Supporting Information

Acyl fluorides: fast, efficient and versatile lysine-based protein conjugation *via* plug-and-play strategy

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General Methods

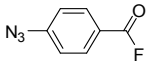
General experimental procedures: Unless otherwise indicated, reactions were carried out under an atmosphere of argon in flame-dried glassware with magnetic stirring. Air and/or moisture-sensitive liquids were transferred via syringe. When required, solutions were degassed by bubbling of argon through a needle. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an appropriate revelation solution. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 μm) from Macherey-Nagel. Melting point of compounds was determined on Büchi B-540. Protein concentration of antibody stock solution (PBS 1/20X, pH 7.3) was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). *Preparative HPLC* procedures were performed on semi-preparative HPLC Shimadzu Auto-injector SIL-10A (pump: Shimadzu LC-8A, UV-Vis detector: Shimadzu SPD-10A, collector: Shimadzu fraction collector FRC-10A) using a Sunfire C18 (150 mm × 19 mm i.d., 5 μm, Waters) at a flow of 17 mL/min. Per sample 1 mL was injected and water/ACN containing 0.05% TFA was used as eluent system. The gradient applied was 5% to 95% ACN in 40 minutes and 10 minutes of re-equilibration. Detection was done at 550 nm for TAMRA derivatives.

Materials: All reagents were obtained from commercial sources and used without prior purifications. Dry solvents were obtained from Sigma-Aldrich. Instrumentation: ^{1}H and ^{13}C NMR spectra were recorded at 23 °C on Bruker 400 and 500 spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follow: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (J, Hz) and integration.

Compounds synthesis

Acylating reagents

4-azidobenzoyl fluoride, ABF



$C_7H_4FN_3O$

MW = 165,13 g/mol

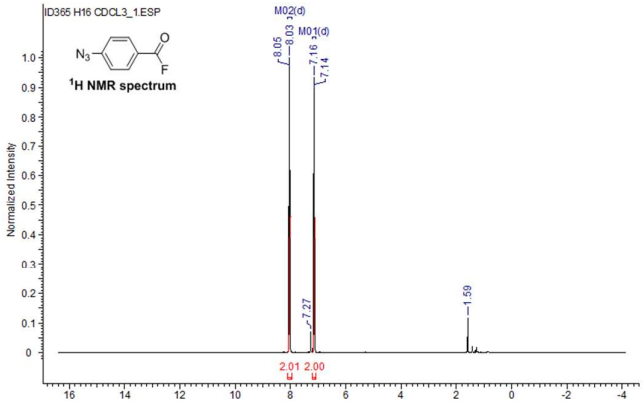
To 4-azidobenzoic acid (1 eq., 300 mg, 1.84 mmol) in acetonitrile (9 mL), was added pyridine (1 eq., 148 µL, 1.84 mmol). The mixture was stirred at room temperature until a homogeneous solution formed and then cyanuric fluoride (1.35 eq., 213.6 µL, 2.48 mmol) was added. The mixture was allowed to stir at room temperature for 16 h. The reaction mixture was poured onto ice water (20 mL) and diluted with diethyl ether (50 mL). The mixture was transferred to a separatory funnel, the aqueous layer was removed, and the organic layer was washed with water (2 x 10 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to provide the crude residue, which was purified by silica gel flash chromatography (Cyclohexane 2 min, then Cyclohexane to EtOAc in 18 min) to yield **ABF** (200 mg, 1.21 mmol, 66 %) as a pale yellow (t_m = 46.6 °C).

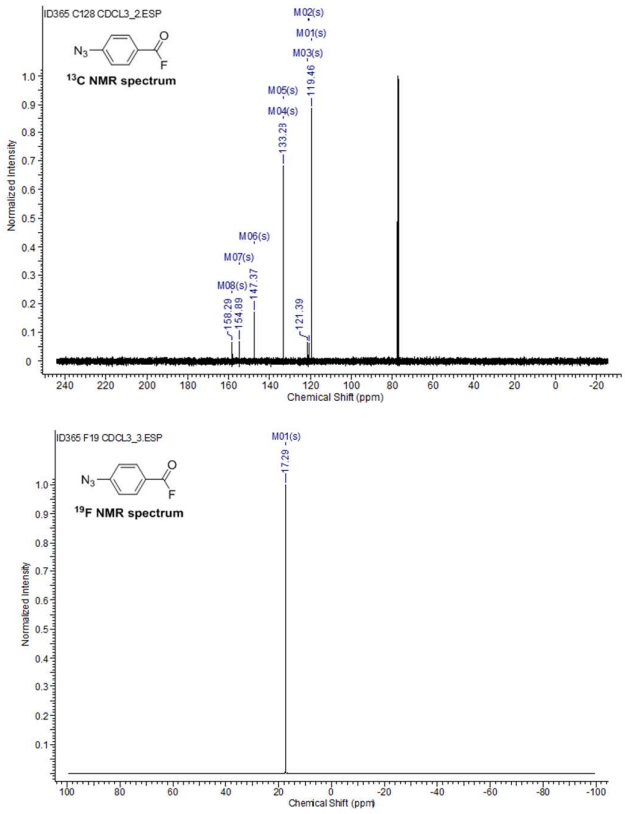
^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.04 (d, *J* = 8.3 Hz, 3 H), 7.15 (d, *J* = 8.3 Hz, 2 H).

^{13}C NMR (101 MHz, $CDCl_3$, δ ppm): 156.6 (d, *J* = 341.9 Hz), 147.4, 133.3 (d, *J* = 3.9 Hz), 121.1 (d, *J* = 62.6 Hz), 119.5.

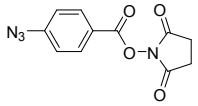
^{19}F NMR (376 MHz, $CDCl_3$, δ ppm): 17.29.

MS (ESI) m/z: 331.05 $[2M+H]^+$.





4-azidobenzoyl *N*-hydroxysuccinimide, ABNHS



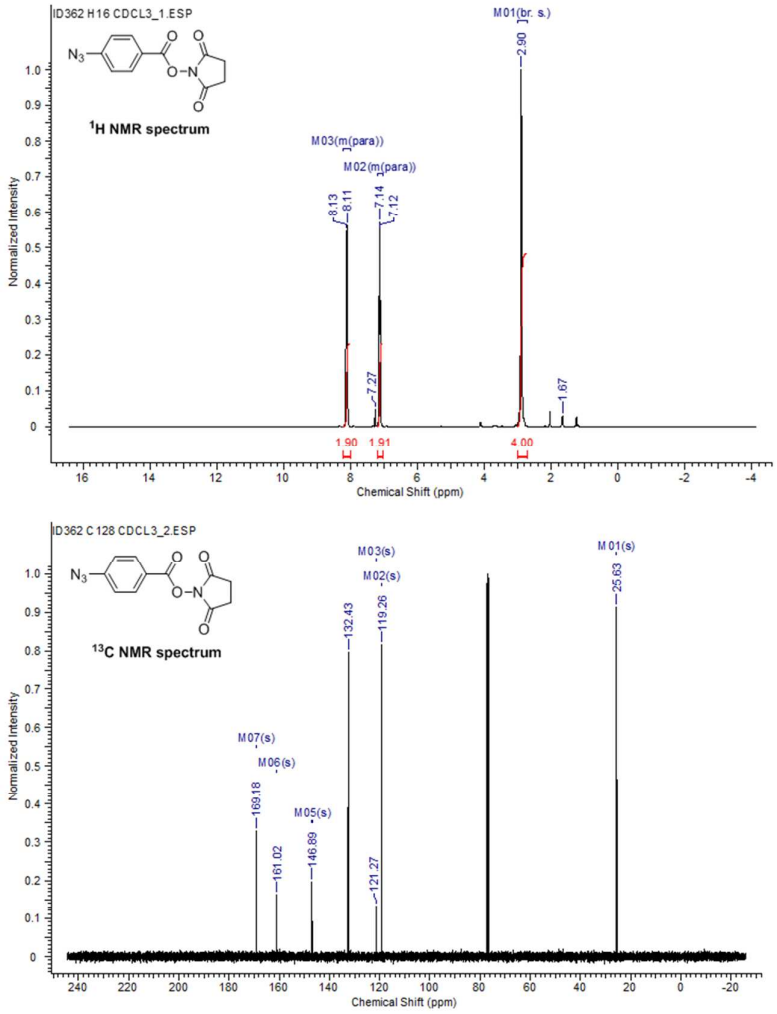
$C_{11}H_8N_4O_4$
MW = 260,21g/mol

4-azidobenzoic acid (1 eq., 500 mg, 3.06 mmol) was dissolved in DMF (9.76 mL) and cooled to 0 °C. To this solution, EDCI (1.2 eq., 705 mg, 3.68 mmol) was added followed by *N*-hydroxysuccinimide (1.2 eq., 423 mg, 3.68 mmol). The reaction was stirred in the dark under argon at 0 °C for approximately 1 h and then at room temperature for 15 h. DMF was removed in vacuo. This concentrated mixture was dissolved in 30 ml of EtOAc and then extracted with water (3x 20 ml). The organic layer was dried over $MgSO_4$, filtered, evaporated and purified by silica gel column chromatography (Cyclohexane 2 min, then Cyclohexane to EtOAc in 18 min) to yield **ABNHS** (745 mg, 2.86 mmol, 93 %) as a white solid (t_m = 172.4 °C, melting with gas formation).

^{1}H NMR (400 MHz, CDCl3, δ ppm): 8.00 - 8.22 (d, *J* = 8.3 Hz, 2 H), 7.03 - 7.21 (d, *J* = 8.3 Hz, 2 H), 2.90 (br. s., 4 H).

^{13}C NMR (101 MHz, $CDCl_3$, δ ppm): 169.2, 161.0, 146.9, 132.4, 121.3, 119.3, 25.6.

MS (ESI) m/z: 283.02 $[M+Na]^+$.

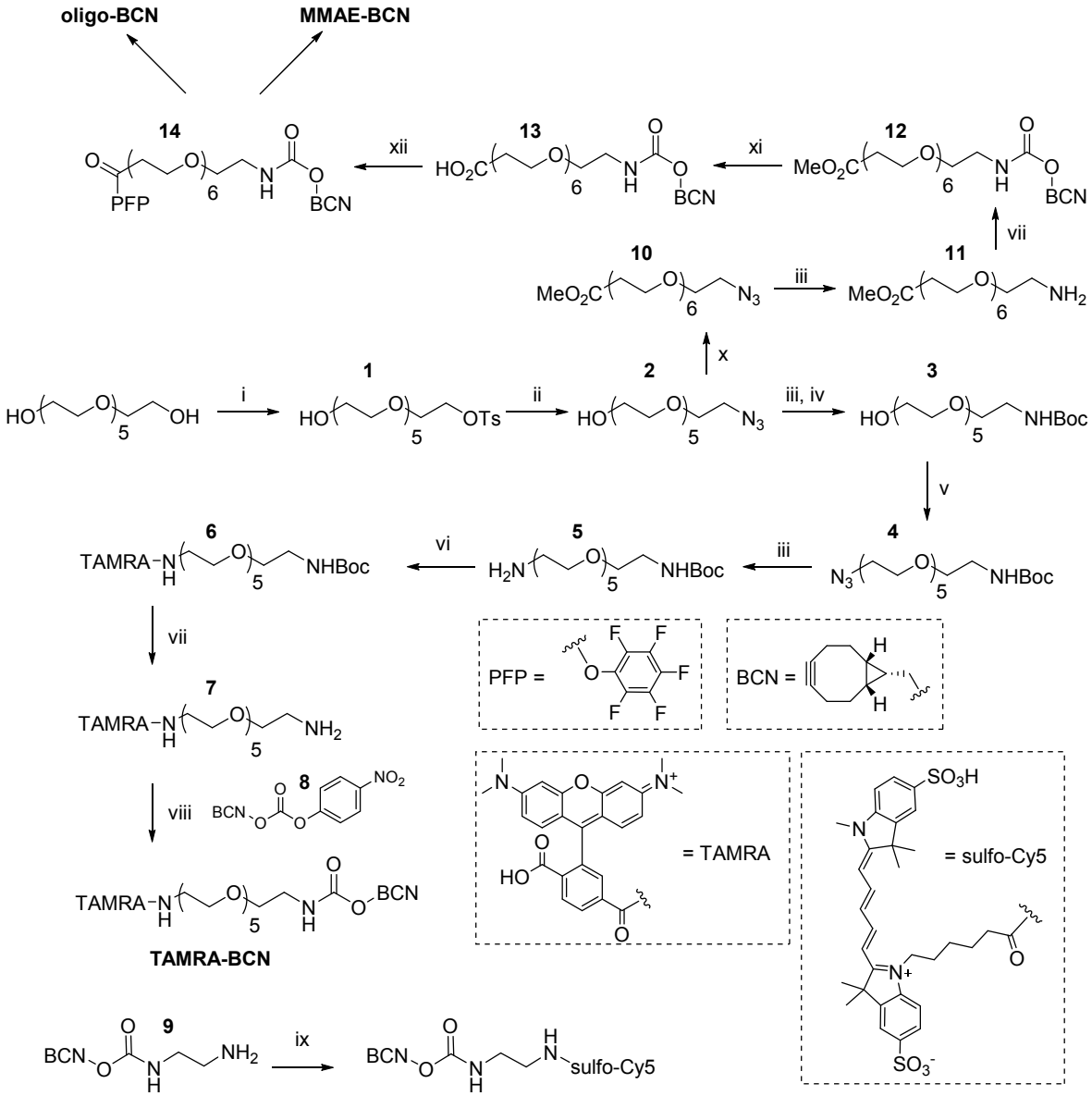


Payloads

Scheme S1 represents the synthetic road towards TAMRA-BCN, Cy5-BCN, MMAE-BCN and oligo-BCN payloads.
The compounds **8**[1] and **9**[2] were synthesized according to procedures described in the literature.

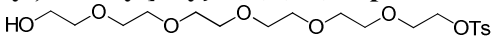
[1] Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L.J.A; Rutjes, F.P.J.T.; van Hest, J.C.M.; Lefeber, D.J.; Friedl, P.; van Delft, F.L. Angew. Chem. Int. Ed., 2010, 49, 9422 –9425

[2] Wang, K., Sachdeva, A., Cox, D.J., Wilf, N.M., Lang, K., Wallace, S., Mehl, R.A., Chin J.W. Nature Chemistry, 2014, 6, 393



Scheme S1. i) KI, Ag_2O, TsCl in DCM at 0 °C; ii) NaN_3 in DMF at 50°C; iii) H_2, 10 wt% Pd/C, in MeOH at 25 °C; iv) Boc_2O, NEt_3 in DCM at 25 °C; v) MsCl, NEt_3, NaN_3 in DMF at 0 °C; vi) TAMRA-6-COOH, NEt_3, HBTU in DMF at 0 °C; vii) HCl in MeOH at 25 °C; viii) **8**, NEt_3 in DMF at 25 °C; ix) sulfoCy5-NHS, DIEA in DMF at 25 °C; x) methyl acrylate, *t*-BuOK in THF at 0 °C; xi) LiOH in $MeOH/H_2O$ at 25 °C; xii) pentafluorophenol, DCC in DCM at 25 °C.

17-{[(4-methylphenyl)sulfonyl]oxy}-3,6,9,12,15-pentaoxaheptadecan-1-ol, 1[3]



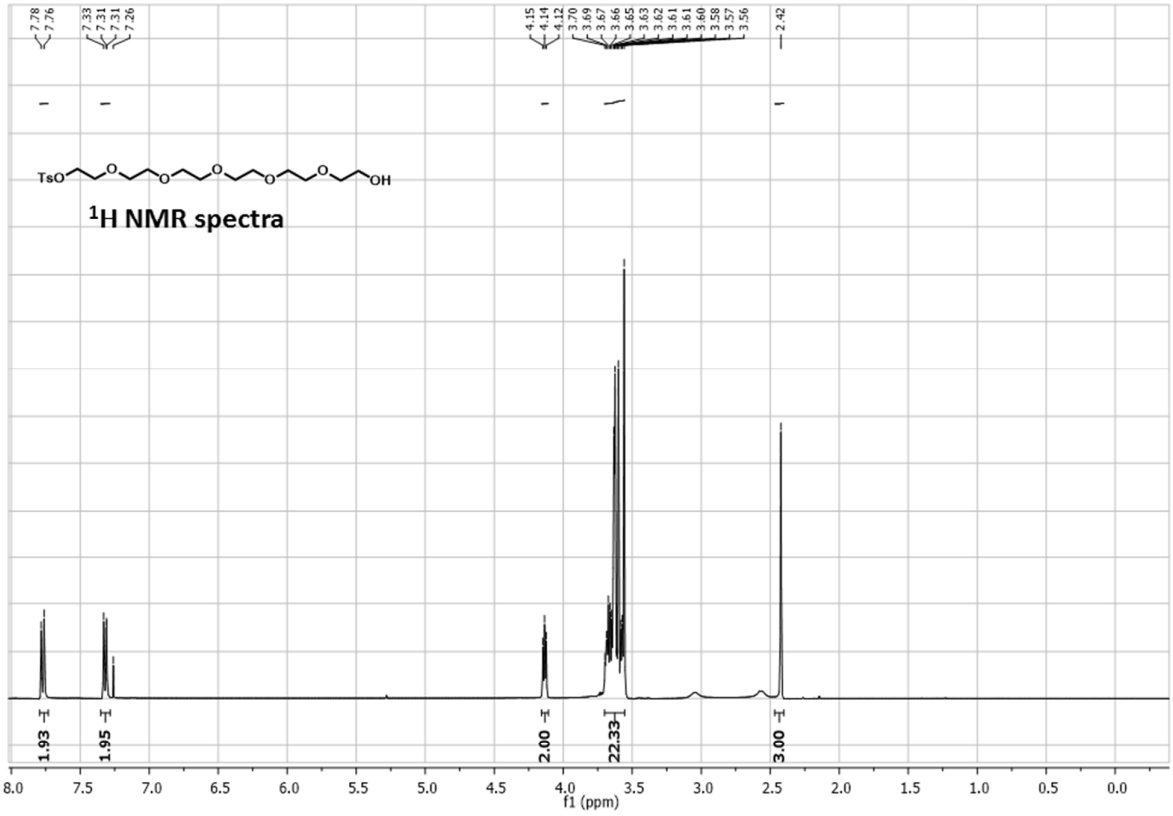
$C_{19}H_{32}O_9S$

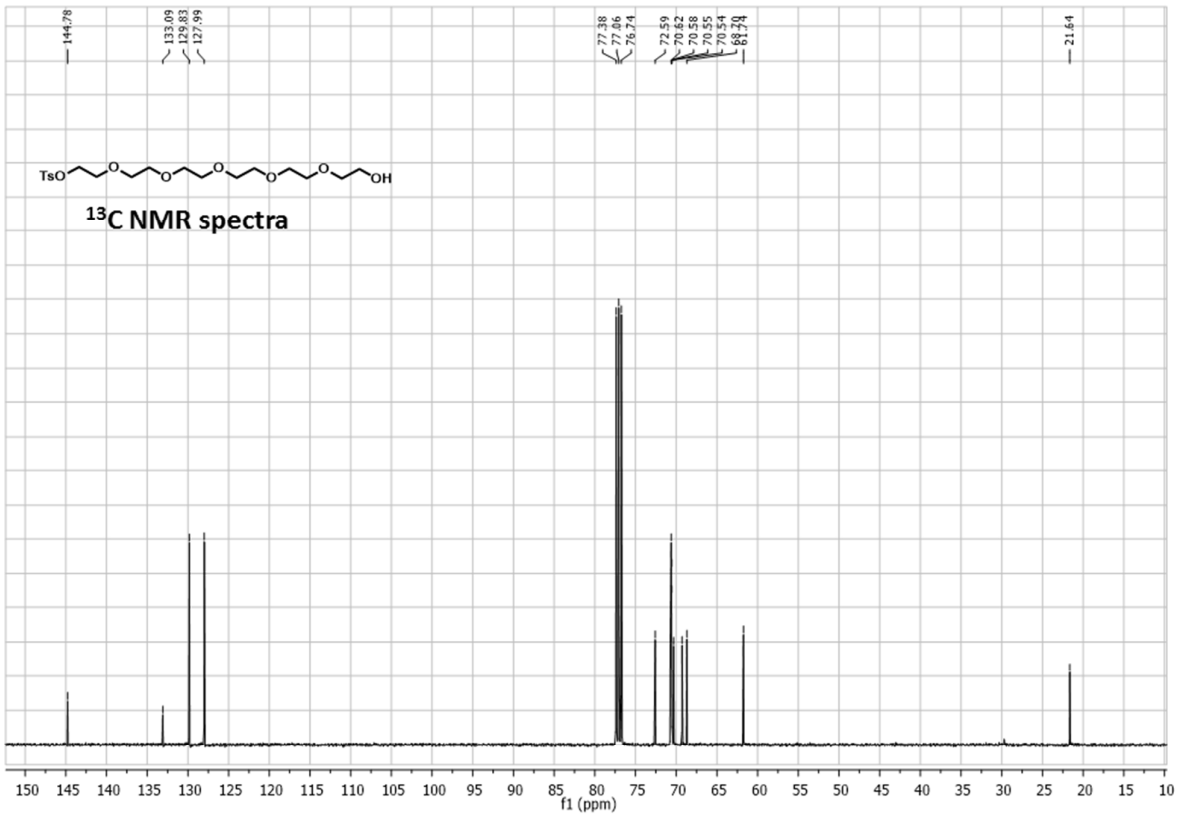
MW = 436.52 g/mol

[3] Bouzide, A.; Sauve G. *Org Lett.* **2002**, *4*, 2329-2332.

