A practical fluorogenic substrate for high-throughput screening of glutathione S-transferase inhibitors

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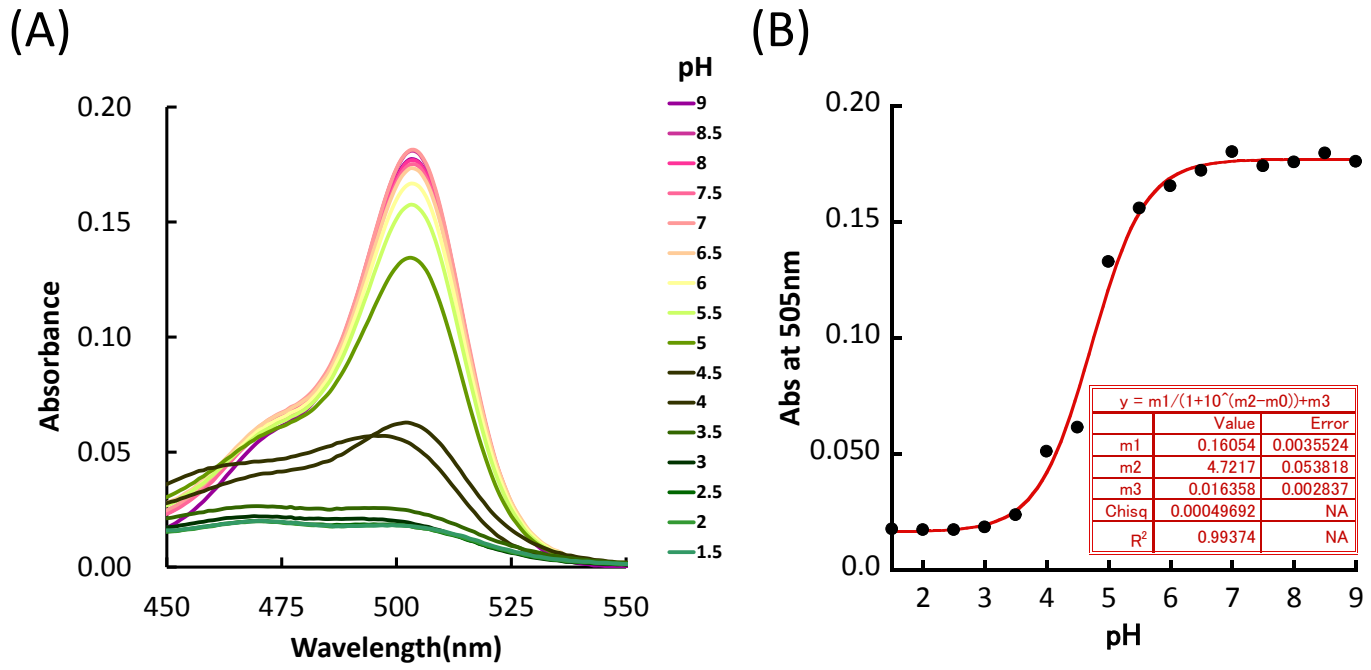


Fig. S1. pH dependence of absorbance of 3,4-DNADCF.

(A) Absorbance spectra of 2 μM 3,4-DNADCF in 100 mM sodium phosphate at various pH values. (B) pH dependence of absorbance at 505 nm. Data were fitted to the Henderson-Hasselbalch equation.

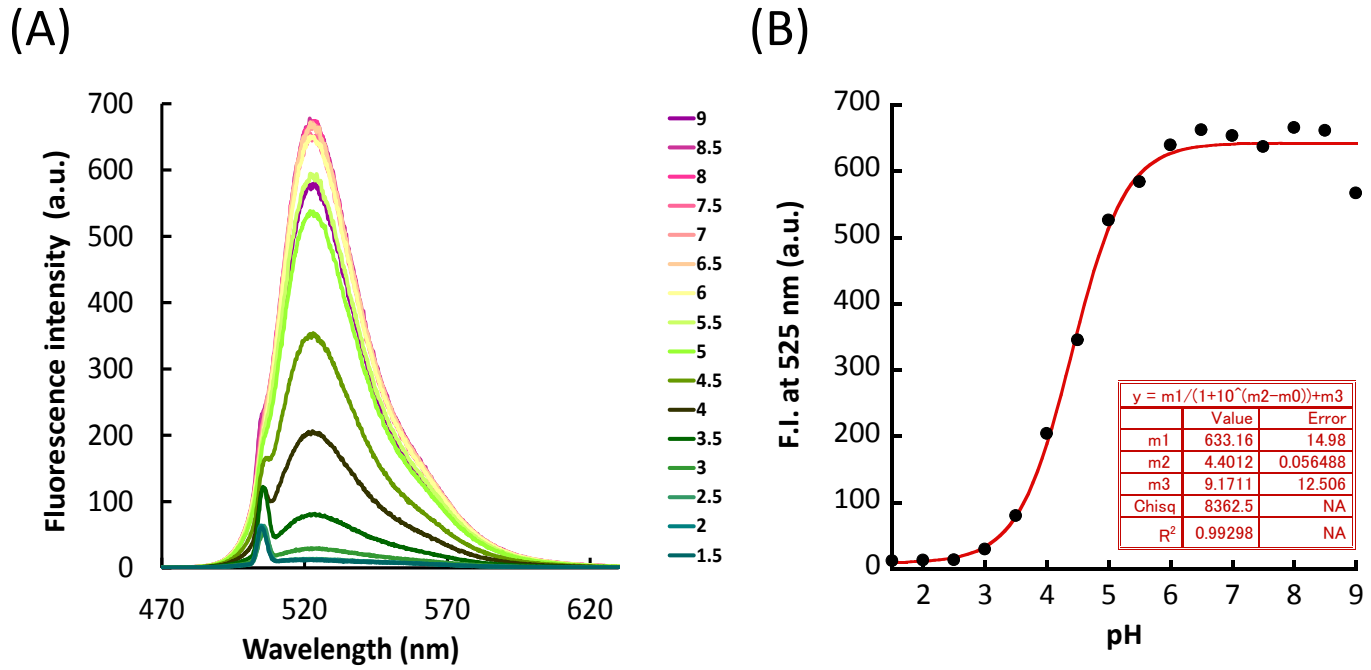


Fig. S2. pH dependence of fluorescence after the reaction.

(A) Fluorescence spectra after the reaction in 100 mM sodium phosphate at various pH values. Excitation was at 490 nm. (B) pH dependence of the fluorescence intensity at 525 nm. Data were fitted to the Henderson-Hasselbalch equation.

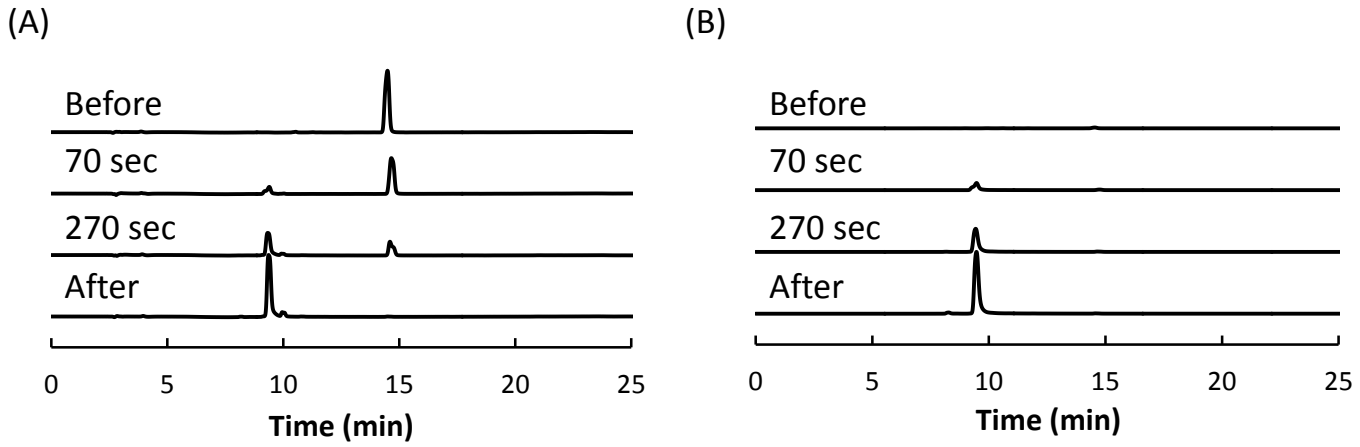


Fig. S3. Confirmation of product formation by means of HPLC.

