Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Molecular Fe-complex as a catalyst probe for in-gel visual detection of proteins via signal amplification

Sushma Kumari[†#], Chakadola Panda[†#], Shyamalava Mazumdar[‡] and Sayam Sen Gupta[†*]

[†] Chemical Engineering and Process Development Division, National Chemical laboratory, Dr Homi Bhabha Road, Pune, India

[‡] Division of Chemical Sciences, Tata Institute of Fundamental Research, Colaba, Mumbai-400005, India

[#]Both authors contributed equally

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Materials and methods

(a) Materials

5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) , Maleic anhydride, Alanine and N-hydroxy succinamide, (diethyl-amino)sulfur trifluoride (DAST), 3 ,3′, 5, 5′-Tetramethylbenzidine (TMB), Aminoguanidine hydrochloride, Acrylamide, N,N'-Methylenebisacrylamide, tris(hydroxymethyl)aminomethane (Tris), Sodiumdodecylsulphate (SDS), Ammonium presulphate (APS), N,N,N′,N′-Tetramethylethylenediamine (TEMED), Glycine, were obtained from Aldrich. O-(2-Aminoethyl)-O′-(2-azidoethyl)pentaethylene glycol (the pegyl amino-azide linker) was obtained from Polypure. Econo-Pac® Chromatography Columns, Bio-Gel P-6DG Gel, centrifugal filter tubes (MW = 10K) were purchased from BIO-RAD. Alkyne-biuret-Fe-TAML[1] and Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) [2] were prepared as reported earlier.

(b) Biological materials

Protein bio-markers (6,500 to 67,000 Da), Human serum albumin (HSA; mol wt. 66.5 kDa), Bovine serum albumin (BSA; mol wt. 66.5 kDa), Pepsin (mol wt. 42 kDa), Carbonic anhydrase (mol wt. 30 kDa), Chymotrypsin (mol wt. 25 kDa), Trypsin (mol wt. 23.3 kDa), Trypsinogen (mol wt. 25 kDa), and Chymotrypsinogen (mol wt. 26 kDa) were obtained from Sigma-Aldrich and used without further purification

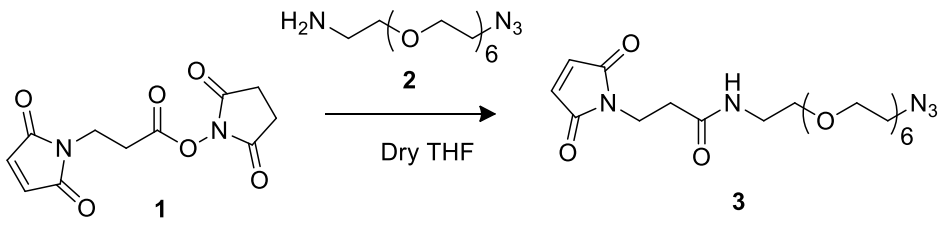
(c) Methods

Thiol estimation (Ellman's test)

Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used for the estimation of free thiol groups in BSA and BSA-N_3 [3]. An excess of DTNB in phosphate buffer (0.1 M, pH 8) was incubated with BSA and BSA-N_3 for 25 min in the dark at room temperature. Free thiols react with DTNB and generates 2-nitro-5-thiobenzoate anion whose absorption co-efficient (13.6 mM^{-1} cm^{-1} at 412 nm) was used to quantify the free thiol in the protein. The intrinsic low absorbance of BSA-N_3 at this wavelength accounted for complete conjugation of free thiols of Bovine Serum Albumin (BSA) to maleimide.

2. Synthesis

(a) Synthesis of maleimide-azide linker (3)



The maleimide-NHS linker (**1**) was prepared by following a procedure reported before [4]. To a 0.75 ml solution of **1** (7 mg; 26 mmol, 1eq) in dry THF was added a solution of azido-peg-amine (**2**; 10 mg; 28.5 mmol, 1.1 eq) prepared in 0.75 ml of dry THF and stirred at room temperature for 1hr. The reaction was monitored by TLC over time. After completion of the reaction, the product maleimide-azide (**3**) linker was taken out and kept at -20 °C for further protein conjugation reaction without any further purification. Considering 100% consumption of **1**; concentration of **3** was assumed to be 17.3 mM. HR-MS showed m/z values 502 corresponding to the M-H^+ species in the positive ion mode of the instrument (Figure S6).

(b) Labeling of the BSA with maleimide-azide

To a solution of BSA (2 mg/mL; 1 mL) in 100 mM phosphate buffer pH 7.4 was added 40 µL of **3** (0.6 mM, 20 eq) in 40 µLDMSO and the resulting solution was shaken overnight at 4 ^{o}C. The reaction mixture was extensively purified by dialysis against 100 mM phosphate buffer pH 7.4 over 24 hour while changing the buffer after every four hours. The concentration of purified BSA-N_3 congugate was confirmed by Bradford assay (~ 1.8 mg/mL) and was further used for protein-conjugation reactions.

(c) Conjugation of biuret-Fe-TAML on to BSA-N_3 using azide-alkyne click reaction (CuAAC)

The following click conjugation protocol was adopted from a previous report by Finn *et al*. with slight modifications [2]. In a 2mL eppendorf, 56 µL of BSA-N_3 (1.8 mg/ml) and 97 µL of 100 mM phosphate buffer pH 7.4 were added. To this solution were added 7.5 µL of alkyne-biuret-Fe-TAML (20 mM), 10 µL of premixed solution of $CuSO_4$ and THPTA ligand (stock solution containing 10 µL of 20mM $CuSO_4$ and 20 µL of 50mM THPTA ligand) and 10 µL of aminoguanidine hydrochloride (100mM). The reaction mixture was well mixed with the help of a vortex and degassed by bubbling with N_2 gas (to remove O_2) followed by addition of 20 µL of freshly prepared sodium ascorbate (100mM) solution under positive flow of N_2. The final concentration of BSA-N_3 in the reaction mixture therefore amounted to 0.5mg/ml. After addition of sodium ascorbate, the reaction mixture was stirred for an additional hour. For analysis by gel electrophoresis, 10 µL of the reaction mixture was added to 90 µL of 100 mM pH 7.4 phosphate buffer and 100 µL of 1X SDS loading buffer. This solution was then analysed by SDS-PAGE.

(d) Gel electrophoresis and substrate development

The click reaction mixture containing SDS loading buffer was further serially diluted and loaded onto a 12% polyacrylamide gel (1.0 mm thickness) to obtain the concentration of protein in the range of 100 - 5 ng/well. The gel was then subjected to electrophoresis at a constant potential of 100 mV for typically 70 min. The bromo phenol blue indicator from the loading gel and the unreacted catalyst being small molecules are expected to run much faster than proteins. Typically electrophoresis was terminated when the bromophenol blue reached the terminal of the gel. After completion of gel electrophoresis, the gel was taken out of the

electrophoresis gel cassette, washed 4 to 5 times with deionized water and the gel was cut into two parts. The first part which included the biomarker was stained using 0.5% Coomassie blue, followed by destaining to remove Coomassie blue background. To the second part of the gel, a mixture of H_2O_2 (100 µL; 80 mM) and TMB (100 µL; 0.5 mM) in 40 mM phosphate buffer pH 7.4 was added with gentle agitation for 2 minutes. A nice blue coloured band appeared on the gel at a position where the BSA-Fe-TAML conjugate is expected based on the protein marker. The gel was then incubated with 50 mM phosphate buffer pH 4.5 and the color of the blue band intensified due to the higher extinction co-efficient of the oxidized TMB at pH 4.5.

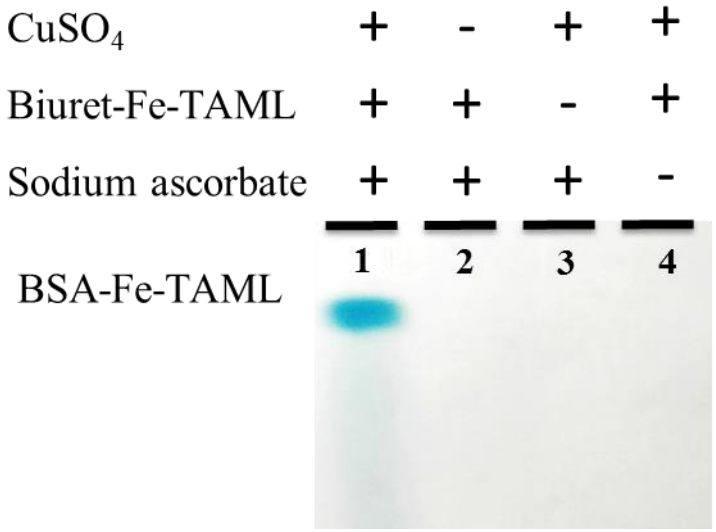


Figure S1. SDS-PAGE analysis to confirm the traizole formation upon click reaction with BSA-N_3 and alkyne-biuret-Fe-TAML. The gel was treated 0.05 mM TMB / 20 mM H_2O_2 for visualisation of the protein bands. Lane 1: presence of all three components; Lane 2: absence of $CuSO_4$; Lane 3: absence of alkyne-biuret-Fe-TAML and; Lane 4: absence of sodium ascorbate.

