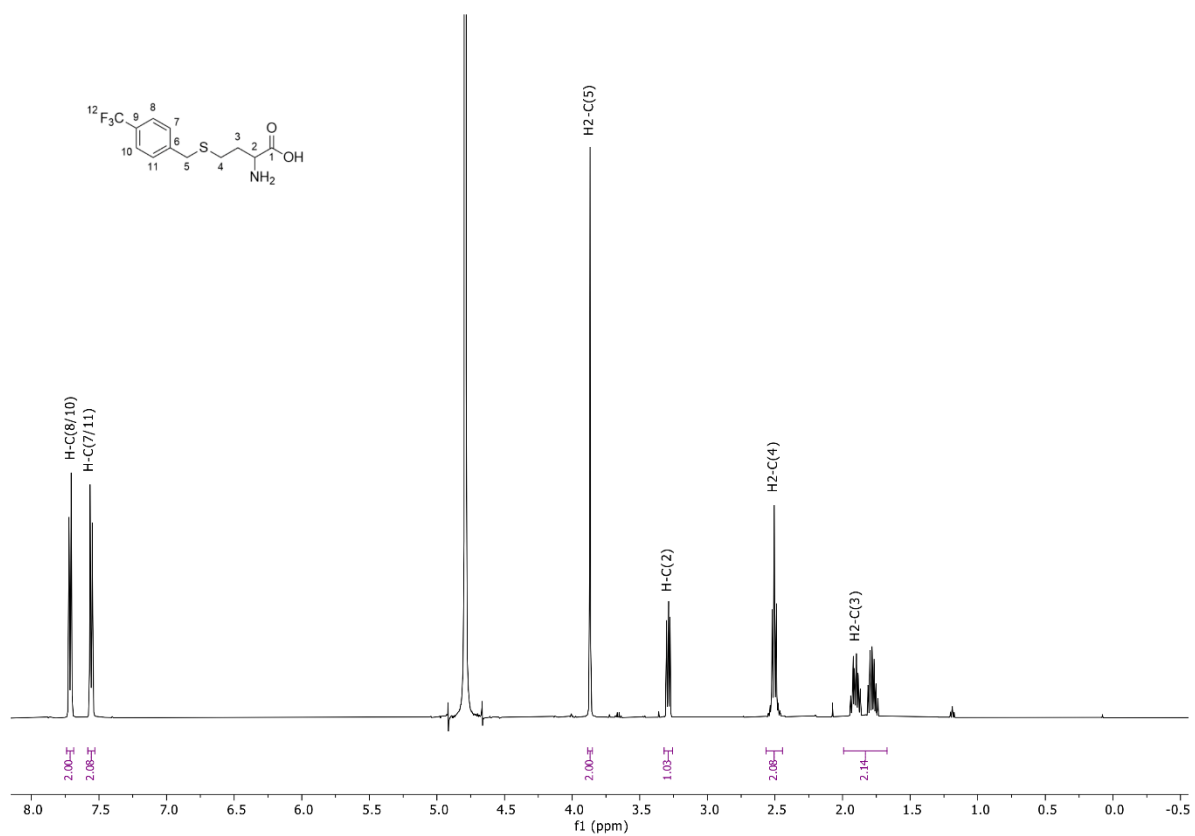


Figure S7: ¹H-NMR spectrum of compound **11b**.

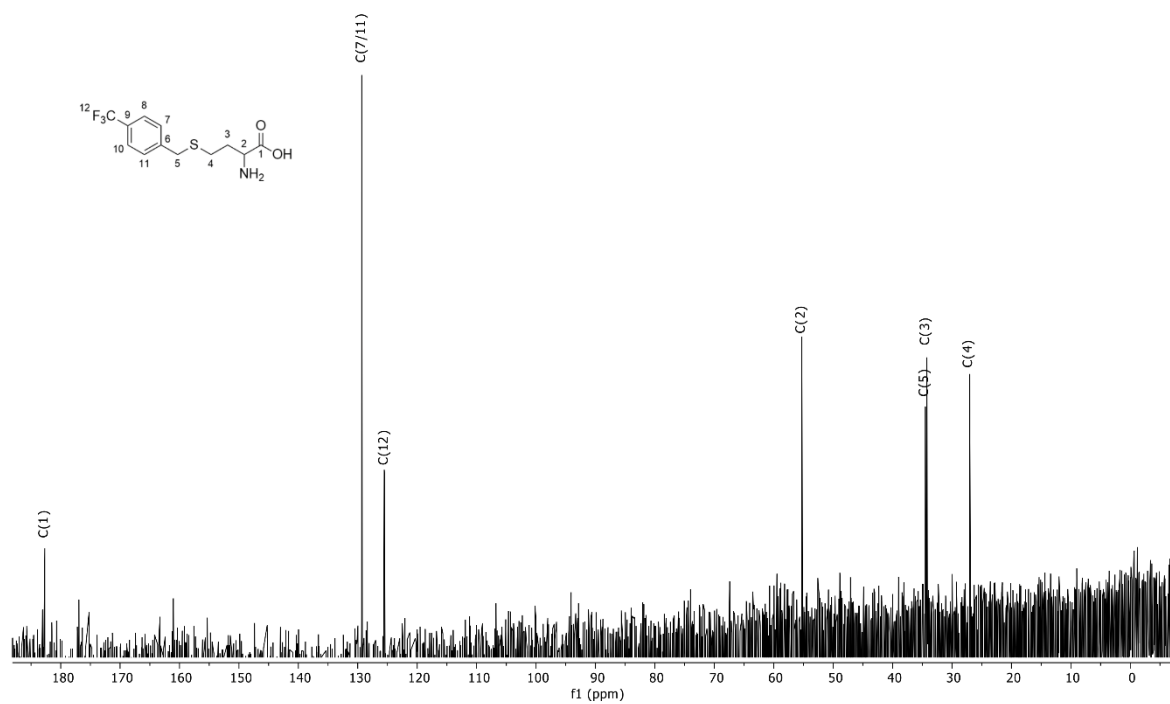


Figure S8: ¹³C-NMR spectrum of compound **11b**.

