Bioorthogonal chemical tagging of protein cholesterylation in living cells

Electronic Supplementary Information

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1. Supplemental Figures

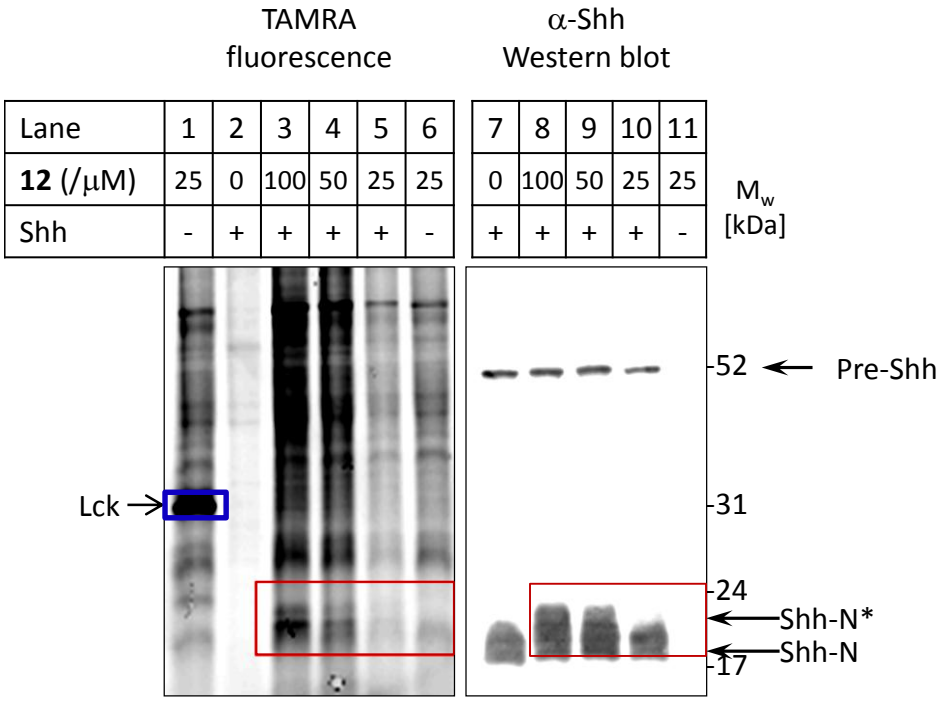


Figure S1. Labeling of HEK293a cells with alkynyl palmitate analogue **12**. Cells were transfected with Shh (Lanes 2–5 and 7–10) or Lck (Lane 1) and tagged by application of **12** at various concentrations from 0 to 100 μM. Cells were then lysed and the lysates subjected to CuAAC with capture reagent **10**. Lck is a palmitoylated tyrosine kinase, and acted as a positive control for labeling (as reported in Refs. 15 and 20). As expected, **12** dose-dependently labels a large number of palmitoylated proteins in the cell; a fluorescent band specific to Shh can be seen at ca. 22 kDa. α-Shh Western blot shows an upward band shift on tagging and labeling (as is seen for Shh cholesterylation, see main text), indicating an increase in molecular weight. The band at 52 kDa is unprocessed pre-Shh that has not undergone autocleavage/cholesterylation.

2. Experimental data:

2.1. Synthesis

All reagents obtained from commercial sources (Sigma-Aldrich, Merck, BDH) were used without further purification. Ultrapure laboratory grade water (18.2 MΩ) was obtained from in-house Elix® and Milli-Q® water purification systems (Millipore, UK). Organic solutions were concentrated *in vacuo* using a Büchi rotary evaporator (Rotavapor R-210, Vacuum controller V-850 heating bath B-491 and a KNF Laboport or Büchi V-700 membrane diaphragm pump). Reactions were followed by means of TLC using aluminium-backed silica plates (Merck, Si60, F254) and, unless otherwise stated, visualised under UV irradiation and/or using acidic *p*-anisaldehyde followed by heating as required. Flash column chromatography was carried out on BDH silica gel (Si60, 43–60 μm). Purity of the compounds was determined by analysis of NMR spectroscopy, accurate mass spectrometry and LC-MS. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AM-400 spectrometer in $CDCl_3$ using residual non-deuterated solvent peak as an internal reference (δ_H = 7.26 ppm). Assignment abbreviations: app (apparent), s (singlet), d (doublet), dd (double doublet), t (triplet), dt (double triplet), q (quarted), qui (quintet), sept (septet), m (multiplet), ster (steroid proton(s) and not further assigned). COSY and DEPT was used as a guide to assign signals in ^{1}H-spectra where required. Chemical shifts (δ) are reported in parts per million (ppm), and the coupling constants (*J*) are given in Hertz (Hz). Mass spectrometry was performed (unless stated otherwise) using electrospray ionisation (ESI) by Mr J. Barton on an *Autospec P673 Spectrometer* at the Chemistry Department Mass Spectrometry Service at Imperial College London (UK).

2.1.1. (3*S*)-3-*tert*-Butyldimethylsilyloxypregn-5-en-20-one (2).

To a stirred solution of 3*β*-hydroxy-5-pregnen-20-one (**1**) (3.00 g, 9.48 mmol) and imidazole (2.00 g, 29.45 mmol) in DMF (10 mL), was added *tert*-butyldimethylsilyl chloride (2.22 g, 14.73 mmol) at rt. After 12 h, the mixture was diluted with EtOAc. The aqueous layer was extracted with EtOAc and the organic layer washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (hexane–EtOAc, 97:3) to afford (**2**) (3.35 g, 82%) as a colorless solid. R_f = 0.75 (hexane/EtOAc, 7:3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (1H, m, *H6*), 3.50 (1H, m, *H3*), 2.55 (1H, dd, *J* = 8, *H17*), 2.35–1.94 (5H, m, ster.), 2.14 (3H, m, *H21*), 1.89–1.35 (10H, m, ster.), 1.35–0.96 (4H, m, ster.), 1.02 (3H, s, *H19*), 0.91 (9H, s, (*t*-Bu)-Si), 0.65 (3H, s, *H18*), 0.08 (6H, s, $(CH_3)_2$-Si); MS *m*/*z* 455 $[M + Na]^+$.

2.1.2. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-hydroxypregn-5-ene (3).

$NaBH_4$ (95 mg, 2.5 mmol) was added to a solution of ketone (**2**) (634 mg, 1.5 mmol) in THF (10 mL) and MeOH (10 mL) at rt, and stirring was continued for 2 h. The reaction mixture was acidified with 1 M HCl (to pH 5) and concentrated to a third of its volume. Water was added to the residue and extracted with dichloromethane. The organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to afford two diastereoisomers of the alcohol (**3**) (536 mg, 83%). R_f = 0.70 and 0.65 (hexane–EtOAc, 8:2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (1H, m, *H6*), 3.75 (1H, m, *H17*), 3.50 (1H, m, *H3*), 2.29 (1H, dt, *J* = 2.1, 13.2, ster.), 2.19 (1H, ddd, *J* = 2.1, 5.1, 13.2, ster.), 2.09 (1H, dt, *J* = 20.7, 12.3, ster.), 1.99 (1H, m, ster), 1.83 (1H, dt, *J* = 3.3, 13.2), 1.78–1.40 (10H, m, ster.), 1.40–0.93 (6H, m, ster.), 1.16 (3H, d, *J* = 8, *H21*), 1.03 (3H, s, *H19*), 0.91 (9H, s, Si(*t*-Bu)), 0.79 (3H, s, *H18*), 0.08 (6H, s, $Si(CH_3)_2$).

2.1.3. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-*N*,*N*-dimethyl-amidoxapropyloxy)pregn-5-ene (4).

To a stirred solution of the alkoxide generated *in situ* from the secondary alcohol (**3**) (294 mg, 0.68 mmol) and NaH (60% dispersion in mineral oil, 55 mg, 1.36 mmol) in dry THF (2.5 mL) was added *N*,*N*-dimethylacrylamide (280 μL, 2.72 mmol) at 0 °C and the stirring was continued for 6 h at the same temperature. The reaction was quenched carefully at 0 °C by the addition of saturated aqueous NH_4Cl (2 mL) and the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane–EtOAc, 70:30) to afford the expected amide (**4**) (197 mg, 54%) and 107 mg of starting material (**3**) (36%). R_f = 0.30 (hexane–EtOAc, 5:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (1H, m, *H6*), 3.87 (1H, dt, *J* = 5.9, 7.8, ster.), 3.65 (1H, q, *J* = 7.3, ½CH_2CON$(CH_3)_2$), 3.50 (1H, app sept, *J* = 4.9, *H3*), 3.35 (1H, dt, J = 5.9, 11.8, ½CH_2CON$(CH_3)_2$), 3.06 (3H, s, ½N$(CH_3)_2$), 2.97 (3H, s, ½N$(CH_3)_2$), 2.67 (2H, m, CH_2CH_2CON$(CH_3)_2$), 2.29 (1H, t, *J* = 11.1, ster.), 2.19 (1H, ddd, *J* = 2.1, 5.1, 13.2, ster.), 2.03 (2H, m, ster), 1.83 (1H, dt, *J* = 3.1, 13.3, ster.), 1.77–1.25 (9H, m, ster.), 1.25–0.80 (6H, m, ster.), 1.10 (3H, d, *J* = 5.9, *H21*), 1.02 (3H, s, *H19*), 0.91 (9H, s, Si(*t*-Bu)), 0.71 (3H, s, *H18*), 0.07 (6H, s, $Si(CH_3)_2$); MS *m*/*z* 554 $[M + Na]^+$, 532 $[M + H]^+$.

2.1.4. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-hydroxypropyl-oxy)-pregn-5-ene (5).

A solution of amide **4** (430 mg, 0.81 mmol) in dry THF (4.5 mL) was treated with Super-hydride® (1.0 M in THF, 4.86 mL, 4.86 mmol) at 0 °C. After 3 h, the reaction mixture was quenched by addition of MeOH (2 mL), diluted with Et_2O and washed with saturated aqueous NH_4Cl solution. After separation, the aqueous phase was extracted twice with Et_2O and the combined organic layers were washed with brine, dried over Na_2SO_4

and concentrated under reduced pressure. The colorless oil was purified by flash column chromatography (hexane–EtOAc, 9:1) to afford the colorless waxy solid (**5**) (354 mg, 89%). R_f = 0.56 (hexane–EtOAc, 7:3); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.33 (1H, m, *H6*), 3.85–3.70 (3H, m, ster. and C*H*$_2$OH), 3.50 (2H, m, *H3* and ½C*H*$_2$(CH$_3$)$_2$OH), 3.29 (1H, m, ½C*H*$_2$(CH$_3$)$_2$OH), 2.29 (1H, dt, *J* = 2.0, 13.2, ster.), 2.15 (2H, m, ster.), 2.04–1.38 (12H, m, ster), 1.38–1.14 (8H, m, ster and C*H*$_2$CH$_2$OH), 1.12 (3H, d, *J* = 5.9, *H21*), 1.02 (3H, s, *H19*), 0.91 (9H, s, Si(*t*-Bu)), 0.72 (3H, s, *H18*), 0.08 (6H, s, Si(CH$_3$)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 121.02, 78.36, 72.65, 67.58, 62.71, 56.54, 56.19, 50.27, 42.84, 42.26, 39.53, 37.35, 36.62, 32.36, 32.11, 31.97, 31.81, 25.94 (3CH$_3$), 25.72, 24.44, 20.98, 19.45, 18.38, 12.63, –4.59; MS *m*/*z* 491 $[M + H]^+$; HRMS *m*/*z* calculated for $C_{30}H_{55}O_3Si$ ($[M + H]^+$) 491.3920 found 491.3912.

2.1.5. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-azidopropyl-oxy)-pregn-5-ene (7).

Methanesulfonyl chloride (53 µL, 0.68 mmol) was added to a mixture of **5** (305 mg, 0.62 mmol) and Et_3N (259 µL, 1.86 mmol) in DCM (1 mL) at 0 °C. The reaction mixture was brought to rt whilst stirring and extracted with DCM. The organic layers combined were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. 250 mg of the crude were directly dissolved in DMF (1 mL) without further purification. After addition of NaN_3 (115 mg, 1.76 mmol), the mixture was heated at 90 °C overnight, poured into water and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. Purification by flash column chromatography (DCM) afforded the azide (**7**) (57 mg, 25%) as a white waxy solid and 100 mg of alcohol **5**. R_f = 0.77 (DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.34 (1H, m, *H6*), 3.66 (1H, dt. *J* = 6.0, 9.2, ster.), 3.50 (1H, app. sept., *J* = 4.7, ster.), 3.43 (2H, m, *H25*), 3.31 (2H, m, *H23* and *H17*), 2.29 (1H, dt, *J* = 2.1, 13.3, ster.), 2.19 (1H, ddd, *J* = 1.9, 4.9, 13.2, ster.), 2.09 (1H, dt, *J* = 3.4, 12.9, ster.), 1.99 (1H, m, ster.), 1.84 (3H, m,), 1.77–1.35 (9H, m, ster.), 1.25–0.89 (6H, m, ster.), 1.10 (3H, d, *J* = 6.0, *H21*), 1.03 (3H, s, *H19*), 0.91 (9H, s, Si(t-Bu)), 0.72 (3H, s, *H18*), 0.08 (6H, s, Si(CH$_3$)$_2$); MS *m*/*z* 488 $[(M - N_2) + H]^+$.

2.1.6. (3*S*)-20-(3-Azidopropyloxy)pregn-5-en-3-ol (8).

