## Traceless cysteine-linchpin enables precision engineering of lysine in native proteins

Neelesh C. Reddy, a Rajib Molla, a Pralhad Namdev Joshi, a Sajeev T. K., b Ipsita Basu, c Jyotsna Kawadkar, b Neetu Kalra, b Ram Kumar Mishra, b Suman Chakraborty, c Sanjeev Shukla, b Vishal Rai* a

aDepartment of Chemistry, and bDepartment of Biological Sciences, Indian Institute of Science Education and Research Bhopal, Bhopal Bypass Road, Bhauri, Bhopal, 462 066 M.P. India cDepartment of Chemical and Biological Sciences, S. N. Bose National Centre for Basic Sciences, Block-JD, Sector-III, Salt Lake, Kolkata-700 106 W.B. India. dSchool of Bioengineering, VIT, Bhopal. 

vrai@iiserb.ac.in

## Table of Contents

| 1. Supplementary Notes 1 | ............................................................. | S4 |
|--------------------------|--------------------------------------------------------------------------------------------------|----|
| 2. Supplementary Methods | ................................................................................. | S10|
| 2.1 LDM_{C,K} reagent – selection of F_C component | ............................................................................. | S10|
| 2.1.1 Identification of F_C (Cys-selective electrophile) | ....................................................................... | S10 |
| 2.1.2 Establishing chemoselectivity of F_C (nitroolefin) | ........................................................................... | S10 |
| 2.1.3 Establishing C-S bond dissociation with F_C | .............................................................................. | S10 |
| 2.2 Peptide labeling | .................................................................................. | S11 |
| 2.2.1 Michael addition with the peptide | ................................................................................. | S11 |
| 2.2.2 Acylation with the peptide | .................................................................................. | S11 |
| 2.2.3 Macrocyclization with the peptide | ................................................................................ | S11 |
| 2.2.4 Site-selectivity with the peptide | ................................................................................. | S11 |
| 2.2.5 LDM_{C,K} workflow with peptide | ................................................................................. | S11 |
| 2.3 Protein labeling | .................................................................................. | S12 |
| 2.3.1 Reactivity and selectivity of nitroolefin (F_C) in protein bioconjugation | ................................................................. | S12 |
| 2.3.2 LDM_{C,K} reagent - selection of F_K component (acyl donor) | .................................................. | S12 |
| 2.3.3 Kinetics of Michael addition and cyclization | ....................................................................... | S12 |
| 2.3.4 Site-selective modification of native proteins | ........................................................................ | S12 |
| 2.3.5 Molecular dynamics simulations | .............................................................................. | S13 |
| 2.3.6 Single-site installation of tags | ................................................................................ | S13 |
| 2.3.7 Dual-probe installation | ................................................................................ | S14 |
| 2.4 Protein digestion | .................................................................................. | S14 |
| 2.5 Single protein (HSA) labeling in the mixture of proteins and its enrichment | .................................................. | S15 |
| 2.6 Modification of HSA in cell lysate and its enrichment | .................................................. | S15 |
| 2.7 Antibody drug conjugate | ................................................................................ | S16 |
| 2.7.1 Site-selective modification of trastuzumab | ....................................................................... | S16 |
Supplementary Table 8: Stability of LDM$_{C,K}$ reagent 9d. .......................................................... S106
8.3 pH screening .......................................................................................................................... S112

Supplementary Table 9. pH screening with β-lactoglobulin A and LDM$_{C,K}$ reagent 9d. .......... S112
8.4 Concentration screening ........................................................................................................ S114

Supplementary Table 10. Concentration screening with β-lactoglobulin A and LDM$_{C,K}$ reagent
9d. ........................................................................................................................................... S114
8.5 $^{19}$F NMR data of labeled β-lactoglobulin A........................................................................ S116
8.6 Fluorescence data of labeled β-lactoglobulin A................................................................. S116
8.7 Circular dichroism spectra of proteins ................................................................................. S117
8.8a Reaction of peptides (competing nucleophile) with LDM$_{C,K}$ reagent 9d .................. S118
8.8b C-S and C-C bond cleavage in peptides ......................................................................... S122
8.8c Linchpin dissociation in cyclic protein (BLGA)................................................................. S125

Supplementary Table 11: Stability of cyclic BLGA at different pH as a function of time....... S125
8.9a Enrichment of protein from E. Coli BL21 cell-lysate (without HSA) using coumarin tag. .. S128
8.9b Enzymatic assay of HSA (Human serum albumin) ........................................................... S129
8.10 Comparative analysis of light chain (LC) and heavy chain (HC) in native and modified
trastuzumab............................................................................................................................... S130
8.11 Experiments with CASLQK tagged Hs-Ubc9 ................................................................. S131

9. Protein sequence ..................................................................................................................... S135
10. Spectral data ......................................................................................................................... S137

Supplementary references ......................................................................................................... S175
1. Supplementary Notes 1

The reagents, proteins, and enzymes were purchased from Sigma-Aldrich (Merck), Alfa Aesar, Novabiochem, Emcure, Roche, Labex, and Thermo Fisher Scientific. Fritted polypropylene chromatography columns were purchased from Bio-Rad. The reagent-grade organic solvents were used. Aqueous buffers were prepared freshly using Merck Millipore Grade I water (Resistivity > 5 MΩ cm, Conductivity < 0.2 μS/cm, TOC < 30 ppb). Mettler Toledo (FE20) pH meter was used to adjust the final pH. The reaction mixture for the small molecules was stirred (Heidolph magnetic stirrer, 480-576 x g). Proteins were either vortexed (336 x g, 25-37 °C) or incubated (25-37 °C) in Thermo Fisher Scientific MaxQ 8000 incubator-shaker or Thermo Scientific Digital Heating Shaking Drybath. Amicon® Ultra-0.5 mL 3-kDa or 10-kDa 30-kDa MWCO centrifugal filters from Merck Millipore were used to remove small molecules from protein mixture, desalting, and buffer exchange. Organic solvents were removed by BUCHI rotavapor R-210/215, whereas aqueous samples were lyophilized by CHRISt ALPHA 2-4 LD plus lyophilizer. Circular Dichroism (CD) measurements were recorded on a JASCO J-815 CD spectropolarimeter equipped with a Peltier temperature controller. All the spectra were measured with a 50 nm/min scan speed, spectral bandwidth 1 nm, using a 10 mm path length cuvette at 25 °C. UV-Vis spectra were recorded in Agilent Carry-100 UV-Vis Spectrophotometer connected with a Peltier temperature controller. Microplate reader measurements were performed on BioTek Cytation 5. Steady-state fluorescence spectra were recorded in HORIBA JOBIN YVON, FLUOROLOG 3-111. The quartz cuvette of 1 cm path length was used to record the fluorescence spectra.

Chromatography: Thin-layer chromatography (TLC) was performed on silica gel coated aluminium TLC plates (Merck, TLC Silica gel 60 F254). The compounds were visualized using a UV lamp (254 nm) and stains such as iodine, ninhydrin, 2,4-diphenylhydrazine. The flash column chromatography was carried out on CombiFlash Rf 200, CombiFlash NextGen 300+, and gravity columns using 230-400 or 100-200 mesh silica gel from Merck.

Nuclear magnetic resonance spectra: 1H, 13C, and 19F NMR spectra were recorded on Bruker Advance III 400 and 500 MHz NMR spectrometer. 1H NMR spectra were referenced to TMS (0 ppm), DMSO-d6 (2.50 ppm), D2O (4.79 ppm), and acetone-d6 (2.05 ppm), whereas 13C NMR spectra were referenced to CDC13 (77.16 ppm), DMSO-d6 (39.52 ppm), and acetone-d6 (29.84 ppm). 19F NMR was referenced to trifluoroacetic acid (-75.70 ppm). The following abbreviations designate peak multiplicities: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; tt, triplet of triplets. All the NMR spectra were recorded at 298 K.

Mass spectrometry: SCIEX X500B qToF platform paired with ExionLC AD UHPLC and Agilent 6130 mass spectrometer with Agilent 1200 series HPLC was used for the MS data of small molecules, peptides, and proteins. HRMS data for small molecules were recorded on Bruker Daltonics MicroTOF-Q-II with electrospray ionization (ESI). Matrix-assisted laser desorption/ionization time of flight mass spectrometry was performed with Bruker Daltonics UltraFlleXtreme Software-Flex control version 3.4, using sinapic acid and α-cyano-4-hydroxycinnamic acid (HCCA) matrix. Data collection and analysis was performed using Open LAB CDS A.01, SCIEX OS 1.7, Bruker Daltonics ESI Compass 1.3, and BioPharmaView Flex 2.1. Peptide mass and fragment ion calculator (proteomicsToolkit, FragIonServlet) were used for peptide mapping and sequencing.
Acetonitrile and H\textsubscript{2}O were buffered with 0.01\% formic acid and used as the mobile phase. Method A was used to record the LC-ESI-MS data for proteins. The stability of LDM reagents in aqueous buffer is analyzed through LC-ESI-MS using Method B. All the Amino acid and peptide related data is analyzed through LC-ESI-MS using Method C.

**Method A:** Intact mass of proteins on Agilent 6130 Quadrupole paired with Agilent 1200 infinity series HPLC (Column: Agilent, Poroshell 300 SB-C18 5 μm 2.1 × 75 mm, flow rate 0.5 ml/min)

| Time (min) | H\textsubscript{2}O (%) | Acetonitrile (%) |
|------------|------------------------|-----------------|
| 0          | 90                     | 10              |
| 1          | 90                     | 10              |
| 8          | 40                     | 60              |
| 9          | 90                     | 10              |
| 10         | 90                     | 10              |

**Method B:** Intact mass of small molecules on Agilent 6130 Quadrupole paired with agilent 1200 infinity series HPLC (Column: Agilent, Poroshell 120 EC-C18 2.7 μM 3.0 × 50 mm, flow rate 0.3 ml/min)

| Time (min) | H\textsubscript{2}O (%) | Acetonitrile (%) |
|------------|------------------------|-----------------|
| 0          | 75                     | 25              |
| 4          | 65                     | 35              |
| 5          | 65                     | 35              |
| 5.5        | 10                     | 90              |
| 8          | 10                     | 90              |
| 12         | 10                     | 90              |

**Method C:** Intact mass of peptides and amino acids on Agilent 6130 Quadrupole paired with Agilent 1200 infinity series HPLC (Column: Agilent, AdvanceBio Peptide 2.7 μm 4.6 × 150 mm, flow rate 0.5 ml/min)

| Time (min) | H\textsubscript{2}O (%) | Acetonitrile (%) |
|------------|------------------------|-----------------|
| 0          | 90                     | 10              |
| 3          | 50                     | 50              |
| 5          | 25                     | 75              |
| 10         | 10                     | 90              |
| 15         | 10                     | 90              |
| 25         | 10                     | 90              |

**Determination of percentage conversion in the protein labeled experiments**

**ESI-MS:** Conversion for protein labeled was calculated based on the relative peak intensity of native protein and labeled protein in the deconvoluted mass spectrum.

\[
\% \text{ Conversion} = \frac{I_{\text{desired product}}}{I_{\text{all relevant species}}}, \text{ where } I_{\text{desired product}} \text{ is the peak intensity of labeled protein, and } I_{\text{all relevant species}} \text{ is the sum of the peak intensities of native protein and labeled protein in the deconvoluted mass spectra.}
\]

**MALDI-ToF-MS:** Conversion for protein labeling was calculated based on the relative peak intensity of native protein and labeled protein in the mass spectrum.
For all the examples, we preferred ESI-MS data over MALDI-ToF-MS for the estimation of conversions due to deviations less than ±5%.

**Method D:** Peptide mapping and MS-MS of proteins on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Peptide XB-C18 (2.5 x 150 mm, 1.7 µm, 100 Å) flow rate 0.3 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|----------------|---------|------------------|
| 0              | 95      | 5                |
| 2              | 95      | 5                |
| 25             | 50      | 50               |
| 26             | 20      | 80               |
| 27             | 20      | 80               |
| 28             | 95      | 5                |
| 29             | 95      | 5                |
| 30             | 95      | 5                |

**Method E:** Intact antibody conjugate analysis on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Protein XB-C8 (4.6 x 150 mm, 1.8 µm, 300 Å) flow rate 0.5 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|----------------|---------|------------------|
| 0              | 75      | 25               |
| 5              | 75      | 25               |
| 7              | 55      | 45               |
| 9              | 10      | 90               |
| 11             | 10      | 90               |
| 13             | 75      | 25               |
| 15             | 75      | 25               |

**Method F:** Light and heavy chains analysis of antibody on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Protein XB-C8 (4.6 x 150 mm, 1.8 µm, 300 Å) flow rate 0.5 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|----------------|---------|------------------|
| 0              | 95      | 5                |
| 2              | 95      | 5                |
| 35             | 70      | 30               |
Method G: Peptide mapping and MS-MS of antibody on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Peptide XB-C18 (2.5 x 150 mm, 1.7 µm, 100 Å) flow rate 0.3 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|---------------|---------|-----------------|
| 0             | 95      | 5               |
| 5             | 95      | 5               |
| 50            | 50      | 50              |
| 55            | 20      | 80              |
| 60            | 20      | 80              |
| 62            | 95      | 5               |
| 65            | 95      | 5               |

Method H: Intact mass of protein on SCIEX X500B paired with ExonLC AD UPLC [Column: Phenomenex, bioZen, Peptide XB-C8 (4.6 x 150 mm, 1.8 µm, 300 Å) flow rate 0.6 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|---------------|---------|-----------------|
| 0             | 80      | 20              |
| 1             | 80      | 20              |
| 5             | 20      | 80              |
| 7             | 20      | 80              |
| 9             | 80      | 20              |
| 10            | 80      | 20              |

Method I: Intact mass of peptide on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Peptide PS-C18 (2.1 x 150 mm, 3 µm, 100 Å) flow rate 0.5 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|---------------|---------|-----------------|
| 0             | 90      | 10              |
Method J: MS-MS of small peptide on SCIEX X500B paired with ExionLC AD UHPLC [Column: Phenomenex, bioZen, Peptide PS-C18 (2.1 x 150 mm, 3 µm, 100 Å) flow rate 0.5 ml/minutes. Acetonitrile and H₂O were buffered with 0.1% formic acid and used as the mobile phase.]

| Time (minutes) | H₂O (%) | Acetonitrile (%) |
|----------------|---------|-----------------|
| 0              | 90      | 10              |
| 3              | 90      | 10              |
| 5              | 50      | 50              |
| 10             | 50      | 50              |
| 15             | 25      | 75              |
| 20             | 10      | 90              |
| 22             | 90      | 10              |
| 25             | 90      | 5               |

MS parameters (variables) for intact antibody, light, and heavy chains, and peptides

Parameter set A: Intact antibody
Curtain gas (psi): 40
Ion source gas 1 (psi): 60
Ion source gas 2 (psi): 60
Temperature (°C): 500
Declustering potential (V): 225
Time bins to sum: 80

Parameter set B: HC and LC
Curtain gas (psi): 35
Ion source gas 1 (psi): 45
Ion source gas 2 (psi): 40
Temperature (°C): 600
Declustering potential (V): 120
Time bins to sum: 20
Parameter set C: Peptide mapping and MS-MS

Curtain gas (psi): 30
Ion source gas 1 (psi): 45
Ion source gas 2 (psi): 40
Temperature (°C): 550
Declustering potential (V): 80
Time bins to sum: 4

Parameter set D: Intact mass of protein

Curtain gas (psi): 35
Ion source gas 1 (psi): 45
Ion source gas 2 (psi): 40
Temperature (°C): 400
Declustering potential (V): 120
Time bins to sum: 20

Parameter set E: Intact Peptide MS

Curtain gas (psi): 30
Ion source gas 1 (psi): 45
Ion source gas 2 (psi): 40
Temperature (°C): 550
Declustering potential (V): 80
Time bins to sum: 4

Parameter set F: small Peptide MS-MS

Curtain gas (psi): 30
Ion source gas 1 (psi): 45
Ion source gas 2 (psi): 40
Temperature (°C): 550
Declustering potential (V): 80
Time bins to sum: 4

SPPS (Solid Phase Peptide Synthesis): All the Fmoc amino acids were derivatized to amide with rink amide resin using SPPS with 95% purity. Similarly, the synthesis of all the peptide were done using the same resin i.e. rink amide.

The peptides were designed to be conformationally flexible to avoid biases towards a specific secondary structure. It was important that they present an extreme case to the LDMC_K reagents so that we can anticipate the potential challenges with protein-based substrates. Besides, the selection ensured that C-S and C-N bond formation steps were well deconvoluted. This attribute was critical to establish the sequential order of these reactions. Further, we anticipated that even if the selectivity with such peptides is moderate, the methods would be appropriate for proteins as they display additional conformational constraints.
Cleavage of peptide from resin: The peptide was cleaved from the resin by mixing it with TFA/H₂O/TIPS (95:2.5:2.5, 4 ml) for 2 h. Subsequently, TFA was removed by evaporation on rotatory evaporator. The crude mixture was triturated (diethyl ether) and purified by preparative HPLC. [Hint: To cleave all cysteine (after cleavage it is free) containing peptide from resin TFA/H₂O/TIPS/DTT/Phenol (90:2.5:2.5:2.5:2.5, 4 ml) for 2 h was used.]

Purification of peptides: The purification was done using reverse-phase HPLC using Agilent column ZORBAX 300SB-C18 (5 μm, 9.4*250 mm)

Purification method: Mobile phase A: H₂O; mobile phase B: ACN

| Time | Mobile phase A (%) | Mobile phase B (%) | Flow rate (ml/min) |
|------|---------------------|---------------------|---------------------|
| 0    | 85.0                | 15.0                | 4.0                 |
| 25   | 5.0                 | 95.0                | -                   |
| 30   | 5.0                 | 95.0                | -                   |

MD simulations: The structure for β-lactoglobulin was obtained from the Protein Data Bank (PDB ID: 3BLG). The Cys121 residue was modified by the LDM_{C,K} reagent (9d) manually using Pymol. The force field parameters for the modified Cys121 (linchpin) were generated using the Antechamber utility of AmberTools 20 software suite. The generated parameters were consistent with the GAFF2 atom types and AM1-BCC partial charges.

2. Supplementary Methods

2.1 LDM_{C,K} reagent – selection of F_C component

2.1.1 Identification of F_C (Cys-selective electrophile)

The electrophile for conjugate addition (1a-1l, 5 µmol each in DMSO-d⁶ (300 µl) was taken separately in 5 ml glass vial having 150 µl of D₂O. To this solution, reagent 2 (0.6 mg, 5 µmol) in DMSO-d⁶ (50 µl) from a freshly prepared stock solution was added and stirred at 25 °C. The overall concentration of the reaction was 10 mM. The progress of the reaction was monitored by 1H NMR within 10 min. (Related: Fig. 2a, Supplementary Fig. 15-26, Supplementary Table1)

2.1.2 Establishing chemoselectivity of F_C (nitroolefin)

All the twenty Fmoc-amino acids (4a-4t, 100 nmol each) in DMF: acetonitrile (3:7, total vol. 40 µl) from a freshly prepared stock solution were taken into 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, the reagent 1l (28 µg, 100 nmol) in acetonitrile (20 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the reaction was monitored by ESI-MS within 10 min. (Related: Fig. 2b, Supplementary Figs. 27-28, Supplementary Table 2)

2.1.3 Establishing C-S bond dissociation with F_C

The reagent 3l (40 µg, 100 nmol) in acetonitrile (60 µl) from a freshly prepared stock solution was taken into a 1.5 ml microcentrifuge tube containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, a selected base (6a/6b/6c/6d/6e/6f, 100 nmol) was added followed by vortexing at 25 °C. In parallel, another reaction was performed using NaHCO₃ buffer (140 µl, 0.1 M, pH 8.5) in the absence of base while keeping other parameters constant. The overall
concentration of the reaction was 500 µM. The progress of all the reactions were monitored by ESI-MS for 12-24 h. (Related: Fig. 2c, Supplementary Figs. 29-37, Supplementary Table 3)

2.2 Peptide labeling
The peptide stock solution was freshly prepared using acetonitrile with two drops of HPLC grade methanol to enhance the solubility (2.2.1-2.2.4).

2.2.1 Michael addition with the peptide
Peptide containing free cysteine 12a (68 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent 11 (28 µg, 50 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the Michael addition was monitored by LC-ESI-MS up to 1 h.

2.2.2 Acylation with the peptide
Peptide containing protected cysteine and free lysine 12c (80 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent 9d (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the reaction was monitored by LC-ESI-MS up to 1 h.

2.2.3 Macrocyclization with the peptide
Peptide containing free cysteine and lysine 12S1-S3 and 12b (75 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution were taken separately into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent 9d (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the Michael addition followed by macrocyclization was monitored by LC-ESI-MS up to 1 h. This protocol was also used for peptides 12e-12g for examining the competition from Tyr, Ser, and His.

2.2.4 Site-selectivity with the peptide
Peptide containing one free cysteine and two lysine residues 12d (116 µg, 100 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was taken into a 1.5 ml HPLC vial containing phosphate buffer (140 µl, 50 mM, pH 7.0). To this solution, reagent 9d (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C. The overall concentration of the reaction was 500 µM. The progress of the reaction was monitored by LC-ESI-MS up to 1 h. The site-of-modification was confirmed by MS-MS.

