

Supporting Information

**Further Exploration of Hydrazine-Mediated Bioconjugation Chemistries**

Melrose Mailig, David Hymel & Fa Liu\*

Novo Nordisk Research Center  
530 Fairview Avenue North, Seattle, WA 98109  
e-mail: [liufasioc@live.com](mailto:liufasioc@live.com)

## Table of Contents

1. General Remarks on Material and Method .....	S2
2. Synthesis of Peptides .....	S3
3. General Procedures for Hydrazine Ligation Optimization .....	S9

## The List of Figures

Figure S1	UPLC-MS of purified peptide hydrazine <b>1</b>
Figure S2	UPLC-MS traces of synthesizing peptide <b>2</b>
Figure S3	UPLC-MS traces of synthesizing peptide <b>3</b>
Figure S4	UPLC trace of peptide acylhydrazine <b>4</b>
Figure S5	UPLC-MS traces of synthesizing peptide <b>5</b>
Figure S6	UPLC-MS traces of synthesizing peptide <b>6</b>
Figure S7	UPLC-MS of peptide <b>7</b>
Figure S8	UPLC-MS of peptide <b>12</b>
Figure S9	UPLC-MS of peptide <b>13</b>
Figure S10	The UPLC traces of the treating peptide <b>5</b> with 5 mM glucose
Figure S11	The UPLC traces of the treating peptide <b>7</b> with 5 mM glucose
Figure S12	Peptide pyrazolone <b>7</b> 100 $\mu$ M and 5 mM glucose were incubated in a 50 mM phosphate buffer pH 7.4 for 48 h, then followed by 4 h treatment of 5 mM $\beta$ -mercaptoethanol
Figure S13	NMR Spectrum of pyrazolone-glucose adduct
Figure S14	UPLC trace of incubating peptides trifluoromethylated hydrazone <b>12</b> and pyrazole <b>13</b> with 5 mM glucose.
Figure S15	UPLC trace of incubating peptides hydrazone <b>5</b> and pyrazolone <b>7</b> with 5 mM glucose and 1 mM reduced glutathione.
Figure S16	UPLC trace of incubating peptides trifluoromethylated hydrazone <b>12</b> and pyrazole <b>13</b> with 5 mM glucose and 1 mM reduced glutathione.
Figure S17	UPLC traces for the reaction of peptide GLP1-ABDCon with 5 mM Glucose and 1 mM GSH at neutral pH, 37°C.
Figure S18	The UPLC traces of the chemical stability of peptide <b>5</b>
Figure S19	The UPLC traces of the chemical stability of peptide <b>7</b>
Figure S20	Mass Spec. of the degradation products of peptide pyrazolone <b>7</b> at neutral pH, 37 °C, day 4; and the proposed structures.
Figure S21	The UPLC traces of the chemical stability of peptide <b>12</b>
Figure S22	The UPLC traces of the chemical stability of peptide <b>13</b>
Figure S23	The UPLC traces of treating 5 arginine-containing peptide PrRP with 5 equiv. diketone 1-phenyl-1,3-butanedione in a neutral pH buffer.
Figure S24	The impact of 5 mM EDTA on peptide hydrazine ABDcon <b>14</b> stability in a 50 mM phosphate buffer, pH 7.4, 37°C

## 1. General Remarks on Material and Method

### Abbreviations

DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide, ESI-TOP, electrospray ionization time-of-flight; equiv., equivalent; GSH, glutathione reduced; HCl, hydrochloric acid; H<sub>2</sub>O, water; LC-MS, liquid chromatography-mass spectrometry; MBHA, 4-Methylbenzhydrylamine; MeCN, acetonitrile; MeOH, Methanol; MS, mass spectrometry;; Na<sub>2</sub>SO<sub>4</sub>, sodium sulfate; oxyma, ethyl cyanohydroxyiminoacetate; RP-HPLC, reverse-phase high performance liquid chromatography; rt, room temperature; SPPS, solid phase peptide synthesis; TIS, triisopropylsilane; TFA, trifluoroacetic acid.

### Materials and Method

Reagents and solvents were obtained and used without purification unless specified from the commercial sources: *Sigma-Aldrich, Acros Organics, TCI America, 1-Click Chemistry, Fisher Scientific, VWR Internationals, Santa Cruz Biotechnology, Combi Blocks, BDH and Alfa Aesar*. Orthogonally protected Fmoc-amino acids were purchased from Iris Biotech as pre-weighed cartridges and SPPS resins were purchased from NovaBioChem. Aqueous buffers were prepared freshly using Millipore Grade I water. Mettler Toledo pH meter was used to adjust the final pH. The reaction mixtures for small molecule was heated and stirred using IKA IKAMAG RET Basic Stainless-Steel Hotplate Stirrer. The reactions with peptide were vortexed (200 rpm, rt) and incubated at 25 °C or 37 °C (VWR Gravity Convection Incubator, Basics 120V). TLC was performed on silica gel coated with aluminum TLC plates (Merck, TLC Silica gel 60 F254). The compounds were visualized using a UV lamp (254 nm) and Potassium Permanganate stain. Organic solvents were removed by BUCHI rotavapor R-210/215, whereas aqueous samples were lyophilized by CHRiST APLHA 3-4 LSC Basic. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained on a Bruker Aeon 400 MHz NMR spectrometer. <sup>1</sup>H NMR spectra were referenced to TMS (0 ppm), CDCl<sub>3</sub> (7.26 ppm) whereas <sup>13</sup>C NMR spectra referenced to CDCl<sub>3</sub> (77.16 ppm). Peak multiplicities are designated by the following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. Chemical shifts (δ) are reported in ppm relative to solvent residual peaks and coupling constants are reported in Hz. All the NMR spectra were recorded at 298 K. For LC-MS and HR-MS analysis, MS detection was obtained using Waters Xevo G2-XS QToF mass spectrometer in ESI+ mode. Preparative HPLC was performed using a Waters Prep 150 LC system equipped with a Waters XSelect CSH C18 column (19 x 250 mm, 5 μm) at a flow rate of 20 mL/min [Buffer A: 89.9% water / 10% acetonitrile with 0.1% TFA; Buffer B: 89.9% acetonitrile / 10% water with 0.1% TFA].

A 50 mM sodium acetate buffer pH 4.5 or a 50 mM sodium phosphate buffer pH 7.4 was prepared directly before each experiment.

### Automated Solid Phase Peptide Synthesis

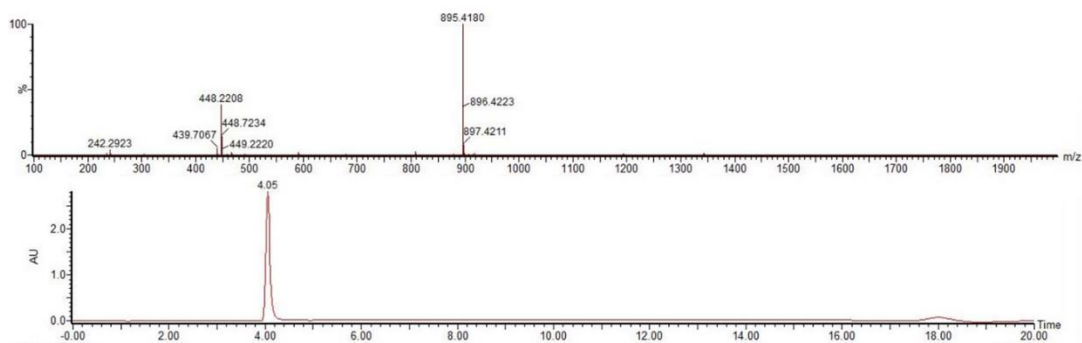
The automated SPPS was performed on a Symphony X peptide synthesizer (Gyros Biotechnologies). Peptides were synthesized using Fmoc-SPPS chemistry on rink amide MBHA resin (100-200 mesh, ~0.34 mmol/g loading). Couplings were carried out for 2-4 h using Fmoc amino acid (1.2 mmol/g loading, 12 equiv.), DIC (12 equiv.), Oxyma (12 equiv.). Fmoc protecting group was removed using 20% piperidine in DMF.

Peptides were cleaved from the resin by treatment with freshly made solution of TFA/TIS/H<sub>2</sub>O (95:2.5:2.5, v/v/v, 15 mL) or TFA/H<sub>2</sub>O (97.5:2.5, v/v, 15mL) for 3-4 h at room temperature. The eluted cleavage solution was precipitated in cold diethyl ether. The crude peptide samples were agitated on a vortex and spun in a centrifuge. The supernatant was removed and the crude peptide pellet was washed with cold ether twice. The crude peptide pellet was dissolved in 10% MeCN/H<sub>2</sub>O and then filtered via a 0.2 μm filter unit. The resulting solution was loaded onto a preparative RP-HPLC column and resolved using one of the following methods: Method A (0-40 min, 15-35% B, flow rate 20 mL/min), Method B (0-40 min, 18-38%

B, flow rate 20 mL/min) Method C (0-40 min, 20-40% B, flow rate 20 mL/min), Method D (0-40 min, 10-40% B, flow rate 5 mL/min), and Method E (0-40 min, 20-50% B, flow rate 5 mL/min).

