# Supporting information

## A pyridinium-based strategy for Lysine-selective protein modification and chemoproteomic profiling in live cells

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# 1. Supplementary Figures and Discussions

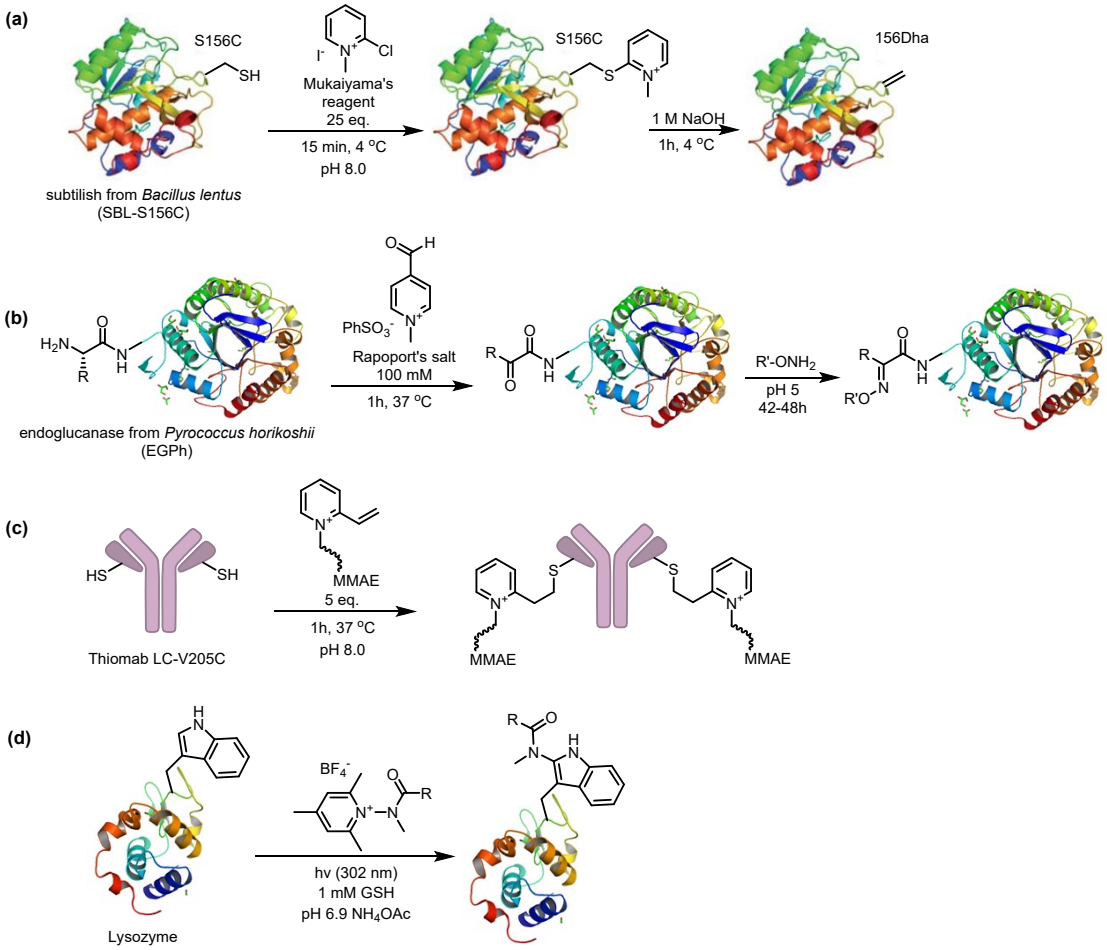
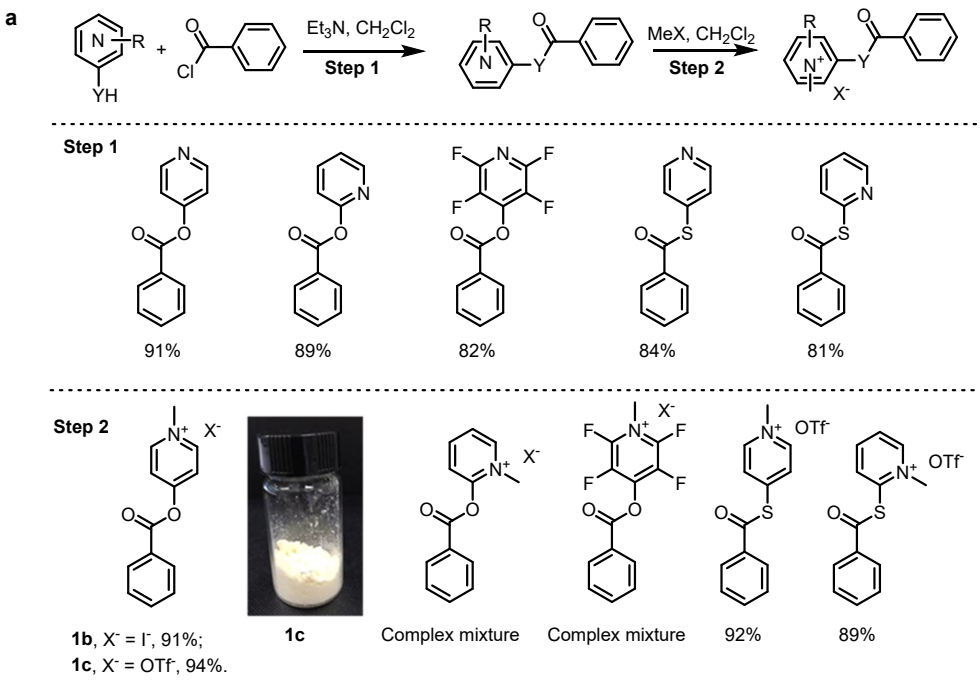
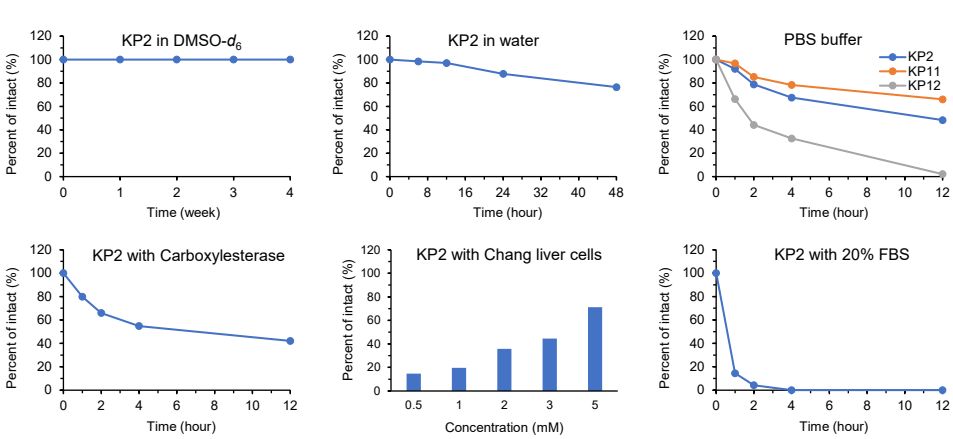
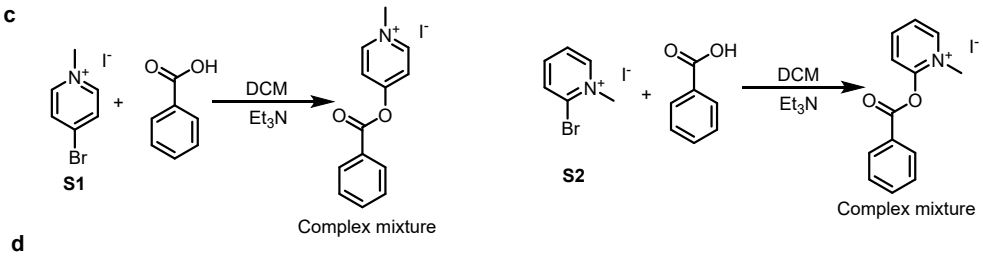



**Figure S1**. Recent examples of application of pyridinium salt to protein chemical modification. (**a**) Elimination of protein cysteine to dehydroalanine (Dha) after reaction with 2-halogenated N-methylpyridinium (Mukaiyama's reagent)[1]; (**b**) N-Methylpyridinium-4-carboxaldehyde mediated transamination of protein N-terminal residue[2]; (**c**) Cysteine-selective protein modification by 2-vinyl N-Methylpyridinium[3]. MMAE: monomethyl auristatin E; (**d**) Photoinduced electron transfer between tryptophan residues and N-carbamoylpyridinium salts in peptides and proteins[4].


a
Et3N, CH2Cl2
Step 1
MeX, CH2Cl2
Step 2
Step 1
91%
89%
82%
84%
81%
Step 2
1b, X- = I-, 91%;
1c, X- = OTf-, 94%.
1c
Complex mixture
Complex mixture
92%
89%


b
KP2 in DMSO-d6
KP2 in water
PBS buffer
KP2
KP11
KP12
Percent of intact (%)
Time (week)
Time (hour)
KP2 with Carboxylesterase
KP2 with Chang liver cells
KP2 with 20% FBS
Concentration (mM)


c
DCM
Et3N
S1
Complex mixture
S2
Complex mixture

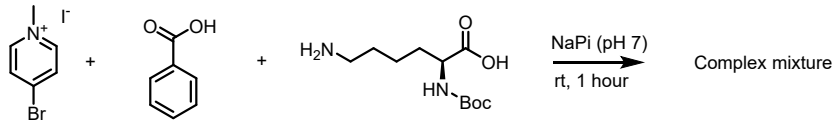

d
NaPi (pH 7)
rt, 1 hour
Complex mixture

**Figure S2**. The preparation of pyridinium activated esters. (**a**) The two steps synthetic route of pyridinium activated esters. (**b**) Solution and digestion stability assay of pyridinium ester. **Top left**: pyridinium **1c** was dissolved in DMSO-*d6* and scanned with NMR spectrometer at room temperature. **Top middle**: **KP2** (1 mM) was dissolved in water and analyzed with HPLC at 37 ˚C with an internal standard (dibenzyl sulfoxide, labelled with the red asterisk). **Top right**: pyridinium ester **KP2** and NHS ester **(KP11 and KP12)** (1 mM) was dissolved in PBS buffer (pH 7.4) and analyzed with HPLC at 37 ˚C. Around 20% hydrolysate were observed for pyridinium ester **KP2** and NHS ester **KP11** in PBS buffer within 2 hours. **Bottom left**: **KP2** (1 mM) was dissolved in PBS buffer (pH 7.4, 0.1 mg/mL Carboxylesterase) and analyzed with HPLC at 37 ˚C. Around 20% hydrolysate were observed for **KP2** with carboxylesterase within 1 hour. **Bottom middle**: **KP2** (5 mM, 3 mM, 2 mM, 1mM and 0.5 mM) was incubated with Chang liver cells (rich in Carboxylesterase 1 (CE1)) in PBS buffer (pH 7.4) for 1 hour at 37 ˚C and analyzed with HPLC. >50% Effective ester was detected in the high centration (5 mM) **KP2** with Chang liver cells within 1 hour. **Bottom right**: **KP2** (1 mM) was dissolved in 20% Foetal Bovine Serum (FBS) and analyzed with HPLC at 37 ˚C. Around 90% hydrolysate were observed for **KP2** with carboxylesterase within 1 hour. (**c**) Using Mukaiyama's reagents for the preparation of pyridinium activated esters. (**d**) The using of an analogue of Mukaiyama's reagent (2-bromide N-methylpyridinium) for a three component amidation led to a complex mixture.

The methylation of 4-pyridinol ester gave basically quantitative yields of methylated-pyridinium salt **1b-1c** with an easy precipitation step and could be scaled up to 10 grams. However, complex mixtures were precipitated for 2-pyridinol and 4-tetrafluoropyridinol ester, indicating the highly unstable nature of these salts. The reaction between 4-pyridinethiol ester/2-pyridinethiol ester and MeOTf gave high yields of pyridinium salts, respectively. Mukaiyama's reagents are 2-halogenated N-methyl-pyridinium, -benzoxazolium, -benzothiazolium and -pyrimidinium salts for the activation of carboxylic acids leading to ester and amide *via* a *in situ* generated 2-acyloxy intermediates under heating condition[5], which are commonly applied for synthesis of ester and amide in organic chemistry[6]. These reactions are usually carried out in an aprotic anhydrous solvent due to its highly reactive and unstable intermediate, which is consistent with our investigation of the methylation of eater (**Figure S2a**). 2- And 4-bromo-N-methylpyridinium iodide were prepared to react with benzoic acid in DCM solvent, respectively. Target products were detected, but the mixtures were too complicated to purified (**Figure S2c**). We also tested 4-bromo-N-methylpyridinium iodide as a *in situ* activating agent for the reaction between benzoic acid and Boc-Lys-OH in water solvent, but complex mixture was obtained (**Figure S2d**). Therefore, there are practical difficulty to use Mukaiyama's reagent as substrate for the synthesis of pyridinium activated esters.

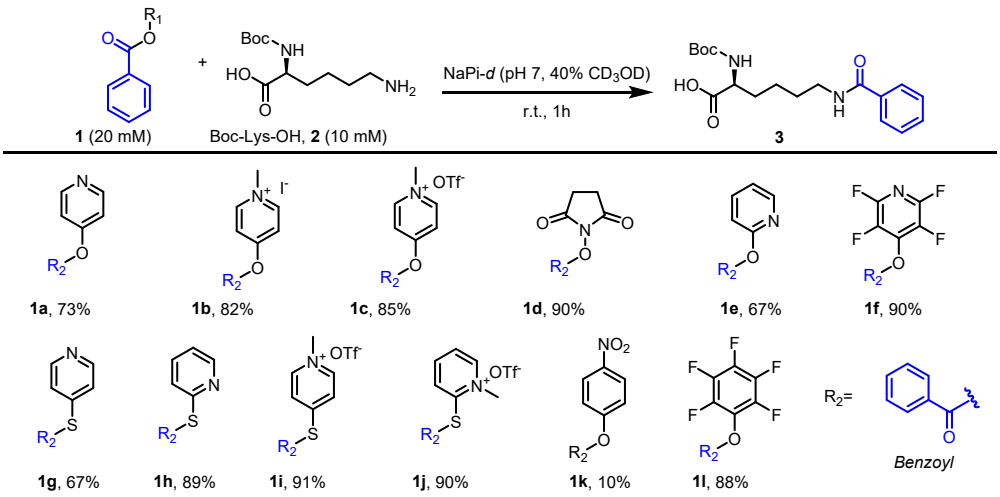



**Figure S3**. Screening of the reaction between activated esters and Boc-Lys-OH. Condition: activated ester **1** (20 mM), Boc-Lys-OH **2** (10 mM) in NaPi (pH 7, in $D_2O$ with 40% $CD_3OD$) solvent at room temperature for 1 hour. The yields were determined by $^1H$ NMR.

The preliminary screening of activating groups demonstrated that the reaction of pyridinium iodized salt **1b** (82%), triflate salt **1c** (85%), tetrafluoropyridinol ester **1f** (90%), pentafluorophenol ester **1l** (88%) as well as the two pyridinium thiol esters **1i** (91%) and **1j** (90%) gave comparable conversions to that of NHS ester **1d** (90%). Generally, the conversions of pyridinol ester (73% for **1a** and 67% for **1e**) and pyridinethiol ester (67% for **1g** and 89% for **1h**) were lower than their onium salt counterparts, and the reaction of nitrophenol ester **1k** was inefficient at this condition (10% yield). Although, the pyridinium thiol esters are efficient activated esters for amidation, but the highly unstable nature of these thioesters is hindered their application for bioconjugation.

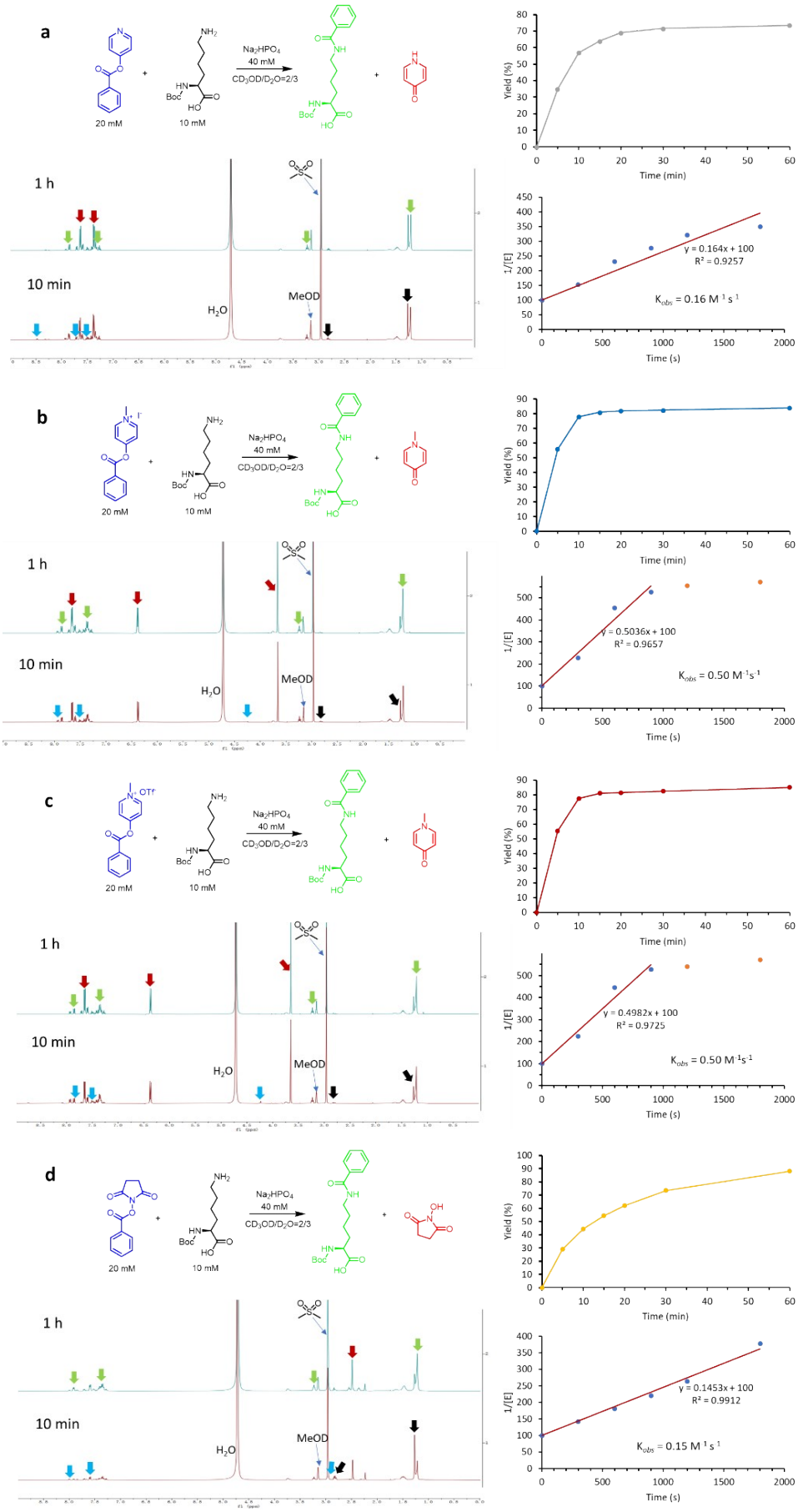

a
20 mM
10 mM
Na2HPO4
40 mM
CD3OD/D2O=2/3
1 h
10 min
H2O
MeOD
Yield (%)
Time (min)
1/[E]
y = 0.164x + 100
R² = 0.9257
Kobs = 0.16 M-1 s-1
Time (s)
b
20 mM
10 mM
Na2HPO4
40 mM
CD3OD/D2O=2/3
1 h
10 min
H2O
MeOD
Yield (%)
Time (min)
1/[E]
y = 0.5036x + 100
R² = 0.9657
Kobs = 0.50 M-1s-1
Time (s)
c
20 mM
10 mM
Na2HPO4
40 mM
CD3OD/D2O=2/3
1 h
10 min
H2O
MeOD
Yield (%)
Time (min)
1/[E]
y = 0.4982x + 100
R² = 0.9725
Kobs = 0.50 M-1s-1
Time (s)
d
20 mM
10 mM
Na2HPO4
40 mM
CD3OD/D2O=2/3
1 h
10 min
H2O
MeOD
Yield (%)
Time (min)
1/[E]
y = 0.1453x + 100
R² = 0.9912
Kobs = 0.15 M-1 s-1
Time (s)

**Figure S4**. $^{1}$H NMR monitored reaction kinetics of ester **1a-1d**. Overlay of $^{1}$H NMR (400 MHz) spectra for reaction time 10 mins and 1 hour at 298 K. Blue, black, green and red arrows point to signals of activated ester **1a-1d**, Boc-Lys-OH, product **3** and leaving group. Conditions: activated ester **1a-1d** (20 mM), Boc-Lys-OH **2** (10 mM) in NaPi (pH 7, in $D_2O$ with 40% $CD_3OD$) at room temperature. Two replicates were conducted for the calculation of observed rate constants ($k_2$, $M^{-1}$ $s^{-1}$). For each sub-figure: Top right charts: Estimation of the yield versus time plot by $^{1}$H NMR. Bottom right charts: Estimation of the observed reaction constant ($k_{obs}$) using the linearly-fitted region of the 1/[Boc-Lys-OH]) versus time plot.

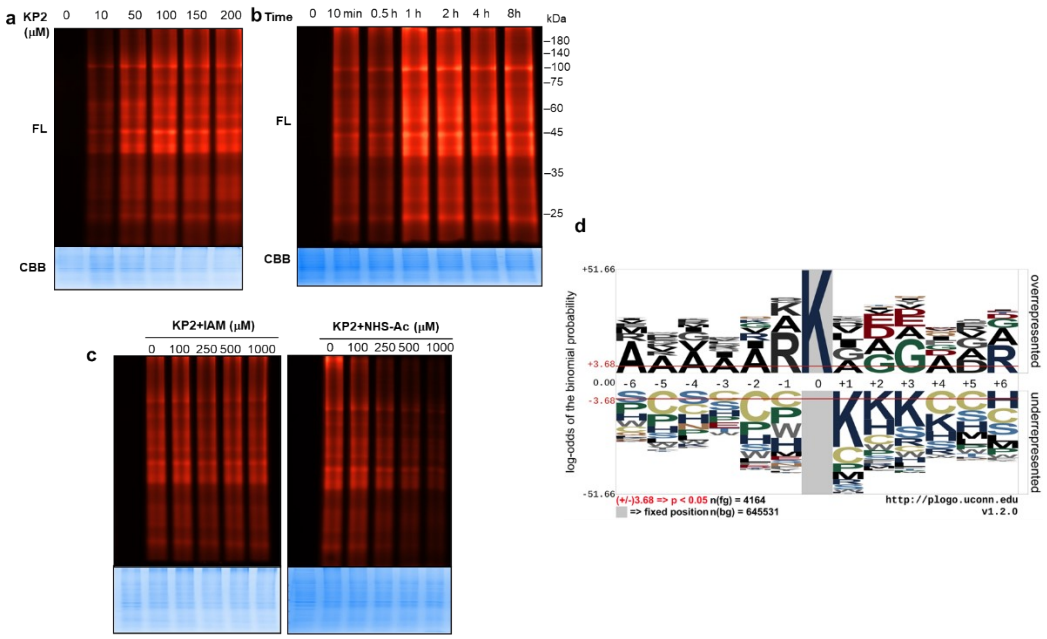



**Figure S5**. (**a**) Dose- and (**b**) Time- dependent labeling of MCF-7 cells with **KP2**. (**c**) **KP2** preferentially labels lysine residues in MCF-7 cell proteomes. FL, in-gel fluorescence scanning. CBB, Coomassie gel. (**d**) A pLogo analysis of the **KP2** labeling pattern in MCF-7 cell lysates.

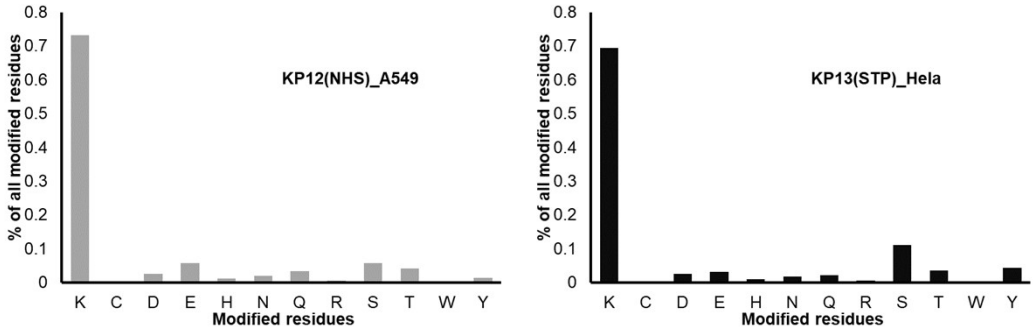



**Figure S6**. The distribution of labeling residues of NHS-Yne (**KP12**) and STP-Yne (**KP13**).

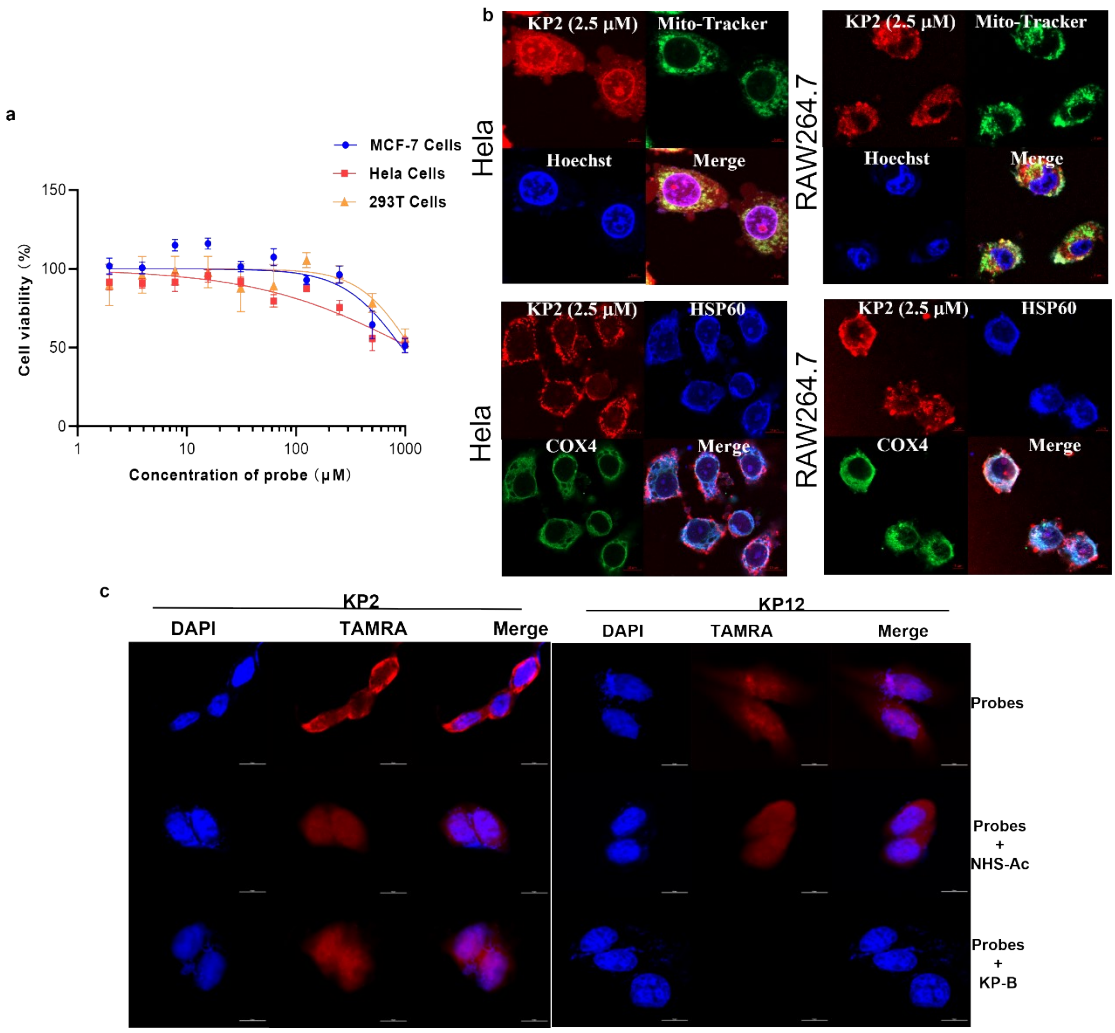


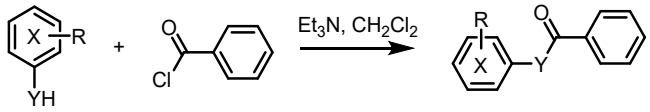

**Figure S7**. (**a**) $IC_{50}$ values of the probe **KP2** against MCF-7/Hela/293T cells. Data represent means ± standard deviation for three experiments. (**b**) Cellular imaging of **KP2** (2.5 μM) with live Hela/RAW264.7 macrophages. **KP2** exhibited the strongest in *situ* labeling in live cells after 1 hour of treatment, colocalized with that of MitoTracker Green FM (100 nM) and DAPI (blue). Scale bar = 10 μm. (**c**) Cellular imaging of **KP2** or **KP12** (2.5 μM) with MCF-7 cells under 1 hour of treatment; Competitive modification profiles of **KP2** with MCF-7 cells in the presence of competitors. Scale bar = 10 μm.

# 2. Supplementary Materials and Methods for Chemistry

## 2.1. General Information

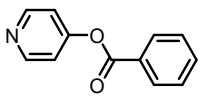
All chemical reagents are commercially available from *Energy Chemical* without purification. The reactions were monitored by TLC (silica gel-G). **Nuclear Magnetic Resonance (NMR)** spectra were recorded on Bruker 400 MHz or 500 MHz spectrometer using trimethylsilane (TMS) as internal standard under ambient temperature (20 ˚C). **High-Resolution Mass Spectrometry (HRMS)** were measured on a Q-Exactive Focus. **Reverse Phase High Performance Liquid Chromatography** (**HPLC**) was performed on SHIMAZU prominence LC-20AT instrument equipped with Kromasil 100-5-C18 column (4.6 × 250 mm, 5 μm). $H_2O$ (containing 0.1% TFA) and pure $CH_3CN$ were used as solvents in linear gradient mixtures. **Mass Spectrometry** (**MS**) to screen the molecular weight of HPLC fractions were carried out on SHIMAZU LC-MS 8030 in positive ion mode.

## 2.2. General procedure A for the synthesis of esters




To a round-bottomed flask was charged with hydroxyl or sulfhydryl substrate (1 equiv.) and $Et_3N$ (1 equiv.). The flask was sealed and refiled with nitrogen for 3 times. $CH_2Cl_2$ was added by a syringe, and the flask was cooled in an ice bath. Benzoyl chloride (1 equiv.) was added dropwise. After the addition of benzoyl chloride, the reaction solution was allowed to stir for 2 h at room temperature. The reaction completion was monitored by TLC. The organic phase was washed with saturated brine for two times and dried over anhydrous $Na_2SO_4$, and concentrated on a rotary evaporator. The crud product was purified by flash column chromatography using eluent solution Hexane/EtOAc= 10/1 in volume.

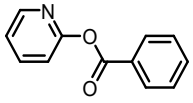
The synthesis of **1a** was followed **General Procedure A** using pyridin-4-ol as substrate.




white powder, 91% yield. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.66 (dd, *J* = 4.8, 1.3 Hz, 2H), 8.21 – 8.13 (m, 2H), 7.69 – 7.63 (m, 1H), 7.51 (t, *J* = 7.9 Hz, 2H), 7.24 (dd, *J* = 4.7, 1.6 Hz, 2H).

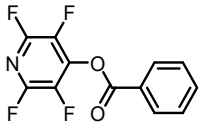
$^{13}$C NMR (126 MHz, $CDCl_3$) δ 163.90, 157.87, 151.48, 134.20, 130.34, 128.80, 128.72, 117.15.

The synthesis of **1e** was followed **General Procedure A** using pyridin-2-ol as substrate.

 colourless oil, 89%. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.53 – 8.46 (m, 1H), 8.29 – 8.22 (m, 2H), 7.90 – 7.81 (m, 1H), 7.66 (tt, *J* = 7.1, 1.3 Hz, 1H), 7.56 – 7.50 (m, 2H), 7.32 – 7.21 (m, 2H).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ 164.88, 158.25, 148.68, 139.74, 133.98, 130.44, 129.11, 128.68, 122.25, 116.78.

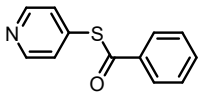
The synthesis of **1f** was followed **General Procedure A** using 2,3,5,6-tetrafluoropyridin-4-ol as substrate.

 colourless oil, 82%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.15 (dt, *J* = 8.5, 1.6 Hz, 2H), 7.70 – 7.63 (m, 1H), 7.55 – 7.48 (m, 2H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 162.43, 134.63, 130.61, 128.96, 128.88.

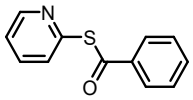
$^{19}$F NMR (376 MHz, $CDCl_3$) δ -86.93 – -87.13 (m), -142.76 (d, *J* = 23.5 Hz), -149.57 (d, *J* = 23.5 Hz).

The synthesis of **1g** was followed **General Procedure A** using pyridine-4-thiol as substrate.

 yellow powder, 84%. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.70 (d, *J* = 4.5 Hz, 2H), 8.03 (d, *J* = 7.8 Hz, 2H), 7.66 (t, *J* = 7.2 Hz, 1H), 7.60 – 7.42 (m, 4H).

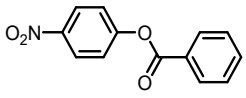
$^{13}$C NMR (101 MHz, $CDCl_3$) δ 187.68, 150.11, 138.64, 136.14, 134.32, 129.03, 128.63, 127.67.

The synthesis of **1h** was followed **General Procedure A** using pyridine-2-thiol as substrate.

 yellow powder, 81%. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.74 – 8.66 (m, 1H), 8.07 – 8.00 (m, 2H), 7.79 (td, *J* = 7.6, 1.8 Hz, 1H), 7.74 (d, *J* = 7.8 Hz, 1H), 7.65 – 7.60 (m, 1H), 7.50 (t, *J* = 7.7 Hz, 2H), 7.34 (ddd, *J* = 7.2, 4.9, 1.2 Hz, 1H).

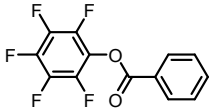
$^{13}$C NMR (101 MHz, $CDCl_3$) δ 189.35, 151.27, 150.52, 137.38, 136.55, 134.04, 131.01, 128.92, 127.63, 123.79.

The synthesis of **1k** was followed **General Procedure A** using 4-nitrophenol as substrate.

 white powder, 92% yield. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.36 – 8.27 (m, 2H), 8.20 (dt, *J* = 8.5, 1.4 Hz, 2H), 7.72 – 7.64 (m, 1H), 7.59 – 7.50 (m, 2H), 7.46 – 7.38 (m, 2H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 164.29, 155.79, 145.46, 134.31, 130.38, 128.85, 128.59, 125.33, 122.70.

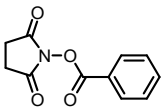
The synthesis of **1l** was followed **General Procedure A** using 2,3,4,5,6-pentafluorophenol as substrate.

 colourless oil, 87% yield. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.11 – 8.06 (m, 2H), 7.62 – 7.55 (m, 1H), 7.47 – 7.41 (m, 2H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 162.11, 134.36, 130.22, 128.67, 128.47.

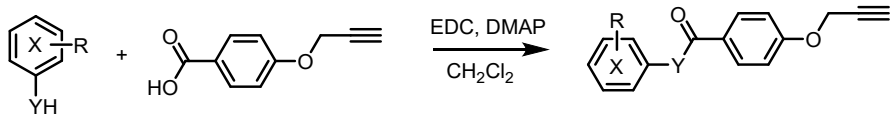
$^{19}$F NMR (376 MHz, $CDCl_3$) δ -152.31 (d, *J* = 19.1 Hz), -157.08 – -158.54 (m), -162.14 (t, *J* = 20.5 Hz).

The synthesis of **1d** was followed **General Procedure A** using N-hydroxysuccinimide (NHS) as substrate.

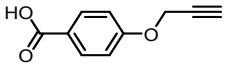
 white powder, 90% yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 7.7 Hz, 2H), 7.70 (t, *J* = 7.5 Hz, 1H), 7.53 (t, *J* = 7.7 Hz, 2H), 2.92 (s, 4H).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ 169.48, 161.99, 135.05, 130.62, 128.97, 125.16, 25.77.

## 2.3. General procedure B for the synthesis of esters




The preparation of 4-(prop-2-yn-1-yloxy)benzoic acid was followed the same procedure as described by previous literature.

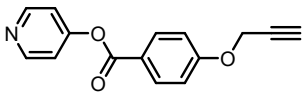



**S4**, white powder, 81%. $^1$H NMR (400 MHz, MeOD) δ 8.06 – 7.98 (m, 2H), 7.12 – 7.05 (m, 2H), 4.85 (d, $J$ = 2.3 Hz, 2H), 3.03 (t, $J$ = 2.4 Hz, 1H).

$^{13}$C NMR (101 MHz, MeOD) δ 168.26, 161.58, 131.36, 123.43, 114.26, 77.83, 75.91, 55.36.

4-Dimethylaminopyridine (DMAP, 5% mol) was added to 4-(prop-2-yn-1-yloxy)benzoic acid (1 equiv.) in $CH_2Cl_2$ solvent, followed by 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 2 equiv.). After stirring for 10 min, hydroxyl or sulfhydryl substrate (1 equiv.) was added and stirred at room temperature for 16 h under nitrogen atmosphere. The reaction completion was monitored by TLC. The solution was washed successively with brine for three times, then dried over $Na_2SO_4$. The solvent was then removed in vacuum to afford the crude product, which was purified by flash column chromatography using eluent solution Hexane/EtOAc= 10/1 in volume.

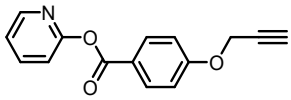
The synthesis of **KP3** was followed **General Procedure B** using pyridin-4-ol as substrate.




white powder, 78%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.66 (dd, $J$ = 4.7, 1.6 Hz, 2H), 8.21 – 8.10 (m, 2H), 7.23 (dd, $J$ = 4.7, 1.6 Hz, 2H), 7.13 – 7.01 (m, 2H), 4.79 (d, $J$ = 2.4 Hz, 2H), 2.57 (t, $J$ = 2.4 Hz, 1H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 163.45, 162.17, 157.96, 151.43, 132.50, 121.80, 117.17, 114.97, 77.58, 77.32, 77.06, 76.81, 76.40, 55.99.

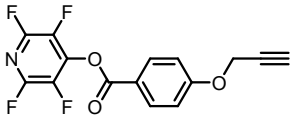
The synthesis of **KP4** was followed **General Procedure B** using pyridin-2-ol as substrate.



white powder, 74%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.41 (s, 1H), 8.15 (d, *J* = 8.8 Hz, 2H), 7.77 (td, *J* = 8.0, 1.6 Hz, 1H), 7.24 – 7.18 (m, 1H), 7.16 (d, *J* = 7.9 Hz, 1H), 7.02 (d, *J* = 8.8 Hz, 2H), 4.73 (d, *J* = 2.2 Hz, 2H), 2.55 (t, *J* = 2.3 Hz, 1H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 164.39, 161.96, 158.29, 148.61, 139.55, 132.51, 122.21, 122.09, 116.77, 114.78, 77.73, 76.39, 55.94.

The synthesis of **KP5** was followed **General Procedure B** using 2,3,5,6-tetrafluoropyridin-4-ol as substrate.

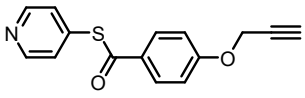


white powder, 70%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.18 – 8.13 (m, 2H), 7.10 (d, *J* = 9.0 Hz, 2H), 4.80 (d, *J* = 2.4 Hz, 2H), 2.59 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 162.97, 160.86, 144.71, 144.61, 144.50, 142.76, 142.67, 142.55, 139.97, 139.87, 137.79, 137.72, 137.56, 137.49, 135.70, 135.64, 135.47, 135.40, 133.17, 119.19, 115.29, 77.33, 76.57, 56.04.

$^{19}$F NMR (376 MHz, Chloroform-*d*) δ -88.44 – -88.77 (m), -151.81 – -152.10 (m).

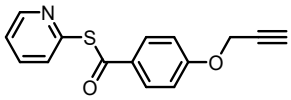
The synthesis of **KP8** was followed **General Procedure B** using pyridine-4-thiol as substrate.



yellow powder, 65%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.66 (d, *J* = 5.8 Hz, 2H), 8.00 (d, *J* = 8.8 Hz, 2H), 7.46 (dd, *J* = 4.7, 1.3 Hz, 2H), 7.06 (d, *J* = 9.0 Hz, 2H), 4.78 (d, *J* = 2.4 Hz, 2H), 2.57 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ 186.12, 162.26, 150.09, 138.86, 129.91, 129.62, 128.66, 115.08, 77.52, 76.51, 56.04.

