Supporting Information

An efficient reagent for covalent introduction of alkyne into proteins

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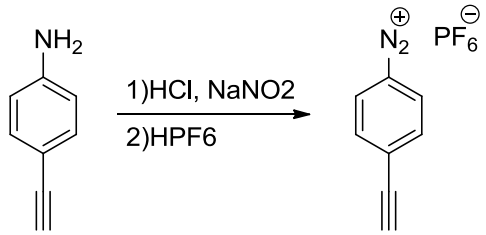
Experimental part

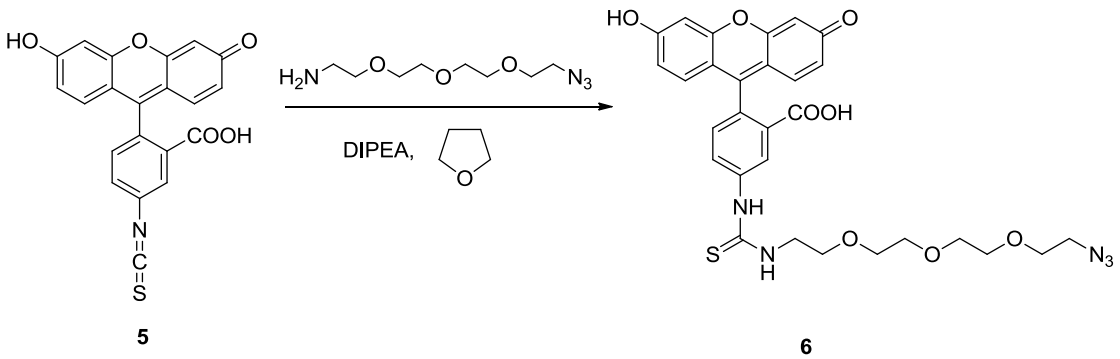
1. Synthesis of the diazonium reagent 2



1 **2**

4-Ethynyl Benzene Diazonium Hexafluorophosphate (2). 4-Ethynylaniline (1.00 g, 8.5 mmol) was dissolved in 30 mL cold concentrated HCl. The water solution of $NaNO_2$ (1.77 g, 25.6 mmol) was slowly added to the mixture at -10 ℃. After 1.5 h reaction, 60% HPF_6 in water (2.5 mL, 17.0 mmol) was added at -10 ℃ and stirred for 1 h. Then the mixture was stirred at room temperature for 30 min. The product were collected by filtration and washed with ice-cold water, yielding a light yellow solid of **2** (1.34 g, 57%). ^{1}H NMR (600 MHz, DMSO-d_6), δ 8.70-8.65 (m, 2H), 8.01 (d, J = 8.4 Hz, 2H), 5.04 (s, 1H). ^{13}C NMR (151 MHz, DMSO-d_6), δ 133.9, 133.8, 132.9, 115.3, 91.2, 81.1. ^{31}P NMR (243 MHz, DMSO-d_6), δ -144.23 (h, $J_{P\text{-}F}$= 711.2 Hz). ^{19}F NMR (376 MHz, DMSO-d_6), δ -70.30 (d, $J_{P\text{-}F}$ = 711.2 Hz). HRMS (ESI): *m/z* $[M]^+$ calcd. for $C_6H_5N_2$: 129.0447; found: 129.0447.

2. Synthesis of fluorescence probe (6).

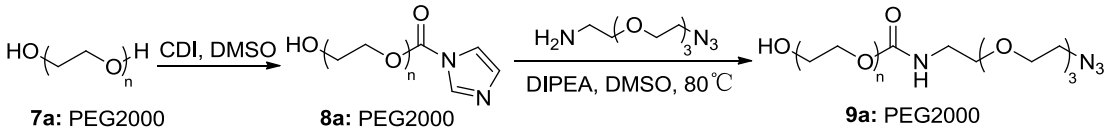


5 **6**

A mixture of fluorescein isothiocyanate isomer I **5** (77.9 mg, 0.2 mmol) and

11-azido-3,6,9-trioxaundecan-1-amine (40 μL, 0.2 mmol) was dissolved in 9 mL dry tetrahydrofuran. DIPEA (38 μL, 0.22 mmol) was added to the reaction mixture and stirred at room temperature for 2 h. The crude product was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH). TLC R_f = 0.74 (CH_2Cl_2/CH_3OH = 9:1). ^{1}H NMR (400 MHz, MeOD), δ 8.17 (s, 1H), 7.73 (d, J = 7.7 Hz, 1H), 7.10 (d, J = 8.1 Hz, 1H), 6.64 (d, J = 7.3 Hz, 4H), 6.50 (d, J = 8.5 Hz, 2H), 3.70 (bs, 2H), 3.64-3.55 (m, 12H), 3.26 (s, 2H). ^{13}C NMR (101 MHz, MeOD), δ 182.6, 171.2, 161.8, 154.3, 148.8, 142.3, 131.4, 130.8, 130.4, 129.2, 125.8, 119.8, 113.9, 111.6, 103.5, 71.5, 71.2, 70.9, 70.1, 51.6, 45.4.

