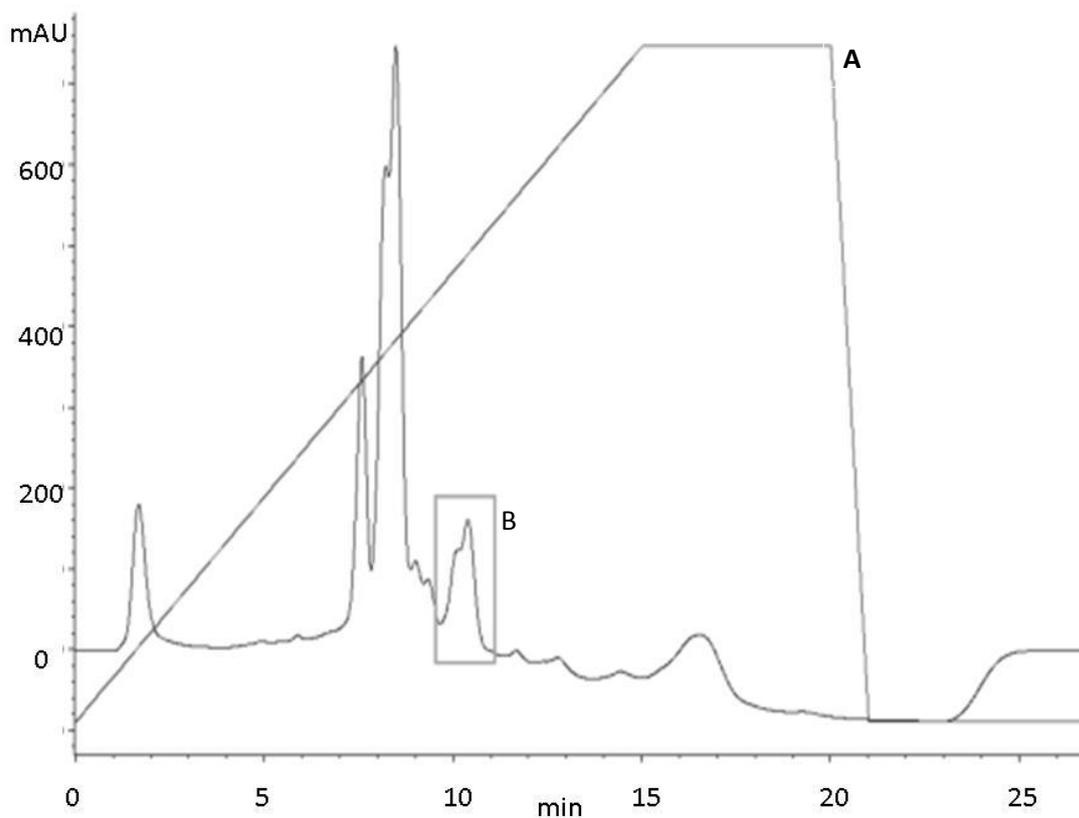
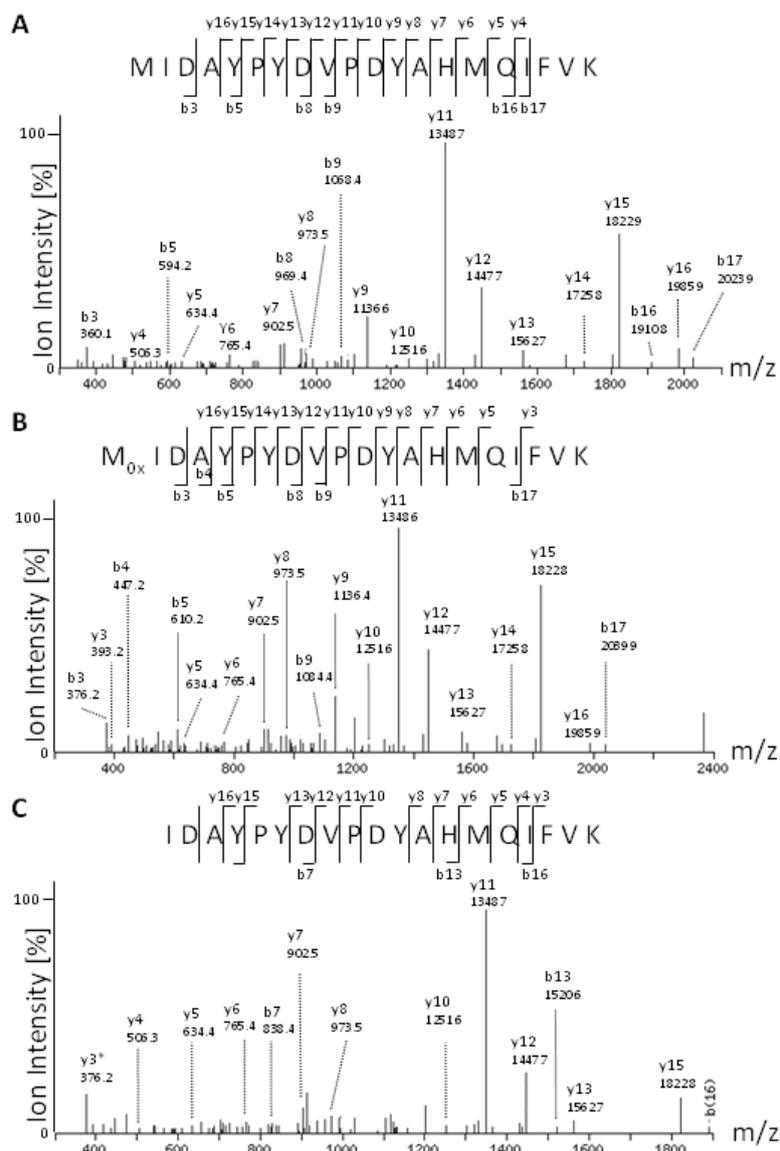


## SUPPORTING INFORMATION



**Supplementary Figure 1:** HPLC purification of HA-Ub-Alkyne probe **2**. Alkyne probe **2** was purified to >95% purity using a SCX biomonomolith column (5.2 x 4.95 mm, Agilent) with a linear gradient (line A) from 0% to 100% B, 50 mM NaOAc (pH 4.5, buffer A), 50 mM NaOAc (pH 4.5) and 1 M NaCl (buffer B) at a flow rate of 0.5ml/min with subsequent washing and re-equilibration steps. The highlighted area (box B) was collected and determined by MALDI analysis to be Alkyne probe **2**.

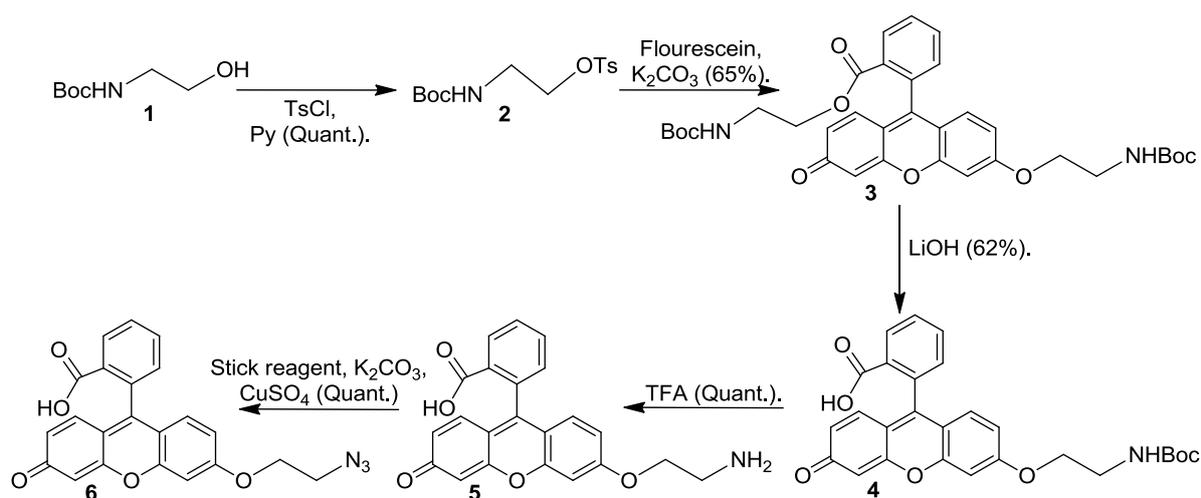


**Supplementary Figure 2:** Characterisation of different HA-Ub-alkyne probe **2** variants. **(A)** MS/MS spectrum of the N-terminal HA-Ub tryptic peptide 1-20 (Ub 1-6) with a precursor ion mass of 1208.7  $[M+2H]^{2+}$  (Mr 2415.1 Da) derived from probe **2** with an intact mass of 10,682 Da. Identified b and y fragment ions are indicated. **(B)** MS/MS spectrum of the N-terminal HA-Ub tryptic peptide 1-20 (Ub 1-6) with a precursor ion mass of 1217.1  $[M+2H]^{2+}$  (Mr 2431.1 Da) containing a methionine oxidation at position 1 derived from probe **2** with an intact mass of 10,698 Da. Identified b and y fragment ions are indicated. **(C)** MS/MS spectrum of the N-terminal HA-Ub tryptic peptide lacking the N-terminal methionine 2-20 (Ub 1-6) with a precursor ion mass of 1143.6  $[M+2H]^{2+}$

(Mr 2284.1 Da) derived from probe **2** with an intact mass of 10,550 Da. Identified b and y ions are indicated. The \* indicates a loss of ammonia/deamination (-17 Da).