The reaction was monitored by absorbance at 505 nm (A) and fluorescence at 525 nm with excitation at 505 nm (B). 'Before' and 'After' represent 3,4-DNADCF before the reaction and the reaction mixture after completion of the reaction, respectively. The reaction was quenched by addition of acetic acid at each time point (70 and 270 sec) and analysed. HPLC conditions were as follows. Eluents: A, 100 mM triethylamine acetate (pH 7.0); B, 80% acetonitrile. Gradient conditions: A:B = 80:20 (0 min) → 0:100 (20 min).

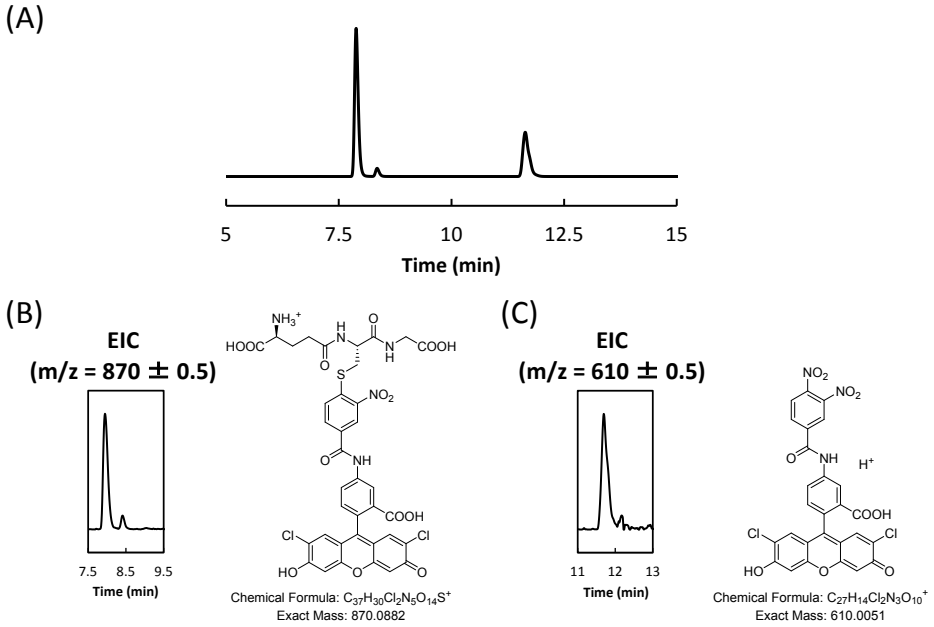


Fig. S4. Confirmation of product formation by means of LC-MS.

(A) HPLC chromatograms of reaction mixture at 270 sec in Fig. S3. Absorbance at 490 nm was recorded. (B and C) Extracted ion chromatograms (EIC) and molecular structures corresponding to (B) product (m/z = 870 ± 0.5) and (C) 3,4-DNADCF (m/z = 610 ± 0.5). LC conditions were as follows. Eluents: A, 0.1% formic acid-H_2O; B, 0.1% formic acid/20% H_2O/80% acetonitrile. Gradient conditions: A:B = 95:5 (0 min) → 5:95 (30 min).

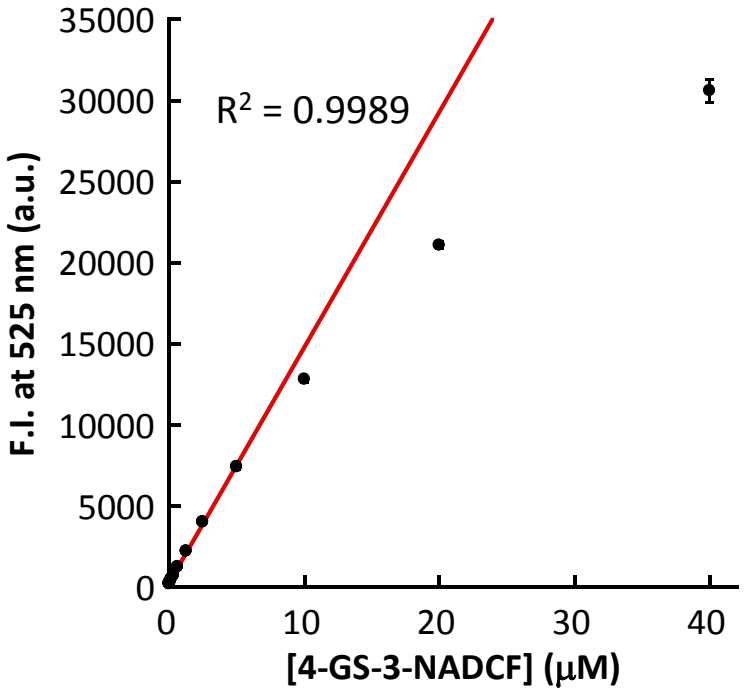


Fig. S5. Relationship between fluorescence intensity and 4-GS-3-NADCF concentration in a 100 mM sodium phosphate buffer. After 40 μM 3,4-DNADCF was fully reacted in a 100 mM sodium phosphate buffer (pH 6.5, 1% DMSO) containing 1 mM GSH and 6xHis-hGSTP1-1, the reaction mixture was serially diluted two-fold with the same buffer and the fluorescence intensity measured after excitation at 505 nm. Measurements were done in triplicate. The dots and error bars respectively represent means and standard deviations. Linearity was ensured up to 5 μM 4-GS-3-NADCF with $R^2 = 0.9989$.

