Supporting Information

for

Site-Selective and Inducible Acylation of Thrombin using Aptamer-Catalyst Conjugates

by

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Table of Contents

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

BSA = Bovine Serum Albumin

$CDCl_3$ = deuterated chloroform

ddH_2O = double-distilled water

DMAP = dimethylaminopyridine

DCM = dichloromethane

DIPEA = *N,N*-diisopropylethylamine

DMSO = dimethyl sulfoxide

DTT = dithiothreitol

EtOAc = ethyl acetate

ESI = Electron Spray Ionisation

FA = formic acid

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC = high-performance liquid chromatography

IAA = iodoacetic acid

LC-MS = liquid chromatography–mass spectrometry

^{LC} = on the light chain

MeOD = methanol-d_4

PyOx = pyridinium oxime

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

Temp = template

Thrombin = human alpha thrombin

THPTA = Tris(3-hydroxypropyltriazolylmethyl)amine

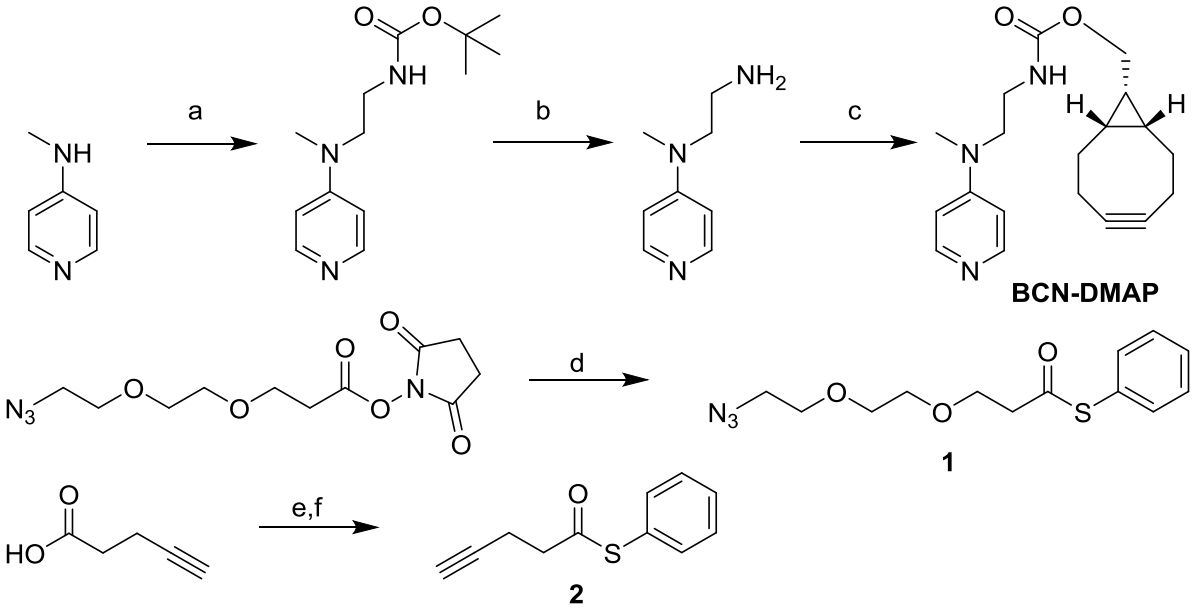
General Information

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.
- Sulforhodamine B PEG_3 azide was purchased from Tenova Pharmaceuticals.
- BCN-PEG(2000) was purchased from Synaffix BV.
- Lysozyme was purchased from Sigma Aldrich.
- Human alpha-thrombin was purchased from Haematologic Technologies.
- BSA was purchased from Millipore Corporation.
- Trastuzumab was purchased from Bio-connect Services BV.
- Trypsin Gold was purchased from PROMEGA BENELUX BV.
- DNA strands were purchased from Integrated DNA technologies.
- BCN-DNA was purchased from Eurogentec.
- MabPac™ RP Column (4 µm, 3.0 mm x 100 mm) was purchased from Thermo Fischer.
- Reprosil-Gold 300 C4, 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).

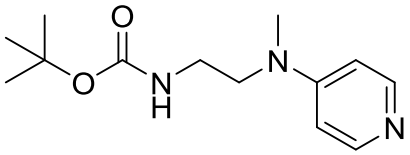
Experimental Procedures

2.1 Synthesis of Organic Compounds



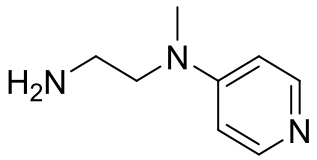
Scheme S1. Synthesis of BCN-DMAP and thioesters **1** and **2**. (a) NaH, *tert*-butyl (2-bromoethyl)carbamate, anh. THF, rt, 24 h, **9%**; (b) TFA (60 vol%), DCM, rt, 36 h, **quant**; (cBCN-succinimidyl carbonate, NEt_3, anh. THF, rt, 18 h, **69%**; (d) thiophenol, toluene, rt, 16 h, **85%**; (e) Isobutyl chloroformate, NEt_3, DCM, rt, 10 min; (f) thiophenol, NEt_3, DCM, rt, 16 h, **54%**.

2.1.1 Boc-ethylamine-DMAP:



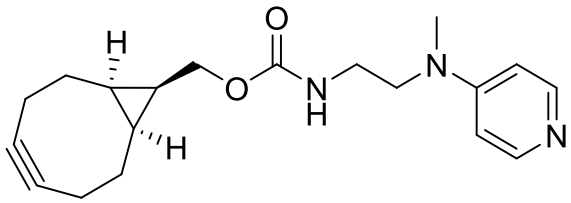
4-(methylamino)-pyridine (200 mg, 1.85 mmol, 1.0 eq) and NaH (592 mg, 14.8 mmol, 8.0 eq) were dissolved in 2 mL of anh. THF and the mixture stirred at rt for 90 min until H_2 evolution was no longer evident. To this, *tert*-butyl (2-bromoethyl)carbamate (622 mg, 2.77 mmol, 1.5 eq), which was dissolved in 1 mL anh. THF, was added and the mixture was stirred at rt for 24 h. The reaction was quenched with saturated NH_4Cl solution (5 mL) and the product was extracted with DCM (6 × 10 mL). The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The target compound was purified via flash column chromatography (SiO_2, eluent: 5–9% [10% (25% NH_3 in H_2O) in MeOH] in DCM), yielding a white solid (41 mg, 9%). HRMS (ESI): calculated for $[M+H]^+$ 252.1712; found: 252.1709. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 5.8 Hz, 2H), 6.51 (d, *J* = 5.8 Hz, 2H), 4.86 (s, 1H), 3.48 (dd, *J* = 12.2, 5.8 Hz, 2H), 3.28 (q, *J* = 6.4 Hz, 2H), 2.97 (s, 3H), 1.41 (s, 9H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.1, 153.7, 149.7, 106.5, 79.5, 50.7, 37.9, 37.6, 28.4 ppm.

2.1.2 ethylamine-DMAP:



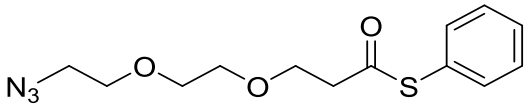
Tert-butyl (2-(methyl(pyridin-4-yl)amino)ethyl)carbamate (41 mg, 163 µmol, 1.0 eq) was dissolved in 1.2 mL CH_2Cl_2 and cooled to 0 °C. Trifluoroacetic acid (1.8 mL 60 vol%) was added dropwise and the resulting mixture was stirred at rt for 36 h. The mixture was concentrated under reduced pressure and the target compound was purified via flash column chromatography [SiO_2, eluent: 6–14% (10% NH_3 in MeOH) in DCM], yielding a light-brown sticky oil (20 mg, 81%). ^{1}H NMR (400 MHz, D_2O) δ 8.04–7.97 (m, 2H), 6.61 (d, *J* = 6.0 Hz, 2H), 3.41 (t, *J* = 6.7 Hz, 2H), 2.94 (s, 3H), 2.79 (t, *J* = 6.2 Hz, 2H) ppm. ^{13}C NMR (101 MHz, D_2O) δ 156.3, 147.8, 106.1, 53.0, 37.7, 35.8 ppm.

2.1.3 BCN-DMAP:



N^1-methyl-N^1-(pyridin-4-yl)ethane-1,2-diamine (20 mg, 132 µmol, 1.0 eq) and NEt_3 (148 µL, 1.06 mmol, 8.0 eq) were dissolved in 0.7 mL anh. THF and stir for 10 min. BCN-succinimidyl carbonate (47.8 mg, 164 µmol, 1.24 eq) was dissolved in 0.7 mL anh. THF and added dropwise to the solution. The mixture was allowed to warm up to rt and stirred for 18 h. The mixture was concentrated under reduced pressure and the target compound was purified via flash column chromatography (SiO_2, eluent: 5–7% [10% (25% NH_3 in H_2O) in MeOH] in DCM), yielding a colourless sticky oil (30 mg, 69%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19–8.11 (m, 2H), 6.54–6.48 (m, 2H), 5.27 (t, *J* = 6.0 Hz, 1H), 4.11 (d, *J* = 8.1 Hz, 2H), 3.48 (t, *J* = 6.7 Hz, 2H), 3.33 (q, *J* = 6.5 Hz, 2H), 2.97 (s, 3H), 2.32–2.03 (m, 6H), 1.53 (q, *J* = 11.2, 10.4 Hz, 2H), 1.30 (p, *J* = 8.7 Hz, 1H), 0.90 (t, *J* = 10.0 Hz, 2H) ppm.^{13}C NMR (101 MHz, $CDCl_3$) δ 157.0, 153.7, 149.7, 106.6, 98.9, 63.1, 50.7, 38.2, 37.7, 29.1, 21.5, 20.2, 17.8 ppm.

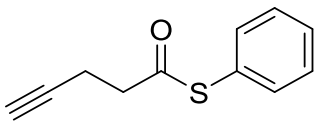
2.1.4 azido-thioester (**1**):



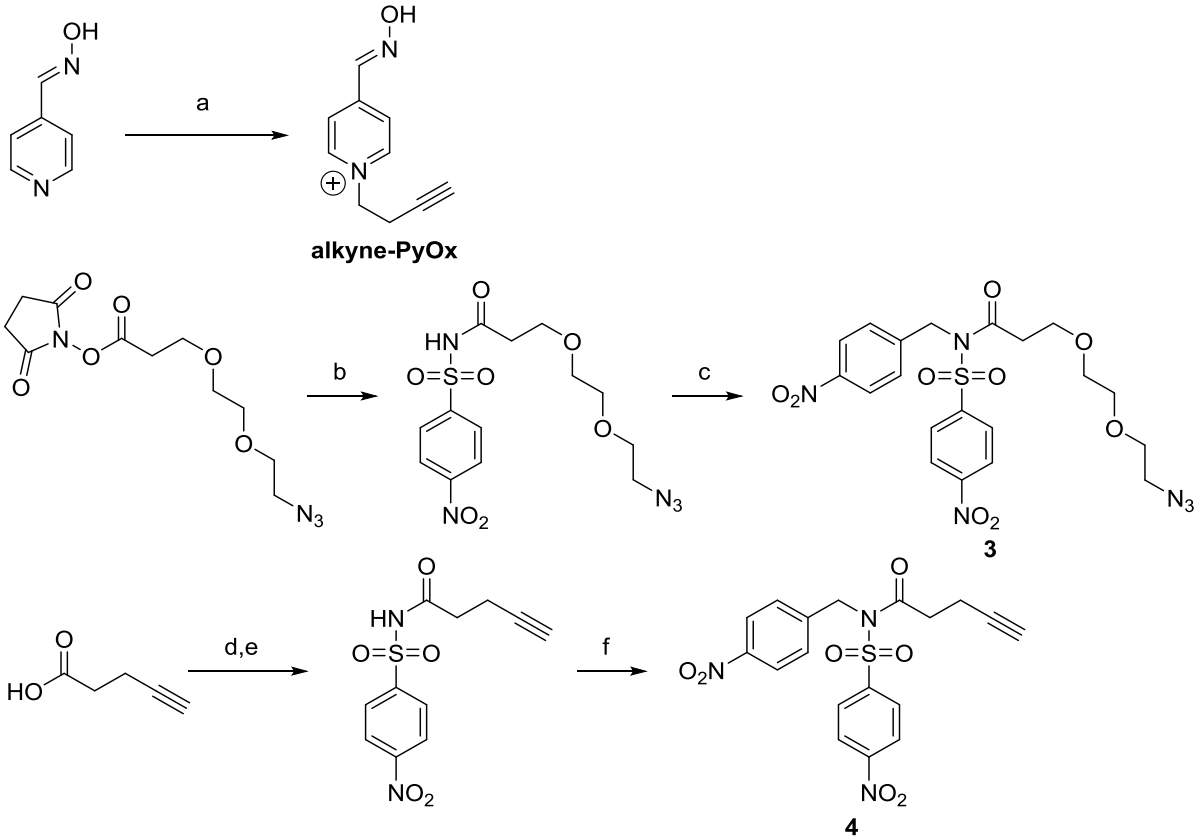
2,5-dioxopyrrolidin-1-yl 3-(2-(2-azidoethoxy)ethoxy)propanoate (150 mg, 500 µmol, 1.03 eq) was dissolved in 2 mL toluene. Thiophenol (49 µL, 480 µmol, 1.0 eq) was added. Triethylamine (81 µL, 580 µmol, 1.2 eq) was added dropwise. The mixture was stirred at rt for 16 h. The reaction was quenched with 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.

2.1.5 alkyne-thioester (**2**):

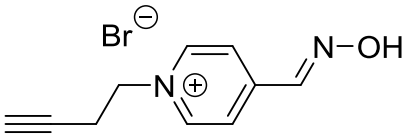


Pent-5-ynoic acid (1.03 g, 10 mmol, 1.0 eq) were dissolved in 40 mL DCM. Isobutyl chloroformate (1.5 mL, 11 mmol, 1.1 eq) and triethylamine (1.4 mL, 10 mmol, 1.0 eq) were added and the mixture was stirred at rt for 10 min under argon atmosphere. Thiophenol (2.1 mL, 20 mmol, 2.0 eq) and triethylamine (1.4 mL, 10 mmol, 1.0 eq) were added and the mixture was stirred at rt for 16 h. The mixture was filtered, the solids washed with DCM. The mixture was washed with 1M $KHSO_4$, water and brine. The organic layer was then dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via automated flash column chromatography (SiO_2, 5% EtOAc in petroleum ether (40/60)), yielding a yellow oil (1.04 g, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (s, 5H), 2.90 (dd, *J* = 7.9, 6.8 Hz, 2H), 2.58 (td, *J* = 7.4, 2.6 Hz, 2H), 2.02 (t, *J* = 2.7 Hz, 1H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.4, 135.8, 129.7, 129.4, 127.4, 82.0, 69.6, 42.2, 14.7 ppm.



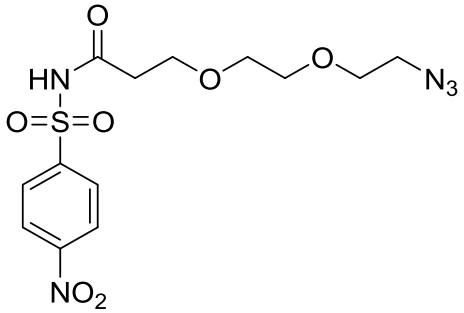
Scheme S2. Synthesis of alkyne-PyOx and ANANS **3** and **4**. (a) 4-bromobut-1-yne, anh. ACN, 82°C, 25 h, **92%**; (b) 4-nitrobenzenesulfonamide, DIPEA, DCM, rt, 20 h, **90%**; (c) DIPEA, 1-(bromomethyl)-4-nitrobenzene, anh. THF, 50°C, 17 h, **72%**; (d) isobutyl chloroformate, NEt_3, DCM, rt, 10 min; (e) 4-nitrobenzenesulfonamide, NEt_3, DCM, rt, 18 h, **11%**; (f) DIPEA, 1-(bromomethyl)-4-nitrobenzene, anh. THF, 50 °C, 18 h, **72%**.

2.1.6 alkyne-functionalized PyOx:



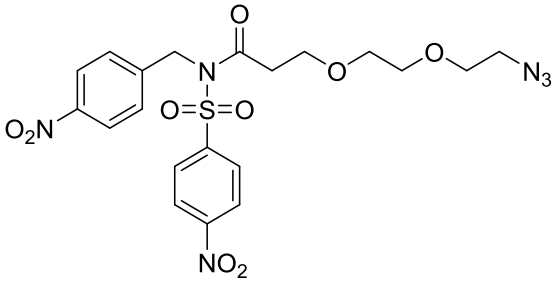
Pyridine-4-aldoxime (370 mg, 3.03 mmol, 1.0 eq) and 4-bromobut-1-yne (600 µL, 6.67 mmol, 2.2 eq) were dissolved in 6 mL anh. ACN and the mixture was refluxed for 25 h. The mixture was filtered, the solids washed with ACN and the residues was left to dry overnight, yielding a light-brown solid (488 mg, 92%). HRMS (ESI) calculated for $[M]^+$: 175.0871; found $[M]^+$: 175.0863. ^{1}H NMR (400 MHz, D_2O) δ 8.96–8.89 (m, 2H), 8.42 (s, 1H), 8.29–8.22 (m, 2H), 4.77 (s, 2H), 3.01 (td, *J* = 6.3, 2.6 Hz, 2H), 2.54 (q, *J* = 2.6, 2.2 Hz, 1H) ppm. ^{13}C NMR (101 MHz, D_2O) δ 150.0, 146.3, 144.0, 125.7, 78.6, 74.7, 59.3, 20.0 ppm.

2.1.7 azido-ANANS precursor:



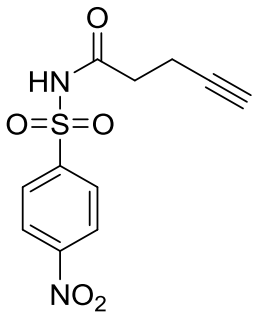
4-nitrobenzenesulfonamide (162 mg, 799 µmol, 1.2 eq) was dissolved in 1 mL DCM and *N,N*-diisopropylethylamine (232 µL, 1.33 mmol, 2.0 eq) was added to the mixture and stirred for 5 min. 2,5-dioxopyrrolidin-1-yl 3-(2-(2-azidoethoxy)ethoxy)propanoate (200 mg, 666 µmol, 1.0 eq) was added and the mixture 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.

2.1.8 azido-ANANS (**3**):



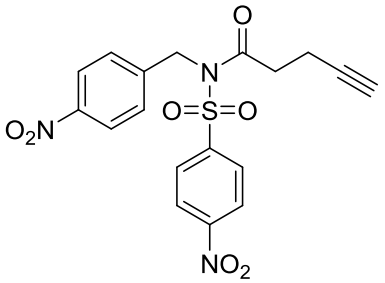
3-(2-(2-azidoethoxy)ethoxy)-*N*-((4-nitrophenyl)sulfonyl)propanamide (120 mg, 307 µmol, 1.0 eq) was dissolved in 1 mL anh. THF. *N,N*-diisopropylethylamine (268 µL, 1.54 mmol, 5.0 eq) was added to the mixture and stirred at rt for 5 min. 1-(bromomethyl)-4-nitrobenzene (265 mg, 1.23 mmol, 4.0 eq) was 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.

2.1.9 alkyne-ANANS precursor:



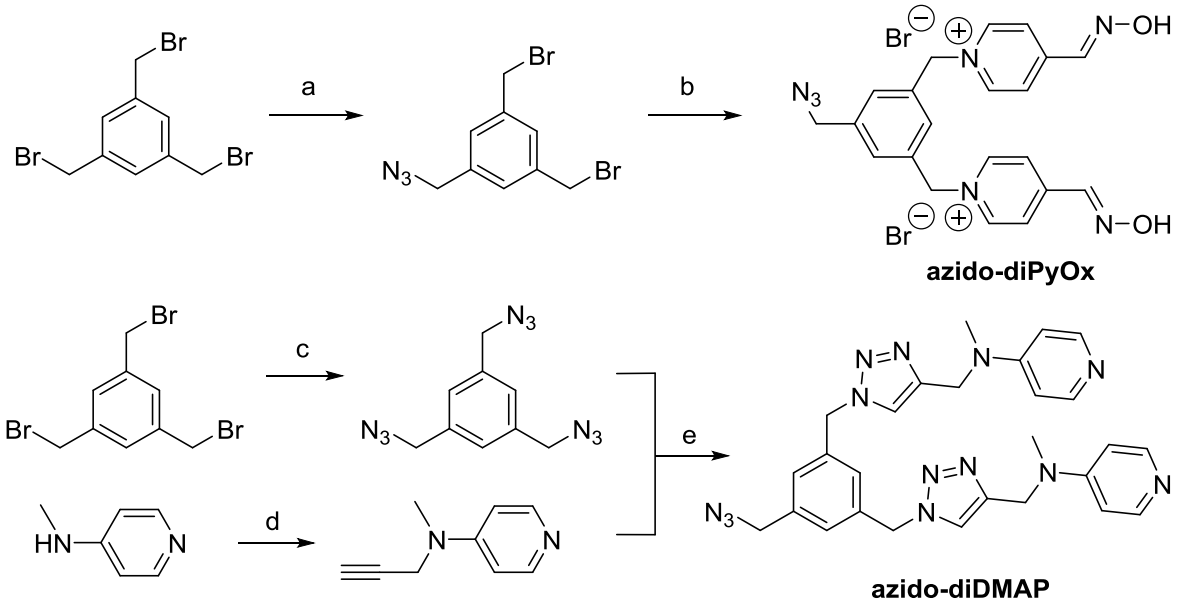
Pent-5-ynoic acid (0.5 g, 5 mmol, 1 eq) were dissolved in 20 mL DCM. Isobutyl chloroformate (0.75 mL, 5.5 mmol, 1.1 eq) and triethylamine (0.7 mL, 5 mmol, 1 eq) were added and the mixture was stirred at rt for 10 min under argon atmosphere. 4-nitrobenzenesulfonamide (1.31 g, 6.50 mmol, 1.3 eq) and triethylamine (0.7 mL, 5 mmol, 1 eq) were added to the mixture and stirred at rt for 18 h. The mixture was filtered and concentrated under reduced pressure. The solution was purified via automated flash column chromatography (SiO_2, 0–5% [10% (25% NH_3 in H_2O) in MeOH] 5% MeOH in DCM), yielding a white solid (157 mg, 11%). ^{1}H NMR (400 MHz, MeOD) δ δ 8.42–8.37 (m, 2H), 8.25–8.18 (m, 2H), 2.44 (td, *J* = 6.8, 1.5 Hz, 2H), 2.39–2.32 (m, 2H), 2.19 (t, *J* = 2.6 Hz, 1H) ppm. ^{13}C NMR (101 MHz, MeOD) δ 174.4, 151.7, 147.7, 130.5, 124.9, 83.1, 69.8, 36.3, 13.8 ppm.

2.1.10 alkyne-ANANS (**4**):



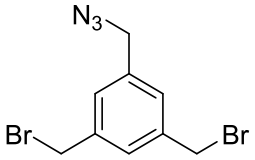
N-((4-nitrophenyl)sulfonyl)pent-4-ynamide (157 mg, 556 µmol, 1.0 eq) and was dissolved in 4 mL anh. THF. *N,N*-diisopropylethylamine (0.5 mL, 3 mmol, 5 eq) was added to the mixture and stirred at rt for 10 min. 1-(bromomethyl)-4-nitrobenzene (481 mg, 2.23 mmol, 4.0 eq) was added to the solution and the mixture was stirred at 50°C for 18 h. The mixture was washed with brine (10 mL)

and extracted with EtOAc (3 × 8 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified via automated flash column chromatography (SiO_2, 20–40% EtOAc in petroleum ether(40-60)) yielding a pale-yellow solid (168 mg, 72%). HRMS (ESI) calculated for $[M+Na]^+$: 440.0528; found $[M+Na]^+$: 440.0511. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48–8.32 (m, 2H), 8.23 (dt, *J* = 6.9, 3.0 Hz, 2H), 8.14–8.03 (m, 2H), 7.56 (dt, *J* = 6.6, 3.1 Hz, 2H), 5.18–5.12 (m, 2H), 2.82 (td, *J* = 7.0, 3.0 Hz, 2H), 2.45 (tt, *J* = 6.4, 3.1 Hz, 2H), 1.89 (q, *J* = 3.0 Hz, 1H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 150.9, 147.9, 144.5, 143.0, 129.4, 128.5, 124.8, 124.3, 81.8, 69.7, 49.5, 35.8, 14.1 ppm.



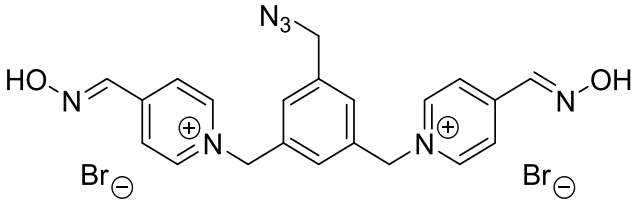
Scheme S3. Synthesis of the divalent catalysts. (a) NaN_3, DMF, rt, 16 h, **57%**; (b) pyridine-4-aldoxime, ACN, 65°C, 32 h, **64%**. (c) NaN_3, DMF, rt, 16 h, **98%**; (d) n-BuLi, propargylbromide, anh. THF, -80°C-0°C, 30 min, **57%**; (e) $Cu(I)(ACN)_4PF_6$, O_2-poor THF, rt, 18 h, **11%**.

2.1.11 1-(azidomethyl)-3,5-bis(bromomethyl)benzene:



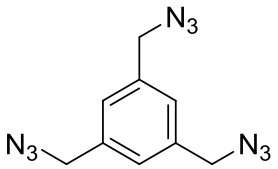
1,3,5-tris(bromomethyl)benzene (30 mg, 84 µmol, 1.0 eq) and sodium azide (5.5 mg, 84 µmol, 1.0 eq) were dissolved in 100 µL DMF and stirred at rt for 16 h. The mixture was blow-dried and the residue dissolved in 400 µL DCM. The products were separated by means of preparative TLC (5% diethylether in petroleum 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.

2.1.12 azido-diPyOx:



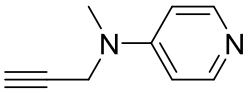
1-(azidomethyl)-3,5-bis(bromomethyl)benzene (8.0 mg, 25 µmol, 1.0 eq) and pyridine-4-aldoxime (15 mg, 125 µmol, 5 eq.) were dissolved in 500 µL ACN and the mixture was stirred at 65 °C for 32 h. The mixture was transferred to an Eppendorf tube, washing the vessel with ACN and centrifuged with a tabletop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

2.1.13 1,3,5-tris(azidomethyl)benzene:



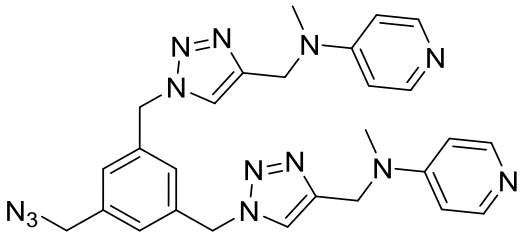
1,3,5-tris(bromomethyl)benzene (200 mg, 560 µmol, 1.0 eq) and sodium azide (219 mg, 3.36 mmol, 6.0 eq) were dissolved in 0.5 mL DMF and stirred at rt for 16 h. The mixture was blow-dried and the residue dissolved in 1 mL DCM. The mixture was washed with H_2O and extracted with DCM (3 × 5mL). The organic 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.

2.1.14 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.

2.1.15 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) hexafluorophosphate (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.

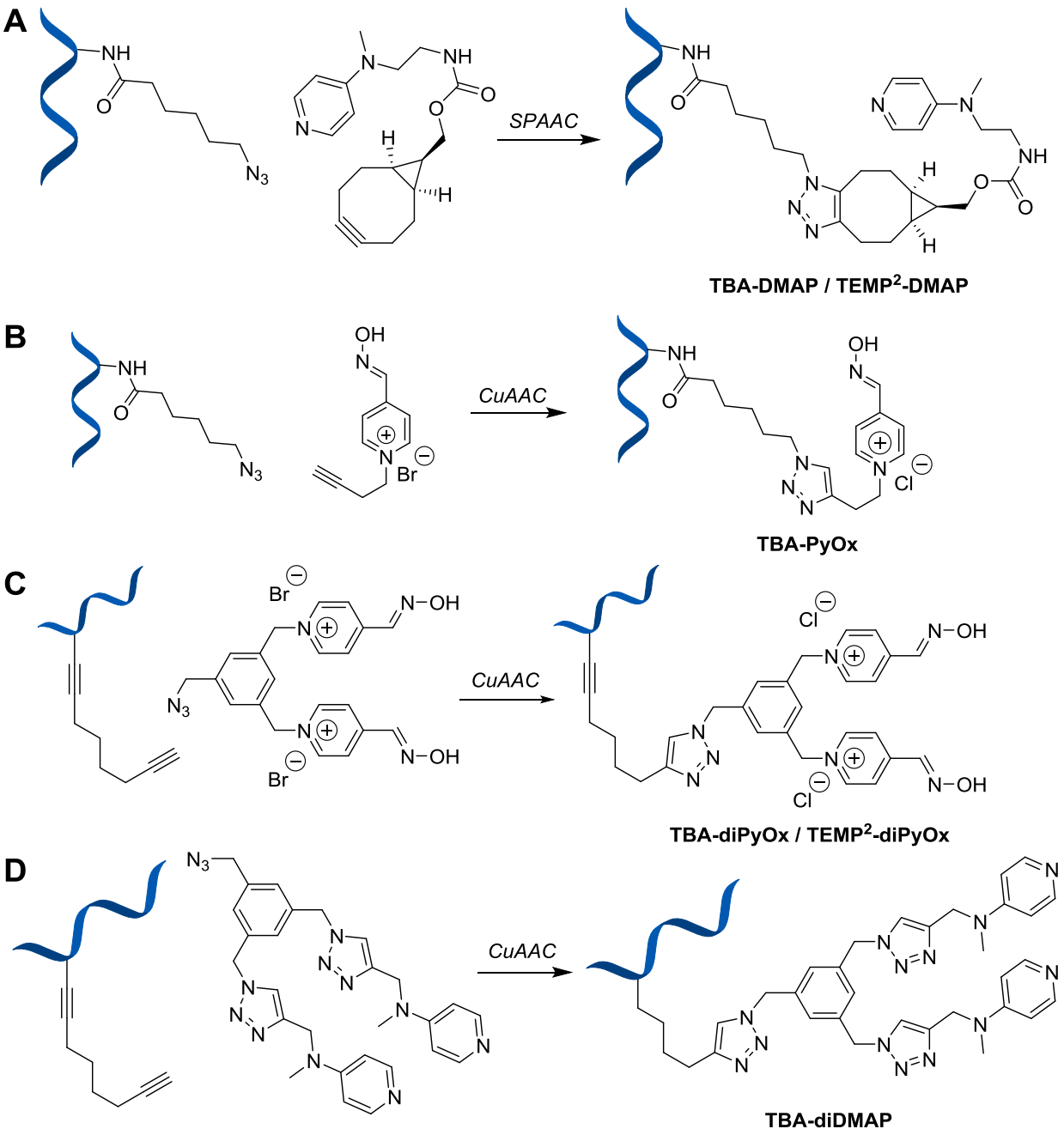
2.2 Synthesis of DNA constructs

2.2.1 Synthesis of TBA-DMAP constructs

DNA sequences with azido-thymine modification were purchased as HPLC-purified lyophilized powders from Integrated DNA Technologies (IDT). The DNA was treated with 50 equivalents of compound **1** with respect to the DNA concentration and the reaction mixture was incubated in the dark at 4 °C for 16-20 hours. The synthesized DNA construct was purified by spin-filtration over 3 kDa MWCO Amicon® Ultra-15 Centrifugal Filter Units, washing 5 times with 400 mM NaCl solution in ddH_2O. Purity and concentration were determined by HPLC-MS and UV-Vis.

2.2.2 Synthesis of DNA-PyOX, DNA-diPyOx and DNA-diDMAP

DNA sequences with azido/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 **4**, **7** or **8** (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 DNA 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 constructs. (**A**) Commercially available DNA-azide and BCN-DMAP are coupled via strain-promoted alkyne-azide click chemistry. (**B**) DNA-azide and alkyne-PyOx are coupled via copper-catalyzed alkyne-azide click chemistry. (**C**) DNA-alkyne and azido-diPyOx or (**D**) azido-diDMAP are coupled via copper-catalyzed alkyne-azide click chemistry.

2.3 Protein Modification Studies

2.3.1 Protocol for modification of thrombin with 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, 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 (reaction times: 2 hrs for DMAP catalyst, 5 hrs for PyOx).

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.

2.3.2 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, 1.2 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.

2.3.3 Protocol for SDS-PAGE analysis

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.

2.3.4 Protocol for the analysis of protein 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, the gradient varying per protein. For thrombin, the 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.

2.3.5 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).