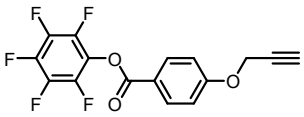
The synthesis of **KP9** was followed **General Procedure B** using pyridine-2-thiol as substrate.



yellow powder, 54%. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.74 – 8.68 (m, 1H), 8.06 (d, *J* = 8.9 Hz, 2H), 7.87 – 7.73 (m, 2H), 7.37 (ddd, *J* = 7.3, 4.9, 1.1 Hz, 1H), 7.09 (d, *J* = 8.9 Hz, 2H), 4.81 (d, *J* = 2.3 Hz, 2H), 2.61 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ 187.87, 162.04, 151.58, 150.56, 137.20, 131.01, 130.10, 129.86, 123.63, 114.95, 77.63, 76.43, 56.01.

The synthesis of **KP10** was followed **General Procedure B** using 2,3,4,5,6-pentafluorophenol as substrate.

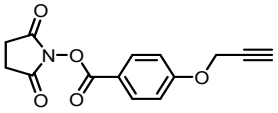


white powder, 85%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.20 – 8.13 (m, 2H), 7.13 – 7.06 (m, 2H), 4.80 (d, *J* = 2.5 Hz, 2H), 2.57 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 162.61, 162.16, 132.97, 119.99, 115.14, 77.45, 76.48, 56.01.

$^{19}$F NMR (376 MHz, $CDCl_3$) δ -152.53 (d, *J* = 19.1 Hz), -158.19 (t, *J* = 22.0 Hz), -162.45 (t, *J* = 19.8 Hz).

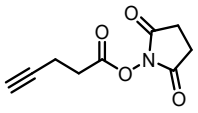
The synthesis of **KP11** was followed **General Procedure B** using NHS as substrate.



white powder, 83%. $^1$H NMR (500 MHz, $CDCl_3$) δ 8.13 – 8.07 (m, 2H), 7.08 – 7.02 (m, 2H), 4.77 (d, *J* = 2.4 Hz, 2H), 2.89 (s, 4H), 2.56 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 169.42, 162.72, 161.41, 132.86, 118.05, 115.15, 77.39, 76.53, 56.01, 25.72.

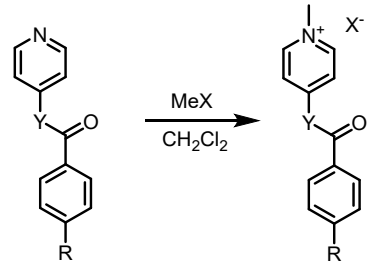
The synthesis of **KP12** was followed **General Procedure B** using NHS and 4-pentynoic acid as substrate.



white solid, 83%. $^{1}$H NMR (300 MHz, $CDCl_3$) δ 2.92 – 2.79 (m, 6H), 2.65 – 2.53 (m, 2H), 2.04 (t, $J$ = 2.7 Hz, 1H).

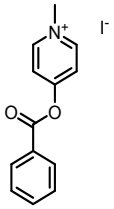
$^{13}$C NMR (75 MHz, $CDCl_3$) δ 169.04, 167.06, 80.88, 70.03, 30.24, 25.55, 14.03.

## 2.4. General procedure C for the methylation of pyridinol esters




To a round-bottomed flask was charged with pyridinol ester (1 equiv.). The flask was sealed and refiled with nitrogen for 3 times. $CH_2Cl_2$ was added by a syringe, and the flask was cooled in an ice bath. MeI or MeOTf (3 equiv.) was added dropwise. The reaction solution was allowed to stir for 24 h at room temperature, and white or yellow solid was precipitated. The product was filtered and washed with ether and $CH_2Cl_2$. The obtained powder was dried under vacuum, and the pure pyridinium salt was provided.

The synthesis of **1b** was followed **General Procedure C** using and MeI as substrate.

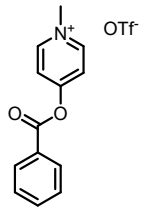



off-white powder, 91%. $^{1}$H NMR (500 MHz, MeOD) δ 8.99 (d, $J$ = 7.1 Hz, 2H), 8.25 (dt, $J$ = 8.5, 1.5 Hz, 2H), 8.15 (d, $J$ = 7.3 Hz, 2H), 7.78 (tt, $J$ = 7.3, 1.3 Hz, 1H), 7.69 – 7.57 (m, 2H), 4.41 (s, 3H).

$^{13}$C NMR (126 MHz, MeOD) δ 163.66, 162.44, 147.77, 134.93, 130.38, 128.88, 127.39, 120.34, 47.04.

HRMS, (ESI): [$M^+$] Calcd. m/z 214.08626, found m/z 214.08636, Error: 0.51 ppm.

The synthesis of **1c** was followed **General Procedure C** using and MeOTf as substrate.



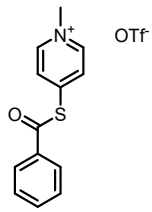
white powder, 94%. $^{1}$H NMR (400 MHz, MeOD) δ 9.00 (d, *J* = 7.1 Hz, 2H), 8.32 – 8.25 (m, 2H), 8.17 (d, *J* = 7.2 Hz, 2H), 7.86 – 7.77 (m, 1H), 7.70 – 7.61 (m, 2H), 4.44 (s, 3H).

$^{13}$C NMR (101 MHz, MeOD) δ 163.58, 162.42, 147.72, 134.90, 130.38, 128.87, 127.37, 120.27, 46.90.

$^{19}$F NMR (376 MHz, MeOD) δ -80.03.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 214.08626, found m/z 214.08636, Error: 0.51 ppm.

The synthesis of **1i** was followed **General Procedure C** using and MeOTf as substrate.



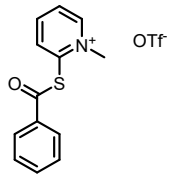
yellow powder, 92%. $^{1}$H NMR (400 MHz, MeOD) δ 8.93 (d, *J* = 6.7 Hz, 2H), 8.39 (d, *J* = 6.7 Hz, 2H), 8.16 – 8.09 (m, 2H), 7.86 – 7.77 (m, 1H), 7.70 – 7.62 (m, 2H), 4.46 (s, 3H).

$^{13}$C NMR (101 MHz, MeOD) δ 184.58, 152.24, 144.77, 135.18, 130.25, 129.23, 127.66, 118.87.

$^{19}$F NMR (376 MHz, MeOD) δ -80.05.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 230.06341, found m/z 230.06387, Error: 2.01 ppm.

The synthesis of **1j** was followed **General Procedure C** using and MeOTf as substrate.



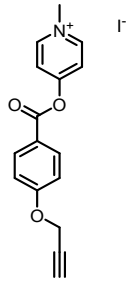
yellow powder, 89%. $^{1}$H NMR (500 MHz, MeOD) δ 8.06 (d, *J* = 7.3 Hz, 1H), 8.01 – 7.96 (m, 2H), 7.66 – 7.55 (m, 2H), 7.42 (dt, *J* = 28.7, 7.5 Hz, 3H), 6.85 (t, *J* = 6.1 Hz, 1H), 3.96 (s, 3H).

$^{13}$C NMR (101 MHz, MeOD) δ 177.35, 167.21, 142.58, 135.78, 133.99, 132.91, 129.99, 129.14, 128.27, 114.81, 45.10.

$^{19}$F NMR (376 MHz, MeOD) δ -80.02.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 230.06341, found m/z 230.06381, Error: 1.74 ppm.

The synthesis of **KP1** was followed **General Procedure C** using and MeI as substrate.

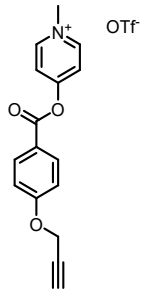


white powder, 86%. $^{1}$H NMR (500 MHz, MeOD) δ 8.93 (d, *J* = 6.9 Hz, 2H), 8.20 (d, *J* = 9.0 Hz, 2H), 8.10 (d, *J* = 7.1 Hz, 2H), 7.18 (d, *J* = 9.0 Hz, 2H), 4.88 (d, *J* = 2.2 Hz, 2H), 4.38 (s, 3H), 3.04 (t, *J* = 2.3 Hz, 1H).

$^{13}$C NMR (126 MHz, MeOD) δ 163.81, 163.29, 161.91, 147.61, 132.68, 120.16, 119.89, 115.10, 77.45, 76.34, 55.67, 46.82.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 268.09682, found m/z 268.09616, Error: -2.46 ppm.

The synthesis of **KP2** was followed **General Procedure C** using and MeOTf as substrate.



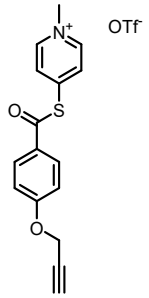
white powder, 89%. $^{1}$H NMR (500 MHz, MeOD) δ 8.93 (d, *J* = 7.1 Hz, 2H), 8.23 – 8.17 (m, 2H), 8.10 (d, *J* = 7.4 Hz, 2H), 7.21 – 7.15 (m, 2H), 4.88 (d, *J* = 2.4 Hz, 2H), 4.37 (s, 3H), 3.04 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (101 MHz, MeOD) δ 163.82, 163.30, 161.92, 147.63, 132.70, 120.18, 119.90, 115.10, 77.46, 76.38, 55.66, 46.81.

$^{19}$F NMR (376 MHz, MeOD) δ -80.08.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 268.09682, found m/z 268.09616, Error: -2.46 ppm.

The synthesis of **KP6** was followed **General Procedure C** using and MeOTf as substrate.



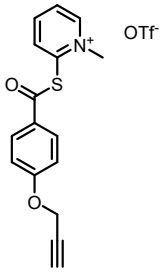
yellow powder, 89%. $^{1}$H NMR (400 MHz, MeOD) δ 8.90 (d, *J* = 6.7 Hz, 2H), 8.37 (d, *J* = 6.8 Hz, 2H), 8.16 – 8.08 (m, 2H), 7.27 – 7.20 (m, 2H), 4.93 (d, *J* = 2.4 Hz, 2H), 4.44 (s, 3H), 3.10 (t, *J* = 2.4 Hz, 1H).

$^{13}$C NMR (101 MHz, MeOD) δ 182.65, 163.48, 152.84, 144.60, 130.08, 130.03, 128.16, 115.33, 77.37, 76.50, 55.75.

$^{19}$F NMR (376 MHz, MeOD) δ -80.06.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 284.07398, found m/z 284.07462, Error: 2.25 ppm.

The synthesis of **KP7** was followed **General Procedure C** using and MeOTf as substrate.



yellow powder, 85%. $^{1}$H NMR (400 MHz, MeOD) δ 9.28 (d, *J* = 6.1 Hz, 1H), 8.68 (td, *J* = 7.9, 1.2 Hz, 1H), 8.51 – 8.44 (m, 1H), 8.27 – 8.19 (m, 1H), 8.15 – 8.07 (m, 2H), 7.30 – 7.22 (m, 2H), 4.95 (d, *J* = 2.3 Hz, 2H), 4.51 (s, 3H), 3.11 (t, *J* = 2.4 Hz, 1H).

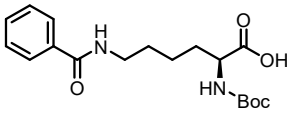
$^{13}$C NMR (101 MHz, MeOD) δ 182.28, 163.77, 149.15, 145.58, 137.42, 130.52, 128.40, 127.36, 115.48, 114.39, 77.30, 76.57, 55.81, 47.37.

$^{19}$F NMR (376 MHz, MeOD) δ -80.04.

HRMS, (ESI): [$M^{+}$] Calcd. m/z 284.07398, found m/z 284.07440, Error: 1.50 ppm.

## 2.5. General Procedure D for the reaction between activated esters and Boc-Lys-OH

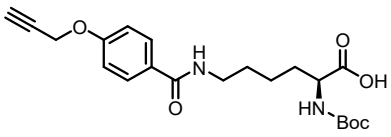
To a 5 mL EP tube was added Boc-Lys-OH (25 mg, 0.1 mmol), **1c** or **KP2** (0.2 mmol), $Na_2HPO_4$ 12 $H_2O$ (143 mg, 0.4 mmol) and 4 mL MeCN/H2O = 1/1 solvent. The tube was then sealed and shook on a shaker at 37 °C for 1h. After filtration, the resulting reaction mixture was then purified directly *via* HPLC. Desired distillates were identified by MS and lyophilized to obtain target products.



**3**, white powder, isolated yiled 74%. $^{1}$H NMR (500 MHz, $CDCl_3$) δ 8.10 (s, 2H), 7.72 (t, *J* = 8.5 Hz, 2H), 7.45 (t, *J* = 7.3 Hz, 1H), 7.37 (t, *J* = 7.5 Hz, 2H), 5.38 (d, *J* = 6.9 Hz, 1H), 4.32 – 4.14 (m, 1H), 3.39 (q, *J* = 6.5 Hz, 2H), 1.87 – 1.65 (m, 2H), 1.66 – 1.56 (m, 2H), 1.38 (d, *J* = 7.1 Hz, 11H).

$^{13}$C NMR (126 MHz, $CDCl_3$) δ 175.63, 168.93, 156.07, 133.98, 131.77, 128.63, 127.11, 80.36, 53.22, 39.89, 31.95, 28.78, 28.29, 22.50.

HRMS, (ESI): $[M+Na^+]^+$ Calcd. m/z 373.17339, found m/z 373.17340, Error: 0.02 ppm.

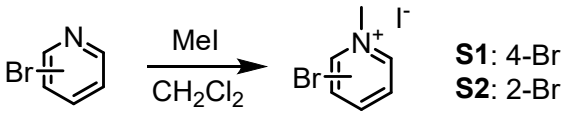


**S5**, white powder, isolated yiled 71%. $^1$H NMR (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.7 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 2H), 6.63 (s, 1H), 5.36 (d, *J* = 7.7 Hz, 1H), 5.21 (s, 1H), 4.70 (d, *J* = 2.2 Hz, 2H), 4.34 – 4.21 (m, 1H), 3.39 (d, *J* = 6.3 Hz, 2H), 2.53 (t, *J* = 2.4 Hz, 1H), 1.89 – 1.66 (m, 2H), 1.63 – 1.55 (m, 2H), 1.40 (s, 11H).

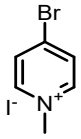
$^{13}$C NMR (101 MHz, $CDCl_3$) δ 175.41, 167.89, 160.14, 156.03, 128.95, 127.36, 114.73, 80.26, 77.98,, 76.14, 55.88, 53.21, 39.75, 31.99, 29.00, 28.36, 22.48.

HRMS, (ESI): $[M-H^+]^-$ Calcd. m/z 403.18746, found m/z 403.18759, Error: 3.05 ppm.

## 2.6. Preparation of bromo pyridinium S1, S2 and the three component amidation



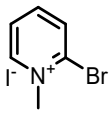

To a round-bottomed flask was charged with 2-Br or 4-Br pyridine (1 equiv.). The flask was sealed and refiled with nitrogen for 3 times. $CH_2Cl_2$ was added by a syringe, and the flask was cooled in an ice bath. MeI (3 equiv.) was added dropwise. The reaction solution was allowed to stir for 24 h at room temperature, and solid was precipitated. The product was filtered and washed with ether and $CH_2Cl_2$. The obtained powder was dried under vacuum, and the pure pyridinium salt was provided.



**S1**: black powder, 87%. $^1$H NMR (400 MHz, Deuterium Oxide) δ 8.59 – 8.53 (m, 2H), 8.23 – 8.17 (m, 2H), 4.24 (s, 3H).

$^{13}$C NMR (101 MHz, $D_2O$) δ 145.44, 143.24, 131.69, 47.99.

HRMS, (ESI): [$M^+$] Calcd. m/z 171.97564, found m/z 171.97572, Error: 0.49 ppm.



**S2**: light yellow powder, 85%. $^1$H NMR (400 MHz, Deuterium Oxide) δ 8.91 (dd, *J* = 6.1, 1.7 Hz, 1H), 8.51 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.96 (td, *J* = 7.9, 1.7 Hz, 1H), 7.87 (ddd, *J* = 7.8, 6.1, 1.5 Hz, 1H), 4.37 (s, 3H).

$^{13}$C NMR (101 MHz, $D_2O$) δ 147.77, 144.02, 141.40, 127.16, 117.24, 55.48.

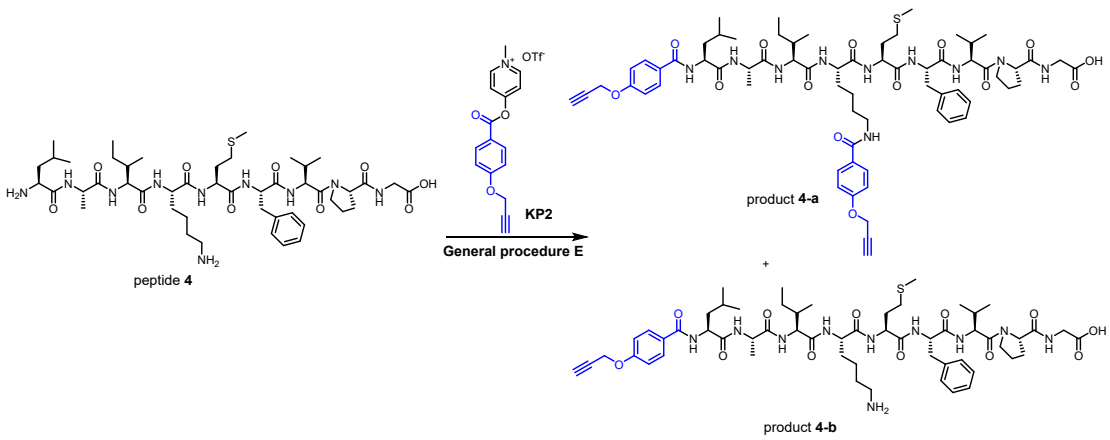
HRMS, (ESI): [$M^+$] Calcd. m/z 171.97564, found m/z 171.97578, Error: 0.85 ppm.

## 2.7. General procedure E and characterization data for the reaction between pyridinium activated ester and peptide

**General procedure E for the reaction of peptide**

A 20 mM MeCN stock solution of **KP2** was made up of 84 mg **KP2** (0.2 mmol) and 10 mL MeCN. This stock solution can store in a capped glass for more than one month in 4 °C refrigerator. To a 500 uL EP tube was added specific peptide, 100 uL PBS (pH 7.4) buffer and 100 uL **KP2** stock solution. The tube was then sealed and shook on a shaker at room temperature for 30 min. The resulting solution was then analyzed directly *via* HPLC after filtration. Desired distillates were identified by MS and lyophilized to obtain target products.

### 2.7.1 Characterization data for the reaction between KP2 and peptide 4

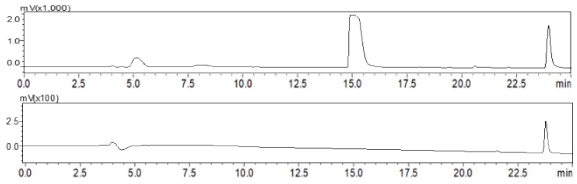



The reaction was followed **General Procedure E** using peptide **4** (0.5 umol), sequence: $NH_2$-LAI**K**MFVPG-OH.

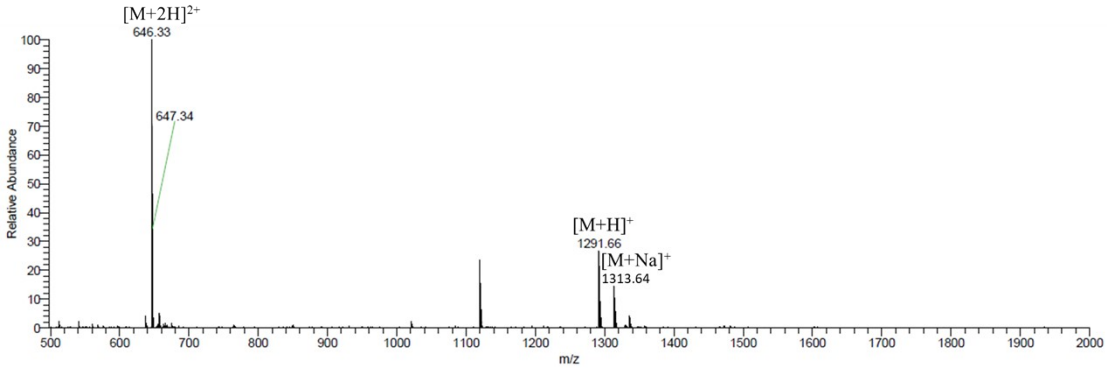
**Table S1.** Kinetic study of the reaction of peptide **4** and **KP2**

| Entry | pH | Reaction time (min) | Conversion (%) 4-a (4-b) |
|---|---|---|---|
| 1 | | 10 | 7 (51) |
| 2 | 6.0 | 30 | 50 (48) |
| 3 | | 60 | 62 (38) |
| 4 | | 10 | 43 (55) |
| 5 | 6.5 | 30 | 98 |
| 6 | | 60 | 99 |
| 7 | | 10 | 98 |
| 8 | 7.0 | 30 | 99 |
| 9 | | 60 | 99 |
| 10 | 7.4 | 10 | 98 |

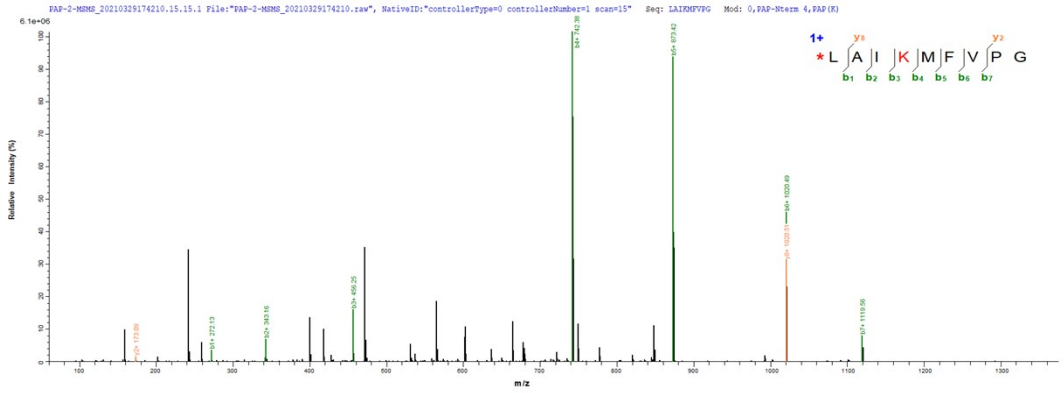
| | | | |
|---|---|---|---|
| 11 | | 30 | 99 |
| 12 | | 60 | 99 |
| 13 | | 10 | 99 |
| 14 | 8.0 | 30 | 99 |
| 15 | | 60 | 99 |



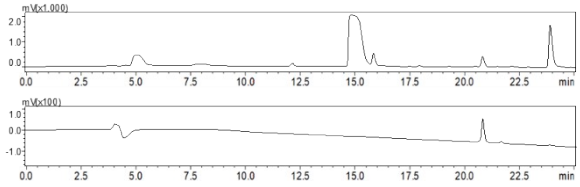

LC trace of the reaction of peptide **4** after 60 mins and purified product **4-a** at pH 7.4.



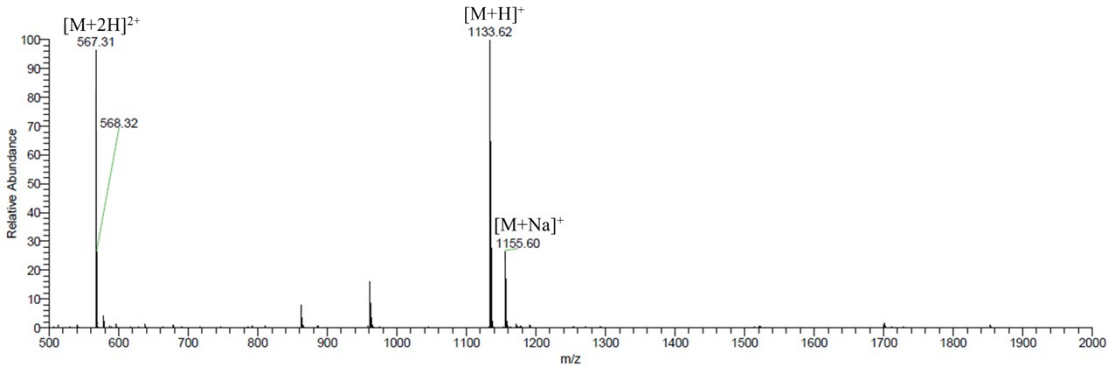

ESI Mass spectrum of purified product **4-a**. Calculated Mass $[M+H]^+$: 1291.66; $[M+2H]^{2+}$: 646.33; Mass Found (ESI+) $[M+H]^+$: 1291.66; $[M+2H]^{2+}$: 646.33.



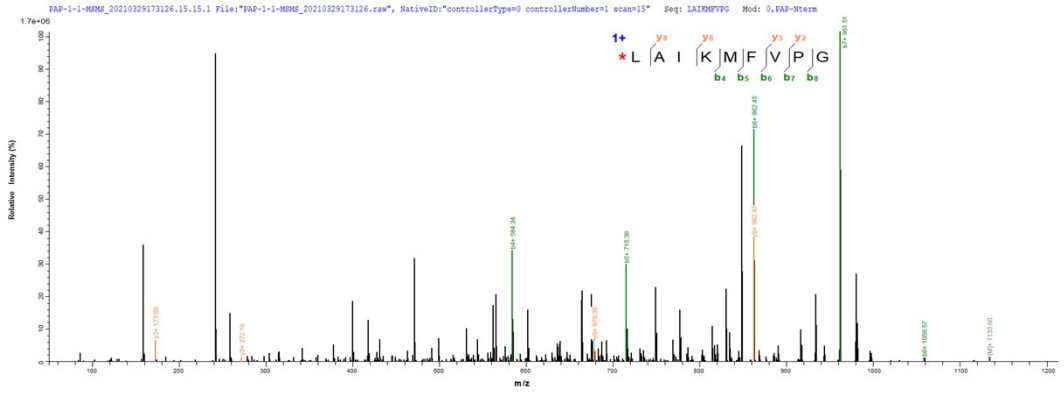

De novo ms/ms analysis of purified product **4-a**.




LC trace of the reaction of peptide **8** after 60 mins and purified product **4-b** at pH 6.0.

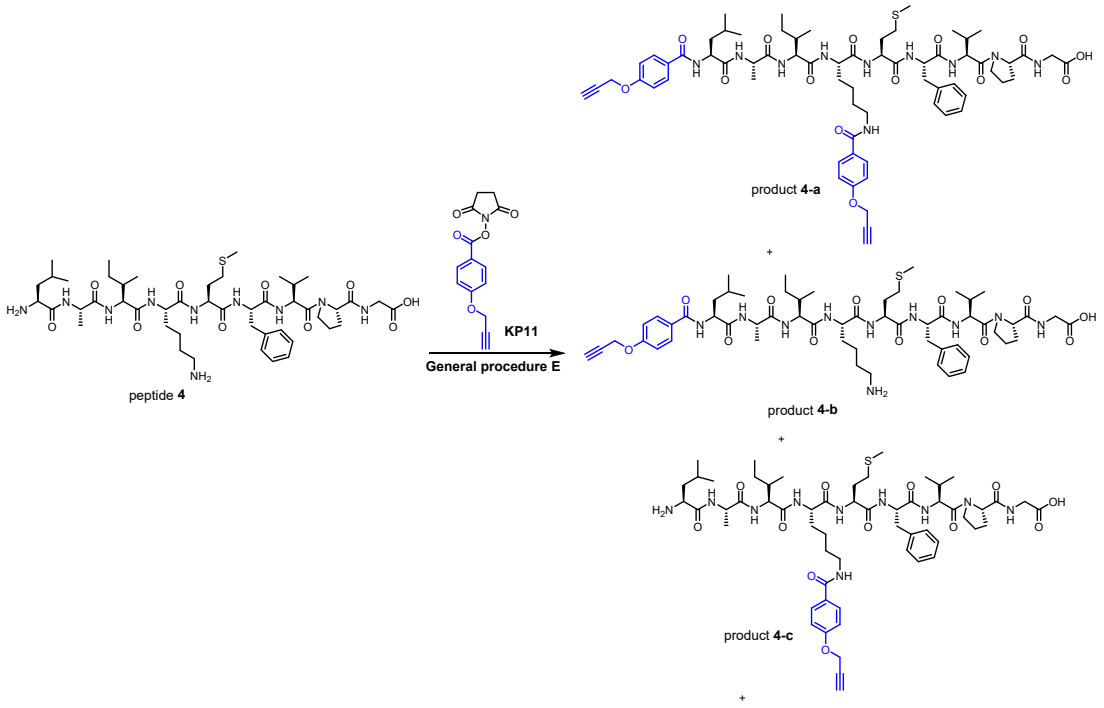



ESI Mass spectrum of purified product **4-b**. Calculated Mass $[M+H]^+$: 1133.62; $[M+2H]^{2+}$: 567.31; Mass Found (ESI+) $[M+H]^+$: 1133.62; $[M+2H]^{2+}$: 567.31.




De novo ms/ms analysis of purified product **4-b**.

### 2.7.2 Characterization data for the reaction between KP11 and peptide 4

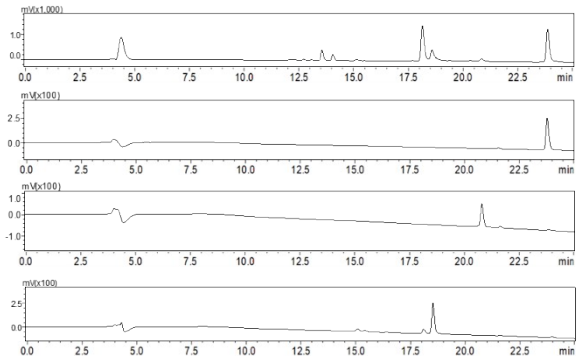



The reaction was followed **General Procedure E** using peptide **4** (0.5 umol) and **KP11** (0.4 mmol), sequence: $NH_2$-LAIKMFVPG-OH.

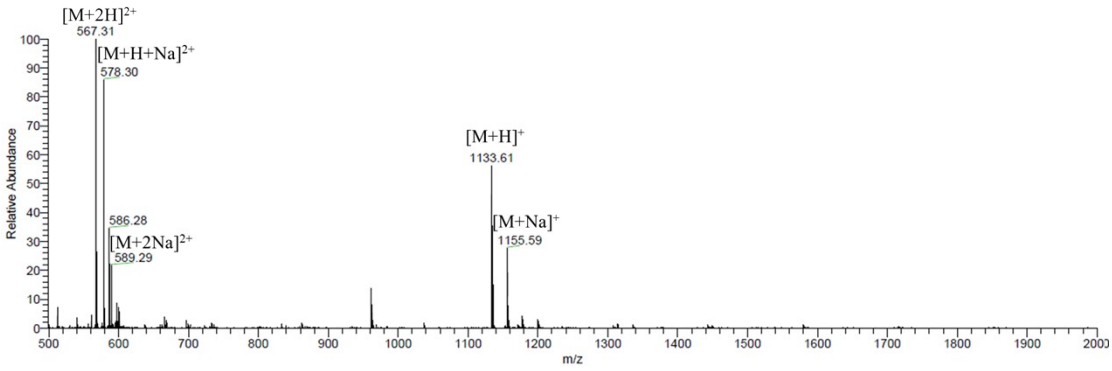
**Table S2.** Kinetic study of the reaction of peptide **4** and **KP11**

| Entry | pH | Reaction time (min) | Conversion (%) 4-a (4-b) (4-c) |
|---|---|---|---|
| 1 | 6.0 | 10 | 0 (1) (5) |
| 2 | | 30 | 8 (21) (28) |
| 3 | | 60 | 17 (26) (27) |
| 4 | 6.5 | 10 | 0 (8) (35) |
| 5 | | 30 | 39 (15) (39) |

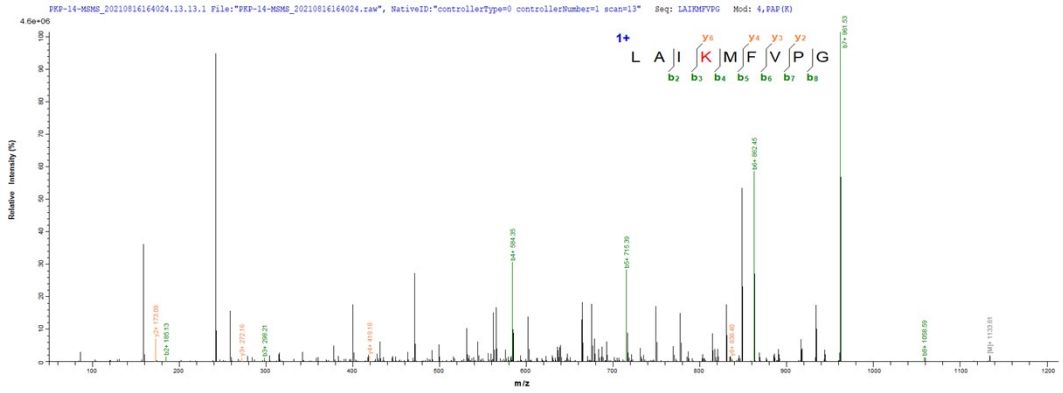
| | | | |
|---|---|---|---|
| 6 | | 60 | 57 (14) (25) |
| 7 | | 10 | 0 (8) (43) |
| 8 | 7.0 | 30 | 54 (5) (38) |
| 9 | | 60 | 73 (3) (24) |
| 10 | | 10 | 5 (6) (54) |
| 11 | 7.4 | 30 | 57 (3) (35) |
| 12 | | 60 | 79 (2) (19) |
| 13 | | 10 | 9 (6) (64) |
| 14 | 8.0 | 30 | 59 (3) (33) |
| 15 | | 60 | 83 (1) (16) |




LC trace of the reaction of peptide **4** and KP 12 after 60 mins and purified product **4-a**, **4-b** and **4-c** at pH 8.0.

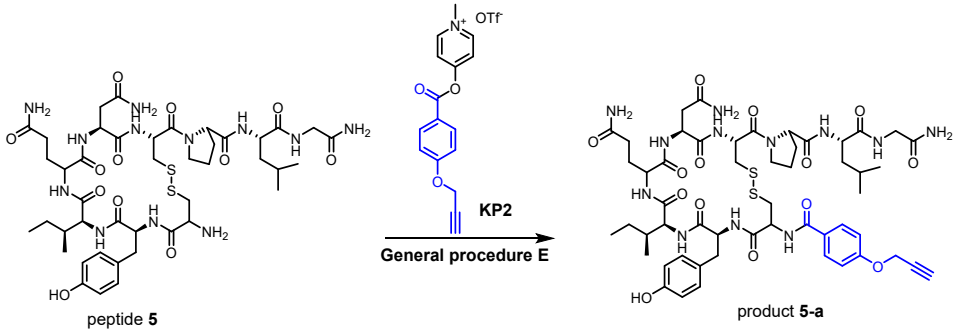



ESI Mass spectrum of purified product **4-c**. Calculated Mass $[M+H]^+$: 1133.62; $[M+2H]^{2+}$: 567.31; Mass Found (ESI+) $[M+H]^+$: 1133.62; $[M+2H]^{2+}$: 567.31.

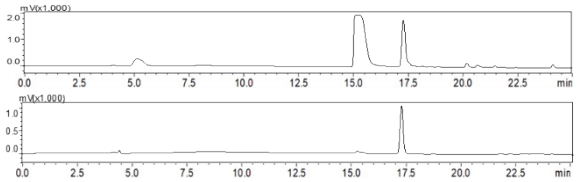



De novo ms/ms analysis of purified product **4-c**.

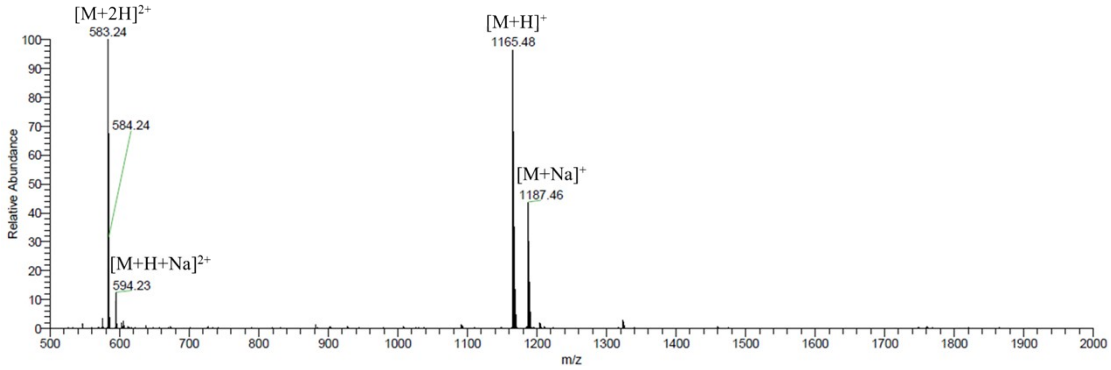
### 2.7.3 Characterization data for the reaction between KP2 and peptide 5



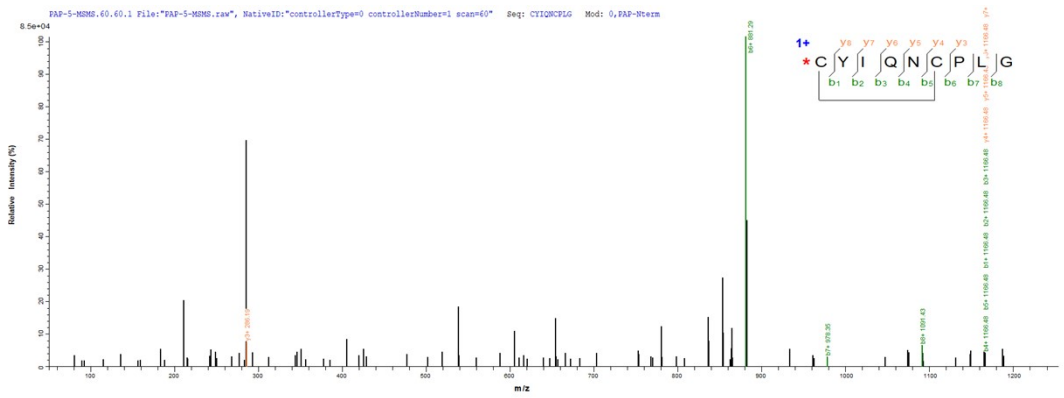

The reaction was followed **General Procedure E** using peptide **5** (0.5 umol), sequence: $NH_2$-CYIQNCPLG-$NH_2$ (disulfide bond: Cys1-Cys6).



LC trace of the reaction of peptide **5** and purified product **5-a** and **5-b** at pH 6.5.

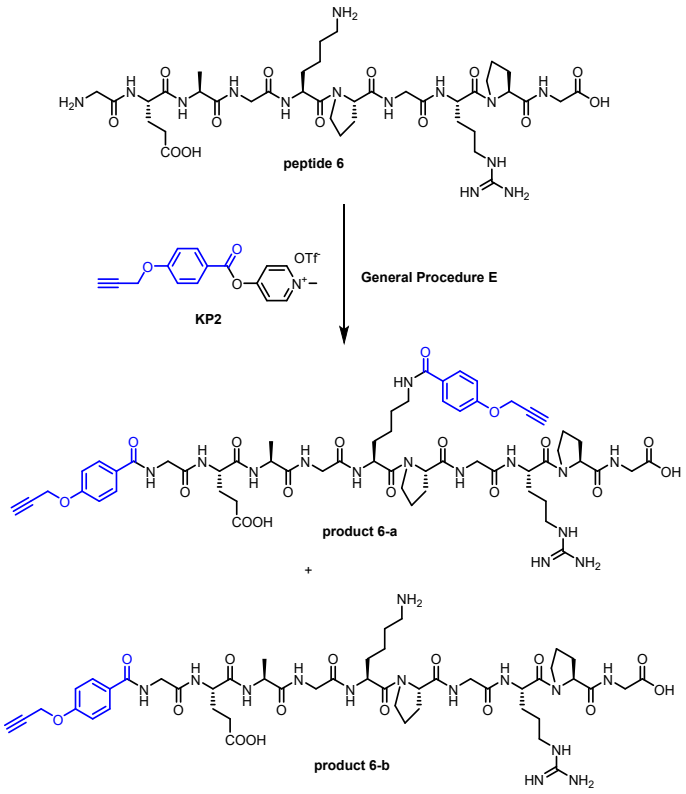



ESI Mass spectrum of purified product **5-a**. Calculated Mass $[M+H]^+$: 1165.48; $[M+2H]^{2+}$: 583.24; Mass Found (ESI+) $[M+H]^+$: 1165.48; $[M+2H]^{2+}$: 583.24.

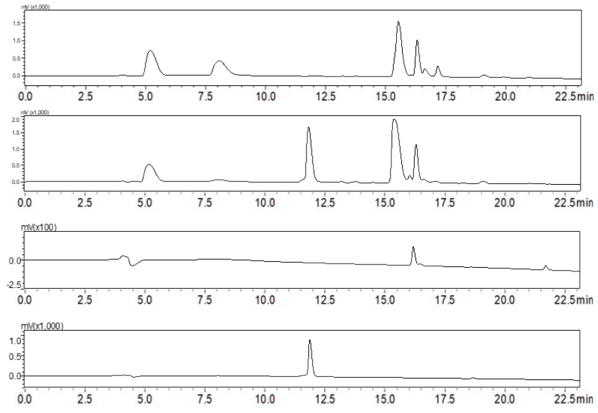



De novo ms/ms analysis of purified product **5-a**.