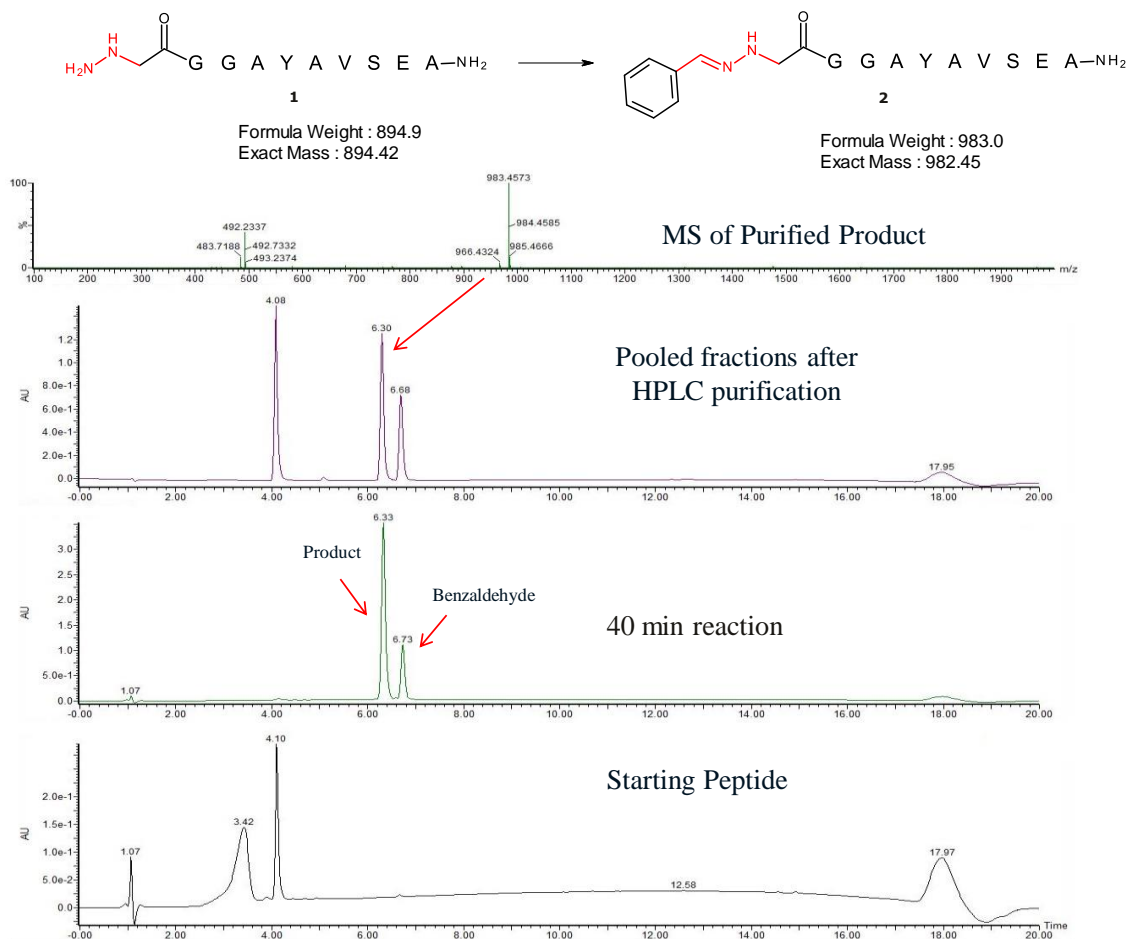
## 2. Synthesis of Peptides

**Synthesis of Peptide Hydrazine 1:** The resin-bound peptide (100.0  $\mu\text{mol}$ ) with unprotected *N*-terminal (from the automated solid-phase peptide synthesizer) was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min). A solution of bromo acetic acid (20.0 equiv.) and DIC (10.0 equiv.) in DMF (5 mL) was added to the resin and left shaking for 45 min. This procedure was repeated for two more times and then the resin was washed successively with DCM (3 x 10 mL) and DMSO (3 x 5 min). A solution of 2% anhydrous hydrazine in DMSO (5 mL) was added to the resin and left shaking for 90 min. The excess reagent was removed by washing the resin with DCM (3 x 10 mL). The hydrazine-bearing peptide was cleaved from the resin using TFA/TIS/ $\text{H}_2\text{O}$  (95:2.5:2.5, v/v/v, 15 mL) for 3 h at room temperature and purified using the general method mentioned above and afforded **1** as a fluffy white powder (30.0 mg, 33.5  $\mu\text{mol}$ , 34%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{37}\text{H}_{59}\text{N}_{12}\text{O}_{14}$  895.4273; Found 895.4180.



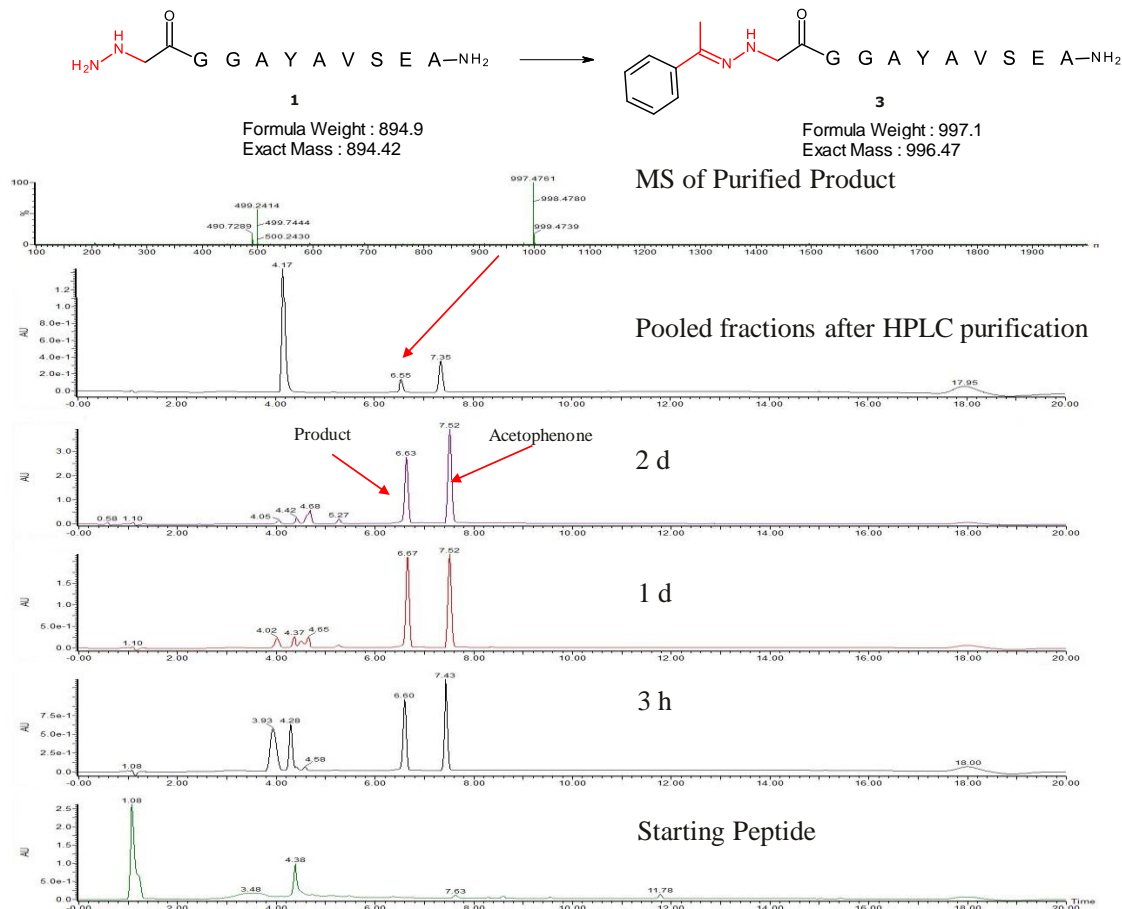
**Figure S1.** UPLC-MS of purified peptide hydrazine **1**.

**Synthesis of Peptide Hydrazone 2:** Peptide **1** (15.0 mg, 17.0  $\mu\text{mol}$ ) was dissolved in 10% MeCN in 50 mM phosphate buffer solution at pH 4.5 and to this solution was added a solution of benzaldehyde (1.5 equiv.) in MeCN. The reaction was monitored by LC-MS and purified using the RP-HPLC. Purification by semi-preparative RP-HPLC afforded a mixture of peptide **2** and its hydrolysis as a fluffy white powder (total 10.8 mg, 11.0  $\mu\text{mol}$ , 68%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{44}\text{H}_{63}\text{N}_{12}\text{O}_{14}$  983.4587; Found 983.4573.



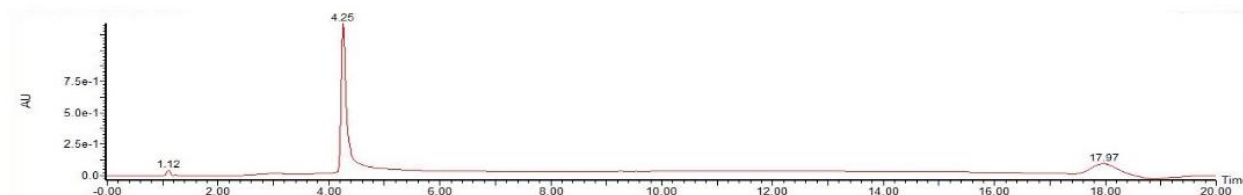
**Figure S2.** UPLC-MS traces of synthesizing peptide **2**.

**Synthesis of Peptide Hydrazone **3**:** Peptide hydrazine **1** (15.0 mg, 17.0  $\mu\text{mol}$ ) was dissolved in 10% MeCN in 50 mM phosphate buffer solution at pH 4.5 and to this solution was added a solution of acetophenone (2.0 equiv.) in MeCN. The reaction was monitored by LC-MS and purified using the RP-HPLC. Purification by semi-preparative RP-HPLC afforded a mixture of **3** and its hydrolysis as a fluffy white powder (8.5 mg, 9.0  $\mu\text{mol}$ , 50%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{45}\text{H}_{65}\text{N}_{12}\text{O}_{14}$  997.4743; Found 997.4761.



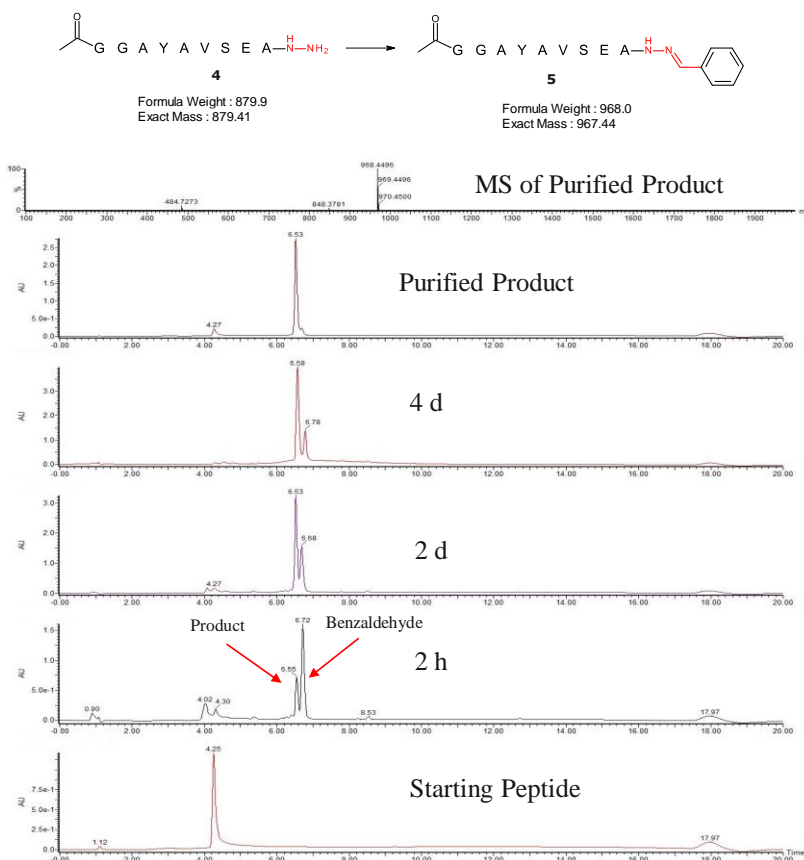
**Figure S3.** UPLC-MS traces of synthesizing peptide **3**.