To a stirred solution of the silyl ether (**7**) (50 mg, 0.097 mmol) in THF (300 µL) was added TBAF (1.0 M in THF, 300 µL, 0.30 mmol) at rt; the stirring was continued for 4 h before the second addition of TBAF (1.0 M in THF, 300 µL, 0.30 mmol). After stirring overnight at the same temperature the mixture was diluted with EtOAc (300 µL), washed sequentially with 1 N HCl (600 µL), saturated aqueous $NaHCO_3$ and brine. The combined aqueous layers were extracted twice with EtOAc and the combined organic layers dried over Na_2SO_4 and concentrated under reduced pressure. The crude was purified by flash column chromatography (DCM–MeOH, 97:3) to obtain the tagged azido-cholesterol analogue (**8**) (35 mg, 90%) as a white waxy solid. R_f = 0.46 (DCM–MeOH, 95:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 (1H, m, H6), 3.65 (1H, dt, *J* = 6.0, 9.2, H23), 3.55 (1H, app. sept, *J* = 5.1, H2), 3.43 (2H, dt, *J* = 1.5, 6.8, H25), 3.31 (2H, m, H23 and H17), 2.30 (2H, m, ster.), 2.06 (1H, dt, *J* = 3.1, 12.7, ster.), 1.99 (1H, m, ster.), 1.85 (5H, m, ster.), 1.57 (10H, m, ster.), 1.31–0.86 (7H, m, ster.), 1.09 (3H, m, ster.), 0.72 (3H, s, H18); ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.88, 121.60, 78.14, 71.81, 64.43, 56.41, 56.19, 50.21, 48.82, 42.29, 39.60, 37.26, 36.56, 31.96–31.81–31.67 (3CH$_3$), 29.68, 25.81, 24.51, 20.99, 19.42, 18.32, 12.53; MS *m*/*z* 374 $[(M - N_2) + H]^+$; HRMS *m*/*z* calculated for $C_{24}H_{40}NO_2$ ($[(M - N_2) + H]^+$) 374.3020 found 374.3053.

2.1.7. Alkynyl trifunctional capture reagent (9).

Biotin-PEG Novatag™ resin (480 µmol/g loading, 104.2 mg, 50.0 µmol, 1.0 eq) was swollen in DMF (2 mL) and deprotected (Fmoc) with 20% v/v piperidine in DMF (2 mL for 3 min × 3) and washed with DMF, DCM and DMF sequentially. *N*-*α*-Fmoc-glycine (74.3 mg, 5.0 eq), HATU (93.1 mg, 4.9 eq) and DIPEA (87.1 µL, 10.0 eq) were dissolved in DMF (2 mL) and stood for 5 min before being added to the deprotected resin and the reaction shaken for 30 min. Coupling was repeated for this and all other steps. Following washing (DMF–DCM–DMF), deprotection (Fmoc, 20% v/v piperidine) of the coupled glycine residue and further washing, a solution of *N*-Fmoc-propargyl-glycine (41.9 mg, 2.5 eq), HATU (46.6 mg, 2.5 eq) and DIPEA (43.5 µL, 5.0 eq) in DMF (2.0 mL) was added and the reaction shaken for 30 min. TAMRA (32.3 mg, 1.5 eq) was activated for 10 min in DMF (2 mL) with DIC (16.3 mg, 1.5 eq) and HOAt (10.1 mg, 1.5 eq) and coupled to the deprotected (Fmoc, 20% v/v piperidine), washed lysine-azide residue for 2 × 2 hours. The resin was washed sequentially with DMF, DCM, MeOH and diethyl ether and dried overnight in a vacuum dessicator. The crude product was cleaved from the resin with 95% TFA, 2.5% water and 2.5% triisopropylsilane and precipitated with cold TBME. The solids were pelleted by centrifugation for 15 min at 4300 rpm at 4 °C and washed (with vortexing and centrifugation) three times with TBME. The pelleted product was dried in a desiccator overnight. The product was purified by semi preparative LC-MS over a gradient of MeOH (0.1% FA) in water (0.1% FA) (0–1 min 5% MeOH, 11–13 min 2% water), with detection over 100–600 nm. The product **9** was obtained by lyphophilisation as a bright pink amorphous solid (28.9 mg, 57% yield). ^{1}H NMR (500 MHz, MeOD) δ 8.71 (1H, d, *J* = 1.7, HD), 8.21 (1H, dd, *J* = 7.9, 1.8, HE), 8.16 (2H, s), 7.45 (1H, d, *J* = 7.9, HF), 7.20 (2H, d, J = 9.4, HC), 7.03 (2H, dd, *J* = 9.5, 2.2, HB), 6.94 (2H, d, *J* = 2.4, HA), 4.69 (1H, dd, *J* = 8.3, 6.2, H20), 4.46 (1H, ddd, *J* = 7.9, 4.9, 0.7, H1), 4.27 (1H, dd, *J* = 7.9, 4.5, H2), 3.97 (1H, d, *J* = 16.8, H19), 3.80 (1H, d, *J* = 16.8, H19), 3.63–3.58 (4H, m), 3.59–3.54 (4H, m), 3.52 (2H, t, *J* = 6.2), 3.48 (2H, t, *J* = 6.2), 3.33 (2H, d, *J* = 7.6), 3.29 (12H, s, NMe$_2$), 3.21 (2H, dd, *J* = 12.4, 5.7), 3.19–3.15 (1H, m, H4), 2.89 (3H, ddd, *J* = 14.7, 8.6, 3.8, H3 (1H) and H21), 2.68 (1H, d, *J* = 12.7, H3 (1H)), 2.47 (1H, t, *J* = 2.6, H22), 2.16 (2H, t, *J* = 7.3), 1.81 (2H, qui, *J* = 6.5), 1.72 (3H, dd, *J* = 13.0, 6.5), 1.66–1.50 (3H, m, H5), 1.45–1.36 (2H, m). ^{13}C NMR (125 MHz, MeOD) δ 175.94, 173.12, 171.44, 170.30, 169.11, 166.08, 165.56, 161.19, 159.04, 158.82, 138.47, 137.84, 136.56, 132.31 (C, B), 131.28 (F), 130.94 (E), 130.67 (D), 115.27 (B), 114.74 (C), 97.42 (A), 80.65, 72.51, 71.53, 71.22, 69.95, 69.77, 63.37 (2), 61.61 (1),

57.01 (4), 55.16 (20), ~49 (2 × NMe_2 under solvent peak), 43.80 (19), 41.05 (3), 40.89, 37.90, 37.84, 36.85, 30.39, 30.35, 29.79, 29.50, 26.89, 22.00 (21); MS *m*/*z* (ESI), 1011 ($[M + H]^+$), 1033 ($[M + Na]^+$), 506 ($[M + 2H]^{2+}$); HRMS *m*/*z* (ESI), calculated for $C_{52}H_{67}N_8O_{11}S$ ($[M + H]^+$) 1011.4650, found 1011.4615.

2.1.8. Azido trifunctional capture reagent (10).

Synthesis and purification as for **9** but with the addition of *N*-Fmoc-Lys-Az-OH (49.3 mg, 2.5 eq) in place of *N*-Fmoc-propargyl-glycine. The product **10** was obtained by lyophilization as a bright pink amorphous solid (15.9 mg, 29% yield). ^{1}H NMR (400 MHz, MeOD) δ 8.71 (1H, d, J = 1.7, HD), 8.23 (1H, s, OH on TAMRA?), 8.22 (1H, dd, J = 7.9, 1.8, HE), 7.45 (1H, d, J = 7.9, HF), 7.23 (2H, d, J = 9.5, HC), 7.05 (2H, dd, J = 9.5, 2.4, HB), 6.96 (2H, d, J = 2.3, HA), 4.51 (2H, m, H20 and H1), 4.30 (1H, dd, J = 7.9, 4.5, H2), 3.99 (1H, d, J = 16.8, ½H19), 3.83 (1H, d, J = 16.8, ½H19), 3.64 (4H, dt, J = 5.7, 3.0), 3.58 (6H, ddd, J = 15.2, 9.0, 4.3), 3.50 (2H, t, J = 6.2), 3.41–3.35 (4H, m), 3.31 (12H, s, H25), 3.27–3.17 (3H, m, incl H4), 2.93 (1H, dd, J = 12.8, 5.0, ½H3), 2.70 (1H, d, J = 12.7, ½H3), 2.19 (2H, t, J = 7.4), 2.09–1.91 (2H, m), 1.85 (2H, p, J = 6.5), 1.79–1.68 (5H, m), 1.61 (5H, ddd, J = 22.2, 16.0, 8.5), 1.49–1.38 (2H, m). Non-assigned 36 H's correspond to 18 × CH_2. ^{13}C NMR (100 MHz, MeOD) δ 175.91, 175.03, 171.52, 170.79, 169.32, 166.12 (6 × C=O), 166.07, 161.29, 159.00, 158.75, 139.30, 137.66, 136.59, 132.38 (C, B), 131.14 (F), 130.68 (E), 130.50 (D), 115.19 (B), 114.73 (C), 97.41 (A), 71.53, 71.23, 71.21, 69.94, 69.79, 63.36, 61.60, 57.02, 56.34, 52.27, ~49 (2 × NMe_2 under solvent peak), 43.72, 41.06, 40.89, 37.92, 37.83, 36.84, 31.90, 30.38, 29.80, 29.60, 29.50, 26.90, 24.47; MS *m*/*z* (ESI), 1070 ($[M + H]^+$), 1092 ($[M + Na]^+$), 536 ($[M + 2H]^{2+}$); HRMS *m*/*z* (ESI), calculated for $C_{53}H_{72}N_{11}O_{11}S$ ($[M + H]^+$) 1070.5134, found 1070.5124.

2.1.9. Heptadec-16-ynoic acid (12).

To a solution of TMS–acetylene (0.60 mL, 4.25 mmol) in dry THF (4.0 mL) under nitrogen and at −78 °C was added a solution of n-BuLi in hexanes (2.0 mL, 2.5 M, 5.0 mmol). The reaction mixture was allowed to warm up to room temperature for a few seconds before cooling to −78 °C. To this mixture was added a solution of 15-bromopentadecanoic acid (453 mg, 1.41 mmol) in dry THF (15 mL) and dry DMPU (7.9 mL) dropwise. The reaction was allowed to warm to room temperature and stirred until complete. Upon completion (after 18 h, followed by TLC) the reaction mixture was returned to −78 °C and quenched by the addition of saturated $NH_4Cl_{(aq.)}$ (8.0 mL). The precipitated salts prevented phase separation, but filtration through celite and washing through with water (8 mL) and diethylether (80 mL) provided two clear layers. The aqueous layer was extracted with diethylether (3 × 50 mL) and the combined organic layers were washed with brine (3 × 20 mL) before drying over $MgSO_4$ and concentration under reduced pressure. A portion of the crude product was purified by flash column chromatography (n-hexane–ethylacetate (1 : 1) + 1% AcOH, R_f = 0.67) to give the TMS protected alkyne (44 mg). The TMS protected alkyne (44 mg, 0.13 mmol) was taken up in MeOH (3.0 mL) and to this was added K_2CO_3 (36 mg). The reaction mixture was stirred at room temperature for 18 h, after which time all volatiles were removed under reduced pressure and the residues partitioned between ethylacetate–$HCl_{(aq.)}$ (1 : 1, 60 mL). The aqueous phase was extracted with ethylacetate (3 × 10 mL) and the combined organic phases dried and concentrated as before. The product (**12**) was isolated as clear colourless oil and required no further purification (34 mg, 98% (last step only)). ^{1}H NMR (400 MHz, D_2O) δ 2.37 (2H, t, J = 7.5, CH_2CO$_2$H), 2.20 (2H, dt, J = 2.6, 7.1 Hz, CH_2C≡CH), 1.96 (1H, t, J = 2.6 Hz, C≡CH), 1.66 (2H, qi, J = 7.2), 1.54 (2H, qi, J = 7.2),1.47–1.22 (20H, m); ^{13}C NMR (100 MHz, D_2O) δ 178.5 (C=O), 77.2 (C≡CH), 68.0 (C≡CH), 33.7 ($HO_2C(CH_2)_{14}$C≡CH), 29.6, 29.6, 29.5, 29.4, 29.2, 29.1, 29.1, 28.8, 28.5, 24.7, 18.4; HRMS *m*/*z* (ESI), calculated for $C_{17}H_{29}O_2$ ($[M - H]^-$) 265.2168, found 265.2170.

2.1.10. Methyl 2-(3-oxo-6-(prop-2-ynyloxy)xanthen-9-yl)-benzoate (13).

Prepared according to the method of Hvilsted *et al.*[2] ^{1}H NMR (400 MHz, MeOD) δ 8.28 (1H, dd, J = 1.3, 7.8, Ar-H), 7.77 (1H, dt, J = 1.4, 7.5, Ar-H), 7.70 (1H, dt, J = 1.4, 7.5, Ar-H), 7.34 (1H, dd, J = 1.2, 7.4, Ar-H), 7.10 (1H, d, J = 2.5, Ar-H), 6.93 (1H, d, J = 8.9, Ar-H), 6.88 (1H, d, J = 9.7, Ar-H), 6.83 (1H, dd, J = 2.5, 8.9, Ar-H), 6.57 (1H, dd, J = 1.9, 9.7, Ar-H), 6.50 (1H, d, J = 1.9, Ar-H), 4.83 (2H, d, J = 2.4, CH_2C≡CH), 3.67 (3H, s, OCH_3), 2.64 (1H, t, J = 2.4, C≡CH); HRMS *m*/*z* (ESI), calculated for $C_{24}H_{16}O_5$ ($[M + H]^+$) 385.1076, found 385.1095.

