

Supplementary Information

Electronically tuned N-terminal glycine selective bioconjugation enables *in vitro* and *in vivo* species-specific *Staphylococcus aureus* targeting

Mohan Kumar,^a Divya Bindra,^b Ashish Verma,^a Sisir Patra,^a Drishiya Vats,^c Kaveri A. S.,^b Nikita Sharma,^b Kartikay Tyagi,^d Rahul Maitra,^e Kamal Vats,^{a,d} Manoj V. Mane,^f Sidharth Chopra,^e Ram Kumar Mishra,^b and Vishal Rai^{a,d*}

^aDepartment of Chemistry, ^bDepartment of Biological Sciences, and ^cAnimal Facility, Indian Institute of Science Education and Research Bhopal, ^dPlabeltech Private Limited, IICE, Bhauri, Madhya Pradesh, 462066, India ^eCSIR-Central Drug Research Institute, Lucknow-226031, India, ^fCentre for Nano and Material Sciences, Jain (Deemed-to-be University), Jain Global Campus, Bangalore, Karnataka 562112, India.

Email: vrai@iiserb.ac.in

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1. Supplementary notes and general information

The reagents, proteins, antibodies, and enzymes were purchased from Sigma-Aldrich (Merck), Alfa Aesar, Plabeltech, Novabiochem, Medchem Express, Thermo Fisher Scientific, Invitrogen, and Cell Signaling Technology. Human serum purchased from Merck, H4522. 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). The 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 a Thermo Fisher Scientific MaxQ 8000 incubator-shaker or a Thermo Scientific Digital Heating Shaking Drybath. Amicon® Ultra-0.5 mL 3-kDa or 10-kDa MWCO centrifugal filters from Merck Millipore were used to remove small molecules from the 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. Microplate reader measurements were performed on BioTek Cytation 5. Confocal laser scanning microscopy was performed using Olympus FV3000 to acquire images of bacterial and HeLa cells. The morphology of samples was investigated using the transmission electron microscopy (TEM)– FEI TALOS 200S instrument, operating at a voltage of 200 kV. HR FESEM from Zeiss-ULTRA Plus was utilized for scanning and capturing images at different magnifications by using 15 kV acceleration voltage. HEK293T and HeLa cell lines were obtained from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, # 11995-065) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen, # 16000044) and antibiotics (100 units/mL Penicillin and Streptomycin, Invitrogen, # 15140122) were used for cell culture. For in vivo detection in an intramuscular infection model in mice, the tissues were harvested and embedded in tissue freezing medium (Sigma, Cat. No. SHH0026). Cryosections were prepared using a Leica cryomicrotome at –20 °C and mounted onto glass slides. For the skin infection model in mice, in vivo imaging was performed using the PerkinElmer IVIS Lumina X5 imaging system and analyzed using Living Image software (PerkinElmer). ImageJ software was used for processing and quantification of images from

confocal laser microscopy and the wound healing assay. Flow cytometry analysis was performed using FACS BD AriaIII and analyzed using Flowjo software.

Chromatography: Thin-layer chromatography (TLC) was performed on silica gel-coated aluminum TLC plates (Merck, TLC Silica gel 60 F254). The compounds were visualized using a UV lamp (254 nm) and stains such as iodine, ninhydrin, and 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 , ^{13}C , and ^{19}F NMR spectra were recorded on a Bruker Advance III 400 and 500 MHz NMR spectrometer. ^1H NMR spectra were referenced to TMS (0 ppm), CDCl_3 (7.26 ppm), DMSO-d_6 (2.50 ppm), and D_2O (4.79 ppm), whereas ^{13}C NMR spectra were referenced to CDCl_3 (77.16 ppm) and DMSO-d_6 (39.52 ppm), ^{19}F NMR was referenced to trifluoroacetic acid (-75.45 ppm). The following abbreviations designate peak multiplicities: s, singlet; bs, broad singlet; (d) doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. 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). Data collection and analysis were performed using Open LAB CDS A.01, SCIEX OS 1.7, Bruker Daltonics ESI Compass 1.3, and BioPharmaView Flex 2.1.

Acetonitrile and H_2O were buffered with 0.1-0.01% formic acid and used as the mobile phase. Method A and Method B were used to record the LC-MS data for proteins. All the Amino acid and peptide-related data are analyzed through LC-MS using method C and method D.

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 $\times$ 75 mm, flow rate 0.5 mL/min)

Time (min)	H_2O (%)	Acetonitrile (%)
0	90	10
1	90	10
8	40	60
9	90	10
12	90	10

Method B: 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)	H ₂ O (%)	Acetonitrile (%)
0	80	20
1	80	20
5	20	80
7	20	80
9	80	20
10	80	20

Method C: 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 ₂ O (%)	Acetonitrile (%)
0	75	25
4	65	35
5	65	35
5.5	10	90
8	10	90
12	10	90

Determination of percentage conversion in the protein labeling 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.

$$\% \text{ Conversion} = I_{\text{desired product}} / I_{\text{all relevant species}}$$

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

Method D: Peptide mapping and LC-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)	H ₂ O (%)	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: LC-MS and 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 μ m, 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)	H ₂ O (%)	Acetonitrile (%)
0	90	10
3	90	10
5	50	50
10	50	50
12	10	90
15	90	10

SPPS (Solid Phase Peptide Synthesis): The synthesis of all the peptides was done using rink amide resin. The peptides **4a-t** were designed from the N-terminus residue of insulin. Peptide **11a** was designed from the Gly₅ interpeptide. N^α-rhodamine N^ε-Fmoc Lys was used for the synthesis of 11a with a fluorophore.

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 a rotatory evaporator. The crude mixture was purified by trituration using chilled diethyl ether.

Nano-LC-MS method for global chemoproteomics

Table S1: The gradient of acetonitrile and water (0.1% formic acid) was used for the animal cell proteomics separation in nano-LC Orbitrap Exploris 240 mass spectrometry.

Sr. No.	Time	Duration [min]	Flow [μl/min]	%B	Volume [μl]	No. of column volumes
1.	0.000	Run				
2.	0.000	0.000	0.250	1.0	0.00	0.00
3.	0.100	0.100	0.250	5.0	0.03	0.06
4.	85.100	85.000	0.250	28.0	21.25	47.86
5.	120.100	35.000	0.250	40.0	8.75	19.71
6.	120.100	Column Wash				
7.	130.000	9.900	0.250	99.0	2.48	5.57
8.	150.000	20.000	0.250	99.0	5.00	11.26
9.	150.000	Stop Run				
10.	150.000	Column Equilibration				

Table S2: Separation column specification

Sr. No.	Property	Value
1.	Inner Diameter	75 [μm]
2.	Length	15.0 [cm]
3.	Void volume	0.444 [μl]
4.	Maximum pressure	1500 [bar]
5.	Maximum flow	100.0 [μl/min]
6.	Maximum Temperature	60.0 [°C]
7.	Maximum pressure changes Up	1000 [bar/min]
8.	Maximum pressure changes Down	1000 [bar/min]

Table S3: Trap column specifications

Sr. No.	Property	Value
1.	Inner Diameter	300 [μm]
2.	Length	0.5 [cm]
3.	Void volume	0.237 [μl]
4.	Maximum pressure	1500 [bar]
5.	Maximum flow	200.0 [μl/min]
6.	Maximum Temperature	60.0 [°C]
7.	Maximum pressure changes up	1000 [bar/min]
8.	Maximum pressure changes down	1000 [bar/min]
9.	Support Backwash flush	Yes

Table S4: Orbitrap Exploris MS methods settings

Sr. No.	Parameters-1	Parameters-2	Specifications	Values
1.	Method settings	NA	Application mode	Peptide
			Method duration (min)	150
2.	Global parameters	Ion Source	Ion source type	NSI
			Spray voltage	Static
			Positive ion (V)	2100
			Negative ion (V)	1500
			Ion transfer tube temp. (°C)	300
			Use ion source setting from tune	False
			FAIMS mode	Not installed
3.		MS global settings	Infusion mode	LC
			Expected LC peak width	10
			Advanced peak determination	True
			Default charge state	2

			Enable Xcalibur	False
			Internal mass calibration	OFF
4.	Experiment#1 [MS]	NA	Start time (min)	0
			End time (min)	150
		Master scan #Full scan	Orbitrap resolution	60000
			Scan range (m/z)	350-1200
			RF lens (%)	70
			AGC target	Standard
			Max injection time mode	Auto
			Micro scans	1
			Data type	Profile
			Polarity	Positive
			Source fragmentation	Disabled
		Filters #MIPS	Monoisotopic peak determination	Peptide
			Relax restriction when too few precursors are found	True
		#Intensity		
			Intensity threshold	1.0e4
		Charge state	Include charge state(s)	2-5
			Include undermined charge states	False
		Dynamic exclusion	Dynamic exclusion mode	Custom
			Exclude after n times	1
			Exclude duration (s)	60
			Mass tolerance	ppm
			Low	10
			High	10
			Exclude isotopes	True
			Perform dependent scan on single charge state per precursor only	True
		Data dependent		
			Data-dependent mode	No. of scans
			No. of dependent scans	20
		ddMS ²	Multiplex ions	False
			Isolation window (m/z)	2
			Isolation offset	Off
			Collision energy type	Normalized
			HCD collision energy (%)	27
			Orbitrap resolution	15000
			Scan range mode	Define first mass
			First mass (m/z)	120

			AGC targets	Standard
			Max injection time mode	Auto
			Microscans	1
			Data type	Profile

Data analysis in Protein Discoverer 2.5

All nano-RPLC-ESI-MS/MS raw files were searched against a non-redundant UniProt *S. aureus* (downloaded from the website of www.uniprot.org) and implemented on the Proteome Discoverer (Thermo Scientific, v2.5). The decoy database was generated by a pre-implemented Mascot search tool. The parameters were set as follows: trypsin enzyme, maximum 2 missed cleavages, precursor-ion mass tolerance was set to 10 ppm; fragment-ion mass tolerance was set to 0.05 Da, carbamidomethyl (C) as static modification, and several dynamic modifications were oxidation (M), acetyl (N term), thioacylation (K and protein N-term), and carbamidomethylthio-propanoylation (K and protein N-term). In case of 2n treatment, Met loss + Δm from modification was added at the protein N-terminus. In case of NHS-alk, Δm from modification was added on Lys and protein N-terminus. The false discovery rate was controlled <1% in every search result. The other settings were the same as the conventional search. All data were combined from three biological replicates.

2. Experimental methods

2.1 Procedure for protein labeling

In a 2 mL microcentrifuge tube, protein (**1**, **6a-m**, 5 nmol) was mixed in sodium bicarbonate buffer (200 μ L, 0.1 M, pH 7.8). To this solution, **2** (2.5 mmol) in DMSO (50 μ L) from a freshly prepared stock solution was added and vortexed at 25 °C. The overall concentration of protein and reagent (**2**) was 20 μ M and 10 mM, respectively. After 2–6 h, NH_2OH (5 mmol) was added and vortexed for 1 h. Next, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed by using a 0.5 mL 3-kDa or 10-kDa MWCO centrifugal spin concentrator. The protein mixture was further washed with grade I water (2×0.4 mL). The sample was analyzed by LC-MS.

2.2 Procedure for labeling of insulin in the presence of additives

In a 2 mL microcentrifuge tube, protein (**1**, 5 nmol) was mixed with sodium bicarbonate buffer (0–180 μ L, 0.1 M, pH 7.8). To this solution, an additive (ADP, 5 mM; or ATP, 5 mM; or 10 % human serum; or 100% DMEM media) was added to compete with the reagents' H-bonding network. Next, **2j** (10 mM) or **2k** (10 mM) in DMSO (50 μ L) from a freshly prepared stock solution was added and vortexed at 25 °C. After 2–24 h, NH_2OH (5 mmol) was added and vortexed for 1 h. The reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and

excess reagent and salts were removed by using a 0.5 mL 3-kDa MWCO centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 × 0.4 mL). The samples were analyzed by LC-MS.

2.3 Kinetics experiments for labeling of insulin

For kinetics experiments, in a 2 mL microcentrifuge tube, protein (**1**, 5 nmol) was mixed with sodium bicarbonate buffer (200 µL, 0.1 M, pH 7.8). Next, **2g-j** (10 mM) and **2k** (10 mM) in DMSO (50 µL) from a freshly prepared stock solution were added and vortexed at 25 °C. Reactions were stopped at 5–120 min by adding NH₂OH (5 mmol) to quench the aldehydes immediately. Then, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 µL), and excess reagent and salts were removed by using a 0.5 mL 3-kDa MWCO centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 × 0.4 mL). Conversion to aminoalcohol formation was monitored by LC-MS. Experiments were performed in triplicate. Further, data for conversion versus time was plotted on Origin.

2.4 Stability of insulin bioconjugate **3j**

In a 2 mL microcentrifuge tube, protein (**1**, 5 nmol) was mixed with sodium bicarbonate buffer (200 µL, 0.1 M, pH 7.8). To this solution, aromatic aldehydes **2j** (10 mM) in DMSO (50 µL) from a freshly prepared stock solution were added and vortexed at 25 °C. After 2 h, NH₂OH (5 mmol) was added and vortexed for an additional 1 h. Subsequently, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 µL), and excess reagent and salts were removed by using a 0.5 mL 3-kDa or 10-kDa MWCO centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 × 0.4 mL). The sample was analyzed by LC-MS. Now, bioconjugate **3j** after MS analysis was stored in water, PBS, or 10% human serum at 4 °C and 25 °C for seven days. MS analysis for bioconjugate stability was performed from 1 to 7 days.

2.5 Procedure for labeling peptides

In a 1.5 mL microcentrifuge tube, peptides (**4a-t**, 50 nmol) were mixed with sodium bicarbonate buffer (90 µL, 0.1 M, pH 7.8). To this solution, **2j** (2.5–5 mmol) in DMSO (10 µL) from a freshly prepared stock solution was added and vortexed at 25 °C. After 2–4 h, NH₂OH (10 mmol) was added and vortexed for another 1 h. Subsequently, the reaction mixture was lyophilized, and the unreacted reagent was removed by DCM wash. The peptide was dissolved in 100 µL of methanol, and the sample was analyzed by LC-MS.

2.6 Rapi-Gly-tag for residue and protein-specific labeling in a mixture of proteins

Representative mixture of seven proteins – insulin (**1**), aprotinin (**6l**), ubiquitin (**6f**), cytochrome C (**6e**), lysozyme (**6i**), β -lactoglobulin (**6j**), and RNase A (**6k**). In a 2 mL microcentrifuge tube, 10 nmol of each protein was mixed in sodium bicarbonate buffer (400 μ L, 0.1 M, pH 7.8). To this solution, **2j** (5 mmol) in DMSO (100 μ L) from a freshly prepared stock solution was added and vortexed at 25 °C. After 2 h, NH_2OH (10 mmol) was added and vortexed for 1 h. The reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed using a 0.5 mL 3-kDa centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 $\times$ 0.4 mL). The sample was analyzed by LC-MS.

2.7 Single-site late-stage installation of tags

Cu-Click: Insulin bioconjugate **3n** was taken in 200 μ L of PBS buffer. To this solution, azide probe (Cy3-azide, 20 μ M in 200 μ L) and click reagent (10 μ L from stock solution: 100 mM THPTA ligand, 20 mM CuSO_4 , 300 mM sodium ascorbate in water) were added. Reaction was vortexed at 25 °C for 1 h. Next, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed by using a 0.5 mL 3-kDa centrifugal spin concentrator. The installation of the fluorophore was analyzed by SDS-PAGE. The same protocol was used for the installation of other azide probes, including desthiobiotin-azide and ^{19}F -azide probe. To confirm the installation of biotin, the protein mixture was incubated with a 1:1 ratio of streptavidin for 1 h to enable streptavidin-desthiobiotin binding. The installation of the biotin tag was analyzed by SDS PAGE, and the ^{19}F NMR tag was validated by ^{19}F -NMR spectroscopy using TFA (0.2 mM) as an internal standard.

Cu-free Click: In a 2 mL microcentrifuge tube, protein (**6b-d**, 5 nmol) was mixed with sodium bicarbonate buffer (200 μ L, 0.1 M, pH 7.8). To this solution, **2p** (2.5 mmol) in DMSO (50 μ L) from a freshly prepared stock solution was added and vortexed at 25 °C. After 2–6 h, NH_2OH (5 mmol) was added and vortexed for an additional 1 h. Next, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed by a 0.5 mL 3-kDa or 10-kDa MWCO centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 $\times$ 0.4 mL). The sample was analyzed by LC-MS. Further, DBCO-Cy5 (1 μ g) was added to the protein mixture and incubated for 1 h at 25 °C. This reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess DBCO-Cy5 was removed by a 0.5 mL 10-kDa MWCO centrifugal spin concentrator. The installation of Cy5 with Cu-free click chemistry was analyzed by SDS-PAGE.

2.8 Bioconjugation site analysis

All the solutions were prepared freshly before the experiments.

Insulin bioconjugate

Insulin (0.1 mg) in 100 μ L of H₂O was taken in a 1.5 mL microcentrifuge tube. To reduce the disulfide bond between chain A and chain (b), dithiothreitol (10 μ L, 0.2 M DTT in H₂O) was added. The reaction mixture was incubated at 37°C for 1 h. The chain A N-terminal Gly modification was confirmed by LC-MS and LC-MS-MS analysis.

Melittin bioconjugate

Melittin (0.1 mg) in H₂O (200 μ L) was taken in a 1.5 mL microcentrifuge tube, incubated with 5 μ L of trypsin solution at 37 °C for 4. LC-MS and LC-MS-MS to confirm the N-terminal Gly modification.

Myoglobin bioconjugate

Myoglobin (0.1 mg) was taken in a 1.5 mL microcentrifuge tube. To this solution, urea (8 M, 10 μ L) and tert-butanol (20 μ L) were added and incubated for 1 h at 37 °C. The urea concentration of the sample was reduced by using a 0.5 mL 3-kDa MWCO centrifugal spin concentrator. The sample was re-suspended in 100 μ L grade I water. To this solution, 5 μ L of trypsin solution was added, and the mixture was incubated at 37 °C for 16 h. The pH of the digested solution was adjusted to < 6 (verified by pH paper) with trifluoroacetic acid (0.5%). Subsequently, the sample was used for peptide mapping by LC-MS and sequencing by LC-MS-MS.

SUMO1 and N-Gly eGFP bioconjugate

Protein (0.1 mg, 7.3 nmol) in grade I water (10 μ L) with urea (8 M) was taken in a 1.5 mL microcentrifuge tube and incubated for 30 minutes. To cleave the disulfide bonds, a reducing agent (5 μ L, 0.2 M DTT in grade I water) was added to the solution, and the sample was incubated for 1 h at 37 °C. To block the free sulfhydryl groups, an alkylating agent (5 μ L, 0.2 M iodoacetamide in grade I water) was added to the solution and incubated (in the dark) for 1 h at ambient temperature. To quench the unreacted iodoacetamide, a reducing agent (5 μ L, 0.2 M DTT in grade I water) was added again to the mixture, and the sample was incubated at 25 °C for 1 h. The urea concentration of the sample was reduced by using a 0.5 mL 3 kDa MWCO centrifugal spin concentrator, and the sample was re-suspended in 100 μ L grade I water. To this solution, 5 μ L of enzyme (trypsin) solution was added, and the mixture was incubated at 37 °C for 16 h. The pH of the digested solution was adjusted to < 6 (verified by pH paper) with trifluoroacetic acid (0.5%). Subsequently, the sample was used for peptide mapping by LC-MS and sequencing by LC-MS-MS.

2.9 Reaction with glycineamide

In a 50 mL round-bottom flask, amino acid amide (0.9 mmol) was dissolved in sodium bicarbonate buffer (9 mL, 0.1 M, pH 7.8). To this solution, **2j** (2.7 mmol) in DMF (9 mL) from a stock solution was added, and the reaction mixture was allowed to stir at 25 °C. After 12 h, the reaction mixture was concentrated by lyophilization. Subsequently, solvent extraction with ethyl acetate and water (1:1, 50 mL) was used to remove the salts and unreacted Gly-NH₂. The gravity column chromatography with DCM/methanol produces an analytically pure product (**9a**, 40%). For XRD analysis, 10 mg of **9a** was dissolved in acetonitrile and crystallized with the solvent diffusion method using ACN/DCM solvent system at 4 °C.

2.10 Computational methods

All calculations were conducted using a DFT method implemented in the Gaussian 16 suite of programs.¹ For geometry optimization and frequency analysis, the B3LYP functional and 6-311G++(d,p) basis set² were employed. No symmetry constraints were imposed during geometry optimizations. Transition states were verified by examining the normal mode corresponding to the imaginary frequency and performing intrinsic reaction coordinate (IRC) analysis³ to ensure proper connection between reactants and products on the potential energy surface. Single-point energy refinements were carried out using the hybrid-meta-GGA M062X functional⁴ with the 6-311++G (d, p) basis set. Solvation energies were calculated for all intermediates and transition states using a self-consistent reaction field (SCRF) approach in water as a solvent and the SMD continuum solvation model.⁵ Free energies (ΔG) reported throughout the article, unless stated otherwise, were determined at the M062X/6-311++G(d,p)//B3LYP/6-311++G(d,p) level. The ΔG values were obtained by adding the in-solvent electronic energy (ΔE) at M062X/6-311++G(d,p) to the free energy corrections computed at B3LYP/6-311G++(d,p) in the gas phase.

2.11 Late-stage bond engineering

C-C bond dissociation enabled by retro-*rap*i-Gly-tag method: At this stage, bioconjugate **3j** (5 nm) was taken, in a 2 mL microcentrifuge tube with sodium bicarbonate buffer (200 μ L, 0.1 M, pH 7.8, 0.5 M NaCl). To this solution, pyridoxal 5'-phosphate (PLP, 250 nm) was added and incubated at 25 °C for 1 h. Subsequently, NH₂OH (5 mmol) was added and vortexed for 30 min. Next, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed by using a 0.5 mL 3-kDa centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 $\times$ 0.4 mL). The sample was analyzed by LC-MS.

N-formyl generation: For oxidation of aminoalcohol to generate N-Gly oxoaldehyde, bioconjugate **3j** (5 nm) was taken in 200 μ L of PB (pH 7.4, 0.1 M, 150 mM NaCl). NaIO₄ (50 nm) and L-Met (100 nm) were added to this solution and incubated at 25 °C for 10 min. Subsequently, 10 μ L of ethylene glycol was added to quench the excess NaIO₄. Next, the reaction mixture was diluted with acetonitrile:water (10:90, 1000 μ L), and excess reagent and salts were removed by using a 0.5 mL 3-kDa centrifugal spin concentrator. The protein mixture was further washed with grade I water (2 $\times$ 0.4 mL). The sample was analyzed by LC-MS.

2.12 Expression of SUMO1 and eGFP

Bacterial transformation: Competent *E. coli* cells were thawed on ice [DH5 α for plasmid extraction and BL21 (DE3) for protein expression]. Approximately 50 ng of plasmid DNA was added to 50-100 μ L of competent cells and incubated on ice for 15-20 min. Heat shock was applied at 42 °C for 40 s, followed by immediate incubation on ice for 2 min. Subsequently, 800 μ L of LB (Luria-Bertani Broth) medium was added for recovery, and the cells were incubated at 37 °C with shaking (180 rpm) for 45 min. The recovered cells were spread onto LB agar plates containing the appropriate antibiotic and incubated overnight at 37 °C.

Protein Purification: A primary culture (2-5 mL) was inoculated from a single bacterial colony and grown overnight in LB medium at 37 °C, 180 rpm. Secondary cultures (desired volume) were then inoculated with 1% of the overnight culture. At $\sim$ 0.6 OD₆₀₀, protein expression was induced (200 μ M IPTG, 30 °C, 4 h for SUMO1 and 0.02% L-arabinose, 25 °C, 8 h for eGFP).⁶ Following induction, cells were harvested by centrifugation at 6000 $\times$ g for 10 min, and the resulting pellet was stored at -80 °C until further use. For lysis, frozen pellets were thawed and resuspended in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mg/mL lysozyme, 0.2% Triton X-100, 1 mM PMSF, 1x LPA mix, 5 mM β -mercaptoethanol (β -ME)], followed by incubation on ice for 10-15 min. The mixture was sonicated (45% amplitude; 10 s ON/10 s OFF pulses) until the lysate cleared. The soluble fraction was obtained by centrifugation at 21000 $\times$ g for 30 min at 4 °C. For affinity purification, the supernatant was incubated with pre-equilibrated GSH beads for 1 h. Beads were washed thrice with wash buffer [20 mM Tris (pH 7.5), 400 mM NaCl, 1 mM EDTA, 5 mM β -ME]. Bound proteins were eluted using elution buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 20 mM glutathione], and protein concentrations were determined by Bradford assay. For tag removal, protein-bound beads were washed three times with PreScission protease buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT, 150 mM NaCl, 0.1% Triton]. PreScission protease was added at a 1:50 ratio, and the mixture was incubated overnight at 4 °C. The cleaved protein was then eluted from the column, flash frozen, and stored at -80°C.

2.13 Protocol for in vitro SUMOylation

In vitro SUMOylation assays were performed using 0.25 µg of GST-SAE2/SAE1 (E1 enzyme), 0.75 µg of (His)₆-Ubc9 (E2 enzyme), 3-4 µg of SUMO1/modified SUMO1, and 3-4 µg of substrate in SUMOylation buffer (50 mM Tris, pH 7.5, 5 mM MgCl₂, 5 mM ATP, and 5 mM DTT). Reactions were incubated at 37 °C for 12 h and terminated by adding 6× Laemmli buffer. Reaction products were analyzed using SDS–PAGE.

2.14 Enzymatic assay of myoglobin

Myoglobin activity was checked before and after the labeling by oxidation of o-phenylenediamine (o-PDA) with hydrogen peroxide. Oxidation of o-phenylenediamine to 2,3-diaminophenazine was monitored at 426 nm (A₄₂₆) using a quartz cuvette (path length, 1 cm at 40 °C). Citric acid-Na₂HPO₄ buffer (pH 5.20) was prepared using grade I water. All solutions were made in an aqueous buffer. The freshly prepared myoglobin (**6c**), labeled myoglobin (**7c**), o-phenylenediamine, and hydrogen peroxide (50%) solutions were used for the assay.

Conditions used for the assay:

1. o-PDA (1 mM) and H₂O₂ (1 mM) in the absence of myoglobin (**6c**)
2. o-PDA (1 mM), H₂O₂ (1 mM), and myoglobin (**6c**, 1 × 10⁻⁴ mM)
3. o-PDA (1 mM), H₂O₂ (1 mM), and purified labeled myoglobin (**7c**, 1 × 10⁻⁴ mM)

2.15 Insulin bioactivity assay

Immunofluorescence: HeLa cells were grown on coverslips in a six-well plate format in DMEM and serum-starved for 24 h in 1% serum-containing DMEM medium. Subsequently, cells were treated with 50 µM LY294002 (PI3K inhibitor) for 30 minutes. Next, cells were washed twice with PBS and treated with either native insulin **1** or modified insulin **3j** (3 µg each) in 1 mL of 1% FBS containing DMEM media for 30 minutes. Post-treatment, cells were washed twice with PBS and fixed using 4% paraformaldehyde (PFA) for 10 min at 25 °C. The cells were then rehydrated and permeabilized with rehydration buffer (10 mM Tris, 150 mM NaCl, 0.1% Triton X-100) for 10 min. After rehydration, cells were blocked with 5% normal goat serum (NGS) for 1 h at 4 °C. The cells were stained for 12 h with pAkt antibody (1:600, anti-pAkt-S473, CST #4060) at 4 °C and for 20 mins with phalloidin-647 (1:100, PHDN1-A, Cytoskeleton Inc.). After primary antibody incubation, cells were washed three times with PBS-T (5 minutes each) and incubated with Alexa Fluor-488 conjugated goat anti-rabbit IgG against pAkt (1:1000, Thermo #A11034) for 1 h. Cells were washed thrice with PBS-T, mounted on slides using DAPI-containing mounting medium, and analyzed by CLSM.

Western blotting: HEK293T cells were cultured and treated as mentioned in the immunofluorescence section. Post-treatment, cells were washed twice with PBS and lysed

directly in the 1X Laemmli buffer (containing 1 mM sodium orthovanadate and 1 mM PMSF) by boiling at 100 °C for 10 minutes. The cleared lysates were obtained by centrifugation and analyzed on 10% SDS-PAGE. Following wet transfer protocols, proteins were transferred onto the methanol-activated 0.2 µm PVDF membrane using 1X transfer buffer (2.5 mM Tris-HCl, pH 7.5, 19.2 mM Glycine). The membrane was blocked for 1 h in 5% BSA. Further, the membrane was incubated overnight with anti-pAkt-S473 (1:2000) and anti-GAPDH (1:3000, CST Cat #5174). The membrane was later washed three times for 10 min each with TBS-T buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). The membrane was incubated with Alexa Fluor Plus 680 secondary antibodies for 1 h. Later, it was washed with TBS-T buffer (three times for 10 min each). Images were taken using the LI-COR IR system.

2.16 Bacterial culture and N-Gly labeling with 2n

The primary culture was grown in LB broth (5 mL) at 37 °C for 12 h using an inoculum from an LB agar plate. The grown primary culture (OD₆₀₀ 0.6) was further used to culture *S. aureus* and *E. coli* BL21 (DE3) at 37 °C, 12 h in 5 mL LB media. The cells were harvested by centrifugation (6000 x g, 10 min, and 4 °C). The pellet was further washed 2 times with cold sodium bicarbonate buffer (pH 7.8, 0.1 M). After that, the pellet was further dissolved in 0.5 mL of bicarbonate buffer (pH 7.8, 0.1 M) and used as a bacterial stock. The 50 µL of bacterial stock was taken in a 1.5 mL microcentrifuge tube, and an additional 140 µL of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, freshly prepared stock solution of **2n** (0.25-5 mM) in 10 µL DMSO was added and vortexed (350 rpm) for 15-180 min at 25 °C. Then, NH₂OH (20 mM) was added to the mixture and incubated for 15 min to quench the excess aldehydes. Now, the N-Gly modified bacteria were washed 5 times with PBS. Next, the pellet was re-suspended in 100 µL of click solution containing CuSO₄ (50 µM), THPTA (250 µM), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and Cy3 azide (2 µM) in ice-cold PBS. Further, this mixture was incubated at ice for 10 min to install the Cy3 fluorophore through Cu-catalyzed click reaction. Further, bacteria were washed 3 times to remove non-conjugated Cy3. These Cy3-labeled bacteria were analyzed by CLSM and flow cytometry. For CLSM, conjugated bacteria were re-suspended in 100 µL of PBS. 2 µL of the mixture was then diluted on a mounting slide and spread well with microtips. The spread bacteria on the slide were heat-dried (60 °C), followed by mounting with mounting media.

2.17 Procedure for additive treatment after N-Gly modification of *S. aureus*

To confirm the covalent conjugation of **2n** and Cy3 in *S. aureus*, the bacterial pellet post Cy3 conjugation was resuspended with NaCl (1 M), SDS (0.1%), urea (2 M), NH₂OH (100 mM),

and pyridoxyl-5'-phosphate (PLP, 2 mM) in PBS and incubated for 1 h at 37 °C. Next, bacteria were washed 3 times before analysis with flow cytometry.

2.18 Procedure for species-specific *S. aureus* modification in bacterial co-culture

The 50 µL of bacterial stock in a 1:1 ratio of *S. aureus* and other bacteria from fresh culture was taken in a 1.5 mL microcentrifuge tube, and an additional 140 µL of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, freshly prepared stock solution of **2n** (2 mM) in 10 µL DMSO was added and vortexed (350 rpm) for 2 h at 25 °C. Then, NH₂OH (20 mM) is added to the mixture and incubated for 15 min to quench the excess aldehydes. Next, the N-Gly modified bacteria were further washed 5 times with PBS. Further, pellet was re-suspended in 100 µL of click solution containing CuSO₄ (50 µM), THPTA (250 µM), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and Cy3 azide (2 µM) in ice-cold PBS. The mixture was incubated at ice for 10 min to install the Cy3 fluorophore through Cu-catalyzed click reaction. Next, bacteria were washed 3 times to remove non-conjugated Cy3. These Cy3-labeled bacteria were analyzed by CLSM.

2.19 Procedure for enrichment and target identification in *S. aureus*

50 µL of *S. aureus* bacterial stock was taken in a 1.5 mL microcentrifuge tube, and an additional 140 µL of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, freshly prepared stock solution of **2n** (2 mM) in 10 µL DMSO was added and vortexed (350 rpm) for 2 h at 25 °C. Then, NH₂OH (20 mM) was added to the mixture and incubated for 15 min to quench the excess aldehydes. Now, the N-Gly modified bacteria were washed 5 times with PBS. Next, the pellet was re-suspended in 100 µL of click solution containing CuSO₄ (50 µM), THPTA (250 µM), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and desthiobiotin azide (20 µM) in ice-cold PBS. Further, this mixture was incubated at ice for 10 min to install the desthiobiotin through Cu-catalyzed click reaction. Now, bacteria were further washed 3 times to remove non-conjugated desthiobiotin. These desthiobiotin-labeled bacteria were further subjected to enzymatic digestion with a mixture of mutanolysin (150 µg/mL) and lysostaphin (100 µg/mL) in 500 µL of Tris-HCl buffer (0.5 mM, pH 6.5.⁷ After gentle mixing, the solution was incubated at 37 °C for 4 h. Samples were then pelleted, and the supernatant was heated at 90 °C for 5 min before isolation of biotinylated adducts. Next, supernatant was added to 50 µL of streptavidin agarose bead and mixed with end-on rotation for 4 h. Further, the bead was pelleted at 1000 x g for 3 min and washed 3 times with PBS. Now, immobilized desthiobiotin-labeled peptides were eluted with 200 µL of elution solution (50% ACN/water, 0.1% formic acid) and analyzed by MS.

2.21 Procedure for in-gel fluorescence analysis and global chemoproteomics

Procedure for N-Gly modification in *S. aureus* cells and in-gel fluorescence analysis

The 50 μ l of bacterial stock was taken in a 1.5 mL microcentrifuge tube, and an additional 140 μ l of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, freshly prepared stock solution of **2n** (2 mM) in 10 μ L DMSO was added and vortexed (350 rpm) for 15-180 min at 25 °C. Then, NH_2OH (20 mM) was added to the mixture and incubated for 15 min to quench the excess aldehydes. Now, the N-Gly modified bacteria were washed 5 times with PBS. Next, the pellet was resuspended in 100 μ L of click solution containing CuSO_4 (50 μ M), THPTA (250 μ M), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and Cy3 azide (2 μ M) in ice-cold PBS. Further, this mixture was incubated on ice for 10 min to install the Cy3 fluorophore via a Cu-catalyzed click reaction. Further, bacteria were washed 3 times to remove non-conjugated Cy3. These Cy3-labeled bacteria were lysed and analyzed by in-gel fluorescence analysis.

Procedure for enrichment of modified proteome and its identification

The 50 μ l of bacterial stock was taken in a 1.5 mL microcentrifuge tube, and an additional 140 μ l of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, a freshly prepared stock solution of **2n** (2 mM) or **NHS-alk** (200 μ M) in 10 μ L DMSO was added, and the mixture was vortexed (350 rpm) for 15-180 min at 25 °C. Then, NH_2OH (20 mM) was added to the mixture and incubated for 15 min to quench the excess aldehydes. Now, the modified bacteria were washed 5 times with PBS. Next, the pellet was resuspended in 100 μ L of click solution containing CuSO_4 (50 μ M), THPTA (250 μ M), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and DADPS-biotin azide (2 μ M) in ice-cold PBS. Further, this mixture was incubated at ice for 10 min to install the biotin through Cu-catalyzed click reaction. Further, bacteria were washed 3 times to remove non-conjugated biotin. These biotin-labeled bacteria were lysed in RIPA buffer. Next, proteins were lyophilized and redissolved in 100 μ l of 8 M urea (in 50 mM NH_4HCO_3). Subsequently, 5 μ l of 200 mM DTT in NH_4HCO_3 (pH 7.5-8.0) was added to the mixture for disulfide reduction, which was incubated for 1 h at 25 °C. Next, alkylation of reduced thiols was performed by adding 20 μ l of 200 mM iodoacetamide (in 50 mM NH_4HCO_3 , pH 8.0), followed by gentle vortexing and incubation for 1 h in the dark at 25 °C. The unutilized iodoacetamide was quenched by the addition of 200 mM DTT (in NH_4HCO_3 , pH 8.0), and the mixture was incubated for 1 h in the dark at 25 °C. After completion of this step, 1 ml of CaCl_2 (1 mM) in 50 mM NH_4HCO_3 buffer (pH 8.0) was added to reduce the urea concentration to 1 M. Next, the trypsin enzyme (1:50 w/w, trypsin: protein) was added to digest the proteins. After the addition of trypsin, the reaction mixture was vortexed and incubated at 37 °C for 16 h. After the digestion of the protein, the pH of the

reaction mixture was adjusted to 3-4 by placing 1 μ L of the solution on pH paper. Next, DADPS biotinylated peptides were enriched with streptavidin agarose beads. Here, the peptide digest of 1 ml was added to 50 μ L of streptavidin agarose beads and mixed with end-on rotation for 4 h to immobilize the biotinylated peptides. Further, the bead was pelleted at 1000 x g for 3 min and washed 6 times with PBS, 3 times with 1 M KCl, and 3 times with grade-I water. Now, the immobilized DADPS-biotin-labeled peptides were eluted with 200 μ L of elution solution (5% ACN/water, 10% formic acid). At this stage, the eluted samples were desalted using a C18 spin column and analyzed by nano-LC coupled with an Orbitrap Exploris 240.

2.22 Procedure for multi-probe installation on *S. aureus*

Multi-probe with 2s: 50 μ L of bacterial stock of *S. aureus* from fresh culture was taken in a 1.5 mL microcentrifuge tube, and an additional 140 μ L of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, freshly prepared stock solution of **2s** (62.5-500 μ M) in 10 μ L DMSO was added and vortexed (350 rpm) for 2 h at 25 $^{\circ}$ C. For dual probe: NH_2OH (20 mM) was added to the mixture and incubated for 15 min to quench the excess aldehydes. For triple probe: Biotin hydroxyl amine (1 mM) was added to the mixture and incubated for 1 h to install biotin on the cell surface. Now, the N-Gly modified bacteria were further washed 5 times with PBS. Next, the pellet was re-suspended in 100 μ L of click solution containing CuSO_4 (50 μ M), THPTA (250 μ M), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and Cy3 azide (2 μ M) in ice-cold PBS. Further, this mixture was incubated at ice for 10 min to install the Cy3 fluorophore through Cu catalyzed click reaction. Now, bacteria were further washed 3 times to remove non-conjugated Cy3. For the triple probe, the bacterial pellet was further treated with 20 μ g/mL of FITC-labeled streptavidin in PBS and incubated for 1 h. The bacterial pellet was further washed 3 times to remove unbound streptavidin. The dual or triple-probe-labeled bacteria were analyzed by CLSM.

Multi-probe with 2t: 50 μ L of bacterial stock of *S. aureus* from fresh culture was taken in a 1.5 mL microcentrifuge tube, and an additional 140 μ L of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, a freshly prepared stock solution of **2t** (250 μ M) in 10 μ L DMSO was added and vortexed (350 rpm) for 2 h at 25 $^{\circ}$ C. For dual probe: NH_2OH (20 mM) is added to the mixture and incubated for 15 min to quench the excess aldehydes. For triple probe: Biotin hydroxyl amine (1 mM) is added to the mixture and incubated for 1 h to install biotin on the cell surface. Now, the N-Gly modified bacteria were further washed 5 times with PBS. Next, it was re-suspended in 100 μ L of click solution DBCO-Cy5 (1 μ M) in ice-cold PBS. Further, this mixture was incubated at ice for 10 min to install Cy5 fluorophore through Cu-free click reaction. Now, bacteria were further washed 3 times to remove non-conjugated Cy5. For the triple probe, the bacterial pellet was further treated with 20 μ g/mL of FITC-labeled

streptavidin in PBS and incubated for 1 h. The bacterial pellet was further washed 3 times to remove unbound streptavidin. The dual or triple probe-labeled bacteria were analyzed by CLSM.

Multi-probe with 2u: 50 μ L of bacterial stock of *S. aureus* from fresh culture was taken in a 1.5 mL microcentrifuge tube, and an additional 140 μ L of bicarbonate buffer (pH 7.8, 0.1 M) was added. To this bacterial suspension, a freshly prepared stock solution of **2u** (250 μ M) in 10 μ L DMSO was added and vortexed (350 rpm) for 2 h at 25 $^{\circ}$ C. For dual probe: NH_2OH (20 mM) is added to the mixture to quench the excess aldehydes. For triple probe: Biotin hydroxyl amine (1 mM) is added to the mixture and incubated for 1 h to install biotin on the cell surface. Now, the N-Gly modified bacteria were further washed 5 times with PBS. At first, the washed pellet was re-suspended in 100 μ L of click solution, DBCO-Cy5 (1 μ M) in ice-cold PBS for Cy5 installation. Further, this mixture was incubated at ice for 10 min to install the Cy5 fluorophore through Cu free click reaction. Then, bacteria were further washed 3 times to remove non-conjugated Cy5. Next, the pellet was re-suspended in 100 μ L of click solution containing CuSO_4 (50 μ M), THPTA (250 μ M), amino guanidine hydrochloride (1 mM), sodium ascorbate (2.5 mM), and Cy3 azide (2 μ M) in ice-cold PBS to install Cy3. Further, this mixture was incubated at ice for 10 min for installation of Cy3 fluorophore through Cu-catalyzed click reaction. Now, bacteria were further washed 3 times to remove non-conjugated Cy3. For the triple probe, the bacterial pellet was further treated with 20 μ g/mL of FITC-labeled streptavidin in PBS and incubated for 1 h. The bacterial pellet was further washed 3 times to remove unbound streptavidin. The dual or triple probe-labeled bacteria were analyzed by CLSM.

2.23 Mammalian cell culture

Cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 units/ mL Penicillin and Streptomycin) in a humidified incubator at 37 $^{\circ}$ C under 5% CO_2 conditions. The cells were grown in 100-mm cell culture plates (8×10^6 cells) for Western blot analysis or in six-well plates (3×10^5 cells per well) for immunofluorescence detection.

2.24 Species-specific *S. aureus* modification in co-culture with mammalian cells

HeLa cells were co-cultured with *S. aureus* by adding 50 μ L of freshly prepared bacterial suspension ($\text{O.D.}_{600} = 0.6$) in DMEM to the culture plate. Following co-culture establishment, a freshly prepared solution of compound **2t** (final concentration: 250 μ M in DMEM) was added, and cells were incubated at 37 $^{\circ}$ C for 2 h. To quench unreacted aldehyde groups, hydroxylamine (NH_2OH) was added to a final concentration of 20 mM, followed by a 15 min incubation. Cells were subsequently washed twice with PBS. For fluorescent labeling, a

solution of DBCO-Cy5 (1 μ M) in DMEM was added and incubated at 4 °C for 10 min to facilitate bioorthogonal conjugation. Cells were washed twice with PBS and imaged using CLSM or harvested using 5% trypsin–EDTA solution for flow cytometry analysis.

2.25 Species-specific *S. aureus* detection in *Drosophila melanogaster* larvae

***Drosophila* husbandry and larval synchronization**

Wild-type *Drosophila melanogaster* (w1118) flies were maintained on standard cornmeal agar medium at 25 °C under a 12 h light/dark cycle. For experimental synchronization, adult flies were transferred onto fresh food vials and allowed to lay eggs for 1 h at 25 °C. Vials containing eggs were subsequently kept at 25 °C for 48 h. Feeding second-instar larvae were collected and used for all experiments.

