A mild TCEP-based *para*-azidobenzyl cleavage strategy to transform reversible cysteine thiol labelling reagents into irreversible conjugates

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General Experimental

All reagents were purchased from Aldrich, AlfaAesar or Lumiprobe and were used as received. Where described below pet. refers to petroleum ether (40–60 °C). All reactions were monitored by thin-layer chromatography (TLC) on pre-coated SIL G/UV254 silica gel plates (254 μm) purchased from VWR. Flash column chromatography was carried out with Kiesegel 60M 0.04/0.063 mm (200–400 mesh) silica gel. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker Avance 500 instrument operating at a frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C, and a Bruker Avance 600 instrument operating at a frequency of 600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$ or CD_3OD (as indicated below). The chemical shifts (δ) for ^{1}H and ^{13}C are quoted relative to residual signals of the solvent on the ppm scale. ^{1}H NMR peaks are reported as singlet (s), doublet (d), triplet (t), quartet (q), quint. (quintet), sext. (sextet), oct. (octet), m (multiplet), br (broad), dd (doublet of doublet), dt (doublet of triplets), ABq (AB quartet). Coupling constants (*J* values) are reported in Hertz (Hz) and are H-H coupling constants unless otherwise stated. Signal multiplicities in ^{13}C NMR were determined using the distortionless enhancement by phase transfer (DEPT) spectral editing technique. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR Spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm^{-1}). Melting points were measured with a Gallenkamp apparatus and are uncorrected. Mass spectra were obtained on a VG70-SE mass spectrometer.

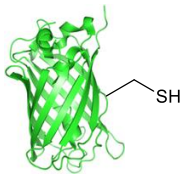
Protein LC-MS

LC-MS was performed on protein samples using a Thermo Scientific uPLC connected to MSQ Plus Single Quad Detector (SQD). Column: Hypersil Gold C4, 1.9 μm, 2.1 × 50 mm. Wavelength: 254 nm. Mobile Phase: 99:1 Water (0.1% formic acid): MeCN (0.1% formic acid) to 1:9 Water (0.1% formic acid): MeCN (0.1% formic acid) gradient over 4.5 min. Flow Rate: 0.3 mL/min. MS Mode: ES+. Scan Range: m/z = 500–2000. Scan time: 1.5 s. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h. Ion series were generated by integration of the total ion chromatogram (TIC) over the 4.2–5.5 min range. Total mass spectra for protein samples were reconstructed from the ion series using the pre-installed ProMass software using default settings for large proteins in m/z range 500–1500.

Cloning and expression of proteins

The gene for GFPS147C in the vector pNIC28-Bsa4 was generated as described previously.[1]

GFPS147C 1[1]



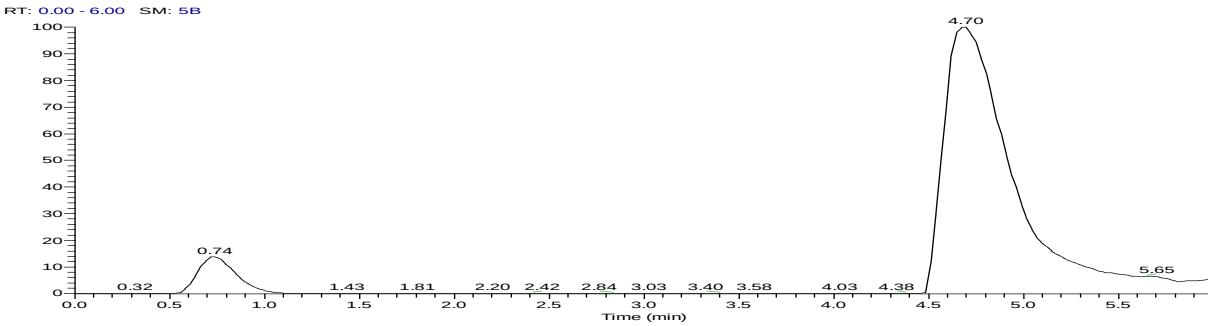
1

Sequence

MHHHHHHSSGVDLGTDNLYFQSMRKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGK
LTLKFICTTGKLPVPWPTLVTTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTY
KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFN**C**HNVYITADKQKNGIKANFKIRHNV
EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDE
LYK

Expected mass: 29,341 Da. Observed mass: 29,332 Da.

(a)



(b)

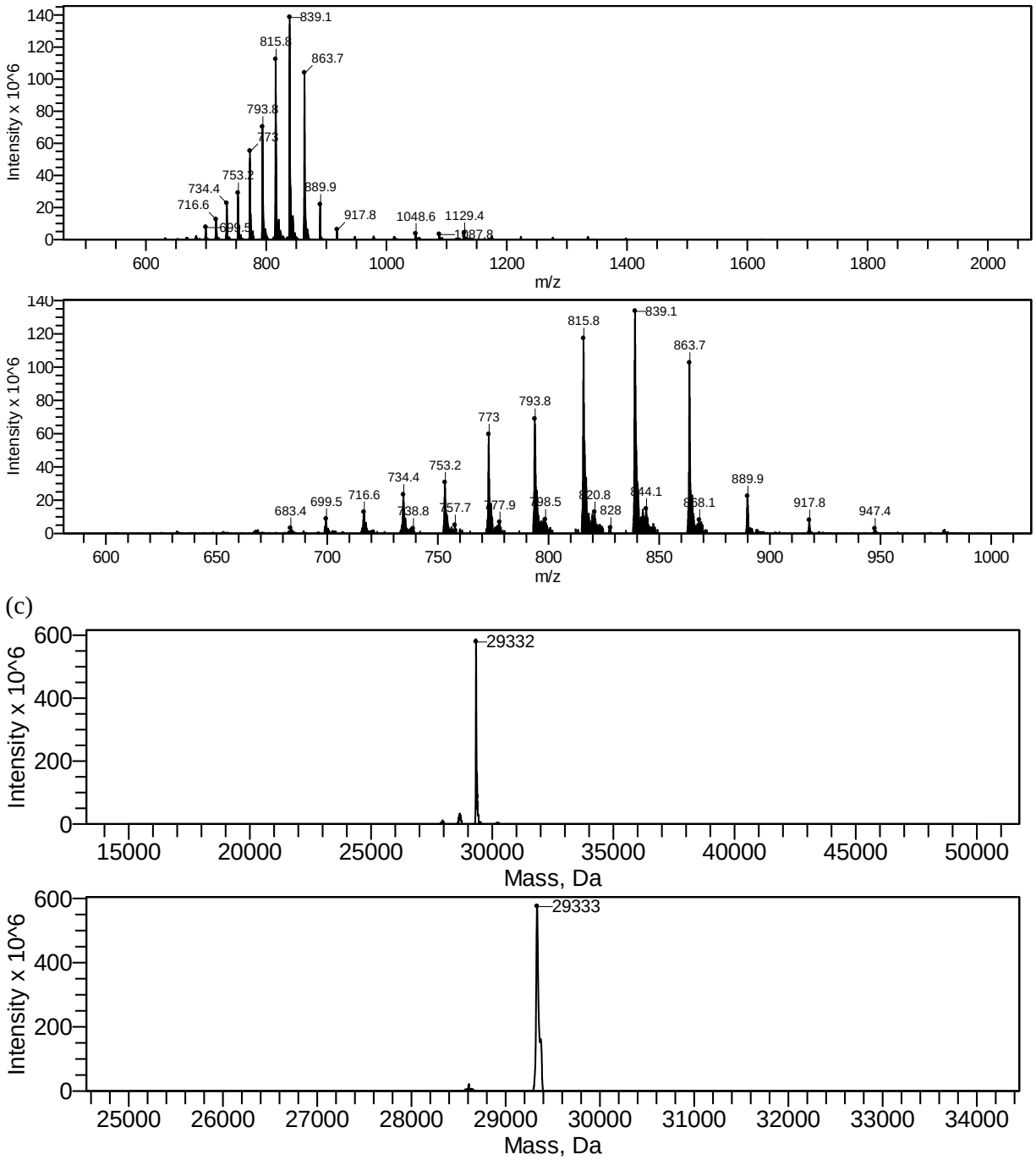


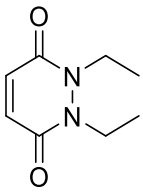
Figure S1. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for GFPS147C **1**.

SDS-PAGE gels

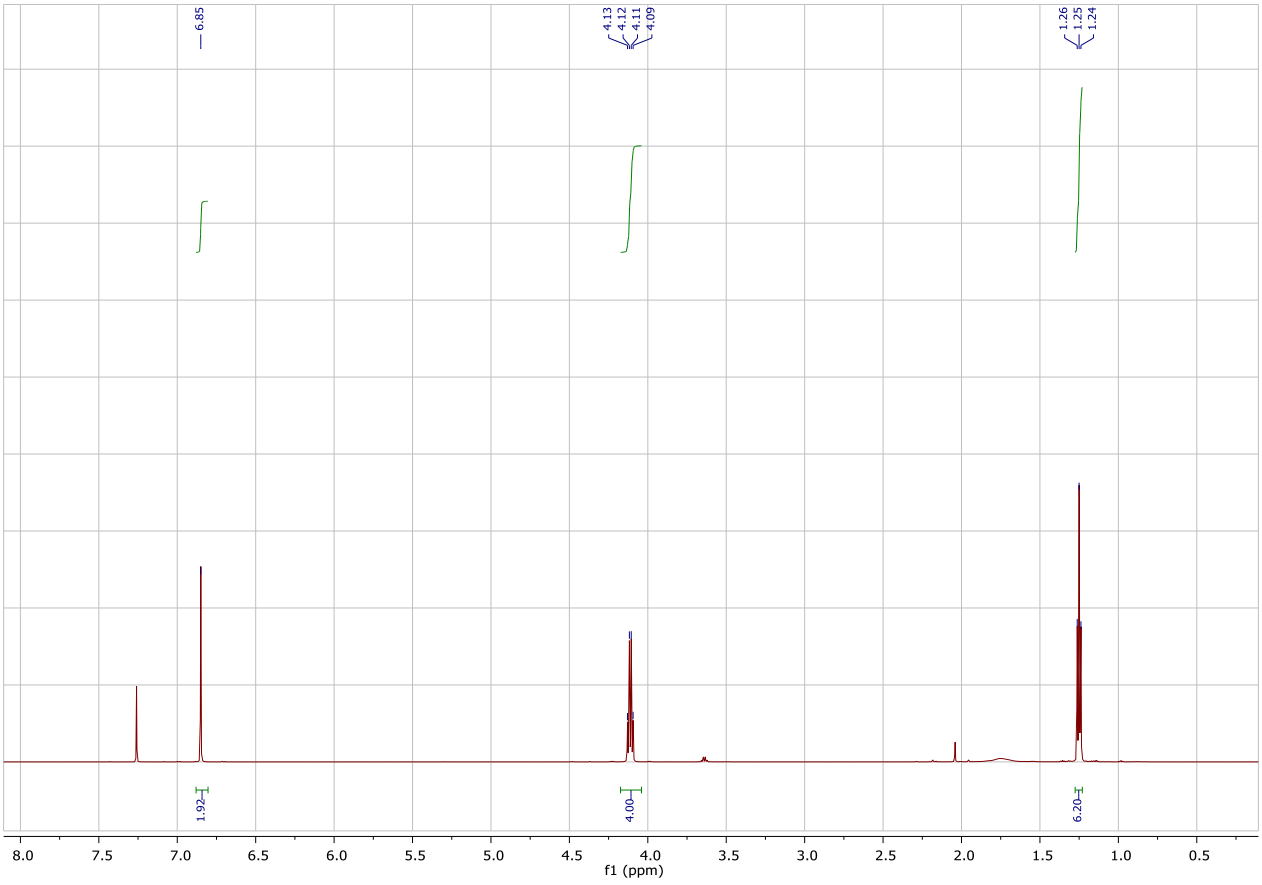
Non-reducing glycine-SDS-PAGE at 16% acrylamide gels were performed following standard lab procedures. A 6% stacking gel was used and a broad-range MW marker (10–250 kDa, Precision Plus Protein Standards, Bio-Rad) was co-run to estimate protein weights. Samples (15 µL at ~12 µM GFP construct) were mixed with loading buffer (3 µL, composition for 6 × SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg bromophenol blue in 10 mL) and heated at 75 °C for 3 min. The gel was run at 150 V for 60 min in 1 × SDS running buffer. The gel was stained with Coomassie dye.

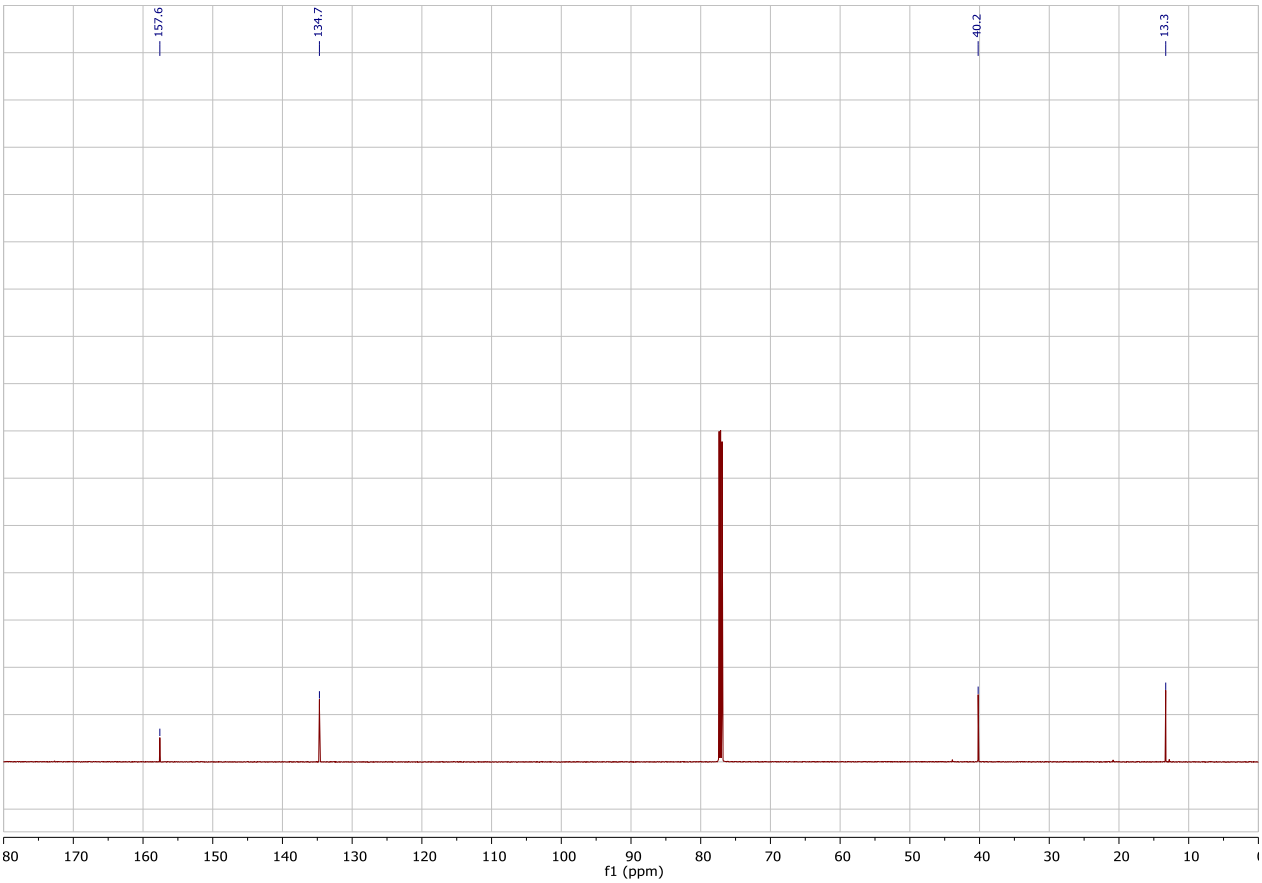
Synthesis of compounds

1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione 2

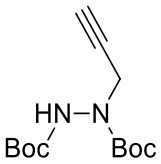


To a solution of maleic anhydride (98 mg, 1.0 mmol) in glacial AcOH (3 mL) was added *N,N'*-diethylhydrazine·2HCl (161 mg, 1.0 mmol) and the reaction mixture heated at 130 °C for 16 h. The solvent was removed *in vacuo* and the crude residue purified by column chromatography (50% EtOAc/pet. to neat EtOAc) to give 1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione (121 mg, 0.72 mmol, 72%) as a white solid: ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.85 (s, 2H), 4.11 (q, *J* = 7.0 Hz, 4H), 1.25 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 157.6 (C), 134.7 (CH), 40.2 (CH_2), 13.3 (CH_3); IR (solid) 2984, 1636, 1590 cm^{-1}; LRMS (CI) 169 (100, $[M+H]^+$); HRMS (CI) calcd for $C_8H_{13}N_2O_2$ $[M+H]^+$ 169.0977, observed 169.0980.

