A Platform for the Detection of Trypanosomes via Selective Small Molecule Recognition

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SUPPORTING INFORMATION

Instruments: Reagents and synthetic intermediates were characterized with ^{1}H and ^{13}C NMR recorded on a Varian Mercury VX-300-MHz high-field NMR. Absorbance spectra were recorded on a Cary 3E UV-Vis Spectrophotometer. Fluorescence measurements were acquired using a Perkin Elmer LS55 Fluorescence spectrometer at 25 °C. All experiments were performed in triplicate to ensure reproducibility.

General Procedures: All reagents were purchased from commercial vendors and used without further purification. FLASH-EDT_2 was synthesized according to literature protocols.[1] ^{1}H and ^{13}C NMR spectra matched those reported for this compound. Trypanothione disulfide (TS_2) was purchased from Bachem. Triscarboxyethylphoshphine hydrochloride (TCEP•HCl) was purchased from Thermo Scientific. All aqueous solutions were prepared with Millipore water. All experiments were performed in triplicate on different days to ensure reproducibility.

Determining λ_{ex} and λ_{em} for FLASH-$(TSH_2)_2$: A MOPS buffer (100 mM) was prepared in water and neutralized to pH 6.94 with NaOH. Stock solutions of FLASH-EDT_2 (1mM) and EDT (1 mM) were prepared in dry DMSO. A stock solution of TCEP•HCl (10 mM) was prepared in water.

A solution of TSH_2 (3.23 mM) was prepared in water by dissolving TS_2 (2.020 mg, MW 721.86 g/mol, 2.80 μmol, 1 eq) in 251 μL of water. To this

solution was added TCEP•HCl (616 μL of a 10 mM solution, 6.16 μmol, 2.2 eq), and the mixture was stirred for 20 min.

To a cuvette containing 2.50 mL of MOPS buffer neutralized to a pH of 6.94 was added FLASH-EDT_2 (2.50 μL of a 1 mM stock solution, 2.50 nmol, 1 eq). Fluorescence intensity measurements were collected for 7 min. No increase in fluorescence was observed. To this cuvette was added TSH_2 (30.96 μL of the 3.23 mM stock solution of TSH_2, 100 nmol, 40 eq). The fluorescence intensity immediately began to rise and reached saturation within 1 h.

An aliquot (1.25 mL) was diluted with 1.25 mL of MOPS buffer. This solution was scanned to determine λ_{ex} and λ_{em} for FLASH-$(TSH_2)_2$. Emission spectra were acquired from 460-800 nm by scanning excitation excitation wavelengths from 450-570 nm in 5 nm increments. These data provided a contour plot (**Figure S1**) to determine the maximum λ_{ex} and λ_{em}. This plot shows that λ_{ex} = 505 nm and λ_{em} = 527 nm for FLASH-$(TSH_2)_2$.

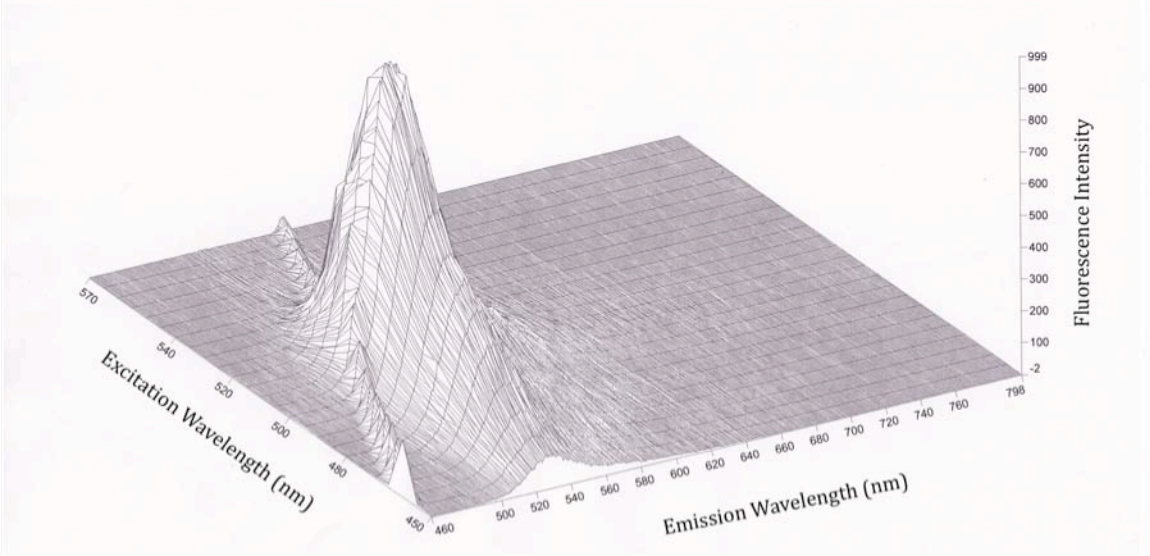


Figure S1. Determination of λ_{ex} and λ_{em} for FLASH-$(TSH_2)_2$: Contour plot of emission specrtra from varied λ_{ex} to determine the optimal λ_{ex} = 505 nm and λ_{em} = 527 nm for FLASH-$(TSH_2)_2$.

Procedure for binding experiment in buffer: A MOPS buffer (100 mM) was prepared in water and neutralized to pH 6.94 with NaOH. Stock solutions of

FLASH-EDT_2 (1.00 mM) and EDT (1.00 M) were prepared in dry DMSO. A stock solution of TCEP•HCl (10.0 mM) was prepared in water.

A solution of TSH_2 (3.23 mM) was prepared in water by dissolving TS_2 (2.020 mg, MW 721.86 g/mol, 2.80 μmol) in 251 μL of water. To this solution was added TCEP•HCl (616 μL of a 10.0 mM solution, 6.16 μmol, 2.2 eq), and the mixture was stirred for 20 min.

Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. Background fluorescence was recorded for 2.500 mL MOPS buffer and subtracted from subsequent measurements. To this cuvette was added FLASH-EDT_2 (2.50 μL of a 1 mM stock solution, 2.50 nmol, 1 eq). No increase in fluorescence was observed for seven minutes. At this time, TSH_2 (31.0 μL of the 3.23 mM stock solution of TSH_2, 100 nmol, 40 eq) was added. The fluorescence intensity immediately began to rise and reached saturation within 1 h. Binding was reversible within 30 seconds by the addition of EDT (12.50 μL of a 1.00 M solution, 12.5 μmol, 5000 eq). Results can be seen in **Figure 1**.

This experiment was repeated by performing the reduction in the presence of the dye. Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. Background fluorescence was recorded for 2.500 mL MOPS buffer and subtracted from subsequent measurements. To this cuvette was added FLASH-EDT_2 (2.50 μL of a 1.00 mM stock solution, 2.50 nmol, 1 eq). The fluorescence was monitored for 5 min with no observed increase in fluorescence. To this solution was added TS_2 (50.0 μL of a 2.00 mM stock solution, 100 nmol, 40 eq). The fluorescence measurements were recorded for 7 min with no observed increase in fluorescence. To this solution was added TCEP•HCl (20.0 μL of a 10.0 mM solution, 200 nmol, 80 eq). The fluorescence intensity immediately began to rise and reached saturation within 45 min (see **Figure 1**). The reaction reached saturation within 1 hour indicating that the rate-determining step for the reaction is the conjugation of two molecules of TSH_2 to the arsenical probe. The reduction of TCEP•HCl was facile in the presence of the arsenical probe.

We performed control experiments to observe any fluorescence attributable to the phosphine or the phosphine oxide. Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. Background fluorescence was recorded for 2.50 mL MOPS buffer and subtracted from subsequent measurements. To this cuvette was added FLASH-EDT_2 (2.50 μL of a 1.00 mM stock solution, 2.50 nmol, 1 eq). The fluorescence was monitored for 5 min with no observed increase in fluorescence. To this solution was added TCEP•HCl (20.0 μL of a 10.0 mM solution, 200 nmol, 80 eq). The fluorescence was monitored for 60 min with no observed increase in fluorescence (see Figure S2).

We also performed control experiments to observe any fluorescence that may be attributable to endogenous monothiols. We used sodium 2-mercaptoethanesulfonate (MES) as the control. Background fluorescence was recorded for 2.50 mL MOPS buffer and subtracted from subsequent measurements. To this cuvette was added FLASH-EDT_2 (2.50 μL of a 1.00 mM stock solution, 2.50 nmol, 1 eq). The fluorescence was monitored for 5 min with no observed increase in fluorescence. To this solution was added MES (25.0 μL of a 1.00 M stock solution of MES in H_2O, 25.0 μmol, 10,000 eq). The fluorescence was monitored for 60 min with increase in fluorescence of <2% (**Figure S2**).

The background reaction of FLASH-EDT_2 with the monothiol MES was also measured. Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. The background fluorescence was recorded for 2.50 mL MOPS buffer and subtracted from subsequent measurements. To this cuvette was added FLASH-EDT_2 (2.50 μL of a 1.00 mM stock solution, 2.50 nmol, 1 eq). The fluorescence was monitored for 5 min with no observed increase in fluorescence. To this solution was added MES (25.0 μL of a 1.0 M stock solution of MES in H_2O, 10 000 eq) and let stand for 60 min with no increase in fluorescence.

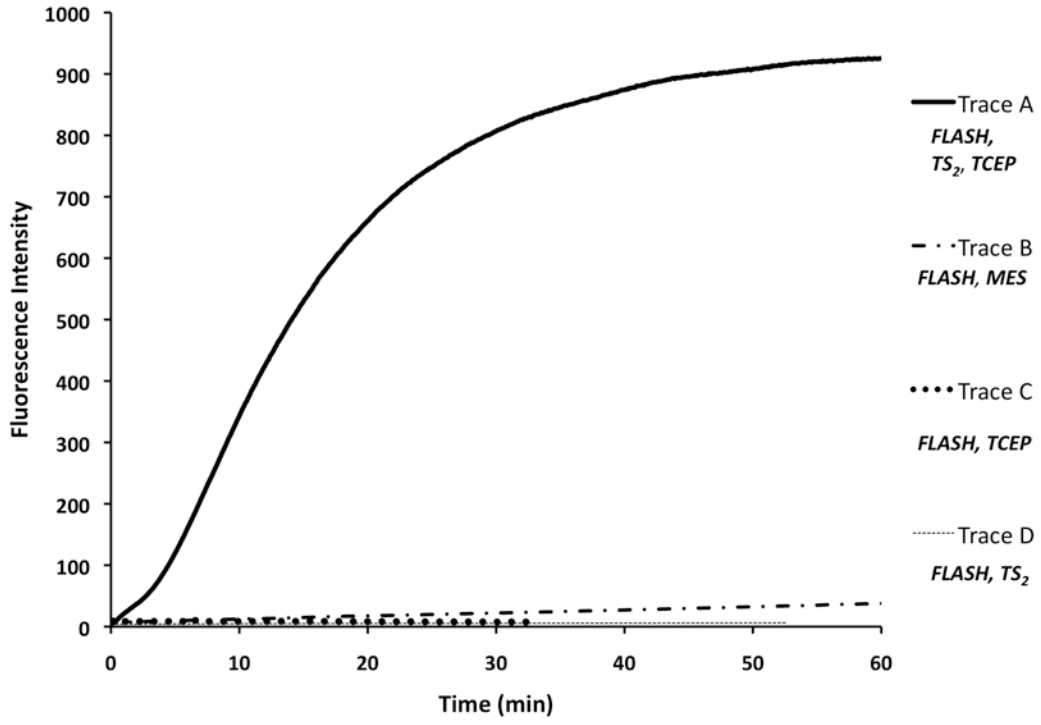


Figure S2. Binding experiments in buffer: (*Trace A*) 1.0 μM FLASH, 40.0 μM TS_2, 80.0 μM TCEP•HCl (*Trace B*) 1.0 μM FLASH, 10.0 mM MES (*Trace C*) 1.0 μM FLASH, 80.0 μM TCEP•HCl (*Trace D)* 1.0 μM FLASH, 40.0 μM TS_2