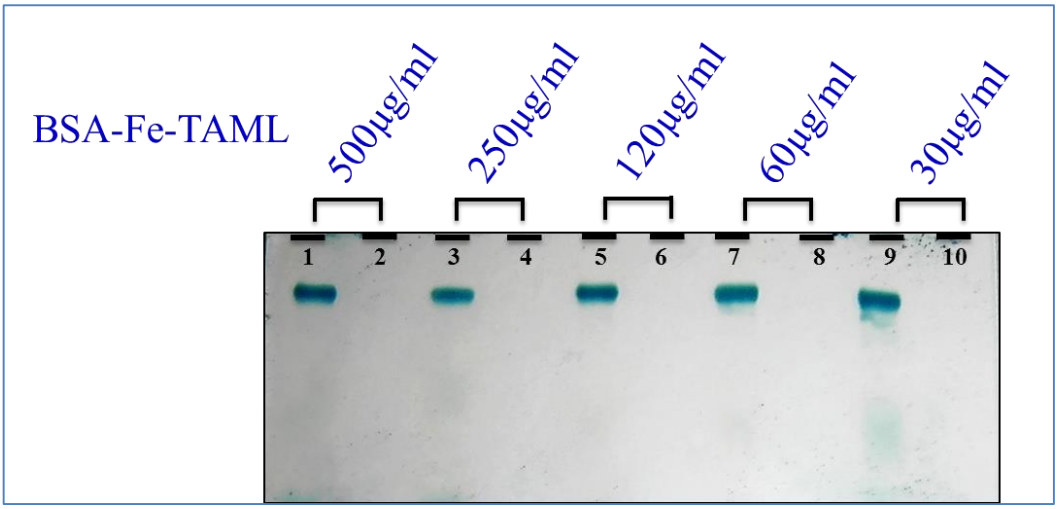


Figure S2. SDS-PAGE analysis for the click reaction between BSA-N_3 and alkyne-biuret-Fe-TAML at various concentrations of BSA-N_3 (500 - 30 µg/mL). The reaction mixture was quenched with SDS-loading buffer and analysed by SDS-PAGE by loading 150 ng of protein in each well. Protein bands were visualized on the gel after treatment with 0.05 mM TMB / 20 mM H_2O_2. Lanes: 1, 3, 5, 7 and 9 correspond to different click reaction mixtures with same amount of protein conjugates loaded; Lane: 2, 4, 6, 8 and 10 correspond to control reactions in which all components of "click reaction" were added with the exclusion of sodium ascorbate.

(e) CuAAC for the conjugation of fluorescein alkyne on to the BSA-N_3 and in-gel fluorescence imaging

Fluoresceine alkyne was prepared by following a procedure as reported by Finn et al. [1] and was used for protein conjugation reaction. The same procedure of click conjugation was followed as was done with alkyne biuret-Fe-TAML and concentrations of the reagents being same as well. After gel electrophoresis the gel was analysed with the help of a Gel Doc to visualize the protein bands corresponding to the fluorescein-BSA conjugates.

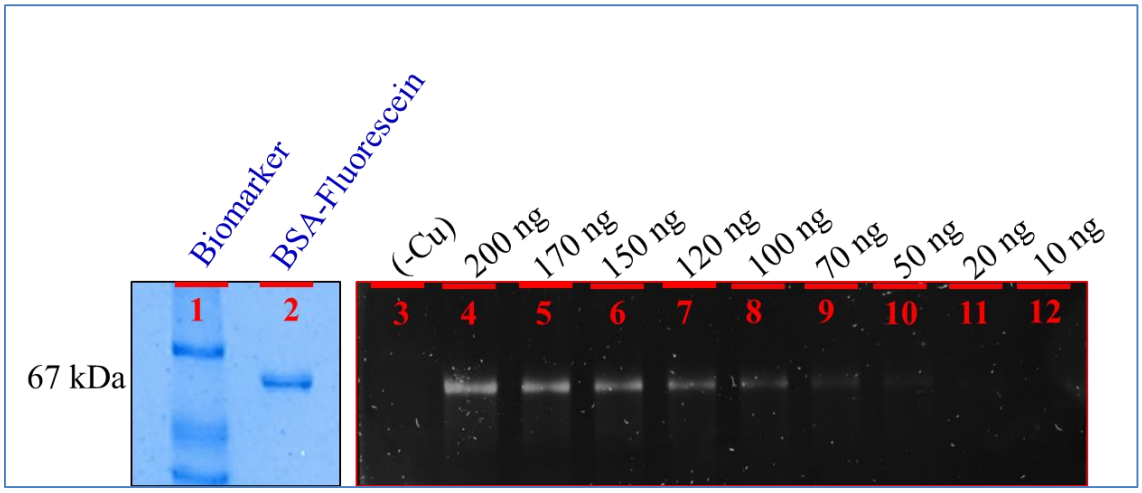
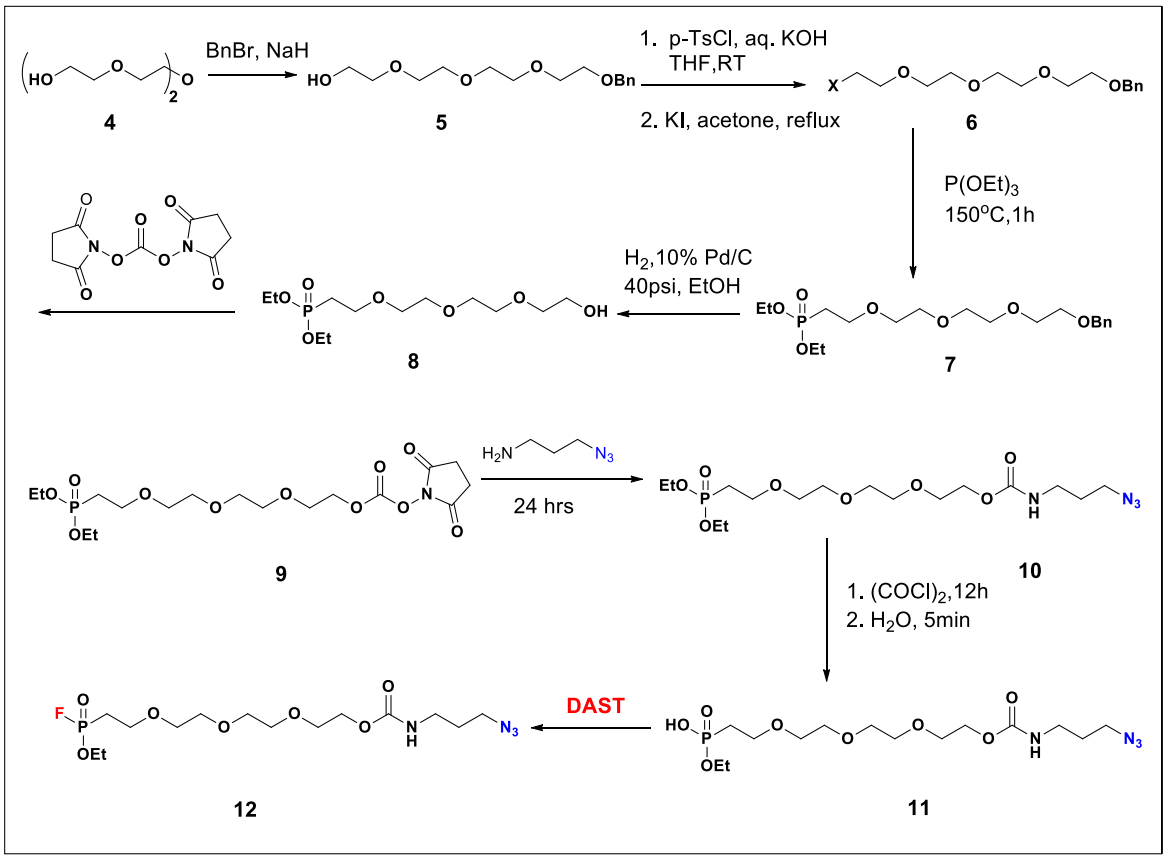


Figure S3. Visualization of the reaction mixture after CuAAC with fluorescein-alkyne and BSA-N_3 by Gel Doc. Lane 1and 2: Coomassie staining of biomarker and fluorescein conjugated BSA-N_3 respectively; Lane 3-12: Fluorescence imaging of protein bands using Gel Doc. Lane 1: biomarker; Lane 2: BSA-Fluorescein (200ng); Lane 3-12: BSA-Fluorescein with varying amounts of protein (200 - 10 ng) loaded in each well.

