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Electronic Supporting Information

Reduction with Tris(2-Carboxyethyl)Phosphine (TCEP) Enables the Use of S-*Sulphonate Protecting Group for Thiol-mediated Bioconjugation*

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1. General Information

All reagents and solvents were purchased from commercial sources (Acros, Aldrich, SDS, Pierce) and were used without further purification. 5(6)-Carboxy-fluorescein diacetate *N*-succinimidyl ester, biotin *N*-succinimidyl ester and benzoic acid *N*-succinimidyl ester (Bz-OSu) were purchased or synthesized according to standard procedures.[1]

Maleimide-activated horseradish peroxidase (HRP) was purchased from Thermo Scientific Pierce.

96-F MaxiSorp 96-well microtitration plates (microplates) were obtained from Nunc.

NMR spectra were recorded on a Bruker AM Avance apparatus at 300 MHz or on a Bruker RDX apparatus at 400 MHz for ^{1}H and 75 or 101 MHz for ^{13}C. Chemical shifts were referenced to Me_4Si as an internal reference. Data are presented as follows; chemical shift (ppm), multiplicity (s = singulet, d = doublet, t = triplet, q = quadruplet, dd = doublet of doublet, dt = doublet of triplet, m = multiplet, br = broad), coupling constant *J* (Hz) and integration. High-resolution mass spectra (HRMS) were obtained using a Q-tof Waters apparatus using negative ion (ESI^-) or positive ion (ESI^+) electrospray ionisation mode. HPLC analyses were carried out on a Waters Alliance system with a E2695 separation module and a 2998 photodiode array detector; Column XTerra MS C18 (5 µm, 2.1×150 mm); Gradient, see table S1.

Table S1. HPLC method.

Flow (mL/min)	Time (min)	%A (H_2O/TFA 0.1%)	%B (MeCN/TFA 0.1%)
0.2	0	100	0
0.2	20	0	100

LC-MS analyses were carried out on a Waters Alliance 2790 apparatus coupled with a Q-tof Waters mass spectrometer using negative ion (ESI^-) or positive ion (ESI^+) electrospray modes with a Column XTerra MS C18 (5 µm, 2.1x150 mm), according conditions described in table S1. RP-HPLC purifications were carried out an Armen SPOT Prep system with Lichroprep C18 40-60 µm column and Dual-wavelength UV-Vis 200-600 nm spectrophotometer. Flash chromatography purifications were performed on an Armen SPOT

[1] Susumu, K. ; Uyeda, H.T ; Medintz, I.L.; Pons, T.; Delehanty, J.B.; Mattousi, H. *J. Am. Chem. Soc.* **2007**, *129*, 13987-13996.

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Prep System with Interchim puriflash cartridges and Dual-wavelength UV-Vis 200-600 nm spectrophotometer.

Automated preparative size exclusion chromatography (SEC) separations were carried out on an ÄKTApurifier 100 system (General Electric) equipped with an UV-900 detector (elution monitored at λ = 210, 280 and 443 nm), using a Superdex 200 10/300GL column. Running buffer was 5 mM phosphate, 150 mM NaCl pH 7.4 (PBS) and chromatography was performed at 0.5 mL/min.

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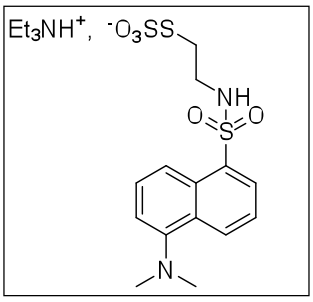
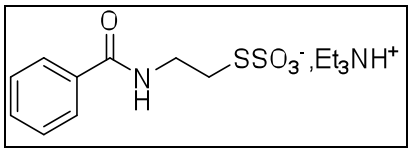
2. Synthesis of starting materials



***S*-(2-(5-(Dimethylamino)naphthalene-1-sulfonamido)ethyl)thiosulfuric acid, triethyl ammonium salt (1a).** To a suspension of *S*-(2-aminoethyl)thiosulfuric acid (0.64 mmol, 100 mg, 1 eq) in acetonitrile (10 mL) was added dropwise a solution of dansyl chloride (0.83 mmol, 223 mg, 1.3 eq) in acetonitrile (12 mL) and triethylamine (1.27 mmol, 175 µL, 2 eq). The reaction mixture was stirred for 18 h at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by flash chromatography on silica gel (eluent: CH_2Cl_2/MeOH gradient 100:0 to 80:20) to afford **1a** as a yellow solid (227 mg, 74%) corresponding to the 0.87-1 triethylammonium salt. ^{1}H NMR (300 MHz, DMSO, δ ppm) δ 8.85 (br s, 1H), 8.47 (d, J = 8.4 Hz, 1H), 8.29 (d, J = 8.6 Hz, 1H), 8.13-8.12 (m, 2H), 7.65 (d, J = 8.4 Hz, 1H), 7.60 (d, J = 8.4 Hz, 1H), 7.27 (d, J = 7.5 Hz, 1H), 3.20 – 3.00 (m, 7H), 2.84-2.80 (m, 8H), 1.16 ($(CH_3CH_2)_3N$, t, J = 7.2 Hz, 7.52H). ^{13}C NMR (75 MHz, DMSO, δ ppm) 151.3, 136.0, 129.3, 129.0, 128.2, 127.8, 123.6, 119.1, 115.1, 45.7, 45.0, 43.3, 33.4, 8.6. HRMS (ESI^-): calcd: 389.0300 for $C_{14}H_{17}N_2O_5S_3$; found: 389.0307. HPLC t_R : 12.8 min (method see table S1, UV detection 286 nm).



***S*-(2-Benzamidoethyl)thiosulfuric acid, triethyl ammonium salt (1b)**

To a solution of benzoic acid *N*-succinimidyl ester (Bz-OSu) (4.56 mmol, 1 g, 1 eq) in DMF (10 mL) was added dropwise a solution of *S*-(2-aminoethyl)thiosulfuric acid (4.56 mmol, 0.72 g, 1 eq) in DMF (30 mL) and triethylamine (9.12 mmol, 1.27 mL, 2 eq). The reaction mixture was stirred for 18 h at room temperature. The mixture was concentrated under

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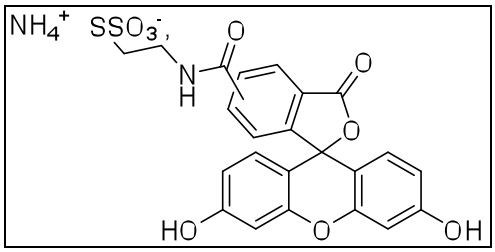
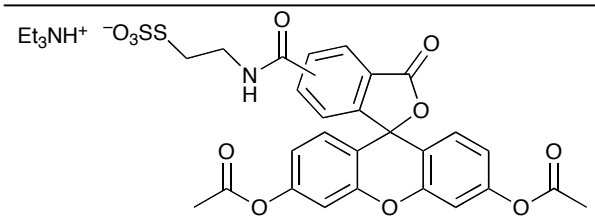
reduced pressure and the residue was purified by flash chromatography on silica gel (eluent: CH_2Cl_2/MeOH gradient 100:0 to 80:20) to afford **1b** as an colorless oil (0.97 g, 72%, purity 89%) corresponding to a stoichiometric mixture with triethylamine. ^{1}H-NMR (DMSO d_6, 300 MHz, δ ppm) 8.70 (br s, NH), 7.80-7.91 (m, 2H), 7.35-7.59 (m, 3H), 3.48-3.64 (m, 2H), 3.10 (q, J = 7.1 Hz , 6H), 2.60 (br s, 2H), 1.18 (t, J = 7.1 Hz, 9H).^{13}C NMR (75 MHz, DMSO, δ ppm) 172.7, 134.3, 131.0, 128.2, 127.1, 62.0, 45.8, 33.0, , 8.6. HRMS (ESI$^-$) calcd: 260.0045 for $C_9H_{10}NO_4S_2$; found: 260.0051. HPLC t_R : 15.4 min (method see table S1,UV detection 228 nm).



_S_-(2-(3',6'-Dihydroxy-3-oxo-3_H_-spiro[isobenzofuran-1,9'-xanthene]-5(6)-ylcarboxamido)ethyl)thiosulfuric acid, ammonium salt (1c)

a. _S_-(2-(3',6'-diacetyl-3-oxo-3_H_-spiro[isobenzofuran-1,9'-xanthene]-5(6)-ylcarboxamido)-ethyl)thiosulfuric acid, triethyl ammonium salt



To a solution of 5(6)-carboxy-fluorescein diacetate _N_-succinimidyl ester (0.54 mmol, 300 mg, 1 eq) in DMF (5 mL) was added dropwise a solution of _S_-(2-aminoethyl)thiosulfuric acid (0.54 mmol, 84 mg, 1 eq) solubilized in DMF (5 mL) then triethylamine (1.18 mmol, 167 µL, 2.2 eq). The reaction mixture was stirred for 18 h at room temperature.

The mixture was concentrated under reduced pressure and the residue was purified by flash chromatography on silica gel (eluent: CH_2Cl_2/MeOH gradient 100:0 to 80:20) to afford the diacetyled product as a yellow solid (178 mg, 49%) corresponding to a non-stoichiometric mixture with triethylamine (0.69:1). ^{1}H-NMR (300 MHz, DMSO d_6, δ ppm) 9.14-9.02 (m, 1H), 8.98-8.88 (m, 0.5H), 8.58-8.47 (m, 1H), 8.35-8.09 (m, 1H), 7.50 (m, 0.5H), 7.35-7.22 (m, 1H), 7.01 – 6.82 (m, 2H), 6.79-6.53 (m, 2H), 4.11 (d, J = 4.8 Hz, 3H), 3.74-3.58 (m, 1H), 3.57-3.35 (m, 3H), 3.09 (q, J = 7.3 Hz, 5H), 2.57 – 2.47 (m, 3H), 2.30 (s, 3H), 1.18 (t, J =

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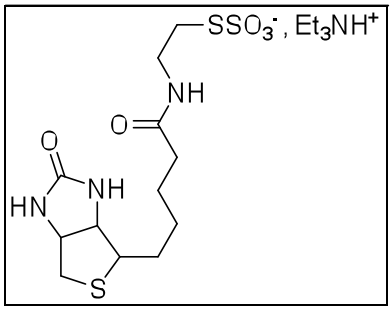
7.3 Hz, 6H), 1.07 (t, J = 7.0 Hz, 1H). HPLC t_R = 15.0, 16.8 and 18.0 min (method see table S1, UV detection 442 nm).

b. Removal of acetyl protecting group

S-(2-(3',6'-diacetyl-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5(6)-ylcarboxamido)ethyl) thiosulfuric acid, triethyl ammonium salt (71.4 mmol, 50 mg) in aqueous ammonium carbonate 0.1 M (5 mL) was stirred for 18 h at room temperature and the reaction progress was monitored by HPLC to check completion. The mixture was concentrated under reduced pressure and lyophilized to afford **1c** as an orange solid (36 mg, 100%, purity 94%, 6% acetamide) corresponding to the ammonium salt. ^{1}H-NMR (400 MHz, DMSO d_6, δ ppm) 9.18-8.76 (m, 1H), 8.50 (s, 0.5H), 8.31-7.99 (m, 1H), 7.69 (s, 0.5H), 7.48-7.04 (m, 1.5H), 6.93-6.29 (m, 5.5H), 3.56 (m, 2H), 3.04 (m, 2H), 1.76 (s, 2H). ^{13}C-NMR (101 MHz, DMSO d_6, δ ppm) : 171.9, 165.1, 152.6, 136.6, 129.7, 113.7, 109.8, 102.8, 46.1, 33.5, 23.0, 11.6. HRMS (ESI^-) calcd: 514.0267 for $C_{23}H_{16}NO_9S_2$; found: 514.0243. HPLC t_R = 15.3 min (method see table S1, UV detection 442 nm).



_S_-(5-[(3a_S_,4_S_,6a_R_)-2-oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl]pentanamido)ethyl)-thiosulfuric acid, triethyl ammonium salt (1d)

To a solution of ((5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoate)*N*-succinimydyl ester (0.26 mmol, 100 mg, 1 eq) in DMF (2.5 mL) was added dropwise a suspension of *S*-(2-aminoethyl)thiosulfuric acid (0.26 mmol, 41 mg, 1 eq) in DMF (2.5 mL) then triethylamine (0.58 mmol, 84 µL, 2 eq). The reaction mixture was stirred for 18 h at room temperature. The mixture was concentrated under reduced pressure and the residue was purified by flash chromatography on silica gel (eluent: CH_2Cl_2/MeOH gradient 100:0 to 70:30) to afford **1d** as a colorless oil (82 mg, 72%) corresponding to a non-stoichiometric mixture with triethylamine (0.5:1).^{1}H NMR (300 MHz, DMSO, δ ppm) 7.98 (br s, 1H), 6.43 (s, 1H), 6.35 (s, 1H), 4.39-4.22 (m, 1H), 4.22-4.06 (m, 1H), 3.23-3.00 (m, 5H), 2.99-2.72 (m, 3H), 2.60-2.56 (m, 2H), 2.05 (t, J = 7.2 Hz, 2H), 1.73-1.23 (m, 6H), 1.18 (t, J = 7.2 Hz, 4H). ^{13}C NMR (75 MHz, DMSO, δ ppm) 171.1, 161.9, 60.2, 58.3, 54.5, 47.7,

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44.9, 34.3, 32.5, 27.3, 27.1, 24.4, 7.9 . HRMS (ESI$^-$) calcd: 382.0565 for $C_{12}H_{20}N_3O_5S_3$; found: 382.0549. HPLC t_R : 13.5 min (method see table S1, UV detection 210 nm).

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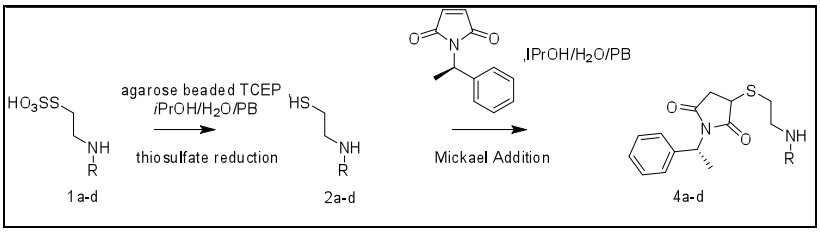
3. Reactions with immobilized TCEP



In-situ reaction on 1a-1d (2 steps without purification)

General protocol for preparation of materials:

The reactions were performed in a 2 mL column with a cellulose filter. This column was washed with *i*PrOH/0.5 M phosphate buffer pH 7.4 1:9 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G). Agarose beaded TCEP (4 µmol, 0.5 mL) was introduced on the column, centrifuged 1min at 1000 G then washed with *i*PrOH /0.5 M phosphate buffer pH 7.4 1:9 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G).

Reduction step:

1a-1d (1 µmol, 100 µL of 10 mM solution in *i*PrOH, 1 eq) were introduced in solution of 0.5 M phosphate buffer pH 7.4 (1 mL) on agarose beaded TCEP column. The mixture was stirred for 30 min then centrifuged (1 min at 1000 G). The filtrate was analyzed by LC-MS.

Table S2. LC-MS analytical data for reactants **1a** and **3** before reduction (Figure S1) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
1a	11.16	519414	2672778	47.5	391.1
3	16.96	530872	2405059	48.6	202.1

Table S3. LC-MS analytical data for the reduction step of **1a** (Figure S2) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
2a	13.84	1944220	1198896	94.8	311.1

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Table S4. LC-MS analytical data for reactants **1b** and **3** before reduction (Figure S3) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
1b	11.08	178710	983037	22.9	262.1
3	16.96	566333	2424480	72.5	220.1

Table S5. LC-MS analytical data for the reduction step of **1b** (Figure S4) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
2b	13.52	140378	866802	97.7	182.1

Table S6. LC-MS analytical data for reactants **1c** and **3** before reduction (Figure S5) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
1c	13.12	1005531	3041912	58.7	516.0
3	16.85	615650	2421881	35.9	202.1

Table S7. LC-MS analytical data for the reduction step of **1c** (Figure S6) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
2c	15.11	1018101	4847811	82.4	436.1

Table S8. LC-MS analytical data for reactants **1d** and **3** before reduction (Figure S7) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
1d	11.16	676980	2499688	51.8	382.1
3	16.91	610076	2499559	46.7	202.1

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Table S9. LC-MS analytical data for the reduction step of **1d** (Figure S8) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
2d	11.72	51999	354049	97.2	304.2

Michael Addition:

To the crude residue **2a**-**2d** was added reactant **3** (2 µmol, 100 µL from a mother solution at 4 mg/mL in *i*PrOH, 2 eq). After 1h, the mixture was analyzed by LC-MS.