## Supplementary Methods

*Analysis by tandem mass spectrometry.* Intact HA-Ub-Alkyne probe (**2**) was subjected to in-solution trypsin digestion as described previously.<sup>1</sup> Digested material was desalted and concentrated using C18 Sep Pack column cartridges (Waters) and dried under reduced pressure. Samples were subsequently analysed by nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Dionex U300 HPLC system coupled to a high capacity iontrap tandem mass spectrometer (HCTplus, Bruker Daltonics) as described previously.<sup>2</sup> MS/MS spectra were search against a custom made database containing the HA-ubiquitin sequence in order to match the N-terminal part using a Mascot search engine (version 2.3).



Scheme S1. Synthesis of fluorescein azide **6**.

*O*-Tosyl-*N*-Boc-ethanolamine (**2**).<sup>3</sup> *N*-Boc-ethanolamine **1** (1.0 g, 6.2 mmol) was dissolved in DCM (5 mL). Pyridine (1.3 mL) was added and the solution cooled to 0 °C. *p*-Toluene sulfonyl chloride (2 g, 10.5 mmol) was added and the reaction stirred for 18 hours. The resultant solution was washed with 6:1 H<sub>2</sub>O:pyridine (2 × 30 mL) and 4:1 H<sub>2</sub>O:37% aq. HCl (30 mL) prior to drying with Na<sub>2</sub>SO<sub>4</sub> and concentration under reduced pressure. This afforded 1.8g (92% yield) of **2** as a white amorphous solid. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): δ 7.79 (dt, *J* = 8.5 and 1.2 Hz, 2H), 7.29 (d, *J* = 8.5 Hz, 2H), 4.85 (bs, 1H), 4.09 (t, *J* = 5.0 Hz, 2H), 3.28–3.25 (m, 2H), 2.45 (s, 3H), 1.44 ppm (s, 9H). <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>): δ 130.4, 128.4, 69.9, 40.2, 28.7 & 22.1 ppm. ESI-MS *m/z* = 653 (2M+H)<sup>+</sup>. IR (neat)  $\nu$ : 3413.5, 1711.2, 1589.5, 1511.3 cm<sup>-1</sup>.

Fluorescein-di-*O,O'*-*N*-Boc Ethylamine (**3**). Fluorescein disodium salt, (300 mg, 1.0 mmol), K<sub>2</sub>CO<sub>3</sub> (400 mg, 2.9 mol) and tosylate **2** (800 mg, 2.5 mmol) were dissolved in DMF (5 mL) and heated to 60 °C for 18 hours. The mixture was then diluted with EtOAc (10 mL) and extracted with brine (10 mL). The aqueous layer was washed with EtOAc (10 mL) and the combined organic extracts further washed with brine (2 × 10 mL). The organic layer was dried with Na<sub>2</sub>SO<sub>4</sub> and concentrated prior to loading onto a silica plug. The product was washed with DCM followed by elution with warm acetone which after concentration afforded desired product **3** in 89% yield as an orange foam. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): δ 8.27 (d, *J* = 7.4 Hz, 1H), 7.66 - 7.65 (m, 2H), 7.29 (d, *J* = 6.0 Hz, 1H), 6.98–6.93 (m, 2H), 6.78 (d, *J* = 7.0 Hz, 1H), 6.62–6.60 (m, 3H), 4.13 (t, *J* = 4.8 Hz, 2H), 4.01 (t, *J* = 5.1 Hz, 2H), 3.57 (t, *J* = 5.4 Hz, 2H), 3.12 (t, *J* = 4.9 Hz, 2H), 1.42 (s, 9H), 1.40 ppm (s, 9H). <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>): δ 184.3, 164.2, 163.1, 156.0, 135.7, 131.9, 130.9, 130.4, 129.6, 113.7, 113.6, 104.8, 102.3, 64.3, 37.0, 31.9, 31.4 & 28.8 ppm. IR (neat)  $\nu$ : 3409.5, 3000.3, 2985.6, 1706.2, 1688.1, 1514.5 cm<sup>-1</sup>. ESI-MS *m/z* = 619 (M+H)<sup>+</sup>. HRMS (ESI) calculated for C<sub>34</sub>H<sub>38</sub>N<sub>2</sub>NaO<sub>9</sub> (M+Na)<sup>+</sup> 641.2470 found 641.2489. Anal. Calcd for C<sub>34</sub>H<sub>38</sub>N<sub>2</sub>O<sub>9</sub> C,66.01; H,6.19; N, 4.53, found C,65.92; H,6.12; N, 4.48.

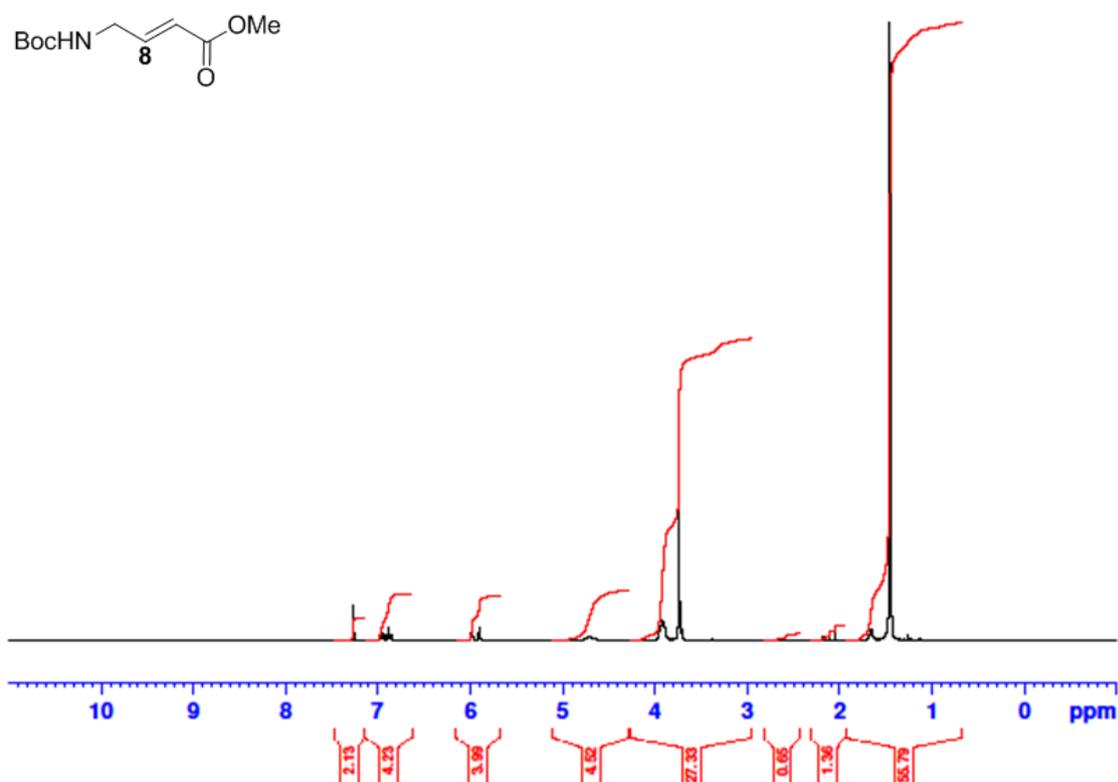
Fluorescein-*O'*-*N*-Boc Ethylamine (**4**). Fluorescein derivate **3**, (500 mg, 0.80 mmol) was dissolved in THF (10 mL). H<sub>2</sub>O (10 mL) was added along with LiOH·H<sub>2</sub>O (600 mg, 25 mmol) and the reaction