2.2.5 LDMC-K workflow with peptide
Peptide containing free cysteine and lysine 12h (122 µg, 100 nmol) in phosphate buffer (160 µl, 50 mM, pH 7.0) from a freshly prepared stock solution was taken separately into a 1.5 ml HPLC vial. To this solution, reagent 9d (40 µg, 100 nmol) in acetonitrile (40 µl) was added and incubated at 25 °C for 15 minutes. The overall concentration of the reaction was 500 µM. Next, n-butyl maleimide (S34, 382 µg, 50 equiv.) solution in acetonitrile (10 µl) was added to the reaction mixture (14i) and incubated at 25 °C for 50 min. to form 15a. The pH of the reaction mixture was readjusted to ~9.0 and incubated further for retro-Henry reaction (15b) at 25 °C
for 36 h. The pH was readjusted back to ~7.0 and NH_2OH.HCl (690 µg, 200 equiv., 10 µl) was added from a freshly prepared stock solution. The reaction mixture was incubated at 37 °C for 3 h to render oxime 15c. The progress of all the reactions was monitored by ESI-MS (XIC).

2.3 Protein labeling
2.3.1 Reactivity and selectivity of nitroolefin (F_C) in protein bioconjugation

Protein 8a (2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, control reagent 11 (20 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and control reagent was 20 µM and 200 µM, respectively. After 30 min, the reaction mixture was diluted with water (500 µl). The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2 x 0.4 ml). The sample was analyzed by LC-ESI-MS or MALDI-ToF-MS. The aqueous sample was concentrated by lyophilization before subjecting it to digestion, peptide mapping, and sequencing by MS-MS.

2.3.2 LDM_C-K reagent - selection of F_C component (acyl donor)

Protein 8a (2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM_C-K reagent (9d-9h, 20 nmol, each separately) in acetonitrile (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM_C-K reagent was 20 µM and 200 µM, respectively. After 6 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600 x g to settle down the unreacted LDM_C-K reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2 x 0.4 ml). The sample was analyzed by LC-ESI-MS.

2.3.3 Kinetics of Michael addition and cyclization

Protein 8a (2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM_C-K reagent (9d, 20 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM_C-K reagent was 20 µM and 200 µM, respectively. The progress of the transformation was ceased at the given time points by diluting the reaction mixture from 100 µl to 1000 µl with Grade I water. A control experiment confirmed that the reaction showed no noticeable progress for thirty minutes after dilution. The sample was immediately subjected to mass analysis at regular intervals (10 sec to 6 h).

2.3.4 Site-selective modification of native proteins

Protein 8a (2 nmol) in phosphate buffer (70-80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM_C-K reagent (9d-9h, 2.4-20 nmol, each separately) in acetonitrile (20-30 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM_C-K reagent was 20 µM and 24-200 µM, respectively. After 1-24 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600 x g to precipitate the unreacted LDM_C-K reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2×0.4 ml). The sample was
analyzed by LC-ESI-MS or MALDI-ToF-MS. The sample was then buffer exchanged using NaHCO$_3$ (100 µl, 0.1 M, pH 8.5) and incubated for 48 h. It resulted in the C-S bond dissociation, and a free aldehyde handle on the protein (17) was generated via the retro-Henry reaction. The sample was then buffer exchanged using phosphate buffer (100 µl, 50 mM, pH 7.0). To this solution, hydroxylamine hydrochloride 18 or O-benzylhydroxylamine hydrochloride 19 (2 µmol) in water (10 µl) from a freshly prepared stock solution was added for late-stage modification (oxime formation) and incubated for 3 h. The excess of hydroxylamine and salts were removed by centrifugal spin concentrator (0.5 ml, 10 kDa MWCO) and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS. The aqueous sample was concentrated by lyophilization before subjecting it to digestion, peptide mapping, and sequencing by MS-MS. [Note: The same protocol was followed for the single-site modification of Bovine Serum Albumin (BSA, 8j) and Human Serum Albumin (HSA, 8k)]

2.3.5 Molecular dynamics simulations

The atomistic classical molecular dynamics (MD) simulation studies were performed the LDM chemistry. The structures were solvated in TIP3P water box, extending 12 Å from the solute in all three directions using the LEAP module in AMBER20. Appropriate numbers of Na$^+$ and Cl$^-$ counterions were added to neutralize the charges and to maintain 150 mM physiological salt concentration. AMBER ff14 force field was used to describe the interaction involving protein and Joung and Cheatham parameters for ions. Resulting solvated structures were subjected to minimization. Then MD was performed under constant pressure-constant temperature conditions (NPT) for 500 ps using a harmonic restraint on the solute with a force constant of 10 kcal/mol/Å². This was followed by production run of 1000 ns. Three independent trajectories were generated for the production runs. The linchpin molecule and the protein were simulated separately in water using the similar protocols as reference systems.

The MD simulations were carried out using the PMEMD module of AMBER20 package with imposed 3D periodic boundary conditions. TIP3P water model was used to solvate the systems. A time step of 2 fs was used to integrate the equation of motion. The temperatures were maintained for the simulations using Langevin dynamics, while pressure was kept constant at 1 atm using Berendsen weak coupling method with anisotropic pressure scaling. The particle mesh Ewald method was used to calculate long-range electrostatic calculations with a real space cut-off distance of 10 Å where the vdW and direct electrostatic interactions were truncated. All bond lengths involving hydrogen atoms were held fixed using the SHAKE algorithm. All analyses were done using CPPTRAJ module of AMBER20 tools. Snapshot generations were done using (Visual Molecular Dynamics).

2.3.6 Single-site installation of tags

After the installation of free aldehyde handle on the protein (17) via retro-Henry process, various derivatives of hydroxylamine (21-23) (20-60 nmol) in H$_2$O:ACN (1:1) from a freshly prepared stock solution was added for the late-stage modification (oxime formation) and incubated for 12-24 h. The excess hydroxylamine and salts were removed by centrifugal spin concentrator (0.5 ml, 10 kDa MWCO), and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS. The aqueous sample was concentrated by lyophilization before subjecting it to digestion, peptide mapping, and sequencing by MS-MS.
2.3.7 Dual-probe installation

Protein 8a (2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, LDM\textsubscript{C,K} reagent (9d, 20 nmol) in acetonitrile (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. The overall concentration of protein and LDM\textsubscript{C,K} reagent was 20 µM and 200 µM, respectively. After 6 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600 x g to precipitate the unreacted LDM\textsubscript{C,K} reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 ml, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2×0.4 ml). The sample was analyzed by LC-ESI-MS.

The sample was then buffer exchanged using NaHCO\textsubscript{3} (100 µl, 0.1 M, pH 8.5) and incubated for 48 h. The C-S bond dissociation and retro-Henry reaction render the free aldehyde handle on the protein (17). The sample was then buffer exchanged using phosphate buffer (100 µl, 50 mM, pH 7.0). To this solution, various derivatives of hydroxylamine (21-23) (20-60 nmol) in ACN (10 µl) from the freshly prepared stock solution were added. The incubation for 12-24 h rendered the late-stage modification through oxime formation. Later to this solution, the CPM dye (27, 20 nmol) in acetonitrile (20 µl) from freshly prepared stock solution was added and incubated for 1 h. The excess hydroxylamine derivative, CPM dye, and salts were removed by centrifugal spin concentrator (0.5 ml, 10 kDa MWCO), and the sample was collected in an aqueous medium. The modification of protein was analyzed by LC-ESI-MS.

2.4 Protein digestion

All the solutions were prepared freshly before use in reactions.\textsuperscript{15}

**Step 1. Denaturation:** Protein (0.13 mg, 7.3 nmol) in grade I water (10 µl) with urea (6 M) was taken in a 1.5 ml microcentrifuge tube and incubated for 30 minutes.

**Step 2. Disulfide reduction:** To cleave the disulfide bonds, a reducing agent (1 µl, 0.2 M DTT in Grade I water) was added to the solution, and the sample was incubated for 1 h at 37 °C.

**Step 3. Sulphydryl alkylation:** To block the free sulfhydryl groups, alkylating agent (5 µl, 0.2 M iodoacetamide in Grade I water) was added to the solution and incubated (in the dark) for 1 h at ambient temperature.

**Step 4. Quenching excess alkylating reagent:** To quench the unreacted iodoacetamide, a reducing agent (DTT, 5 µl) was added again to the mixture and the sample was incubated at 25 °C for 1 h. The dilution of the reaction mixture with Grade I water reduced the urea concentration to 0.6 M.

**Step 5. Desalting:** The unreacted reagent and salts were removed using centrifugal spin concentrator (0.5 ml, 10 kDa MWCO). This step helps in avoiding the signal suppression during subsequent analysis by ESI-MS after step 6.

**Step 6. Enzymatic digestion:** The desalted sample was diluted to 100 µl with Grade I water. To this mixture, 5 µl of α-chymotrypsin or trypsin solution [5 µg, based on ratio of chymotrypsin or trypsin/protein (1:20); α-chymotrypsin or trypsin in Grade I water] was added. After incubation at 37 °C for 18 h, trifluoroacetic acid (0.5%) was used to adjust the pH of digested solution to <6 (verified by pH paper). Subsequently, the sample was subjected to peptide mapping by MS and sequencing by MS-MS.
2.5 Single protein (HSA) labeling in the mixture of proteins and its enrichment

Bioconjugation in a representative mixture of eight proteins – Ubiquitin (17 µg, 2 nmol), cytochrome C (24 µg, 2 nmol), RNase A (27 µg, 2 nmol), insulin (23 µg, 4 nmol), α-lactalbumin (28 µg, 2 nmol), lysozyme C (28 µg, 2 nmol), myoglobin (34 µg, 2 nmol), and HSA (25 µg, 0.4 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) were mixed in a 1.5 ml microcentrifuge tube. Here, the HSA concentration was kept five times lower than the other proteins to present a substantial challenge to the method. To this solution, LDM<sub>C,K</sub> reagent 9d (1.6 µg, 4 nmol) in ACN (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. After 3 h, the reaction mixture was diluted with grade I water (500 µl). The unreacted LDM<sub>C,K</sub> reagent 9d and salts were removed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO) and the protein mixture was collected in the NaHCO<sub>3</sub> buffer (100 µl, 0.1 M, pH 8.5). Next, the incubation for 48 h generates free aldehyde handle on protein (32) via the retro-Henry reaction. After 48 h, the reaction mixture was diluted with phosphate buffer (500 µl, 50 mM, pH 7.0). The buffer exchange was performed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). Subsequently, this reaction mixture was utilized for the enrichment of HSA.

Enrichment protocol: In a 5 ml fritted polypropylene chromatography column with end tip closures, hydrazide beads (400 µl, hydrazide resin loading: 16 µmol/ml) were taken. The beads were washed with phosphate buffer (0.1 M, pH 7.0, 5 x 1 ml) and re-suspended (phosphate buffer, 100 µl, 50 mM, pH 7.0). The reaction mixture containing modified HSA 32 (40 nmol) in phosphate buffer (500 µl, 50 mM, pH 7.0) was added to the beads. Next, the end-to-end rotation (29 x g, rotary mixer) was performed at 25 °C for 24 h. The supernatant was collected, and the beads were washed with KCl (0.5 M, 6 x 1 ml) and phosphate buffer (0.1 M, pH 7.0, 4 x 1 ml) to remove the unreacted and adsorbed proteins from the resin. The beads were further washed with Grade I water (6 x 1 ml) and re-suspended (phosphate buffer, 450 µl, 50 mM, pH 6.5). To release the labeled protein from its immobilized derivative, the O-hydroxylamine derivative of coumarin 23 (20 µM) in ACN:H<sub>2</sub>O (50 µl) was added. The subsequent end-to-end rotation at 25 °C for 24 h led to transoximization. The supernatant was collected while the salts and 23 were removed using the centrifugal spin concentrator (10 kDa MWCO). The purity of the labeled protein 33 was confirmed by in-gel fluorescence, peptide mapping, and MS-MS. The protocol results in coumarin tagged labeled protein 33 with excellent purity.

HSA activity: We have recorded the esterase activity of the labeled and unlabeled protein using p-nitrophenyl acetate (pNPA) as the substrate. It produces p-nitrophenolate as a product after the hydrolysis of pNPA in the presence of the protein, and p-nitrophenolate absorbs at 400 nm. We monitored the change in absorbance at 400 nm over 150 minutes, providing us with the progression of the hydrolysis of pNPA in the presence of the protein. The protein and pNPA concentrations were 5 mM and 200 mM, respectively, for all the experiments. Data were acquired in a Carry 100 UV-Vis spectrophotometer at 25 °C.

2.6 Modification of HSA in cell lysate and its enrichment

E. coli BL21 cell lysate (2 µg/1 µl) in phosphate buffer (75 µl, 50 mM, pH 7.0) spiked with HSA 8k (25 µg, 0.4 nmol, in phosphate buffer, 5 µl, 50 mM, pH 7.0) were taken in a 1.5 ml microcentrifuge tube. To this solution, the LDM<sub>C,K</sub> reagent 9d (1.6 µg, 4 nmol) in ACN (20 µl) from a freshly prepared stock solution was added and incubated at 25 °C. After 3 h, the reaction mixture was diluted with Grade I water (500 µl). The unreacted LDM<sub>C,K</sub> reagent 9d and salts...
were removed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). The reaction mixture was collected in NaHCO₃ buffer (100 µl, 0.1 M, pH 8.5). The subsequent incubation for 48 h renders free aldehyde handle on protein (32) via retro-Henry reaction. Next, the reaction mixture was diluted with phosphate buffer (500 µl, 50 mM, pH 7.0). The buffer exchange was performed by centrifugal spin concentrator (0.5 ml, 3 kDa MWCO). This reaction mixture was utilized for the enrichment of modified HSA in the subsequent step.

**Enrichment protocol:** In a 5 ml fritted polypropylene chromatography column with end tip closures, hydrazide beads (400 µl, hydrazide resin loading: 16 µmol/ml) were taken. The beads were washed with phosphate buffer (0.1 M, pH 7.0, 5 x 1 ml) and re-suspended (phosphate buffer, 100 µl, 50 mM, pH 7.0). The cell lysate containing labeled and unlabeled HSA (combined batch: 2.6 mg, 40 nmol) in phosphate buffer (500 µl, 50 mM, pH 7.0) were added to the beads, followed by end-to-end rotation (39 x g, rotary mixer) at 25 °C for 24 h. The supernatant was collected, and the beads were washed with KCl (0.5 M, 6 x 1 ml) and phosphate buffer (0.1 M, pH 7.0, 4 x 1 ml) to remove the unreacted and adsorbed proteins from resin. The beads were further washed with Grade I water (6 x 1 ml) and re-suspended (phosphate buffer, 450 µl, 50 mM, pH 6.5). To release the labeled protein from its immobilized derivative, the O-hydroxylamine derivative of coumarin 23 (20 µM) in ACN:H₂O (50 µl) was added. Next, the transoximation is facilitated by end-to-end rotation at 25 °C for 24 h. The supernatant was collected while the salts and 23 were removed using the centrifugal spin concentrator (10 kDa MWCO). The purity of the labeled protein 33 was confirmed by in-gel fluorescence, peptide mapping, and MS-MS. The protocol results in coumarin tagged labeled protein 33 with excellent purity.

### 2.7 Antibody drug conjugate

#### 2.7.1 Site-selective modification of trastuzumab

Trastuzumab 34 (300 µg, 2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. TCEP (5 µg, 2 nmol) was added to this solution, and the reaction mixture was vortexed for 1 h. It was followed by the addition of reagent 9d (12 µg, 30 nmol) in ACN (20 µl) from a freshly prepared stock solution and vortexed for 8 h at 25 °C. The overall concentration of the trastuzumab 34 and LDM₇⁻K reagent 9d was 20 µM and 300 µM, respectively. After 8 h, the reaction mixture was diluted with water (500 µl), followed by centrifugation for 1 min at 9600 x g to precipitate the unreacted LDM₇⁻K reagent. The unreacted reagent and salts were removed using a centrifugal spin concentrator (0.5 mL, 10 kDa MWCO). The protein mixture was further washed with Grade I water (2×0.4 ml). The sample was analyzed by ESI-MS. The sample was then buffer exchanged using NaHCO₃ (100 µl, 0.1 M, pH 8.5) and incubated for 24 h. This step resulted in C-S bond dissociation to generate the free aldehyde handle on the protein (37) enabled by the retro-Henry reaction. This trastuzumab conjugate (37) can be stored after lyophilization for late-stage installation of desired probes through their hydroxylamine derivative.

#### 2.7.2 Tagging of trastuzumab

The labelled trastuzumab (37, 2 nmol) in phosphate buffer (80 µl, 50 mM, pH 7.0) was taken in a 1.5 ml microcentrifuge tube. To this solution, the hydroxylamine derivative of coumarin 23 (2 µmol) in Grade I water (10 µl) from a freshly prepared stock solution were added...
separately for the late-stage installation of probes. The reaction mixture was incubated for 3 h to yield antibody-fluorophore conjugate (AFC, 38). The excess hydroxylamine derivative and salts were removed by centrifugal spin concentrator (0.5 ml, 10 kDa MWCO).

**Antibody-drug conjugates (ADC):** The hydroxylamine derivative of DM1 35a (2 µmol) in DMSO (20 µl) and 10% HCl (10 µl) from a freshly prepared stock solution was added to the antibody bioconjugate 37 in phosphate buffer (80 µl, 50 mM, pH 7.0). Subsequently, the reaction mixture was vortexed at 25 °C for 12 h to form the oxime derivative i.e. antibody-drug conjugate (ADC, 39). The reaction mixture was frozen, lyophilized, followed by the addition of 100 µl of water. Under these conditions, the unreacted DM1 derivative 35a is insoluble, enabling its precipitation and removal. The centrifugal spin concentration (10 kDa MWCO) and volume reduction to 250 µl ensured the complete removal of 35a. The sample was lyophilized and stored for further studies.

**Antiproliferative assay:** SKBR-3 cells (10⁴) were seeded in a 96-well plate (tissue culture grade, flat bottom) in a final volume of 100 µl of MacCoy’s 5A culture medium. After seeding for 24 h, the cells were treated with various concentrations (0.25-0.5 nM) of ADC (39), Kadcyla (T-DM1, 40), DM1 (35), and Trastuzumab (34) for the next 48 h and the total volume was kept 200 µl after addition of compounds. All the treatments were given in triplicate. The inhibition of cell proliferation was assessed using the MTT assay from Sigma Aldrich (Sigma Aldrich, Saint Louis, USA). Briefly, MTT reagent (100 µl, final concentration 0.5 mg/ml) was added after removing the medium, and the plates were incubated at 37 °C. After 1-1.5 h (depending upon the formation of crystals) of incubation, DMSO (100 µl) was added, and absorbance was taken on an ELISA plate reader (CYTATION 5, BioTeK) with a test wavelength of 570 nm and a reference wavelength of 630 nm. Relative growth inhibition rates for the untreated control were calculated and expressed as % inhibition of cell proliferation. In order to check the selectivity of ADC (39), we performed the MTT assay in HER-2 negative MDA-MB-231 cells. MDA-MB-231 cells were seeded in the DMEM medium, and the same protocol was followed as mentioned above.
3. Synthesis and characterization

3.1 LDMc-κ reagent synthesis

Supplementary Fig. 1. Synthesis of ethyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 11.

**Synthesis of ethyl 4-(4-formylphenoxy)butanoate S3**

In a 50 ml round bottom flask, p-hydroxybenzaldehyde S1 (611 mg, 5 mmol), ethyl 4-bromobutyrate S2 (859 μl, 6 mmol), K₂CO₃ (1.037 g, 7.5 mmol) and NaI (374 mg, 2.5 mmol) were dissolved in ACN (25 ml). The reaction mixture was stirred at reflux condition for 36 h followed by filtration to remove potassium carbonate. The filtrate was concentrated to remove ACN and the residue was purified using silica gel flash column chromatography ethyl acetate:n-hexane (3:97) to give S3 (969 mg, 82% yield, colourless liquid). TLC (ethyl acetate:n-hexane, 10:90 v/v): Rf = 0.59; 1H NMR (500 MHz, CDCl₃): δ 9.88 (s, 1H), 7.81 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.8 Hz, 2H), 4.15 (q, J = 7.2 Hz, 2H), 4.10 (t, J = 6.2 Hz, 2H), 2.51 (t, J = 7.2 Hz, 2H), 2.14 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H); 13C NMR (101 MHz, CDCl₃): δ 190.9, 173.1, 164.0, 132.1, 130.1, 114.8, 67.2, 60.6, 30.7, 24.5, 14.3; HRMS (ESI): [M+H]^+ calcd. for C₁₃H₁₇O₄, 237.1127; found, 237.1121.

**Synthesis of ethyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate (11)**

In a 100 ml round bottom flask, ethyl 4-(4-formylphenoxy)butanoate S3 (2.36 g, 10 mmol), ammonium acetate (2.31 g, 30 mmol), nitromethane (2.7 ml, 50 mmol) and acetic acid (25 ml) were refluxed for 12 h. The reaction mixture turns into a red-brown solution while heating. The progress of the reaction was followed by TLC and 2,4-DNP was utilized to check the consumption of starting material i.e., aldehyde. After the completion of reaction, reaction mixture was allowed to cool and poured onto the ice to get a yellow colour solid, which was filtered using Büchner funnel. The crude reaction mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (2:98) to afford ethyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 11 (2.51 g, 90% yield, yellow solid). TLC (ethyl acetate:n-hexane, 10:90 v/v): Rf = 0.89; 1H NMR (500 MHz, CDCl₃): δ 7.96 (d, J = 13.6 Hz, 1H), 7.53 – 7.46 (m, 3H), 6.93 (d, J = 8.7 Hz, 2H), 4.15 (q, J = 7.1 Hz, 2H), 4.07 (t, J = 6.2 Hz, 2H), 2.52 (t, J = 7.2 Hz, 2H), 2.14 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H); 13C NMR (126 MHz, CDCl₃): δ
173.4, 162.6, 139.4, 135.5, 131.6, 123.0, 115.8, 67.5, 60.9, 31.0, 24.8, 14.6; HRMS (ESI): [M+Na]+ calcd. for C14H17NNaO5, 302.1107; found, 302.0999.