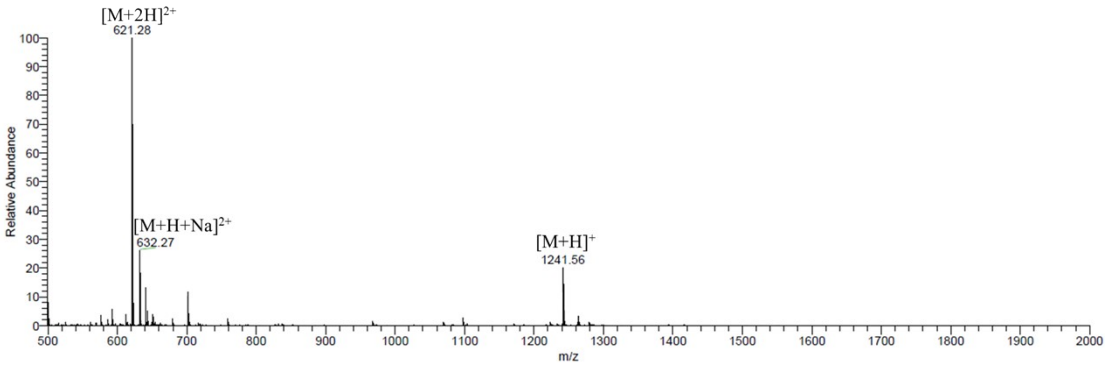
### 2.7.4 Characterization data for the reaction between KP2 and peptide 6



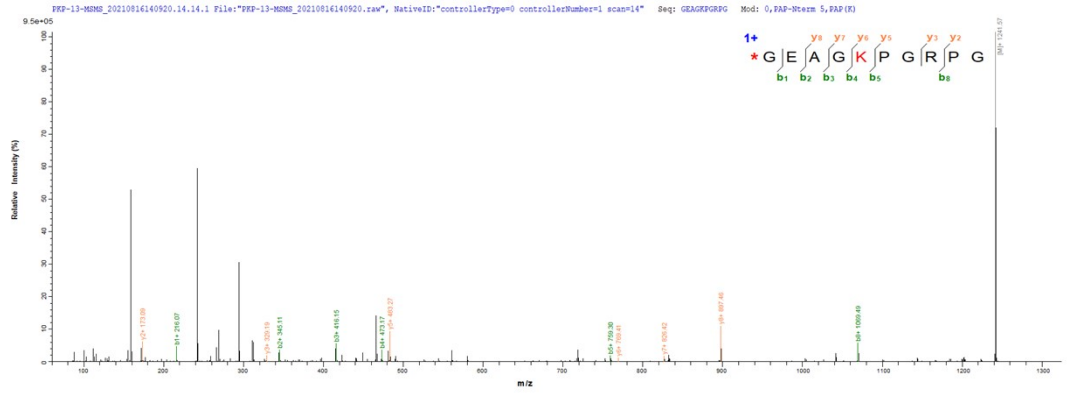

The reaction was followed **General Procedure E** using peptide **6** (0.4 umol), sequence: $NH_2$-GEAGKPGRPG-OH.



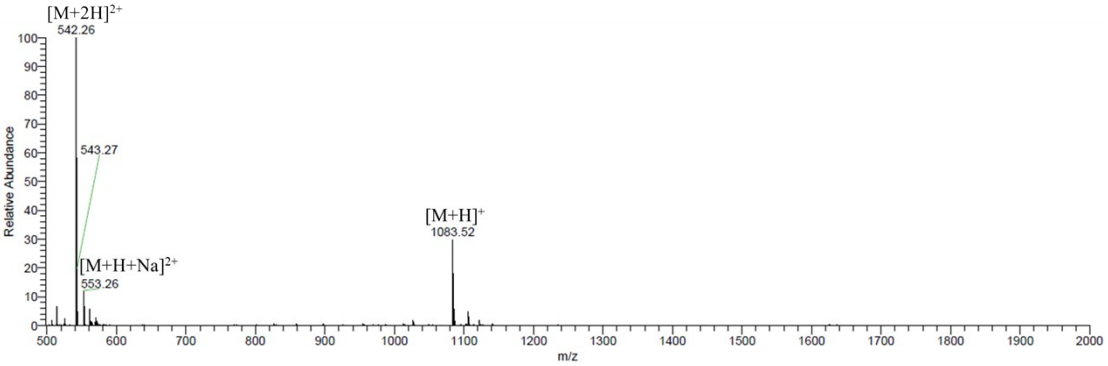

LC trace of the reaction of peptide **6** at pH 8.0 and 7.0 and purified product **6-a** and **6-b**.



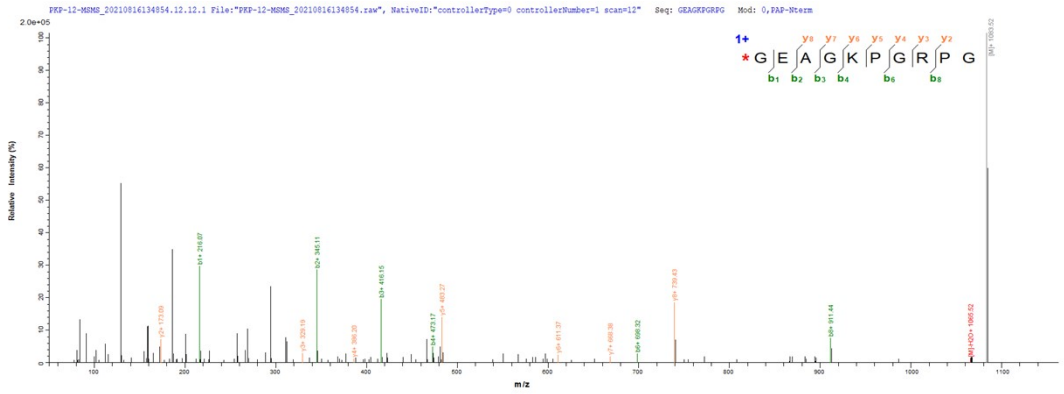

ESI Mass spectrum of purified product **6-a**. Calculated Mass $[M+H]^+$: 1241.56; $[M+2H]^{2+}$: 621.28; Mass Found (ESI+) $[M+H]^+$: 1241.56; $[M+2H]^{2+}$: 621.28.




De novo ms/ms analysis of purified product **6-a**.

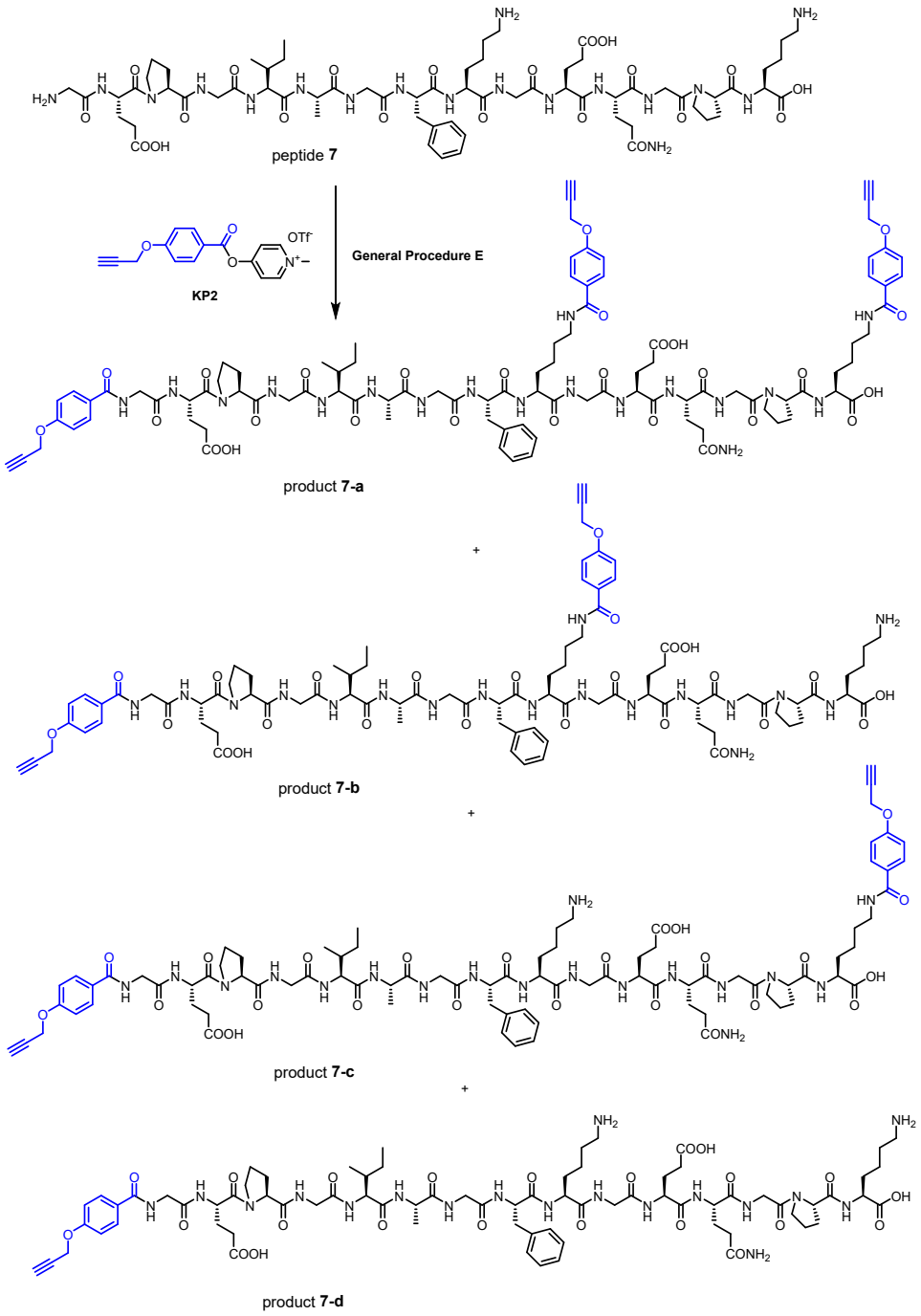


ESI Mass spectrum of purified product **6-b**. Calculated Mass $[M+H]^{+}$: 1083.52; $[M+2H]^{2+}$: 542.26; Mass Found (ESI+)$[M+H]^{+}$: 1083.52; $[M+2H]^{2+}$: 542.26.

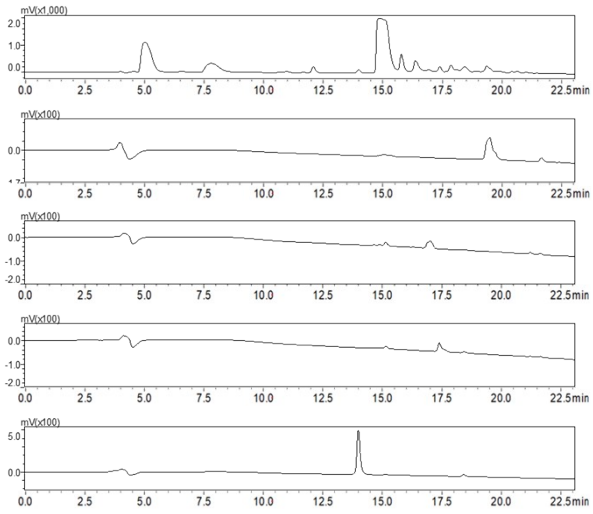



De novo ms/ms analysis of purified product **6-b**.

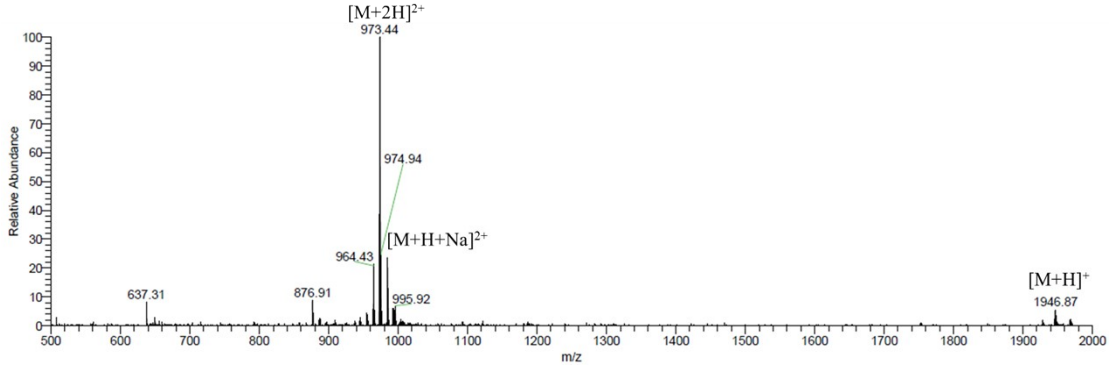
### 2.7.5 Characterization data for the reaction between KP2 and peptide 7



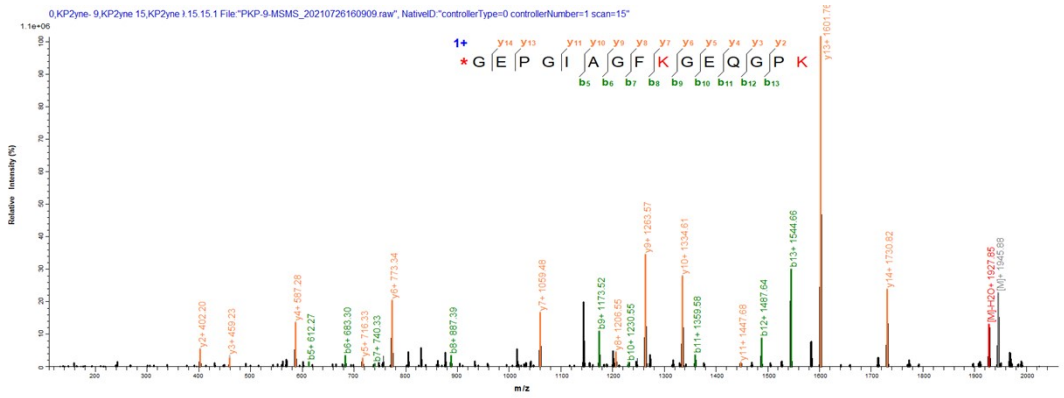

The reaction was followed **General Procedure E** using peptide **7** (0.2 umol), sequence: $NH_2$-GEPGIAGF**K**GEQGP**K**-OH.



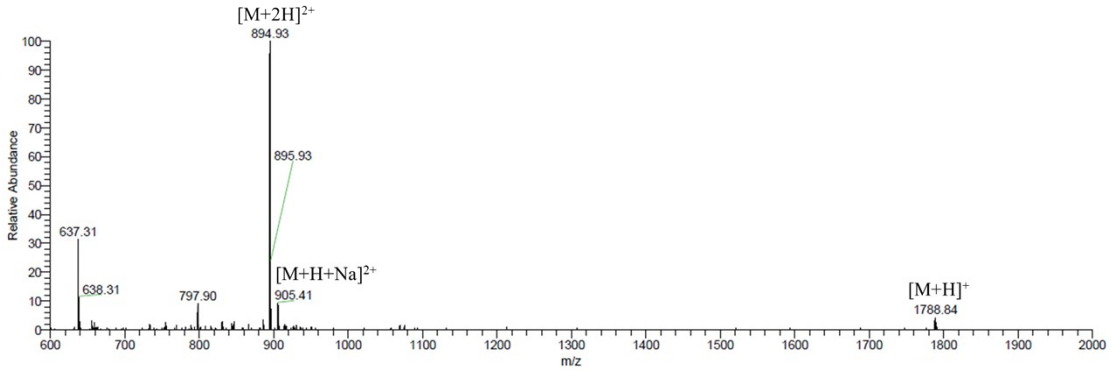

LC trace of the reaction of peptide **7** and purified product **7-a**, **7-b**, **7-c** and **7-d**.



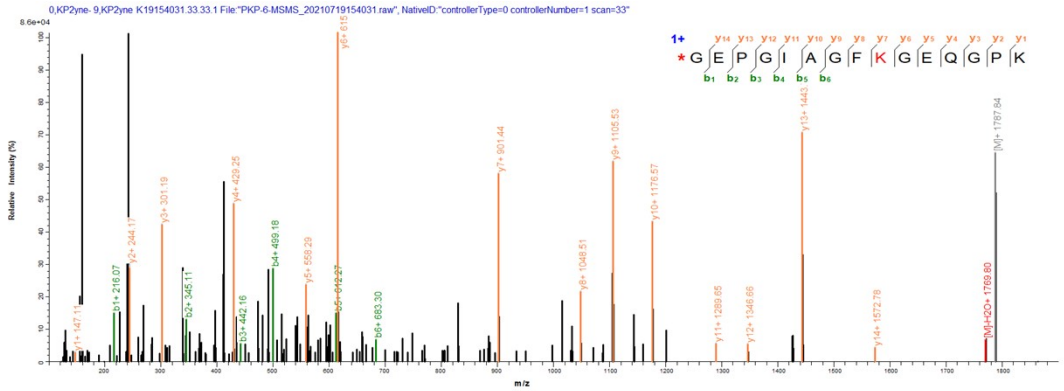

ESI Mass spectrum of purified product **7-a**. Calculated Mass $[M+H]^+$: 1945.87; $[M+2H]^{2+}$: 973.44; Mass Found (ESI+) $[M+H]^+$: 1945.87; $[M+2H]^{2+}$: 973.44.



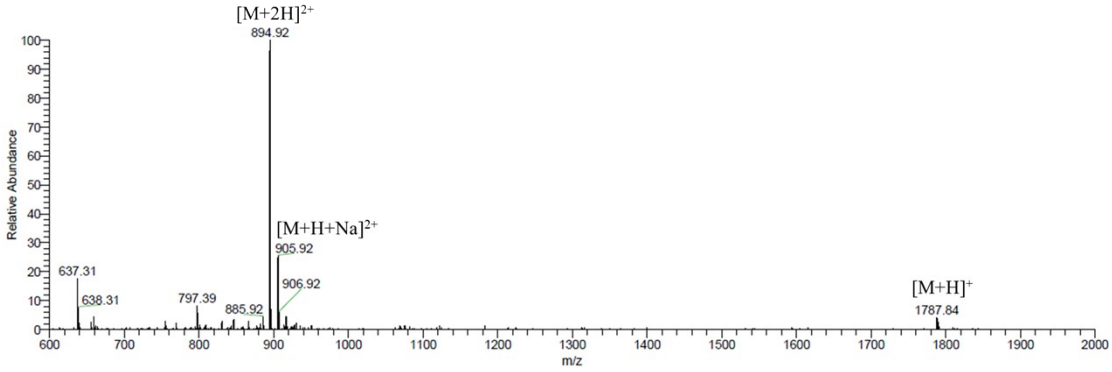
De novo ms/ms analysis of purified product **7-a**.



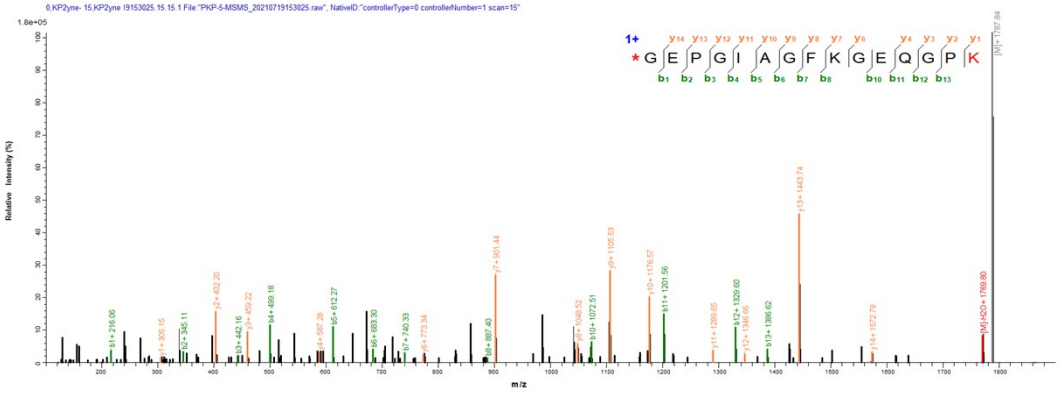

ESI Mass spectrum of purified product **7-b**. Calculated Mass $[M+H]^+$: 1787.84; $[M+2H]^{2+}$: 894.93; Mass Found (ESI+) $[M+H]^+$: 1787.84; $[M+2H]^{2+}$: 894.93.



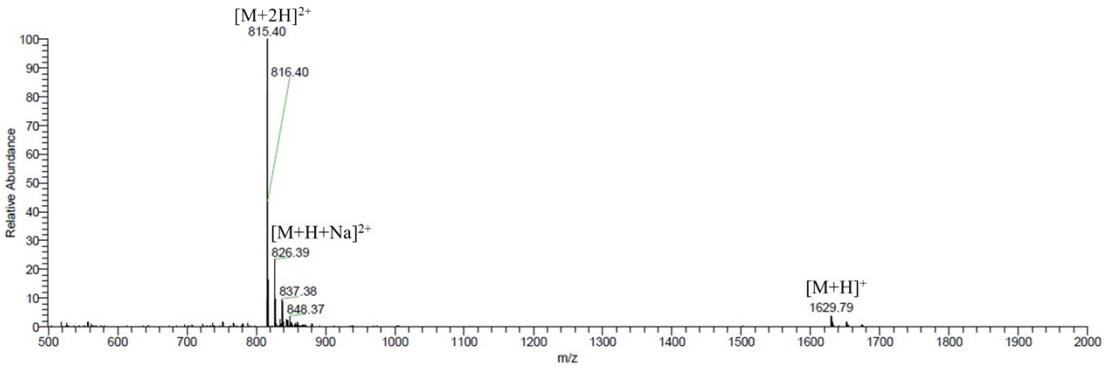
De novo ms/ms analysis of purified product **7-b**.



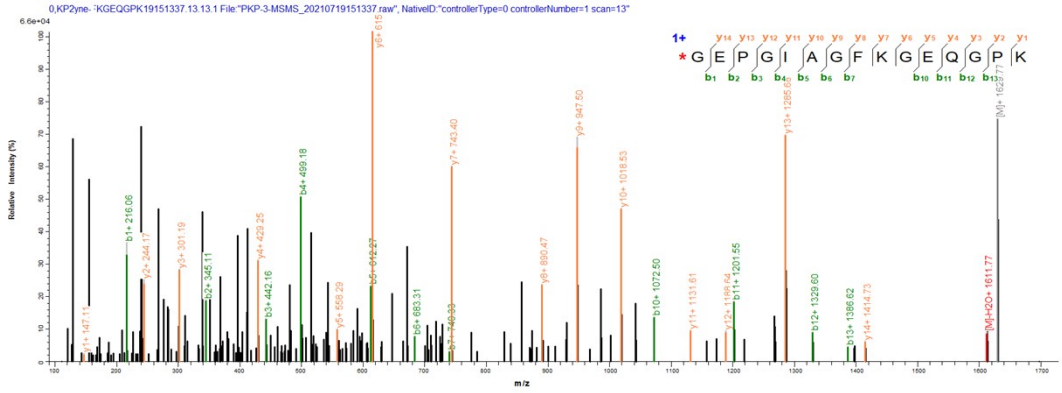

ESI Mass spectrum of purified product **7-c**. Calculated Mass $[M+H]^+$: 1787.84; $[M+2H]^{2+}$: 894.92; Mass Found (ESI+) $[M+H]^+$: 1787.84; $[M+2H]^{2+}$: 894.93.




De novo ms/ms analysis of purified product **7-c**.

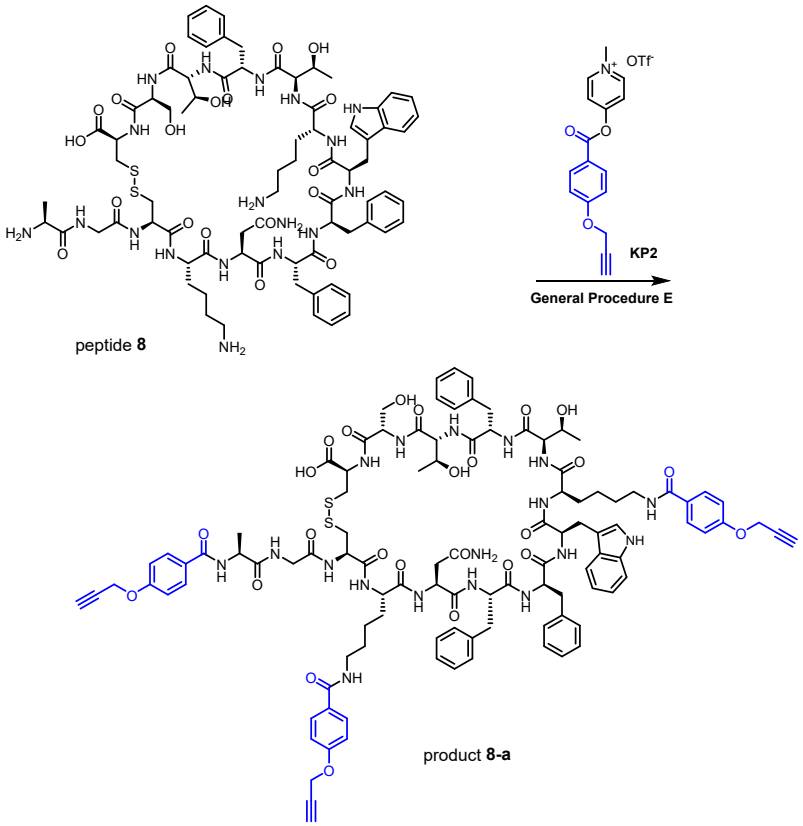



ESI Mass spectrum of purified product **7-d**. Calculated Mass $[M+H]^+$: 1629.79; $[M+2H]^{2+}$: 815.40; Mass Found (ESI+)$[M+H]^+$: 1629.79; $[M+2H]^{2+}$: 815.40.

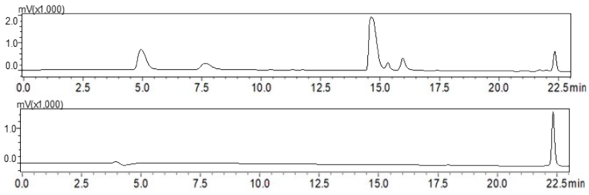



De novo ms/ms analysis of purified product **7-d**.

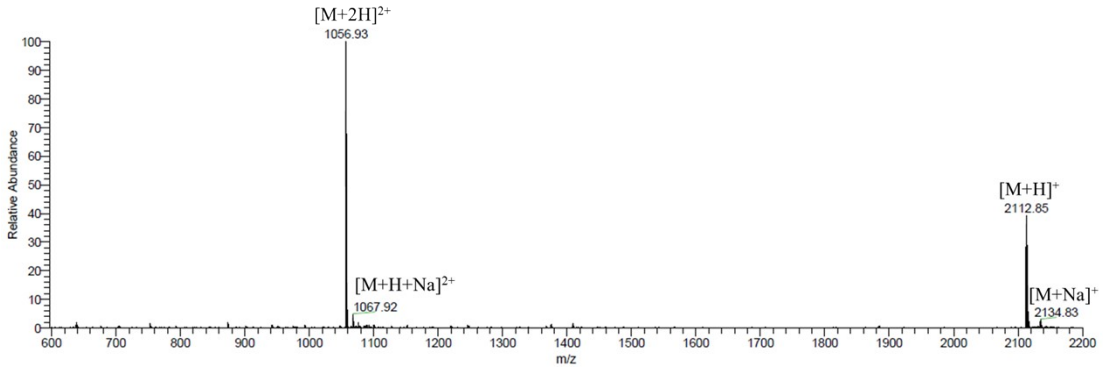
### 2.7.6 Characterization data for the reaction between KP2 and peptide 8



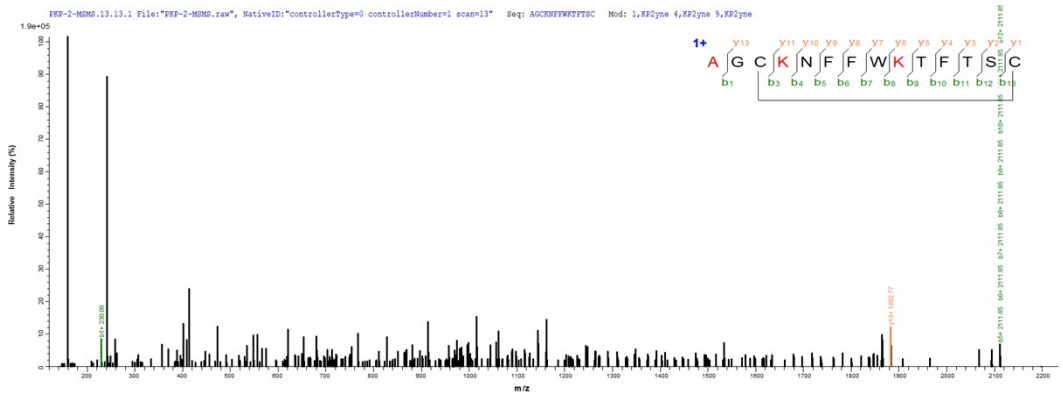

The reaction was followed **General Procedure E** using peptide **8** (0.2 umol), sequence: $NH_2$-AGC**K**NFFW**K**TFTSC-OH (disulfide bond: Cys3-Cys14).




LC trace of the reaction of peptide **8** and purified product **8-a**.

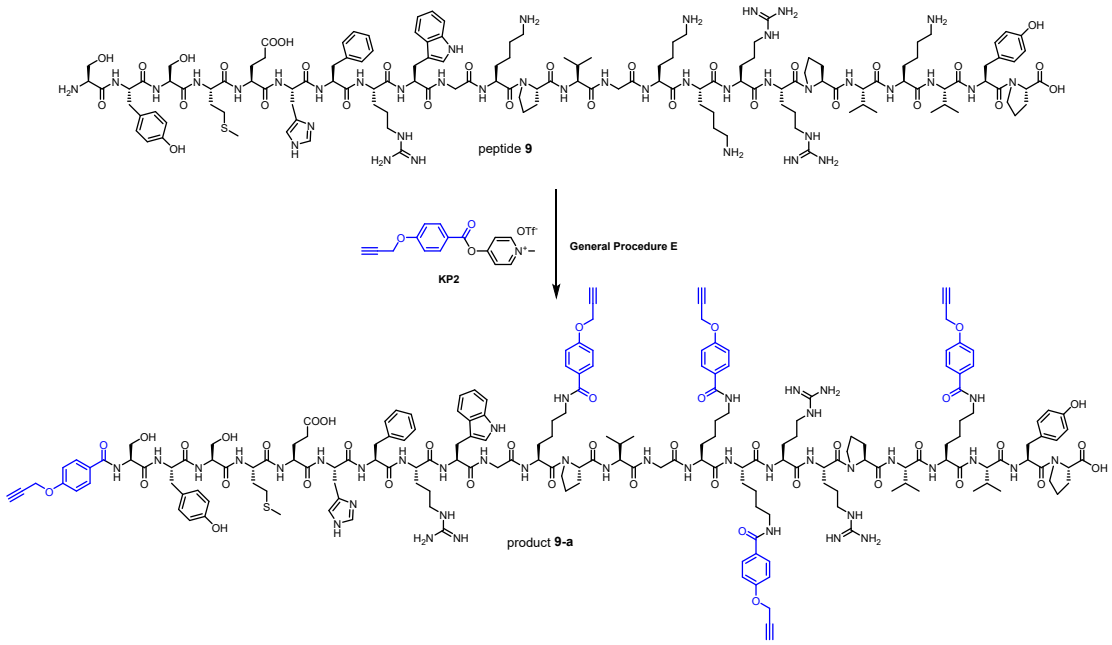



ESI Mass spectrum of purified product **8-a**. Calculated Mass $[M+H]^+$: 2112.85; $[M+2H]^{2+}$: 1056.93; Mass Found (ESI+) $[M+H]^+$: 2112.85; $[M+2H]^{2+}$: 1056.93.

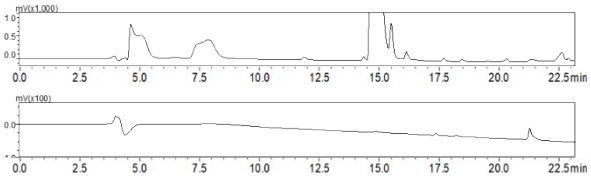



De novo ms/ms analysis of purified product **8-a**.

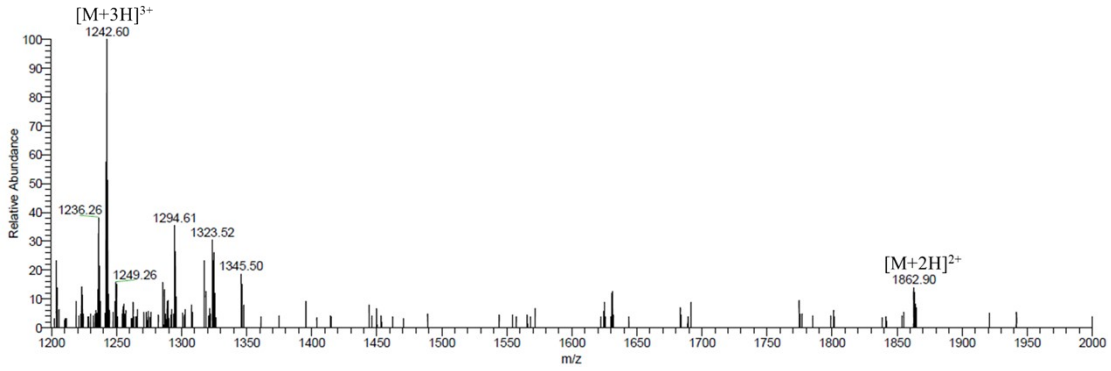
### 2.7.7 Characterization data for the reaction between KP2 and peptide 9



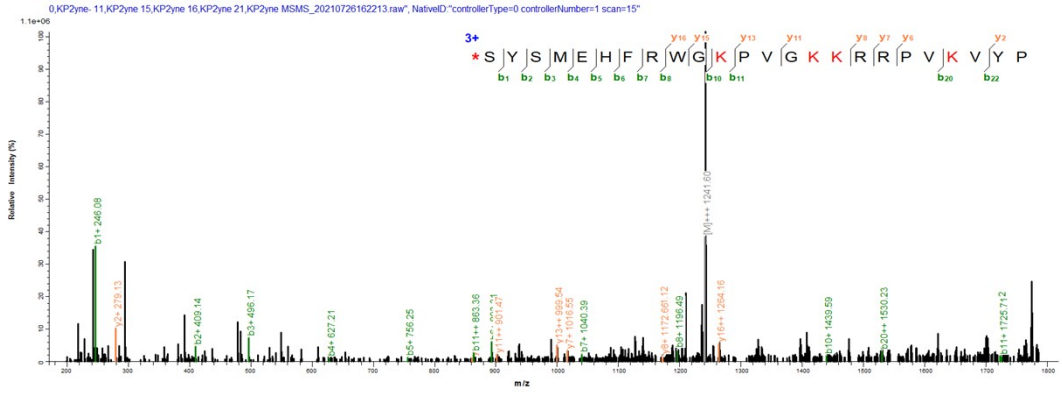

The reaction was followed **General Procedure E** using peptide **9** (0.12 umol), sequence: $NH_2$-SYSMEHFRWG**K**PVG**KK**RRPV**K**VYP-OH.




LC trace of the reaction of peptide **9** and purified product **9-a**.

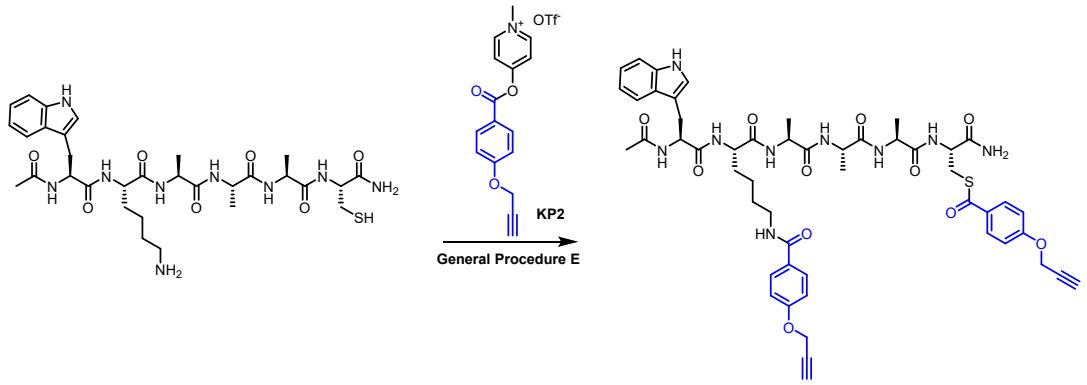



ESI Mass spectrum of purified product **9-a**. Calculated Mass $[M+2H]^{2+}$: 1861.88, $[M+3H]^{3+}$: 1241.60; Mass Found (ESI+) $[M+2H]^{2+}$: 1861.89, $[M+3H]^{3+}$: 1241.59.

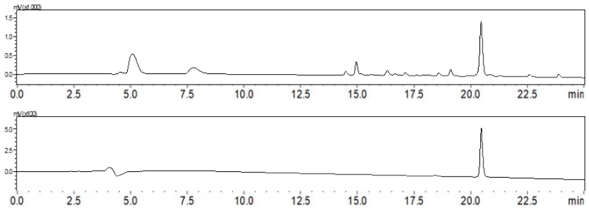



De novo ms/ms analysis of purified product **9-a**.

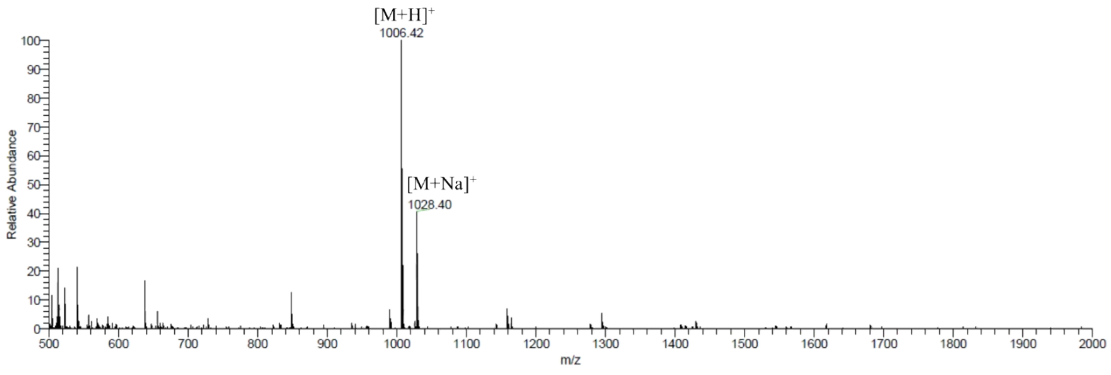
### 2.7.8 Characterization data for the reaction between KP2 and peptide 10



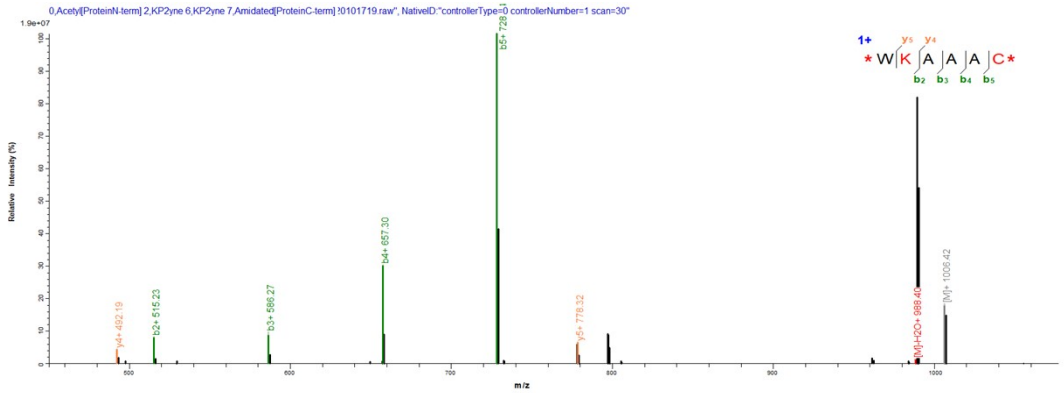

The reaction was followed **General Procedure E** using peptide **10** (0.4 umol), sequence: $NH_2$-W**K**AAA**C**-$NH_2$.




LC trace of the reaction of peptide **10** and purified product **10-a**.

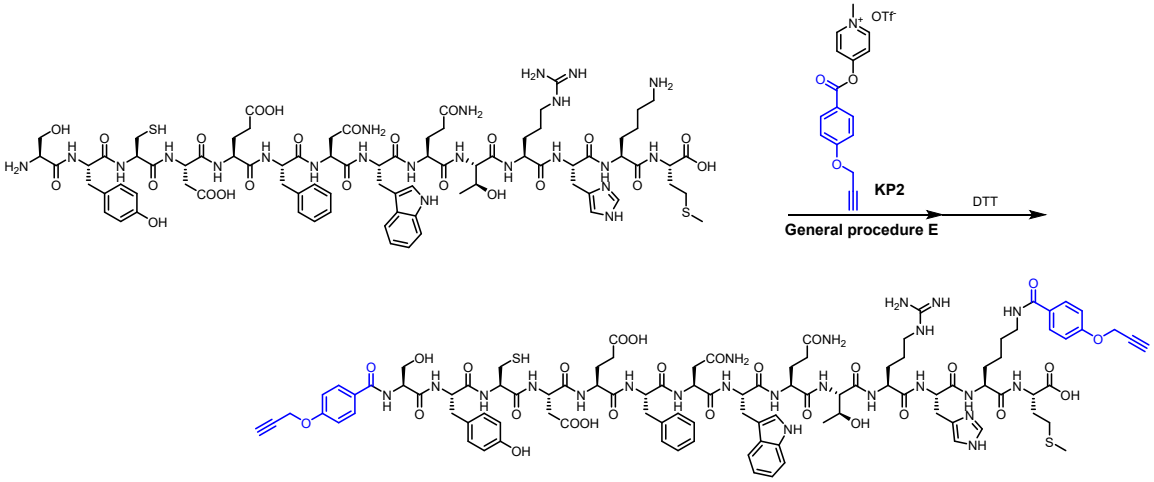



ESI Mass spectrum of purified product **10-a**. Calculated Mass $[M+H]^+$: 1006.42; Mass Found (ESI+) $[M+H]^+$: 1006.42.