3. Synthesis of azido-PEG2000 (9a) and azido-PEG6000 (9b).



7a: PEG2000
7b: PEG6000

8a: PEG2000
8b: PEG6000

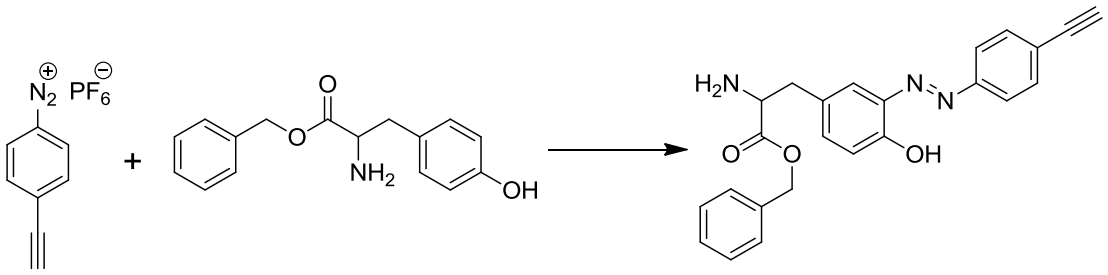
9a: PEG2000
9b: PEG6000

PEG2000-N_3 (9a). 7a (4.0 g, 2.0 mmol) was heated at 80 ℃ under reduced pressure to remove trace water. The solid was dissolved by 40 mL DMSO after cooling down to room temperature. CDI (1.62 g, 10.0mmol) was added to the solution and stirred for 19 h. Then the solution was diluted with 500 mL EtOAc and put at -20 ℃ for 5 h. The resulting white precipitate was collected by filtration and washed by Et_2O to give **8a** (397 mg, 0.2 mmol), which was dissolved in 3 mL DMSO. 11-Azido-3,6,9-trioxaundecan-1-amine (60 μL, 0.3 mmol) and DIPEA (103 μL, 0.6 mmol) were added to the DMSO solution. After heated at 80 ℃ for 24 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 125 mL EtOAc and put at -20 ℃ overnight. After pouring the supernate and diluted with Et_2O, the white precipitate was collected by centrifugation (4000 rpm, 0 ℃, 10 min). Then the solid was washed with Et_2O and was centrifuged again. The product **9a** was obtained by drying the solid under reduced pressure, yield 84.4 mg (18.8%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.18 (t, J = 5.4

Hz, 1H), 4.04 (t, J = 9.2 Hz, 2H), 3.73-3.33 (m, ≈171H), 3.16-3.06 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.2, 72.3, 69.8, 69.7, 69.5, 69.3, 69.1, 68.8, 63.1, 60.2, 50.0.

PEG6000-N_3 (9b). PEG6000 **7b** (12.0 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 PEG6000) after cooling down to room temperature. CDI (1.56 g, 9.6 mmol) was added to the solution and stired for 2 days. 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 **8b** (5.86 g, 48%). **8b** (1.20 g, 0.2 mmol) was dissolved in 5 mL DMSO. 11-Azido-3,6,9-trioxaundecan-1-amine (60 μL, 0.3 mmol) and DIPEA (103 μL, 0.6 mmol) were added to the DMSO solution. After heated at 80 ℃ for 40 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 **9b** was given by drying the solid under reduced pressure, yield 586.3 mg (47.1%).^{1}H NMR (400 MHz, DMSO-d_6) δ 7.18 (t, J = 5.5 Hz, 1H), 4.04 (t, J = 9.2 Hz, 2H), 3.80-3.35 (m, ≈483H), 3.16-3.07 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 156.2, 72.3, 69.8, 69.7, 69.5, 69.3, 69.1, 68.8, 63.1, 60.2, 50.0.

4. Reaction with Tyr-containing small molecule.



2 **3** **4**

Product 4 for HRMS. 10 μL 100 mM **2** in CH_3CN and 10 μL 100 mM **3** in DMSO were co-incubated in 80 μL 50 mM PBS (pH 8.0, containing 30% CH_3CN) for 1 h at

room temperature. Then the reaction mixture was submitted into ESI-MS without purification.

UV-Vis analysis of the reaction. To 2 mL PBS (50 mM, pH 7.0 or pH 8.0, containing 30% CH_3CN) was added 2 μL **2** (100 mM) in CH_3CN and 2 μL **3** (100 mM) in DMSO. The progress of the reaction was monitored by UV-Vis spectrophotometry from 250 to 600 nm. In order to explore the kinetics of the reaction more accurately, the reaction progress was monitored by UV-Vis spectrophotometry at 400 nm. The reaction condition is 2 μL **2** (100 mM) in CH_3CN and 20 μL **3** (100 mM) in DMSO was added to 1.98 mL PBS (50 mM, pH 7.0 or pH 8.0, containing 30% CH_3CN).

