Protein tetrazinylation via diazonium coupling for covalent and catalyst-free bioconjugation

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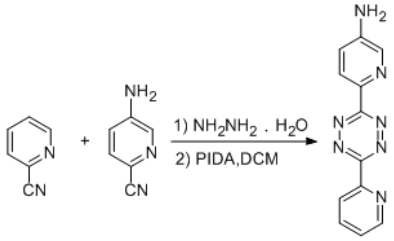
1. General

All chemicals and solvents used for synthesis were purchased from commercial suppliers and applied directly in the experiment without further purification. The progress of the reaction was monitored by TLC on pre-coated silica plates (Merck 60F-254, 250 μm in thickness), and spots were visualized by UV light. Merck silica gel (100-200 mesh) was used for general column chromatography purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethylsilane ($Si(CH_3)_4$ = 0.00 ppm) or residual solvent peaks (DMSO-d_6 = 2.50 ppm, MeOD = 3.310 ppm). ^{1}H NMR coupling constants (J) are reported in Hertz (Hz), and multiplicity is indicated as the following: s (singlet), d (doublet), t (triplet), dd (doublet doublet), m (multiple). High-resolution mass spectra (HRMS) were obtained on a XEVO-G2QTOF (ESI) (Waters, USA) or Bruker Apex IV FTMS. The UV-visible spectra were recorded on a UV-3600 UV-VIS-NIR-spectrophotometer (SHIMADZU, Japan).

2. Synthesis of the diazonium reagent (1)

6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-amine (2)

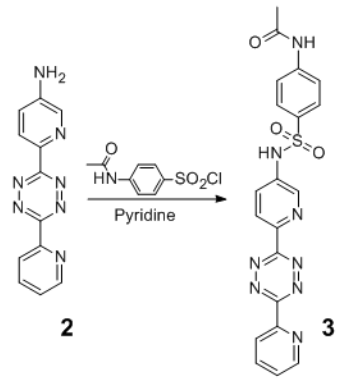


2

Compound **2** was synthesized according to the literature (*Tetrahedron Letters*, 2014, **55**, 4795). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.90 (s, 1H), 8.53 (d, J = 7.6 Hz, 1H), 8.36 (d, J = 8.5 Hz, 1H), 8.23 (s, 1H), 8.12 (s, 1H), 7.69 (s, 1H), 7.12 (d, J = 8.2 Hz, 1H), 6.37 (s, 2H). HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{12}H_{10}N_7$: 252.0992; found: 252.0998.

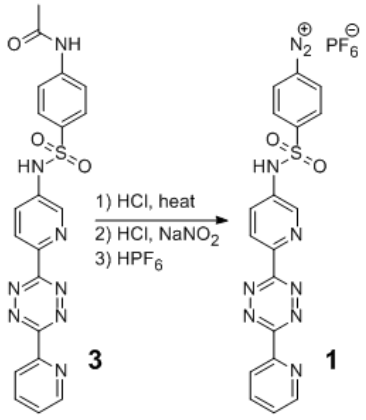
6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-4'-acetamidobenzenesulfona

mide (3)



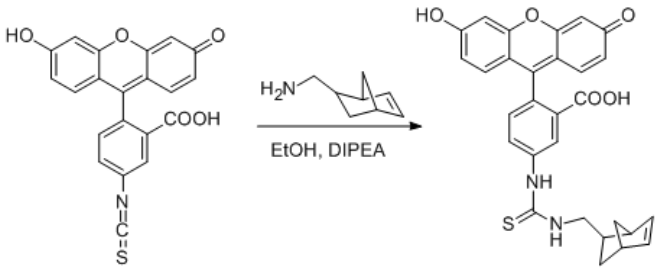
To an ice-cooled solution of **2** (466 mg, 1.86 mmol) in pyridine (8 mL) was slowly added 4-acetamidobenzenesulfonyl chloride (867 mg, 3.72 mmol) in pyridine (2 mL). The mixture was stirred at 0 ℃ for 2 h and allowed to reach room temperature. Water (20 mL) was added and the resulting solution was extracted by ethyl acetate. After removing the solvent under reduced pressure, the crude product was purified by silica gel column chromatography (MeOH:CH_2Cl_2 = 10%) to give pink solid **3** (566 mg, 67.7%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.91 (d, J = 4.3 Hz, 1H), 8.63 (d, J = 2.3 Hz, 1H), 8.55 (dd, J = 11.8, 8.4 Hz, 2H), 8.18 – 8.08 (m, 1H), 7.89 – 7.80 (m, 3H), 7.76 (d, J = 8.8 Hz, 2H), 7.70 (dd, J = 7.2, 5.0 Hz, 1H), 2.04 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.1, 163.0, 162.7, 150.6, 150.1, 144.5, 143.7, 141.2, 137.8, 137.5, 132.3, 128.2, 126.6, 126.2, 125.0, 124.2, 118.8, 24.1. HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{20}H_{17}N_8O_3S$: 449.1139; found: 449.1140.

6-(6-(pyridine-2-yl)-1,2,4,5-tetrazin-3-yl)pyridine-3-4'-diazoniumbenzenesulfonamide hexafluorophosphate (1)



3 (190 mg, 0.42 mmol) was dissolved in 15 mL concentrated HCl and was heat at 50 ℃ overnight. Then the solution was added 10 mL concentrated HCl and 20 mL water and cooled down to 0 ℃. The water solution of $NaNO_2$ (234 mg, 3.4 mmol) was slowly added to the mixture at 0 ℃. After 1.5 h reaction, 60% HPF_6 in water (626 μL, 4.3 mmol) was added at 0 ℃ and stirred for 1 h. The product was collected by filtration and washed by ice-cold diethyl ether, yielding a red solid of **1** (109 mg, 46.1%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 – 8.81 (m, 3H), 8.72 (s, 1H), 8.68 – 8.53 (m, 2H), 8.51 – 8.33 (m, 2H), 8.20 (t, J = 7.5 Hz, 1H), 7.99 – 7.91 (m, 1H), 7.80 – 7.72 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.9, 162.7, 150.3, 149.6, 148.2, 145.9, 142.1, 138.5, 136.1, 134.2, 129.1, 128.6, 128.0, 127.0, 125.2, 124.4, 121.4. ^{31}P NMR (162 MHz, DMSO-d_6) δ -143.22 (h, $J_{P\text{-}F}$ = 711.3 Hz). ^{19}F NMR (376 MHz, DMSO-d_6) δ -70.11 (d, $J_{P\text{-}F}$ = 711.3 Hz). HRMS (ESI): m/z $[M]^+$ calcd. for $C_{18}H_{12}N_9O_2S$: 418.0829; found: 418.0821.

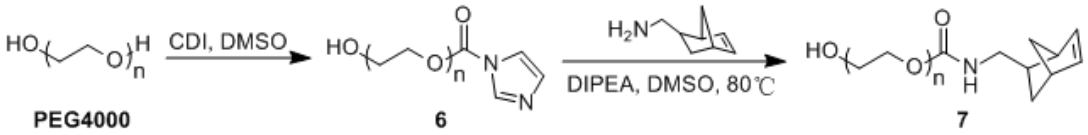
3. Synthesis of norbornylene-containing fluorescent dye (5)



Fluorescein isothiocyanate isomer I (50 mg, 0.13 mmol) was dissolved in ethanol. Then 1-bicyclo[2.2.1]hept-5-ene-2-methylamine (18 μL, 0.14 mmol) and DIPEA (27 μL, 0.15 mmol) were added. The mixture was stirred at room temperature overnight. After removing the solvent under reduced pressure, the crude product was purified by silica gel column chromatography (MeOH:CH_2Cl_2 = 2.5%) to give **5** (13.1 mg, 19.7%). ^{1}H NMR (400 MHz, MeOD) δ 8.17 (s, 1H), 7.77 (dd, J = 8.2, 2.0 Hz, 1H), 7.15 (m, 1H), 6.72 – 6.66 (m, 4H), 6.54 (dd, J = 8.7, 2.3 Hz, 2H), 6.24 – 6.02 (m, 2H), 2.91 (s, 1H), 2.83 (s, 1H), 2.56 – 2.43 (m, 1H), 1.99 – 1.87 (m, 1H), 1.47 (m, 2H), 1.40 (m, 2H), 0.66 (m, 1H). ^{13}C NMR (101 MHz, MeOD) δ 171.3, 154.2, 142.7,

138.6, 137.8, 137.4, 133.3, 130.3, 129.9, 129.0, 125.6, 113.7, 111.6, 103.5, 50.5, 45.6, 43.7, 39.4, 31.1. HRMS (ESI): m/z $[M+H]^+$ calcd. for $C_{29}H_{25}N_2O_5S$: 513.1479; found: 513.1479.