Bacterial cultures and larval exposure

E. coli and *S. aureus* were grown overnight in LB broth at 37 °C with shaking. Secondary cultures were prepared and adjusted to an optical density of 1.0 at 600 nm (OD₆₀₀). PBS was used as a negative control. For the infection assay, 250 μ L of bacterial suspension was mixed with 250 μ L of 20% (w/v) sucrose solution. Synchronized second-instar larvae (n=10) were incubated in this mixture for 2 h at 25 °C. Following incubation, larvae were washed three times with sterile PBS to remove non-ingested bacteria.

In vivo labeling and detection of *S. aureus* in *Drosophila* gut

To detect bacterial uptake, larvae were incubated with **2t** (250 μ M) reagent prepared in 20% sucrose solution for 2 h at 25 °C. After incubation, larvae were again washed three times with PBS. Midguts were dissected in PBS and subsequently incubated with Cy5 dye solution for 10 min on ice in 0.2% PBST (PBS with 0.2% Triton X-100). Dissected guts were fixed in 4% PFA for 40 min at 25 °C. Tissues were washed three times for 10 min each in 0.2% PBST. Samples were mounted on glass slides using mounting medium with DAPI for nuclear staining. Samples were imaged under the confocal microscope.

In vivo labeling and detection of *S. aureus* in *Drosophila* gut with Dlg staining

After *in vivo* labelling with 2t, for immunostaining, fixed guts were blocked in 3% Normal Goat Serum (NGS) prepared in 0.2% PBST for 1 h at room temperature. Samples were then incubated overnight at 4°C with a mouse anti-Discs large (Dlg) primary antibody (1:100 dilution; Developmental Studies Hybridoma Bank) diluted in blocking solution. Following primary antibody incubation, tissues were washed three times for 10 min each in 0.2% PBST and subsequently incubated with anti-mouse Alexa Fluor-conjugated secondary antibody (1:800 dilution, Invitrogen) for 1 h at room temperature in the dark. After secondary antibody incubation, samples were washed three times for 15 min each in 0.2% PBST. Finally, tissues

were mounted on glass slides using DAPI-containing mounting medium to visualize nuclei. Images were acquired using a confocal microscope.

In vivo labeling and detection of *S. aureus* in *Drosophila* gut with **2u**

DBCO-Cy5 was first mixed with **2u** in a 1:1 ratio to generate a pre-conjugated Cy5–**2u** probe, which was then administered to *Drosophila* larvae for *in vivo* labeling. After incubation, larvae were again washed three times with PBS. Midguts were dissected in PBS and subsequently incubated with Cy3 click mix solution for 10 min on ice in 0.2% PBST (PBS with 0.2% Triton X-100). Dissected guts were fixed in 4% PFA for 40 min at 25 °C. Tissues were washed three times for 10 min each in 0.2% PBST. Samples were mounted on glass slides using mounting medium with DAPI for nuclear staining. Samples were imaged under the confocal microscope.

2.26 Growth curve assay for *S. aureus* and *E. coli*

The growth kinetics of *S. aureus* and *E. coli* BL21 were evaluated using a microplate-based assay in the presence of compound **2n**. Overnight bacterial cultures grown in LB medium were inoculated into 24-well plates at a final volume of 1 mL per well. For *S. aureus*, samples included untreated (native control), vehicle-treated (DMSO), and compound **2n**-treated groups at 50, 100, 200, 300, 400, and 500 μ M concentrations. Ampicillin was used as a negative control. For *E. coli* BL21, native, DMSO, ampicillin, and 200 μ M **2n**-treated samples were assessed based on prior results in *S. aureus*. Plates were incubated at 37 °C with orbital shaking at 200 rpm. Optical density at 600 nm (OD₆₀₀) measurements were recorded at 1 h intervals over 18 h using a plate reader. All conditions were independently repeated three times. Growth curves were generated to compare bacterial proliferation across treatments.

2.27 Minimum inhibitory concentration (MIC) assay for different bacterial strains

Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.^{8,9} The MIC was defined as the lowest concentration of the compound at which no visible bacterial growth was detected. Assays were performed in duplicate and repeated independently three times to ensure reproducibility.

2.28 Inhibition of biofilm formation

Crystal violet assay: Biofilm formation by *S. aureus* was evaluated using a crystal violet (CV) assay. An overnight culture of *S. aureus* was diluted in LB medium and grown to mid-log phase (OD₆₀₀ = 0.6). For biofilm assays, 10 μ L of this culture was seeded into each well of a

96-well flat-bottom plate containing 190 μ L of LB medium, resulting in a total volume of 200 μ L per well. Wells were supplemented with compound **2n** at the desired concentrations. Native (untreated) and DMSO-treated cells were taken as controls. Plates were incubated at 37 °C for 24 h under static conditions to allow biofilm development. After incubation, planktonic cells and media were carefully aspirated, and wells were gently washed once with 200 μ L of phosphate-buffered saline (PBS; 0.1 M, pH 7.4) to remove loosely attached cells. To fix the biofilm, 99% methanol was added and incubated for 5 min, after which the methanol was discarded, and the plate was air-dried. Biofilms were stained by adding 1% (w/v) crystal violet solution to each well and incubating for 20 min at 25 °C. Excess stain was removed by washing, and the plate was dried. To solubilize the bound dye, 50 μ L of 33% (v/v) acetic acid was added to each well. Absorbance was measured at 570 nm using a microplate reader. All samples were analyzed in triplicate and quantified.

FE-SEM analysis: Scanning electron microscopy (SEM) was performed on *S. aureus* cultures treated with compound **2n** to visualize biofilm architecture. Bacterial biofilms were grown as mentioned on the crystal violet assay in 24-well plates containing sterile glass coverslips in 1 mL of total volume, with or without **2n** at the indicated concentrations. Untreated (native) and DMSO-treated samples served as controls. After incubation, the medium was gently removed, and the biofilm-coated coverslips were fixed with 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 1 h at 25 °C. Samples were washed twice with cold PBS and dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, and 100%), with each step carried out for 10 min. The final dehydration step in 100% ethanol was extended to 20 min to ensure complete removal of water. Coverslips were mounted onto aluminum SEM stubs and dried overnight at 37 °C. Prior to imaging, samples were sputter-coated with a thin layer of gold. Biofilm morphology was visualized using field-emission scanning electron microscopy (FE-SEM) at various magnifications.

2.29 Inhibition of pre-formed biofilm

Crystal violet assay: Inhibition of pre-formed *S. aureus* biofilm was evaluated by the crystal violet (CV) assay. An overnight culture of *S. aureus* was diluted in LB medium and grown to mid-log phase ($OD_{600} = 0.6$). For pre-formed biofilm assays, 10 μ L of this culture was seeded into each well of a 96-well flat-bottom plate containing LB media and incubated at 37 °C for 24 h under static conditions to allow biofilm development. After 24 h of biofilm development, wells were supplemented with compound **2n** at the desired concentrations for another 24 h. Native (untreated) and DMSO-treated cells were taken as controls. The samples were then processed as stated before (section 2.6).

FE-SEM analysis: Pre-formed *S. aureus* biofilms were treated with **2n** at the indicated concentrations for 24 h, while native (untreated) and DMSO-treated samples served as controls. The samples were processed for FE-SEM as described in Section 2.26.

2.30 TEM analysis

An overnight culture of *S. aureus* was diluted in LB medium and grown at 37 °C with either DMSO (control) or **2n** (2.5 × MIC) for 12 h. After incubation, samples were washed twice with ice-cold PBS and dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, and 100%), with each step carried out for 10 min. The final dehydration step in 100% ethanol was extended to 20 min to ensure complete removal of water. Further, bacteria were stained with freshly prepared uranyl acetate 0.2 % w/v for 5 min. Then, samples for TEM analysis were prepared by drop-casting them over a carbon-coated 400 mesh Cu grid, and they were dried inside a vacuum desiccator at RT for 12 h.

2.31 Animal model, ethics approval, and experimental design

CD-1 mice (6–8 weeks old, healthy) were housed under standard laboratory conditions (20–25 °C, 12 h light/dark cycle, 60–70% relative humidity). The experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of IISER Bhopal (2025-IISERB-7.1-IAEC). Animal experiments were performed per the guidelines provided by the Committee for Control and Supervision of Experiments on Animals (CCSEA, Govt. of India).

2.32 Procedure for *S. aureus* selective detection in mice

Blood infection model: CD-1 mice (6-8 weeks old) were intravenously injected via the retro-orbital sinus with *S. aureus* (10⁸ colony-forming units, CFU) in sterile PBS, in a total volume of 100 µL. *E. coli* (10⁸ CFU) and PBS alone served as negative controls. One hour post-infection, animals received an intraperitoneal injection of **2t** at a dose of 22 mg/kg, while another control group (infected with *S. aureus*) received PBS. Four hours following administration of **2t**, mice were euthanized, and whole blood was collected via cardiac puncture. Plasma was separated by centrifugation at 500 × g for 10 min at 4 °C. Bacteria were pelleted from the plasma fraction by centrifugation at 11,000 × g for 2 min. The resulting bacterial pellet was then incubated with 1 µM DBCO-Cy5 dye in PBS for 10 min on ice. Cells were subsequently washed three times with PBS to remove unbound dye. Labeled bacteria were mounted onto glass coverslips and imaged using CLSM.

Tissue infection model: CD-1 mice (6-8 weeks old) were injected intramuscularly into the thigh region with 10⁸ CFU of *S. aureus*, in a volume of 50 µL. *E. coli* (10⁸ CFU) or sterile PBS

served as negative controls. One hour post-infection, mice received a subcutaneous injection of **2t** (100 μ L) at a dose of 22 mg/kg; another control group with *S. aureus* infection received PBS. After an additional 2 h, animals were euthanized, and infected thigh muscle tissues were harvested and embedded in tissue freezing medium. Cryosections (7 μ m) were cut using a cryomicrotome at -20°C and mounted onto glass slides. Tissue sections were fixed in 4% PFA for 15 min at 25°C , followed by three washes with PBS. For fluorescent labeling, sections were incubated with 1 μ M DBCO-Cy5 in PBS for 10 min at 4°C . Excess dye was removed by washing the sections three times with PBS. Labeled sections were imaged using CLSM. Identical acquisition parameters were used across all samples to enable comparative analysis.

Skin infection model: To establish a skin infection model, full-thickness excisional circular wounds using a biopsy punch (~ 6 mm diameter) were created on the dorsal neck region of anesthetized CD-1 mice (6–8 weeks old). 10^8 CFU of bioluminescent *S. aureus* (Xen36) was topically applied to the wound site in a 10 μ L volume and allowed to adhere for 5–10 min. *E. coli* (10^8 CFU) or sterile PBS served as negative controls. The wound was closed using a 3/0 silk suture to maintain localized infection. One hour post-infection, **2t** was administered intraperitoneally at a dose of 22 mg/kg in a volume of 200 μ L. Control mice received sterile PBS. Twelve hours after compound administration, DBCO-Cy5 was injected via the retro-orbital sinus in a total volume of 100 μ L for systemic delivery of the fluorescent probe. Mice were anesthetized using isoflurane (2–3% in oxygen) during all injections and imaging procedures to ensure immobilization and minimize discomfort. Eight hours after dye injection, *in vivo* imaging was performed. Bioluminescent signals from the *S. aureus* (Xen36) strain and Cy5 fluorescence were sequentially acquired using appropriate filters (bioluminescence: open emission; Cy5: excitation 640 nm, emission 680 nm). Co-localization of bioluminescent and fluorescent signals was analyzed using Living Image software. Identical imaging parameters were used across all animals to enable quantitative comparison.

2.33 Wound healing in mice post *S. aureus* infection

CD-1 mice (6–8 weeks old) were randomly assigned to experimental groups ($n = 3$ per group; total $n = 9$), including control (no *S. aureus*), untreated, and 2% **2n** treatment groups. All procedures were conducted in accordance with institutional ethical guidelines and approved animal care protocols. Mice were singly housed in individually ventilated cages (IVCs) to maintain aseptic conditions throughout the study. For anesthesia, animals received intraperitoneal injections of ketamine (80 mg/kg) and xylazine (8 mg/kg). Dorsal fur was removed using a depilatory agent, and the skin was cleaned with alcohol. Full-thickness excisional circular wounds using a biopsy punch (~ 6 mm diameter) were created on the dorsum of each animal. Wounds were then inoculated with either *S. aureus* (10^7 CFU/mL,

10 µL per wound) or sterile PBS (control). Treatment commenced 24 h post-infection. The test group received a topical application of 2% (w/w) **2n**, while control and untreated animals received the vehicle only. Treatments were applied once daily for nine consecutive days. Wound progression was documented photographically, and wound areas were quantified using ImageJ software. On day 9, animals were euthanized, and wound tissues were harvested for histopathological analysis. The experiment was conducted in triplicate, and wound area data are presented as mean ± S.E.M.

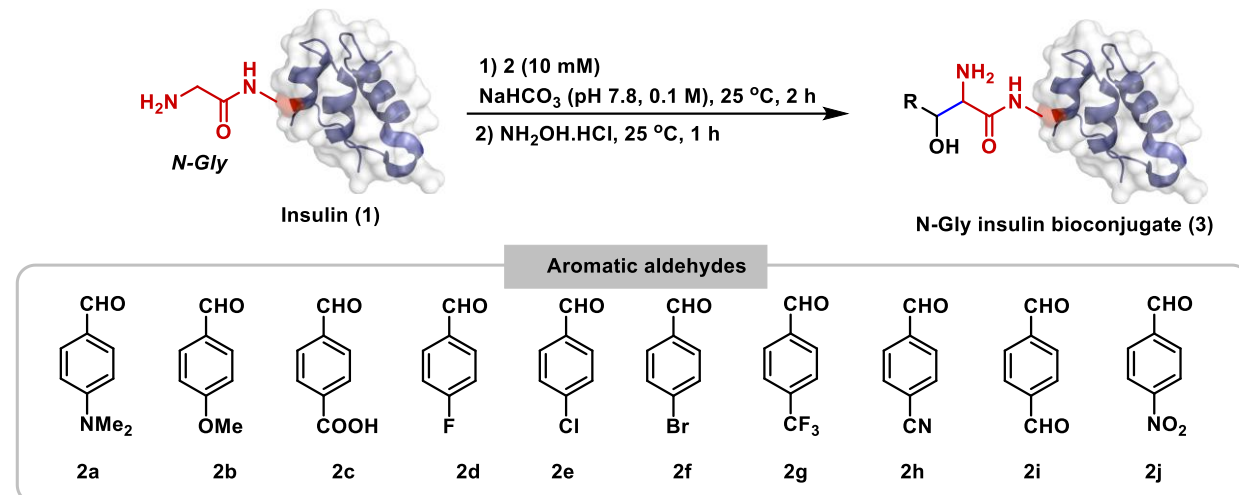
Hematoxylin and Eosin staining. Skin tissue samples from animals of each group on day 9 were collected and fixed in 10% neutral buffered formalin. Following fixation, samples were dehydrated using a graded alcohol series and embedded within paraffin wax at 60 °C. Tissue blocks were sectioned into 5 µm-thick sections using a microtome and mounted on glass slides. Slides were deparaffinized using xylene for 30 minutes, and then they were rehydrated successively with IPA (100%, 90%, and 80%) for 2-3 minutes each, followed by a final 3-minute rehydration in distilled water. Following blotting off the excess water, the slides were stained with hematoxylin for 1-2 minutes. Again, wash the slides with distilled water, and then follow this step by rinsing the slides with 95% alcohol to ensure complete dehydration. Then, the slides were counterstained with an eosin Y solution for 1 minute. Following staining, slides were dehydrated again using 95% alcohol. Finally, slides were cleaned by xylene washing and mounted with DPX mounting medium. The obtained slides were examined and imaged under a light microscope using different magnifications.

Masson Trichome staining. One slide from each group on day 9 was further processed for evaluation of collagen deposition using Masson Trichrome staining. Briefly, the slides were dipped in Bouin's solution for 1 hour at 56 °C and then washed in running tap water to remove the yellow color. They were then stained with Weigert's haematoxylin for 10 minutes and Biebrich scarlet-acid fuchsin for 10 minutes, with intermittent washing in distilled water. Later, the slides were differentiated with phosphomolybdic-phosphotungstic acid solution for 10-15 min to enhance collagen staining. Then, the slides were transferred to aniline blue solution for 5-10 minutes. Following washing, the slides were treated with a 1% acetic acid solution for 2 minutes to facilitate stain differentiation, washed, and then dehydrated using a gradient of alcohol concentrations. Finally, cleared using xylene. Slides were then mounted with a cover slip using DPX and observed under a light microscope.

3. N-Gly bioconjugation

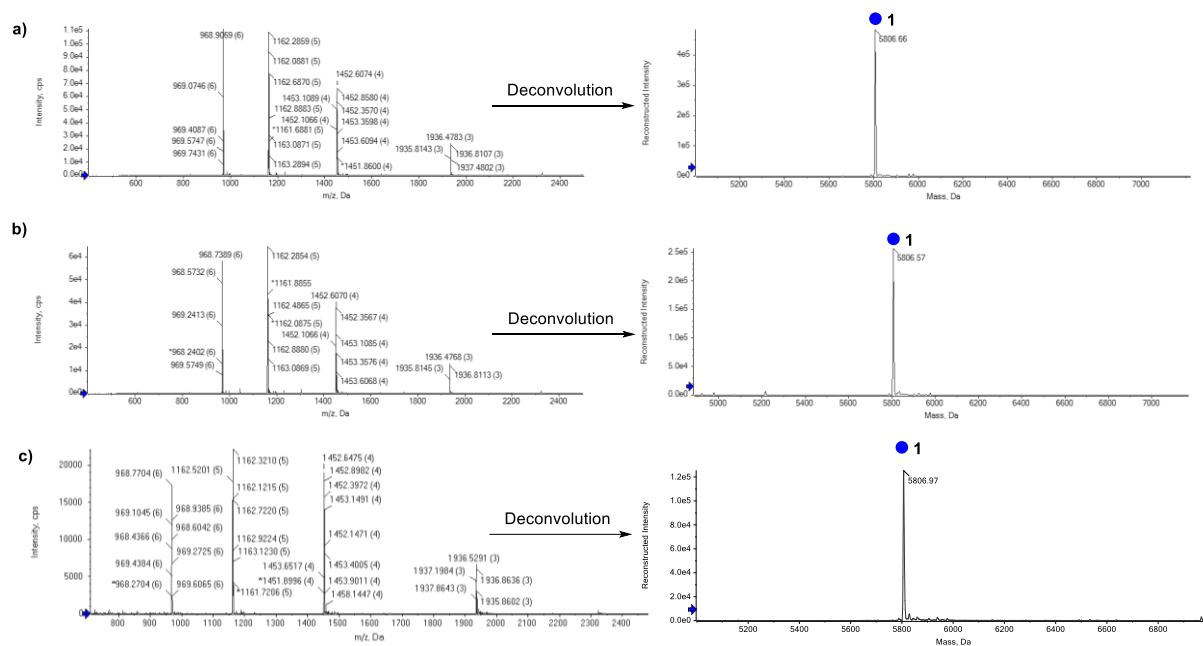
3.1 Initial investigation for N-Gly modification in a model protein

Supplementary Table 1. Screening of aromatic aldehydes **2a-2j** for N-terminus Gly modification of insulin.

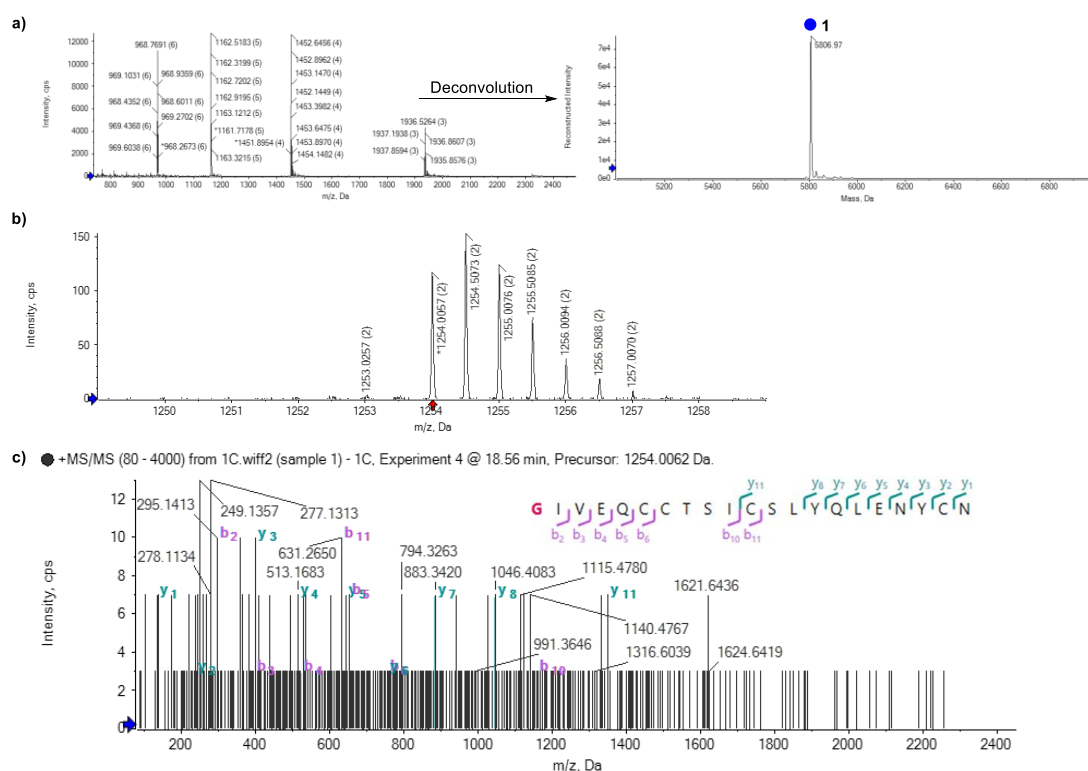


Entries	Aromatic aldehydes (2a-2j)	% Conversion (3)			
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	2a	0	0	0	0
2	2b	0	0	0	0
3	2c	0	0	0	0
4	2d	2	0	0	1
5	2e	7	7	8	7
6	2f	10	10	11	10
7	2g	50	49	42	47
8	2h	82	82	83	82
9	2i	86	87	86	86
10	2j	88	86	89	87

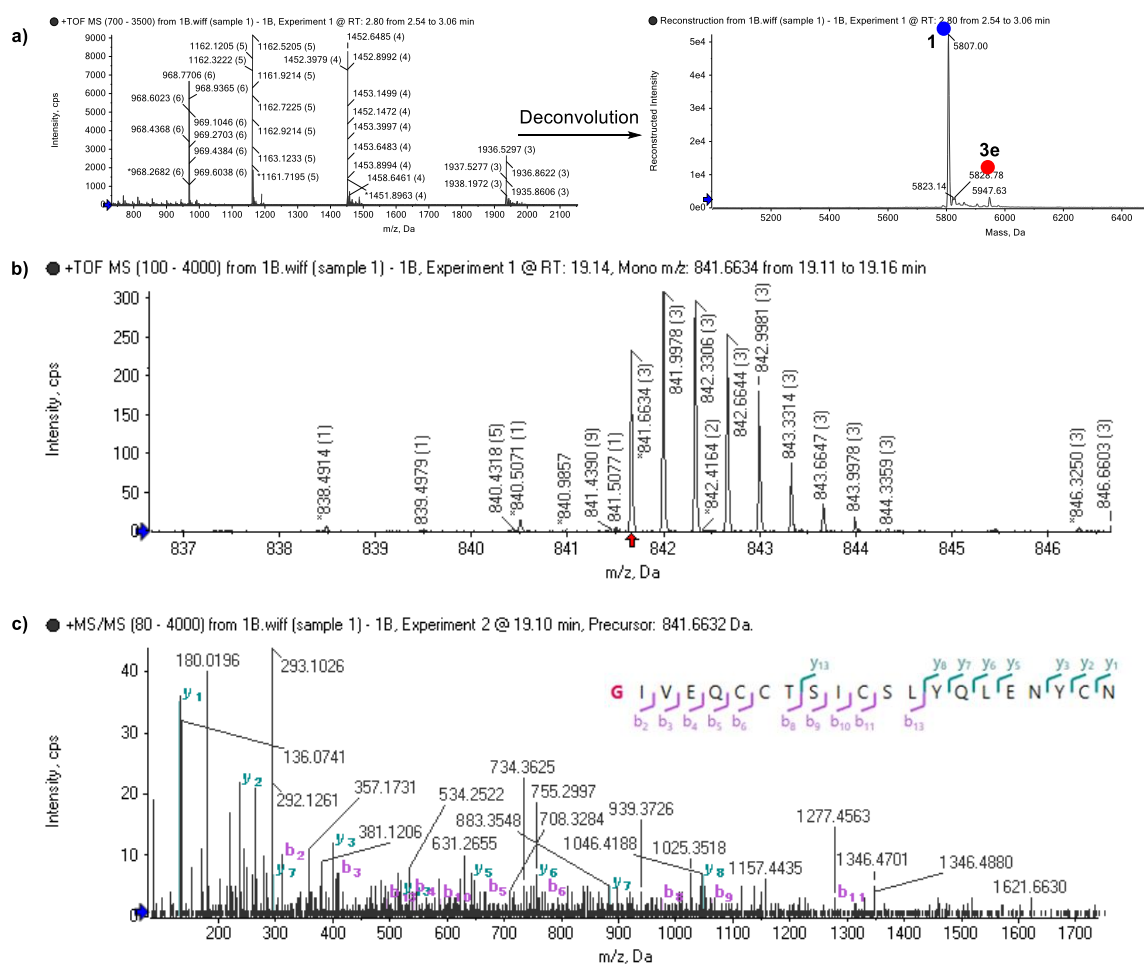
^aFor analytical data of replicates 2 and 3, see the replicate MS data file.



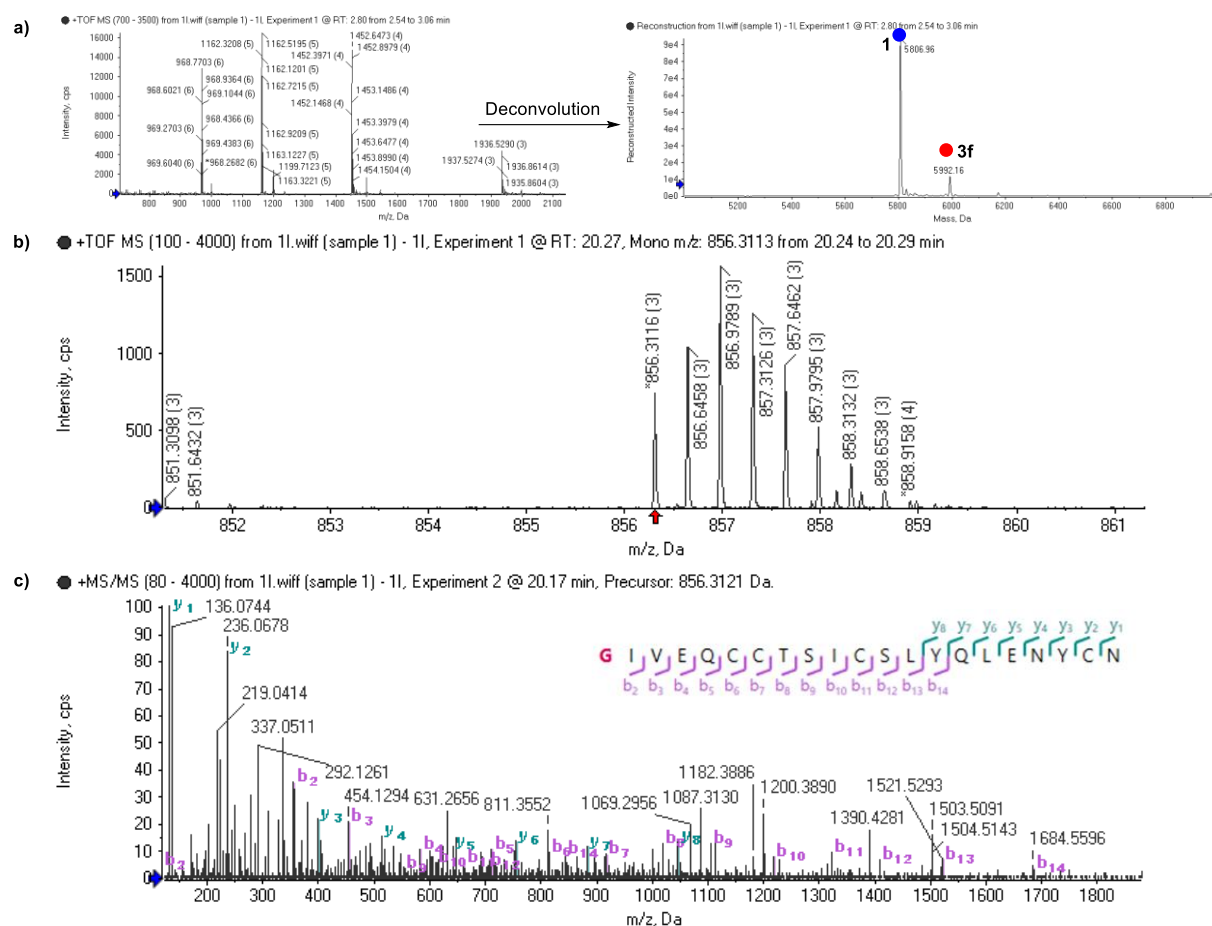
Supplementary Fig. 1. Replicate 1: Charged and deconvoluted MS spectra for insulin (1) after reaction with (a) 2a, (b) 2b, and (c) 2c for N-Gly bioconjugation.



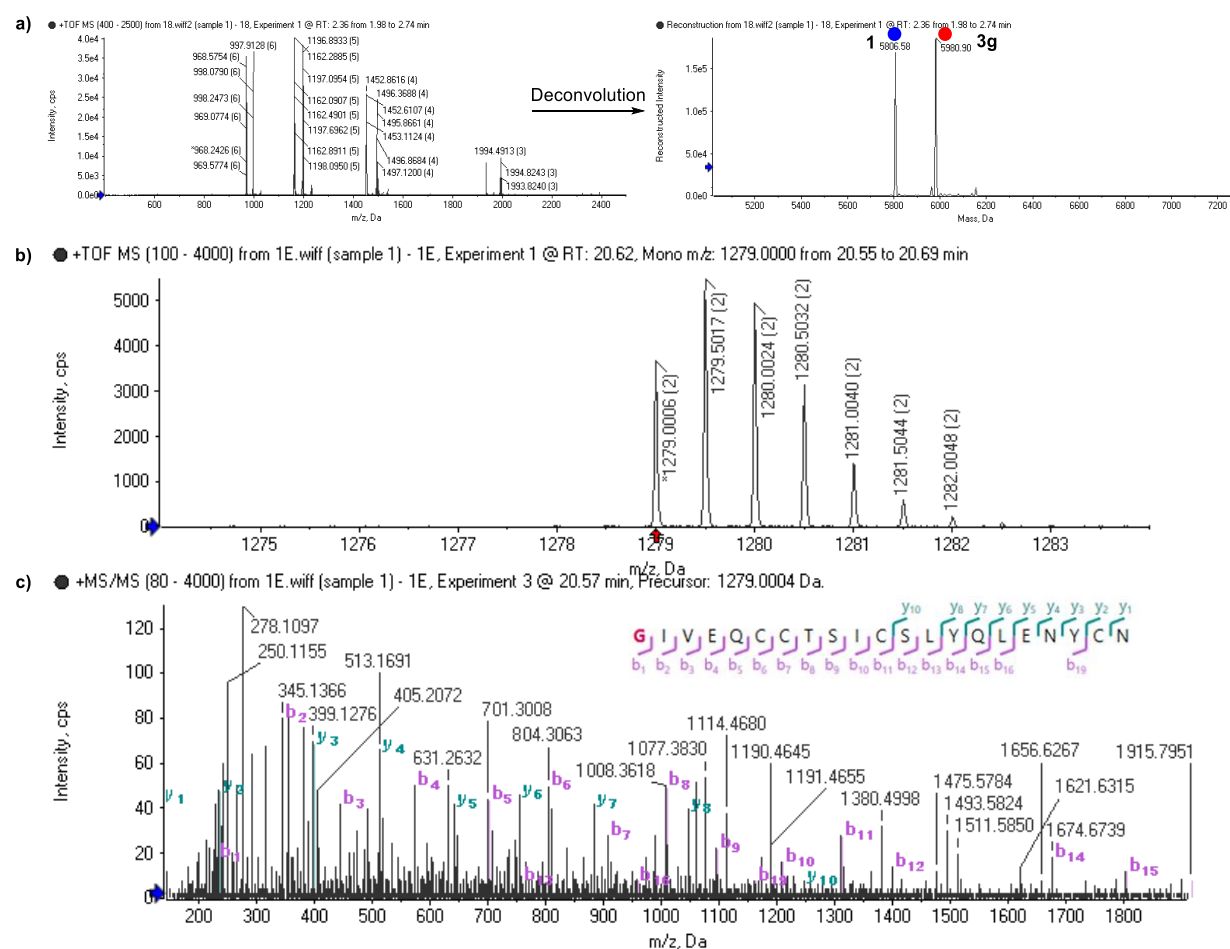
Supplementary Fig. 2. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (1) after reaction with 2d for N-Gly bioconjugation. (b) Peptide mapping of 3d after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSICSLSLYQLENYCN (G1-N21, m/z 1254.0062, [M+2H]²⁺) confirms the site of modification, N-Gly (G1). The low S/N is attributed to 2% conversion in this experiment.



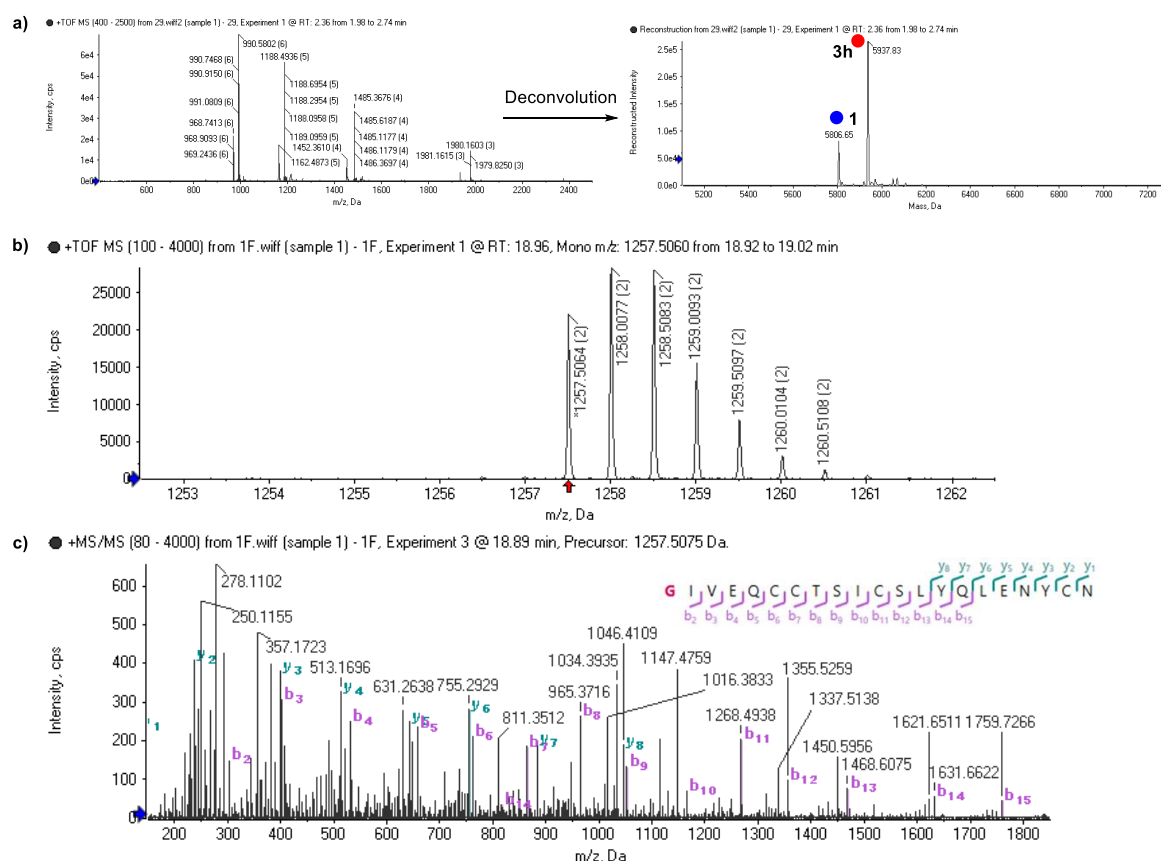
Supplementary Fig. 3. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2e** for N-Gly bioconjugation. (b) Peptide mapping of **3e** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSLCSLYQLENYCN (G1-N21, m/z 841.6632, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



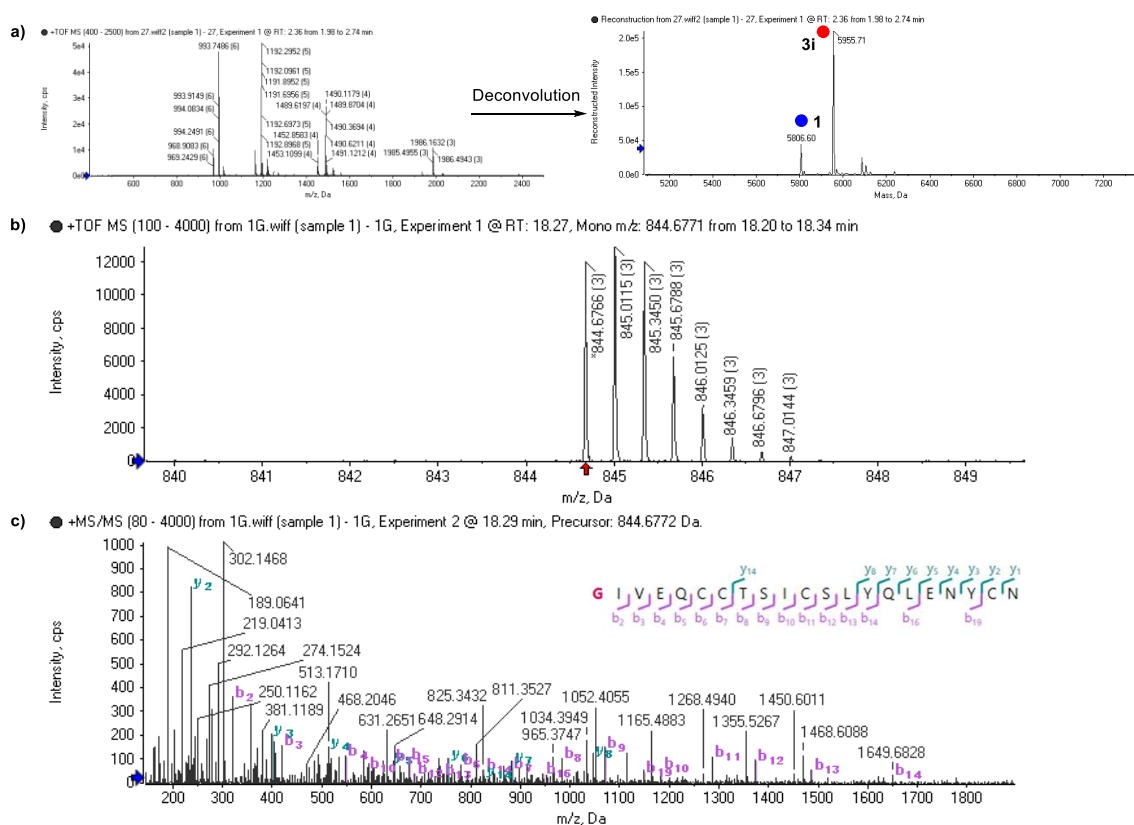
Supplementary Fig. 4. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2f** for N-Gly bioconjugation. (b) Peptide mapping of **3f** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI CSLYQLENYCN (G1-N21, m/z 856.3121, $[M+3H]^3+$) confirms the site of modification, N-Gly (G1).



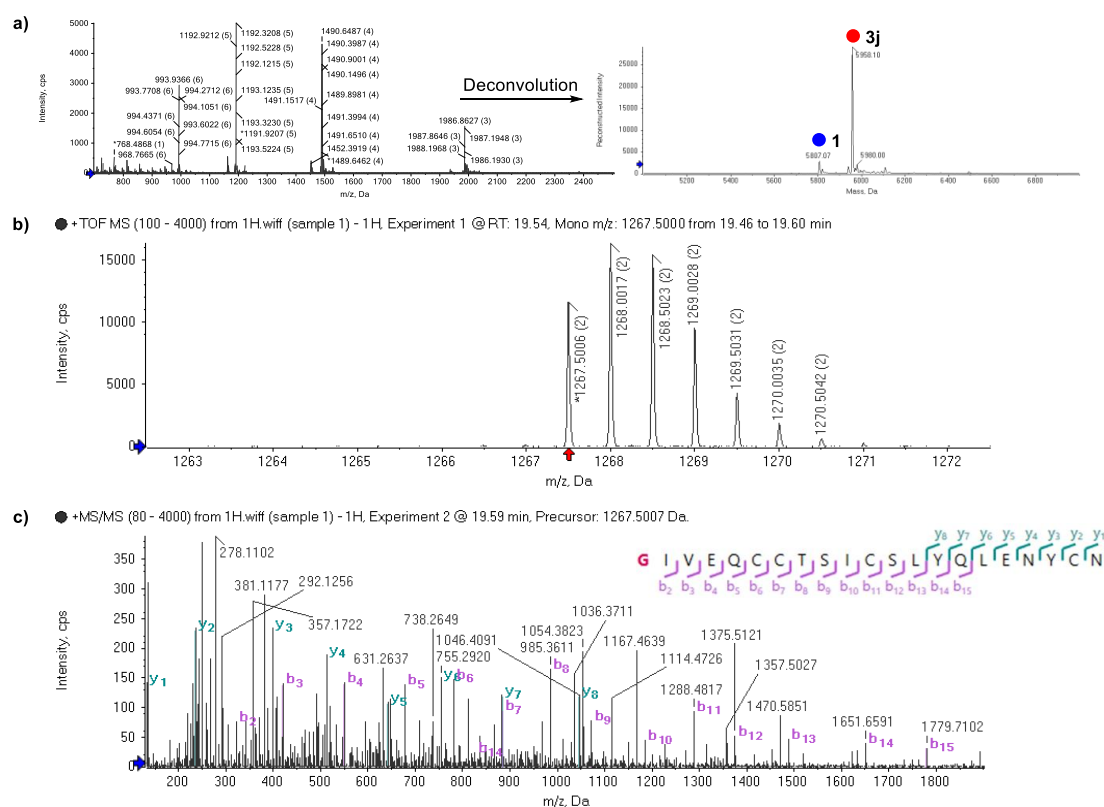
Supplementary Fig. 5. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2g** for N-Gly bioconjugation. (b) Peptide mapping of **3g** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI CS L Y Q L E N Y C N (G1-N21, m/z 1279.0004, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).



Supplementary Fig. 6. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2h** for N-Gly bioconjugation. (b) Peptide mapping of **3h** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSIQSLYQLENYCN (G1-N21, m/z 1257.5075, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).