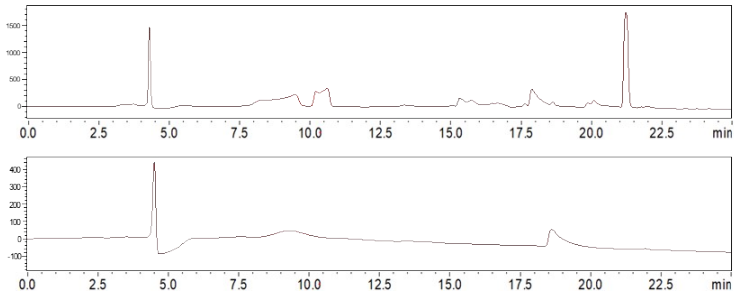



De novo ms/ms analysis of purified product **10-a**.

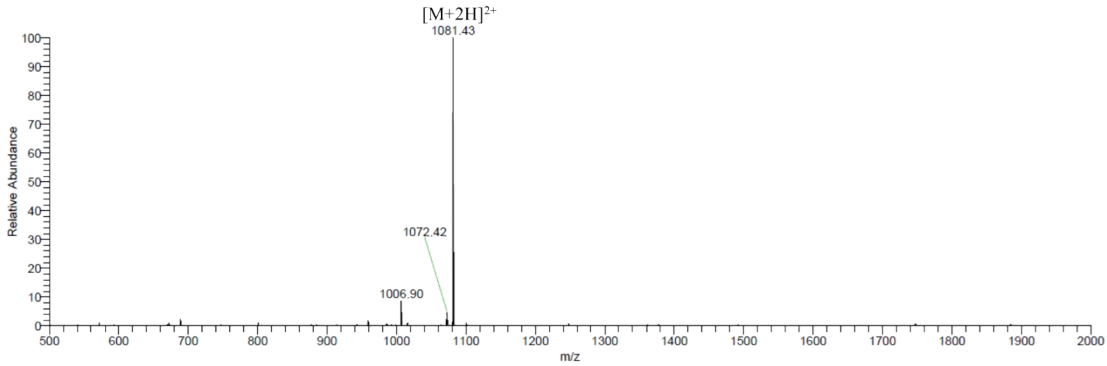
### 2.7.9 Characterization data for the reaction between KP2 and peptide 11



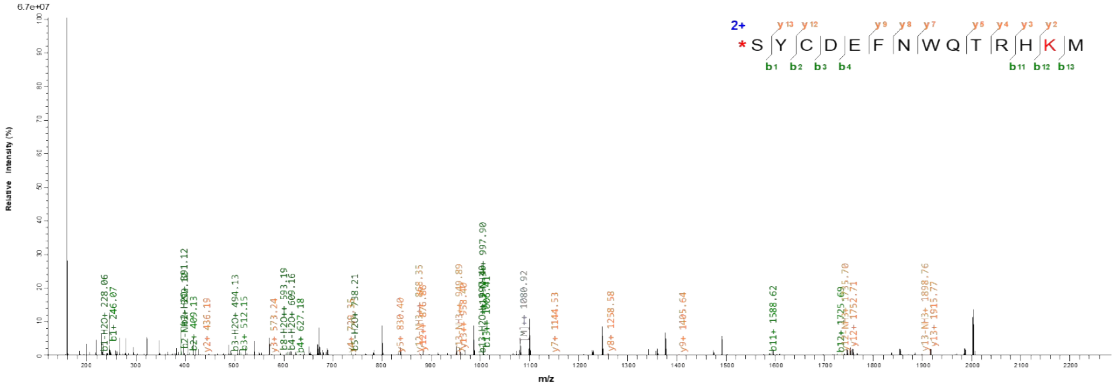

The reaction was followed **General Procedure E** using peptide **11** (0.4 umol), sequence: $NH_2$-SYCDEFNWQTRHKM-OH. After the amidation, DTT (20 mM) was added into the crude reaction.




LC trace of the reaction of peptide **11** and purified product **11-a**.




ESI Mass spectrum of purified product **11-a**. Calculated Mass $[M+2H]^{2+}$: 1081.43; Mass Found (ESI+) $[M+2H]^{2+}$: 1081.43.




De novo ms/ms analysis of purified product **11-a**.

# 3. Supplementary Materials and Methods for Biology

## 3.1. Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. TAMRA-$PEG_4$-azide (named as "TAMRA-$N_3$" in this study), PC biotin-$PEG_3$-azide (named as "PC biotin-$N_3$" in this study) and Dadps biotin-$PEG_4$-azide (named as "Dadps biotin-$N_3$" in this study) were purchased from BIOCONE (Chengdu, CHINA). Sequencing-grade trypsin and NeutrAvidin™ agarose were purchased from Thermo Scientific. All solutions were made with ultrapure Milli-Q water (Millipore, Bedford, MA). Bovine serum albumin (BSA) was dissolved in PBS buffer, pH 7.5. The peptide and probes were dissolved in DMSO to an indicated concentration.

## 3.2. Preparation of human cell line proteomes in lysate or in live cells

All cell lines (a low passage number < 20 passages) were maintained at 37 ˚C in a humidified atmosphere containing 5% carbon dioxide. Hela, HEK293T and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) using supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and glutamine. Jurkat cells were grown in RPMI-1640 medium supplemented with 10% FBS, penicillin, streptomycin and glutamine. For lysate labeling, cells were grown to approximately 80-90% confluence on 100 mm polystyrene tissue culture plates. Cells were washed with cold PBS. For lysate labeling, cells were directly scraped with cold PBS and cell pellets were isolated by centrifugation (1,400g, 3 min, 4 ˚C), and stored at -80 ˚C until use; for in live labeling, 5 mM **KP2** probe was incubated with cells in PBS for one hour at 37 ˚C in a humidified atmosphere containing 5% carbon dioxide, cells were scraped with cold PBS and cell pellets were isolated by centrifugation (1,400g, 3 min, 4 ˚C), and stored at -80 ˚C until use. On ice, pre-cooled microcentrifuge tube resuspended cell pellets in 1 ml cold DPBS buffer and sonicated until pellet is disturbed then centrifuged at x12,000 rpm 10 min. The final supernatant protein (soluble fraction) was transferred to a new tube and quantified at 595 nm using Bradford assay kit. A final protein concentration of 2 mg/ml was used for reactivity measurements by ABPP and gel-based ABPP.

## 3.3. Gel fluorescence analysis of labeled proteins

For probe labeling assay, 30 μM BSA or 100 μg proteins extracted from MCF-7 cells were treated with KP probes (**KP1-KP13**) at indicated concentration at R.T. for 1 hour. A freshly pre-mixed click chemistry reaction cocktail was then added (50 μM TAMRA-PEG4-$N_3$ in DMSO, 100 μM TBTA in DMSO:t-butanol 1:4, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in water, and 1 mM $CuSO_4$ in water) to the mixture above. The reaction was further incubated for another 1 hour at R.T. and then boiled using 4× SDS-PAGE loading buffer at 95 ˚C for 10 min. The samples were analyzed by 12% SDS-PAGE gels (polyacrylamide gel electrophoresis) and visualized by in-gel fluorescence using a BioRad ChemiDoc™

imager. Then the gels were then stained with Coomassie staining (CBB) and scanned. For competition assay, proteins were treated with IAM, NHS-Ac and **KP-B**, respectively at indicated concentration as above processes.

## 3.4. KP2 labeling and click chemistry

For reactivity measurements by ABPP[10], 1 ml 2 mg/ml proteome aliquots were treated at R.T. with 100 mM **KP2** (10 µl of 10 mM stock in water) and 500 mM **KP2** (10 µl of 50 mM stock in water), respectively. Samples were conjugated by CuAAC to PC biotin-$N_3$ for labeling cell lysates or Dadps biotin-$N_3$ for labeling live cell (10 mM stock in DMSO, final concentration = 110 µM) using 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (stock in water) and 100 µM TBTA (stock in DMSO:t-butanol 1:4) and 1 mM $CuSO_4$ (stock in water). The samples were allowed to react for 1 hour at R.T.. The precipitated protein pellets were collected by centrifugation (8000 g, 10 min), washed with cold methanol three times, and resuspended in 1.2% SDS/DPBS. The samples were then diluted to 0.2% SDS/PBS. And incubated with streptavidin-agarose beads slurry (Thermo) for 3 hours at 29 ˚C. The beads were washed in 5 ml DPBS (3x) and water sequentially and suspended in 500 of 6 M urea/Tris. After reduction with 10 mM dithiothreitol (DTT) at 37 ˚C for 30 mins and alkylation with 20 mM iodoacetamide (IAM) at 35 ˚C for 30 mins with shaking in dark, the beads were resuspended in DPBS containing 2 M urea. To this was added trypsin (2 µg) and the samples were allowed to digest overnight at 37 ˚C with shaking. The beads were collected by centrifugation and washed with 2 × 1 ml DPBS and 2 × 1 ml water) and then transferred to fresh Eppendorf tubes with 1 ml water. For the photo-cleavable tag, 1 ml of methanol-$H_2O$ (7-3, vol/vol) was added to the beads, the suspensions were transferred to tubes followed irradiation under UV 365 nm (5000 x 100 µJ/$cm^2$) for 1 hour to release the adducted peptides from cell lysates; For the acid-cleavable tag, the beads were incubated with 2% formic acid for 0.5 hours at 25 ˚C to release probe-modified peptides in live cells. The mixtures were transferred wo a low-binding tube and centrifuged to collect the cleaved peptides in liquid phase. The beads were washed with 2 × 0.5 ml methanol-$H_2O$ (7-3, vol/vol) and the peptides solutions were combined, dried in vacuum centrifuge and stored at -80 ˚C prior to analysis.

## 3.5. Cell viability assay

Cell viability was assessed through the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) colorimetric assay. Briefly, all cells were seeded onto 96-well culture plates at a density of $8x10^3$ cells/well. The probes were added into each well with different gradient concentrations and the cells incubated for 24 hours. At the end of experiments, 20 µl of MTT solution (5 mg/ml in 10 mM PBS Sigma, St. Louis, MO) was added in each well and the cells incubated for 4 hours at 37 ˚C. The culture medium was replaced with 200 µl of DMSO to dissolve the MTT metabolite and the OD measured at

490 nm using a micro-ELISA reader. Cell viability was calculated as the percentage absorbance relative to that of the control cultures.

## 3.6. Site-specific attachment of KP2 to four protein substrates

Site-specific attachment of **KP2** to four protein substrates was characterized using rapifleX MALDI-TOF/TOF. A mount of 50 µM (25 µl of 0.2 mM stock in water) protein substrates were treated with 0, 50, 250, 1000 µM **KP2** (0, 0.5, 2.5, 10 µl of 10 mM stock in water) respectively, in phosphate buffer at pH 7.5 and 37 ˚C for 1 hour. Mixture was subjected to centrifuge (5000 rpm) by using ultrafiltration centrifuge tube (with 3K or 10K molecular weight cutoff), which was pretreated with water. The protein substrates were washed (3 X 200 µl water) to remove the remaining **KP2** and the mixture concentration was determined by Nano drop for LC-MS analysis.

## 3.7. LC-MS/MS Analysis

After dry in the speed VAC, obtained samples were loaded onto a Thermo analytical column (75 µm i.d. × 25 cm) C18 column with an Easy-nLC 1200 chromatography pump coupled with Orbitrap Exploris$^{TM}$480. For each analysis, we reconstituted peptides in 10 µl 0.1% FA and loaded 8 µl onto the column for running. Peptides in each running were separated on a 110 mins (8-40% ACN) gradient. Parameters are as follows in Full MS/ data dependent -MS2 TopN mode: mass analyzer over m/z range of 350−1500 with a mass resolution of 60000 (at m/z=200) in a data-dependent mode, 1.6 m/z isolation window. 20 most intense ions are selected for MS/MS analysis at a resolution of 15000 using collision mode of HCD. Peptides labeled in vitro were analyzed by Orbitrap Exploris$^{TM}$480 and peptide labeled in live cells were analyzed by Lumos, under the same setting parameters.

## 3.8. Peptide and protein identification

Spectral data was searched against the Uniprot/Swiss-prot protein database using PD 2.4 and filtered to 1% FDR (false discovery rate) at the protein level. Default parameters used following exceptions: a minimum of 1 unique peptide was required for quantitation; peptide matching between runs was included and peptides containing oxidation (O), N-terminal acetylation (protein N-term), and carbamidomethyl (C) as variable modifications. For reactivity profiling experiments, lysine residues were searched with up to one differential modification for **KP2** tag (+258.281 as Kpyri-pc for PC biotin-$N_3$, +302.354 as Kpyri-acid for Dadps biotin-$N_3$ or +159.04 Da) as Kpyri for **KP2** direct modification). Only tryptic peptides with a maximum of two missed cleavages were allowed; fragment mass tolerance was set to 0.02 Da for MS/MS fragment ions; mini and max peptide lengths were 6 and 144. GO enrichment was performed for cellular components of labeled

proteins, and Uniport accession numbers of identified reactive-histidine-containing proteins were subjected to GO term analysis after remove Keratin contaminates.

## 3.9. Differential labeling analysis of residues labeled by probe KP2

For analysis of the residues labeled by probe **KP2**, peptide and protein identification were conducted as detailed above with modification for biotin tag (+258.281 Da, named as Kpyri-pc) allowed on lysine, cysteine, arginine, aspartate, glutamate, histidine, serine, threonine, tyrosine, asparagine, glutamine and tryptophan.

## 3.10. $R_{100:10}$ value calculation and processing

The ratios of MS1 for each unique peptide were quantified with label-free. For reactivity measurements by ABPP, the $R_{100:10}$ value was calculated from the ratio of MS1 peak areas comparing the 100 mM KP1 alkyne sample with the 10 mM **KP2** labeling sample. Ratios for unique peptides are calculated for each experiment; overlapping peptides with the same modified lysine are grouped together and the median ratio is reported as the final ratio ($R_{100:10}$).

## 3.11. Cross-data processing for reactivity profiling

For reactivity profiling, the median of biological replicates of the same condition and cell-line was calculated. For peptides containing several possible modified lysines, the lysine with the highest number of quantification events was used for analysis and the remaining, redundant peptides were reported as alternative modification sites. Peptides were required to be detected in at least one 100 mM vs 10 mM and one 10 mM vs 10 mM data set with the latter R value being smaller than 2. All ratios derived from soluble reactivity experiments were averaged. If the final reactivity value was >10, it was set to 10.

## 3.12. Sequence motifs

For all lysine identified in the proteome profiling experiments, the flanking sequence (± 7 amino acids) was determined with an R package (ggseqlogo[11]), parsing the UniProtKB entries for all proteins identified.

## 3.13. Imaging analysis KP2 labeling in *situ* cells

Approximately 800,000 cells (MCF-7/RAW264.7) were seeded in a 24-well glass chamber with cell culture medium and incubated at 37 ˚C with 5% $CO_2$ for 24-48 hours. The cells were washed with PBS and first pre-treated with NHS-AC (2.5 μM), KP-B (2.5 μM) or DMSO in PBS. The medium was changed prior to addition of 2.5 μM probes in PBS. After incubated for 1 hour, the cells were washed 3 times with PBS. A freshly pre-mixed click chemistry reaction cocktail was then added (10 μM TAMRA-PEG4-$N_3$ in DMSO, 10 μM TBTA, 0.1 mM TCEP and 0.1 mM $CuSO_4$) to the mixture above. The reaction was further incubated for another 1

hour at room temperature. They were then washed with PBS for 3 times and 0.1% Tween in PBS for once. And then fixed with 300 µl of 4% formaldehyde in PBS for 15 mins and permeabilized with 300 µl of 0.1% Triton X-100 in PBS for 15 min. Finally, the cells stained with 4', 6-diamidino-2-phenylindole (DAPI) (D1306, Invitrogen) for image. Image processing was done with LSM 980 laser scanning confocal microscope (Zeiss) confocal microscope system and carried out using ZEN 3.2 blue edition (ZEISS).

## 3.14. Immunofluorescence staining

Approximately 800,000 cells (MCF-7 cells) were seeded on coverslips before experiment. After 2.5 µM **KP2** combined with MitoTracker Green FM (M7514 0.1 µM, Invitrogen) treatment (1 hour) or not, cells were washed twice with PBS. A freshly pre-mixed click chemistry reaction cocktail was then added (10 µM TAMRA-PEG4-$N_3$ in DMSO, 10 µM TBTA, 0.1 mM TCEP and 0.1 mM $CuSO_4$) to the mixture above. The reaction was further incubated for another 1 hour at room temperature. They were then washed with PBS for 3 times and 0.1% Tween in PBS for once. Then cells were fixed in 4% formaldehyde for 20 mins at room temperature, washed, and permeabilized in 0.5% PBS-T (Triton X-100) on ice for 10 mins. Next, cells were washed, and blocked in 3% PBS-B (BSA) for 1 hour at 37 ˚C. After incubation with two mitochondrial marker protein antibodies of Cytochrome c oxidase subunit 4 isoform 1 (COX4) (PA5-29992, Invitrogen) and Heat Shock Protein 60 (HSP60) (MA3-013, Invitrogen) at a dilution of 1:200 overnight, cells were washed and probed with anti-mouse/rabbit conjugated secondary antibodies (Jackson ImmunoResearch) (1:1000) in 3% PBS-B (BSA) for 1 hour at room temperature. Cells on the coverslips were then washed, covered with mounting medium, and observed under LSM 980 laser scanning confocal microscope (Zeiss) confocal microscope system.

## 4. References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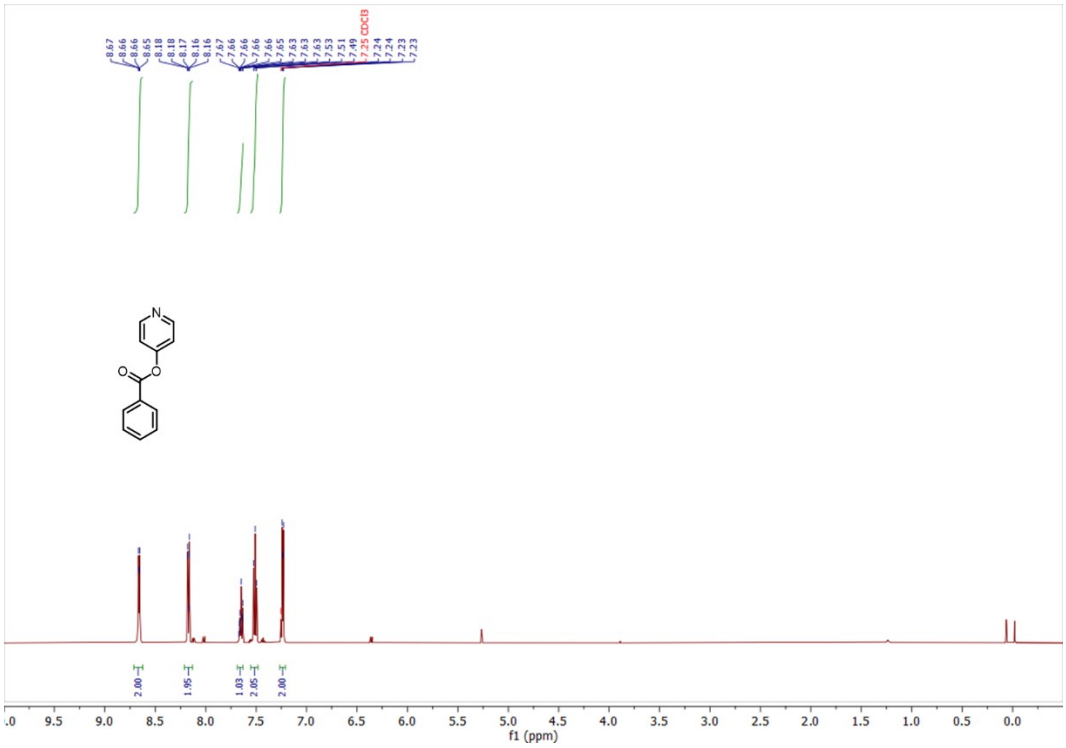
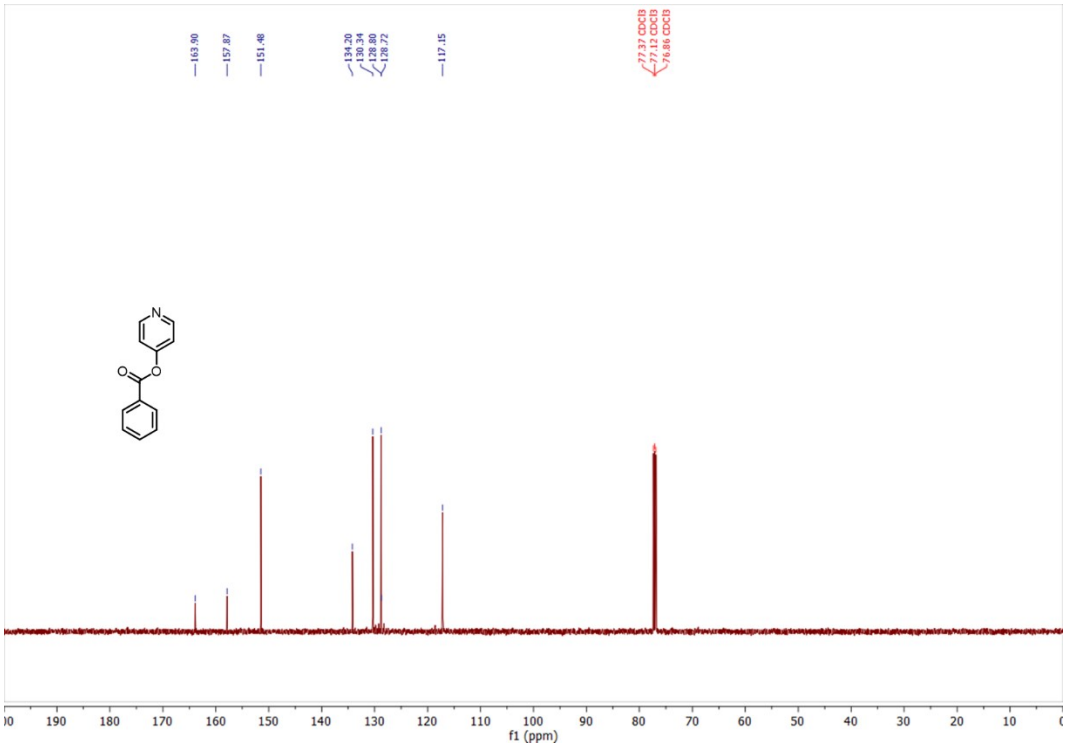
# 5. NMR Spectra




$^{1}$H NMR spectrum of **1a**

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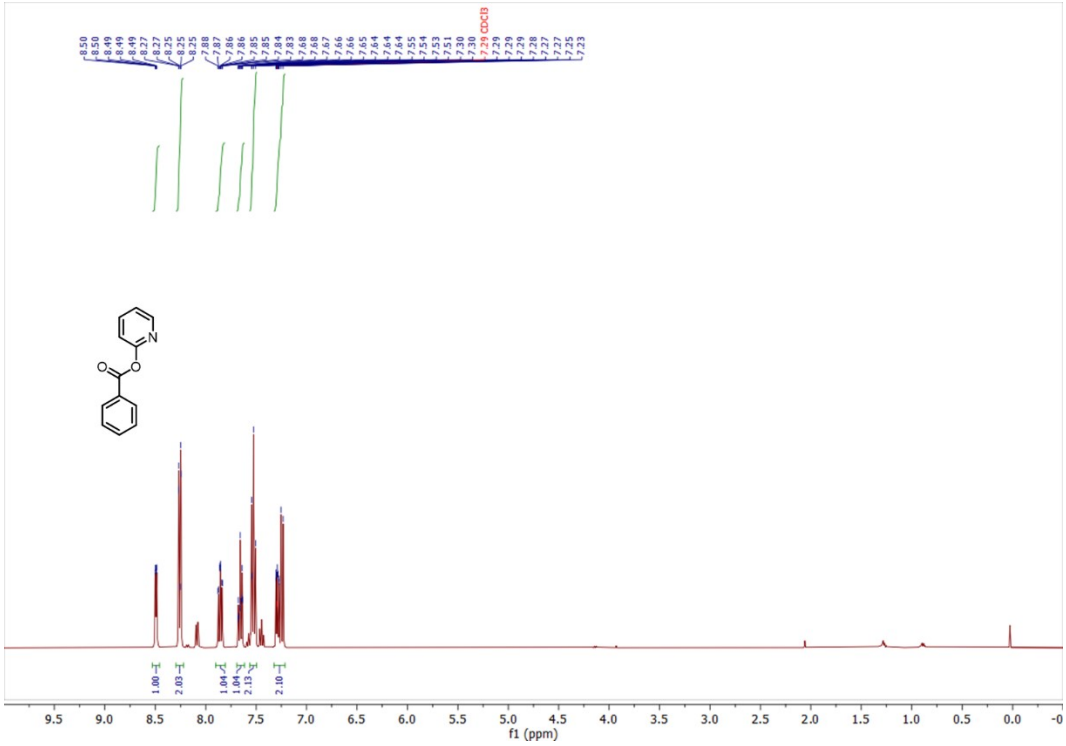
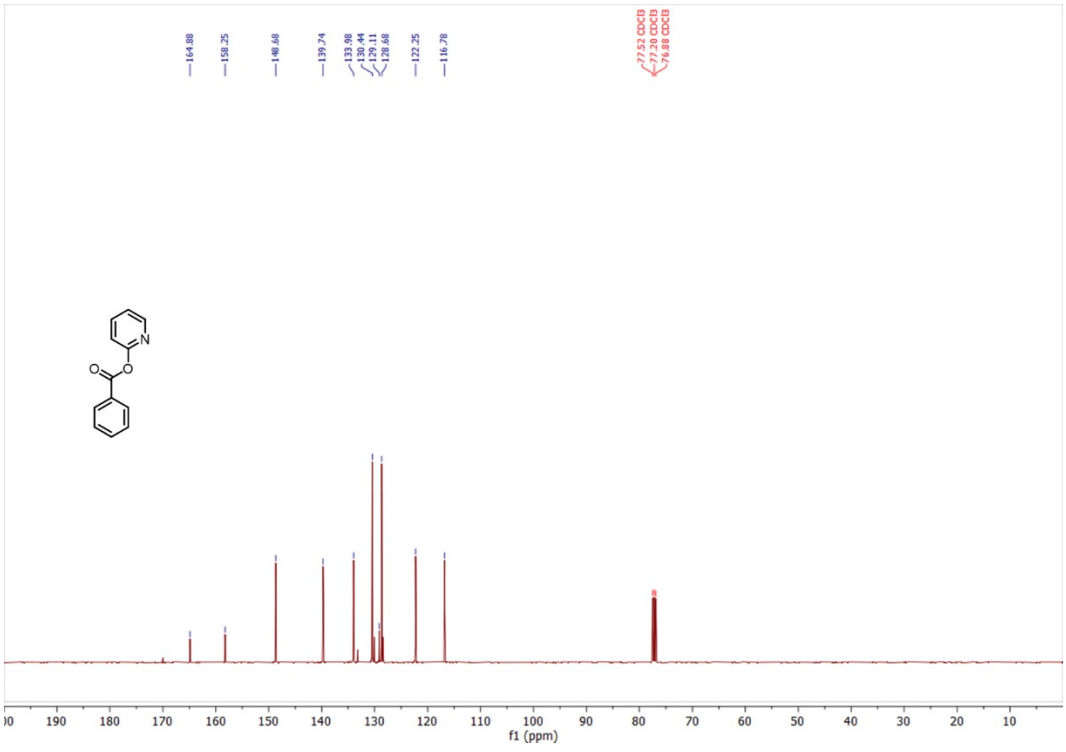



$^{1}$H NMR spectrum of **1b**

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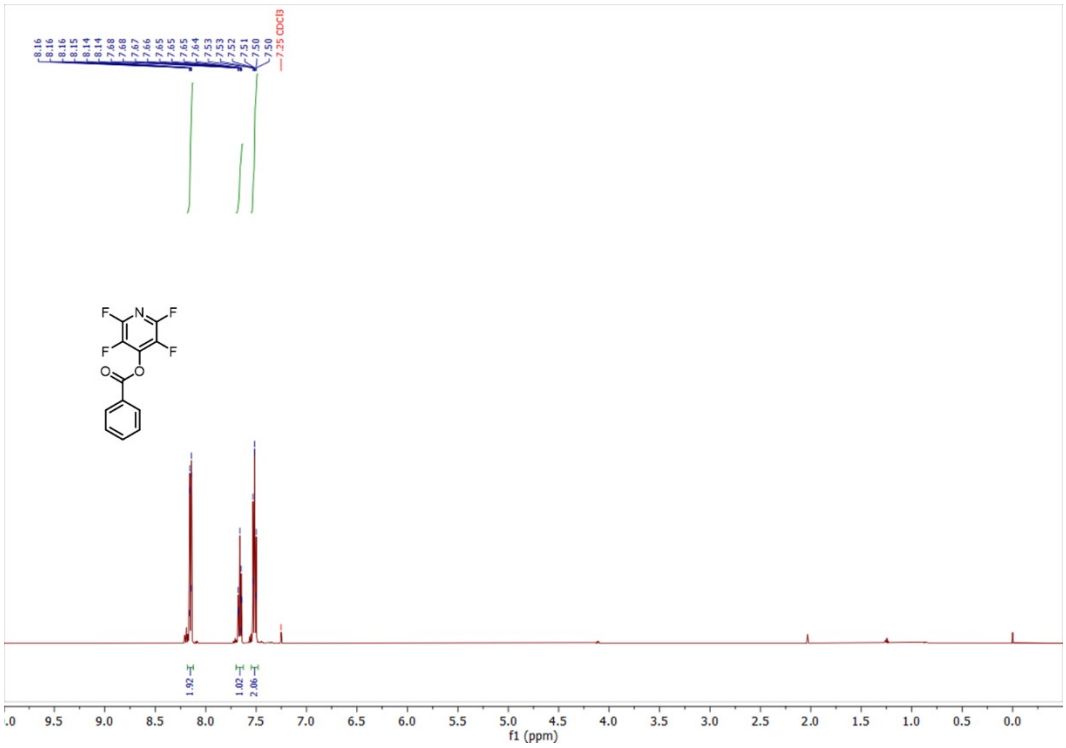


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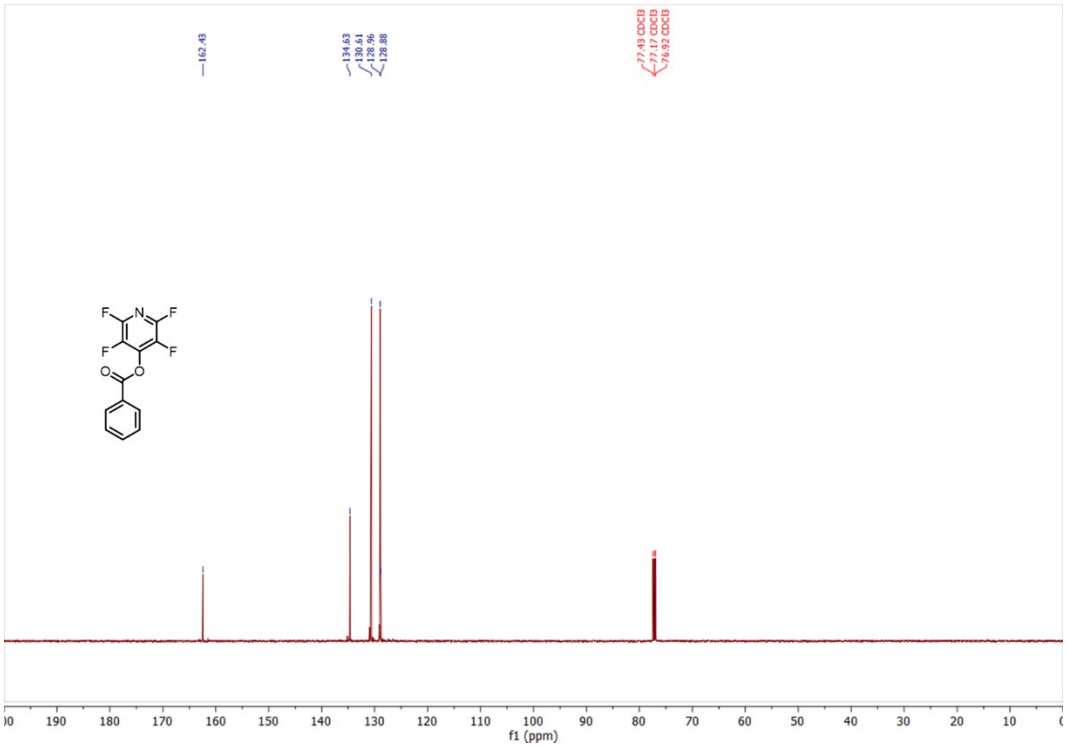


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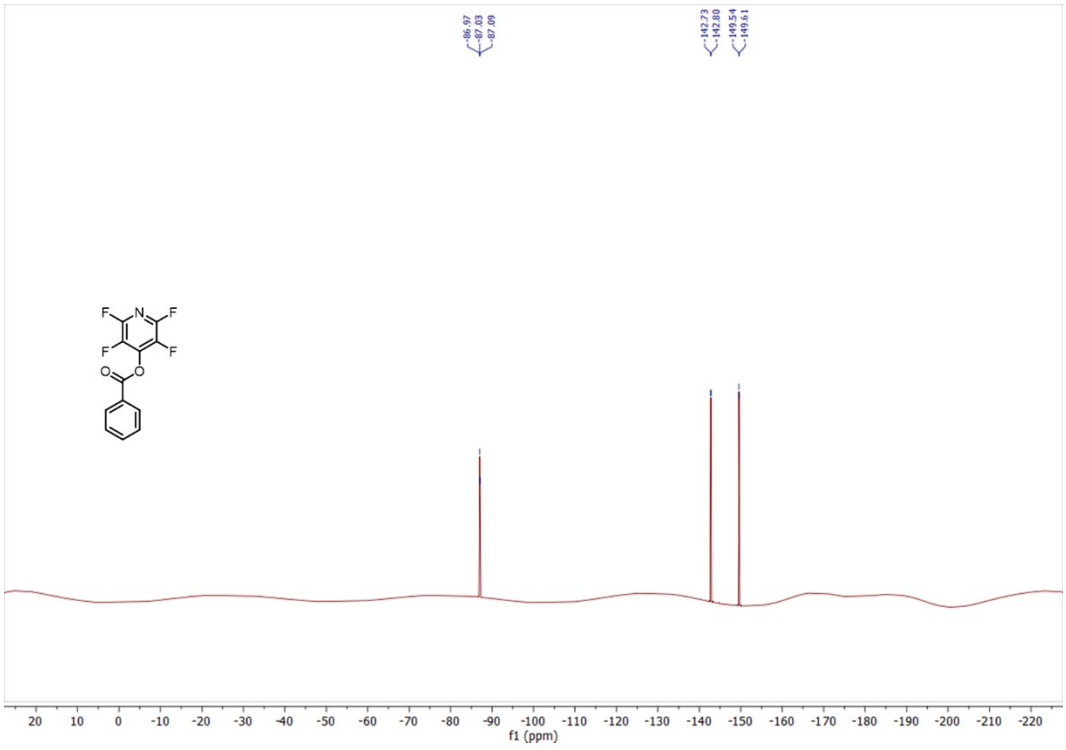


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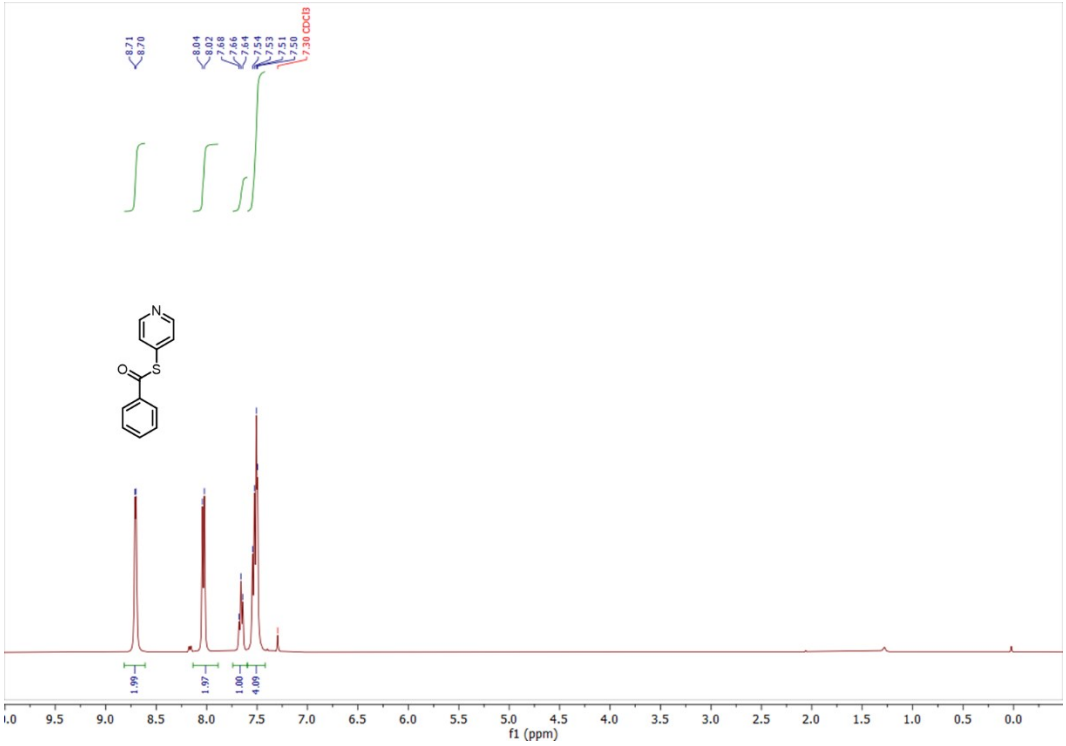
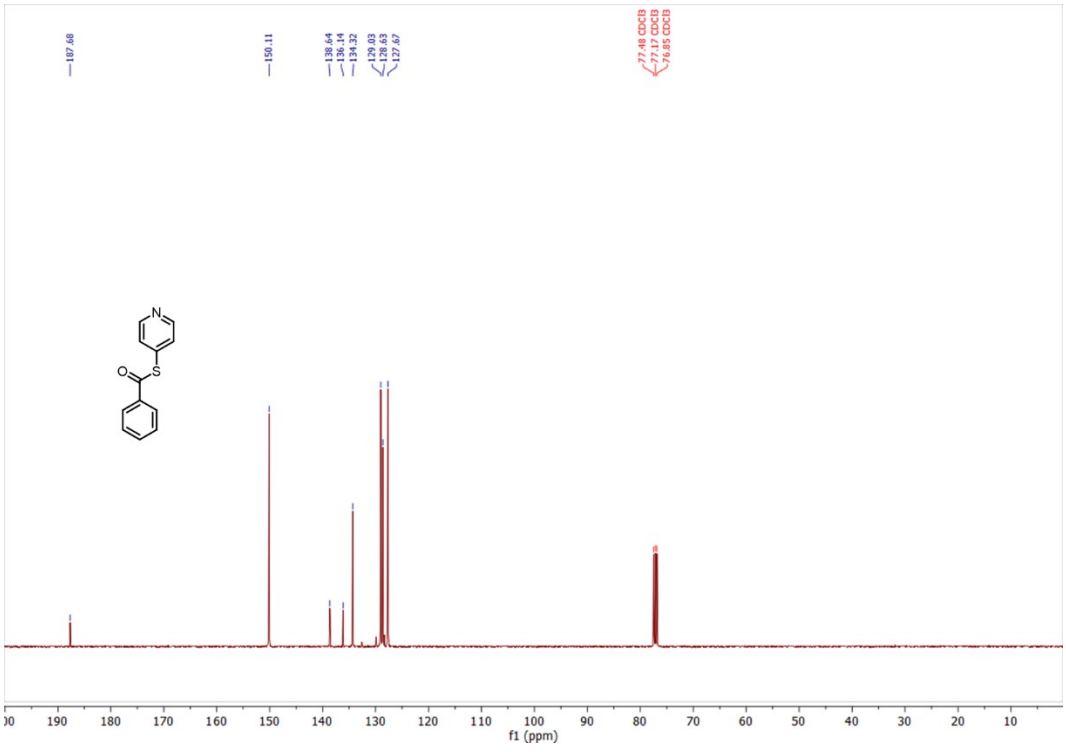



