Supporting Information

Biocompatible and rapid cyclization of peptides with 2,4-difluoro-6-hydroxy-1,3,5-benzenetricarbonitrile for the development of cyclic peptide libraries

Xuejun Zheng, Weidong Liu, Ziyan Liu, Yibing Zhao, and Chuanliu Wu*

Department of Chemistry, College of Chemistry and Chemical Engineering, The MOE Key Laboratory of Spectrochemical Analysis and Instrumentation, Xiamen University, Xiamen, 361005, P.R. China.

*Corresponding Author; Email: chlwu@xmu.edu.cn

Table of Contents

1. Materials and Instruments

Materials: The peptides investigated were supplied by GL Biochem (Shanghai) or Sangon Biotech (Shanghai) at >95% purity. All peptides were N-terminally acetylated and C-terminally amidated and confirmed by analytical liquid chromatogram and MS characterization. 2,4,6-Trifluorobenzene-1,3,5-tricarbonitrile and Silver tetrafluoroborate ($AgBF_4$) was purchased from J&K Scientific (Beijing). Glutathione reduced (GSH), glutathione oxidized (GSSG), acetonitrile (ACN), trifluoroacetic acid (TFA), $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, $NaHCO_3$, Na_2CO_3, and Butylamine was purchased from Sinopharm Chemical Reagent Co.,Ltd (Shanghai). Meta-phosphoric acid (HPO_3, Vetec^{TM} reagent grade), Tween 20 and PEG 8000 were purchased from Sigma-Aldrich (Beijing). Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and 1,3-Bis(bromomethyl)benzene (BBMB) were purchased from Tokyo Chemical Industry (Shanghai). Yeast Extract and Tryptone were purchased from England Oxoid. Tris, Glucose, Bovine Serum Albumin, PMSF, Imidazole, Ampicillin, Kanamycin and Agar were purchased from Sangon Biotech (Shanghai). Sulfo-NHS-LC-biotin and streptavidin-coated magnetic beads (Dynabeads® M-280 Streptavidin, Invitrogen; Cat. No.11205D) were purchased from America Pierce (Nanjing). Isopropyl-β-Dthiogalactopyranoside (IPTG) were purchased from INALCO SPA (Milano, Italy). Anisole and DL-Dithiothreitol (DTT) was purchased from Innochem (Beijing).

Instruments: ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance III instrument (500 MHz). ^{19}F NMR spectrum was recorded on Bruker Avance II instrument (400 MHz). High performance liquid chromatography (HPLC) was performed by a Waters ACQUITY® UPLC HClass system equipped with a quaternary solvent manager (QSM), a sample manager with flow-through needle (SM-FTN), a column manager, and an AQUITY® PDA detector. Chromatograms were recorded by using an AQUITY HPLC® BEH C18 1.7 μm (2.1×100 mm) column at the flow rate of 0.3 mL·min^{-1} by using gradients of ACN +0.1% TFA and water +0.1% TFA. A SCIENTZ JY92-IIN ultrasonic cell disruptor was used to disrupt cells. Fast Protein Liquid Chromatography (FPLC) on a Superdex^{TM} 75

10/300 GL column (GE Healthcare) was used to purify proteins. A Bruker Esquire 3000 plus ion trap ESI mass spectrometry (MS), Bruker autoflex maX MALDI-TOF mass spectrometer and Bruker Impact II QqTOF mass spectrometer were used to identify the products formed.

2. Experimental Procedures

2.1. Synthesis of DFB

2,4,6-Trifluorobenzene-1,3,5-tricarbonitrile is moisture-sensitive. **DFB** was obtained from the immediate and quantitative hydrolysis of 2,4,6-trifluorobenzene-1,3,5-tricarbonitrile by trace water in organic solvents or in aqueous buffers. To prepare a **DFB** stock solution, 20 mg of 2,4,6-trifluorobenzene-1,3,5-tricarbonitrile was dissolved in 200 μL acetonitrile. The stock solution containing **DFB** with a chromatographic purity was then used for cyclization of peptides. DFB was characterized using ^{1}H NMR, ^{13}C NMR, ^{19}F NMR and MS spectrometry (Figure S1).

2.2. Reactions of DFB and GSH

The GSH aqueous stock solution was made fresh and used immediately. The ratio of reactants (**DFB** to GSH) was 1:100. To 90 μL 100 mM phosphate buffer (PB, pH 7.4) containing 50 μM DFB, 10 μL GSH (50 mM) was added. The reaction was incubated at 37 ℃. At pre-defined times, aliquots (20 μL) were taken, quenched with 20 μL of 10% aqueous HPO_3, and analyzed by HPLC (Figure S2).

2.3. General procedures for cyclization of peptides

50 μM peptide and 500 μM **DFB** (10-fold excess) were co-incubated in 100 mM PB (pH 7.4) containing 150 μM TCEP at 37 ℃. TCEP was used to avoid the formation of intramolecular disulfides. The reactions were monitored by HPLC. The products formed were confirmed by ESI mass spectrometry.

2.4. Evaluation of peptide stability in the presence of base, acid or an external thiol nucleophile

The evaluation of the cyclic peptide stability referred to the previous reported methods.[1] Peptide **p1 DFB**-cyclic product (**DFB-p1**) was pre-dissolved in water in an Eppendorf tube to afford the 5 mM stock solution used in the stability evaluation experiments. For each experiment, **DFB-p1** (5 mM; 3 μL) and stability test reagent (297 μL 100 mM in PB, pH 7.4) were combined in an Eppendorf tube and incubated at 37 ℃ for 24 hrs to 96 hrs. After the incubation, the resulting samples were analyzed by HPLC (Figure S5).

Basic condition

Stability test reagent: phosphate buffer (PB, 100 mM, pH 8.0)

Final condition: 50 μM **DFB-p1** in 100 mM phosphate buffer (PB, pH 8.0); 4 d at 37 ℃.

Acidic condition

Stability test reagent: HCl (0.1 M, in H_2O)

Final condition: 50 μM **DFB-p1** in 5 mM HCl (100-fold excess); 4 d at 37 ℃.

External Thiol Nucleophiles: GSH

Stability test reagent: Glutathione (50 mM in 100 mM PB; pH 7.4)

Final condition: 50 μM **DFB-p1** in 250 μM GSH (in 100 mM PB, pH 7.4); 4 d at 37 ℃.

2.5. Stability of peptides cyclized by DFB and BBMB toward oxidation

Peptide **p1** cyclic products (**DFB-p1** and **BBMB-p1**) were pre-dissolved respectively in water in Eppendorf tubes to afford the 5 mM stock solutions and used in the oxidation stability evaluation experiments. For each cyclic peptide (4 μL, 5 mM in H_2O) in an Eppendorf tube, H_5IO_6 (20 μL, 4 mM in H_2O) was added and mixed with a vortexer, then transferred into a preheated water bath at 37 ℃. Each reaction was quenched with Na_2SO_3 (10 μL, 40 mM in H_2O) after 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, and 6 h, the resulting mixtures were kept at room temperature for additional 10 min. Subsequently, the resulting samples were analyzed by UPLC (Figure S5).[1]

Final conditions before quenching: 100 μM **DFB-p1**, 400 μM H_5IO_6, 37 ℃.

2.6. Kinetics of reaction of peptides with DFB or BBMB

50 μM peptide and 50 μM **DFB** (or **BBMB**) were co-incubated in 100 mM PB (pH 7.4) containing 150 μM TCEP at 37 ℃. At pre-defined times, aliquots (20 μL) were taken and quenched with 20 μL of 10% aqueous HPO_3. The reaction was monitored from 0 to 40 min by UPLC (280 nm) to determine the concentration.

2.7. The reaction of bi-cyclization with DFB

50 μM peptide **p7** and 500 μM DFB (10-fold excess) were co-incubated in 100 mM PB (pH 7.4) containing 150 μM TCEP at 37 ℃. TCEP was used to avoid the formation of intramolecular disulfides. The reaction was monitored by HPLC. The products formed were confirmed by ESI mass spectrometry.

