Supporting information

Single and dual functionalization of proteins using site-specific nucleophilic carbon ligations

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Contents

1. Experimental section

1) General

The peptide Ac-YLCTPSRA-NH_2 was purchased from Ontores Biotechnologies (Zhejiang, China). Peptide HPLC analysis was conducted using Liquid chromatography (Agilent Technologies 1200 series) equipped with a Hypersil Gold™ C18 column (250 mm × 4.6 mm, Thermo Fisher Scientific). Peptide and protein MS analysis was conducted using a high-resolution mass spectrometry (LTQ Orbitrap XL, Thermo Fisher Scientific) located in the State Key Laboratory of Fine Chemicals at Dalian University of Technology. All genes encoding proteins were synthesized and ligated to the expression vector by Sangon Biotech (Shanghai) Co., Ltd. Reagent grade chemicals were purchased from Shanghai Macklin Biochemical Co., Ltd unless otherwise stated.

2) Reactions on fGly-containing peptide scaffold

The installation of the aldehyde handle on peptide Ac-YLCTPSRA-NH_2 was performed according to our previous publication.[1] For all reactions, Peptide containing fGly as the reaction acceptor was dissolved in aqueous buffer (50 mM MES buffer, containing 2% DMSO, pH 6.5) at a concentration of 0.1 mM. The reactions were initiated by adding nucleophiles dissolved in DMSO and the reaction can be quenched by adding 10% volume equivalent of 1 M HCl and followed by LC-MS analysis. The gradient of LC was 20–36% acetonitrile (containing 0.1% (v/v) of trifluoroacetic acid) in water (containing 0.1% (v/v) of trifluoroacetic acid) over 30 min. The concentration of the reactant/product and the conversion of the reaction can be calculated according to the integrated peak areas (215 nm absorption).

3) Kinetic analysis

For the Knoevenagel condensation reaction, 0.1 mM peptide and 0.1 mM C-nucleophiles were incubated at room temperature for 0, 5 min, 10 min, 20 min, 30 min, 60 min. The conversion was determined using the HPLC assay and the results were plotted using the equation bellow:[2]

$$y = \frac{\ln \frac{[P]_0[S]_t}{[P]_t[S]_0}}{([S]_0 - [P]_0)} = k_2 t \qquad \text{(eq. 1)}$$

Where $[P]_0$ and $[S]_0$ is the initial concentrations of the peptide and C-nucleophiles, and $[P]_t$ and $[S]_t$ is the concentration of the peptide and C-nucleophiles at corresponding reaction time t.

For the Knoevenagel-Michael tandem reaction, the kinetic parameters of the consecutive reaction were determined under pseudo-first order reaction conditions:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

Where A is the fGly peptide; B is the Knoevenagel intermediate; and C is the Michael adduct. A (0.1 mM) was dissolved in 0.1 ml MES buffer (pH 6.5), and 5 mM C-nucleophiles (50 equiv.) was added to the solution. The mixture was kept at room temperature for 1, 2, 5, 10, 20, 30 min and followed by HPLC analysis. The concentration of the A, B and C was calculated according to the

integrated peak areas, and the concentration of the B was fitted against reaction time using the following equation:

$$[B] = \frac{[A]_0 k_1 (e^{(-k_1 t)} - e^{(-k_2 t)})}{k_2 - k_1} \quad \text{(eq. 2)}$$

Where $[A]_0$ is the initial concentration of A, [B] is the concentration of B at time t.

4) Protein expression and purification

Nanobody 7D12-Cys was expressed and purified as previously described.[1, 3] Briefly, Nanobody 7D12-Cys was expressed in *E.coli* Shuffle T7 cells (pET21a(+) as the expression vector, Nde1/Xho1 as the restriction sites). The transformed cells were first grown in Terrific Broth medium (37 ℃, supplemented with 50 ug/ml kanamycin) for 6 h until OD_{600} reached 2-3. Then isopropyl β-D-1-thiogalactopyranoside (IPTG, 250 μM) was added and the temperature was switched to 18℃. The cells were cultured further for 12 h and the cell pellet was obtained by centrifugation (8000 g, 5 min). The pellet was lysed using AH-NANO high pressure homogenizer (ATS Engineering limited, China) and purified using 5 ml HisTrap HP column (GE Healthcare). The purified protein was exchanged to PBS and stored at -80 ℃ until use.

7D12-Cys sequence:

AEFQVKLEESGGGSVQTGGSLRLTCAASGRTSRSYGMGWFRQAPGKEREFVSGISWRGD
STGYADSVKGRFTISRDNAKNTVDLQMNSLKPEDTAIYYCAAAAGSAWYGTLYEYDYW
GQGTQVTVSSEPKTPKPQPQPQPQPQPNPTTEHHHHHHGGGGSLCTPSR

5) Protein labeling

The protein aldehyde tag conversion of 7D12-Cys to 7D12-fGly was conducted according to the protocol in our previous publication.[1] The Cys residue located in the LCTPSR motif can be specifically recognized by formylglycine generating enzyme (FGE), which catalyzes the transformation of the Cys residue to a biorthogonal fGly residue. Briefly, 7D12-Cys (0.1 mM) was dissolved in triethanolamine buffer (50 mM triethanolamine, 150 mM NaCl, 2 mM DTT) and the solution was flowed through (flow rate of 0.1 mL/min) a 1 mL continuous-flow reactor immobilized with FGE (36 mg protein per g of carrier). The solution of 7D12-fGly in the outlet was collected. The 7D12-fGly was then exchanged to PBS buffer and stored at -80 ℃ until use.

For protein labeling, fGly-containing proteins (0.1 mM) were dissolved in 50 mM MES buffer (pH 6.5, 2% DMSO) and probes in DMSO were added (1 mM, 10 equiv.). The reactions were kept at room temperature. After the reaction, the labelled proteins were purified using ultrafiltration. The isolated yields of protein labeling reactions were calculated based on the relative intensity of the product and substrate using protein MS analysis.

6) Circular dichroism analysis

Proteins were buffer exchanged to 10 mM phosphate buffer and added to a 10 mm cell at a concentration of 0.5 mg/ml, and the circular dichroism spectra of protein was obtained using a MOS-500 circular dichroism spectrometer (Bio-Logic Science Instrument) in steps of 1 nm.

7) Cell imaging

A431 or MCF7 cells (5×10^4) were seeded into cell dishes containing DMEM medium supplemented with 1% penicillin-streptomycin and 10% FBS and cultured overnight. Then 1 μM probes were added to the medium and incubated at 37°C for 1 h. The cells were washed with PBS twice and then imaged using a Confocal laser scanning microscope (Olympus FV1000) under 405 nm or 488 nm excitation.

8) Synthesis.

For the synthesis of Meldrum's acid derivative 3-(2-methyl-4,6-dioxo-1,3-dioxan-2-yl) propanoic acid,[4] pyrazalone derivatives 2-(5-hydroxy-3-methyl-1H-pyrazol-1-yl) acetic acid[5] and 4-(5-hydroxy-3-methyl-1H-pyrazol-1-yl) benzoic acid,[6] Dansyl-PEG_2-NH_2[7] and FITC-PEG_2-NH_2,[8] we followed the route reported in the literature.

(a) Synthesis of **probe 1**: 3-(2-methyl-4,6-dioxo-1,3-dioxan-2-yl) propanoic acid (99 mg, 0.49 mmol) and HATU (190.12 mg, 0.5 mmol) were dissolved in 2 ml DMF. 2,4,6-trimethylpyridine (66.07 μl, 0.5 mmol) was added and the reaction was then stirred at room temperature for 15 min. Dansyl-PEG_2-NH_2 was dissolved in 1 ml DMF supplemented with 2,4,6-trimethylpyridine (132.15 μl, 1 mmol), and was added slowly to the stirred solution. The mixture was stirred for another 2 h and the product was purified using reverse phase column chromatography in a gradient of acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid) 0-100% yielding a viscous solid (138 mg, 50% yield). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.34 (d, *J* = 9.0 Hz, 1H), 8.26 (d, *J* = 8.4 Hz, 1H), 7.57 (m, 1H), 6.79 (s, 1H), 6.05 (t, *J* = 5.6 Hz, 1H), 3.61 (t, *J* = 4.9 Hz, 2H), 3.55 (d, *J* = 5.9 Hz, 1H), 3.48 (d, *J* = 5.1 Hz, 2H), 3.10 (d, *J* = 5.2 Hz, 1H), 2.96 (s, 2H), 2.93 (m, 6H), 2.88 (s, 2H), 2.80 (s,6H), 2.41 (d, *J* = 6.2 Hz, 1H), 2.37 (d, *J* = 6.5 Hz, 1H), 1.66 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.24, 163.01, 145.35, 137.00, 135.56, 130.09, 129.92, 129.67, 129.55, 123.49, 117.12, 117.00, 107.23, 70.35, 70.22, 70.05, 70.01, 69.45, 69.32, 69.11, 46.39, 46.22, 43.11, 43.02, 39.81, 36.65, 33.82, 29.75, 25.82. HRMS (ESI+): m/z calculated for $C_{26}H_{35}N_3O_9S$ $[M+H]^+$: 566.2128, found: 566.2163.

(b) Synthesis of **probe 2**: 4-(5-hydroxy-3-methyl-1H-pyrazol-1-yl) benzoic acid (54.2 mg, 0.24 mmol), HATU (95.06 mg, 0.25 mmol) were dissolved in 5 ml DMF, and 2,4,6-trimethylpyridine (33 μl, 0.25 mmol) was added. The mixture was stirred at room temperature for 15 min. In another vial, Dansyl-PEG_2-NH_2 was dissolved in 1 ml DMF, and 2,4,6-trimethylpyridine (67 μl, 0.5 mmol) was added. The mixture added to the stirring solution dropwise. The reaction was kept at room temperature for 2 h, followed by silica gel column chromatography (dichloromethane: methanol 10:1), yielding probe 2 as a light green solid (101 mg, 70% yield). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.64 (d, *J* = 8.5 Hz, 1H), 8.42 (d, *J* = 8.7 Hz, 1H), 8.26 (dd, *J* = 7.4, 1.2 Hz, 1H), 7.96 – 7.90 (m, 2H), 7.87 – 7.80 (m, 2H), 7.56 (ddd, *J* = 8.7, 7.4, 4.1 Hz, 2H), 7.03 (s, 1H), 5.85 (s, 1H), 3.68 (d, *J* = 2.7 Hz, 4H), 3.53 (dt, *J* = 5.0, 3.1 Hz, 2H), 3.50 – 3.41 (m, 6H), 3.10 (q, *J* = 5.0, 4.5 Hz, 2H), 2.97 (s, 6H), 2.21 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 170.76, 167.11, 156.82, 150.39, 140.63, 135.16, 130.19, 130.07, 129.68, 129.51, 128.16, 127.99, 123.68, 120.05, 117.95, 115.68, 70.38, 70.22, 69.39, 45.93, 45.60, 43.16, 43.06, 39.97, 17.04. HRMS (ESI+): m/z calculated for $C_{29}H_{35}N_5O_6S$ $[M+H]^+$: 582.2342, found: 582.2387.

