Supplementary Material (ESI) for Chemical Communications

Supporting Information

Orthogonal or simultaneous use of disulfide and hydrazone exchange in dynamic covalent chemistry in aqueous solution

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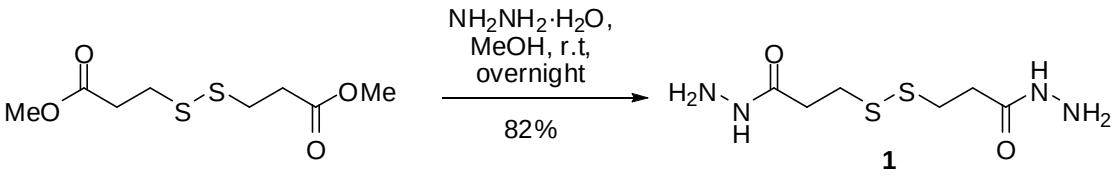
MATERIAL AND METHODS

3,5-Dihydroxybenzaldehyde and hydrazine hydrate were purchased from Aldrich. 2-Furanmethanethiol, sodium acetate, sodium formate, acetic acid and formic acid were purchased from Fluka. 3,3'-Dithiopropionic acid dimethylester was purchased from TCI. Ammonia solution was purchased from Romil. 4-Hydroxybenzaldehyde was purchased from Avocado. Acetonitrile and methanol were purchased from Fisher.

Synthesis of dithiodipropionic acid dihydrazide (1)



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3,3'-Dithiopropionic acid dimethylester (1.19 g , 4.2 mmol) was dissolved in dry MeOH (25 mL). Hydrazine hydrate (1.24 mL, 25 mmol, 6 equiv) was added and reaction stirred overnight at room temperature. The resulting suspension was filtered and the resulting white solid washed with MeOH (10 mL), water (2 mL) and MeOH (5 mL). Compound **1** was obtained in a 80% yield (0.98 g).

^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 9.05 (s, 2H, N-N*H*-C(O)), 4.21 (s, 4H, NH_2-N-C(O)), 2.88 (t, 4H, C(O)-C-CH_2-S), 2.40 (t, 4H, N-C(O)-CH_2-C).

Additional characterisation data can be found in: (a) X. Z. Shu, Y. Liu, Y. Luo, M. C. Roberts and G. D. Prestwich, *Biomacromolecules*, 2002, **6**, 1304; (b) K.P. Vercruysse, D. M. Marecak, J.F. Marecek, G.D. Prestwich, *Bioconjugate Chem.*, 1997, **8**, 686.

General method to set up disulfide-hydrazone exchange experiments

Experiment A

Dithiopropionic acid dihydrazide (**1**) (200 µL, 40 mM in ammonium acetate buffer 20 mM at pH 8.5), 4-hydroxybenzaldehyde (**2**) (200 µL, 80 mM in acetonitrile), 2-furanmethanethiol (**3**) (1.61 µL), ammonium acetate buffer 20 mM at pH 8.5 (600 µL) and acetonitrile (600 µL) were mixed in a 2 mL HPLC vial. The resulting solution (1.6 mL, 1:1 acetonitrile water) that contained the following concentrations of each reactant: 5 mM of **1**, 10 mM of **2** and **3**, 10 mM of buffer was equilibrated for 3 days. Half of the volume (800 µL) was placed in a different vial and acidified to pH 2.5 with formic acid (60 µL). The mixture was allowed to equilibrate for 2 days.

Experiment B

Dithiopropionic acid dihydrazide (**1**) (200 µL, 40 mM in ammonium formate buffer 20 mM at pH 2.5), 4-hydroxybenzaldehyde (**2**) (200 µL, 80 mM in acetonitrile), 2-furanmethanethiol (**3**) (1.61 µL), ammonium formate buffer 20 mM at pH 2.5 (600 µL) and acetonitrile (600 µL) were mixed in a 2 mL HPLC vial. The resulting solution (1.6 mL, 1:1 acetonitrile water) that contained the following concentrations of each reactant: 5 mM of **1**, 10 mM of **2** and **3**, 10 mM of buffer was equilibrated for 3 days. Half of the volume (800 µL) was placed in a different vial and the pH was adjusted to 8.5 with triethylamine (130 µL). The mixture was allowed to equilibrate for 2 days.

Experiment C

Hydrazone exchange at pH 8.5: dihydrazone **5** was obtained at pH 4.5 mixing dithiopropionic acid dihydrazide (**1**) (187.5 µL, 40 mM in ammonium acetate buffer 20 mM at pH 4.5), 4-hydroxybenzaldehyde (**2**) (187.5 µL, 80 mM in acetonitrile), ammonium acetate buffer 20 mM at pH 4.5 (562.5 µL) and acetonitrile (562.5 µL) in a 2 mL HPLC vial. Compound **5** was obtained after 2 days. The mixture was alkalinised to pH 8,5 with ammonia solution (20-22%, 3.75 µL) and 3,5-dihydroxybenzaldehyde (**4**) (1.04 mg, 1 equiv) was added. The resulting solution (1.5 mL, 1:1 acetonitrile/water) that contained the following concentrations of each reactant: 5 mM of **1** and **4**, 10 mM of **2** and 10 mM of buffer was equilibrated for 5 days.

