Supplementary information

Triazin-pyridine Chemistry for Protein Labeling on Tyrosine

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Supplementary figures

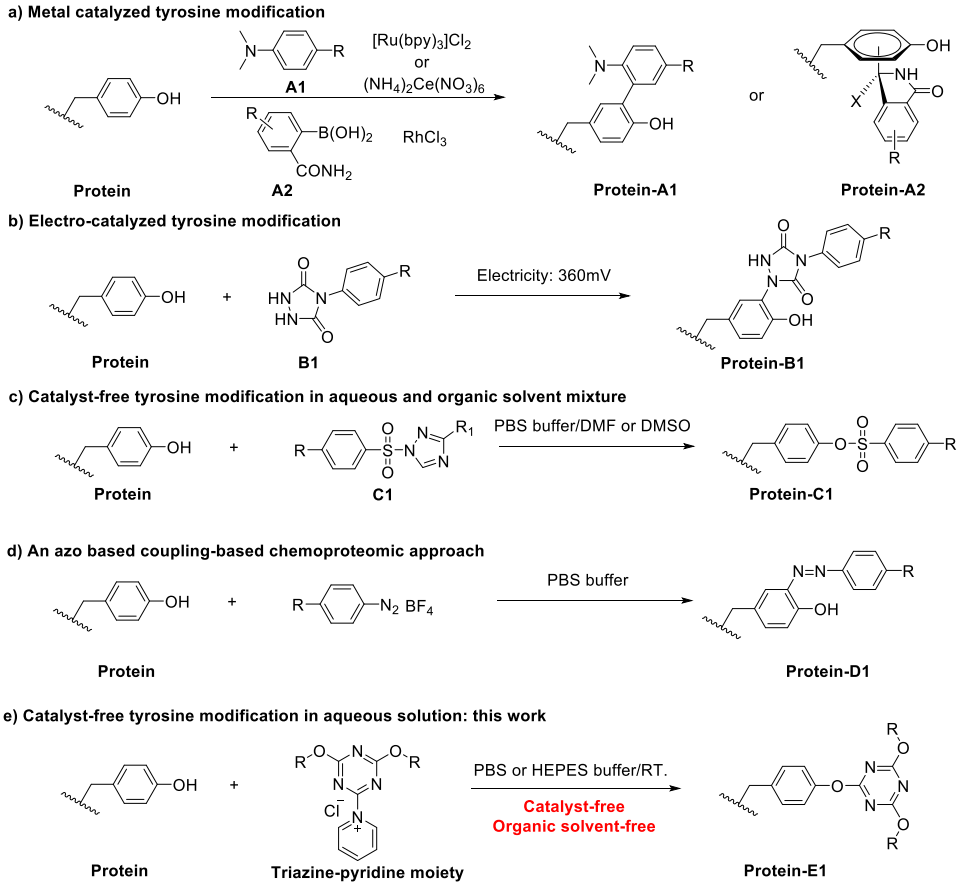
Materials and methods for bioanalysis

- **Cu(I)-catalyzed cycloaddition/ click chemistry**
- **In-gel fluorescence scanning**
- **In solution digestion**
- **Cell culture, lysis and labeling with probes**
- **Stability of probe 8 and conjugates**
- **Streptavidin affinity enrichment of probe labeled peptides**
- **On beads digestion**
- **MS analysis for peptides**
- **MS analysis of probe 8 labeling in whole proteome**
- **Domain enrichment analysis**
- **Live cell labeling**
- **Imaging of probe 8 labeled live cells by confocal microscopy**
- **Labeling of probe 8 on recombinant protein**

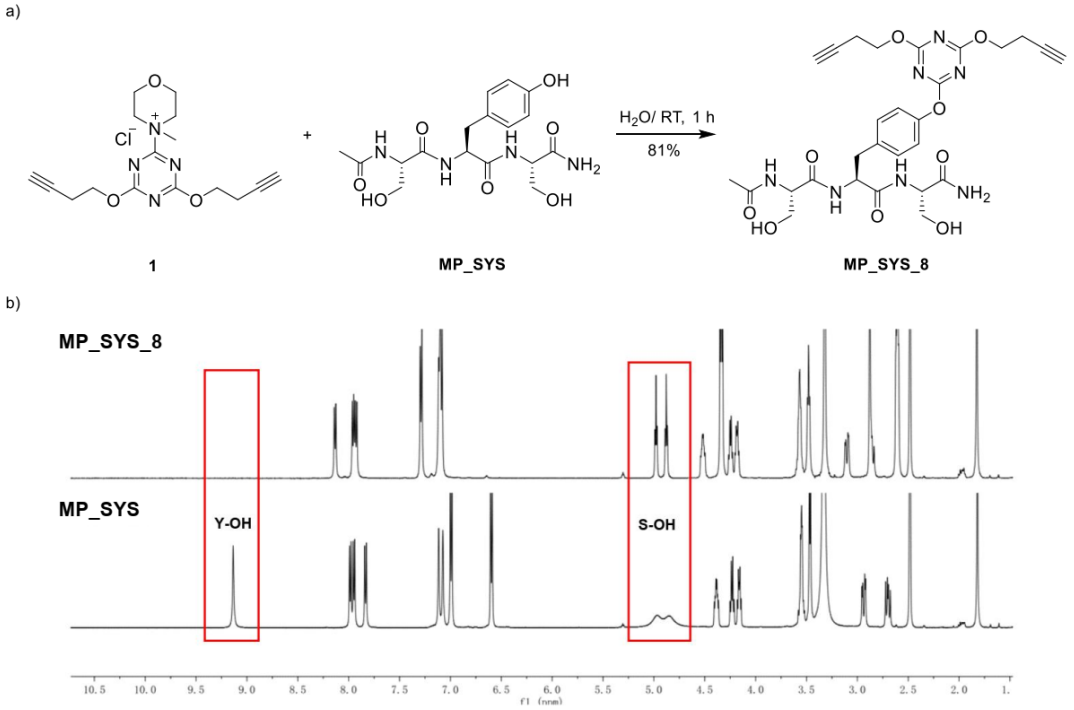
Chemistry

- **Peptide synthesis**
- **General procedures for RP-LC-MS detection**
- **General procedures for reaction screening on model peptides**
- **Chemoselectivity between tyrosine and cysteine**
- **Kinetic study of TPC modification on aromatic hydroxyl group**
- **General information for TPC probes synthesis**
- **NMR Spectra**

Supplementary figures



SI Scheme 1. Tyrosine targeted protein chemical modification strategies.



SI Figure 1. Probe **1** label **MP_SYS**. a) The reaction of probe **1** with **MP_SYS**; b) ^{1}H-NMR analysis (up: ^{1}H-NMR of **MP_SYS_8**, down: ^{1}H-NMR of **MP_SYS**, Y-OH: hydroxyl group of tyrosine, S-OH: hydroxyl group of serine). **MP_SYS:** CH_3CONH-SYS-$CONH_2$, **MP_SYS_8**: probe **1** modified **MP_SYS**.

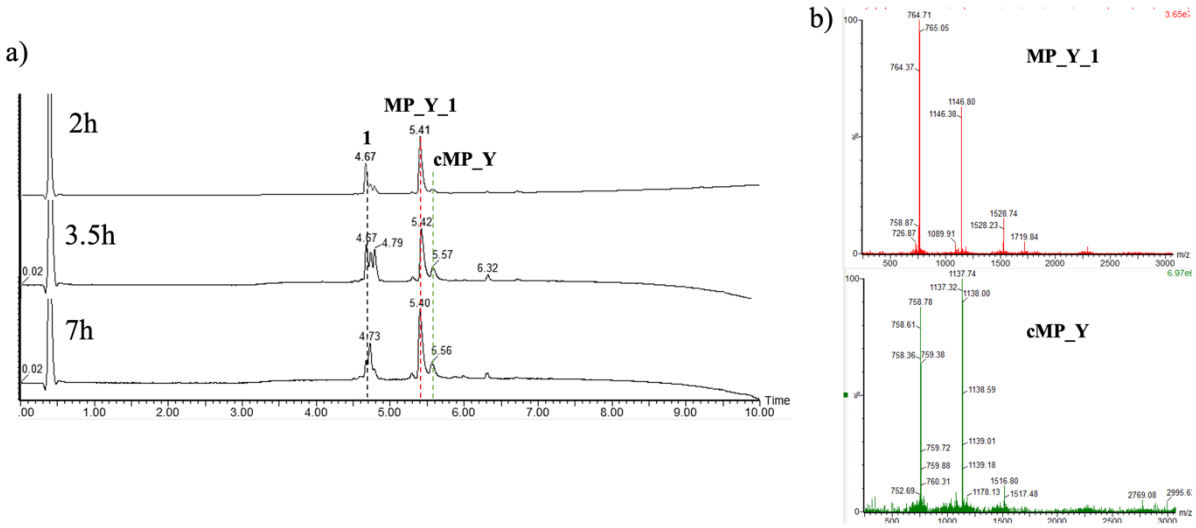
SI Table 1. Reaction screening on model peptide (**MP_Y**: NH_2-NSTKNLTFAMRSSGDYGEV-$CONH_2$)

Entry	Compounds	Equiv.	Conditions[a]	Conversion[b]	Side Reaction[c]
1	**1**	1.3 equiv.	RT, 1h, HEPES buffer, pH 6.8	8.2%	N/A
2	**1**	1.3 equiv.	RT, 1h, PBS buffer, pH 7.4	13.7%	N/A
3	**1**	1.3 equiv.	RT, 1h, Borate buffer, pH 7.4	40.4%	N/A
4	**1**	1.3 equiv.	30 °C, 2.5 h, PBS buffer, pH 7.4	65.9%	N/A
5	**1**	2.5 equiv.	30 °C, 3.5 h, PBS buffer, pH 7.4	71.4%	7.5%
6	**1**	3 equiv.	RT, 4 h, HEPES buffer, pH 6.8	95%	2.1%
7	**2**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	31%	Yes some impurity
8	**3**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	0%	N/A
9	**4**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	34%	Messy
10	**5**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	93%	Low intensity
11	**6**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	70%	Low intensity
12	**7**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	93%	N/A
13	**7**	2 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	92%	N/A
14	**7**	3 equiv.	RT, 4 h, HEPES buffer, pH 6.8	95%	N/A
15	**8**	3 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	84%	Trace amount
16	**8**	2 equiv.	30 °C, 4 h, HEPES buffer, pH 6.8	88%	N/A
17	**8**	3 equiv.	RT, 4 h, HEPES buffer, pH 6.8	87%	N/A

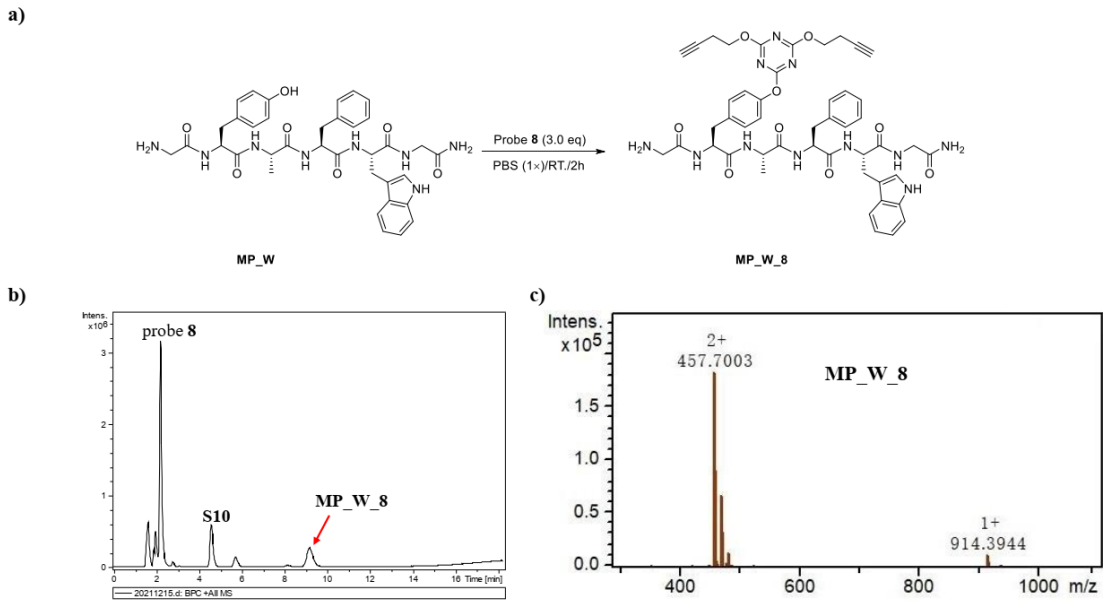
[a]: The reaction concentration is 0.5mM, [b]: The conversion was calculated based on the LCMS, [c]: Side reaction was obtained as coupling reaction.

SI Table 2. Sequences of the model peptides and related conjugates

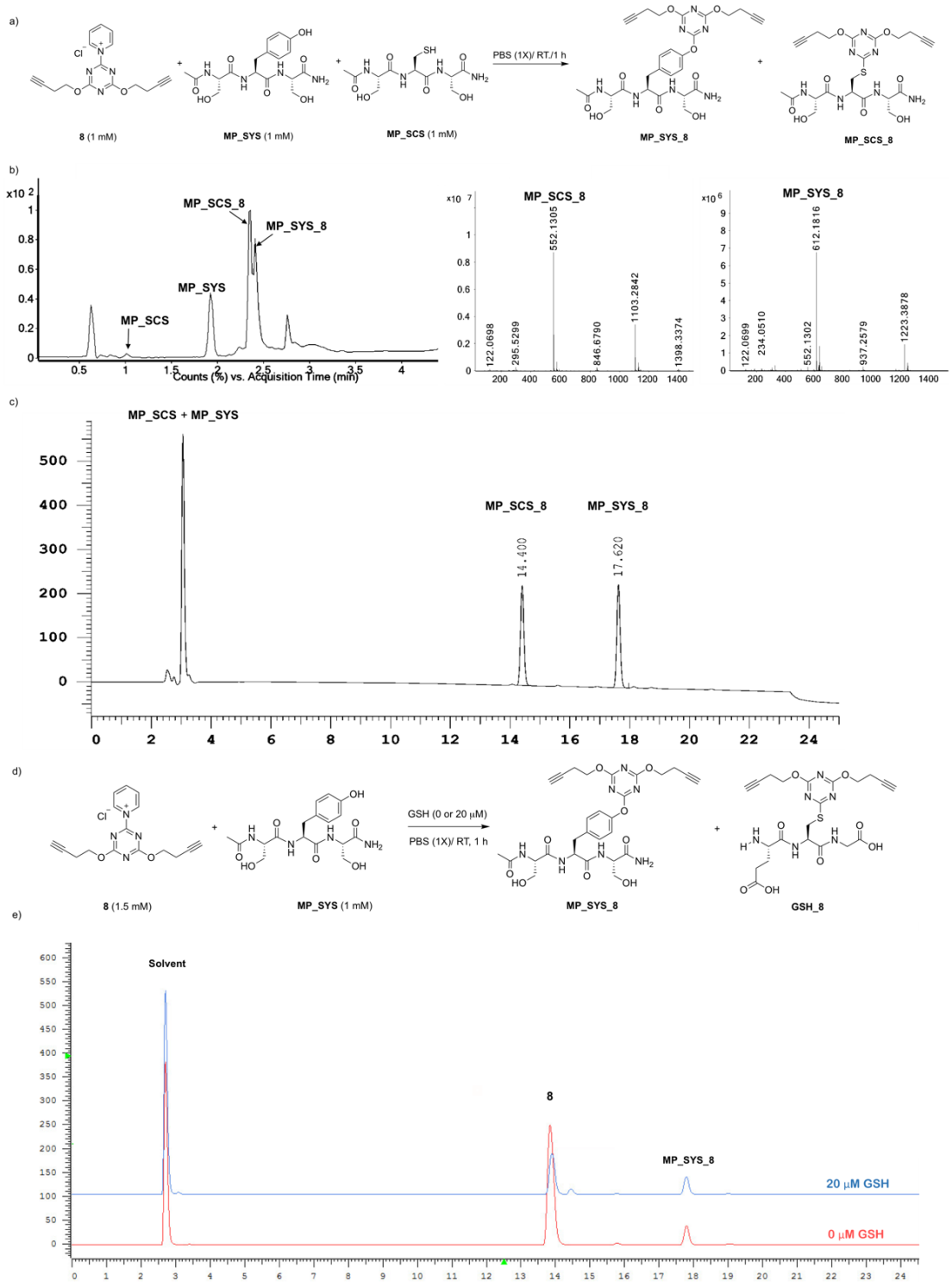
Abbreviation	Sequences
MP_SYS	CH_3CONH-SYS-$CONH_2$
MP_SCS	CH_3CONH-SCS-$CONH_2$
MP_Y	NH_2-NSTKNLTFAMRSSGDYGEV-$CONH_2$
MP_F	NH_2-NSTKNLTFAMRSSGDFGEV-$CONH_2$
MP_SY	NH_2-SFEDIHHYR-$CONH_2$
MP-W	NH_2-GYAFWG-$CONH_2$
MP_SYS_8	CH_3CONH-S**Y**S-$CONH_2$ (Probe **8** modified)
MP_SCS_8	CH_3CONH-S**C**S-$CONH_2$ (Probe **8** modified)
MP_Y_1	NH_2-NSTKNLTFAMRSSGD**Y**GEV-$CONH_2$ (Probe **1** modified)
MP_Y_8	NH_2-NSTKNLTFAMRSSGD**Y**GEV-$CONH_2$ (Probe **8** modified)
MP_SY_8	NH_2-SFEDIHH**Y**R-$CONH_2$ (Probe **8** modified)



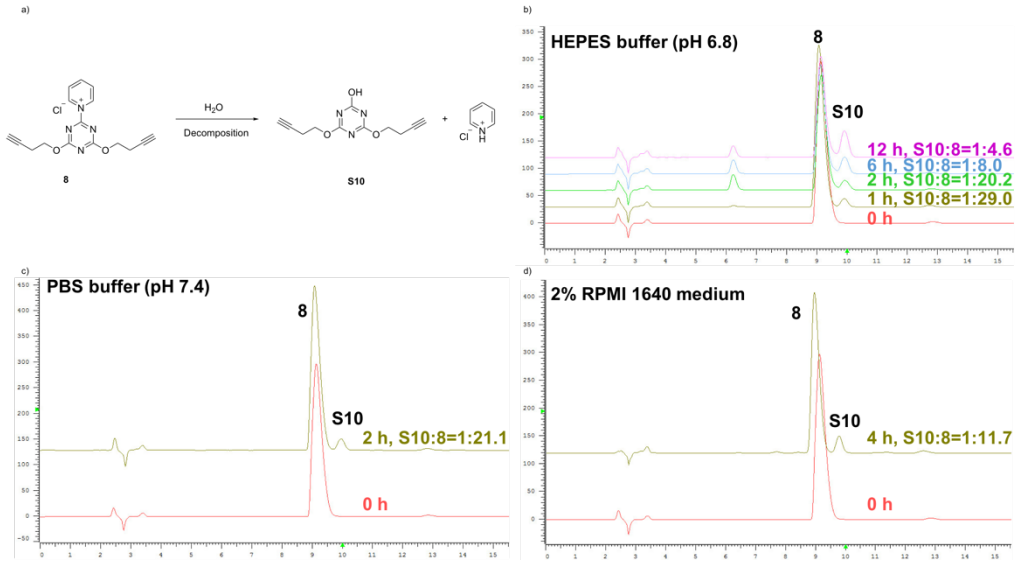
SI Figure 2. Side reaction of probe **1** with **MP_Y**. a) LC trace of probe **1** (2.5 eq) with **MP_Y** (1 eq) in PBS buffer (pH 7.4) at 30℃. b) Mass spectra of probe **1** labeled peptide (**MP_Y_1**) and side-reaction cycylic peptide (**cMP_Y**). **MP_Y_1**: probe **1** modified **MP_Y**.



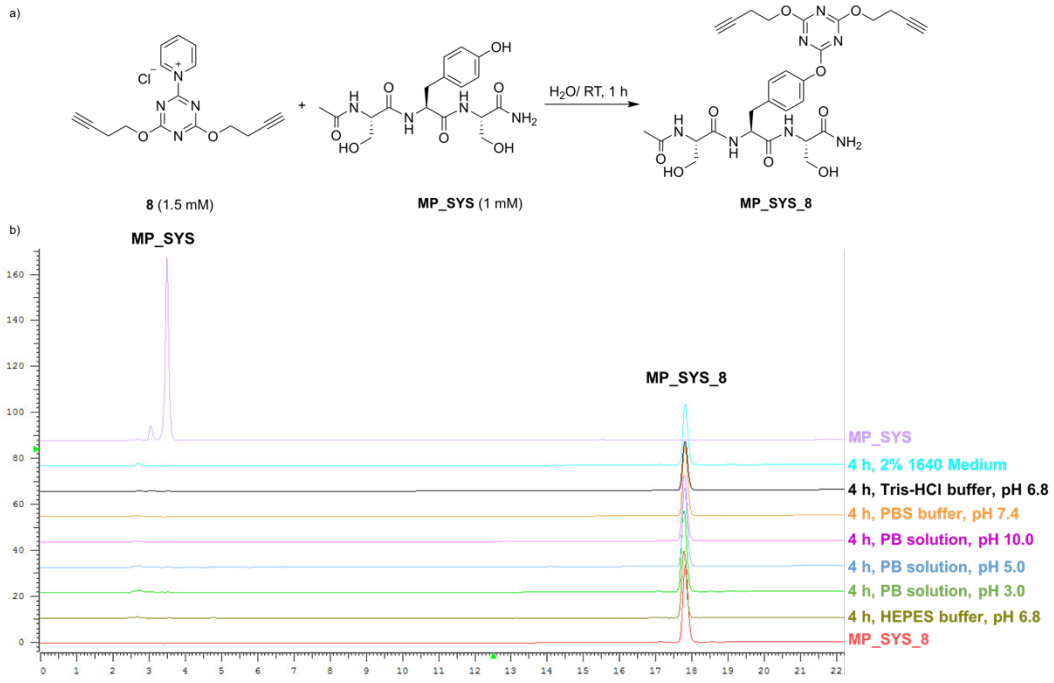
SI Figure 3. Reaction of probe **8** with **MP_W** (NH_2-GYAFWG-$CONH_2$). a) Reaction of probe **8** with **MP_W**. b) LC trace of probe **8** (3 eq) with **MP_W** (1 eq) in PBS buffer (pH 7.4) at 25℃. b) Mass spectra of probe **8** labeled peptide (**MP_W_8**).



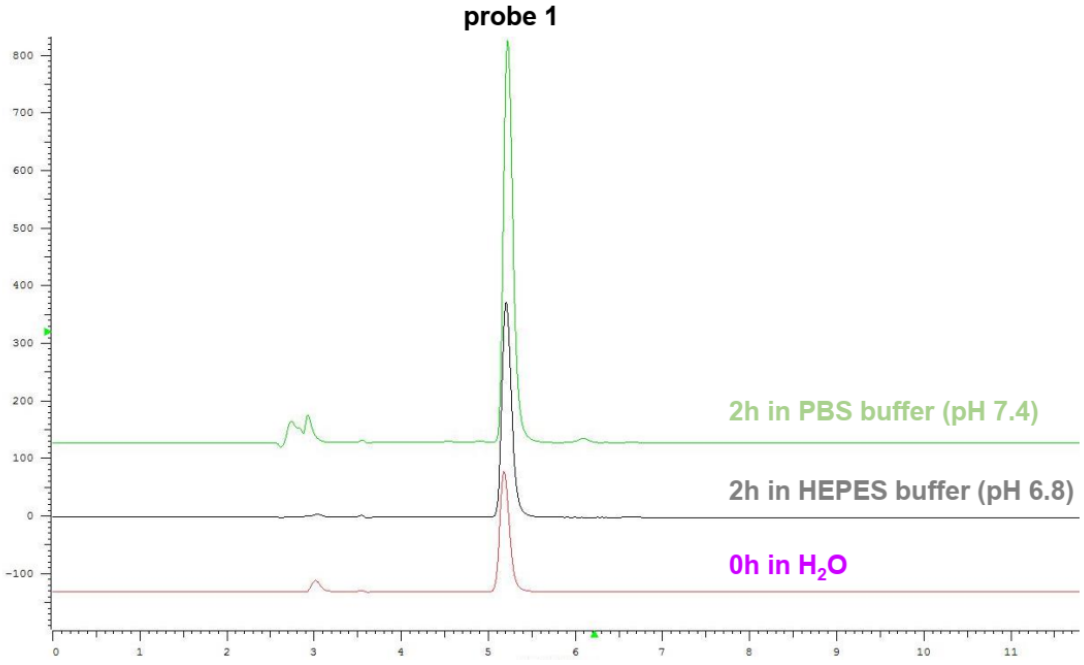
SI Figure 4. Chemoselectivity of probe **8** towards tyrosine and cysteine. a) Reaction of probe **8** with **MP_SCS** and **MP_SYS**; b) LC-MS analysis of the reaction mixture; c) HPLC of the reaction mixture; d) Reaction of probe **8** with **MP_SYS** and **GSH**; e) The influence of **GSH** on the labeling of tyrosine. **MP_SCS:** CH_3CONH-SCS-$CONH_2$, **MP_SYS:** CH_3CONH-SYS-$CONH_2$, **MP_SCS_8**: probe **8** modified **MP_SCS**, **MP_SYS_8**: probe **8** modified **MP_SYS**.