Table S10. LC-MS analytical data for the Michael addition of **2a** to **3** (Figure S9) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
5	12.13	165508	903662	16.0	Not detected
4a	16.96	830423	2471971	80.4	512.2

Table S11. LC-MS analytical data for the Michael addition of **2b** to **3** (Figure S10) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
5	12.15	83662	483576	10.2	Not detected
3	16.97	476547	2359743	58.3	202.1
4b	17.57	229594	1361319	28.1	383.2

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Table S12. LC-MS analytical data for the Michael addition of **2c** to **3** (Figure S11) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
5	12.17	88742	903662	4.9	Not detected
3	16.89	579165	2395723	32	202.1
4c	17.30	994450	2957085	54.8	637.1

Table S13. LC-MS analytical data for the Michael addition of **2d** to **3** (Figure S12) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
5	12.21	208507	1067764	21.3	Not detected
4d	15.17	321289	1868564	32.8	505.3
3	16.92	4081499	2197088	41.7	202.1

In-situ reaction on HRP-maleimide (2 steps without purification)

General protocol for preparation of materials:

The reaction was performed in a 2 mL column with a cellulose filter. This column was washed with *i*PrOH/0.5 M phosphate buffer pH 7.4 1:9 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G). Agarose beaded TCEP (4 µmol, 0.5 mL) was introduced on the column, centrifuged 1min at 1000 G then washed with *i*PrOH/0.5 M phosphate buffer pH 7.4 1:9 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G).

Reduction step:

1c (1 µmol, 100 µL of 10 mM solution in *i*PrOH, 1 eq) was introduced in solution of 0.5 M phosphate buffer pH 7.4 (1 mL) on agarose beaded TCEP column. The mixture was stirred for 30 min and centrifuged (1 min at 1000 G).

Michael Addition:

To 58 µL of the crude residue **2c** was added maleimide-activated horseradish peroxidase (600 µL of a mother solution at 1 mg/mL in *PBS 1X,* 0.2 eq). After 1 h, 500 µL of each sample was analyzed and purified by SEC.

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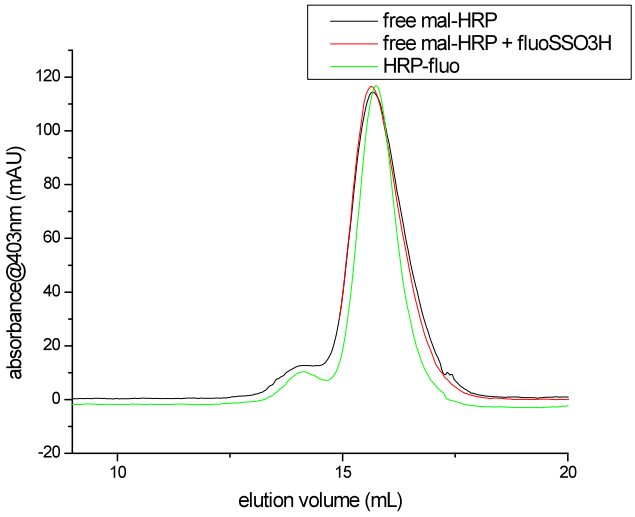
Table S14. SEC chromatogram for the Michael addition of **2c** to maleimide-activated horseradish peroxidase (mal-HRP) (UV detection 403 nm) (in comparison with free maleimide-activated horseradish peroxidase -free mal-HRP- and free maleimide-activated horseradish peroxidase -free mal-HRP-in solution with **1c**)



Fraction between 14 and 17 mL were collected.

The specific fluorescence of fluorescein grafted onto purified HRP was measured using a Berthold Tristar LB941 multimode microplate reader and standard black, 96-well Elisa microplates (λ_{exc} = 485 nm; λ_{em} = 535 nm). Wells were filled with 100 µL in duplicate.

Table S15. Fluorescence analytical data for the Michael addition of **2c** to maleimide-activated horseradish peroxidase (free mal-HRP) (in comparison with free maleimide-activated horseradish peroxidase -free mal-HRP- and free maleimide-activated horseradish peroxidase -free mal-HRP-in solution with **1c**) (λ_{exc} = 485 nm; λ_{em} = 535 nm)

Fluorescence (RFU)	Free maleimide activated HRP	Maleimide activated HRP + reactant **1c**	Maleimide activated HRP + residue **2c**
Measure 1	1711	1049	252265
Measure 2	1691	1018	266808

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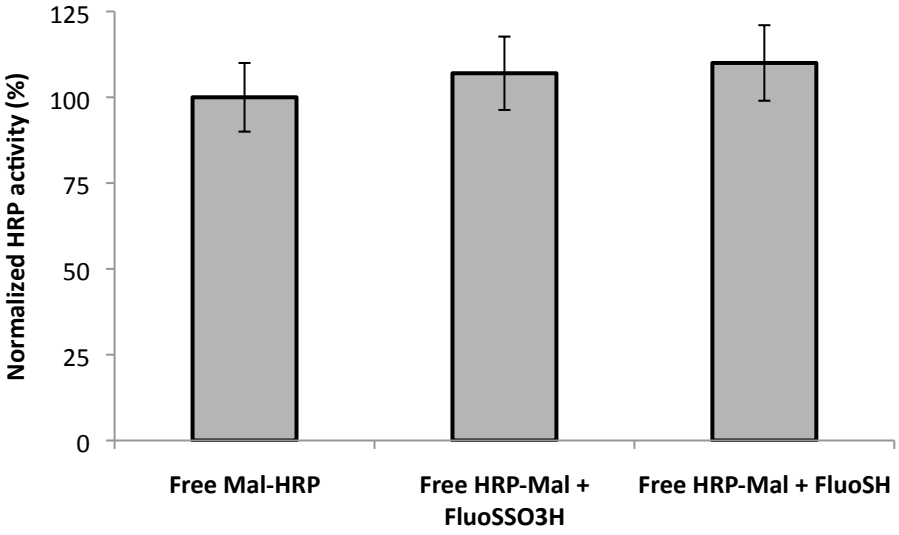
An enzymatic activity test was performed on the same samples

MaxiSorpTM 96-well plates were first saturated with BSA (3% in PBS) for 1 h to prevent destructive adsorption of the enzyme. After saturation, wells were rinsed five times with PBST and once with PBS. Wells were filled in duplicate with purified enzymes diluted in PBS from 1/2 to 1/1024. Revelation was performed by adding 100 µL by well of a solution made of 9 mL of 0.1 M citrate buffer / ethanol 50/50 (v/v) + 400 µL H_2O_2 30% + 1 mL of orthophenylene diamine (OPD) at 40 mg/mL. After 3 min of incubation the reaction was stopped by addition of 50 µL of 4 M sulfuric acid, then the optical density of the mixture was measured at λ = 492 nm (OPD absorbance) using a Tecan Sunrise Microplate reader.

Table S16. The comparison of HRP enzymatic activity data after the Michael reaction of **2c** to maleimide-activated horseradish peroxidase (free mal-HRP) (in comparison with free maleimide-activated horseradish peroxidase -free mal-HRP- and free maleimide-activated horseradish peroxidase -free mal-HRP- in solution with **1c**). Activities were normalized by free mal-HRP and after correction by the HRP concentrations in each collected fraction determined using micro BCA protein assay kit (Pierce, Ref 23235).



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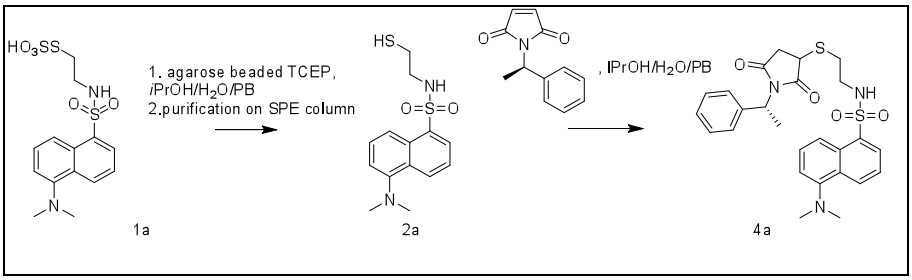
With SPE purification for reduction step on 2a-2d

Example:



General protocol for the preparation of material:

- Preparation of agarose beaded TCEP column: Reaction was performed in a 2 mL column equipped with a cellulose filter. This column was washed with *i*PrOH /1 M phosphate buffer /H_2O 10:40:50 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G). Agarose beaded TCEP (0.5 mL, 4 µmol) was introduced on the column, centrifuged 1min at 1000G then washed with *i*PrOH /0.5 M phosphate buffer pH 7.4 1:9 (2 × 1 mL) then centrifuged (2 × 1 min at 1000 G).

-SPE Column pre-treatment/ conditioning: *i)* percolation of MeOH (1 mL) through the SPE column then *ii)* equilibration H_2O (1 mL) and *i*PrOH/0.5 M phosphate buffer pH 7.4 1:9 (1 mL).

Reduction step:

A solution of **1a** (1 µmol, 100 µL of 10 mM solution in *i*PrOH, 1 eq) was introduced in 0.5 M phosphate buffer pH 7.4 (1 mL) on Agarose beaded TCEP column. The mixture was stirred for 30 min and centrifuged (1 min at 1000G). The filtrate was loaded on a SPE column then washed with *i*PrOH / 0.5 M phosphate buffer pH 7.4 1:9 (2 mL) and eluted with *i*PrOH / 0.5 M phosphate buffer pH 7.4 9:1 (2 mL). 2 mL of thiol **2a** eluted was detected by fluorescence and analyzed by LC-MS.

Table S17. LC-MS analytical data for the reduction step of **1a** (Figure S13) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
	11.16	967	5499	3.68	
2a	13.71	21379	130082	81.37	311.1
	15.88	979	6445	3.73	
	21.34	2949	15921	11.22	

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Michael addition:

To the filtrate containing **2a** was added reactant **3** (2 µmol, 100 µL of a mother solution at 4 mg/mL in *i*PrOH). After 1h, the mixture was analyzed by LC-MS.

Table S18. LC-MS analytical data for the Michael addition of **2a** to **3** (Figure S14) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)	ESI^+-MS
2d	16.93	698489	2566494	98.5	512.3

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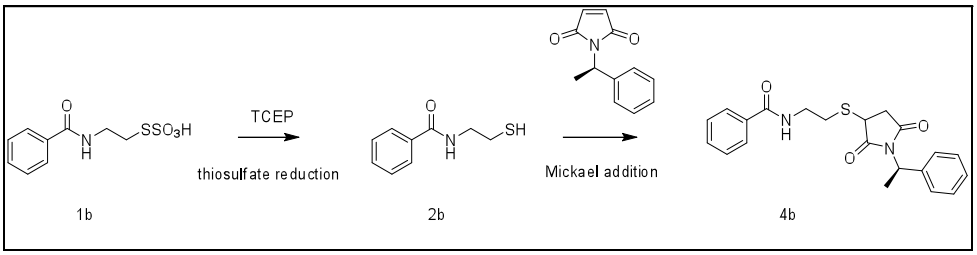
4. Reactions with soluble TCEP

Example:



In situ reaction from *S*-sulfonate 1b (without purification)

Reduction step:

A solution of *S*-sulfonate **1b** (1 µmol, 100 µL of 10 mM solution in *i*PrOH, 1 eq) was introduced in a solution of TCEP (2 µmol) in 0.1 M phosphate buffer pH 7.4 (0.9 mL). The mixture was stirred for 30 min then analyzed by HPLC.

Table S19. HPLC analytical data for the reduction step (Figure S15)(UV detection 210 nm).

Compound	t_R (min)	Area (Vsec)	Height	Area (%)
1b	12.51	737585	89534	7.8
2b	15.33	8679391	1005701	92.2

Michael addition:

To the crude mixture was directly added reactant **3** (1 µmol, 100 µL of a mother solution at 4 mg/mL in *i*PrOH). After 1h, the mixture was analyzed by HPLC.

Table S20. HPLC analytical data for side-product **6** (Figure S16)(UV detection 210 nm).

Compound	t_R (min)	Area (Vsec)	Height	Area (%)
6	15.17	34036009	2051959	100

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Table S21. HPLC analytical data for the Michael addition (Figure S17)(UV detection 210 nm).

Compound	t_R (min)	Area (Vsec)	Height	Area (%)
1b	12.51	n.d.	n.d.	n.d.[2]
	13.80	13106230	1405479	13.0
6	15.11	300070850	1999470	30.0
	16.52	28440105	355642	2.7
	19.11	32490912	2264408	31.8
	19.83	21378959	1955281	21.3

Reaction involving a RP-HPLC purification step after the reduction of 1b

Reduction step: Formation of *N*-(2-mercaptoethyl)benzamide **2b**

To a solution of *S*-(2-benzamidoethyl)thiosulfuric acid, triethyl ammonium salt **1b** (0.2 mmol, 52 mg, 1 eq) in 0.1 M phosphate buffer pH 7.4 (5 mL) was added a solution of TCEP (0.8 mmol, 228 mg, 4 eq) in H_2O (5 mL). The mixture was stirred for 30 min at 30°C. The reaction progress was controlled by HPLC analysis.

Table S22. HPLC analytical data for the reduction step monitoring of **1b** (Figure S18) (UV detection 214 nm)

Compound	t_R (min)	Area (Vsec)	Height	Area (%)
1b	14.48	1927700	195935	12.9
2b	15.47	13055270	1349515	87.1

The crude product was purified by RP-HPLC C18 (eluent: 100% H_2O-0.1%TFA to 100% MeCN-0.1%TFA) to afford **2b** as a white solid (18 mg, 50%). HPLC t_R = 15.4 min (UV detection 214 nm). ^{1}H NMR (400 MHz, DMSO, δ ppm) 8.61 (s, 1H), 7.85 (d, J = 7.0 Hz, 2H), 7.58-7.39 (m, 3H), 3.42 (m, 2H), 2.66 (q, J = 7.1 Hz, 2H), 2.43 (s, 1H).

Michael addition step: Synthesis of *N*-(2-(2,5-dioxo-1-((R)-1-phenylethyl)pyrrolidin-3-ylthio)ethyl)benzamide **4b**

[2] n.d.: not detected

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To a solution of *N*-(2-mercaptoethyl)benzamide **2b** (10 mmol, 18 mg, 1 eq) in 0.12 M phosphate buffer pH 7.4 (37 mL) was added a solution of reactant **3** (20 mmol, 40 mg, 2 eq) in *i*PrOH (2.5 mL). The mixture was stirred for 1 h at room temperature.

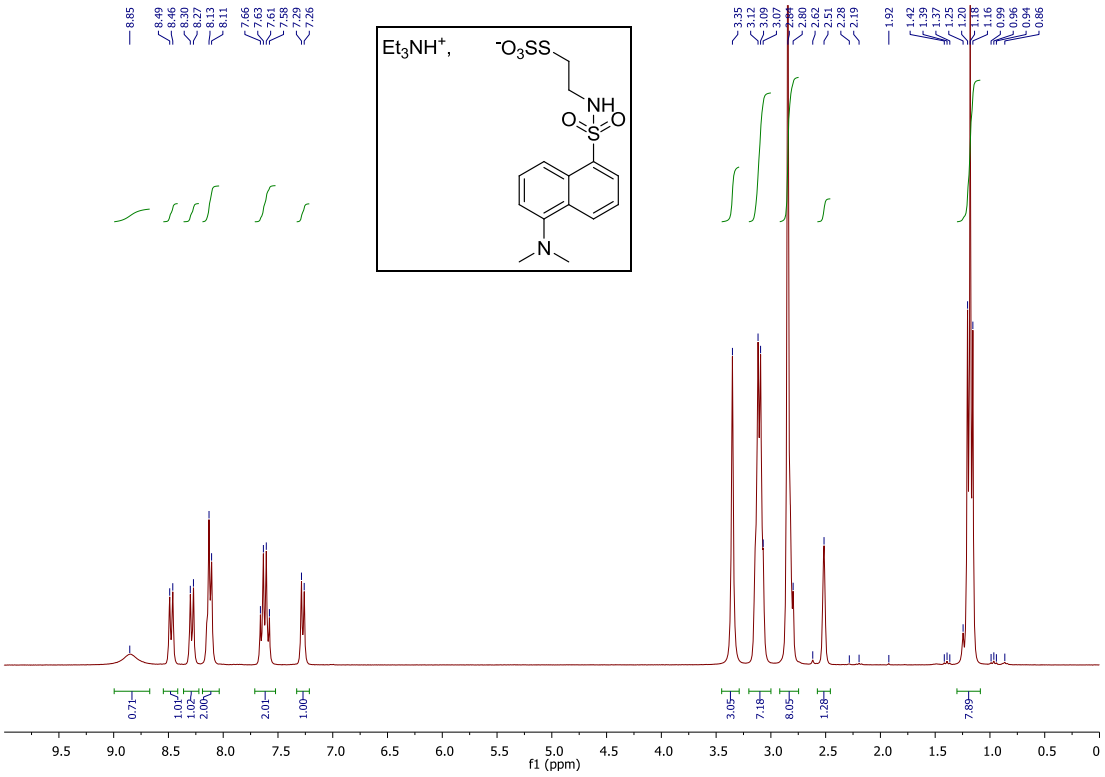
The reaction progress was monitored by HPLC analysis.