4. Synthesis of norbornylene–PEG4000 (7)



PEG4000 6 7

PEG4000 (8.0 g, 2.0 mmol) was heated at 80 ℃ under reduced pressure to remove water. The solid was dissolved by 40 mL DMSO (with 5% CH_2Cl_2 to facilitate the dissolution of PEG4000) after cooling down to room temperature. CDI (1.56 g, 9.6 mmol) was added to the solution and stired for 40 h. After removing CH_2Cl_2 under reduced pressure, the solution was diluted with 500 mL EtOAc and put at -20 ℃ overnight. The resulting white precipitate was collected by filtration and washed by Et_2O to give **6** (5.65 g, 69.0%). **6** (5.40 g, 1.3 mmol) was dissolved in 5 mL DMSO. 1-bicyclo[2.2.1]hept-5-ene-2-methylamide (390 μL, 1.95 mmol) and DIPEA (670 μL, 3.9 mmol) were added to the DMSO solution. After heated at 80 ℃ for 30 h, the mixture was washed by brine and extracted with CH_2Cl_2 (3 × 25 mL). The CH_2Cl_2 solution was dried by Na_2SO_4 and concentrated under reduced pressure. And the resulting oil-like mixture was diluted with 150 mL EtOAc and put at -20 ℃ overnight. The resulted white precipitate was collected by centrifugation. The product **7** was given by drying the solid under reduced pressure, yield 4.54 g (83.9%). ^{1}H NMR (400 MHz, DMSO) δ 7.22 (t, J = 5.6 Hz, 1H), 6.16 – 5.93 (m, 2H), 3.67 (t, J = 9.6 Hz, 2H), 3.51 (s, 332H), 3.35 – 3.31 (m, 2H), 2.23 – 2.10 (m, 1H), 1.79 – 1.69 (m, 1H), 1.29 (t, J = 7.9 Hz, 2H), 1.19 (t, J = 9.2 Hz, 2H), 0.51 – 0.38 (m, 1H). ^{13}C NMR (101 MHz, DMSO) δ 156.0, 136.8, 132.5, 69.8, 68.9, 63.0, 48.9, 44.3, 43.6, 41.8, 29.6. The integration of chemical shift at 3.51 in ^{1}H NMR spectrum in **7** contained about 332H (83 CH_2CH_2 moieties), which is consistent with the PEG4000 moiety (83*44 = 3652 Da (mass for OCH_2CH_2 is 44 Da)). And the HRMS spectrum of **7** showed the increase mass compared with the HRMS spectrum of the starting PEG4000.

5. UV-Vis absorbance analysis

The solubility of the diazonium reagent **1**: **1** was dissolved in PBS (20 mM, pH 7.4, containing 5% DMSO) with different concentrations (50, 100, 200, 250 or 300 μM). The solution was monitored by UV-Vis spectrophotometry from 250 to 600 nm.

Reaction with Tyr-containing molecule: To 2 mL PBS (20 mM, pH 7.4, containing 2% DMSO) was added 2 μL **1** (20 mM solution in DMSO) and 4 μL **4** (100 mM solution in DMSO). The progress of the reaction was monitored by UV-Vis spectrophotometry from 250 to 600 nm.

The stability of the diazonium reagent **1** in PBS: 10 μL **1** (20 mM solution in DMSO) was added to 1990 μL PBS (20 mM, pH 7.4, containing 30% DMSO). The solution was monitored by UV-Vis spectrophotometry from 250 to 600 nm.

The stability of the solid diazonium reagent **1**: The solid reagent **1** was firstly stored at -20℃ and stored at room temperature for 1, 4 or 7 day(s). Then 10 μL **1** (20 mM solution in DMSO) was added to 1990 μL PBS (20 mM, pH 7.4, containing 30% DMSO). The solution was monitored by UV-Vis spectrophotometry from 250 to 600 nm.

The selectivity of the diazonium reagent **1**: To 1990 μL PBS (20 mM, pH 7.4, containing 30% DMSO) was added 10 μL **1** (20 mM solution in DMSO) and amino acid (Arg, Cys, Glu, His, Lys, Met, Phe, Pro, Ser, Thr, or Tyr) solution whose final concentration was 0.5 mM. The reaction was incubated for 10 min at room temperature. Then the solution was monitored by UV-Vis spectrophotometry from 250 to 600 nm.

In order to explore the kinetics of the reaction of **1** and His or Tyr, the reaction progress was monitored by UV-Vis spectrophotometry at 365 nm. The reaction condition was 10 μL **1** (20 mM solution in DMSO) and 2 μL His or Tyr (100 mM solution in H_2O) was added to 1988 μL PBS (20 mM, pH 7.4, containing 30% DMSO).

Modification of lysozyme by **1**: To 1970 μL PBS (20 mM, pH 7.4, containing 30% DMSO) was added 10 μL **1** (20 mM solution in DMSO) and 20 μL Lysozyme (1 mM solution in PBS). The progress of the reaction was monitored by UV-Vis

spectrophotometry from 0 to 55 min.

6. Fluorescence labelling of model proteins

One-pot reaction: 100 μL protein (BSA or lysozyme, 100 μM) solution in PBS (20 mM, pH 7.4) was added 1 μL **1** (100 mM in DMSO) and the mixture was incubated for 2 h at room temperature. Then 1 μL **5** (100 mM in DMSO) was added to the mixture and incubated for another 2 h. The proteins treated with **5** only were set as control group. After reaction, the protein samples were directly electrophored in 15% SDS-PAGE. The PAGE was firstly imaged under the 365 nm UV light to detect whether the proteins were labelled. After the UV imaging, the PAGE was stained by Coomassie brilliant blue and destained by glacial acetic acid/alcohol/water mixture (2:1:17).

7. Protein PEGylation

PEGylation without SDS: 100 μL lysozyme (100 μM) solution in PBS (20 mM, pH 7.4) was added 1 μL **1** (100 mM in DMSO) and the mixture was incubated for 2 h at room temperature. Then the mixture was added 2 μL **7** (250 mM in DMSO). The reaction was incubated overnight. The lysozyme treated with **1** or **7** only was set as control group. After reaction, the protein samples were directly electrophored in 15% SDS-PAGE. The PAGE was stained by Coomassie brilliant blue and destained by glacial acetic acid/alcohol/water mixture (2:1:17).

PEGylation with SDS: 300 μL lysozyme (400 μM) solution in PBS (20 mM, pH 7.4) was pretreated with 300 μL SDS (40 mM in PBS) for 5 min to obtain the Lys-SDS solution. The diazonium solution was prepared by adding 8 μL **1** (100 mM in DMSO) to 200 μL PBS and the PEG4000 solution was prepared by adding 8 μL **7** (500 mM in DMSO) to 200 μL PBS. The 200 μL Lys-SDS solution in ice bath was added the 100 μL diazonium solution and incubated for 2 h at room temperature. Then the 100 μL PEG4000 solution was added to the mixture in ice bath and incubated for another 2 days at room temperature. The Lys-SDS solution treated with the diazonium solution or the PEG4000 solution only was set as control group. After reaction, the protein

samples were directly electrophored in 15% SDS-PAGE. The PAGE was stained by Coomassie brilliant blue and destained by glacial acetic acid/alcohol/water mixture (2:1:17).

The similar operations were also carried out of which the reaction time treated with **7** were set as 3, 6, 9 h. Final concentrations in reaction: Lysozyme: 100 μM; SDS: 10 mM; reagent **1**: 1 mM; norbornylene-PEG4000 (**7**): 5 mM.

8. Modification of TMV

Preparing of tetrazine-containing TMV: 1 mL TMV suspension (10 mg/mL) was incubated with 20 μL **1** (250 mM in DMSO) in PBS (20 mM, pH 7.4) for one day at room temperature. The reaction mixture was then transferred into a centrifugal filtration device (10 KDa, Millipore) and eluted by PBS (20 mM, pH 7.4) twice. After desalination, the tetrazine-containing TMV was submitted into ESI-MS.

Fluorescence labeling of TMV: 100 μL tetrazine-containing TMV (2 mg/mL) was added 1 μL **5** (100 mM in DMSO) and incubated for overnight at room temperature. The TMV treated with **5** only was set as control group. After reaction, the TMV samples were directly electrophored in 15% SDS-PAGE. The PAGE was firstly imaged under the UV light to detect whether the TMV was labelled. After the UV imaging, the PAGE was stained by Coomassie brilliant blue and destained by glacial acetic acid/alcohol/water mixture (2:1:17).

9. Fluorescence labelling of *E. coli* cell lysates

Cell cultures of Escherichia coli (E. coli) strain DH5α were grown at 37 °C under 225 rpm in 2.5% Luria-Bertani medium (LB) over night. Cells were harvest by centrifugation at 4000 rpm for 10 min at 4 ℃ and resuspended in PBS on ice. Cells were lysed using SDS (5% w/v) to form cell lysates. 100 μL cell lysates (50 mg/mL) was was incubated with **1** whose final concentration was set as 2 mM or 5 mM for 2 h at room temperature. Then 3 μL **5** (100 mM in DMSO) was added to the mixture and incubated for overnight. The cell lysates treated with **5** only was set as control group. After reaction, the samples were directly electrophored in 10% SDS-PAGE. The

PAGE was firstly imaged by tanon-1600 fluorescent scanner to detect the fluorescence labeled cell lysates. After the UV imaging, the PAGE was stained by Coomassie brilliant blue and destained by glacial acetic acid/alcohol/water mixture (2:1:17).