$^{1}$H NMR spectrum of **1g**

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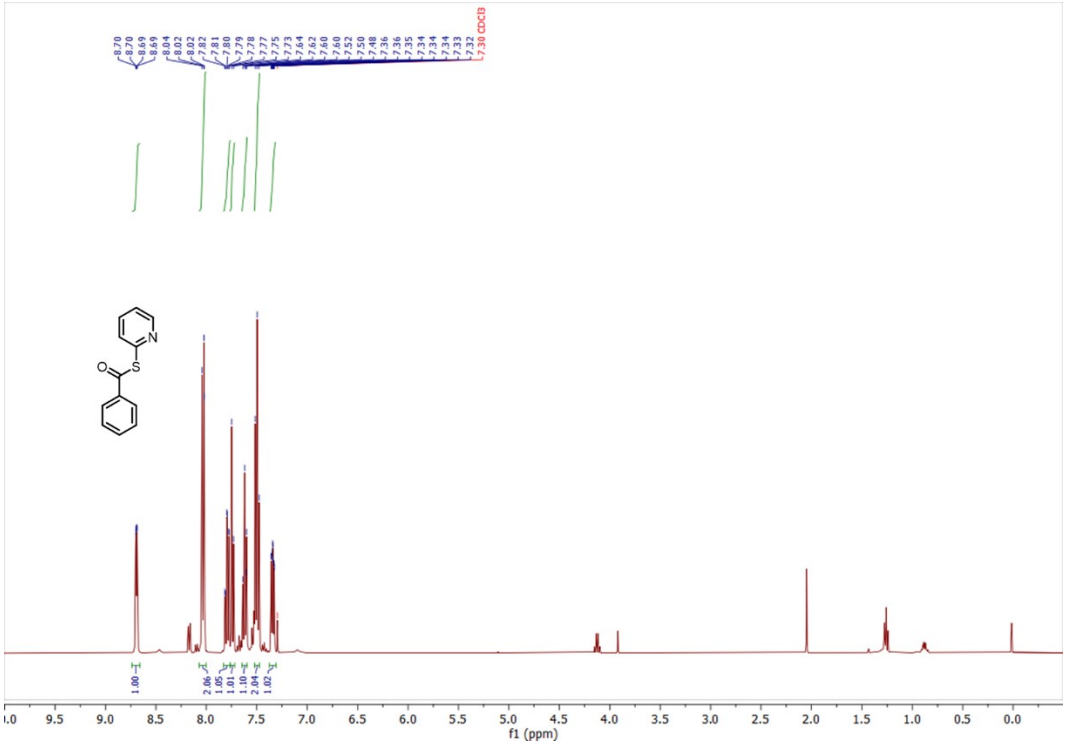


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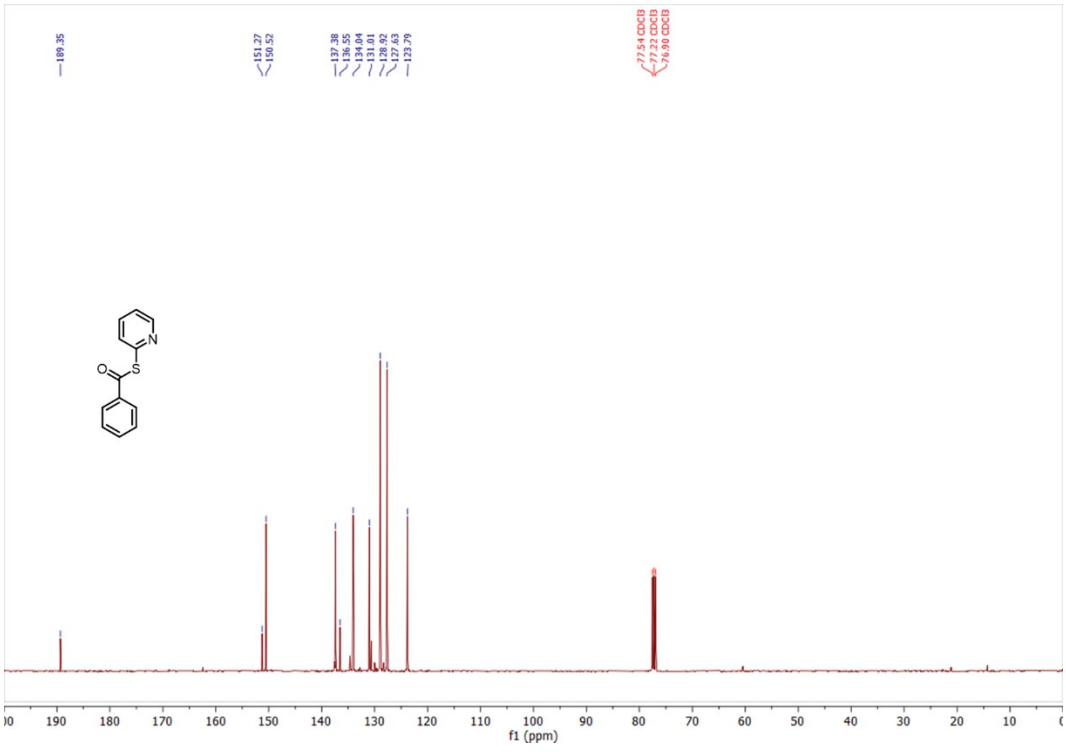


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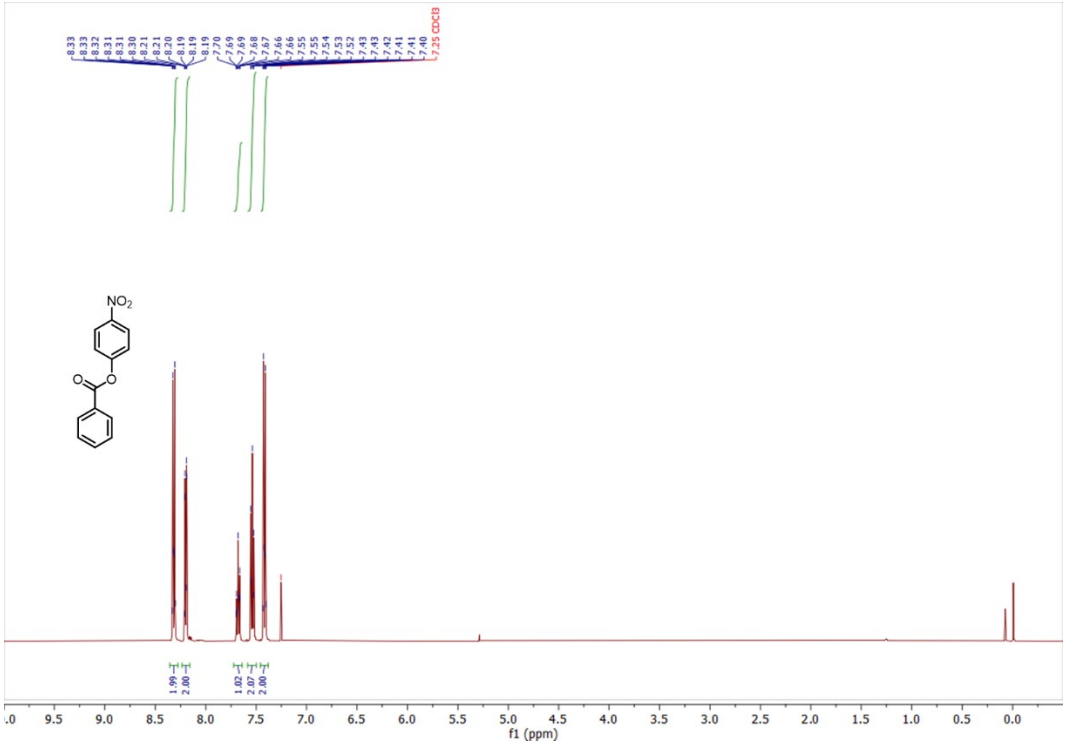


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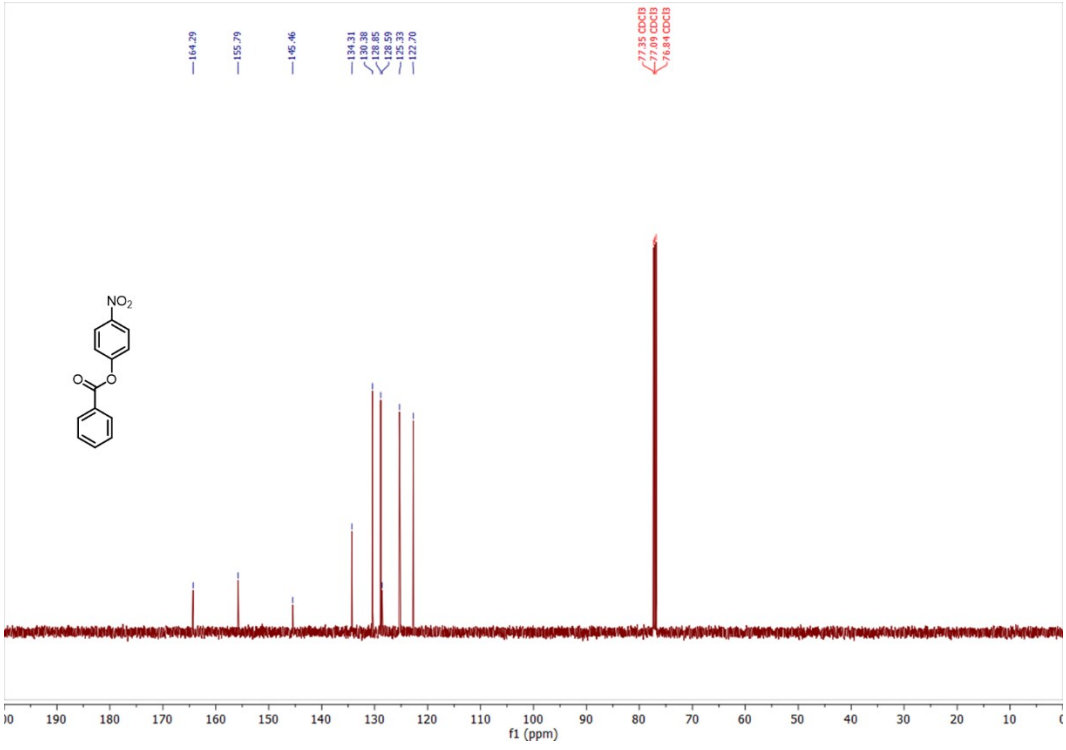


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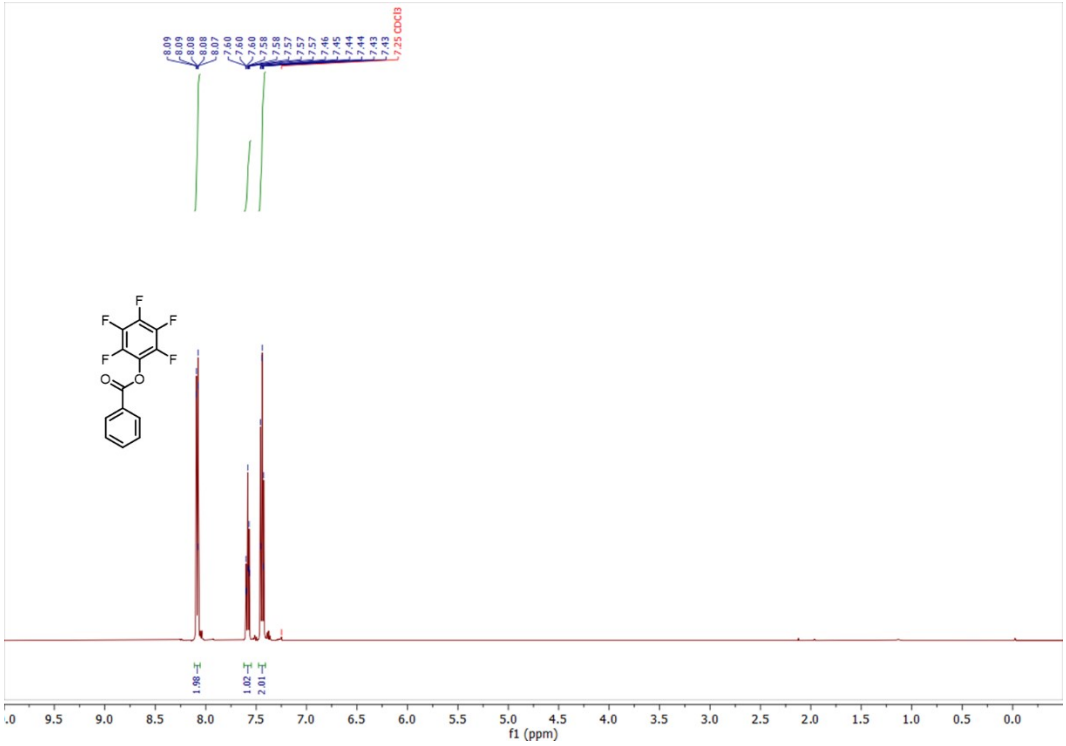


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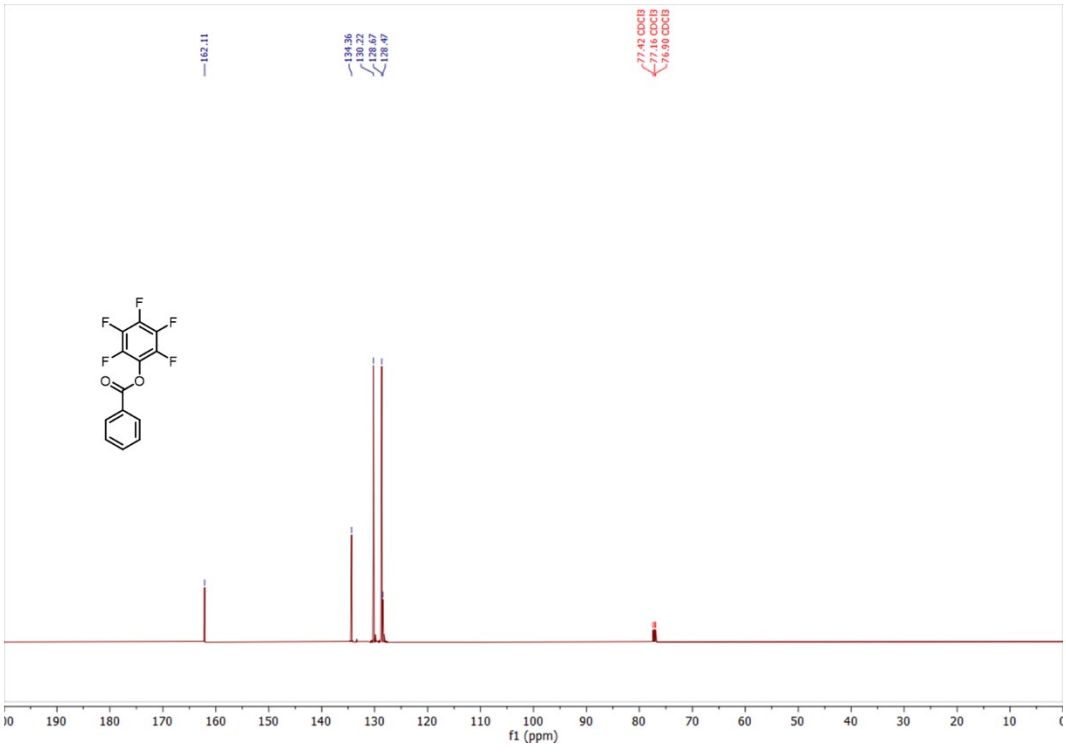
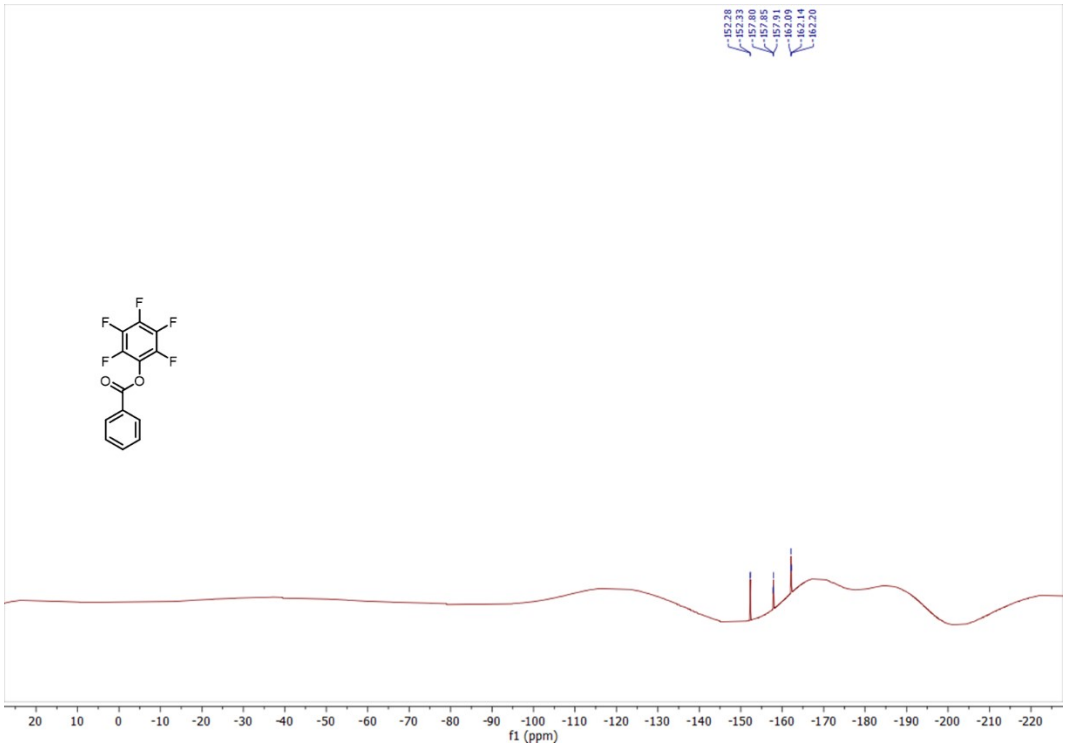



$^{13}$C NMR spectrum of **1l**

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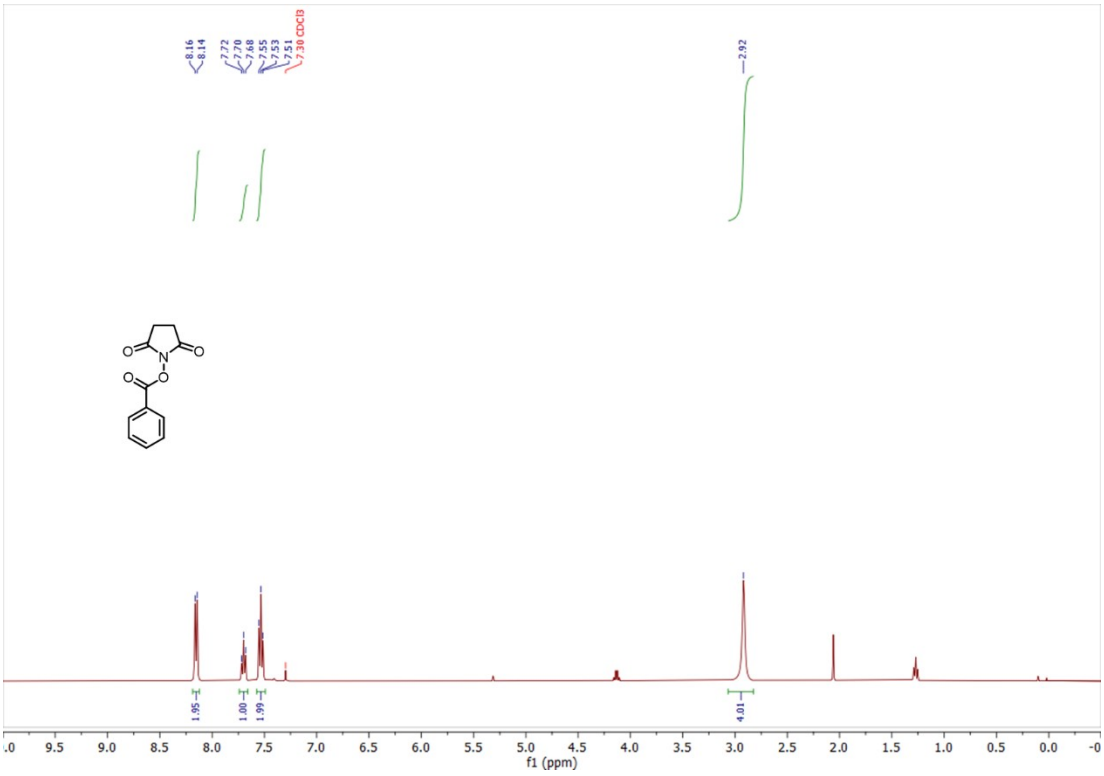


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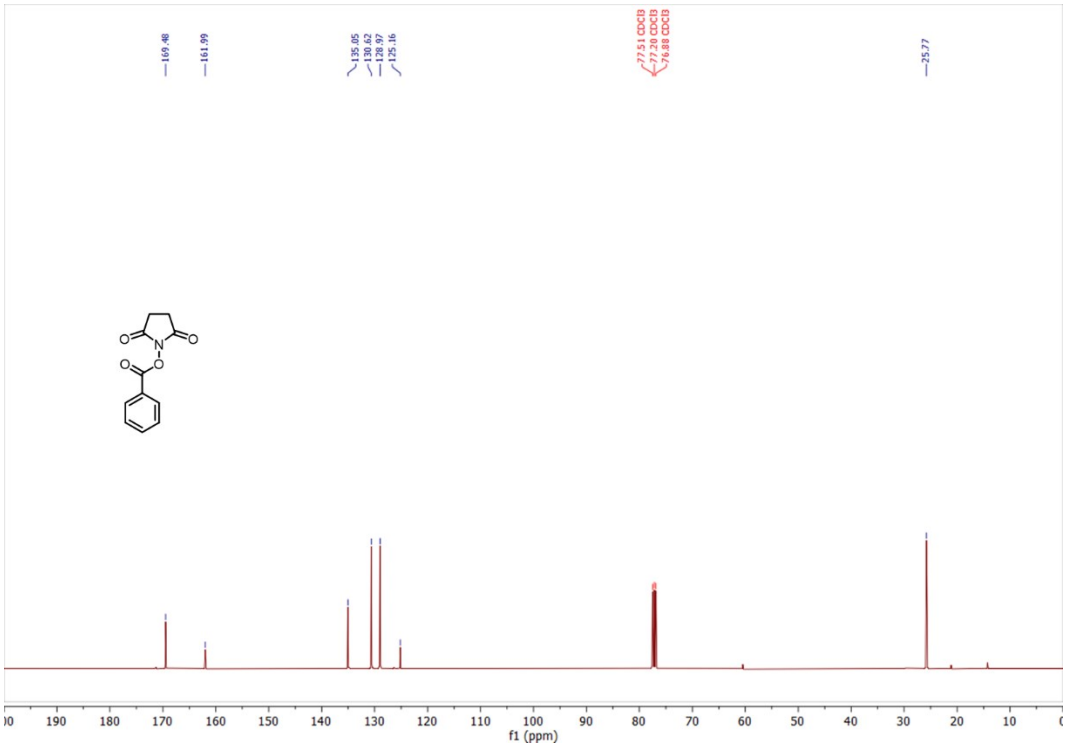
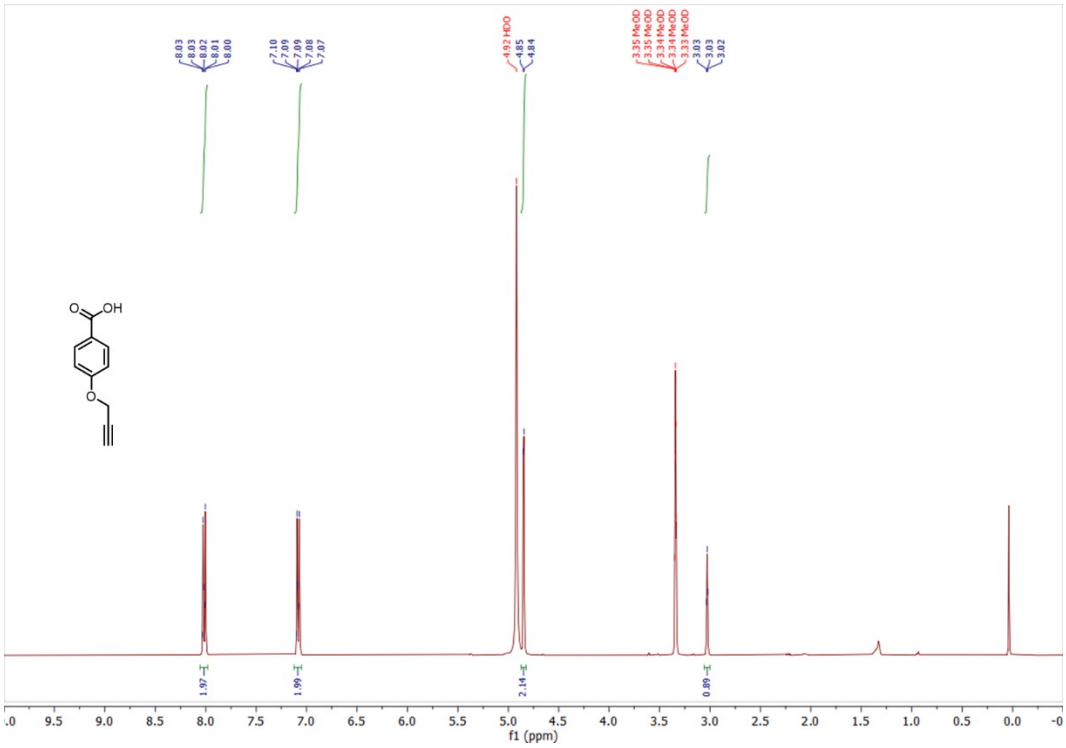
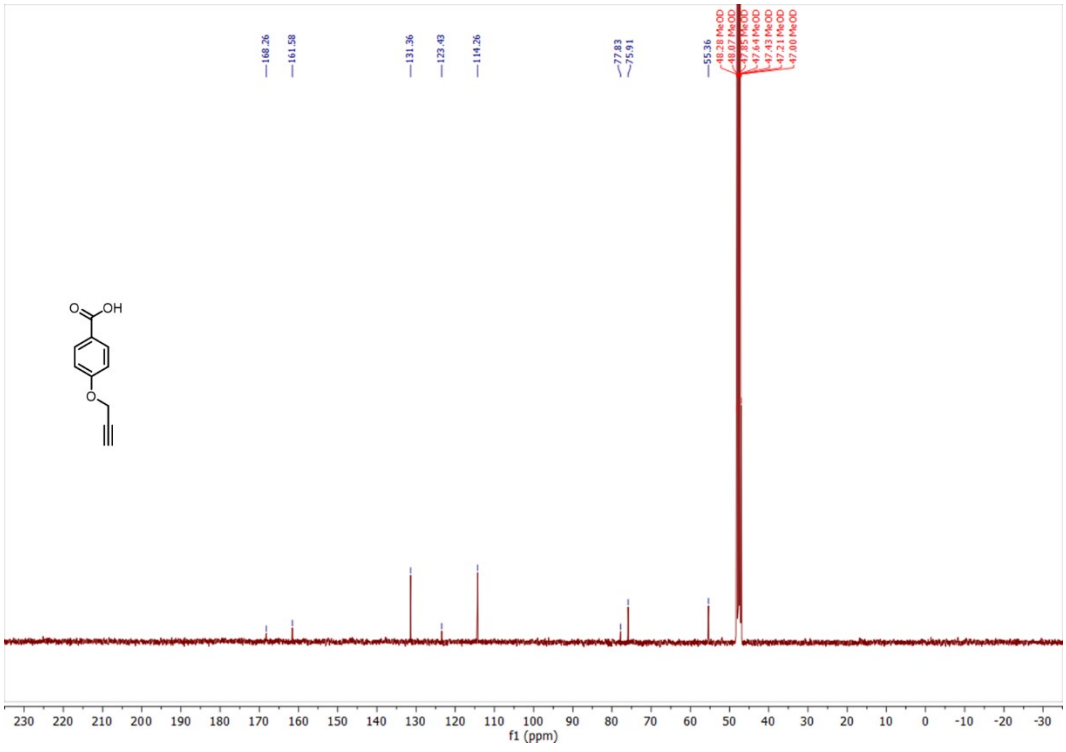