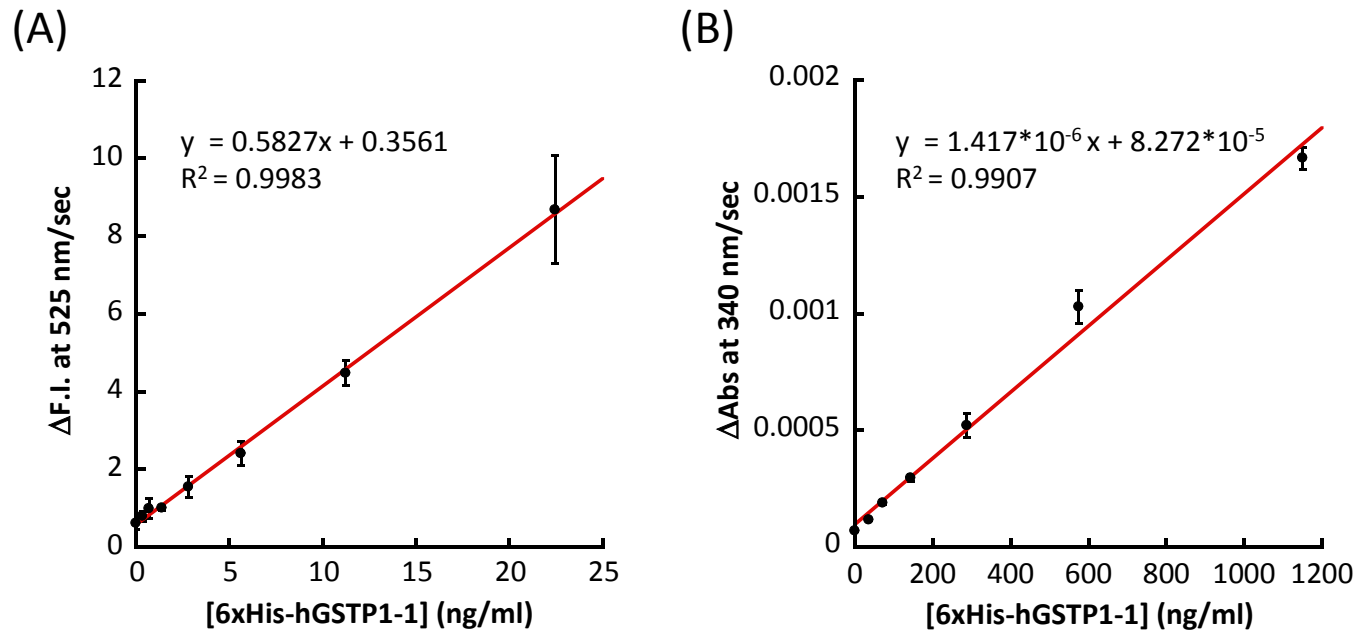


Fig. S6. Relationship between initial rate and 6xHis-hGSTP1-1 concentration.

1 μM 3,4-DNADCF (A) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (B) were used as substrates. Each reaction was carried out in a 100 mM sodium phosphate buffer (0.1% DMSO or 4% ethanol as cosolvent) containing 1 mM GSH.- Enzyme concentration at the detection limit was calculated from $3SD_0$/slope; where SD_0 is standard deviation of the initial rate in the absence of the enzyme: it was 1.26 ng/ml for 3,4-DNADCF (A) and 6.63 ng/ml for CDNB (B). Measurements were done in quadruplicate. The dots and error bars respectively represent means and standard deviations.

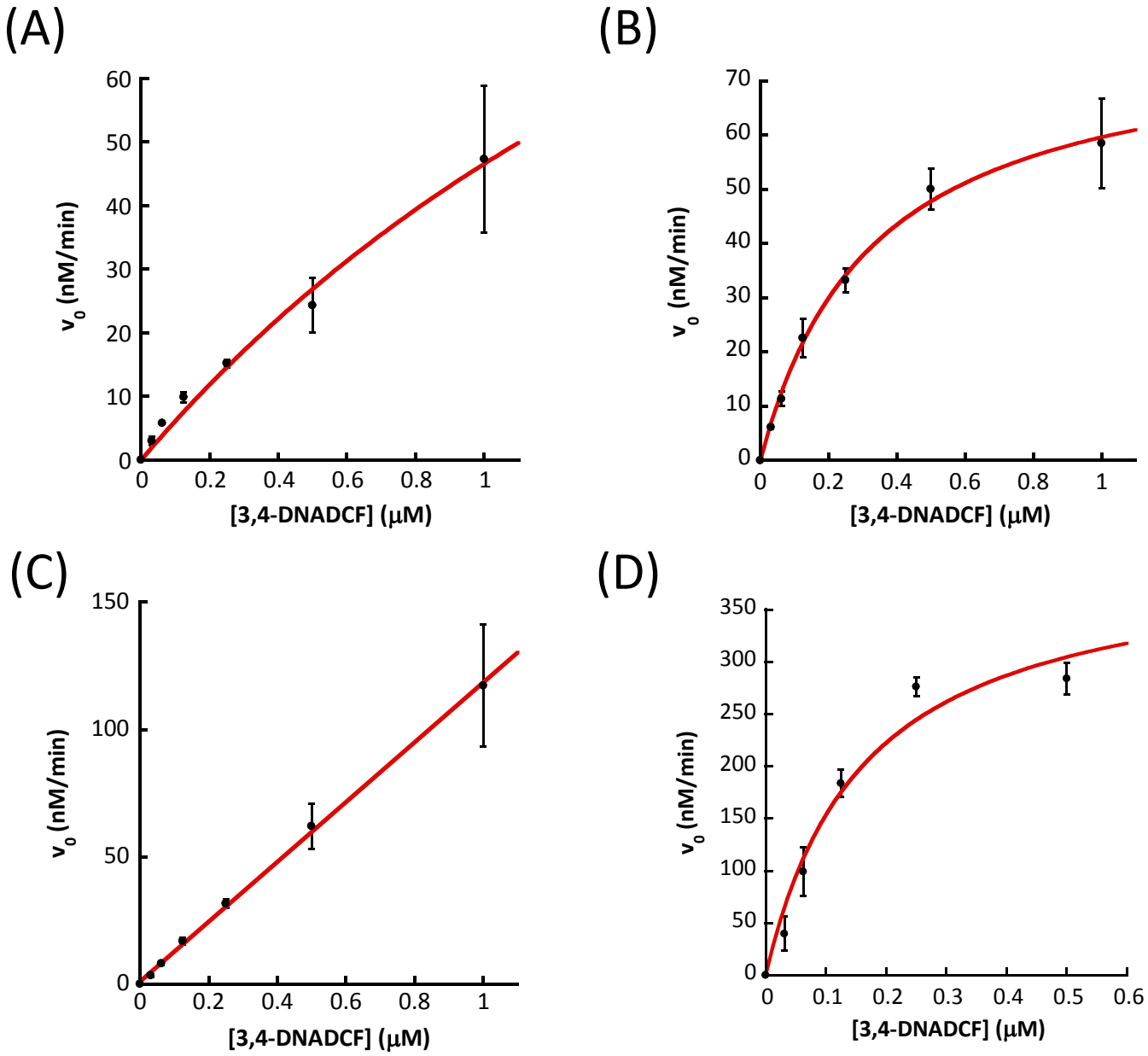


Fig. S7. Relationship between initial velocity and 3,4-DNADCF concentration in a 100 mM sodium phosphate buffer (pH 6.5). The initial velocities of 6xHis-hGSTA1-1 (A), 6xHis-hGSTM1-1 (B), 6xHis-hGSTP1-1 (C), and Nobo-Dm/GSTe14 (D) in the presence of 1 mM GSH at 28°C are plotted. Measurements were done in triplicate. The dots and error bars respectively represent means and standard error of the mean (n = 3). In (B) and (D), data were fitted to the Michaelis-Menten equation.