(c) Synthesis of **probe 3**: 2-(5-hydroxy-3-methyl-1H-pyrazol-1-yl) acetic acid (153 mg, 0.98 mmol), HATU (380.24 mg, 1 mmol) were dissolved in 5 ml DMF and 2,4,6-trimethylpyridine (132

µl, 1 mmol) was added. The mixture was stirred at room temperature for 15 min. In a separate vial, FITC-PEG_2-NH_2 (537.59 mg, 1 mmol) was dissolved in 5 ml DMF, and 2,4,6-trimethylpyridine (264 µl, 2 mmol) was added. The mixture was added to the stirring solution dropwise and the reaction mixture was stirred at room temperature for 2 h. The product was purified using reversed phase column chromatography in a gradient of acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid) 0-100%, giving **probe 3** as an orange solid (298 mg, 45% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.11 (s, 2H), 8.29 (s, 1H), 8.00 (s, 1H), 7.74 (d, J = 8.2 Hz, 1H), 7.18 (d, J = 8.3 Hz, 1H), 6.67 (d, J = 2.3 Hz, 2H), 6.63 – 6.53 (m, 5H), 5.26 (s, 1H), 4.43 (s, 2H), 3.66 – 3.54 (m, 8H), 3.45 (t, J = 5.8 Hz, 2H),, 3.24 (q, J = 5.9 Hz, 2H), 2.06 (s, 2H), 1.26 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 180.53, 168.46, 166.28, 159.43, 151.80, 146.98, 146.22, 141.37, 129.30, 128.92, 126.39, 123.93, 116.21, 112.51, 109.62, 102.15, 87.75, 69.54, 69.48, 68.81, 68.33, 64.82, 53.43, 47.43, 43.56, 41.68, 38.65, 16.61. HRMS (ESI+): m/z calculated for $C_{33}H_{33}N_5O_9S$ $[M+H]^+$: 676.2033, found: 676.2068.

(d) Synthesis of **probe 4**: Tris(2-carboxyethyl)phosphine Hydrochloride (150 mg, 528 µmol, 3 equiv.) and DIPEA (148 µl, 704 µmol, 4 equiv.) was dissolved in 1.5 ml degassed DMF. In a separate vial, Dansyl-PEG_2-NH_2 (58.5 mg, 176 µmol, 1 equiv.), EDCI (33.7 mg, 176 µmol, 1 equiv.) and HOAt (47.9 mg, 352 µmol, 2 equiv.) was dissolved in 1.5 ml degassed DMF. The two solutions were then combined and the reaction was stirred under an inert atmosphere for 30 min. The product was purified using reverse phase column chromatography in a gradient of acetonitrile (0.1% trifluoroacetic acid): water (0.1% trifluoroacetic acid) 0-70%, giving probe 4 as a viscous solid (82 mg, 40%). ^{1}H NMR (500 MHz, Methanol-d_4) δ 8.58 (dd, J = 8.6, 1.2 Hz, 1H), 8.42 (dd, J = 8.7 Hz, 1H), 8.25 (dd, J = 7.3, 1.3 Hz, 1H), 7.64 (ddd, J = 8.6, 7.5, 4.4 Hz, 2H), 7.40 (d, J = 7.5 Hz, 1H), 3.53 (t, J = 5.4 Hz, 2H), 3.52 – 3.46 (m, 2H), 3.46 – 3.34 (m, 6H), 3.07 (t, J = 5.5 Hz, 2H), 2.97 (s, 6H), 2.88 (t, J = 6.1, 5.3 Hz, 2H), 2.84 (t, J = 9.5, 6.9 Hz, 2H), 2.79 (t, J = 6.8 Hz, 2H), 2.51 (dq, J = 14.0, 7.1 Hz, 6H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 175.68, 175.62, 173.37, 151.65, 137.41, 130.95, 130.75, 130.61, 130.22, 129.11, 124.85, 121.62, 117.01, 71.19, 71.12, 70.67, 70.41, 46.07, 43.81, 40.55, 29.04, 17.75, 17.34. HRMS (ESI-): m/z calculated for $C_{27}H_{40}N_3O_9PS$ $[M-H]^-$: 612.2223, found: 612.2164.

2. Supplementary figures

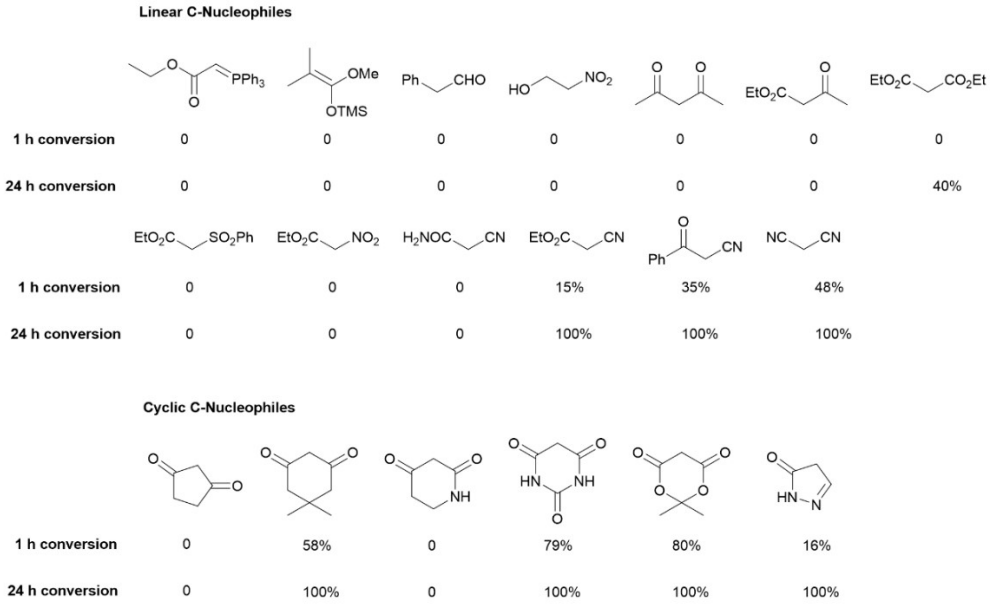


Fig. S1 Conversion of the reactions between C-nucleophiles and fGly peptide in 1 h or 24 h. 1 mM C-nucleophiles in DMSO was added to fGly-containing peptide (0.1 mM) dissolved in 50 mM MES buffer (pH 6.5, 2% DMSO). The reaction was kept at room temperature and followed by LC-MS analysis.

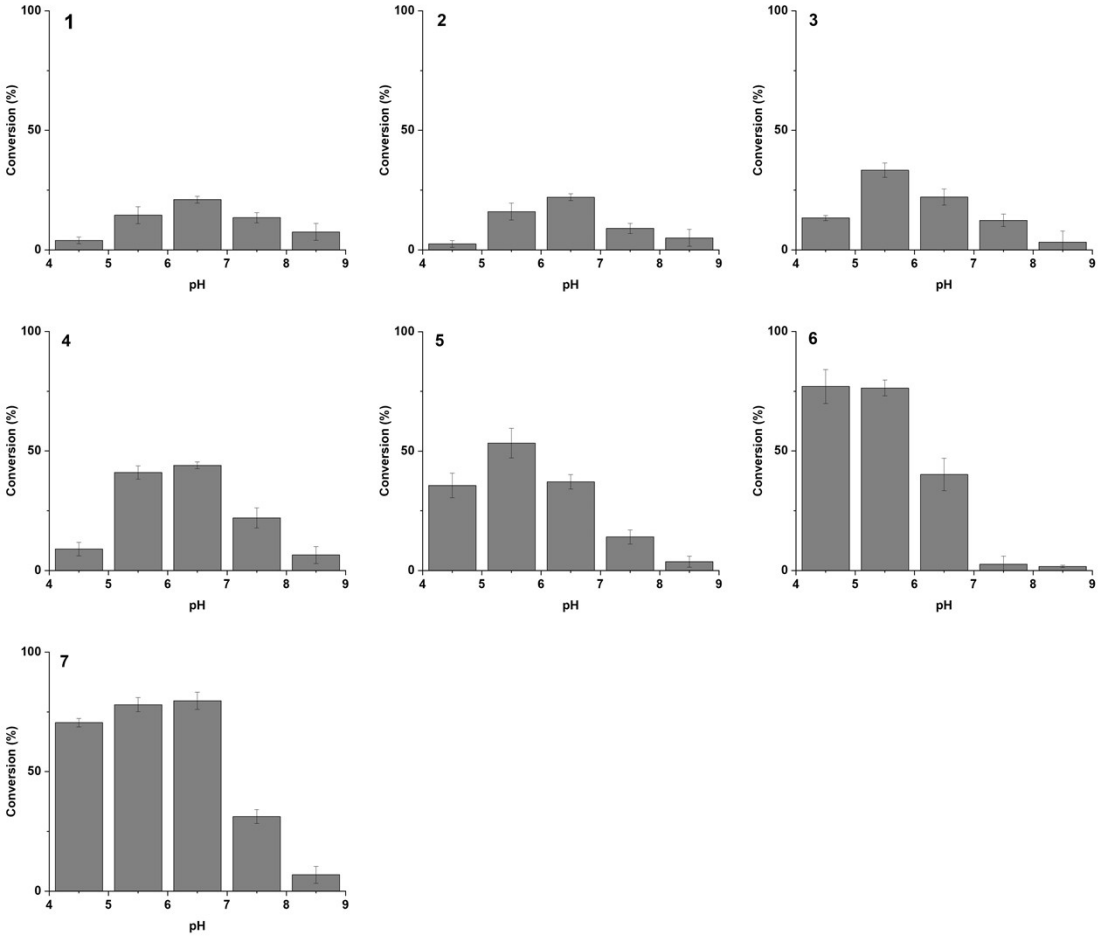


Fig. S2 Conversion of the **1-7** Knoevenagel Condensation at different pH. Peptide containing fGly (0.1 mM) was dissolved in buffer solution with different pH (pH 4.5 acetate buffer, pH 5.5 MES buffer, pH 6.5 MES buffer, pH 7.5 PBS buffer, pH 8.5 Tris buffer). C-nucleophiles (1 mM) was added, respectively, and the conversion was determined using HPLC after 1 h of incubation at room temperature.

