Electronic Supplementary Information for

A Covalent G-site Inhibitor for Glutathione *S*-Transferase Pi ($GSTP_{1\text{-}1}$)

Yuko Shishido,[a,b] Fumiaki Tomoike,[a] Yasuaki Kimura,[a] Keiko Kuwata,[c] Takato Yano,[d] Kenji Fukui,[d] Haruka Fujikawa,[a] Yoshitaka Sekido,[e] Yuko Murakami-Tonami,[e] Tomoshi Kameda,[f] Satoshi Shuto,[b] Hiroshi Abe*[a]

a. *Graduate School of Science, Nagoya University, Fro-cho, Chikusa-Ku, Nagoya, 464-8602, Japan.*

b. *Faculty of Pharmaceutical Science, Hokkaido University, Kita-12, Nishi-6, Kita-Ku, Sapporo, 060-0812, Japan.*

c. *Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Fro-cho, Chikusa-Ku, Nagoya, 464-8602, Japan.*

d. *Department of Biochemistry, Osaka Medical College, 2-7, Daigakumachi, Takatsuki, Osaka 569-8686, Japan.*

e. *Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1, Kanokoden, Chikusa-Ku, Nagoya, 464-8681, Japan.*

f. *Artificial Intelligence Research Center, National Institute of Advanced Industrial Science and Technology, 2-4-7, Aomi, Koutou-ku, Tokyo, 135-0064, Japan*

*E-mail: h-abe@chem.nagoya-u.ac.jp

Experimental Procedures

General Information:

Analytical separations for all compounds were performed on a HPLC system from JASCO equipped with a PU-2080 Plus solvent pump, a MX-2080-32 Dynamic Mixer, a MD-2010 Plus multi-wavelength detector and a LC-Net II / ADC controller. Analytical HPLC was performed using YMC Hydrosphere C-18 (250 × 4.6 mm, particle size: S-5 µm) column with water containing 0.1% (v/v) trifluoroacetic acid as solvent A and acetonitrile containing 0.1% (v/v) trifluoroacetic acid as solvent B at a flow rate of 1.0 mL / min. The gradient program was as follows: 0% B (0–5 min), 0–100% B (5–30 min), and 100% B (30–35 min). High-resolution mass spectra (HRMS) data were acquired in positive ion mode using micrOTOF-QII (Bruker) with an electrospray ionization (ESI) source. Nuclear magnetic resonance (NMR) spectra were acquired on a JMM ECS-400 (JEOL) spectrometer with 400 MHz for proton (^{1}H NMR) and 100 MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ). Preparative HPLC was performed on a HPLC system from JASCO equipped with a PU-4086-Binary solvent pump, a MD-2010 Plus multi-wavelength detector and a LC-Net II / ADC controller. Samples were injected onto YMC Hydrosphere C-18 (250 × 20 mm, particle size: S-5 µm) column at room temperature with water containing 0.1% (v/v) trifluoroacetic acid as solvent A and acetonitrile containing 0.1% (v/v) trifluoroacetic acid as solvent B at a flow rate of 1.0 mL / min. The gradient program was as follows: 0% B (0–5 min), 0–100% B (5–30 min), and 100% B (30–35 min). HPLC was used to establish the purity of target compounds. All final compounds had > 95% purity using the HPLC methods described above.

General preparation of samples:

***S*-glutathionyl ethylenesulfonyl fluoride (1)**

To a round-bottom flask was added glutathione (1.53 g, 4.97 mmol) and H_2O (18.0 mL) and Ar bubbling for 5 min. To a stirred solution was added ethenesulfonyl fluoride (453.4 µL, 5.47 mmol) in acetonitrile (2.0 mL). The reaction was stirred for 24 h at room temperature. The reaction mixture was filtered through a membrane filter (0.45 µm) and purified by preparative RP-HPLC to afford the desired product (1.05 g, 51%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ: 4.64 (1H, dd, J = 8.8, 5.2 Hz), 4.07–3.98 (5H, m), 3.17–3.10 (3H, m), 2.97 (1H, dd, J = 14.4, 8.8 Hz), 2.62–2.55 (2H, m), 2.26–2.20 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ: 174.5, 173.0, 172.5, 172.0, 52.9, 52.6, 50.4, 50.3, 41.2, 32.9, 31.0, 25.6, 24.6; HRMS (ESI) m/z calculated for $C_{12}H_{21}FN_3O_8S_2$ $[M+H]^+$ 418.0749, found 418.0749.

***S*-glutathionyl propyl boronicanhydride (2)**

To a round-bottom flask was added 2-(bromopropyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (32 µL, 0.15 mmol) and DMF (800 µL) and Ar bubbling for 5 min. To a stirred solution was added glutathione (30.7 mg, 0.1 mmol) in H_2O (100 µL) and 2.5 M NaOH (100 µL). The reaction was

stirred during 24 h at rt. The product was purified by preparative RP-HPLC to afford the desired product (15.8 mg, 33%) as a colorless oil. ^{1}H NMR (400 MHz, CD_3OD) δ: 4.56 (1H, dd, *J* = 9.2, 5.2 Hz), 4.04 (1H, t, *J* = 6.4 Hz), 3.97 (2H, s), 3.01 (1H, dd, *J* = 14.2, 5.6 Hz), 2.74 (1H, dd, *J* = 14.2, 9.2 Hz), 2.61–2.49 (4H, m), 2.28–2.13 (2H, m), 1.69–1.61 (2H, m), 0.88 (2H, t, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ: 174.5, 173.4, 172.7, 171.7, 54.3, 53.7, 49.8, 41.8, 35.5, 34.4, 32.5, 27.2, 25.0; HRMS (ESI) m/z calculated for $C_{13}H_{23}BN_3O_7S$ $[M+H]^+$ 376.1347, found 376.1346.

***S*-glutathionyl propyl iodide (3)**

To a round-bottom flask was added glutathione diisopropylethylamine salt (329.3 mg, 0.582 mmol) and DMF (4.3 mL) and Ar bubbling for 5 min. To a stirred solution was added DBU (173.5 µL, 1.16 mmol) in CH_3OH (1.5 mL) and 1, 3-diiodo propane (344.4 mg, 1.16 mmol) in THF (0.5 mL). The reaction was stirred during 30 min at 50 °C. The reaction mixture was quenched with 1N HCl aq. to pH = 4 and concentrated in vacuo. The product was purified by preparative RP-HPLC to afford the desired product (303.8 mg, 89%) as pale yellow oil. ^{1}H NMR (400 MHz, D_2O) δ: 4.54 (1H, dd, *J* = 8.6, 5.2 Hz), 4.07 (1H, t, *J* = 6.8 Hz), 3.98 (2H, s), 3.28 (2H, t, *J* = 6.8 Hz), 3.02 (1H, dd, *J* = 14.4, 5.6 Hz), 2.84 (1H, dd, *J* = 14.4, 8.8 Hz), 2.68–2.54 (4H, m), 2.26–2.17 (2H, m), 2.03–1.96 (2H, m); ^{13}C NMR (100 MHz, CD_3OD) δ: 174.4, 173.2, 172.7, 171.5, 54.3, 53.5, 41.8, 34.5, 34.0, 33.4, 32.4, 27.1, 4.98; HRMS (ESI) m/z calculated for $C_{13}H_{23}IN_3O_6S$ $[M+H]^+$ 476.0347, found 476.0349.