5. Fluorescence labeling of BSA.

500 μL BSA (100 μM) solution in 50 PBS (mM, pH 8.0) was added 1 μL **2** (100 mM) in DMSO and the mixture was co-incubated for 2 h at room temperature. The fluorescence labelling was based on click reaction by copper catalyst. The **click catalyst** (10 mM) was prepared as a mixture of 50 μL TBTA (20 mM), 30 μL deionized water, 10 μL Cu^{2+} (100 mM) and 10 μL ascorbic acid (vitamin C, 200 mM, fresh preparation). 1 μL fluorescence probe **6** (100 mM in DMSO) and 25 μL catalyst was added to 475 μL alkyne-modified BSA or native BSA (negative control) for 2 h reaction at room temperature. The labeled BSA suspension was directly electrophored in 15% SDS-PAGE. The PAGE was firstly imaged under the UV light to detect whether the BSA was labelled. After the UV imaging, the PAGE was stained by Coomassie brilliant blue for 1 h and destained by the glacial acetic acid/alcohol/water mixture (1:1:8).

6. Fluorescence labeling of TMV particles.

To label the TMV particles, 10 mg/mL TMV suspension (99 μL) was incubated with 1 μL **2** (100 mM) in PBS buffer (0.1 M, pH 7.0) for 2 h at room temperature. The reaction mixture was then transferred into a centrifugal filtration device (3 KDa, Millipore) and eluted by PBS. 80 μL **2**-labelled TMV was incubated with 1 μL fluorescence probe **6** (100 mM in DMSO) and 10 μL **click catalyst** for 2 h at room

temperature. The same reaction except the native TMV suspention was set as the negative control. The labelled TMV suspension was denatured by base and then electrophored in 10% SDS-PAGE. The PAGE was firstly imaged under the UV light and then stained by Coomassie brilliant blue.

7. Protein PEGylation.

To achive the protein PEGylation, we selected a small protein lysozyme (14.4 KDa). A 10 μL lysozyme (2 mM) solution was incubated with 1 μL **2** (100 mM) in 89 μL PBS buffer (50 mM, pH 8.0) for 2 h at room temperature. The reaction mixture was transferred into a centrifugal filtration device (10 KDa, Millipore) and eluted by PBS buffer (50 mM, pH 8.0). 85 μL lysozyme elution was then incubated overnight with 5 μL 20 mM PEG2000-N_3 (or PEG6000-N_3) dissolved in DMSO and 10 μL **click catalyst** at 4 ℃. The bioconjugations were analyzed by 10% SDS-PAGE. The gel was stained by R250 (Coomassie brilliant blue) for 1 h and destained by the glacial acetic acid/alcohol/water mixture (1:1:8). The band shift was imaged by the Gel Doc XRi instructment (Bio-Rad) and processed with the Quantity One software.