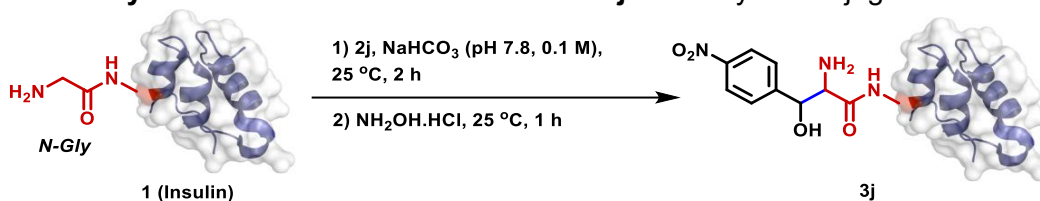
Supplementary Fig. 7. Replicate 1: (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2i** for N-Gly bioconjugation. (b) Peptide mapping of **3i** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSLICSLYQLNLYCN (G1-N21, m/z 844.6772, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



Supplementary Fig. 8. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** for N-Gly bioconjugation. (b) Peptide mapping of **3j** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSICSLYQLENYCN (G1-N21, m/z 1267.5007, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).

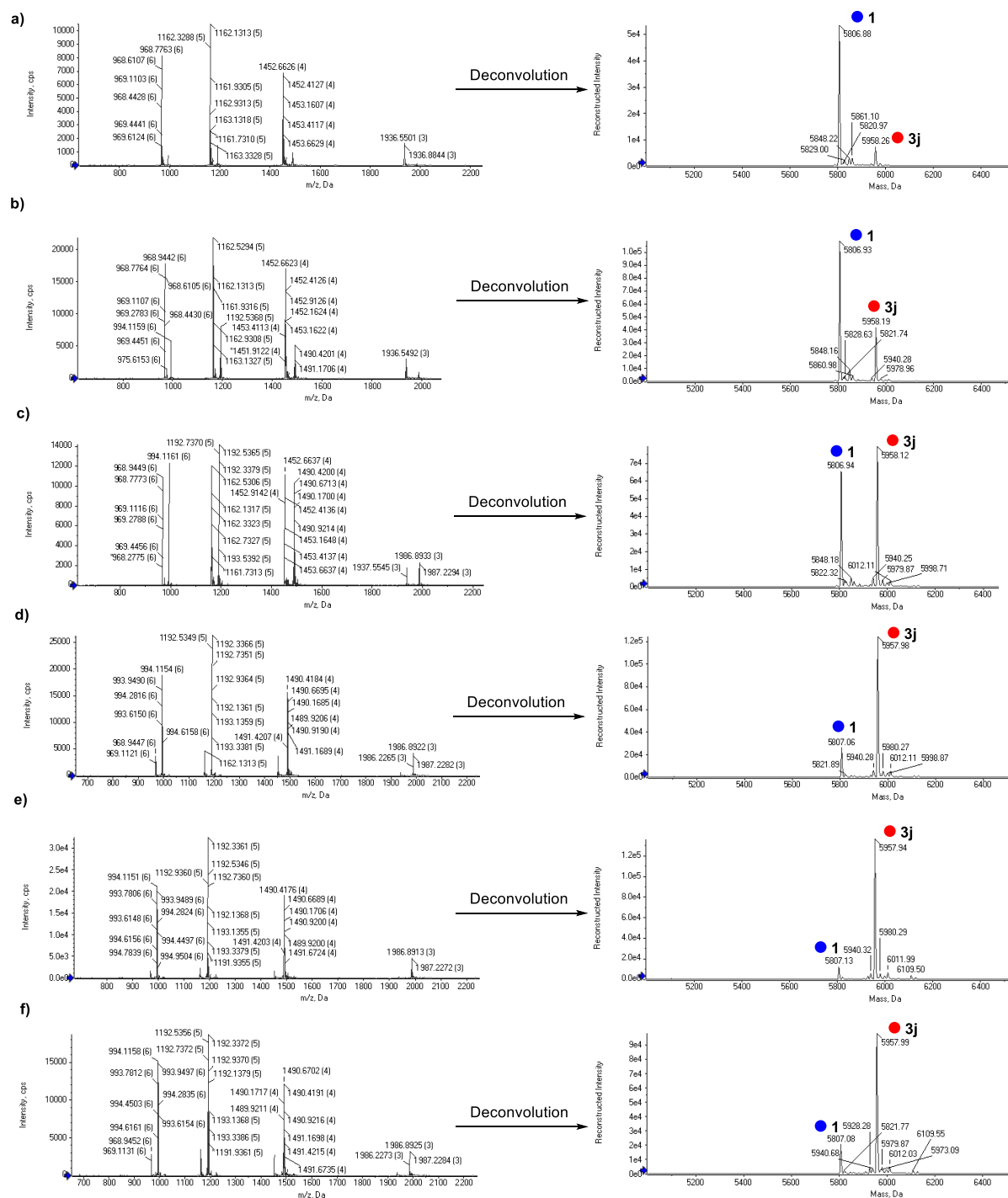
3.2 Optimization of reaction attributes

Supplementary Table 2. Effect of concentration of **2j** on N-Gly bioconjugation of insulin.



Entries	Concentration of 2j (mM)	% Conversion (3j)			
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	0.5	13	16	16	15
2	1	30	32	32	31
3	2	56	57	58	57
4	5	83	83	83	83
5	10	92	92	92	92
6	20	84	85	85	84

^aFor analytical data of replicates 2 and 3, see the replicate MS data file.



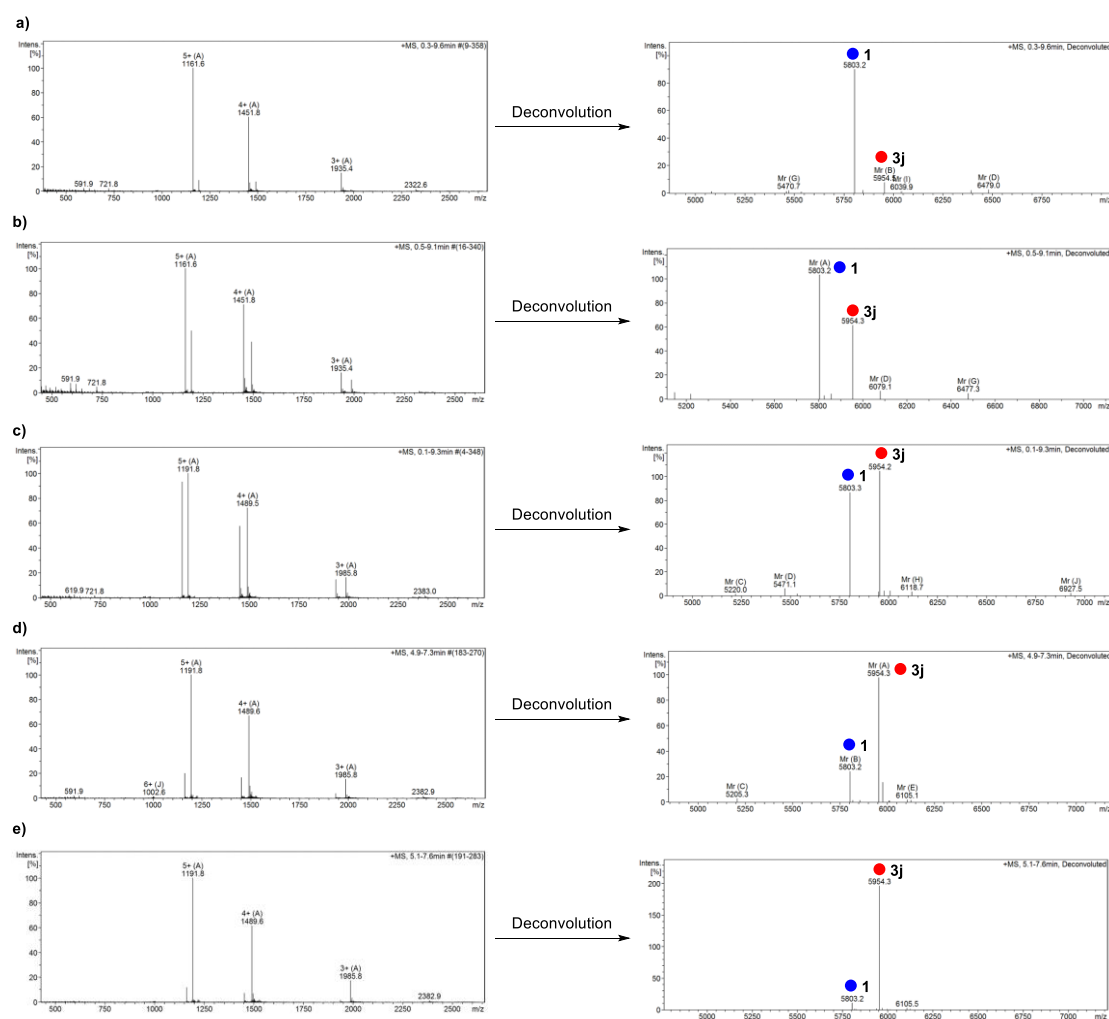
Supplementary Fig. 9. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction at different concentrations of **2j**; (a) 0.5 mM, (b) 1 mM, (c) 2 mM, (d) 5 mM, (e) 10 mM, and (f) 20 mM.

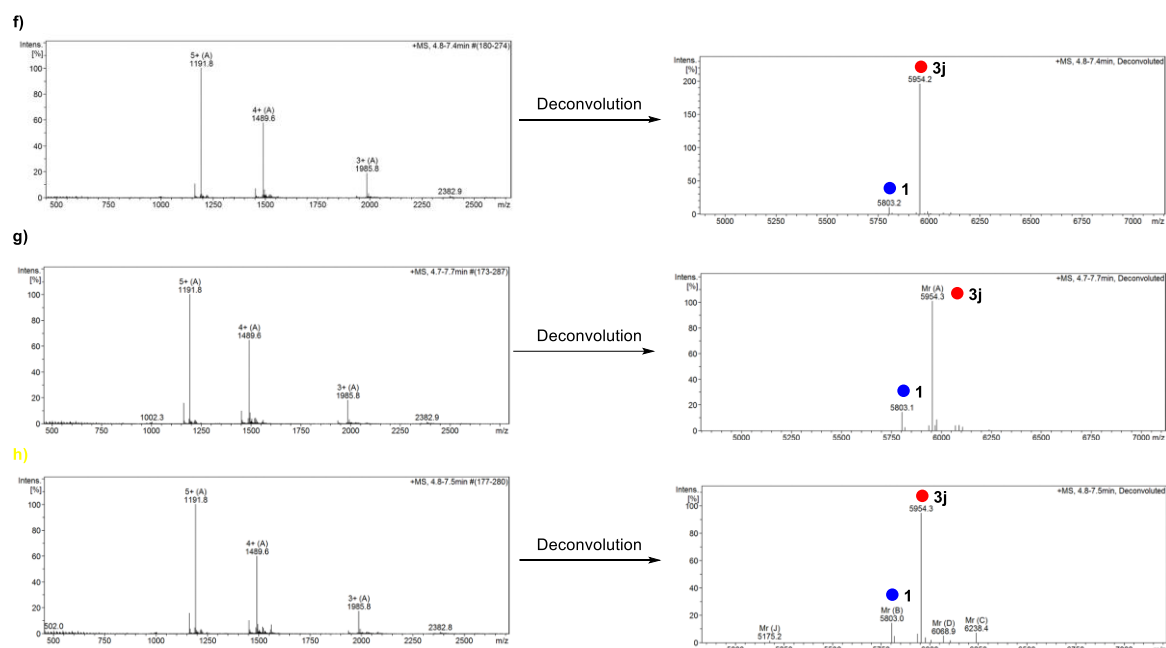
Supplementary Table 3. Progress of N-Gly bioconjugation of insulin as a function of time.

1) 2j (10 mM)
NaHCO₃ (pH 7.8, 0.1 M),
25 °C, 2 h
2) NH₂OH.HCl, 25 °C, 1 h

1 (Insulin)		3j			
Entries	Time (min)	% Conversion (3j)			
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	2	11	9	8	9
2	10	40	40	31	37
3	20	60	64	63	62
4	40	78	77	76	77
5	60	86	86	86	86
6	120	88	86	89	87
7	240	92	92	84	89
8	360	91	93	93	92

^aFor analytical data of replicates 2 and 3, see the replicate MS data file.





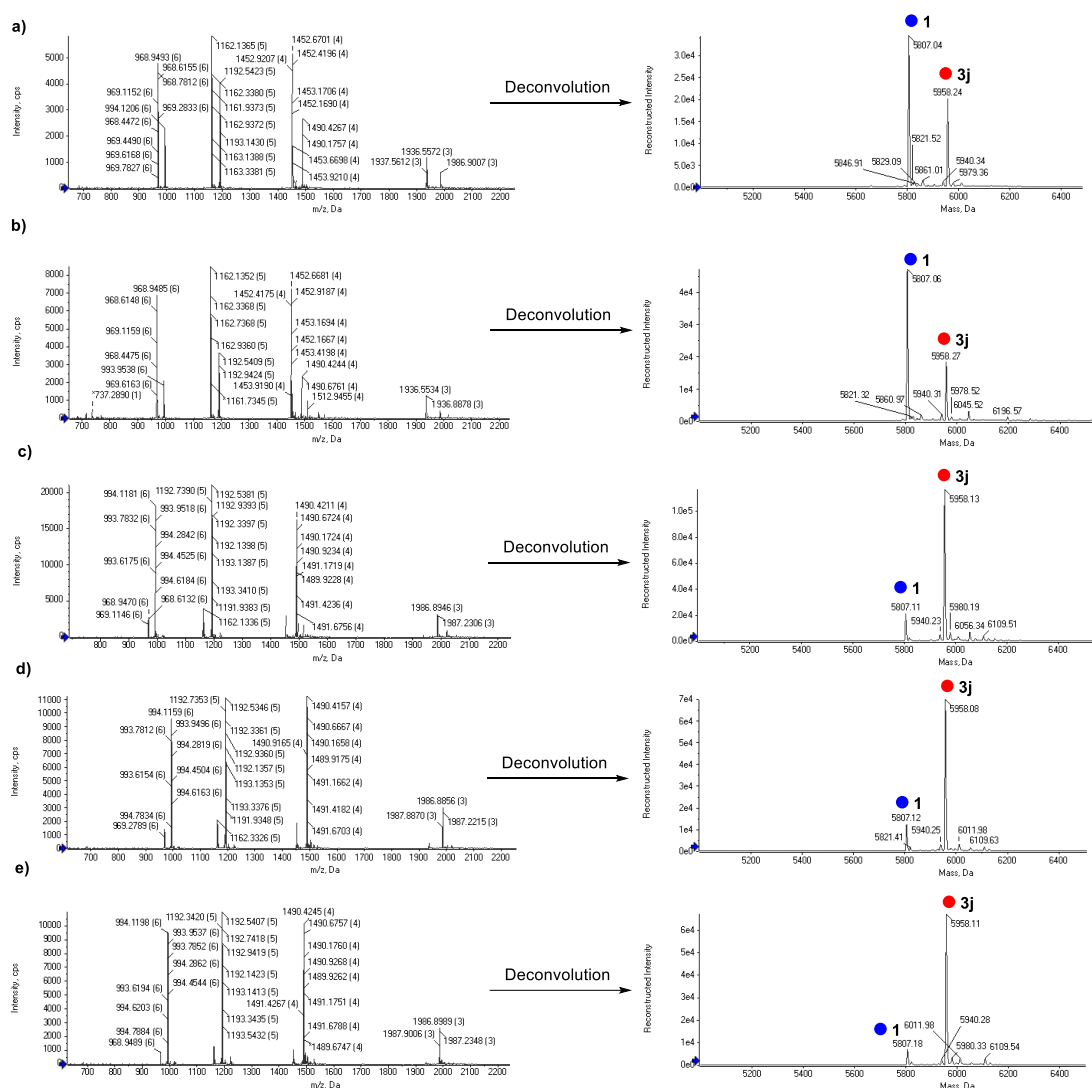
Supplementary Fig. 10. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** at different time intervals; (a) 2 min, (b) 10 min, (c) 20 min, (d) 40 min, (e) 60 min, (f) 120 min, (g) 240 min, and (h) 360 min.

Supplementary Table 4. Effect of buffer composition on N-Gly bioconjugation of insulin.

1) **2j** (10 mM),
NaHCO₃ (pH 7.8, 0.1 M)
25 °C, 2 h
2) NH₂OH.HCl, 25 °C, 1 h

Entries	Buffer (0.1 M, pH 7.8)	% Conversion (3j)			
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	Tris	37	39	38	38
2	HEPES	28	29	31	29
3	PB	85	85	86	85
4	NH ₄ HCO ₃	85	84	86	85
5	NaHCO ₃	89	89	90	89

^aFor analytical data of replicates 2 and 3, see the replicate MS data file.

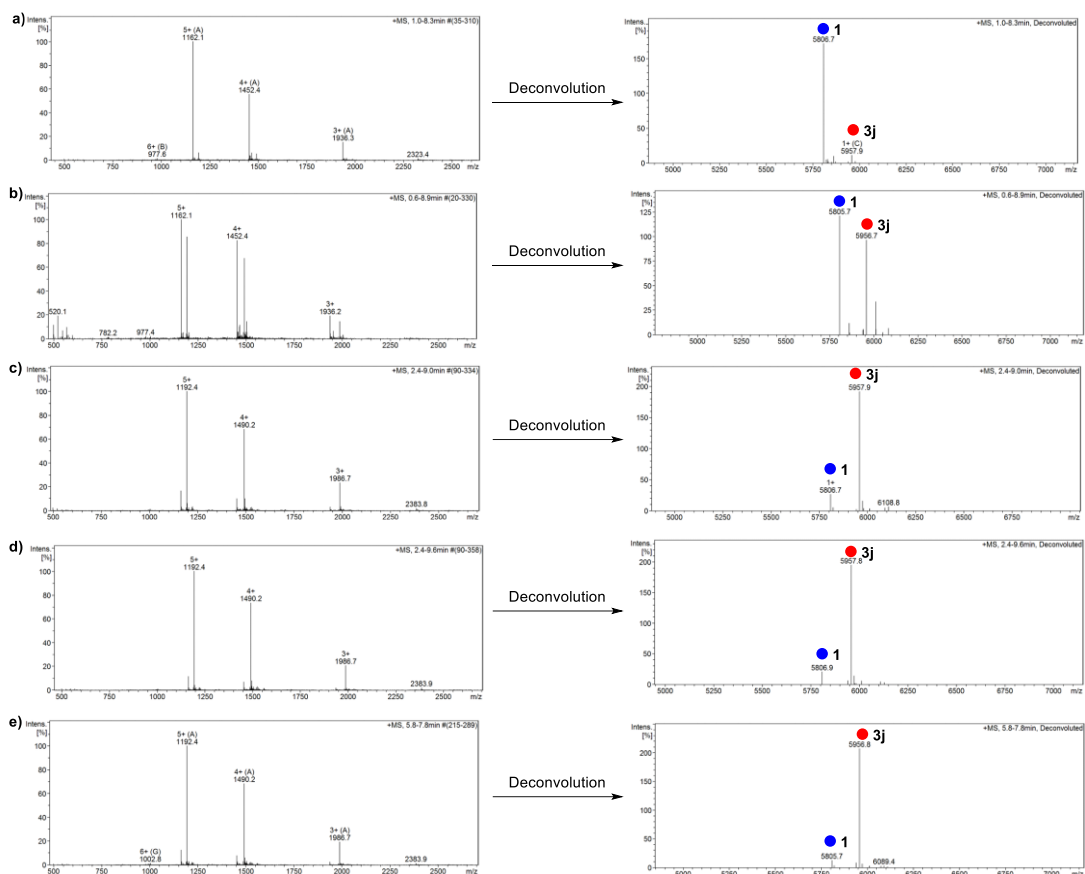


Supplementary Fig. 11. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** using: (a) Tris buffer, (b) HEPES buffer, (c) PB buffer, (d) NH_4HCO_3 buffer, and (e) NaHCO_3 buffer.

Supplementary Table 5. Effect of pH on N-Gly bioconjugation of insulin.

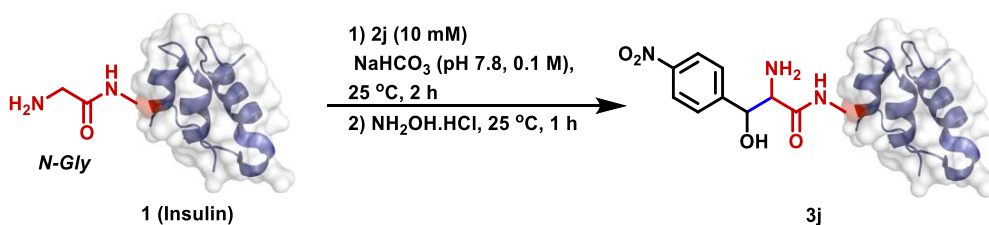
Entries	pH of NaHCO_3 buffer (0.1 M)	% Conversion (3j)			
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	5	5	11	12	7
2	6	45	47	44	46
3	7	86	89	90	88
4	7.8	91	90	91	91
5	9	90	91	91	90

^aFor analytical data of replicates 2 and 3, see the replicate MS data file.

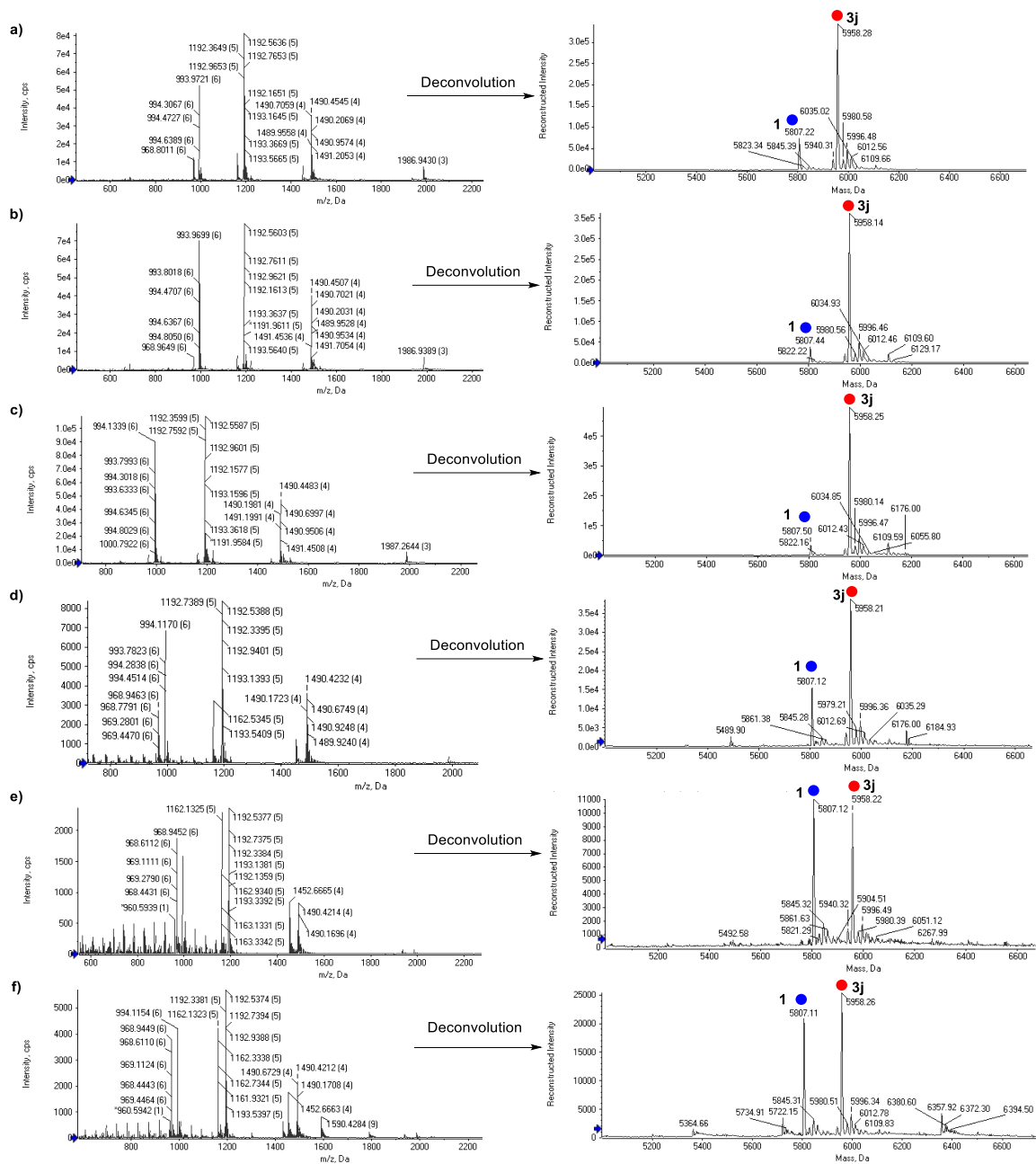


Supplementary Fig. 12. Replicate 1: Charged and deconvoluted MS spectra of insulin (1) after reaction with 2j at: (a) pH 5.0, (b) pH 6.0, (c) pH 7.0, (d) pH 7.8, and (e) pH 9.0.

Supplementary Table 6. Effect of protein concentration on N-Gly bioconjugation of insulin.

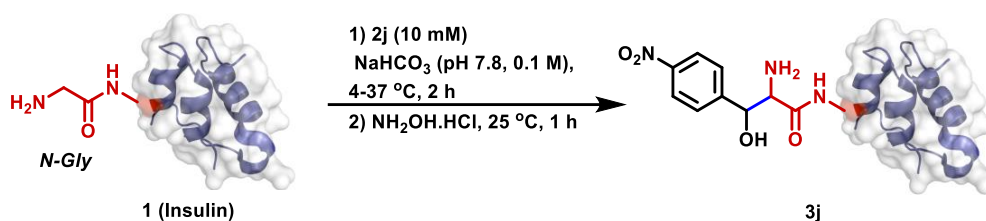


Entries	Concentration of 1 (μM)	% Conversion (3j)
1	10	84
2	20	91
3	30	92
4	50	74
5	75	51
6	100	57

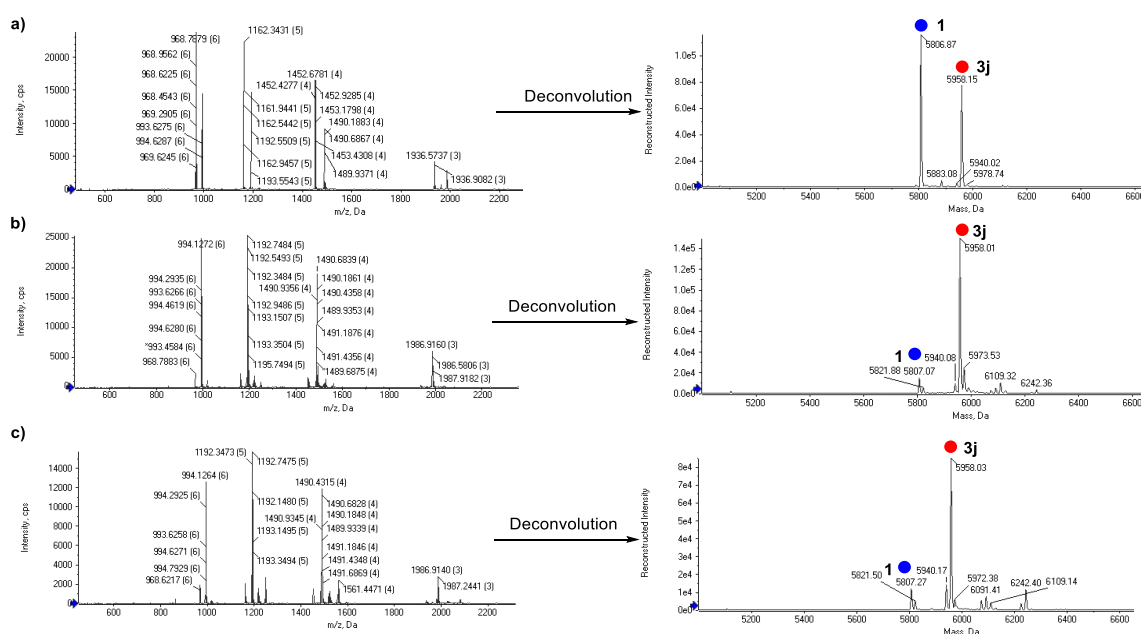


Supplementary Fig. 13. Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** at different protein concentrations: (a) 10 μM , (b) 20 μM , (c) 30 μM , (d) 50 μM , (e) 75 μM , and (f) 100 μM .

Supplementary Table 7. Effect of reaction temperature on N-Gly bioconjugation of insulin.



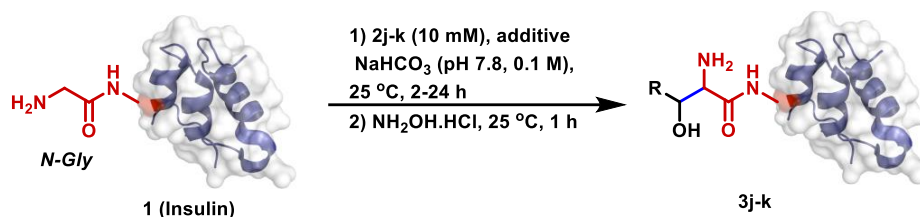
Entries	Temperature (°C)	% Conversion (3j)
1	4	39
2	25	91
3	37	86



Supplementary Fig. 14. Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j at: (a) 4 °C, (b) 25 °C, and (c) 37 °C.

3.3 Control experiments

Supplementary Table 8. N-Gly bioconjugation of insulin with **2j** versus **2k** in the presence of biological additives.

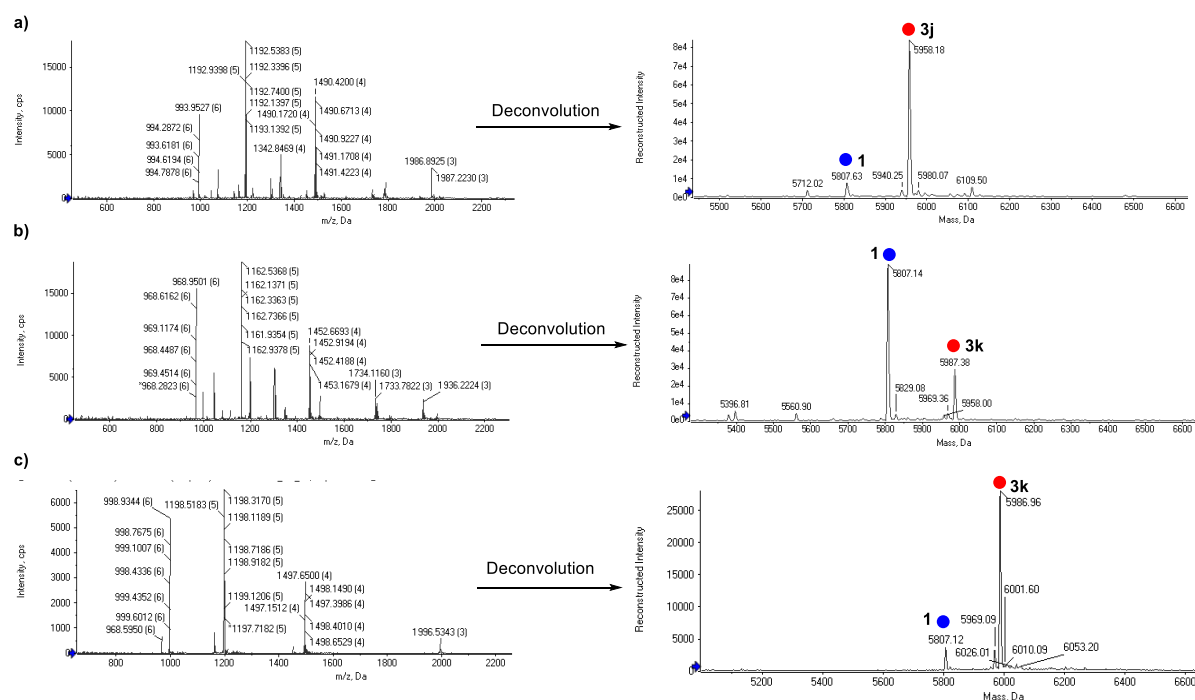


Entries	Additives	% Conversion (3)			
		2j (2 h)			Mean
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	
1	None	94	91	92	92
2	ADP	93	89	87	89
3	ATP	92	89	91	90
4	Human serum	74	88	98	91
5	DMEM media	87	88	87	75

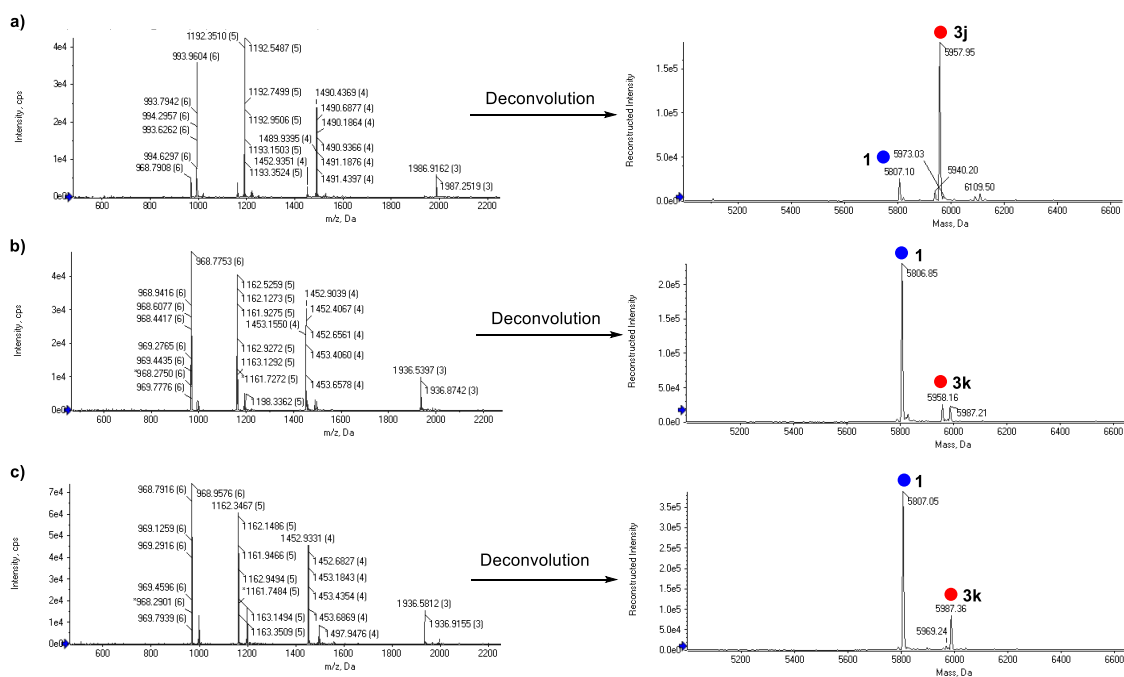
Entries	Additives	% Conversion (3)			
		2k (2 h)			Mean
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	
1	None	20	22	22	21
2	ADP	7	6	7	6
3	ATP	3	1	1	1
4	Human serum	0	1	1	1
5	DMEM media	3	5	5	4

Entries	Additives	% Conversion (3)			
		2k (24 h)			Mean
		Replicate 1	Replicate 2 ^a	Replicate 3 ^a	
1	None	87	85	87	86
2	ADP	14	19	18	17
3	ATP	19	17	17	17
4	Human serum	0	5	6	3
5	DMEM media	14	8	9	10

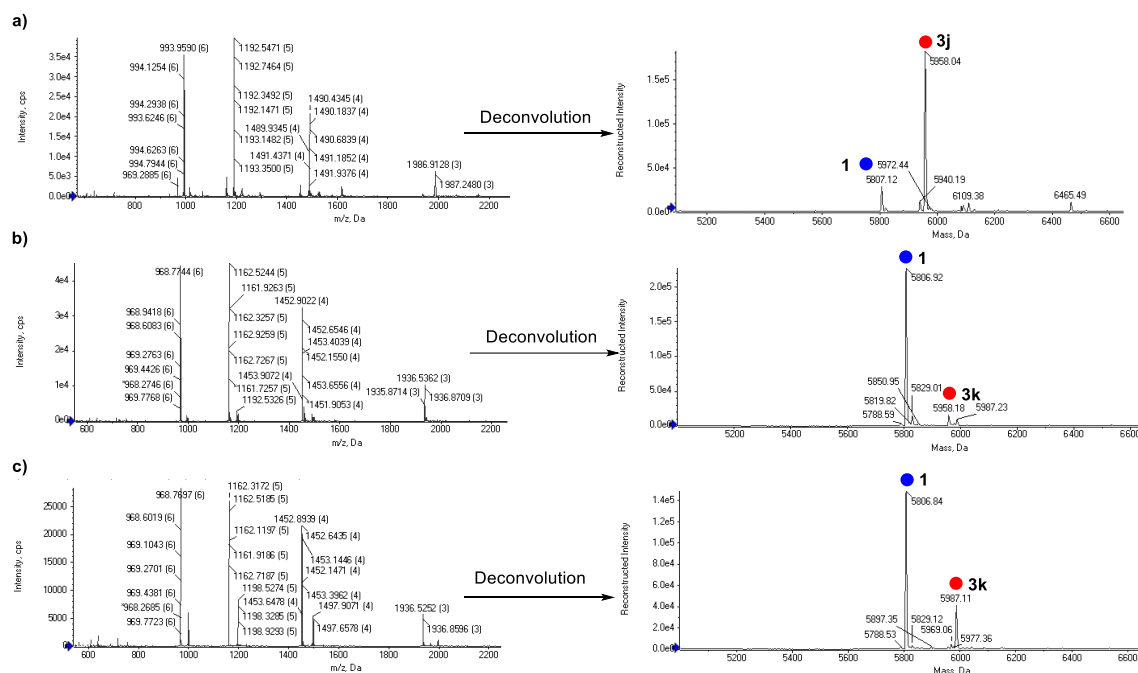
^aFor analytical data of replicates 2 and 3, see the replicate MS data file.



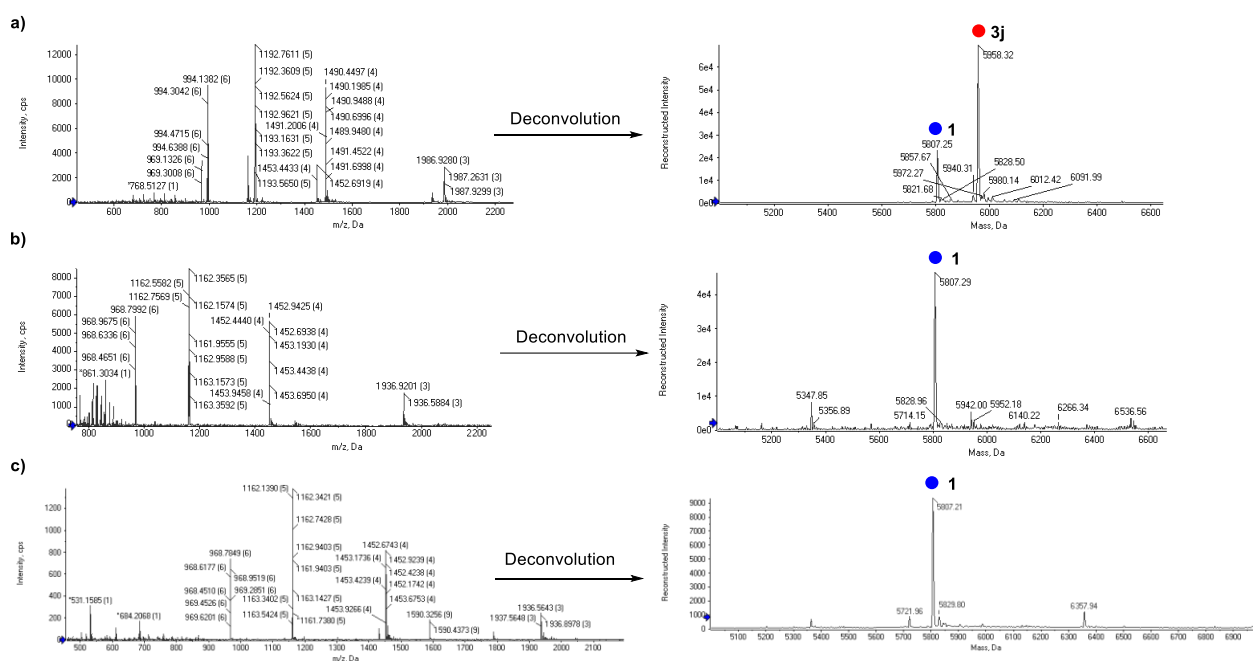
Supplementary Fig. 15. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) without any additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



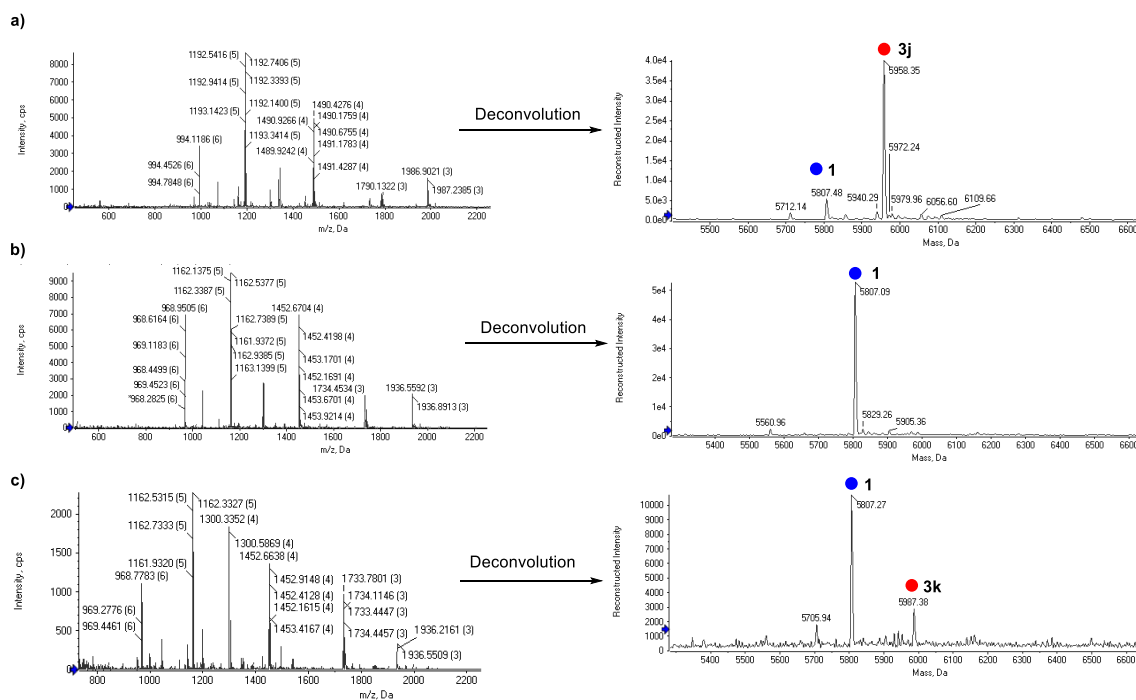
Supplementary Fig. 16. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of ADP (5 mM) as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h



Supplementary Fig. 17. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of ATP (5 mM) as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



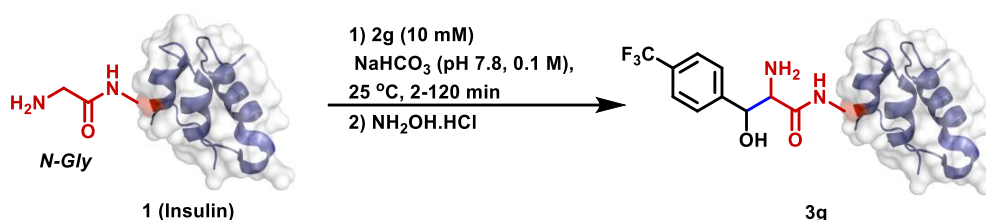
Supplementary Fig. 18. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) in the presence of 10% human serum as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.