10. Supporting figures

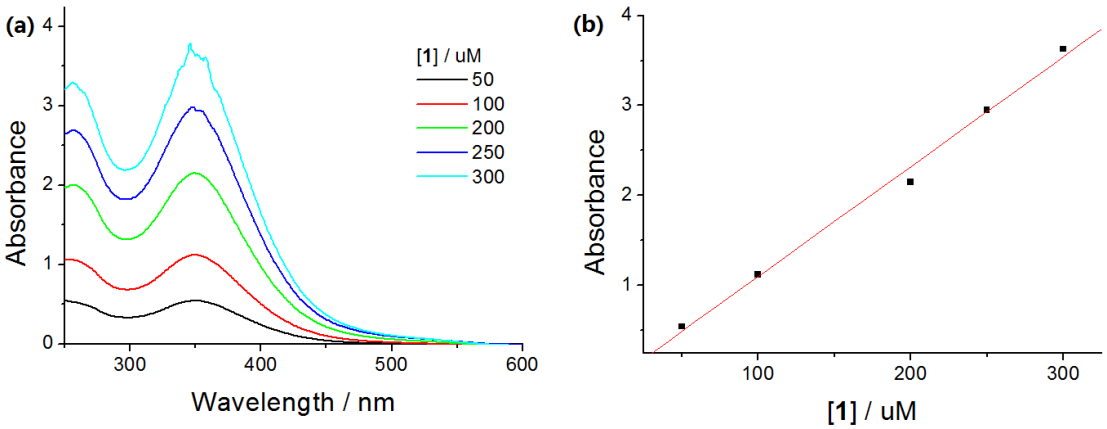


Fig. S1 (a) The UV-Vis absorbance spectra of different concentrations of **1** in PBS (20 mM, pH 7.4, containing 5% DMSO) at room temperature. The concentration was indicated in the inset. (b) Linear regression curve fitted according to the absorbance at 350 nm (R=0.997). The result indicated the good solubility of **1** in aqueous buffer.

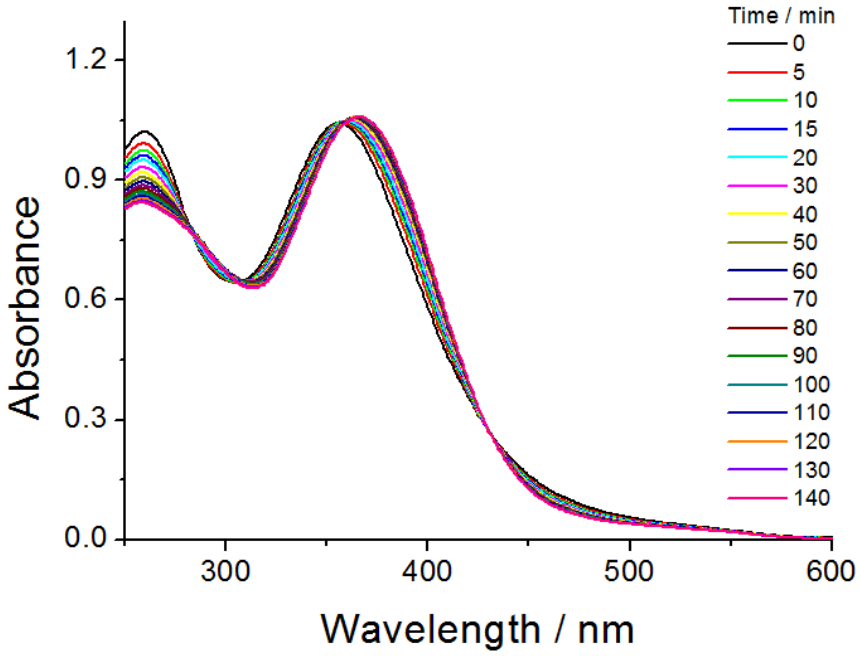


Fig. S2 Time-dependent UV-Vis absorbance spectra of **1** (100 μM) in PBS (20 mM, pH 7.4, containing 30% DMSO) at room temperature. The time was indicated in the inset.

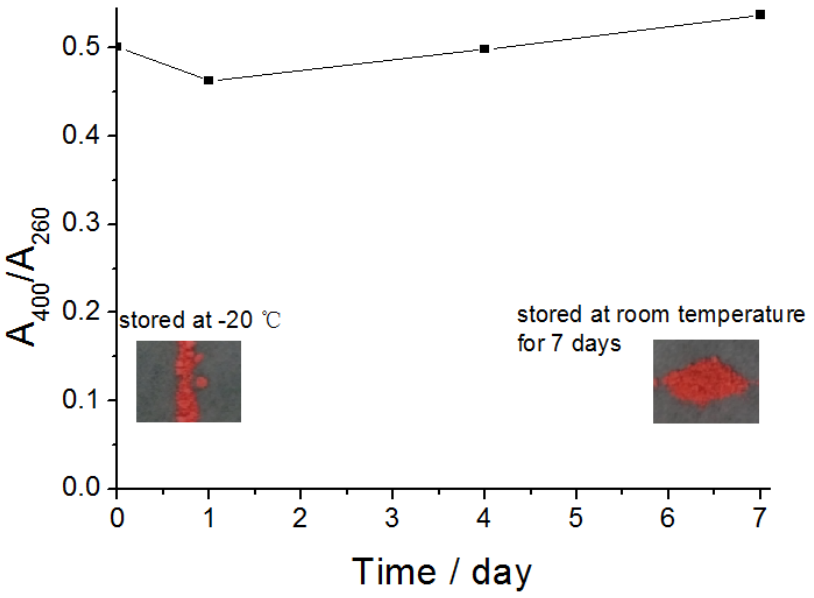


Fig. S3 The stability study of the solid reagent **1** was carried out by stored **1** at -20℃ and stored at room temperature for 1, 4 or 7 day(s). Then UV-Vis absorbance spectrum of **1** (100 μM) in PBS (20 mM, pH 7.4, containing 30% DMSO) at room temperature was tested and the absorbance ratio A_{400}/A_{260} was calculated. The little change of the ratio indicated little decomposition of **1**. And the insets showed that there was almost no change of the state of the solid reagent **1** after stored at room temperature for 7 days.

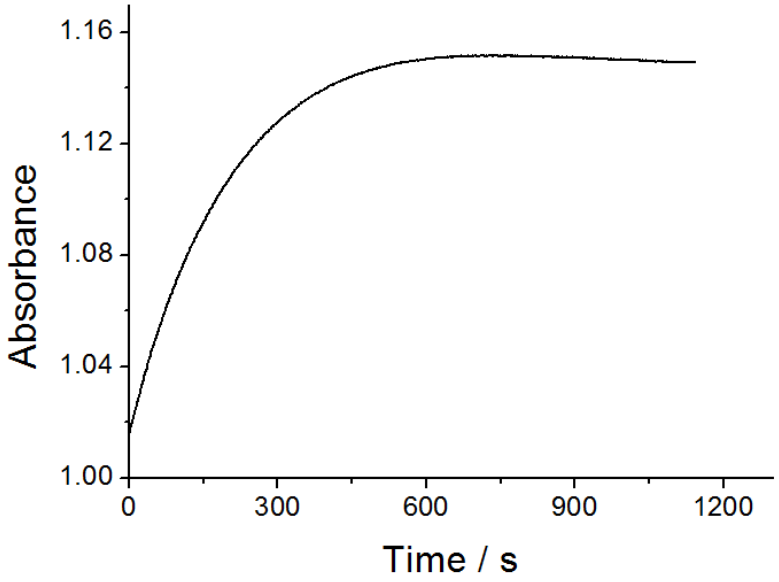


Fig. S4 The absorbance at 365 nm versus reaction time for **1** (100 μM) upon treatment with Tyr-containing small molecule **4** (100 μM) in PBS (20 mM, pH 7.4, containing 30% DMSO) at room temperature.

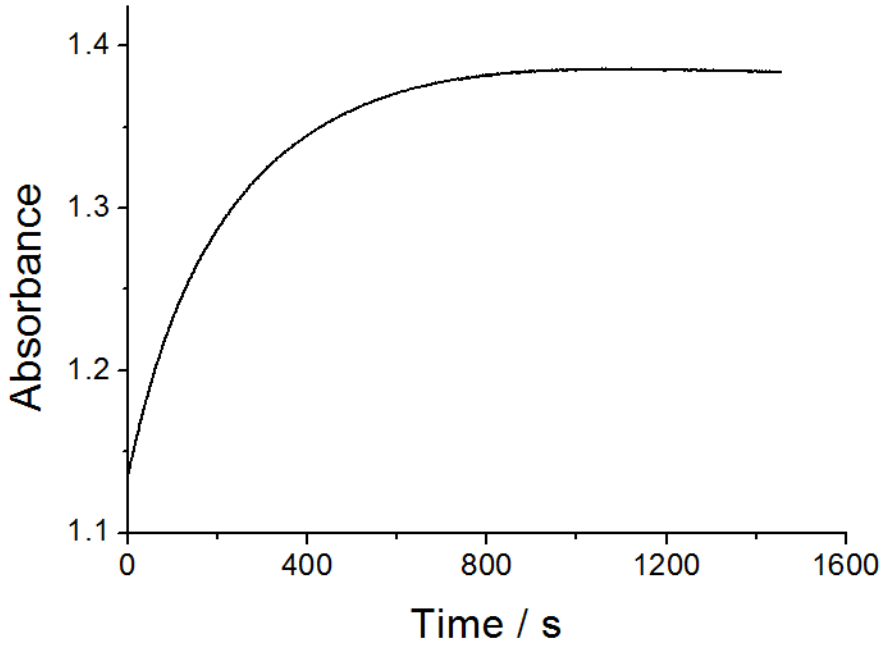


Fig. S5 The absorbance at 365 nm versus reaction time for **1** (100 μM) upon treatment with histidine (100 μM) in PBS (20 mM, pH 7.4, containing 30% DMSO) at room temperature.