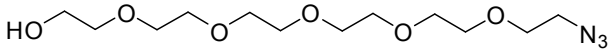
To a solution of hexaethylene glycol (1 eq., 12.1 g, 42.9 mmol) in DCM (380 mL) at 0 °C were added KI (0.2 eq., 1.42 g, 8.6 mmol) and Ag_2O (1.5 eq., 14.9 g, 64.3 mmol). Tosyl chloride (1.05 eq., 8.58 g, 45.0 mmol) was then added portionwise and the reaction mixture was stirred at 0 °C for 30 minutes. The mixture was then filtered through a pad of celite and concentrated in vacuo. The crude material was purified by silica gel flash chromatography (DCM to DCM/MeOH 95:5) to give **1** (16.4 g, 37.6 mmol, 88 %) as a clear yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.77 (d, *J* = 8.2 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 4.14 (t, *J* = 4.8 Hz, 2H), 3.72–3.53 (m, 22H), 2.42 (s, 3H), the OH signal is missing.
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 144.8, 133.1, 129.8 (2C), 128.0 (2C), 72.6, 70.7-70.3, 69.3, 68.7, 61.7, 21.6.





17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol, 2[4]

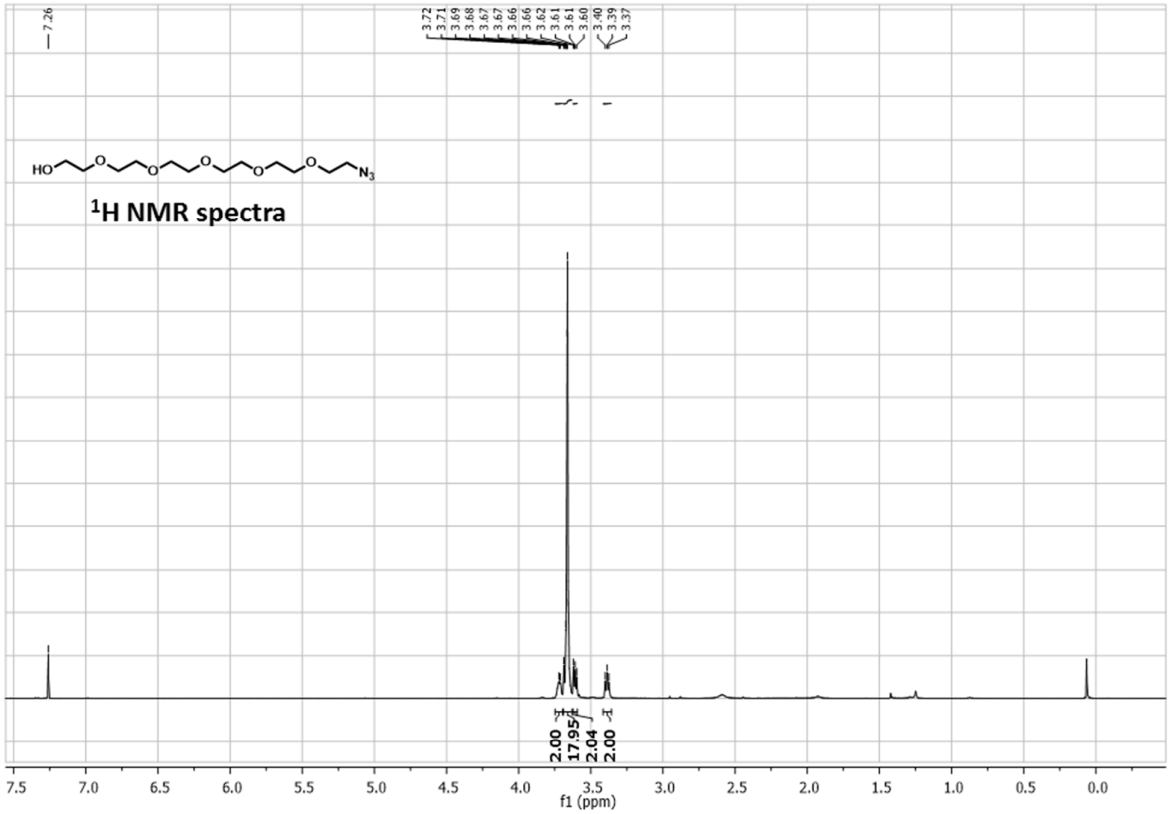


$C_{12}H_{25}N_3O_6$
MW = 307.34 g/mol

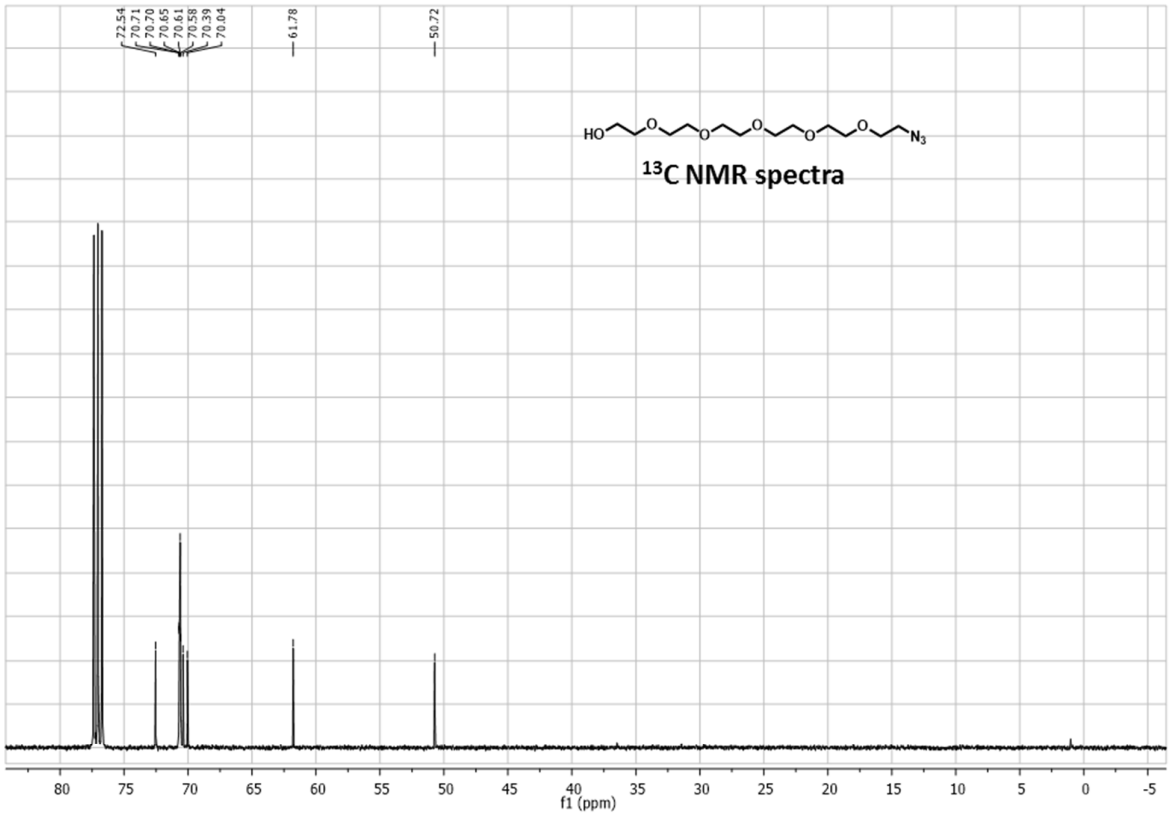
To a mixture of **1** (1 eq., 9.1 g, 20.9 mmol) in 25 mL of DMF was added NaN_3 (1.5 eq., 2.03 g, 31.3 mmol). The reaction was stirred at 50°C for 5 hours and filtered through a pad of celite. After evaporation, 200 mL of DCM were added and the solution was washed with brine (3 x 150 mL). The organic layer was dried over $MgSO_4$ and concentrated. The crude material was purified by silica gel flash chromatography (EtOAc to EtOAc/MeOH 9:1 in 25 min) to afford **2** (6.16 g, 20.04 mmol, 96 %) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 3.75 – 3.70 (m, 2H), 3.69 – 3.63 (m, 18H), 3.63 – 3.57 (m, 2H), 3.39 (t, *J* = 5.1 Hz, 2H). The OH signal is missing.
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 72.5, 70.7–70.6 (7C), 70.4, 70.0, 61.8, 50.7.

[4] Müller, M. K.; Brunsveld, L. *Angew. Chem. Int. Ed.*, **2009**, *48*, 2921-2924.

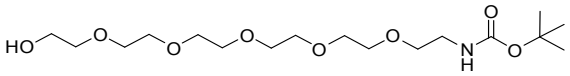


^{1}H NMR spectra



^{13}C NMR spectra

tert-butyl (17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl)carbamate, 3

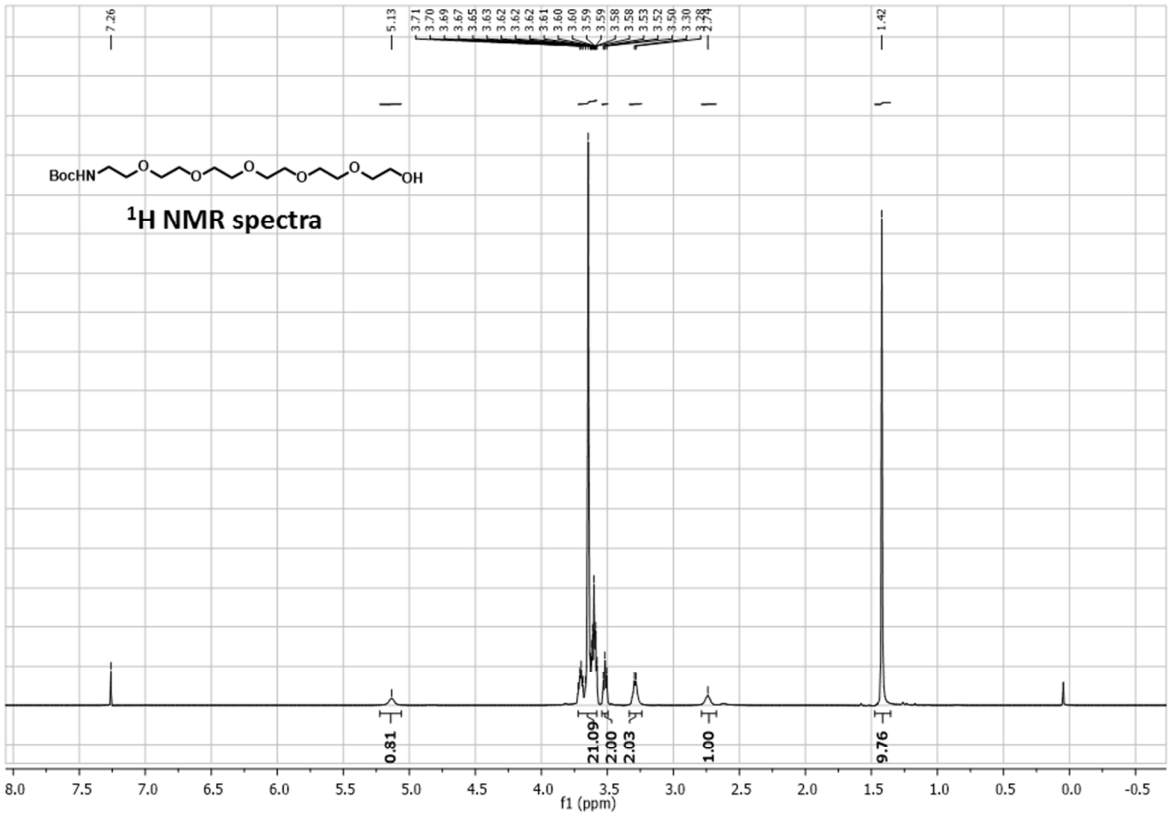


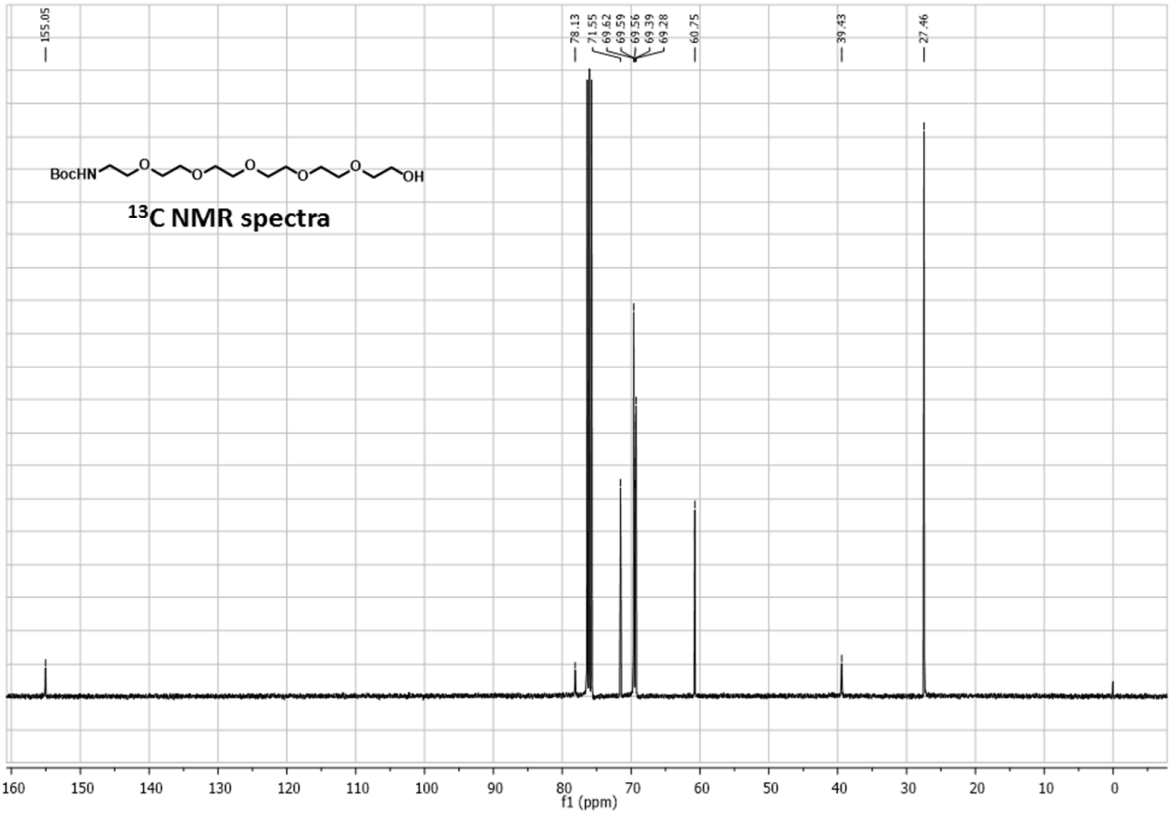
$C_{17}H_{35}NO_8$
MW = 381.46 g/mol

To a solution of **2** (1 eq., 6.80 g, 17.83 mmol) in MeOH (250 mL) was added 10 wt% Pd/C (0.02 eq., 0.379 g, 0.35 mmol) and the reaction mixture was stirred at room temperature under atmospheric pressure of hydrogen. After 12 hours, the mixture was filtered through a pad of celite and concentrated. The crude material was dissolved in DCM (100 mL) followed by the addition of Boc_2O (1.2 eq., 5.66 mL, 26.4 mmol) and NEt_3 (2 eq., 6.13 mL, 44.1 mmol). The reaction mixture was stirred at room temperature for 12 hours. 200 mL of water were added and the mixture was extracted with DCM (4 x 150 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude was purified by flash chromatography (EtOAc 5 min then DCM to DCM/MeOH 9:1 in 30 min) to afford *tert*-butyl (17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl)carbamate **3** (6.05 g, 15.9 mmol, 72%) as a yellowish oil.

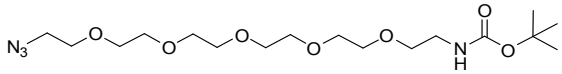
^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 5.13 (brs, 1H), 3.85–3.55 (m, 20H), 3.49 (dt, *J* = 5.8, 3.9 Hz, 2H), 3.29 (d, *J* = 4.9 Hz, 2H), 2.74 (brs, 1H), 1.42 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 156.0, 79.1, 77.4, 77.2, 77.0, 76.7, 72.6, 70.6–70.5 (3C), 70.4, 70.3, 61.7, 40.4, 28.4 (3C).





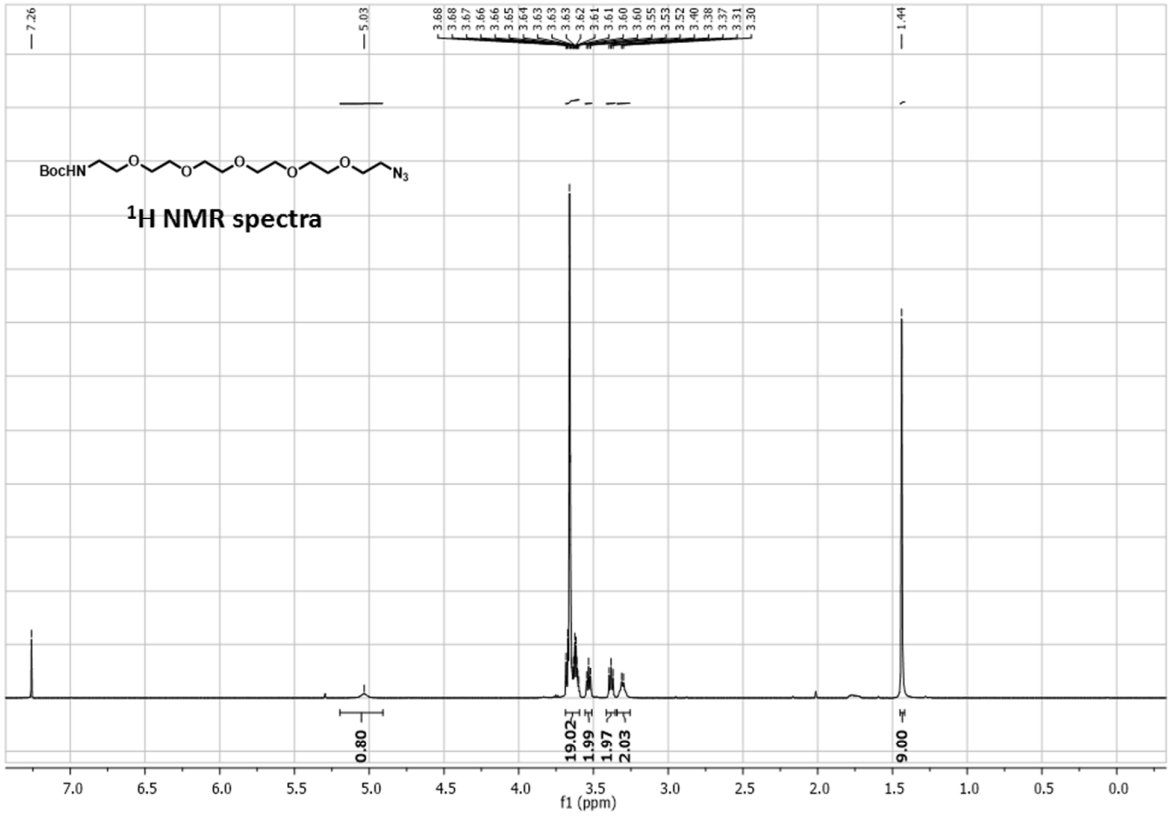
***tert*-butyl *N*-(17-azido-3,6,9,12,15-pentaoxaheptadecyl)carbamate, 4**

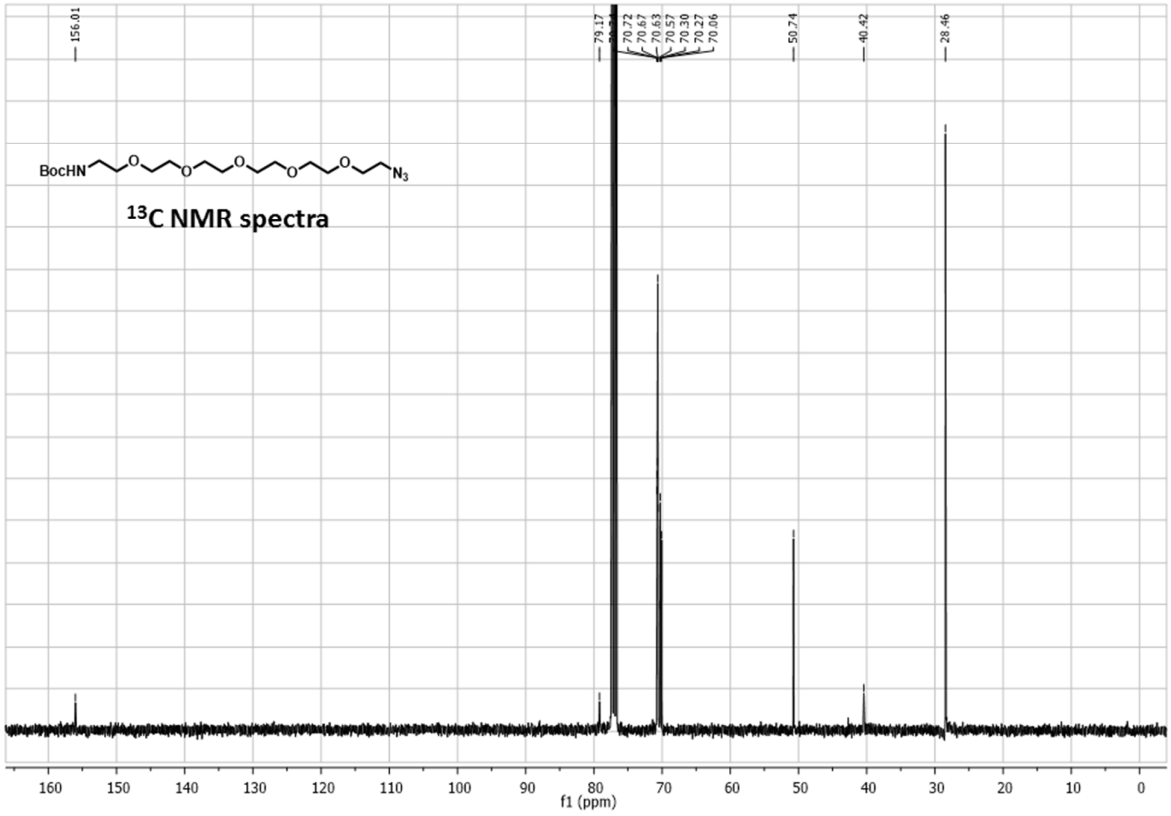


$C_{17}H_{34}N_4O_7$
MW = 406.47 g/mol

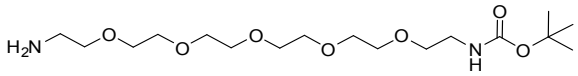
To a solution of *tert*-butyl (17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl)carbamate **3** (1 eq., 6 g, 15.7 mmol) and NEt_3 (5 eq., 10.9 mL, 78.6 mmol) in DMF (30 mL) under argon at 0 °C was added MsCl (2 eq., 2.43 mL, 31.5 mmol) dropwise. The mixture was stirred at room temperature for 2 hours then NaN_3 (2.2 eq., 2.25 g, 1.22 mL, 34.6 mmol) was added. The reaction was stirred at room temperature for 12 hours. After concentration, 150 mL of water were added and the mixture was extracted with DCM (3 x 100 mL). The combined organic layers were dried over $MgSO_4$ and concentrated. The crude was purified by flash chromatography (DCM to DCM/MeOH 9:1 in 30 min) to afford *tert*-butyl *N*-(17-azido-3,6,9,12,15-pentaoxaheptadecan-1-yl)carbamate **4** (4.86 g, 12 mmol, 76%) as a yellowish oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 5.03 (s, 1H), 3.74–3.58 (m, 18H), 3.53 (t, *J* = 5.1 Hz, 2H), 3.38 (t, *J* = 5.1 Hz, 2H), 3.35–3.26 (m, 2H), 1.44 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 156.0, 79.2, 77.3, 77.2, 77.0, 76.7, 70.7–70.6 (3C), 70.3, 70.3, 70.1, 50.7, 40.4, 28.5 (3C).