We also wanted to compare the effect of the introduction of MES to a solution of the FLASH-$(TSH_2)_2$ complex. Tsien has reported that the conjugation of FLASH-EDT_2 to tetracysteine peptides proceeded faster in the presence of low millimolar concentrations of monothiols such as MES. Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. The background fluorescence was recorded for 3.00 mL MOPS buffer and subtracted from subsequent measurements. To test the stability of FLASH-$(TSH_2)_2$ to MES, added FLASH-EDT_2 (2.10 μL of a 1.00 mM stock solution, 2.1 nmol, 1 eq) to this cuvette. To this solution was added TS_2 (42.0 μL of the 2.00 mM stock solution of TS_2, 84.0 nmol, 40 eq) followed by TCEP•HCl (16.8 μL of the 10.00 mM stock solution of TCEP•HCl, 168.0 nmol, 80 eq). The fluorescence intensity

immediately began to rise and reached saturation within 45 min. Once the reaction reached saturation, MES was added (30.0 μL of a 1.0 M stock solution of MES in H_2O, 30.0 μmol, 14 285 eq) (see *Trace A,* **Figure S3**). The measureable fluorescence in the sample immediately decreased and reached a new equilibrium with 76% the fluorescence of the original sample. We repeated this experiment for 1.0 mM final concentration of MES (3.0 μL of a 1.0 M stock solution of MES in H_2O, 3.0 μmol, 1 428 eq). We observed a slower decrease in observable fluorescence intensity (*Trace B,* **Figure S3**), the fluorescence in the sample still decayed to 80% of the maximal response over 2 h. We compared these results to competitive binding in the presence of low millimolar concentrations of MES. In this experiment, FLASH-EDT_2 (2.10 μL of a 1.00 mM stock solution, 2.1 nmol, 1 eq) was dissolved in 3.00 mL of MOPS buffer. To this solution was added MES (30.0 μL of a 1.0 M stock solution of MES in H_2O, 30.0 μmol, 14 285 eq). To this solution was added TS_2 (42.0 μL of the 2.00 mM stock solution of TS_2, 84.0 nmol, 40 eq) followed by the addition of TCEP•HCl (16.8 μL of the 10.00 mM stock solution of TCEP•HCl, 168 nmol, 80 eq). The fluorescence intensity immediately began to rise and reached saturation within 100 min at 75% the maximum fluorescence intensity (see *Trace C,* **Figure S3**). We repeated this experiment with 1.0 mM final concentration of MES (*Trace D,* **Figure S3**) and found that the reaction reached its maximum intensity of only 70% the maximum value within 50 minutes, but the fluorescence in the sample then began to decrease. In each case, the maximum fluorescence intensity was over 20% less than the intensity of that achieved without MES suggesting that pre-exposure to the large excess of a monothiol will occupy some of the binding sites, but that the probe is selective for the dithiol metabolite. In addition, the reaction does not proceed at a faster rate in the presence of millimolar concentrations of the monothiol. The background reaction with 10.0 mM MES showed an 8% increase in fluorescence over 2 h (*Trace E*, **Figure S3**).