**Supplementary Fig. 2.** 2,5-dioxopyrrolidin-1-yl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9a.

**Synthesis of (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid (S4)**

In a 10 ml round bottom flask, ethyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 11 (279 mg, 1 mmol) and trifluoroacetic acid (307 µl, 4 mmol) were dissolved in water (5 ml). The reaction mixture was stirred at 100 °C for 12 h. The reaction mixture is allowed to cool and resulting into green yellow solid, which was filter using Büchner funnel. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to isolate gave (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid S4 (240 mg, 95% yield, yellow solid). TLC (ethyl acetate:n-hexane, 30:70 v/v): Rf = 0.30; 1H NMR (500 MHz, CDCl3): δ 7.97 (d, J = 13.6 Hz, 1H), 7.54 – 7.46 (m, 3H), 6.94 (d, J = 8.6 Hz, 2H), 4.09 (t, J = 6.1 Hz, 2H), 2.60 (t, J = 7.1 Hz, 2H), 2.16 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 178.3, 162.2, 139.1, 135.3, 131.3, 122.9, 115.5, 67.0, 30.3, 24.3; HRMS (ESI): [M+H]+ calcd. for C12H14NO5, 252.0872; found, 252.0900.

**Synthesis of 2,5-dioxopyrrolidin-1-yl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate (9a)**

In a 50 ml round bottom flask, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, N-hydroxysuccinimide (920 mg, 8 mmol) was added and stirred at 25 °C, under an inert atmosphere. The progress of the reaction was followed by thin-layer chromatography. After 12 h, the product was extracted using DCM (50 ml × 2). The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to isolate 2,5-dioxopyrrolidin-1-yl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9a (487.2 mg, 70% yield, yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rf = 0.62; 1H NMR (500 MHz, DMSO-
S

20

δ 8.11 (d, J = 13.6 Hz, 1H), 8.07 (d, J = 13.6 Hz, 1H), 7.82 (d, J = 8.9 Hz, 2H), 7.06 (d, J = 8.8 Hz, 2H), 4.14 (t, J = 6.2 Hz, 2H), 2.86 (t, J = 7.3 Hz, 2H), 2.82 (s, 4H), 2.10 (m, 2H); ¹³C NMR (126 MHz, DMSO-d₆): δ 171.0, 168.6, 161.5, 139.2, 135.7, 131.9, 122.7, 115.1, 66.1, 26.9, 25.3, 23.7; HRMS (ESI): [M+Na]⁺ calcd. for C₁₆H₁₆N₂O₇, 371.0855; found, 371.0846.

Supplementary Fig. 3. Synthesis of 2,4,6-tribromophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9b.

Synthesis of 2,4,6-tribromophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate (9b)

In a 50 ml round bottom flask, (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid S₄ (502 mg, 2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were kept on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under inert atmosphere and ice-cold conditions. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,4,6-tribromophenol (1320 mg, 4 mmol) was added and stirred at 25 °C, under an inert atmosphere. The progress of the reaction was followed by TLC. After 12 h, the product was extracted using DCM (50 ml × 2). The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 30:70) to isolate 2,4,6-tribromophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9b (487.2 mg, 70% yield, yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rₜ = 0.65; ¹H NMR (500 MHz, CDCl₃): δ 7.98 (d, J = 13.6 Hz, 1H), 7.71 (s, 2H), 7.54 – 7.49 (m, 3H), 7.50 (d, J = 8.7 Hz, 2H), 4.17 (t, J = 6.0 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H), 2.31 (m, 2H); ¹³C NMR (126 MHz, DMSO-d₆): δ 169.6, 162.0, 139.7, 136.2, 135.1, 132.4, 123.1, 120.2, 118.5, 115.6, 66.8, 29.9, 24.3; HRMS (ESI): [M+H]⁺ calcd. for C₁₈H₁₅Br₃O₅, 561.8500; found, 561.8495.
Synthesis of (3,5-dibromo-4-hydroxyphenyl)(morpholino)methanone (S9)

In a 50 ml round bottom flask, 3,5-dibromo-4-hydroxybenzoic acid S7 (888 mg, 3 mmol), 3-(((ethylimino)methylene)amino)-N,N-dimethylpropan-1-amine (EDC·HCl, 863 mg, 4.5 mmol), and HOBt (405 mg, 3 mmol) were mixed in anh. CH₂Cl₂ (15 ml) at 25 °C. To this solution, DIPEA (1306 µl, 7.5 mmol) and morpholine S8 (775 µl, 9 mmol) were added and stirred for 6 h. The reaction mixture was concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 15:85) to isolate (3,5-dibromo-4-hydroxyphenyl)(morpholino)methanone S9 (yield 83%, white solid). TLC (ethyl acetate:n-hexane, 80:20 v/v): Rf = 0.60; ¹H NMR (400 MHz, CDCl₃): 6 7.46 (s, 2H), 3.90-3.25 (m, 8H); ¹³C NMR (101 MHz, CDCl₃) δ 167.5, 151.2, 131.4, 129.7, 110.4, 66.9 (2C); HRMS (ESI): [M+H]+ calcd. for C₁₁H₁₂Br₂NO₃, 363.9184; found, 363.9173.

Synthesis of 2,6-dibromo-4-(morpholine-4-carbonyl)phenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate (9c)

In a 50 ml round bottom flask, (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid S4 (502 mg, 2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were kept on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under inert atmosphere and ice-cold conditions. Subsequently, the reaction mixture was allowed to achieve the ambient temperature. To this solution, (3,5-dibromo-4-hydroxyphenyl)(morpholino)methanone (1451 mg, 4 mmol) was added and stirred at 25 °C, under an inert atmosphere. The progress of the reaction was followed by TLC. After 12 h, the product was extracted using DCM (50 ml × 2). The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 30:70) to isolate...
2,6-dibromo-4-morpholinophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9c (760 mg, 67% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): R<sub>f</sub> = 0.58; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.97 (d, J = 13.6 Hz, 1H), 7.60 (s, 2H), 7.55 – 7.49 (m, 3H), 6.97 (d, J = 8.8 Hz, 2H), 4.18 (t, J = 6.0 Hz, 2H), 3.87 – 3.40 (m, 8H), 2.94 (t, J = 7.2 Hz, 2H), 2.32 (m, 2H); <sup>13</sup>C NMR (126 MHz, DMSO-d<sub>6</sub>): δ 169.3, 166.2, 165.4, 161.7, 146.1, 139.4, 136.5, 135.8, 132.1, 131.2, 122.8, 117.3, 115.3, 111.6, 66.5, 65.9, 29.7, 24.0; HRMS (ESI): [M+H]<sup>+</sup> calcd. for C<sub>23</sub>H<sub>23</sub>Br<sub>2</sub>N<sub>2</sub>O<sub>7</sub>, 596.9872; found, 596.9867.

Supplementary Fig. 5. Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9d.

Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate (9d)

In a 50 ml round bottom flask, (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid S4 (502 mg, 2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,3,5,6-tetrafluorophenol (474 µl, 4 mmol) was added and stirred at 25 °C, under an inert atmosphere for 12 h. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography (chloroform) to isolate 2,3,5,6-tetrafluorophenyl (E)-4-(4-(2-nitrovinyl)phenoxy)butanoate 9d (542 mg, 68% yield, yellow solid). TLC (CHCl<sub>3</sub>): R<sub>f</sub> = 0.63; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 7.97 (d, J = 13.6 Hz, 1H), 7.56 – 7.47 (m, 3H), 7.01 (m, 1H), 6.97 (d, J = 8.7 Hz, 2H), 4.15 (t, J = 5.9 Hz, 2H), 2.93 (t, J = 7.2 Hz, 2H), 2.30 (m, 2H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 169.1, 162.1, 146.3 (dtd, J = 248.7, 11.8, 4.1 Hz, 1C), 140.9 (m, 1C), 139.0, 135.3, 131.3, 129.9 (tt, J = 14.4, 4.1 Hz, 1C), 123.0, 115.5, 103.4 (t, J = 22.8 Hz, 1C), 66.6, 30.1, 24.4; HRMS (ESI): [M+H]<sup>+</sup> calcd. for C<sub>18</sub>H<sub>14</sub>F<sub>4</sub>NO<sub>5</sub>, 400.0808; found, 400.0803.
Supplementary Fig. 6. Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(2-(2-nitrovinyl)phenoxy)butanoate 9e.

Synthesis of ethyl 4-(2-formylphenoxy)butanoate (S12)

In a 50 ml round bottom flask, 2-hydroxybenzaldehyde S11 (611 mg, 5 mmol), ethyl 4-bromobutyrate S2 (859 μl, 6 mmol), K₂CO₃ (1.037 g, 7.5 mmol) and NaI (374 mg, 2.5 mmol) were dissolved in ACN (25 ml). The reaction mixture was stirred at reflux condition for 24 h followed by filtration to remove potassium carbonate. The filtrate was concentrated to remove ACN and the residue was purified using silica gel flash column chromatography ethyl acetate:n-hexane (3:97) to give S12 (851 mg, 72% yield, colorless liquid).

TLC (ethyl acetate:n-hexane, 10:90 v/v): Rᵣ = 0.60; ¹H NMR (500 MHz, CDCl₃): δ 10.46 (s, 1H), 7.79 (dd, J = 7.7, 1.8 Hz, 1H), 7.53 – 7.46 (m, 1H), 7.01 – 6.93 (m, 2H), 4.16 – 4.08 (m, 4H), 2.51 (t, J = 7.2 Hz, 2H), 2.20 – 2.11 (m, 2H), 1.22 (t, J = 7.2 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 189.7, 173.0, 161.2, 136.0, 128.4, 125.0, 120.8, 112.5, 67.4, 60.6, 30.7, 24.5, 14.2; HRMS (ESI): [M+Na]⁺ calcd. for C₁₃H₁₆NaO₄, 259.0946; found, 259.0934.

Synthesis of ethyl (E)-4-(2-(2-nitrovinyl)phenoxy)butanoate (S13)

In a 100 ml round bottom flask, ethyl 4-(2-formylphenoxy)butanoate S12 (2.36 g, 10 mmol), ammonium acetate (2.31 g, 30 mmol), nitromethane (2.7 ml, 50 mmol) and acetic acid (25 ml) were refluxed for 12 h. The reaction mixture turns into a red-brown solution while heating. The progress of the reaction was followed by TLC and 2,4-DNP was utilized to check the consumption of starting material i.e., aldehyde. After the completion of reaction, reaction mixture was allowed to cool and poured onto the ice to get a yellow colour solid, which was filtered using Büchner funnel. The crude reaction mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (2:98) to afford ethyl (E)-4-(2-(2-
nitrovinyl)phenoxy)butanoate S13 (2.51 g, 90% yield, yellow solid). TLC (ethyl acetate:n-hexane, 10:90 v/v): Rf = 0.91; 1H NMR (700 MHz, CDCl3): δ 8.16 (d, J = 13.6 Hz, 1H), 7.82 (d, J = 13.6 Hz, 1H), 7.47 – 7.41 (m, 2H), 7.01 (m, 1H), 6.97 (d, J = 8.3 Hz, 1H), 4.16 (t, J = 6.3 Hz, 2H), 4.14 (q, J = 7.1 Hz, 2H), 2.54 (t, J = 7.1 Hz, 2H), 2.22 (m, 2H), 1.26 (t, J = 7.2 Hz, 3H); 13C NMR (126 MHz, CDCl3): δ 172.9, 158.7, 138.2, 135.5, 133.6, 132.2, 121.3, 119.3, 112.3, 67.7, 60.8, 30.7, 24.4, 14.3; HRMS (ESI): [M+Na]+ calcd. for C14H17NNaO5, 302.1004; found, 302.0999.

**Synthesis of (E)-4-(2-(2-nitrovinyl)phenoxy)butanoic acid (S14)**

\[
\text{O} \quad \begin{array}{l}
\text{O} \\
\text{O}
\end{array}
\]

In a 10 ml round bottom flask, ethyl (E)-4-(2-(2-nitrovinyl)phenoxy)butanoate S13 (279 mg, 1 mmol) and trifluoroacetic acid (307 µl, 4 mmol) were dissolved in water (5 ml). The reaction mixture was stirred at 100 °C for 12 h. The reaction mixture is allowed to cool and results into green yellow solid, which was filtered using Büchner funnel. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to isolate gave (E)-4-(2-(2-nitrovinyl)phenoxy)butanoic acid S14 (202 mg, 80% yield, yellow solid). TLC (ethyl acetate:n-hexane, 30:70 v/v): Rf = 0.32; 1H NMR (500 MHz, CDCl3): δ 8.14 (d, J = 13.6 Hz, 1H), 7.80 (d, J = 13.6 Hz, 1H), 7.47 – 7.37 (m, 2H), 7.01 – 6.92 (m, 2H), 4.13 (t, J = 6.3 Hz, 2H), 2.54 (d, J = 7.0 Hz, 2H), 2.20 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 158.7, 138.1, 135.6, 133.6, 132.1, 121.3, 119.2, 112.3, 67.7, 30.5, 24.4; HRMS (ESI): [M+H]+ calcd. for C12H14NO3, 252.0872; found, 252.0866.

**Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(2-(2-nitrovinyl)phenoxy)butanoate (9e)**

\[
\text{O} \quad \begin{array}{l}
\text{O} \\
\text{O}
\end{array}
\]

In a 50 ml round bottom flask, (E)-4-(2-(2-nitrovinyl)phenoxy)butanoic acid S14 (100 mg, 0.25 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (96 mg, 0.5 mmol) were dissolved in anh. DCM (2.5 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,3,5,6-tetrafluorophenol S10 (59 µl, 0.5 mmol) was added and stirred at 25 °C, under an inert atmosphere for 12 h. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography (chloroform) to isolate 2,3,5,6-tetrafluorophenyl (E)-4-(2-(2-nitrovinyl)phenoxy)butanoate 9e (115 mg, 73% yield, yellow solid). TLC (CHCl3): Rf = 0.64; 1H NMR (500 MHz, CDCl3): δ 8.20 (d, J = 13.7 Hz, 1H), 7.81 (d, J = 13.6 Hz, 1H), 7.51 – 7.43 (m, 2H), 7.07 – 6.95 (m, 2H), 4.24 (t, J = 6.2 Hz, 2H), 2.95 (t, J = 7.0 Hz, 2H), 2.38 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 168.9, 158.5, 158.7, 138.1, 135.6, 133.6, 132.1, 121.3, 119.2, 112.3, 67.7, 30.5, 24.4; HRMS (ESI): [M+H]+ calcd. for C12H14NO3, 252.0872; found, 252.0866.
146.5 (dtd, J = 248.4, 12.2, 3.7 Hz, 1C), 140.7 (m), 138.2, 135.2, 133.7, 131.9, 129.7 (m, 1C), 121.6, 119.4, 112.4, 103.5 (t, J = 22.8 Hz, 1C), 67.1, 30.0, 24.4; HRMS (ESI): [M+H]+ calcd. for C18H14F4NO5, 400.0808; found, 400.0803.

Supplementary Fig. 7. Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate 9f.

**Synthesis of ethyl 4-(3-formylphenoxy)butanoate (S16)**

In a 50 ml round bottom flask, 3-hydroxybenzaldehyde **S15** (611 mg, 5 mmol), ethyl 4-bromobutyrate **S2** (859 μl, 6 mmol), K$_2$CO$_3$ (1.037 g, 7.5 mmol) and NaI (374 mg, 2.5 mmol) were dissolved in ACN (25 ml). The reaction mixture was stirred at reflux condition for 24 h followed by filtration to remove potassium carbonate. The filtrate was concentrated to remove ACN and the residue was purified using silica gel flash column chromatography ethyl acetate:n-hexane (3:97); to give ethyl 4-(3-formylphenoxy)butanoate **S16** (921 mg, 78% yield, colorless liquid). TLC (ethyl acetate:n-hexane 10:90 v/v): Rf = 0.60; $^1$H NMR (500 MHz, CDCl$_3$): δ 9.94 (s, 1H), 7.46 – 7.37 (m, 2H), 7.35 (d, J = 1.5 Hz, 1H), 7.14 (dt, J = 7.0, 2.4 Hz, 1H), 4.13 (q, J = 7.1 Hz, 2H), 4.05 (t, J = 6.2 Hz, 2H), 2.50 (t, J = 7.3 Hz, 2H), 2.16 – 2.07 (m, 2H), 1.23 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (126 MHz, CDCl$_3$): δ 192.1, 173.1, 159.5, 137.9, 130.1, 123.5, 121.9, 112.9, 67.1, 60.6, 30.8, 24.6, 14.3; HRMS (ESI): [M+H]$^+$ calcd. for C13H17O4, 237.1127; found, 237.1032.
Synthesis of ethyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate (S17)

In a 100 ml round bottom flask, ethyl 4-(3-formylphenoxy)butanoate S16 (2.36 g, 10 mmol), ammonium acetate (2.31 g, 30 mmol), nitromethane (2.7 ml, 50 mmol) and acetic acid (25 ml) were refluxed for 12 h. The reaction mixture turns into a red-brown solution while heating. The progress of the reaction was followed by TLC and 2,4-DNP was utilized to check the consumption of starting material i.e., aldehyde. After the completion of reaction, reaction mixture was allowed to cool and poured onto the ice to get a yellow colour solid, which was filtered using Büchner funnel. The crude reaction mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (2:98) to afford ethyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate S17 (2.56 g, 92% yield, yellow solid). TLC (ethyl acetate:n-hexane 10:90 v/v): Rf = 0.90; ¹H NMR (700 MHz, CDCl₃): δ 7.95 (d, J = 13.6 Hz, 1H), 7.57 (d, J = 13.6 Hz, 1H), 7.35 (m, 1H), 7.13 (d, J = 7.5 Hz, 1H), 7.05 (s, 1H), 7.02 (d, J = 8.3 Hz, 1H), 4.15 (q, J = 7.1 Hz, 2H), 4.04 (t, J = 6.1 Hz, 2H), 2.52 (t, J = 7.2 Hz, 2H), 2.13 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H); ¹³C NMR (176 MHz, CDCl₃): δ 173.2, 159.5, 139.2, 137.5, 131.5, 130.6, 122.0, 118.6, 114.6, 67.1, 60.7, 30.8, 24.6, 14.4; HRMS (ESI): [M+Na]⁺ calcd. for C₁₄H₁₇NNaO₅, 302.1004; found, 302.1004.

Synthesis of (E)-4-(3-(2-nitrovinyl)phenoxy)butanoic acid (S18)

In a 10 ml round bottom flask, ethyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate S17 (279 mg, 1 mmol) and trifluoroacetic acid (307 µl, 4 mmol) were dissolved in water (5 ml). The reaction mixture was stirred at 100 °C for 12 h. The reaction mixture is allowed to cool results into green yellow solid, which was filter using Büchner funnel. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to isolate gave (E)-4-(3-(2-nitrovinyl)phenoxy)butanoic acid S18 (202 mg, 80% yield, yellow solid). TLC (ethyl acetate:n-hexane 30:70 v/v): Rf = 0.34; ¹H NMR (700 MHz, CD₃OD): δ 8.00 (d, J = 13.6 Hz, 1H), 7.89 (d, J = 13.6 Hz, 1H), 7.35 (m, 1H), 7.24 (d, J = 7.5 Hz, 1H), 7.25 (s, 1H) 7.06 (d, J = 8.4 Hz, 1H), 4.06 (t, J = 2.0 Hz, 2H), 2.50 (t, J = 7.3 Hz, 2H), 2.07 (m, 2H); ¹³C NMR (176 MHz, CD₃OD): δ 177.2, 161.2, 140.2, 139.2, 133.4, 131.6, 123.4, 119.8, 115.7, 68.5, 31.6, 26.0; HRMS (ESI): [M+Na]⁺ calcd. for C₁₂H₁₃NNaO₅, 274.0691; found, 274.0686.
Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate (9f)

In a 50 ml round bottom flask, (E)-4-(3-(2-nitrovinyl)phenoxy)butanoic acid S18 (100 mg, 0.25 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (96 mg, 0.5 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (2.5 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,3,5,6-tetrafluorophenol S10 (59 µl, 0.5 mmol) was added and stirred at 25 °C, under an inert atmosphere for 12 h. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography (chloroform) to isolate 2,3,5,6-tetrafluorophenyl (E)-4-(3-(2-nitrovinyl)phenoxy)butanoate 9f (108 mg, 69% yield, yellow solid). TLC (CHCl3): Rf = 0.65; 1H NMR (500 MHz, CDCl3): δ 7.97 (d, J = 13.7 Hz, 1H), 7.57 (d, J = 13.6 Hz, 1H), 7.37 (m, 1H), 7.16 (d, J = 7.6 Hz, 1H), 7.09 – 6.96 (m, 3H), 4.13 (t, J = 6.0 Hz, 2H), 2.93 (t, J = 7.2 Hz, 2H), 2.29 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 169.2, 159.4, 146.2 (tdt, J = 248.6, 12.1, 4.3 Hz, 1C), 141.8 (m, 1C), 139.0, 137.6, 131.6, 130.7, 129.7 (m, 1C), 122.2, 118.5, 114.7, 103.4 (t, J = 22.8 Hz, 1C), 66.6, 30.1, 24.5; HRMS (ESI): [M+H]+ calcd. for C18H14F4NO5, 400.0808; found, 400.0803.