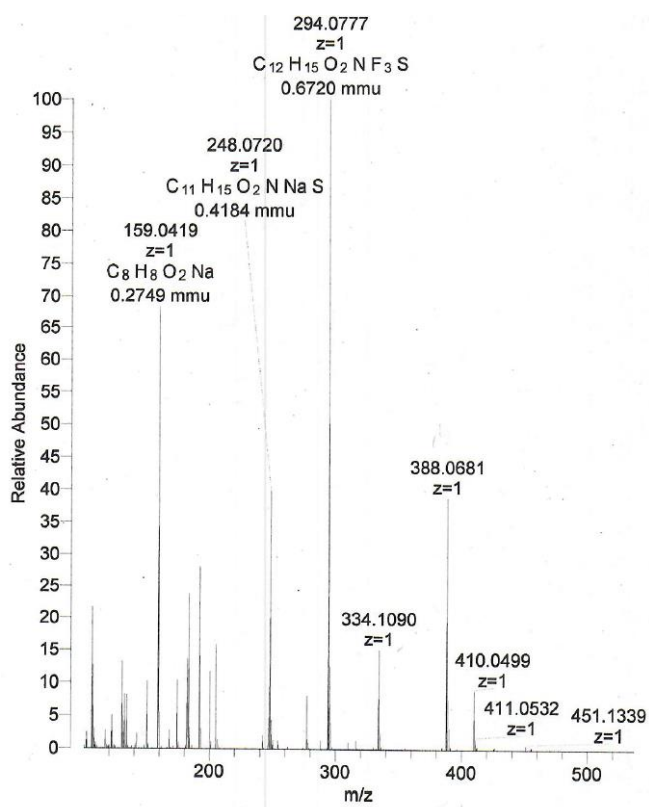


Figure S9: Mass spectrum of compound **11b**. Calculated mass of $[C_{12}H_{15}NF_3O_2S]^+ = 294.0776 = [M+H]^+$; found: 294.0777

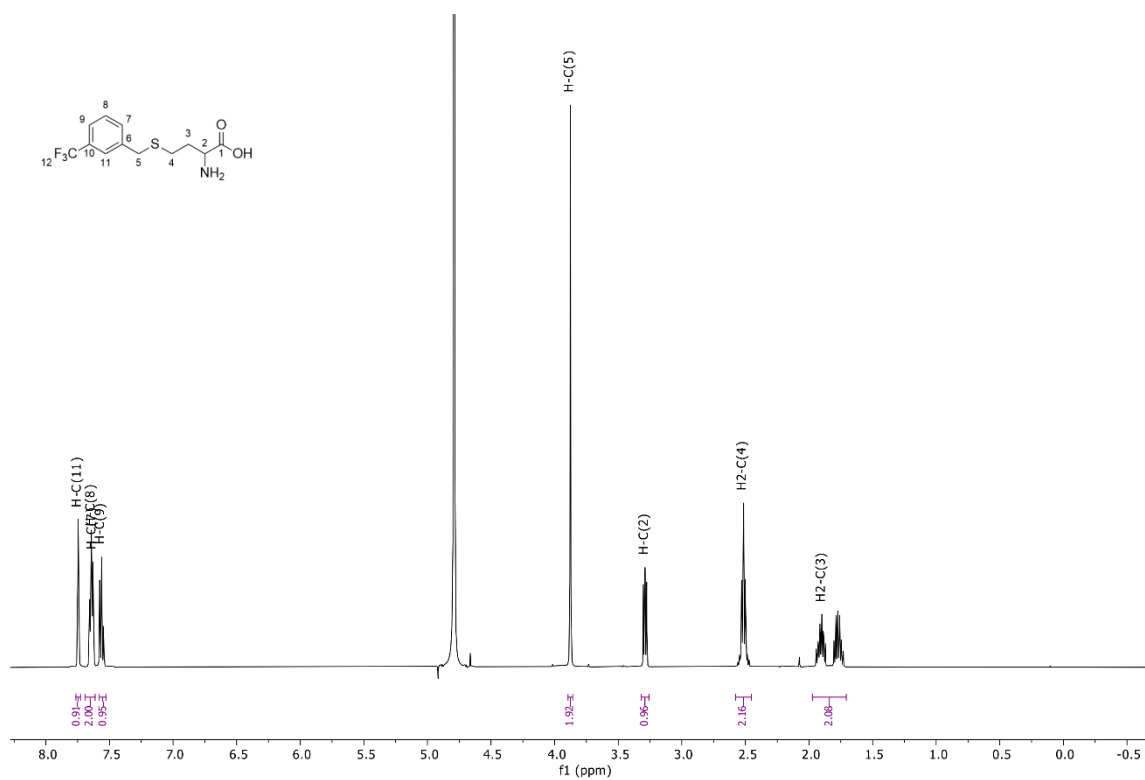


Figure S10: 1H -NMR spectrum of compound **16b**.