Hydrazone exchange at pH 4.5: dihydrazone **5** was obtained at pH 4.5 mixing dithiopropionic acid dihydrazide (**1**) (50 µL, 40 mM in acetonitrile), 4-hydroxybenzaldehyde (**2**) (50 µL, 80 mM in acetonitrile), ammonium acetate buffer 20 mM at pH 4.5 (200 µL) and acetonitrile (100 µL) in a 2 mL HPLC vial. Compound **5** was obtained after 4 days. 3,5-dihydroxybenzaldehyde **4** (0.27 mg, 1 equiv) was added. The resulting solution (0.8 mL, 1:1 acetonitrile water) that

contained the following concentrations of each reactant: 5mM of **1** and **4**, 10 mM of **2** and 10 mM of buffer was equilibrated for 11 days.

Hydrazone exchange at pH 2.5: dihydrazone **5** was obtained at pH 2.5 mixing in a 2 mL HPLC vial dithiopropionic acid dihydrazide (**1**) (50 µL, 40 mM in ammonium formate buffer 20 mM at pH 2.5), 4-hydroxybenzaldehyde **2** (50 µL, 80 mM in acetonitrile), ammonium acetate buffer 20 mM at pH 4.5 (150 µL) and acetonitrile (150 µL). Compound **5** was obtained after 2 days. 3,5-Dihydroxybenzaldehyde (**4**) (0.27 mg, 1 equiv) was added. The resulting solution (0.4 mL, 1:1 acetonitrile water) that contained the following concentrations of each reactant: 5 mM of **1** and **4**, 10 mM of **2** and 10 mM of buffer was equilibrated for 1 day.

Experiment D

Dithiopropionic acid dihydrazide (**1**) (200 µL, 40 mM in ammonium acetate buffer 20 mM at pH 4.5), 4-hydroxybenzaldehyde (**2**) (200 µL, 80 mM in acetonitrile), 2-furanmethanethiol (**3**) (1.61 µL), ammonium formate buffer 20 mM at pH 4.5 (600 µL) and acetonitrile (600 µL) were mixed in a 2 mL HPLC vial. The resulting solution (1.6 mL, 1:1 acetonitrile water) that contained the following concentrations of each reactant: 5 mM of **1**, 10 mM of **2** and **3**, 10 mM of buffer was equilibrated for 5 days

HPLC ANALYSIS

HPLC was performed using an Agilent 1100 series HPLC equipped with a multiwavelength UV detector. Acetonitrile was acquired from Fisher and formic acid from Romil.

HPLC Parameters
Injection volume: 2 µL
Flow rate: 1.000 mL/min
Column: Perkin Elmer cartridge column, Pecosphere 3 C18, 33 × 4.6 mm, 3 µm, 80 Å. Spherical monofunctional bonded silica phase.
Mobile phase: milliQ water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B)

Gradient elution

Time (mins)	Solvent B(%)
0	10
1	20
1.5	40
3	40
4	95
7	95

HPLC/ESI-MS ANALYSIS

LC-MS was performed using an Agilent 1100 series HPLC equipped with a diode array UV detector and an Agilent XCT ion trap mass spectrometer. Solvents and formic acid were acquired from Romil. The LC parameters are as described in the previous section.

MS Parameters
Mass range mode: Standard Enhanced
Ion polarity: Negative mode
Ion Source: ESI
Dry temperature: 350 ºC
Nebuliser pressure: 32.00 psi
Dry gas flow: 9 L/min
HV capillary: 3500 V

Representative base peak chromatogram (Figure S1) and extracted ion traces (Figure S2) of a mixture made of dihydrazide/disulfide (1), 5 mM, 4-hydroxybenzaldehyde (2) 10 mM and furanmethanethiol (3) 10 mM in 1:1 acetonitrile/ ammonium acetate buffer pH 4.5 after 7 days.

Structures were identified and correlated to UV peaks by analysis of the base peak chromatogram.

