

# PEGylation and Dimerization of Expressed Proteins Under Near Equimolar Conditions with Potassium 2-Pyridyl Acyltrifluoroborates

## Supporting Information

Christopher J. White and Jeffrey W. Bode\*

Laboratorium für Organische Chemie, Department of Chemistry and Applied Biosciences  
ETH Zürich, Zürich, 8093, Switzerland

bode@org.chem.ethz.ch

### Table of Contents

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1. General Information.....	S3
1.1 General synthetic methods, solvents & reagents.....	S3
1.2 Gel electrophoresis.....	S3
1.3 UV-Vis spectroscopy – protein quantification.....	S4
1.4 Fast Protein Liquid Chromatography (FPLC).....	S4
1.5 Characterization instrumentation.....	S5
2. Preparation of small molecule reagents and PEG KATs.....	S6
2.1 Methylsulfonophenylloxadiazole-hydroxylamine <b>4</b> .....	S6
2.2 5-fluoropyridin-2-yl potassium acyltrifluoroborate <b>11</b> .....	S11
2.3 mPEG KATs <b>9a-d</b> and diKAT <b>12</b> .....	S12
2.4 Additional small molecule reagents.....	S20
3. Protein expression and purification.....	S23
3.1 sfGFP(S147C) <b>5</b> .....	S23
3.2 Ubc9(C93A).....	S25
3.3 T4L(V131C).....	S27
3.4 HTP(S261C).....	S29
4. Preparation of protein – hydroxylamine conjugates.....	S31
5. PEGylation of protein – hydroxylamine conjugates.....	S35
5.1 General procedure for PEGylation.....	S35
5.2 PEGylated protein substrates.....	S36

6. Control experiment to confirm PEGylation through a KAT ligation.....	S53
7. Protein homodimerization.....	S55
7.1 Homodimerization of T4L(V131C) <b>20</b> .....	S55
7.2 Homodimerization of sfGFP(S147C) <b>21</b> .....	S58
8. Control experiment – one-step PEGylation of sfGFP(S147C) with a methylsulfonophenyloxadiazole-mPEG.....	S59
9. NMR data.....	S63
10. References.....	S83

## 1. General Information

### 1.1 General synthetic methods, solvents & reagents

Unless otherwise stated, all reactions were carried out in oven-dried glassware sealed with rubber septa under an atmosphere of dry N<sub>2</sub> and were stirred with Teflon-coated magnetic stir bars. Thin layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm) pre-coated with silica gel 60 F254 and visualized by UV quenching and staining with KMnO<sub>4</sub> or I<sub>2</sub>. Flash column chromatography was performed under a forced-flow of air using Silicycle SiliaFlash F60 (40–63 μm particle size).

All organic solvents (EtOH, MeOH, DMF, THF, CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, hexanes, CH<sub>3</sub>CN, DMSO) were used as supplied (ACS or HPLC grade) unless otherwise noted. THF was purified by distillation from sodium benzophenone ketyl prior to use. CH<sub>2</sub>Cl<sub>2</sub> and Et<sub>3</sub>N were purified by distillation from CaH<sub>2</sub>. Anhydrous DMF and toluene were obtained by passage through two columns of anhydrous neutral A-2 alumina under an atmosphere of N<sub>2</sub> and then stored over activated 4 Å molecular sieves. MeOH and 1,4-dioxane were dried over activated 3 Å and 4 Å molecular sieves, respectively, and stored under an inert atmosphere of N<sub>2</sub>. All reagents were purchased from Sigma Aldrich and used as received, without further purification, unless otherwise stated. 2-bromo-5-fluoropyridine was purchased from Apollo Scientific Limited. The 5.0 kDa mPEG and 15.0 kDa 4-arm PEG reagents were purchased from JenKem Technology USA. All restriction enzymes, T4 polynucleotide kinase, Phusion® High-Fidelity DNA Polymerase and DpnI were obtained from New England Biolabs (MA, USA). Oligonucleotides were synthesized by Microsynth AG (Balgach, Switzerland). Kanamycin sulphate BioChemica, ampicillin sodium salt and lysozyme (22500 U/ mg) were obtained from Axon Lab AG (Baden, Switzerland). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from Fisher Scientific (Geel, Belgium). DNase I was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Ni(II) – NTA Agarose was obtained from Qiagen GmbH (Hilden, Germany). The 10 kDa mPEG *para*-phenyl KAT 7 was prepared as previously described in the literature.<sup>5</sup>

### 1.2 Gel electrophoresis

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini-PROTEAN Tetra Cell system (Bio-Rad) connected to a PowerPac Basic (Bio-Rad) programmable power supply, following slight modifications of the protocol of Laemmli.<sup>1</sup> Protein electrophoresis samples (2 – 5 μg protein, desalted) were treated with an equal volume of a Sample

Buffer, Laemmli 2x Concentrate (Sigma) containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl at a pH of approximately 6.8. Samples were then heated to 60 °C for 10 minutes to denature the protein sample and ensure reduction of any disulfide bonds. Samples were loaded (typically a 10 µL loading volume) onto commercially available 15-well Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad) with a gradient gel percentage of either 8 – 16% or 4 – 20%. A commercially available 10 – 180 kDa pre-stained protein ladder (Thermo Fisher) was applied to at least one well (5 µL) of each gel for the assignment of apparent molecular masses. Gels were run for 30 minutes at 200 V in a running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3). Protein bands were subsequently visualized by incubating the gels in a staining solution (0.1% Coomassie Brilliant Blue R 250, 40% MeOH, 10% acetic acid) with gentle agitation for 1 hour. This was followed by a destaining protocol in which the gels were gently agitated in a destaining solution (40% MeOH, 10% acetic acid) for several hours, replenishing the solution every hour until the background of the gel became fully destained. ImageJ was used to calculate ligation conversions by optical densitometry.

### **1.3 UV-Vis spectroscopy – protein quantification**

OD<sub>600</sub> and protein concentration measurements were executed using a NanoDrop 2000c UV-Vis spectrophotometer. Protein concentrations for quantification after expression and purification were determined by the absorption at 280 nm using extinction coefficients calculated by ProtoParam (<http://expasy.org/tools/protparam.html>) based on the amino acid sequence of the protein.

### **1.4 Fast Protein Liquid Chromatography (FPLC)**

All protein purifications were performed on an ÄKTA pure chromatography system (GE Healthcare) using the UNICORN 6.3 Workstation system control software. All purifications were carried out at 4 °C. All buffers were freshly prepared, filtered and degassed immediately prior to use. Size exclusion chromatography (SEC) purifications were performed using a Superdex 75 Increase 10/300 GL column. Cation and Anion exchange chromatography purifications were performed using the strong ion exchange Mono S 5/50 GL and Mono Q 5/50 GL columns respectively. All FPLC columns were purchased from GE Healthcare. All protein purifications were monitored at wavelengths of 254 nm, 280 nm and 490 nm (the latter for purifications involving GFP).

### 1.5 Characterization instrumentation

LC-MS analysis was performed on a Dionex UltiMate 3000 RSLC connected to a Surveyor MSQ Plus mass spectrometer; a reversed-phase RESTEK Pinnacle II C18 (4.6 x 50 mm) column was used, running a gradient of 5 to 100% CH<sub>3</sub>CN in H<sub>2</sub>O over 6.5 min and 100% CH<sub>3</sub>CN for 2.5 min for analysis of small molecules.

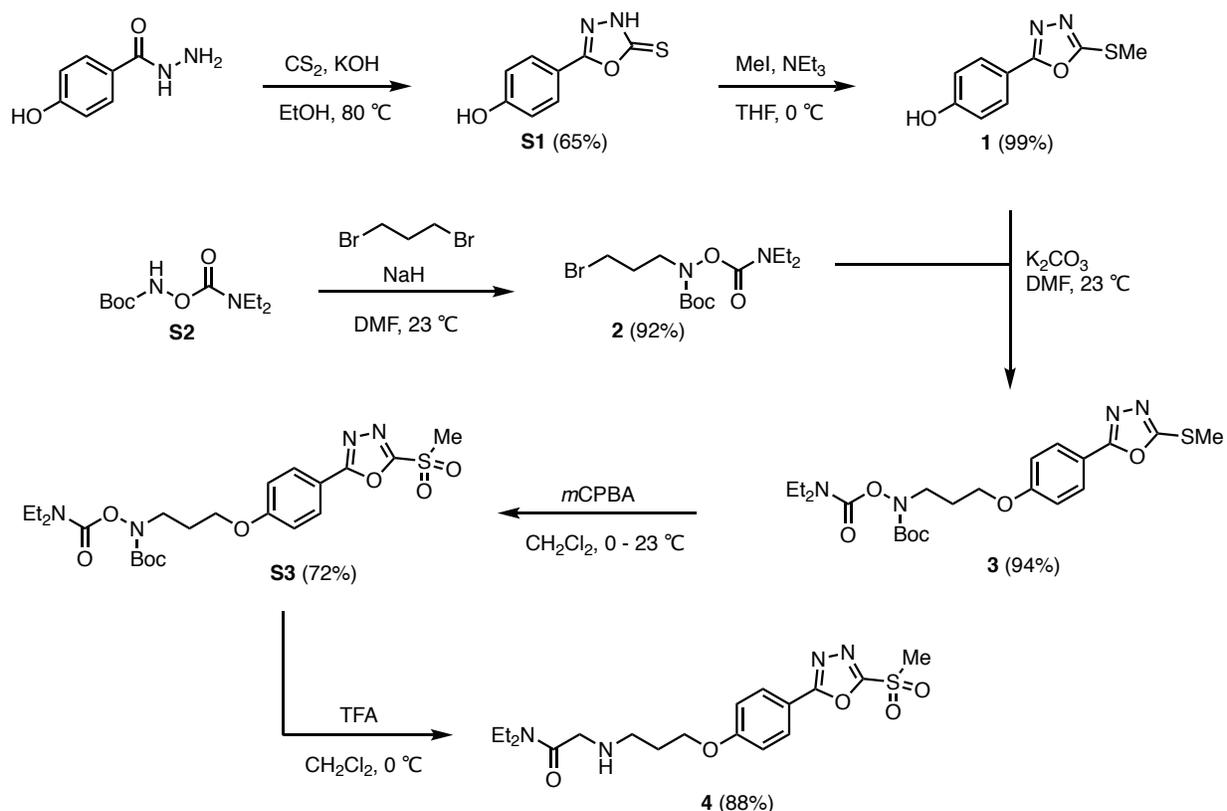
High-resolution mass spectra were obtained by the mass spectrometry service of the ETH Zürich Laboratorium für Organische Chemie on a Varian IonSpec FT-ICR (ESI), a Bruker Daltonics maXis ESI-QTOF spectrometer (ESI), a Bruker Daltonics SOLARIX spectrometer (MALDI), or a Bruker Daltonics UltraFlex II spectrometer (MALDI-TOF). For ESI (+MS) an enhanced quadratic calibration mode was used with the following reference mass peaks: 118.0863, 322.0481, 622.0290, 922.0098, 1221.9906, 1521.9715, 1821.9523, 2121.9332, 2421.9140, 2721.8948.

NMR spectra were recorded on a Bruker AV-300, a Bruker AV-400, a Bruker AV-III-500, a Bruker DRX-II-500 or a Bruker AV-III-600. Data for <sup>1</sup>H NMR are reported as follows: chemical shift (multiplicity, coupling constants where applicable, number of hydrogens). Abbreviations are as follows: s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), dd (doublet of doublet), dt (doublet of triplet), ddt (doublet of doublet of triplet), m (multiplet), br (broad), bs (broad singlet), bd (broad doublet) bq (broad quartet).

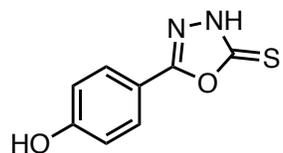
## 2. Preparation of small molecule reagents and PEG KATs

### 2.1 Methylsulfonylphenyloxadiazole-hydroxylamine 4

#### Scheme S1. Synthesis of bifunctional reagent 4.



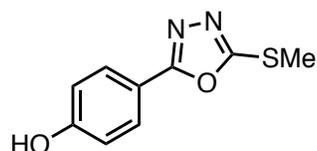
#### 5-(4-Hydroxyphenyl)-1,3,4-oxadiazole-2(3H)-thione (S1):



Compound **S1** was prepared as previously described in the literature with slight modifications.<sup>2</sup> Potassium hydroxide (8.42 g,  $1.50 \times 10^2$  mmol, 3.00 equiv) was dissolved in absolute EtOH (200 mL) and cooled to 0 °C in an ice bath with stirring. 4-Hydroxybenzhydrazide (7.61 g, 50.0 mmol, 1.00 equiv) was added and the resulting solution was stirred at 0 °C for 5 min. Carbon disulfide (6.04 mL,  $1.00 \times 10^2$  mmol, 2.00 equiv) was added and the resulting solution was warmed to RT, fitted with a reflux condenser and stirred at 95 °C for 18 hours. The resulting orange solution was cooled to room temperature and concentrated under reduced pressure. H<sub>2</sub>O (200 mL) was added and the solution was cooled to 0 °C in an ice bath with stirring. 6N HCl (aq) was added drop-wise until the solution became acidic (pH = 2.0). At this point, the resulting precipitate that formed was isolated via vacuum filtration and washed with

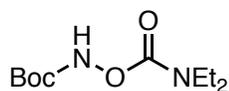
ice-cold H<sub>2</sub>O. The precipitate was dried *in vacuo* to yield an off-white, beige amorphous solid. Recrystallization from 1 : 5 EtOH : H<sub>2</sub>O (v/v) afforded **S1** as off-white needles (6.32g, 65.1% yield). The spectral data of **S1** was in agreement with that reported in the literature.<sup>2</sup> <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 14.55 (s, 1H), 10.38 (s, 1H), 7.71 (d, *J* = 8.5 Hz, 2H), 6.93 (d, *J* = 8.5 Hz, 2H); <sup>13</sup>C NMR (101 MHz, DMSO) δ 177.0, 161.1, 160.9, 128.1, 116.2, 113.1.

#### 4-(5-(Methylthio)-1,3,4-oxadiazol-2-yl)phenol (**1**):



Compound **1** was prepared as previously described in the literature with slight modifications.<sup>3,4</sup> Compound **S1** (2.55 g, 13.1 mmol, 1.00 equiv) was dissolved in anhydrous THF (40 mL) and cooled to 0 °C in an ice bath with stirring. Methyl iodide (0.90 mL, 14.4 mmol, 1.10 equiv) was added, followed by NEt<sub>3</sub> (1.84 mL, 13.2 mmol, 1.01 equiv). The reaction mixture was stirred at 0 °C for 4 hours and quenched with saturated aqueous NH<sub>4</sub>Cl (25 mL). The resulting suspension was warmed to room temperature and diluted with EtOAc (50 mL) and H<sub>2</sub>O (25 mL). The phases were separated and the aqueous phase was extracted with EtOAc (3 x 50 mL). The combined organic phases were washed with brine (50 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography, eluting with 50% EtOAc in hexanes to afford product **1** as a white solid (2.68 g, 98.3% yield). The spectral data of **1** was in agreement with that reported in the literature.<sup>4</sup> <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 10.30 (s, 1H), 7.83 – 7.75 (m, 2H), 6.98 – 6.85 (m, 2H), 2.74 (s, 3H); <sup>13</sup>C NMR (101 MHz, DMSO) δ 165.2, 163.4, 160.7, 128.3, 116.1, 113.8, 14.3.

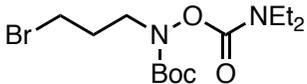
#### *N*-*tert*-Butoxycarbonyl-*O*-diethylcarbamoylhydroxylamine (**S2**):



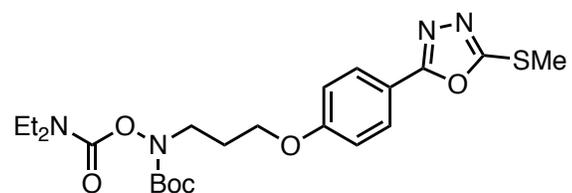
Compound **S2** was prepared as previously described in the literature with slight modifications.<sup>5</sup> To a solution of *N*-*tert*-butoxycarbonylhydroxylamine (6.10 g, 45.8 mmol, 1.30 equiv) and Et<sub>3</sub>N (6.30 mL, 45.8 mmol, 1.30 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (70 mL) was added slowly *N,N*-diethylcarbamoylchloride (4.60 mL, 35.2 mmol, 1.00 equiv) over 5 min, followed by DMAP (0.43 g, 3.5 mmol, 0.10 equiv) at room temperature. The resulting mixture was fitted with a reflux condenser and stirred at 40 °C for 40 hours or until TLC indicated full conversion of *N,N*-diethylcarbamoylchloride (20% EtOAc in hexanes). After cooling to RT, the mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with 1 M HCl (aq). The aqueous phase was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x). The combined organic phases were washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under

reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 10% to 20% EtOAc in hexanes. This afforded the product **S2** as a colorless oil which slowly crystallized into a waxy white solid (7.29 g, 89.2% yield). The spectral data of **S2** was in agreement with that reported in the literature.<sup>5</sup> **<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>) δ 7.78 (s, 1H), 3.34 (q, *J* = 7.2 Hz, 4H), 1.48 (s, 9H), 1.28-1.11 (m, 6H); **<sup>13</sup>C NMR** (101 MHz, CDCl<sub>3</sub>) δ 156.7, 155.7, 82.9, 43.1, 41.6, 28.2, 14.1, 13.4; **HRMS** (ESI) calc'd for C<sub>10</sub>H<sub>20</sub>N<sub>2</sub>NaO<sub>4</sub> [M + Na]<sup>+</sup>: 255.1315, found: 255.1317.

***tert*-Butyl (3-bromopropyl)((diethylcarbamoyl)oxy)carbamate (2):**

 Sodium hydride (0.568 g, 14.2 mmol, 1.20 equiv) was suspended in anhydrous DMF (10 mL) and cooled to 0 °C in an ice bath with stirring. A solution of hydroxylamine **S2** (2.75 g, 11.8 mmol, 1.00 equiv) in DMF (15 mL) was added to the stirring suspension of NaH over 5 minutes. The resulting mixture was stirred at 0 °C for 30 min, at which point 1,3-dibromopropane (3.00 mL, 29.6 mmol, 2.51 equiv) was added. The reaction solution was warmed to room temperature and stirred overnight for 16 hours. The solution was cooled back down to 0 °C and the reaction was quenched with saturated aqueous NH<sub>4</sub>Cl (50 mL). The resulting suspension was subsequently warmed to room temperature and diluted with H<sub>2</sub>O (50 mL) and EtOAc (300 mL). The aqueous phase was separated and the organic phase was washed with H<sub>2</sub>O (50 mL) and brine (4 x 50 mL). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 20% to 30% EtOAc in hexanes. This afforded the product **2** as a colorless oil (3.82 g, 91.9% yield). **<sup>1</sup>H NMR** (400 MHz, Chloroform-*d*) δ 3.77 (t, *J* = 6.4 Hz, 2H), 3.50 (t, *J* = 6.6 Hz, 2H), 3.31 (q, *J* = 7.1 Hz, 4H), 2.13 (p, *J* = 6.5 Hz, 2H), 1.46 (s, 9H), 1.22 – 1.12 (m, 6H); **<sup>13</sup>C NMR** (101 MHz, CDCl<sub>3</sub>) δ 155.1, 154.2, 132.7, 118.1, 82.2, 49.1, 43.1, 41.8, 30.9, 30.8, 28.3, 14.3, 13.5. **HRMS** (ESI) calc'd for C<sub>13</sub>H<sub>25</sub>BrN<sub>2</sub>NaO<sub>4</sub> [M + Na]<sup>+</sup>: 375.0887, found: 375.0890; **IR** (ν/cm<sup>-1</sup>, neat) 2970, 2938, 1740, 1422, 1366, 1228, 1217, 1140, 1094, 919.

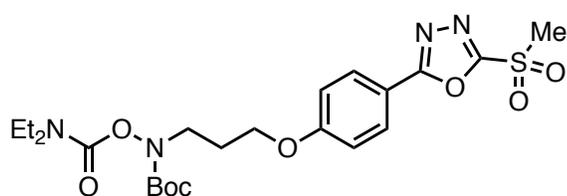
***tert*-Butyl ((diethylcarbamoyl)oxy)(3-(4-(5-(methylthio)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)carbamate (3):**



To a solution of phenol **1** (1.93 g, 9.27 mmol, 1.00 equiv) in anhydrous DMF (10 mL) was added K<sub>2</sub>CO<sub>3</sub> (1.41 g,

10.2 mmol, 1.10 equiv). The resulting suspension was stirred at room temperature for 20 min, at which point a solution of alkyl bromide **2** (3.70 g, 10.5 mmol, 1.13 equiv) in DMF (10 mL) was added. The reaction mixture was stirred at room temperature for 18 hours, diluted with EtOAc (200 mL) and washed with H<sub>2</sub>O (2 x 50 mL) and brine (3 x 50 mL). The organic phase was collected and dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 30 to 40% EtOAc in hexanes to afford the product **3** as a colorless syrup (4.12 g, 92.6% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 7.95 – 7.86 (m, 2H), 7.02 – 6.94 (m, 2H), 4.14 (t, *J* = 5.9 Hz, 2H), 3.84 (t, *J* = 6.5 Hz, 2H), 3.29 (bs, 4H), 2.76 (s, 3H), 2.10 (p, *J* = 6.3 Hz, 2H), 1.43 (s, 9H), 1.15 (t, *J* = 7.1 Hz, 6H); <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 165.9, 164.4, 161.8, 155.1, 154.3, 128.5, 116.2, 115.1, 82.1, 65.5, 47.5, 43.1, 41.7, 28.3, 27.4, 14.8, 14.3, 13.5. HRMS (ESI) calc'd for C<sub>22</sub>H<sub>33</sub>N<sub>4</sub>O<sub>6</sub>S [M + H]<sup>+</sup>: 481.2115, found: 481.2117; IR (ν/cm<sup>-1</sup>, neat) 2971, 2935, 1739, 1612, 1503, 1475, 1422, 1366, 1255, 1217, 1172, 1129, 1068, 949, 838.

***tert*-Butyl ((diethylcarbamoyloxy)(3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy) propyl)-carbamate (S3):**

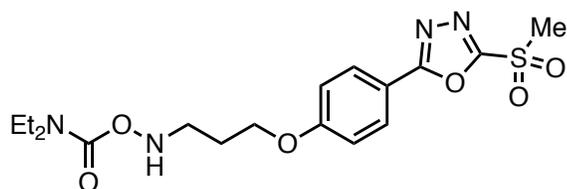


Compound **3** (1.92 g, 4.00 mmol, 1.00 equiv) was dissolved in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (60 mL) and cooled to 0 °C in an ice bath with stirring. *m*-CPBA (2.96 g, 12.0 mmol, 3.00 equiv) was added as a solid in batches over 2

min. The ice bath was removed and the resulting reaction mixture was stirred at room temperature for 18 hours. The reaction was quenched with saturated aqueous Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (40 mL) and continued to stir at room temperature for 30 min. The resulting suspension was poured into H<sub>2</sub>O (100 mL), the phases were separated and the aqueous phase was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3 x 50 mL). The combined organic phases were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude material was dissolved in EtOAc (100 mL) and washed with saturated aqueous NaHCO<sub>3</sub> (4 x 50 mL), H<sub>2</sub>O (3 x 50 mL) and brine (50 mL). The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure onto celite. The crude material (dry-loaded onto celite) was purified by flash chromatography using a gradient elution of 30% to 50% EtOAc in hexanes. A common by-product in this reaction is the partially oxidized sulfoxide, which is more polar than the desired sulfone. The product **S3** was isolated as a colorless syrup (1.47 g, 71.7% yield). <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.10 – 8.00 (m, 2H), 7.08 – 6.99 (m, 2H), 4.18 (t, *J* = 6.1 Hz, 2H), 3.85 (t, *J* = 6.4 Hz, 2H), 3.29 (bs,

4H), 2.11 (p,  $J = 6.3$  Hz, 2H), 2.04 (s, 3H), 1.43 (s, 9H), 1.16 (t,  $J = 7.1$  Hz, 6H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  166.8, 163.2, 161.7, 155.1, 154.3, 129.8, 115.5, 114.3, 82.1, 65.7, 47.4, 43.1, 41.7, 28.3, 27.4, 14.3, 13.5. **HRMS** (ESI) calc'd for  $\text{C}_{22}\text{H}_{33}\text{N}_4\text{O}_8\text{S}$   $[\text{M} + \text{H}]^+$ : 513.2019, found: 513.2010; **IR** ( $\nu/\text{cm}^{-1}$ , neat) 2971, 2935, 1739, 1610, 1495, 1424, 1366, 1350, 1260, 1228, 1217, 1154, 1129, 948, 841.

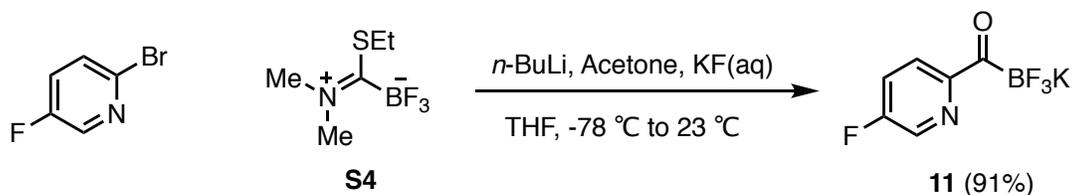
***N*-ethyl-*N*-((((3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)amino)oxy)carbonyl)ethanamine (4):**



Compound **S3** (1.44 g, 2.81 mmol, 1.00 equiv) was dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$  (15 mL) and cooled to 0 °C in an ice bath with stirring. Trifluoroacetic acid (6.50 mL, 84.3 mmol, 30.0 equiv) was added and the reaction was stirred at 0 °C for 5 hours.  $\text{CH}_2\text{Cl}_2$  (20 mL) was added followed by the *careful* addition of saturated aqueous  $\text{NaHCO}_3$  (50 mL), along with solid  $\text{NaHCO}_3$  with stirring, until the solution became weakly alkaline (pH = 8.0). The resulting suspension was warmed to room temperature, the phases were separated and the aqueous phase was extracted with  $\text{CH}_2\text{Cl}_2$  (4 x 25 mL). The combined organic phases were dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 50% to 80% EtOAc in hexanes. A short column should be used, as the product tends to streak on silica gel. The product **4** was isolated as a pale yellow oil that slowly solidified into a white solid at -20 °C (1.12 g, 96.6% yield).  $^1\text{H}$  NMR (400 MHz, Chloroform-*d*)  $\delta$  8.09 – 8.01 (m, 2H), 7.08 – 7.00 (m, 2H), 4.19 (t,  $J = 6.2$  Hz, 2H), 3.50 (s, 3H), 3.29 (bs, 4H), 3.20 (t,  $J = 6.6$  Hz, 2H), 2.09 (p,  $J = 6.5$  Hz, 2H), 1.14 (t,  $J = 7.1$  Hz, 6H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  166.8, 163.0, 161.7, 156.8, 129.8, 115.4, 114.4, 66.2, 49.6, 43.1, 42.5, 41.2, 27.1, 13.8; **HRMS** (ESI) calc'd for  $\text{C}_{17}\text{H}_{25}\text{N}_4\text{O}_6\text{S}$   $[\text{M} + \text{H}]^+$ : 413.1495, found: 413.1489; **IR** ( $\nu/\text{cm}^{-1}$ , neat) 3236, 2973, 2926, 1697, 1608, 1587, 1494, 1478, 1460, 1421, 1346, 1258, 1162, 1144, 956, 840, 772, 742, 663.

2.2 5-fluoropyridin-2-yl potassium acyltrifluoroborate **11**

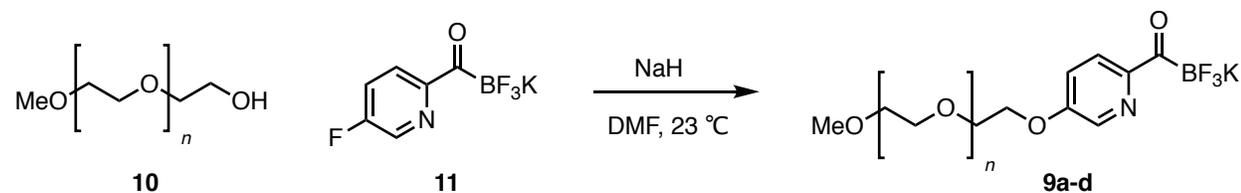
**Scheme S2.** Synthesis of 2-pyridyl KAT **11** with Bode KAT reagent **S4**.



2-Bromo-5-fluoropyridine (1.41 g, 8.00 mmol, 1.00 equiv) was dissolved in anhydrous toluene (20 mL) and cooled to  $-78\text{ }^\circ\text{C}$  in a dry ice/acetone bath. *n*-Butyllithium (1.6 M in hexanes, 5.0 mL, 8.0 mmol, 1.0 equiv) was added dropwise over 30 minutes with the aid of a syringe pump. The resulting yellow-brown solution was stirred for 1 hour at  $-78\text{ }^\circ\text{C}$ . Reagent **S4**<sup>6</sup> (1.48 g, 8.00 mmol, 1.00 equiv) was dissolved separately in anhydrous toluene (15 mL) and subsequently added to the reaction mixture dropwise over 30 minutes with the aid of a syringe pump. The reaction was stirred for an additional 1.5 hours at  $-78\text{ }^\circ\text{C}$ , at which point acetone (0.60 mL, 8.0 mmol, 1.0 equiv) was added to quench any residual *n*-butyllithium. After 10 minutes, an aqueous solution of KF (8.00 M, 3.00 mL, 24.0 mmol, 3.00 equiv) was added dropwise over 1 minute. The reaction flask was removed from the dry ice/acetone bath and stirred for 2 hours, warming to room temperature. To the resulting yellow, heterogeneous solution was added  $\text{CH}_2\text{Cl}_2$  (40.0 mL). The mixture was stirred and filtered. The resulting yellow precipitate was washed with  $\text{CH}_2\text{Cl}_2$  (3 x 50 mL) and acetone (3 x 50 mL). The remaining filter cake (containing **11** and inorganic salts) was washed multiple times with DMF (typically 200 – 250 mL) until the resulting filtrate was colorless. The DMF solution was concentrated under reduced pressure to yield the product **11** as a yellow solid (1.68 g, 90.9% yield). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  8.58 (d,  $J = 2.9$  Hz, 1H), 7.98 (dd,  $J = 8.6, 4.9$  Hz, 1H), 7.77 (td,  $J = 8.8, 2.9$  Hz, 1H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  233.3 – 229.2 (m, C(O)), 159.4 (d,  $J = 257.1$  Hz, CF), 154.6 (s, C), 137.0 (d,  $J = 23.2$  Hz, CH), 124.7 (dd,  $J = 5.1, 2.6$  Hz, CH), 122.9 (d,  $J = 17.9$  Hz, CH); <sup>19</sup>F NMR (471 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  -124.8 (dd,  $J = 8.9, 4.8$  Hz, 1F), -142.8 (dd,  $J = 94.3, 36.7$  Hz, 3F); <sup>11</sup>B NMR (160 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  -1.20 (q,  $J = 51.9$  Hz); HRMS (ESI) calc'd for C<sub>6</sub>H<sub>3</sub>BF<sub>3</sub>NO [M – K]<sup>+</sup>: 192.0249, found: 192.0250; IR ( $\nu/\text{cm}^{-1}$ , neat) 1657, 1587, 1578, 1476, 1291, 1220, 1127, 1086, 1037, 1020, 997, 882, 843, 815, 762, 664.

### 2.3 mPEG KATs **9a-d** and diKAT **12**

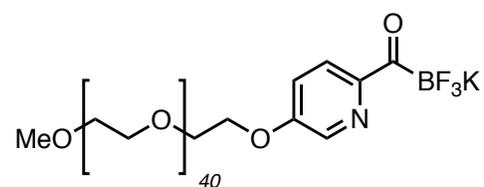
**Scheme S3.** General procedure for the synthesis of 2-pyridyl mPEG-KATs (**9a-d**)



#### General procedure

The poly(ethylene glycol) mono-methyl ether **10** (0.20 mmol, 1.0 equiv) was dissolved in anhydrous toluene (60 mL) and 60 °C and the toluene was evaporated under reduced pressure. The azeotropic distillation was repeated to a total of 3 times (3 x 60 mL) and subsequently dried under high vacuum. The dried PEG reagent was dissolved in anhydrous DMF (5.0 mL) at 60 °C. Sodium hydride (60% disperse in mineral oil, 0.40 mmol, 2.0 equiv) was added to the homogenous solution at room temperature and stirred for 30 minutes. To the deprotonated poly(ethylene glycol) mono-methyl ether solution was added **11** (0.14 g, 0.60 mmol, 3.0 equiv) (also dried by azeotropic distillation with toluene) and the reaction was stirred for 14 hours. Conversion was shown to be greater than 95% according to the  $^1\text{H}$  NMR spectrum of the crude reaction mixture. The excess sodium hydride was quenched by cooling the reaction solution to 0 °C and adding saturated aqueous  $\text{KHF}_2$  (0.50 mL) drop-wise. The reaction mixture was warmed to room temperature and transferred to a dialysis membrane tube (MWCO: 1.0, 2.0 or 3.5 kDa depending on the size of **10** used) and was subsequently dialyzed against aqueous 10 mM  $\text{KF} : \text{CH}_3\text{CN}$  (1 : 1, v/v) (1 x 2.0 L) and  $\text{H}_2\text{O} : \text{CH}_3\text{CN}$  (1 : 1, v/v) (2 x 2.0 L). The final dialyzed solution was lyophilized to yield 2-pyridyl mPEG KAT **9a-d** as a yellow or white solid.

#### (5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 2000 (**9a**):



Prepared from 2.0 kDa poly(ethylene glycol) mono-methyl ether (0.38 g, 0.20 mmol, 1.0 equiv, average  $M_n \sim 1900$ ) by the general procedure for 2-pyridyl mPEG KATs **9a-d**. After dialysis with a 1.0 kDa MWCO dialysis membrane tube and subsequent lyophilization, the product **9a** was isolated as a yellow solid (0.28 g, 50% yield).  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.28 (dd,  $J = 2.9, 0.6$  Hz, 1H), 7.92 (d,  $J = 8.6$  Hz, 1H), 7.40 (dd,  $J = 8.7, 3.0$

Hz, 1H), 4.23 – 4.19 (m, 2H), 3.79 – 3.74 (m, 2H), 3.61 – 3.58 (m, 2H), 3.51 (s, 200H), 3.44 – 3.42 (m, 2H), 3.24 (s, 3H);  $^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )  $\delta$  231.3, 155.3, 151.1, 137.1, 124.8, 119.8, 71.3, 69.9, 69.8, 69.6, 68.7, 67.5, 58.0;  $^{19}\text{F}$  NMR (470 MHz, DMSO- $d_6$ )  $\delta$  -141.8 – -143.5 (m, 3F);  $^{11}\text{B}$  NMR (160 MHz, DMSO- $d_6$ )  $\delta$  -1.15 (bd,  $J = 59.5$  Hz); MALDI-TOF see spectrum.

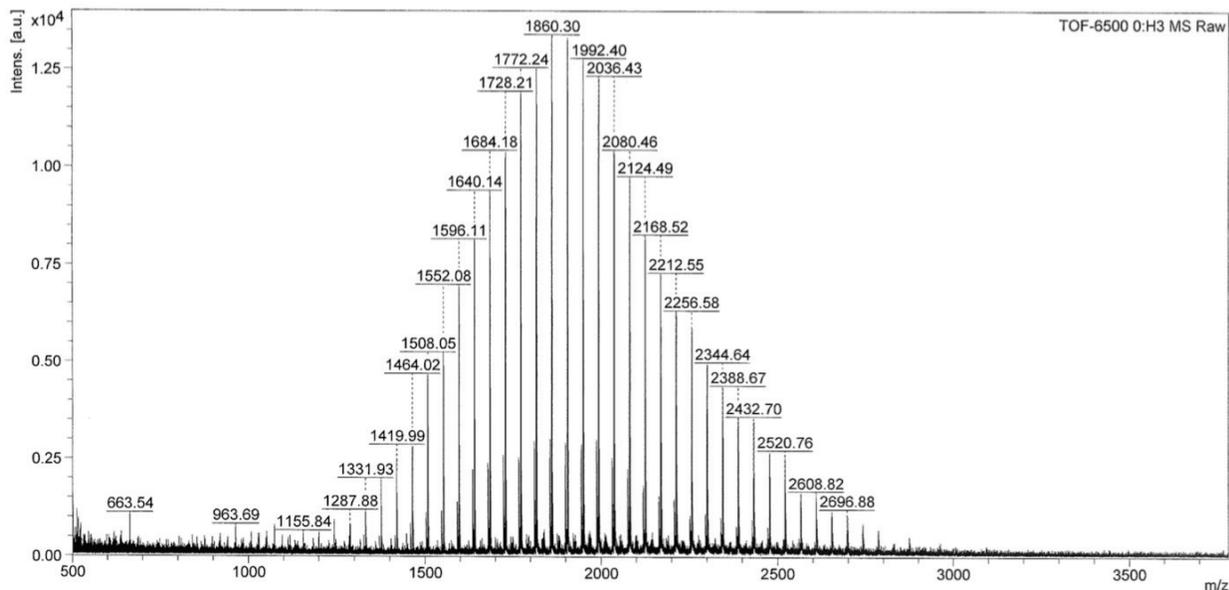


Figure S1. MALDI-TOF of 2.0 kDa mPEG-OH (**10a**)

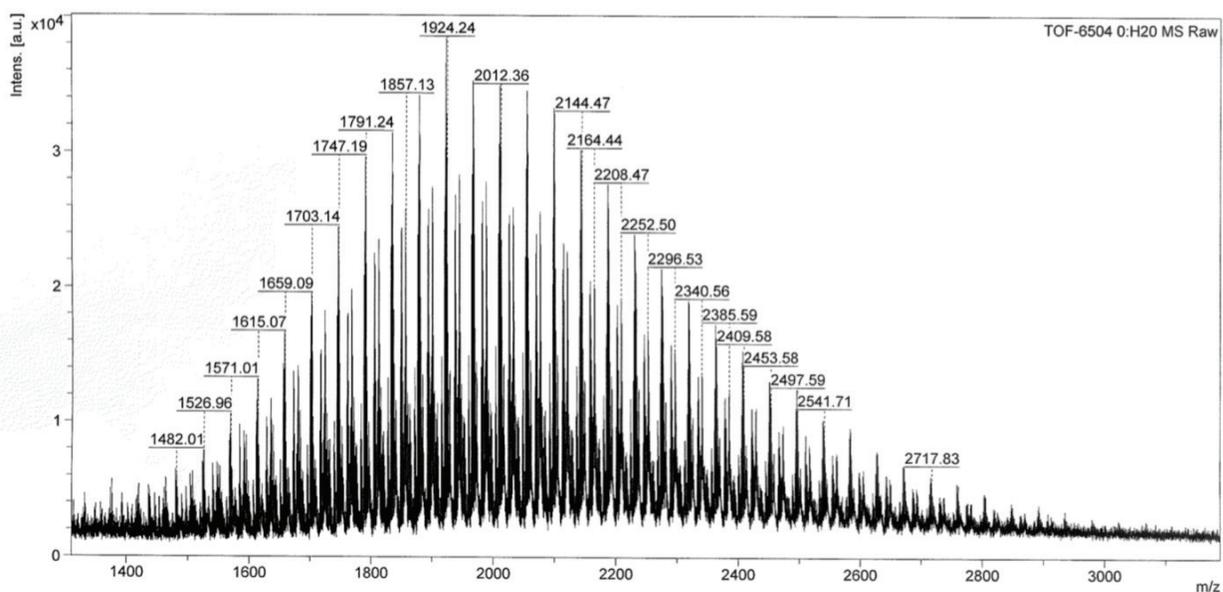
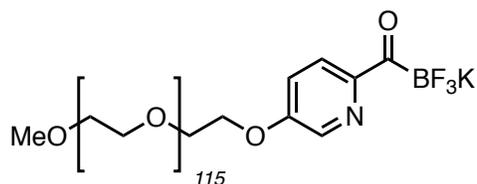


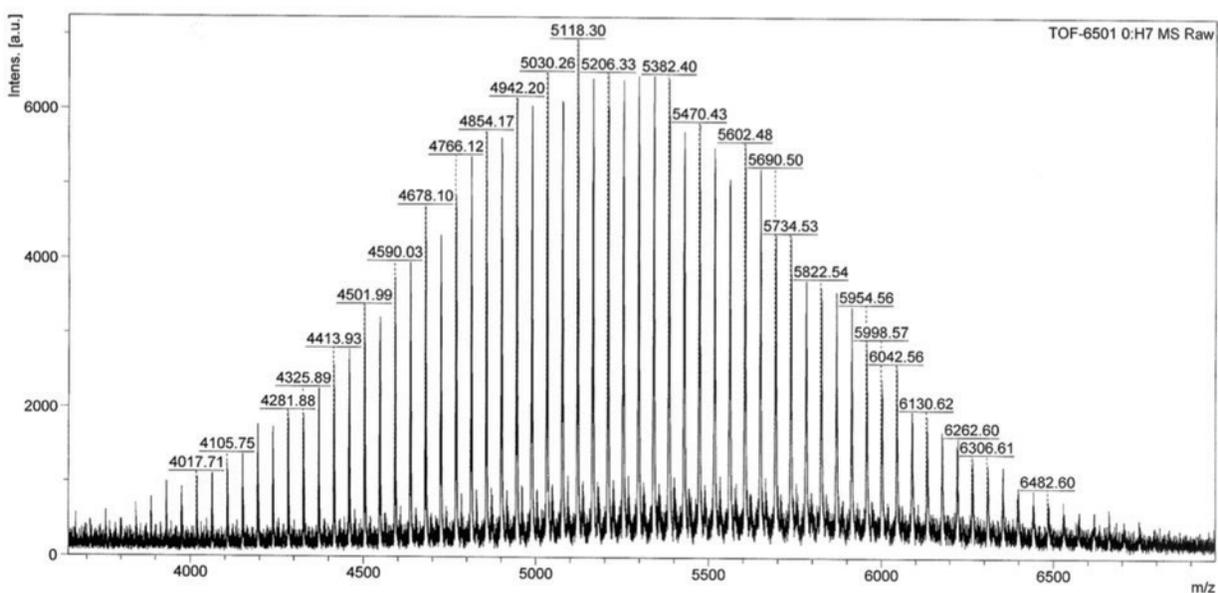
Figure S2. MALDI-TOF of 2.0 kDa 2-pyridyl mPEG-KAT (**9a**)

**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 5000 (9b):**

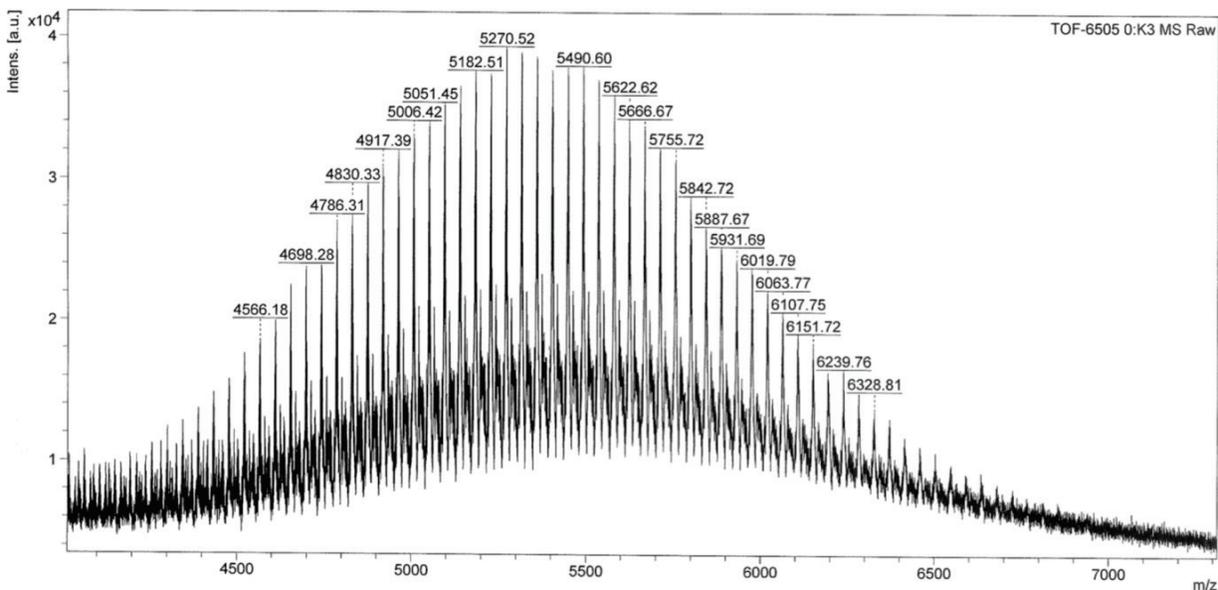


Prepared from 5.0 kDa poly(ethylene glycol) mono-methyl ether (1.0 g, 0.20 mmol, 1.0 equiv, average  $M_n \sim 5000$ ) by the general procedure for 2-pyridyl mPEG KATs **9a-d**. After dialysis with a 2.0 kDa MWCO dialysis membrane tube and subsequent

lyophilization, the product **9b** was isolated as a light yellow solid (685 mg, 65% yield).  $^1\text{H NMR}$  (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.27 (dd,  $J = 2.9, 0.6$  Hz, 1H), 7.92 (d,  $J = 8.6$  Hz, 1H), 7.40 (dd,  $J = 8.7, 2.9$  Hz, 1H), 4.22 – 4.19 (m, 2H), 3.79 – 3.75 (m, 2H), 3.51 (s, 542H), 3.24 (s, 3H);  $^{13}\text{C NMR}$  (151 MHz,  $\text{DMSO-}d_6$ )  $\delta$  155.3, 151.1, 137.1, 124.8, 119.8, 71.3, 69.9, 69.8, 69.6, 68.8, 67.5, 58.0;  $^{19}\text{F NMR}$  (470 MHz,  $\text{DMSO-}d_6$ )  $\delta$  -141.96 – -142.87 (m, 3F);  $^{11}\text{B NMR}$  (160 MHz,  $\text{DMSO-}d_6$ )  $\delta$  -1.15 (bd,  $J = 55.7$  Hz); **MALDI-TOF** see spectrum.