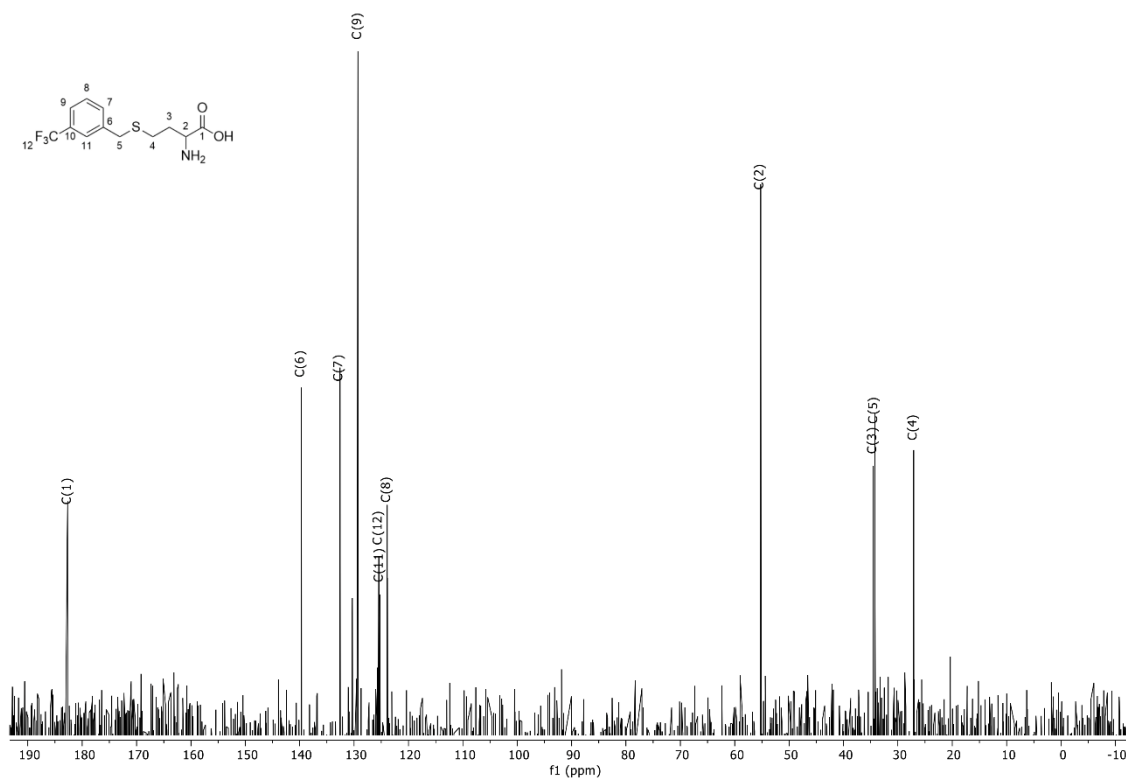


Figure S11: ^{13}C -NMR spectrum of compound **16b**.

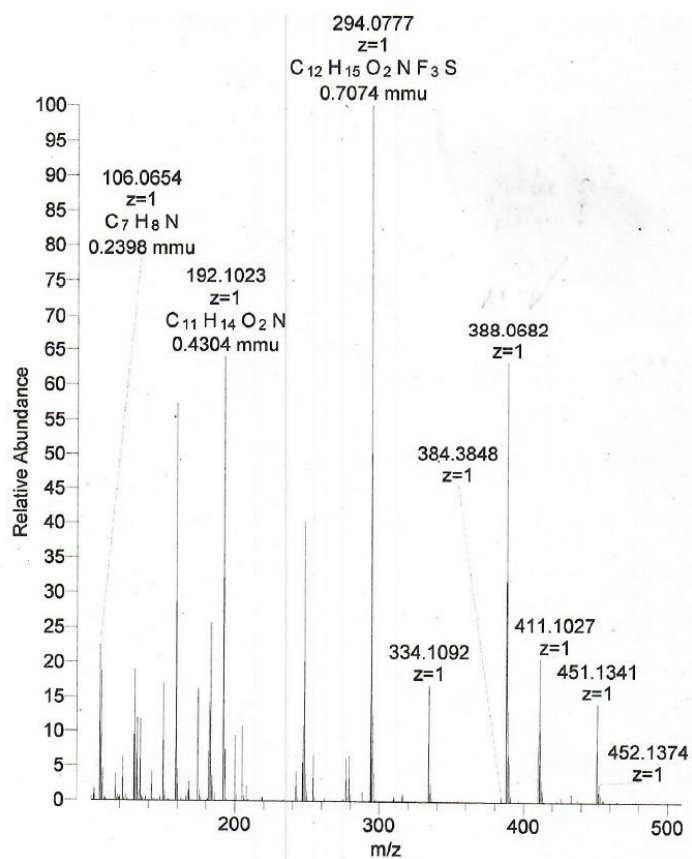


Figure S12: Mass spectrum of compound **16b**. Calculated mass of $[\text{C}_{12}\text{H}_{15}\text{NF}_3\text{O}_2\text{S}]^+ = 294.0776 = [\text{M}+\text{H}]^+$; found: 294.0777