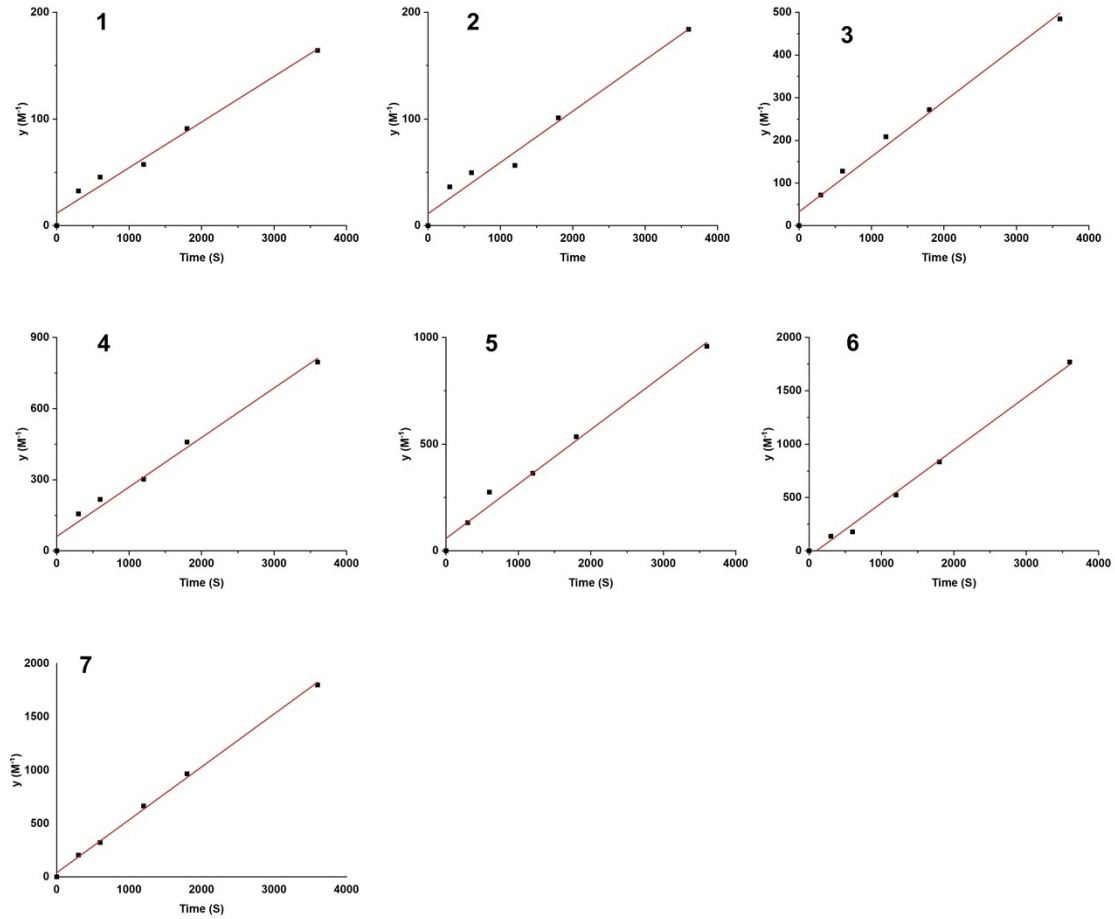
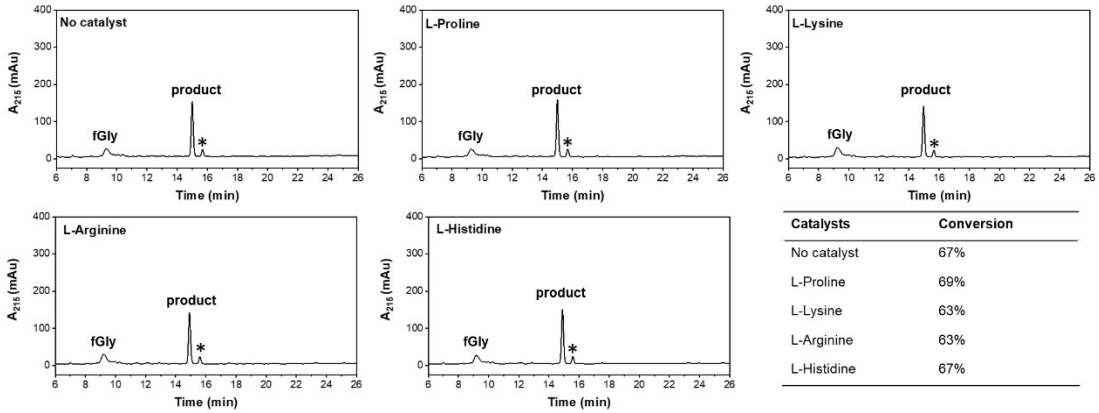


Fig. S3 Kinetic data for the Knoevenagel condensation reactions between the peptide and C-nucleophiles **1-7**. The data points were fitted using linear regression.



Catalysts	Conversion
No catalyst	67%
L-Proline	69%
L-Lysine	63%
L-Arginine	63%
L-Histidine	67%

Fig. S4 HPLC analysis of Meldrum's acid Knoevenagel condensation in the presence of 5 mM organo-catalysts (L-proline, L-Lysine, L-Arginine and L-Histidine). Peptide (0.1 mM) was dissolved in 50 mM MES buffer (pH 6.5), 1 mM Meldrum's acid was added and the reaction mixture was supplemented with 5 mM organo-catalysts. The reaction was kept at room temperature for 30 min and followed by HPLC analysis. 'fGly' indicates unreacted fGly bearing peptide, '*' indicates unidentified impurities.

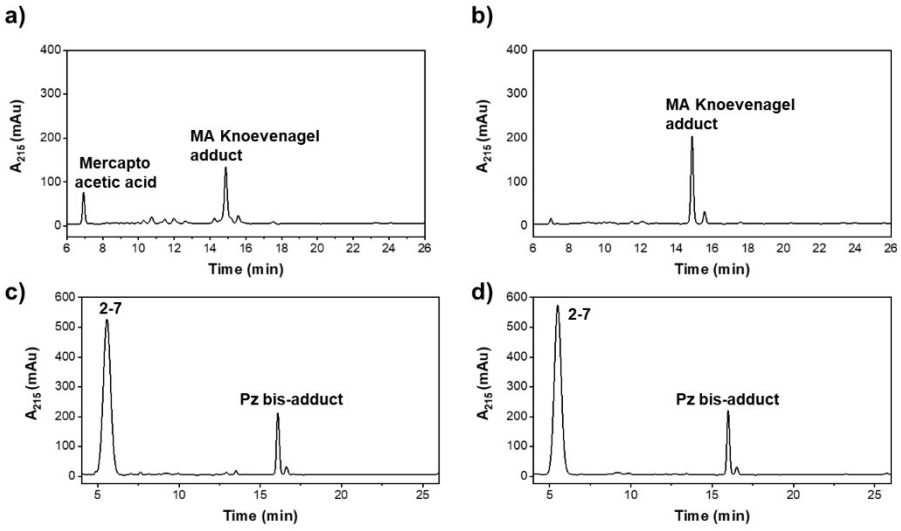


Fig. S5 HPLC analysis of the reactivity of MA and Pz enone to thiol and amine nucleophiles. Peptide containing fGly (0.1 mM) was dissolved in 50 mM MES buffer (pH 6.5), 1 mM MA or Pz **2g** in DMSO was added and the reaction was kept at room temperature for 2 h followed by adding 10 mM Mercapto acetic acid or benzylamine to the reaction mixture. The reactions were kept at room temperature for 24 h and followed by HPLC-HRMS analysis. The results showed minimal side products in the reaction between a) MA and Mercapto acetic acid, b) MA and benzylamine, c) **2g** and Mercapto acetic acid, d) **2g** and benzylamine.

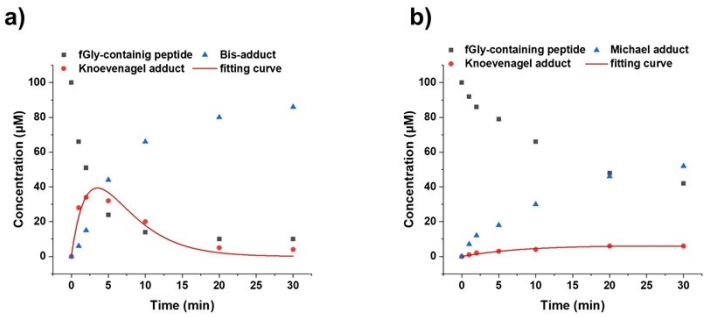


Fig. S6 a) Kinetic data for the Knoevenagel- Michael tandem reaction b) Kinetic data for Knoevenagel-phospha-Michael tandem reaction.

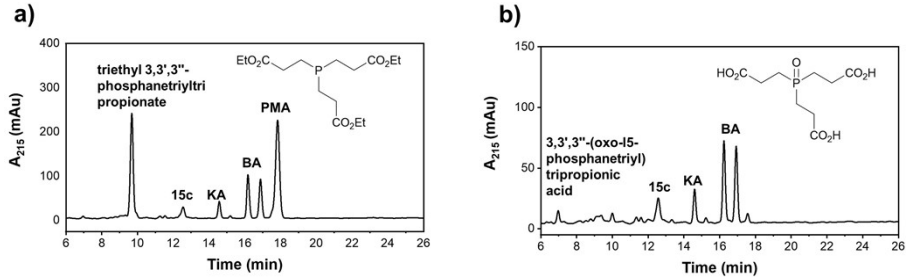


Fig. S7 HPLC analysis of the Knoevenagel-Phospha-Michael tandem reaction between **2c** and a) triethyl 3,3',3"-phosphanetriyltripropionate, b) 3,3',3"-(oxo-l5-phosphanetriyl)tripropionic acid. KA: Knoevenagel adduct; BA: bis-adduct; PMA: phospha-Michael adduct.

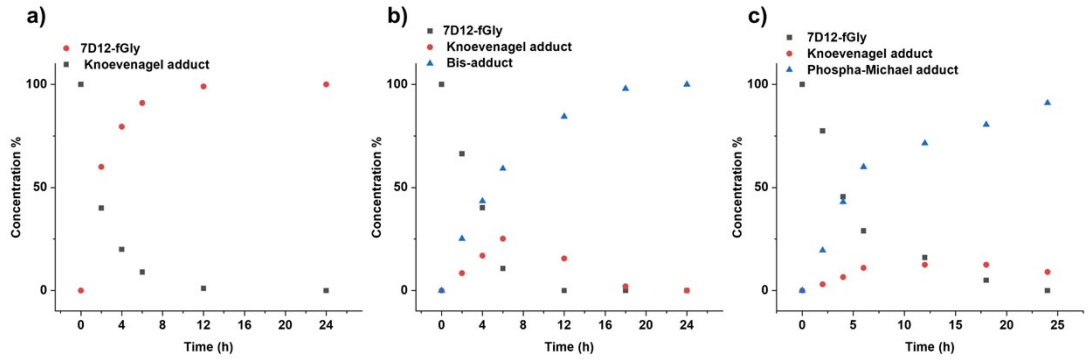
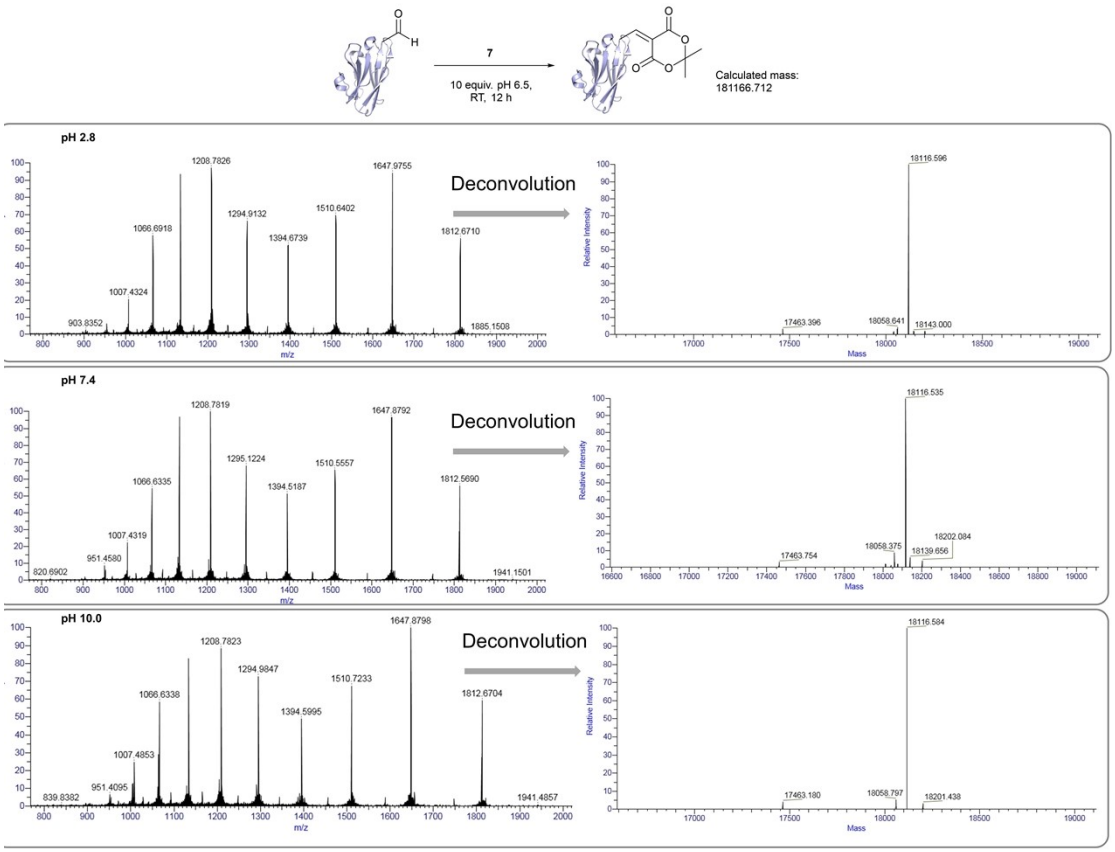


Fig. S8 Tracking over time of 7D12-fGly modification.


