Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.
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Supporting Information

for

DNA Nanocrane-Based Catalysts for Region-Specific Protein Modification

by

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List of Abbreviations

ACN	=	acetonitrile
ANANS	=	alkylated *N*-acyl-*N*-sulfonamide
Anh.	=	anhydrous
BCN	=	*endo*-bicyclo[6.1.0]nonyne
BME	=	2-mercaptoethanol / β-mercaptoethanol
Brine	=	concentrated NaCl in H_2O
BSA	=	Bovine Serum Albumin
CA2	=	Carbonic Anhydrase 2
$CDCl_3$	=	deuterated chloroform
ddH_2O	=	double-distilled water
diDMAP	=	dD = divalent DMAP catalyst
diPyOx	=	dP = divalent PyOx catalyst
DMAP	=	dimethylaminopyridine
DCM	=	dichloromethane
DIPEA	=	*N,N*-diisopropylethylamine
DMSO	=	dimethyl sulfoxide
DNA_{cat}	=	DNA bearing the catalyst (*see DNA sequences*)
DTT	=	dithiothreitol
EtOAc	=	ethyl acetate
ESI	=	Electron Spray Ionisation
FA	=	formic acid
HCl	=	hydrochloric acid
HEPES	=	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	=	high-performance liquid chromatography
HRMS	=	high resolution mass spectrometry
IAA	=	iodoacetic acid
LC-MS	=	liquid chromatography–mass spectrometry
^{LC}	=	on the light chain
Lig	=	ligandside (*see DNA sequences*)
MeOD	=	methanol-d_4
NMR	=	nuclear magnetic resonance
PyOx	=	pyridinium oxime
SA	=	sulfonamide
SAA	=	surface accessible area
SDS-PAGE	=	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPAAC	=	strain-promoted alkyne-azide click
TAMN	=	(0.5% trifluoroacetic acid + 30% ACN + 70% ddH_2O + 400 mM NaCl)
TBA	=	thrombin binding aptamer
TBA2	=	thrombin binding aptamer 2, a.k.a. HD22
TBA3	=	thrombin binding aptamer 3
Temp	=	template
TRM	=	thrombin = human alpha thrombin
THPTA	=	Tris(3-hydroxypropyltriazolylmethyl)amine

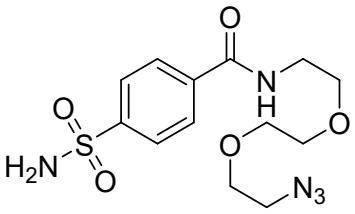
1.0 Materials

- Solvents were purchased from VWR-TS and used without prior purification, unless otherwise specified.
- Reagents were purchased from Sigma Aldrich and used without prior purification, unless otherwise specified.
- Azido-lissamine was purchased from Tenova Pharmaceuticals.
- BCN-lissamine was supplied by dr. J. Bruins.
- BCN-PEG(2000) was purchased from Synaffix BV.
- Lysozyme and Carbonic Anhydrase were purchased from Sigma Aldrich.
- Human alpha-thrombin was purchased from Haematologic Technologies.
- BSA was purchased from Millipore Corporation.
- Trypsin Gold was purchased from PROMEGA BENELUX BV.
- DNA strands were purchased from Integrated DNA technologies.

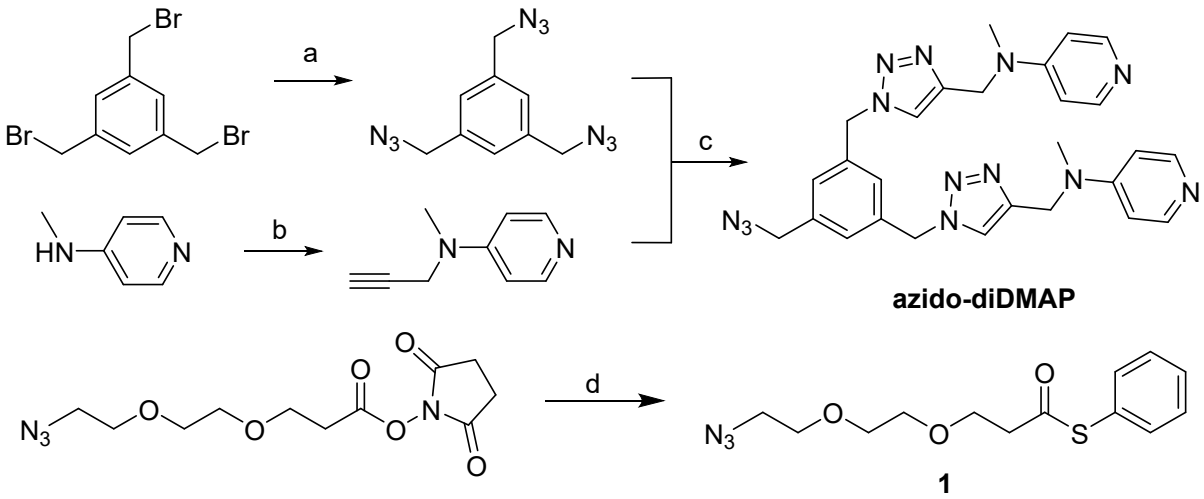
- MabPac™ RP Column (4 µm, 3.0 mm x 100 mm) was purchased from Thermo Fischer.
- Reprosil-Gold 300 C18, 3 µm. 250 mm x 4.0 mm was purchased from Screening Devices.
- HPLC runs were performed using a Finnigan Surveyor Plus HPLC system (Thermo Fisher).
- MS measurements were performed using a Q Exactive Mass Spectrometer (Thermo Fisher).
- Absorption measurements to determine DNA and protein concentrations were performed using a Scientific™ Nanodrop 2000 (Thermo Fisher).

2.1 Synthesis of molecular compounds

2.1.1 azido-EG_2-sulfonamide:

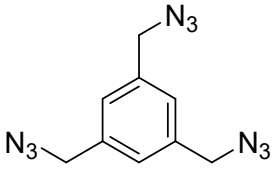


4-sulfamoylbenzoic acid (100 mg, 500 µmol, 1.0 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (85 mg, 550 µmol, 1.1 eq), hydroxybenzotriazole (84 mg, 550 µmol, 1.1 eq), azido-PEG_2-amine (82 mg, 470 µmol, 0.95 eq) and DIPEA (0.13 mL, 750 µmol, 1.5 eq) were dissolved in DMF (5 mL) and stirred at RT for 18 h. The reaction mixture was washed with brine (15 mL) and extracted with DCM (3 × 10 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash column chromatography The residue was purified by flash column chromatography (SiO_2, 5% (10% [25% NH_3 in H_2O] in MeOH) in DCM) yielding a brown oil (27 mg, 16%). HRMS (ESI) calculated for $[M+Na]^+$: 380.1005; found $[M+Na]^+$: 380.1005. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.2 Hz, 2H), 7.71 (d, *J* = 8.2 Hz, 2H), 6.02 (s, 2H), 3.63 (ddt, *J* = 14.2, 9.8, 5.0 Hz, 10H), 3.32 (t, *J* = 4.9 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.79, 144.98, 137.88, 127.98, 126.43, 70.46, 70.23, 69.94, 69.59, 50.67, 40.15 ppm.



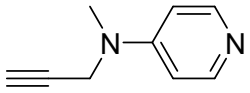
*Scheme S1. Synthesis of azido-diDMAP and thioester **1**. (a) NaN_3, DMF, rt, 16 h, **98%**; (b) n-BuLi, propargylbromide, anh. THF, -80°C-0°C, 30 min, **57%**; (c) $Cu(I)(ACN)_4PF_6$, O_2-poor THF, rt, 18 h, **11%**; (d) thiophenol, toluene, rt, 16 h, **85%**.*

2.1.2 1,3,5-tris(azidomethyl)benzene:



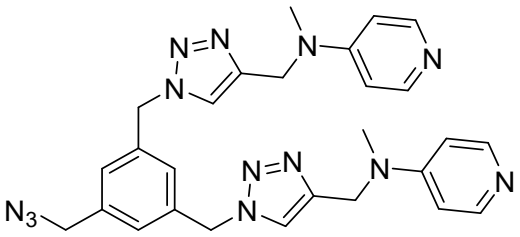
1,3,5-tris(bromomethyl)benzene (200 mg, 560 µmol, 1.0 eq) and sodium azide (219 mg, 3.36 mmol, 6.0 eq) were dissolved in 0.5 mL DMF and stirred at rt for 16 h. The mixture was blow-dried and the residue dissolved in 1 mL DCM. The mixture was washed with H_2O and extracted with DCM (3 × 5mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure, yielding a clear oil (134 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (s, 3H), 4.38 (s, 6H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.0, 127.4, 54.3 ppm.

2.1.3 alkyne-DMAP:



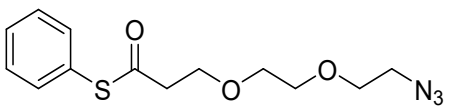
4-(methylamino)-pyridine (200 mg, 1.9 mmol, 1.0 eq) was placed in a flame-dried 25 mL flask under argon and dissolved in anhydrous THF (1.5 mL). The solution was cooled to -90 °C and n-butyllithium (0.8 mL, 2.0 mmol, 1.1 eq) was added and the mixture was stirred for 15 min @ -90 °C. Propargyl bromide (200 µL, 2.77 mmol, 1.5 eq) was added and the reaction mixture was stirred for 15 min at -90 °C and 15 min at 0 °C. The mixture was quenched with sat. NH_4Cl (5 mL) and extracted with THF (3 × 6 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (SiO_2, 5% (10% [25% NH_3 in H_2O] in MeOH) in EtOAc) yielding a brown oil (152.8 mg, 57%). HRMS (ESI) calculated for $[M+H]^+$: 147.0922; found [M+H]+: 147.0917. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (s, 2H), 6.44 (d, *J* = 5.8 Hz, 2H), 3.90 (d, *J* = 2.6 Hz, 2H), 2.85 (s, 3H), 2.14 (t, *J* = 2.5 Hz, 1H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.0, 149.7, 107.4, 78.0, 72.4, 40.5, 37.4 ppm.

2.1.4 azido-diDMAP:

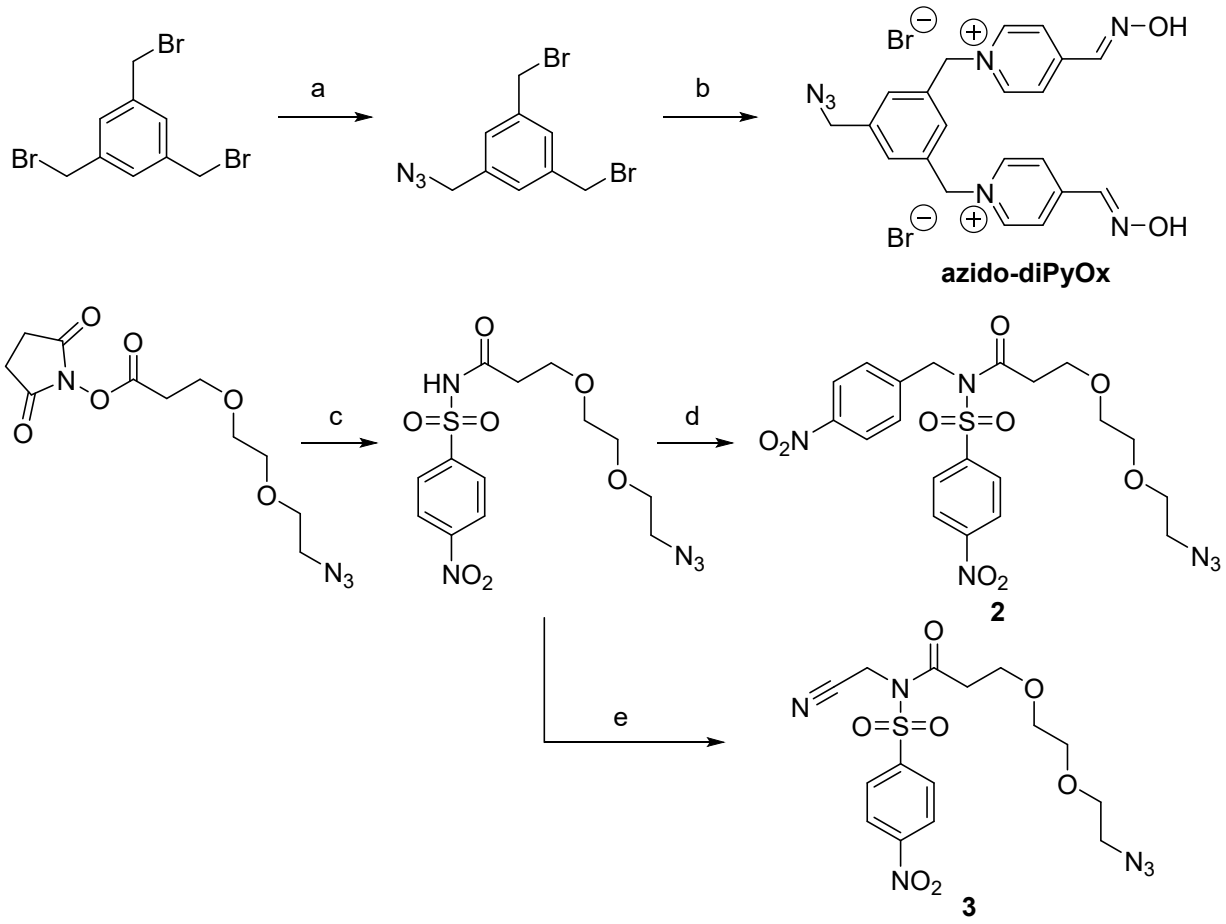


1,3,5-tris(azidomethyl)benzene (30 mg, 123 µmol, 1.0 eq), N-methyl-N-(prop-2-yn-1-yl)pyridin-4-amine (36 mg, 247 µmol, 2.0 eq) and diisopropylethylamine (107 µL, 617 µmol, 5.0 eq) were dissolved in acetonitrile 3 mL) and the mixture was bubbled with argon for 30 minutes. Tetrakis(acetonitrile)copper(I)hexafluoro-phosphate (230 mg, 617 µmol, 5.0 eq) was added and the reaction was stirred at rt overnight. The mixture was washed with 10% 3M NaOH in brine (5 mL) and extracted with EtOAc (3 × 10 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 8-12% (10% [25% NH_3 in H_2O] in MeOH) in DCM)) yielding a pale-yellow solid (7 mg, 11 %). HRMS (ESI) calculated for $[M+H]^+$: 536.2747; found $[M+H]^+$: 536.2740. ^{1}H NMR (400 MHz, CD_3CN) δ 8.14 (s, 4H), 7.70 (s, 2H), 7.19 (d, *J* = 1.7 Hz, 2H), 7.13 (d, *J* = 1.8 Hz, 1H), 6.75 (s, 4H), 5.49 (s, 4H), 4.66 (s, 4H), 4.35 (s, 2H), 3.08 (s, 6H) ppm. ^{13}C NMR (101 MHz, CD_3CN) δ 155.0, 148.6, 144.7, 138.5, 138.2, 128.5, 128.0, 123.7, 108.3, 54.5, 53.8, 47.4, 38.2 ppm.

2.1.5 azido-thioester (1):

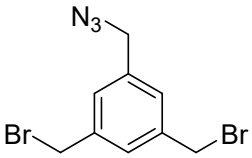


2,5-dioxopyrrolidin-1-yl 3-(2-(2-azidoethoxy)ethoxy)propano-ate (150 mg, 500 µmol, 1.03 eq) was dissolved in 2 mL toluene. Thiophenol (49 µL, 480 µmol, 1.0 eq) was added. Triethylamine (81 µL, 580 µmol, 1.2 eq) was added dropwise. The mixture was stirred at rt for 16 h. The reaction was quenched with brine (8 mL) and the product was extracted with EtOAc (3 × 6 mL). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 20% EtOAc in petroleum ether (40/60)), yielding a colourless oil (121 mg, 85%). HRMS (ESI) calculated for $[M+Na]^+$: 318.0888; found $[M+Na]^+$: 318.0877. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.35 (m, 5H), 3.81 (t, *J* = 6.3 Hz, 2H), 3.71–3.58 (m, 6H), 3.35 (t, *J* = 5.1 Hz, 2H), 2.92 (t, *J* = 6.3 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.3, 134.4, 129.4, 129.1, 127.6, 70.5, 70.5, 70.0, 66.6, 50.6, 43.9 ppm.



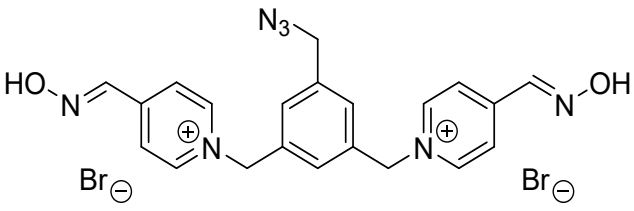
Scheme S2. Synthesis of the divalent catalysts. (a) NaN_3, DMF, rt, 16 h, ***57%****; (b) pyridine-4-aldoxime, ACN, 65°C, 32 h,* ***64%****; (c) 4-nitrobenzenesulfonamide, DIPEA, DCM, rt, 20 h,* ***90%****; (d) DIPEA, 1-(bromomethyl)-4-nitrobenzene, anh. THF, 50°C, 17 h,* ***72%****; (e) DIPEA, 2-bromoacetonitrile, anh. THF, 50°C, 20 h,* ***15%.***

2.1.6 1-(azidomethyl)-3,5-bis(bromomethyl)benzene:



1,3,5-tris(bromomethyl)benzene (30 mg, 84 µmol, 1.0 eq) and sodium azide (5.5 mg, 84 µmol, 1.0 eq) were dissolved in 100 µL DMF and stirred at rt for 16 h. The mixture was blow-dried and the residue dissolved in 400 µL DCM. The products were separated by means of preparative TLC (5% diethylether in petroleum ether (40-60)) and the desired product recovered with diethyl ether, filtered and concentrated under reduced pressure, yielding a white solid (15.1 mg, 57%). HRMS (ESI) calculated for $[M+H]^+$: 317.9241 / 319.9240; found $[M+H]^+$: 317.9318 / 319.9297. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 (s, 1H), 7.27 (d, *J* = 8.3 Hz, 2H), 4.46 (d, *J* = 7.5 Hz, 4H), 4.37 (s, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.3, 137.1, 129.6, 128.7, 54.3, 32.4 ppm.

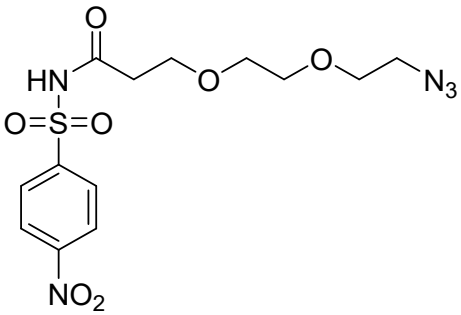
2.1.7 azido-diPyOx:



1-(azidomethyl)-3,5-bis(bromomethyl)benzene (8.0 mg, 25 µmol, 1.0 eq) and pyridine-4-aldoxime (15 mg, 125 µmol, 5 eq.) were dissolved in 500 µL ACN and the mixture was stirred at 65 °C for 32 h. The mixture was transferred to an Eppendorf tube, washing the

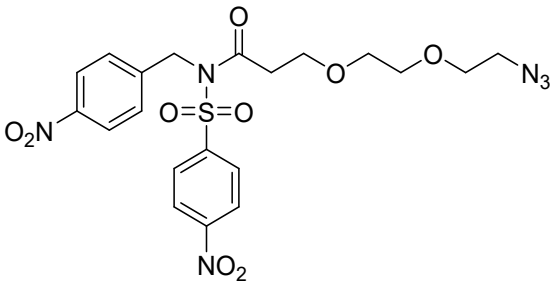
vessel with ACN and centrifuged with a tabletop centrifuge. The ACN was carefully removed and the solid washed with clean ACN (3 × 1 mL). The residue was allowed to dry overnight, yielding a brown solid (6.5 mg, 64%). HRMS (ESI) calculated for $[M–H]^+$: 402.1678; found $[M–H]^+$: 402.1678. ^{1}H NMR (400 MHz, D_2O) δ 8.86 (dt, *J* = 9.9, 4.8 Hz, 4H), 8.39 (d, *J* = 2.3 Hz, 2H), 8.25–8.19 (m, 4H), 7.55 (d, *J* = 2.8 Hz, 2H), 7.51 (s, 1H), 5.86 (d, *J* = 2.4 Hz, 4H), 4.49 (d, *J* = 2.3 Hz, 2H) ppm. ^{13}C NMR (101 MHz, D_2O) δ 149.3, 146.2, 144.8, 144.7, 138.7, 134.8, 129.9, 129.0, 125.0, 63.4, 53.3 ppm.

2.1.8 azido-ANANS precursor:



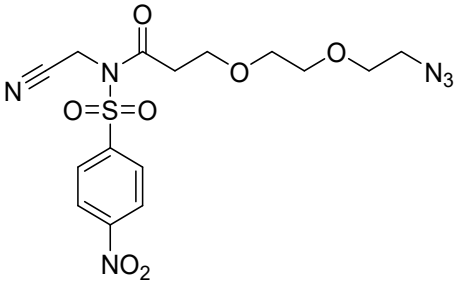
4-nitrobenzenesulfonamide (162 mg, 799 µmol, 1.2 eq) was dissolved in 1 mL DCM and *N,N*-diisopropylethylamine (232 µL, 1.33 mmol, 2.0 eq) was added to the mixture and stirred for 5 min. 2,5-dioxopyrrolidin-1-yl 3-(2-(2-azidoethoxy)ethoxy)propanoate (200 mg, 666 µmol, 1.0 eq) was added and the mixture was stirred at rt for 20 h. The reaction was washed with 1 M HCl (8 mL) and the product extracted with DCM (3 × 8 mL). The organic layer was washed with brine (25 mL) and the product extracted with DCM (2 × 15 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, starting with 3% [10% (25% NH_3 in H_2O) in MeOH] in DCM, then 5% MeOH in DCM), yielding a yellow oil (233 mg, 90%). HRMS (ESI) calculated for $[M+H]^+$: 410.0746; found $[M+H]^+$: 410.0730. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, *J* = 8.5 Hz, 2H), 8.16 (d, *J* = 8.4 Hz, 2H), 3.60 (dt, *J* = 12.1, 5.4 Hz, 8H), 3.45 (t, *J* = 4.9 Hz, 2H), 2.49 (t, *J* = 5.7 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.3, 149.8, 147.9, 128.7, 123.9, 70.3, 70.0, 69.7, 67.7, 50.7, 39.0 ppm.

2.1.9 azido-ANANS (2):



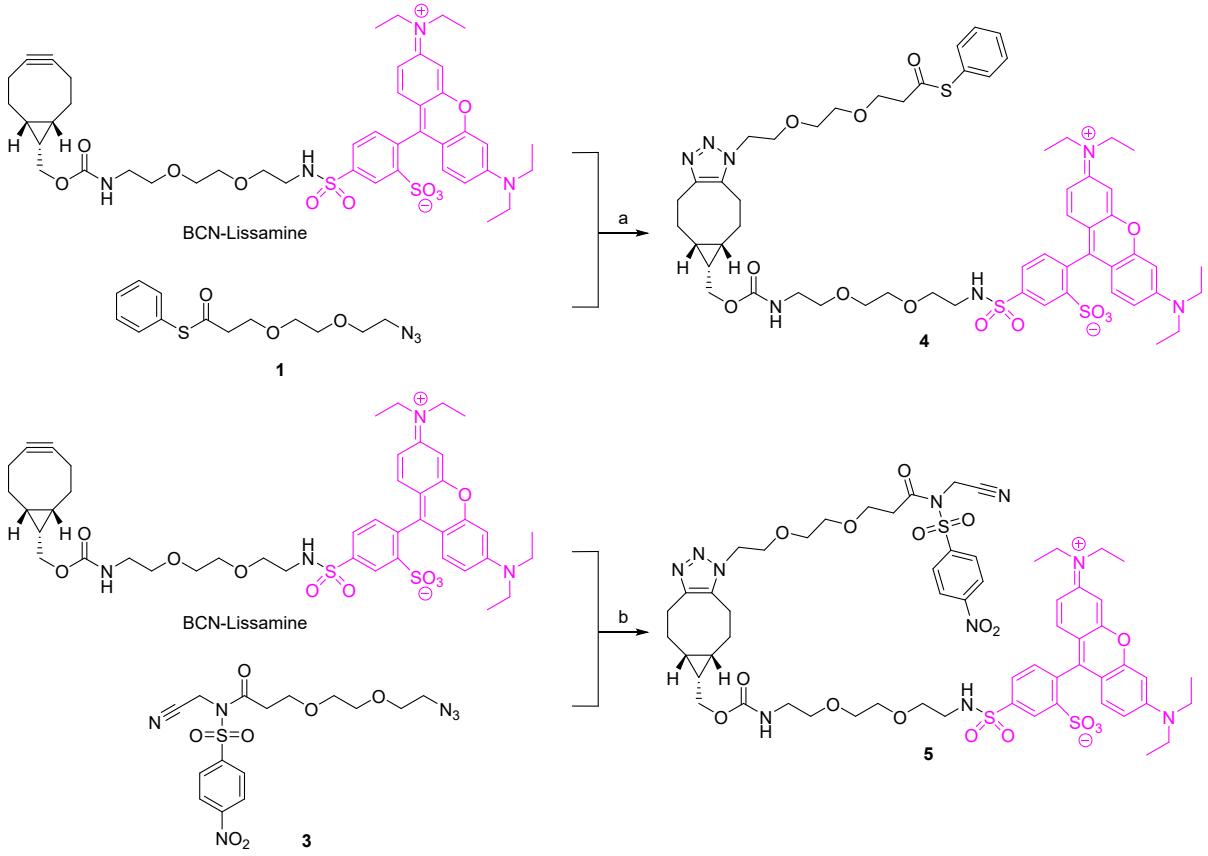
3-(2-(2-azidoethoxy)ethoxy)-*N*-((4-nitrophenyl)-sulfonyl)propanamide (120 mg, 310 µmol, 1.0 eq) was dissolved in 1 mL anh. THF. *N,N*-diisopropylethylamine (268 µL, 1.54 mmol, 5.0 eq) was added to the mixture and stirred at rt for 5 min. 1-(bromomethyl)-4-nitrobenzene (265 mg, 1.23 mmol, 4.0 eq) was dissolved in 0.7 mL anh. THF and added to the solution and the mixture was stirred at 50 °C for 17 h. The mixture was washed with brine (5 mL) and extracted with EtOAc (3 × 5 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 30–40% EtOAc in petroleum ether (40-60)) yielding a yellow oil (121 mg, 76%). HRMS (ESI) calculated for $[M+H]^+$: 545.1067; found $[M+H]^+$: 545.1049. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (d, *J* = 8.5 Hz, 2H), 8.23 (d, *J* = 8.4 Hz, 2H), 8.11 (d, *J* = 8.6 Hz, 2H), 7.55 (d, *J* = 8.3 Hz, 2H), 5.17 (s, 2H), 3.70 (t, *J* = 6.0 Hz, 2H), 3.62 (t, *J* = 4.9 Hz, 2H), 3.54 (q, *J* = 5.3, 4.2 Hz, 4H), 3.35 (t, *J* = 4.9 Hz, 2H), 2.84 (t, *J* = 6.0 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.4, 150.9, 147.8, 144.7, 143.3, 129.5, 128.4, 124.6, 124.2, 70.6, 70.1, 66.4, 50.8, 49.5, 37.0 ppm.

2.1.10 azido-ANANS (3):



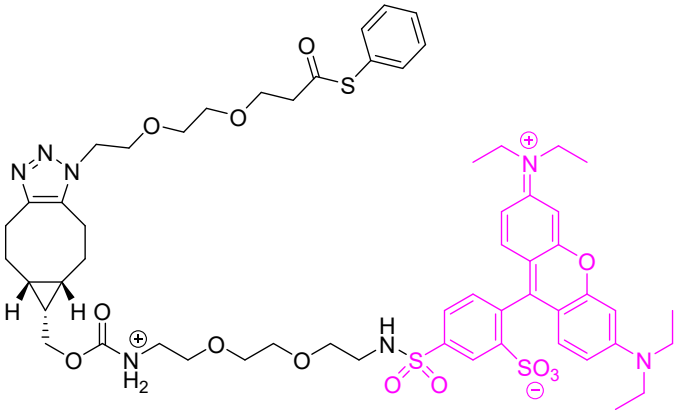
3-(2-(2-azidoethoxy)ethoxy)-N-((4-nitrophenyl)sulfonyl)-propanamide (120 mg, 310 µmol, 1.0 eq) was dissolved in 1 mL anhydrous THF and DIPEA (270 µL, 1.6 mmol, 5.0 eq) was

added to the mixture and stirred at RT for 5 min. 2-bromoacetonitrile (150 mg, 1.2 mmol, 4.0 eq) was dissolved in 0.7 mL anhydrous THF and added to the solution and the mixture was stirred at 50°C for 17 h. The mixture was washed with 1 M HCl (10 mL) and extracted with DCM (3x8 mL). The organic layer was washed with brine (20 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 30% -> 50% EtOAc in hexane) yielding an brownish oil (21 mg, 16%). HRMS (ESI) calculated for (M + Na+): 449.0855; found (M + Na+): 449.0865. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (d, *J* = 8.9 Hz, 2H), 8.23 (d, *J* = 9.0 Hz, 2H), 4.83 (s, 2H), 3.77 (t, *J* = 5.9 Hz, 2H), 3.63 (dd, *J* = 5.6, 4.3 Hz, 2H), 3.57 (m, 4H), 3.37 (t, *J* = 5.0 Hz, 2H), 2.96 (t, *J* = 5.9 Hz, 2H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.24, 151.27, 143.54, 129.65, 124.93, 114.49, 70.67, 70.52, 70.10, 66.32, 50.78, 36.97, 33.52 ppm.