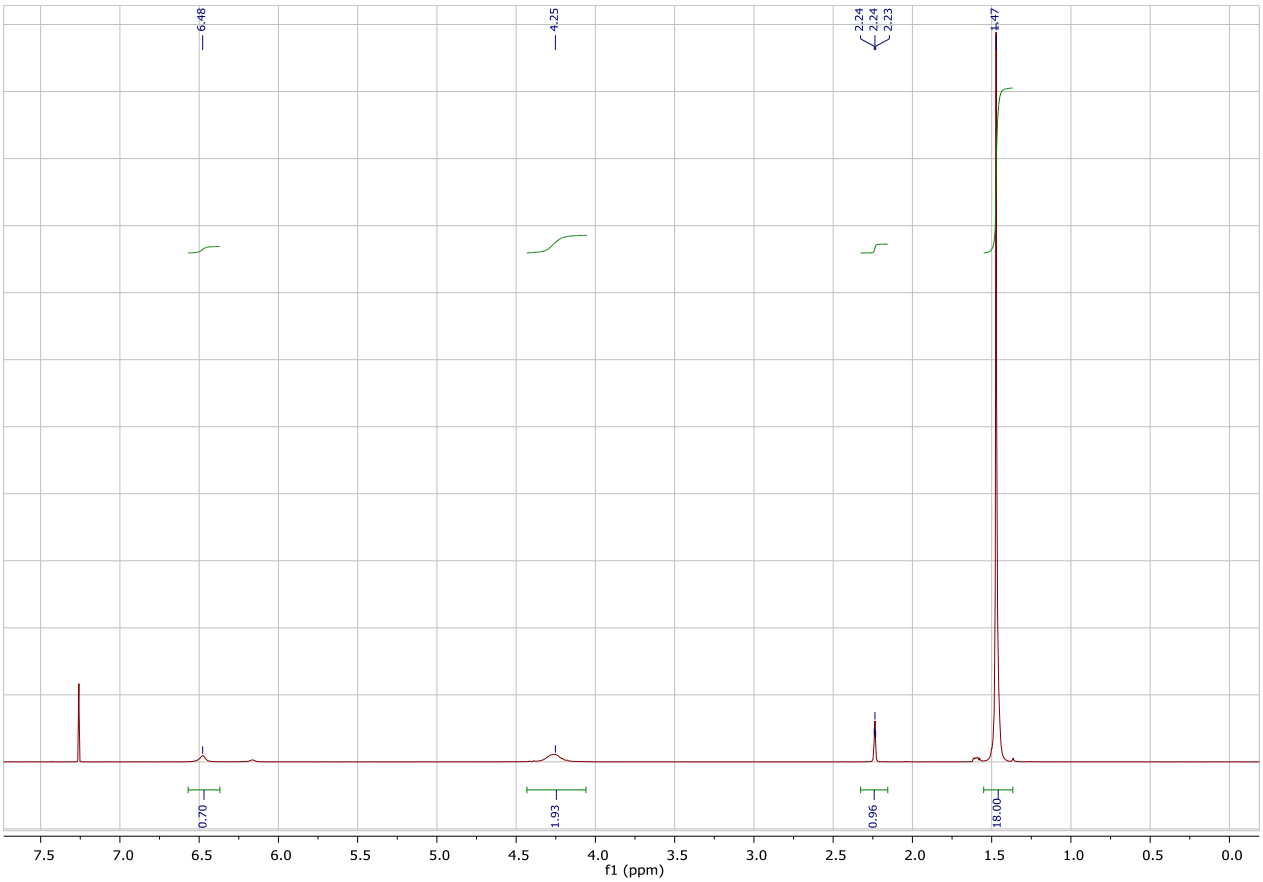


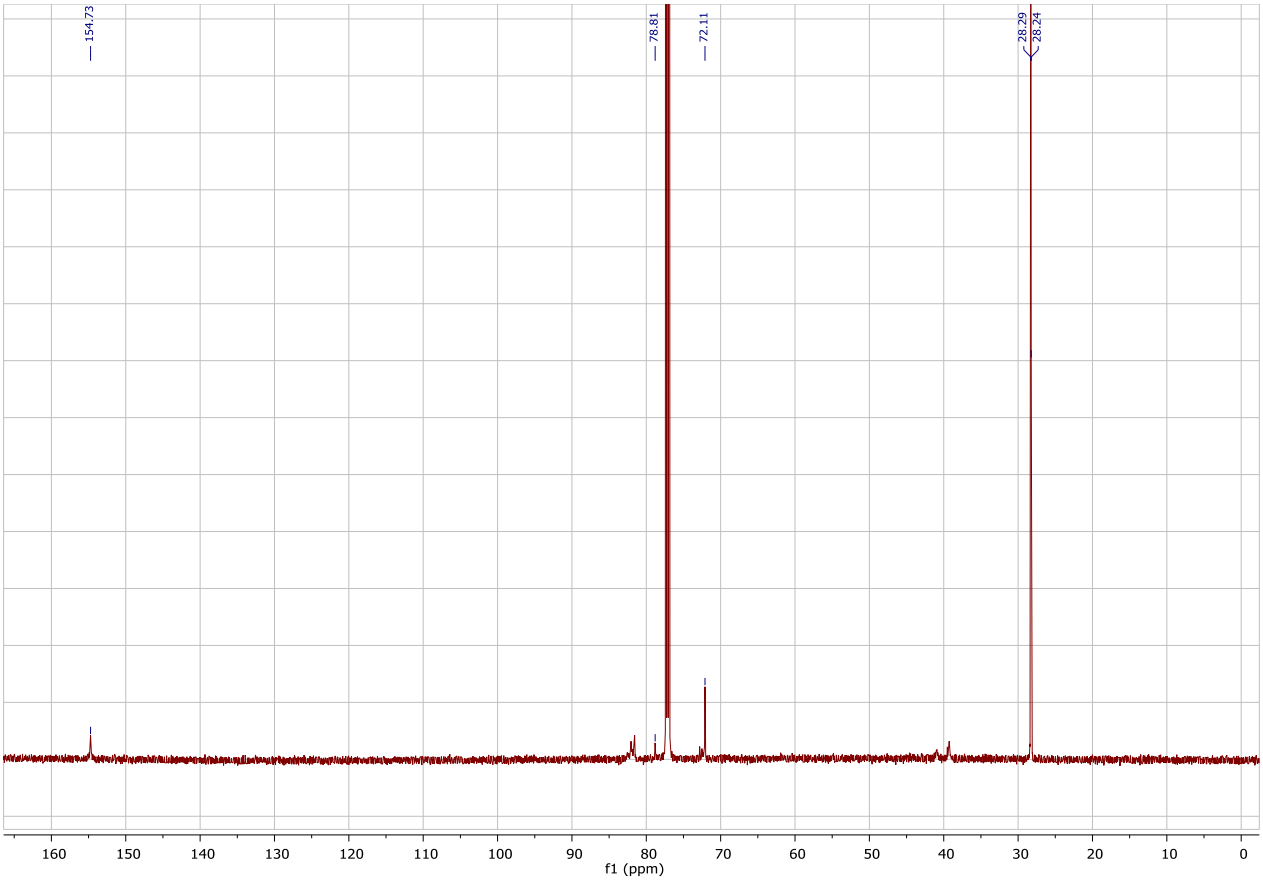


Di-*tert*-butyl 1-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate[2]

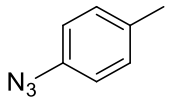


To a solution of di-*tert*-butyl hydrazine-1,2-dicarboxylate (300 mg, 1.29 mmol) in a mixture of toluene (2 mL) and 5% aq. NaOH (2 mL) was added tetra-*n*-butylammonium bromide (13 mg, 0.03 mmol) and propargyl bromide (461 mg, 3.87 mmol). The reaction mixture was stirred at 21 °C for 16 h. After this time, H_2O (20 mL) was added and the mixture was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were washed with brine (15 mL), dried ($MgSO_4$), and concentrated *in vacuo*. Purification by flash column chromatography (20% EtOAc/pet.) yielded di-*tert*-butyl 1-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate (435 mg, 1.61 mmol, 85%) as a white solid: m.p. 101-103 ºC (*lit. m.p.* 103.1-103.4 °C)[2]; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.47 (br s, 0.78H), 6.18 (br s, 0.22H,), 4.28-4.22 (m, 2H), 2.24 (t, *J* = 2.4 Hz, 1H), 1.48 (s, 18H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 154.7 (C), 82.2 (C), 81.7 (C), 78.8 (C), 72.1 (CH), 39.7 (CH_2), 28.3 (CH_3), 28.2 (CH_3); IR (solid) 3310, 2112, 1703 cm^{-1}.

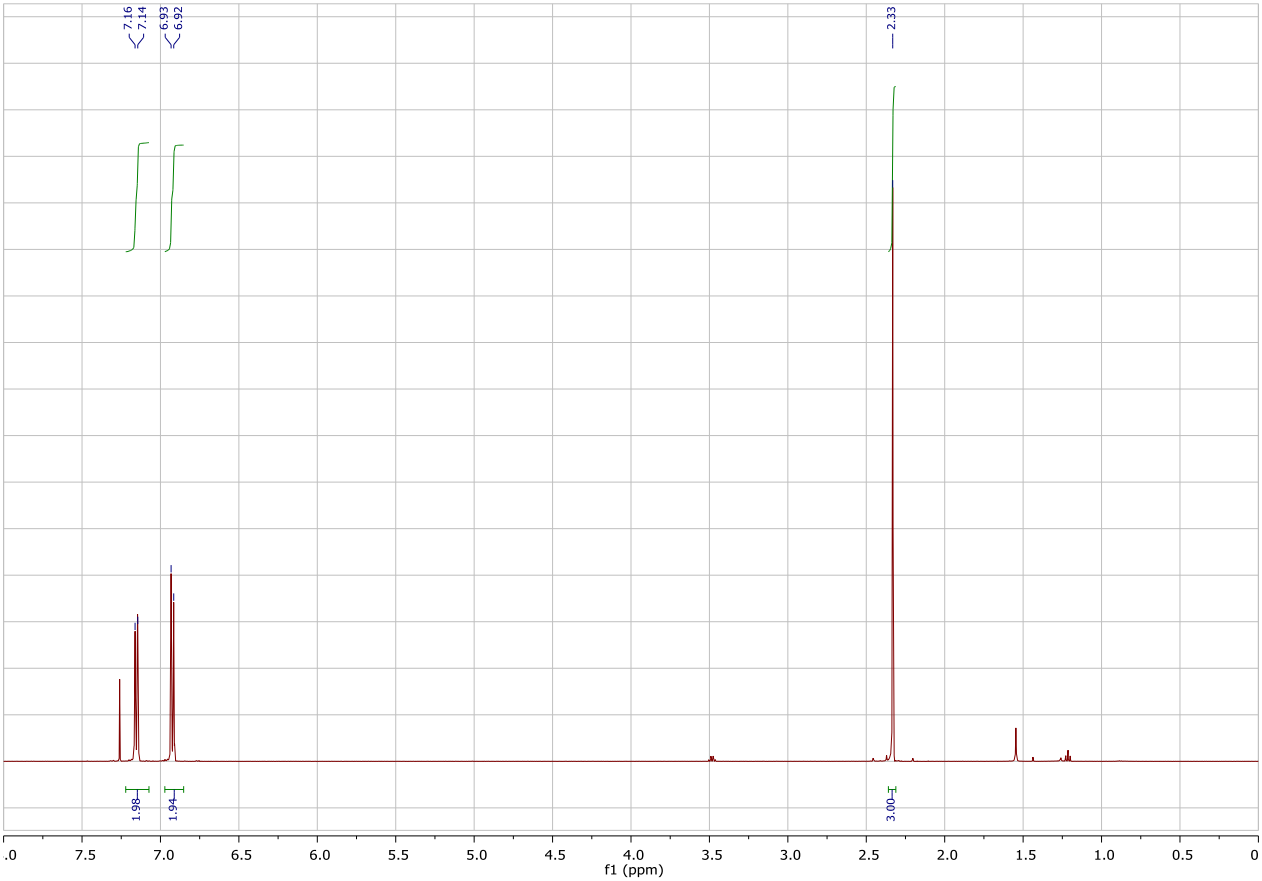

6.48
4.25
2.24
2.24
2.23
1.47
0.70
1.93
0.96
18.00
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

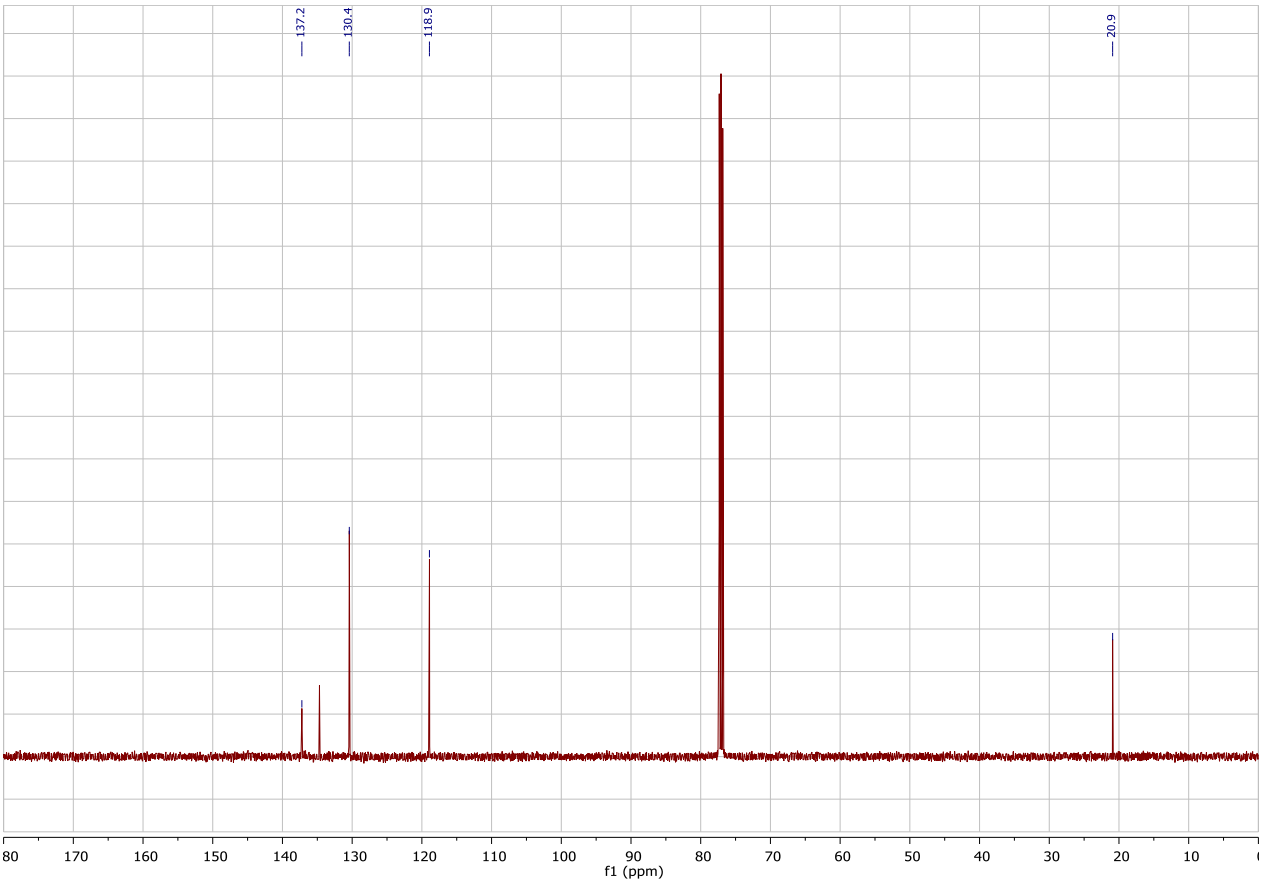

154.73
78.81
72.11
28.29
28.24
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

1-Azido-4-methylbenzene[3]

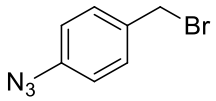


To a solution of *p*-toluidine (2.0 g, 18.4 mmol) in 2N HCl (28 mL) at −5 °C was added slowly a solution of sodium nitrite (1.5 g, 22.4 mmol) in H_2O (5 mL) over 5 min making sure that the internal temperature did not rise above 0 °C. After completion of addition, the reaction mixture was stirred at −5 °C for 5 min to form a diazonium salt. Then urea (130 mg, 2.2 mmol) was added to neutralise the diazonium salt solution. Following this, the diazonium salt solution was added to a solution of sodium azide (2.4 g, 37.2 mmol) and sodium acetate (4.6 g, 56 mmol) in 30 mL of H_2O at 0 °C over 5 min. The mixture was stirred for 2 h at 0 °C. The mixture was extracted into Et_2O (2 × 60 mL), the combined organic layers dried ($MgSO_4$) and concentrated *in vacuo* to afford 1-azido-4-methylbenzene (2.3 g, 17.3 mmol, 94%) as a yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (d, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 8.4 Hz, 2H), 2.33 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 137.2 (CH), 134.7 (CH), 130.4 (CH), 118.9 (CH), 21.0 (CH_3). IR (thin film) 2104, 1609, 1521 cm^{-1}.

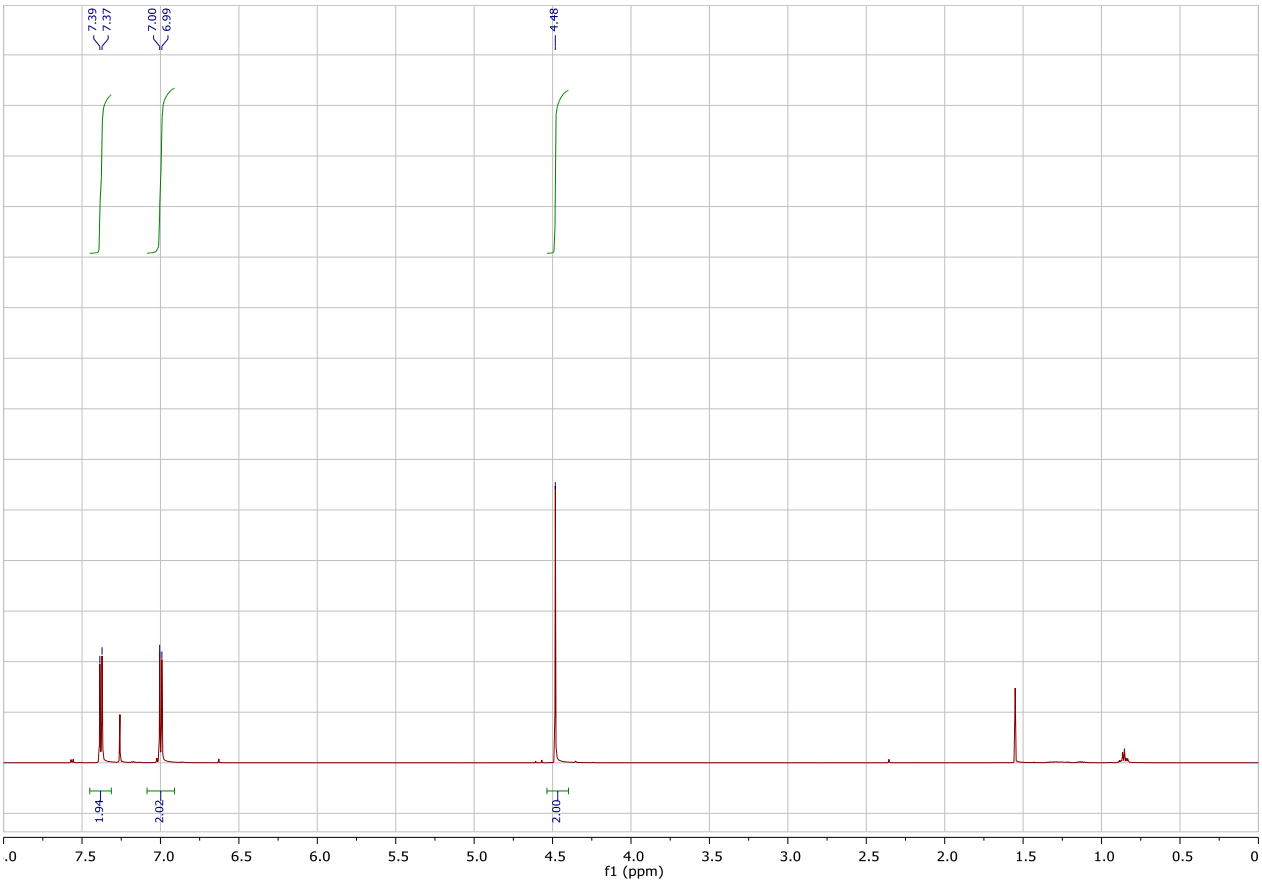




1-Azido-4-(bromomethyl)benzene[4]