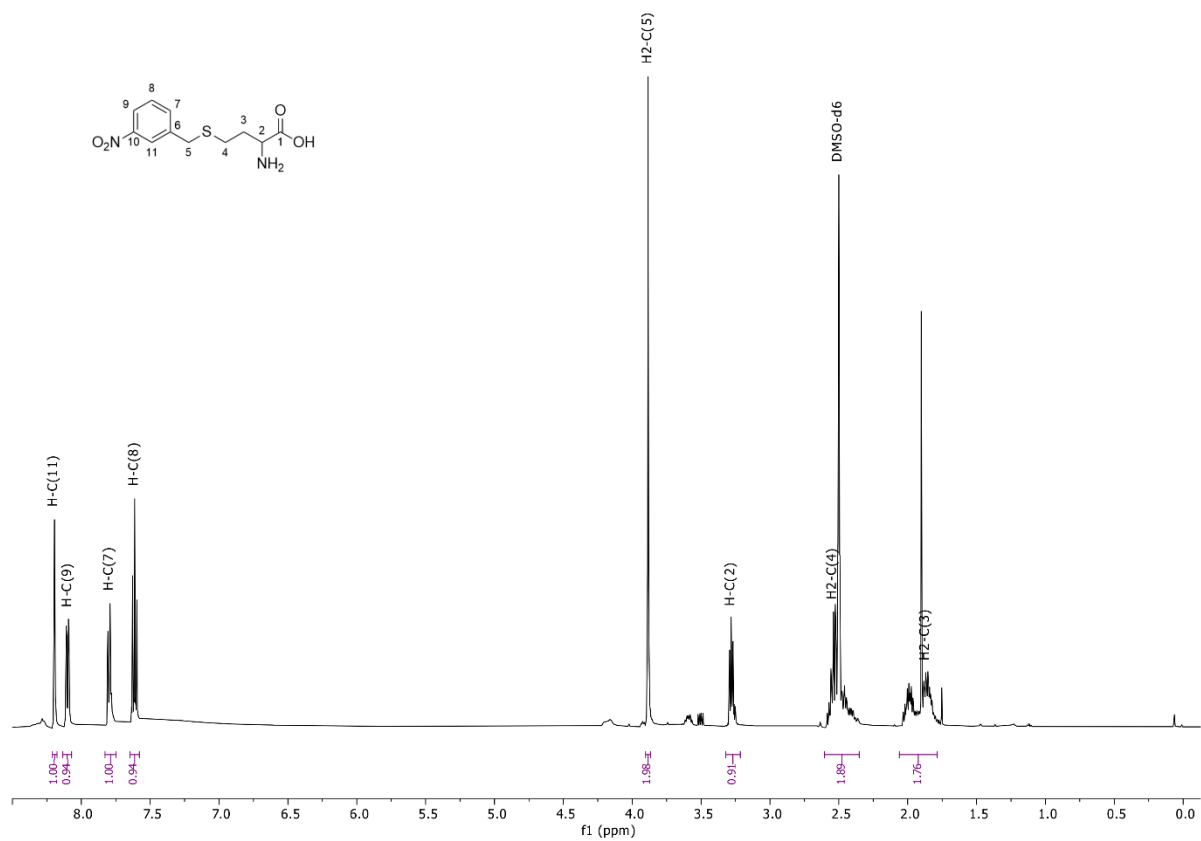


Figure S13: ¹H-NMR spectrum of compound 17b.

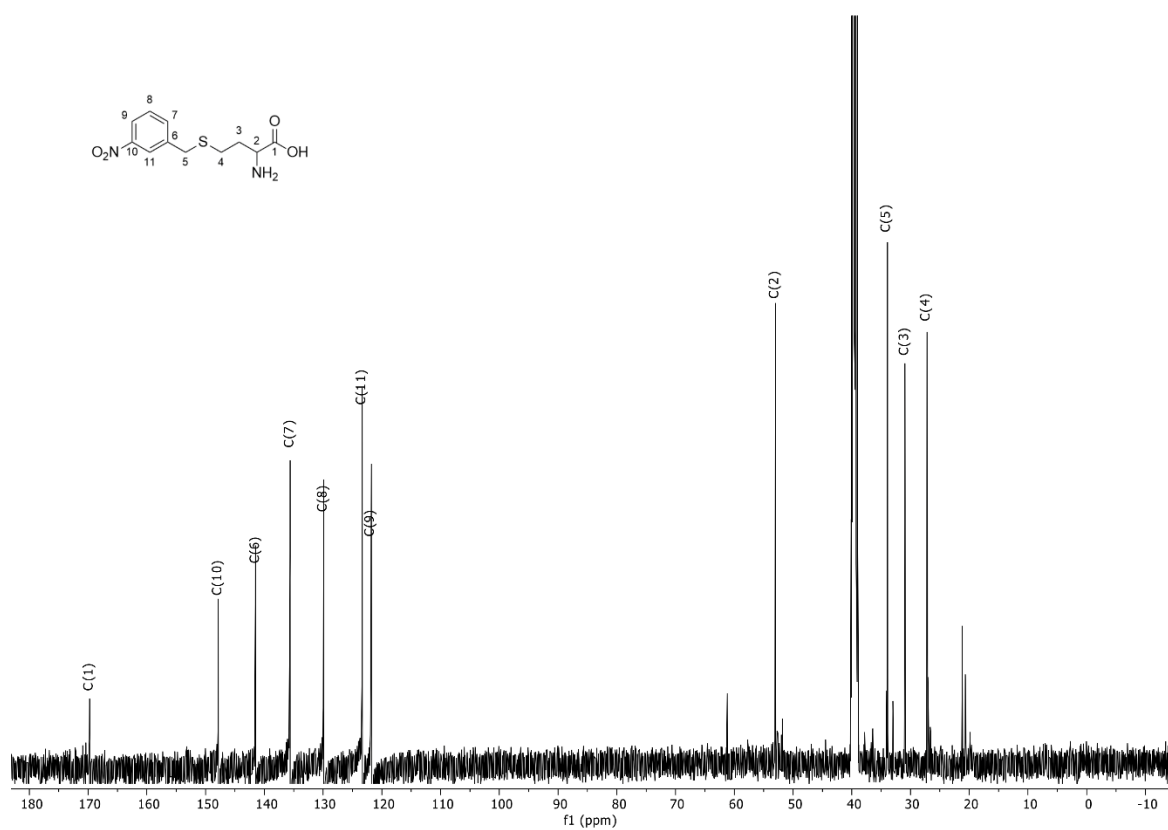


Figure S14: ¹³C-NMR spectrum of compound 17b.