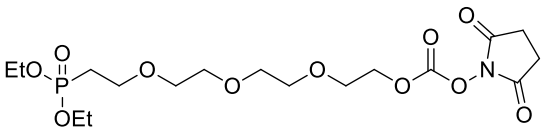
(f) Synthesis of FP-N_3 (12)



Scheme S1: Synthetic scheme to FP-N_3 probe

Synthesis of FP-N_3 probe

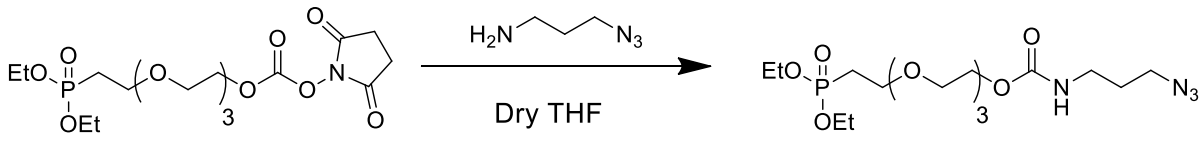
The synthetic scheme for the probe FP-N_3 is delineated in scheme SI XX.



9

As illustrated in scheme **1**, compound **9** was synthesized by following the procedure reported by Showalter et al [5]. The structural assignment of this compound was supported by diagnostic peaks in the ^{1}H NMR (Figure S12).

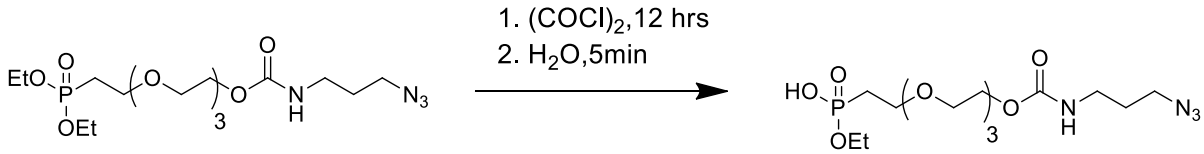
1H NMR ($CDCl_3$, 200 MHz,) δ/ppm: 4.47-4.42 (m, 2H), 4.11-4.00 (m, 4H), 3.76-3.61 (m, 12H, 2.82 (s, 4H), 2.20-2.02 (m, 2H), 1.33-1.26 (t, 6H).



9 **10**

Compound 10. Azido propyl amine was synthesized as reported before [6]. To a solution of **9** (0.50 g, 1.1mmol, 1.0 equiv) in 5mL dry THF was added 3-azidopropyl amine (0.330 g, 3.29 mmol, 3 eq) and the reaction mixture was stirred at room temperature for12 hrs. The solvent was then removed, the resulting residue was dissolved in CH_2Cl_2 (10 mL) and subsequently washed with H_2O (10 mL) and saturated aqueous NaCl (10 mL). The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The compound was purified by silica-gel chromatography while eluting with ethyl acetate/methanol (90:10) followed by concentration of product fractions to afford **10** as light brown oil. Yield: 0.400 g (82%)

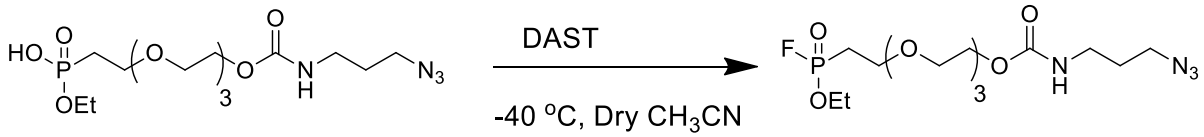
1H NMR ($CDCl_3$, 200 MHz,) δ/ppm: 5.93 (s, 1H), 4.20- 4.14 (m, 2H), 4.10-3.99 (m, 4H), 3.75-3.54 (m, 12H), 3.37-3.21 (m, 4H), 2.18-2.00 (m, 2H), 1.79-1.72 (m, 2H), 1.32-1.25 (t, 6H)



10 **11**

Compound 11: To a solution of **10** (100 mg, 0.227 mmol, 1.0equiv) in dry DCM at 0 °C was added oxalyl chloride (0.078µL, 0.908 mmol, 3.7 equiv). After the addition, the mixture was stirred at room temperature for 18 h. The reaction mixture was then concentrated under a stream of gaseous nitrogen and the remaining residue was treated with H_2O (500µL) for 5 min. The solvent was evaporated under a stream of gaseous nitrogen and the remaining residue dried by vacuum to provide **11** Yield: 80mg (85%)

1H NMR ($CDCl_3$, 200 MHz,) δ/ppm: 8.89 (s, 1H), 4.39- 4.08 (m, 4H), 3.77-3.63 (m, 12H), 3.37-3.20 (m, 4H), 2.17-2.04 (m, 2H), 1.80-1.86 (m, 2H), 1.33-1.26 (t, 3H)



11 **12**

Compound 12: To a solution of monoethyl phosphonate polyether carbamate **11** (50 mg, 0.121 mmol, 1.0 eq) in 1.5 mL of anhydrous dichloromethane at − 42 °C was added (diethyl-amino) sulphur trifluoride (DAST; 48 µL, 0.364 mmol, 3.0 eq) and stirred for 30 min. The reaction mixture was quenched with water at −42 °C and stirring at room temperature for another 10 min. The reaction mixture was extracted with dichloromethane (3X). The obtained organic phase was dried and concentrated, which was further evacuated under high vacuum to afford **12** as a yellow oil which was used directly as inhibitor for serine proteases. Yield: 40mg (80%)

1H NMR ($CDCl_3$, 200 MHz,) δ/ppm: 4.41- 4.21 (m, 4H), 3.84-3.63 (m, 12H), 3.4-3.32 (m, 2H), 2.19-2.34 (m, 2H), 1.74-1.92 (m, 2H), 1.32-1.39 (t, 3H); 19F NMR (282 MHz, $CDCl_3$) δ −58.66, −61.49

(g) Selective labeling of serine hydrolases with FP-N_3 and subsequent CuAAC for the conjugation of biuret-Fe-TAML

Samples of each protein BSA, HSA, pepsin, carbonic anhydrase, trypsin, chymotrypsin, trypsinogen, chymotrypsinogen (1µM each) were incubated separately with FP-N_3 (40 µM) in 50 mM tris buffer (pH 8.0) at room temperature for 30min. The samples were quickly purified by size-exclusion chromatography using Bio-Spin disposable chromatography column filled with Bio-Gel-P-10 to remove unreacted FP-N_3. 100 µL of the reaction mixture was loaded onto 1 mL of Bio-Gel-P-10 centrifuged one time at 500 rpm for 30 sec. The purified protein solution (protein labelled with FP-N_3) was clicked with the alkyne-biuret-Fe-TAML complex. 200 µL of FP-N_3 labelled protein (1 µM) was added to an eppendorf. To this was added 163 µL of 100 mM phosphate buffer pH 7.4 followed by addition of 4 µL of alkyne tailed biuret-Fe-TAML (20 mM), 3 µL of premixed solution of $CuSO_4$ and THPTA ligand (10 µL of 20 mM $CuSO_4$ and 20 µL of 50 mM THPTA ligand stock) and 10 µL of 100 mM aminoguanidine hydrochloride. The reaction mixture was well mixed with the help of a vortex and degassed by bubbling with N_2 gas (to remove O_2) followed by addition of 10 µL of freshly prepared sodium ascorbate solution under positive flow of N_2. For analysis by gel electrophoresis, 10 µL of the reaction mixture was added to 90 µL of 100 mM pH 7.4 phosphate buffer and 100 µL of 1X SDS loading buffer. This solution was then analysed by SDS-PAGE. The gel was removed from the electrophoresis cassette and treated with a mixture of H_2O_2 (100 µL; 80 mM) and TMB (100 µL; 0.5 mM) in 40 mM phosphate buffer pH 7.4 with gentle agitation for 2 minutes. A nice blue coloured band appeared for the serine proteases labelled with FP-Fe-TAML on the gel. The colour of the blue band was further intensified by incubating with 50 mM phosphate buffer pH 4.5. However, such blue bands were not seen for the non-serine proteases like BSA, HSA, pepsin, carbonic anhydrase and the zymogen of the serine proteases like chymotrypsinogen and trypsinogen although their presence in the gel was confirmed by Coomassie staining (Figure S5).

Other control experiment where the serine proteases (trypsin) were subjected to auto-digestion for 30 minutes (Lane 4; Figure S4 below) were carried out to rule out nonspecific binding of FP-N_3 to the protein.

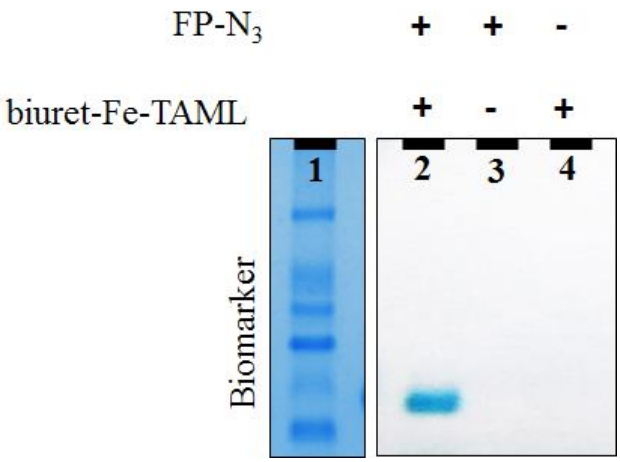


Figure S4. SDS-PAGE analysis to confirm the covalent attachment of FP-N_3 labelled trypsin with alkyne-biuret-Fe-TAML using CuAAC. The gel was treated 0.05 mM TMB / 20 mM H_2O_2 for visualisation of the protein bands. Lane 1: biomarker with Coomassie staining; Lane 2: trypsin labelled with FP-Fe-TAML; Lane 3: trypsin conjugation reaction in absence of biuret-Fe-TAML; Lane 4: trypsin conjugation reaction in absence of FP-N_3 (auto digested trypsin).