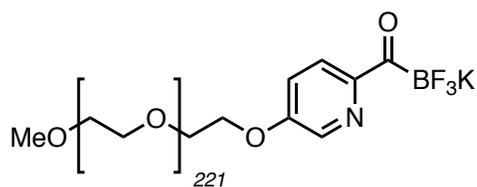


**Figure S3.** MALDI-TOF of 5.0 kDa mPEG-OH (**10b**)



**Figure S4.** MALDI-TOF of 5.0 kDa 2-pyridyl mPEG-KAT (**9b**)

**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 10000 (**9c**):**



Prepared from 10 kDa poly(ethylene glycol) mono-methyl ether (2.0 g, 0.20 mmol, 1.0 equiv, average  $M_n \sim 10000$ ) by the general procedure for 2-pyridyl mPEG KATs **9a-d**. After dialysis with a 3.5 kDa MWCO dialysis membrane tube and subsequent lyophilization, the product **9c** was isolated as a faintly yellow solid (1.86 g, 91% yield).  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.28 (dd,  $J = 3.0, 0.6$  Hz, 1H), 7.93 (d,  $J = 8.6$  Hz, 1H), 7.41 (dd,  $J = 8.7, 2.8$  Hz, 1H), 4.23 – 4.19 (m, 2H), 3.79 – 3.76 (m, 2H), 3.51 (s, 948H), 3.24 (s, 3H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )  $\delta$  155.3, 151.0, 137.0, 124.8, 119.9, 71.3, 69.9, 69.8, 69.6, 68.8, 67.6, 58.0;  $^{19}\text{F}$  NMR (470 MHz,  $\text{DMSO-}d_6$ )  $\delta$  -142.2 (bs, 3F);  $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )  $\delta$  0.38 – -2.44 (m); MALDI-TOF see spectrum.

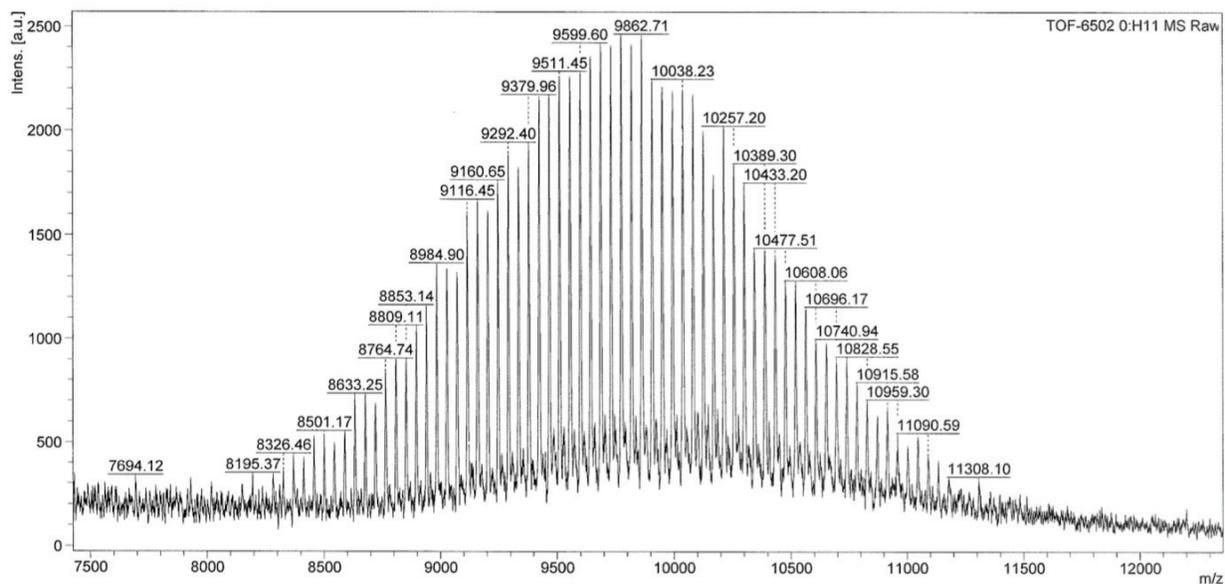


Figure S5. MALDI-TOF of 10 kDa mPEG-OH (10c)

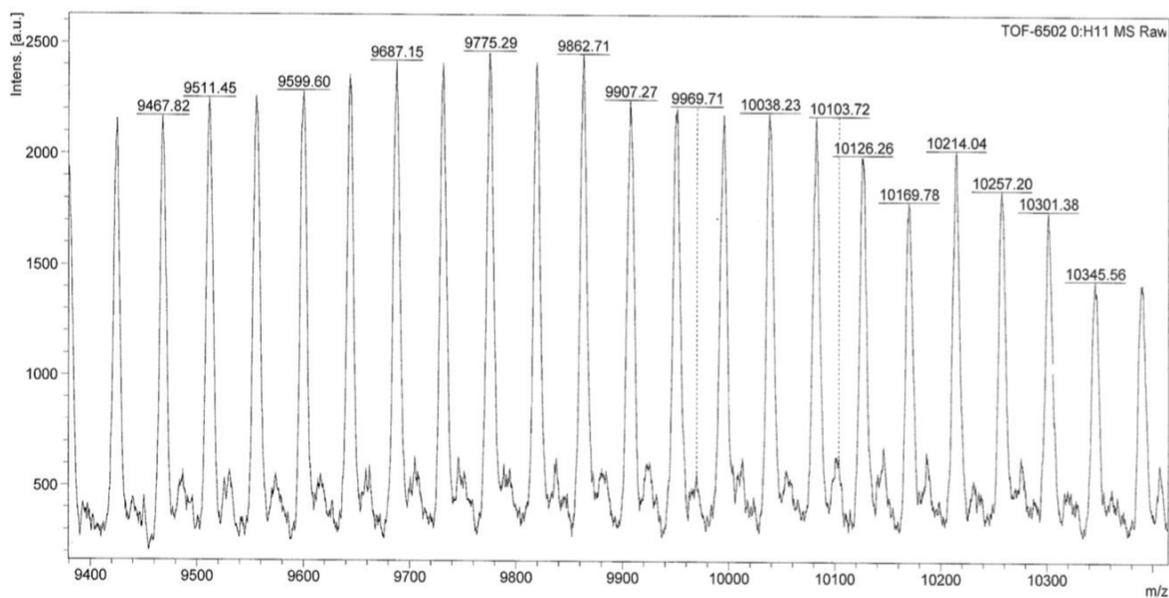
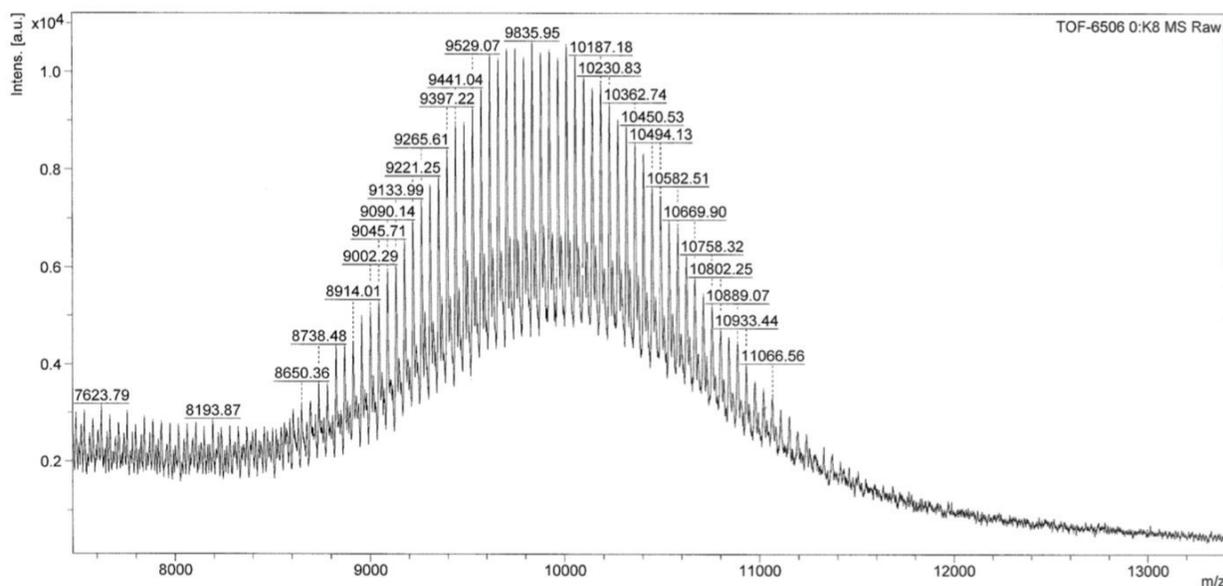
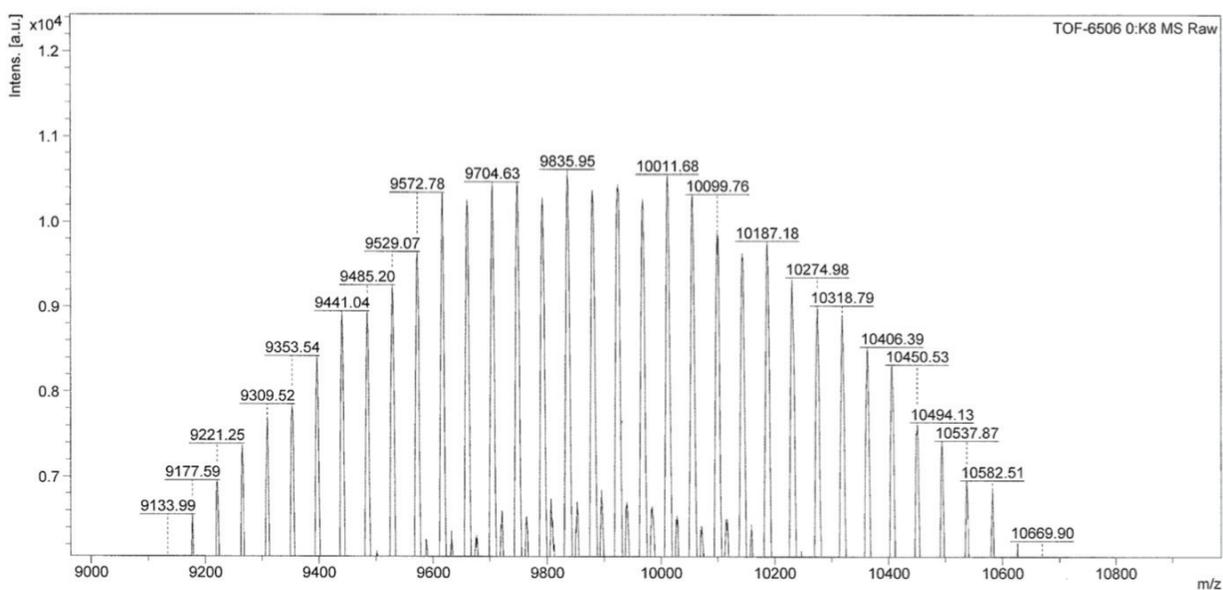


Figure S6. MALDI-TOF of 10 kDa mPEG-OH (10c) – zoomed

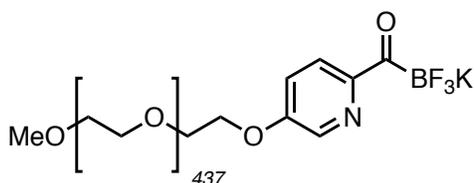


**Figure S7.** MALDI-TOF of 10 kDa 2-pyridyl mPEG-KAT (**9c**)



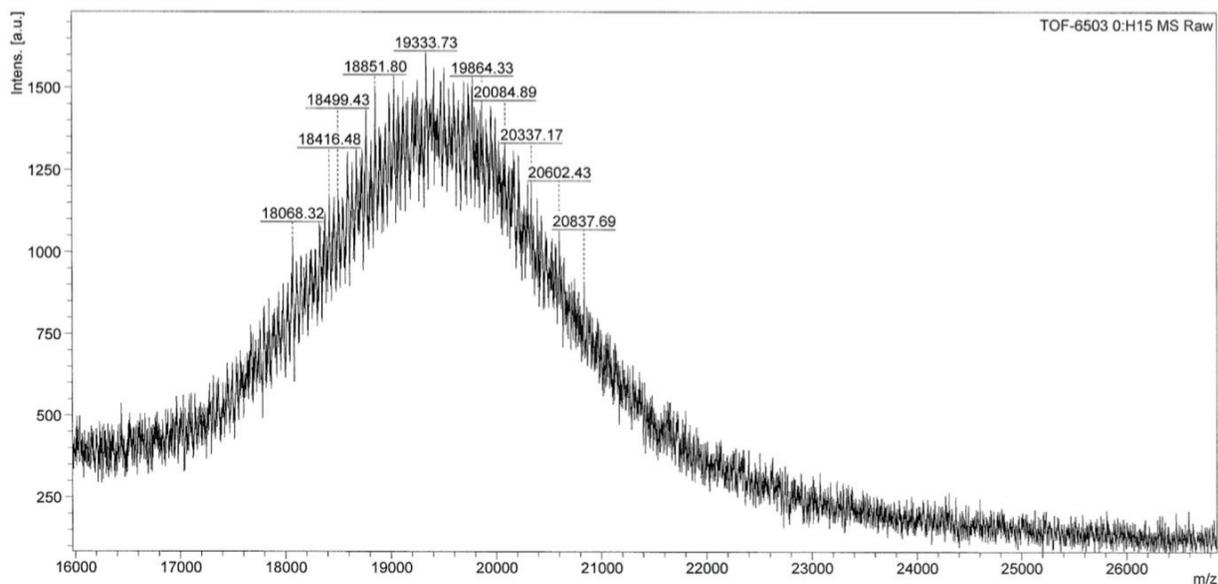
**Figure S8.** MALDI-TOF of 10 kDa 2-pyridyl mPEG-KAT (**9c**) – zoomed

**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 20000 (**9d**):**

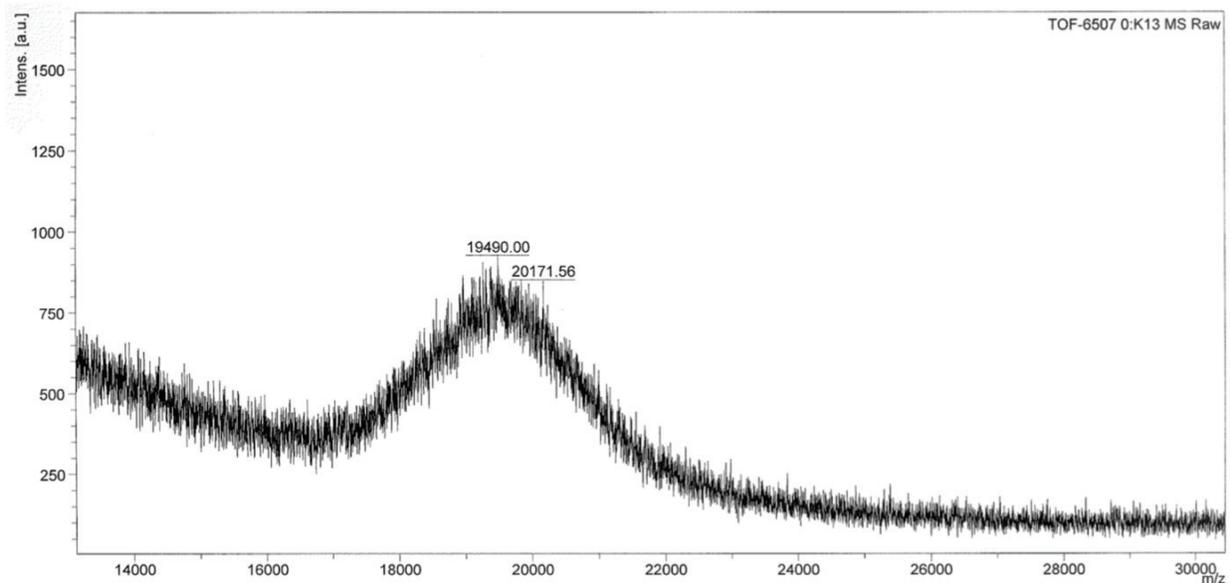


Prepared from 20 kDa poly(ethylene glycol) mono-methyl ether (2.00 g, 0.10 mmol, 1.0 equiv, average  $M_n \sim 20000$ ) by the general procedure for 2-pyridyl mPEG KATs **9a-d**. After dialysis with a 3.5 kDa MWCO dialysis membrane tube and

subsequent lyophilization, the product **9d** was isolated as a white solid (1.87 g, 94% yield).  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta$  8.27 (d,  $J = 2.9$  Hz, 1H), 7.92 (d,  $J = 8.6$  Hz, 1H), 7.39 (dd,  $J = 8.6, 3.0$  Hz, 1H), 4.23 – 4.18 (m, 2H), 3.79 – 3.75 (m, 2H), 3.51 (br s, 1760H), 3.24 (s, 3H);  $^{13}\text{C}$  NMR (151 MHz, DMSO)  $\delta$  155.3, 151.2, 137.1, 124.8, 119.7, 71.3, 69.9, 69.8, 69.6, 68.8, 67.5, 58.0;  $^{19}\text{F}$  NMR (470 MHz, DMSO- $d_6$ )  $\delta$  -142.2 (bs, 3F);  $^{11}\text{B}$  NMR (160 MHz, DMSO- $d_6$ )  $\delta$  -1.11 (bs); MALDI-TOF see spectrum.

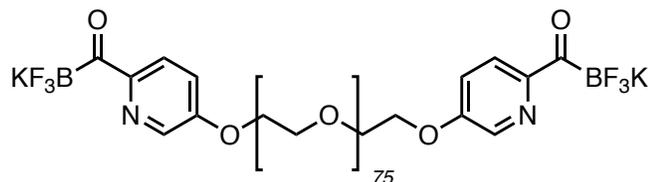


**Figure S9.** MALDI-TOF of 20 kDa mPEG-OH (**10d**)



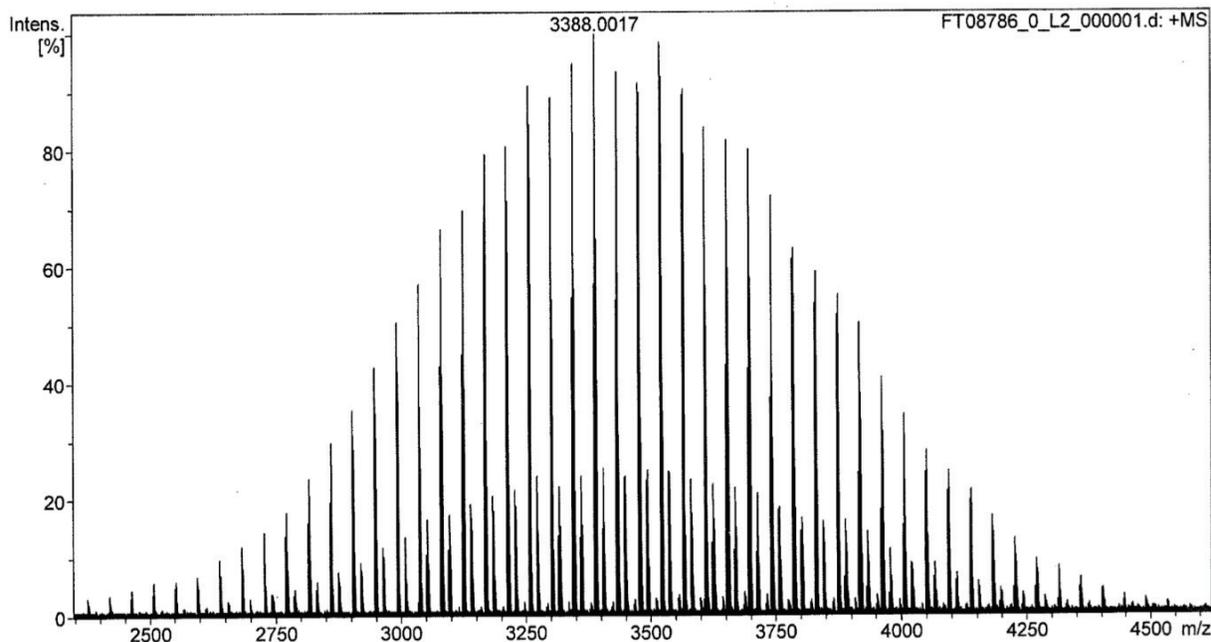
**Figure S10.** MALDI-TOF of 20 kDa 2-pyridyl mPEG-KAT (**9d**)

**(((oxybis(ethane-2,1-diyl))bis(oxy))bis(pyridine-5,2-diyl))bis((trifluoro- $\lambda^4$ -boranyl)methanone), dipotassium salt – PEG 3350 (**12**):**

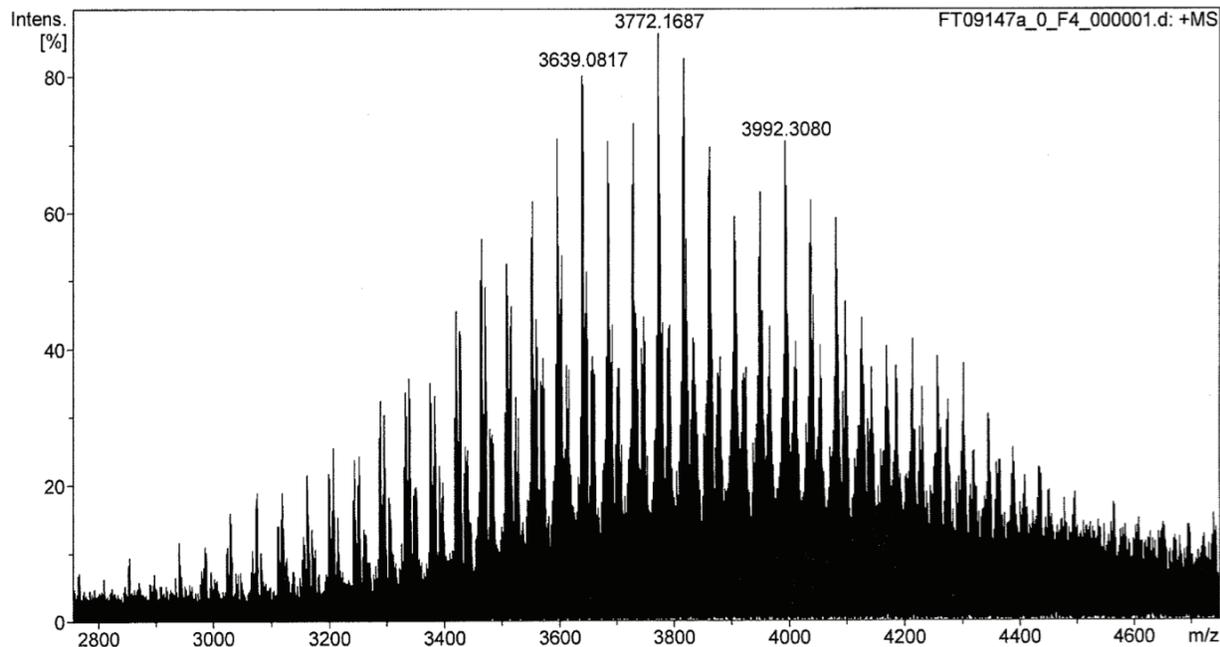


Prepared from 3.4 kDa poly(ethylene glycol) (0.70 g, 0.20 mmol, 1.0 equiv, average  $M_n \sim 3350$ ) by the general procedure for 2-pyridyl mPEG KATs **9a-d**. 2-pyridyl KAT **11** (462 mg, 2.00 mmol, 10.0

equiv) and sodium hydride (32 mg, 0.80 mmol, 4.0 equiv) were used in appropriate excess. After dialysis with a 1.0 kDa MWCO dialysis membrane tube and subsequent lyophilization, the diKAT product **12** was isolated as a light yellow solid (658 mg, 91% yield).  $^1\text{H NMR}$  (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.28 (d,  $J = 2.9$  Hz, 2H), 7.92 (d,  $J = 8.6$  Hz, 2H), 7.40 (dd,  $J = 8.7, 2.9$  Hz, 2H), 4.24 – 4.18 (m, 4H), 3.81 – 3.74 (m, 4H), 3.51 (s, 328H);  $^{13}\text{C NMR}$  (151 MHz,  $\text{DMSO-}d_6$ )  $\delta$  231.0, 155.3, 151.1, 137.1, 124.8, 119.8, 69.9, 69.7, 68.7, 67.5;  $^{19}\text{F NMR}$  (470 MHz,  $\text{DMSO-}d_6$ )  $\delta$  -141.92 – -142.59 (m, 6F);  $^{11}\text{B NMR}$  (160 MHz,  $\text{DMSO-}d_6$ )  $\delta$  -1.14 (bd,  $J = 60.6$  Hz); **MALDI-TOF** see spectrum.



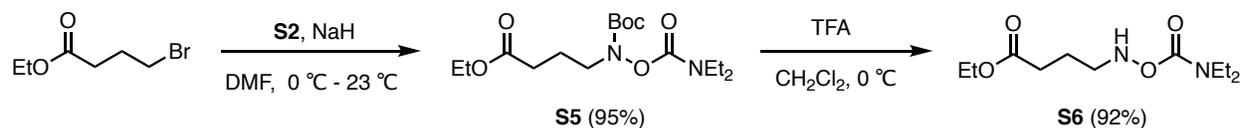
**Figure S11.** MALDI-TOF of 3.4 kDa PEG-diol



**Figure S12.** MALDI-TOF of 3.4 kDa bis 2-pyridyl PEG-KAT(12)

## 2.4 Additional small molecule reagents

**Scheme S4.** Synthesis of hydroxylamine ester **S6** for KAT ligation quenching.

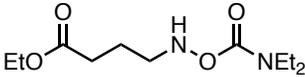


### Ethyl 4-((*tert*-butoxycarbonyl)((diethylcarbamoyl)oxy)amino)butanoate (**S5**)

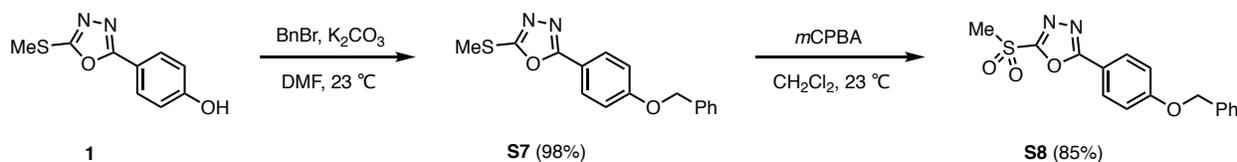
CCOC(=O)CCCN(C(=O)OCC)C(=O)OCC
 Sodium hydride (440 mg, 11.0 mmol, 1.10 equiv) was suspended in anhydrous DMF (20.0 mL) and cooled to 0 °C in an ice bath with stirring. A solution of **S2** (2.32 g, 10.0 mmol, 1.00 equiv) in anhydrous DMF (20 mL) was added to the stirring suspension of NaH over 5 min. The resulting mixture was stirred at 0 °C for 30 min, at which point ethyl 4-bromobutanoate (1.9 mL, 13 mmol, 1.3 equiv) was added. The reaction solution was warmed to room temperature and stirred for 20 hours. The solution was cooled back down to 0 °C and the reaction was quenched with saturated aqueous NH<sub>4</sub>Cl (50 mL). The resulting suspension was subsequently warmed to room temperature and diluted with H<sub>2</sub>O (50 mL) and EtOAc (300 mL). The aqueous phase was separated and the organic phase was washed with H<sub>2</sub>O (50 mL) and brine (4 x 50 mL). The organic

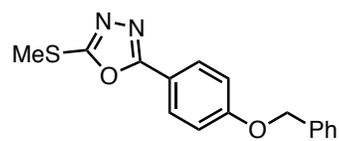
phase was dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 10% to 20% EtOAc in hexanes. The product **S5** was isolated as a pale yellow oil (3.29 g, 95% yield). The spectral data of **S5** was in agreement with that reported in the literature.<sup>7</sup>  $^1\text{H NMR}$  (400 MHz, Chloroform-*d*)  $\delta$  4.11 (q,  $J = 7.1$  Hz, 2H), 3.65 (bt,  $J = 6.8$  Hz, 2H), 3.30 (bq,  $J = 7.1$  Hz, 4H), 2.39 (t,  $J = 7.5$  Hz, 2H), 1.89 (dd,  $J = 7.8$ , 6.9 Hz, 2H), 1.44 (s, 9H), 1.23 (t,  $J = 7.1$  Hz, 3H), 1.16 (t,  $J = 7.1$  Hz, 6H);  $^{13}\text{C NMR}$  (101 MHz, Chloroform-*d*)  $\delta$  173.2, 155.1, 154.2, 81.9, 60.4, 49.7, 43.1, 41.7, 31.5, 28.3, 22.9, 14.3, 13.5.

### Ethyl 4-(((diethylcarbamoyl)oxy)amino)butanoate (**S6**)

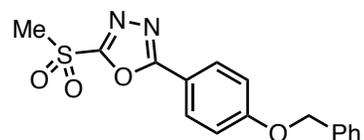

 Compound **S5** (1.91 g, 5.50 mmol, 1.00 equiv) was dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$  (10 mL) and cooled to 0 °C in an ice bath with stirring. Trifluoroacetic acid (9.0 mL,  $1.2 \times 10^2$  mmol, 22 equiv) was added and the reaction was stirred at 0 °C for 2 hours.  $\text{CH}_2\text{Cl}_2$  (10 mL) was added followed by the *careful* addition of saturated aqueous  $\text{NaHCO}_3$  (50 mL), along with solid  $\text{NaHCO}_3$  with stirring, until the solution became weakly alkaline (pH = 8.0). The resulting suspension was warmed to room temperature, the phases were separated and the aqueous phase was extracted with  $\text{CH}_2\text{Cl}_2$  (2 x 50 mL). The combined organic phases were washed with brine (50 mL), dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated under reduced pressure. The product **S6** (1.25 g, 92%) was isolated as a yellow oil and used without further purification.  $^1\text{H NMR}$  (400 MHz, Chloroform-*d*)  $\delta$  6.06 (bs, 1H), 4.11 (q,  $J = 7.2$  Hz, 2H), 3.27 (s, 4H), 2.99 (t,  $J = 6.9$  Hz, 2H), 2.41 (t,  $J = 7.4$  Hz, 2H), 1.86 (p,  $J = 7.2$  Hz, 2H), 1.23 (t,  $J = 7.1$  Hz, 3H), 1.12 (t,  $J = 7.1$  Hz, 5H);  $^{13}\text{C NMR}$  (101 MHz, Chloroform-*d*)  $\delta$  173.3, 156.8, 60.5, 52.0, 42.4, 41.1, 31.8, 22.7, 14.3, 13.8; **HRMS** (ESI) calc'd for  $\text{C}_{11}\text{H}_{23}\text{N}_2\text{O}_4$   $[\text{M} + \text{H}]^+$ : 247.1652, found: 247.1654; **IR** ( $\nu/\text{cm}^{-1}$ , neat) 3237, 2976, 2936, 1732, 1701, 1477, 1420, 1378, 1272, 1179, 1157, 1097, 1032, 961, 781, 756.

### Scheme S5. Synthesis of methylsulfone phenyloxadiazole **S8** for cysteine capping.



**2-(4-(benzyloxy)phenyl)-5-(methylthio)-1,3,4-oxadiazole (S7)**

To a solution of compound **1** (1.3 g, 6.3 mmol, 1.00 equiv) in anhydrous DMF (12 mL) was added  $K_2CO_3$  (0.87 g, 6.3 mmol, 1.0 equiv). The resulting suspension was stirred at room temperature for 20 min at which point benzyl bromide (0.75 mL, 6.3 mmol, 1.0 equiv) was added. The reaction mixture was stirred at room temperature for 20 hours, diluted with EtOAc (100 mL) and washed with  $H_2O$  (2 x 50 mL) and brine (3 x 50 mL). The organic phase was collected, dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 20% to 40% EtOAc in hexanes. This afforded the product **S7** as a white, crystalline solid (1.8 g, 98% yield).  $^1H$  NMR (400 MHz, Chloroform-*d*)  $\delta$  7.97 – 7.91 (m, 2H), 7.50 – 7.29 (m, 5H), 7.10 – 7.03 (m, 2H), 5.13 (s, 2H), 2.76 (s, 3H);  $^{13}C$  NMR (101 MHz, Chloroform-*d*)  $\delta$  165.9, 164.4, 161.5, 136.3, 128.8, 128.6, 128.4, 127.6, 116.6, 115.5, 70.3, 14.8. HRMS (ESI) calc'd for  $C_{16}H_{15}N_2O_2S$  [ $M + H$ ] $^+$ : 299.0849, found: 299.0851; IR ( $\nu/cm^{-1}$ , neat) 3028, 2930, 1739, 1613, 1504, 1481, 1453, 1378, 1262, 1198, 1176, 1040, 1028, 832, 732, 694, 673.

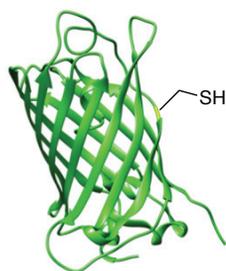
**2-(4-(benzyloxy)phenyl)-5-(methylsulfonyl)-1,3,4-oxadiazole (S8)**

Compound **S7** (1.23 g, 4.10 mmol, 1.00 equiv) was dissolved in anhydrous  $CH_2Cl_2$  (20 mL) and cooled to 0 °C in an ice bath with stirring. *m*-CPBA (2.96 g, 12.0 mmol, 2.93 equiv) was dissolved separately in  $CH_2Cl_2$  (40 mL) and added to **S7** drop-wise over 30 min. The ice bath was removed and the resulting reaction mixture was stirred at room temperature for 16 hours. The reaction was quenched with saturated aqueous  $Na_2S_2O_5$  (40 mL) and continued to stir at room temperature for 30 minutes. The resulting suspension was poured into  $H_2O$  (50 mL), the phases were separated and the aqueous phase was extracted with  $CH_2Cl_2$  (3 x 50 mL). The combined organic phases were dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure. The crude material was dissolved in EtOAc (100 mL) and washed with saturated aqueous  $NaHCO_3$  (4 x 50 mL),  $H_2O$  (3 x 50 mL) and brine (50 mL). The organic phase was dried over  $Na_2SO_4$ , filtered and concentrated under reduced pressure. The crude material was purified by flash chromatography using a gradient elution of 20 to 50% EtOAc in hexanes. This afforded the product **S8**, as a white solid (1.16 g, 3.50 mmol, 85.3% yield).  $^1H$  NMR (400 MHz, Acetone-*d*<sub>6</sub>)  $\delta$  8.13 – 8.06 (m, 2H), 7.52 (ddt,  $J = 7.5, 1.4, 0.7$  Hz, 2H), 7.42 (ddt,  $J = 8.1, 6.5, 1.1$  Hz, 2H), 7.39 – 7.33 (m, 1H), 7.31 – 7.25 (m, 2H), 5.28 (s, 2H), 3.62 (s, 3H);  $^{13}C$  NMR (101 MHz,

Acetone)  $\delta$  167.2, 163.7, 163.2, 137.6, 130.4, 129.5, 129.1, 128.7, 116.8, 116.1, 71.0, 43.6; **HRMS** (ESI) calc'd for  $C_{16}H_{15}N_2O_4S$   $[M + H]^+$ : 331.0747, found: 331.0749; **IR** ( $\nu/cm^{-1}$ , neat) 3028, 2924, 1739, 1609, 1585, 1429, 1383, 1343, 1299, 1251, 1183, 1147, 1082, 992, 950, 843, 757, 740, 704, 664.

### 3. Protein expression and purification

#### 3.1 sfGFP(S147C) (5)



##### **Molecular subcloning:**

The plasmid, pBAD24-sfGFPx1 was a gift from Sankar Adhya and Francisco Malagon<sup>8</sup> (Addgene plasmid # 51558). Following an overnight liquid culture inoculation in lysogeny broth (LB) – Miller (10 mL) (Ampicillin 100  $\mu g/ mL$ ), the plasmid was isolated from DH5 $\alpha$  cells using a GeneJET Plasmid Miniprep Kit (Thermo Fisher) following the manufacturer's instructions. Site-directed mutagenesis for the S147C mutation was carried out following the QuikChange II Site-Directed Mutagenesis protocol (Agilent). The following primers were used to for the S147C mutation<sup>9</sup>:

Forward: 5' – CATAAGCTGGAATACAATTTAACTGCCACAATGTTTACATCACCGCC – 3'

Reverse: 5' – GGCGGTGATGTAAACATTGTGGCAGTTAAAATTGTATTCCAGCTTATG – 3'

A 3' – GSGHHHHHH tag was inserted using Overhang PCR with the following primers:

Forward: 5' – TCATCACCACTGATGACCC – 3'

Reverse: 5' – TGATGACCAGAACCTTTGTACAGTACAGTTCATCCATACCATG – 3'

The resulting sequence of pBAD24–sfGFP(S147C)–His<sub>6</sub> was confirmed by DNA Sanger sequencing performed by MicroSynth AG (Balgach, Switzerland).

##### **Protein expression and purification:**

A dense overnight culture of *E. coli* strain DH10B (Thermo Fisher) transformed with pBAD24–sfGFP(S147C)–His<sub>6</sub> was used to inoculate 1.0 L of LB-Miller medium supplemented with 100  $\mu g/ mL$  of ampicillin in 2.0 L beveled Erlenmeyer flasks. The culture was incubated at 37 °C, 100 rpm until

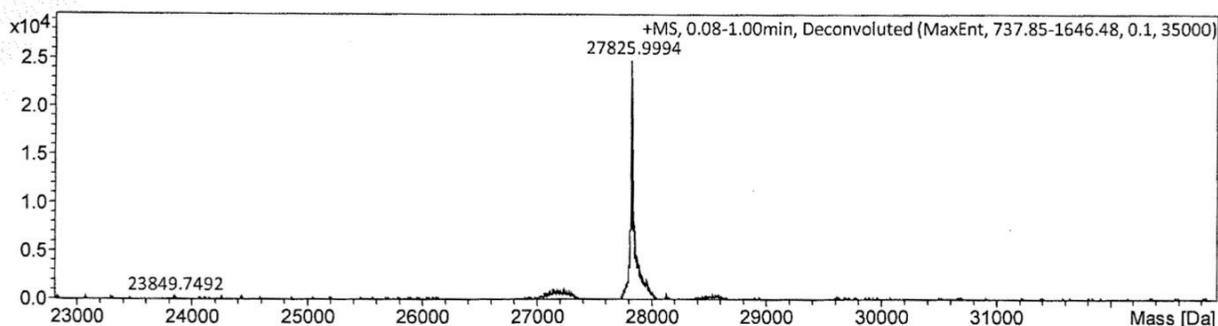
OD<sub>600</sub> reached 0.6 – 0.7, at which point protein expression was induced by the addition of 0.2% (w/v) L-(+)-arabinose. After an additional 21 h of culturing, the cells were harvested by centrifugation at 5000 rpm and 4 °C for 45 minutes. The cell pellet was washed with 20 mM sodium phosphate (Na-Phos) buffer pH 7.4 (100 mL), re-suspended in 20 mM Na-Phos, 500 mM NaCl, 35 mM imidazole, pH 7.4 (15 mL) and stored at -80 °C.

Following thawing on ice, the suspension was treated with DTT (1 mM), lysozyme (1 mg/ mL) and a spatula tip of DNase I and incubated on ice for 1.0 hour. The cells were lysed by sonication (5 x 1 min at 0 °C) and the solution cleared by centrifugation at 14000 rpm and 4 °C for 30 minutes. The supernatant was filtered via passage through a 0.2 µm membrane filter and loaded onto 4 mL of Ni(II)-NTA agarose resin equilibrated with 20 mM Na-Phos, 500 mM NaCl, 35 mM imidazole, 1 mM DTT, pH 7.4. Following washing with the same buffer (50 mL), the protein was eluted with 15 mL of the equilibration buffer containing 500 mM imidazole. The eluent containing **5** was dialyzed 3 times against 2.0 L of 20 mM Tris HCl, 1 mM DTT, pH 8.5 at 4 °C. The dialyzed protein solution was further purified by anion exchange chromatography (Mono Q 5/50 GL) with buffer A (20 mM Tris HCl, 1 mM DTT, pH 8.5) and a gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, 1 mM DTT, pH 8.5). The purified protein **5** was concentrated and exchanged into degassed 20 mM Tris HCl, 1 mM DTT, pH 7.4 buffer through spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) to a final concentration of 500 µM. The resulting solution containing **5** may be stored for up to one month on ice at 0 °C, without appreciable, irreversible oxidative decomposition. The expression yield of **5** was found to be approximately 15 mg/ 1.0 L culture.