2.2. Biochemistry

2.2.1. Media, buffers and solutions

Cell culture reagents; media, buffers, fetal calf serum (FCS), sodium pyruvate and trypsin, were from PAA Laboratories, Germany. Water used was deionized to 18 MΩ and sterilized by UV irradiation (Millipore™ Milli-Q® system). LB medium contained tryptone 10 g/L, yeast extract 5 g/L and NaCl 5 g/L. Transformation solution I contained 80 mM $CaCl_2$ and 50 mM $MgCl_2$. Transformation solution II contained 75 mM $CaCl_2$ and 25% glycerol. TE buffer contained 10 mM Tris (pH 8.0) and 1 mM EDTA. TBE buffer contained 89 mM Tris (pH 8.0), 89 mM boric acid and 2 mM EDTA. 10 × PBS contained KH_2PO_4 2 g/L, KCl 2 g/L, NaCl 80 g/L and Na_2HPO_4 11.5 g/L, pH 7.2–7.4. PBS-T contained 1 × PBS and 0.05% Tween®-20, TBS-T contained 1 × TBS and 0.1% Tween®-20. Antibiotic solutions were prepared at 1000-fold stock concentrations and sterile filtered. Ampicillin (100 mg/mL) was prepared in water, spectinomycin (100 mg/mL) was prepared in water–DMSO 1:1, and chloramphenicol (30 mg/mL) was prepared in ethanol. Click-chemistry lysis buffer contained 1 × PBS, 0.1% SDS, 1% Triton® X-100, 1 × EDTA-free Complete protease inhibitor (Roche Diagnostics). IP lysis buffer contains 50 mM Tris HCl (pH 8.0), 150 mM NaCl and 1% Triton® X-100. IP low-salt buffer contains 20 mM Tris (pH 7.5).

2.2.2. Plasmids and bacterial strains.

pENTR223.1-hSHH (DNAFORM) was propagated in *E. coli* using spectinomycin (100 μg/mL) for selection. pcDNA-DEST40 (Invitrogen) was propagated in the *E. coli* strain OneShot ccdB Survival 2 T1 (Invitrogen) using chloramphenicol (30 μg/mL) for selection. pcDNA-DEST40-hShh was propagated in the *E. coli* strain DH5α using ampicillin (100 μg/mL) for selection.

2.2.3. Expression vector cloning

Expression vector pcDNA-DEST40-hSHH was made using Gateway cloning (Invitrogen) based on recombination between an entry vector and a destination vector. 150 ng of the entry vector ENTR223.1-hShh were mixed with 150 ng of the destination vector pcDNA-DEST4 in TE buffer to a total volume of 8 μL. 2 μL of the LR Clonase II enzyme mix were added and the recombination was allowed to take place for 1.5 h at 25 °C. To end the reaction, 2 μL of Proteinase K solution were added for 10 min at 37 °C. 2 μL were used to transform *E. coli* DH5α cells. Transformants were selected on LB agar and ampicillin (100 μg/mL) plates. Recombined clones were further negatively selected on LB agar plates with chloramphenicol (30 μg/mL). A colony that was ampicillin resistant but chloramphenicol sensitive was used for plasmid purification. The plasmid was sent for sequencing to confirm correct gene insertion. All plasmids were sent to MWG Eurofins (UK) for sequencing.

2.2.4. DNA plasmid preparation

Cloning plasmid pcDNA-DEST40-hSHH for mammalian expression was purified using the EndoFree Plasmid Maxi kit (Qiagen). DNA concentration and purity were determined by measuring absorbance at 260 nm and 280 nm. Purity was assessed by the A260/A280 ratio where ratios of 1.8–2.0 were considered free from contaminants.

2.2.5. Preparation of competent cells

A single colony of *E. coli* cells was inoculated in 6 mL LB medium for growth overnight at 37 °C. 5 mL were used to scale up the culture to 100 mL. The cells were grown until the optical density at 600 nm (OD_{600}) reached 0.9 AU. The culture was cooled on ice and cells were pelleted by centrifugation (2000 rpm, 4 °C, 20 min). The supernatant was discarded and the cell pellet resuspended in 20 mL ice-cold transformation solution I and incubated on ice for 15 min. The cells were pelleted, resuspended with 10 mL ice-cold transformation solution I and incubated on ice for 20 min. After a final centrifugation, cells were resuspended in pre-chilled transformation solution II, aliquoted into pre-chilled tubes, flash-frozen in dry ice–ethanol and stored at –80 °C.

2.2.6. Transformation of bacteria

50 μL chemo-competent *E. coli* cells thawed on ice were mixed with 2 μL plasmid DNA and incubated on ice for 15 min. The mixture was heat-shocked at 42 °C for 45 sec and returned to ice for 2 min. 250 μL pre-heated SOC medium (Invitrogen) were added to the cells, which were incubated at 37 °C for 1 h. Finally, 200 μL of the culture were plated on 9 cm selective LB-agar plates and incubated at 37 °C overnight.

2.3. Cell Culture

2.3.1. Cell lines

Cell lines HEK293a (transfected and untransfected), A549 and PANC1 were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin. All cell lines were maintained at 37 °C and 5% CO_2.

2.3.2. Transfection of HEK293a

HEK293a cells were transfected with pcDNA-DEST40-hShh using Lipofectamine™ 2000 transfection reagent (Invitrogen). Transfection was typically carried out in a 6-well plate. 8×10^5 cells were seeded in antibiotic-free medium. 24 h later, the medium was replaced with fresh medium followed by addition of the transfection mixture. The transfection mixture contained 5 µL Lipofectamine™ 2000, 2 µg DNA in 200 µL Opti-MEM® medium (Gibco) and was prepared according to manufacturer's instructions.

2.3.3. Cell lysis

For harvesting, cells were rinsed with ice-cold PBS, incubated in 400–500 µL ice-cold IP or click-chemistry lysis buffer and incubated on ice for 15 minutes. After lysis, cells were scraped off the surface using a rubber cell scraper. DNA was sheared by passing the lysate multiple times though 0.8 mm and 0.5 mm syringe needles. The lysate was centrifuged at 16,000 × g for 10 min to remove insoluble material. The supernatant was used for further experiments or stored at –80 °C.

2.4. Protein content assays

Total protein content in cell lysates was determined using *DC* Protein Assay (Bio-Rad). BSA dissolved in sample lysis buffer was used as standard in all measurements (0.2–3.0 mg/mL). Samples were prepared in duplicates and at different dilutions in microtiter plates. Absorbance was measured at 750 nm.

2.5. SDS-PAGE

Proteins were separated by SDS-PAGE using 12% Bis-Tris gels and NuPAGE® MES SDS running buffer from Invitrogen (Tris, SDS, 4-morpholinoethane sulfonic acid).samples were prepared by boiling for 5 min in NuPAGE® LDS 4 × sample loading buffer (141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5) containing 20% 2-mercaptoethanol. Seeblue® Pre-Stained standard (Invitrogen), biotinylated protein ladder (Cell Signalling Technology) and Amersham ECL™ Plex™ Fluorescent Rainbow Markers (G. E. Healthcare) were used where appropriate to allow molecular weight comparison. Gels were run using the Mini-PROTEAN® Tetra Cell system (Bio-Rad) and a Bio-Rad power supply unit at 180–200 V and typically took 40 min to run.

2.6. Western blotting

Proteins were transferred from SDS-PAGE gels to a nitrocellulose membrane (Hybond™-ECL™, 0.45 µM, G. E. Healthcare) using a Novex® Semi-Dry Blotter (Invitrogen). Tris-glycine transfer buffer (NuPAGE, Invitrogen) was used to soak the blotting paper and membrane prior to transfer. Transfer was carried out at 20 V for 27 min. Membranes were blocked for 1 hour at room temperature (or overnight at 4 °C) with BSA (5% w/v in 10 mL TBS-T) and washed with TBS-T (3 × 10 mL for 5 min). For Shh detection, primary antibody (1:200 in 10 mL TBS-T, Shh (H-160) rabbit polyclonal IgG, 200 µg/ml, Insight Biotechnology) was applied, followed by washing (3 × TBS-T). Secondary conjugation (1:10,000 in 10 mL TBS-T, goat anti-rabbit IgG (HRP), Insight Biotechnology) was followed by washing (3 × TBS-T). Visualisation was carried out using the enhanced chemiluminescence (ECL) kit according to the manufactures instructions and on a Fujifilm LAS 3000 imager.

2.7. Cell labeling with lipid analogues and label detection

All labeling using bioorthogonal labels was carried out on recombinantly expressed Shh in HEK293a cells, or natively expressed Shh in HEK293a, A549 or PANC1 cells.

2.8. Palmitate analogue labeling

To label overexpressed Shh (along with any other natively expressed palmitoylated entities) with the palmitate analogue, cells were transfected with Shh expression vector and allowed to express for 24 h prior to changing medium to labeling medium (DMEM, 5% FCS, 1 mM sodium pyruvate and the specified concentration of palmitate analogue **12**). Cells were harvested after 16 hours. This protocol was optimized by changing to labeling medium immediately before transfection and harvesting the cells after 24 hours.

2.9. Cholesterol analogue labeling

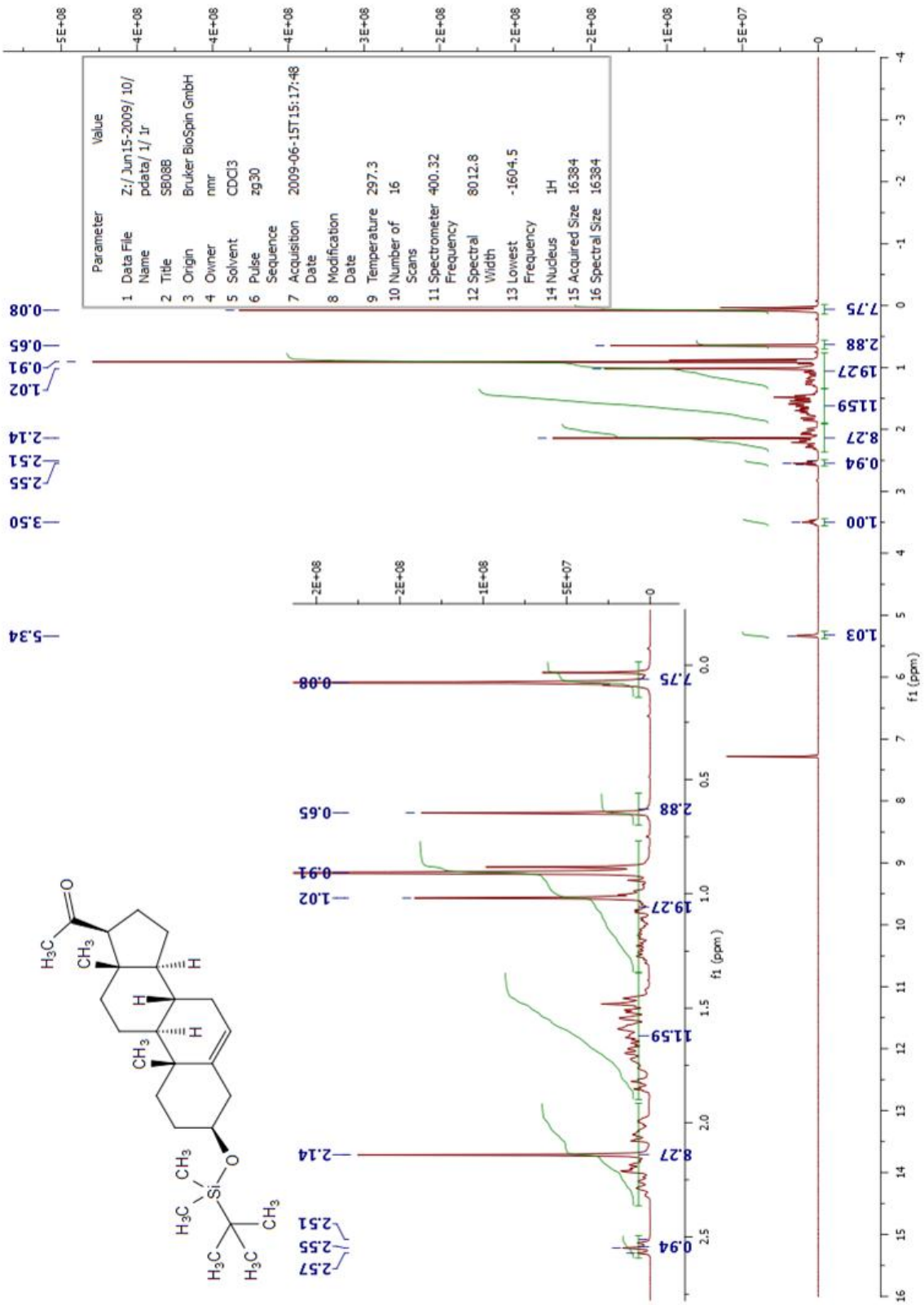
To label Shh (along with any other natively expressed cholesterolated entities) with the cholesterol analogue, cells were transfected with the Shh expression vector. After 6 hours, the medium was exchanged for low-serum medium (± mevastatin) (DMEM, 1% FCS). After an additional 2 h, medium was exchanged again for labeling medium (DMEM, 1% FCS, and a specific concentration of **8**). Cells were harvested and lysed after 16 h in labeling medium.