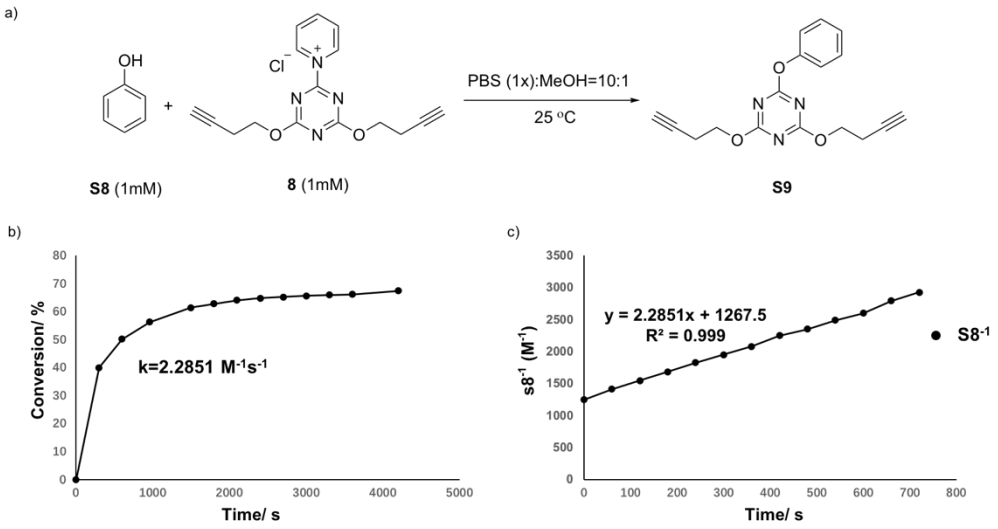
SI Figure 5. Stability of probe **8** in different buffers. a) Proposed hydrolysis of probe **8**; b) Stability of probe **8** in HEPES buffer (pH 6.8); c) Stability of probe **8** in PBS buffer (pH 7.4); d) Stability of probe **8** in 2% RPMI 1640 medium.



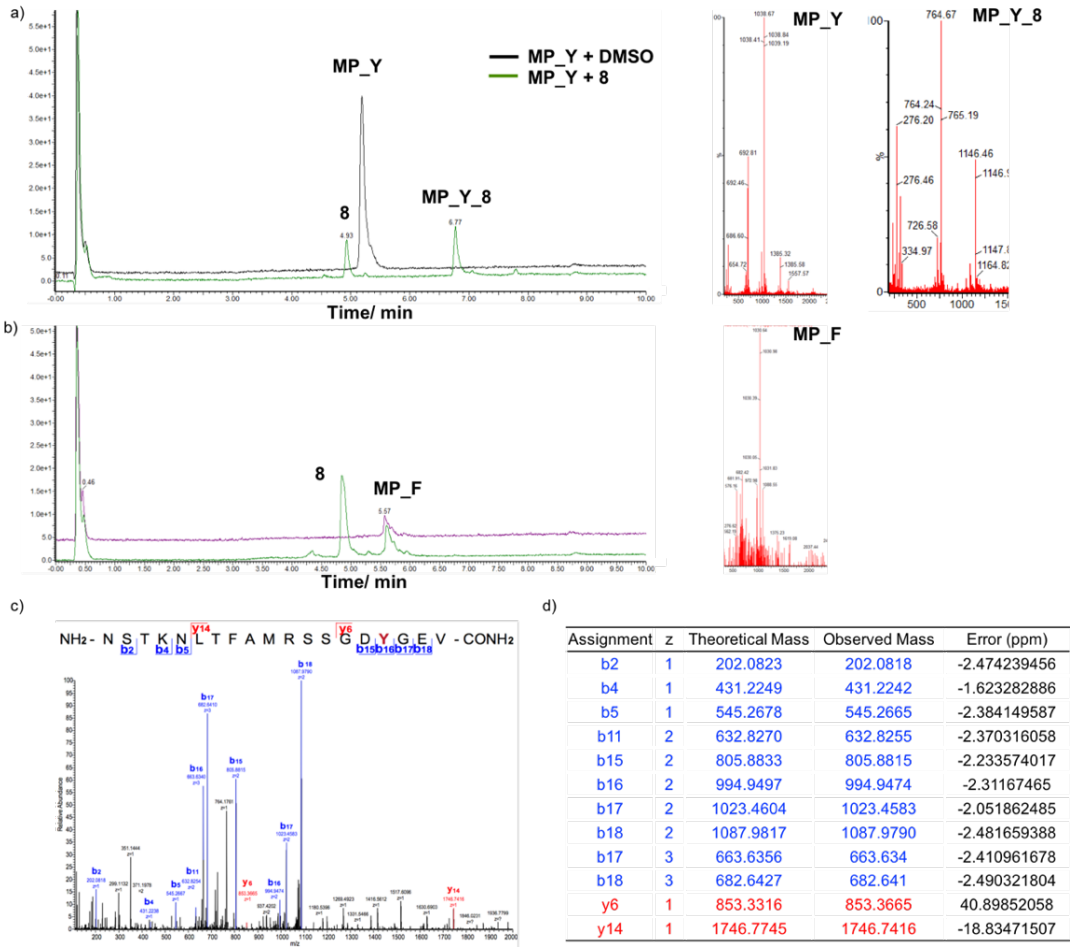
SI Figure 6. Stability of the probe **8** conjugate in different solutions for 4 hours. a) Reaction of probe **8** with **MP_SYS**; b) HPLC of **MP_SYS_8** in different solutions. **MP_SYS:** CH_3CONH-SYS-$CONH_2$, **MP_SYS_8**: probe **8** modified **MP_SYS**.



SI Figure 7. Stability of probe **1** in HEPES buffer (pH 6.8) and PBS buffer (pH 7.4).

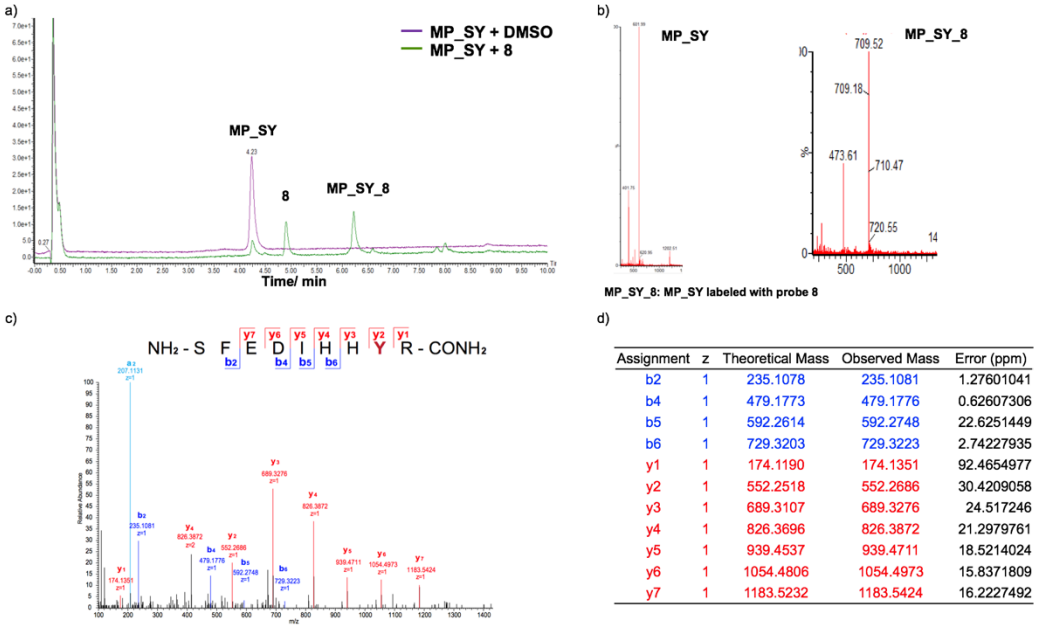


SI Figure 8. Kinetic study of TPC. a) Conversion of **S8** to **S9**. b) Calculation of second order rate constant of **S8** with probe **8** (The initial concentration of **s8** was 0.8 mM, **s8**$^{-1}$ from 0-12 minutes are 1250, 1413, 1547, 1685, 1826, 1954, 2079, 2252, 2354, 2491, 2600, 2792, 2929 M^{-1} separately).



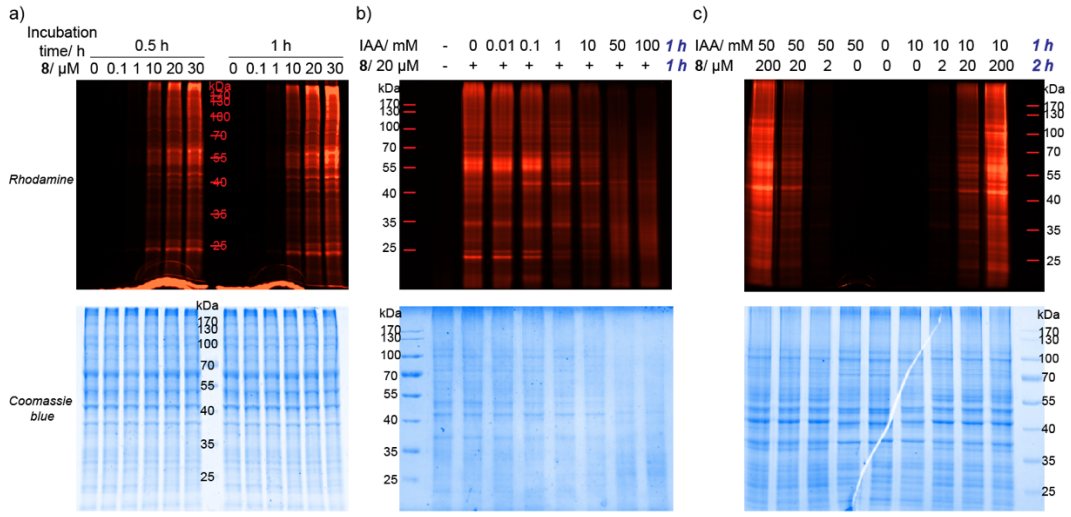
Assignment	z	Theoretical Mass	Observed Mass	Error (ppm)
b2	1	202.0823	202.0818	-2.474239456
b4	1	431.2249	431.2242	-1.623282886
b5	1	545.2678	545.2665	-2.384149587
b11	2	632.8270	632.8255	-2.370316058
b15	2	805.8833	805.8815	-2.233574017
b16	2	994.9497	994.9474	-2.31167465
b17	2	1023.4604	1023.4583	-2.051862485
b18	2	1087.9817	1087.9790	-2.481659388
b17	3	663.6356	663.634	-2.410961678
b18	3	682.6427	682.641	-2.490321804
y6	1	853.3316	853.3665	40.89852058
y14	1	1746.7745	1746.7416	-18.83471507

SI Figure 9. Characterization of probe **8** labeled model peptide (**MP_Y**). a) LC-MS spectra of the reaction mixture of probe **8** with the model peptide (**MP_Y**); b) LC-MS spectra of the reaction mixture of probe **8** with the model peptide (**MP_F**); c) and d) MS/MS annotation of probe **8** labeled model peptide (**MP_Y**). **MP_Y:** NH_2-NSTKNLTFAMRSSGD**Y**GEV-$CONH_2$, **MP_F:** NH_2-NSTKNLTFAMRSSGD**F**GEV-$CONH_2$, **MP_Y_8**: probe **8** modified **MP_Y**.

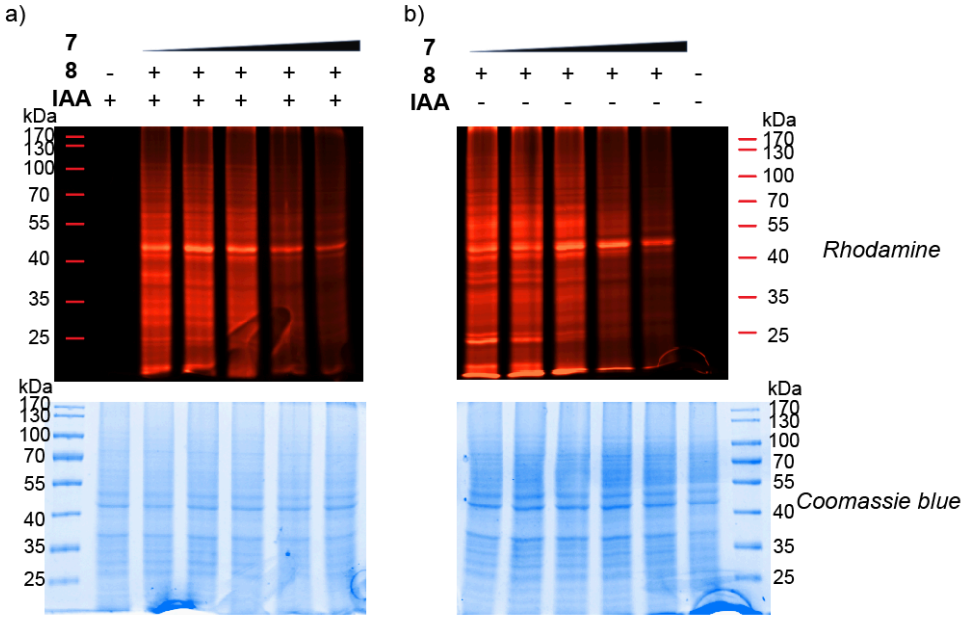


Assignment	z	Theoretical Mass	Observed Mass	Error (ppm)
b2	1	235.1078	235.1081	1.27601041
b4	1	479.1773	479.1776	0.62607306
b5	1	592.2614	592.2748	22.6251449
b6	1	729.3203	729.3223	2.74227935
y1	1	174.1190	174.1351	92.4654977
y2	1	552.2518	552.2686	30.4209058
y3	1	689.3107	689.3276	24.517246
y4	1	826.3696	826.3872	21.2979761
y5	1	939.4537	939.4711	18.5214024
y6	1	1054.4806	1054.4973	15.8371809
y7	1	1183.5232	1183.5424	16.2227492

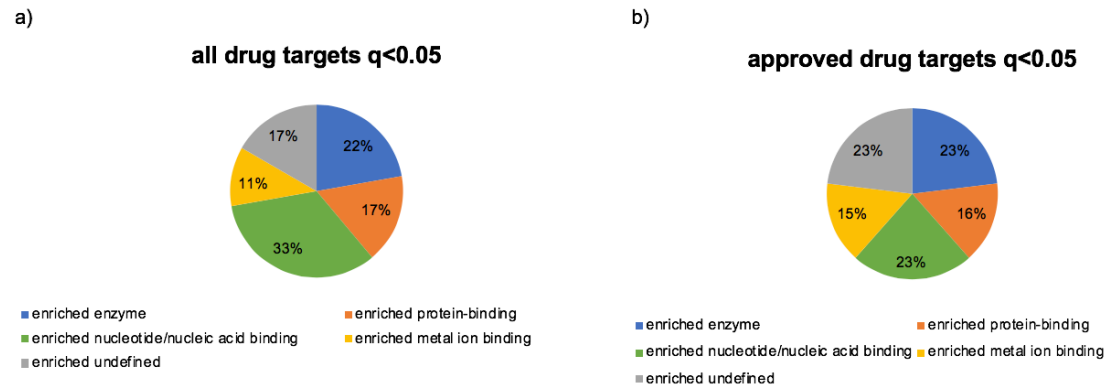
SI Figure 10. Characterization of probe **8** labeled model peptide (**MP_SY**). a) and b) LC-MS spectra of the reaction mixture of probe **8** (or **DMSO**) with model peptide (**MP_SY**); c) and d) MS/MS annotation of **MP_SY_8**. **MP_SY:** NH_2-SFEDIHH**Y**R-$CONH_2$, **MP_SY_8**: probe **8** modified **MP_SY**.



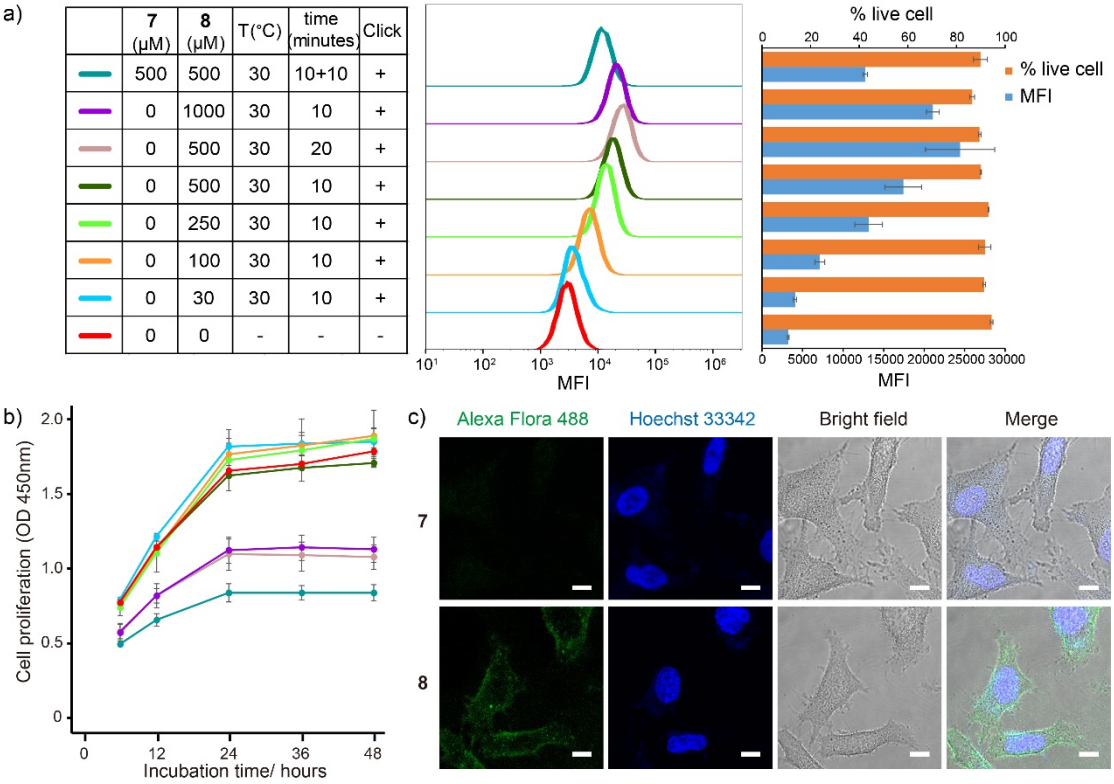
SI Figure 11. Concentration and time dependent labeling of probe **8** in HeLa total cell lysates. a) Concentration-dependent labeling of probe **8** in HeLa cell lysates for 0.5 hour and 1 hour. b) Influence of concentration-dependent treatment of IAA to probe **8** labeling in HeLa cell lysates. c) Concentration-dependent labeling of probe **8** in iodoacetamide (IAA, final concentration 10 mM/ 50 mM) treated HeLa cell lysates.



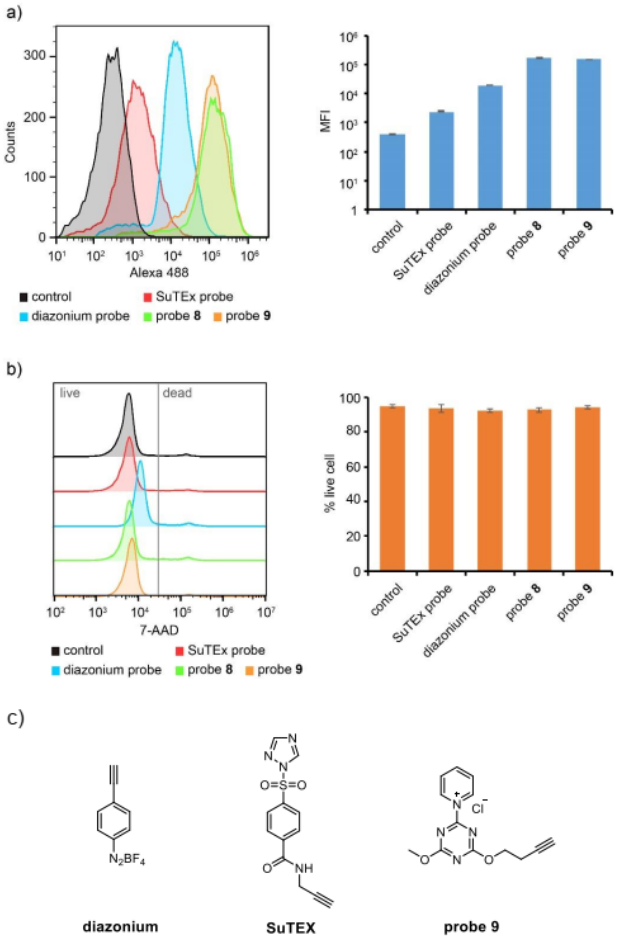
SI Figure 12. Labeling competition of probe **8** (20 μM) in the whole proteome by compound **7**. a) Concentration-dependent competition of compound **7** (0, 20, 200, 2000, 10000 μM) for the labeling of probe **8** (20 μM) after IAA (20 mM) blocking; b) Concentration-dependent competition of compound **7** for the labeling of probe **8** without IAA blocking.



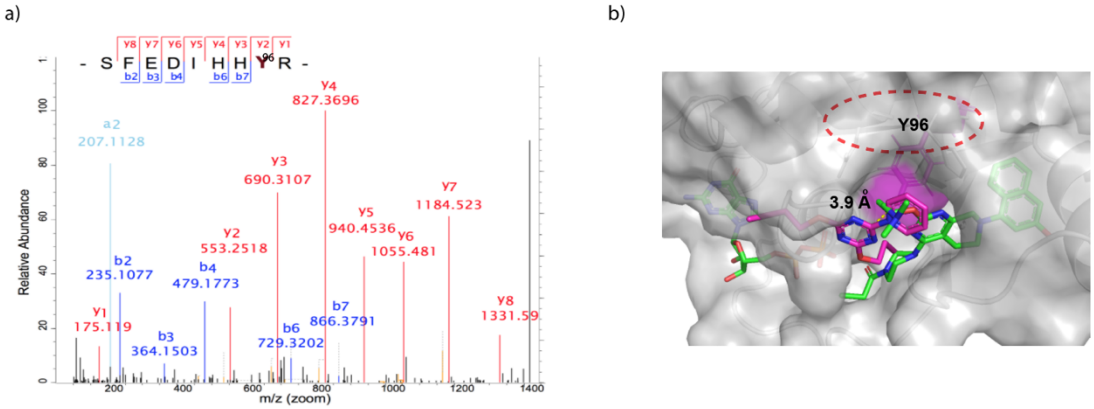
SI Figure 13. Functional annotation of probe **8** modified peptides in Drugbank Database. a) Functional annotation of probe **8** modified peptides in all drug targets; b) Functional annotation of probe **8** modified peptides in approved drug targets.



SI Figure 14. Biocompatibility of probe **8** in live cell labelling. a) Flow cytometry data of live cell labelling and cell viability after labelling with probe **8** or compound **7** at designated conditions (incubation concentration and time) in HeLa; b) Time-dependent cell viability treating with designated conditions shown in a); c) Live cell imaging of probe **8** (500 μM)) or compound **7** (500 μM) labelled HeLa, Scale bar: 10 μm. MFI: mean fluorescence intensity.