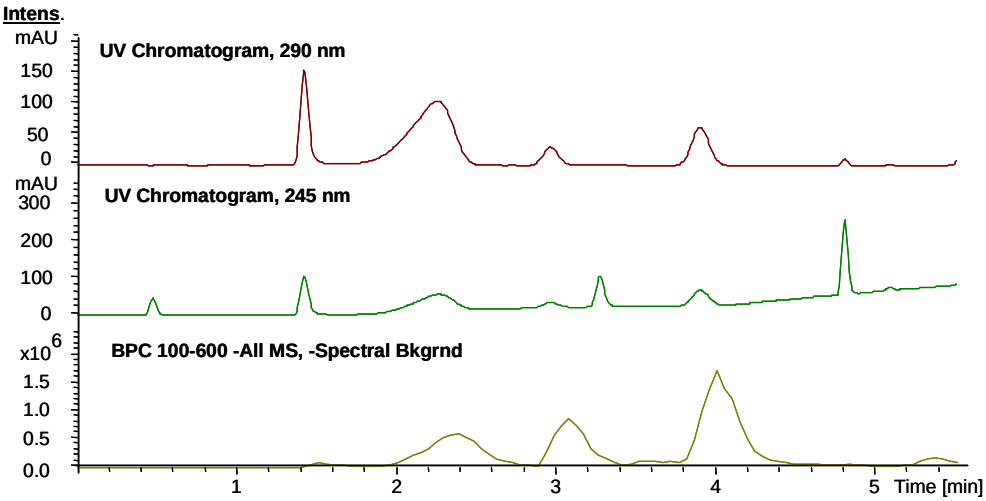

Figure S1

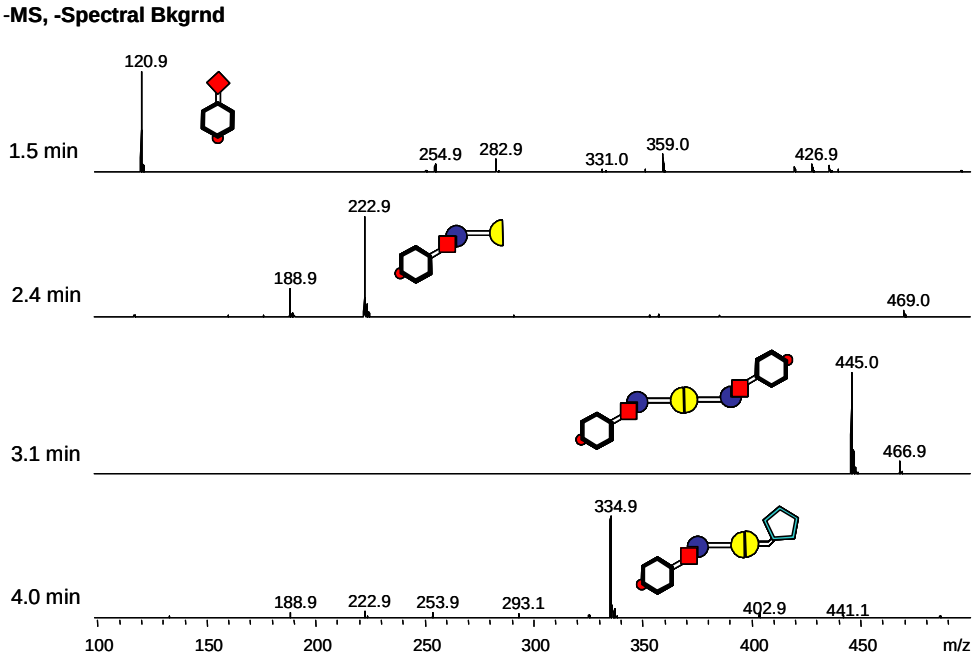

Figure S2

Representative base peak chromatogram (Figure S3) and extracted ion traces (Figure S4) of a mixture made of dihydrazone/ disulfide (5), 5 mM, and 3,5-dihydroxybenzaldehyde (4), 5 mM in 1.1 acetonitrile/ammonium formate buffer pH 2.5 after 1 day.