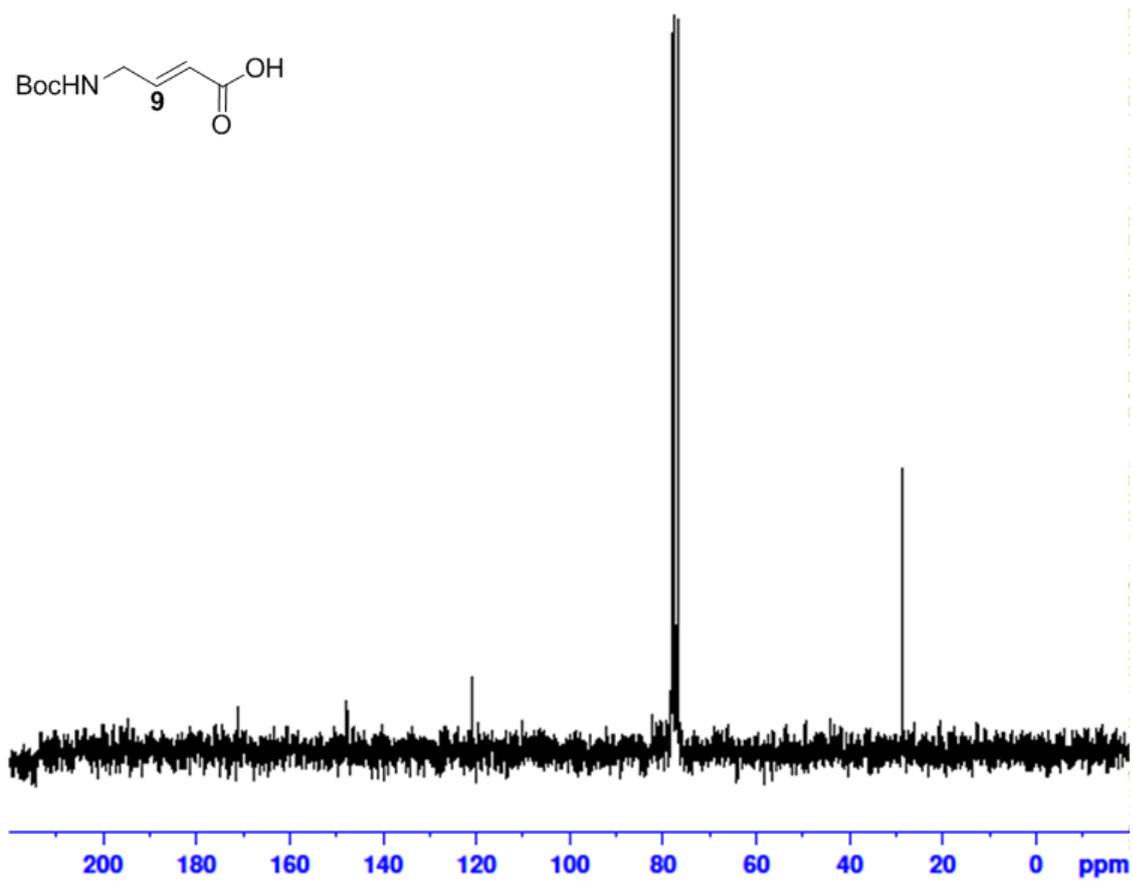
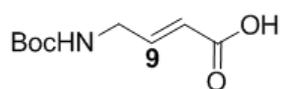
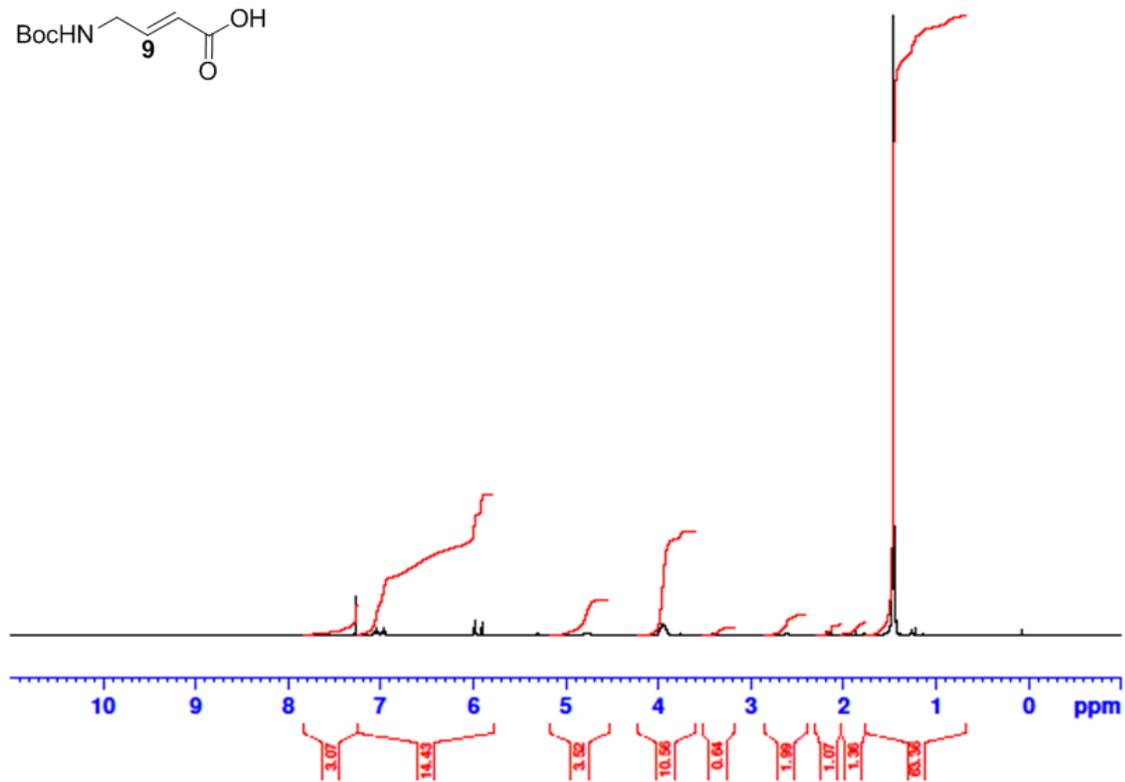
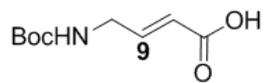
stirred for 3 hours. The reaction progression was checked after this time by t.l.c. (3:2 petrol:EtOAc) showing complete starting material consumption ( $R_f$  0.1) and product formation ( $R_f$  0.3). THF was removed under reduced pressure after which the aqueous solution was adjusted to pH 3 by the addition of 1 M HCl (aq.). The product was extracted with DCM (3 x 10 mL) and the combined organic layers dried with  $\text{Na}_2\text{SO}_4$ . Concentration under reduced pressure followed by flash column chromatography on silica (3:2 petrol:EtOAc to EtOAc) afforded 260 mg (68% yield) of desired product **4** as a viscous orange oil.  $^1\text{H NMR}$  (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.98 (dd,  $J$  = 6.1 and 1.5 Hz, 1H), 7.62-7.61 (m, 2H), 7.13 (dd,  $J$  = 6.1 and 1.4 Hz, 1H), 6.64–6.61 (m, 3H), 6.53-6.50 (m, 3H), 3.99 (t,  $J$  = 4.6 Hz, 2H), 3.76 (t,  $J$  = 4.5 Hz, 2H), 1.42 ppm (s, 9H).  $^{13}\text{C NMR}$  (50 MHz,  $\text{CDCl}_3$ ):  $\delta$  170.4, 158.6, 157.8, 153.5, 135.6, 130.2, 129.5, 125.4, 124.5, 113.2, 112.2, 103.5, 101.9, 68.4, 53.9 & 28.8 ppm. IR (neat)  $\nu$ : 3367.9, 2998.7, 2980.2, 1688.4, 1611.3, 1504.0  $\text{cm}^{-1}$ . ESI-MS  $m/z$  = 474 (M-H) $^-$ . HRMS (ESI) calculated for  $\text{C}_{27}\text{H}_{25}\text{NaO}_7$  (M+Na) $^+$  498.1523 found 498.1519.