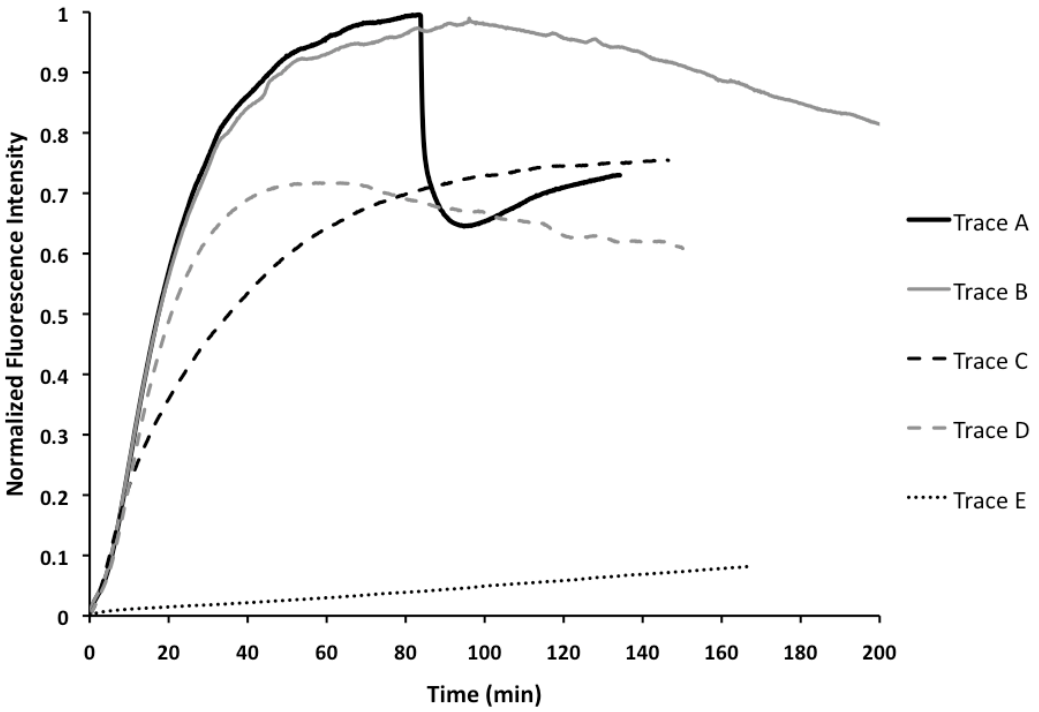


Figure S3. Binding experiments in buffer: Our competitive binding experiments with millimolar concentrations of the monothiol MES illustrate that the monothiol decreased the rate of conjugation and lowered the observable fluorescence intensity. Therefore, MES was not adopted for the assay protocol. (*Trace A*) 0.70 μM FLASH-EDT_2, 28.0 μM TS_2, 56.0 μM TCEP•HCl then 10.0 mM MES (*Trace B*) 0.70 μM FLASH-EDT_2, 28.0 μM TS_2, 56.0 μM TCEP•HCl, 10.0 mM MES (*Trace C*) 0.70 μM FLASH-EDT_2, 28.0 μM TS_2, 56.0 μM TCEP•HCl followed by 1.0 mM MES (*Trace D*) 1.0 μM FLASH-EDT_2, 28.0 μM TS_2, 56.0 μM TCEP•HCl, 1.0 mM MES (*Trace E)* 1.0 μM FLASH-EDT_2, 10.0 mM MES.

Procedure for determining stoichiometry of fluorescent species:

To determine the nature of the fluorescent species, we performed a series of HPLC studies with fluorescence and mass spectrometry detection. Analyses were performed on a Waters 2690XE separation module with a 996 photodiode

array detector and a 2475 multifluorescence detector and an Thermo Scientific LCQ Fleet LC/ MS^n.

For analysis with fluorimetric detection, we used an ACE 3 C18 column (50 x 2.1 mm) and used a gradient for separation using the following method, flow rate of 750 μL/min:

Table S1. Gradient for HPLC studies with fluorimetric detection.

Time (min)	H_2O with 0.1% TFA	MeCN
0	100 %	0 %
3	100 %	0 %
10	0%	100%
13	0%	100%
16	100%	0%
20	100%	0%

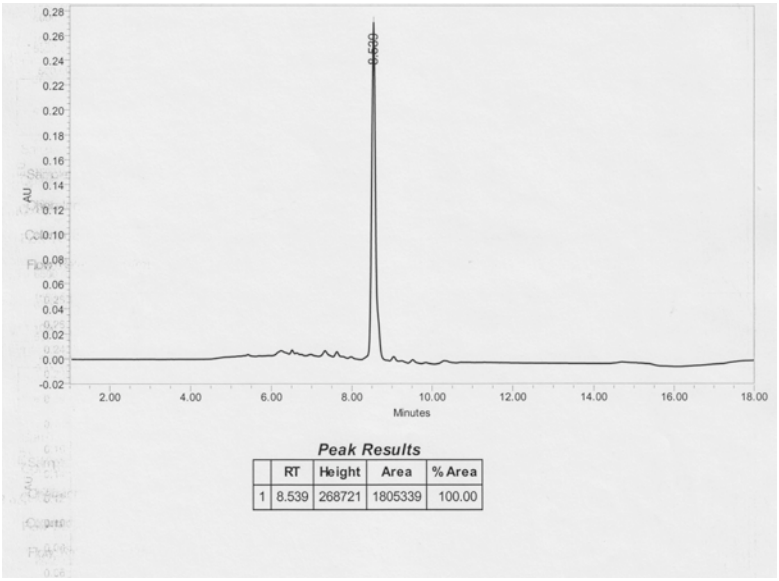
For analysis with mass spectral detection, we used a Varian Pursuit 3 C18 column (50 x 2.0 mm) and performed a gradient separation using the following method, flow rate 500 μL/min:

Table S2. Gradient for HPLC studies with mass spectral detection.

Time (min)	H_2O with 0.1% TFA	MeCN
0	100 %	0 %
0.25	100 %	0 %
6	0%	100%
7.85	0%	100%
8	100%	0%
10	100%	0%

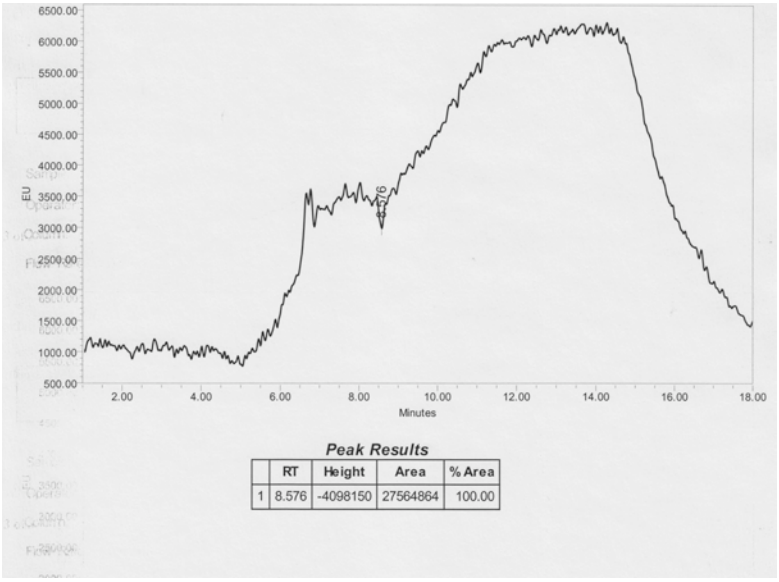
Procedure for separation of FLASH-$(TSH_2)_2$ using HPLC with UV, Fluorescence, and Mass Spectral detection:

To 80.0 μL of MOPS buffer pH 6.95 was added 100.0 μL of a 2.0 mM solution of TS_2 and 20.0 μL of a 0.5 mM solution of FLASH-EDT_2. This solution was injected on the Waters HPLC and showed a single peak with UV detection (254 nm, RT = 8.54 min, **Figure S4**) and showed no peak in with fluorescence detection (**Figure S5**).