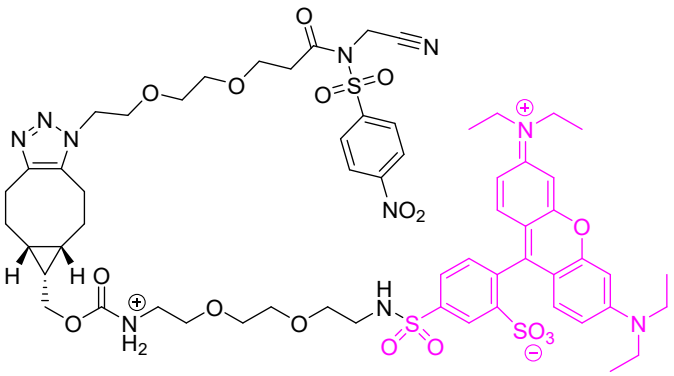
*Scheme S3. Synthesis of the fluorescent tag molecules: lissamine-thioester **4** and lissamine-ANCNS **5**. (a) DMSO, rt, 17 h, **quant.**; (b) DMSO, rt, 19 h, **quant.***

2.1.11 lissamine-thioester (**4**):



10 mM BCN-lissamine in DMSO (58 µL, 0.58 µM, 1.0 eq) and 10 mM azido-thioester **1** in DMSO (58 µL, 0.58 µM, 1.0 eq) were added to a 0.5 mL vial and stirred at r.t. for 19 h. The resulting mixture was analyzed with HPLC-MS to confirm >95% product formation and the subsequent solution was used. HRMS (ESI) calculated for (M + H+): 1160.4507; found (M + Na+): 1160.4519.

2.1.12 lissamine-ANANS (**5**):

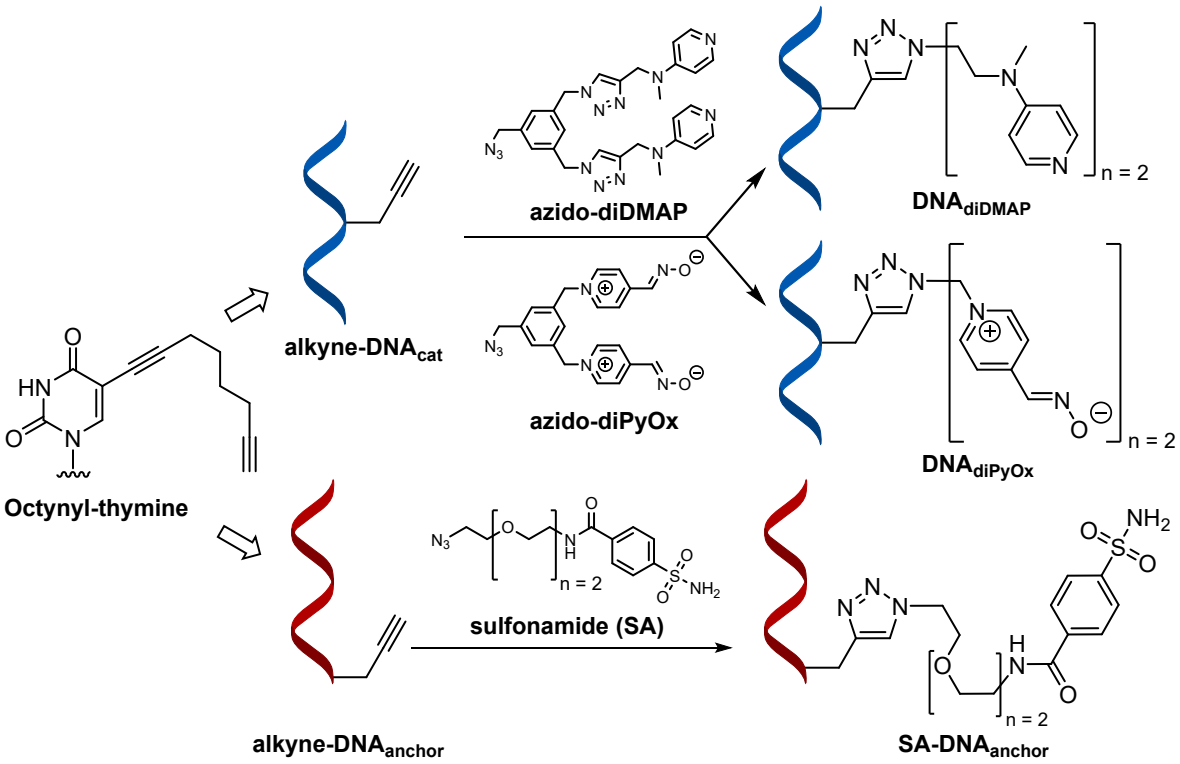


10 mM BCN-lissamine in DMSO (58 µL, 0.58 µM, 1.0 eq) and 10 mM azido-ANCNS **3** in DMSO (58 µL, 0.58 µM, 1.0 eq) were added to a 0.5 mL vial and stirred at r.t. for 19 h. The resulting mixture was analyzed with HPLC-MS to confirm >95% product formation and the subsequent solution was used. HRMS (ESI) calculated for (M + H+): 1291.4473; found (M + Na+): 1291.4447.

2.2 Synthesis of DNA constructs

2.2.1 Synthesis of DNA-catalyst constructs

DNA-alkyne sequences with alkyne-thymine modification were purchased as HPLC-purified lyophilized powders from Integrated DNA technologies. The powders were dissolved in oxygen-poor ddH_2O. The DNA was treated with 10 equivalents of compound azido-diDMAP or azido-diPyOx (from 100 mM stock in DMSO) with respect to the DNA concentration, 100 µM [Cu•THPTA] (complex of $CuSO_4$ and THPTA mixed in a ratio of 1:5 in ddH_2O) and 10 mM sodium ascorbate (from a freshly made stock of 100 mM in ddH2O) and incubated in the dark at 12 °C for 16–20 hours. The synthesized PMET-catalyst construct was purified by spin filtration over 3 kDa MWCO Amicon® Ultra-15 Centrifugal Filter Units, washing 3 times with 400 mM NaCl solution in ddH_2O. Purity and concentration were determined by HPLC-MS and UV-Vis.

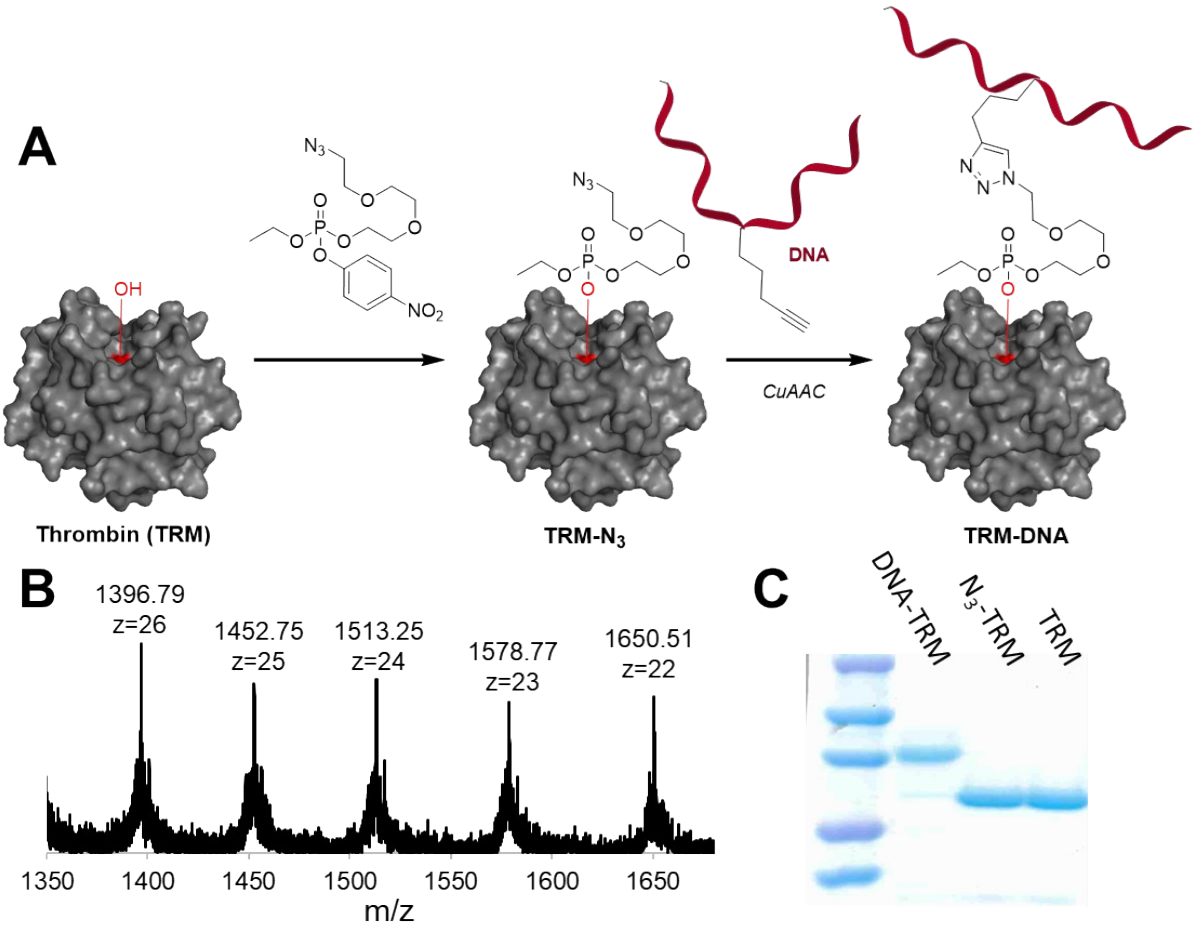


Scheme S4. Syntheses of $DNA_{catalyst}$ and DNA_{anchor} constructs. Alkyne-DNA_{cat} and azido-diDMAP or azido-diPyOx are coupled via copper-catalysed alkyne-azide click chemistry and purified via spin filtration. The same applies for the synthesis of the SA-DNA strand.

2.2.2 Synthesis of DNA-protein construct

Thrombin was incubated with azido-EG_2-paraoxon (20 eq.) in 20% glycerol in 50 mM HEPES (pH: 7.2) at 37 °C for 3 h and subsequently purified by spin filtration over 10 kDa MWCO Amicon® Ultra-15 Centrifugal Filter Units, washing 3 times with 20% glycerol in 50 mM NaCl solution in 25 mM Tris (pH: 8.0). The respective azido-protein was treated with 2–3 equivalents of DNA_{temp} (from 500 µM stock in oxygen-poor ddH_2O) with respect to the protein concentration, 100–150 µM [Cu•THPTA] (complex of $CuSO_4$ and THPTA mixed in a ratio of 1:5 in ddH_2O) and 7–10 mM sodium ascorbate (from a freshly made stock of 100 mM in oxygen-poor ddH_2O) and incubated in the dark at 12 °C for 16–20 hours. The

synthesized protein-DNAtemp constructs were purified by spin filtration over 10 kDa MWCO Amicon® Ultra-15 Centrifugal Filter Units, washing 3 times with 20% glycerol in 50 mM NaCl solution in 25 mM Tris (pH: 8.0). The formed TRM-DNA construct was purified by FPLC, using an ion-exchange MonoQ column (Vol: 1 mL) using a gradient from 0–1 M NaCl in 20 mM Tris (pH: 8.0). The collected fractions were and concentrated by spin filtration over 10 kDa MWCO Amicon® Ultra-15 Centrifugal Filter Units, washing 3 times with 50 mM NaCl solution in 25 mM Tris (pH: 8.0). The concentration of GRX-DNA_{temp} was quantified from absorption values determined with a ScientificTM Nanodrop 2000, using a 1:1 mixture of native TRM with DNA as a reference.



Scheme S5. (A) Synthesis of thrombin-DNA (TRM-DNA) by using a paraoxon derivative (B) Mass spectrometry data of TRM-EG_2-N_3. Calculated peaks: 1396.8 (z=26), 1452.7(z=25), 1513.1(z=24), 1578.9(z=23), 1650.6(z=22). (C) SDS-PAGE comparison of TRM, TRM-N_3 and TRM-DNA.

2.3 Protein Modification Studies

2.3.1 Protocol for modification of thrombin with DNA-bound diDMAP

A mixture was typically prepared containing 10 µM protein (from a 200 µM stock solution in 50% glycerol in ddH_2O), 10-30 µM DNA construct (from varying stock concentrations in ddH_2O) and in HEPES buffer [50 mM, pH=8.0, with 350 mM NaCl and 50 mM KCl]. This mixture was incubated in the dark for 60 min at 37 °C, after which acyl donor (from varying stock concentrations in DMSO) was added. The reaction mixture was again incubated in the dark at 37 °C, shaking the tubes at 500 rpm for 2 h. Prior to SDS-PAGE analysis, additional functionalization is required to visualize the modifications. See further on, section "*2.3.4 Protocol for SDS-PAGE analysis*".

These conditions were also used when performing the reaction in cell lysate, albeit with half the concentration of HEPES buffer replaced by Tris buffer [10 mM, pH=7.0, with 400 mM NaCl].

2.3.2 Protocol for modification of thrombin with DNA-bound diPyOx

A mixture was typically prepared containing 10 µM protein (from a 200 µM stock solution in 50% glycerol in ddH_2O), 10-30 µM DNA construct (from varying stock concentrations in ddH_2O) and in HEPES buffer [50 mM, pH=7.2, with 350 mM NaCl and 50 mM KCl]. This mixture was incubated in the dark for 60 min at 37 °C, after which acyl donor (from varying stock concentrations in DMSO) was added. The reaction mixture was again incubated in the dark at 37 °C, shaking the tubes at 500 rpm for 5-6 h. Prior to SDS-PAGE analysis, additional functionalization is required to visualize the modifications. See further on, section "*2.3.4 Protocol for SDS-PAGE analysis*".

These conditions were also used when performing the reaction in cell lysate, albeit with half the concentration of HEPES buffer replaced by Tris buffer [10 mM, pH=7.0, with 400 mM NaCl].

2.3.3 Protocol for switchable activity of DNA-bound acyl transfer catalyst

A mixture was typically prepared containing 10 µM thrombin (from a 200 µM stock solution in 50% glycerol in ddH_2O), 30 µM DNA construct (from varying stock concentrations in ddH_2O) and in HEPES buffer [50 mM, pH=8.0, with 350 mM NaCl and 50 mM KCl]. This mixture was incubated in the dark for 60 min at 37 °C. To switch OFF the activity of TBA-catalyst construct, 1.0 equivalents of TBA-OFF DNA was added after which the mixture was incubated at 37 °C for 15 min. Similarly, the TBA-catalyst construct was switched back ON by adding 1.5 equivalents (with respect to the original GQ concentration, *i.e.*, 1.25 equivalents with respect to the OFF strand) of TBA-ON DNA after which the mixture was incubated at 37 °C for 15 min. The acyl donor (from varying stock concentrations in DMSO) was added after each switching event, and the reaction mixture was incubated in the dark at 37 °C, shaking the tubes at 500 rpm. Reaction times were 2 h when using the DMAP catalyst and 5 h when using the PyOx catalyst. Switching can also be performed while the reaction is ongoing, which was demonstrated in Keijzer et al. *Chem. Commun.* **2021**, *57*, 12960-12963.

These conditions were also used when performing the reaction in cell lysate, albeit with half the concentration of HEPES buffer replaced by Tris buffer [10 mM, pH=7.0, with 400 mM NaCl].

2.3.4 Protocol for SDS-PAGE analysis

Prior to SDS-PAGE analysis, additional functionalization is required to visualize the modifications. Two approaches were used: band shifting or fluorescent staining.

- Band shifting: thrombin modified with an azide-carrying acyl donor was treated with 6 equivalents of BCN-PEG2000 (purchased from Synaffix B.V.) with respect to the concentration of acyl donor and incubated at 12 °C overnight.
- Fluorescent staining: Thrombin modified with an alkyne-carrying acyl donor was treated with 6 equivalents of azido-sulphorhodamine B (purchased from Tenova Chemicals B.V.) with respect to the acyl donor, 100 µM [Cu•THPTA] (complex of $CuSO_4$ and tris(3-hydroxypropyltriazolylmethyl)amine mixed in a ratio of 1:5 in ddH_2O) and 1 mM sodium ascorbate (from a freshly made stock of 10 mM in ddH_2O) and incubated at 12 °C overnight.

Acrylamide gels (12%) were prepared according to Bio-Rad bulletin 6201 protocol. Specifically, reaction mixtures containing 2–5 µg of protein were diluted with one volume equivalent of SDS-PAGE sample buffer (2×) containing 10% BME and incubated for 10 minutes at 95 °C. The denatured sample was then used for SDS-PAGE analysis (12% acrylamide gel). Precision Plus Protein™ Dual Color Standards was used as a reference protein ladder. After running, if one of the proteins was modified with a fluorophore, a UV-photo of the gel was taken. Gels were then stained using Coomassie brilliant blue (0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol and 40% demineralized water) by shaking gently for 0.5 hours, and destained with destaining solution (10% acetic acid, 50% methanol, and 40% demineralized water) by shaking gently for 1 hour. Afterwards, the destaining solution was replaced with H_2O and shaken gently overnight at room temperature. When the BCN-PEG2000 mass-tag was used, quantification was performed by integrating the intensity of the Coomassie stained bands of de SDS-PAGE gel using ImageJ software.

2.3.5 Protocol for the analysis of thrombin modification on HPLC(-MS)

The reaction mixture was aspirated three times with a pipette, after which 10 µL was added to an HPLC vial insert that already contained 10 µL of buffer (200 mM Citrate and 400 mM NaCl; pH: 5.5). The resulting mixture was also aspirated three times. This sample was then run over a Thermo Fischer MAbPAC RP column 3.0 × 100 mm, at 80 °C using for thrombin a gradient started with 23% (ACN + 0.1% FA) ending with 33% (ACN + 0.1% FA) in (95% H_2O + 5% ACN + 0.1% FA) (flow rate 0.5 mL/min) over 25 min. The system used was an Agilent 1220 Infinity LC system with DAD detector. For mass spectrometry analysis, reaction mixtures were diluted to a final protein concentration of 0.25 mg/mL. Protein samples were then analysed on a Thermo Scientific™ Q Exactive Focus Orbitrap using the same gradient as was used for the HPLC analyses.

2.3.6 Tryptic digestion of protein and subsequent analysis to determine site-specificity of the modification

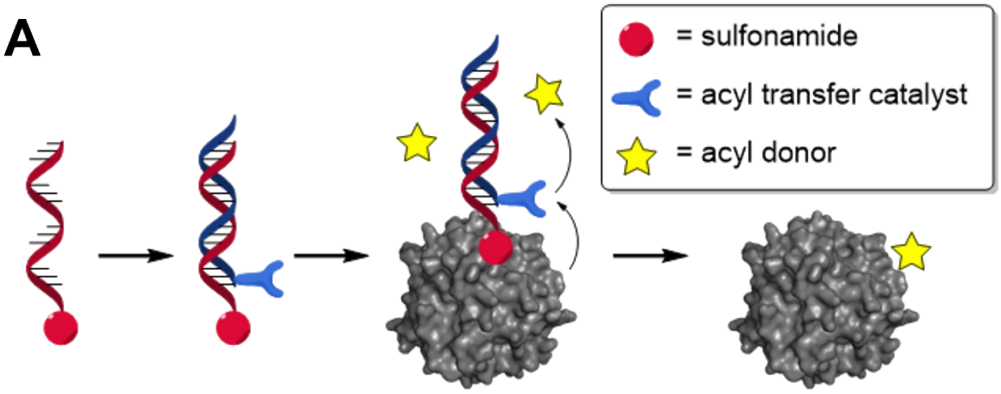
Modified protein samples were subjected to SDS-PAGE separation and the desired protein bands cut from the gel and cut up to small pieces. The pieces were washed by incubating three times with 50 mM NH_4HCO_3 (pH: 8.0) in 50% ACN in ddH_2O and subsequently dried in a Speedyvac vacuum centrifuge. The dry pieces were swollen in 50 µL DTT [10 mM in 100 mM NH_4HCO_3 (pH: 8.0)] and incubated for 45 minutes at 56 °C. The supernatant was removed and 50 µL of IAA (55 mM in 100 mM NH_4HCO_3 (pH: 8.0)) was added and the pieces were incubated in the dark at rt for 30 min. The supernatant was removed and the pieces were washed by incubating once with 50 mM NH_4HCO_3 (pH: 8.0) in 50% ACN in ddH_2O and subsequently dried in a vacuum centrifuge. The gel pieces were swollen in 40 µL trypsin gold (125 ng/µL) and incubated at 37 °C for 16-18 h. The initial supernatant was collected and the gel pieces were washed by incubating 15 min at 37 °C with 20 µL NH_4HCO_3 (100 mM,

pH: 8.0) and 15 min at 37 °C when diluted with 20 µL. The collected supernatants were combined and dried in a vacuum centrifuge and the dry peptide digest dissolved in 20 µL 0.1% FA.

Peptide digests were analysed on an EASY nanoLC connected to Thermo Scientific™ Q Exactive PLUS. Peptides were trapped onto a PepSep trap column (2 cm × 100 µm ID, 5 µm C18 ReproSil) and subsequently separated on a PepSep analytical column (8 cm × 75 µm ID, 3 µm C18 ReproSil, PepSep). Elution was achieved using a gradient that started with 5% (ACN + 0.1% FA) ending with 40% (ACN + 0.1% FA) in (H_2O + 0.1% FA), washing the column with 80% (ACN + 0.1% FA) afterwards. The eluted peaks were analysed using MaxQuant software, searching for peptides with mass modification corresponding to H(11)O(3)C(7)N(3) (*i.e.*, the substitution of a proton on the protein by the acyl group of thioester **1** or ANANS **3**) and limiting criteria of 1% PSM FDR and a minimal peptide score of 80. As protein database human proteome was used, obtained from www.unitprot.org (code: UP000005640).

3.1 Primary supporting data

3.1.1 Sulfonamide-guided modification of CA2:



B

Lane#	1	2	3	4	5	6	7
SA-DNA	-	x	x	-	x	x	-
DNA-dP	-	-	x	-	-	x	-
Conv%	**-**	**-**	**-**	**8**	**6**	**44**	**-**

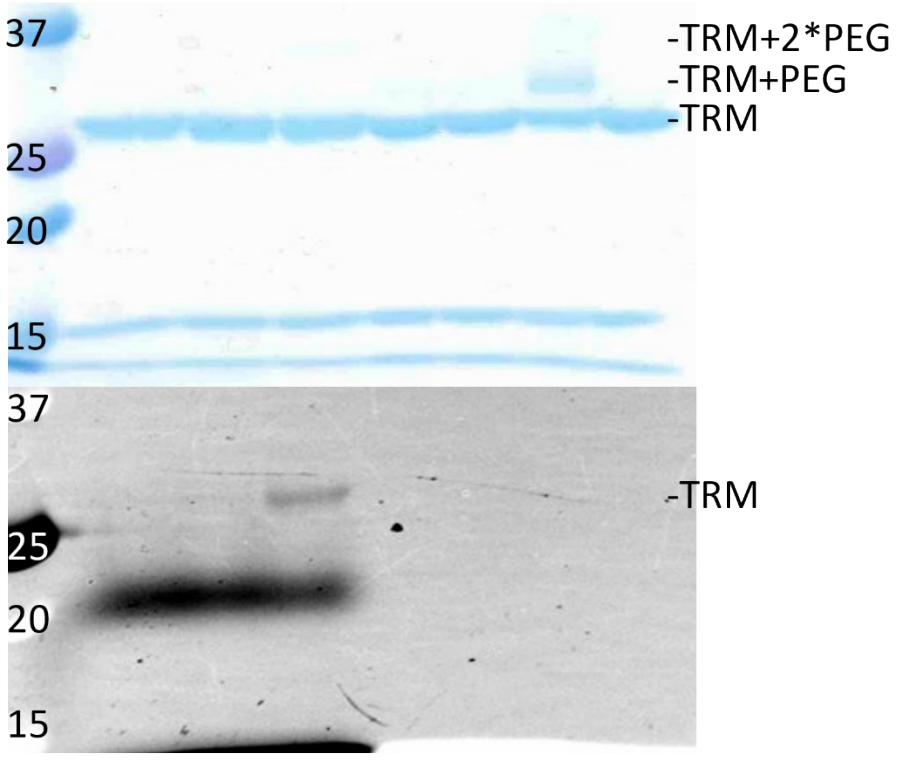


*Figure S1. (**A**) Schematic of how the DNA-bound CA2 inhibitor guides the DNA-bound catalyst and results in modification. (**B**) CA2 modified by SA-DNA/DNA$_{diPyOx}$ system with ANANS **2** and afterwards subjected to SPAAC. Lane 1-3: SPAAC with BCN-lissamine. Lane 4-6: SPAAC with BCN PEG$_{2000}$. Conversions are normalized and were calculated using ImageJ. The numbers on the left indicate the mass of the marker proteins in kDa.*

3.1.2 DNA tower crane

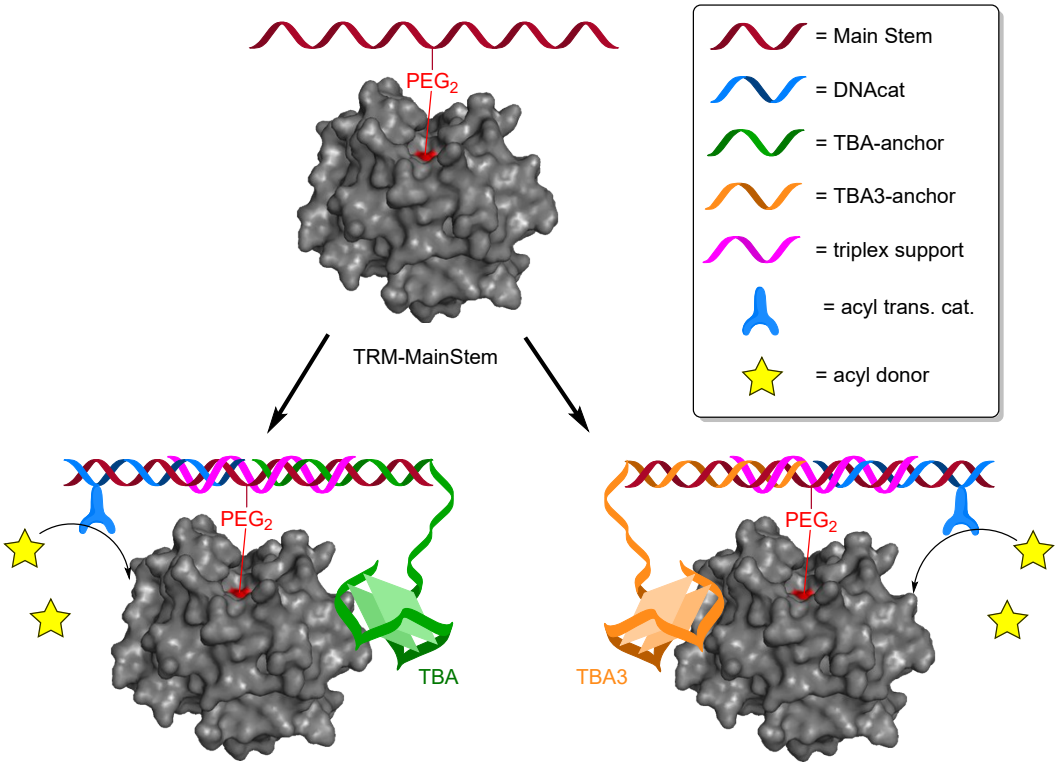


Figure S2 Schematic of the DNA Tower Crane. The Main Stem is attached via the covalent thrombin inhibitor that was attached to the 13th nucleobase. The DNA sequence of the main stem was designed such that it can pair with two additional strands: the catalyst-carrying strand DNAcat and an aptamer-anchor-carrying strand. The type of aptamer anchor determines the orientation and consequently the site of modification.