BocHN
N3
1H NMR spectra
f1 (ppm)


BocHN
N3
13C NMR spectra
f1 (ppm)

tert-butyl _N_-(17-amino-3,6,9,12,15-pentaoxaheptadecyl)carbamate, 5[5]

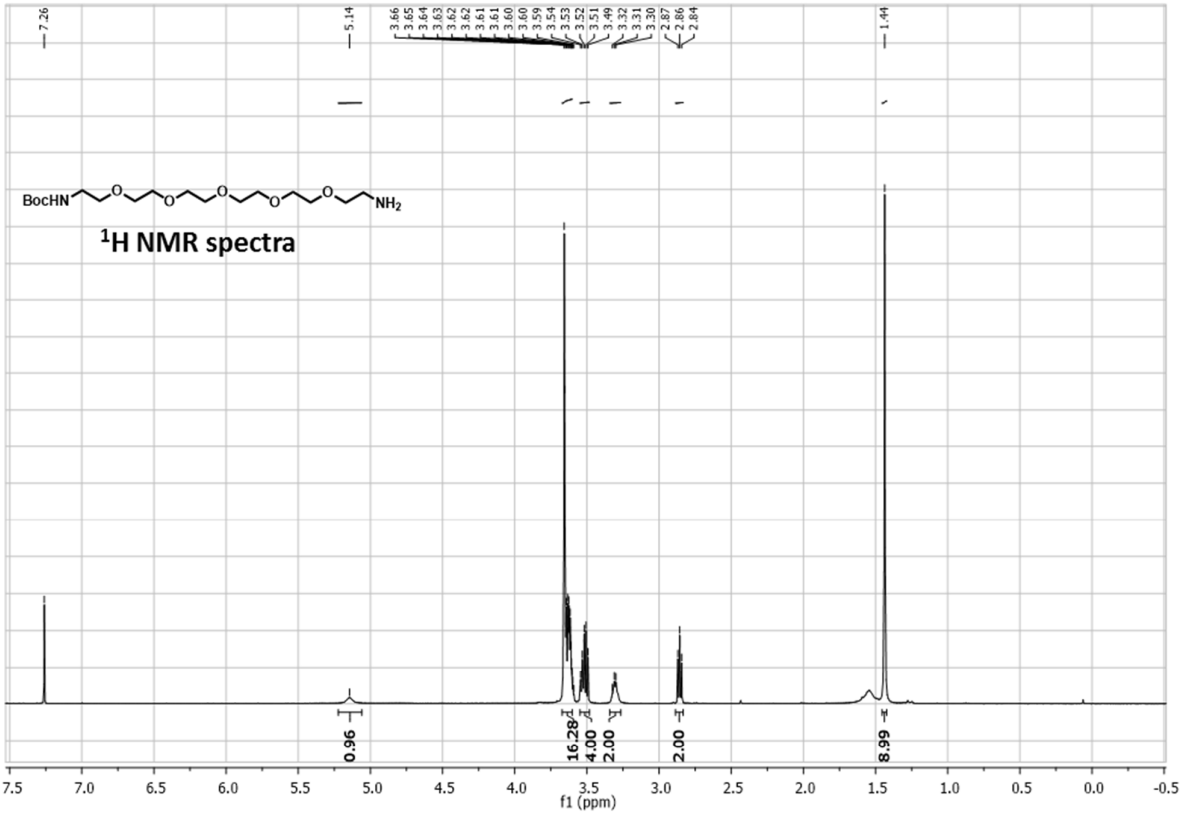


$C_{17}H_{36}N_2O_7$
MW = 380.48 g/mol

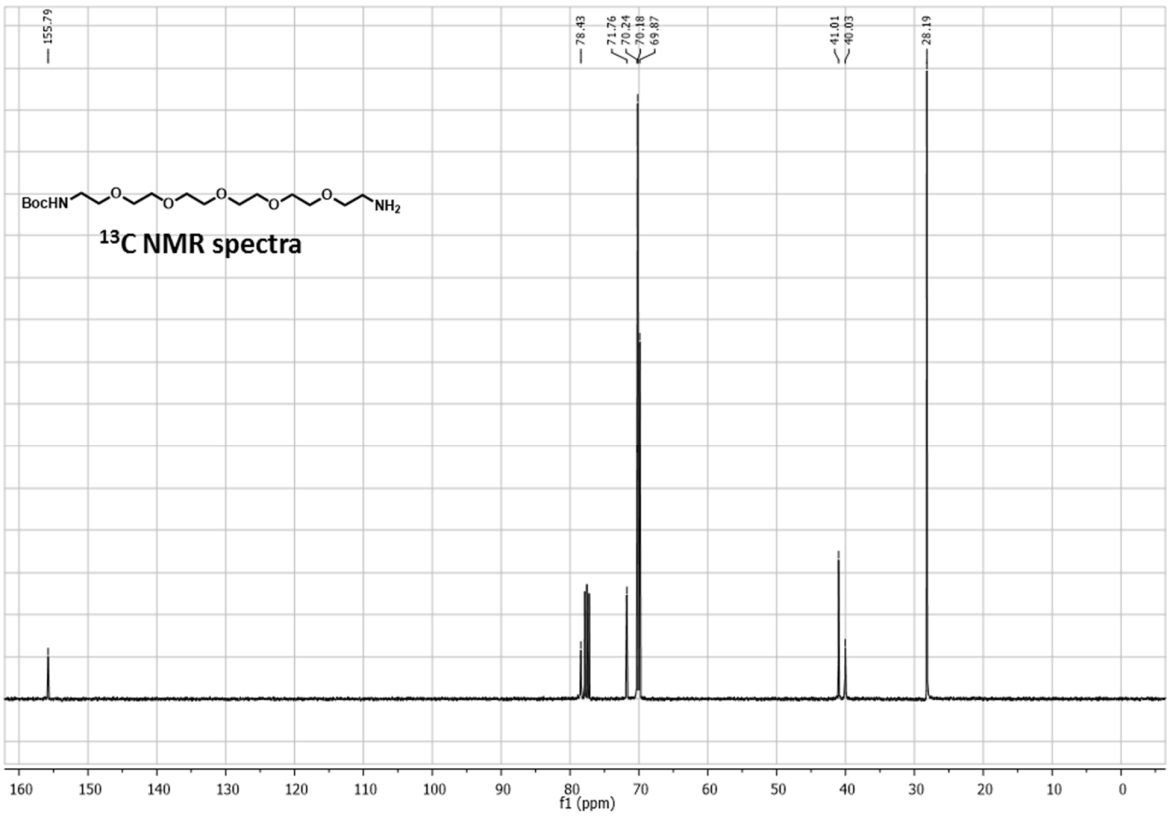
tert-Butyl *N*-(17-azido-3,6,9,12,15-pentaoxaheptadecan-1-yl)carbamate **4** (1 eq., 2.6 g, 6.4 mmol) was dissolved in MeOH (243 mL). 10 wt% Pd/C (0.02 eq., 0.136 g, 0.128 mmol) was added and the mixture was stirred at 0 °C under an atmospheric pressure of H_2 for 24 hours. The mixture was filtered through celite and concentrated *in vacuo*. The crude was purified by flash chromatography (DCM to DCM/MeOH (10% NH_4OH) 9:1 in 30 min) to afford *tert*-butyl *N*-(17-amino-3,6,9,12,15-pentaoxaheptadecan-1-yl)carbamate **5** (2.15 g, 5.65 mmol, 88 %) as a yellowish oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 5.14 (s, 1H), 3.75–3.58 (m, 16H), 3.57–3.45 (m, 4H), 3.37–3.21 (m, 2H), 2.86 (t, *J* = 5.2 Hz, 2H), 1.44 (s, 9H). The NH_2 signal is missing.

^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 155.8, 78.4, 71.8, 70.2–70.1 (8C), 69.9, 41.0, 40.0, 28.2 (3C).

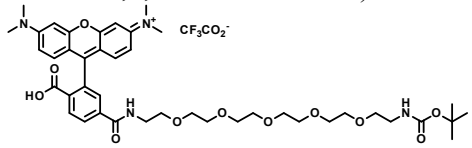


[5] Walton, J.G.A.; Patterson, S.; Liu, G.; Haraldsen,J.D.; Hollick, J.J.; Slawin, A.M.Z.; Ward, G.E.; Westwood, N.J. *Org. Biomol. Chem.*, **2009**, *7*, 3049-3060.



TAMRA-peg$_6$-NHBoc (TFA salt), 6

(*N*-(9-(2-Carboxy-5-((2,2-dimethyl-4-oxo-3,8,11,14,17,20-hexaoxa-5-azadocosan-22-yl)carbamoyl)phenyl)-6-(dimethylamino)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium 2,2,2-trifluoroacetate)



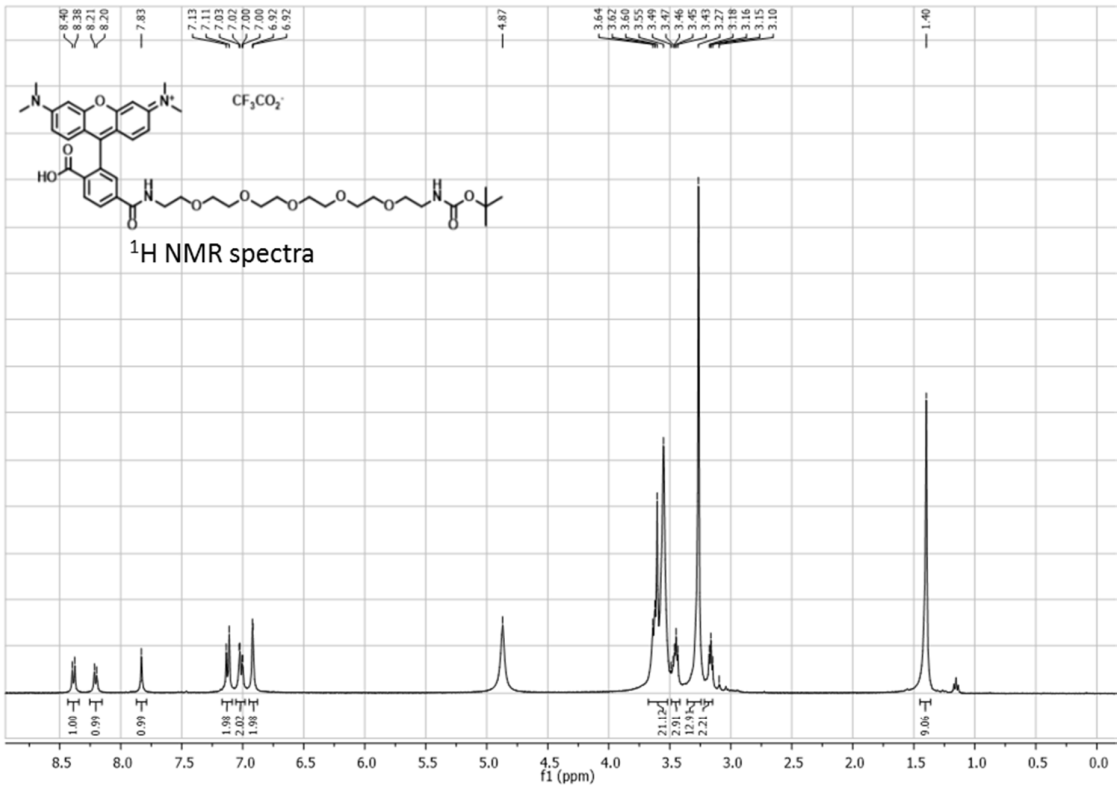
$C_{44}H_{57}F_3N_4O_{13}$

MW = 906.94 g/mol

To a solution of TAMRA-6-COOH (1 eq., 60.0 mg, 0.14 mmol) and NEt_3 (3.3 eq., 60 µL, 0.46 mmol) in DMF (1 mL) cooled to 0 °C was added HBTU (1.5 eq., 79.3 mg, 0.21 mmol). After 5 minutes a solution of *tert*-butyl *N*-(17-amino-3,6,9,12,15-pentaoxaheptadecan-1-yl)carbamate **5** (1.5 eq., 79.6 mg, 0.209 mmol) in DMF (1 mL) was added and the mixture was stirred for 2 hours at room temperature. Water was added (5 mL) and the mixture was concentrated under reduced pressure. The residue was dissolved in a minimum of MeOH and purified by flash chromatography (RP 16 g, H_2O (0.05% TFA) to ACN, 30 minutes) to afford TAMRA-peg$_6$-NHBoc **6** (67.9 mg, 0.0856 mmol, 61 %) as a pink solid.

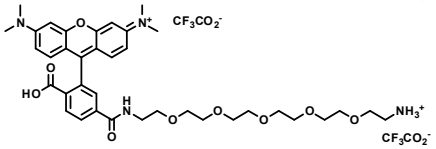
^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.39 (d, *J* = 8.2 Hz, 1H), 8.21 (d, *J* = 7.0 Hz, 1H), 7.83 (brs, 1H), 7.12 (d, *J* = 9.5 Hz, 2H), 7.01 (dd, *J* = 2.0 and 9.4 Hz, 2H), 6.92 (d, *J* = 1.9 Hz, 2H), 3.78–3.49 (m, 20H), 3.47–3.42 (m, 2H), 3.27 (s, 12H), 3.16 (t, *J* = 5.5 Hz, 2H), 1.40 (s, 9H), CO_2H and NH signals are missing.

MS (ESI) m/z: 793.4 $[M]^+$.



TAMRA-peg$_6$-NH$_2$ (TFA salt), 7

(1-(4-Carboxy-3-(6-(dimethylamino)-3-(dimethyliminio)-3*H*-xanthen-9-yl)phenyl)-1-oxo-5,8,11,14,17-pentaoxa-2-azanonadecan-19-aminium 2,2,2-trifluoroacetate)



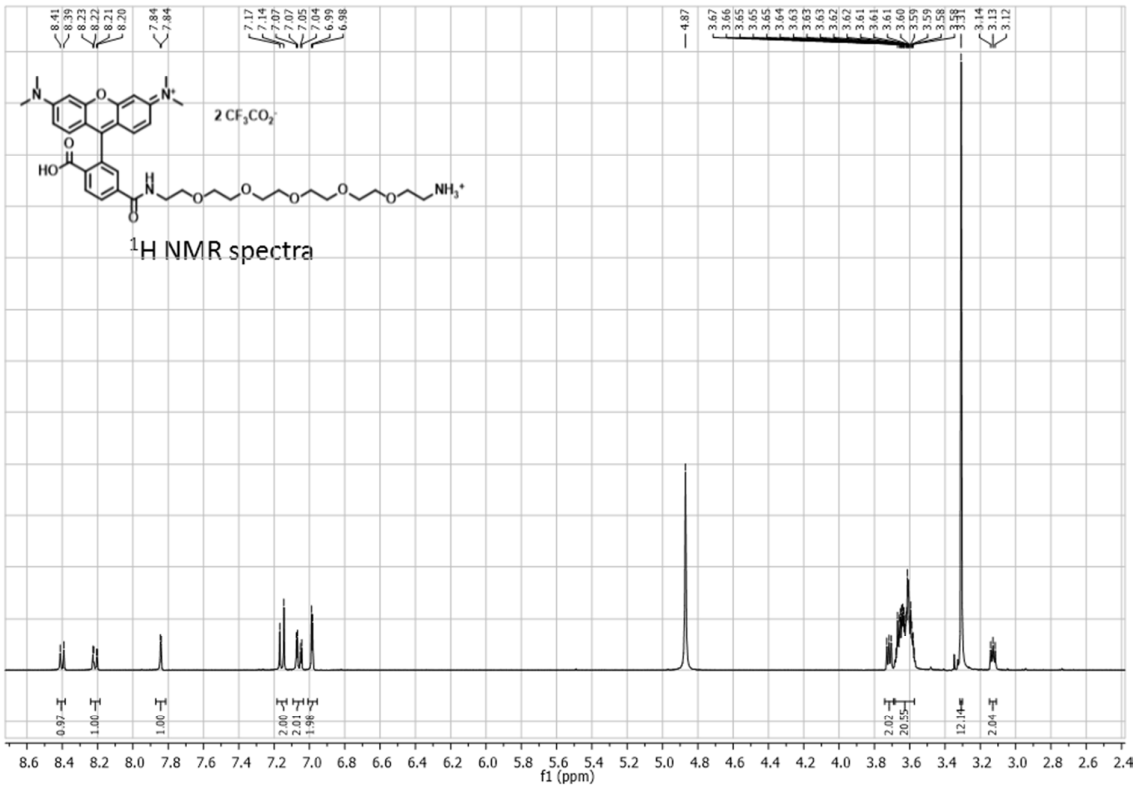
$C_{41}H_{50}F_6N_4O_{13}$

MW = 920.84 g/mol

To a solution of TAMRA-peg$_6$-NHBoc **6** (1 eq., 60 mg, 0.076 mmol) in MeOH (3 mL) was added a 4N solution of HCl in dioxane (15 eq., 0.28 mL, 1.14 mmol) and the reaction was stirred at room temperature for 3 hours. After concentration under reduced pressure, the mixture was dissolved in a minimum of MeOH and purified by flash chromatography (RP 16 g, H_2O (0.05% TFA) to ACN, 30 minutes) to afford TAMRA-peg$_6$-NH$_2$ (TFA salt) **7** (59.2 mg, 0.0643 mmol, 85 %) as a pink solid.

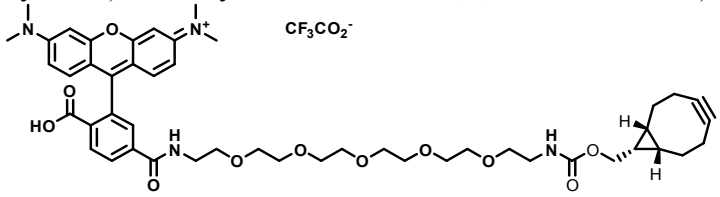
^{1}H NMR (400 MHz, CD_3OD, δ ppm): 8.40 (d, *J* = 8.2 Hz, 1H), 8.21 (dd, *J* = 1.8, 8.2 Hz, 1H), 7.88 (dd, *J* = 33.9, 10.5 Hz, 1H), 7.16 (d, *J* = 9.5 Hz, 2H), 7.06 (dd, *J* = 2.4, 9.5 Hz, 2H), 6.99 (d, *J* = 2.4 Hz, 2H), 3.75–3.70 (m, 2H), 3.69–3.55 (m, 20H), 3.31 (s, 12H), 3.15–3.11 (m, 2H). CO_2H, NH and NH_2 signals are missing.

MS (ESI) m/z: 693.2 $[M]^+$.