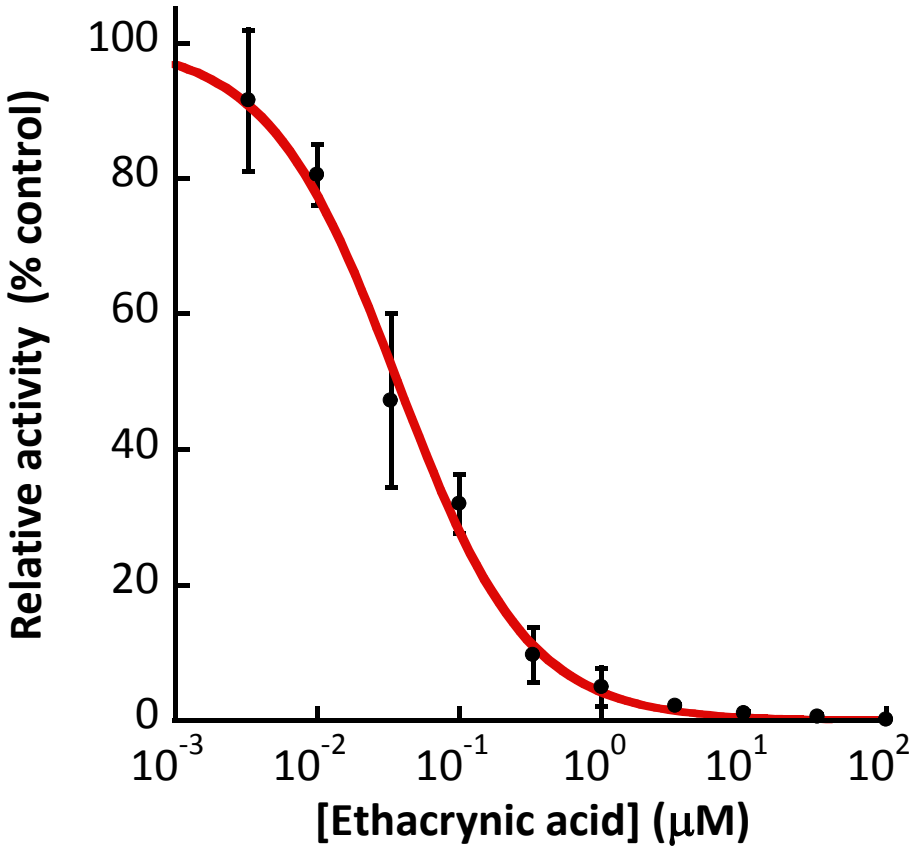


Fig. S8. Inhibition of Nobo-Dm/GSTe14 by ethacrynic acid.

Dose-response curve of ethacrynic acid toward Nobo-Dm/GSTe14. Assay was performed in sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20) containing 1.1% DMSO as a cosolvent and 1 mM GSH in the presence or absence of the respective concentrations of ethacrynic acid. Relative activity represents the percentage of Nobo-Dm/GSTe14 activity in the presence of ethacrynic acid with respect to that in its absence. The dots and error bars respectively represent means and standard error of the mean (n = 3). IC_{50} was determined to be 31.9 ± 3.3 nM.

Table S1 PCR primers for subcloning GSTs into vector pCOLD-I

Primer Name	Sequence (5'-3')
hGSTA1_S_pCOLD	AAAAACATATGGCAGAGAAGCCCAAGC
hGSTA1_AS_pCOLD	AAAAAGAATTCCATGACTGCGTTATTAAAACC
hGSTA2_S_pCOLD	AAAAAACATATGGCAGAGAAGCCCAAGC
hGSTA2_AS_pCOLD	AAAGAATTCGTTCTTGACCTCTATGGCTGG
hGSTA3_S_pCOLD	AAAAAACATATGGCAGGGAAGCCCAAG
hGSTA3_AS_pCOLD	AAAGAATTCCTTAGCCTCCATGGCTGC
hGSTA4_S_pCOLD	AAAAAACATATGGCAGCAAGGCCCAAG
hGSTA4_AS_pCOLD	AAAGAATTCCATGGATGTGTTGTTTTATGGCC
hGSTM1_S_pCOLD	AAAGAGCTCATGCCCATGATACTGGGGTAC
hGSTM1_AS_pCOLD	AAAAAGAATTCACTACTTGTTGCCCCAG
hGSTM2_S_pCOLD	AAAAAACATATGCCCATGACACTGGGGTAC
hGSTM2_AS_pCOLD	AAAGAATTCGCCCTACTTGTTGCCCCAG
hGSTM3_S_pCOLD	AAAAAACATATGTCGTGCGAGTCGTCTATG
hGSTM3_AS_pCOLD	AAAGAATTCCTCCTGCTCAGCATACAGG
hGSTK1_S_pCOLD	AAAAAACATATGGGGCCCCTGCC
hGSTK1_AS_pCOLD	AAAGAATTCGCTTCCTCCGGGCAATC
hGSTO1_S_pCOLD	AAAAAACATATGTCCGGGGAGTCAGC
hGSTO1_AS_pCOLD	AAAGAATTCCTGACTCCTGCCCCCT
hGSTZ1_S_pCOLD	AAAAAACATATGCAGGCGGGGAAGC
hGSTZ1_AS_pCOLD	AAAGAATTCCTAGGCCCTCAGCTCAG
hGSTT1_S_pCOLD	AAAAAACATATGGGCCTGGAGCTGTAC
hGSTT1_AS_pCOLD	AAAGAATTCTCACCGGATCATGGCCAG
hGSTT2_S_pCOLD	AAAAAACATATGGGCCTAGAGCTGTTTCTTG
hGSTT2_AS_pCOLD	AAAGAATTCCATCCCAGACCCTTCAGG

Table S2 Specific activities (μmol min^{-1} mg $protein^{-1}$) toward 3,4-DNADCF of various GSTs.

Isoenzymes[a]	Specific activity (μmol min^{-1} mg $protein^{-1}$)
hGSTA1-1	0.039 ± 0.005
hGSTA2-2	0.097 ± 0.020
hGSTA3-3	0.060 ± 0.021
hGSTA4-4	0.019 ± 0.007
hGSTM1-1	3.0 ± 0.2
hGSTM2-2	0.13 ± 0.04
hGSTM3-3	0.11 ± 0.05
hGSTP1-1	2.2 ± 0.3
hGSTT1-1	~ 0.001
hGSTK1-1	~ 0.004
Nobo-Dm/GSTe14	2.4 ± 0.8

a. hGSTs are N-terminal hexahistidine tagged proteins

Table S3 Kinetics parameters (mean ± s.e.m.) of GSTs for 3,4-DNADCF in 100 mM sodium phosphate buffer (pH 6.5 or 7.4).

GSTs	pH	k_{cat} (s^{-1})	$K_M^{3,4\text{-DNADCF}}$ (nM)	app. $k_{cat}/K_M^{3,4\text{-DNADCF}}$ (x 10^6 $M^{-1}s^{-1}$)
hGSTP1-1[a]	6.5	-	-	6.1 ± 1.0[b]
	7.4	-	-	6.9 ± 1.0[b]
hGSTM1-1[a]	6.5	1.0 ± 0.0	314 ± 16	3.1 ± 0.1[c]
hGSTA1-1[a]	6.5	-	-	2.7 ± 0.4[b]
Nobo-Dm/GSTe14	6.5	3.8 ± 0.1	162 ± 18	23 ± 2[c]

a. N-terminal hexahistidine-tagged GSTs. b. Apparent k_{cat}/K_M (app. k_{cat}/K_M) value was directly determined by equation (4).