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lig-T5-TBA	x	x	x	x	-	-	-	-	-	-	-	-	TBA^{12}	-
Lig-T5-TBA3	-	-	-	-	x	x	x	x	-	-	-	-	-diPy	-
DNA_{dP} (5'e)	x	-	x	-	x	-	x	-	x	-	x	-	-	-
DNA_{dP} (T6)	-	x	-	x	-	x	-	x	-	x	-	x	-	-
Triplex Supp	x	x	-	-	x	x	-	-	-	-	-	-	-	-
Conv%	**13**	**26**	**14**	**31**	**13**	**27**	**13**	**28**	**28**	**28**	**7**	**3**	**56**	**0**

*Figure S3 TRM-MainStem modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor, a triplex support and/or a DNA_{diPyOx} strand. TBA^{12}-diPyOx was used as positive control. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h. Incubation time to assemble system: 2 h.*

Lane#	1	2	3	4	5	6	7	8	9	10	11
Lig-T3-TBA	x	-	-	-	-	-	-	-	-	-	TBA^{12}
Lig-T5-TBA	-	x	-	-	-	-	-	x	-	-	-diPy
Lig-T7-TBA	-	-	x	-	-	-	-	-	-	-	-
Lig-T3-TBA3	-	-	-	x	-	-	-	-	-	-	-
Lig-T5-TBA3	-	-	-	-	x	-	-	-	-	-	-
Lig-T7-TBA3	-	-	-	-	-	x	-	-	-	-	-
DNA_{dP}(T6)	x	x	x	x	x	x	x	-	x	-	-
Conv%	**14**	**17**	**14**	**14**	**15**	**16**	**14**	**28**	**6**	**1**	**69**

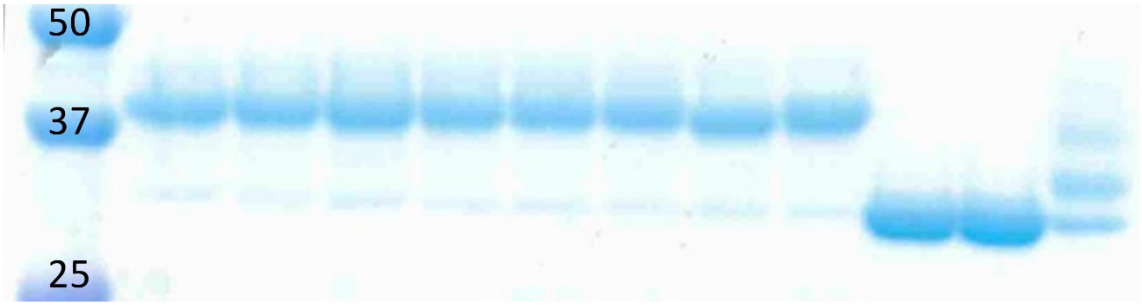


*Figure S4. TRM-MainStem modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor and/or a DNA_{diPyOx} strand. TBA^{12}-diPyOx was used as positive control. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h. Incubation time to assemble system: 2 h.*

Lane#	1	2	3	4	5	6	7
Lig-T7-TBA	x	x	-	-	-	-	-
Lig-T7-TBA3	-	-	x	x	-	-	-
DNA_{dP} (5'e)	x	-	x	-	x	-	-
DNA_{dP} (T6)	-	x	-	x	-	x	-
Conv%	**11**	**12**	**11**	**20**	**12**	**12**	**4**

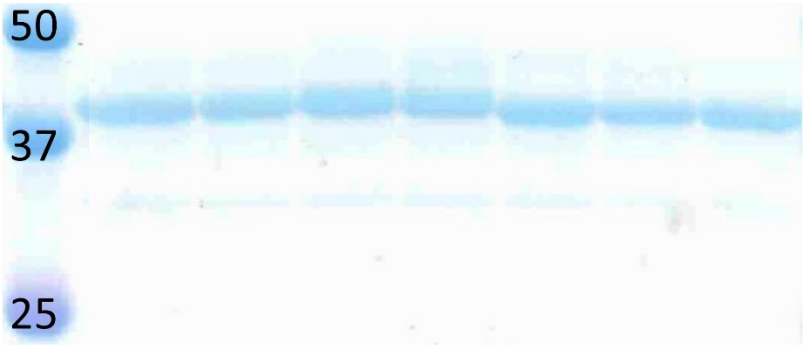


*Figure S5. TRM-MainStem modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor and/or a DNA_{diPyOx} strand. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h. Incubation time to assemble system: 2 h.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lig-T5-TBA	x	x	-	-	-	-	x	x	-	-	-	-	x	-
Lig-T5-TBA3	-	-	x	x	-	-	-	-	x	x	-	-	-	x
DNA_{dP} (5'e)	x	-	x	-	x	-	x	-	x	-	x	-	-	-
DNA_{dP} (T6)	-	x	-	x	-	x	-	x	-	x	-	x	-	-
Inc. time (h)	14	14	14	14	14	14	13+1	13+1	13+1	13+1	13+1	13+1	14	14
Conv%	**7**	**14**	**6**	**27**	**14**	**15**	**7**	**13**	**6**	**24**	**5**	**12**	**3**	**2**

*Figure S6. TRM-MainStem modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor and/or a DNA_{diPyOx} strand. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h. Incubation time to assemble system: 14 h or 13 h without DNA_{diPyOx} and 1 h with DNA_{diPyOx}.*

Lane#	1	2	3	4	5	6	7
Lig-T7-TBA	x	-	-	-	-	x	-
Lig-T7-TBA3	-	x	x	-	-	-	x
DNA_{dD} (5'e)	-	x	-	x	-	-	-
DNA_{dD} (T6)	x	-	x	-	x	-	-
Conv%	**23**	**37**	**19**	**42**	**24**	**31**	**10**

*Figure S7. TRM-MainStem modified by DNA_{diDMAP} with thioester **1** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor and/or a DNA_{diDMAP} strand. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diDMAP} and 200 µM thioester **1**, pH: 8.0, at 37 °C for 2 h. Incubation time to assemble system: 14 h.*

3.1.3 DNA tower crane selectivity

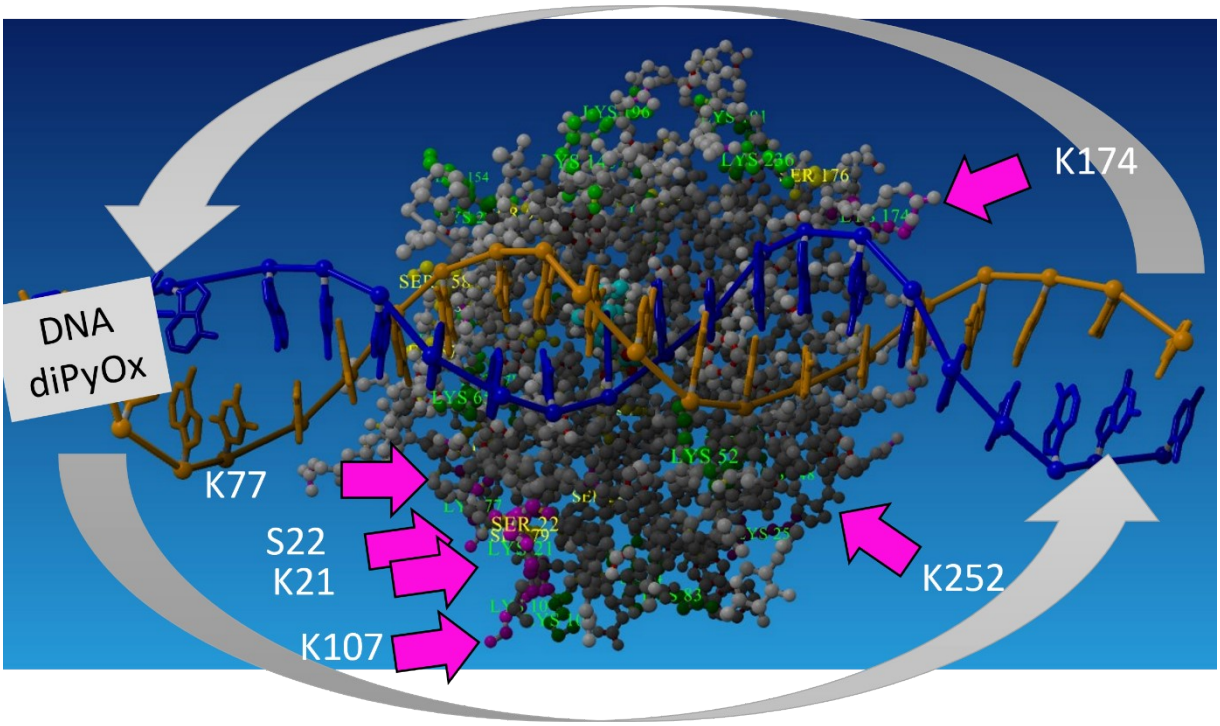


Figure S8. Acylated residues with TRM-MainStem + DNA_{diPyOx}(5'end). A 3D schematic of the tower crane with a duplex (orange/blue) on top and the catalyst (grey box) on the 5'end of DNA_{diPyOx}, without an aptamer anchor, able to freely rotate. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows.

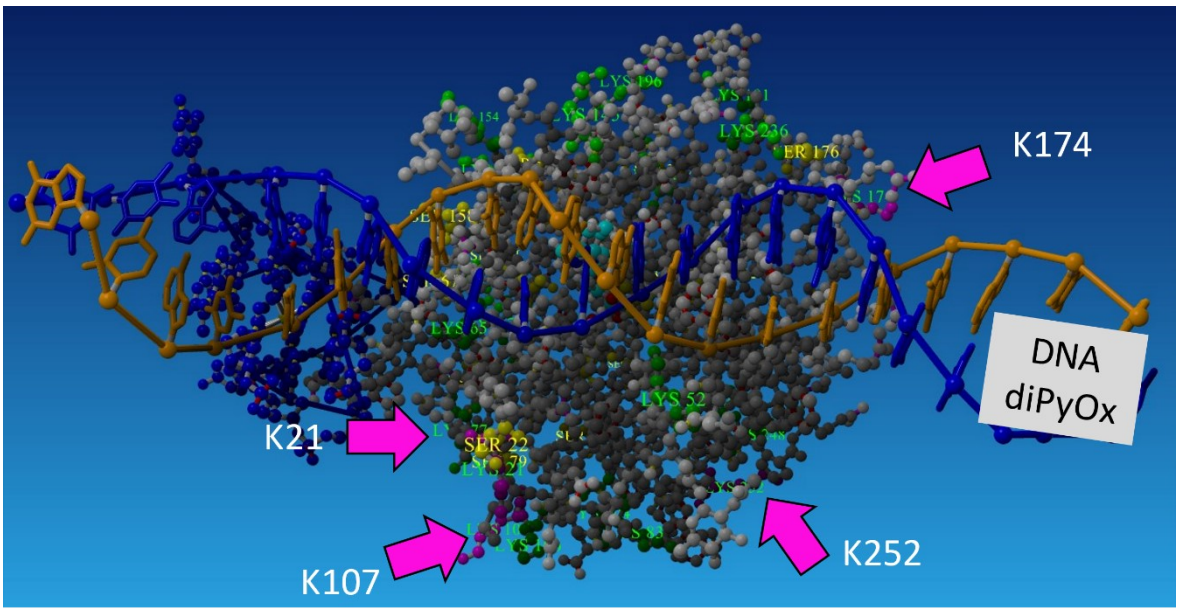


Figure S9. Acylated residues with TRM-MainStem + DNA_{diPyOx}(5'end) + Lig-T7-TBA. A 3D schematic of the tower crane with a duplex (orange/blue) on top, TBA2 (blue) on the righthand side and the catalyst on the left (grey box) on the 5'end of DNA_{diPyOx}. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows.

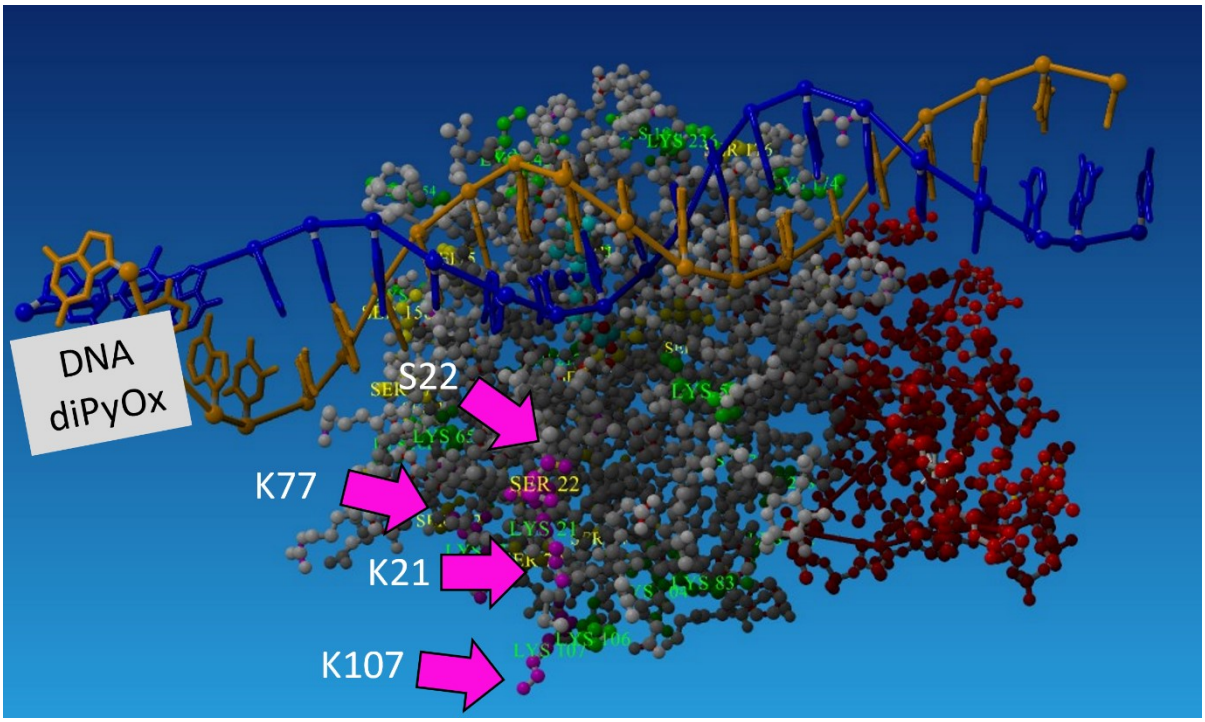


Figure S10. Acylated residues with TRM-MainStem + DNA_{diPyOx}(5'end) + Lig-T7-TBA3. A 3D schematic of the tower crane with a duplex (orange/blue) on top, TBA3 (red) on the righthand side and the catalyst on the left (grey box) on the 5'end of DNA_{diPyOx}. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows. The modification appears localized on the opposite site of the TBA3 binding site.

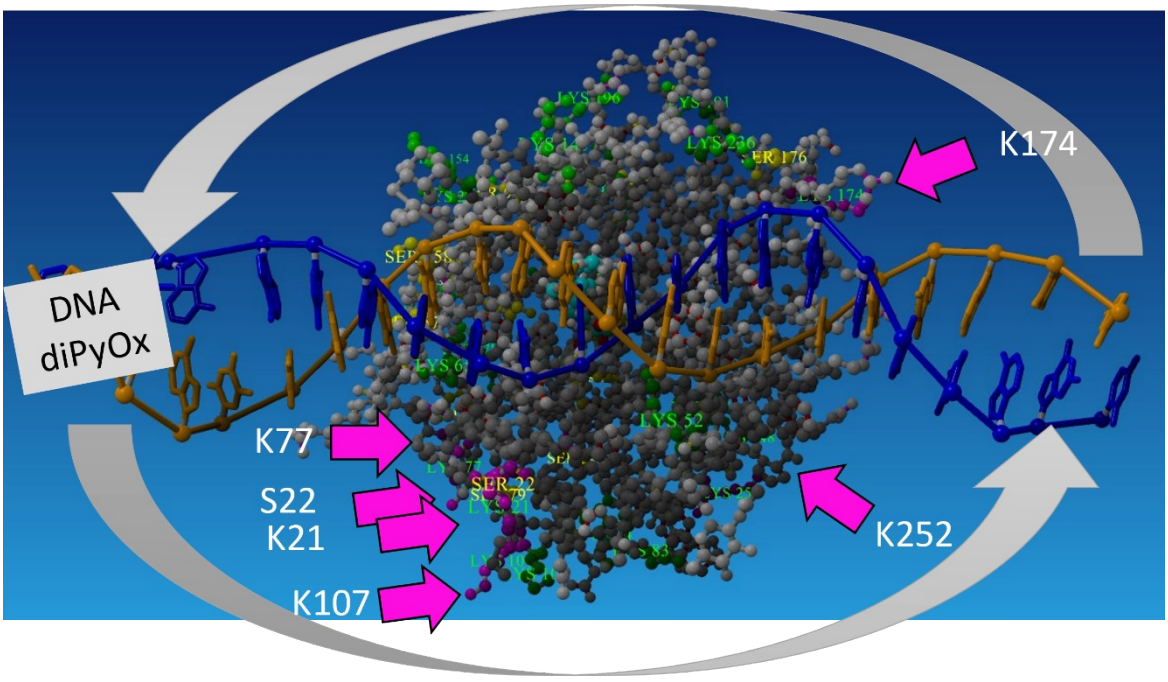


Figure S11. Acylated residues with TRM-MainStem + DNA_{diPyOx}(T6). A 3D schematic of the tower crane with a duplex (orange/blue) on top and the catalyst (grey box) on the 5'end of DNA_{diPyOx}, without an aptamer anchor, able to freely rotate. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows.

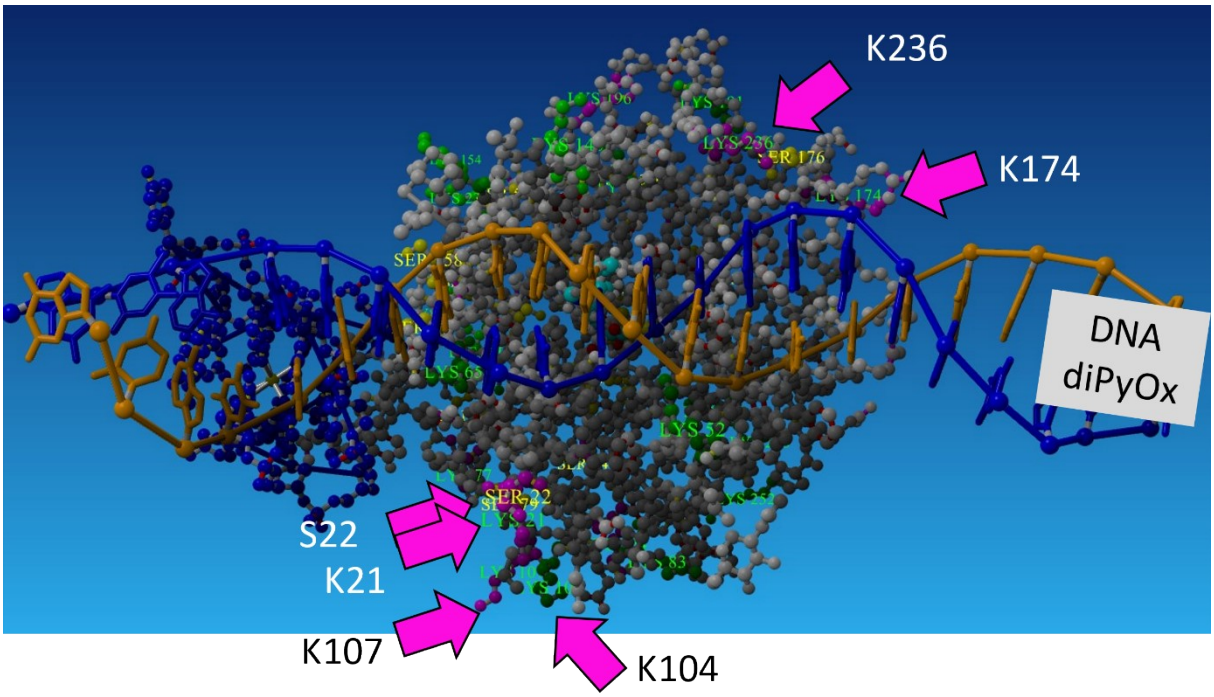


Figure S12. Acylated residues with TRM-MainStem + DNA$_{diPyOx}$(T6) + Lig-T7-TBA. A 3D schematic of the tower crane with a duplex (orange/blue) on top, TBA2 (blue) on the righthand side and the catalyst on the left (grey box) on the 5'end of DNA$_{diPyOx}$. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows.

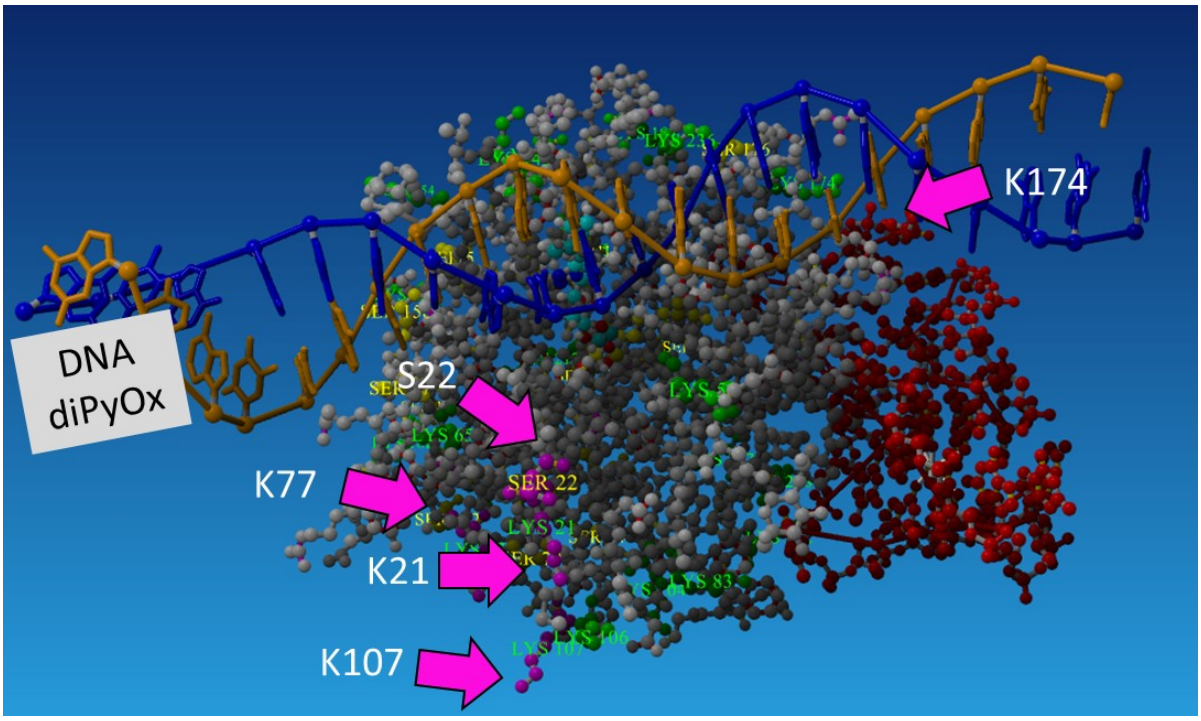


Figure S13. Acylated residues with TRM-MainStem + DNA$_{diPyOx}$(T6) + Lig-T7-TBA3. A 3D schematic of the tower crane with a duplex (orange/blue) on top, TBA3 (red) on the righthand side and the catalyst on the left (grey box) on the 5'end of DNA$_{diPyOx}$. Of all the targetable residues (Lys in green/Ser in yellow), the modified ones (pink) are indicated by pink arrows.

3.1.4 DNA gantry crane

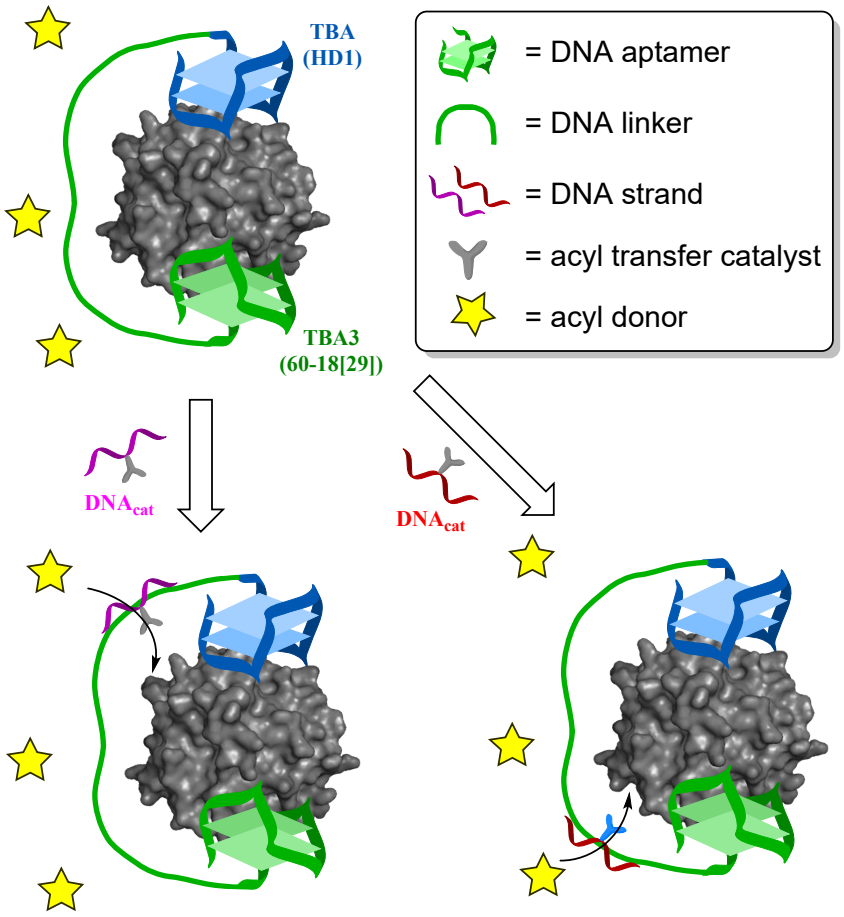


Figure S14. Schematic of the DNA Gantry Crane. The DNA nanostructure (green) binds with aptamers TBA and TBA3 on either side of thrombin. The DNA linker that connects the two aptamers then functions as the attachment point for a second catalyst-bearing strand (blue/red). By designing the sequence of the DNA linker, different strands can hybridize at different sites along the linker and thereby modify different sites of the protein.