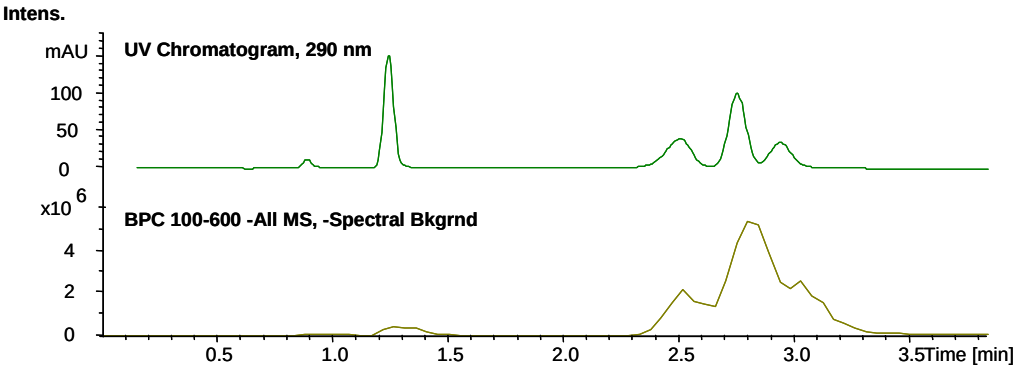

Figure S3

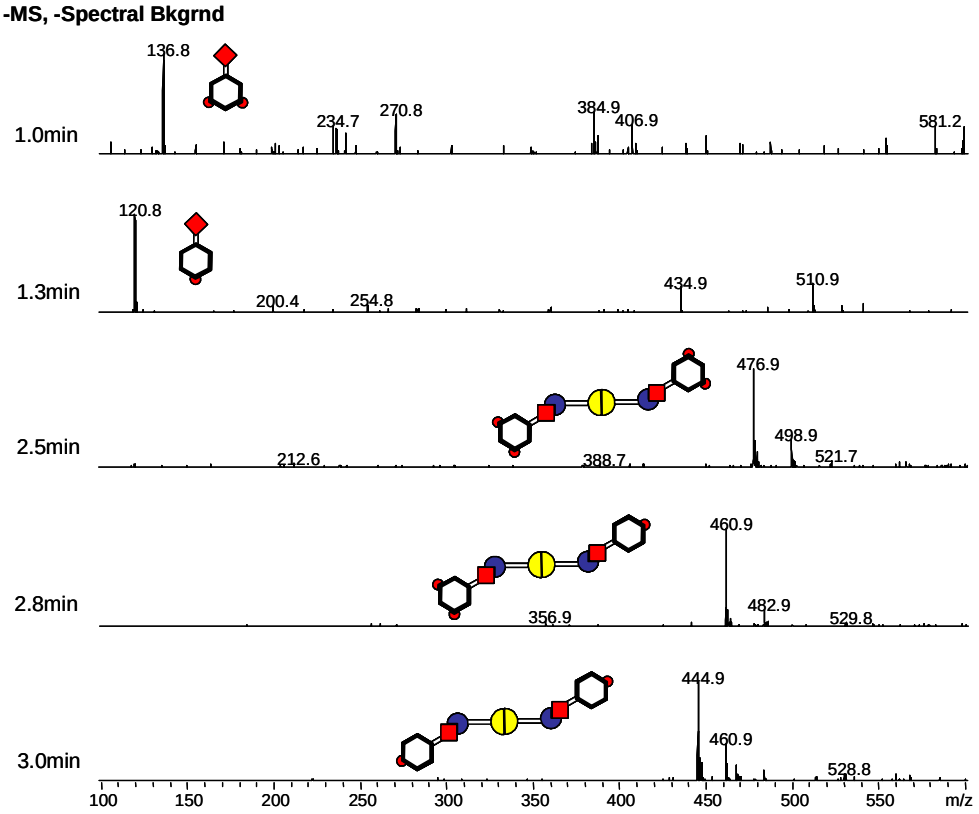

Figure S4

DISULFIDE EXCHANGE WITH DIFFERENT THIOLS

Experiments involved the species represented in Figure S5. In all the experiments dihydrazone **12** was formed at different acidic pHs (2.5, 3.0, 3.5, 4.0 and 4.5) starting from dithioproionic acid dihydrazide (**1**), 5 mM, and 3,5-dihydroxybenzaldehyde (**4**)**,** 10 mM, in 1:1 acetonitrile/10 mM ammonium acetate (pH 4.0, 4.5) or formate (pH 2.5, 3.0, 3.5) buffer. After 24 hours 2 equiv. of thiol, 10 mM, were added (**14** as a solid and **3** and **15** as liquids) and the equilibration process followed by HPLC during 3-4 days.

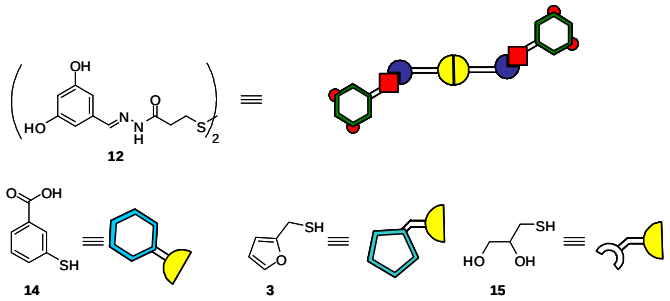

Figure S5

The pK_a values for the thiol groups were estimated using Marvin Sketch from ChemAxon:

14, pKa = 6.20

3, pKa = 9.71

15, pKa = 9.92

Figure S6, Figure S7 and Figure S8 show the HPLC traces for the disulfide exchange reactions between compound **12** and thiols **14**, **3** and **15** respectively in the pH range 2.5 to 4.5.