An aliquot of **5** (0.01 µmol) was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass determination (**ESI-MS** *see* Figure S13).

Calculated mass: 27826.1 Da (27846.1 Da for sequence -20 Da for GFP chromophore formation)

Observed mass: 27826.0 Da

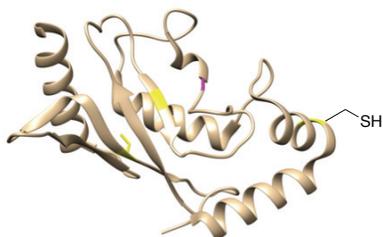


**Figure S13.** Deconvoluted (ESI) mass spectrum of sfGFP(S147C) 5

Protein sequence:

MRKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTCLKFICTTGKLPVPWPTLVTT  
 LTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIEL  
 KGIDFKEDGNILGHKLEYNFNCHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPI  
 GDGPVLLPDNHYLSTQSVLSKDPNEKRDHMLLEFVTAAGITHGMDELTKGSGHHHHHH

### 3.2 Ubc9(C93A)



#### Molecular subcloning:

A codon-optimized gene encoding for wild type Ubc9 was purchased from ATG Biosynthetics GmbH (Merzhausen, Germany) and subcloned into the vector pET-28b (+) in frame with an *N*-terminal His<sub>6</sub> tag, using the restriction enzymes *Nde*I (5') and *Xho*I (3'). Site-directed mutagenesis for the C93A mutation was carried out

following the QuikChange II Site-Directed Mutagenesis protocol (Agilent). The following primers were used for the C93A mutation:

Forward: 5' – CCCAGCGGTACGGTTGCCCTGAGCATCCTTG – 3'

Reverse: 5' – CAAGGATGCTCAGGGCAACCGTACCGCTGGG – 3'

The resulting sequence of pET-28b (+)-His<sub>6</sub>-UBC9(C93A) was confirmed by DNA Sanger sequencing performed by MicroSynth AG (Balgach, Switzerland).

**Protein expression and purification:**

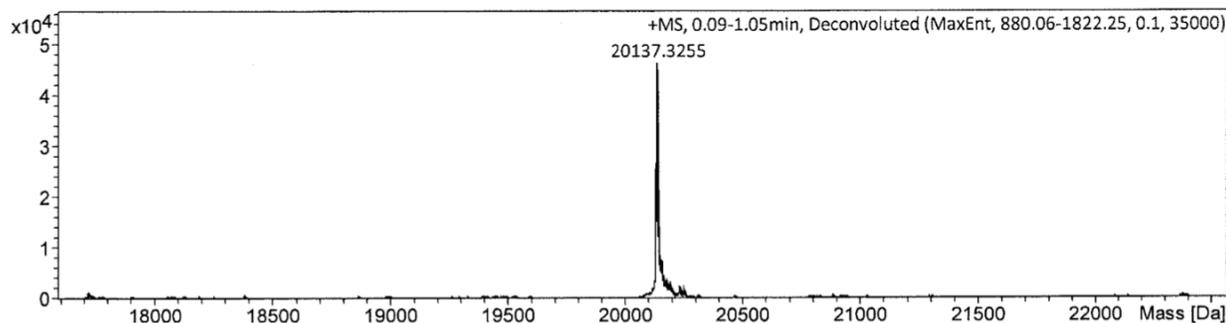
A dense overnight culture of *E. coli* strain BL21-gold(DE3) competent cells transformed with pET-28b (+)-His<sub>6</sub>-UBC9(C93A) was used to inoculate 1.0 L of LB-Miller medium supplemented with 50 µg/mL of kanamycin in 2.0 L beveled Erlenmeyer flasks. The culture was incubated at 37 °C, 100 rpm until OD<sub>600</sub> reached 0.6 – 0.7, at which point protein expression was induced by the addition of 1.0 mM IPTG. After an additional 3 h of culturing, the cells were harvested by centrifugation at 5000 rpm and 4 °C for 45 minutes. The cell pellet was re-suspended in 50 mM HEPES, 350 mM NaCl, 20 mM imidazole, pH 8.0 (20 mL) and stored at -80 °C.

Following thawing on ice, the suspension was treated with DTT (1 mM), lysozyme (20 µg/mL), phenylmethylsulfonyl fluoride (PMSF) (1 mM) and a spatula tip of DNase I and incubated on ice for 1.0 hour. The cells were lysed by sonication (5 x 1 min at 0 °C) and the solution cleared by centrifugation at 14000 rpm and 4 °C for 30 minutes. The supernatant was filtered via passage through a 0.2 µm membrane filter and loaded onto 4 mL of Ni(II)-NTA agarose resin equilibrated with 20 mM HEPES, 350 mM NaCl, 20 mM imidazole, 1 mM DTT, pH 8.0. Following washing with the same buffer (50 mL), the protein was eluted with 15 mL of the equilibration buffer containing 400 mM imidazole. The eluent containing Ubc9 (C93A) was dialyzed 3 times against 2.0 L of 50 mM sodium phosphate, 1 mM DTT, pH 7.0 at 4 °C. The dialyzed protein solution was further purified by cation exchange chromatography (Mono S 5/50 GL) with buffer A (50 mM Na-Phos, 1 mM DTT, pH 7.0) and a gradient of buffer B (50 mM Na-Phos, 1.0 M NaCl, 1.0 mM DTT, pH 7.0). The purified protein was concentrated via spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) to a final concentration of 200 µM and dialyzed 3 times against 2.0 L of 50 mM HEPES, 50 mM NaCl, 1 mM DTT, pH 7.6. The protein was then portioned into aliquots (0.05 µmol) and stored at -80 °C. The expression yield of Ubc9(C93A) was found to be approximately 30 mg/ 1.0 L culture.

An aliquot of Ubc9(C93A) (0.01 µmol) was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass determination (**ESI-MS** see Figure S14).

Calculated mass: 20138.1 Da

Observed mass: 20137.3 Da

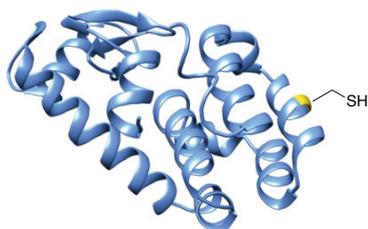


**Figure S14.** Deconvoluted (ESI) mass spectrum of Ubc9(C93A)

Protein sequence:

GSSHHHHHSSGLVPRGSHMMSGIALSRLAQRKAWRKDHPFGFVAVPTKNPDGTMNLMNW  
 ECAIPGKKGTPWEGGLFKLRMLFKDDYPSSPPKCKFEPPLFHPNVYPSGTV~~A~~LSILEEDKDWRP  
 AITIKQILLGIQELLNEPNIQDPAQAEAYTIYCQNRVEYEKRVRAQAKKFAPS

### 3.3 T4L(V131C)



#### Molecular subcloning:

A codon-optimized gene encoding for WT\* (no cysteines: C54T, C97A) T4 lysozyme incorporating the mutation V131C was purchased from ATG Biosynthetics GmbH (Merzhausen, Germany) and subcloned into the vector pET-28b (+) using the restriction enzymes, *Xba*I (5') and *Xho*I (3').

The resulting sequence of pET-28b (+)-T4L(V131C) was confirmed by DNA Sanger sequencing performed by MicroSynth AG (Balgach, Switzerland).

#### Protein expression and purification:

A dense overnight culture of *E. coli* strain BL21-gold(DE3) competent cells transformed with pET-28b (+)-T4L(V131C) was used to inoculate 1.0 L of LB-Miller medium supplemented with 50 µg/ mL of kanamycin in 2.0 L beveled Erlenmeyer flasks. The culture was incubated at 37 °C, 100 rpm until OD<sub>600</sub> reached 0.6 – 0.7, at which point protein expression was induced by the addition of 1.0 mM IPTG. After an additional 2 h of culturing, the cells were harvested by centrifugation at 5000 rpm and 4

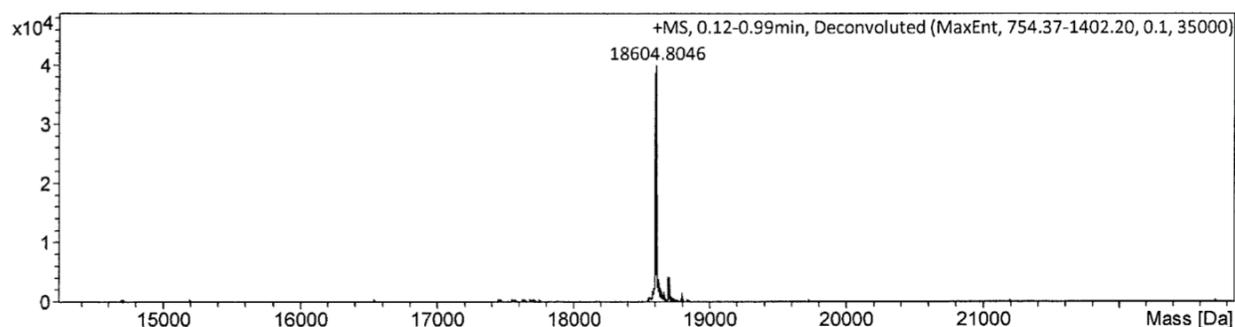
°C for 25 minutes. The cell pellet was re-suspended in 25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6 buffer (20 mL) and stored at -20 °C.

Following thawing on ice, the suspension was treated with DTT (1 mM) and Benzonase A (1 µL/ 10 mL culture) and incubated on ice for 1.0 hour. To ensure quantitative lysis, the cells were sonicated (2 x 1 min at 0 °C) and the solution cleared by centrifugation at 14000 rpm and 4 °C for 30 minutes. The cell lysis supernatant containing T4L(V131C) was filtered through a 0.2 µm membrane filter and directly purified by cation exchange chromatography (Mono S 5/50 GL) with buffer A (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6) and a 0 – 20% step-wise gradient of buffer B (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, 1.0 M NaCl, pH 7.6). Fractions containing T4L(V131C) (which eluted at 20% buffer B) were pooled and passed through a 30 kDa Molecular weight cutoff filter via spin diafiltration (Amicon® Ultra – 15, 30 kDa MWCO) to remove higher molecular weight impurities. The isolated T4L(V131C) was concentrated (500 µM) and exchange into degassed 0.1 M potassium phosphate (K-Phos), 5 mM DTT, pH 6.5 buffer by spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO). The resulting solution was stored at 0 °C on ice. The expression yield of T4L(V131C) was approximately 20 mg/ 1.0 L culture.

An aliquot of T4L(V131C) (0.01 µmol) was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass determination (**ESI-MS** see Figure S15).

Calculated mass: 18605.4 Da

Observed mass: 18604.8 Da

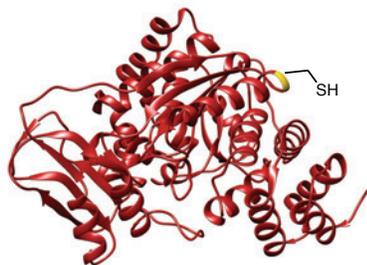


**Figure S15.** Deconvoluted (ESI) mass spectrum of T4L(V131C)

Protein sequence:

MNIFEMLRIDEGLRLKIYKDTEGYTYTIGIGHLLTKSPSLNAAKSELDKAIGRNTNGVITKDEAEK  
LFNQDVDAAVRGILRNAKLKPVYDSLDAVRRRAALINMVFQMGETGVAGFTNSLRMLQQKRW  
DEAACNLAKSRWYNQTPNRAKRVITTFRTGTWDAYKNL

### 3.4 HTP(S261C)



The plasmid, pYSBLIC containing the gene encoding for HTP(S261C) with an *N*-terminal His<sub>6</sub>-Tag was a gift from Marcel Grogg and Donald Hilvert, which was transformed into Rosetta<sup>TM</sup>(DE3)pLysS Competent Cells (Novagen). A dense overnight culture was used to inoculate 1.0 L of an auto-induction media<sup>10</sup> supplemented with 50 µg/ mL of kanamycin in 2.0 L beveled Erlenmeyer flasks. The culture was incubated at 37 °C, 100

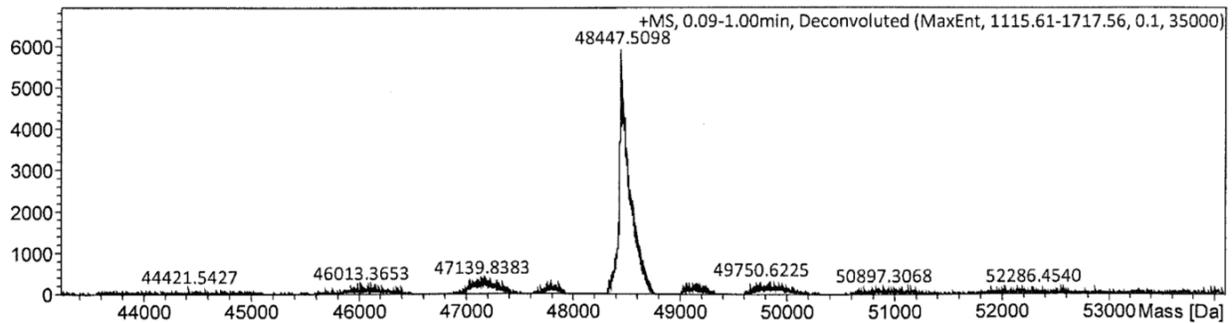
rpm for 6.0 hours, at which point the cells were harvested by centrifugation at 5000 rpm and 4 °C for 45 minutes. The cell pellet was re-suspended in 20 mM Na-Phos, 500 mM NaCl, 35 mM imidazole buffer (30 mL) and stored at -20 °C.

Following thawing on ice, the suspension was treated with DTT (1 mM), lysozyme (1 mg/ mL) and a spatula tip of DNase I and incubated on ice for 1.0 hour. The cells were lysed by sonication (5 x 1 min at 0 °C) and the solution cleared by centrifugation at 14000 rpm and 4 °C for 30 minutes. The supernatant was filtered via passage through a 0.2 µm membrane filter and loaded onto 4 mL of Ni(II)-NTA agarose resin equilibrated with 20 mM Na-Phos, 500 mM NaCl, 35 mM imidazole, 1 mM DTT, pH 7.4. Following washing with the same buffer (50 mL), the protein was eluted with 15 mL of the equilibration buffer containing 500 mM imidazole. The eluent containing HTP(S261C) was dialyzed 2 times against 2.0 L of 20 mM Tris HCl, 1 mM DTT, pH 8.0 at 4 °C. The dialyzed protein solution was further purified by anion exchange chromatography (Mono Q 5/50 GL) with buffer A (20 mM Tris HCl, 1 mM DTT, pH 8.0) and a gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, 1 mM DTT, pH 8.0). Fractions containing the purified protein were pooled and dialyzed 2 times against 2.0 L 20 mM Tris HCl, 2 mM DTT, pH 7.4 at 4 °C. The resulting solution containing HTP(S261C) may be stored for up to 1.5 weeks on ice at 0 °C, without appreciable, irreversible oxidative decomposition. The expression yield of HTP(S261C) was found to be approximately 5 mg/ 1.0 L culture.

An aliquot of HTP(S261C) (0.01 µmol) was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass determination (**ESI-MS** see Figure S16).

Calculated mass: 48447.8 Da

Observed mass: 48447.5 Da

**Figure S16.** Deconvoluted (ESI) mass spectrum of HTP(S261C)

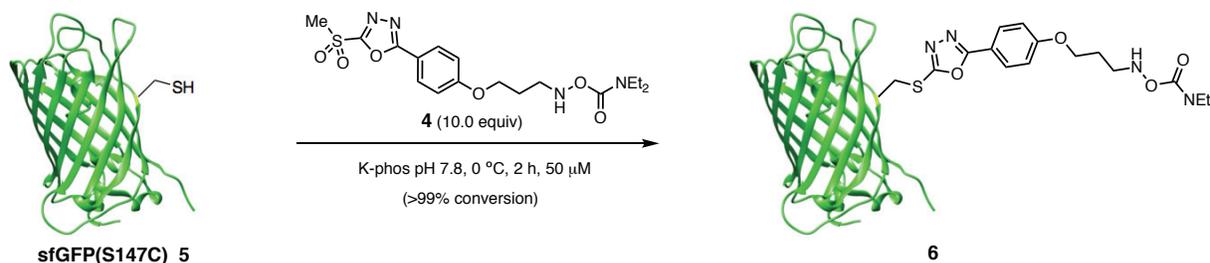
Protein sequence:

GSSHHHHHMSPEPKQLPELIRMKRDGGRLSEADIRGFVAAVVNGSAQGAQIGAMLMAIRLR  
GMDLEETSVLTQALAQSGQQLWPEAWRQQLVDKHSTGGVGDVSLVLPALAACGCKVP  
MISGRGLGHTGGTLDKLESIPGFNVIQSPEQMQVLLDQAGCCIVGQSEQLVPADGILYAARDVT  
ATVDSLPLITASILSKKLVEGLSALVVDVKFGGAAVFPNQEQAARELAKTLVGVGACLGLRVAA  
ALTAMDKPLGR**C**VGHAVEVEEALLCMDGAGPPDLRDLVTTLGGALLWLSGHAGTQAQGAAR  
VAAALDDGSALGRFERMLAAQGVDPGLARALCSGSPAERRQLLPRAREQEELLAPADGTVEL  
VRALPLALVLHELGAGRSRAGEPLRLGVGAELLVDVVGQRLRRGTPWLRVHRDGPALSGPQSR  
ALQEALVLSRAPFAAPSPFAELVLPPQQ

#### 4. Preparation of protein – hydroxylamine conjugates

##### sfGFP(S147C) – hydroxylamine (6)

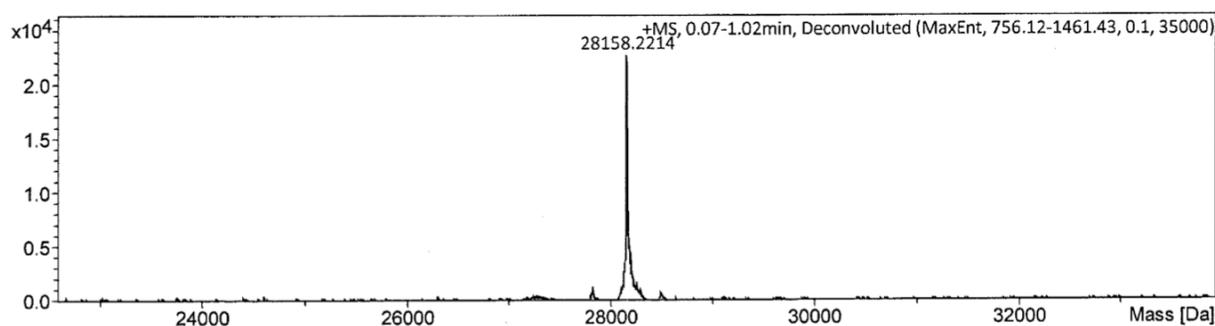
##### Scheme S6. Synthesis of sfGFP(S147C) – hydroxylamine bioconjugate 6



An aliquot of sfGFP(S147C) **5** (0.025 μmol, 1.0 equiv) was treated with DTT (25.0 μL, 12.5 μmol, 5.00 x10<sup>2</sup> equiv, 0.5 M in H<sub>2</sub>O) and diluted to a concentration of 200 μM with MilliQ H<sub>2</sub>O. The resulting solution was vortexed quickly and incubated at room temperature for 1 h. Excess DTT was removed through repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M potassium phosphate (K-Phos), 1 mM EDTA, pH 7.8 buffer at 4 °C. The resulting solution containing reduced **5** (devoid of DTT) was diluted (50 μM) with additional pH 7.8 buffer and to it was added hydroxylamine **4** (25.0 μL, 0.250 μmol, 10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0 °C for 2 h. The reaction mixture was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine conjugate **6** (ESI-MS *see* Figure S17).

Calculated mass: 28158.4 Da

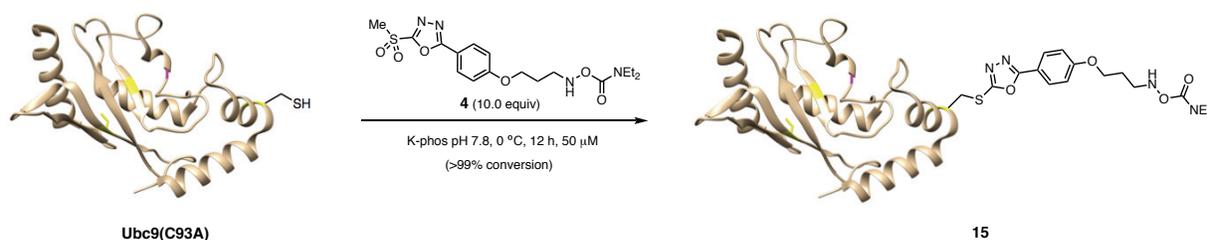
Observed mass: 28158.2 Da



**Figure S17.** Deconvoluted (ESI) mass spectrum of hydroxylamine bioconjugate **6**

### Ubc9(C93A) – hydroxylamine (**14**)

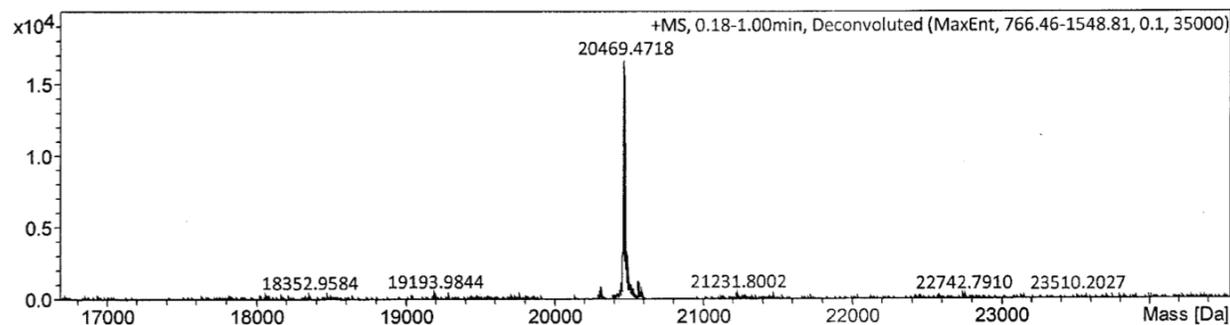
#### Scheme S7. Synthesis of Ubc9(C93A) – hydroxylamine bioconjugate **14**



An aliquot of Ubc9(C93A) (0.050  $\mu\text{mol}$ ,  $2 \times 10^2 \mu\text{M}$ , 1.0 equiv) in 50 mM HEPES, 50 mM NaCl, 1 mM DTT, pH 7.6 was thawed on ice and treated with DTT (10.0  $\mu\text{L}$ , 5.00  $\mu\text{mol}$ ,  $1.0 \times 10^2$  equiv, 0.5 M in  $\text{H}_2\text{O}$ ). The resulting solution was vortexed quickly and incubated at room temperature for 1 h. Excess DTT was removed through repetitive (x3) spin dialfiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M K-Phos, 1 mM EDTA, pH 7.8 buffer at 4 °C. The resulting solution of Ubc9(C93A) was diluted (50  $\mu\text{M}$ ) with additional pH 7.8 buffer and to it was added hydroxylamine **4** (50.0  $\mu\text{L}$ , 0.500  $\mu\text{mol}$ , 10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0 °C for 12 h. The reaction mixture was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ  $\text{H}_2\text{O}$  for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine conjugate **14** (ESI-MS see Figure S18).

Calculated mass: 20471.3 Da

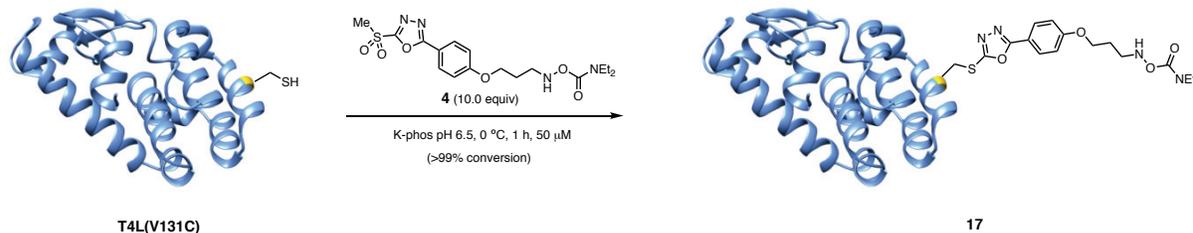
Observed mass: 20469.5 Da



**Figure S18.** Deconvoluted (ESI) mass spectrum of hydroxylamine bioconjugate **14**

### T4L(V131C) – hydroxylamine (**16**)

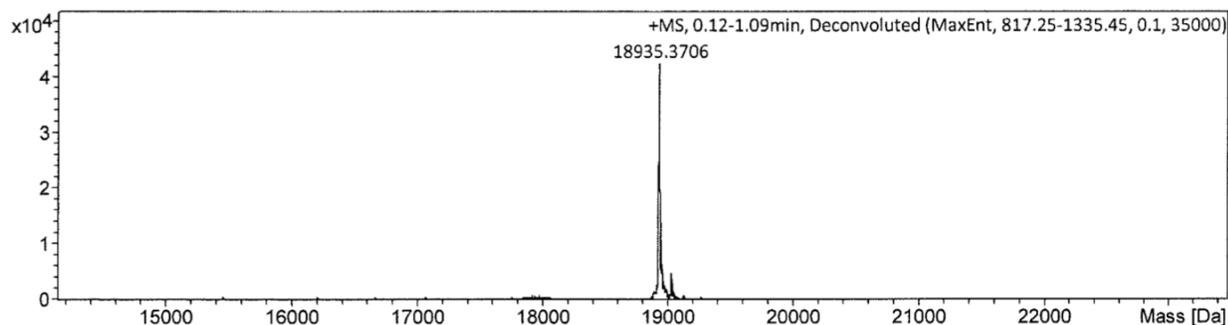
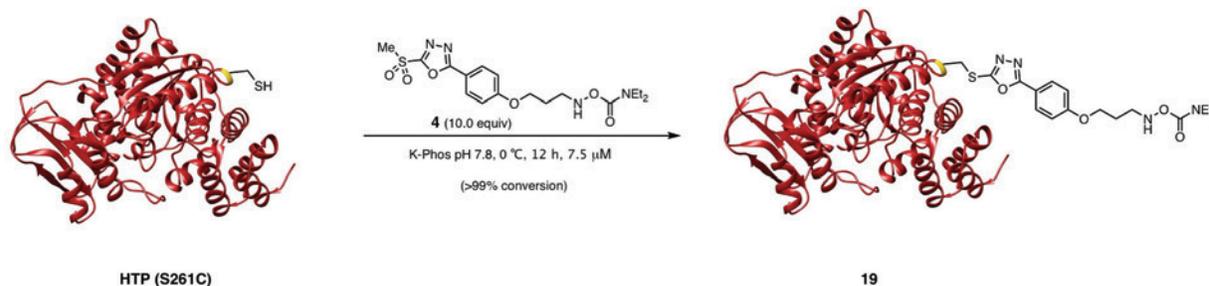
### Scheme S8. Synthesis of T4L(V131C) – hydroxylamine bioconjugate **16**



An aliquot of T4L(V131C) (0.050  $\mu\text{mol}$ , 1.0 equiv) was diluted (200  $\mu\text{M}$ ) with 0.1 M K-Phos, pH 6.5 and treated with DTT (50.0  $\mu\text{L}$ , 25.0  $\mu\text{mol}$ ,  $5.00 \times 10^2$  equiv, 0.5 M in  $\text{H}_2\text{O}$ ). The resulting solution was vortexed quickly and incubated at room temperature for 1 h. Excess DTT was removed through repetitive (x3) spin dialfiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M K-Phos, pH 6.5 buffer at 4  $^\circ\text{C}$ . The resulting solution of T4L(V131C) was diluted (50  $\mu\text{M}$ ) with additional pH 6.5 buffer and to it was added hydroxylamine **4** (50.0  $\mu\text{L}$ , 0.500  $\mu\text{mol}$ , 10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0  $^\circ\text{C}$  for 1 h. The reaction mixture was passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ  $\text{H}_2\text{O}$  for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine bioconjugate **16** (ESI-MS see Figure S19).

Calculated mass: 18937.5 Da

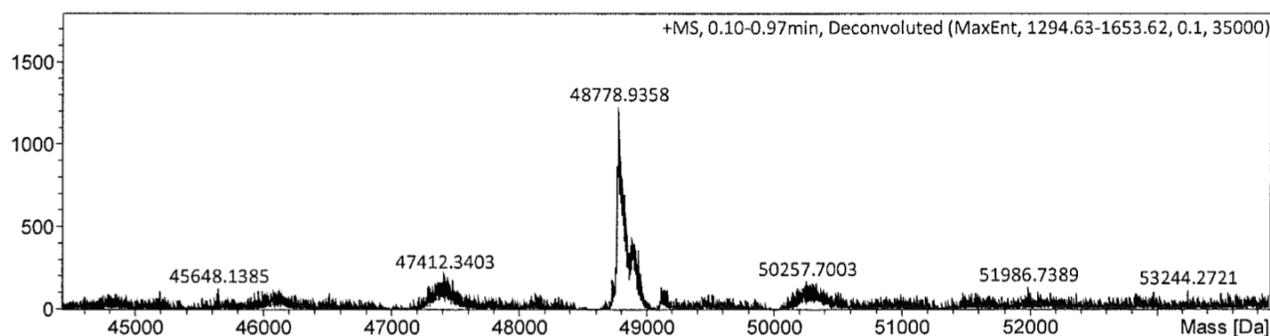
Observed mass: 18935.4 Da

**Figure S19.** Deconvoluted (ESI) mass spectrum of hydroxylamine bioconjugate **16**.**HTP(S261C) – hydroxylamine (18)****Scheme S9.** Synthesis of HTP(S261C) – hydroxylamine bioconjugate **18**

An aliquot of HTP(S261C) (0.010  $\mu\text{mol}$ , 1.0 equiv) was treated with a spatula tip of DTT (>500 equiv), shaken gently and incubated at 4  $^{\circ}\text{C}$  for 1 h. The resulting solution was passed through a PD-MidiTrap G-25 desalting column (GE Healthcare) to remove excess DTT and exchanged into a freshly degassed 0.1 M K-Phos, 1 mM EDTA, pH 7.8 buffer (1.5 mL). The concentration of HTP(S261C) in solution was subsequently measured by UV-Vis spectroscopy (typically 5 – 10  $\mu\text{M}$ ) and to the solution was added hydroxylamine **4** (10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0  $^{\circ}\text{C}$  for 12 h. The reaction mixture was concentrated to 500  $\mu\text{L}$  through a spin diafiltration (Amicon® Ultra – 4, 3.0 kDa MWCO) and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ  $\text{H}_2\text{O}$  for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine bioconjugate **18** (ESI-MS *see* Figure S20).

Calculated mass: 48779.7 Da

Observed mass: 48778.9 Da



**Figure S20.** Deconvoluted (ESI) mass spectrum of hydroxylamine bioconjugate **18**

## 5. PEGylation of protein – hydroxylamine conjugates

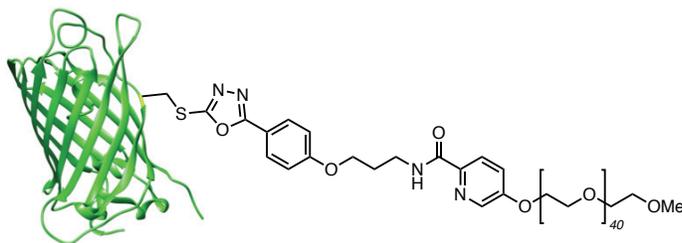
### 5.1 General procedure for PEGylation

Following the cysteine-methylsulfone reaction to append an *O*-carbamoyl hydroxylamine onto the surface of the corresponding protein substrate (see section 4), the crude reaction mixture (not desalted) was diluted (15  $\mu$ M) with either a 20 mM K-Phos, pH 7.4 buffer (for sfGFP and Ubc9) or a 100 mM K-Phos, pH 6.5 buffer (for T4L) containing 5% DMF. Excess methylsulfone hydroxylamine **4** was removed through repetitive spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C with the corresponding buffer containing 5% DMF (x5) and then once more with MilliQ H<sub>2</sub>O to remove a majority of the buffer salts in the reaction mixture. The concentrated crude reaction solution containing the protein – hydroxylamine conjugate (>250  $\mu$ M) was diluted to 25  $\mu$ M with freshly prepared 50 mM glycine hydrochloride (Gly-HCl), 50 mM KF, 5% DMF, pH 3.6 buffer. To this was added the appropriate mPEG-KAT **9** (1.5 equiv or 4.0 equiv with respect to protein – hydroxylamine, 10 mM in DMF) and the resulting solution was vortexed quickly and gently agitated at room temperature for either 1 h (with 4.0 equiv **9** – Condition A) or 5 h (with 1.5 equiv **9** – Condition B). The ligation was quenched by the addition of a large excess of hydroxylamine **S6** (200 equiv, 0.1 M in DMF) and gently agitated at room temperature for an additional 30 min. The reaction mixture was subsequently exchanged into the appropriate buffer for ion exchange chromatography through repetitive (x3) spin

diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C and filtered through a 0.2 µm membrane filter prior to purification. A small aliquot (25 µL) of the crude reaction mixture was taken for SDS-PAGE analysis.

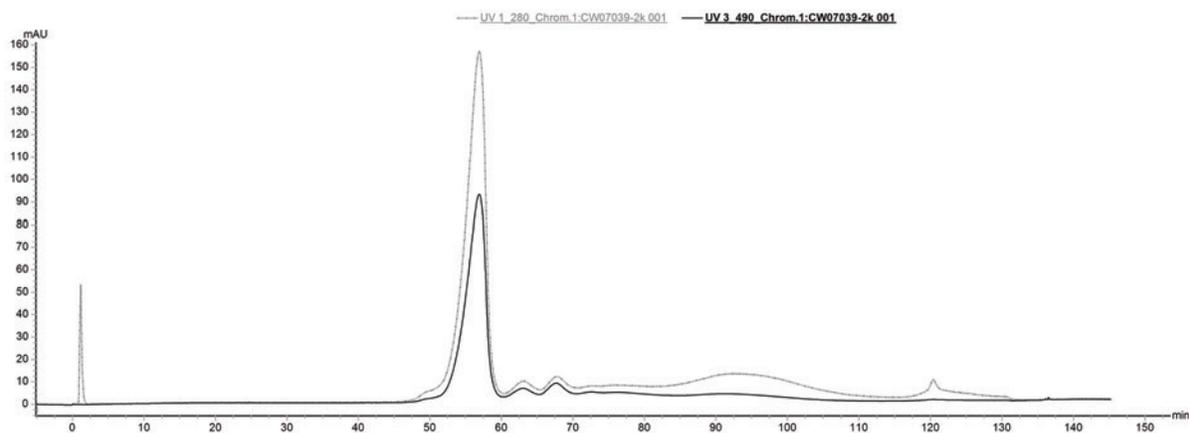
## 5.2 PEGylated protein substrates

### sfGFP(S147C) – mPEG2k (13a)

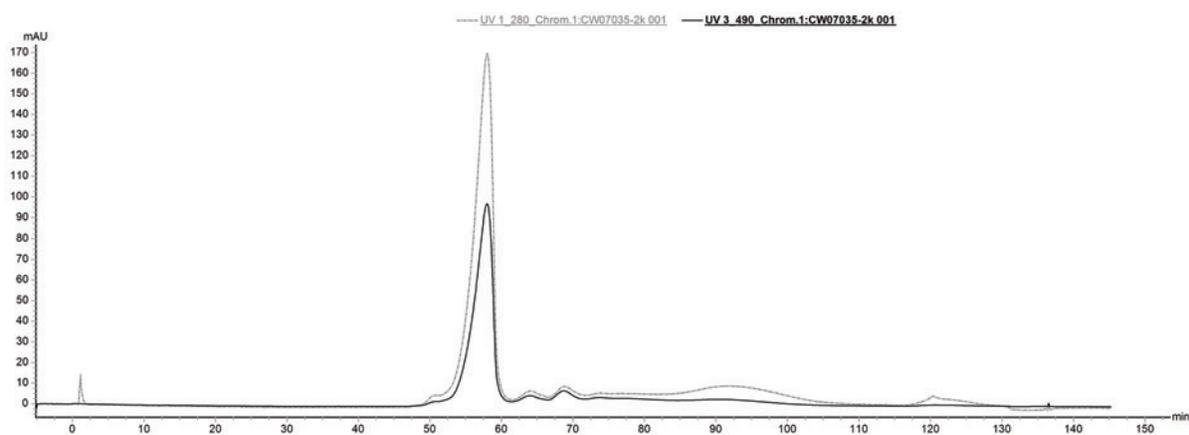


Prepared following the general procedure for PEGylation through a KAT ligation of sfGFP(S147C) – hydroxylamine **6** with mPEG KAT **9a**. After quenching with hydroxylamine **S6**, a 20 mM Tris HCl, pH 8.5 buffer was used

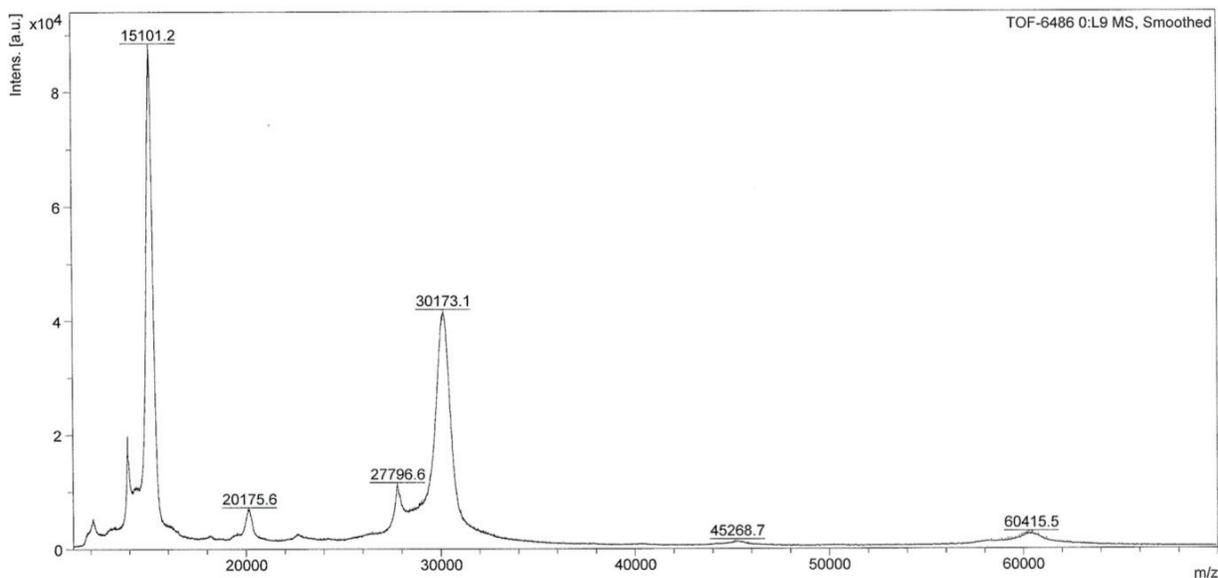
for the repetitive spin filtrations prior to FPLC purification. The crude reaction was purified by anion exchange chromatography (Mono Q 5/50 GL) with an isocratic washing of buffer A (20 mM Tris HCl, pH 8.5) for 10 column volumes (CVs), followed by a 0 – 25% linear gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, pH 8.5) over 25 CVs with a flow rate of 0.25 mL/min. Retention time of **13a** = 23.5 mL, 58.0 min, 11% buffer B (*see* Figures S21 and S22 for traces). Fractions containing the desired monoPEGylated product **13a** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (**MALDI-TOF** *see* Figure S23).



**Figure S21.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG2k **13a** using optimized Condition A (4.0 equiv **9a**, 1.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.

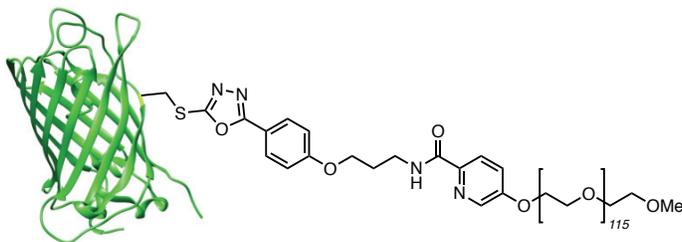


**Figure S22.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG2k **13a** using optimized Condition B (1.5 equiv **9a**, 5.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.



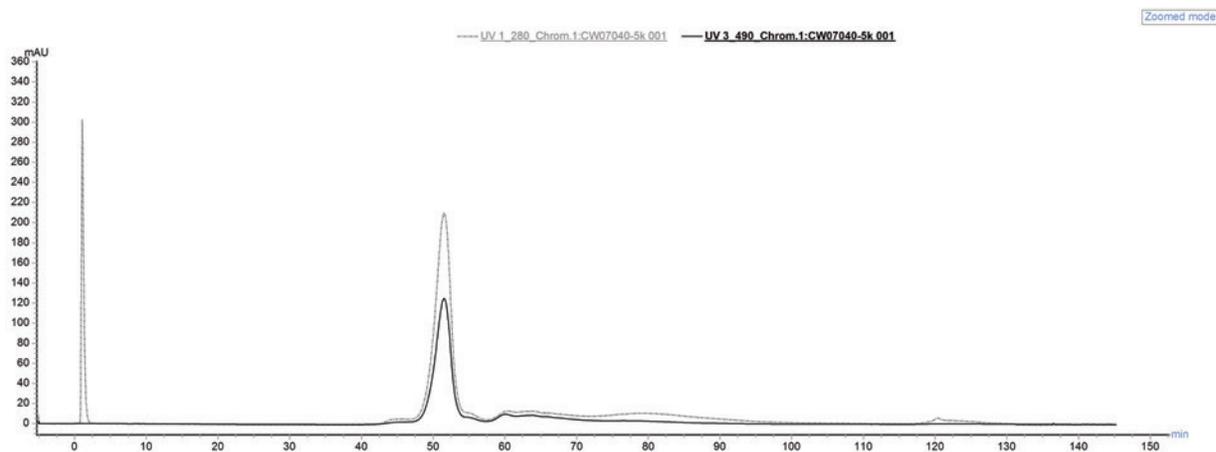
**Figure S23.** MALDI-TOF of sfGFP(S147C) – mPEG2k (**13a**)

### sfGFP(S147C) – mPEG5k (**13b**)

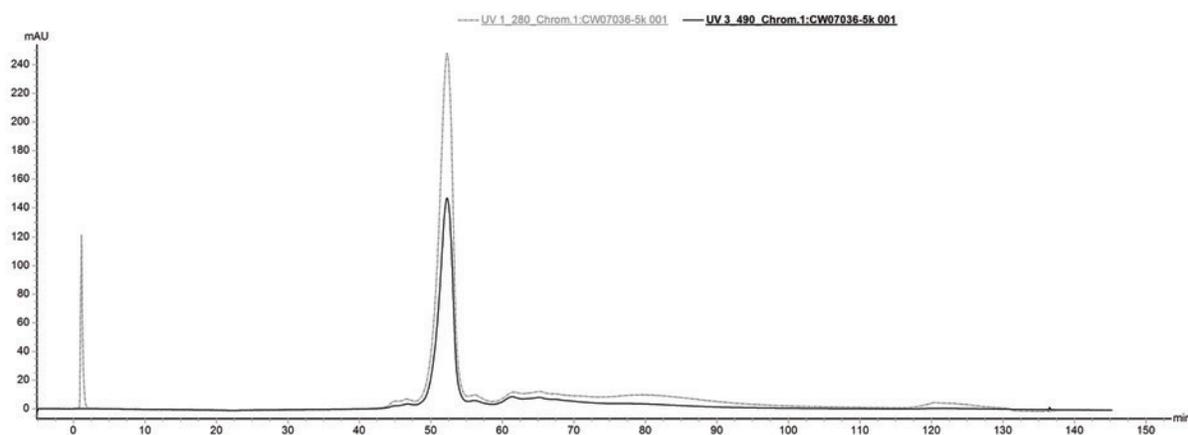


Prepared by KAT ligation of sfGFP(S147C) – hydroxylamine **6** with mPEG KAT **9b**, following the general procedure for PEGylation and purified using an identical protocol for sfGFP(S147C) – mPEG2k **13a**.