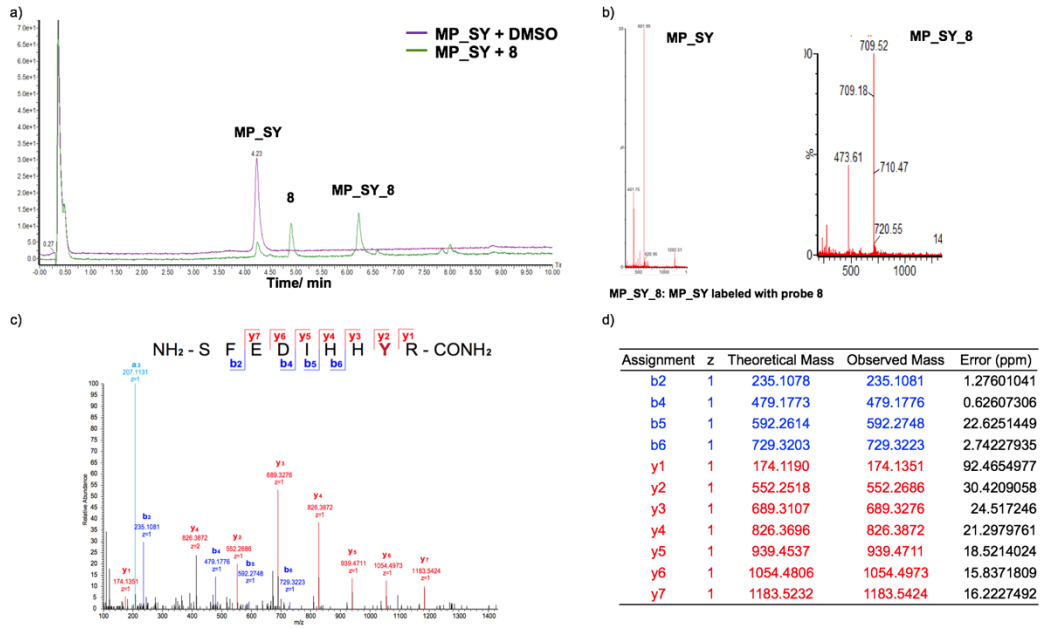


SI Figure 15. Labelling efficiency and biocompatibility of 3 kinds of tyrosine labelling probes in Hela cells. a) Flow cytometry data of live cell labelling after probe reaction and CuAAC; b) Flow cytometry data of 7-AAD staining after probe reaction. The labelling condition of all probes is at concentration of 500 μM for labelling at 30 ℃ for 10 minutes. Control group represents cells that are not treated with any tyrosine labelling probes; c) The structure of diazonium (synthesized according to the previous report[1]), SuTEX (synthesized according to previous report[2]) and probe **9**.



SI Figure 16. Y^{96} labeled by probe **8** on recombinant KRas. a) MS^2 spectra of the modified tyrosine on peptide (SFEDIHHYR) of KRas; b) Superimpose of the noncovalent docked conformation of probe **8** with KRas (pink) and the protein

cocrystal complex covalently bounded (PDB: 6N2K) (green). Region containing SFEDIHHYR peptides is shown in a red dash circle. The distance between Y96 and the carbon of 1,3,5- triazin moiety was 3.9 Å in yellow dashed line.



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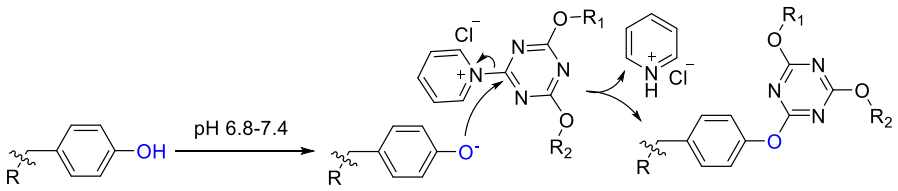
y6	1	1054.4806	1054.4973	15.8371809
y7	1	1183.5232	1183.5424	16.2227492

SI Figure 17. Characterization of probe **8** labeled model peptide (**MP_SY**). a) and b) LC-MS spectra of the reaction mixture of probe **8** (or **DMSO**) with model peptide (**MP_SY**); c) and d) MS/MS annotation of **MP_SY_8**.



SI Figure 18. Proposed mechanism of action of TPC.

Materials

Rhodamine-azide and TBTA were synthesized in house. Biotin-DADPS-Azide, Biotin-azide, BTTAA were purchased from Click Chemistry Tools, Scottsdale, AZ. DMEM (high glucose), FBS, PBS, Alexa Fluor 488-streptavidin, 7-AAD, Hoechst 33342, high capacity streptavidin agarose, and MS grade trypsin were purchased from Thermo, Waltham, MA. Cell Counting Kit 8 (CCK8) was purchased from Abcam, Cambridge, UK.

Other chemicals are from Sigma Aldrich.

All compounds were solved in DMSO to make stock solutions and diluted in the related buffer to make working solutions.

Cell culture, lysis and labeling with probes

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific) supplemented with 10% FBS (FBS; ThermoFisher Scientific), 100 U/ml penicillin and 100 g/ml streptomycin (ThermoFisher Scientific). Cells were maintained in a humidified 37 ℃ incubator with 5% CO_2.

HeLa cells were lysed in HEPES buffer (50 mM HEPES, 150 mM NaCl, 0.1% Tween 20, 20% glycerol, 2% $MgCl_2$, pH 7.5). The cell lysates protein concentration was measured by BCA assays (ThermoFisher Scientific) and normalized to 2 mg/mL.

For concentration-dependent labeling of whole proteome, 50 μL of HeLa total cell lysates (2 mg/mL) was labeled with indicated concentrations of the probe at 30 ℃ for designated incubation time. For iodoacetamide (IAA) pretreated cell lysates labeling, designated final concentrations of IAA were incubated with total cell lysates at 30 ℃ for designated incubation time (1 hour or 2 hours) in the dark before incubation with the probe. For concentration-dependent competition of compound **7** (designated concentrations) to probe **8** (20 μL) labeling in HeLa cell lysates, HeLa cell lysates were pretreated with 20 mM IAA at 30 ℃ for 1 hour, followed by incubation of designated concentrations of the competitor at 30 ℃ for 2 hours and the incubation of the probe at 30 ℃ for 2 hours.

Cu(I)-catalyzed cycloaddition/ click chemistry

The procedure was modified from the previous report[3]. For the fluorescent labeling experiment, 10 μL of labeled protein sample was subjected to click reaction. Rhodamine-azide was added to a final concentration of 100 μM, followed by 1 mM TCEP and 100 μM TBTA, and the reaction was initiated by the addition of 1 mM $CuSO_4$. The reaction mixture was incubated for 2 hours at room temperature. After that, 2.5 μL 5×loading buffer was added and boiled at 95 ℃ for 10 minutes before SDS-PAGE.

In-gel fluorescence scanning

The protein samples after clicking with rhodamine were resolved by SDS-PAGE and visualized by scanning the gel under Bio-Rad ChemiDoc imaging system. Coomassie blue staining was performed as the reference of equal sample loading.

Proteome labeling by TPC probe

For proteome sample preparation, HeLa cell lysates were pretreated with 50 mM IAA at 30 ℃ for 1 hour, followed by incubation with probe **8** (100 μM) at 30 ℃ for 2 hours. After the whole proteome labeling by the probes, the reaction mixtures were subjected to Cu(I)-catalyzed cycloaddition with rhodamine-azide at room temperature for 2 hours and separated by SDS-PAGE. Fluorescence gel scanning displayed the labeling of the whole proteome.

Stability of probe 8 and conjugates

10 μL Probe **8** (100 mM in DMSO) was incubated in 1 mL HEPES buffer (50 mM HEPES, 150 mM NaCl, 0.1% Tween 20, 20% glycerol, 2% $MgCl_2$, pH 7.5) at 30 ℃. UPLC was detected after 1 hour and 2 hours separately.

40 μL Probe **8** (5 mM in H_2O) and 20 μL of the corresponding buffer solutions (0.1M HEPES buffer (pH 6.8), 0.1M PBS buffer (pH 7.4), 20% RPMI 1640 medium) were added into 140 μL H_2O and incubated at 30 ℃ for designated time (0 hour, 1 hour, 2

hours, 4 hours, 6 hours, 12 hours). The solutions were analyzed by HPLC. The decomposition product **S10** of probe **8** was detected by LC-MS.

40 μL **MP_SYS_8** and 20 μL of the the corresponding buffer solutions (0.1M HEPES buffer (pH 6.8), 0.1M Phosphate buffer (pH 3.0-10.0), 0.1M PBS buffer (pH 7.4), 0.1M Tris-HCl buffer (pH 6.8), 20% RPMI 1640 medium in H_2O) were incubated at 30 ℃ for 4 hours, the solutions were analyzed by HPLC.

40 μL **MP_SYS_8** and 20 μL of the the corresponding buffer solutions (0.1M HEPES buffer (pH 6.8), 0.1M Phosphate buffer (pH 3.0-10.0), 0.1M PBS buffer (pH 7.4), 0.1M Tris-HCl buffer (pH 6.8), 20% RPMI 1640 medium in H_2O) were incubated at 30 ℃ for 4 hours, the solutions were analyzed by HPLC.

Stability of probe 1

40 μL Probe **1** (5 mM in H_2O) and 20 μL of the corresponding buffer solutions (0.1M HEPES buffer (pH 6.8), 0.1M PBS buffer (pH 7.4) were added into 140 μL H_2O and incubated at 30 ℃ for designated time (0 hour, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours). The solutions were analyzed by HPLC.

Streptavidin affinity enrichment of probe labeled peptides in whole proteome

Total HeLa cell lysates (2 mg/mL, 2 mL, pretreated with 20 mM IAA) were incubated with the probe (final concentration: 10 μM and 100 μM, 2 μL 10 mM and 100 mM in DMSO separately) at 30 ℃ for 2 hours. The labeled proteome was then subjected to Cu(I)-catalyzed cycloaddition with Biotin-DADPS-Azide (4 equiv.) at room temperature for 2 hours according to the manufacture. After clicking with Biotin-DADPS-Azide, the labeled proteome was precipitated by 4 volumes of ice-cold acetone at -20 ℃ overnight. After washing with ice-cold methanol twice, the air-dried protein pellet was dissolved in PBS with 4% SDS, 20 mM EDTA and 10% glycerol by vortexing and heating. The solution was then diluted with 10 volumes of PBS. High-capacity streptavidin agarose beads (ThermoFisher Scientific) were added to bind the biotinylated proteins with rotating for 2 hours at room temperature. To remove nonspecific binding, the beads were then washed with PBS with 0.2% SDS, 6 M urea in PBS with 0.1% SDS and 250 mM NH_4HCO_3 with 0.05% SDS sequentially.

On beads digestion

The beads were soaked in 100 μL 10 mM NH_4HCO_3 and heated at 55 ℃ for 10 minutes. Then the beads were incubated with 20 mM dithiothreitol (DTT) in 100 μL 10 mM NH_4HCO_3 at 55 ℃ for 40 minutes and 50 mM iodoacetamide in 100 μL 10 mM NH_4HCO_3 in the dark at room temperature for 40 minutes. The beads were incubated with 2 μg trypsin in 20 μL trypsin buffer at 37 ℃ for 16 hours. After trypsin digestion, the supernatant was discarded and the beads were washed with 500 μL H_2O twice. Then the beads were eluted with 50 μL 10% formic acid at room temperature for 0.5 hour twice. The eluted enriched peptides were collected, desalted with StageTips, and dried by SpeedVac.

MS/MS analysis for peptides

Model peptides (**MP_Y_8** and **MP_SY_8**) and probe **8** were dissolved in DMSO to make 100 mM stock solutions. 5 μL 100 mM model peptide (**MP_Y_8** or **MP_SY_8**) was diluted in 1 mL HEPES buffer (pH 6.8) buffer to make 0.5 mM model peptide

reaction solution separately. 5 μL 100 mM probe **8** stock solution in DMSO was added into the model reaction solution above mentioned. After rotating at room temperature for 4 hours, the reaction mixtures were lyophilized. Crude **MP_Y_8** and **MP_SY_8** (reaction of **MP_Y_8** and **MP_SY_8** with probe **8** separately) were directly desalted using C18 stage tips. After concentrating, the crude peptide was analyzed using a linear ion trap Orbitrap Velos mass spectrometer (LTQ Orbitrap Velos, Thermo-Scientific). Briefly, the crude peptide was dissolved in MeOH and directly injected into ESI source using a syringe with flow rate of 4 μL/minute. The spray voltage was set to 3000 V and capillary temperature was set to 275 ℃. The MS/MS scans were recorded at a resolution of 30000 with a mass range from 100 to 1800 m/z. Peak at 709.32 m/z **(MP_SY_8)** or 764.69 m/z **(MP_Y_8)** was isolated as parent mass and fragmentation type was set to HCD (40.00). The normalized collision energy was set to 25, Act Q was set to 0.25 and Act time was set to 0.1 ms. Ppm was calculated based on this: Error (ppm) = ((Observed m/z -theoretical m/z)/theoretical m/z)*10^6.

MS/MS analysis of probe 8 labeling in whole proteome

Crude peptides after desalting were further analyzed on an Orbitrap Fusion^{TM} Tribrid^{TM} mass spectrometer (Thermo-Scientific) with a Nanospray Flex^{TM} ion source (Thermo-Scientific) connected to an Easy-nLC 1200 system (Thermo-Scientific). A 15-cm-long fused silica emitter (75 μm I.D., 360 μm O.D., 15 μm Tip I.D.; New Objective, Inc.) was packed with Reprosil-Pur C18-AQ 2.4 μm resin (Dr. Maisch GmbH). TMT labeled peptide was resuspended in buffer A (LC-MS grade H_2O with 0.1 % FA (v/v)) and introduced into the column through the autosample of the Easy-nLC 1200 system. Peptides were eluted, further separated by a 240 minutes organic solvent gradient at a flow rate of 300 nL/minute and sprayed directly into MS. The Orbitrap Fusion was operated with a positive ion spray voltage of 2000 V and a transfer tube temperature of 320 ℃. Data acquisition on the Orbitrap Fusion was carried out using a data-dependent method. The Full scans were recorded at a resolution of 120K with a mass range from 350 to 1800 m/z. The HCD MS/MS scans (isolation mode was quadrupole; isolation window was 0.7 Da; collision energy (%) was 38 with stepped collision energy (%) of 5; first mass was set to 100) were recorded at a resolution of 50 K.

The raw data files were searched with MaxQuant software (version 1.5.5.1) (Cox J, Mann M (2008) against *homo sapiens* UniProt FASTA database (downloaded on April 7th, 2020, 188357 entries), Drugbank all drug targets and Drugbank approved drug targets (Drugbank database 5.0) separately[4]. The false discovery rate (FDR) evaluation was done by searching a reverse database and was set to 0.01 for proteins and peptides. The parameters were used as follow: cysteine carbamidomethylation was set as a fixed modification, whereas methionine oxidation, glutamine deamidation were set as variable modifications. **8**_B1 and **8**_B2 were set as variable modifications with a mass shift of + 358.1753 Da and + 501.2812 Da. The maximum number of missed cleavages was set to two for trypsin digestion. The "Match between runs" option was selected, and the other parameters were set to default values. Contaminants, proteins identified by reverse identification, and proteins with ≤ 1 unique peptide were excluded from further analysis. For probe **8** modified peptides, only peptides with a minimum length of 7 amino acids, PEP ≤ 0.01, modification sites ≥ 1, and intensity > 0 were

accepted as bona fide modified peptides. Bona fide modified peptides in at least one of the three technical replicates were counted in the final modified peptides. Protein numbers used in the percentage calculation in this manuscript were all based on the proteins from modification sites after database searching and the strict screening (as attached file 'Modified peptides_proteins_homo sapiens', 'Modified peptides_proteins_All Drug Targets').

All the raw mass spectrometry data have been deposited in the public proteomics repository MassIVE and are accessible at ftp://massive.ucsd.edu/MSV000086762.

Domain enrichment analysis

Probe **8**-modified sites were scanned for ProRule domains[5] by InterProScan v81.0[6]. Annotated Uniprot[7] human proteome (accessed date: 15th Sep. 2020) was used as a reference database for domain enrichment analysis. A probe-modified site that was annotated as a ProRule domain was considered an "enriched hit". If a site was annotated to more than one domain, each one was considered as a hit. Probe-modified sites with large chunks of shared sequences were regarded as duplications and only counted once. Counts in reference database were determined by the number of nonoverlapping occurrences of the domain (e.g. CALM1 was accounted for four EF-hand domains (PRU00448)). P-value of each domain that was annotated in probe-modified sites was calculated by two-sided Fisher's exact test using R. The probability of observing such distribution can be calculated by:

$$P = \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

where K is the number of all domain occurrences in the experimental dataset (probe), k is the number of a certain domain occurrence in the experimental dataset; N is the number of all domain occurrences in the reference dataset (Uniprot), n is the number of a certain domain occurrence in the reference dataset. The P values were then corrected by Benjamini–Hochberg correction for multiple hypothesis testing. A domain was considered significantly enriched if it had q value <0.05 (i.e. false discovery rate < 5%). Enriched domains were grouped into 5 categories by their functions based on GO numbers: nucleotide/nucleic acid binding (GO:0000166, GO:0003676), enzyme (GO:0003824), protein-binding (GO:0005515), metal ion binding (GO:0046872) and undefined (other or no GO). Domains are considered significantly overrepresented if they have q values < 0.05 after Benjamini – Hochberg correction of a two-sided Fisher's exact test.

Labeling on HeLa cells with probes and analyzing by flow cytometry (FACS)

HeLa cells were grown in monolayer in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were then harvested by trypsinization and distributed into a 96-well U-bottom plate (0.5 M cells/well). The cells were washed with PBS and pelleted (400 × g, 3 minutes), followed by resuspension in 100 μL of PBS containing different concentrations of probe **8** (0 μM, 30 μM, 100 μM, 250 μM, 500 μM, 1000 μM) with or without compound **7** (500 μM). The reactions were stopped by adding 100 μL of culture medium at various time points. The cells were then pelleted, washed two times

with PBS (1% FBS), and resuspended in the same buffer containing 100 μM biotin-azide, BTTAA-$CuSO_4$ complex ([BTTAA]: [$CuSO_4$] = 300 μM:50 μM), 2.5 mM sodium ascorbate. After a 5 minutes CuAAC reaction on ice, the cells were pelleted, washed two times with PBS (1% FBS), and incubated in Alexa Fluor 488-streptavidin (1 μg/mL) on ice for 30 minutes. After washing for two times with PBS (1% FBS), the cells were resuspended in 200 μL of the same buffer containing 2 μg/mL 7-AAD for flow cytometry analysis. Flow cytometry experiments were performed on an ACEA NovoCyte flow cytometer using a 488 nm argon laser. For each sample, at least 30000 cells were recorded. Flow cytometry data was analyzed using Flowjo. Cell viability was determined by gating the sample on the basis of forward scatter and 7-AAD channel. Mean fluorescence intensity (MFI) for Alexa Fluor 488 was calculated for live cells.

Similar procedures were performed for probe diazonium, SuTEX and probe **9**.

Cell proliferation assay

HeLa cells grown in monolayer were trypsinized, washed and distributed into a 96-well plate (5000 cells/well). Allowed the plate overnight a humidified incubator (37 °C, 5% CO_2) to settle down the cells. The cells then were incubated in 200 μL of PBS containing different concentrations of probe **8**. The reactions were stopped by adding 100 μL of culture medium at various time points. After incubating the plate for an appropriate length of time, 10 μl of CCK-8 solution was added to each well, followed by 1 hour incubation. The absorbance at 450 nm was measured using a microplate reader.

Imaging of probe 8 labeled live cells by confocal microscopy

HeLa cells were cultured in 35 mm glass bottom dishes in DMEM supplemented with 10% FBS. The cells (roughly 70% to 90% confluent) then were washed with PBS two times and incubated in 200 μL of PBS containing 1 μL 100 mM compound **7** or probe **8** (final conc. 500 μM) at 30 ℃ for 10 minutes. The cells were then washed twice with PBS (1% FBS), followed by CuAAC reaction and Alexa Fluor 488-streptavidin incubation as described above. After washing twice, the cells were treated with Hoechst 33342 (1 μg/mL) to stain the nucleus. Following incubation for 10 minutes, the cells were washed three times with PBS (1% FBS), and imaged using a Leica TCS SP8 confocal microscope with a 63×/1.4 oil objective. All images were acquired using a 0.1 μm step, and the composite figures were prepared by ImageJ.

Expression of GST-KRas

Recombinant GST-tagged human KRAS G12C (1-188) was expressed in *Escherichia coli* and purified using affinity chromatography and size-exclusion chromatography. Briefly, human KRas4B (Met1-Met188) (Uniprot identifier: P01116-2) was cloned into pET-28 a (+) vector with a N-terminal His-GST-tag. G12C mutants was generated by site-directed mutagenesis. *E.coli* BL21(DE3) cells were transformed with corresponding plasmid and grown on LB agar plates containing 50 μg/mL Kanamycin. A single colony was inoculated into LB medium with 50 μg/mL Kanamycin at 37 ℃, 220 rpm. When OD600 reached 0.6, the temperature was cooled to 16 ℃. Then IPTG was added to final concentration of 200 μM and incubated for 20 h. The cell was collected by centrifugation (13000 × g, 3 min) and lysed by ultrasonic liquid processors

with lysis buffer (20 mM Tris, 500 mM NaCl, pH = 7.5) before being centrifuged at 20000 × g for 30 min. The protein was purified by GST-affinity column (BeyoGold, China) and size-exclusion chromatography. The buffer was then substituted to HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 6.8).

Labeling on recombinant protein GST-KRas

GST-KRas in HEPES pH 6.8 was incubated with 110 μM probe **8** (0.5 equiv of all Y on GST-KRas) at 30 ℃ for 30 minutes separately. After the incubation, the samples were subjected to click reaction and LC-MS sample preparation.

MS analysis for probe 8 modified recombinant protein KRas

Crude peptides were subjected to LC-MS/MS. MS analysis was performed on a linear ion trap Orbitrap Velos mass spectrometer (LTQ Orbitrap Velos, Thermo-Scientific) with a nanoelectrospray ion source (Shortgun Proteomics Inc.) connected to HPLC (Finnigan, Thermo-Scientific). A 12-cm-long fused silica emitter (75 μm I.D., 360 μm O.D., 15 μm Tip I.D.; New Objective, Inc.) was packed with Reprosil-Pur C18-AQ 5-μm resin (Dr. Maisch GmbH). Digested peptides were resuspended in buffer A (99.9% water and 0.1% formic acid (v/v)) and injected into the column by a thermostated micro-autosampler (Finnigan, Thermo-Scientific). Peptides were eluted, further separated by a 100 min organic solvent gradient and sprayed directly into MS. Unattended data acquisition mode was applied in which the spectrometer automatically alternated between a high resolution (R = 60,000) full scan and a lower resolution (R = 7,500) fragment scan of the ten most abundant ions of peptides.

The raw data files were searched with MaxQuant software (version 1.5.5.1) (Cox J, Mann M (2008) against the KRas from *homo sapiens* UniProt FASTA database (downloaded on Dec. 31th, 2019, 78 entries). The false discovery rate (FDR) evaluation was done by searching a reverse database and was set to 0.01 for proteins and peptides. The parameters were used as follow: cysteine carbamidomethylation was set as a fixed modification, whereas methionine oxidation, glutamine deamidation were set as variable modifications. Probe **8** was set as a variable modification with a mass shift of + 215.0695 Da. The maximum number of missed cleavages was set to two for trypsin digestion. The "Match between runs" option was selected, and the other parameters were set to default values. Contaminants, proteins identified by reverse identification, proteins identified only by site and proteins with ≤ 1 unique peptide were excluded from further analysis. For probe **8** modified peptides, only peptides with a minimum length of 7 amino acids, PEP ≤ 0.01, modification sites ≥ 1, intensity > 0, location probability >0.5 and typical MS/MS spectrum were accepted as bona fide modified peptides. Bona fide modified peptides in at least one of the three technical replicates were counted in the final modified peptides.

Chemistry

Peptide Synthesis

All commercial materials (Sigma-Aldrich, CSBio, Chem-Impex and GL Biochem) were used without further purification. All solvents were reagent grade or HPLC grade (RCI or DUKSAN). The following Fmoc amino acids were purchased from GL Biochem and Chemimpex. The solid phase peptide synthesis was following previous reported procedures[8]. Briefly, Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Pro-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Trp(Boc)-OH were loaded on resin according to designated peptides. All separations involved a mobile phase of 0.1% trifluoroacetic acid (TFA) (*v/v*) in acetonitrile (ACN) and H_2O.

Synthesis was performed manually on rink amide resin (GL Biochem) under the standard Fmoc protocol. Removal of Fmoc protecting group was performed using a mixture of 20/80 (*v/v*) of piperidine/DMF for 10 minutes. Coupling was performed using Fmoc-Amino acids (4.0 equiv.), HATU (4.0 equiv.) and DIPEA (8.0 equiv.) in DMF for 1 h at room temperature. Upon completion of the synthesis, 9.5:0.25:0.25 of TFA: TIPS: H_2O (*v:v:v*) were used to perform global deprotection. The peptides were then precipitated in diethyl ether and purified by semi-preparative RP-HPLC.

General procedures for RP-LC-MS detection

For all the RP-LC-MS and RP-UPLC detection of OPA-peptide conjugates, all separations were conducted in a mobile phase of 0.1% TFA (*v/v*) in acetonitrile and 0.1% TFA (*v/v*) in H_2O. RP-LC-MS analysis was performed with Waters HPLC system equipped with photodiode array detector (Waters 2996) using Vydac 218TP C18 column (5 μm, 300 Å, 4.6 × 250 mm) at a flow rate of 0.6 mL/minute.

General Procedures for Reaction Screening on Model Peptides

For all experiments, model peptide (e.g., NH_2-NSTKNLTFAMRSSGDYGEV-$CONH_2$, 0.5 μmol, 1.0 equiv.) was dissolved in aqueous buffer solution with a final reaction concentration of 0.5 mM. Synthetic TPC probes in DMSO with designated amount were added and the reaction solution was stirred at room temperature or heated to 30 ℃ by RS9000 heater/shaker reaction station. The reaction was monitored at 0 hour, 1 hour, 2.5 hours, 3.5 hours and 4 hours. At each time point, the reaction was monitored by RP-UPLC. The reaction was monitored by RP-UPLC and the conversion percentage was calculated based on consumption of model peptide which has been converted to the final desired product.