N-Biotinyl-3,6-dioxaoctane-1,8-diamine trifluoroacetate salt

(+)-Biotin (101.9 mg, 0.417 mmol), *N*-Boc-3,6-dioxa-1,8-octanediamine (129.5 mg, 0.522 mmol) and HOBt (112.7 mg, 0.834 mmol) were dissolved in DMF (4.2 mL) and were cooled to 0 °C under argon. EDC•HCl (159.9 mg, 0.834 mmol) was added and the reaction mixture was stirred for 1.5 h at 0 °C and then was allowed to reach room temperature. After 16 h of stirring, the solvent was evaporated, and the resulting yellow oil was purified by column chromatography (C/M 15/1 to 9/1) to afford *N*-Boc-*N'*-biotynyl-3,6-dioxaoctane-1,8-diamine (227.9 mg, quant.) as white solid. The compound (202.7 mg, 0.427 mmol) was solved in CH_2Cl_2 (4.2 mL) and to the solution was added trifluoroacetic acid (TFA) (2.1 mL). After 30 min of stirring at room temperature, the solvent was evaporated, and the resulting oil was dissolved in CH_3OH (5 mL) and was evaporated to remove any residual TFA. This process was repeated twice again leading to the titled compound (226.7 mg, 100%) as brown oil that was used in the next step without any further purification.

9-BBN protected GS-ESF biotin conjugate

GS-ESF (613.6 mg, 1.47 mmol) was suspended in CH_3OH (9.8 mL) under Ar. 9-BBN (3.2 mL, 0.5 M in THF) was added dropwise. The resulting solution was stirred at reflux for 3 h, the conversion to the dual-protected amino acid was nearly quantitative, as assessed by ninhydrin stained TLC (4:1:1 ^{n}BuOH/AcOH/H_2O). The residue was purified by preparative RP-HPLC to afford the desired product (616.8 mg, 78%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.39–6.29 (2H, m), 4.64 (1H, dd, *J* = 8.8, 5.6 Hz), 3.99–3.88 (4H, m), 3.80–3.74 (1H, m), 3.14–3.01 (3H, m), 2.84 (1H, dd, *J*

= 14.4, 8.8 Hz), 2.65–2.55 (2H, m), 2.26–2.19 (1H, m), 2.14–2.07 (1H, m), 1.91–1.65 (10H, m), 1.48–1.45 (2H, m), 0.56 (2H, s); ^{13}C NMR (100 MHz, CD_3OD) δ: 176.9, 175.3, 172.8, 56.2, 53.8, 51.7, 51.6, 41.9, 34.7, 33.1, 32.5, 32.4, 32.3, 27.3, 26.0, 25.7, 25.2. *N*-biotinyl-3,6-dioxaoctane-1,8-diamine TFA salt (281.5 mg, 0.752 mmol) was dissolved in DMF (5.0 mL) and the pH adjusted to 8 using trimethylamine (85.0 µL, 0.610 mmol). 9-BBN protected GS-ESF (269.3 mg, 0.501 mmol), HOBt (101.6 mg, 0.752 mmol) and EDC•HCl (144.1 mg, 0.752 mmol) were added to above mixture and stirred for 40 h at room temperature. The mixture was concentrated *in vacuo* and purified by flash column chromatography to afford product as a white solid (191.0 mg, 43%). ^{1}H NMR (400 MHz, CD_3OD) δ: 8.55–8.48 (1H, m), 6.39–6.25 (2H, m), 4.59 (1H, dd, *J* = 7.2, 6.0 Hz), 4.50 (1H, dd, *J* = 7.8, 5.2 Hz), 4.32 (1H, dd, *J* = 7.8, 4.8 Hz), 3.99–3.94 (2H, m), 3.89 (2H, s), 3.84–3.77 (1H, m), 3.62 (4H, s), 3.56 (4H, q, *J* = 5.2 Hz), 3.41–3.37 (4H, m), 3.21 (1H, dt, *J* = 7.2, 4.4 Hz), 3.13–3.04 (3H, m), 2.96–2.86 (2H, m), 2.72 (1H, d, *J* = 12.8 Hz), 2.63–2.59 (2H, m), 2.27–2.21 (3H, m), 2.14–2.07 (1H, m), 1.89–1.85 (4H, m), 1.79–1.56 (10H, m), 1.48–1.41 (4H, m), 0.57 (2H, s); ^{13}C NMR (100 MHz, CD_3OD) δ: 174.9, 174.1, 173.4, 170.9, 169.2, 164.0, 69.3, 69.2, 68.6, 68.4, 61.3, 59.6, 55.0, 54.1, 52.2, 49.7, 49.5, 41.5, 39.1, 38.4, 38.3, 34.8, 32.4, 31.0, 30.5, 30.4, 30.3, 27.7, 27.5, 25.4, 24.9, 24.0, 23.7, 23.2; HRMS (ESI) m/z calculated for $C_{36}H_{62}BFN_7O_{11}S_3$ $[M+H]^+$ 894.3747, found 894.3749.

N-Biotinyl-3,6-dioxaoctane-1,8-diamine-N'-glutathionyl ethylenesulfonyl fluoride (4)

9-BBN protected GS-ESF biotin conjugate (5.2 mg, 5.82 µmol) was dissolved in 1,4-dioxane : H_2O (1 : 2.7) mixture and 12 N HCl aq. (24.0 µL, 0.291 mmol) was added. The resulting solution was stirred for 2 days at 40 °C. The reaction mixture was concentrated and purified by preparative RP-HPLC to give the titled compound (0.6 mg, 12%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ: 4.62 (2H, dd, *J* = 8.2, 5.6 Hz), 4.43 (1H, dd, *J* = 8.2, 4.8 Hz), 4.07–4.02 (2H, m), 3.94 (2H, s), 3.84 (1H, t, *J* = 6.8 Hz), 3.69 (4H, s), 3.64 (4H, dd, *J* = 5.2 Hz), 3.45–3.39 (4H, m), 3.37–3.32 (1H, m), 3.17–3.12 (3H, m), 3.03–2.94 (2H, m), 2.79 (1H, d, *J* = 13.2 Hz), 2.55 (2H, q, *J* = 7.6 Hz), 2.29 (2H, t, *J* = 7.6 Hz), 2.18 (2H, dd, *J* = 14.6, 7.6 Hz), 1.75–1.60 (4H, m), 1.46–1.41 (2H, m); ^{13}C NMR (100 MHz, D_2O) δ: 177.0, 174.4, 172.5, 171.6, 171.0, 165.4, 69.5, 68.9, 68.8, 62.1, 60.3, 55.4, 53.1, 52.3, 50.4, 50.3, 42.6, 39.7, 39.0, 38.9, 35.5, 32.8, 30.9, 27.9, 27.7, 25.5, 25.2, 24.6; HRMS (ESI) m/z calculated for $C_{28}H_{49}FN_7O_{11}S_3$ $[M+H]^+$ 774.2631, found 774.2633.