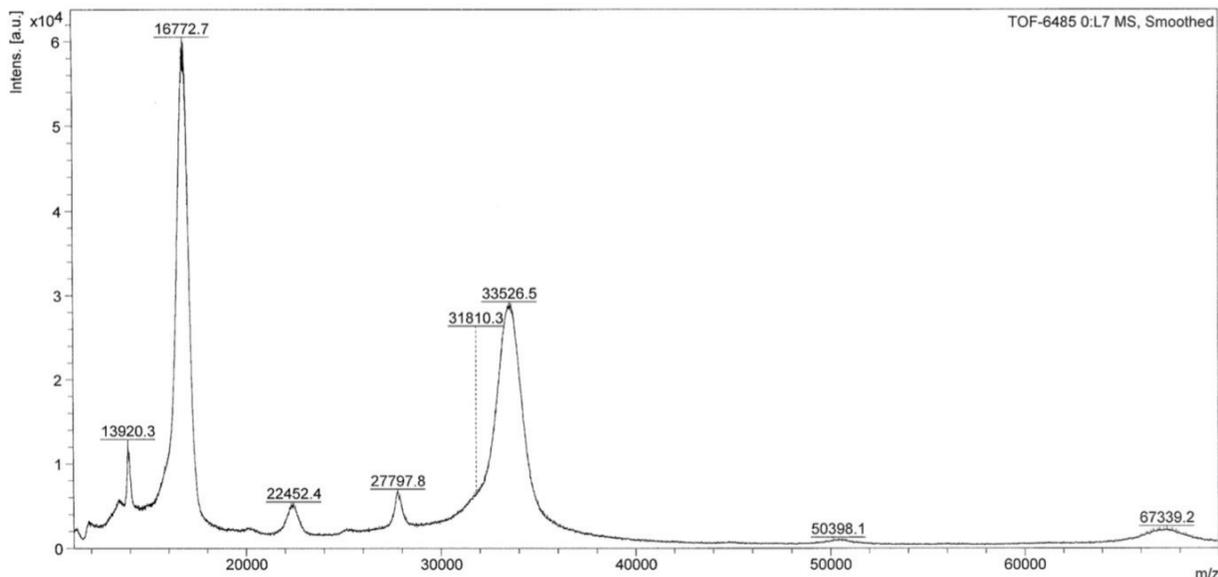
Retention time of **13b** = 22.3 mL, 52.2 min, 10% buffer B (*see* Figures S24 and S25 for traces). Fractions containing the desired monoPEGylated product **13b** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (MALDI-TOF *see* Figure S26).



**Figure S24.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG5k **13b** using optimized Condition A (4.0 equiv **9b**, 1.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.

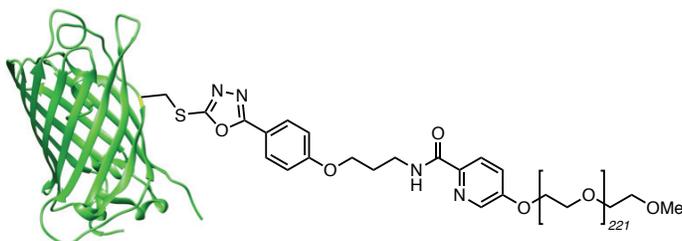


**Figure S25.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG5k **13b** using optimized Condition B (1.5 equiv **9b**, 5.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.



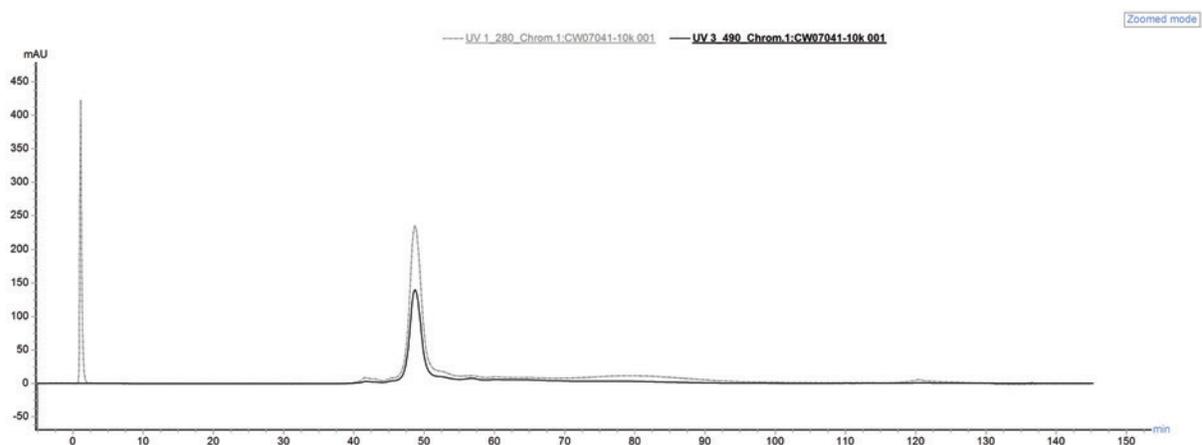
**Figure S26.** MALDI-TOF of sfGFP(S147C) – mPEG5k (**13b**)

### sfGFP(S147C) – mPEG10k (**13c**)

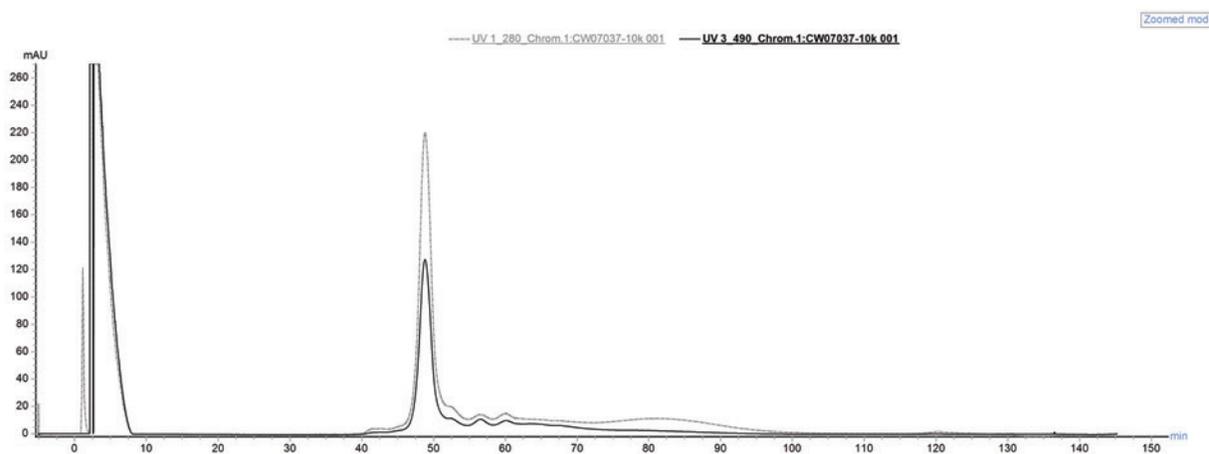


Prepared by KAT ligation of sfGFP(S147C) – hydroxylamine **6** with mPEG KAT **9c**, following the general procedure for PEGylation and purified using an identical protocol for sfGFP(S147C) – mPEG2k **13a**.

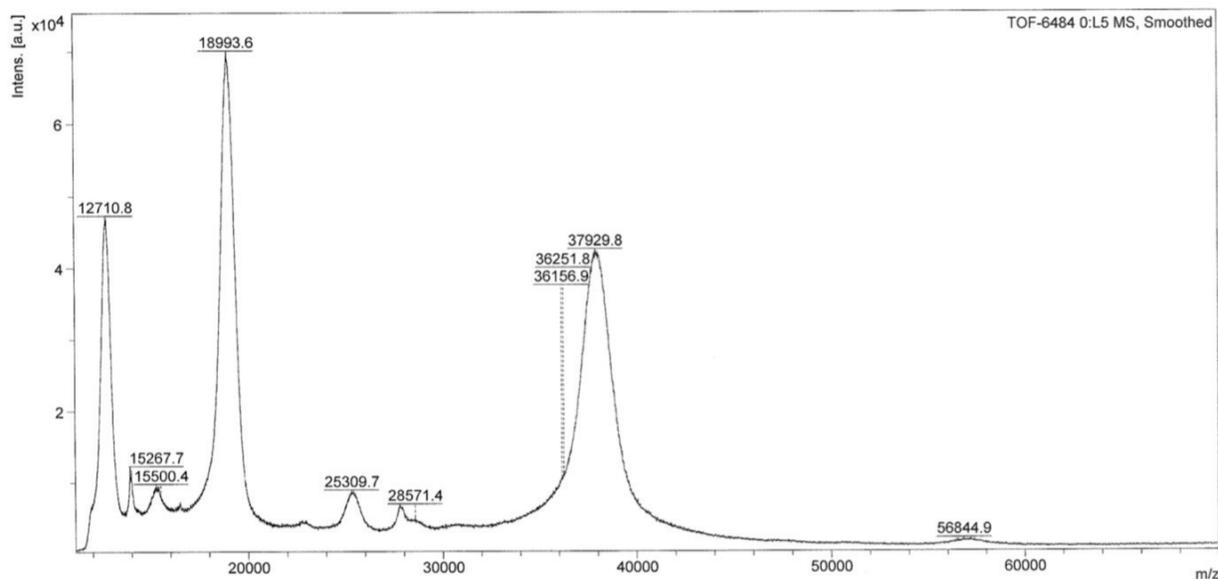
Retention time of **13c** = 21.4 mL, 48.8 min, 9% buffer B (*see* Figures S27 and S28 for traces). Fractions containing the desired monoPEGylated product **13c** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (MALDI-TOF *see* Figure S29).



**Figure S27.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG10k **13c** using optimized Condition A (4.0 equiv **9c**, 1.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.

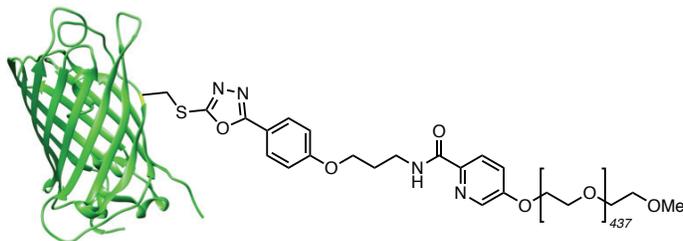


**Figure S28.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG10k **13c** using optimized Condition B (1.5 equiv **9c**, 5.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.



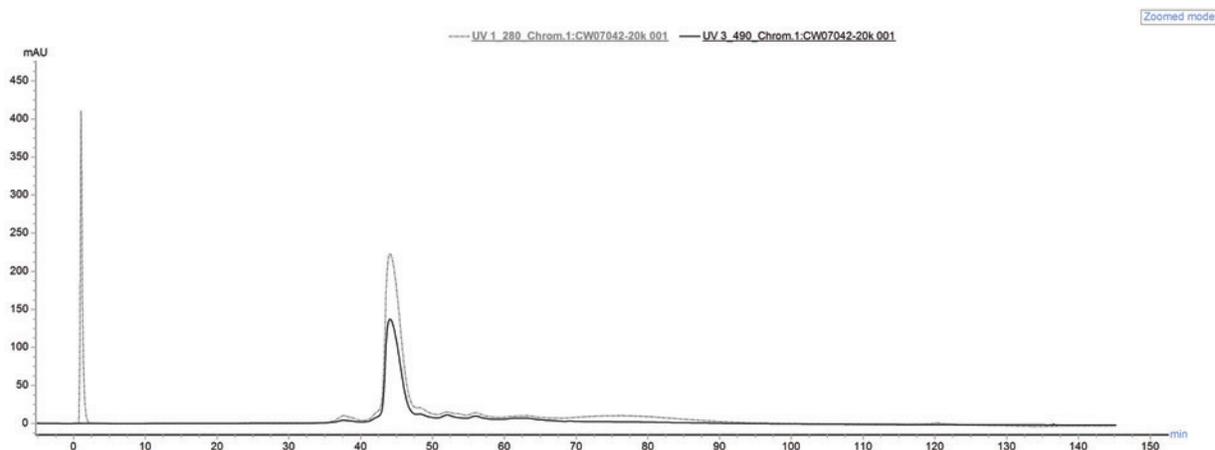
**Figure S29.** MALDI-TOF of sfGFP(S147C) – mPEG10k (**13c**)

### sfGFP(S147C) – mPEG20k (**13d**)

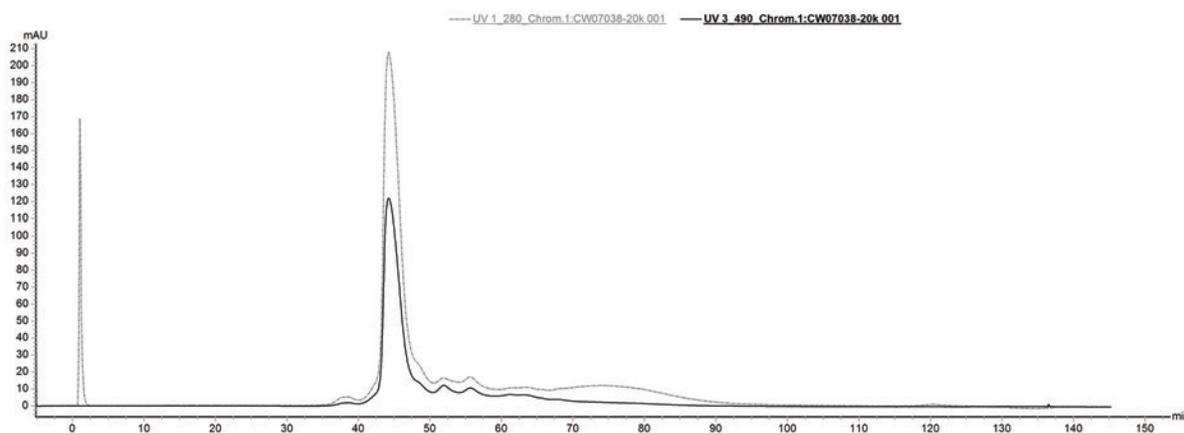


Prepared by KAT ligation of sfGFP(S147C) – hydroxylamine **6** with mPEG KAT **9d**, following the general procedure for PEGylation and purified using an identical protocol for sfGFP(S147C) – mPEG2k **13a**.

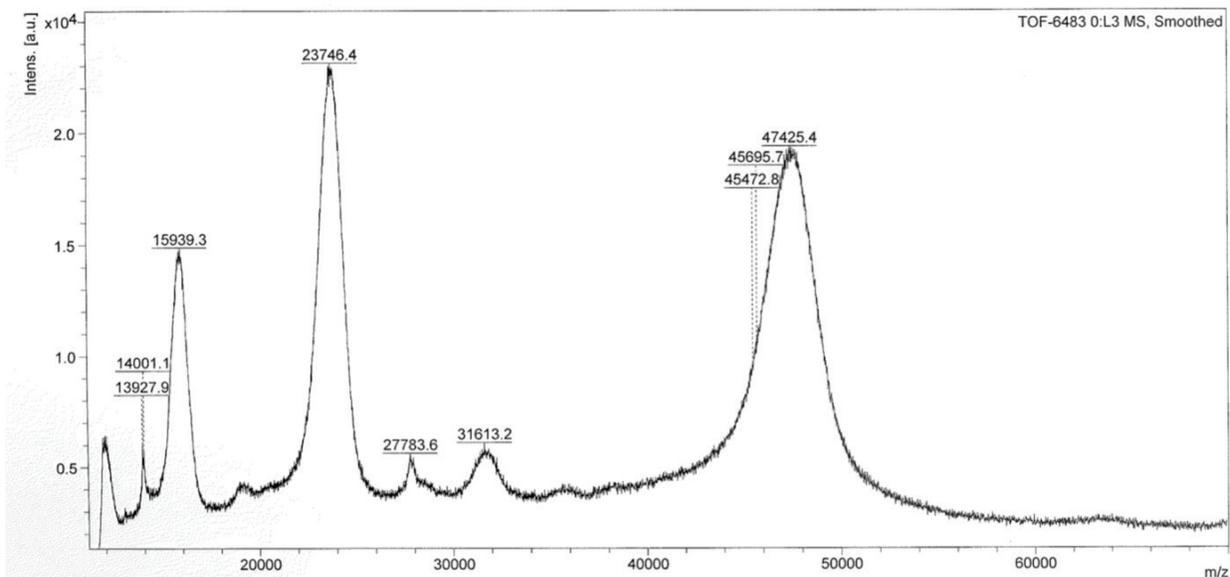
Retention time of **13d** = 20.3 mL, 44.2 min, 8% buffer B (*see* Figures S30 and S31 for traces). Fractions containing the desired monoPEGylated product **13d** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (MALDI-TOF *see* Figure S32).



**Figure S30.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG20k **13d** using optimized Condition A (4.0 equiv **9d**, 1.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.

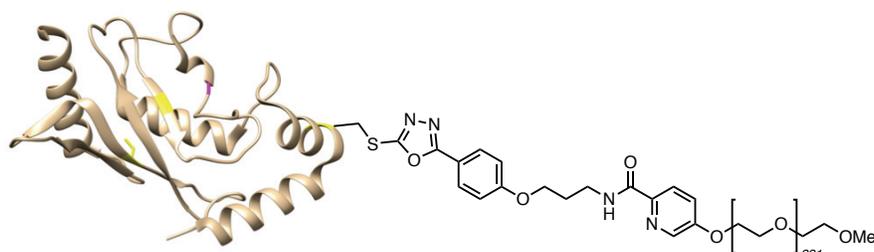


**Figure S31.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG20k **13d** using optimized Condition B (1.5 equiv **9d**, 5.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.



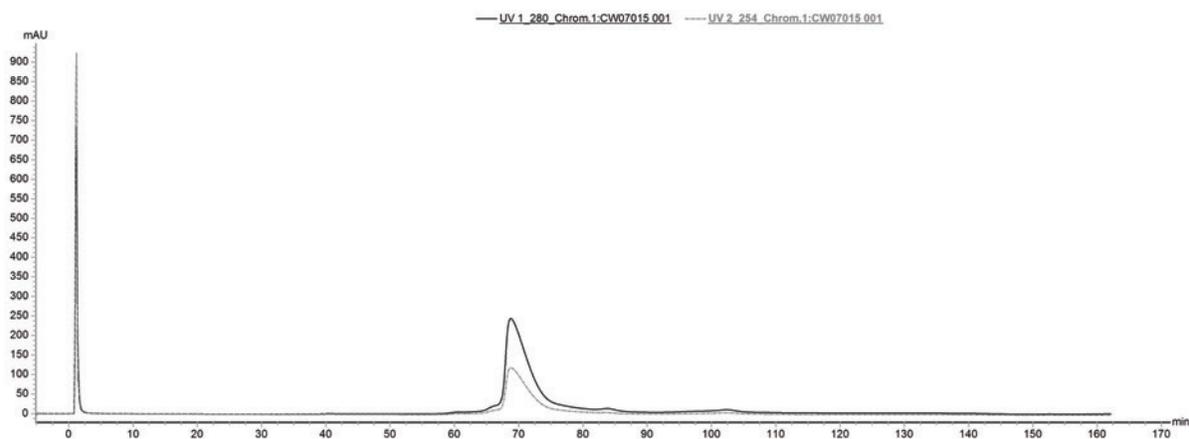
**Figure S32.** MALDI-TOF of sfGFP(S147C) – mPEG20k (**13d**)

### Ubc9(C93A) – mPEG10k (**15c**)

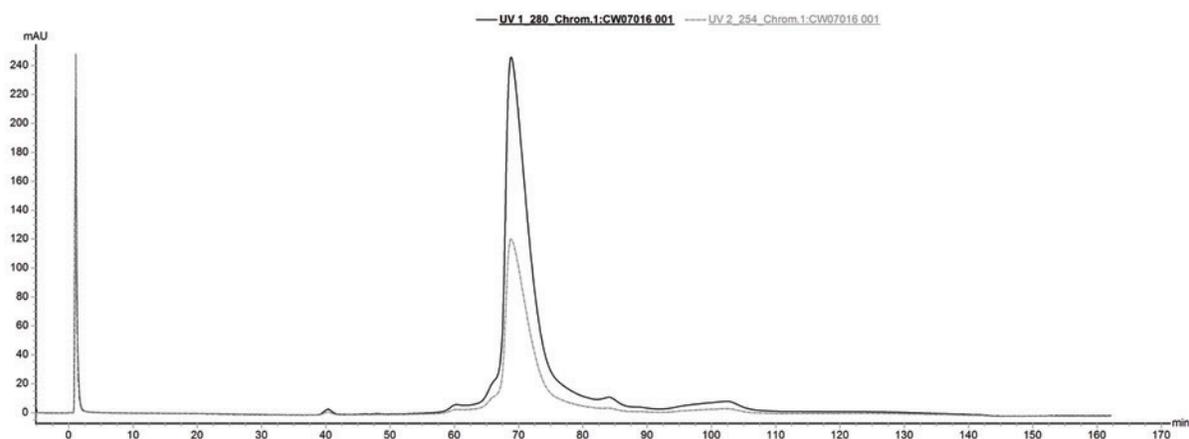


Prepared following the general procedure for PEGylation by KAT ligation of Ubc9(C93A) – hydroxylamine **14** with mPEG KAT **9c**. After

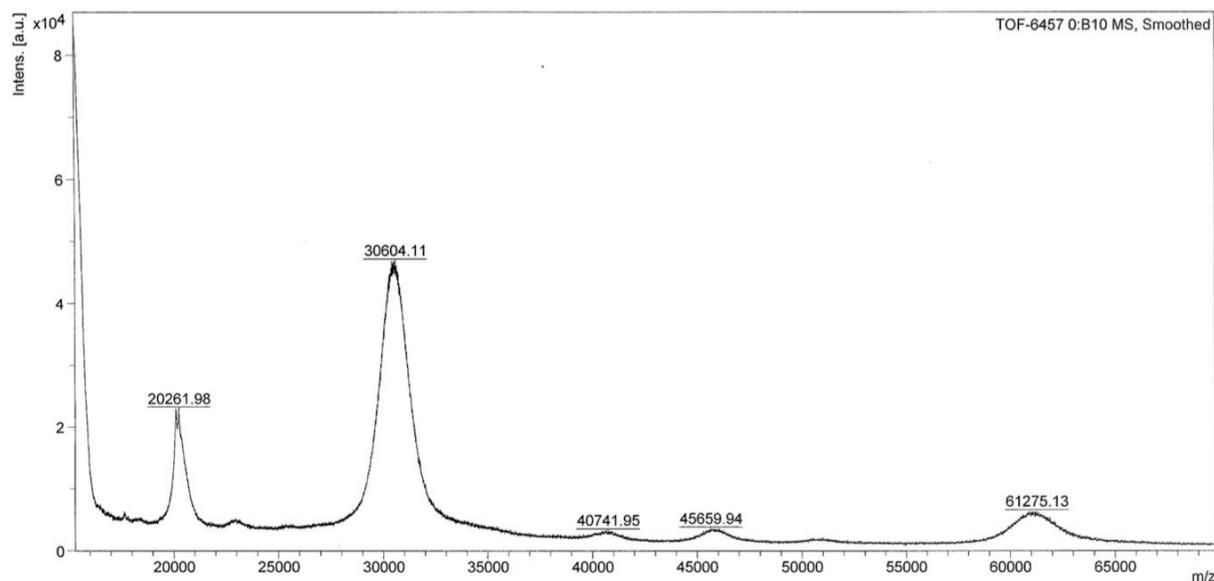
quenching with hydroxylamine **S6**, a 50 mM Na-Phos, pH 7.0 buffer was used for the repetitive spin filtrations prior to FPLC purification. The crude reaction was purified by cation exchange chromatography (Mono S 5/50 GL) with an isocratic washing of buffer A (50 mM, Na-Phos, pH 7.0) for 10 CVs, followed by a 0 – 50% linear gradient of buffer B (50 mM Na-Phos, 1.0 M NaCl, pH 7.0) over 20 CVs with a flow rate of 0.25 mL/min. Retention time of **15c** = 26.4 mL, 68.8 min, 36% buffer B (*see* Figures S33 and S34 for traces). Fractions containing the desired monoPEGylated product **15c** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (**MALDI-TOF** *see* Figure S35).



**Figure S33.** FPLC trace of the crude reaction mixture to form Ubc9(C93A) – mPEG10k **15c** using optimized Condition A (4.0 equiv **9c**, 1.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.

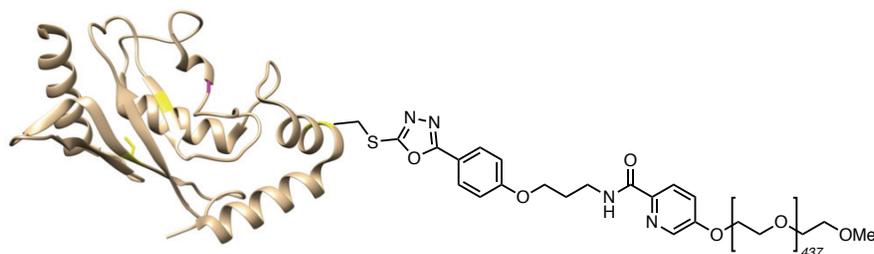


**Figure S34.** FPLC trace of the crude reaction mixture to form Ubc9(C93A) – mPEG10k **15c** using optimized Condition B (1.5 equiv **9c**, 5.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.



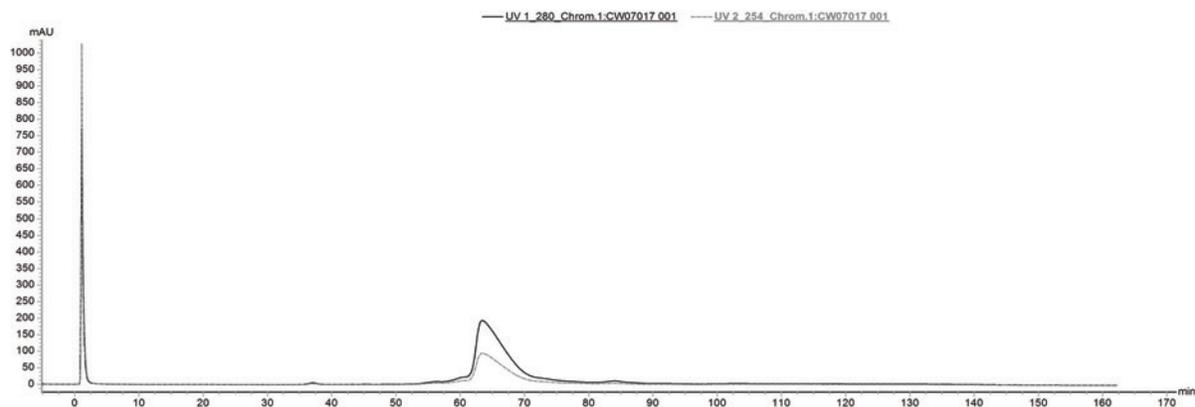
**Figure S35.** MALDI-TOF of Ubc9(C93A) – mPEG10k (**15c**)

#### Ubc9(C93A) – mPEG20k (**15d**)

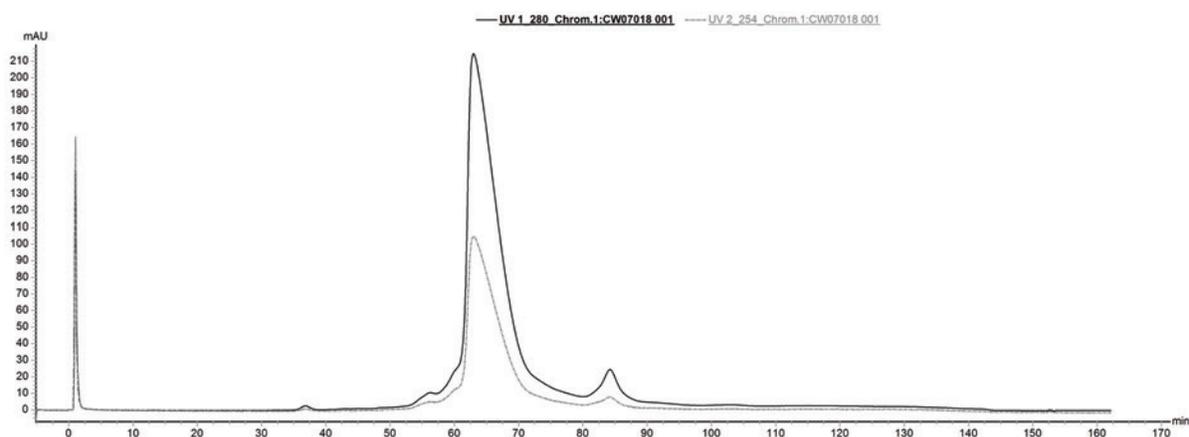


Prepared by KAT ligation of Ubc9(C93A) – hydroxylamine **14** with mPEG KAT **9d**, following the general procedure for PEGylation and purified using an identical

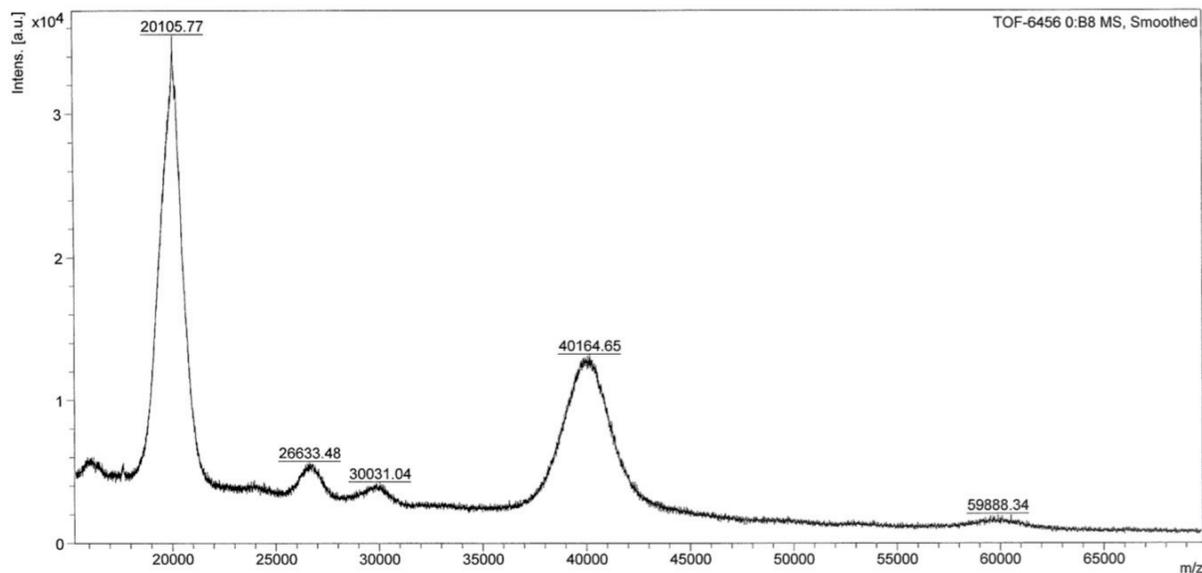
protocol for Ubc9(C93A) – mPEG10k **15c**. Retention time of **15d** = 25.1 mL, 63.4 min, 32% buffer B (*see* Figures S36 and S37 for traces). Fractions containing the desired monoPEGylated product **15d** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (**MALDI-TOF** *see* Figure S38).



**Figure S36.** FPLC trace of the crude reaction mixture to form Ubc9(C93A) – mPEG20k **15d** using optimized Condition A (4.0 equiv **9d**, 1.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.

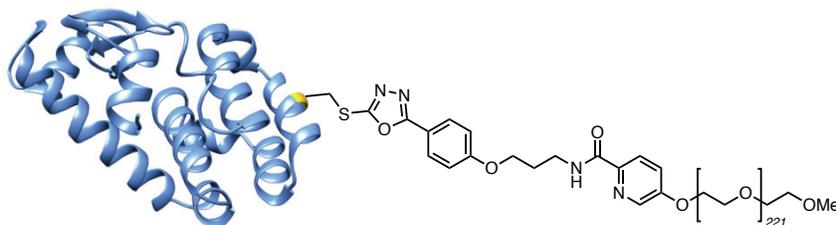


**Figure S37.** FPLC trace of the crude reaction mixture to form Ubc9(C93A) – mPEG20k **15d** using optimized Condition B (1.5 equiv **9d**, 5.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.



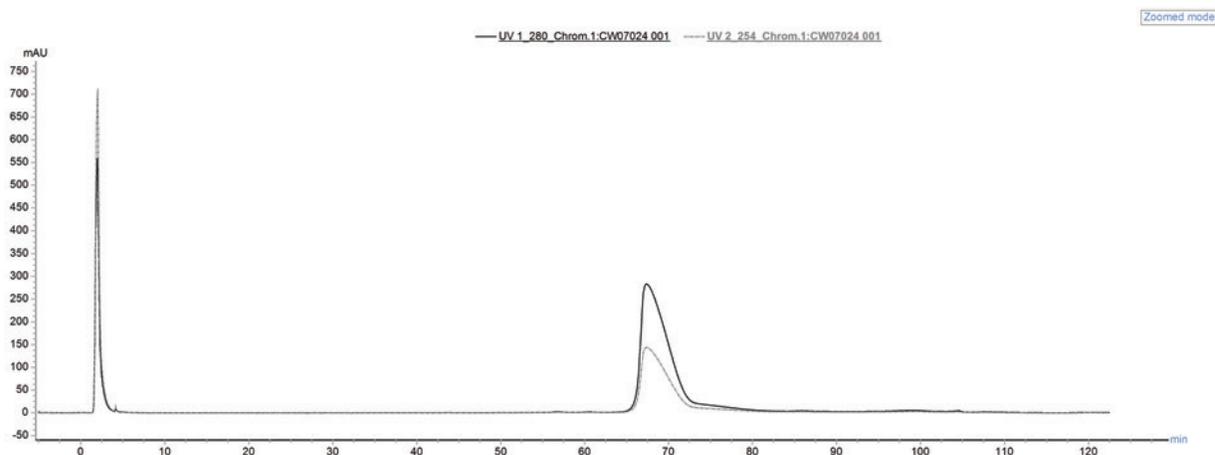
**Figure S38.** MALDI-TOF of Ubc9(C93A) – mPEG20k (**15d**)

#### T4L(V131C) – mPEG10k (**17c**)

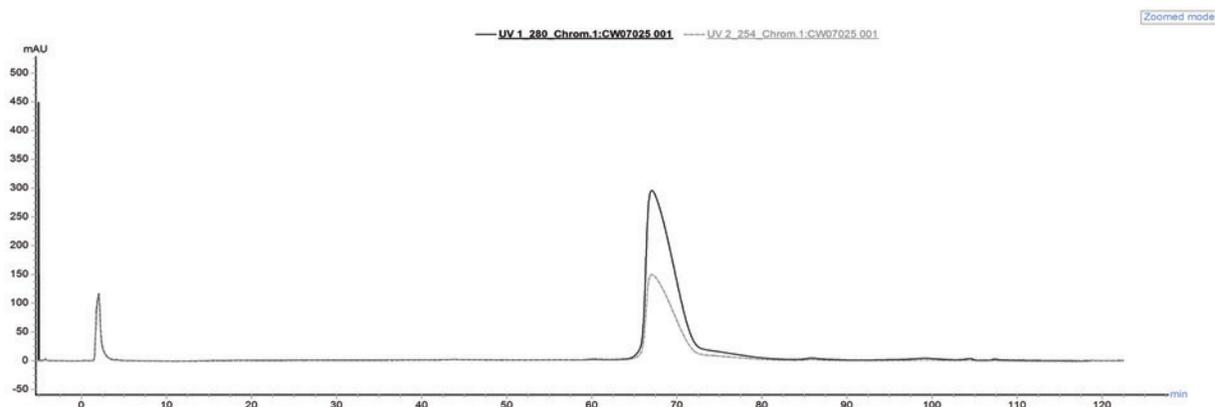


Prepared following the general procedure for PEGylation by KAT ligation of T4L(V131C) – hydroxylamine **16** with mPEG KAT **9c**. After quenching with hydroxylamine **S6**, a 25 mM

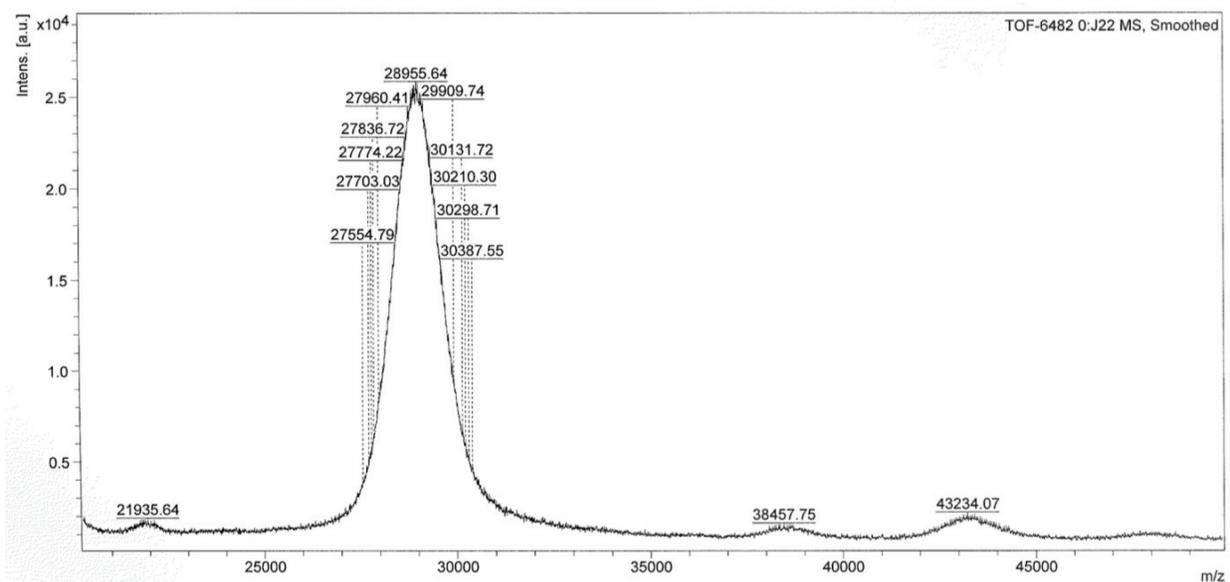
Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6 buffer was used for the repetitive spin filtrations prior to FPLC purification. The crude reaction was purified by cation exchange chromatography (Mono S 5/50 GL) with an isocratic washing of buffer A (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6) for 10 CVs, followed by a 0 – 20% linear gradient of buffer B (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, 1.0 M NaCl, pH 7.6) over 20 CVs with a flow rate of 0.25 mL/min. Retention time of **17c** = 25.2 mL, 67.5 min, 14% buffer B (*see* Figures S39 and S40 for traces). Fractions containing the desired monoPEGylated product **17c** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (MALDI-TOF *see* Figure S41).



**Figure S39.** FPLC trace of the crude reaction mixture to form T4L(V131C) – mPEG10k **17c** using optimized Condition A (4.0 equiv **9c**, 1.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.

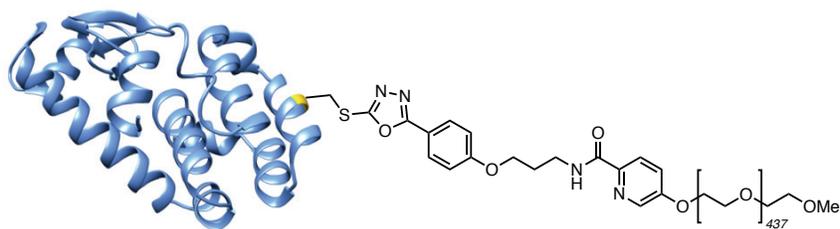


**Figure S40.** FPLC trace of the crude reaction mixture to form T4L(V131C) – mPEG10k **17c** using optimized Condition B (1.5 equiv **9c**, 5.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.