TAMRA-BCN

(*N*-(9-(5-((1-((1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13,16,19-hexaoxa-4-azahenicosan-21-yl)carbamoyl)-2-carboxyphenyl)-6-(dimethylamino)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium 2,2,2-trifluoroacetate)



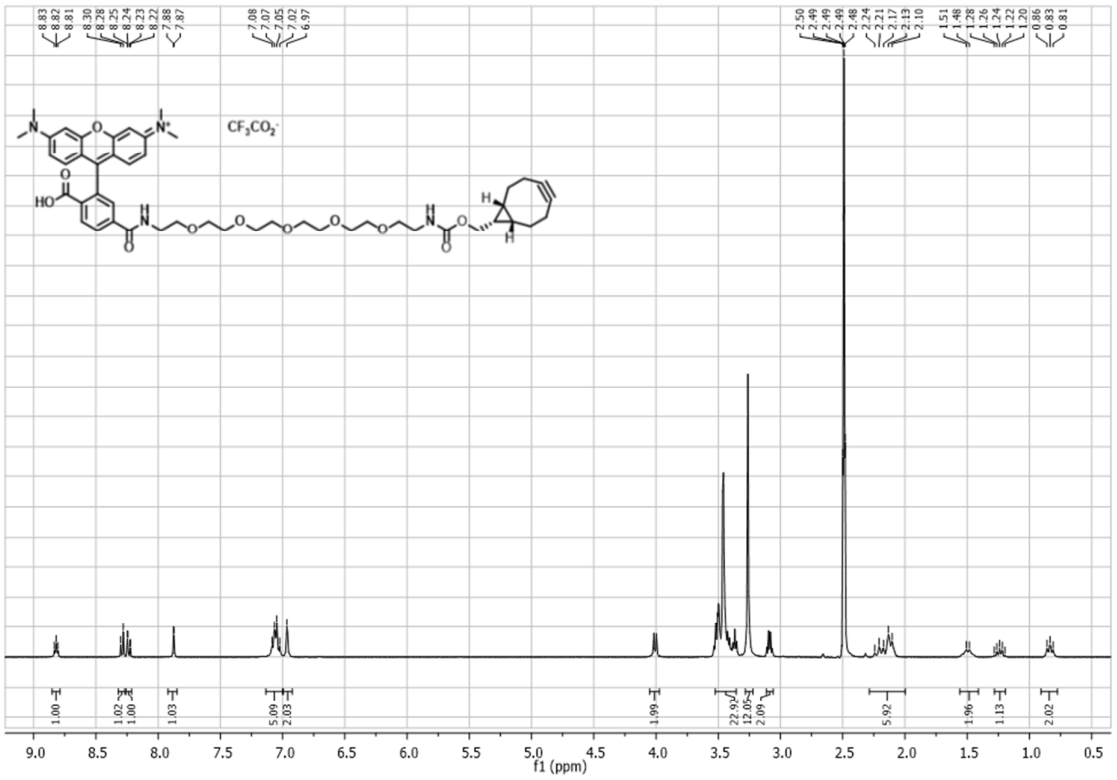
$C_{50}H_{61}F_3N_4O_{13}$

MW = 983.03 g/mol

To a solution of TAMRA-peg$_6$-NH$_2$ **7** (1 eq., 17 mg, 0.018 mmol) and NEt$_3$ (5 eq., 0.013 mL, 0.092 mmol) in DMF (2 mL) was added (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate **8** (1.1 eq., 6.4 mg, 0.020 mmol). The reaction was stirred at room temperature for 3 hours. After evaporation under reduced pressure, the crude material was purified by preparative HPLC (H$_2$O (0.05% TFA)/ACN from 95:5 to 5/95 in 40 min) to afford **TAMRA-BCN** (12.2 mg, 0.012 mmol, 67 %) as a pink solid.

^{1}H NMR (400 MHz, d_6-DMSO, δ ppm): 8.82 (t, *J* = 5.5 Hz, 1H), 8.29 (d, *J* = 8.2 Hz, 1H), 8.24 (dd, *J* = 1.7 and 8.2 Hz, 1H), 7.88 (d, *J* = 1.3 Hz, 1H), 7.12–7.00 (m, 5H), 6.97 (s, 2H), 4.01 (d, *J* = 8.0 Hz, 2H), 3.63–3.33 (m, 22H), 3.26 (s, 12H), 3.09 (q, *J* = 6.0 Hz, 2H), 2.30–2.01 (m, 6H), 1.64–1.40 (m, 2H), 1.33–1.13 (m, 1H), 0.83 (t, *J* = 9.6 Hz, 2H). CO_2H signal is missing.

MS (ESI) m/z: 869.4 $[M]^+$.

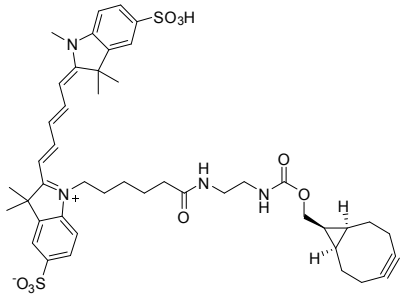


Cy5-BCN

1-(6-((2-((((1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*,5*E*)-5-(1,3,3-trimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium-5-sulfonate

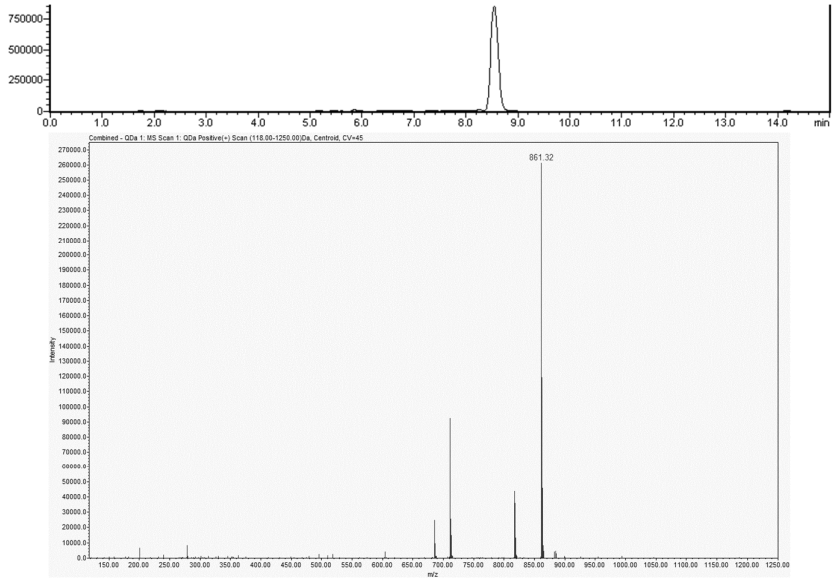
$C_{45}H_{56}N_4O_9S_2$

MW = 861.08 g/mol



To a solution of sulfoCy5-NHS (1 eq., 8.60 mg, 0.0113 mmol) and DIEA (3 eq., 0.0056 mL, 0.0339 mmol) in DMF (2 mL) was added (1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (2-aminoethyl)carbamate **9** (1.2 eq., 3.2 mg, 0.0135 mmol). The reaction was stirred 3 hours at room temperature. After concentration under reduced pressure the crude material was purified by flash chromatography (RP 5 g, H_2O to ACN, 30 minutes) to afford **Cy5-BCN** (7.70 mg, 0.0087 mmol, 77 %) as a dark solid.

MS (ESI) m/z: 861.3 $[M + H]^+$.

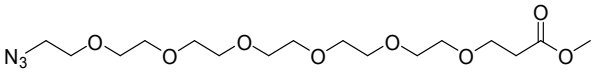


LCMS Analysis of Cy5-BCN

methyl 1-azido-3,6,9,12,15,18-hexaoxahenicosan-21-oate, 10

$C_{16}H_{31}N_3O_8$

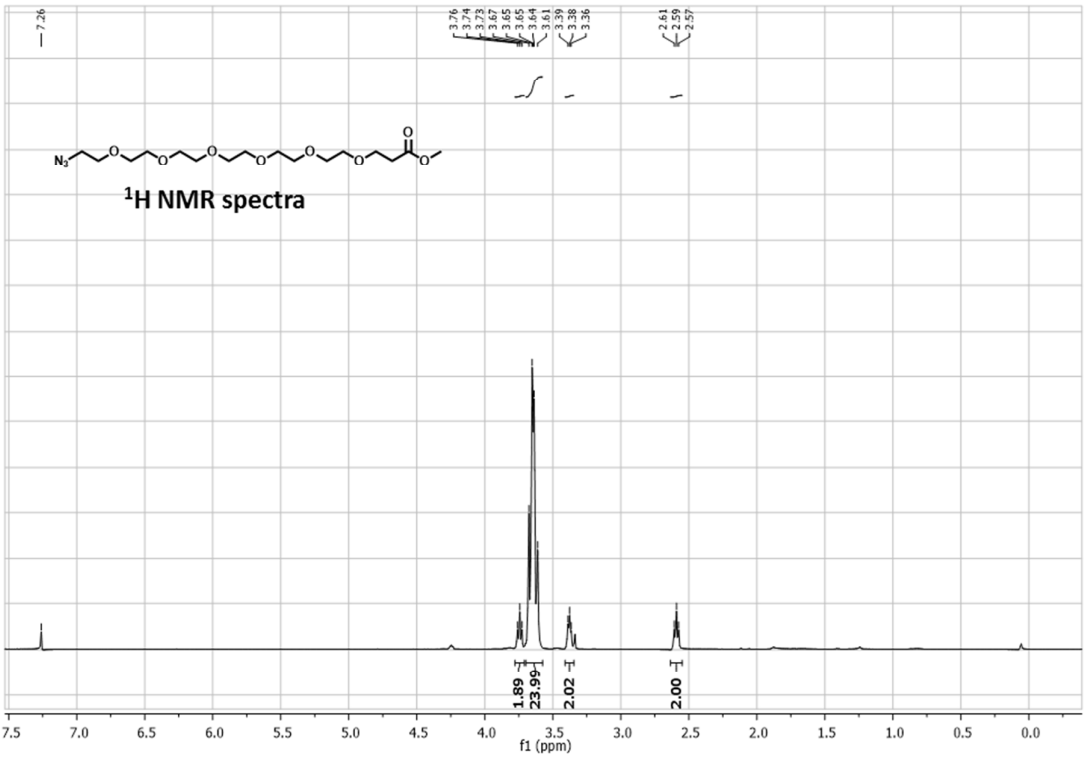
MW = 393.44 g/mol

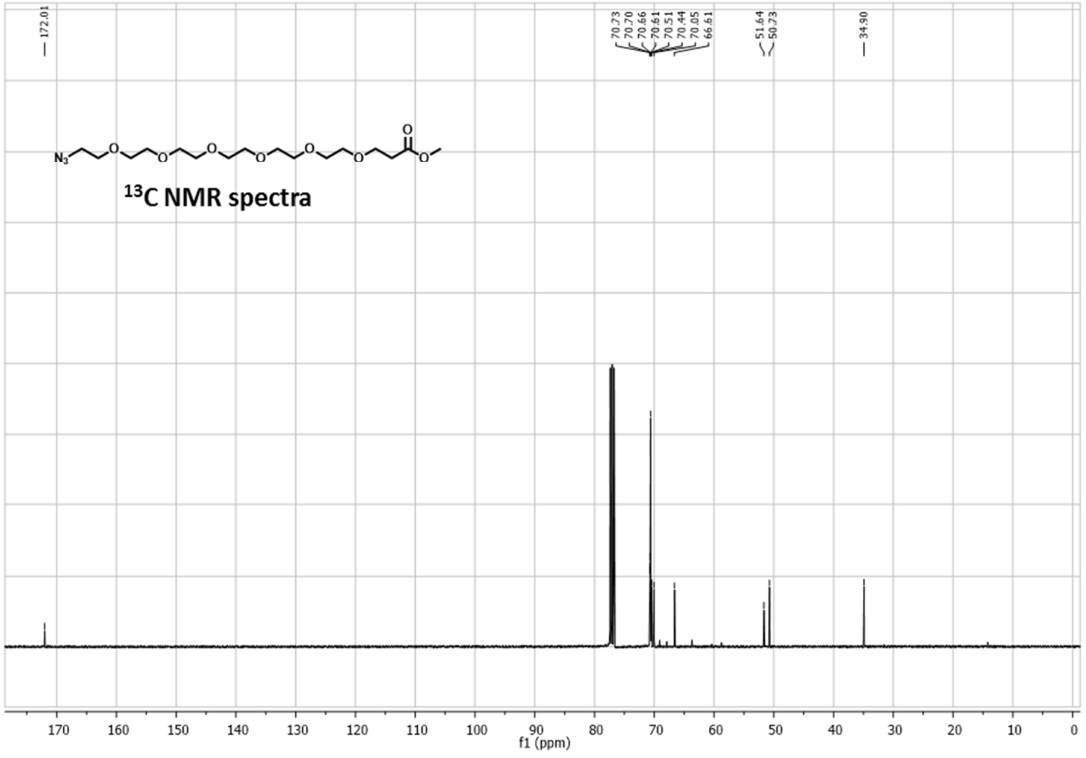


To a solution of 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol **2** (1 eq., 3.40 g, 11.10 mmol)) and methyl acrylate (1.5 eq., 1.49 mL, 16.60 mmol) in THF (30 mL) at 0°C was added *t*-BuOK (0.1 eq., 135 mg, 1.20 mmol). The reaction was stirred at room temperature for 5 hours. After concentration, H_2O (100mL) was added and the mixture was extracted with EtOAc (150 mL). The combined organic layers were dried over $MgSO_4$ and evaporated. The crude was purified by silica gel flash chromatography (Cyclohexane to EtOAc in 35 minutes) to afford **10** (1.80 g, 4.58 mmol, 41 %) as a yellowish oil.

^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 3.74 (t, *J* = 6.4 Hz, 2H), 3.70 – 3.58 (m, 25H), 3.38 (t, *J* = 4.7 Hz, 2H), 2.59 (t, *J* = 6.4 Hz, 2H).

^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 172.0, 70.7 – 70.4, 70.0, 66.6, 51.6, 50.7, 34.9.

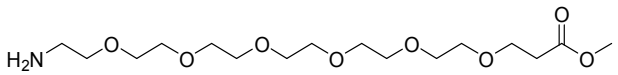

7.26
3.76
3.74
3.73
3.67
3.65
3.65
3.64
3.61
3.39
3.38
3.36
2.61
2.59
2.57
N3
O
1H NMR spectra
1.89
23.99
2.02
2.00
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


172.01
70.73
70.70
70.66
70.61
70.51
70.44
70.05
66.61
51.64
50.73
34.90
N3
O
13C NMR spectra
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

methyl 1-amino-3,6,9,12,15,18-hexaoxahenicosan-21-oate, 11

$C_{16}H_{33}NO_8$

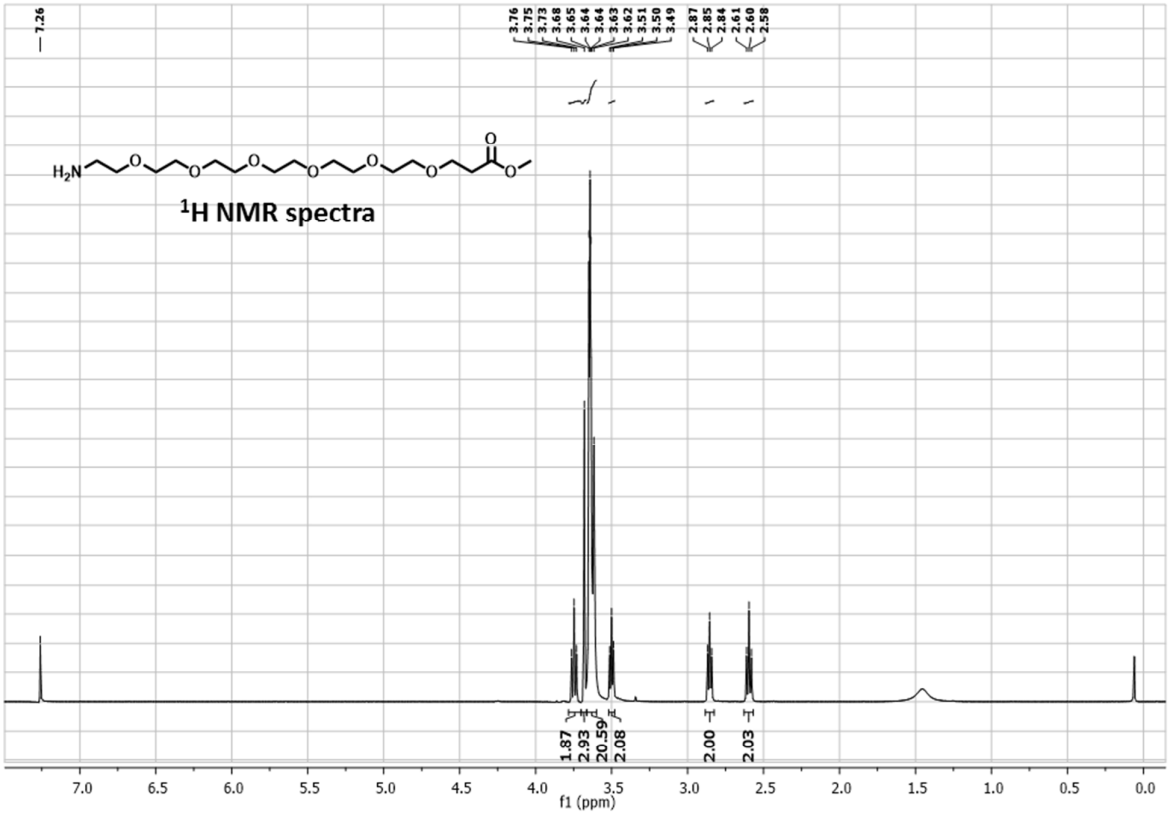
MW = 393.44 g/mol

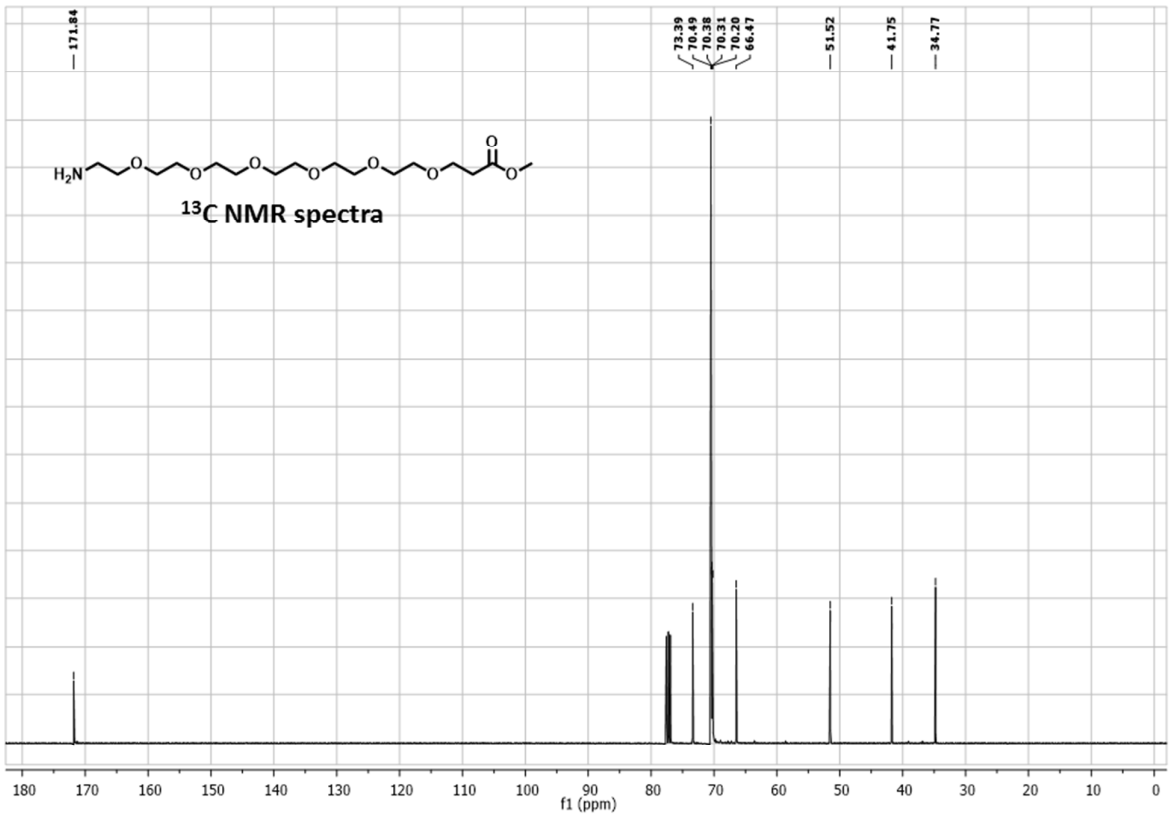


To a solution of **10** (1 eq., 600 mg, 1.53 mmol) in MeOH (30 mL) was added Pd/C (1 %, 16.2 mg, 0.0153 mmol) and the mixture was stirred at room temperature under atmospheric pressure of H_2 for 14 hours. The mixture was filtered through celite, concentrated and purified by silica gel flash chromatography (DCM to DCM/MeOH/NH_4OH 9/0.9/0.1 in 30 minutes) to afford **11** (455 mg, 1.24 mmol, 81 %) as a yellowish oil.

^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 3.75 (t, *J* = 6.5 Hz, 2H), 3.68 (s, 3H), 3.67 – 3.59 (m, 20H), 3.50 (t, *J* = 5.2 Hz, 2H), 2.85 (t, *J* = 5.2 Hz, 2H), 2.60 (t, *J* = 6.5 Hz, 2H). The NH_2 signal is missing.

^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 171.8, 73.4, 70.5 – 70.2, 66.5, 51.5, 41.7, 34.8.