A solution of 1-azido-4-methylbenzene (0.85 g, 6.4 mmol), *N*-bromosuccinimide (1.5 g, 8.3 mmol) and azobis(isobutyronitrile) (0.31 g, 1.9 mmol) in dry benzene (20 mL) was heated under reflux under argon in the dark for 8 h. After this time, the mixture was poured into H_2O (20 mL), extracted into Et_2O (2 × 20 mL), the combined organic layers dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash column chromatography (neat pet.) yielded 1-azido-4-(bromomethyl)benzene (1.1 g, 5.1 mmol, 80%) as a light brown solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.4 Hz, 2H), 7.00 (d, *J* = 8.4 Hz, 2H), 4.48 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 140.3 (CH), 134.6 (CH), 130.7 (CH), 119.5 (CH), 33.0 (CH_2); IR (solid) 2107, 1607, 1505 cm^{-1}; LRMS (EI) 213 (100, $[M^{81}Br]^{+\bullet}$), 211 (100, $[M^{79}Br]^{+\bullet}$); HRMS (EI) calcd for $C_7H_6N_3Br$ $[M^{79}Br]^{+\bullet}$ 210.9740, observed 210.9743.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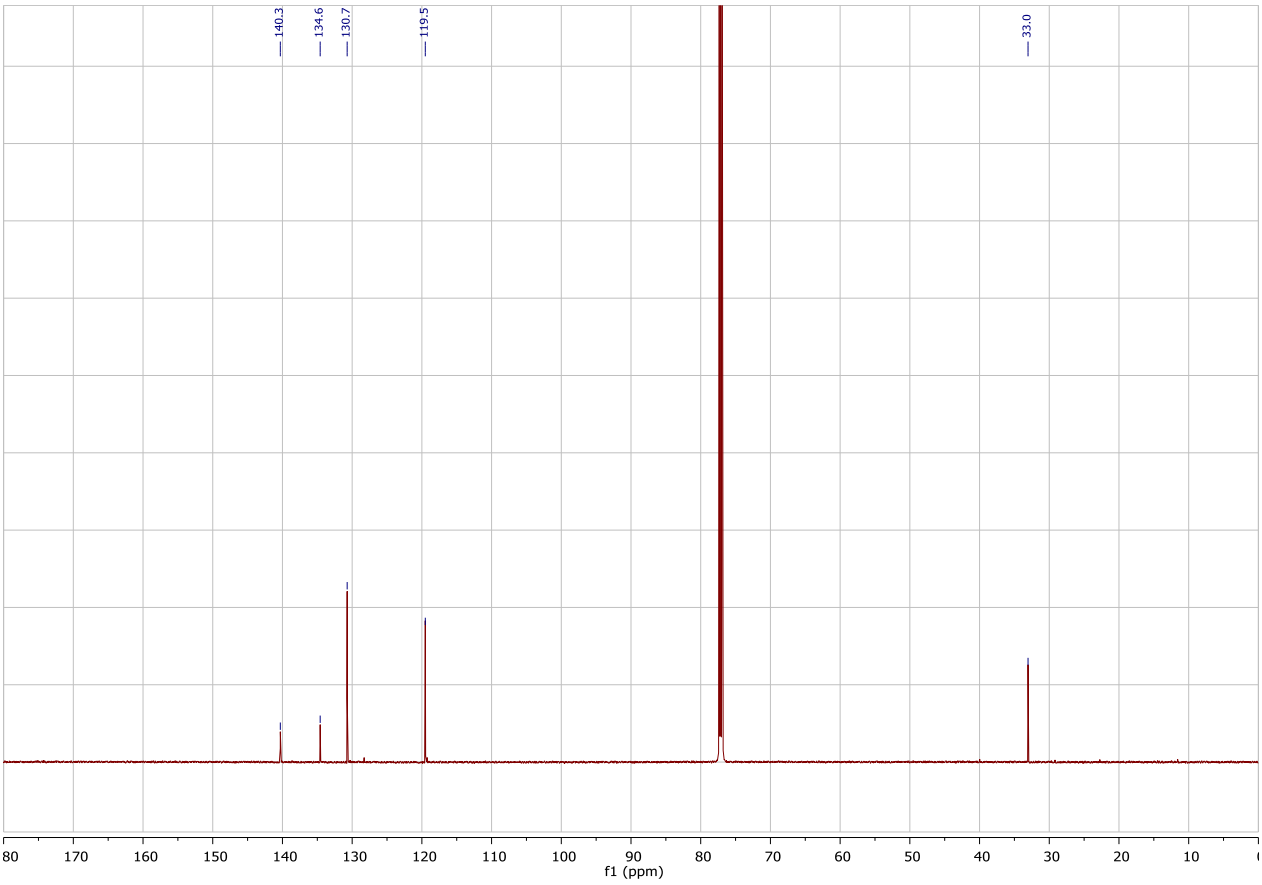
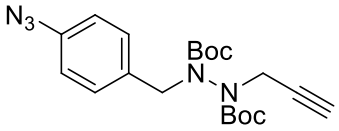
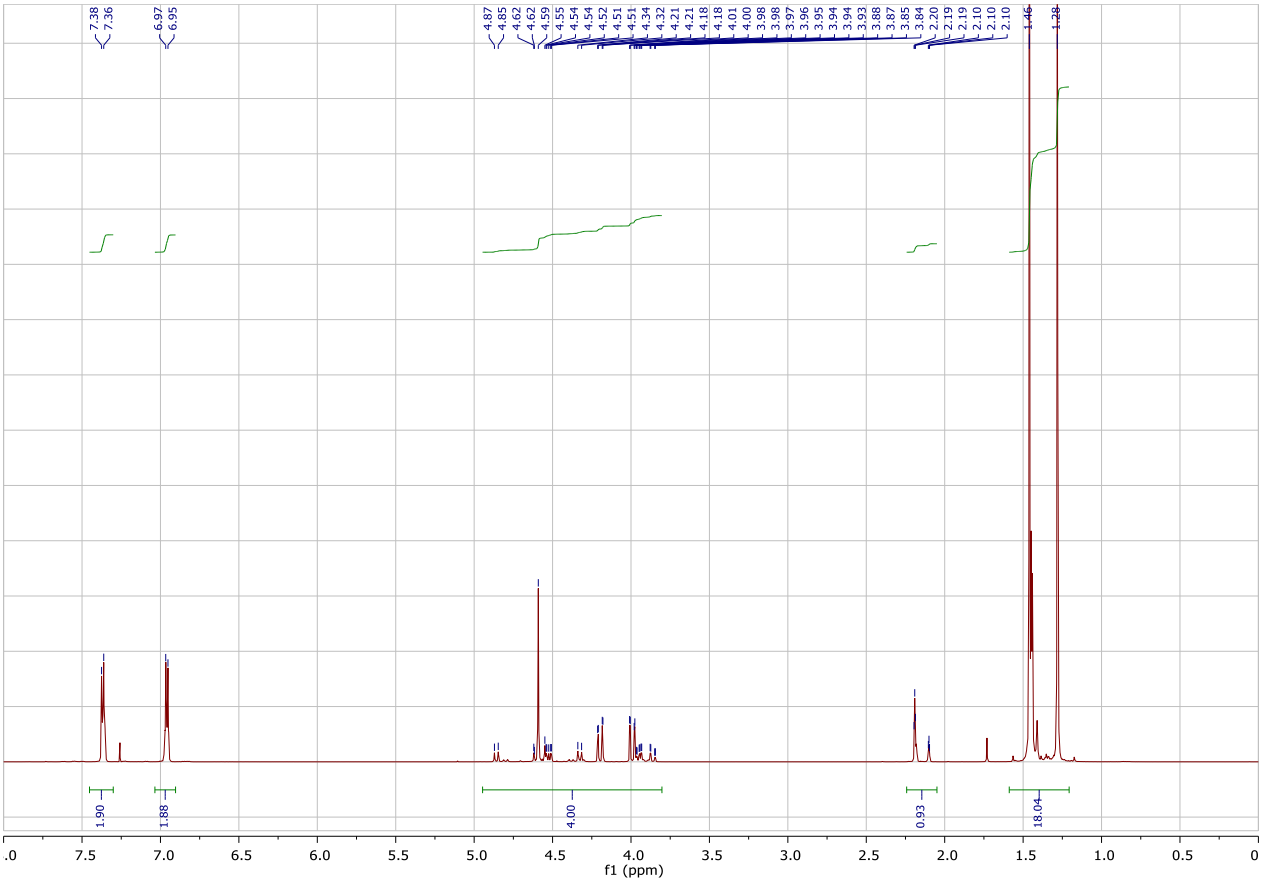
4.48
1.94
2.02
2.00
f1 (ppm)

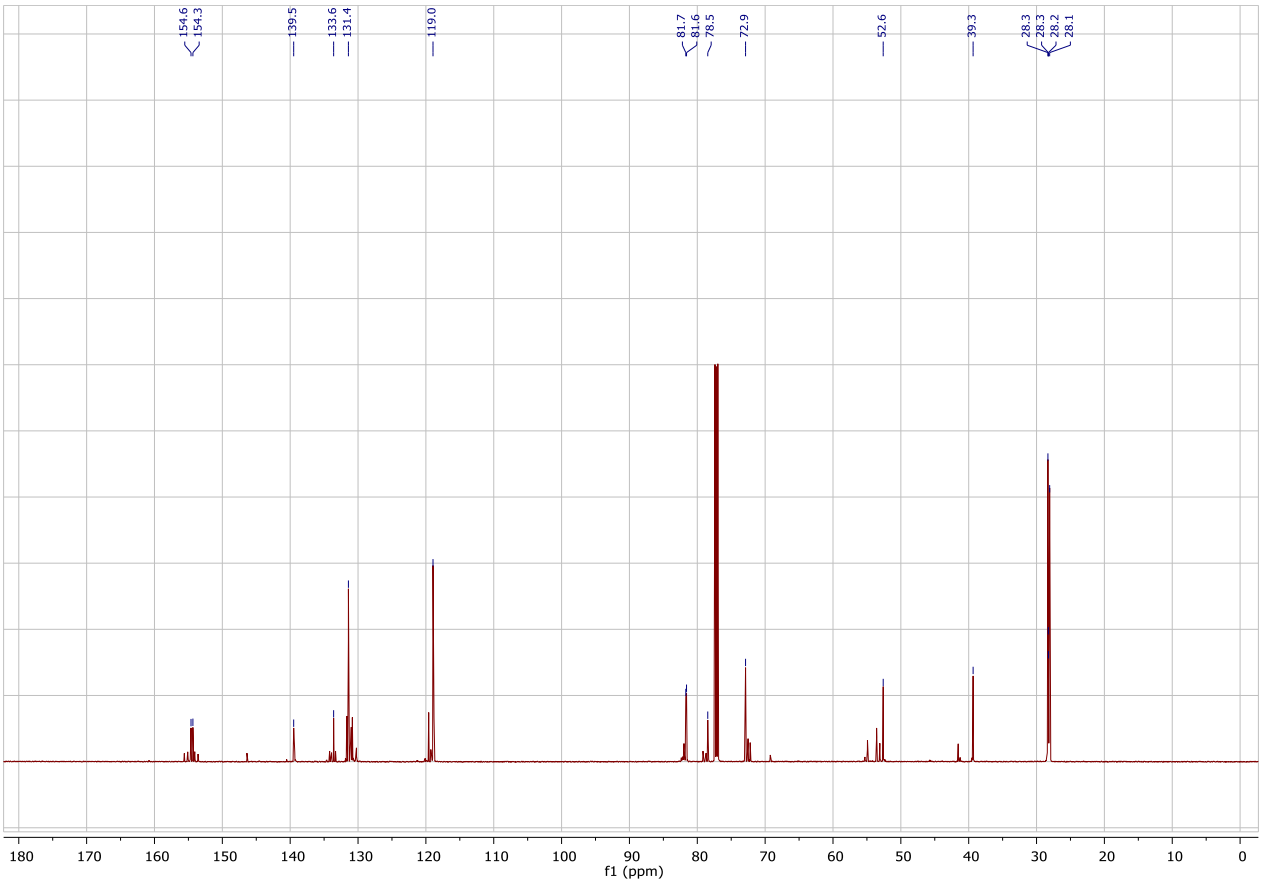

140.3
134.6
130.7
119.5
33.0
f1 (ppm)

Di-*tert*-butyl 1-(4-azidobenzyl)-2-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate

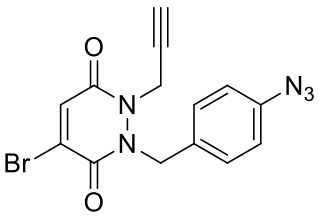


To a solution of di-*tert*-butyl 1-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate (200 mg, 0.70 mmol) in DMF (10 mL) was added caesium carbonate (480 mg, 1.50 mmol) and 1-azido-4-(bromomethyl)benzene (230 mg, 1.10 mmol). The reaction mixture was stirred at 21 °C for 16 h. After this time, the reaction mixture was diluted with H_2O (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (15 mL), dried ($MgSO_4$), and concentrated *in vacuo*. Purification by flash column chromatography (20% Et_2O/pet.) yielded di-*tert*-butyl 1-(4-azidobenzyl)-2-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate (261 mg, 0.65 mmol, 93%) as a viscous dark yellow liquid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.4 Hz, 2H), 6.97 (d, *J* = 8.4 Hz, 2H), 4.63–3.98 (m, 4H), 2.19 (t, *J* = 2.4 Hz, 1H), 1.47 (s, 9H), 1.30 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) (major rotamer) δ 154.6 (C), 154.3 (C), 139.5 (C), 133.6 (C), 131.4 (CH), 118.9 (CH), 81.7 (C), 81.6 (C), 78.5 (C), 72.9 (CH), 52.6 (CH_2), 39.3 (CH_2), 28.3 (CH_3), 28.1 (CH_3); IR (thin film) 3257, 2110, 1705, 1600, 1507 cm^{-1}; LRMS (CI) 424 (100, $[M+Na]^+$), 365 (70); HRMS (CI) calcd for $C_{20}H_{27}N_5O_4Na$ $[M+Na]^+$ 424.1961, observed 424.1965.

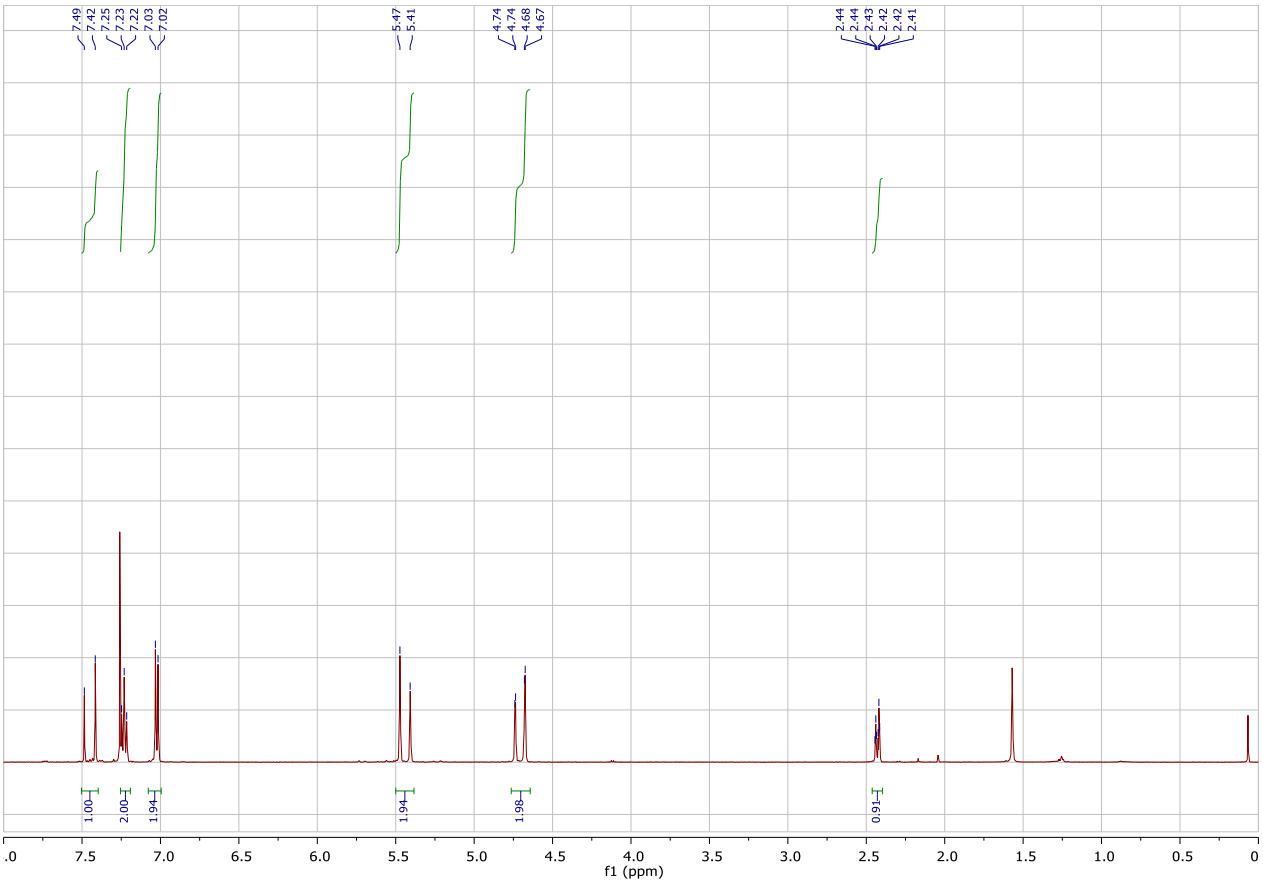




2-(4-Azidobenzyl)-4-bromo-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione 5



To a solution of di-*tert*-butyl 1-(4-azidobenzyl)-2-(prop-2-yn-1-yl)hydrazine-1,2-dicarboxylate (236 mg, 0.59 mmol) in CH_2Cl_2 (9 mL) was added TFA (3 mL) and the reaction mixture stirred at 21 °C for 2 h. After this time, all volatile material was removed *in vacuo* using toluene as an azeotrope. The crude residue was added to a solution of bromomaleic anhydride (125 mg, 0.71 mmol) in glacial AcOH (15 mL), and the reaction mixture heated at 130 °C for 16 h. Then the reaction mixture was concentrated *in vacuo*, and purification by flash column chromatography (20% EtOAc/pet.) yielded an inseparable mixture of regioisomers 2-(4-azidobenzyl)-4-bromo-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione and 1-(4-azidobenzyl)-4-bromo-2-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione (126 mg, 0.35 mmol, 60%) as an orange oil: ^{1}H NMR (600 MHz, $CDCl_3$) (major regioisomer) δ 7.42 (s, 1H), 7.23 (d, *J* = 8.3 Hz, 2H), 7.02 (d, *J* = 8.3 Hz, 2H), 5.47 (s, 2H), 4.68 (d, *J* = 2.5 Hz, 2H), 2.42 (t, *J* = 2.5 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) (major regioisomer) δ 155.8 (C), 154.5 (C), 140.7 (C), 135.9 (CH), 134.6 (C), 131.2 (C), 128.5 (CH), 120.0 (CH), 76.1 (C), 74.7 (CH), 49.9 (CH_2), 35.4 (CH_2); IR (solid) 3299, 3140, 3066, 2970, 2249, 2110, 1637, 1596 cm^{-1}; LRMS (ESI) 384 (100, $[M^{81}Br+Na]^+$), 382 (100, $[M^{79}Br+Na]^+$); HRMS (ESI) calcd for $C_{14}H_{10}N_5O_5{}^{81}BrNa$ $[M^{81}Br+Na]^+$ 383.9897, observed 383.9721.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4.67
2.44
2.44
2.43
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2.42
2.41
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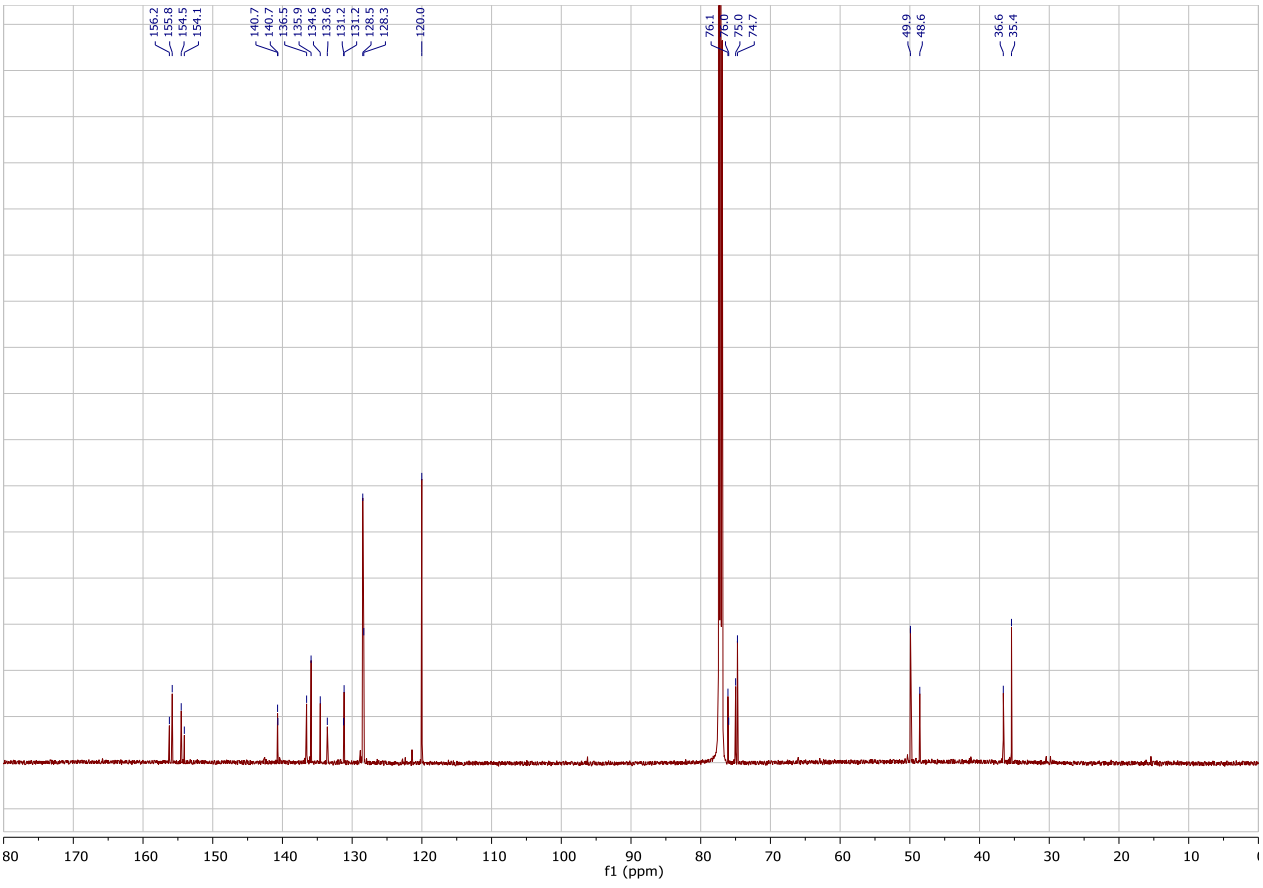
2.00
1.94
1.94
1.98
0.91
f1 (ppm)