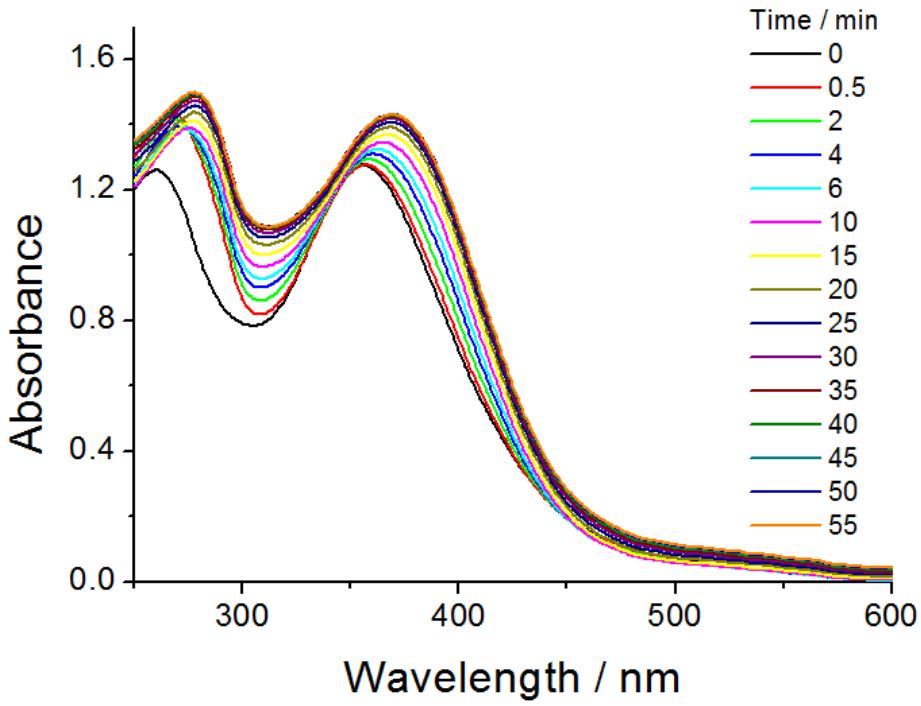


Fig. S6 Time-dependent UV-Vis absorbance spectra of **1** (100 μM) upon treatment with lysozyme (10 μM) in PBS (20 mM, pH 7.4, containing 30% DMSO) at room temperature. The reaction time is indicated in the inset.

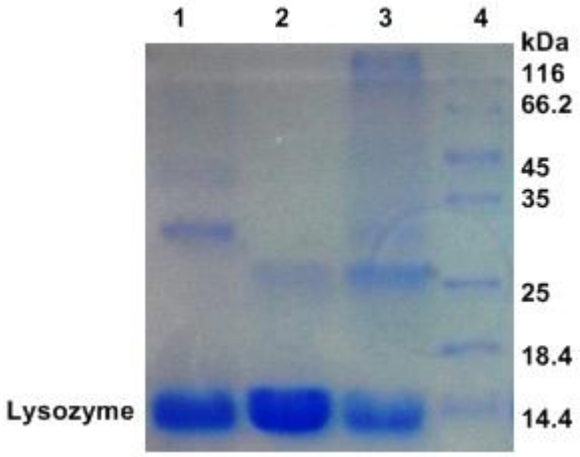


Fig. S7 15% SDS PAGE of lysozyme after PEGylation which without pretreating the lysozyme with SDS. Lane 1, lysozyme + **1**; lane 2, lysozyme + **7**; lane 3, lysozyme + **1** + **7**; lane 4, protein marker.

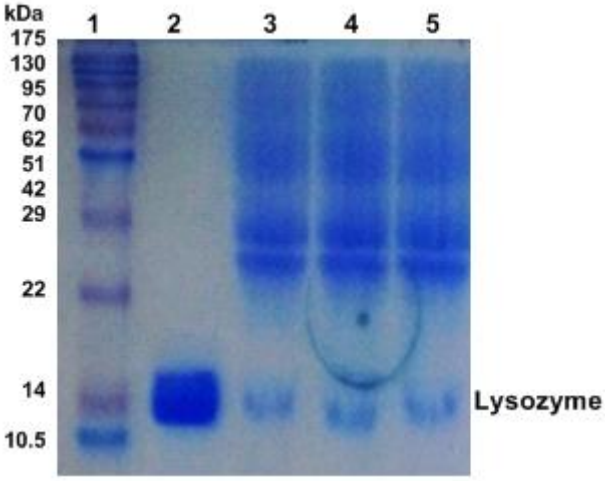


Fig. S8 15% SDS PAGE of lysozyme after PEGylation which pretreated the lysozyme with SDS and incubated for different time after added the norbornylene-PEG4000. Lane 1, protein marker; lane 2, lysozyme; lane 3, lysozyme + SDS + **1** + **7** for 3 h; lane 4, lysozyme + SDS + **1** + **7** for 6 h; lane 5, lysozyme + SDS + **1** + **7** for 9 h.

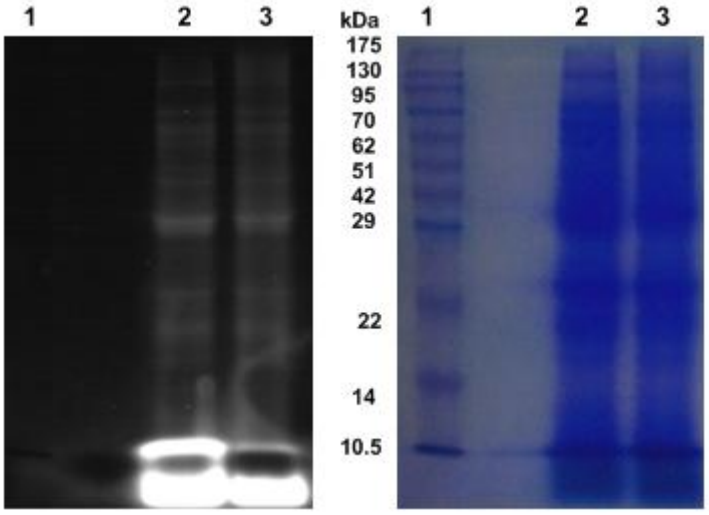


Fig. S9 10% SDS PAGE of E. coli cell lysates scanned by tanon-1600 fluorescent scanner (left) or Coomassie blue staining (right). Lane 1, protein marker; lane 2, cell lysates treated with 2 mM **1** and then treated with **5**; lane 3, cell lysates treated with 5 mM **1** and then treated with **5**.

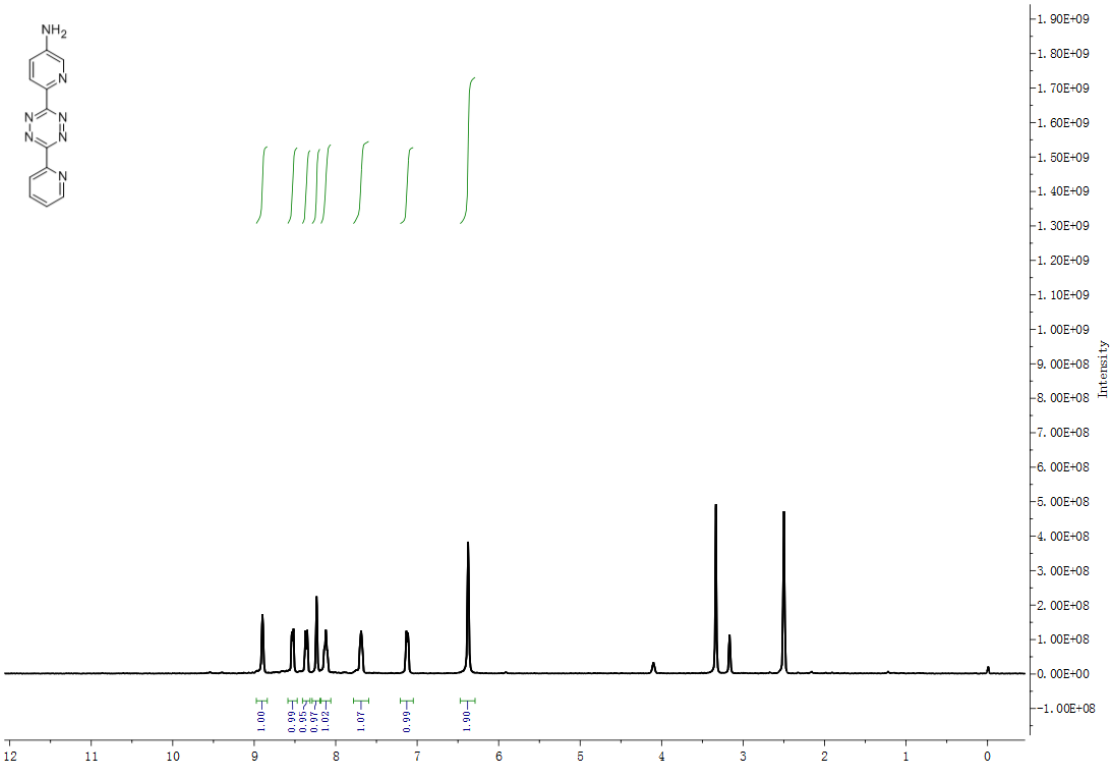


Fig. S10 ^{1}H NMR spectrum of **2**.