Supplementary Fig. 19. Replicate 1: Charged and deconvoluted MS spectra for insulin (1) in the presence of DMEM media as an additive after reaction; (a) with **2j** for 2 h, (b) with **2k** for 2 h, and (c) with **2k** for 24 h.

3.4 Kinetics experiments

Supplementary Table 9. Kinetics experiments: N-Gly bioconjugation of insulin with **2g**.



Entries	Time (min)	% Conversion (3g)			
		Replicate 1	Replicate 2	Replicate 3	Mean
1	2	0	0	0	0
2	5	0	0	0	0
3	10	7	6	6	6
4	15	10	11	13	11
5	20	16	16	13	15
6	25	19	19	16	18
7	30	20	25	20	22
8	35	29	23	22	25
9	40	30	24	25	26
10	45	30	26	30	29
11	50	34	28	32	31

12	55	35	31	35	34
13	60	37	38	35	37
14	75	43	48	43	45
15	90	49	46	51	49
16	120	50	49	42	47

R = Replicate; For replicate 2 and 3, see replicate MS data file.

a) **Calculation of rate constant for pseudo-first-order reaction**

$$\text{Rate} = k [A] [B] = k' [A]$$

where, [A] = concentration of the insulin (**1**)

and [B] = concentration of **2g-2k**

Since B is in excess, $k' = k [B]$

Hence, $\text{Rate} = k' [A]$ under pseudo-first-order reaction conditions

According to integrated rate law for first order reaction:

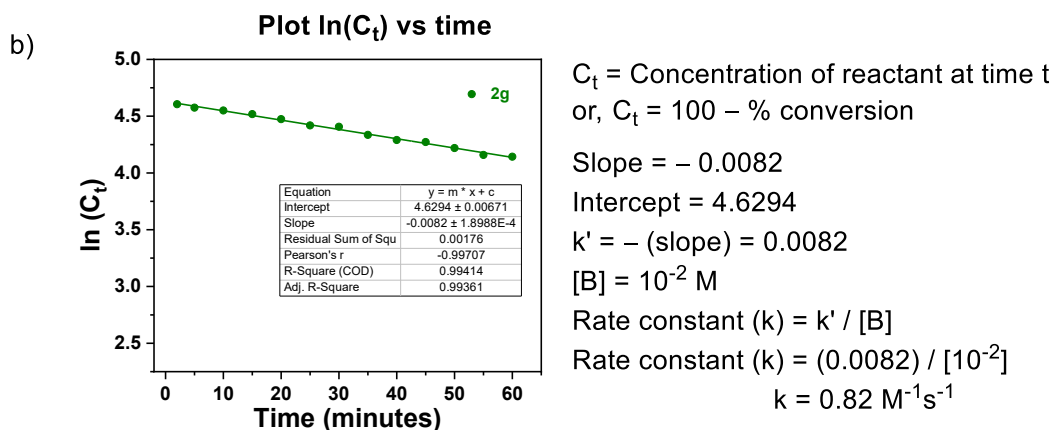
$$\ln(C_t) = -k't + \ln(C_0)$$

In the form of a linear equation, $y = mx + b$;

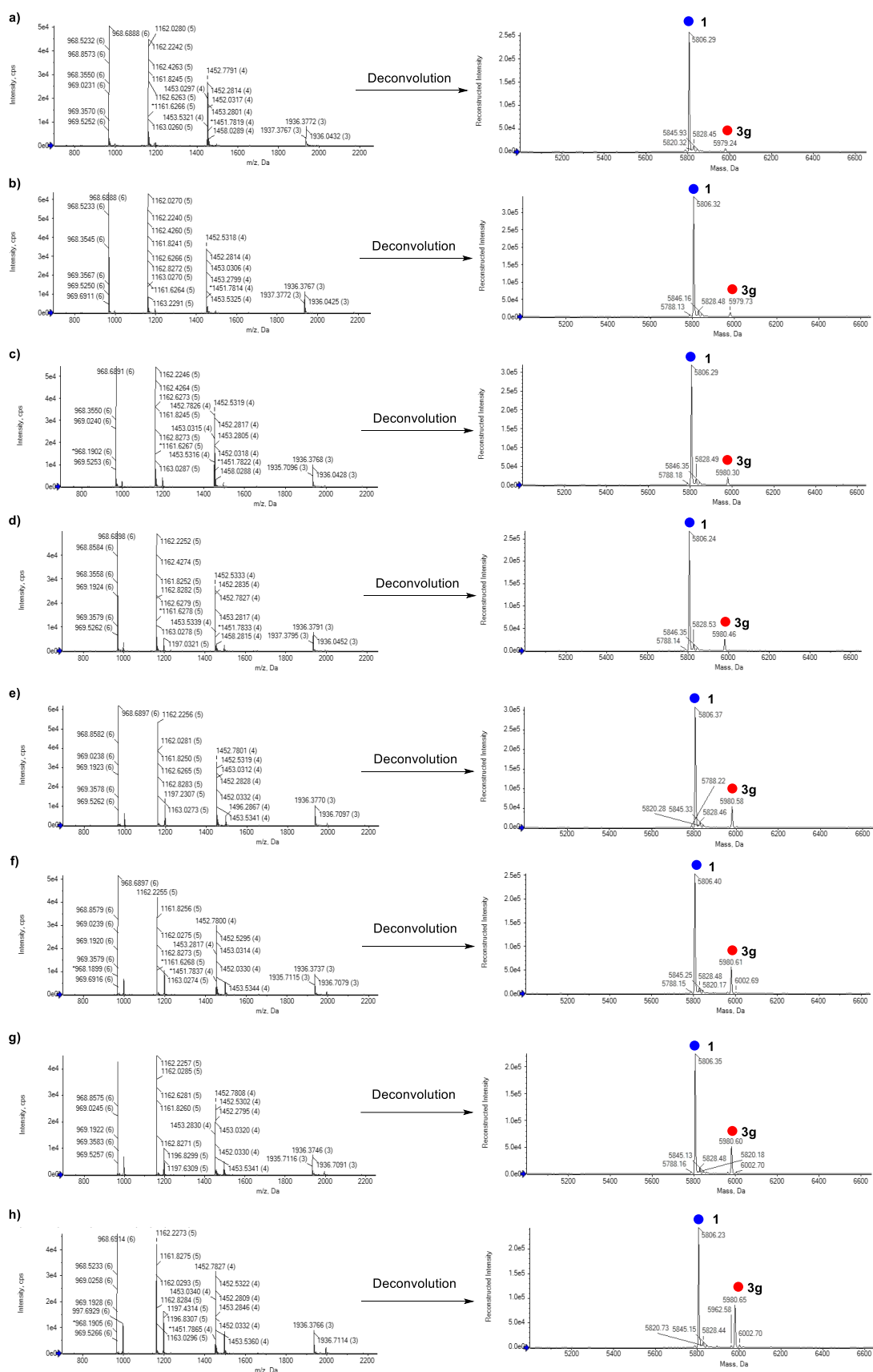
$y = \ln(C_t)$; $x = t$; slope, $m = -k'$; intercept, $b = \ln(C_0)$

Therefore, rate constant, $k' = -m$

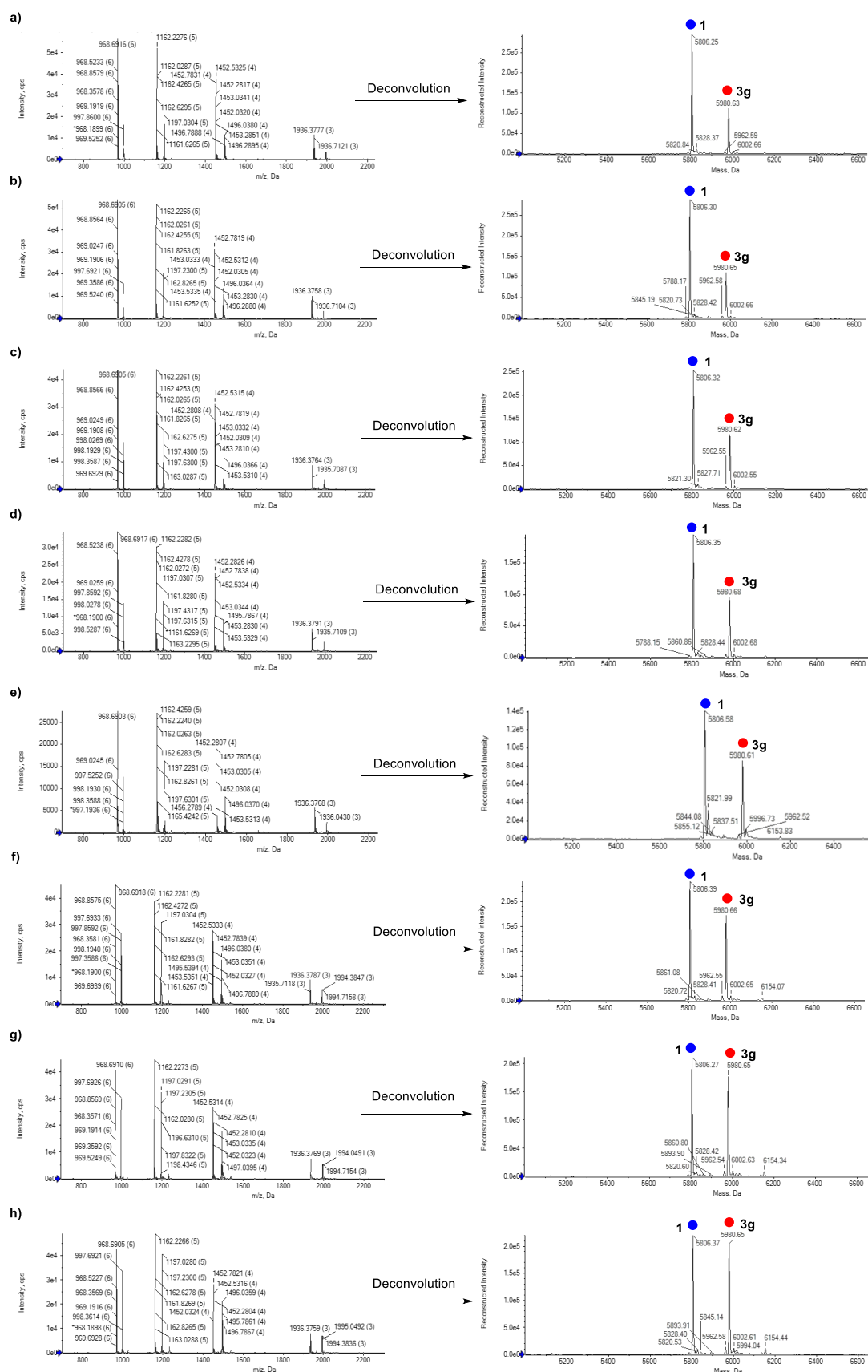
Also, rate constant of pseudo-first-order reaction, $k = k' / [B]$



Supplementary Fig. 20. (a) Calculation of rate constant for N-Gly bioconjugation of insulin (**1**) after reaction with **2g-k**.^{10,11,12} (b) Plot for $\ln(C_t)$, where C_t is % unreacted insulin (**1**), versus time to estimate pseudo-first-order rate constant for the reaction of insulin (**1**) with **2g**.

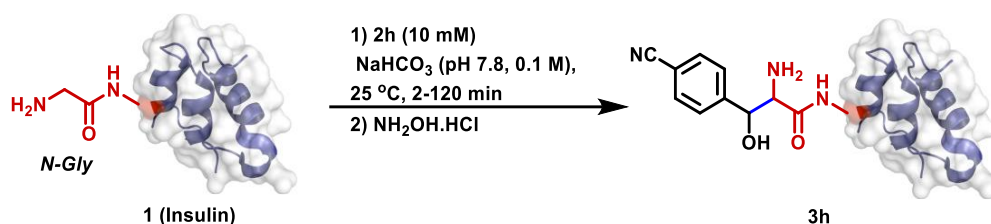


Supplementary Fig. 21. Replicate 1: Charged and deconvoluted MS spectra for insulin (1) after reaction with 2g for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



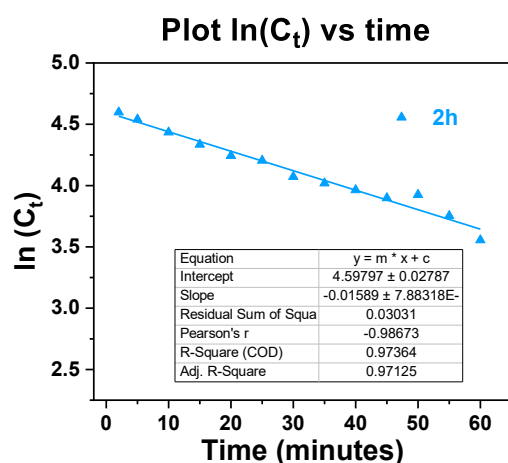
Supplementary Fig. 22. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2g** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

Supplementary Table 10. Kinetics experiments: N-Gly bioconjugation of insulin with **2h**.



Entries	Time (min)	% Conversion (3h)			
		Replicate 1	Replicate 2	Replicate 3	Mean
1	2	2	0	0	1
2	5	9	5	5	6
3	10	19	14	14	16
4	15	25	26	20	24
5	20	32	31	26	30
6	25	42	41	42	42
7	30	45	38	41	41
8	35	49	46	38	44
9	40	55	46	41	47
10	45	57	46	49	51
11	50	56	45	47	49
12	55	62	53	57	57
13	60	68	63	64	65
14	75	75	67	72	71
15	90	76	74	75	75
16	120	82	82	83	82

For replicate 2 and 3, see replicate MS data file.



C_t = Concentration of reactant at time t

$C_t = 100 - \% \text{ conversion}$

Slope = -0.01589

Intercept = 4.59797

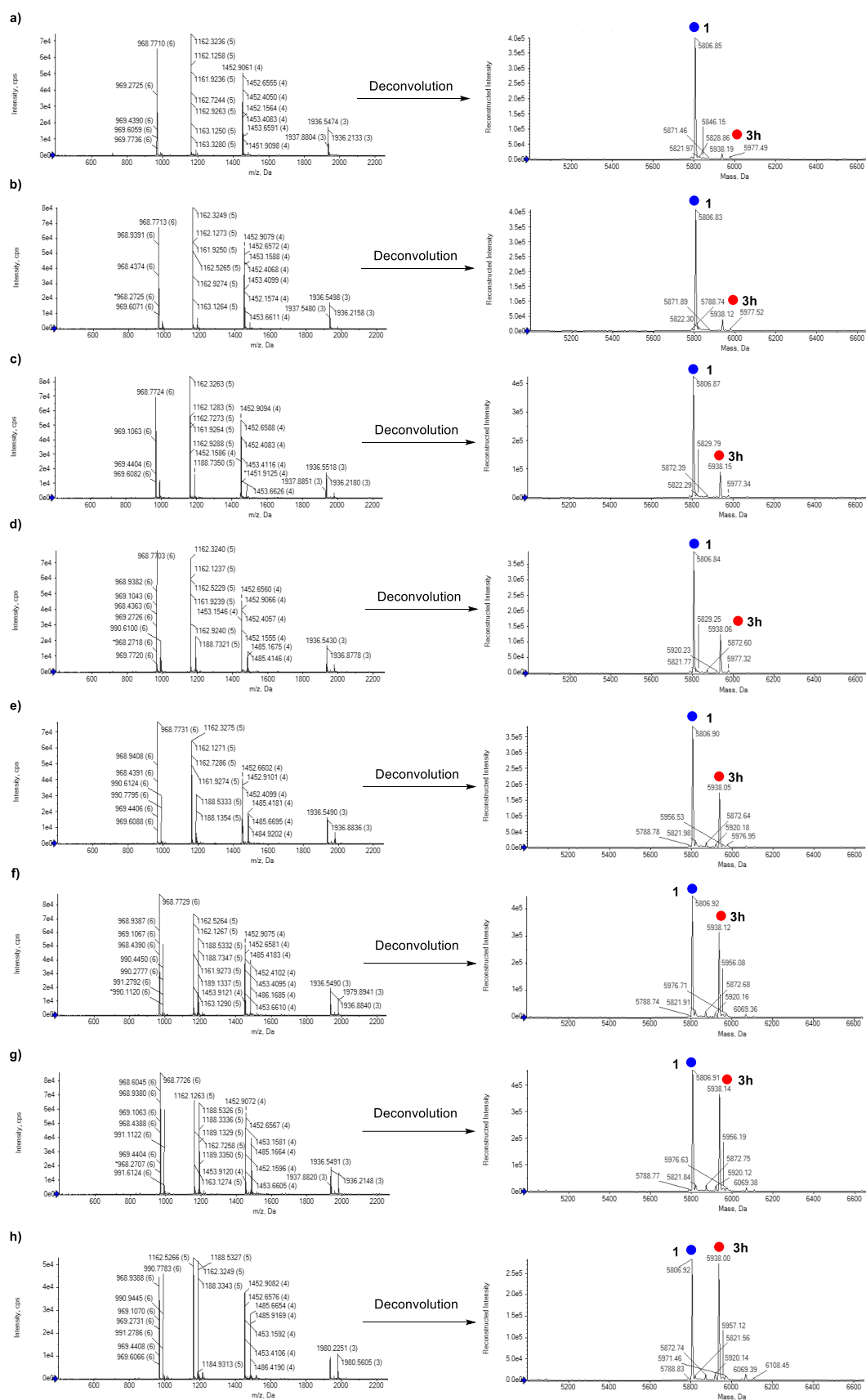
$k' = -(\text{slope}) = 0.01589$

$[\text{B}] = 10^{-2} \text{ M}$

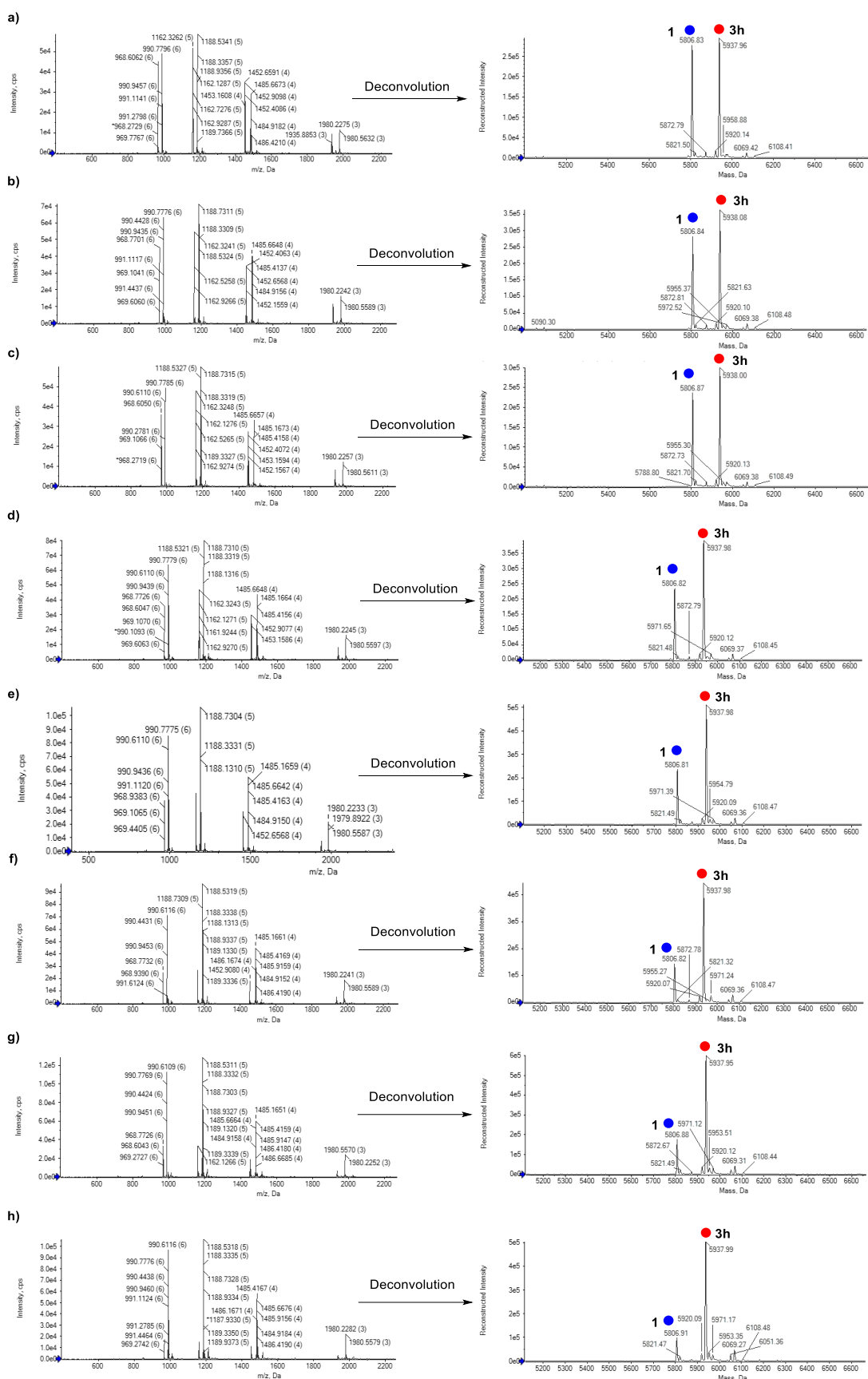
Rate constant (k) = $k' / [\text{B}]$

Rate constant (k) = $(0.01589) / [10^{-2}]$
 $k = 1.589 \text{ M}^{-1}\text{s}^{-1}$

Supplementary Fig. 23. Plot for $\ln(C_t)$, where C_t is % unreacted insulin (**1**), versus time to estimate pseudo-first-order rate constant for reaction of insulin **1** with **2h**.

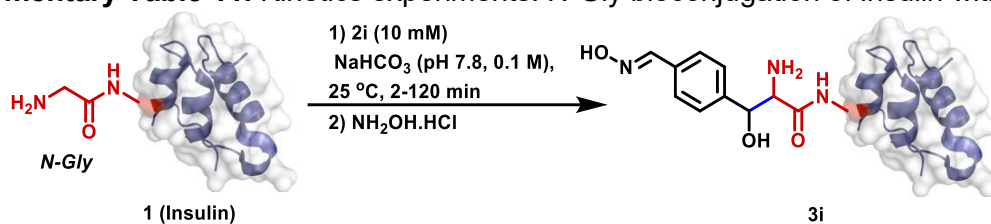


Supplementary Fig. 24. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2h** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



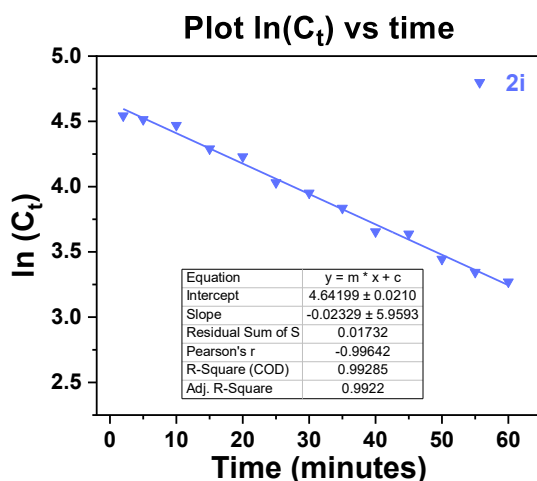
Supplementary Fig. 25. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2h** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

Supplementary Table 11. Kinetics experiments: N-Gly bioconjugation of insulin with **2i**.



Entries	Time (min)	% Conversion (3i)			
		Replicate 1	Replicate 2	Replicate 3	Mean
1	2	6	7	6	6
2	5	15	10	9	11
3	10	20	15	13	16
4	15	33	31	26	30
5	20	41	34	32	36
6	25	47	46	40	44
7	30	57	54	46	52
8	35	55	52	51	53
9	40	64	63	61	63
10	45	67	65	62	65
11	50	72	68	71	70
12	55	72	73	74	73
13	60	75	73	76	75
14	75	80	81	79	80
15	90	83	83	83	83
16	120	86	87	86	86

For replicate 2 and 3, see replicate MS data file.



C_t = Concentration of reactant at time t
 $C_t = 100 - \% \text{ conversion}$

Slope = -0.02329

Intercept = 4.64199

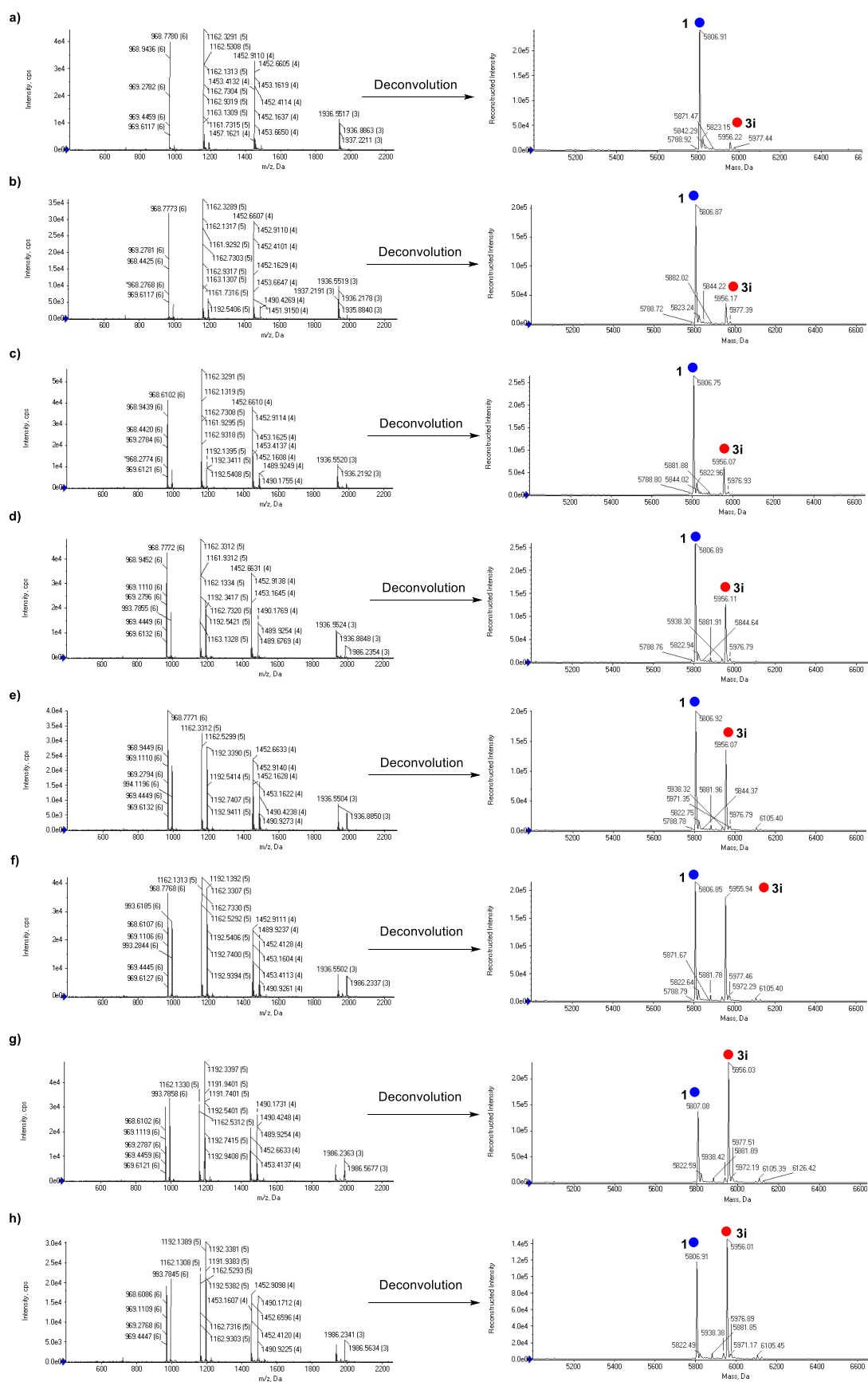
$k' = -(\text{slope}) = 0.02329$

$[B] = 10^{-2} \text{ M}$

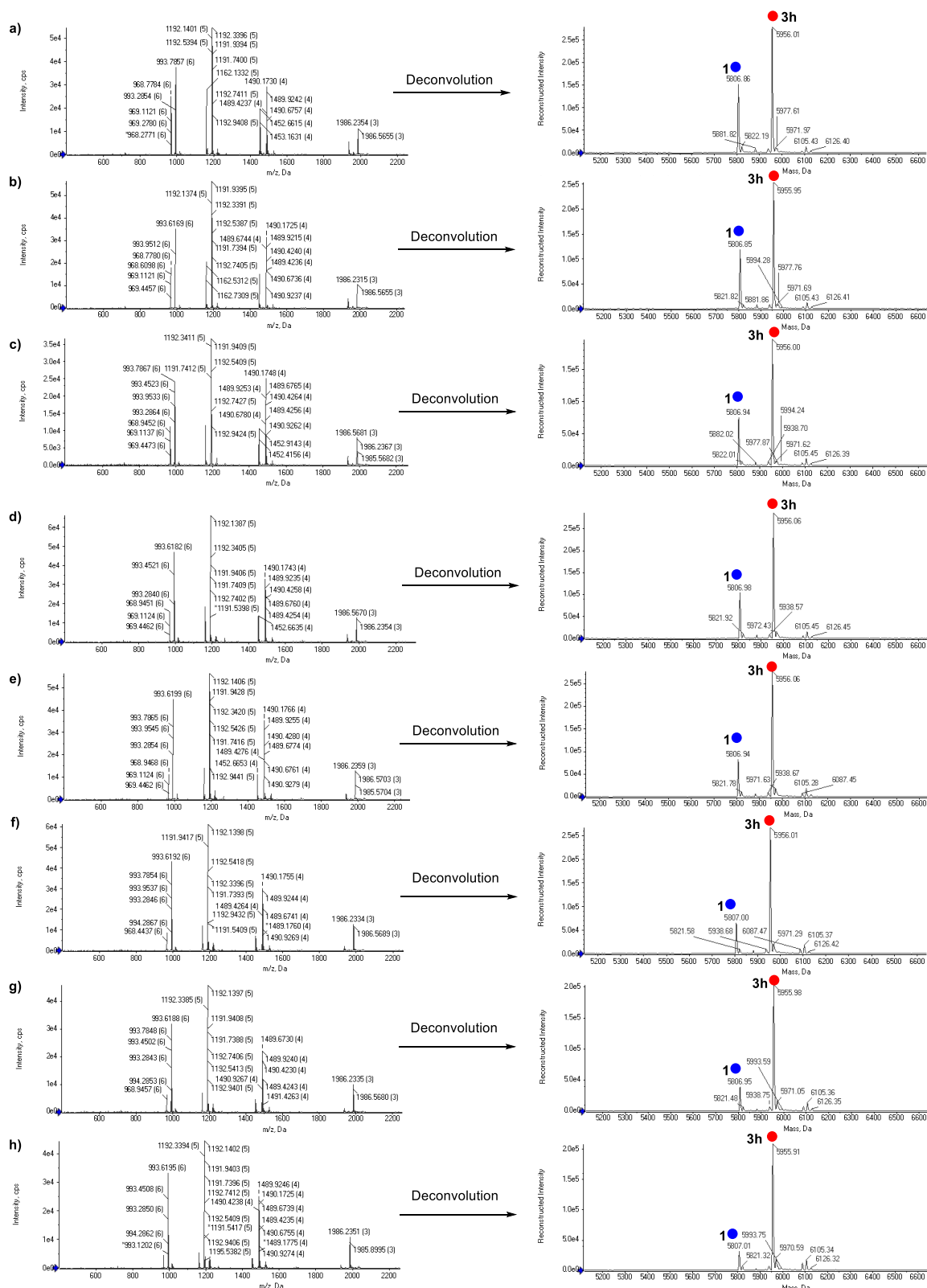
Rate constant (k) = $k' / [B]$

Rate constant (k) = $(0.02329) / [10^{-2}]$
 $k = 2.329 \text{ M}^{-1}\text{s}^{-1}$

Supplementary Fig. 26. Plot for $\ln(C_t)$, where C_t is % unreacted insulin (**1**), versus time to estimate pseudo-first-order rate constant for reaction of insulin **1** with **2i**.

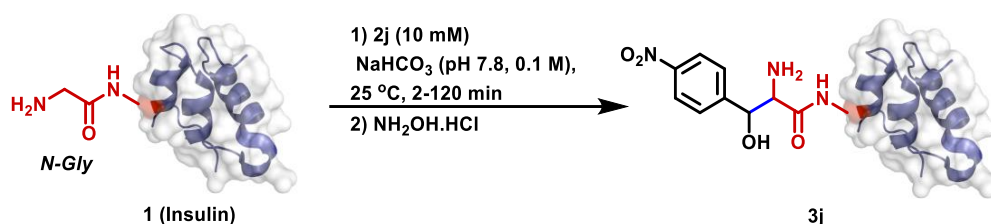


Supplementary Fig. 27. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2i** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.



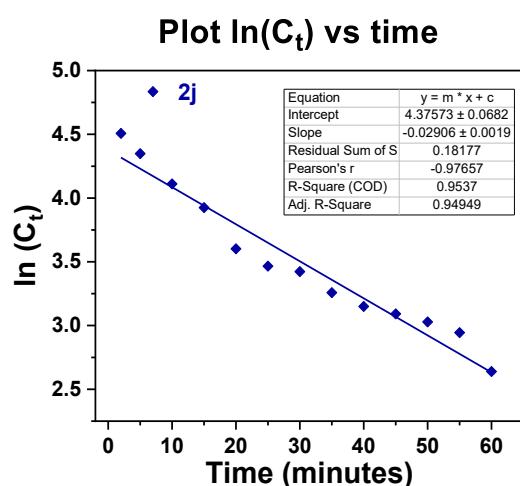
Supplementary Fig. 28. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2i** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

Supplementary Table 12. Kinetics experiments: N-Gly bioconjugation of insulin with **2j**.



Entries	Time (min)	% Conversion (3j)			
		Replicate 1	Replicate 2	Replicate 3	Mean
1	2	11	9	8	9
2	5	22	24	22	23
3	10	40	40	31	37
4	15	46	50	48	48
5	20	60	64	63	62
6	25	68	68	68	68
7	30	69	68	69	69
8	35	75	76	74	75
9	40	78	77	76	77
10	45	80	75	76	77
11	50	82	78	80	80
12	55	80	81	82	81
13	60	86	86	86	86
14	75	88	89	86	88
15	90	83	87	88	86
16	120	88	86	89	88

For replicate 2 and 3, see replicate MS data file.



C_t = Concentration of reactant at time t

C_t = 100 – % conversion

Slope = – 0.02906

Intercept = 4.37573

$K' = -(\text{slope}) = 0.02906$

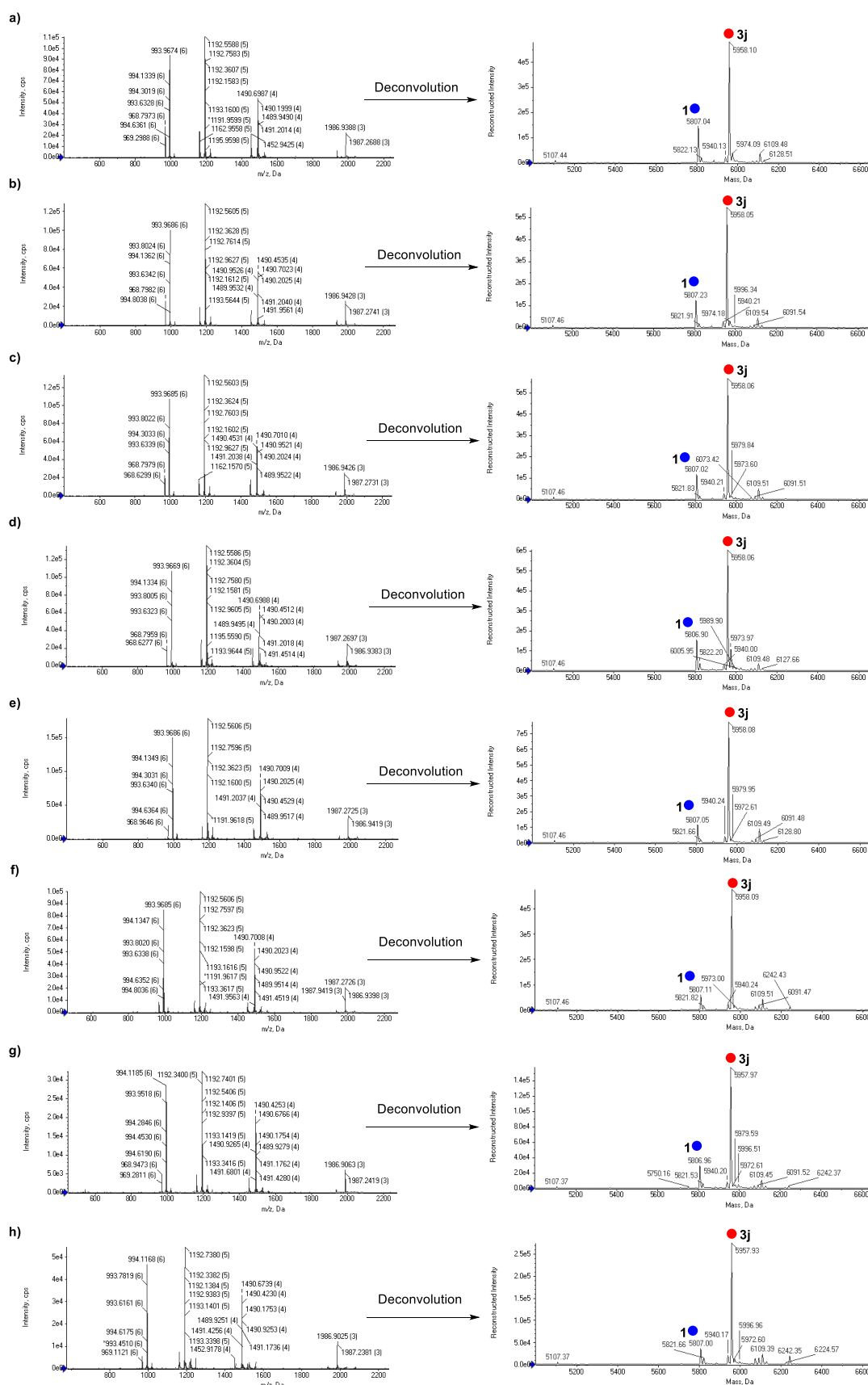
$[B] = 10^{-2} \text{ M}$

Rate constant (k) = $k' / [B]$

Rate constant (k) = $(0.02906) / [10^{-2}]$

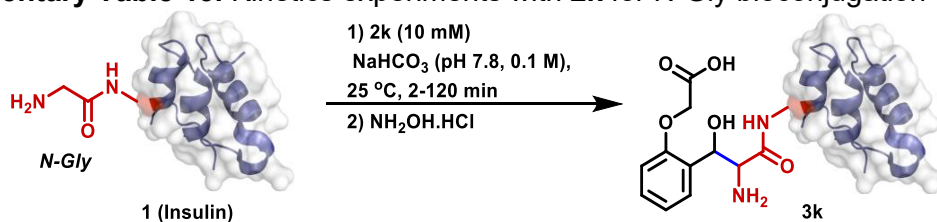
$k = 2.906 \text{ M}^{-1}\text{s}^{-1}$

Supplementary Fig. 29. Plot for $\ln(C_t)$, where C_t is % unreacted insulin (**1**), versus time to estimate pseudo first-order-rate constant for reaction of insulin **1** with **2j**.



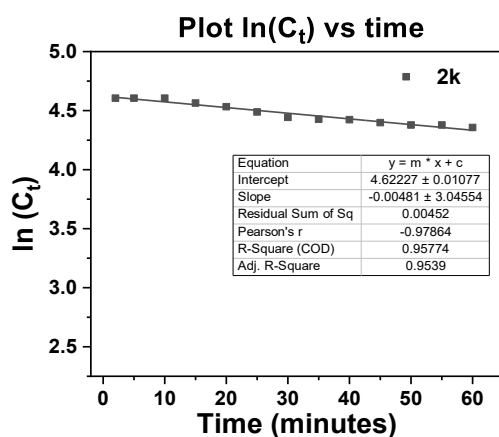
Supplementary Fig. 31. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2j** for kinetics at different time intervals (40-120 min). (a) 40 min, (b) 45 min, (c) 50 min, (d) 55 min, (e) 60 min, (f) 75 min, (g) 90 min, and (h) 120 min.

Supplementary Table 13. Kinetics experiments with **2k** for N-Gly bioconjugation of insulin.



Entries	Time (min)	% Conversion (3k)			
		Replicate 1	Replicate 2	Replicate 3	Mean
1	2	0	0	0	0
2	5	0	0	0	0
3	10	1	0	0	0
4	15	3	0	0	1
5	20	7	8	6	7
6	25	11	12	0	8
7	30	16	14	15	15
8	35	17	16	16	16
9	40	18	8	15	14
10	45	20	18	14	17
11	50	15	21	20	19
12	55	19	16	12	16
13	60	11	17	12	13
14	75	12	25	19	19
15	90	26	26	24	25
16	120	31	35	27	31

For replicate 2 and 3, see replicate MS data file.



C_t = Concentration of reactant at time t

$C_t = 100 - \% \text{ conversion}$

Slope = - 0.00481

Intercept = 4.62227

$k' = - (\text{slope}) = 0.00481$

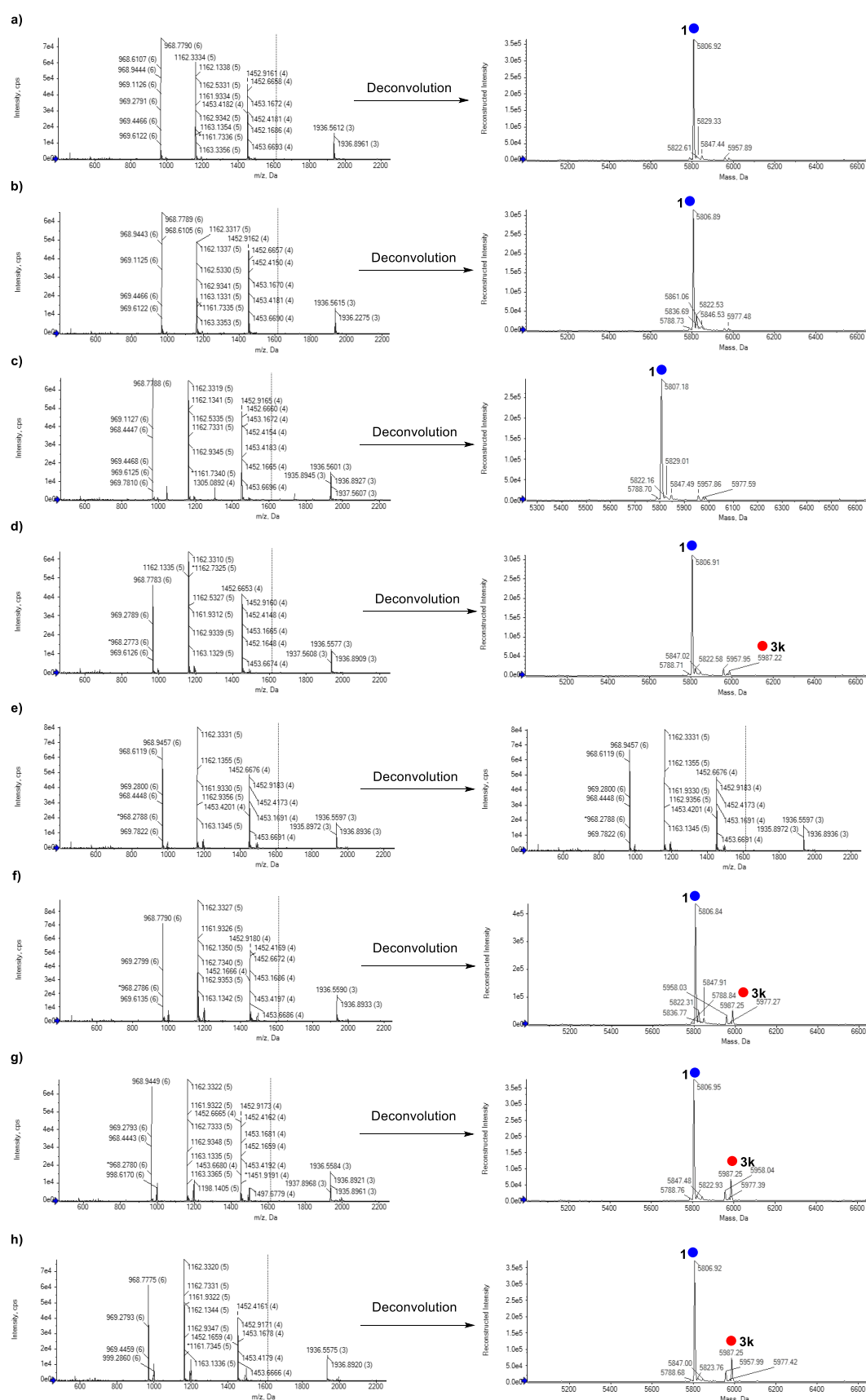
$[B] = 10^{-2} \text{ M}$

Rate constant (k) = $k' / [B]$

Rate constant (k) = $(0.00481) / [10^{-2}]$

$k = 0.481 \text{ M}^{-1}\text{s}^{-1}$

Supplementary Fig. 32. Plot for $\ln(C_t)$, where C_t is % unreacted insulin (**1**), versus time to estimate pseudo-first-order rate constant for reaction of insulin **1** with **2k**.