**Synthesis of Peptide Acylhydrazine 4:** The resin bound peptide with unprotected *N*-terminal (from the automated solid-phase peptide synthesizer using 200  $\mu\text{mol}$  Fmoc-NHNH-2CTC-resin) was washed successively with DCM (3 x 10 mL) and DMF (3 x 5 min). A solution acetic anhydride (10.0 equiv.) and Hunig's base (11.0 equiv.) in DMF (10 mL) was added to the resin and left shaking for 1 h. This procedure was repeated for one more time and then the resin was washed successively with DCM (3 x 10 mL). The acetylated peptide was cleaved from the resin using TFA/H<sub>2</sub>O (97.5:2.5, v/v, 15 mL) for 3 h at room temperature and purified using the general method mentioned above providing 70 mg peptide acylhydrazine **4** as a fluffy white powder at 40% yield. HRMS (ESI-TOF) *m/z*: [M + H]<sup>+</sup> Calcd for C<sub>37</sub>H<sub>58</sub>N<sub>11</sub>O<sub>14</sub> 880.4164; Found 880.4160.



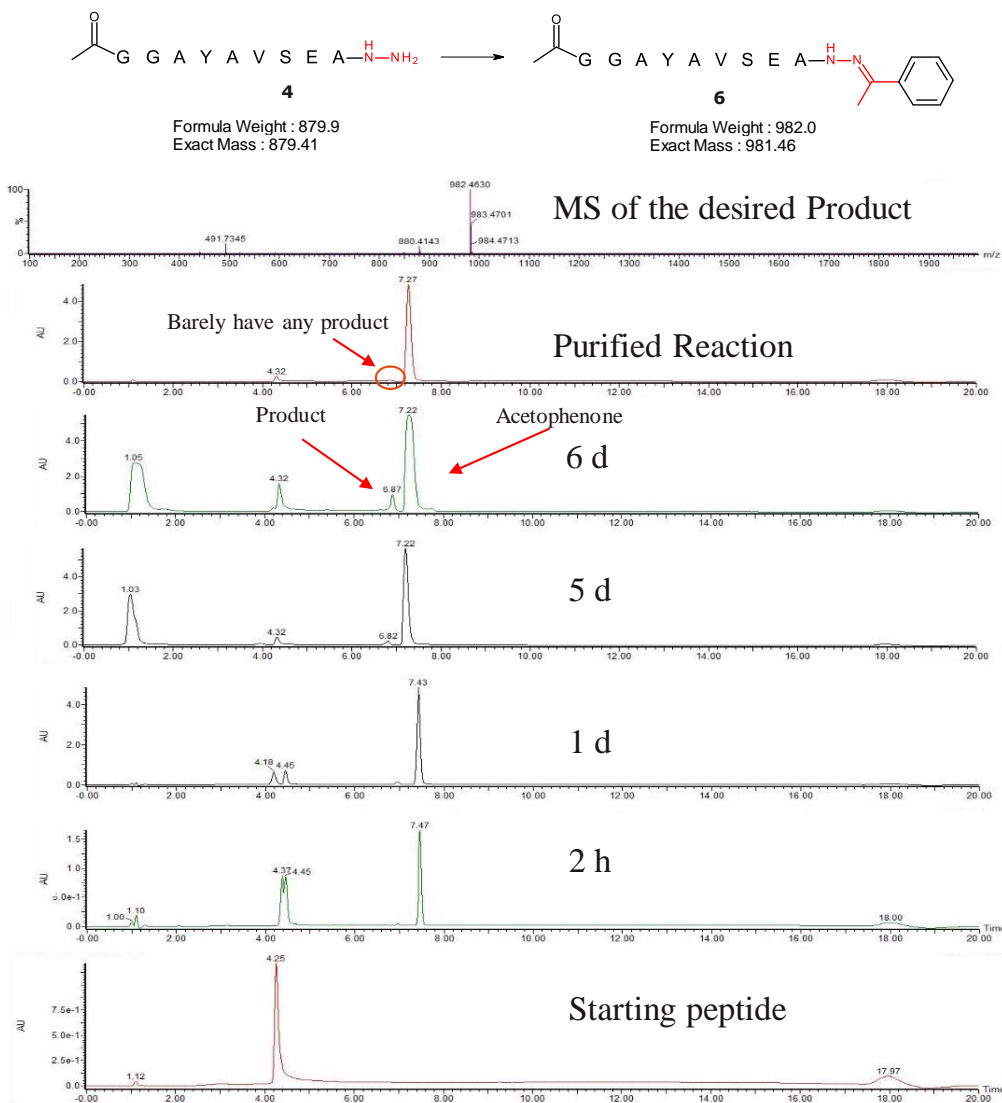
**Figure S4.** UPLC trace of peptide acylhydrazine **4**.

**Synthesis of Peptide Acyl Hydrazone 5:** The purified acetylated peptide **4** (30.0 mg, 30.0  $\mu\text{mol}$ ) was dissolved in 10% MeCN in 50 mM phosphate buffer solution at pH 4.5 and to this solution was added a solution of benzaldehyde (1.5 equiv.) in MeCN. The reaction was monitor by LC-MS and purify using the RP-HPLC. Purification by semi-preparative RP-HPLC afforded **5** as a fluffy white powder (20 mg, 21  $\mu\text{mol}$ , 61%). HRMS (ESI-TOF) *m/z*: [M + H]<sup>+</sup> Calcd for C<sub>44</sub>H<sub>62</sub>N<sub>11</sub>O<sub>14</sub> 968.4477; Found 968.4496.



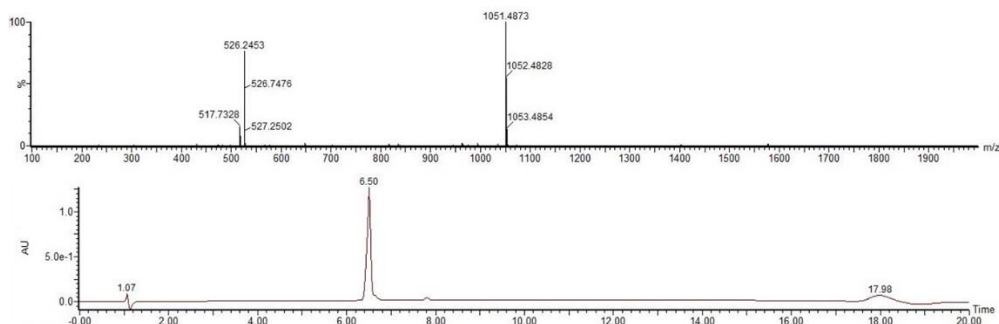
**Figure S5.** UPLC-MS traces of synthesizing peptide **5**.

**Synthesis of Peptide Acylhydrazone **6**:** The purified peptide **4** (15.0 mg, 15.0  $\mu$ mol) was dissolved in 1 mL DMSO and to this solution was added acetophenone (20.0 equiv.) and acetic acid (10  $\mu$ L). The reaction was monitored by LC-MS. The reaction was purified after 6 d, even the reaction did not go into completion. The purification using the semi-preparative RP-HPLC led to the hydrolysis of the desired product. HRMS (ESI-TOF) m/z:  $[M + H]^+$  Calcd for  $C_{45}H_{64}N_{11}O_{14}$  982.4634; Found 982.4630.



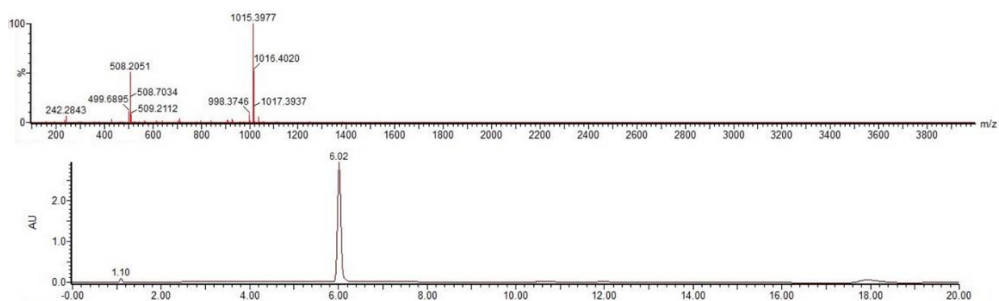
**Figure S6.** UPLC-MS traces of synthesizing peptide **6**.

**Synthesis of Peptide 7:** The synthesis was set up with peptide hydrazine **1** (8.0  $\mu\text{mol}$ ) using general procedure below for the optimization of  $\beta$ -keto ester substrates. Purification by semi-preparative RP-HPLC afforded **7** as a fluffy white powder (3.2 mg, 3.0  $\mu\text{mol}$ , 38%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{48}\text{H}_{67}\text{N}_{12}\text{O}_{15}$  1051.4848; Found 1051.4873.



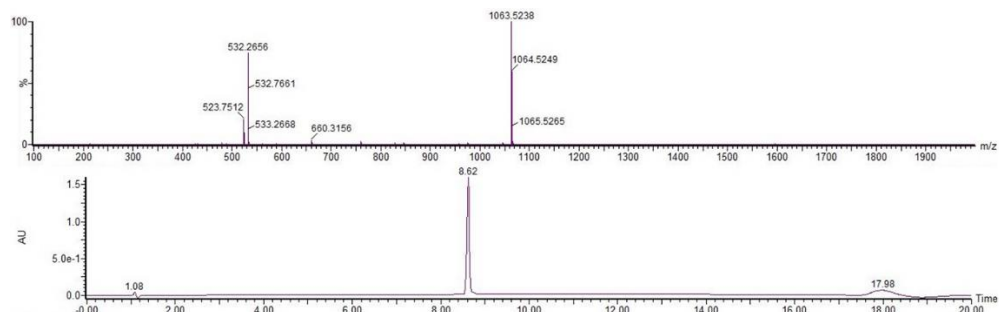
**Figure S7.** UPLC-MS of peptide **7**.