Enzyme preparation:

Human GSTs were expressed essentially as described previously[1] and purified from bacterial lysate using GSTrap HP (GE Healthcare UK Ltd, Little Chalfont, England) according to the manufacturer's instructions. The high purity of the enzyme was confirmed by SDS-PAGE with Commassie Brilliant Blue G-250. Protein concentration was determined from the absorbance at 280 nm.

Measurement of GST Activity with Standard Substrates:

The specific activity of the purified cytosolic GSTs was measured using 1 mM GSH (PEPTIDE INSTITUTE. INC., Osaka, Japan) and 1 mM CDNB (KISHIDA CHEMICAL Co., Ltd., Osaka, Japan) as second substrate in a 96 well plate by absorbance spectrometry (Mithras LB940; Berthold Technologies, Bad Wildbad, Germany) by following the change in absorbance at 340 nm. The molar extinction coefficients used for CDNB conjugation was 9.6 $mM^{-1}cm^{-1}$. All enzyme activity measurements were performed at 37 °C. The cytosolic GSTs were assayed in 10 mM PBS buffer pH 7.4. Enzymatic activities were calculated after correction for the nonenzymatic reaction. These measurements were performed in order to validate the activity and stability of the enzymes. The specific activities were in general agreement with the values reported previously.[2]

Determination of inhibition of GSTs activity (IC_{50} determination):

Inhibition experiments with GSTs were carried out by incubating various amounts of **1**, **2**, **3** and *S*-hexyl GSH, predissolved in DMSO (10 mM) for 6 h with a fixed enzyme concentration (4 µg/mL) in 150 µL final volume of 10 mM PBS buffer pH 7.4 containing 1 mM GSH. The residual activity of GSTs was determined spectrophotometrically at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) upon the addition of the substrate 1-chloro-2,4-dinitrobenzene (CDNB, final concentration 1 mM) at 37 °C. The IC_{50} value for the inhibition of GST activity was determined by fitting the plot of residual GST activity against the inhibitor concentrations (log scale) with a sigmoidal dose-response function using ImageJ software. The reported data are average of at least three independent experiments. These measurement were performed on a microplate reader.

Time-course inactivation by 1, 2 and 3:

GSTs (4 µg/mL) were incubated with **1**, **2** and **3** (0.1 mM), pre-dissolved in DMSO, in 10 mM PBS buffer containing 1 mM GSH pH 7.4 at 37 °C. At fixed time intervals, over a period of 60 or 120 min, aliquots of the sample mixture were assayed for residual GST activity in 10 mM PBS buffer pH 7.4 containing 1 mM CDNB (Figure 4 and S4). The reported data are average of at least three independent experiments. These measurements were performed on V-650 spectrometer (JASCO, Tokyo, Japan).

Circular Dichroic Studies:

CD experiments utilizing a J-720WN (JASCO) were performed at room temperature using a quartz cell with a 1 cm path length. CD spectra were collected from 200 to 300 nm and with a scanning speed of 200 nm/min. The bandwidth was 1.0 nm and the response time was 0.5 sec. All CD spectra

were baseline-corrected for signal contributions due to the buffer. The concentration of $GSTP_{1-1}$ was 0.1 mg/mL.

Reversibility of GST inhibitors:

For each reversibility assay, $GSTP_{1-1}$ (4 μg/mL) was treated with **1**, **2**, **3** and *S*-hexyl GSH (0.1 mM) or without an inhibitor at 30 °C for 6 h in 10 mM PBS buffer pH 7.4 containing 1 mM GSH. 1 mM CDNB was then added as a substrate to start GST "standard assay" reaction. For each "wash-out assay" reaction, to remove inhibitors unbound to $GSTP_{1-1}$ before beginning the reaction with the substrate, each pre-incubated reaction mixture was loaded onto Amicon Ultra-0.5 mL Centrifugal Filters (10 kDa molecular-weight cut off) and washed three times with 10 mM PBS buffer (pH 7.4) by centrifuging at 14,000 × *g* for 20 min.

Measurement of protein by liquid chromatography-mass spectrometry (LC-MS):

Protein was subjected to LC-MS analysis (Dionex Ultimate 3000 HPLC system equipped with an autosampler, and an EXACTIVE Plus mass spectrometer (Thermo Fisher Scientific, MA, USA) fitted with a PLRS-S column [5 μm, 2.1 × 150 mm; Agilent, CA, USA]) at 60 °C using 100% water containing 0.1% (v/v) formic acid, and B, acetonitrile. The column was developed at a flow rate of 200 μL/min with an acetonitrile concentration gradient of: 5% B for 5 min, then 5% B to 90% B for 10 min, sustaining 90% B for 3 min, 90% B to 5% B for 1 min, and finally re-equilibrating with 5% B for 10 min.

Affinity Purification of Labeled $GSTP_{1-1}$ and Protein Digestion:

$GSTP_{1-1}$ (4.0 mg/mL) were pretreated with or without biotinylated GS-ESF (**4**) (final conc. 1.25 mM) at 37 °C for 60 min in 40 μL final volume of 10 mM PBS buffer pH 7.4. Samples then centrifuged (14,000 × *g*, 30 min, 4 ℃) to exclude unreacted ligands. The solution was rotated with 50 μL of streptavidin beads (TAMAGAWA SEIKI Co., Ltd., Nagano, Japan, washed three times with PBS to remove storage buffer) for 60 min at 4 °C before being washed by washing buffer (0.2% (w/v) SDS, 6M urea, 50 mM Tris (pH 7.4), 50 mM DTT). 2 × SDS-PAGE loading buffer was added to the samples and the mixture was boiled at 90 °C for 10 min. 10 μL of this solution was separated by SDS-PAGE at 40 mA for 90 min on 15% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue G-250. Stained gel was visualized on ChemiDoc XRS (Bio-Rad, CA, USA). The specific band was excised from the Coomassie Brilliant Blue-stained gel and subjected to in-gel tryptic digestion[3] and subsequent MS analysis.