Reaction of Probe 8 with MP_W (NH_2-GYAFWG-$CONH_2$)

40 μL **MP_W** (5 mM in H_2O) and 120 μL probe **8** (5 mM in H_2O) and 20 μL PBS (pH = 7.4, 100 mM) were added into 20 μL H_2O and incubated at 25 ℃ for 1 hour, the reaction was monitored by LC-MS.

Chemoselectivity towards tyrosine and cysteine

40 μL **MP_SCS** (5 mM in H_2O), 40 μL **MP_SYS** (5 mM in H_2O) and 40 μL probe **8** (5 mM in H_2O) and 20 μL PBS (pH = 7.4, 100 mM) were added into 60 μL H_2O and

incubated at 25 ℃ for 1 hour, the reaction was monitored by LC-MS, the ratio of **MP_SCS_8**:**MP_SYS_8** = 1:1.08 was predicted by HPLC.

The concentration of glutathione (GSH) is about 2-20 μM under physiological conditions.[9] The labeling of **MP_SYS** with probe **8** in the presence of 20 μM GSH proceeded successfully. 40 μL **MP_SYS** (5 mM in H_2O), 60 μL probe **8** (5 mM in H_2O), 0-20 μL GSH (200 μM in H_2O) and 20 μL PBS (pH = 7.4, 100 mM) were added into 60-80 μL H_2O (80 μL H_2O for 0 μL GSH and 60 μL H_2O for 20 μL GSH) and incubated at 25 ℃ for 1 hour. HPLC analysis shows that **MP_SYS** fully converted to **MP_SYS_8** in the presence of 0 or 20 μM GSH in the reaction solutions, the GSH labeled compound **GSH_8** was detected on LC-MS.

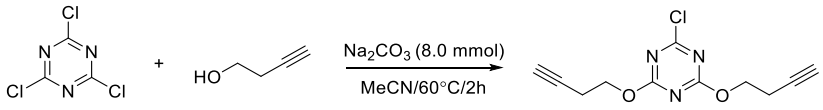
Kinetic study of TPC modification on aromatic hydroxyl group

80 μL Phenol (**S8**, 2 mM in ACN), 40 μL probe **8** (4 mM in H_2O) and 20 μL PBS (pH 7.4, 0.1 M) were added into 60 μL H_2O and incubated at 25 ℃ and the reaction was monitored by LC-MS and HPLC at 1 minute intervals. Second order reaction rate constant of 2.2851 $M^{-1}s^{-1}$ was calculated from 1/[sub] value. Conversion was calculated by HPLC.

General information for TPC probes synthesis

All commercially available chemicals were of reagent grade quality. NMR spectra were measured on a Bruker 600 spectrometer with tetramethylsilane (Me_4Si) as the internal standard. Mass spectra were measured on a Q-TOF Global mass spectrometer. 2-Chloro-4,6-dimethoxy-1,3,5-triazine and 2,4-ichloro-6-methoxy-1,3,5-triazine were purchased from Aladdin and peptide MP_SYS was purchased from GLPBIO.

Synthesis of 2-Chloro-4,6- bis(3-butyn-1-yloxy)-1,3,5-triazine S1



TCT (3.0 mmol) 12.0 mmol **S1**

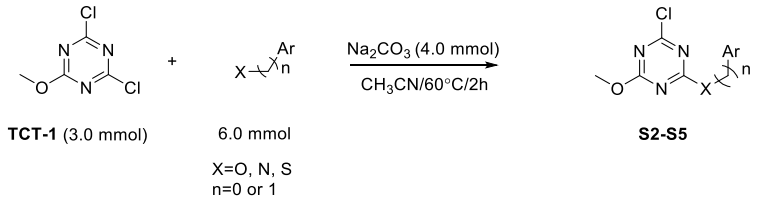
To a solution of 2,4,6-trichloro-1,3,5-triazine (**TCT**, 552 mg, 3.0 mmol) in 15 mL CH_3CN was added 3-butyn-1-ol (840 mg, 12.0 mmol) and Na_2CO_3 (848 mg, 8.0 mmol), the reaction mixture was stirred at 60 ℃ for 2 hours.[10] Upon reaction completion, the reaction was cooled to room temperature, 100 mL H_2O was added and the mixture was extracted with EtOAc (3 ×50 mL). The combined organic phases were filtered through Na_2SO_4, concentrated under vacuum, and the residue was purified by column chromatography on silica gel (PE: EA=50:1) to give the target **S1** (527 mg, 70%) as white solid.

^{1}H NMR (500 MHz, Chloroform-*d*): δ 4.54 (t, *J* = 7.0 Hz, 4H), 2.71 (td, *J* = 6.9, 2.6 Hz, 4H), 2.03 (t, *J* = 2.6 Hz, 2H).

^{13}C NMR (126 MHz, Chloroform-*d*): δ 172.88, 171.81, 79.07, 70.60, 66.68, 18.86.

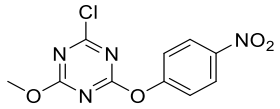
HRMS (ESI): *m/z* calcd for [$C_{11}H_{10}ClN_3O_2$+H^+]: 252.0534, found 252.0539.

Synthesis of S2-S5 (General procedure A)



To a solution of 2,4-dichloro-6-methoxy-1,3,5-triazine (**TCT-1**, 540 mg, 3.0 mmol) in 15 mL CH_3CN was added the aromatic derivatives (6.0 mmol) and Na_2CO_3 (424 mg, 4.0 mmol), the reaction mixture was stirred at 60 ℃ for 2 hours. Upon reaction completion, the reaction was cooled to room temperature, 100 mL H_2O was added and the mixture was extracted with EtOAc (3 ×50 mL). The combined organic phases were filtered through Na_2SO_4, concentrated under vacuum, and the residue was purified by column chromatography on silica gel to give the target **S2-S5**.

2-Chloro-4-methoxy-6-(4-nitrophenoxy)-1,3,5-triazine S2



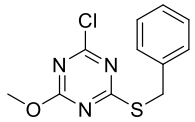
Prepared from **TCT-1** (540 mg, 3.0 mmol) and 4-nitrophenol (816 mg, 6.0 mmol) according to General Procedure A and purified by flash column chromatography (PE: EA=30:1) to give **S2** (719 mg, 85%) as white solid.

^{1}H NMR (400 MHz, Chloroform-*d*): δ 8.34 (d, *J* = 8.9 Hz, 2H), 7.38 (d, *J* = 8.9 Hz, 2H), 4.06 (s, 3H).

^{13}C NMR (101 MHz, Chloroform-*d*): δ 173.58, 172.83, 171.46, 155.72, 145.87, 125.59, 122.46, 56.56.

HRMS (ESI): *m/z* calcd for $[C_{10}H_7ClN_4O_4+H^+]$: 283.0229, found 283.0235.

2-Chloro-4-methoxy-6-(phenylmethyl)thio-1,3,5-triazine S3



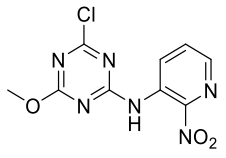
Prepared from **TCT-1** (540 mg, 3.0 mmol) and benzyl mercaptan (744 mg, 6.0 mmol) according to General Procedure A and purified by flash column chromatography (PE: EA=50:1) to give **S3** (673 mg, 84%) as colorless liquid.

^{1}H NMR (400 MHz, Chloroform-*d*): δ 7.40 (d, *J* = 7.3 Hz, 2H), 7.33 (t, *J* = 7.3 Hz, 2H), 7.28 (t, 1H), 4.40 (s, 1H), 4.05 (s, 3H).

^{13}C NMR (101 MHz, Chloroform-*d*): δ 185.18, 170.60, 169.82, 135.90, 129.12, 128.72, 127.73, 56.05, 35.16.

HRMS (ESI): *m/z* calcd for $[C_{11}H_{10}ClN_3OS+H^+]$: 268.0306, found 268.0312.

2-Chloro-4-methoxy-6- *N*-(2-nitro-3-pyridinyl)-1,3,5-triazine S4



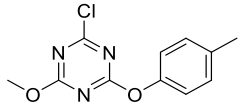
Prepared from **TCT-1** (540 mg, 3.0 mmol) and 2-nitro-3-pyridinamine (834 mg, 6.0 mmol) according to General Procedure A and purified by flash column chromatography (PE: EA=10:1) to give **S4** (380 mg, 45%) as light yellow solid.

^{1}H NMR (400 MHz, Chloroform-*d*): δ 9.86 (s, 1H), 8.79 (d, *J* = 3.5 Hz, 1H), 8.51 (d, *J* = 8.0 Hz, 1H), 7.32 (dd, *J* = 8.1, 4.7 Hz, 1H), 4.03 (s, 3H).

^{13}C NMR (101 MHz, Chloroform-*d*): δ 171.93, 171.59, 165.20, 153.48, 144.49, 135.62, 134.86, 120.05, 55.96.

HRMS (ESI): *m/z* calcd for [$C_9H_7ClN_6O_3$+H^+]: 283.0341, found 283.0345.

2-Chloro-4-methoxy-6-(4-methylphenoxy)-1,3,5-triazine S5



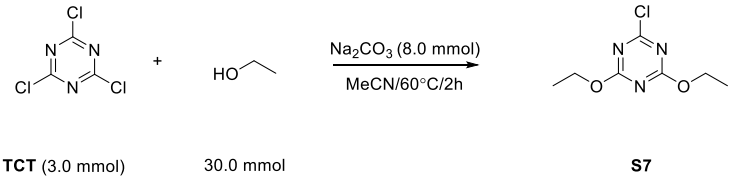
Prepared from **TCT-1** (540 mg, 3.0 mmol) and p-methylphenol (648 mg, 6.0 mmol) according to General Procedure A and purified by flash column chromatography (PE: EA=50:1) to give **S5** (662 mg, 88%) as white solid.

^{1}H NMR (400 MHz, Chloroform-*d*): 7.22 (d, *J* = 8.2 Hz, 2H), 7.05 (d, *J* = 8.3 Hz, 2H), 4.02 (s, 3H), 2.37 (s, 3H).

^{13}C NMR (101 MHz, Chloroform-*d*): δ 173.23, 172.77, 172.37, 149.21, 136.17, 130.21, 120.89, 56.20, 20.96.

HRMS (ESI): *m/z* calcd for [$C_{11}H_{10}ClN_3O_2$+H^+]: 252.0534, found 252.0539.

2-Chloro-4,6- diethoxy-1,3,5-triazine S7



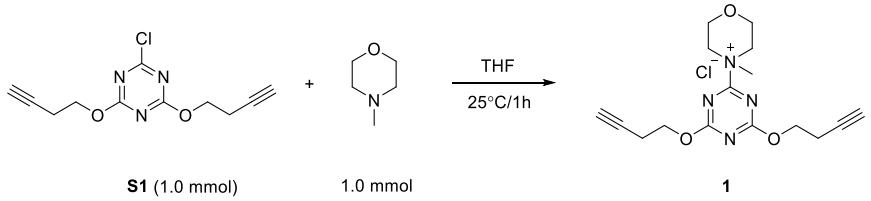
To a solution of 2,4,6-trichloro-1,3,5-triazine (**TCT**, 540 mg, 3.0 mmol) in 15 mL CH_3CN was added ethanol (1.75 mL, 30.0 mmol) and Na_2CO_3 (848 mg, 8.0 mmol), the reaction mixture was stirred at 60 ℃ for 2 hours. Upon reaction completion, the reaction was cooled to room temperature, 100 mL H_2O was added and the mixture was extracted with EtOAc (3 ×50 mL). The combined organic phases were filtered through Na_2SO_4, concentrated under vacuum, and the residue was purified by column chromatography on silica gel (PE: EA=50:1) to give the target **S7** (500 mg, 82%) as white solid.

^{1}H NMR (500 MHz, Chloroform-*d*): δ 4.50 (q, *J* = 7.1 Hz, 4H), 1.43 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (126 MHz, Chloroform-*d*): δ 172.62, 172.00, 65.33, 14.12.

HRMS (ESI): *m/z* calcd for [$C_7H_{10}ClN_3O_2$+H^+]: 204.0534, found 204.0539.

4-[4,6-Bis(3-butyn-1-yloxy)-1,3,5-triazin-2-yl]-4-methylmorpholinium chloride 1



S1 (1.0 mmol) 1.0 mmol **1**

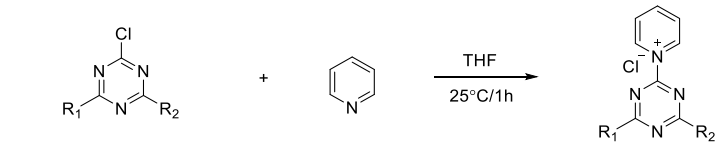
To a solution of **S1** (251 mg, 1.0 mmol) in 10 mL THF was added 4-methylmorpholine (101 mg, 1.0 mmol), the reaction mixture was stirred at 25 ℃ for 1 hour, the product was collected via centrifuging to give the target **1** (288 mg, 82%) as white solid.[11]

^{1}H NMR (600 MHz, D_2O): δ 4.54 (d, *J* = 6.2 Hz, 4H), 4.47 (d, *J* = 12.8 Hz, 2H), 4.01 (d, *J* = 13.8 Hz, 2H), 3.78 (t, *J* = 11.6 Hz, 2H), 3.74 – 3.65 (m, 2H), 3.40 (s, 3H), 2.64 (m, *J* = 6.0 Hz, 4H), 2.29 (s, 2H).

^{13}C NMR (151 MHz, D_2O): δ 173.29, 170.25, 80.85, 71.03, 68.15, 62.10, 60.15, 55.83, 18.23.

HRMS (ESI): *m/z* calcd for [$C_{16}H_{21}N_4O_3^+$]: 317.1608, found 317.1608.

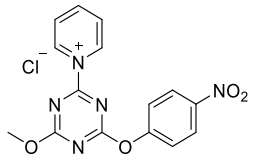
Synthesis of 2-8 (General procedure B)



Triazine derivatives(1.0 mmol) 1.0 mmol **2-8**

To a solution of triazine derivatives (1.0 mmol) in 10 mL THF was added pyridine (79 mg, 1.0 mmol), the reaction mixture was stirred at 25 ℃ for 1 hour, the product was collected via centrifuging to give the target **2-8** as solid.

1-[4-Methoxy-6-(4-nitrophenoxy)-1,3,5-triazin-2-yl]pyridinium chloride 2



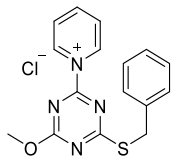
Prepared from **S2** (282 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **2** (260 mg, 72%) as white solid.

^{1}H NMR (400 MHz, Methanol-*d_4*): δ 10.06 (d, *J* = 6.4 Hz, 2H), 9.04 (t, *J* = 7.7 Hz, 1H), 8.47 (d, *J* = 8.9 Hz, 2H), 8.41 (t, *J* = 7.1 Hz, 2H), 7.65 (d, *J* = 9.0 Hz, 2H), 4.22 (s, 3H).

^{13}C NMR (101 MHz, Methanol-*d_4*): δ 174.19, 172.88, 165.55, 155.84, 152.36, 146.24, 140.99, 128.09, 125.37, 122.45, 56.61.

HRMS (ESI): *m/z* calcd for [$C_{15}H12N_5O_4^+$]: 326.0884, found 326.0884.

1-[4-Methoxy-6-(phenylmethyl)thio-1,3,5-triazin-2-yl]pyridinium chloride 3



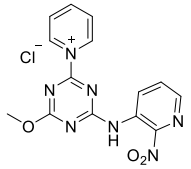
Prepared from **S3** (267 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **3** (242 mg, 70%) as white solid.

^{1}H NMR (500 MHz, Methanol-*d*$_4$): δ 10.08 (d, *J* = 5.7 Hz, 2H), 9.00 (t, *J* = 7.7 Hz, 1H), 8.38 (t, *J* = 7.3 Hz, 2H), 7.50 (d, *J* = 7.4 Hz, 2H), 7.34 (t, *J* = 7.5 Hz, 2H), 7.27 (t, *J* = 7.4 Hz, 1H), 4.64 (s, 2H), 4.25 (s, 3H).

^{13}C NMR (126 MHz, Methanol-*d*$_4$): δ 187.10, 170.97, 162.69, 151.99, 140.93, 136.01, 128.76, 128.47, 128.01, 127.47, 56.37, 34.95.

HRMS (ESI): *m/z* calcd for [$C_{16}H_{15}N_4OS^+$]: 311.0961, found 311.0960.

1-[4-Methoxy-6-*N*-(2-nitro-3-pyridinyl)-1,3,5-triazin-2-yl]pyridinium chloride 4



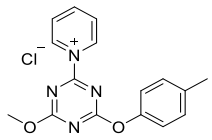
Prepared from **S4** (282 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **4** (188 mg, 52%) as light yellow solid.

^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 10.03 (d, *J* = 6.2 Hz, 2H), 9.00 (t, *J* = 7.7 Hz, 1H), 8.79 (d, *J* = 4.5 Hz, 1H), 8.59 (d, *J* = 8.1 Hz, 1H), 8.38 (t, *J* = 7.1 Hz, 2H), 7.61 (dd, *J* = 8.1, 4.7 Hz, 1H), 4.10 (s, 3H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 172.40, 166.48, 163.97, 152.52, 151.70, 142.72, 140.66, 139.99, 134.54, 127.77, 122.09, 55.65.

HRMS (ESI): *m/z* calcd for [$C_{14}H_{12}N_7O_3^+$]: 326.0996, found 326.1002.

1-[4-Methoxy-6-(4-methylphenoxy)-1,3,5-triazin-2-yl]pyridinium chloride 5



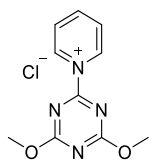
Prepared from **S5** (251 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **5** (251 mg, 76%) as white solid.

^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 10.02 – 9.96 (m, 2H), 8.99 (tt, *J* = 7.7, 1.4 Hz, 1H), 8.41 – 8.31 (m, 2H), 7.33 (d, *J* = 8.2 Hz, 2H), 7.22 – 7.16 (m, 2H), 4.16 (s, 3H), 2.41 (s, 3H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 174.12, 173.60, 165.39, 152.11, 149.49, 140.86, 136.56, 130.04, 128.00, 120.56, 56.31, 19.54.

HRMS (ESI): *m/z* calcd for [$C_{16}H_{15}N_4O_2^+$]: 295.1190, found 295.1188.

1-(4,6-Dimethoxy-1,3,5-triazin-2-yl)pyridinium chloride 6



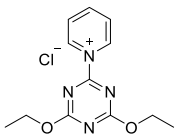
Prepared from 2-chloro-4,6-dimethoxy-1,3,5-triazine (**S6**, 175 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **6** (206 mg, 81%) as white solid.

^{1}H NMR (500 MHz, Methanol-*d*$_4$): δ 10.13 (d, *J* = 5.9 Hz, 2H), 9.00 (t, *J* = 7.7 Hz, 1H), 8.38 (t, *J* = 7.2 Hz, 2H), 4.25 (s, 6H).

^{13}C NMR (126 MHz, Methanol-*d*$_4$): δ 173.85, 151.92, 140.93, 127.88, 56.13.

HRMS (ESI): *m/z* calcd for [$C_{10}H_1N_4O_2^+$]: 219.0877, found 219.0878.

1-(4,6-Diethoxy-1,3,5-triazin-2-yl)pyridinium chloride 7



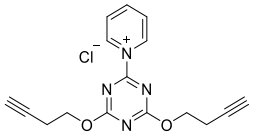
Prepared from **S7** (203 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **7** (237 mg, 84%) as white solid.

^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 10.11 (d, *J* = 6.3 Hz, 2H), 9.00 (t, *J* = 7.7 Hz, 1H), 8.38 (t, *J* = 7.1 Hz, 2H), 4.69 (q, *J* = 7.1 Hz, 4H), 1.50 (t, *J* = 7.1 Hz, 6H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 173.21, 165.03, 151.88, 140.91, 127.91, 66.12, 13.01.

HRMS (ESI): *m/z* calcd for [$C_{12}H_{15}N_4O_2^+$]: 247.1190, found 247.1188.

1-[4,6-Bis(3-butyn-1-yloxy)-1,3,5-triazin-2-yl]pyridinium chloride 8



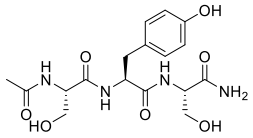
Prepared from **S1** (251 mg, 1.0 mmol) and pyridine (79 mg, 1.0 mmol) according to General Procedure B to give **8** (234 mg, 71%) as white solid.

^{1}H NMR (400 MHz, Methanol-*d*$_4$): δ 10.12 (d, *J* = 6.3 Hz, 2H), 9.01 (t, *J* = 7.7 Hz, 1H), 8.38 (t, *J* = 7.1 Hz, 2H), 4.73 (t, *J* = 6.5 Hz, 4H), 2.80 (td, *J* = 6.5, 2.4 Hz, 4H), 2.41 (t, *J* = 2.2 Hz, 2H).

^{13}C NMR (101 MHz, Methanol-*d*$_4$): δ 173.16, 152.04, 140.99, 127.95, 79.11, 70.27, 67.84, 18.13.

HRMS (ESI): *m/z* calcd for [$C_{16}H_{15}N_4O_2^+$]: 295.1190, found 295.1186.

NMR and MS data of Ac-Ser-Tyr-Ser-NH$_2$ MP_SYS



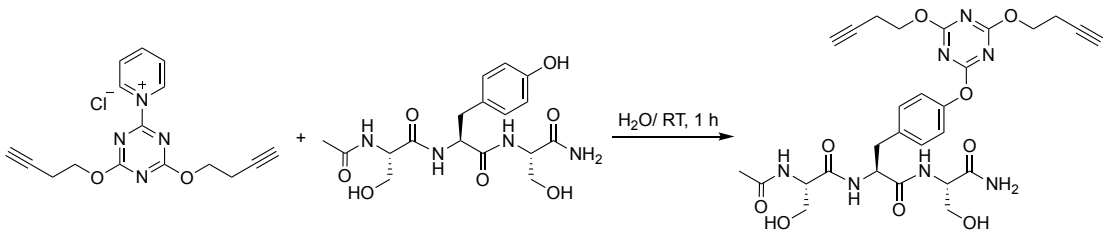
^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 9.14 (s, 1H), 7.97 (dd, *J* = 18.4, 7.7 Hz, 2H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 20.3 Hz, 2H), 6.99 (d, *J* = 8.3 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 2H), 4.92 (d, *J* = 56.5 Hz, 2H), 4.39 (td, *J* = 8.6, 4.5 Hz, 1H), 4.23 (dd, *J* = 13.5,

6.3 Hz, 1H), 4.16 (dd, *J* = 13.2, 5.5 Hz, 1H), 3.57 – 3.53 (m, 2H), 3.47 (d, *J* = 6.1 Hz, 2H), 2.94 (dd, *J* = 14.0, 4.1 Hz, 1H), 2.70 (dd, *J* = 13.9, 9.3 Hz, 1H), 1.82 (s, 3H).

^{13}C NMR (126 MHz, DMSO-*d*$_6$): δ 172.27, 171.28, 170.81, 170.08, 156.19, 130.56, 128.20, 115.28, 62.07, 55.63, 55.59, 54.97, 36.55, 22.96.

HRMS (ESI): *m/z* calcd for [$C_{17}H_{24}N_4O_7$+H^+]: 397.1718, found 397.1718.

Synthesis of MP_SYS_8



8 (0.1 mmol) **MP_SYS** (0.05 mmol) **MP_SYS_8**

To a solution of **MP_SYS** (20 mg, 0.05 mmol) in 5 mL H_2O was added **8** (29.5 mg, 0. mmol), the reaction mixture was stirred at 25 ℃ for 1 hour, 0.1M HOAc (1mL) was added to quench the reaction. Semi-preparative HPLC purification (10%—50% ACN/H_2O with 0.1% TFA over 40 minutes), then concentrated under vacuum and lyophilization to give the target **MP_SYS_8** (23 mg, 74%) as white solid.