2.4 Primary supporting data

2.4.1 Overview of modified nucleotides and their proximal AA residues

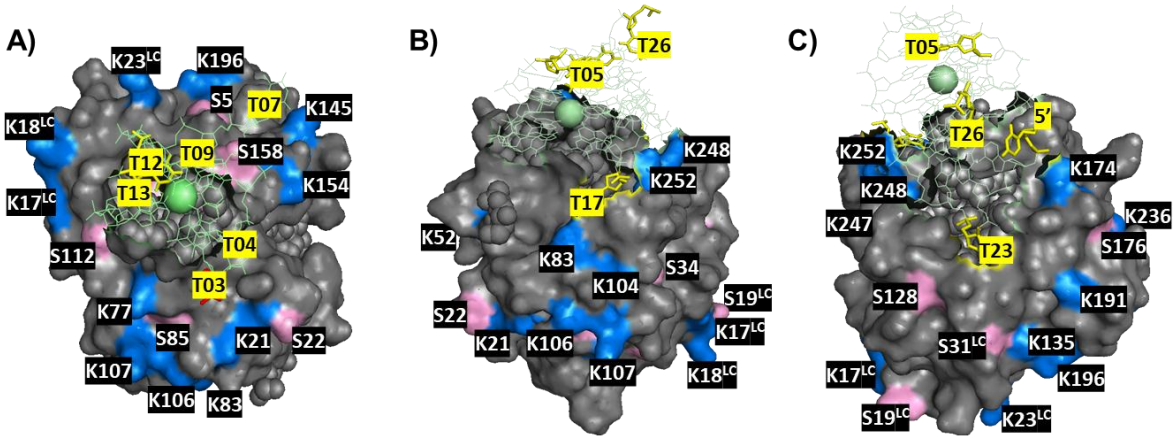


*Figure S1. Crystal structures of thrombin with aptamers. (**A**) TBA and its proximal residues and (**B/C**) TBA2 and proximal residues on either side of the aptamer. Lysine residues are blue, serine residues are pink, aptamer is light green. Nucleobases to which catalysts were attached are yellow. (PDB-code: 5EW1)*[16]

2.4.2 TBA-DMAP conversions & tryptic digestion data

*Table S1. Conversions (calculated from SDS-PAGE data) and targeted residues for the different TBA-DMAP constructs, in comparison to the absence of catalyst or with free DMAP. The red X highlights modifications that are also found in the absence of catalyst or with free DMAP and are background acylation. Conditions: 10 µM thrombin, 30 µM catalyst, 150 µM azide-thioester **1**, pH: 8.0, 37 °C, 2 h.*

Catalyst	Conv. (%)	K21	K77	K83	K106	K107	K145	K154	K196	K236	K252	$K18^{LC}$	$K23^{LC}$
-	5-8			X			X		X	X	X		X
free DMAP	5-10			X			X		X	X	X		X
TBA^{3}-DMAP	27	X		X	X	X	X	X	X	X	X		X
TBA^{4}-DMAP	12	X		X	X				X	X	X		X
TBA^{7}-DMAP	16			X			X	X	X	X	X		X
TBA^{9}-DMAP	8			X					X	X	X		X
TBA^{12}-DMAP	36-49		X	X	X	X			X	X	X	X	X
TBA^{13}-DMAP	17		X	X	X	X			X	X	X	X	X

2.4.3 TBA-DMAP and TBA2-DMAP conversions

*Table S2. Conversions (calculated from SDS-PAGE data) of all DMAP and diDMAP constructs as calculated from SDS-PAGE results using ImageJ. Conditions: 10 µM thrombin, 30 µM catalyst, azido-thioester **1**, pH: 8.0, 37 °C, 2 h.*

Catalyst	1 (µM)	Conv (%)	Catalyst	1 (µM)	Conv (%)
-	150	5-8	-	300	12-20
DMAP	150	5-10	DMAP	300	18-20
TBA^{3}-DMAP	150	27	TBA^{12}-DMAP	300	53-61
TBA^{4}-DMAP	150	12	TBA^{12}-diDMAP	300	59-65
TBA^{7}-DMAP	150	16	TBA2^{1}-DMAP	300	22
TBA^{9}-DMAP	150	8	TBA2^{5}-DMAP	300	12
TBA^{12}-DMAP	150	36-49	TBA2^{8}-DMAP	300	21
TBA^{13}-DMAP	150	17	TBA2^{17}-DMAP	300	14-18
TBA^{12}-diDMAP	150	48	TBA2^{18}-DMAP	300	19
TBA2^{17}-DMAP	150	5	TBA2^{23}-DMAP	300	20
TBA2^{17}-diDMAP	150	9	TBA2^{26}-DMAP	300	10
			TBA2^{17}-diDMAP	300	15

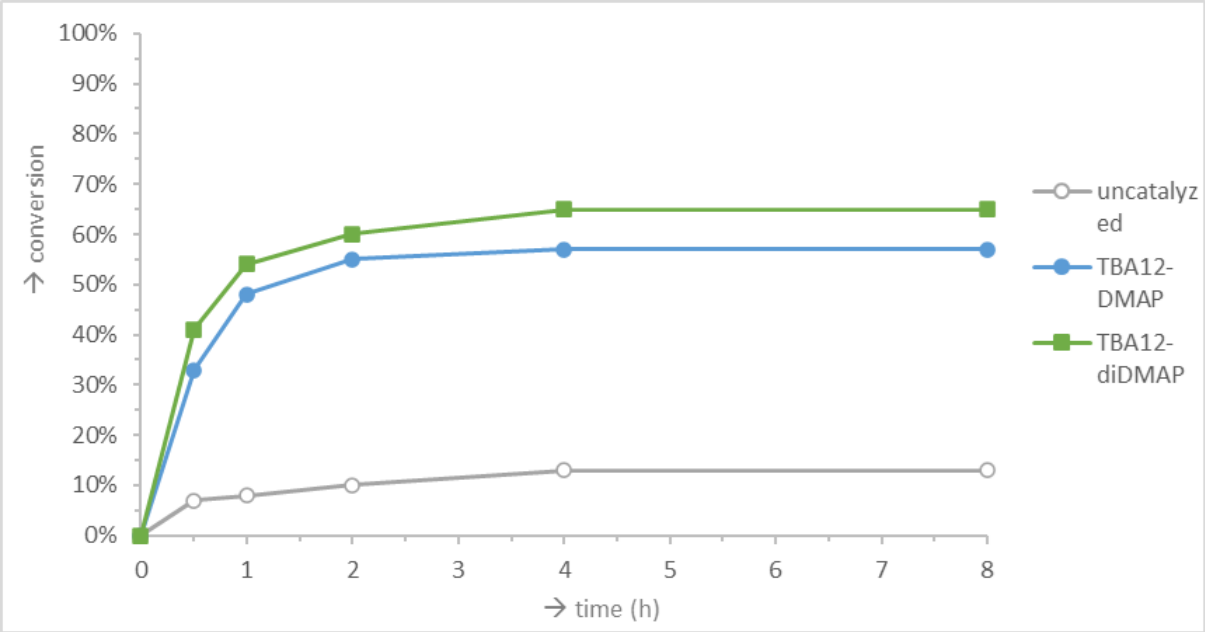


*Figure S2. Kinetics graph of uncatalyzed acylation with thioester **1** compared to TBA^{12}-DMAP and TBA^{12}-diDMAP driven acylation of thrombin. After 2 h, the reaction appears to be mostly complete.*

2.4.4 TBA-PyOx and TBA-diPyOx conversions & tryptic digestion data

*Table S3. Details of the modification by the various catalyst-aptamer constructs, including conversion and the position(s) of the modification(s). The red X highlights modifications that are also found in the absence of catalyst or with free PyOx and are background acylation. Conditions: 10 µM thrombin, 30 µM catalyst, 300 µM azido-ANANS **3**, 37 °C, 6 h.*

pH	Catalyst	Conv. (%)	K21	K77	K83	K106	K107	K145	K154	K196	K18[LC]	K23[LC]	S5	S22	S158
7.2	free PyOx	0					X	X							
	TBA^{3}-PyOx	18	X	X	X	X	X	X	X					X	X
	TBA^{12}-PyOx	28	X	X	X	X	X	X	X					X	X
	TBA^{3}-diPyOx	71-93	X			X	X	X		X		X	X	X	X
	TBA^{12}-diPyOx	75-91	X	X			X	X	X	X	X	X		X	X
	$TBA^{3,12}$-bis(diPyOx)	73	not analyzed												
8.0	free PyOx	2		X			X	X							
	TBA^{3}-PyOx	29	X	X	X	X	X	X	X		X			X	X
	TBA^{12}-PyOx	38	X	X	X	X	X	X	X		X			X	X
	TBA^{12}-diPyOx	83	X	X		X	X	X	X	X	X	X			X
	$TBA^{3,12}$-bis(diPyOx)	84	not analyzed												

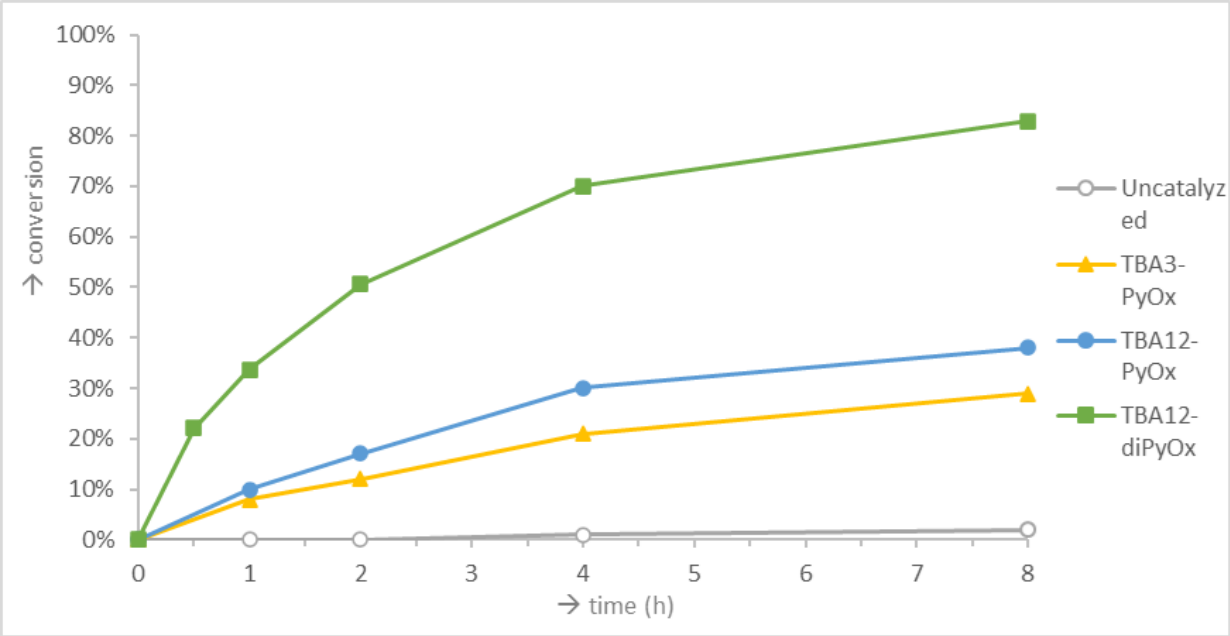


*Figure S3. Kinetics graph of uncatalyzed acylation with ANANS **3** compared to TBA^{3}-PyOx, TBA^{12}-PyOx and TBA^{12}-diPyOx driven acylation of thrombin. After 8 h, the reaction is still ongoing, but detectable levels of uncatalyzed acylation appears.*

2.4.5 TBA-diPyOx competitive assay

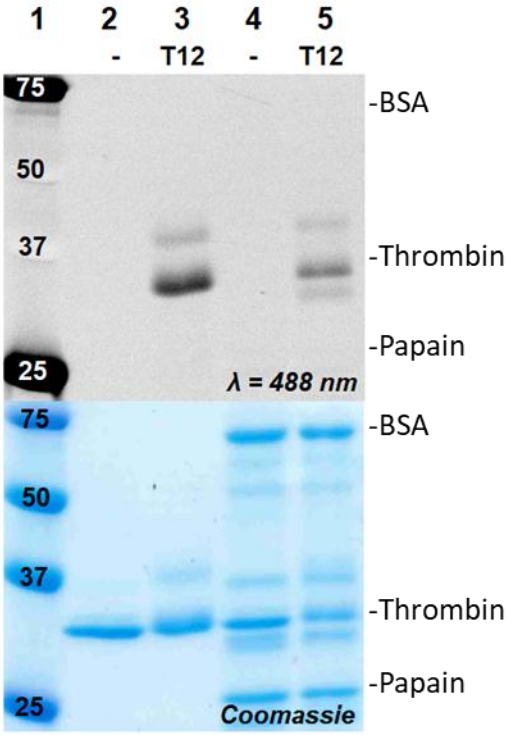


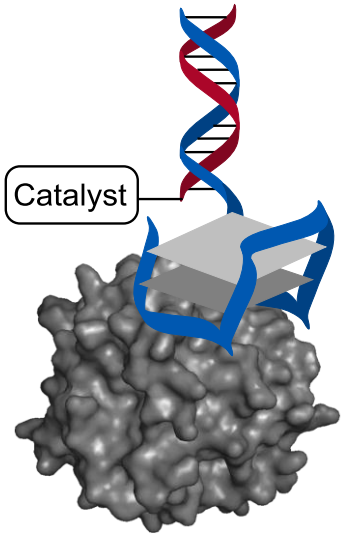
*Figure S4. SDS-PAGE results of thrombin modified by TBA^{12}-diPyOx with alkyne-ANANS **4** and subsequent CuAAC with an azido-PEG-lissamine. The only band that becomes fluorescent is thrombin, indicating specificity of TBA-diPyOx12 over the proteins BSA, lysozyme and papain.*

2.4.6 TBA2-diPyOx conversions & tryptic digestion data

*Table S4. Conversions (calculated from SDS-PAGE data) and modified residues for different TBA2-diPyOx constructs, in comparison to free diPyOx. The red X highlights modifications that are also found in the absence of catalyst or with free diPyOx and are background acylation. Conditions: 10 µM thrombin, 30 µM catalyst, 300 µM azido-ANANS **3**, pH: 7.2, 37 °C, 6 h.*

Catalyst	Conv (%)	K21	K77	K83	K104	K106	K107	K135	K145	K174	K236	K247	K248	K252	S22	S128	S19^{LC}	S31^{LC}
free diPyOx	0		X						X									
TBA2$^{5'end}$ -diPyOx	15		X					X	X	X	X							X
TBA2^{5}-diPyOx	6	X	X	X			X		X	X		X	X	X	X			
TBA2^{17}-diPyOx	27	X	X	X	X	X	X		X					X	X			
TBA2^{23}-diPyOx	20		X					X	X	X	X					X	X	X
TBA2^{26}-diPyOx	20		X	X		X	X		X	X	X	X	X	X			X	

2.4.7 Templated catalyst approach



Lane#	1	2	3	4	5	6	7	8	9
DNA-cat.	-	$T^{12}dP$	$T^{3,12}dP$	-	DMAP	D_{temp}	D_{temp}	-	dP_{temp}
Aptamer	-	-	-	-	-	TBA_{temp}	$TBA2_{temp}$	-	TBA_{temp}
pH	7.2	7.2	7.2	8.0	8.0	8.0	8.0	7.2	7.2
Substrate	(3)	(3)	(3)	(1)	(1)	(1)	(1)	(3)	(3)
Conv%	**2**	**73**	**69**	**14**	**14**	**11**	**11**	**5**	**5**

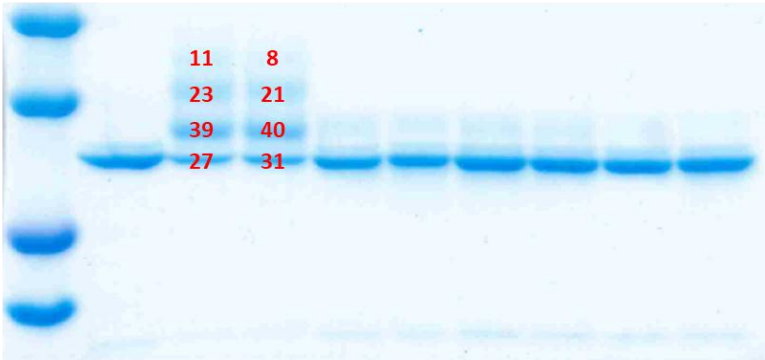


*Figure S5. SDS-PAGE results of thrombin and lysozyme modified with azido-thioester **1** or azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Lane 1–3: comparison of diPyOx and bis(diPyOx). Lanes 4–7: DNA-templated DMAP acylation of thrombin. Lanes 8–9: DNA-templated diPyOx acylation of thrombin. Conditions: 10 µM protein, 30 µM DNA, 300 µM acyl donor, 37 °C in the dark for 6 h. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Abbreviations: $T^{12}dP$ = TBA^{12}-diPyOx; $T^{3,12}dP$ = $TBA^{3,12}$-bis(diPyOx); D_{temp} = DMAP-template; TBA_{temp} = TBA-template; $TBA2_{temp}$ = TBA2-template; dP_{temp} = diPyOx-template.*

2.4.8 TBA-cat vs TBA competition experiment

Lane#	1	2	3	4	5	6	7	8	9	10
Catalyst	$T^{12}dP$	$T^{12}dP$	$T^{12}dP$	$T^{12}d$	$T^{12}dP$	$T2^{17}dP$	$T2^{17}dP$	$T2^{17}dP$	$T2^{17}dP$	$T2^{17}dP$
TBA (eq)		0.5	1.0	2.0						2.0
TBA2 (eq)					2.0		0.5	1.0	2.0	
Conv%	**37**	**31**	**20**	**7**	**34**	**11**	**8**	**6**	**4**	**11**

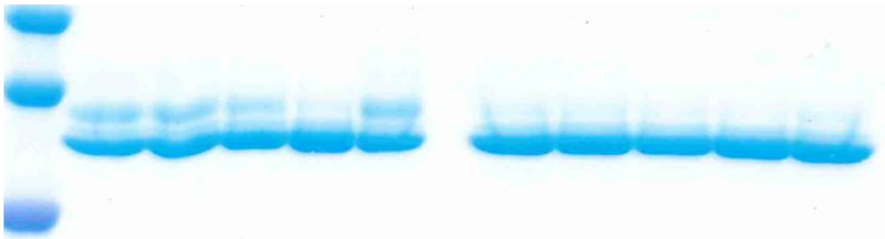


*Figure S6. SDS-PAGE results of thrombin modified with azido-thioester **1** or azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Varying equivalents of native aptamer were added to observe the effect on the yield and estimate whether the affinity of the modified aptamer was affected by the catalyst. Conditions: 20 µM protein, 30 µM DNA, 300 µM acyl donor, 37 °C in the dark for 8 h. The changes in modification percentages indicate that the affinity of the catalyst-aptamer construct is comparable to that of the native aptamer. Conversions were calculated with ImageJ.*

2.4.9 TBA-DMAP activity switch

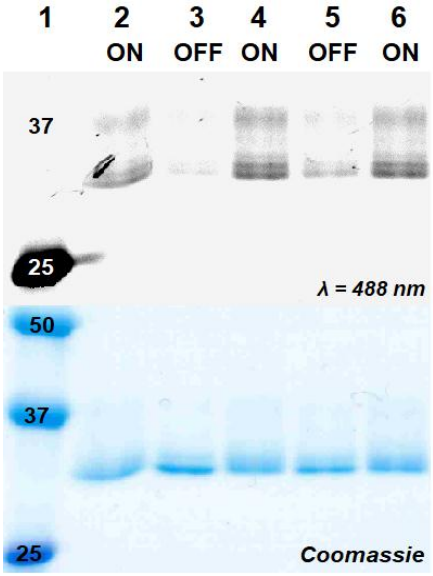
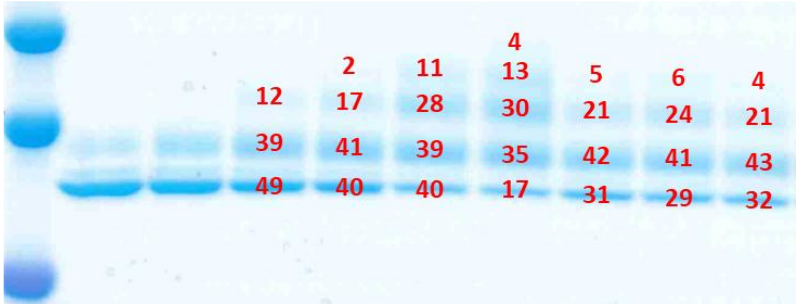


*Figure S7. SDS-PAGE results of thrombin modified with alkyne-thioester **2** and subsequent CuAAC with an azido-PEG-lissamine. Lane 2: TBA^{12}-DMAP; Lane 3: TBA^{12}-DMAP + TBA-OFF; Lane 4: TBA^{12}-DMAP + TBA-OFF + TBA-ON; Lane 5: TBA^{12}-DMAP + TBA-OFF + TBA-ON + TBA-OFF; Lane 6: TBA^{12}-DMAP + TBA-OFF + TBA-ON + TBA-OFF + TBA-ON. Conditions: 10 µM thrombin, 30 µM TBA^{12}-DMAP, 150 µM **2**, pH: 8.0, 37 °C, 2 h.*

2.4.10 TBA-diPyOx activity switch performed in situ

Lane#	1	2	3	4	5	6	7	8	9
t_{ON} (h)	0.5	1	2	4	6	8	2		2
t_{OFF} (h)	7.5	7	6	4	2		4	4	2
t_{ON} (h)							2	4	2
t_{OFF} (h)									2
Conv%	**22**	**34**	**51**	**60**	**77**	**83**	**69**	**71**	**68**

Lane#	10	11	12
time prior to **3** (h)	2	2	6
$t_{reaction}$ (h)	6	4	2
Conv%	**78**	**70**	**51**



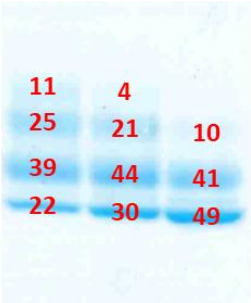


*Figure S8. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Lane 1–6: reactions run with TBA^{12}-diPyOx for 'x' hours in ON and stopped with TBA-OFF after 'y' hours. Lanes 7–9: similar to 1-6, but with more in situ switch steps. Lane 10–12: azido-ANANS **3** was added after waiting for 'x' hours to compare 'x' hour reaction time to 'x' hour OFF time. Conversions match, thus the switch works. Conditions: 10 µM protein, 30 µM DNA, 1.1 eq TBA ON/OFF, 300 µM acyl donor, 37 °C in the dark for 8 h. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers in the gel indicate the various percentages of labelled products.*

2.5 Secondary supporting data

2.5.1 Additional SDS-PAGE data

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cat	-	TBA	DMAP	$T^{12}D$	T+D	TBA	DMAP	$T^{12}D$	T+D	-	TBA	DMAP	$T^{12}D$	-
cat (uM)	-	30	30	30	30	60	60	60	60	-	30	30	30	-
1 (uM)	150	150	150	150	150	150	150	150	150	300	300	300	300	-
Conv%	**8**	**10**	**10**	**49**	**10**	**12**	**11**	**53**	**13**	**20**	**18**	**19**	**61**	-

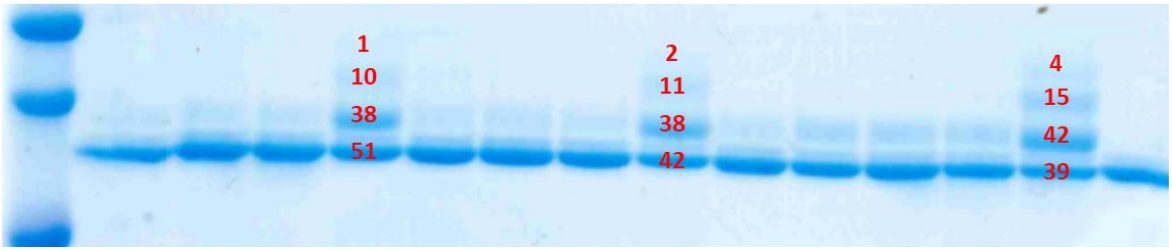


*Figure S9. SDS-PAGE results of thrombin modified using azido-thioester **1**, followed by and SPAAC derivatization with a BCN-functionalized 2 kDa masstag (BCN-PEG$_{2000}$). Different catalytic constructs were used: DMAP, unmodified TBA, TBA with DMAP attached to nucleobase T12 and TBA and DMAP (T+D) together. Conditions: 10 µM thrombin, 30 or 60 µM DNA construct, 150 or 300 µM thioester **1**, pH 8.0, at 37 °C in the dark for 2 h and quenched with an excess of aminoethanol. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9
cat	DMAP	TBA	$T^{3}D$	$T^{4}D$	$T^{7}D$	$T^{9}D$	$T^{12}D$	$T^{13}D$	-
Conv%	**5**	**5**	**27**	**12**	**16**	**8**	**36**	**17**	**5**

*Figure S10. SDS-PAGE results of thrombin modified using azido-thioester 1 and subsequently SPAACed with a BCN-functionalized 2 kDa masstag (BCN-PEG$_{2000}$). Different catalytic constructs were used: DMAP, unmodified TBA and TBA with DMAP attached to nucleobase T3, T4, T7, T9, T12 or T13. Conditions: 10 µM thrombin, 30 µM DNA construct, 150 µM thioester **1**, pH 8.0, at 37 °C in the dark for 2 h and quenched with aminoethanol. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cat	DMAP	$T^{12}D$	$T2^{17}D$	dD	$T^{12}dD$	$T2^{17}dD$	-	DMAP	$T^{12}D$	$T2^{17}D$	dD	$T^{12}dD$	$T2^{17}dD$	-
1 (uM)	150	150	150	150	150	150	150	300	300	300	300	300	300	300
Conv%	**6**	**40**	**5**	**5**	**48**	**9**	**4**	**8**	**53**	**14**	**11**	**59**	**15**	**6**

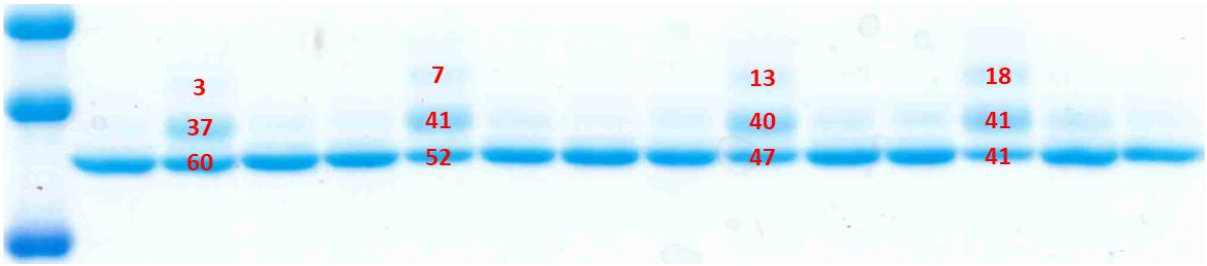


*Figure S11. SDS-PAGE results of thrombin modified with azido-thioester **1** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Different catalytic constructs were used: DMAP, TBA^{12}-DMAP ($T^{12}D$), $TBA2^{17}$-DMAP ($T2^{17}D$), diDMAP (dD), TBA^{12}-diDMAP ($T^{12}dD$) and $TBA2^{17}$-diDMAP ($T2^{17}dD$). Conditions: 10 µM thrombin, 30 µM DNA construct and 300 µM azido-thioester **1**, pH 8.0, at 37 °C in the dark for 2 h and quenched with aminoethanol. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Catalyst	-	$T^{12}D$	$T^{12}dD$	-	$T^{12}D$	$T^{12}dD$	-	$T^{12}D$	$T^{12}dD$	-	$T^{12}D$	$T^{12}dD$	-	$T^{12}D$	$T^{12}dD$
T_{react}(h)	8	8	8	4	4	4	2	2	2	1	1	1	1	1	1
Conv%	**12**	**57**	**65**	**13**	**57**	**65**	**10**	**55**	**60**	**8**	**48**	**54**	**7**	**33**	**41**

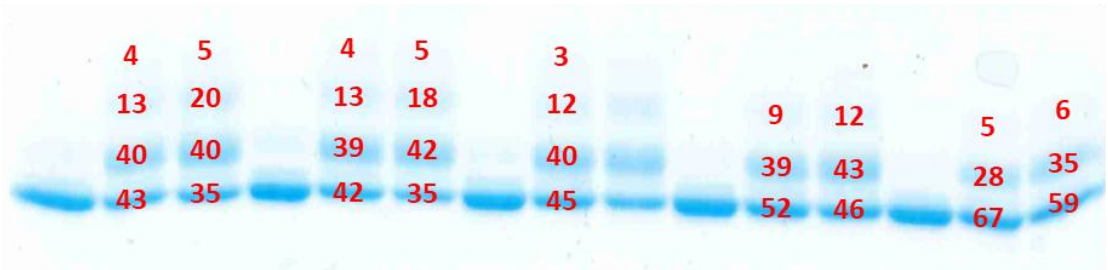


*Figure S12. SDS-PAGE results of thrombin modified with azido-thioester **1** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Different catalytic constructs were used: TBA^{12}-DMAP ($T^{12}D$), and TBA^{12}-diDMAP ($T^{12}dD$). Conditions: 10 µM thrombin, 30 µM DNA construct and 300 µM azido-thioester **1**, pH 8.0, at 37 °C in the dark for 'x' h and quenched with aminoethanol. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cat	-	T^3P	$T^{12}P$	-	T^3P	$T^{12}P$	-	T^3P	$T^{12}P$	-	T^3P	$T^{12}P$	T^3P	$T^{12}P$
t_{reac} (h)	24	24	24	8	8	8	4	4	4	2	2	2	1	1
Conv%	**9**	**47**	**44**	**1**	**29**	**38**	**0**	**21**	**30**	**0**	**12**	**17**	**10**	**10**

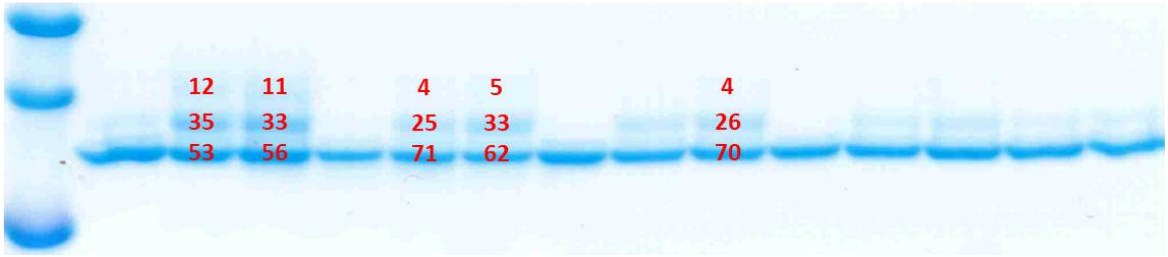


*Figure S13. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. TBA with PyOx attached to nucleobase T3 or T12. Conditions: 10 µM thrombin, 30 µM DNA construct and 300 µM ANANS **3**, pH 7.2, 37 °C in the dark for increasing reaction times. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12
cat	-	$T^{12}P$	-	$T^{12}P$	-	$T^{12}P$	-	$T^{12}P$	-	$T^{12}P$	-	$T^{12}P$
pH	7.2	7.2	8.0	8.0	7.2	7.2	7.2	7.2	8.0	8.0	7.2	7.2
DMSO	10%	10%	10%	10%	20%	20%	-	-	-	-	-	-
DMF	-	-	-	-	-	-	20%	20%	20%	20%	20%	20%
Substr.	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(1)	(1)
Conv%	**0**	**22**	**5**	**49**	**3**	**21**	**2**	**25**	**6**	**48**	**11**	**22**

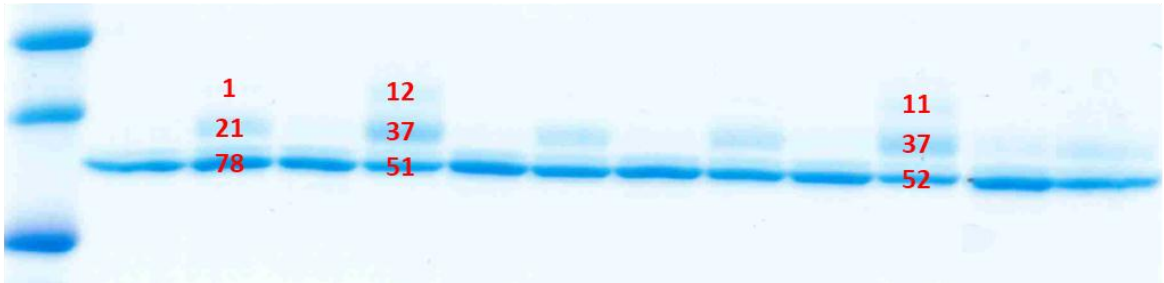


*Figure S14. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. TBA with PyOx attached to nucleobase T12. Conditions: 10 µM thrombin, 30 µM DNA construct and 300 µM substrate, 37 °C in the dark for 5 h. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12
cat	-	$T^{3}P$	$T^{12}P$	-	$T^{3}P$	$T^{12}P$	-	$T^{12}dP$	-	$T^{12}dP$	$T^{3}P$	$T^{12}P$
pH	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2	8.0	8.0	7.2	7.2
3 (uM)	150	150	150	300	300	300	300	300	300	300	300	300
t_{reac} (h)	4	4	4	4	4	4	6	6	6	6	6	6
Conv%	**0**	**16**	**22**	**3**	**20**	**34**	**0**	**75**	**4**	**83**	**15**	**28**