**Synthesis of Peptide 12:** The synthesis was set up with peptide hydrazine **1** (20.0  $\mu\text{mol}$ ) using general procedure below for the optimization of  $\beta$ -keto esters. Purification by semi-preparative RP-HPLC afforded **12** as a fluffy white powder (8.2 mg, 10.0  $\mu\text{mol}$ , 53%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{41}\text{H}_{58}\text{F}_3\text{N}_{12}\text{O}_{15}$  1015.4097; Found 1015.3977.



**Figure S8.** UPLC-MS of peptide **12**.

**Synthesis of Peptide 13:** The synthesis was set up with peptide hydrazine **1** (10.0  $\mu\text{mol}$ ) using general procedure below for the optimization of 1,3-diketone substrates. Purification by semi-preparative RP-HPLC afforded **13** as a fluffy white powder (6.0 mg, 5.6  $\mu\text{mol}$ , 57%). HRMS (ESI-TOF)  $m/z$ :  $[\text{M} + \text{H}]^+$  Calcd for  $\text{C}_{50}\text{H}_{71}\text{N}_{12}\text{O}_{14}$  1063.5213; Found 1063.5238.



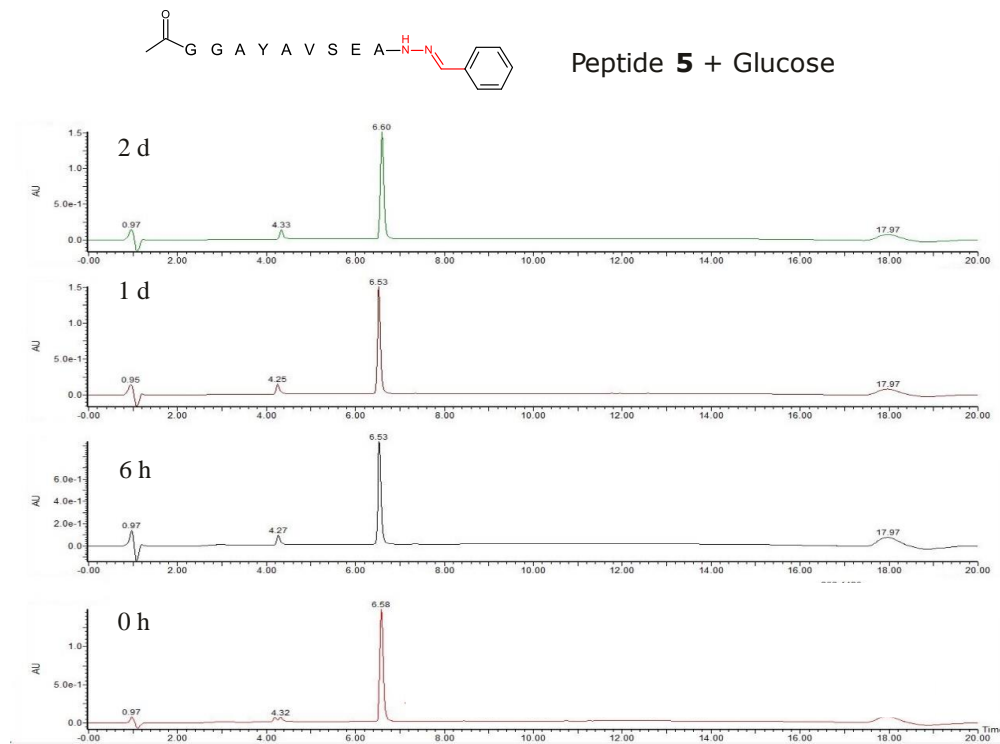
**Figure S9.** UPLC-MS of peptide **13**.

### 3. General procedures for the optimization of hydrazine-mediated bioconjugations

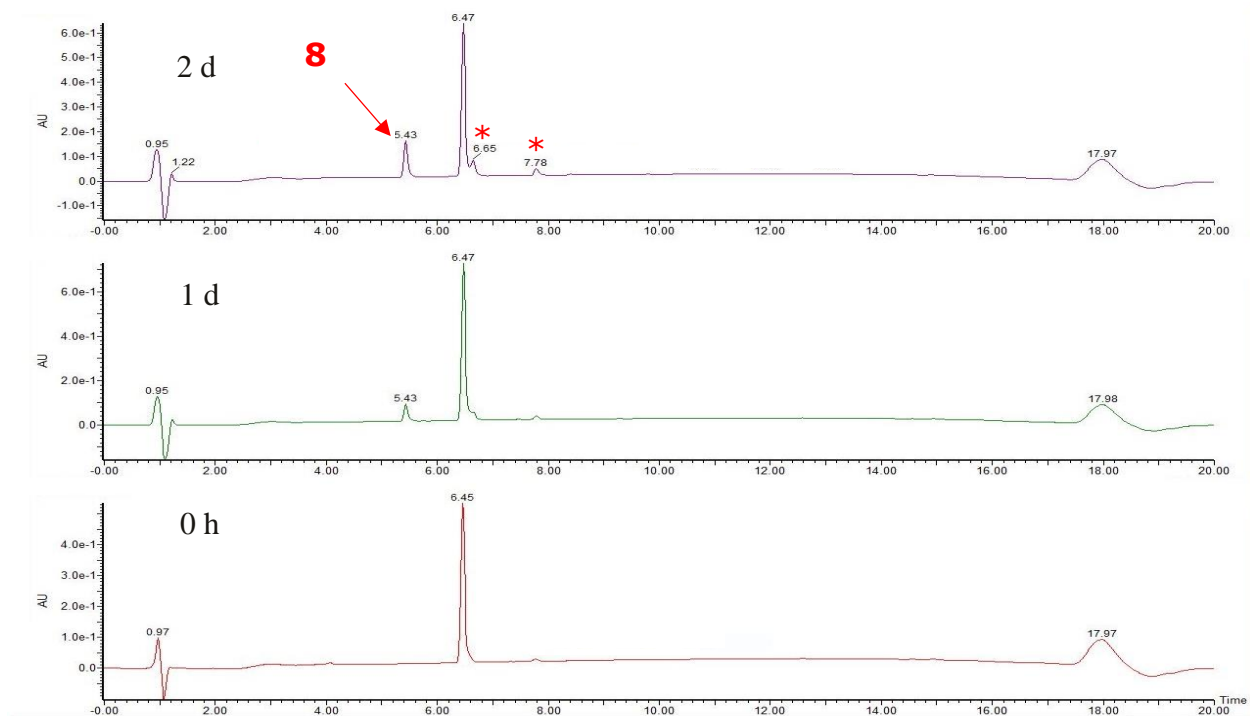
General procedure for screening  $\beta$ -keto ester substrates with peptide hydrazine **1** (product: peptide **9**): A stock solution of  $\beta$ -keto ester (9.0  $\mu$ mol, 750  $\mu$ L MeOH) was prepared before the experiment. A 4 mL vial equipped with a stir bar was charged with peptide hydrazine **1** (1.3 mg, 1.50  $\mu$ mol, 1.0 equiv.), buffer solution (350  $\mu$ L, pH 4.5), MeOH (450  $\mu$ L), and 250  $\mu$ L stock solution of  $\beta$ -keto ester (3.0  $\mu$ mol, 2.0 equiv.) respectively. The reaction mixture was placed on a hot plate stirrer and stirred (150 rpm) at 40  $^{\circ}$ C. Aliquots were taken at indicated time point for LC-MS analysis.

General procedure for screening diketone substrates with peptide hydrazine **1** (products: peptide **10** and **11**): A stock solution of diketone (6.75  $\mu$ mol, 750  $\mu$ L MeOH) was prepared before the experiment. A 4 mL vial equipped with a stir bar was charged with peptide hydrazine **1** (1.3 mg, 1.50  $\mu$ mol, 1.0 equiv.), buffer solution (350  $\mu$ L, pH 7.4), MeOH (450  $\mu$ L), and 250  $\mu$ L stock solution of diketone (2.25  $\mu$ mol, 1.5 equiv.) respectively. The reaction was placed in a gravity convection incubator and incubated at 37  $^{\circ}$ C. Aliquots were taken at indicated time point for LC-MS analysis.