Protein Target Identification by LC-MS/MS:

HeLa cells were grown in 60-80% confluence DMEM media with FBS on 10 cm cell culture plates. Cells were harvested and sonicated to lyse to form whole cell lysates. These lysates were separated by centrifugation at 12000 rpm for 10 min at 4 °C to yield soluble and membrane proteins. The supernatant was collected and the pellet was discarded. Protein concentrations for soluble lysates were determined using a standard BCA assay (Pierce™ BCA Protein Assay Kit #23225). HeLa soluble protein lysates (1196.5 μg/mL) were yield and concentrated to 7121 μg/mL using Amicon Ultra-0.5 mL Centrifugal Filters (3K). HeLa lysates (70 μL, 7121 μg/mL) were pretreated with biotinylated GS-ESF (final conc. 1 mM) or PBS buffer (pH 7.4) at 37 °C for 1 hr. Samples then centrifuged (14000 × *g*, 30 min, 4 ℃) to exclude unreacted ligands. The solution was rotated with 50 μL of streptavidin beads for 90 min at rt before being washed by washing buffer (0.2% (w/v) SDS, 6M urea, 50 mM Tris (pH 7.4), 50 mM DTT). 2 × SDS-PAGE loading buffer was added to the samples and 10 μL of this solution was separated by SDS-PAGE at 40 mA for 90 min on 12.5% polyacrylamide gel. The same sample was analyzed in two gels. One gel underwent a typical procedure for Oriole staining. Stained gel was visualized on ChemiDoc XRS. The other gel used for western blot analysis and Streptavidin-Horseradish Peroxidase Conjugates detected specific proteins labeled by biotinylated ligand in a western blot. The specific band was excised from the Oriole-stained gel and subjected to in-gel tryptic digestion[3] and subsequent MS analysis.

Protein Digestion and Analysis by LC-MS/MS:

Samples were analyzed by nano-flow reverse phase liquid chromatography followed by tandem MS, using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, MA, USA). A capillary reverse-phase HPLC-MS/MS system was composed of a Dionex U3000 gradient pump equipped with a VICI CHEMINERT valve and the Q Exactive equipped with a Dream Spray nano-electrospray ionization (NSI) source (AMR, Tokyo, Japan). Samples were automatically injected using a PAL System autosampler (CTC Analytics, Zwingen, Switzerland) and a peptide L-trap column (Trap and Elute mode, Chemical Evaluation Research Institute, Tokyo, Japan) attached to an injector valve for desalinating and concentrating peptides. After washing the trap with MS-grade water containing 0.1% (v/v) trifluoroacetic acid and 2% (v/v) acetonitrile (solvent C), the peptides were loaded onto a separation capillary C18 reverse-phase column (NTCC-360/100-3-125, 125×0.1 mm, Nikkyo Technos, Tokyo, Japan) by switching the valve. The eluents used were: A, 100% water containing 0.5% (v/v) acetic acid, and B, 80% (v/v) acetonitrile containing 0.5% (v/v) acetic acid. The column was developed at a flow rate of 0.5 μL/min with an acetonitrile concentration gradient of: 5% B to 35% B for 20 min, then 35% B to 95% B for 1 min, sustaining 95% B for 3 min, 95% B to 5% B for 1 min, and finally re-equilibrating with 5% B for 10 min. Xcalibur 3.0.63 (Thermo Fisher Scientific) was used to record peptide spectra over the mass range of m/z 350–1800. Repeatedly, MS spectra were recorded followed by 20 data-dependent high-energy

collisional dissociation (HCD) MS/MS spectra generated from the 20 highest intensity precursor ions. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by Proteome Discoverer 2.0.0.802 (Thermo Fisher Scientific). Searches were performed using SEQUEST (Thermo Fisher Scientific) against $GSTP_{1-1}$ peptide sequence database or homo sapience (taxon identifier 9606 (uniprot)). Search parameters were set as follows: enzyme selected with two maximum missing cleavage sites, a mass tolerance of 10 ppm for peptide tolerance, 0.02 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of oxidation (M) and biotinylated GS-ESF (S, T, Y). Peptide identifications were based on a significant Xcorr value (high confidence filter). Peptide identification and modification information returned from SEQUEST were manually inspected and filtered to obtain confirmed peptide identification and modification lists of HCD MS/MS.

Crystallization and Structural determination:

The protein solution, containing 7.4 mg/mL $GSTP_{1-1}$ and 3.3 mM GS-ESF (**1**), was prepared. The crystals were prepared by mixing the protein solution and the crystallization solution, containing 100 mM MES (pH 6.0), 40 mM $CaCl_2$, 20 mM DTT, 17.6% (w/v) PEG8000 using the hanging drop vapor-diffusion method at 277 K. An X-ray diffraction data set was collected at Spring-8 BL38B1 (Hyogo, Japan).[4] The reflections were processed using the HKL-2000 program suite.[5] Structure was solved by using a molecular replacement method with Molrep in CCP4i software suits.[6] The ligand-bound human $GSTP_{1-1}$ structure (PDB code 2A2R) was used as the reference model. Initial model was refined by using programs, Phenix[7] and Coot.[8] The refinement statistics were given in Table S2.

Molecular Dynamics Simulation of a $GSTP_{1-1}$ dimer with and without an inhibitor 1:

The microscopic state of a $GSTP_{1-1}$ dimer with and without GS-ESF (**1**) was studied in a water box using the molecular dynamics (MD) simulations. The 100-ns calculations were performed three times for each system. The system contained a $GSTP_{1-1}$, **1**, 7 sodium ions, and ca. 19,000 water molecules are added. A $GSTP_{1-1}$ was described using AMBER14SB force field[9] and **1** was done using the general AMBER force field (GAFF)[10] and the partial charge of **1** atoms was determined as RESP charge.[11] The model of amino-terminal γ-glutamyl residue from GSH was used from the paper written by Rigsby et al.[12] The water molecules were described using the TIP3P model.[13] The ion parameter were described using Joung and Cheatham model.[14]

The simulations were conducted with the NPT ensemble with a temperature of 300 K and a pressure of 1 bar in a truncated dodecahedron box with dimensions of approximately 96.8 Å. The temperature was controlled using a Langevin thermostat with a viscosity of 0.5 ps^{-1}. The pressure was controlled by a Parrinello-Rahman barostat[15] with relaxation times of 2.0 ps. The electrostatics were treated

using the particle mesh Ewald (PME) method[16] with a 10.0-Å cutoff distance. The van der Waals interactions were expressed using the cutoff method with 10.0-Å cutoff distances. The covalent bonds for the hydrogen atoms in the surfactants were constrained using the linear constraint solver (LINCS).[17] The covalent bonds in the water were constrained using the SETTLE algorithm.[18] The integration time step was 2 fs. The simulations were conducted using the GROMACS 2016 simulator.[19]

Prediction of pK_a shifts:

PROPKA3.1 was used to predict the effects of the mutations on the pK_a of $GSTP_{1\text{-}1}$.[20,21] The structure after 100-ns MD simulation was used for the prediction. The natural amino acid was modeled by amber force field.[9] The amino-terminal γ-glutamyl residue and compound **1** were described by the same model used in MD simulation.[11,12]