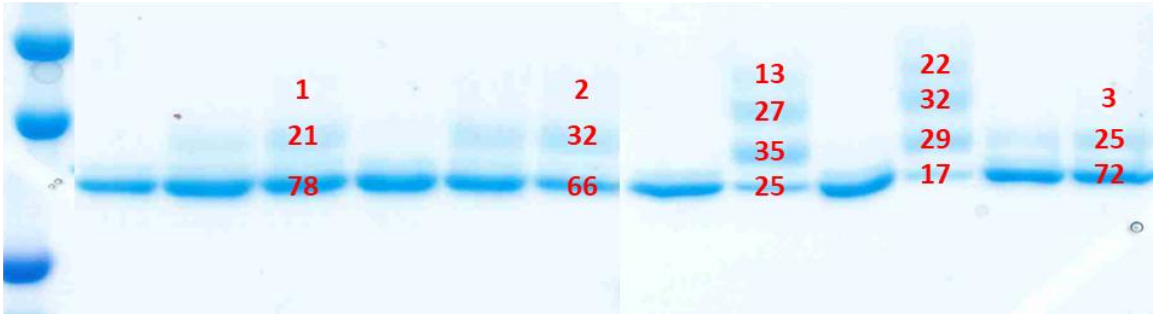


*Figure S15. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. TBA with PyOx attached to nucleobase T3 or T12 and diPyOx attached to T12 ($T^{12}dP$). Conditions: 10 µM thrombin, 30 µM DNA construct, azido-ANANS **3**, 37 °C in the dark. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9
cat	DMAP	TBA2	$T^{1}D$	$T^{5}D$	$T^{8}D$	$T^{17}D$	$T^{18}D$	$T^{23}D$	$T^{26}D$
Conv%	**18**	**16**	**22**	**22**	**21**	**18**	**19**	**20**	**8**

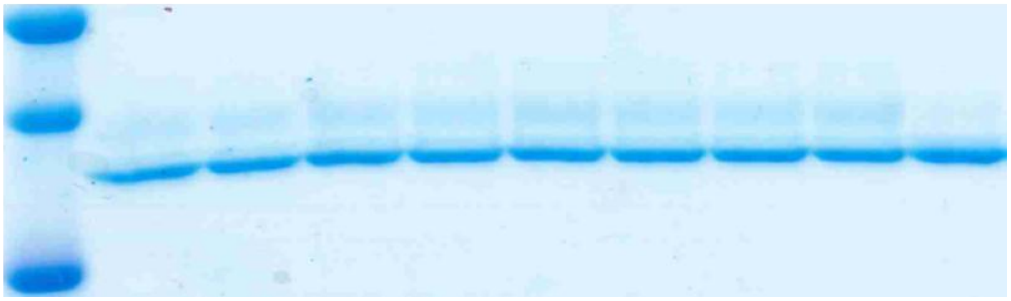


*Figure S16. SDS-PAGE results of thrombin modified with azido-thioester **1** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Different catalytic constructs were used: DMAP, unmodified TBA2 and TBA2 with DMAP attached to nucleobase T1, T5, T8, T17, T18, T23 or T26. Conditions: 10 µM thrombin, 30 µM DNA construct and 300 µM **1**, pH 8.0, at 37 °C in the dark for 2 h and quenched with aminoethanol. The bottom row displays the modification percentages of each lane as calculated with ImageJ.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13
cat	-	Py	dPy	b(dPy)	-	Py	dPy	b(dPy)	-	Py	dPy	b(dPy)	-
3 (uM)	300	300	300	300	600	600	600	600	1200	1200	1200	1200	-
Conv%	**3**	**25**	**64**	**64**	**8**	**30**	**69**	**73**	**11**	**35**	**70**	**71**	**0**

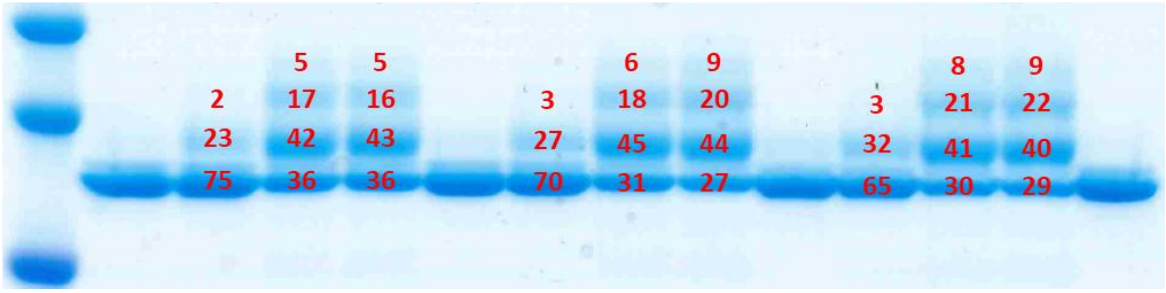


*Figure S17. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. Activities of PyOx (Py) or diPyOx (dPy) attached to T12 of TBA and bis(diPyOx) (bdPy) attached to T3 and T12 of TBA using increasing concentrations of azido-ANANS **3**. Conditions: 10 µM thrombin, 20 µM DNA construct, 37 °C in the dark for 6 h. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

Lane#	1	2	3	4	5	6	7	8	9	10	11	12	13	14
cat	-	T2-5'	T2-5	T2-17	T2-23	T2-26	-	T2-5'	T2-5	T2-17	T2-23	T2-26	T1-3	T1-12
pH	7.2	7.2	7.2	7.2	7.2	7.2	8.0	8.0	8.0	8.0	8.0	8.0	7.2	7.2
Conv%	**0**	**15**	**6**	**27**	**20**	**20**	**7**	**19**	**15**	**41**	**33**	**30**	**94**	**91**

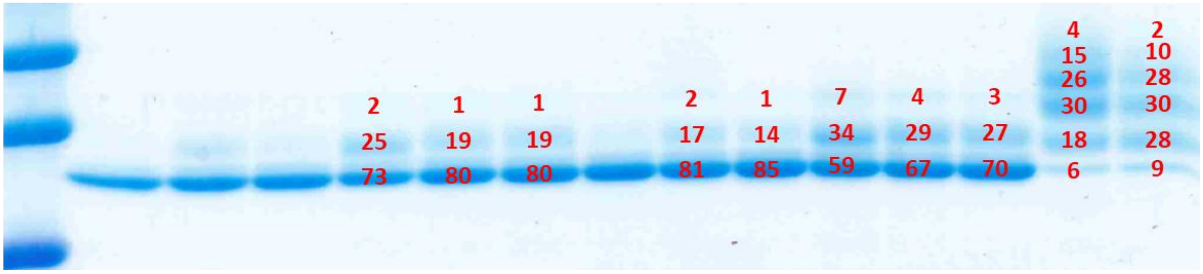


*Figure S18. SDS-PAGE results of thrombin modified with azido-ANANS **3** and subsequently SPAACed with a BCN-functionalized 2 kDa masstag. diPyOx attached to TBA2 at the 5' end (T2-5'), T5 (T2-5), T17, (T2-17), T23 (T2-23) or T26 (T2-26) and attached to TBA nucleobase T3 (T1-3) or T12 (T1-12). Conditions: 10 µM protein, 30 µM DNA, 300 µM acyl donor, 37 °C in the dark for 6 h. The bottom row displays the modification percentages of each lane as calculated with ImageJ. Red numbers indicate the percentages of mono-, di and more-labelled products.*

2.5.2 DNA sequences

Table S5. The codes and nucleobase sequences for each DNA strand that was used in this study. The abbreviation "iAzideN" indicates the inclusion of a thymine nucleobase with a hexylazide attached. The abbreviation "i5OctdU" indicates the inclusion of a thymine nucleobase with a octynyl attached.

Code	DNA Sequence (5' to 3')
TBA	GGT TGG TGT GGT TGG
TBA2 (HD22)	TCC GTG GTA GGG CAG GTT GGG GTG AC
TBA-3-azide	GG/iAzideN/ TGG TGT GGT TGG
TBA-4-azide	GGT /iAzideN/GG TGT GGT TGG
TBA-7-azide	GGT TGG /iAzideN/GT GGT TGG
TBA-9-azide	GGT TGG TG/iAzideN/ GGT TGG
TBA-12-azide	GGT TGG TGT GG/iAzideN/ TGG
TBA-13-azide	GGT TGG TGT GGT /iAzideN/GG
TBA-3-alkyne	GG/i5OctdU/ TGG TGT GGT TGG
TBA-12-alkyne	GGT TGG TGT GG/i5OctdU/ TGG
TBA-3,12-dialkyne	GG/i5OctdU/ TGG TGT GG/i5OctdU/ TGG
TBA2-1-azide	/5AzideN/CC GTG GTA GGG CAG GTT GGG GTG AC
TBA2-5-azide	TCC G/iAzideN/G GTA GGG CAG GTT GGG GTG AC
TBA2-8-azide	TCC GTG G/iAzideN/A GGG CAG GTT GGG GTG AC
TBA2-17-azide	TCC GTG GTA GGG CAG G/iAzideN/T GGG GTG AC
TBA2-18-azide	TCC GTG GTA GGG CAG GT/iAzideN/ GGG GTG AC
TBA2-23-azide	TCC GTG GTA GGG CAG GTT GGG G/iAzideN/G AC
TBA2-26-azide	TCC GTG GTA GGG CAG GTT GGG GTG A/3AzideN/
TBA2-5'end-alkyne	/5Hexynyl/TCC GTG GTA GGG CAG GTT GGG GTG AC
TBA2-5-alkyne	TCC G/i5OctdU/G GTA GGG CAG GTT GGG GTG AC
TBA2-17-alkyne	TCC GTG GTA GGG CAG G/i5OctdU/T GGG GTG AC
TBA2-23-alkyne	TCC GTG GTA GGG CAG GTT GGG G/i5OctdU/G AC
TBA2-26-alkyne	TCC GTG GTA GGG CAG GTT GGG GTG A/35OctdU/
hexynyl-TEMP[1]	/5Hexynyl/A AAA TAT ATA TAT ATA AAA
TEMP[2]-TBA	TTT TAT ATA TAT ATA TTT T GGT TGG TGT GGT TGG
TEMP[2]-TBA2	TTT TAT ATA TAT ATA TTT T TCC GTG GTA GGG CAG GTT GGG GTG AC
TBA-OFF	ATG CCC AAC CAC ACC AAC CAT GC
TBA-ON	GCA TGG TTT GTG TGT TTG GGC AT

2.5.3 HPLC and MS data of DNA constructs

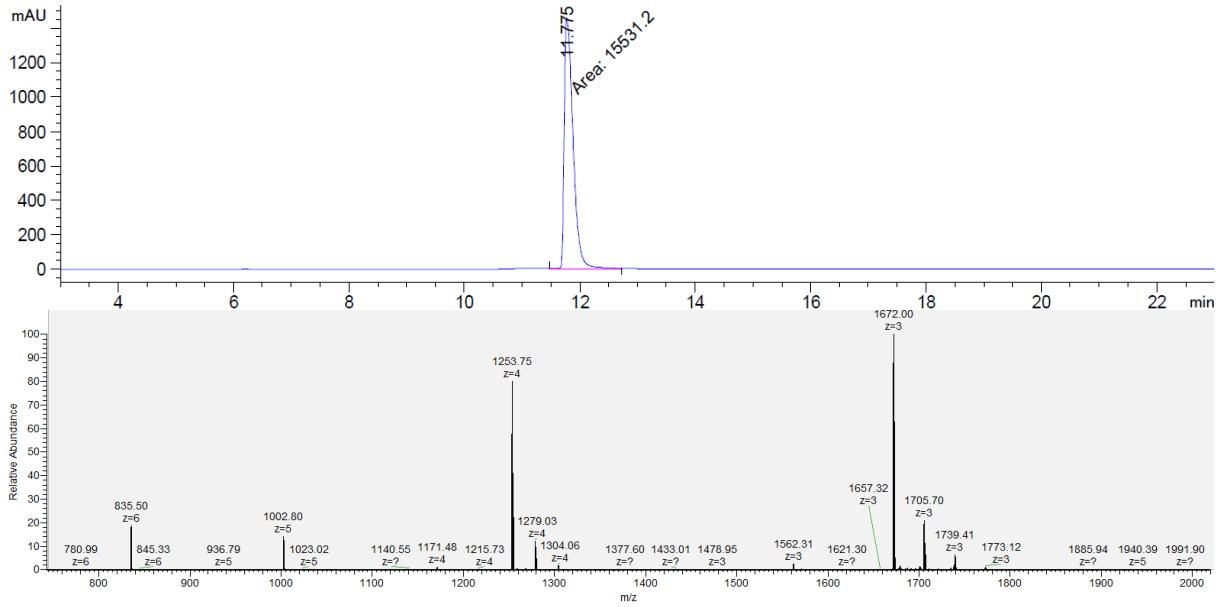


Figure S19. TBA-azide HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 835.5(6), 1002.8(5), 1253.7(4), 1672.0(3).

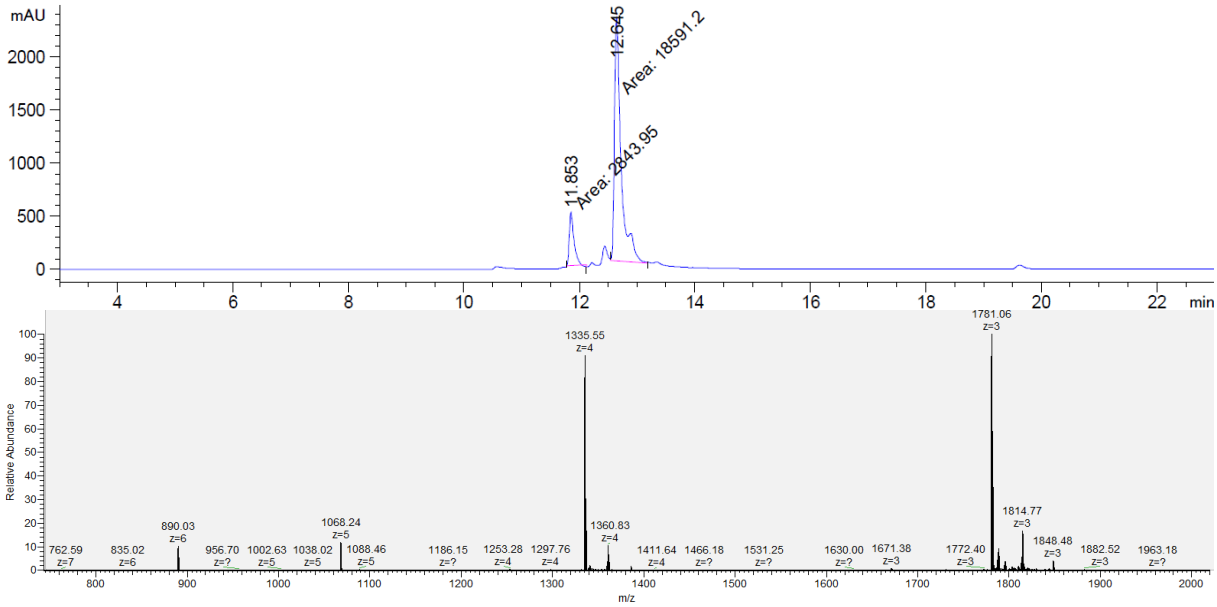


Figure S20. TBA^3-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.85 min corresponds to product. The peak at 12.65 corresponds to product with 1x BCN-DMAP as adduct.

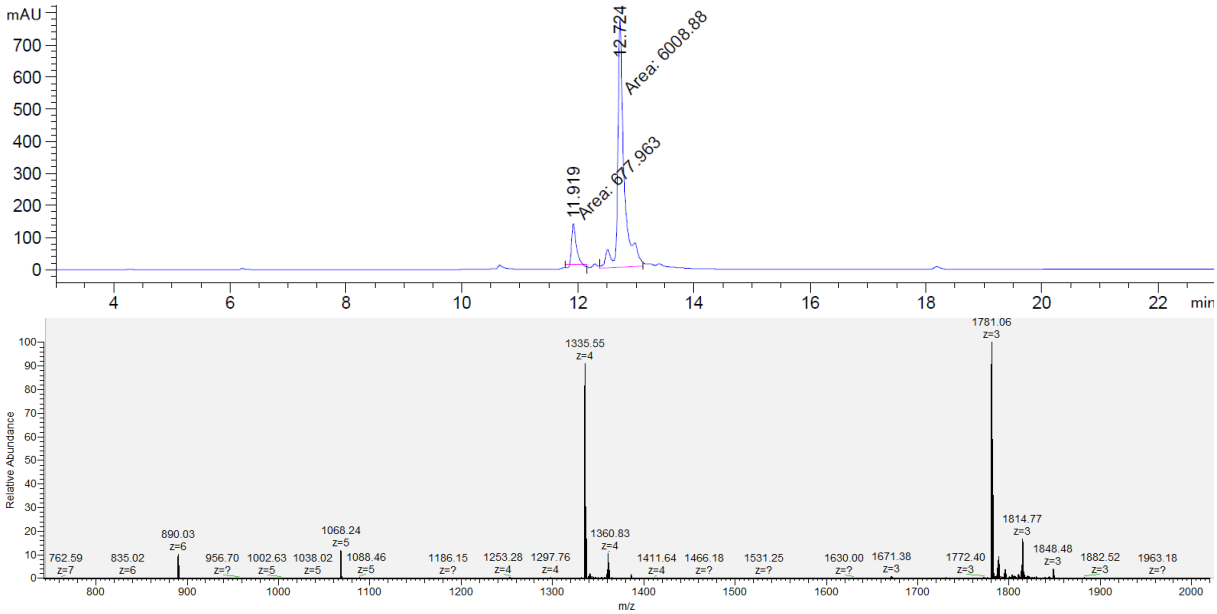


Figure S21. TBA[4]-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.92 min corresponds to product. The peak at 12.72 corresponds to product with 1x BCN-DMAP as adduct.

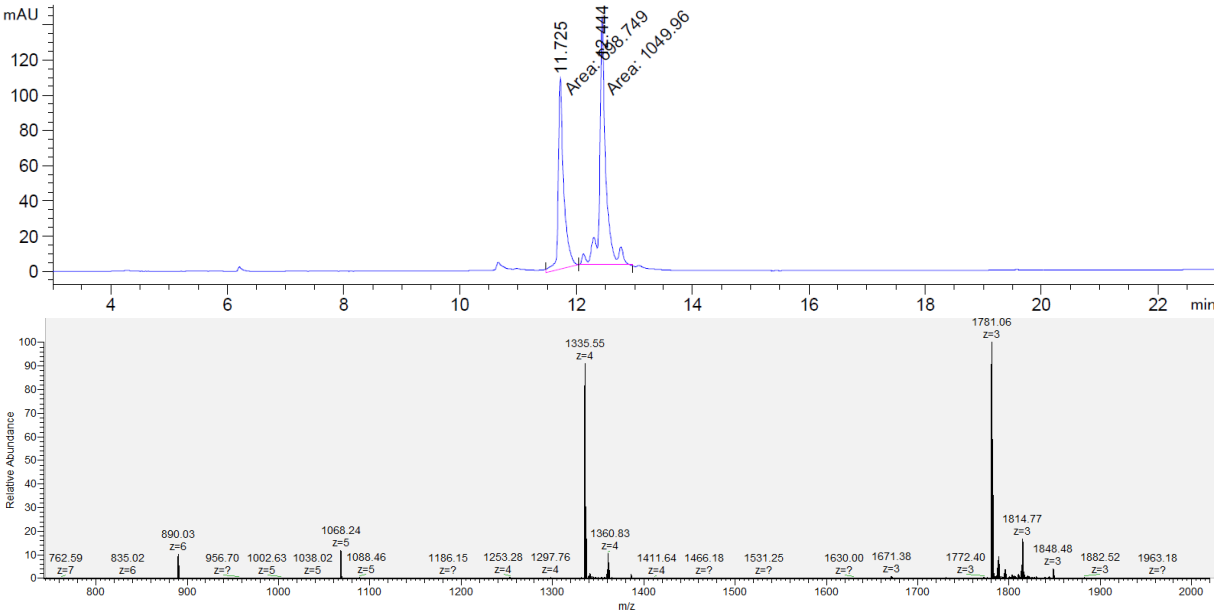


Figure S22. TBA[7]-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.73 min corresponds to product. The peak at 12.44 corresponds to product with 1x BCN-DMAP as adduct.

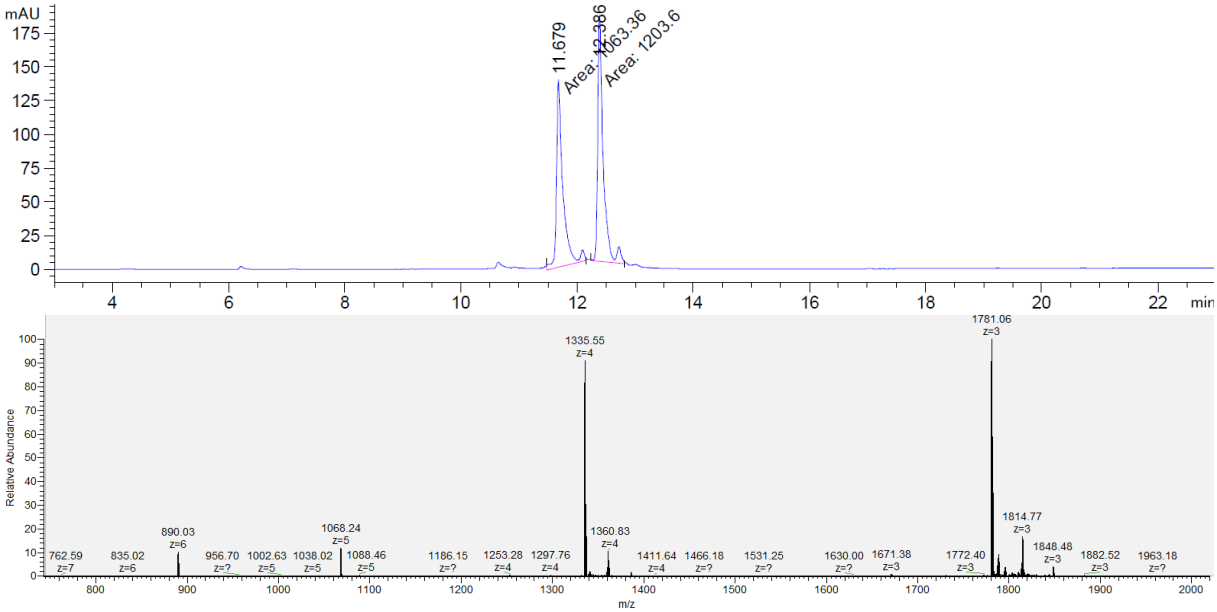


Figure S23. TBA[9]-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.68 min corresponds to product. The peak at 12.39 corresponds to product with 1x BCN-DMAP as adduct.

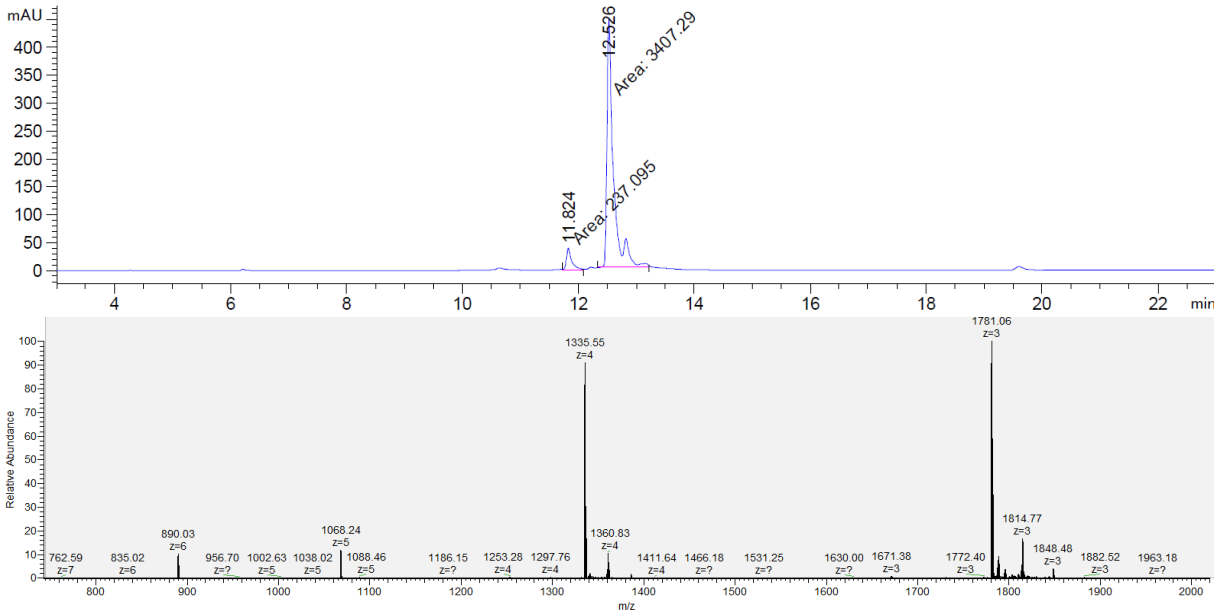


Figure S24. TBA[12]-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.82 min corresponds to product. The peak at 12.53 corresponds to product with 1x BCN-DMAP as adduct.

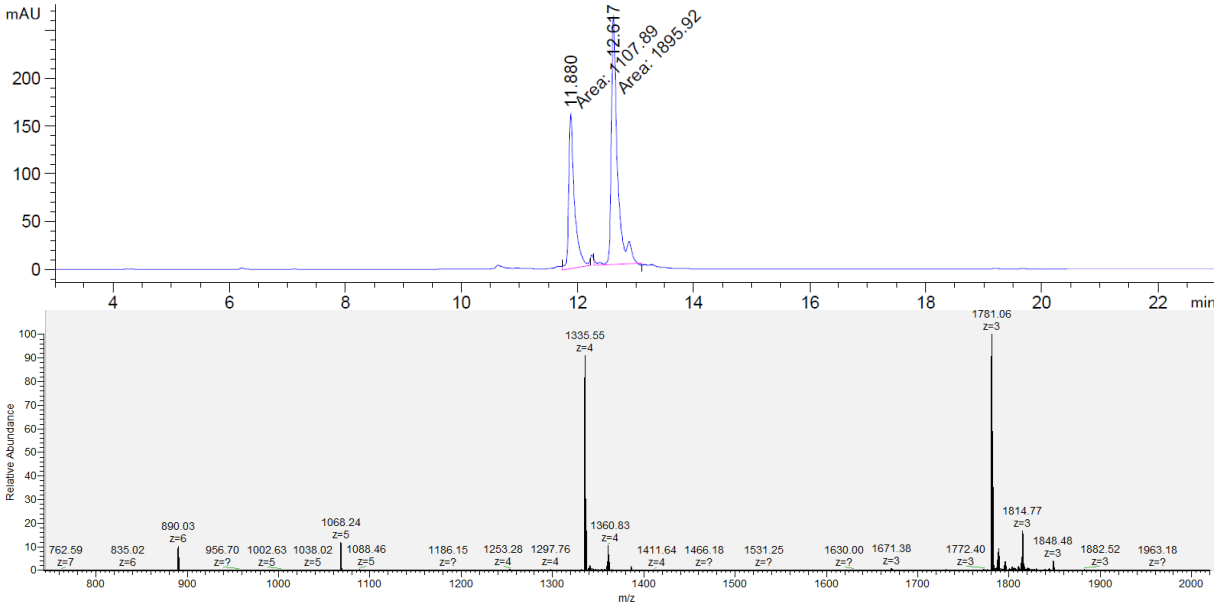


Figure S25. TBA[13]-DMAP HPLC traces (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 890.0(6), 1068.2(5), 1335.5(4), 1781.1(3). The peak at 11.88 min corresponds to product. The peak at 12.65 corresponds to product with 1x BCN-DMAP as adduct.

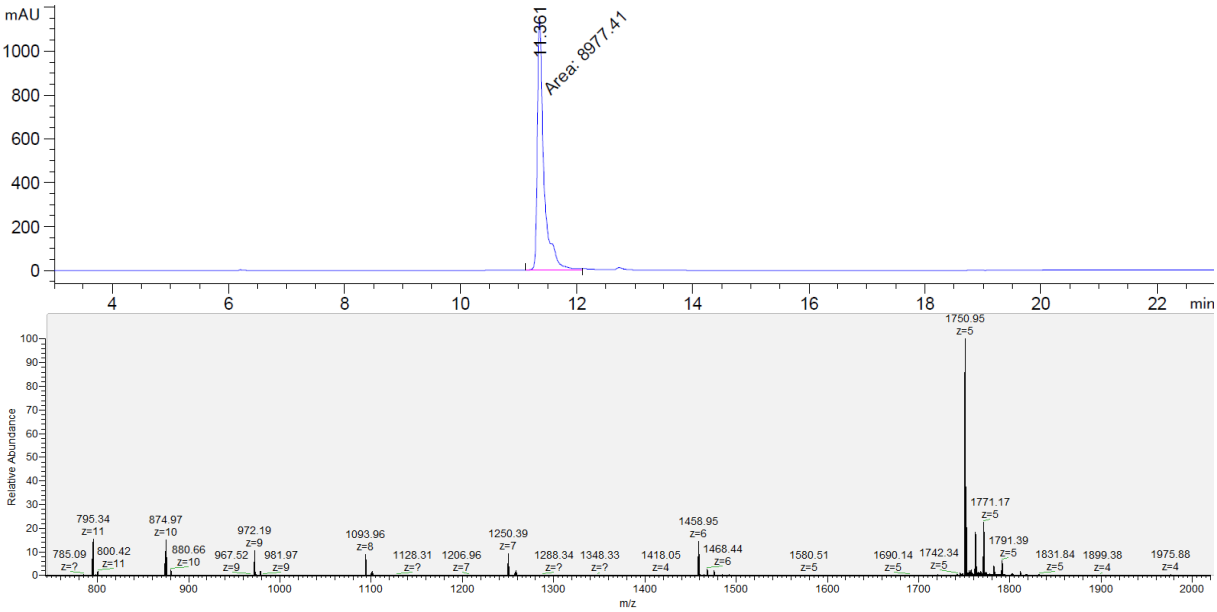


Figure S26. TBA2[1]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5).

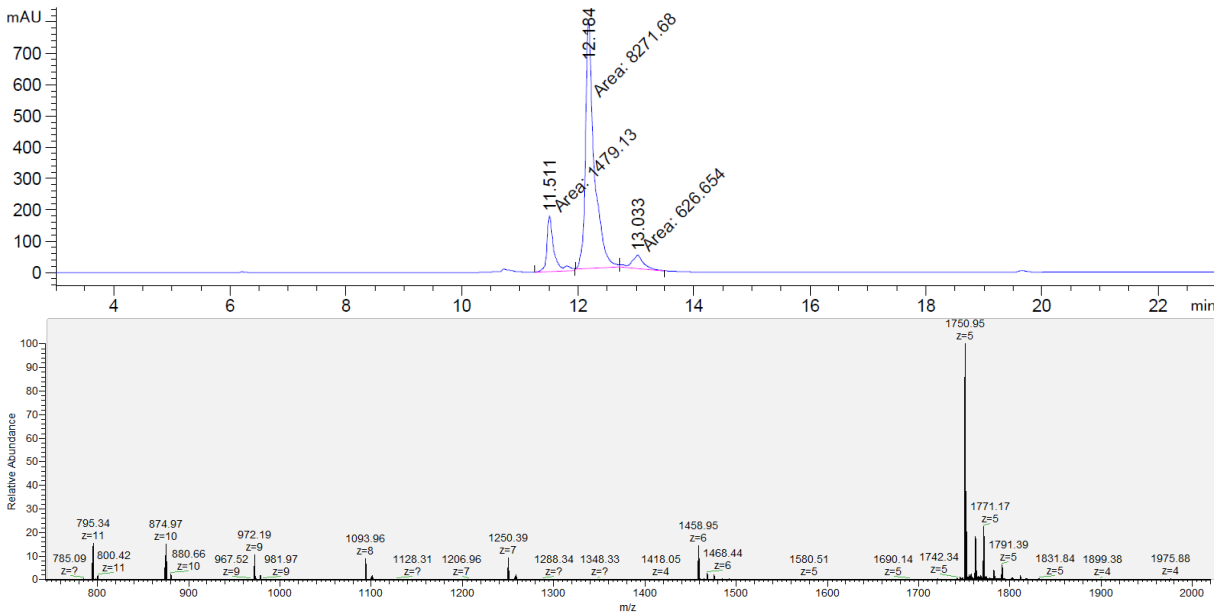


Figure S27. TBA2[5]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5). The peak at 11.51 min corresponds to product. The peak at 12.18 corresponds to product with 1x BCN-DMAP as adduct.