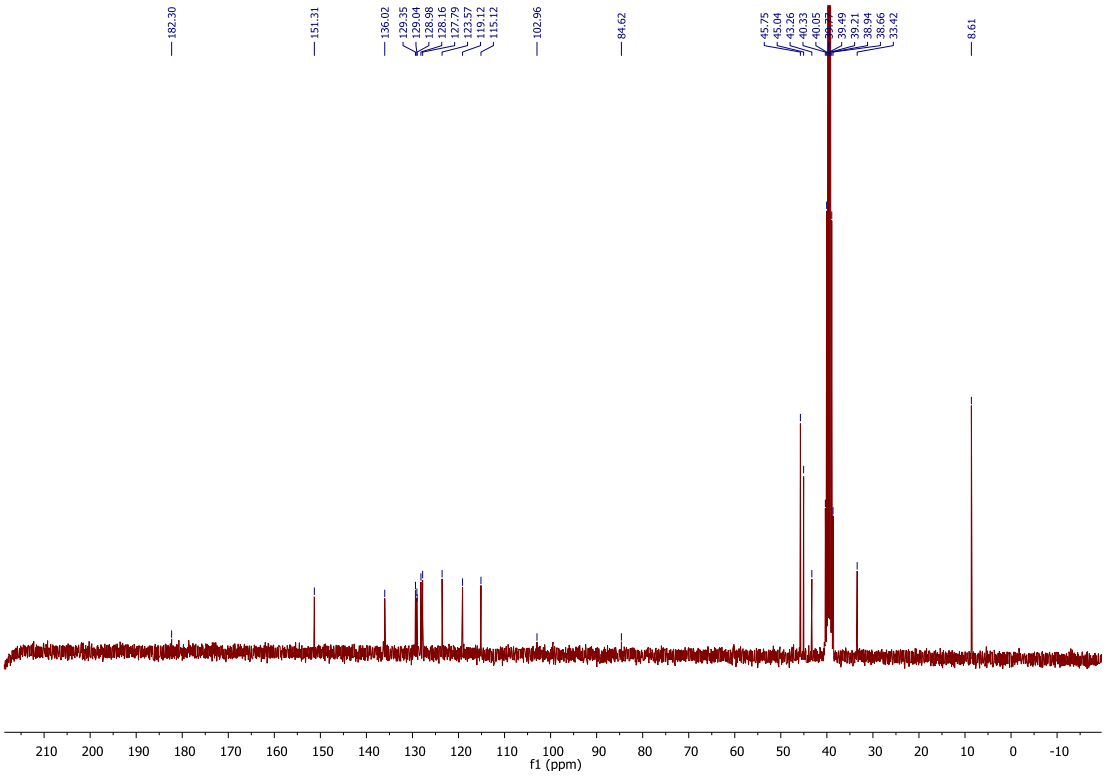
Table S23. HPLC analytical data for the Michael addition step monitoring of **2b** (Figure S19) (UV detection 214nm)

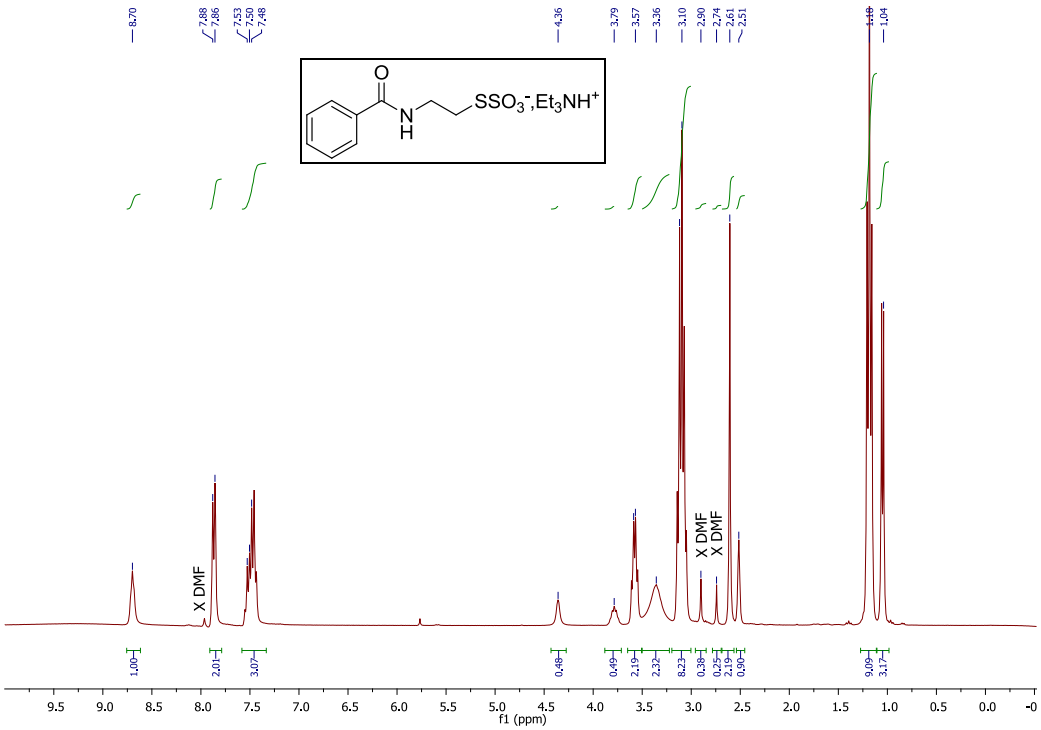
Compound	t_R (min)	Area (Vsec)	Height	Area (%)
	17.00	1335531	152718	5.7
4b	19.20	19200240	19773042	82.1
3	19.92	2850108	291272	12.2

The mixture was extracted with ethyl acetate (3 × 40 mL). The organic layers were assembled, dried over $MgSO_4$, filtrated and concentrated *in vacuo*. The crude was purified by flash chromatography on silica gel (eluent: petroleum ether/ ethyl acetate) to afford a mixture of diastereoisomers 0.55:0.45 **4b** (34 mg, 89%, purity: 92%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm) 7.80-7.66 (m, 2H), 7.49-7.11 (m, 8H), 6.97 (br s, 1H), 6.90 (br s, 1H), 5.41-5.28 (m, 1H), 3.77-3.45 (m, 3H), 3.21-2.94 (m, 2H), 2.92-2.70 (m, 1H), 2.50-2.30(m, 1H), 1.74 (d, J = 7.3 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$, δ ppm) δ 174.8, 174.7, 172.0, 165.6, 136.9, 132.2, 129.5, 126.6, 125.9, 125.4, 125.1, 58.3, 48.7, 37.2, 37.0, 33.7, 30.4, 30.3, 19.0, 14.4, 14.2, 12.2. HRMS (ESI^+): calcd: 383.1429 for $C_{21}H_{23}N_2O_3S$; found: 389.1447. HPLC t_R : 19.2 min (UV detection 228 nm).

5. Reproduction of 1H and 13C spectra



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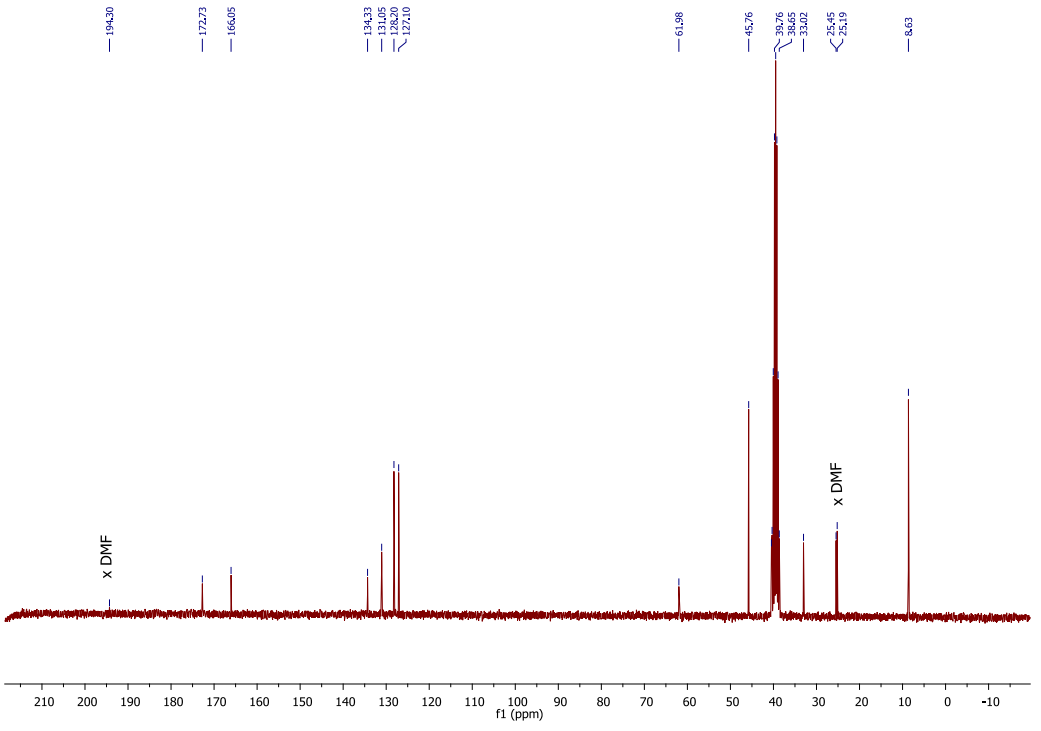
7.88
7.86
7.53
7.50
7.48
4.36
3.79
3.57
3.36
3.10
2.90
2.74
2.61
2.51
1.19
1.04
O
N
H
SSO3-,Et3NH+
X DMF
X DMF
X DMF
1.00
2.01
3.07
0.48
0.49
2.19
2.32
8.23
0.36
0.25
2.54
0.90
9.09
3.17
f1 (ppm)


194.30
172.73
166.05
134.33
131.05
128.20
127.10
61.98
45.76
39.76
38.65
33.02
25.45
25.19
8.63
x DMF
x DMF
f1 (ppm)

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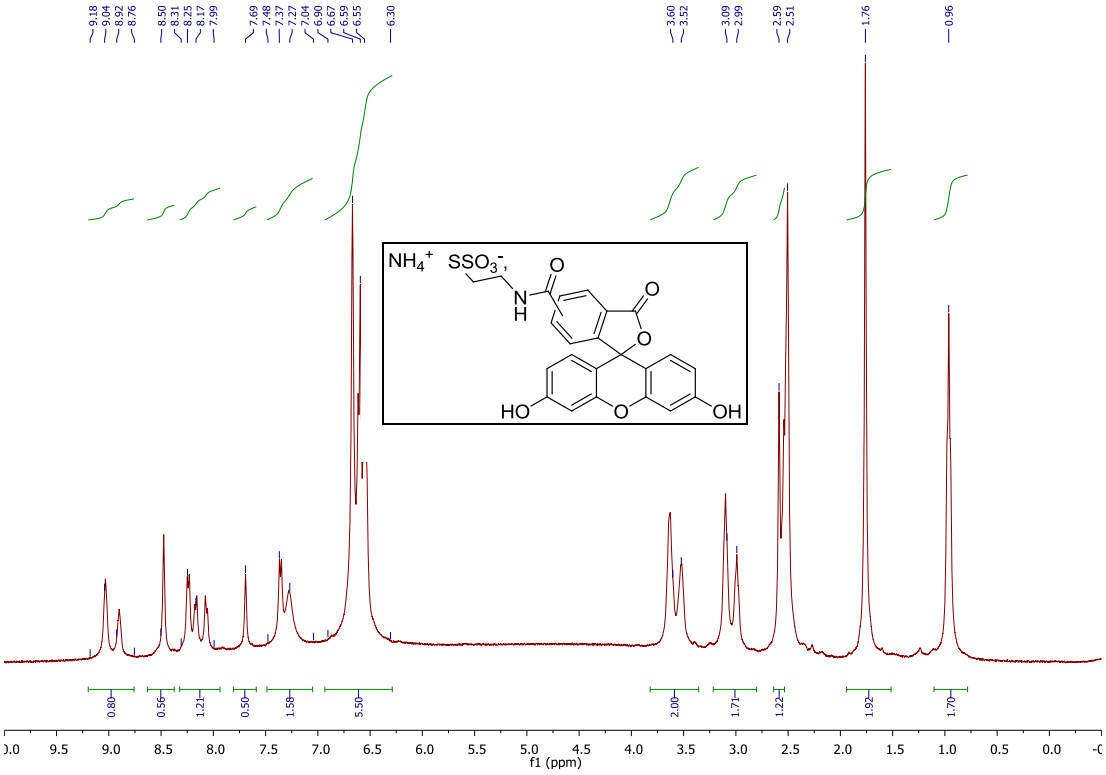

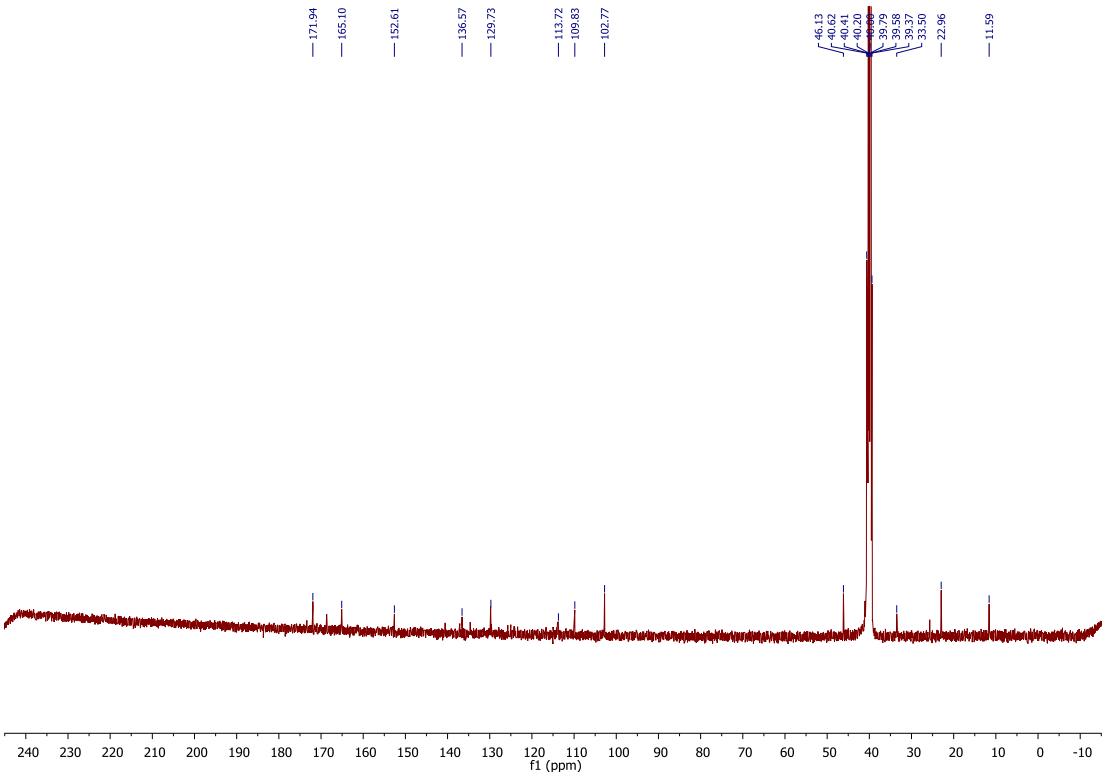

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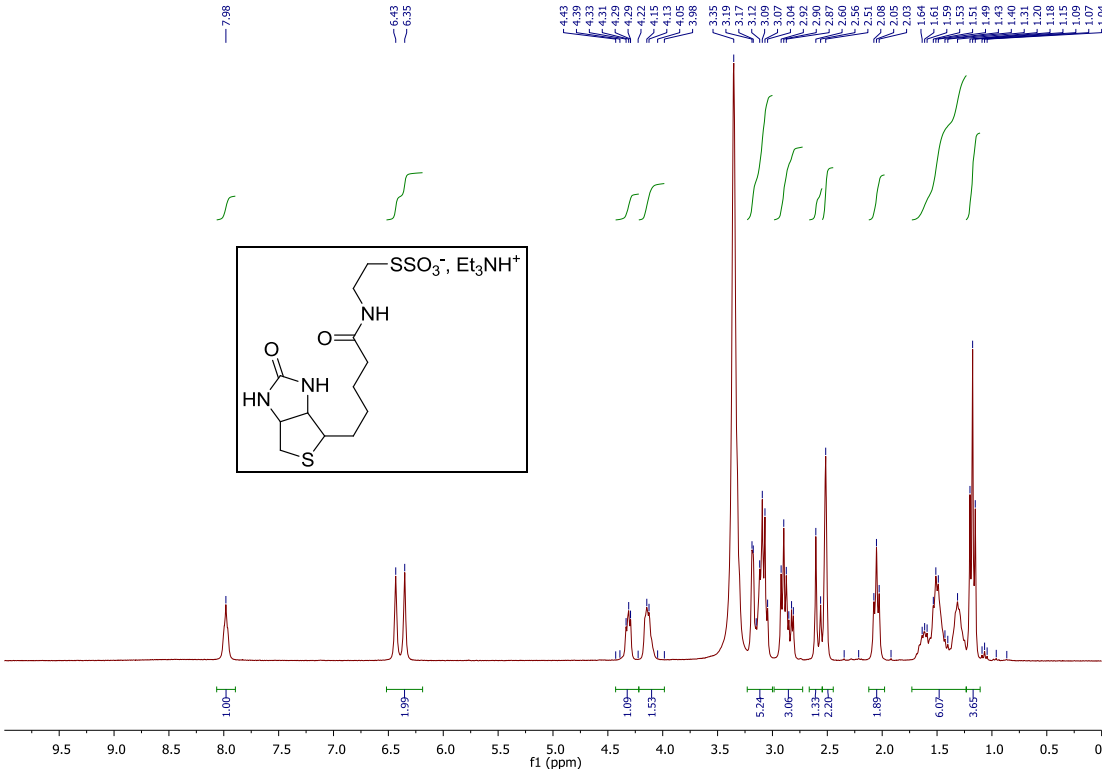

