Supplementary information for:

Direct screening of a target-specific covalent binder: stringent regulation of warhead reactivity at a matchmaking environment

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Contents

1. General

All the reagents, kits, and solvents were purchased commercially and used without further purification. *S. japonicum* glutathione-*S*-transferase (GST) was prepared according to the reported procedure.[1] Peptides (*i.e.*, LESCAW, Fam-GG-LESCAW, LNYCDGW, and Fam-GG-LNYCDGW; Fam represents carboxyfluorescein) were synthesized, purified, and characterized by GenScript Inc. (NJ, USA). Warhead-modified peptides were a purified with reverse-phase high performance liquid chromatography (LC-20AD, Shimadzu, Japan) equipped with a Xterra prep MS C18 column (10 × 50 nm, Waters). Each conjugate was separated using a 0–100% liner gradient of methanol containing 0.1% (v/v) formic acid during 10 minutes at a flow rate of 4 mL per minute.

NMR experiments were performed at 25℃ using a 500 MHz spectrometer (JNM-ECA500, Jeol Resonance, Japan). Liquid chromatography (LC) analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies, USA) using a 0–100% gradient of acetonitrile containing 0.1% (v/v) formic acid at a flow rate of 300 μL per minutes, equipped with a C18 reverse-phase column (Hypersil GOLD, 2.1 × 100 mm, Thermo Fisher Scientific, USA) connected to a photodiode array (PDA) and/or a LCQ-Fleet ion trap mass spectrometer. Electrospray ionization-time-of-flight-mass spectrometry (ESI-TOF-MS; JMS-T100 AccuTOF, Jeol Resonance, Japan) was performed by dissolving the analyte in methanol and directly injecting into the instrument.

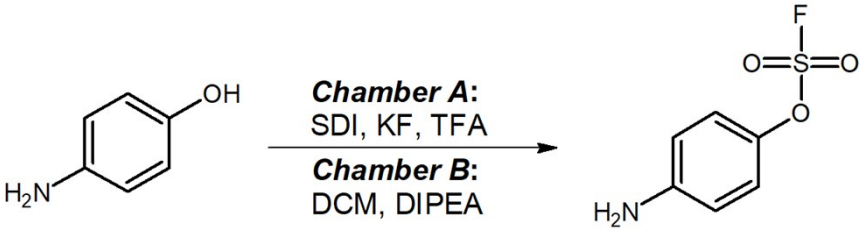
All images of stained gel and in-gel fluorescence were captured by ChemDoc XRS+ (BioRad Laboratories Inc., USA), and band intensities were quantified using Image Lab software (BioRad Laboratories Inc., USA).

For protein digestion and analysis by LC-MS/MS, 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, and the gel was stained with Rapid Stain CBB kit (Nacalai tesque, #30035-14, Japan). The stained protein bands were excised from the gel. Proteins in the gel were reduced with 25 mM dithiothreitol (DTT) at 60℃ for 10 minutes, and then alkylated with 55 mM iodoacetamide at room temperature for 60 minutes in the dark. Digestion was carried out with modified trypsin (Promega, #V5111, USA) at 37℃ overnight. The resulting peptides were analyzed using the above LC-MS/PDA system. The trypsinized peptides were separated using a 0%–50% gradient of acetonitrile containing 0.1% (v/v) formic acid during 55 minutes at a flow rate of 300 μL per minute, and then eluted peptides were directly sprayed into the mass spectrometer. The mass spectrometer was operated

in the data-dependent mode and externally calibrated. Survey MS scans were fragmented with collision-induced dissociation in the ion trap. A dynamic exclusion window was applied within 30 s. All tandem mass spectra were collected using normalized collision energy of 40%. Data were acquired and analyzed with Xcalibur software v. 2.07 (Thermo Fisher Scientific, USA).

2. Syntheses of turn-on warheads

2.1. Synthesis of warhead precursor[2]



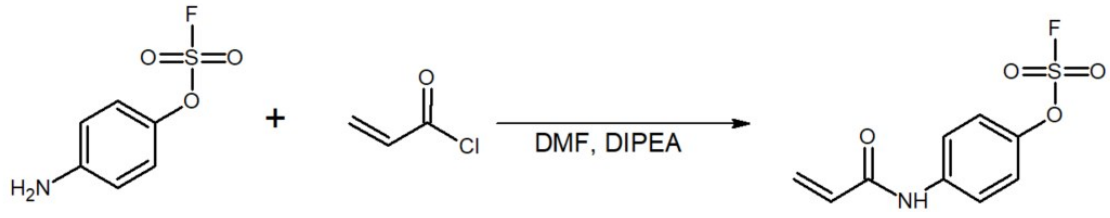
The warhead precursor was synthesized on a preparative scale according to the following procedure. Chamber A of a flame-dried small two-chamber was filled with 1,1'-sulfonyldiimidazole (SDI, 1.5 g, 7.5 mmol, 1.5 eq. TCI, #S0803, Japan), and potassium fluoride (KF, 1.2 g, 20 mmol, 4.0 eq., Wako, #165-03762, Japan). Next, chamber B was charged with 4-aminophenol (0.5 g, 5.0 mmol, 1.0 eq., TCI, #A0384, Japan), *N,N*-diisopropylethylamine (DIPEA, 2.5 mL, 15 mmol, 3.0 eq., Watanabe Chem., #HK290507, Japan), and dichloromethane (DCM, 10 mL). Finally, 5 mL trifluoroacetic acid was added by injection through a septum in chamber A. The reaction was stirred for 18 hours at room temperature. The crude reaction mixture was mixed with ethyl acetate (15 mL), and successively washed with saturated $NaHCO_3$ aq. (5.0 mL), brine (5.0 mL), dried over Na_2SO_4, and evaporated. Then, it was purified by a flash column chromatography on silica gel (hexane/ethyl acetate, 2/1). The title compound was obtained as a white solid (0.76 g, yield 80%). Identification of the purified warhead precursor was performed by ^{1}H, ^{13}C, and ^{19}F NMR (Fig. S1). Warhead precursor: ^{1}H NMR ($CDCl_3$, 500 MHz,) δ 7.11 (d, J = 9.5 Hz, 2H), 6.68 (dd, J = 2.0 and 6.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 146.8, 142.2, 121.9, 115.6; ^{19}F NMR ($CDCl_3$, 470 MHz) δ 36.08.

2.2. Synthesis of Br-AFS

A cysteine reactive warhead, 4-bromoacetamide-benzenfluorosulfate (Br-AFS), was synthesized on a preparative scale according to a following procedure. The warhead precursor (33 mg, 0.17 mmol, 1.0 eq.), DIPEA (0.10 mL, 0.59 mmol, 3.4 eq.) and bromoacetyl bromide (22 μL, 0.25 mmol, 1.5 eq., TCI, #B0539, Japan) were mixed in 3.0 mL of DCM. After stirring for 12 hours at room temperature, the reaction mixture was mixed with ethyl acetate (10 mL), and successively washed with water (5 mL), 0.10 M HCl aq. (5 mL), saturated $NaHCO_3$ aq. (5 mL), brine (5 mL), dried over Na_2SO_4, and evaporated. Then, it was purified by the flash column chromatography on silica gel (hexane/ethyl acetate, 2/1). The title compound was obtained as a brown solid (15 mg, yield 30%). Identification of the purified Br-AFS was performed by ^{1}H, ^{13}C, and ^{19}F NMR (Fig. S2).