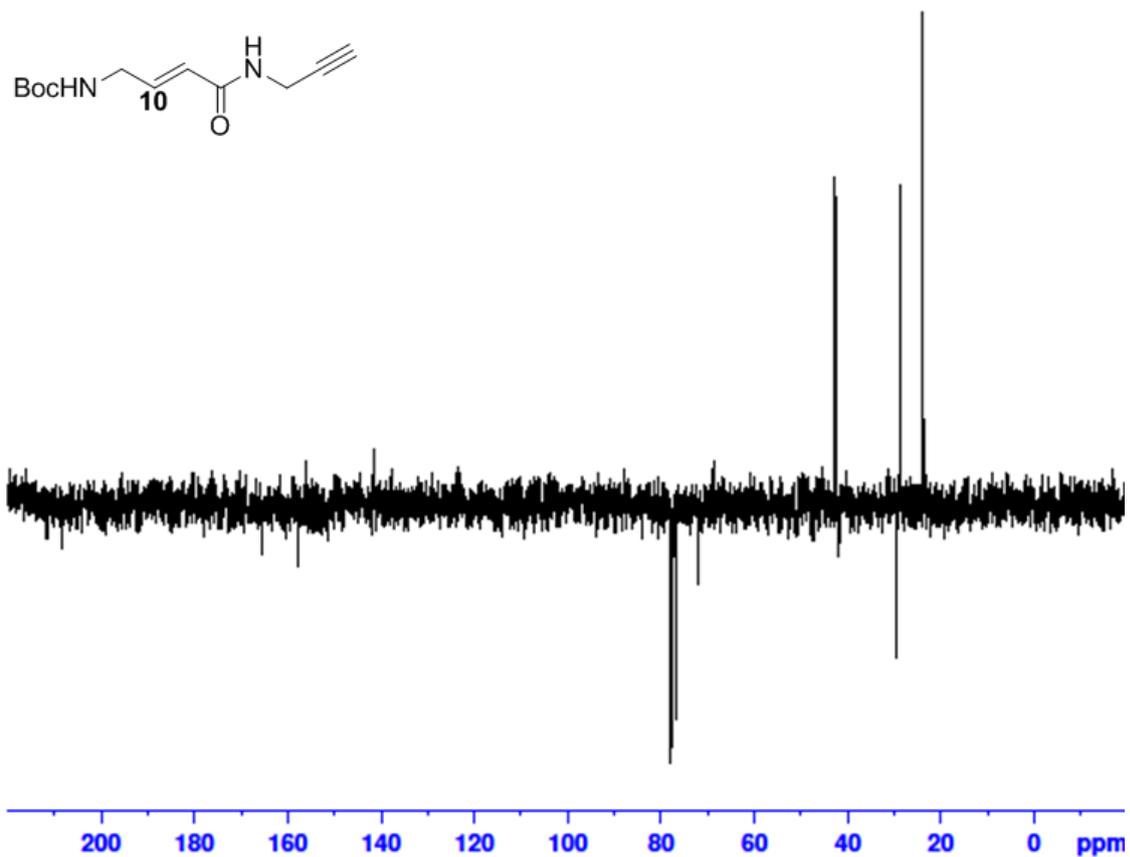
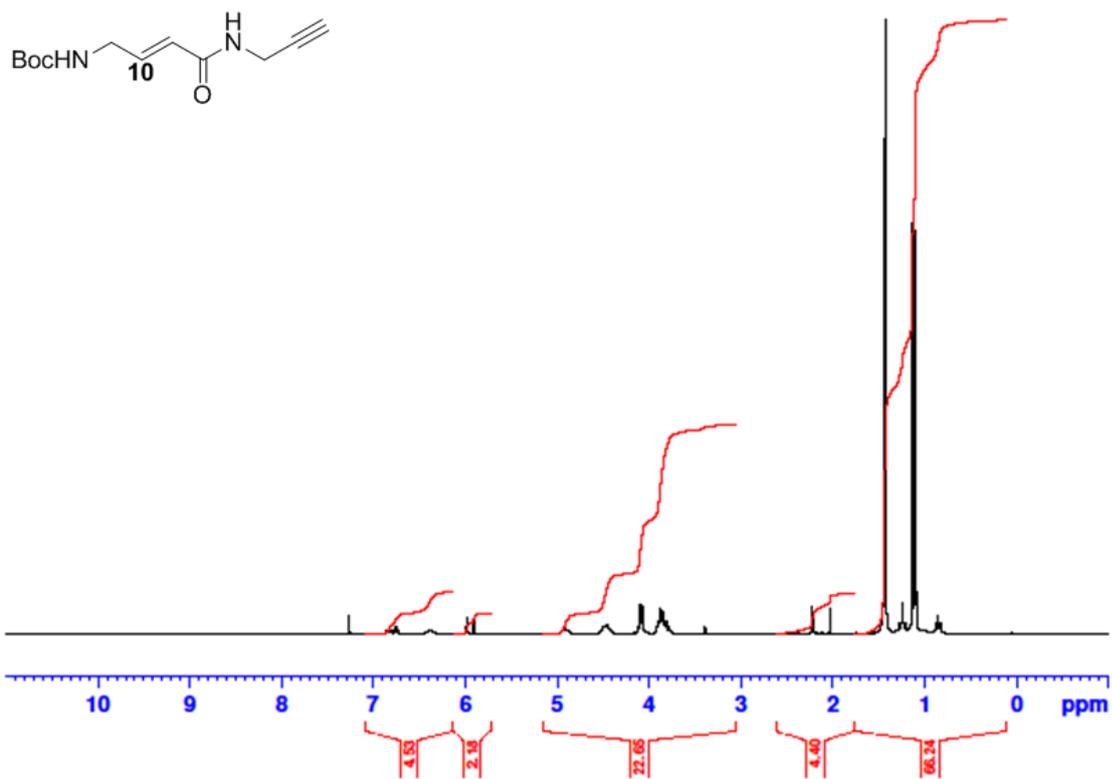
Fluorescein-*O'*-Ethylamine (**5**). *N*-Boc protected **4**, (160 mg, 0.34 mmol) was dissolved in DCM (6 mL). TFA (2 mL) was added and the reaction stirred for 1 hour, after this time by t.l.c. (3:2 petrol:EtOAc) showed complete starting material consumption ( $R_f$  0.3) and product formation ( $R_f$  0.01). The solution was concentrated under reduced pressure, with re-suspension and co-evaporation with toluene (3 x 5 mL) to afford the desired product **5** in quantitative yield which was reacted immediately without further purification.  $^1\text{H NMR}$  (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.04 (d,  $J$  = 7.6 Hz, 1H), 7.76 - 7.68 (m, 2H), 7.16 – 7.09 (m, 3H), 6.74 (d,  $J$  = 4.1 Hz, 1H), 6.70-6.64 (m, 3H), 4.44 (m, 2H), 3.87 ppm (t,  $J$  = 5.2 Hz, 2H).  $^{13}\text{C NMR}$  (50 MHz,  $\text{CDCl}_3$ ):  $\delta$  172.0, 162.8, 154.4, 130.7, 129.7, 128.4, 126.4, 125.7, 113.6, 113.4, 104.2, 103.4, 66.1 & 54.9 ppm.