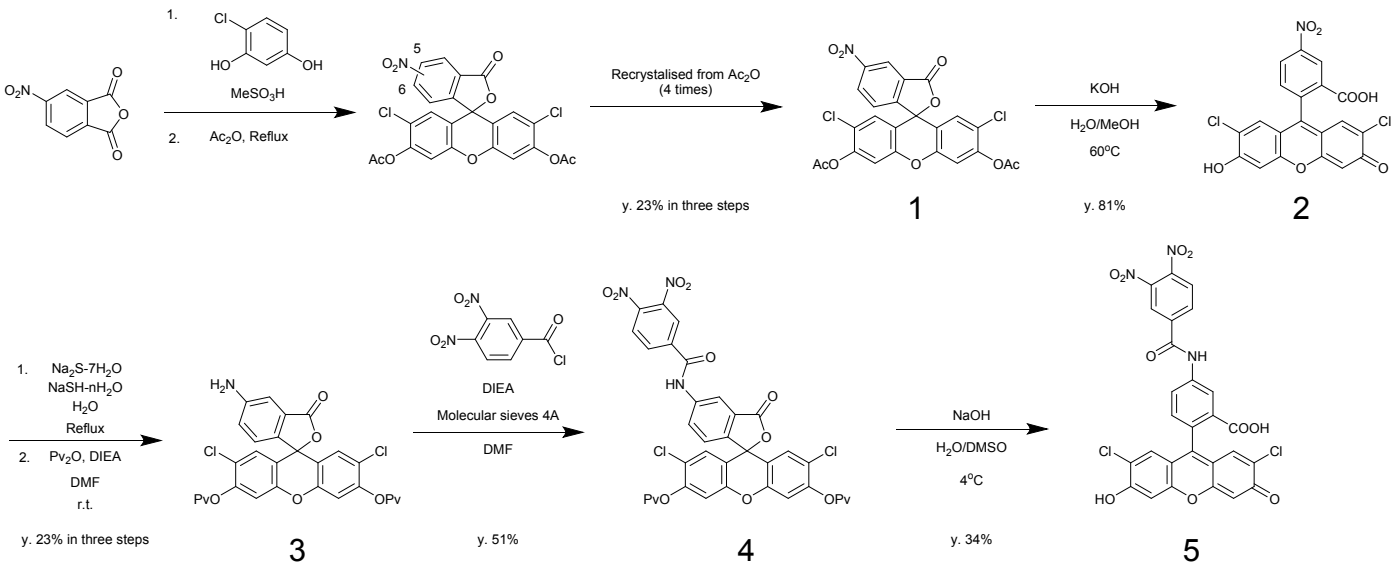
c. Determined by Michaelis-Menten plot.

Material and Methods

Materials and general instrumentation. All chemicals and solvents for synthesis were purchased from TCI (Tokyo, Japan), Wako Pure Chemical (Osaka, Japan), and Kanto Chemical (Tokyo, Japan) and used as received without further purification. Thin layer chromatography (TLC) was performed using Silica Gel 60 F254 (Merck, 0.25 mm thick) with UV detection (254 nm). Column chromatography was performed using Silica Gel 60N (spherical, neutral, 63−210 μm, Cat. No. 37565-79, Kanto Chemical). Reduced glutathione (Cat. No. 071-02014) and β-estradiol (Cat. No. 052-04041) was obtained from Wako Pure Chemical. Ethacrynic acid was purchased from TCI (Cat. No. E0526). NMR spectra were recorded on a Bruker DRX-400 spectrometer at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR. Chemical shifts (δ values) in ^{1}H- and ^{13}C-NMR were calibrated to the residual solvent resonance at 7.26 and 77.16 ppm for $CDCl_3$, 2.05 and 29.84 ppm for acetone-d_6, and 2.5 and 39.52 ppm for DMSO-d_6. Mass spectra were obtained using a Micromass LCT spectrometer (Waters, Milford, MA, USA) in ESI positive mode. UV-visible spectra were obtained on a V-550 UV/VIS spectrophotometer (JASCO Corp., Tokyo, Japan). Fluorescence photometric studies were performed on an RF-5300PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan), SH-9000 microplate reader (Corona Electric, Ibaraki, Japan), and PHERAstar Plus reader (BMG Labtech, Offenburg, Germany).

Synthesis of 3,4-DNADCF

Scheme S1



Compounds **1** and **2** were synthesised according to the procedures previously described[1].

3',6'-Dipivaloyl-2',7'-dichloro-5-aminospiro[isobenzofuran-1(3*H*),9'-[9*H*]xanthen]-3-one (3)

2',7'-Dichloro-5-nitrofluorescein (**2**) (0.87 g, 1.96 mmol), sodium sulphide nonahydrate (1.6 g, 6.67 mmol), and sodium monohydrogensulphide *n*-hydrate (0.89 g) were dissolved in 30 ml H_2O. The resulting mixture was stirred at 110°C for 3.5 h.

After cooling to 4°C, concentrated hydrochloric acid was added dropwise to give an orange precipitate, which was stirred for 20 min at ambient temperature. The mixture was filtered and washed with H_2O. The filtrate was dried *in vacuo* to give crude 2',7'-dichloro-5-aminofluorescein. The crude product and caesium carbonate (1.7 g, 5.1 mmol) were dissolved in 8 ml DMF. At 4°C, pivalic anhydride (1.08 g, 5.78 mmol) was added dropwise, and the reaction mixture was stirred at ambient temperature under argon. After 2 h of stirring, pivalic anhydride (0.092 g, 0.49 mmol) was added dropwise and the reaction mixture was stirred for 1 h. Then, the reaction mixture was diluted with 100 ml AcOEt and washed with 1 N HCl and saturated NaH_2PO_4 aq. brine. The AcOEt layer was dried with anhydrous magnesium sulphate, filtered, and concentrated *in vacuo*. The material was purified by silica gel chromatography (Silica Gel 60N, AcOEt: hexane = 1:2) to give the title compound **3** (772 mg, yield 67% in two steps). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.21 (1H, d, J = 2.1 Hz), 7.08 (2H, s), 6.99 (1H, J = 8.2, 2.2 Hz), 6.93 (1H, d, J = 8.2 Hz), 6.91 (2H, s), 4.13 (2H, br), 1.39 (18H, s) ^{13}C-NMR (100 MHz, DMSO-d_6) δ 175.6, 168.9, 149.9, 148.7, 141.6, 129.0, 127.5, 124.7, 122.7, 122.5, 118.0, 112.5, 108.9, 80.5, 39.4, 27.1; HRMS (ESI-TOF) *m*/*z* calculated for $[C_{30}H_{27}Cl_2NO_7+H]^+$ 584.1243, found 584.1252 (+1.0 mmu).

3,4-DNADCF dipivaloate (4)

3,4-Dinitrobenzoic acid (130 mg, 0.61 mmol) was suspended in 5 ml thionyl chloride. A small amount of DMF (a few drops) was added and the mixture was refluxed for 2.5 h. After cooling to ambient temperature, the thionyl chloride was evaporated off with benzene three times. The resultant oil was mixed with 4 ml DMF, which was added to 4 ml DMF containing **3** (65.9 mg, 0.11 mmol) and activated molecular sieves 4A at 4°C. *N*,*N*-diisopropylethylamine (159 mg, 1.23 mmol) was added and the reaction mixture was stirred at 4°C for 1.5 h. The reaction mixture was diluted with 50 ml AcOEt and washed with 1 N HCl three times and once with brine. The AcOEt layer was dried with anhydrous magnesium sulphate, filtered, and concentrated *in vacuo*. The material was purified by silica gel chromatography (Silica Gel 60N, AcOEt: hexane = 1:3) to give the title compound **4** (128 mg, yield 51%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.34 (1H, s), 8.57 (1H, d, J = 1.9 Hz), 8.50-8.47 (2H, m), 8.44 (1H, dd, J = 8.4, 1.9 Hz), 8.04 (1H, d, J = 8.3 Hz), 7.35 (1H, dd, J = 8.4 Hz), 7.10 (2H, s), 6.81 (2H, s), 1.41 (18H, s) ^{13}C-NMR (100 MHz, DMSO-d_6) δ 176.1, 168.9, 161.5, 150.0, 149.1, 147.1, 144.5, 142.7, 140.3, 138.4, 132.5, 128.9, 128.2, 127.1, 125.8, 125.2, 124.6, 122.9, 116.8, 116.5, 112.9, 81.9, 39.5, 27.1; HRMS (ESI-TOF) *m*/*z* calculated for $[C_{37}H_{29}Cl_2N_3O_{12}+H]^+$ 778.1207, found 778.1217 (+1.0 mmu)

3,4-DNADCF (5)