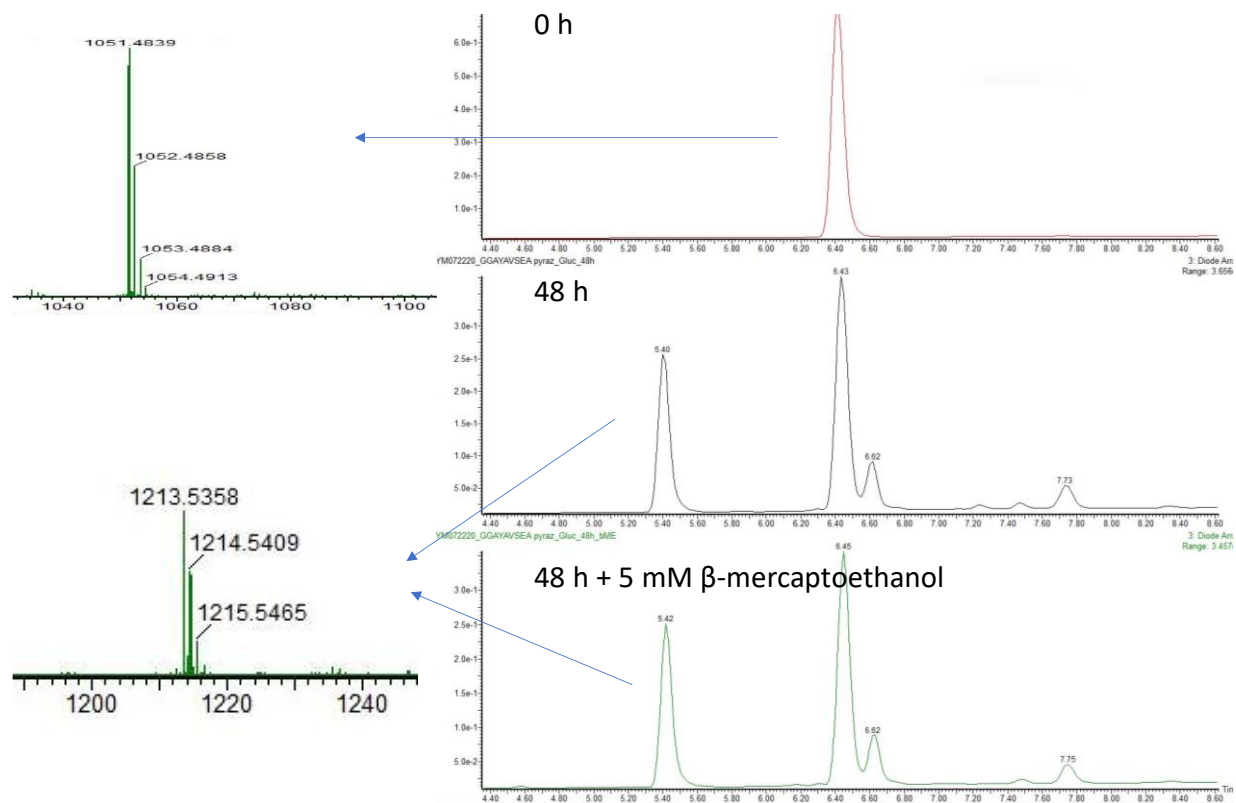
General procedure for reaction of pyrazolone peptide or pyrazole peptide with glucose. A stock solution of glucose (9 mg, 50  $\mu$ mol, 500  $\mu$ L of 50 mM phosphate buffer solution pH 7.4) and pyrazolone or pyrazole (1.0  $\mu$ mol, 2000  $\mu$ L of 50 mM phosphate buffer solution pH 7.4) were prepared respectively before the experiment. A 1.5 mL vial was charged with 200  $\mu$ L stock solution of pyrazolone or pyrazole peptide (0.10  $\mu$ mol, 1.0 equiv.), 50 mM phosphate buffer solution pH 7.4 (750  $\mu$ L), and 50  $\mu$ L stock solution of glucose (5.0  $\mu$ mol, 50.0 equiv.) respectively. The reaction mixture was placed on a vortex for 2 second and placed in a gravity convection incubator and incubated at 37  $^{\circ}$ C. Aliquots were taken for LC-MS analysis at the indicated time point.



**Figure S10.** The UPLC traces of the treating peptide **5** with 5 mM glucose

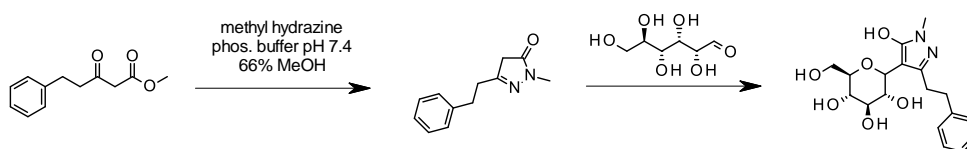


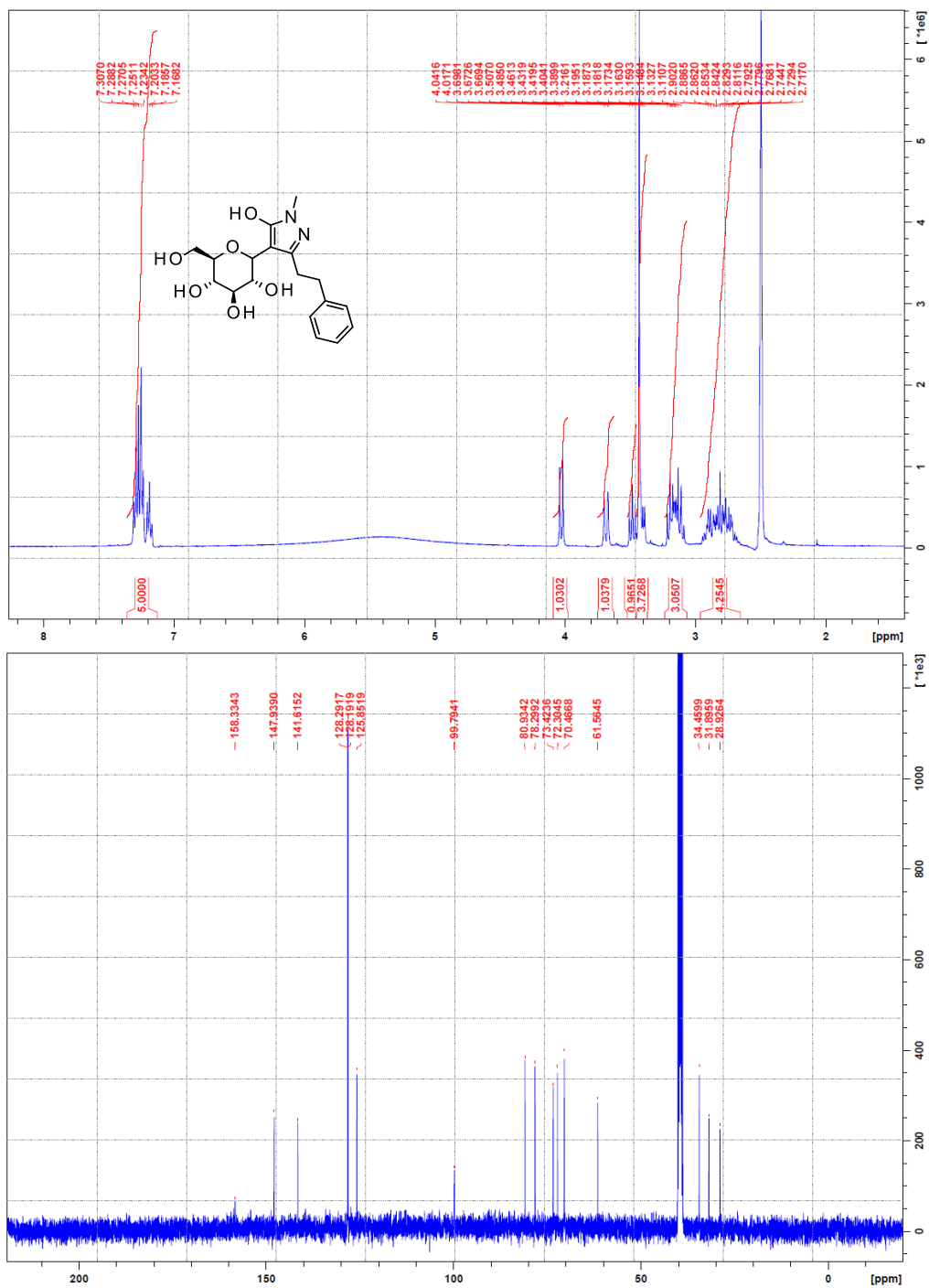
**Figure S11.** The UPLC traces of the treating peptide **7** with 5 mM glucose



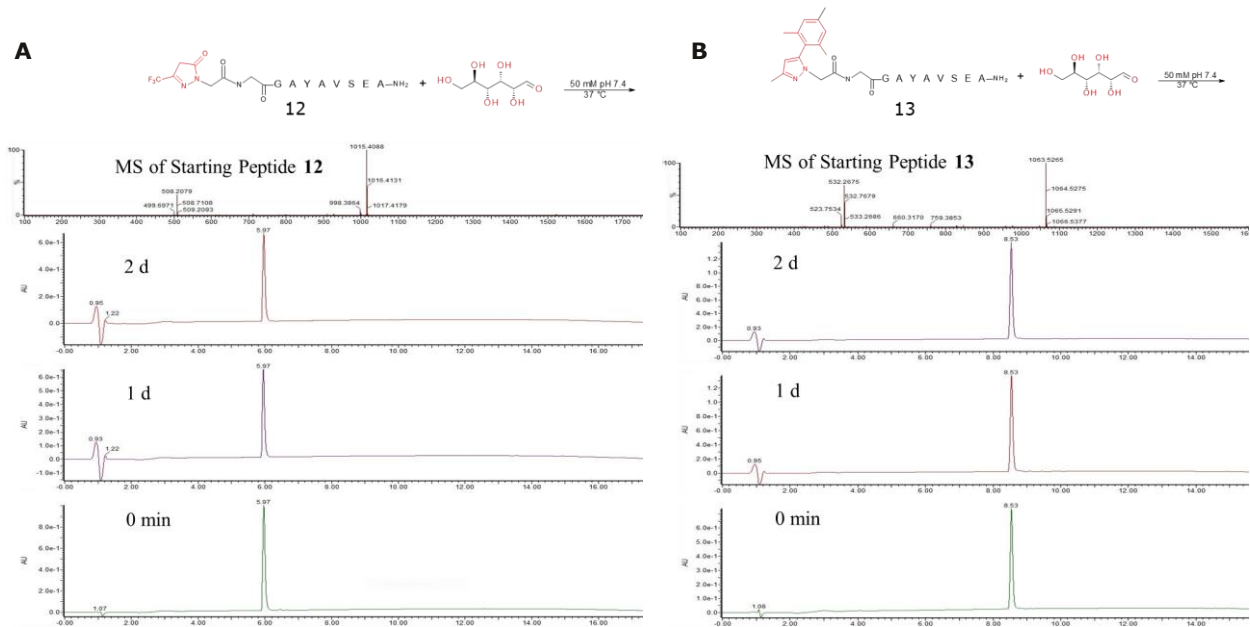
**Figure S12.** Peptide pyrazolone **7** (100  $\mu$ M) and 5 mM glucose were incubated in 50 mM phosphate buffer pH 7.4 for 48 h, then followed by 4 h treatment of 5 mM  $\beta$ -mercaptoethanol

2-methyl-5-(2-phenylethyl)-4-[(3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]-4H-pyrazol-3-one. 10 mL of MeOH and 5 mL of 50 mM phosphate pH 7.4 buffer was added to a 20 mL scintillation vial with stir bar. Methyl 3-oxo-5-phenyl-pentanoate (50 mg, 242  $\mu$ mol) was added with stirring, followed by methyl hydrazine (19  $\mu$ L, 364  $\mu$ mol, 1.5 equiv.). The pyrazolone product was allowed to stir overnight at r.t. 3.6 mL of 1 M D-Glucose in 50 mM phosphate pH 7.4 buffer (3.6 mmol, 10 equiv.) was added and allowed to continue to stir at r.t. for 72 h. The reaction was then diluted with 20 mL of water and purified by reverse-phase preparative HPLC [0-40 min, 0-40% B, flow rate 20 mL/min]. Fractions containing pure product were pooled, evaluated for purity and identity, and lyophilized to provide 14 mg (38  $\mu$ mol, 16% yield) as a fluffy white powder.  $^1\text{H}$  NMR ( $d_6$ -DMSO, 400 MHz)  $\delta$  7.35 – 7.10 (m, 5H), 4.03 (d,  $J$  = 9.8 Hz, 1H), 3.68 (d,  $J$  = 11.7 Hz, 1H), 3.48 (t,  $J$  = 9.3 Hz, 1H), 3.45 – 3.37 (m, 4H), 3.24 – 3.07 (m, 3H), 2.97 – 2.66 (m, 4H).  $^{13}\text{C}$  NMR ( $d_6$ -DMSO, 100 MHz)  $\delta$  158.3, 147.9, 141.6, 128.3, 128.2, 125.9, 99.8, 80.9, 78.3, 73.4, 72.3, 70.5, 61.6, 34.5, 31.9, 28.9. HR-MS (ESI-TOF) calculated for  $\text{C}_{18}\text{H}_{25}\text{N}_2\text{O}_6^+$  [ $\text{M}+\text{H}^+$ ] 365.1707, found 365.1724.