Supplementary Fig. 33. Replicate 1: Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2k** for kinetics at different time intervals (2-35 min). (a) 2 min, (b) 5 min, (c) 10 min, (d) 15 min, (e) 20 min, (f) 25 min, (g) 30 min, and (h) 35 min.

3.5 Stability of bioconjugate

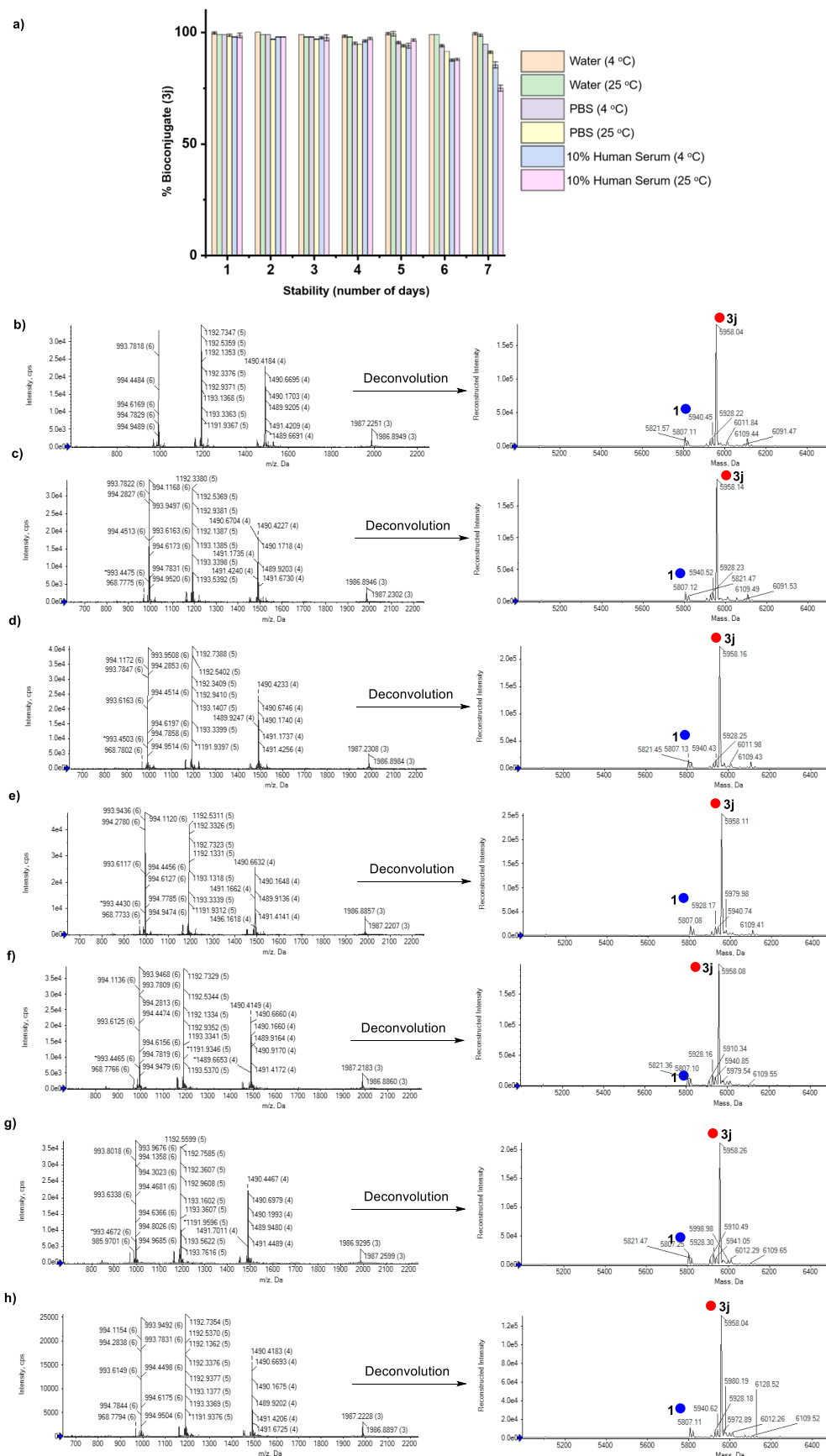
Supplementary Table 14. Stability of bioconjugate **3j**.

Day	% Conversion (3j)							
	Water							
	4 °C				25 °C			
	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	93	93	92	93	92	92	92	92
2	93	93	93	93	92	92	92	92
3	92	92	92	92	91	91	91	91
4	92	91	91	91	91	91	91	91
5	92	93	92	92	91	93	93	91
6	92	92	92	92	92	92	92	92
7	92	92	93	92	91	92	92	91

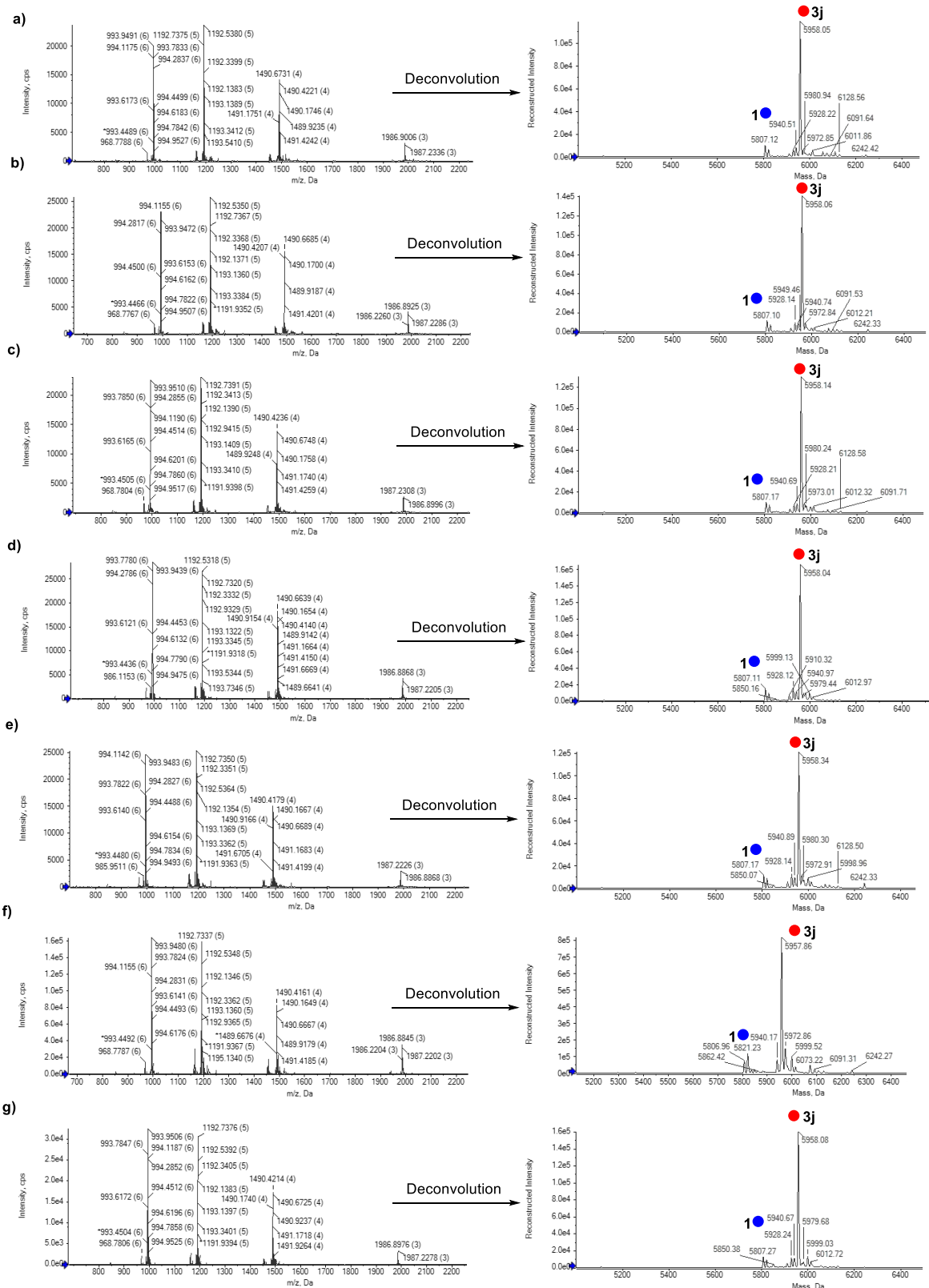
Day	% Conversion (3j)							
	PBS							
	4 °C				25 °C			
	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	92	92	92	92	92	92	91	91
2	92	92	92	92	90	90	90	90
3	91	91	91	91	90	90	90	90
4	89	88	88	88	88	88	88	88
5	89	89	88	88	88	87	87	87
6	88	87	87	87	85	85	85	85
7	88	88	88	88	85	85	84	84

Day	% Conversion (3j)							
	10% Human serum							
	4 °C				25 °C			
	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean	Replicate 1	Replicate 2 ^a	Replicate 3 ^a	Mean
1	91	91	91	91	91	91	93	91
2	91	91	91	91	91	91	91	91
3	90	91	91	90	92	91	89	90
4	90	89	89	89	90	90	91	90
5	86	88	88	87	90	90	89	89
6	81	81	82	81	81	82	82	81
7	79	78	81	79	68	70	71	69

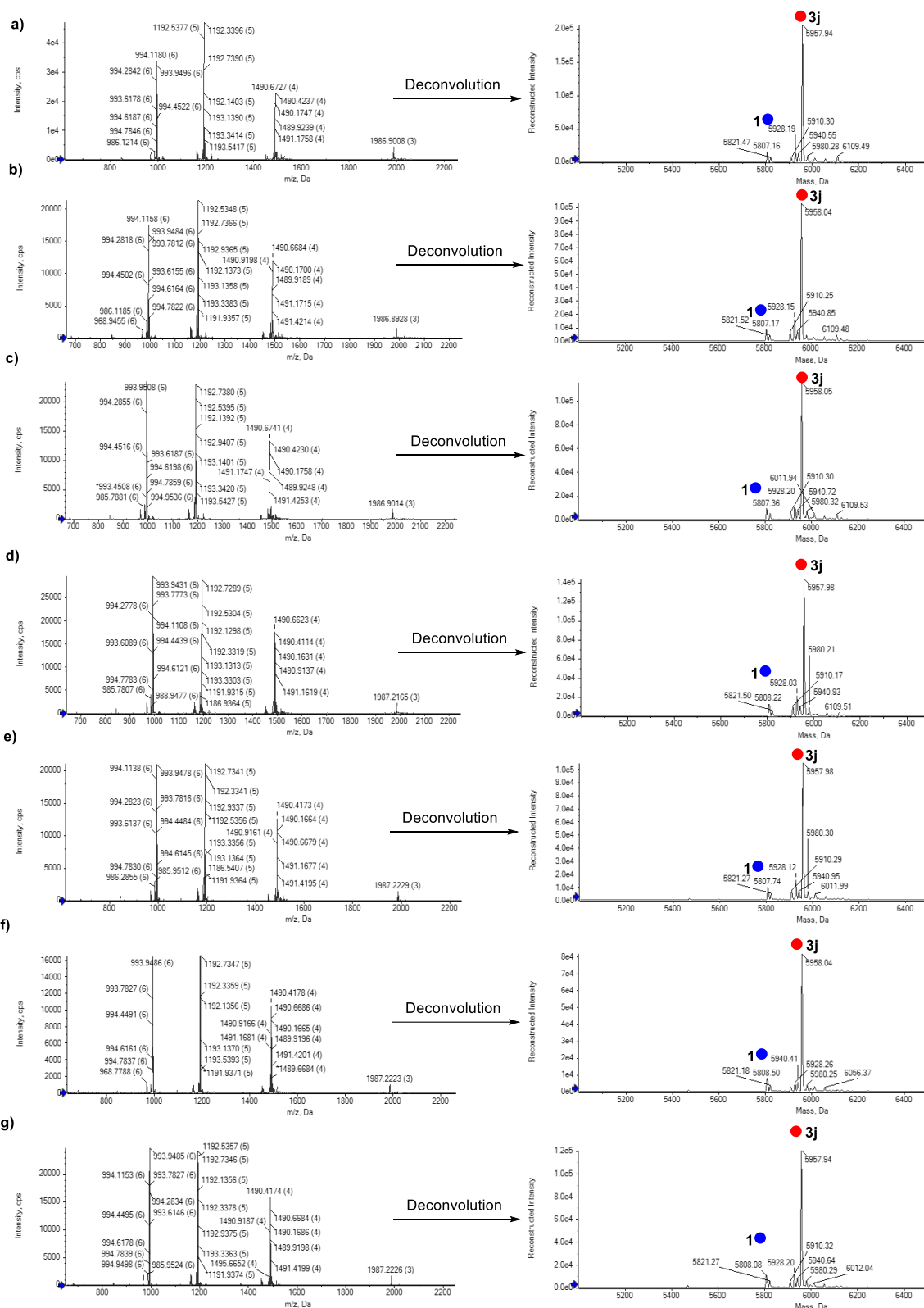
^aFor analytical data of replicates 2 and 3, see the replicate MS data file.



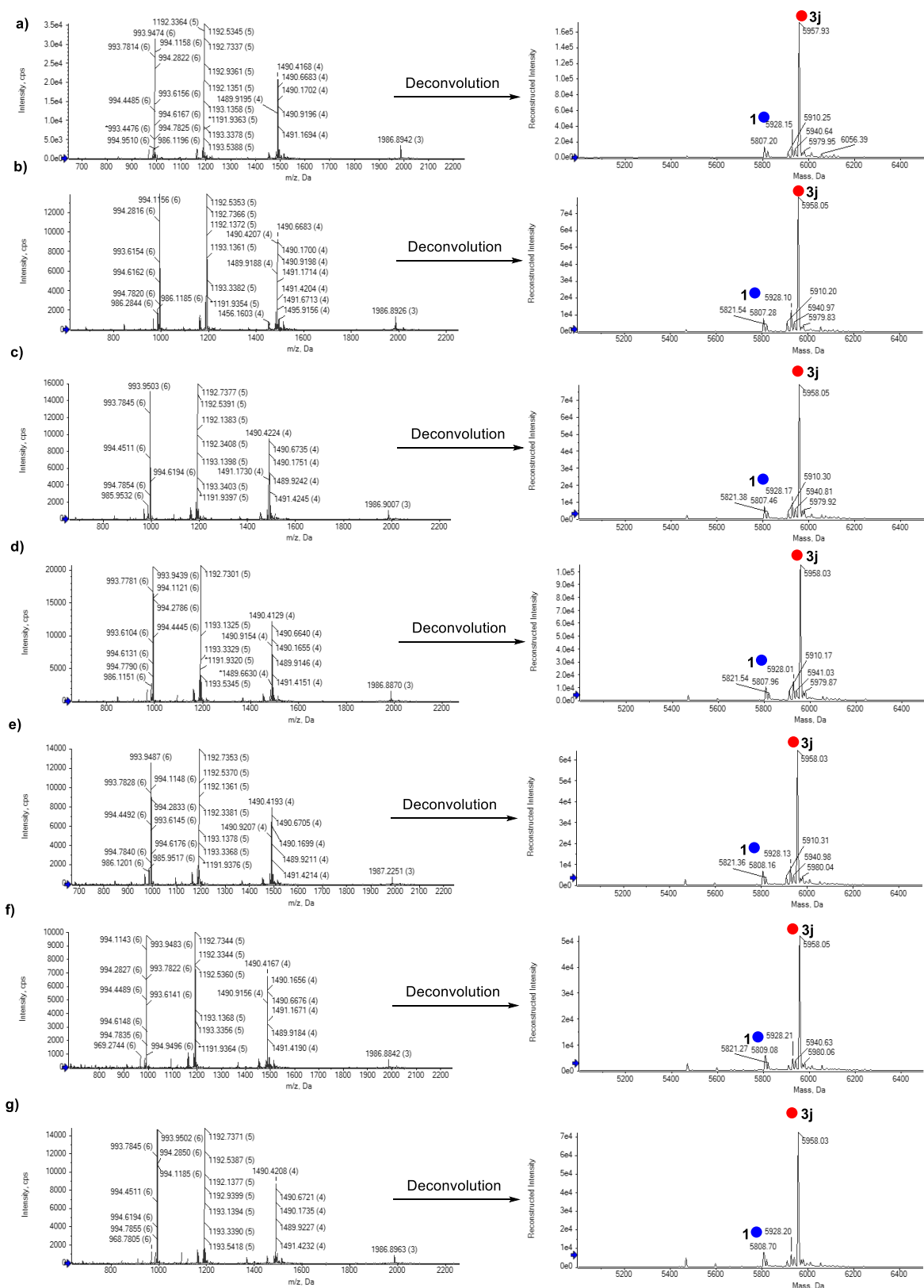
Supplementary Fig. 35. (a) Bar plot of combined bioconjugate stability data. Replicate 1: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in water at 4 °C for (b) day 1, (c) day 2, (d) day 3, (e) day 4, (f) day 5, (g) day 6, and (h) day 7.



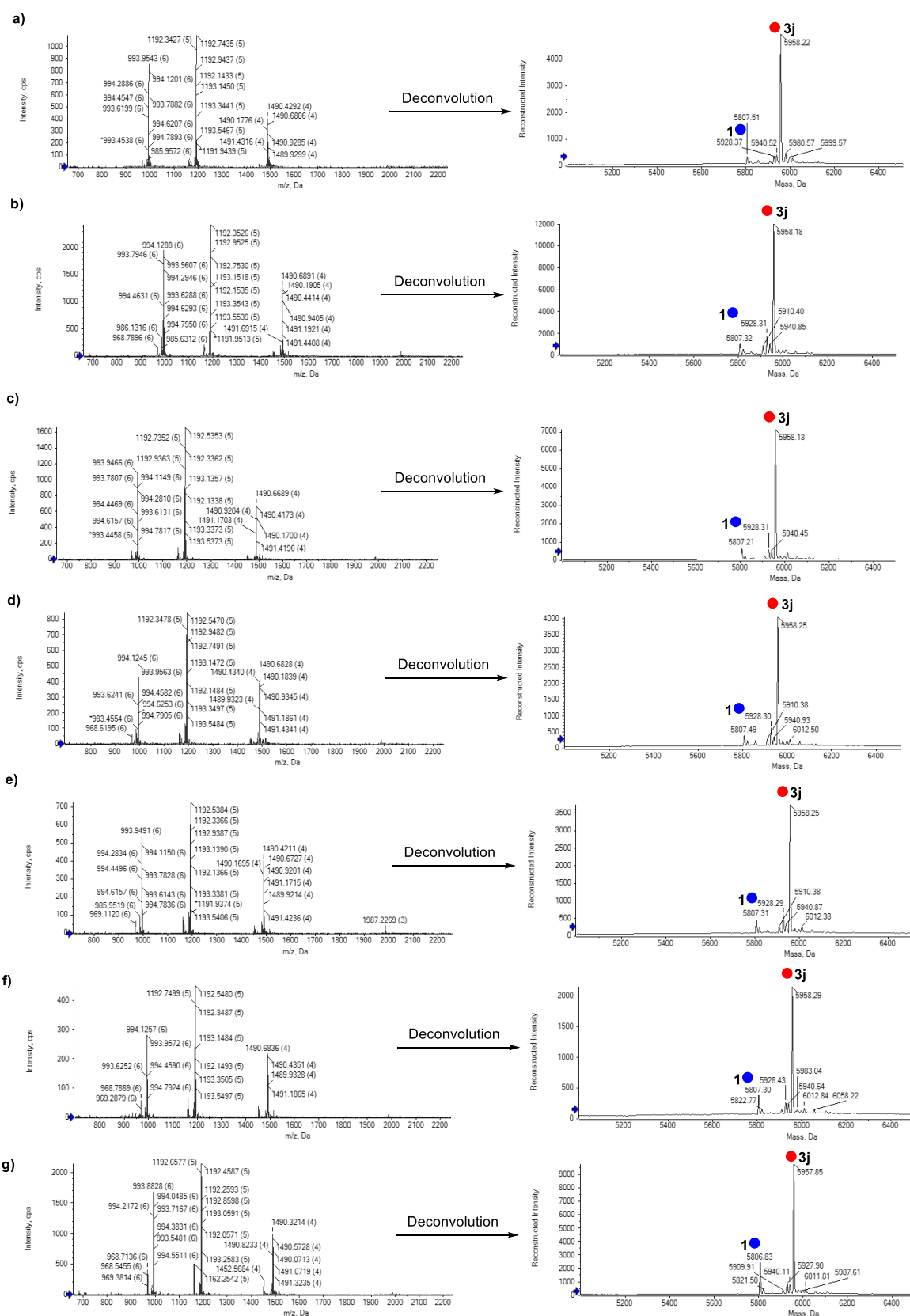
Supplementary Fig. 36. Replicate 1: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in water at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



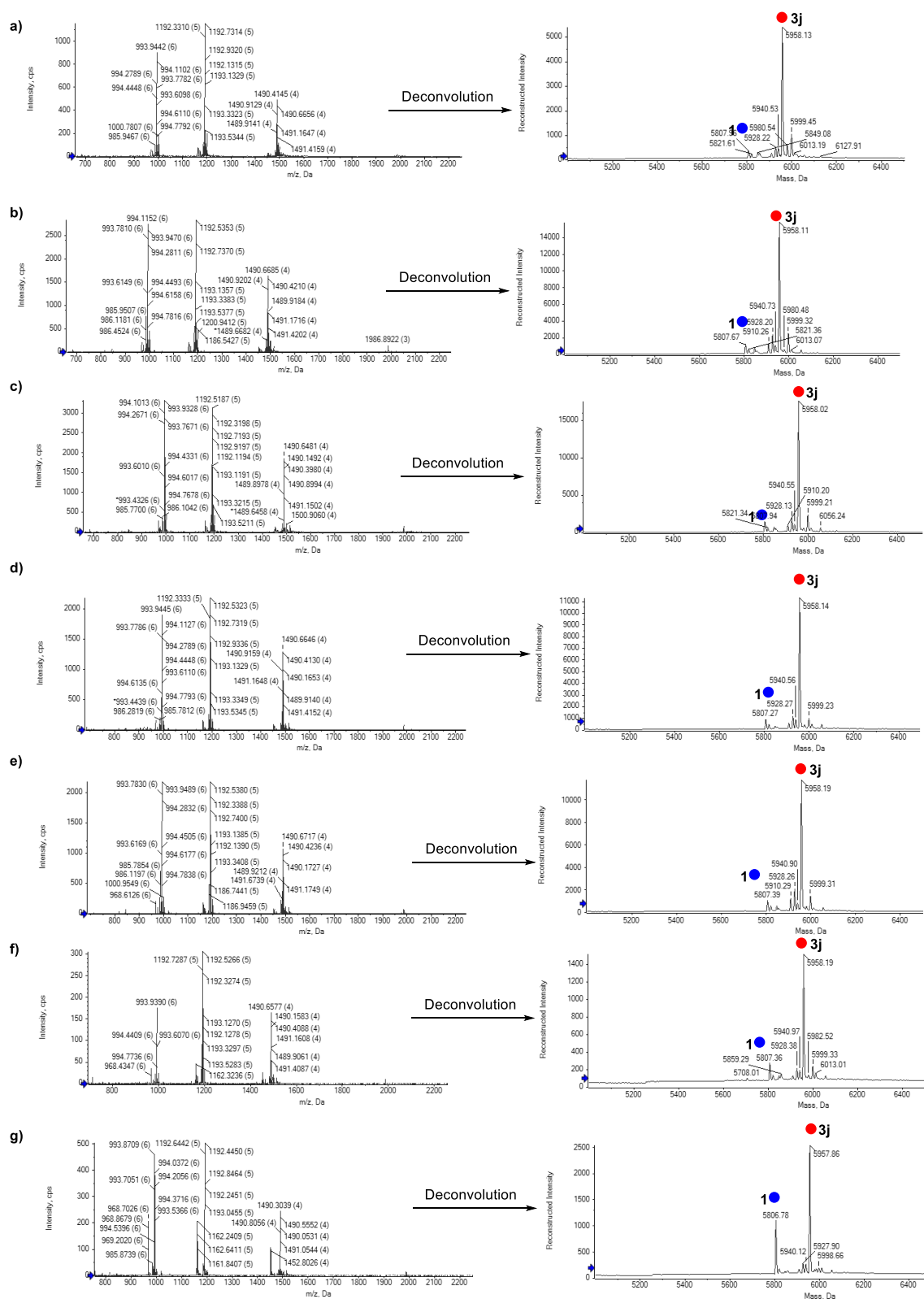
Supplementary Fig. 37. Replicate 1: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in PBS at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



Supplementary Fig. 38. Replicate 1: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in PBS at 25 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



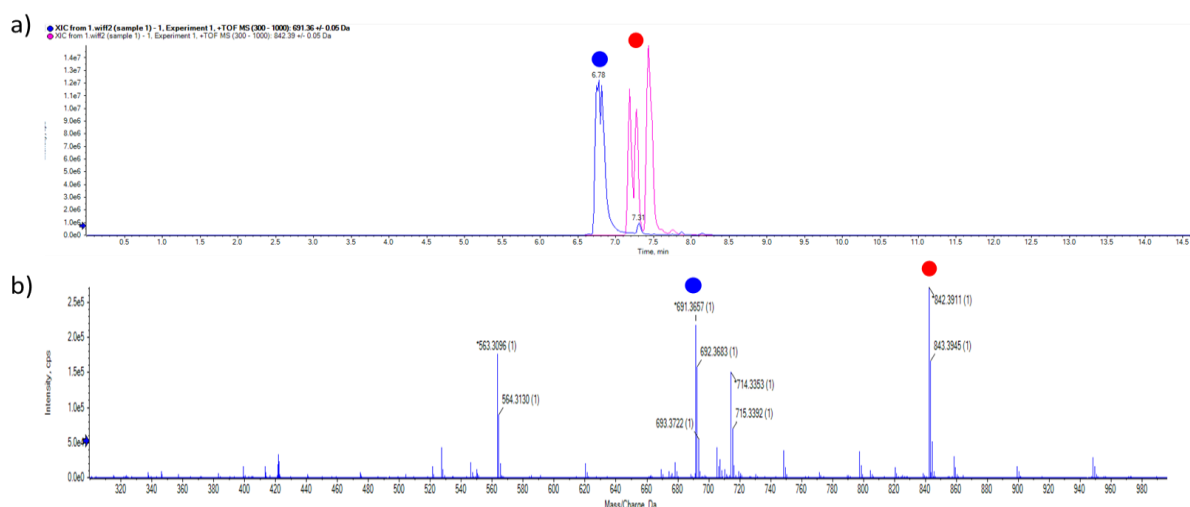
Supplementary Fig. 39. Replicate 1: Charged and deconvoluted MS spectra for stability of insulin bioconjugate **3j** in 10% human serum at 4 °C for (a) day 1, (b) day 2, (c) day 3, (d) day 4, (e) day 5, (f) day 6, and (g) day 7.



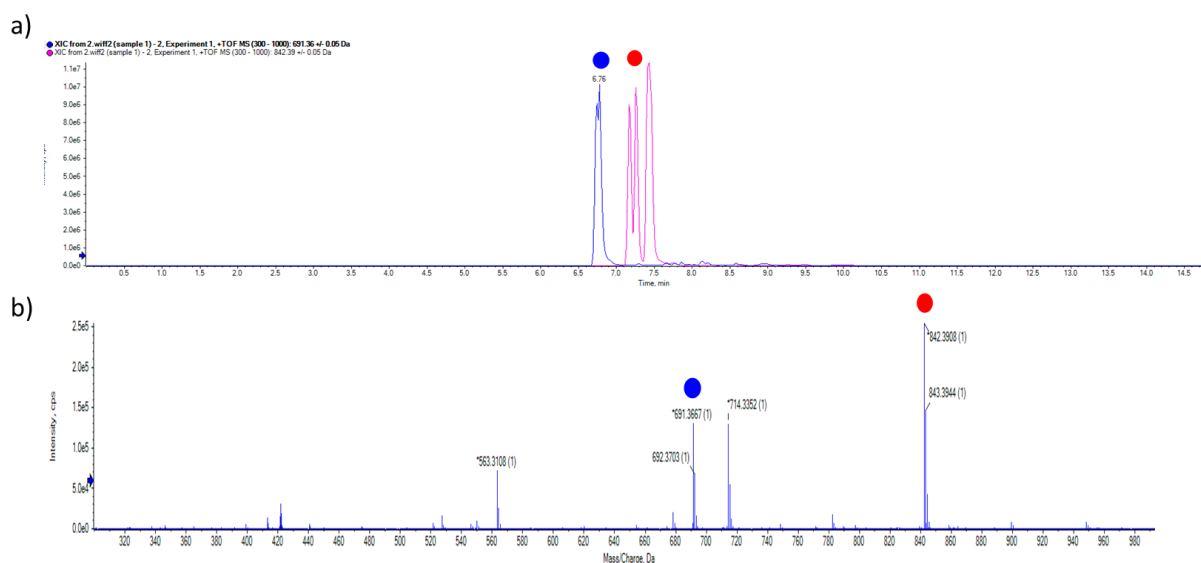
3.6 Residue specificity with peptides

Supplementary Table 15. Optimization of reaction attributes.

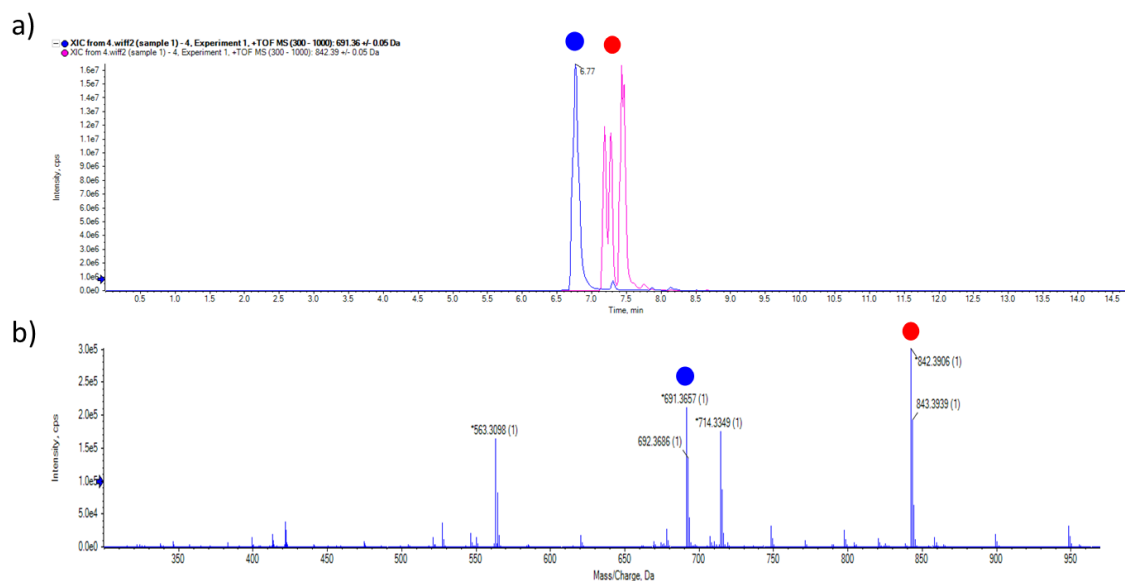
<chem>N[C@@H](C(=O)I[V]E[Q]F)C(=O)O</chem> 4a + 1. 2j (25-50 mM) NaHCO₃ buffer (0.1 M, pH 7.8) 500 μM, 25 °C, 2-4 h 2. NH₂OH, 25 °C, 1 h <chem>N[C@@H](C(=O)I[V]E[Q]F)C(=O)Oc1ccc([N+](=O)[O-])cc1</chem> 5a		
2j (mM)	% Conversion (5a)	
	Time (2 h)	Time (4 h)
25	58	70
37.5	63	77
50	66	84



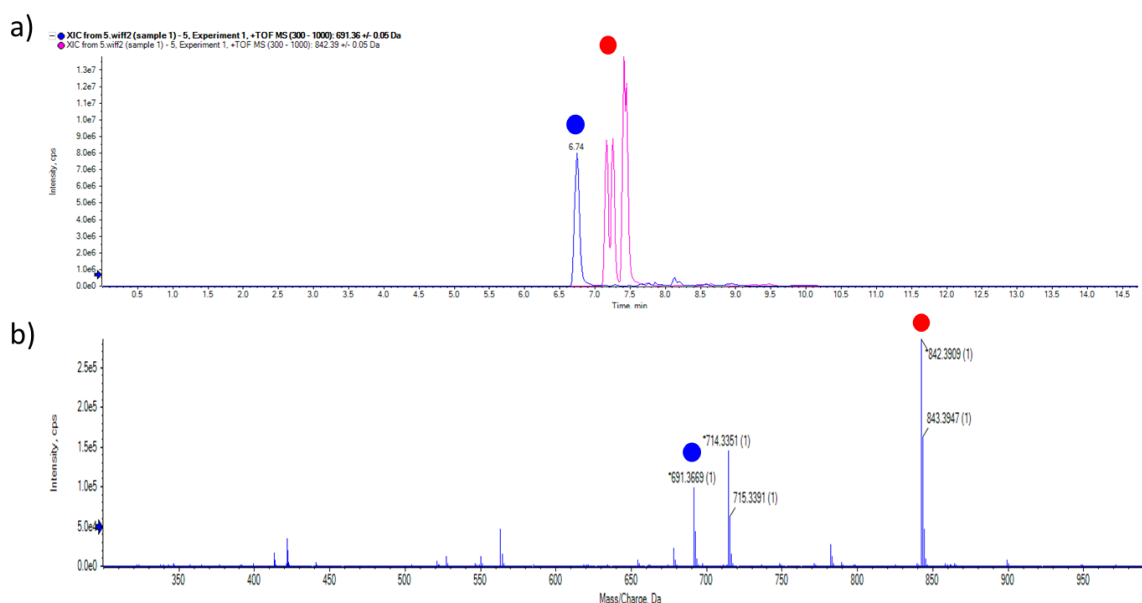
Supplementary Fig. 41. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (25 mM) for 2 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**.



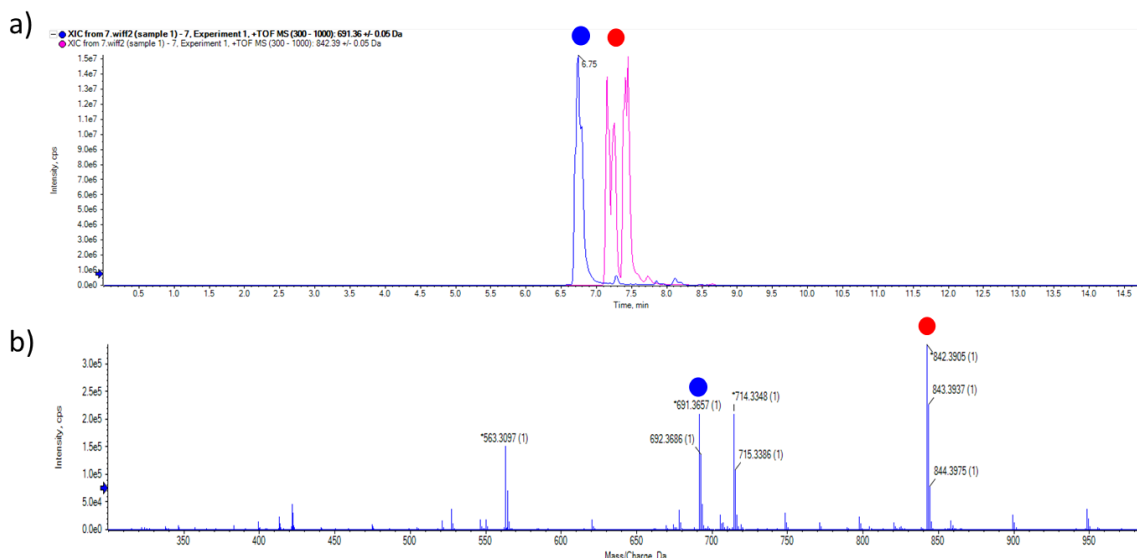
Supplementary Fig. 42. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (25 mM) for 4 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**.



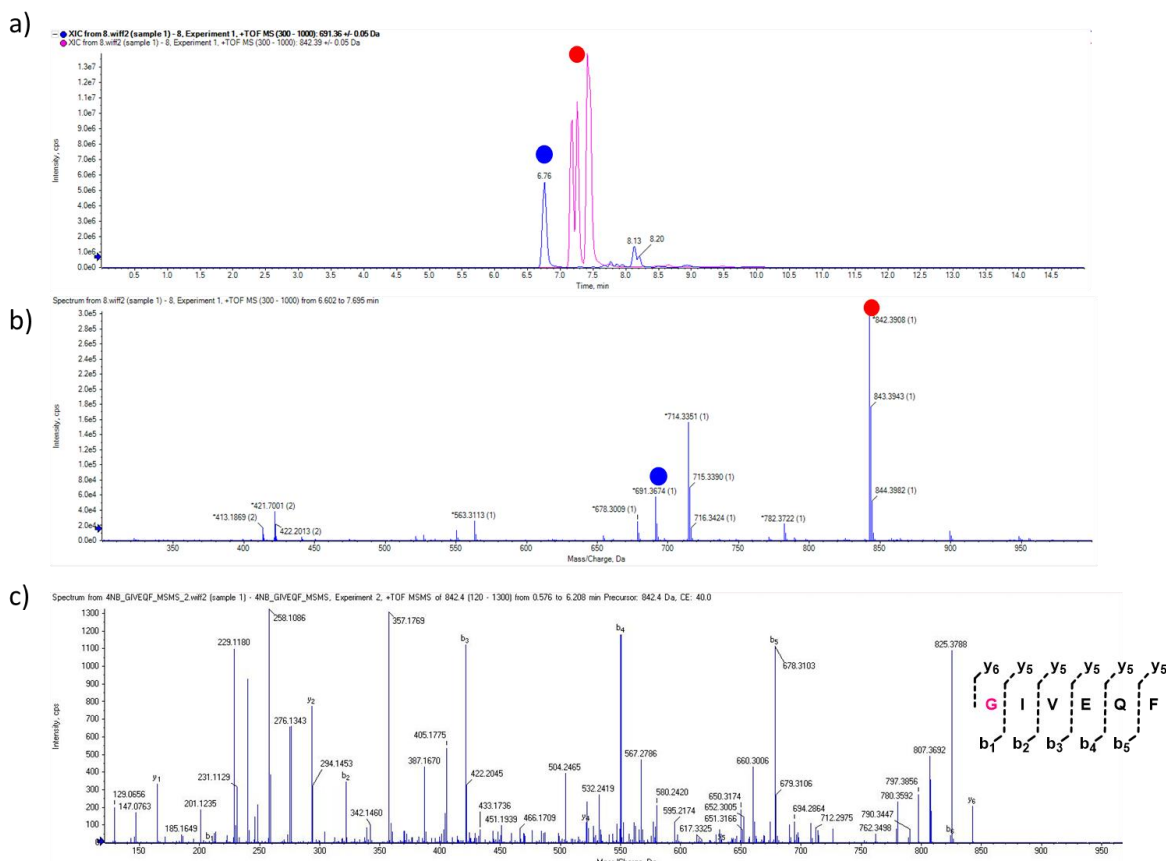
Supplementary Fig. 43. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (37.5 mM) for 2 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**.



Supplementary Fig. 44. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (37.5 mM) for 4 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**.

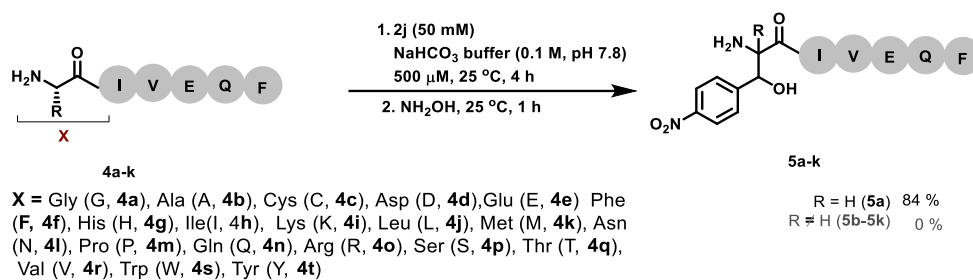


Supplementary Fig. 45. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (50 mM) for 2 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**.



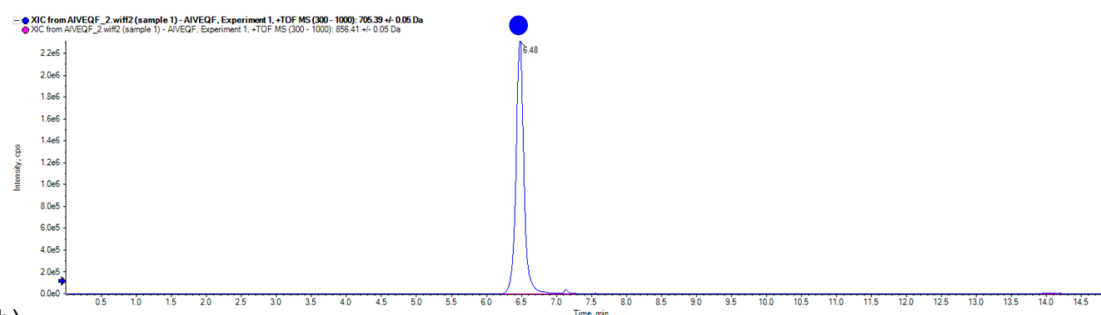
Supplementary Fig. 46. LC-MS analysis for modification of GIVEQF (**4a**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4a** (m/z 691.36) and modified peptide **5a** (m/z 842.39). (b) MS data for peptides **4a** and **5a**. (c) MS-MS of labeled peptide **5a** confirms the site of modification, N-Gly (G1).

Supplementary Table 16. Residue-specific reaction of **2j** with peptide XIVEQF.