Supporting figures

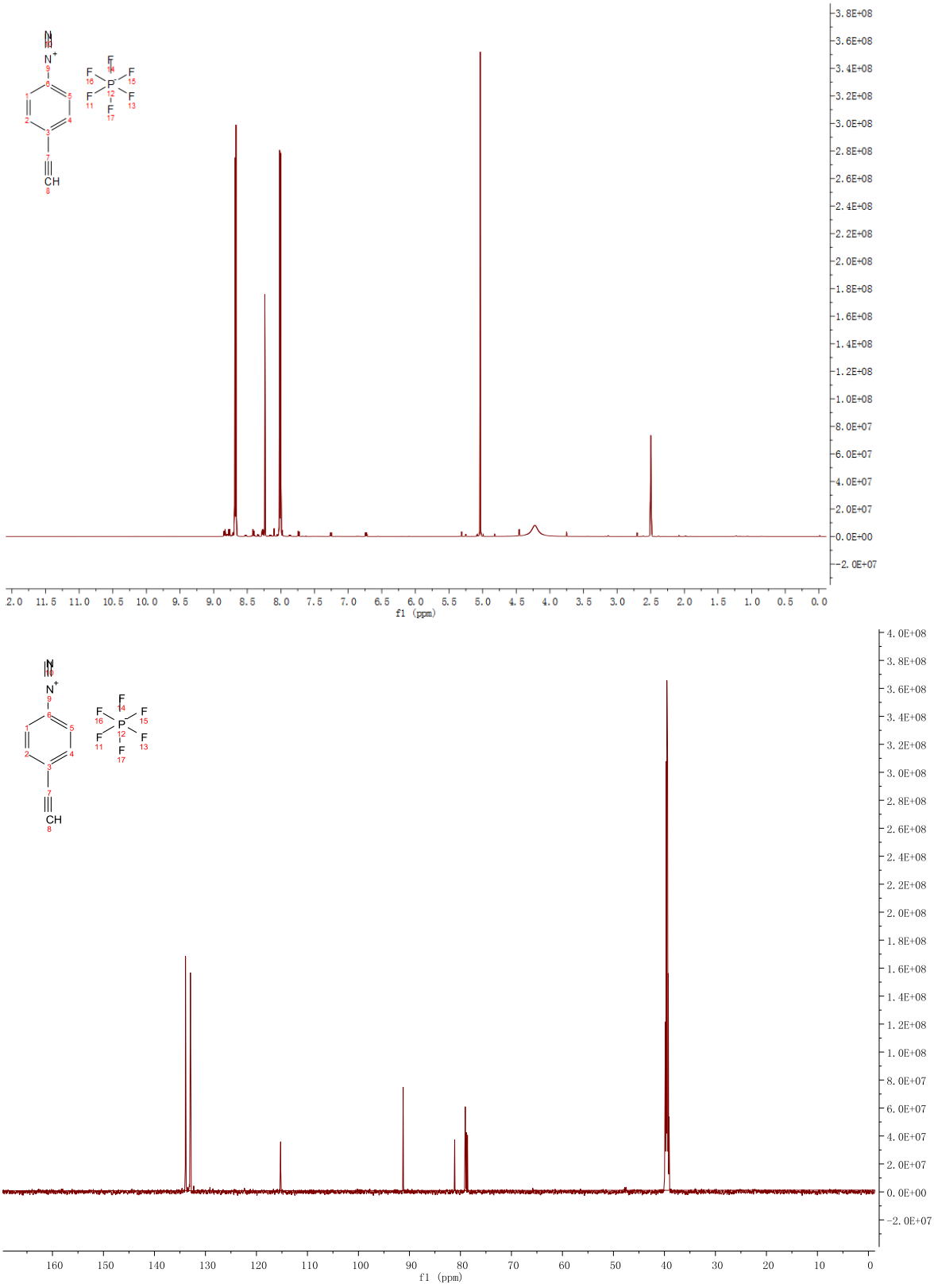


Fig. S1. ^{1}H-NMR and ^{13}C-NMR spectra of **2**.

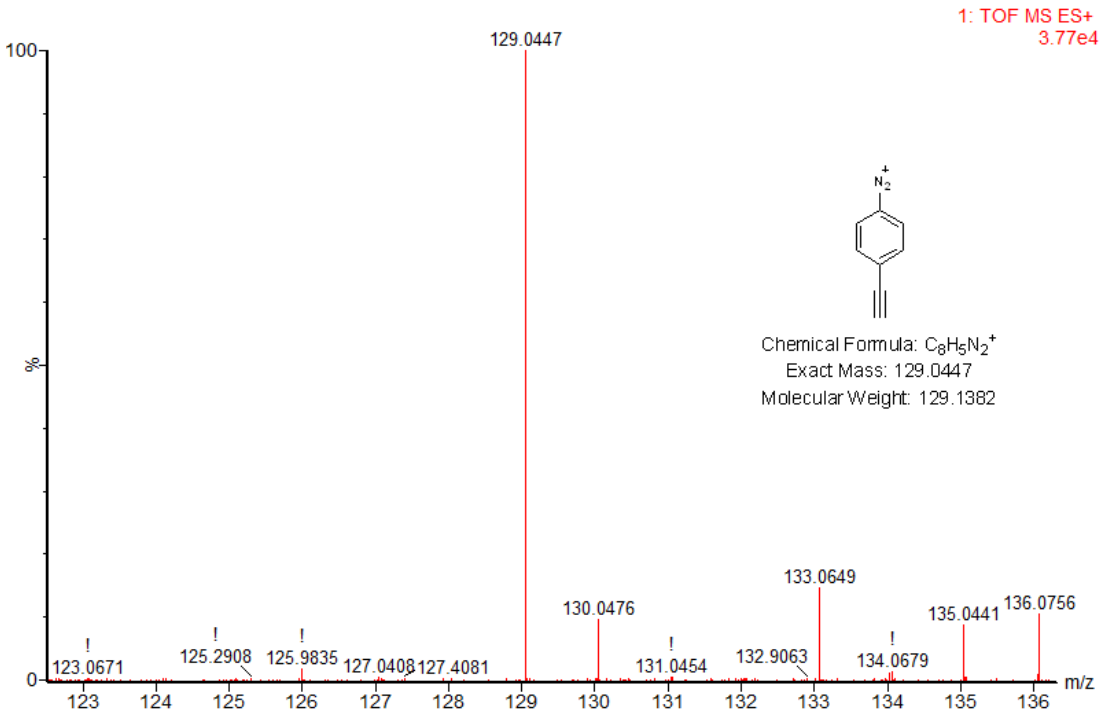


Fig. S2. HRMS spectrum of **2**.

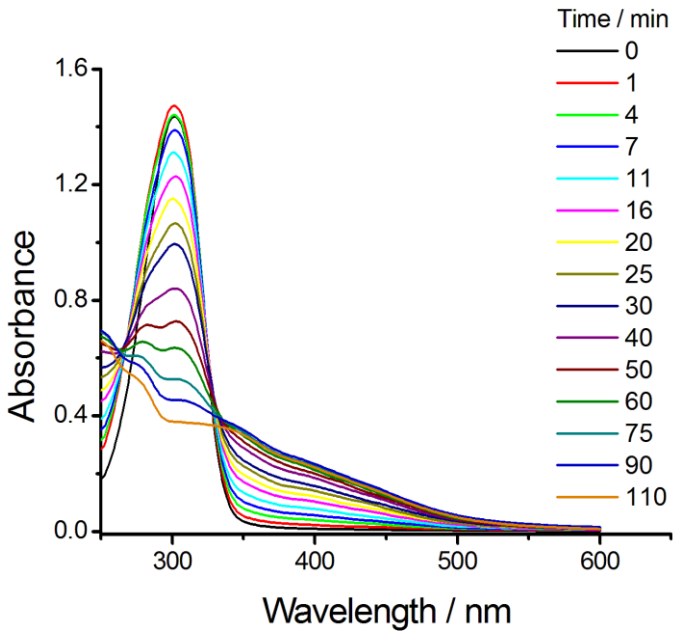


Fig. S3 Time-dependent UV-Vis absorbance spectra of **2** (100 μM) upon treatment with **3** (100 μM) in 50 mM PBS (pH 7.0, containing 30% CH_3CN) at room temperature. The different reaction time is indicated inset.