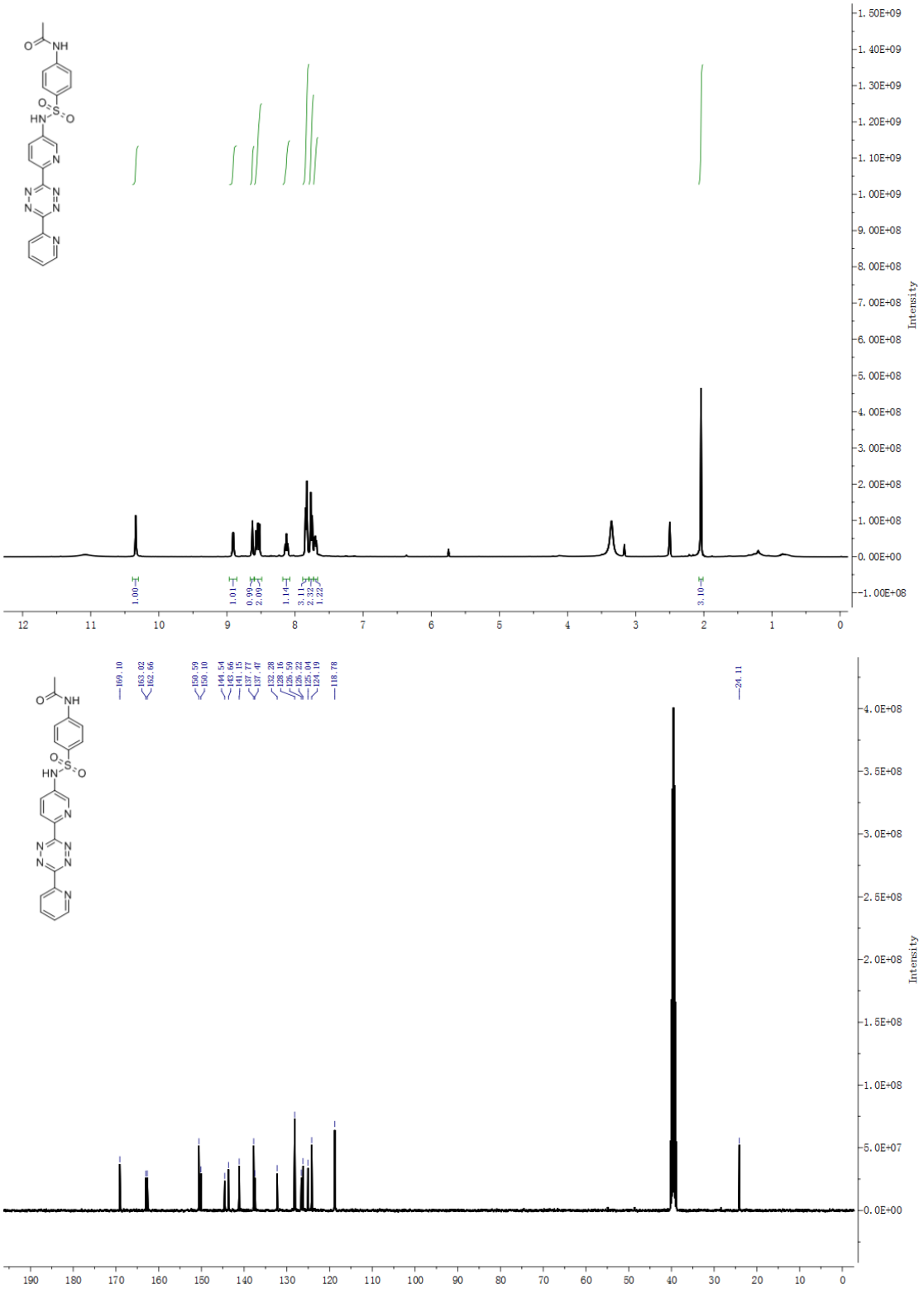
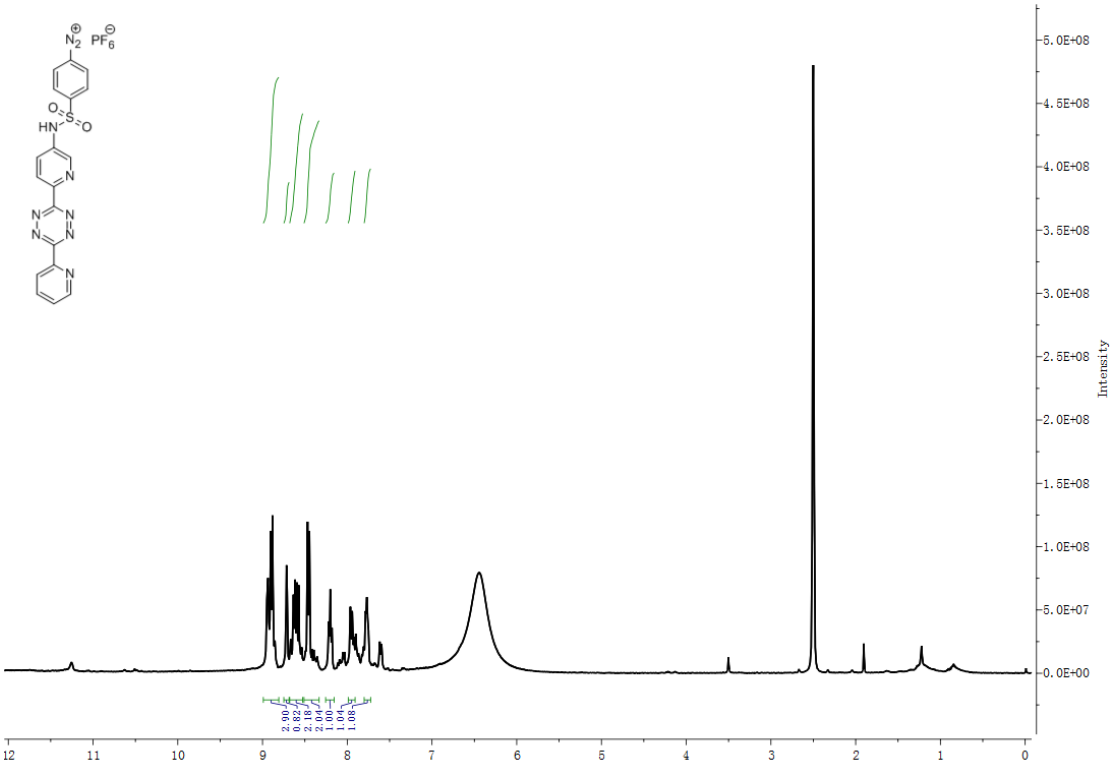