2.8. Synthesis of standard products for the identification of bicyclic p7

To identify the crosslink connectivity of bicyclic products of **p7**, standard bicyclic products were synthesized via orthogonal protection strategy. Half of the thiols in **p7** were protected by acetamidomethyl group (Acm), two unprotected thiols reacted with the first **DFB** and formed an monocyclic peptide. The Acm groups were then removed and the remaining two thiols were recovered, followed by reacting with the second **DFB**. The bicyclic **p7** with specific crosslink connectivity were obtained and served as standard samples. The specific connectivity was then assigned by comparing the retention time of the standard samples and those of the bicyclic **p7**. The reaction scheme and chromatograph spectra were shown in Figure S11. The detailed experiment procedures were as follows.

a) Reaction of the first DFB with Acm protected peptide p7

100 μM Acm protected peptide **p7** and 1 mM **DFB** (10-fold excess) were co-incubated in 100 mM PB (pH 7.4) containing 300 μM TCEP at 37 ℃. The cyclic product was purified and collected by HPLC.

b) Removal of the Acm groups

The cyclic peptide from the previous step was dissolved in cold trifluoroacetic acid. Anisole (20 equiv.) was added followed by addition of $AgBF_4$ (40 equiv.). The mixture was

incubated at 4 ℃ for 1.5 h, then cold ether was added. After centrifuge to isolate the precipitate, the silver salt was treated by 1 M acetic acid, to which dithiothreitol (80 equiv.) was then added and mix at room temperature for 3 h. the Acm-free cyclic peptide was purified and collected by HPLC.

c) Reaction of the second DFB with Acm-free cyclic peptide p7

50 μM Acm-free cyclic peptide **p7** obtained from the above steps and 500 μM **DFB** (10-fold excess) were co-incubated in 100 mM PB (pH 7.4) containing 150 μM TCEP at 37 ℃. The bicyclic **p7** with specific crosslink connectivity were obtained and served as standard samples.

2.9. Fluorescence polarization assay

Both fluorescence polarization binding assay and competition assay were performed in 1× PBS, and fluorescence anisotropy was measured in a black opaque 96-well microplate (PerkinElmer) using an Infinite® 200 PRO multimode microplate readers (TECAN). The polarization data were fitted with the equation (1) in Origin 8.5, which was based on one-site competitive model.[2] The inhibition constant K_i was calculated by equation (2).[3]

$$y = A_2 + \frac{A_1 - A_2}{1+10^{(x-logEC50)}} \quad (1)$$

$$K_i = [I]_{50}/(\frac{[L]_{50}}{K_d} + \frac{[P]_0}{K_d} + 1) \quad (2)$$

where x is the logmolar concentration of the **DFB**-cyclized peptides, y is the measured fluorescence anisotropy, A_1 is the top plateaus of anisotropy, A_2 is the bottom plateaus of anisotropy and K_d is the dissociation constant. $[I]_{50}$ denotes the concentration of the **DFB**-cyclized peptides at 50% inhibition, $[L]_{50}$ is the concentration of the free FITC labeled peptides at 50% inhibition and $[P]_0$ is the concentration of the free protein at 0% inhibition.

Fluorescence polarization assay of Bcl-xl and Bcl-2

A peptide with the sequence of CEFLDWEMDGC, which was obtained from the screening of a **DFB**-cyclic peptide library, was synthesized and labeled fluorescently via conjugation

of the β-Ala residue with fluorescein isothiocyanate (FITC) (FITC-βA-CEFLDWEMDGC-NH_2), which was then modified by **DFB**. The stock solutions of **DFB**-cyclized peptides were prepared in DMSO and then diluted stepwise by PBS (The final concentration of DMSO is less than 1%). For the FP binding assay, **F-DFB-M1** solution with the final concentration of 50 nM was added into Bcl-xl protein (or Bcl-2 protein) solution ranging from 20 nM to 1μM. Samples were incubated at room temperature for 10 minutes before measurement. For the FP competition assay, **F-DFB-M1**/Bcl-xl (or **F-DFB-M1**/Bcl-2) complexes (50/500 nM) were prepared and then added the testing peptides ranging from 50 nM to 10 μM. Samples were incubated at room temperature for 10 minutes and measured on a plate reader.

Fluorescence polarization assay of Keap1

A peptide (DEETGEF), which can effectively bind with Keap1, was labeled with FITC via conjugation of the β-Ala residue (FITC-ETGE, FITC-[β-Ala]-DEETGEF). The peptide solution was prepared prior to use by diluting the 1 mM stock solution in DMSO using 1×PBS (The final concentration of DMSO is less than 1%). For the FP competition assay, FITC-ETGE/Keap1 complexes (50/500 nM) were prepared and treated with peptides ranging from 10 nM to 10 μM. Samples were incubated at room temperature for 10 minutes and measured on the microplate readers.

2.10. Protein expression

B cell lymphoma-xl (Bcl-xl)

Recombinant plasmids that contain the DNA region coding for Bcl-xl (human, residues 1-209) with His-SUMO tag were purchased from Zoonbio Biotechnology Co., Ltd (Nanjing, China) and transfected into the *E. coli* strain BL21 (DE3) competent cells. The transformed cells were cultured in LB medium with 50 mg/mL kanamycin at 37 ℃ to logarithmic phase. The protein expression was induced by 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 16 ℃ for 12 hours. Then the cells were harvested, resuspended in lysis buffer (160 mM Tris, 500 mM NaCl, 1 mM PMSF, pH 7.9) and sonicated on ice. The lysate was pelleted by centrifugation, and the protein was purified by

Ni^{2+} Sepharose column with gradient elution and followed by Fast Protein Liquid Chromatography (FPLC) on a Superdex^{TM} 75 10/300 GL column (GE Healthcare). Finally, the purified Bcl-xl protein was quantified by UV-Vis spectrophotometry and stored at −80 ℃ for subsequent use.

B cell lymphoma-2 (Bcl-2)

The DNA region coding for Bcl-2 protein was the same as that used in previous reports,[4] which was composed of residues 1-34 of human Bcl-2, residues 35-50 of human Bcl-xl, and residues 92-207 of human Bcl-2. Recombinant plasmids containing the DNA encoding region with His-SUMO tag were purchased from Zoonbio Biotechnology Co., Ltd (Nanjing, China) and transfected into the *E. coli* strain BL21 (DE3) competent cells. The transformed cells were cultured in LB medium with 50 mg/mL kanamycin at 37 ℃ to logarithmic phase. The protein expression was induced by 0.2 mM isopropyl-β-D- thiogalactopyranoside (IPTG) at 16 ℃ for 12 hours. Then the cells were harvested, resuspended in lysis buffer (160 mM Tris, 500 mM NaCl, 1 mM PMSF, pH 7.9) and sonicated on ice. The lysate was pelleted by centrifugation, and the protein was purified by Ni^{2+} Sepharose column with gradient elution and followed by Fast Protein Liquid Chromatography (FPLC) on a Superdex^{TM} 75 10/300 GL column (GE Healthcare). Finally, the purified Bcl-2 protein was quantified by UV-Vis spectrophotometry and stored at −80 ℃ for subsequent use.

Keap1 kelch domain (Keap1)

Recombinant plasmids that contain the DNA region coding for Keap1 (residues 321-609) with 6×His tag were purchased from Zoonbio Biotechnology Co., Ltd (Nanjing, China) and transfected into the *E. coli* strain BL21 (DE3) competent cells. Keap1 protein was expressed in *E. coli* BL21 (DE3) grown in fresh LB medium containing 100 μg/mL ampicillin and induced by 0.2 mM isopropyl-β-Dthiogalactopyranoside (IPTG) for 12 hours at 16 ℃. Then the cells were harvested and lysed in lysis buffer (160 mM Tris, 500 mM NaCl, 1 mM PMSF, pH 7.9) by sonication on ice. The lysate was pelleted by centrifugation, and the protein was purified by Ni^{2+} Sepharose column with gradient elution by varying the concentration of imidazole, followed by Fast Protein Liquid

Chromatography (FPLC) on a Superdex^{TM} 75 10/300 GL column (GE Healthcare). Finally, the purified Keap1 protein was quantified by UV-Vis spectrophotometer and stored at −80 ℃ for subsequent use.

2.11. Cyclization of phage library

The phage library was produced and purified as described previously.[5] To 480 μL phage solution of 10^{13} *pfu*/mL in a 1.5 mL microcentrifuge tube (Eppendorf, Germany), 10 μL of 50 mM TCEP was added, the tube was treated under vortexes for several seconds and incubated at 37 °C with shaking for 30 min. Then 10 μL of 50 mM **DFB** in water was added into the reduced phage solution. The reaction was mixed with vortexes for several seconds and incubated at 37 ℃ with shaking for 1 hour. Then 100 μL ice-cold PEG/NaCl solution was added to precipitate the phages. The tube was inverted several times and placed on ice for 1 hour. The phage pellet was re-suspended in 1 mL 1×PBS after centrifuging, and the supernatant was discarded. The re-suspended phage was diluted and allowed to infect the TG1 cells in exponential growth period to monitor the phage titer. Finally, the modified phage solution was stored at 4 ℃ for the phage selection.