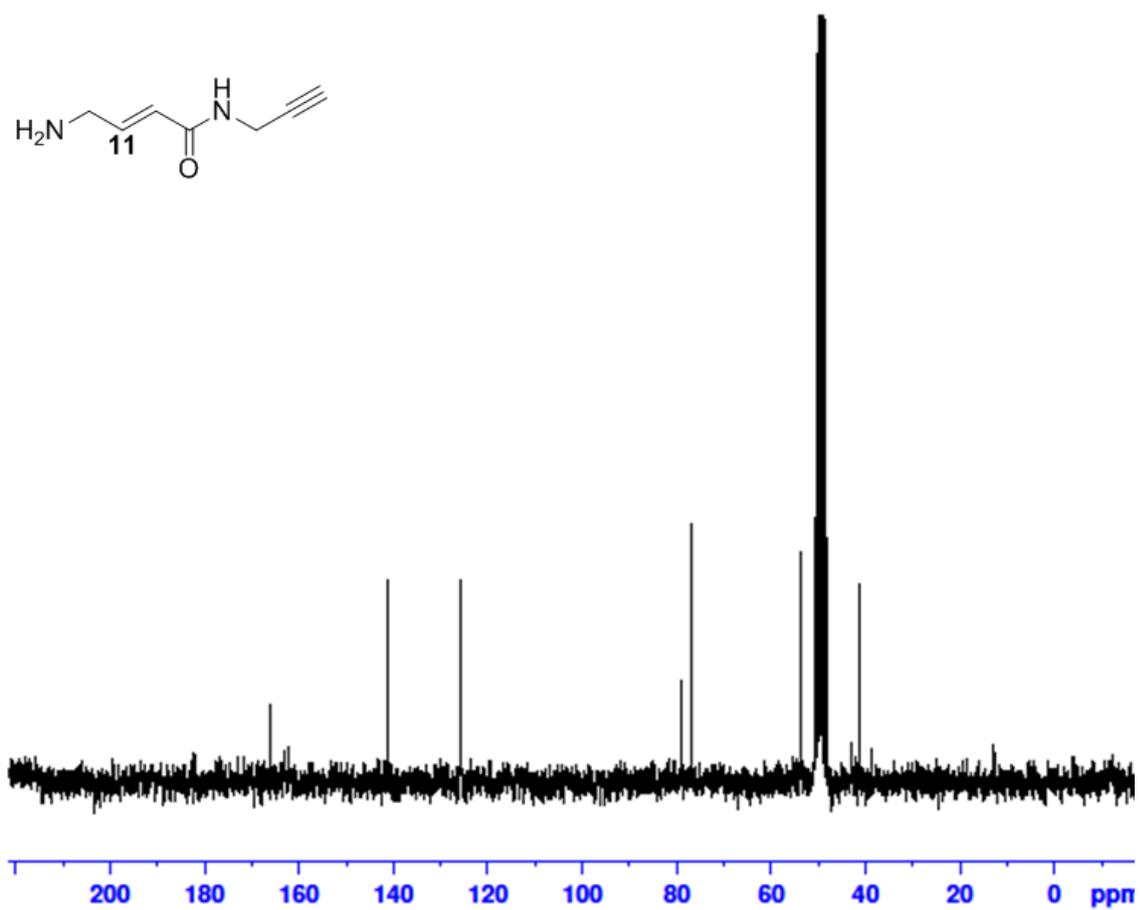
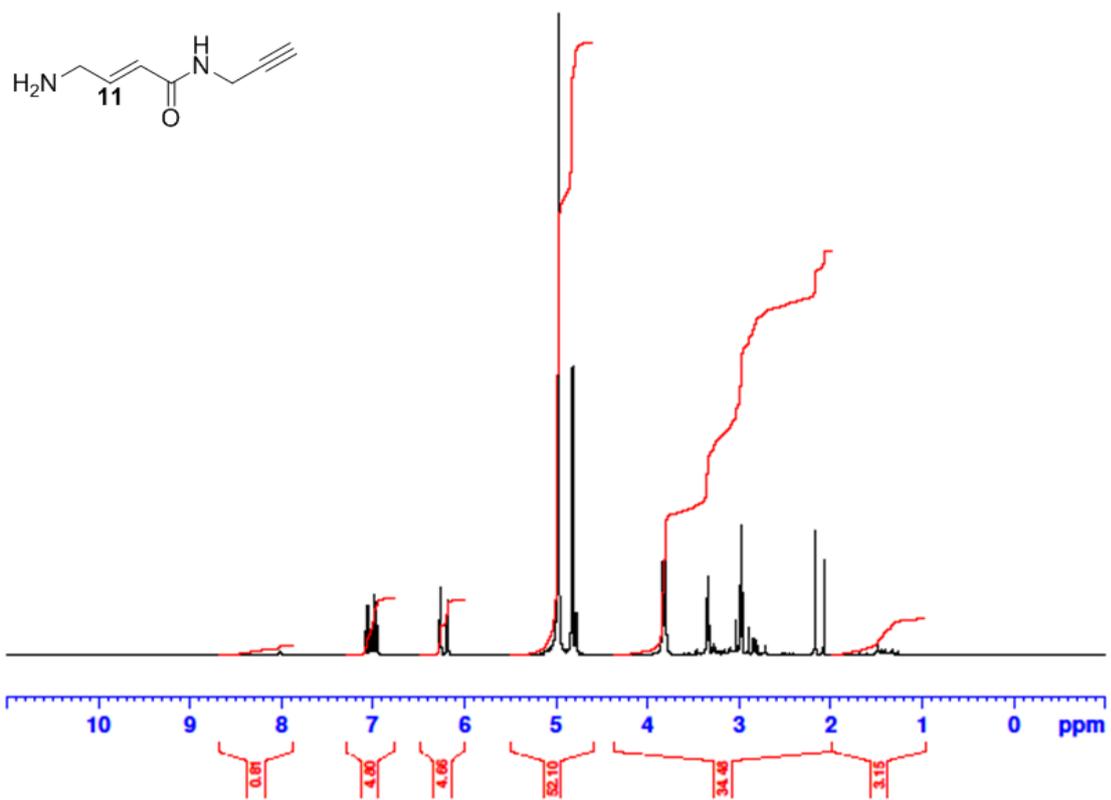
Fluorescein-*O'*-*N*-azido Ethylamine (**6**). Amine **5**, (140 mg, 0.34 mmol) was dissolved in MeOH (15 mL).  $\text{K}_2\text{CO}_3$  (225 mg, 1.7 mmol) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (6 mg, 0.024 mmol). Imidazole-1-sulfonyl Azide Hydrochloride[6] (150 mg, 0.41 mmol) was added and the reaction mixture stirred at room temperature. After 18 hours t.l.c (9:1 EtOAc:MeOH) showed complete starting material

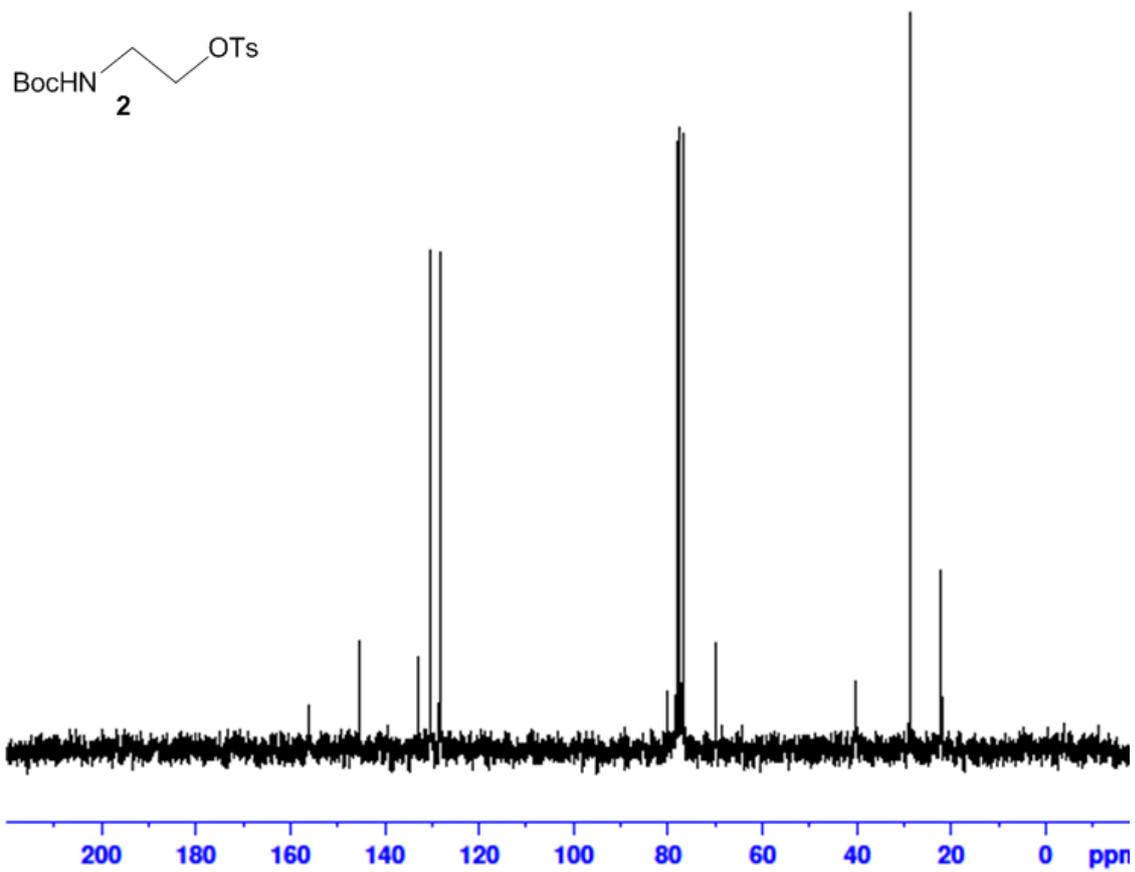
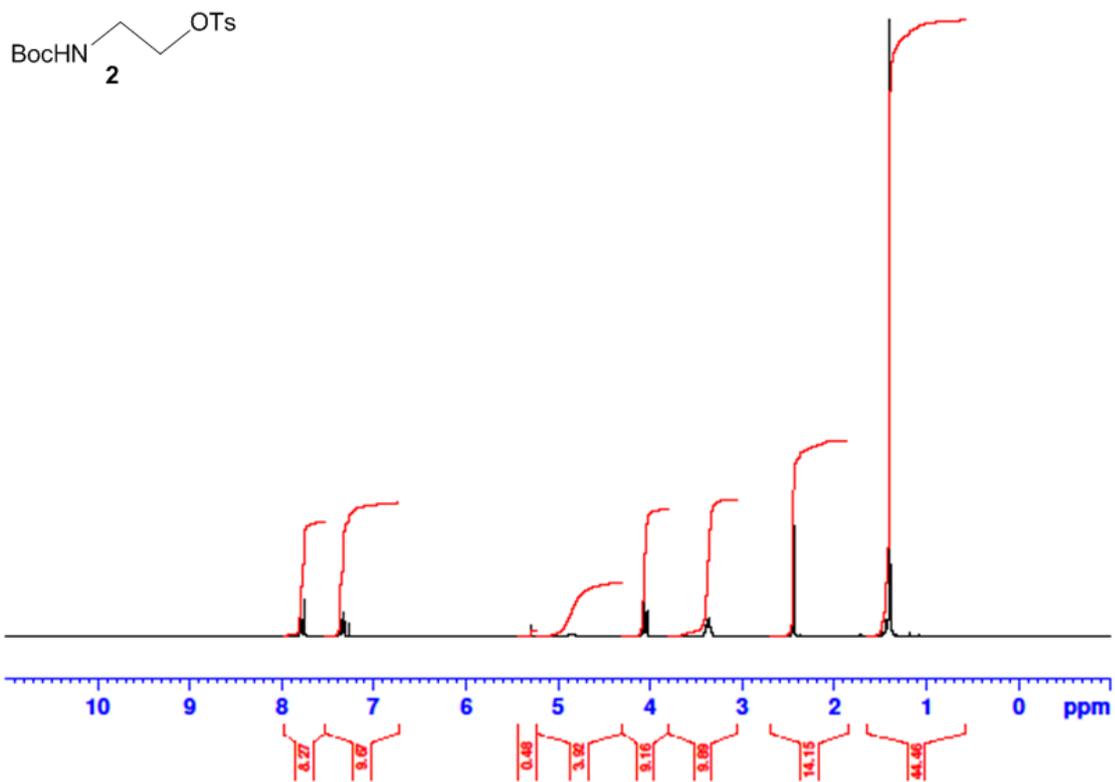
consumption ( $R_f$  0.2) and product formation ( $R_f$  0.8). The solvent was removed under reduced pressure and the resulting mixture taken up in  $H_2O$  (5 mL), the aqueous solution was adjusted to pH 3 by the addition of 1 M HCl (aq.). The product was extracted with EtOAc ( $3 \times 10$  mL) and the combined organic layers dried with  $Na_2SO_4$ . Concentration under reduced pressure afforded 120 mg (88% yield) of desired product **6** as an orange oil.  $^1H$  NMR (200 MHz,  $CDCl_3$ ):  $\delta$  8.04 (dd,  $J = 5.3$  and  $1.5$  Hz, 1H), 7.67–7.66 (m, 2H), 7.17 (dd,  $J = 6.4$  and  $1.4$  Hz, 2H), 6.81–6.79 (m, 2H), 6.69–6.65 (m, 4H), 4.18 (t,  $J = 5.0$  Hz, 2H), 3.62 ppm (t,  $J = 5.2$  Hz, 2H).  $^{13}C$  NMR (50 MHz,  $CDCl_3$ ):  $\delta$  185.3, 176.4, 153.2, 135.4, 130.3, 129.7, 126.0, 124.9, 113.7, 113.0, 103.6, 102.0, 67.8 & 50.4 ppm. IR (neat)  $\nu$ : 3234.6, 2998.4, 2981.7, 2106.22, 1752.9, 1610.2, 1503.9  $cm^{-1}$ . ESI-MS  $m/z = 402$  ( $M+H$ ) $^+$ . HRMS (ESI) calculated for  $C_{22}H_{16}N_3O_5$  ( $M+H$ ) $^+$  402.1084, found 402.1076