Supplementary Fig. 8. Synthesis of 2,3,5,6-tetrafluorophenyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate 9g.
Synthesis of ethyl 6-(4-formylphenoxy)hexanoate (S20)

In a 50 ml round bottom flask, 4-hydroxybenzaldehyde S1 (610 mg, 5 mmol), ethyl 6-bromohexanoate S19 (1 ml, 6 mmol), K$_2$CO$_3$ (1.037 g, 7.5 mmol) and NaI (374 mg, 2.5 mmol) were dissolved in ACN (25 ml). The reaction mixture was stirred at reflux condition for 24 h followed by filtration to remove potassium carbonate. The filtrate was concentrated to remove ACN and the residue was purified using silica gel flash column chromatography (ethyl acetate:n-hexane 3:97) to give ethyl 6-(4-formylphenoxy)hexanoate S20 (1.05 g, 80% yield, colorless liquid). TLC (ethyl acetate:n-hexane 10:90 v/v): $R_f = 0.57$; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 9.85 (s, 1H), 7.80 (d, J = 8.7 Hz, 2H), 6.96 (d, J = 8.7 Hz, 2H), 4.11 (q, J = 7.1 Hz, 2H), 4.02 (t, J = 6.3 Hz, 2H), 2.32 (t, J = 7.5 Hz, 2H), 1.82 (m, 2H), 1.69 (m, 2H), 1.50 (m, 2H), 1.23 (t, J = 7.2 Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 190.9, 173.6, 164.2, 132.1, 129.9, 114.8, 68.1, 60.4, 34.3, 28.8, 25.6, 24.7, 14.3; HRMS (ESI): [M+H]$^+$ calcd. for C$_{15}$H$_{21}$O$_4$, 265.1440; found, 265.1434.

Synthesis of ethyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate (S21)

In a 100 ml round bottom flask, ethyl 6-(4-formylphenoxy)hexanoate S20 (1.32 g, 5 mmol), ammonium acetate (1.15 g, 15 mmol), nitromethane (1.35 ml, 25 mmol) and acetic acid (12.5 ml) were refluxed for 12 h. The reaction mixture turns into a red-brown solution while heating. The progress of the reaction was followed by TLC and 2,4-DNP was utilized to check the consumption of starting material i.e., aldehyde. After the completion of reaction, reaction mixture was allowed to cool and poured onto the ice to get a yellow colour solid, which was filtered using Büchner funnel. The crude reaction mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (2:98) to afford ethyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate S21 (1.34 g, 88% yield, yellow solid). TLC (ethyl acetate:n-hexane 10:90 v/v): $R_f = 0.85$; $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.97 (d, J = 13.6 Hz, 1H), 7.51 (d, J = 13.6 Hz, 1H), 7.48 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H), 4.13 (q, J = 7.1 Hz, 2H), 4.01 (t, J = 6.4 Hz, 2H), 2.34 (t, J = 7.4 Hz, 2H), 1.82 (m, 2H), 1.71 (m, 2H), 1.52 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H); $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 173.7, 162.6, 139.2, 135.1, 131.3, 122.6, 115.5, 68.2, 60.4, 34.3, 28.9, 25.7, 24.8, 14.4; HRMS (ESI): [M+Na]$^+$ calcd. for C$_{16}$H$_{21}$NNaOs, 330.1317; found, 330.1312.
Synthesis of (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoic acid (S22)

In a 10 ml round bottom flask, ethyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate S21 (307 mg, 1 mmol) and trifluoroacetic acid (307 µl, 4 mmol) were dissolved in water (5 ml). The reaction mixture was stirred at 100 °C for 12 h. The reaction mixture is allowed to cool results into green yellow solid, which was filter using Büchner funnel. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to isolate gave (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoic acid S22 (237 mg, 85% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane 30:70 v/v): Rf = 0.28; 1H NMR (500 MHz, CDCl3): δ 7.90 (d, J = 13.6, 1H), 7.53 – 7.39 (m, 3H), 6.86 (m, 2H), 3.95 (m, 2H), 2.28 (m, 2H), 1.76 (m, 2H), 1.62 (m, 2H), 1.45 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 162.5, 139.3, 134.9, 131.3, 122.4, 115.4, 68.1, 33.9, 25.5, 24.6; HRMS (ESI): [M+H]+ calcd. for C14H18NO5, 280.1185; found, 280.1179.

Synthesis of 2,3,5,6-tetrafluorophenyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate (9g)

In a 50 ml round bottom flask, (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoic acid S22 (100 mg, 0.35 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (134 mg, 0.7 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (3.5 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,3,5,6-tetrafluorophenol S10 (83 µl, 0.5 mmol) was added and stirred at 25 °C, under an inert atmosphere for 12 h. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography (Chloroform) to isolate 2,3,5,6-tetrafluorophenyl (E)-6-(4-(2-nitrovinyl)phenoxy)hexanoate 9g (101 mg, 67% yield, yellow solid). TLC (CHCl3): Rf = 0.60; 1H NMR (500 MHz, CDCl3): δ 7.97 (d, J = 13.6 Hz, 1H), 7.56 – 7.46 (m, 3H), 7.00 (m, 1H), 6.94 (d, J = 8.6 Hz, 2H) 4.05 (t, J = 6.3 Hz, 2H), 2.73 (t, J = 7.3 Hz, 2H), 1.93 – 1.82 (m, 4H), 1.6 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 169.4, 162.5, 146.2 (dtd, J = 248.6, 12.1, 4.1 Hz, 1C), 141.7 (m, 1C), 139.2, 135.2, 131.3, 129.8 (m, 1C), 122.7, 115.5, 103.3 (t, J = 22.8 Hz), 68.0, 33.4, 28.8, 25.5, 24.6; HRMS (ESI): [M+H]+ calcd. for C20H18F4NO5, 428.1121; found, 428.1116.
Supplementary Fig. 9. Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoate 9h.

Synthesis of tert-butyl (4-hydroxyphenyl)carbamate (S25)

In a dry 100 ml round bottom flask, 4-aminophenol S23 (2.18 g, 20 mmol) was dissolved in THF (50 ml). To this solution, di-tert-butyl dicarbonate S24 (4.59 ml, 24 mmol) in THF (50 ml) was added slowly at 0 °C by a dropping funnel. After 2 h, the reaction mixture was brought to 25 °C and stirred for 10 h. The reaction mixture was concentrated in vacuo. Subsequently, washing of the crude solid by hexane rendered the analytically pure product tert-butyl (4-hydroxyphenyl)carbamate S25 (3.75 g, 90% yield, white solid). TLC (ethyl acetate:n-hexane, 20:80 v/v): Rf = 0.40; 1H NMR (500 MHz, CDCl3): δ 7.20-7.10 (m, 2H), 6.73 (d, J = 8.7 Hz, 2H), 6.34 (s, 1H), 5.36 (d, J = 7.0 Hz, 1H), 5.15 (s, 1H); 13C NMR (126 MHz, CDCl3): δ 153.6, 152.1, 131.0, 121.4, 115.7, 80.4, 28.4; HRMS (ESI): [M+Na]+ calcd. for C11H15NNaO3, 232.0950; found, 232.0938.

Synthesis of ethyl 4-(4-((tert-butoxycarbonyl)amino)phenoxy)butanoate (S26)

In a 100 ml round bottom flask, tert-butyl (4-hydroxyphenyl)carbamate S25 (2.092 g, 10 mmol), ethyl 4-bromobutanoate S2 (1.717 ml, 12 mmol), K2CO3 (2.764 g, 20 mmol) and NaI (749 mg, 5 mmol) were dissolved in ACN (50 ml) and refluxed for 24 h. The reaction mixture
was concentrated in vacuo and subjected to partial purification by solvent-solvent extraction using ethyl acetate and water. The organic layer was separated, dried over anh. sodium sulfate and filtered. The filtrate was concentrated under reduced pressure. The crude compound was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (20:80) to afford ethyl 4-(4-((tert-butoxycarbonyl)amino)phenoxy)butanoate S26 (2.70 g, 84% yield, white solid). TLC (ethyl acetate:n-hexane, 20:80 v/v): Rf = 0.21; 1H NMR (500 MHz, CDCl3): δ 7.26-7.19 (m, 2H), 6.81 (d, J = 9.0 Hz, 2H), 6.36 (s, 1H), 4.14 (q, J = 7.1 Hz, 2H), 3.96 (t, J = 6.0 Hz, 2H), 2.50 (t, J = 7.3 Hz, 2H), 2.14-2.04 (m, 2H), 1.50 (s, 9H), 1.25 (t, J = 7.1 Hz, 3H); 13C NMR (126 MHz, CDCl3): δ 173.4, 155.1, 153.3, 131.6, 120.7, 115.0, 80.4, 67.2, 60.6, 31.0, 28.5, 24.8, 14.4; HRMS (ESI): [M+Na]⁺ calcd. for C17H25NNaO5, 346.1630; found, 346.1649.

Synthesis of ethyl 4-(4-aminophenoxy)butanoate (S27)

In a 100 ml round bottom flask, ethyl 4-(4-((tert-butoxycarbonyl)amino)phenoxy)butanoate S26 (1.62 g, 5 mmol) was dissolved in CH2Cl2 (25 ml). To this solution, trifluoroacetic acid (1.53 ml, 20 mmol) was added slowly over 15 minutes and stirred at 25 °C for 12 h. The reaction mixture was quenched with sodium bicarbonate (slow addition), followed by addition of CH2Cl2. The organic layer was separated, dried with anh. sodium sulfate, filtered, and concentrated in vacuo to give ethyl 4-(4-aminophenoxy)butanoate S27 (1.05 g, 95% yield, pale yellow liquid). TLC (ethyl acetate:n-hexane, 30:70 v/v): Rf = 0.25; 1H NMR (500 MHz, CDCl3): δ 6.76-6.70 (m, 2H), 6.66-6.61 (m, 2H), 4.14 (q, J = 7.1 Hz, 2H), 3.92 (t, J = 6.1 Hz, 2H), 3.41 (s, 2H), 2.49 (t, J = 7.3 Hz, 2H), 2.12-2.01 (m, 2H), 1.25 (t, J = 7.1 Hz, 3H); 13C NMR (126 MHz, CDCl3): δ 173.5, 152.1, 140.2, 116.5, 115.8, 67.6, 60.5, 31.0, 24.9, 14.4; HRMS (ESI): [M+H]⁺ calcd. for C12H17NO3, 224.1287; found, 224.1265.

Synthesis of 4-(4-formylphenoxy)butanoic acid (S28)

In a 25 ml round bottom flask, ethyl 4-(4-formylphenoxy)butanoate S3 (236 mg, 1 mmol) and 1 N LiOH (aq., 3 ml) were dissolved in THF (0.3 ml) and stirred at room temperature. The progress of the reaction was followed by TLC. After 3 h, the reaction mixture was concentrated to remove THF and rest part was quenched with 4 N HCl (aq.) and compounds were extracted with ethyl acetate. The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography using ethyl acetate:hexane (70:30) to isolate 4-(4-formylphenoxy)butanoic acid S28 (187 mg, 90% yield, crystalline solid). TLC (ethyl acetate:n-
hexane, 50:50 v/v): Rf = 0.52; 1H NMR (500 MHz, CDCl₃): δ 9.89 (s, 1H), 7.83 (d, J = 8.7 Hz, 2H), 6.99 (d, J = 8.7 Hz, 2H), 4.12 (t, J = 6.1 Hz, 2H), 2.61 (t, J = 7.2 Hz, 2H), 2.17 (m, 2H); 13C NMR (176 MHz, CDCl₃): δ 191.0, 175.4, 164.0, 132.2, 130.2, 114.9, 67.1, 29.8, 24.4; HRMS (ESI): [M+H]+ calcd. for C₁₁H₁₃O₄, 209.0814; found, 209.0808.

Synthesis of ethyl 4-(4-(4-formylphenoxy)butanamido)phenoxy)butanoate (S29)

In a 50 ml round bottom flask 4-(4-formylphenoxy)butanoic acid S28 (416 mg, 2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution ethyl 4-(4-aminophenoxy)butanoate (892 mg, 4 mmol) was added and stirred at 25 °C, under an inert atmosphere. The progress of the reaction was followed by thin-layer chromatography. After 12 h, the product was extracted using DCM (50 ml × 2). The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 30:70) to obtain ethyl 4-(4-(4-(4-formylphenoxy)butanamido)phenoxy)butanoate S29 (677 mg, 82% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rf = 0.68; 1H NMR (500 MHz, CDCl₃): δ 9.87 (s, 1H), 7.81 (d, J = 7.6 Hz, 2H), 7.37 (d, J = 8.8 Hz, 2H), 7.28 (s, 1H), 6.98 (d, J = 8.4 Hz, 2H), 6.82 (d, J = 8.8 Hz, 2H), 4.19 – 4.10 (m, 4H), 3.97 (t, J = 6.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 2.49 (t, J = 7.3 Hz, 2H), 2.28 – 2.18 (m, 2H), 2.13 – 2.04 (m, 2H), 1.25 (t, J = 7.1 Hz, 4H); 13C NMR (126 MHz, CDCl₃): δ 191.0, 173.4, 170.2, 164.0, 155.8, 132.2, 131.0, 130.1, 121.8, 114.9, 67.4, 67.2, 60.6, 33.6, 30.9, 25.0, 24.8, 14.4; HRMS (ESI): [M+H]+ calcd. for C₂₃H₂₈NO₆, 414.1917; found, 414.1965.

Synthesis of (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoic acid (S30)

In a 100 ml round bottom flask, ethyl 4-(4-(4-formylphenoxy)butanamido)phenoxy)butanoate S29 (770.24 mg, 2 mmol), 1 (N) aq. LiOH were added into THF solvent and kept the whole reaction mixture at room temperature with constant stirring. Reaction was monitored by thin-layer chromatography. After 8 h THF was completely concentrated in vacuo and neutralized the whole reaction mixture with 4 (N) aq. HCl solution, the product was extracted using ethyl acetate (50 ml × 2). The collected organic fractions were dried over anh. sodium sulfate and concentrated in vacuo to obtain the product.

S32
Followed by obtained product was treated with ammonium acetate (462 mg, 6 mmol), nitromethane (535 µl, 10 mmol) and acetic acid (5 ml) were refluxed for 12 h. The reaction mixture turns into a red-brown solution while heating. The progress of the reaction was followed by TLC and 2,4-DNP was utilized to check the consumption of starting material i.e., aldehyde. After the completion of reaction, reaction mixture was allowed to cool and poured onto the ice to get a yellow colour solid, which was filtered using Büchner funnel. The crude reaction mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (2:98) to (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoic acid S30 (770 mg, 71% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 10:90 v/v): Rf = 0.95; 1H NMR (500 MHz, CDCl3): δ 7.86 (d, J = 13.5 Hz, 1H), 7.44 (d, J = 13.5 Hz, 1H), 7.39 (d, J = 8.2 Hz, 1H), 7.12 (s, 1H), 7.06 (t, J = 8.0 Hz, 1H), 6.93 (d, J = 8.3 Hz, 1H), 6.84 (d, J = 8.4 Hz, 2H), 6.51 (d, J = 8.4 Hz, 1H), 4.00 (t, 2H), 3.88 (t, 2H), 2.53 – 2.42 (m, 2H), 2.41 – 2.34 (m, 1H), 2.13 – 2.04 (m, 2H), 2.00 – 1.91 (m, 2H), 1.16 (t, 4H); 13C NMR (126 MHz, CDCl3): δ 175.8, 162.3, 159.19, 139.3, 134.9, 131.3, 129.5, 122.5, 115.3, 112.2, 110.2, 106.3, 67.3, 66.7, 33.2, 30.5, 24.8, 24.5; HRMS (ESI): [M+H]+ calcd. for C22H25N2O7, 429.1662; found, 429.1656.

Synthesis of 2,3,5,6-tetrafluorophenyl (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoate (9h)

In a 50 ml round bottom flask, (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoic acid S30 (856 mg, 2 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (764 mg, 4 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (20 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. 2,3,5,6-tetrafluorophenol S10 (474 µl, 4 mmol) was added and stirred at 25°C, under an inert atmosphere for 12 h. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 30:70) to isolate 2,3,5,6-tetrafluorophenyl (E)-4-(4-(4-(2-nitrovinyl)phenoxy)butanamido)phenoxy)butanoate 9h (793 mg, 69% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rf = 0.67; 1H NMR (500 MHz, CDCl3): δ 7.96 (d, J = 13.6 Hz, 1H), 7.51 (d, J = 13.6 Hz, 1H), 7.49 (d, J = 8.7 Hz, 2H), 7.33 (s, 1H), 7.24 – 7.17 (m, 2H), 6.97 – 6.89 (m, 3H), 6.67 (d, J = 8.2 Hz, 1H), 4.13 (t, J = 5.9 Hz, 2H), 4.07 (t, J = 5.9 Hz, 2H), 2.90 (t, J = 7.3 Hz, 2H), 2.59 (t, J = 7.0 Hz, 2H), 2.29 – 2.18 (m, 4H), 0.88 (t, J = 7.1 Hz, 2H); 13C NMR (126 MHz, CDCl3): δ 170.4, 169.3, 162.3, 159.4, 134.9, 131.3, 129.5, 122.5, 115.3, 112.2, 110.2, 67.3, 66.7, 33.2, 30.5, 24.8, 24.5; HRMS (ESI): [M+H]+ calcd. for C28H24F4N2O7, 577.1520; found, 577.1592.
Supplementary Fig. 10. Synthesis of 2,3,5,6-tetrafluorophenyl 4-(4-(2-nitroethyl)phenoxy)butanoate 11a.

Synthesis of 4-(4-(2-nitroethyl)phenoxy)butanoic acid (S31)

In a 50 ml round bottom flask, (E)-4-(4-(2-nitrovinyl)phenoxy)butanoic acid S4 (300 mg, 1.19 mmol) was dissolved in DMSO (23.8 ml). To this solution, slowly sodium borohydride (90 mg, 2.38 mmol) was added under ice cold condition and stirred at 25 °C for 8 h. the progress of the reaction was monitored by TLC. After 8 h, the reaction mixture was concentrated in vacuum. This was followed by solvent-solvent extraction using 40% hexane:ethyl acetate and water. The organic fractions were combined, dried on anh. sodium sulfate, filtered, and concentrated on rotary evaporator. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:hexane, 60:40) to isolate 4-(4-(2-nitroethyl)phenoxy)butanoic acid S31 (160 mg, 52% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): \( R_f = 0.52 \); \( ^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta 7.11 \) (d, \( J = 8.6 \) Hz, 2H), \( 6.84 \) (d, \( J = 8.6 \) Hz, 2H), \( 4.56 \) (t, \( J = 7.4 \) Hz, 2H), \( 4.00 \) (t, \( J = 6.1 \) Hz, 2H), \( 3.25 \) (t, \( J = 7.4 \) Hz, 2H), \( 2.58 \) (t, \( J = 7.2 \) Hz, 2H), \( 2.11 \) (m, 2H); \( ^{13}\)C NMR (126 MHz, CDCl\(_3\)): \( \delta 179.3, \) 158.2, 129.8, 127.9, 115.1, 76.7, 66.7, 32.8, 30.6, 24.5; HRMS (ESI): [M+H]\(^+\) calcd. for C\(_{12}\)H\(_{16}\)NO\(_5\), 254.1028; found, 254.1023.

Synthesis of 2,3,5,6-tetrafluorophenyl 4-(4-(2-nitroethyl)phenoxy)butanoate (11a).

In a 10 ml round bottom flask, 4-(4-(2-nitroethyl)phenoxy)butanoic acid S31 (100 mg, 0.39 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (149 mg, 0.78 mmol) were dried on high vacuum for 30 min. Next, the reagents were dissolved in anh. DCM (3.9 ml) under the presence of an inert atmosphere and ice-cold condition. Subsequently, the reaction mixture is allowed to achieve the ambient temperature. To this solution, 2,3,5,6-tetrafluorophenol (82.5 µl, 0.78 mmol) was added and stirred at 25 °C, under an inert atmosphere for 12 h. The progress of the reaction was followed by TLC. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:n-hexane, 25:65) to isolate 2,3,5,6-tetrafluorophenyl 4-(4-(2-nitroethyl)phenoxy)
butanoate 11a (110 mg, 70% yield, crystalline yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rf = 0.70; ¹H NMR (500 MHz, CDCl₃): δ 7.13 (d, J = 8.6 Hz, 2H), 7.00 (m, 1H), 6.87 (d, J = 8.6 Hz, 2H), 4.57 (t, J = 7.4 Hz, 2H), 4.06 (t, J = 5.9 Hz, 2H), 3.26 (t, J = 7.4 Hz, 2H), 2.91 (t, J = 7.3 Hz, 2H), 2.25 (m, 2H); ¹³C NMR (126 MHz, CDCl₃): δ 169.3, 158.2, 129.8, 128.1, 115.1, 103.4, 76.7, 66.4, 32.9, 30.2, 24.7; HRMS (ESI): [M+Na]⁺ calcd. for C₁₈H₁₅F₄NaO₅, 424.0784; found, 424.0779.

Supplementary Fig. 11. Synthesis of 1-butyl-1H-pyrrole-2,5-dione S34.

Synthesis of 1-butyl-1H-pyrrole-2,5-dione (S34)

To a 250 ml round bottom flask containing maleic anhydride S32 (784 mg, 8 mmol) in ethanol (80 ml), dropwise addition of n-Butylamine (948 µl, 9.6 mmol) and triethyl amine (2.24 ml, 16 mmol) in ethanol (80 ml) was done at ice cold condition followed by stirring for 4 h. The progress of the reaction was followed by TLC. The reaction mixture was concentrated to remove solvent and content were redissolve in acetic anhydride (6 ml) with sodium acetate (327 mg, 3.99 mmol). The reaction mixture was heated over 70 °C for 30 mins and allow to cool down followed by addition of water (10 ml). Subsequently extraction was carried out with ethyl acetate (30 ml × 3). The organic fractions were combined, dried on anh. sodium sulfate, filtered, and concentrated on rotary evaporator. The purification of crude reaction mixture was performed by silica gel flash column chromatography (ethyl acetate:hexane, 30:70) to isolate 1-butyl-1H-pyrrole-2,5-dione S34 (120 mg, 29% yield, pale yellow solid). TLC (ethyl acetate:n-hexane, 50:50 v/v): Rf = 0.48; ¹H NMR (500 MHz, CDCl₃): δ 6.66 (s, 2H), 3.49 (t, J = 7.3 Hz, 2H), 1.54 (m, 2H), 1.27 (m, 2H), 0.90 (t, J = 7.4 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 171.0, 134.1, 37.7, 30.7, 20.0, 13.7; HRMS (ESI): [M+H]⁺ calcd. for C₈H₁₂NO₂, 154.0868; found, 154.0863.
3.2 Derivatives of O-hydroxyl amine

![Diagrams of synthesis processes](image)

Supplementary Fig. 12. Synthesis of 1-butyl-1H-pyrrole-2,5-dione 9b.