	RT	Height	Area	% Area
1	8.539	268721	1805339	100.00

Figure S4. HPLC chromatogram with UV detection for FLASH-EDT_2.



	RT	Height	Area	% Area
1	8.576	-4098150	27564864	100.00

Figure S5. HPLC chromatogram with fluorescence (λ_{ex} = 505 nm and λ_{em} = 525 nm) detection for FLASH-EDT_2.

This solution was injected in the LC/MS system using the generic gradient, and showed a single peak in UV and MS chromatograms (RT = 5.64 min, m/z = 665.0 [FLASH-EDT$_2$ +H]$^+$, **Figure S6**).

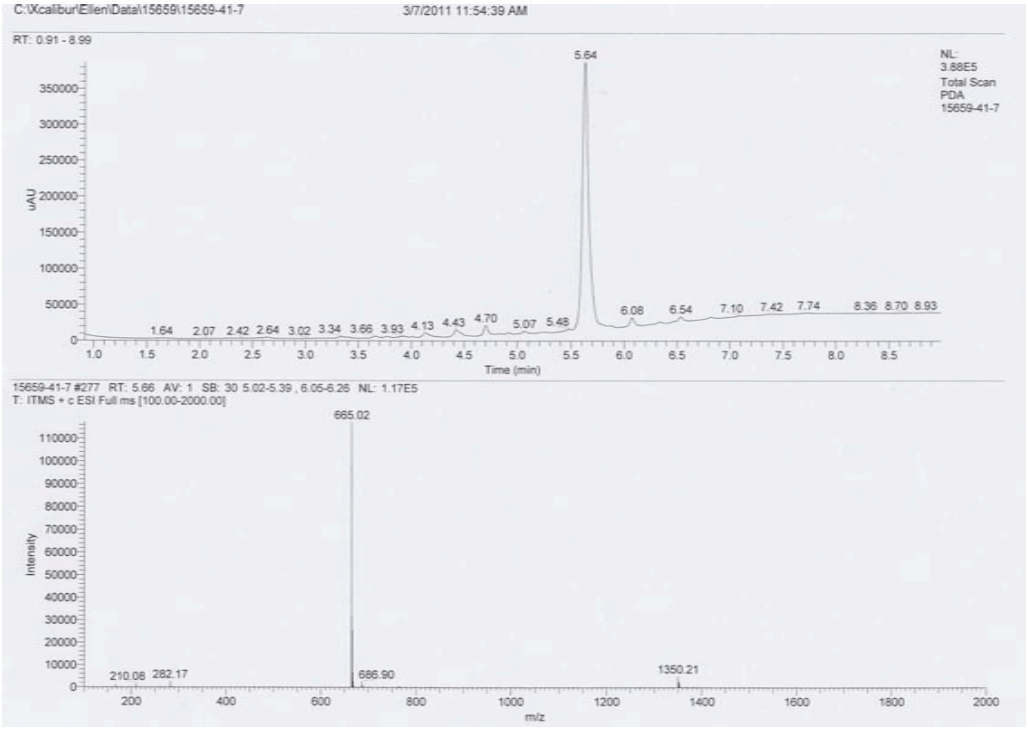


Figure S6. LCMS chromatogram for FLASH-EDT$_2$.

To determine the nature of the fluorescent species formed upon exposure to TSH$_2$, we subjected the reaction mixture to the same analysis. To 80.0 μL of MOPS buffer, pH 6.95 was added 100.0 μL of a 2.0 mM solution of TS$_2$, 20.0 μL of 0.50 mM FLASH-EDT$_2$, and 40.0 μL of TCEP•HCl. This solution was allowed to reach saturation (60 min at rt). The sample was injected in the HPLC with UV and fluorescence detection and revealed three UV active species (**Figure S7**) and three fluorescent species (**Figure S8**). The first species at (RT = 5.52) corresponds to 34.5% of UV active material but 96.3% of fluorescence in the sample. The second species (RT = 6.40 min) corresponds to 9.3% of the UV active material and 2.1% of the fluorescence in the sample. The third UV active species (RT = 8.53 min) corresponds to 56.2% of the UV active material but does not contribute to the fluorescence of the sample.

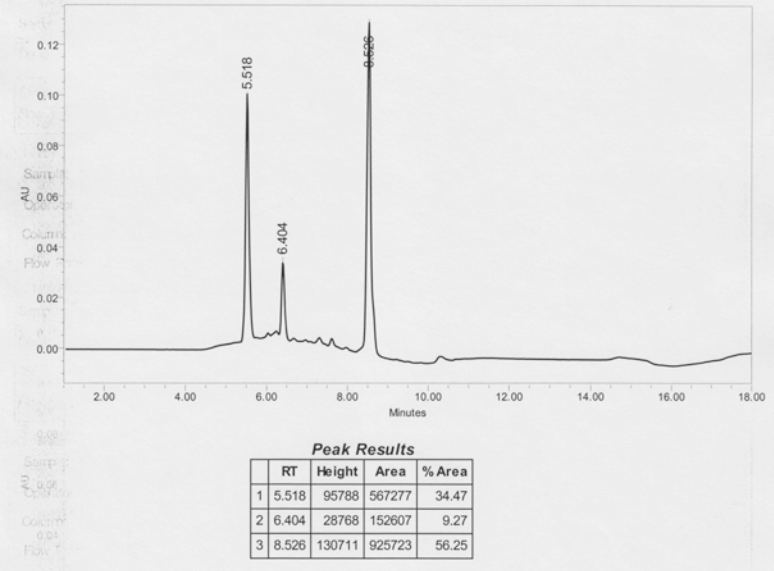


Figure S7. HPLC chromatogram with UV detection for FLASH-$(TSH_2)_2$ binding experiments.