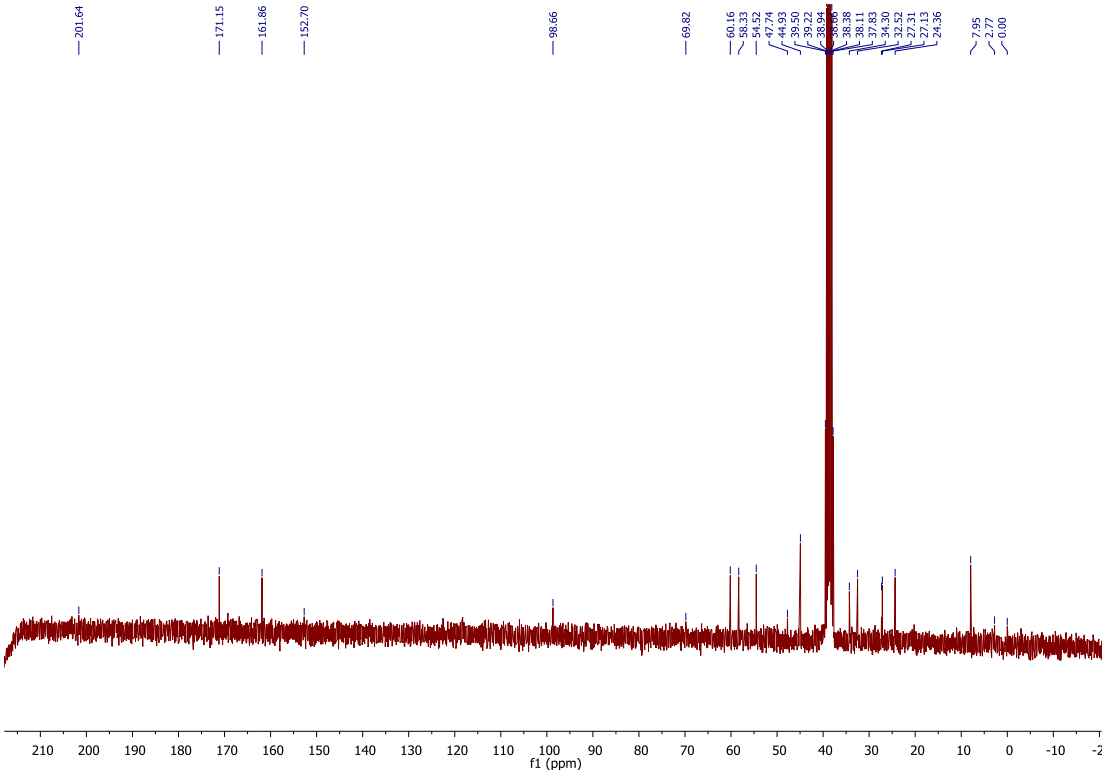

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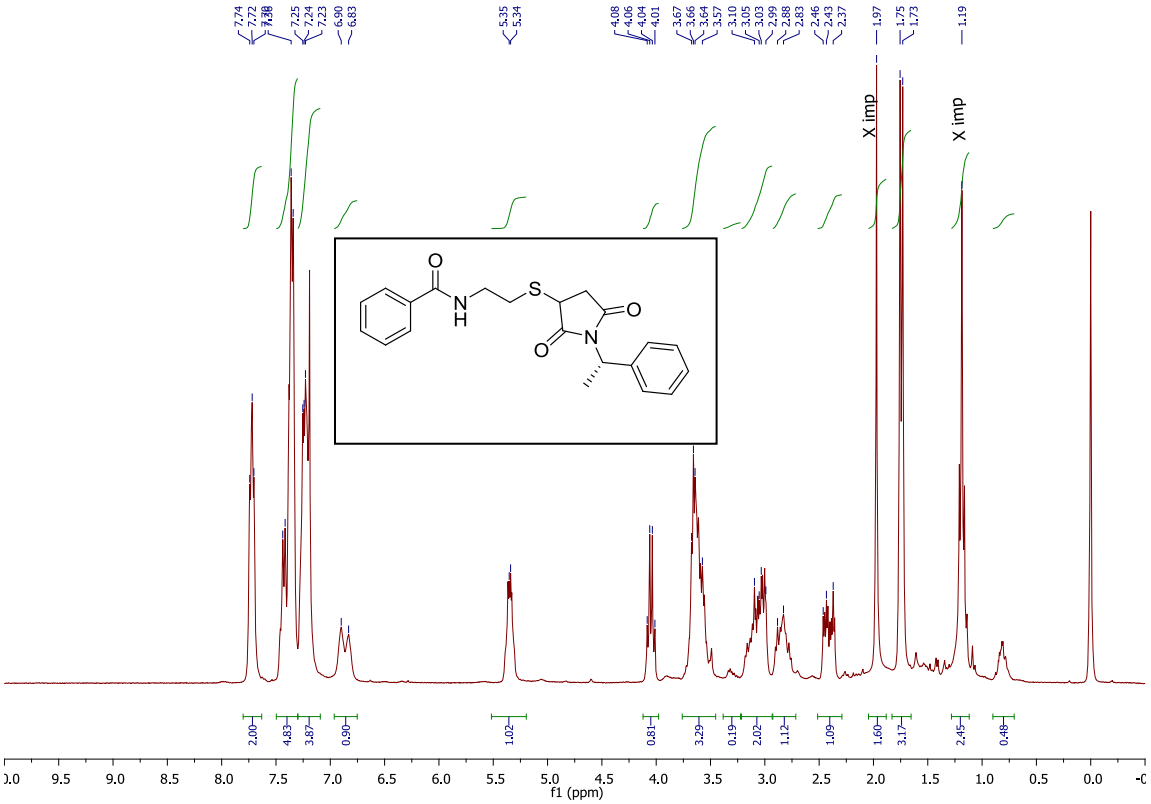

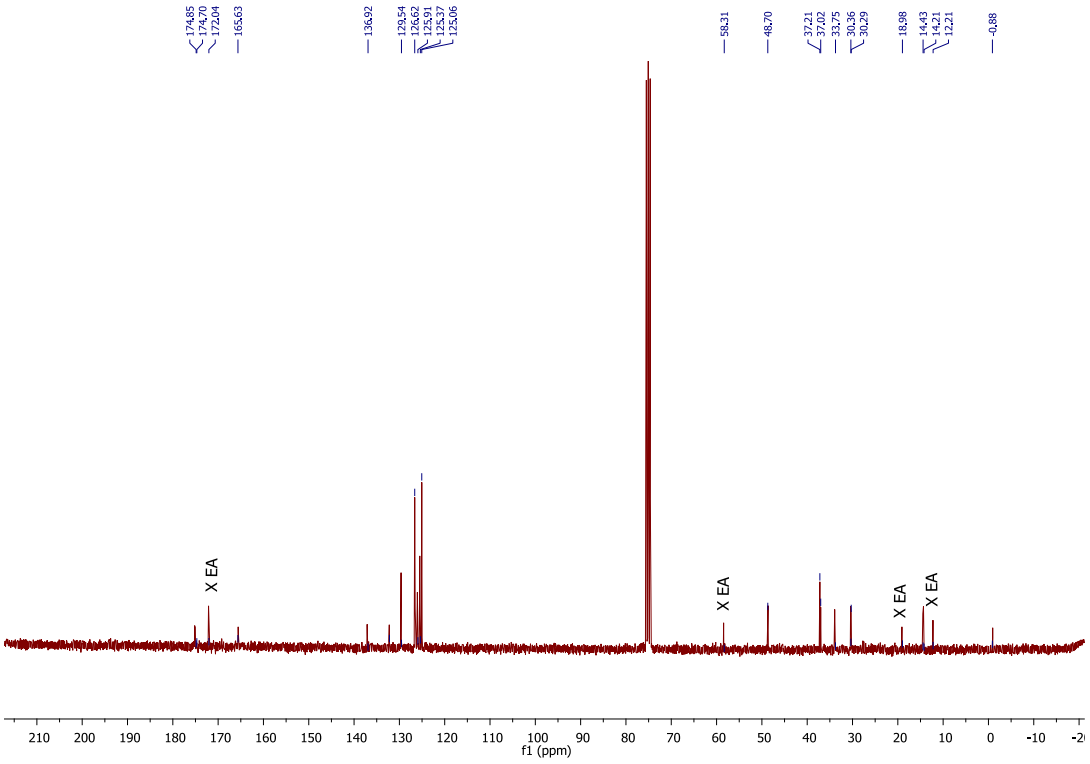

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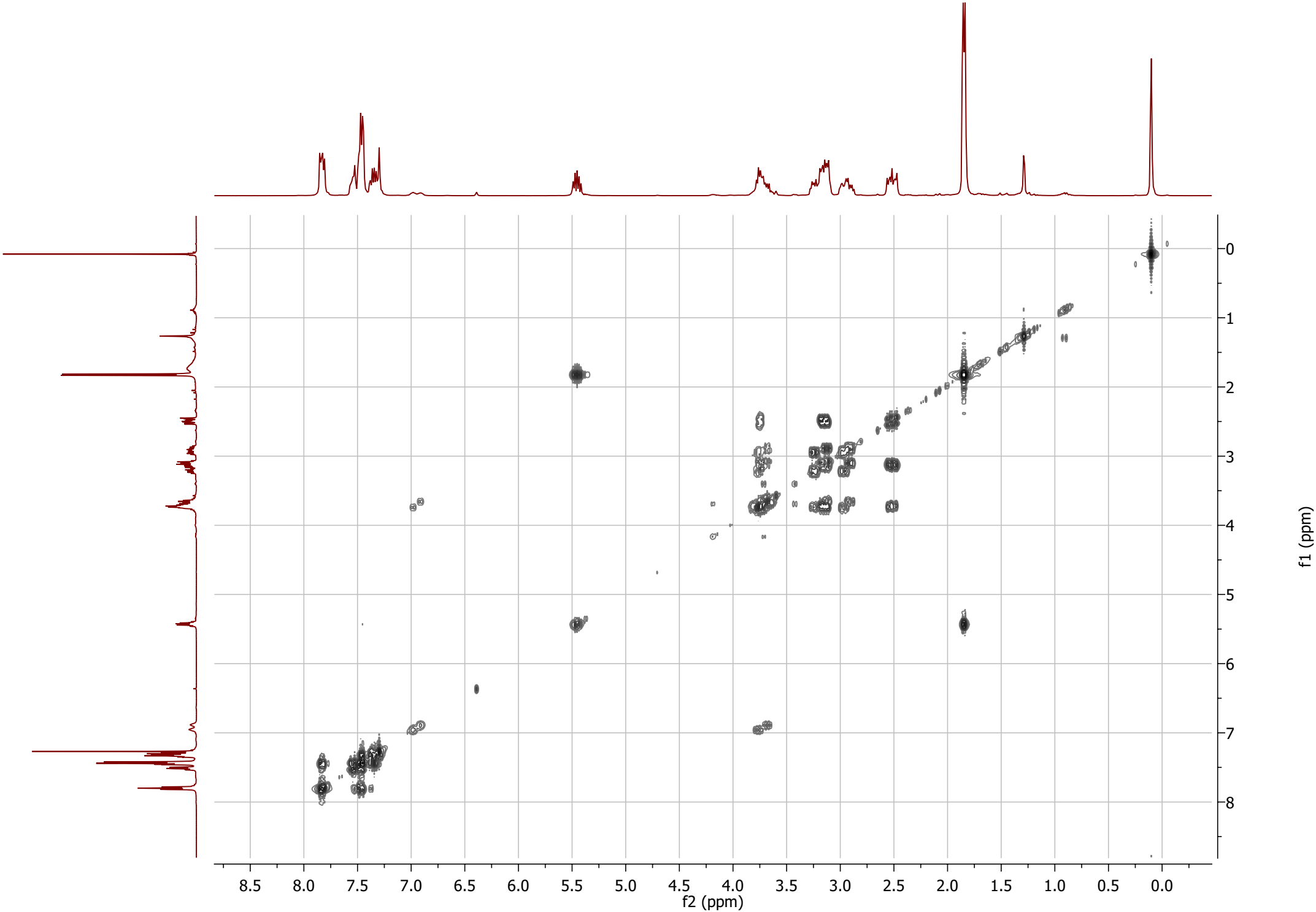
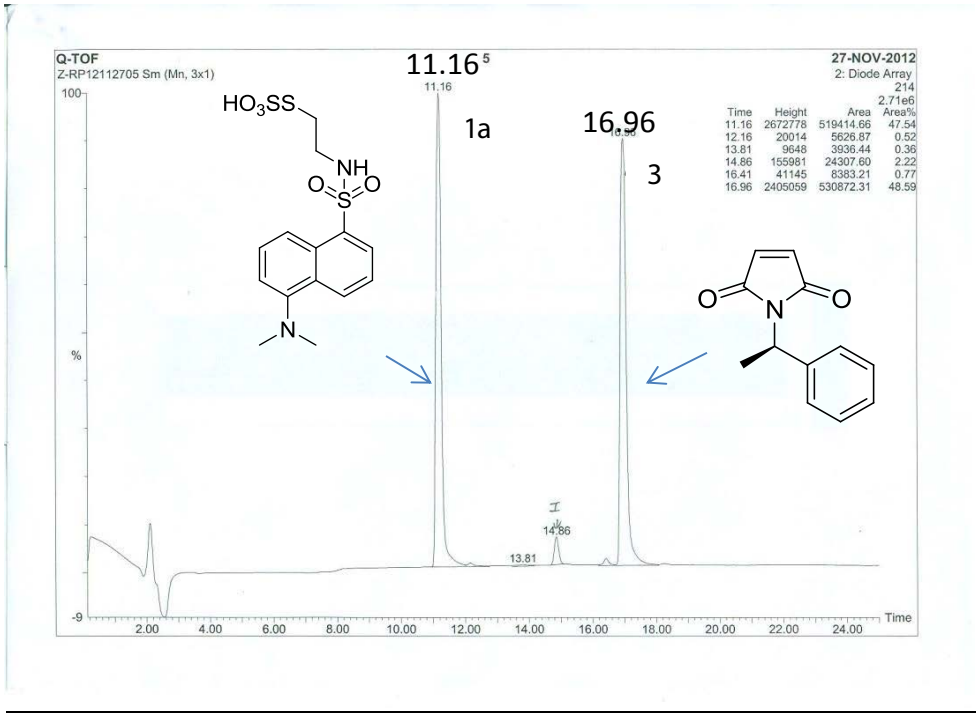


6. Reproduction of LC-MS chromatograms

Figure S1. LC-MS analytical data for reactants **1a** and **3** before reduction (UV detection 214 nm)