**Synthesis of 2-(3-bromopropoxy)isoindoline-1,3-dione (S37)**

In a 250 ml round bottom flask charged with magnetic stir bar, N-hydroxyphthalimide S35 (4.89 g, 30 mmol) and K$_2$CO$_3$ (8.29 g, 60 mmol) were dissolved in ACN (60 ml). Next, 1,3-dibromopropane S36 (8.34 ml, 60 mmol) was added in the reaction mixture was reflux for 16 h. The reaction mixture was concentrated in vacuo followed by addition of 1 N NaOH solution and ethyl acetate for extraction. The organic layer was separated, dried over anh. sodium sulfate, filtered, and concentrated in vacuo. The purification of crude mixture was performed by flash column chromatography using ethyl acetate:hexane (3:97) to render S37 (50% yield, white solid). TLC (ethyl acetate:n-hexane, 30:70 v/v): R$_f$ = 0.57; $^1$H NMR (400 MHz, CDCl$_3$): δ 7.85-7.82 (m, 2H), 7.77-7.74 (m, 2H), 4.36 (t, J = 5.8 Hz, 2H), 3.70 (t, J = 6.5 Hz, 2H), 2.30-2.28 (m, 2H); $^{13}$C NMR (101 MHz, CDCl$_3$): δ 163.7, 134.7, 129.0, 123.7, 76.2, 31.6, 29.4; HRMS (ESI): [M+H]$^+$ calcd. for C$_{11}$H$_{11}$BrNO$_3$, 286.0; found, 285.9.

**Synthesis of 3-((1,3-dioxoisooindolin-2-yl)oxy)propyl 3,5-bis(trifluoromethyl)benzoate S39**

In a 25 ml round bottom flask, 3,5-bis(trifluoromethyl)benzoic acid S40 (258 mg, 1 mmol), 2-(3-bromopropoxy)isoindoline-1,3-dione S38 (312 mg, 1.1 mmol) and K$_2$CO$_3$ (414 mg, 3 mmol) were dissolved in acetonitrile (5 ml). The reaction mixture was refluxed and the progress of the reaction was followed by TLC. After 8 h, the reaction mixture was concentrated and purification by silica gel flash column chromatography (ethyl acetate:n-hexane, 2:98) led to the
isolation of 3-((1,3-dioxoisooindolin-2-yl)oxy)propyl 3,5-bis(trifluoromethyl)benzoate S39 (335 mg, 73% yield, white solid). TLC (ethyl acetate:n-hexane, 30:70 v/v): Rf = 0.67; 1H NMR (400 MHz, CDCl3): δ 8.51 (s, 2H), 8.05 (s, 1H), 7.91-7.81 (m, 2H), 7.80-7.72 (m, 2H), 4.72 (t, J = 6.3 Hz, 2H), 4.41 (t, J = 6.0 Hz, 2H), 2.39-2.21 (m, 2H); 13C NMR (101 MHz, CDCl3): δ 164.0, 163.7, 134.7, 132.5, 132.3 (q, J = 34.1 Hz, 2C), 130.1-129.8 (m, 2C), 129.0, 126.6-126.6 (m, 1C), 123.7, 123.0 (q, J = 272.8 Hz, 2C), 74.9, 62.7, 27.8; 19F NMR (376 MHz, CDCl3) δ -62.94 (TFA was used as an internal standard, -75.70 ppm); HRMS (ESI): [M+H]+ calcd. for C20H14F6NO5, 462.0775; found, 462.0775.

Synthesis of 3-(aminooxy)propyl 3,5-bis(trifluoromethyl)benzoate (21)

In a 5 ml round bottom flask, 3-((1,3-dioxoisooindolin-2-yl)oxy)propyl 3,5-bis(trifluoromethyl)benzoate S39 (138 mg, 0.3 mmol) in DCM (3 ml) and hydrazine monohydrate (80%, 37 µl, 0.75 mmol) were stirred at room temperature. The progress of the reaction was followed by TLC. After 3 h, the reaction mixture was filtered and concentration of the filtrate in vacuo led to the isolation of 3-(aminooxy)propyl 3,5-bis(trifluoromethyl)benzoate 21 (80 mg, 81% yield, white solid). TLC (ethyl acetate:n-hexane, 30:70 v/v): Rf = 0.33; 1H NMR (400 MHz, CDCl3): δ 8.48 (s, 2H), 8.07 (s, 1H), 5.43 (bs, 2H), 4.50 (t, J = 6.5 Hz, 2H), 3.83 (t, J = 6.1 Hz, 2H), 2.19-2.04 (m, 2H); 13C NMR (101 MHz, CDCl3): δ 164.1, 132.6, 132.4 (q, J = 33.9 Hz, 2C), 130.0-129.7 (m, 2C), 126.6-126.3 (m, 1C), 123.02 (q, J = 273.0 Hz, 2C), 72.2, 63.6, 27.9; 19F NMR (376 MHz, CDCl3) δ -62.54 (TFA was used as an internal standard, -75.70 ppm); HRMS (ESI): [M+H]+ calcd. for C12H12F6NO3, 332.0721; found, 332.0699.

Supplementary Fig. 13. Synthesis of 7-((3-(aminooxy)propyl)thio)-4-methyl-2H-chromen-2-one 22.

Synthesis of 3-((1,3-dioxoisooindolin-2-yl)oxy)propyl 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (S41)

In a 5 ml round bottom flask, biotin S40 (244 mg, 1 mmol), 2-(3-bromopropoxy)isoindoline-1,3-dione S37 (568 mg, 2 mmol), and DBU (304 µl, 2 mmol) were dissolved in acetonitrile (20 ml). The reaction mixture was refluxed and the progress of the reaction was monitored by
TLC. After 16 h, the reaction mixture was concentrated in vacuum. This was followed by solvent-solvent extraction using ethyl acetate and water. The organic fractions were combined, dried on anh. sodium sulfate, filtered, and concentrated on rotary evaporator. The purification of crude reaction mixture was performed by silica gel flash column chromatography (MeOH:DCM, 0.5-5%) to isolate 3-((1,3-dioxoisindolin-2-yl)oxy)propyl 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate S41 (224 mg, 50% yield, white solid). TLC (MeOH:DCM 5:95 v/v): Rf = 0.33; 1H NMR (500 MHz, CDCl3): δ 7.89-7.82 (m, 2H), 7.80-7.73 (m, 2H), 5.96 (s, 1H), 5.48 (s, 1H), 4.56-4.47 (m, 1H), 4.38-4.26 (m, 5H), 3.23-3.13 (m, 1H), 2.91 (dd, J = 12.8, 5.0 Hz, 1H), 2.74 (dd, J = 12.8 Hz, 1H), 2.36 (t, J = 7.4 Hz, 2H), 2.16-2.09 (m, 2H), 1.80-1.60 (m, 4H), 1.54-1.39 (m, 2H); 13C NMR (126 MHz, CDCl3): δ 173.7, 163.8, 163.7, 134.7, 129.0, 123.7, 75.1, 62.0, 60.7, 60.2, 55.5, 40.7, 34.0, 28.4, 28.3, 27.8, 24.9; HRMS (ESI): [M+H]+ calcd. for C21H26N3O6S, 448.1542; found, 448.1548.

Synthesis of 3-(aminooxy)propyl 5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (22)

This compound was synthesized according to the procedure for synthesis of 21. (yield 71%, white solid). TLC (MeOH:DCM 5:95 v/v): Rf = 0.21; 1H NMR (500 MHz, D2O): δ 4.63 (dd, J = 7.9, 4.9 Hz, 1H), 4.45 (dd, J = 7.9, 4.5 Hz, 1H), 4.21 (t, J = 6.3 Hz, 2H), 3.90 (t, J = 6.2 Hz, 2H), 3.45-3.27 (m, 1H), 3.02 (dd, J = 13.1, 5.0 Hz, 1H), 2.80 (d, J = 13.0 Hz, 1H), 2.44 (t, J = 7.3 Hz, 2H), 2.08-1.94 (m, 2H), 1.84-1.55 (m, 4H), 1.53-1.37 (m, 2H); 13C NMR (126 MHz, D2O): δ 176.9, 165.3, 72.4, 62.1, 62.0, 60.3, 55.3, 39.7, 33.6, 27.9, 27.6, 26.7, 24.1; HRMS (ESI): [M+H]+ calcd. for C13H24N3O4S, 318.1488; found, 318.1467.

Supplementary Fig. 14. Synthesis of 7-((3-(aminooxy)propyl)thio)-4-methyl-2H-chromen-2-one 23.

Synthesis of 2-((3-((4-methyl-2-oxo-2H-chromen-7-yl)thio)propoxy)isoindoline-1,3-dione (S43)

In a 25 ml round bottom flask, 7-mercaptop-4-methylcoumarin S42 (192 mg, 1 mmol), K2CO3 (276 mg, 2 mmol), and 2-((3-bromopropoxy)isoindoline-1,3-dione S30 (568 mg, 2 mmol) were dissolved in degassed acetonitrile (5 ml) and refluxed for 16 h. The reaction mixture was concentrated in vacuo and purified by silica gel flash column chromatography using ethyl
acetate:hexane (7:3) to give S43 (375 mg, 95% yield, white solid). TLC (ethyl acetate:n-hexane 50:50 v/v): Rf = 0.37; ¹H NMR (400 MHz, CDCl₃): δ 7.90-7.81 (m, 2H), 7.80-7.73 (m, 2H), 7.48 (d, J = 8.2 Hz, 1H), 7.26-7.20 (m, 2H), 6.22 (d, J = 0.8 Hz, 1H), 4.36 (t, J = 5.8 Hz, 2H), 3.35 (t, J = 7.1 Hz, 2H), 2.41 (d, J = 0.9 Hz, 3H), 2.23-2.08 (m, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 163.8, 160.7, 154.0, 152.3, 142.6, 134.7, 129.0, 124.9, 123.8, 123.4, 117.5, 114.8, 114.1, 76.6, 28.7, 27.8, 18.7; HRMS (ESI): [M+H]⁺ calcd. for C₂₁H₁₈NO₅S, 396.0906; found, 396.0925.

Synthesis of 7-((3-(aminoxy)propyl)thio)-4-methyl-2H-chromen-2-one (23)

![Chemical structure of 7-((3-(aminoxy)propyl)thio)-4-methyl-2H-chromen-2-one (23)](image)

2-(3-((4-methyl-2-oxo-2H-chromen-7-yl)thio)propoxy)isoindoline-1,3-dione S43 (237 mg, 0.6 mmol) was dissolved in CH₂Cl₂ (12 ml) in a 50 ml round bottom flask. To this solution, hydrazine monohydrate (80%, 29 µl, 0.6 mmol) was added and stirred at 25 °C for 3 h. The reaction mixture was filtered and the filtrate was concentrated. The purification of crude reaction mixture was performed by reverse phase preparative HPLC to isolate 23 (76 mg, 45% yield, pale green viscous liquid). TLC (ethyl acetate:n-hexane 50:50 v/v): Rf = 0.60; ¹H NMR (400 MHz, CDCl₃): δ 7.46 (d, J = 8.3 Hz, 1H), 7.23-7.13 (m, 2H), 6.18 (d, J = 0.9 Hz, 1H), 3.79 (t, J = 5.9 Hz, 2H), 3.07 (t, S41 J = 7.3 Hz, 2H), 2.40 (d, J = 0.8 Hz, 3H), 2.02-1.90 (m, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 160.7, 154.0, 152.3, 143.3, 124.7, 123.1, 117.2, 114.1, 113.9, 73.9, 29.0, 27.8, 18.6; HRMS (ESI): [M+H]⁺ calcd. for C₁₃H₁₆NO₃S, 266.0851; found, 266.0841.

The compounds 35a was synthesized according to the reported procedure.¹⁷
4. Reference table: characterization data for protein labeling

| Figure          | Related data in supplementary information                                                                 | Page(s)       |
|-----------------|-----------------------------------------------------------------------------------------------------------|---------------|
| Fig. 2a         | Sec. 5.1, Supplementary Figs. 15-26, Supplementary Table 1                                                | S41-S47       |
| Fig. 2b         | Sec. 5.1, Supplementary Figs. 27-28, Supplementary Table 2                                                | S48-S50       |
| Fig. 2c         | Sec. 5.1, Supplementary Figs. 29-37, Supplementary Table 3                                                | S51-S55       |
| Fig. 3a         | Sec. 5.2, Supplementary Figs. 38-43, Supplementary Figs. 1-10                                            | S56-S57       |
| Fig. 3b         | Sec. 5.3, Supplementary Figs. 44-50, Supplementary Table 4                                                | S58-S67       |
|                 | Sec. 8.8a, Supplementary Fig. 99-101, Supplementary Fig. 102-106                                         | S118-S121     |
|                 |                                                                                                          | S122-S124     |
| Fig. 4          | Sec. 6.4 and 6.5, Supplementary Fig. 55 and 56                                                          | S72-S73       |
| Fig. 5a         | Sec. 6.8, Supplementary Figs. 66-70                                                                    | S76-S85       |
| Fig. 5b         | Sec. 6.7, Supplementary Fig. S65, Supplementary Table 7                                                 | S75-S76       |
| Fig. 5d-5f      | Sec. 8.1, Supplementary Fig. 91 and 92                                                                  | S105-S106     |
| Fig. 6a-6b      | Sec. 6.9 and 6.10, Supplementary Figs. 71-77                                                           | S86-S92       |
|                 | Supplementary Figs. 12-14                                                                              |               |
| Supplementary Fig. 51 | Supplementary Table 5                                                                                     | S36-S38       |
| Supplementary Fig. 52 | Sec. 6.1, Supplementary Table 5                                                                            | S68           |
| Supplementary Fig. 53 | Sec. 6.1.1, Supplementary Table 6                                                                              | S69-S70      |
| Supplementary Fig. 54 | Sec. 6.2, Supplementary Table 6                                                                              | S70-S71      |
| Supplementary Figs. 57-64 | Sec. 6.3, Supplementary Table 6                                                                             | S71           |
| Supplementary Table 6 | Supplementary Table 6                                                                                       |               |
| Supplementary Figs. 78-84 | Sec. 6.6, Supplementary Table 6                                                                             | S73-S75      |
| Supplementary Fig. 96 | Sec. 6.11, Supplementary Table 6                                                                            | S92-S97      |
| Supplementary Fig. 97 | Sec. 8.5, Supplementary Table 6                                                                              | S116          |
| Supplementary Fig. 98 | Sec. 8.6, Supplementary Table 6                                                                              | S116          |
| Supplementary Figs. 114-188 | Sec. 8.7, Supplementary Table 6                                                                             | S117          |
| Supplementary Fig. 93 | Sec. 10, Supplementary Table 6                                                                              | S137-S174     |
| Supplementary Table 8 | Supplementary Table 6                                                                                       |               |
| Supplementary Fig. 111-113 | Sec. 8.11, Supplementary Table 6                                                                            | S131-S134    |
| Supplementary Fig. 94 | Sec. 8.3, Supplementary Table 6                                                                              | S112-S113    |
| Supplementary Table 9 | Supplementary Table 6                                                                                       |               |
| Supplementary Fig. 108-109 | Sec. 8.9a & Sec 8.9b, Supplementary Table 6                                                                | S128-S129    |
| Supplementary Fig. 95, | Sec. 8.4, Supplementary Table 6                                                                              | S114-S115    |
| Supplementary Table 10 | Supplementary Table 6                                                                                       |               |
5. Supplementary Notes 2: small molecules and peptides

5.1 Small molecule characterization

Supplementary Table 1. Screening of Cys-selective electrophiles (Fc) for C-S bond formation

| Entry | Electrophile (E) | Product (3a-3l) | % Conversion<sup>b</sup> |
|-------|-----------------|-----------------|--------------------------|
| 1     | 1a              | 3a              | 0                        |
| 2     | 1b              | 3b              | 0                        |
| 3     | 1c              | 3c              | 0                        |
| 4     | 1d              | 3d              | >99                      |
| 5     | 1e              | 3e              | 60                       |
| 6     | 1f              | 3f              | 0                        |
| 7     | 1g              | 3g              | 0                        |
| 8     | 1h              | 3h              | 0                        |
| 9     | 1i              | 3i              | 0                        |
| 10    | 1j              | 3j              | 83                       |
| 11    | 1k              | 3k              | >99                      |
| 12    | 1l              | 3l              | >99                      |

<sup>a</sup> DMSO-d<sub>6</sub>:D<sub>2</sub>O (7:3), 10 mM, 10 min.<sup>b</sup>% Conversion based on <sup>1</sup>H-NMR.<sup>b</sup>% Conversions by <sup>1</sup>H NMR.
Supplementary Fig. 15. Screening of electrophile (1a) for identification of Fc \( ^1 \)H-NMR (DMSO-d6:D2O, 7:3) of electrophile (1a), benzyl mercaptan (2), and (1a+2) to form the adduct 3a (0\%, 10 min).

Supplementary Fig. 16. Screening of electrophile (1b) for identification of Fc \( ^1 \)H-NMR (DMSO-d6:D2O, 7:3) of electrophile (1b), benzyl mercaptan (2), and (1b+2) to form the adduct 3b (0\%, 10 min).
Supplementary Fig. 17. Screening of electrophile (1c) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1c), benzyl mercaptan (2), and (1c+2) to form the adduct 3c (0%, 10 min).

Supplementary Fig. 18. Screening of electrophile (1d) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1d), benzyl mercaptan (2), and (1d+2) to form the adduct 3d (>99%, 10 min).
**Supplementary Fig. 19.** Screening of electrophile (1e) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1e), benzyl mercaptan (2), and (1e+2) to form the adduct 3e (60%, 10 min).

**Supplementary Fig. 20.** Screening of electrophile (1f) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1f), benzyl mercaptan (2), and (1f+2) to form the adduct 3f (0%, 10 min).
Supplementary Fig. 21. Screening of electrophile (1g) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1g), benzyl mercaptan (2), and (1g+2) to form the adduct 3g (0%, 10 min).

Supplementary Fig. 22. Screening of electrophile (1h) for identification of Fc. $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1h), benzyl mercaptan (2), and (1h+2) to form the adduct 3h (0%, 10 min).
Supplementary Fig. 23. Screening of electrophile (1i) for identification of Fc $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1i), benzyl mercaptan (2), and (1i+2) to form the adduct 3i (0%, 10 min).

Supplementary Fig. 24. Screening of electrophile (1j) for identification of Fc $^1$H-NMR (DMSO-d$_6$:D$_2$O, 7:3) of electrophile (1j), benzyl mercaptan (2), and (1j+2) to form the adduct 3j (83%, 10 min).
**Supplementary Fig. 25.** Screening of electrophile (1k) for identification of Fc. \(^1\)H-NMR (DMSO-d\(_6\):D\(_2\)O, 7:3) of electrophile (1k), benzyl mercaptan (2), and (1k+2) to form the adduct 3k (>99%, 10 min).

**Supplementary Fig. 26.** Screening of electrophile (1l) for identification of Fc. \(^1\)H-NMR (DMSO-d\(_6\):D\(_2\)O, 7:3) of electrophile (1l), benzyl mercaptan (2), and (1l+2) to form the adduct 3l (>99%, 10 min).
Supplementary Table 2. Chemoselectivity of nitroolefin (Fc) with cysteine.

| Entry | Fmoc-AA-NH₂ | Product (5a-5t) | % Conversion<sup>b</sup> |
|-------|-------------|----------------|--------------------------|
| 1     | Fmoc-Ala-NH₂ (4a) | 5a            | 0                        |
| 2     | Fmoc-Arg-NH₂ (4b) | 5b            | 0                        |
| 3     | Fmoc-Asn-NH₂ (4c) | 5c            | 0                        |
| 4     | Fmoc-Asp-NH₂ (4d) | 5d            | 0                        |
| 5     | Fmoc-Cys-NH₂ (4e) | 5e            | >99                      |
| 6     | Fmoc-Glu-NH₂ (4f) | 5f            | 0                        |
| 7     | Fmoc-Gln-NH₂ (4g) | 5g            | 0                        |
| 8     | Fmoc-Gly-NH₂ (4h) | 5h            | 0                        |
| 9     | Fmoc-His-NH₂ (4i) | 5i            | 0                        |
| 10    | Fmoc-Ile-NH₂ (4j) | 5j            | 0                        |
| 11    | Fmoc-Leu-NH₂ (4k) | 5k            | 0                        |
| 12    | Fmoc-Lys-NH₂ (4l) | 5l            | 0                        |
| 13    | Fmoc-Met-NH₂ (4m) | 5m            | 0                        |
| 14    | Fmoc-Phe-NH₂ (4n) | 5n            | 0                        |
| 15    | Fmoc-Pro-NH₂ (4o) | 5o            | 0                        |
| 16    | Fmoc-Ser-NH₂ (4p) | 5p            | 0                        |
| 17    | Fmoc-Thr-NH₂ (4q) | 5q            | 0                        |
| 18    | Fmoc-Trp-NH₂ (4r) | 5r            | 0                        |
| 19    | Fmoc-Tyr-NH₂ (4s) | 5s            | 0                        |
| 20    | Fmoc-Val-NH₂ (4t) | 5t            | 0                        |

<sup>a</sup> 30% ACN, NaP (pH 7.0, 50 mM), 500 µM, 25 °C, 30 min. <sup>b</sup>% Conversions by LC-ESI-MS.
Supplementary Fig. 27. Chemoselective labeling of Fmoc-Cys-NH$_2$ (4e) with nitroolefin (1I) monitored by LC-ESI-MS. (a) LC profile for Chemoselective labeling of Fmoc-Cys-NH$_2$ (4e, 1 equiv.) enabled by reagent (1I, 1 equiv.) resulted into adduct (5e, 93%) after 30 min. (b) ESI-MS data for Fmoc-Cys-NH$_2$ (4e). (c) ESI-MS data of Cys adduct with 1I (5e).
Supplementary Fig. 28. Chemoselective labeling of Fmoc-Cys-NH$_2$ (4e) in presence of all twenty natural amino acid with nitroolefin (I) monitored by LC-ESI-MS. (a) LC profile for all the twenty natural amino acids (4a-4t). (b) LC profile for Chemoselective labeling of Fmoc-Cys-NH$_2$ (4e, 1 equiv.) enabled by reagent (I, 1 equiv.) in presence of all twenty natural amino acid resulted into adduct (5e, >99%) after 30 min. (c) ESI-MS data for all the twenty natural amino acids (4a-4t). (d) ESI-MS data of Cys adduct with I (5e).
Supplementary Table 3. C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l).

| Entry | Additive               | % Conversion (1l)^b | % Conversion (7a)^b |
|-------|------------------------|---------------------|---------------------|
| 1     | None                   | 27                  | 0                   |
| 2     | DABCO (6a)             | 33                  | 0                   |
| 3     | DBU (6b)               | 40                  | 14                  |
| 4     | DIPEA (6c)             | 42                  | 22                  |
| 5     | Tetramethylguanidine (6d) | 33                  | 0                   |
| 6     | Triethylamine (6e)     | 40                  | 18                  |
| 7     | Urea (6f)              | 31                  | 0                   |
| 8^c   | 0.1 M NaHCO₃           | 44                  | 55                  |

^c 30% ACN, NaHCO₃ (pH 8.5, 0.1 M), 25 °C, 500 µm, 12 h. ^d % Conversions by LC-ESI-MS.