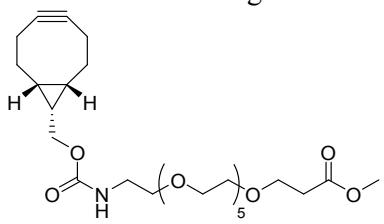




methyl 1-((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13,16,19,22-heptaoxa-4-azapentacosan-25-oate, 12

$C_{27}H_{45}NO_{10}$

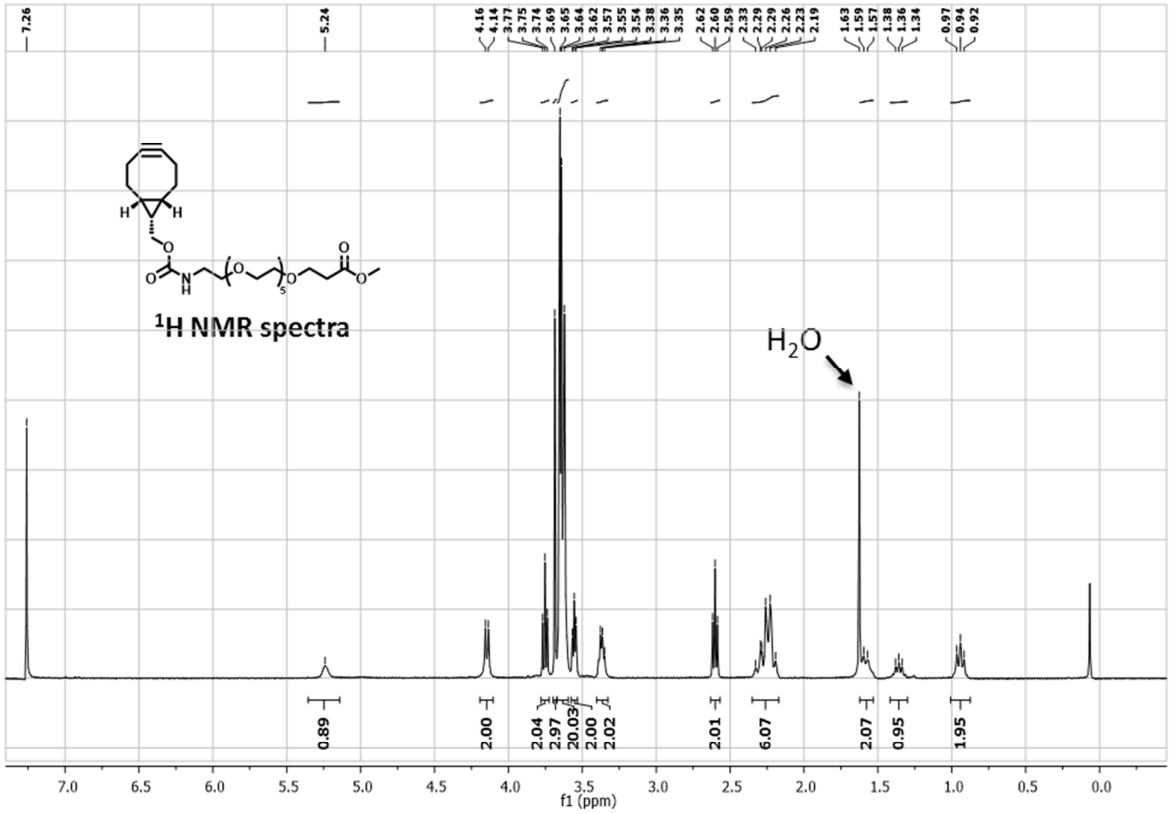
MW = 543.65 g/mol

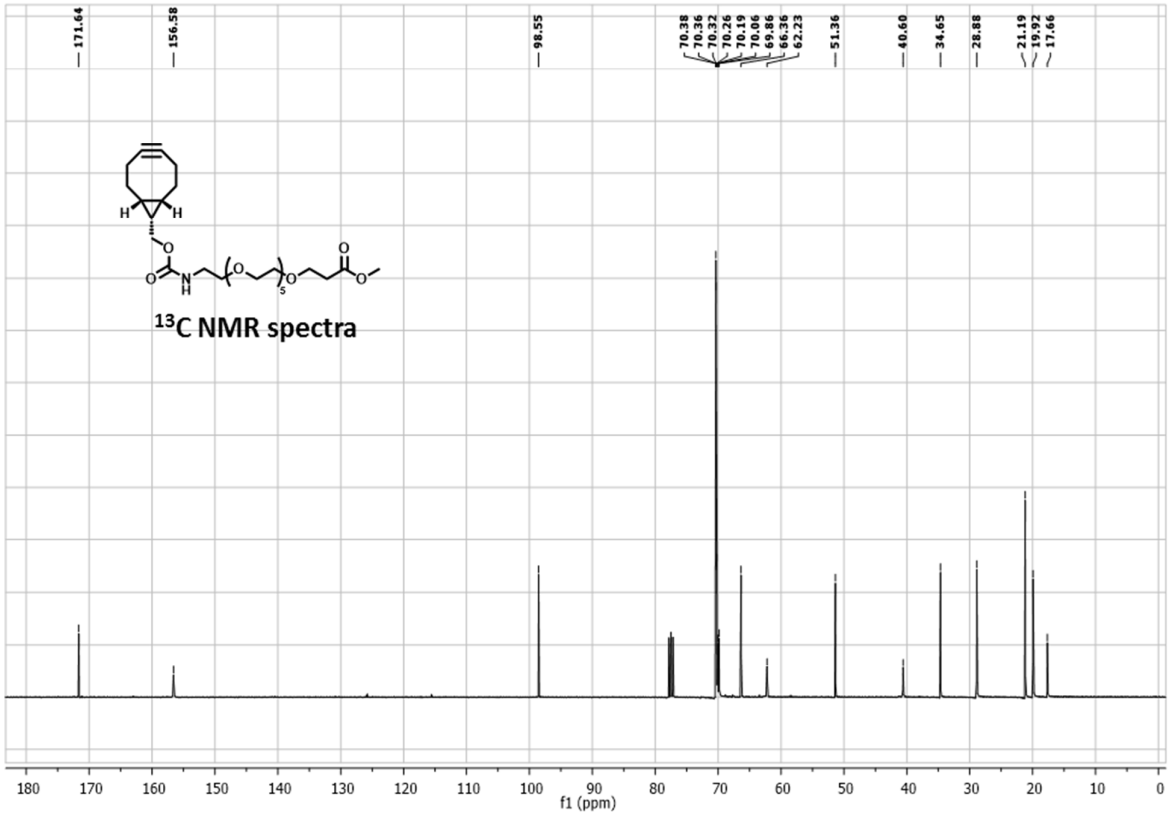


To a solution of (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate **11** (1 eq., 250 mg, 0.79 mmol) in 0.5 mL of DMF was added a solution of **8** (1.1 eq., 320 mg, 0.87 mmol) and NEt_3 (3 eq., 0.331 mL, 2.38 mmol) in 0.5 mL of DMF. The reaction mixture was stirred overnight at room temperature. After evaporation, 20 mL of an aqueous solution of $NaHPO_4$ (1M) were added and the mixture was extracted with EtOAc (3 x 40 mL). The organic layer was dried over $MgSO_4$ and concentrated. The crude was purified by silica gel flash chromatography (DCM to DCM/MeOH 85/15 in 30 minutes) to afford **12** (360 mg, 0.66 mmol, 84 %) as a colorless oil.

^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 5.24 (bs, 1H), 4.15 (d, *J* = 8.0 Hz, 2H), 3.75 (t, *J* = 6.5 Hz, 2H), 3.69 (s, 3H), 3.67 – 3.60 (m, 20H), 3.55 (t, *J* = 5.0 Hz, 2H), 3.42 – 3.30 (m, 2H), 2.60 (t, *J* = 6.5 Hz, 2H), 2.35 – 2.15 (m, 6H), 1.62 – 1.51 (m, 2H), 1.44 – 1.29 (m, 1H), 0.94 (t, *J* = 9.8 Hz, 2H).

^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 171.6, 156.6, 98.5, 70.4, – 70.2, 70.1, 69.9, 66.4, 62.2, 51.4, 40.6, 34.6, 28.9, 21.2, 19.9, 17.7.

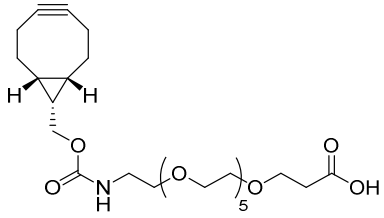

7.26
5.24
4.16
4.14
3.77
3.75
3.74
3.69
3.65
3.64
3.62
3.57
3.55
3.54
3.38
3.36
3.35
2.62
2.60
2.59
2.33
2.29
2.29
2.26
2.23
2.19
1.63
1.59
1.57
1.38
1.36
1.34
0.97
0.94
0.92
0.89
2.00
2.04
2.97
20.03
2.00
2.02
2.01
6.07
2.07
0.95
1.95
H2O
1H NMR spectra
f1 (ppm)


171.64
156.58
98.55
70.38
70.36
70.32
70.26
70.19
70.06
69.86
66.36
62.23
51.36
40.60
34.65
28.88
21.19
19.92
17.66
13C NMR spectra
f1 (ppm)

1-((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13,16,19,22-heptaoxa-4-azapentacosan-25-oic acid, 13

$C_{26}H_{43}NO_{10}$

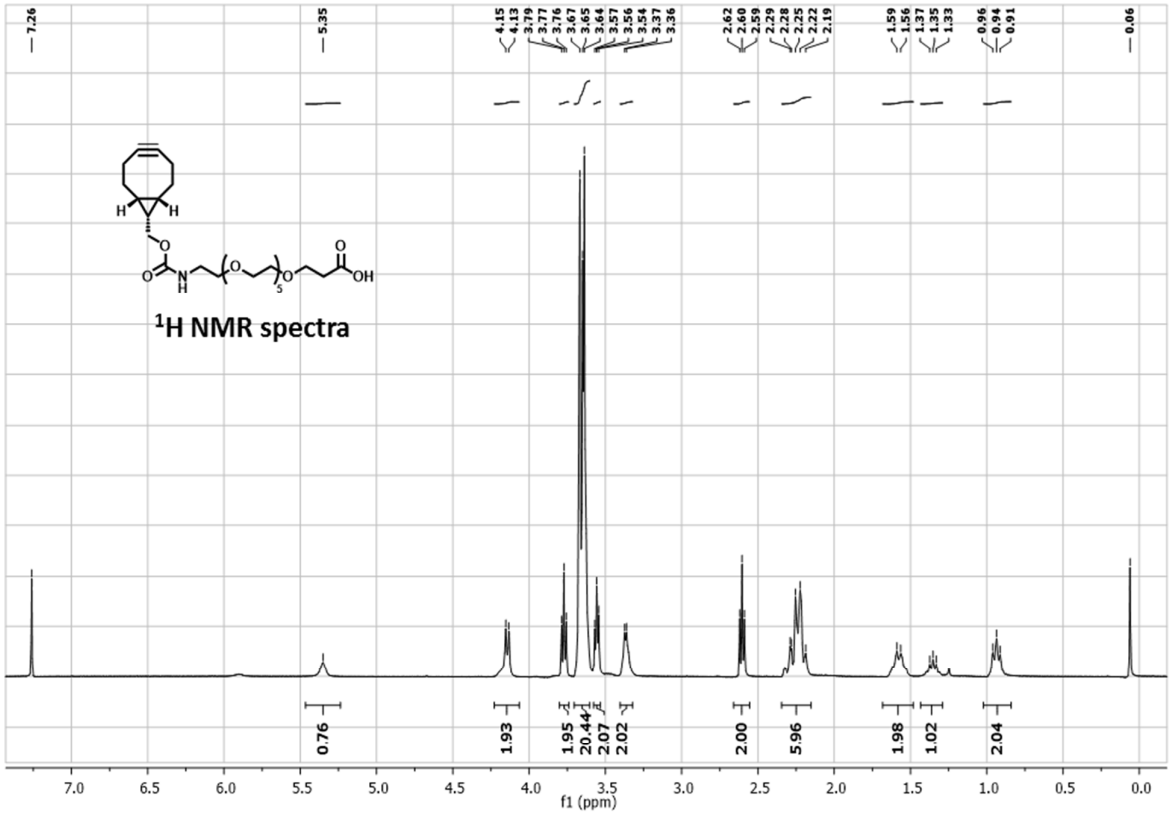
MW = 529.63 g/mol



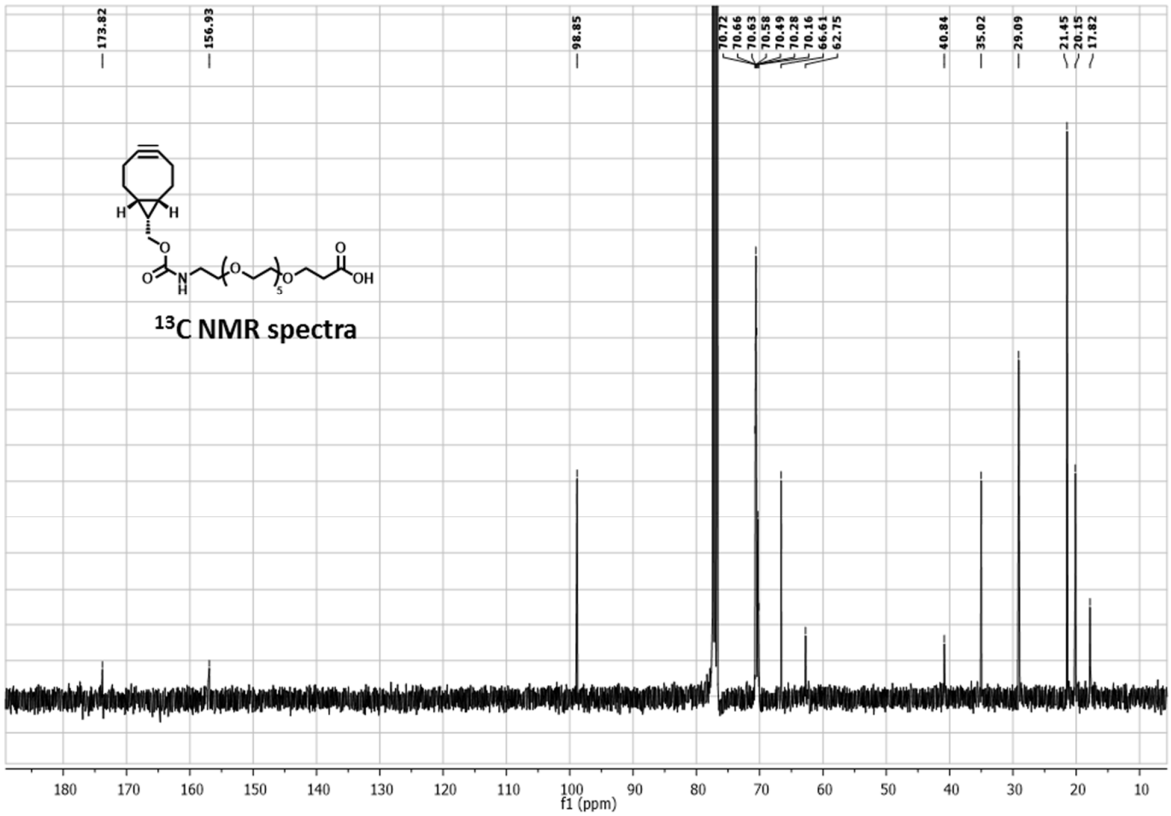
To a solution of **12** (1 eq., 915 mg, 1.68 mmol) in 10 mL of MeOH/H_2O 1/1 was added LiOH (5 eq., 201 mg, 8.42 mmol). The reaction mixture was stirred at room temperature overnight. After MeOH evaporation, the aqueous layer was acidified by addition of 50 mL of an aqueous solution of NaH_2PO_4 (1M) and extracted with DCM (4 x 50mL). The combined organic layer was dried over $MgSO_4$ and concentrated to afford **13** (815 mg, 1.54 mmol, 91 %) as a yellowish oil.

^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 5.35 (bs, 1H), 4.14 (d, *J* = 8.0 Hz, 2H), 3.77 (t, *J* = 6.0 Hz, 2H), 3.71 – 3.58 (m, 20H), 3.56 (t, *J* = 5.0 Hz, 2H), 3.41 – 3.30 (m, 2H), 2.60 (t, *J* = 6.0 Hz, 2H), 2.38 – 2.13 (m, 6H), 1.66 – 1.50 (m, 2H), 1.41 – 1.28 (m, 1H), 0.94 (t, *J* = 9.7 Hz, 2H). The CO_2H signal is missing.

^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 173.8, 156.9, 98.8, 70.2, 70.7 – 70.2, 66.6, 62.7, 40.8, 35.0, 29.1, 21.4, 20.1, 17.8.



^{1}H NMR spectra

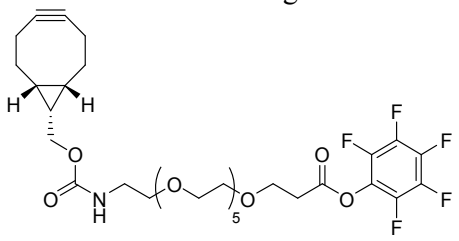


^{13}C NMR spectra

perfluorophenyl 1-((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)-3-oxo-2,7,10,13,16,19,22-heptaoxa-4-azapentacosan-25-oate, 14

$C_{32}H_{42}F_5NO_{10}$

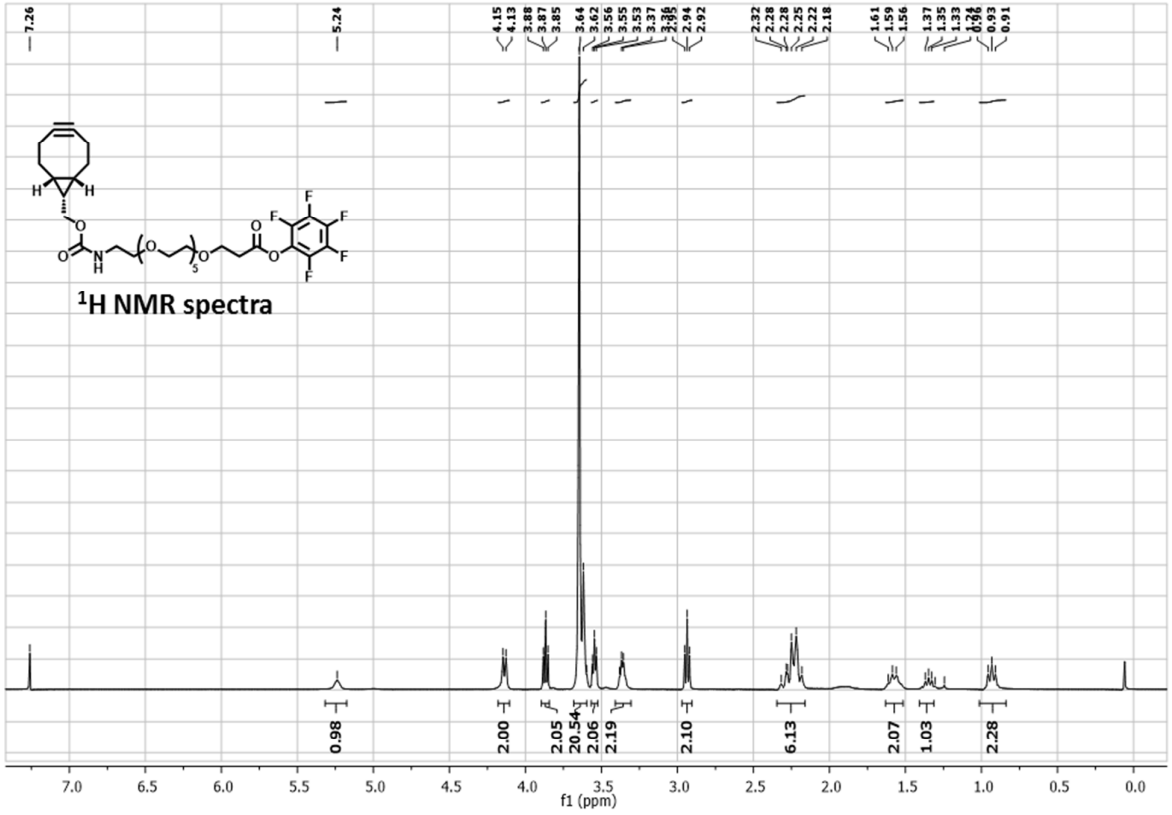
MW = 695.68 g/mol

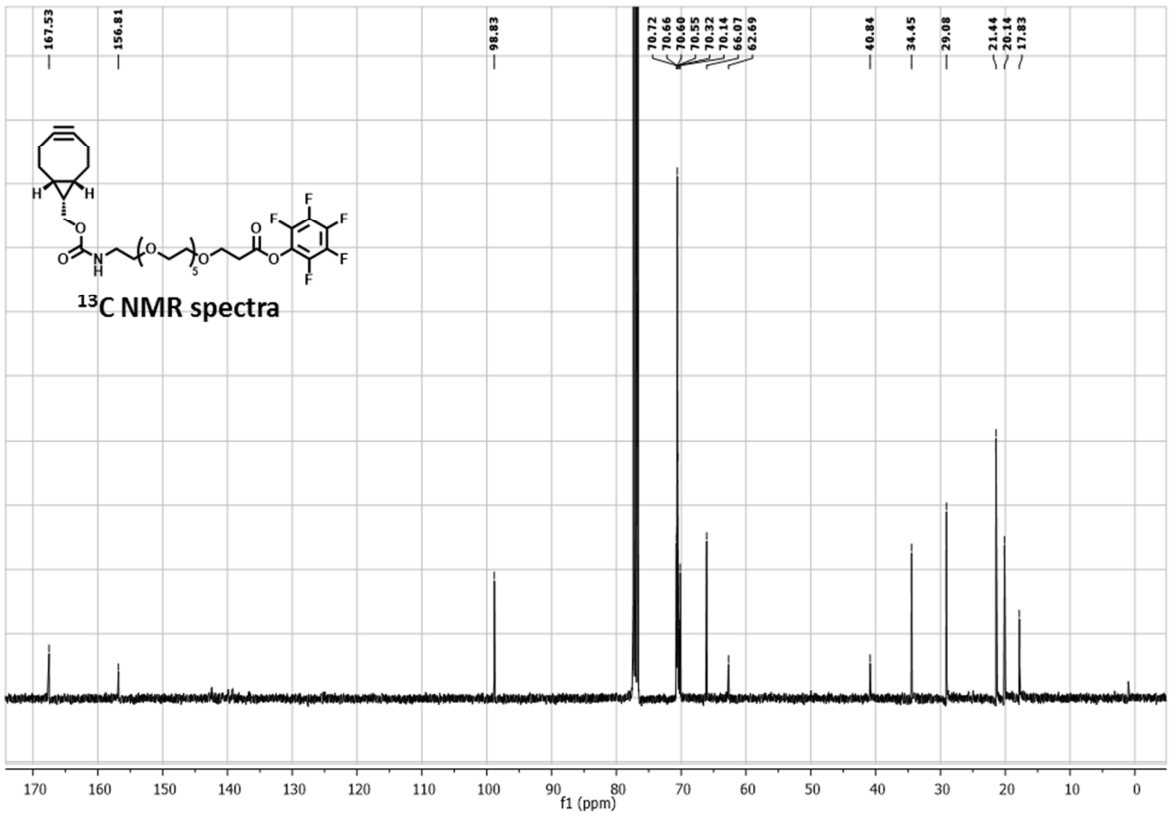


To a solution of **13** (1 eq., 800 mg, 1.51 mmol) and pentafluorophenol (1.2 eq., 333 mg, 1.81 mmol) in DCM (20 mL) was added DCC (1.1 eq., 342 mg, 1.66 mmol). The reaction mixture was stirred at room temperature for 4 hours. After concentration, the crude was dissolved in DCM (75 mL), filtered through a pad of celite and washed with an aqueous solution of $NaHCO_3$ (2 x 75mL). The organic layer was dried over $MgSO_4$ and purified by silica gel flash chromatography (Cyclohexane/EtOAc 1/1 to EtOAc in 30 minutes then EtOAc/MeOH 95/5 in 10 minutes) to afford **14** (875 mg, 1.26 mmol, 83 %) as a yellowish oil.