Lane#	1	2	3	4	5	6	7	8	9	10*	11	12	14	14
DNA_{dp}(5'e)	x	x	x	x	x	x	x	x	x	x	x	x	-	TBA^{12}
Code	5Y5	6Y6	7Y7	11Y3	3Y11	8Y8	11Y5	5Y11	9Y9	12Y6	6Y12	-	-	-diPy
Conv%	**5**	**9**	**8**	**4**	**9**	**9**	**5**	**13**	**9**	**6**	**10**	**2**	0	**57**

*Figure S15. Thrombin modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The code indicates the type of DNA anchor that was used, where Y is the binding sequence for DNA_{diPyOx} (catalyst positioned on 5' end) and the numbers on represent the total nucleobases on either side of that sequence. TBA^{12}-diPyOx was used as positive control. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h.*

Lane#	1	2	3	4	5	6	7	8	9	10*	11	12	14	14
DNA_{dP} (T6)	x	x	x	x	x	x	x	x	x	x	x	x	-	TBA^{12}
Code	5Y5	6Y6	7Y7	11Y3	3Y11	8Y8	11Y5	5Y11	9Y9	12Y6	6Y12	-	-	-diPy
Conv%	**14**	**16**	**17**	**12**	**13**	**16**	**11**	**15**	**9**	**12**	**14**	**1**	0	**56**

*Figure S16. Thrombin modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The code indicates the type of DNA anchor that was used, where Y is the binding sequence for DNA_{diPyOx} (catalyst positioned on T6) and the numbers on represent the total nucleobases on either side of that sequence. TBA^{12}-diPyOx was used as positive control. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 5 h.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	12	14
DNA_{dP} (5'e)	x	x	x	-	-	-	-	-	-	-	-	-	-	TBA^{12}
DNA_{dP} (T3)	-	-	-	x	x	x	-	-	-	-	-	-	-	-diPy
DNA_{dP} (T6)	-	-	-	-	-	-	x	x	x	-	-	-	x	-
DNA_{dP} (T10)	-	-	-	-	-	-	-	-	-	x	x	x	-	-
Code	5Y11	8Y8	11Y5	5Y11	8Y8	11Y5	5Y11	8Y8	11Y5	5Y11	8Y8	11Y5	-	-
Conv%	**11**	**9**	**9**	**24**	**19**	**11**	**21**	**21**	**11**	**12**	**11**	**0**	**0**	**80**

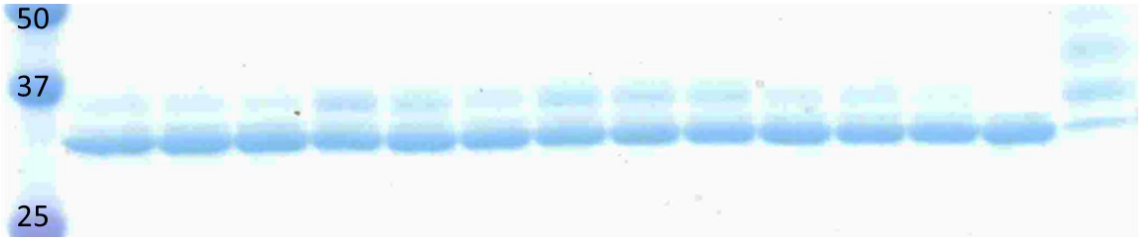


*Figure S17. Thrombin modified by DNA_{diPyOx} with ANANS **2** and afterwards PEGylated with BCN-PEG_{2000}. The code indicates the type of DNA anchor that was used, where Y is the binding sequence for DNA_{diPyOx} (catalyst positioned on ##) and the numbers on represent the total nucleobases on either side of that sequence. TBA^{12}-diPyOx was used as positive control. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diPyOx} and 300 µM ANANS **2**, pH: 7.2, at 37 °C for 8 h.*

Lane#	1	2	3	4	5	6	7
5Y11	x	x	-	-	-	-	-
8Y8	-	-	x	x	-	-	-
11Y5	-	-	-	-	x	x	-
DNA_{dD} (5'e)	x	-	x	-	x	-	-
DNA_{dD} (T6)	-	x	-	x	-	x	x
Conv%	**0**	**0**	**0**	**0**	**0**	**0**	**0**

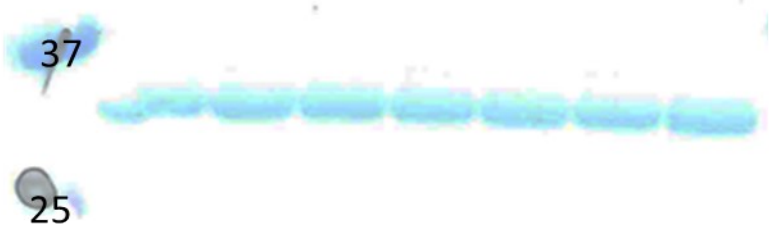


*Figure S18. Thrombin modified by DNA_{diDMAP} with thioester **1** and afterwards PEGylated with BCN-PEG_{2000}. The modification was performed with(out) an aptamer anchor and/or a DNA_{diDMAP} strand. Conversions are normalized and were calculated using ImageJ. Conditions: 10 µM TRM, 30 µM DNA_{diDMAP} and 200 µM thioester **1**, pH: 8.0, at 37 °C for 2 h. Incubation time to assemble system: 14 h.*

3.1.5 DNA gantry crane selectivity:

*Table S1. The position(s) of the modification(s) resulting from different DNA Gantry Crane systems. The code works as follows: the letter Y is the binding sequence for DNA_{diPyOx} and the numbers on represent the total nucleobases on either side of that sequence. The /code indicates the position of the catalyst on the DNA_{diPyOx} strand. The red X highlights modifications that are background acylation. The data in the highlighted column is described in detail in the main text and shown in Figure 3F. Conditions: 10 µM thrombin, 30 µM catalyst, 300 µM azido-ANANS **2**, 37 °C, 6 h.*

		PDB	backg	11Y5/T6	5Y11/T6	8Y8/T6	8Y8/5'	8Y8/T3	8Y8/T10
LC	S			X	X	X	X	X	X
LC	K	9		X	X				
LC	K	10		X	X		X		
	K	36	X	X	X	X	X	X	X
	S	36A	X	X	X	X	X		X
	K	81		X	X	X	X	X	X
	K	87					X		
	K	109		X	X	X	X	X	
	K	110	X	X	X	X	X	X	X
	K	135					X	X	X
	K	145	X	X	X	X	X	X	X
	S	153		X		X	X	X	
	K	169	X	X	X	X	X	X	X
	K	185		X	X				X
	K	186D		X	X	X	X	X	X
as	S	195			X			X	X
	K	224		X	X	X		X	X
	K	240	X						

3.1.6 Cell Lysate results

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TBA^{12}-dPy	x	-	-	-	-	-	-	x	-	-	-	-	-	-
8Y8	-	x	-	-	-	-	-	-	x	-	-	-	-	-
DNA_{dPy} (T6)	-	x	x	-	-	-	-	-	x	x	-	-	-	-
SA-DNA	-	-	-	-	x	-	-	-	-	-	-	x	-	-
CatDNA-dPy	-	-	-	-	x	x	-	-	-	-	-	x	x	-
Target prot.	TRM	TRM	TRM	TRM	CA2	CA2	CA2	TRM	TRM	TRM	TRM	CA2	CA2	CA2
5 (uM)	100	100	100	100	100	100	100	500	500	500	500	500	500	500

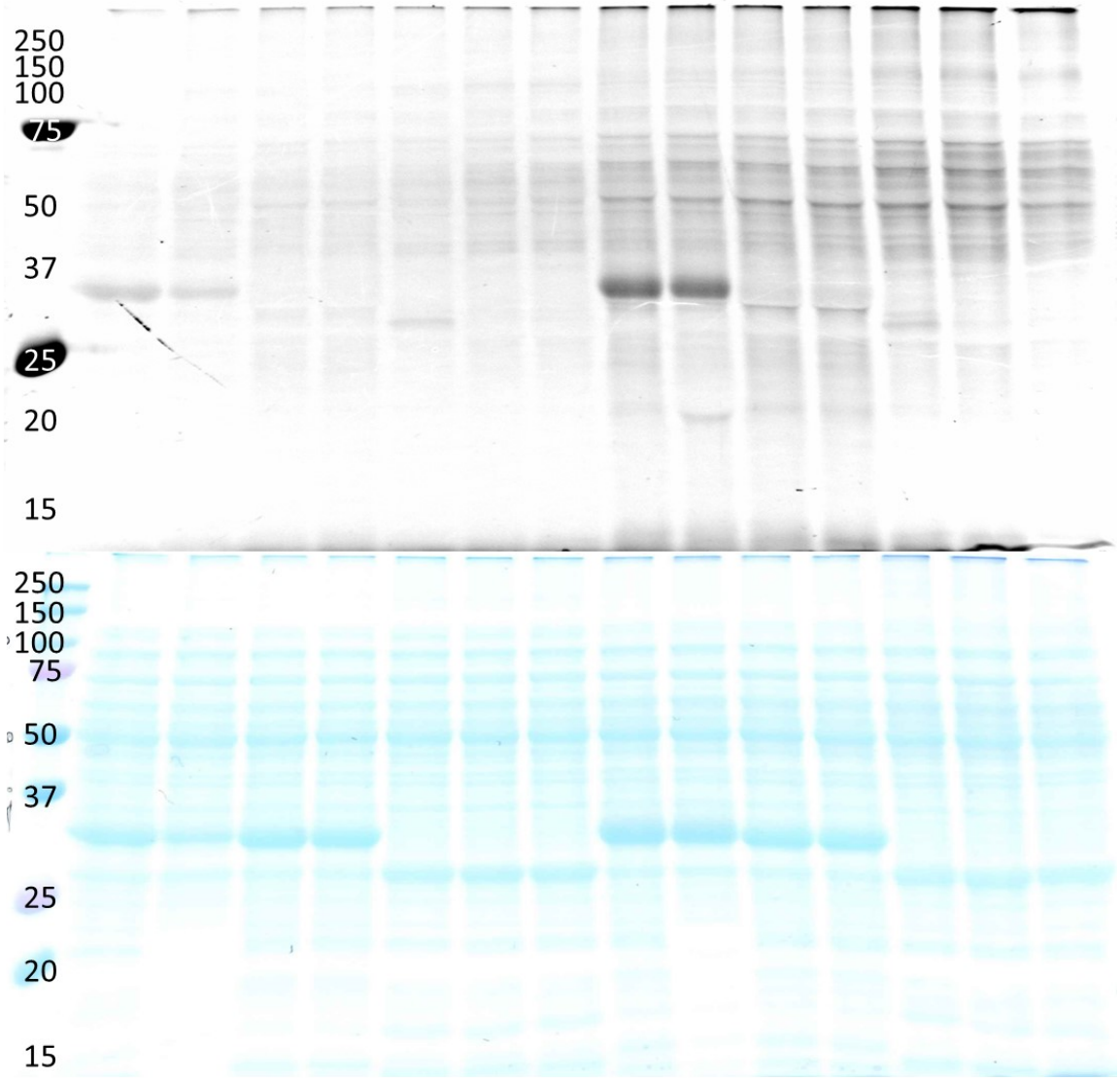


*Figure S19. Modification of TRM and CA2 modified in cell lysate (E.Coli) by DNA_{diPyOx} with ANANS **5**. Conditions: 10 µM TRM/CA2 and 30 µM DNA_{diDMAP}, pH: 7.2, at 37 °C for 6 h.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
TBA^{12}-dDM	x	-	-	-	-	-	-	x	-	-	-	-	-	-
8Y8	-	x	-	-	-	-	-	-	x	-	-	-	-	-
DNA_{dD} (T6)	-	x	x	-	-	-	-	-	x	x	-	-	-	-
SA-DNA	-	-	-	-	x	-	-	-	-	-	-	x	-	-
CatDNA-dD	-	-	-	-	x	x	-	-	-	-	-	x	x	-
Target prot.	TRM	TRM	TRM	TRM	CA2	CA2	CA2	TRM	TRM	TRM	TRM	CA2	CA2	CA2
4 (uM)	100	100	100	100	100	100	100	500	500	500	500	500	500	500

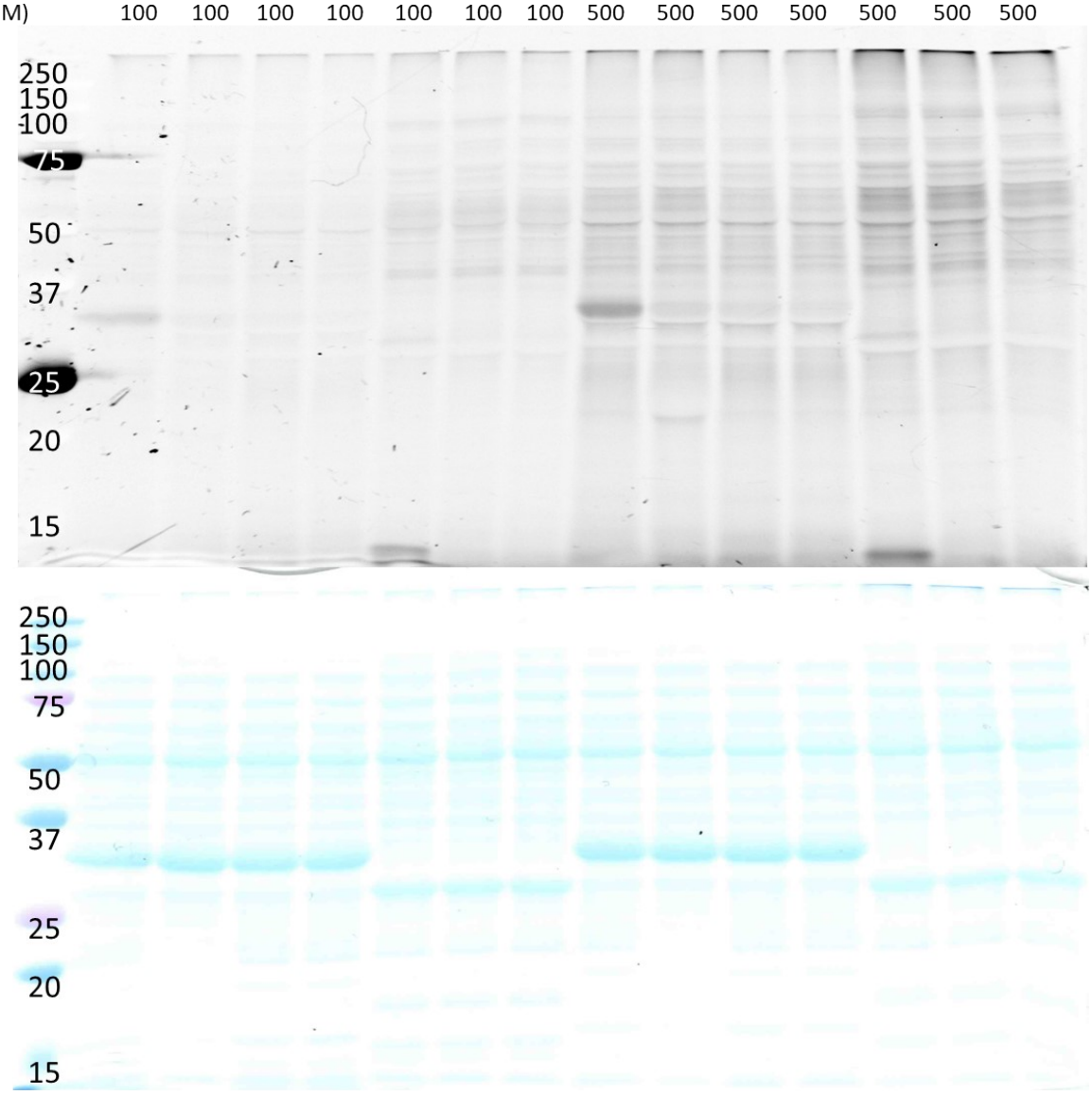


*Figure S20. Modification of TRM and CA2 in cell lysate (E. coli) by DNA_{diDMAP} with thioester **4**. Conditions: 10 µM TRM/CA2 and 30 µM DNA_{diDMAP}, pH: 8.0, at 37 °C for 2 h.*

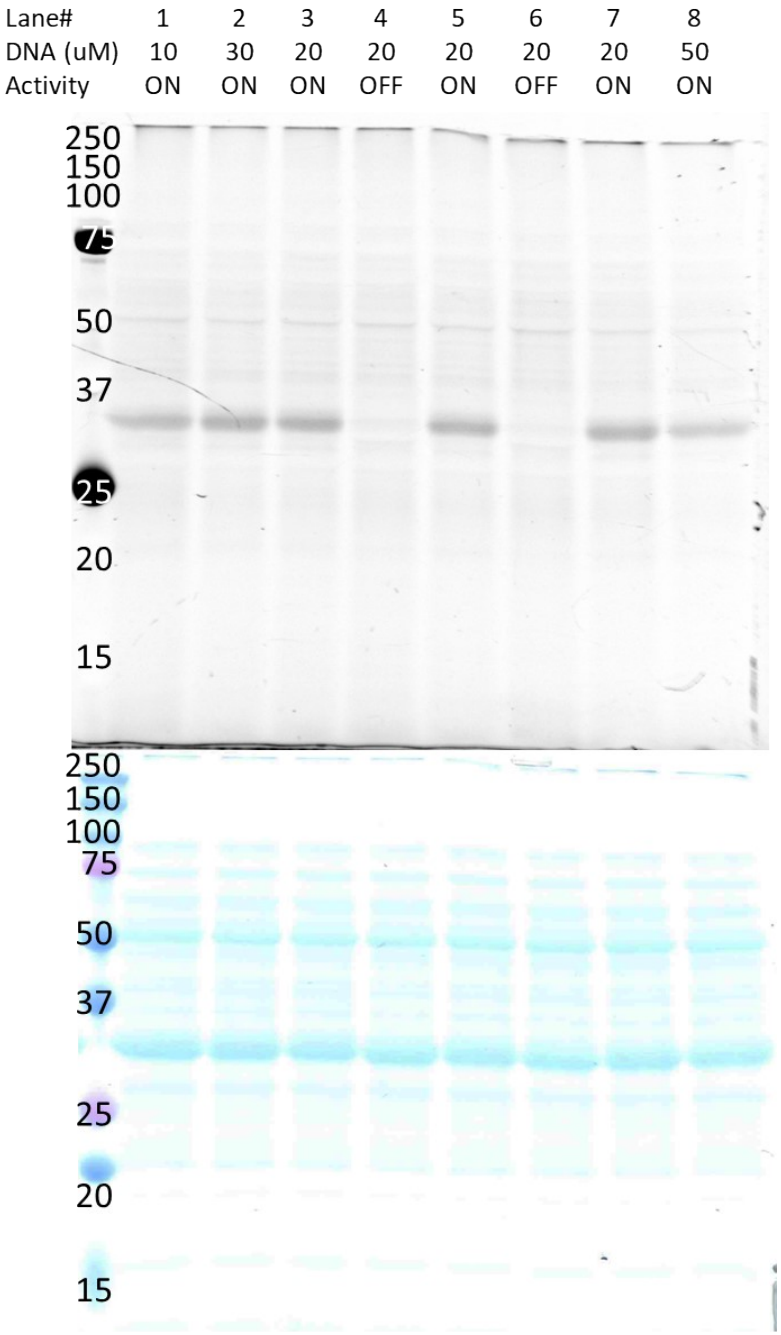


*Figure S21. Control over the modification of TRM in cell lysate (E. coli) by TBA^{12}-diPyOx with ANANS **5**. Conditions: 10 µM TRM, 100 µM ANANS **5**, pH: 7.2, at 37 °C for 6 h.*

3.2 Secondary supporting data

3.2.1 DNA sequences

Table S2. The codes and nucleobase sequences for each DNA strand that was used in this study. The abbreviation "i5OctdU" indicates the inclusion of a thymine nucleobase with a octynyl attached. The abbreviation "5Hexynyl" indicates the inclusion of a hexynyl at the 5'end phosphate group.

Code	DNA Sequence (5' to 3')
	CA2 inhibitor strands
DNA-Catalyst	AC ATG TAT CTA TTT /35OctdU/
Inhibitor-DNA	/5Hexynyl/ A AAA TAG ATA CAT GT
	Gantry Crane strands:
TBA3-T5YT5-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTT GTA TCT ACG ATT TTTTT GGT TGG TGT GGT TGG
TBA3-T6YT6-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTT GTA TCT ACG ATT TTTTTT GGT TGG TGT GGT TGG
TBA3-T11YT3-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTTTTTT GTA TCT ACG ATT TTT GGT TGG TGT GGT TGG
TBA3-T7YT7-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTT GTA TCT ACG ATT TTTTTTT GGT TGG TGT GGT TGG
TBA3-T3YT11-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTT GTA TCT ACG ATT TTTTTTTTTT GGT TGG TGT GGT TGG
TBA3-T11YT5-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTTTTT GTA TCT ACG ATT TTTTT GGT TGG TGT GGT TGG
TBA3-T8YT8-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTT GTA TCT ACG ATT TTTTTTT GGT TGG TGT GGT TGG
TBA3-T5YT11-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTT GTA TCT ACG ATT TTTTTTTTTT GGT TGG TGT GGT TGG
TBA3-T12YT6-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTTTTTT GTA TCT ACG ATT TTTTT GGT TGG TGT GGT TGG
TBA3-T8YT8-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTTTTT GTA TCT ACG ATT TTTTTTTT GGT TGG TGT GGT TGG
TBA3-T6YT12-TBA	AGT CCG TGG TAG GGC AGG TTG GGG TGA CT TTTTT GTA TCT ACG ATT TTTTTTTTTTT GGT TGG TGT GGT TGG
DNAcat-5'-alkyne	/5Hexynyl/AA TCG TAG ATA C
DNAcat-T3-alkyne	AA/i5OctdU/ CGT AGA TAC
DNAcat-T6-alkyne	AAT CG/i5OctdU/ AGA TAC
DNAcat-T10-alkyne	AAT CGT AGA /i5OctdU/AC
	Crane strands:
MainStem-T13-alkyne	GAT TGT TCG AAT /i5OctdU/ GTA TCT ACG ATT
Ligandside-T3-TBA	ATT CGA ACA ATC TTT GGT TGG TGT GGT TGG
Ligandside-T3-TBA3	ATT CGA ACA ATC TTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
Ligandside-T5-TBA	ATT CGA ACA ATC TTTTT GGT TGG TGT GGT TGG
Ligandside-T5-TBA3	ATT CGA ACA ATC TTTTT AGT CCG TGG TAG GGC AGG TTG GGG

	TGA CT
Ligandside-T7-TBA	ATT CGA ACA ATC TTTTTTT GGT TGG TGT GGT TGG
Ligandside-T7-TBA3	ATT CGA ACA ATC TTTTTTT AGT CCG TGG TAG GGC AGG TTG GGG TGA CT
DNAcat-5'-alkyne	/5Hexynyl/AA TCG TAG ATA C
DNAcat-T6-alkyne	AAT CG/i5OctdU/ AGA TAC
	Other strands:
TBA-12-alkyne	GGT TGG TGT GG/i5OctdU/ TGG
TBA-OFF	ATG CCC AAC CAC ACC AAC CAT GC
TBA-ON	GCA TGG TTT GTG TGT TTG GGC AT

3.2.2 HPLC and MS data of DNA constructs

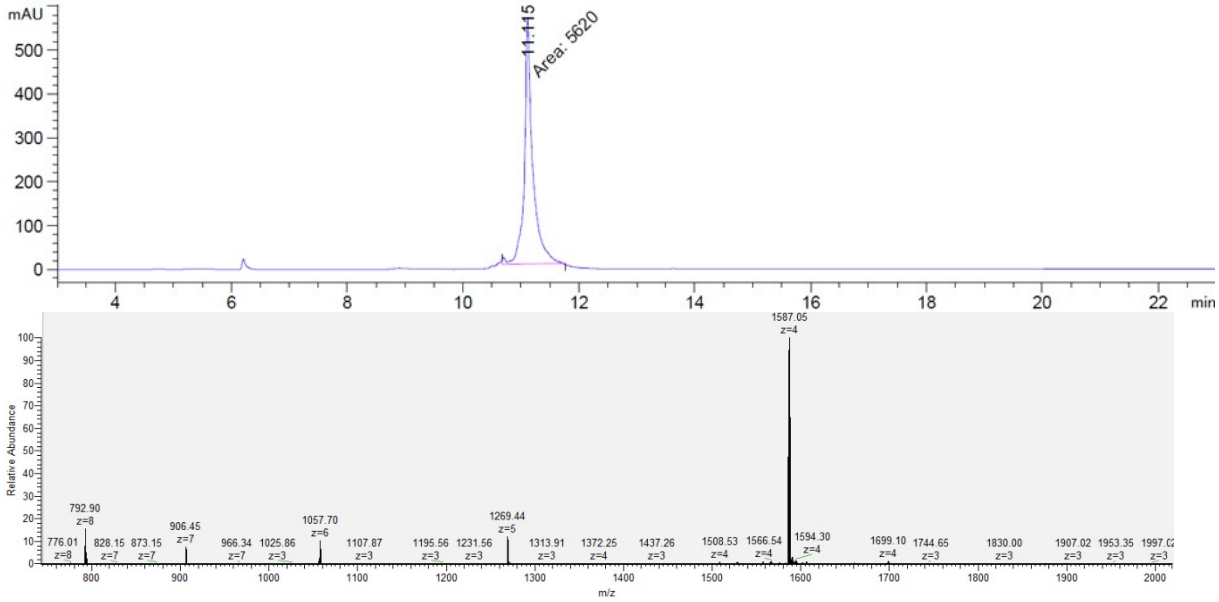


Figure S24. Inhibitor-DNA HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 793.0(8), 906.4(7), 1057.6(6), 1269.4(5), 1587.0(4).

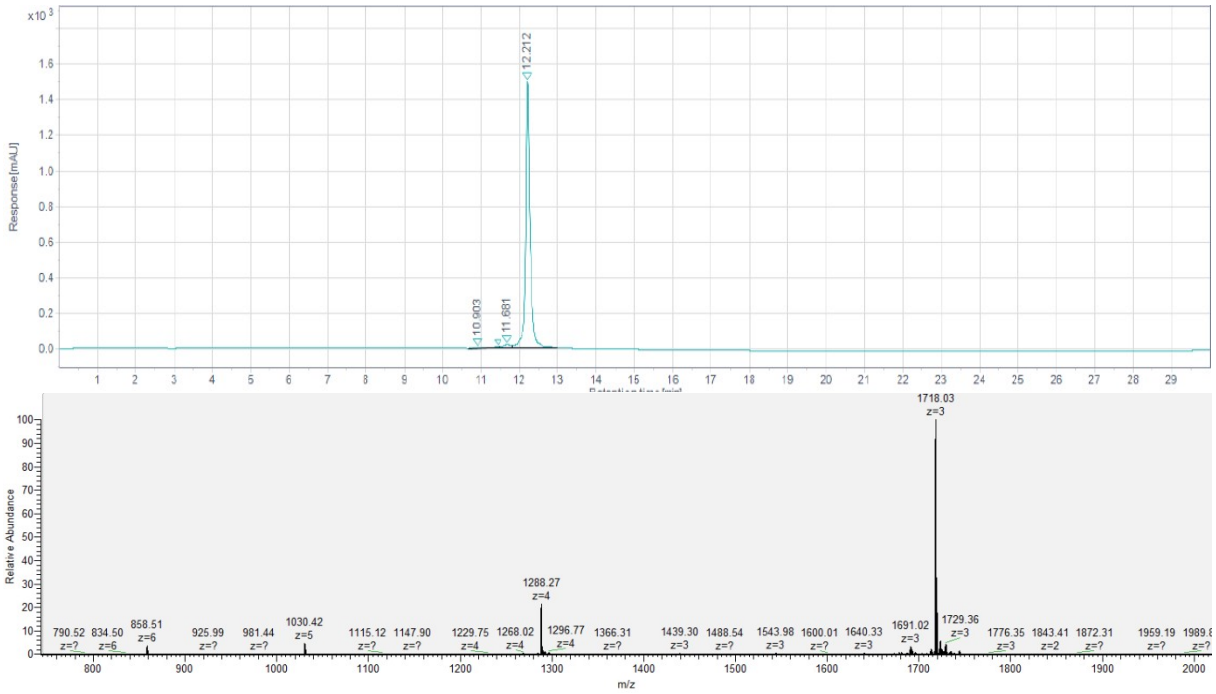


Figure S23. Catalyst-DNA-dD HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 858.7(6), 1030.4(5), 1288.3(4), 1718.0(3).

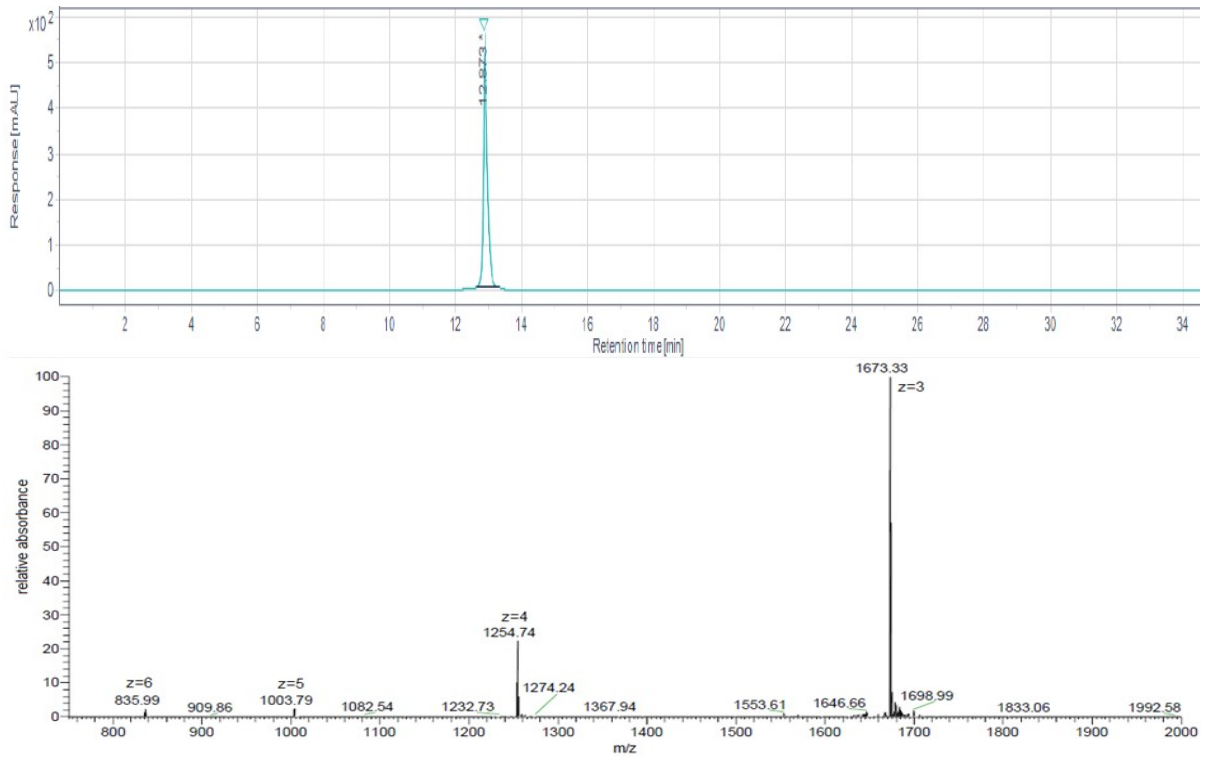


Figure S24. Catalyst-DNA-dP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 836.1(6), 1003.6(5), 1254.7 (4), 1673.(3).

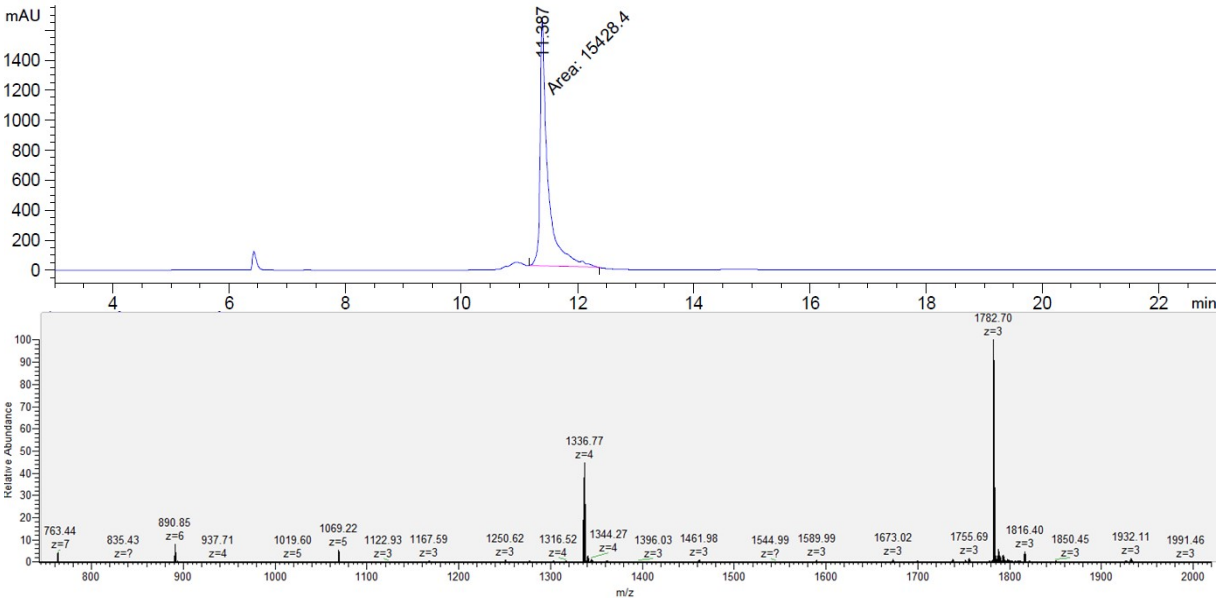


Figure S25. TBA[12]-diDMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 763.4(7), 890.9 (6), 1069.2(5), 1336.8(4), 1782.7(3).