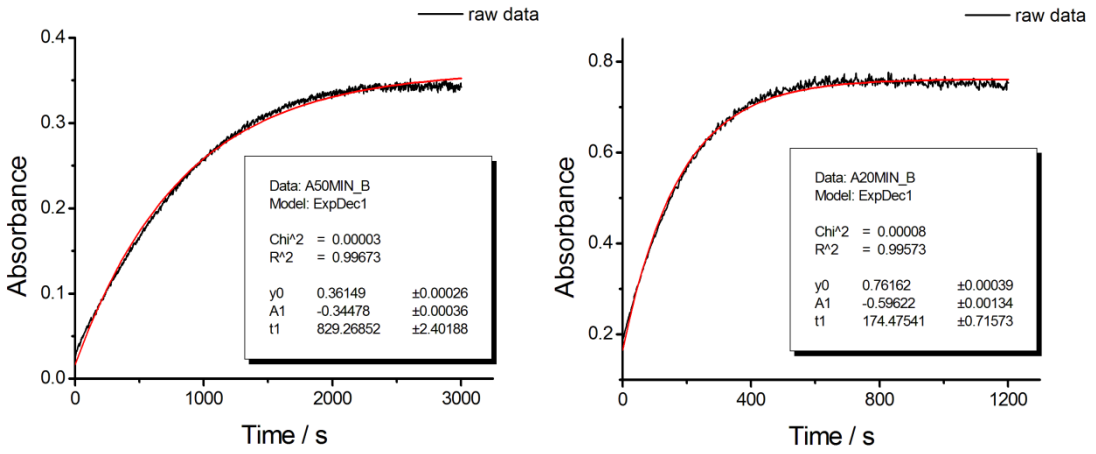


Fig. S4 The absorbance at 400 nm versus reaction time of **2** (100 μM) upon treatment with **3** (1 mM) in 50 mM PBS (pH 7.0 (left) or pH 8.0 (right), containing 30% CH_3CN) at room temperature. The red line represents the best fitting with single-exponential function.

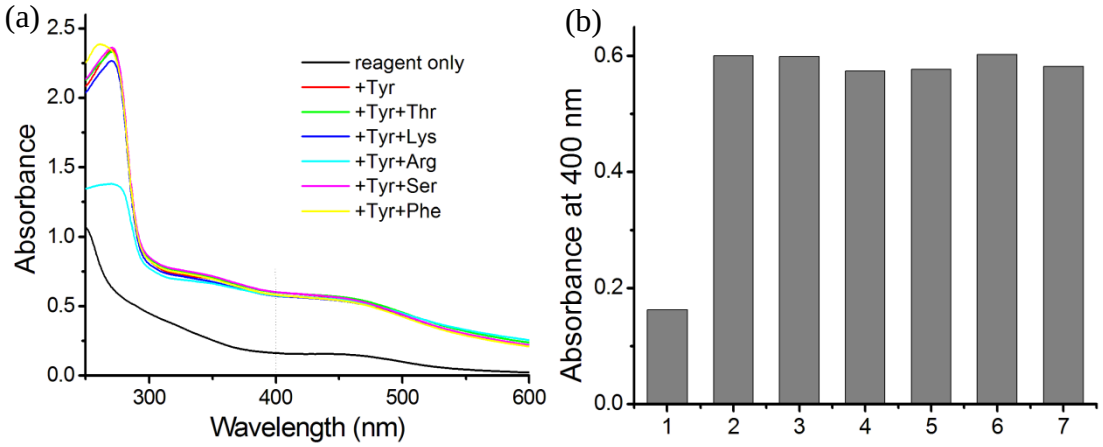


Fig. S5 Competitive reaction tests of **2** (0.1 mM) and **3** (1 mM) in the presence of some amino acids (1 mM). The reaction was performed at pH 8.0 for 30 min. Absorbance spectra of each reaction solution were checked (a) and the absorbance at 400 nm was shown in (b). Lane 1, only **2**; lane 2, **2** and **3**; lanes 3-7, **2** and **3** in the presence of Thr, Lys, Arg, Ser, Phe, respectively. Because the specific reaction of **2** and **3** gives azo with unique absorbance at 400 nm, the results of similar absorbance value at 400 nm for lanes 2-7 imply that the reaction of **2** and **3** was nearly not disrupted in the presence of amino acids Thr, Lys, Arg, Ser and Phe.