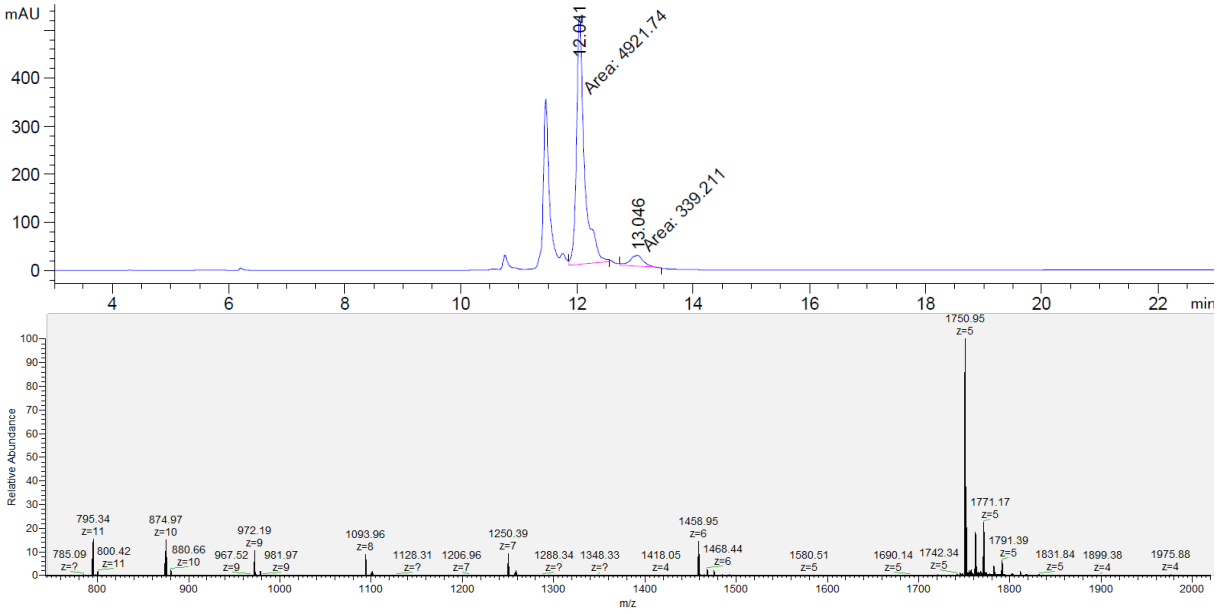


Figure S28. TBA2[8]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5). The peak at 11.45 min corresponds to product. The peak at 12.04 corresponds to product with 1x BCN-DMAP as adduct.

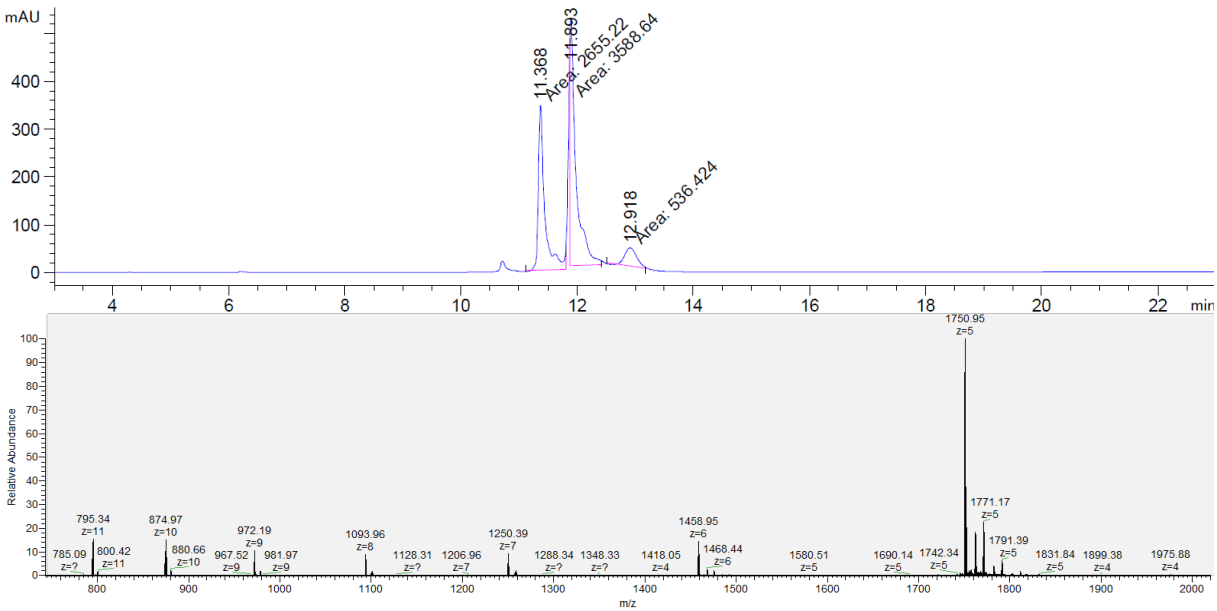


Figure S29. TBA2[17]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5). The peak at 11.34 min corresponds to product. The peaks at 11.89 and 12.92 correspond to product with 1x BCN-DMAP as adduct.

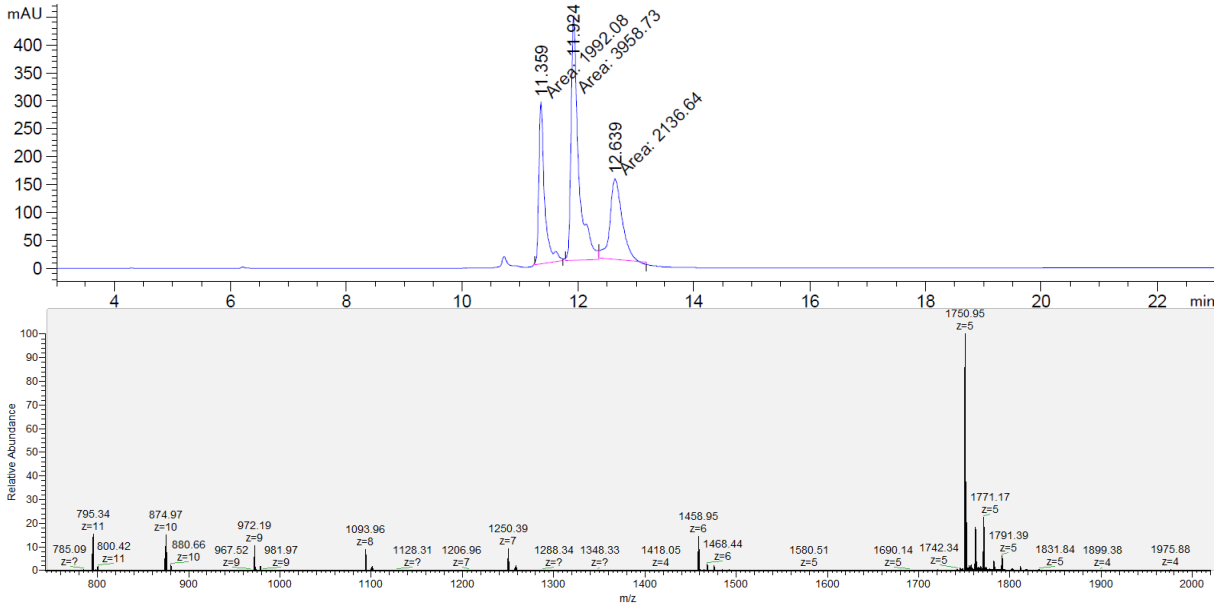


Figure S30. TBA2[18]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5). The peak at 11.36 min corresponds to product. The peaks at 11.92 and 12.64 correspond to product with 1x BCN-DMAP as adduct.

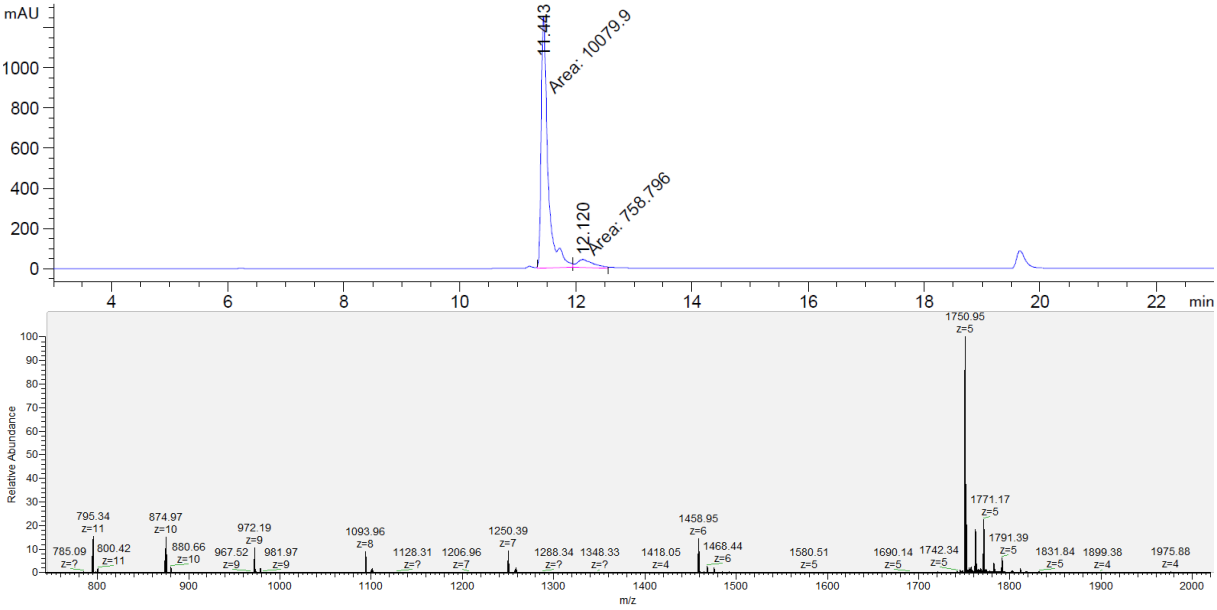


Figure S31. TBA2[23]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5).

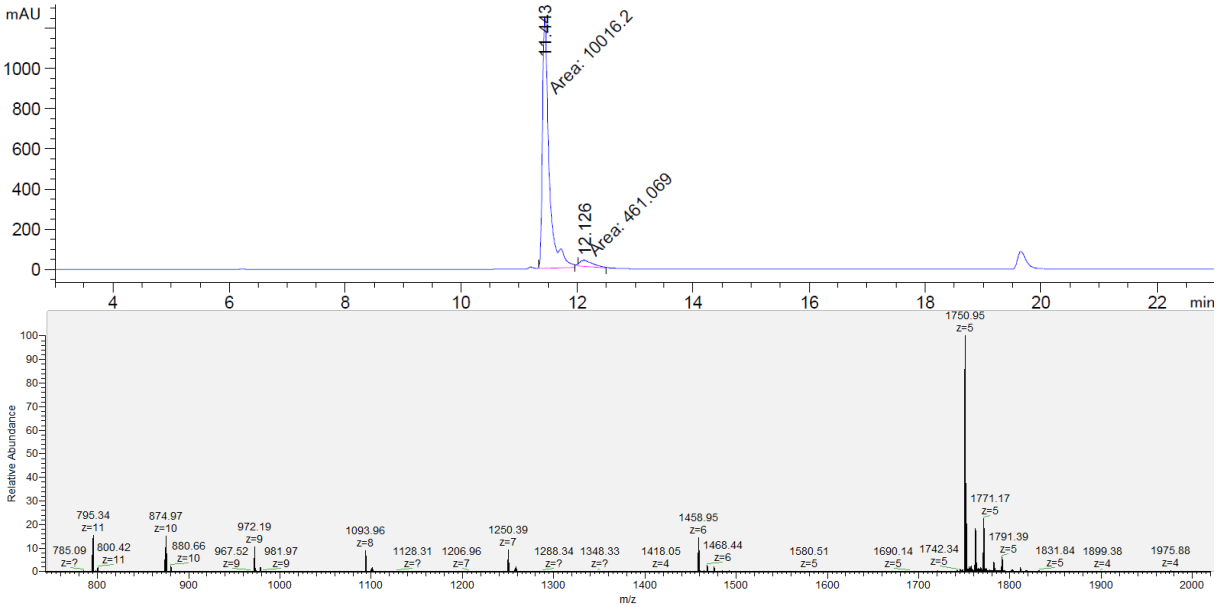


Figure S32. TBA2[26]-DMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.4(11), 875.0(10), 972.3(9), 1094.0(8), 1250.4(7), 1455.0(6), 1750.9(5).

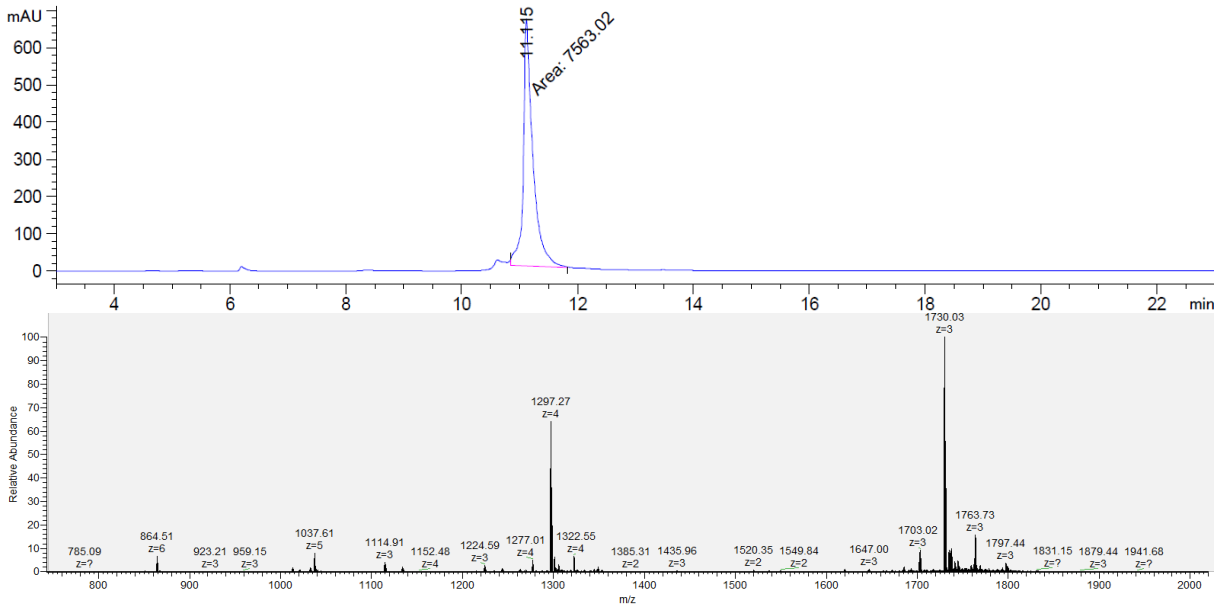


Figure S33. TBA[3]-PyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 864.6(6), 1037.6(5), 1297.3(4), 1730.1(3).

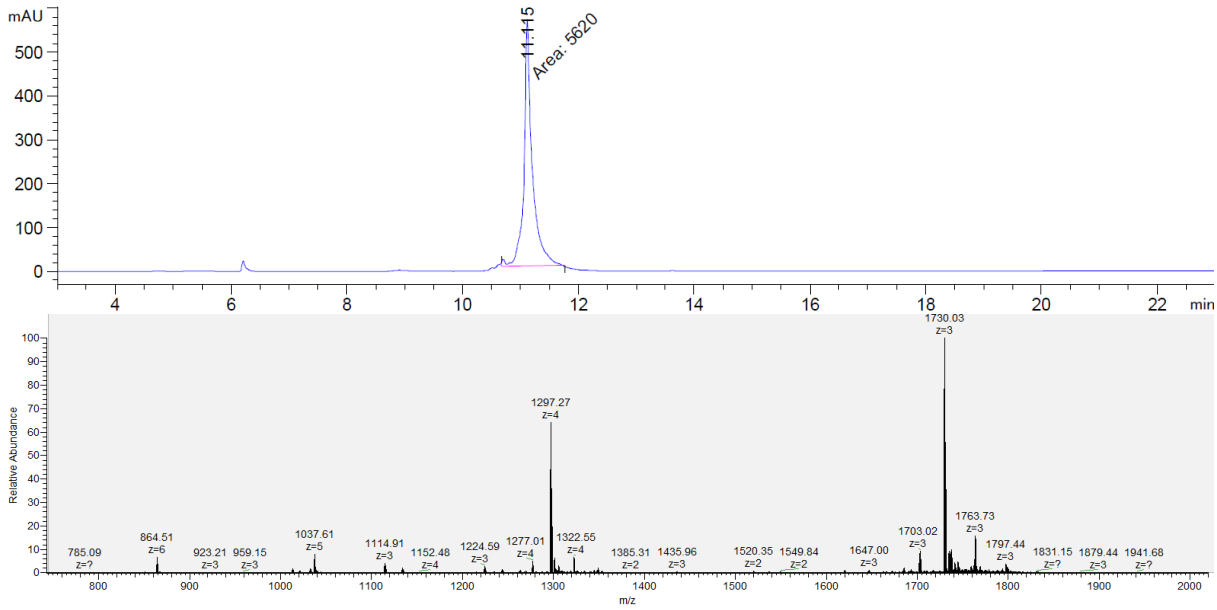


Figure S34. TBA[12]-PyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 864.6(6), 1037.6(5), 1297.3(4), 1730.1(3).

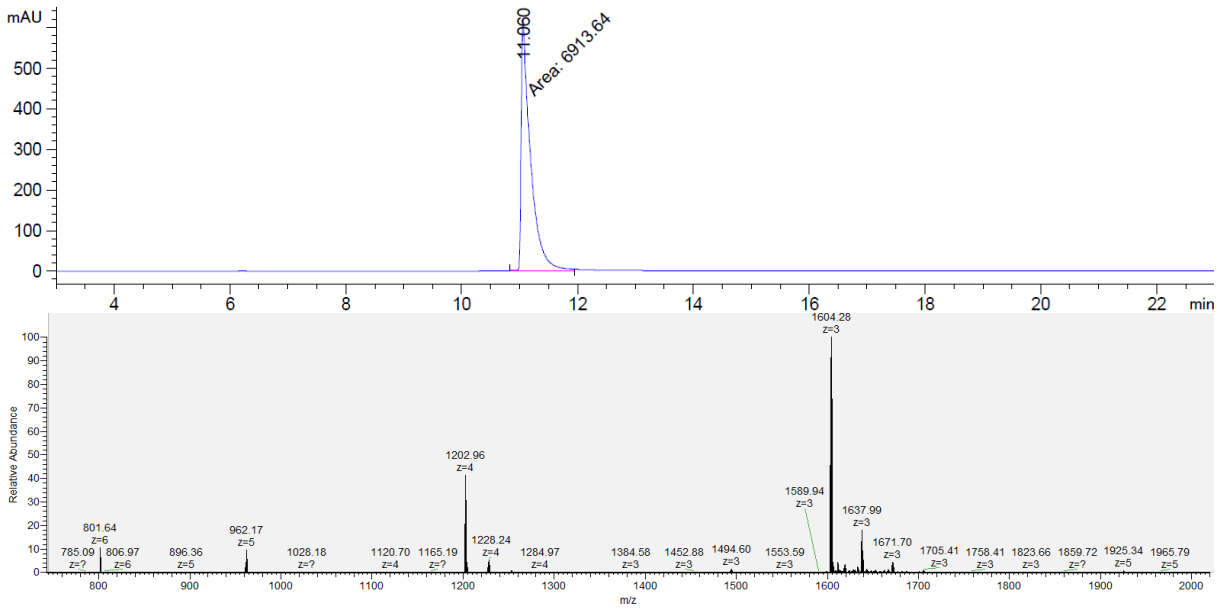


Figure S35. TBA-alkyne HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 801.6(6), 962.2(5), 1202.9(4), 1604.3(3).

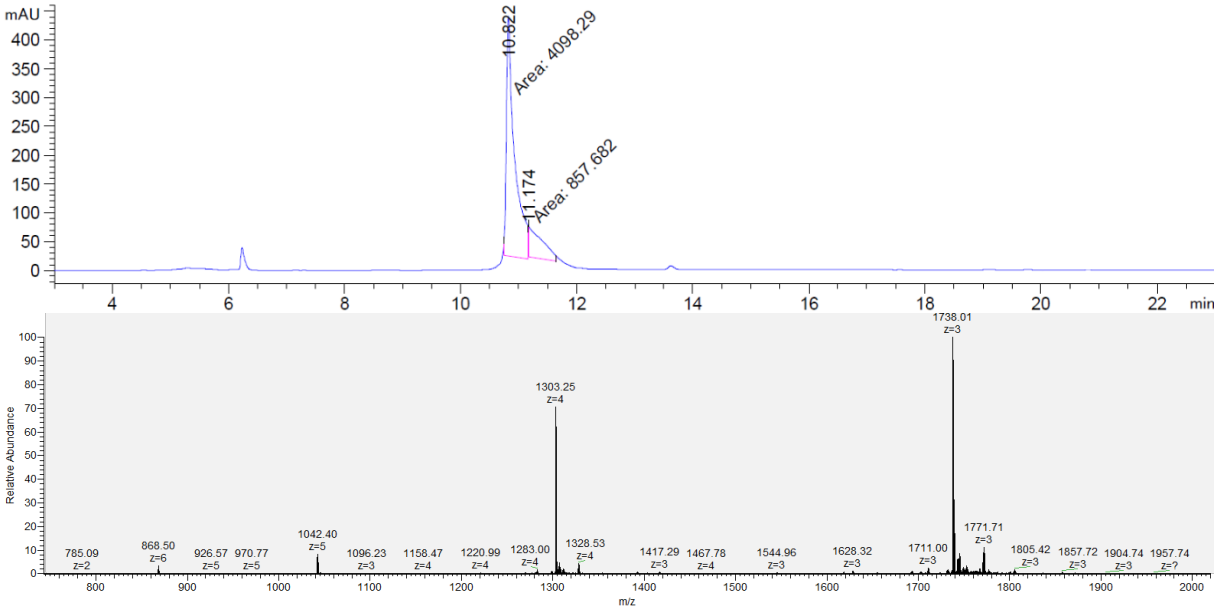


Figure S36. TBA^3-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 868.5(6), 1042.4(5), 1303.3(4), 1738.0(3).

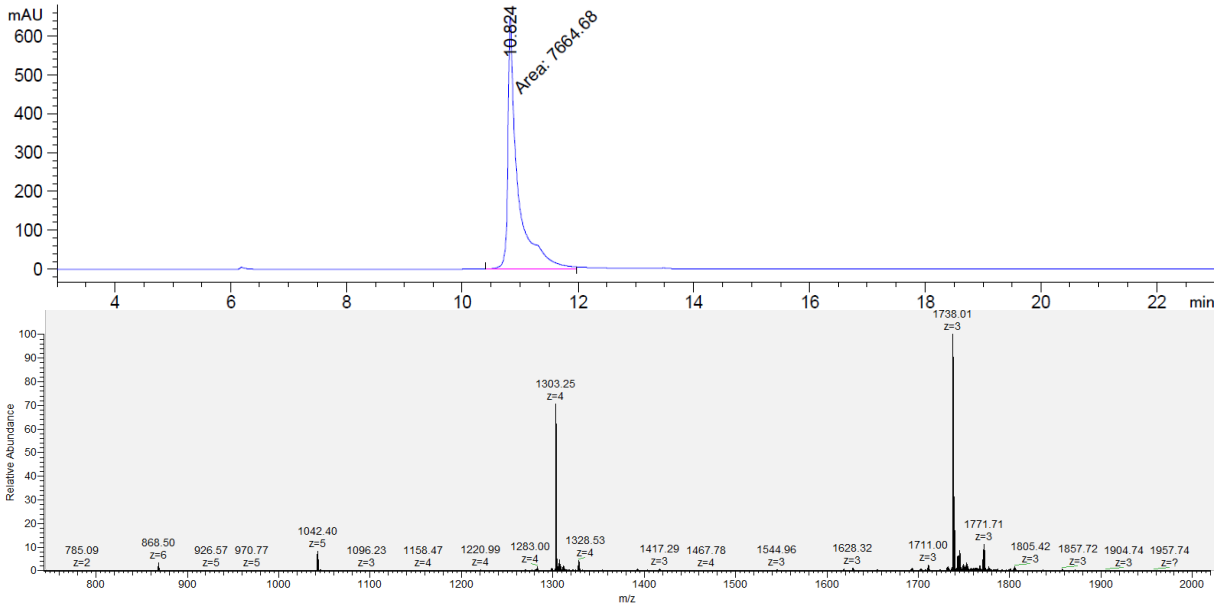


Figure S37. TBA[12]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 868.5(6), 1042.4(5), 1303.3(4), 1738.0(3).

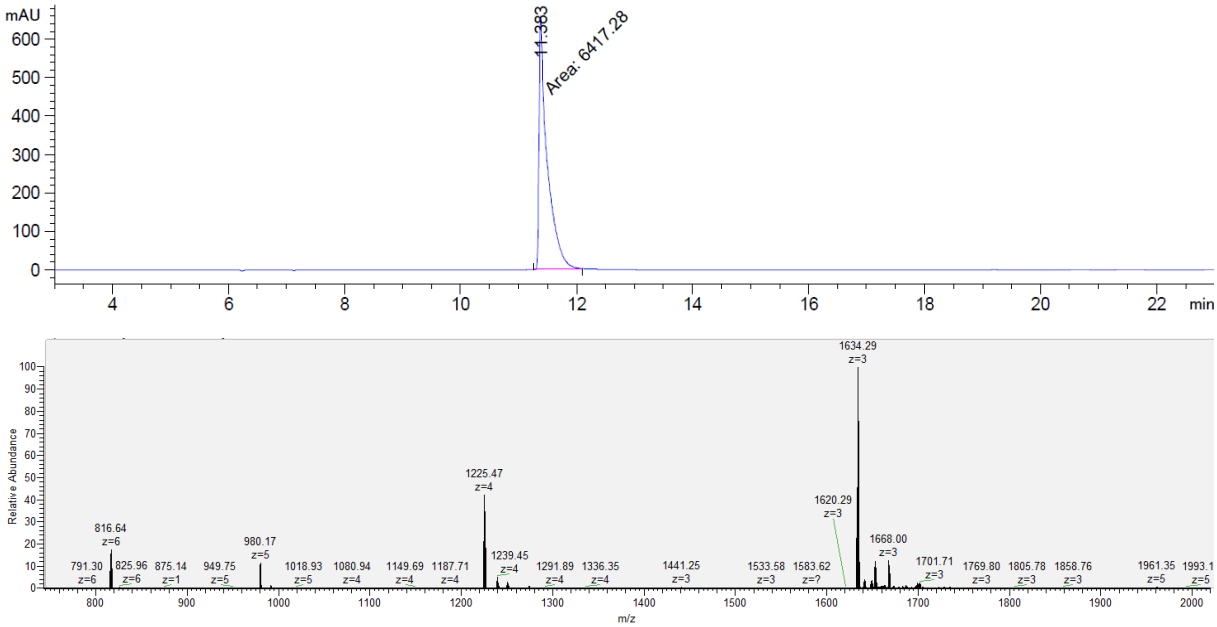


Figure S38. TBA[3,12]-dialkyne HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 816.6(6), 980.2(5), 1225.5(4), 1634.3(3).

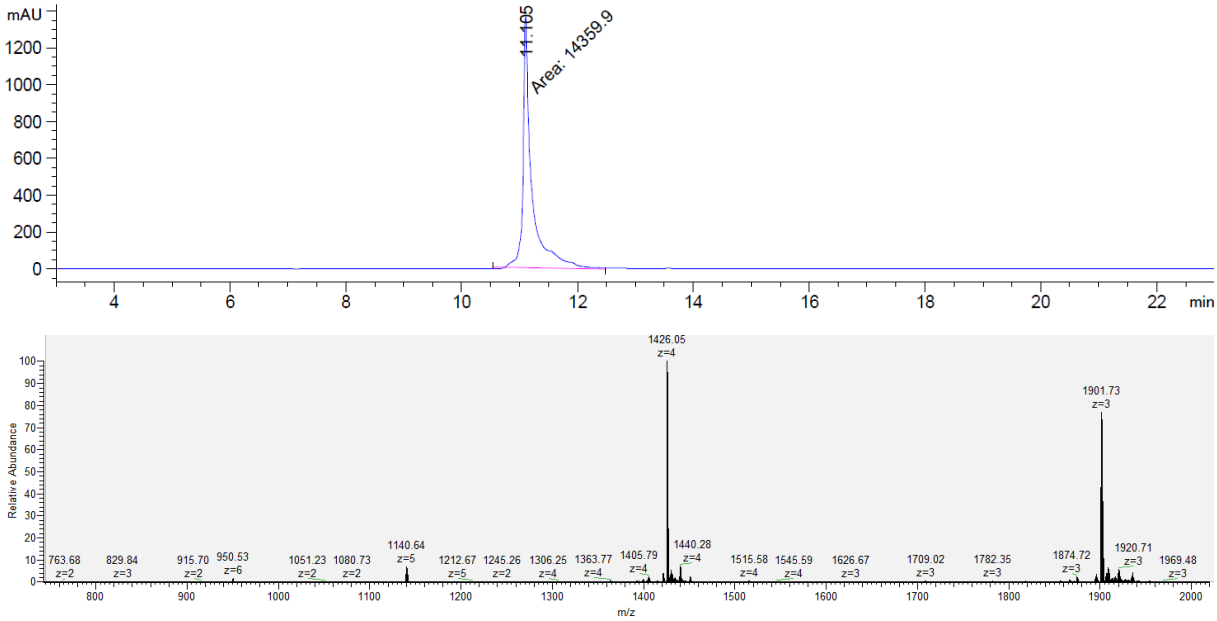


Figure S39. TBA^{3,12}-bis(diPyOx) HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 1140.6(5), 1426.1(4), 1901.7(3).

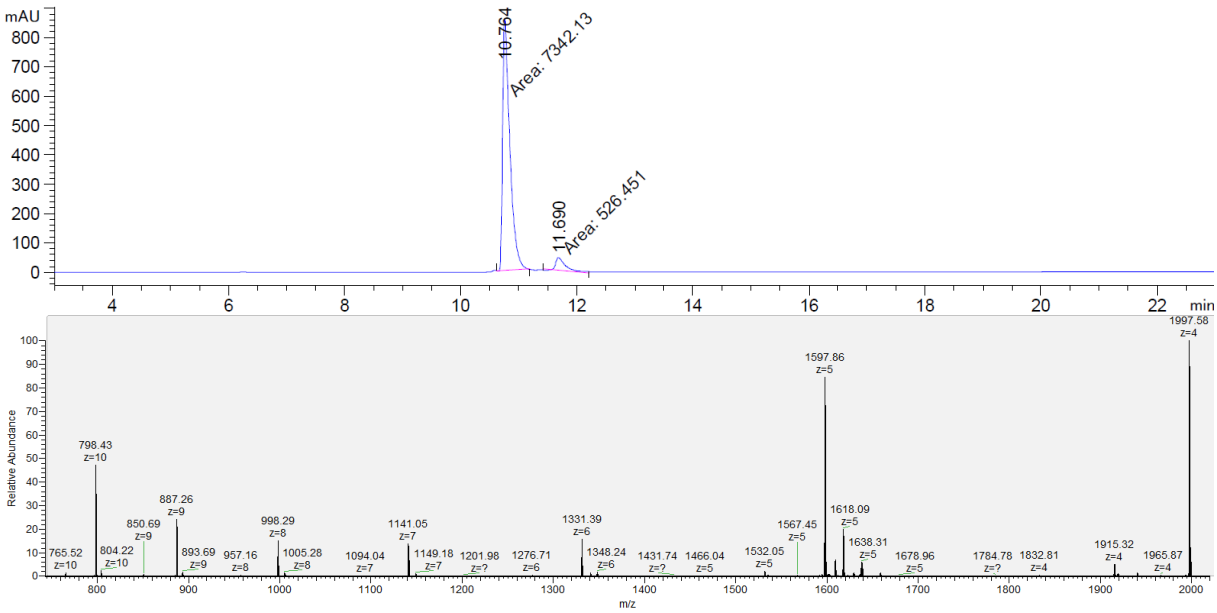


Figure S40. TBA2-5'end-alkyne HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 798.4(10), 887.3(9), 998.3(8), 1141.1(7), 1331.4(6), 1597.9(5), 1997.6(4). Peak at t_R 10.76 min corresponds to the TBA2-5'end-alkyne, peak at t_R 11.69 min corresponds an unknown impurity with similar mass.

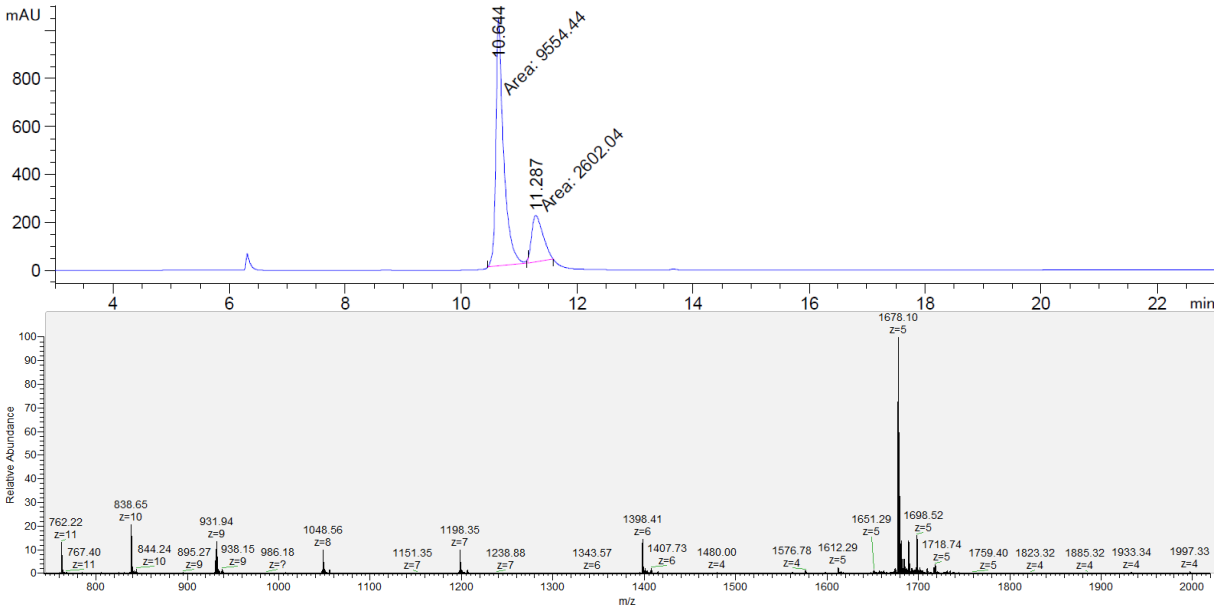


Figure S41. TBA2-5'end-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 762.2(11), 838.6(10), 931.8 (9), 1048.4(8), 1198.4(7), 1398.3(6), 1678.1(5). Peak at t_R 10.64 min corresponds to the TBA2-5'end-diPyOx, peak at t_R 11.29 min corresponds the same impurity as in the starting material (Fig. S40).