Br-AFS: ^{1}H NMR ($CDCl_3$, 500 MHz,) δ 7.68 (dd, J = 2.0 and 7.0 Hz, 2H), 7.34 (d, J = 8.5 z, 2H), 4.04 (s, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 163.7, 146.5, 137.3, 121.9, 121.6, 29.3; ^{19}F NMR ($CDCl_3$, 470 MHz) δ 37.50; HRMS (ESI) Found *m/z* 309.9229, 311.9245 [$(M-H)^-$; calculated for $C_8H_7BrFNO_4S$: 309.9190, 311.9170].

2.3. Synthesis of MAFS



The Michael-acceptor type aryl-fluorosulfate (MAFS) warhead was synthesized on a preparative scale according to a following procedure. The warhead precursor (0.12 g, 0.60 mmol, 1.0 eq.), DIPEA (0.17 ml, 1.8 mmol, 3.0 eq.) and acryloyl chloride (54 μl, 0.70 mmol, 1.1 eq., TCI, #A0147, Japan) were mixed in 1.2 mL of *N,N*-dimethylformamide (DMF). After stirring for 3 hours at room temperature, the reaction

mixture was mixed with ethyl acetate (10 mL), washed successively with saturated $NaHCO_3$ aq. (5 mL), 0.10 M HCl aq. (5 mL), brine (5 mL), dried over Na_2SO_4, and evaporated to yield a white solid pure product (0.11 g, yield 76%). Identification of the purified warhead was performed by ^1H, ^{13}C, and ^{19}F NMR (Fig. S6).
MAFS: ^1H NMR (CD_3OD, 500 MHz,) δ 7.82 (dt, J = 10.1 and 2.6 Hz, 2H), 7.41 (dd, J = 11.7 and 3.2 Hz, 2H), 6.46–6.37 (m, 2H), 5.80 (dd, J = 9.2 and 2.9 Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ 164.9, 145.9, 139.1, 130.8, 127.2, 121.3, 121.2; ^{19}F NMR (CD_3OD, 470 MHz) δ 35.21; HRMS (ESI) Found *m/z* 244.0087 [$(M-H)^-$; calculated for $C_9H_8FNO_4S$: 244.0085].

3. Identification of covalent-binding position on GST with AFS-modified LNYCDGW peptide

3.1. Synthesis of AFS-modified LNYCDGW peptide

The peptide (LNYCDGW, 50 mM) was dissolved in 20 mM phosphate buffer (PB, Na_2HPO_4/NaH_2PO_4, pH 7.4 at 25℃) / 50% (v/v) DMSO, and reacted with Br-AFS (80 mM) in the presence of neutralized tris(2-carboxyethyl)phosphine (TCEP; 2.0 mM). The mixture was reacted for more than 4 hours at room temperature in the dark with vigorous shaking, and purified by the reverse-phase HPLC (yield 30%), and identified with MS and MS/MS analyses (Fig S3A).

3.2. Identification of covalent-binding position by tandem MS analysis

The AFS-modified LNYCDGW peptide (0.50 mM) was mixed with GST (0.15 mM) in Dulbecco's Phosphate-Buffered Saline (D-PBS) and incubated for 12 hours at 37℃. It was mixed with 1 × sample buffer, denatured at 95℃, separated by 12% (w/v) SDS-PAGE, followed by trypsinization/LC-MS/MS analysis. The covalent-binding site was determined to be the glutathione binding pocket; the covalently bound amino acid was tyrosine located at the 111th position[5] from the N-terminus (Fig. S3B).

3.3. Molecular docking simulation using MolDesk Basic

For the docking simulation, the structure of the covalent binder (*i.e.*, LNYC*DGW, C* represents AFS-modified cysteine) was created by ChemDraw Ultra (version 11.0) and converted to mol file. Docking of the GST (PDB: 1UA5) was performed with MolDesk Basic (version 1.1.45, Imsbio Inc., Japan) under a graphical-user interface (GUI) of several myPresto programs[3, 4] as follows. First, the mol file was further converted to a mol2 file. Second, the target GST protein was stripped out of the

co-crystalized glutathione and converted to a pdb file format. Finally, the binder and the glutathione-subtracted GST input file were entered and docked using sievgene program of myPresto (version 5.000).[3, 4] For the docking, thirty separate poses were taken. The binding geometry of the best docking model was supported experimentally by an exclusive covalent-binding of the warhead, which was proved by MS/MS analysis of the trypsinized fragment of the conjugated GST (Fig. S3C).

4. Evaluation of reaction conversion yield of each warhead-modified LNYCDGW peptide

AFS- or ethene sulfonyl fluoride- (ESF) modified LNYCDGW peptide (1.0 mM; the latter has been already reported in a previous work[5]) and GST (0.15 mM) were mixed in D-PBS, respectively, and incubated for 12 hours at 37℃. It was mixed with 1 × sample buffer, denatured at 95℃, separated by 12% (w/v) SDS-PAGE, followed by trypsinization/LC-MS/MS analysis (Fig. S4).

5. Evaluation of thioetherification between each warhead and designated cysteines of model peptide on T7 phage

For evaluation of a successful introduction of the turn-on warhead to designated cysteines (underlined) of a model monoclonal peptide (-GSRVS-C-GGRDRPG-C-LSV; fused at the C-terminal region of gp10) on T7 phage via the gp10 based-thioetherification ($10BASE_d$-T)[1], we mixed each warhead (*i.e.*, Br-AFS or MAFS; 1.0 mM) and the model peptide on T7 phage (1.0×10^{11} plaque forming units) in 0.70 mL of 1.0 M NaCl / 0.50 mM neutralized TCEP in D-PBS containing 10% (v/v) DMSO at 4℃ or 55℃. After 3 hours of the reaction, the peptide was further treated with 5-idodoacetamide-fluorescein (FL-IA; 0.20 mM) for 3 hours at 4℃. The latter reaction with FL-IA blocks all of the unreacted cysteines of the model peptide on T7 phage after the introduction with Br-AFS. Our previous study demonstrated that FL-IA was conjugated to at least 95% of the designated cysteines of the model peptide on T7 phage under the condition.[6] Thus, we can indirectly estimate introduction yield of Br-AFS by the fluorescent densitometric analysis. After the $10BASE_d$-T, the T7 phage particles were precipitated by centrifugation with a mixture of polyethylene glycol 6000 and sodium chloride to final concentrations of 5% (w/v) and 0.5 M, respectively.[1] The precipitate was dissolved in the sample buffer, and whole T7 phage proteins were subjected to 12% (w/v) SDS-PAGE after the denaturation, followed by the in-gel fluorescence imaging and CBB staining (Fig. S7).

6. Direct screening of covalent binder

6.1. Biotinylation of GST[1]

GST (1.4 mM) in 20 mM PB (pH 7.4) was mixed with *N*-succinimidyl 6-(biotinamido)hexanoate (TCI, #S0490, Japan) at a final concentration of 2.8 mM and incubated at 4℃ overnight. For desalination, Zeba™ Spin Desalting Columns (Thermo Fisher Scientific, USA) was used with centrifugation at 800*g* for 4 minutes at 4℃. The biotinylation of GST was confirmed by western blotting; from the densitometric quantification reported previously[6], it was estimated that approximately 0.5 molecules of biotin were conjugated to a single GST molecule (data not shown). The biotinylated GST was incubated with streptavidin-coupled magnetic nanoparticles (Dynabeads™ MyOne™ Streptavidin T1, Invitrogen, #65601, USA), to afford GST-immobilized beads.