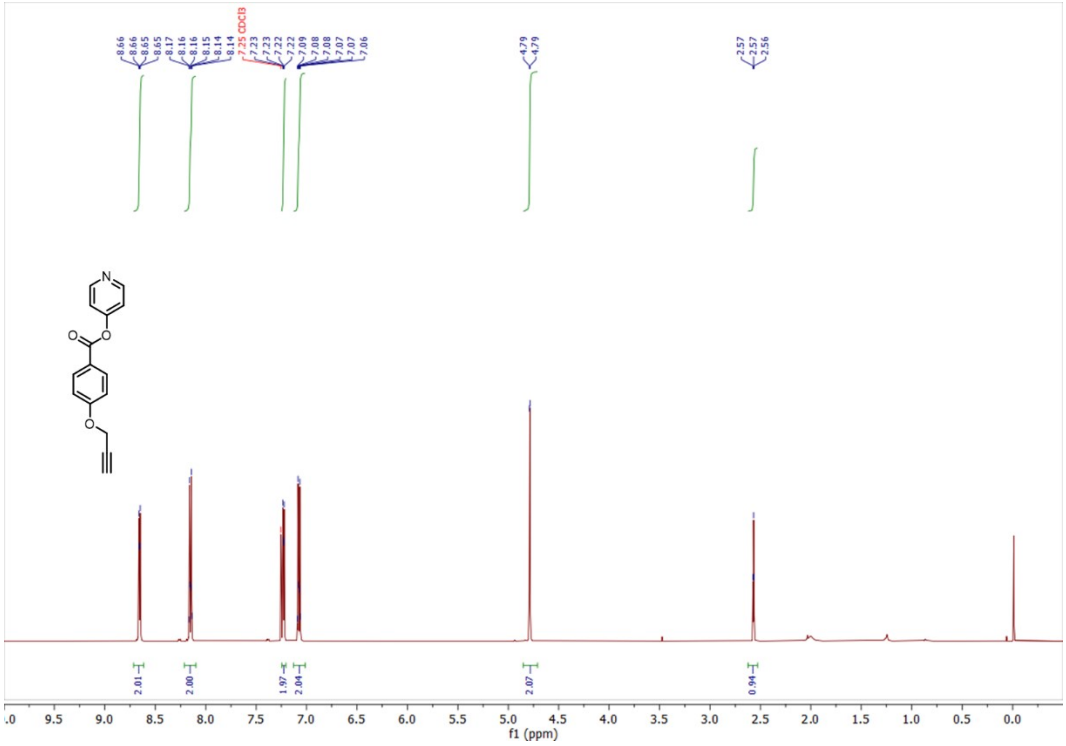

$^{13}$C NMR spectrum of **1d**



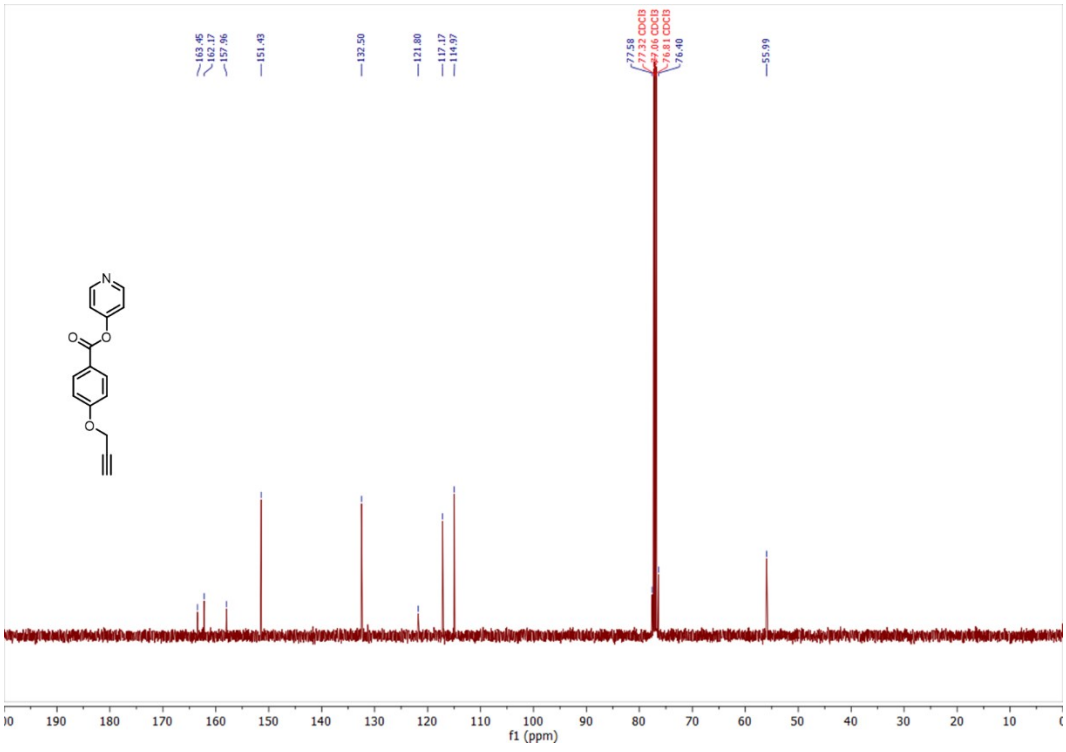

$^1$H NMR spectrum of **S4**



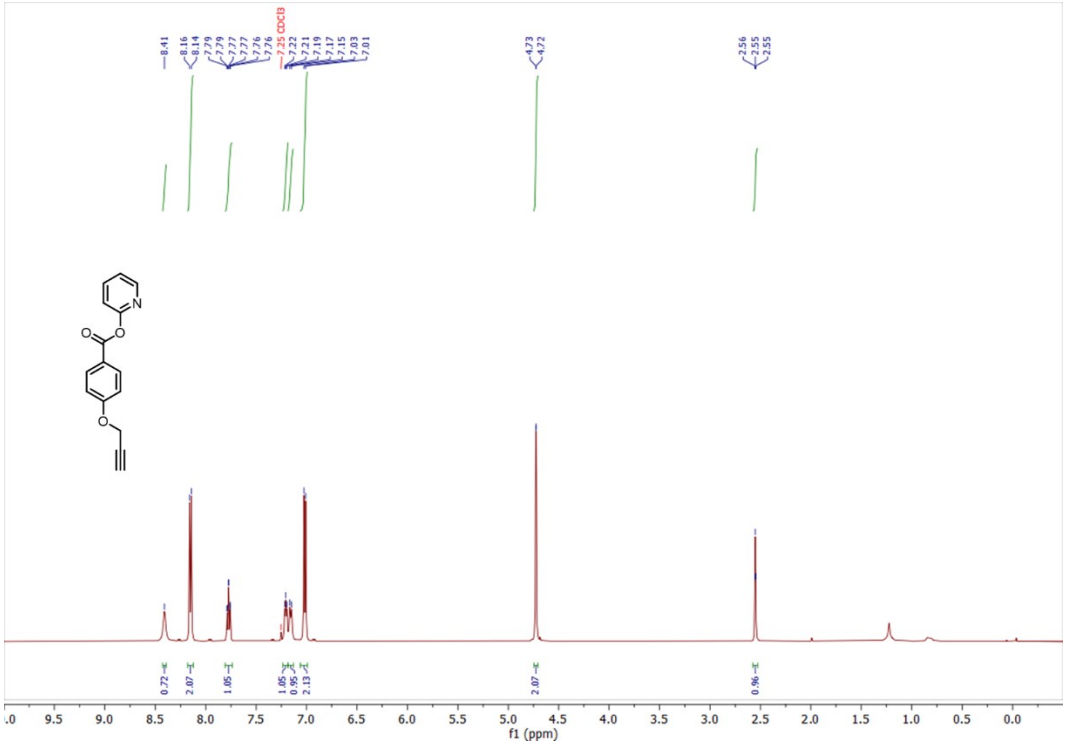

$^{13}$C NMR spectrum of **S4**



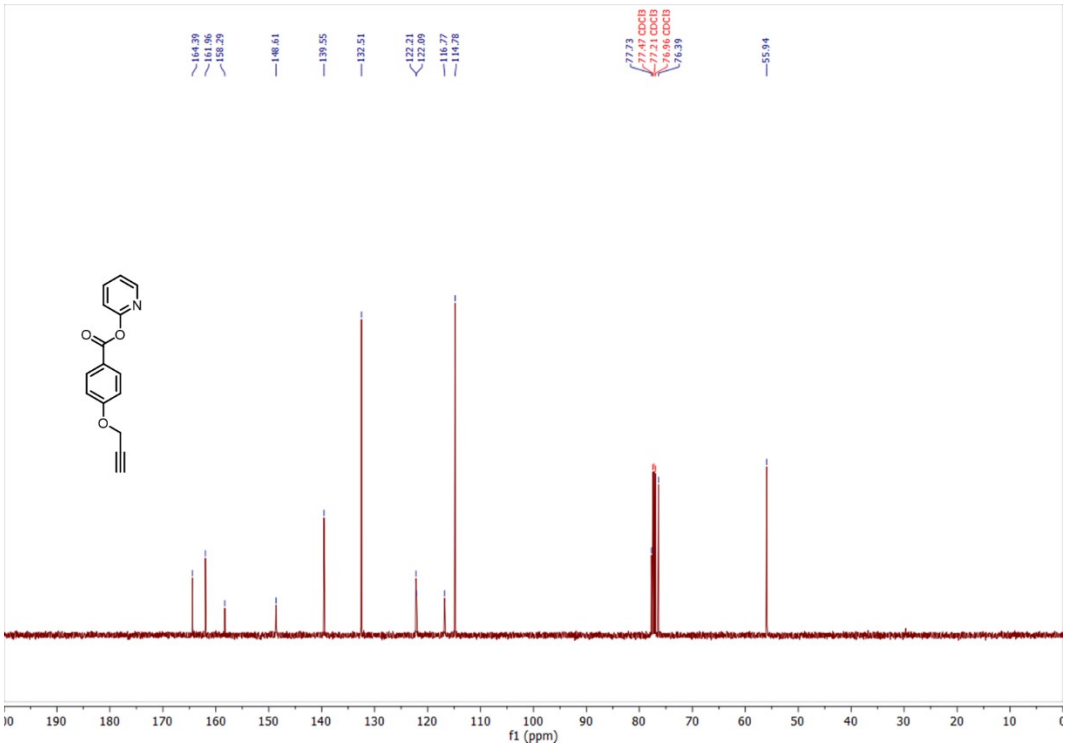

$^{1}$H NMR spectrum of **KP3**



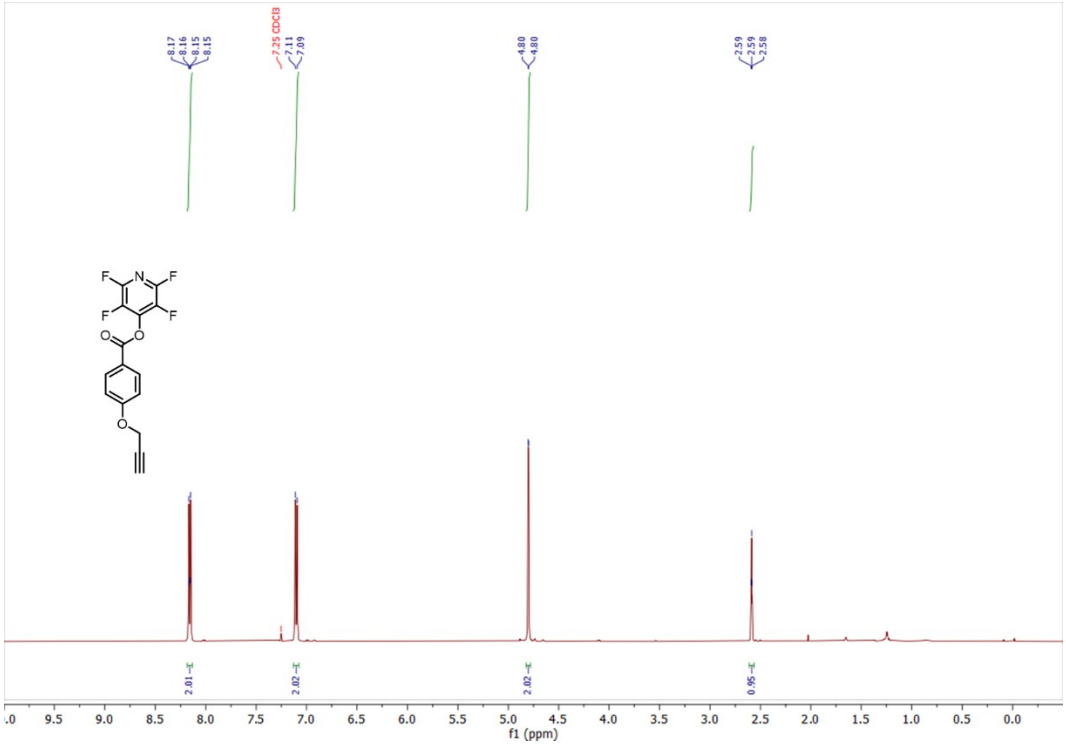

$^{13}$C NMR spectrum of **KP3**



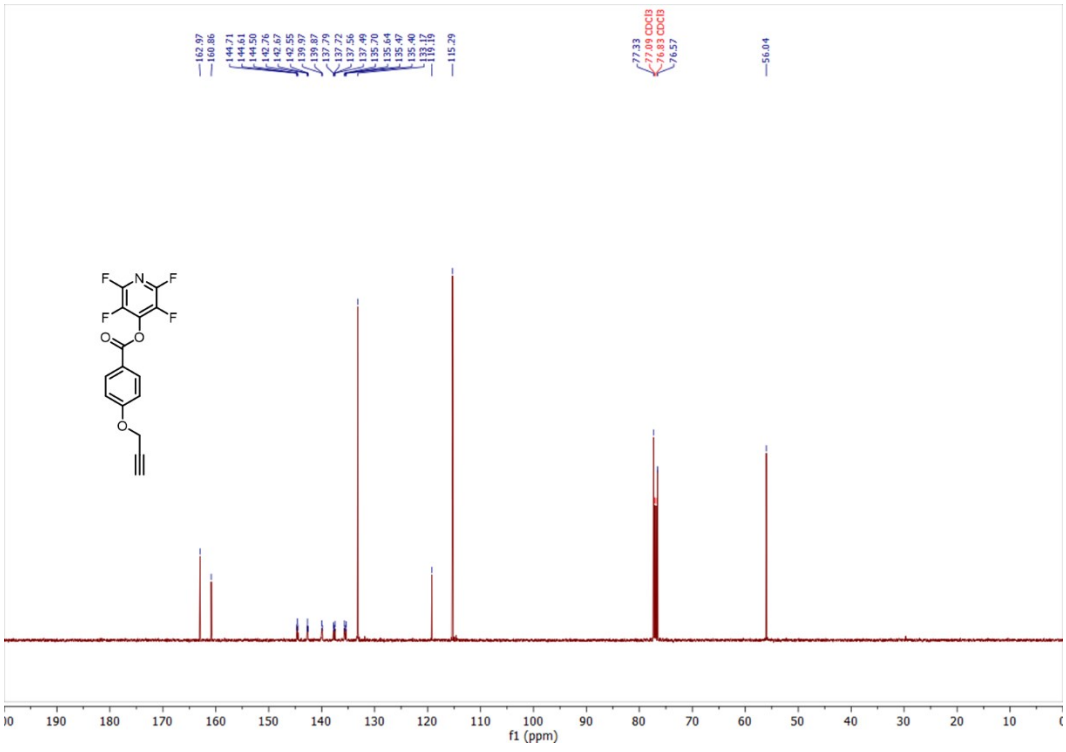

$^{1}$H NMR spectrum of **KP4**



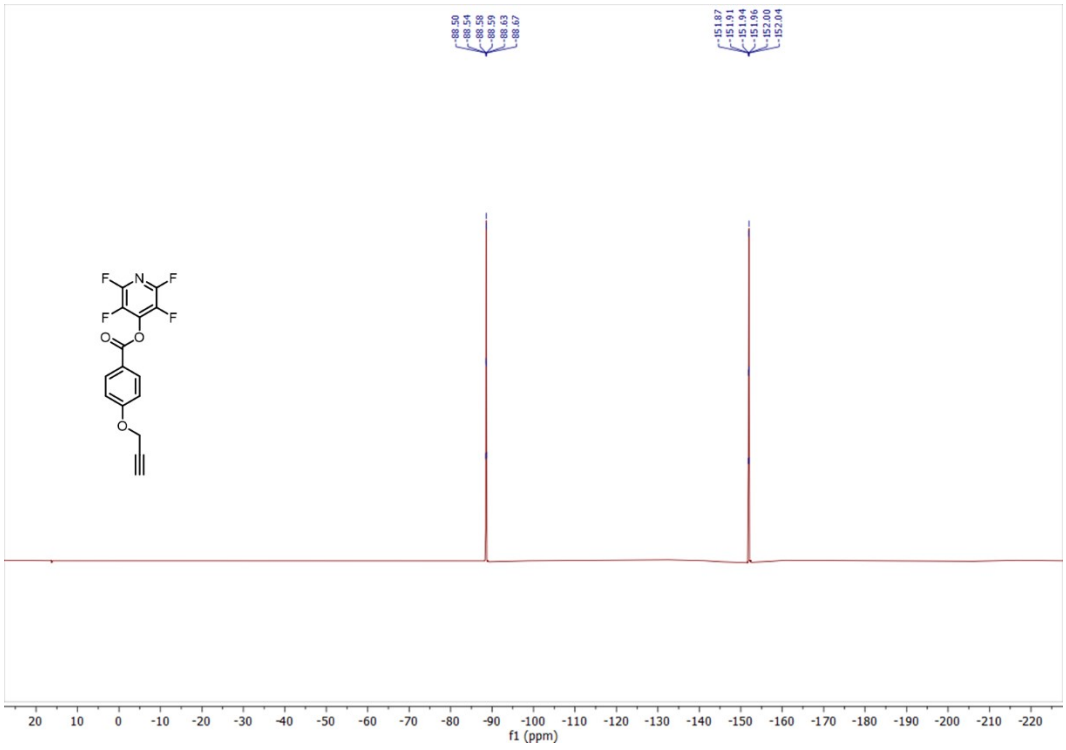

$^{13}$C NMR spectrum of **KP4**



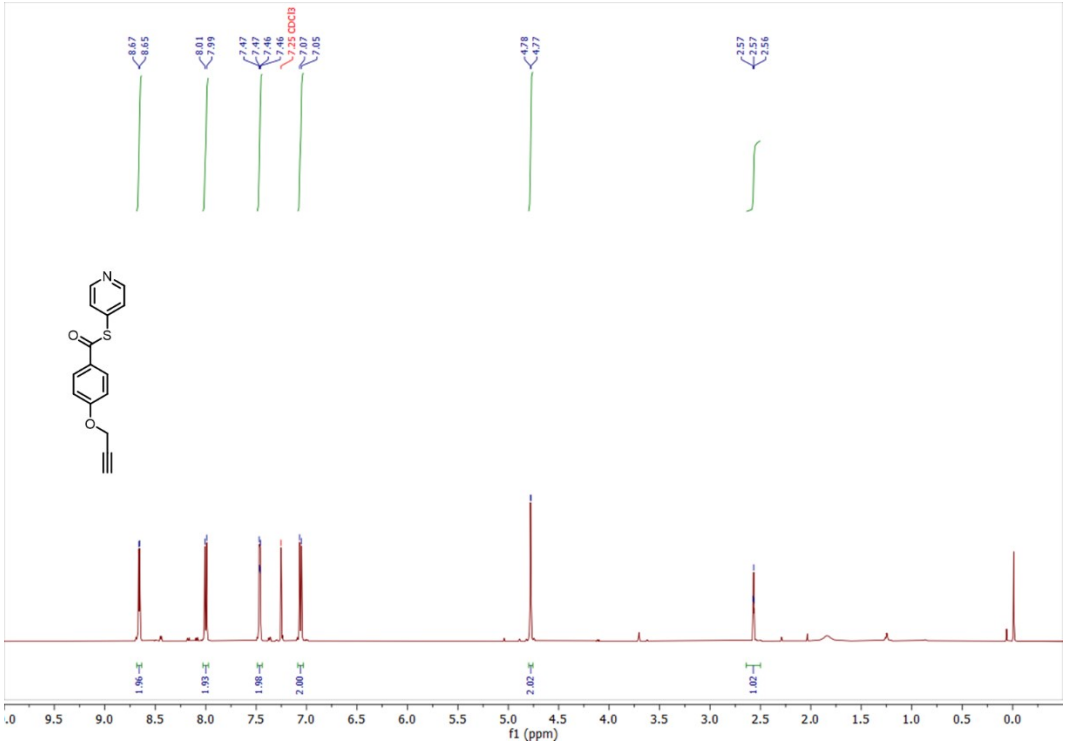

$^{1}$H NMR spectrum of **KP5**



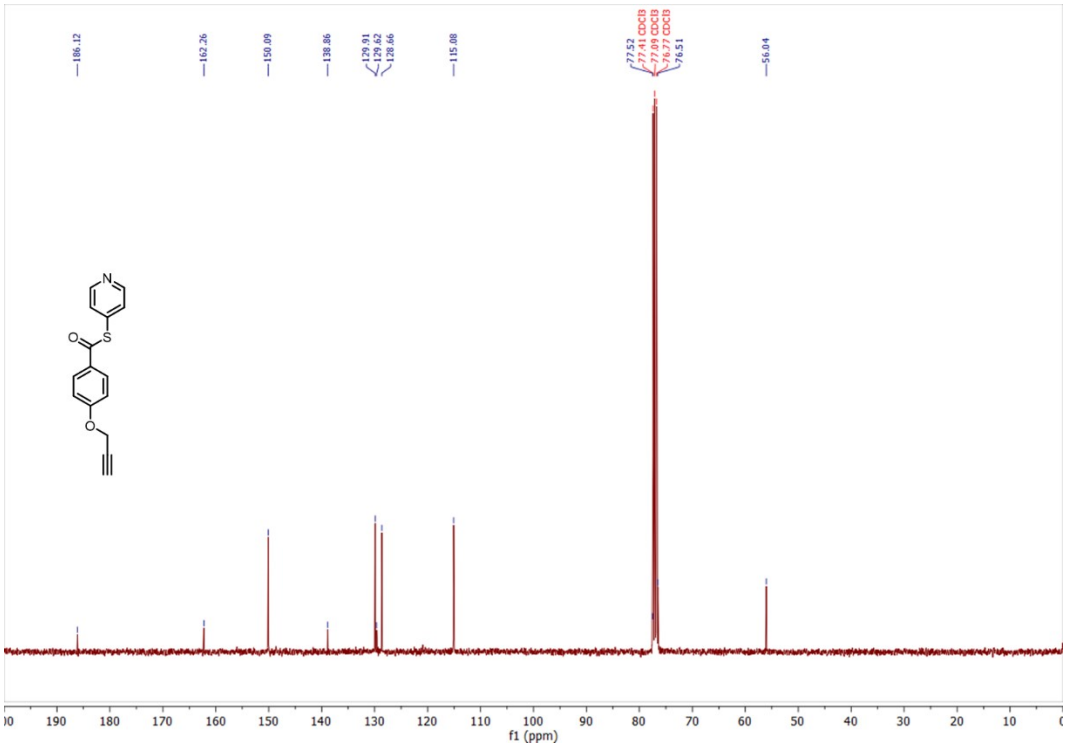

$^{13}$C NMR spectrum of **KP5**



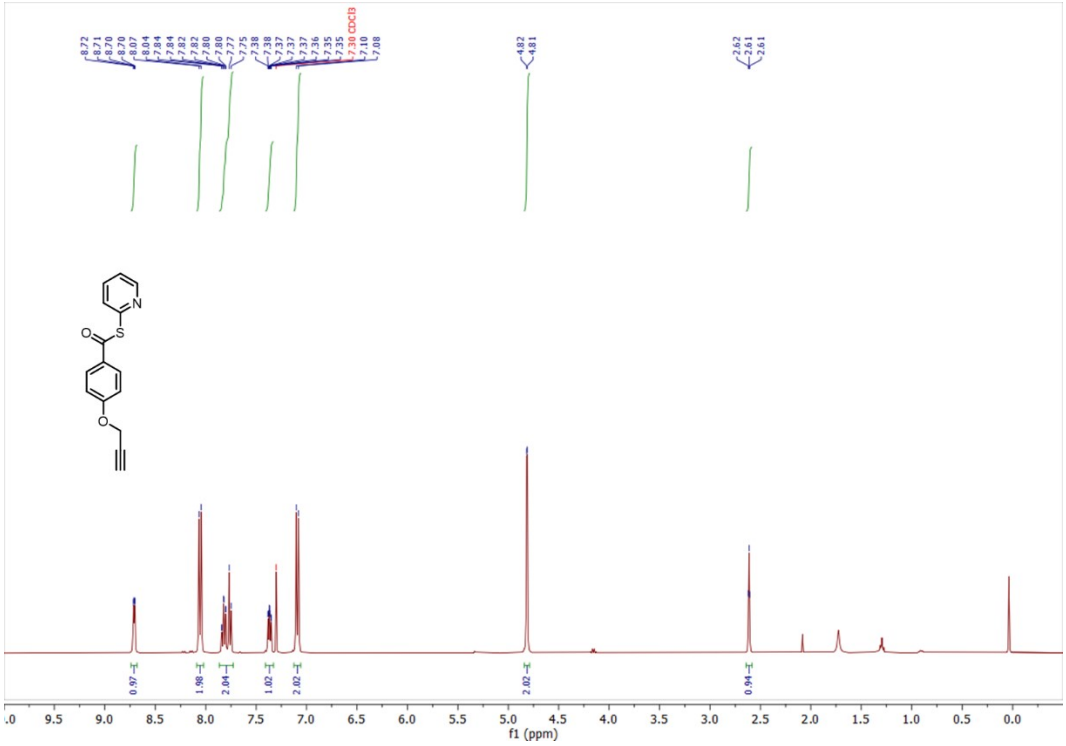

$^{19}$F NMR spectrum of **KP5**



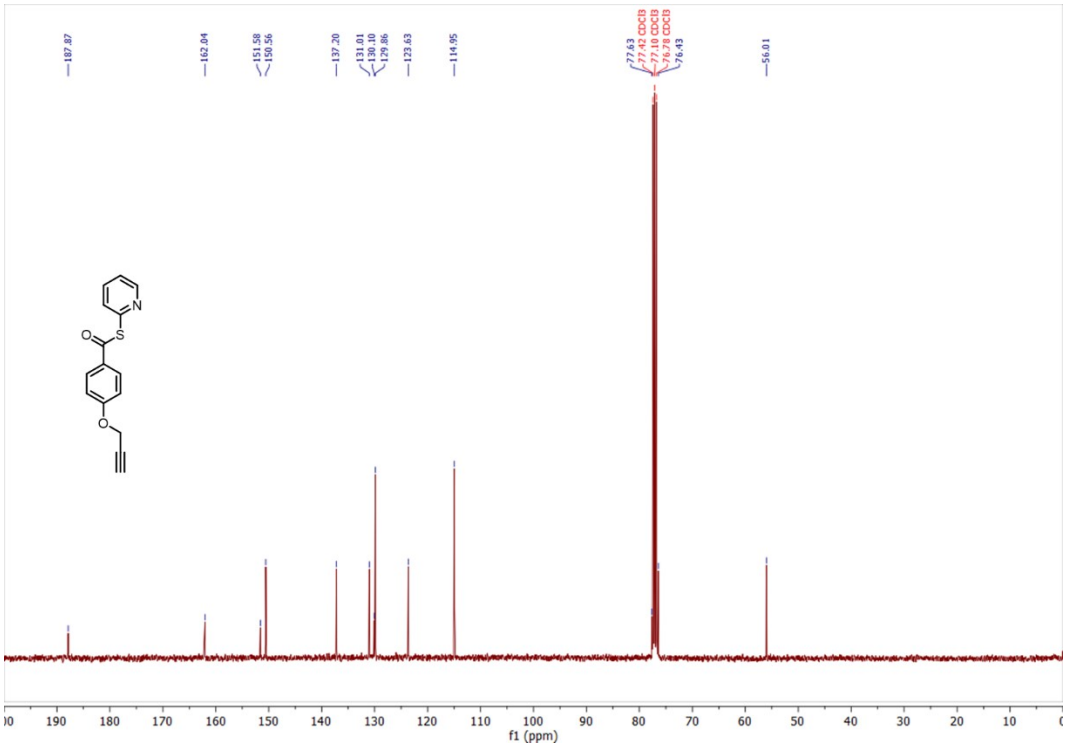

$^{1}$H NMR spectrum of **KP8**



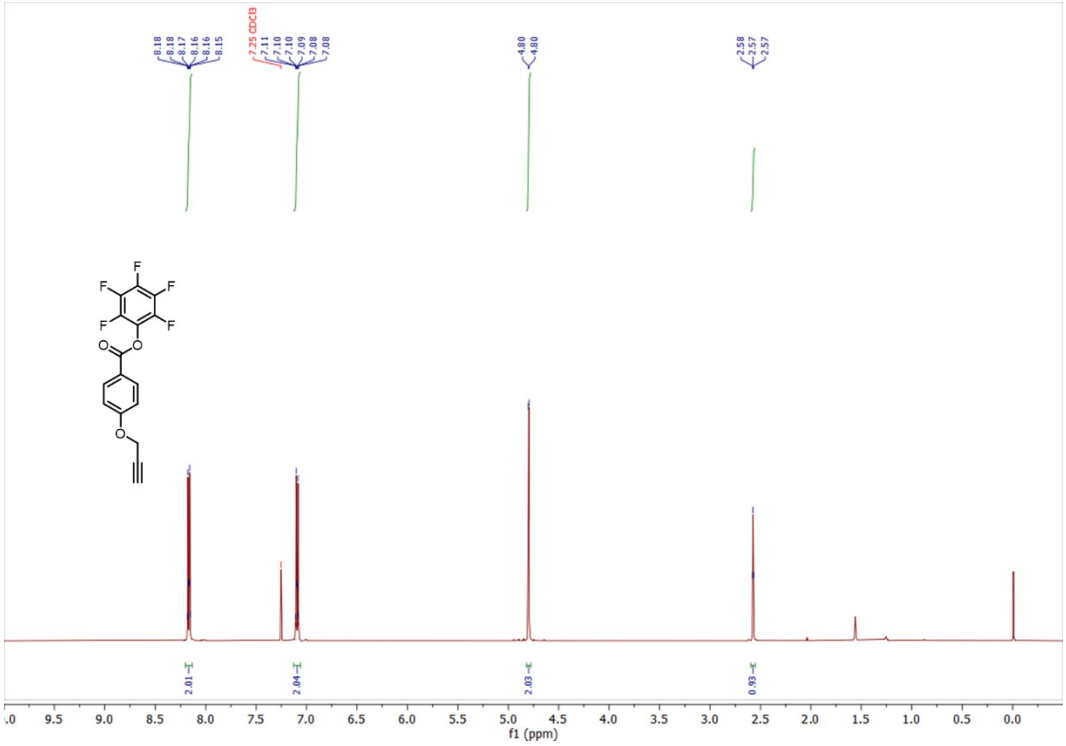

$^{13}$C NMR spectrum of **KP8**



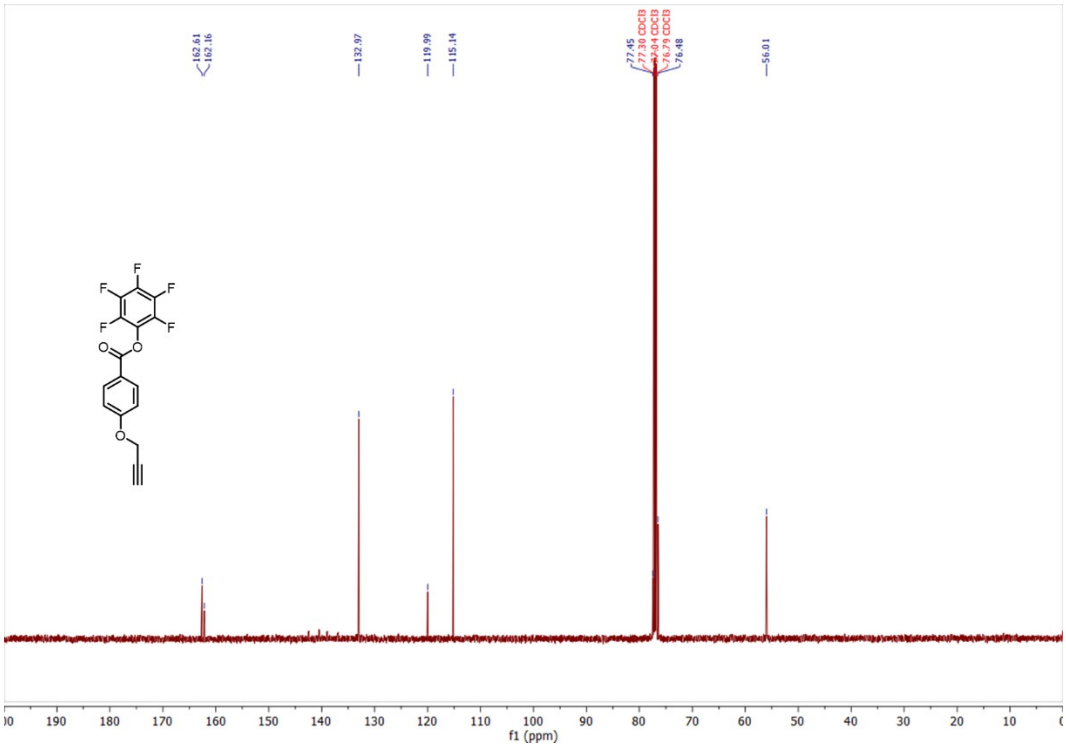

$^{1}$H NMR spectrum of **KP9**



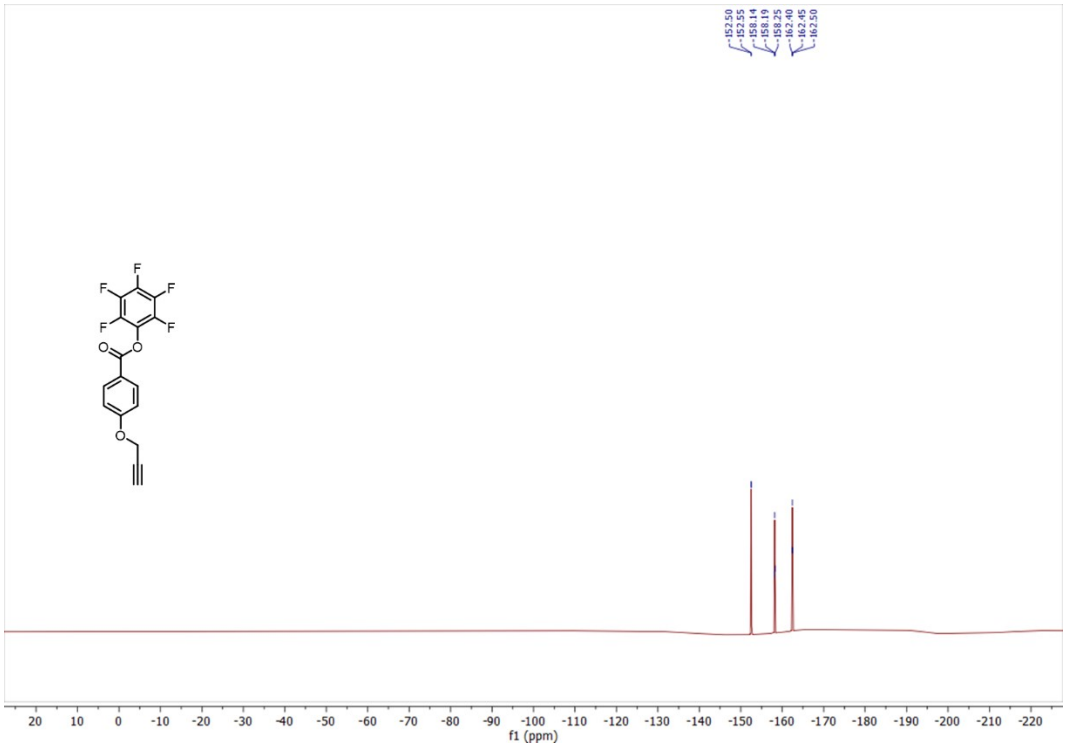

$^{13}$C NMR spectrum of **KP9**



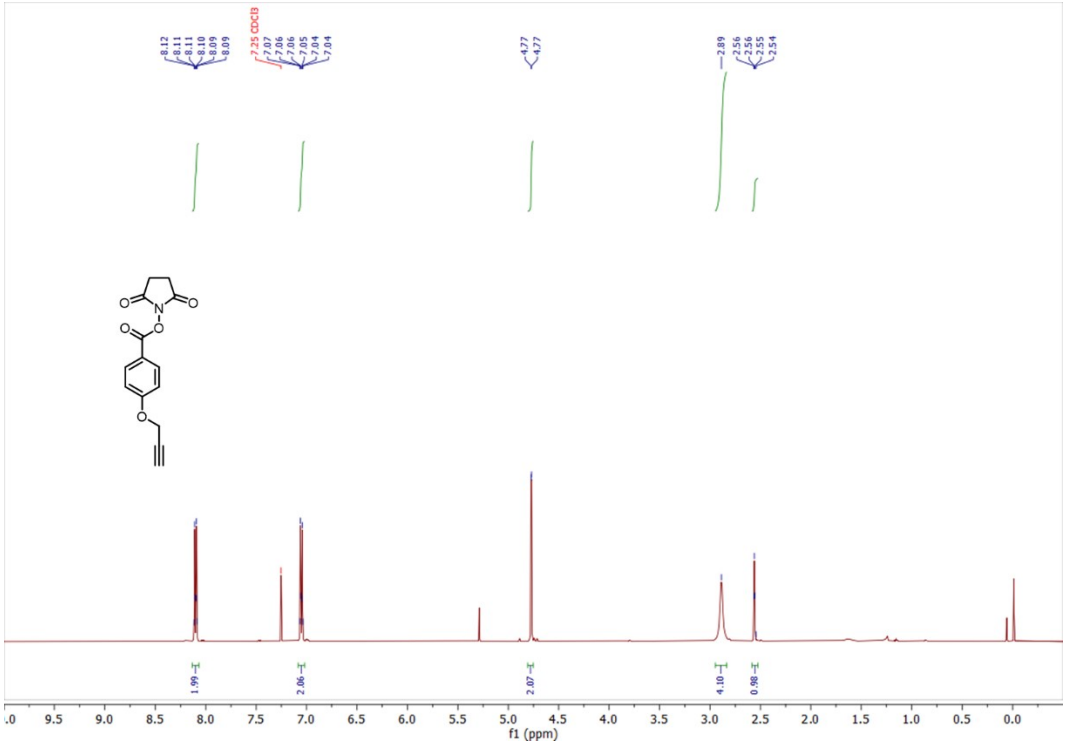

$^{1}$H NMR spectrum of **KP10**



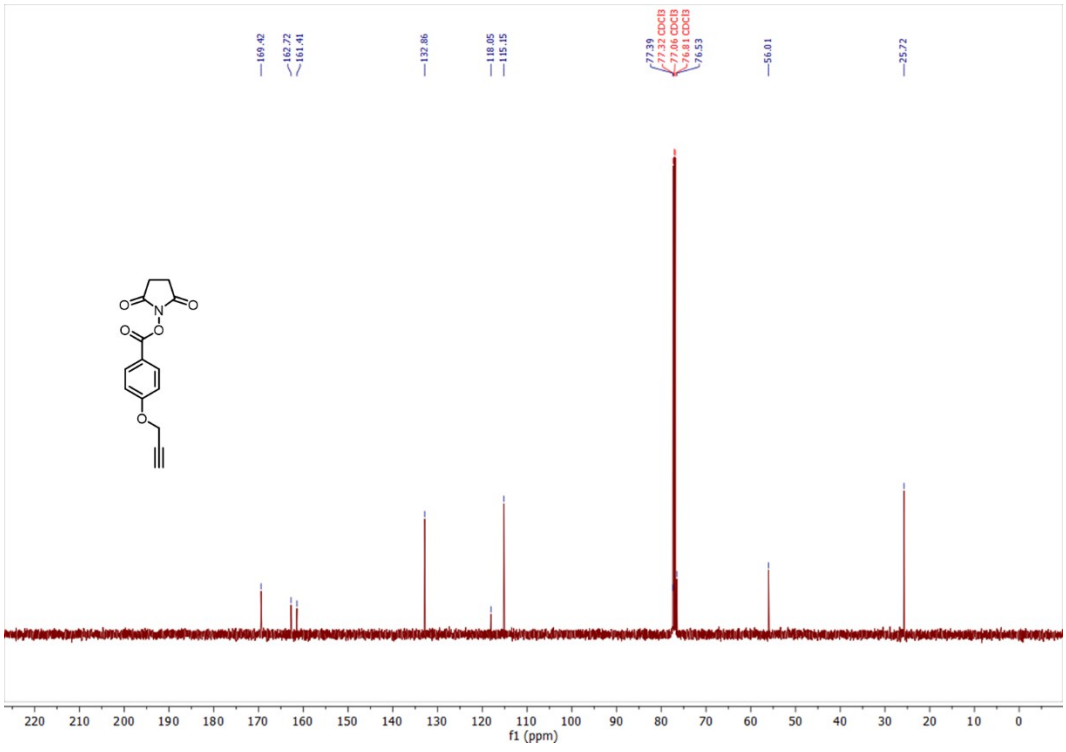

$^{13}$C NMR spectrum of **KP10**



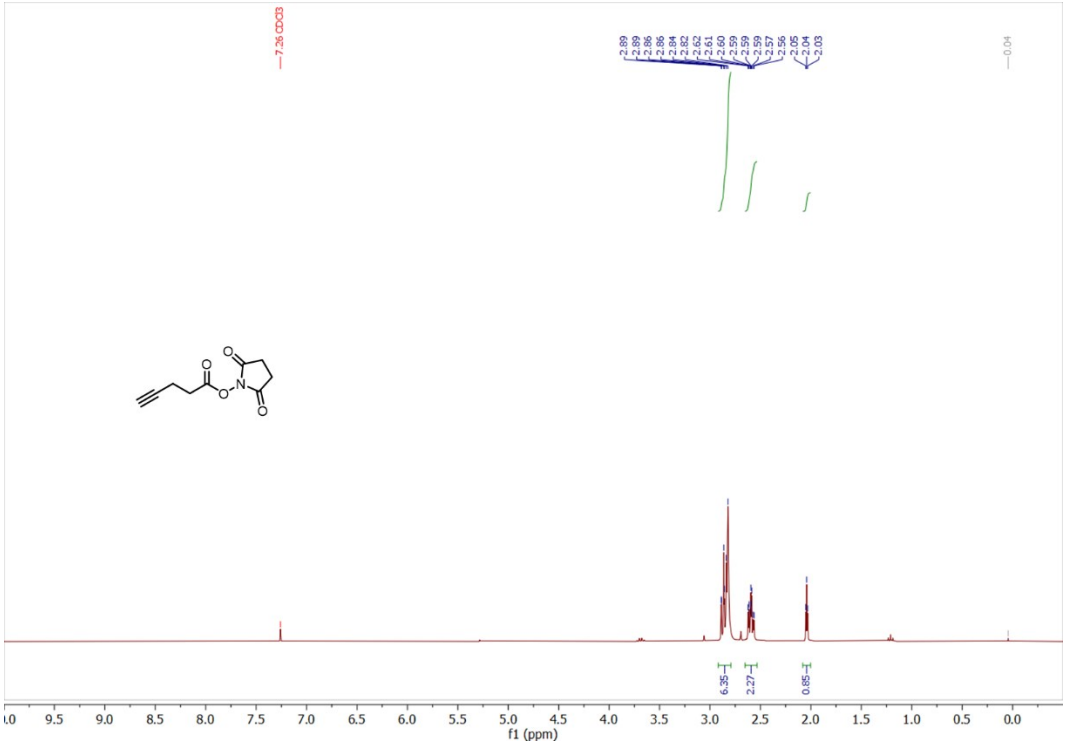

$^{19}$F NMR spectrum of **KP10**



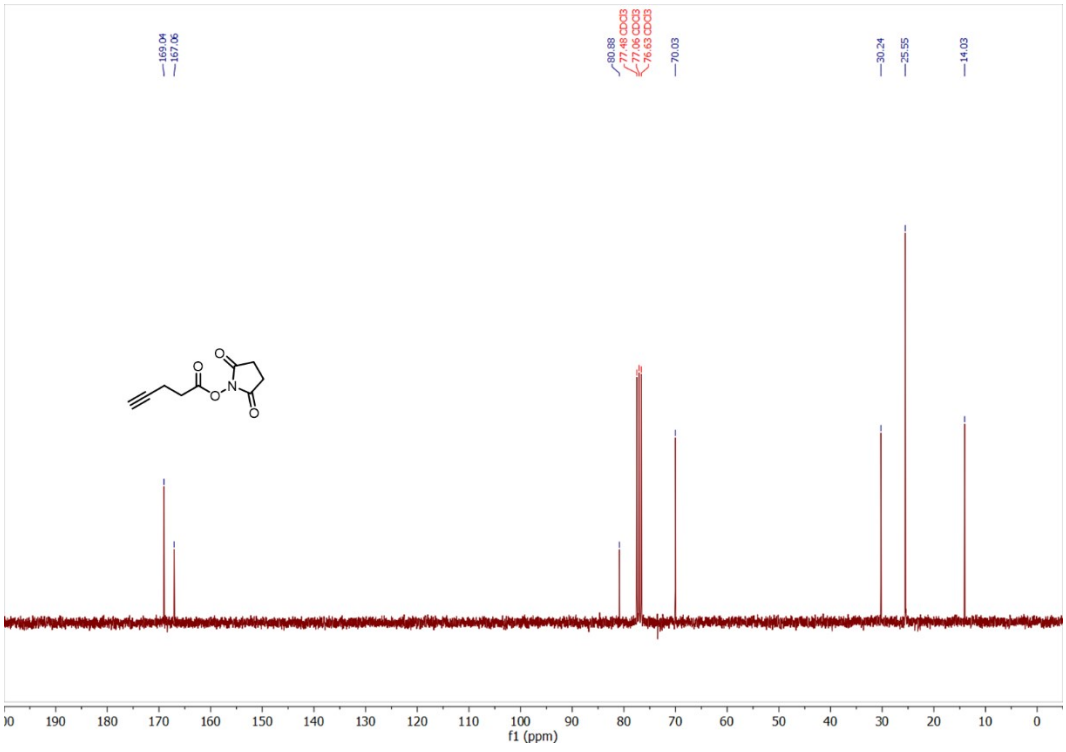

$^{1}$H NMR spectrum of **KP11**



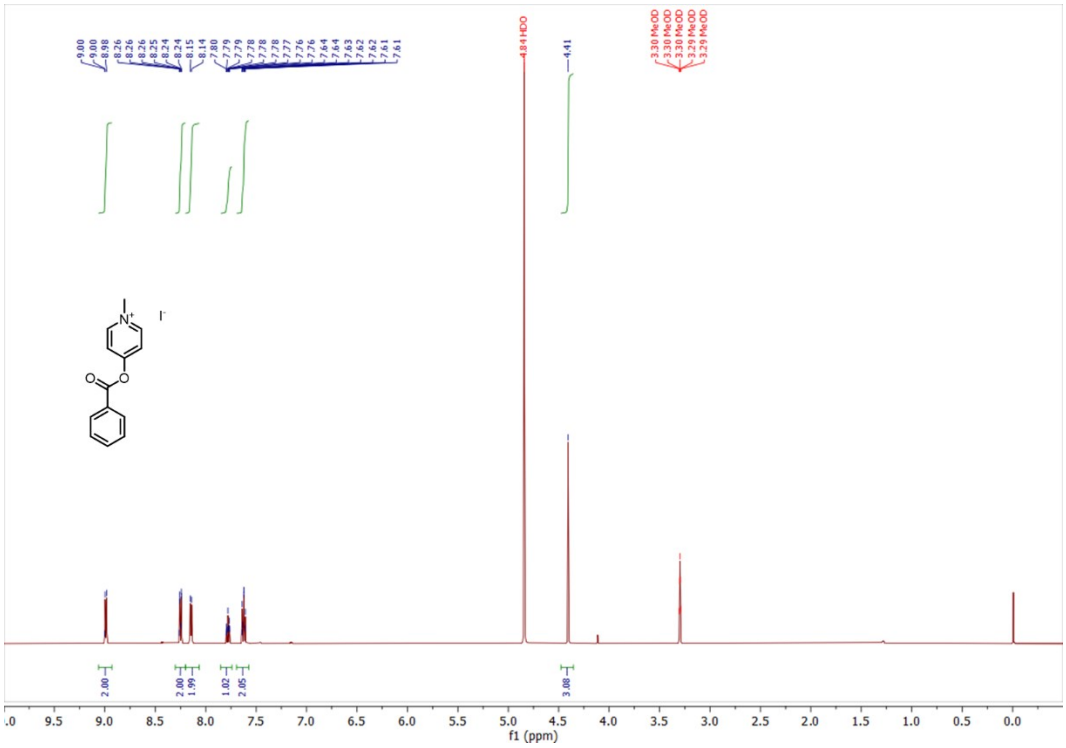