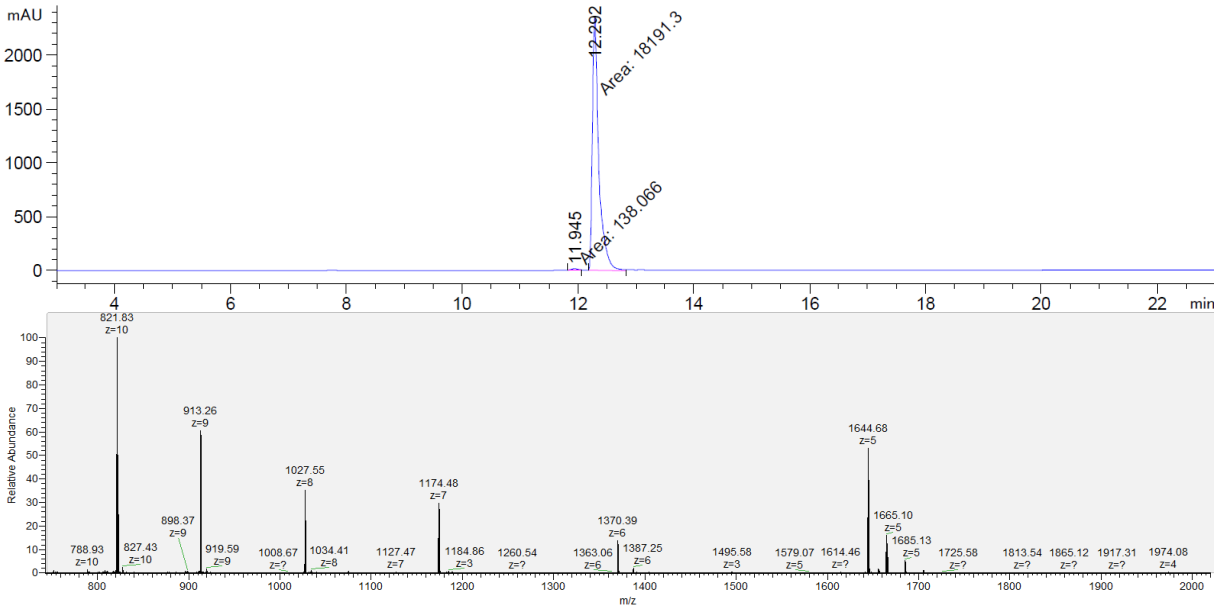


Figure S42. TBA2-alkyne HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 821.9(10), 913.3(9), 1027.6(8), 1174.6(7), 1370.5(6), 1644.8(5).

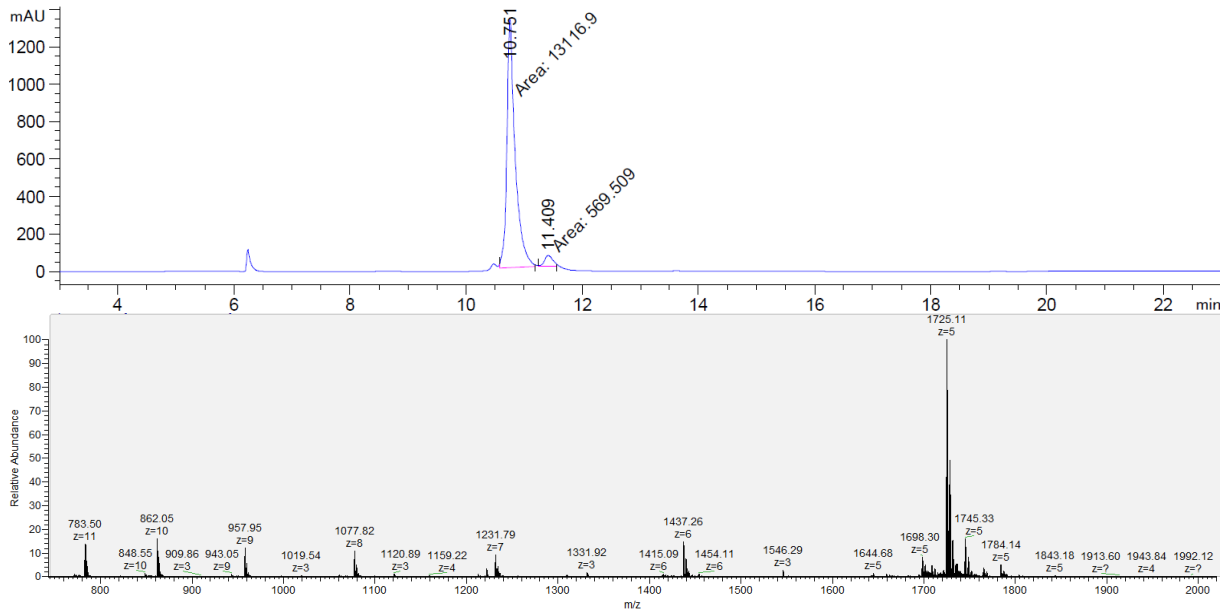


Figure S43. TBA2[5]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 783.6(11), 862.0(10), 957.9(9), 1077.8(8), 1231.9(7), 1437.4(6), 1725.0(5).

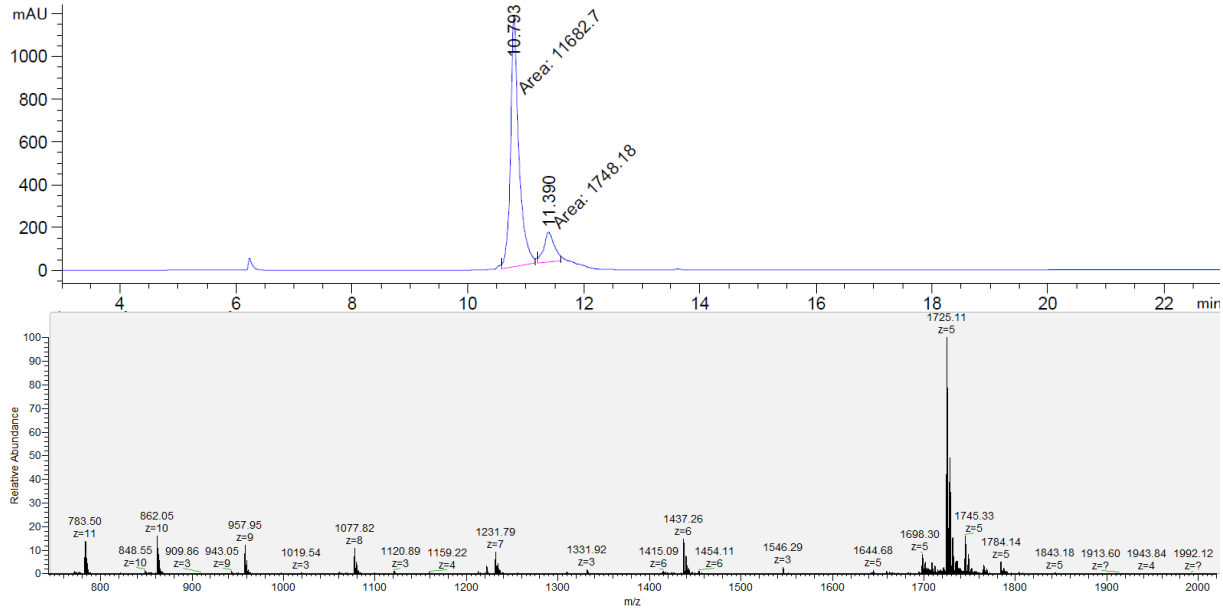


Figure S44. TBA2[17]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 783.6(11), 862.0(10), 957.9(9), 1077.8(8), 1231.9(7), 1437.4(6), 1725.0(5).

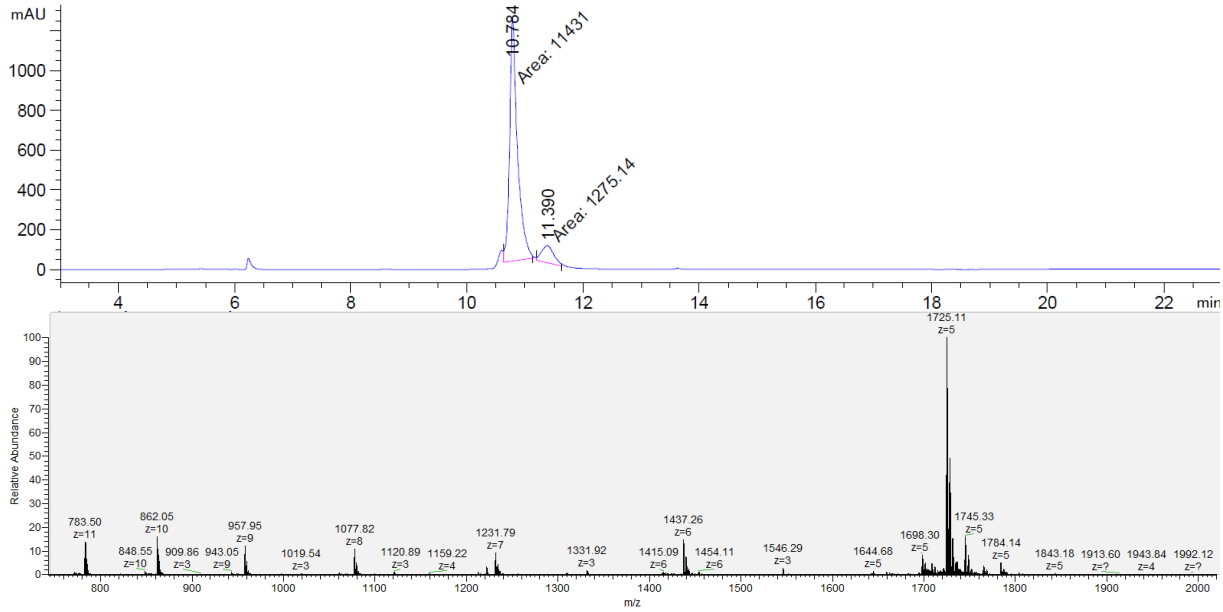


Figure S45. TBA2[23]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 783.6(11), 862.0(10), 957.9(9), 1077.8(8), 1231.9(7), 1437.4(6), 1725.0(5).

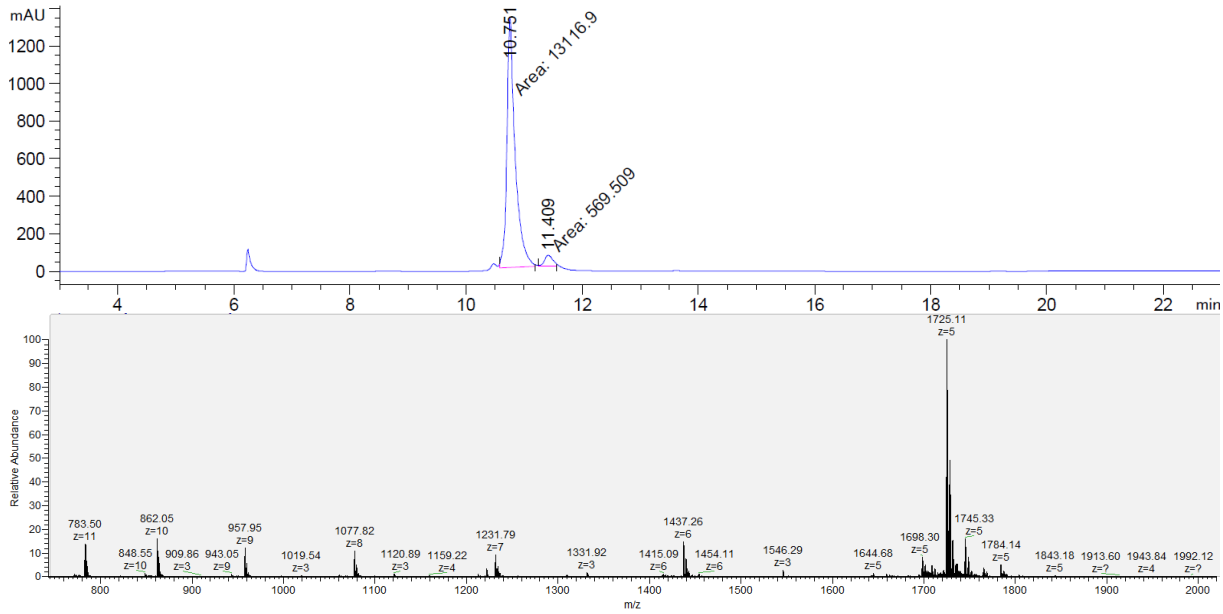


Figure S46. TBA2[26]-diPyOx HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 783.6(11), 862.0(10), 957.9(9), 1077.8(8), 1231.9(7), 1437.4(6), 1725.0(5).

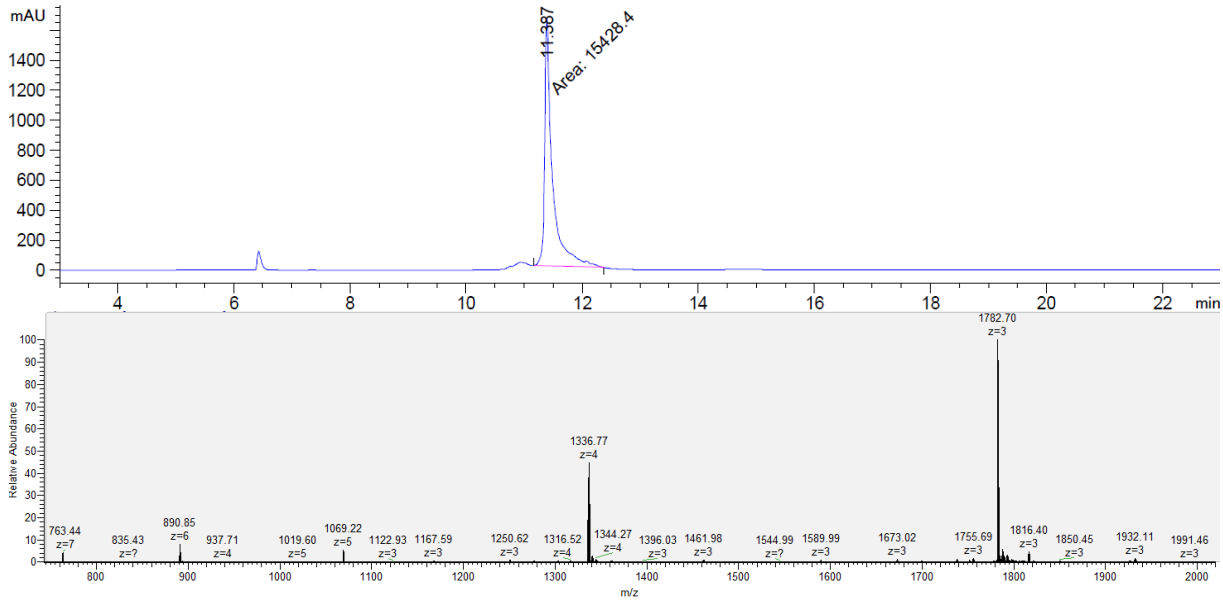


Figure S46. TBA[12]-diDMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 763.4(7), 890.9 (6), 1069.2(5), 1336.8(4), 1782.7(3).

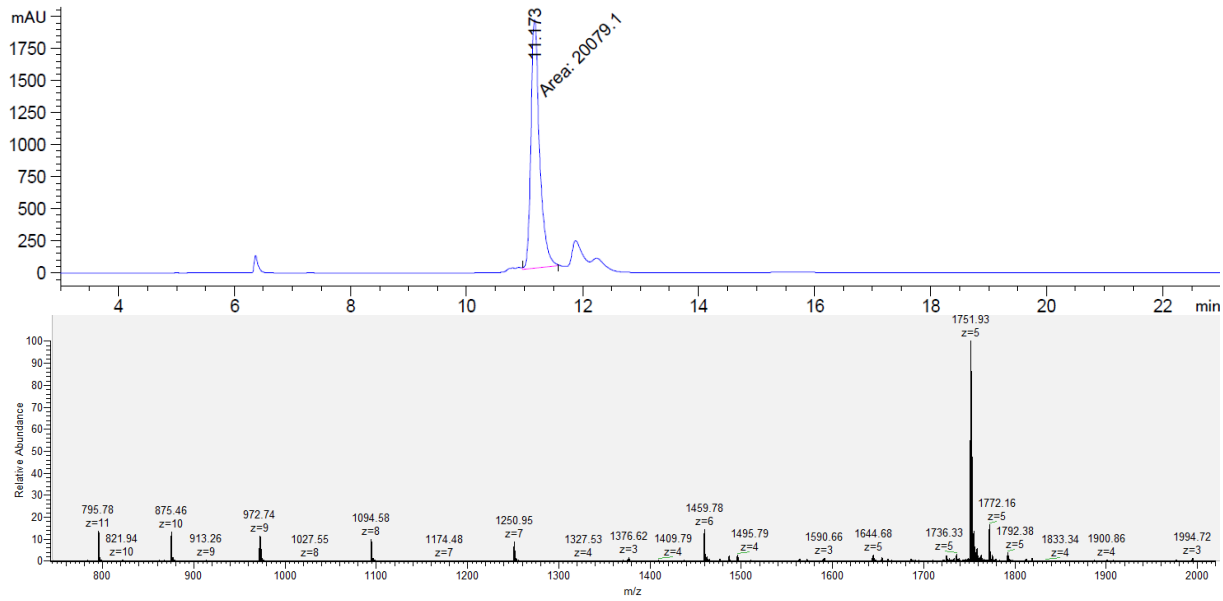


Figure S47. TBA2[17]-diDMAP HPLC trace (Abs 260 nm) and mass spectrometry data. Calculated mass(z) = 795.7(11), 875.4(10),972.8(9), 1094.5(8), 1251.0(7), 1459.7(6), 1751.9(5). Peak at t_R 11.85 min corresponds to the TBA[3]-DMAP, peak at t_R 12.65 min corresponds to an unknown impurity.

2.5.4 MS data of modified thrombin

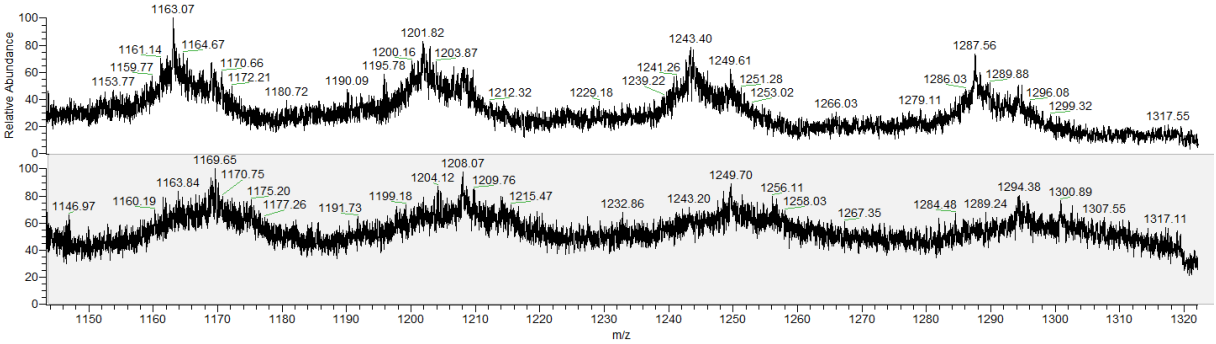


*Figure S48. The m/z values of native thrombin and thrombin modified with acyl donor **1** or **3** (both result in the same increase in mass). Native thrombin has a mass of 36025 Da, acylation with acyl donor **1** or **3** leads to a nett mass addition of 185 Da per molecule. The first panel shows 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.*

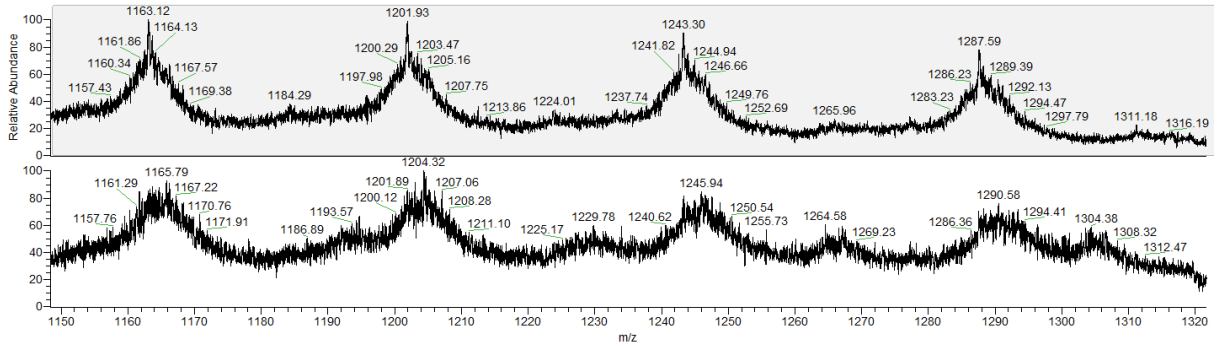


*Figure S49. The m/z values of native thrombin and thrombin modified with acyl donor **2** or **4** (both result in the same increase in mass). Native thrombin has a mass of 36025 Da, acylation with compound **2** or **4** leads to a nett mass addition of 80 Da per molecule. The first panel shows mass(z) peaks: 1163(31), 1202(30), 1243(29) and 1288(28) corresponding to unmodified thrombin. The second panel shows mass(z) peaks: 1166(31), 1204(30), 1246(29) and 1291(28) corresponding to mono-modified thrombin.*

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

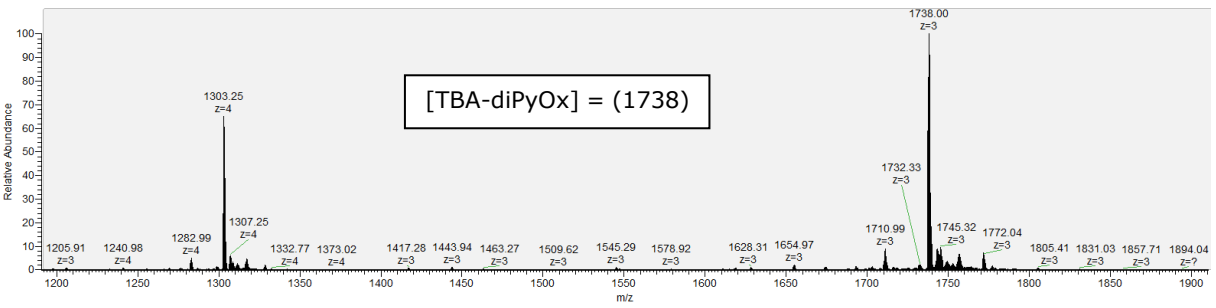


Figure S50. Mass data of TBA[12]-diPyOx in HEPES (pH 7.2) after 8 h at 37°C.

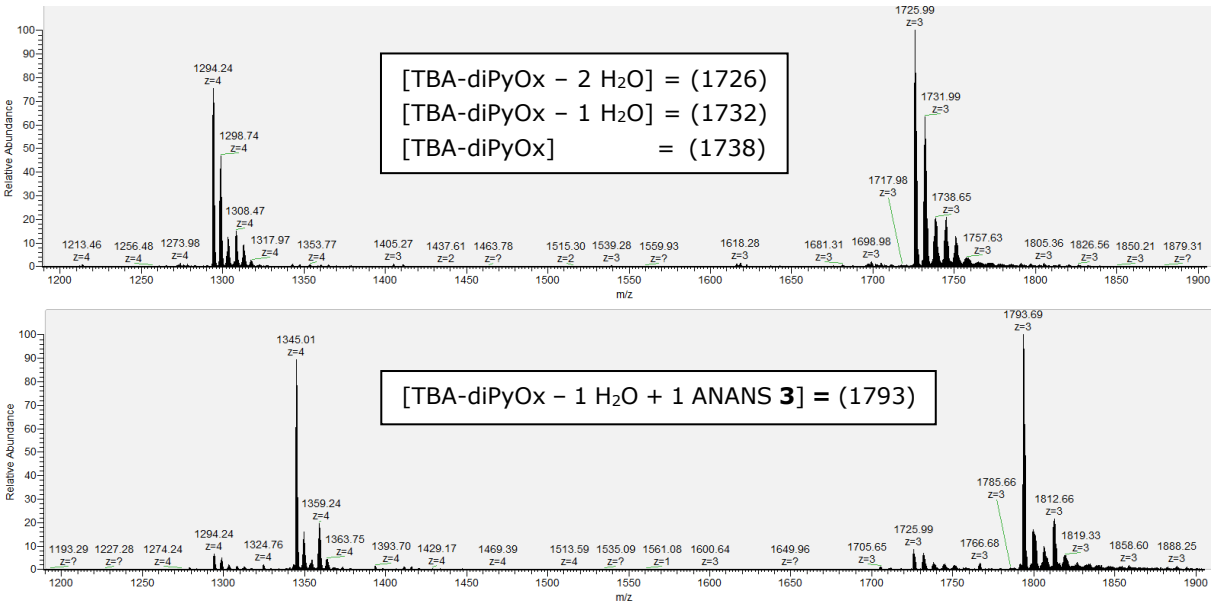


*Figure S51. Mass data of TBA[12]-diPyOx with ANANS **3** in HEPES (pH 7.2) after 8 h at 37 °C. As the acyl group cannot be donated to, dehydration of the catalyst occurs. Only non-functional and mono-functional TBA-diPyOx is detected.*

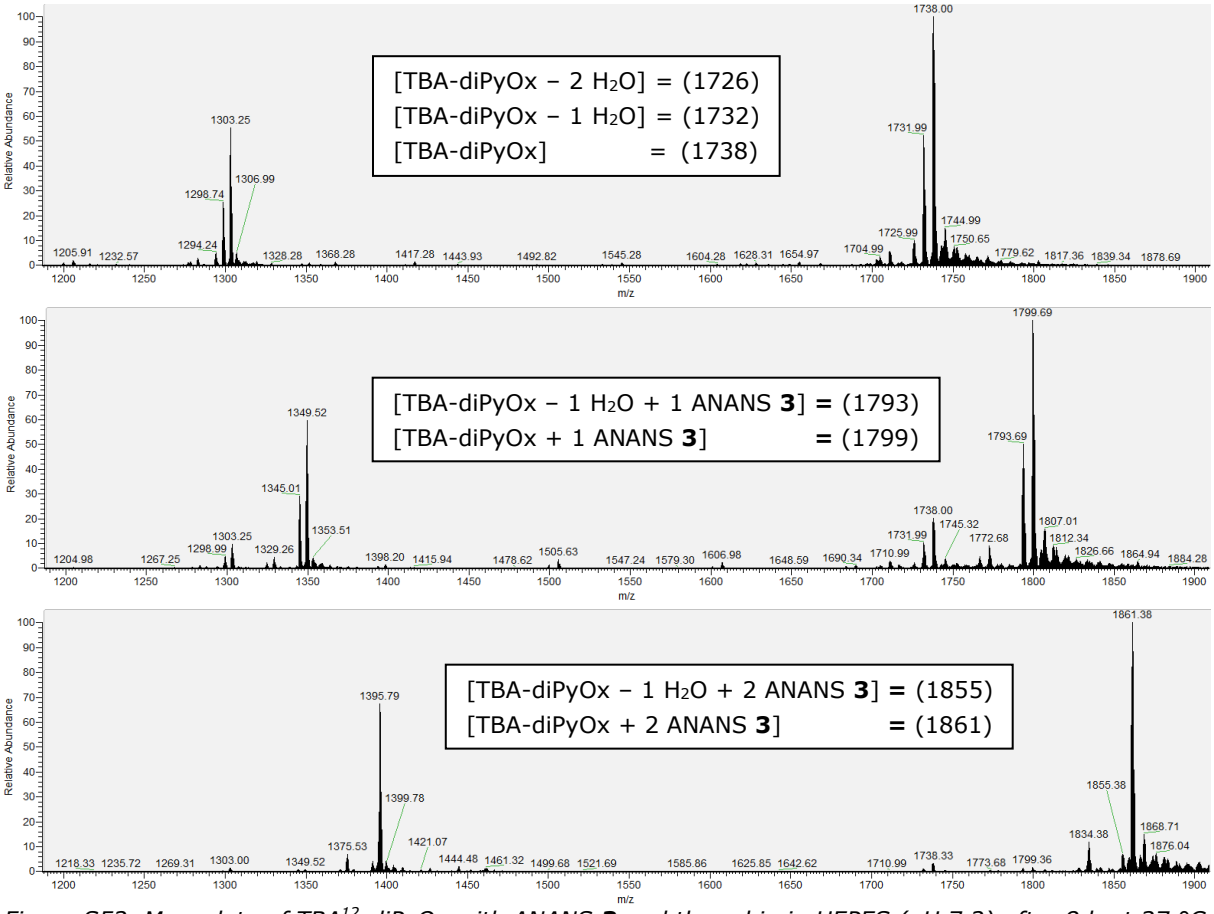


*Figure S52. Mass data of TBA[12]-diPyOx with ANANS **3** and thrombin in HEPES (pH 7.2) after 8 h at 37 °C. As the acyl group can now be donated to thrombin, dehydration of the catalyst occurs much less and di-functional diPyOx is predominantly detected.*

2.5.5 NMR data of organic compounds

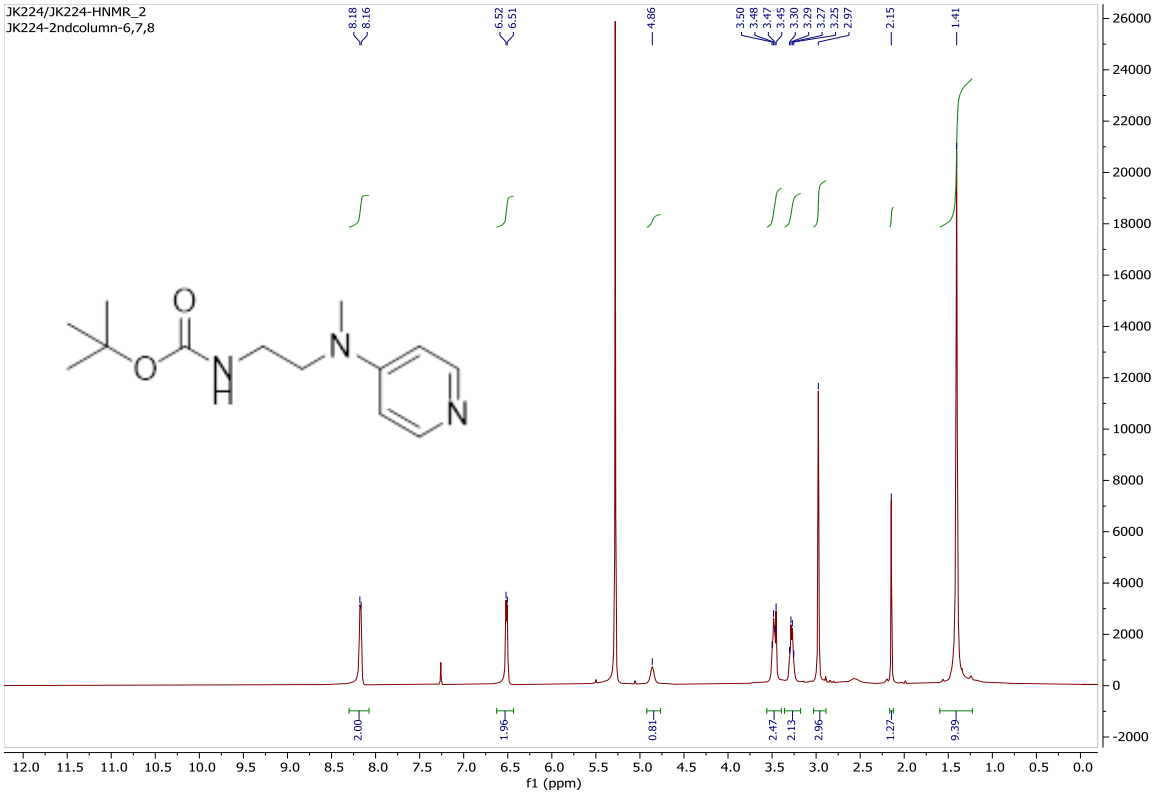


Figure S53. ^{1}H NMR spectrum of Boc-ethylamine-DMAP.