7
10 equiv. pH 6.5,
RT, 12 h
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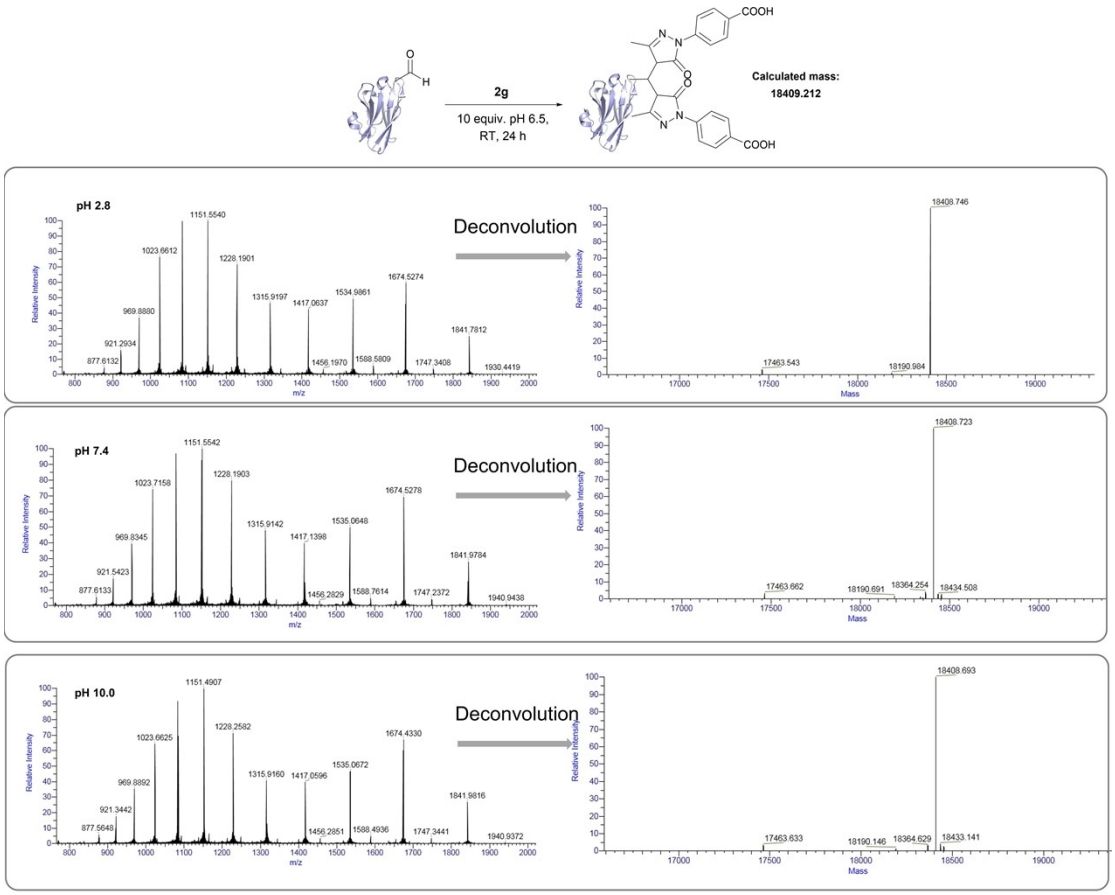
pH 2.8
Deconvolution
pH 7.4
Deconvolution
pH 10.0
Deconvolution
m/z
Mass
Relative Intensity


2g
10 equiv. pH 6.5,
RT, 24 h
COOH
COOH
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pH 10.0
Deconvolution
m/z
Mass
Relative Intensity

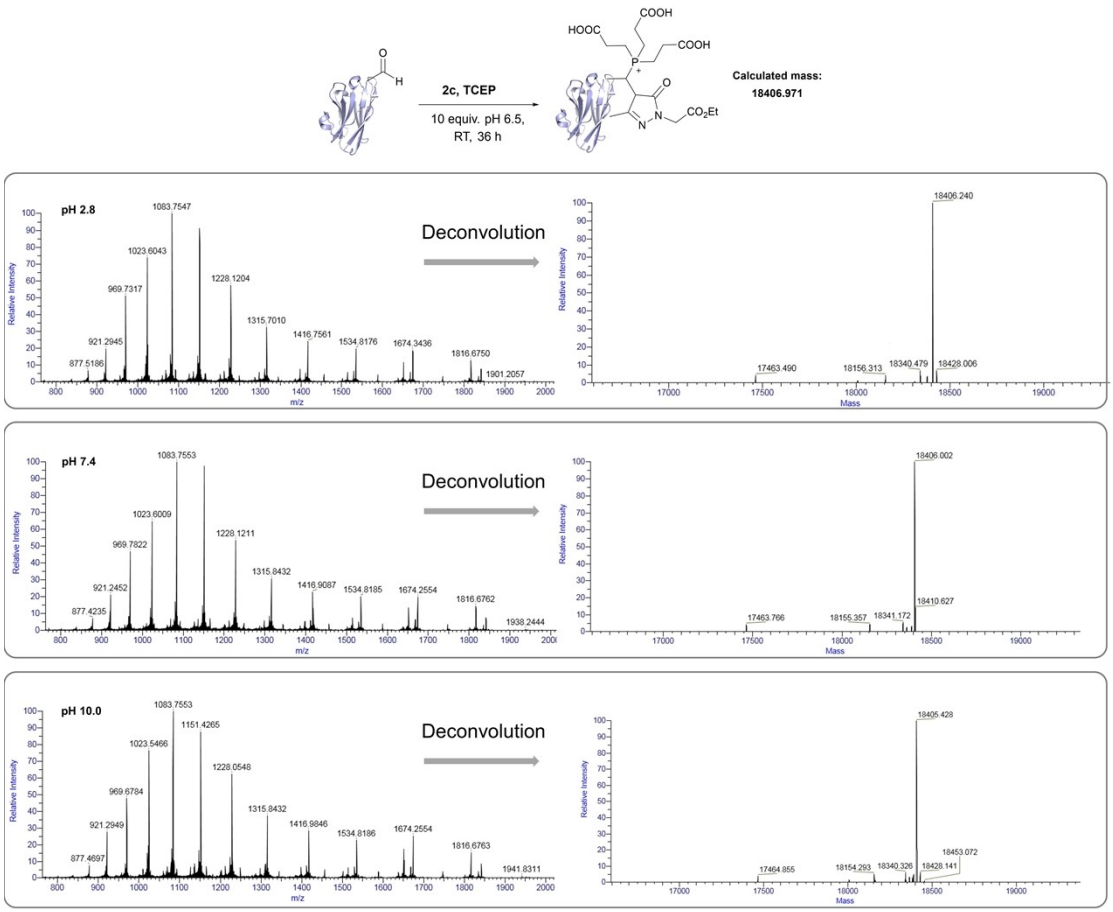

Fig. S9 Stability study of the conjugate in acidic, neutral and basic conditions. The protein conjugates were purified and incubated for 48 h at 37 ℃, followed by protein MS analysis.