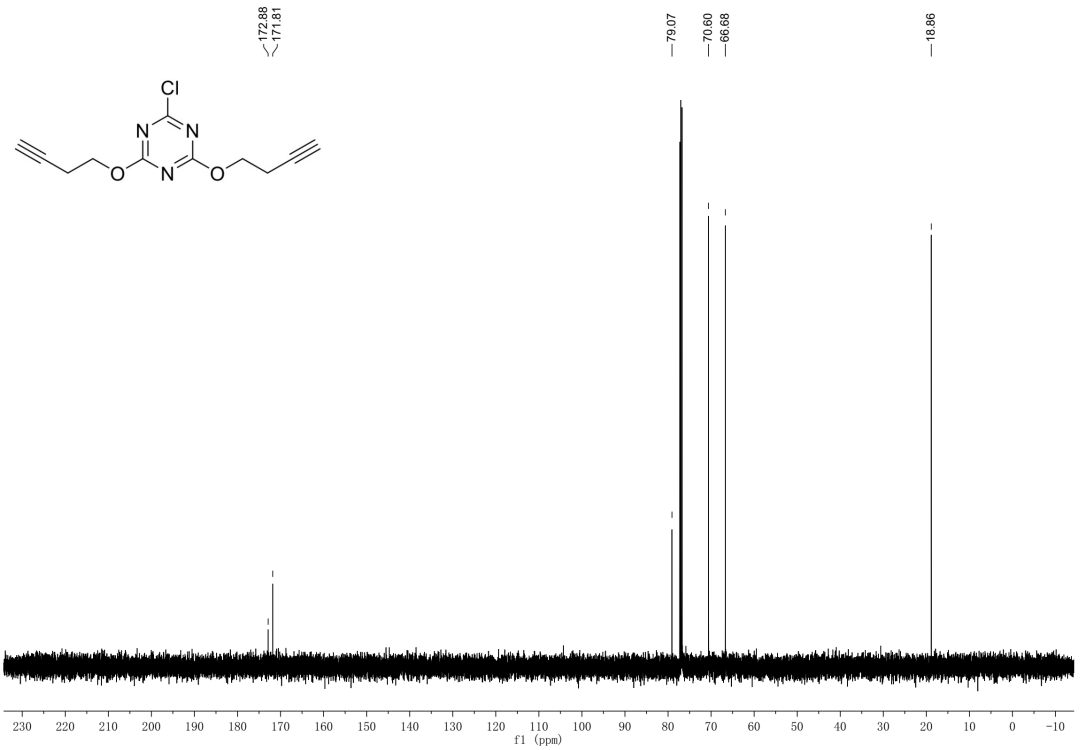
^{1}H NMR (500 MHz, DMSO-*d*$_6$): δ 8.13 (d, *J* = 7.8 Hz, 1H), 7.94 (dd, *J* = 15.0, 7.8 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 7.10 (t, *J* = 8.7 Hz, 4H), 4.98 (t, *J* = 5.5 Hz, 1H), 4.88 (t, *J* = 5.9 Hz, 1H), 4.52 (td, *J* = 8.5, 4.4 Hz, 1H), 4.34 (t, *J* = 6.5 Hz, 4H), 4.25 (dd, *J* = 13.8, 6.1 Hz, 1H), 4.21 – 4.15 (m, 1H), 3.61 – 3.53 (m, 2H), 3.48 (t, *J* = 6.1 Hz, 2H), 3.10 (dd, *J* = 14.0, 3.8 Hz, 1H), 2.88 (dd, *J* = 5.0, 2.8 Hz, 2H), 2.61 (td, *J* = 6.5, 2.8 Hz, 4H), 1.83 (s, 3H).

^{13}C NMR (126 MHz, DMSO-*d*$_6$): δ 173.24, 173.01, 172.23, 171.11, 170.93, 170.12, 150.44, 136.01, 130.77, 121.53, 81.27, 73.16, 66.39, 62.09, 62.06, 55.67, 55.64, 54.51, 36.68, 22.97, 18.65.

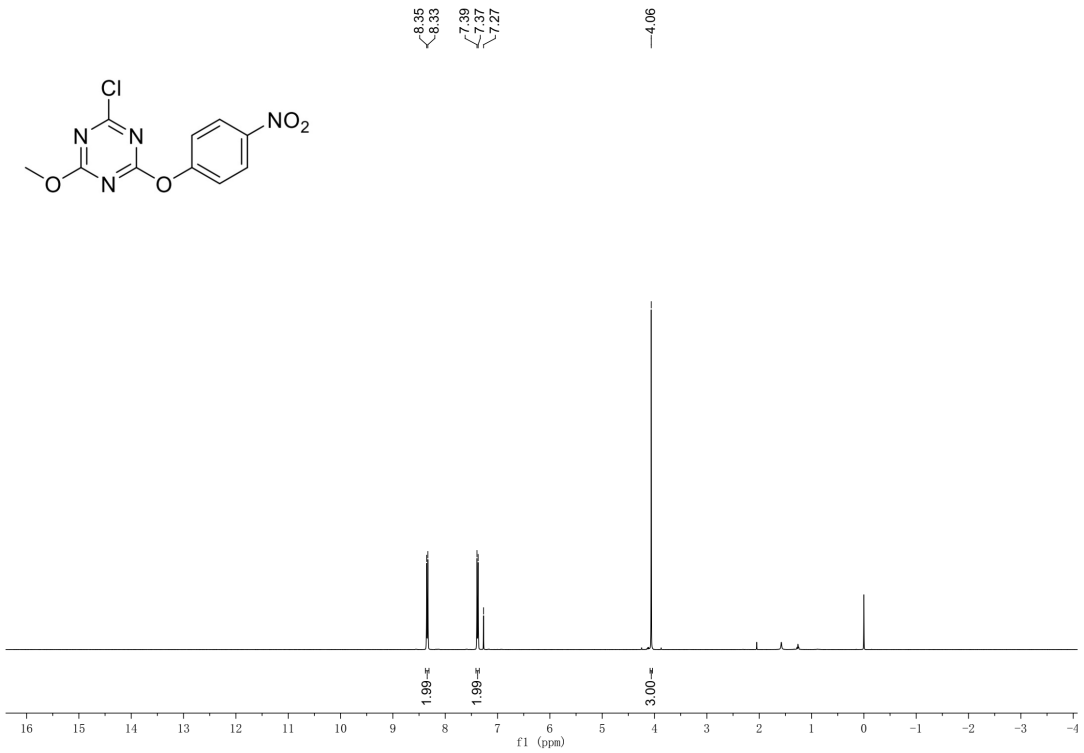
HRMS (ESI): *m/z* calcd for [$C_{28}H_{33}N_7O_9$+H^+]: 612.2413, found 612.2425.

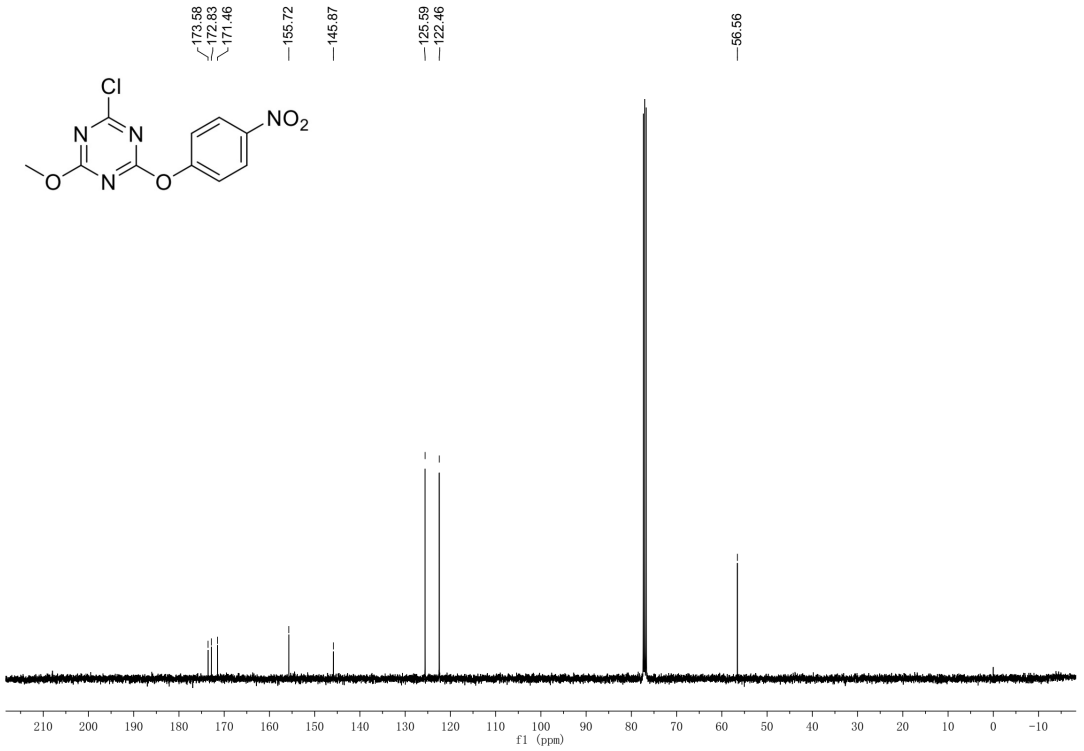
The diazonium probe[1] and SuTEX[1] were synthesized according to previously reported.

NMR Spectra
Compound S1

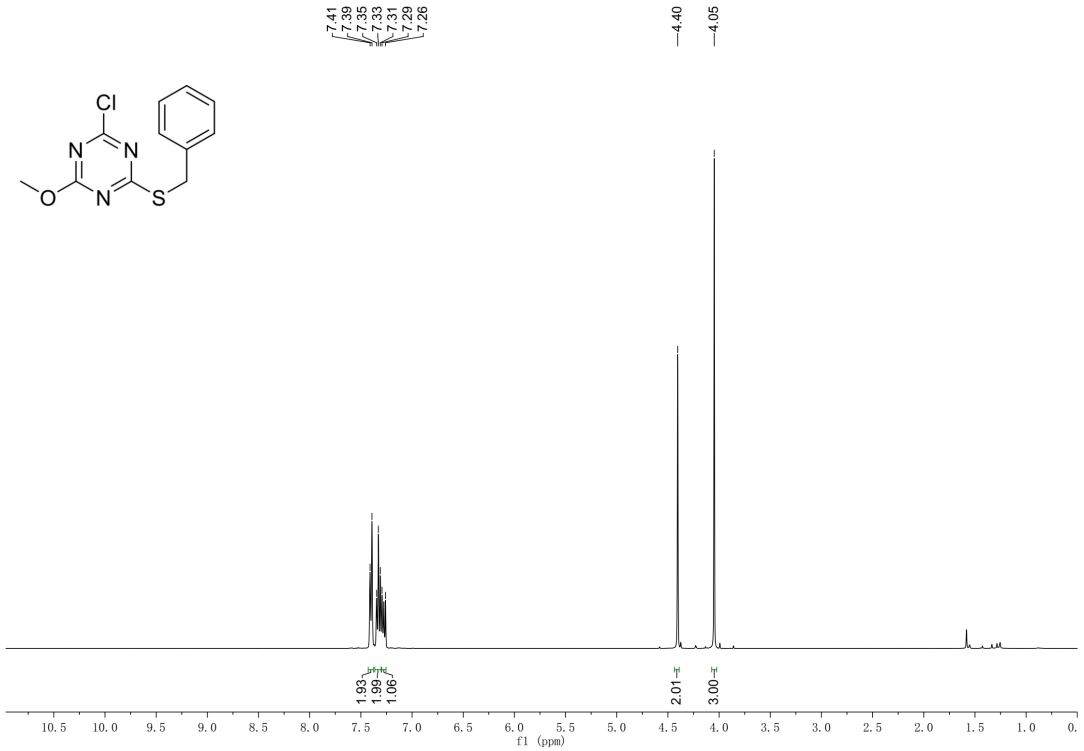


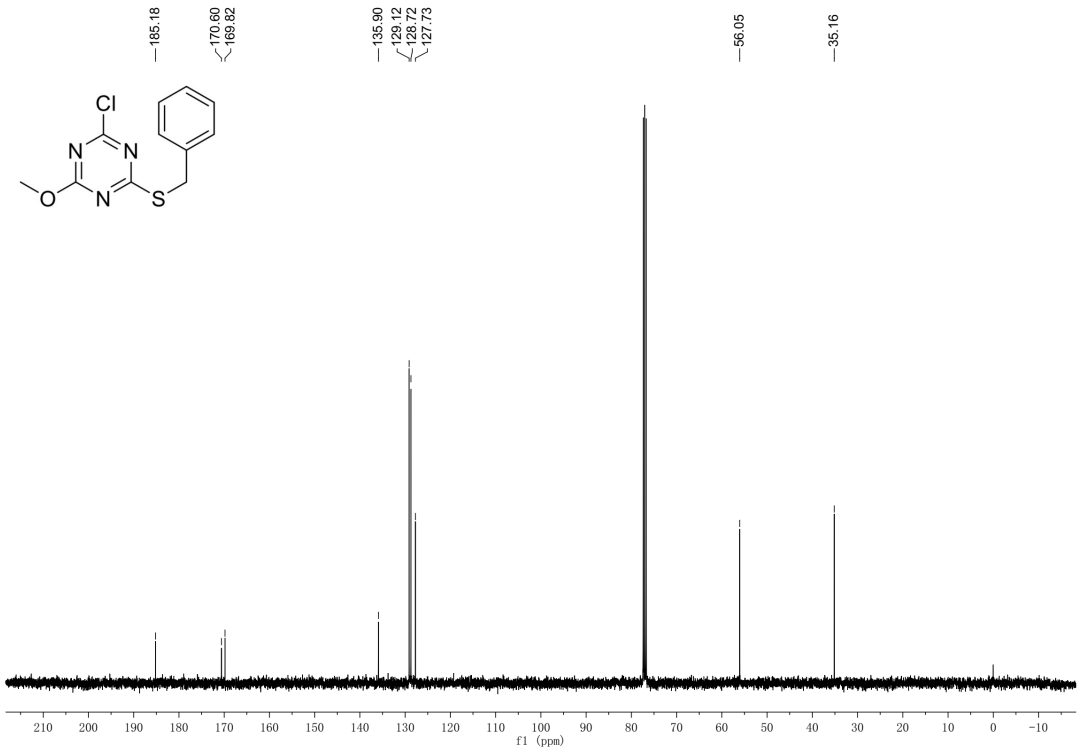
Compound S2


8.35
8.33
7.39
7.37
7.27
4.06
Cl
N
N
O
N
O
NO2
1.99
1.99
3.00
f1 (ppm)

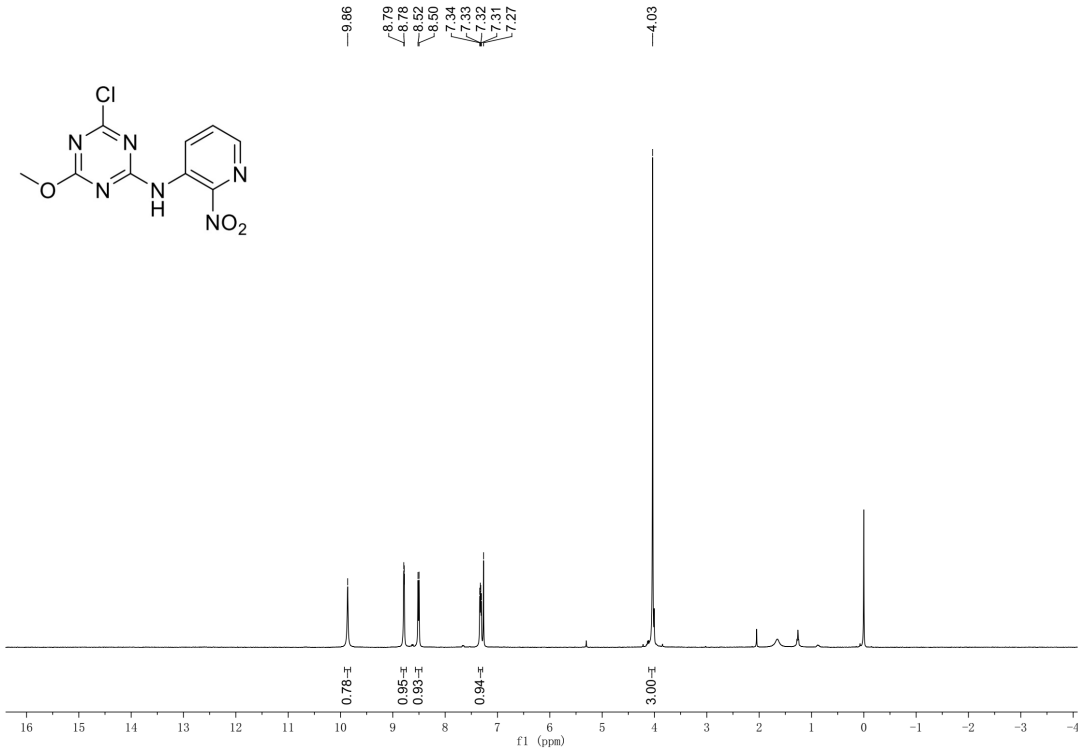

173.58
172.83
171.46
155.72
145.87
125.59
122.46
56.56
Cl
N
N
O
N
O
NO2
f1 (ppm)

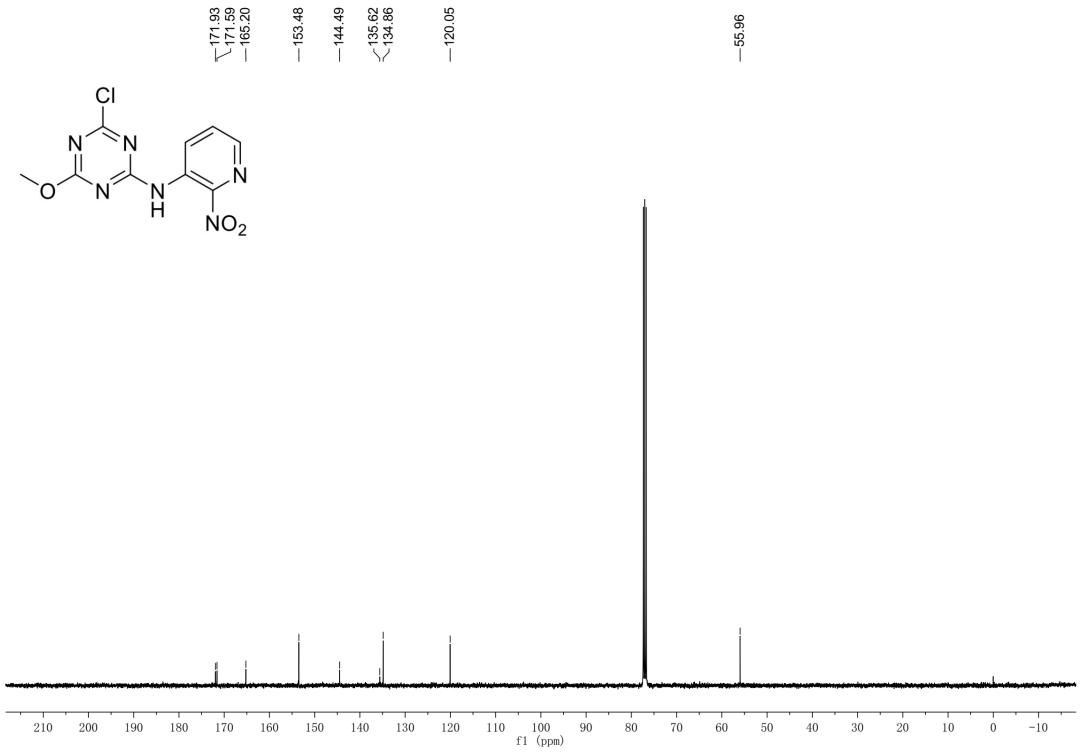
Compound S3


7.41
7.39
7.35
7.33
7.31
7.29
7.26
4.40
4.05
Cl
N
N
O
N
S
1.93
1.99
1.06
2.01
3.00
f1 (ppm)

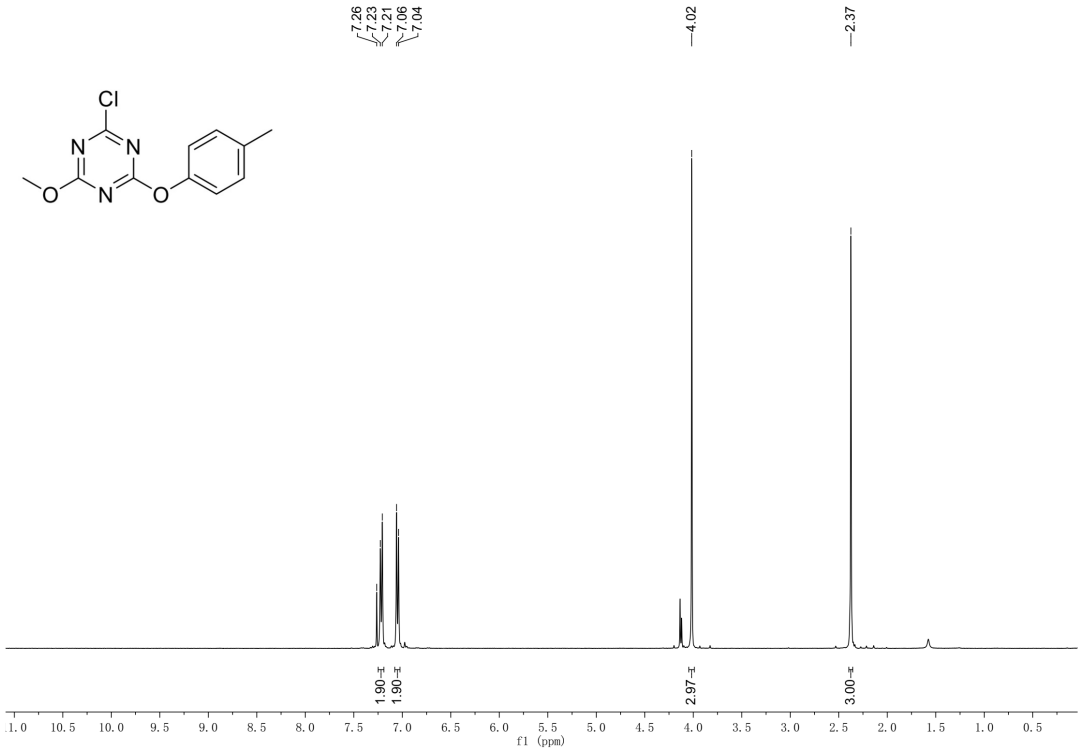

185.18
170.60
169.82
135.90
129.12
128.72
127.73
56.05
35.16
Cl
N
N
O
N
S
f1 (ppm)

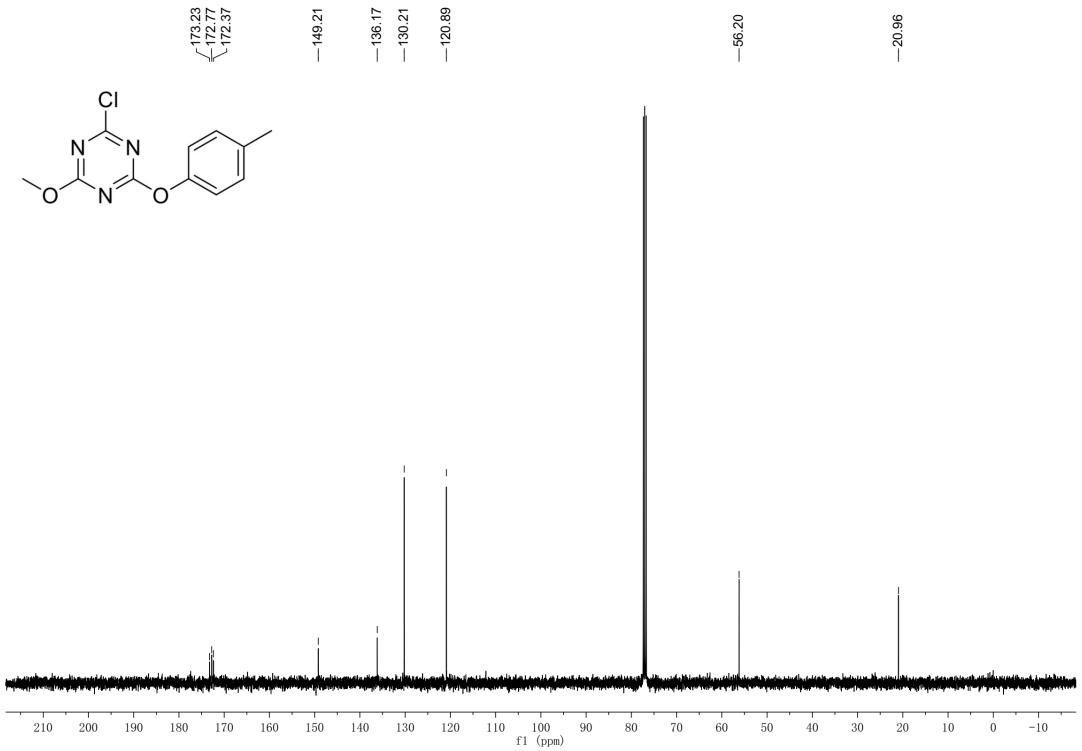
Compound S4


9.86
8.79
8.78
8.52
8.50
7.34
7.33
7.32
7.31
7.27
4.03
Cl
N
N
O
N
H
N
NO2
0.78
0.95
0.93
0.94
3.00
f1 (ppm)