6.2. Determination of stringent washing condition during biopanning

The model monoclonal- or library (-SGGG-X_3-C-$X_{4–10}$-C-X_3; X represents any randomized amino acid; diversity: 8.6×10^9)-peptide on T7 phage (1.0×10^{11} plaque forming units)[1] was reacted with MAFS (1.0 mM) for 3 hours at 37℃. After the 10BASE$_d$-T, the warhead-modified monoclonal- or polyclonal phage was precipitated as described in Section 5, and dissolved in a selection buffer (D-PBS supplemented with 1.0% (v/v) Tween-20). Then, each phage was incubated with the GST-immobilized beads for 1 hour at 37℃ using a rotator. The beads were washed with 0.20 mL of a stringent wash buffer (1.0% (w/v) SDS, 4.0 M urea, 0.15 M NaCl, 50 mM DTT in D-PBS) under sonication (10 minutes), and this washing process was repeated four times. Then, numbers of the GST-conjugated phages on the beads were directly counted by a plaque assay[1].

6.3. Biopanning against GST by using turn-on type covalent-binding peptide library

The peptide library on T7 phage (1.0×10^{11} plaque forming units) was modified via the 10BASE$_d$-T. After the MAFS modification and the phage precipitation, it was dissolved in the selection buffer. To remove non-specific binders (*i.e.*, beads-, streptavidin-, plastic-, and BSA-binders), the library was pre-incubated with streptavidin-coupled beads for 1 hour at 37 ℃, and then the supernatant was further

incubated with the GST-immobilized beads for 1 hour at 37 ℃. The latter beads were washed four times as described in Section 6.2. Then, the GST-bound phages were directly infected with *E. coli* BLT5403 strain, and the amplified phages were used for the next round of biopanning. In the second round, biopanning was performed as same above, except containing 1% (w/v) BSA in the selection buffer.

7. Next generation sequencing (NGS) analysis

The phages (1.0×10^{11} plaque forming units) recovered from the second round of biopanning were twice subjected to the phenol / chloroform treatment to purify phage DNA. After the ethanol precipitation, DNA was dissolved in water and 10 ng of it was used for polymerase chain reaction (PCR; 18 cycles repeating three steps of denaturation at 94℃ for 30 seconds, annealing at 58℃ for 30 seconds, and extension at 72℃ for 1 minutes) as a template to amplify the library-coding region using a forward primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCTAAGTACGCAATGGGCC) and a reverse primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTCAACGTTCATATGGTATGAGCG). The product was twice subjected to the phenol / chloroform treatment to purify. After the ethanol precipitation, DNA was dissolved in water. The purified DNA was ligated with adapter DNA (Nextera XT Index Kit, illumine, USA) harbored with the appropriate index sequences and purified on Agencourt AMPure XP Beads, according to the protocols of QIA seq 1-Step Amplificon Library Kit (QIAGEN). The adaptor-ligated amplicon was again amplified by PCR (8 cycles repeating three steps of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C, 30 seconds) using the primer mix in the kit and purified on Agencourt AMPure XP Beads. The adaptor-ligated DNA prepared thus was supplied to NGS analysis on Illumina Miseq using Miseq v3 reagent. About twenty amplicon samples, which were indexed by the barcode, were mixed, and analyzed. The NGS data collected on Miseq were transformed to FastQ format, and sorted by the barcode. The nucleotide data were quality-checked, merged, and translated into amino acid sequences to determine the representative peptide.

8. Identification of covalent-binding position of covalent binder

8.1. Synthesis of covalent binder

The representative peptide (*i.e.*, LESCAWY, 50 mM) was dissolved in 20 mM PB (pH 7.4) / 50% (v/v) DMSO, and reacted with MAFS (80 mM) in the presence of neutralized TCEP (2.0 mM). The mixture was reacted for more than 12 hours at room temperature in the dark with vigorous shaking, purified by the reverse-phase HPLC (yield 60%), and identified with MS and MS/MS analyses (Fig. S8).

8.2. Identification of covalent-binding position by tandem MS analysis

The covalent binder (0.40 mM) was mixed with GST (0.10 mM) in D-PBS and incubated for 12 hours at 37℃. It was mixed with 1 × sample buffer, denatured at 95℃, separated by 12% (w/v) SDS-PAGE, followed by trypsinization/LC-MS/MS analysis. As shown in Fig. 3 in the main text, the covalent-binding site was determined to be the glutathione binding pocket; the covalently bound amino acid of GST was tyrosine which was located at the 111^{th} position from the N-terminus.

8.3. Molecular docking simulation of covalent binder

The docking simulation was performed as Section 3.3 except using a structure of the covalent binder (*i.e.*, LEYC*AWY, C* represents MAFS-modified cysteine). The binding geometry of the best docking model was supported experimentally by the exclusive covalent conjugation of the warhead, which was proved by MS/MS analysis of the trypsinized fragment of the conjugated GST (Section 8.2).

9. Evaluation of reaction conversion yield and protein-specificity of covalent binder

9.1. Evaluation of concentration-dependent conversion

For assessment of concentration-dependent conversion of the covalent binder, we mixed it at various molar concentrations with GST (0.10 mM) in D-PBS and incubated it for 24 hours at 37℃. It was mixed with the sample buffer, denatured at 95℃, separated by 12% (w/v) SDS-PAGE followed by trypsinization/LC-MS/MS analysis. The reaction conversion yield was calculated as mean ± SD (n = 3) from the absorbance peak ratios between the remaining GST fragment containing 111^{th} tyrosine (*i.e.*, IA<u>Y</u>SK at 6.7 minutes in Fig. 4A; <u>Y</u> should react with the warhead) and a different GST fragment as an internal standard (*i.e.*, YGVSR at 6.9 minutes).

9.2. Evaluation of time-dependent conversion

For assessment of time-dependent conversion of the covalent binder, we incubated the covalent binder (0.20 mM) and GST (0.10 mM) for arbitrary time in D-PBS at 37℃. After the incubation, it was mixed with the sample buffer, and quantified in the same procedure as described in Section 9.1.

9.3. Kinetic evaluation of covalent binder[7, 8]

GST activity inhibition by the covalent binder was measured by a colorimetric assay. GST catalyzes conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the resulting reaction product has a molar absorption at 340 nm [9]. The enzymatic activity was determined by monitoring the change in NanoPhotometer (Implen, Germany) using a 1 mm lid for the NanoPhotometer submicroliter cell.