$^{13}$C NMR spectrum of **KP11**



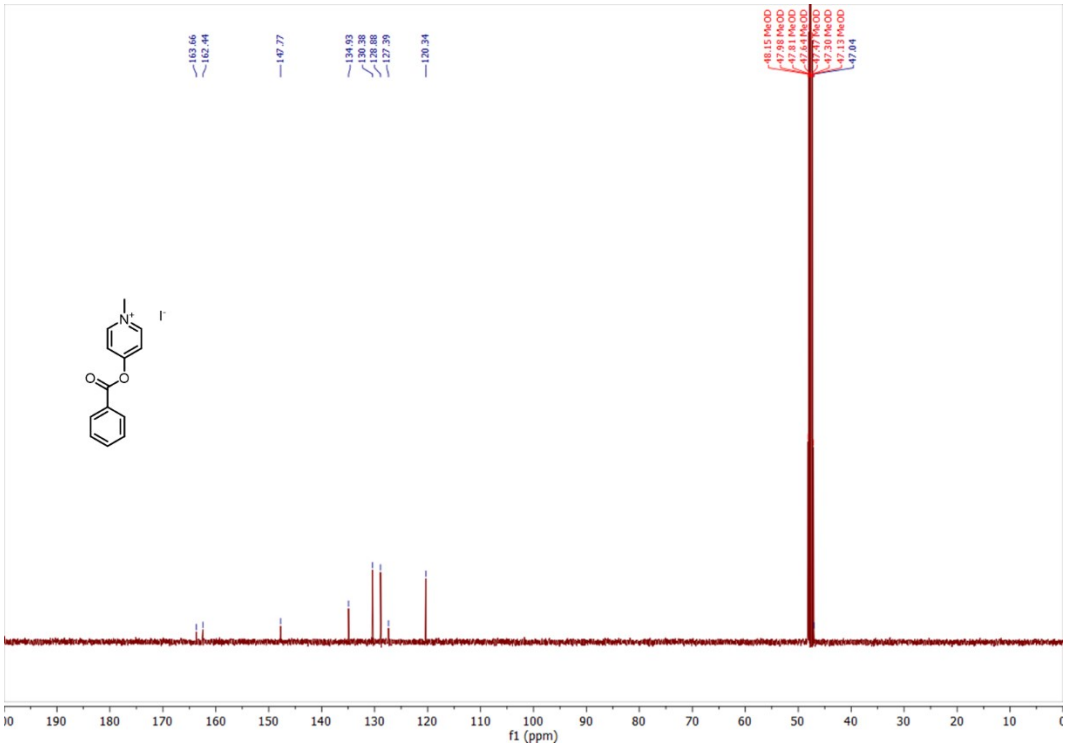

$^{1}$H NMR spectrum of **KP12**



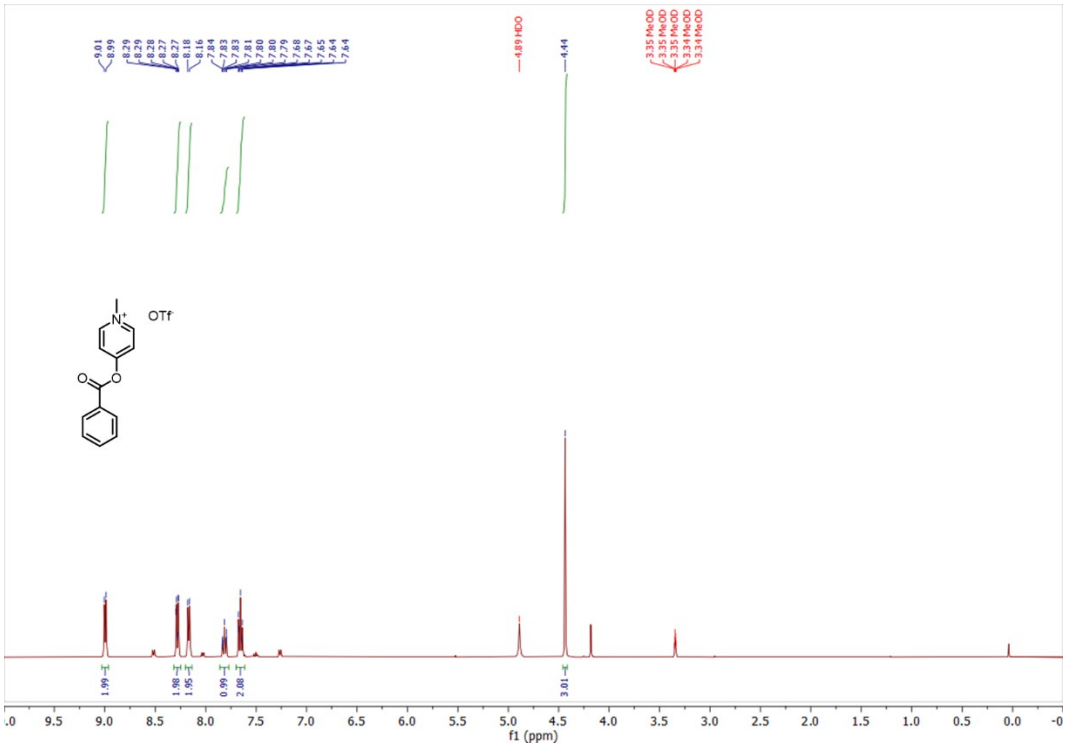

$^{13}$C NMR spectrum of **KP12**



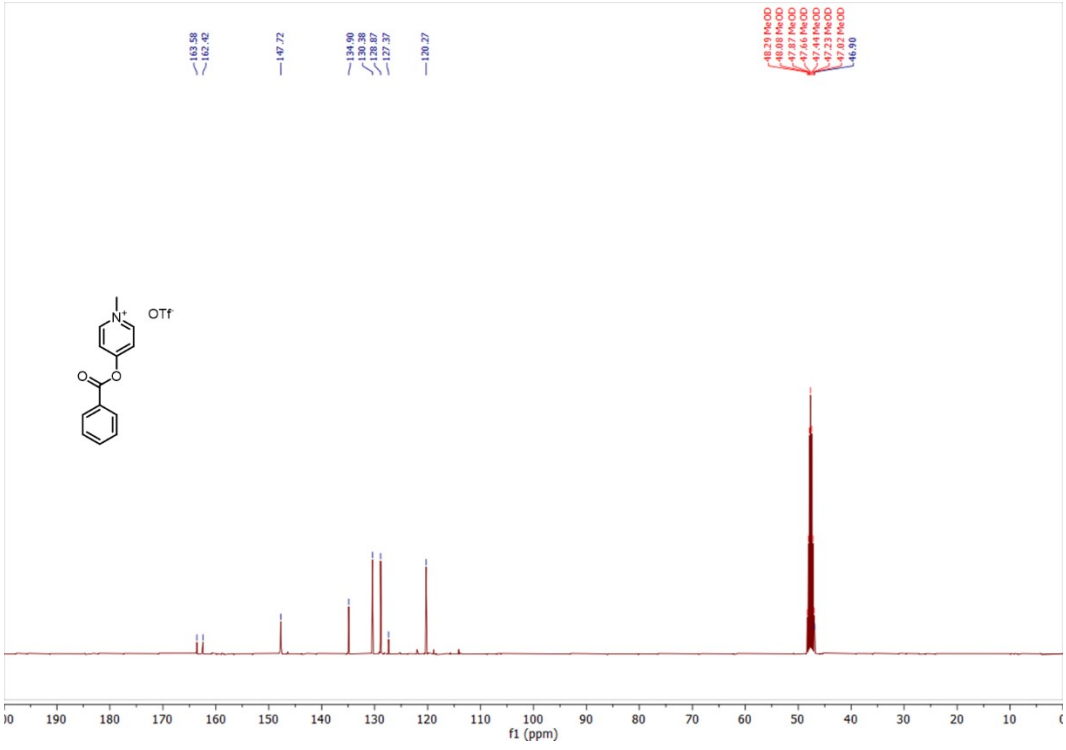

$^{1}$H NMR spectrum of **1b**



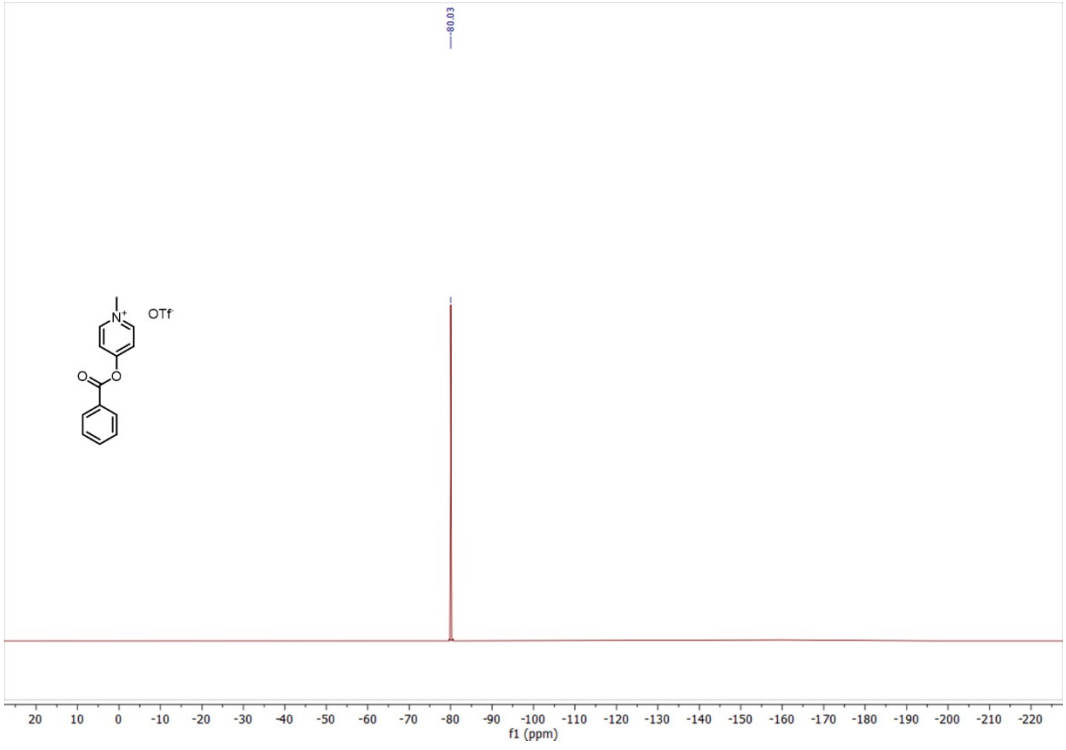

$^{13}$C NMR spectrum of **1b**



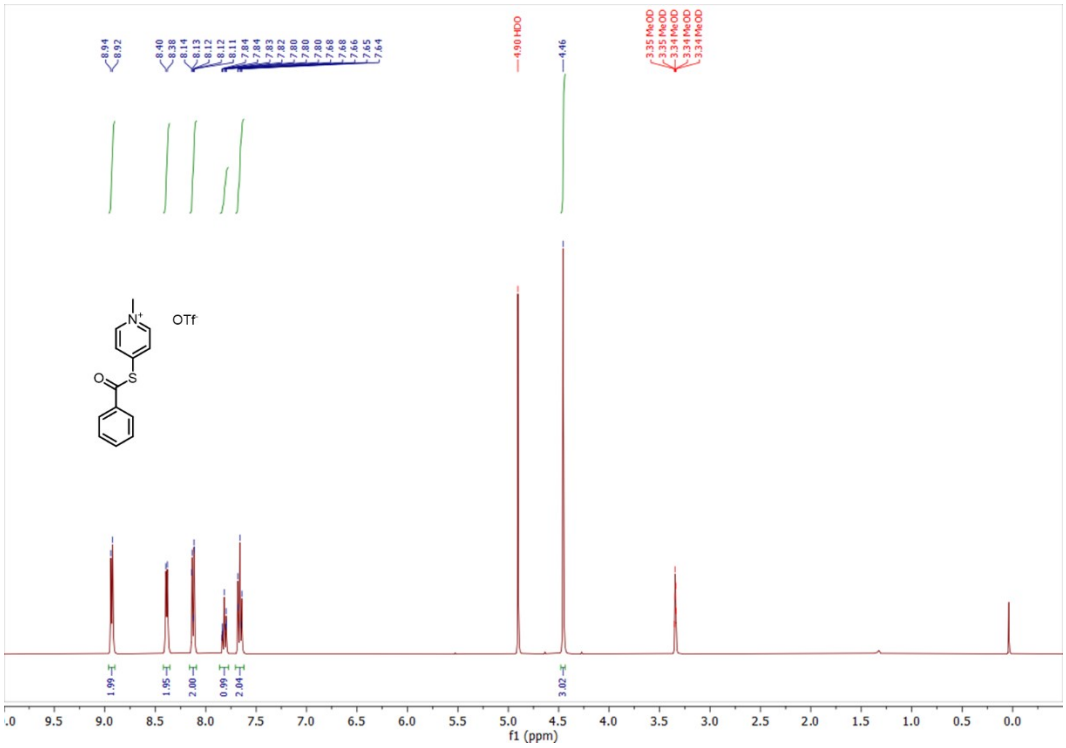

$^{1}$H NMR spectrum of **1c**



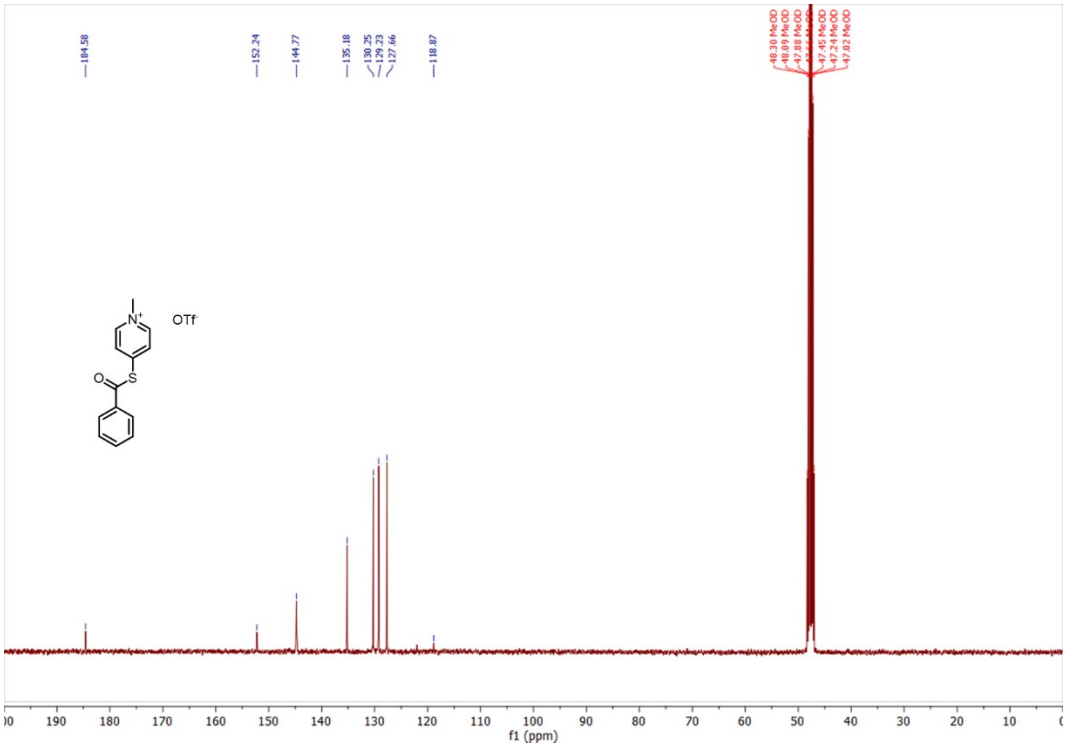

$^{13}$C NMR spectrum of **1c**



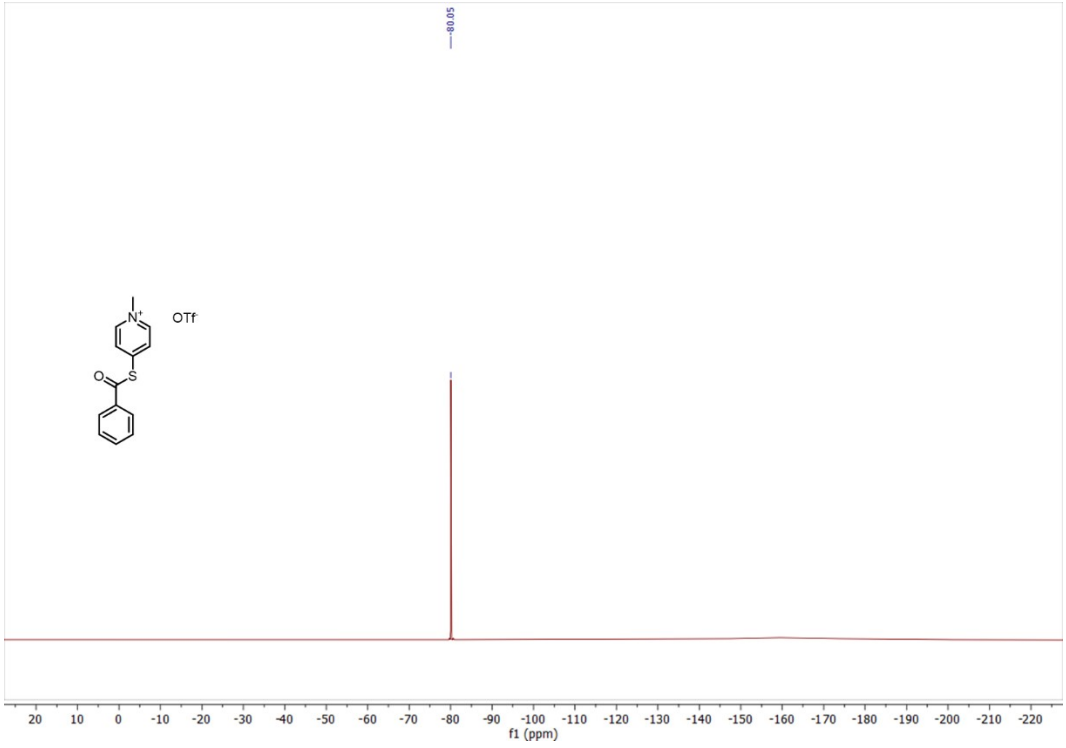

$^{19}$F NMR spectrum of **1c**



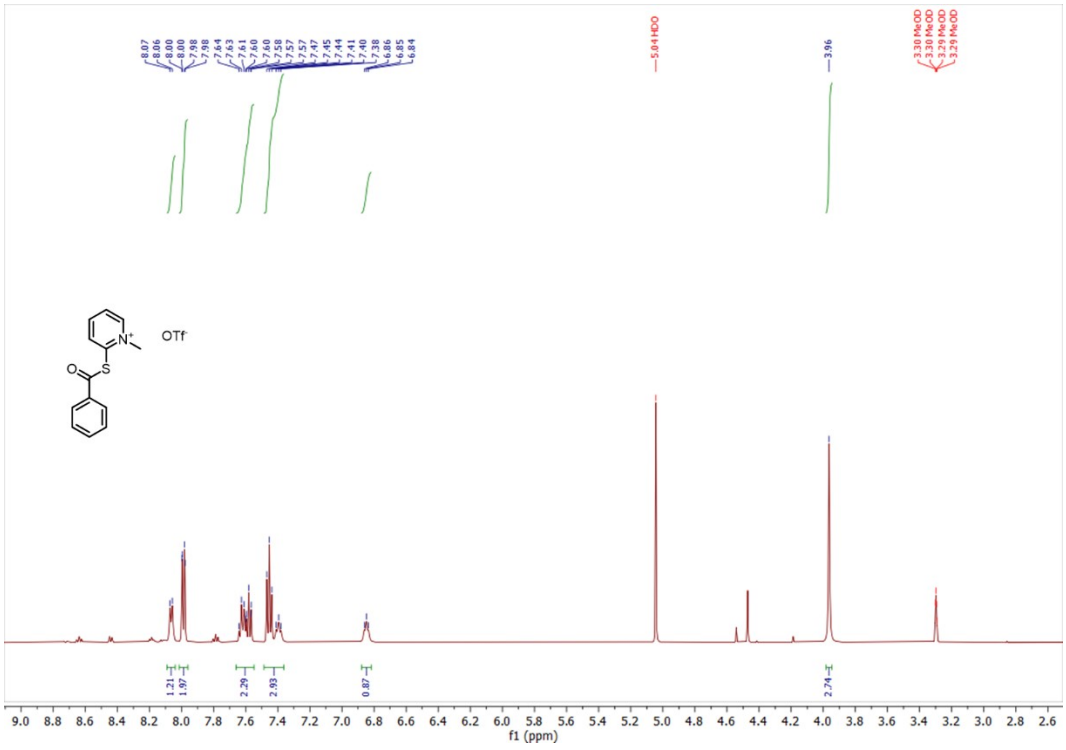

$^{1}$H NMR spectrum of **1i**



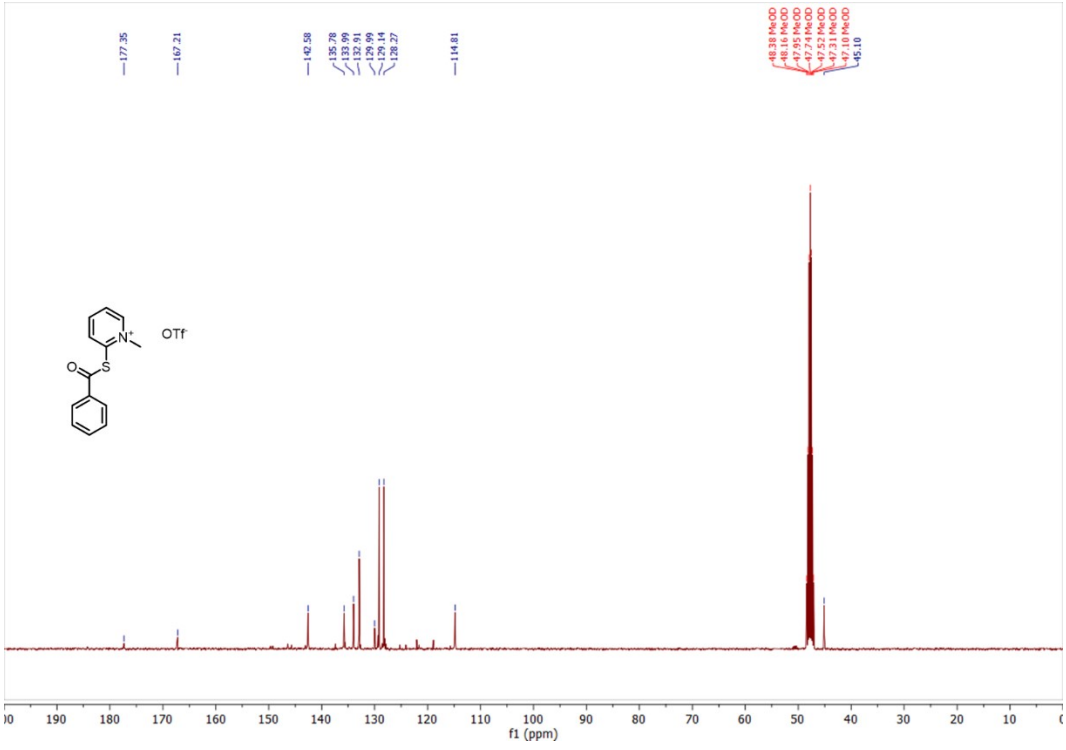

$^{13}$C NMR spectrum of **1i**



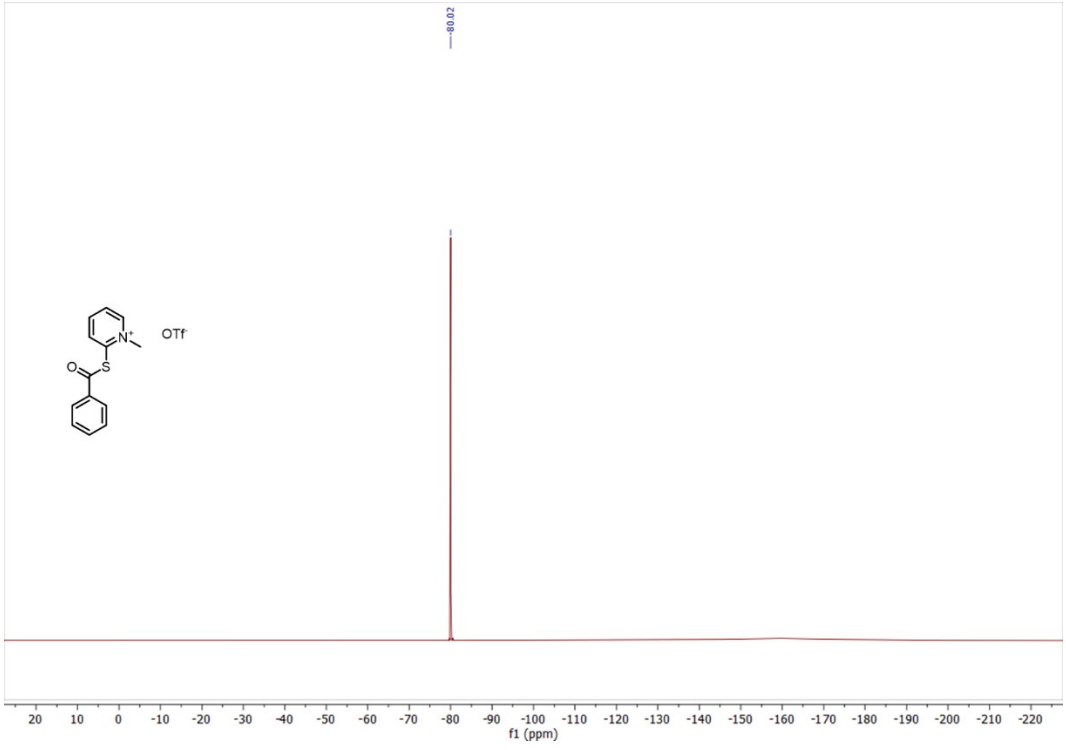

$^{19}$F NMR spectrum of **1i**



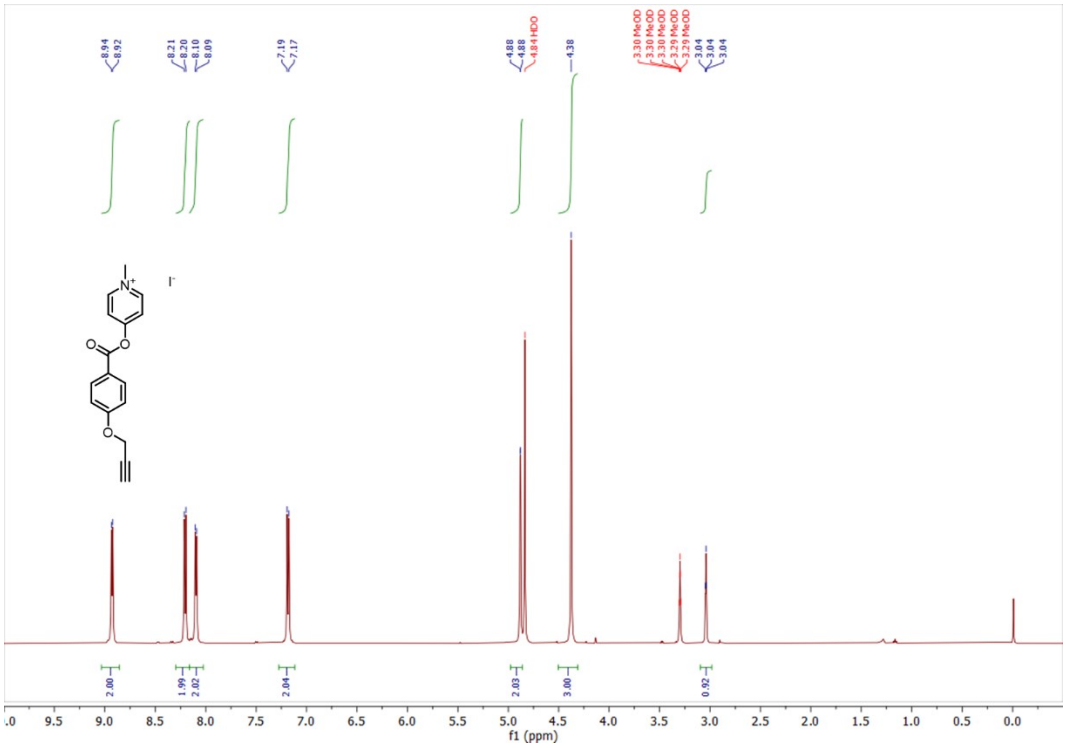

$^{1}$H NMR spectrum of **1j**



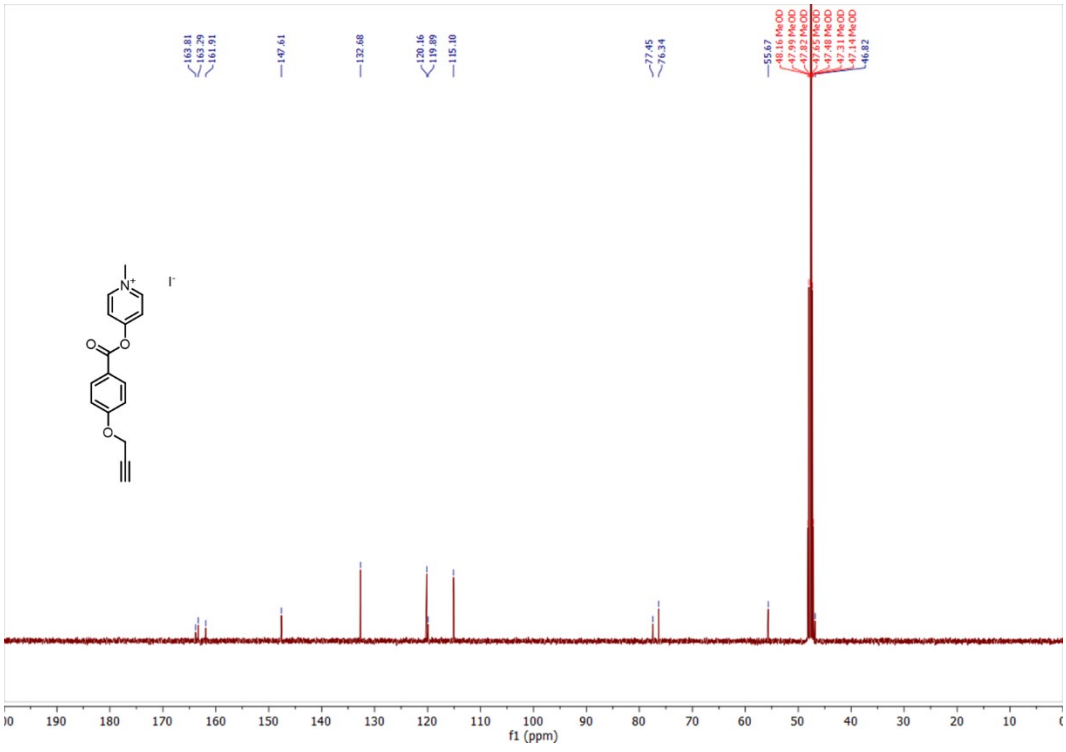

$^{13}$C NMR spectrum of **1j**



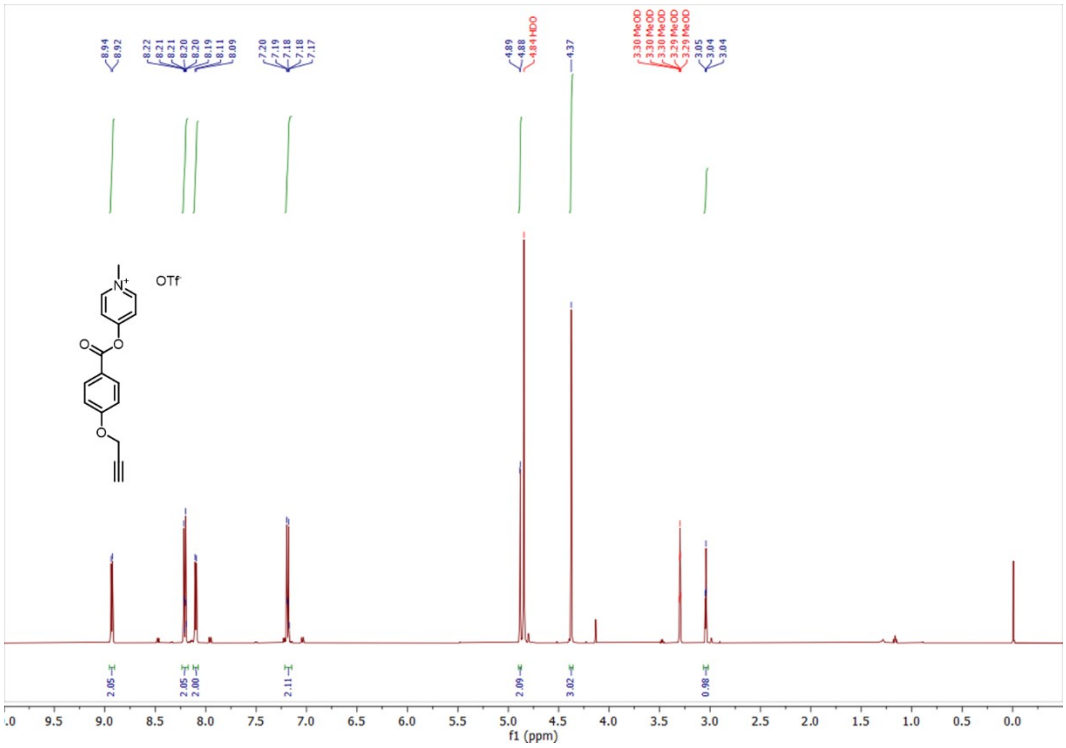

$^{19}$F NMR spectrum of **1j**



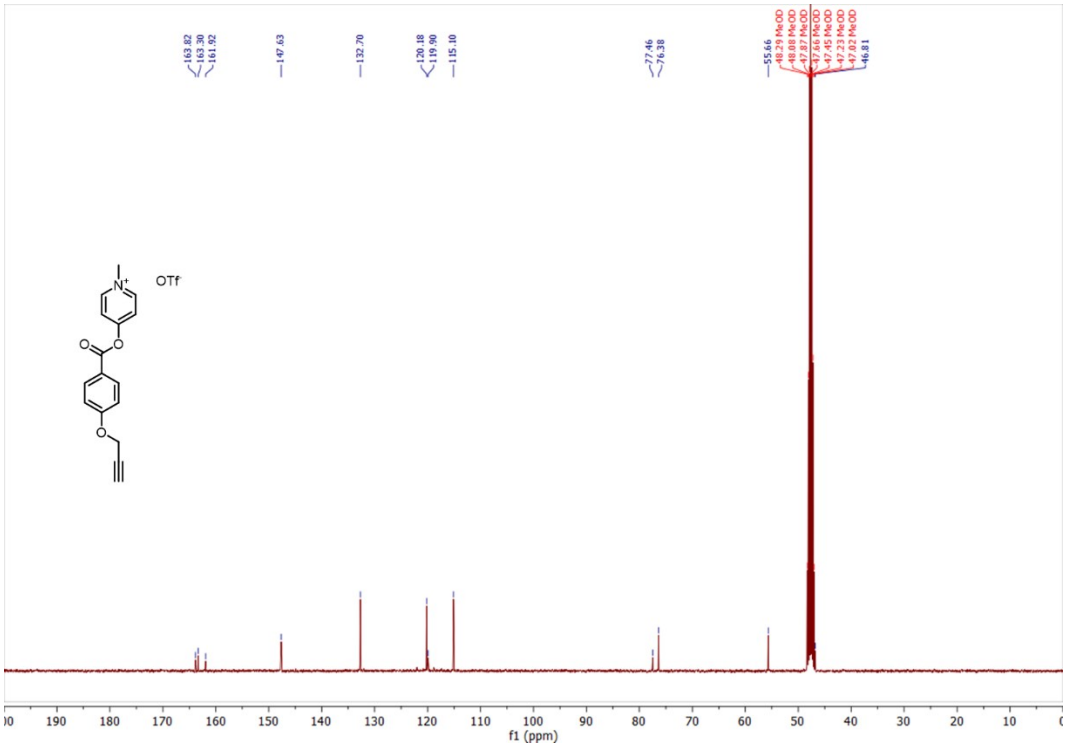

$^{1}$H NMR spectrum of **KP1**



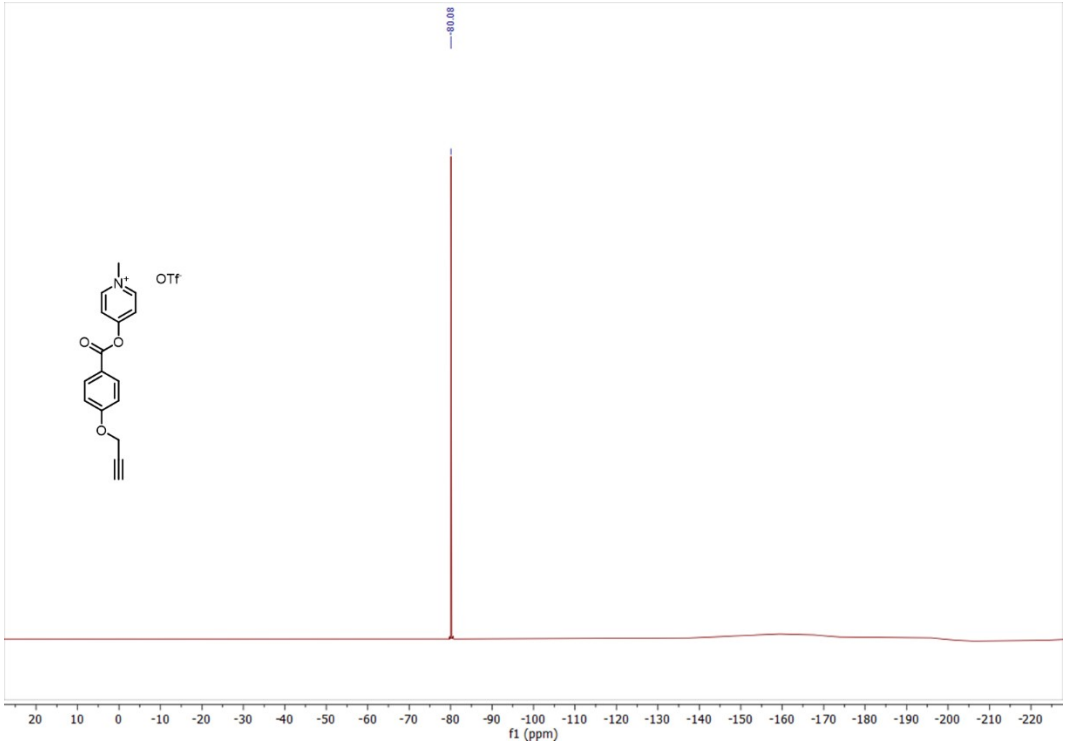

$^{13}$C NMR spectrum of **KP1**



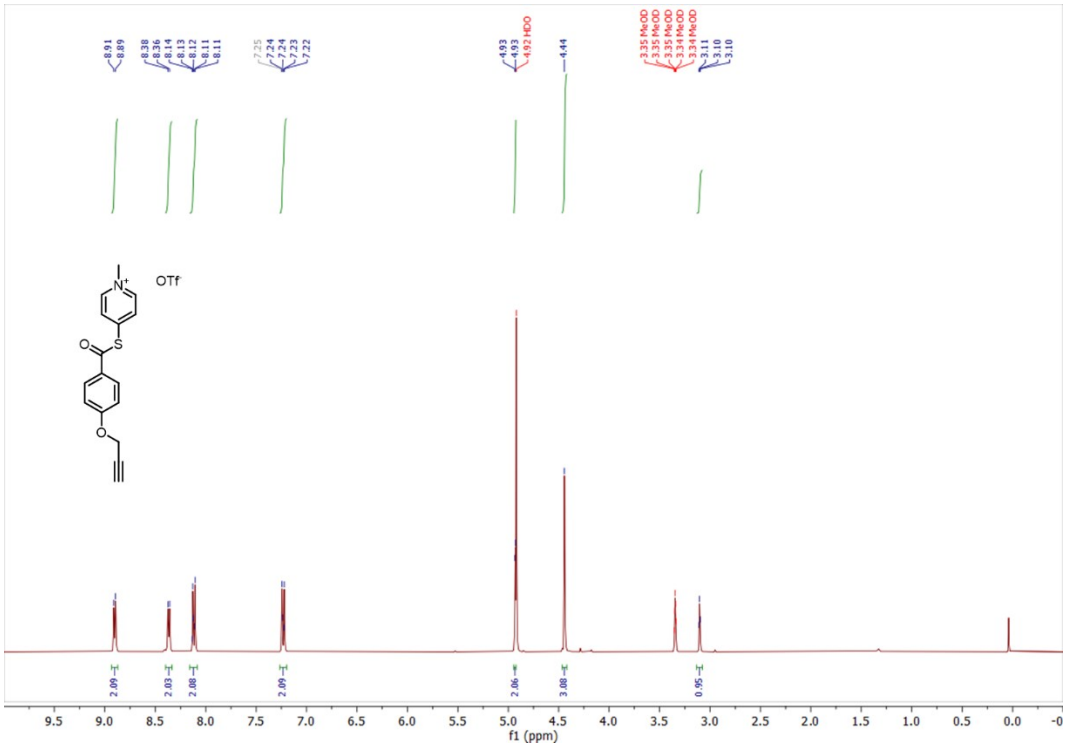

$^{1}$H NMR spectrum of **KP2**



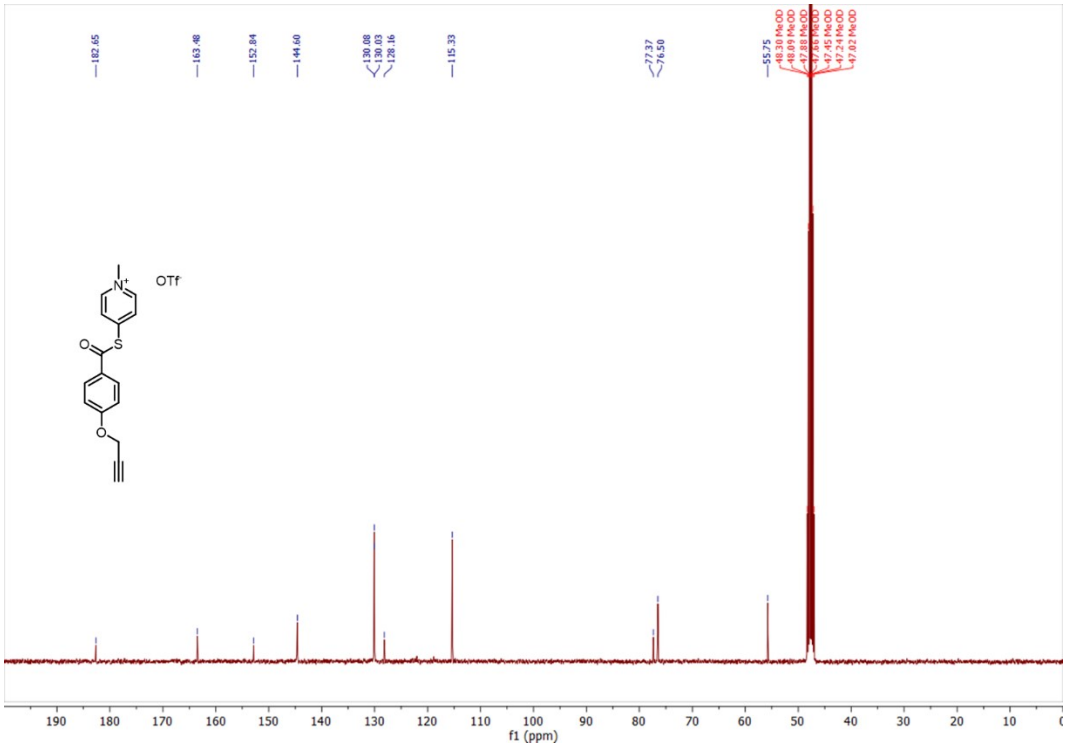

$^{13}$C NMR spectrum of **KP2**



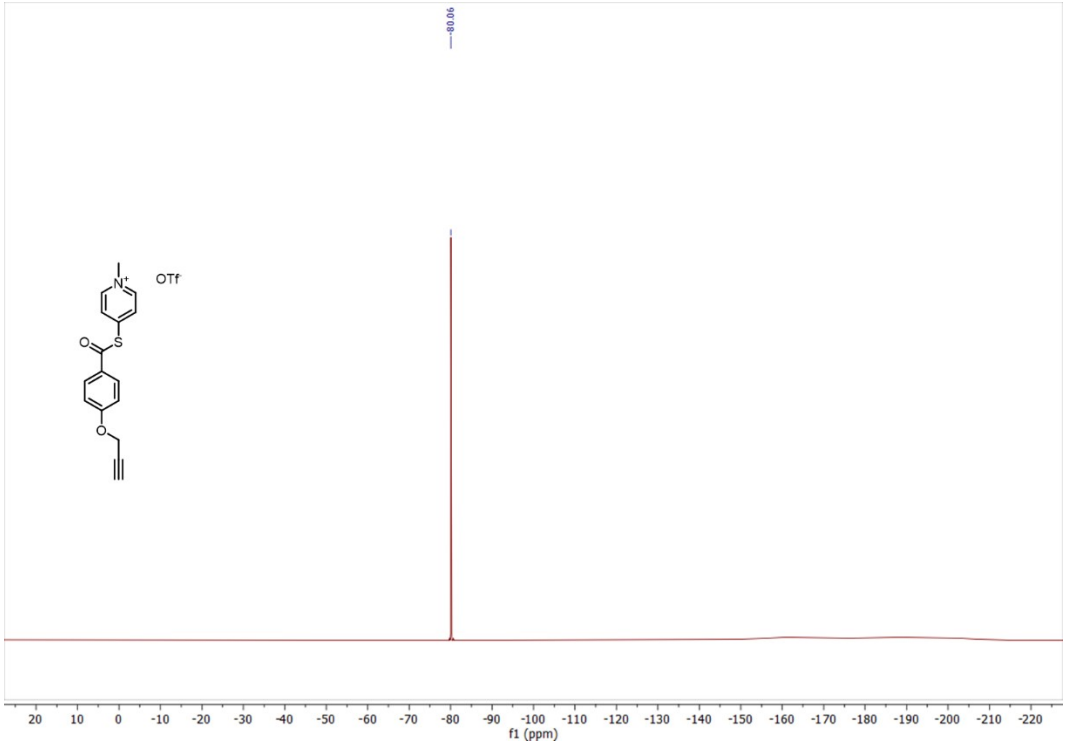

$^{19}$F NMR spectrum of **KP2**