^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 5.24 (bs, 1H), 4.14 (d, *J* = 8.0 Hz, 2H), 3.87 (t, *J* = 6.2 Hz, 2H), 3.69 – 3.57 (m, 20H), 3.55 (t, *J* = 5.0 Hz, 2H), 3.42 – 3.28 (m, 2H), 2.94 (t, *J* = 6.2 Hz, 2H), 2.35 – 2.11 (m, 6H), 1.67 – 1.50 (m, 2H), 1.41 – 1.29 (m, 1H), 0.93 (t, *J* = 9.7 Hz, 2H).

^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm): 167.5, 156.8, 98.8, 70.7 – 70.1, 66.0, 62.7, 40.8, 34.4, 29.1, 21.4, 20.1, 17.8.

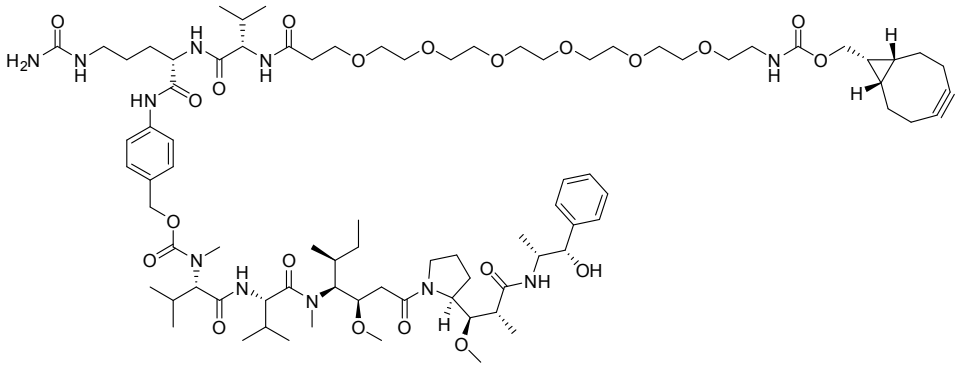




MMAE-BCN

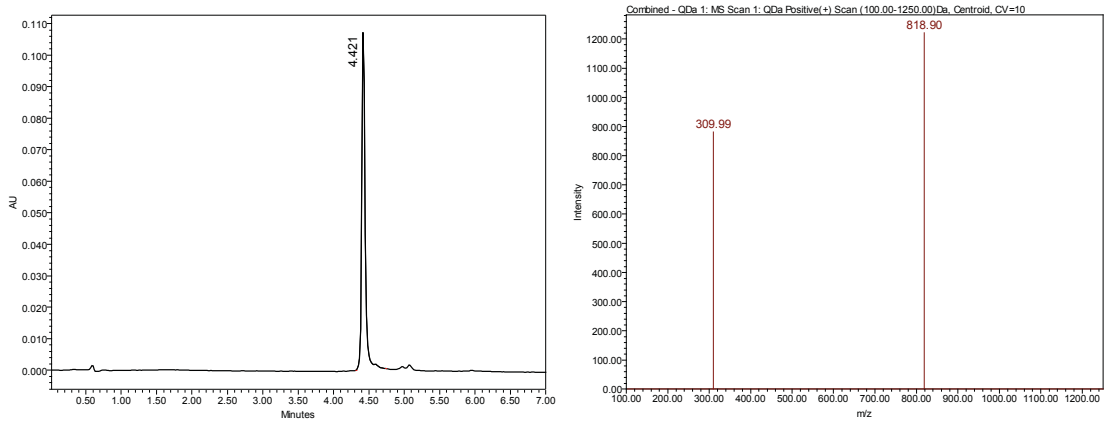
$C_{84}H_{135}N_{11}O_{21}$

MW = 1633.98 g/mol



To the solution of *4-((S)-2-((S)-2-amino-3-methylbutanamido)-5-ureidopentanamido)benzyl ((S)-1-(((S)-1-(((3R,4S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamate* (**Val-Cit-PAB-MMAE**, 5 mg, 1eq., 100 mM in DMSO) was added a solution of **14** (3.7 mg, 1.2 eq, 100 mM in DMSO) followed by a solution of DIEA (2.9 mg, 5 eq., 0.5 M in DMF). The mixture was incubated at r.t. for 30 min and the product was purified by semi-preparative HPLC to afford **MMAE-BCN** (4.5 mg, 62%).

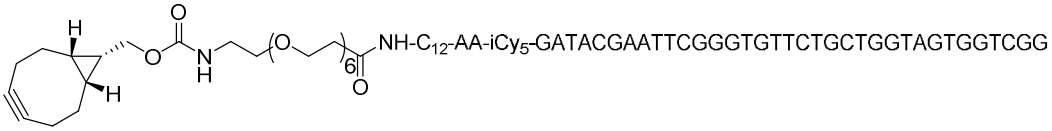
MS (ESI) *m/z*: 818.9 $[M+2H]^{2+}/2$.



LCMS Analysis of MMAE-BCN

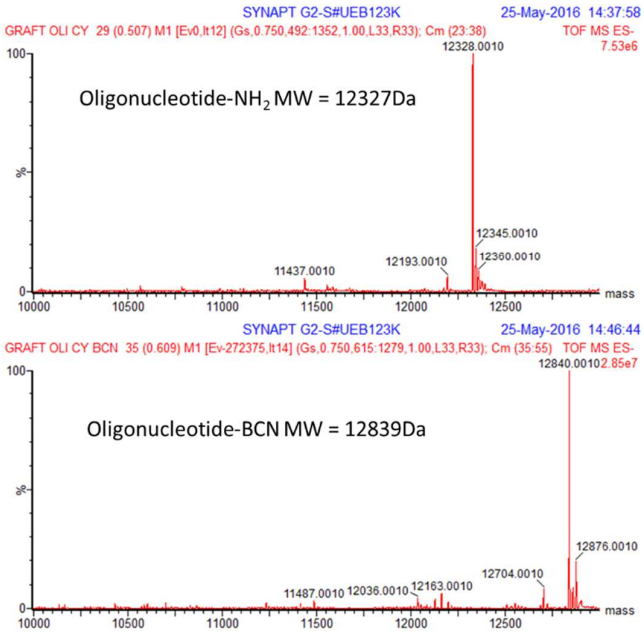
Preparation of oligonucleotide-BCN (ON1)

MW=12839 g/mol



Materials and methods: Amino-modified oligonucleotides were purchased from IDT. The purifications after oligonucleotide conjugation were carried out on a Shimadzu system (pump: LC 20-AD, detector: SPD 20-A, autosampler: SIL 20-A) using a SunFire™ C18 5 µM 4.6 × 150 mm column (Waters). HPLC parameters were as follows: flow rate 1 mL/min, mobile phase A was triethylammonium acetate (TEAA) in water (50 mM), and mobile phase B was TEAA in acetonitrile (50 mM). The detection was done at 260 nm. Gradient: from 15 to 40% of mobile phase B from 0 to 30 min.

General procedure for oligonucleotide conjugations: In a 2 mL Eppendorf tube, amino-modified oligonucleotide (1 nmol/µL in water, 1 eq.), a solution of conjugation reagent **14** in DMSO (10 eq.) and $NaHCO_3$ (1 M in water, 250 eq.) were introduced. The final volume was adjusted with DMSO to obtain a 1/1 water/DMSO ratio. The mixture was incubated at room temperature overnight under argon atmosphere and was then directly injected in HPLC for purification. After lyophilization, the oligonucleotide conjugate **ON1** was dissolved in water and concentration was measured by spectrophotometry. Mass spectrometry confirmed the successful conjugation.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25-May-2016 14:37:58
GRAFT OLI CY 29 (0.507) M1 [Ev0,It12] (Gs,0.750,492:1352,1.00,L33,R33); Cm (23:38)
TOF MS ES-
7.53e6
Oligonucleotide-NH2 MW = 12327Da
12328.0010
12345.0010
12360.0010
12193.0010
11437.0010
mass
SYNAPT G2-S#UEB123K
25-May-2016 14:46:44
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TOF MS ES-
2.85e7
Oligonucleotide-BCN MW = 12839Da
12840.0010
12876.0010
12704.0010
12163.0010
12036.0010
11487.0010
mass

Kinetics

Hydrolysis in PBS 1x buffer (pH 7.3)

The acylating reagents (1 mM or 0.5 mM) in PBS 1x buffer (pH 7.3) containing 5% of DMSO were analyzed by LC-MS at 254 nm (Figure S1). Pseudo-first-order rate constant for the reaction was determined by plotting $\ln(A_t)$ versus time and analyzing by linear regression, where A is the area under the peak of acylating reagents. The observed pseudo-first-order rate constant, k_1, was reproducible to within ±5% and was measured for two different concentrations of the acylating reagent. The average of two or more runs were normally taken. The resulting data is contained in Table S1.

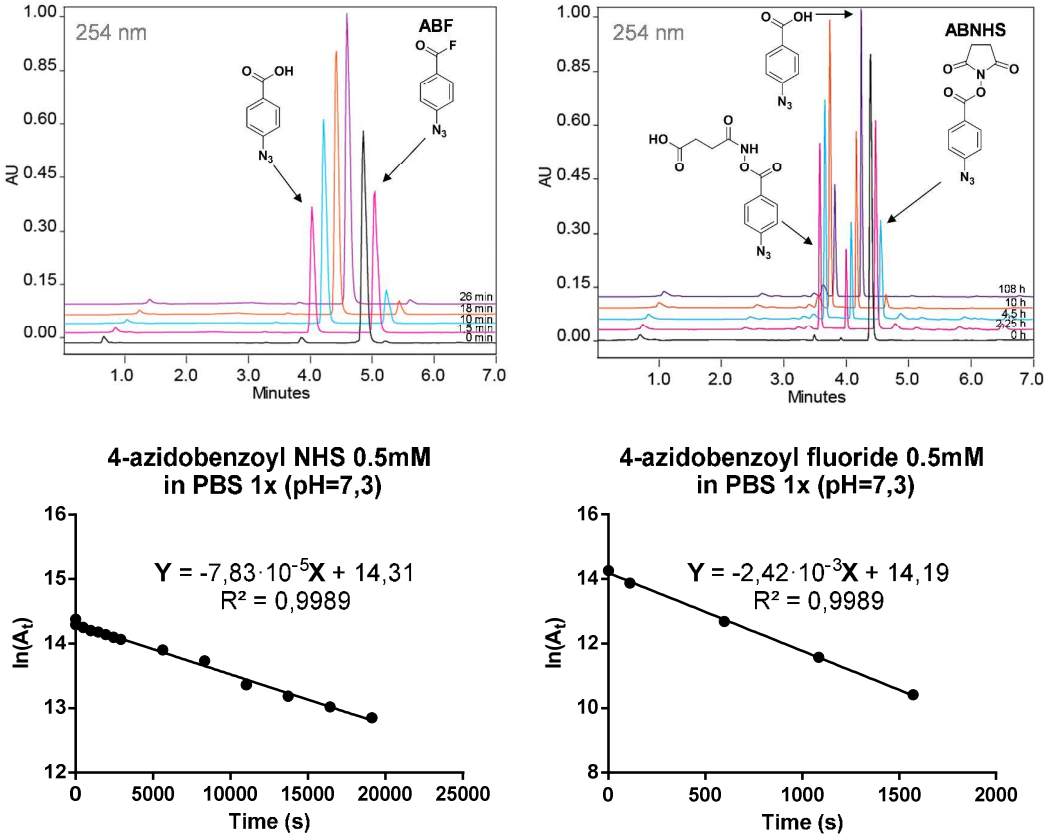


Figure S1. Hydrolysis of **ABF** and **ABNHS** in PBS 1x (pH 7.3) monitored by LC-MS at 254 nm and plot of kinetic data.

Aminolysis with benzylamine (pKa 9.4)

The acylation was conducted under pseudo-first order conditions with benzylamine in a 10-fold excess of the acylating reagent in PBS 1x buffer (pH 7.3) or ACN with 2% of DMSO in the final solution. Aliquots of reaction mixture were taken after certain intervals of time and quenched with the same volume of water containing 5% v/v of TFA. The samples were then analyzed by LC-MS at 254 nm (Figure S2). The second order rate constant, k_2, for the

reaction was determined from linear plot of $\ln(A_\infty - A_t)$ versus time using the following formula:

$\ln (A_\infty - A_t) = -kt + \ln A_\infty$, where $k = k_1 + k_2 \cdot C_0(BnNH_2)$;

A is the area under the peak of the amide product;

k_1 is a pseudo-first order rate constant of hydrolysis in PBS 1x (pH 7.3);

$C_0(BnNH_2)$ is a concentration of benzylamine.

The determined second order constant, k_2, was reproduced for two or more different concentrations of acylating reagent, and the average of two runs were normally taken. The resulting data is contained in Table S1.

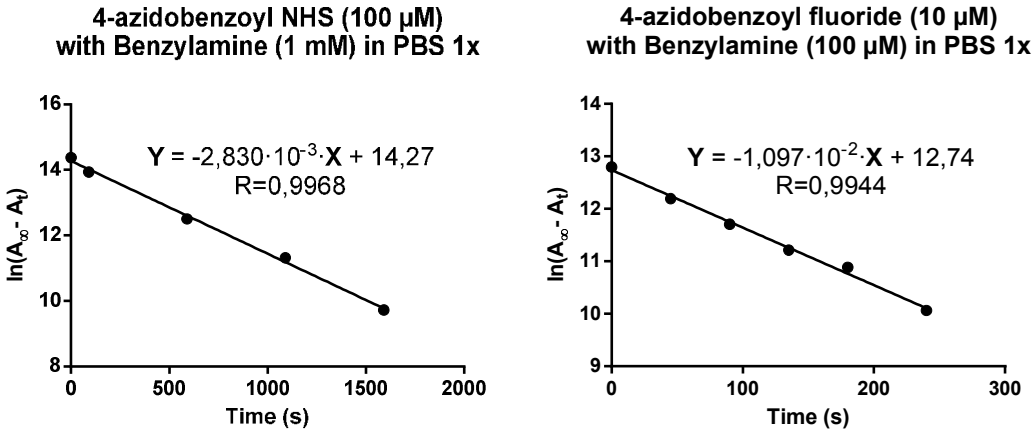


Figure S2. Plot of kinetic data of aminolysis in PBS 1x (pH 7.3)

Table S1. Rate constants of hydrolysis and aminolysis of the acylating reagents

Compound	Hydrolysis in PBS 1x[a]	Aminolysis with benzylamine in PBS 1x[a]	
	$k_1/10^{-4}$, s^{-1}	k_2, $L \cdot mol^{-1} \cdot s^{-1}$	$t_{1/2}$, min[b]
4-azidobenzoyl NHS, **ABNHS**	0.77	2.72	62
4-azidobenzoyl fluoride, **ABF**	24.59	87.95	1.9

[a] reproduced to within ± 5%; [b] $C_0(BnNH_2) = C_0$(**ABF** or **ABNHS**)=100 µM

Chemical biology

Preparation of antibody conjugates

Conjugation step: To a solution of trastuzumab (1 eq, 1 mg/mL, 90 µL in PBS 1x (pH 7.3)) was added DMSO (5 µL) and **ABF** or **ABNHS** (2,3,4,6,8 or 10 eq. in 2.47 µL of DMSO) at 25 °C and the reaction mixture was incubated at 25 °C for 18 h. The excess of reagents was then removed by gel filtration chromatography using Bio-spin P-30 Columns (Bio-Rad, Hercules, U.S.A.) pre-equilibrated with PBS 1/20x (pH 7.3) to give a solution of trastuzumab-azide (**T-N_3**), which was used in the functionalization step.

Functionalization step: To the solution of **T-N_3** in PBS 1/20x was added TAMRA-BCN **1** or MMAE-BCN (1.5 eq per 1 eq. of acylating reagent on conjugation stage, 1 mM in DMSO) or Oligonucleotide1-BCN (4.5 eq., 8.8 µL, 350 µM in H_2O) and the reaction mixture was incubated at 25 °C for 20 h. The excess of reagents was then removed by gel filtration chromatography using Bio-spin P-30 Columns (Bio-Rad, Hercules, U.S.A.) equilibrated with PBS 1/20x (pH=7.3). The average yield over two steps was 65-80%. The protein concentration of antibody conjugates was determined by BCA assay (ref 23225, ThermoFisher Scientific).

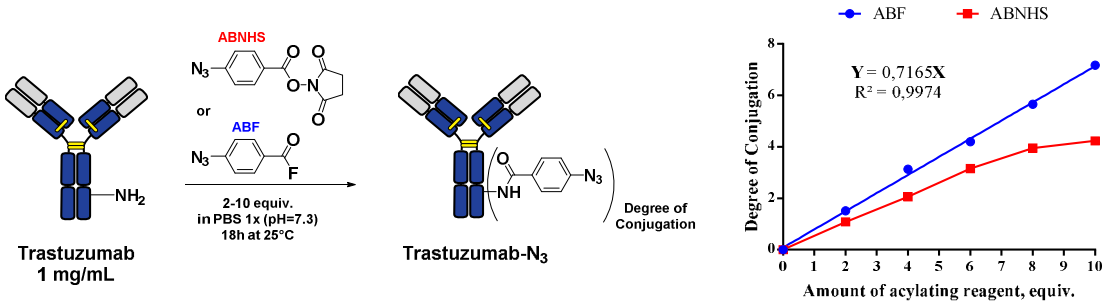


Figure S3. Efficiency of acylating reagents evaluated from plot of DoC vs amount of acylating reagent

Dual conjugation with mixture of two fluorophores

To a solution of trastuzumab (1 eq, 1 mg/mL, 2000 µL in PBS 1x (pH 7.3)) was added **ABF** (3 eq., 41.1 µL, 1 mM in DMSO) at 25 °C and the reaction mixture was incubated at 25 °C for 30 min. The aliquots of reaction mixture (100 µL) were taken and were reacted with mixture of TAMRA-BCN and Cy-BCN (4.5 eq., 6.17 µL, 0.5 mM in DMSO), where the ratio between two fluorophores was 0/10; 2/8; 4/6; 5/5; 6/4; 8/2 and 10/0 respectively. The samples were incubated at 25 °C for 20 h. The excess of reagents was then removed by gel filtration chromatography using Bio-spin P-30 Columns (Bio-Rad, Hercules, U.S.A.) equilibrated with PBS 1/20x (pH 7.3) to yield (65-80%) trastuzumab-TAMRA/Cy5 conjugates (**T-TAMRA/Cy5**), that were further analyzed by UV-Vis spectroscopy (SAFAS Xenius XC) at 558 nm and 650 nm (100 µL of antibody conjugates, 0.5 mg/mL in 96-well plate). The protein concentration of antibody conjugates was determined by BCA assay (ref 23225, ThermoFisher Scientific).

Kinetic study of antibody acylation with ABF and ABNHS

Kinetic study was performed with **ABF** and **ABNHS** in parallel. Further described is the protocol for **ABF**; the same protocol was used for **ABNHS**.

Conjugates preparation: To a solution of trastuzumab (1 eq, 1 mg/mL, 1000 µL in PBS 1x (pH 7.3)) was added DMSO (10 µL) and **ABF** (4 or 3 eq., 1 mM in DMSO) at 4 °C or 25 °C. After incubation at 4 °C or 25 °C for 1 min, 7.5 min, 15 min, 30 min, 60 min, or 120 min, aliquots (100 µL) were taken and purified by gel filtration chromatography using Bio-spin P-30 Columns pre-equilibrated with PBS 1/20x (pH 7.3). The resulting conjugates were subjected to SPAAC with TAMRA-BCN **1** (6 eq., 4.11 µL, 1 mM in DMSO) at 25 °C for 20 h. The excess of reagents was then removed by gel filtration chromatography using Bio-spin P-30 Columns pre-equilibrated with PBS 1/20x (pH 7.3) to yield (65-80%) trastuzumab-TAMRA conjugates (**T-TAMRA**), that were further analyzed by SDS PAGEs and native-HRMS. The protein concentration of antibody conjugates was determined by BCA assay (ref 23225, ThermoFisher Scientific).