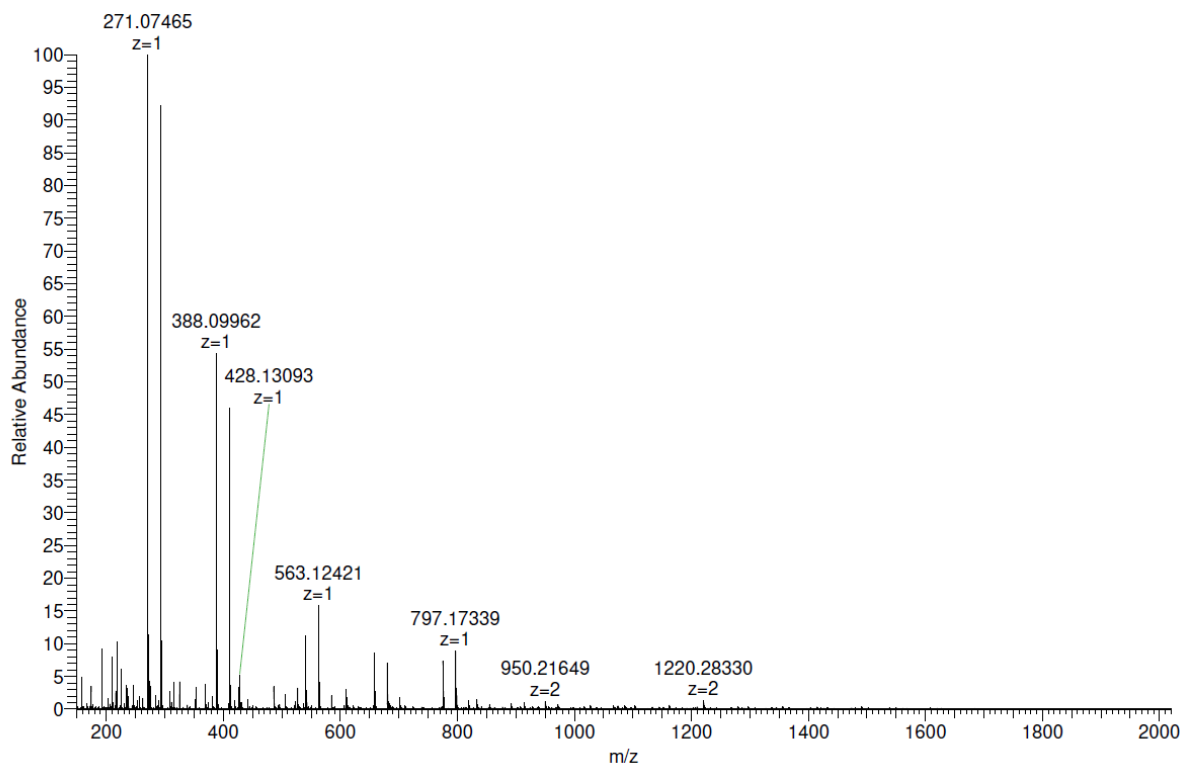


Figure S15: Mass spectrum of compound **17b**. Calculated mass of $[C_{11}H_{15}N_2O_4]^+$ = 271.07470 = $[M+H]^+$; found: 271.07465; Calculated mass of $[C_{11}H_{14}N_2O_4Na]^+$ = 293.05665 = $[M+Na]^+$; found: 293.05665.

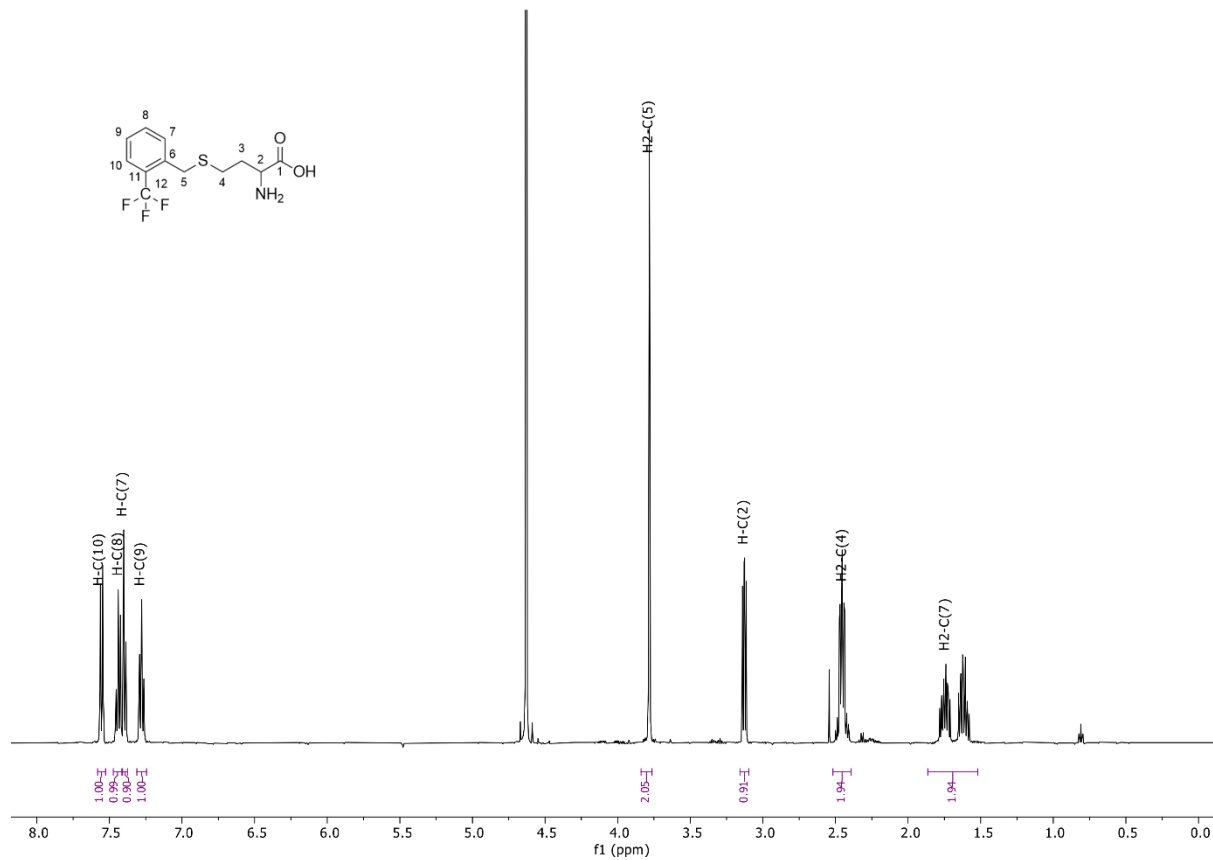


Figure S16: ¹H-NMR spectrum of compound **22b**.