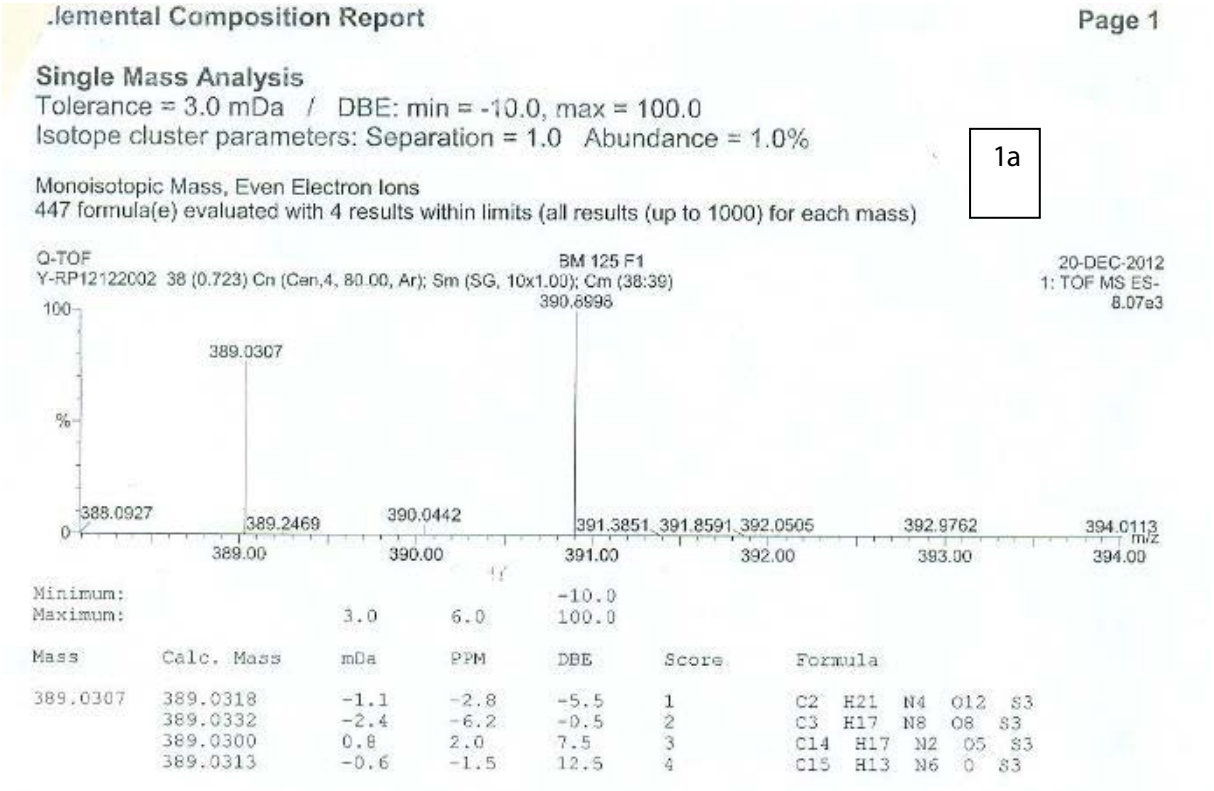
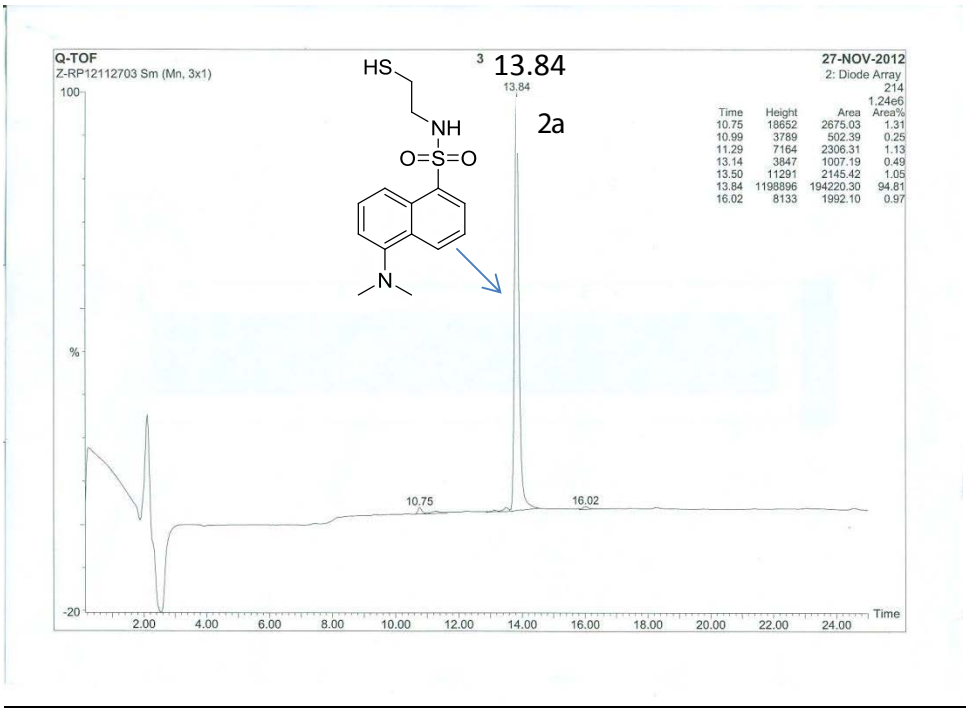


Figure S2. LC-MS analytical data for the reduction step of **1a** (UV detection 214 nm)



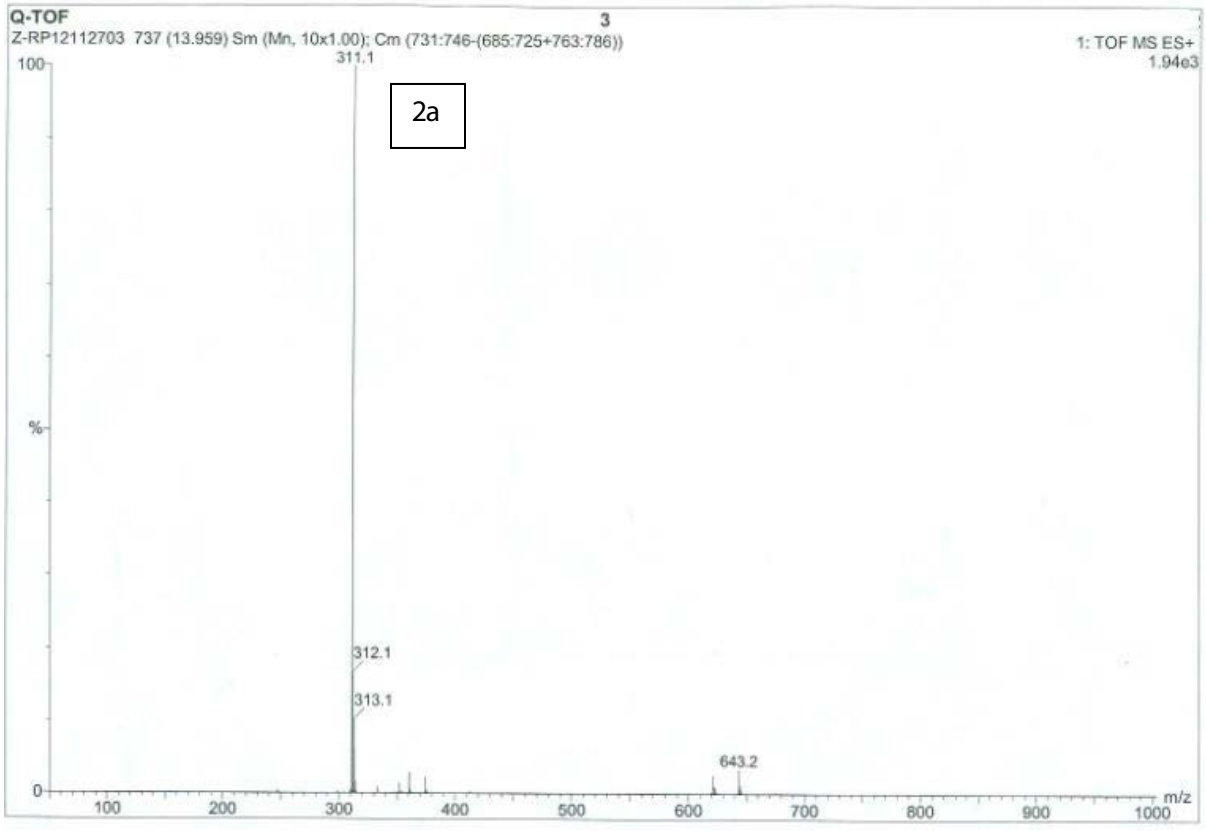
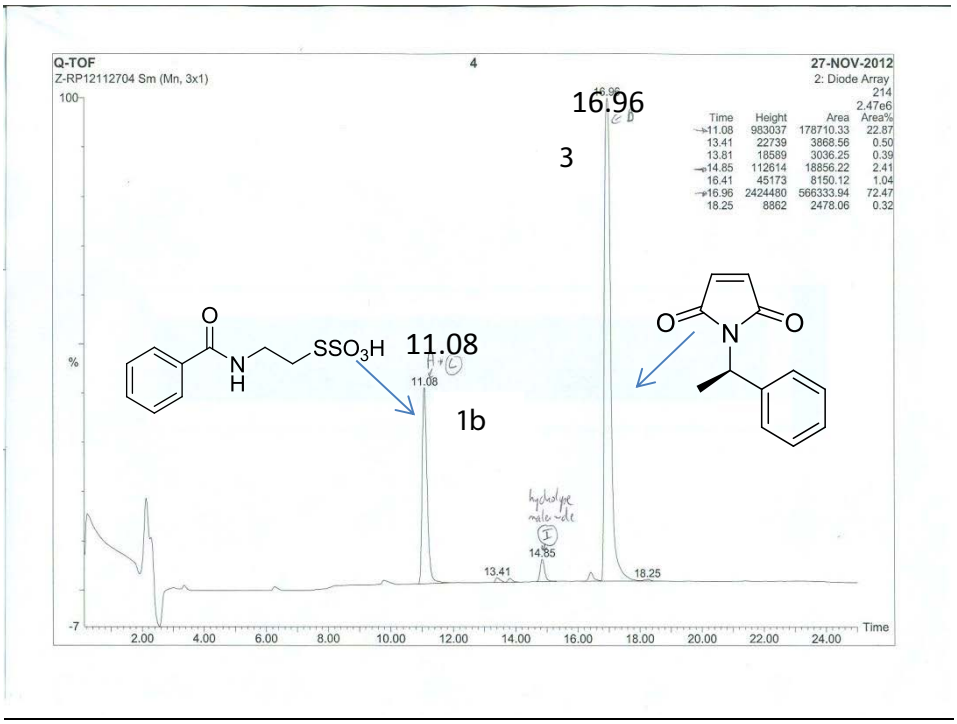


Figure S3. LC-MS analytical data for reactants **1b** and **3** before reduction (UV detection 214 nm)



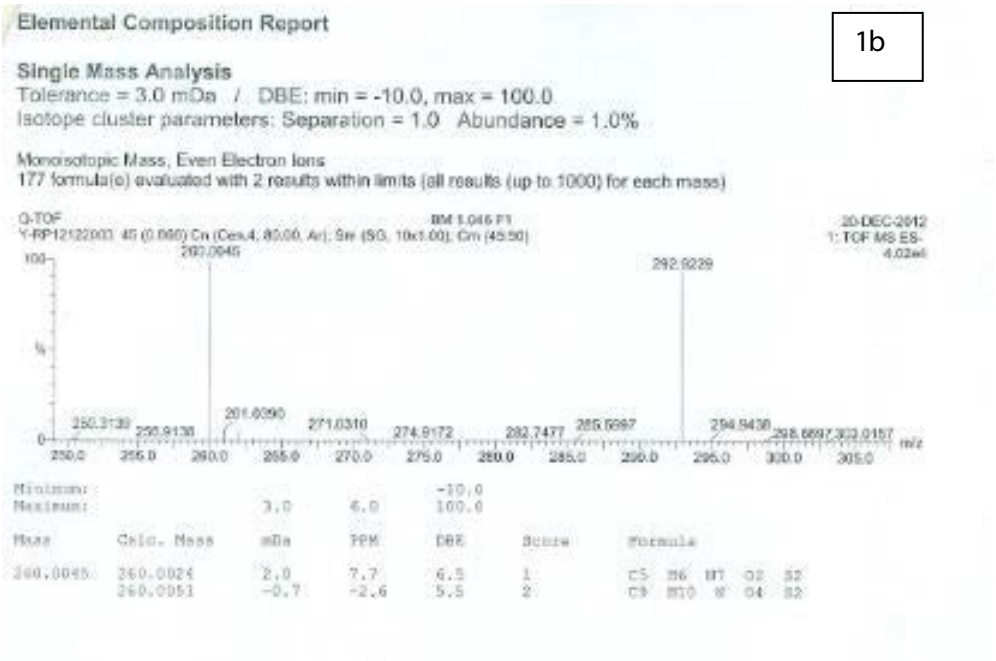
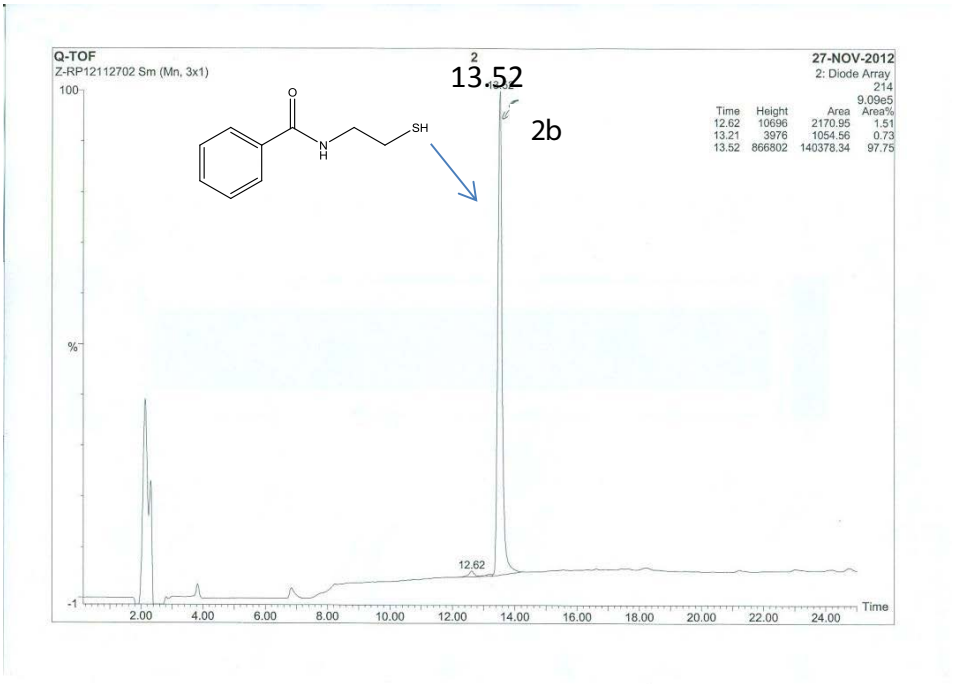


Figure S4. LC-MS analytical data for the reduction step of **1b** (UV detection 214 nm).



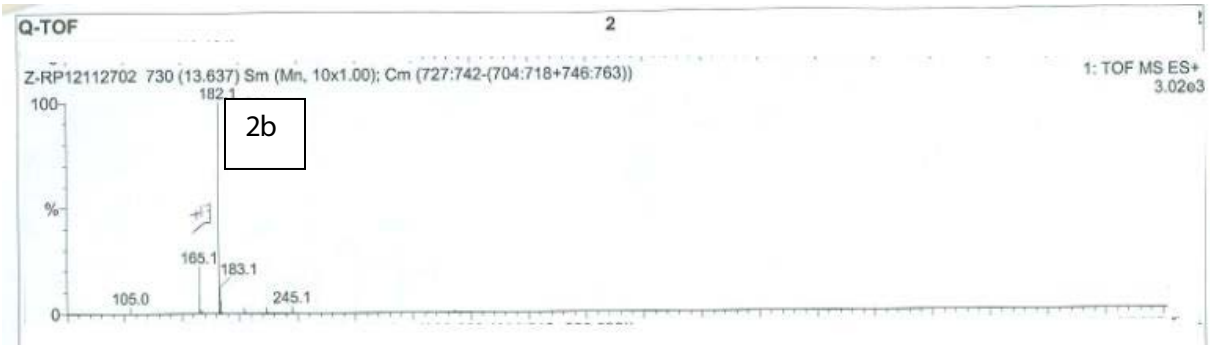
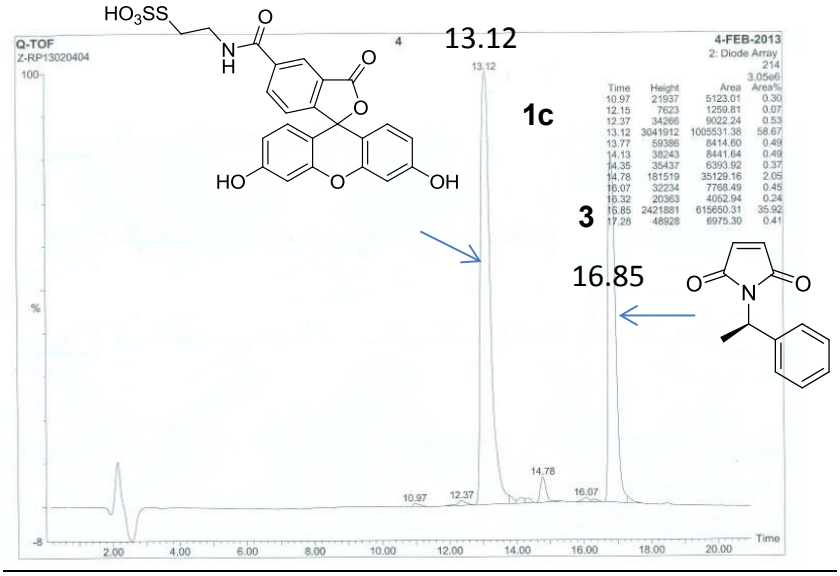


Figure S5. LC-MS analytical data for reactants **1c** and **3** before reduction (UV detection 214 nm)

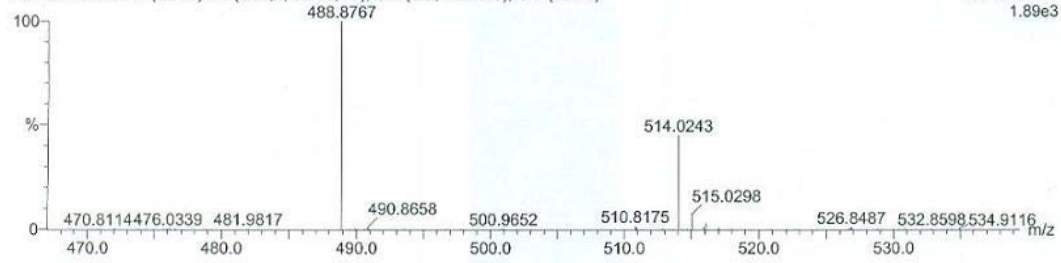


Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 3.0 mDa / DBE: min = -10.0, max = 100.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

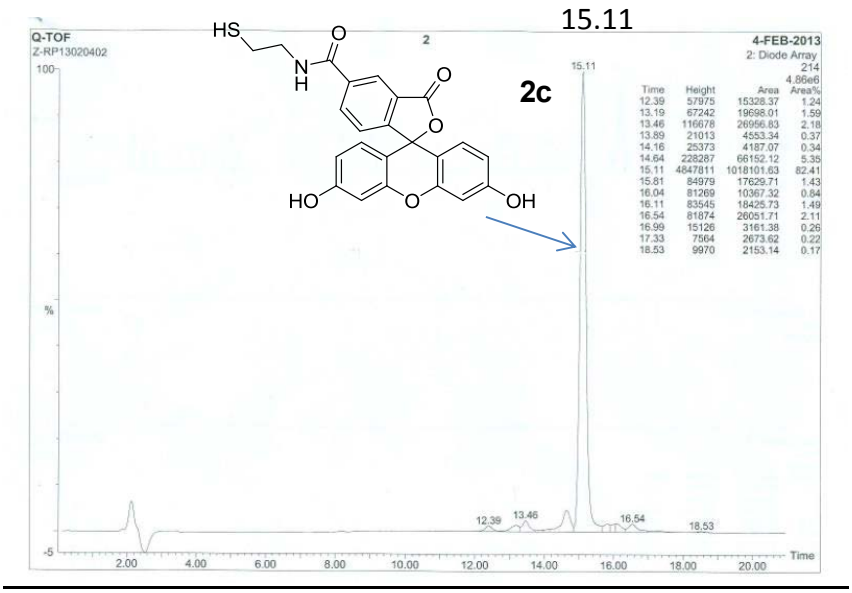
Monoisotopic Mass, Even Electron Ions
578 formula(e) evaluated with 2 results within limits (all results (up to 1000) for each mass)

Q-TOF BM1.165 31-JAN-2013
Y-RP13013102 45 (0.870) Cn (Cen,4, 80.00, Ar); Sm (SG, 10x1.00); Cm (45:46) TOF MS ES-
1.89e3



Minimum:				-10.0		
Maximum:		3.0	6.0	100.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
514.0243	514.0240	0.3	0.6	17.5	1	C19 H12 N7 O7 S2
	514.0267	-2.4	-4.6	16.5	2	C23 H16 N O9 S2

Figure S6. LC-MS analytical data for the reduction step of **1c** (UV detection 214 nm).