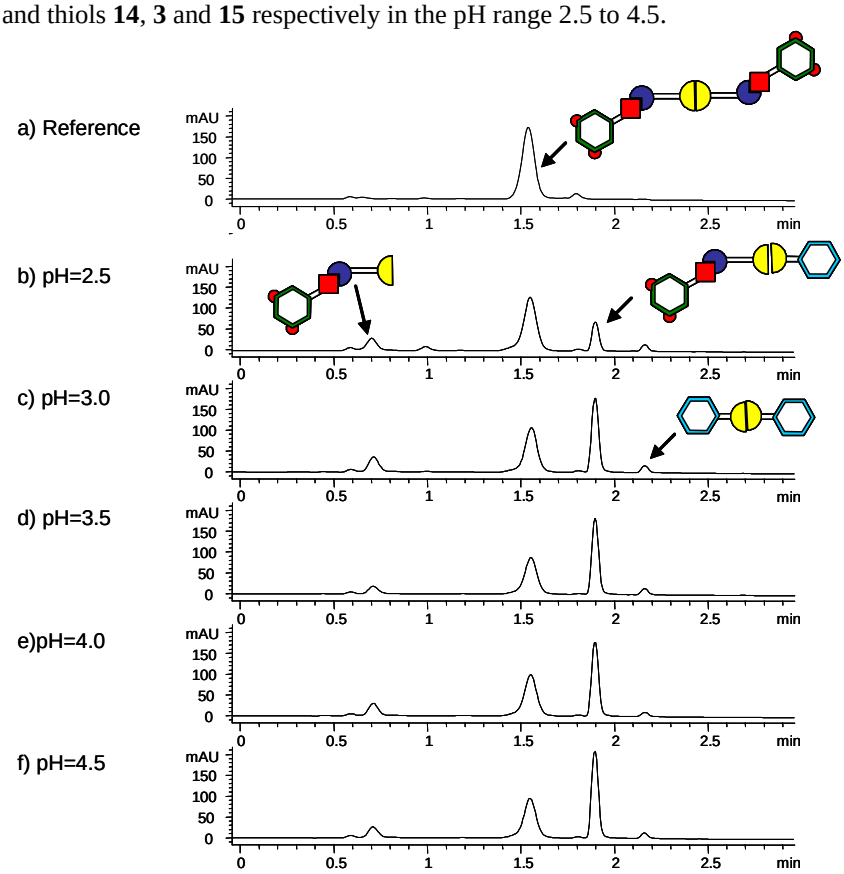

Figure S6: HPLC traces of 3,5-dihydroxybenzaldehyde dihydrazone before (a) and 4 days after the addition of 3,5-mercaptobenzoic acid (**14**) at (b) pH 2.5; (c) pH 3.0; (d) pH 3.5; (e) pH 4.0; and (f) pH 4.5.

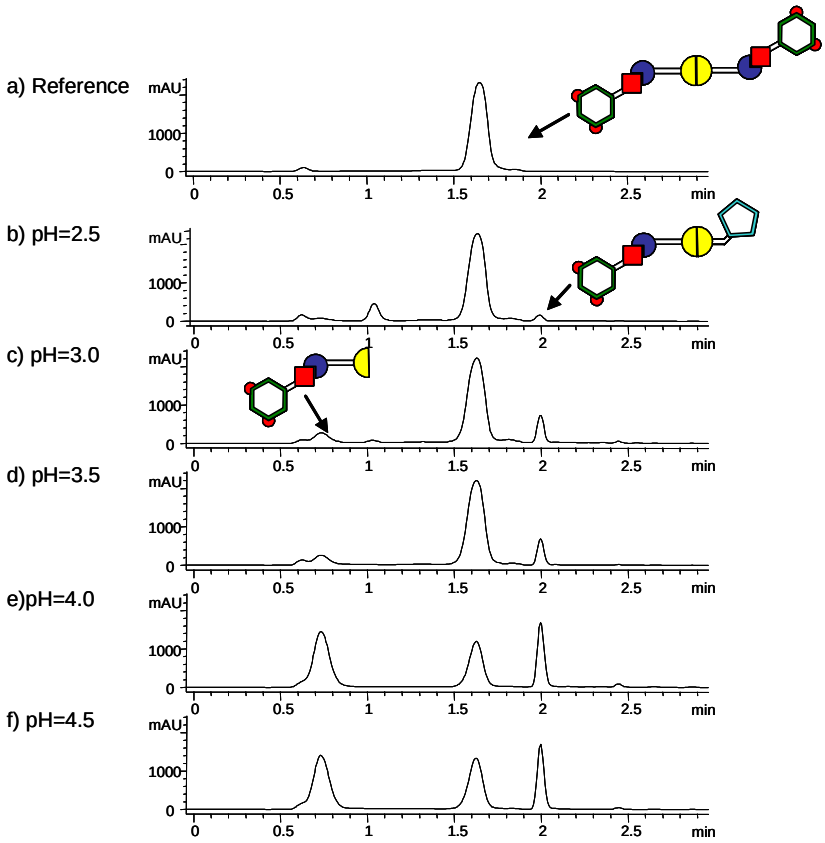

Figure S7: HPLC traces of 3,5-dihydroxybenzaldehyde dihydrazone before (a) and 4 days after the addition of furanmethanethiol (**3**) at (b) pH 2.5; (c) pH 3.0; (d) pH 3.5; (e) pH 4.0; and (f) pH 4.5.

