Electronic Supplementary Material (ESI) for Chemical Science
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A Novel Approach to the Site-Selective Dual Labelling of a Protein via Chemoselective Cysteine Modification

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Surface and stick representation of subtilisin showing the α-proton of S156

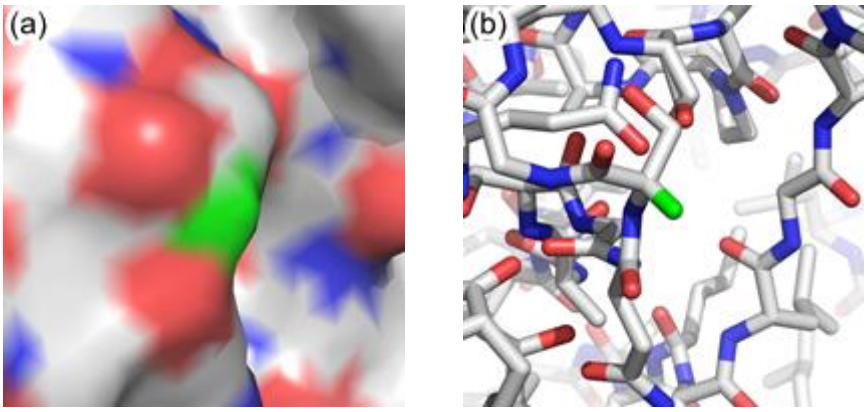

Figure S1. (a) Surface and (b) stick representation of subtilisin (PDB ID: 1GCI) showing the α-proton (added with PyMOL) of S156 (green).[1]

General Procedures

Dibenzylcyclooctyne-PEG_4-Fluor 545 was purchased from Jena Bioscience GmbH. All other reagents were purchased from Sigma-Aldrich or Fisher Scientific and were used without further purification. 2,5-Dibromohexanediamide was synthesized as reported by Davis.[2]

Protein Mass Spectroscopy

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 x 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 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 3.4-4.8 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 superfolder GFP and GFP(S147C) in the vector pNIC28-Bsa4 were generated as described previously.[3]

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GFP(T230C,233Δ) was generated by ligation independent subcloning of superfolder GFP into pNIC28 Bsa4 using the primers:

5’ - TACTTCCAATCCATGCGTAAAGGCGAAGAGCTGTTCAC - 3’
5’ - TATCCACCTTTACTGCTAACCATGACAGATGCCCGCTGCGGTTACG - 3’

GFP(S147C,T230C,233Δ) was generated by ligation independent subcloning of GFP(S147C) into pNIC28 Bsa4 using the primers:

5’ - TACTTCCAATCCATGCGTAAAGGCGAAGAGCTGTTCAC - 3’
5’ - TATCCACCTTTACTGCTAACCATGACAGATGCCCGCTGCGGTTACG - 3’

The vector was transformed into BL21(DE3)plysS cells, and proteins were expressed at 30 °C using standard methods. Proteins were purified by nickel chromatography, and the buffer was exchanged by repeated diafiltration into sodium phosphate (100 mM, pH 8.0) at 4 °C. Prior to analysis by LCMS, dithiothreitol (10 µL, 340 mM as a solution in water, 1000 equivalents per 100 µL of protein (1 mg/mL solution)) was added, the mixture incubated at 21 °C for 1 h and excess reducing agent was removed by repeated diafiltration with sodium phosphate (100 mM, pH 8.0).

Solvent accessibility calculations

Solvent accessibilities of α-protons in subtilisin and superfolder GFP were calculated using Naccess using the default parameters.[4]

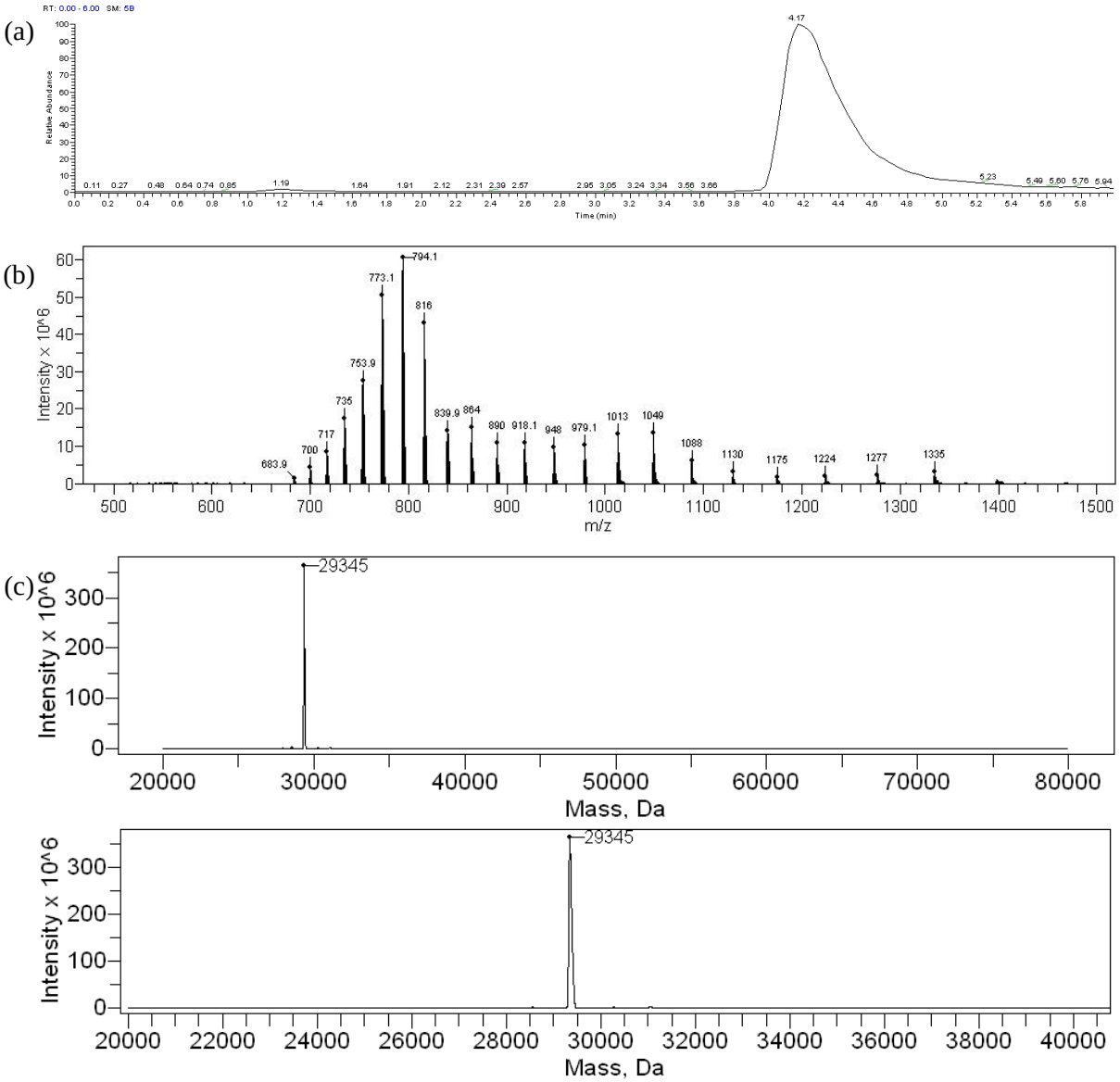
Fluorescence measurements

Fluorescence properties were measured using a Cary Eclipse fluorescence spectrophotometer using 1 µM of fluorescent compound, in PBS pH 7.4 at 20 ºC. The sample was excited at 494 nm, and the emission intensity was scanned at 120 nm/min with an averaging time of 0.5 s and a data interval of 1 nm. The excitation and emission slits were set to 5 nm.

Superfolder GFP

Sequence

MHHHHHHSSGVDLGTENLYFQSMRKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGK
LTLKFICTTGKLPVPWPTLVTTLTYGVQCFARYPDHMKQHDFFKSAMPEGYVQERTISFKDDGTY
KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNV
EDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDE
LYK



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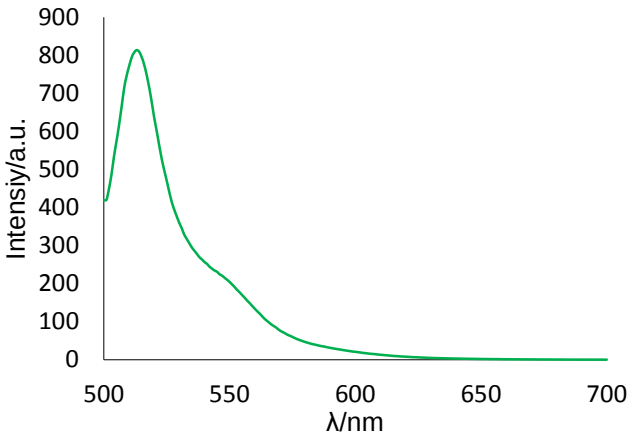
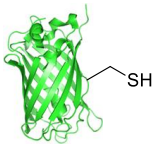


Figure S2. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for Superfolder GFP.

GFP(S147C)[3]



Sequence

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KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNCHNVYITADKQKNGIKANFKIRHNV
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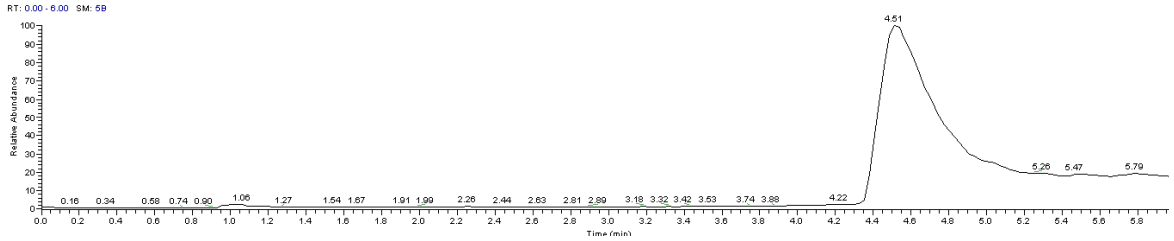
LYK

(a)



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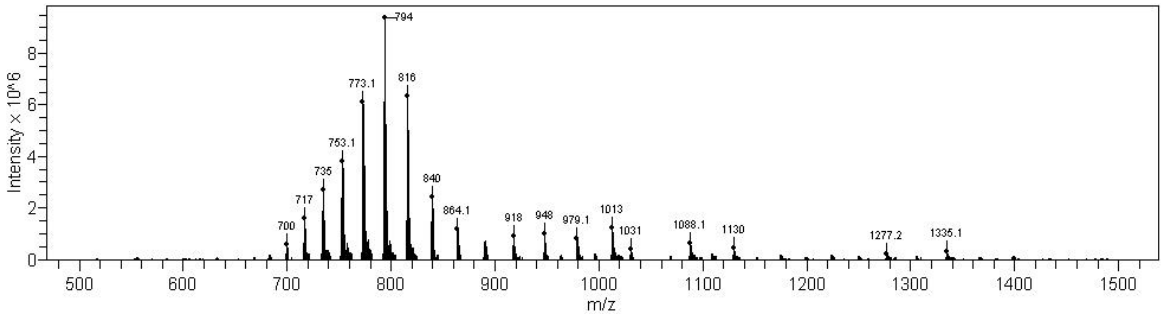