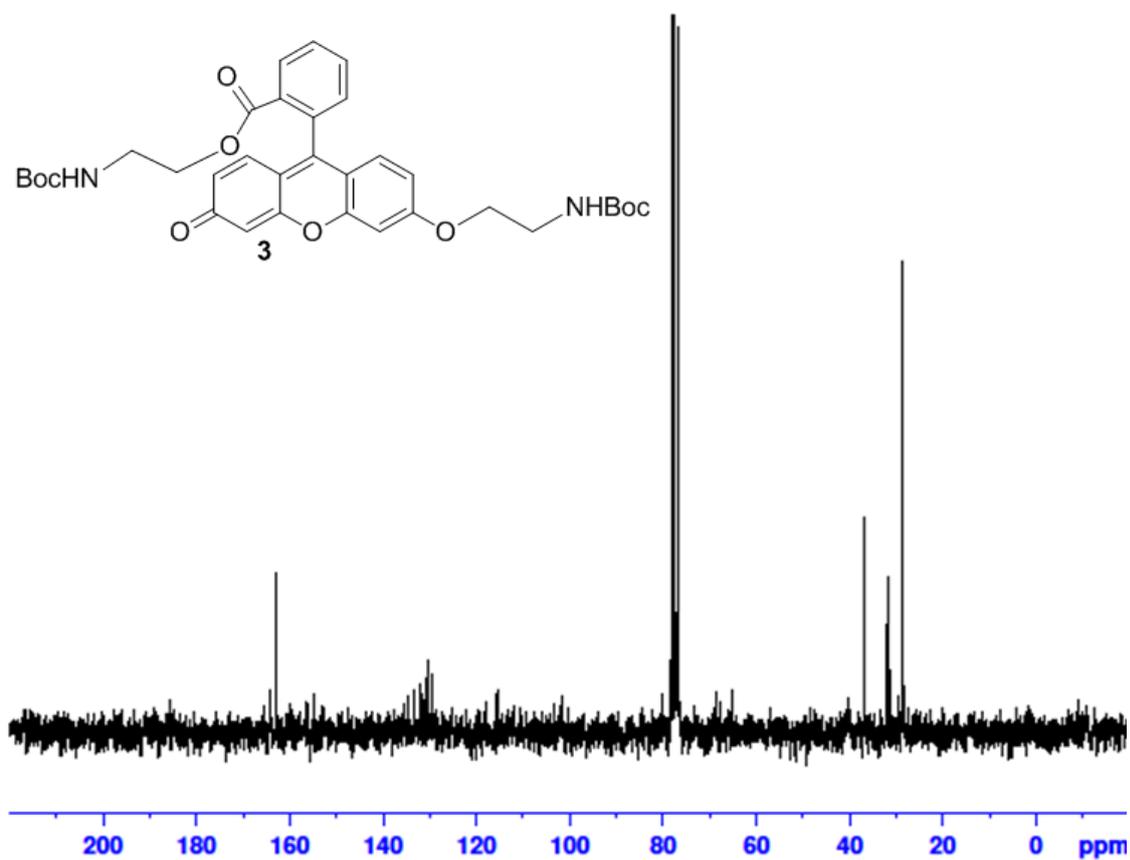
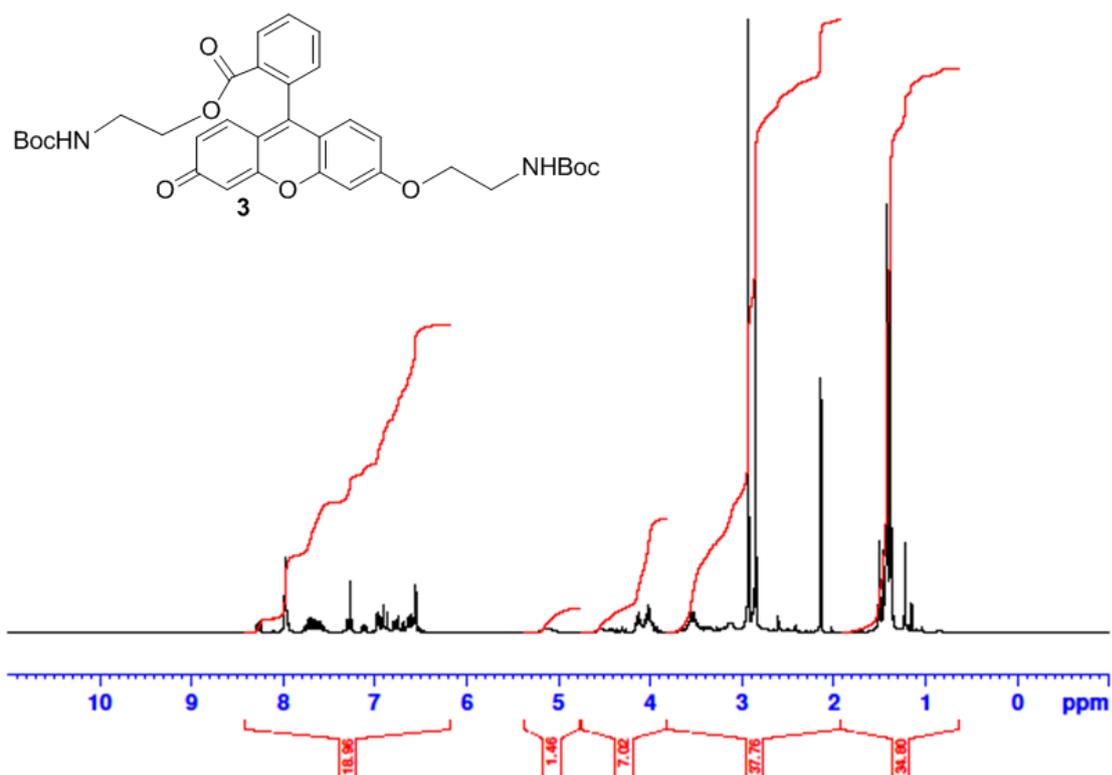