2.12. Phage bio-panning

The target proteins were biotinylated as previously described.[5] 100 μL streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Invitrogen, Thermo Fisher Scientific; neutravidin-coated magnetic beads for the second round) were washed three times with binding buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) in a 1.5 mL microcentrifuge tube and the washed beads were re-suspended completely (the tube was removed from the magnet) with 100 μL binding buffer, followed by distributing into two 1.5 mL microcentrifuge tubes equally. Then the biotinylated target protein (2-10 μg; 1st round 10 μg, 2nd round 5 μg and 3rd round 2μg) was added to one of the two microcentrifuge tubes and the same volume of 1×PBS (without protein) was added to the other one. The tubes were incubated on a slowly rotating wheel for 15 minutes at room temperature. These beads were washed three times with the binding buffer to remove

unbounded proteins and re-suspended with 300 μL binding buffer together with 150 μL blocking buffer (binding buffer with 0.3% v/v Tween-20 and 3% w/v BSA), then incubated on a slowly rotating wheel at room temperature for 2 hours. In parallel, the modified phage library (>10^{12} t.u.) dissolved in 3 mL binding buffer was blocked by addition of 1.5 mL blocking buffer and incubated on a slowly rotating wheel at room temperature for 2 hours. Then the blocked phages were split equally into two 10 mL tubes, into which the blocked beads with and without the immobilized proteins added, respectively. The two tubes were incubated on a slowly rotating wheel at room temperature for 30 minutes. After that, the unbound phages in the supernatant were removed (the co-incubated solution were transferred to 1.5 mL tubes placed in a magnet). The beads were washed for nine times with the washing buffer (binding buffer with 0.1% v/v Tween-20) and twice with binding buffer. During the period, the tubes were replaced at least three times. The buffer was removed completely in the last washing step, and then 200 μL elution buffer (50 mM glycine, pH 2.2) was added to elute the phages that bounded on the beads by re-suspending the beads on a mini-vortex finder and incubated for 5 minutes. Then the supernatant was transferred into a 1.5 mL microcentrifuge tube containing 50 μL neutralization buffer (1 M Tris-HCl, pH 8.0). The eluted phage was diluted to infect exponential TG1 cells for monitoring the phage titer, followed by amplification and purification.

2.13. Surface plasmon resonance (SPR) assays

SPR assays were performed using a Biacore T200. Proteins (Sumo-Bcl-xl and Sumo-Bcl-2) were immobilized on the chip surface using standard amine-coupling procedures as instructed by the Biacore sensor surface handbook. Proteins (1 μM) were dissolved 10 mM acetate buffer at pH 4.5. 10 mM Phosphate buffer saline (PBS) with 0.05 % Tween 20 and 0.05 mM EDTA was used as the running buffer. Flow cell Fc2 was activated with a mixture of EDC and NHS. Proteins were then coupled to reach a target response of 1000 RU. Ethanolamine was then passed over the surface to deactivate the excess esters. Flow cell Fc1 not immobilized with proteins was used the reference channel. Serially diluted samples (0, 80, 160, 320, 640, 1280, 2560, 5120 and 1280 nM for **DBF-M1**; 0, 320, 640, 1280,

2560, 5120, 10240 and 1280 nM for reduced and oxidized M1) were passed over the flow cells (Fc1 and Fc2) at a flow rate of 30 μL/min. For data analysis, all of the sensorgrams were processed using double referencing. Kinetics data for the binding of **DFB-M1** with Bcl-xl were analyzed using a 1:1 binding model and local fit to obtain kinetic rate constant (K_a and K_d). The dissociation constant can then be determined by K_d/K_a). For oxidized M1, kinetic fit to the data is impossible because the binding and dissociation is too fast. The dissociation constant is thus determined using steady state affinity fit. The reproducibility of the assays was confirmed by performing replicate determinations.

3. Results

Table S1 MS data of peptides

Name	m/z expected	m/z found
p1	779.3082 ($[M+H]^+$)	779.3093[#]
DFB-p1	944.3050 ($[M+H]^+$)	944.3218[#]
p2	1675.7902 ($[M-H]^-$)	1675.6429[#]
DFB-p2	921.8998 ($[M+2H]^{2+}$)	921.8[&]
p3	1095.4716 ($[M+H]^+$)	1095.4933[#]
DFB-p3	630.7382 ($[M+2H]^{2+}$)	630.7514[#]
p4	656.7448 ($[M+2H]^{2+}$)	656.7563[#]
DFB-p4	738.2432 ($[M+2H]^{2+}$)	738.2573[#]
p5	701.3586 ($[M+2H]^{2+}$)	701.3567[#]
DFB-p5	783.8572 ($[M+2H]^{2+}$)	783.8741[#]
p6	787.8700 ($[M+2H]^{2+}$)	787.8708[#]
DFB-p6	870.3684 ($[M+2H]^{2+}$)	870.3928[#]

#: obtained on a Bruker Esquire 3000 Plus mass spectrometer

&: obtained on a Bruker Impact II QqTOF mass spectrometer

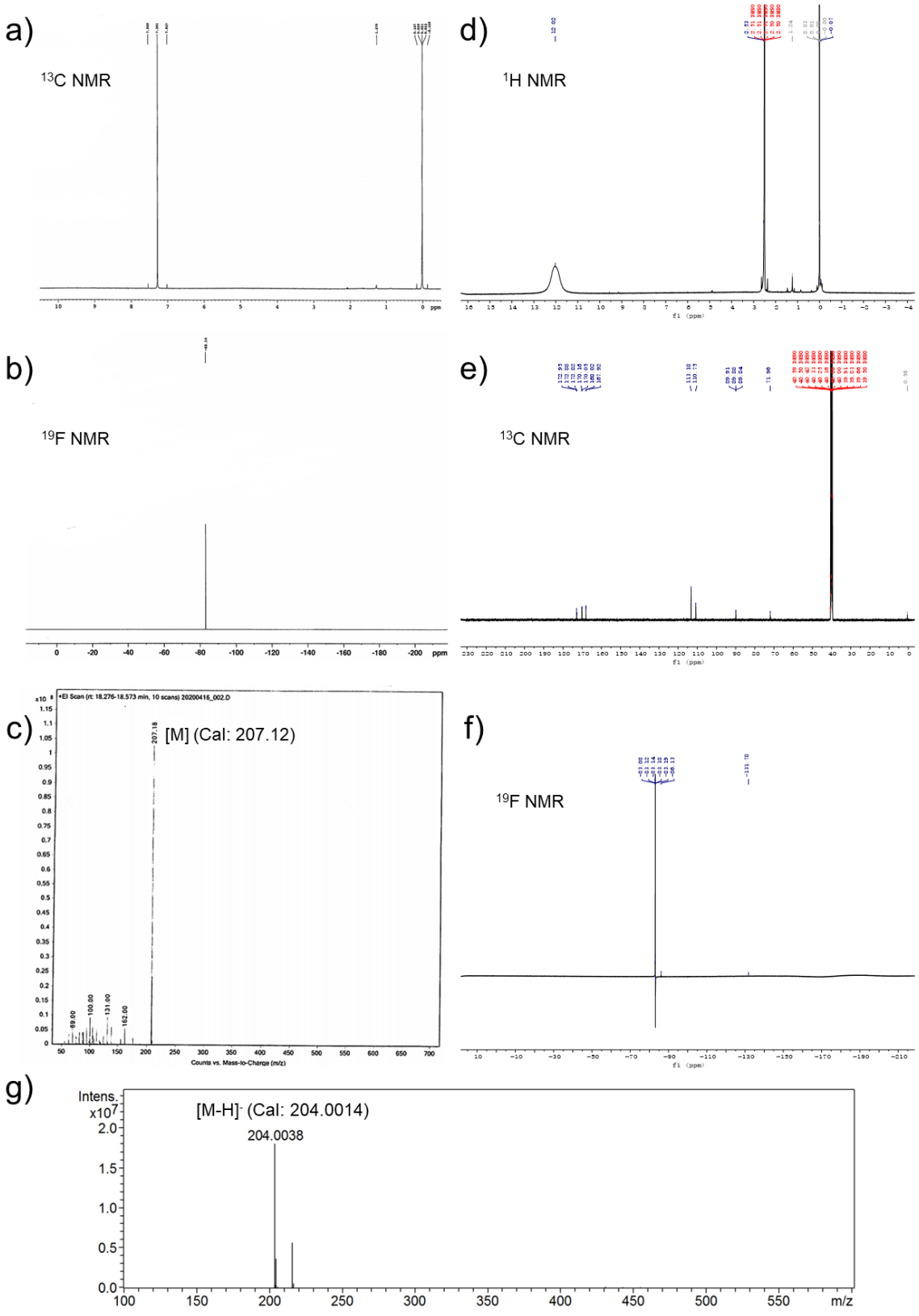

Figure S1. (a)-(c) ^{13}C NMR, 19F NMR and MS characterization of 2,4,6-trifluorobenzene-1,3,5-tricarbonitrile, provided by J&K Scientific (Guangzhou). (d)-(g) ^{1}H NMR, ^{13}C NMR, ^{19}F NMR and MS characterization of **DFB**.