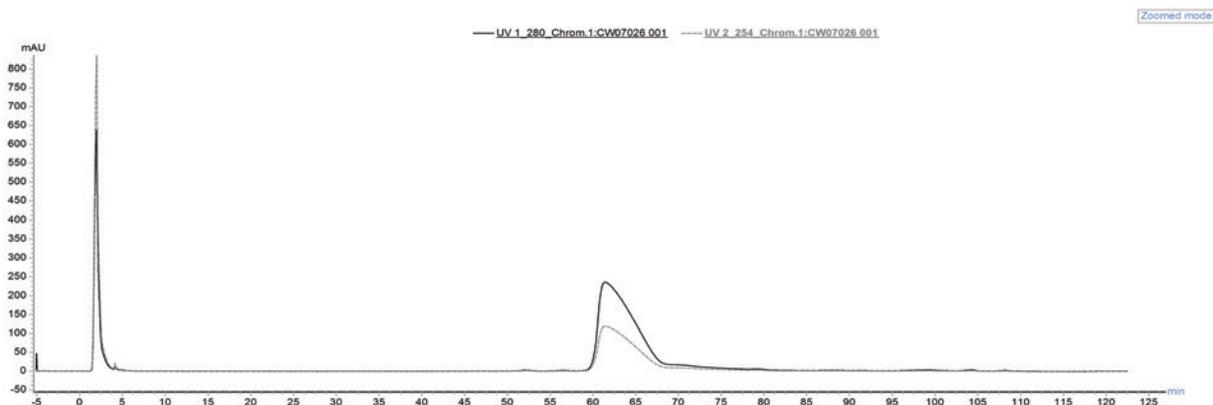
**Figure S41.** MALDI-TOF of T4L(V131C) – mPEG10k (**17c**)

#### T4L(V131C) – mPEG20k (**17d**)

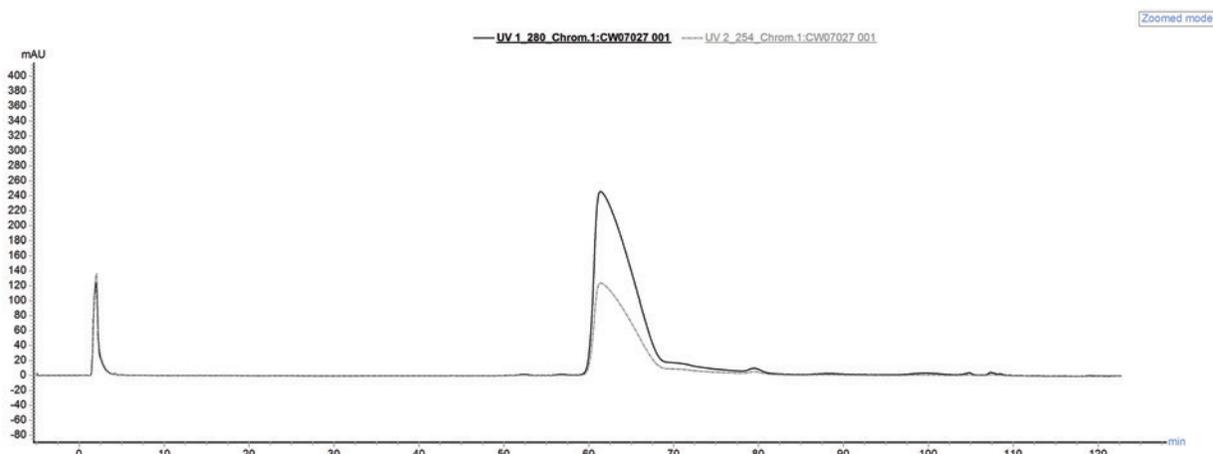


Prepared by KAT ligation of T4L(V131C) – hydroxylamine **16** with mPEG KAT **9d**, following the general procedure for PEGylation and purified using an identical protocol for

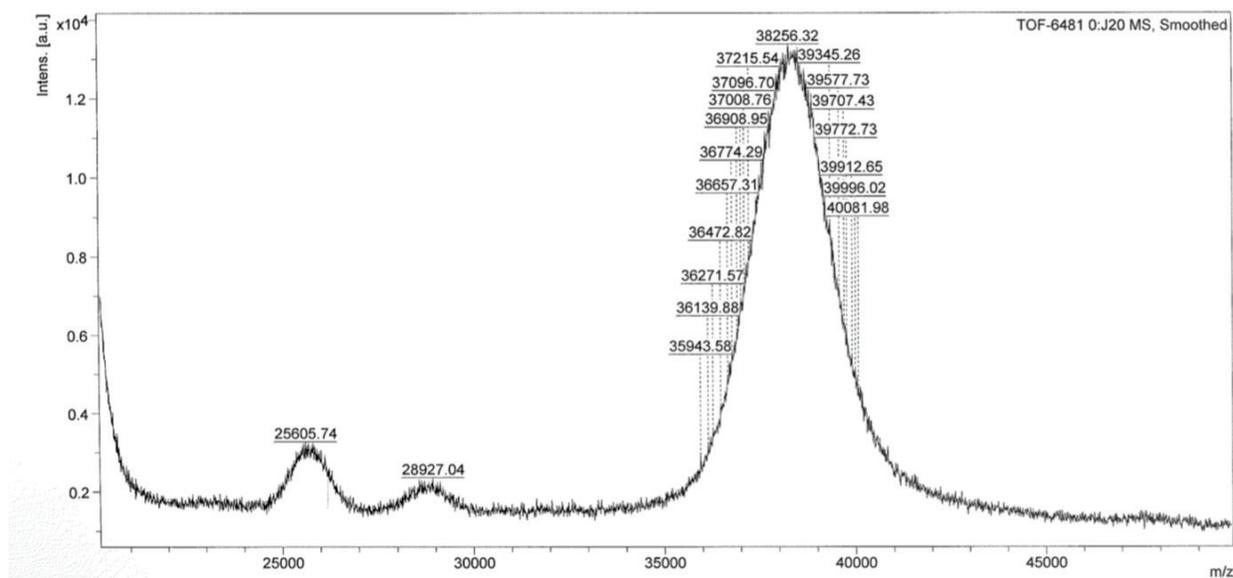
T4L(V131C) – mPEG10k **17c**. Retention time of **17d** = 23.7 mL, 61.5 min, 12% buffer B (*see* Figures S42 and S43 for traces). Fractions containing the desired monoPEGylated product **17d** were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS PAGE analysis (**MALDI-TOF** *see* Figure S44).



**Figure S42.** FPLC trace of the crude reaction mixture to form T4L(V131C) – mPEG20k **17d** using optimized Condition A (4.0 equiv **9d**, 1.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.

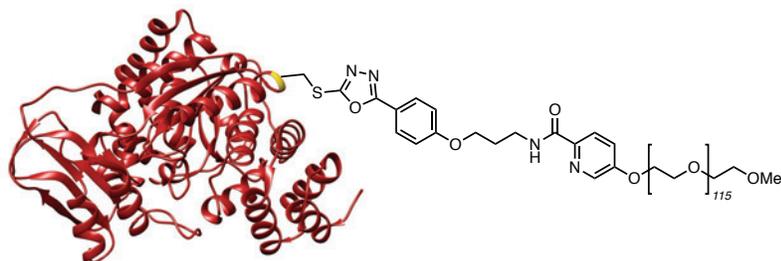


**Figure S43.** FPLC trace of the crude reaction mixture to form T4L(V131C) – mPEG20k **17d** using optimized Condition B (1.5 equiv **9d**, 5.0 h ligation time). Black (solid): 280 nm, grey: 254 nm.



**Figure S44.** MALDI-TOF of T4L(V131C) – mPEG20k (**17d**)

#### HTP(S261) – mPEG5k (**19b**)



Following the cysteine-methylsulfone coupling to append an *O*-carbamoyl hydroxylamine onto the surface of HTP(S261C) (see section 4), the reaction mixture was subjected to repetitive (x4) spin diafiltrations (Amicon® Ultra – 4, 3.0 kDa MWCO)

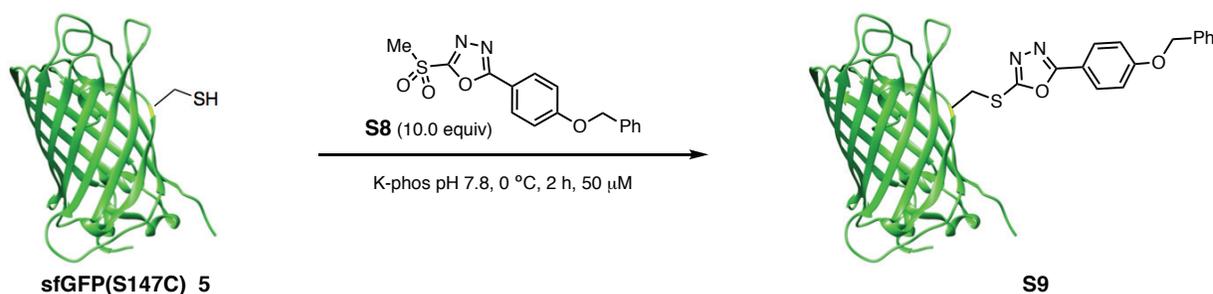
at 4 °C with a 20 mM K-Phos, 5% DMF, pH 7.4 buffer to remove excess **4**. The concentrated crude solution containing **18** was diluted (10 μM) with freshly prepared 50 mM Gly-HCl, 50 mM KF, 5% DMF, pH 3.6 buffer. To this was added mPEG KAT **9b** (4.0 equiv, 10 mM in DMF) and the resulting solution was vortexed quickly and gently agitated at room temperature. Aliquots were taken after 1.0, 2.0, 4.0 and 6.0 h (quenching with **S6**) for SDS-PAGE analysis.

## 6. Control experiment to confirm PEGylation through a KAT ligation

A control experiment was designed in order to confirm that our results for protein PEGylation (Section 5) were indeed the result of a site-specific KAT ligation between the 2-pyridyl KAT functionality of the mPEG chain and the *O*-carbamoyl hydroxylamine appended onto the surface of protein substrate. This experiment was designed to rule out the possibility of non-specific interactions of 2-pyridyl KAT with other protein functionalities that might give false positives in SDS PAGE and FPLC analyses. To do this, we stirred an excess of 2-pyridyl mPEG KAT **9c** with a protein substrate devoid of any hydroxylamine functionality under our optimized ligation conditions (Gly-HCl buffer, pH 3.6).

For this, we selected sfGFP(S147C) **5** as our protein substrate. To mimic the protein substrates used for KAT ligation (*see* sections 4 and 5), we first treated **5** with methyl sulfone phenyloxadiazole **S8** (lacking a hydroxylamine) to yield the resulting Cys147-capped bioconjugate **S9** (Scheme S10).

### Scheme S10. Synthesis of sfGFP(S147C) – phenyloxadiazole bioconjugate **S9**



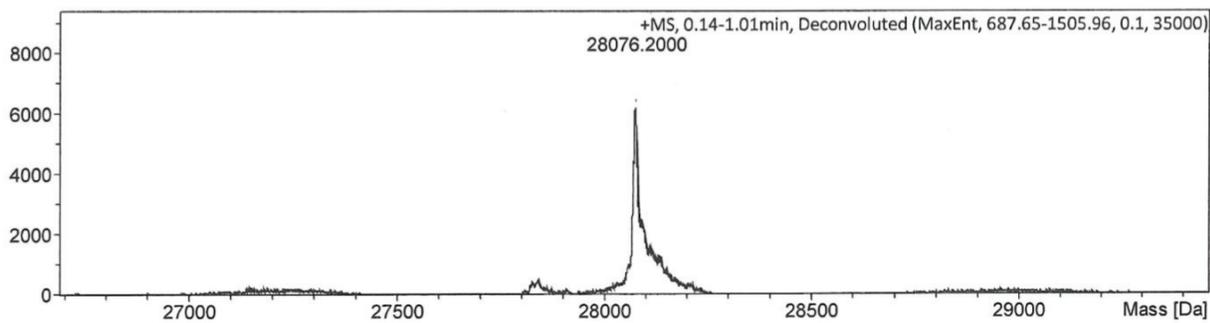
### sfGFP(S147C) – phenyloxadiazole (**S9**)

An aliquot of sfGFP(S147C) **5** (0.025 μmol, 1.0 equiv) was treated with DTT (25.0 μL, 5 x 10<sup>2</sup> equiv, 0.5 M in H<sub>2</sub>O) and diluted to a concentration of 200 μM with MilliQ H<sub>2</sub>O. The resulting solution was vortexed quickly and incubated at room temperature for 1.0 h. Excess DTT was removed through repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M potassium phosphate, 1 mM EDTA, pH 7.8 buffer at 4 °C. The resulting solution containing reduced **5** (devoid of DTT) was diluted (50 μM) with additional pH 7.8 buffer and to it was added reagent **S8** (25.0 μL, 10.0 equiv, 10 mM in DMF). The solution was vortexed quickly and incubated on ice at 0 °C for 2.0 hours. The reaction mixture was passed through a PD MiniTrap G-25

desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O for mass analysis. ESI-MS indicated full conversion to the corresponding hydroxylamine conjugate **S9** (ESI-MS *see* Figure S45).

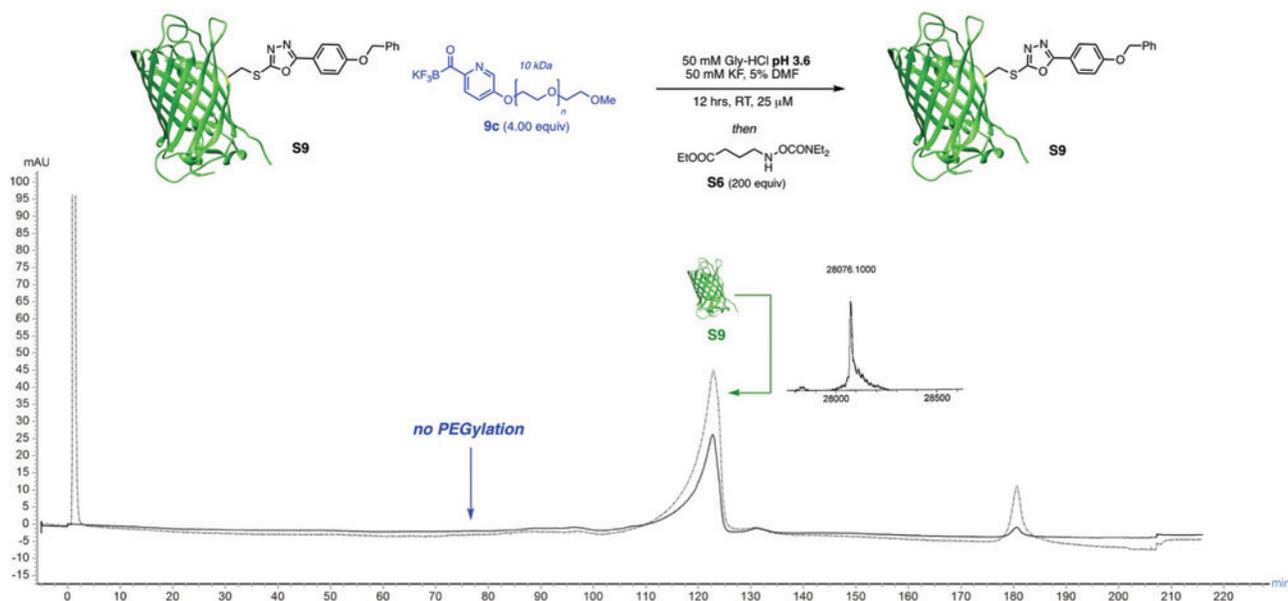
Calculated mass: 28076.3 Da

Observed mass: 28076.2 Da



**Figure S45.** Deconvoluted (ESI) mass spectrum of bioconjugate **S9**

Bioconjugate **S9** was used without further purification. The crude desalted reaction mixture was concentrated (>250  $\mu$ M) via spin diafiltration (Amicon® Ultra – 4, 10 kDa MWCO) and diluted to a concentration of 25.0  $\mu$ M with freshly prepared 50 mM Gly-HCl, 50 mM KF, 5% DMF, pH 3.6 buffer. To this was added an excess (4.0 equiv) of 2-pyridyl mPEG KAT **9c** and the resulting solution was gently stirred for 12 h. The reaction was quenched by the addition of a large excess of hydroxylamine **S6** (200 equiv, 0.1 M in DMF) and gently stirred at room temperature for an additional 30 min. The reaction mixture was subjected to multiple (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a 20 mM Tris HCl, pH 8.5 buffer at 4 °C. The crude reaction was filtered through a 0.2  $\mu$ m membrane filter and directly purified by anion exchange chromatography (Mono Q 5/50 GL) with an isocratic washing of buffer A (20 mM Tris HCl, pH 8.5) for 10 CVs, followed by a 0 – 20% linear gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, pH 8.5) over 40 CVs with a flow rate of 0.25 mL/min. Analysis of the chromatogram and subsequent ESI MS analysis of the major peak revealed no PEGylation of **S9** (*see* Figure S46 for trace) confirming that 2-pyridyl KATs do not, to any significant extent, interact with other proteinaceous functionalities under our optimized ligation conditions (Gly-HCl, pH 3.6 buffer).



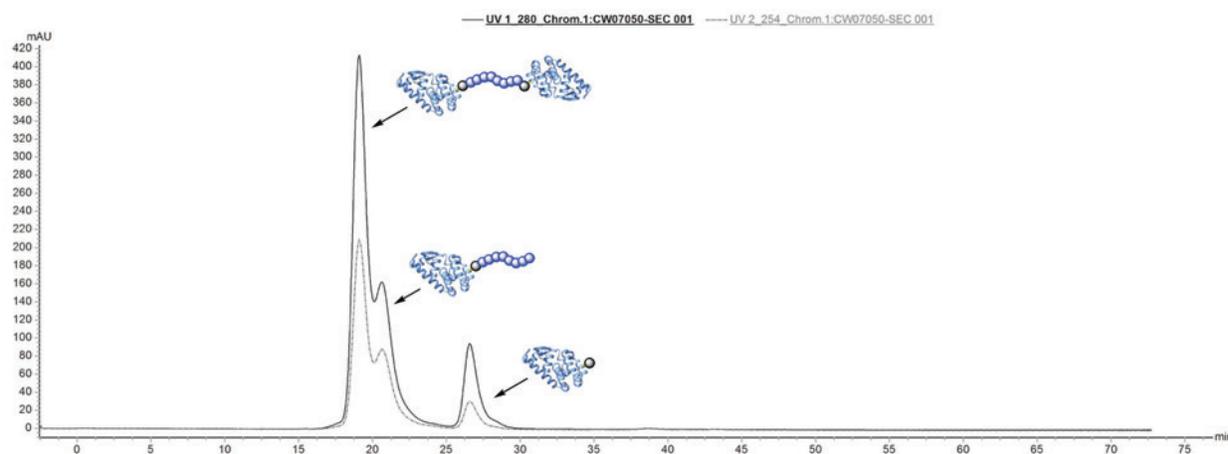
**Figure S46.** FPLC trace of the crude reaction mixture between **S9** and **9c** indicating no protein PEGylation. The deconvoluted (ESI) mass spectrum of the isolated major peak is shown. Black (solid): 490 nm, grey (dotted): 280 nm.

## 7. Protein homodimerization and oligomerization

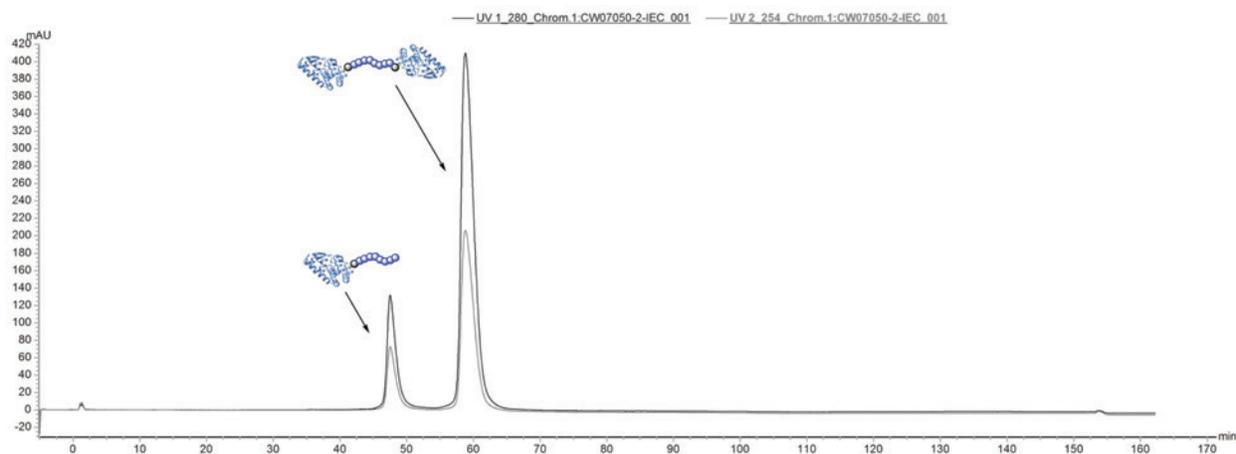
### 7.1 Homodimerization of T4L(V131C) (20)

Following the cysteine-methylsulfone ligation to append *O*-carbamoyl hydroxylamine **4** onto the surface of T4L(V131C) to form bioconjugate **16** (see section 4), the crude reaction mixture was diluted (15  $\mu$ M) a 100 mM K-Phos, pH 6.5 buffer containing 5% DMF. Excess methylsulfone hydroxylamine **4** was removed through repetitive spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C with 100 mM K-Phos 6.5, 5% DMF (x5) and once more with MilliQ H<sub>2</sub>O to remove a majority of the buffer salts in the reaction mixture. The concentrated crude reaction solution containing **16** (>250  $\mu$ M) was diluted to either 50  $\mu$ M or 100  $\mu$ M (with respect to **16**) with freshly prepared 50 mM Gly-HCl, 50 mM KF, 5% DMF, pH 3.6 buffer. To this was added diKAT **12** (0.5 equiv with respect to **16**, 5 mM in DMF). The resulting solution was vortexed quickly and gently agitated at room temperature for 0.5, 1.0 or 5.0 h. The ligation was quenched by the addition of a large excess of hydroxylamine **S6** (200 equiv,

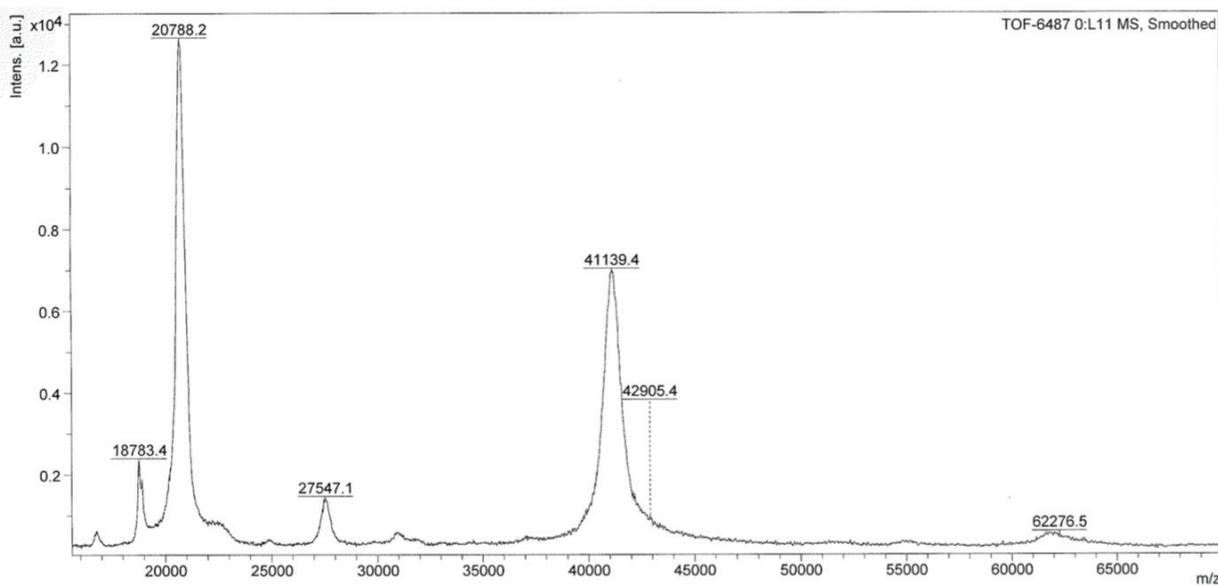
0.1 M in DMF) and gently agitated at room temperature for an additional 30 min. The reaction mixture was diluted (15  $\mu$ M) with a 25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, 150 mM NaCl, pH 7.6 buffer and subjected to repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C with the same buffer. Upon concentration (200  $\mu$ M), the crude reaction was filtered through a 0.2  $\mu$ m membrane filter and aliquots (25  $\mu$ L) were taken for SDS-PAGE analysis. The reaction was initially subjected to purification by size exclusion chromatography (Superdex 75 10/300 GL). The first major peak, containing both the monoligated intermediate and the desired homodimer product **20** (see Figure S47 for trace) was collected and exchanged into a 25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6 buffer through repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C. This was subjected to a second purification by cation exchange chromatography (MonoS 5/50 GL) with an isocratic washing of buffer A (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, pH 7.6) for 10 CVs, followed by a 0 – 50% linear gradient of buffer B (25 mM Tris, 25 mM MOPS, 0.2 mM EDTA, 1.0 M NaCl, pH 7.6) over 25 CVs with a flow rate of 0.25 mL/min. Fractions containing the desired homodimer product **20** (see Figure S48, second major peak) were pooled and passed through a PD MiniTrap G-25 desalting column (GE Healthcare) eluting with MilliQ H<sub>2</sub>O, prior to mass and SDS-PAGE analysis (MALDI-TOF see Figure S49).



**Figure S47.** FPLC trace (size exclusion chromatography) of the crude reaction mixture to form T4L – homodimer **20** with a 1.0 hour ligation time, at a protein concentration of 50  $\mu$ M. The identities of each peak shown. Black (solid): 280 nm, grey: 254 nm.



**Figure S48.** FPLC trace (cation exchange chromatography) of the pooled fractions from the first major peak (with shoulder) of the above size exclusion trace (Figure S47). Identities of each peak are shown. Black (solid): 280 nm, grey: 254 nm.



**Figure S49.** MALDI-TOF of T4L(V131C) – homodimer (**20**)

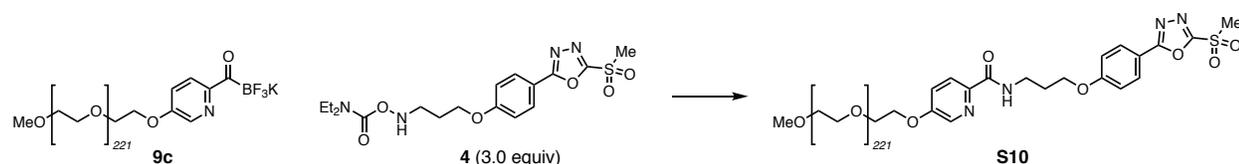
## 7.2 Homodimerization of sfGFP(S147C) (21)

Following the cysteine-methylsulfone ligation to append *O*-carbamoyl hydroxylamine **4** onto the surface of sfGFP(S147C) **5** to form bioconjugate **6** (*see* section 4), the crude reaction mixture was diluted (15  $\mu$ M) a 20 mM K-Phos, pH 7.4 buffer containing 5% DMF. Excess methylsulfone hydroxylamine **4** was removed through repetitive spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C with 20 mM K-Phos 7.4, 5% DMF (x5) and once more with MilliQ H<sub>2</sub>O to remove a majority of the buffer salts in the reaction mixture. The concentrated crude reaction solution containing **6** (>250  $\mu$ M) was diluted to 25  $\mu$ M or 100  $\mu$ M with freshly prepared 50 mM Gly-HCl, 50 mM KF, 5% DMF, pH 3.6. To this was added diKAT **12** (0.5 equiv with respect to **6**, 5 mM in DMF). The resulting solution was vortexed quickly and gently agitated at room temperature for 0.5, 1.0 or 5.0 h. The ligation was quenched by the addition of a large excess of hydroxylamine **S6** (200 equiv, 0.1 M in DMF) and gently agitated at room temperature for an additional 30 minutes. The reaction mixture was diluted (15  $\mu$ M) with a 20 mM Tris HCl, pH 8.5 buffer and subjected to repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C with the same buffer. Upon concentration (200  $\mu$ M), the crude reaction was filtered through a 0.2  $\mu$ m membrane filter and aliquots (25  $\mu$ L) were taken for SDS-PAGE analysis.

### 8. Control experiment – one-step PEGylation of sfGFP(147C) with a methylsulfonylphenyloxadiazole-mPEG

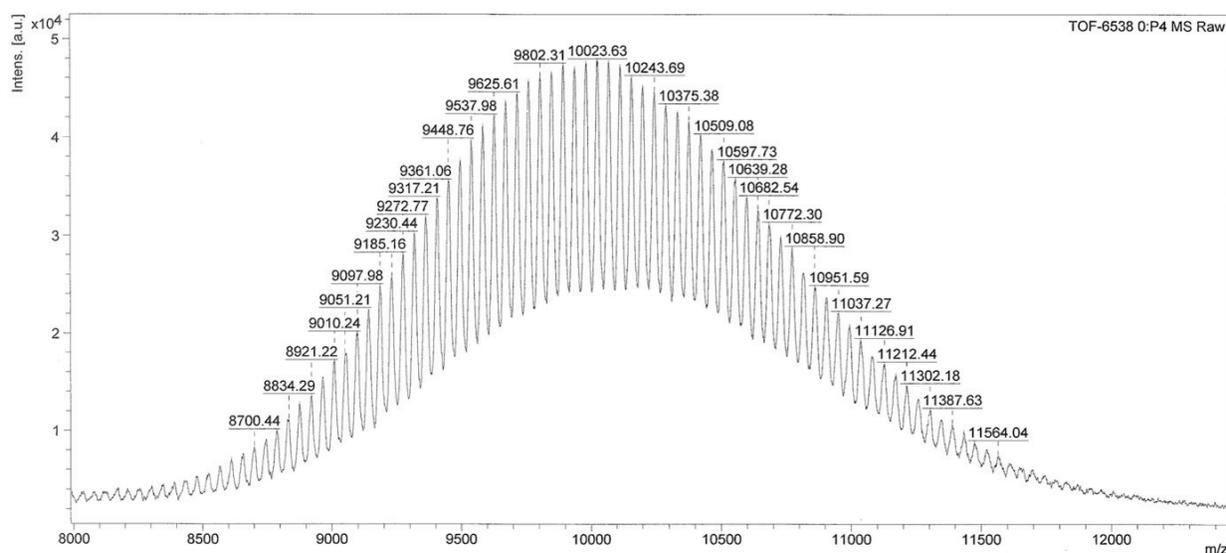
It should be noted that our KAT-mediated protein PEGylation is, in actuality, a two-step process in which a protein of interest is first appended with a small molecule reagent and treated with an orthogonally reactive PEG reagent in a subsequent, second step. With our success in KAT-mediated protein PEGylation, we sought to investigate the effectiveness of the corresponding one-step process (i.e. the direct treatment of protein bearing a solvent-exposed cysteine residue with a thiol-reactive PEG reagent) and how it would compare to our optimized KAT-mediated PEGylation protocol. To do this methylsulfonylphenyloxadiazole-mPEG **S10** was first prepared through a KAT-ligation between 10 kDa 2-pyridyl-mPEG-KAT **9c** and an excess of bifunctional reagent **4** (Scheme S11).

#### Scheme S11. Synthesis of 10 kDa methylsulfonylphenyloxadiazole-mPEG **S10**



#### 5-(2-(2-methoxyethoxy)ethoxy)-N-(3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)picolinamide – PEG 10000 (**S10**).

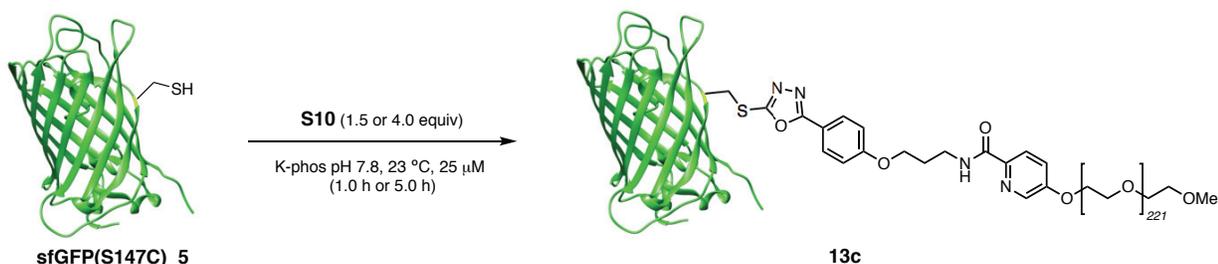
10 kDa 2-pyridyl mPEG-KAT **9c** (256 mg,  $2.50 \times 10^{-2}$  mmol, 1.00 equiv) was dissolved in a mixture of *t*-BuOH : H<sub>2</sub>O (5 : 1, v/v) containing 0.1 M oxalic acid (2.5 mL) at room temperature with stirring. Bifunctional reagent **4** (15.5 mg,  $3.75 \times 10^{-2}$  mmol, 1.50 equiv) was added as a solid. The resulting solution was stirred at room temperature for 15 hours. The reaction mixture was transferred to a 3.5 kDa MWCO dialysis membrane tube and subsequently dialyzed against aqueous H<sub>2</sub>O : CH<sub>3</sub>CN (1 : 1, v/v) (6 x 2.0 L) at room temperature. The final dialyzed solution was lyophilized to yield methylsulfonylphenyloxadiazole-mPEG **S10** as a white solid (247 mg, 96% yield). **<sup>1</sup>H NMR** (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.76 (t, *J* = 6.0 Hz, 1H), 8.33 (d, *J* = 2.8 Hz, 1H), 8.08 – 8.00 (m, 2H), 7.98 (d, *J* = 8.7 Hz, 1H), 7.55 (dd, *J* = 8.8, 2.9 Hz, 1H), 7.24 – 7.15 (m, 2H), 4.30 – 4.23 (m, 2H), 4.16 (t, *J* = 6.1 Hz, 2H), 3.83 – 3.76 (m, 2H), 3.69 (s, 3H), 3.68 (dd, *J* = 3.8, 2.2 Hz, 2H), 3.51 (s, 948H), 3.24 (s, 3H), 2.04 (p, *J* = 6.4 Hz, 2H); **<sup>13</sup>C NMR** (101 MHz, DMSO) δ 165.8, 163.7, 162.4, 161.7, 156.8, 142.7, 136.7, 129.4, 123.0, 121.4, 115.5, 114.1, 71.3, 70.2, 69.8, 69.6, 68.7, 67.9, 66.4, 58.0, 42.9, 36.1, 28.8; **MALDI-TOF** see spectrum.



**Figure S50.** MALDI-TOF of 10 kDa methylsulfonephenyloxadiazone-mPEG (**S10**)

With **S10** in hand, we were set to investigate the effectiveness of a one-step PEGylation protocol and chose sfGFP(S147C) **5**, our model substrate from our KAT ligation studies, as a suitable candidate. In trying to maintain an effective comparison, we ran two experiments mirroring our optimized conditions A & B for the KAT ligation, in which only the equivalency of **S10** and reaction time were varied. Thus sfGFP(S147C) was treated with 1) 4.0 equivalents of **S10** for 1.0 hour at room temperature and 2) 1.5 equivalents of **S10** for 5.0 hours at room temperature. Both reactions were carried out in a potassium phosphate buffer at pH 7.8, at a protein concentration of 25  $\mu$ M (Scheme S12).

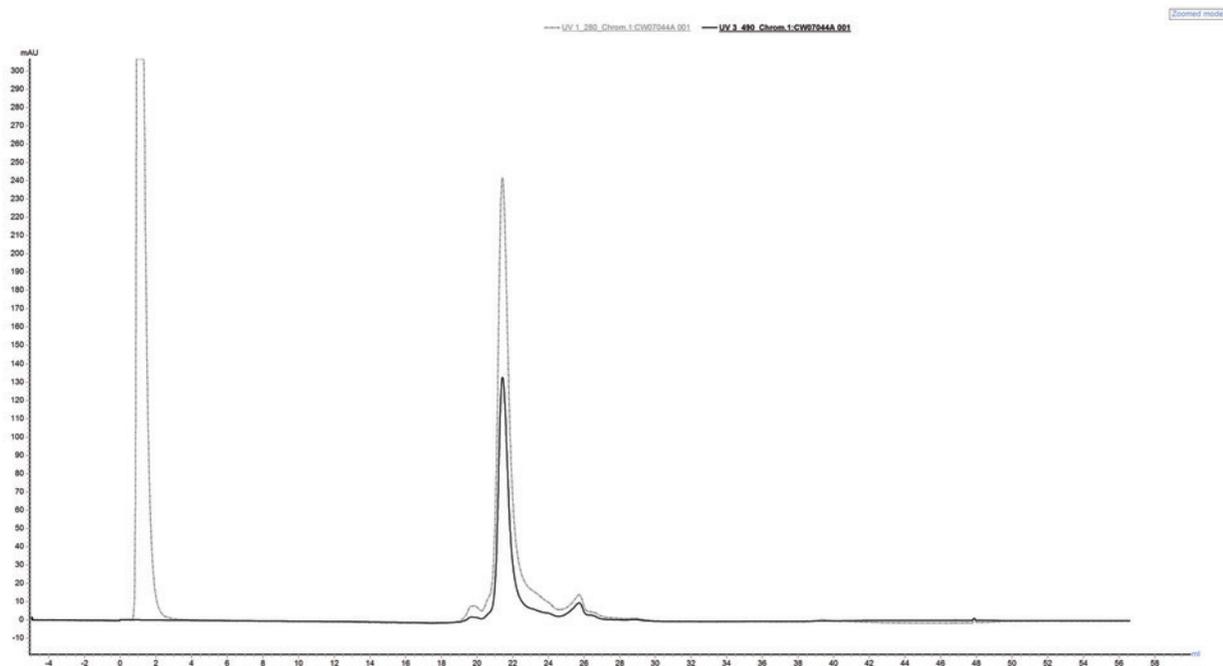
**Scheme S12.** Synthesis of sfGFP(S147C) – mPEG 10k **13c**



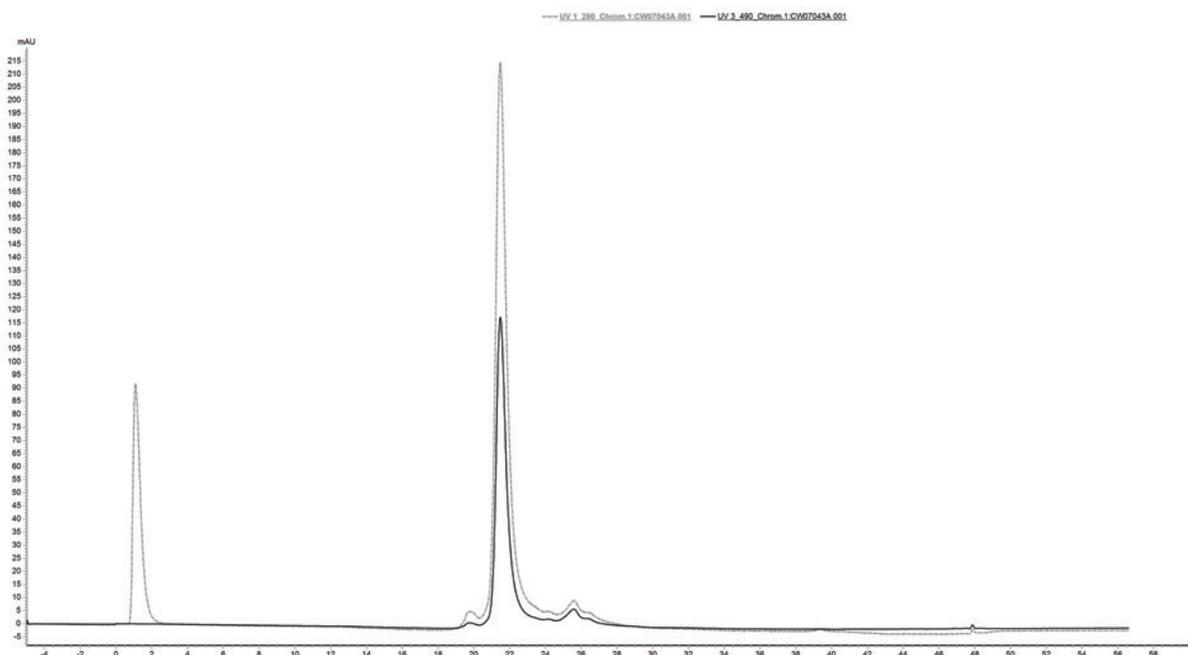
**sfGFP(S147C) – mPEG10k (13c)**

An aliquot of sfGFP(S147C) **5** (0.025  $\mu$ mol, 1.0 equiv) was treated with DTT (25.0  $\mu$ L, 5  $\times 10^2$  equiv, 0.5 M in H<sub>2</sub>O) and diluted to a concentration of 200  $\mu$ M with MilliQ H<sub>2</sub>O. The resulting solution was vortexed quickly and incubated at room temperature for 1.0 h. Excess DTT was removed through

repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) with a freshly degassed (argon balloon) 0.1 M potassium phosphate, 1 mM EDTA, pH 7.8 buffer at 4 °C. The resulting solution containing reduced **5** (devoid of DTT) was diluted (25 μM) with additional pH 7.8 buffer and to it was added reagent **S10** (3.8 μL, 1.5 equiv, or 10 μL, 4.0 equiv, 10 mM in DMF). The solution was vortexed quickly and gently agitated at room temperature for either 1.0 h (with 4.0 equiv **S10** – *reaction 1*, mirroring Condition A) or 5.0 h (with 1.5 equiv **S10** – *reaction 2*, Condition B) under an argon atmosphere. The reactions were quenched by the addition of a large excess of β-mercaptoethanol (500 equiv, 0.1 M in DMF) and gently agitated at room temperature for an additional 1.0 h. The reaction mixtures were subsequently exchanged into a 20 mM Tris HCl, pH 8.5 buffer through repetitive (x3) spin diafiltrations (Amicon® Ultra – 4, 10 kDa MWCO) at 4 °C. The crude reactions were purified by anion exchange chromatography (Mono Q 5/50 GL) with an isocratic washing of buffer A (20 mM Tris HCl, pH 8.5) for 10 column volumes (CVs), followed by a 0 – 25% linear gradient of buffer B (20 mM Tris HCl, 1.0 M NaCl, pH 8.5) over 25 CVs with a flow rate of 0.25 mL/min. Retention time of **13c** = 23.5 mL, 58.0 min, 11% buffer B (*see* Figures S51 and S52 for traces of reactions 1 and 2 respectively). Ligation efficiencies were estimated from the FPLC traces as a ratio of the peak area of the PEGylated product **13c** over areas of all peaks at 490 nm.



**Figure S51.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG10k **13c** with **S10** for reaction 1 (4.0 equiv **9c**, 1.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.