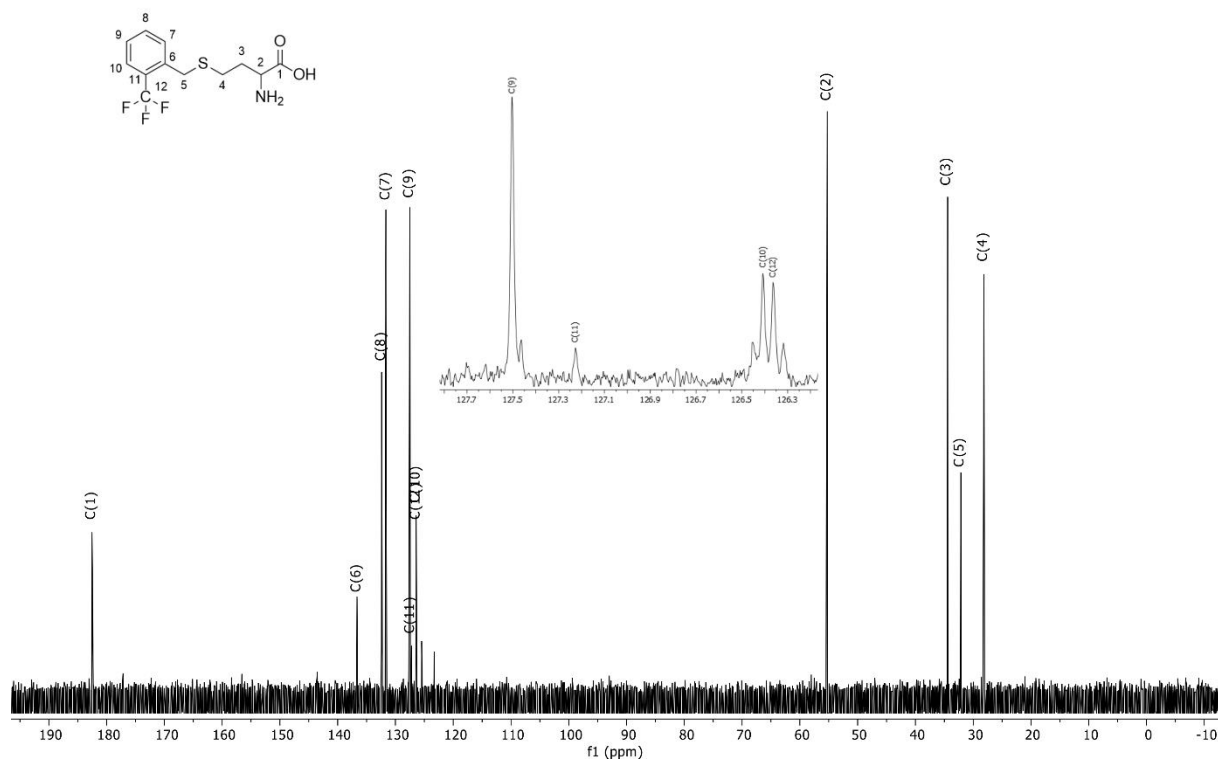


Figure S17: ¹³C-NMR spectrum of compound **22b**.

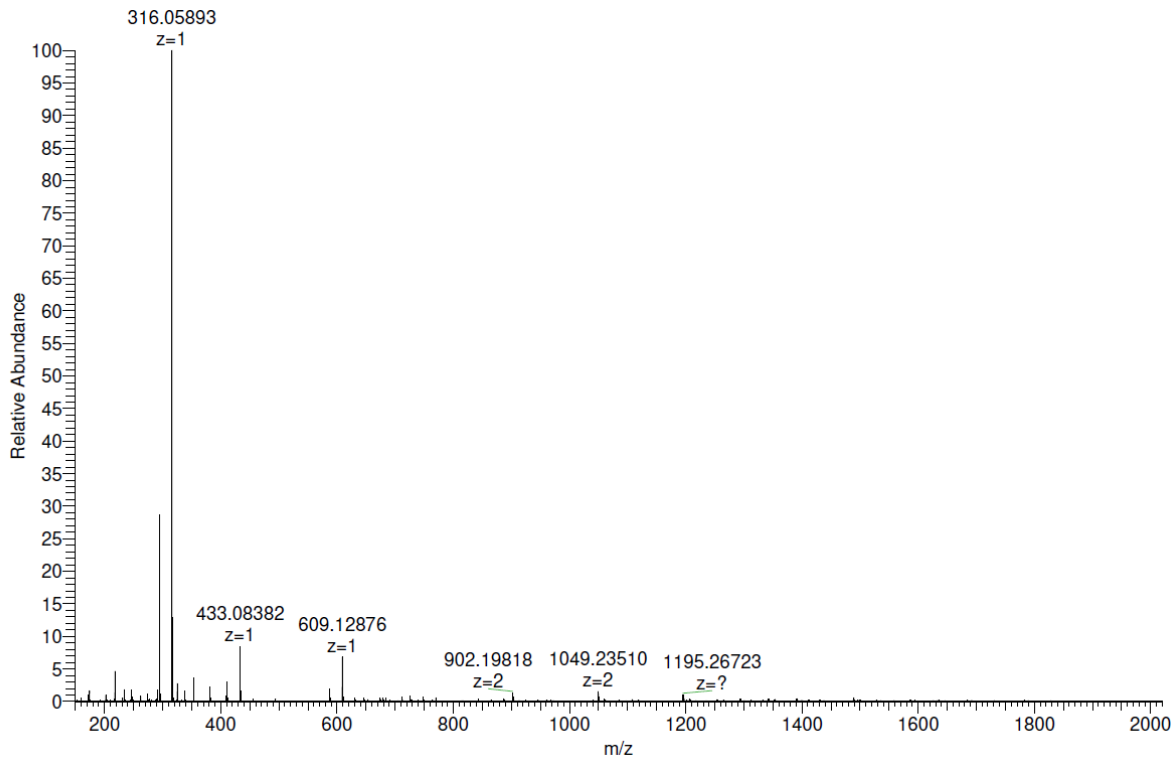
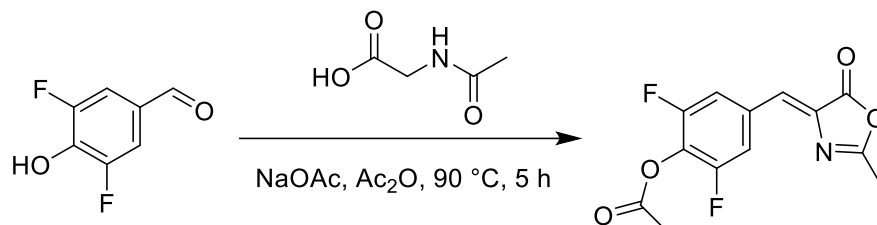


Figure S18: Mass spectrum of compound **22b**. Calculated mass of $[C_{12}H_{14}N_1O_1S_1F_3Na]^+$ = 316.05896 = $[M+Na]^+$; found: 316.05893.