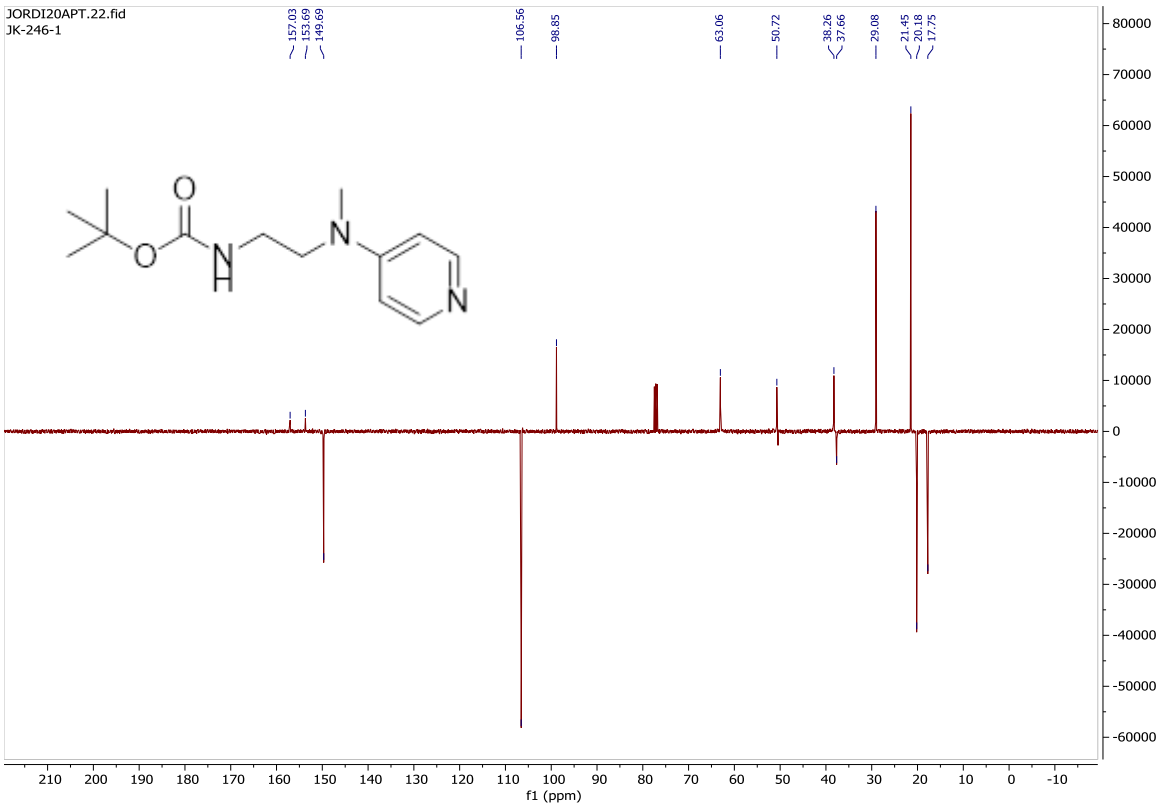


Figure S54. ^{13}C APT NMR spectrum of Boc-ethylamine-DMAP.

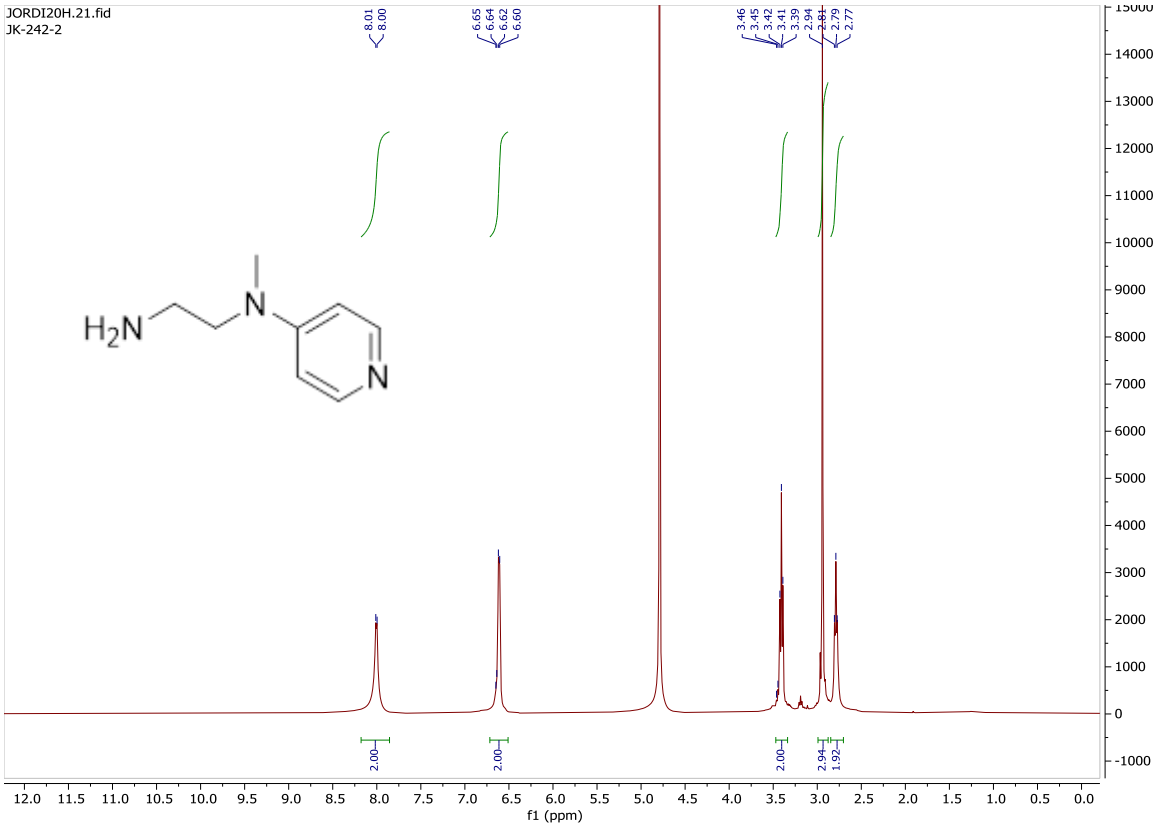


Figure S55. ^{1}H NMR spectrum of ethylamine-DMAP.

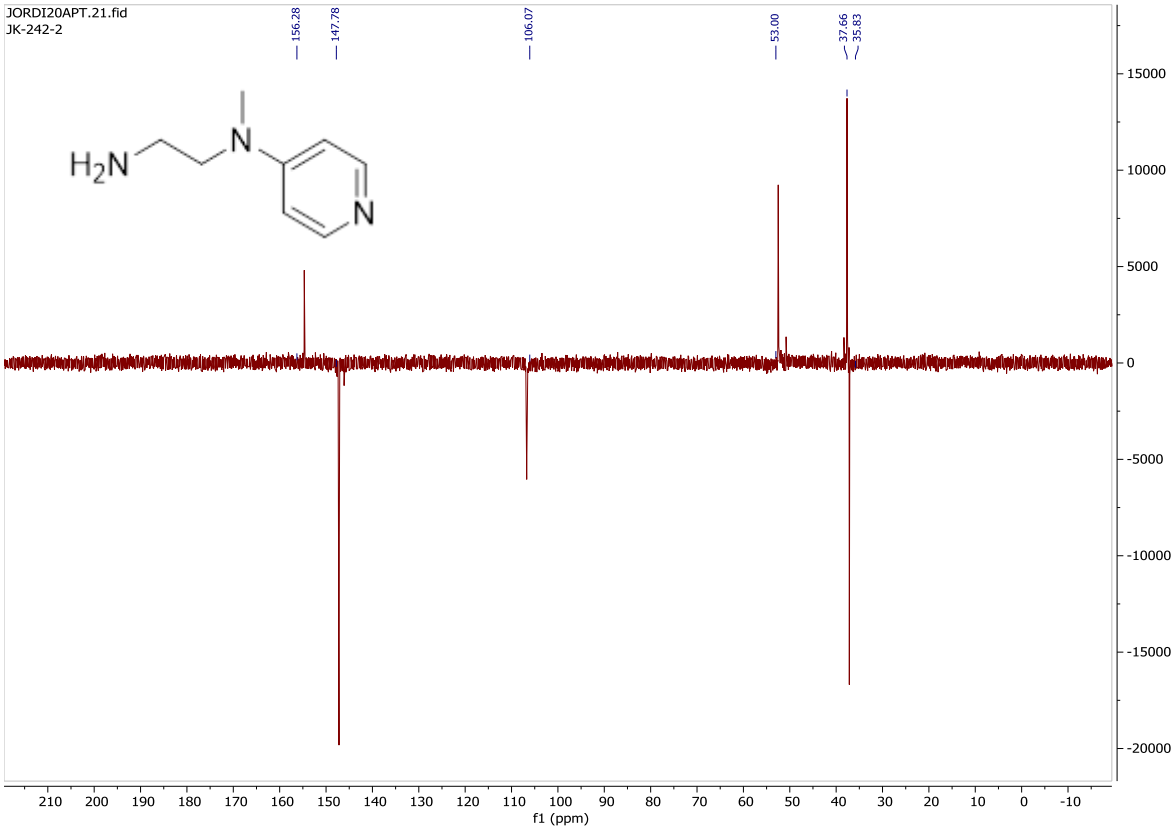


Figure S56. ^{13}C APT NMR spectrum of ethylamine-DMAP.

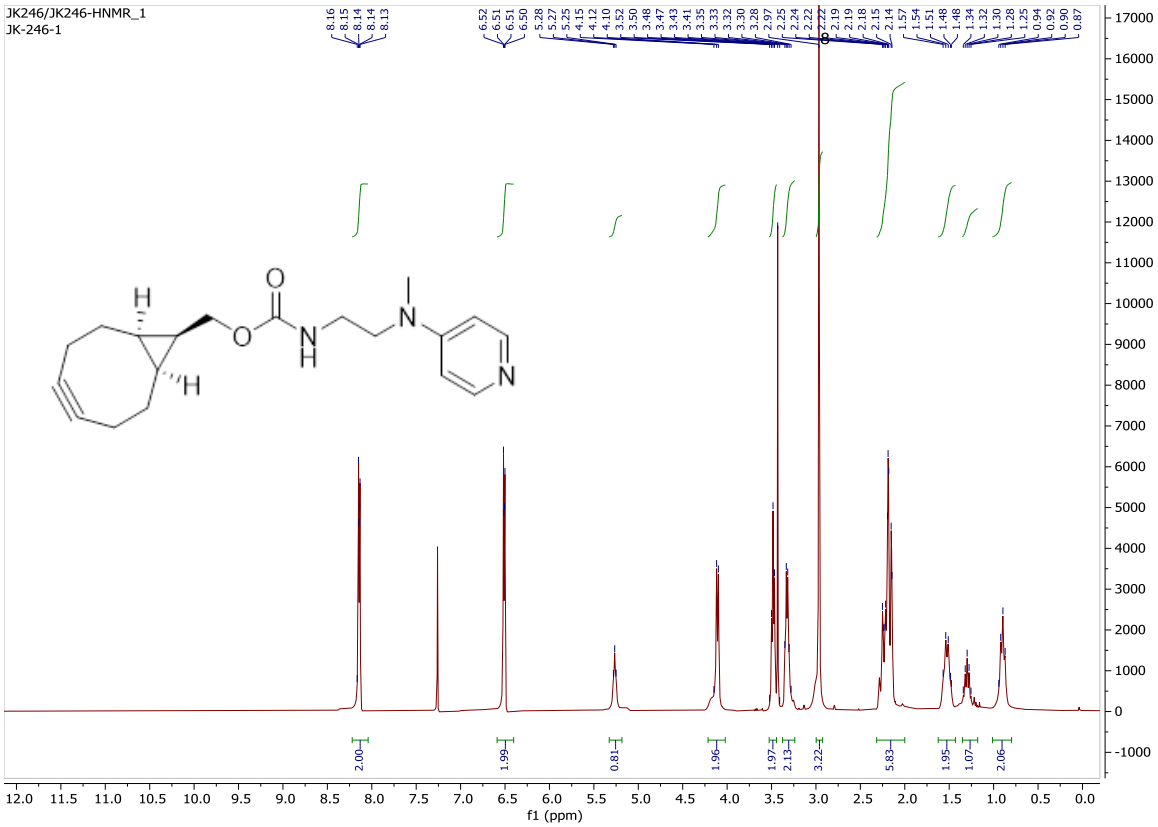


Figure S57. ^{1}H NMR spectrum of BCN-DMAP.

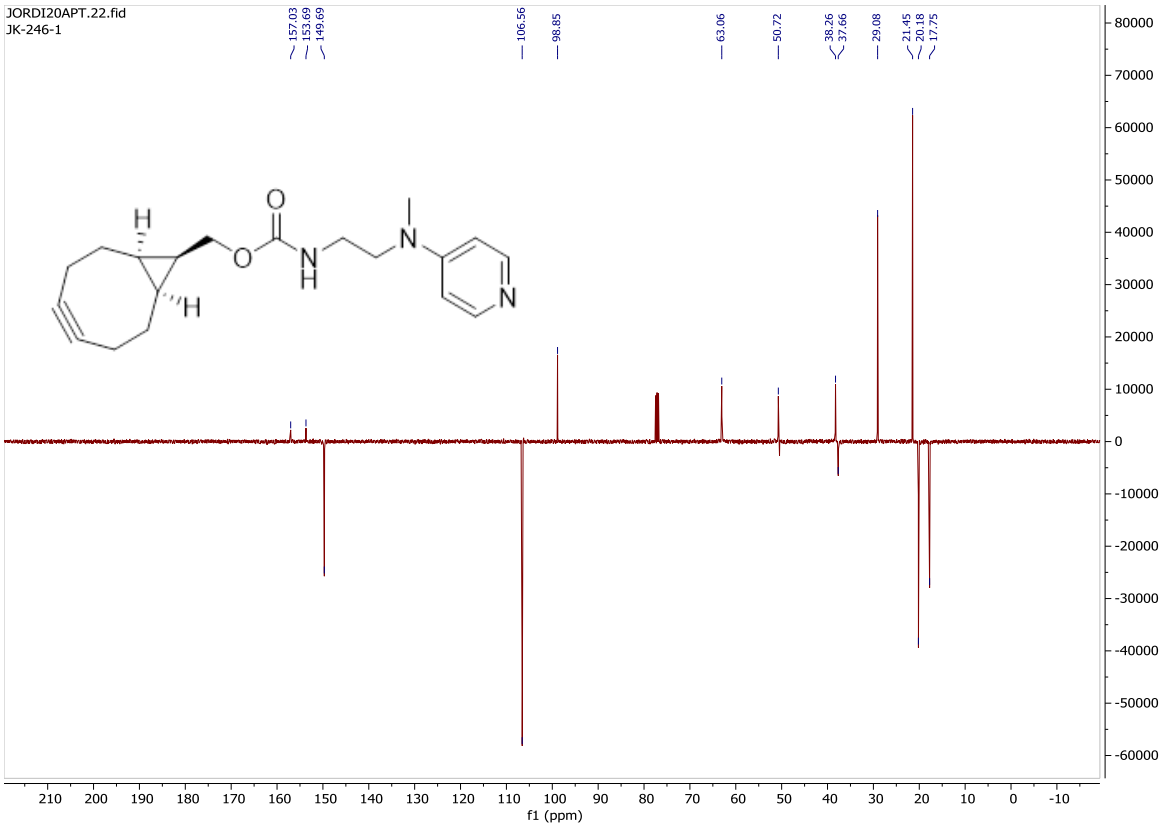


Figure S58. ^{13}C APT NMR spectrum of BCN-DMAP.

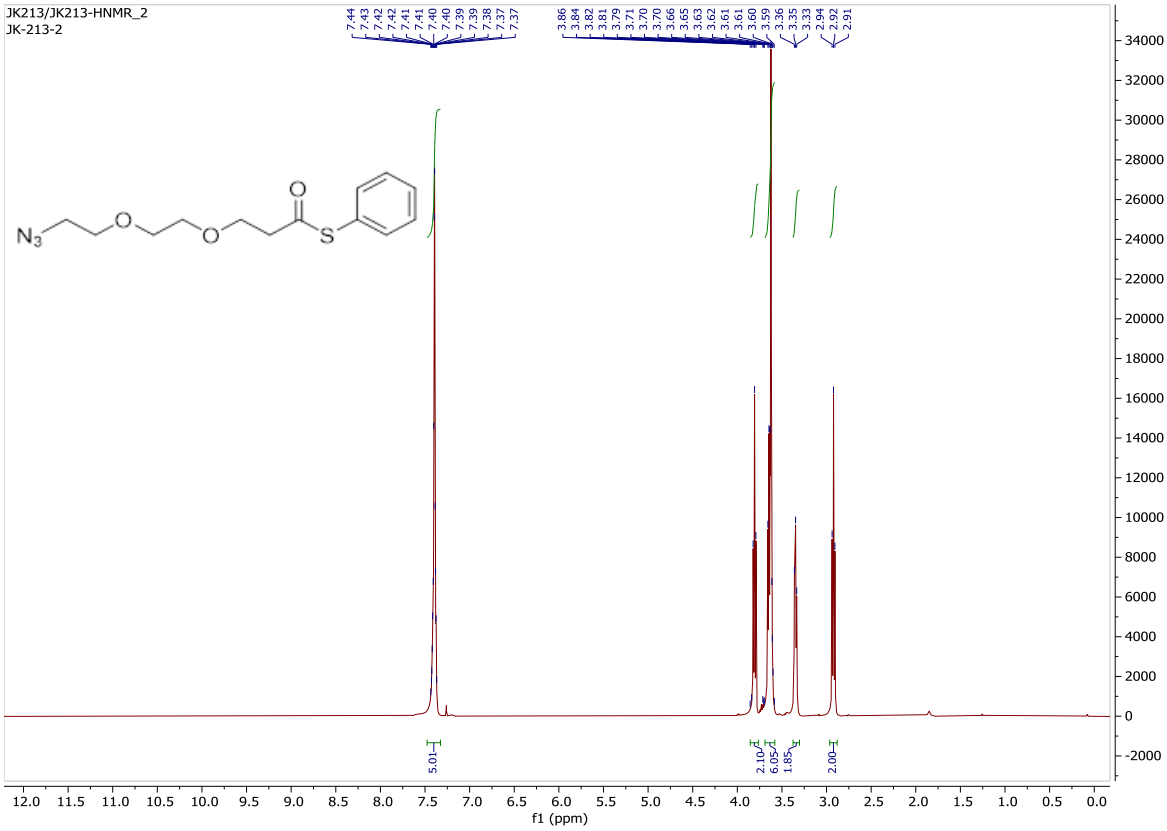


Figure S59. ^{1}*H NMR spectrum of azido-thioester* ***1.***

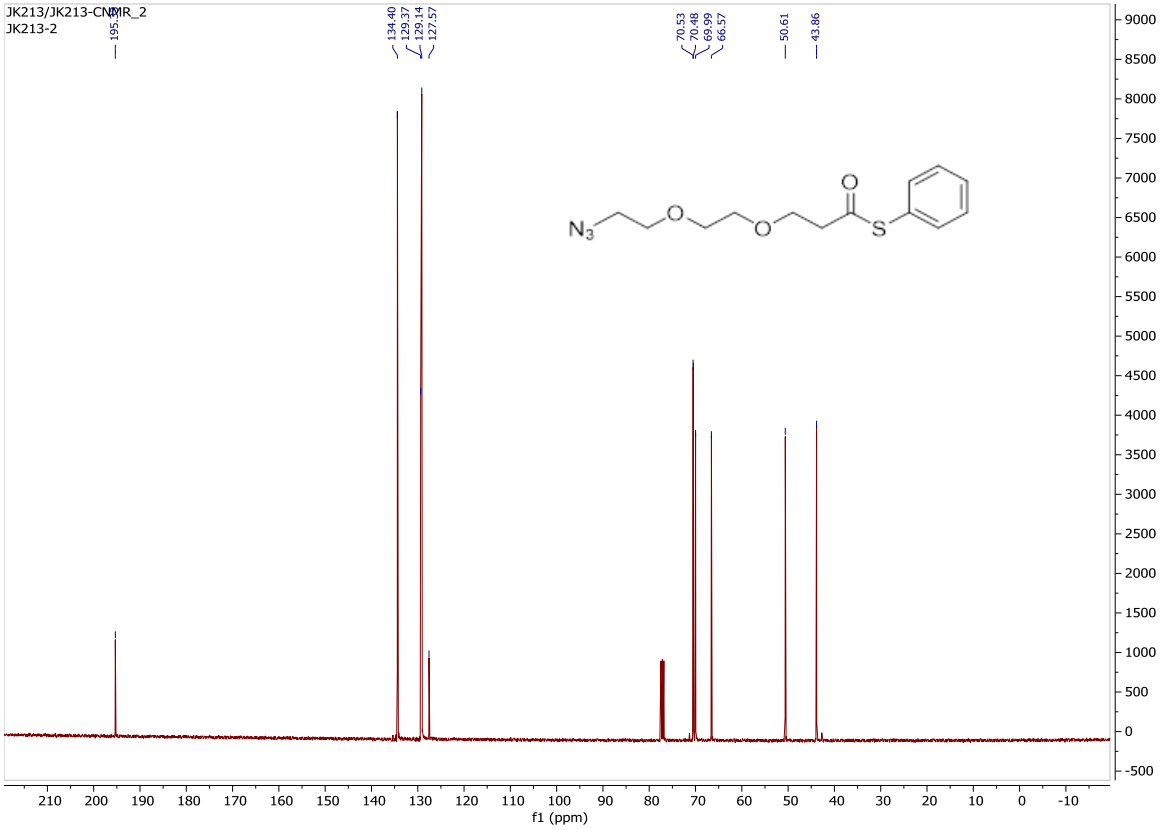


Figure S60. ^{13}*C NMR spectrum of azido-thioester* ***1.***

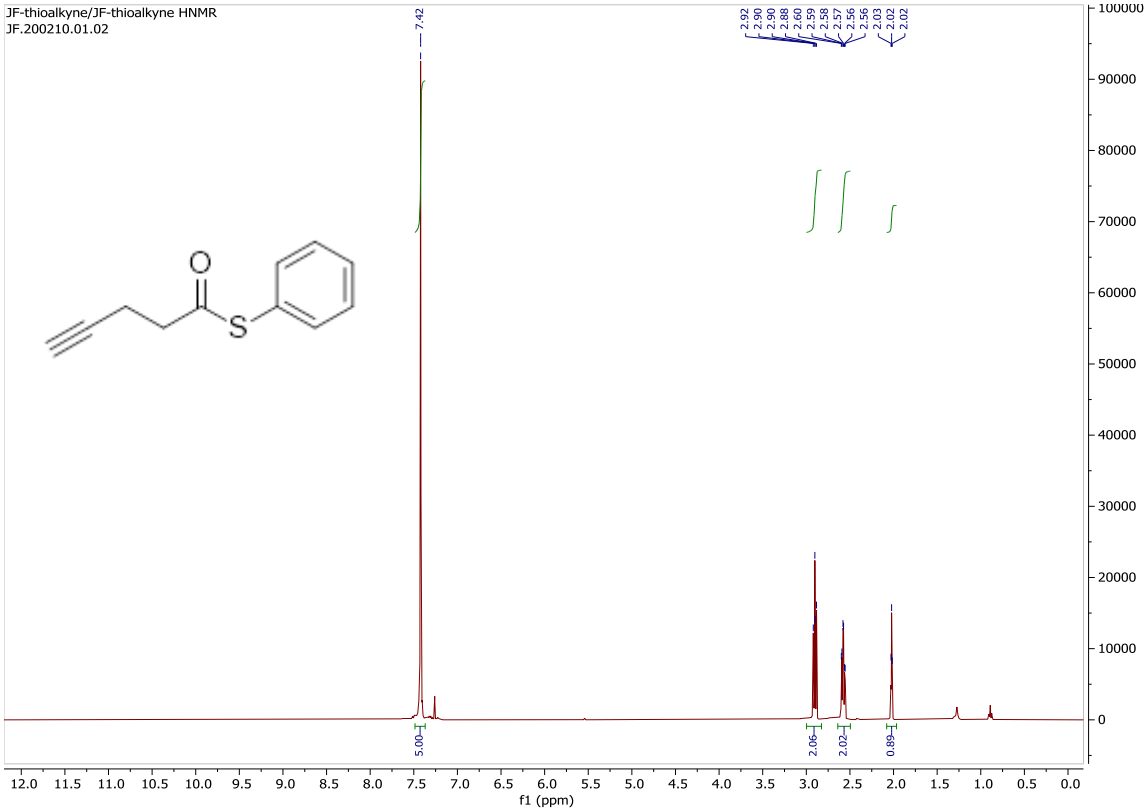


Figure S61. ^{1}*H NMR spectrum of alkyne-thioester* ***2.***

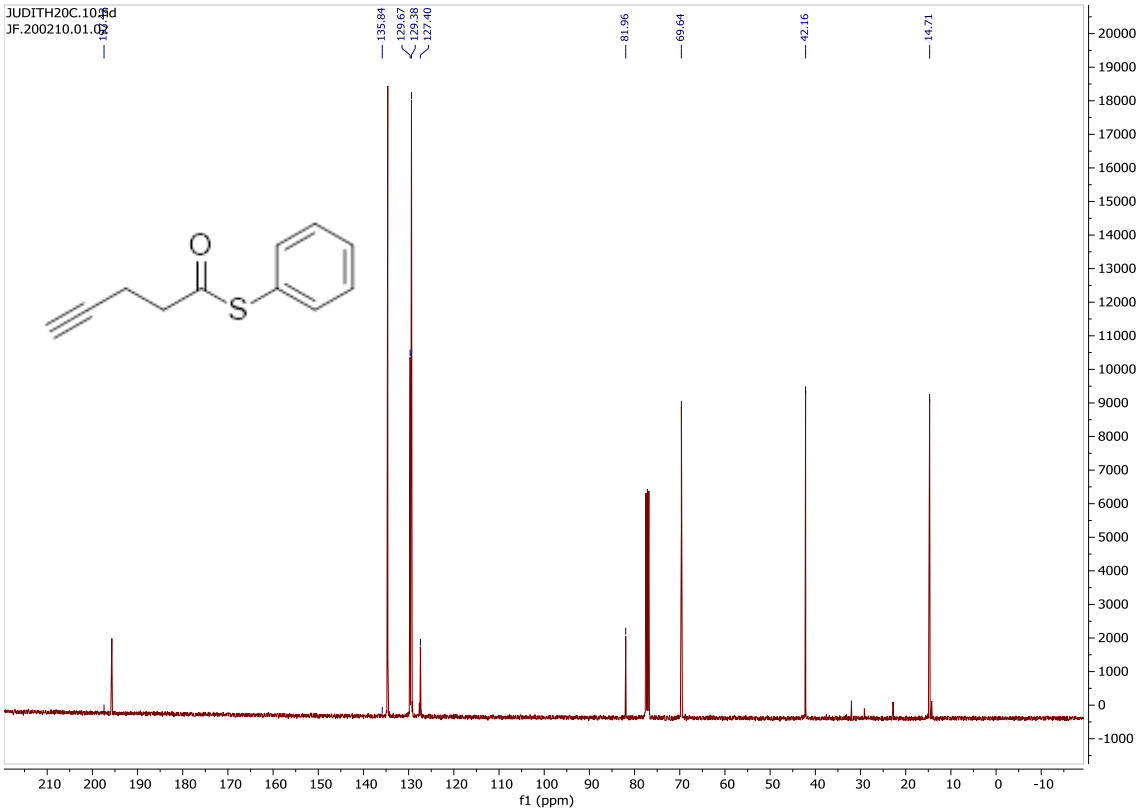


Figure S62. ^{13}*C NMR spectrum of alkyne-thioester* ***2.***

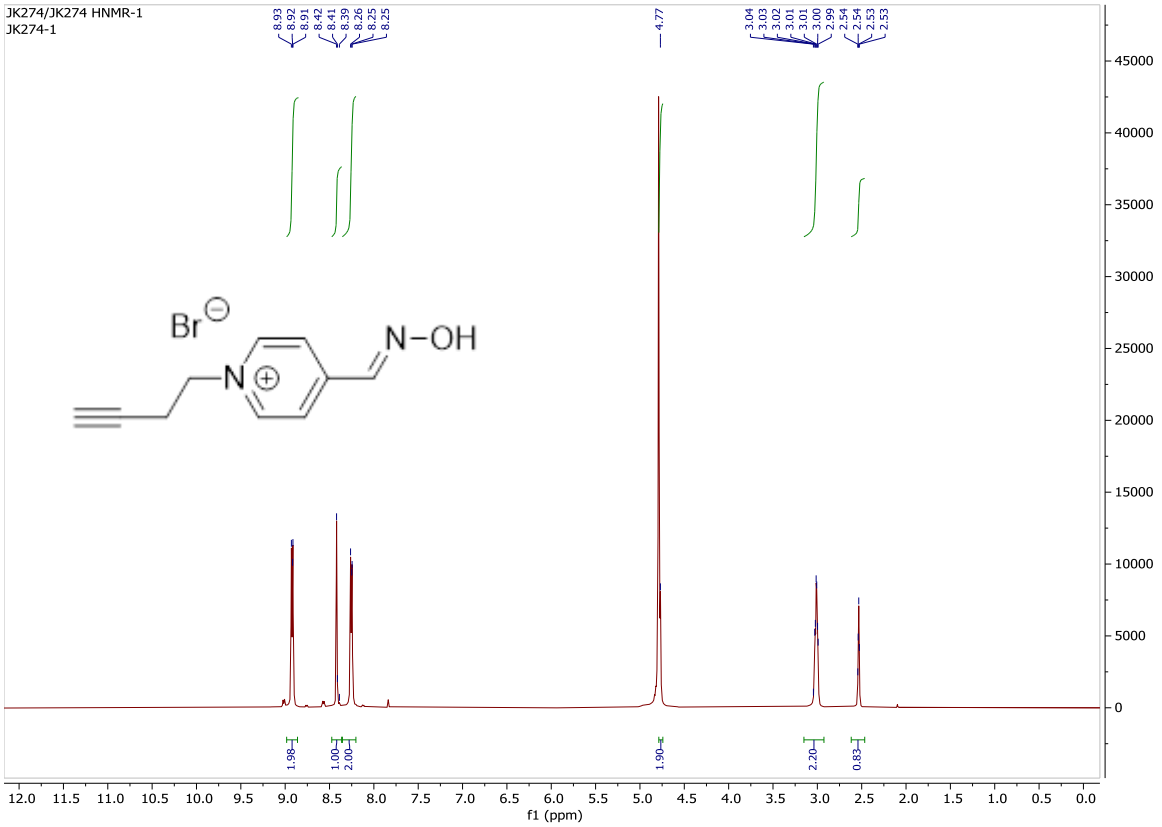


Figure S63. ^{1}H NMR spectrum of alkyne-PyOx.

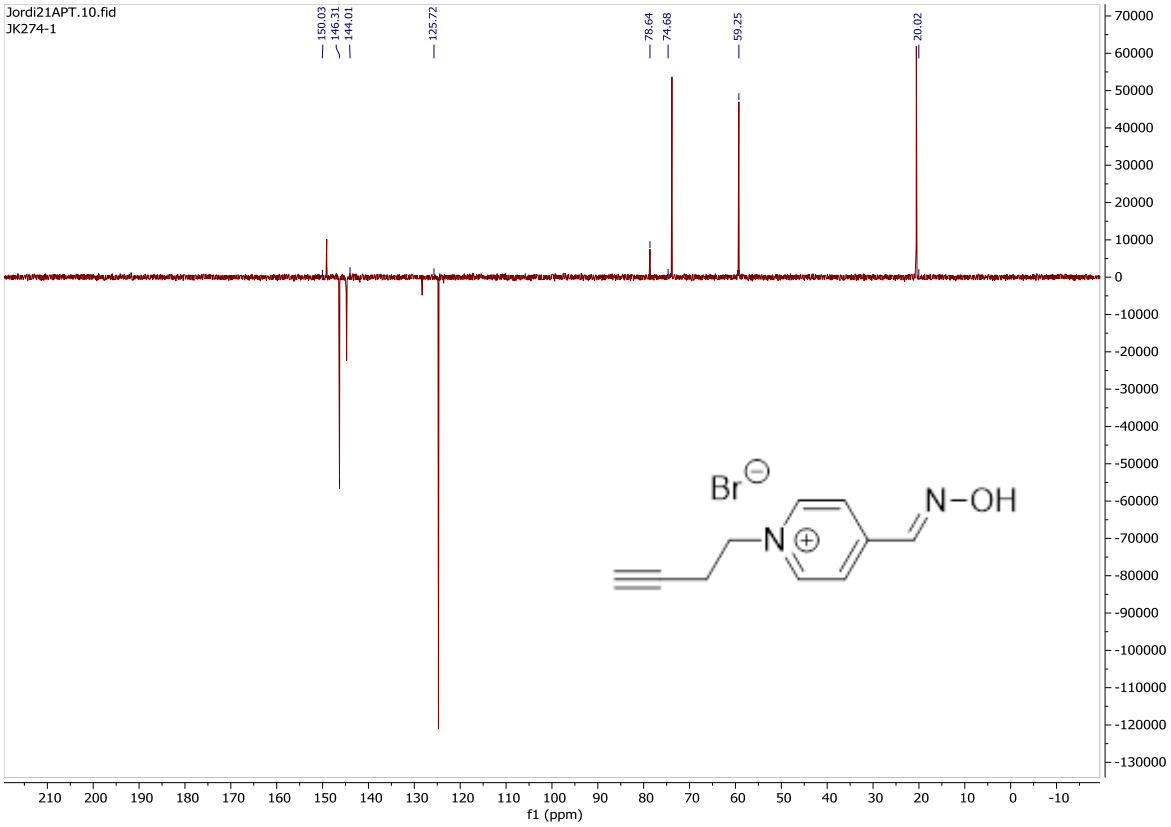


Figure S64. ^{13}C APT NMR spectrum of alkyne-PyOx.