Fig. S11 ^{1}H NMR, ^{13}C NMR spectra of **3**.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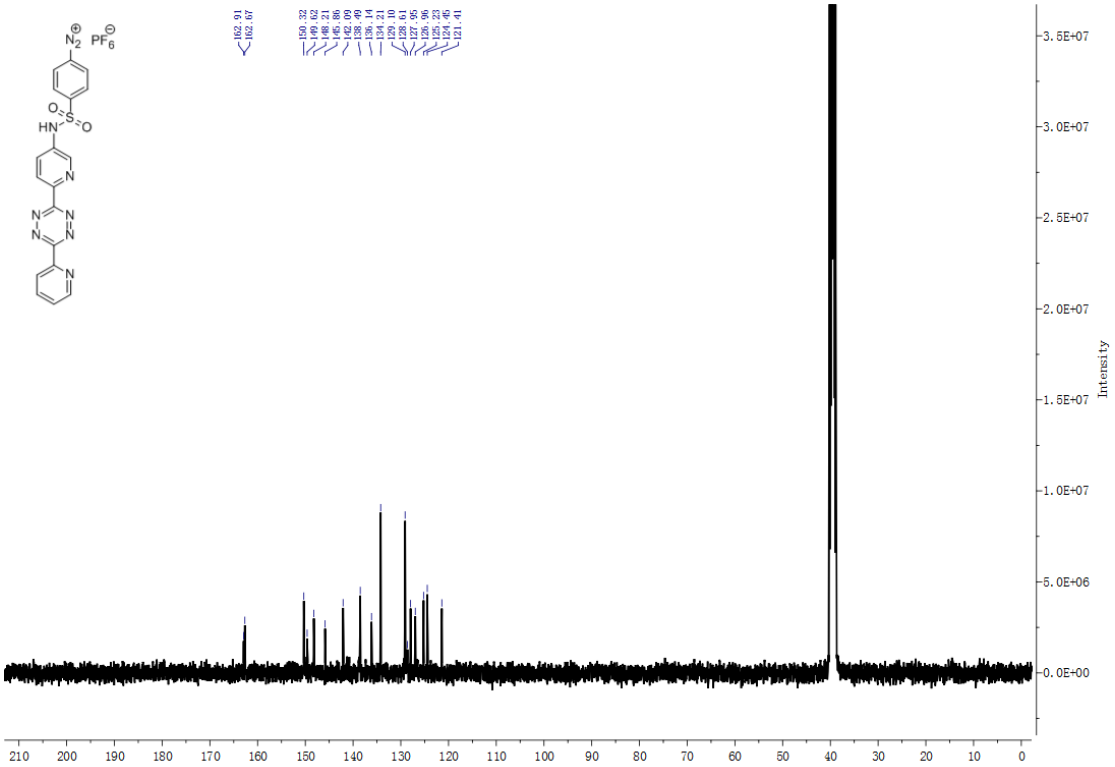
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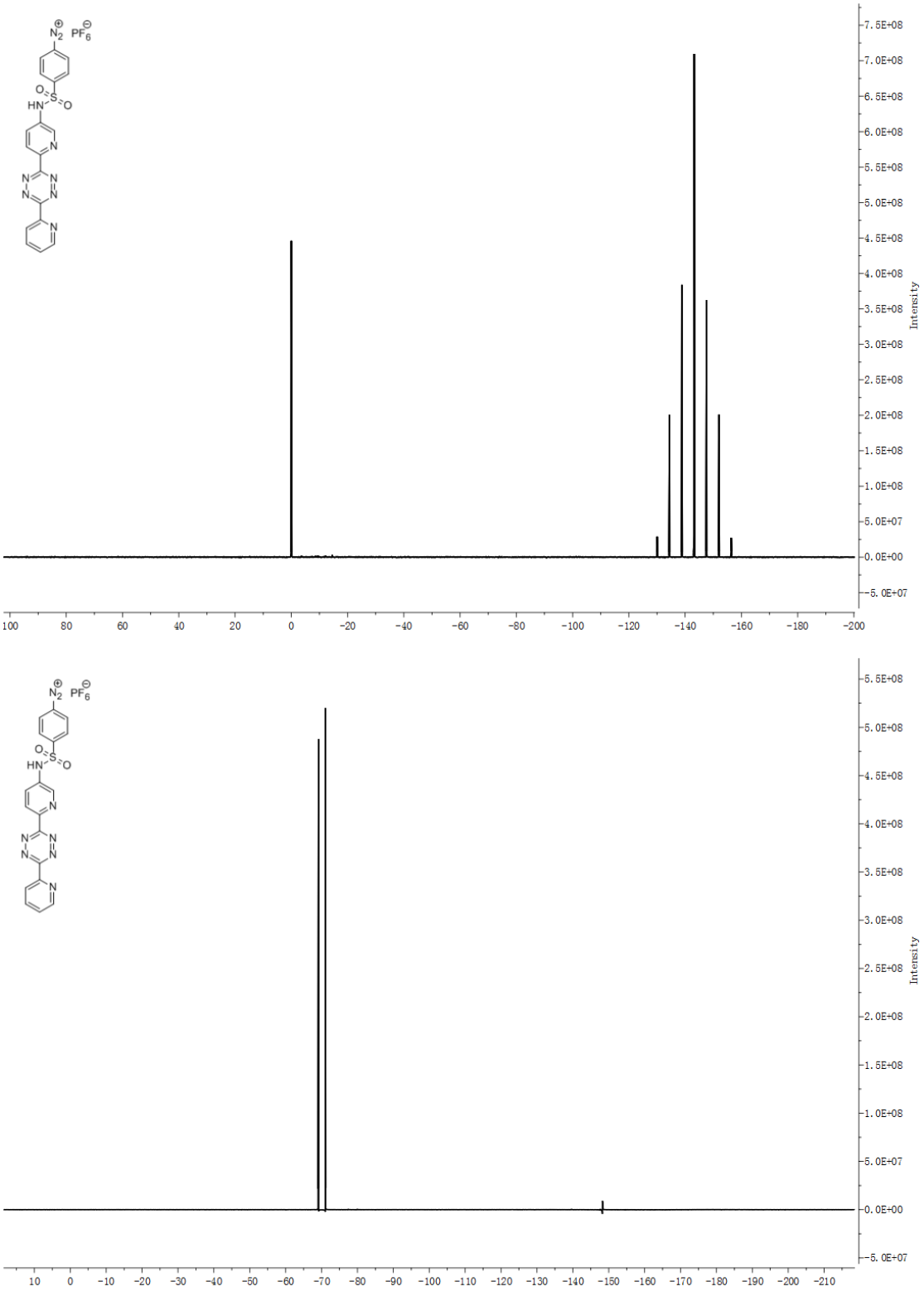

Fig. S12 ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, ^{19}F NMR spectra of **1**.

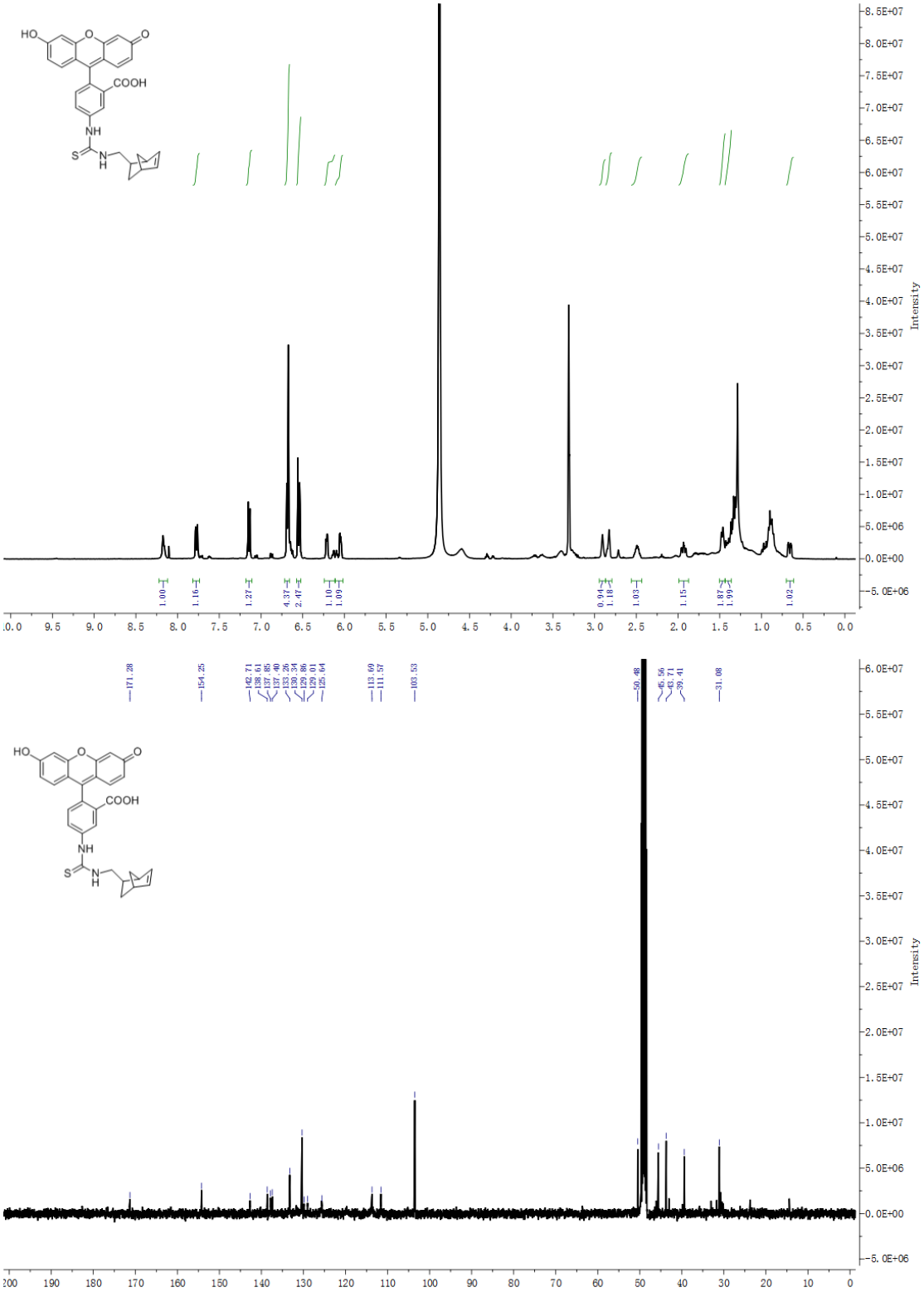

Fig. S13 ^{1}H NMR, ^{13}C NMR spectra of **5**.