The formation of disulfide adduct 2a in the control experiments with small molecules can be ignored as it remains negligible upon translation of reaction to large biomolecules such as proteins.

Supplementary Fig. 29. C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for benzylmercaptan-nitrolefin adduct (3l). (b) ESI-MS data for benzylmercaptan-nitrolefin adduct (3l) with various fragment pattern of intact mass.
**Supplementary Fig. 30.** C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3I) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3I) in NaP (pH 7.0, 50 mM) resulted into products (1I, 27% and 7a, 0%) after 24 h. (b) ESI MS data for product 1I.

**Supplementary Fig. 31.** C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3I) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3I) with of DABCO (6a, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1I, 33% and 7a, 0%) after 24 h. (b) ESI-MS data for product 1I.
Supplementary Fig. 32. C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) with of DBU (6b, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1l, 40% and 7a, 14%) after 24 h. (b) ESI-MS data for product 7a.

Supplementary Fig. 33. C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) with of DIPEA (6c, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1l, 42% and 7a, 22%) after 24 h. (b) ESI-MS data for product 7a.
**Supplementary Fig. 34.** C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) with of tetramethylguanidine (6d, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1l, 33% and 7a, 0%) after 24 h. (b) ESI-MS data for product 1l.

**Supplementary Fig. 35.** C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) with of triethylamine (6e, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1l, 40% and 7a, 18%) after 24 h. (b) ESI-MS data for product 7a.
Supplementary Fig. 36. C-S bond dissociation of benzylmercaptan–Fₖ adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) with of urea (6f, 1 equiv.) in NaP (pH 7.0, 50 mM) resulted into products (1l, 31% and 7a, 0%) after 24 h. (b) ESI-MS data for product 1l.

Supplementary Fig. 37. C-S bond dissociation of benzylmercaptan-nitrolefin adduct (3l) monitored by LC-ESI-MS. (a) LC profile for reaction of benzylmercaptan-nitrolefin adduct (3l) in NaHCO₃ (pH 8.5, 0.1 M) resulted into products (1l, 44% and 7a, 55%) after 12 h. (b) ESI-MS data for product 7a.
5.2 Selection of acyl-donor (Fₗ)

Supplementary Fig. 38. Screening of various electrophiles for selection of acyl-donor (Fₗ).

Native BLG A (β-lactoglobulin variant A)

Supplementary Fig. 39. ESI-MS spectrum for β-lactoglobulin variant A [8a, (8a+324)].

Supplementary Fig. 40. (a) ESI-MS spectrum for highly heterogenous-labeled β-lactoglobulin A from the reaction of β-lactoglobulin A 8a (1 equiv.) and LDM<sub>C,K</sub> reagent 9a (10 equiv.) after 6 h. (b) ESI-MS spectrum for highly multi-labeled β-lactoglobulin A 10a from the reaction of β-lactoglobulin A 8a (1 equiv.) and LDM<sub>C,K</sub> reagent 9a (10 equiv.) after 30 sec.
Supplementary Fig. 41. ESI-MS spectrum for mono-labeled β-lactoglobulin A 10b from the reaction of β-lactoglobulin A 8a (1 equiv.) with LDM<sub>C,K</sub> reagent 9b (10 equiv.). 10ba indicates the Michael adduct of β-lactoglobulin A with reagent 9b.

Supplementary Fig. 42. ESI-MS spectrum for mono-labeled β-lactoglobulin A 10c from the reaction of β-lactoglobulin A 8a (1 equiv.) and LDM<sub>C,K</sub> reagent 9c (10 equiv.). 10ca and 10cb indicates mono and bis-labeled Michael adduct of β-lactoglobulin A with reagent 9c.

Supplementary Fig. 43. ESI-MS spectrum for mono-labeled β-lactoglobulin A 10d from the reaction of β-lactoglobulin A 8a (1 equiv.) with LDM<sub>C,K</sub> reagent 9d (10 equiv.).
5.3 Peptide modification

Supplementary Table 4. Peptide screening

| Entry | Peptides                  | % Conversion<sup>b</sup> | 13a-13d | 14a-14d |
|-------|---------------------------|--------------------------|---------|---------|
| 1<sup>c</sup> | Bz-LCGLLG-NH₂ (12a) | 77 (13a) | 0       |         |
| 2<sup>d</sup> | Bz-LCKGLL-NH₂ (12S1) | 0 (13S1) | 65 (14S1) |         |
| 3<sup>d</sup> | Bz-LCGKLL-NH₂ (12S2) | 0 (13S2) | 33 (14S2) |         |
| 4<sup>d</sup> | Bz-LCGJKL-NH₂ (12S3) | 0 (13S3) | 75 (14S3) |         |
| 5<sup>d</sup> | Bz-LCGLLK-NH₂ (12b) | 3 (13b) | 75 (14b) |         |
| 6<sup>e</sup> | Bz-C('Bu)GGGK-NH₂ (12c) | 0 (13c) | 0 (14c) |         |
| 7<sup>f</sup> | Bz-LCGLLKGGLK-NH₂ (12d) | 5 (13d) | 33 (14d) (K6) | 0 (K10) |

<sup>a</sup>30% ACN, NaP (pH 7.0, 50 mM), 500 µM, 25 °C, 10-60 min. <sup>b</sup>% Conversions by LC-ESI-MS.<sup>c</sup> Peptide containing only Cys 12a resulted in F-C adduct 13a with 1I reagent. <sup>d</sup>Peptide containing Cys (i<sup>th</sup>) and Lys (i+1 to i+4) 12s1-12b position were treated with LDM<sub>C,K</sub> reagent 9d resulted into F-C adduct and cyclization. The high conformational flexibility of a small peptide counters the effect from relative Cys/Lys position. However, this effect becomes more pronounced in the case of proteins. <sup>e</sup>Peptide having Cys protected with 'Bu and free Lys 12c was reacted with LDM<sub>C,K</sub> reagent 9d to check the rate of acylation. <sup>f</sup>Peptide containing Cys (i<sup>th</sup>) and Lys at two distinct position (i+4 and i+8) 12d were treated with LDM<sub>C,K</sub> reagent 9d for site-selectivity. All the peptides used for the study here, were N-terminus protected with benzoyl group to get better LC profile and the C-terminus carboxylate were converted to amide using rink amide resin via solid phase peptide synthesizer.
Supplementary Fig. 44. (a) C-S bond formation for Cys containing peptide 12a with reagent 11. (b) LC data for Fc adduct 13a of Cys containing peptide 12a enabled by reagent 11 after 10 min. (c) ESI-MS data for 12a. (d) ESI-MS data for Fc adduct 13a.
Supplementary Fig. 45. (a) C-S bond formation and cyclization for peptide containing Cys (i^th) and lysine (i+1) 12S1 enabled by LDM_{C-K} reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i^th) and lysine (i+1) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for 12S1. (d) ESI-MS data for Michael adduct 13S1. (e) ESI-MS data for cyclized peptide 14S1.
Supplementary Fig. 46. (a) C-S bond formation and cyclization for peptide containing Cys (i^th) and lysine (i+2) 12S2 enabled by LDM_{C-K} reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i^th) and lysine (i+2) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for 12S2a. (d) ESI-MS data for Michael adduct 13S2. (e) ESI-MS data for cyclized peptide 14S2.
Supplementary Fig. 47 (a) C-S bond formation and cyclization for peptide containing Cys (i^{th}) and lysine (i+3) 12S3 enabled by LDM-C-K reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i^{th}) and lysine (i+3^{th}) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for 12S3. (d) ESI-MS data for Michael adduct 13S3. (e) ESI-MS data for cyclized peptide 14S3.
Supplementary Fig. 48. (a) C-S bond formation and cyclization for peptide containing Cys (i^{th}) and lysine (i+4^{th}) 12b enabled by LDM_{C.K} reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i^{th}) and lysine (i+4^{th}) enabled by reagent 9d with different interval time (10-60 min). (c) ESI-MS data for 12b1. (d) ESI-MS data for Michael adduct 13b. (e) ESI-MS data for cyclized peptide 14b. (f) MS-MS data for K6 modification in 14b.
**Supplementary Fig. 49.** (a) Acylation of Lys containing peptide (in which Cys protected with 'Bu group) 12c enabled by LDM\(_{C\text{-K}}\) reagent 9d. (b) LC data for acylation of Lys containing peptide 12c enabled by reagent 9d. (c) ESI-MS data for 12c.

\* 30% ACN, NaP (pH 7.0, 50 mM), 500 μM, 25 °C, 60 min. \(\text{b} \) % Conversions by LC-ESI-MS.
Peptide having Lys at two distinct positions

(a) LDM$_{C-K}$ reagent 9d (0.50 mM) → C-S bond formation

Cyclization Condition

12d → 12d1

33% conversion

14d (K6)

(b) (c)

60 min

13d $[M+2H]^{2+}$ 780.5

134.2

134.2

235.2

580.8

833.5

1088.4

1233.4

1393.4

(d)

+MS, 11.5-11.7 min #491-500

13d $[M+2H]^{2+}$ 897.4

14d 1393.6

134.2

235.2

30% ACN, NaP (pH 7.0, 50 mM), 500 µM, 25 °C, 60 min. b % Conversions by LC-ESI-MS.

12d1 indicates oxidised and other masses observed in the reaction mixture after 60 min but not the mass of 12d.
Supplementary Fig. 50. (a) Macrocyclization of Cys and two Lys containing peptide (i+4 and i+8) 12d enabled by LDM<sub>CPK</sub> reagent 9d. (b) LC data for macrocyclization of cysteine and two Lys containing peptide (i+4 and i+8) 12d enabled by LDM<sub>CPK</sub> reagent 9d. (c) ESI-MS data for 12d. (d) ESI-MS data for cyclized peptide 14d. (e) MS-MS of cyclic product 14d further confirmed the site-selective modification i.e., K6.
6. Supplementary Notes 3: protein labelling

6.1 Screening of proteins with nitroolefin

Supplementary Table 5. Screening of proteins with nitroolefin

| Entry | Proteins                          | Product (S44-S51) | % Conversion\(^a\) |
|-------|-----------------------------------|-------------------|--------------------|
| 1\(^b\) | \(\beta\)-Lactoglobulin A (8a)    | S44               | 88 (m)             |
| 2     | Lysozyme C (8b)                   | S45               | 0                  |
| 3     | Myoglobin (8c)                    | S46               | 0                  |
| 4     | Cytochrome C (8d)                 | S47               | 0                  |
| 5     | \(\alpha\)-lactalbumin (8e)      | S48               | 0                  |
| 6     | Insulin (8f)                      | S49               | 0                  |
| 7     | Ubiquitin (8g)                    | S50               | 0                  |
| 8     | RNase A (8h)                      | S51               | 0                  |
| 9     | Aprotinin (8i)                    | S52               | 0                  |

\(^a\) % Conversion based on MALDI-MS. \(^b\) m-mono-labeled \(\beta\)-lactoglobulin A (S44) with reagent 1l within 10 min.

Supplementary Fig. 51. MALDI-MS spectrum for various proteins (8a-8i) including mono-labeled \(\beta\)-lactoglobulin A (S44, 88% conversion) after the reaction with reagent 1l.
6.1.1 C-S bond formation of nitroolefin with β-lactoglobulin

(a) 
\[
\begin{align*}
\text{β-lactoglobulin A} & \quad (8a) \\
(20 \ \mu M) & \\
\text{Nitroolefin} & \quad (1I) \\
(0.2 \ mM) & \\
\text{C-S bond formation} & \\
20\% \ ACN & \\
\text{NaP (50 mM, pH 7.0)} & \\
25^\circ \text{C}, 20 \ \mu M, 10 \text{ min} & \\
\rightarrow & \\
\text{β-lactoglobulin A} & \quad (S44)
\end{align*}
\]

(b) 
Deconvoluted

(c) 

Supplementary Fig. 52. (a) Michael addition of β-lactoglobulin A 8a (1 equiv.) enabled by reagent 11 (10 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A S44. (b) Peptide mapping of S44 after the digestion with α-chymotrypsin. (d) MS-MS spectrum of labeled VCQCL (V118-L122, m/z 844.7 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A S44 is C121.

6.2 Acylation of lysine in BLG-A (Cys-Protected)
### Supplementary Fig. 53

(a) Formation of C-S bond (Cys protection) of β-lactoglobulin A 8a (1 equiv.) enabled by reagent S34 (10 equiv.). (b) ESI-MS spectrum mono-labeled β-lactoglobulin A S53 after C-S bond formation (Cys protection). (c) Acylation of Cys-protected β-lactoglobulin A S53 with LDM_{CK} reagent 9d. (d) ESI-MS spectrum of mono-labeled β-lactoglobulin A S53 after no acylation with LDM_{CK} reagent 9d.

### 6.3 Acylation of Cysteine in BLG-A

(a) Reaction of β-lactoglobulin A 8a with control reagent 11a. (b) ESI-MS spectrum penta-labeled β-lactoglobulin A S55 from 8a (1 equiv.) and 11a (10 equiv.).
6.4 Retro-Michael addition with protein

Supplementary Fig. 55. (a) Reaction of \( \text{LDM}_{\text{C,K}} \) reagent 9d with \( \beta \)-lactoglobulin A 8a followed by C-S bond dissociation via retro-Michael reaction. (b) ESI-MS spectrum mono-labeled \( \beta \)-lactoglobulin A 10d after C-S bond formation and cyclization with a mass impurity of 324 (10d+324). (c) ESI-MS spectrum of mono-labeled \( \beta \)-lactoglobulin A (16, 29% conversion) after C-S bond dissociation via retro-Michael reaction.

6.5 Retro-Henry reaction with protein
Supplementary Fig. 56. (a) Reaction of LDM$_{C-K}$ reagent 9d with β-lactoglobulin A 8a followed by C-S bond dissociation via retro-Henry reaction. (b) ESI-MS spectrum mono-labeled β-lactoglobulin A 10d with mass impurity of 324 (10d+324) after C-S bond formation and cyclization. (c) ESI-MS spectrum of mono-labeled β-lactoglobulin A (17, 88% conversion) after C-S bond dissociation via retro-Henry reaction.

6.6 Screening of proteins with LDM$_{C-K}$ reagent (9d)

Supplementary Table 6. Screening of proteins with LDM$_{C-K}$ reagent 9d.

| Entry | Proteins | Product (10d-10di) | % Conversion$^a$ |
|-------|----------|--------------------|-----------------|
| 1$^b$ | β-Lactaglobulin A (8a) | 10d | >99 (m) |
| 2     | Lysozyme C (8b) | 10db | 0 |
| 3     | Myoglobin (8c) | 10dc | 0 |
| 4     | Cytochrome C (8d) | 10dd | 0 |
| 5     | Insulin (8f) | 10df | 0 |
| 6     | Ubiquitin (8g) | 10dg | 0 |
| 7     | RNase A (8h) | 10dh | 0 |
| 8     | Aprotinin (8i) | 10di | 0 |

$^a$ % Conversion based on ESI-MS. $^b$ m-mono-labeled β-lactoglobulin A 10d with reagent 9d.

Supplementary Fig. 57. ESI-MS spectrum of mono-labeled β-lactoglobulin A 10d after the reaction of β-lactoglobulin A 8a with LDM$_{C-K}$ reagent 9d.
Supplementary Fig. 58. ESI-MS spectrum of Lysozyme C 8b after no reaction with LDM_{C,K} reagent 9d.

Supplementary Fig. 59. ESI-MS spectrum of Myoglobin 8c after no reaction with LDM_{C,K} reagent 9d.

Supplementary Fig. 60. ESI-MS spectrum of Cytochrome C 8d after no reaction with LDM_{C,K} reagent 9d.

Supplementary Fig. 61. ESI-MS spectrum of Insulin 8f after no reaction with LDM_{C,K} reagent 9d.

Supplementary Fig. 62. ESI-MS spectrum of Ubiquitin 8g after no reaction with LDM_{C,K} reagent 9d.
Supplementary Fig. 63. ESI-MS spectrum of RNase A 8h after no reaction with LDM<sub>C,K</sub> reagent 9d.

Supplementary Fig. 64. ESI-MS spectrum of Aprotinin 8i after no reaction with LDM<sub>C,K</sub> reagent 9d.

6.7 Kinetics of C-S bond formation and cyclization

Supplementary Table 7. Time-wise monitoring of C-S bond formation and cyclization with reagent 9d.

| Entry | Time  | % Conversion<sup>a,b</sup> | % Conversion<sup>a,b</sup> |
|-------|-------|---------------------------|---------------------------|
|       |       | Michael adduct (10S1)     | Cyclization (10d)         |
|       |       | I    | II   | III | I    | II   | III |
| 1     | 10 sec| 0    | 6    | 10  | 0    | 0    | 0   |
| 2     | 30 sec| 26   | 19   | 20  | 4    | 0    | 0   |
| 3     | 1 min | 18   | 19   | 19  | 5    | 0    | 0   |
| 4     | 2 min | 40   | 44   | 39  | 18   | 18   | 20  |
| 5     | 5 min | 26   | 21   | 24  | 29   | 25   | 24  |
| 6     | 10 min| 27   | 16   | 21  | 49   | 31   | 41  |
| 7     | 30 min| 12   | 11   | 15  | 33   | 42   | 58  |
| 8     | 60 min| 7    | 6    | 4   | 68   | 71   | 57  |
| 9     | 3 h   | 0    | 6    | 6   | 80   | 79   | 78  |
| 10    | 6 h   | 0    | 0    | 0   | 92   | >99  | >99 |

<sup>a</sup> % Conversion based on ESI-MS.  
<sup>b</sup> This experiment was done in triplicate (I-III).
Supplementary Fig. 65. (a) Time-wise monitoring of C-S bond formation and cyclization with LDM<sub>CK</sub> reagent 9d. (b) ESI-MS deconvoluted spectrum for various time interval of C-S bond formation and cyclization for β-lactoglobulin A 8a with LDM<sub>CK</sub> reagent 9d (for trial I).