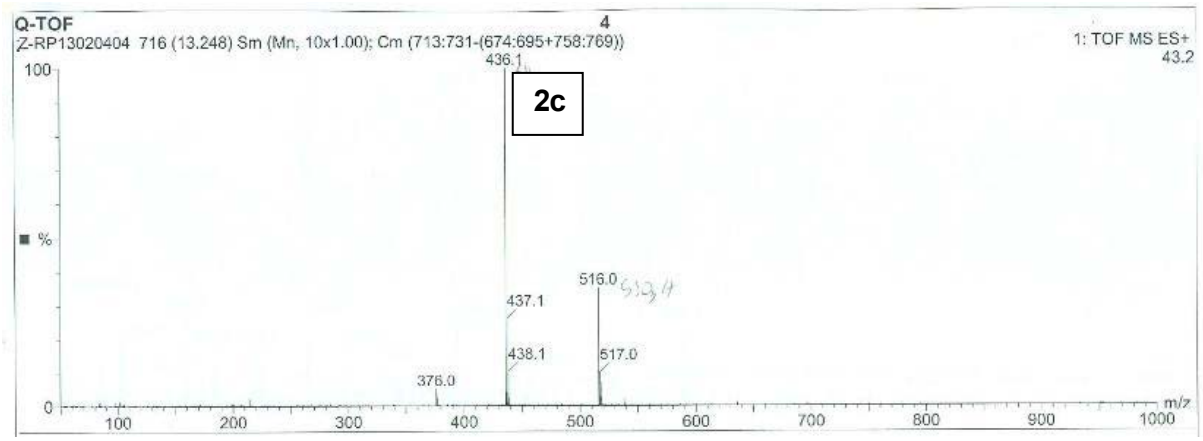
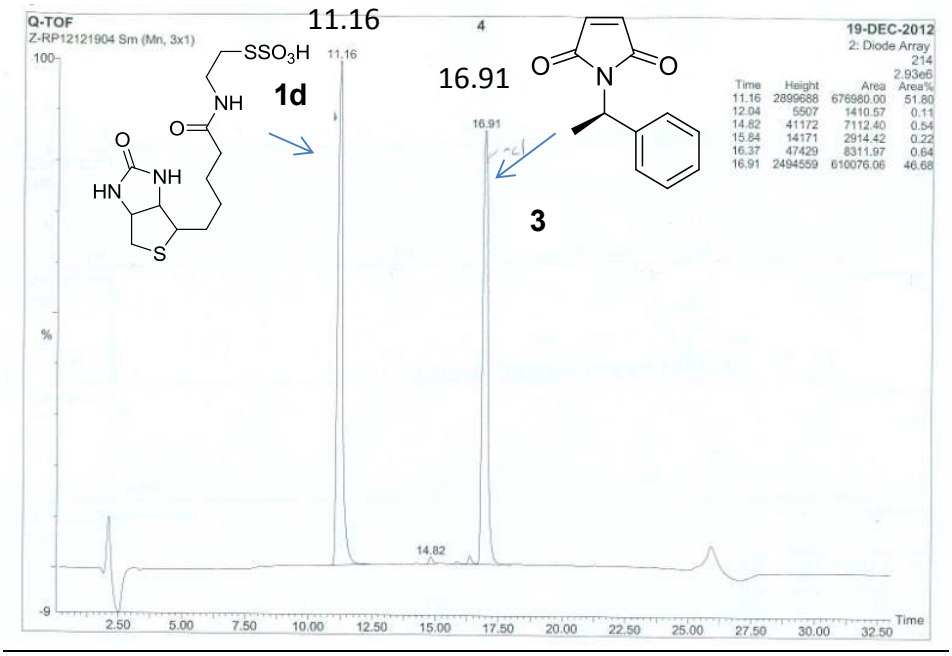


Figure S7. LC-MS analytical data for reactants **1d** and **3** before reduction (UV detection 214 nm).



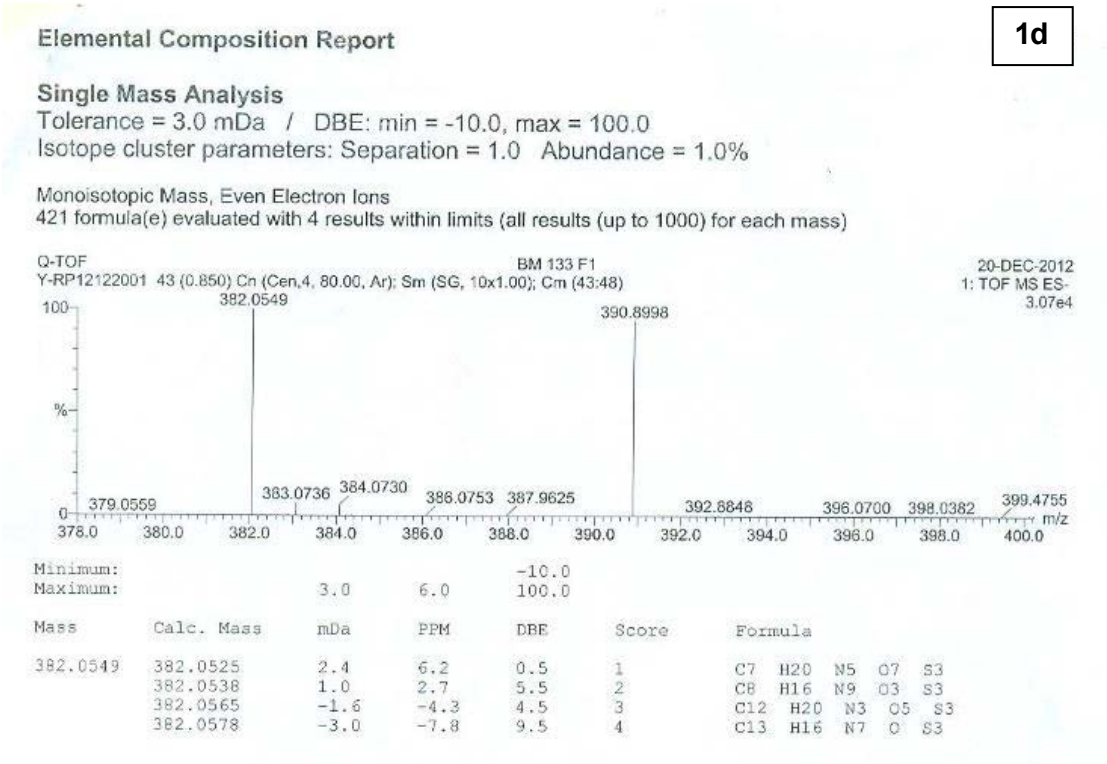
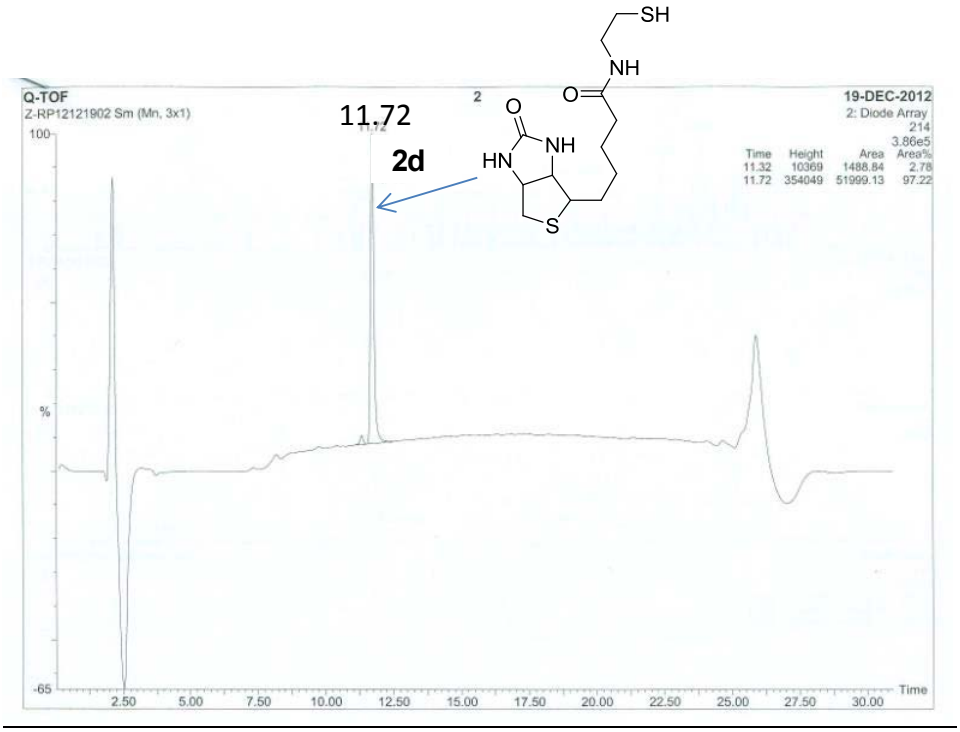


Figure S8. LC-MS analytical data for the reduction step of **1d** (UV detection 214 nm).



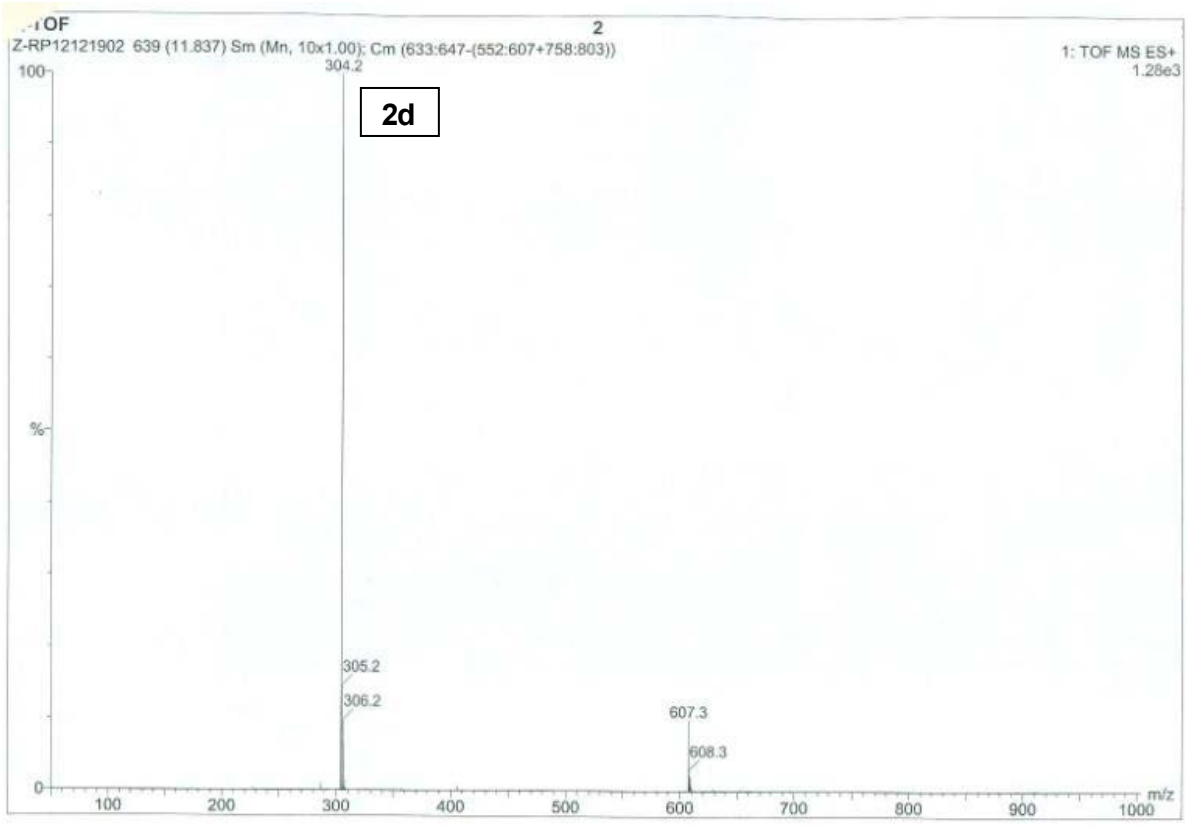
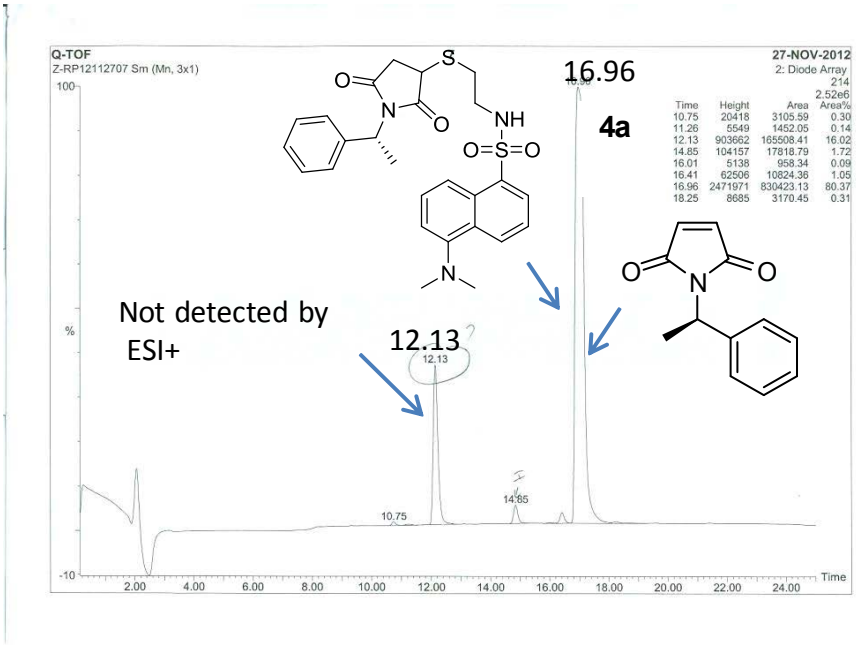


Figure S9. LC-MS analytical data for the Michael addition of **2a** to **3** (UV detection 214 nm).