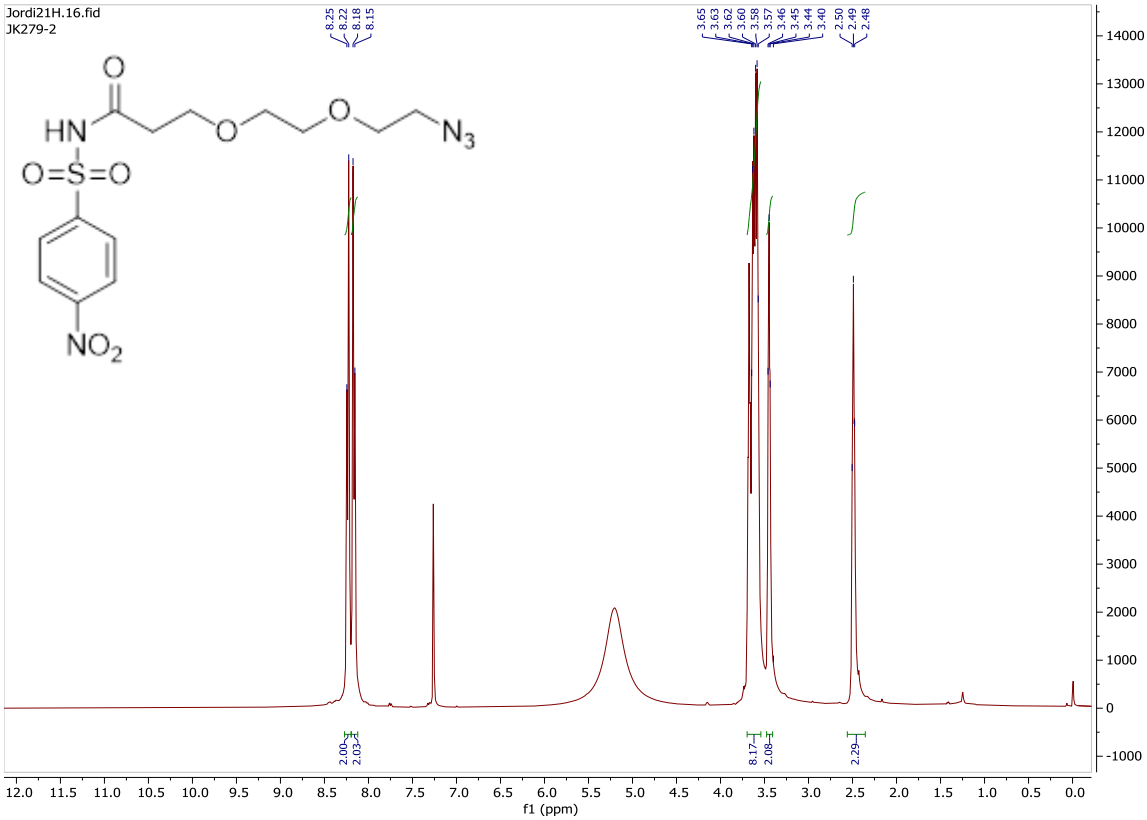


Figure S65. ^{1}H NMR spectrum of azido-ANANS precursor.

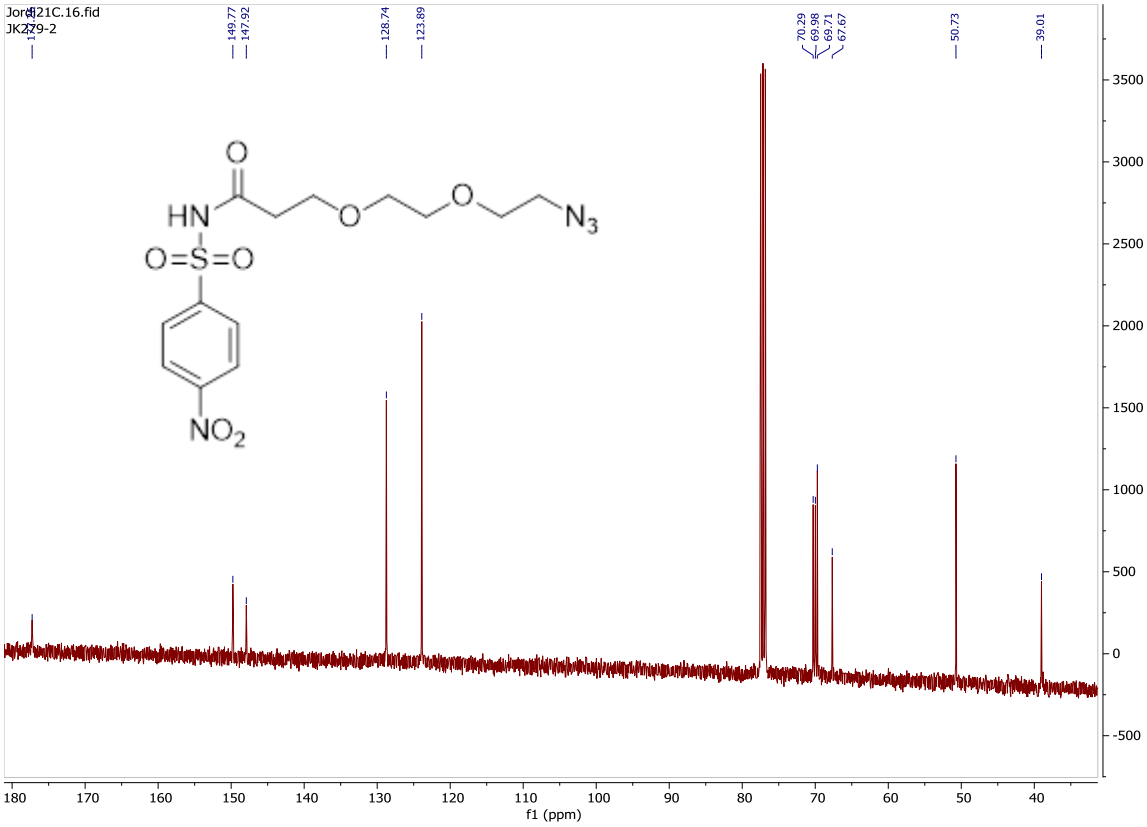


Figure S66. ^{13}C NMR spectrum of azido-ANANS precursor.

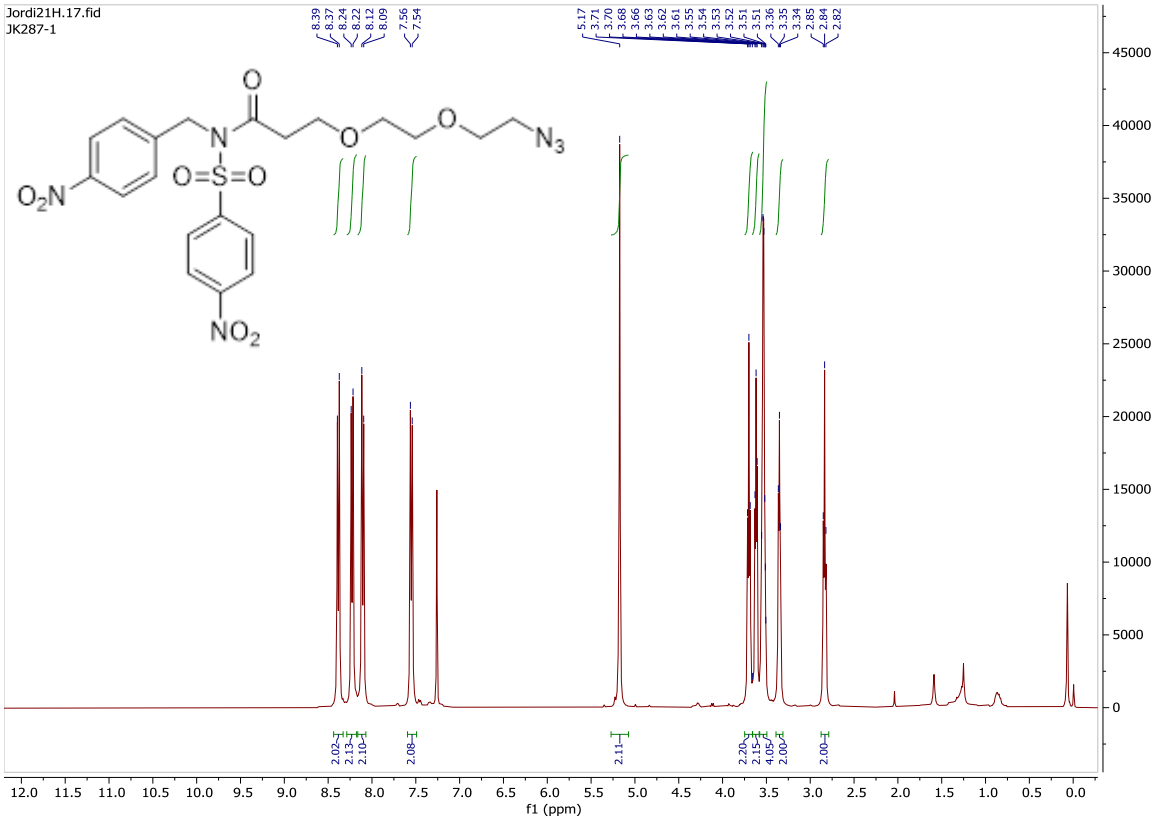


Figure S67. ^{1}*H NMR spectrum of azido-ANANS* ***3.***

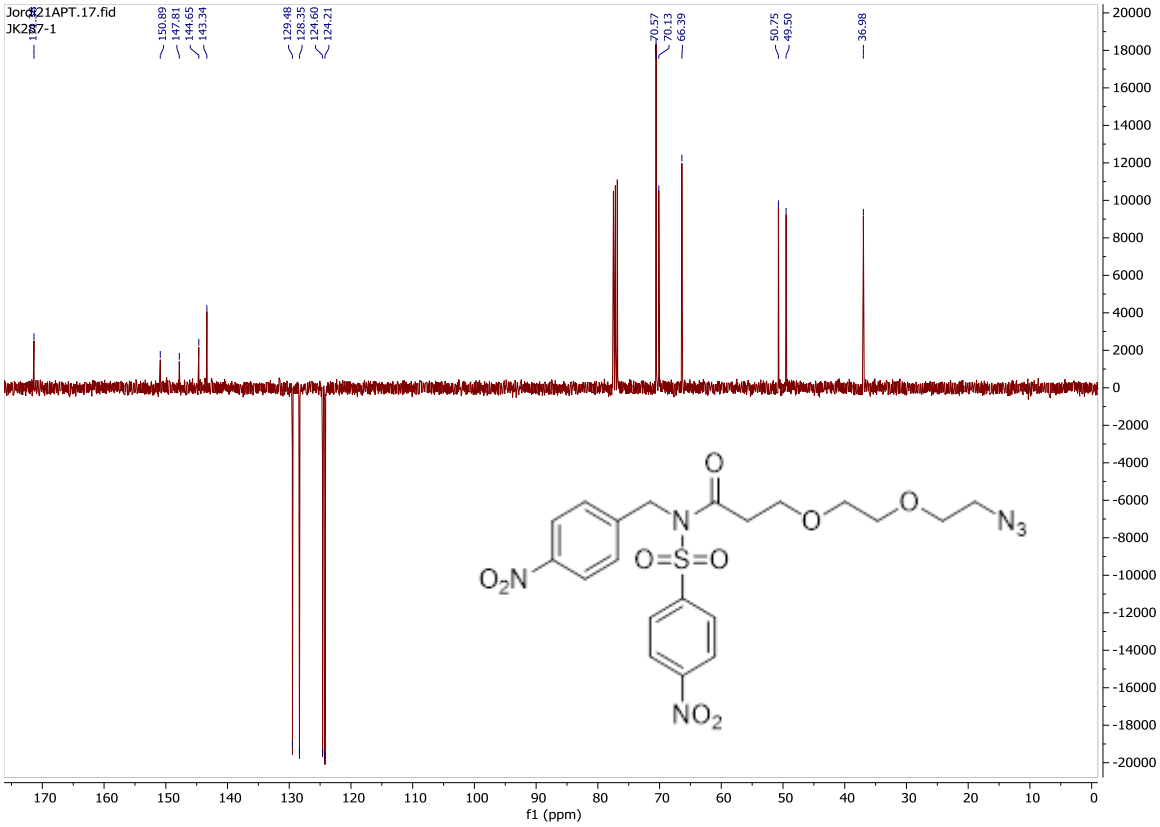


Figure S68. ^{13}*C APT NMR spectrum of azido-ANANS* ***3.***

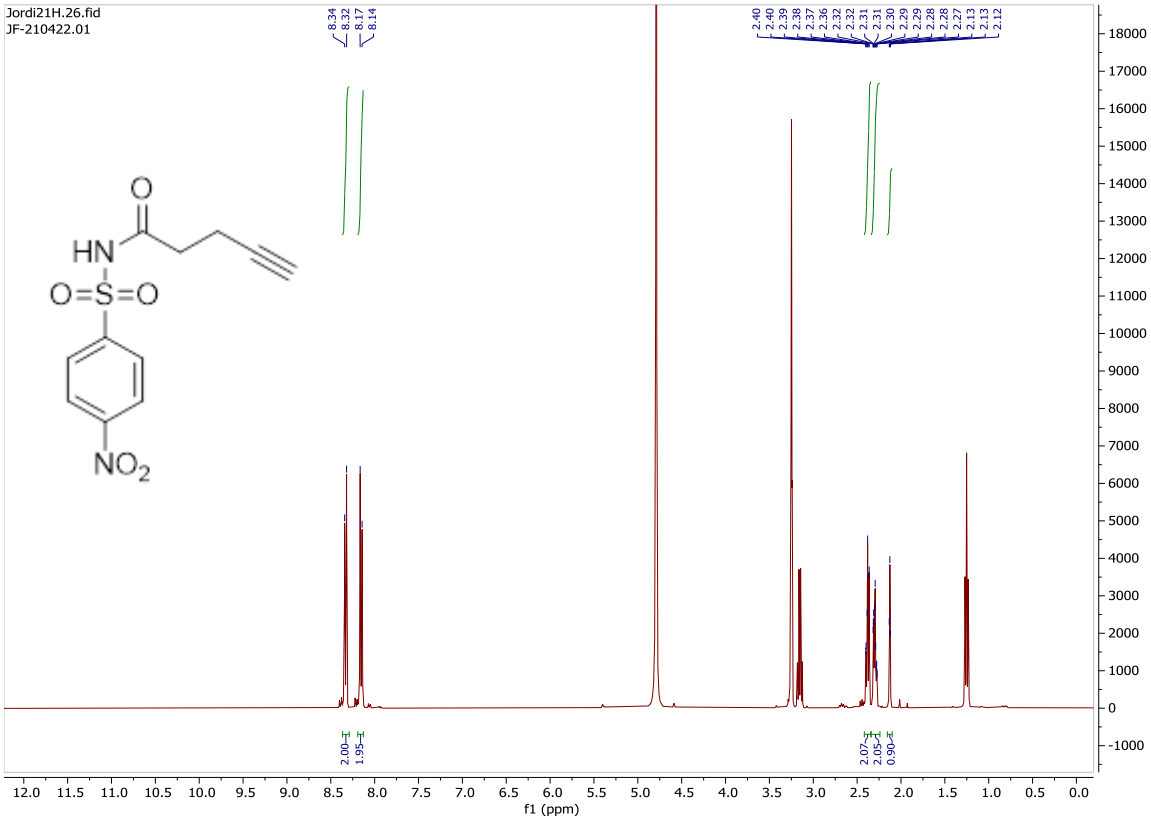


Figure S69. ^{1}H NMR spectrum of alkyne-ANANS precursor (with EtOAc).

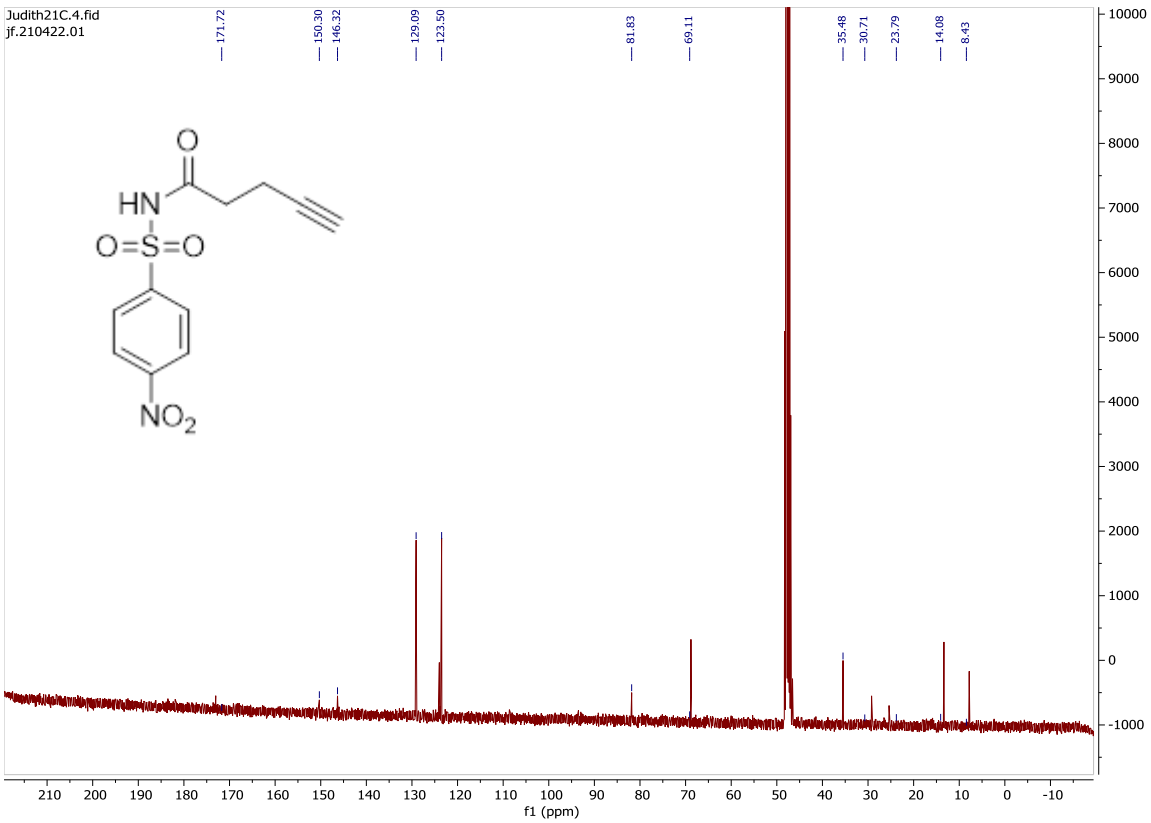


Figure S70. ^{13}C NMR spectrum of alkyne-ANANS precursor (with EtOAc).

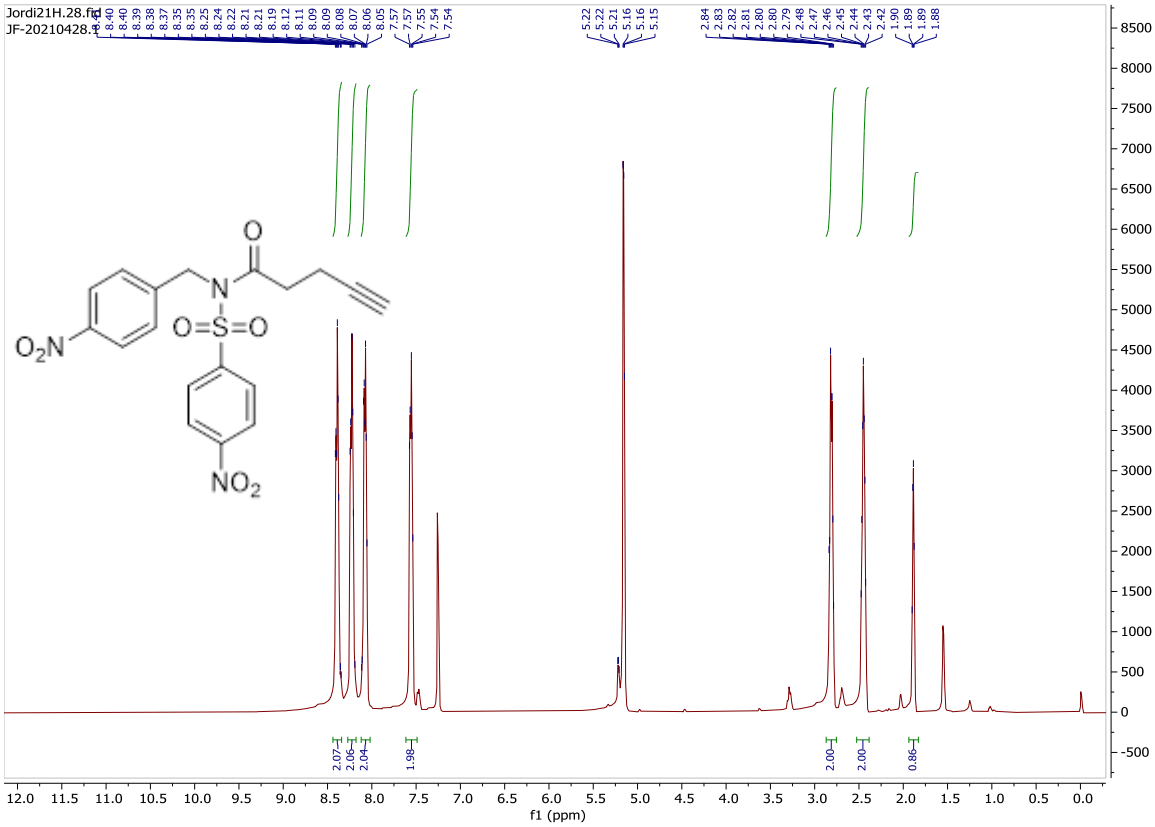


Figure S71. ^{1}*H NMR spectrum of alkyne-ANANS* ***4****.*

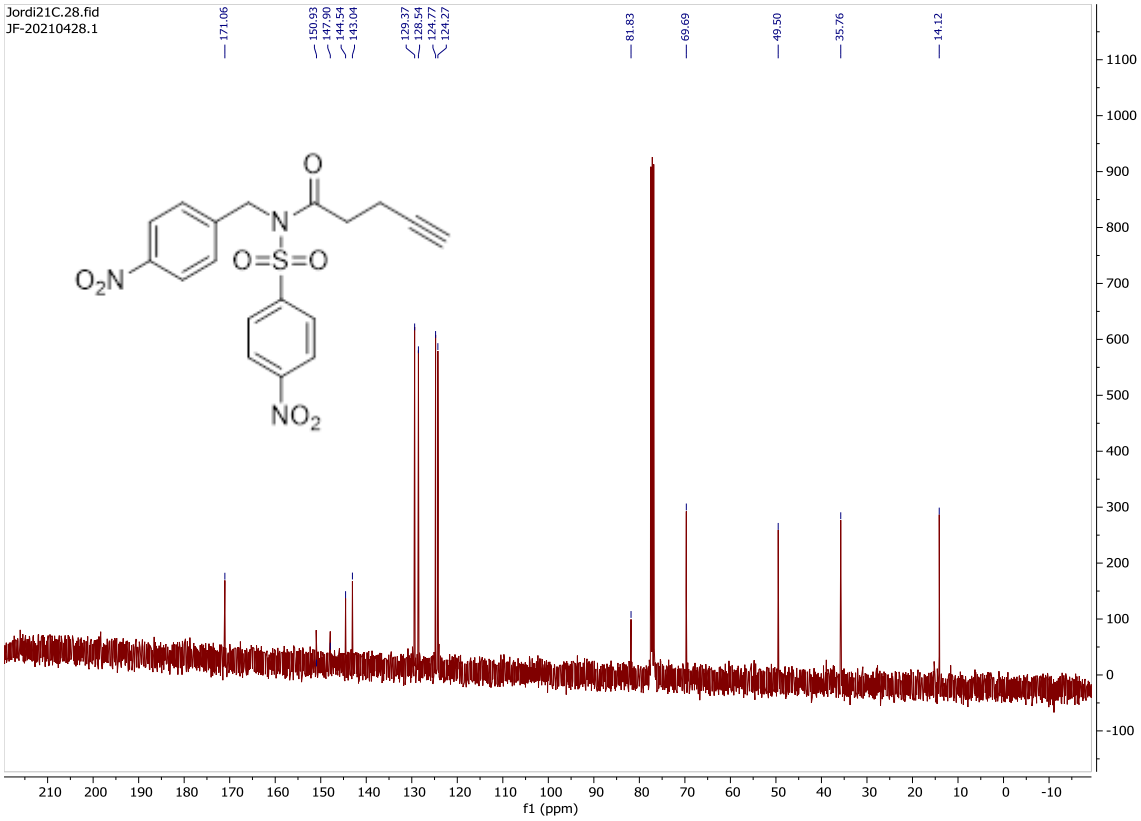


Figure S72. ^{13}*C NMR spectrum of alkyne-ANANS* ***4****.*

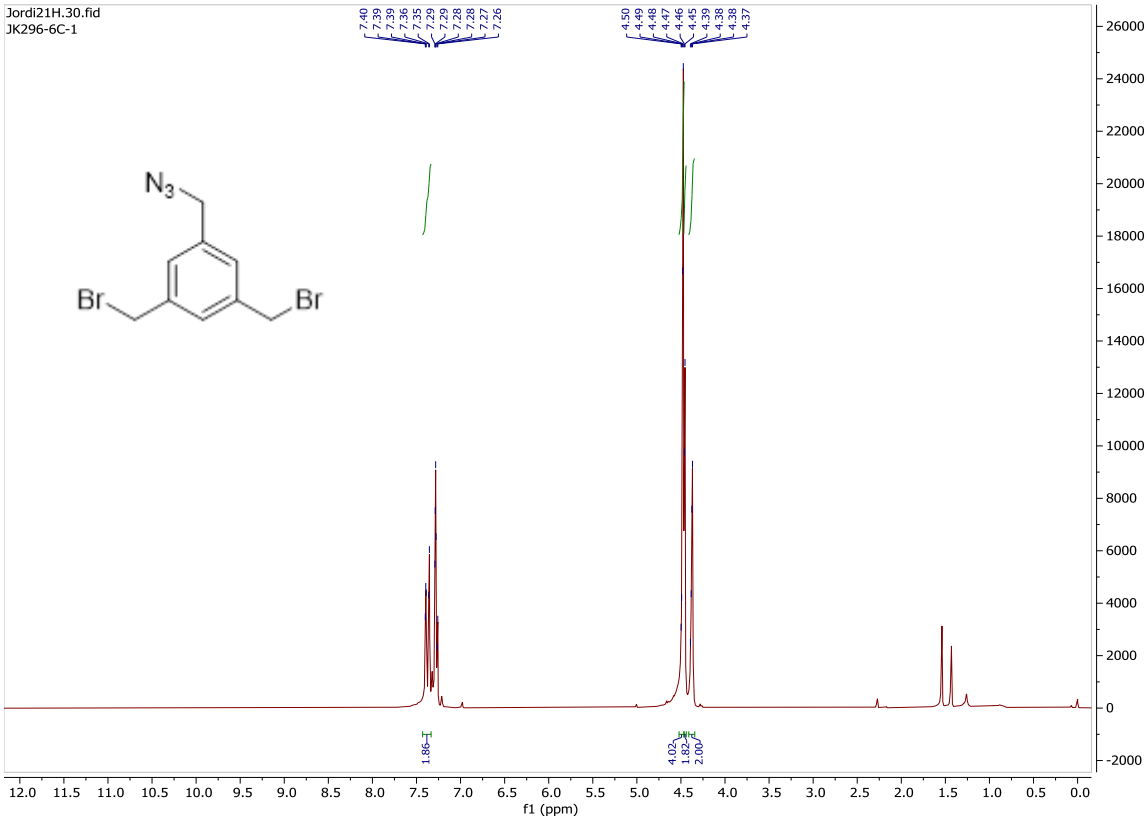


Figure S73. ^{1}H NMR spectrum of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene.

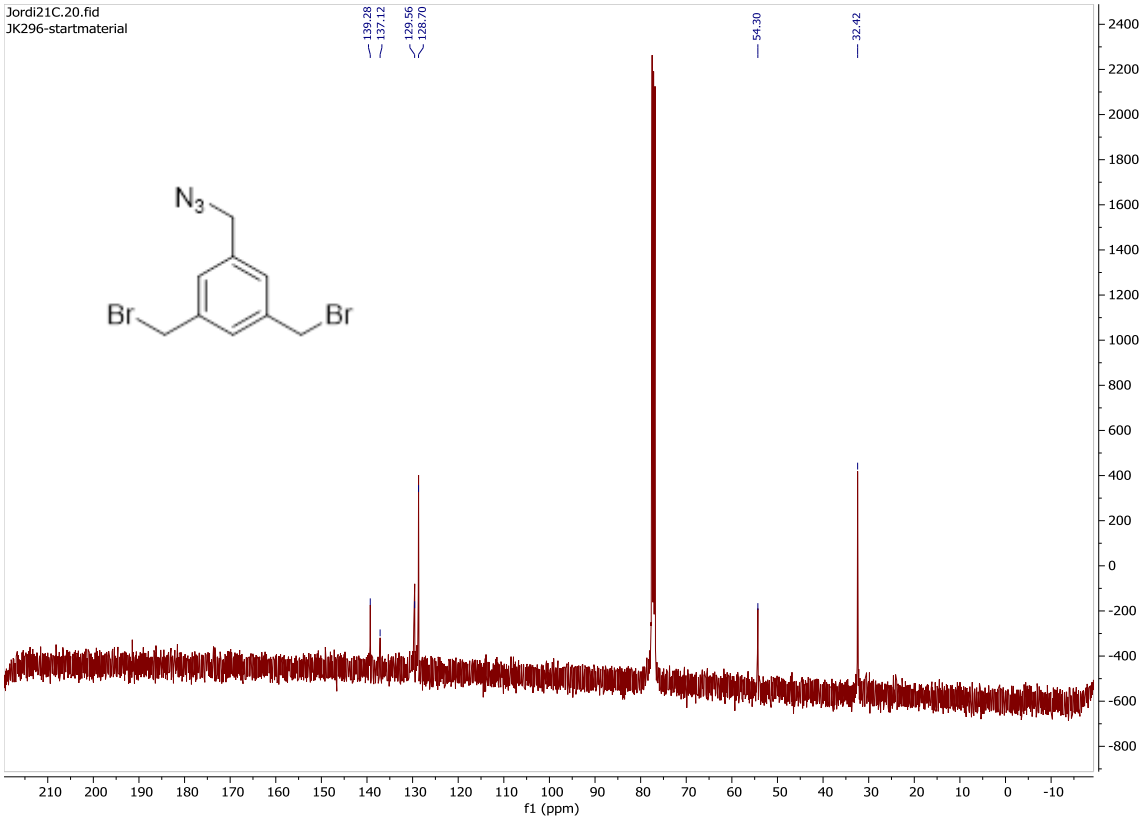


Figure S74. ^{13}C NMR spectrum of 1-(azidomethyl)-3,5-bis(bromomethyl)benzene.

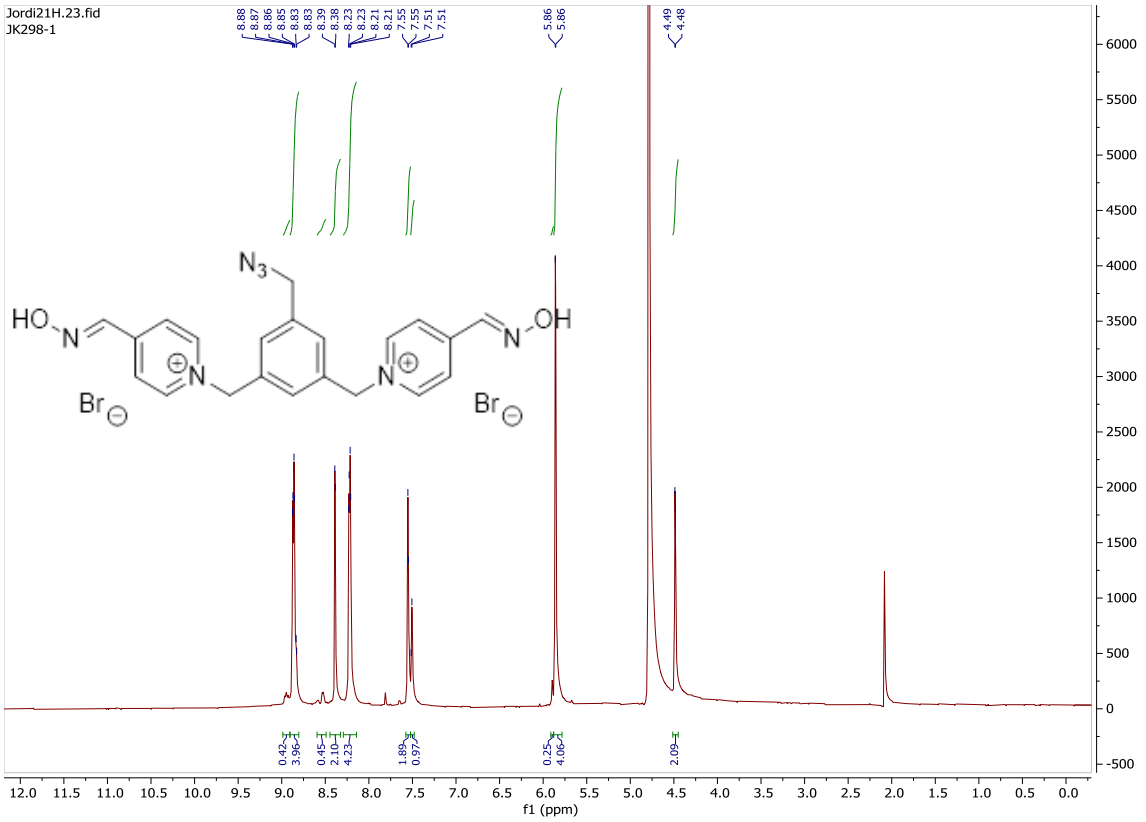


Figure S75. ^{1}H NMR spectrum of azido-diPyOx.