6.8 Single-site labeling of native proteins
Supplementary Fig. 66. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9d (10 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A 10d after cyclization. (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A 20d after oxime formation. (d) Peptide mapping of 20d after the digestion with α-chymotrypsin. (e) MS-MS spectrum of labeled KKY (K100-Y102, m/z 643.3 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A 20d is K101.
Supplementary Fig. 67. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9e (1.2 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A 10e after cyclization with mass impurity of 324 (10e+324). (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A 20e after oxime formation. (d) Peptide mapping of 20e after the digestion with α-chymotrypsin. (e) MS-MS spectrum of labeled KKY (K100-Y102, m/z 733.3 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A 20e is K101.
Supplementary Fig. 68. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9f (1.2 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A 10f after cyclization with mass impurity of 324 (10f+324). (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A 20f after oxime formation. (d) Peptide mapping of 20f after the digestion with α-chymotrypsin. (e) MS-MS spectrum of labeled KKY (K100-Y102, m/z 733.3 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A 20f is K101.
Supplementary Fig. 69. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDM<sub>CK</sub> reagent 9g (1.2 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A 10g after cyclization with mass impurity of 324 (10g+324). (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A 20g after oxime formation. (d) Peptide mapping of 20g after the digestion with α-chymotrypsin. (e) MS-MS spectrum of labeled KGL (K8-L10, m/z 550.4 [M+H]<sup>+</sup>). The site of modification in mono-labeled β-lactoglobulin A 20g is K8.
Supplementary Fig. 70. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDM\textsubscript{CK} reagent 9h (10 equiv.). (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A 10h after cyclization with mass impurity of 324 (10h+324). (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A 20h (with oxidised product 20ha) after oxime formation. (d) Peptide mapping of 20h after the digestion with α-chymotrypsin. (e) MS-MS spectrum of labeled EKF (E134-F136, m/z 806.4 [M+H]\textsuperscript{+}). The site of modification in mono-labeled β-lactoglobulin A 20h is K135.
6.9 Late-stage modification

C-S bond formation
Cyclization
20% ACN, NaP (50 mM, pH 7.0), 25 °C, 20 μM, 6 h
>99% conversion

Late-stage modification
1. NaHCO₃ buffer (pH 8.5, 0.1 M)
   25 °C, 20 μM, 48 h
2. NaP (50 mM, pH 7.0), 25 °C
   RON₃ (21, 10 equiv.), 20 μM, 12 h
   >99% conversion

8a
(20 μM)

10d

24

S86
Supplementary Fig. 71. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9d (10 equiv.) followed by oxime formation with reagent 21. (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A (24, >99% conversion). (c) Peptide mapping of 24 after the digestion with α-chymotrypsin. (d) MS-MS spectrum of labeled KKY (K100-K102, m/z 941.9 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A 24 is K101.
Supplementary Fig. 72. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9d (10 equiv.) followed by oxime formation with reagent 22. (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A (25, 86% conversion). (c) Peptide mapping of 25 after the digestion
with α-chymotrypsin. (c) MS-MS spectrum of labeled KKY (K100-K102, m/z 927.9 [M+H]+). The site of modification in mono-labeled β-lactoglobulin A 25 is K101.
Supplementary Fig. 73. (a) Site-selective labeling of β-lactoglobulin A 8a (1 equiv.) enabled by LDMCK reagent 9d (10 equiv.) followed by oxime formation with reagent 23. (b) ESI-MS spectrum for mono-labeled β-lactoglobulin A (26, 91% conversion). (c) Peptide mapping of 26 after the digestion with α-chymotrypsin. (c) MS-MS spectrum of labeled KKY (K100-K102, m/z 875.3 [M+H]^+). The site of modification in mono-labeled β-lactoglobulin A 26 is K101.
6.10 Dual probe installation

Supplementary Fig. 74. (a) Dual probe installation on β-lactoglobulin A 8a enabled by LDM<sub>CK</sub> reagent 9d (10 equiv.) followed by oxime formation with various hydroxyl amine derivative (21-23, 10-30 equiv.) and Michael addition by CPM dye (27, 10equiv.). (b) Reaction of CPM dye 27 with β-lactoglobulin A 8a. (c) ESI-MS spectrum for mono-labeled β-lactoglobulin A (S56, >99% conversion).
Supplementary Fig. 75. ESI-MS spectrum for mono-labeled β-lactoglobulin A (28, 78% conversion) after dual probe installation with hydroxyl amine derivative 21, and CPM dye 27.

Supplementary Fig. 76. ESI-MS spectrum for mono-labeled β-lactoglobulin A (29, 73% conversion) after dual probe installation with hydroxyl amine derivative 22, and CPM dye 27.

Supplementary Fig. 77. ESI-MS spectrum for mono-labeled β-lactoglobulin A (30, 71% conversion) after dual probe installation with hydroxyl amine derivative 23, and CPM dye 27.

6.11 Single-site modification of serum albumins (BSA and HSA)

Supplementary Fig. 78. ESI-MS spectrum of native BSA 8j.
**Supplementary Fig. 79.** (a) Site-selective labeling of BSA 8j (1 equiv.) enabled by LDM<sub>CK</sub> reagent 9d (10 equiv.). (b) Peptide mapping of S57 after the digestion with trypsin. (c) MS-MS spectrum of labeled KVPQVSTPTLVEVSR (K413-R427, m/z 645.6 [M+3H]<sup>3+</sup>). The site of modification in mono-labeled BSA S57 is K413.
Supplementary Fig. 80. (a) Site-selective labeling of BSA 8j (1 equiv.) enabled by LDMCK reagent 9g (10 equiv.). (b) Peptide mapping of S58 after the digestion with trypsin. (c) MS-MS spectrum of labeled SLGKVGR (S428-R435, m/z 570.8 [M+2H]^{2+}). The site of modification in mono-labeled BSA S58 is K431.

Supplementary Fig. 81. ESI-MS spectrum of native HSA 8k.
Supplementary Fig. 82. (a) Site-selective labeling of HSA 8k (1 equiv.) enabled by LDM_{CK} reagent 9d (10 equiv.). (b) Peptide mapping of S59 after the digestion with trypsin. (c) MS-MS spectrum of labeled LKCASLQK (L198-K205, m/z 621.8 [M+2H]^2+). The site of modification in mono-labeled HSA S59 is K199.
Supplementary Fig. 83. (a) Site-selective labeling of HSA 8k (1 equiv.) enabled by LDM$_{CK}$ reagent 9e (1.2 equiv.). (b) Peptide mapping of S60 after the digestion with trypsin. (c) MS-MS spectrum of labeled LKCASLQK (L198-K205, m/z 414.8 [M+3H]$^{3+}$). The site of modification in mono-labeled HSA S60 is K199.
Supplementary Fig. 84. (a) Site-selective labeling of HSA 8k (1 equiv.) enabled by LDM\textsubscript{CK} reagent 9f (1.2 equiv.). (b) Peptide mapping of S61 after the digestion with trypsin. (c) MS-MS spectrum of labeled LKCASLQK (L198-K205, m/z 621.8 \([M+2H]^2+\)). The site of modification in mono-labeled HSA S61 is K199.
6.12 Modification and enrichment of HSA from mixture of proteins

(a)

(b)

(c)

(d)

- NaP buffer (pH 7.0, 50 mM), 20% ACN, LDMC reagent (9d)
- NaHCO₃ buffer (pH 8.5, 0.1 M), 25 °C, 48 h
- NaP buffer (pH 7.0, 50 mM), 25 °C, 24 h
- NaP buffer (pH 6.0, 50 mM), 25 °C, ROH₂ (23, 100 equiv.), 10% ACN, 24 h
**Supplementary Fig. 85.** (a) Modification and enrichment of HSA 8k in mixture of proteins enabled by LDMCK reagent 9d (10 equiv.). (b) TIC showing mixture of proteins (8b-8h) also modified HSA (S62) eluted at different time interval after the treatment with LDMCK reagent 9d. (c-i) ESI-MS spectra of various f proteins (8b-8h) including modified HSA (S62) suggesting the selective modification of HSA (S62) after the reaction with LDMCK reagent 9d.

1: Ladder  
2: Mixture of proteins  
3: Purified tagged protein (33)

**Supplementary Fig. 86.** (a) Gradient 10-20% SDS-PAGE showing fluorescence imaging and Coomassie staining. MW - Molecular Weight, SDS-PAGE: lane 1 - ladder, lane 2 - mixture of proteins containing HSA 8k, lane 3 - coumarin-tagged purified HSA 33. (b) Peptide mapping of coumarin-tagged purified HSA 33 after the digestion with α-chymotrypsin. (c) MS-MS spectrum of labeled KCASL (K199-L203, m/z 508.2 [M+2H]²⁺). The site of modification in coumarin-tagged purified HSA 33 is K199.
6.13 Modification and enrichment of HSA from cell lysate

(a) Modification and enrichment of HSA 8k in cell lysate derived from BL21 cells, E. coli enabled by LDM_{CK} reagent 9d (10 equiv.). (b) 10% SDS-PAGE showing fluorescence imaging and Coomassie staining. MW - Molecular Weight, SDS-PAGE: lane 1 - ladder, lane 2 - cell lysate containing HSA 8k, lane 3 - coumarin-tagged purified HSA 33. (c) Peptide mapping of coumarin-tagged purified HSA 33 after the digestion with trypsin. (d) MS-MS spectrum of labeled LKCASLQK (L198-K205, m/z 692.8 [M+2H]^2+). The site of modification in coumarin-tagged purified HSA 33 is K199.

Supplementary Fig. 87.
7. Trastuzumab modification

step 1: C-S bond formation and cyclization. *TCEP (1 equiv.), 1 h, NaF (pH 7.0, 50 mM), 25 °C, 20 µM, [29% ACN, LOMe reagent 9d (15 equiv.)], 8 h.
step 2: C-S and C-C bond disassociation (Atrio-Henry). *NaHCO$_3$ buffer (pH 8.5, 0.1 M), 25 °C, 20 µM, 24 h.
step 3: Late-stage modification (caliche formation). *NaF (pH 7.0, 50 mM), 25 °C, 20 µM, 5% TFA (0.01-0.1% equiv.), 3-12 h.
**Supplementary Fig. 88.** (a) Labeling of trastuzumab 34 (1 equiv.) enabled by TCEP (1 equiv.) followed by LDM_C-K reagent 9d (15 equiv.) followed by oxime formation with (23/35a). (b) ESI-MS data for light chain in native trastuzumab. (c) ESI-MS data for light chain in modified trastuzumab. (d) ESI-MS data for heavy chain in native trastuzumab, showing all the post-translational modification. (d) ESI-MS data for heavy chain in modified trastuzumab, showing all the post-translational modification. (e) The bar graph suggesting the percentage modification of light chain (10%) and heavy chain (41%), which sum up the aldehyde antibody ratio (AAR) 1.0.
Supplementary Fig. 89. (a) Peptide mapping of light chain of aldehyde-antibody conjugate 37 after the digestion with α-chymotrypsin and trypsin both. (b) MS-MS spectrum of labeled TLSK (T180-K183, m/z 639.3 [M+H]+). The site of modification in light chain of modified trastuzumab 37 is K183. (c) Peptide mapping of heavy chain of 37 after the digestion with α-chymotrypsin and trypsin both. (d) MS-MS spectrum of labeled TISK (T338-K341, m/z 639.3 [M+H]+). The site of modification in heavy chain of modified trastuzumab 37 is K341.
Supplementary Fig. 90. 12% SDS-PAGE of trastuzumab (34) and coumarin-tagged trastuzumab (38) after DTT (10 mM, 15 min, 37 °C) reduction followed by Coomassie staining and fluorescence imaging. MW - Molecular Weight, SDS-PAGE: lane 1 - ladder, lane 2 - trastuzumab (34), lane 3 - coumarin-tagged trastuzumab (AFC, 38). The band at ~25 kDa and ~50 kDa correspond to the reduced light chain and heavy chain, respectively. The presence of band ~25 kDa and ~50 kDa shown by fluorescence imaging confirms the labeling of light and heavy chain.

8. Supplementary Notes 4

8.1 Molecular dynamics simulations

Supplementary Fig. 91. Probability distribution of the distance between the Cys121-SG atom and the NZ atom of nearby lysine residues in the protein without LDM<sub>C-K</sub> reagent in water. As an example, the distance for Lys101 is shown in the inset.
Supplementary Fig. 9.2. Probability distribution of the distance between the C11 atom of the LDM\textsubscript{C,K} reagent and the NZ atom of nearby lysine residues in the protein in all the three sets of independent runs. The structural snapshots show the interaction of those lysine residues with the LDM\textsubscript{C,K} reagent.

8.2 Stability of the LDM\textsubscript{C,K} reagent 9d

Supplementary Table 8: Stability of LDM\textsubscript{C,K} reagent 9d.

| Entry | pH (X) | 0 h | 6 h | 12 h | 24 h |
|-------|--------|-----|-----|------|------|
| 1     | 6.0    | 0   | 0   | 0    | 0    |
| 2     | 7.0    | 0   | 0   | 0    | 0    |
| 3     | 7.4    | 0   | 0   | 0    | 0    |
| 4     | 8.0    | 0   | 0   | 0    | 0    |
| 5     | 9.0    | 0   | 13  | 20   | 46   |

\[ a \] % Conversion based on LC-ESI-MS. For data, see Supplementary Fig. 9.3 below.
(b) NaP buffer (pH 7.0, 50 mM)

0 h

6 h

12 h

24 h
(c) NaP buffer (pH 7.4, 50 mM)
(d) NaP buffer (pH 8.0, 50 mM)

0 h

6 h

12 h

24 h

Time [min]
Supplementary Fig. 93. (a) LC data of LDM$_{C,K}$ reagent 9d at pH 6.0 after 0, 6, 12, and 24 h. (b) LC data of LDM$_{C,K}$ reagent 9d at pH 7.0 after 0, 6, 12, and 24 h. (c) LC data of LDM$_{C,K}$ reagent 9d at pH 7.4 after 0, 6, 12, and 24 h. (d) LC data of LDM$_{C,K}$ reagent 9d at pH 8.0 after 0, 6, 12, and 24 h. (e) LC data of LDM$_{C,K}$ reagent 9d at pH 9.0 after 0, 6, 12, and 24 h.
8.3 pH screening

Supplementary Table 9. pH screening with β-lactoglobulin A and LDMC-K reagent 9d.

| Entry | pH (X) | % Conversion<sup>a</sup> (10d) | % Conversion<sup>a</sup> (17) |
|-------|-------|-------------------------------|-------------------------------|
| 1     | 6.0   | 28                            | 0                             |
| 2     | 7.0   | >99                           | 0                             |
| 3     | 7.4   | 82 (mono), 18 (bis)           | 0                             |
| 4<sup>b</sup> | 8.0   | 66 (mono), 16 (bis)           | 5 (mono), 12 (bis)           |
| 5<sup>b</sup> | 9.0   | 51 (mono), 17 (bis)           | 25 (bis)                      |

<sup>a</sup> % Conversion based on ESI-MS.  
<sup>b</sup> In this case the retro-Henry product (C-S and C-C bond dissociation) 17 (mono) and 17<sup>b</sup> (bis) was also observed.
Supplementary Fig. 94. ESI-MS spectrum of labeled β-lactoglobulin enabled by LDM_{C,K} reagent 9d (10 equiv.) at various pH (pH 6.0-9.0).
### 8.4 Concentration screening

**Supplementary Table 10.** Concentration screening with β-lactoglobulin A and LDMc-K reagent 9d.

| Entry | Concentration (μM) | % Conversion$^a$ |
|-------|--------------------|------------------|
| 1     | 5.0                | 85               |
| 2     | 10.0               | 87               |
| 3     | **20.0**           | >99              |
| 4     | 50.0               | 81 (mono), 8 (bis) |
| 5     | 73.0               | 89 (mono), 10 (bis) |

$^a$ % Conversion based on ESI-MS.
Supplementary Fig. 95. ESI-MS spectrum of labeled β-lactoglobulin A enabled by $\text{LDM}_{\text{C,K}}$ reagent 9d (10 equiv.) at various concentrations (5 µM- 73 µM).
8.5 $^{19}$F NMR data of labeled $\beta$-lactoglobulin A

**Supplementary Fig. 96.** $^{19}$F-NMR spectrum of labeled BLGA 24 (a sharp signal at -62.65 ppm, internal standard: trifluoroacetic acid (0.2 mM) signal at -75.45 ppm). Sample was recorded in phosphate buffer (0.1 M, pH 7.0):D$_2$O (9:1).

8.6 Fluorescence data of labeled $\beta$-lactoglobulin A

**Supplementary Fig. 97.** Steady-state fluorescence spectra of coumarin tagged BLGA (26) in phosphate buffer (0.1 M, pH 7.0). 26 exhibits emission band peaked at 428 nm (excitation at 333 nm). For MS data, see Supplementary Fig. 73.
8.7 Circular dichroism spectra of proteins

**Supplementary Fig. 98.** Effect of the labeling on structure of proteins. (a) Circular Dichroism (CD) spectra of BLGA 8a (black line), modified BLGA 20d (red line) at concentration 0.1 mg/ml. (b) Circular Dichroism (CD) of BSA 8j (black line), modified BSA S57 (red line) at concentration 0.1 mg/ml. (c) Circular Dichroism (CD) of HSA 8k (black line), modified HSA S59 (red line) at concentration 0.1 mg/ml.
8.8a Reaction of peptides (competing nucleophile) with LDM<sub>C,K</sub> reagent 9d

Supplementary Fig. 99. (a) C-S bond formation and cyclization for peptide containing Cys (i<sup>th</sup>) and Tyr (i+4) 12e enabled by LDM<sub>C,K</sub> reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i<sup>th</sup>) and Tyr (i+4<sup>th</sup>) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for Michael adduct 13e. (d) ESI-MS data for 12e<sup>·</sup>

Step 1: C-S bond formation; step 2: C-X (X= O) bond formation; [Na] (pH 7.5, 50 mM), 25 °C, 500 mM, 20-30% ACN, 9d, 10 min – 1 h; % conversion estimated by LC-ESI-MS.
Supplementary Fig. 100. (a) C-S bond formation and cyclization for peptide containing Cys (i<sup>th</sup>) and Ser (i+4) 12f enabled by LDM<sub>C,K</sub> reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i<sup>th</sup>) and Ser (i+4<sup>th</sup>) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for 12f. (d) ESI-MS data for Michael adduct 13f. (e) ESI-MS data for S-acylation 14f<sup>a</sup>. (f) MS-MS data for S-acylation 14f<sup>b</sup>.
Supplementary Fig. 101. (a) C-S bond formation and cyclization for peptide containing Cys (i\textsuperscript{th}) and His (i+4\textsuperscript{th}) 12g enabled by LDM\textsubscript{Cg} reagent 9d. (b) LC data showing C-S bond formation and cyclization for peptide containing Cys (i\textsuperscript{th}) and His (i+4\textsuperscript{th}) enabled by reagent 9d for different interval time (10-60 min). (c) ESI-MS data for 12g. (d) ESI-MS data for Michael adduct 13g and cyclization 14g. (e) ESI-MS data for hydrolysed Michael adduct 14h.
8.8b C-S and C-C bond cleavage in peptides

Supplementary Fig. 102. Linchpin-directed modification of Lys and downstream synthetic manipulations in a peptide. The selected MS data for 14i, 15a, 15b, and 15c are given in the inset.

Supplementary Fig. 103. (a) C-S bond formation and cyclization with peptide 12h enabled by LDMc.κ reagent 9d resulted into 14i. (b) XIC data for 14i. (c) ESI-MS data for cyclized product 14i.
Supplementary Fig. 104. (a) C-S bond dissociation and formation enabled by maleimide derivative from 14i to yield 15a. (b) XIC data for 15a. (c) ESI-MS data 15a.

step 4: nitroolefin to aldehyde formation (retro-Henry); \(^{b}\)% conversion estimated by LC-ESI-MS; \(^{c}\) re-adjustment of pH \((\sim 9.0)\), 25 \(^{\circ}\)C, 36 h.
Supplementary Fig. 105. Generation of Aldehyde (15b) from nitro-olefin containing peptide 15a by readjustment of pH. (a) XIC data for aldehyde formation 15b. (b) ESI-MS data for.

step 5: oxime formation; b% conversion estimated by LC-ESI-MS; c re-adjustment of pH (~6.5), NH₂OH.HCl (200 equiv.), 25 °C, 3 h.

Supplementary Fig. 106. Oxime formation 15c. resulted from 15b and NH₂OH (a) XIC data for 15c. (b) ESI-MS data for 15c.
8.8c Linchpin dissociation in cyclic protein (BLGA)

Supplementary Table 11: Stability of cyclic BLGA at different pH as a function of time.

| Entry | pH (X) | % Conversiona (with time in min) | 30 | 60 | 180 |
|-------|--------|---------------------------------|----|----|-----|
|       |        |                                 | 16 | 16a| 16  | 16a| 16  | 16a|
| 1     | 7.0    |                                 | 15 | 0  | 22  | 0  | 30  | 22 |
| 2     | 8.0    |                                 | 48 | 0  | 44  | 8  | 43  | 10 |
| 3     | 9.0    |                                 | 42 | 6  | 43  | 11 | 46  | 14 |

a % Conversion based on ESI-MS.
Supplementary Fig. 107. ESI-MS data for retro-Michael reaction of stapled construct at (a) pH 7.0 from 30 min to 3 h, (b) pH 8.0 from 30 min to 3 h, (c) pH 7.0 from 30 min to 3 h.
8.9a Enrichment of protein from E. Coli BL21 cell-lysate (without HSA) using coumarin tag

![Diagram of enrichment process]

Step 1: C-S bond formation
Cyclization
Step 2: C-S bond dissociation
Single protein modification

| Step | Description |
|------|-------------|
| 1    | C-S bond formation |
| 2    | Cyclization |
| 3    | Step 3: Loading |
| 4    | Step 4: Release |

Supplementary notes:
- NaP buffer (pH 7.0, 50 mM), 20 μM ACN, LDM-ε reagent (9d).
- NaHCO₃ buffer (pH 8.5, 0.1 M), 25 °C, 20 μM, 48 h.
- NaP buffer (pH 7.0, 50 mM), 25 °C, 24 h.
- NaP buffer (pH 6.5, 50 mM), 25 °C, RONH₂ (23, 100 equiv.), 10% ACN, 24 h.

---

S128
Supplementary Fig. 108. (a) Modification and enrichment of proteins in cell lysate derived from BL21 cells, E. coli enabled by LDM_{C,K} reagent 9d (10 equiv.). (b) TIC showing a few peaks eluted at different time interval after the treatment with LDM_{C,K} reagent 9d. The ESI-MS data confirms the absence of protein within the MS detection limits. (c) ESI-MS data for S63. (d) ESI-MS data for S64. (e) ESI-MS data for S65. (f) 10% SDS-PAGE showing fluorescence imaging and Coomassie staining. MW - Molecular Weight, SDS-PAGE: lane 1 - ladder, lane 2 - cell lysate (without HSA), lane 3 - coumarin-tagged purified protein.