For kinetic evaluation of the covalent binder on the basis of the assay, we mixed the covalent binder at various molar concentrations with GST (60 μM) in D-PBS and incubated for arbitrary time at 37℃. After the incubation, it was 50-fold diluted by 100 mM PB (K_2HPO_4/KH_2PO_4, pH 6.5 at 25℃) and mixed with CDNB (1.0 mM) and glutathione (0.30 g/L). Then, it was incubated for 30 seconds at room temperature, and the maximum absorbance (340 nm) was measured by the absorbance spectrophotometer. In each incubation time, the maximum absorbance in the absence of the covalent binder was normalized to 100%, and the relative absorbance of each concentration was quantified. The degree of GST-activity inhibition (DoI) was calculated as mean ± SD (n = 3) from the quantified values and plotted against time. Exponential curve fitting of the plot by GraphPad Prism 6 (MDF Co., Ltd.) gave us the value of k_{obs} at each covalent-binder concentration by using an equation of DoI (%) = v_i / k_{obs} × [1 - exp(-k_{obs} × t)]; v_i = initial velocity (Fig. 4D). Finally, the values of k_{obs} (*i.e,* pseudo-first-order rate constant) were plotted against the covalent-binder concentrations, and the values of K_i and k_{inact} (*i.e.,* inhibition constant and inactivation rate constant, respectively) were determined by the curve fitting of k_{obs} = (k_{inact} × [I]) / (K_i + [I]); [I] = covalent-binder concentration (Fig. 4E).

9.4. Evaluation of protein-specificity

The fluorescent peptide possessing the representative sequence (*i.e*., Fam-GG-LESCAWY, 10 mM) was dissolved in 20 mM PB (pH 7.4) / 50% (v/v) DMSO, and reacted with MAFS (20 mM) in the presence of neutralized TECP (2.0 mM) for more than 4 hours at room temperature with vigorous shaking. Then it was monitored / purified by the reverse-phase HPLC. The purified Fam-modified covalent binder (Fig. S10; 0.30 mM) was mixed with GST (0.30 mM) in D-PBS in the presence of 40% (v/v) human serum (Sigma, H4522), and incubated for 12 hours at 37℃. It was mixed with 1 × sample buffer, denatured at 95℃, and separated by 12% (w/v) SDS-PAGE. Proteins, which were conjugated with the fluorescent covalent binder, were detected by the fluorescence imaging, and the whole proteins were visualized by CBB staining.

10. Supplementary figures

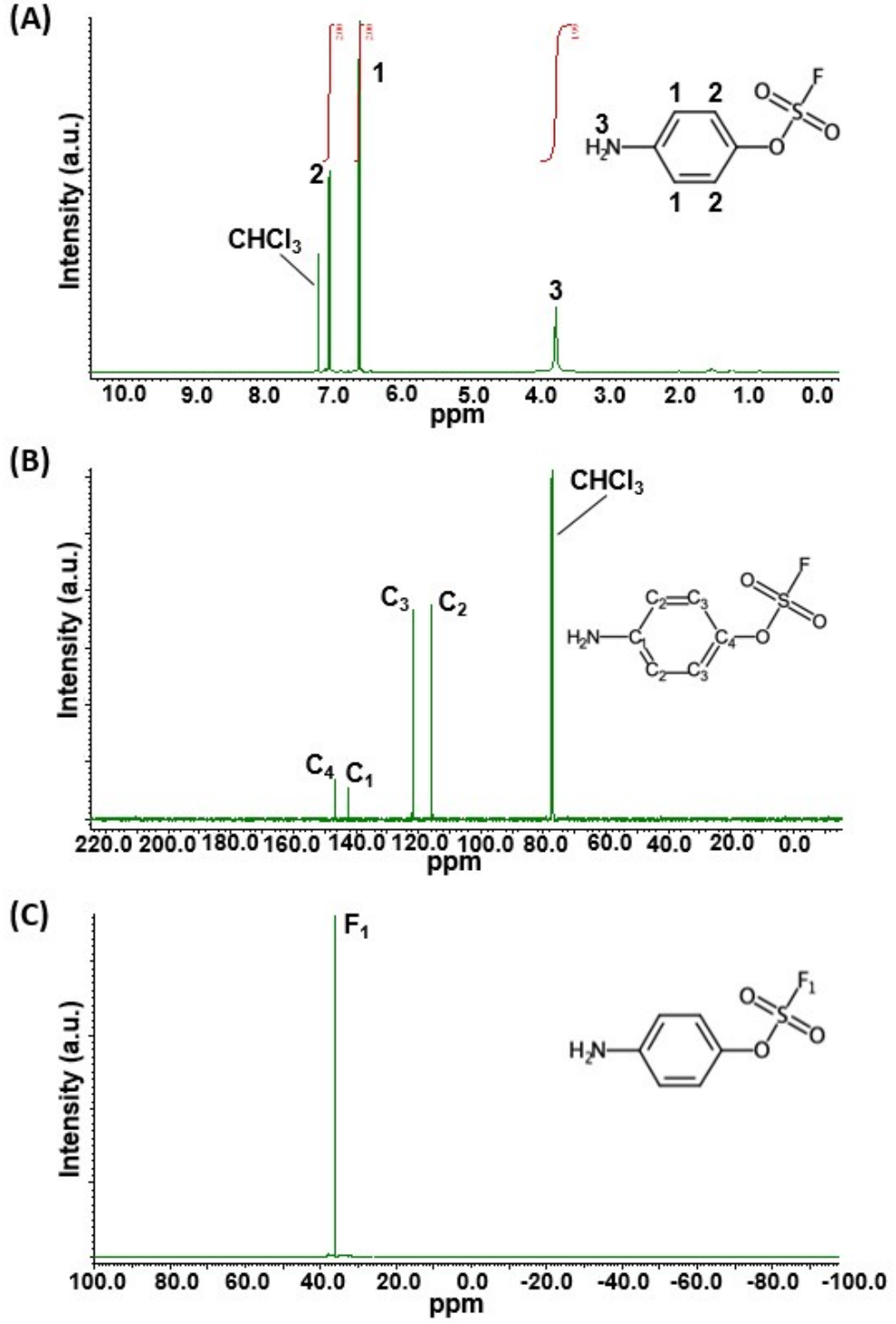


Fig. S1 Identification of the purified warhead precursor.[2] (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) ^{19}F NMR spectrum.