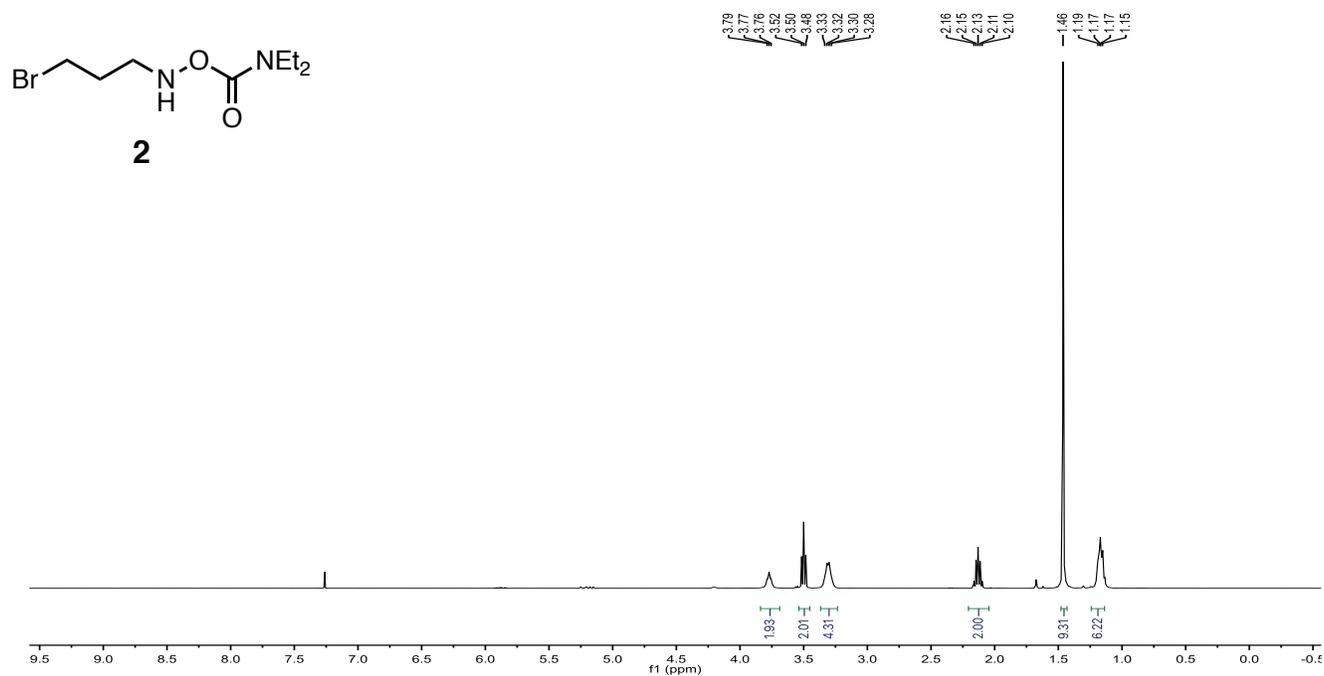
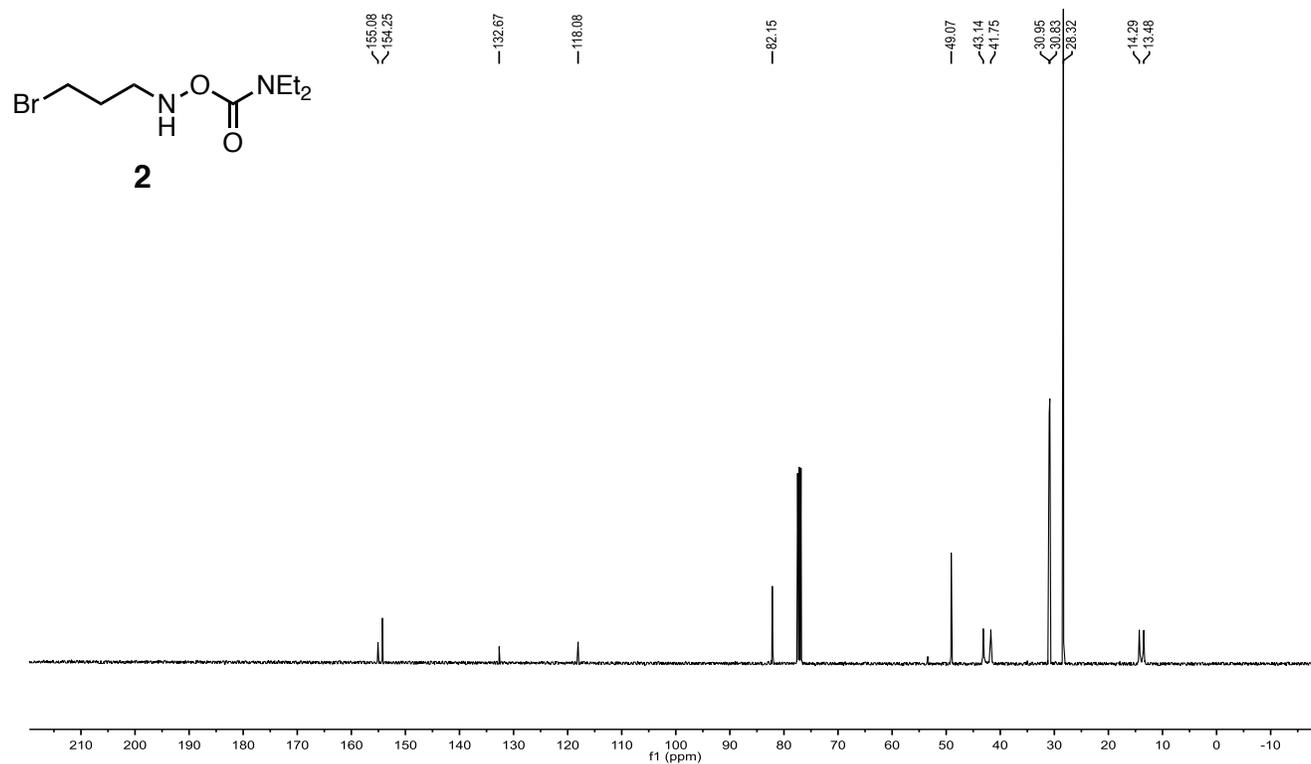
**Figure S52.** FPLC trace of the crude reaction mixture to form sfGFP(S147C) – mPEG10k **13c** with **S10** for reaction 2 (1.5 equiv **9c**, 5.0 h ligation time). Black (solid): 490 nm, grey: 280 nm.

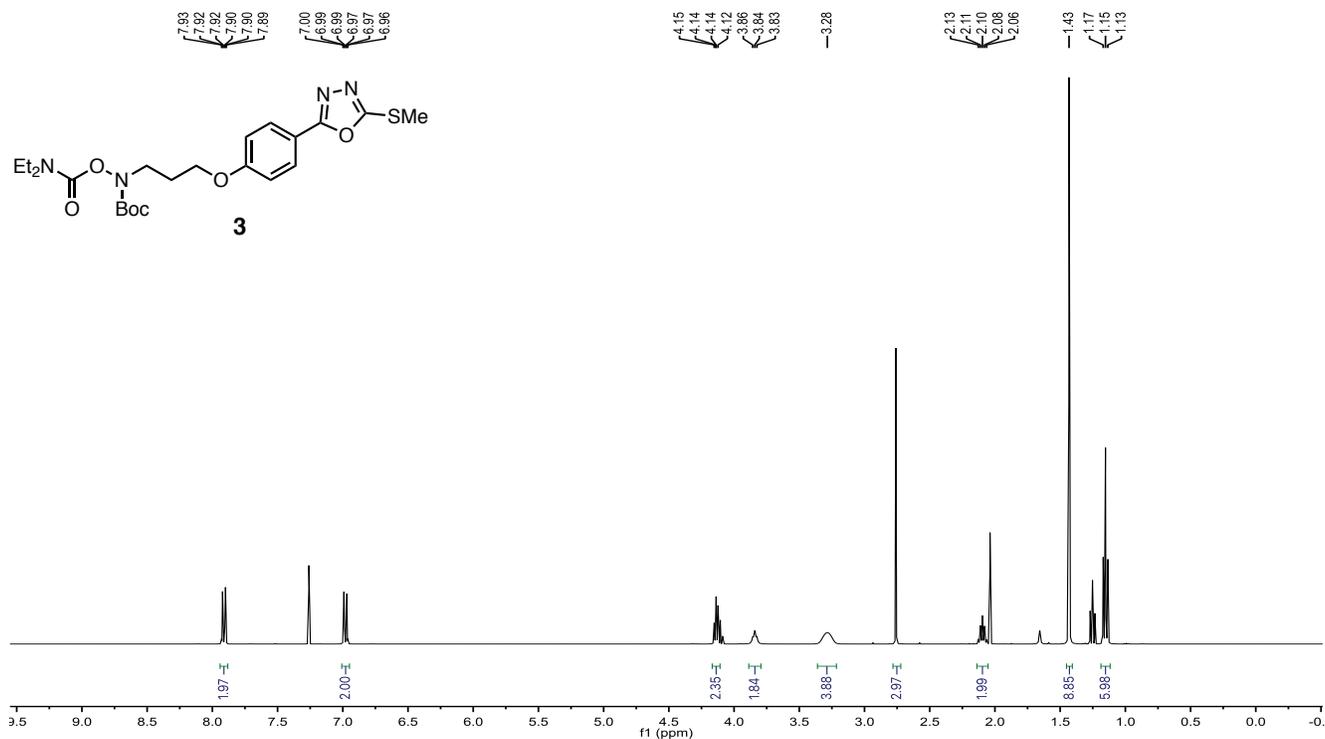
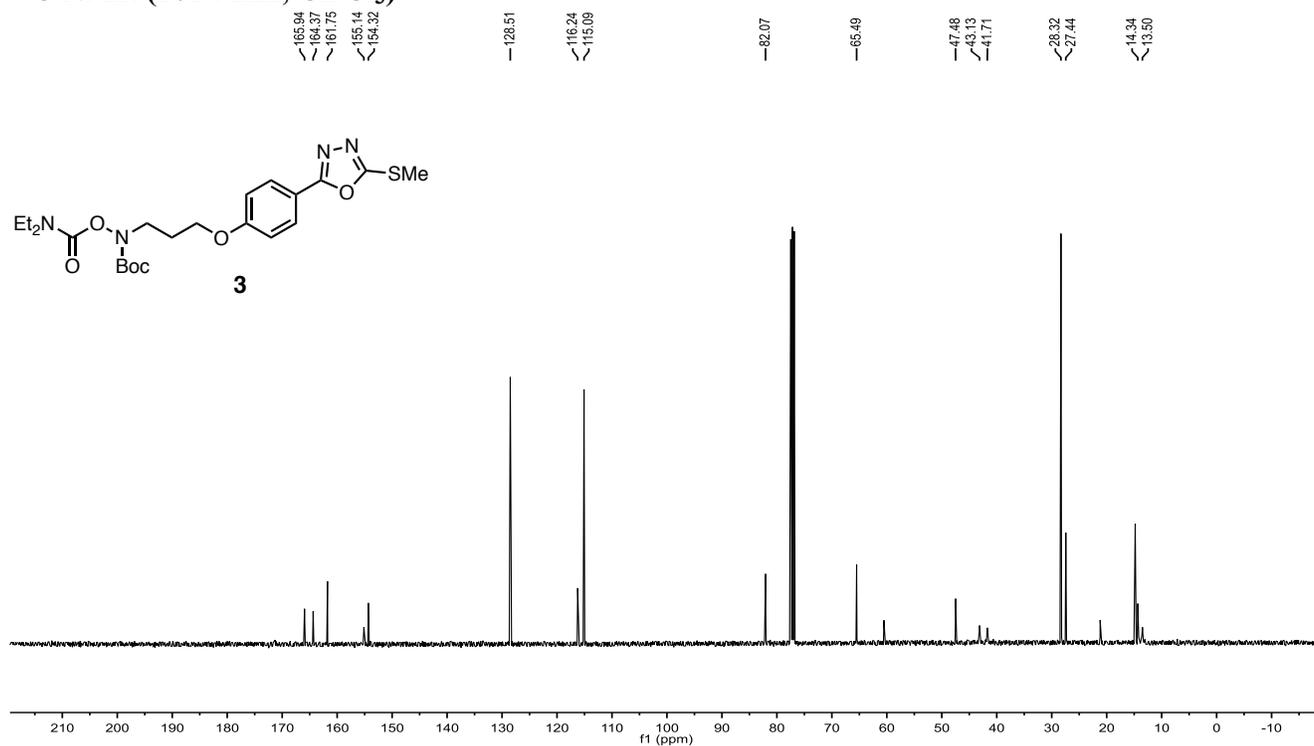
Ligation efficiency (reaction 1: 4.0 equiv **S10**, 1.0 h) = 92%

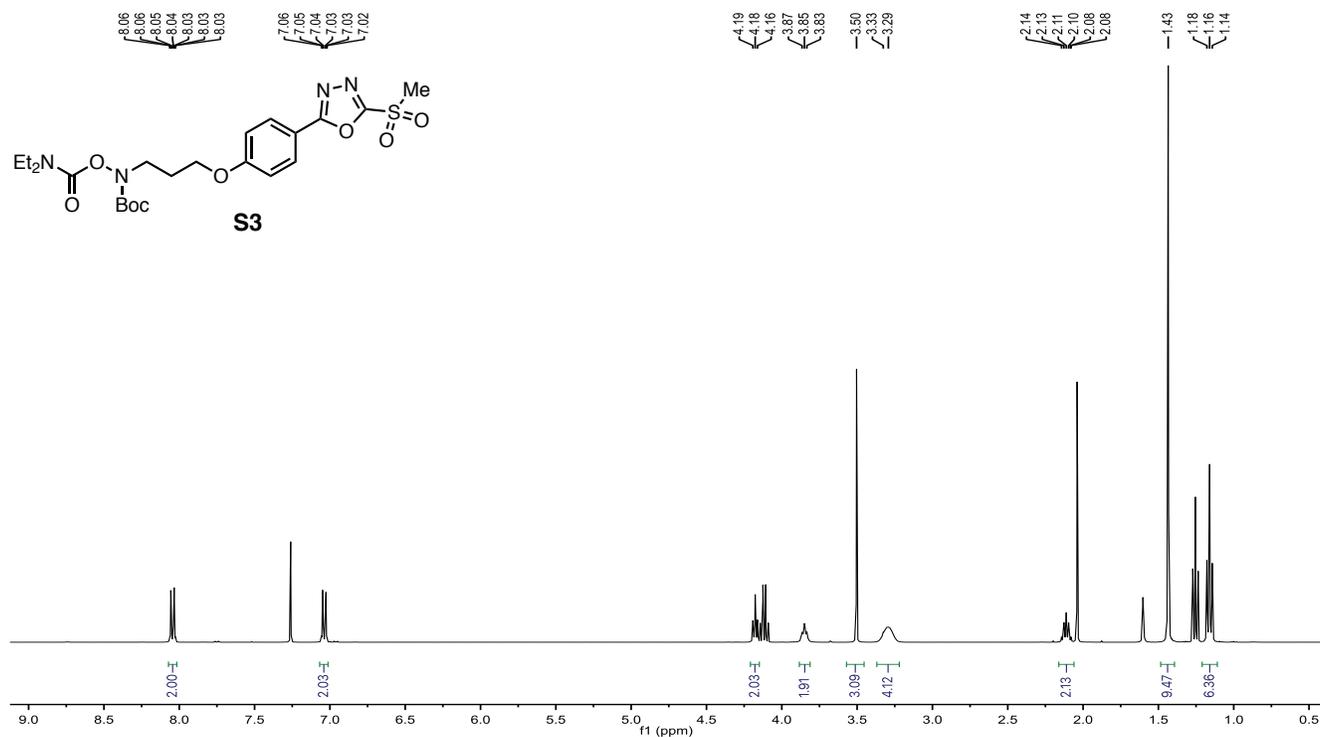
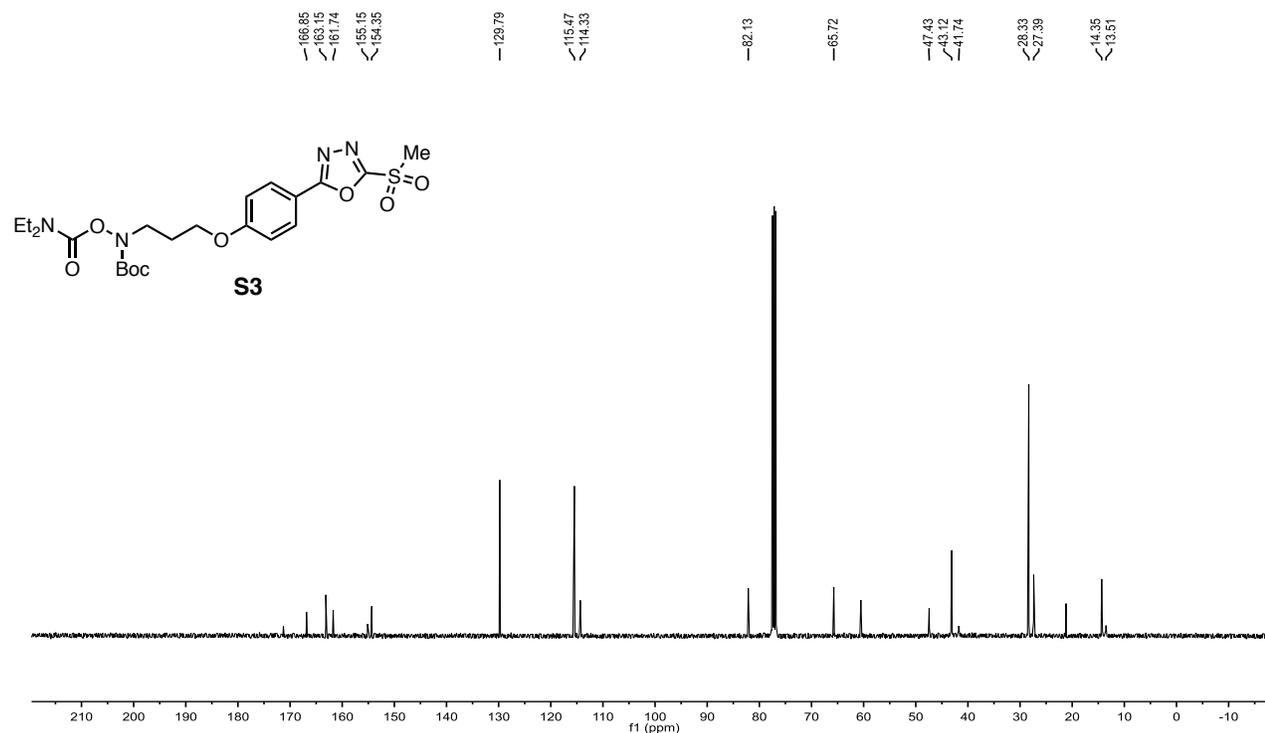
Ligation efficiency (reaction 2: 1.5 equiv **S10**, 5.0 h) = 92%

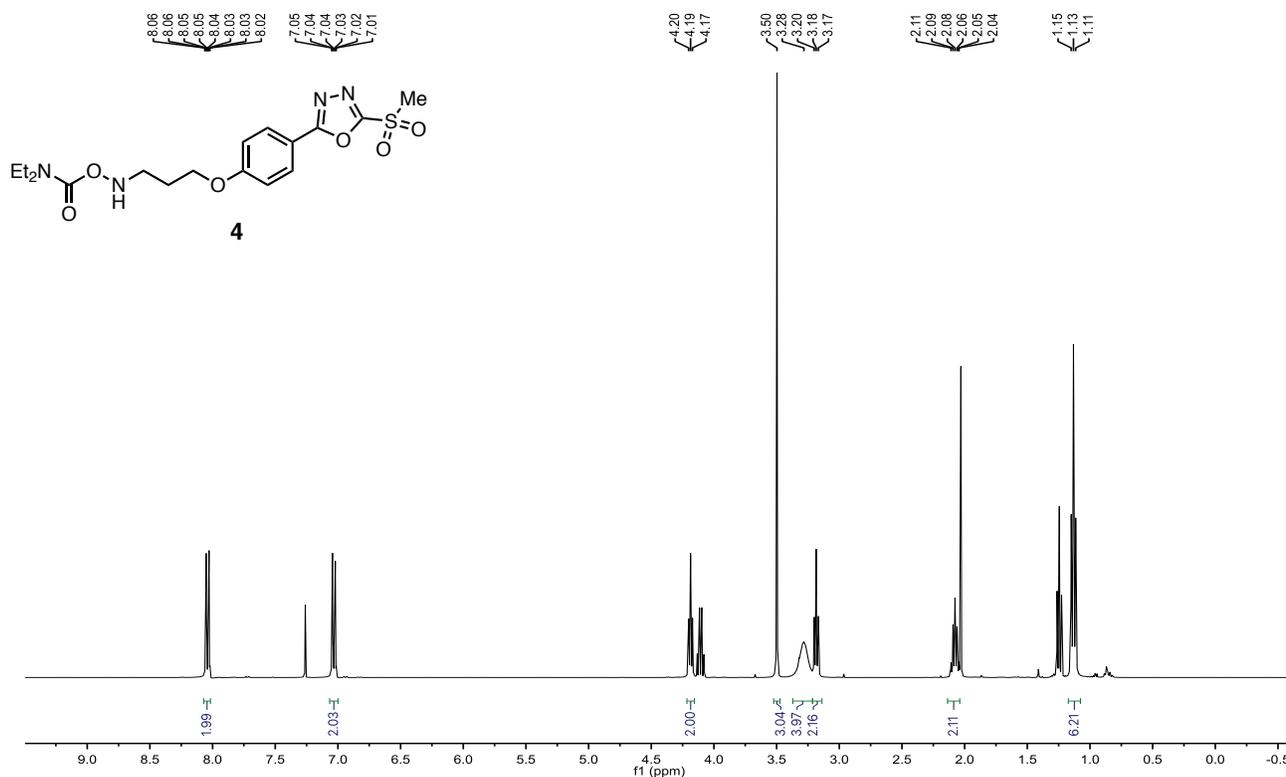
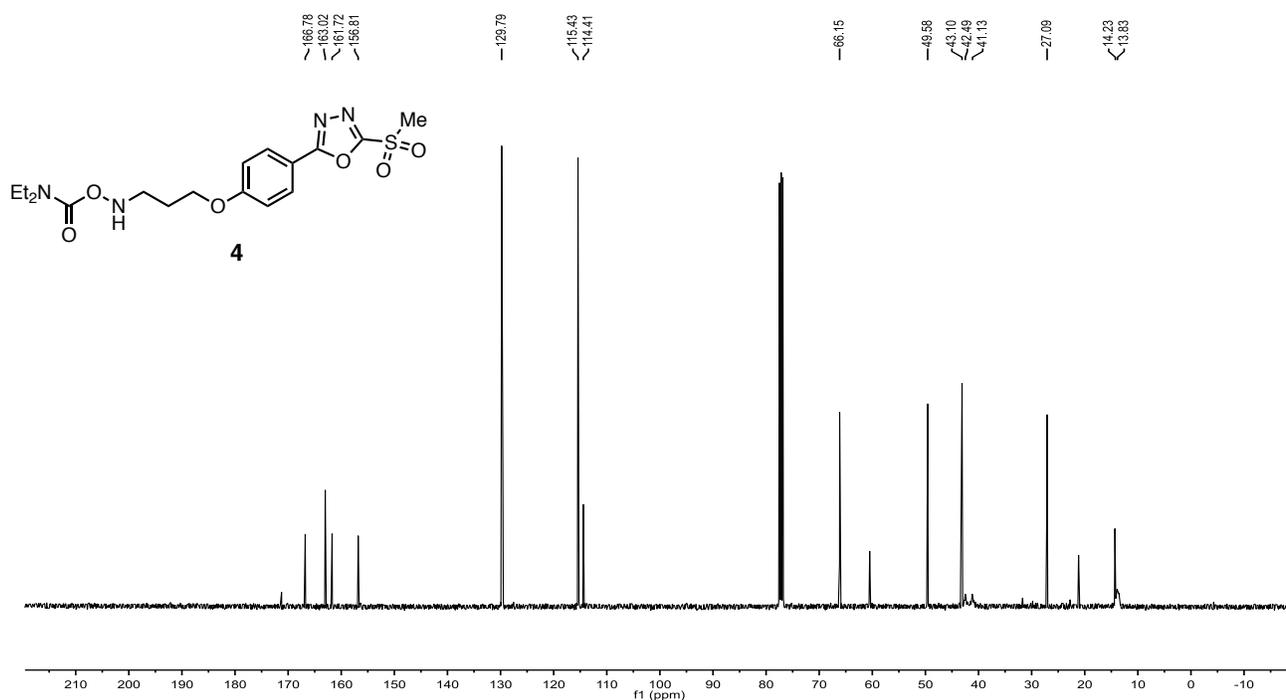
Interestingly, both reactions gave near identical outcomes with a calculated ligation efficiency of 92% by FPLC. This is slightly less than for the corresponding KAT ligation-mediated, two-step strategy (see table 1, entries 6 and 9). Furthermore, it should be noted that the stringent use of degassed buffers was required here to reach these high conversion values. If buffers were not degassed and the reactions were not performed under an argon atmosphere, a decrease in the ligation efficiency between sfGFP(S147C) **5** and **S10** was observed, likely due to competitive thiol oxidation and/or disulfide formation. Taking these factors into account, it can be seen that our two-step, KAT-ligation mediated approach to protein PEGylation is the superior method of choice as the conversions observed are higher and the KAT ligation does not hold the strict requirement to exclude oxygen, making it more practical and operationally efficient.

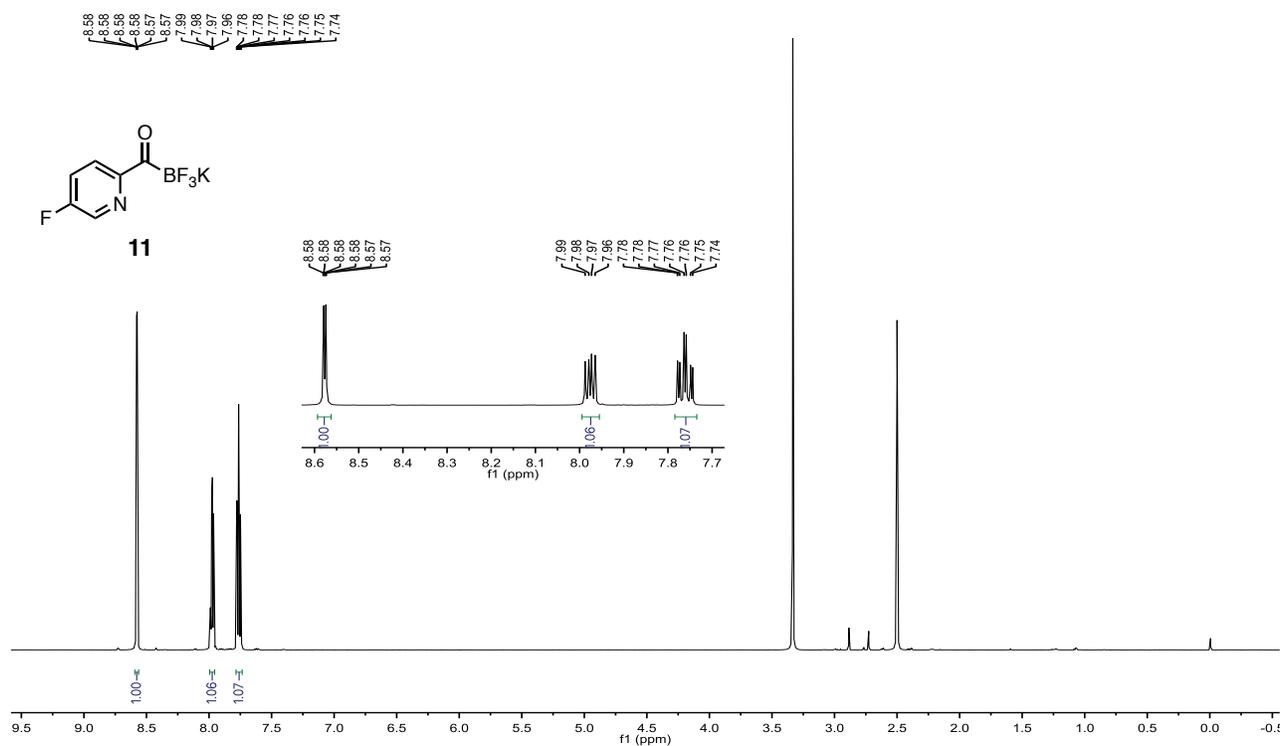
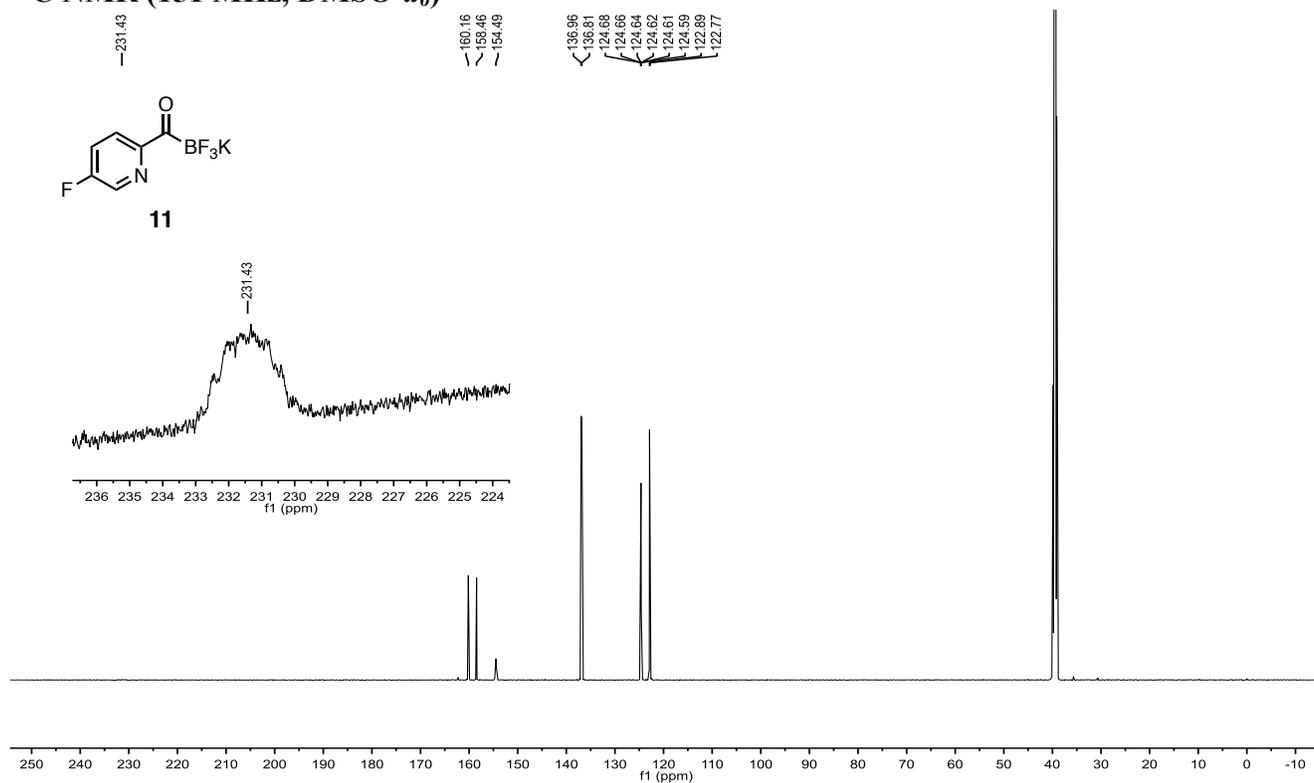
## 9. NMR Data

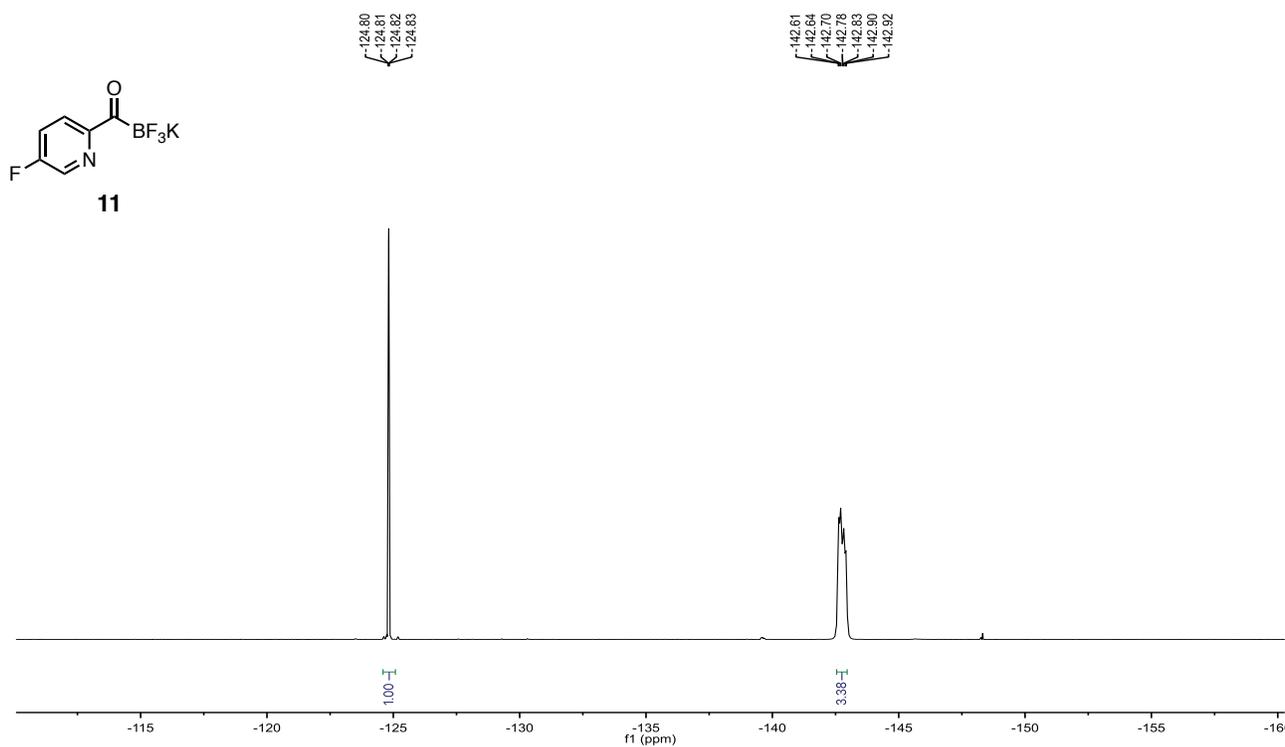
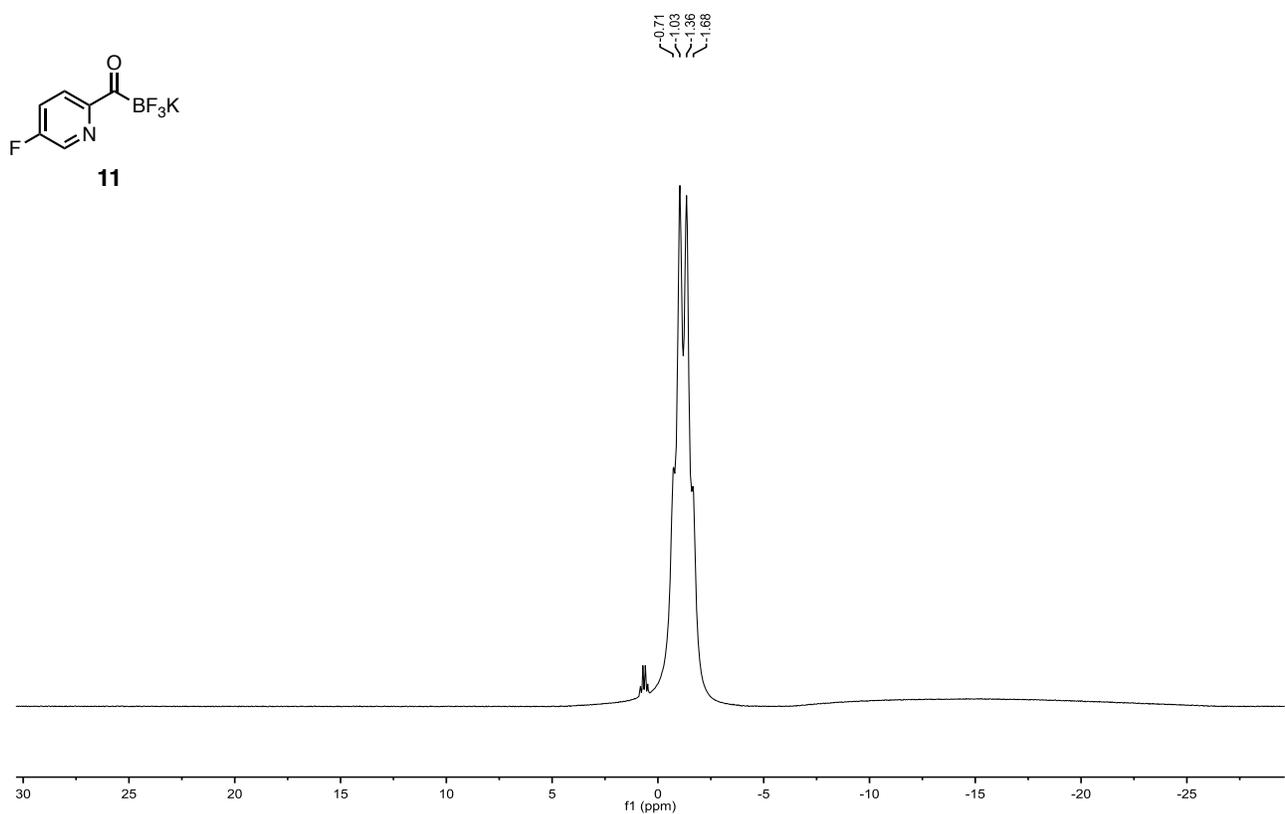
***tert*-Butyl (3-bromopropyl)((diethylcarbamoyl)oxy)carbamate (2):  
<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

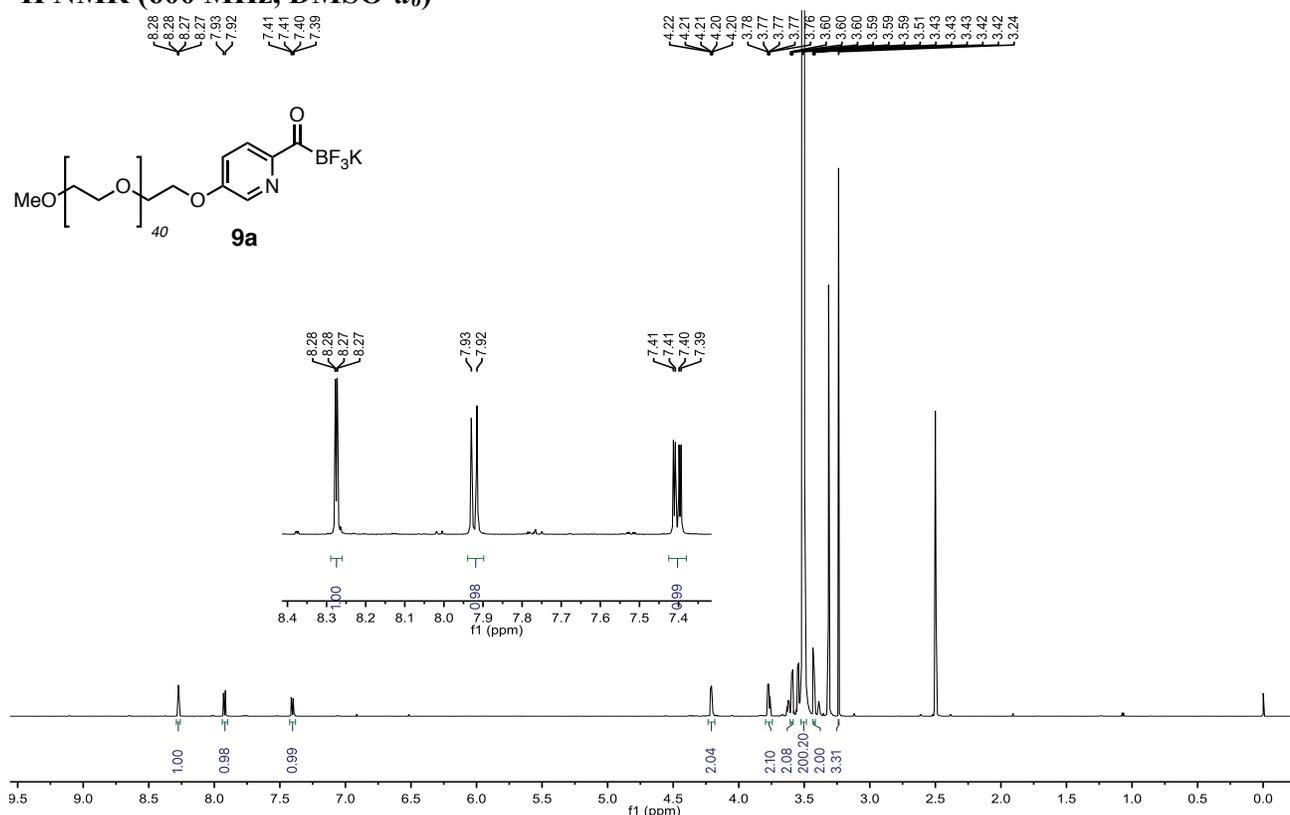
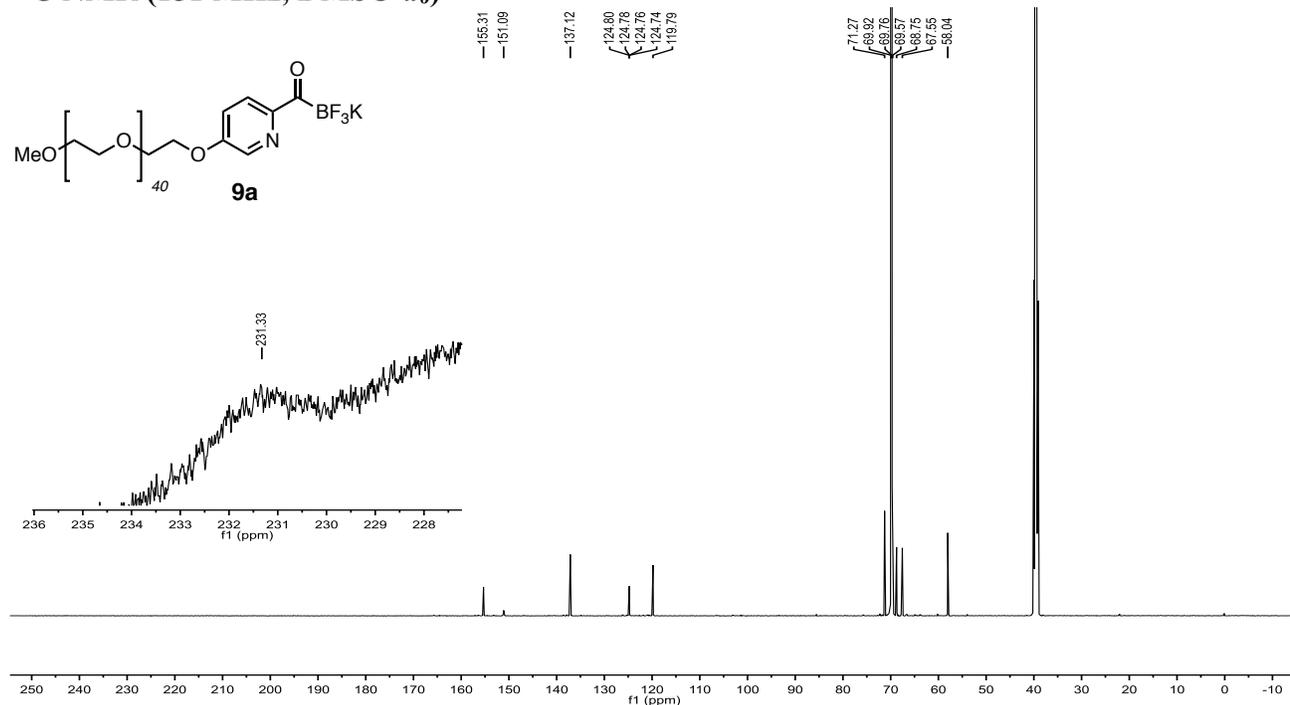
***tert*-Butyl ((diethylcarbamoyloxy)(3-(4-(5-(methylthio)-1,3,4-oxadiazol-2-yl)phenoxy)propyl) carbamate (3):****<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