2.10. Bio-orthogonal ligation reaction

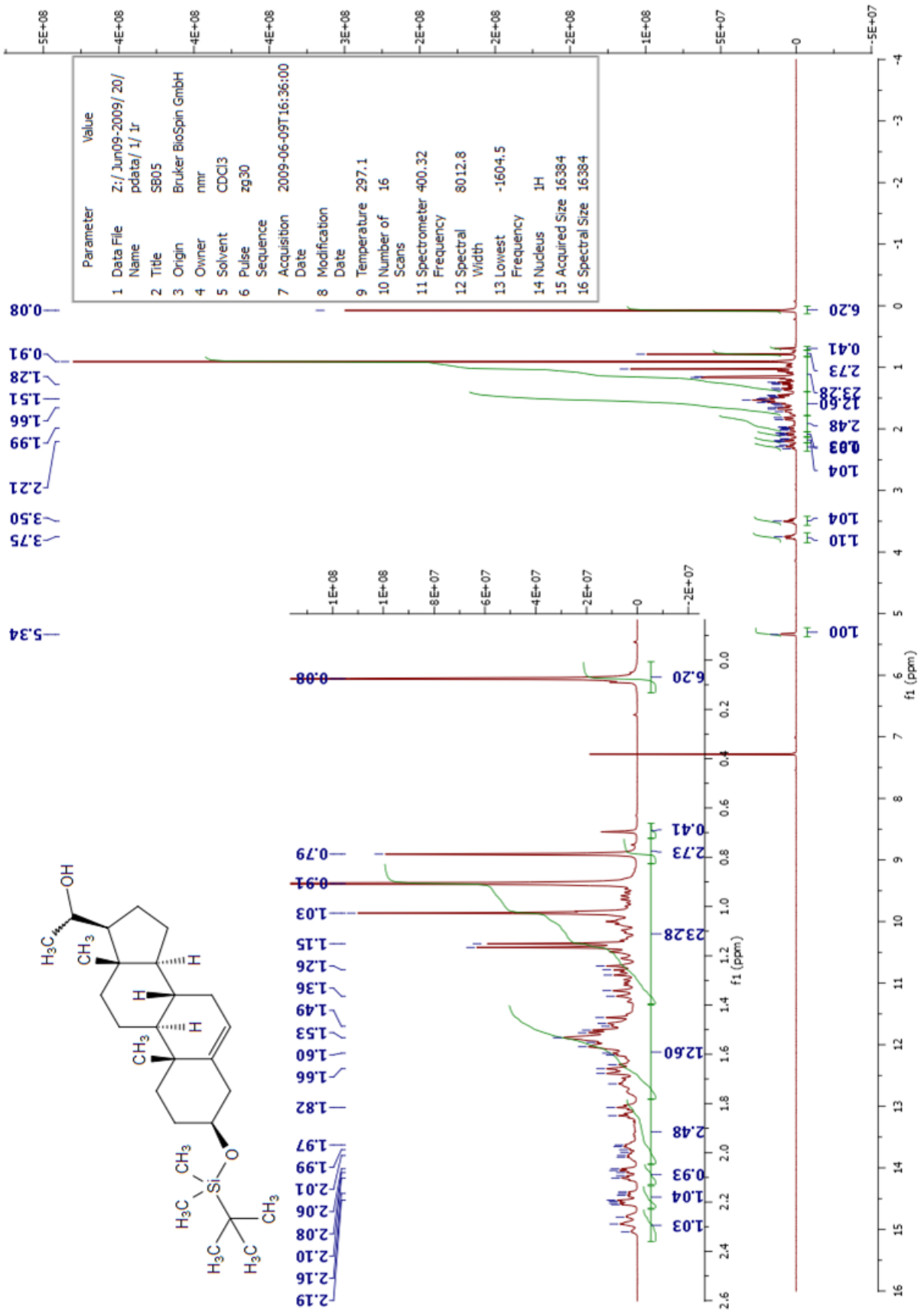
Capture chemistry with cell lysates using 1 mg/mL of total protein in the final reaction volume (typically 100 uL, lysate volume adjusted with lysis buffer as needed). **9** or **10** (10 mM stock in DMSO) at 50 μM final, $CuSO_4$ (40 mM stock in H_2O) at 500 μM final, TCEP (50 mM stock in H_2O) at 500 μM final and TBTA (10 mM stock in DMSO) at 50 μM final. The Capture reagent (**9** or **10**), $CuSO_4$, TCEP and TBTA stocks were pre-mixed in the appropriate quantities and aliquoted into each click reaction as required. The reactions were vortexed for 1 hour at room temperature, the protein precipitated by addition of 10 vols ice cold methanol, frozen at –80 °C overnight, pelleting, and washing with ice cold methanol a further two times. Precipitated proteins were either resuspended in NuPage LDS sample buffer (Invitrogen) with 20% *β*-mercaptoethanol) and 10 μL were loaded onto an SDS-PAGE gel for analysis, or pull-down of biotinylated proteins was carried out using beads (Streptavidin-coupled Dynabeads from Invitrogen). Fluorescently-tagged protein was detected by fluorescence imaging (540 nm excitation and 595 nm emission) using an Ettan DIGE Imager (GE Healthcare) and by Western blotting against Shh as described (*vida supra*).

3. ^{1}H NMR Spectra of compounds.

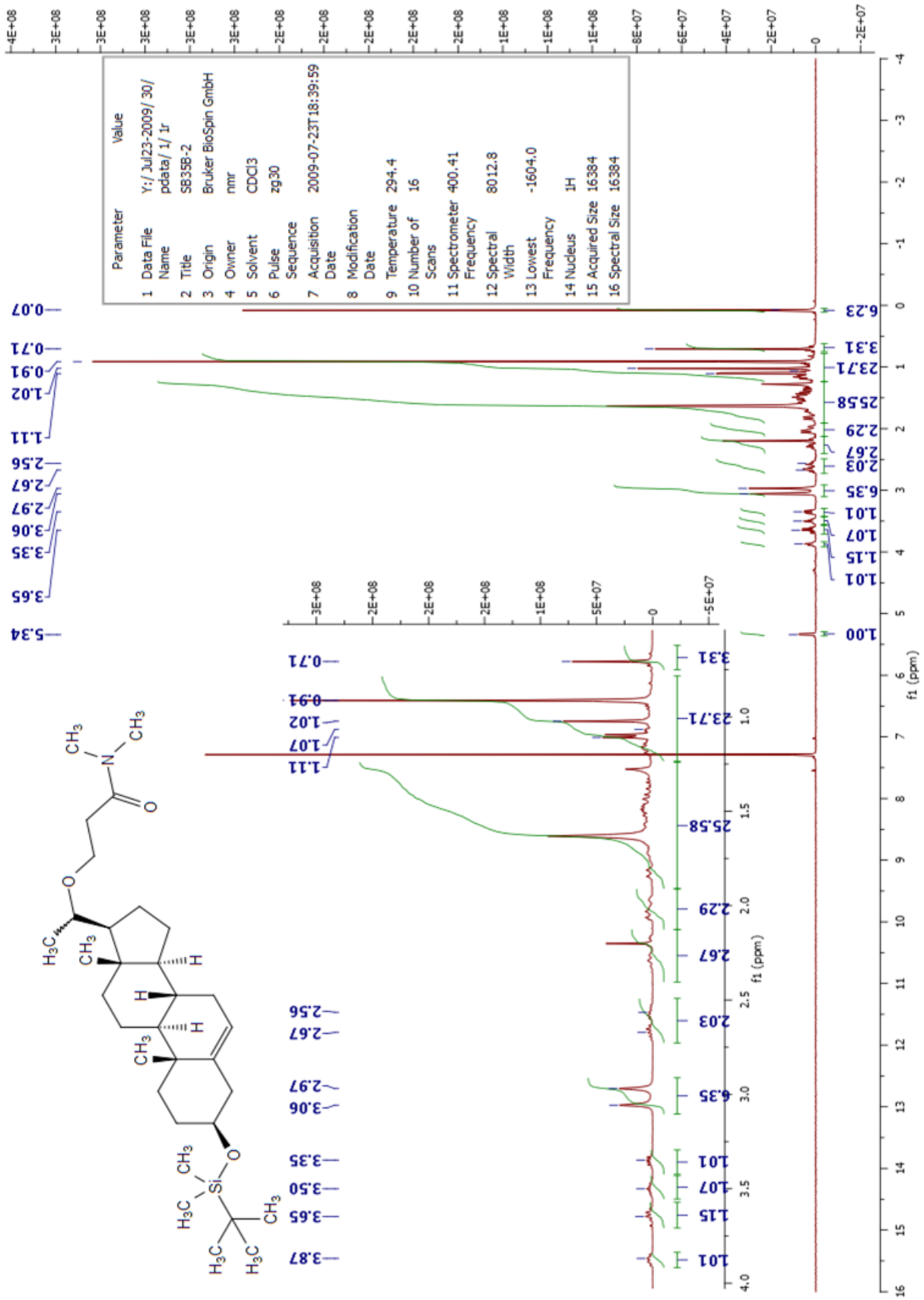
3.1. (3S)-3-tert-Butyldimethylsilyloxypregn-5-en-20-one (2).



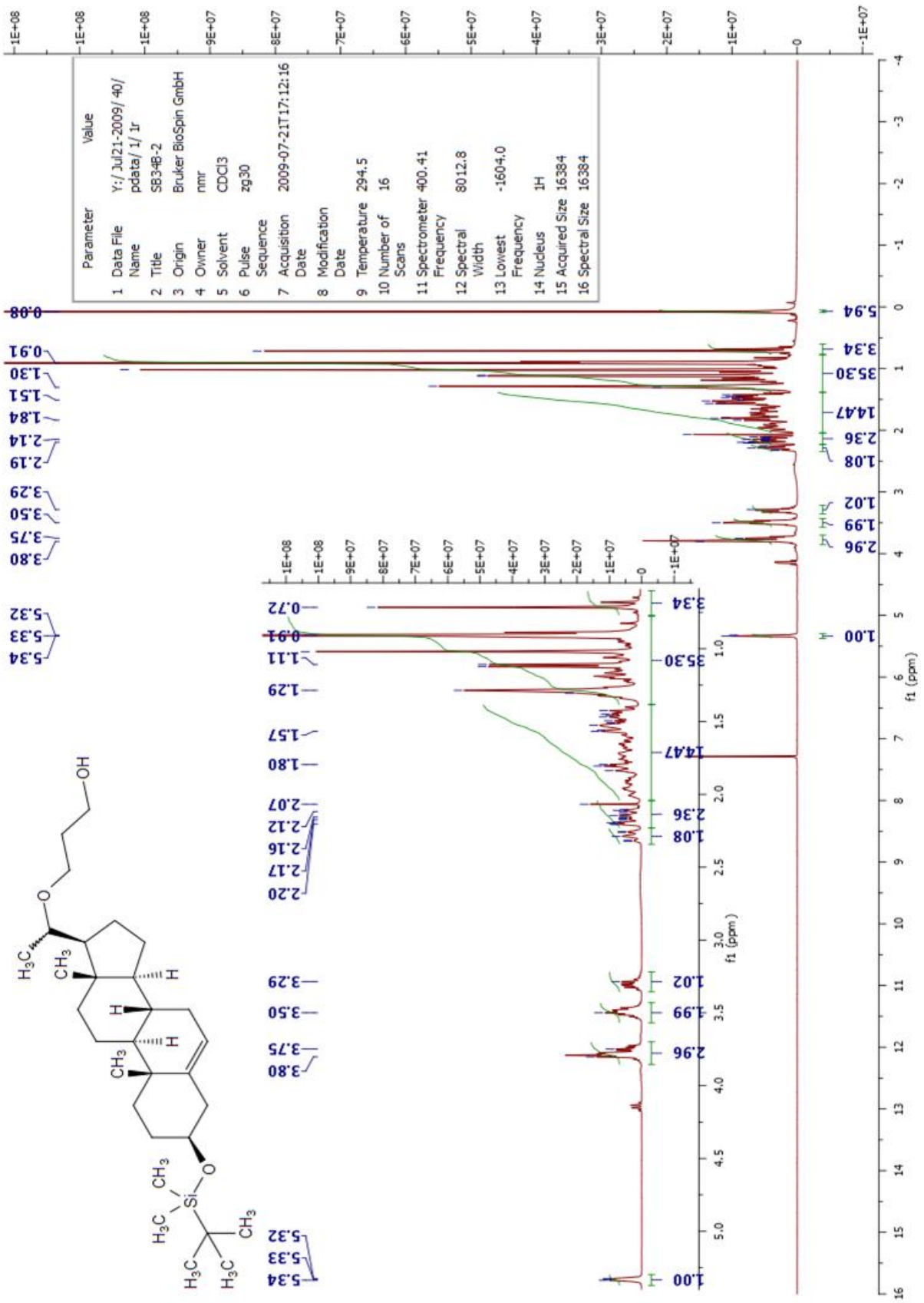
3.2. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-hydroxypregn-5-ene (3).