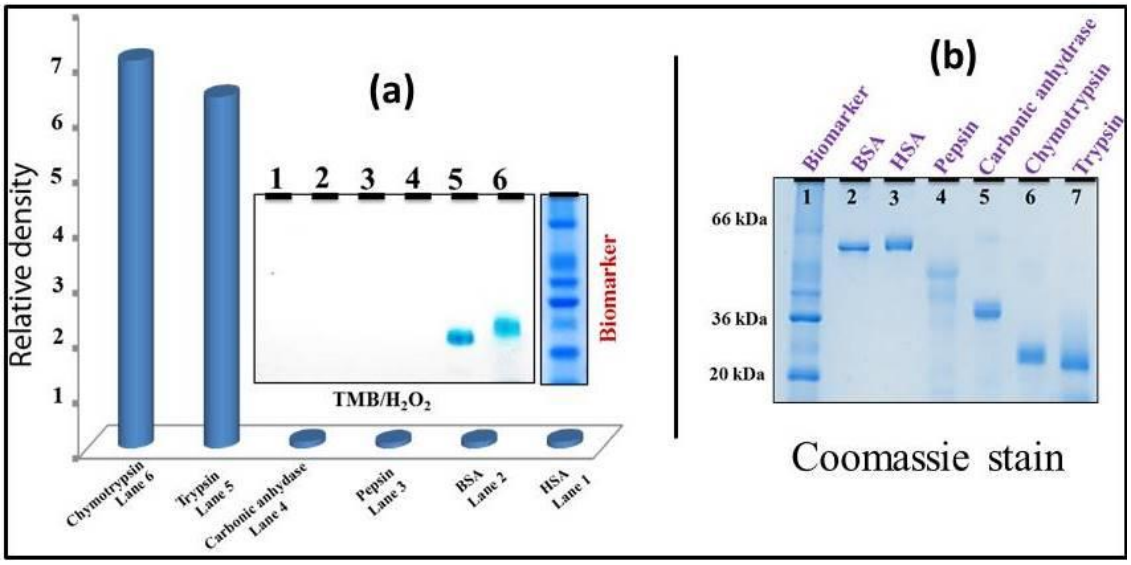


Figure S5. (a) Selectivity of FP-N_3/alkyne-biuret-Fe-TAML towards labelling of serine proteases over Carbonic Anhydrase, Pepsin, BSA and HSA. Relative intensity of the bands observed after treatment with TMB/H_2O_2 for Trypsin and Chymotrypsin (lane 5 and 6) was much higher than that of the other proteins (Lanes 1 – 4). The band intensity was analysed using Image J. Inset: SDS-PAGE gel of Trypsin, Chymotrypsin, Carbonic Anhydrase, Pepsin,

BSA and HSA after treatment with FP-N_3/alkyne-biuret-Fe-TAML and subsequently probed by TMB/H_2O_2. Lane 1- 4: HSA, BSA, Pepsin, Carbonic Anhydrase (100 ng/well). Lane 5 and 6: Trypsin and chymotrypsin (100 ng /well). (b) SDS-PAGE for the Coomassie stained proteins. Each well was loaded with 10µg of protein. Lane 1: Biomarker; Lane 2: BSA; Lane 3: HSA; Lane 4: Pepsin; Lane 5: Carbonic anhydrase; Lane 6: Chymotrypsin and; Lane 7: Trypsin.

(h) Selective labelling of serine proteases in presence of a mixture of proteins

A mixture of different proteins (BSA, pepsin, carbonic anhydrase and trypsin; 1 µM each) was incubated with FP-N_3 (100µM) in 50 mM tris buffer (pH 8.0) for 30min. The sample was purified by run through a PD-10 column to remove unreacted FP- N_3. To the purified protein solution, click reaction was subsequently performed to covalently attach the alkyne-biuret-Fe-TAML complex using CuAAC. The same procedure as said in the above experiment was followed to visualize the selectivity of this methodology. A nice blue coloured band was appeared for the trypsin labelled with FP-Fe-TAML on the gel. The colour of the blue band was further intensified by incubating with 50 mM phosphate buffer pH 4.5. However, no colored bands were observed for the non-serine protease proteins although their presence was confirmed by Coomassie staining with reference to the standard bio-marker ladder.

Characterizations

Instrumentations

Physical measurements

All the synthetic products were characterized by ^{1}H and ^{19}F NMR spectra recorded on a Bruker (200 MHz) spectrometer & these data are reported in δ (ppm) *vs*. $(CH_3)_4Si$ with the deuterated solvent proton residuals as internal standards. High Resolution Mass Spectrometry (HR-MS) was done in positive ion mode of a Thermo Scientific Q-Exactive, using electron spray ionization source, Orbitrap as analyzer and connected with a C18 column (150m × 4.6mm × 8µm). Electron spray ionization mass spectrometry (ESI-MS) was carried out in MS- Agilient 6540 UHD Accurate Mass QTOF. Mass Analyzer: Quadrupole Time Of Flight (QTOF).

Gel electrophoresis was carried out using a mini dual vertical electrophoresis unit from Tarson with a power supply unit from Genie, Bangalore, India.