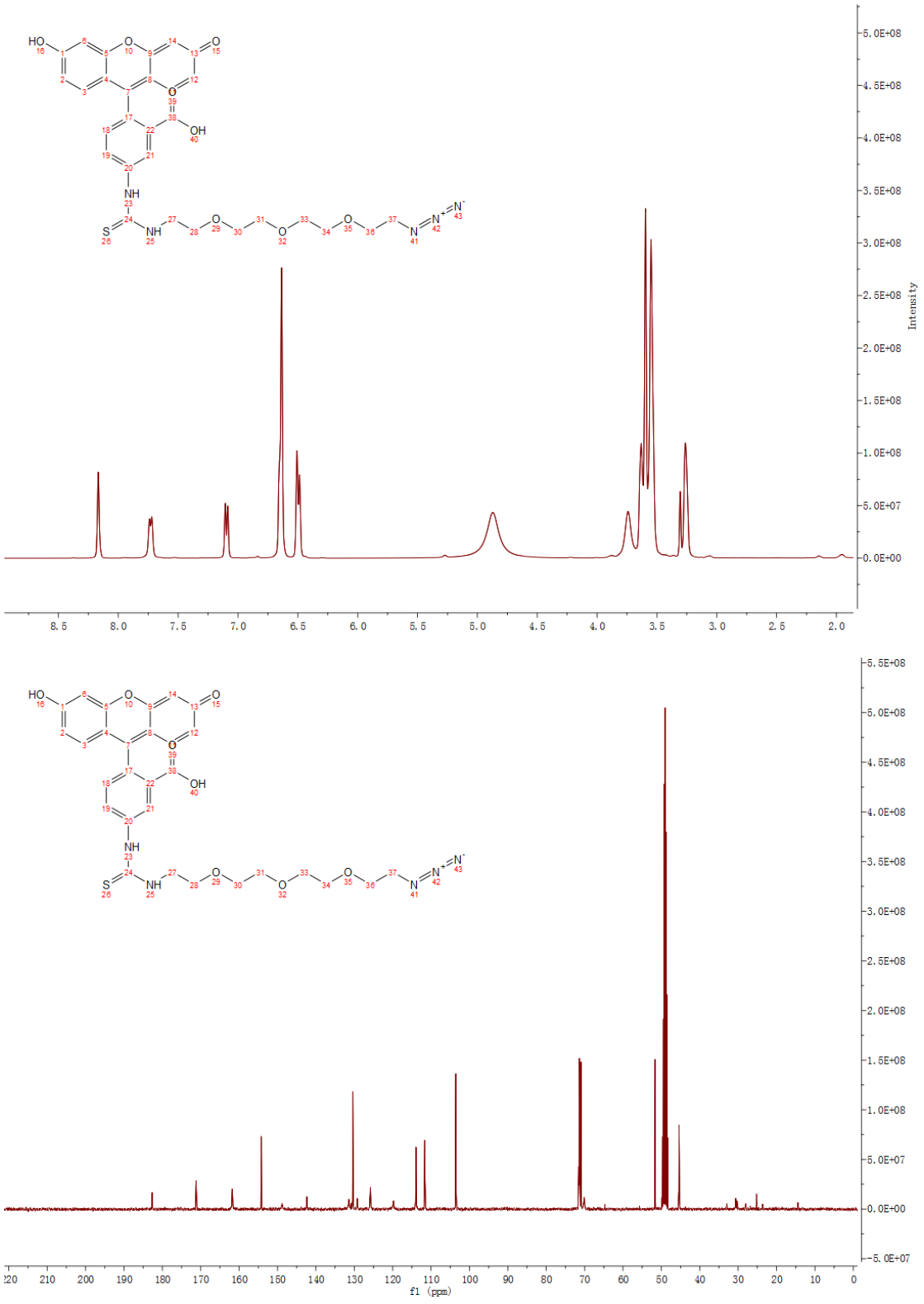


Fig. S6 ^{1}H NMR and ^{13}C NMR spectra of **6**.