Table 2S. Efficacy of conjugation step using **ABF** or **ABNHS** determined from native-HRMS of trastuzumab-TAMRA conjugates **T-TAMRA**

Conditions			Compound			
			ABNHS		**ABF**	
Quantity of acylating reagent, equiv.	t, °C	Time, min	DAR[a]	Efficacy[b], %	DAR	Efficacy, %
4	4	15	0	0	2.19	54.8
		120	0.16	4.0	2.74	68.5
4	25	15	0.27	6.8	2.87	71.8
		120	1.81	45.3	2.94	73.5
3	25	15	0.19	6.3	2.34	78.0
		120	0.79	26.3	2.31	77.0

[a] DAR = average dye to antibody ratio; [b] Efficacy = DAR/Quantity of acylating reagent (equiv.)·100%

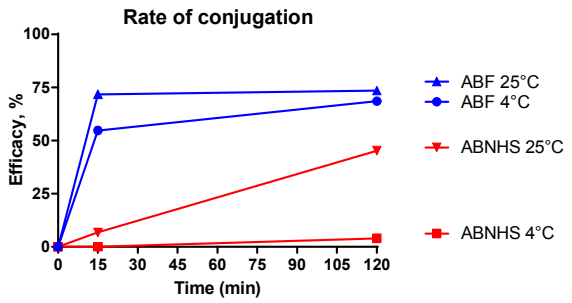


Figure S3. Efficiency of acylating reagents vs time of conjugation step at 4 °C and 25 °C.

Characterization of conjugates

SDS PAGE analysis

Reducing or non-reducing glycine-SDS-PAGE was performed on 4–15% Mini-PROTEAN® TGX™ Gel (Bio-Rad ref 4561084) following standard lab procedures. To samples containing antibody conjugates (24 µL, 0.1 mg/mL in H_2O) was added 8 µL of loading buffer (4x reducing (ref J60015) or non-reducing (ref J63615) Laemmli SDS sample buffer, Alfa Aesar) and heated at 95 °C for 5 minutes. The gel was run at constant voltage (200 V) for 40 min using TRIS 0.25 M - Glycine 1.92 M - SDS 1% as a running buffer.

Fluorescence was visualized on GeneGenius bio-imaging system (Syngene) prior to staining with Coomassie Blue.

Samples preparation for HRMS analysis

Prior to native MS experiments, antibody conjugates (AC) were desalted against 150 mM ammonium acetate solution buffered at pH 7.4 using six cycles of concentration/dilution on micro-concentrators (Vivaspin, 30 kD cutoff, Sartorius, Gottingen, Germany). Protein concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). AC deglycosylation was achieved by incubating (37 °C – 2 h) 0.4 units of Remove-iT® Endo S (New England Biolabs, Ipswich, U.S.A.) per microgram of AC prior to the buffer exchange desalting step.

HRMS analysis

High resolution native mass spectrometry (native-HRMS) was performed on an Exactive Plus EMR (Thermo Fisher Scientific, Bremen, Germany) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion, Ithaca, USA). Electrospray ionization was conducted at a capillary voltage of 1.86 kV and nitrogen nanoflow of 0.15 psi. Native MS experiments were performed using classical interface tuning parameters of the mass spectrometer with a nominal resolution of either 17,500 or 35,000 and in the positive ion mode. The in-source collision-induced dissociation and the higher-energy collisional dissociation cells were set to 200 eV and 50 eV respectively. The trapping gas pressure was set to 3 a.u. (which corresponds to an Ultra High Vaccum of 4.10^{-10} mbar approximatively). In order to improve the transmission of the high mass species the voltages on the injection, inter and bent flatapole were fixed to 8, 7 and 6 V respectively.

External calibration was performed using singly-charged ions produced by a 2 mg/mL solution of cesium iodide in 2-propanol/water (50/50 v/v) and samples were infused at 5 µM in NH_4OAc 150 mM pH 7.5. MS data interpretations and deconvolutions were performed using Protein Deconvolution 4.0 available on BiopharmaFinder SP1 (Thermo Fisher, Bremen, Germany). The parameters of software were optimized for each spectrum.

DoC calculation

The average Degree of Conjugation (DoC) values from native MS were calculated by using eq. 1. These results were derived from the relative peak intensities in deconvoluted mass spectra.

$$\textbf{Eq.1}\ \mathrm{DoC} = \frac{\sum_{k=0}^{k=n} k * I(\mathrm{DoC_k})}{\sum_{k=0}^{k=n} I(\mathrm{DoC_k})},$$

where $I(DoC_k)$ is relative peak intensity of conjugates with k add-on molecules per antibody.

ADC affinity

The antibody affinity of the different T-TAMRA was determined using flow cytometry on two breast adenocarcinoma cell lines: (i) $HER2^+$ SKBR-3 cells; (ii) $HER2^-$ MDA-MB-231 cells. A single cell suspension was obtained after incubating the adherent cells in 0.25 % trypsine for 1-2 min at 37 °C. Subsequent steps were performed at 4 °C. Briefly, 2 x 10^5 cells were blocked in 10 % BSA for 15 min and washed in FACS buffer (5 % BSA, 0.1 % NaN_3).

Then the cells were incubated for 15 min with the following antibodies/ADCs (20 µg/mL in FACS buffer): Trastuzumab, T-DM1, T-TAMRA or IgG1 isotype control. Subsequently, the cells were washed and incubated for 15 min with DyLight649-conjugated goat anti-human IgG antibody (Novus Biologicals, Littleton, CO, USA). The samples were analysed on the Guava® easyCyte 12HT (Merck Millipore, Molsheim, France) and the data analysis was performed using FlowJo X.0.7 (Tree Star, Ashland, OR, USA).

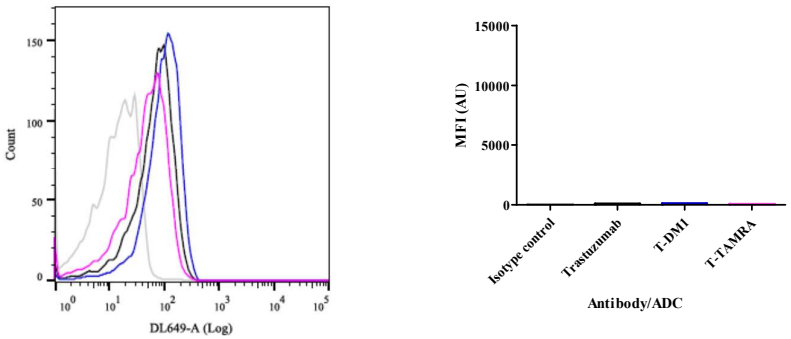
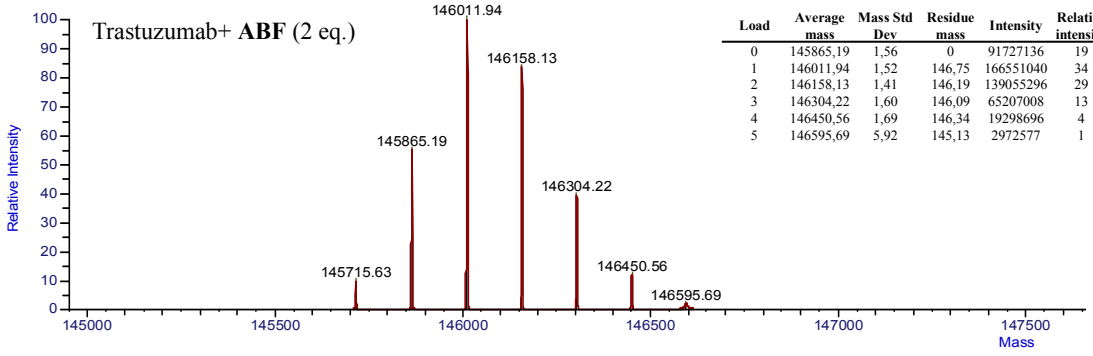


Figure S4. Median fluorescence intensities (MFIs) of T-TAMRA (pink), the benchmark T-DM1 (blue) and the native antibody trastuzumab (black) in HER2$^-$ MDA-MB-231 cells. Rituximab was used as isotype control (grey). The scale of the bar-plot was adapted to that of Fig. 4

Mass spectra and structure of conjugates



Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC
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1	146011,94	1,52	146,75	166551040	34	
2	146158,13	1,41	146,19	139055296	29	
3	146304,22	1,60	146,09	65207008	13	
4	146450,56	1,69	146,34	19298696	4	
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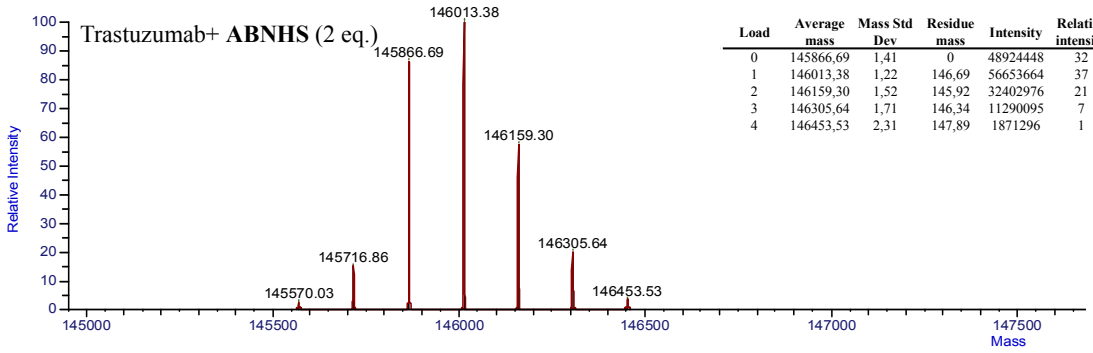

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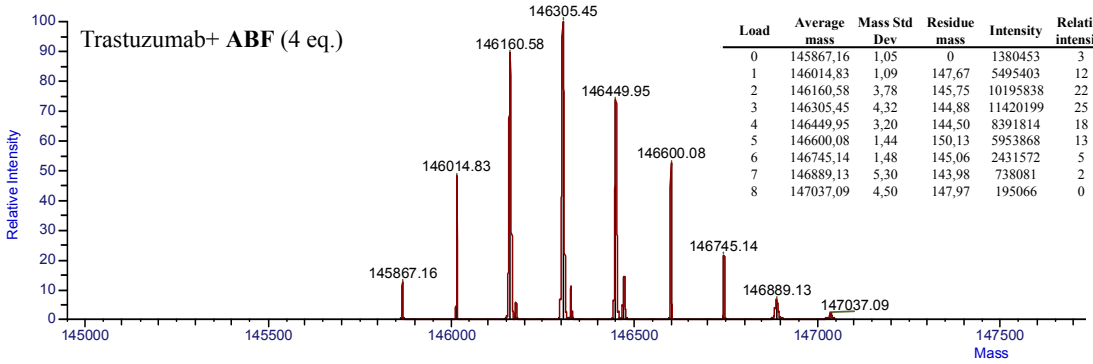
1	146013,38	1,22	146,69	56653664	37	
2	146159,30	1,52	145,92	32402976	21	
3	146305,64	1,71	146,34	11290095	7	
4	146453,53	2,31	147,89	1871296	1	



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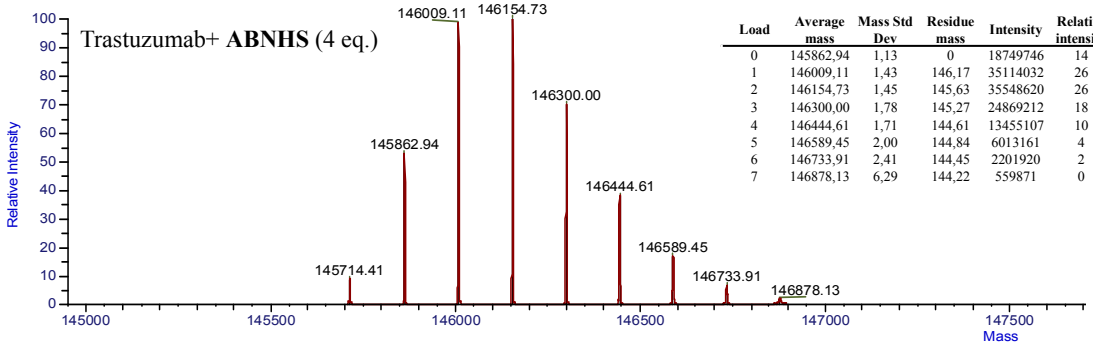
2	146160,58	3,78	145,75	10195838	22	
3	146305,45	4,32	144,88	11420199	25	
4	146449,95	3,20	144,50	8391814	18	
5	146600,08	1,44	150,13	5953868	13	
6	146745,14	1,48	145,06	2431572	5	
7	146889,13	5,30	143,98	738081	2	
8	147037,09	4,50	147,97	195066	0	



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2	146154,73	1,45	145,63	35548620	26	
3	146300,00	1,78	145,27	24869212	18	
4	146444,61	1,71	144,61	13455107	10	
5	146589,45	2,00	144,84	6013161	4	
6	146733,91	2,41	144,45	2201920	2	
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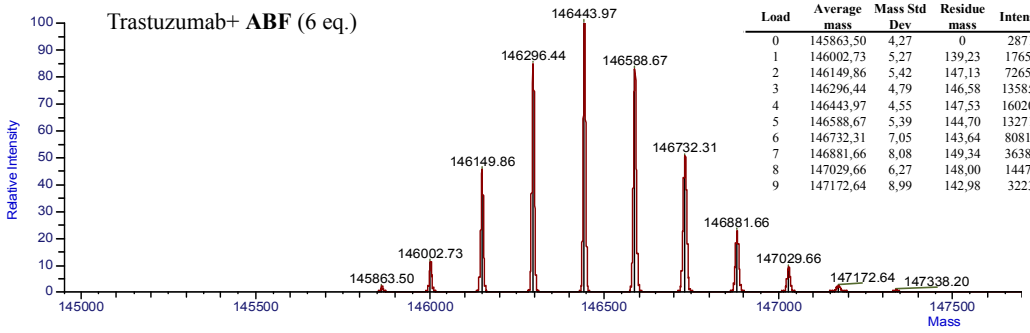

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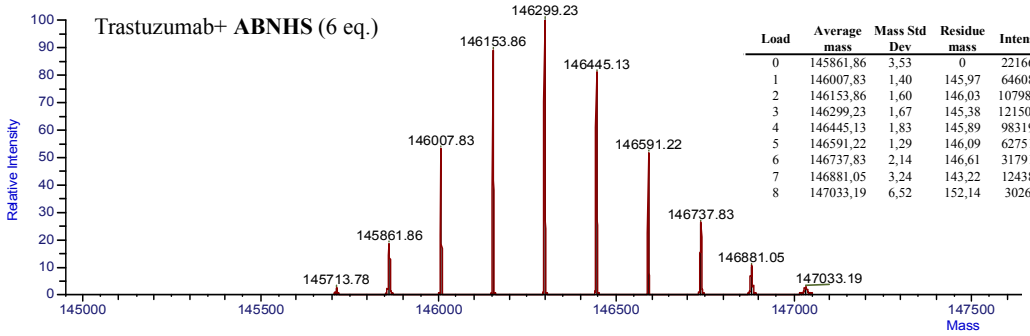
3	146296,44	4,79	146,58	1358575	21	
4	146443,97	4,55	147,53	1602045	24	
5	146588,67	5,39	144,70	1327134	20	
6	146732,31	7,05	143,64	808127	12	
7	146881,66	8,08	149,34	363866	6	
8	147029,66	6,27	148,00	144799	2	
9	147172,64	8,99	142,98	32234	0	



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2	146153,86	1,60	146,03	10798820	21	
3	146299,23	1,67	145,38	12150925	23	
4	146445,13	1,83	145,89	9831958	19	
5	146591,22	1,29	146,09	6275147	12	
6	146737,83	2,14	146,61	3179175	6	
7	146881,05	3,24	143,22	1243852	2	
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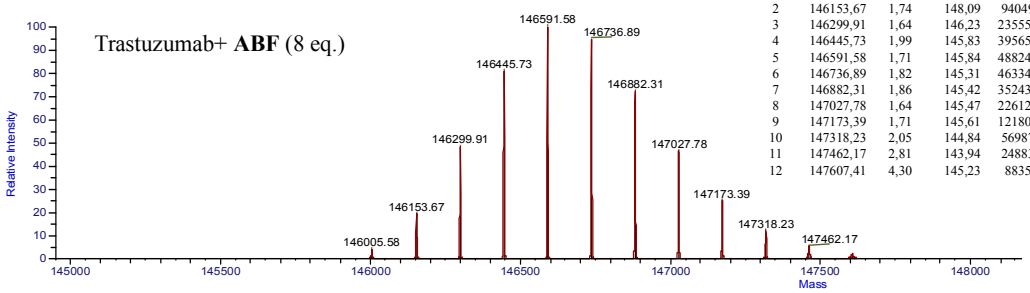

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2	146153,67	1,74	148,09	9404909	4	
3	146299,91	1,64	146,23	23555698	9	
4	146445,73	1,99	145,83	39565972	16	
5	146591,58	1,71	145,84	48824264	20	
6	146736,89	1,82	145,31	46334680	19	
7	146882,31	1,86	145,42	35243904	14	
8	147027,78	1,64	145,47	22612072	9	
9	147173,39	1,71	145,61	12180388	5	
10	147318,23	2,05	144,84	5698744	2	
11	147462,17	2,81	143,94	2488301	1	
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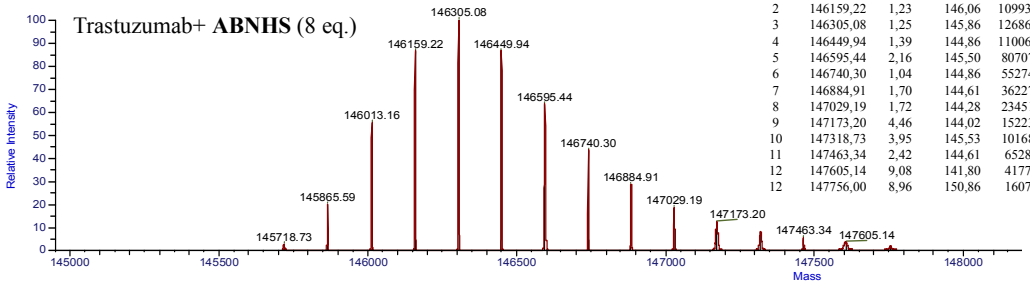

Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC
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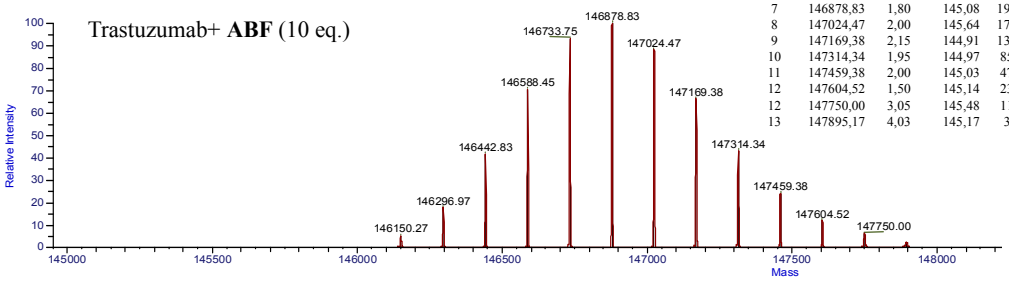
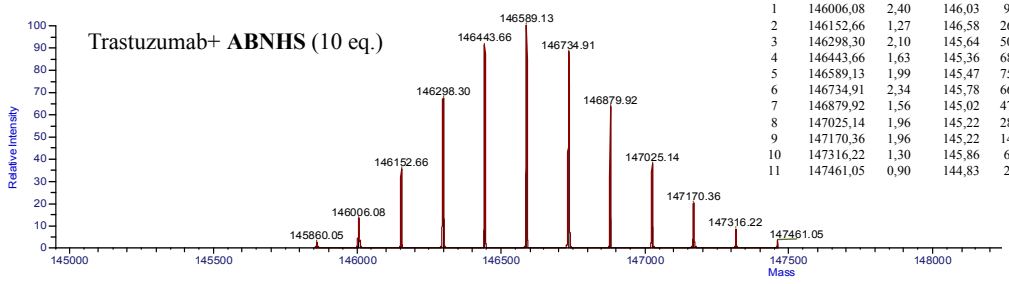
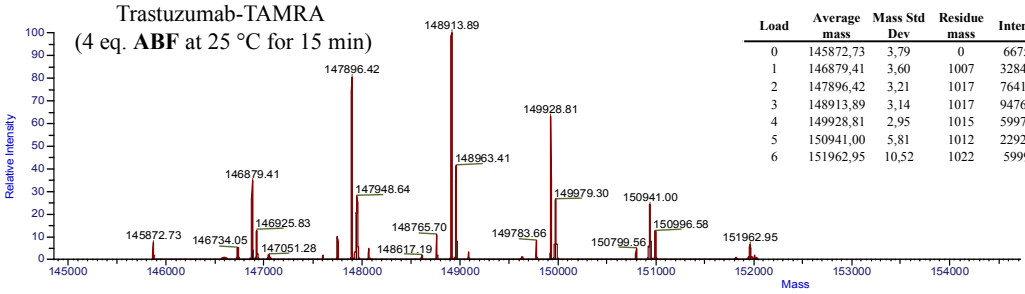
2	146159,22	1,23	146,06	10993333	16	
3	146305,08	1,25	145,86	12686611	19	
4	146449,94	1,39	144,86	11006936	16	
5	146595,44	2,16	145,50	8070716	12	
6	146740,30	1,04	144,86	5527499	8	
7	146884,91	1,70	144,61	3622781	5	
8	147029,19	1,72	144,28	2345116	3	
9	147173,20	4,46	144,02	1522356	2	
10	147318,73	3,95	145,53	1016800	2	
11	147463,34	2,42	144,61	652895	1	
12	147605,14	9,08	141,80	417737	1	
12	147756,00	8,96	150,86	160721	0	