3. Reference

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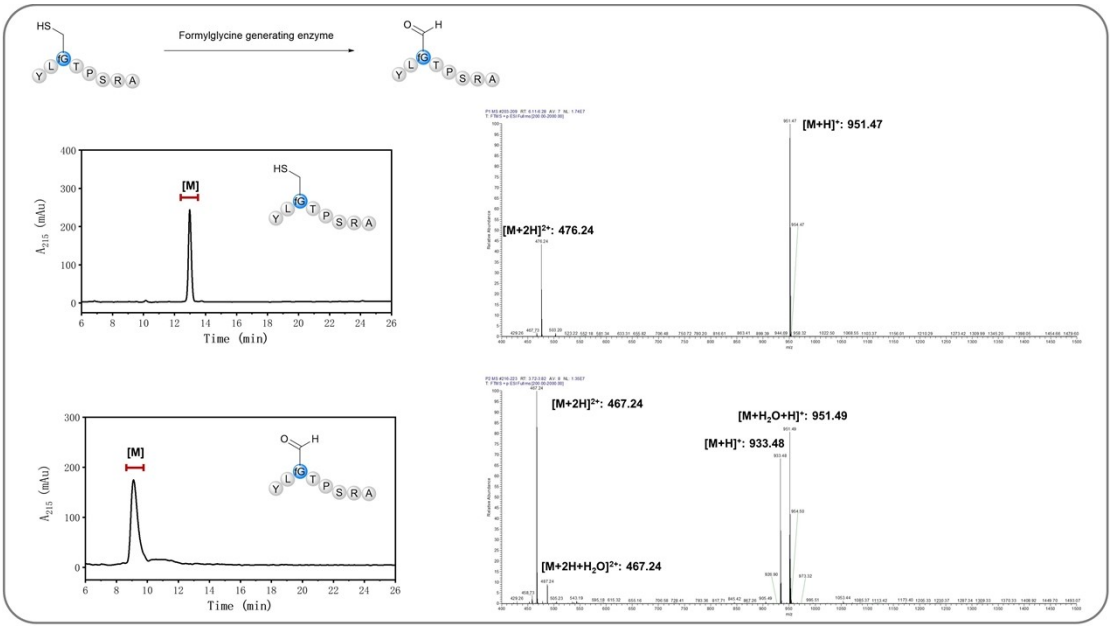
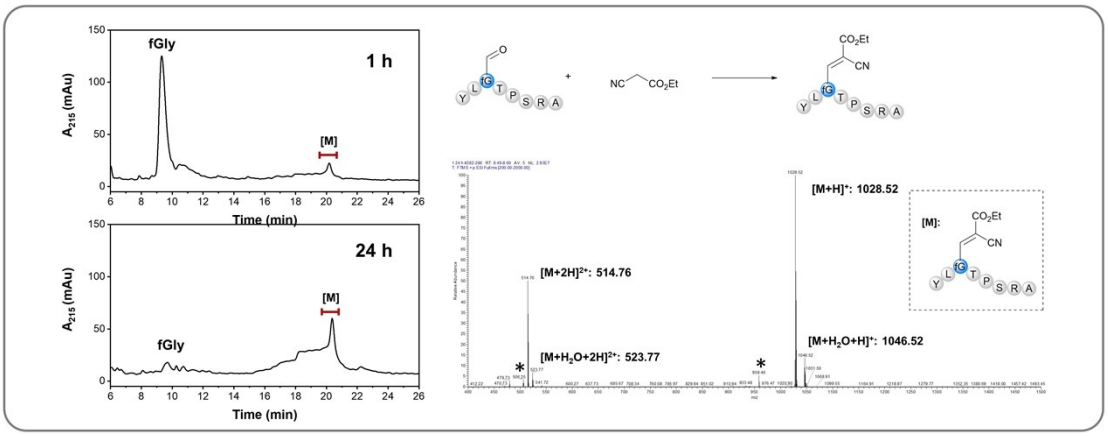
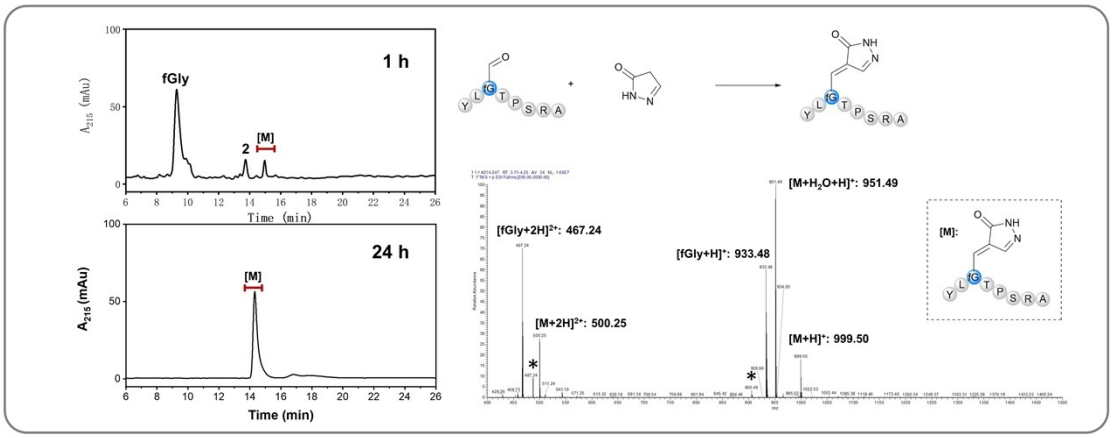
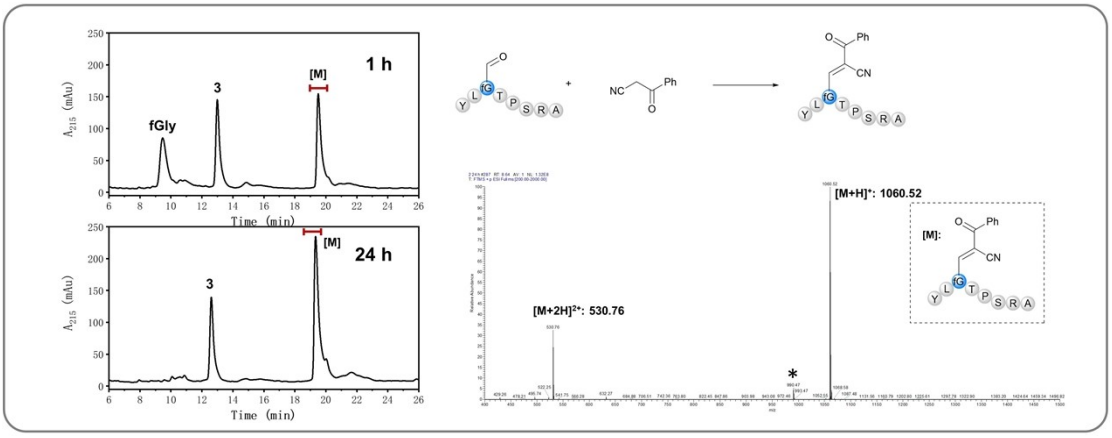
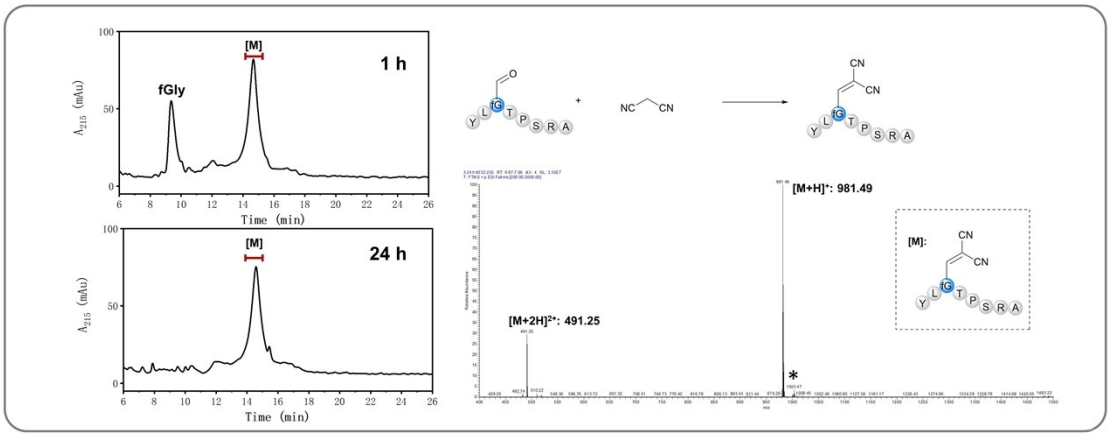
4. LC-MS identification of the peptide reactions








1 h
24 h
fGly
3
[M]
Time (min)
[M+H]⁺: 1060.52
[M+2H]²⁺: 530.76
[M]:


1 h
24 h
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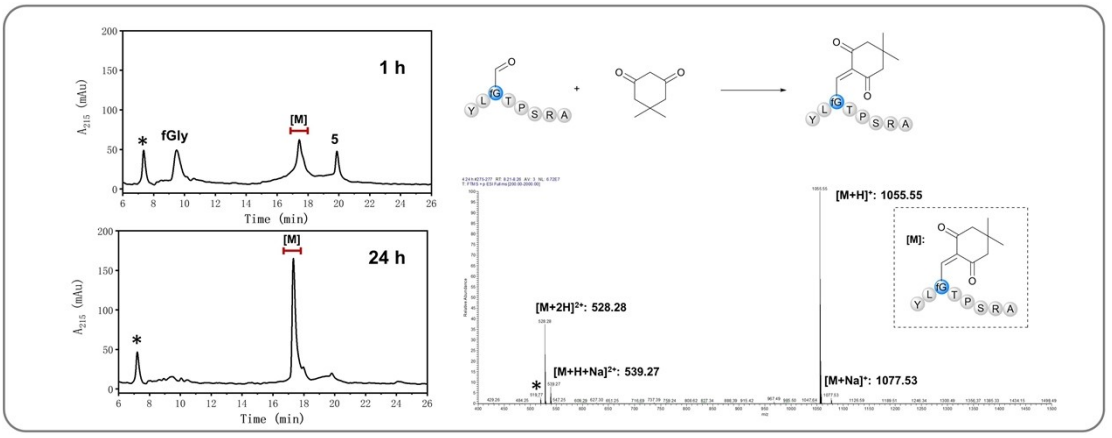
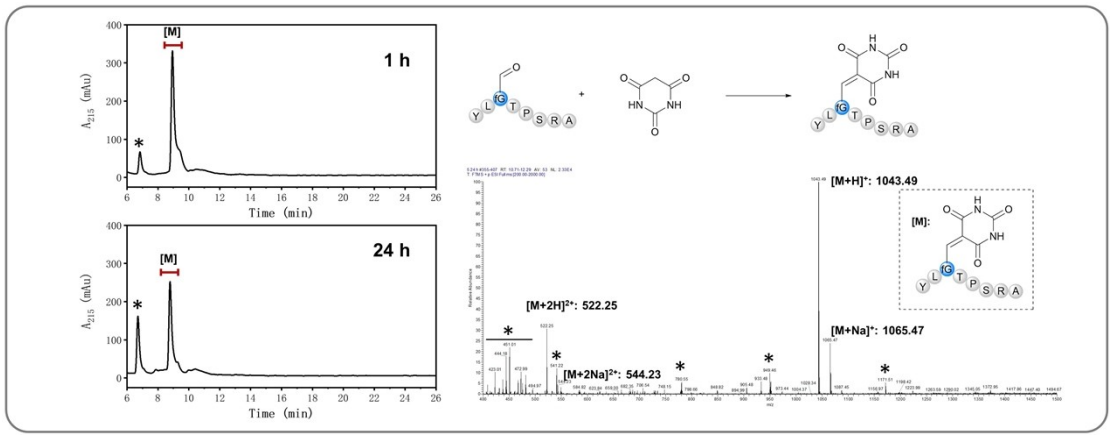
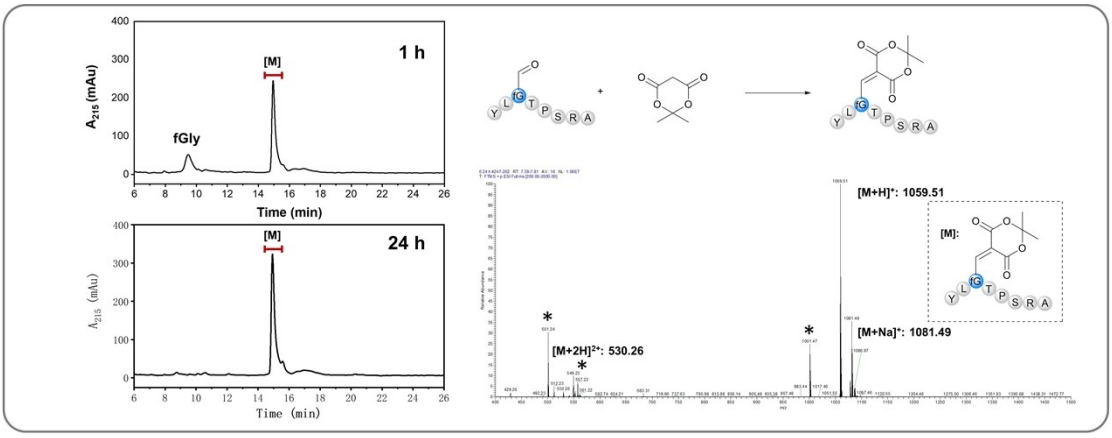
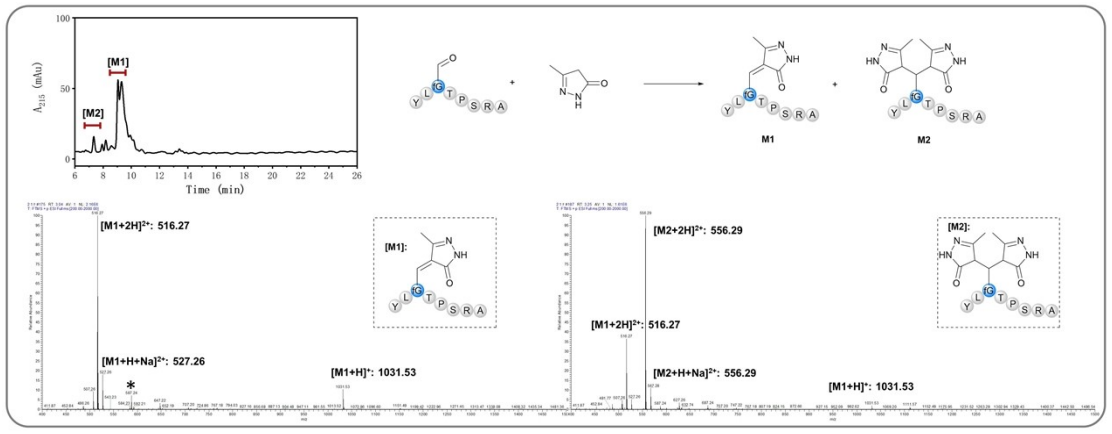
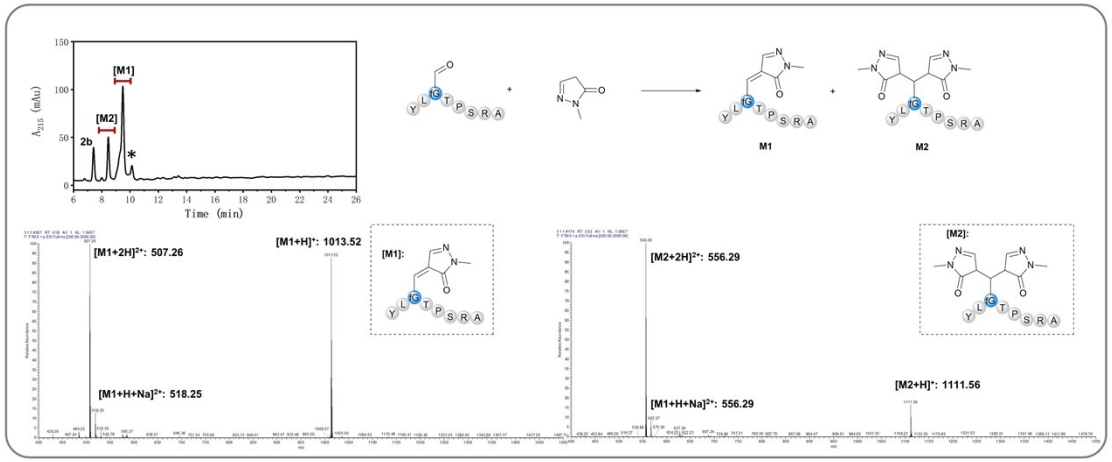
Time (min)
[M+H]⁺: 981.49
[M+2H]²⁺: 491.25
[M]:

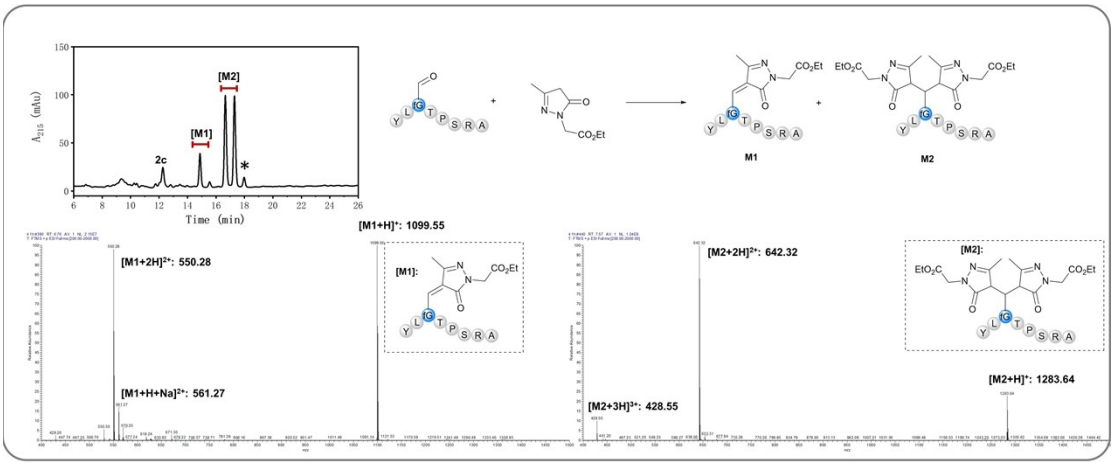

1 h
24 h
fGly
[M]
5
Time (min)
[M+H]⁺: 1055.55
[M+2H]²⁺: 528.28
[M+H+Na]²⁺: 539.27
[M+Na]⁺: 1077.53
[M]:

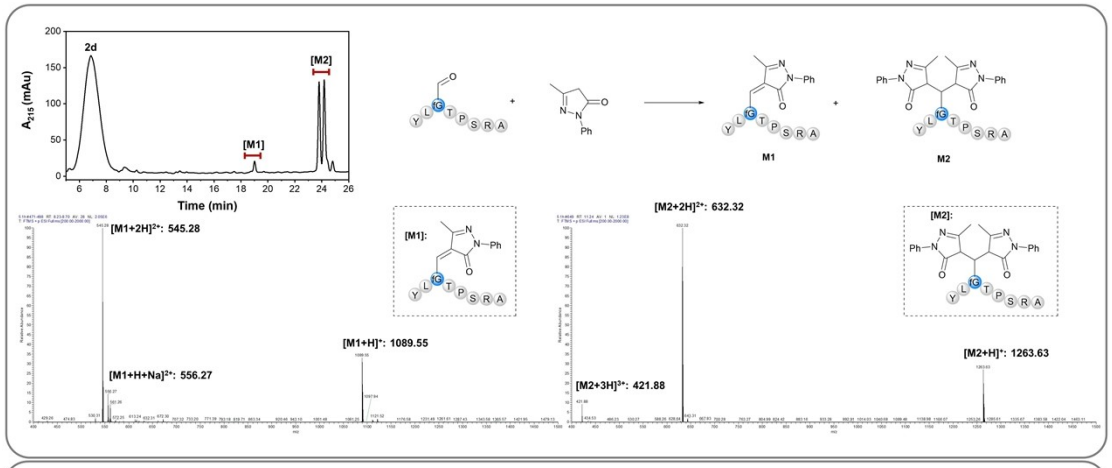

1 h
24 h
[M]
Time (min)
[M+H]⁺: 1043.49
[M+2H]²⁺: 522.25
[M+2Na]²⁺: 544.23
[M+Na]⁺: 1065.47
[M]:

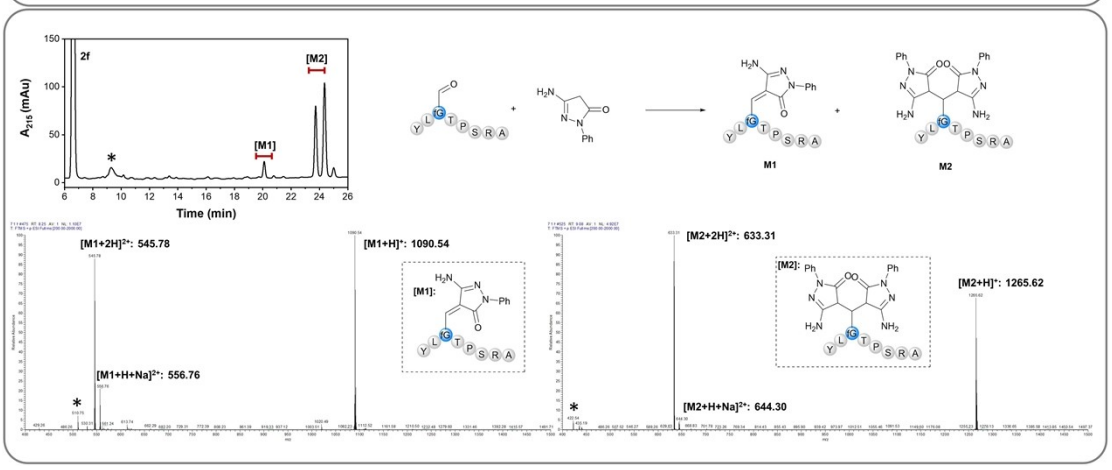

1 h
[M]
fGly
Time (min)
24 h
[M]
Time (min)
[M+H]⁺: 1059.51
[M]:
[M+2H]²⁺: 530.26
[M+Na]⁺: 1081.49

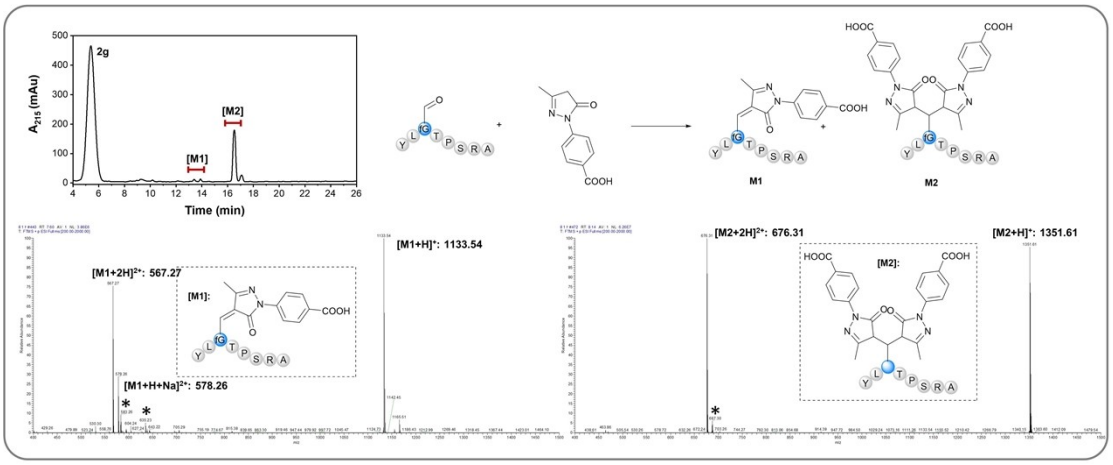

[M1]
[M2]
Time (min)
M1
M2
[M1+2H]²⁺: 516.27
[M1+H+Na]²⁺: 527.26
[M1+H]⁺: 1031.53
[M1]:
[M2+2H]²⁺: 556.29
[M1+2H]²⁺: 516.27
[M2+H+Na]²⁺: 556.29
[M1+H]⁺: 1031.53
[M2]:

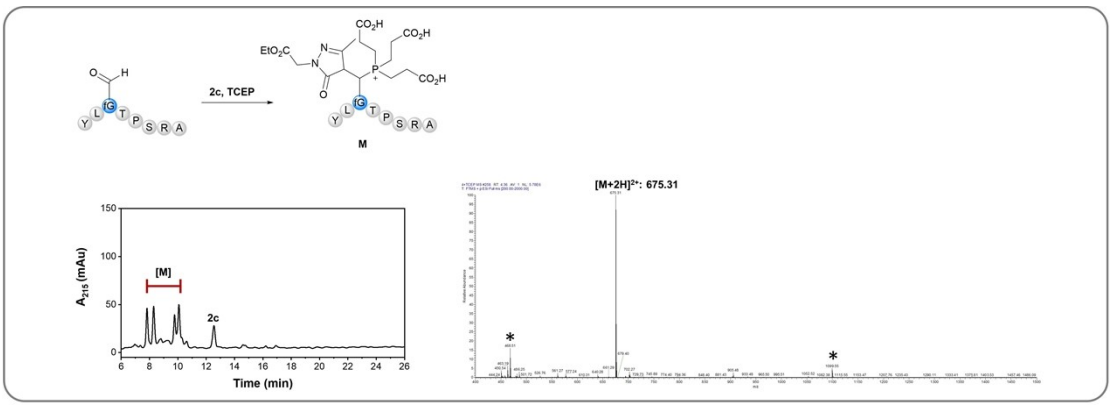

[M1]
[M2]
2b
Time (min)
M1
M2
[M1+2H]²⁺: 507.26
[M1+H]⁺: 1013.52
[M1]:
[M1+H+Na]²⁺: 518.25
[M2+2H]²⁺: 556.29
[M2]:
[M2+H]⁺: 1111.56
[M1+H+Na]²⁺: 556.29