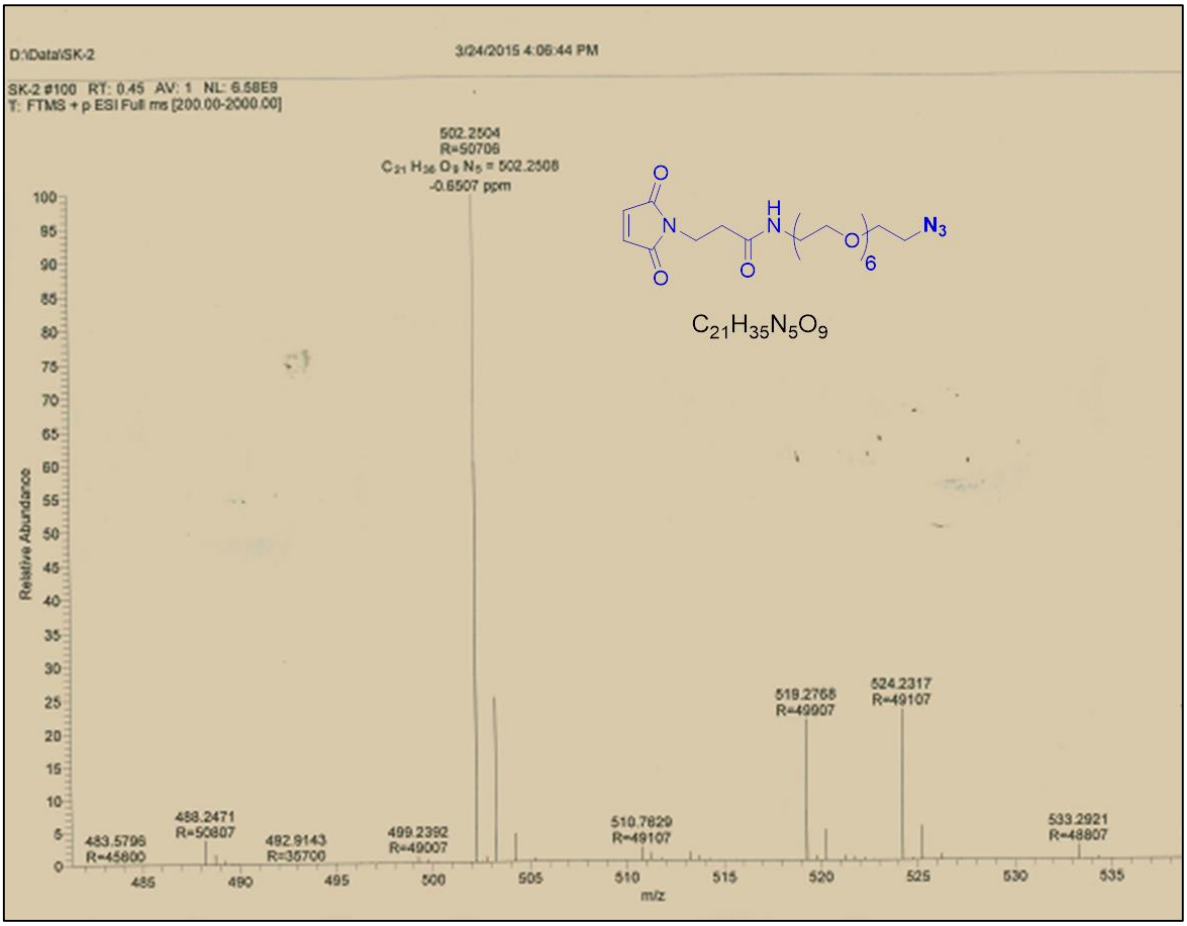


Figure S6. HR-MS spectrum of a maleimide-azide linker

Mass spectrometric characterizations of BSA-N_3 and BSA-Fe-TAML conjugate

To determine the labelling site, we subjected the BSA–maleimide-N_3 conjugate to a tryptic digestion and subsequently analysed by liquid chromatography/tandem mass spectrometry. Forty-five unique peptides were identified, and 65.57% sequence coverage was obtained. The molecular weight of the peptide containing amino acids 21 to 41 [GLVLIAFSQYLQQ***C***PFDEHVK] had an increase of 501 Da (corresponding to maleimide-N_3), thus showing that the modification occurred at Cys34 upon reaction with maleimide–N_3 (Figure S7) . Labelling of maleimide-N_3 to BSA was further confirmed by ESI-MS (Figure S10), which showed an increase of molecular mass 501 Da. For the subsequent covalent attachment of biuret-Fe-TAML, CuAAC reaction was performed with BSA-N_3 (0.5 mg/mL) and alkyne biuret-Fe-TAML in the presence of $CuSO_4$, THPTA and sodium ascorbate for one hour at 4°C. Successful labelling of BSA with biuret-Fe-TAML was confirmed by ESI-MS,

which showed an increase of mass by 1009 Da from the parent BSA (Figure S11). This increase corresponds to addition of both maleimide-N_3 linker and alkyne- biuret-Fe-TAML.

Proteomics of BSA-N_3 conjugate

Stock solution preparation

BSA-N_3: 10µg/µL

Ammonium bicarbonate: 50mm

Dithiothriotol (DTT): 100mm

Iodoacetamide (IAA): 200mm

Trypsin: 1µg/µl

100µg of BSA-N_3 was reduced with 10 mM DTT, alkylated with 55 mM IAA and then kept for overnight digestion with trypsin at 37°C. Digested peptides were extracted with 5% formic acid in 50% acetonitrile and were reconstituted in 5 µl of 0.1% formic acid in 3% acetonitrile. The digested sample was analysed by liquid chromatography-mass spectrometry LC-MS/MS.

BSA-N_3:

DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTL
FGDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGK
YLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQK
FGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTIS
SKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEY
AVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIV
RYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCT
ESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTV
MENFVAFVDKCCAADDKEACFAVEGPKLVVSTQTALA

Total No Cysteine residues: 35
Modified Cysteine site No: C34

Modified Peptide: GLVLIAFSQYLQQCPFDEHVK
Sequence: GLVLIAFSQYLQQCPFDEHVK
C14-Azido (501.24000 Da) Charge: +3, Monoisotopic m/z: 979.50238 Da, MH+: 2936.49259 Da, RT: 33.47 min.