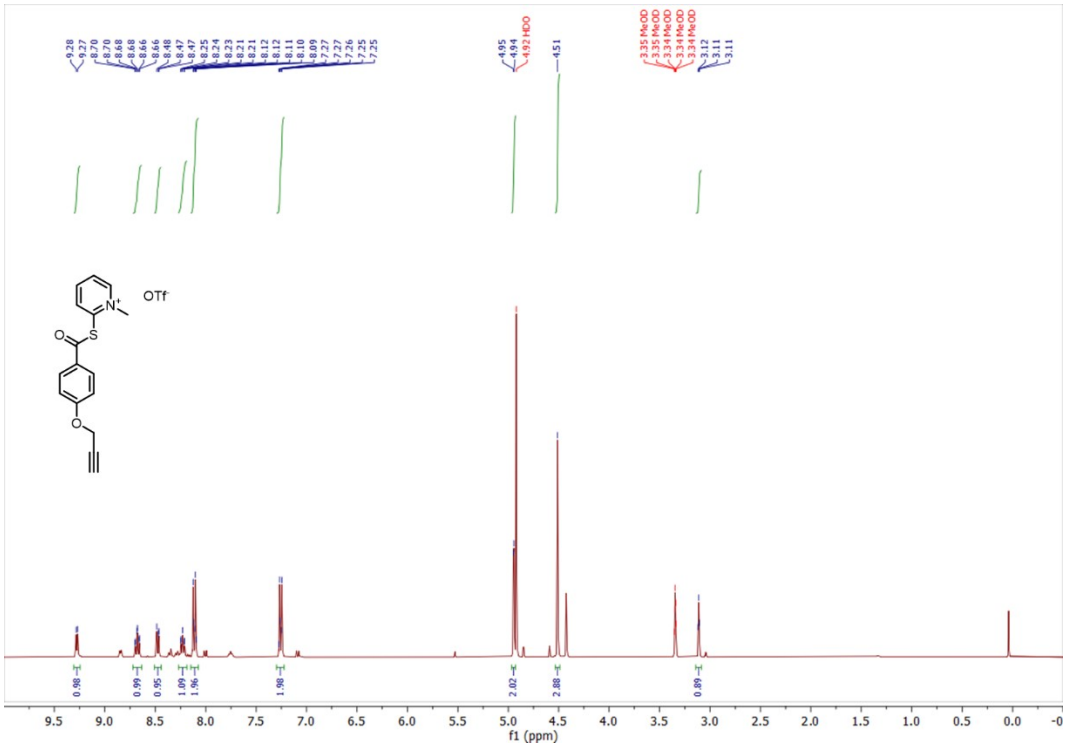

$^{1}$H NMR spectrum of **KP6**



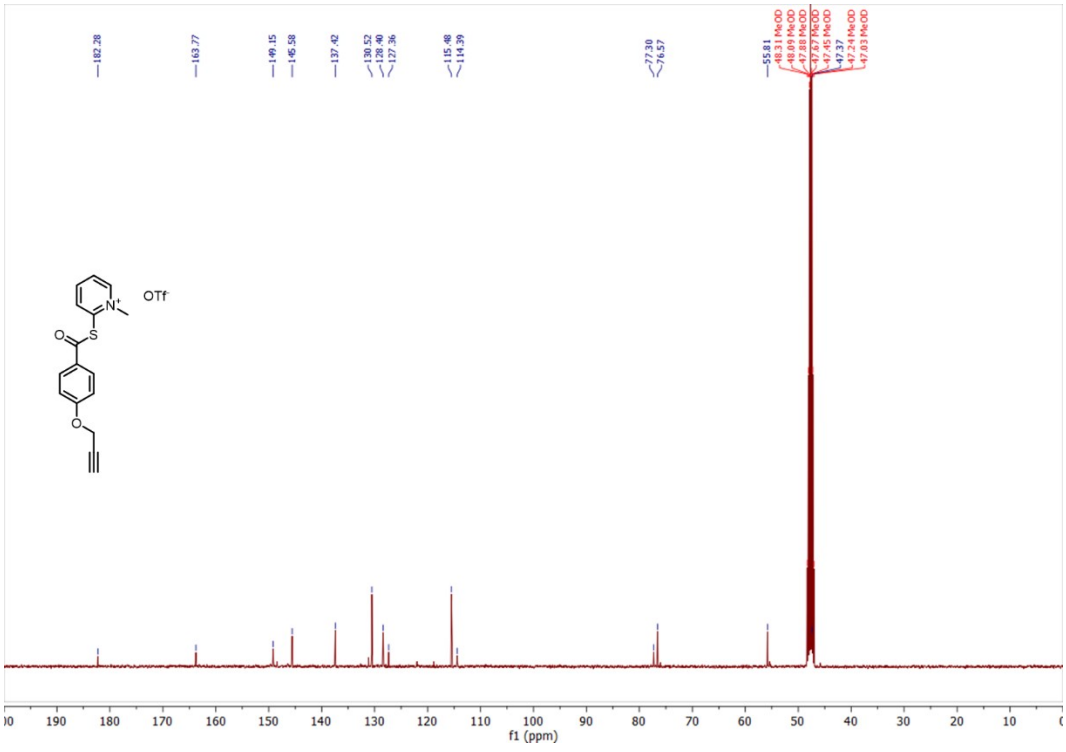

$^{13}$C NMR spectrum of **KP6**



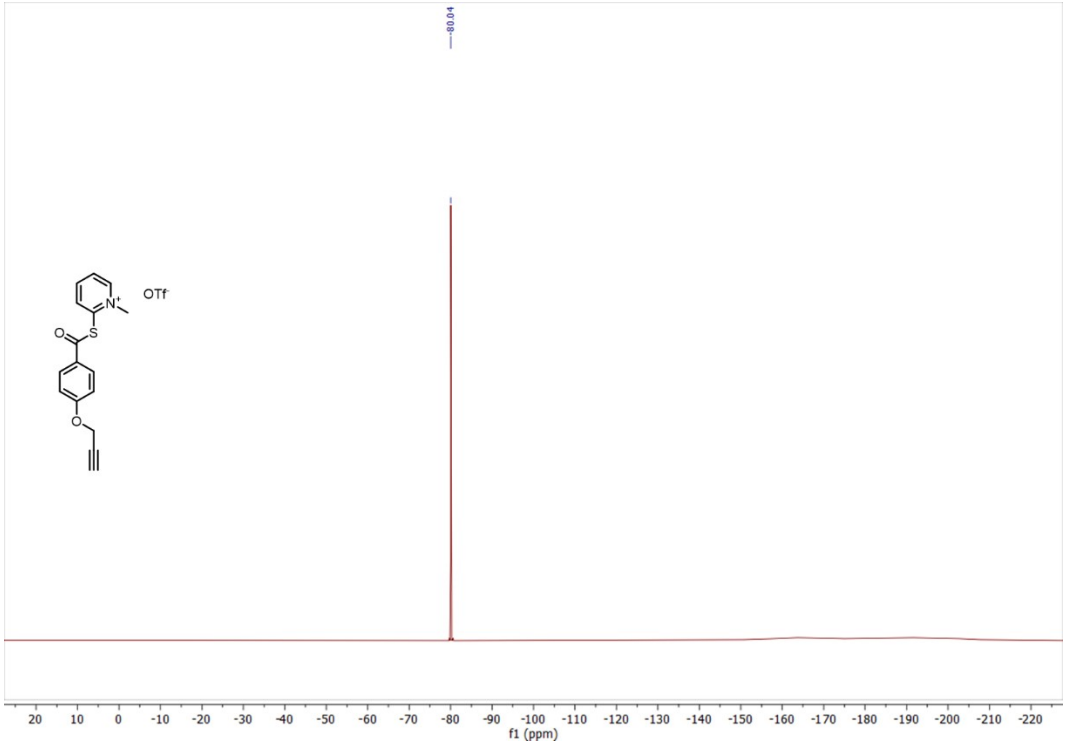

$^{19}$F NMR spectrum of **KP6**



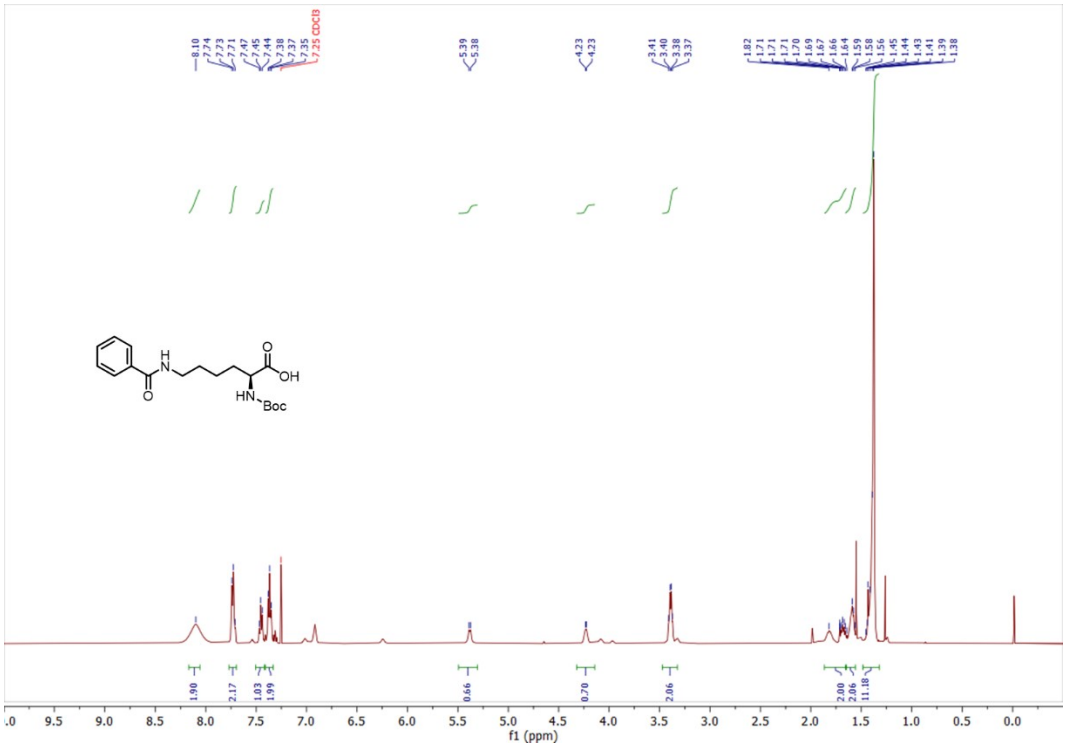

$^{1}$H NMR spectrum of **KP7**



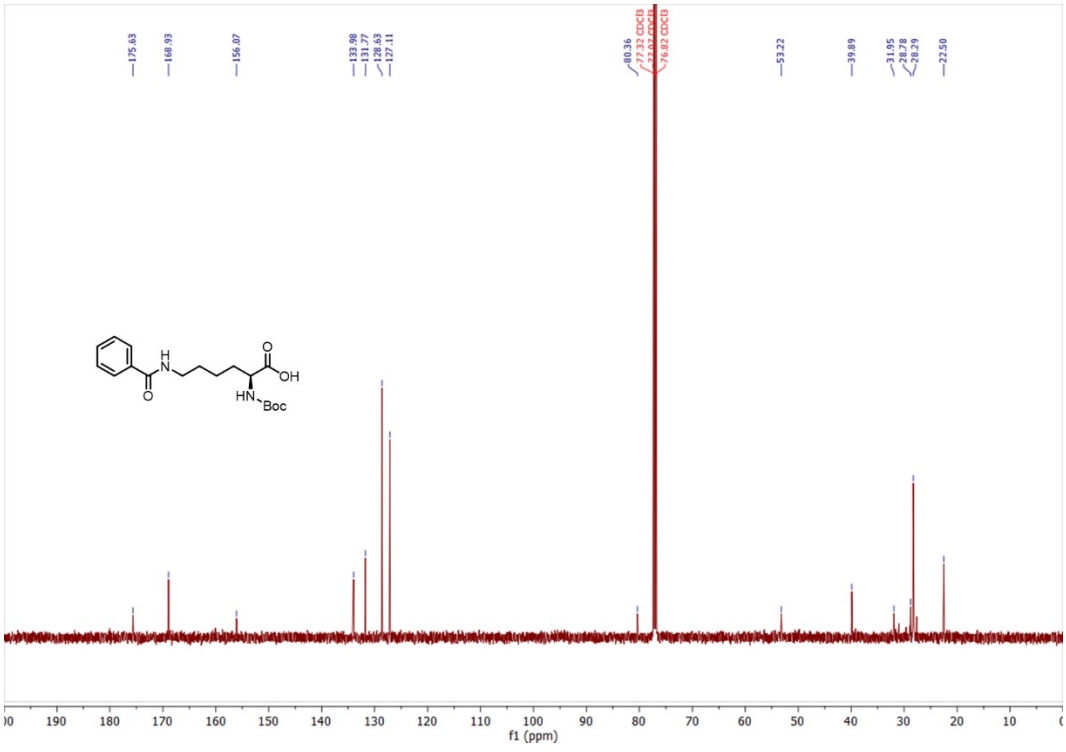

$^{13}$C NMR spectrum of **KP7**



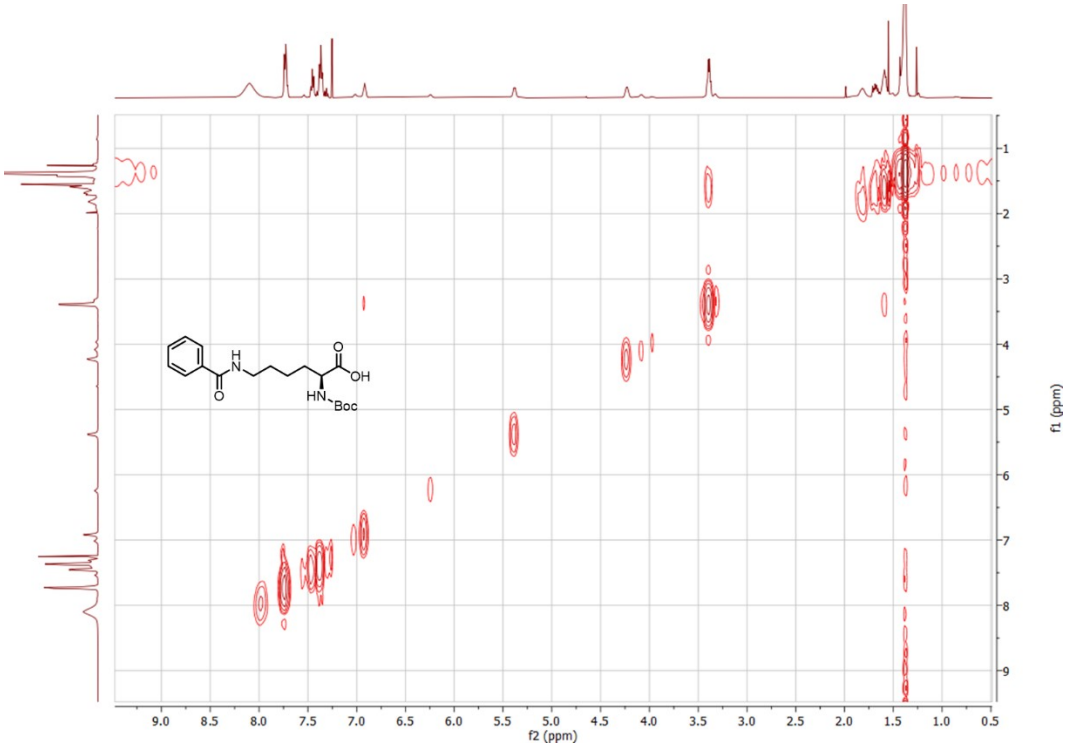

$^{19}$F NMR spectrum of **KP7**



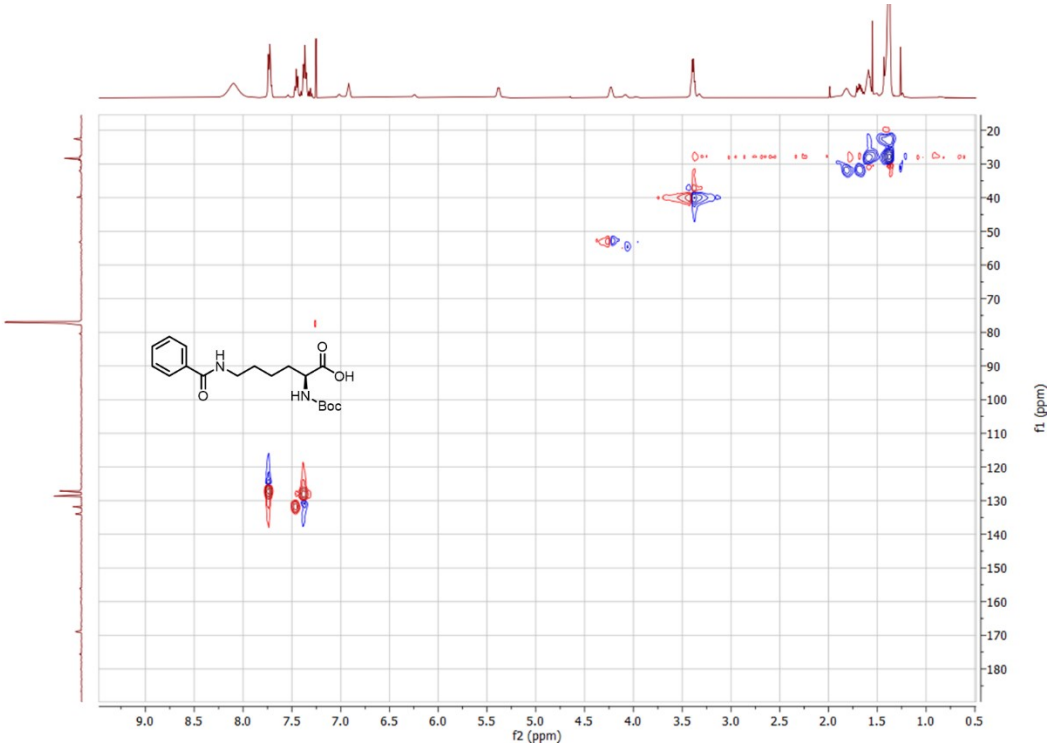

$^{1}$H NMR spectrum of **3**



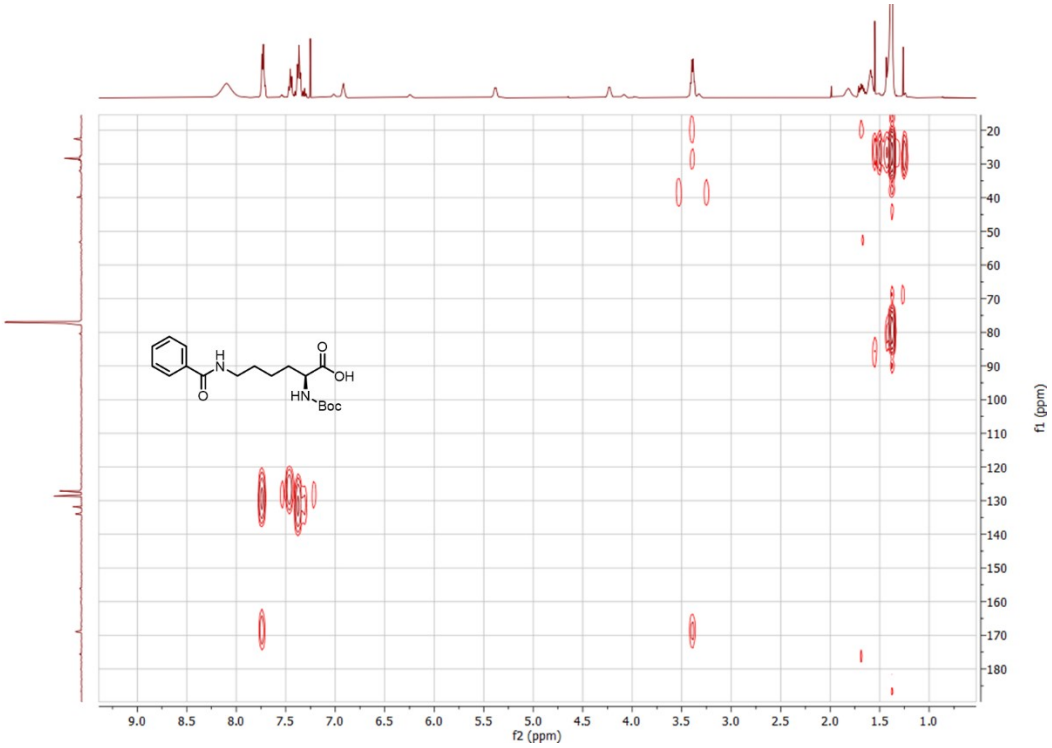

$^{13}$C NMR spectrum of **3**



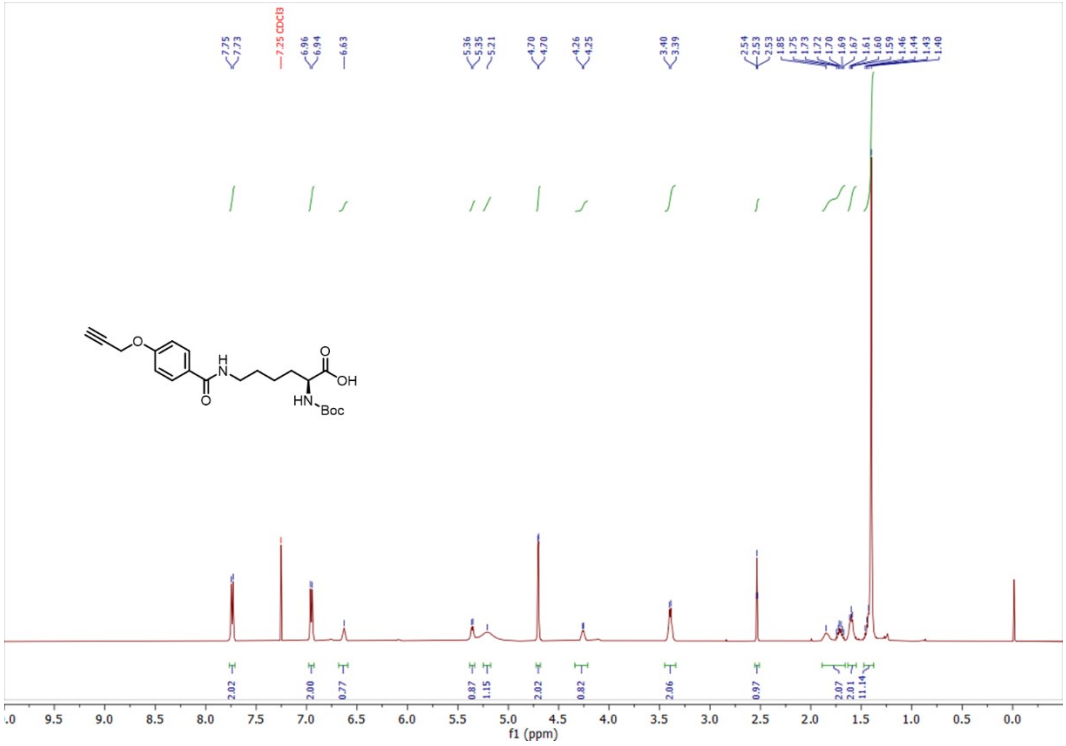

$^{1}$H-$^{1}$H COSY NMR spectrum of **3**



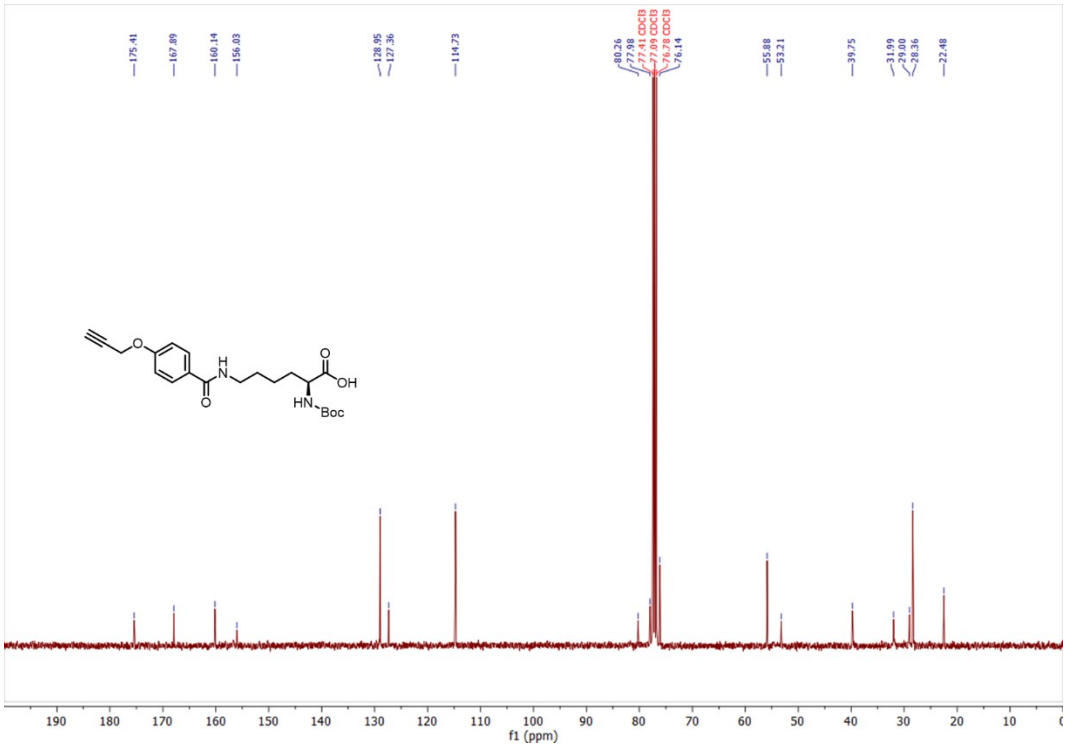

$^{1}$H-$^{13}$C HSQC NMR spectrum of **3**



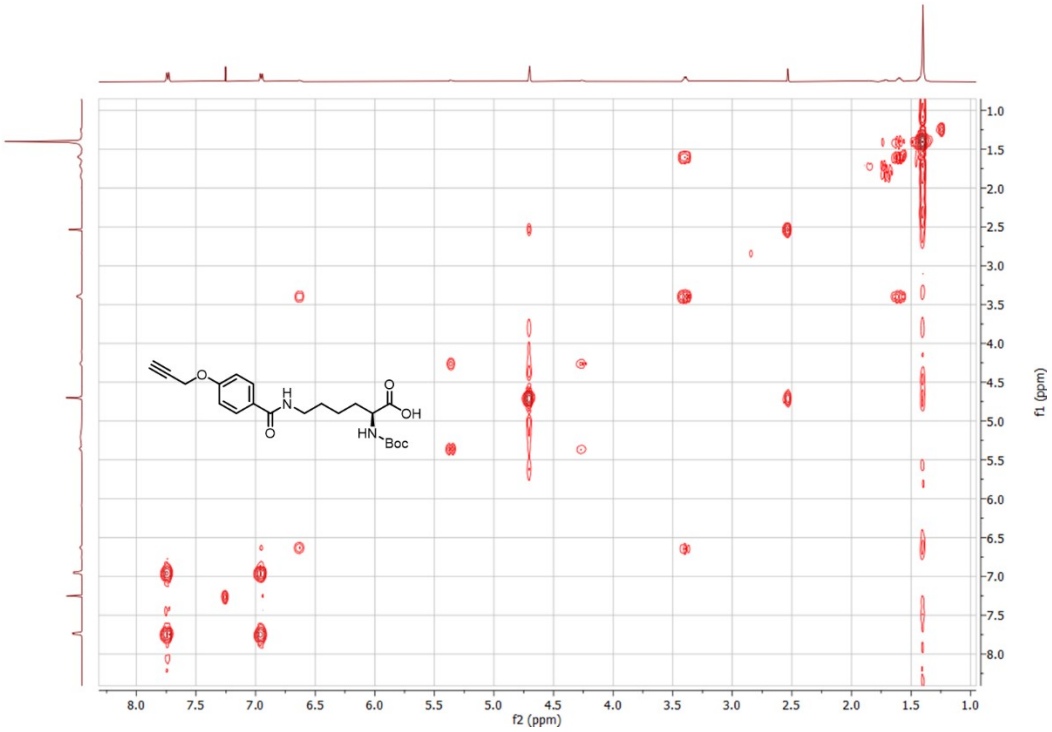

$^{1}H$-$^{13}C$ HMBC NMR spectrum of **3**



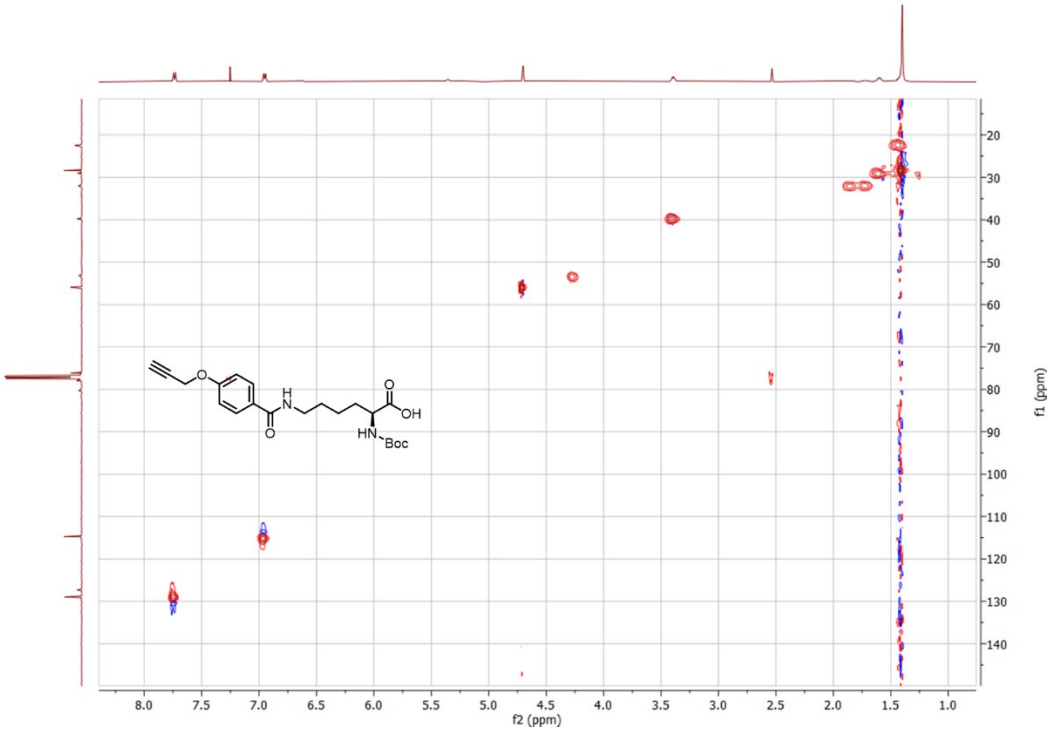

$^{1}$H NMR spectrum of **S5**



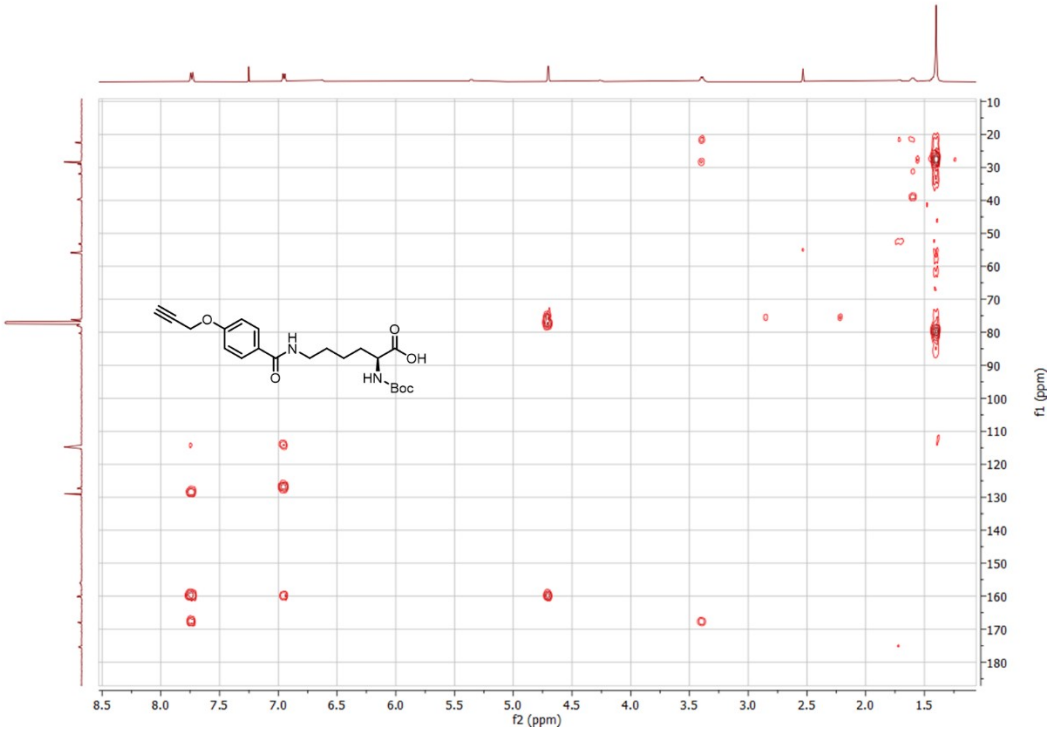

$^{13}$C NMR spectrum of **S5**



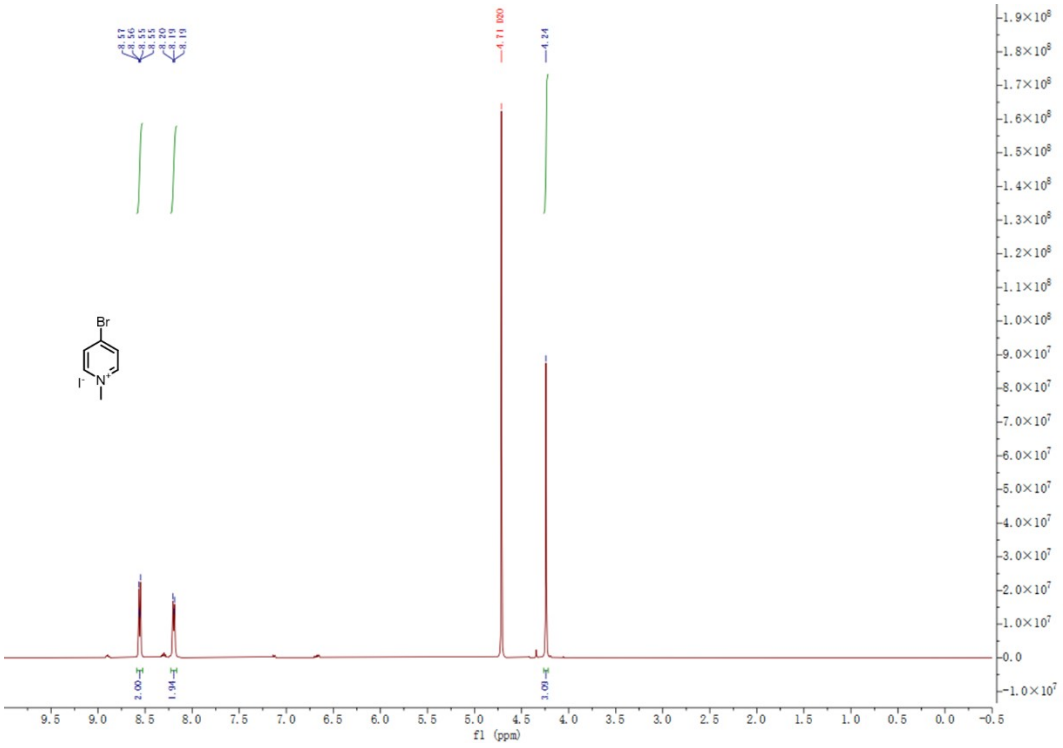

$^{1}$H-$^{1}$H COSY NMR spectrum of **S5**



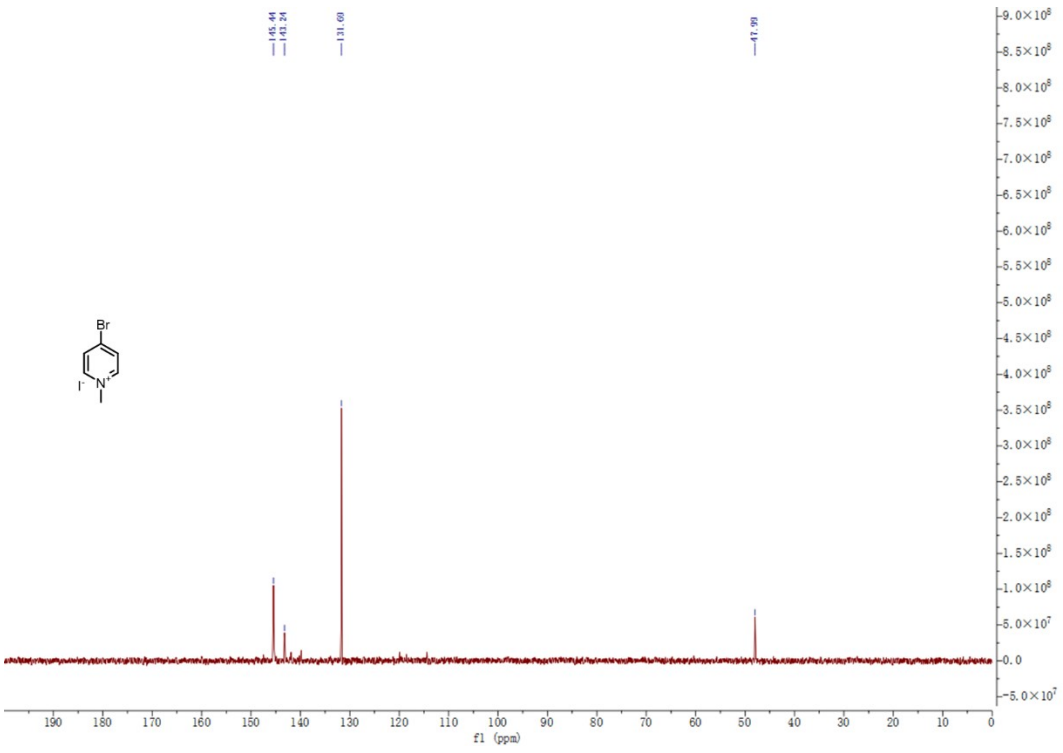

$^{1}$H-$^{13}$C HSQC NMR spectrum of **S5**



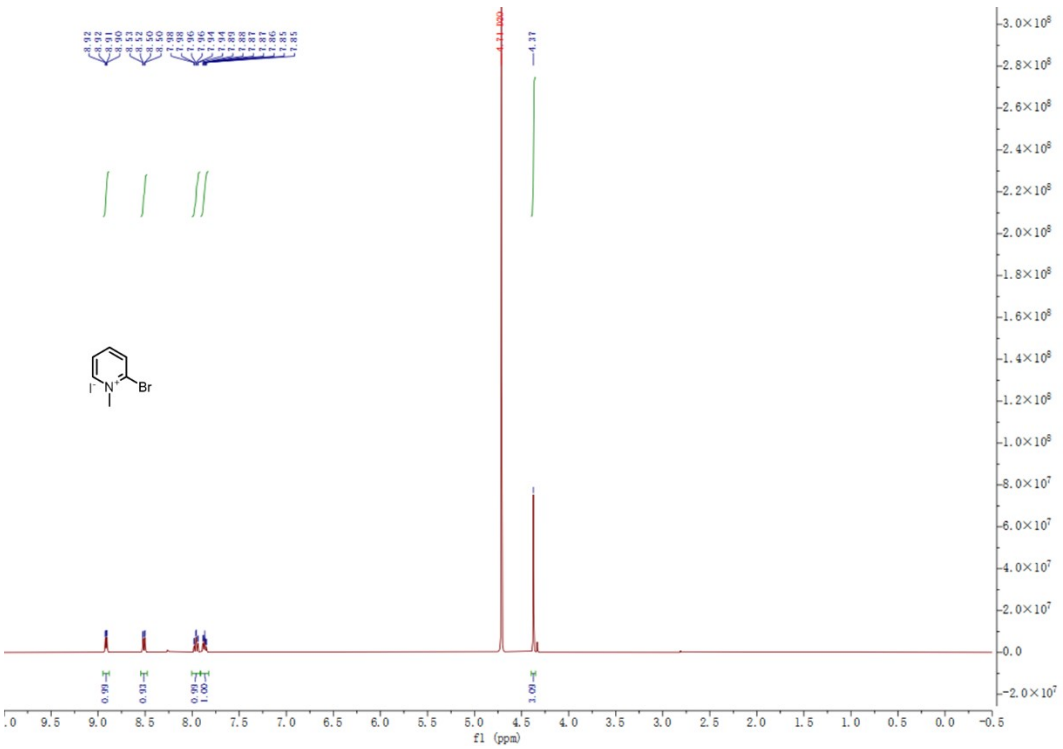

$^{1}$H-$^{13}$C HMBC NMR spectrum of **S5**




$^{1}$H NMR spectrum of **S1**




$^{13}$C NMR spectrum of **S1**




$^{1}$H NMR spectrum of **S2**

$^{13}$C NMR spectrum of **S2**