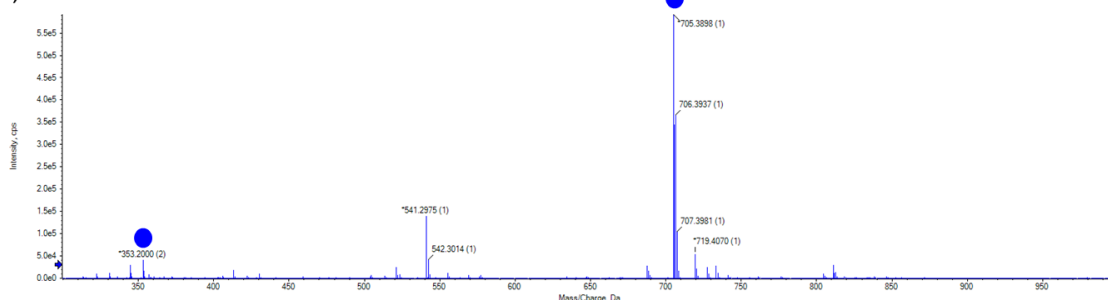


Entries	Peptide sequence (XIVEQF)	% Conversion to 5
1	AIVEQF (4b)	0
2	CIVEQF (4c)	0
3	DIVEQF (4d)	0
4	EIVEQF (4e)	0
5	FIVEQF (4f)	0
6	GIVEQF (4a)	84
7	HIVEQF (4g)	0
8	IIVEQF (4h)	0
9	KIVEQF (4i)	0
10	LIVEQF (4j)	0
11	MIVEQF (4k)	0
12	NIVEQF (4l)	0
13	PIVEQF (4m)	0
14	QIVEQF (4n)	0
15	RIVEQF (4o)	0
16	SIVEQF (4p)	0
17	TIVEQF (4q)	0
18	VIVEQF (4r)	0
19	WIVEQF (4s)	0
20	YIVEQF (4t)	0

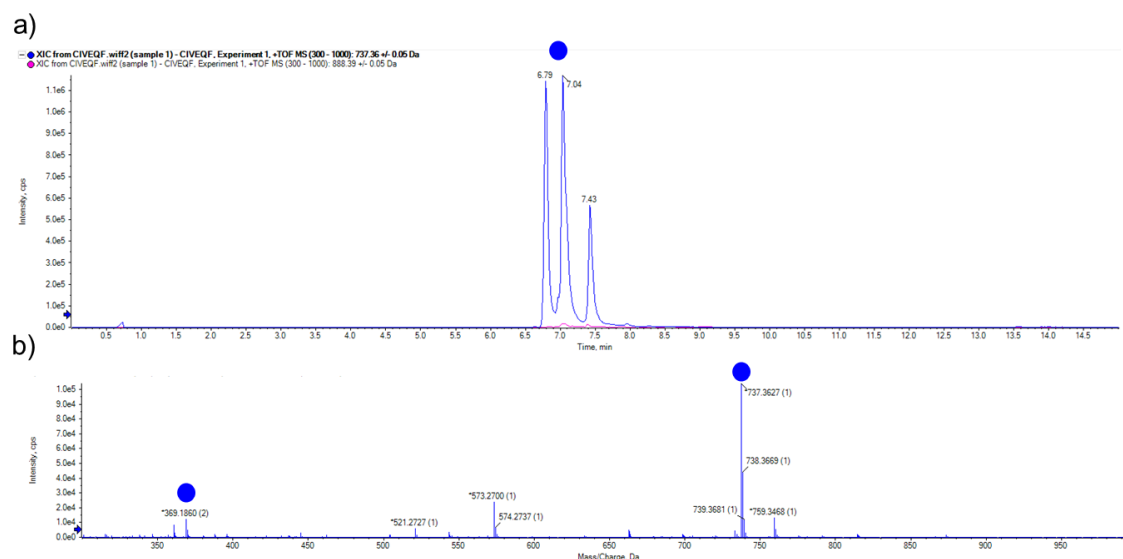
a)



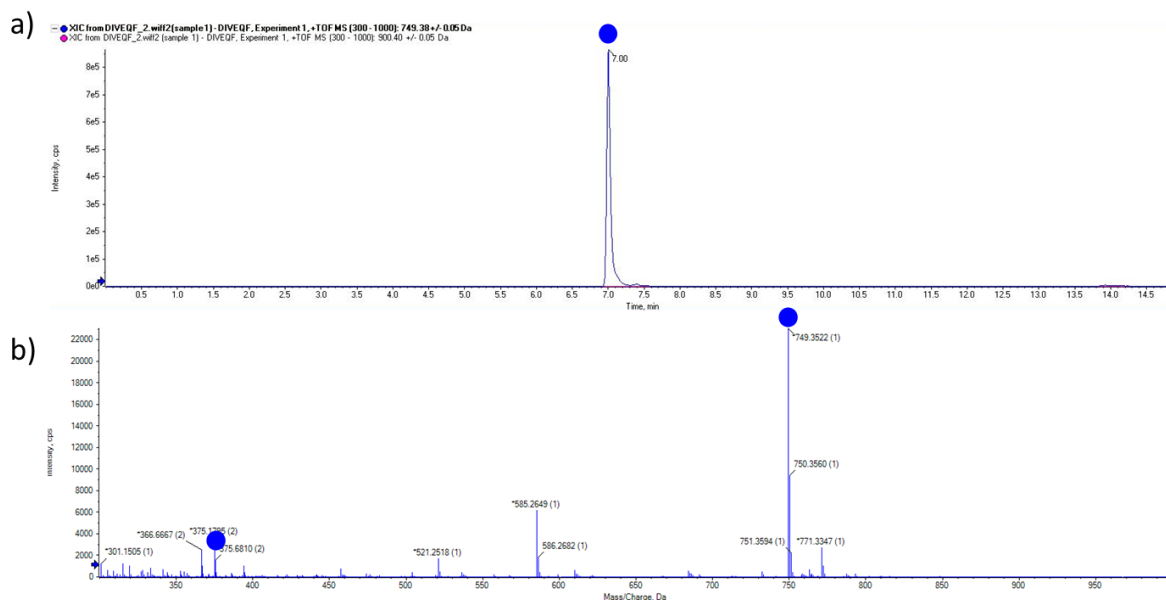
b)



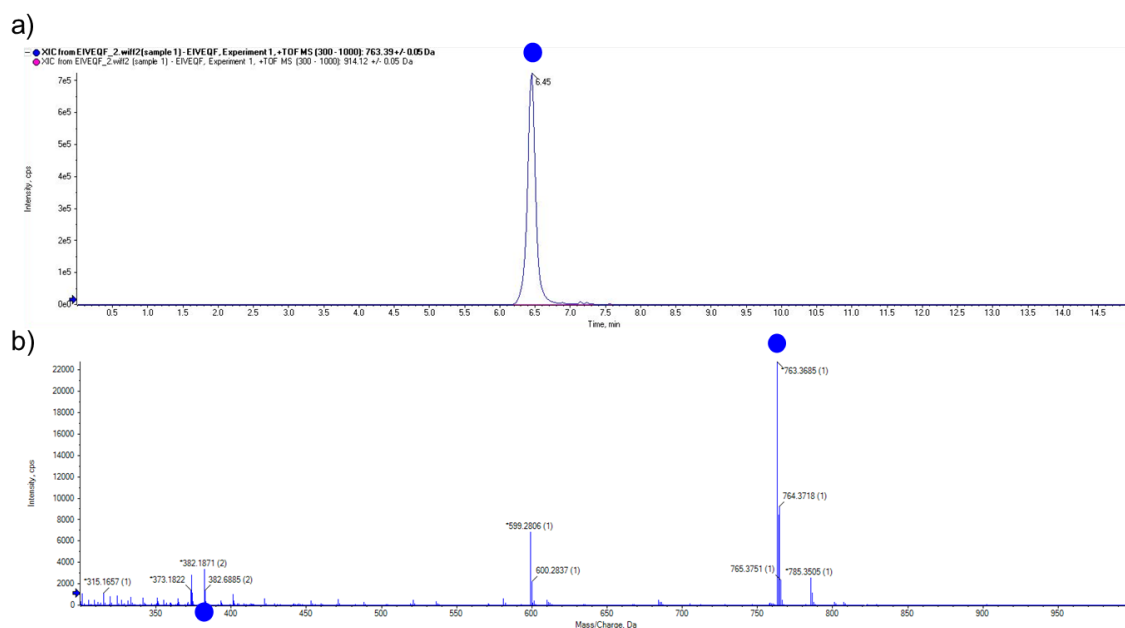
Supplementary Fig. 47. LC-MS analysis for modification of AIVEQF (**4b**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4b** (m/z 707.39) and modified peptide **5b** (m/z 856.41). (b) MS data for peptides **4b** and **5b**. LC-MS analysis confirms no modification of **4b**.



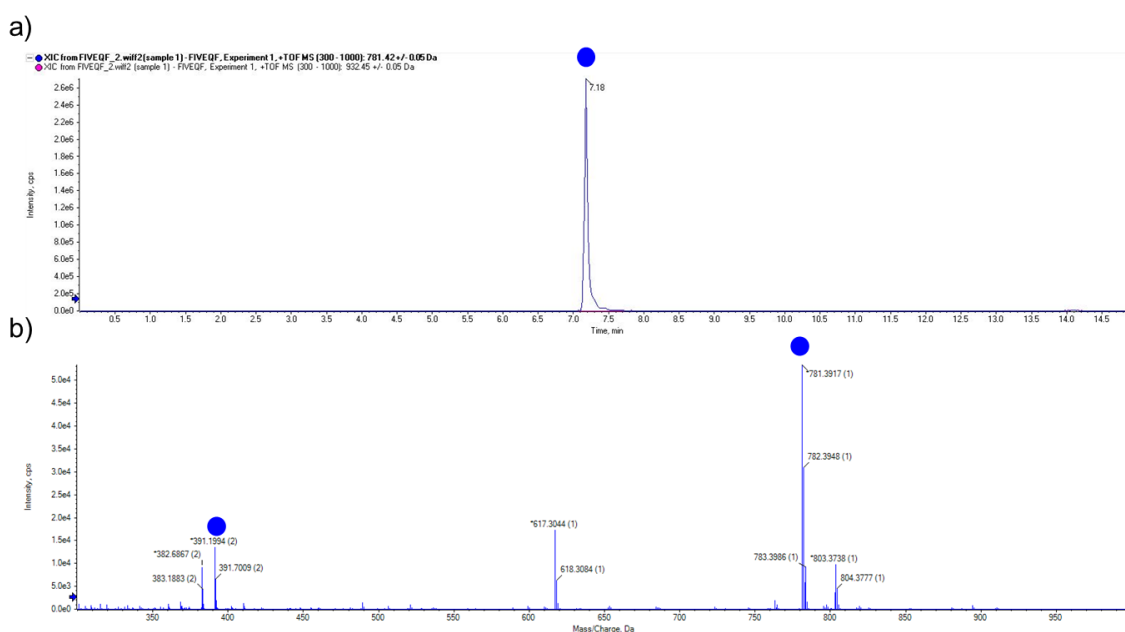
Supplementary Fig. 48. LC-MS analysis for modification of CIVEQF (**4c**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4c** (m/z 737.36) and modified peptide **5c** (m/z 888.39). (b) MS data for peptides **4c** and **5c**. LC-MS analysis confirms no modification of **4c**.



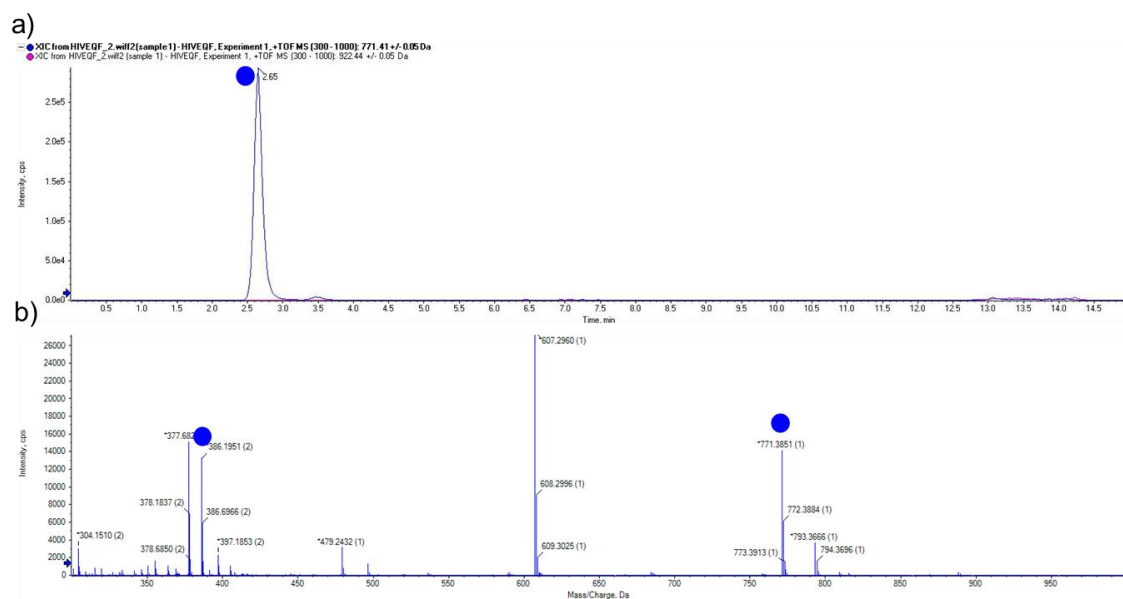
Supplementary Fig. 49. LC-MS analysis for modification of DIVEQF (**4d**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4d** (m/z 749.38) and modified peptide **5d** (m/z 900.40). (b) MS data for peptides **4d** and **5d**. LC-MS analysis confirms no modification of **4d**.



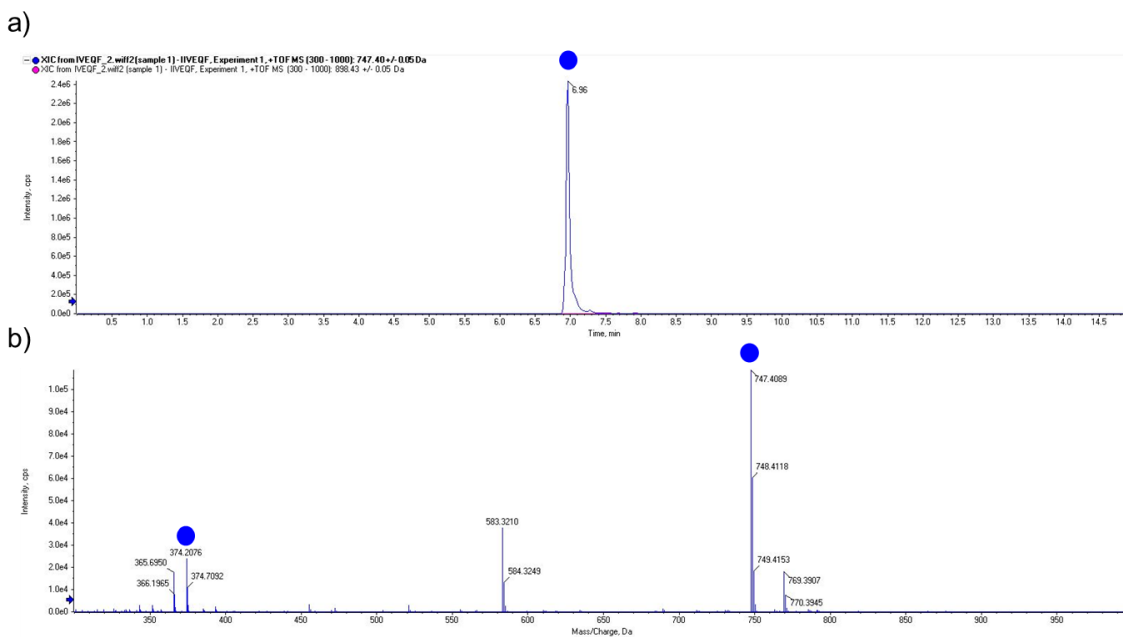
Supplementary Fig. 50. LC-MS analysis for modification of EIVEQF (**4e**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4e** (m/z 763.39) and modified peptide **5e** (m/z 914.12). (b) MS data for peptides **4e** and **5e**. LC-MS analysis confirms no modification of **4e**.



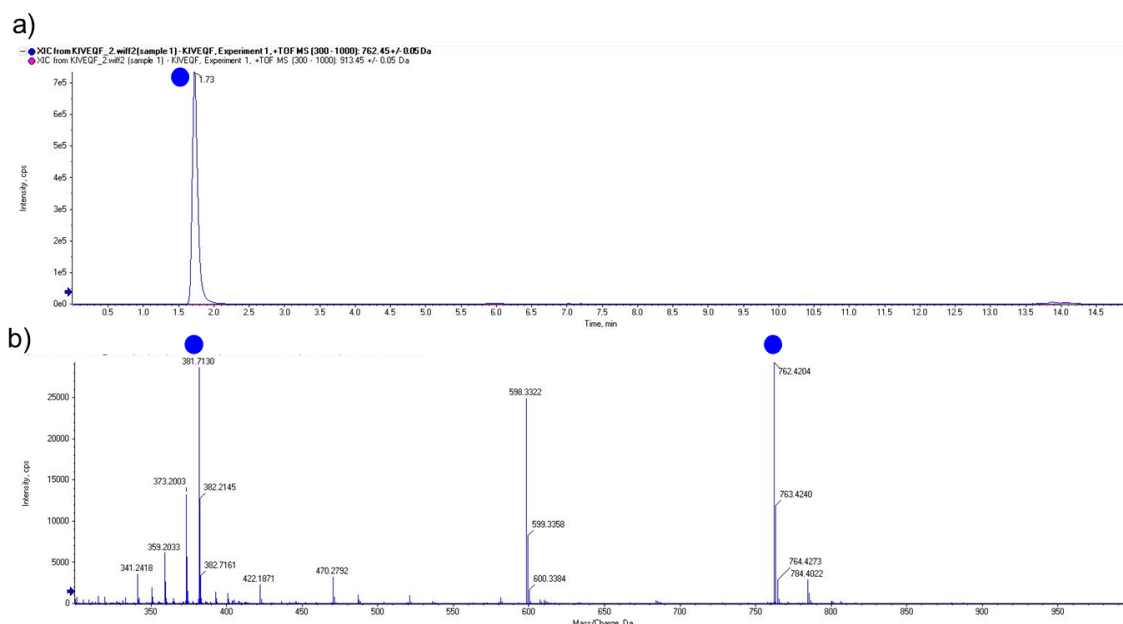
Supplementary Fig. 51. LC-MS analysis for modification of FIVEQF (**4f**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4f** (m/z 781.42) and modified peptide **5f** (m/z 932.45). (b) MS data for peptides **4f** and **5f**. LC-MS analysis confirms no modification of **4f**.



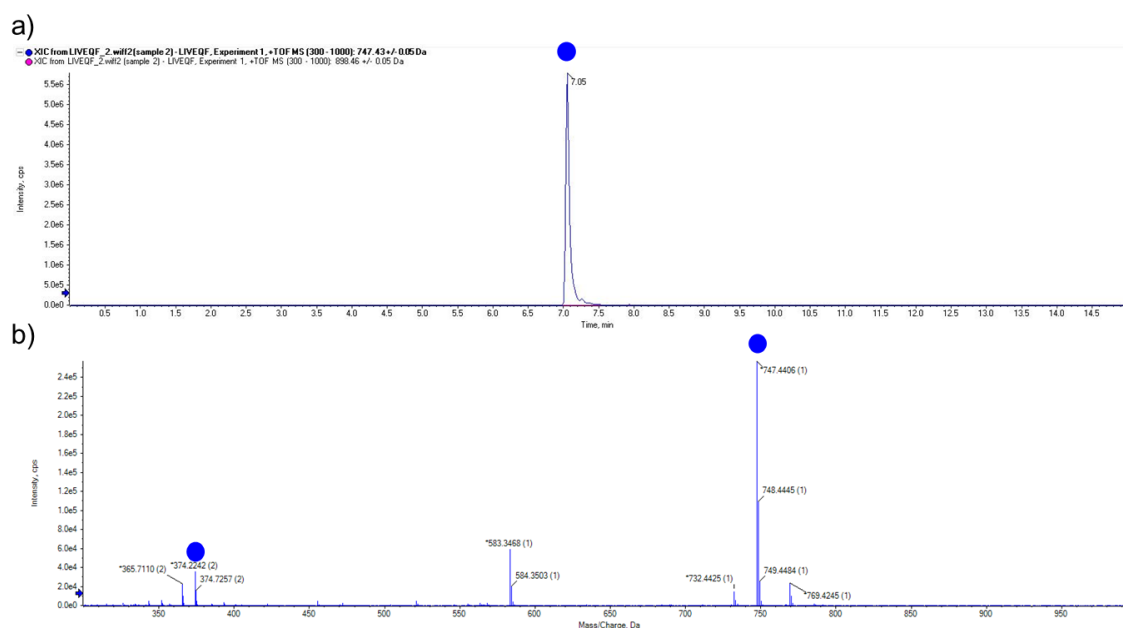
Supplementary Fig. 52. LC-MS analysis for modification of HIVEQF (**4g**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4g** (m/z 771.41) and modified peptide **5g** (m/z 922.44). (b) MS data for peptides **4g** and **5g**. LC-MS analysis confirms no modification of **4g**.



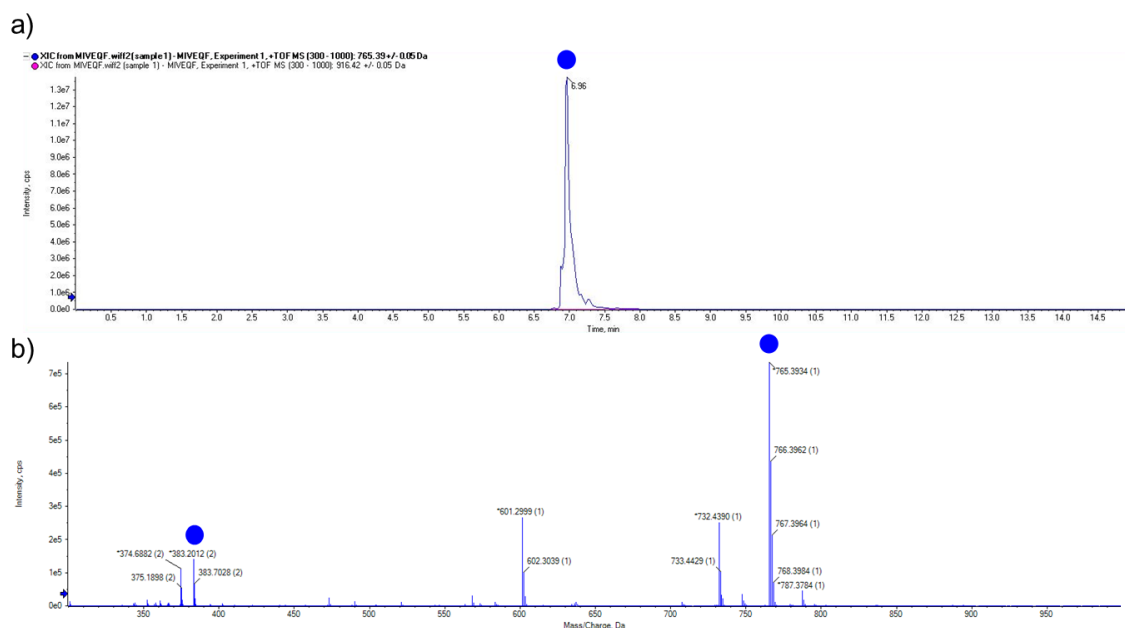
Supplementary Fig. 53. LC-MS analysis for modification of IIVEQF (**4h**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4h** (m/z 747.40) and modified peptide **5h** (m/z 898.43). (b) MS data for peptides **4h** and **5h**. LC-MS analysis confirms no modification of **4h**.



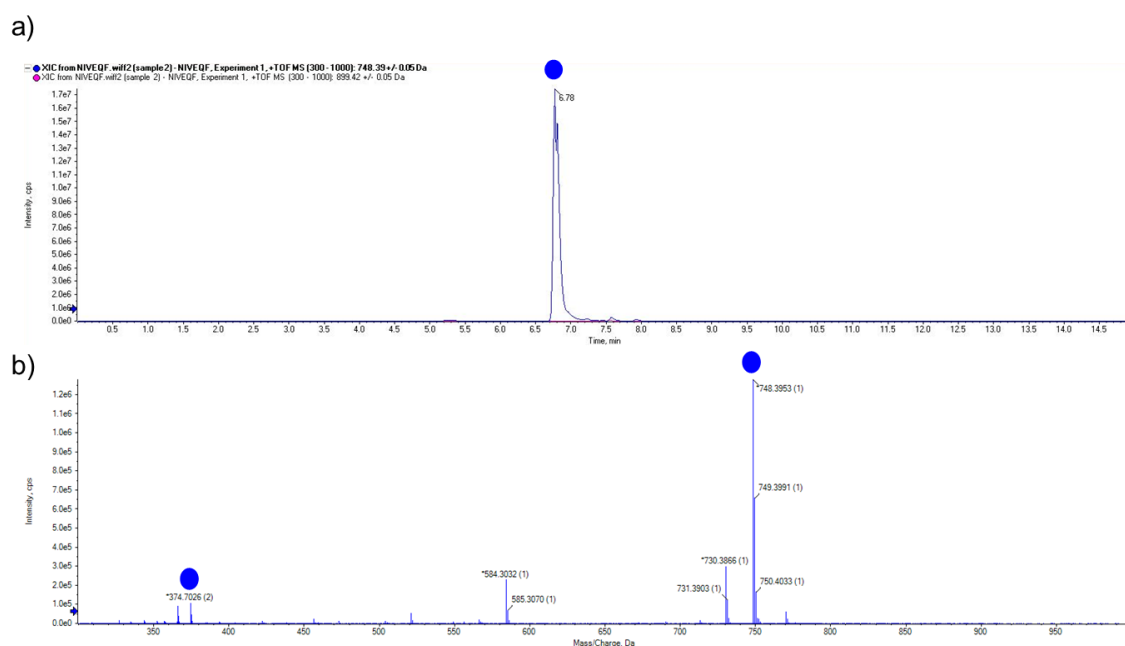
Supplementary Fig. 54. LC-MS analysis for modification of KIVEQF (**4i**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4i** (m/z 762.45) and modified peptide **5i** (m/z 913.45). (b) MS data for peptides **4i** and **5i**. LC-MS analysis confirms no modification of **4i**.



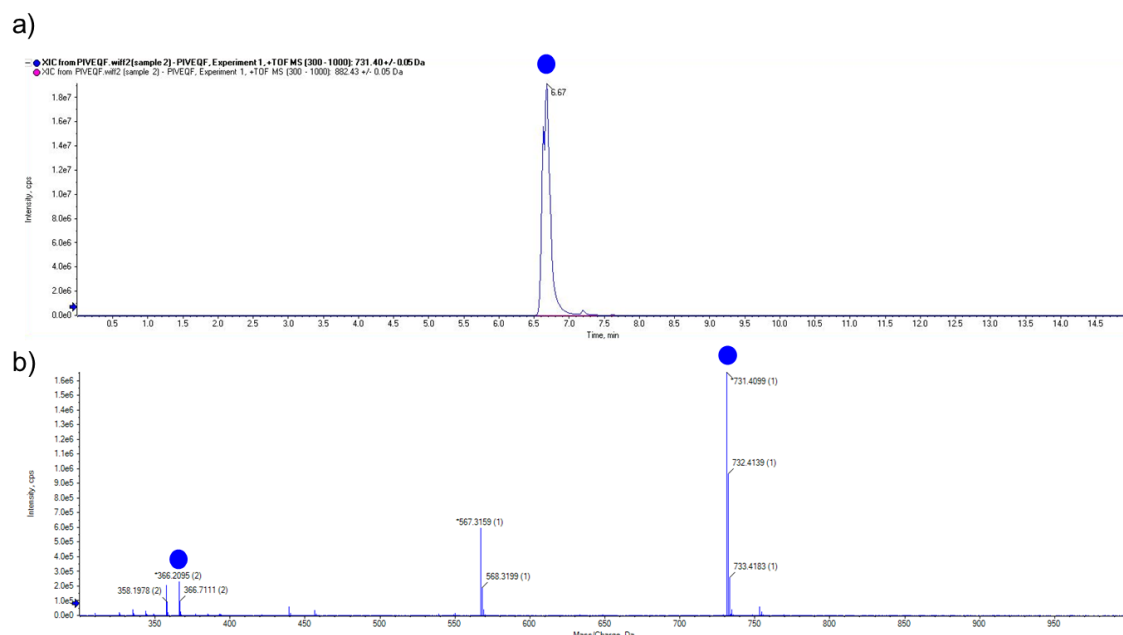
Supplementary Fig. 55. LC-MS analysis for modification of LIVEQF (**4j**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4j** (m/z 747.43) and modified peptide **5j** (m/z 898.46). (b) MS data for peptides **4j** and **5j**. LC-MS analysis confirms no modification of **4j**.



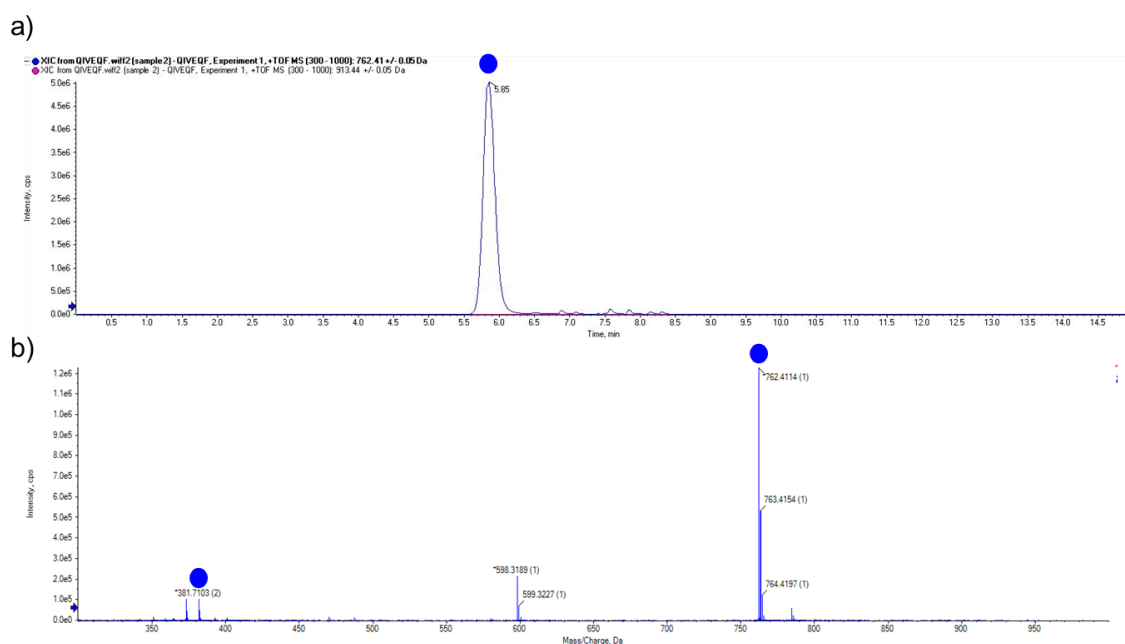
Supplementary Fig. 56. LC-MS analysis for modification of MIVEQF (**4k**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4k** (m/z 765.39) and modified peptide **5k** (m/z 916.42). (b) MS data for peptides **4k** and **5k**. LC-MS analysis confirms no modification of **4k**.



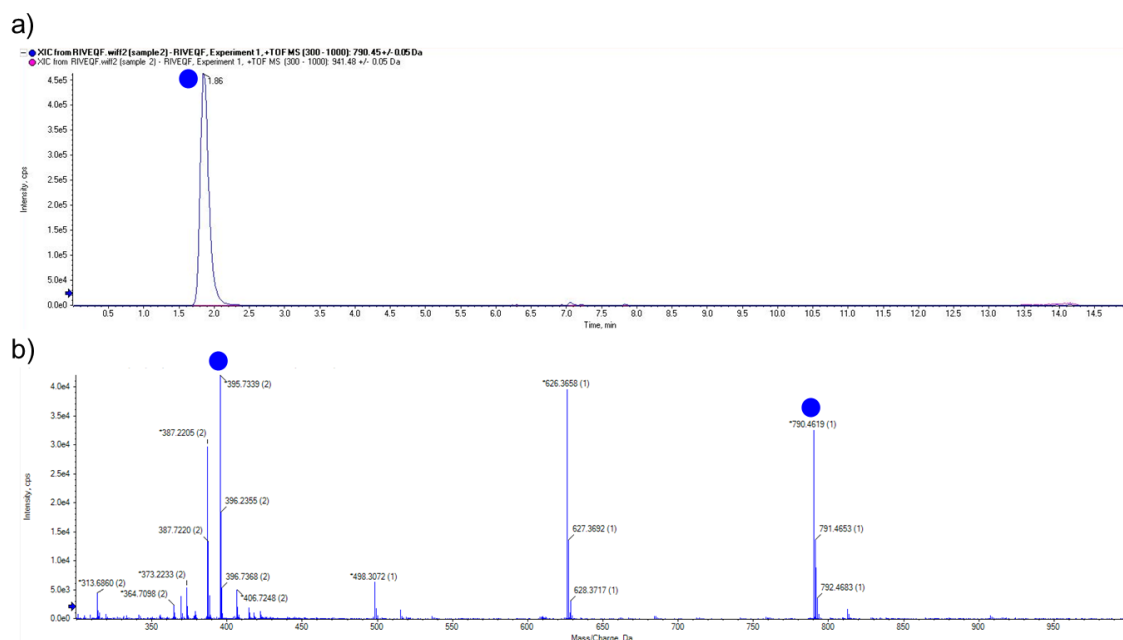
Supplementary Fig. 57. LC-MS analysis for modification of NIVEQF (**4l**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4l** (m/z 748.39) and modified peptide **5l** (m/z 899.42). (b) MS data for peptides **4l** and **5l**. LC-MS analysis confirms no modification of **4l**.



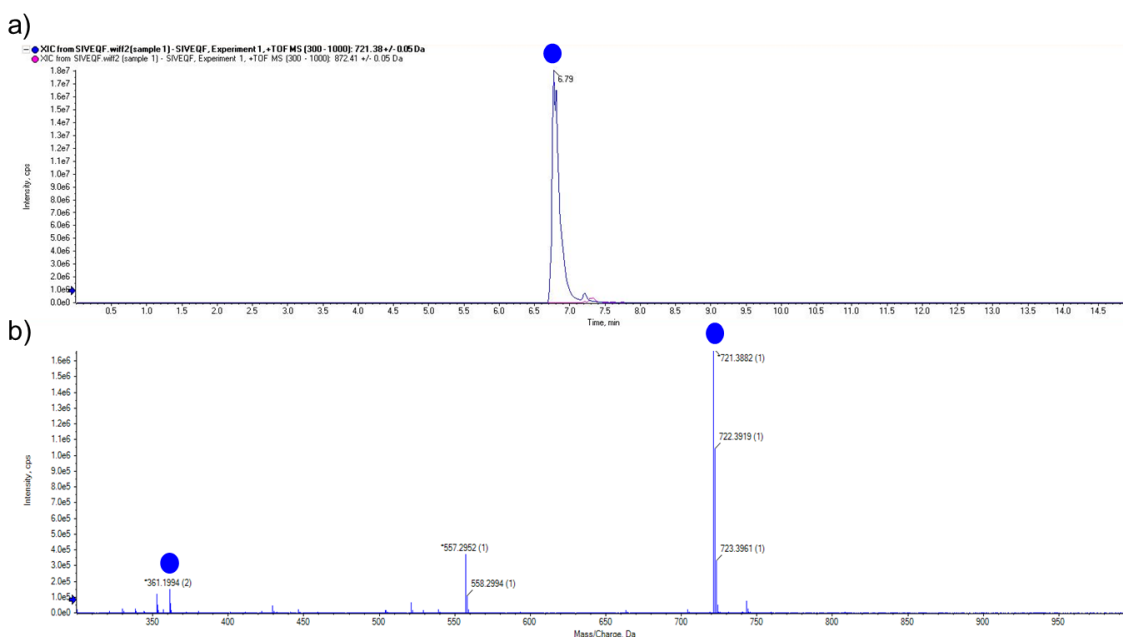
Supplementary Fig. 58. LC-MS analysis for modification of PIVEQF (**4m**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4m** (m/z 731.40) and modified peptide **5m** (m/z 882.43). (b) MS data for peptides **4m** and **5m**. LC-MS analysis confirms no modification of **4m**.



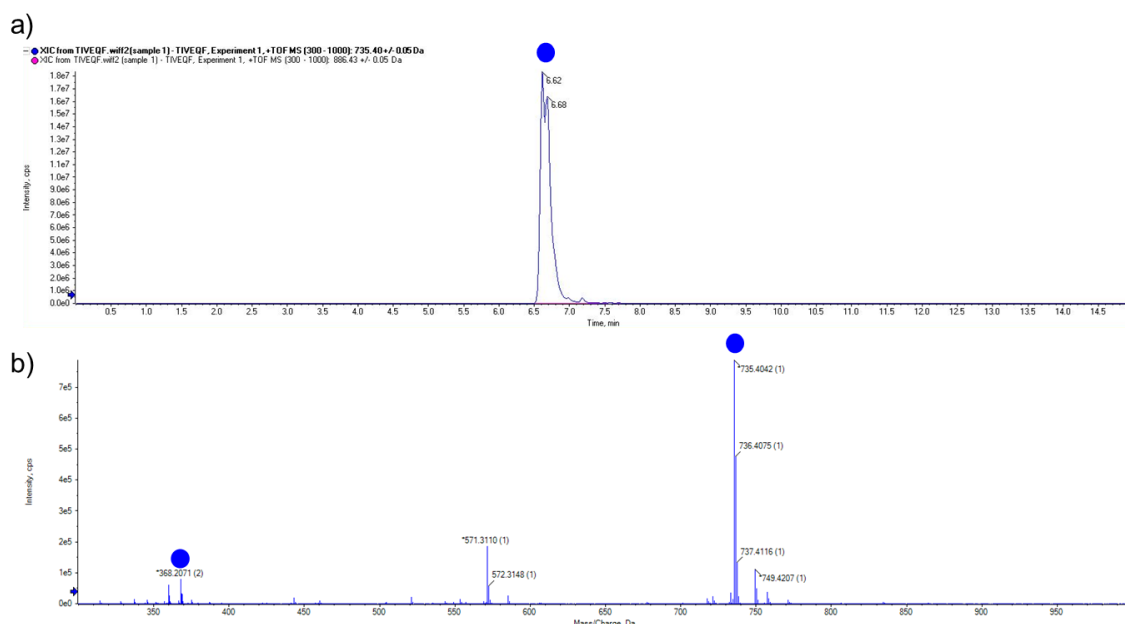
Supplementary Fig. 59. LC-MS analysis for modification of QIVEQF (**4n**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4n** (m/z 762.41) and modified peptide **5n** (m/z 913.44). (b) MS data for peptides **4n** and **5n**. LC-MS analysis confirms no modification of **4n**.



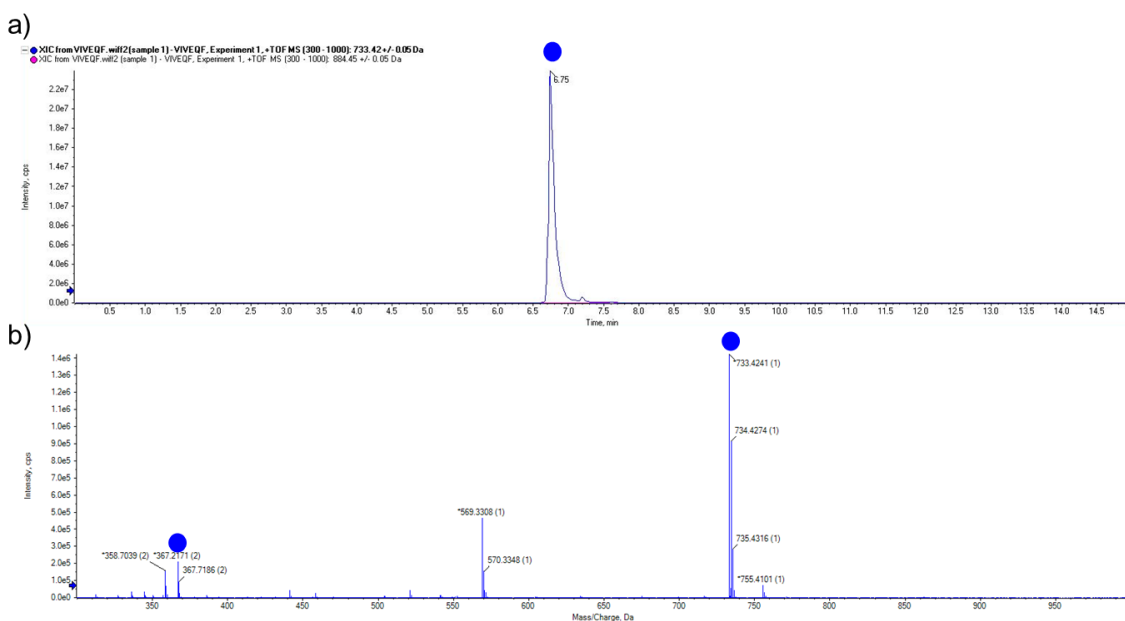
Supplementary Fig. 60. LC-MS analysis for modification of RIVEQF (**4o**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4o** (m/z 790.46) and modified peptide **5o** (m/z 941.48). (b) MS data for peptides **4o** and **5o**. LC-MS analysis confirms no modification of **4o**.



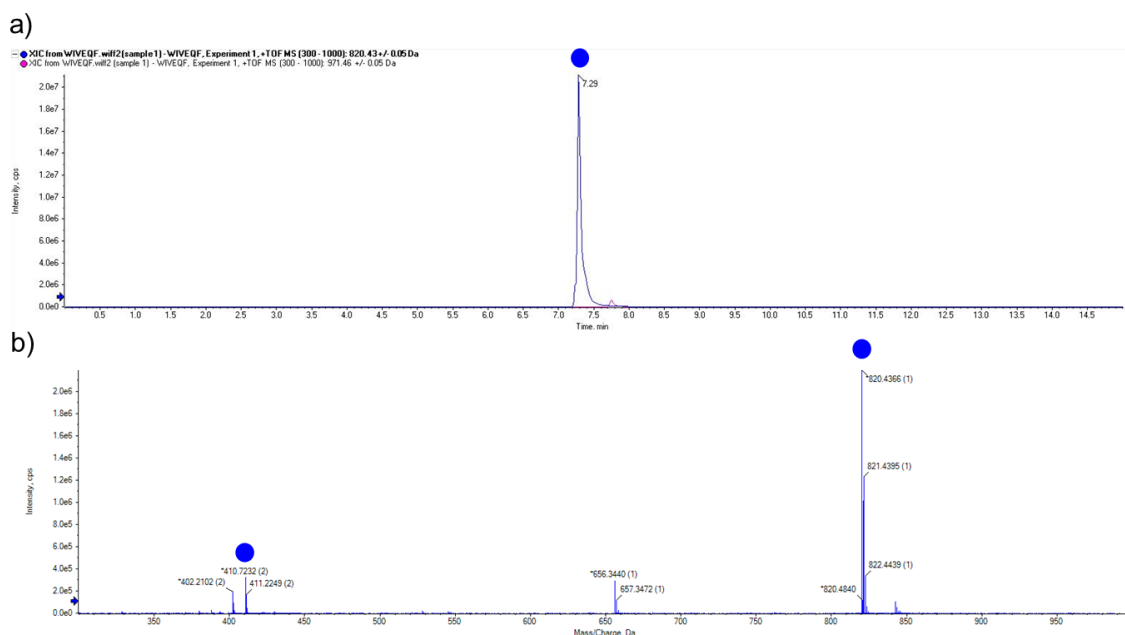
Supplementary Fig. 61. LC-MS analysis for modification of SIVEQF (**4p**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4p** (m/z 721.38) and modified peptide **5p** (m/z 872.41). (b) MS data for peptides **4p** and **5p**. LC-MS analysis confirms no modification of **4p**.