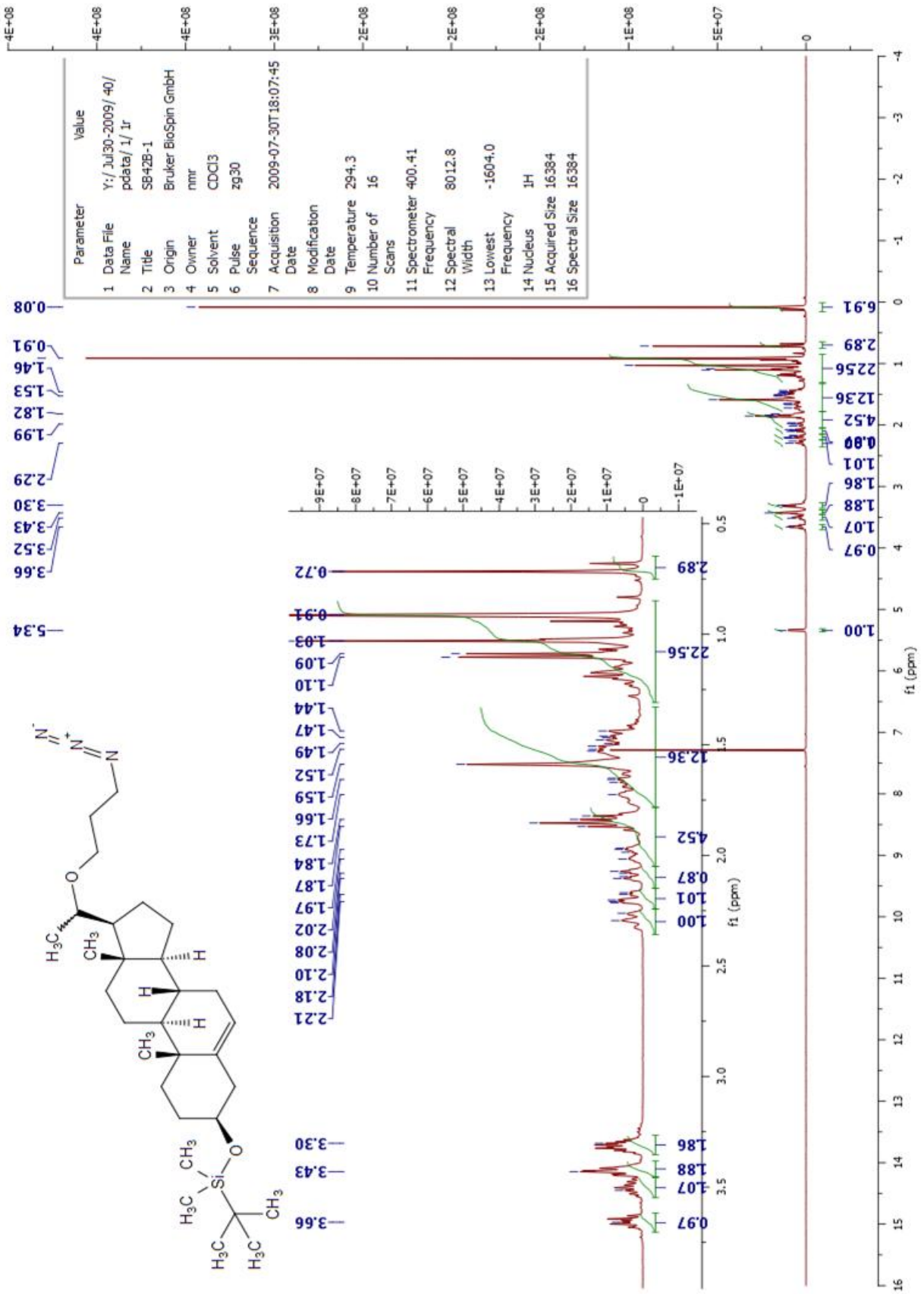
3.3. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-*N*,*N*-dimethyl-amidoxapropyloxy)pregn-5-ene (4).



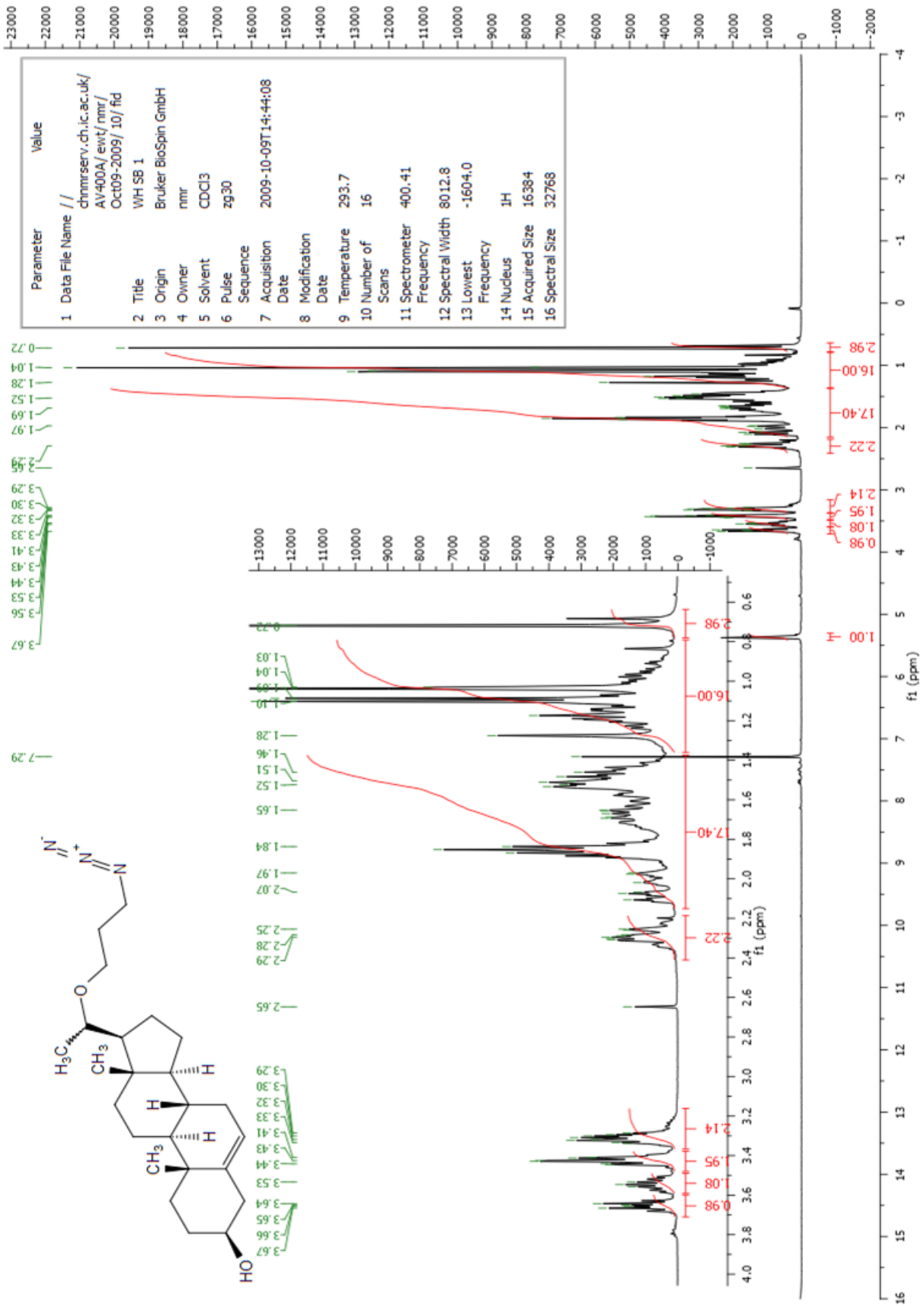
3.4. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-hydroxypropyl-oxy)-pregn-5-ene (5).



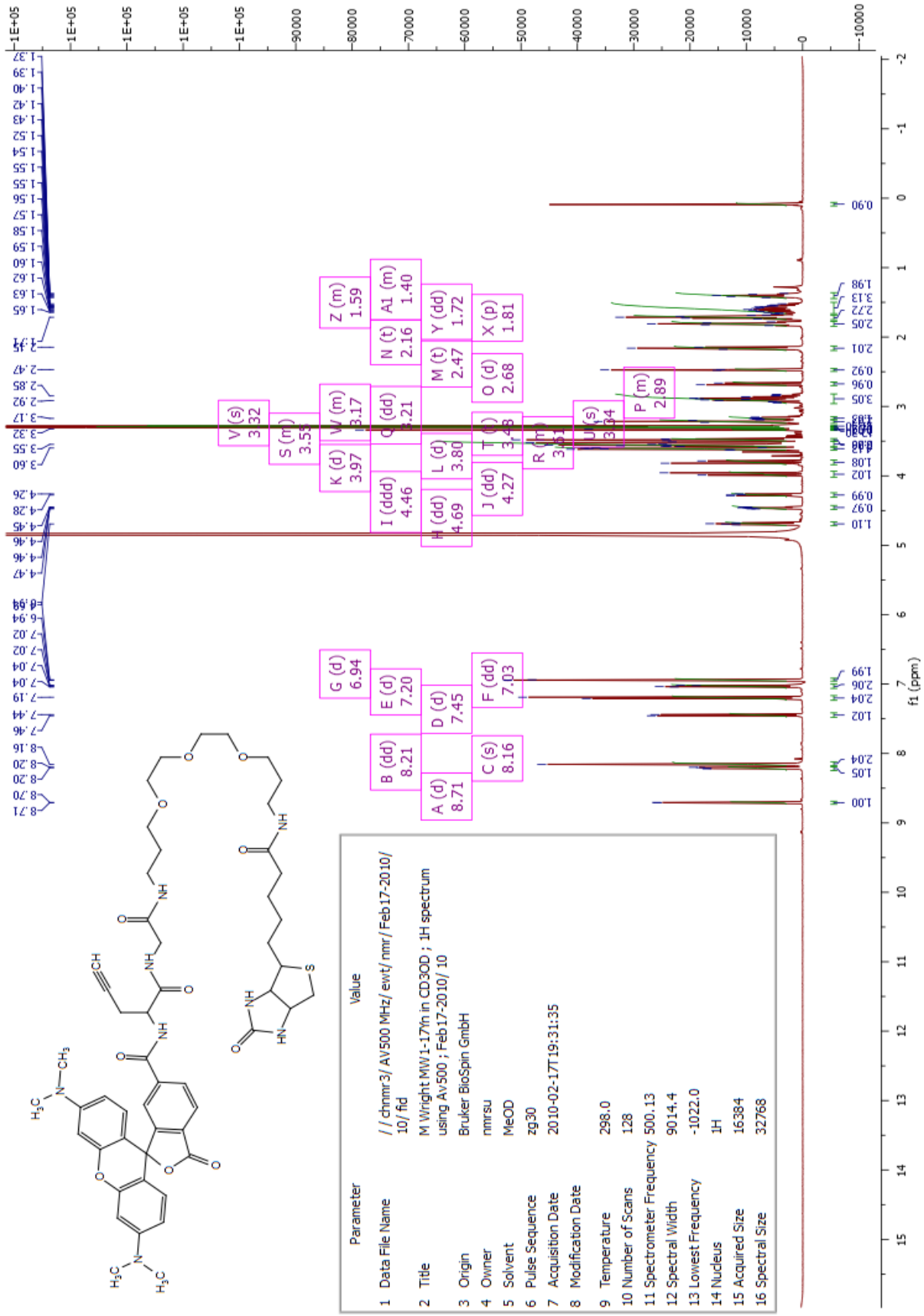
3.5. (3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-azidopropyl-oxy)-pregn-5-ene (7).



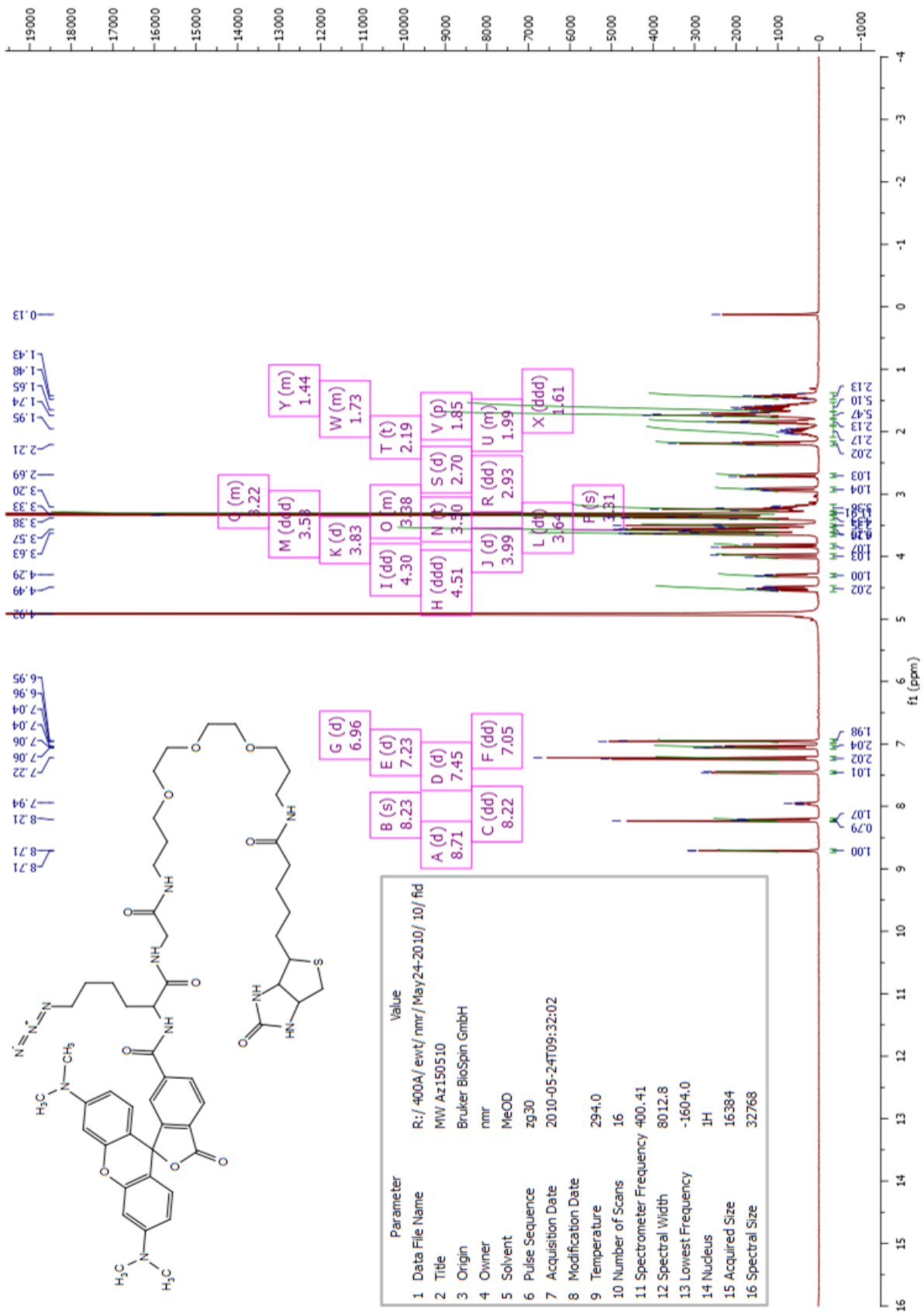
3.6. (3S)-20-(3-azidopropyloxy)pregn-5-en-3-ol (8).