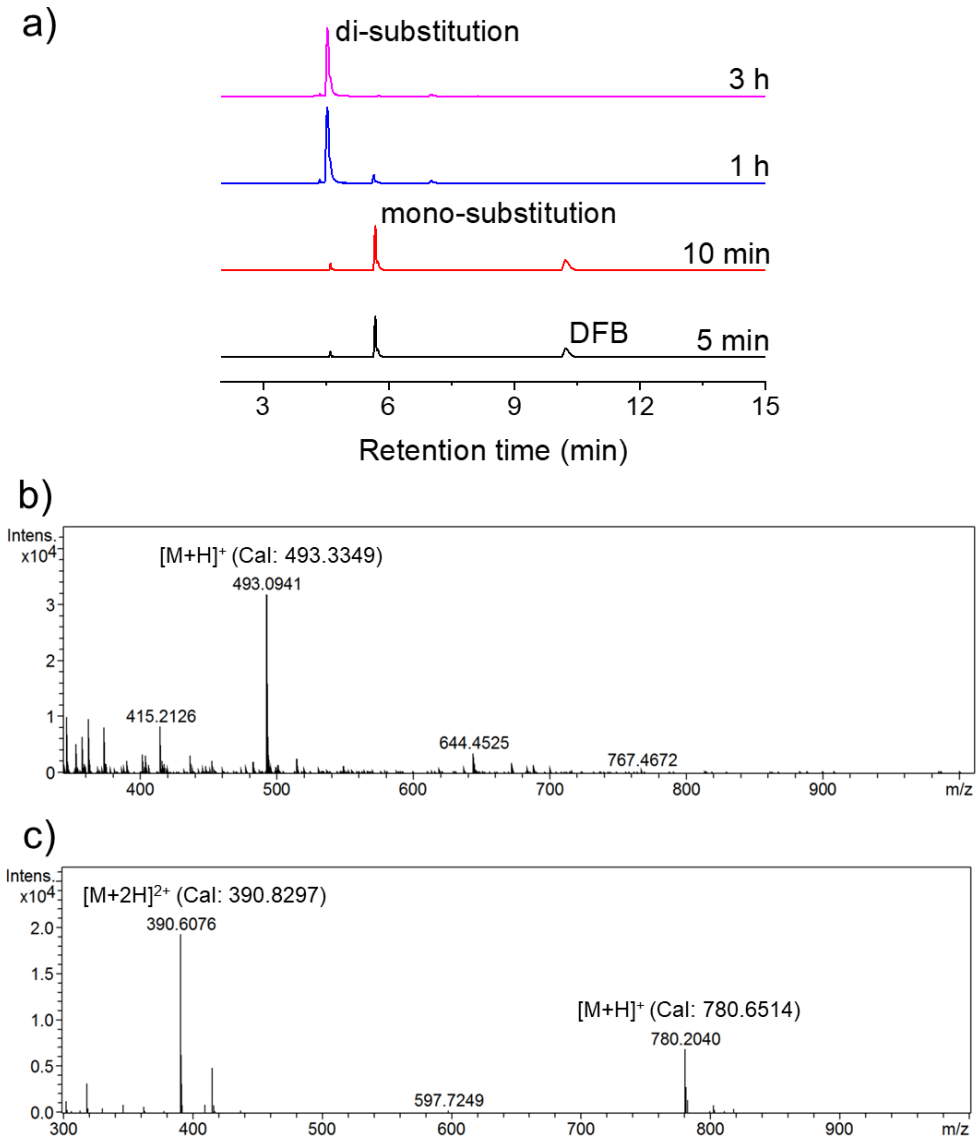

Figure S2. (a) Chromatograms (280 nm) of the reaction of **DFB** and GSH with the ratio of 1:100 after different reaction time. (b) MS characterization of the UPLC peak at 5.8 min, indicating the formation of mono-substituted product. (c) MS characterization of the UPLC peak at 4.8 min, indicating the formation of di-substituted product.

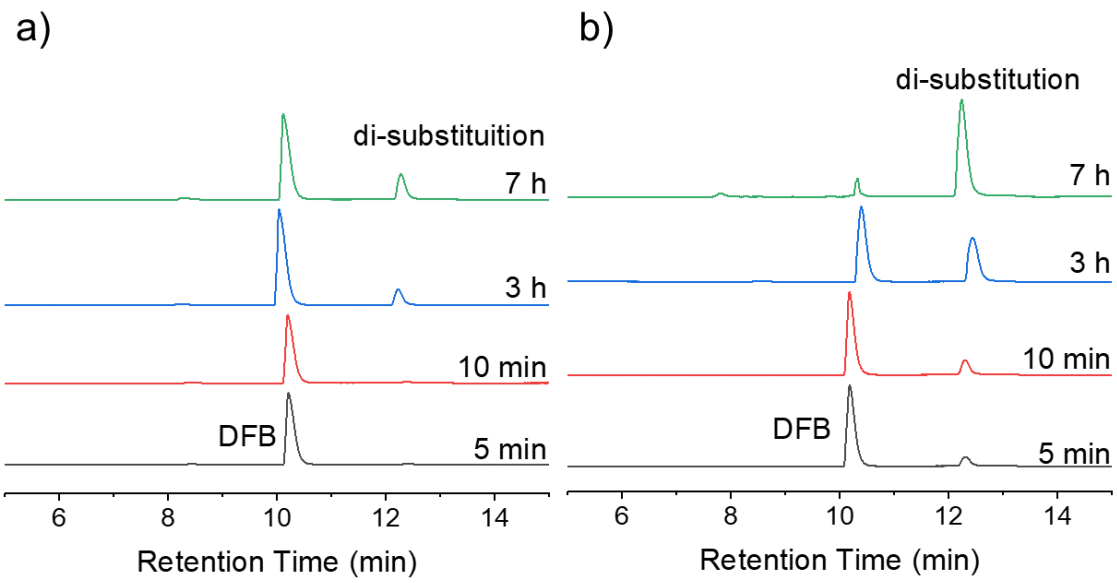

Figure S3. (a) Chromatograms (280 nm) of the reaction of **DFB** and butylamine under neutral conditions (pH 7.4) with the ratio of 1:100 after different reaction time. (b) Chromatograms (280 nm) of the reaction of **DFB** and butylamine under basic conditions (pH 9.2) with the ratio of 1:100 after different reaction time.

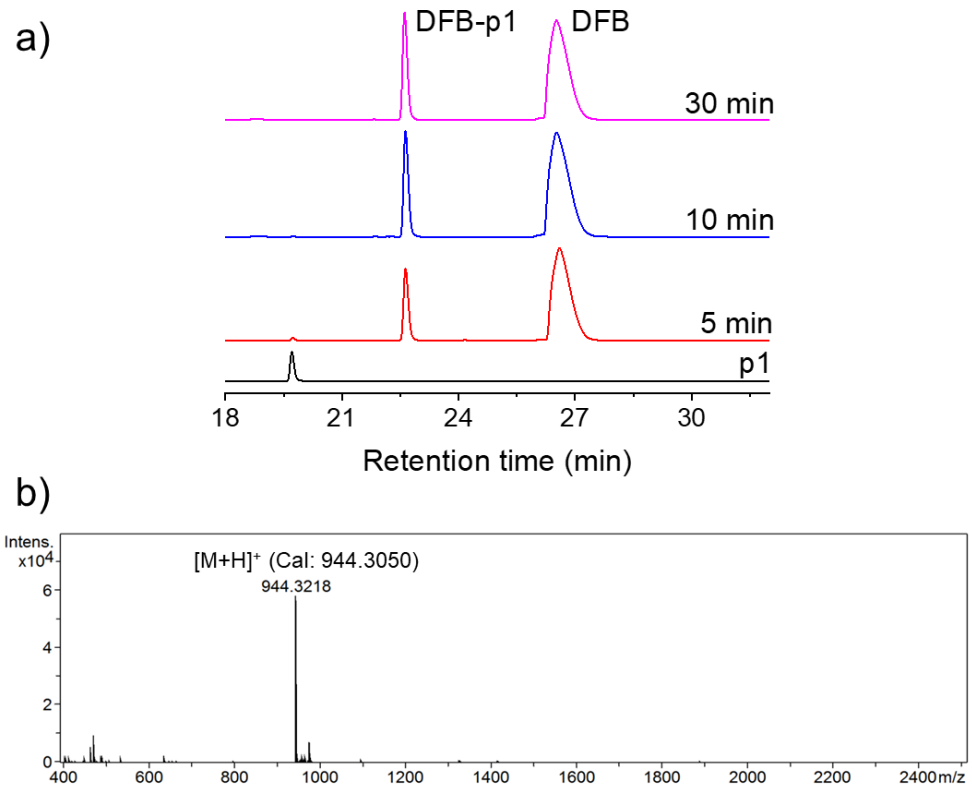

Figure S4. (a) Chromatograms (280 nm) of the reaction of **DFB** and **p1** with the ratio of 10:1 for different reaction time. The reaction was incubated in PB (100 mM, pH 7.4) at 37 ℃. (b) MS characterization of **DFB-p1**.