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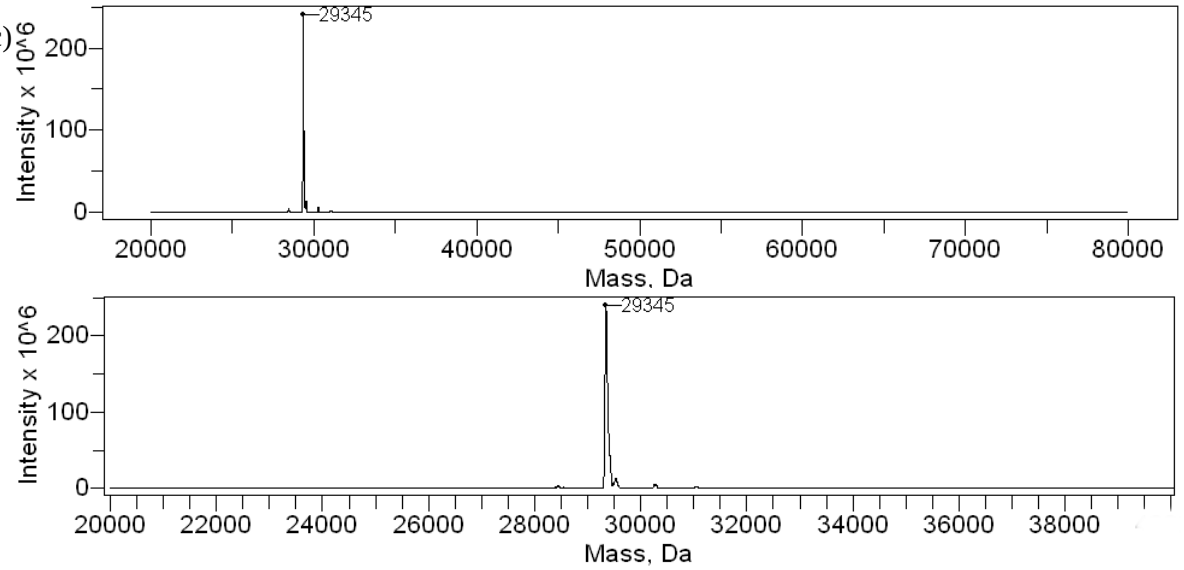

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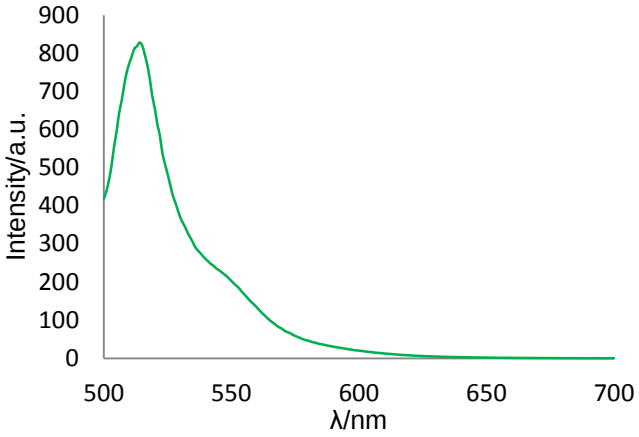
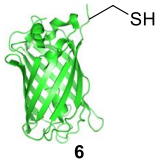


Figure S3. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147C).

GFP(T230C,233Δ) 6



6

Sequence

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KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNSHNVYITADKQKNGIKANFKIRHNV
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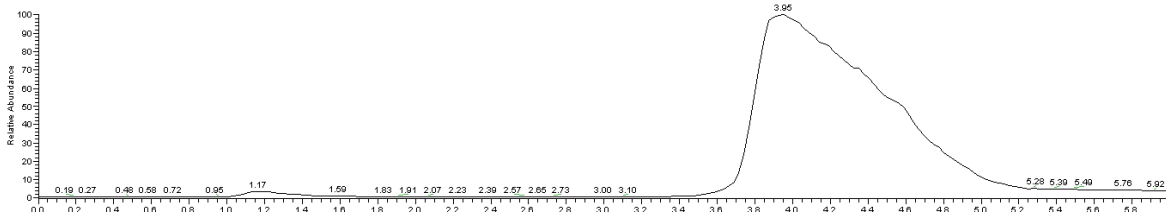
(a)



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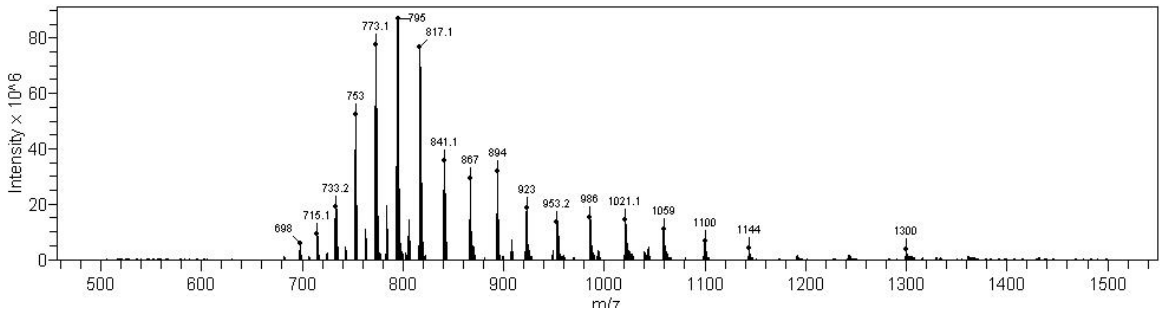

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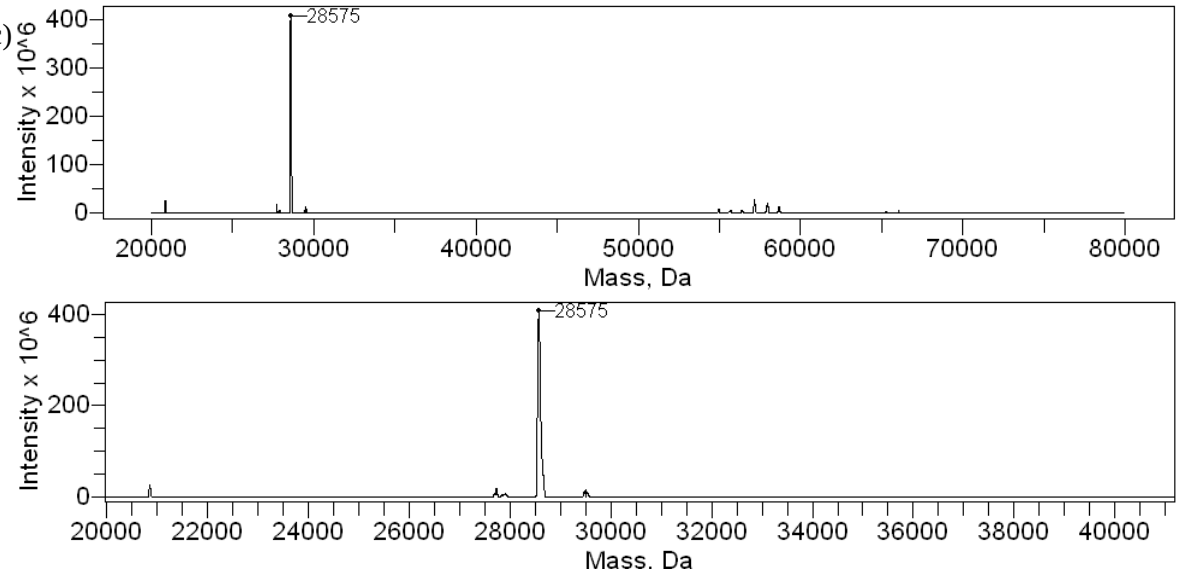

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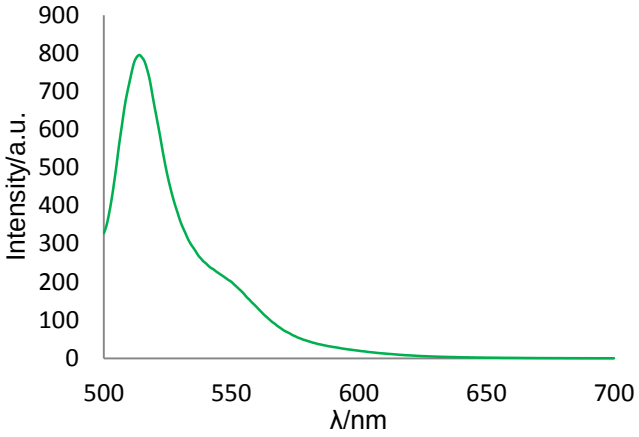
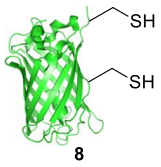


Figure S4. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(T230C,233Δ) **6**.

GFP(S147C,T230C,233Δ) 8



8

Sequence

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KTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNFNCHNVYITADKQKNGIKANFKIRHNV
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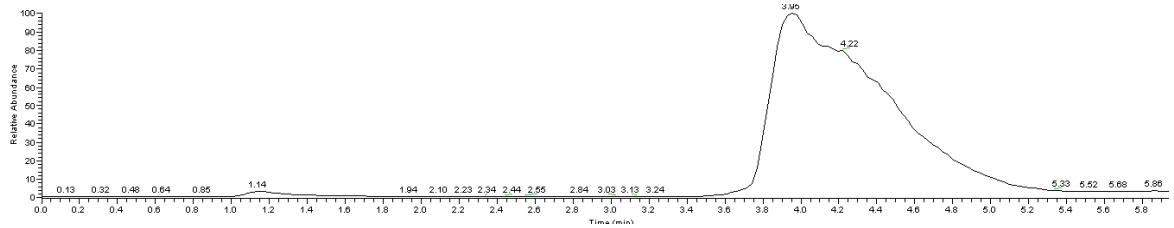
(a)



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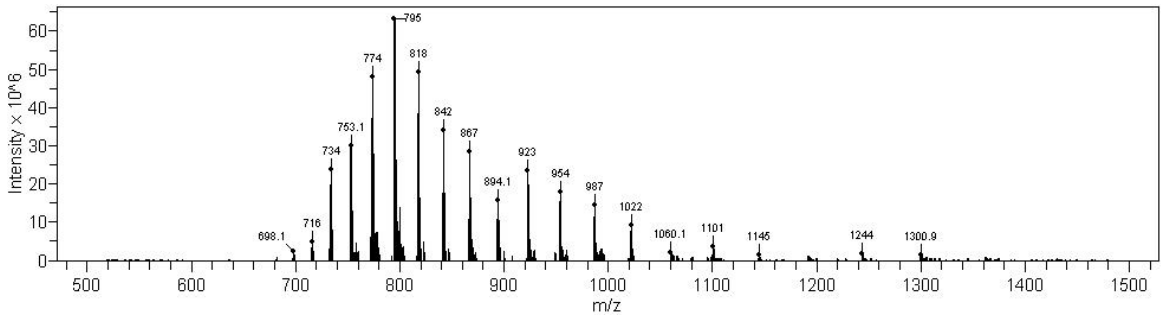