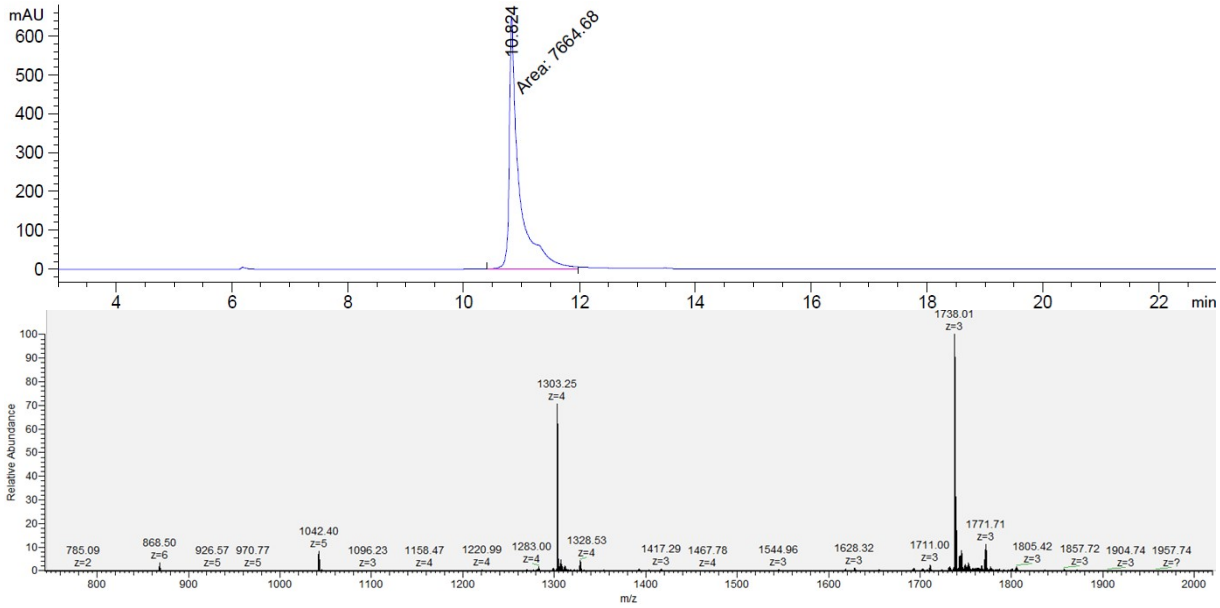


Figure S26. TBA[12]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 868.5(6), 1042.4(5), 1303.3(4), 1738.0(3).

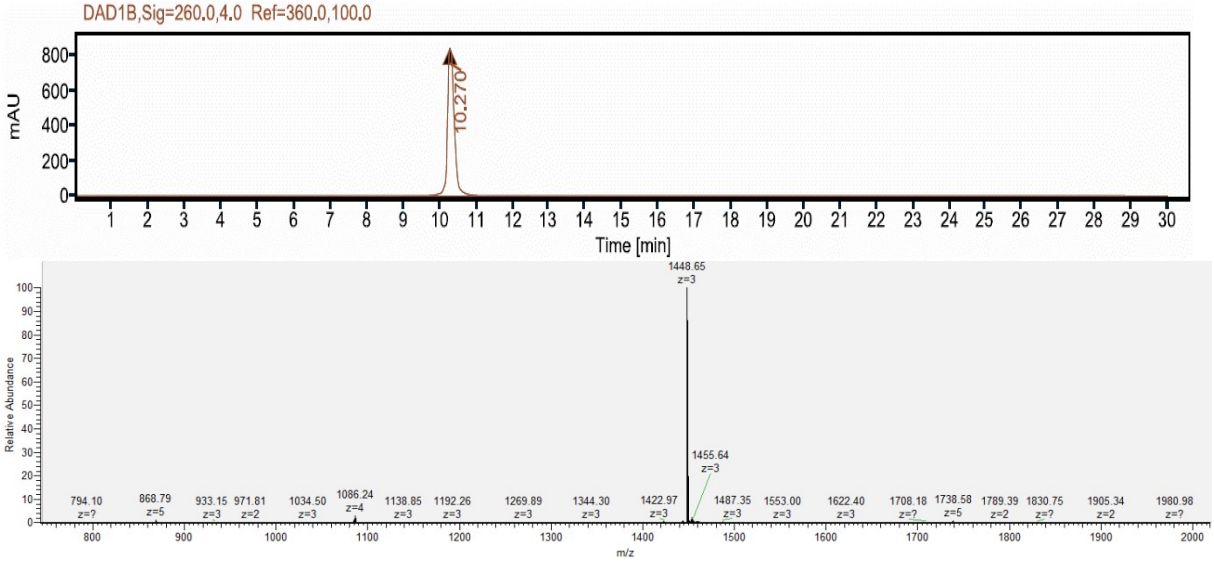


Figure S27. DNAcat-5'-diDMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 868.8(5), 1086.4(4), 1448.6(3).

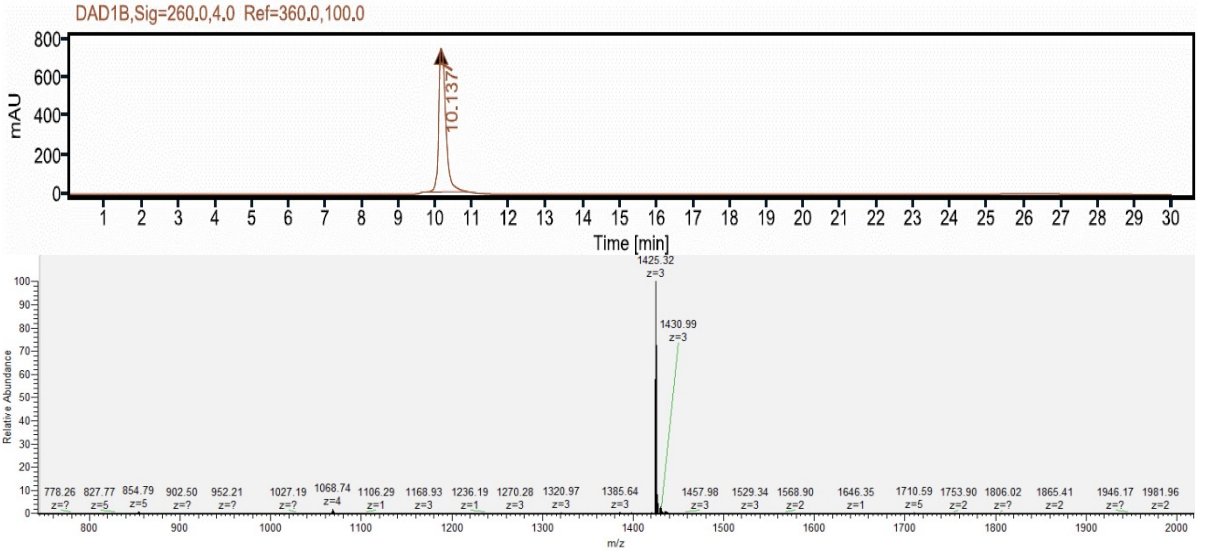


Figure S28. DNAcat-T6-diDMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 854.7 (5), 1068.7(4), 1425.3(3).

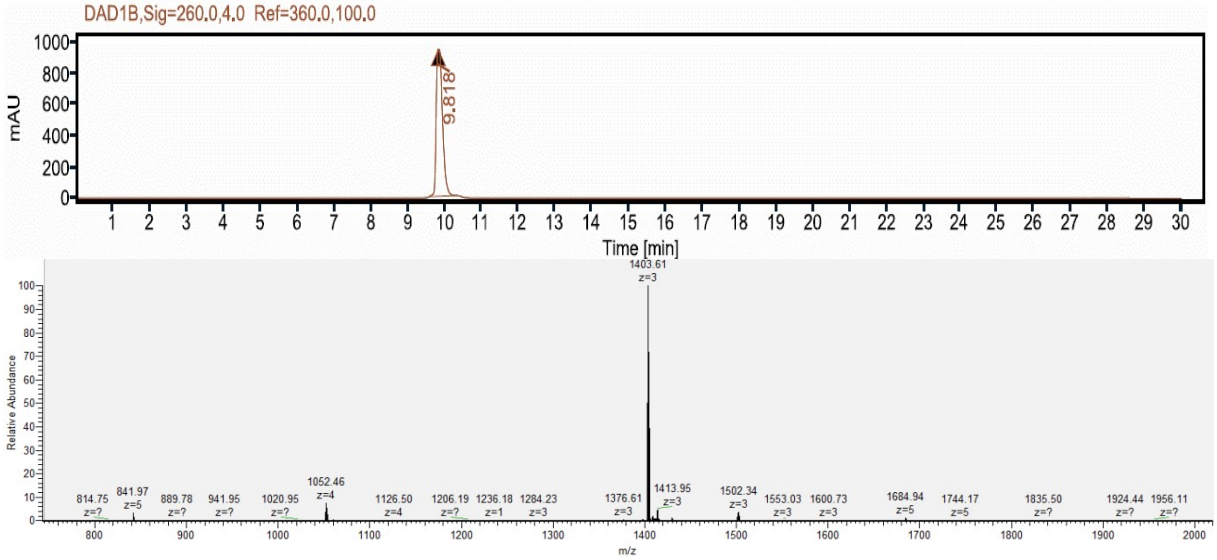


Figure S29. DNAcat-5'-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 842.0(5), 1052.4(4), 1403.6(3).

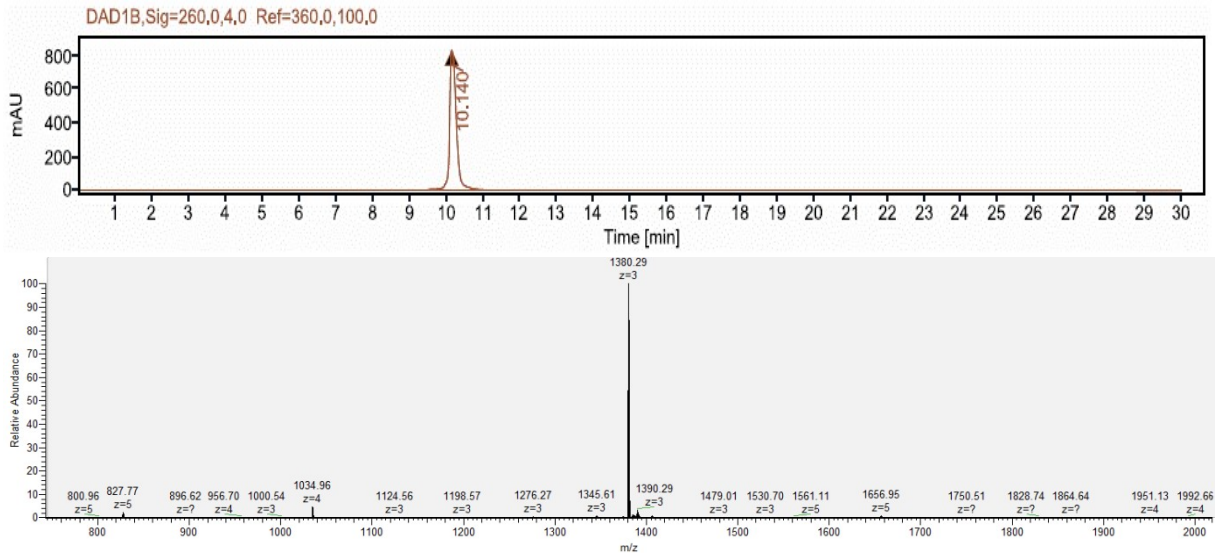


Figure S30. DNAcat-T3-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 827.8 (5), 1035.0(4), 1380.3(3).

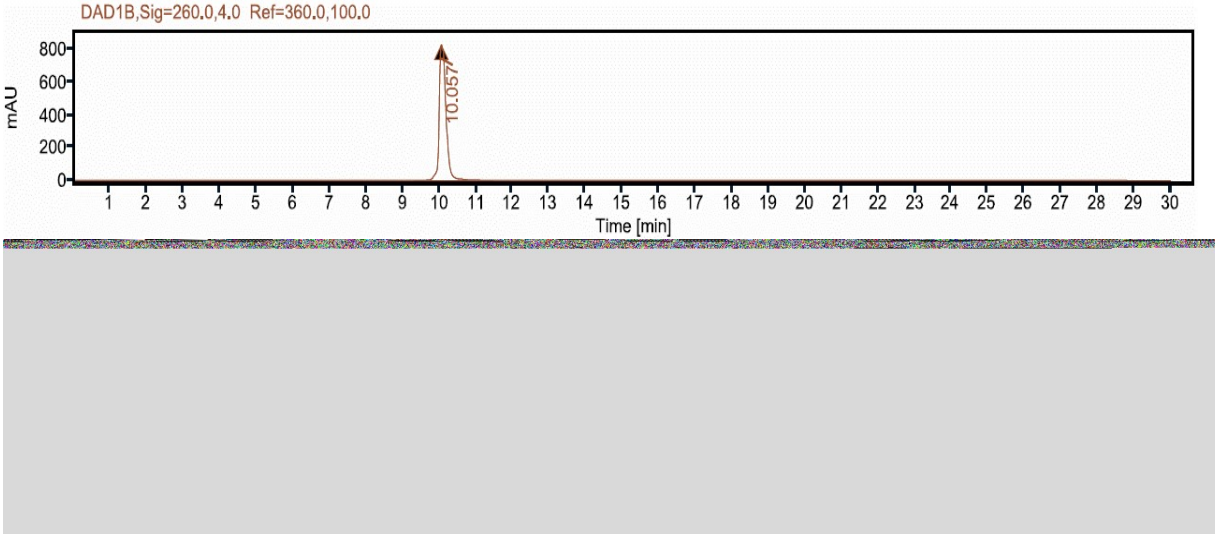


Figure S31. DNAcat-T6-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 827.8 (5), 1035.0(4), 1380.3(3).

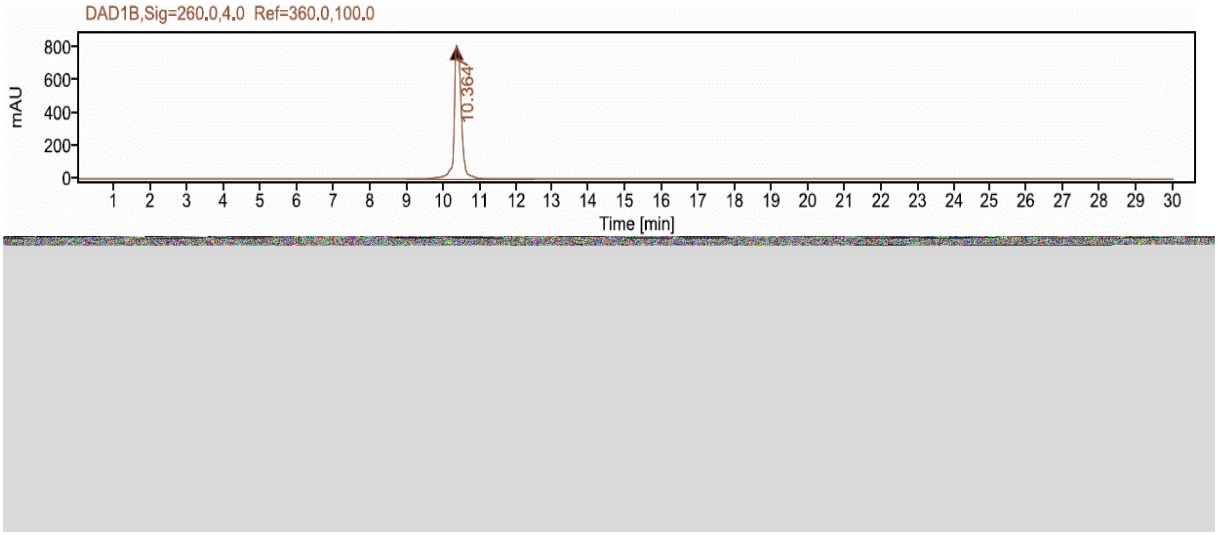


Figure S32. DNAcat-T10-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 827.8 (5), 1035.0(4), 1380.3(3).

3.2.3 MS data of modified thrombin

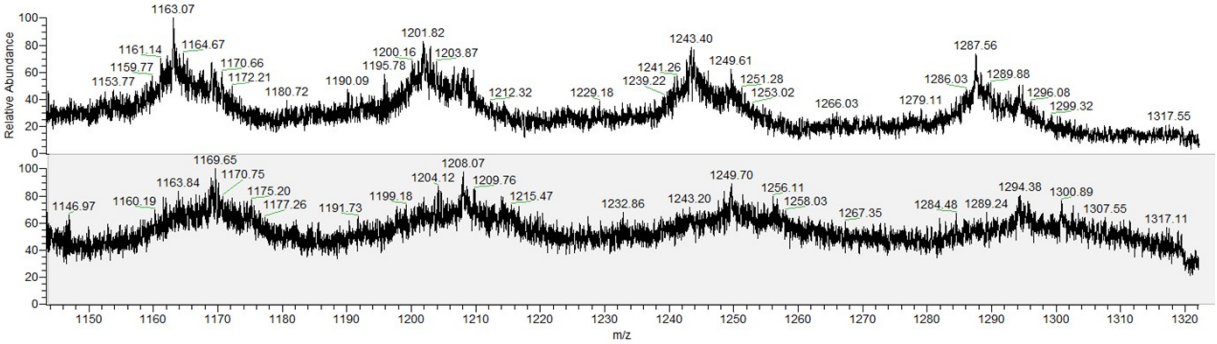


*Figure S33. The m/z values of native thrombin and thrombin modified with acyl donor **1** or **2** (both result in the same increase in mass). Native thrombin has a mass of 36025 Da, acylation with acyl donor **1** or **2** leads to a nett mass addition of 185 Da per molecule. The first panel shows mass(z) peaks: 1163(31), 1202(30), 1243(29) and 1288(28) corresponding to unmodified thrombin. The second panel shows mass(z) peaks: 1170(31), 1208(30), 1250(29) and 1294(28) corresponding to mono-modified thrombin.*

3.2.4 MS data of TBA[12]-diPyOx after reaction

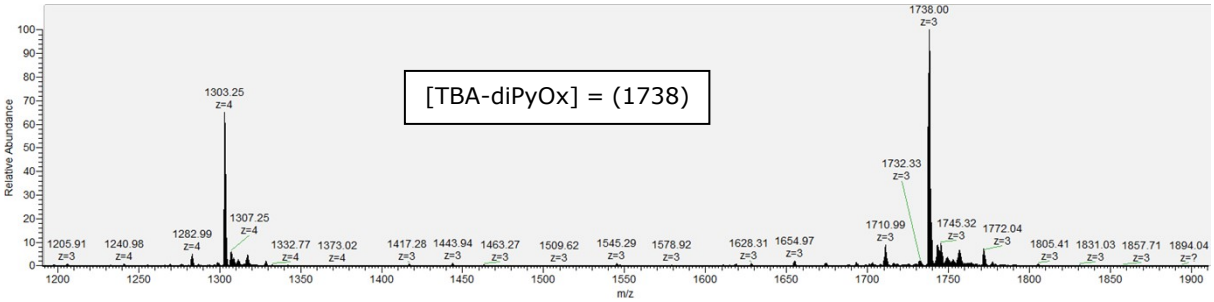


Figure S34. Mass data of TBA[12]-diPyOx in HEPES (pH 7.2) after 8 h at 37°C.

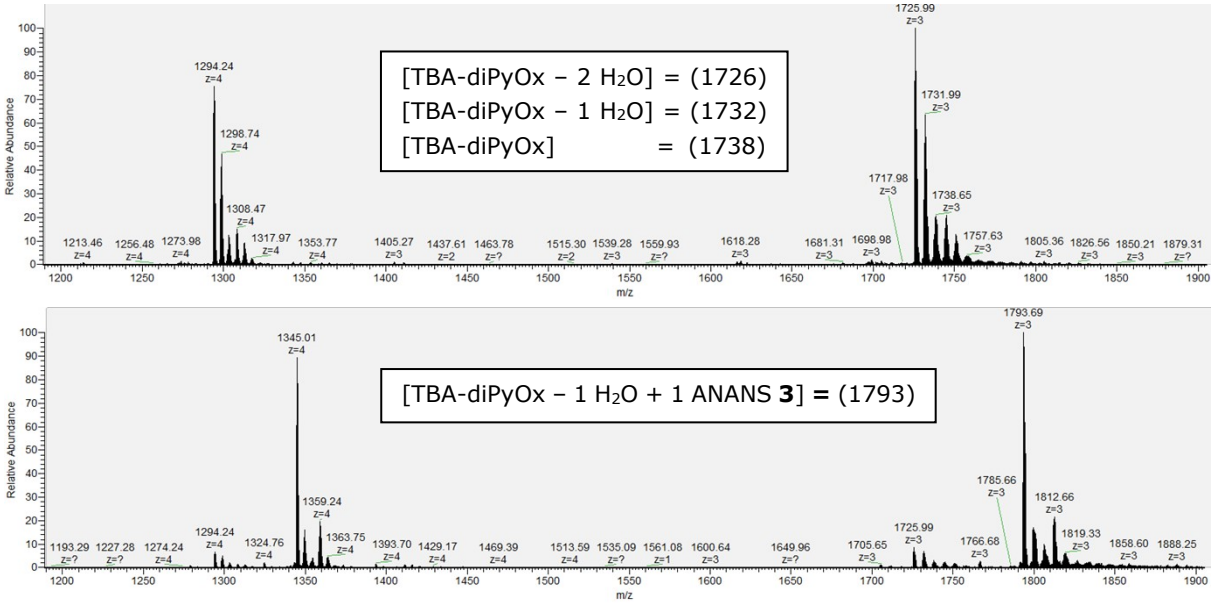


*Figure S35. Mass data of TBA[12]-diPyOx with ANANS **3** in HEPES (pH 7.2) after 8 h at 37 °C. As the acyl group cannot be donated to, dehydration of the catalyst occurs. Only non-functional and mono-functional TBA-diPyOx is detected.*

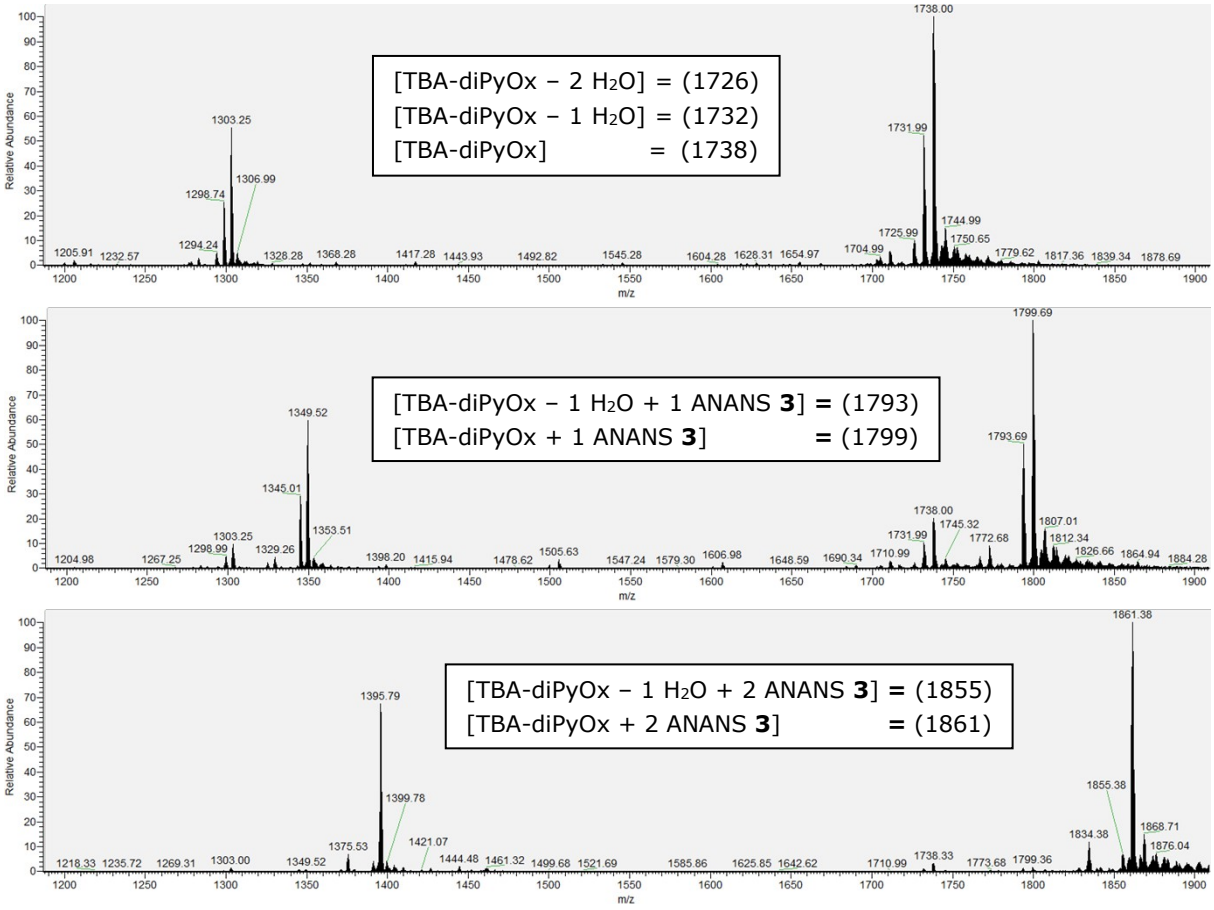


*Figure S36. Mass data of TBA[12]-diPyOx with ANANS **3** and thrombin in HEPES (pH 7.2) after 8 h at 37 °C. As the acyl group can now be donated to thrombin, dehydration of the catalyst occurs much less and di-functional diPyOx is predominantly detected.*

3.2.5 NMR data of organic compounds

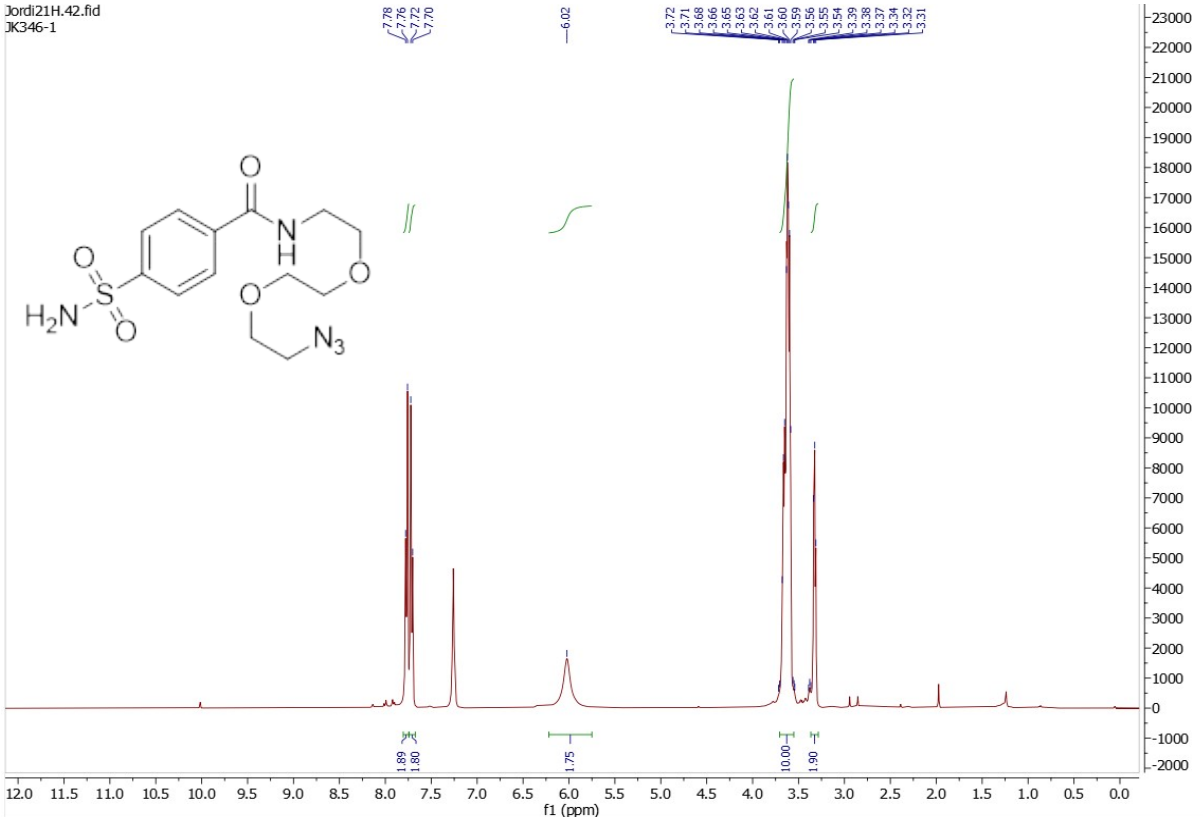


Figure S37. ^{1}H NMR spectrum of azido-EG_2-sulfonamide.