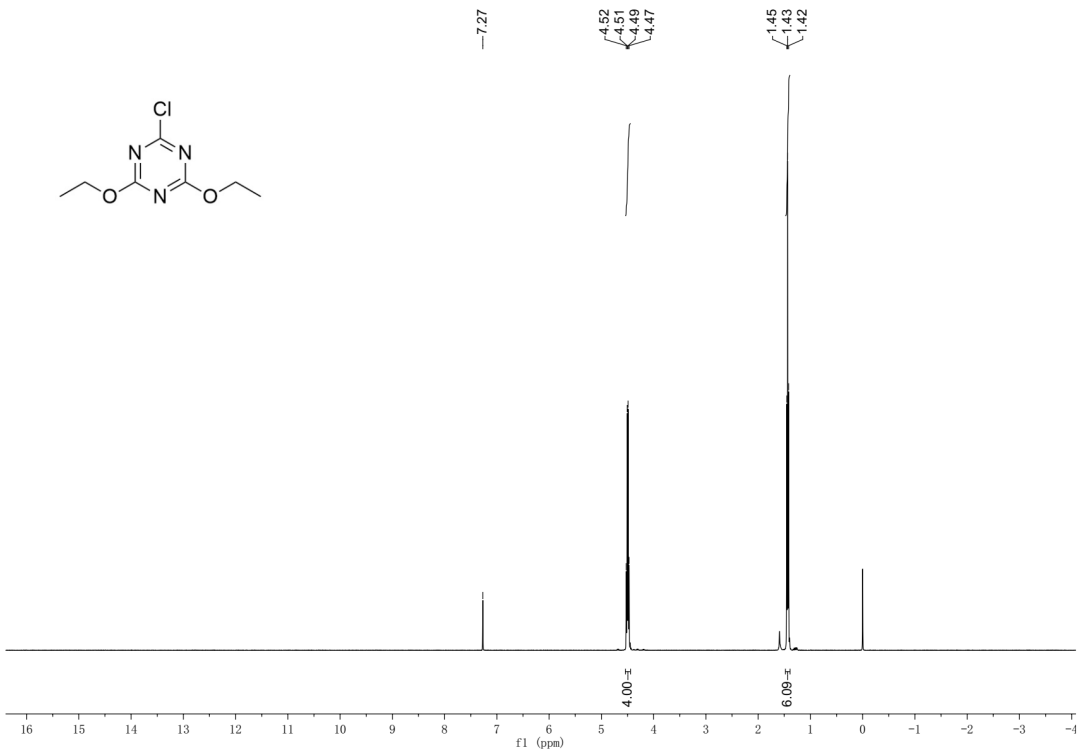

171.93
171.59
165.20
153.48
144.49
135.62
134.86
120.05
55.96
Cl
N
N
O
N
H
N
NO2
f1 (ppm)

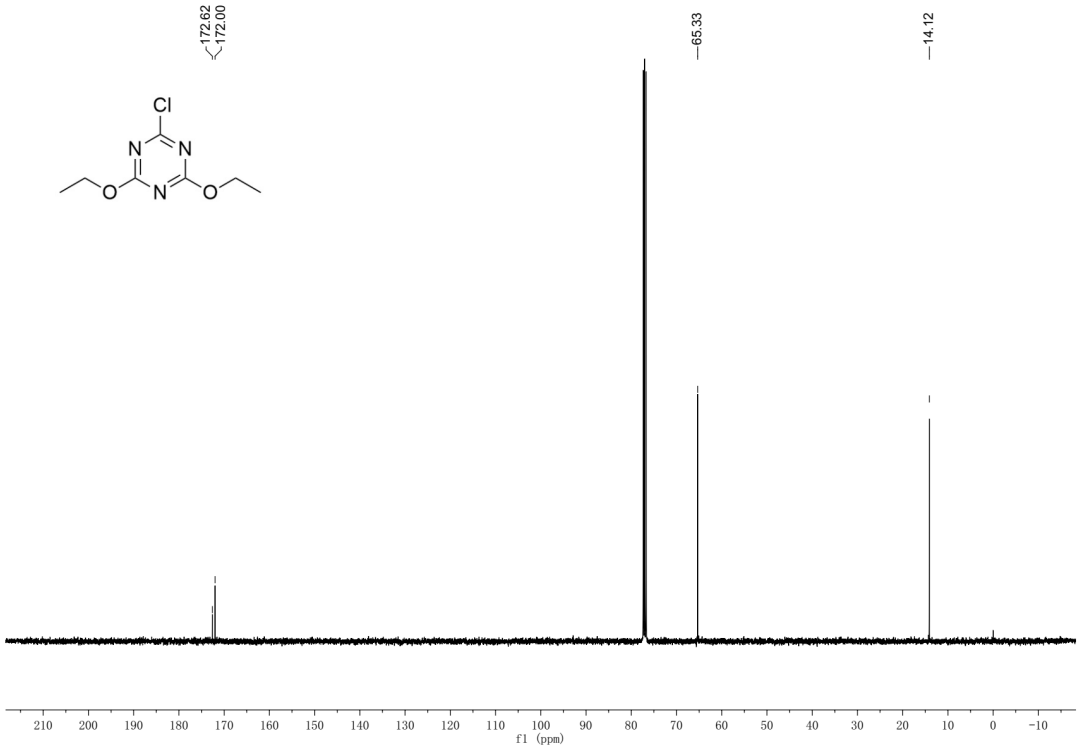
Compound S5


7.26
7.23
7.21
7.06
7.04
4.02
2.37
Cl
N
N
O
N
O
1.90
1.90
2.97
3.00
f1 (ppm)

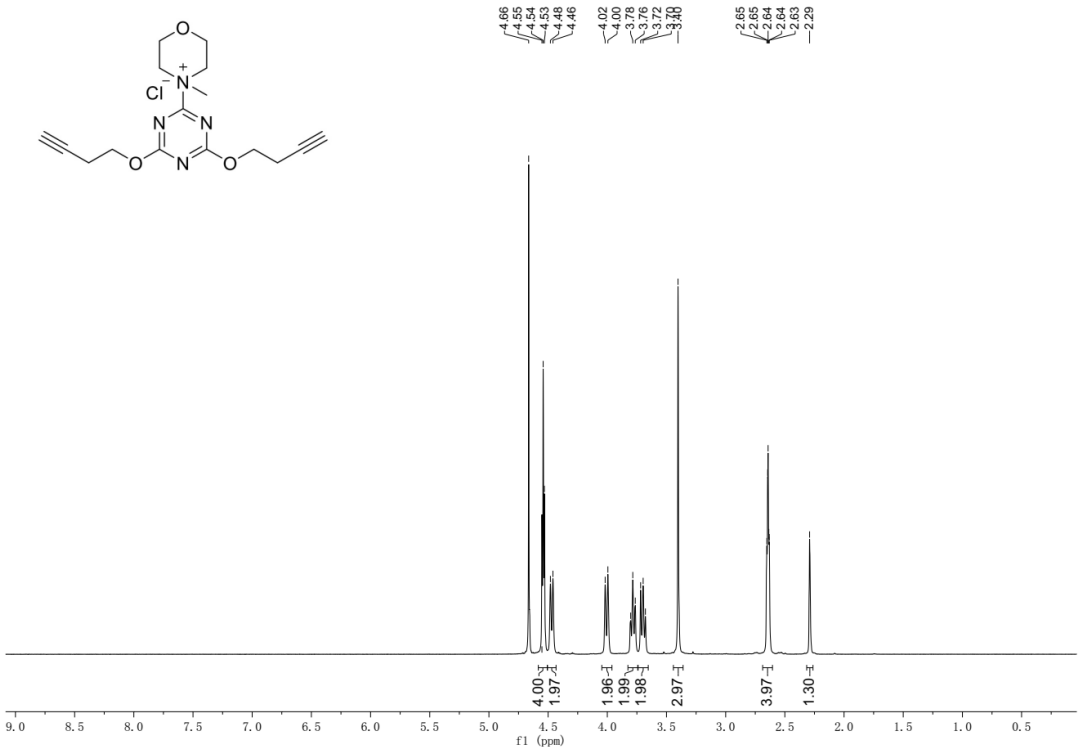

173.23
172.77
172.37
149.21
136.17
130.21
120.89
56.20
20.96
Cl
N
N
O
N
O
f1 (ppm)

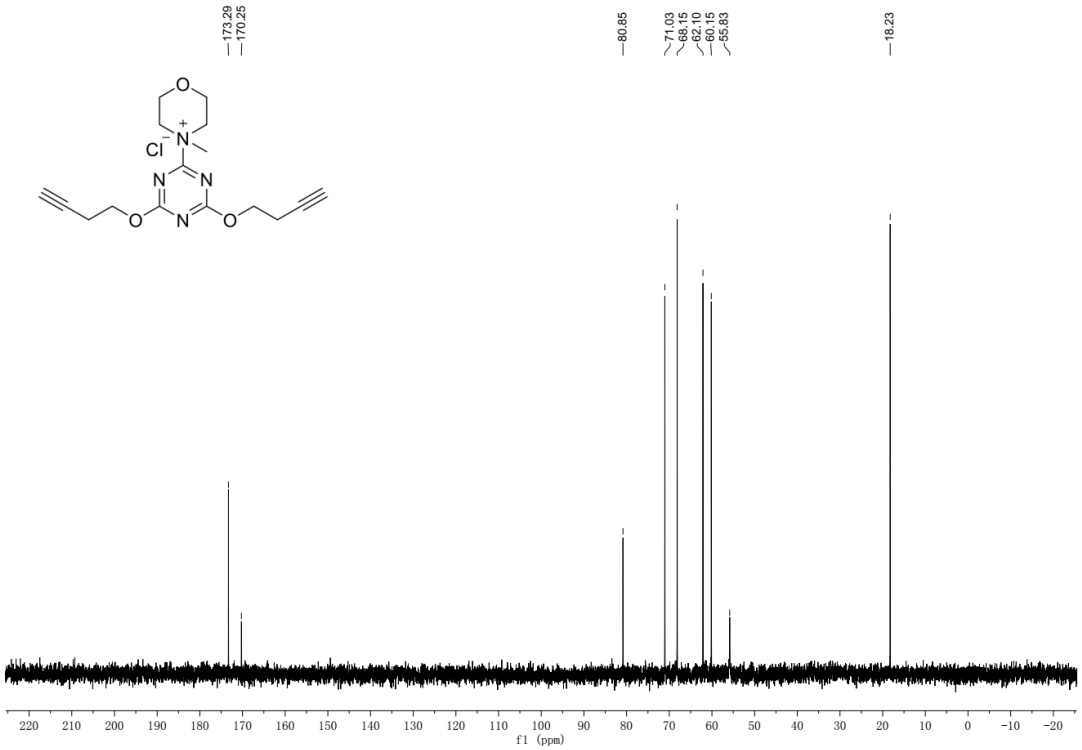
Compound S7


7.27
4.52
4.51
4.49
4.47
1.45
1.43
1.42
Cl
N
N
O
N
O
4.00
6.09
f1 (ppm)

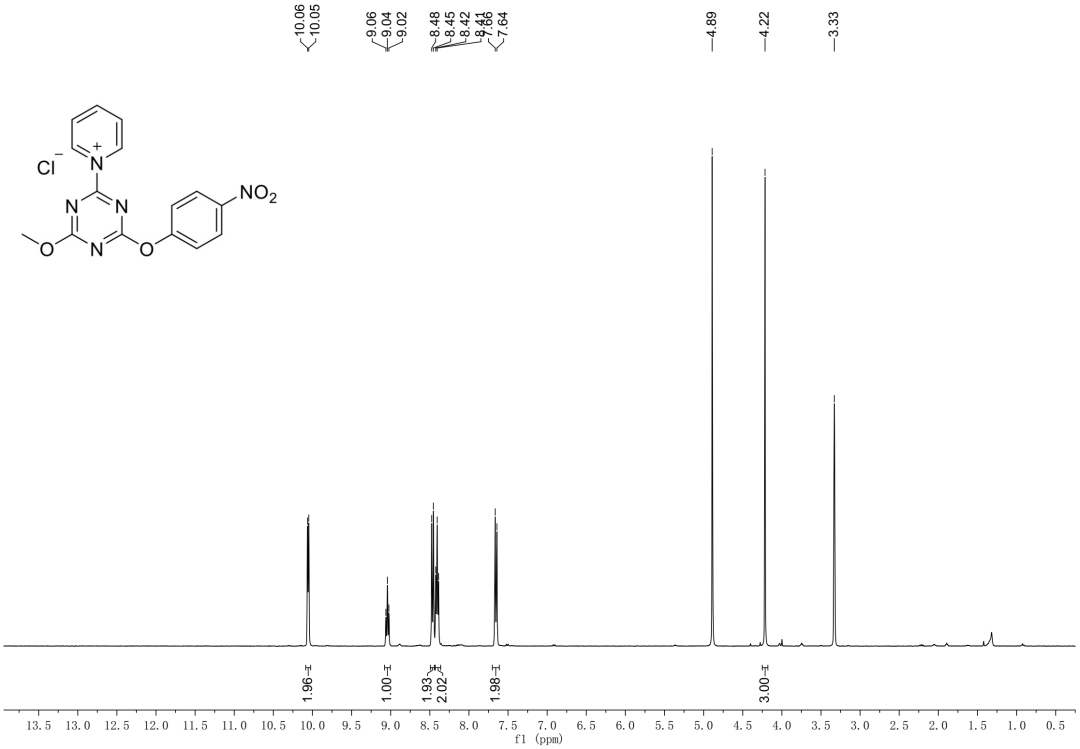

172.62
172.00
65.33
14.12
Cl
N
N
O
N
O
f1 (ppm)

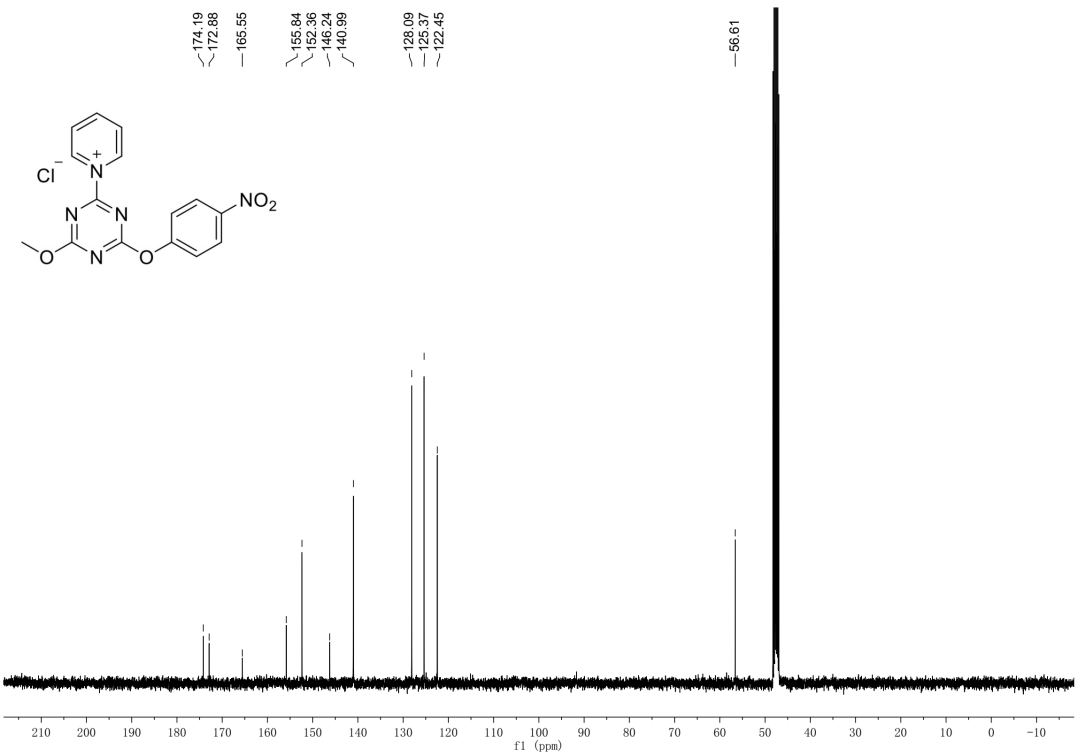
Compound 1


4.66
4.55
4.54
4.53
4.48
4.46
4.02
4.00
3.78
3.76
3.72
3.71
3.48
2.65
2.65
2.64
2.64
2.63
2.29
4.00
1.97
1.96
1.99
1.98
2.97
3.97
1.30
f1 (ppm)

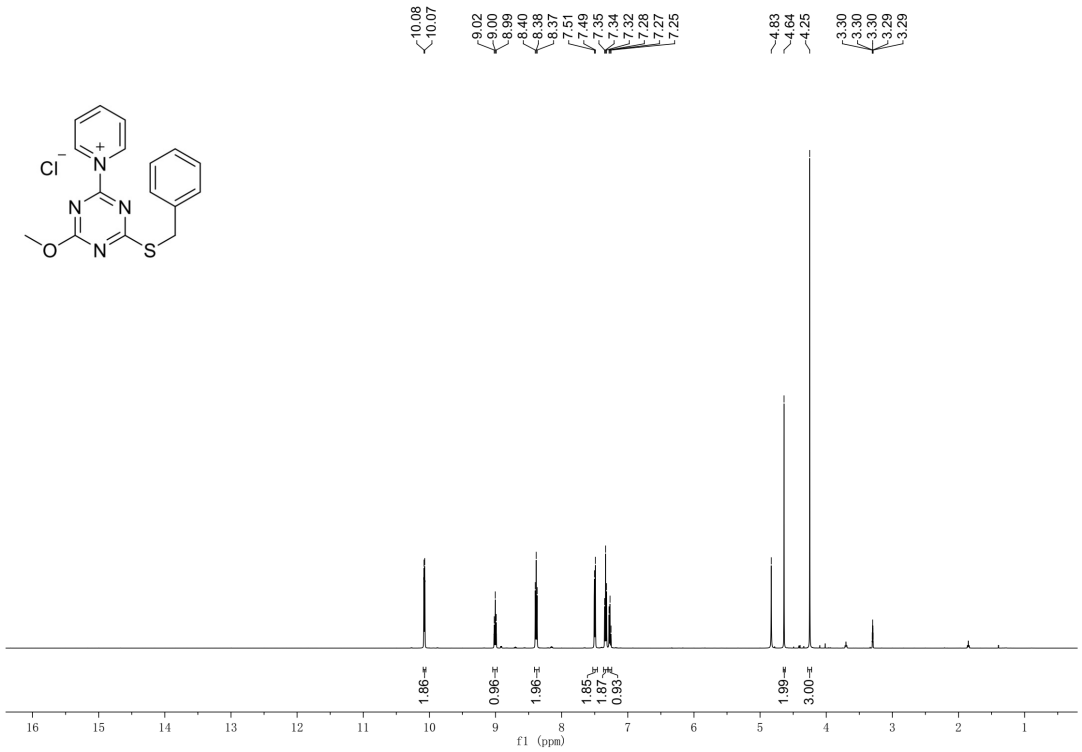

173.29
170.25
80.85
71.03
68.15
62.10
60.15
55.83
18.23
f1 (ppm)

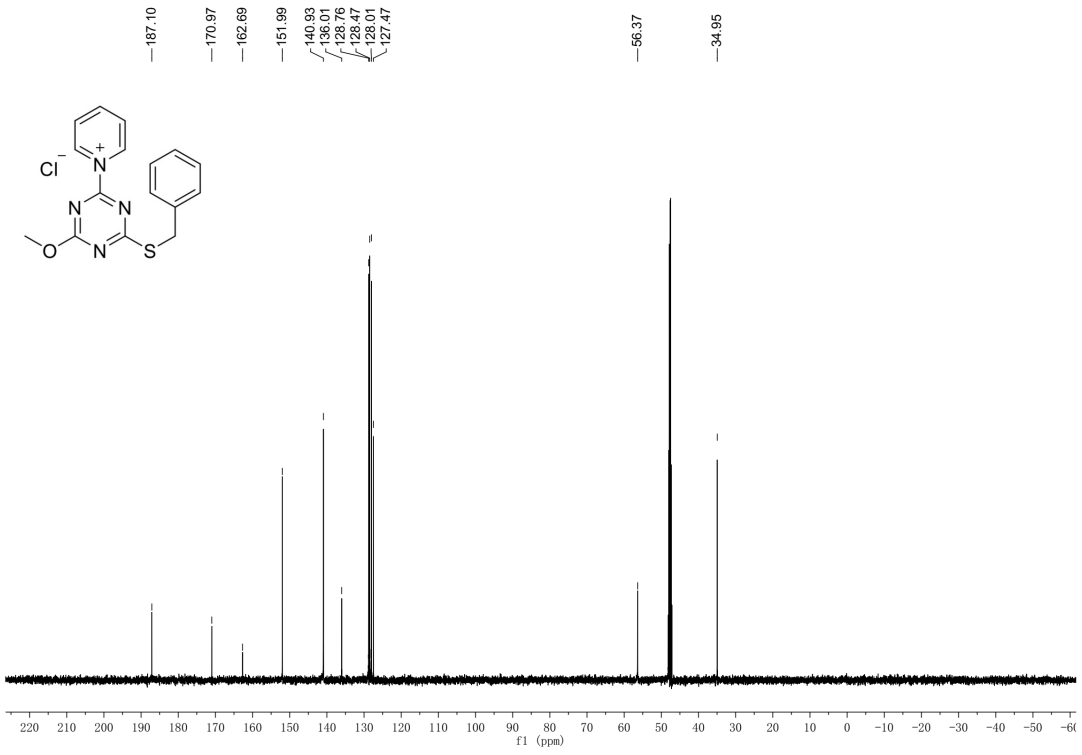
Compound 2


10.06
10.05
9.06
9.04
9.02
8.48
8.45
8.42
8.41
7.66
7.64
4.89
4.22
3.33
Cl⁻
NO2
1.96
1.00
1.93
2.02
1.98
3.00
f1 (ppm)

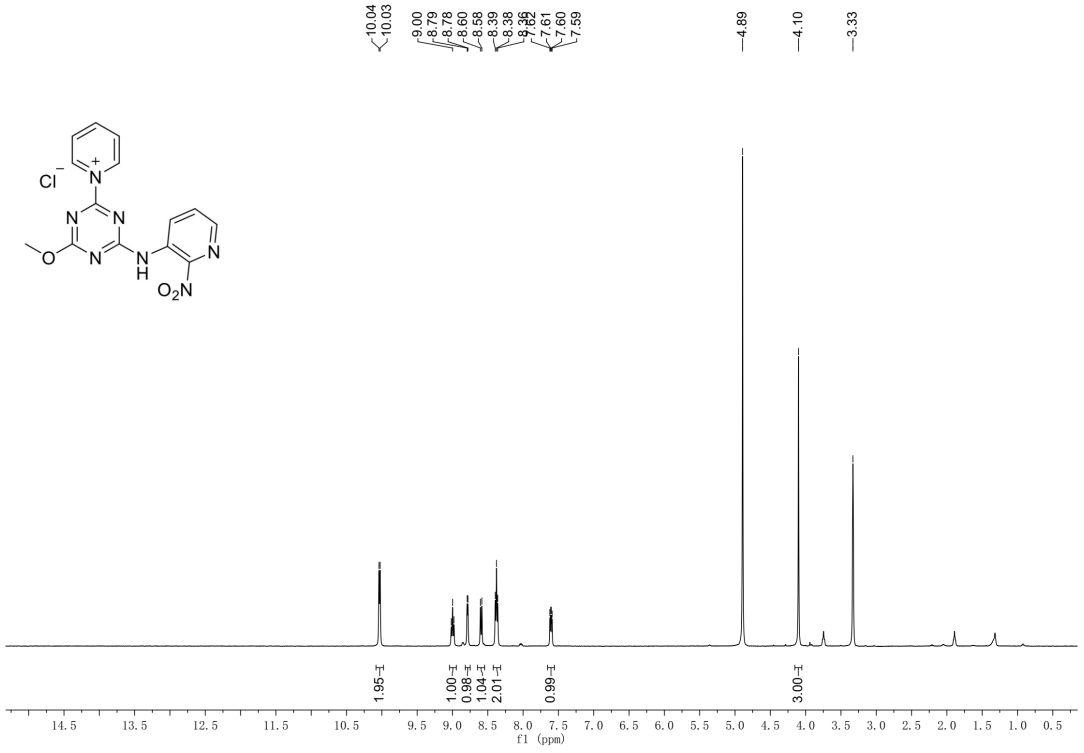

174.19
172.88
165.55
155.84
152.36
146.24
140.99
128.09
125.37
122.45
56.61
Cl⁻
NO2
f1 (ppm)