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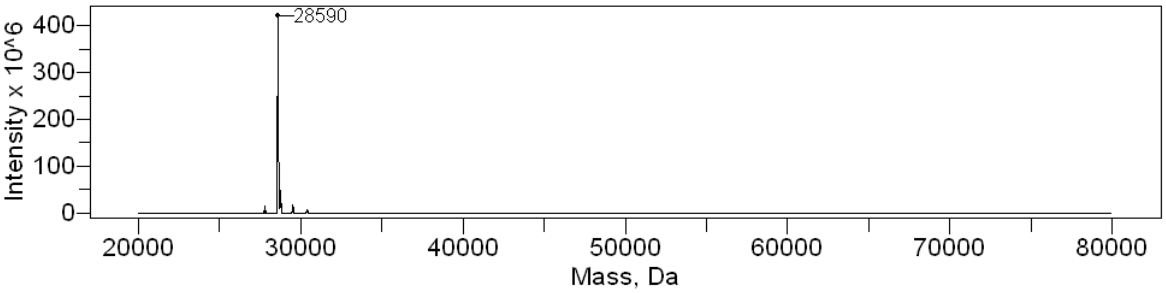

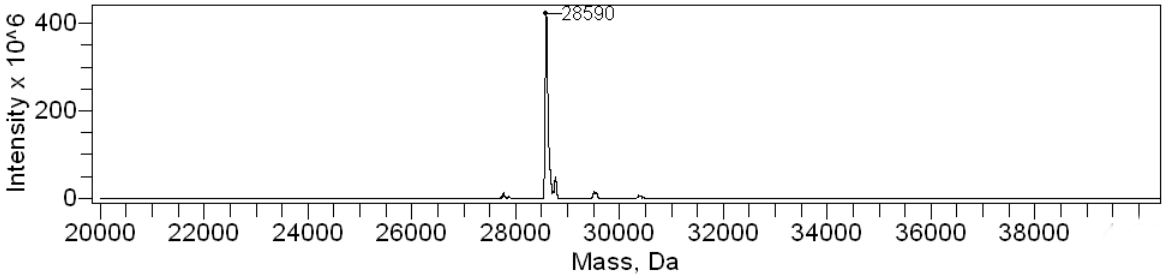

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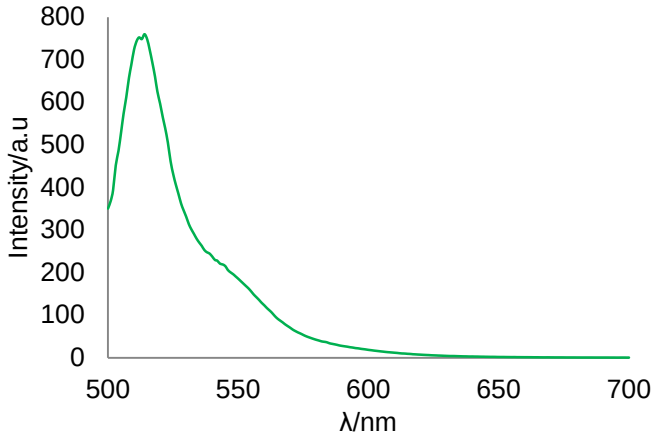

Figure S5. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147C,T230C,233Δ) **8**.

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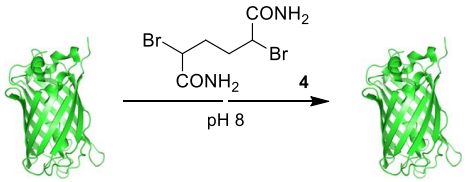
Pre-treatment procedure

Immediately prior to bioconjugation sequences, dithiothreitol (10 μL, 340 mM as a solution in water, 1000 equivalents per 100 μL of protein (1 mg/mL solution)) was added, incubated at 21 °C 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, 10,000 MWCO).

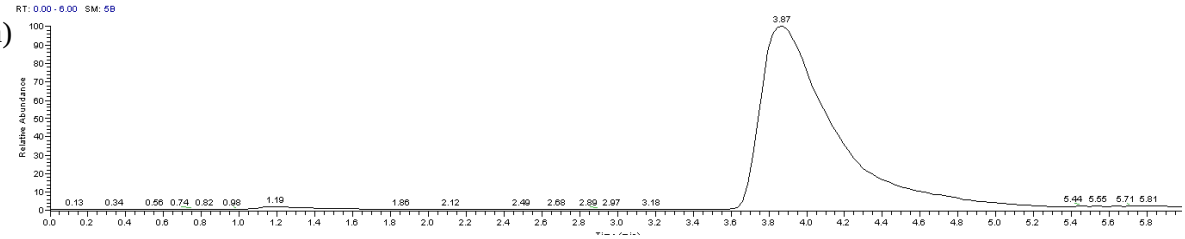
Reaction of GFP with 2,5-dibromohexanediamide 4



Expected mass: 29341

2,5-Dibromohexanediamide (**4**, 10 µL, 17 mM solution in DMF, 50 equivalents) was added to a solution of GFP (100 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2 h and analysed by LCMS.

(a)



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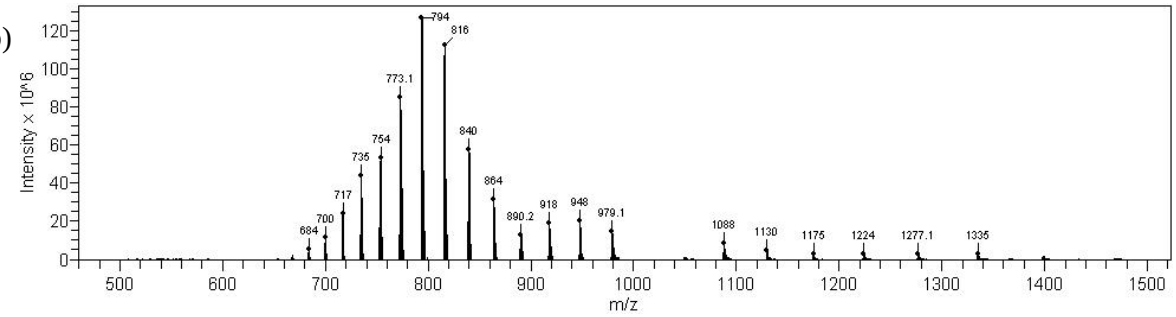

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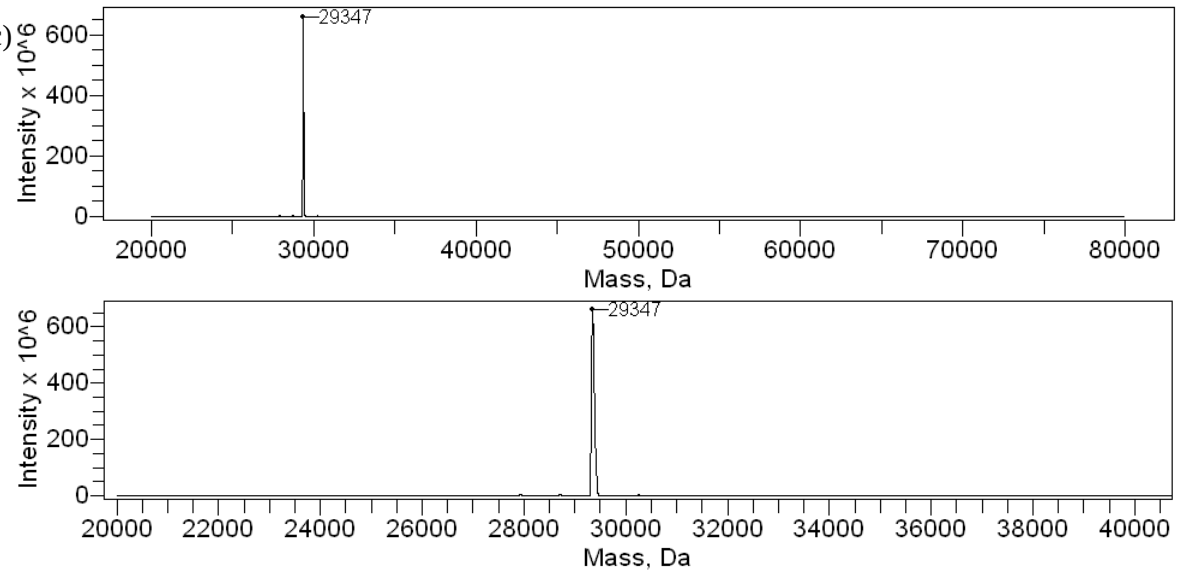

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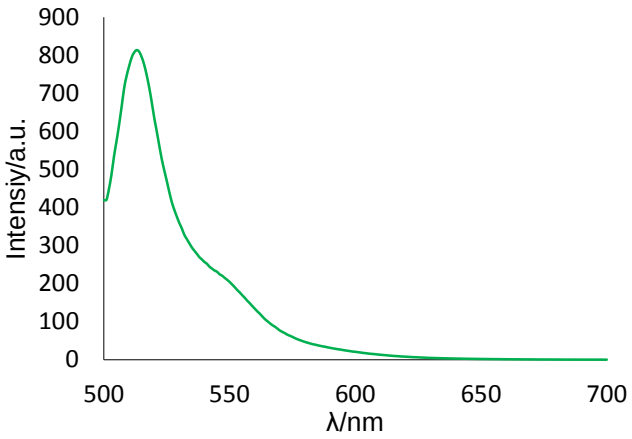
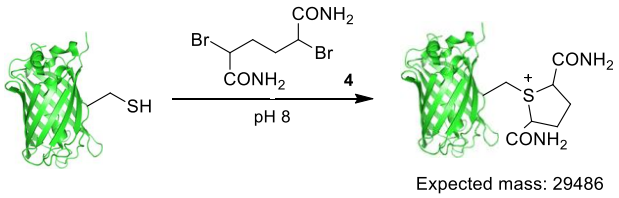


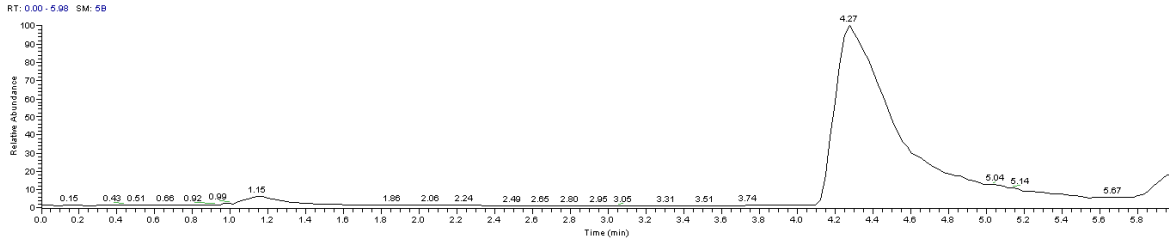
Figure S6. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for reaction of GFP with 2,5-dibromohexanediamide **4**.

Reaction of GFP(S147C) with 2,5-dibromohexanediamide 4[3]



2,5-Dibromohexanediamide (**4**, 10 µL, 17 mM solution in DMF, 50 equivalents) was added to a solution of GFP(S147C) (1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2 h and analysed by LCMS.