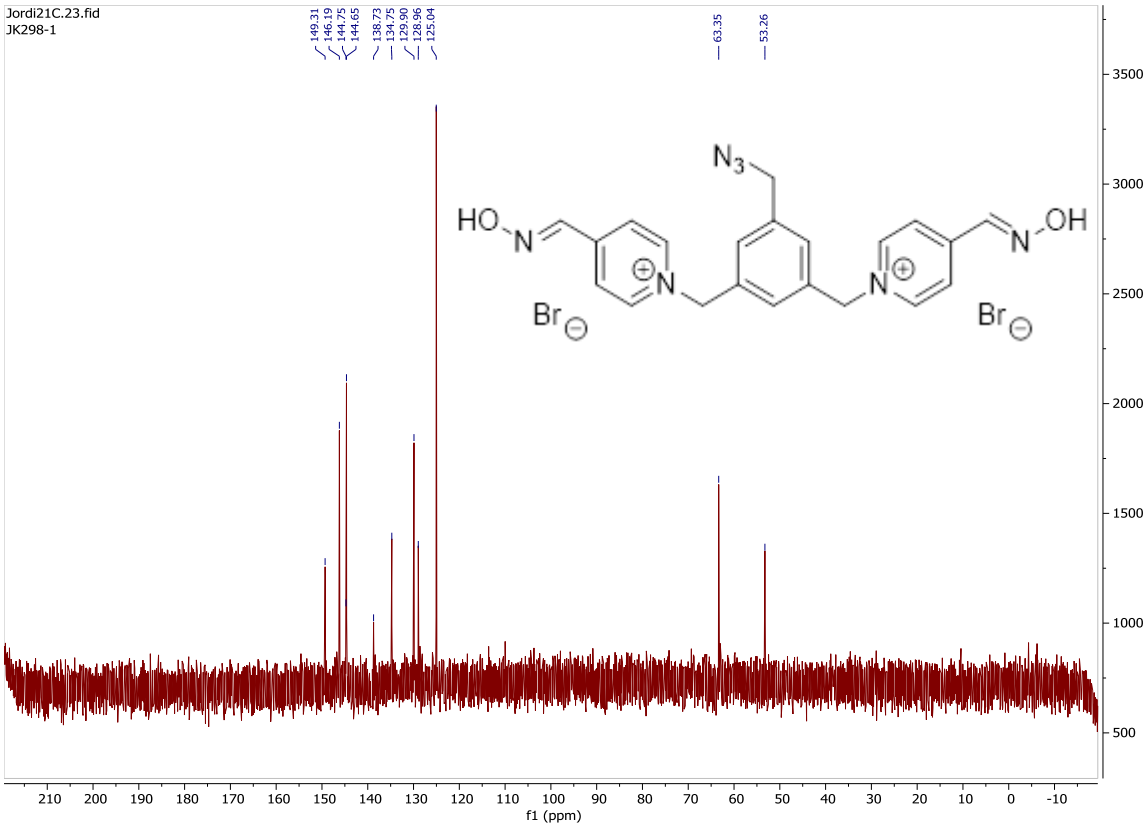


Figure S76. ^{13}C NMR spectrum of azido-diPyOx.

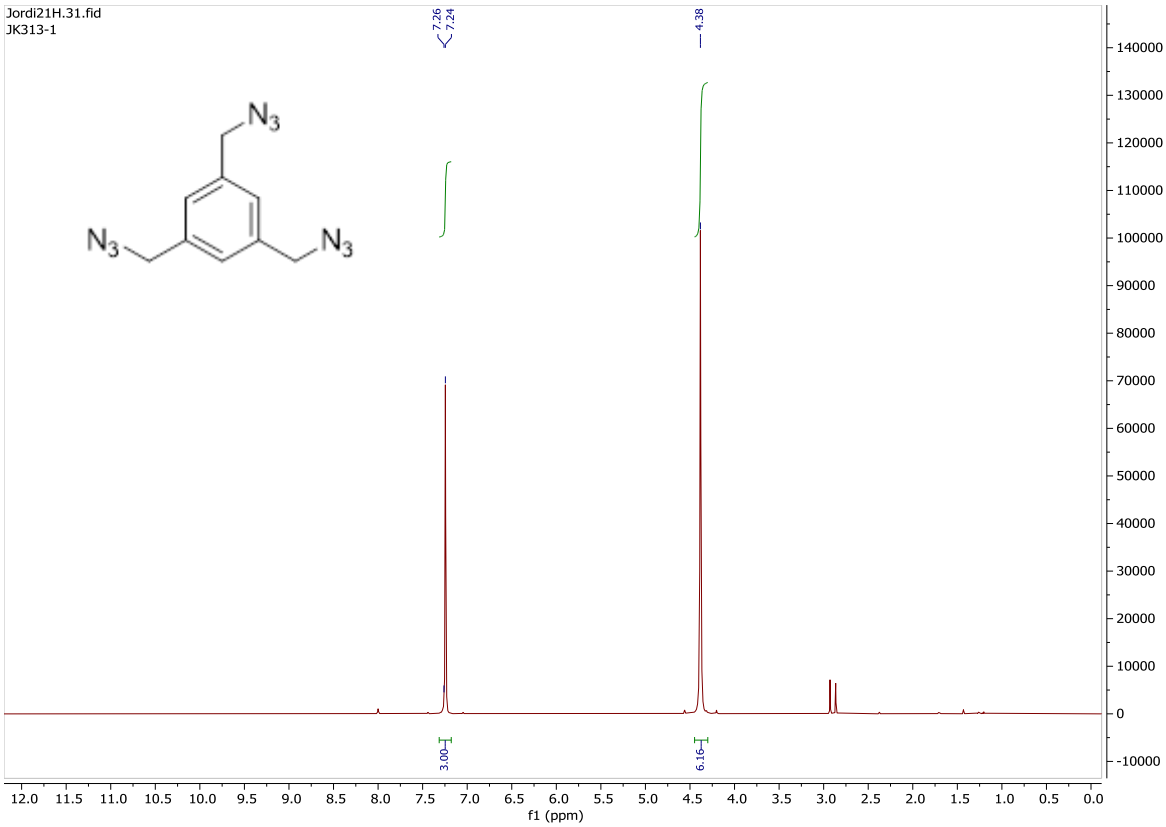


Figure S77. ^{1}H NMR spectrum of 1,3,5-tris(azidomethyl)benzene.

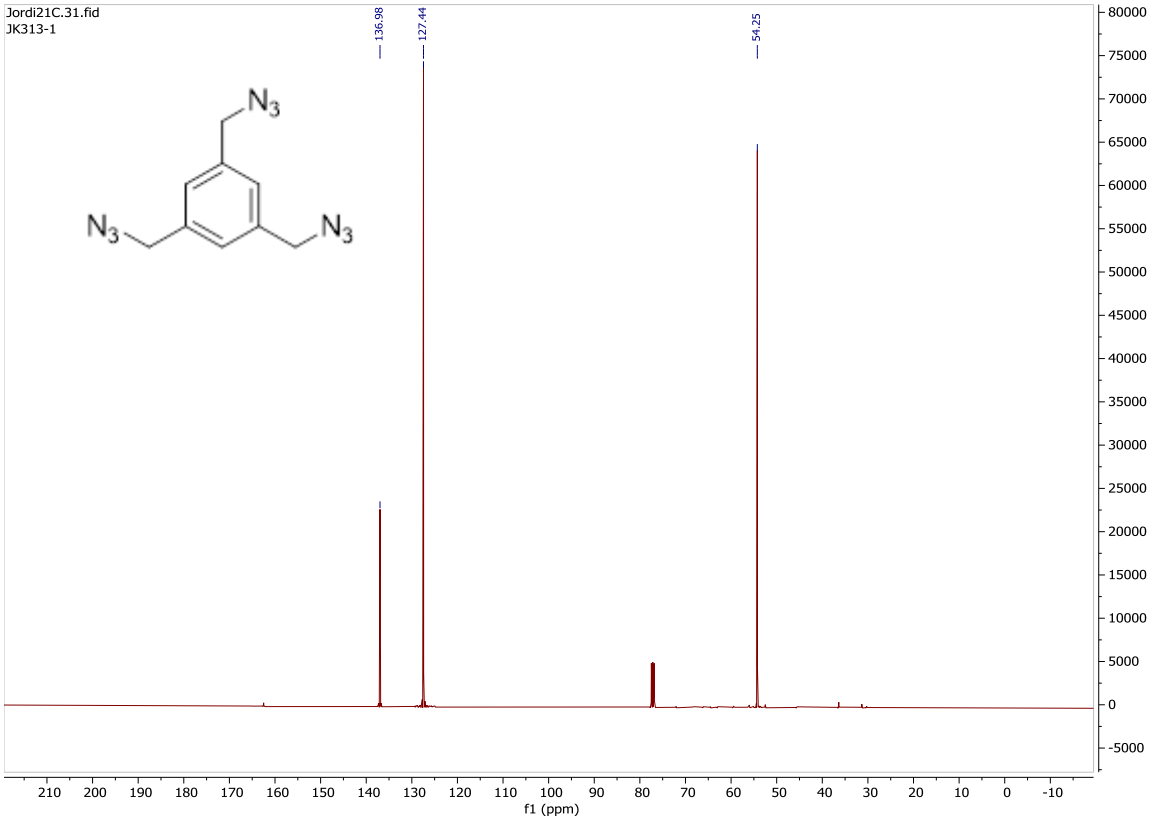


Figure S78. ^{13}C NMR spectrum of 1,3,5-tris(azidomethyl)benzene.

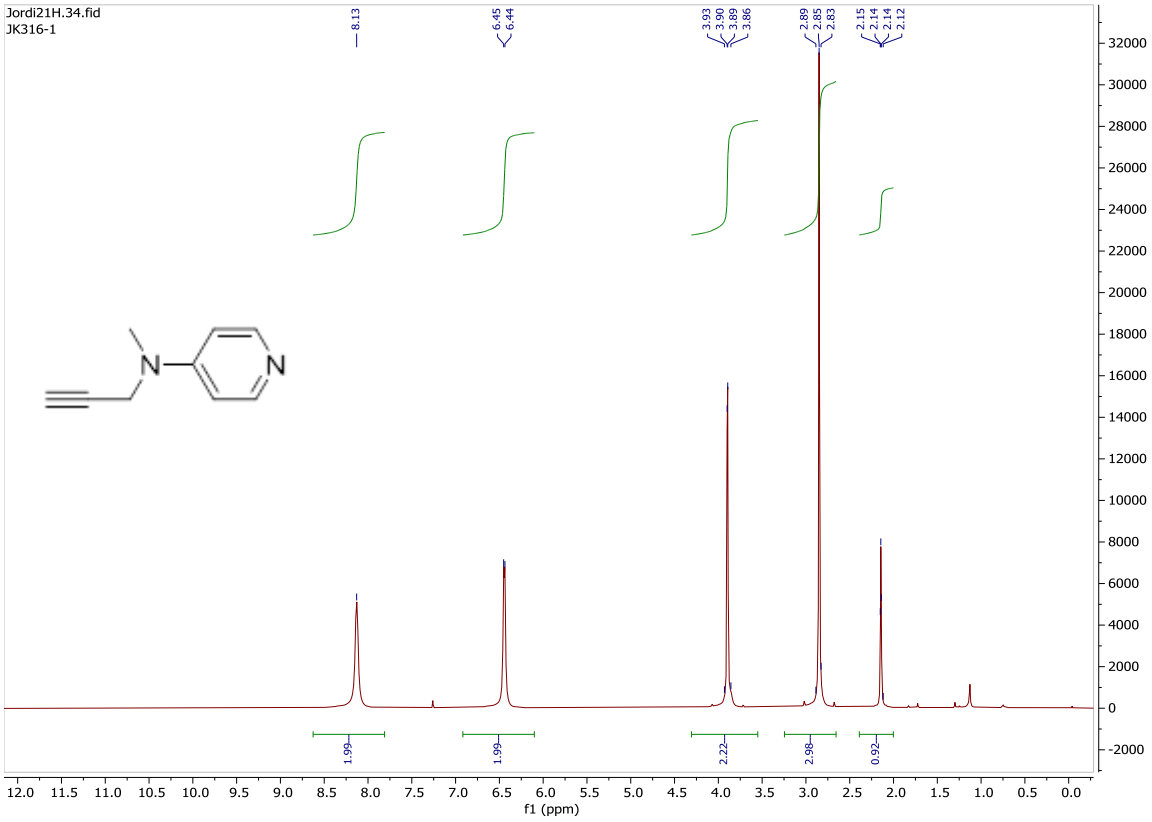


Figure S79. ^{1}H NMR spectrum of alkyne-DMAP.

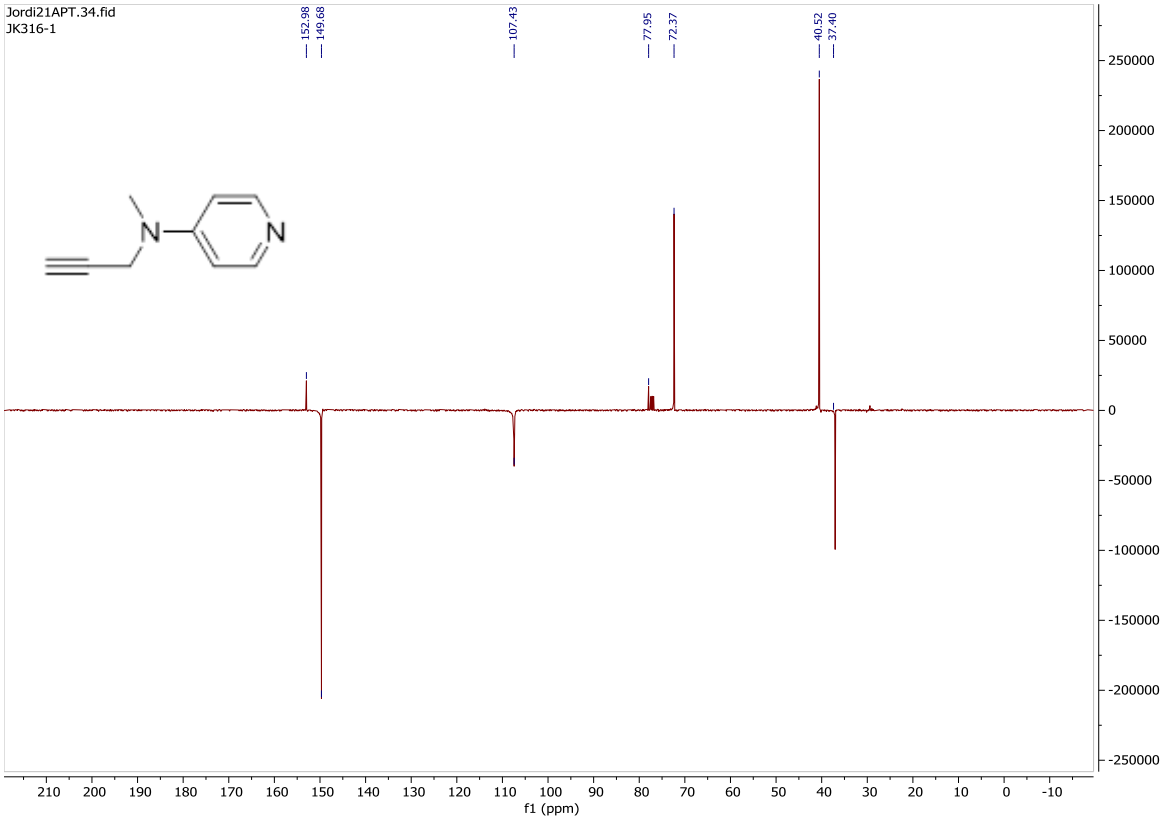


Figure S80. ^{13}C NMR spectrum of alkyne-DMAP.

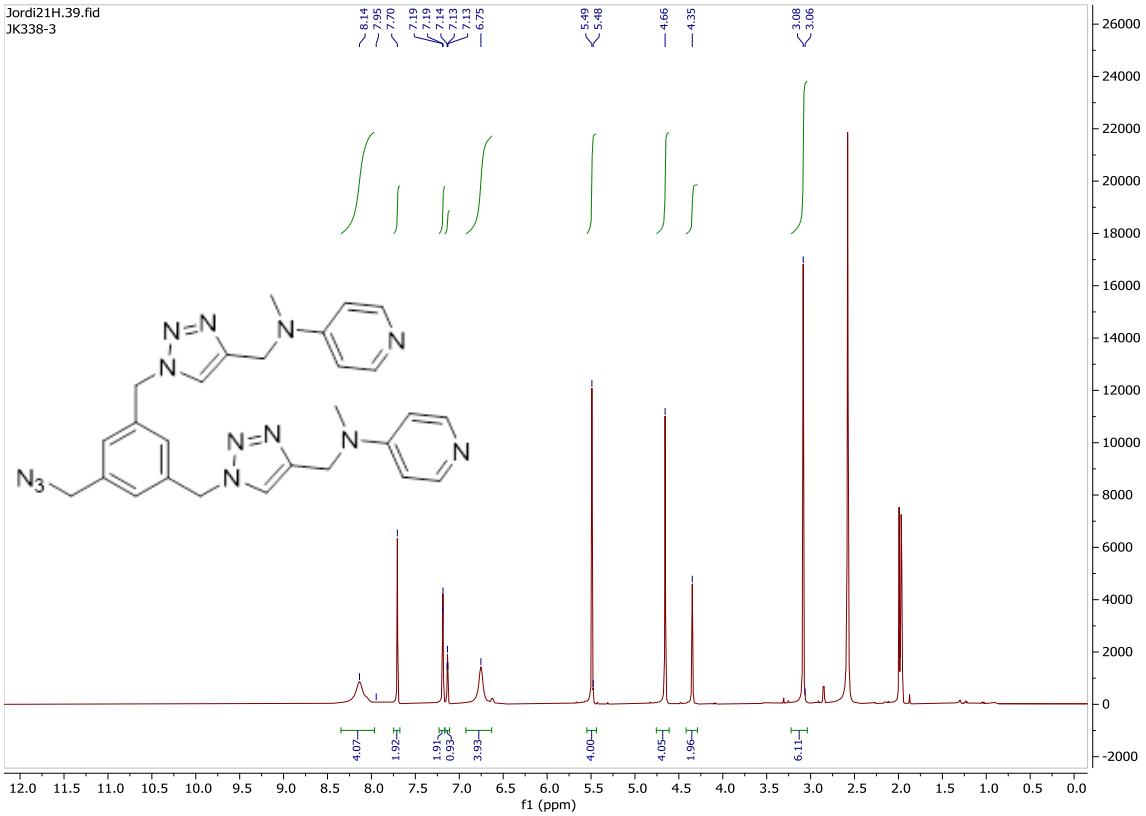


Figure S81. ^{1}H NMR spectrum of azido-diDMAP.

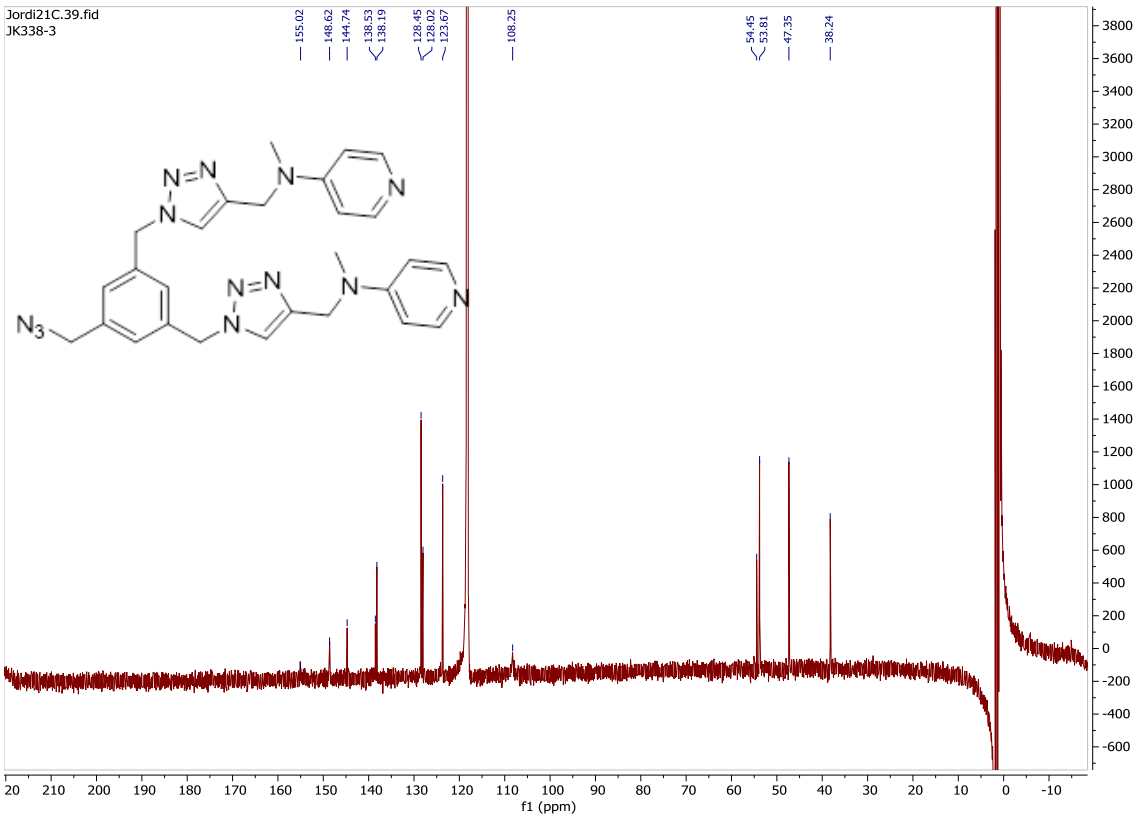


Figure S82. ^{13}C NMR spectrum of azido-diDMAP.

2.5.6 HPLC traces and purity of final compounds

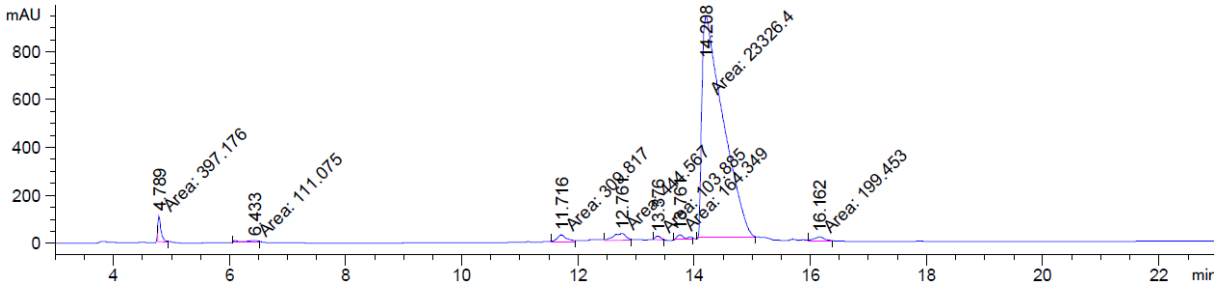


Figure S83. HPLC trace of BCN-DMAP (elution @ 14.3 min). Calculated purity = 93%.

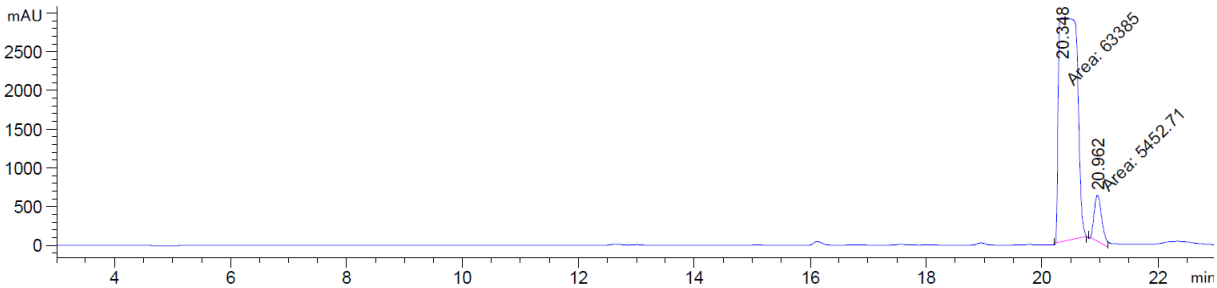


*Figure S84. HPLC trace of azido-thioester **1** (elution @ 20.3 and 21.0 min). Calculated purity = 99%.*

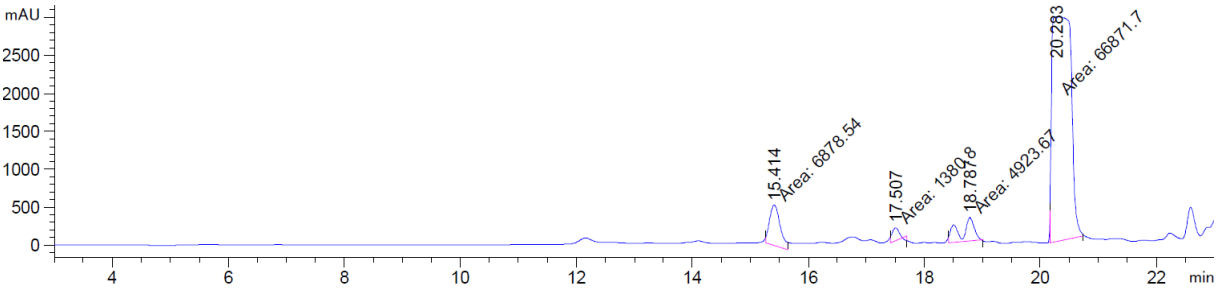


*Figure S85. HPLC trace of alkyne-thioester **2** (elution @ 20.3 min). Calculated purity = 83%.*

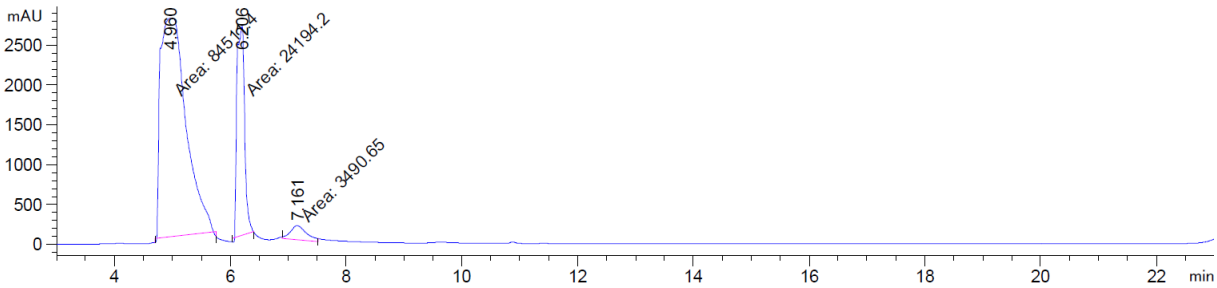


Figure S86. HPLC trace of alkyne-PyOx (elution @ 5.0 and 6.2 min). Calculated purity = 97%.

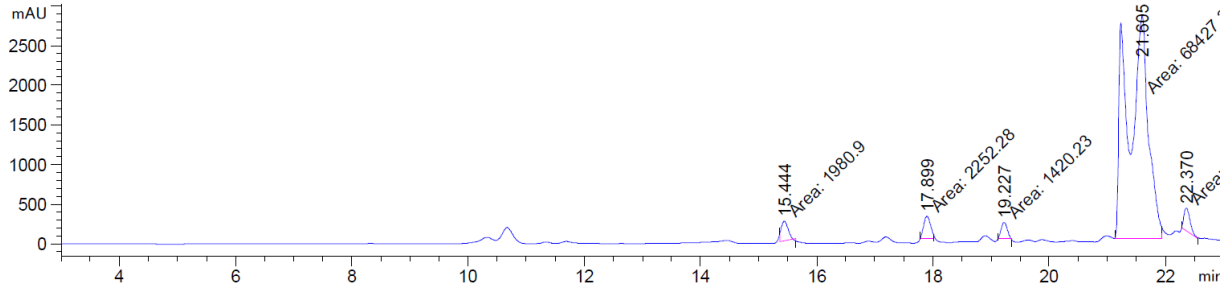


*Figure S86. HPLC trace of azido-ANANS **3** (elution @ 21.1 and 21.6 min). Calculated purity = 90%. Peak at ~10 min is contamination found which was also found in blanc runs.*

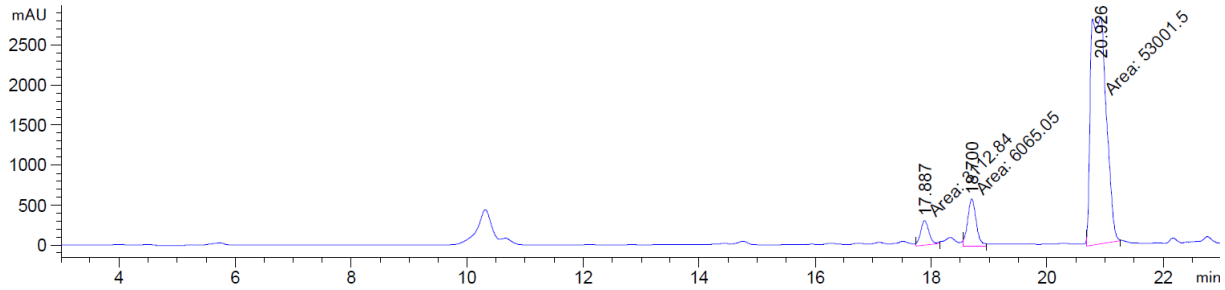


*Figure S87. HPLC trace of alkyne-ANANS **4** (elution @ 20.9). Calculated purity = 85%. Peak at ~10 min is contamination found which was also found in blanc runs.*

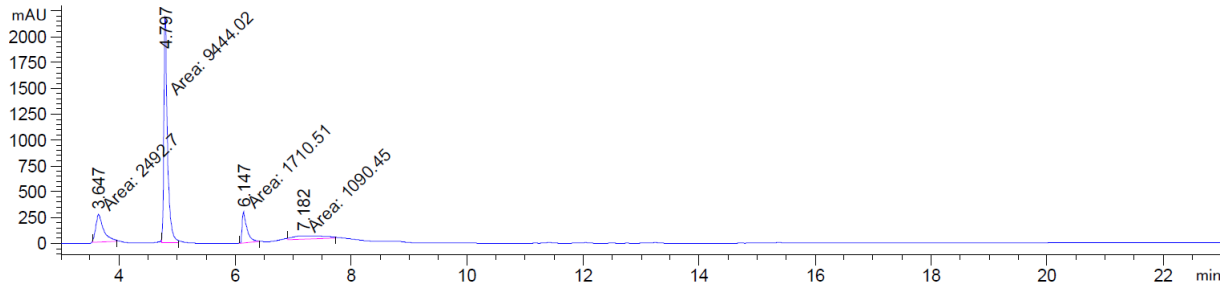


Figure S88. HPLC trace of azido-diPyOx (elution @ 3.6, 4.8 and 6.1 min). Calculated purity = 93%.

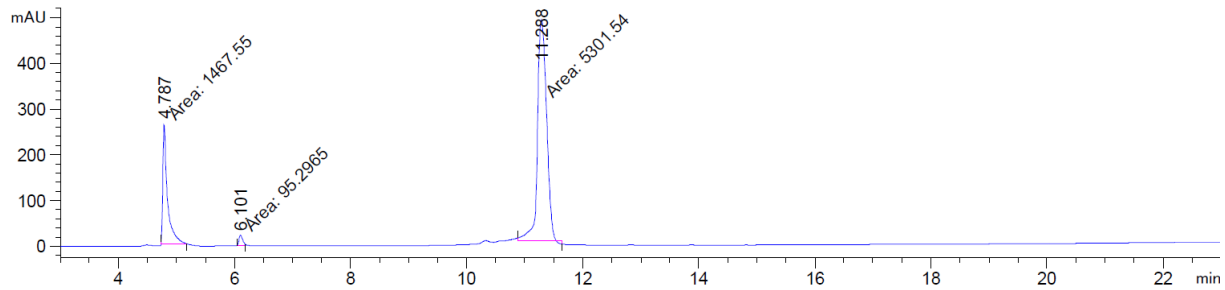


Figure S89. HPLC trace of azido-diDMAP (elution @ 4.8 and 11.3 min). Calculated purity = 99%.