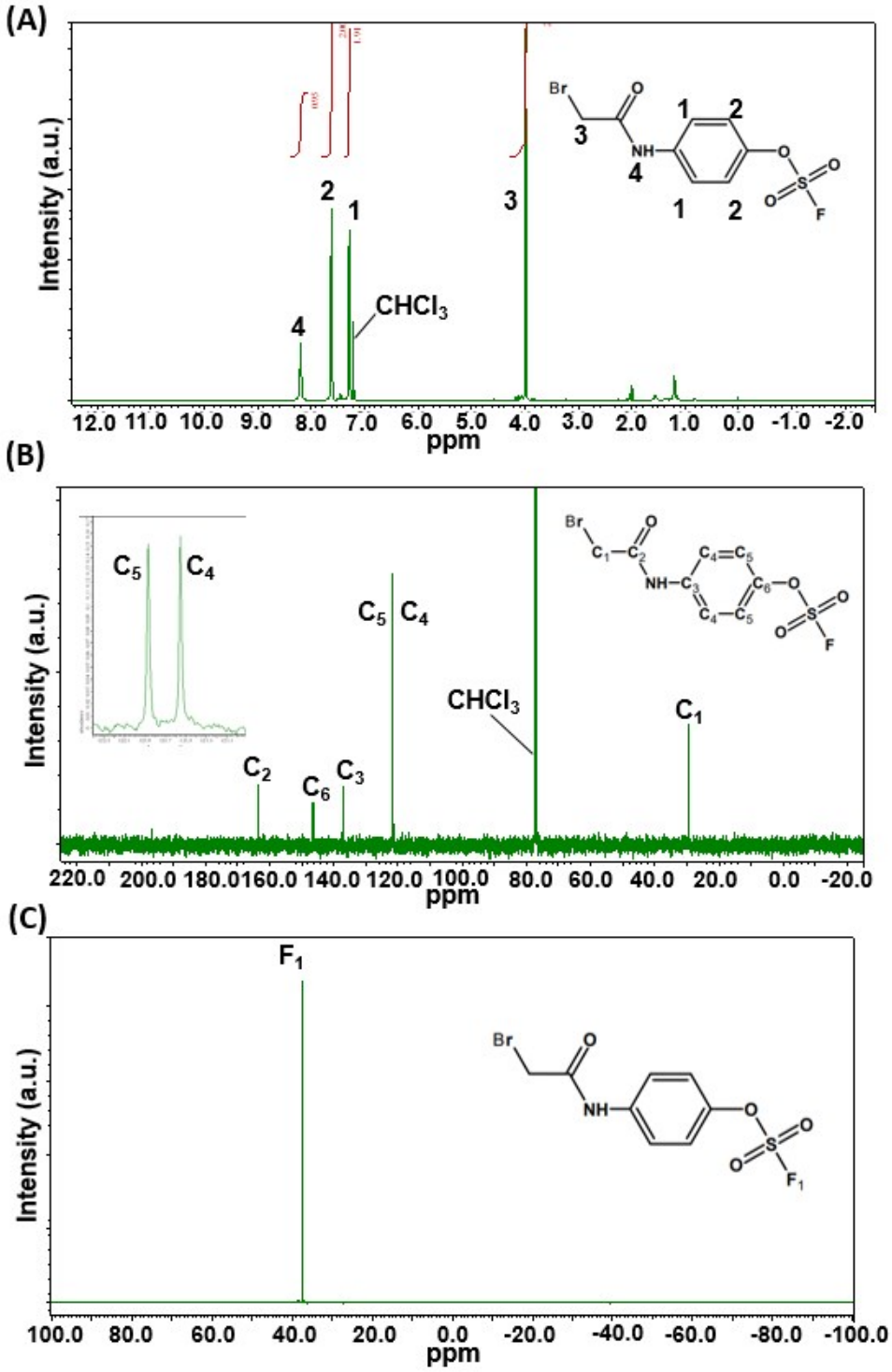


Fig. S2 Identification of the purified Br-AFS. (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) ^{19}F NMR spectrum.

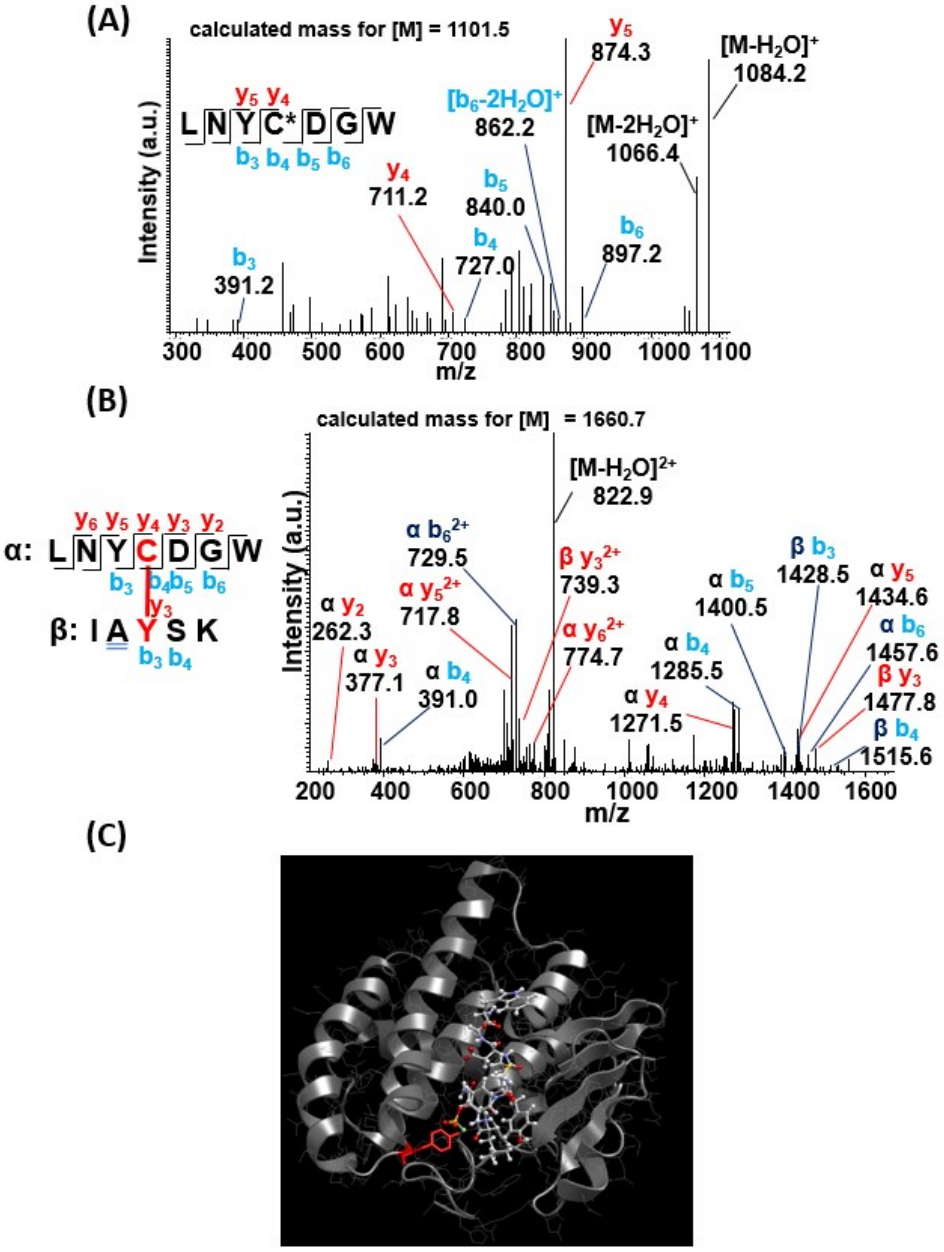


Fig. S3 (A) Identification of the purified LNYC*DGW peptide via MS/MS analysis; C* represents AFS-modified cysteine. (B) Identification of covalent-binding position on GST using the AFS-modified binder by the trypsinization/LC-MS/MS analysis; 111th tyrosine of GST was exclusively modified. (C) Molecular docking simulation of the AFS-modified binder (shown as a stick) to GST (PDB ID: 1UA5) using sievgene of myPrest. Fluorine atom in the warhead and the conjugated 111th tyrosine were coloured in cyan and dark red, respectively. GST was shown as a cartoon with side chains as a line description. The distance between fluorine atom in the warhead and the oxygen atom in 111th tyrosine was deduced to be 3.4 Å.