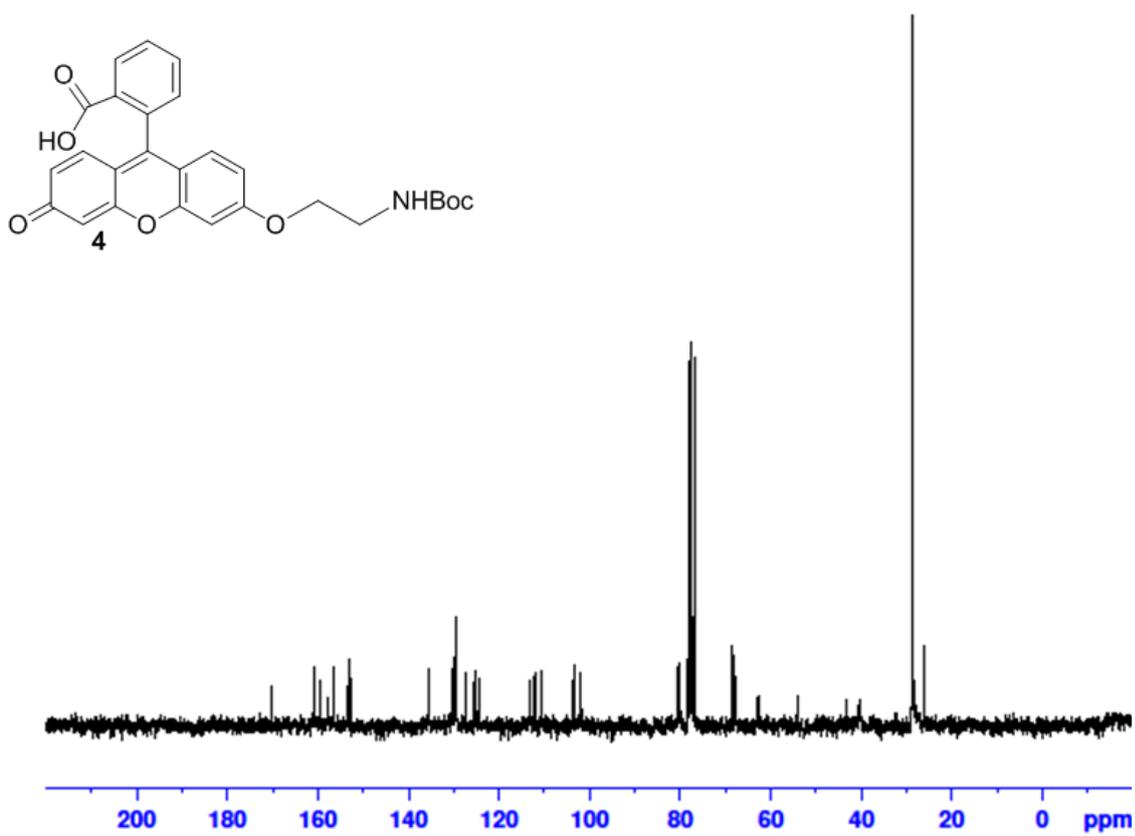
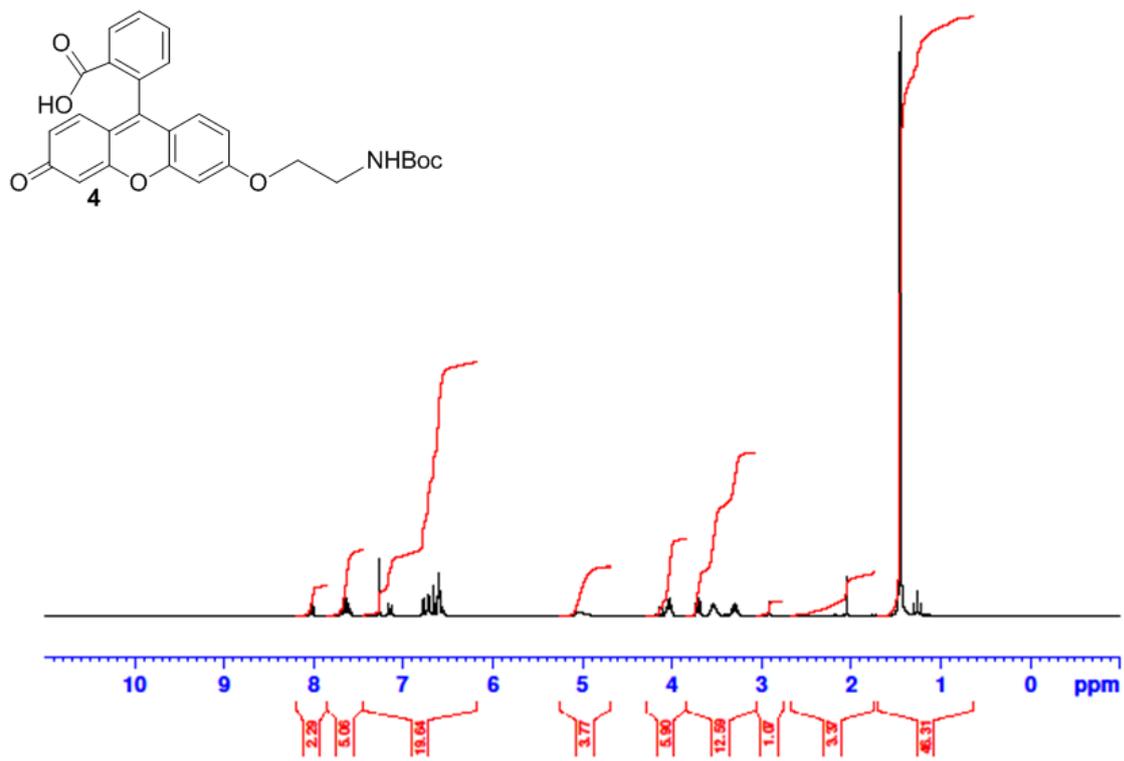


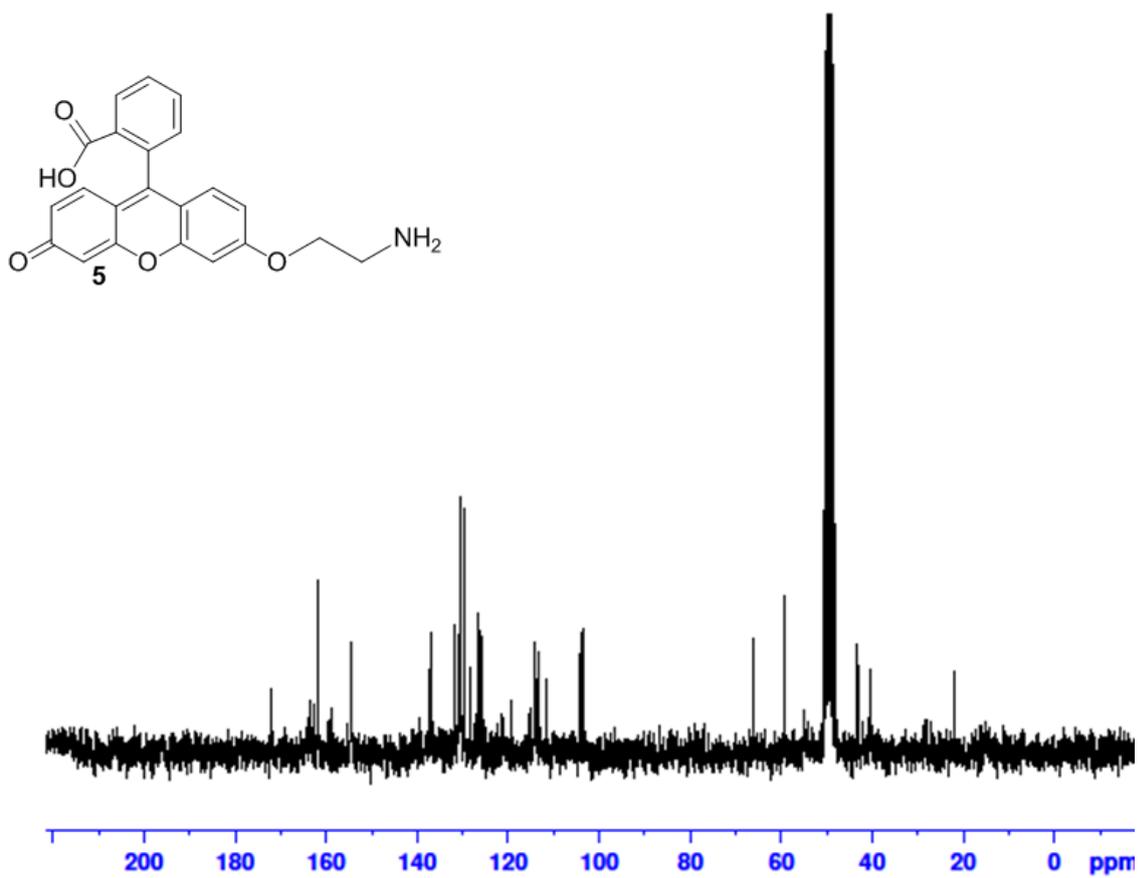
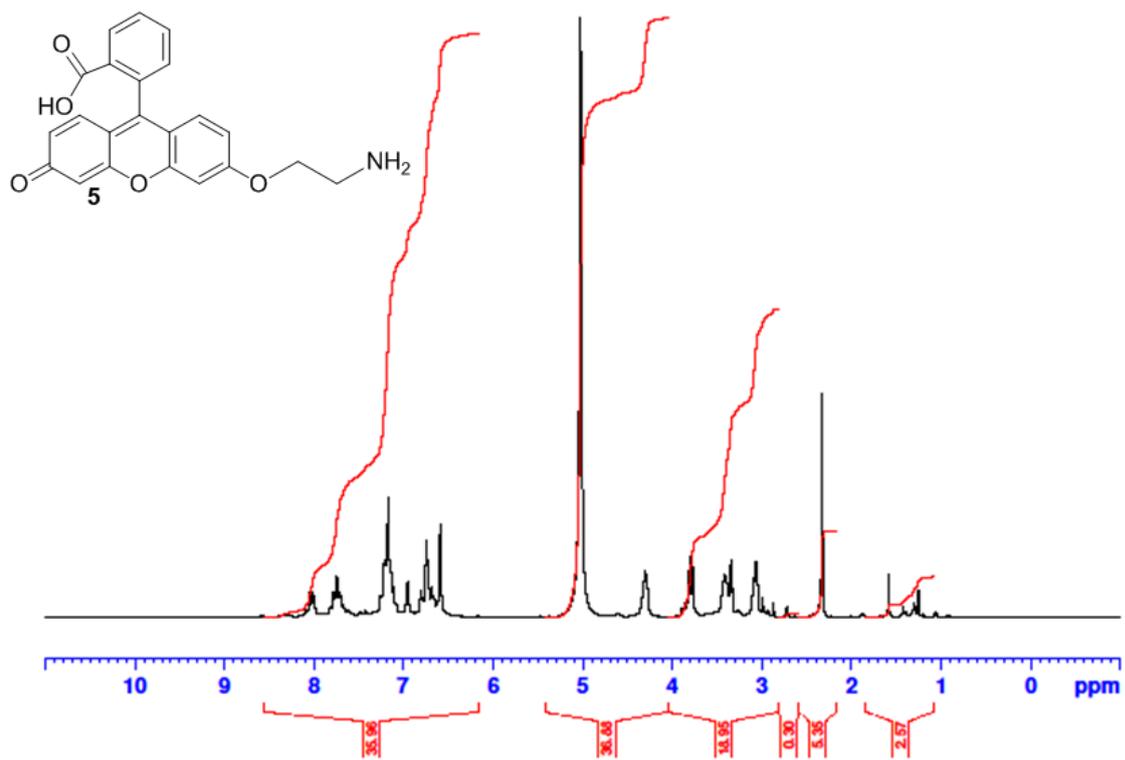