156.2
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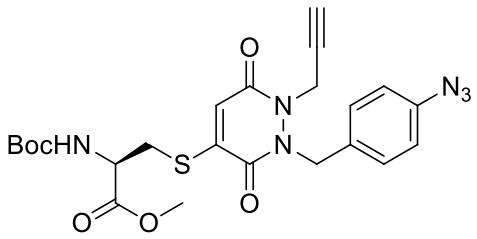
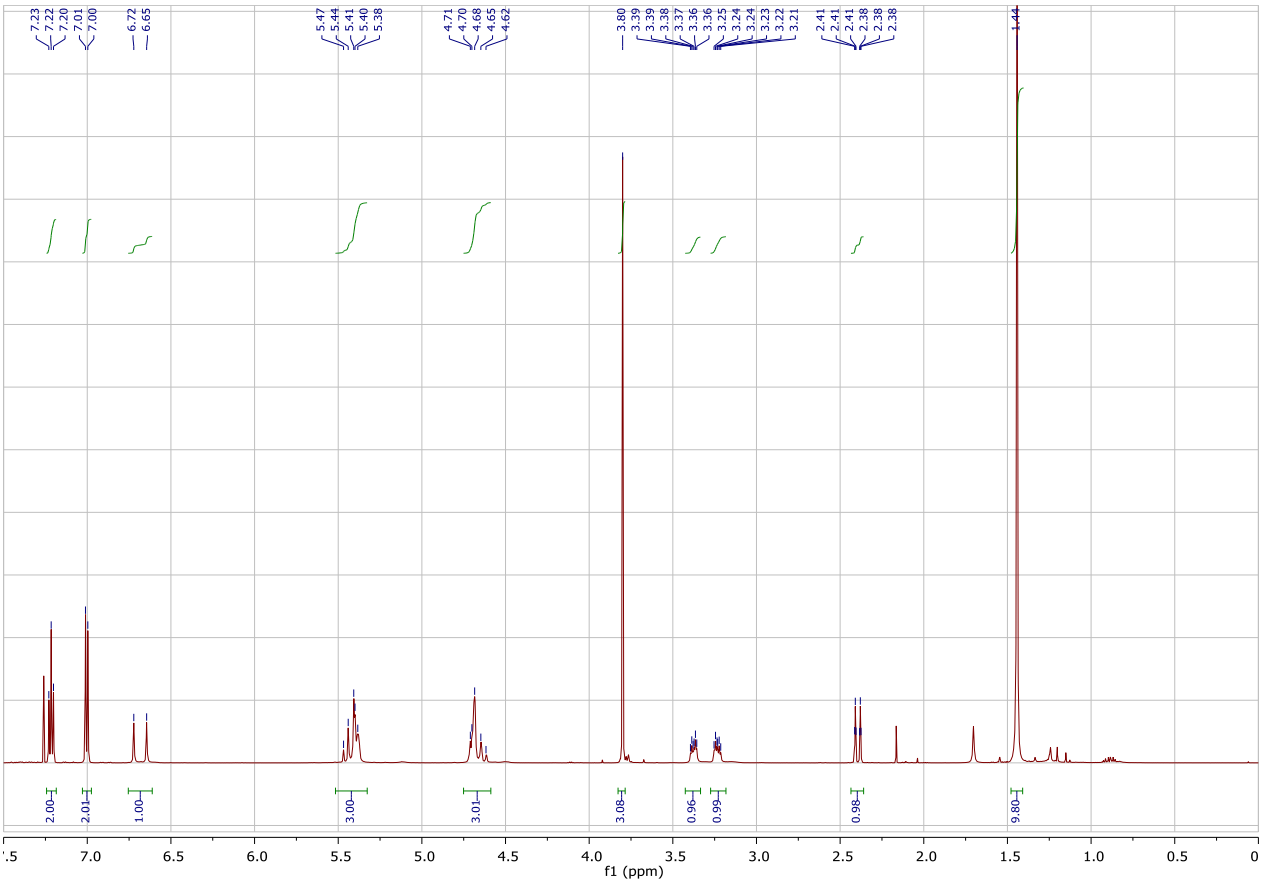
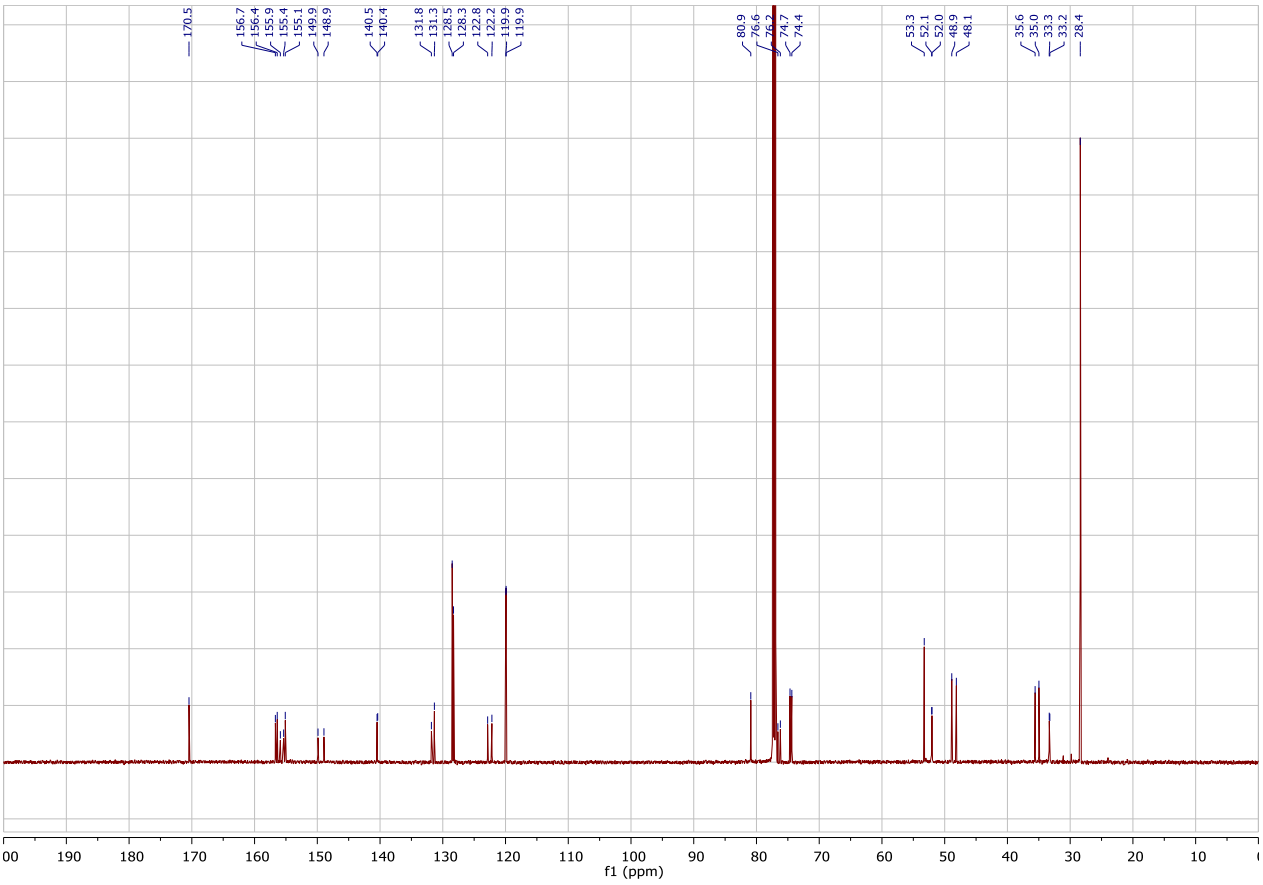
154.5
154.1
140.7
140.7
136.5
135.9
134.6
133.6
131.2
128.5
128.3
120.0
76.1
76.0
75.0
74.7
49.9
48.6
36.6
35.4
f1 (ppm)

(*R*)-Methyl 3-((2-(4-azidobenzyl)-3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate 6

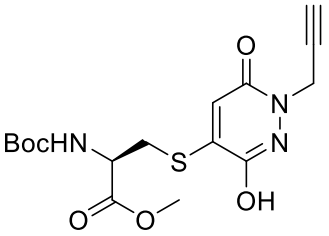


To a mixture of regioisomers 2-(4-azidobenzyl)-4-bromo-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione and 1-(4-azidobenzyl)-4-bromo-2-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione **5** (65 mg, 0.19 mmol) in CH_2Cl_2 (5 mL) was added NEt_3 (0.22 mg, 0.22 mmol) and *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (53 mg, 0.22 mmol) and the mixture was stirred at 21 °C for 2 h. The reaction mixture was then concentrated *in vacuo* and purification by flash column chromatography (20% EtOAc/pet.) yielded an inseparable mixture of regioisomers (*R*)-methyl 3-((2-(4-azidobenzyl)-3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate and (*R*)-methyl 3-((1-(4-azidobenzyl)-3,6-dioxo-2-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate (86 mg, 0.17 mmol, 89%) as a yellow viscous oil: ^{1}H NMR (600 MHz, $CDCl_3$) (major regioisomer) δ 7.22 (t, *J* = 8.4 Hz, 2H), 7.00 (d, *J* = 8.4 Hz, 2H), 6.65 (s, 1H), 5.52–5.31 (m, 3H), 4.75–4.58 (m, 3H), 3.80 (s, 3H), 3.36 (t, *J* = 5.0 Hz, 1H), 3.24 (t, *J* = 5.0 Hz, 1H), 2.38 (t, *J* = 2.5 Hz, 1H), 1.44 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) (major regioisomer) δ 170.5 (C), 156.4 (C), 155.1 (C), 149.9 (C), 140.5 (C), 131.3 (C), 128.5 (CH), 122.8 (CH), 119.9 (CH), 80.9 (C), 76.2 (C), 74.4 (CH), 53.3 (CH_3), 52.0 (CH), 48.1 (CH_2), 35.6 (CH_2), 33.3 (CH_2), 28.4 (CH_3); IR (film) 3289, 2974, 2241, 2109, 1744, 1710, 1628 cm^{-1}; LRMS (ESI) 537 (100, $[M+Na]^+$); HRMS (ESI) calcd for $C_{23}H_{26}N_6O_6SNa$ $[M+Na]^+$ 537.1532, observed 537.1533.

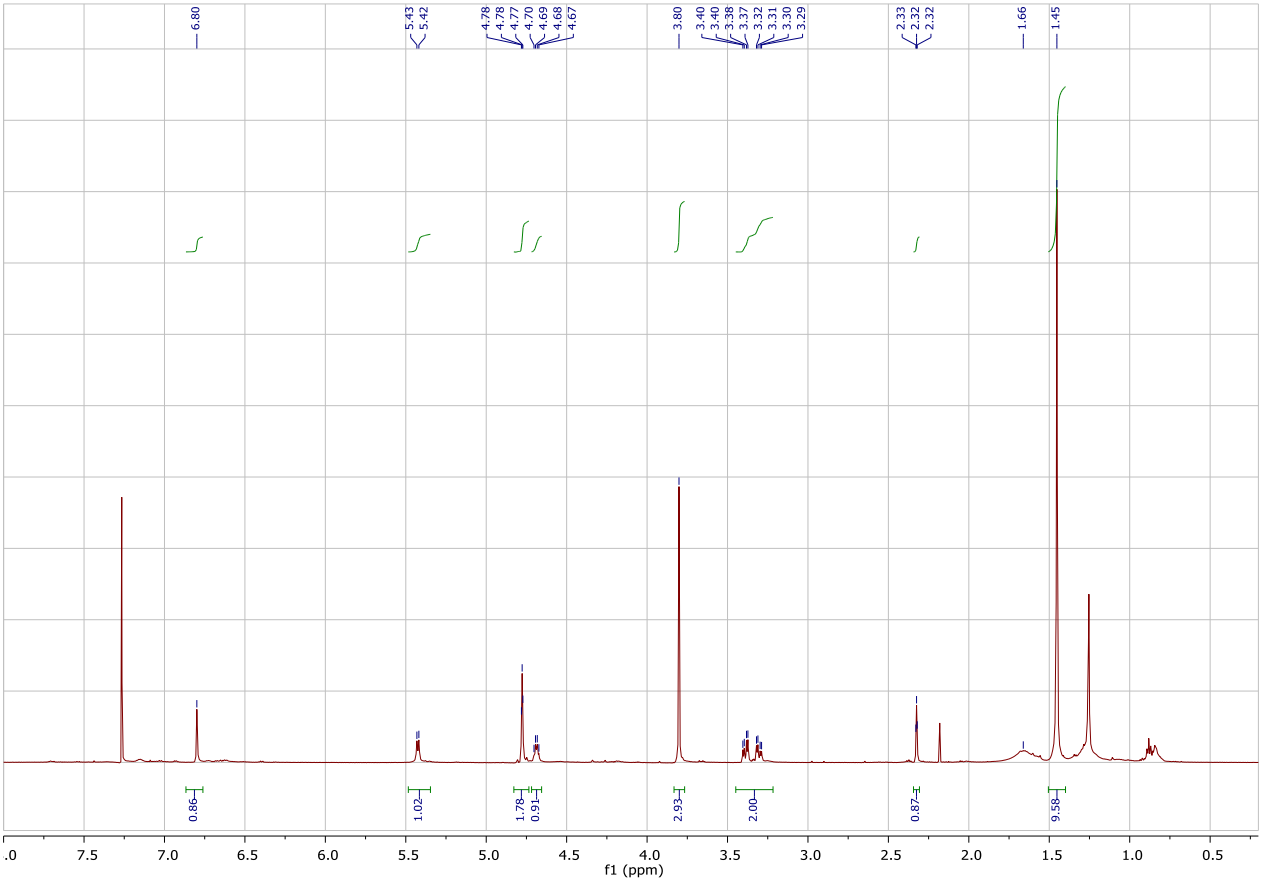

7.23
7.22
7.20
7.01
7.00
6.72
6.65
5.47
5.44
5.41
5.40
5.38
4.71
4.70
4.68
4.65
4.62
3.80
3.39
3.39
3.38
3.37
3.36
3.36
3.25
3.24
3.24
3.23
3.22
3.21
2.41
2.41
2.41
2.38
2.38
2.38
1.44
2.00
2.01
1.00
3.00
3.01
3.08
0.96
0.99
0.98
9.80
f1 (ppm)

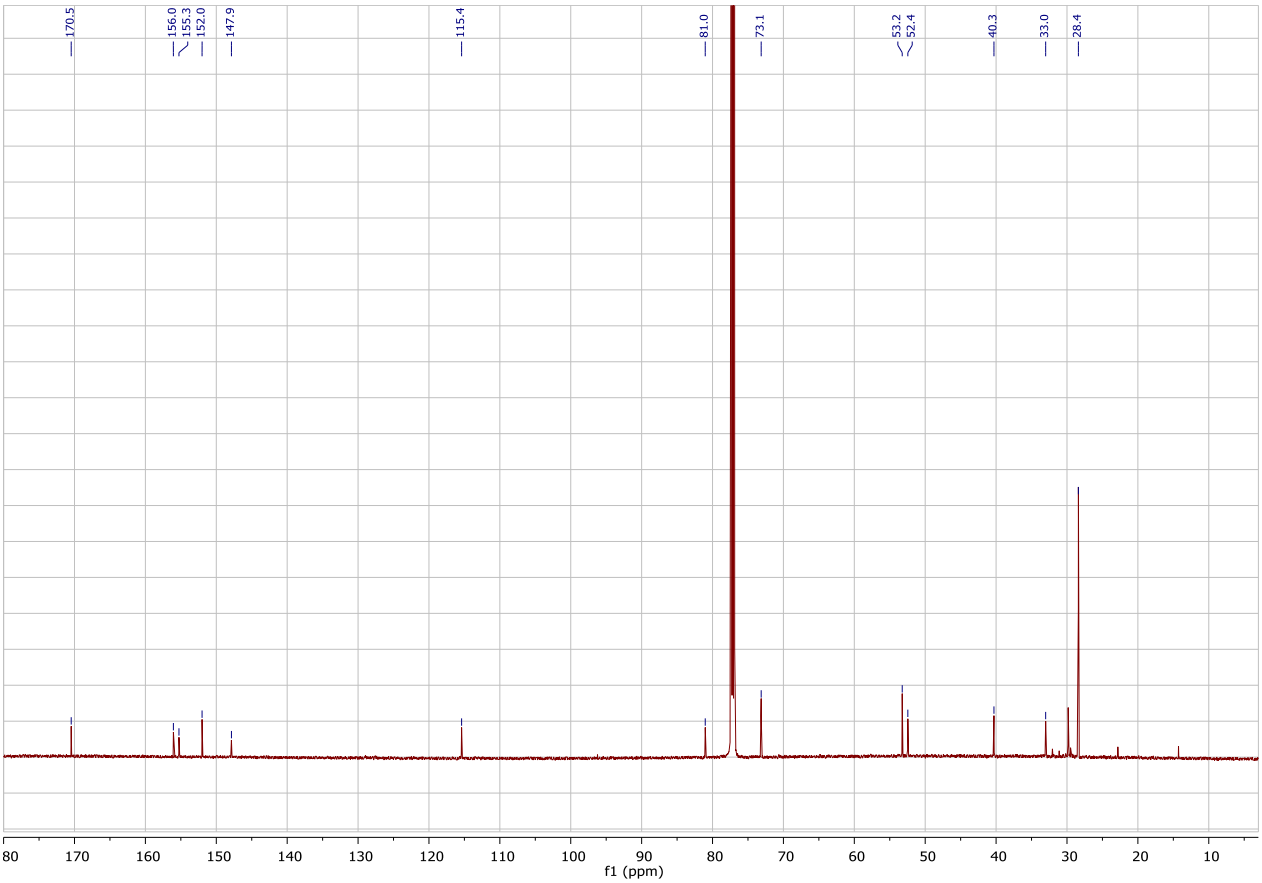

170.5
156.7
156.4
155.9
155.4
155.1
149.9
148.9
140.5
140.4
131.8
131.3
128.5
128.3
122.8
122.2
119.9
119.9
80.9
76.6
76.2
74.7
74.4
53.3
52.1
52.0
48.9
48.1
35.6
35.0
33.3
33.2
28.4
f1 (ppm)

(*R*)-Methyl 2-((*tert*-butoxycarbonyl)amino)-3-((3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)propanoate 7

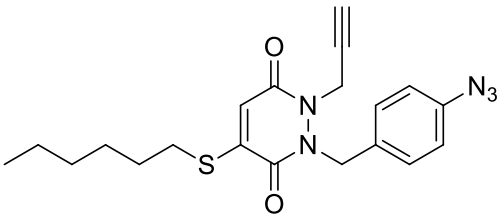


To a solution of (*R*)-methyl 3-((2-(4-azidobenzyl)-3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate **6** (20 mg, 44 µmol) in DMF (2 mL) was added tris(2-carboxyethyl)phosphine hydrochloride (13 mg, 53 µmol) and the mixture was stirred at 21 °C for 2 h. The reaction mixture was then concentrated *in vacuo* and purification by flash column chromatography (5% MeOH/CH_2Cl_2) yielded (*R*)-methyl 3-((2-(4-azidobenzyl)-3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate (14 mg, 36 µmol, 82%) as an yellow oil: ^{1}H NMR (600 MHz, $CDCl_3$) (major regioisomer) δ 6.80 (s, 1H), 5.42 (d, *J* = 7.4 Hz, 1H), 4.81–4.74 (m, 2H), 4.71–4.66 (m, 1H), 3.80 (s, 3H), 3.42–3.28 (m, 2H), 2.32 (t, *J* = 2.5 Hz, 1H), 1.45 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.5 (C), 156.0 (C), 155.3 (C), 152.0 (C), 147.9 (C), 115.4 (CH), 81.0 (C), 73.1 (CH), 53.2 (CH_3), 52.4 (CH), 40.3 (CH_2), 33.0 (CH_2), 28.4 (CH_3); IR (film) 3295, 2960, 2922, 2851, 2109, 1743, 1708, 1637 cm^{-1}; LRMS (ESI) 406 (100, $[M+Na]^+$); HRMS (ESI) calcd for $C_{16}H_{18}N_3O_6SNa$ $[M+Na]^+$ 406.1049, observed 406.1049.