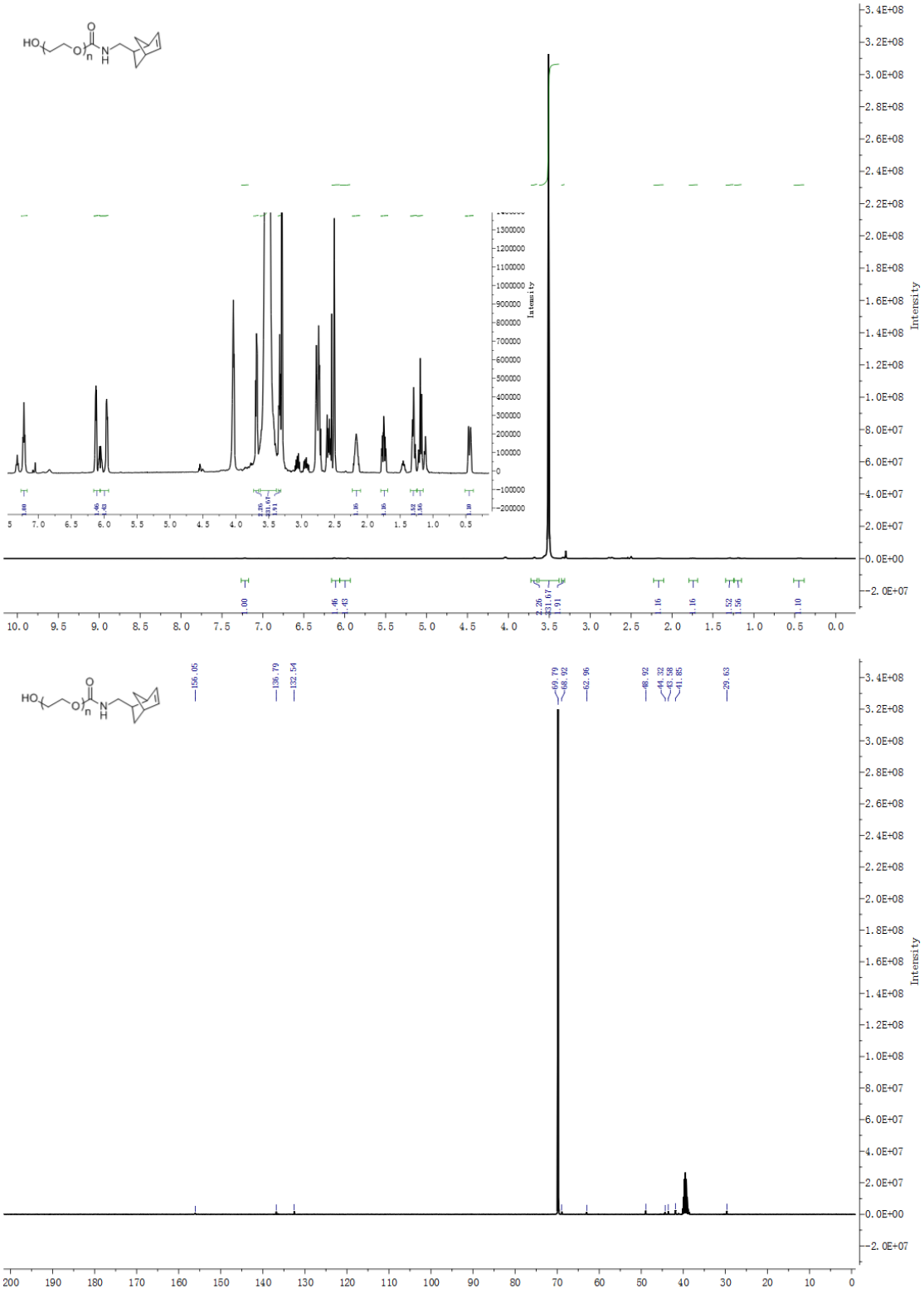

Fig. S14 ^{1}H NMR, ^{13}C NMR spectra of **7**.

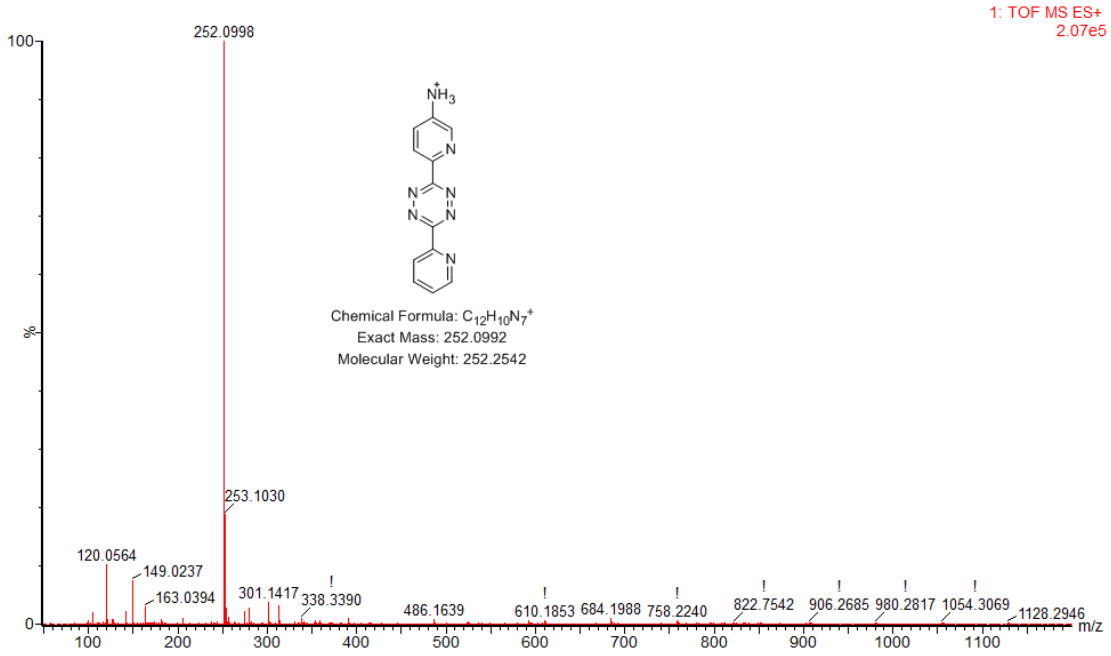

Fig. S15 HRMS spectrum of **2**.

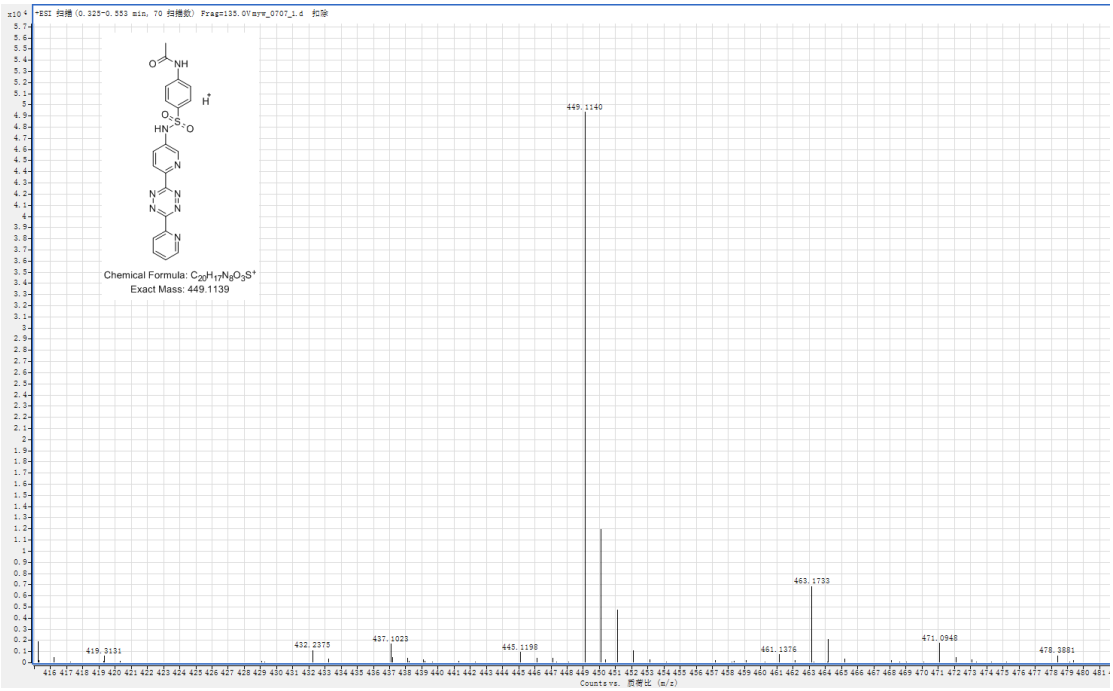

Fig. S16 HRMS spectrum of **3**.