Supplementary Experiments

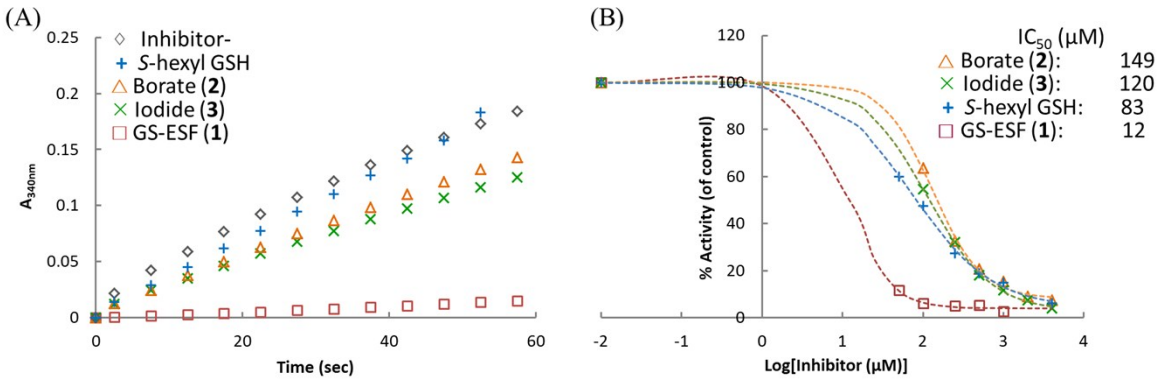


Fig. S1 (A) *In vitro* inhibitory activity of each compound (0.1 mM) to $GSP_{1\text{-}1}$ function. Absorbance (340 nm) of 0.5 mM GSH reacting with 0.2 mM CDNB in the presence of $GSP_{1\text{-}1}$ was measured at a pH of 7.4. (B) Concentration dependency of the inhibitory activity for $GSTP_{1\text{-}1}$ *in vitro*. Inhibitors were pre-incubated in the presence of 0.5 mM of GSH and 4 μg/mL of $GSTP_{1\text{-}1}$ for 0.5 h. After pre-incubation, 0.2 mM of CDNB was added and UV absorbance measured at 340 nm. All measurements were carried out at a pH of 7.4, 37 °C, in 10 mM PBS buffer.

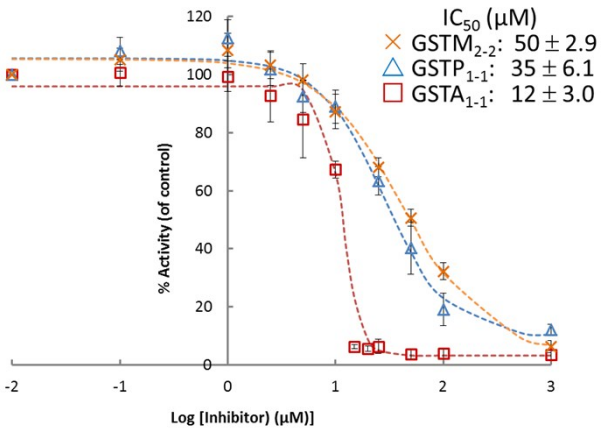


Fig. S2 *In vitro* each GST subtype activity data monitored in the presence of increasing concentrations of **1** demonstrated a concentration-dependent decrease in GSTs activity. Inhibitors of each concentration were pre-incubated in the presence of 1.0 mM of GSH and 4 μg/mL of GST for 6 h. After that 1.0 mM of CDNB was added and UV absorbance was measured at 340 nm. All measurements were carried out at a pH of 6.9, 30 °C, in 200 mM sodium phosphate buffer. Data are the mean ± SEM (n = 3).

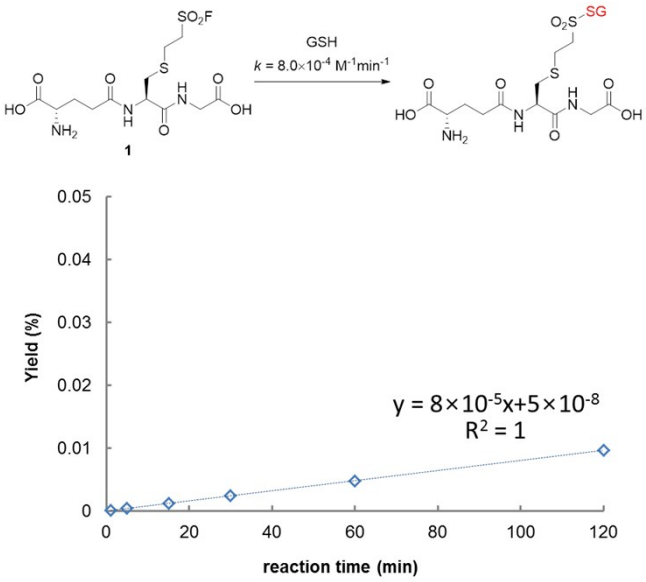


Fig. S3 The yield of non-enzymatic reaction between GSH and **1**. Compound **1** (50 mM) and GSH (0.4 M, 1.0 M, 1.4 M) were incubated at 37 °C in 10 mM PBS buffer (pH 7.4) for each time. After that 20 μL of reaction mixture was sampled and HPLC analysis was performed.

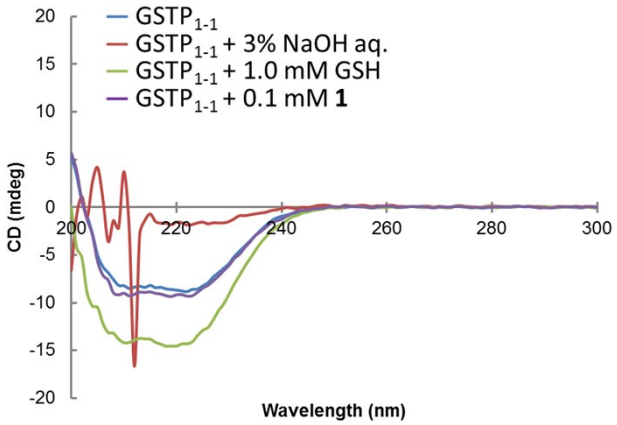


Fig. S4 CD spectra of $GSTP_{1\text{-}1}$ in the absence or presence of 3% (w/v) NaOH aq. (as a denaturant), GSH or **1**.