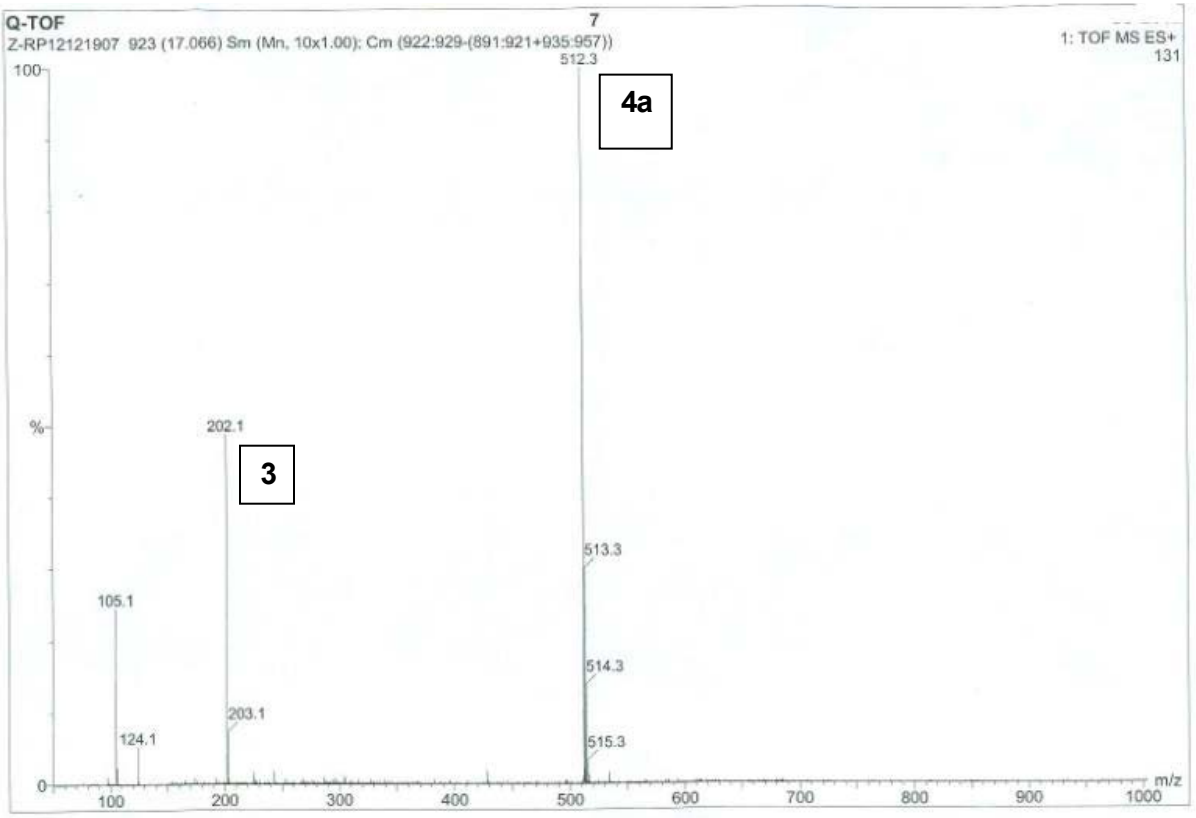
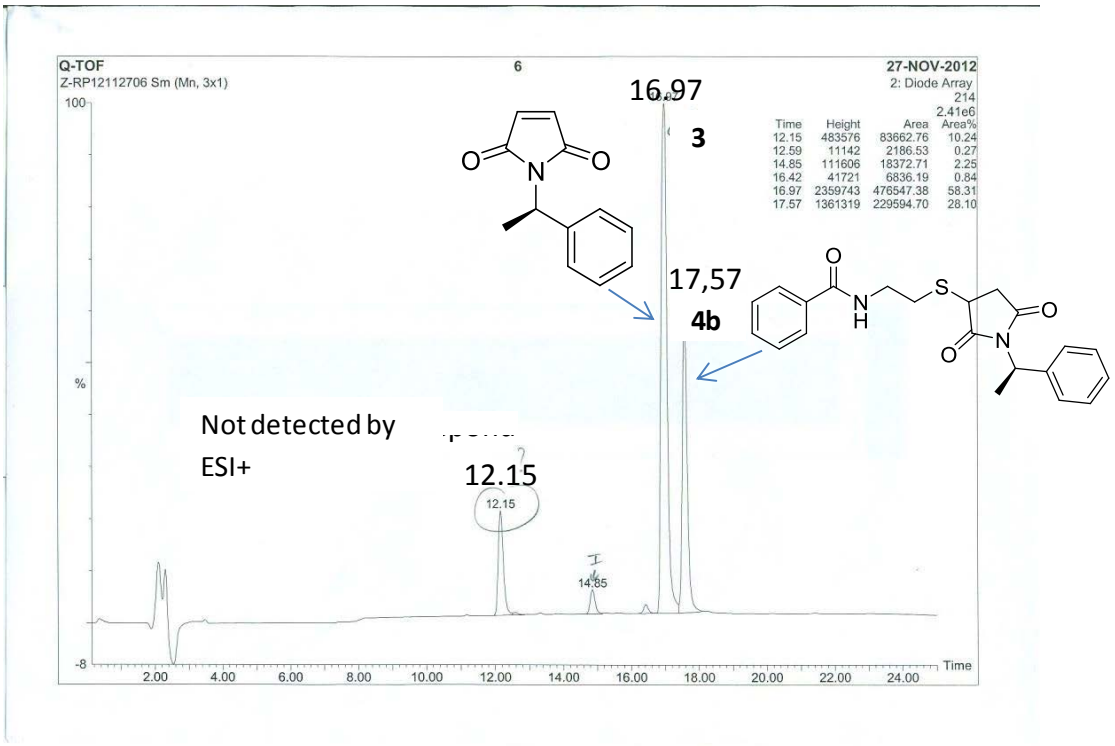


Figure S10. LC-MS analytical data for the Michael addition of **2b** to **3** (UV detection 214 nm).



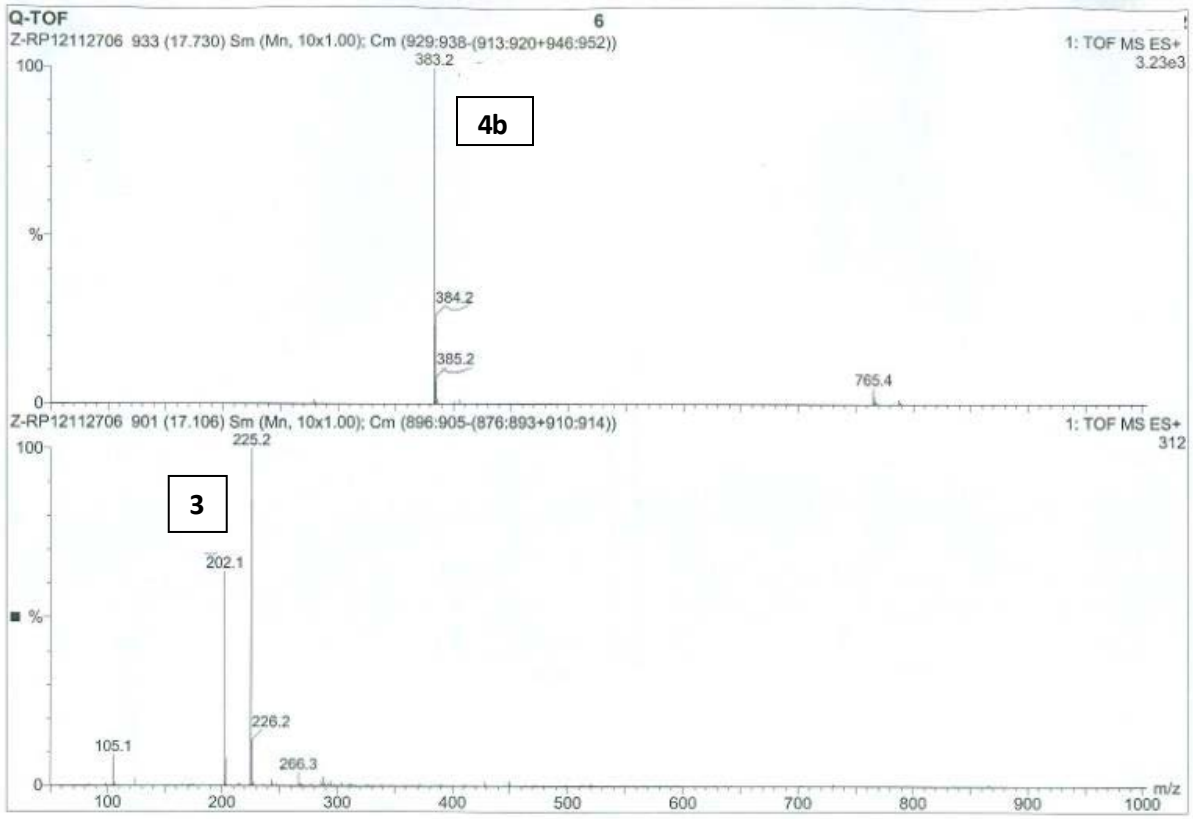


Figure S11. LC-MS analytical data for the Michael addition of **2c** to **3** (UV detection 214 nm).

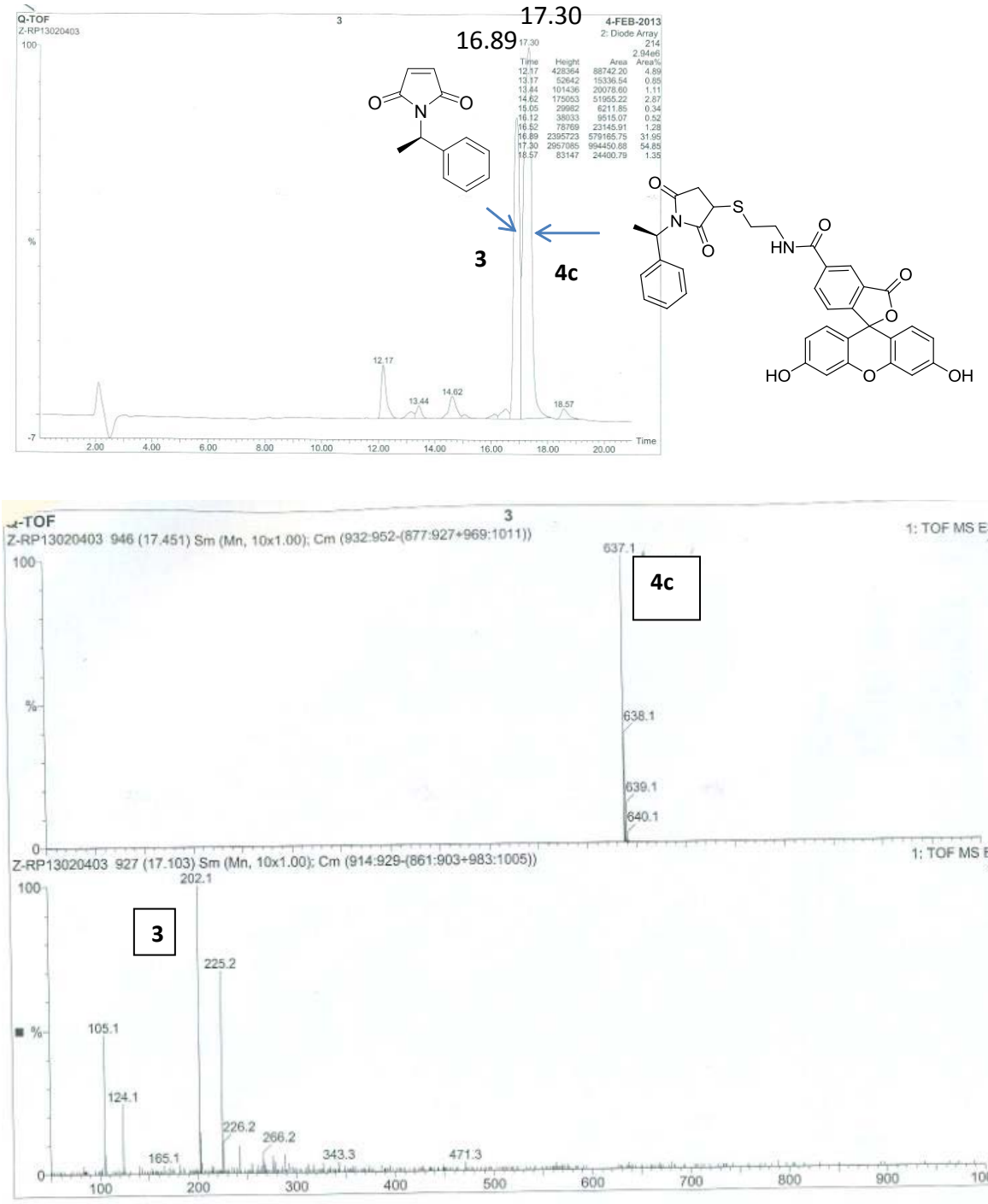
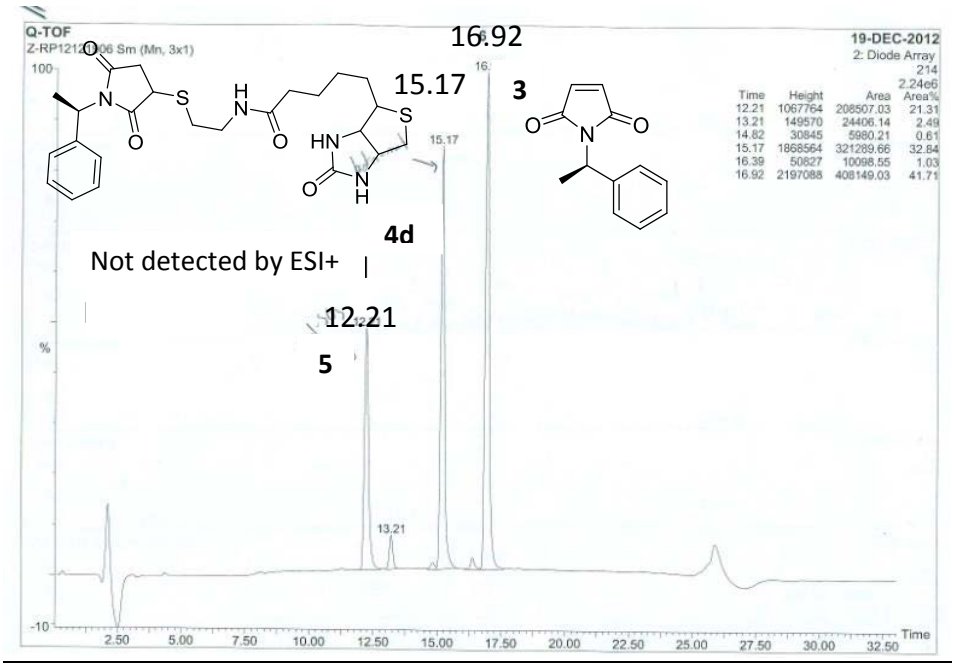


Figure S12. LC-MS analytical data for the Michael addition of **2d** to **3** (UV detection 214 nm).



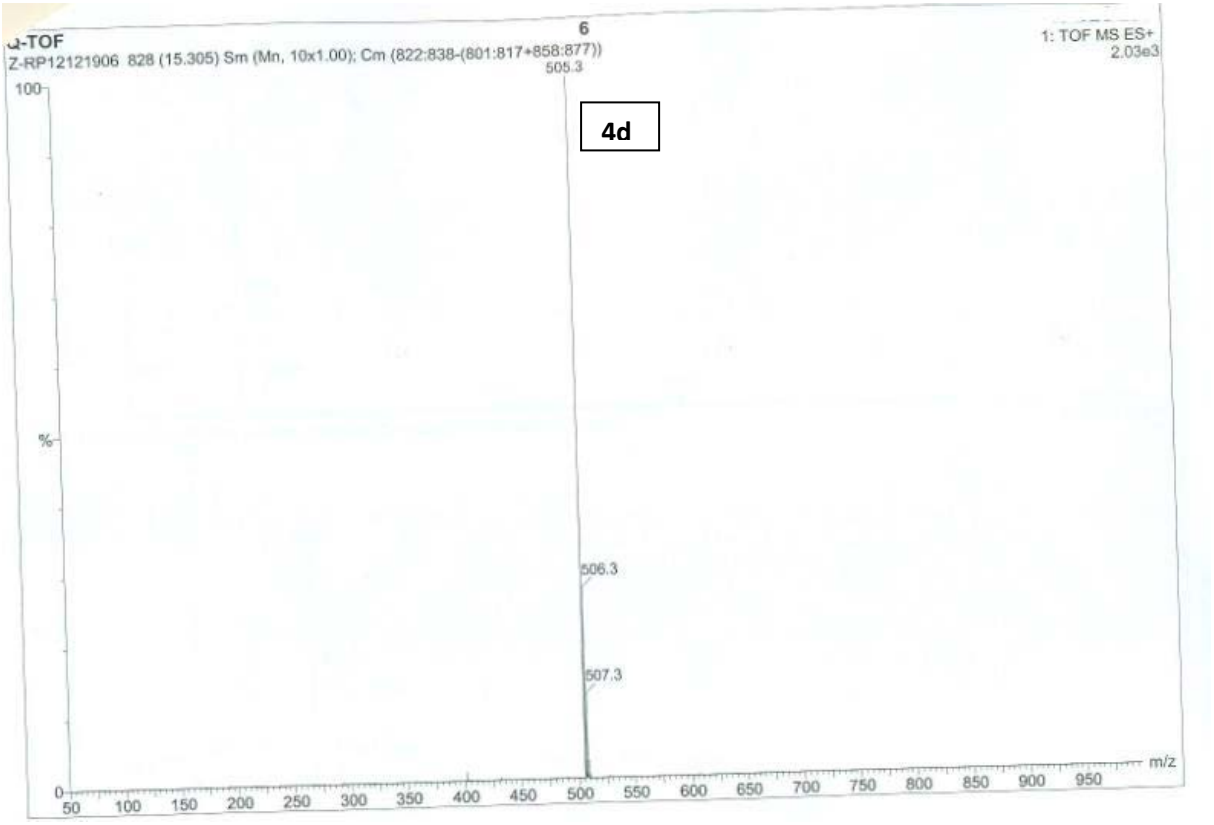
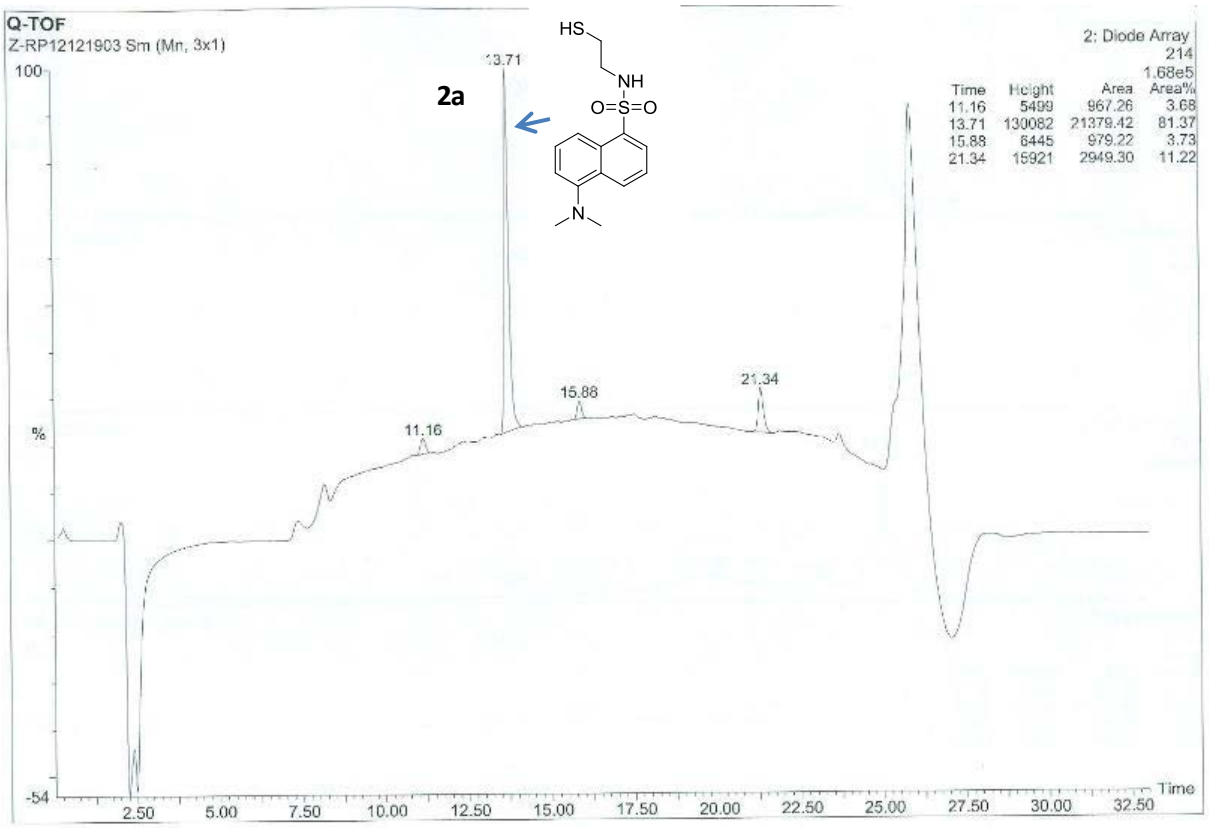