Modified peptide sequence

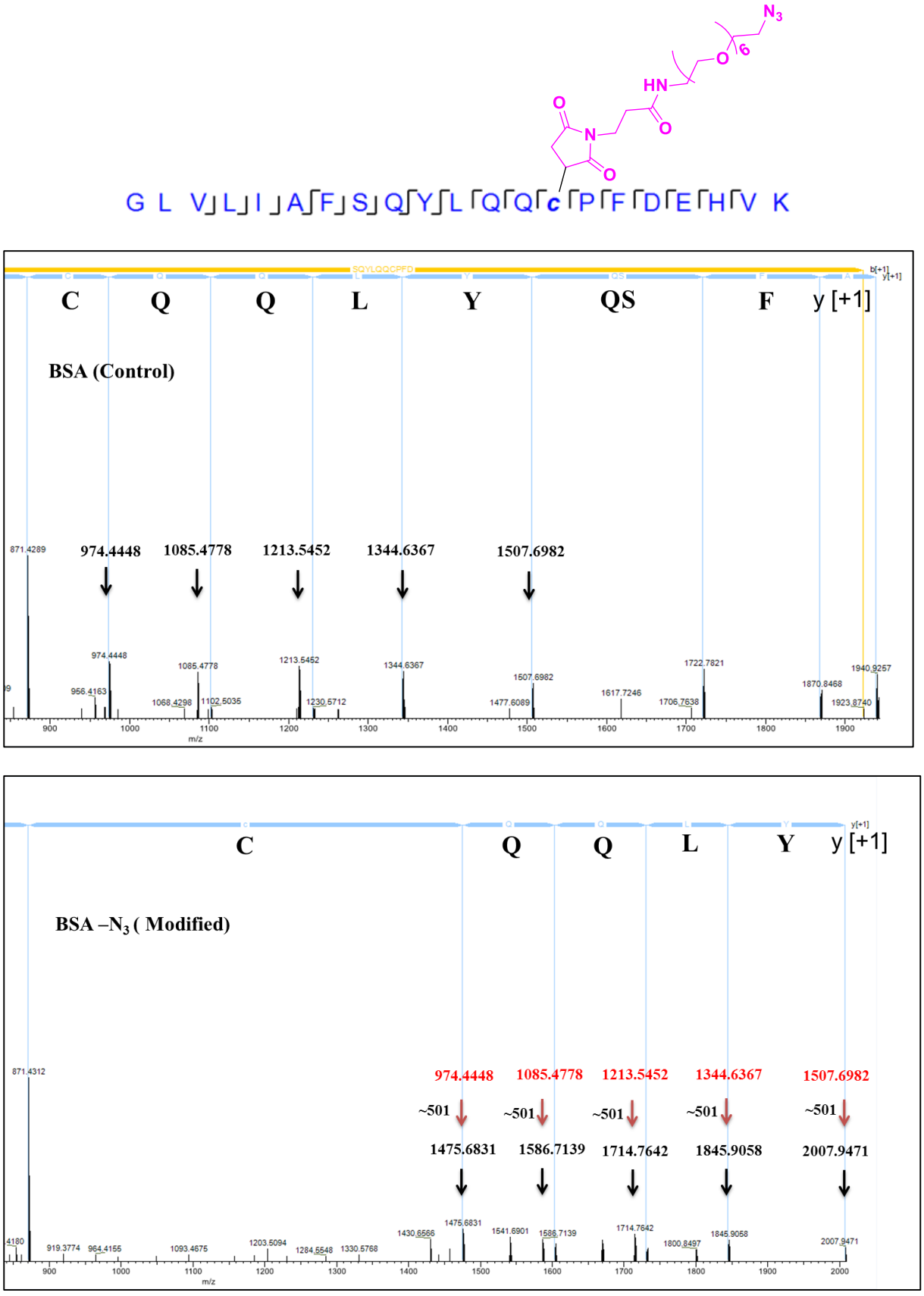


Figure S7. Product ion spectrum of modified peptide obtained on HR-MS/MS

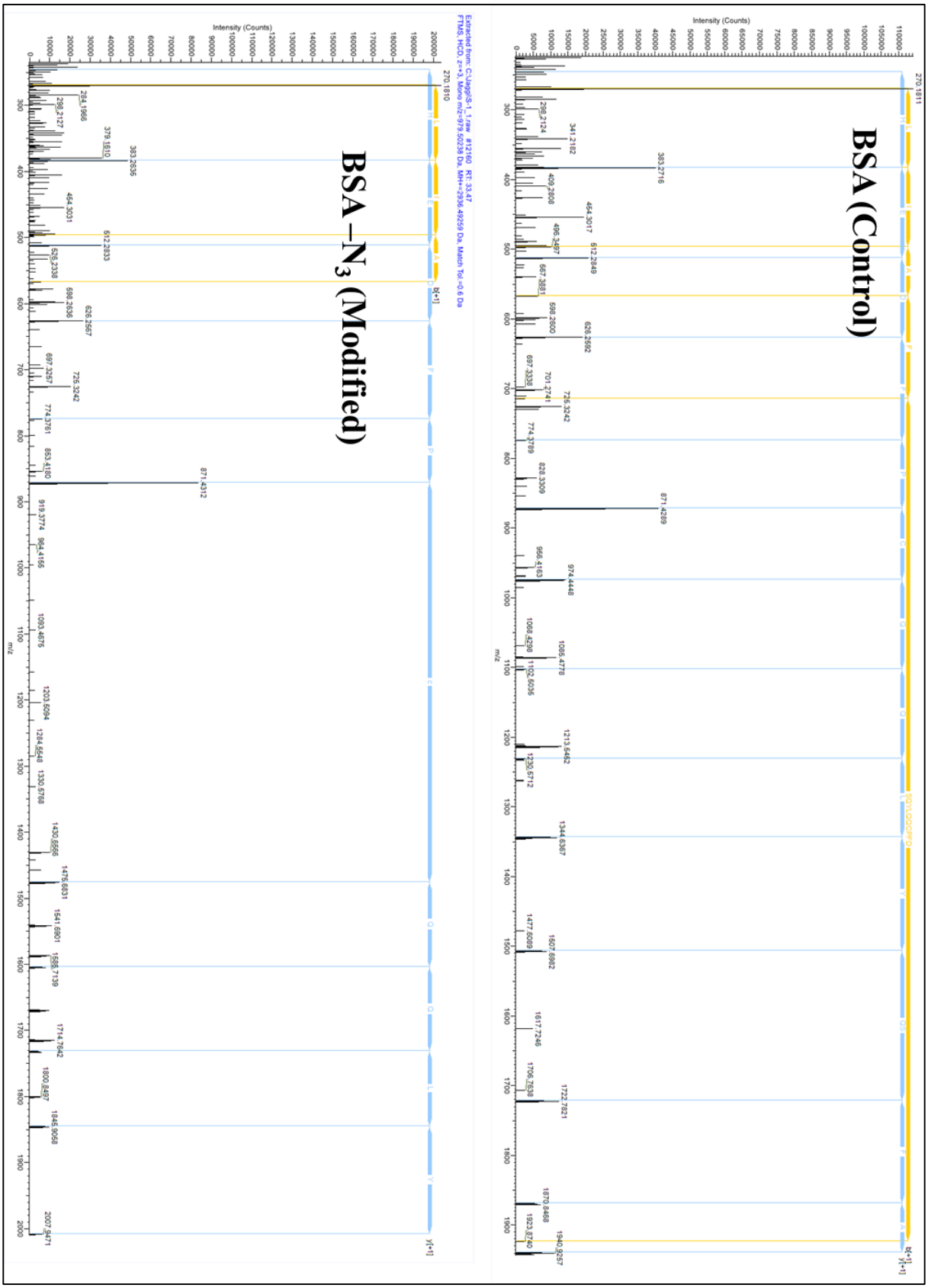


Figure S8. Product ion spectrum of modified peptide obtained on HR-MS/MS

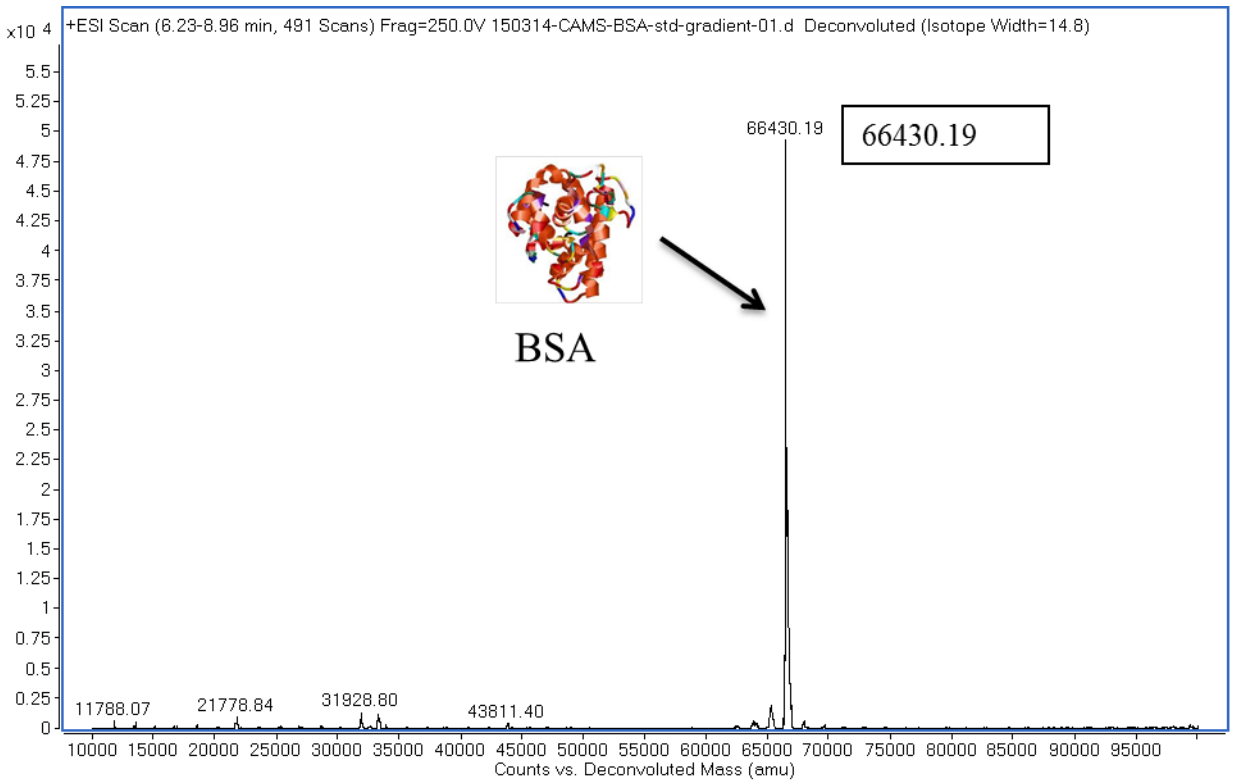


Figure S9. LC-ESI-TOF-MS characterisation of BSA (samples analysed as mobile phase of 0.1% formic acid in water and acetonitrile). Deconvoluted mass spectra of BSA showing peak at 66430.19.

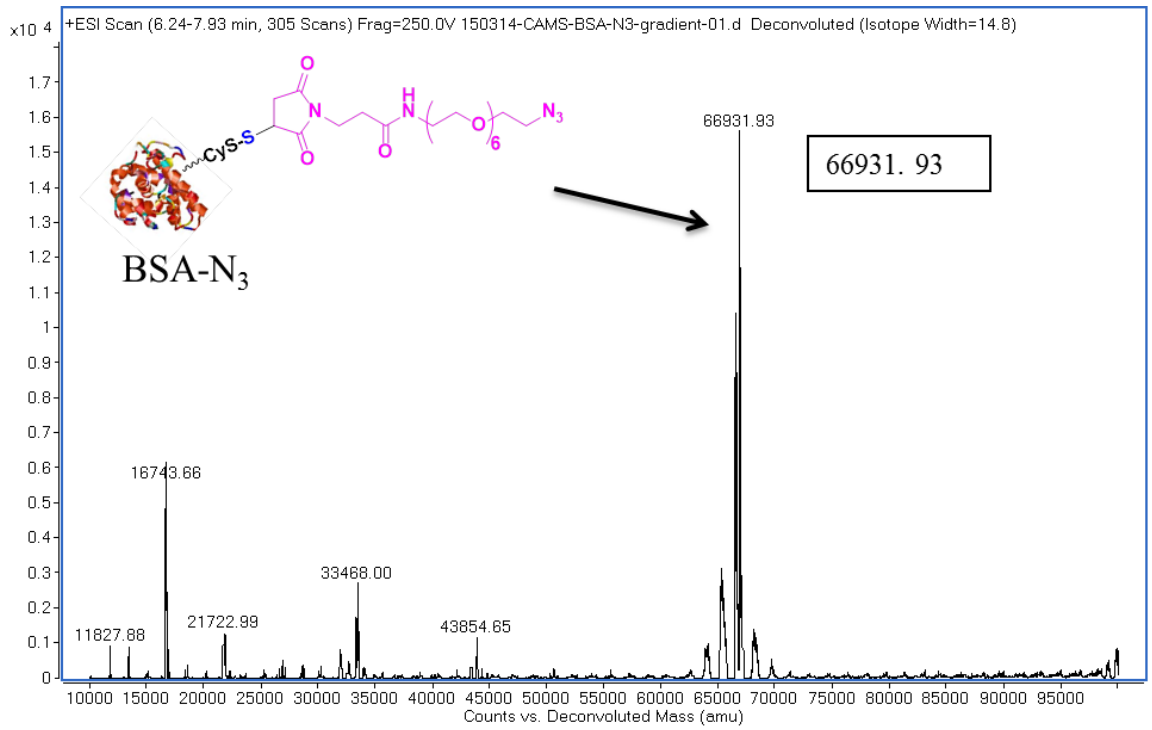


Figure S10. LC-ESI-TOF-MS characterisation of BSA-N_3 (samples analysed as mobile phase of 0.1% formic acid in water and acetonitrile). Deconvoluted mass spectra of BSA-N_3 showing peak at 66931.93.