3,4-DNADCF dipivaloate (**4**) (30.0 mg, 0.04 mmol) was dissolved in 0.5 ml DMSO and cooled to 4°C. Then, 0.5 ml 1 N NaOH aq. was added and the reaction mixture was stirred at 4°C for 30 min. The reaction was quenched by addition of 0.5 ml potassium phosphate buffer (200 mM, pH 7.0). The residue was purified by semi-preparative reverse-phase HPLC. The target fraction was collected and lyophilised to give the title compound **5** (8.1 mg, 34%). The lyophilised product was confirmed to be pure by analytical HPLC. ^{1}H-NMR (400 MHz, acetone-d_6) δ 8.74 (1H, d, J = 1.7 Hz), 8.60 (1H, dd, J = 8.4, 1.7 Hz), 8.56 (1H, m), 8.34 (1H, d, J = 8.4 Hz), 8.13 (1H, dd, J = 8.3, 1.9 Hz), 7.34 (1H, d, J = 8.3 Hz), 6.88 (2H, s), 6.84 (2H, s) ^{13}C-NMR (100 MHz, DMSO-d_6) δ 168.1, 162.0, 156.1, 143.4, 141.7, 139.8, 138.8, 129.6, 127.6, 126.1, 125.1, 121.7, 108.9, 103.0; HRMS (ESI-TOF) *m*/*z* calculated for $[C_{27}H_{13}Cl_2N_3O_{10}+H]^+$ 61.0056, found 610.0048 (-0.9 mmu).

Determination of quantum efficiency. 3,4-DNADCF or DNAF1 was incubated in sodium phosphate buffer (100 mM, pH 7.4, <0.5% acetonitrile as a cosolvent) containing hGSTP1-1 and 0.1 mM GSH for 10 min to let the fluorescence intensity reach a plateau, then was analysed. For the determination of quantum efficiency (QE), fluorescein in 0.1 M NaOH (QE = 0.85) was used as a standard. QE was calculated according to the following equation (1).

$$\frac{QE_S}{QE_R} = \frac{A_R}{A_S} * \frac{D_S}{D_R} * \frac{n_S^2}{n_R^2} \qquad (1)$$

S: sample, R: reference (fluorescein), A: absorbance at the excitation wavelength (490 nm), D: area under the fluorescence spectrum on an energy scale, *n*: refractive index.

Determination of p*K*a value of 3,4-DNADCF and reaction product (Figs. S1 and S2).

The absorbance spectrum of 1 μM 3,4-DNADCF was measured in sodium phosphate buffer (100 mM, 0.1% DMSO as a cosolvent) at various pH values. An aliquot of 10 μM 3,4-DNADCF was reacted with 0.1 mM GSH by incubation in 5 mM phosphate buffer (pH 7.4) containing 1.2 μg/ml 6xHis-hGSTP1-1 at ambient temperature for 10 min. The reaction mixture was diluted 1:10 with 100 mM phosphate buffer at various pH values and the absorbance and fluorescence spectra measured. Absorbance at 505 nm and fluorescence intensity at 525 nm (excitation at 505 nm) were plotted with KaleidaGraph software (Synergy Software, Reading, PA, USA) and fitted to the following equation (2) to determine the p*K*a value[2].

$$S = \frac{S_{max}}{1+10^{(pKa-pH)}} + S_{min} \qquad (2)$$

S: absorbance or fluorescence intensity at indicated pH, S_{max}: maximum absorbance or fluorescence intensity,

S_{min}: minimum absorbance or fluorescence intensity.

HPLC analysis (Fig. S3). HPLC was performed on an Inertsil ODS-3 column (250 mm × 4.6 mm; GL Sciences Inc.) at a flow rate of 1.0 ml/min using an HPLC system composed of a pump (PU-980, JASCO) and a detector (MD-2015 or FP-2025, JASCO). ChromNaV software was used for operation of the HPLC system and data analysis. Eluent: A: B = 80:20 → 0:100 (20 min) A: 100 mM triethylamine acetate, B: 20% H_2O/80% CH_3CN.

Semi-preparative HPLC. Semi-preparative HPLC was performed on a Mightysil RP-18 GP column (250 mm × 20 mm (5 μm); Kanto Chemical, Tokyo, Japan) at a flow rate of 5.0 ml/min using an HPLC system composed of a pump (PU-980, JASCO) and a detector (UV-975, JASCO). Data were acquired by Clarity Lite software (DataApex, Prague, Czech Republic). Eluent: A:B = 80:20 → 5:95 (20 min), A: 10 mM ammonium acetate, B: acetonitrile. Subsequently, a fraction containing 3,4-DNADCF was

analysed by reverse-phase HPLC on a Mightysil RP-18 column (250 mm × 4.6 mm (5 mm); Kanto Chemical, Tokyo, Japan) at a flow rate of 1.0 ml/min using an HPLC system composed of two pumps (LC-10AT, Shimadzu) and a detector (SPD-M20A and RF-10AxL, Shimadzu) operated by Clarity software through a system controller (CVM-20A, Shimadzu). Eluent: A: B = 80:20 → 5:95 (20 min), A: 10 mM ammonium acetate, B: acetonitrile. A fraction confirmed to give a single HPLC peak was lyophilised.

LC-MS analysis (Fig. S4). 3,4-DNADCF (20 μM) was incubated for the indicated time at 37°C in 100 μl sodium phosphate buffer (100 mM, pH 6.5) containing 6xHis-hGSTP1-1 and 0.1 mM GSH. The reaction was quenched by addition of 2 μl acetic acid. An aliquot of the solution (20 μl) was analysed by LC-MS analysis on an Inertsil ODS-3 column (250 mm length and 2.1 mm diameter) (GL Sciences, Tokyo, Japan) using an LC-MS (Agilent 1200 series/6130 Quadrupole LC-MS). Eluent: A:B = 95:5 → 5:95 (30 min), A: 0.1% formic acid, B: 0.1% formic acid/20% H_2O/80% acetonitrile.

Construction of expression plasmids for human GSTs. Human GSTA1 and GSTM1 cDNA clones were purchased from ATCC (Manassas, VA, USA) and a GSTM2 cDNA clone was purchased from Sino Biological Inc. (Beijing, China). cDNA clones of the other human GSTs were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan[3]. For the cDNA clones with base substitutions in the coding sequence, the reference sequences for hGSTA3 (NM_000847.4), hGSTM2 (NM_000848.3) and hGSTT1 (NM_000853.2) were restored by site-directed mutagenesis.

The PCR product obtained using each primer set (Table S1) was digested with *Nde*I and *Eco*RI and ligated into the corresponding sites of vector pCOLD-I. For hGSTP1 and hGSTM1, *Sac*I was used in place of *Nde*I. The nucleotide sequences of the coding regions were confirmed by direct sequencing.