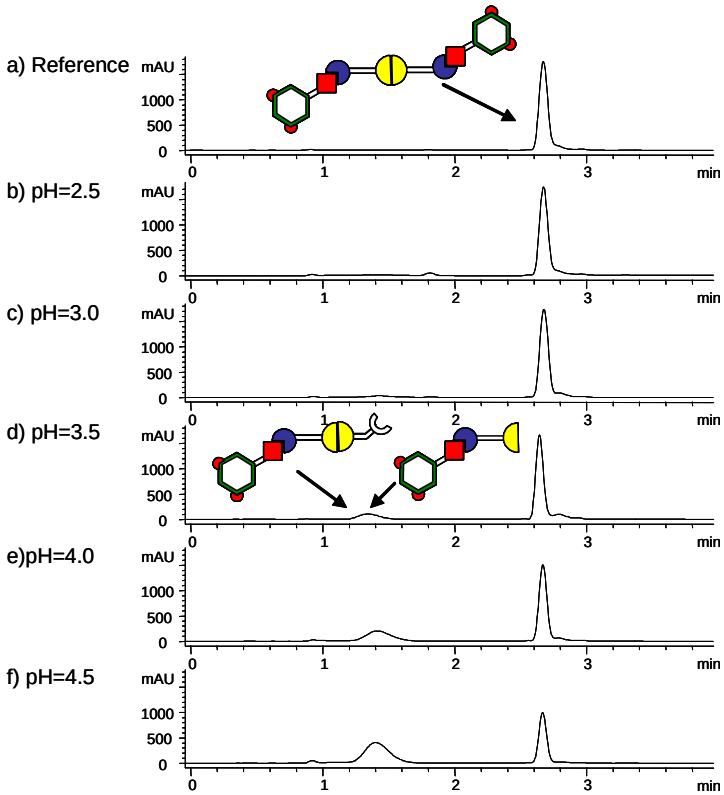

Figure S8: HPLC traces of 3,5-dihydroxybenzaldehyde dihydrazone before (a) and 96 hours after the addition of thioglycerol (**15**) at pH 2.5; (c) pH 3.0; (d) pH 3.5; (e) pH 4.0; and (f) pH 4.5.

Figure S9 and Figure S10 show the base peak chromatogram and extracted ion traces of the mixtures made from **12**/**14** and **12**/**15** respectively.