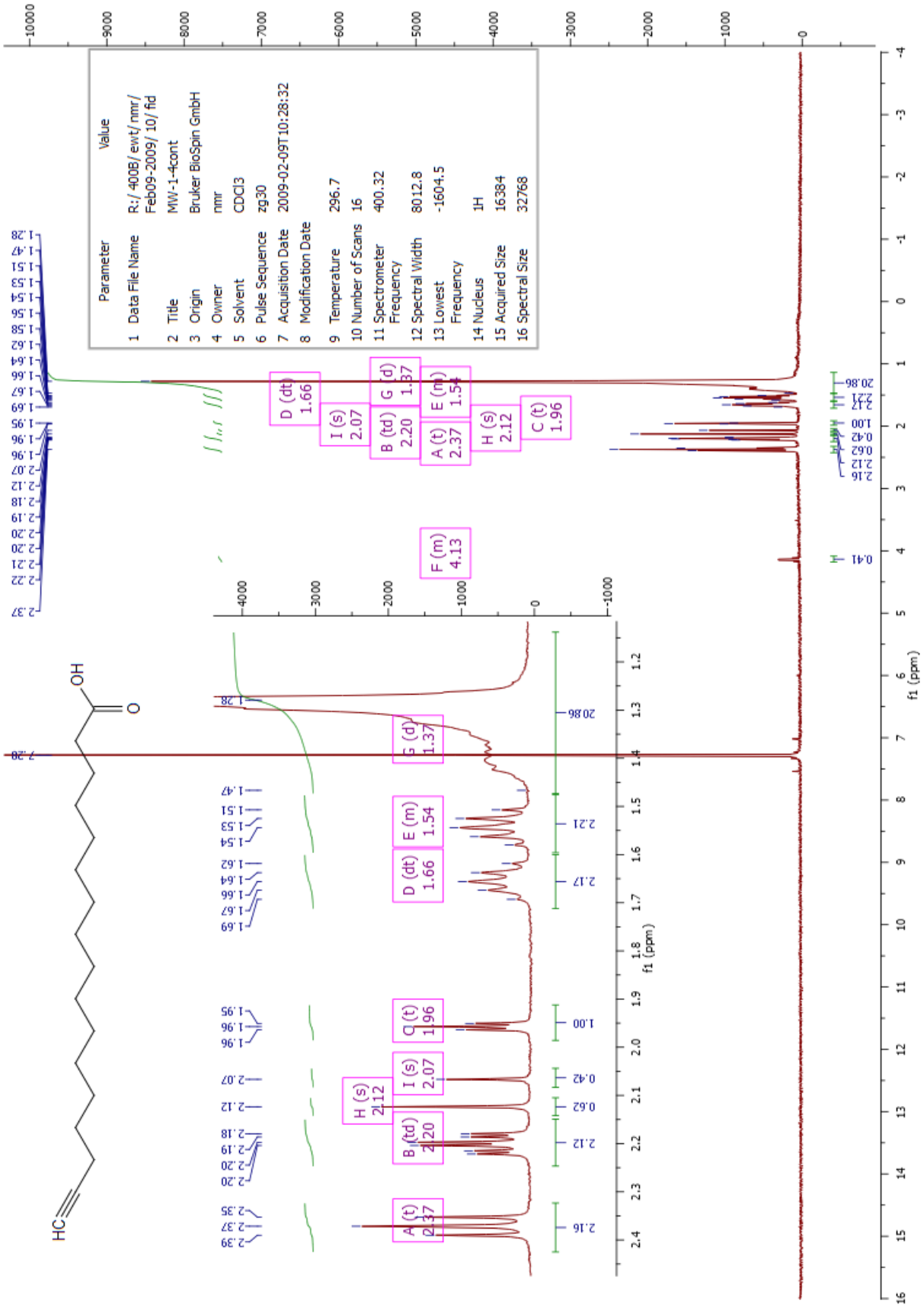
3.7. Alkynyl trifunctional capture reagent (10).



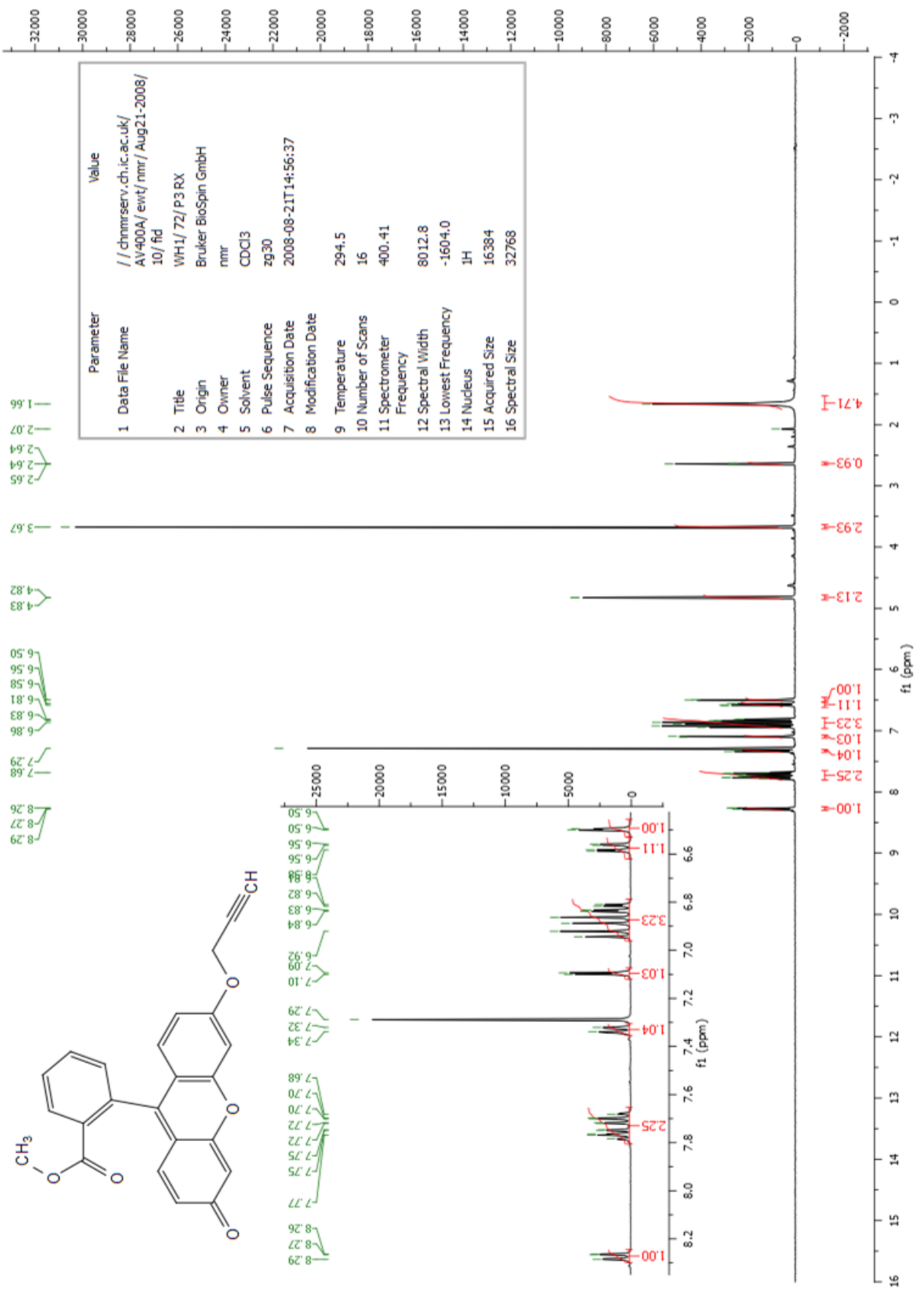
3.8. Azido trifunctional capture reagent (11).



3.9. Hexadec-15-ynoic acid (21).



3.10. Methyl 2-(3-oxo-6-(prop-2-ynyloxy)xanthen-9-yl)-benz-oate (13).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4 Owner
nmr
5 Solvent
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6 Pulse Sequence
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9 Temperature
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13 Lowest Frequency
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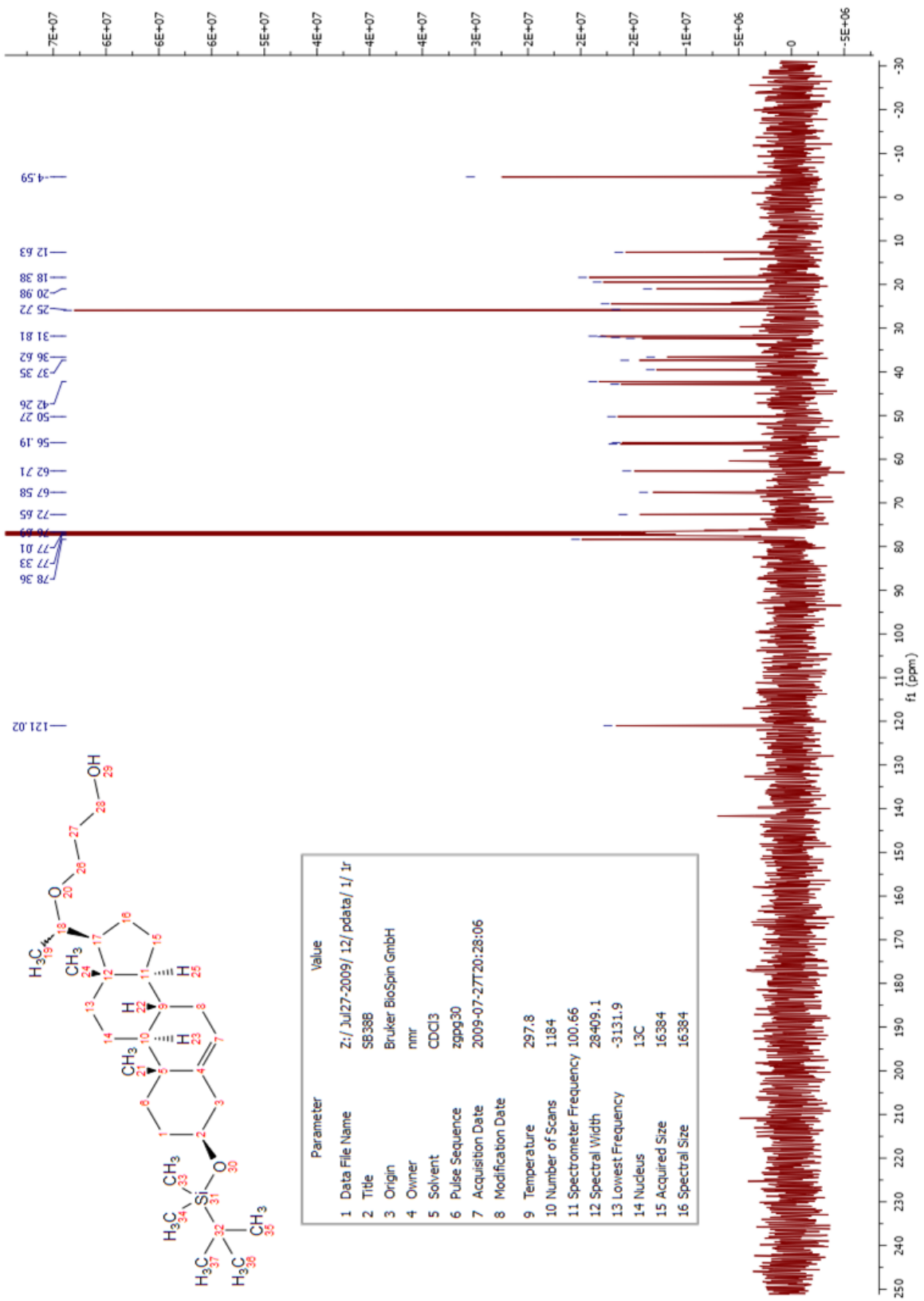
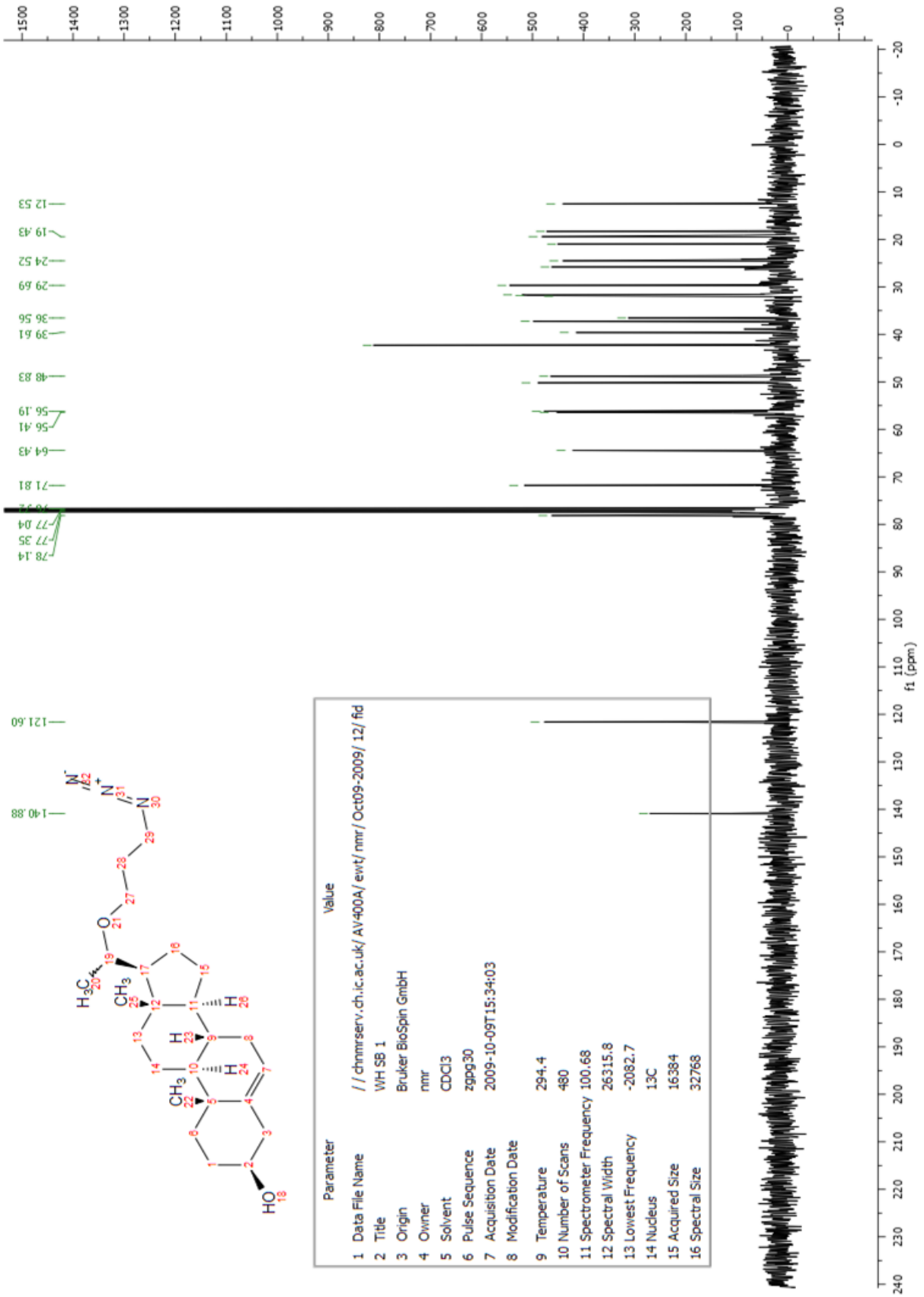
14 Nucleus
1H
15 Acquired Size
16384
16 Spectral Size
32768
f1 (ppm)
CH3
O
CH