Expression and purification of recombinant 6xHis-hGSTs. The expression plasmids for *N*-terminal hexahistidine-tagged (6xHis) human GSTs were each introduced into *Escherichia coli* BL21(DE3)pLys competent cells. A single colony was inoculated into 2 ml LB supplemented with ampicillin (Amp) and chloramphenicol (CP) (50 and 34 μg/ml, respectively) and incubated overnight at 37°C with shaking. The overnight culture was seeded in 200 ml fresh LB-Amp/CP, then incubated at 37°C with shaking. When the OD600 reached 0.4-0.6, the culture solution was cooled at 15°C for 30 min, then isopropyl thio-β-D-galactoside (IPTG) (0.5 mM final concentration) was added to induce expression of the recombinant protein. After agitation at 15°C for 24 h, the cells were collected by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0) containing 300 mM NaCl. The cell suspension was sonicated on ice (10 s sonication pulse at 30 s intervals for 10 min). After cell debris was removed by

centrifugation at 4°C for 30 min, the supernatant was collected. All recombinant human GSTs were purified with cOmplete His-Tag Purification Resin (Cat. No. 05893682001, Roche Diagnostics GmbH, Mannheim, Germany). Recombinant proteins were eluted from the resin with 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 200 mM imidazole. The eluate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) containing 3 mM EDTA and 3.75 mM β-mercaptoethanol at 4°C overnight, then against 25 mM potassium phosphate buffer (pH 7.0) containing 3.75 mM EDTA and 3.75 mM β-mercaptoethanol at 4°C for 6 h. After the second dialysis, glycerol was added to 20% (v/v). The enzyme solution was frozen and kept at -80°C until use. Protein concentration was determined by the standard Bradford assay (Bio-Rad Cat. No. 500-0006; BSA was used for calibration). Activities of purified recombinant GSTs were confirmed with standard methods.

Construction of expression plasmids for Nobo-Dm/GSTe14

pET-22b (+)Nobo-Dm/GSTe14-6xHis: The coding region of Nobo-Dm/GSTe14 was amplified by PCR using a forward primer with an *Nde*I site (5'-CAGTCATATGATGTCTCAGCCCAAGCCGATTTTG-3') and a reverse primer with an *Xho*I site (5'-CTGACTCGAGCTCCACCTTCTCGGTGACTACCGCTG-3'). The amplified product was digested with these restriction enzymes and subcloned into the pET-22b (+) bacterial expression vector.

pCOLD-III- Nobo-Dm/GSTe14: The coding region of Nobo-Dm/GSTe14 was amplified by PCR using a forward primer with a *Kpn*I site (5'-GGTACCATGTCTCAGCCCAAGCCGATTTTG-3') and a reverse primer with a *Xho*I site (5'-CTCGAGCTACTCCACCTTCTCGGTGACTACCG-3'). The amplified product was inserted into the *Sma*I site of vector pBluescript II SK(-), then introduced into *E. coli* DH5α and cloned. A plasmid that had an insert with appropriate nucleotide sequence was selected by DNA sequencing. The plasmid was digested with *Kpn*I and *Hin*dIII and subcloned into pCOLD-III.

Expression and purification of recombinant Nobo-Dm/GSTe14. *E. coli* BL21-CodonPlus-RIL competent cells (Stratagene) were transformed with pCOLD-III Nobo-Dm/GSTe14 or pET-22b(+)-Nobo-Dm/GSTe14. A single colony was inoculated into 20 ml LB supplemented with 0.1 mg/ml Amp and cultured overnight at 37°C with shaking to act as a seed culture.

Nobo-Dm/GSTe14-6xHis: The seed culture was inoculated into 2 L LB-Amp and incubated at 37°C for 12 h. Expression of recombinant protein was induced by addition of 0.1 mM IPTG and agitation at 15°C for 5 h. Cells were collected by centrifugation and suspended into 10 ml phosphate buffered saline (pH 7.4). The suspension was sonicated on ice (5 s sonication pulse at 45 s intervals for 20 min). Cell debris was removed by centrifugation (25,000 x *g*) at 10°C for 20 min and the supernatant was collected. To 10 ml of supernatant, 3 ml TALON Metal Affinity Resin (Clontech Laboratories Inc., Mountain View, CA) was

added and the mixture was incubated overnight at 4°C with gentle shaking, then washed with a washing buffer (20 mM HEPES buffer, pH 7.9, 500 mM KCl, 10% glycerol, 0.036% β-mercaptoethanol, 0.01% NP-40, cOmplete Mini protease inhibitor (Roche Applied Science), 10 mM imidazole) eight times. Protein was eluted with elution buffer (50 mM HEPES, pH 7.0, 140 mM NaCl, 1.5 mM Na_2HPO_4, 200 mM imidazole). To the eluate, an equal volume of glycerol was added and kept at -80°C until use. Protein concentration was determined by a Qubit 2.0 fluorometer using Quant-iT reagent and BSA as a standard (Life Technologies, Carlsbad, CA).

Nobo-Dm/GSTe14: The seed culture was inoculated into 1 L LB-Amp and incubated at 37°C for 3 h, then cooled at 15°C for 35 min. Expression of the recombinant protein was induced by addition of 0.1 mM IPTG and agitation at 15°C for 24 h. The cells were collected by centrifugation and suspended into 35 ml 100 mM sodium phosphate buffer (pH 6.5). The suspension was sonicated on ice (5 s sonication pulse at 10 s intervals for 10 min on ice). Cell debris was removed by centrifugation at 4°C for 15 min and then filtered (0.45 mm filter). Recombinant Nobo-Dm/GSTe14 was purified with an AKTA start system (GE Healthcare UK Ltd, Buckinghamshire, England) equipped with a GSTrap FF column. Binding buffer was phosphate buffered saline (pH 7.3) and elution buffer was 50 mM Tris-HCl (pH 8.0) containing 10 mM GSH. The flow rate was 1.0 ml/min. Target fractions were identified by SDS-PAGE and collected, then dialysed against 50 mM HEPES buffer (pH 7.0) at 4°C overnight. The dialysed protein solution was mixed with glycerol (50% final concentration) and then stored at -20°C before use. Protein concentration was determined by a Qubit 2.0 fluorometer using Quant-iT reagent and BSA as a standard (Life Technologies).

Determination of enzymatic activity.

Assays were typically performed in sodium phosphate buffer (100 mM, pH 6.5, 0.1% DMSO as a cosolvent) containing 3,4-DNADCF and GSH in the presence or absence of 6xHis-GSTs. The activity was determined fluorophotometrically at 525 nm with excitation at 505 nm. In all experiments, the nonenzymatic background reaction rate was subtracted prior to calculation. The rate of product formation was estimated by the following equation (3).

$$\text{Initial rate} = \frac{F_t - F_0}{t} * \frac{P}{F_{\max} - F_0} \quad (3)$$

F_t and F_0: fluorescence intensity at time t and zero, respectively, P: the amount of product of a known concentration in solution, $F_{\max}$: fluorescence intensity after completion of the reaction.

In each experiment, $F_{\max}$ was determined by incubation of 1.0 or 0.5 μM, corresponding to P, of 3,4-DNADCF with 0.1 mM GSH and ≥1.2 μg/ml 6xHis-hGSTP1-1 or Nobo-Dm/GSTe14.

Determination of enzyme kinetic parameters (Tables 2 and 3 and Fig. S5).

Specific activity: Assays were performed in sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20, 0.1% DMSO as a cosolvent) containing 1 μM 3,4-DNADCF and 0.1 mM GSH in the presence or absence of 6xHis-GSTs. GST concentrations affording a linear relationship with the initial rate were used for the determination of specific activity.

6xHis-hGSTM1-1 and Nobo-Dm/GSTe14: Dependency of the enzymatic velocity upon 3,4-DNADCF concentration was investigated at a constant concentration of GSH (1 mM) by varying 3,4-DNADCF concentration from 0.03 to 0.5 or 1 μM. The data were fitted to the Michaelis-Menten equation using KaleidaGraph software (Synergy Software, Reading, PA, USA).

Apparent specificity constant (for 6xHis-hGSTA1-1 and 6xHis-hGSTP1-1). Due to quenching of fluorescence caused by an inner filter effect at high concentrations of fluorogenic substrate, the apparent specificity constant (app. k_{cat}/K_M) was determined using the Michaelis-Menten relationship at much lower 3,4-DNADCF concentrations (0.03-1 μM) than those used for approximating K_M, as shown in equation (4).

$$v_0 = \frac{v_{max} * [S]}{K_M + [S]} \approx \frac{v_{max} * [S]}{K_M} = \frac{k_{cat}}{K_M} * [E] * [S] \qquad (4)$$

v_0: velocity of the reaction, v_{max}: maximum velocity, [S]: substrate concentration, K_M: Michaelis constant, k_{cat}: catalytic constant, [E]: enzyme concentration.