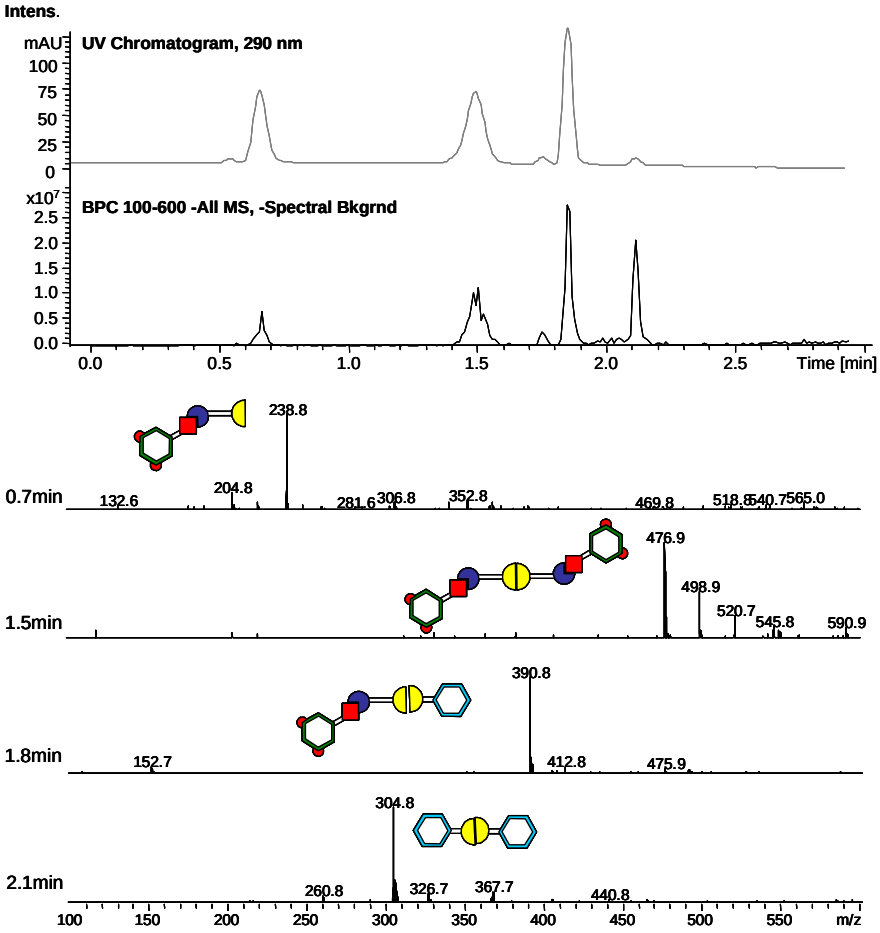

Figure S9

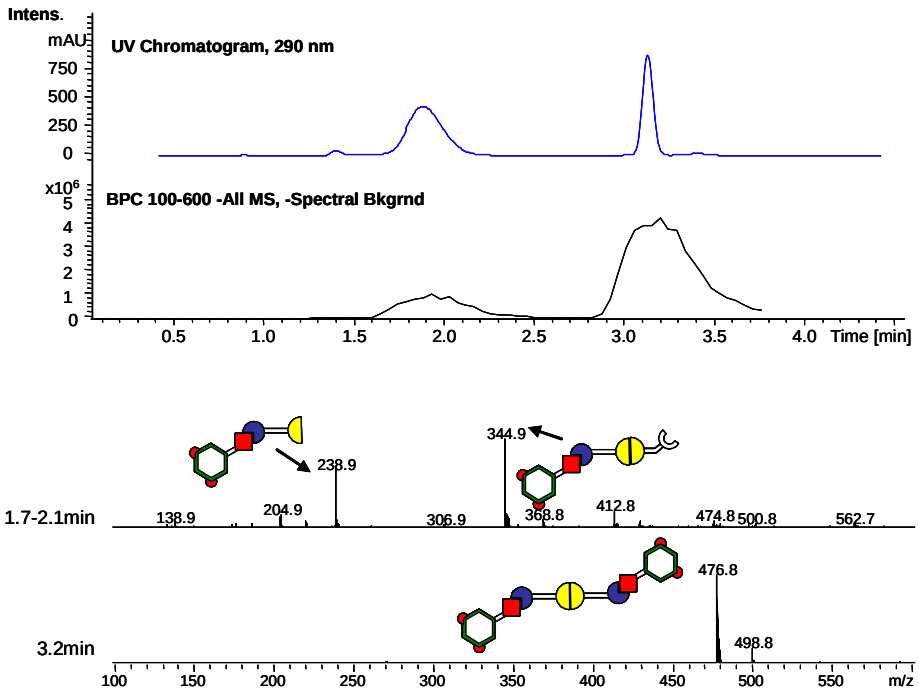

Figure S10

Figure S11, Figure S12 and Figure S13 show the kinetics of the exchange of the compound **12** with thiols **14**, **3** and **15**, respectively, at different pHs. The concentration of starting dihydrazone was quantified from the HPLC peak area, normalised with respect to the total peak area of starting material and disulfide exchange products.

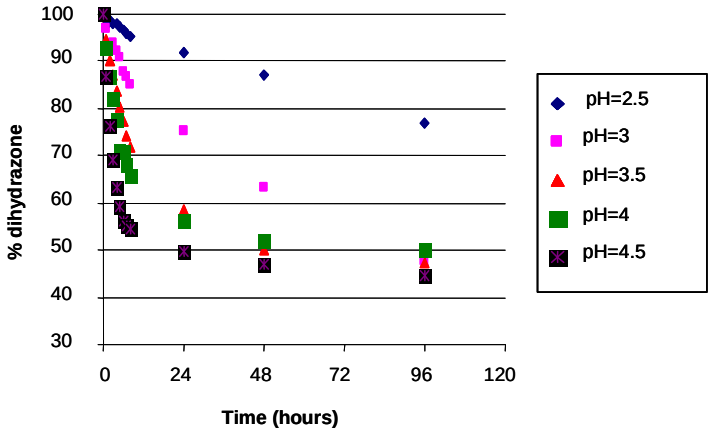

Figure S11. Kinetics of the disappearance of dihydrazone **12** after the addition of 3,5-dimercaptobenzoic acid (**14**) at different pHs.