8.9b Enzymatic assay of HSA (Human serum albumin)

Supplementary Fig. 109. Effect of K199-bioconjugation on the esterase-like activity of HSA. Time dependent UV-Vis spectra was recorded to compare the enzymatic activity of native HSA (8k) and labeled HSA (S59). Esterase-like activity of HSA before and after the labeling are examined by monitoring the catalytic reactivity of HSA using p-nitrophenyl acetate (pNPA) as the substrate. The enzymatic activity of HSA remains unperturbed after the chemical modification.
8.10 Comparative analysis of light chain (LC) and heavy chain (HC) in native and modified trastuzumab

Supplementary Fig. 110. Comparative analysis of light and heavy chain in native and modified trastuzumab by using ESI-MS. (c) ESI-MS data for light chain in native trastuzumab. (d) ESI-MS data for light chain in modified trastuzumab. (c) ESI-MS data for heavy chain in native trastuzumab, showing all the post-translational modification. (d) ESI-MS data for heavy chain in modified trastuzumab, showing all the post-translational modification.
8.11 Experiments with CASLQK tagged Hs-Ubc9

Construction of plasmid for tagged protein production: The pET28a(+)–Hs-Ubc9 plasmid was used to generate a construct capable of adding -CASLQK- hexameric tag to the N-terminus of the protein. Two partially complementary oligonucleotides (5′-CTAGCTGTGCTTCACTCCAGAAAG-3′ and 5′-GATCCTTCTGGAGTGAAGCAGAGCAGAG-3′) providing a NheI and BamHI overhangs respectively at 5′ and 3′ ends were synthesized and annealed in a thermocycler. The pET28-a(+)-Hs-Ubc9 plasmid was digested with NheI and BamHI restriction enzymes, gel purified, and used in a ligation reaction for 2 h at 25 °C with oligos prepared earlier. The resultant construct (pET28-a(+)-Hs-Ubc9_tag) was transformed into E. coli DH5α cells. Isolated plasmids were sent for Sanger sequencing for the correctness of generated clones and confirmation of oligonucleotide addition.

Recombinant protein expression and purification: To express -CASLQK- tagged Hs-Ubc9 protein, pET28-a(+)–Hs-Ubc9_tag construct was transformed into Escherichia coli BL21 (DE3) cells. A single transformed colony was inoculated in 5 ml primary culture and kept for overnight growth at 37°C at 192 x g. The secondary culture was initiated by 1% of the primary culture in antibiotic containing media and was allowed to grow at 37°C. The protein production was induced by 100 µM of IPTG was added in secondary culture for induction of recombinant protein for 4 hours at 30°C. Subsequently, the induced culture was centrifuged at 7680 x g for 10 minutes at 4°C. Bacterial pellets were lysed in lysis buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl) containing 0.2% Triton X-100, 1 mg/mL of lysozyme, 1X PIC (Protease inhibitor cocktail) and 1 mM PMSF (phenyl methane sulfonyl fluoride). The resuspended pellet was incubated on ice for 20 minutes and sonicated (45% amplitude, 10 s on pulse, 10 s off pulse) for 5 minutes on ice. After sonication 10 mM imidazole was added into cell lysate and centrifuged at 17280 x g for 30 minutes at 4°C to pellet down cell debris. The resultant supernatant was incubated with Ni-NTA beads for 1 h at 4°C for binding of His-tagged recombinant protein carrying hexameric -CASLQK- tag. Beads were washed with a wash buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, and 30 mM imidazole) to remove nonspecifically bound proteins. The recombinant protein was eluted with an elution buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, and 250 mM imidazole). Purified and dialysed proteins (Fig. S109) were used for further enzymatic experiments.
Supplementary Fig. 11. Purification of recombinant Hs-UBC9 carrying an N-terminal hexa-histidine and CASLQK peptide sequence. The asterisk on the left denotes induced protein present in different fractions during purification. Lanes after ladder (L) indicate elutions of recombinant protein from the Ni-NTA column.

**LDMc-K modification and in vitro SUMOylation:** Subsequently, we treated the His-tagged recombinant Hs-Ubc9 carrying CASLQK tag with the LDMc-K reagent 9d to render the monolabeled CASLQK tagged Hs-Ubc9 (also see ESI Supplementary Fig. 111). Subsequently, we performed in vitro SUMOylation assay and established that the activity remains unperturbed post-bioconjugation. The purified CASLQK-hexameric-tag bearing Hs-Ubc9 and its monolabeled bioconjugate were used in an in vitro SUMOylation reaction. 0.25 µg of GST tagged SAE2/SAE1 (E1 enzyme), 1.0 µg of Hs-Ubc9 (E2 enzyme), 4 µg GST-SUMO1, and 4 µg of GST-tagged standard peptide substrate were taken in a reaction buffer (50 mM Tris pH 7.5, 5 mM MgCl2, 5 mM ATP and 5 mM dithiothreitol). *In vitro* SUMOylation reactions proceeded for 4 h at 37 °C and were terminated by adding 6X Laemmli buffer. Before analyzing the reaction contents on an SDS-PAGE gel to visualize the activity (Fig. S110), samples were heated for 10 minutes at 100°C.
Supplementary Fig. 112. In vitro SUMOylation with recombinant Hs-Ubc9 and its bioconjugate. Sumoylation of GST-dynamin peptide substrate in the presence of all components of a SUMOylation reaction with (a) native Hs-Ubc9, (b) CASLQK tagged Hs-Ubc9, and (c) labeled CASLQK tagged Hs-Ubc9. Reactions in (d) and (e) are carried out in the presence of GST alone as substrate and serve as a negative control corresponding to (a) and (b), respectively. The asterisk denotes SUMO-modified substrate.
Supplementary Fig. 113. (a) Site-selective labeling of CASLQK tagged Hs-Ubc9 enabled by LDM_{CK} reagent 9d. (b) ESI-MS data of CASLQK tagged Hs-Ubc9 S66. (c) ESI-MS data mono-labeled CASLQK tagged Hs-Ubc9 S67. (d) Peptide mapping of S67 after the digestion with trypsin. (c) MS-MS spectrum of labelled DHPGFVAVPTKNPDGMNLMNWECAIPGK (D50-K79, m/z 716.3 [M+5H]^{5+}). The site of modification in mono-labeled CASLQK tagged Hs-Ubc9 S67 is K79.
9. Protein sequence

**PDB ID:** 3BLG

[https://www.rcsb.org/structure/3BLG; PDB DOI: 10.2210/pdb3BLG/pdb]

LIVTQTMKGLDIQVKAGTVWSLAMAASDILLDAQSPAPLRYVEELKPTPEGDILEL
LQKWENDVCAQQKIIAEKTPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPE
QSLVCQCLVRTPEVDDEALEKFKDKALPMHRLSFNPTQLEQCHI

**PDB ID:** 1AO6 Human Serum Albumin

[https://www.rcsb.org/structure/1AO6; PDB DOI: 10.2210/pdb1AO6/pdb]

DAHKSEVAHRFKDLGEEHFKGLVLIAFASYQLQQPCPFEDHVHKLVNTEFAKTCVAD
ESAENCDKSLHTFLGDOLCVALETYGMADCCAKQEPEPERNCEFLQHKDDNPNL
PRLVRPNTLMCTAFHDNEETFLLKYYEIAIRRHPYFYPYAPELLEFKAHYKAAFECQ
QAADKAAACLLPKLDELRDENGKASSAKQRLLCASLQKFGERAKFKAVALARLSQRPF
KAFAFVESVKLTVTDLTKVHECHGDLLLACADDRALAKYICENQDSISSKLECECEK
PPLKEKSHCIAEVENEMPADLPSLAADFVESKDVCNKYAEAKDFGLMFLYEARR
HPYSVVLVLLRLAKYTEETTECKCAAADPHECAKVFDEKPLVEEQNLKQNCEL
FEQLGEYKFQNALLVRYTTRKVQPVSTPTLVEVRNLKGVGKCCKEHPEAKRMPCAE
DYLSSVLNQLCLVEKTPVSRTKCTESTLVNRPCFASLEVETYPKFENATEFT
TFHADICTYLESERAQIKKQTALVHELKHPKATKEQLKAVMDDFAAFVEKCEADD
KETCFAEEGKVLAASSQAALGL

**PDB ID:** 3V03 Bovine Serum Albumin

[https://www.rcsb.org/structure/3V03; PDB DOI: 10.2210/pdb3V03/pdb]

DTHKSEIAHRFKDLGEEHFKGLVLIAFASYQLQQPCPFDEHVKLIVNLEMENTFAKTCVADE
SHAIGCEEKLHTLFQDGLCVALETYGMADCECKQEPEPERNCEFLSHKDDSPDLP
KLKPDPTLMCTAFHDNETFGLKYELIEAIRRHPYFYPYAPELLEYANKYNGVFQECQC
AEDKGACLLPKIETMEMRKLVTASSARQLRCASIQKFGERALKAWSVARLSQKFPKAE
FVEVTKLVTLTKVHECHGDLLLACADDRALAKYICDNQDTSIKLKECECKPLLP
EKSCHICAEVEKDAIPNLPLTADFADKDVKNYQEAKDLAGSFLYERSSHPEY
AVSVNLRLAKLEYATEECACKKDAPHCAYSTFDKLHKLVDEPQNLKQCNQFEEK
LGEYGFQNALIVRYTTRKVQPVSTPTLVEVSRLGKVGTRETKPSERMPCTEDYL
SLNLRLNQLCLVEKTPVSRTKCTESTLVNRPCFASLEVETYPKFENATEFT
TFHADICTLOYNQRTQTKTALVHELKHPKATKEQLKAVMDDFAAFVEKCEADD
KETCFAEEGKVLAAASQAALGL

**native Hs-Ubc9**

MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSMSGIALSRLAQERKAWRKHDPF
PFGFVAVPTKNPDGMNLMNWECAIPKGKTPWEGGLFKLRFKDDYPSPPCK
FEPFPLFHPNVYPSGTVCNCSLEDEKDWRPAITIKQILLQELNEPIQDPAQAEAYTI
YQCNRVEYERKVRAPAKKAFPS

**CASLQK tagged Hs-Ubc9**

MGSSHHHHHHSSGLVPRGSHMASCASLQKGSMGIALSRLAQERKAWRKHDPF
GFVFVAVPTKNPDGMNLMNWECAIPKGKTPWEGGLFKLRFKDDYPSPPCK
FEPFPLFHPNVYPSGTVCNCSLEDEKDWRPAITIKQILLQELNEPIQDPAQAEAYTI
YQCNRVEYERKVRAPAKKAFPS
2. Trastuzumab (recombinant humanized monoclonal antibody)

*Light chain amino acid sequence (1-214)*

DIQMTQSPSSLSASVGDRVITCRASQDVNTAWYQQKPGKAPKLLIYSASFLYSVGP
SRFGSRSGTDFLTILSSLQPEDFATYCYCQHYTTPFGTVEIKRTVAAPSVFIFPPS
DEQLKSGTASVCLLNNFYPREAVGVKVDNALQSGNSQESVTEQDSKDSYLSSTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

*Heavy chain amino acid sequence (1-449)*

EVQLVESGGGLVQPGGLSCLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPNTGYT
RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFDYAMDFWYGQT
LVTVDSSASTGVSFPLAPSSKGSTSGTEALGCLVLKDYFPEPVTQSWNSGALTSGVHTFP
AVLQSSGLYSLSSVITPVSSSLGTQQTYICNVNKHPSNTKVDKKVEPSCDKTHTCCPPCPA
PELLGGPSVFLFPPKDLTISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNATKT
PREEQNYSTVRVSILTVLHQDNLWNGKEYCKVSNKAPIEKTSAKGGGRPREPQV
YTLPPSREEMTKQNVSLTCLVKGFYPSDIAVWESNGQPENNYKTTPVLDSGDFLY
SKLTVDKSRWQQGNSFCSVMHEALHNHTQKSLSPG
10. Spectral data

Supplementary Fig. 114. $^1$H NMR spectrum in CDCl$_3$ of compound S3.

Supplementary Fig. 115. $^{13}$C NMR spectrum in CDCl$_3$ of compound S3.
Supplementary Fig. 116. $^1$H NMR spectrum in CDCl$_3$ of compound 11.

Supplementary Fig. 117. $^{13}$C NMR spectrum in CDCl$_3$ of compound 11.
Supplementary Fig. 118. $^1$H NMR spectrum in CDCl$_3$ of compound S4.

Supplementary Fig. 119. $^{13}$C NMR spectrum in CDCl$_3$ of compound S4.
Supplementary Fig. 120. $^1$H NMR spectrum in DMSO-$d_6$ of compound 9a.

Supplementary Fig. 121. $^{13}$C NMR spectrum in DMSO-$d_6$ of compound 9a.
Supplementary Fig. 122. $^1$H NMR spectrum in CDCl$_3$ of compound 9b.

Supplementary Fig. 123. $^{13}$C NMR spectrum in DMSO-d$_6$ of compound 9b.
Supplementary Fig. 124. $^1$H NMR spectrum in CDCl$_3$ of compound 9c.

Supplementary Fig. 125. $^{13}$C NMR spectrum in DMSO-d$_6$ of compound 9c.
Supplementary Fig. 126. $^1$H NMR spectrum in CDCl$_3$ of compound 9d.

Supplementary Fig. 127. $^{13}$C NMR spectrum in CDCl$_3$ of compound 9d.
Supplementary Fig. 128. $^1$H NMR spectrum in CDCl$_3$ of compound S12.

Supplementary Fig. 129. $^{13}$C NMR spectrum in CDCl$_3$ of compound S12.
Supplementary Fig. 130. $^1$H NMR spectrum in CDCl$_3$ of compound S13.

Supplementary Fig. 131. $^{13}$C NMR spectrum in CDCl$_3$ of compound S13.
Supplementary Fig. 132. $^1$H NMR spectrum in CDCl$_3$ of compound S14.

Supplementary Fig. 133. $^{13}$C NMR spectrum in CDCl$_3$ of compound S14.
Supplementary Fig. 134. $^1$H NMR spectrum in CDCl$_3$ of compound 9e.

Supplementary Fig. 135. $^{13}$C NMR spectrum in CDCl$_3$ of compound 9e.
Supplementary Fig. 136. $^1$H NMR spectrum in CDCl$_3$ of compound S16.

Supplementary Fig. 137. $^{13}$C NMR spectrum in CDCl$_3$ of compound S16.
Supplementary Fig. 138. $^1$H NMR spectrum in CDCl$_3$ of compound S17.

Supplementary Fig. 139. $^{13}$C NMR spectrum in CDCl$_3$ of compound S17.
Supplementary Fig. 140. $^1$H NMR spectrum in CDCl$_3$ of compound S18.

Supplementary Fig. 141. $^{13}$C NMR spectrum in CDCl$_3$ of compound S18.
Supplementary Fig. 142. $^1$H NMR spectrum in CDCl$_3$ of compound 9f.

Supplementary Fig. 143. $^{13}$C NMR spectrum in CDCl$_3$ of compound 9f.
Supplementary Fig. 144. $^1$H NMR spectrum in CDCl$_3$ of compound S20.

Supplementary Fig. 145. $^{13}$C NMR spectrum in CDCl$_3$ of compound S20.
Supplementary Fig. 146. $^1$H NMR spectrum in CDCl$_3$ of compound S21.

Supplementary Fig. 147. $^{13}$C NMR spectrum in CDCl$_3$ of compound S21.
Supplementary Fig. 148. $^1$H NMR spectrum in CDCl$_3$ of compound S22.

Supplementary Fig. 149. $^{13}$C NMR spectrum in CDCl$_3$ of compound S22.
Supplementary Fig. 150. $^1$H NMR spectrum in CDCl$_3$ of compound 9g.

Supplementary Fig. 151. $^{13}$C NMR spectrum in CDCl$_3$ of compound 9g.
Supplementary Fig. 1.5. H NMR spectrum in CDCl$_3$ of compound S25.

Supplementary Fig. 1.53. $^{13}$C NMR spectrum in CDCl$_3$ of compound S25.
Supplementary Fig. 154. $^1$H NMR spectrum in CDCl$_3$ of compound S26.

Supplementary Fig. 155. $^{13}$C NMR spectrum in CDCl$_3$ of compound S26.
Supplementary Fig. 156. $^1$H NMR spectrum in CDCl$_3$ of compound S27.

Supplementary Fig. 157. $^{13}$C NMR spectrum in CDCl$_3$ of compound S27.
Supplementary Fig. 158. $^1$H NMR spectrum in CDCl$_3$ of compound S28.

Supplementary Fig. 159. $^{13}$C NMR spectrum in CDCl$_3$ of compound S28.
Supplementary Fig. 160. $^1$H NMR spectrum in CDCl$_3$ of compound S29.

Supplementary Fig. 161. $^{13}$C NMR spectrum in CDCl$_3$ of compound S29.
Supplementary Fig. 162. $^1$H NMR spectrum in CDCl$_3$ of compound S30.

Supplementary Fig. 163. $^{13}$C NMR spectrum in CDCl$_3$ of compound S30.
Supplementary Fig. 164. $^1\text{H}$ NMR spectrum in CDCl$_3$ of compound 9h.

Supplementary Fig. 165. $^{13}\text{C}$ NMR spectrum in CDCl$_3$ of compound 9h.
Supplementary Fig. 166. $^1$H NMR spectrum in CDCl$_3$ of compound S31.

Supplementary Fig. 167. $^{13}$C NMR spectrum in CDCl$_3$ of compound S31.
Supplementary Fig. 168. $^1$H NMR spectrum in CDCl$_3$ of compound 11a.

Supplementary Fig. 169. $^{13}$C NMR spectrum in CDCl$_3$ of compound 11a.
Supplementary Fig. 170. $^1$H NMR spectrum in CDCl$_3$ of compound S34.

Supplementary Fig. 171. $^{13}$C NMR spectrum in CDCl$_3$ of compound S34.
Supplementary Fig. 172. $^1$H NMR spectrum in CDCl$_3$ of compound S9.

Supplementary Fig. 173. $^1$H NMR spectrum in CDCl$_3$ of compound S37.
Supplementary Fig. 174. $^{13}$C NMR spectrum in CDCl$_3$ of compound S37.

Supplementary Fig. 175. $^1$H NMR spectrum in CDCl$_3$ of compound S39.
Supplementary Fig. 176. $^{13}$C NMR spectrum in CDCl$_3$ of compound S39.

Supplementary Fig. 177. $^1$H NMR spectrum in CDCl$_3$ of compound S39.
Supplementary Fig. 178. $^1$H NMR spectrum in CDCl$_3$ of compound 21.

Supplementary Fig. 179. $^{13}$C NMR spectrum in CDCl$_3$ of compound 21.
Supplementary Fig. 180. $^{19}$F NMR spectrum in CDCl$_3$ of compound 21.

Supplementary Fig. 181. $^1$H NMR spectrum in CDCl$_3$ of compound S41.
Supplementary Fig. 182. $^{13}$C NMR spectrum in CDCl$_3$ of compound S41.

Supplementary Fig. 183. $^1$H NMR spectrum in D$_2$O of compound 22.
Supplementary Fig. 184. $^{13}$C NMR spectrum in D$_2$O of compound 22.

Supplementary Fig. 185. $^1$H NMR spectrum in CDCl$_3$ of compound S43.
Supplementary Fig. 186. $^{13}$C NMR spectrum in CDCl$_3$ of compound S43.

Supplementary Fig. 187. $^1$H NMR spectrum in CDCl$_3$ of compound 23.
Supplementary Fig. 188. $^{13}$C NMR spectrum in CDCl$_3$ of compound 23.
Supplementary references

1. The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.
2. Hanwell, M. D. et al. Avogadro: An advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics* **2012**, 4:17.
3. Wang, J., Wang, W., Kollman, P. A. & Case, D. A. Automatic atom type and bond type perception in molecular mechanical calculations. *Journal of Molecular Graphics and Modelling* **25**, 247260 (2006).
4. Wang, J., Wolf, R. M.; Caldwell, J. W. & Kollman, P. A. & Case, D. A. Development and testing of a general AMBER force field. *Journal of Computational Chemistry* **25**, 1157–1174 (2004).
5. Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W. & Klein, M. L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **79**, 926–935 (1983).
6. Case, D. A. et al. Amber, University of California, San Francisco (2021).
7. Case, D. A. et al. Amber, University of California, San Francisco (2015).
8. Joung, I. S. & Cheatham, T. E. III Determination of alkali and halide monovalent ion parameters for use in explicitly solvated biomolecular simulations. *J. Phys. Chem. B* **112**, 9020-9041 (2008).
9. Case, D. A. et al. The amber biomolecular simulation programs. *J. Comput. Chem.* **26**, 1668–1688 (2005).
10. Van Gunsteren, W. F. & Berendsen, H. J. C. A leap-frog algorithm for stochastic dynamics. *Mol. Simul.* **1**, 173–185 (1988).
11. Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W. F.; Dinola, A. & Haak, J. R. Molecular-dynamics with coupling to an external bath. *J. Chem. Phys.* **81**, 3684–3690 (1984).
12. Darden, T.; York, D. & Pedersen, L. Particle mesh Ewald: an N.log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).
13. Ryckaert, J.P.; Ciccotti, G. & Berendsen, H. J. C. Numerical integration of cartesian equations of motion of a system with constraints \( \pi \) molecular-dynamics of N-alkanes. *J. Comput. Phys.* **23**, 327–341 (1977).
14. Humphrey, W.; Dalke, A. & Schulten, K. VMD: Visual molecular dynamics. *J. Mol. Graphics Modell.* **14**, 33–38 (1996).
15. Kinter, M. & Sherman, N. E. *Wiley InterScience* (2000).
16. Nandy, A., Chakraborty, S., Nandy, S., Bhattacharyya, K. & Mukherjee, S. Structure activity, and dynamics of human serum albumin in a crowded pluronic F127 hydrogel. *J. Phys. Chem. B* **123**, 3397–3408 (2019).
17. Lelle, M.; Frick, S. U.; Steinbrink, K. & Peneva, K. Novel cleavable cell-penetrating peptide drug conjugates: synthesis and characterization. *J. Pept. Sci.* **20**, 323–333 (2014).

General: The figures were made using ChemOffice, PyMol, and Biorender.