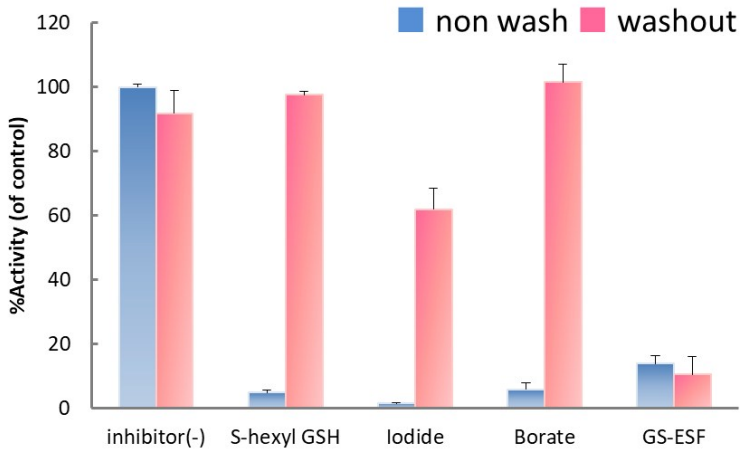


Fig. S5 $GSTP_{1\text{-}1}$ activity treated with each inhibitor before and after washout. For each reversibility assay, 4 μg/mL of $GSTP_{1\text{-}1}$ was pre-incubated with each inhibitor (*S*-hexyl GSH and GS-ESF: 0.1 mM, Iodide and Borate: 1.0 mM) at 30 °C for 6 h. 1.0 mM CDNB was then added as substrate to start the GST "standard assay" reaction and the absorbance at 340 nm of each reaction mixture was measured. For each "wash-out assay" reaction, to remove the inhibitor unbound to GST before beginning the reaction with the substrate, each pre-incubated reaction mixture was loaded onto amicon ultra -0.5 (10 kDa molecular-weight cut off) and washed thrice with the assay buffer by centrifugation at 14,000 × *g* for 20 min. Data are the mean ± SEM (n = 3).

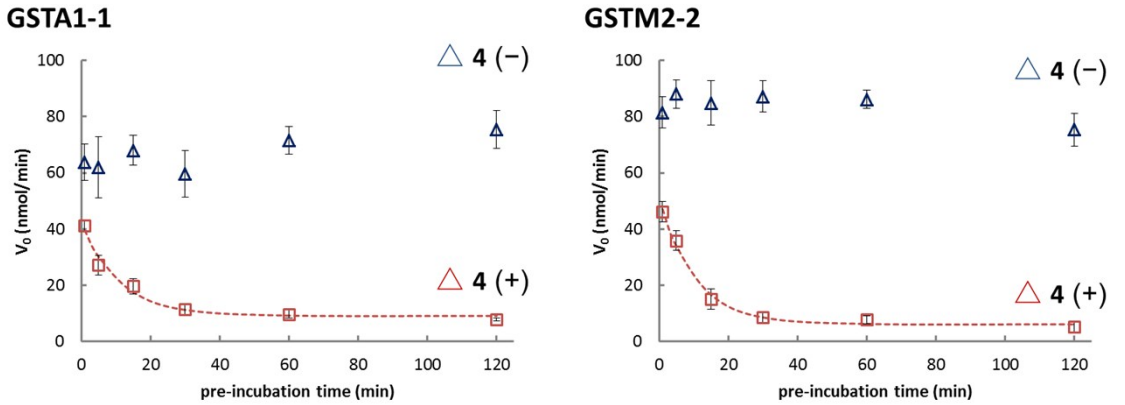


Fig. S6 Time-dependent inhibition of GSTs in the presence or absence of **4**. $GSTA_{1\text{-}1}$ and $GSTM_{2\text{-}2}$ (4 μg/mL) was pre-incubated for different periods of time with GSH (1 mM) in the presence or absence of **4** (0.1 mM). After the pre-incubation, 0.2 mM of CDNB was added and UV absorbance was measured at 340 nm. All measurements were carried out at a pH of 7.4, 37 °C, in 10 mM PBS buffer. Data are the mean ± SEM (n = 3).

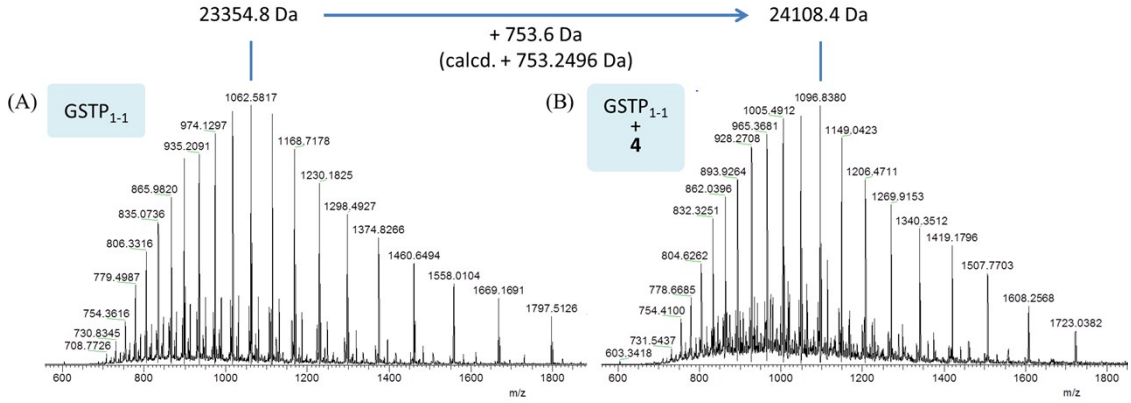


Fig. S7 Intact-protein MS spectra of $GSTP_{1\text{-}1}$ (A) and **4**-$GSTP_{1\text{-}1}$ mixture (B) before deconvolution. The average mass of monovalent ions is shown above each peak. The difference in average mass was consistent with the calculated values before and after addition of **4**.

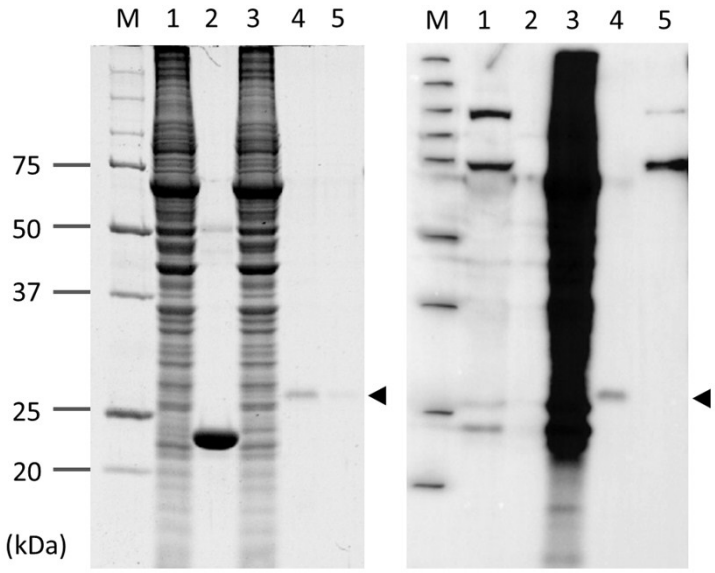
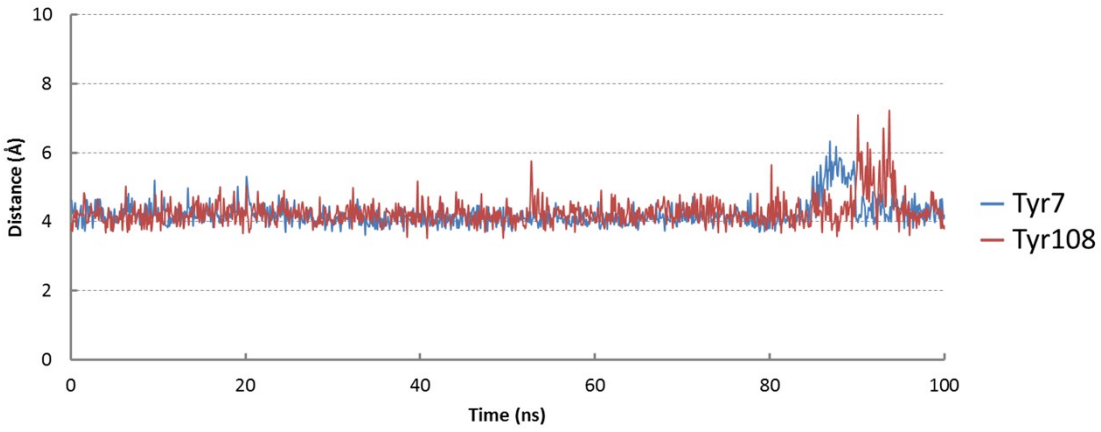
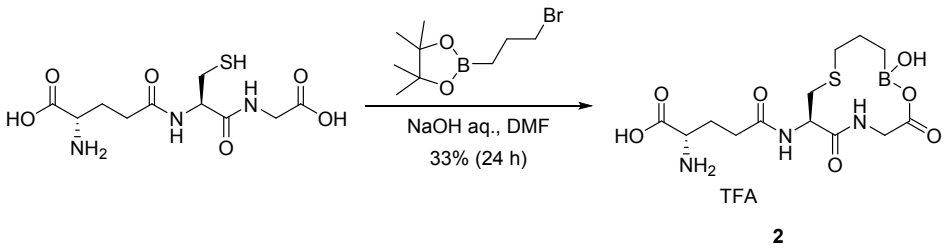