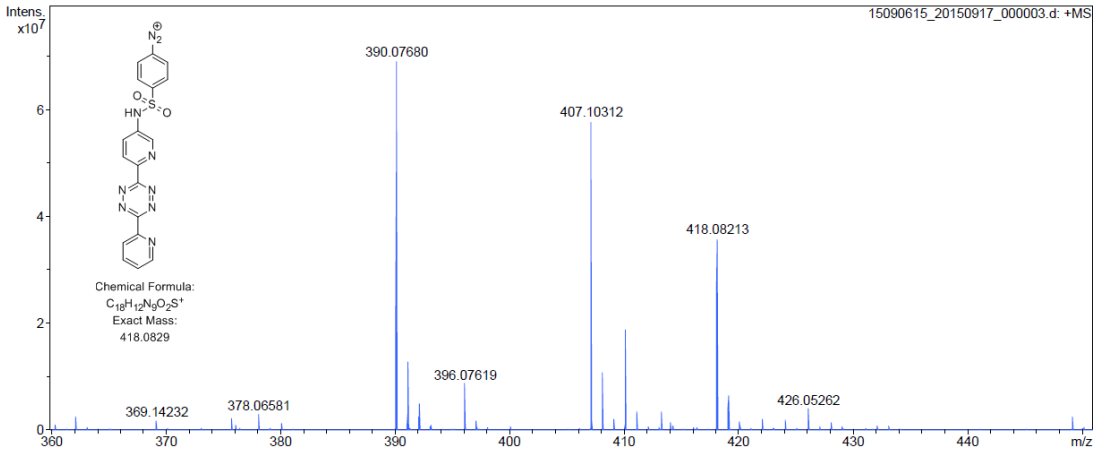

Fig. S17 HRMS spectrum of **1**.

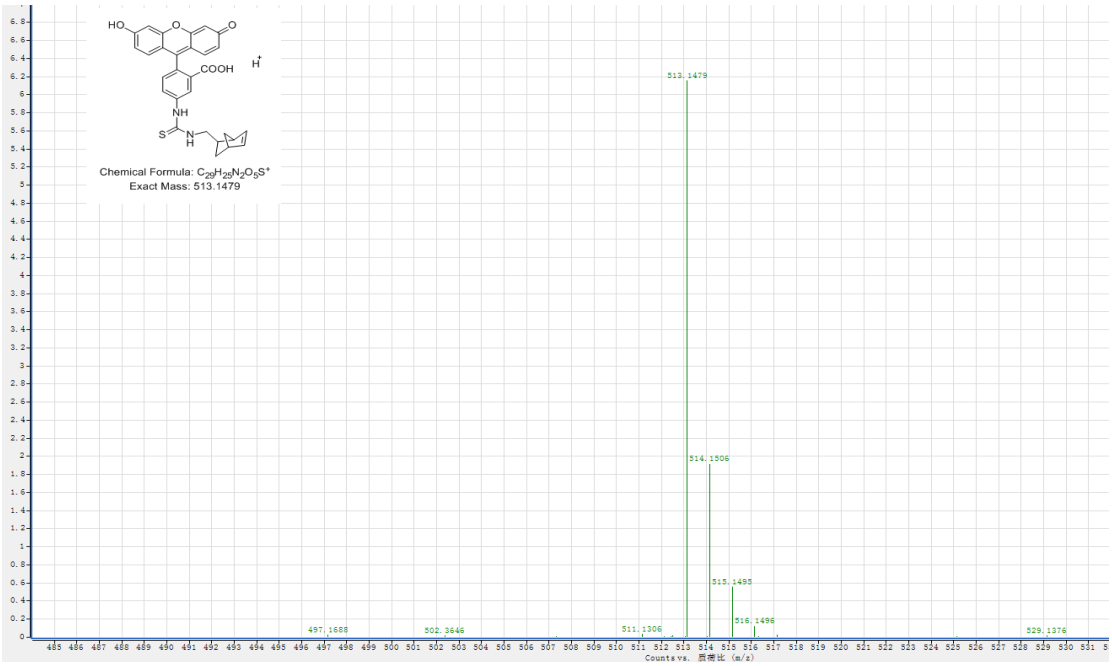

Fig. S18 HRMS spectrum of **5**.

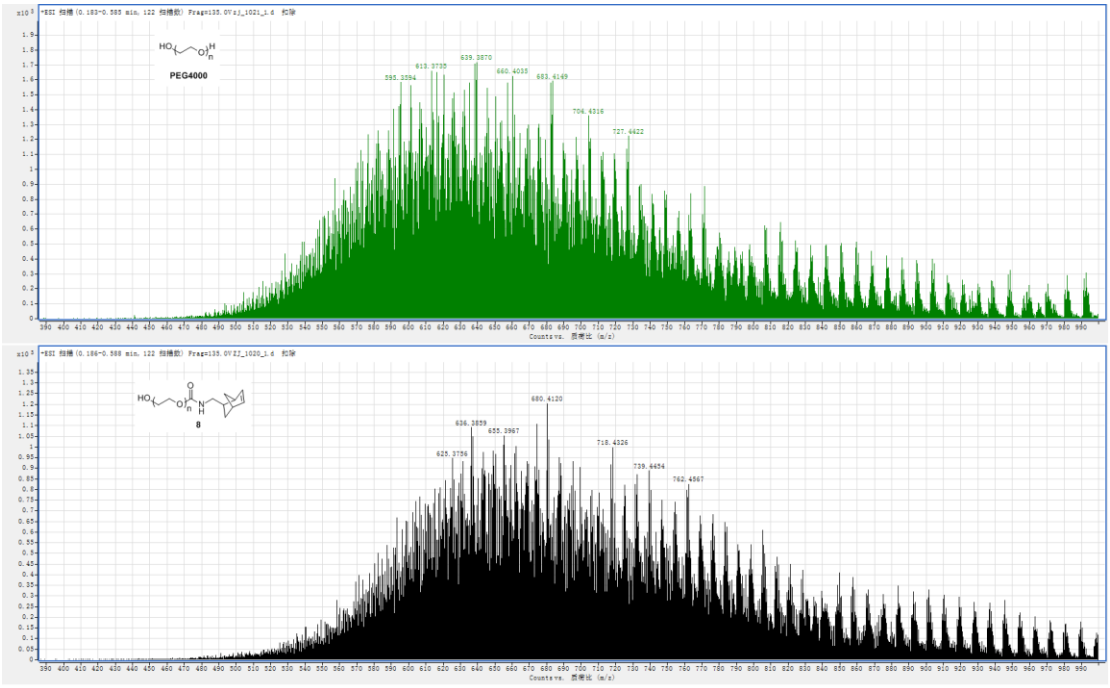

Fig. S19 HRMS spectra of **PEG4000** and **7**.

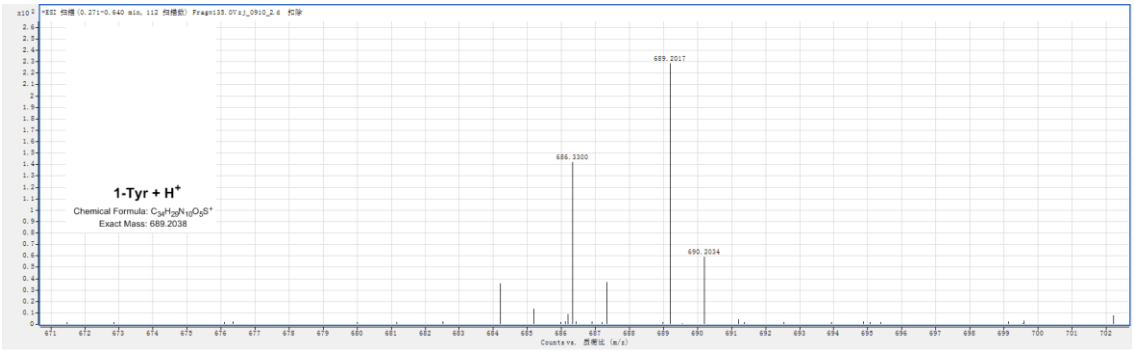

Fig. S20 HRMS spectrum of **1**-Tyr conjugate.

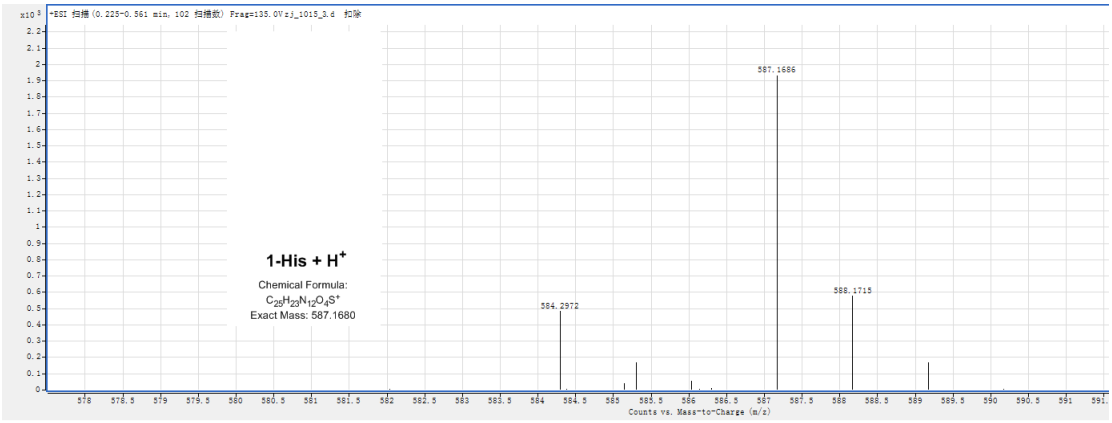

Fig. S21 HRMS spectrum of the **1**-His conjugate.