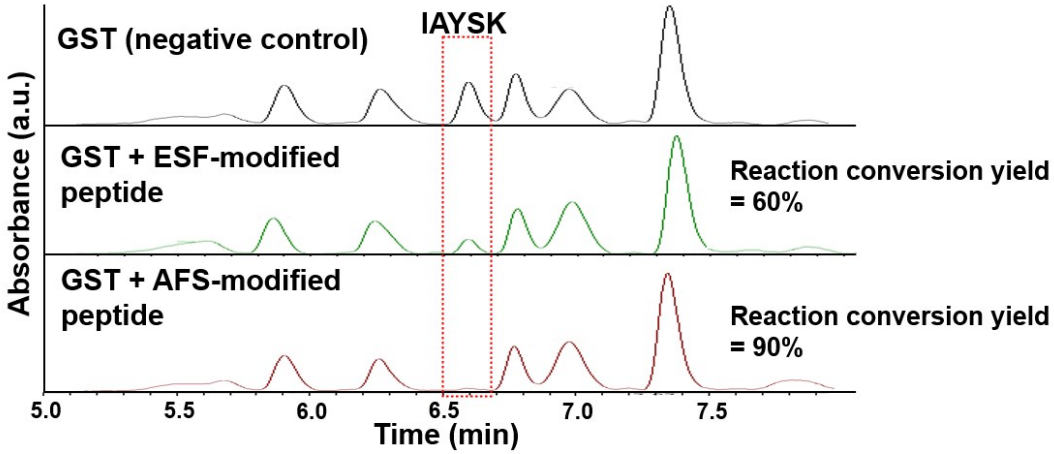


Fig. S4 LC absorbance profiles of trypsinized fragments of GST before (top) and after the reaction with the ESF- (middle) or AFS- (bottom) modified covalent binder (*i.e.*, LNYC*DGW). The reaction conversion yield was calculated from a disappearance of the GST fragment containing 111th tyrosine (*i.e.*, IAYSK at 6.6 minutes; Y should react with each warhead[5]). Other detected GST fragments were used as internal standards.

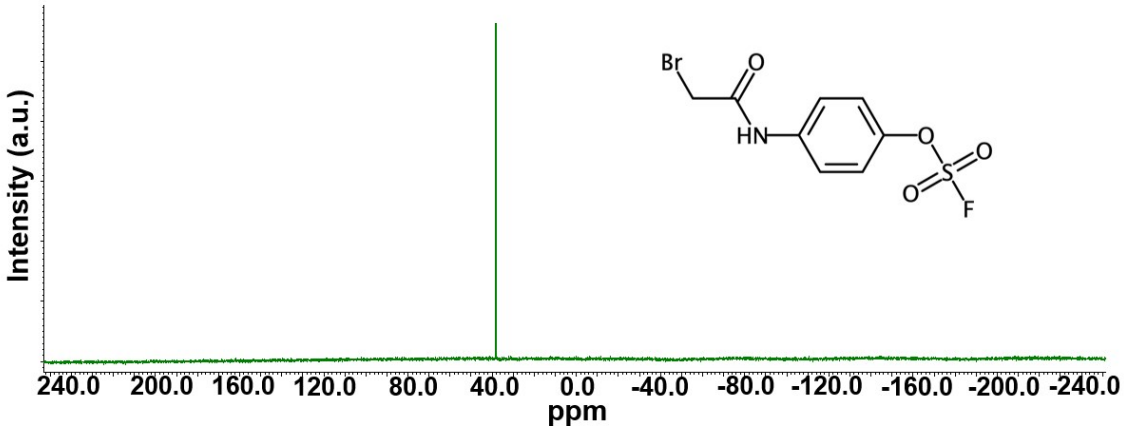


Fig. S5 ^{19}F NMR spectrum of Br-AFS after incubation for 48 hours at 37℃ in 50 mM PB (Na_2HPO_4/NaH_2PO_4 in D_2O, pH 7.4) / 50% (v/v) DMSO-d_6, to prove its excellent stability against hydrolysis of the warhead. Any unfavorable peaks of hydrolyzed products (*e.g.*, hydrogen fluoride; around -200 ppm), could not be seen.

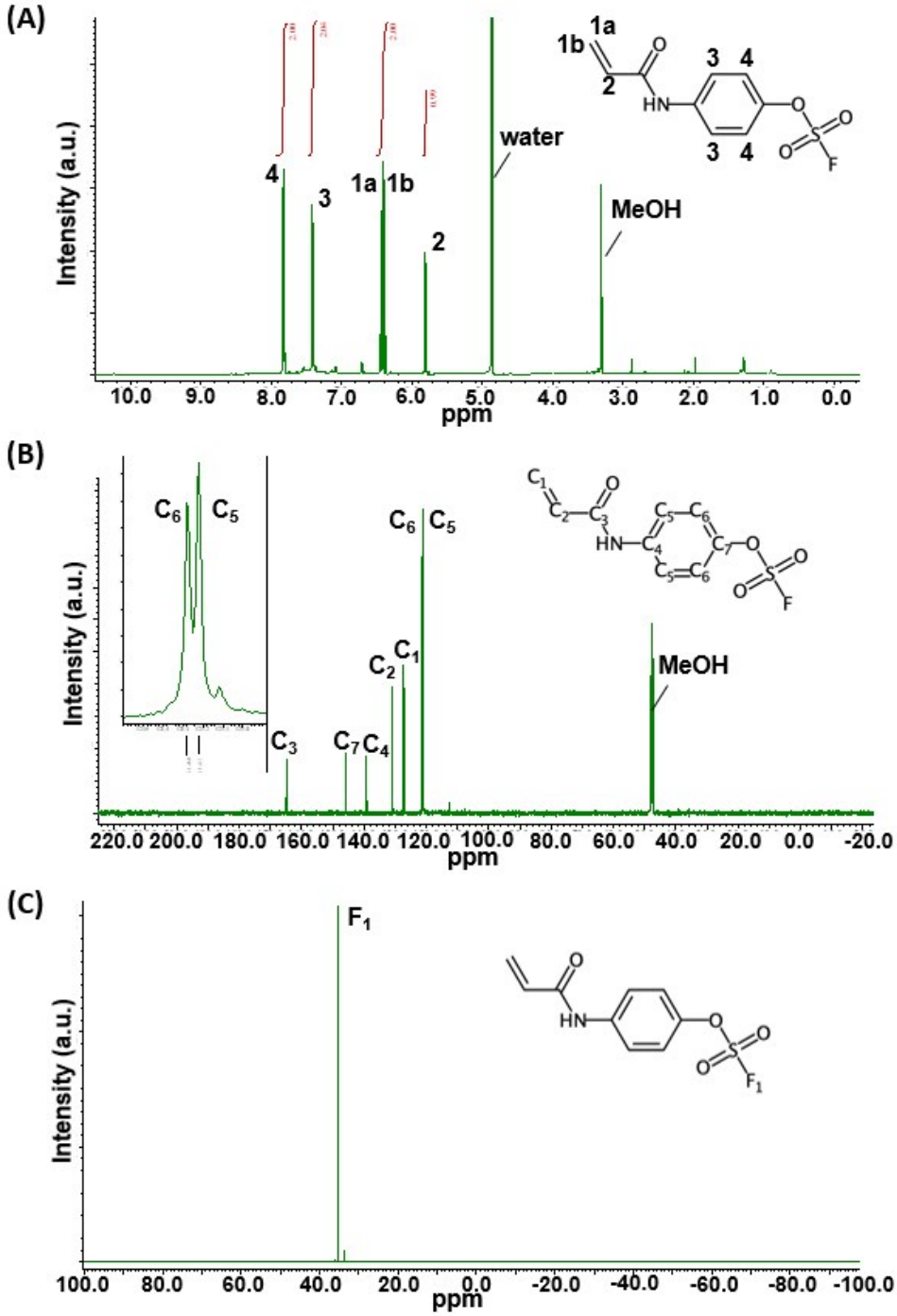


Fig. S6 Identification of the purified MAFS. (A) ^{1}H NMR spectrum. (B) ^{13}C NMR spectrum. (C) ^{19}F NMR spectrum.