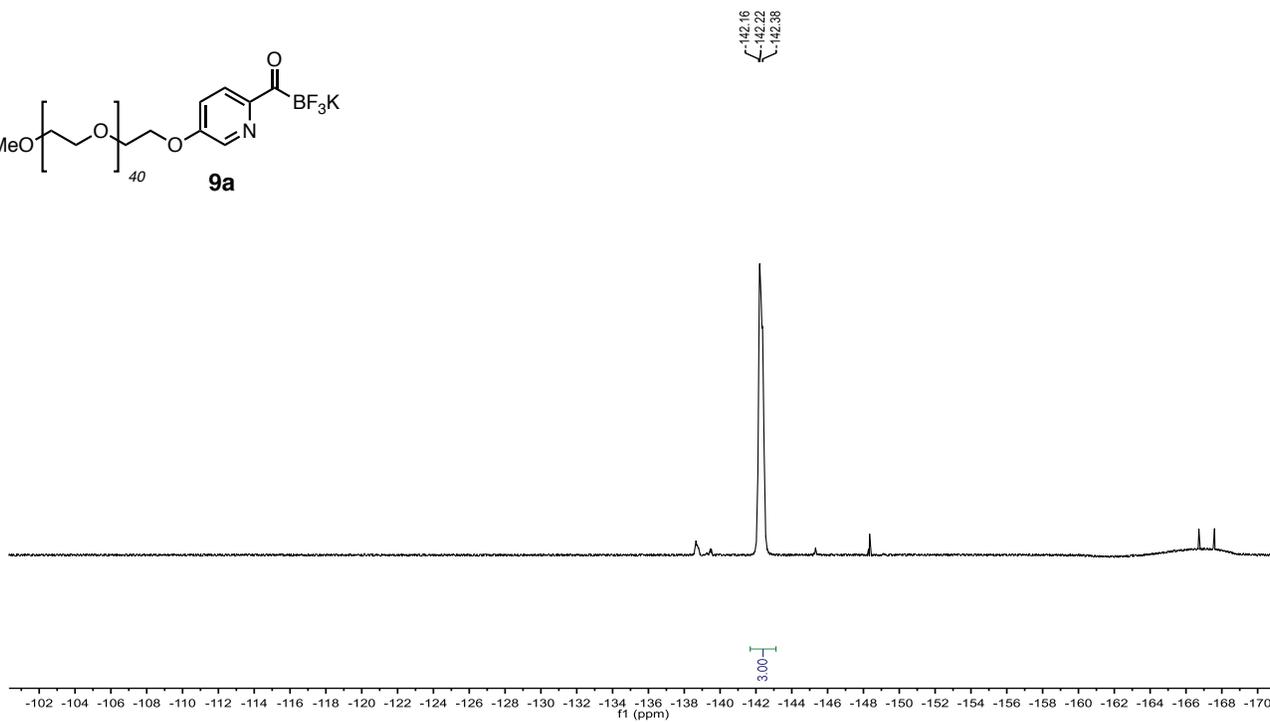
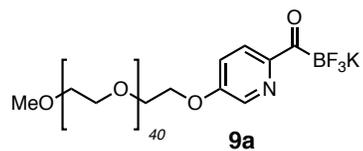
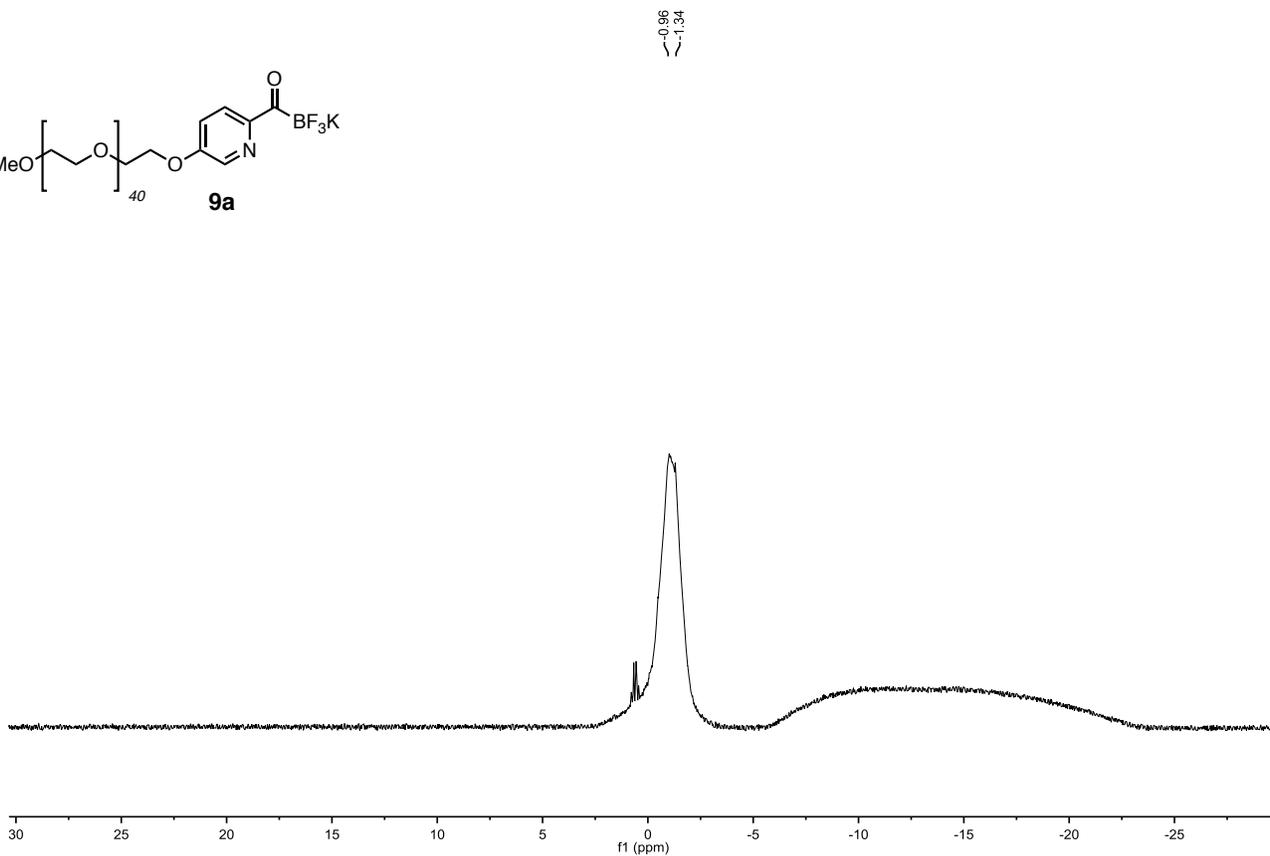
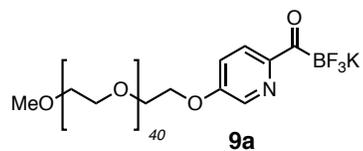
***tert*-Butyl ((diethylcarbamoyloxy)(3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)-carbamate (S3):****<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

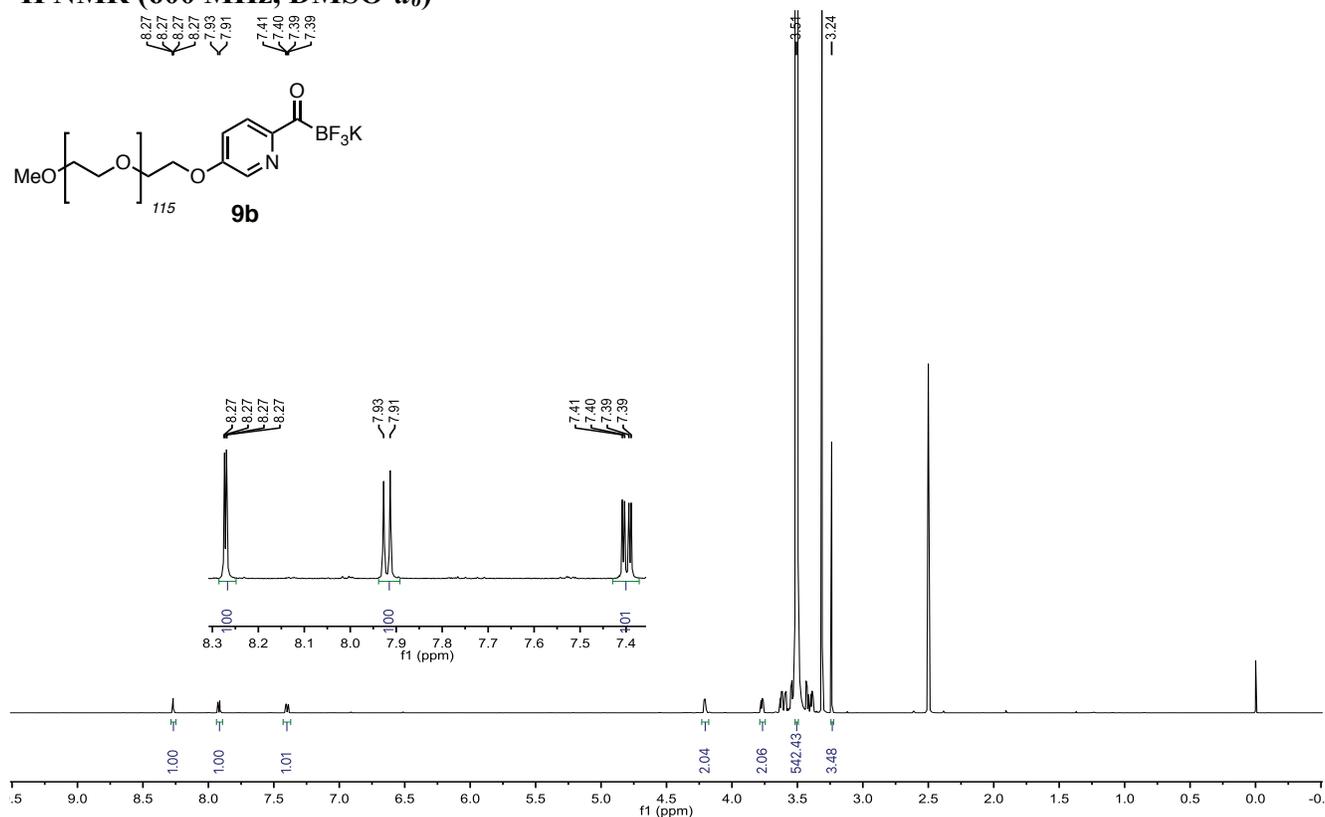
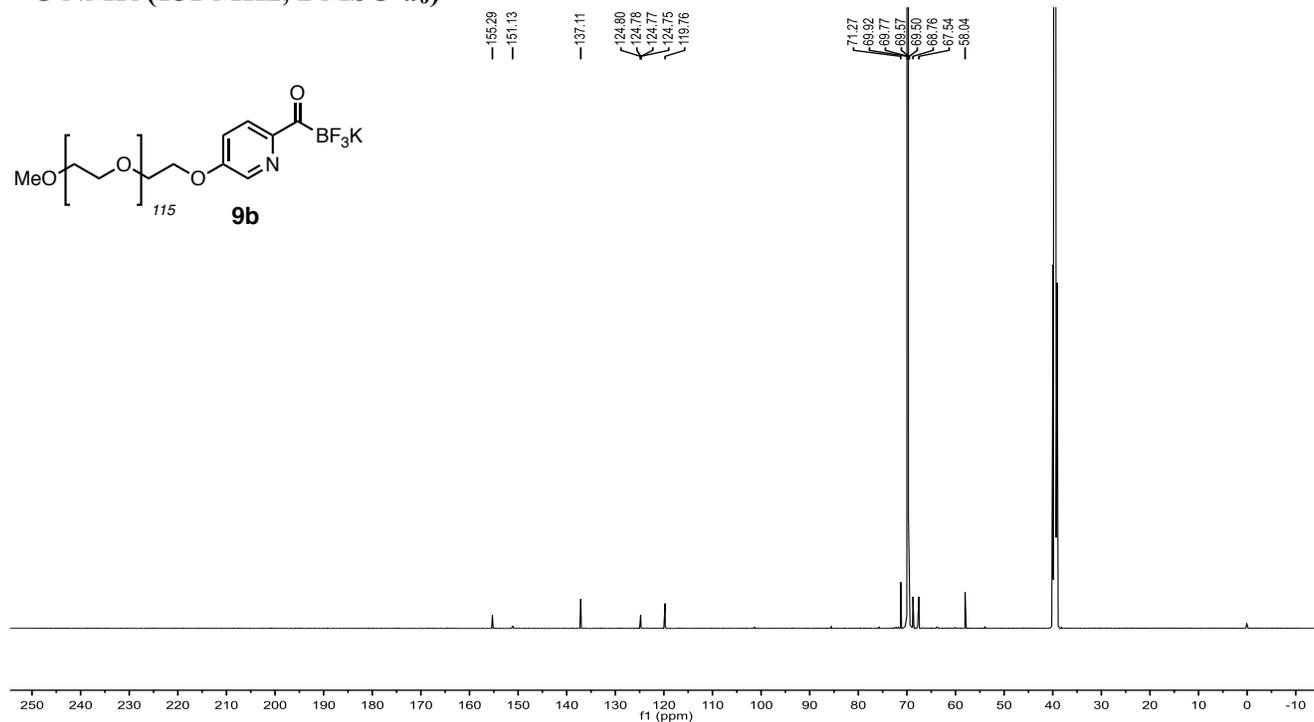
***N*-ethyl-*N*-((((3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)amino)oxy)carbonyl)ethanamine (4):****<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

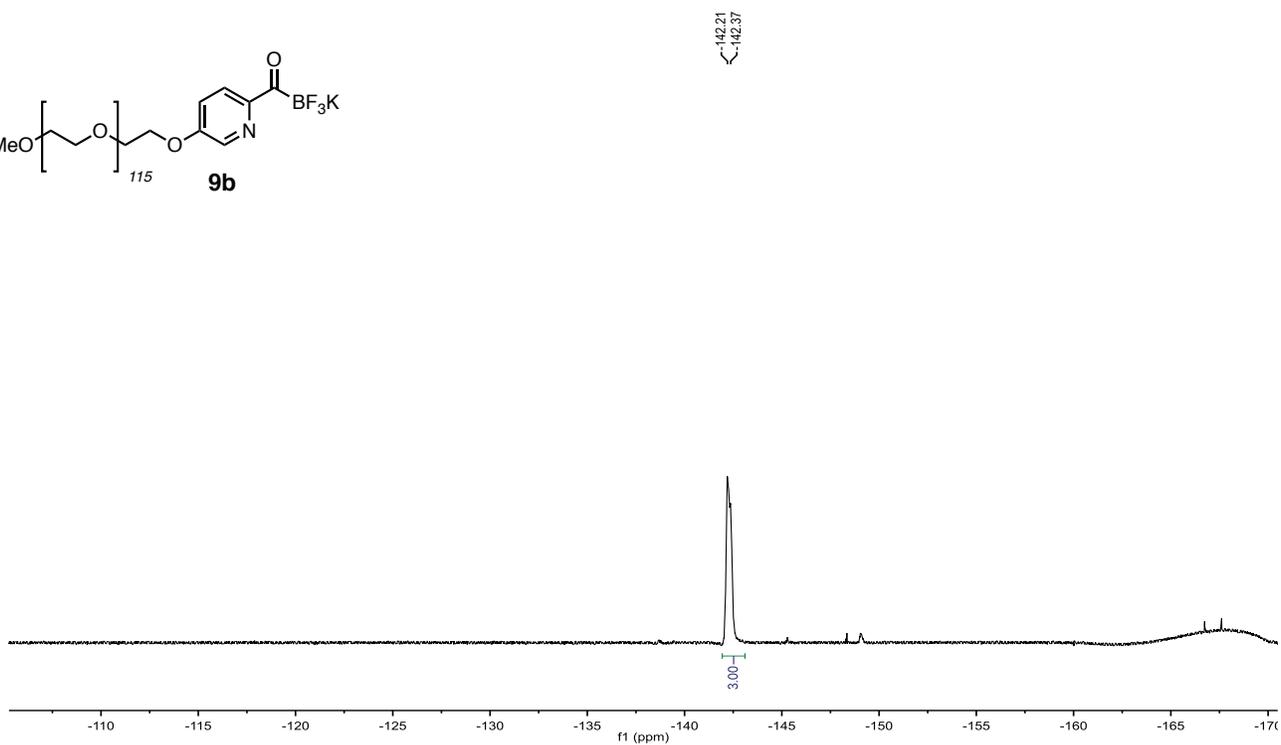
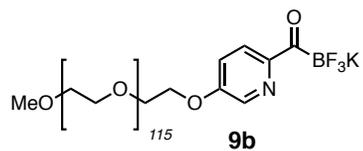
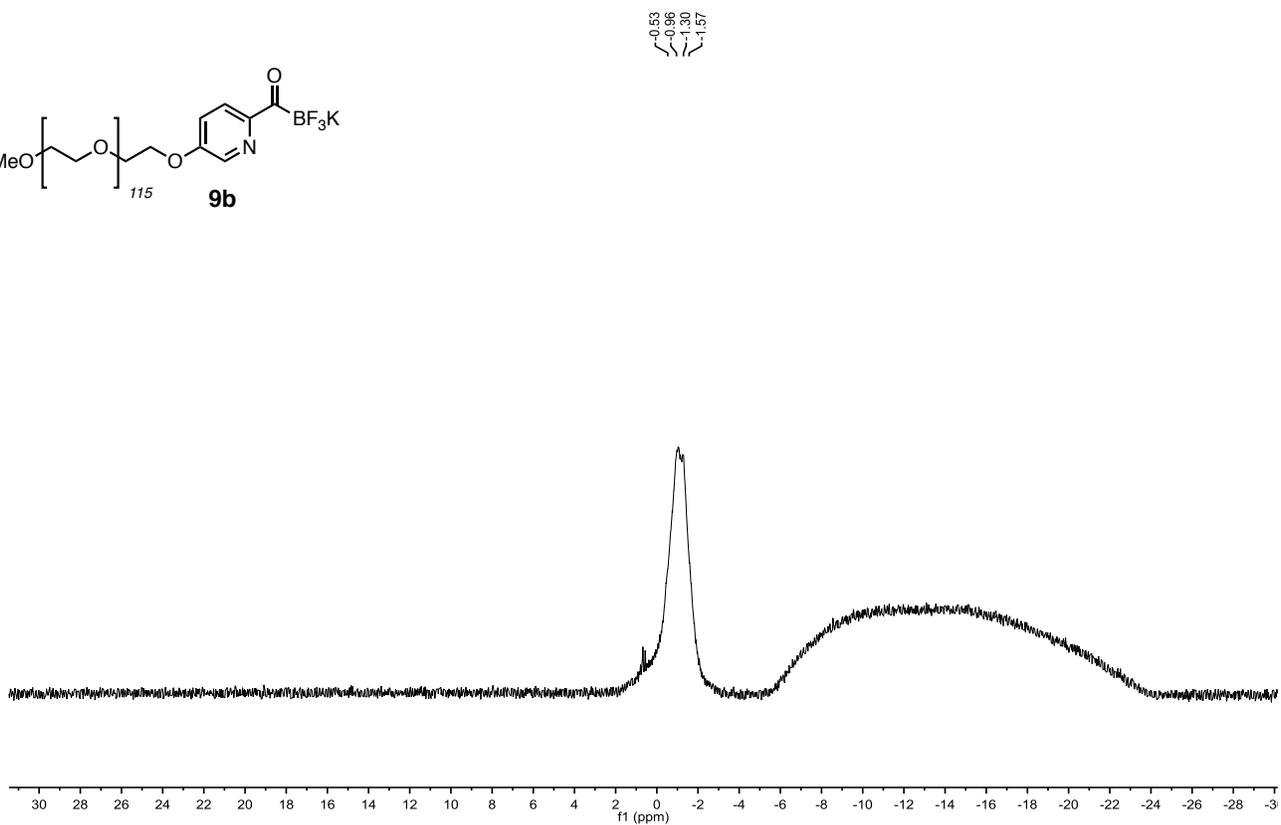
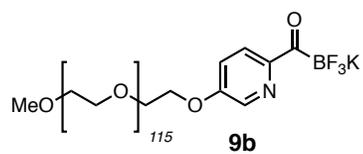
**5-fluoropyridin-2-yl potassium acyltrifluoroborate (11):** **$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )** **$^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )**

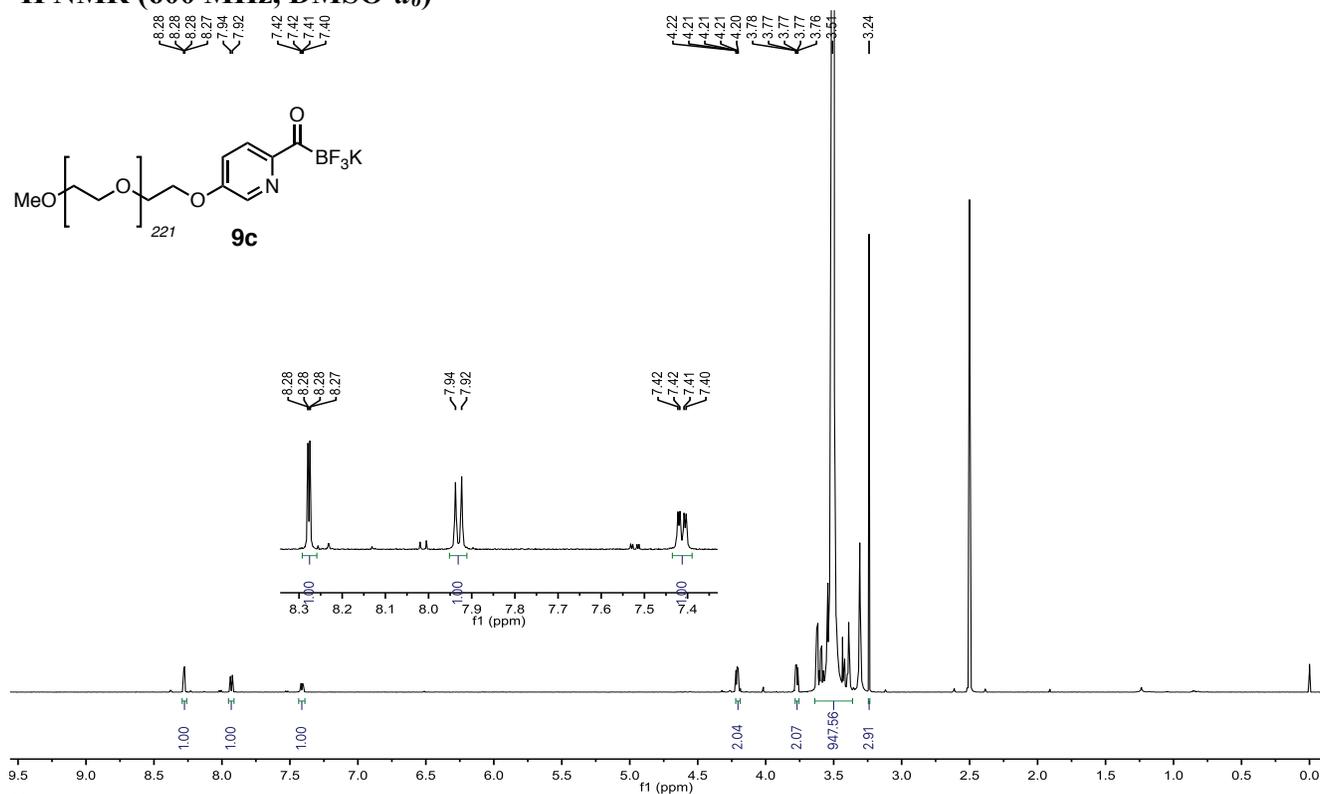
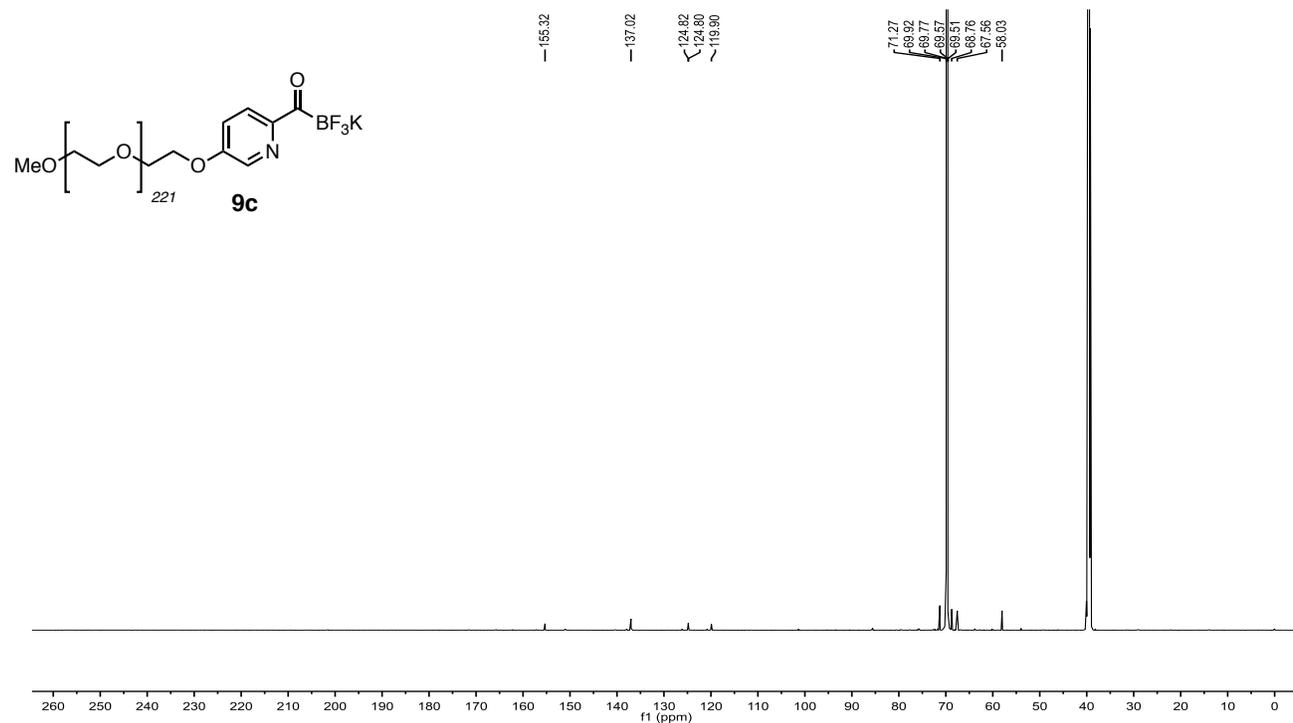
$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

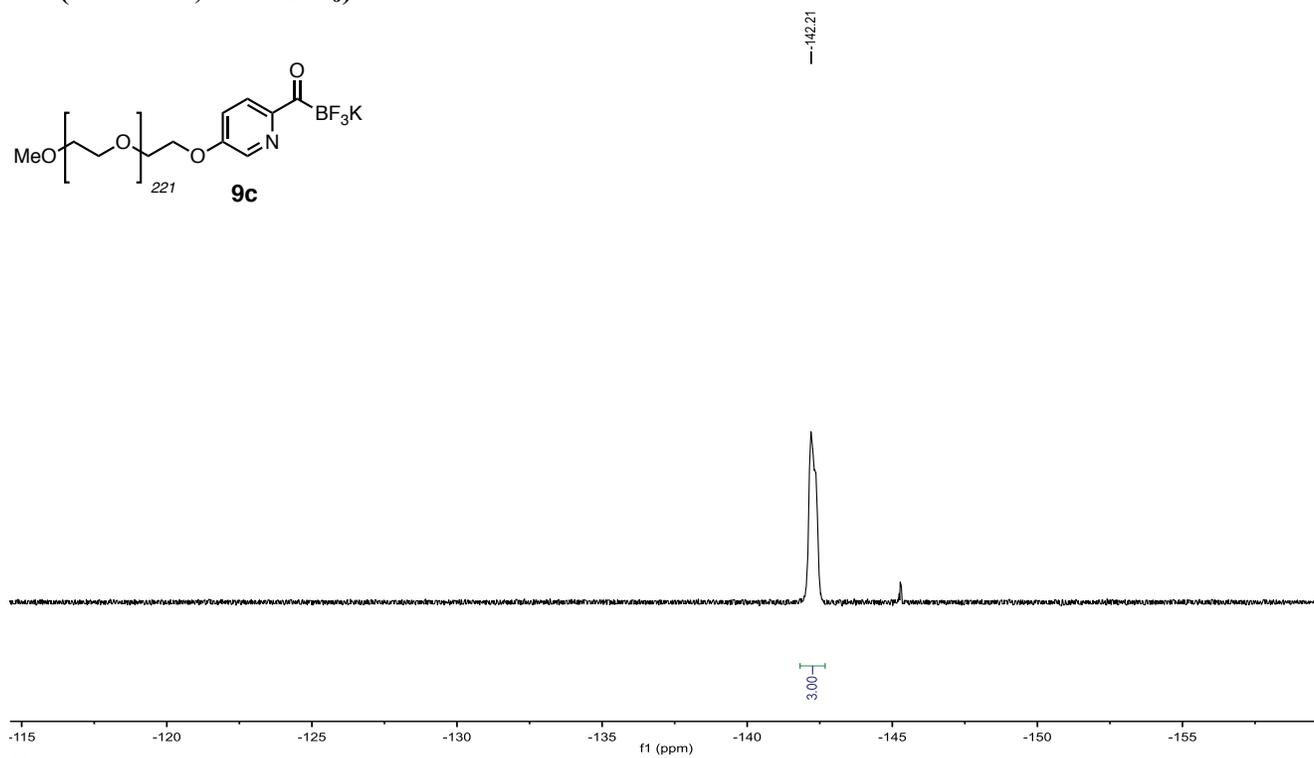
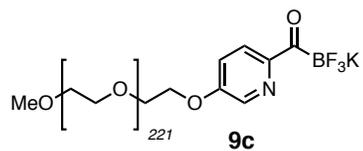
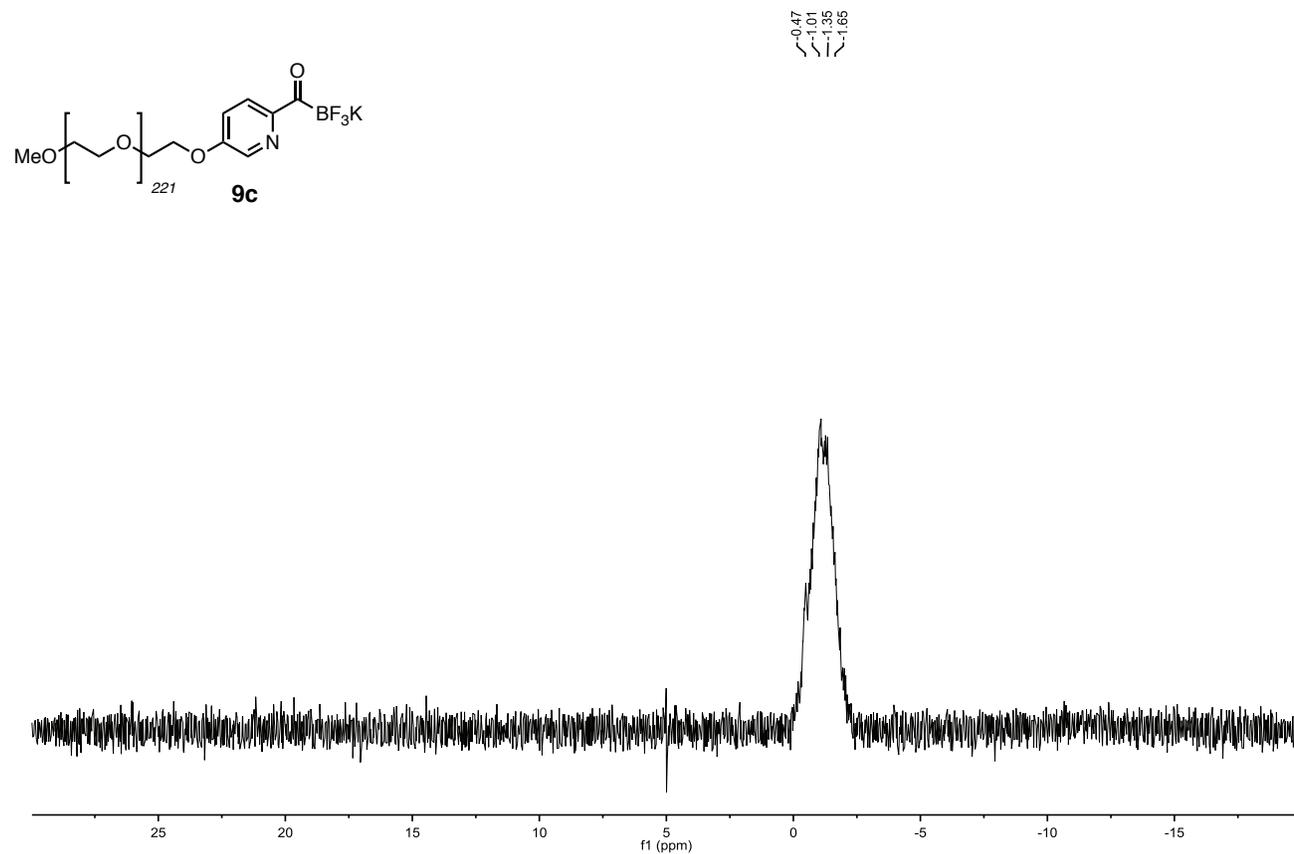
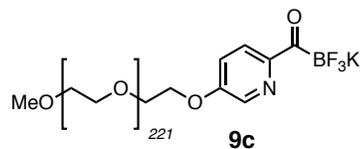
**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 2000 (9a):** **$^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )** **$^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )**

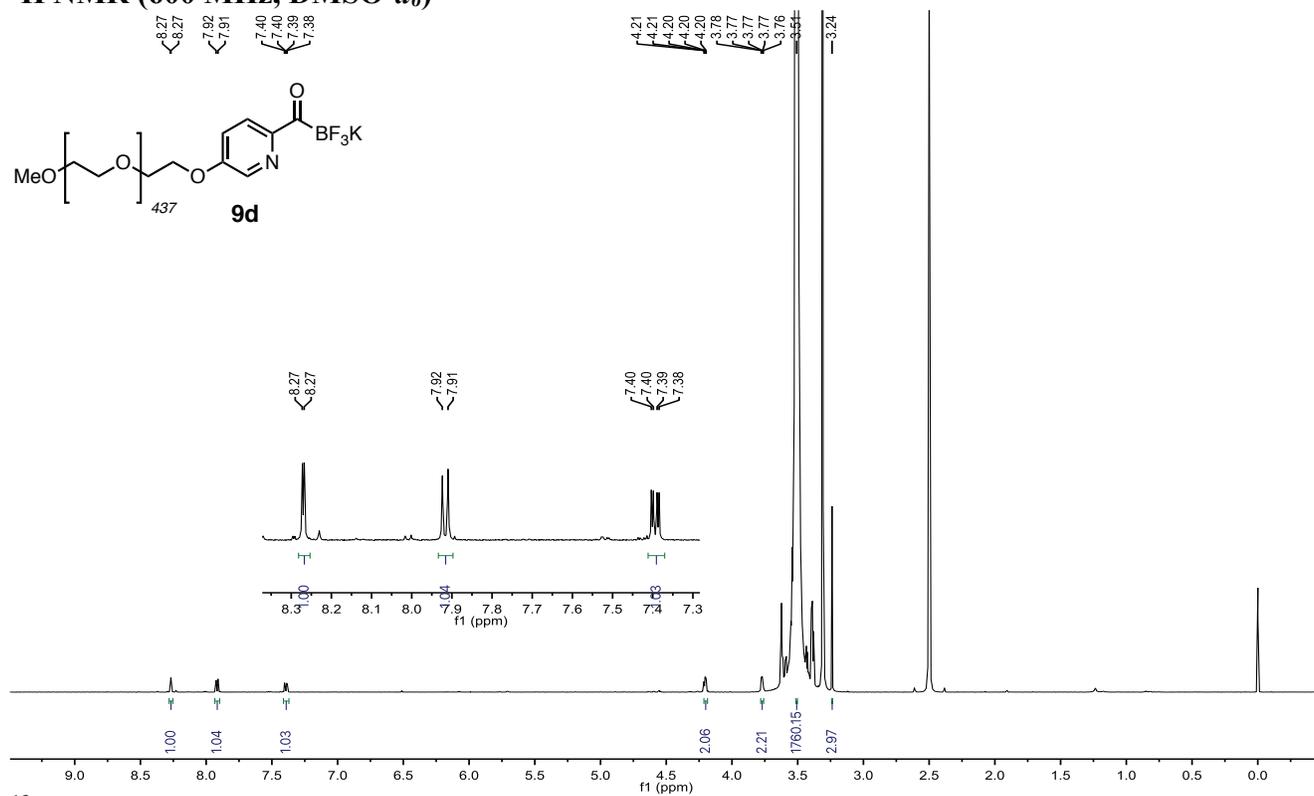
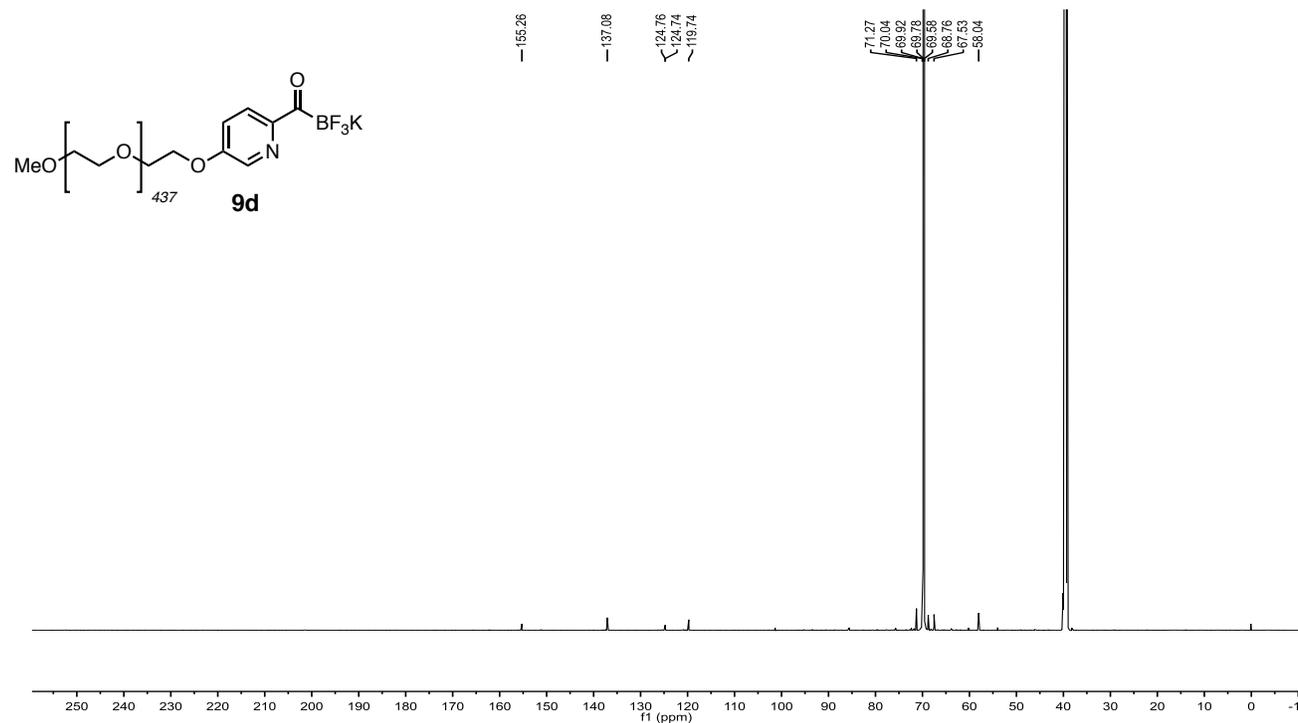
$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