(a)



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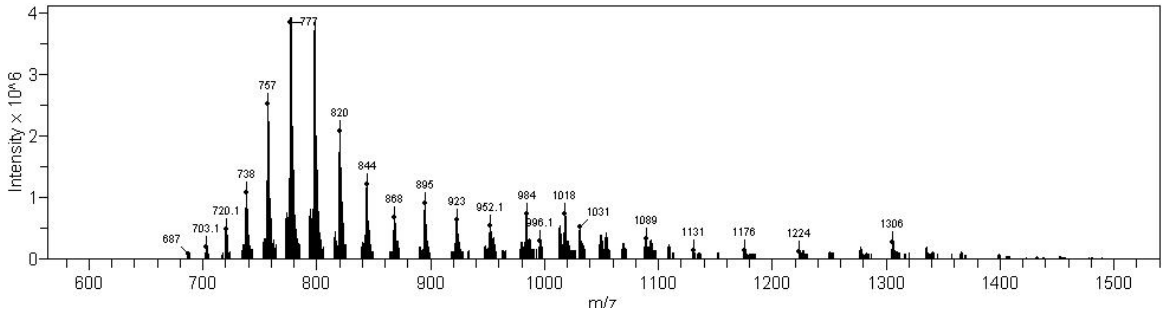

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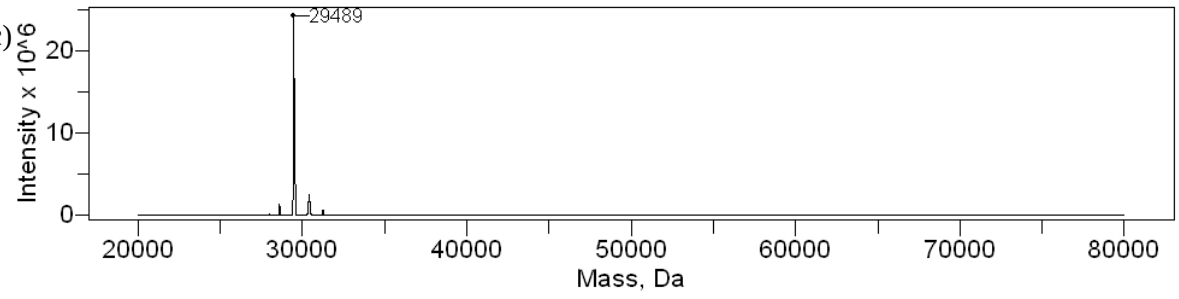

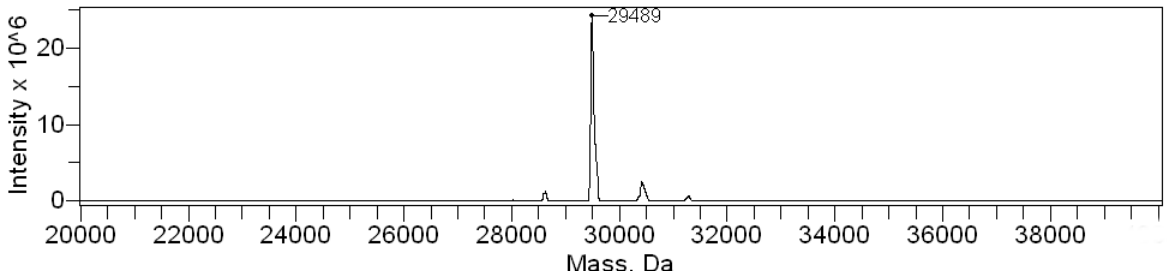

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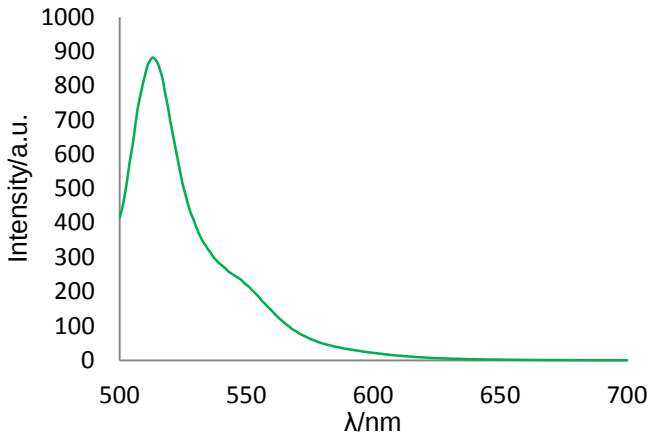
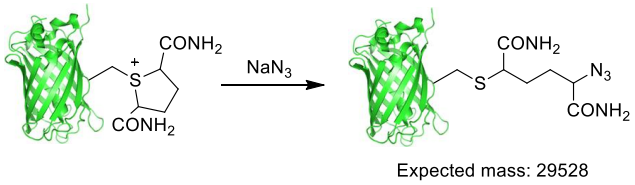


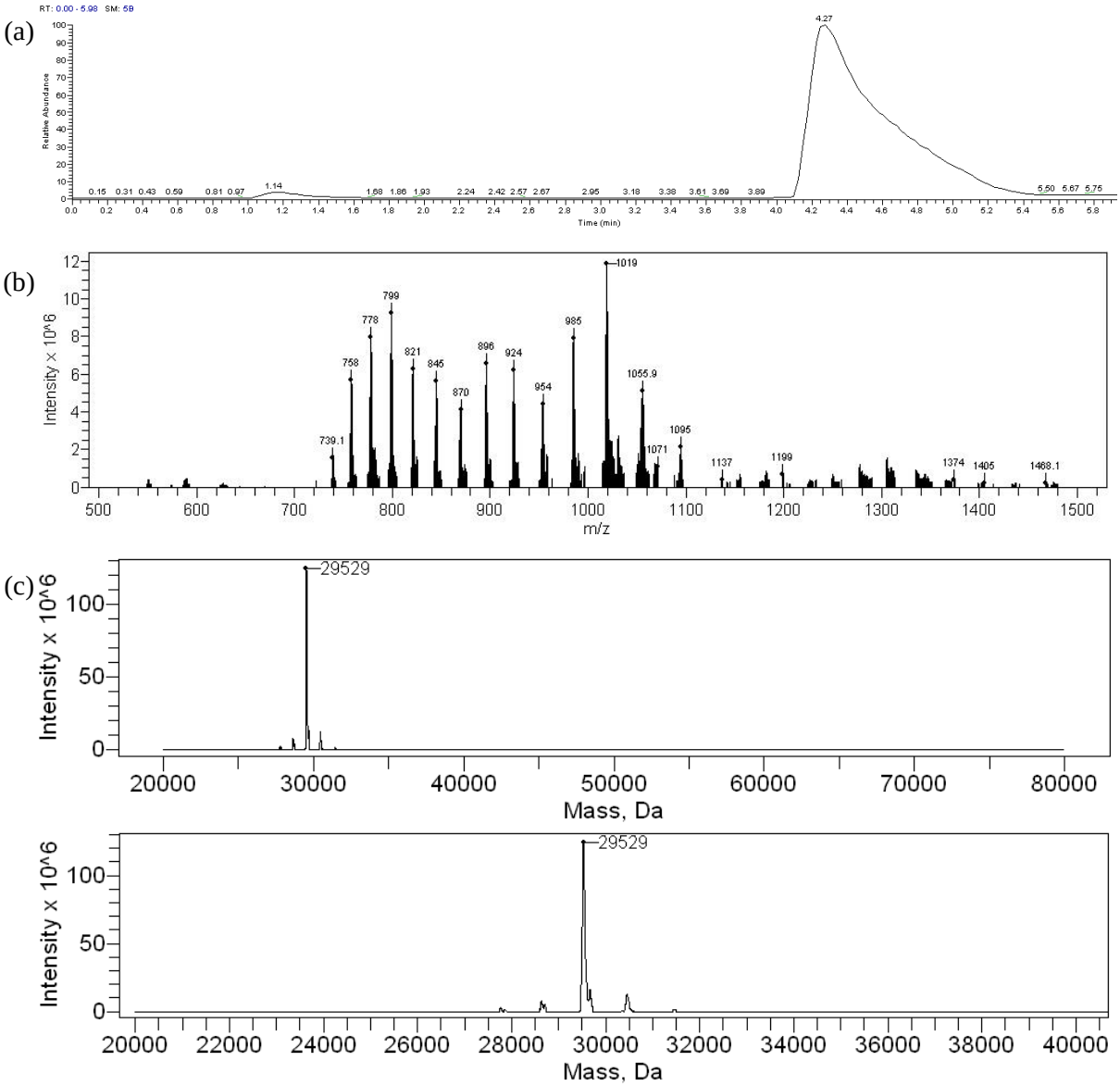
Figure S7. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Sulf).

Reaction of GFP(S147Sulf) with sodium azide[3]



Expected mass: 29528

Sodium azide (10 µL, saturated solution in water) was added to GFP(S147Sulf) (100 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2.5 h and analysed by LCMS.



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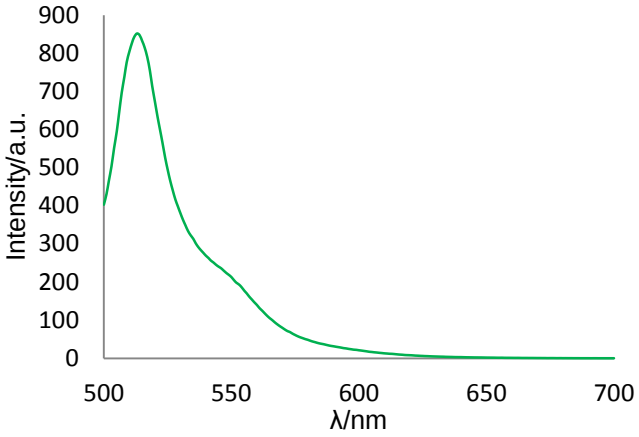
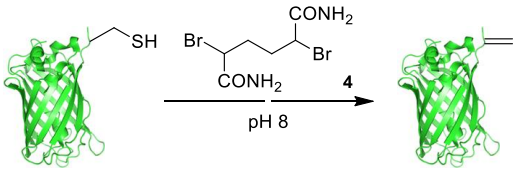


Figure S8. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Azide).

Reaction of GFP(T230C,233Δ) 6 with 2,5-dibromohexanediamide 4

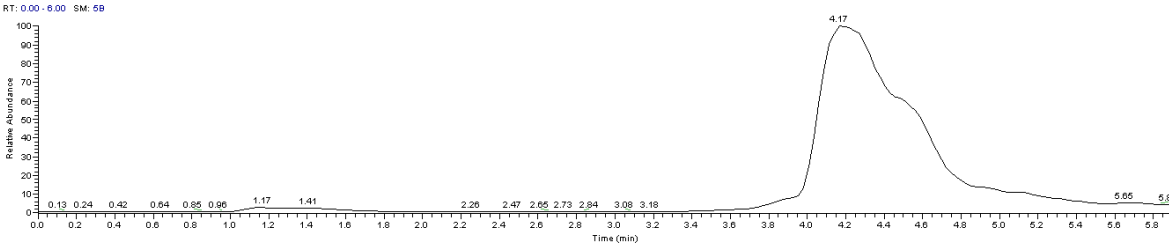


6 **7**

Expected mass: 28541

2,5-Dibromohexanediamide (**4**, 10 μL, 17 mM solution in DMF, 50 equivalents) was added to a solution of GFP(T230C,233Δ) **6** (1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2.5 h and analysed by LCMS.