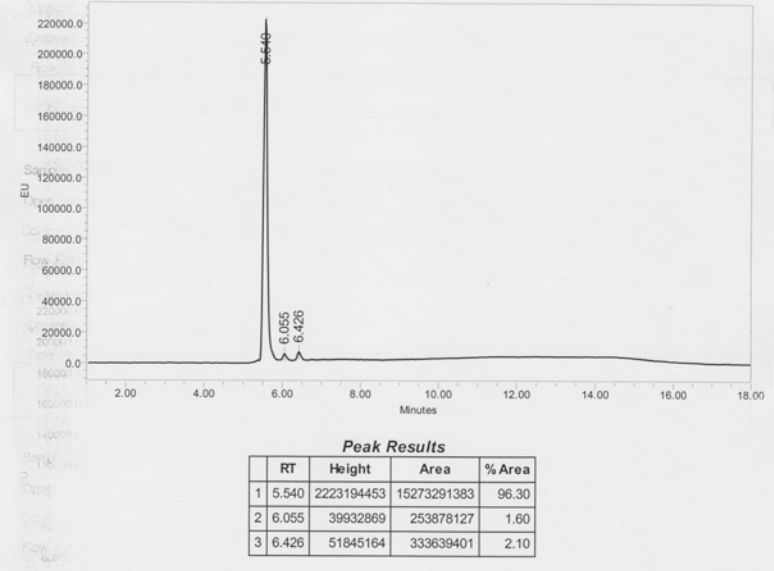


Figure S8. HPLC chromatogram with fluorescence detection (λ_{ex} = 505 nm and λ_{em} = 525 nm) for FLASH-$(TSH_2)_2$ binding experiments.

This sample was then injected in the LCMS to obtain the mass for each UV active species. The first species (RT = 2.67 min, m/z = 1924.2 [FLASH-$(TSH_2)_2$ + $H]^+$, 962.5 [FLASH-$(TSH_2)_2$ + $2H]^{2+}$, **Figure S9**) corresponds to FLASH-$(TSH_2)_2$

and is the major contributor of fluorescence in the sample, confirming the hypothesis that two molecules of TSH_2 must bind for a fluorimetric response.

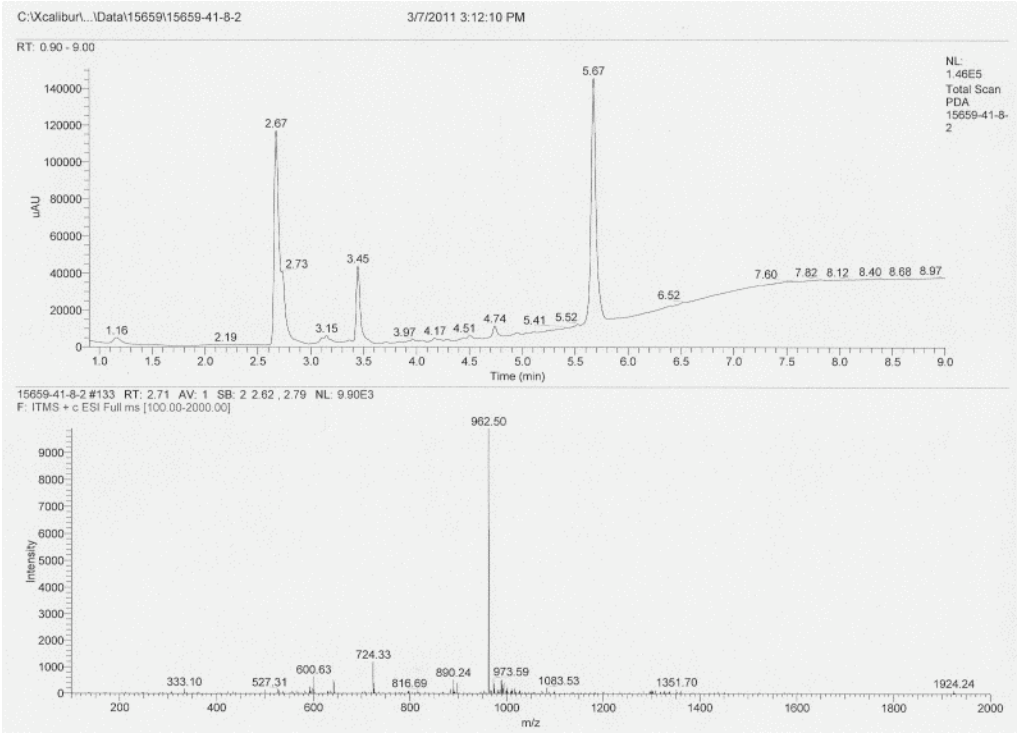


Figure S9. LCMS chromatogram and mass spectrum for FLASH-$(TSH_2)_2$.

The second species (RT = 3.45 min, m/z = 1294.14 [FLASH-(TSH_2, EDT) + H]$^+$, 647.6 [FLASH-(TSH_2, EDT)+ 2H]$^{2+}$, **Figure S10**) corresponds to FLASH-(TSH_2, EDT) and is the minor contributor of fluorescence in the sample.