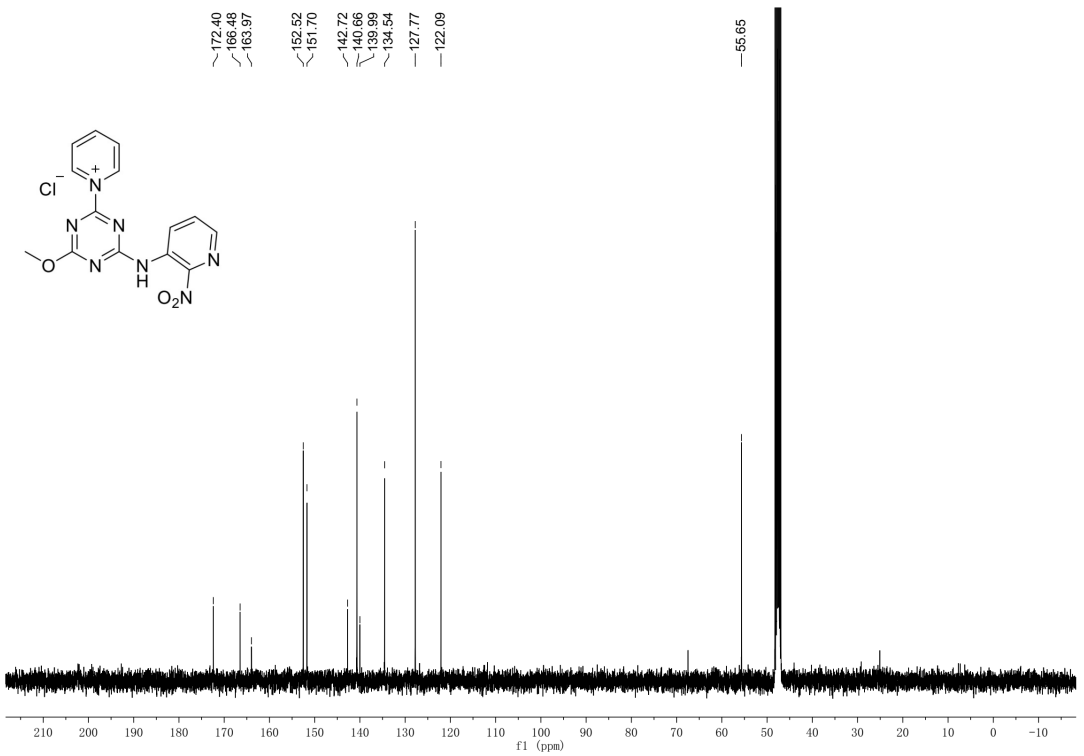
Compound 3


10.08
10.07
9.02
9.00
8.99
8.40
8.38
8.37
7.51
7.49
7.35
7.34
7.32
7.28
7.27
7.25
4.83
4.64
4.25
3.30
3.30
3.30
3.29
3.29
Cl⁻
N
N
N
O
N
S
1.86
0.96
1.96
1.85
1.87
0.93
1.99
3.00
f1 (ppm)

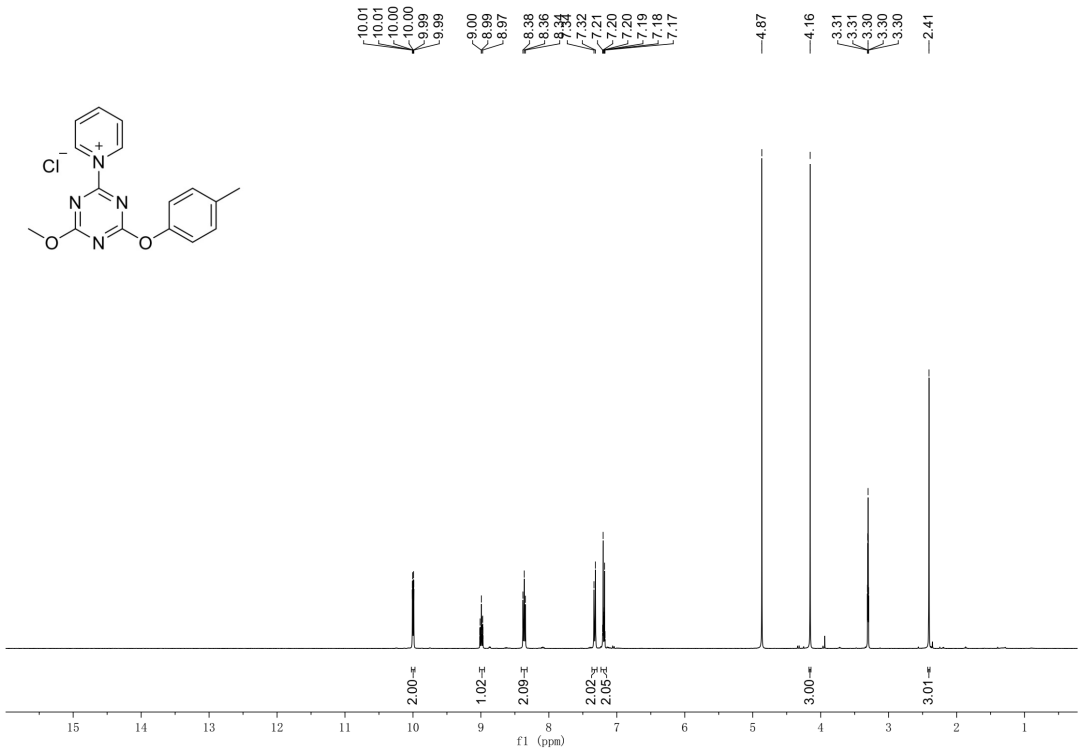

187.10
170.97
162.69
151.99
140.93
136.01
128.76
128.47
128.01
127.47
56.37
34.95
Cl⁻
N
N
N
O
N
S
f1 (ppm)

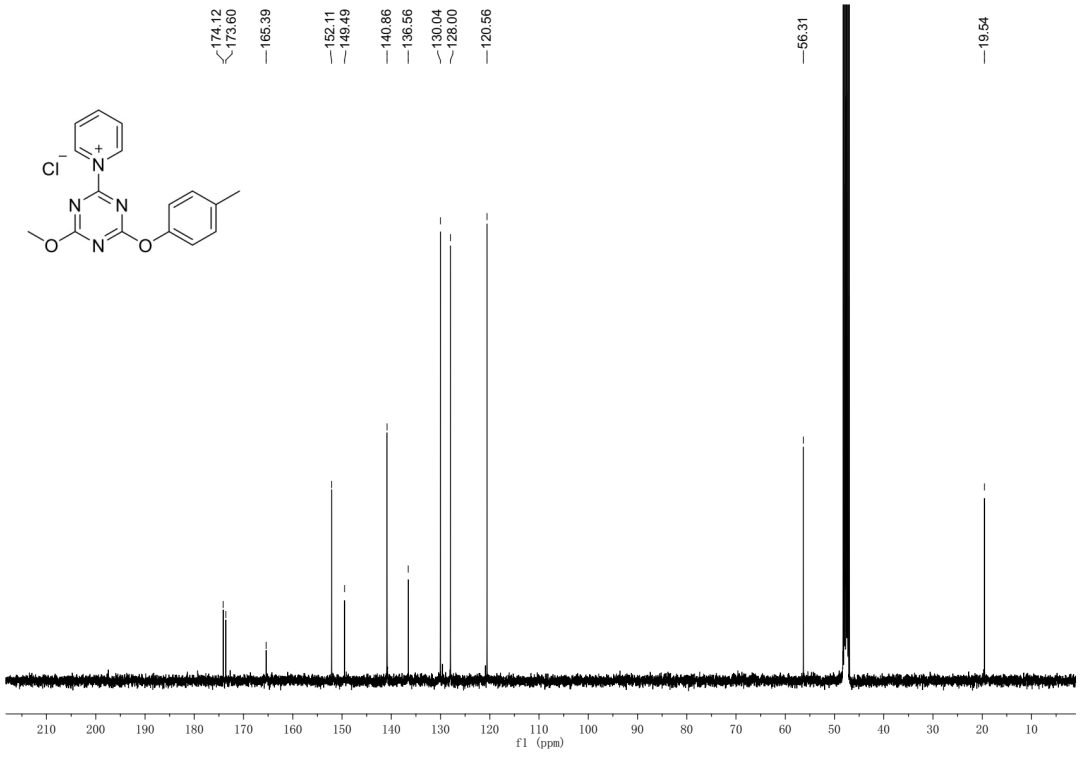
Compound 4


10.04
10.03
9.00
8.79
8.78
8.60
8.58
8.39
8.38
7.61
7.60
7.59
4.89
4.10
3.33
1.95
1.00
0.98
1.04
2.01
0.99
3.00
f1 (ppm)

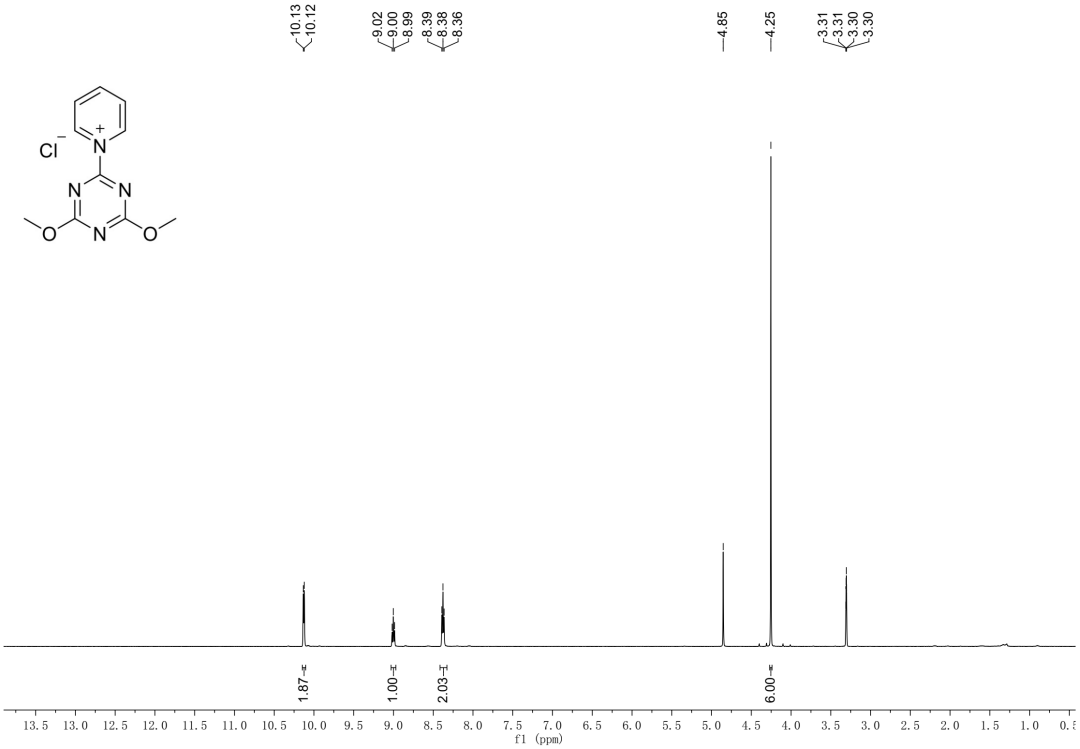

172.40
166.48
163.97
152.52
151.70
142.72
140.66
139.99
134.54
127.77
122.09
55.65
f1 (ppm)

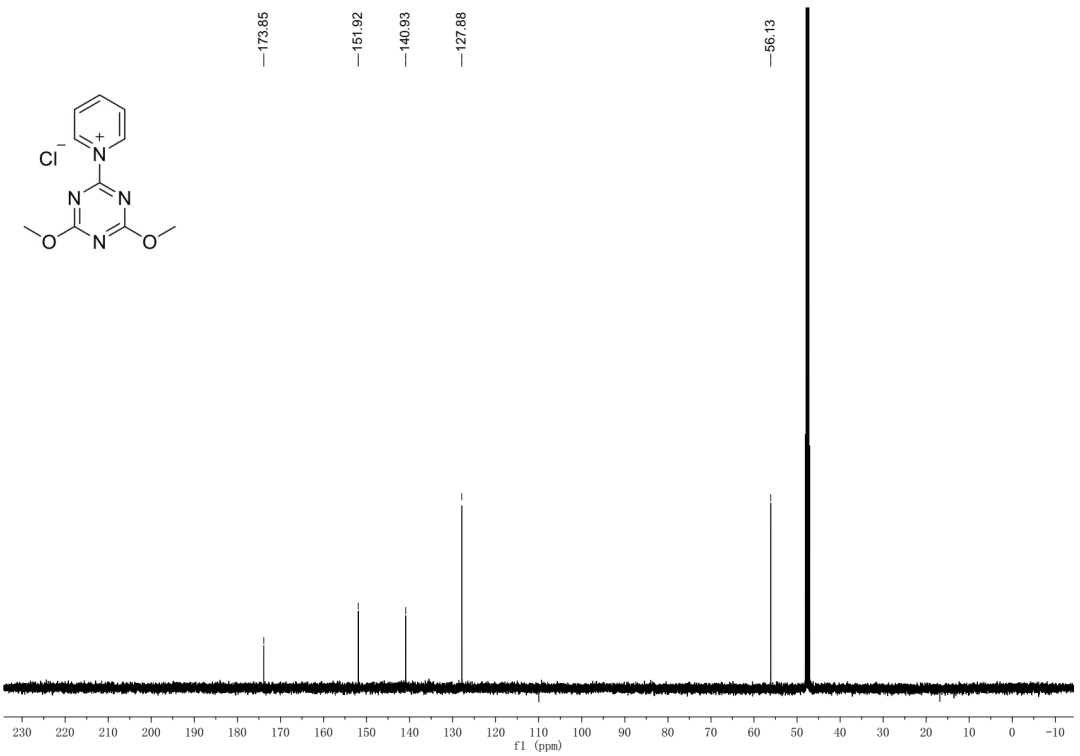
Compound 5


10.01
10.01
10.00
10.00
9.99
9.99
9.00
8.99
8.97
8.38
8.36
7.32
7.21
7.20
7.20
7.19
7.18
7.17
4.87
4.16
3.31
3.31
3.30
3.30
3.30
2.41
Cl
N
N
N
O
N
O
2.00
1.02
2.09
2.02
2.05
3.00
3.01
f1 (ppm)

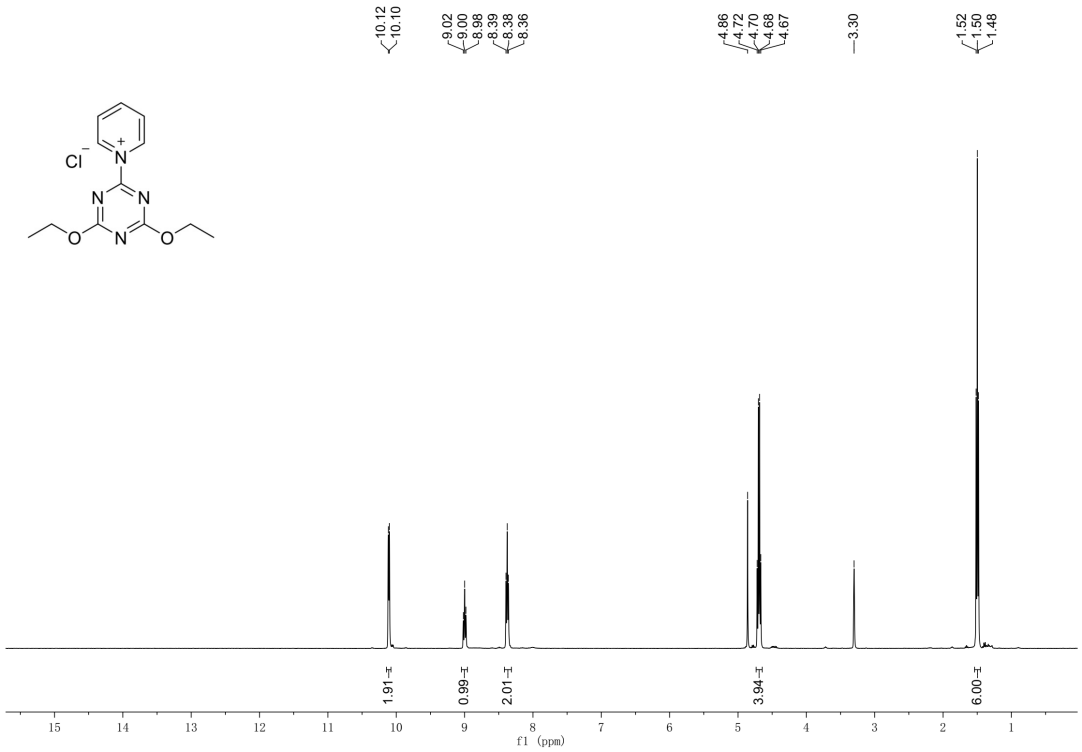

174.12
173.60
165.39
152.11
149.49
140.86
136.56
130.04
128.00
120.56
56.31
19.54
Cl
N
N
N
O
N
O
f1 (ppm)

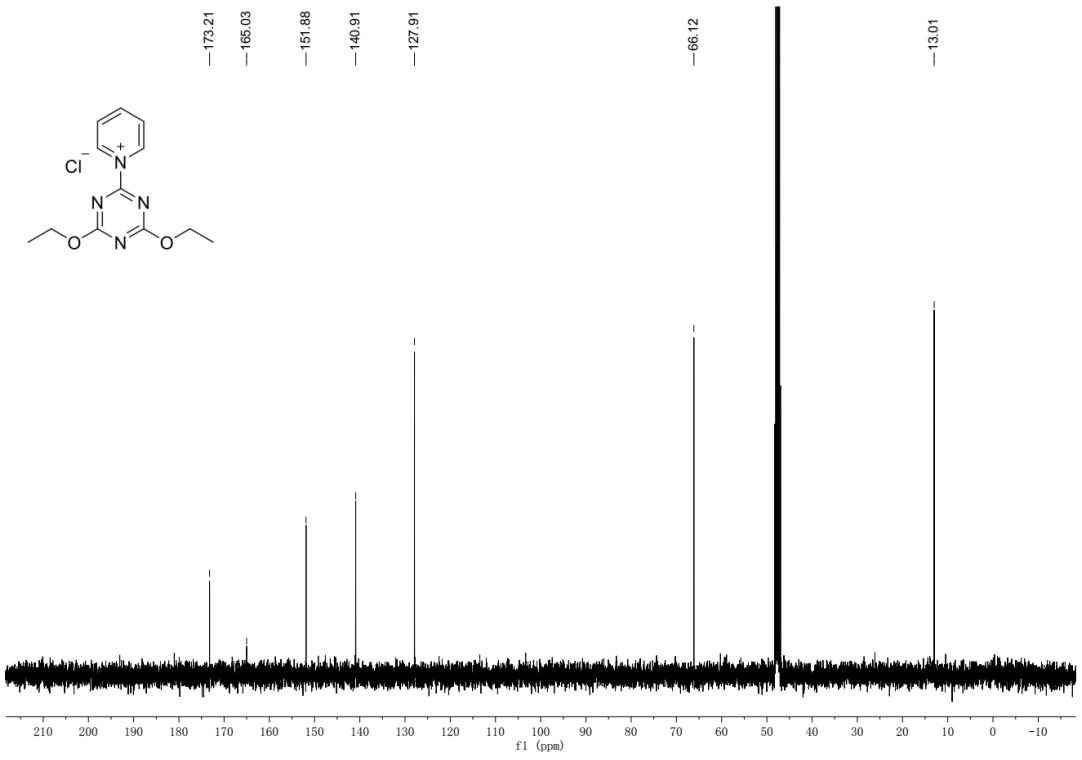
Compound 6


10.13
10.12
9.02
9.00
8.99
8.39
8.38
8.36
4.85
4.25
3.31
3.31
3.30
3.30
Cl⁻
1.87
1.00
2.03
6.00
f1 (ppm)

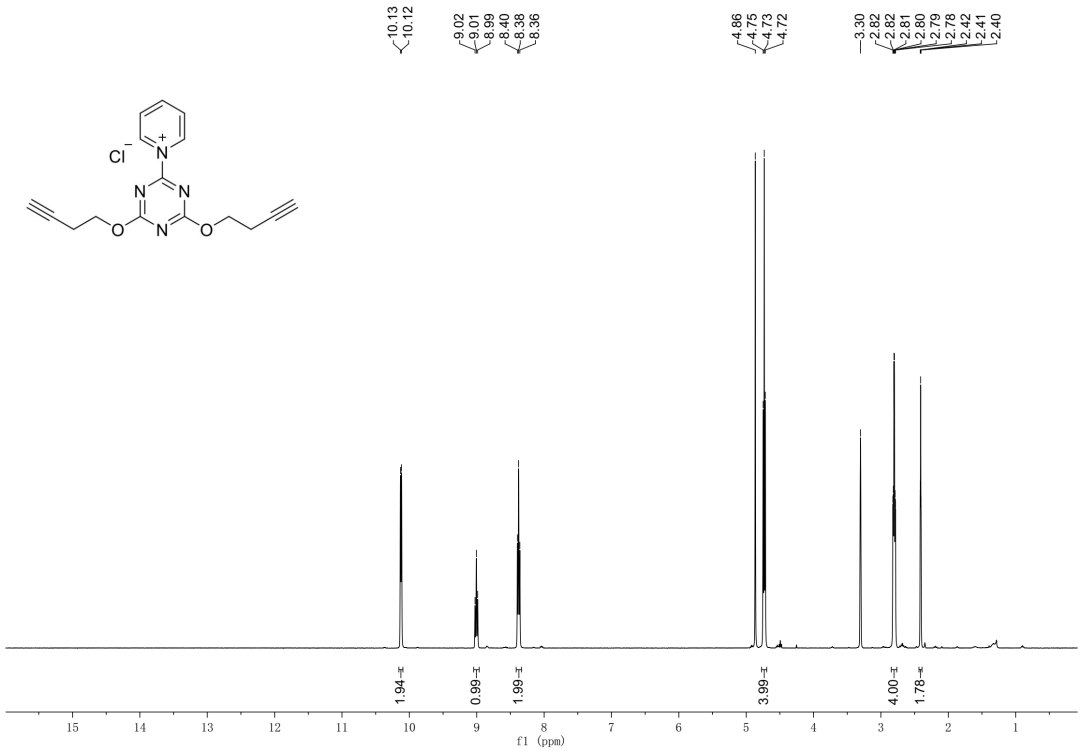

173.85
151.92
140.93
127.88
56.13
Cl⁻
f1 (ppm)

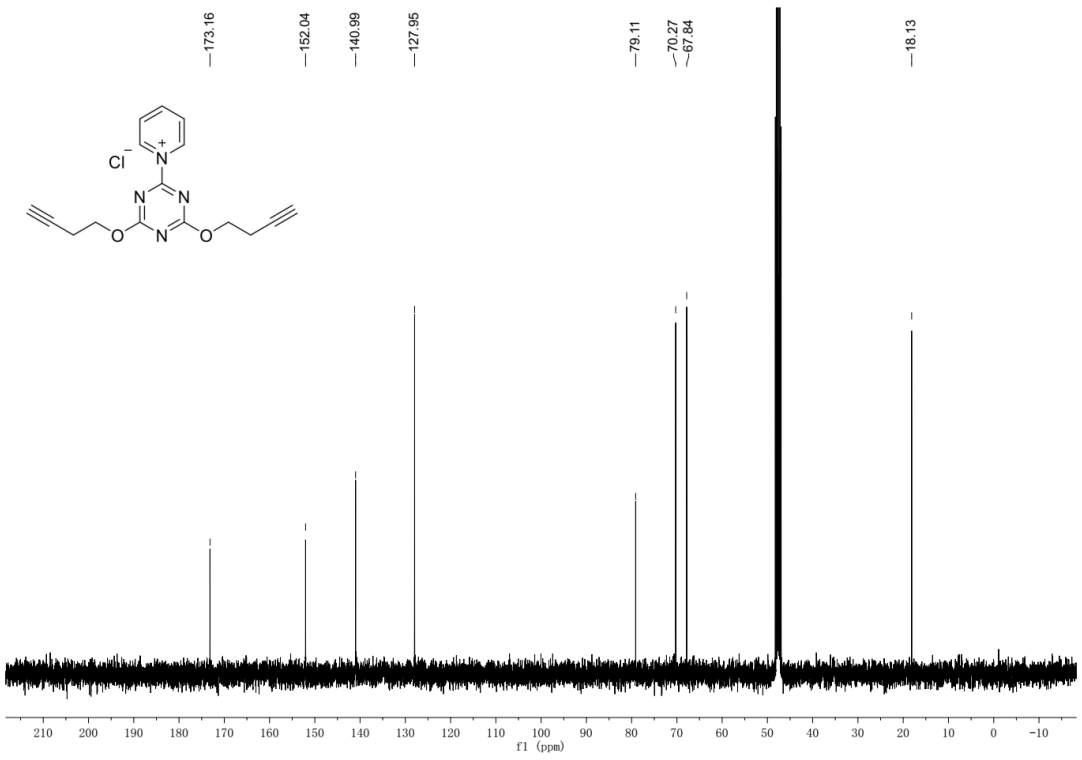
Compound 7


10.12
10.10
9.02
9.00
8.98
8.39
8.38
8.36
4.86
4.72
4.70
4.68
4.67
3.30
1.52
1.50
1.48
1.91
0.99
2.01
3.94
6.00
f1 (ppm)