Figure S13. LC-MS analytical data for the reduction step of **1a** (UV detection 214 nm).



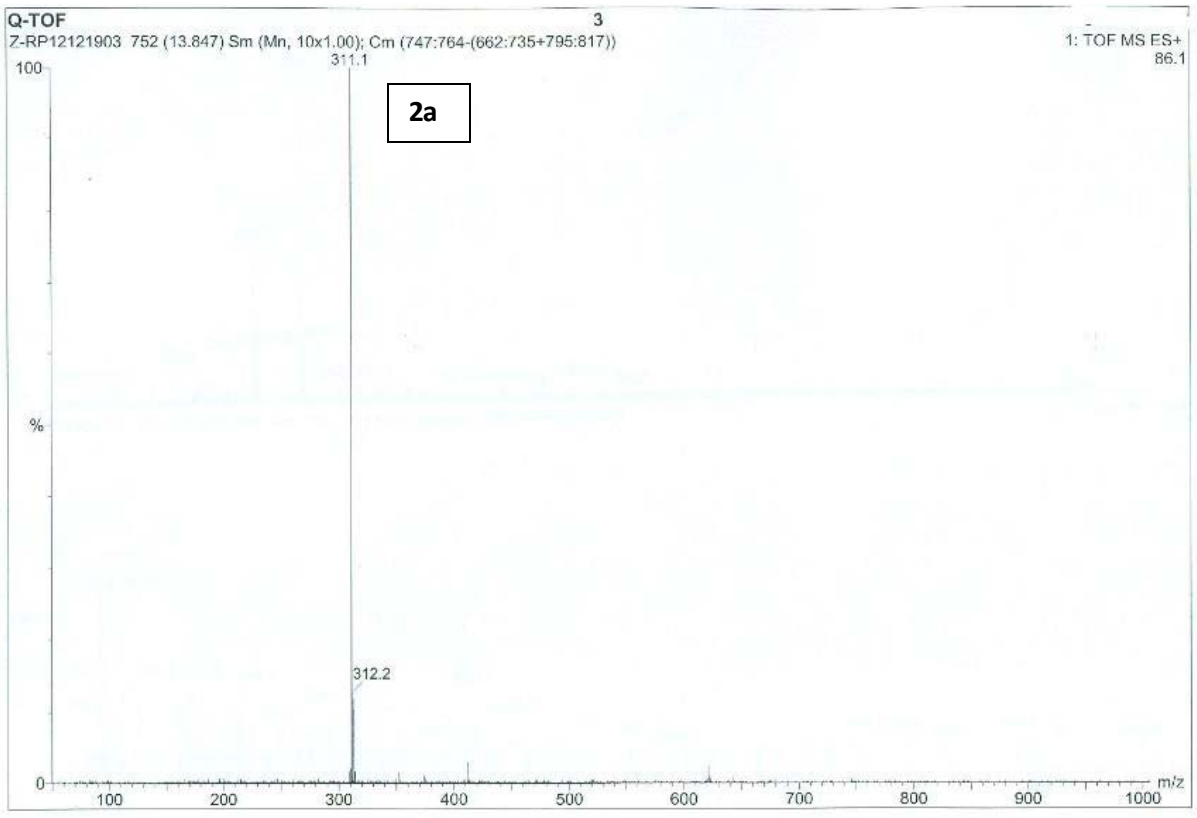
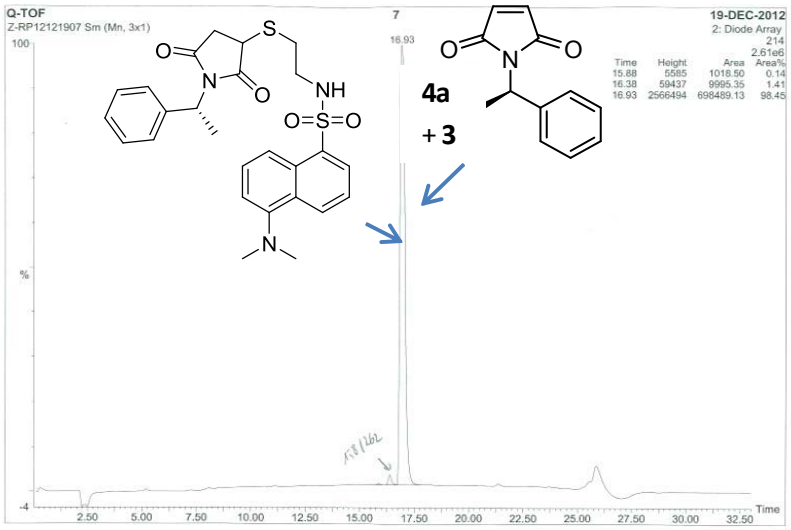


Figure S14. LC-MS analytical data for the Michael addition of **2a** to **3** (UV detection 214 nm).



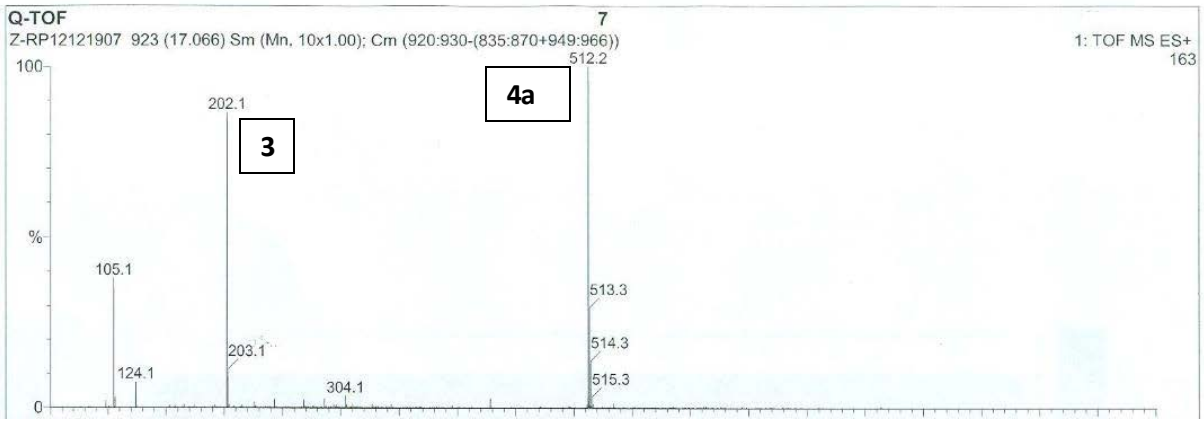


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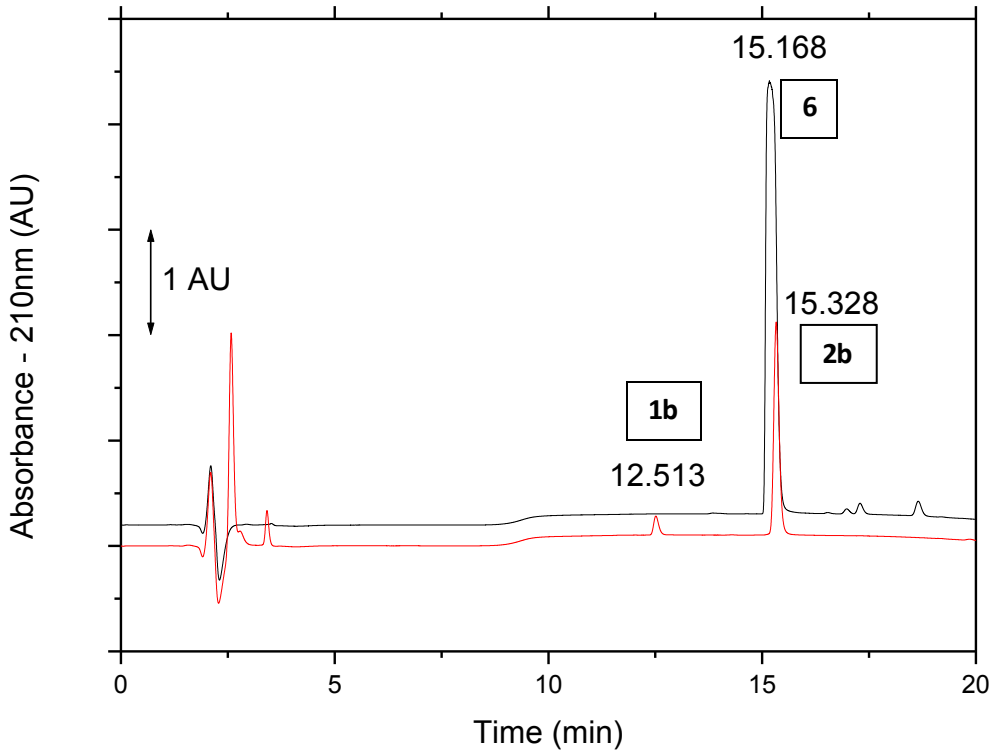
Figure S15. HPLC analytical data for the reduction step (UV detection 210 nm)
Figure S16. HPLC analytical data for side-product **6** (UV detection 210 nm)



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FigureS17. HPLC analytical data for the michael addition (UV detection 210 nm).

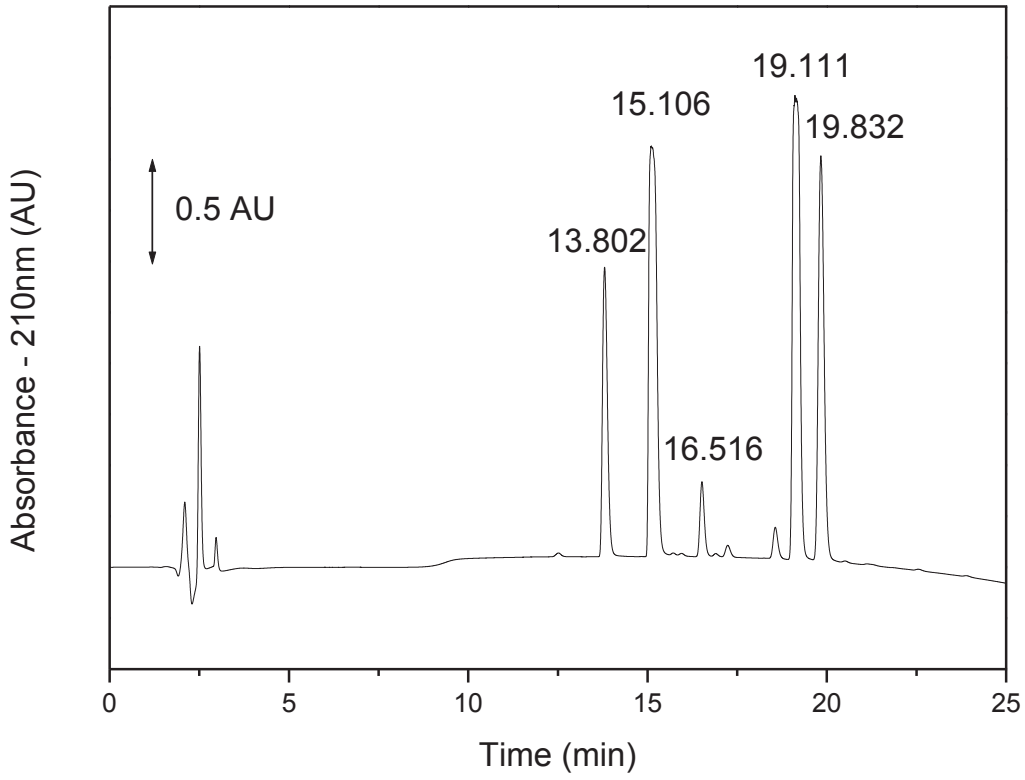

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Figure S18. HPLC analytical data for the reduction step monitoring of **1b** (UV detection 214 nm)

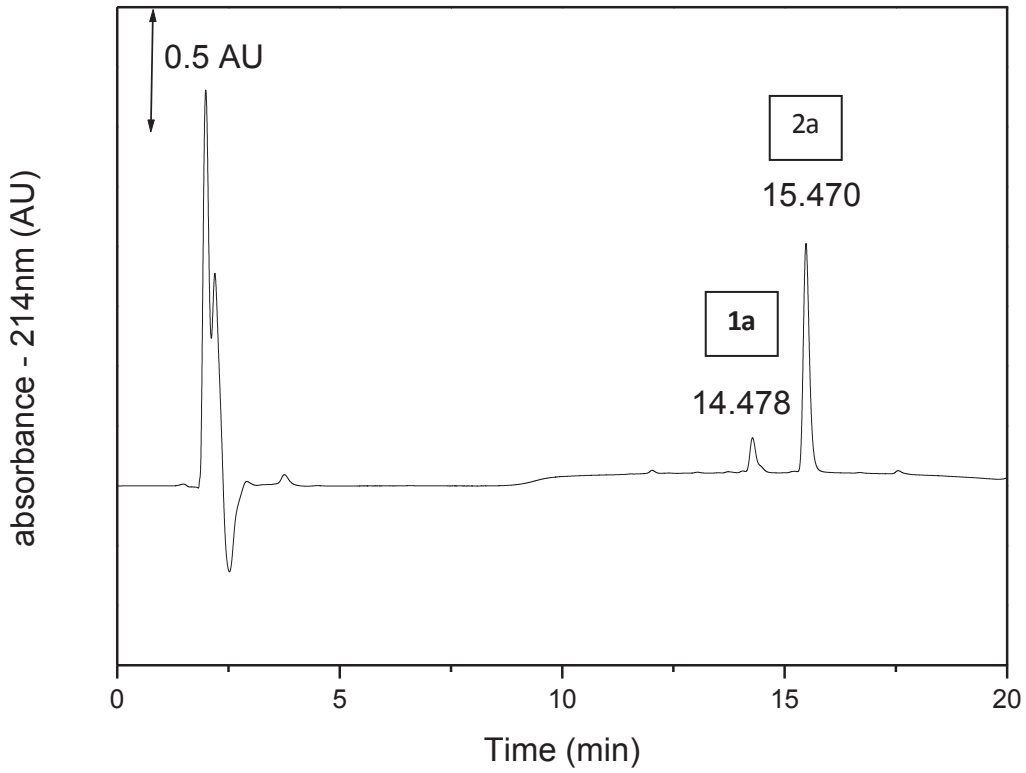

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Figure S19. HPLC analytical data for the Michael addition step monitoring of **2b** (UV detection 214 nm)