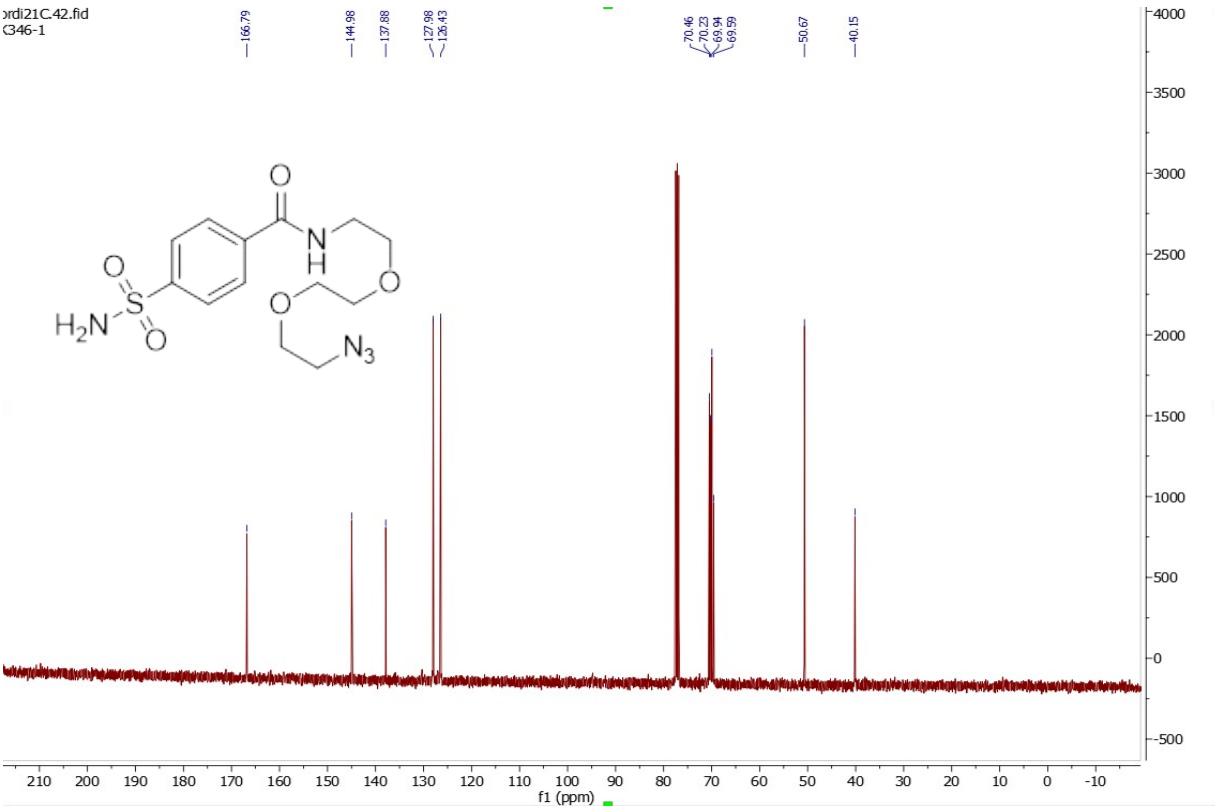


Figure S38. ^{13}C NMR spectrum of azido-EG_2-sulfonamide.

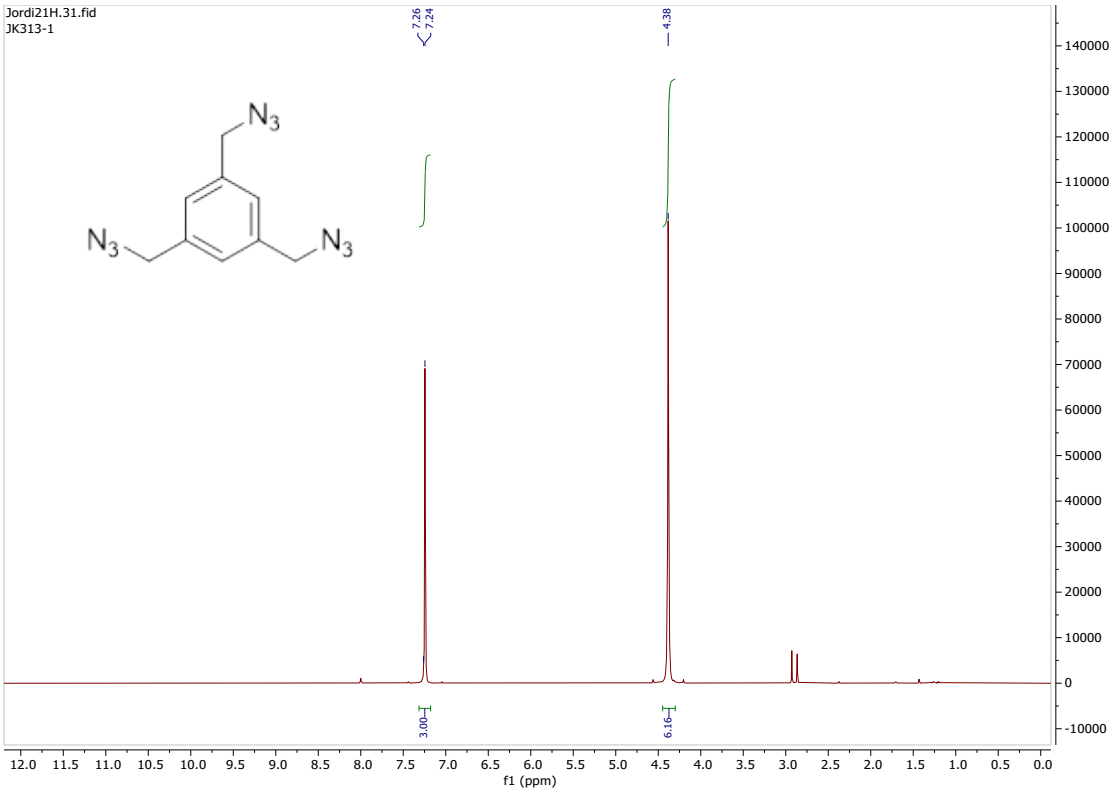


Figure S39. ^{1}H NMR spectrum of 1,3,5-tris(azidomethyl)benzene.

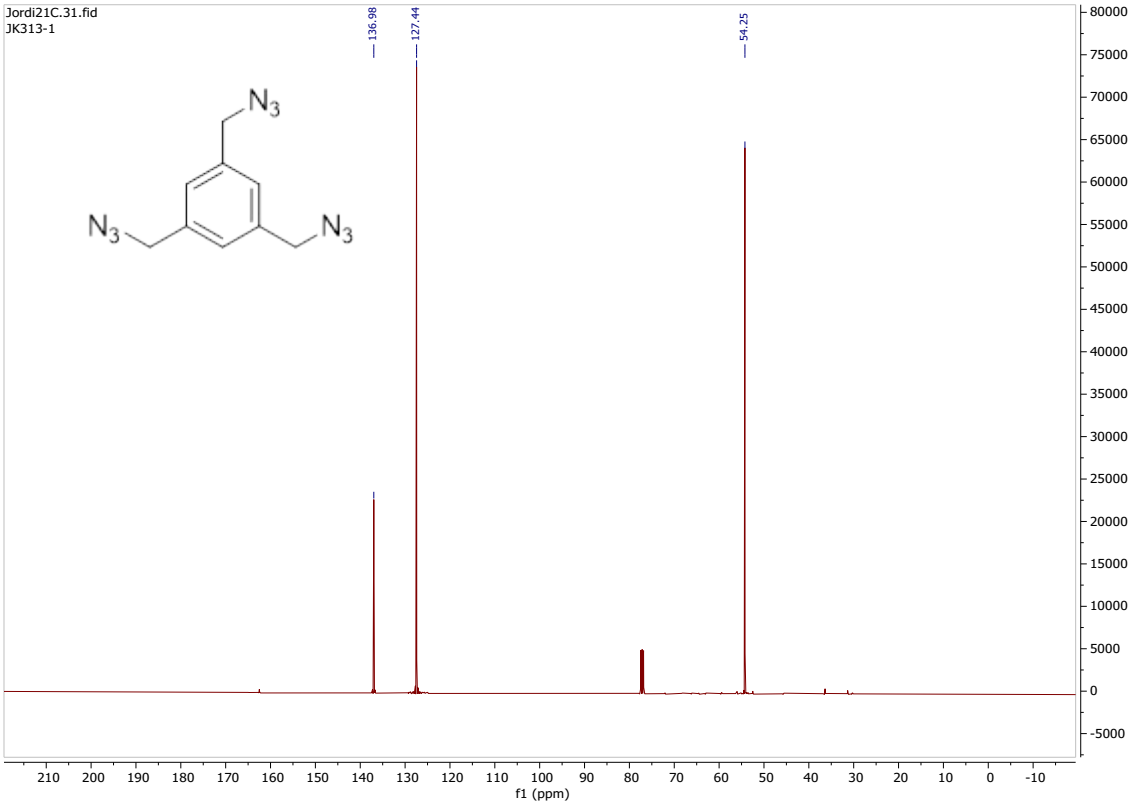


Figure S40. ^{13}C NMR spectrum of 1,3,5-tris(azidomethyl)benzene.

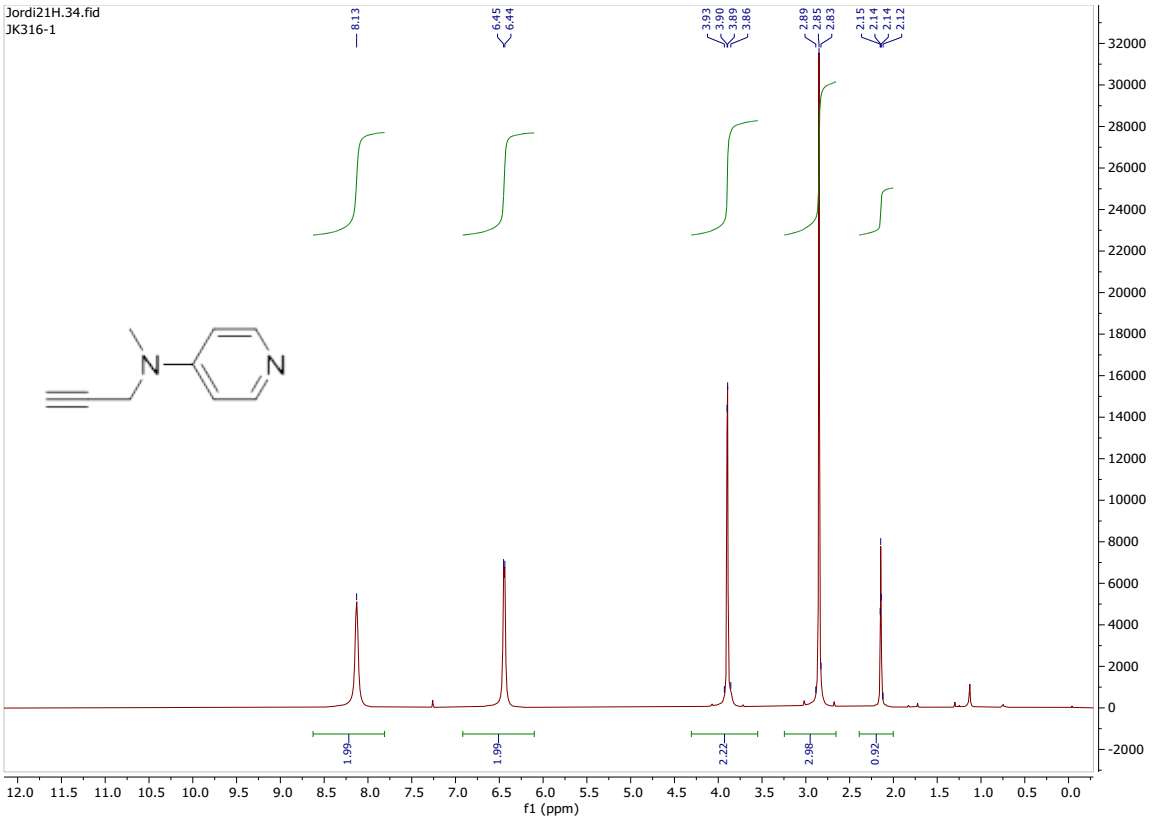


Figure S41. [1]H NMR spectrum of alkyne-DMAP.

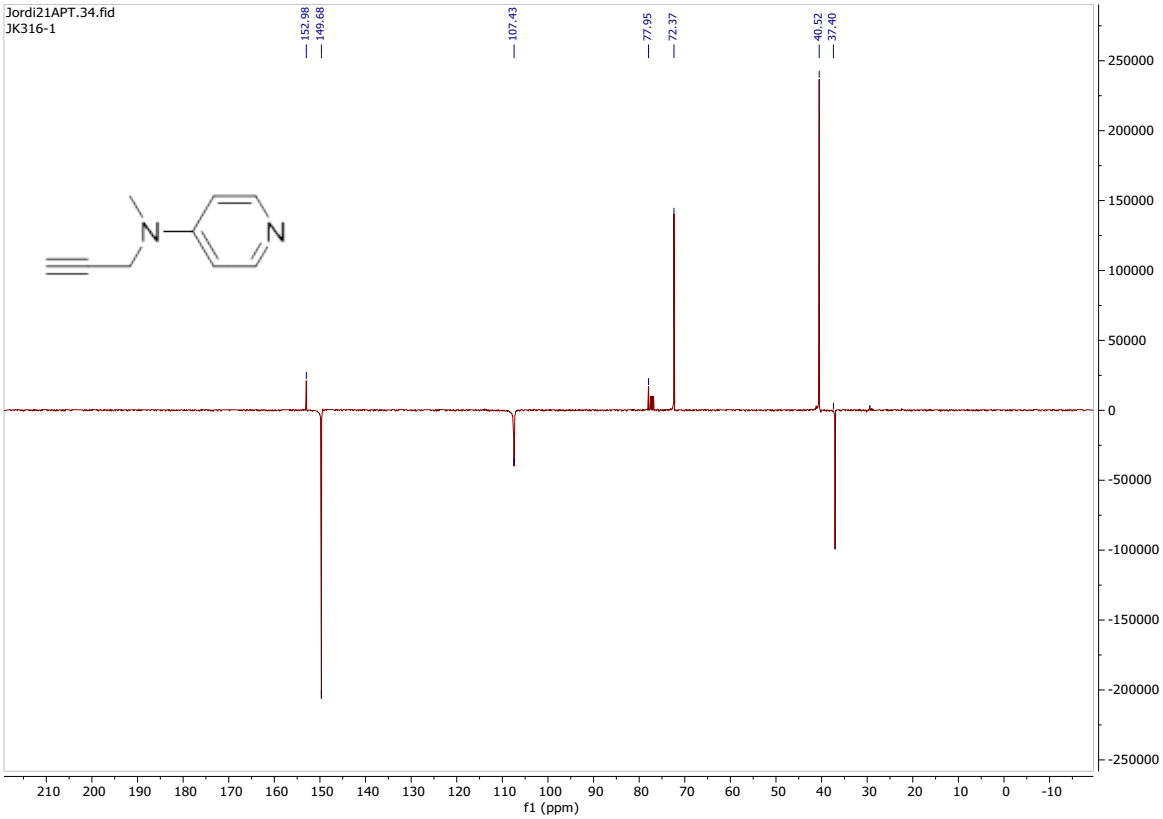


Figure S42. [13]C NMR spectrum of alkyne-DMAP.

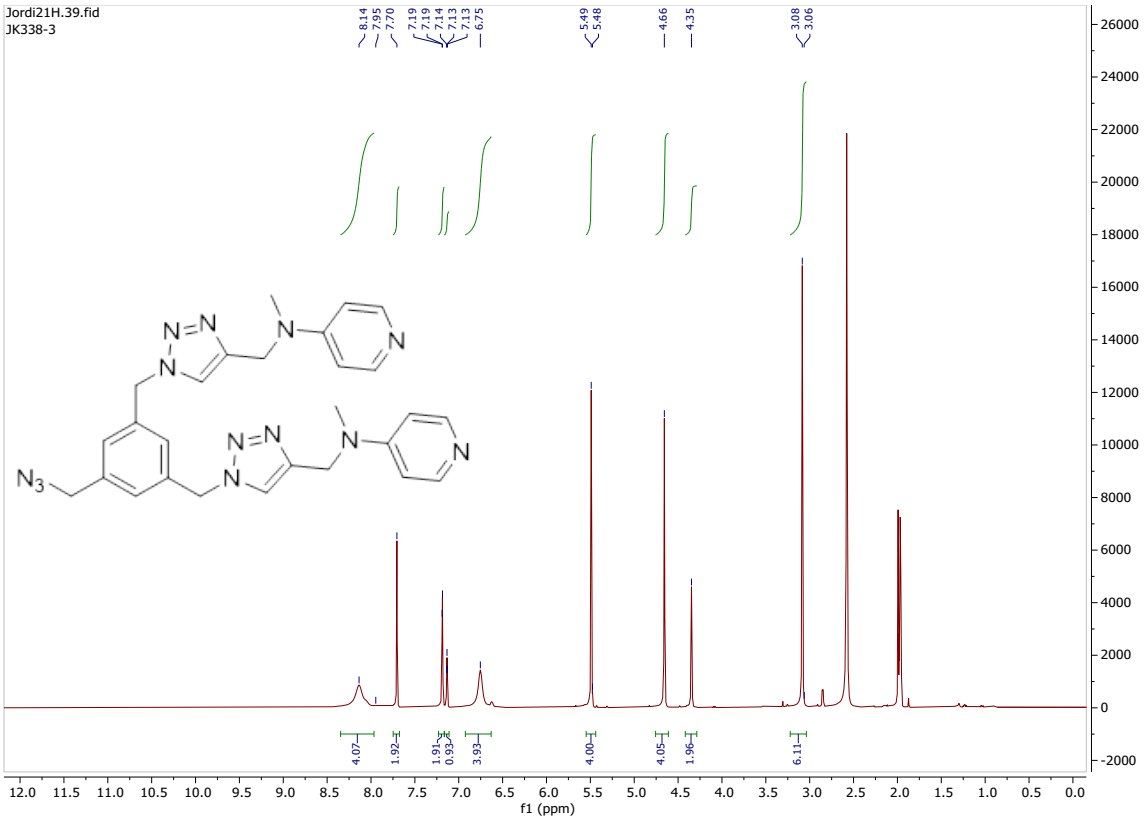


Figure S43. [1]H NMR spectrum of azido-diDMAP.

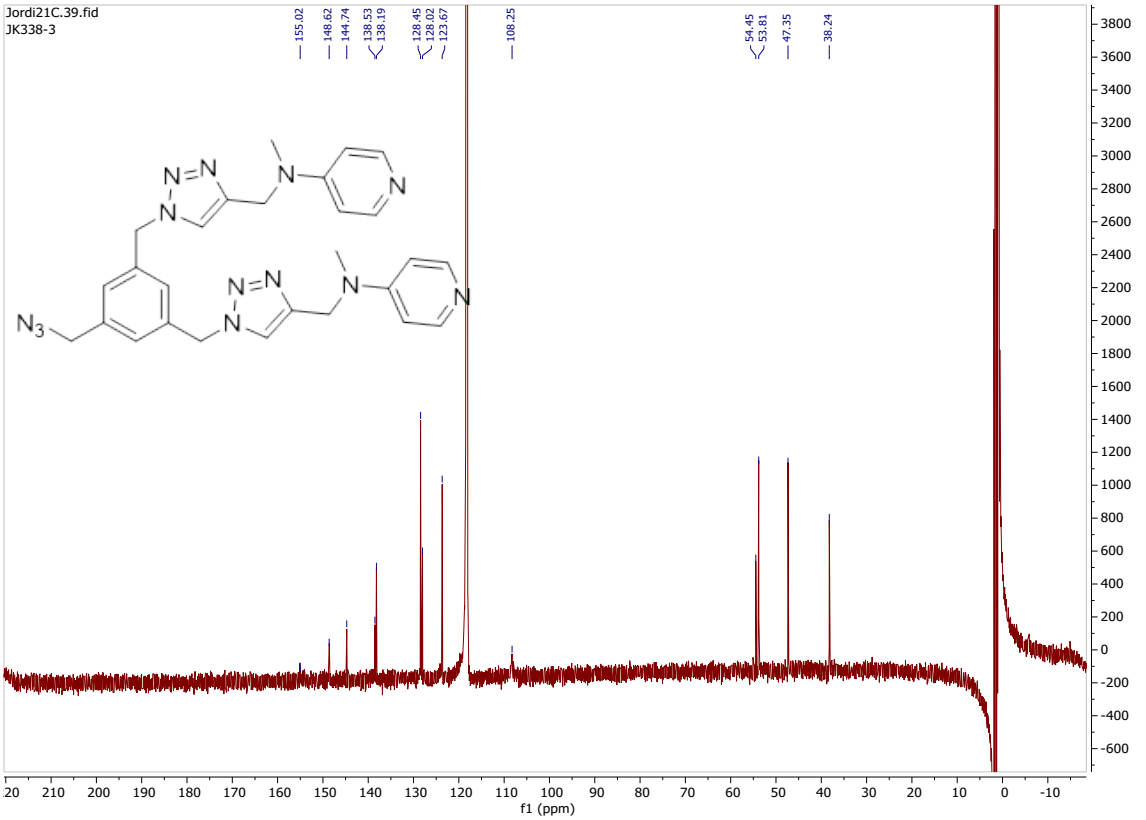


Figure S44. [13]C NMR spectrum of azido-diDMAP.

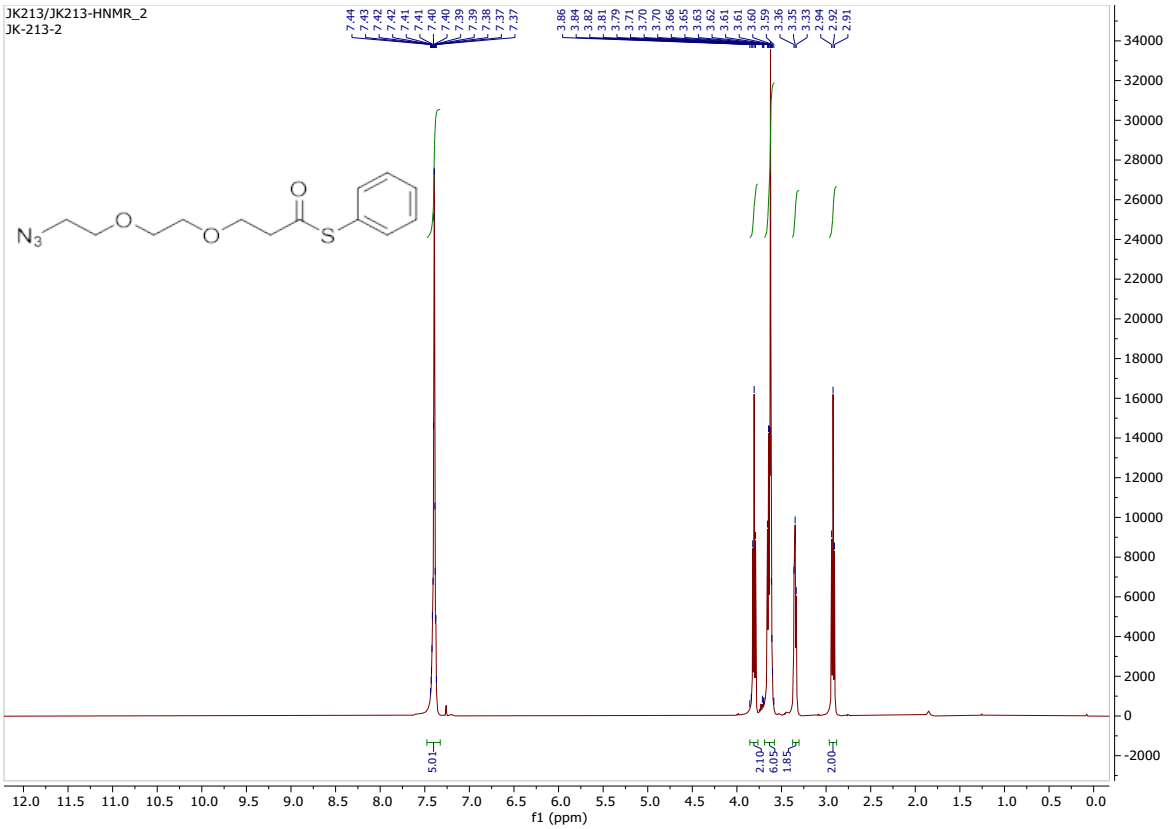


*Figure S45. [1]H NMR spectrum of azido-thioester **1**.*

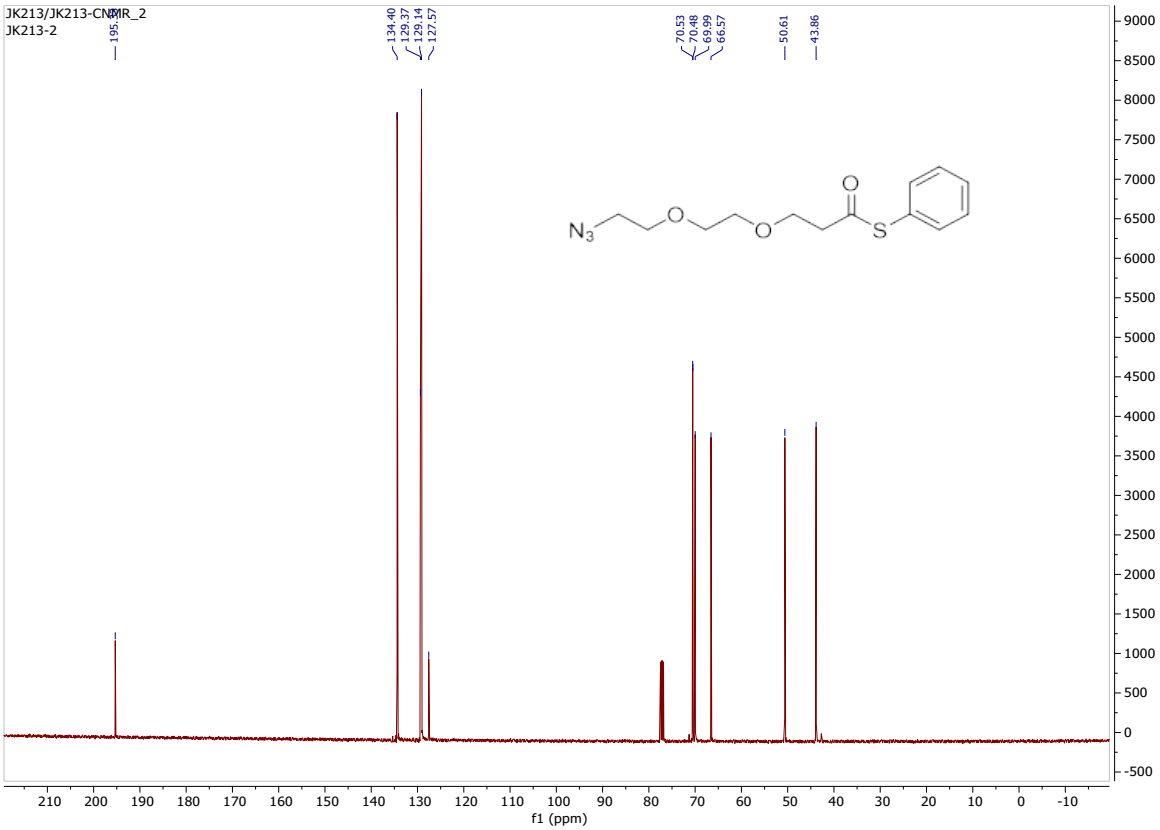


*Figure S46. [13]C NMR spectrum of azido-thioester **1**.*

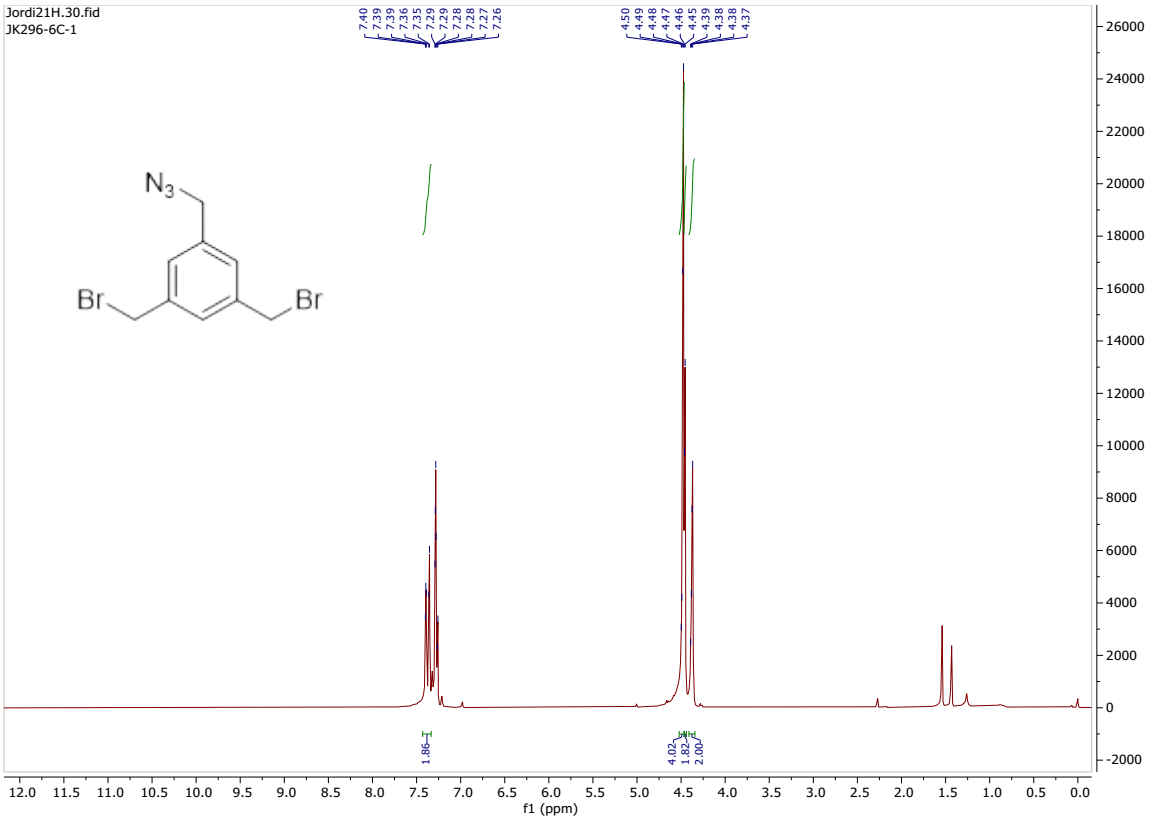


Figure S47. [1]H NMR spectrum of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene.