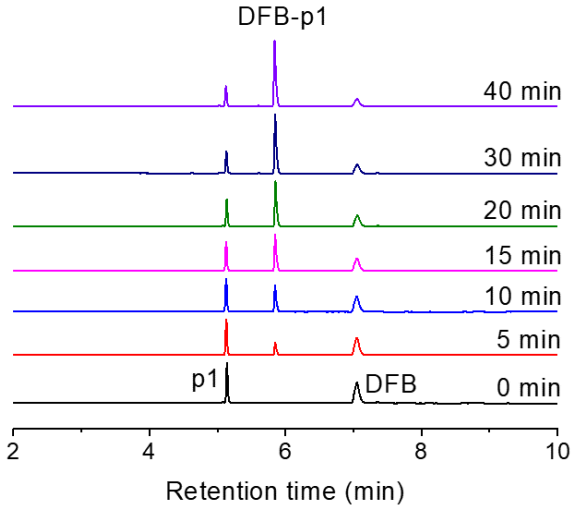

Figure S5. Kinetics of reaction between **DFB** (50 μM) and **p1** (50 μM) in PB (pH 7.4) was monitored by HPLC (280 nm).

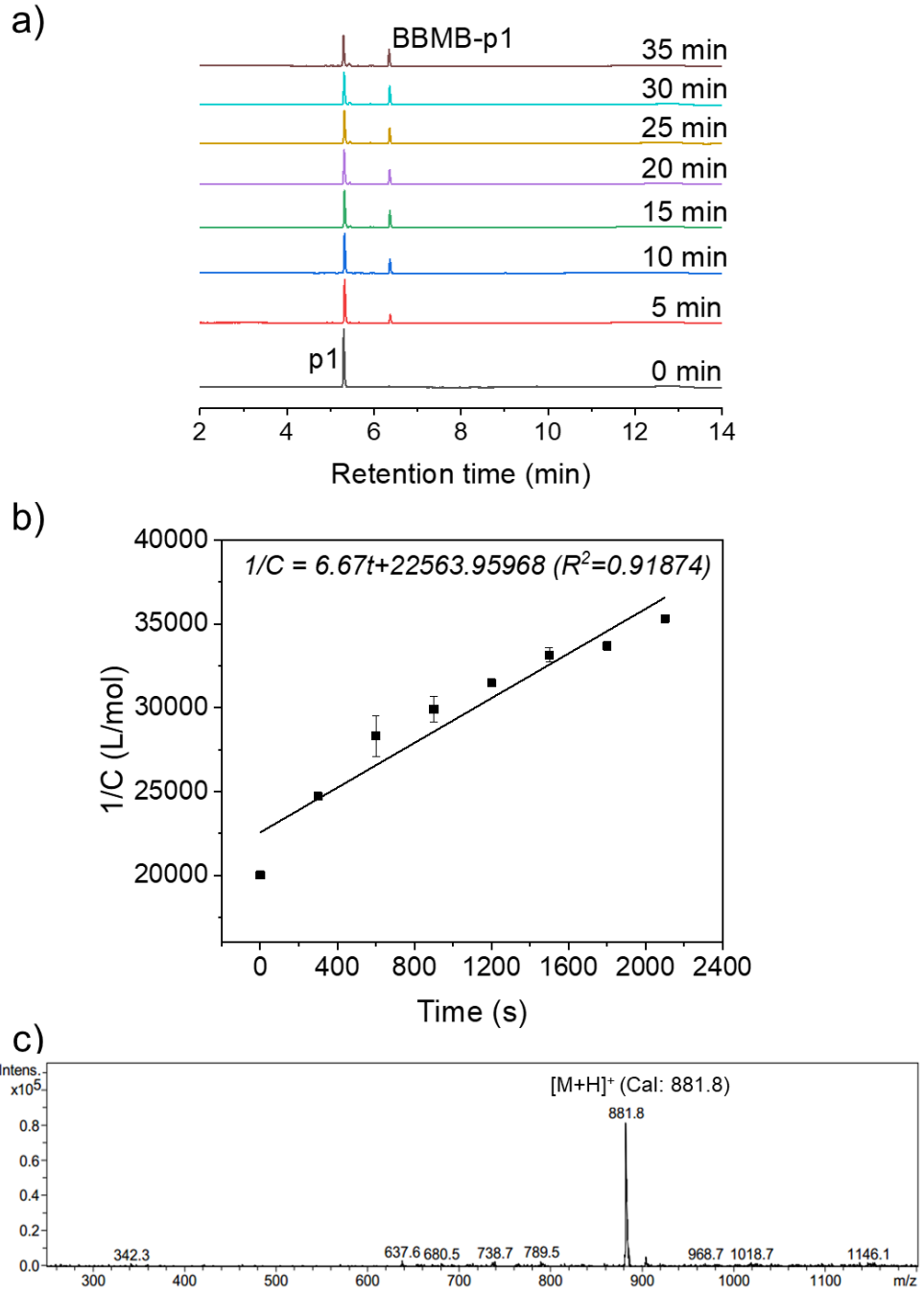

Figure S6. (a) UPLC chromatograms showing the reaction between **BBMB** and **p1**. (b) Curve of t~1/C; data are presented as mean ± s.d. (n=3). To determine second order rate constant, **p1** (50 μM) was reacted with **BBMB** (50 μM) in phosphate buffer (pH 7.4) at 37 ℃. The reaction was monitored from 0 to 40 min by HPLC (280 nm) to determine the concentration (C) of **BBMB-p1**. 1/C was plotted against time (t) to calculate second-order rate constant as 6.67 $M^{-1}s^{-1}$. (c) MS characterization of **BBMB-p1**.

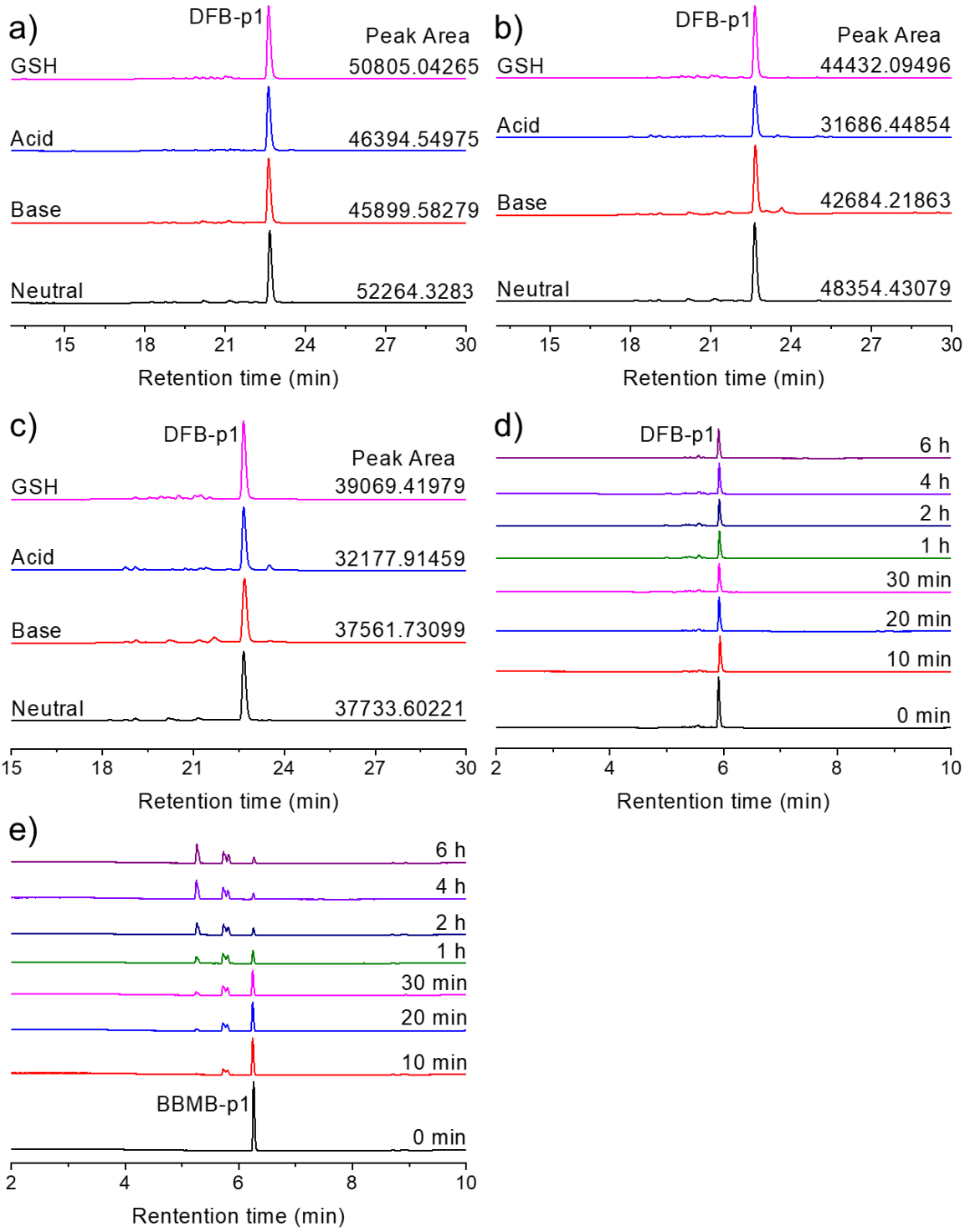

Figure S7. Stability of the cysteine conjugates in the presence of base, acid, external thiol nucleophiles, and under oxidative conditions (a) The stability in different environment after 24 h, (b) 48 h, and (c) 96 h. (d) The oxidation stability of **DFB-p1**. (e) The oxidation stability of **BBMB-p1**. **DFB-p1** stayed stable in the 6-hour testing period, while **BBMB-p1** started to be decomposed after 10 min.