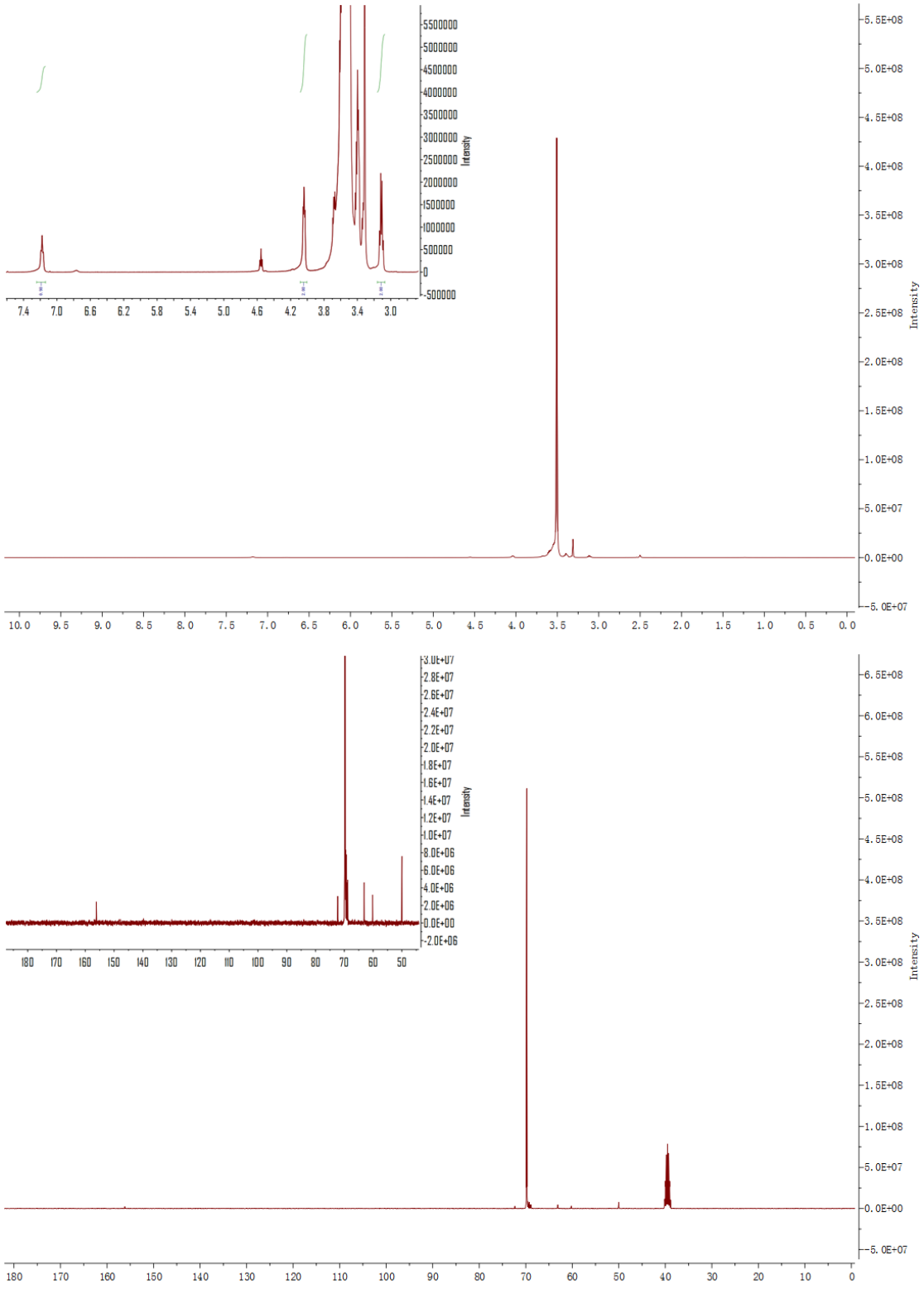


Fig. S7 ^{1}H NMR and ^{13}C NMR spectra of azido-PEG **9a**.