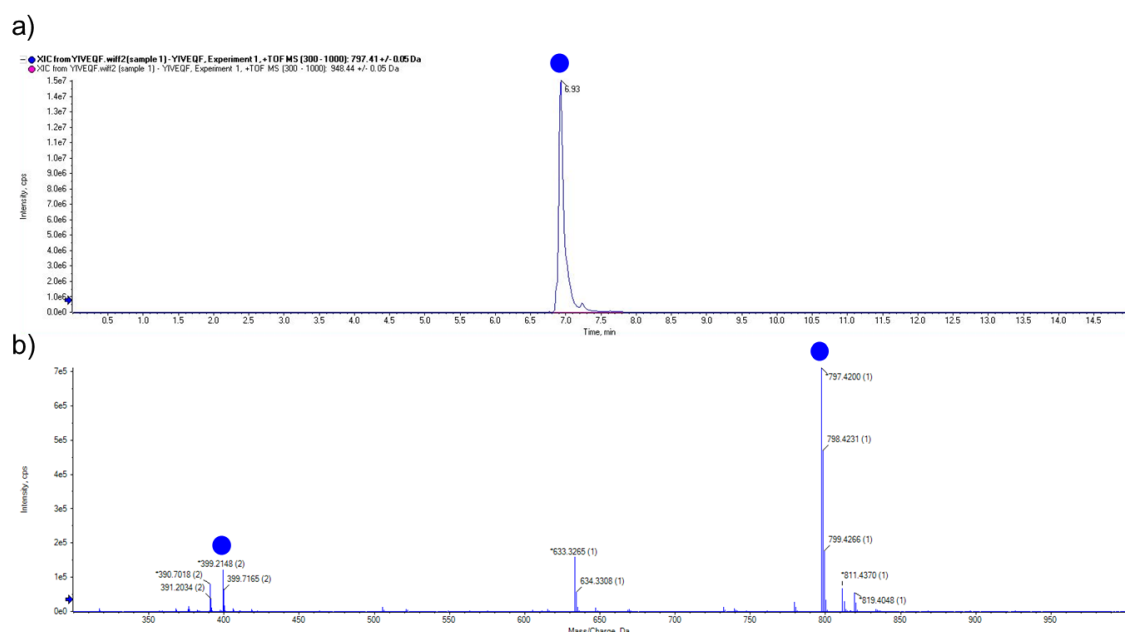
Supplementary Fig. 62. LC-MS analysis for modification of TIVEQF (**4q**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4q** (m/z 735.40) and modified peptide **5q** (m/z 886.43). (b) MS data for peptides **4q** and **5q**. LC-MS analysis confirms no modification of **4q**.



Supplementary Fig. 63. LC-MS analysis for modification of VIVEQF (**4r**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4r** (m/z 733.42) and modified peptide **5r** (m/z 884.45). (b) MS data for peptides **4r** and **5r**. LC-MS analysis confirms no modification of **4r**.

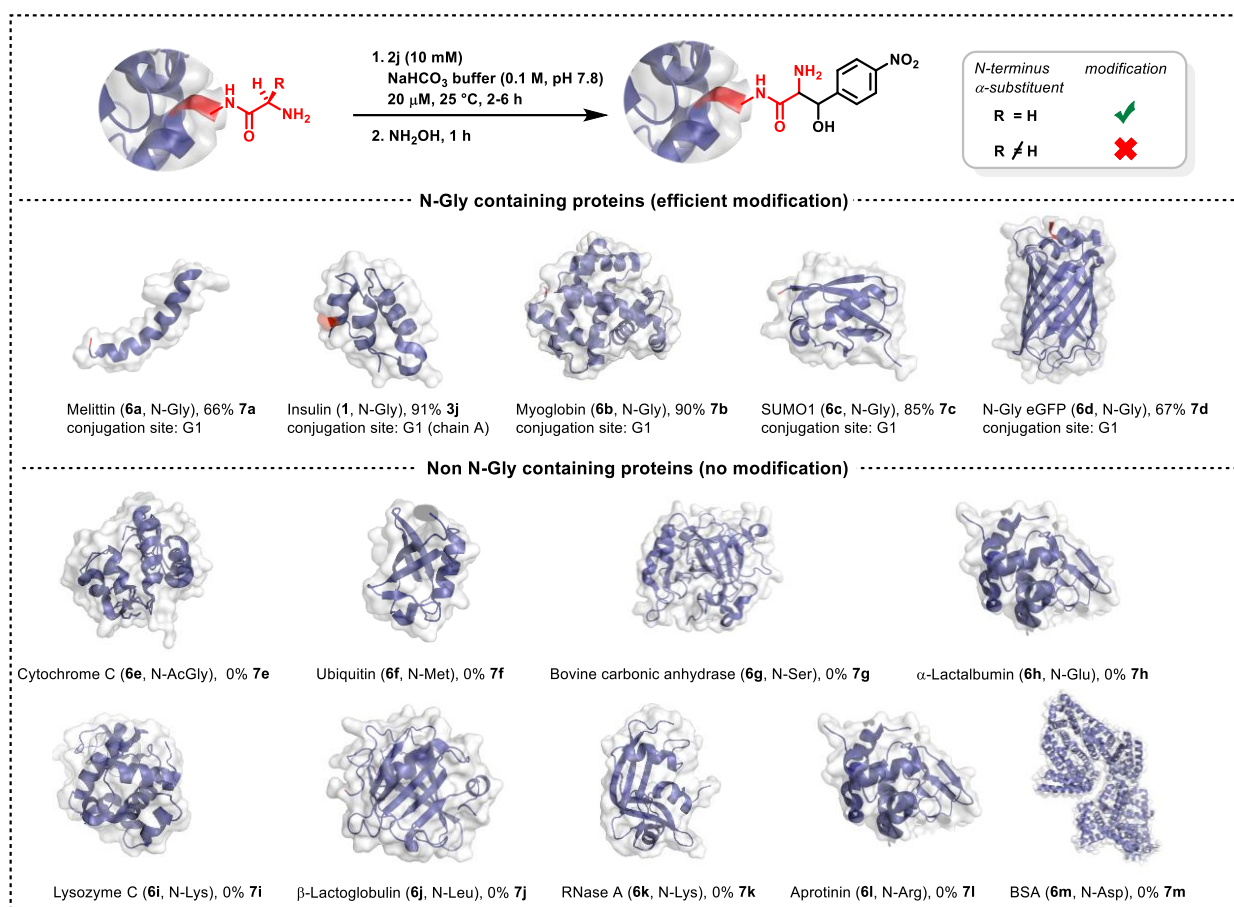


Supplementary Fig. 64. LC-MS analysis for modification of WIVEQF (**4s**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4s** (m/z 820.43) and modified peptide **5s** (m/z 971.46). (b) MS data for peptides **4s** and **5s**. LC-MS analysis confirms no modification of **4s**.

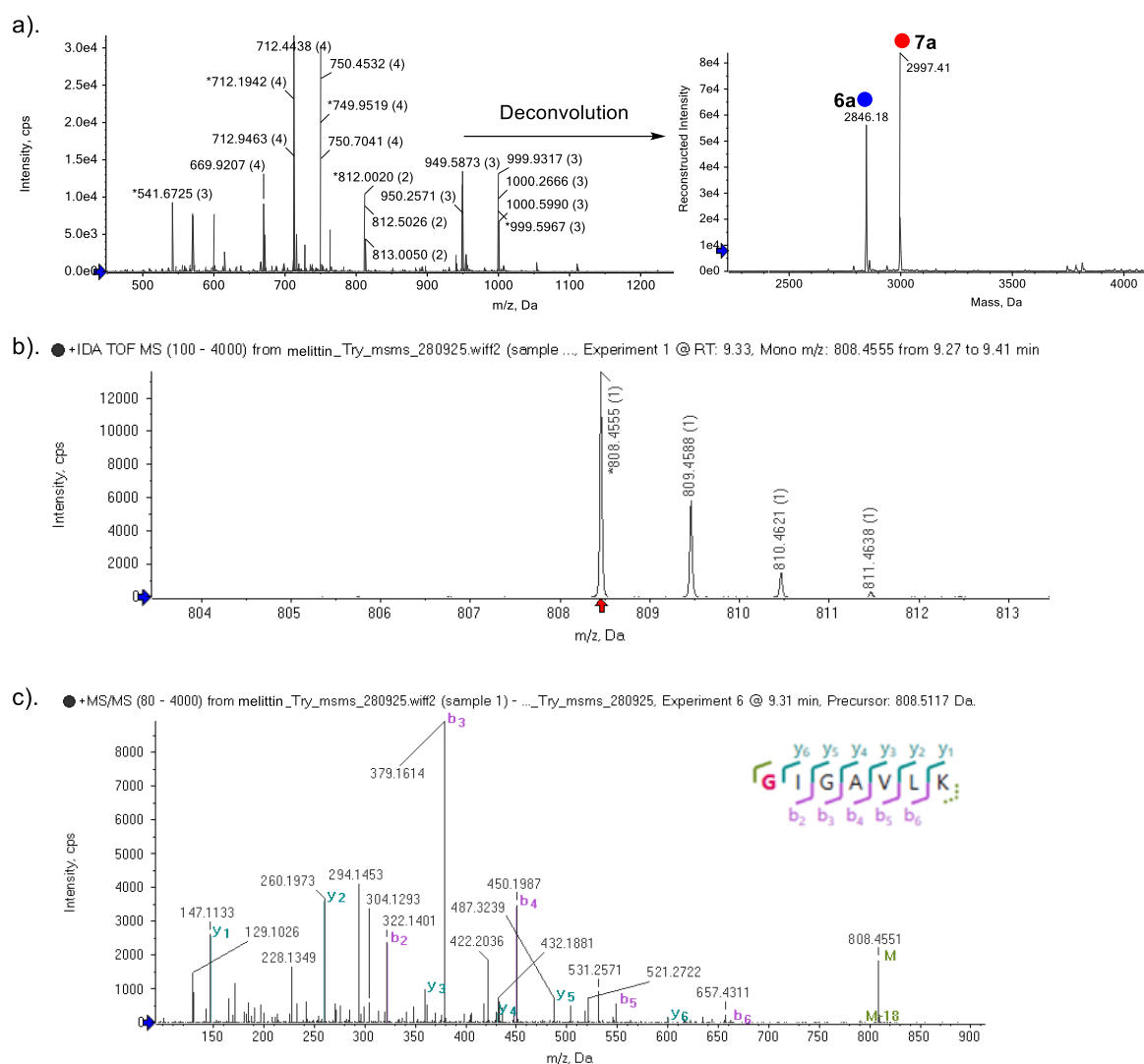


Supplementary Fig. 65. LC-MS analysis for modification of YIVEQF (**4t**) after reaction with **2j** (50 mM) for 4 h. (a) XIC data for native peptide **4t** (m/z 797.41) and modified peptide **5t** (m/z 948.44). (b) MS data for peptides **4t** and **5t**. LC-MS analysis confirms no modification of **4t**.

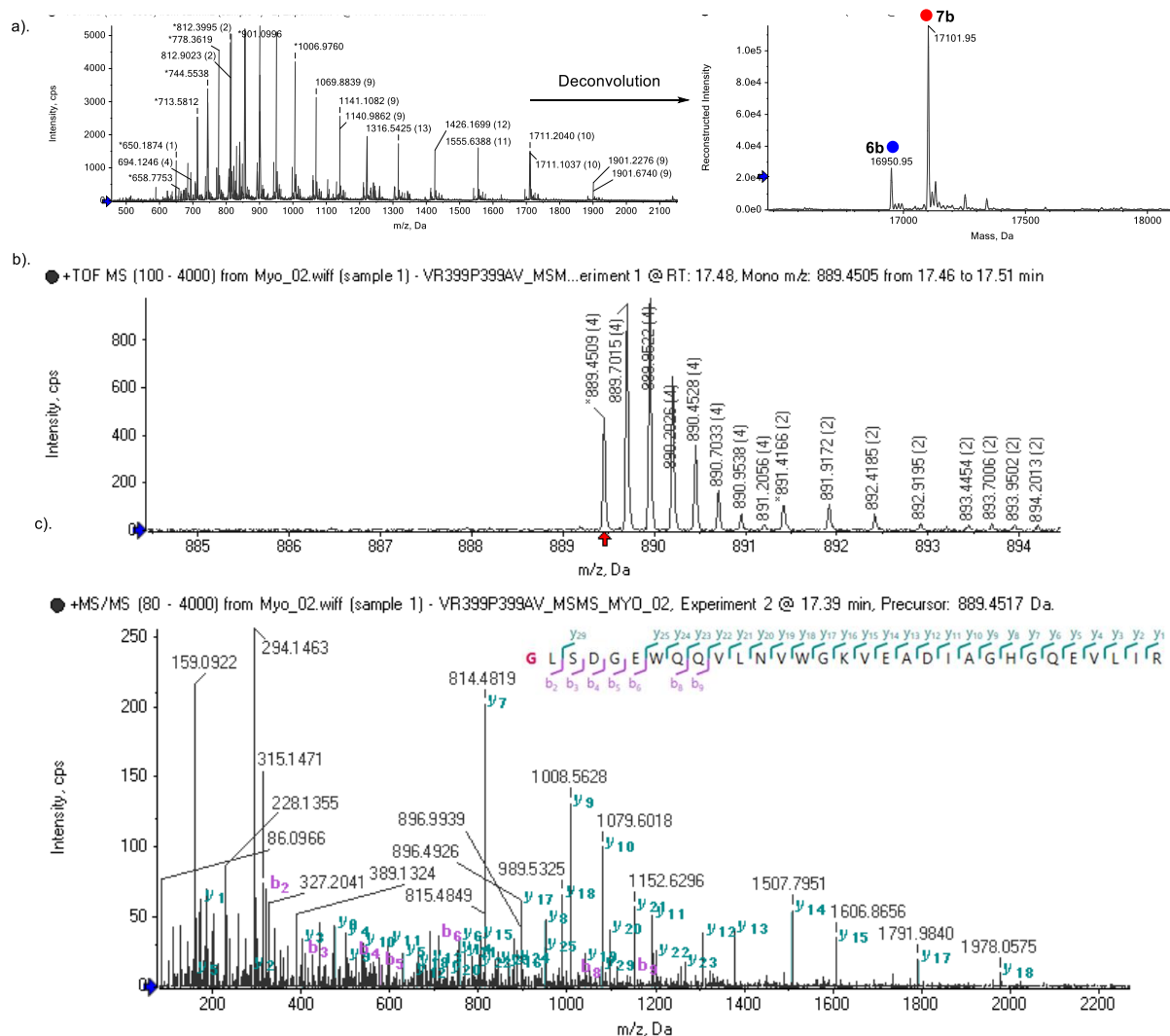
3.7 N-Gly residue-specificity with proteins



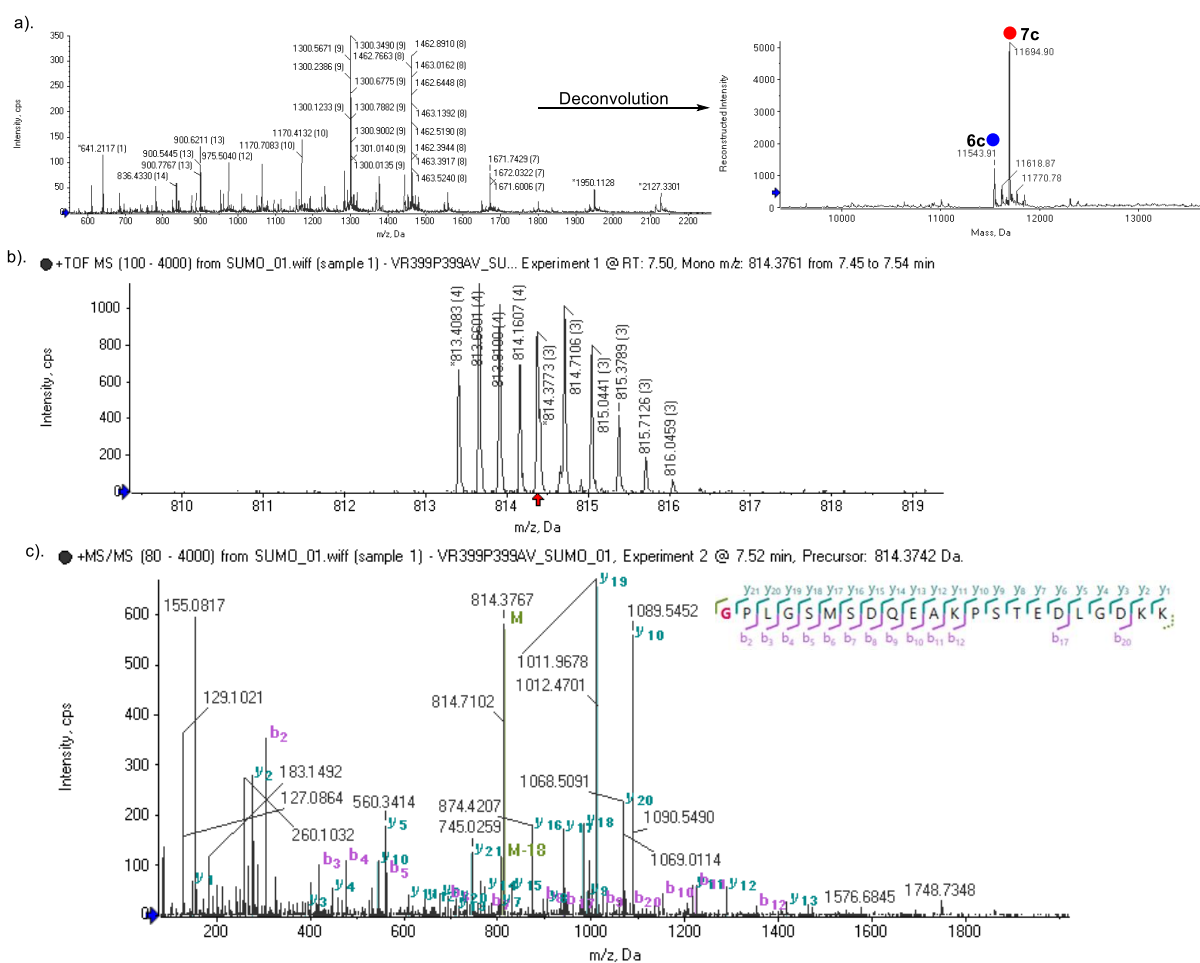
Supplementary Fig. 66. Highly specific N-Gly modification in structurally diverse proteins. Other N-terminal residues do not render any irreversible transformation under the reaction conditions. % Conversions were estimated by ESI-MS.



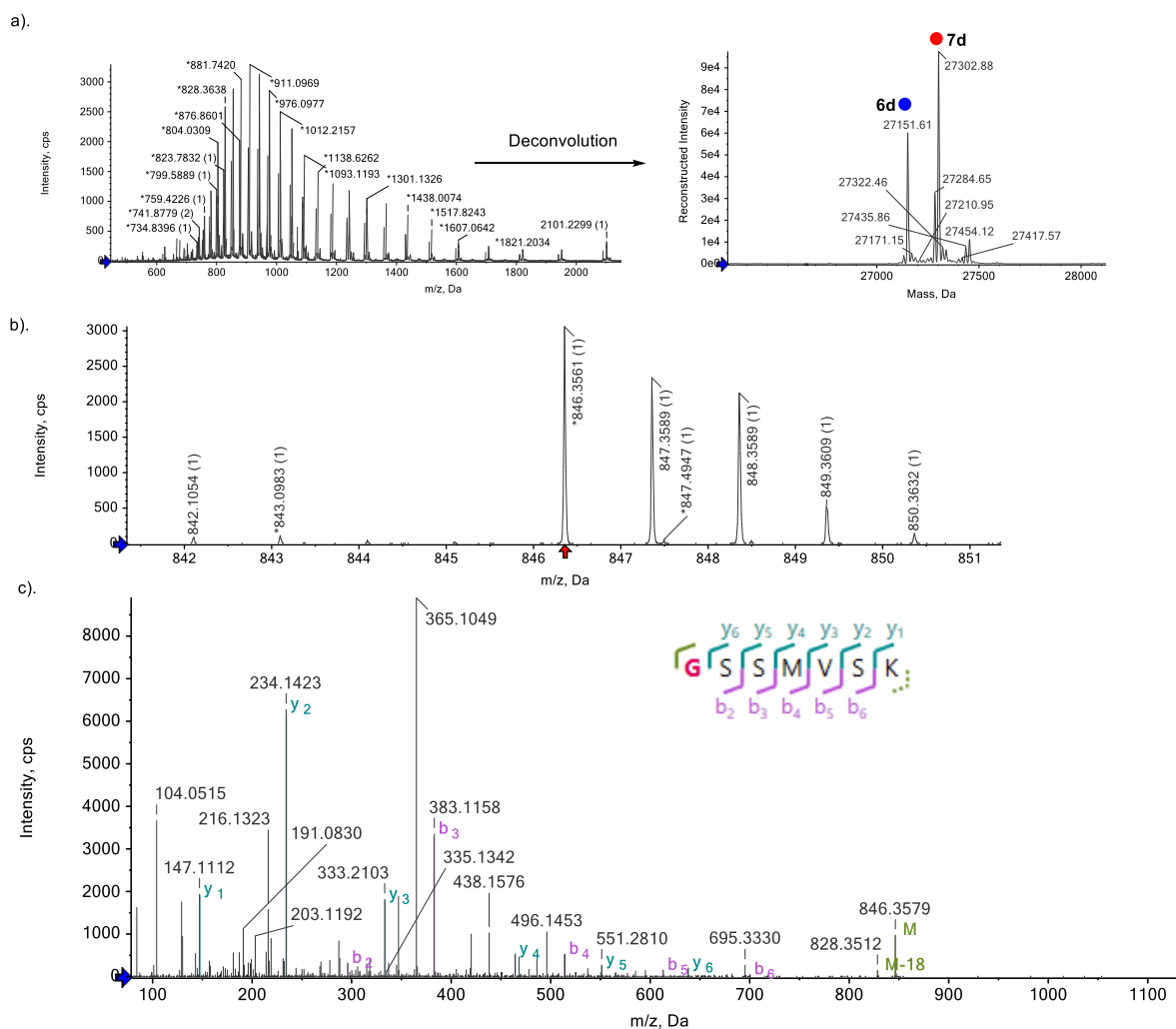
Supplementary Fig. 67. (a) Charged and deconvoluted MS spectra for melittin (**6a**) after reaction with **2j** (10 mM, 2 h) for N-Gly bioconjugation. (b) Peptide mapping of **7a** after trypsin digestion. (c) MS-MS of labeled peptide GIGAVLK (G1-K7, m/z 808.51, $[M+H]^+$) confirms the site of modification, N-Gly (G1).



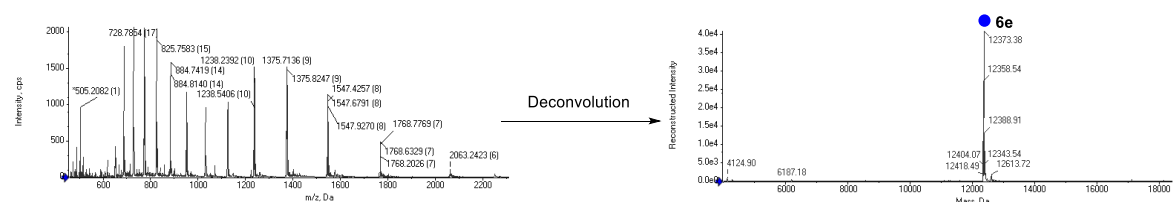
Supplementary Fig. 68. (a) Charged and deconvoluted MS spectra for myoglobin (**6b**) after reaction with **2j** (10 mM, 6 h) for N-Gly bioconjugation. (b) Peptide mapping of **7b** after trypsin digestion. (c) MS-MS of labeled peptide GLSDGEWQQVLNVWGKVEADIAGHGGQEVLIIR (G1-R31, m/z 889.4517, $[M+4H]^4+$) confirms the site of modification, N-Gly (G1).



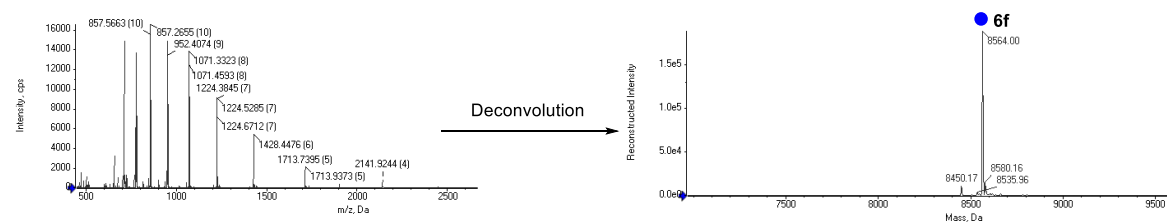
Supplementary Fig. 69. (a) Charged and deconvoluted MS spectra for SUMO1 (**6c**) after reaction with **2j** (10 mM, 4 h) for N-Gly bioconjugation. (b) Peptide mapping of **7c** after trypsin digestion. (c) MS-MS of labeled peptide GPLGSMDSQEA KPSTEDLGDKK (G1-K22, m/z 814.3742, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



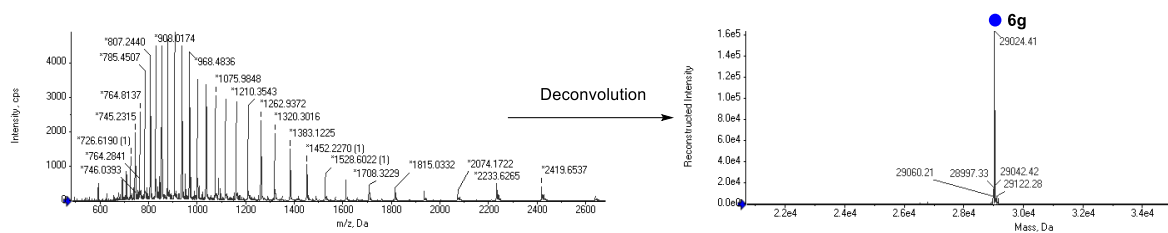
Supplementary Fig. 70. (a) Charged and deconvoluted MS spectra for N-Gly eGFP (**6d**) after reaction with **2j** (10 mM, 6 h) for N-Gly bioconjugation. (b) Peptide mapping of **7d** after trypsin digestion. (c) MS-MS of labeled peptide GSSMVSK (G1-K7, m/z 846.3561, $[M+H]^+$) confirms the site of modification, N-Gly (G1).



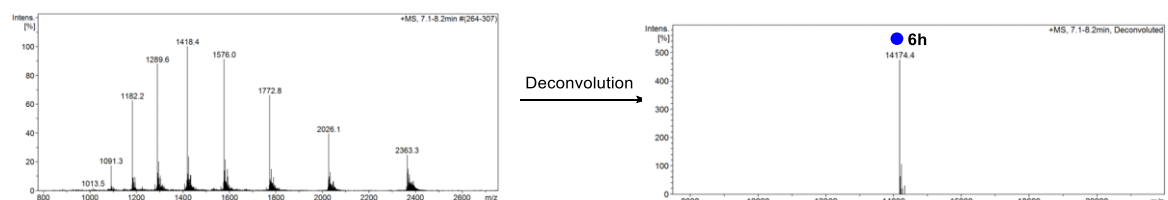
Supplementary Fig. 71. Charged and deconvoluted MS spectra for cytochrome C (**6e**) after reaction with **2j** (10 mM, 2 h) confirm no irreversible bioconjugation.



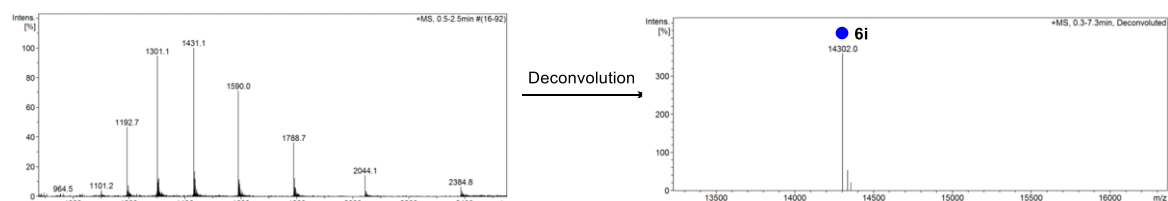
Supplementary Fig. 72. Charged and deconvoluted MS spectra for ubiquitin (**6f**) after reaction with **2j** (10 mM, 2 h) confirm no irreversible bioconjugation.



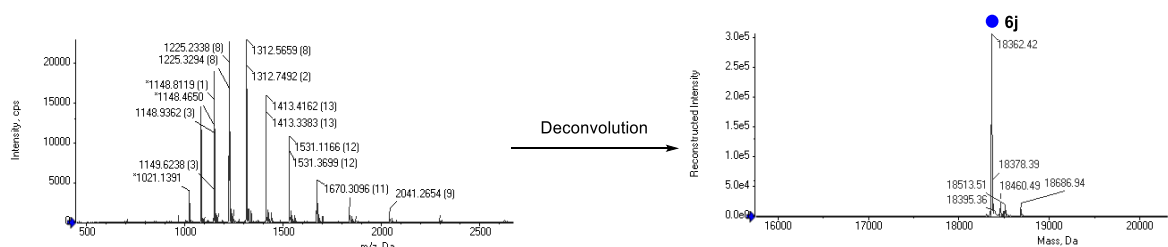
Supplementary Fig. 73. Charged and deconvoluted MS spectra for bovine carbonic anhydrase (6g) after reaction with 2j (10 mM, 2 h) confirm no irreversible bioconjugation.



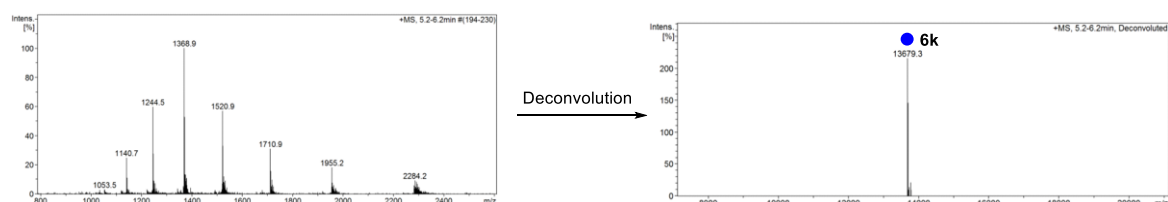
Supplementary Fig. 74. Charged and deconvoluted MS spectra for α-lactalbumin (6h) after reaction with 2j (10 mM, 2 h) confirm no irreversible bioconjugation.



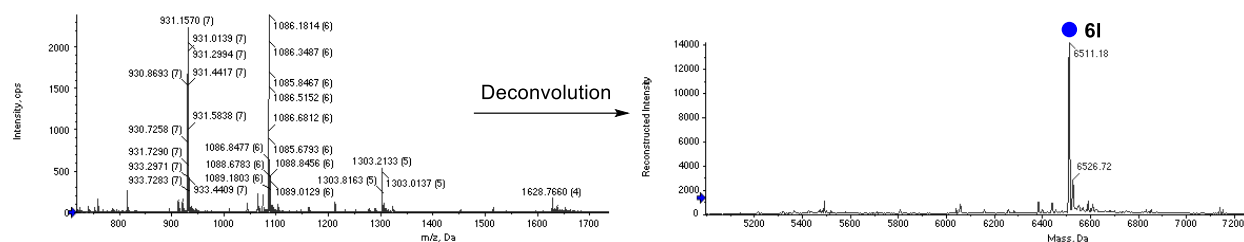
Supplementary Fig. 75. Charged and deconvoluted MS spectra for lysozyme C (6i) after reaction with 2j (10 mM, 2 h) confirm no irreversible bioconjugation.



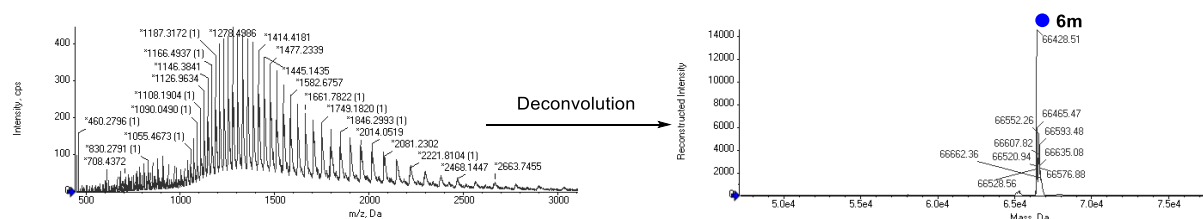
Supplementary Fig. 76. Charged and deconvoluted MS spectra for β-lactoglobulin (6j) after reaction with 2j (10 mM, 2 h) confirm no irreversible bioconjugation.



Supplementary Fig. 77. Charged and deconvoluted MS spectra for RNase A (6k) after reaction with 2j (10 mM, 2 h) confirm no irreversible bioconjugation.

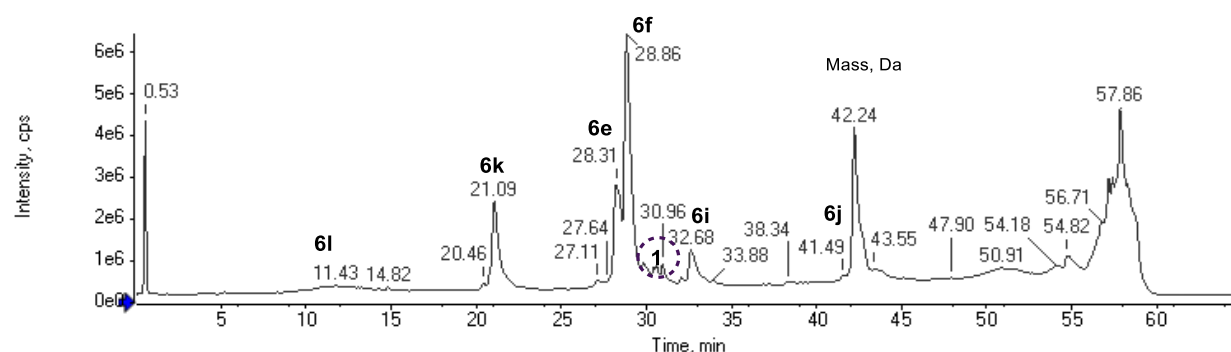


Supplementary Fig. 78. Charged and deconvoluted MS spectra for aprotinin (**6l**) after reaction with **2j** (10 mM, 2 h) confirm no irreversible bioconjugation.

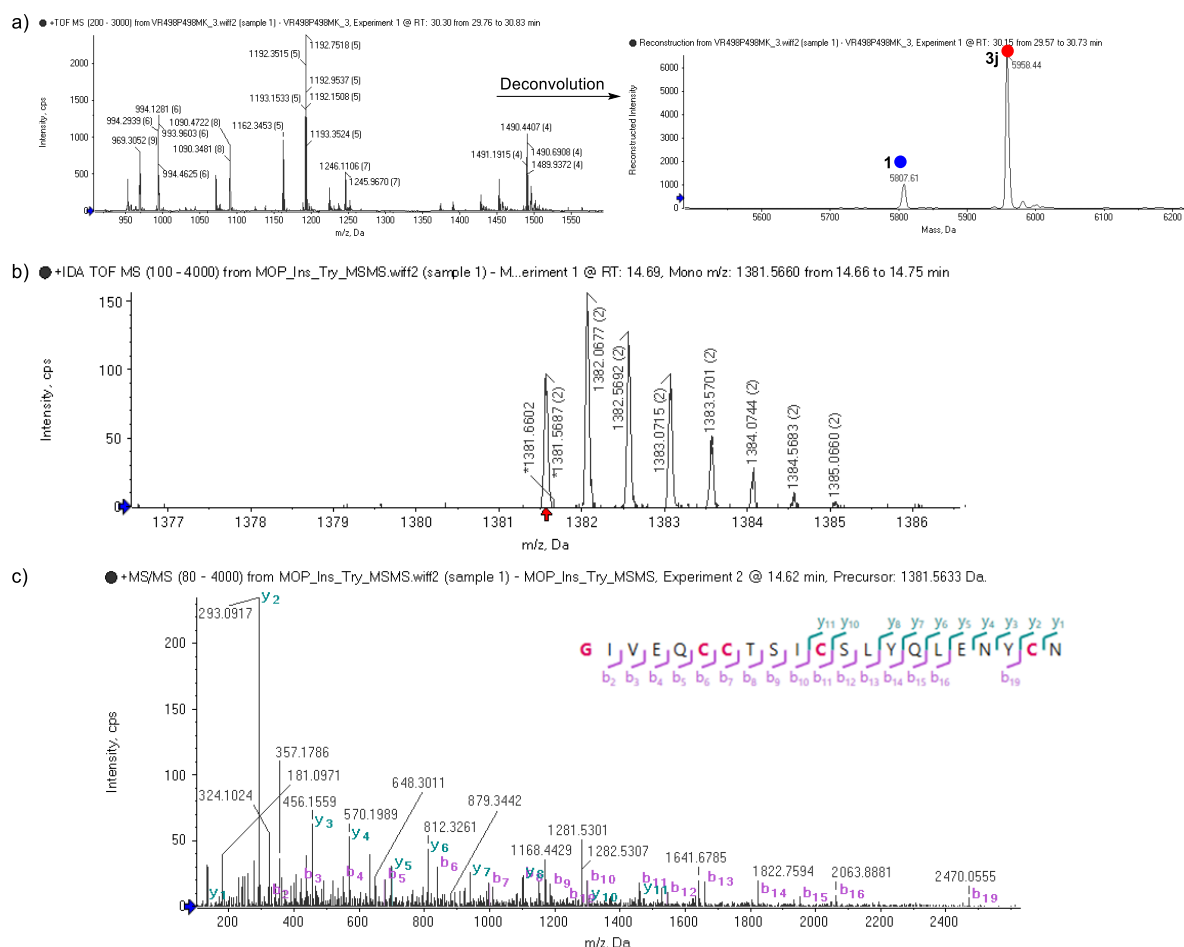


Supplementary Fig. 79. Charged and deconvoluted MS spectra for bovine serum albumin (BSA, **6m**) after reaction with **2j** (10 mM, 2 h) confirm no irreversible bioconjugation.

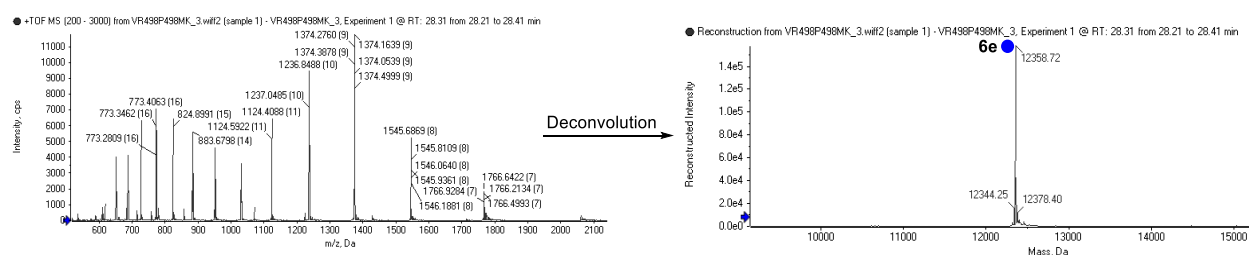
3.8 Residue specificity with a mixture of proteins



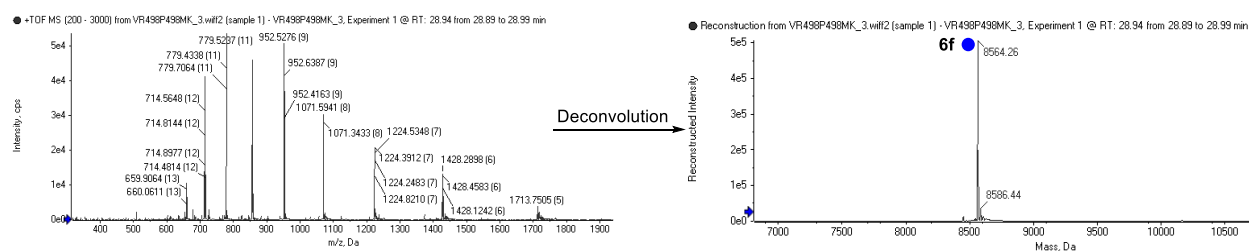
Supplementary Fig. 80. TIC data for N-Gly protein modification in a complex mixture of proteins after reaction with **2j** (10 mM, 2 h).



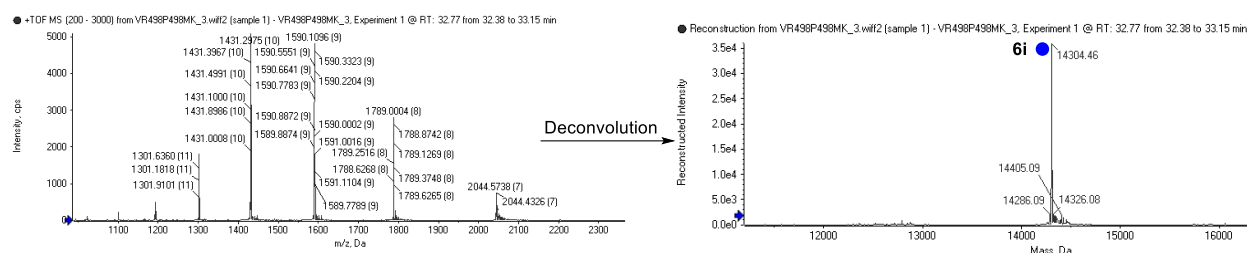
Supplementary Fig. 81. (a) Charged and deconvoluted MS spectra for the labeled insulin (**3j**) in a complex mixture of proteins after reaction with **2j**. (b) Peptide mapping of **3j** after trypsin digestion. (c) MS-MS of labeled peptide GIVEQCCTSICSLSYQLENYCN (G1-N21, m/z 1381.56, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).



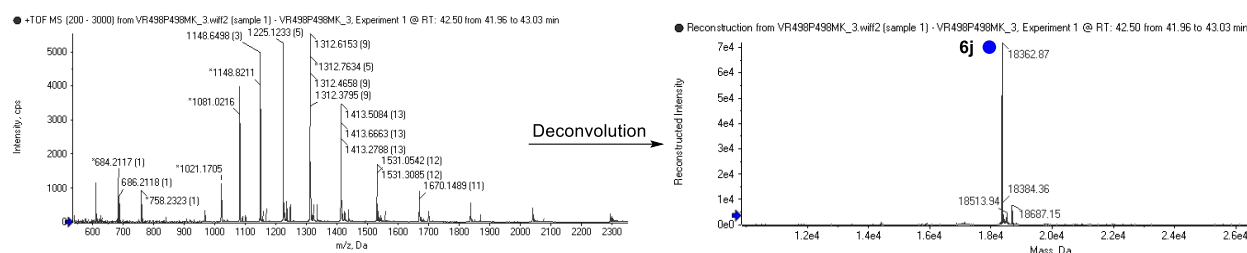
Supplementary Fig. 82. Charged and deconvoluted MS spectra for the unlabeled cytochrome C (**6e**) in a complex mixture of proteins after reaction with **2j**.