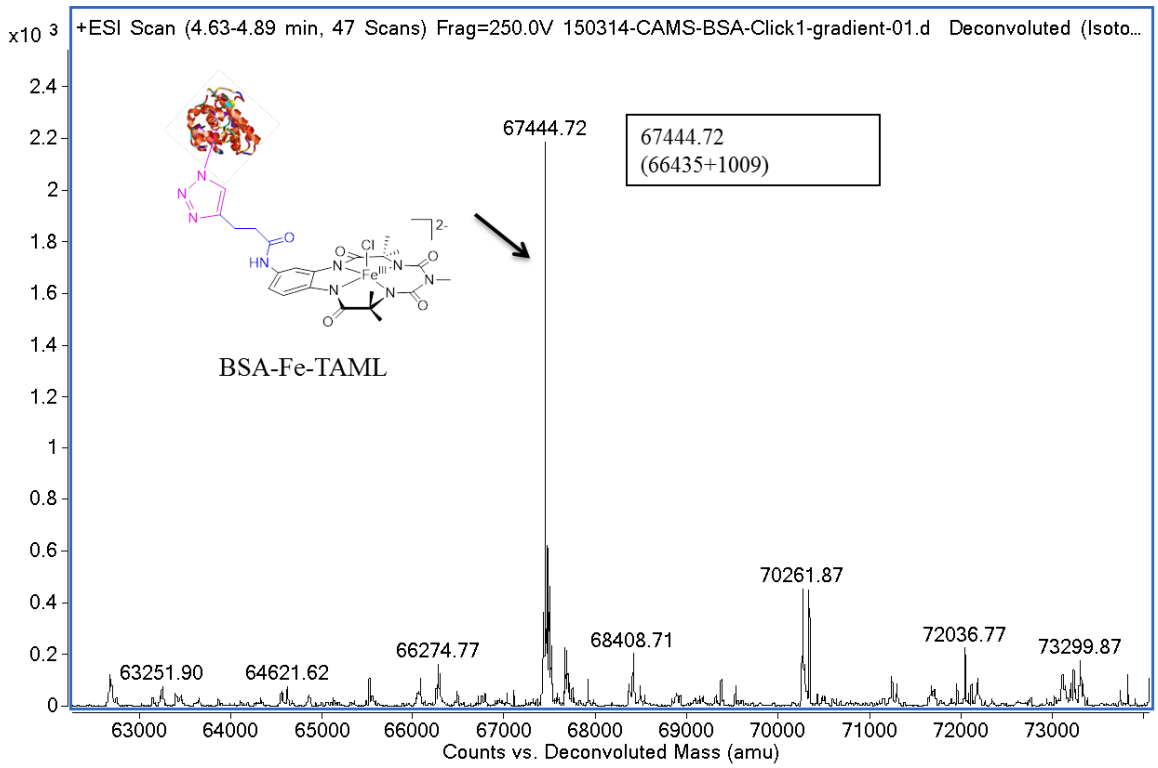


Figure S11. LC-ESI-TOF-MS characterisation of BSA-Fe-TAML (samples analysed as mobile phase of 0.1% formic acid in water and acetonitrile). Deconvoluted mass spectra of BSA-Fe-TAML showing peak at 67444.72.

^{1}H NMR and ^{19}F NMR spectra of PF-N_3 probe

Figure S12. ^{1}H NMR of 2-(2-(2-(2-(diethoxyphosphoryl)ethoxy)ethoxy)ethoxy)ethyl (2,5-dioxopyrrolidin-1-yl) carbonate

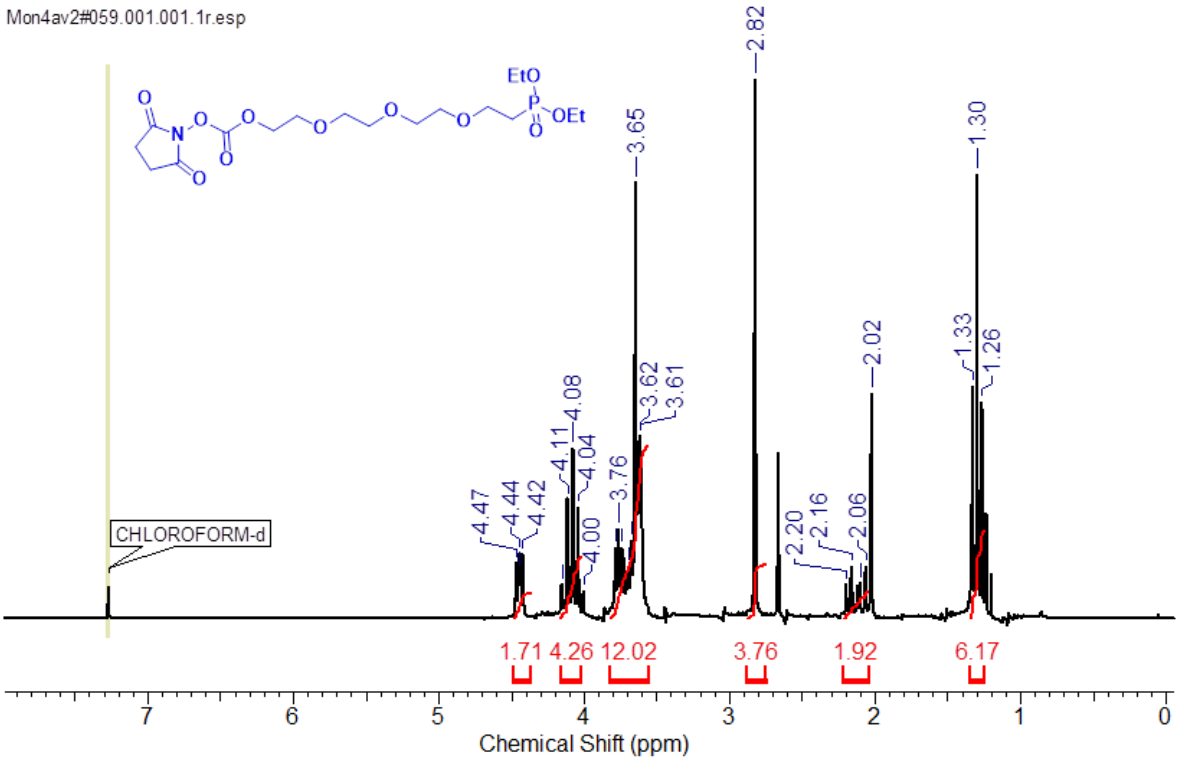


Figure S13. ^{1}H NMR of 2-(2-(2-(2-(diethoxyphosphoryl)ethoxy)ethoxy)ethoxy)ethyl (3-azidopropyl)carbamate

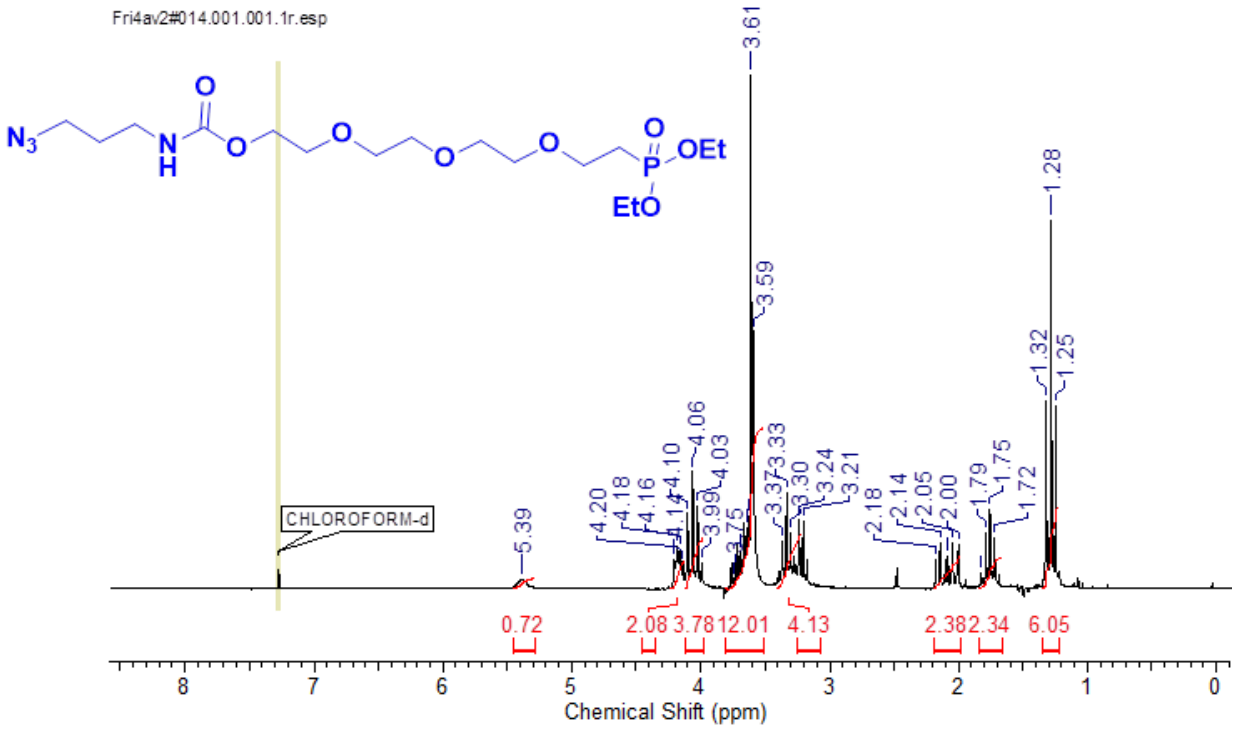


Figure S14. ^{1}H NMR of 2-(2-(2-(2-(ethoxy(hydroxy)phosphoryl)ethoxy)ethoxy)ethoxy) ethyl (3-azidopropyl)carbamate