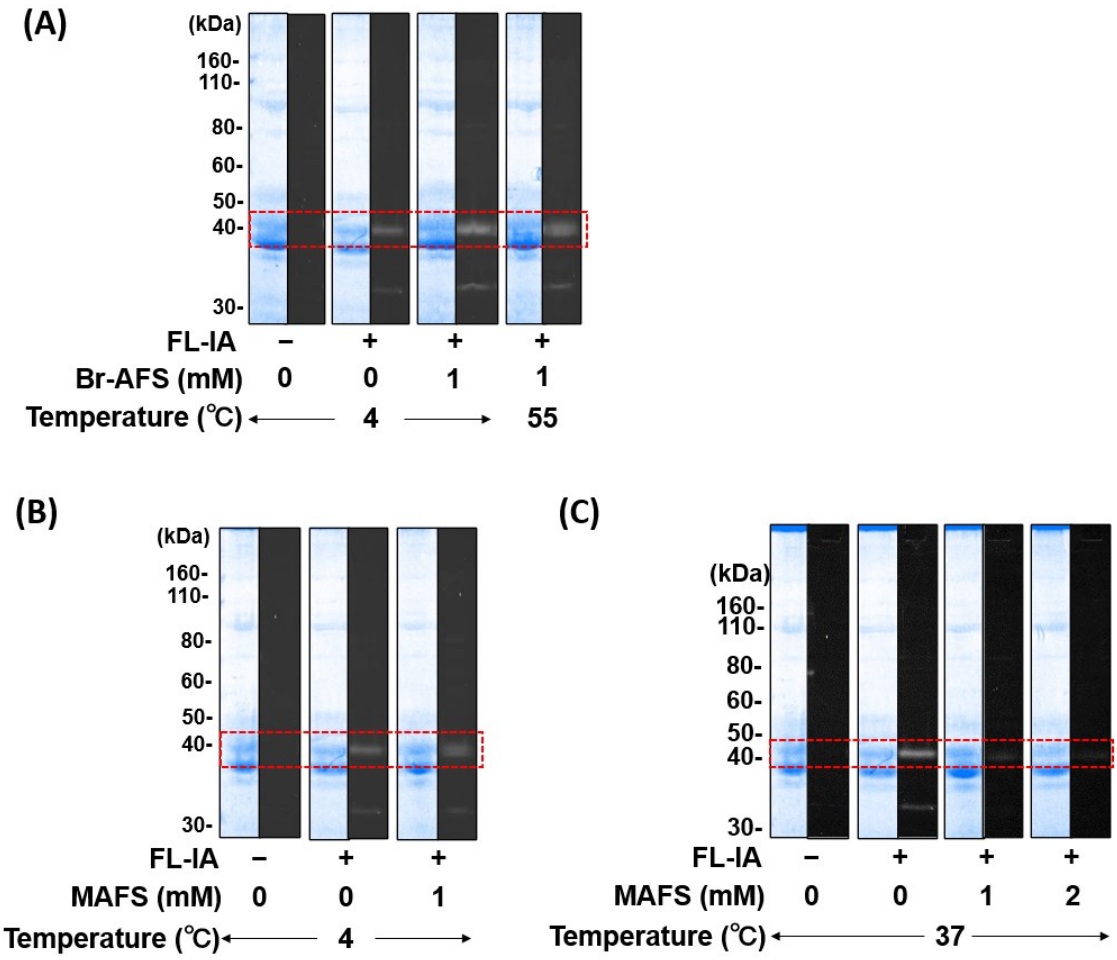


Fig. S7 Evaluation of thioetherification between each warhead and designated cysteines of the model peptide on T7 phage by fluorescent densitometric analysis / CBB staining. A single fluorescent band could be seen at an appropriate molecular weight (ca. 44 kDa; red rectangle) of the peptide-fused gp10 when the warhead (*i.e.*, Br-AFS or MAFS) was absent. This control experiment indicates that the thioetherification with FL-IA exclusively occurred at the peptide-fused gp10.[6] (A) Br-AFS introduction. Even when Br-AFS was present, the fluorescent band did not disappear at each temperature. This indicates that Br-AFS did not react with the designated cysteines even at the elevated temperature. (B, C) MAFS introduction at 4℃ and 37℃, respectively. When incubated at 37℃, the fluorescent band disappeared in a concentration-dependent manner. This indicates that the introduction was successful at an optimal molar concentration of 1.0 mM, and FL-IA no longer reacted with the designated cysteines.[6]