4. ^{13}C NMR Spectra.

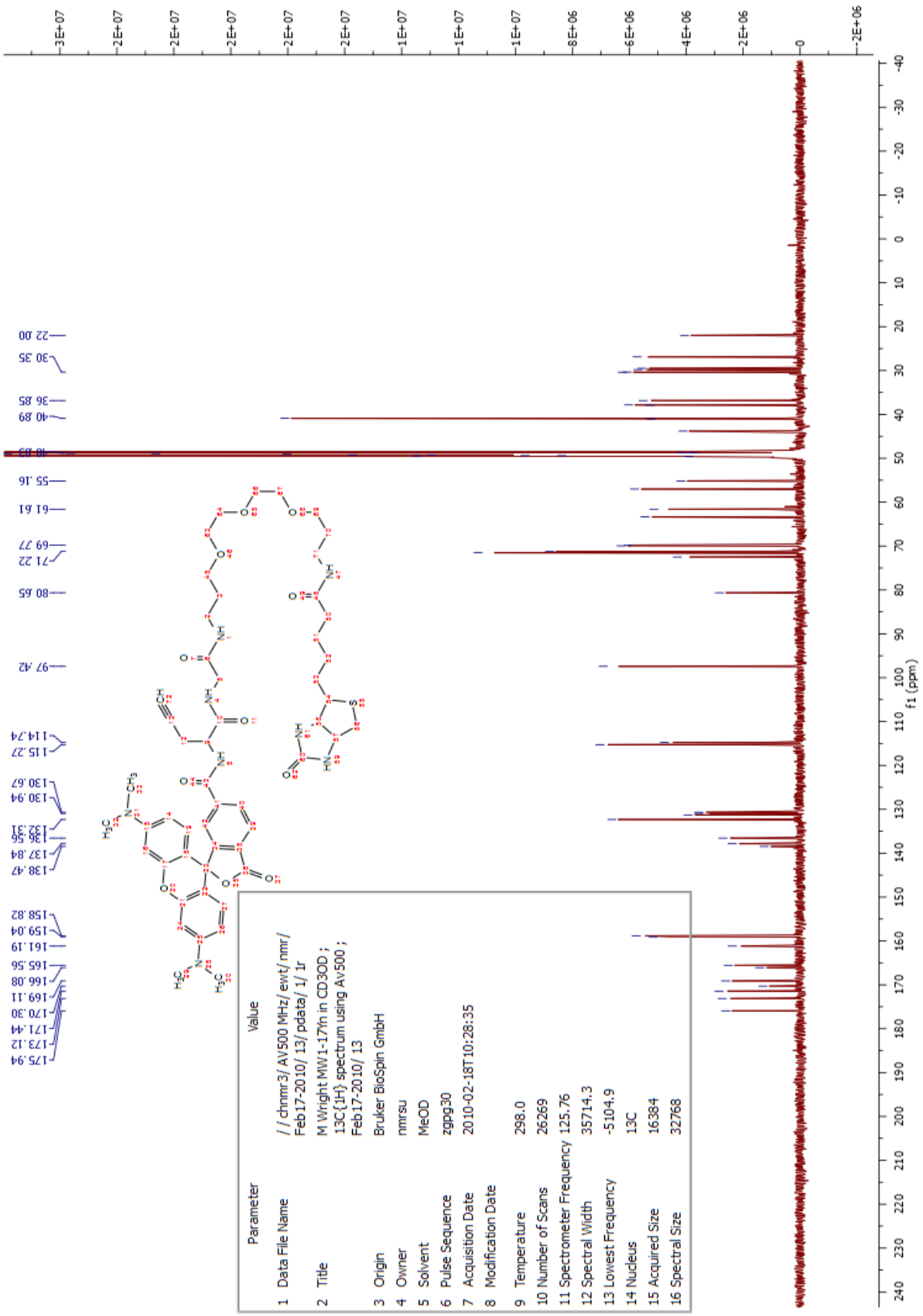
4.1. 3*S*)-3-*tert*-Butyldimethylsilyloxy-20-(3-hydroxypropyl-oxy)-pregn-5-ene (5).



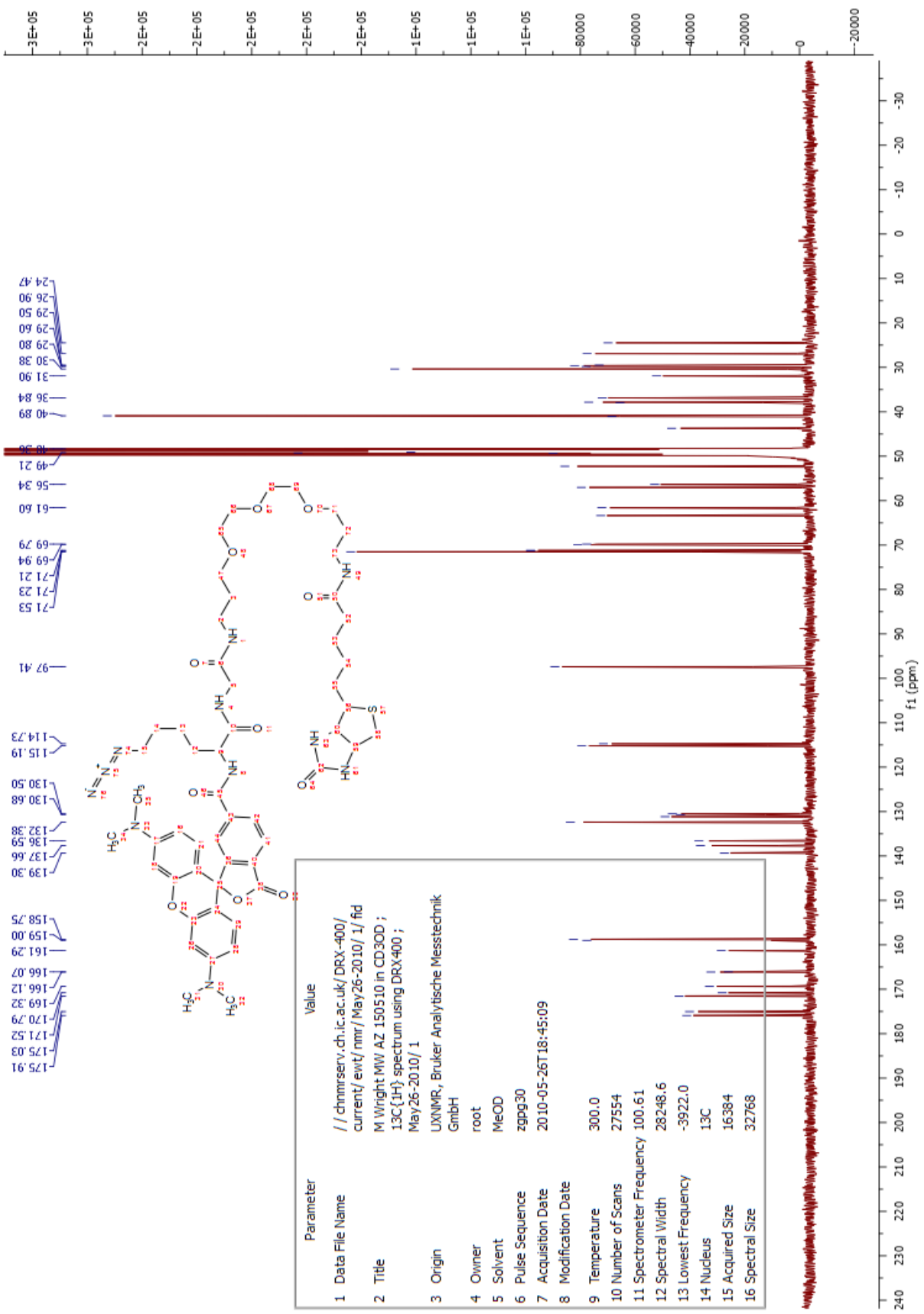
4.2. (3S)-20-(3-azidopropyloxy)pregn-5-en-3-ol (8).



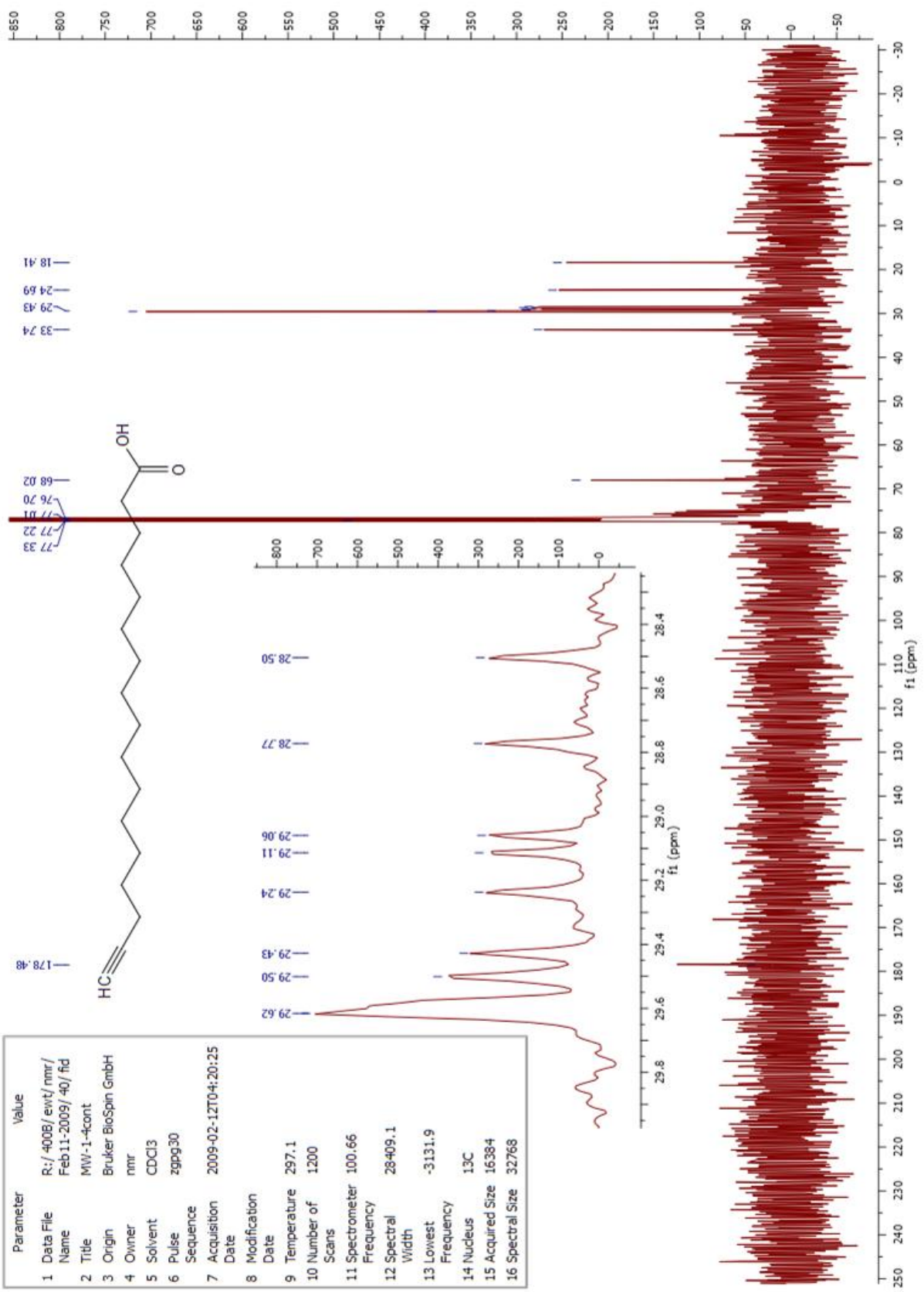
4.3. Alkynyl trifunctional capture reagent (10).