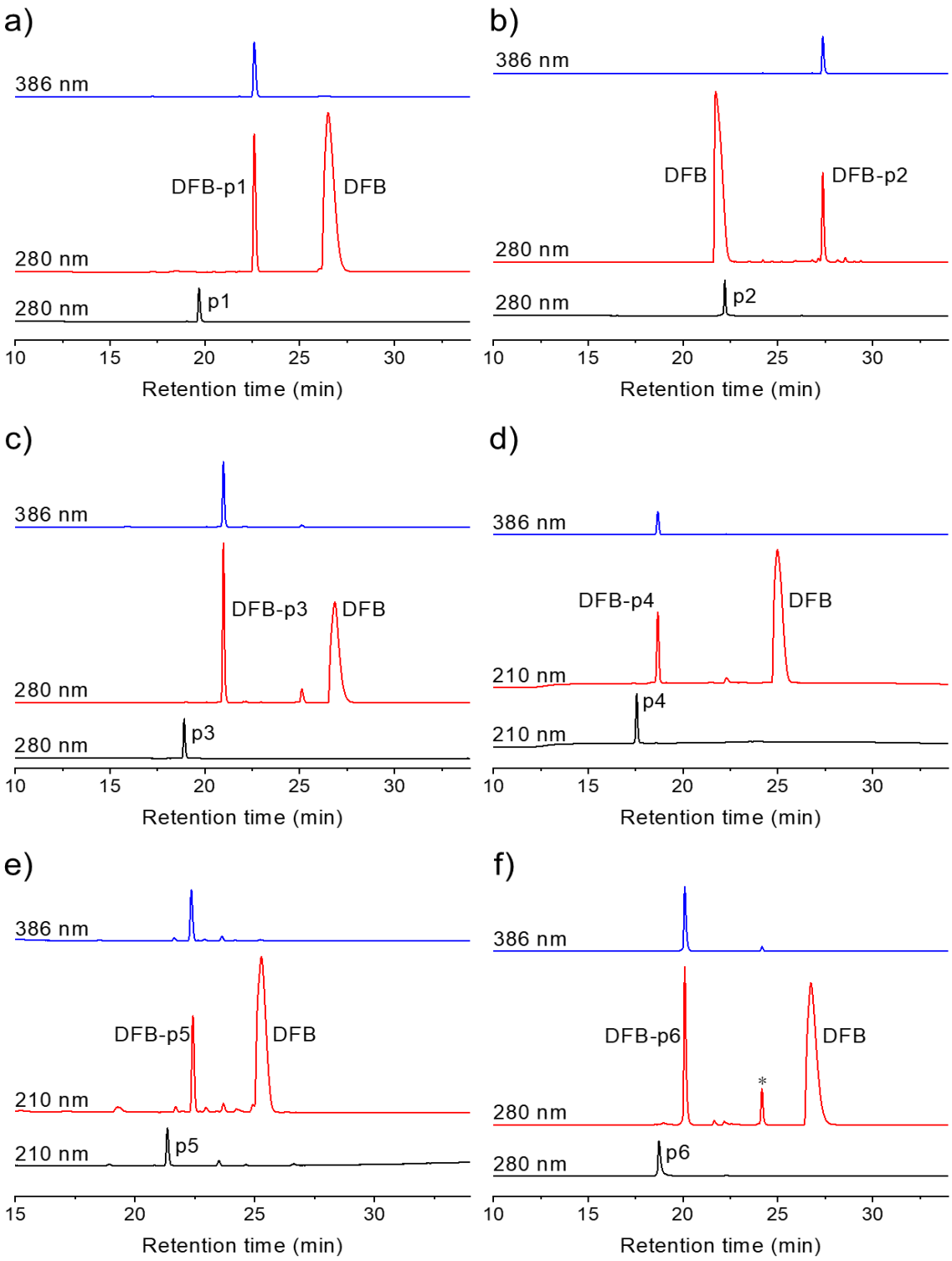

Figure S8. (a)-(f) Chromatograms of the reactions between peptides (**p1-p6**) of different number of space residues with **DFB**. The sequences of **p1-p6** and the space number of residues were shown in Figure 2d. *represents the mono-substituted intermediate (characterized by mass spectrometry; m/z: $[M+2H]^{2+}$ Calcd for $C_{74}H_{105}FN_{26}O_{20}S_2$: 880.3414; Found 880.48).

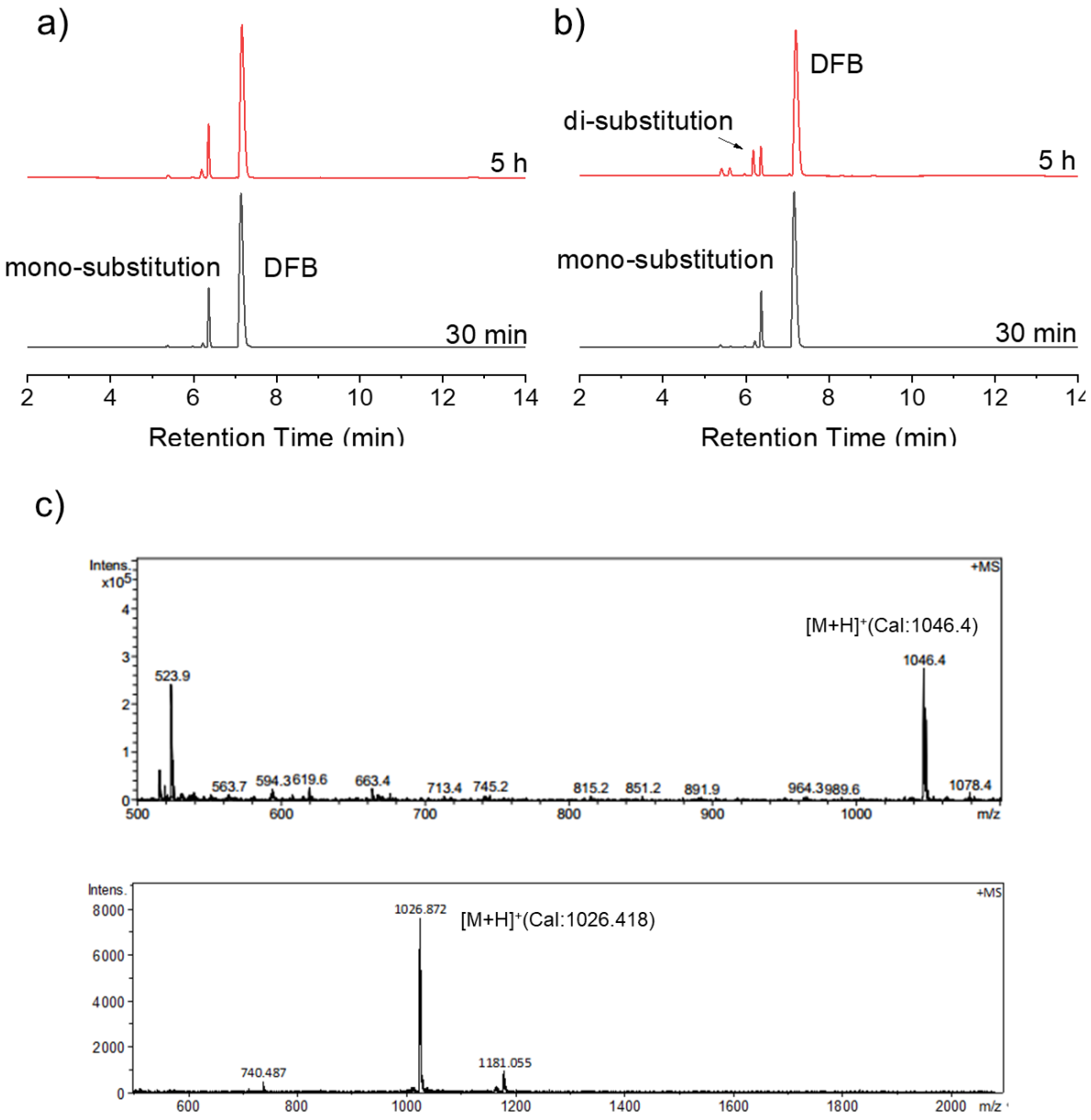

Figure S9. (a) Chromatograms (280 nm) of the reactions of **DFB** and peptide (AcNH-GGKGRGCW-NH_2) with the ratio of 10:1 in phosphate buffer (pH 7.4) at 37 ℃. (b) Chromatograms (280 nm) of the reactions of **DFB** and the peptide with the ratio of 10:1 in phosphate buffer (pH 8.0) at 37 ℃. (c) MS characterization of the UPLC peak at 6.4 min, indicating the formation of mono-substituted product. (d) MS characterization of the UPLC peak at 6.2 min, indicating the formation of di-substituted product.