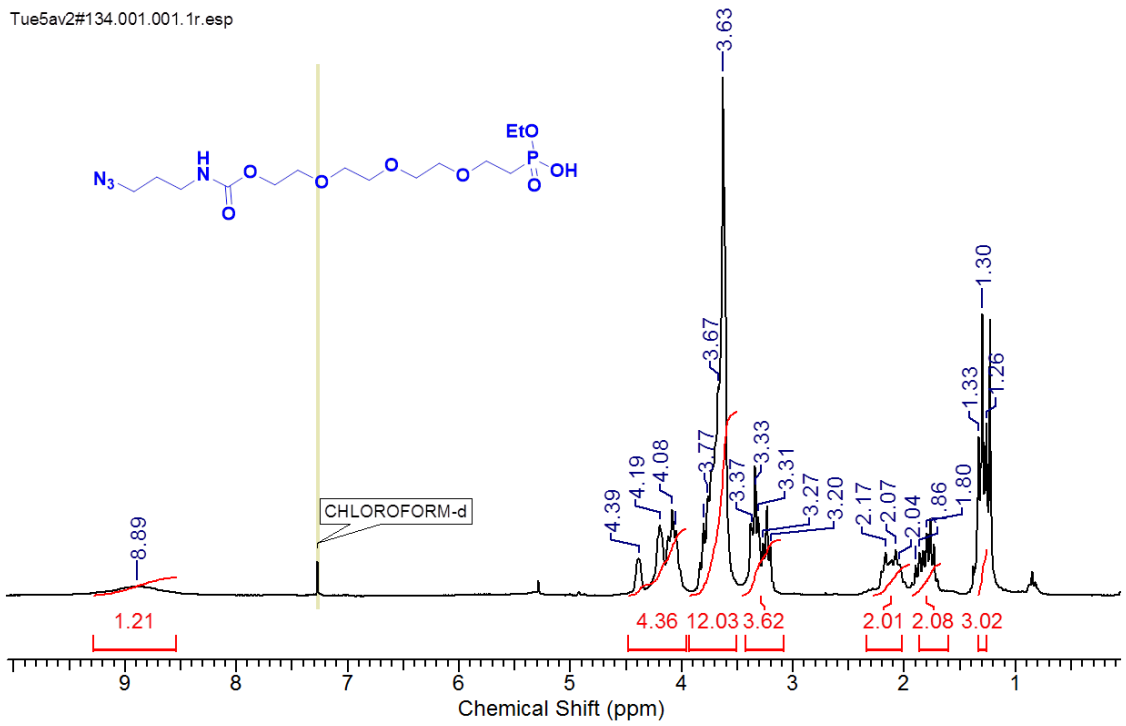


Figure S15. ^{1}H NMR of 2-(2-(2-(2-(ethoxyfluorophosphoryl)ethoxy)ethoxy)ethoxy)ethyl (3-azidopropyl)carbamate

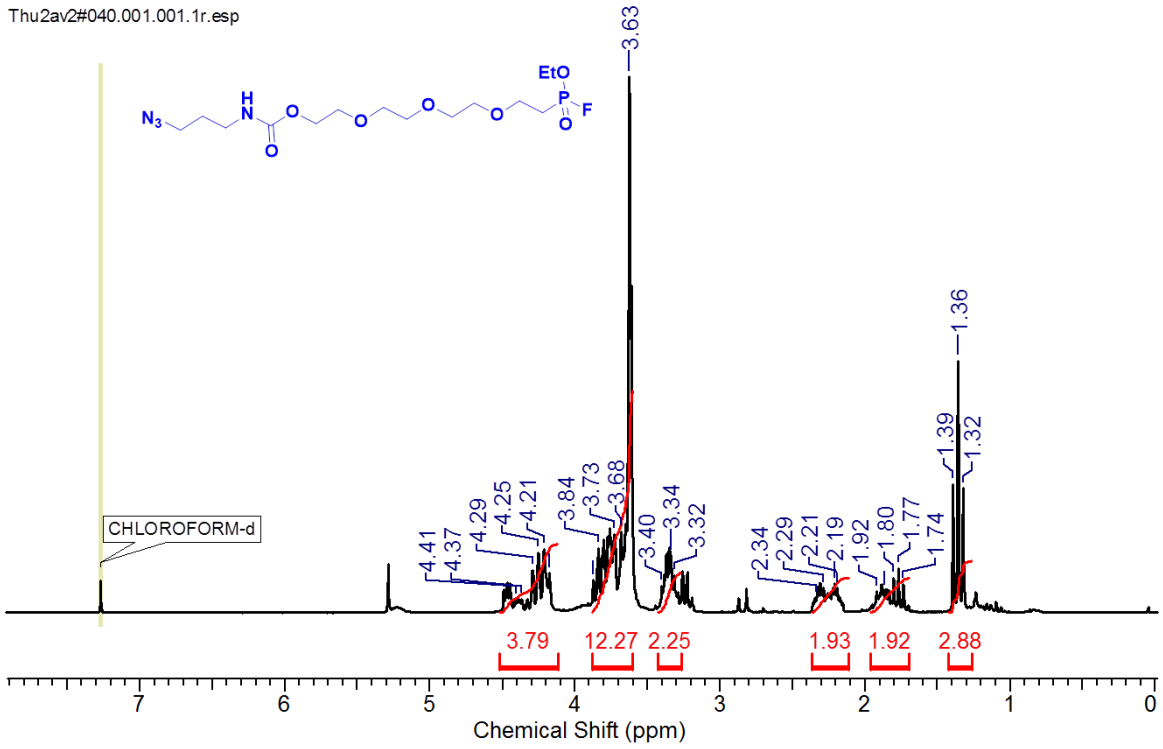
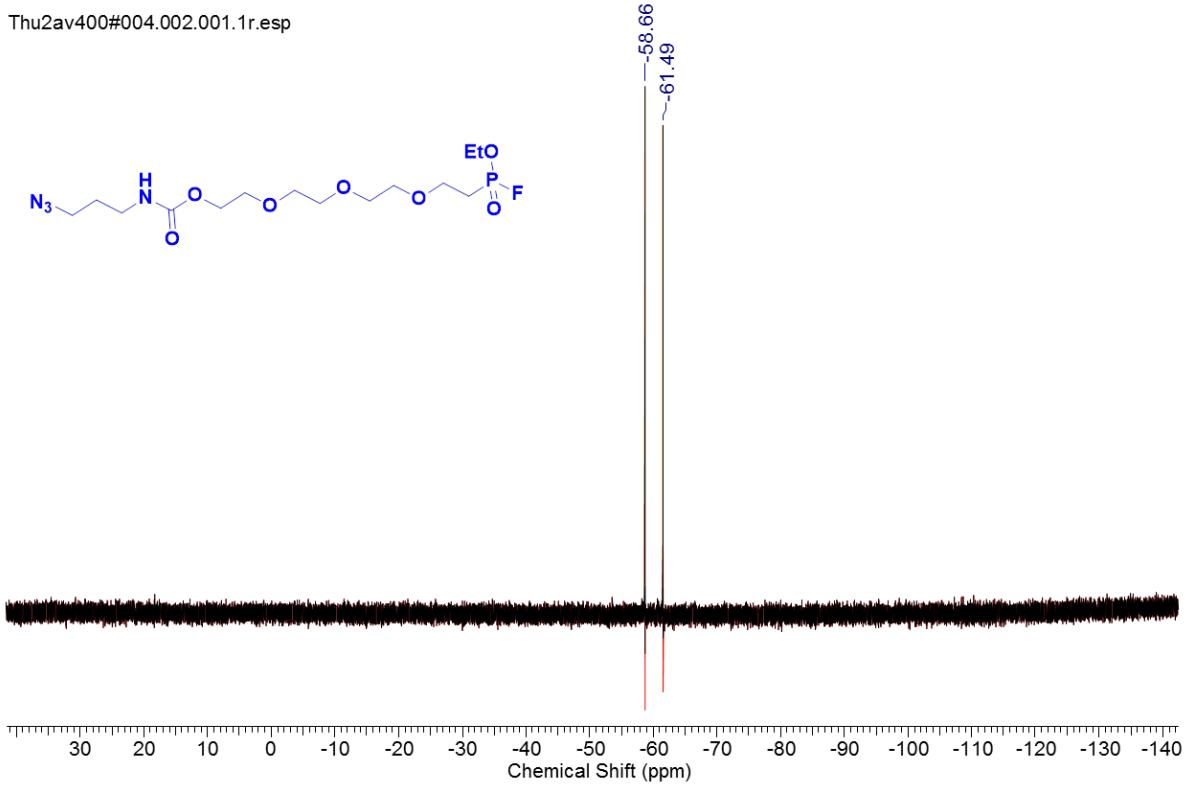


Figure S16. ^{19}F NMR of 2-(2-(2-(2-(ethoxyfluorophosphoryl)ethoxy)ethoxy)ethoxy)ethyl (3-azidopropyl)carbamate



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