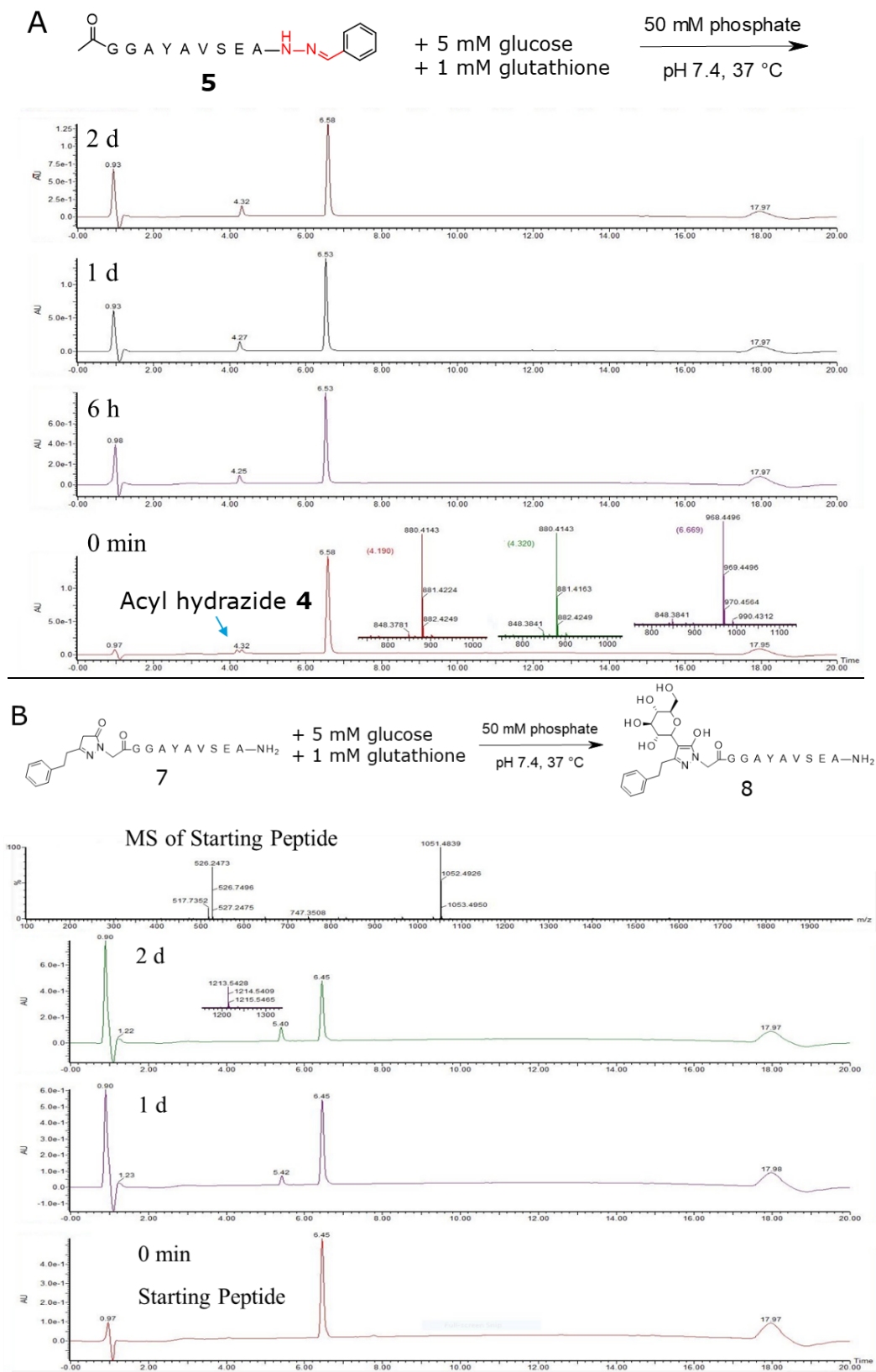


**Figure S13.**  $^1\text{H}$  NMR spectra (400 MHz,  $d_6$ -DMSO) and  $^{13}\text{C}$  NMR spectra (100 MHz,  $d_6$ -DMSO) of the pyrazolone-glucose adduct



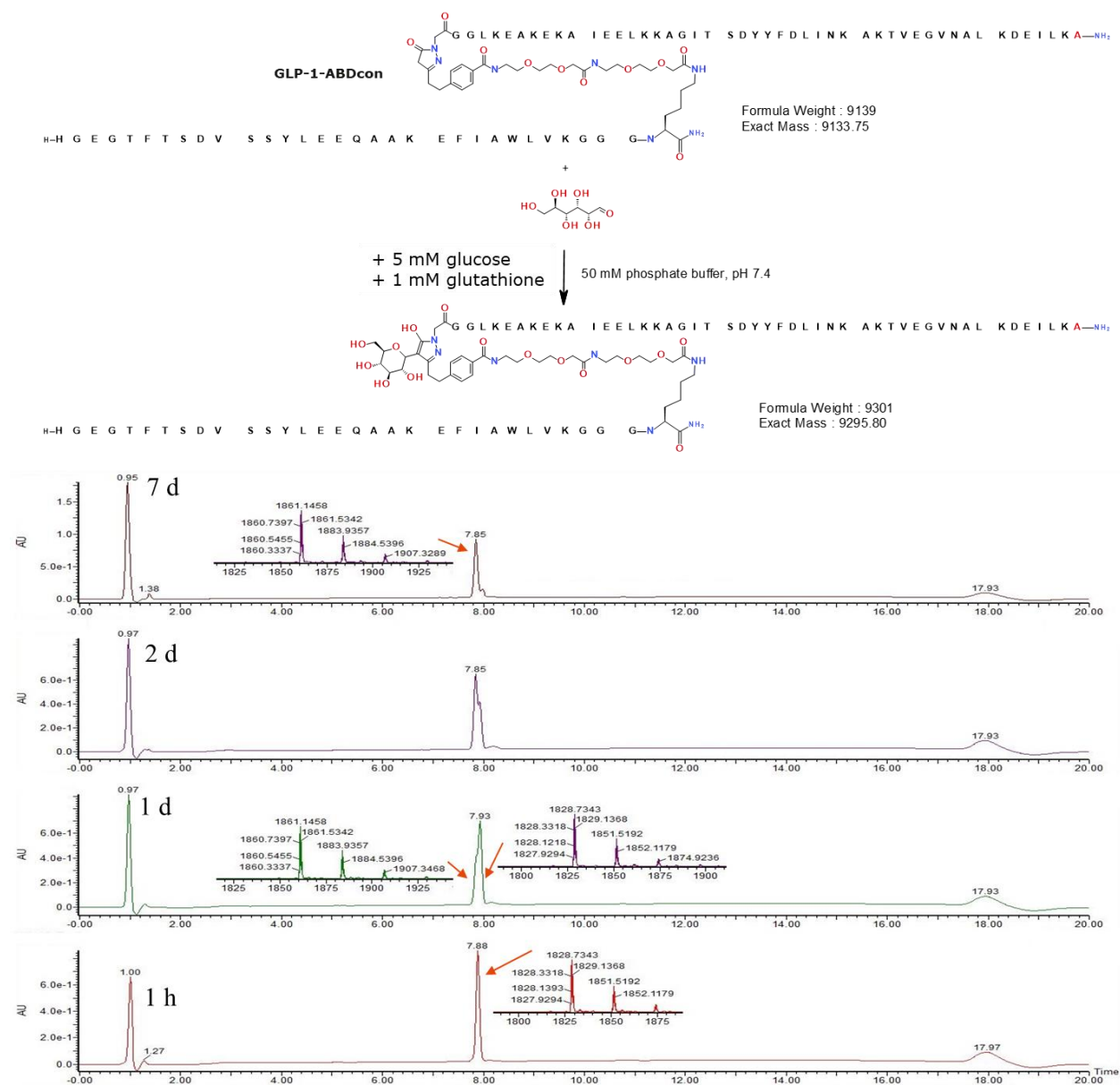
**Figure S14.** UPLC trace of incubating peptides trifluoromethylated hydrazone **12** and pyrazole **13** with 5 mM glucose.

General procedure for reaction of pyrazolone peptide or pyrazole peptide with glucose and reduced glutathione. A stock solution of glucose (9 mg, 50  $\mu\text{mol}$ , 500  $\mu\text{L}$  phosphate buffer solution pH 7.4), GSH (3 mg, 10  $\mu\text{mol}$ , 500  $\mu\text{L}$  of 50 mM phosphate buffer solution pH 7.4) and pyrazolone or pyrazole (1.0  $\mu\text{mol}$ , 2000  $\mu\text{L}$  in 50 mM phosphate buffer solution pH 7.4) were prepared respectively before the experiment. A 1.5 mL vial was charged with 200  $\mu\text{L}$  stock solution of pyrazolone or pyrazole peptide (0.10  $\mu\text{mol}$ , 1.0 equiv.), 50 mM phosphate buffer solution pH 7.4 (700  $\mu\text{L}$ ), 50  $\mu\text{L}$  stock solution of glucose (5.0  $\mu\text{mol}$ , 50.0 equiv.) and 50  $\mu\text{L}$  stock solution of GSH (1.0  $\mu\text{mol}$ , 10.0 equiv.) respectively. The reaction mixture was placed on a vortex for 2 second and placed in a gravity convection incubator and incubated at 37  $^{\circ}\text{C}$ . Aliquots were taken for LC-MS analysis at the indicated time point.