(a)



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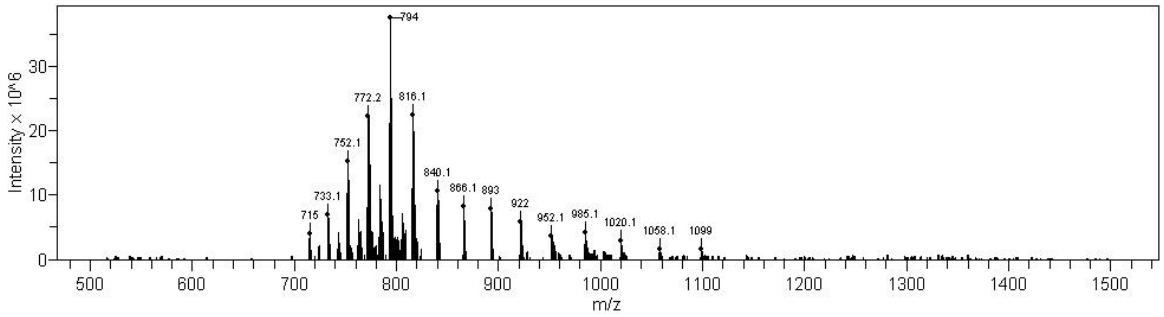
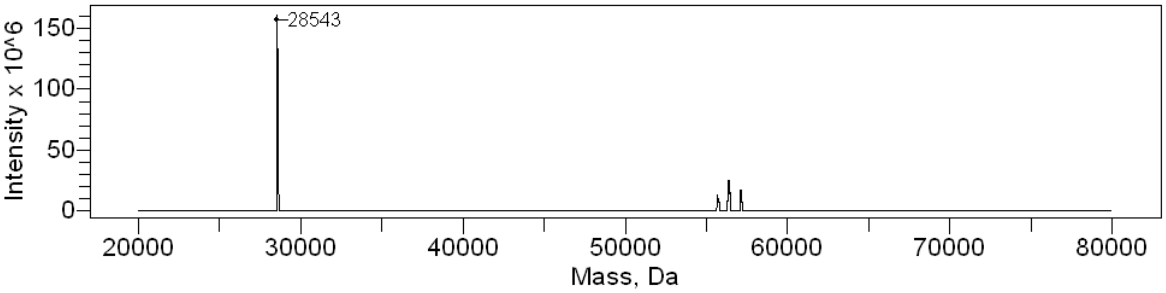
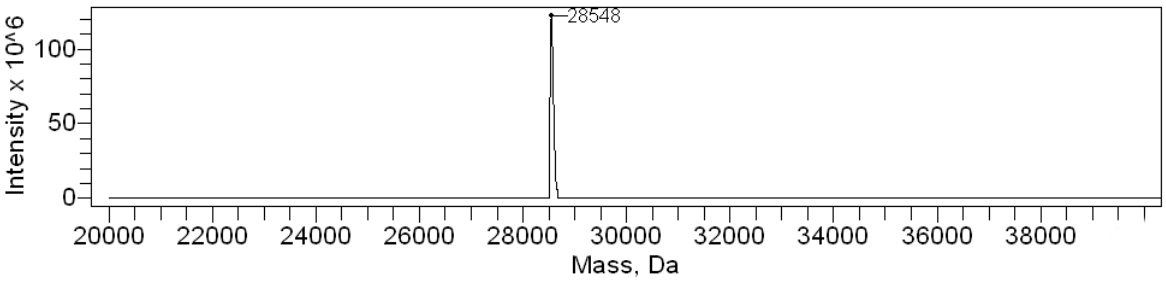


(c)





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(d)

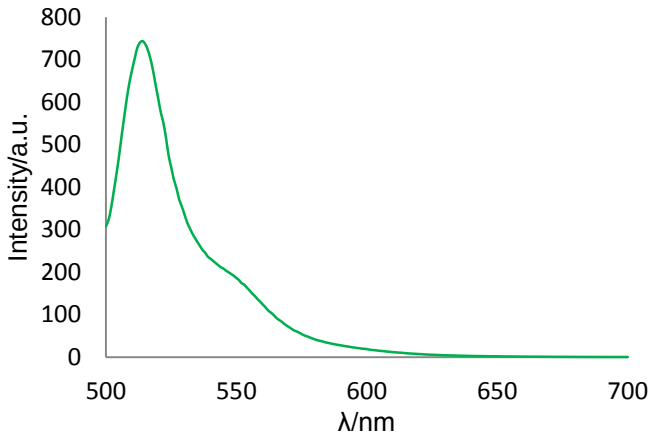

Figure S9. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(T230Dha,233Δ) **7**.

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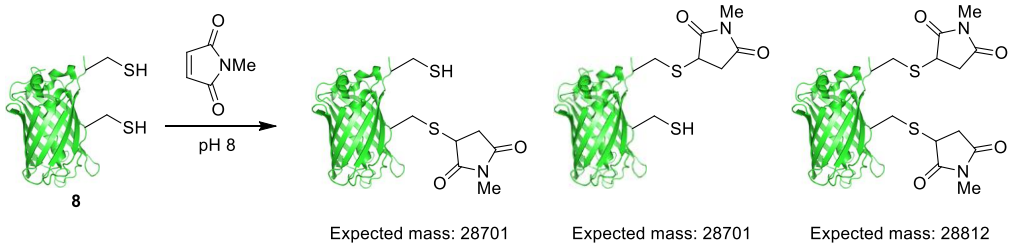
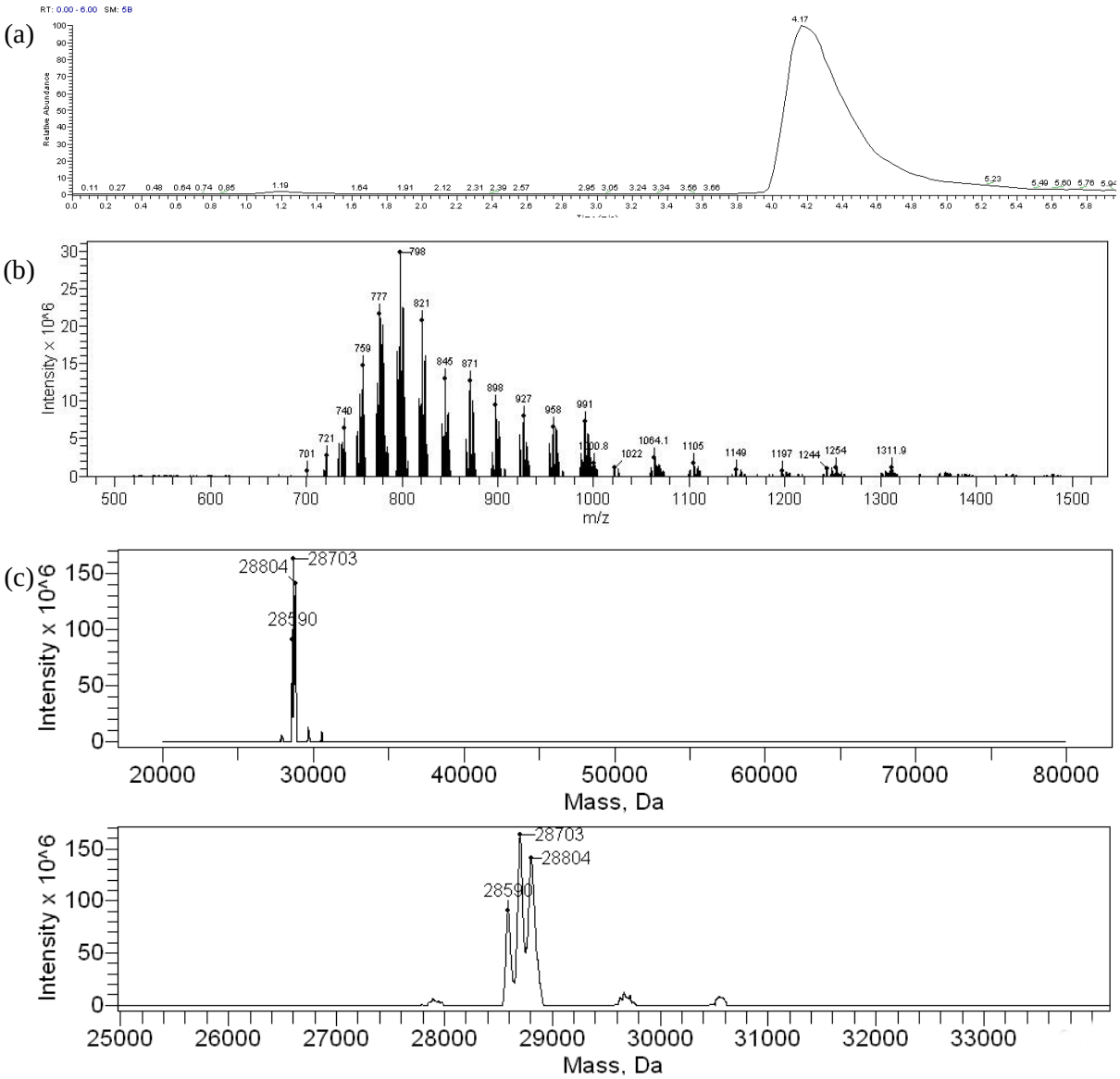
Reaction of GFP(S147C,T230C,233Δ) 8 with N-methylmaleimide



N-Methylmaleimide (5 µL, 6.8 mM solution in DMF, 1 equivalent) was added to a solution of GFP(S147C,T230C,233Δ) **8** (100 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 21 °C for 30 min and analysed by LCMS.