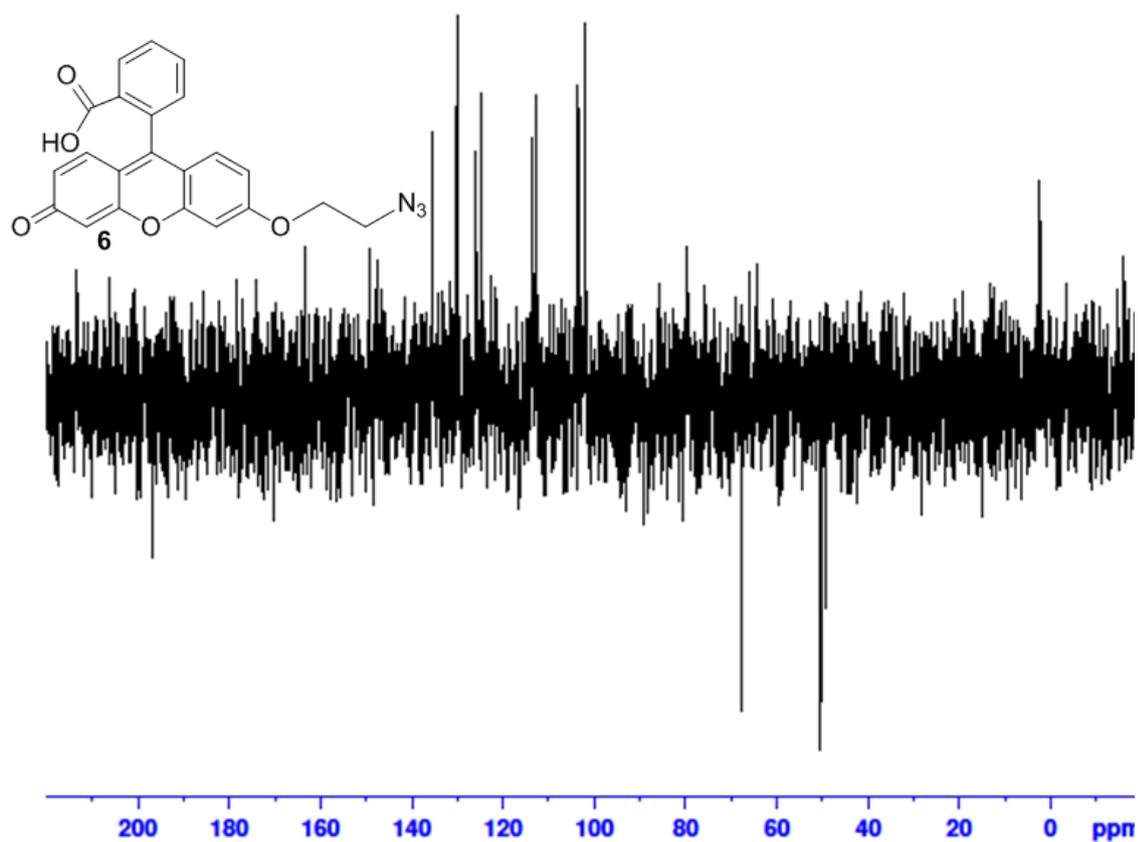
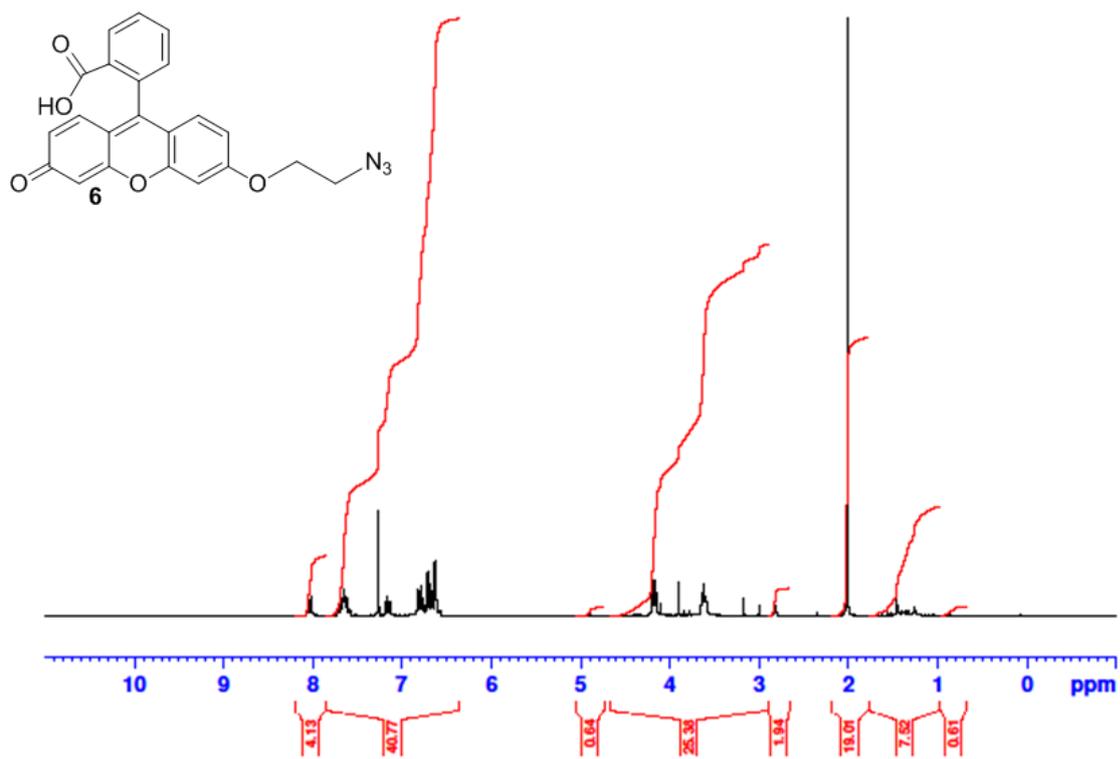












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