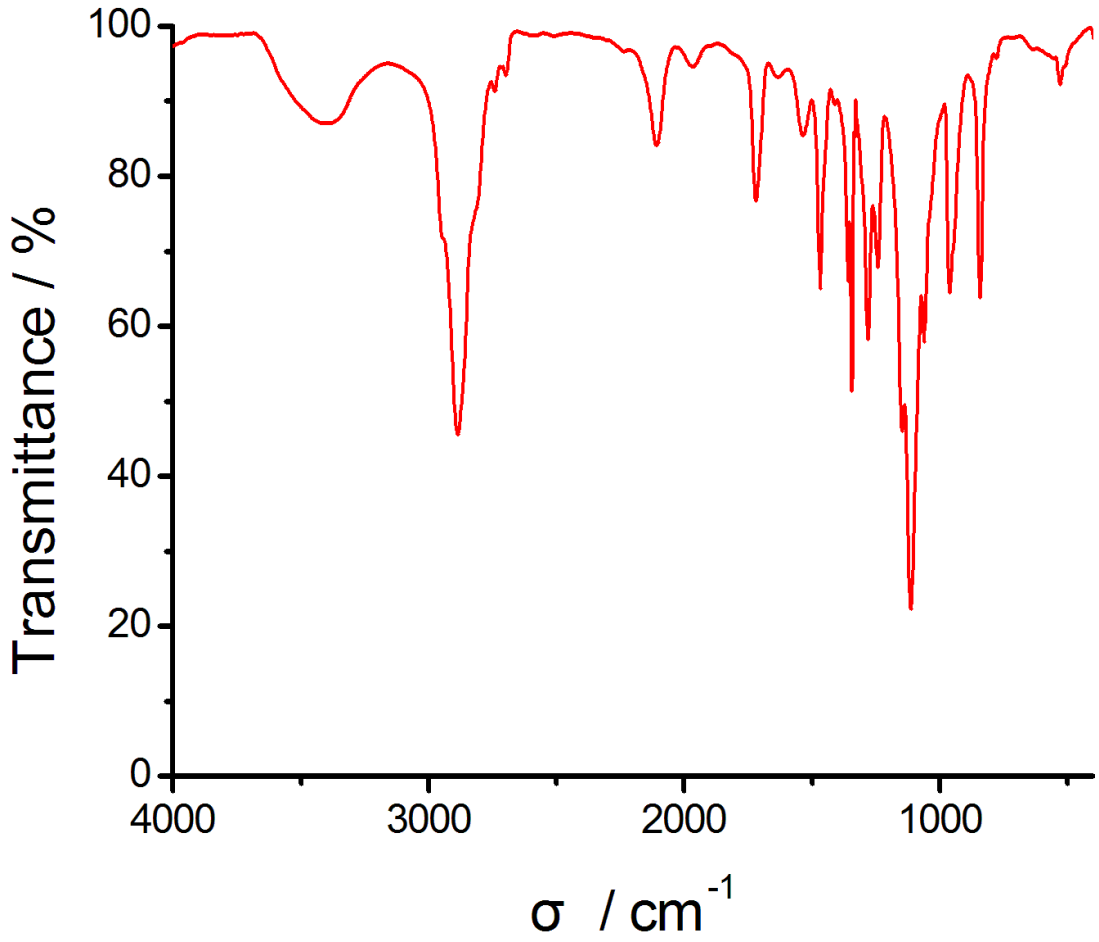


Fig. S8 Infrared Spectrum of azido-PEG **9a**.

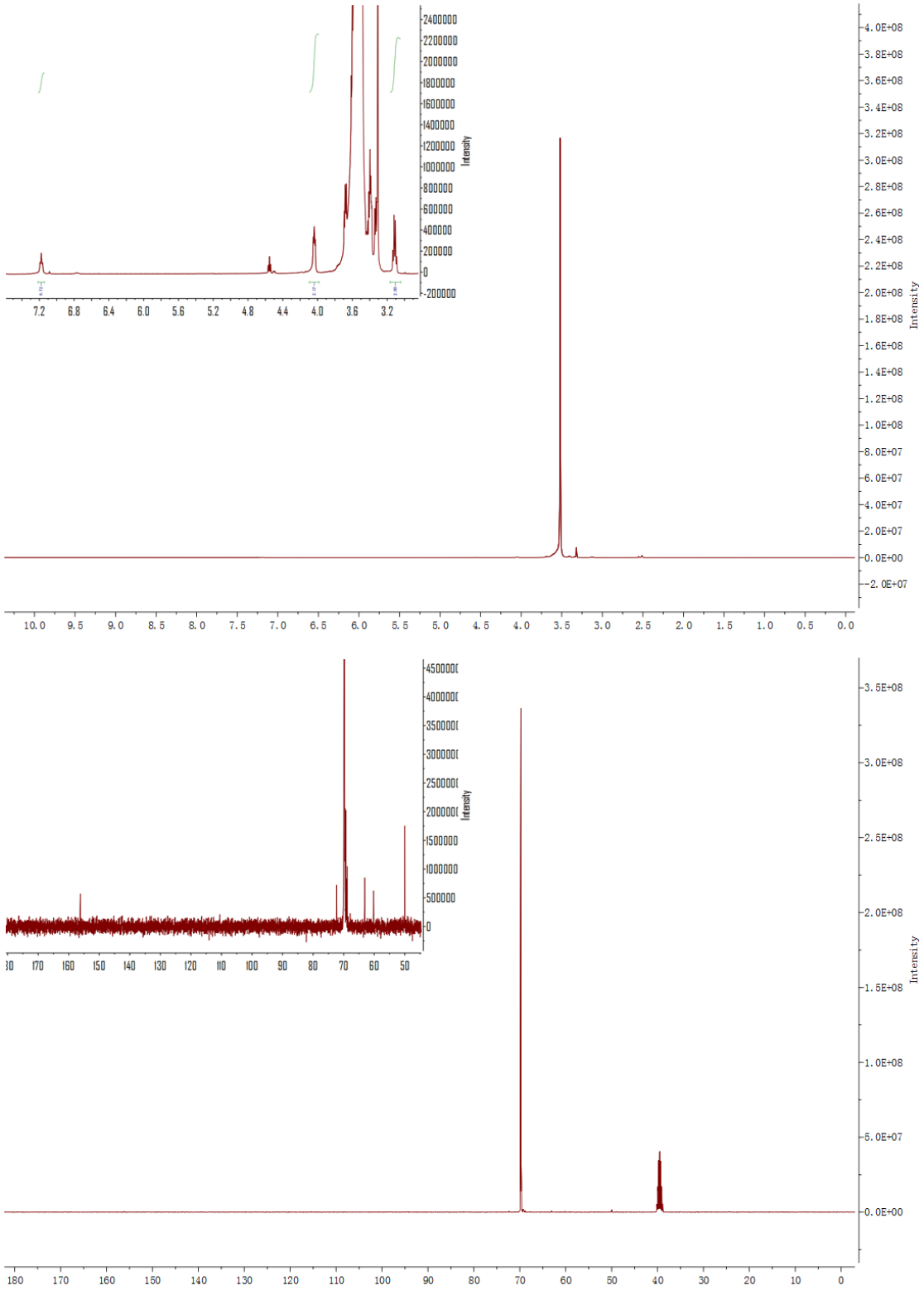


Fig. S9 ^{1}H NMR and ^{13}C NMR spectra of azido-PEG **9b**.