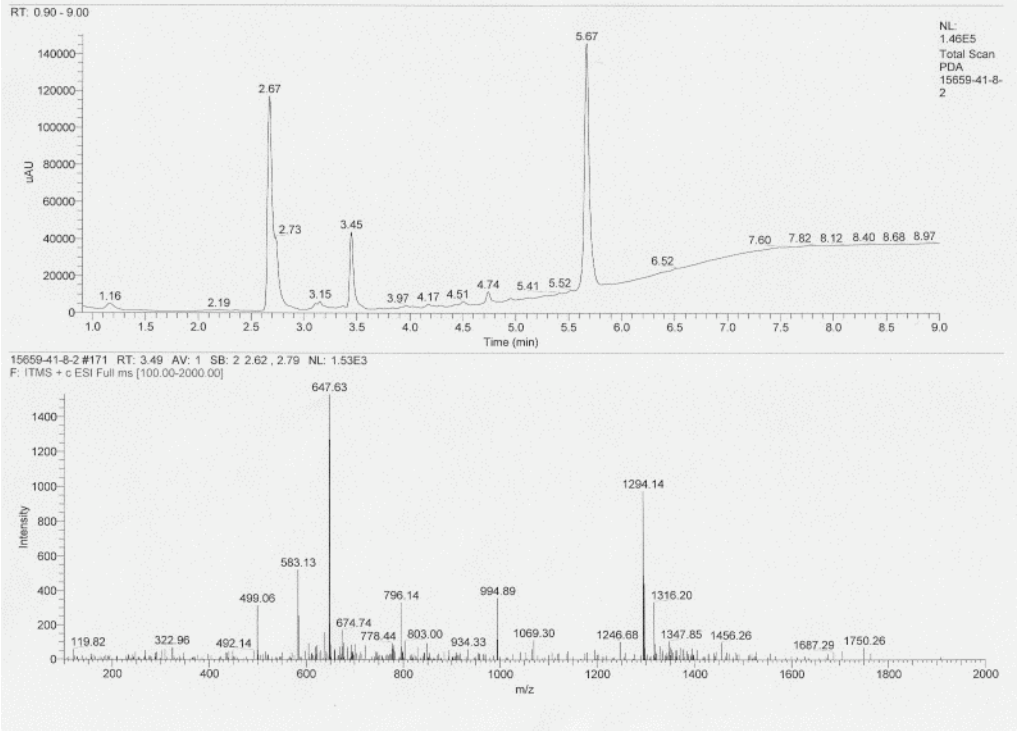


Figure S10. LCMS chromatogram and mass spectrum for FLASH-(TSH_2, EDT).

The third UV active species (RT = 5.67 min, m/z = 664.9 [FLASH-EDT_2 +H]$^+$, **Figure S11**) corresponds to unreacted FLASH-EDT_2 and does not contribute to fluorescence in the sample.

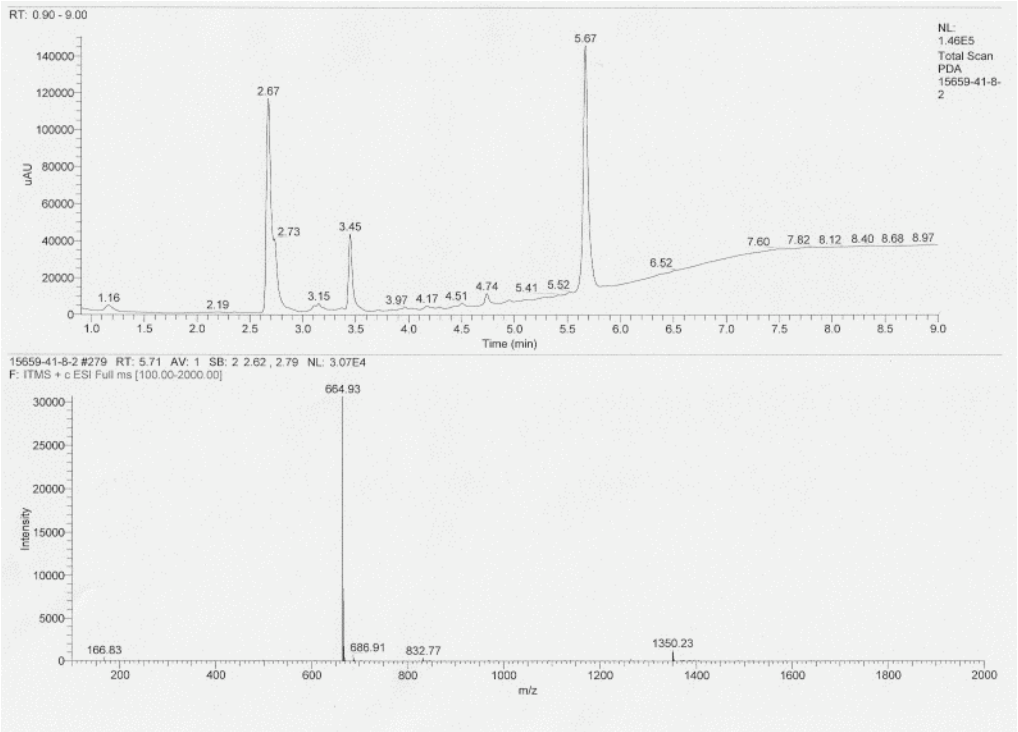


Figure S11. LCMS chromatogram and mass spectrum for FLASH-EDT$_2$.

Procedure for binding experiments in serum extracts: To a vial containing 400.0 μL of rat serum was added TS$_2$ (50.0 μL of a 2.00 mM stock solution, 100 nmol, 40 eq). To this solution was added 800.0 μL of MeCN. This solution was vortexed and centrifuged (10,000 rpm for 3 min). The supernatant was diluted with 1.50 mL of MOPS buffer, pH 6.94. Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. A background was recorded with the diluted supernatant and substracted from subsequent intensity measurements. To this solution was added FLASH-EDT$_2$ (2.50 μL of a 1.00 mM stock solution, 2.50 nmol, 1 eq) followed by the addition of TCEP•HCl (40.0 μL of a 10.0 mM solution, 400 nmol, 160 eq), and the fluorescence intensity was monitored.

A control vial was prepared by adding 400.0 μL of rat serum to a vial containing 800.0 μL of MeCN. This solution was vortexed and centrifuged (10,000 rpm for 3 min). The supernatant was diluted with 1.50 mL of MOPS buffer, pH 6.94. Fluorescence intensity measurements were collected with λ_{ex} =

505 nm and λ_{em} = 527 nm. A background was recorded with the diluted supernatant and subtracted from subsequent measurements. To this solution was added FLASH-EDT_2 (2.50 μL of a 1.0 mM stock solution, 2.50 nmol, 1 eq) followed by the addition of TCEP•HCl (40.0 μL of a 10.0 mM solution, 400 nmol, 160 eq), and the fluorescence intensity was monitored. There was no observed increase in fluorescence intensity (See **Figure 2**).