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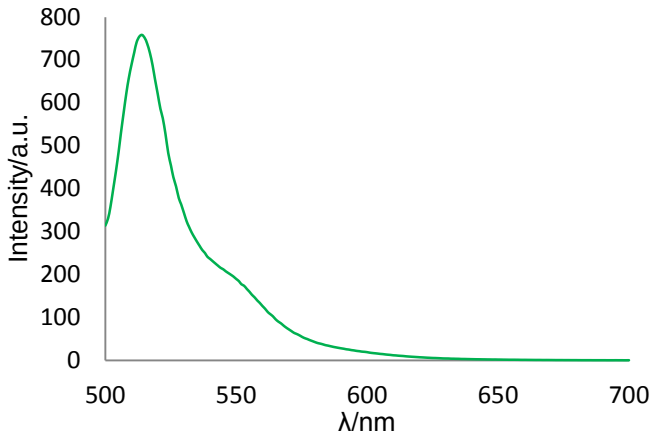
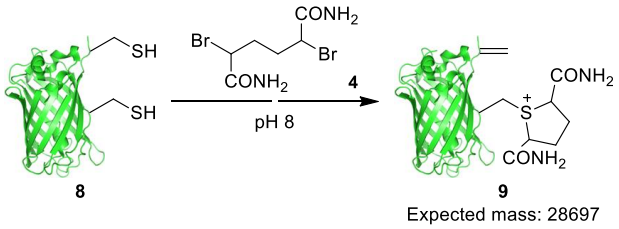


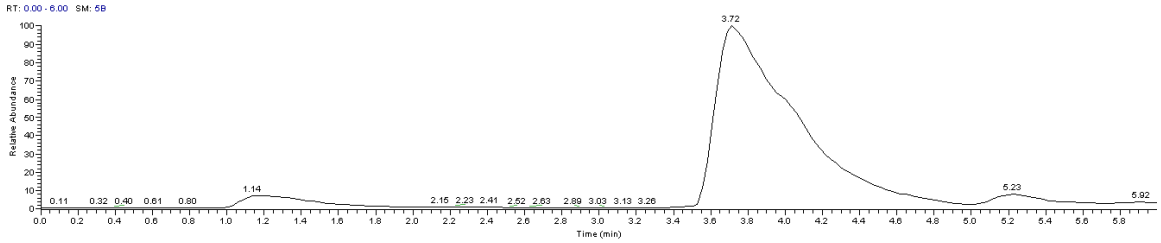
Figure S10. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for reaction of GFP(S147C,T230C,233Δ) **8** with N-methylmaleimide.

Reaction of GFP(S147C,T230C,233Δ) 8 with 2,5-dibromohexanediamide 4

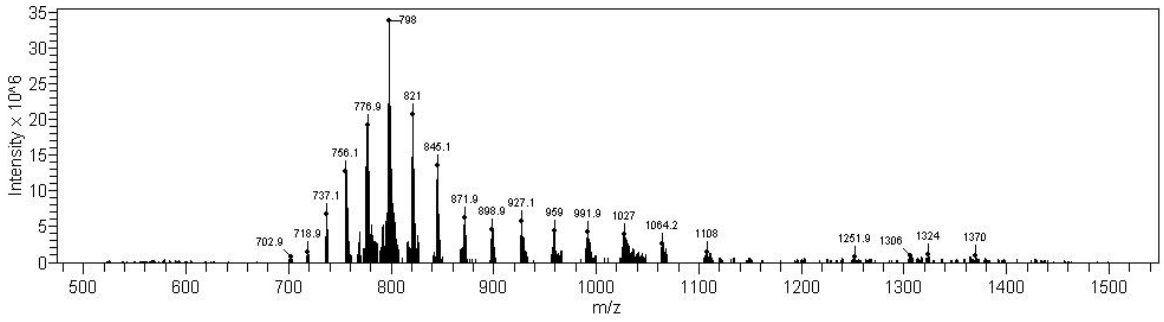


2,5-Dibromohexanediamide (**4**, 30 µL, 17 mM solution in DMF, 50 equivalents) was added to a solution of GFP(S147C,T230C,233Δ) **8** (300 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2 h and analysed by LCMS.

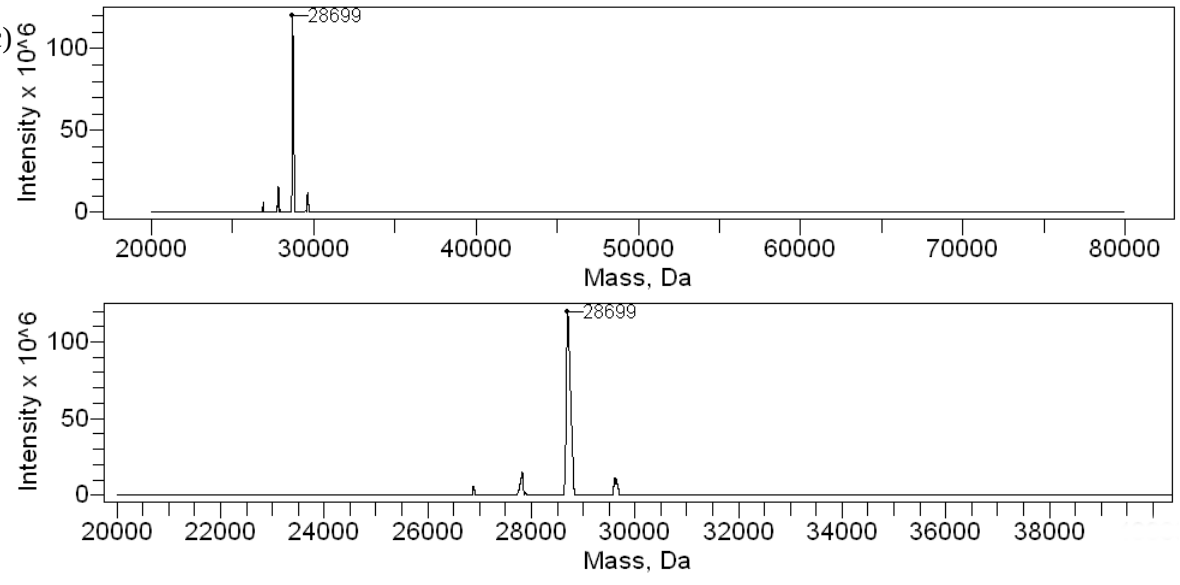
(a)



(b)



(c)



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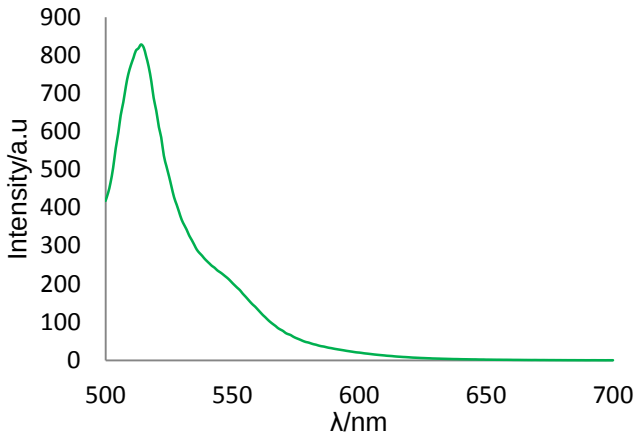
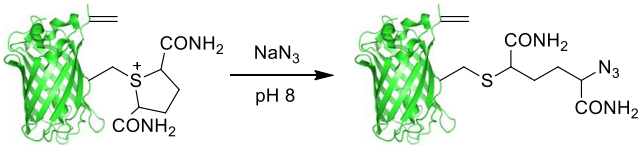


Figure S11. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Sulf,T230Dha,233Δ) **9**.

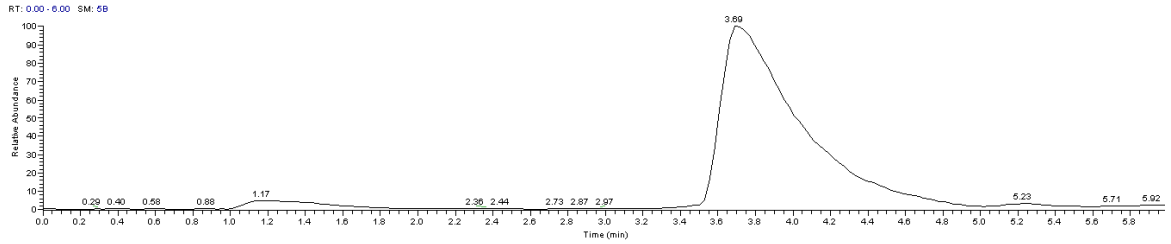
Reaction of GFP(S147Sulf, T230Dha, 233Δ) 9 with sodium azide



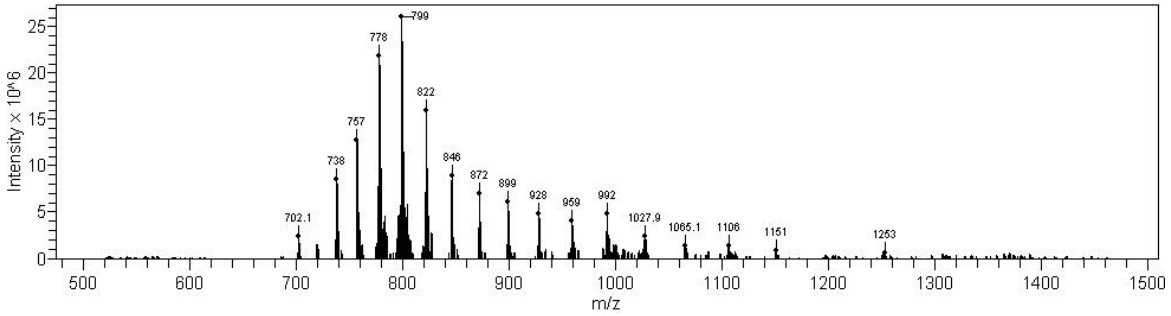
9 **10**
Expected mass: 28739

Sodium azide (30 µL, saturated solution in water) was added to a solution of GFP(S147C,T230C,233Δ) **9** (300 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 37 °C for 2 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 10,000 MWCO) and the mixture analysed by LCMS.

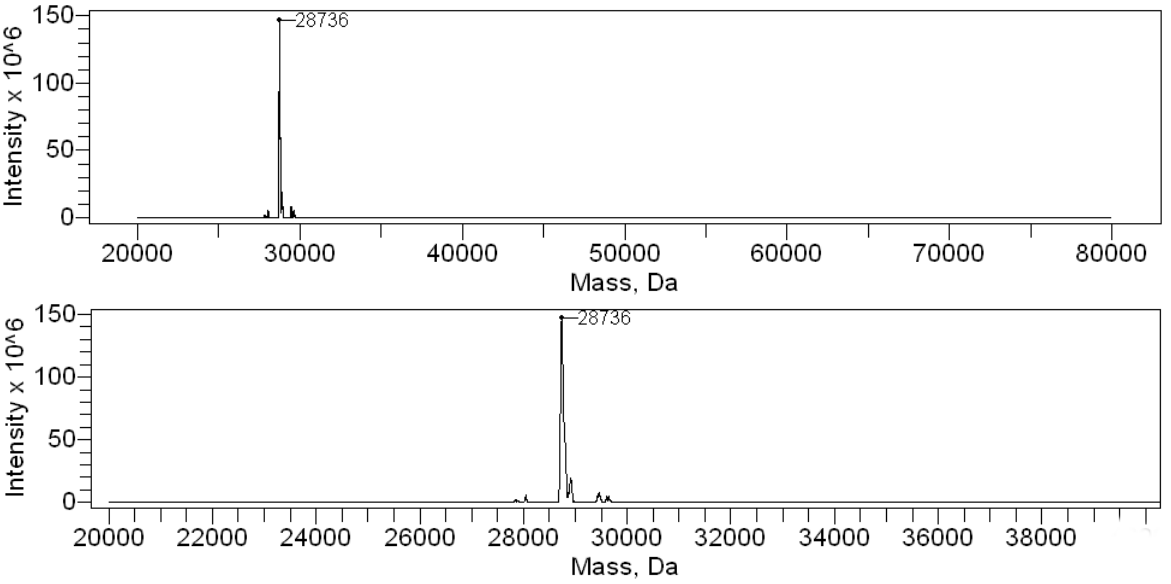
(a)



(b)



(c)



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(d)