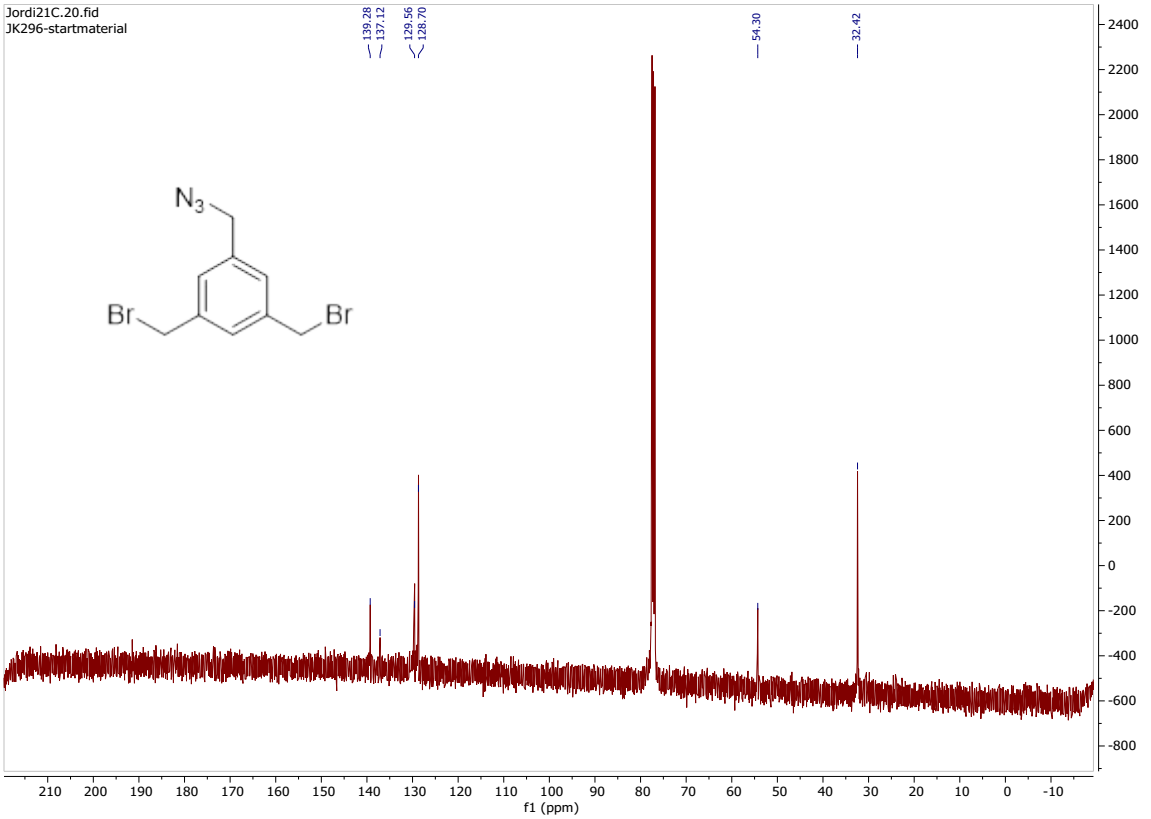


Figure S48. [13]C NMR spectrum of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene.

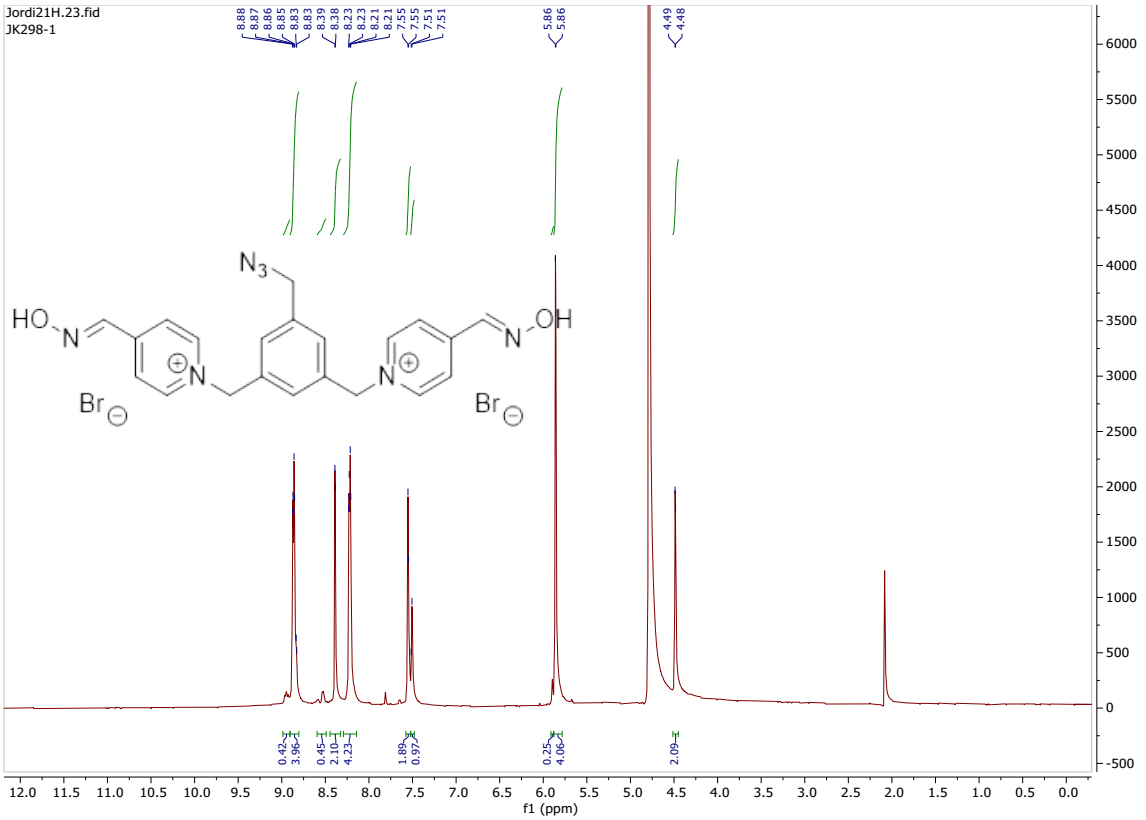


Figure S49. ^{1}H NMR spectrum of azido-diPyOx.

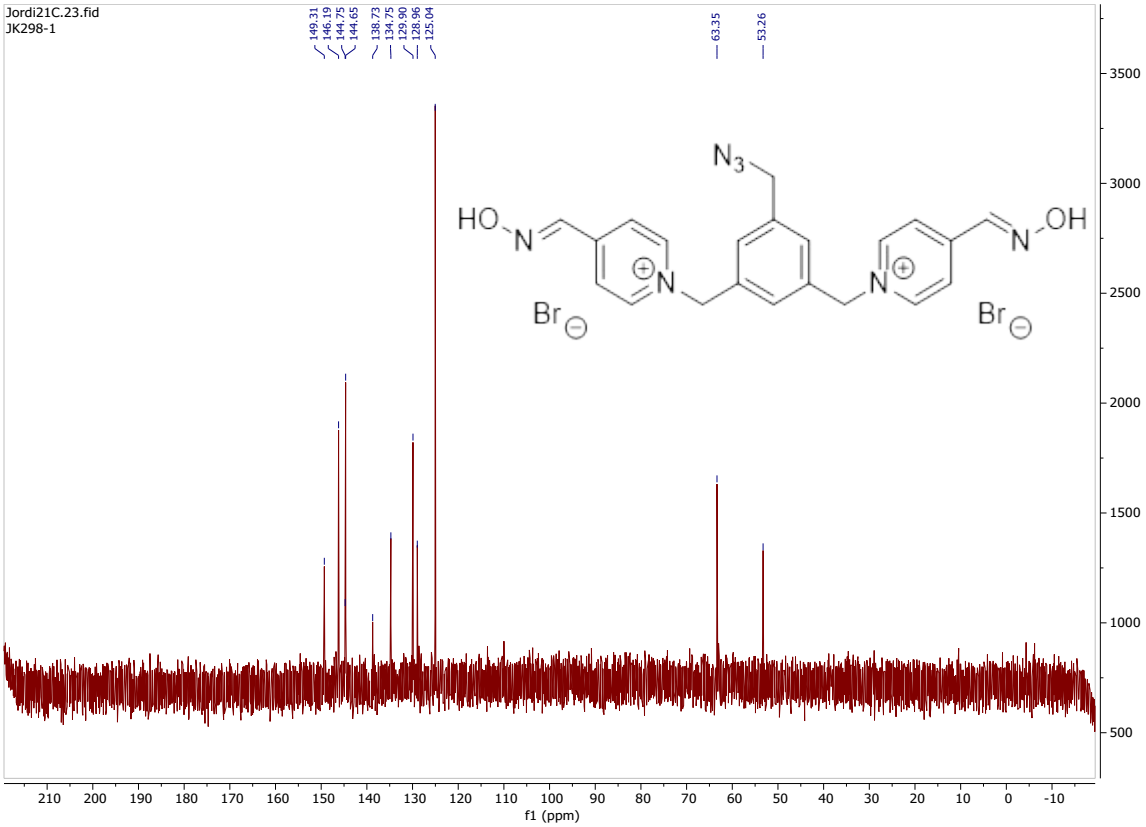


Figure S50. ^{13}C NMR spectrum of azido-diPyOx.

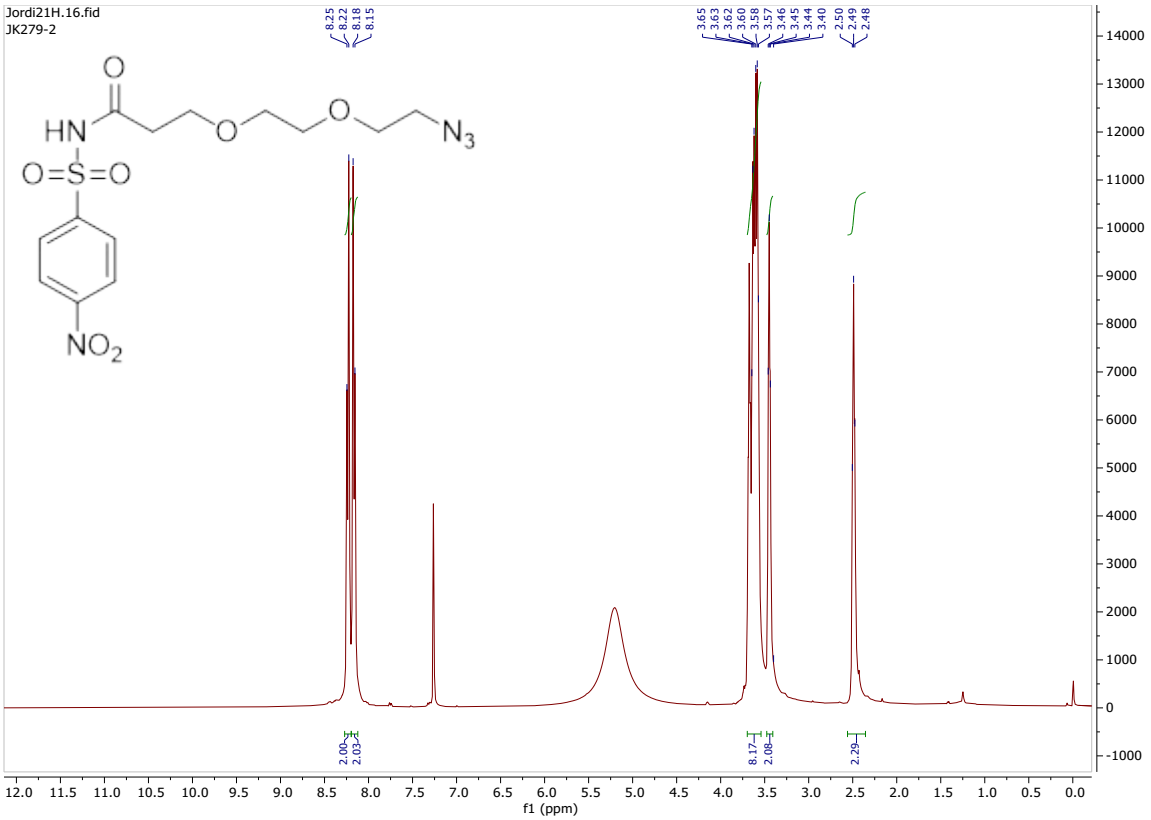


Figure S51. ^{1}H NMR spectrum of azido-ANANS precursor.

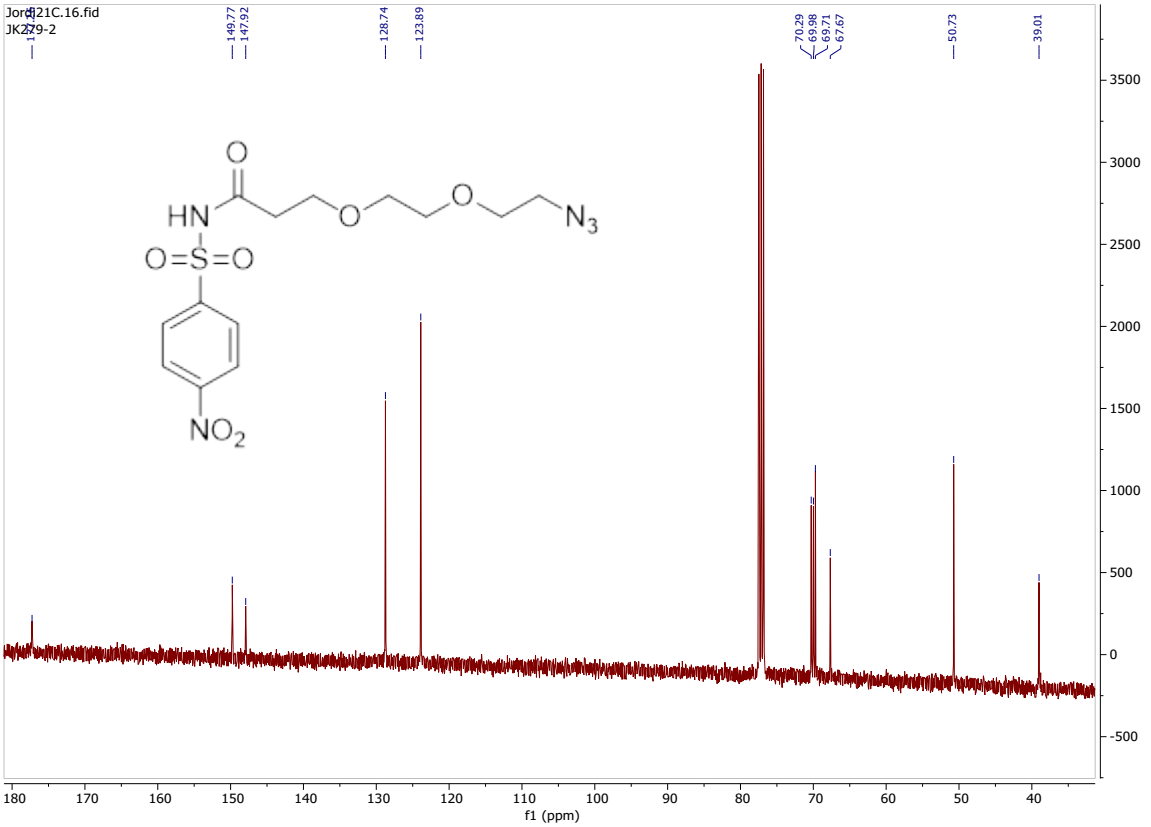


Figure S52. ^{13}C NMR spectrum of azido-ANANS precursor.

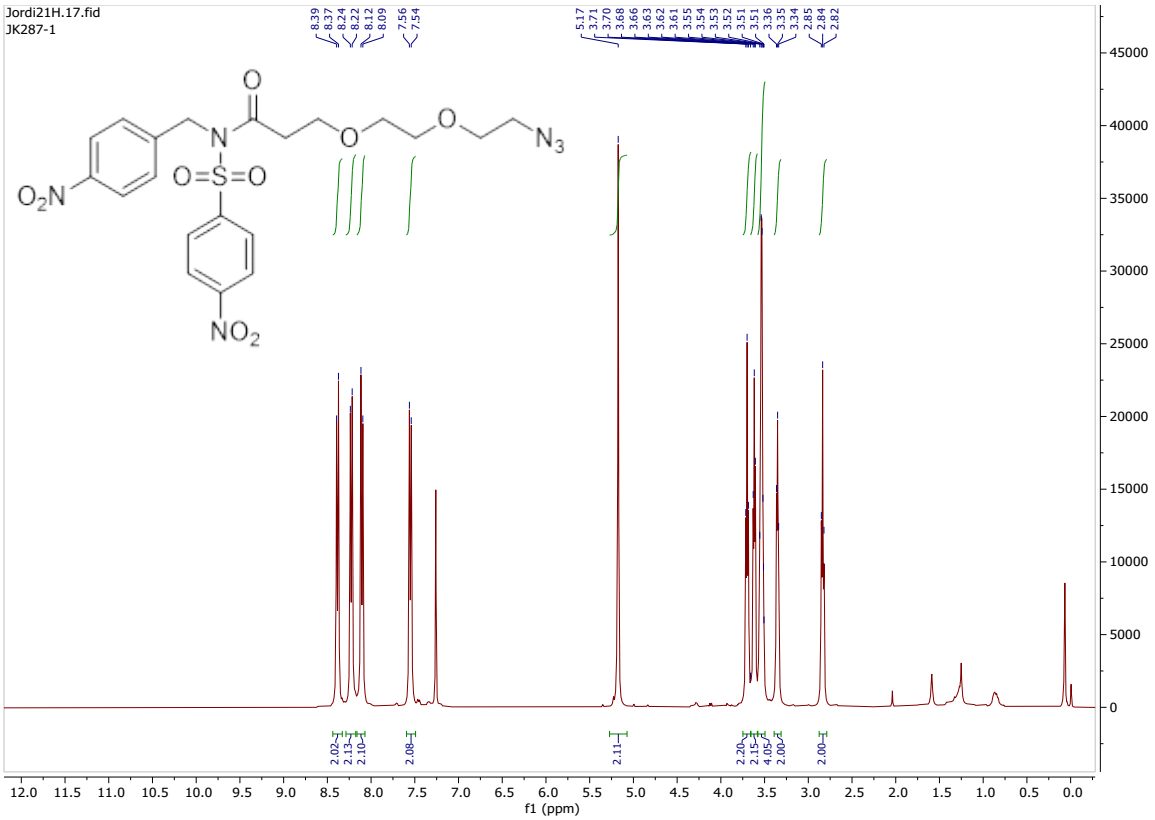


*Figure S53. [1]H NMR spectrum of azido-ANANS **2**.*

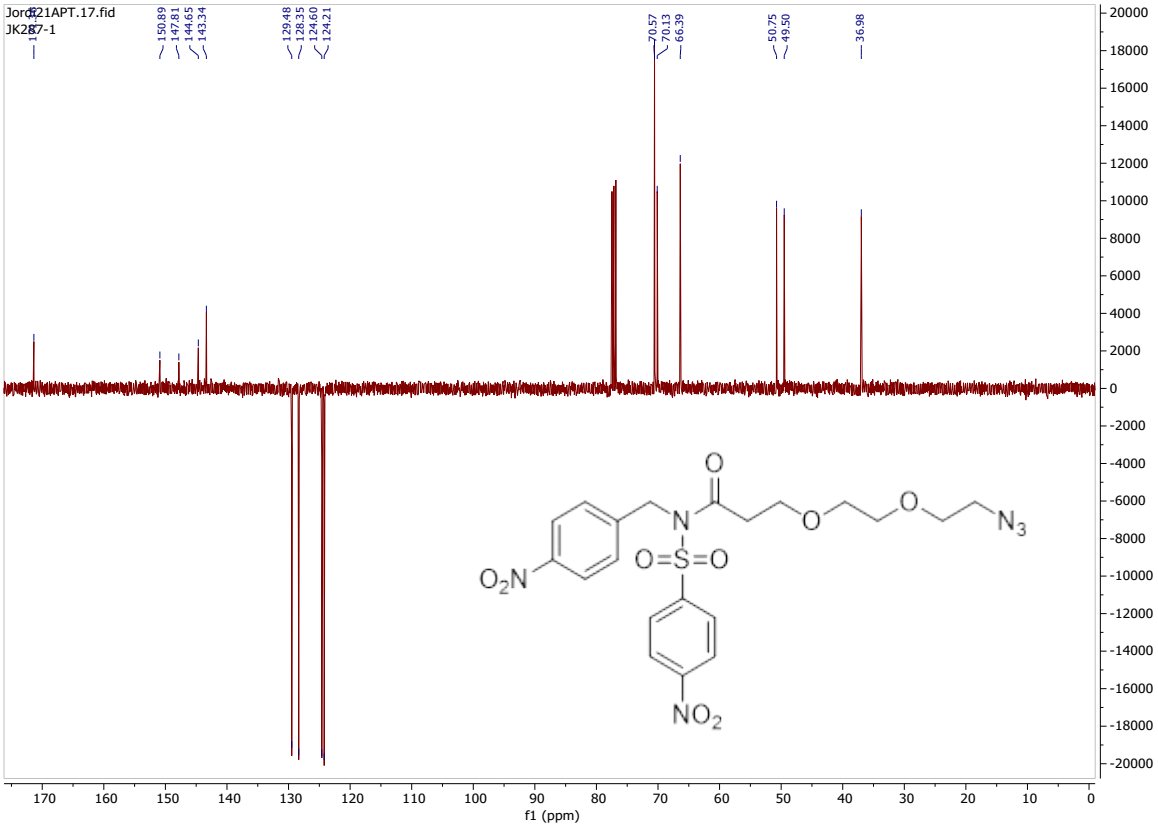


*Figure S54. [13]C APT NMR spectrum of azido-ANANS **2**.*

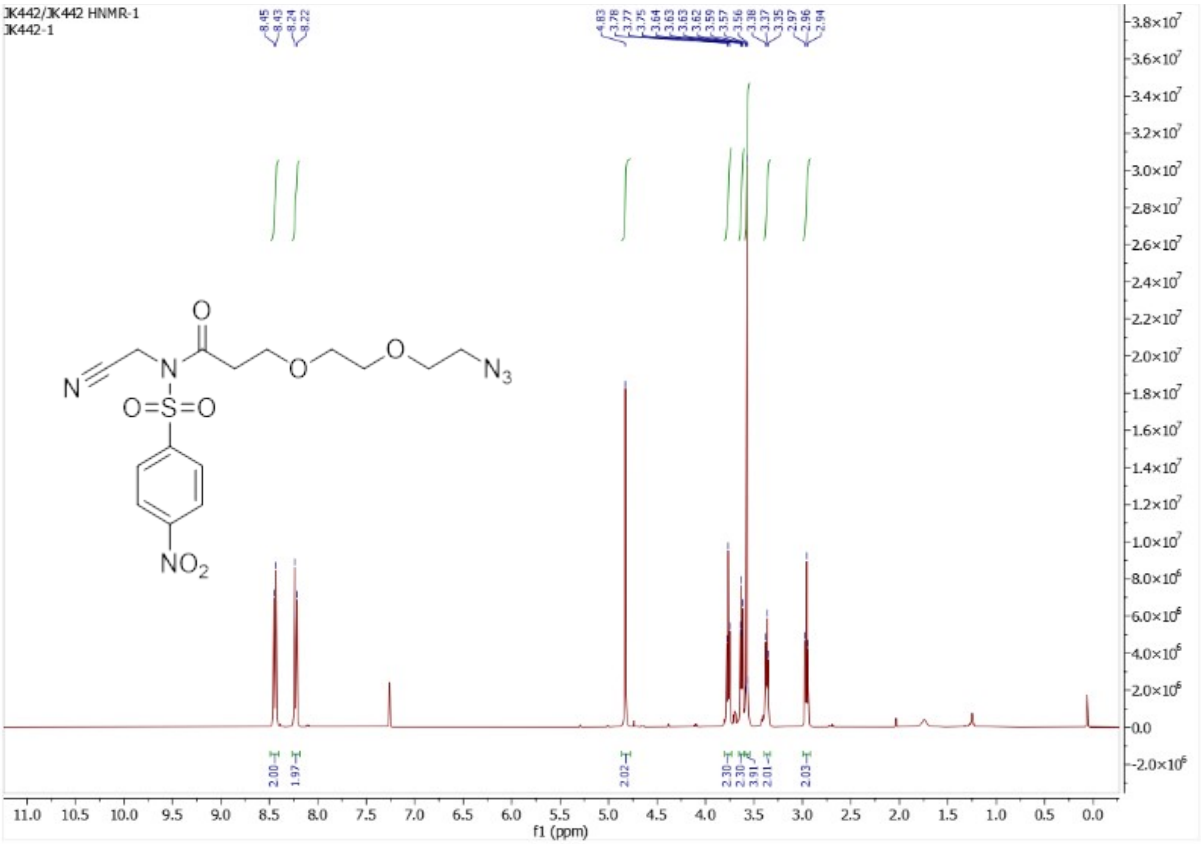


*Figure S55. ^{1}H NMR spectrum of azido-ANANS **3**.*

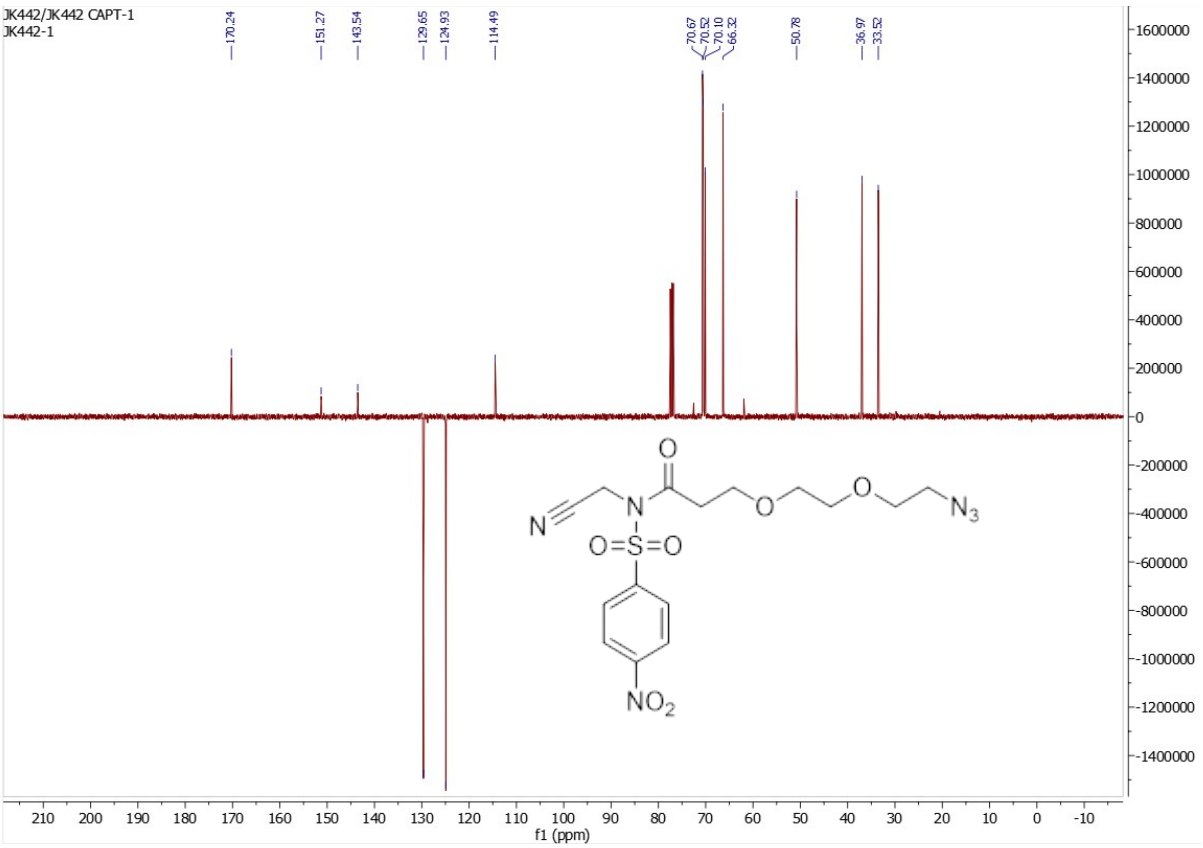


*Figure S56. ^{13}C APT NMR spectrum of azido-ANANS **3**.*

3.2.6 HPLC traces and purity of final compounds

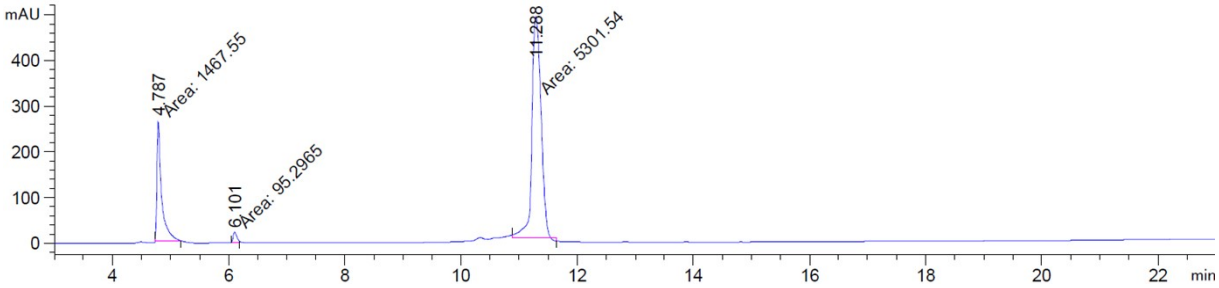


Figure S57. HPLC trace of azido-diDMAP (elution @ 4.8 and 11.3 min). Calculated purity = 99%.