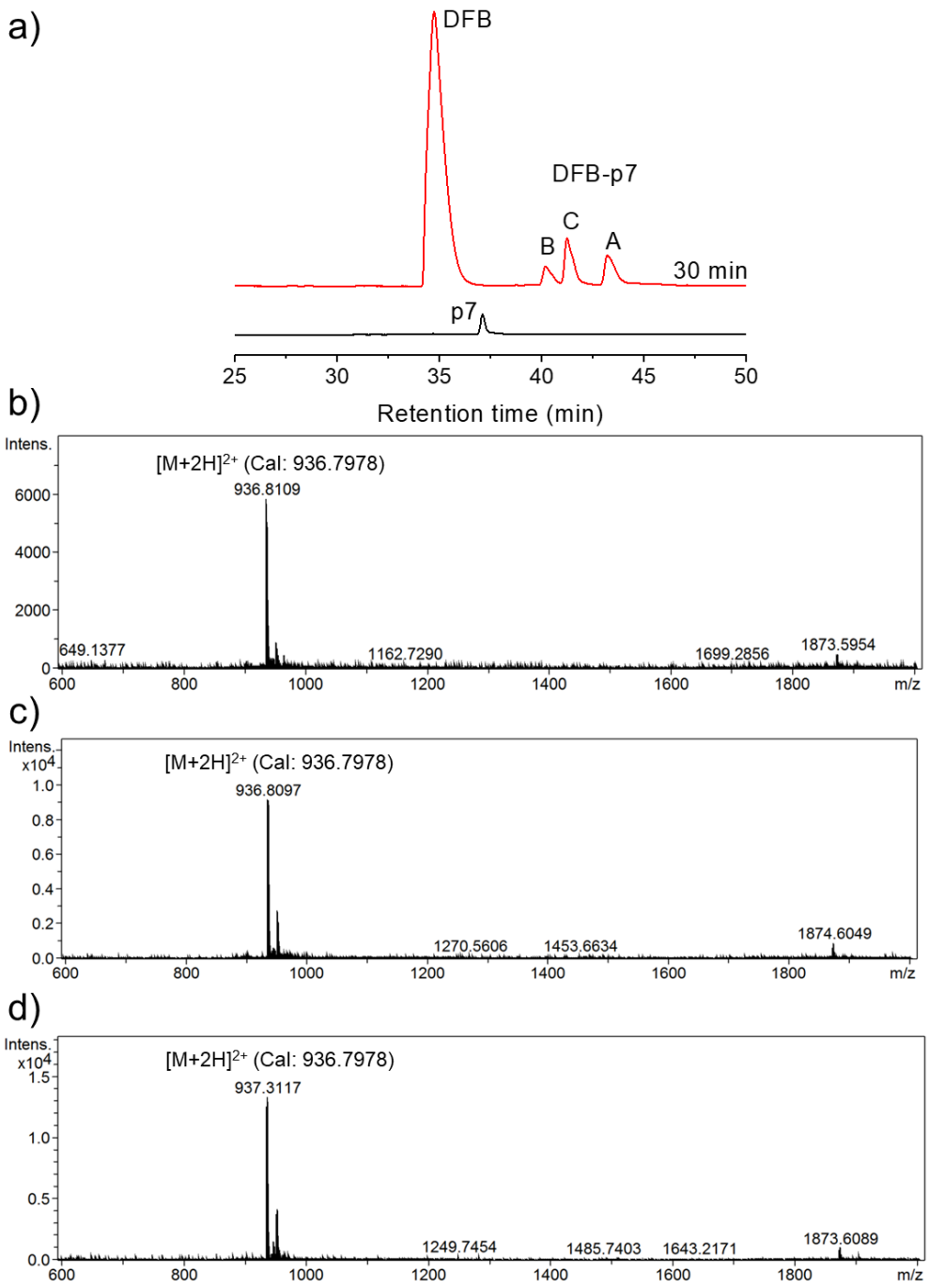

Figure S10. (a) Chromatograms (280 nm) of the reaction of **DFB** and **p7** in pH 7.4, at 37 °C. (b)-(d) MS characterization of products A–C respectively.

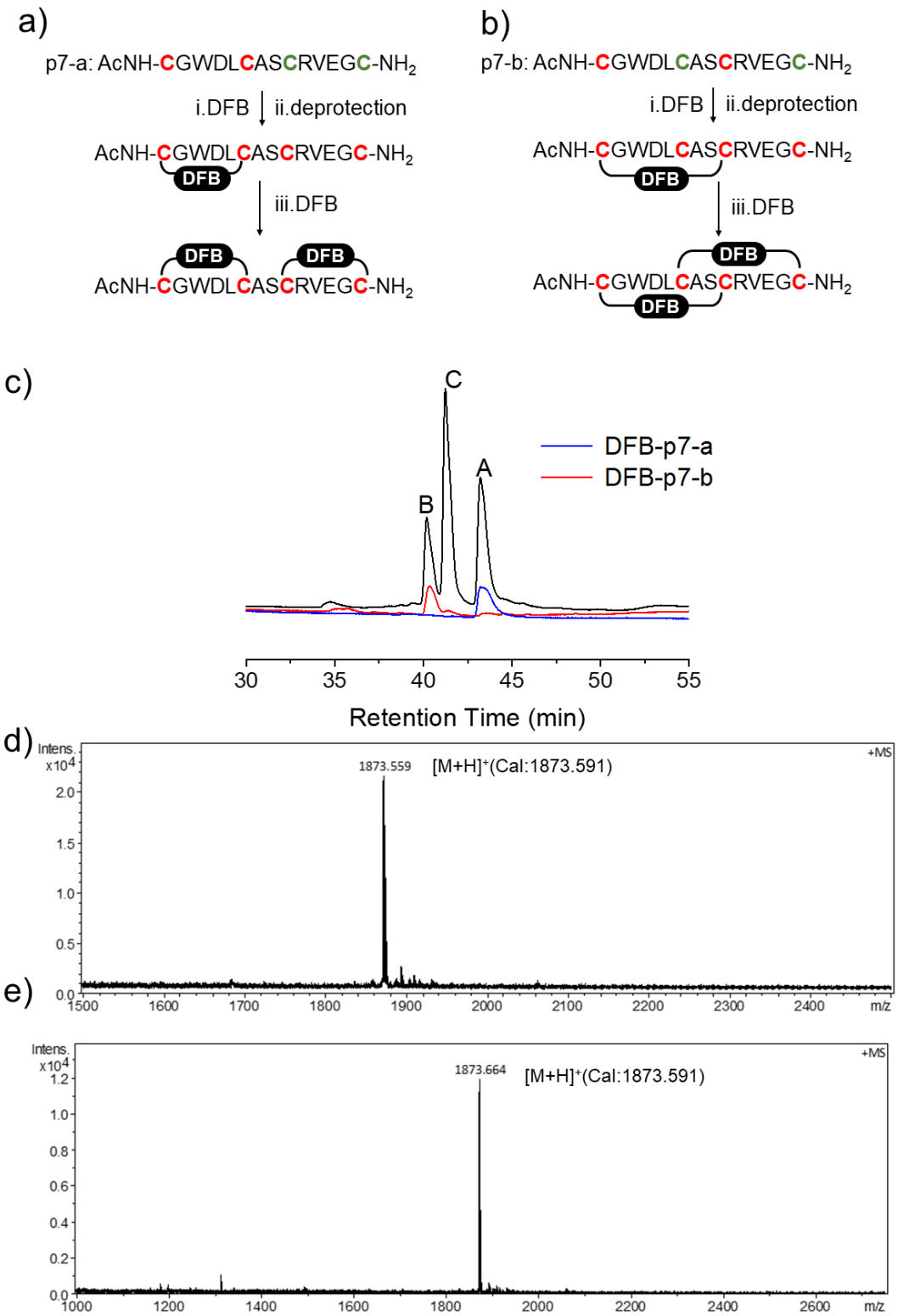

Figure S11. Synthesis of standard products for the identification of bicyclic p7. (a) (b) Reaction scheme of the three steps for synthesis of the standard products (**DFB-p7-a** and **DFB-p7-b**) Cysteine residues in red were unprotected and cysteine residues in green were protected by Acm groups. (i) Reaction of **p7-a** or **p7-b** with the first **DFB**. (ii) Removal of

the Acm groups of the protected cysteine residues in **p7-a** or **p7-b**. (iii) Formation of bicyclic peptides after reaction with the second **DFB**. (c) Chromatograms (386 nm) of the reaction of **p7** with **DFB** (black line), **p7-a** with **DFB** (red line) and **p7-b** with **DFB** (blue line). (d) MS characterization of **DFB-p7-a**. (e) MS characterization of **DFB-p7-b**.