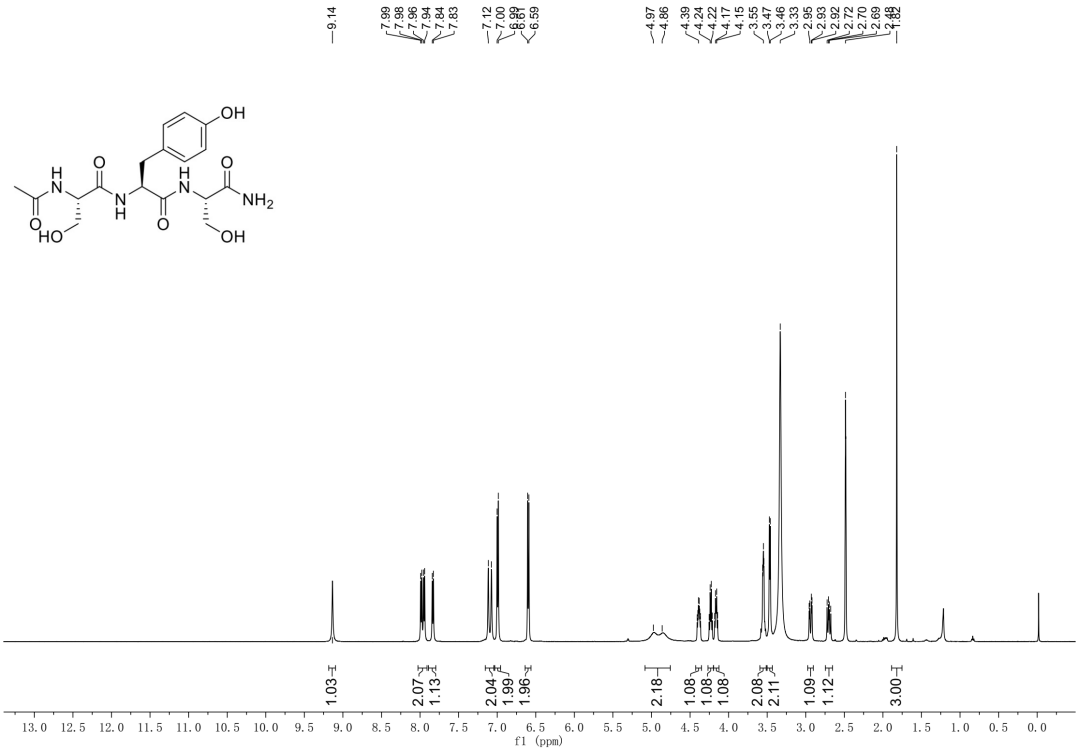

173.21
165.03
151.88
140.91
127.91
66.12
13.01
f1 (ppm)

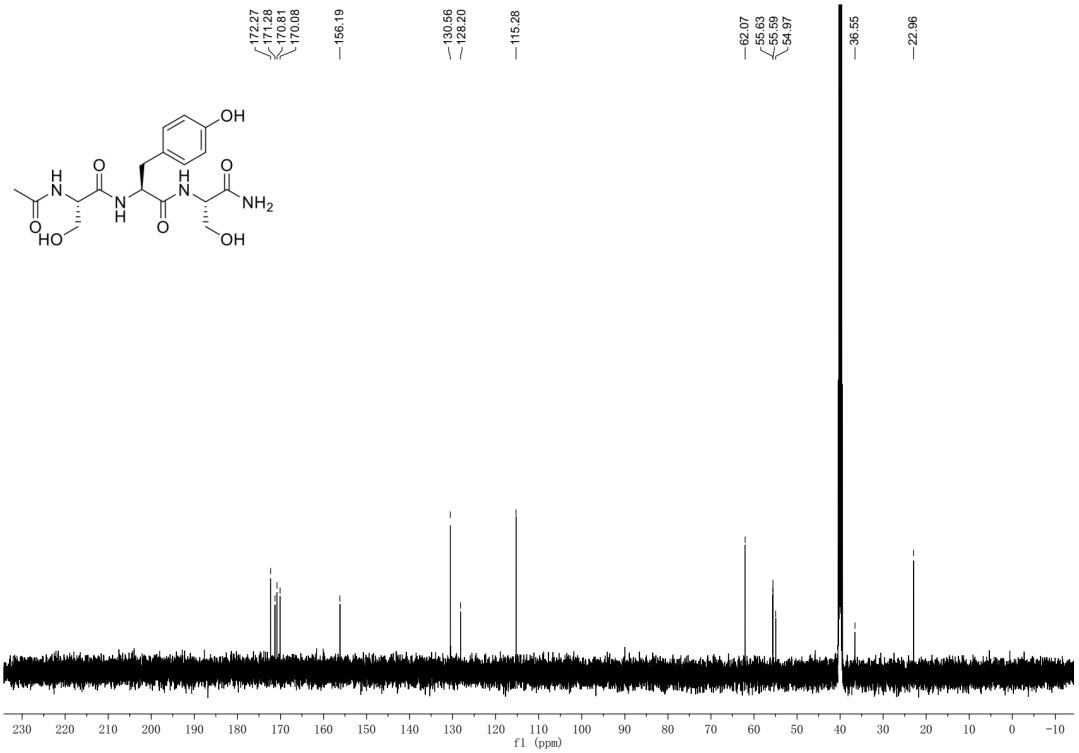
Probe 8


10.13
10.12
9.02
9.01
8.99
8.40
8.38
8.36
4.86
4.75
4.73
4.72
3.30
2.82
2.82
2.81
2.80
2.79
2.78
2.42
2.41
2.40
1.94
0.99
1.99
3.99
4.00
1.78
f1 (ppm)

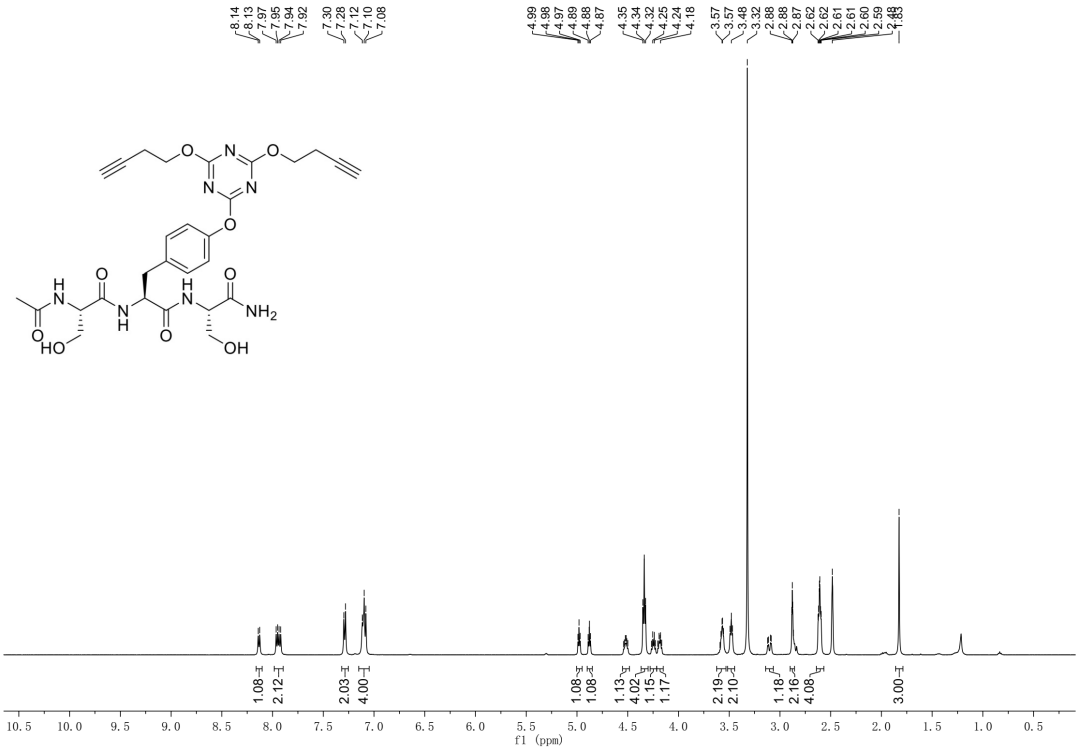

173.16
152.04
140.99
127.95
79.11
70.27
67.84
18.13
f1 (ppm)

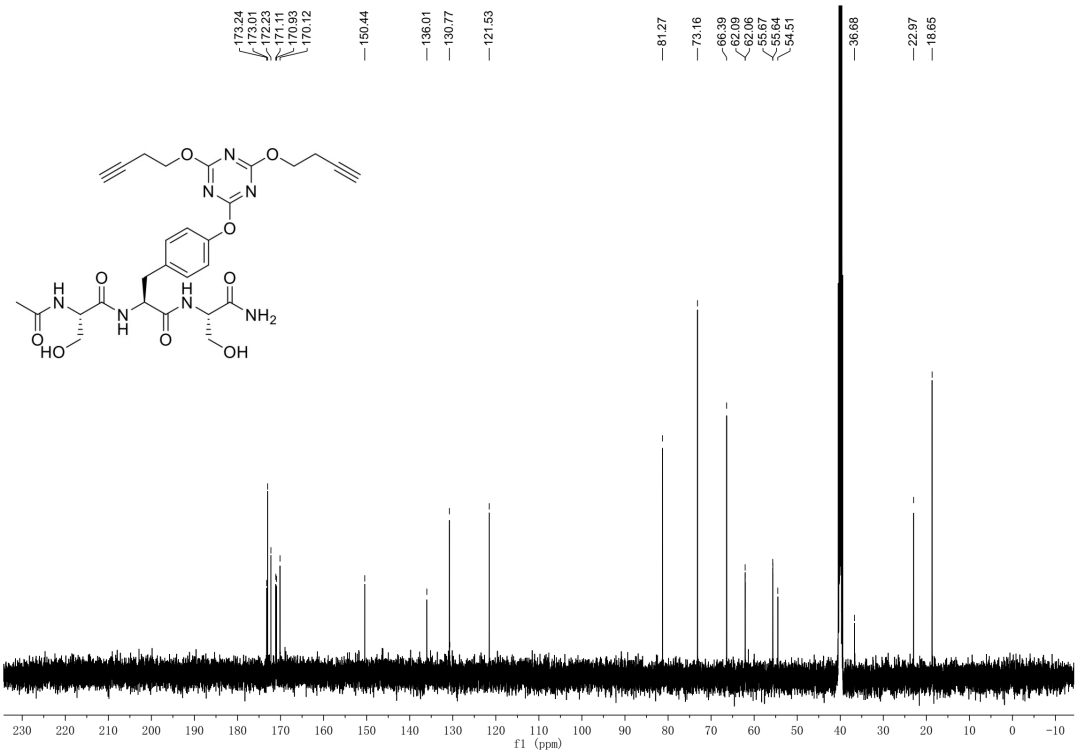
Compound MP_SYS



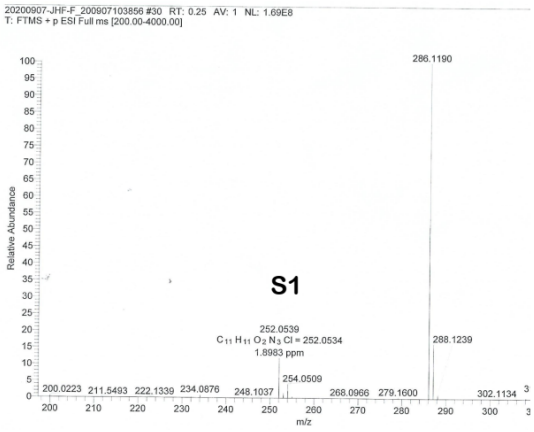


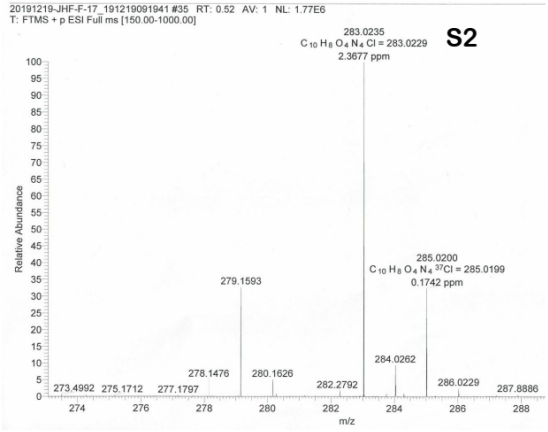
Compound MP_SYS_8

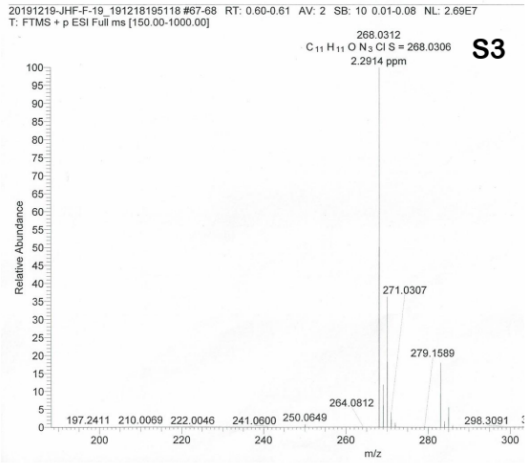


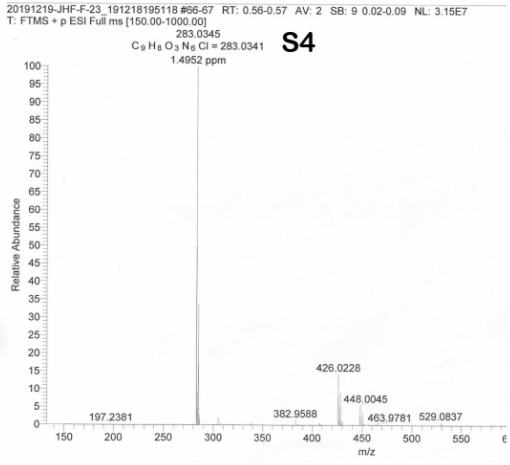


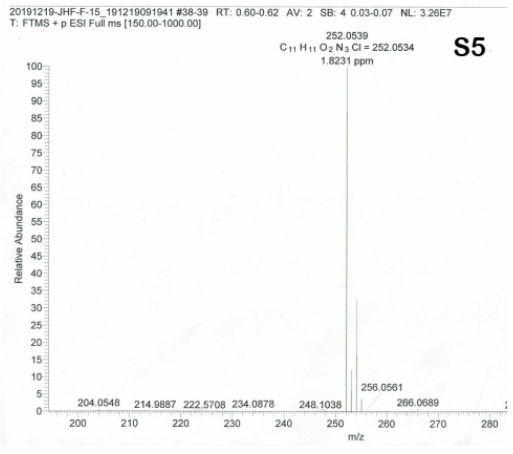
HRMS Spectra
Compound S1-S7

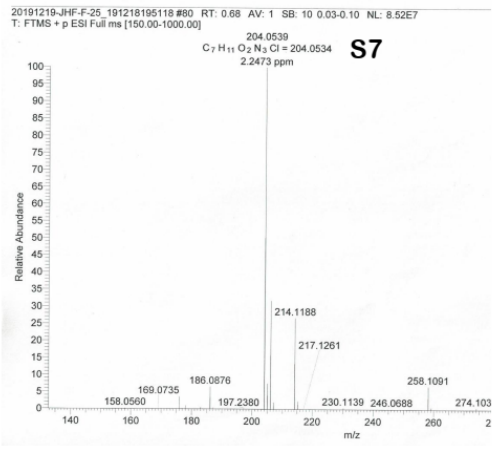




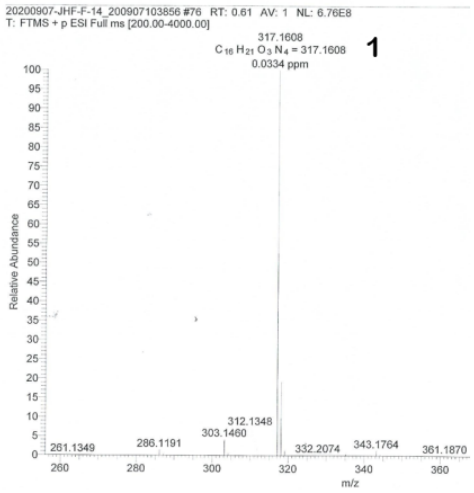


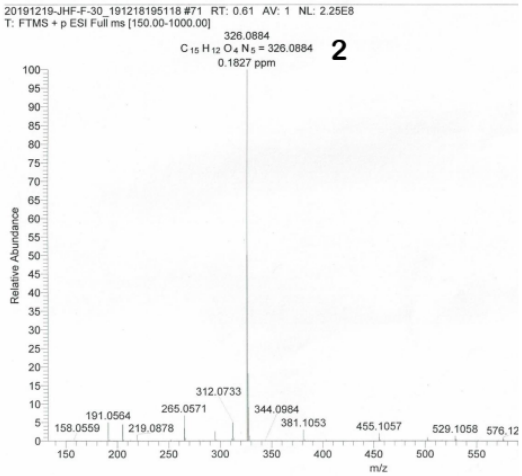


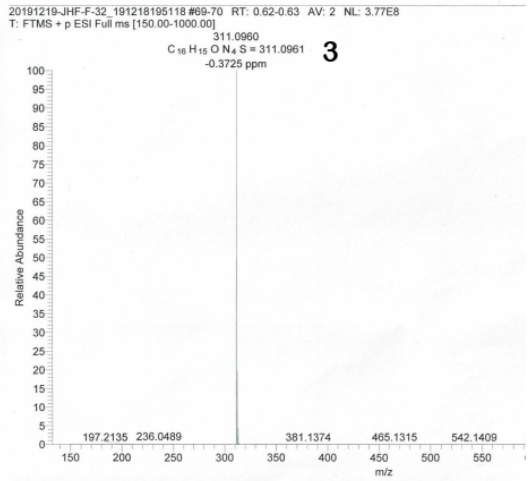


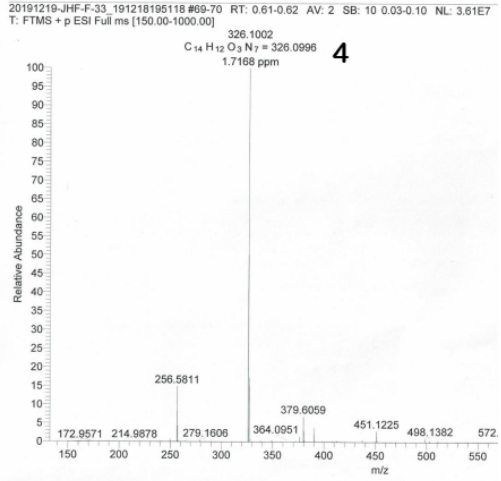


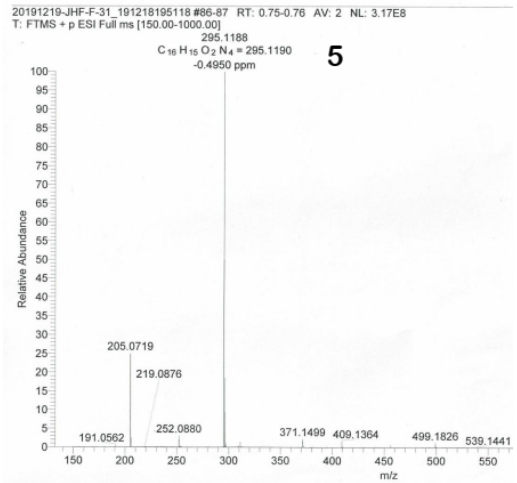
HRMS Spectra
Compound 1-8, MP-SYS, MP-SYS-8

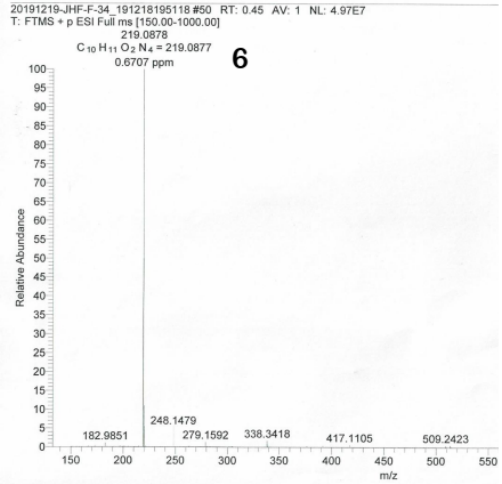


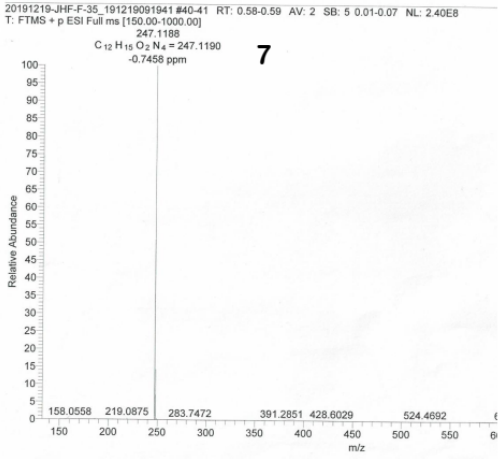


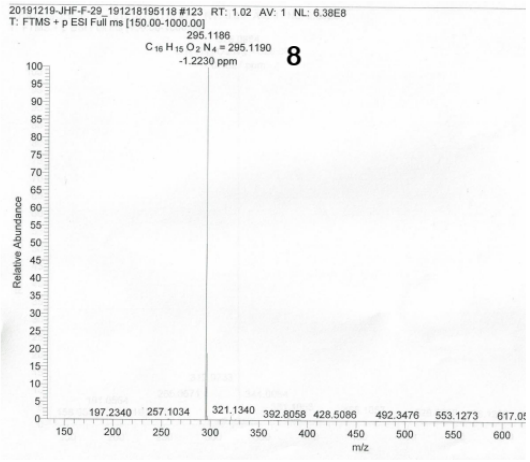


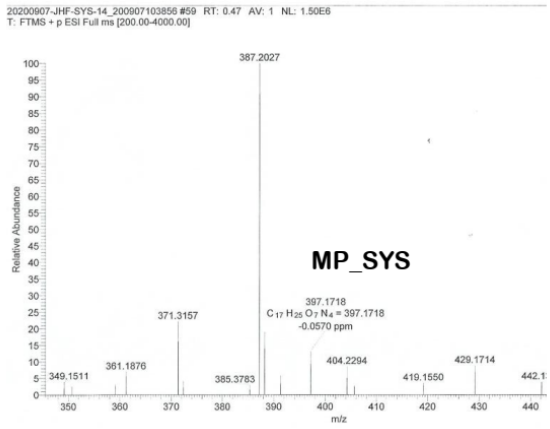


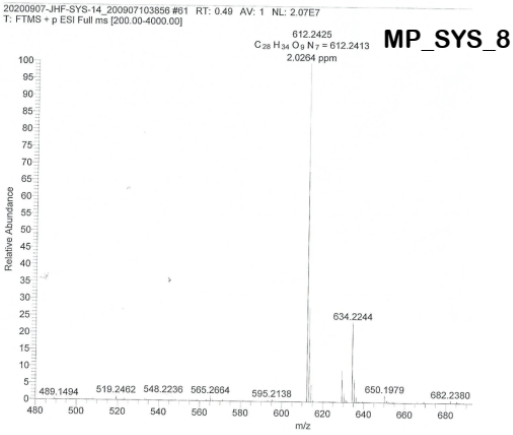





20191219-JHF-F-35_191219091941 #40-41 RT: 0.58-0.59 AV: 2 SB: 5 0.01-0.07 NL: 2.40E8
T: FTMS + p ESI Full ms [150.00-1000.00]
247.1188
C 12 H 15 O 2 N 4 = 247.1190
-0.7458 ppm
7
Relative Abundance
m/z
158.0558
219.0875
283.7472
391.2851
428.6029
524.4692


20191219-JHF-F-29_191218195118 #123 RT: 1.02 AV: 1 NL: 6.38E8
T: FTMS + p ESI Full ms [150.00-1000.00]
295.1186
C 16 H 15 O 2 N 4 = 295.1190
-1.2230 ppm
8
Relative Abundance
m/z
197.2340
257.1034
321.1340
392.8058
428.5086
492.3476
553.1273


20200907-JHF-SYS-14_200907103856 #59 RT: 0.47 AV: 1 NL: 1.50E6
T: FTMS + p ESI Full ms [200.00-4000.00]
MP_SYS
387.2027
397.1718
C 17 H 25 O 7 N 4 = 397.1718
-0.0570 ppm
371.3157
361.1876
349.1511
385.3783
404.2294
419.1550
429.1714
Relative Abundance
m/z


20200907-JHF-SYS-14_200907103856 #61 RT: 0.49 AV: 1 NL: 2.07E7
T: FTMS + p ESI Full ms [200.00-4000.00]
MP_SYS_8
612.2425
C 28 H 34 O 9 N 7 = 612.2413
2.0264 ppm
634.2244
489.1494
519.2462
548.2236
565.2664
595.2138
650.1979
682.2380
Relative Abundance
m/z

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