4.4. Azido trifunctional capture reagent (11).

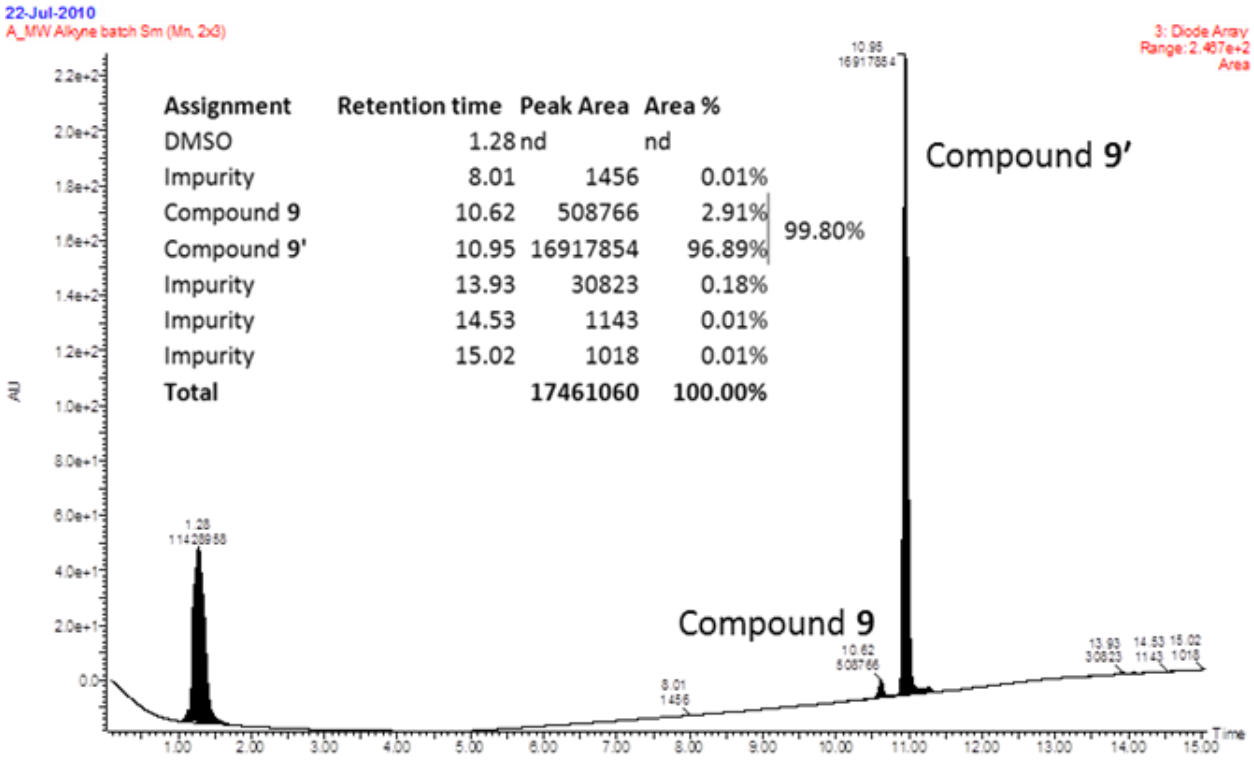


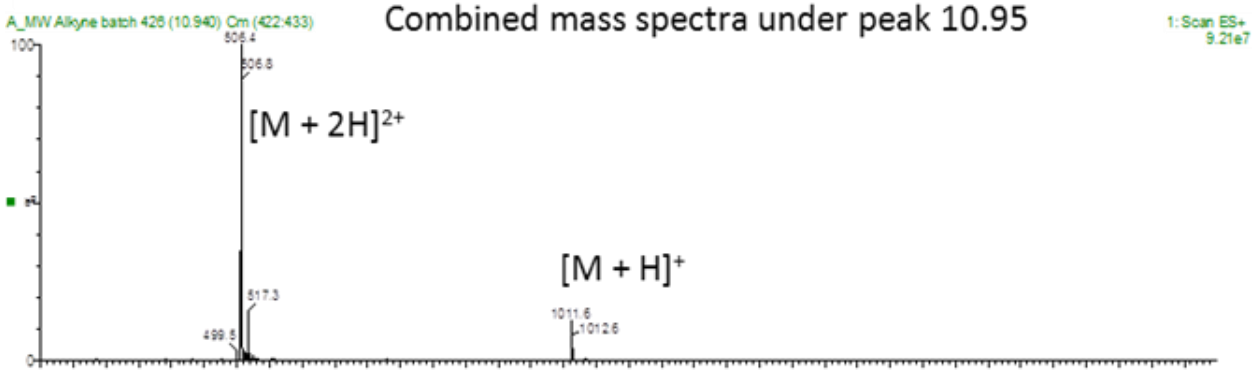
4.5. Hexadec-15-ynoic acid (12).

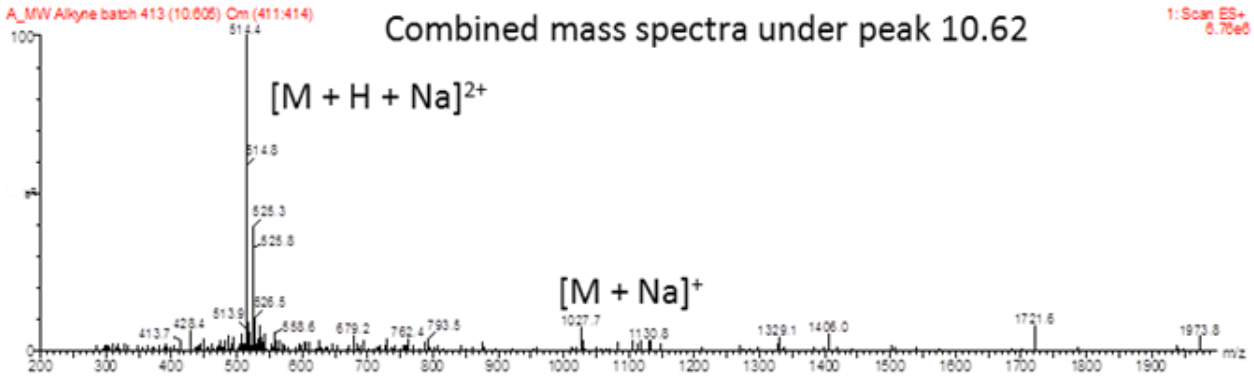


5. LC-MS Spectra.

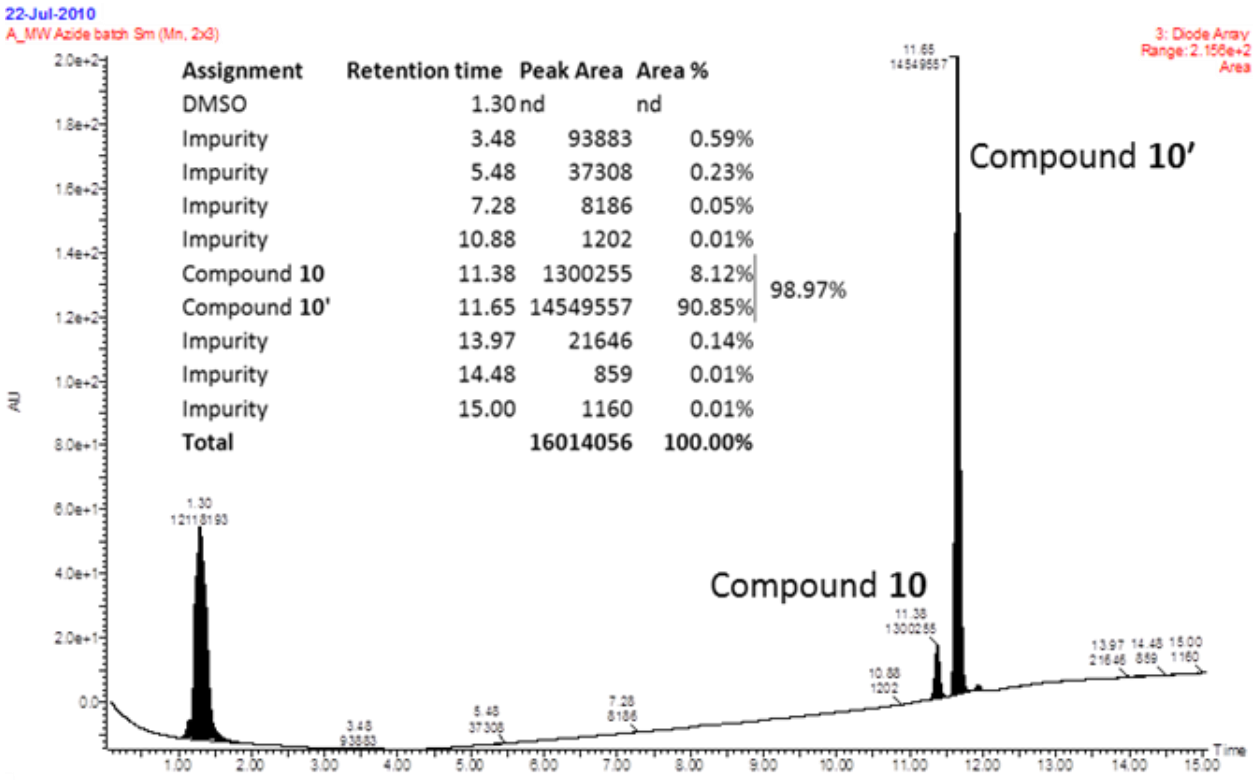
5.1. Trifunctional Capture Reagents 9 and 9' (5 and 6 isomers of TAMRA).



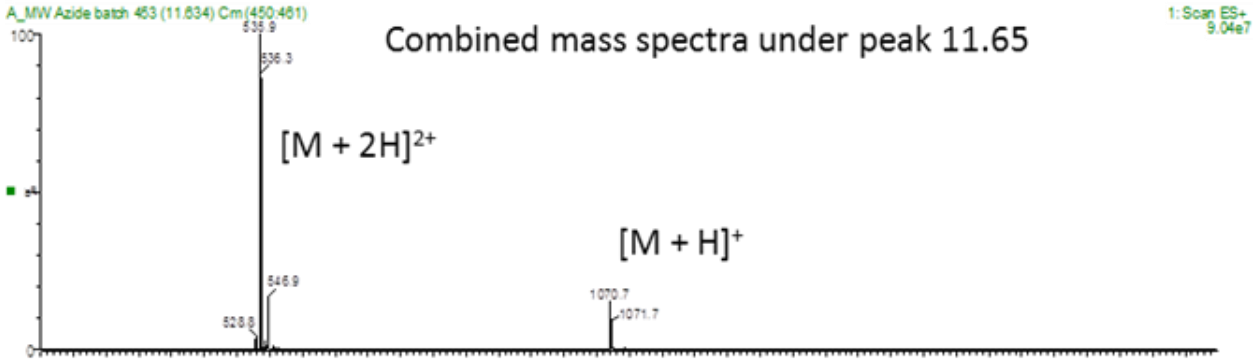


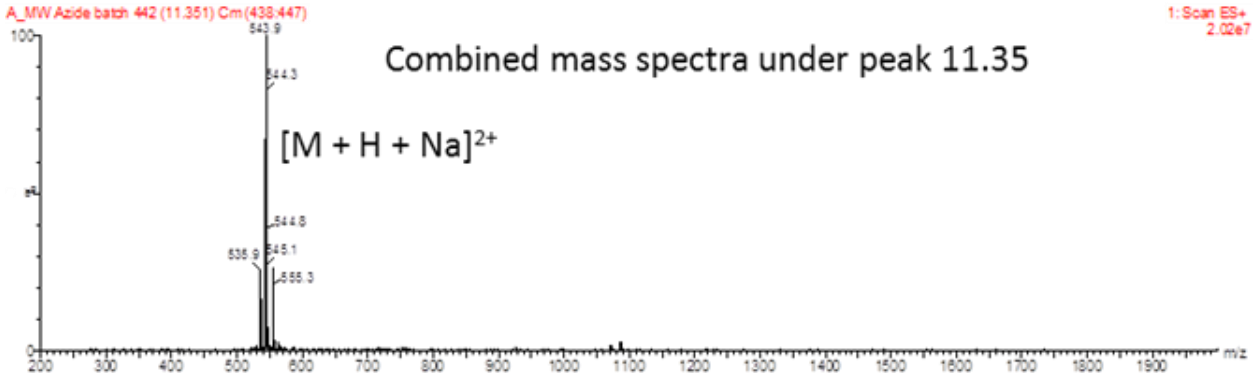


5.2. Trifunctional Capture Reagents 10 and 10' (5 and 6 isomers of TAMRA).



Assignment	Retention time	Peak Area	Area %	
DMSO	1.30	nd	nd	
Impurity	3.48	93883	0.59%	
Impurity	5.48	37308	0.23%	
Impurity	7.28	8186	0.05%	
Impurity	10.88	1202	0.01%	
Compound **10**	11.38	1300255	8.12%	98.97%
Compound **10'**	11.65	14549557	90.85%	
Impurity	13.97	21646	0.14%	
Impurity	14.48	859	0.01%	
Impurity	15.00	1160	0.01%	
Total		**16014056**	**100.00%**	





6. References.

(1) Heal, W. P.; Wickramasinghe, S. R.; Leatherbarrow, R. J.; Tate, E. W. *Org. Biomol. Chem.* **2008**, *6*, 2308-2315.
(2) Daugaard, A. E.; Hvilsted, S.; Hansen, T. S.; Larsen, N. B. *Macromolecules* **2008**, *41*, 4321-4327.