[M2]
[M1]
2c
Time (min)
M1
M2
[M1+H]⁺: 1099.55
[M1+2H]²⁺: 550.28
[M1]:
[M1+H+Na]²⁺: 561.27
[M2+2H]²⁺: 642.32
[M2]:
[M2+H]⁺: 1283.64
[M2+3H]³⁺: 428.55


2d
[M1]
[M2]
A215 (mAu)
Time (min)
M1
M2
[M1+2H]2+: 545.28
[M1+H+Na]2+: 556.27
[M1+H]+: 1089.55
[M2+2H]2+: 632.32
[M2+3H]3+: 421.88
[M2+H]+: 1263.63

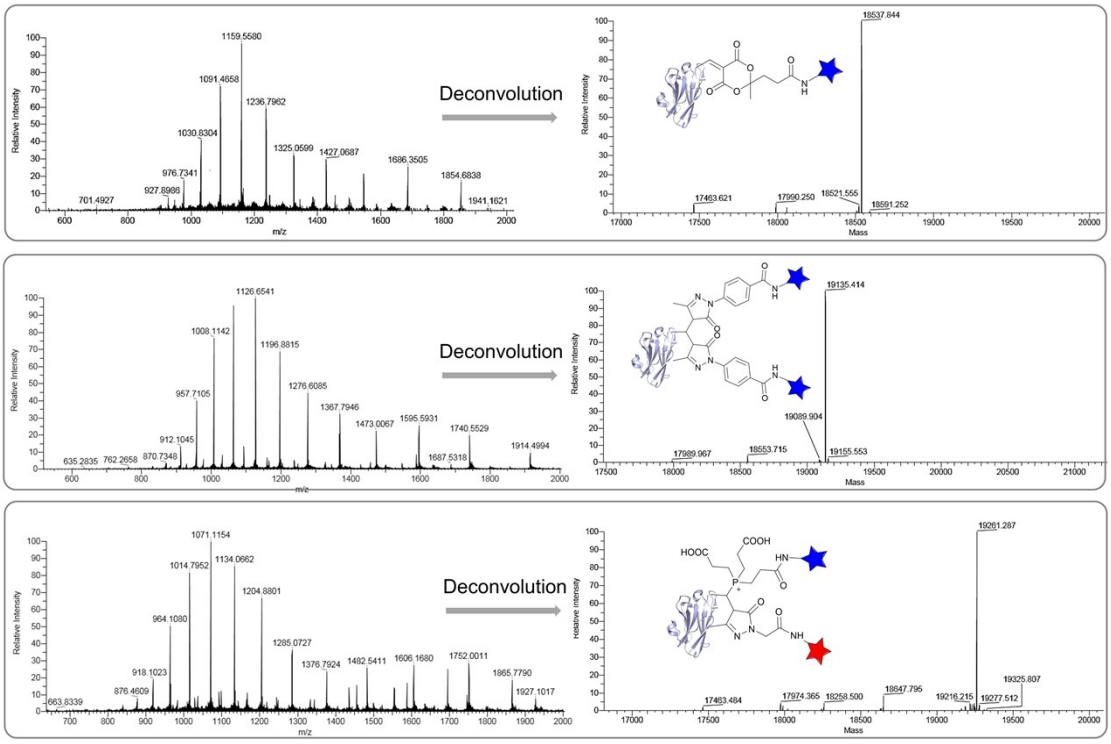

2f
[M1]
[M2]
A215 (mAu)
Time (min)
M1
M2
[M1+2H]2+: 545.78
[M1+H]+: 1090.54
[M1+H+Na]2+: 556.76
[M2+2H]2+: 633.31
[M2+H+Na]2+: 644.30
[M2+H]+: 1265.62


2g
[M1]
[M2]
A215 (mAu)
Time (min)
M1
M2
[M1+2H]2+: 567.27
[M1+H]+: 1133.54
[M1+H+Na]2+: 578.26
[M2+2H]2+: 676.31
[M2+H]+: 1351.61


2c, TCEP
M
[M]
2c
A215 (mAu)
Time (min)
[M+2H]2+: 675.31

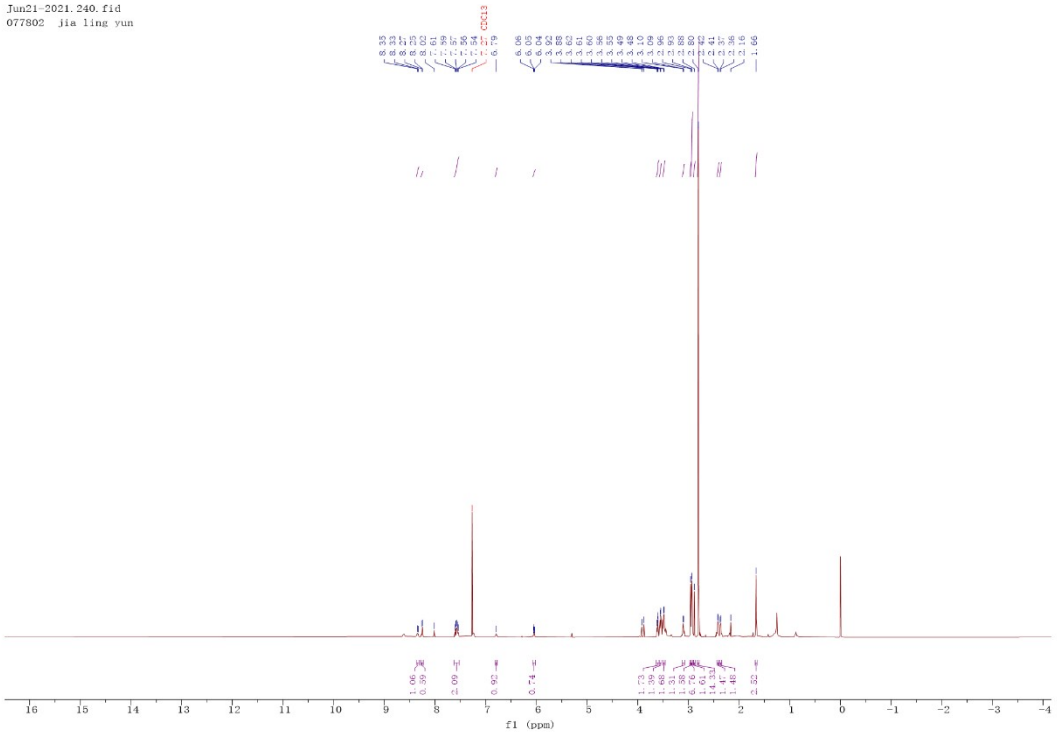
‘*’ indicates unidentified impurities.