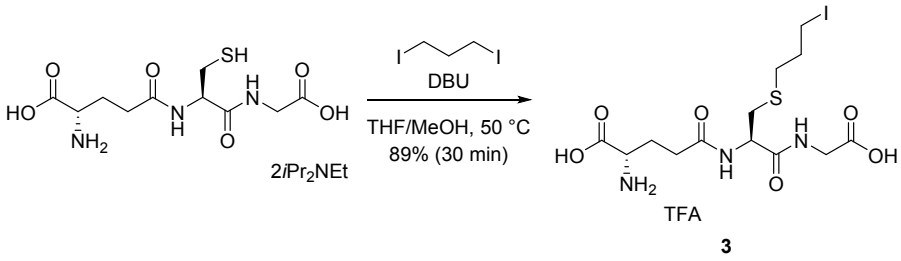
Fig. S8 HeLa lysates (70 μL, 7.1 mg/mL) were treated with 4 (1 mM) at 37 °C for 1 h. Lysates were subjected to pull-down assay using avidin beads, and separated by SDS-PAGE and analyzed by in-gel fluorescence and western blotting. The arrowhead indicates a 25 kDa signal, presumably caused by labeling of GSTs. (left) 12.5% SDS-PAGE [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 20 mA (c.c.), 100 min, Stained with oriole], $GSTP_{1\text{-}1}$ monomer: 23 kDa; (right) Western Blot analysis; Biotinylated proteins were detected using HRP-labelled Streptavidin ($\times 10^4$ dilution) and assessed using ECL Western blotting reagent. In this figure, the chemiluminescence image and the live image are superimposed. Lane 1, HeLa lysate, Lane 2, purified $GSTP_{1\text{-}1}$, Lane 3: **4**-treated samples untreated with avidin beads, Lane 4: **4**-treated samples after pull-down assay, Lane 5: negative control, M: protein molecular weight markers.



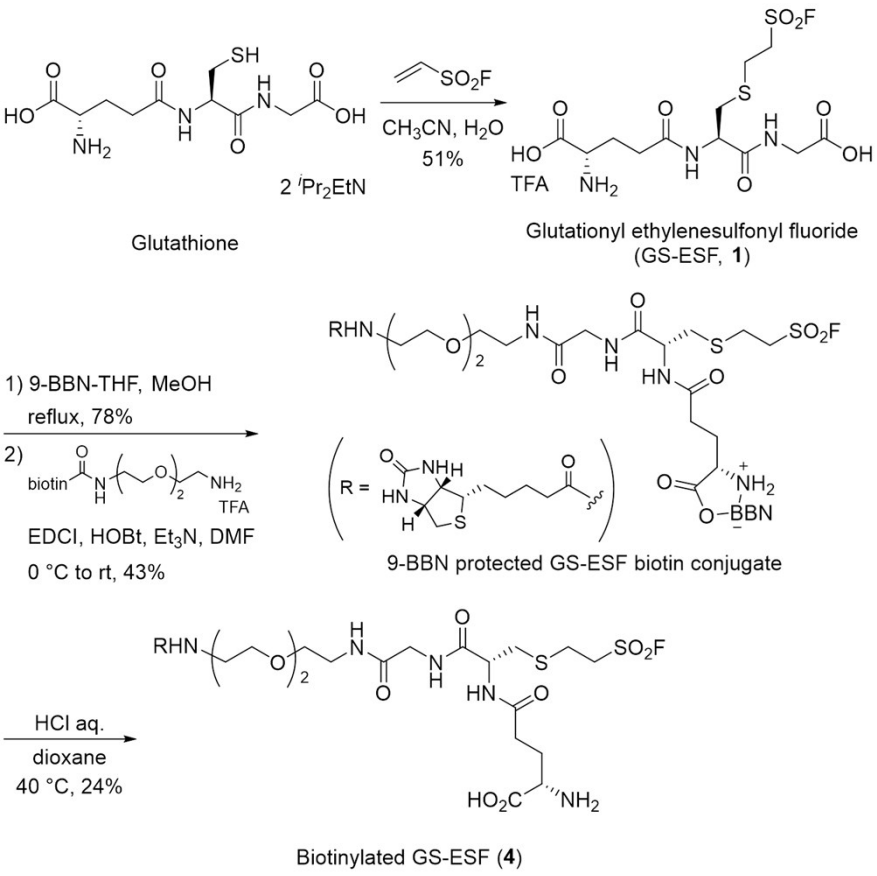
Fig, S9 Change in the distance between each of the side chain oxygen atoms of Tyr7 and Tyr108 and the sulfur atom of GS-ESF in 100 ns. The average distance was 4.2 Å in each case.



Scheme S1. Synthesis of **2**.



Scheme S2. Synthesis of **3**.



Scheme S3. Synthesis of **1** and **4**.

Table S1. HeLa lysates treated with **4** (1 mM) were subjected to pull-down assay using avidin beads and LC-MS/MS analysis. The all proteins detected only in **4**-treated samples compared to the negative control are listed. It is listed in the order of the number of unique peptides.