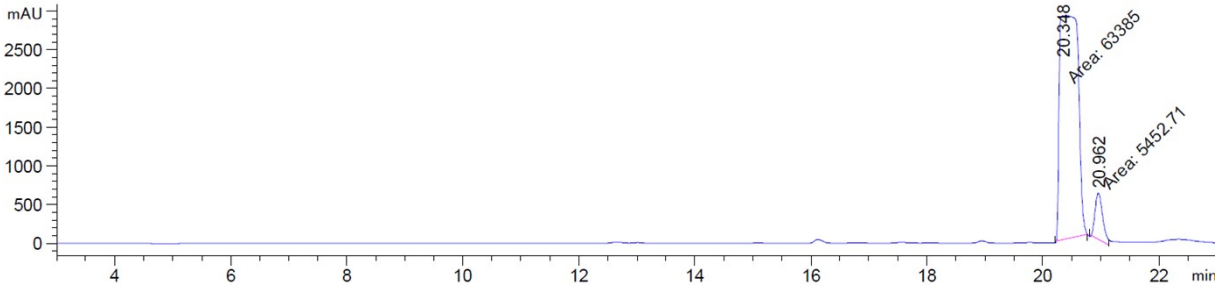


*Figure S59. HPLC trace of azido-thioester **1** (elution @ 20.3 and 21.0 min). Calculated purity = 99%.*

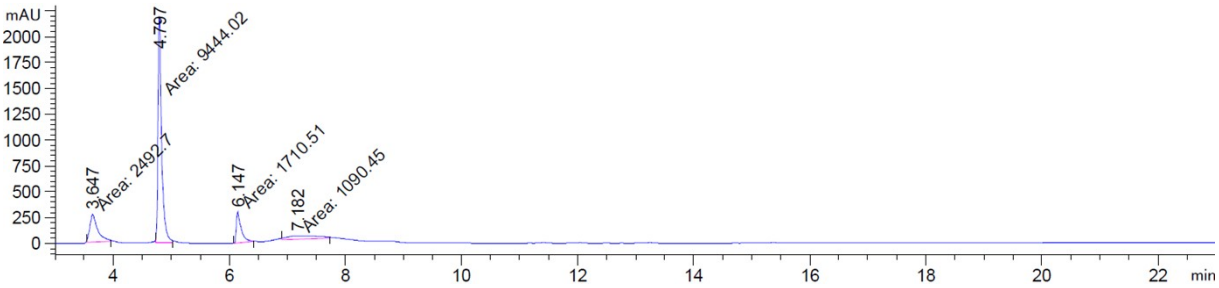


Figure S59. HPLC trace of azido-diPyOx (elution @ 3.6, 4.8 and 6.1 min). Calculated purity = 93%.

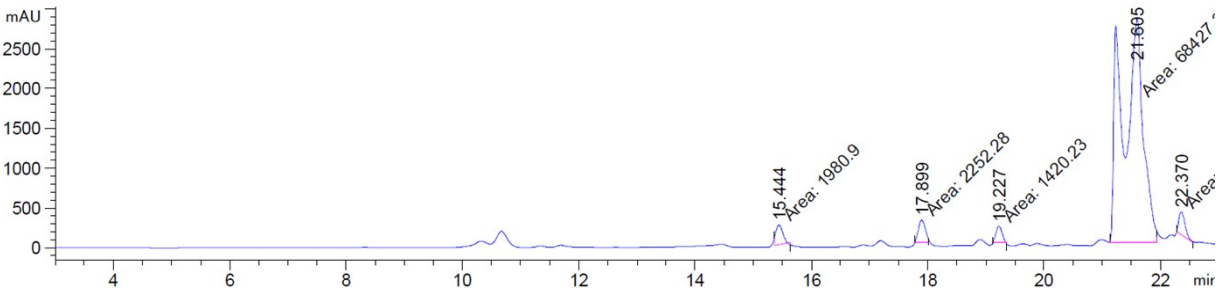


*Figure S60. HPLC trace of azido-ANANS **2** (elution @ 21.1 and 21.6 min). Calculated purity = 90%. Peak at ~10 min is contamination found which was also found in blanc runs.*

3.2.7 Raw gel data

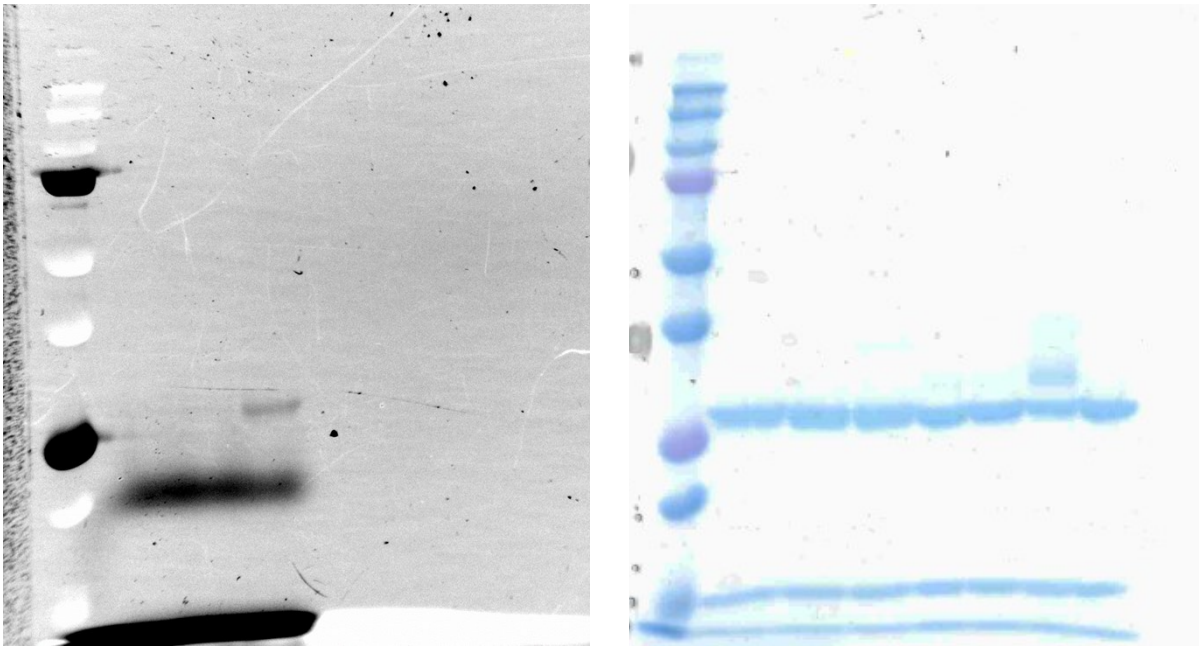


Figure S61. Raw gel images of Figure S1.

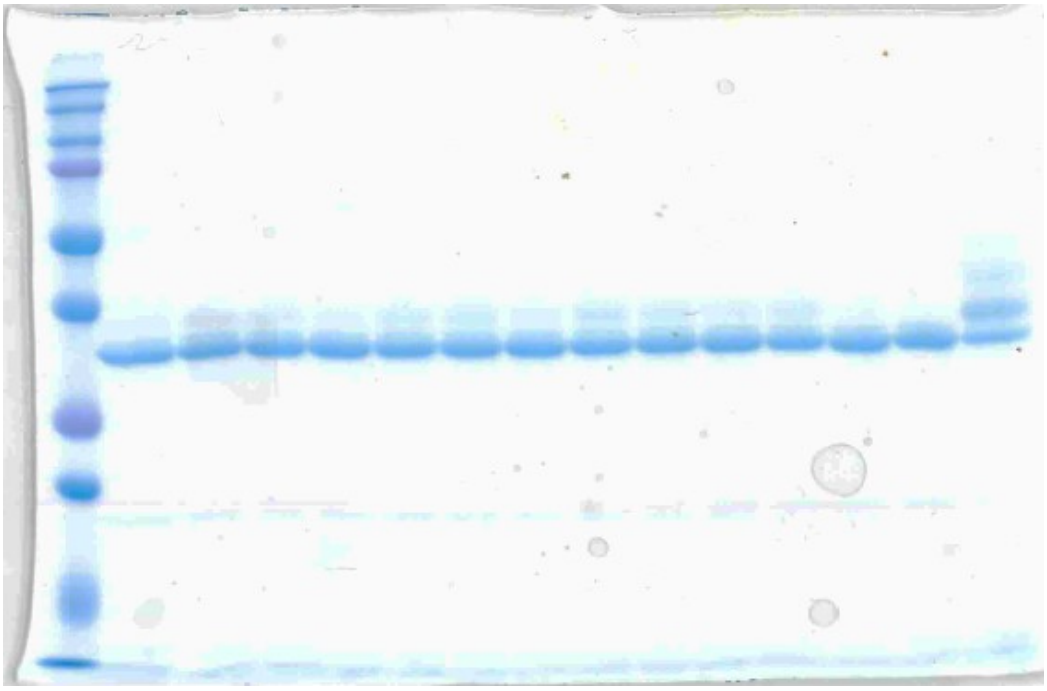


Figure S63. Raw gel image of Figure S3.

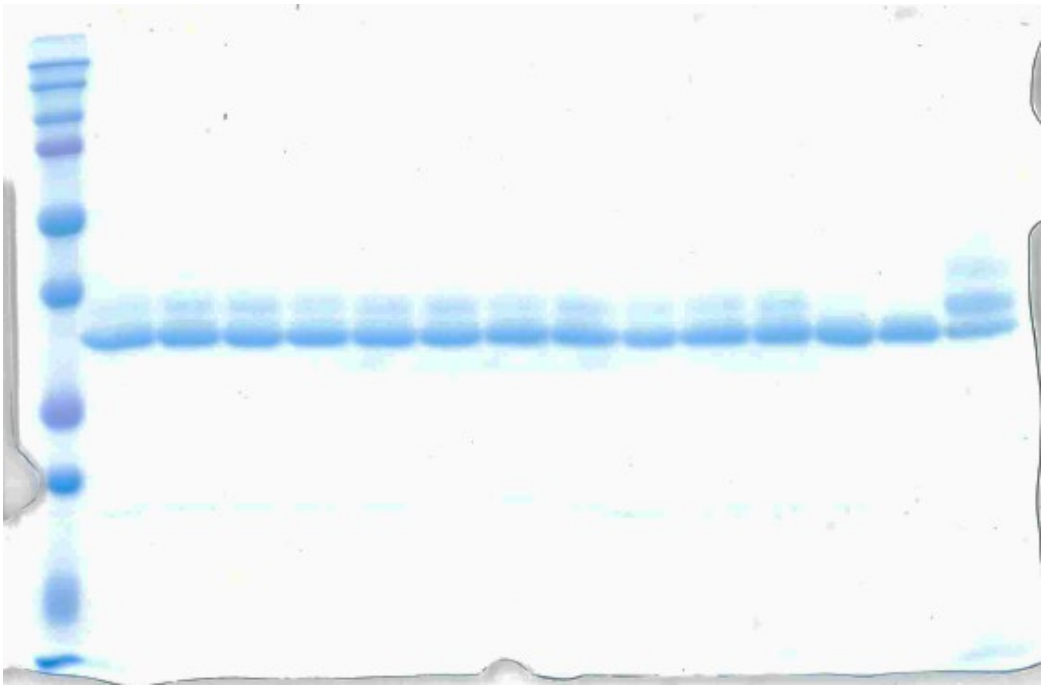


Figure S63. Raw gel image of Figure S4.

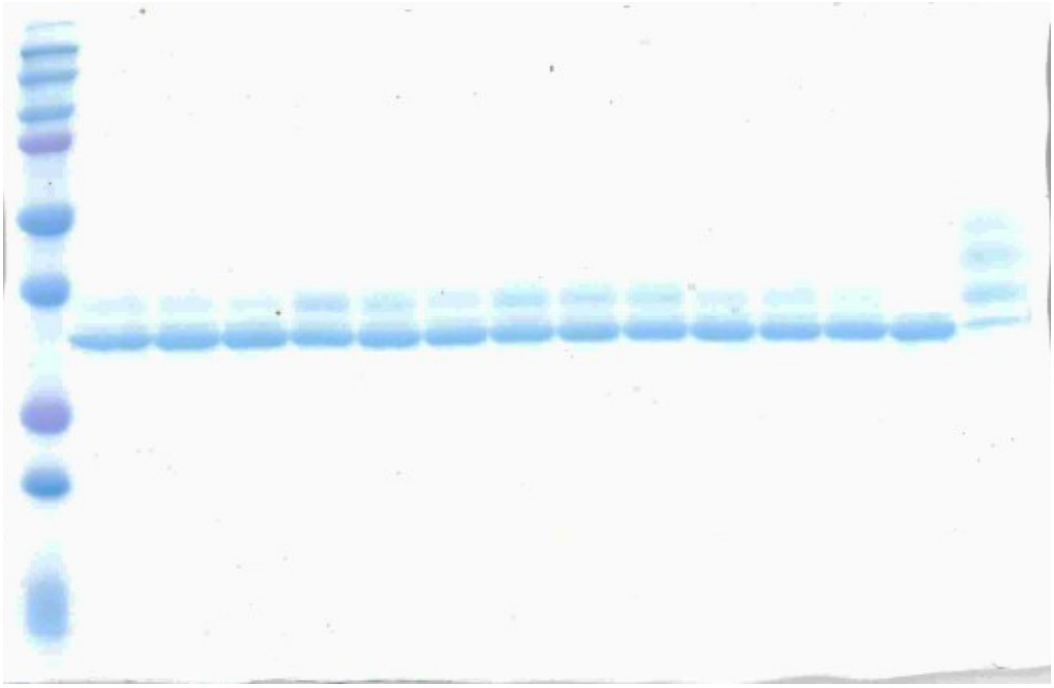


Figure S64. Raw gel image of Figure S5.

Figure S65. Raw gel image of Figure S6. Only the first 7 bands after the ladder.

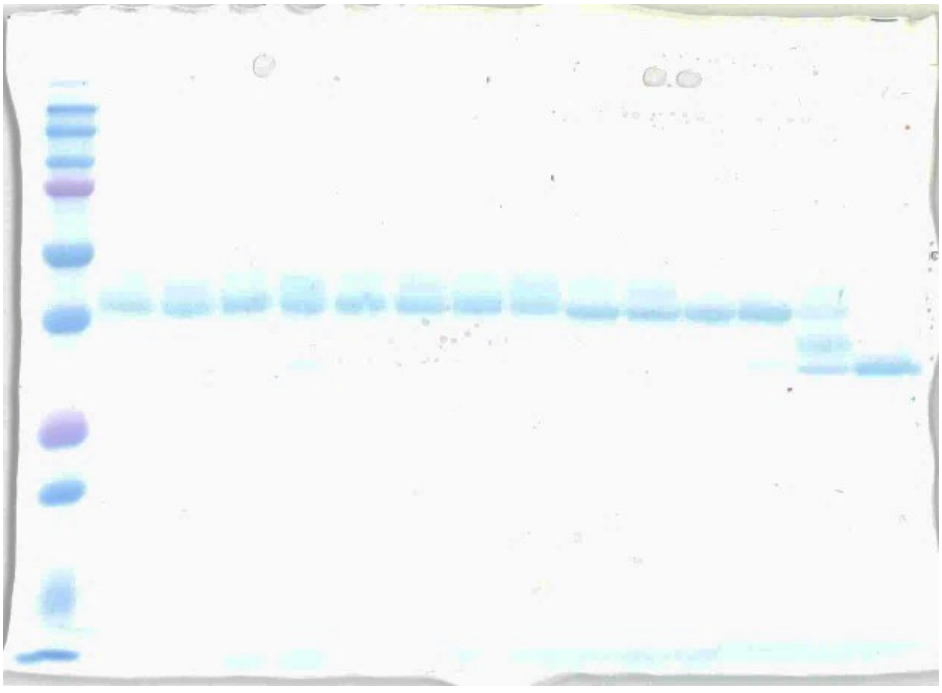


Figure S66. Raw gel image of Figure S8.

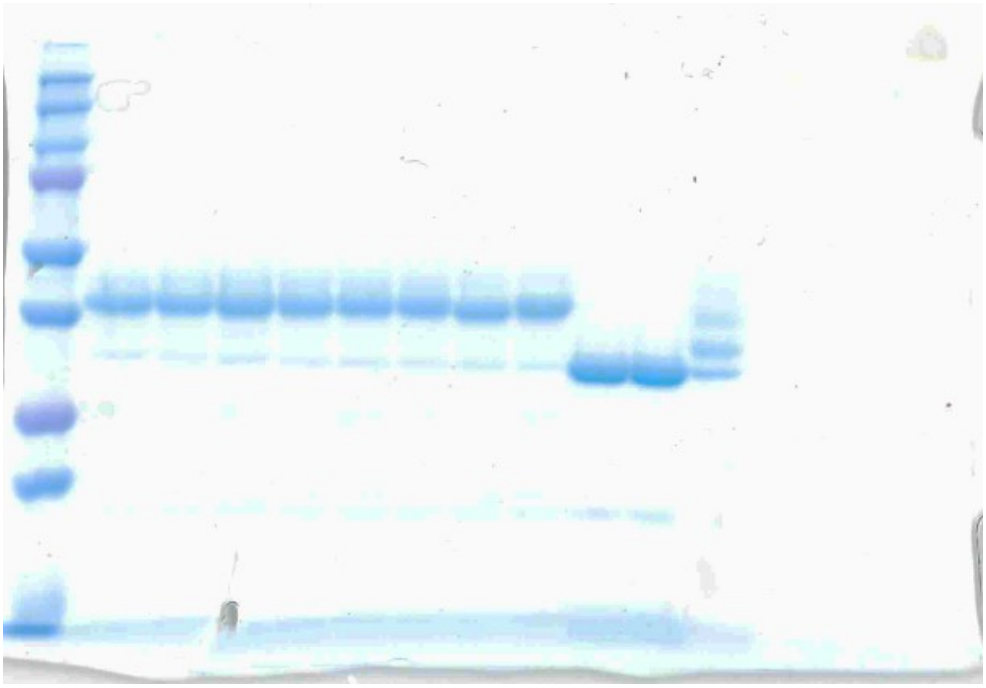


Figure S67. Raw gel image of Figure S9.

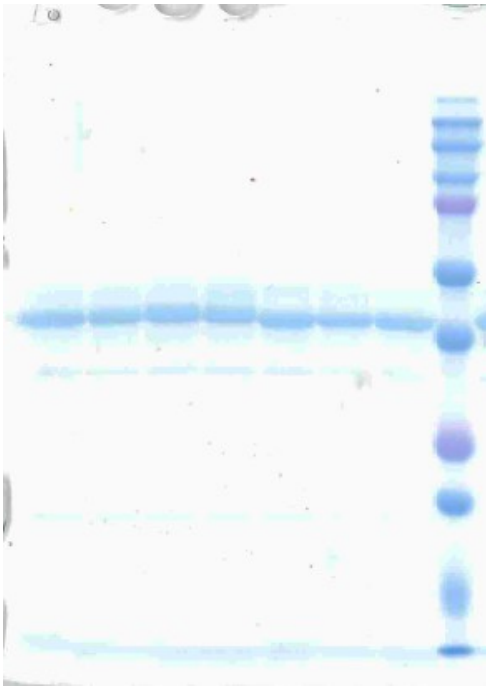


Figure S68. Raw gel image of Figure S10.

Figure S69. Raw gel image of Figure S11.

Figure S70. Raw gel image of Figure S12. Only the last 7 bands after the ladder.

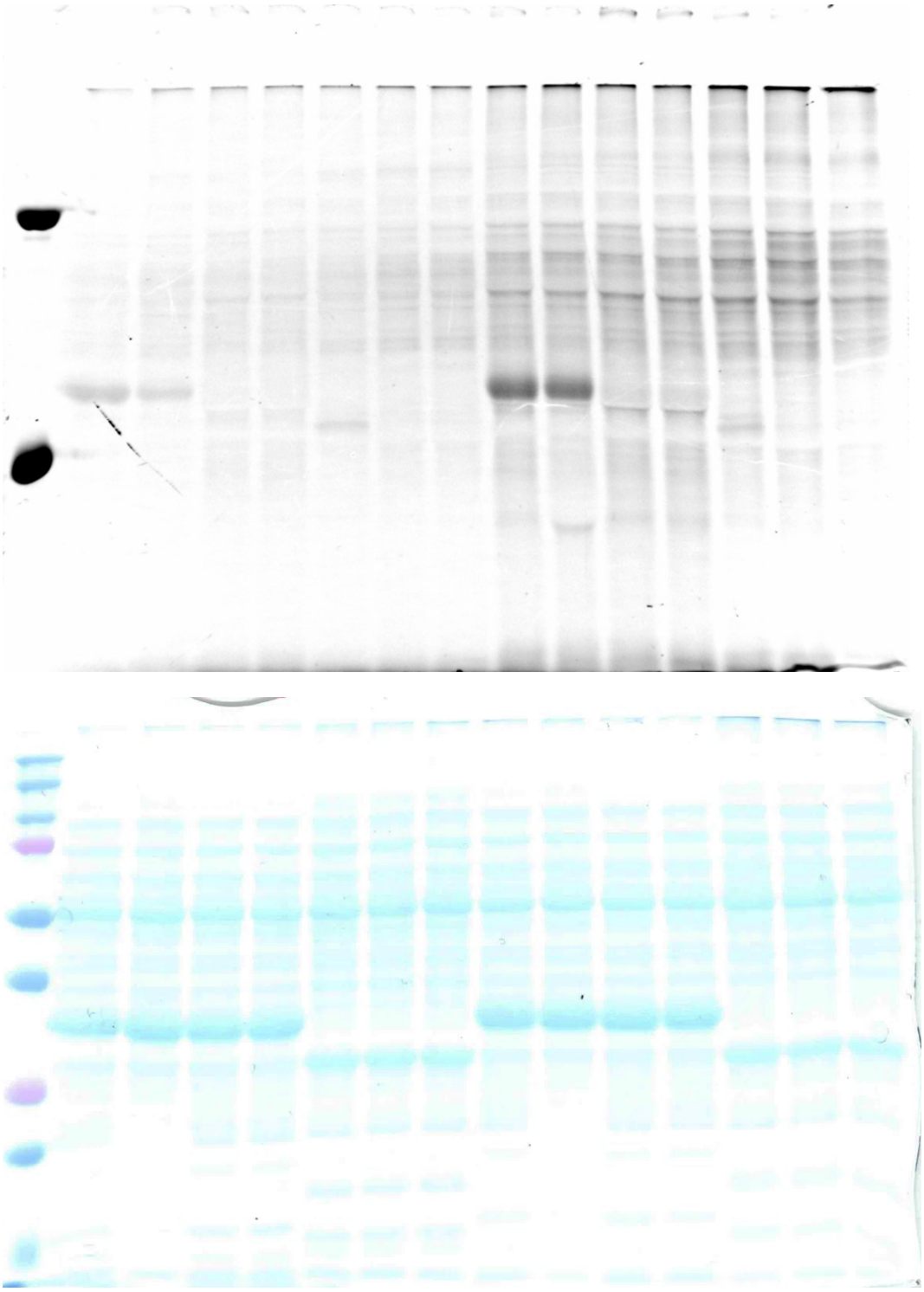


Figure S71. Raw gel images of Figure S19.

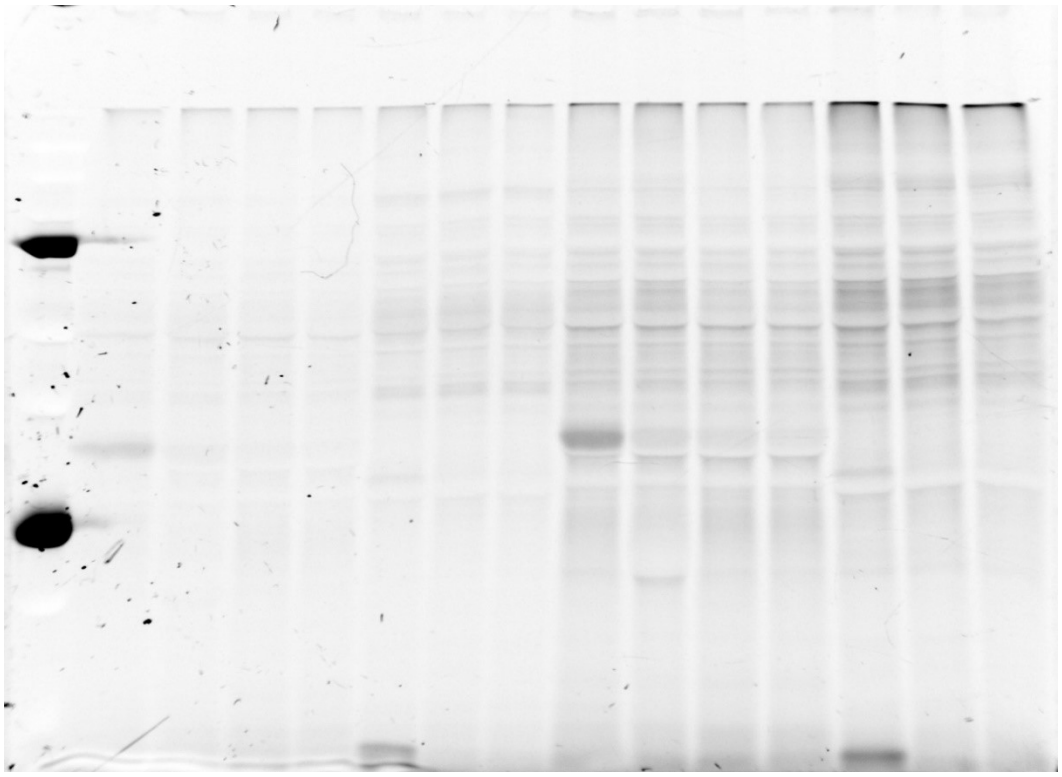


Figure S72. Raw gel images of Figure S20.

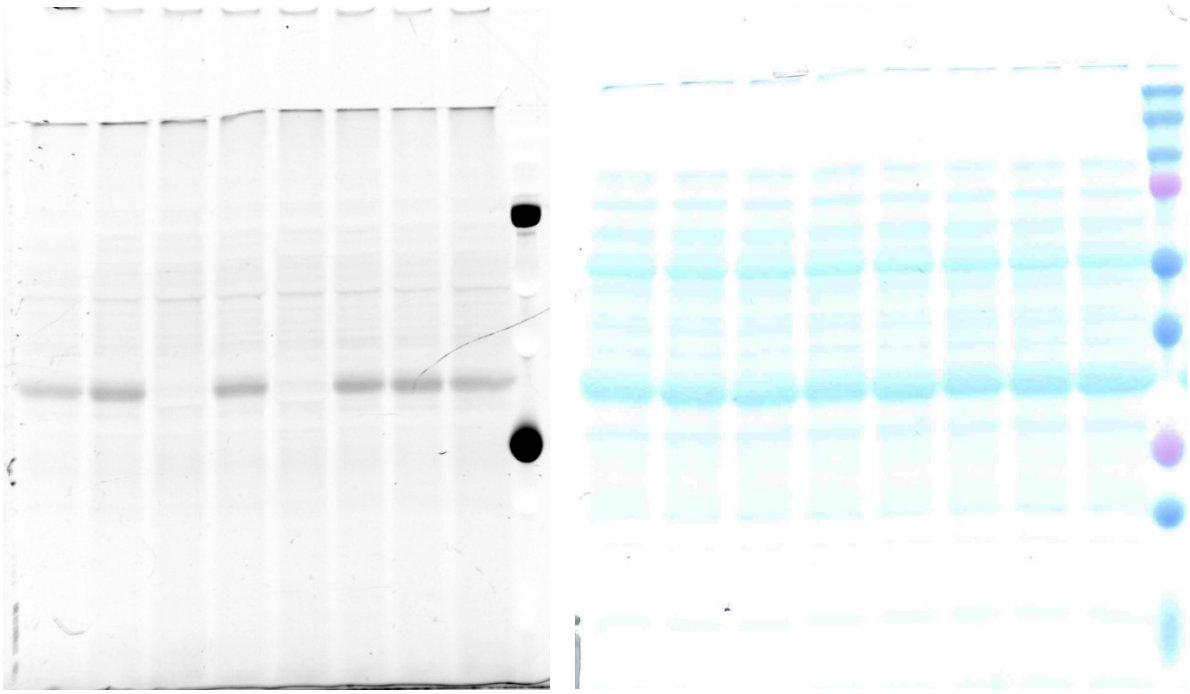


Figure S73. Raw gel images of Figure S21.