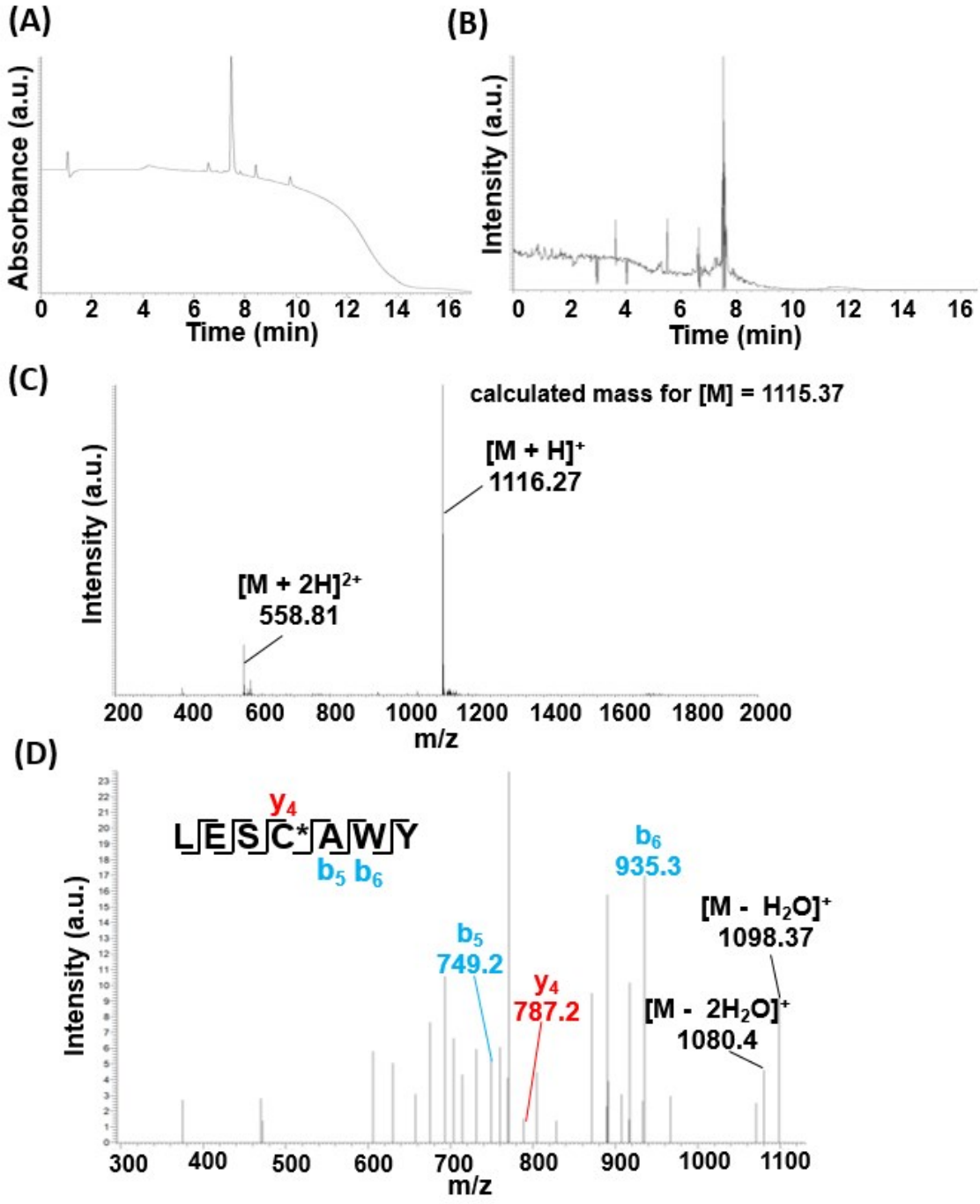


Fig. S8 Identification of the purified covalent binder (*i.e.*, LESC*AWY, C* represents MAFS-modified cysteine) via (A) LC absorbance profile, (B) LC-MS total ion profile, (C) MS, and (D) MS/MS analyses.

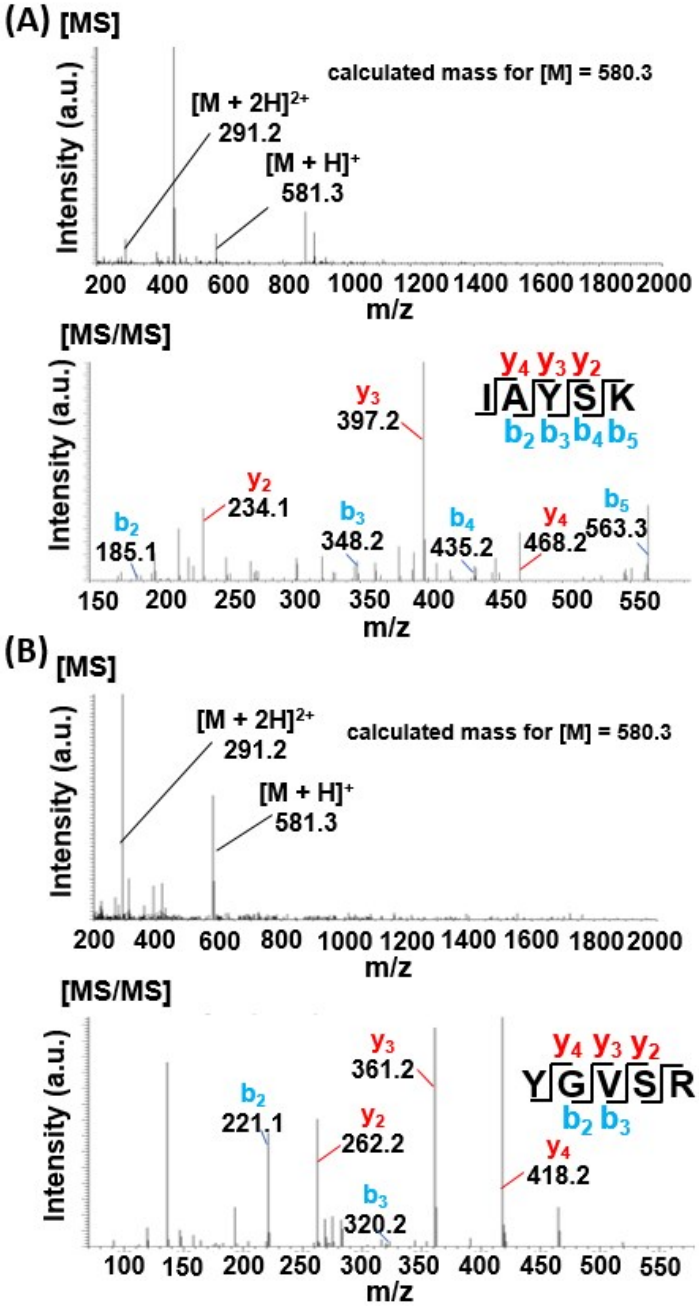


Fig. S9 MS and MS/MS identifications of trypsinized peptide fragments of GST, (A) IAYSK and (B) YGVSR, which correspond to the LC absorbance profiles in Fig. 4A (*i.e.*, 6.7 and 6.9 minutes, respectively).

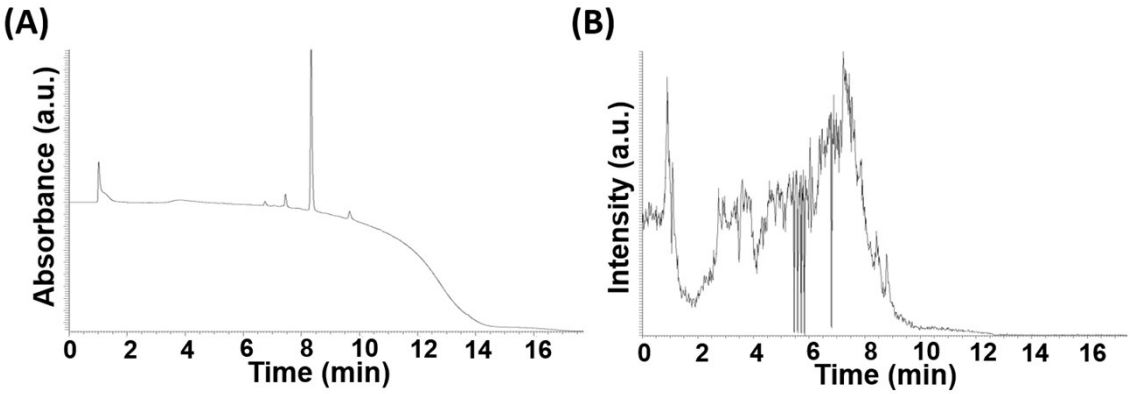


Fig. S10 Identification of the purified Fam-modified covalent binder (Fam-GG-LESC*AWY; Fam, GG, and C* represent carboxyfluorescein, glycylglycine spacer, and MAFS-modified cysteine, respectively) via (A) LC absorbance profile, and (B) LC-MS total ion profile. We could not observe an expected *m/z* value of the purified Fam-modified covalent binder because of the low ionization efficiency of the fluorine derivative both in positive and negative ionization mode.[10] Nevertheless, we believe MAFS was successfully introduced, because a single peptide peak of Fam-GG-LESCAWY on the LC absorbance shifted to a longer retention time after the reaction.

11. Abbreviation list

10BASE$_d$-T: Gp10 based-thioetherification
GST: *S. japonicum* glutathione-*S*-transferase
NMR: nuclear magnetic resonance
LC: liquid chromatography
HPLC: high performance liquid chromatography
PDA: photodiode array
ESI-TOF-MS: electrospray ionization-time-of-flight-mass spectrometry
MS/MS: tandem mass spectrometry
HRMS: high resolution mass spectrometry
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
CBB: Coomassie Brilliant Blue
D-PBS: Dulbecco's Phosphate-Buffered Saline
PB: phosphate buffer
NGS: next generation sequencing
PDB ID: Protein Data Bank identification code
SD: standard deviation
DoI: degree of intensity

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