Protein	Molecular Weight (kDa)
Triosephosphate isomerase	26.7
Zymogen granule protein 16 homolog B	22.7
Ig alpha-1 chain C region	36.5
Glyceraldehyde-3-phosphate dehydrogenase	31.5
Glutathione *S*-transferase P	23.3
Isoform 2 of Membrane-associated progesterone receptor component 2	26.2
Isoform 2 of Cysteine-rich protein 2	30.2
BPI fold-containing family A member 1	26.7

Table S2. Data collection and refinement statistics

Crystal parameters	
Space group	C121
Unit-cell parameters (Å)	
a, b, c	78.04, 89.43, 68.97
α, β, γ	90.00, 98.43, 90.00
Data processing	
Wavelength (Å)	1.00
Resolution (Å)	50–1.90
No. of measured reflection	137,352
completeness (%)	100 (100)
Redundancy	3.7 (3.7)
I/σ	10.7 (1.7)
R_{merge} (%)	16.7 (80.3)
Refinement parameters	
Resolution range	42.13–1.900
No. of reflection	36,842
R	0.198
R_{free}	0.252
No. of. atom	3,998
Protein	3,264

Water	627
Ligand	86
Average *B* value (Å^2)	20.3
r.m.s.d.	
bond lengths (Å)	0.02
bond angles (°)	1.41
PDB accession code	5X79

Values in parentheses are for the outermost shell.

Supplementary text:

The molecular information of compound 1 used in MD simulation and pK_a calculation. It is written in Amber prep format.

```
    0      0      2

This is a remark line
esf.res
ESF     INT    0
CORRECT         OMIT DU      BEG
  0.0000
   1   DUMM   DU       M       0   -1   -2        0.000          .0            .0          .00000
   2   DUMM   DU       M       1    0   -1        1.449          .0            .0          .00000
   3   DUMM   DU       M       2    1    0        1.523      111.21            .0          .00000
   4   N          N            M        3        2     1        1.540      111.208    -180.000    -0.415700
   5   H          H            E        4        3     2        0.996       63.454     -94.507     0.271900
   6   CA         CT           M        4        3     2        1.438       77.713      32.650     0.153604
   7   CB         CT           3        6        4     3        1.542      114.368    -162.329 -0.036314
   8   SG         S            S        7        6     4        1.824      113.891      55.449    -0.266646
   9   CD         CT           3        8        7     6        1.815      100.054    -147.659 -0.030388
  10   CE         CT           3        9        8     7        1.530      112.160      80.422    -0.168853
  11   SZ         S            3       10        9     8        1.773      112.858    -177.295     1.146125
  12   OH1    O            E       11      10      9        1.413      110.459    -172.836 -0.530392
  13   FH         F            E       11      10      9        1.567       98.723     -61.849     -0.219734
  14   OH2    O            E       11      10      9        1.415      110.897      49.043 -0.530392
  15   HE1    H1           E       10       9      8        1.083      112.571     -58.948    0.127481
  16   HE2    H1           E       10       9      8        1.081      111.725      62.962    0.127481
```

```
 17  HD1   H1    E     9     8     7     1.081   109.957   -43.324  0.094200
 18  HD2   H1    E     9     8     7     1.081   105.955  -159.346 0.094200
 19  HB1   H1    E     7     6     4     1.083   109.367   177.723  0.070471
 20  HB2   H1    E     7     6     4     1.077   107.293   -65.121  0.070471
 21  HA    H1    E     6     4     3     1.087   108.847    79.722  0.013084
 22  C     C     M     6     4     3     1.531   107.214   -37.635  0.597300
 23  O     O     E    22     6     4     1.203   121.230   -18.853 -0.567900


LOOP

IMPROPER
  -M   CA   N    H
  CA   +M   C    O

DONE
STOP
```

References

1 J. Zhang, A. Shibata, M. Ito, S. Shuto, Y. Ito, B. Mannervik, H. Abe and R. Morgenstern, *J. Am. Chem. Soc.*, 2011, **133**, 14109–14119.

2 A. S. Johansson, G. Stenberg, M. Widersten and B. Mannervik, *J. Mol. Biol.*, 1998, **278**, 687–98.

3 J. Rosenfeld, J. Capdevielle, J. C. Guillemot and P. Ferrara, *Anal. Biochem.*, 1992, **203**, 173–179.

4 G. Ueno, H. Kanda, R. Hirose, K. Ida, T. Kumasaka and M. Yamamoto, *J. Struct. Funct. Genomics*, 2006, **7**, 15–22.

5 Z. Otwinowski and W. Minor, *Methods Enzymol.*, 1997, 276, 307–326.

6 A. Vagin and A. Teplyakov, *J. Appl. Crystallogr.*, 1997, **30**, 1022–1025.

7 P. D. Adams, P. V. Afonine, G. Bunkóczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L. W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger and P. H. Zwart, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2010, **66**, 213–221.

8 P. Emsley, B. Lohkamp, W. G. Scott and K. Cowtan, *Acta Crystallogr. Sect. D Biol. Crystallogr.*, 2010, **66**, 486–501.

9 J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser and C. Simmerling, *J. Chem. Theory Comput.*, 2015, **11**, 3696–3713.

10 J. M. Wang, R. M. Wolf, J. W. Caldwell, P. a Kollman and D. a Case, *J. Comput. Chem.*, 2004, **25**, 1157–1174.

11 C. I. Bayly, P. Cieplak, W. Cornell and P. A. Kollman, *J. Phys. Chem.*, 1993, **97**, 10269–10280.

12 R. E. Rigsby, D. W. Brown, E. Dawson, T. P. Lybrand and R. N. Armstrong, *Arch. Biochem. Biophys.*, 2007, **464**, 277–283.

13 W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey and M. L. Klein, *J. Chem. Phys.*, 1983, **79**, 926–935.

14 I. S. Joung and T. E. Cheatham, *J. Phys. Chem. B*, 2008, **112**, 9020–9041.

15 M. Parrinello and A. Rahman, *J. Appl. Phys.*, 1981, **52**, 7182–7190.

16 U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee and L. G. Pedersen, *J. Chem. Phys.*, 1995, **103**, 8577–8593.

17 B. Hess, H. Bekker, H. J. C. Berendsen and J. G. E. M. Fraaije, *J. Comput. Chem.*, 1997, **18**, 1463–1472.

18 S. Miyamoto and P. A. Kollman, *J. Comput. Chem.*, 1992, **13**, 952–962.

19 M. J. Abraham, T. Murtola, R. Schulz, S. P??ll, J. C. Smith, B. Hess and E. Lindah, *SoftwareX*, 2015, **1–2**, 19–25.

20 C. R. Søndergaard, M. H. M. Olsson, M. Rostkowski and J. H. Jensen, *J. Chem. Theory Comput.*, 2011, **7**, 2284–2295.

21 M. H. M. Olsson, C. R. SØndergaard, M. Rostkowski and J. H. Jensen, *J. Chem. Theory Comput.*, 2011, **7**, 525–537.