Synthesis of DFHBI

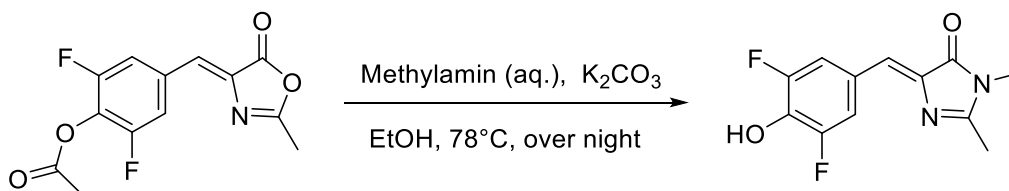
Synthesis of DFHBI (**24**) and its precursor (**23**)

Synthesis of (Z)-2,6-difluoro-4-((2-methyl-5-oxooxazol-4(5H)-ylidene)methyl)phenyl acetate (**23**)



3,5-Difluoro-hydroxybenzaldehyde (399.5 mg, 2.53 mmol, 1 eq.), dry sodium acetate (207.4 mg, 2.53 mmol, 1 eq.) and N-acetylglycine (296.0 mg, 2.53 mmol, 1 eq.) were stirred in 2 mL acetic anhydride at 90 °C for 5 h. The orange liquid was extracted with water/ethyl acetate and the organic phase was removed by rotary evaporation. Product was purified by preparative HPLC and was obtained as a yellow solid (580.5 mg, 2.06 mmol, 82 %).

Synthesis of DFHBI ((Z)-5-(3,5-difluoro-4-hydroxybenzylidene)-2,3-dimethyl-3,5-dihydro-4H-imidazol-4-one) (**24**)



Compound **21** (580.5 mg, 2.064 mmol, 1 eq.), potassium carbonate (427.4 mg, 3.093 mmol, 1.51 eq.) and methylamine (1.337 mL, 4.608 mmol, 1 eq.) were dissolved in 2 mL ethanol and stirred at 72 °C for 19 h. The product was extracted with 1:1 ethyl acetate/sodium acetate aqueous solution (500 mM, pH = 3) and the organic phase was purified by preparative HPLC. DFHBI was obtained as a yellow-orange solid (402 mg, 1.593 mmol, 77 %)

¹H-NMR (599 MHz, DMSO-*d*₆, 299 K): δ [ppm] = 10.95 (s, 1H, H-C(8)), 7.96 (dd, *J* = 8.4, 1.7 Hz, 2H, H-C(1/5)), 6.89 (s, 1H, H-C(9)), 3.08 (s, 3H, H₃-C(17)), 2.35 (s, 3H, H₃-C(15)).

¹³C-NMR (151 MHz, DMSO-*d*₆, 299 K): δ [ppm] = 169.57 C(14), 164.42 C(12), 152.63 C(2/4), 151.03 C(2/4), 138.40 C(10), 135.67 C(arom), 124.69 C(arom), 122.75 C(9), 115.12 C(1/5), 26.23 C(17), 15.39 C(15).

MS (ESI-pos): Calculated mass of [C₁₂H₁₀N₂O₂F₂Na]⁺ = 275.05979; found: 275.05979; calculated mass of [C₁₂H₁₀N₂O₂F₂]⁺ = 253.07831; found: 253.07792

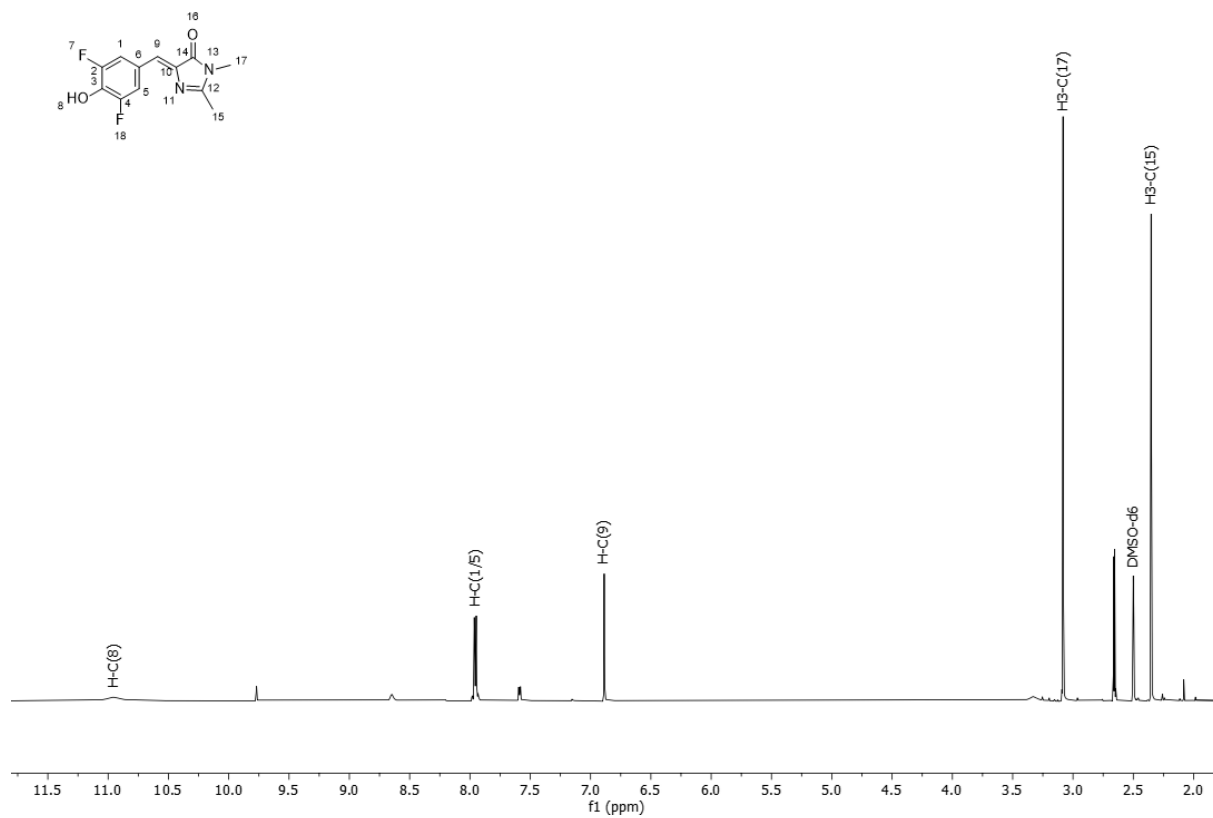


Figure S19: $^1\text{H-NMR}$ spectrum of compound 24.