Validation of assay system in a 384-well format (Fig. 3). Into individual wells of the assay plates, 10 μl sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20) containing 2% DMSO, 2x the indicated concentration of final Nobo-Dm/GSTe14 and 0.2 mM GSH (all at twice the final concentration) was dispensed. Then, 10 μl sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20, 0.2% DMSO) containing 2 μM 3,4-DNADCF was added to each well and the plates were incubated at room temperature for 2 h. At each time point, the fluorescence was measured for all plates with a PHERAstar Plus microplate reader. Background control wells (without Nobo-Dm/GSTe14) or inhibitor-containing wells (20 μM ethacrynic acid) were also prepared.

Validation of assay system with Z'-factor (Fig 3). 10 μl sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20, 2% DMSO) containing 0.1% BSA, 12.8 ng/ml Nobo-Dm/GSTe14, and 0.2 mM GSH was dispensed into each well of the assay plates. Then, 10 μl sodium phosphate buffer (100 mM, pH 6.5, 0.005% Tween 20) containing 1 μM 3,4-DNADCF was added and the

plates were incubated at 25°C for 70 min. The fluorescence was measured by a PHERAstar Plus microplate reader. Z'-factor was calculated according to the following equation (5).

$$\text{Z'-factor} = 1 - \frac{3(SD_s + SD_c)}{Av_s - Av_c} \quad (5)$$

Av_s and Av_c: average of the signal derived from wells with enzymatic (s) and nonenzymatic (c) reaction. SD_s and SD_c: standard deviation of the signal derived from sample and background control.

HTS. Assays were performed in 20 μl sodium phosphate buffer (200 mM, pH 6.5, 0.005% Tween 20, and 1.1% DMSO as a cosolvent) containing 0.1 mM GSH and 35 ng/ml Nobo-Dm/GSTe14-6xHis in the presence or absence of a test compound (0.2 μl of a 2 mM compound in 100% DMSO) at 25°C. Nobo-Dm/GSTe14-6xHis was omitted from the wells for the measurement of the nonenzymatic background reaction. The detailed procedure was as follows: test compounds were predispensed into 384-well microtitre plates, and then 10 μl phosphate buffer (200 mM, pH 6.5, 0.005% Tween 20) containing 0.2 mM GSH and Nobo-Dm/GSTe14-6xHis was dispensed into the plates. Finally, 10 μl phosphate buffer (200 mM, pH 6.5, 0.005% Tween 20) containing 2 μM 3,4-DNADCF was dispensed to start the reaction. After incubation of the plates at 25°C for 90 min, fluorescence intensity was measured at 520 nm with excitation at 485 nm by a PHERAstar microplate reader. Inhibition percentage was calculated according to the following equation (6).

$$Inhibition\ (\%) = \left(\frac{\text{Fl sample - Fl back}}{\text{Fl control - Fl back}}\right) * 100 \quad (6)$$

FI_{sample}: fluorescence intensity of wells containing library compound at 90 min, $FI_{control}$: fluorescence intensity of wells without compounds at 90 min, FI_{back}: fluorescence intensity of wells without Nobo-Dm/GSTe14-6xHis.

Nobo-Dm/GSTe14 inhibitors (Figs. 4 and S6). 3,4-DNADCF (1 μM) was incubated with 1 mM GSH and various concentrations of GST inhibitors. Relative activity (percentage of no-inhibitor control) was plotted against inhibitor concentration and fitted to the following equation (7) to obtain IC_{50} values.

$$\text{Relative activity}\,(\%) = \frac{A_{max} - A_{min}}{1 + ([I]/IC_{50})^n} + A_{min} \quad (7)$$

A_{max}: activity without inhibitors, A_{min}: activity with 100% inhibition, [I]: concentration of inhibitor, IC_{50}: inhibitor concentration giving half maximum enzymatic activity, *n*: Hill constant.

Supporting references

S1. J. Castro, A. Malakhov, and K. Burgess, *Synthesis (Stuttg).*, 2009, **7**, 1224–1226.

S2. T. Shoda, K. Kikuchi, H. Kojima, Y. Urano, H. Komatsu, K. Suzuki, and T. Nagano, *Analyst*, 2003, **128**, 719-723.

S3. T. Ota, Y. Suzuki, T. Nishikawa, T. Otsuki, T. Sugiyama, R. Irie, A. Wakamatsu, K. Hayashi, H. Sato, K. Nagai, K. Kimura, H. Makita, M. Sekine, M. Obayashi, T. Nishi, T. Shibahara, T. Tanaka, S. Ishii, J. Yamamoto, K. Saito, Y. Kawai, Y. Isono, Y. Nakamura, K. Nagahari, K. Murakami, T. Yasuda, T. Iwayanagi, M. Wagatsuma, A. Shiratori, H. Sudo, T. Hosoiri, Y. Kaku, H. Kodaira, H. Kondo, M. Sugawara, M. Takahashi, K. Kanda, T. Yokoi, T. Furuya, E. Kikkawa, Y. Omura, K. Abe, K. Kamihara, N. Katsuta, K. Sato, M. Tanikawa, M. Yamazaki, K. Ninomiya, T. Ishibashi, H. Yamashita, K. Murakawa, K. Fujimori, H. Tanai, M. Kimata, M. Watanabe, S. Hiraoka, Y. Chiba, S. Ishida, Y. Ono, S. Takiguchi, S. Watanabe, M. Yosida, T. Hotuta, J. Kusano, K. Kanehori, A. Takahashi-Fujii, H. Hara, T. Tanase, Y. Nomura, S. Togiya, F. Komai, R. Hara, K. Takeuchi, M. Arita, N. Imose, K. Musashino, H. Yuuki, A. Oshima, N. Sasaki, S. Aotsuka, Y. Yoshikawa, H. Matsunawa, T. Ichihara, N. Shiohata, S. Sano, S. Moriya, H. Momiyama, N. Satoh, S. Takami, Y. Terashima, O. Suzuki, S. Nakagawa, A. Senoh, H. Mizoguchi, Y. Goto, F. Shimizu, H. Wakebe, H. Hishigaki, T. Watanabe, A. Sugiyama, M. Takemoto, B. Kawakami, M. Yamazaki, K. Watanabe, A. Kumagai, S. Itakura, Y. Fukuzumi, Y. Fujimori, M. Komiyama, H. Tashiro, A. Tanigami, T. Fujiwara, T. Ono, K. Yamada, Y. Fujii, K. Ozaki, M. Hirao, Y. Ohmori, A. Kawabata, T. Hikiji, N. Kobatake, H. Inagaki, Y. Ikema, S. Okamoto, R. Okitani, T. Kawakami, S. Noguchi, T. Itoh, K. Shigeta, T. Senba, K. Matsumura, Y. Nakajima, T. Mizuno, M. Morinaga, M. Sasaki, T. Togashi, M. Oyama, H. Hata, M. Watanabe, T. Komatsu, J. Mizushima-Sugano, T. Satoh, Y. Shirai, Y. Takahashi, K. Nakagawa, K. Okumura, T. Nagase, N. Nomura, H. Kikuchi, Y. Masuho, R. Yamashita, K. Nakai, T. Yada, Y. Nakamura, O. Ohara, T. Isogai, and S. Sugano, *Nat. Genet.*, 2004, **36**, 40–45.