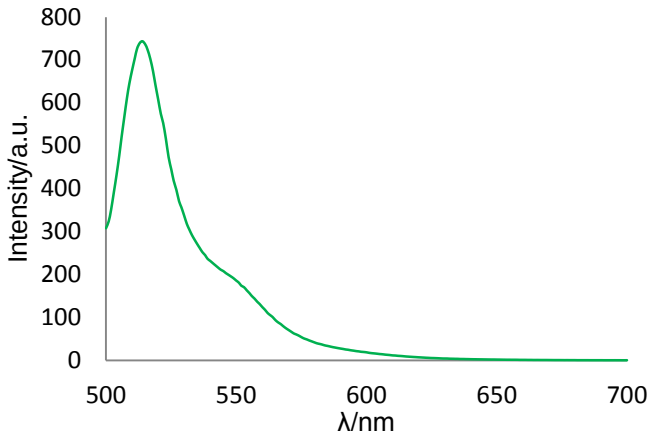
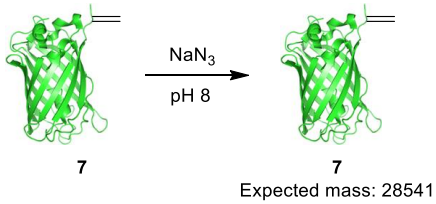


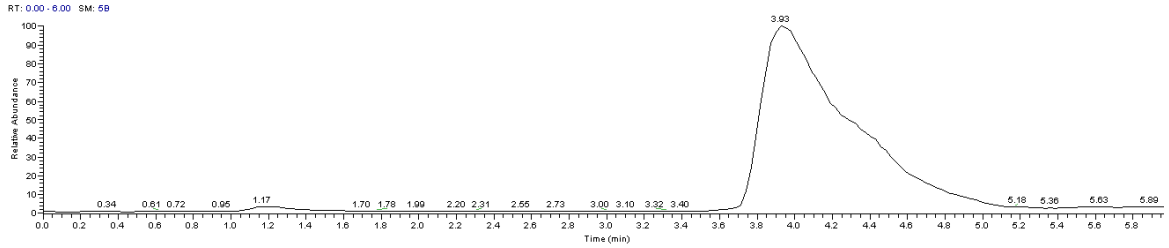
Figure S12. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Azide,T230Dha,233Δ) **10**.

Reaction of GFP(T230Dha,233Δ) 7 with sodium azide



Sodium azide (10 μL, saturated solution in water) was added to a solution of GFP(T230C,233Δ) **7** (1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, and maintained at 37 °C for 4 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 10,000 MWCO) and the mixture analysed by LCMS.

(a)



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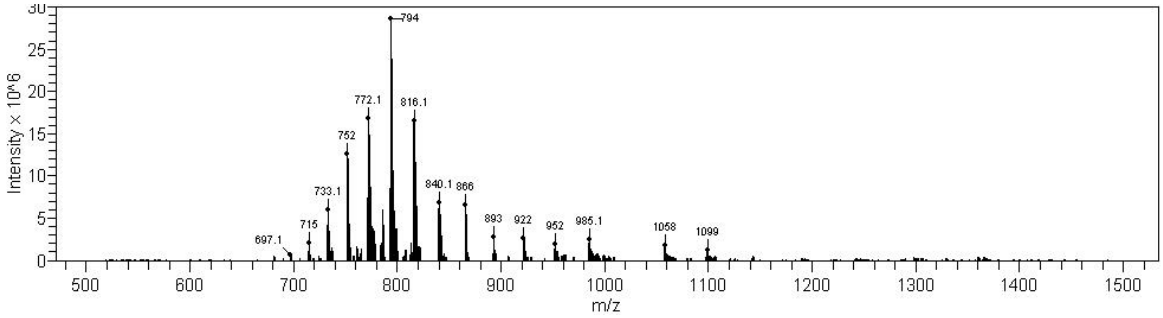
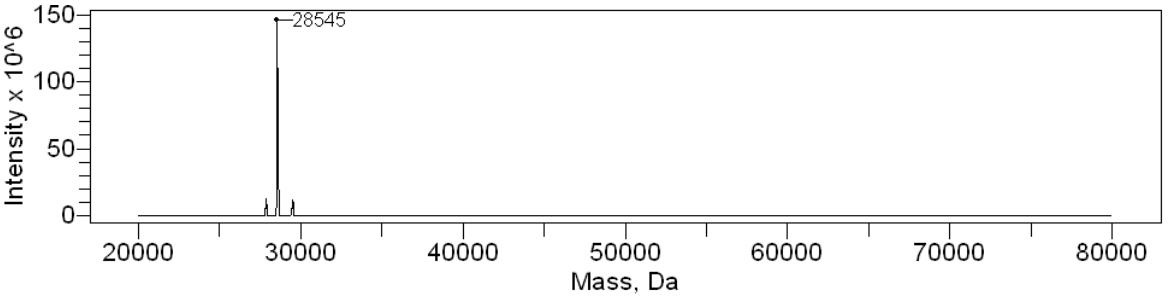
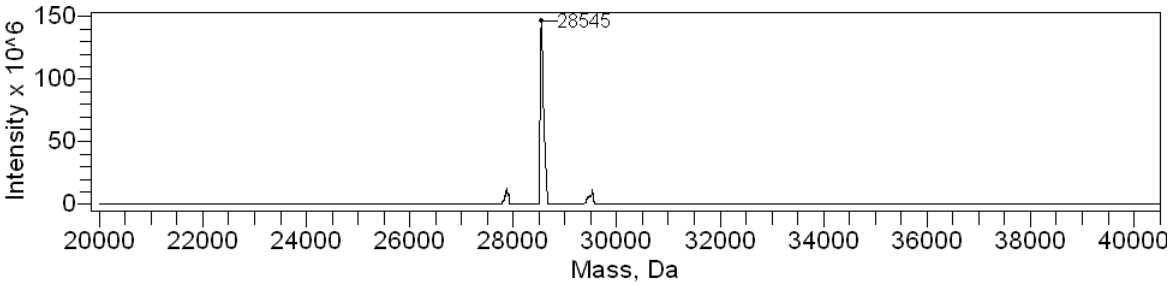


(c)





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(d)

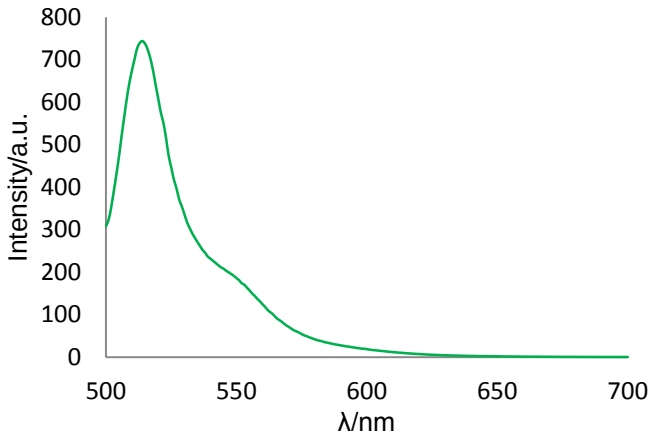
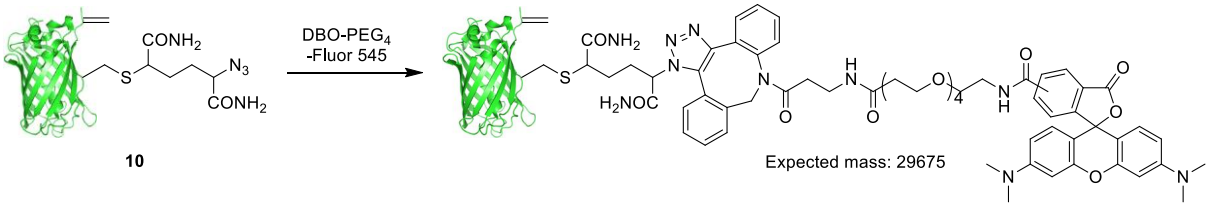


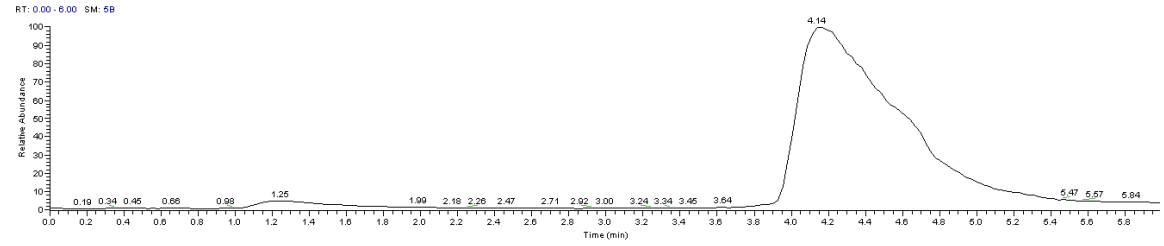
Figure S13. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for reaction of GFP(T230Dha,233Δ) **7** with sodium azide.

Reaction of GFP(S147Azide,T230Dha,233Δ) 10 with dibenzylcyclooctyne-PEG$_4$-Fluor 545



Dibenzylcyclooctyne-PEG$_4$-Fluor 545 (10 µL, 34 mM solution in DMF, 100 equivalents) was added to a solution of GFP(S147Azide,T230Dha,233Δ) **10** (100 µL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 21 °C for 3.5 h. Excess reagents were removed by repeated diafiltration into fresh buffer (100mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 10,000 MWCO) and the mixture analysed by LCMS.