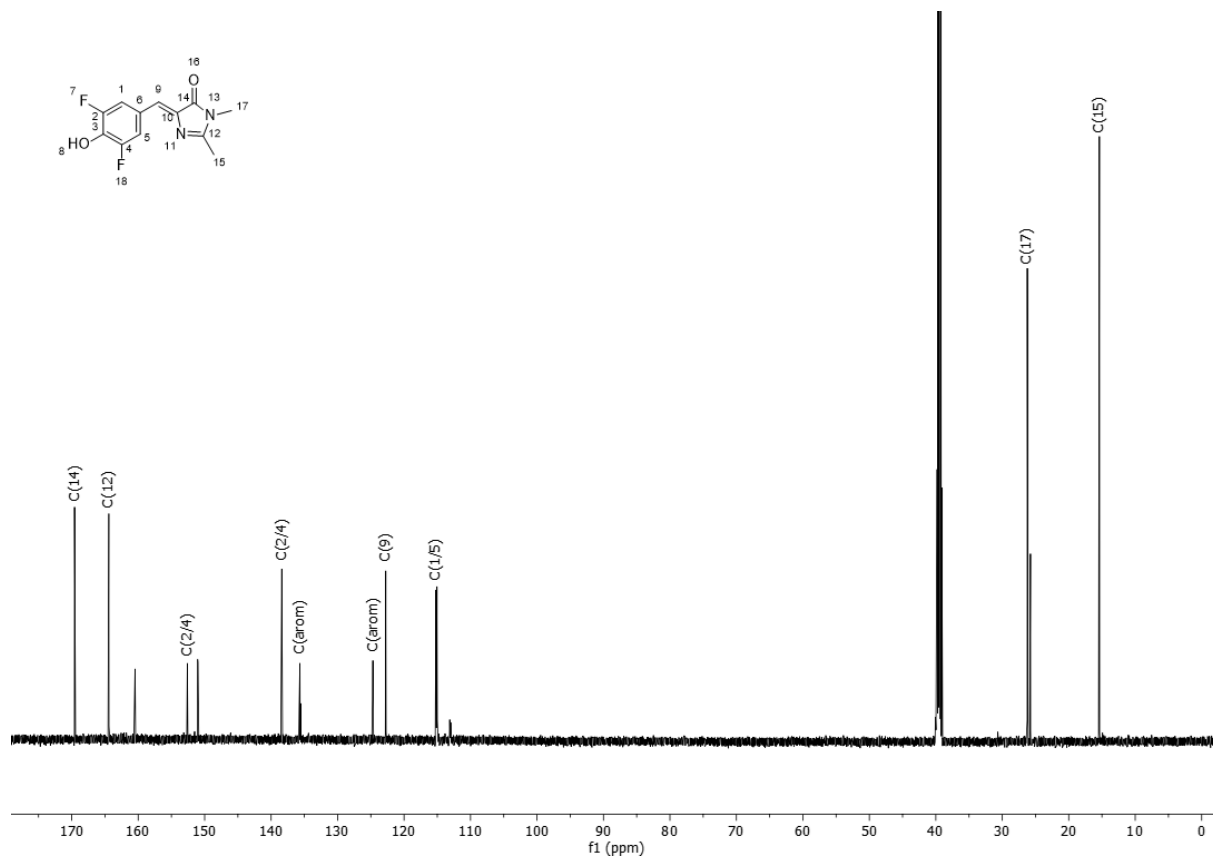


Figure S20: $^{13}\text{C-NMR}$ spectrum of compound 24.

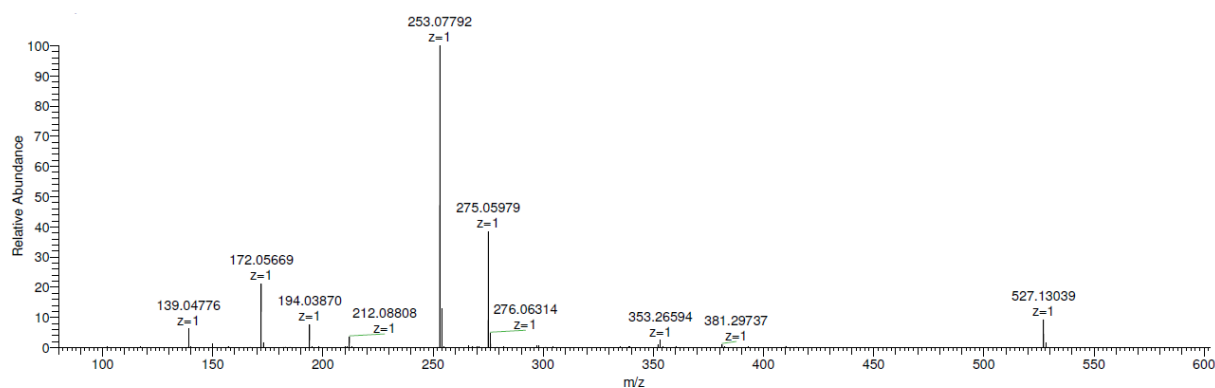


Figure S21: Mass spectrum of compound **24**. Calculated mass of $[\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_2\text{F}_2\text{Na}]^+ = 275.05979 = [\text{M}+\text{Na}]^+$; found: 275.05979; calculated mass of $[\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_2\text{F}_2]^+ = 253.07831 = [\text{M}+\text{H}]^+$; found: 253.07792.

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