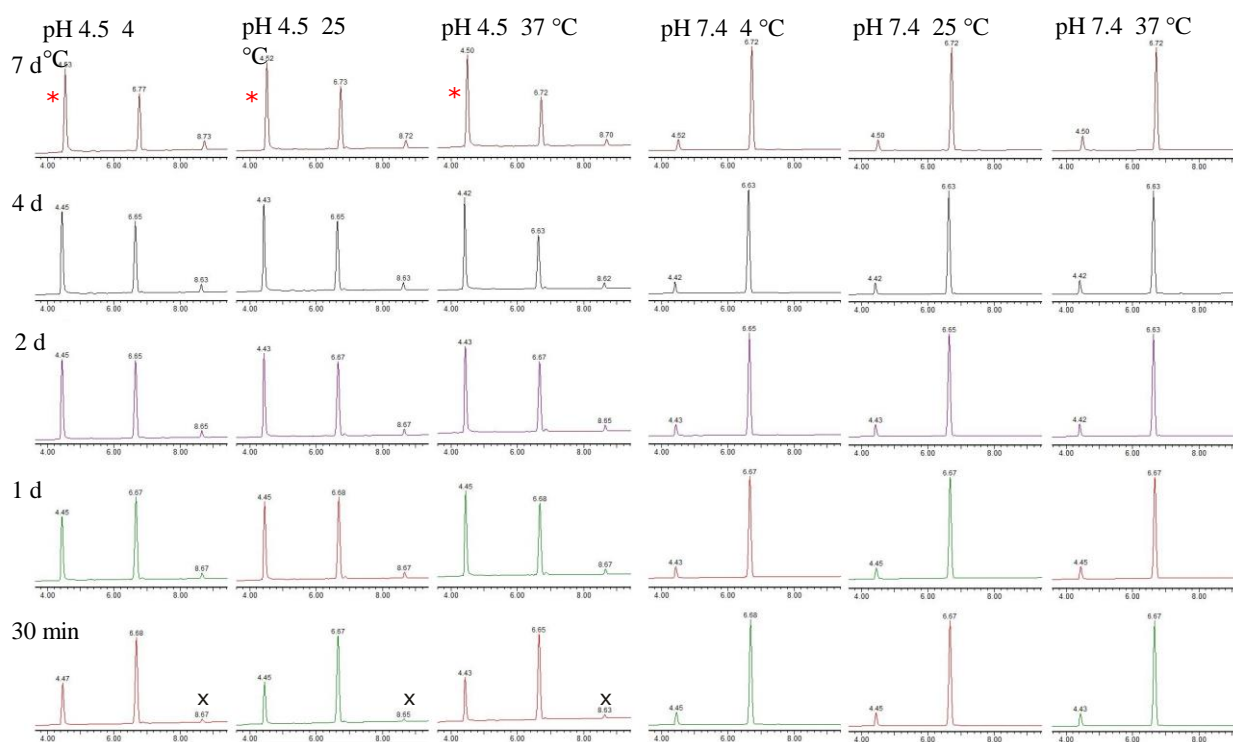
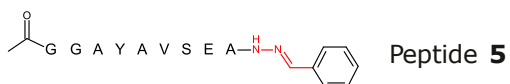
**Figure S15.** UPLC trace of incubating peptides hydrazone **5** (A) and pyrazolone **7** (B) with 5 mM glucose and 1 mM reduced glutathione.





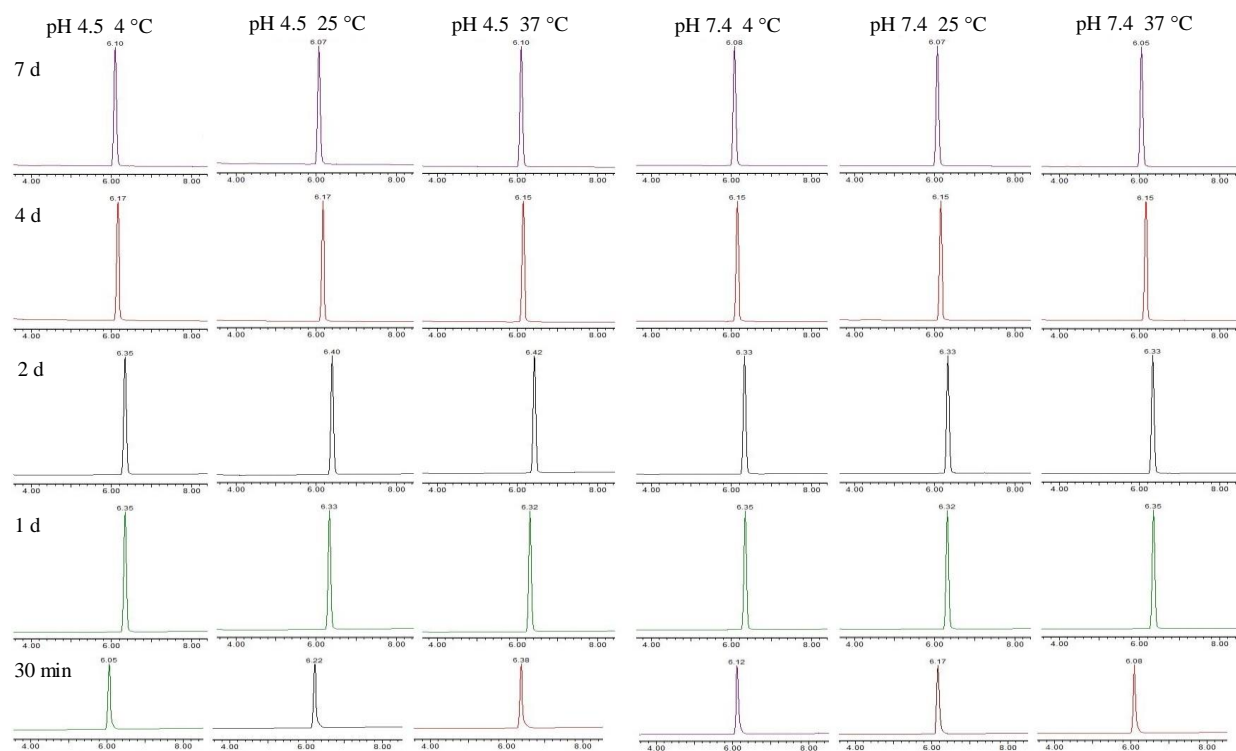
**Figure S17.** UPLC traces for the reaction of peptide GLP1-ABDCon with 5 mM Glucose and 1 mM GSH at neutral pH, 37°C.

**General procedure for chemical stability test.** A 4 mL vial was charged with 1.5 mg of peptide and 50 mM acetate buffer (3 mL, pH 4.5). The solution was mixed by repeatedly pipetting up and down before aliquoting into three 1.5 mL vials. The samples were incubated in dark according to the indicated temperatures (by placing them in a 4 °C refrigerator, a 25 °C bench top, and a 37 °C gravity convection incubator). Aliquots were taken at 30 min, 1 d, 2 d, 4 d, and 7 d. This procedure was repeated using 50 mM phosphate buffer pH 7.4.