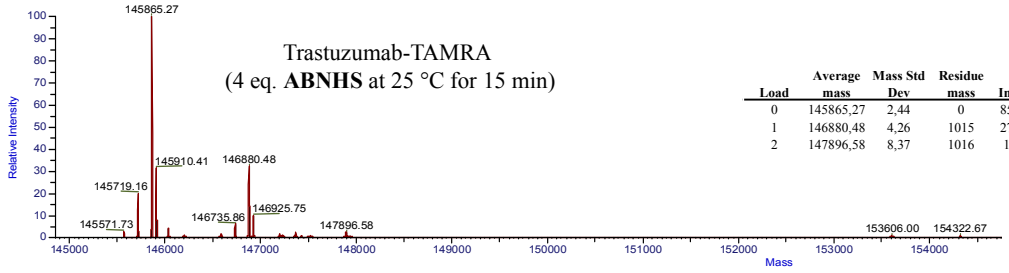
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC
0	145865,59	1,34	0	0	0	7,17
1	146013,16	1,24	147,56	0	0	
2	146150,27	2,41	142,63	930007	1	
3	146296,97	1,69	146,70	3450435	3	
4	146442,83	1,53	145,86	8207216	7	
5	146588,45	1,68	145,63	14004047	12	
6	146733,75	1,80	145,30	18470938	16	
7	146878,83	1,80	145,08	19827514	18	
8	147024,47	2,00	145,64	17493746	16	
9	147169,38	2,15	144,91	13156222	12	
10	147314,34	1,95	144,97	8568676	8	
11	147459,38	2,00	145,03	4710431	4	
12	147604,52	1,50	145,14	2343124	2	
12	147750,00	3,05	145,48	1158928	1	
13	147895,17	4,03	145,17	397590	0	



Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC
0	145860,05	1,79	0	171564	0,4	5,13
1	146006,08	2,40	146,03	973553	2	
2	146152,66	1,27	146,58	2645857	7	
3	146298,30	2,10	145,64	5052115	13	
4	146443,66	1,63	145,36	6875697	17	
5	146589,13	1,99	145,47	7514539	19	
6	146734,91	2,34	145,78	6632034	17	
7	146879,92	1,56	145,02	4758658	12	
8	147025,14	1,96	145,22	2821494	7	
9	147170,36	1,96	145,22	1484383	4	
10	147316,22	1,30	145,86	632995	2	
11	147461,05	0,90	144,83	208822	1	

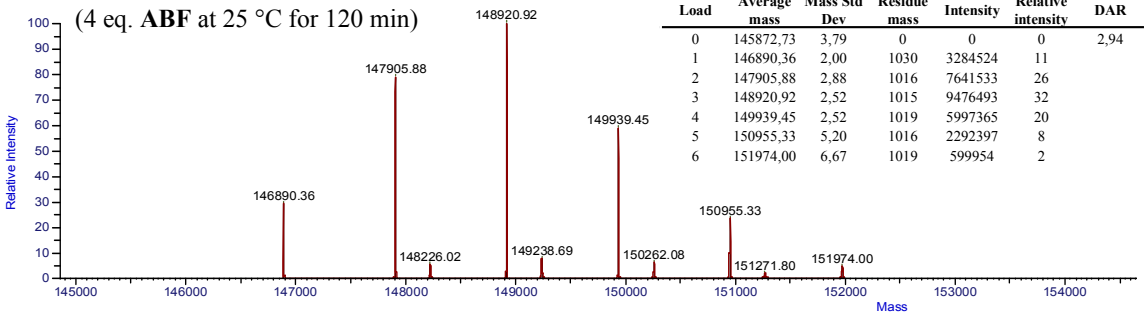


Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145872,73	3,79	0	667534	2	2,87
1	146879,41	3,60	1007	3284524	11	
2	147896,42	3,21	1017	7641533	26	
3	148913,89	3,14	1017	9476493	32	
4	149928,81	2,95	1015	5997365	20	
5	150941,00	5,81	1012	2292397	8	
6	151962,95	10,52	1022	599954	2	



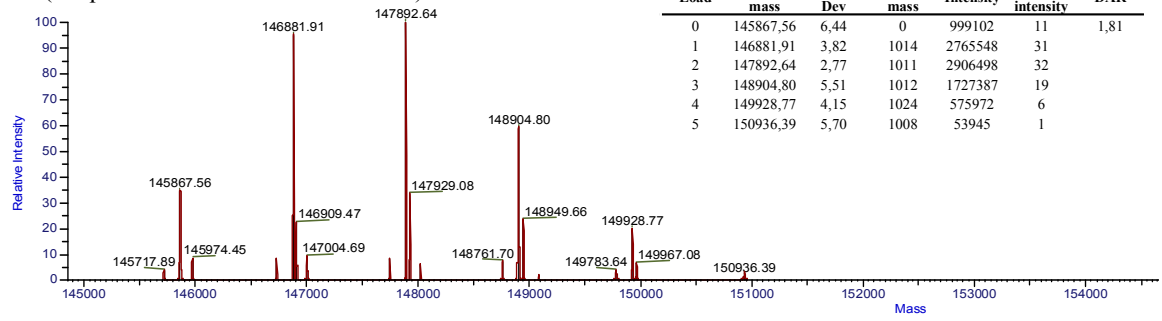
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145865,27	2,44	0	8593097	74	0,27
1	146880,48	4,26	1015	2768487	24	
2	147896,58	8,37	1016	188123	2	

Trastuzumab-TAMRA
(4 eq. **ABF** at 25 °C for 120 min)



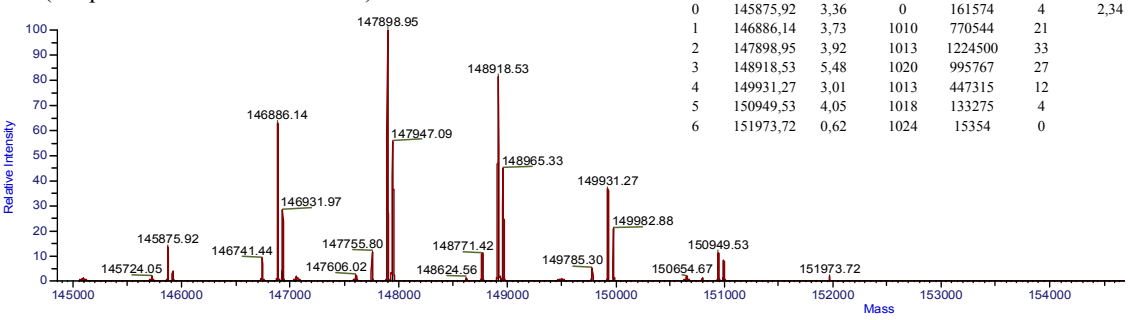
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145872,73	3,79	0	0	0	2,94
1	146890,36	2,00	1030	3284524	11	
2	147905,88	2,88	1016	7641533	26	
3	148920,92	2,52	1015	9476493	32	
4	149939,45	2,52	1019	5997365	20	
5	150955,33	5,20	1016	2292397	8	
6	151974,00	6,67	1019	599954	2	

Trastuzumab-TAMRA
(4 eq. **ABNHS** at 25 °C for 120 min)



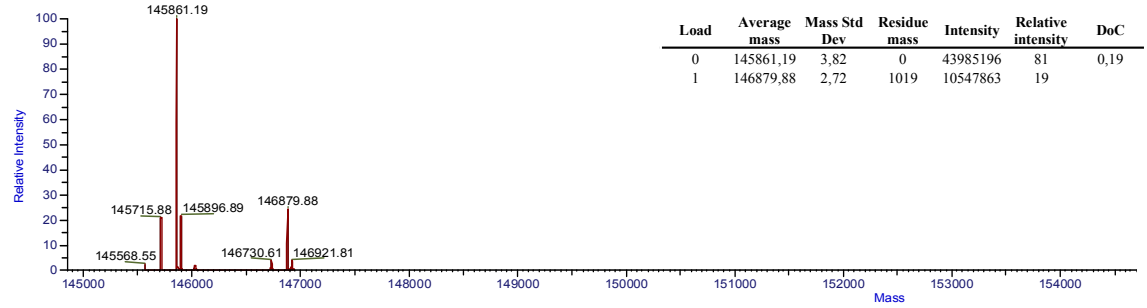
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145867,56	6,44	0	999102	11	1,81
1	146881,91	3,82	1014	2765548	31	
2	147892,64	2,77	1011	2906498	32	
3	148904,80	5,51	1012	1727387	19	
4	149928,77	4,15	1024	575972	6	
5	150936,39	5,70	1008	53945	1	

Trastuzumab-TAMRA
(3 eq. **ABF** at 25 °C for 15 min)



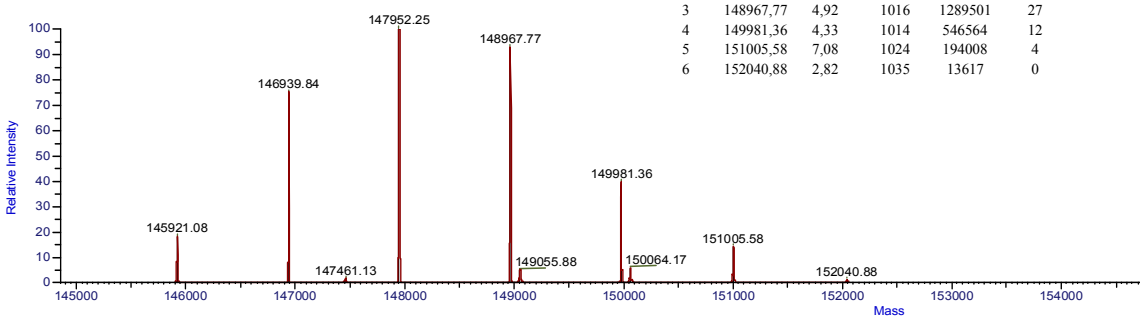
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145875,92	3,36	0	161574	4	2,34
1	146886,14	3,73	1010	770544	21	
2	147898,95	3,92	1013	1224500	33	
3	148918,53	5,48	1020	995767	27	
4	149931,27	3,01	1013	447315	12	
5	150949,53	4,05	1018	133275	4	
6	151973,72	0,62	1024	15354	0	

Trastuzumab-TAMRA
(3 eq. **ABNHS** at 25 °C for 15 min)



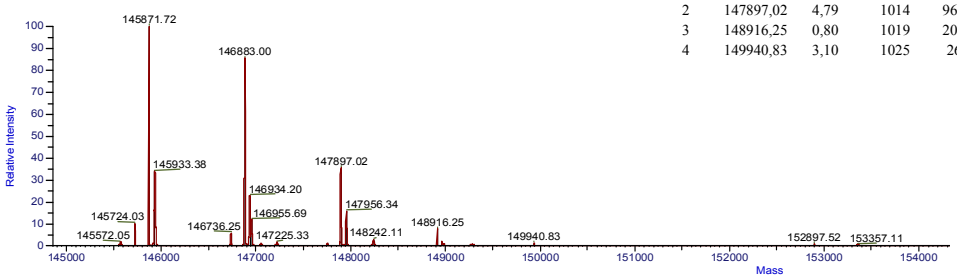
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC
0	145861,19	3,82	0	43985196	81	0,19
1	146879,88	2,72	1019	10547863	19	

Trastuzumab-TAMRA
(3 eq. **ABF** at 25 °C for 120 min)



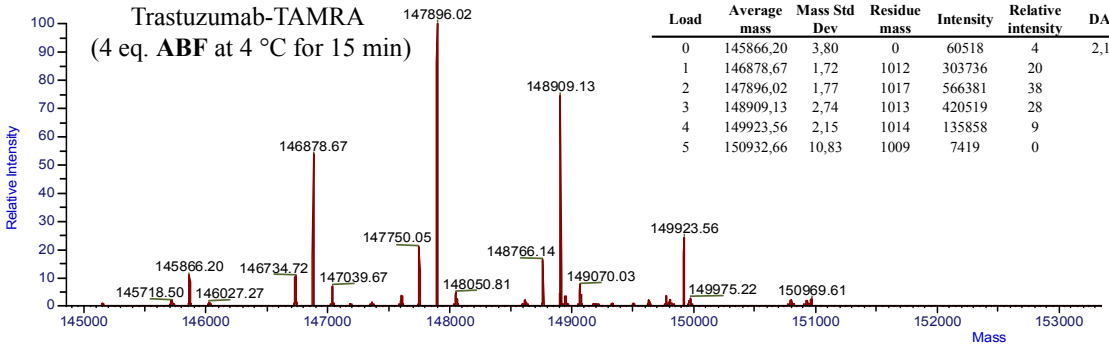
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145921,08	5,14	0	248942	5	2,31
1	146939,84	3,42	1019	1043288	22	
2	147952,25	3,45	1012	1387507	29	
3	148967,77	4,92	1016	1289501	27	
4	149981,36	4,33	1014	546564	12	
5	151005,58	7,08	1024	194008	4	
6	152040,88	2,82	1035	13617	0	

Trastuzumab-TAMRA
(3 eq. **ABNHS** at 25 °C for 120 min)



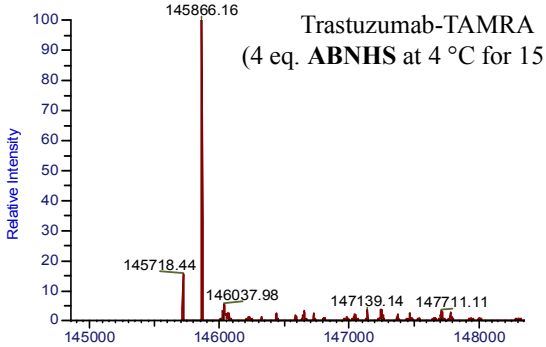
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145871,72	2,10	0	2740313	44	0,79
1	146883,00	4,09	1011	2343337	37	
2	147897,02	4,79	1014	965235	15	
3	148916,25	0,80	1019	204268	3	
4	149940,83	3,10	1025	26369	0	

Trastuzumab-TAMRA
(4 eq. **ABF** at 4 °C for 15 min)



Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145866,20	3,80	0	60518	4	2,19
1	146878,67	1,72	1012	303736	20	
2	147896,02	1,77	1017	566381	38	
3	148909,13	2,74	1013	420519	28	
4	149923,56	2,15	1014	135858	9	
5	150932,66	10,83	1009	7419	0	

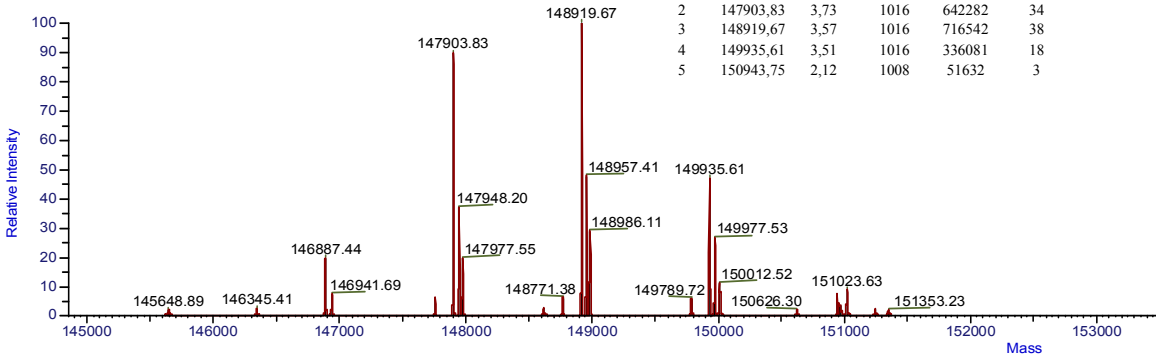
Trastuzumab-TAMRA
(4 eq. **ABNHS** at 4 °C for 15 min)



Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145866,20	3,80	0	477534	100	0

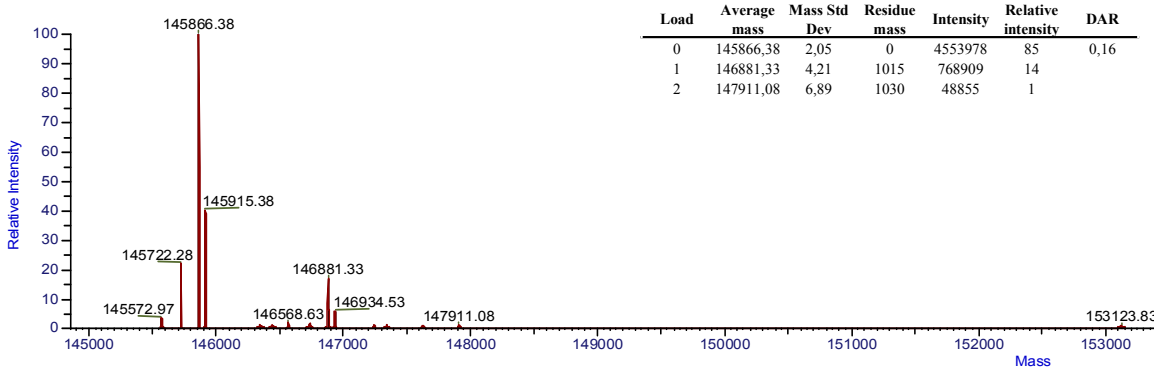
Trastuzumab-TAMRA
(4 eq. **ABF** at 4 °C for 120 min)

Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145866,20	3,80	0	0	0	2,74
1	146887,44	4,82	1021	139638	7	
2	147903,83	3,73	1016	642282	34	
3	148919,67	3,57	1016	716542	38	
4	149935,61	3,51	1016	336081	18	
5	150943,75	2,12	1008	51632	3	



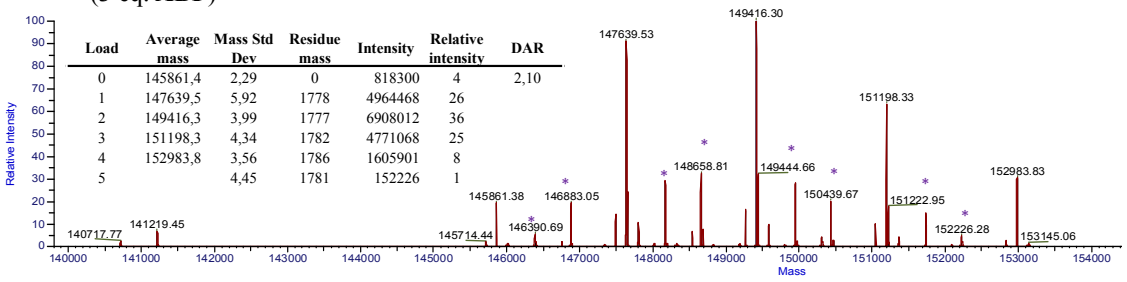
Trastuzumab-TAMRA
(4 eq. **ABNHS** at 4 °C for 120 min)

Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145866,38	2,05	0	4553978	85	0,16
1	146881,33	4,21	1015	768909	14	
2	147911,08	6,89	1030	48855	1	

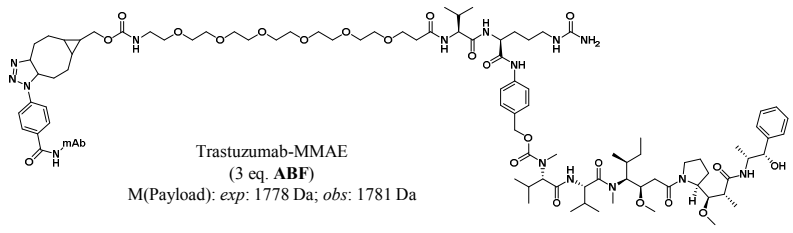


Trastuzumab-MMAE
(3 eq. **ABF**)

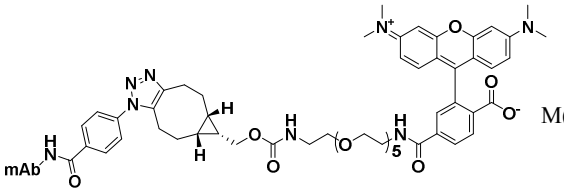
Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DAR
0	145861,4	2,29	0	818300	4	2,10
1	147639,5	5,92	1778	4964468	26	
2	149416,3	3,99	1777	6908012	36	
3	151198,3	4,34	1782	4771068	25	
4	152983,8	3,56	1786	1605901	8	
5		4,45	1781	152226	1	



* BCN-PEG$_6$-COOH (by-product in BCN-MMAE) conjugates with T-N$_3$

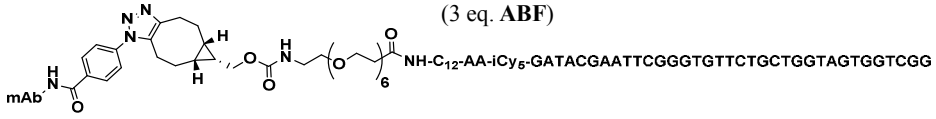


Trastuzumab-MMAE
(3 eq. **ABF**)
M(Payload): *exp*: 1778 Da; *obs*: 1781 Da



Trastuzumab-TAMRA conjugates
M(Payload): *exp*: 1013 Da; *obs*: 1015 Da

Trastuzumab-ON1 conjugate **C1**
(3 eq. **ABF**)



Fluorescence of line on SDS PAGE
Trastuzumab-ON1 conjugate (3 eq. **ABF**)

Load	Signal	DoC without load 0
1	9110000	2,41
2	17800000	
3	7030000	
4	6600000	
5	2080000	

HRMS analysis of Trastuzumab-TAMRA conjugate (3 eq. **ABF)**

Load	Average mass	Mass Std Dev	Residue mass	Intensity	Relative intensity	DoC without load 0
0	145921,08	5,14	0	248942	5	2,44
1	146939,84	3,42	1019	1043288	22	
2	147952,25	3,45	1012	1387507	29	
3	148967,77	4,92	1016	1289501	27	
4	149981,36	4,33	1014	546564	12	
5	151005,58	7,08	1024	194008	4	
6	152040,88	2,82	1035	13617	0	

Oligonucleotide 2 (ON2)

3’-C TAT GCT TAA GAC CCC CAC AAG ACG ACC ATC ACC/5Atto488N/-5’