(a)



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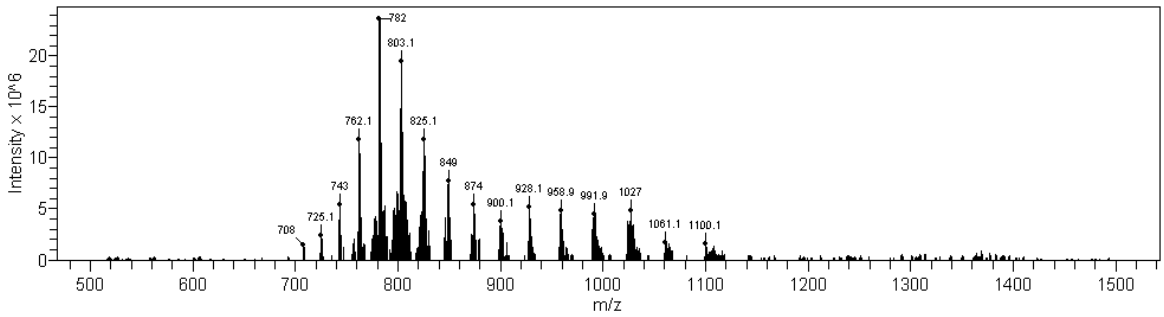
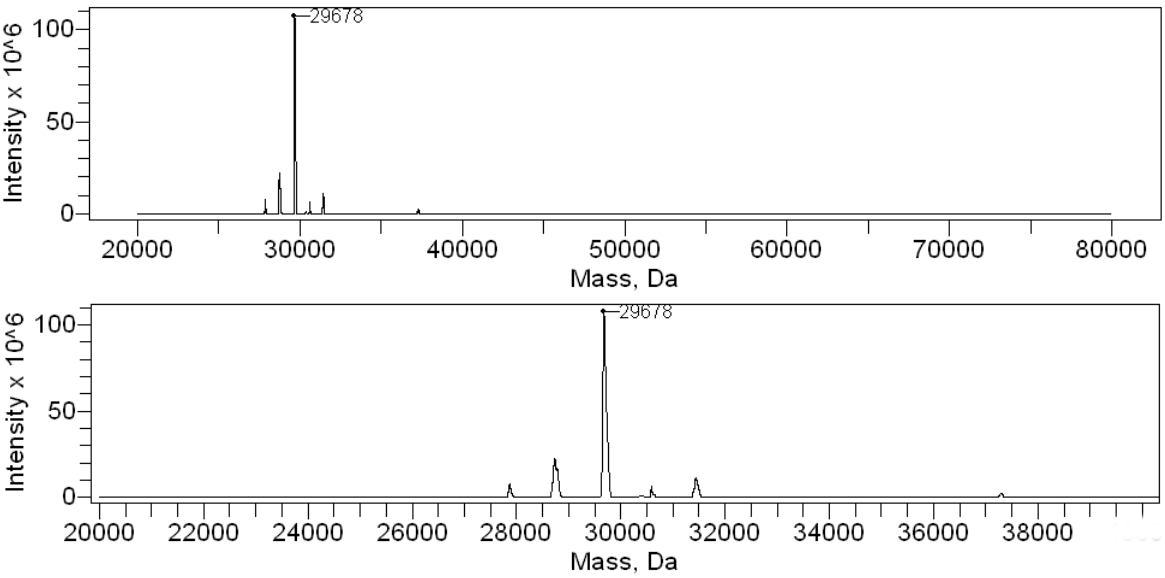


(c)



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(d)

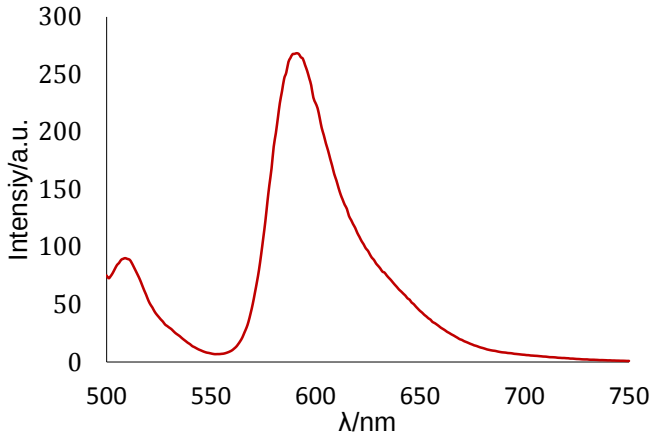
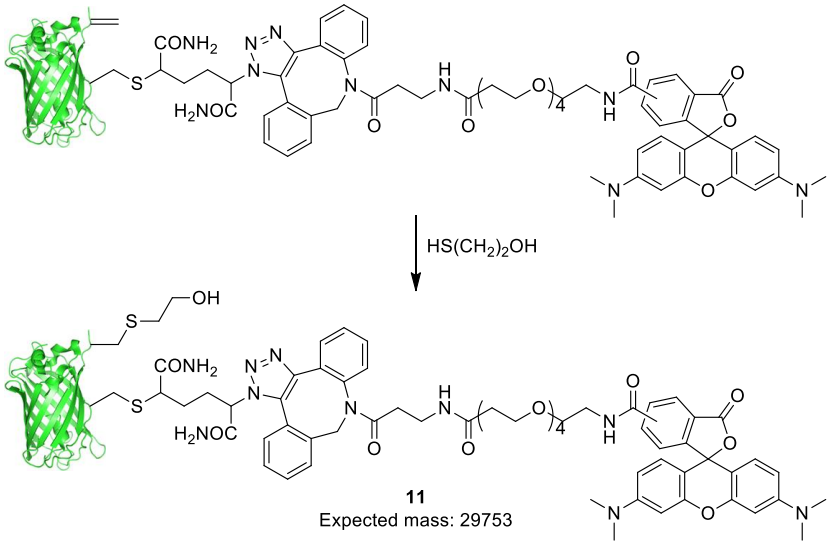


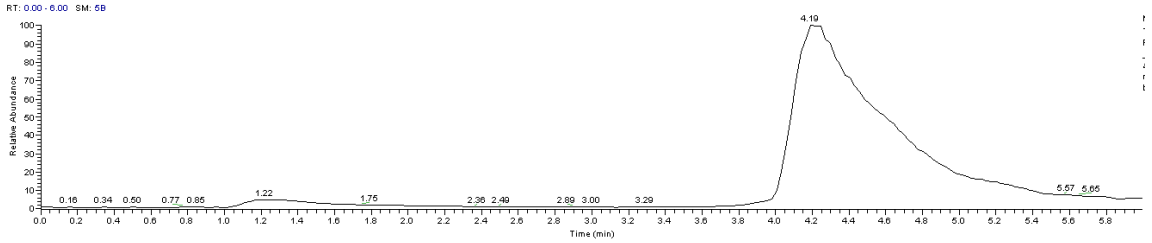
Figure S14. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Fluor545,T230Dha,233Δ).

Reaction of GFP(S147Fluor545,T230Dha,233Δ) with 2-mercaptoethanol



2-Mercaptoethanol (2 μL) was added to a solution GFP(S147Fluor545,T230Dha,233Δ) **10** (100 μL, 1.0 mg/mL) in sodium phosphate (100 mM, pH 8.0) at 21 °C. The mixture was vortexed for 1 s, maintained at 21 °C for 1 h. Excess reagent was removed by repeated diafiltration into fresh buffer (100mM sodium phosphate, pH 8.0) using VivaSpin sample concentrators (GE Healthcare, 10,000 MWCO) and the mixture analysed by LCMS.

(a)



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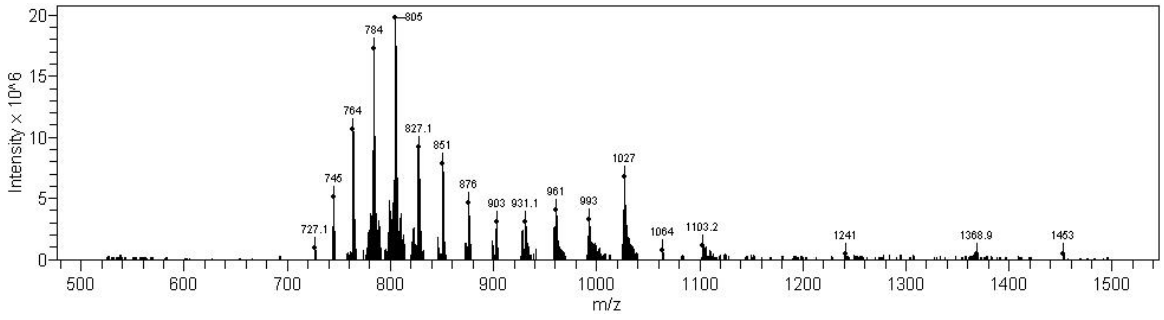

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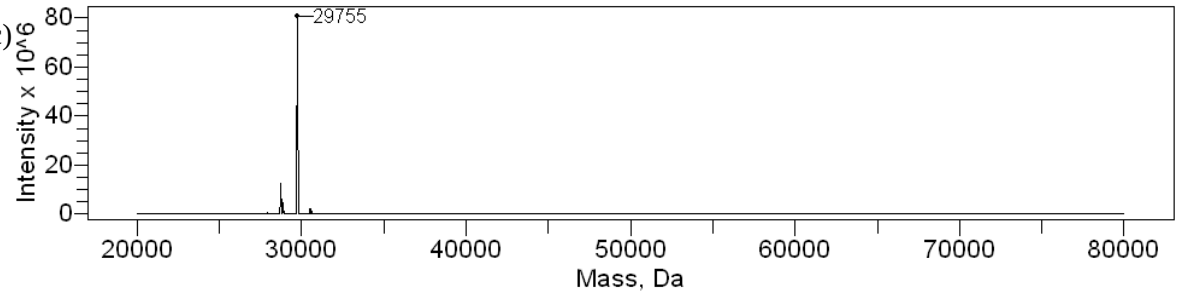

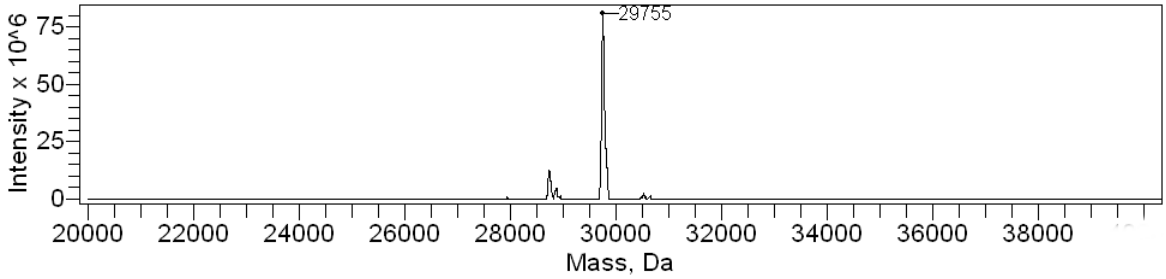

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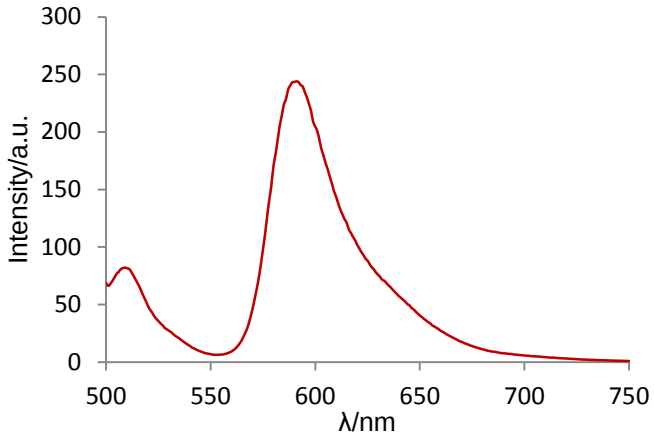

Figure S15. (a) TIC, (b) non-deconvoluted, (c) deconvoluted MS data, and (d) fluorescence emission spectrum for GFP(S147Fluor545,T230BME,233Δ) **11**.

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1. (a) Images generated using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC; and (b) P. Kuhn, M. Knapp, S. M. Soltis, G. Ganshaw, M. Thoene and R. Bott, *Biochemistry*, 1998, **37**, 13446-13452.

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3. (a) R. Nathani, P. Moody, M. E. B. Smith, R. J. Fitzmaurice and S. Caddick, *ChemBioChem*, 2012, **13**, 1283-1285; and (b) P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy and S. Caddick, *ChemBioChem*, 2012, **13**, 39-41.

4. S. Hubbard and J. Thornton, Naccess, 1993, University of Manchester, Manchester, U.K. based on B. Lee, F. M. Richards, *J. Mol. Biol.*, 1971, **55**, 379-400.