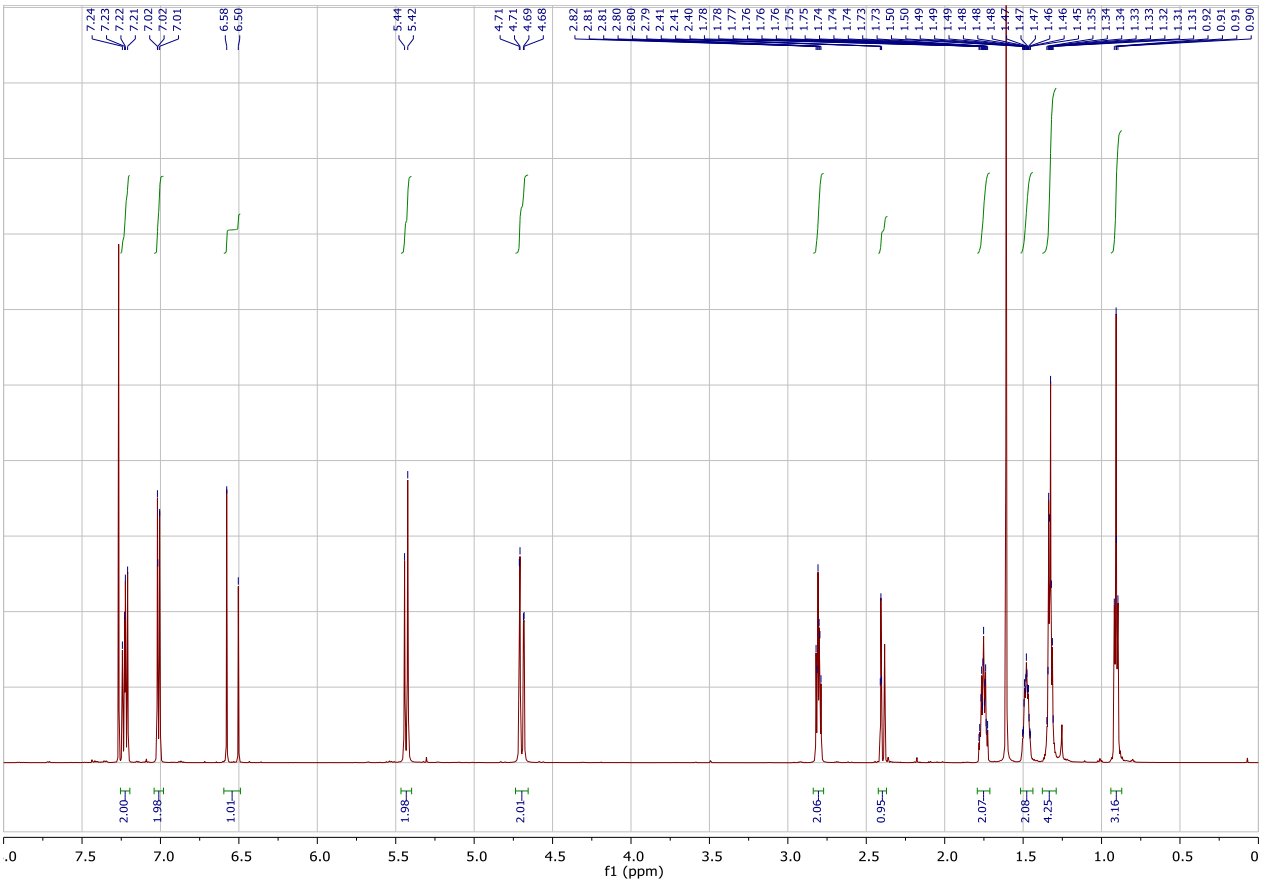


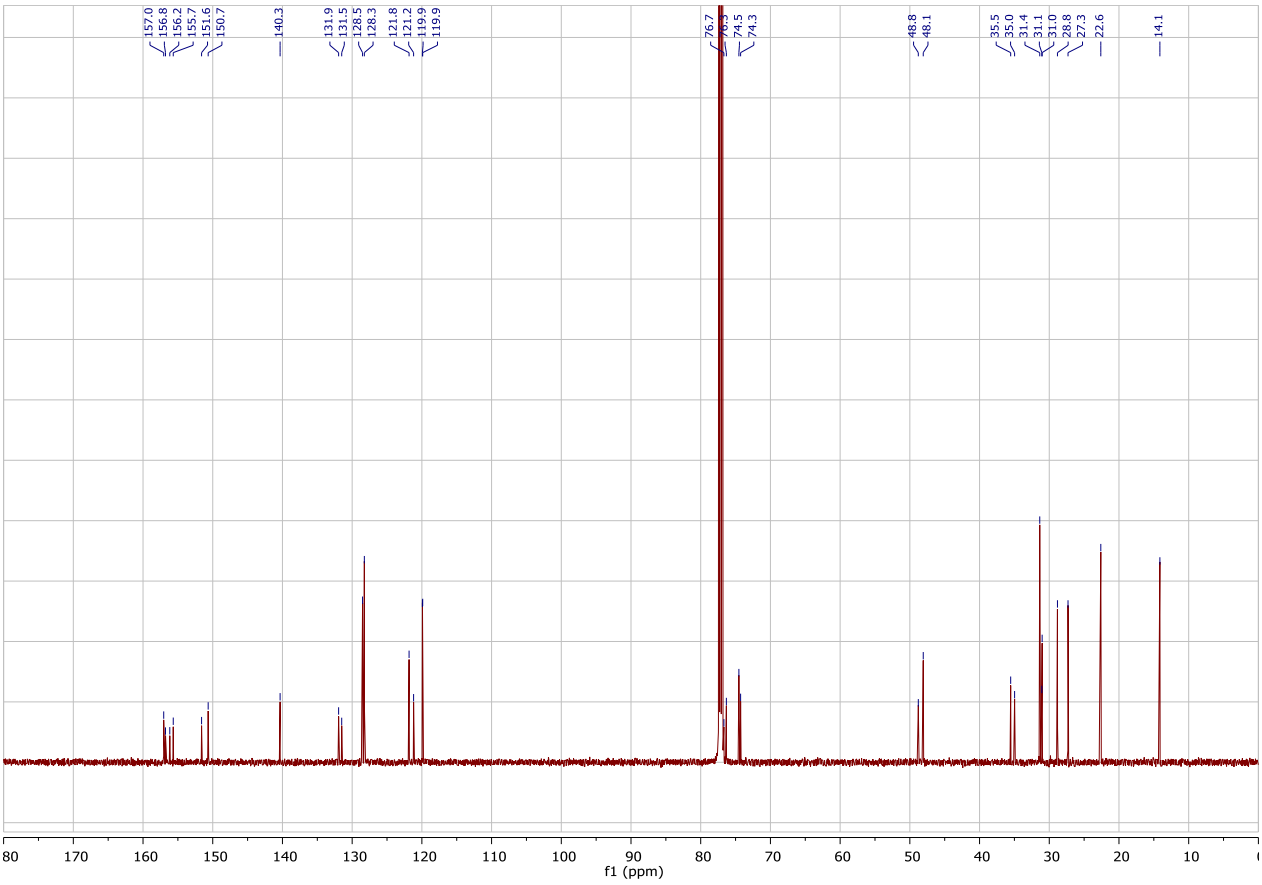


2-(4-Azidobenzyl)-4-(hexylthio)-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione



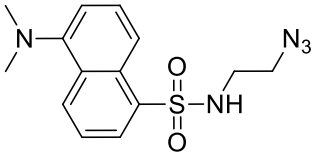
To a an mixture of regioisomers (*R*)-methyl 3-((2-(4-azidobenzyl)-3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((tert-butoxycarbonyl)amino)propanoate and (*R*)-methyl 3-((1-(4-azidobenzyl)-3,6-dioxo-2-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)-2-((*tert*-butoxycarbonyl)amino)propanoate **6** (86 mg, 0.17 mmol) in THF/PBS at pH 7.4 (1:1, 2 mL) was added hexane thiol (301 mg, 2.55 mmol) and the mixture was stirred at 21 °C for 72 h. The reaction mixture was then concentrated *in vacuo* and purification by flash column chromatography (20% EtOAc/pet.) yielded an inseparable mixture of regioisomers 2-(4-azidobenzyl)-4-(hexylthio)-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione and 1-(4-azidobenzyl)-4-(hexylthio)-2-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione (47 mg, 0.12 mmol, 71%) as a colourless oil: ^{1}H NMR (600 MHz, $CDCl_3$) (major regioisomer) δ 7.26–7.19 (m, 2H), 7.04–6.98 (m, 2H), 6.58 (s, 1H), 5.42 (s, 2H), 4.71 (d, *J* = 2.5 Hz, 2H), 2.80 (t, *J* = 7.4 Hz, 2H), 2.41 (t, *J* = 2.5 Hz, 1H), 1.79–1.70 (m, 2H), 1.52–1.44 (m, 2H), 1.37–1.30 (m, 4H), 0.94–0.88 (m, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) (major regioisomer) δ 157.0 (C), 155.7 (C), 150.7 (C), 140.3 (C), 131.9 (C), 128.3 (CH), 121.8 (CH), 119.9 (CH), 76.3 (C), 74.5 (CH), 48.1 (CH_2), 35.5 (CH_2), 31.4 (CH_2), 31.0 (CH_2), 28.8 (CH_2), 27.3 (CH_2), 22.6 (CH_2), 14.1 (CH_3); IR (film) 3245, 2955, 2928, 2856, 2243, 2108, 1680, 1631 cm^{-1}; LRMS (EI) 398 (100, $[M+H]^+$); HRMS (ESI) calcd for $C_{20}H_{24}N_5O_2S$ $[M+H]^+$ 398.1651, observed 398.1654.


7.24
7.23
7.22
7.21
7.02
7.02
7.02
7.01
6.58
6.50
5.44
5.42
4.71
4.71
4.69
4.68
2.82
2.81
2.81
2.80
2.80
2.80
2.79
2.41
2.41
2.40
1.78
1.78
1.77
1.76
1.76
1.76
1.75
1.75
1.74
1.74
1.74
1.73
1.73
1.50
1.50
1.49
1.49
1.49
1.48
1.48
1.48
1.47
1.47
1.46
1.46
1.45
1.35
1.34
1.34
1.33
1.33
1.32
1.31
1.31
0.92
0.91
0.91
0.90
2.00
1.98
1.01
1.98
2.01
2.06
0.95
2.07
2.08
4.25
3.16
f1 (ppm)

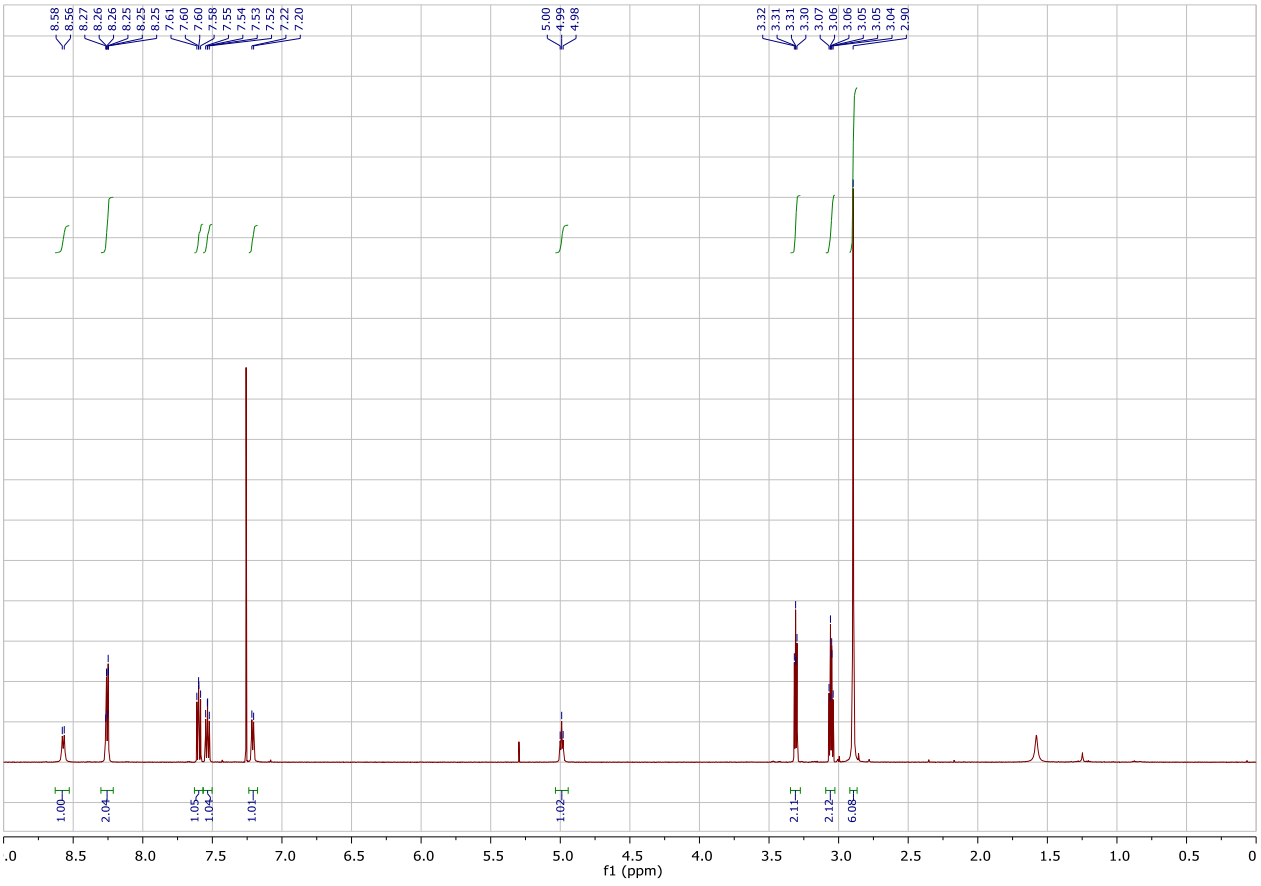

157.0
156.8
156.2
155.7
151.6
150.7
140.3
131.9
131.5
128.5
128.3
121.8
121.2
119.9
119.9
76.7
76.3
74.5
74.3
48.8
48.1
35.5
35.0
31.4
31.1
31.0
28.8
27.3
22.6
14.1
f1 (ppm)

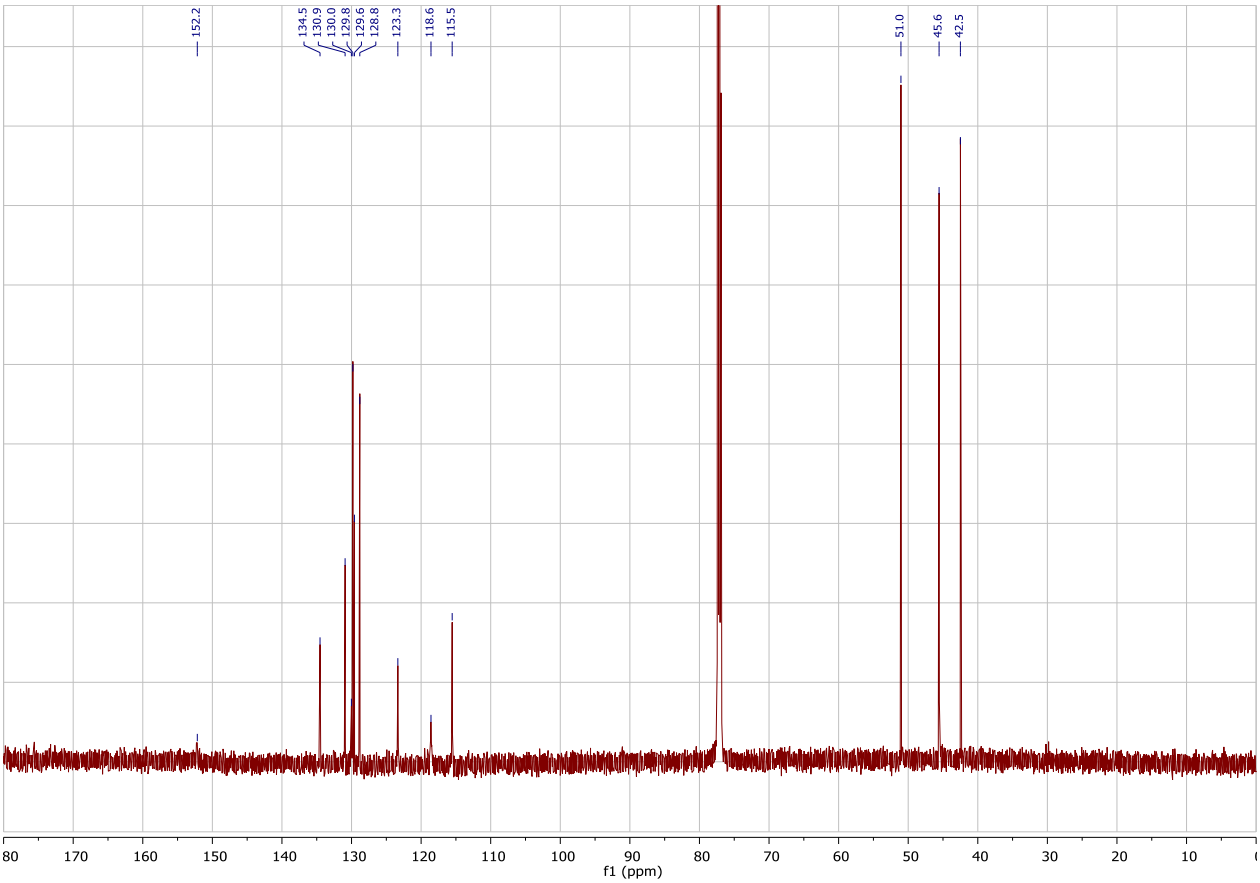
Analogous reaction conditions were applied to (*R*)-Methyl 2-((tert-butoxycarbonyl)amino)-3-((3,6-dioxo-1-(prop-2-yn-1-yl)-1,2,3,6-tetrahydropyridazin-4-yl)thio)propanoate **7** but no reaction was observed by TLC and there was 95% recovery of starting material by flash column chromatography (20% EtOAc/pet.).