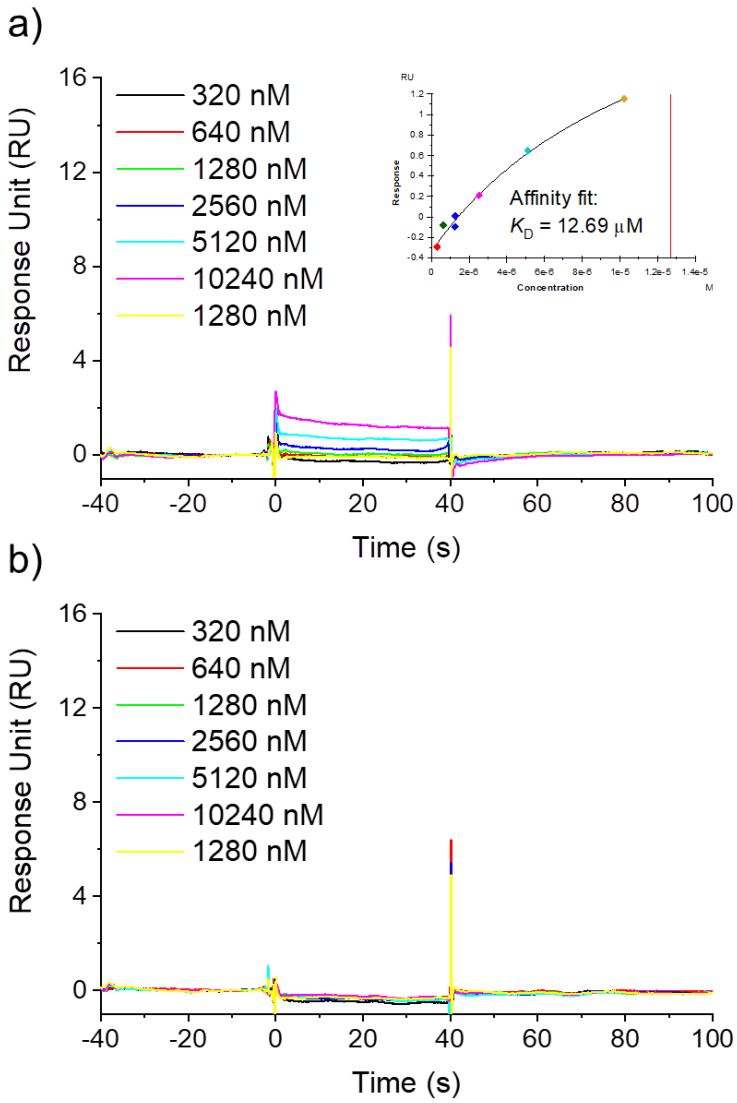

Figure S12. a) Sensorgrams recorded by surface plasmon resonance (SPR) showing the direct binding kinetics of oxidized disulfide-cyclic **M1** (0.32–10.24 μM) with Bcl-xl. Since the binding and dissociation are too fast, the dissociation constant is thus determined using steady state affinity fit (K_D=12.69 μM). b) Sensorgrams showing no obvious binding of linear **M1** (0.32–10.24 μM) with Bcl-xl.

No.	Sequences											Abundance
keap1-M1	C	E	P	E	T	G	E	E	E	E	C	1
keap1-M2	C	V	N	E	A	T	G	E	N	E	C	1
keap1-M3	C	Y	H	V	E	T	G	E	H	G	C	1
keap1-M4	C	P	D	R	E	T	G	E	M	E	C	1
keap1-M5	C	E	D	P	E	T	G	E	R	C	C	1
keap1-M6	C	I	D	P	E	T	G	E	E	Q	C	1
keap1-M7	C	N	D	R	E	T	G	E	E	I	C	1
keap1-M8	C	V	D	A	E	T	G	E	S	E	C	1
keap1-M9	C	E	D	A	E	T	G	E	E	V	C	2
keap1-M10	C	E	A	A	E	T	G	E	W	D	C	1
keap1-M11	C	G	R	D	E	T	G	E	R	T	C	1
keap1-M12	C	E	T	N	E	E	T	G	E	A	C	2
keap1-M13	C	A	Q	L	E	E	T	G	E	S	C	1
keap1-M14	C	P	I	L	E	E	T	G	E	E	C	1
keap1-M15	C	P	F	S	R	E	T	G	E	Y	C	1
keap1-M16	C	D	E	N	A	E	T	G	E	A	C	1
keap1-M17	C	L	R	D	P	E	T	G	E	G	C	1
keap1-M18	C	Q	T	D	P	E	T	G	V	E	C	1
keap1-M19	C	R	E	L	C	Q	E	T	G	E	C	1
keap1-M20	C	G	P	V	E	P	D	T	G	E	C	1
keap1-M21	C	P	N	Q	N	E	E	T	G	E	C	1
keap1-M22	C	P	S	G	M	E	Q	E	T	G	C	1

Figure S13. Sequences obtained from screening of peptide libraries against Keap1 after three rounds of panning.

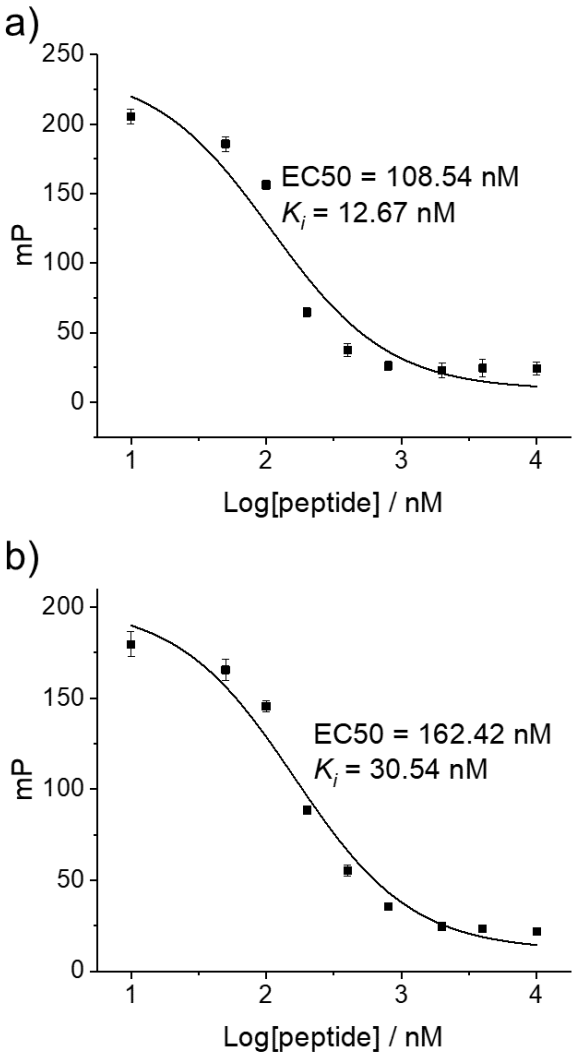

Figure S14. Fluorescence polarization competition assays determining the binding of **DFB**-cyclic peptides with Keap1. (a) Fluorescence polarization competition assays determining the binding of **DFB**-keap1-**M4**. (b) Fluorescence polarization competition assays determining the binding of **DFB**-cyclic peptides with **DFB**-keap1-**M9**.

References

[1]. Vinogradova, E. V.; Zhang, C.; Spokoyny, A. M.; Pentelute, B. L.; Buchwald, S. L., Organometallic palladium reagents for cysteine bioconjugation. *Nature* **2015,** *526* (7575), 687-691.

[2]. Jeong, W.-j.; Lee, M. S.; Lim, Y.-b., Helix Stabilized, Thermostable, and Protease-Resistant Self-Assembled Peptide Nanostructures as Potential Inhibitors of Protein–Protein Interactions. *Biomacromolecules* **2013,** *14* (8), 2684-2689.

[3]. Nikolovska-Coleska, Z.; Wang, R.; Fang, X.; Pan, H.; Tomita, Y.; Li, P.; Roller, P. P.; Krajewski, K.; Saito, N. G.; Stuckey, J. A.; Wang, S., Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Analytical Biochemistry* **2004,** *332* (2), 261-273.

[4]. Petros, A. M.; Medek, A.; Nettesheim, D. G.; Kim, D. H.; Yoon, H. S.; Swift, K.; Matayoshi, E. D.; Oltersdorf, T.; Fesik, S. W., Solution structure of the antiapoptotic protein bcl-2. *Proceedings of the National Academy of Sciences* **2001,** *98* (6), 3012-3017.

[5]. Zha, M.; Lin, P.; Yao, H.; Zhao, Y.; Wu, C., A phage display-based strategy for the de novo creation of disulfide-constrained and isomer-free bicyclic peptide affinity reagents. *Chemical communications* **2018,** *54* (32), 4029-4032.