5. Source protein MS data of protein conjugates labeled with fluorescent probes

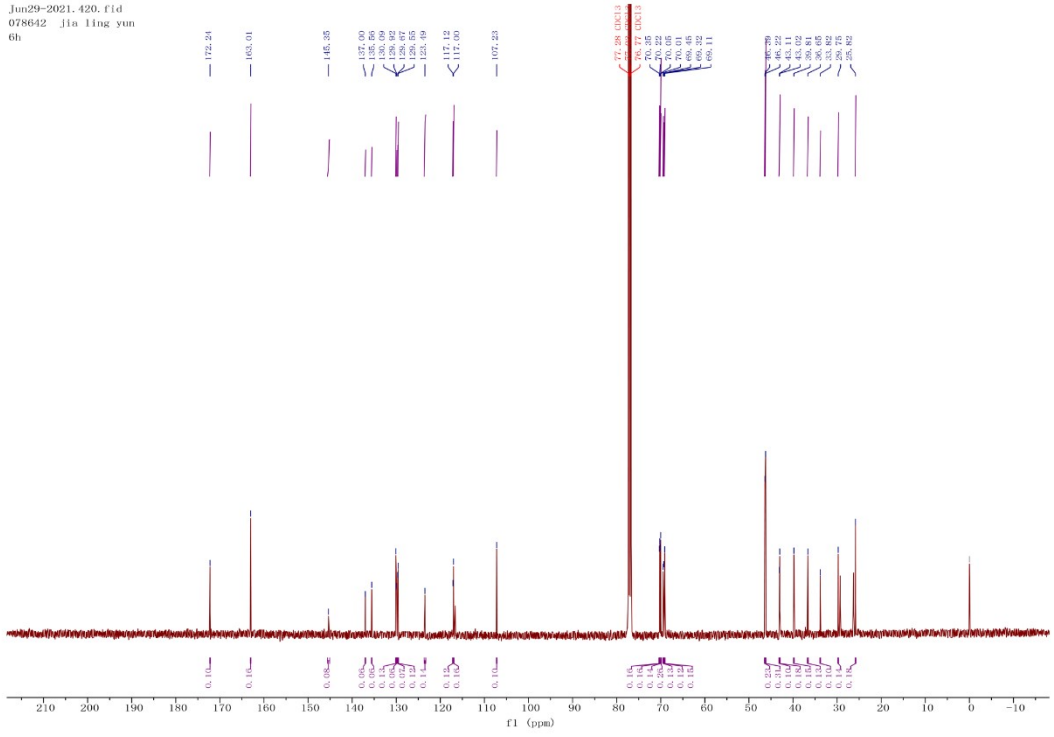


6. NMR data

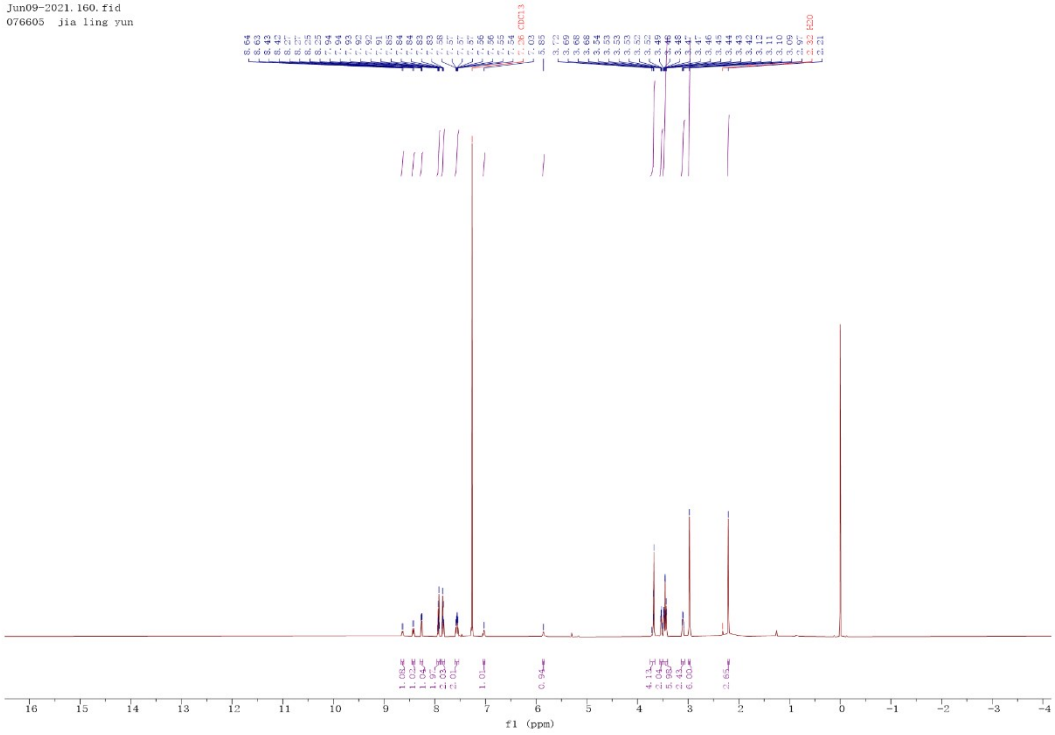
Probe 1 ^{1}H NMR



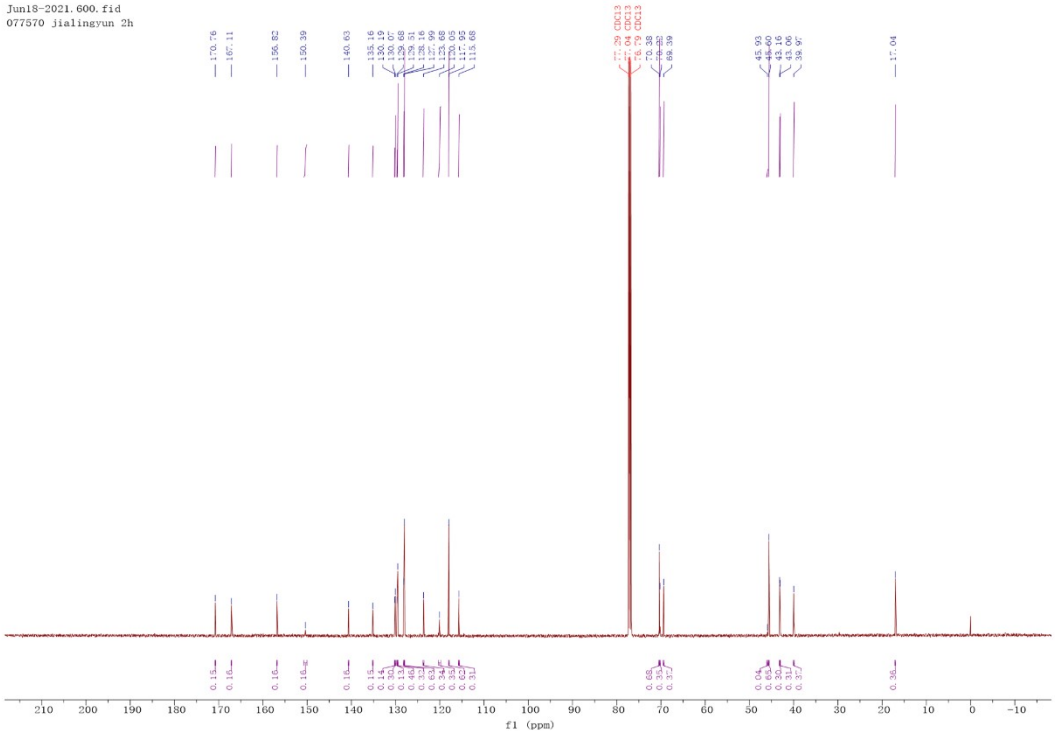
Probe 1 ^{13}C NMR



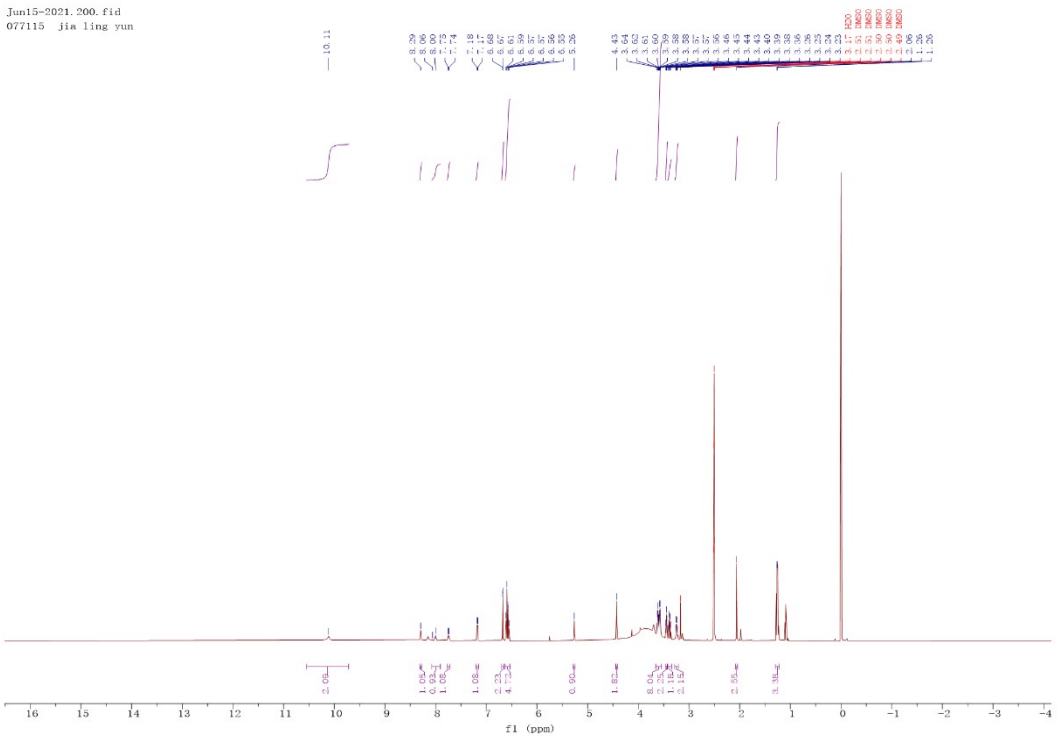
Probe 2 ^{1}H NMR



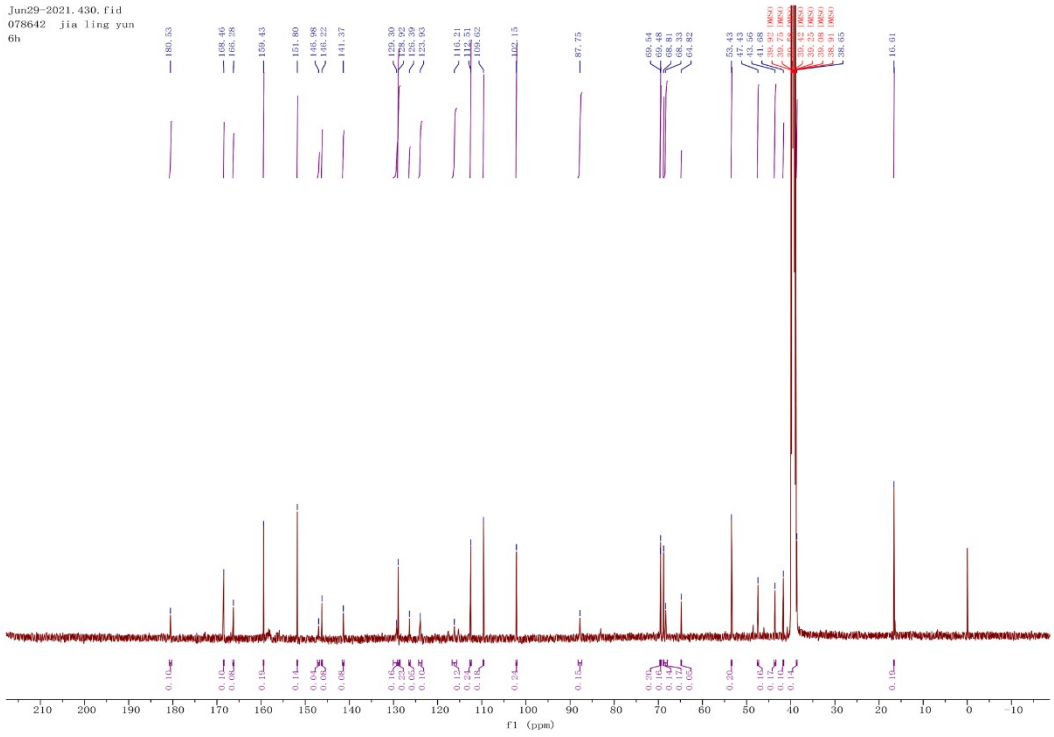
Probe 2 ^{13}C NMR



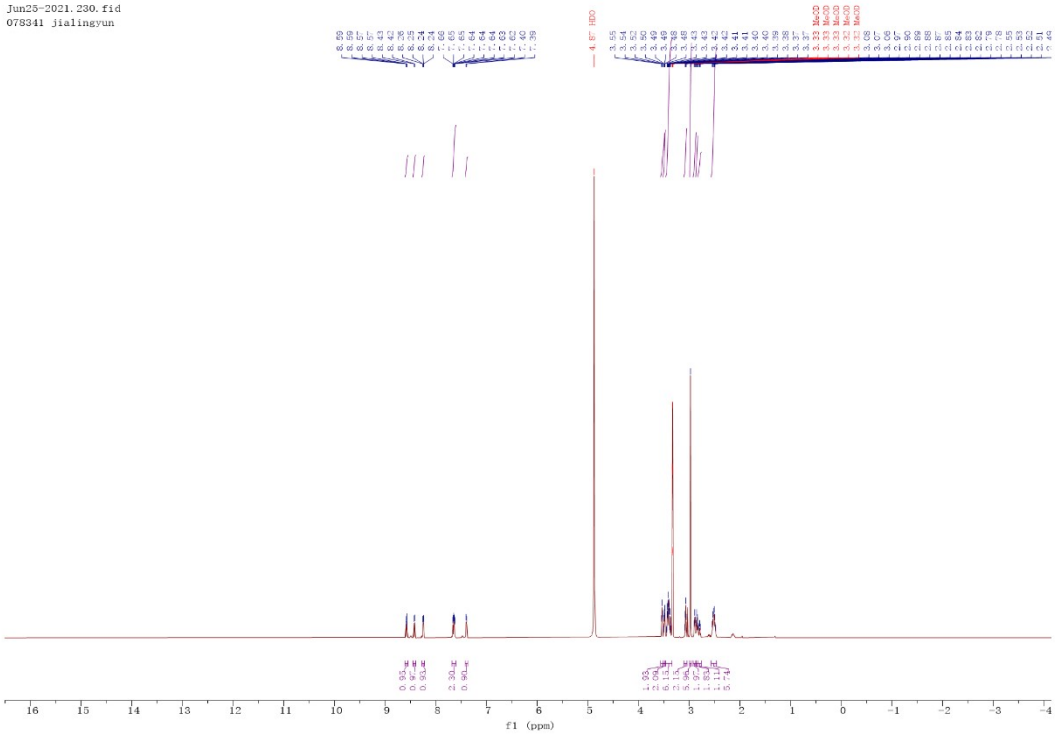
Probe 3 ^{1}H NMR



Probe 3 ^{13}C NMR



Probe 4 ^{1}H NMR



Probe 4 ^{13}C NMR