_N_-(2-Azidoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide[5]



To a solution of dansyl chloride (0.27 g, 1.0 mmol) in CH_2Cl_2 (10 mL) was added 2-bromoethylamine hydrobromide (0.21 g, 1.0 mmol) and NEt_3 (0.28 mL, 2.0 mmol). The reaction mixture was stirred at 21 °C for 4 h. After this time, all volatile material was removed *in vacuo*. The crude residue was then added to a solution of NaN_3 (0.16 g, 2.5 mmol) in MeCN (10 mL), and the reaction mixture heated at 90 °C for 16 h. Then the reaction mixture was concentrated *in vacuo*, and purification by flash column chromatography (50% Et_2O/pet.) yielded *N*-(2-azidoethyl)-5-(dimethylamino)naphthalene-1-sulfonamide as a light green oil (0.19 g, 59%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.57 (d, *J* = 8.5 Hz, 1H), 8.28–8.23 (m, 2H), 7.60 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.53 (dd, *J* = 8.5, 7.3 Hz, 1H), 7.21 (d, *J* = 7.3 Hz, 1H), 4.99 (t, *J* = 6.4 Hz, 1H), 3.34–3.28 (m, 2H), 3.09–3.02 (m, 2H), 2.90 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 152.2 (C), 134.5 (C), 130.9 (CH), 130.0 (C), 129.8 (CH), 129.6 (C), 128.8 (CH), 123.3 (CH), 118.6 (CH), 115.5 (CH), 51.0 (CH_2), 45.6 (CH_3), 42.5 (CH_2); HRMS (ESI) calcd for $C_{14}H_{18}N_5O_2S$ $[M+H]^+$ 320.1181, observed 320.1184.



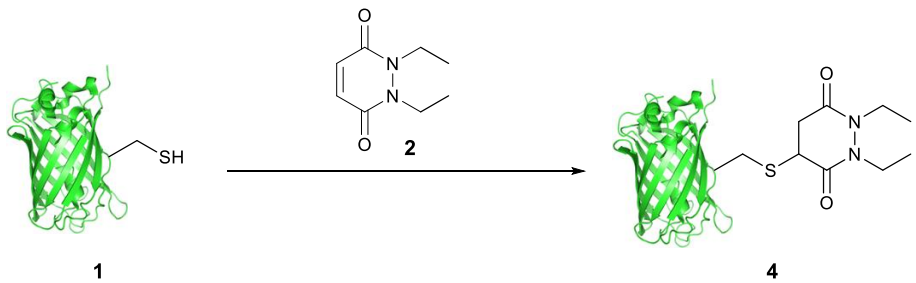


Bioconjugation reactions involving GFPS147C

Pre-treatment procedure for reaction with GFPS147C 1

Immediately prior to bioconjugation with GFPS147C **1**, TCEP (10 µL, 10 mM as a solution in water, per 100 µL of protein (1 mg/mL solution)) was added, incubated on ice for 1 h and the excess reducing agent removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO).

Reaction of GFPS147C 1 with 1,2-diethyl-1,2-dihydropyridazine-3,6-dione 2

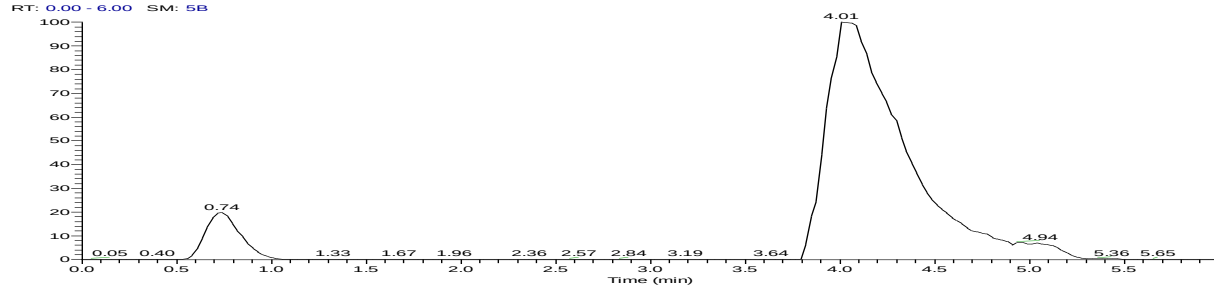


1 **4**

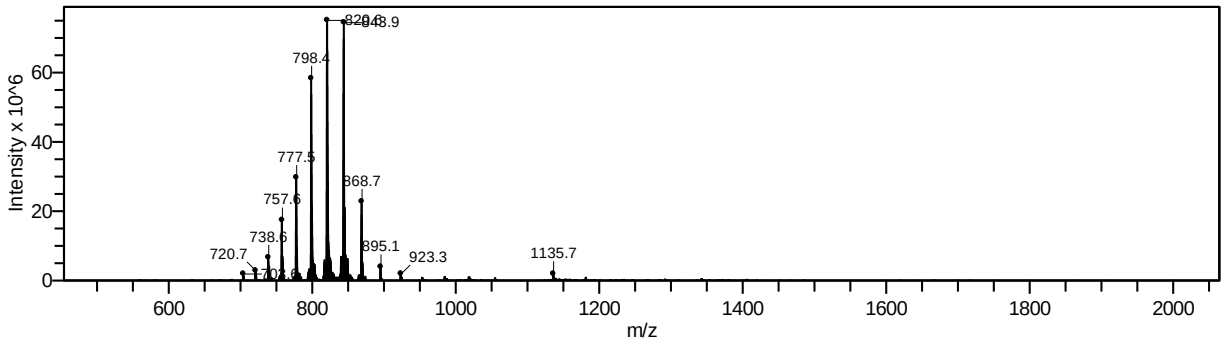
1,2-Diethyl-1,2-dihydropyridazine-3,6-dione **2** (5 µL, 340 µM in DMF, 10 eq) was added to GFPS147C **1** (100 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0). The reaction mixture was incubated at 37 °C for 1 h. Excess reagents were removed by repeated diafiltration into fresh buffer

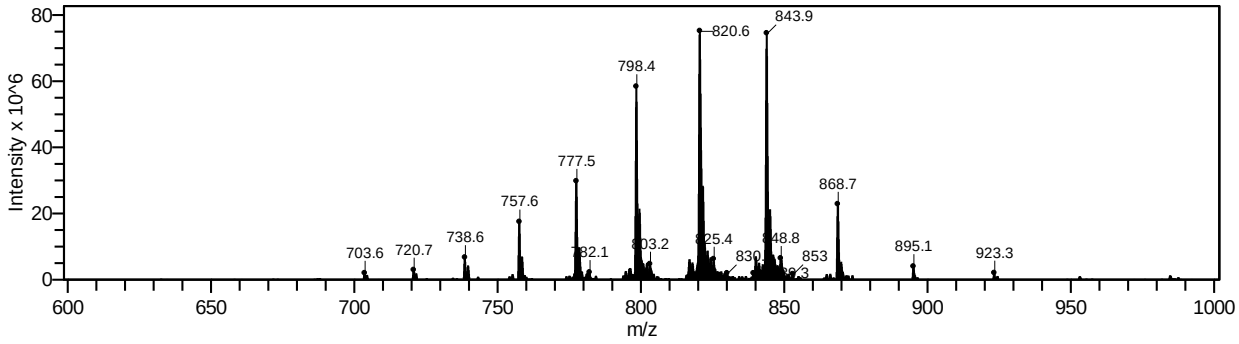
(100 mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 29,501 Da. Observed mass: 29,503 Da.

(a)



(b)





(c)

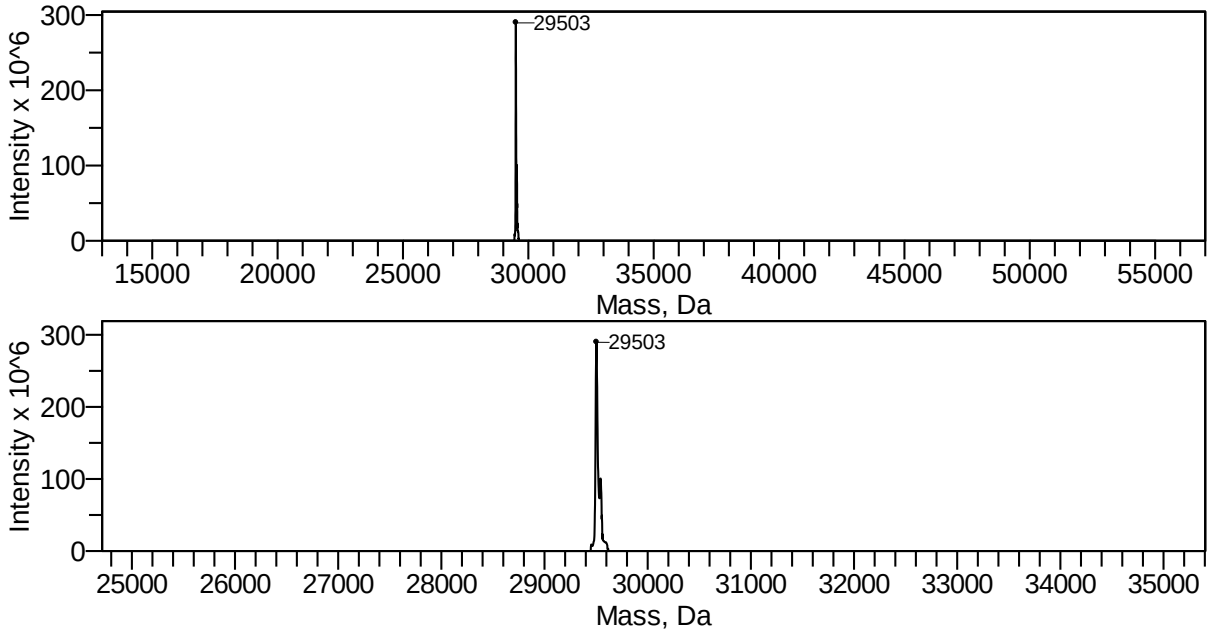
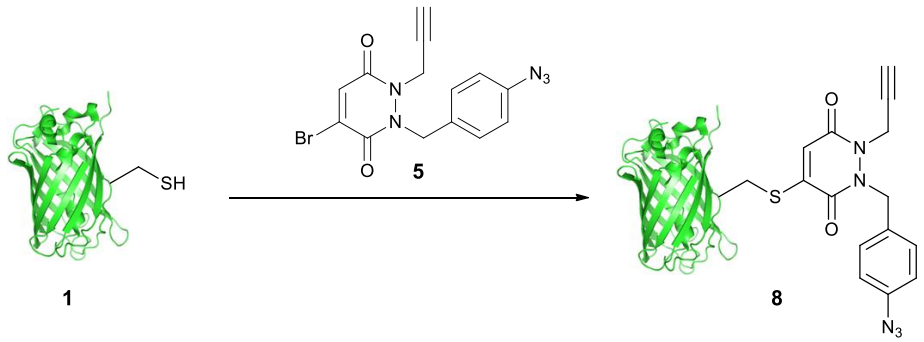


Figure S2 (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **4**.

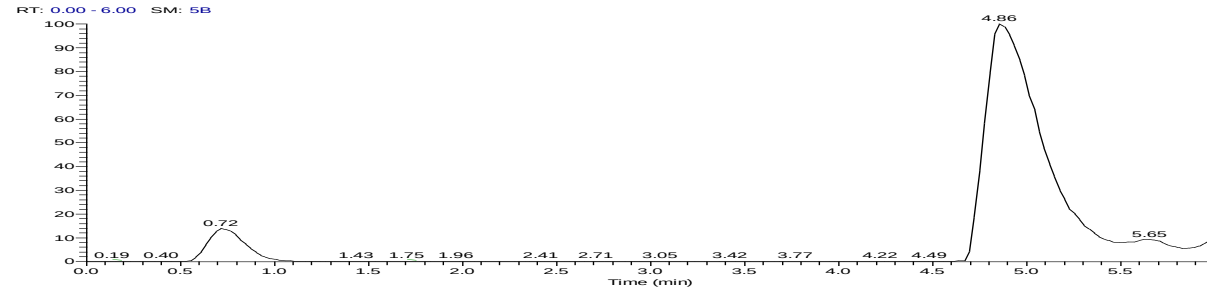
Analogous reaction conditions when applied to 1-methyl-1,2-dihydropyridazine-3,6-dione **3** in place of 1,2-diethyl-1,2-dihydropyridazine-3,6-dione **2** showed no reaction, even after 16 h and 72 h time-points.

Reaction of GFPS147C 1 with 2-(4-azidobenzyl)-4-bromo-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione 5

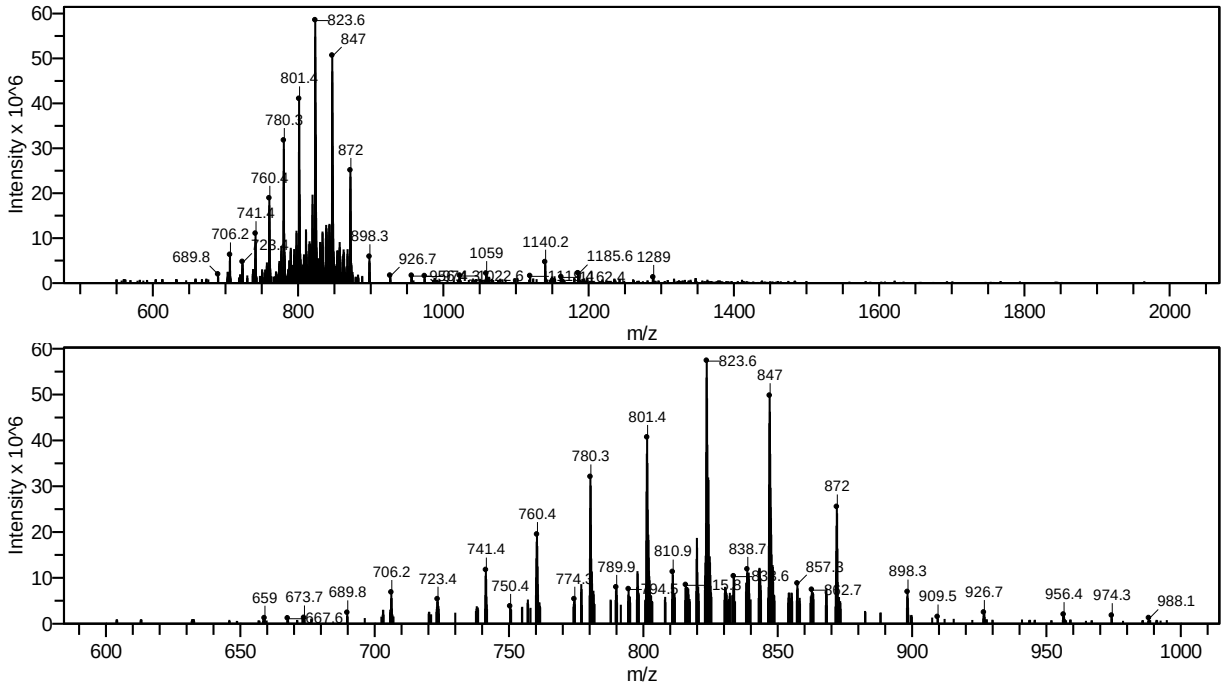


2-(4-Azidobenzyl)-4-bromo-1-(prop-2-yn-1-yl)-1,2-dihydropyridazine-3,6-dione **5** (5 µL, 340 µM in DMF, 10 eq) was added to GFPS147C **1** (100 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0). The reaction mixture was incubated at 37 °C for 1 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 29,612 Da. Observed mass: 29,613 Da.

(a)



(b)



(c)

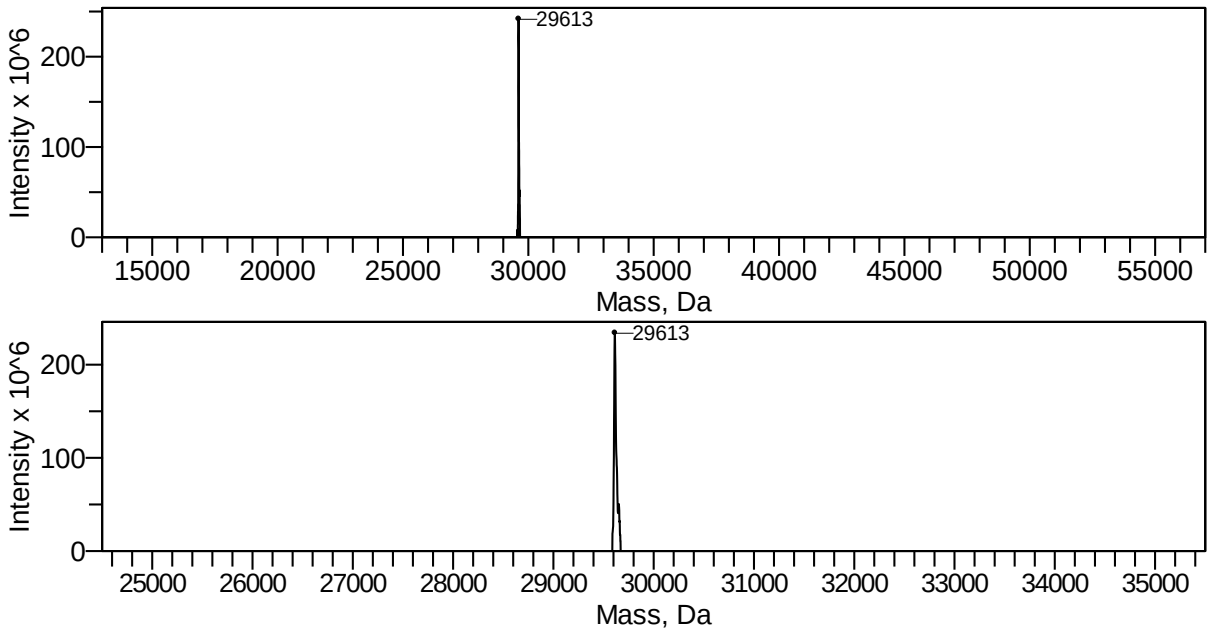
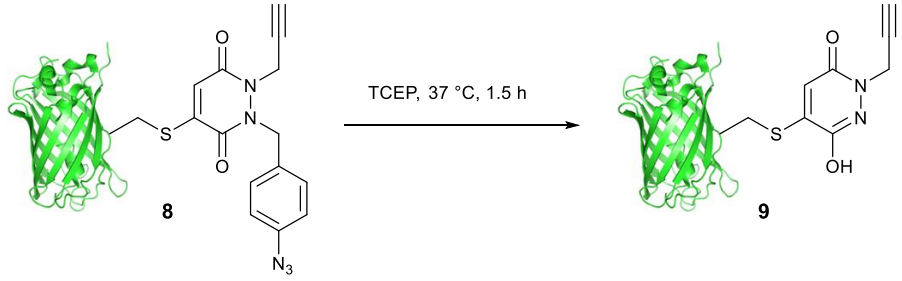


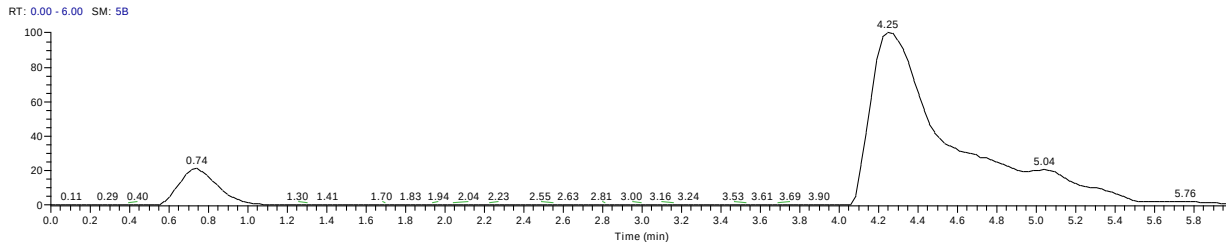
Figure S3. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **8**.