Quantum Yield Determination: The quantum yield of FLASH-$(TSH_2)_2$ was determined using fluorescein as the standard.[2] A stock solution of fluorescein (1.00 mM in 0.10 N NaOH) was prepared. The absorbance spectrum of 0.10 N NaOH was recorded from 400-600 nm in a 1 cm x 1cm cuvette and was subtracted as a background for the absorbance spectrum of fluorescein. Fluorescein (50.0 μL of 1.0 mM stock solution) was diluted in 3.00 mL of 0.10 N NaOH. The absorbance spectrum was recorded from 400 – 600 nm. A 10.0 μL aliquot of this solution was diluted with 3.00 mL of 0.10 N NaOH. The emission spectrum from 450-700 nm was recorded with λ_{ex} = 470 nm. The area under the curve from 480 – 700 nm was corrected by subtracting the area for the blank 0.10 N NaOH solution. The values obtained in these experiments were used to determine the quantum yield of the FLASH probe and conjugate with TSH_2.

To determine the quantum yield of FLASH-EDT_2, 100.0 μL of a 1.00 mM stock solution was diluted with 3.00 mL of MOPS buffer at pH 6.94. The absorbance spectrum was recorded from 400 – 600 nm in a 1 cm x 1cm cuvette. The absorbance spectrum of 3.00 mL of MOPS buffer at pH 6.94 was subtracted as a background correction.The emission spectrum of this sample from 450 – 700 nm was recorded with λ_{ex} = 470 nm excitation. The area under the curve from 480 – 700 nm was corrected by subtracting the area for the blank MOPS buffer solution. The quantum yield for FLASH-EDT_2 was measured as 0.0013 using this procedure.

To determine the quantum yield of FLASH-$(TSH_2)_2$, 20.0 μL of a 1.00 mM stock solution of FLASH-EDT_2 (20.0 nmol, 1 eq) was diluted with 2.50 mL of

MOPS buffer at pH 6.94. To this mixture was added TS_2 (200.0 μL of a 2.00 mM stock solution, 400.0 nmol, 20 eq) and TCEP•HCl (80.0 μL of a 10 mM solution, 800.0 nmol, 40 eq). This mixture was allowed to react for 1.5 h. The absorbance spectrum was recorded from 400 – 600 nm. A 125.0 μL aliquot of this solution was diluted with 2.375 mL of MOPS buffer. The emission spectrum of this solution was recorded with λ_{ex} = 470 nm excitation. The area under the curve from 480 – 700 nm was corrected by subtracting the area for the blank MOPS buffer solution. The quantum yield for FLASH-$(TSH_2)_2$ was measured as 0.24. Quantum yields were determined using the following equation:

$$\Phi_X = \frac{A_S}{A_X} \times \frac{F_x}{F_S} \times \Phi_S$$

A = absorbance at λ_{ex}

F = Integrated area from emission spectrum

Φ = quantum yield

X = analyte, S = standard

Procedure for determining the limit of detection for TSH_2: A solution of TSH_2 (3.23 mM) was prepared in water by dissolving TS_2 (2.020 mg, MW 721.96 g/mol, 2.80 μmol) in 251 μL of water. To this solution was added TCEP•HCl (616.0 μL of a 10.0 mM solution, 6.16 μmol, 2.2 eq), and the mixture was stirred for 20 min.

Fluorescence intensity measurements were collected with λ_{ex} = 505 nm and λ_{em} = 527 nm. A background was recorded with 2.50 mL of MOPS buffer neutralized to a pH of 6.94, and this value was subtracted from subsequent measurements. To this solution was added FLASH-EDT_2 (2.00 μL of a 1.00 mM stock solution, 2.00 nmol, 1 eq). To this solution was added TSH_2 (31.0 μL of the 3.23 mM stock solution of TSH_2, 100 nmol, 50 eq). This reaction reached saturation within 45 min. This corresponds to the 40 μM TSH_2 trace in **Figure 3**.

To a cuvette containing 2.50 mL of MOPS buffer neutralized to a pH of 6.94 was added FLASH-EDT_2 (2.00 μL of a 1.00 mM stock solution, 2.00 nmol, 1 eq). To this solution was added TSH_2 (15.5 μL of the 3.23 mM stock solution of TSH_2, 50 nmol, 25 eq). The fluorimetric response was observable both with the fluorescence spectrometer and with a handheld UV lamp with λ_{ex} = 365 nm after 1 h. This corresponds to the 20 μM TSH_2 trace in **Figure 3**.

To a cuvette containing 2.50 mL of MOPS buffer neutralized to a pH of 6.94 was added FLASH-EDT_2 (2.00 μL of a 1.00 mM stock solution, 2.00 nmol, 1 eq). To this solution was added TSH_2 (7.75 μL of the 3.23 mM stock solution of TSH_2, 25 nmol, 12.5 eq). The fluorimetric response was observable both with the fluorescence spectrometer and with a handheld UV lamp with λ_{ex} = 365 nm after 1 h. This corresponds to the 10 μM TSH_2 trace in **Figure 3**.

To a cuvette containing 2.50 mL of MOPS buffer neutralized to a pH of 6.94 was added FLASH-EDT_2 (2.00 μL of a 1.00 mM stock solution, 2.00 nmol, 1 eq). To this solution was added TSH_2 (3.09 μL of the 3.23 mM stock solution of TSH_2, 10 nmol, 5 eq). The fluorimetric response was only visible with the fluorescence spectrometer after 1 h. This corresponds to the 4 μM TSH_2 trace in **Figure 3**.

(1) Adams, S. R.; Tsien, R. Y. *Nature Protocols* **2008**, *3*, 1527-1534.
(2) Fery-Forgues, S.; Lavabre, D. *J. Chem. Educ.* **1999**, *76*, 1260-1264.