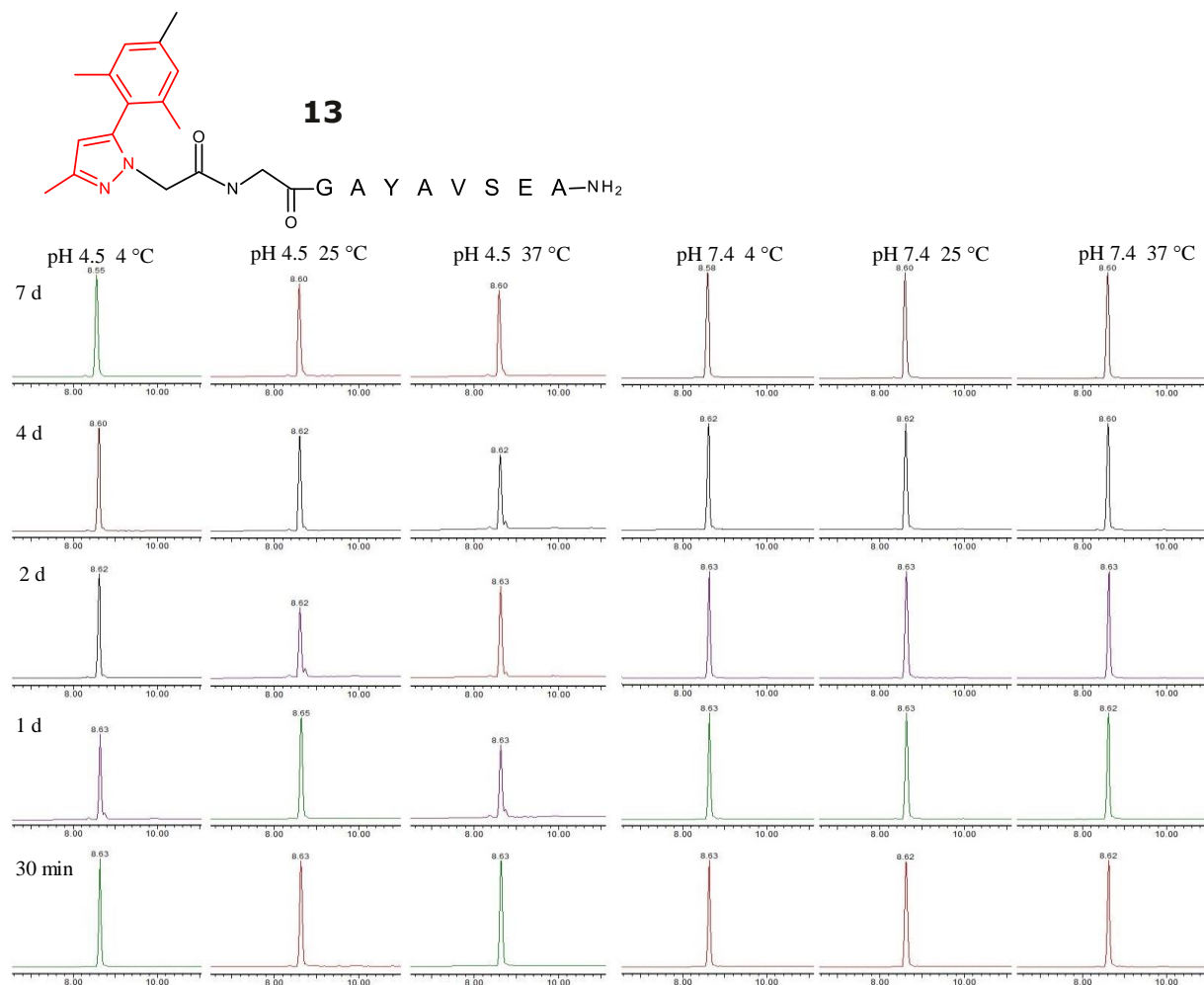


**Figure S18.** The UPLC traces of the chemical stability of peptide 5





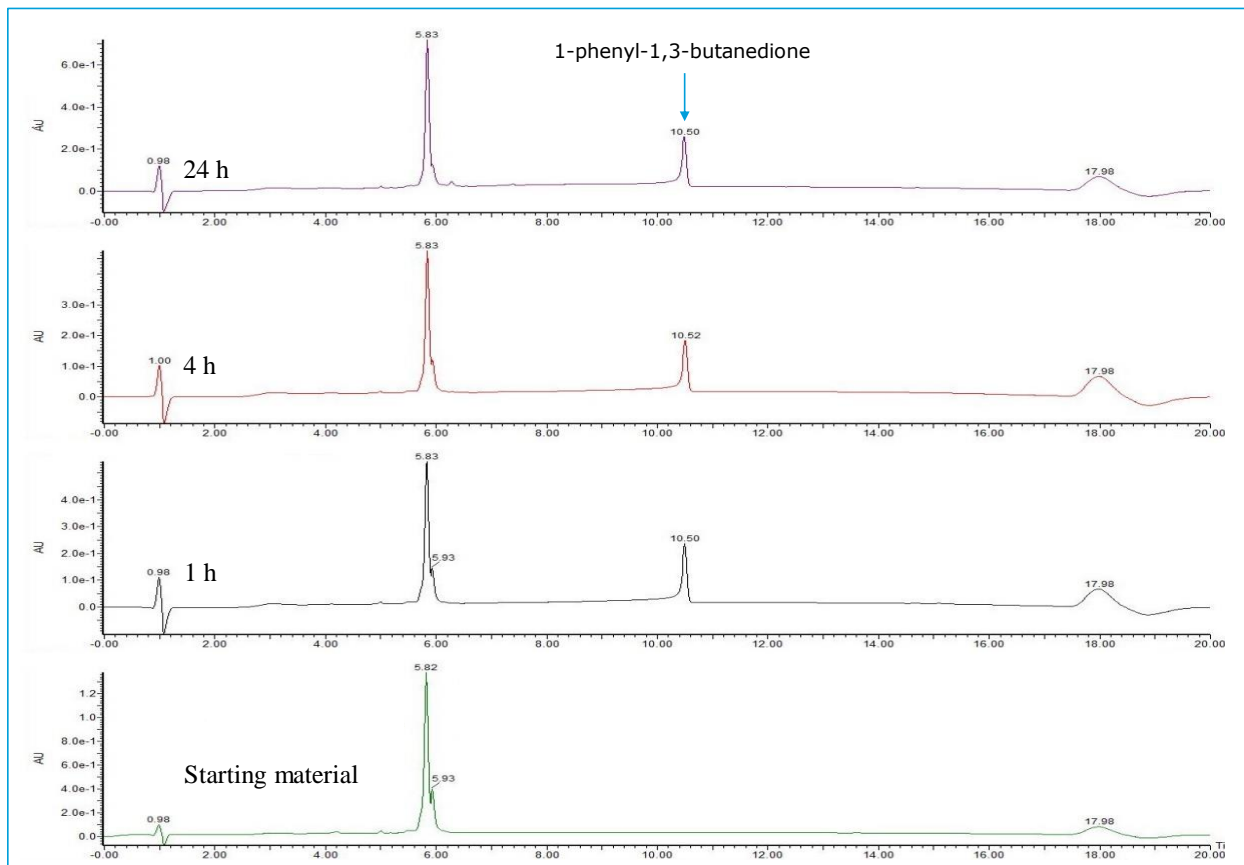
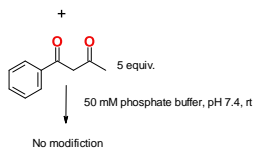
**Figure S21.** The UPLC traces of the chemical stability of peptide **12**



**Figure S22.** The UPLC traces of the chemical stability of peptide **13**

H-S R A H Q H S M E T R T P D I N P A W Y T G R G I R P V G R F-NH<sub>2</sub>

Formula Weight : 3594  
Exact Mass : 3591.81



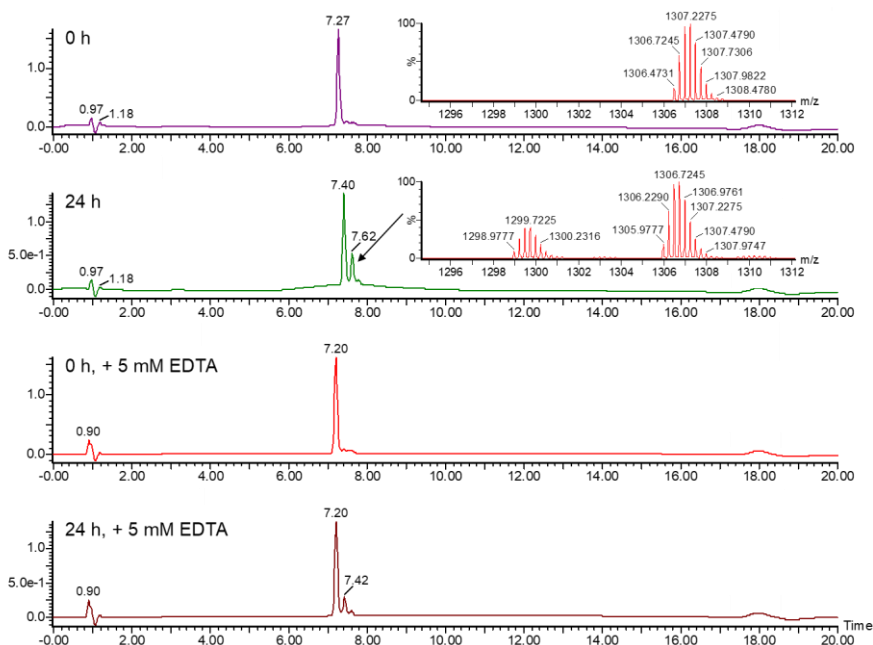
**Figure S23.** The UPLC traces of treating 5 arginine-containing peptide PrRP with 5 equiv. diketone 1-phenyl-1,3-butanedione in a neutral pH buffer rt.

### Conditions for ligation with ABDcon peptide **14**:

**ABDcon N-terminal hydrazine conjugation via pyrazolone ligation.** ABDcon N-terminal hydrazine **14** was dissolved in 50 mM phosphate pH 7.4 buffer at 625  $\mu$ M (3.26 mg/mL). 200  $\mu$ L of this peptide solution was added to an Eppendorf tube, followed by 2.5  $\mu$ L of 100 mM EDTA in water, and 50  $\mu$ L of 5 mM ethyl 4,4,4-trifluoromethylacetoacetate in MeOH. Final concentrations are 500  $\mu$ M peptide **14**, 1 mM reagent, 5 mM EDTA, and 20% MeOH. The ligation was incubated at 37  $^{\circ}$ C in a gravity convection incubator, and aliquots were taken for UPLC-MS analysis at 0 h (prior to reagent addition), 6 h, and 24 h. Conversion to the ligation product **15** was measured by integration of the UV chromatogram at 214 nm. HRMS (ESI-TOF) m/z:  $[M + 4H^+]/4$  Calcd for  $C_{238}H_{392}N_{61}O_{74}F_3^{+4}$  1336.4679; Found 1336.4758.

**ABDcon N-terminal hydrazine conjugation via pyrazole ligation.** ABDcon N-terminal hydrazine **14** was dissolved in 50 mM acetate pH 4.5 buffer at 625  $\mu$ M (3.26 mg/mL). 200  $\mu$ L of this peptide solution was added to an Eppendorf tube, followed by 50  $\mu$ L of 5 mM 1-(2-Mesitylene)-1,3-butanedione in MeOH. Final concentrations are 500  $\mu$ M peptide **14**, 1 mM reagent, and 20% MeOH. The ligation was incubated at 37  $^{\circ}$ C in a gravity convection incubator, and aliquots were taken for UPLC-MS analysis at 0 h (prior to reagent addition), 6 h, and 24 h. Conversion to the ligation product **16** was measured by integration of the UV chromatogram at 214 nm. HRMS (ESI-TOF) m/z:  $[M + 4H^+]/4$  Calcd for  $C_{247}H_{405}N_{61}O_{73}^{+4}$  1348.4958; Found 1348.4926.

**Stability of ABDcon N-terminal hydrazine in neutral buffer.** ABDcon N-terminal hydrazine **14** was dissolved in a 50 mM phosphate pH 7.4 buffer at 600  $\mu$ M (3.13 mg/mL). A separate aliquot of **14** was taken and spiked with 5 mM EDTA (from 100 mM aq. stock). The conditions with and without EDTA were incubated at 37  $^{\circ}$ C in a gravity convection incubator, and samples were taken for UPLC-MS analysis at 0, 6, and 24 h timepoints. Side reactions of the peptide were evaluated by integration of the UV chromatogram at 214 nm.



**Figure S24.** UPLC-MS analysis of the stability of ABDcon N-terminal hydrazine **14** in 50 mM phosphate pH 7.4 buffer with (top two traces) and without 5 mM EDTA (bottom two traces). Hydrazine oxidation (-2 Da) and loss of hydrazine via  $N_2$  (-30 Da) is observed following 24 h, which is lessened by the addition of EDTA.