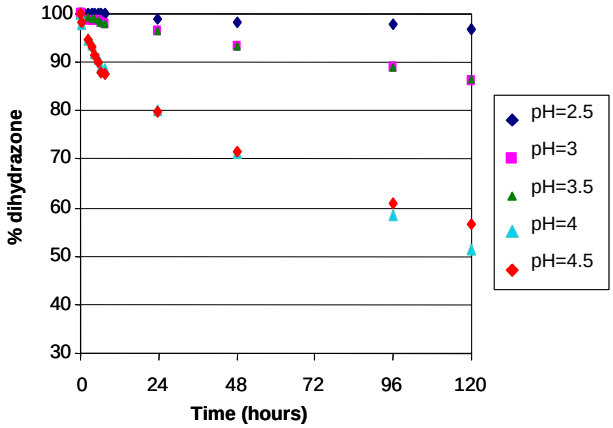

Figure S12 Kinetics of the disappearance of dihydrazone **12** after the addition of 2-furanmethanethiol (**3**) at different pHs

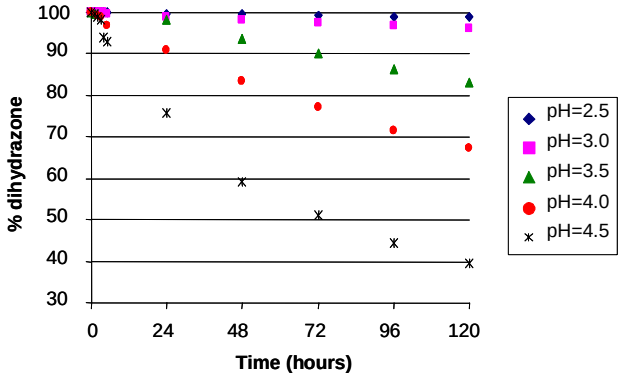

Figure S13: Kinetics of the disappearance of dihydrazone **12** after the addition of thioglycerol **15** at different pHs

The comparison of the rate of exchange of the different thiols with dihydrazone **12** at pH 2.5 is shown in Figure S13. The thiol acidity is crucial to the disulfide exchange: the more acidic aromatic thiol **14** exchanged with **12** even at pH 2.5 while the less acidic thiols (allylic **3** and aliphatic **15**) did not show significant exchange at this pH (Figure S13).

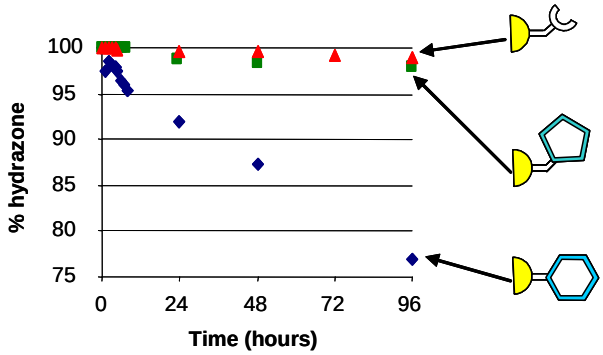

Figure S13: Comparison of the kinetics of disulfide exchange of the different monothiols with the starting dihydrazone **12** at pH 2.5.

HYDRAZONE EXCHANGE AT pH 4.5

The dihydrazone **5** was formed starting from dithioproionic acid dihydrazide (**1**), 5 mM, and 4-hydroxybenzaldehyde (**2**), 10 mM, in 1:1 acetonitrile/10 mM ammonium acetate pH 4.5 or formate pH 2.5 buffer. After 24 hours 2 equiv. of aldehyde **4**, 10 mM and aniline, 50 mM were added as a solid and as liquid, respectively, and the equilibration process monitored by HPLC during 4 hours.

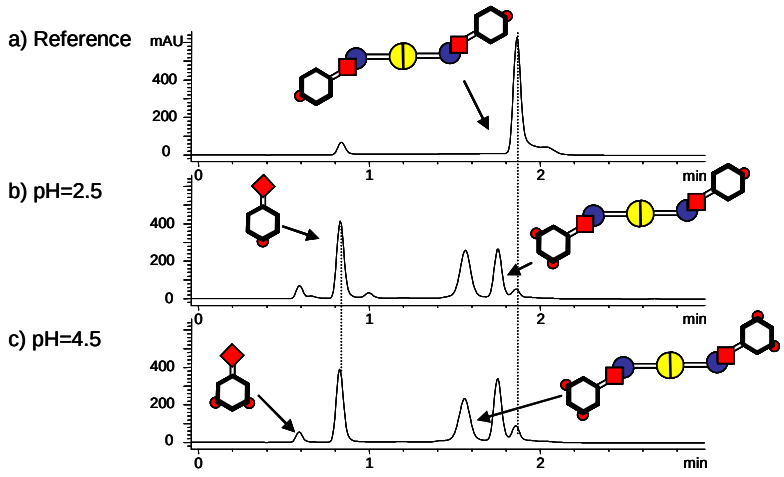

Figure S14: HPLC traces of 4-dihydroxybenzaldehyde dihydrazone before (a) and 4 h after the addition of 2 equiv. of 3,5-dihydroxybenzaldehyde and aniline (**4**) at (b) pH 2.5; (c) pH 4.5 after 4 hours