Reduction of GFPS147CPD 8 with TCEP

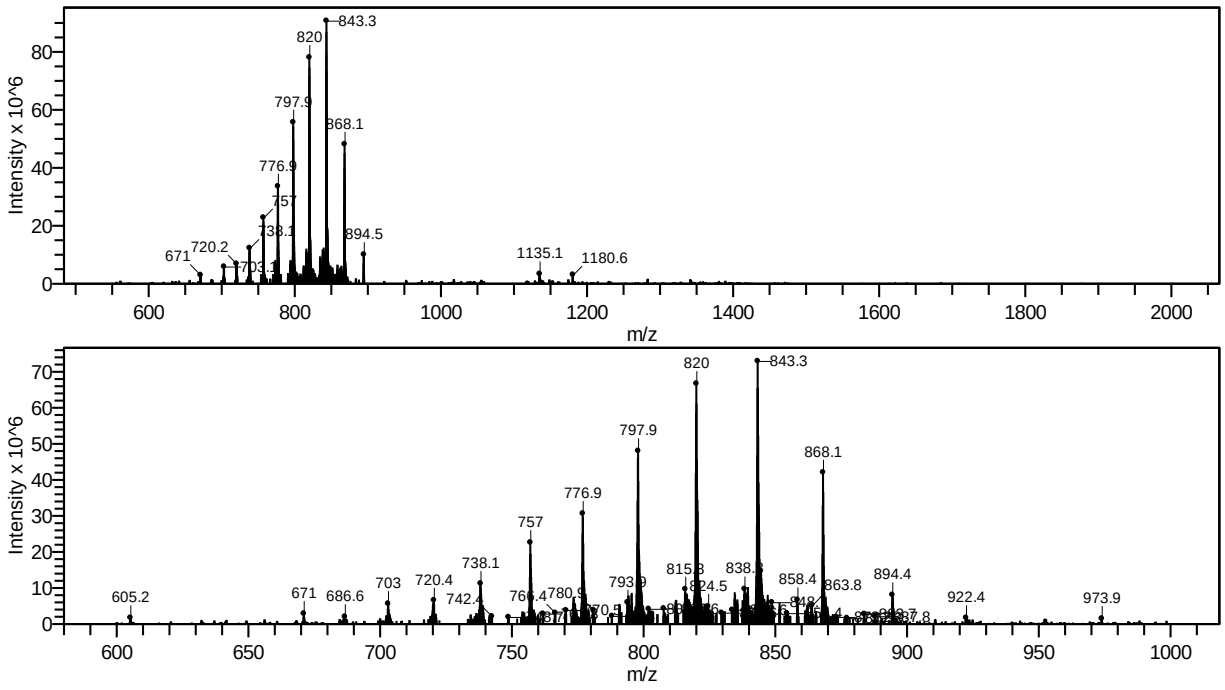


TCEP (10 µL, final concentration 340 µM, 10 eq) was added to the GFPS147CPD conjugate **8** (90 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0). The reaction was incubated at 37 °C for 1.5 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 29,482 Da. Observed mass: 29,484 Da.

(a)



(b)



(c)

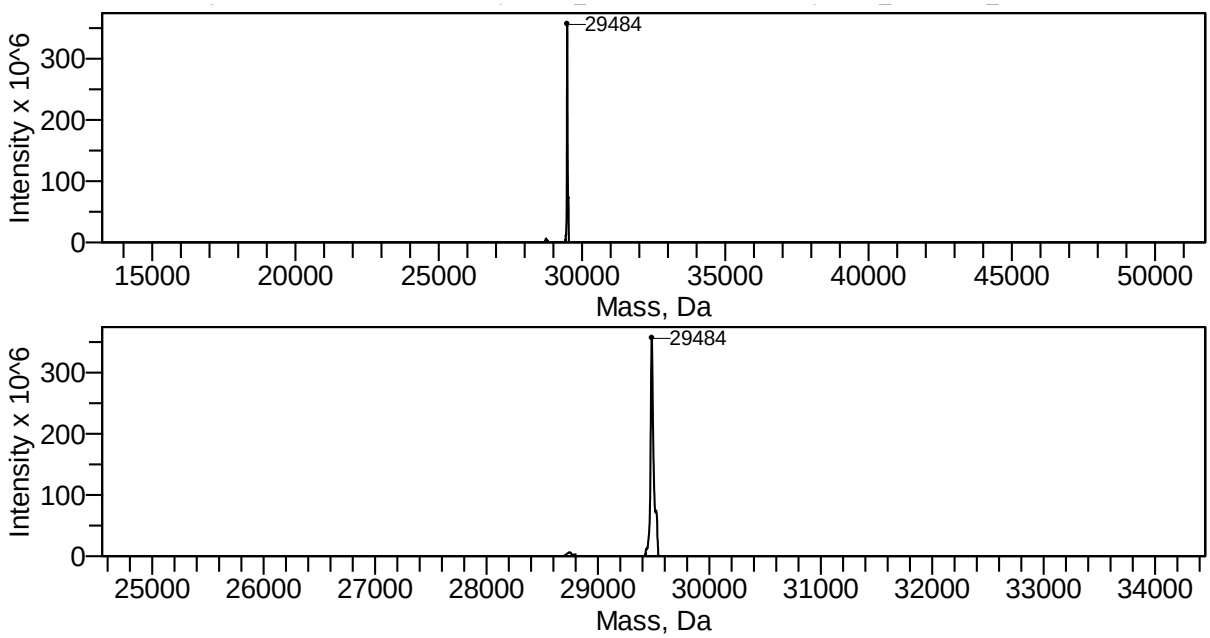
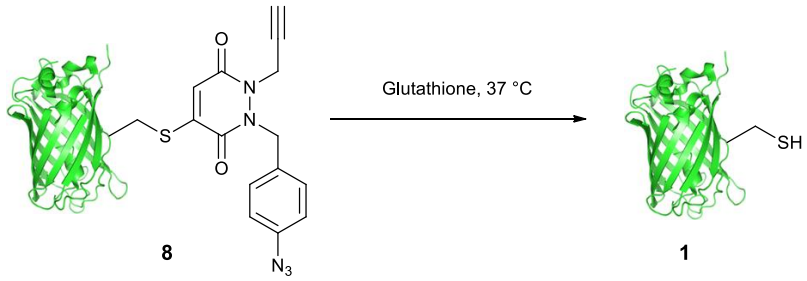


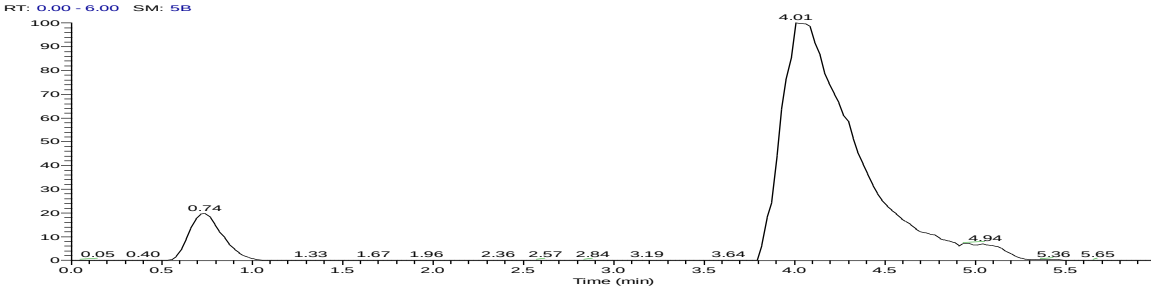
Figure S4. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **9**.

Reaction of GFPS147CPD 8 with glutathione



Glutathione (10 µL, 5 mM) was added to the GFPS147CPD conjugate **8** (90 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 7.4). The reaction was incubated at 37 °C for 72 h and then analysed by LCMS. Expected mass: 29,332 Da. Observed mass: 29,336 Da.

(a)



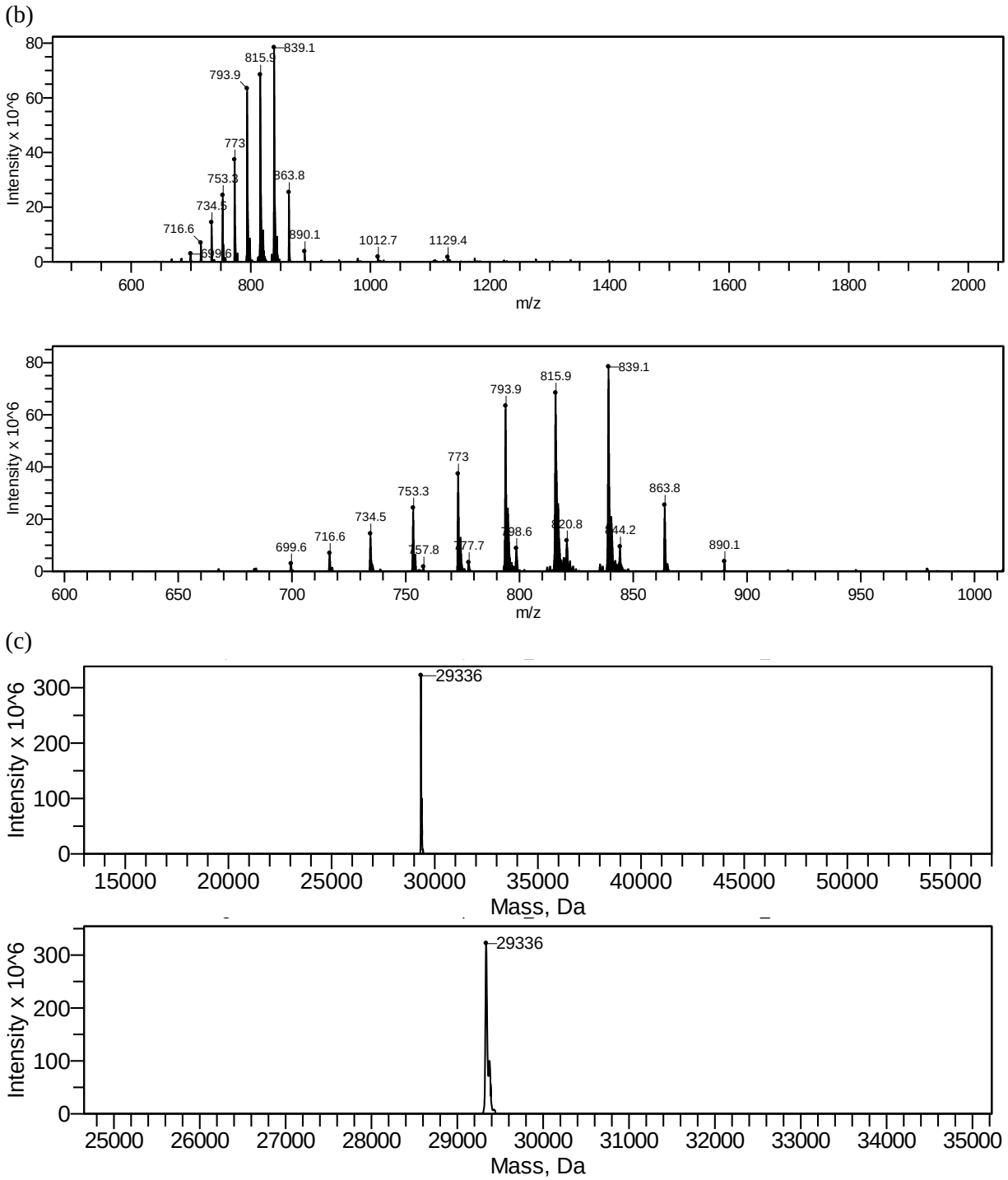
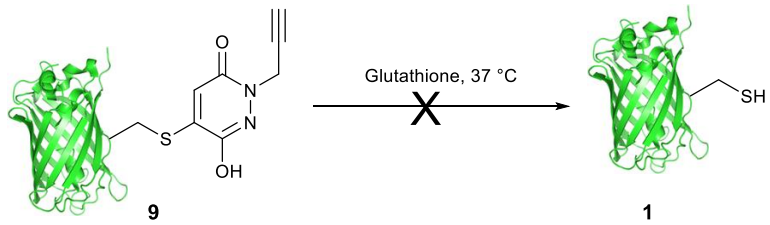


Figure S5. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for reaction of GFPS147CPD **8** with glutathione.

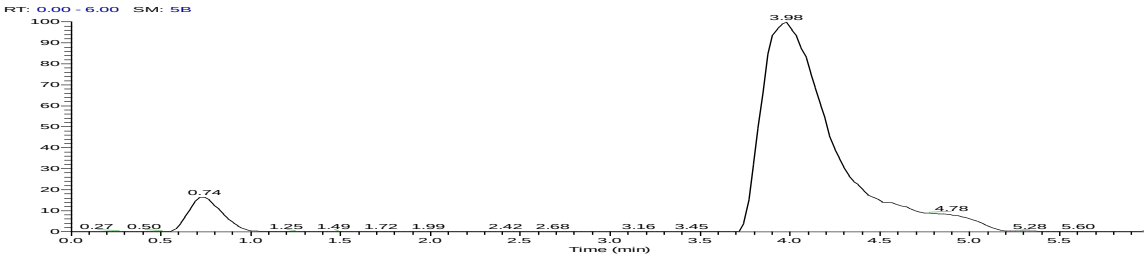
Reaction of GFPS147CPDOH 9 with glutathione



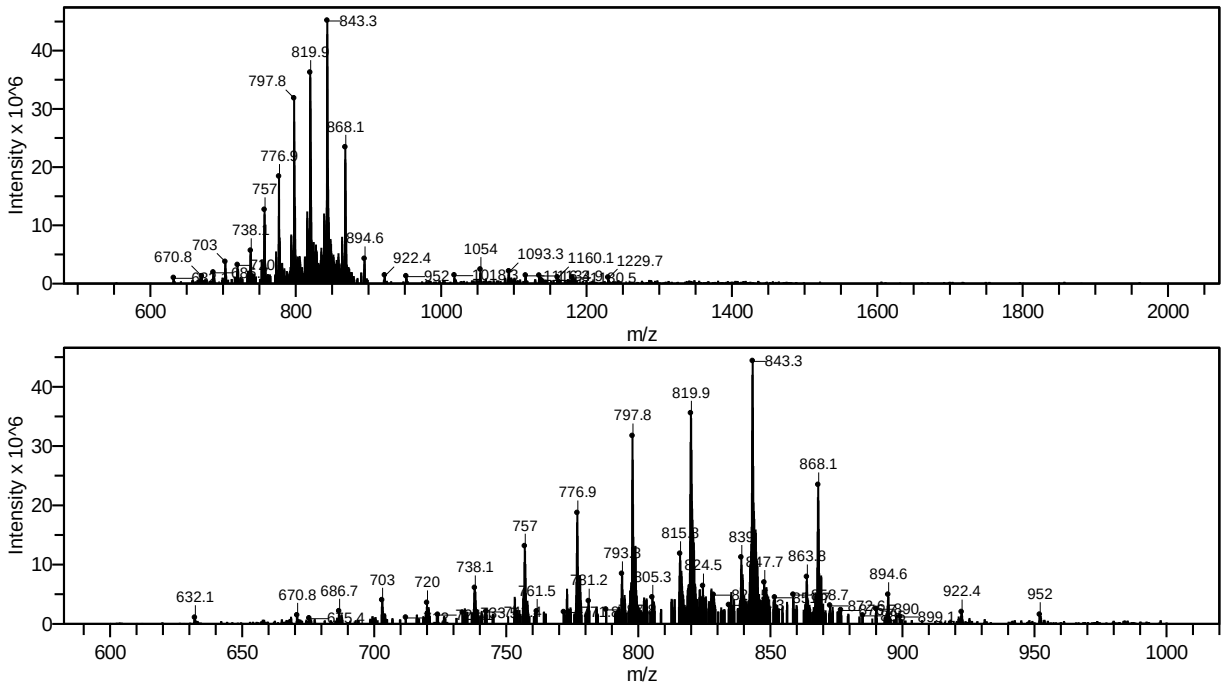
Glutathione (10 µL, 5 mM) was added to the GFPS147CPDOH conjugate **9** (90 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 7.4). The reaction was incubated at 37 °C for 72 h and then analysed by LCMS. Expected mass: 29,482 Da. Observed mass: 29,481 Da.

72 h

(a)



(b)



(c)

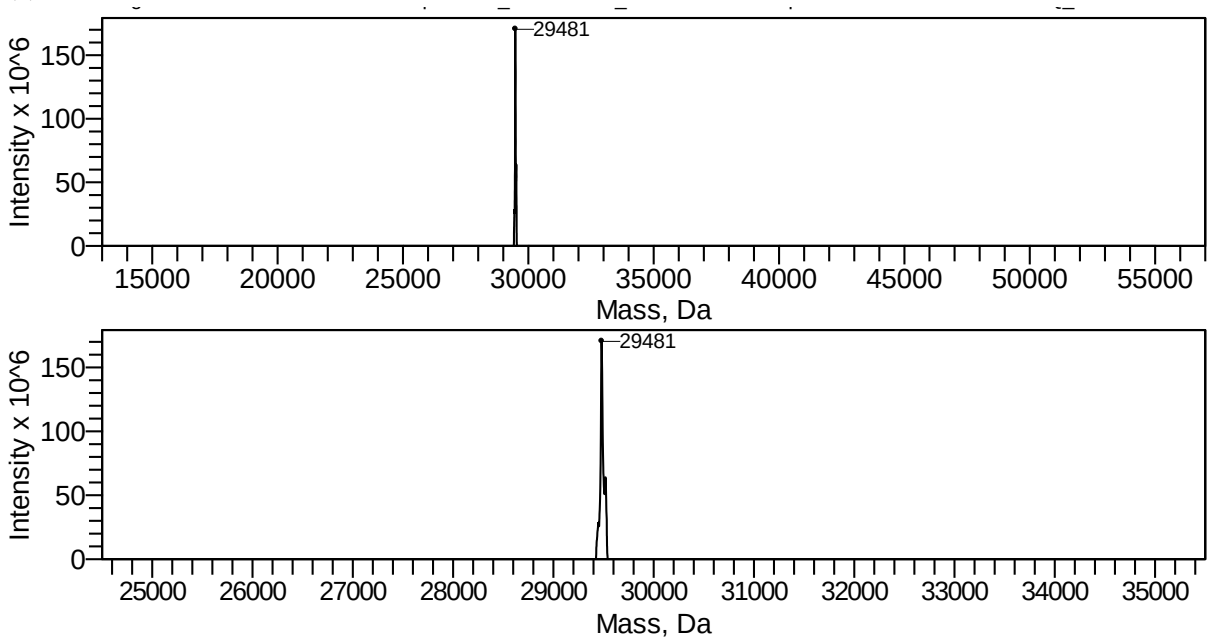
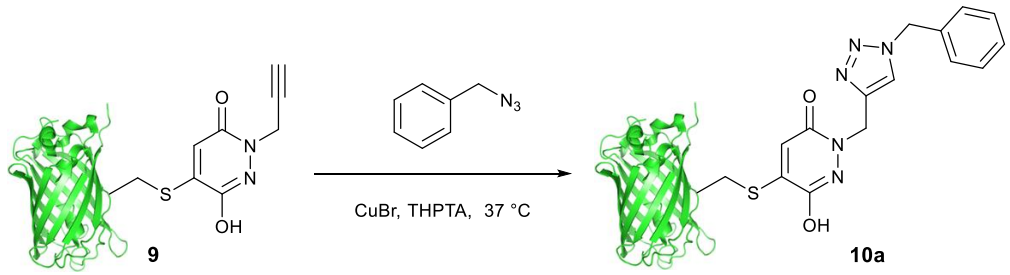


Figure S6. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for reaction of GFPS147CPDOH **9** with glutathione.