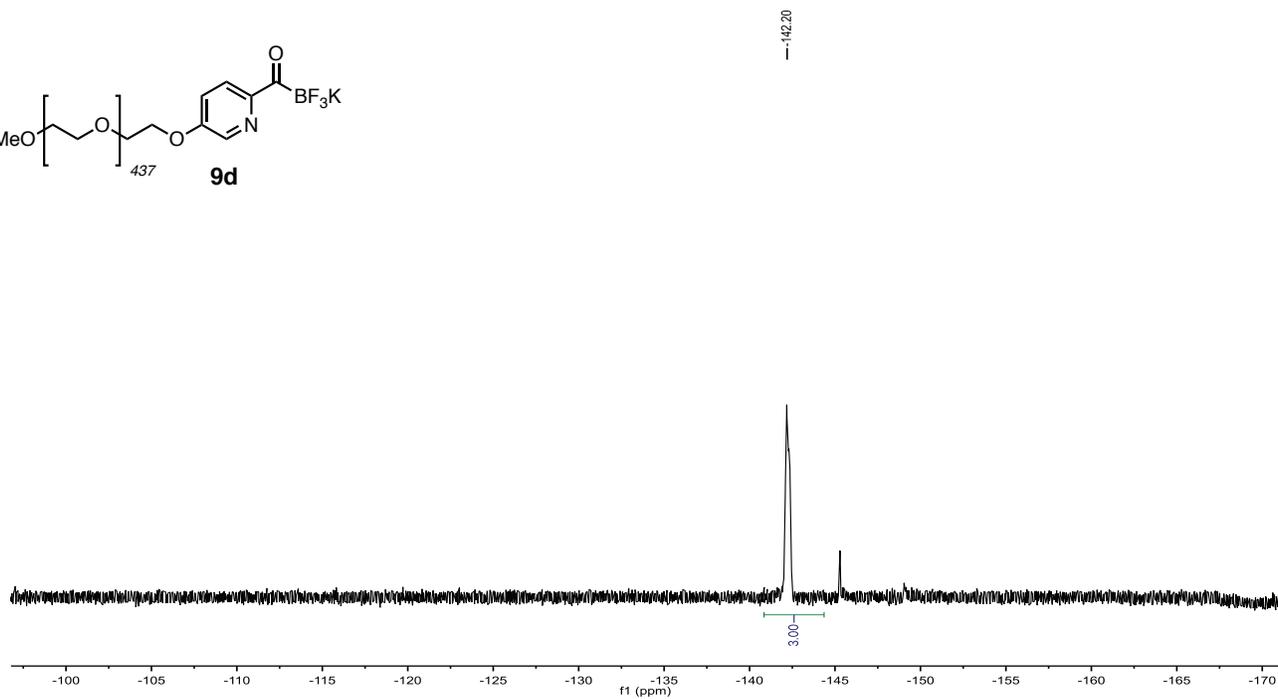
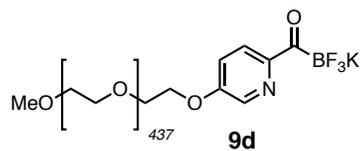
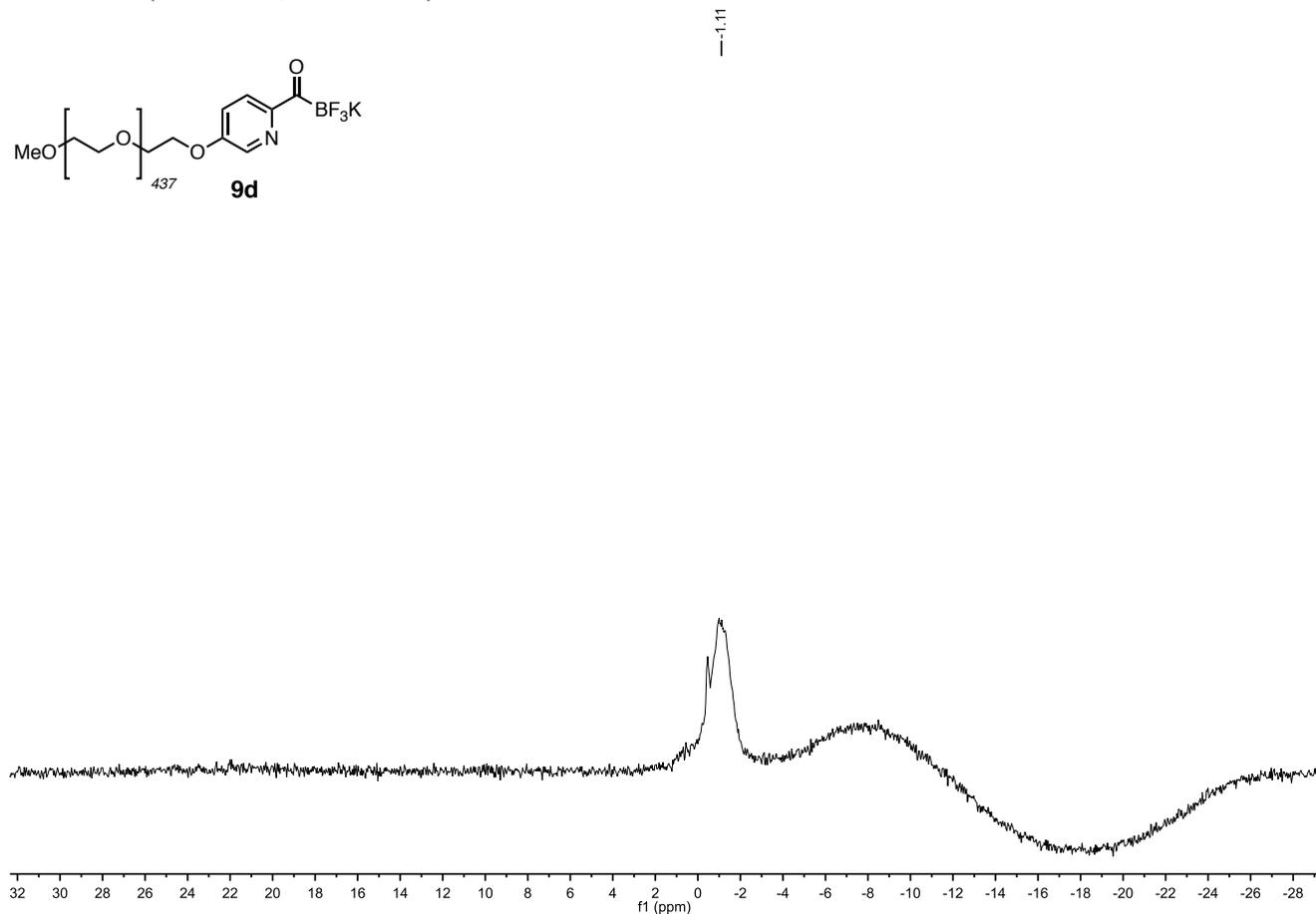
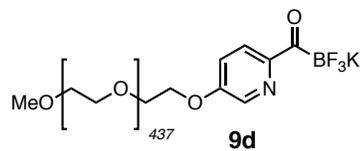
**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 5000 (9b):** **$^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )** **$^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )**

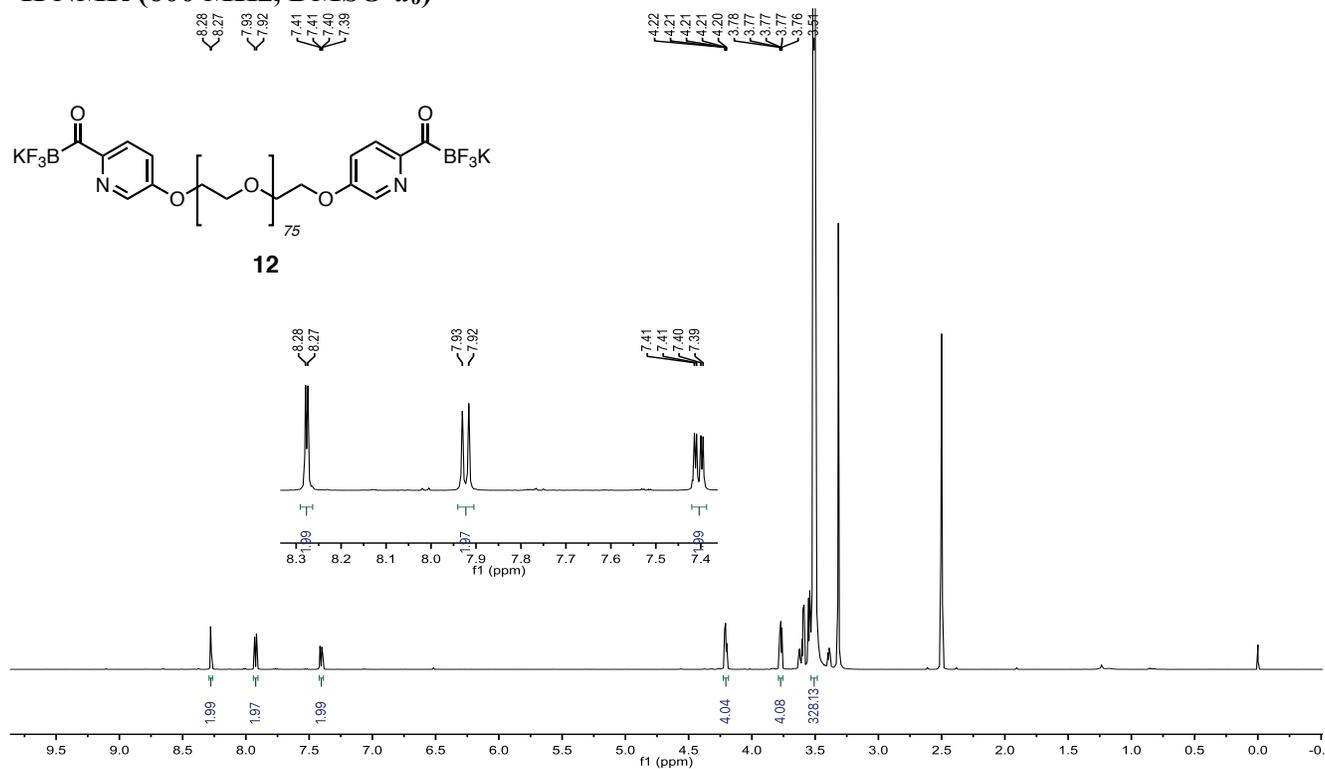
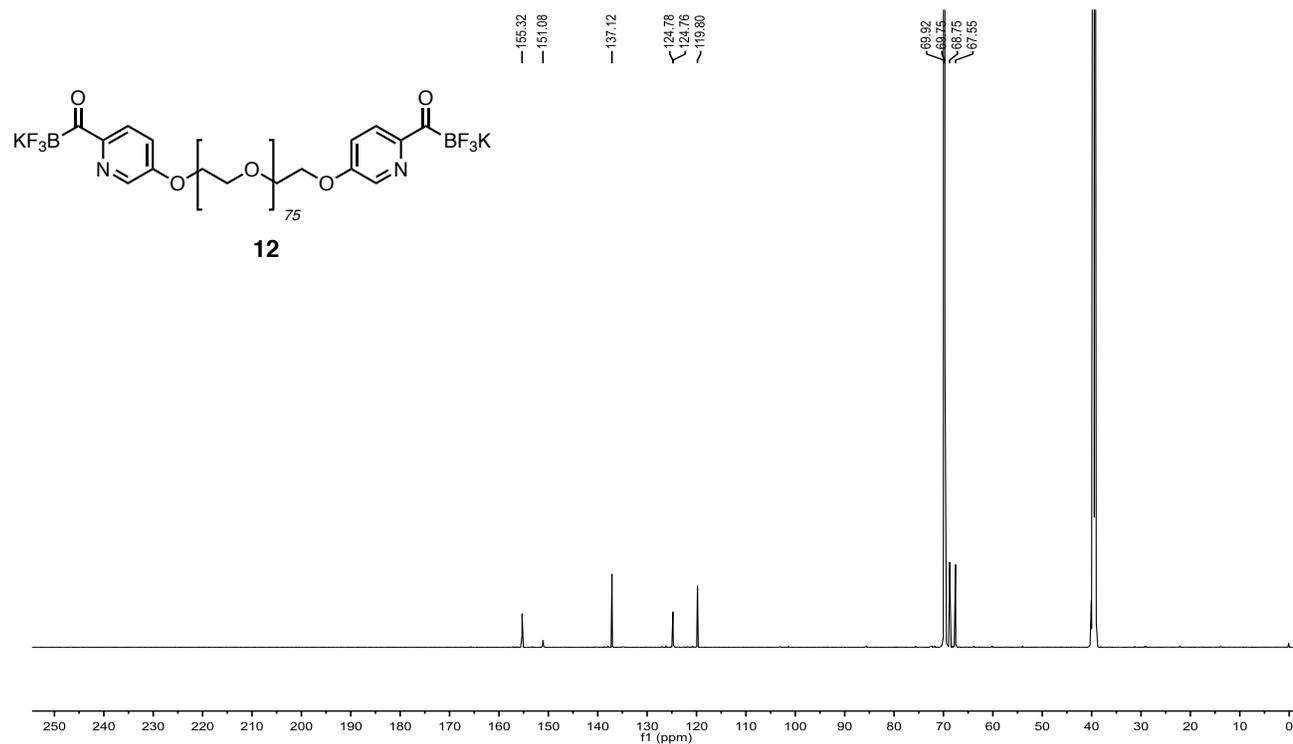
$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

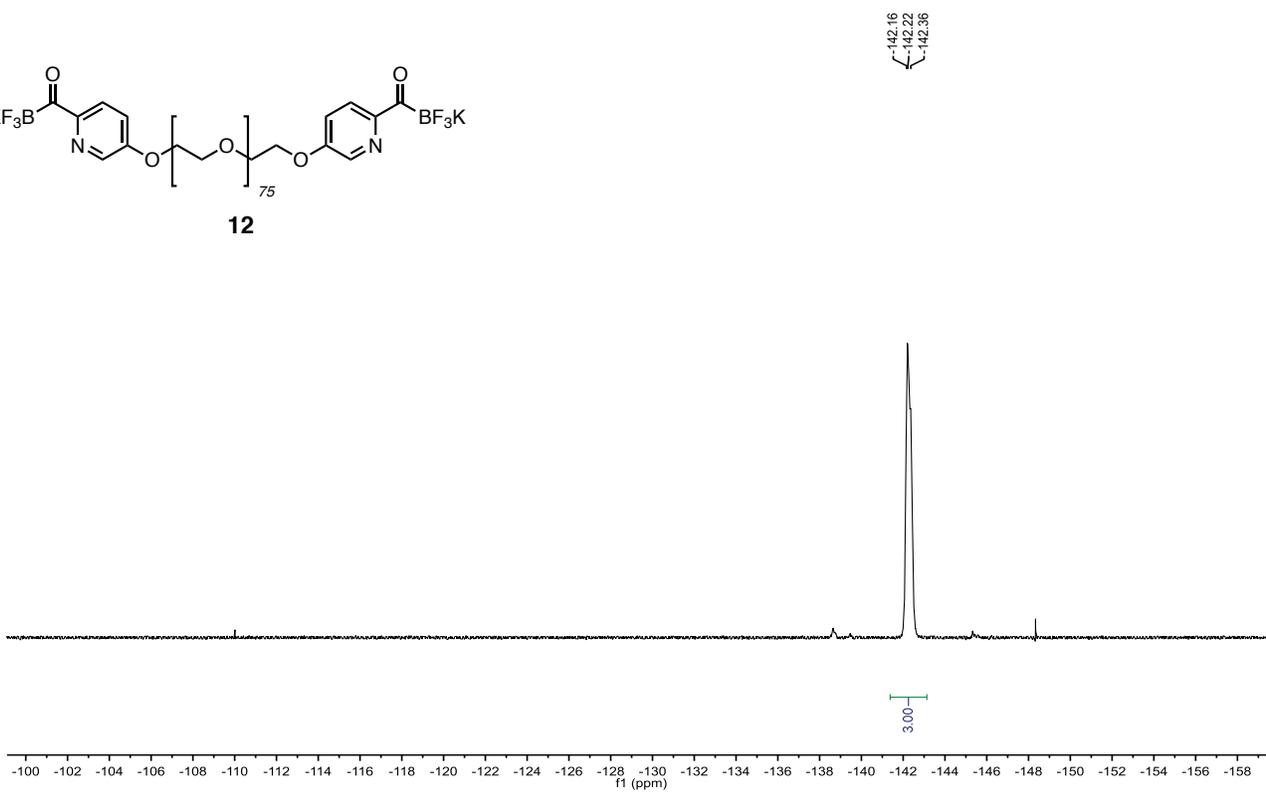
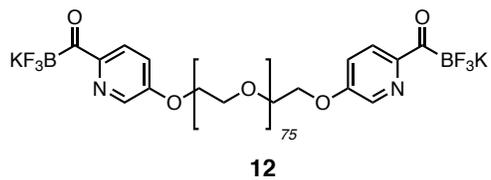
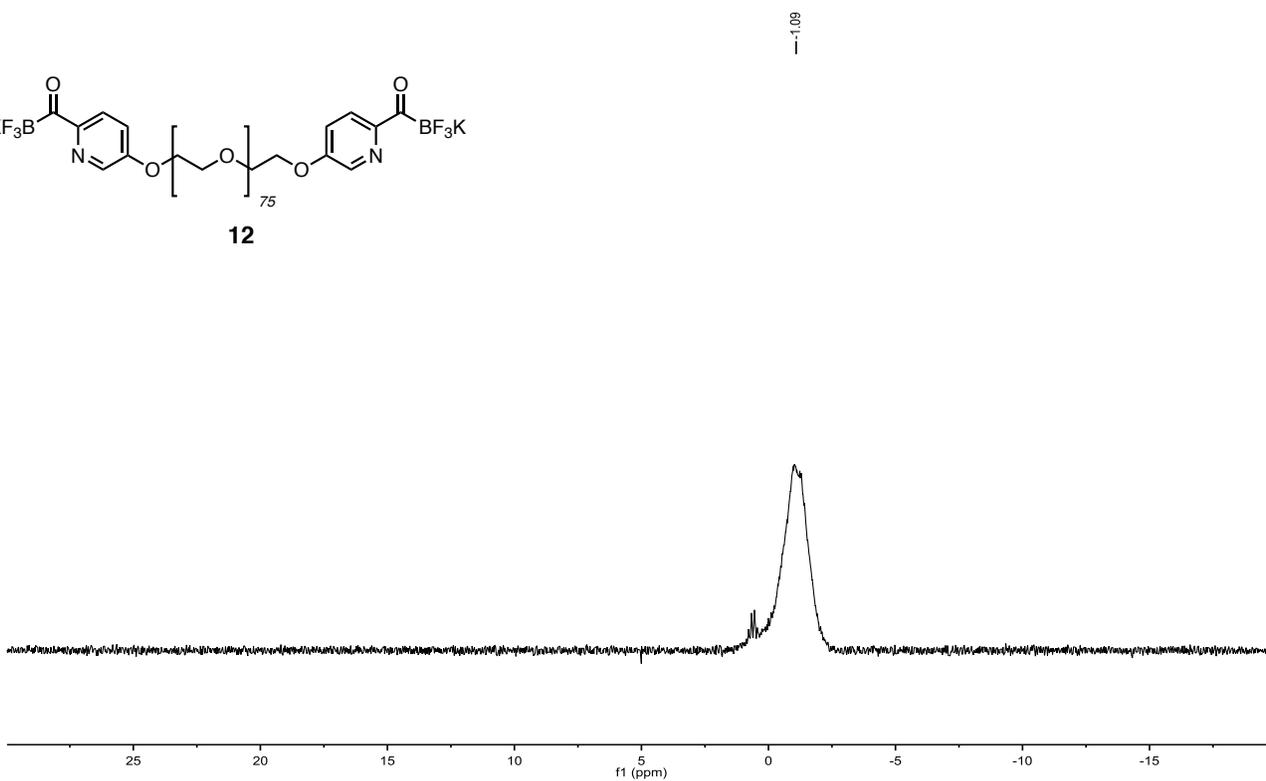
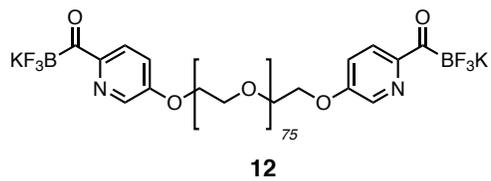
**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 10000 (9c):** **$^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )** **$^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )**

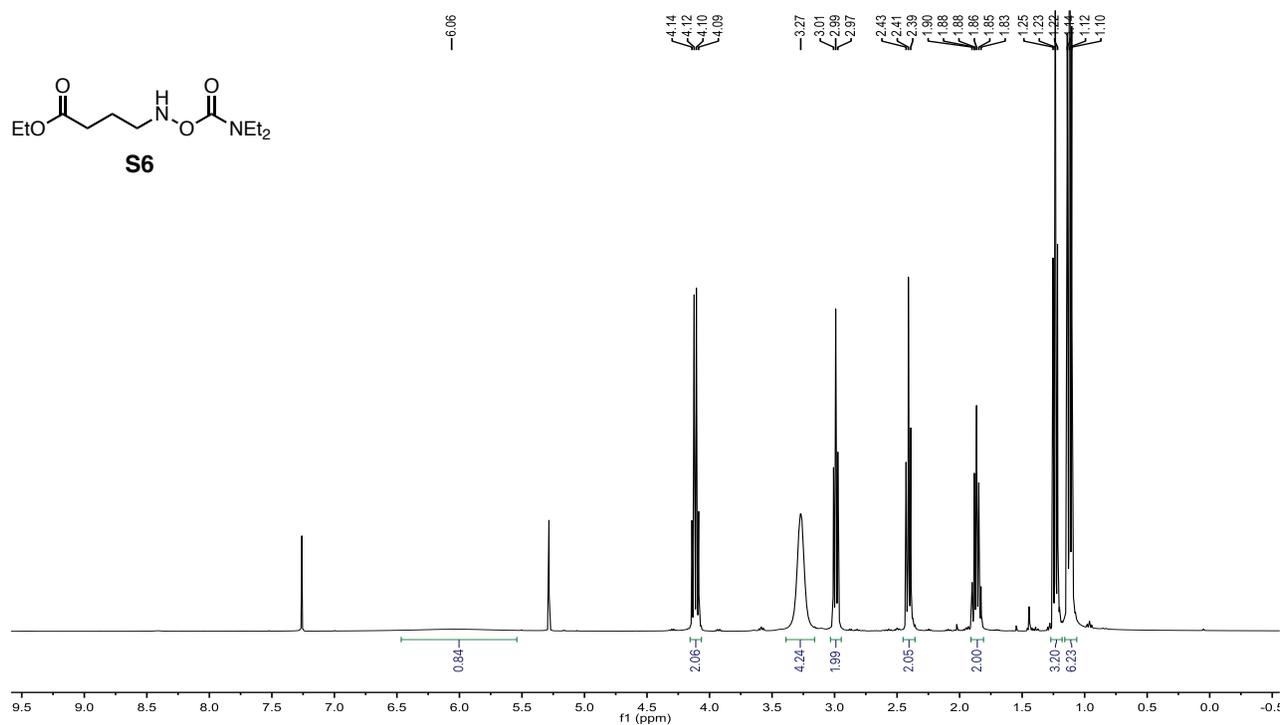
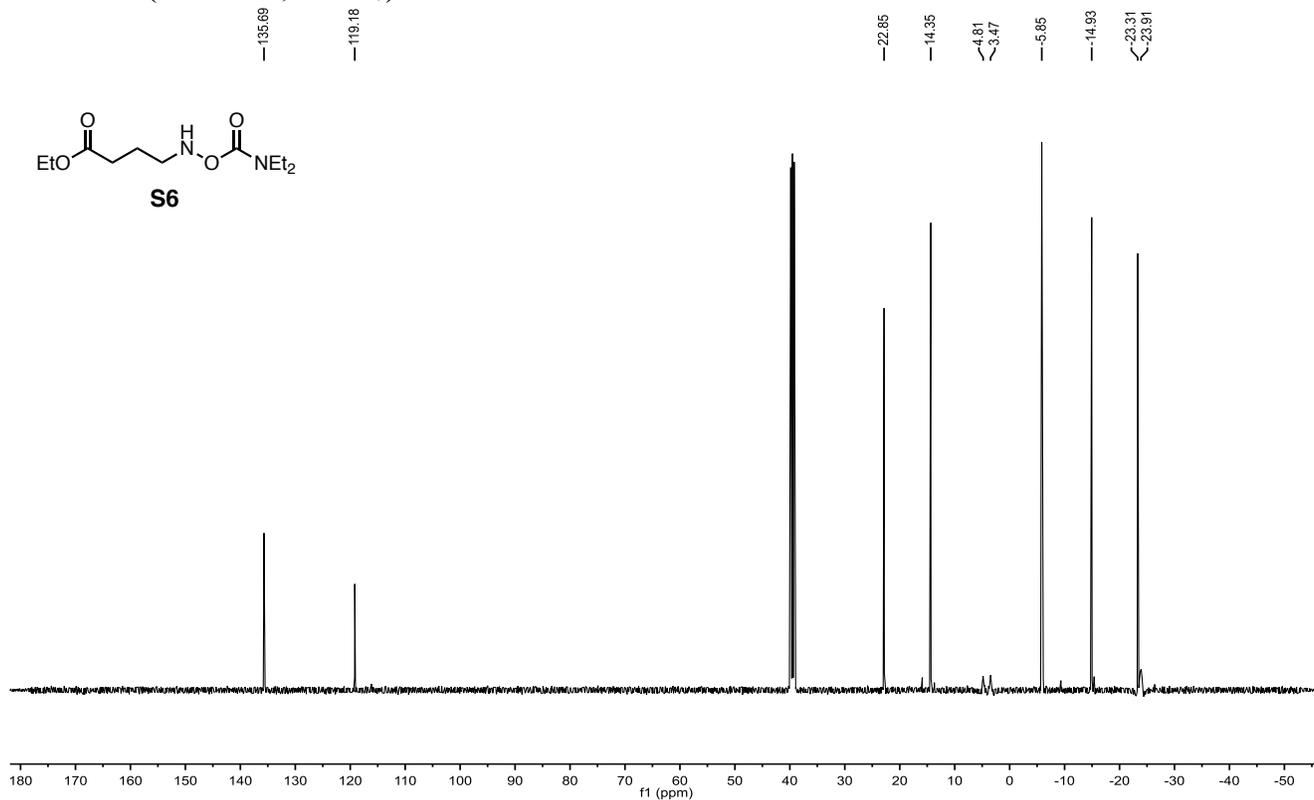
$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

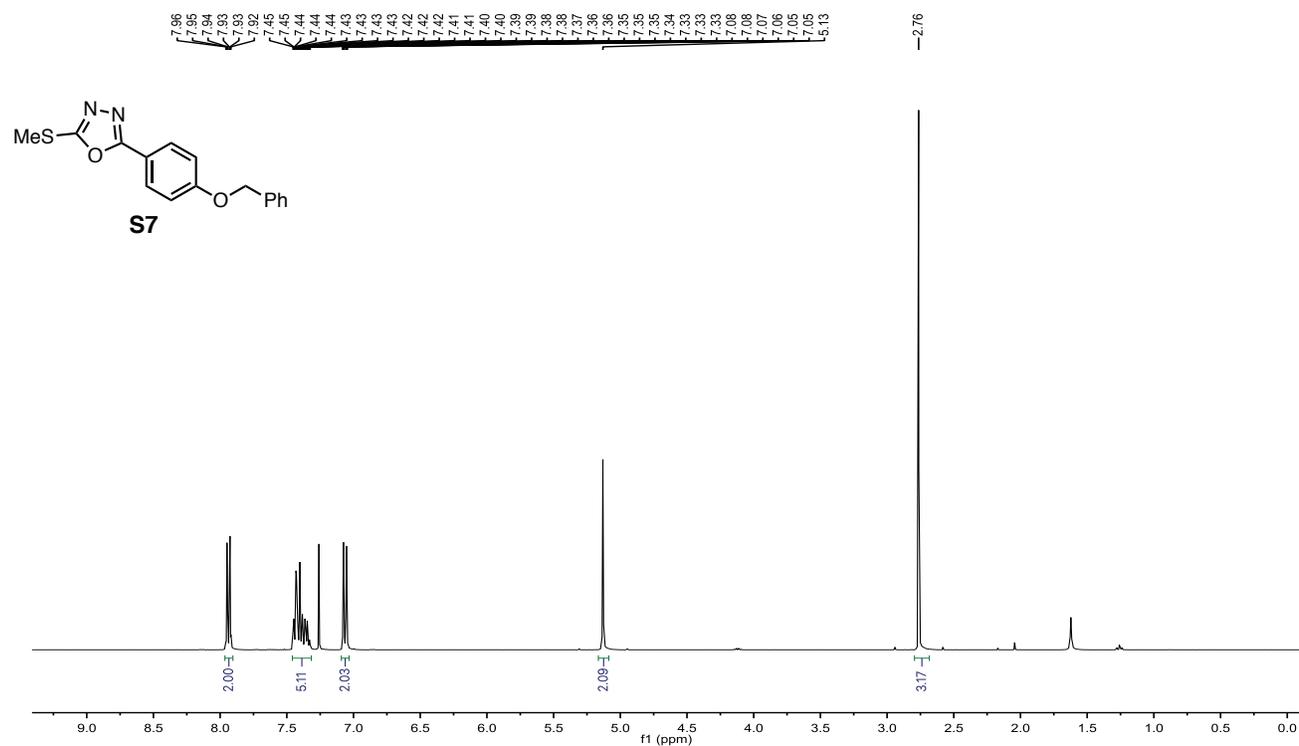
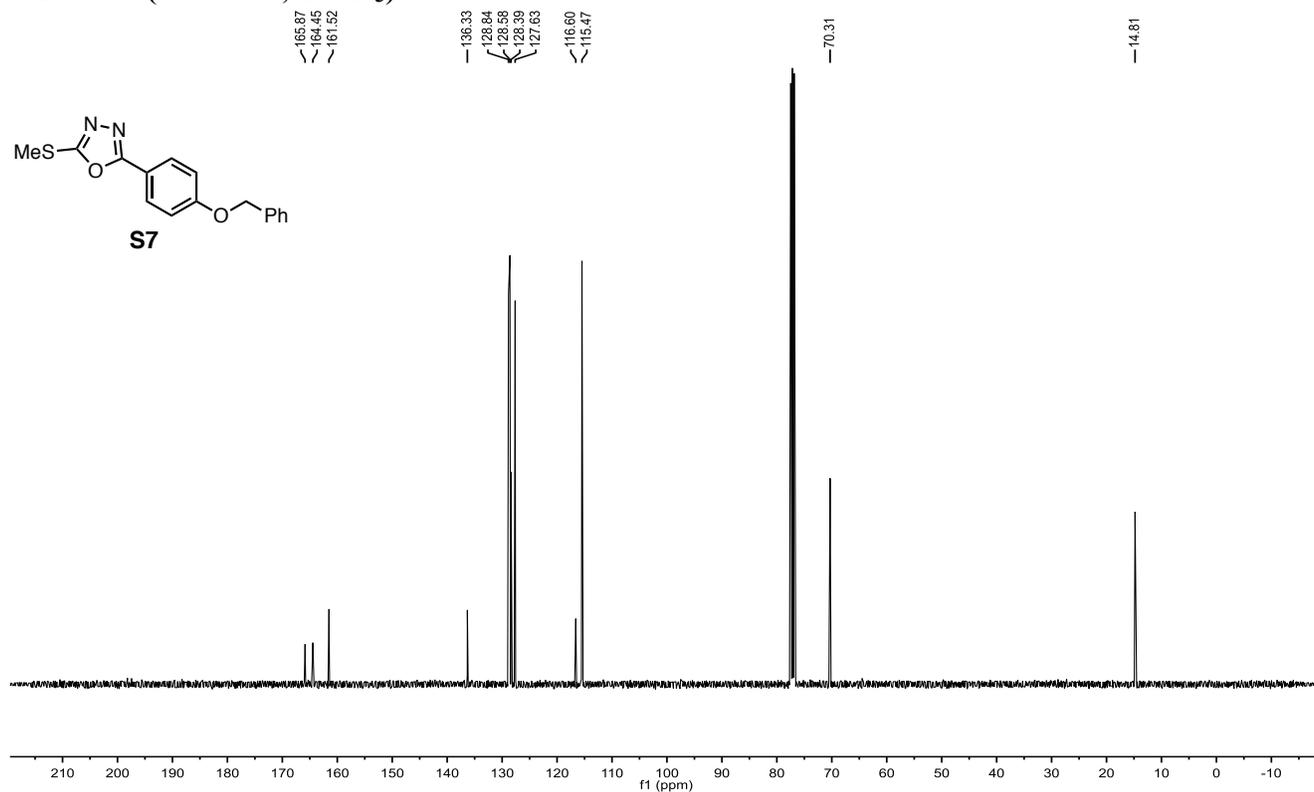
**(5-(2-(2-methoxyethoxy)ethoxy)pyridin-2-yl)(trifluoro- $\lambda^4$ -boranyl)methanone, potassium salt - PEG 20000 (9d):** **$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )** **$^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO-}d_6$ )**

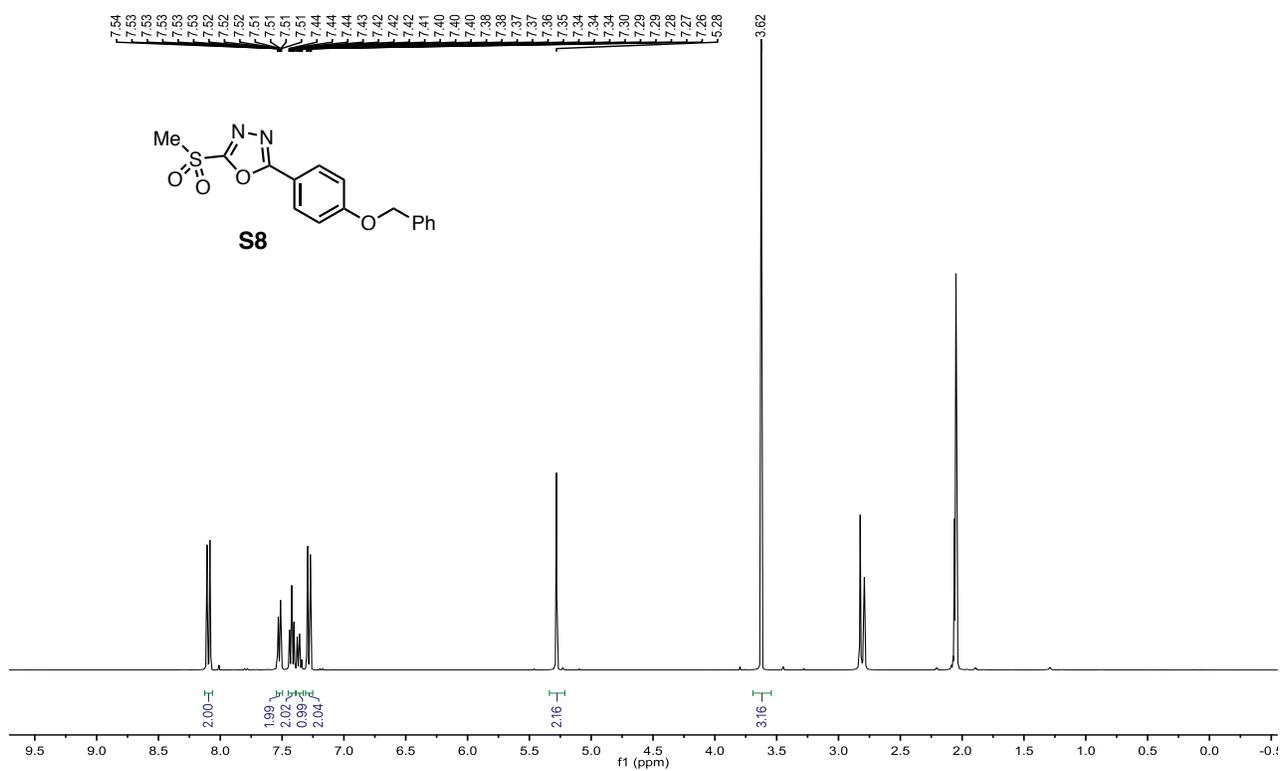
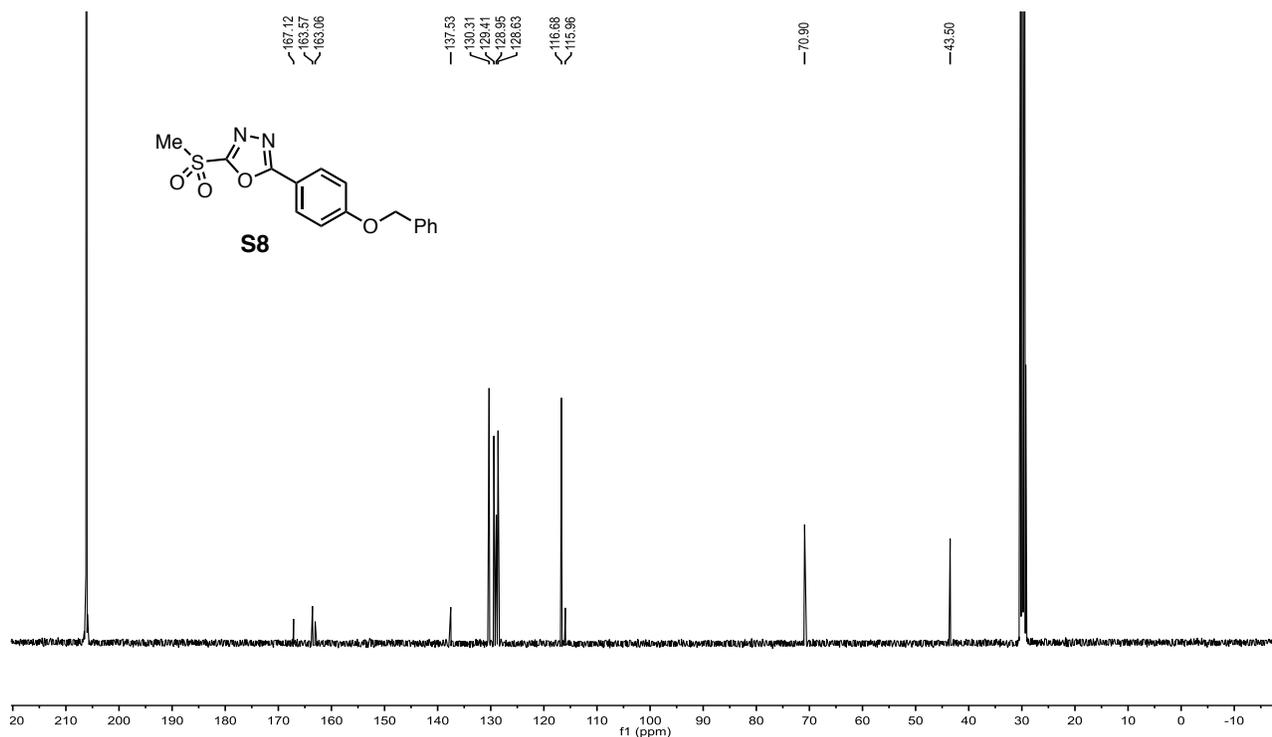
$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

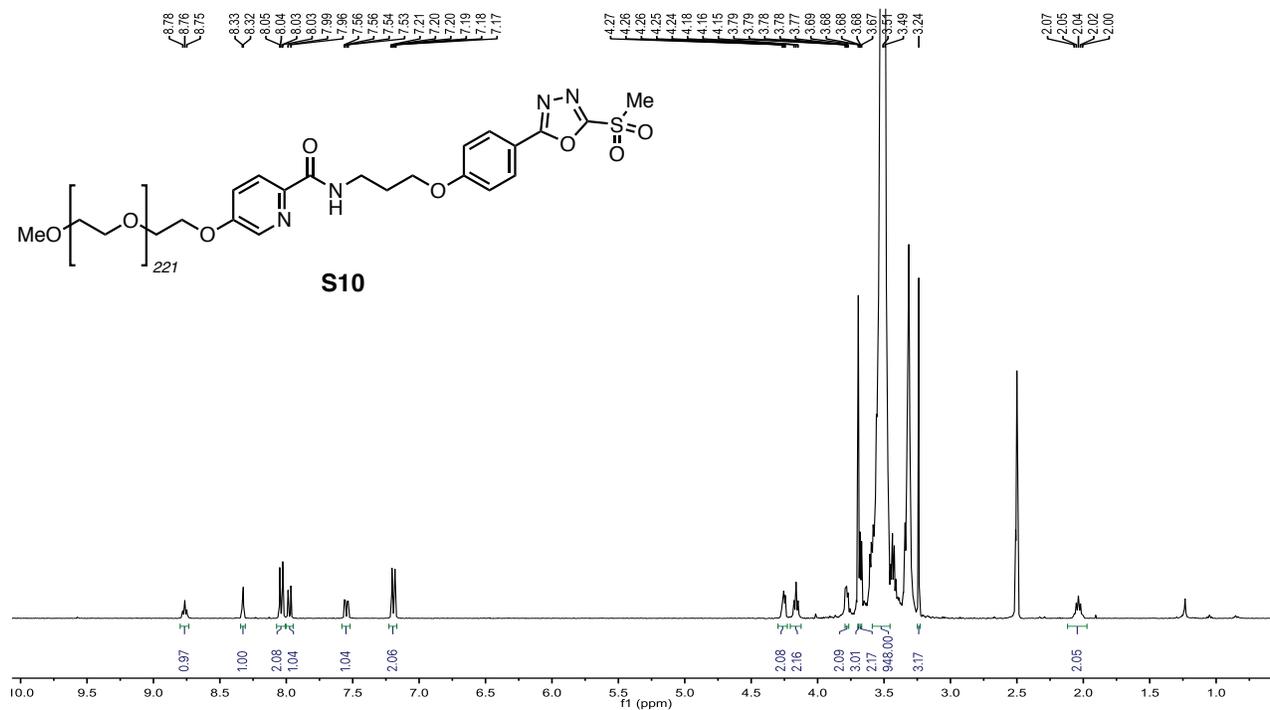
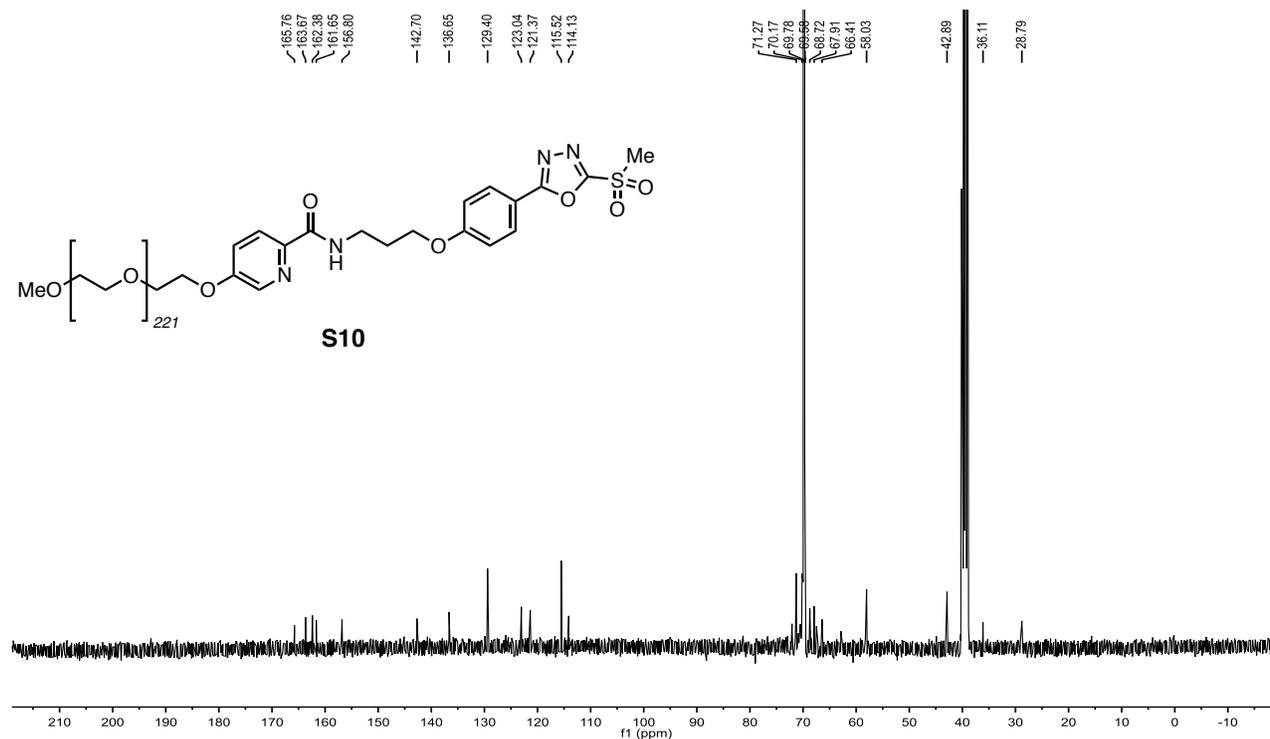
**(((oxybis(ethane-2,1-diyl))bis(oxy))bis(pyridine-5,2-diyl))bis((trifluoro- $\lambda^4$ -boranyl)methanone), dipotassium salt – PEG 3350 (**12**):** **$^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )** **$^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )**

$^{19}\text{F}$  (471 MHz,  $\text{DMSO-}d_6$ ) $^{11}\text{B}$  NMR (160 MHz,  $\text{DMSO-}d_6$ )

**Ethyl 4-(((diethylcarbamoyl)oxy)amino)butanoate (S6):****<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

**2-(4-(benzyloxy)phenyl)-5-(methylthio)-1,3,4-oxadiazole (S7):****<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)****<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)**

**2-(4-(benzyloxy)phenyl)-5-(methylsulfonyl)-1,3,4-oxadiazole (S8)****<sup>1</sup>H NMR (400 MHz, Acetone-*d*<sub>6</sub>)****<sup>13</sup>C NMR (101 MHz, Acetone-*d*<sub>6</sub>)**

**5-(2-(2-methoxyethoxy)ethoxy)-*N*-(3-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenoxy)propyl)picolinamide – PEG 10000 (S10):****<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)****<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>)**

## 10. References

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