Reaction of GFPS147CPDOH 9 with benzyl azide



Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (1.2 µL, 20 mM) was added to a solution of CuBr (2.4 µL, 3 mg/mL in acetonitrile). GFPS147CPDOH **9** (60 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0) was added to the premixed copper solution. Benzyl azide (3 µL, 6.8 mM, 10 eq) was added to the reaction mixture and the mixture was incubated at 37 °C for 4 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, 5 mM EDTA, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 29,615 Da. Observed mass: 29,614 Da.

(a)

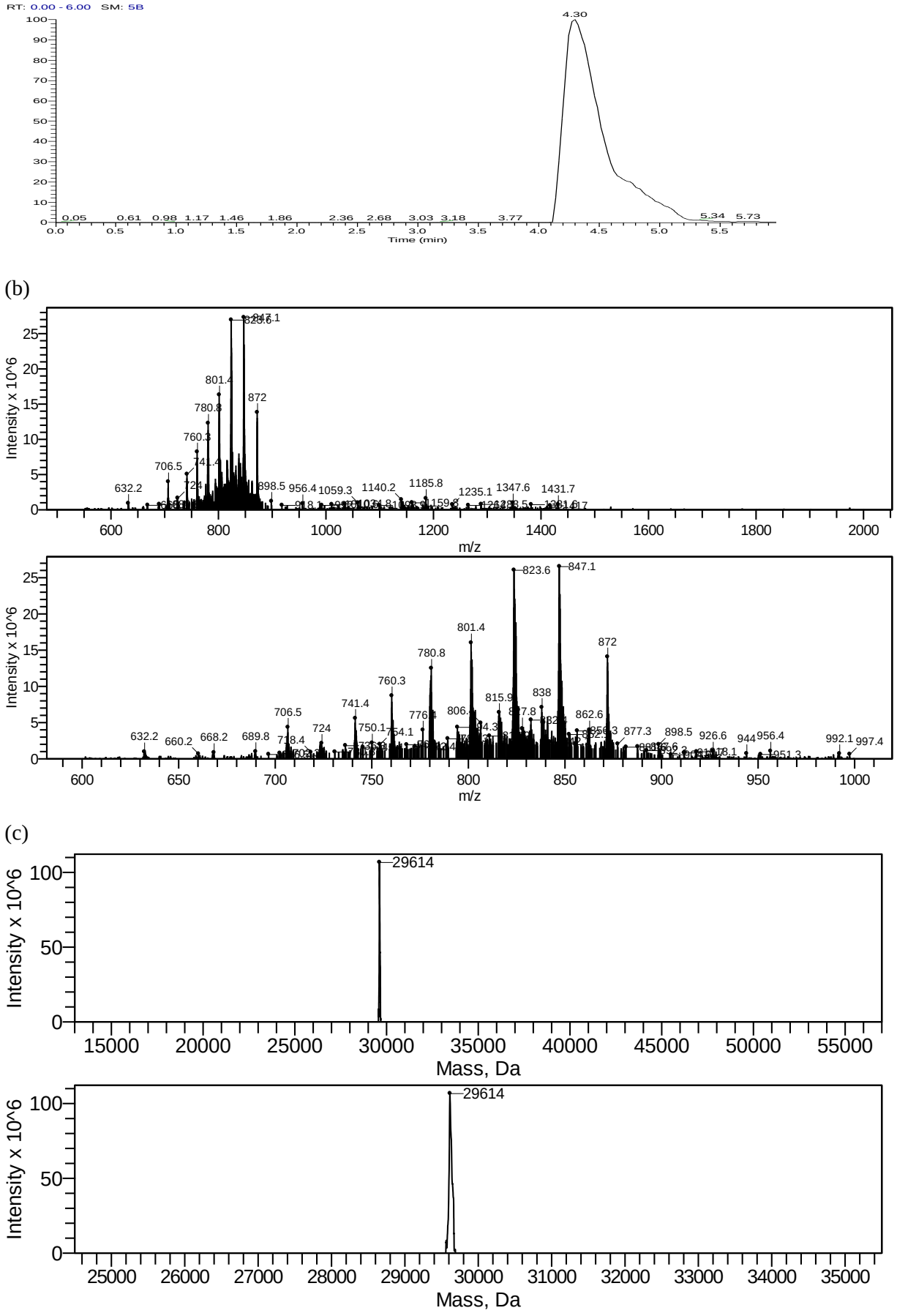
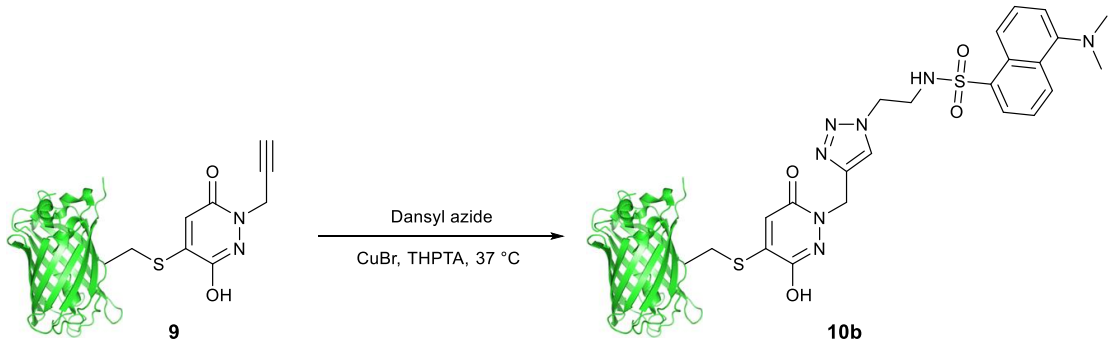


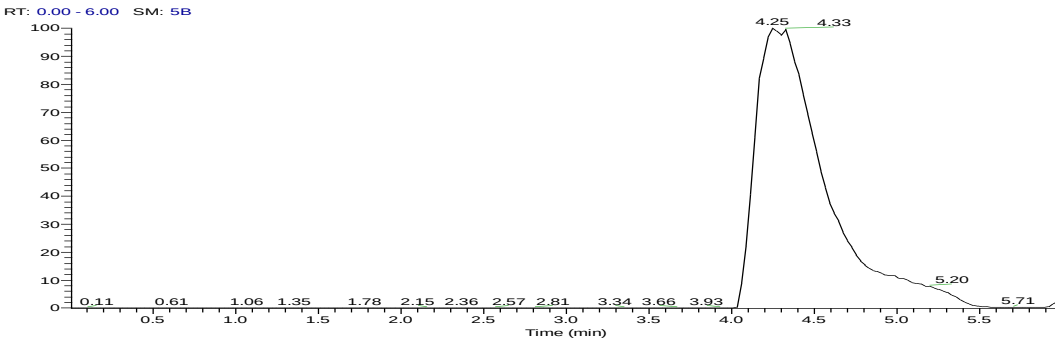
Figure S7. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **10a**.

Reaction of GFPS147CPDOH 9 with dansyl azide



Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (1.2 µL, 20 mM) was added to a solution of CuBr (2.4 µL, 3 mg/mL in acetonitrile). GFPS147CPDOH **9** (60 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0) was added to the premixed copper solution. Dansyl azide (3 µL, 6.8 mM in DMF, 10 eq) was added to the reaction mixture and the mixture was incubated at 37 °C for 4 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, 5 mM EDTA, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 29,801 Da. Observed mass: 29,800 Da.

(a)



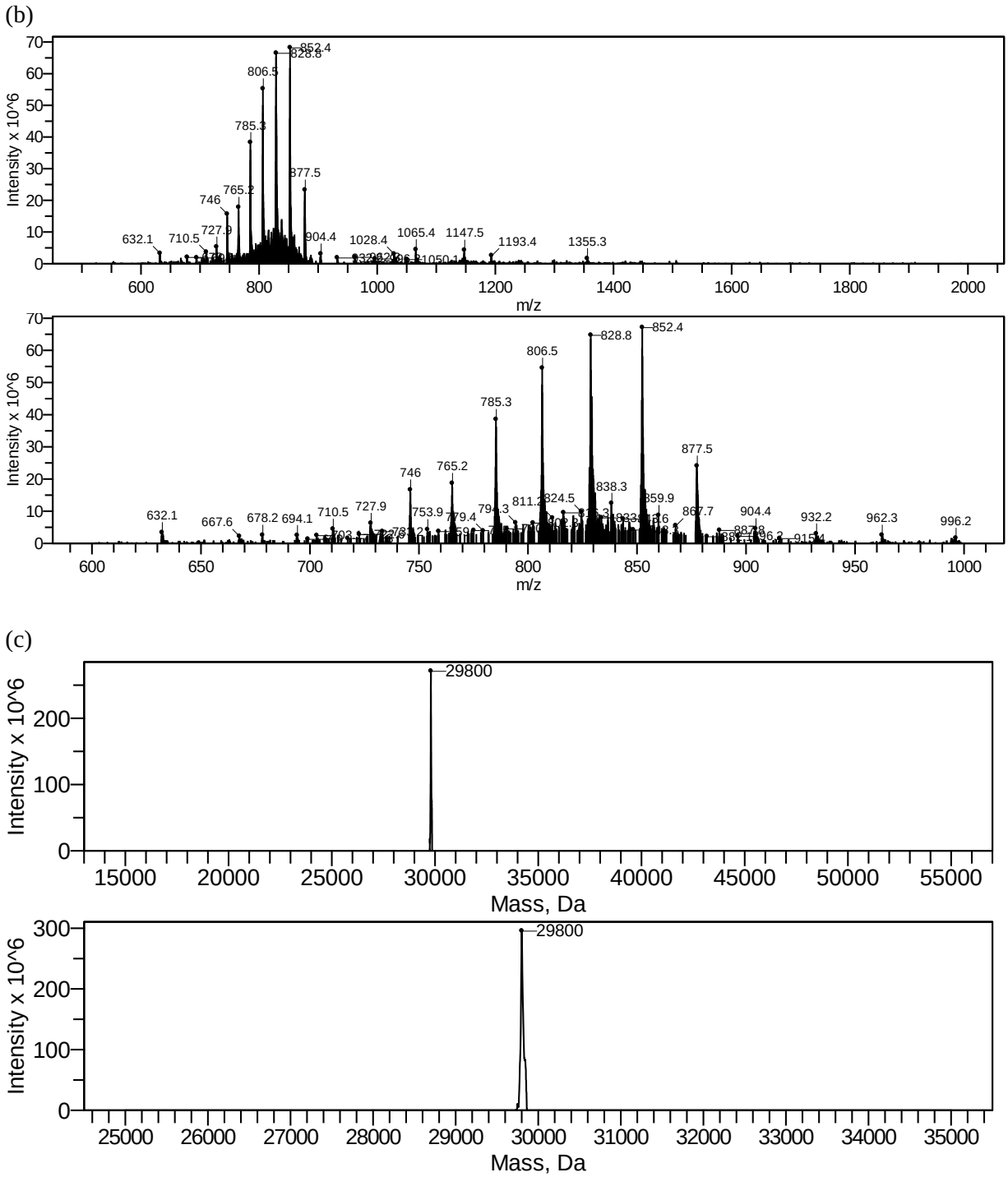
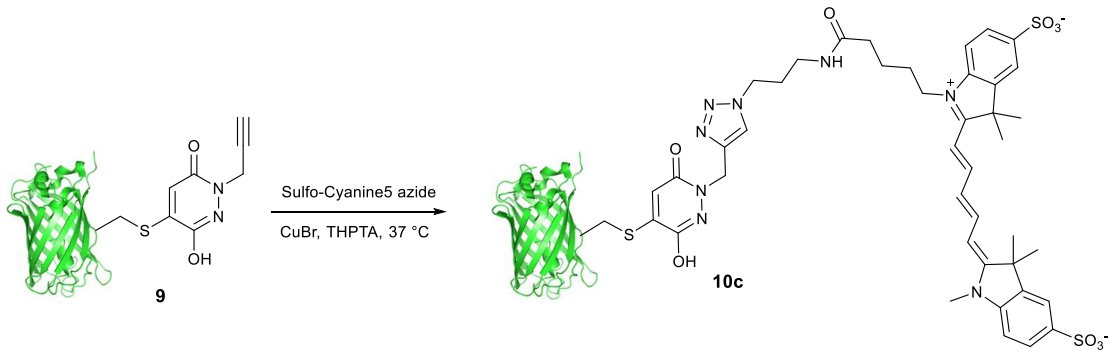


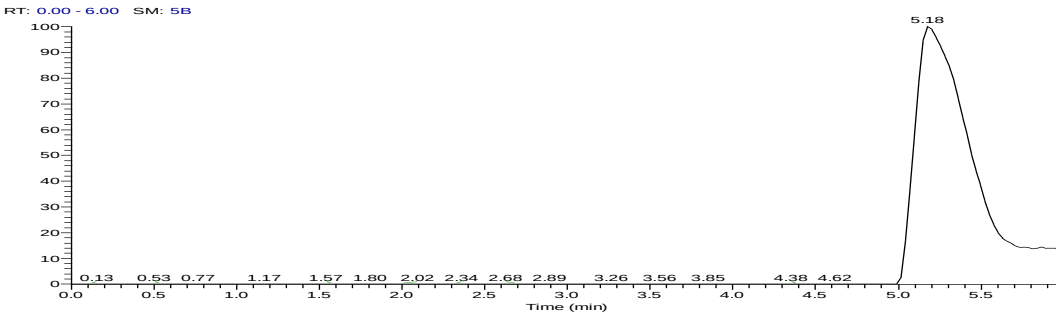
Figure S8. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **10b**.

Reaction of GFPS147CPDOH 9 with sulfo-cyanine5 azide



Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) (1.2 µL, 20 mM) was added to a solution of CuBr (2.4 µL, 3 mg/mL in acetonitrile). GFPS147CPDOH **9** (60 µL, 1 mg/mL, 34 µM) in sodium phosphate buffer (100 mM, pH 8.0) was added to the premixed copper solution. Sulfo-cyanine5 azide (3 µL, 6.8 mM in DMF, 10 eq) was added to the reaction mixture and the mixture was incubated at 37 °C for 4 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100 mM sodium phosphate, 5 mM EDTA, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 3,000 MWCO). The samples were analysed by LCMS. Expected mass: 30,206 Da. Observed mass: 30,204 Da.

(a)



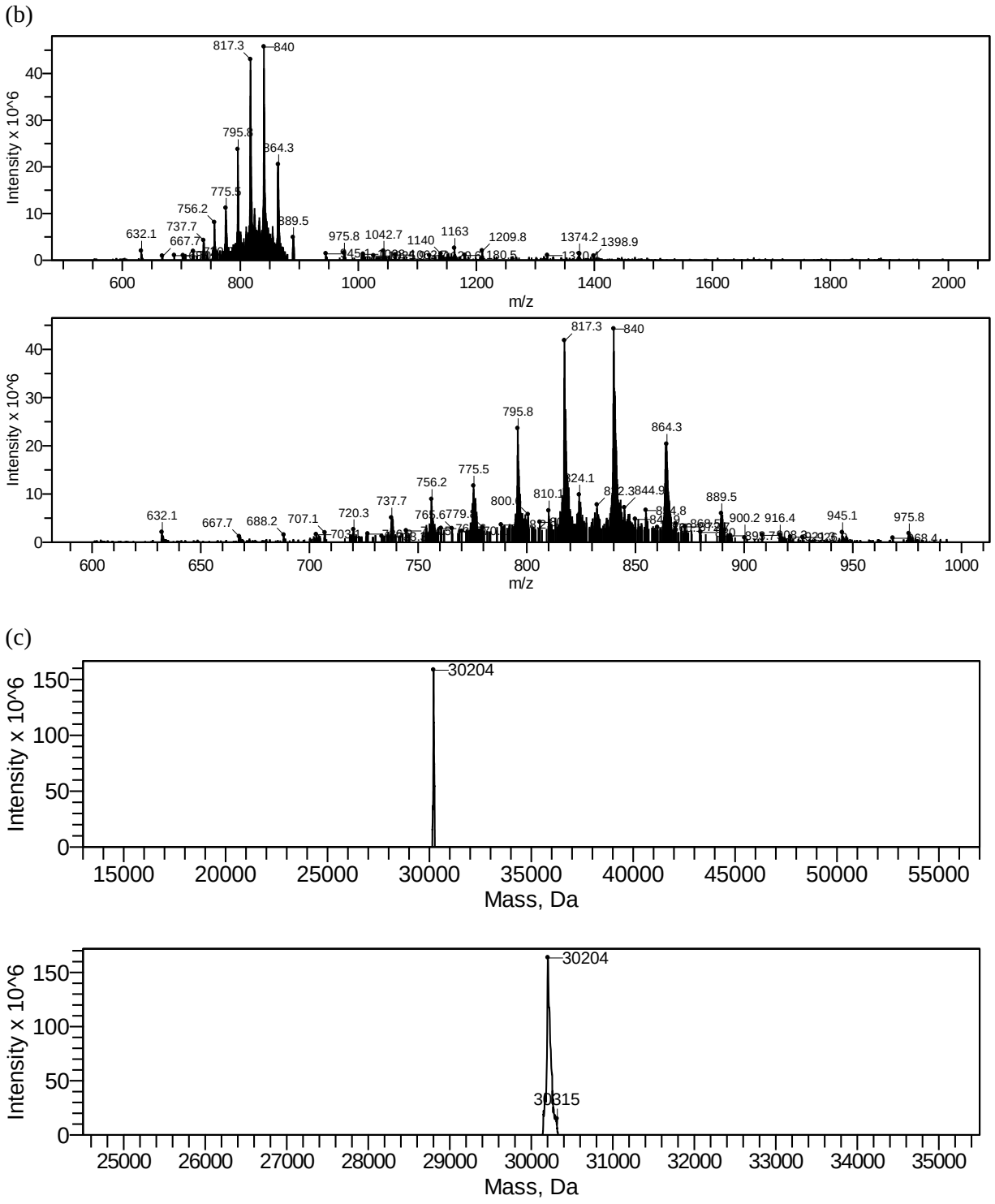


Figure S9. (a) TIC, (b) non-deconvoluted and (c) deconvoluted MS data for **10c**.

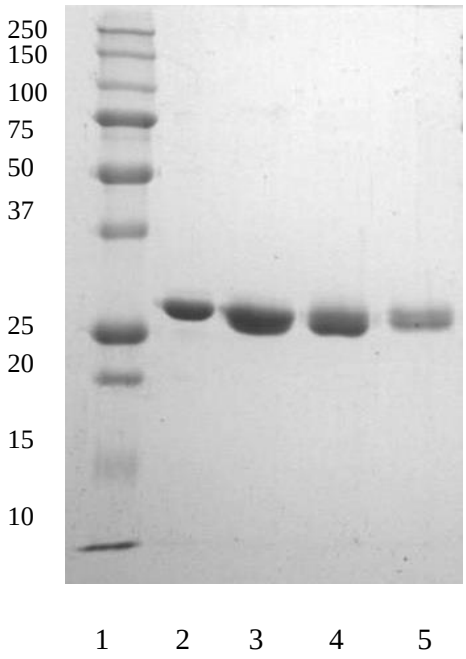


Figure S10. SDS-PAGE gel showing various GFP constructs. Lane 1: Precision Plus Protein Standards ladder, 2: GFPS147C **1**, 3: GFPS147CPD **8**, 4: GFPS147CPDOH **9**, 5: GFPS147CPDOHBenzyl **10a**.

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