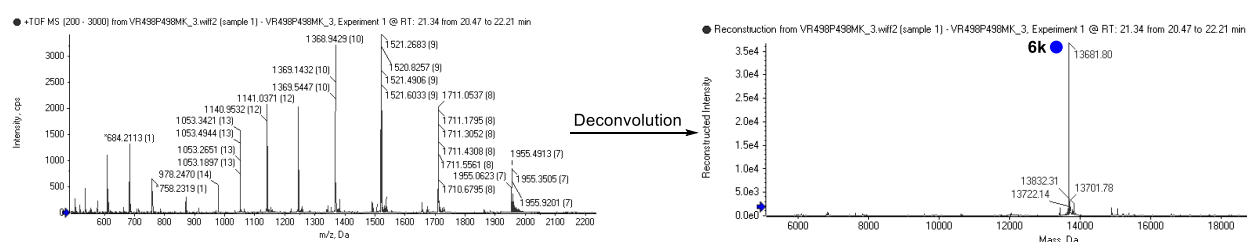
Supplementary Fig. 83. Charged and deconvoluted MS spectra for the unlabeled ubiquitin (**6f**) in a complex mixture of proteins after reaction with **2j**.



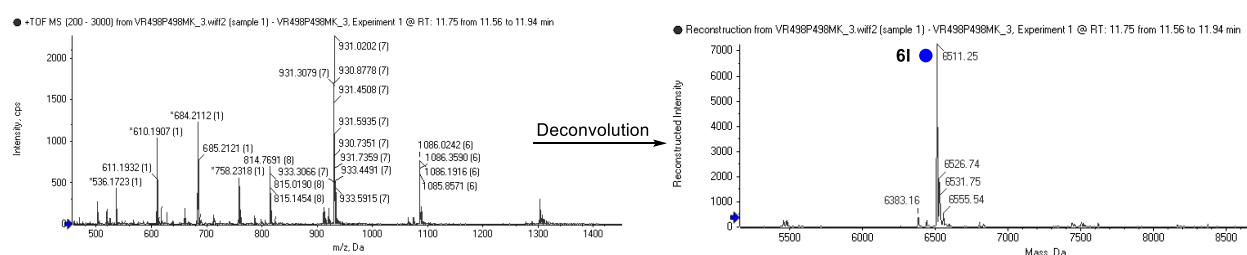
Supplementary Fig. 84. Charged and deconvoluted MS spectra for the unlabeled lysozyme C (**6i**) in a complex mixture of proteins after reaction with **2j**.



Supplementary Fig. 85. Charged and deconvoluted MS spectra for the unlabeled β -lactoglobulin (**6j**) in a complex mixture of proteins after reaction with reagent **2j**.

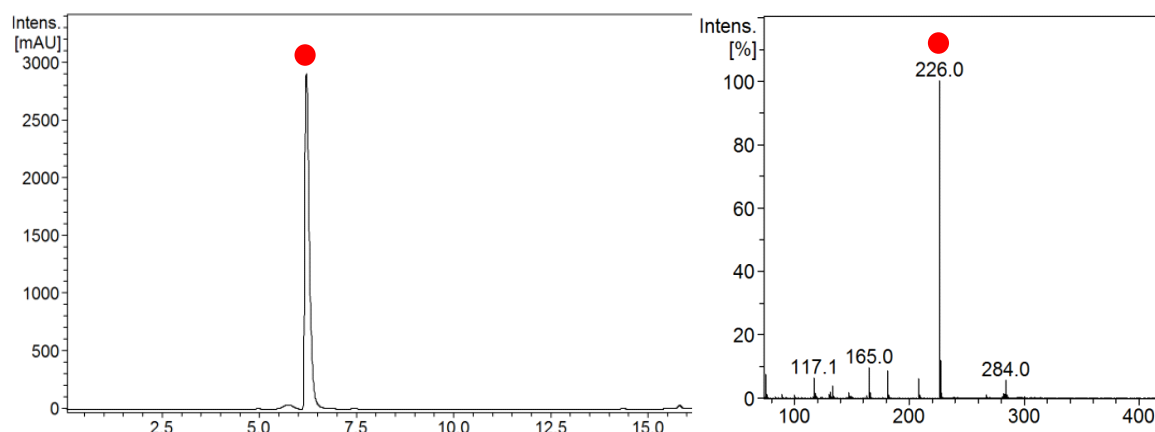
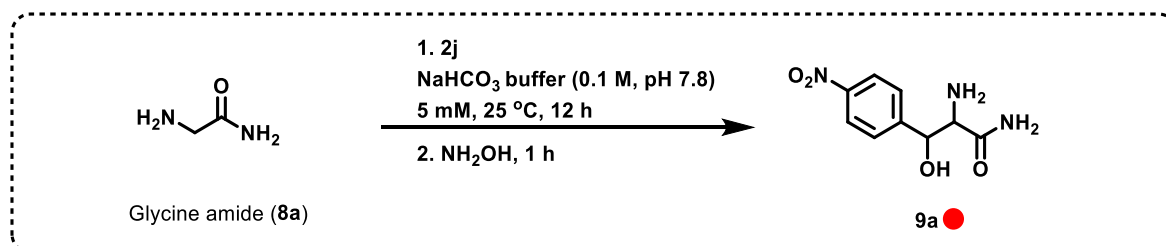


Supplementary Fig. 86. Charged and deconvoluted MS spectra for the unlabeled RNase A (**6k**) in a complex mixture of proteins after reaction with **2j**.

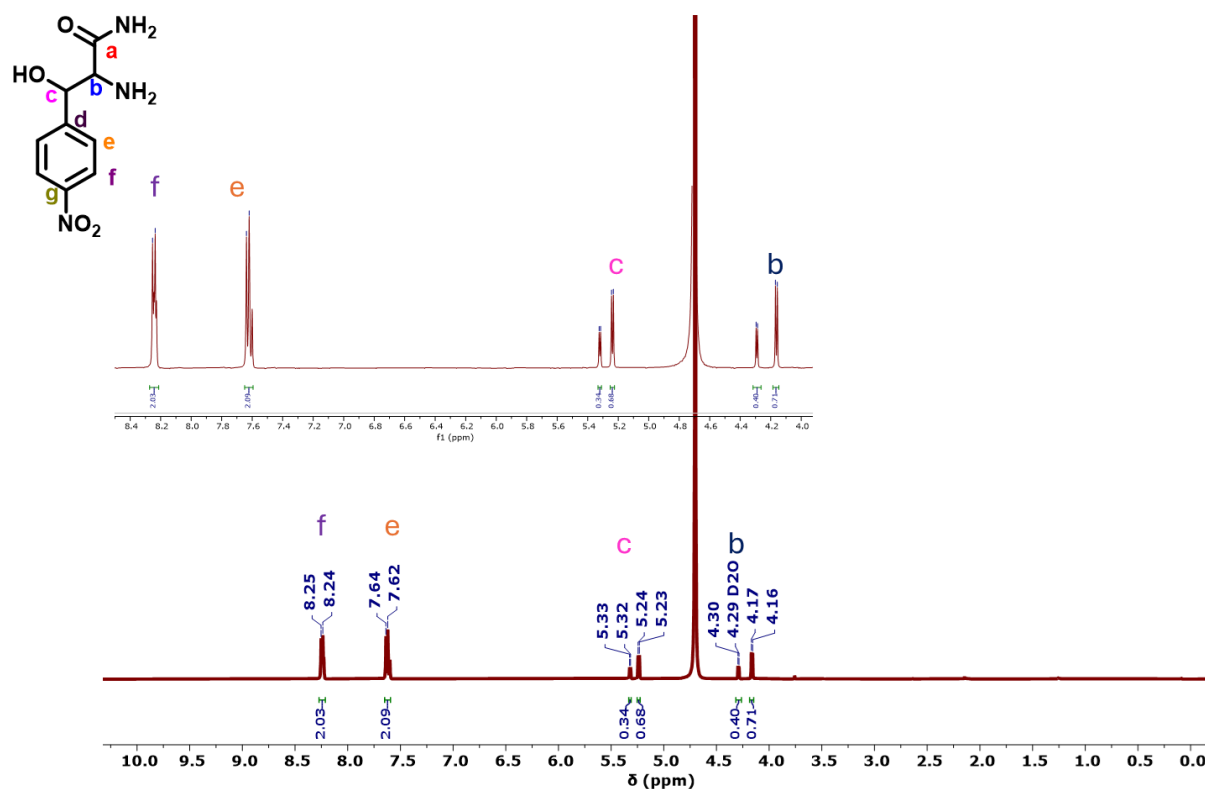


Supplementary Fig. 87. Charged and deconvoluted MS spectra for the unlabeled aprotinin (**6l**) in a complex mixture of proteins after reaction with **2j**.

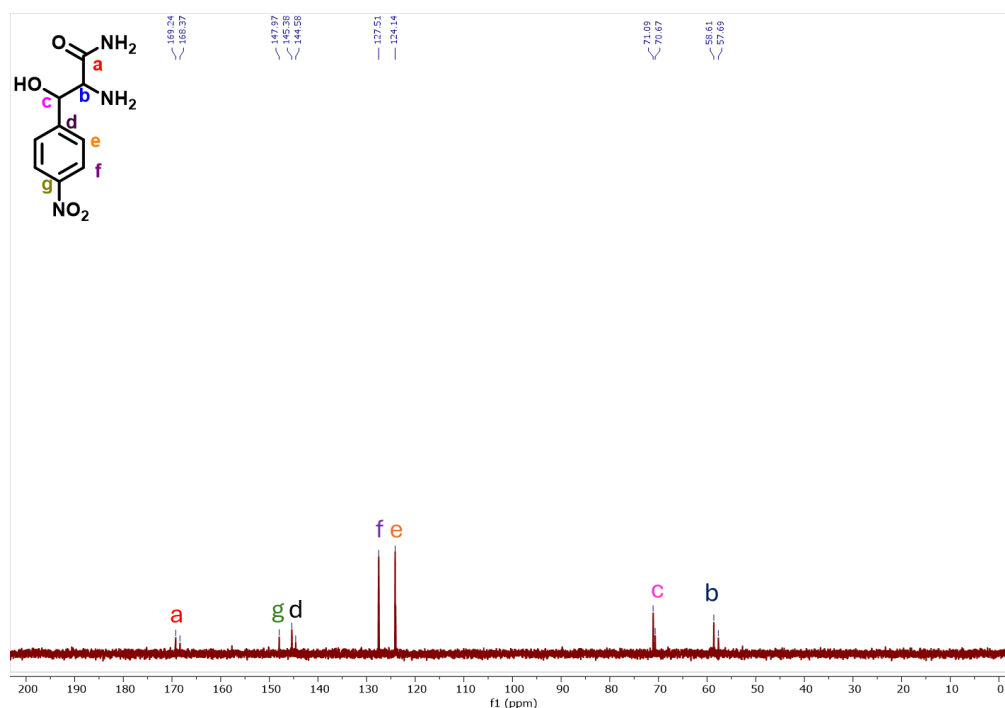
3.9 Confirmation of glycineamide-based aminoalcohol structure by LC-MS, NMR, and XRD



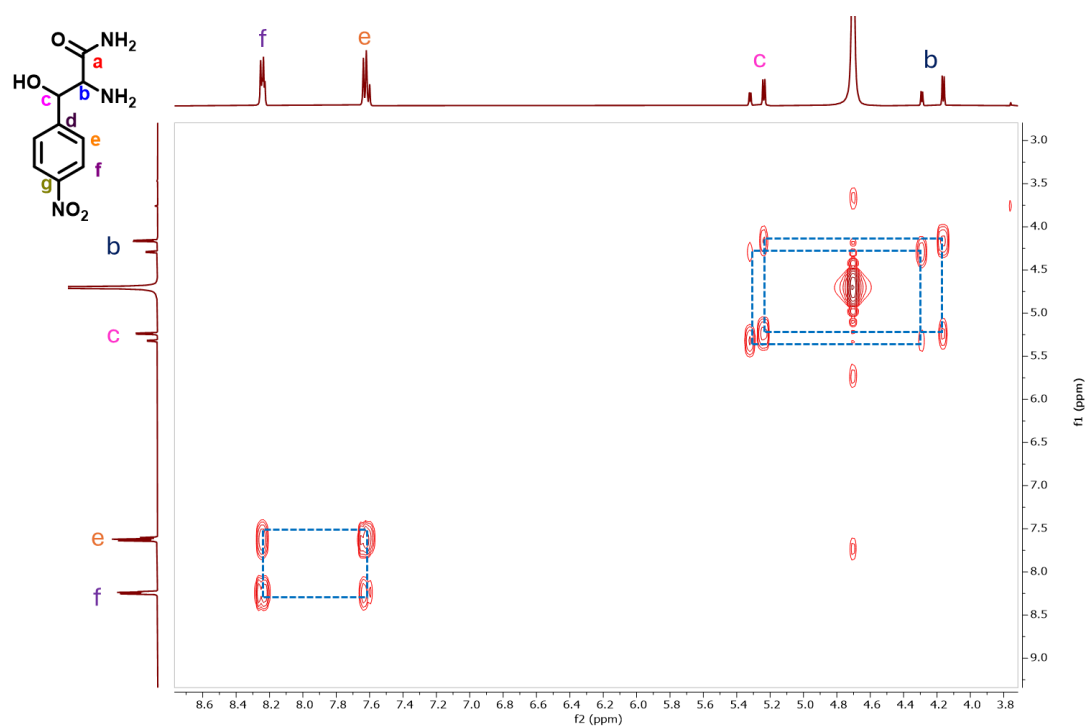
Supplementary Fig. 88. LC (first panel) and MS (second panel) data of the isolated product **9a** (m/z 226.0) after reaction of glycineamide (**8a**) with **2j** supports the formation of aminoalcohol.



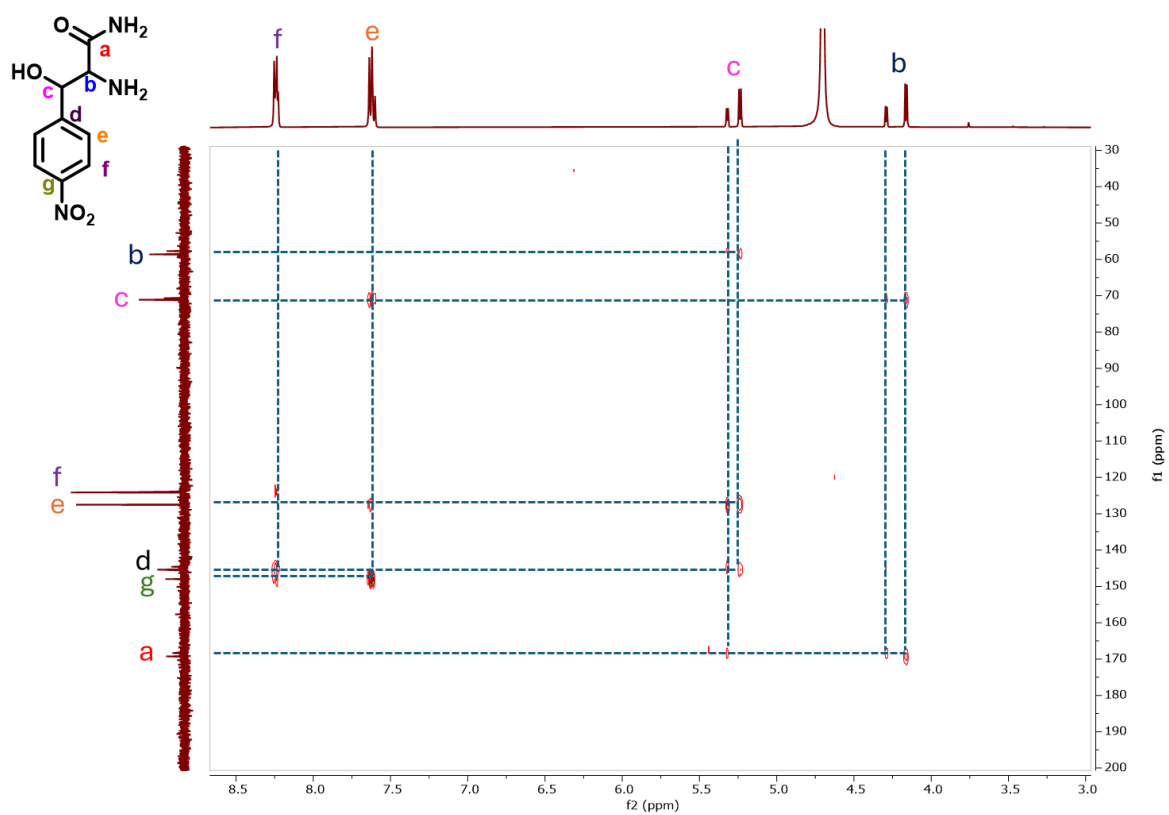
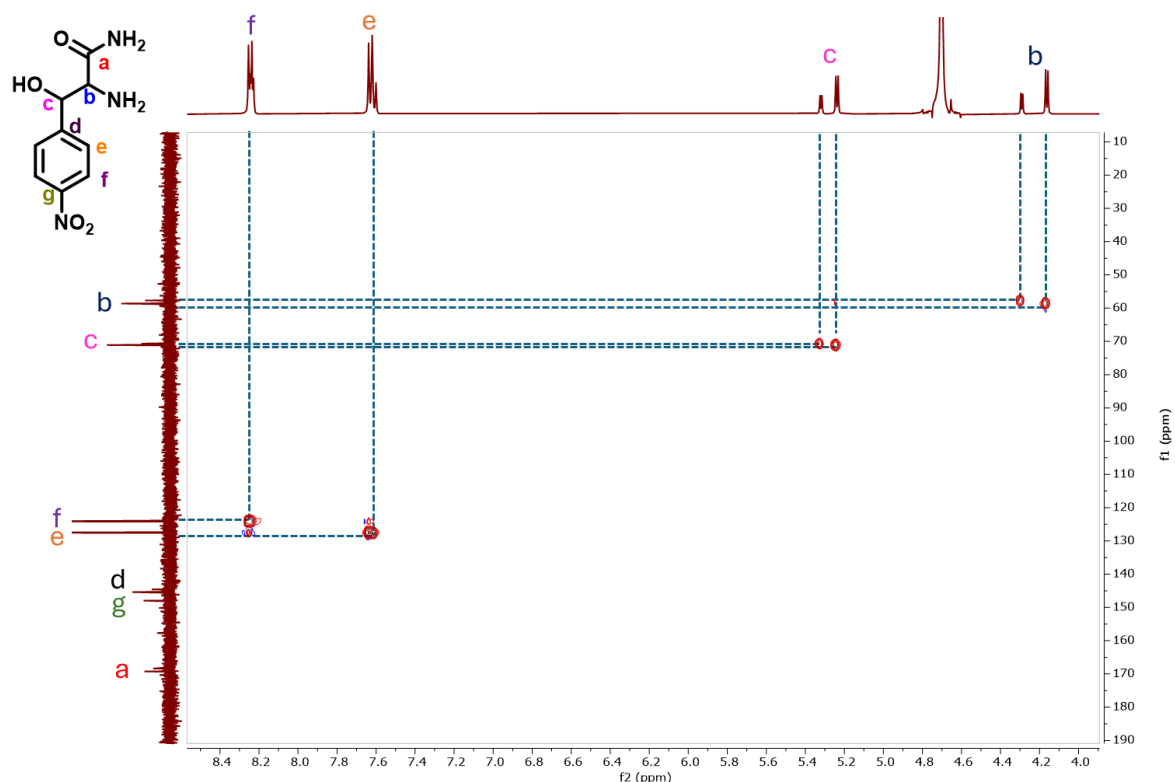
Supplementary Fig. 89. ¹H NMR spectrum of compound **9a** in D₂O.

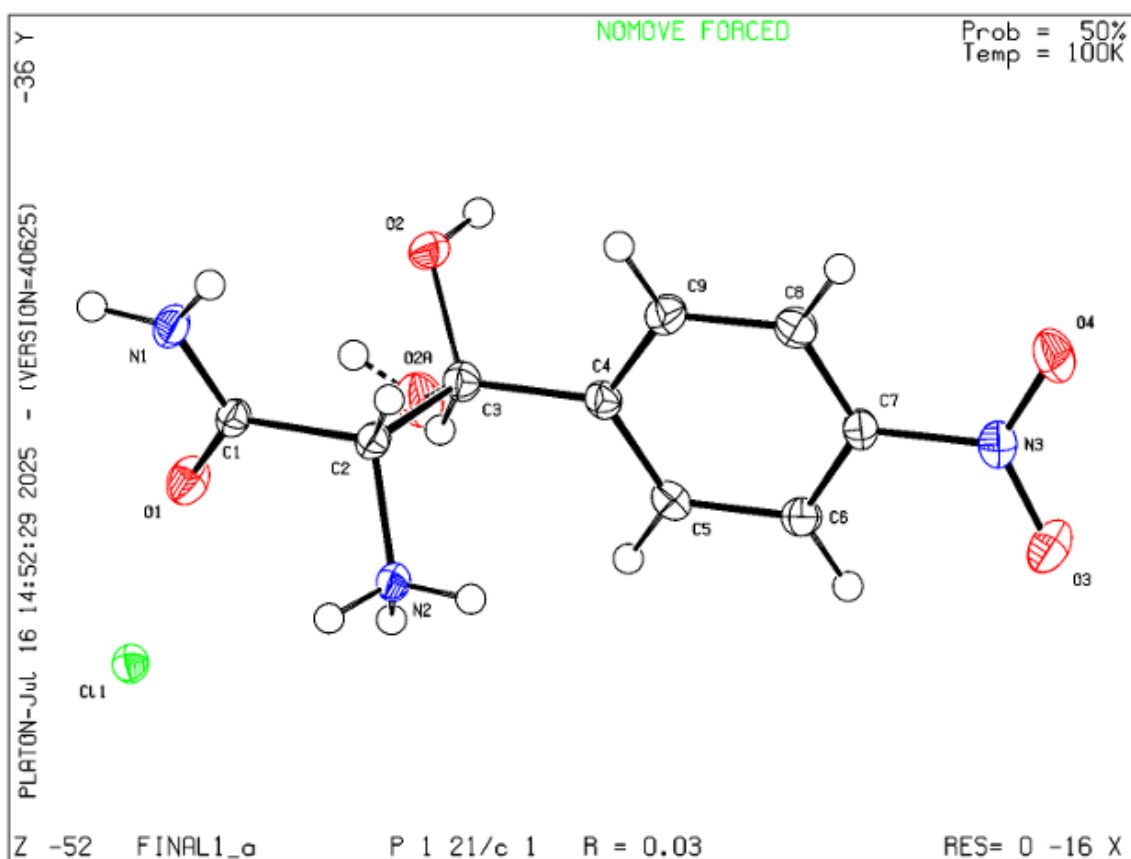


Supplementary Fig. 90. ^{13}C NMR spectrum of compound **9a** in D_2O .



Supplementary Fig. 91. ^1H - ^1H correlation (DQF-COSY) spectrum of compound **9a** in D_2O .





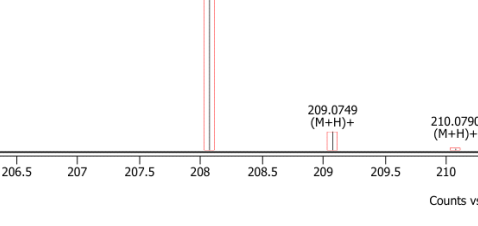
Supplementary Fig. 94. Single crystal X-ray structure of **9a**, CCDC number: 2493333.

Supplementary Table 17. Crystal data and structure refinement

CCDC No.	2493333
Molecule name	9a
Empirical formula	C ₉ H ₁₂ N ₃ O ₄ Cl
Formula weight	261.66
Temperature/K	100
Crystal System	monoclinic
Space group	P 21/c
a/Å	14.9582
b/Å	7.5711

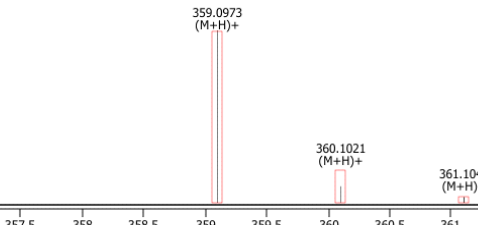
c/Å	10.0347
a/°	90
b/°	107.349
g/°	90
Volume /Å ³	1084.73
Z	4
R(reflections)	0.0334
ρ_{calc} g/cm ³	1.602
μ/mm^{-1}	0.361
F(000)	544.0
Radiation	MoK α (λ = 0.71073)
$\theta(\text{max})$	29.570
h, k, l max	20,10,13
No. of reflections	3048
No. of parameters	181
Goodness-of-fit on F ²	1.039

$$\begin{array}{c}
 \text{R}' \\
 | \\
 \text{H}_2\text{N}-\text{C}(=\text{O})-\text{R} \xrightarrow{\text{R}'-\text{CHO} (2j)} \text{R}'-\text{CH}(\text{OH})-\text{C}(=\text{O})-\text{R} \rightleftharpoons \text{R}'-\text{CH}(\text{OH})-\text{C}(=\text{O})-\text{R} \rightleftharpoons \text{R}'-\text{CH}(\text{OH})-\text{C}(=\text{O})-\text{R} \rightleftharpoons \text{R}'-\text{CH}(\text{OH})-\text{C}(=\text{O})-\text{R} \rightleftharpoons \text{R}'-\text{CH}(\text{OH})-\text{C}(=\text{O})-\text{R} \\
 \text{R} = \text{H}, \text{8a} \quad \text{R} = \text{Me}, \text{8b} \quad \text{R} = \text{H}, \text{8a-IN1} \quad \text{R} = \text{Me}, \text{8b-IN1} \quad \text{R} = \text{H}, \text{8a-IN2} \quad \text{R} = \text{Me}, \text{8b-IN2} \quad \text{R} = \text{H}, \text{8a-IN3} \quad \text{R} = \text{Me}, \text{8b-IN3} \quad \text{R} = \text{H}, \text{9a} \quad \text{R} = \text{Me}, \text{9b}
 \end{array}$$

a) 

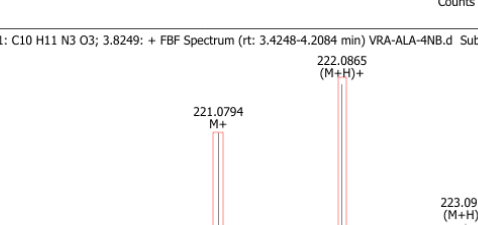
Cpd 1: C9 H9 N3 O3; 1.3927; + FBF Spectrum (rt: 1.1933-1.7748 min) VR-GLY-4NB.d Subtract

Intermediate: **8a-IN1**
Calculated m/z: 208.0717
Observed m/z: 208.0716

b) 

Cpd 1: C16 H14 N4 O6; 3.8185; + FBF Spectrum (rt: 3.7520-4.3169 min) VR-GLY-4NB.d Subtract

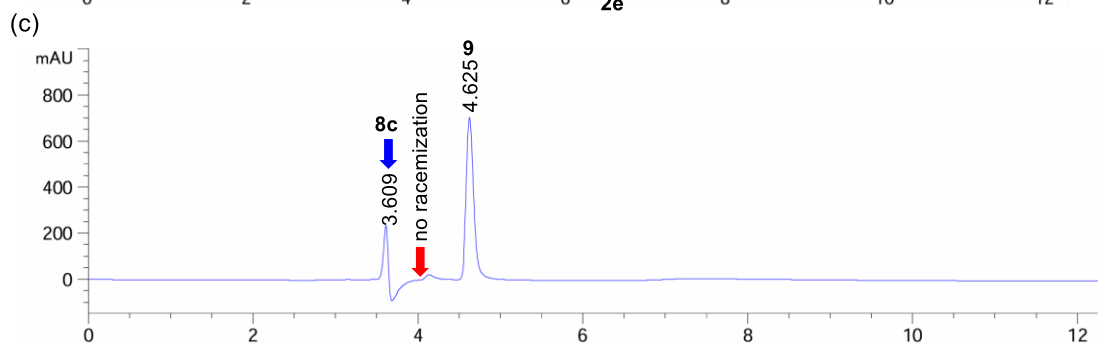
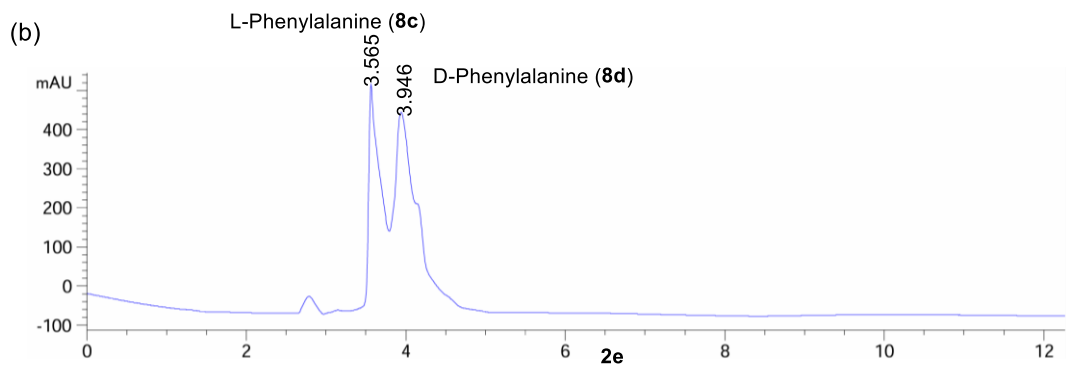
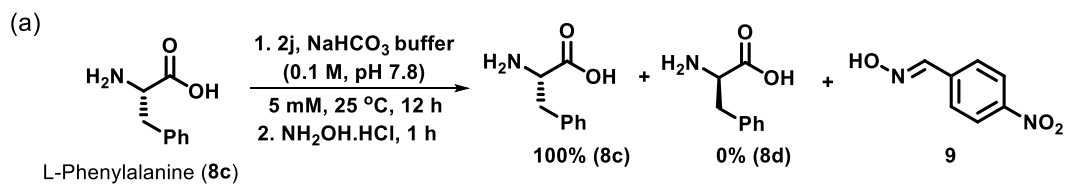
Intermediate: **8a-IN3**
Calculated m/z: 359.0986
Observed m/z: 359.0973

c) 

Cpd 1: C10 H11 N3 O3; 3.8249; + FBF Spectrum (rt: 3.4248-4.2084 min) VRA-ALA-4NB.d Subtract

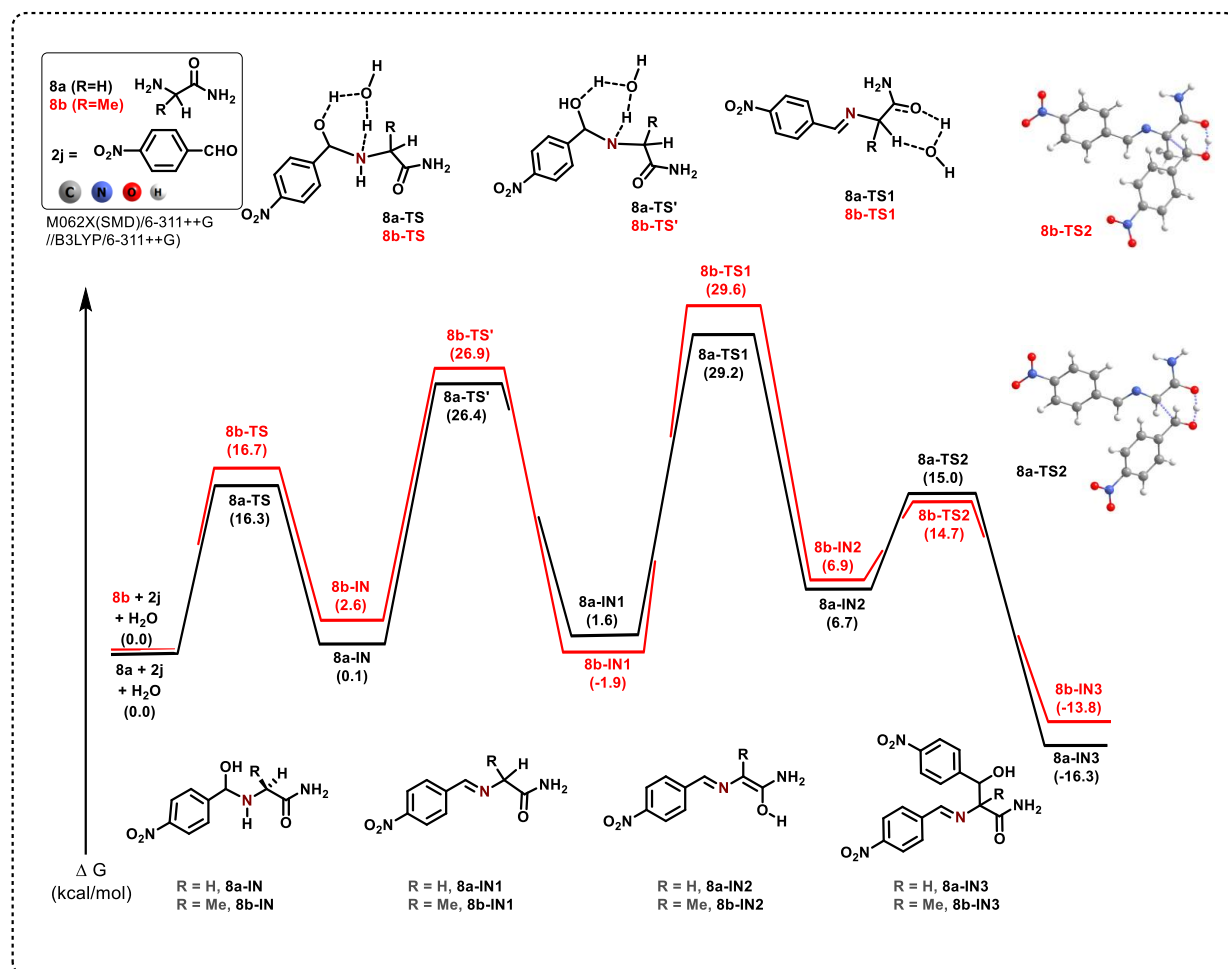
Intermediate: **8b-IN1**
Calculated m/z: 221.0795
Observed m/z: 221.0794

S95

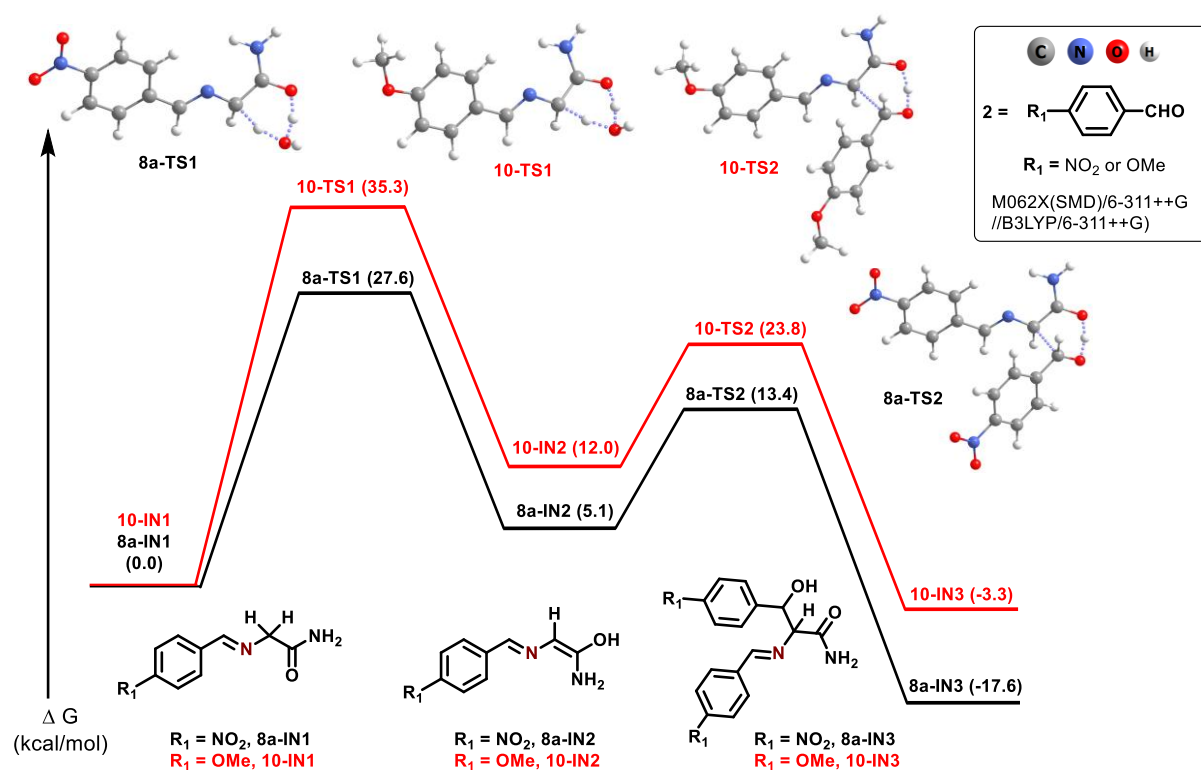


Supplementary Fig. 97. (a) Control experiment to investigate nucleophilic intermediate led **8c** racemization under the reaction conditions. (b) Standard HPLC profile for L-phenylalanine **8c** and D-phenylalanine **8d** (1:1) ratio. (c) Stereo-stability of substituted amino acid (**8c**) under the reaction conditions.

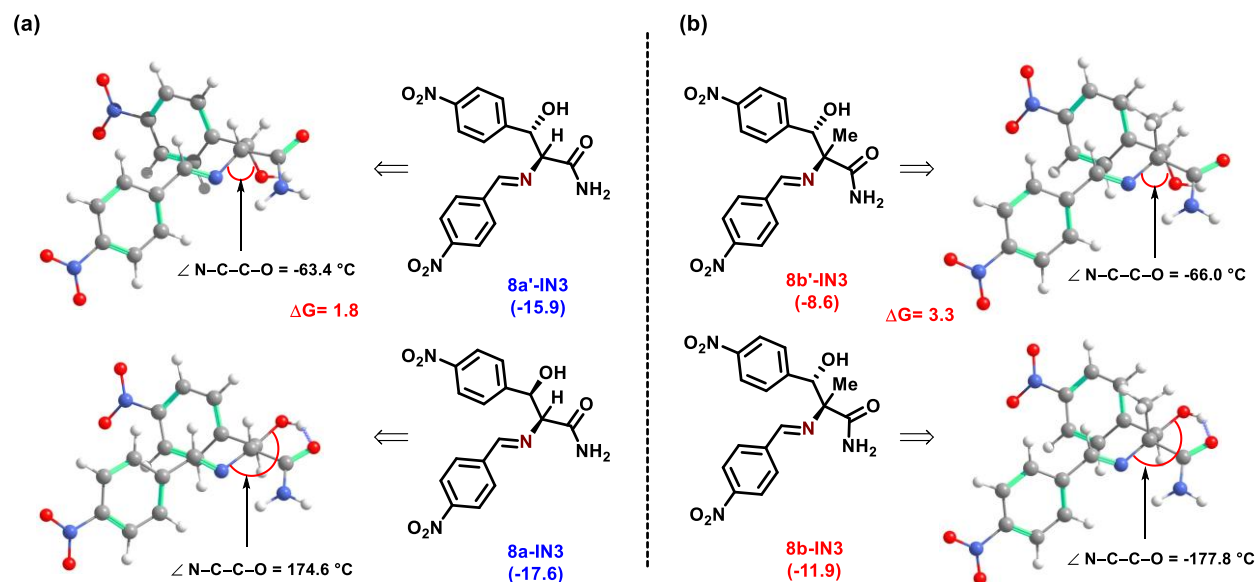
3.10 Computational data



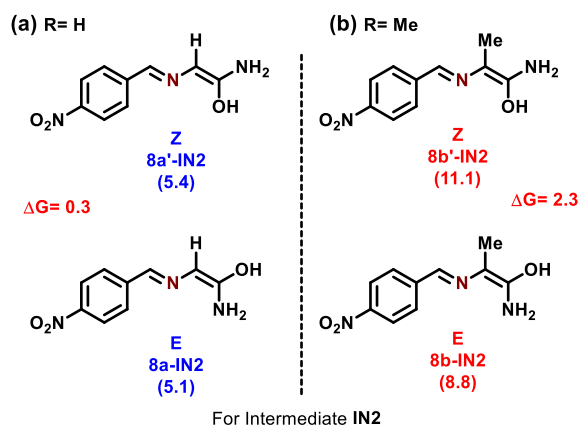
Supplementary Fig. 98. Potential energy profile for the complete pathway.



Supplementary Fig. 99 | Comparative free energy profile for 4-nitrobenzaldehyde (**2j**) and 4-methoxybenzaldehyde (**2b**). Mechanistic insights by DFT calculation using M062X/6-311++G(d,p)//B3LYP/6-311++G(d,p) method. Non-essential hydrogen atoms are omitted for clarity.

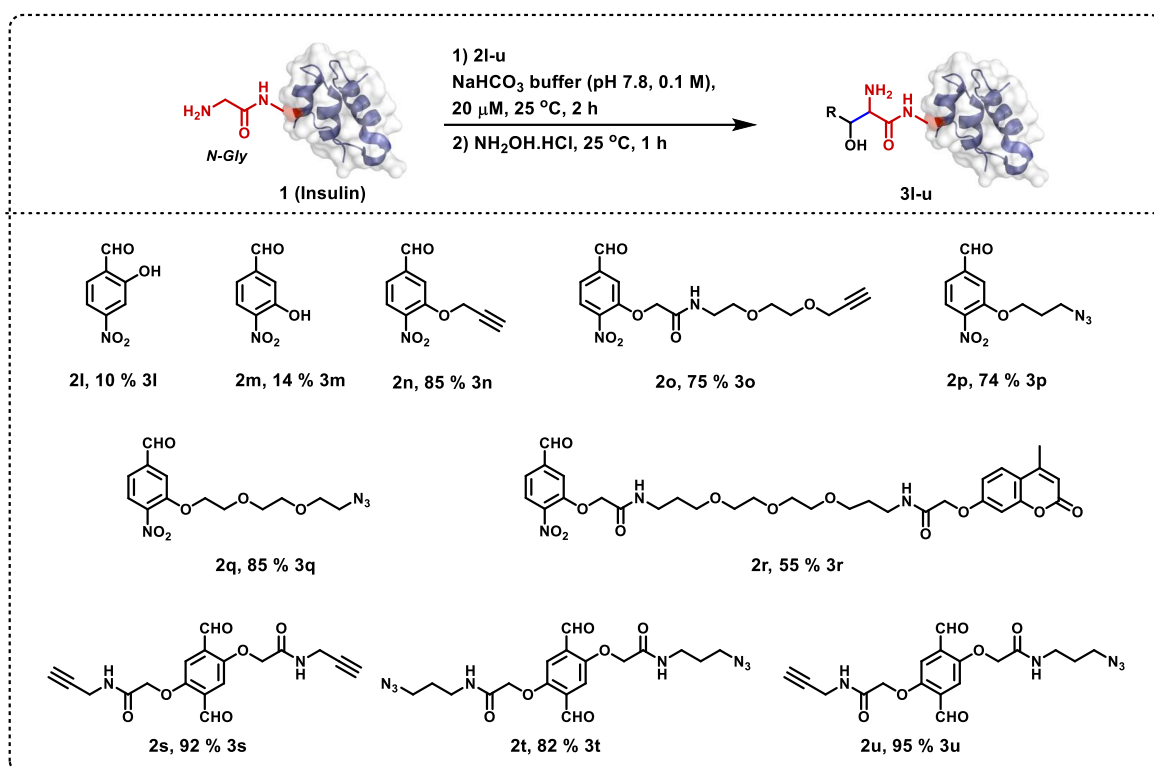


Supplementary Fig. 100 | The conformation of syn- and anti-isomers structures for the intermediates (A) **8a-IN3**, and **8a'-IN3**; (B) **8b-IN3** and **8b'-IN3**.

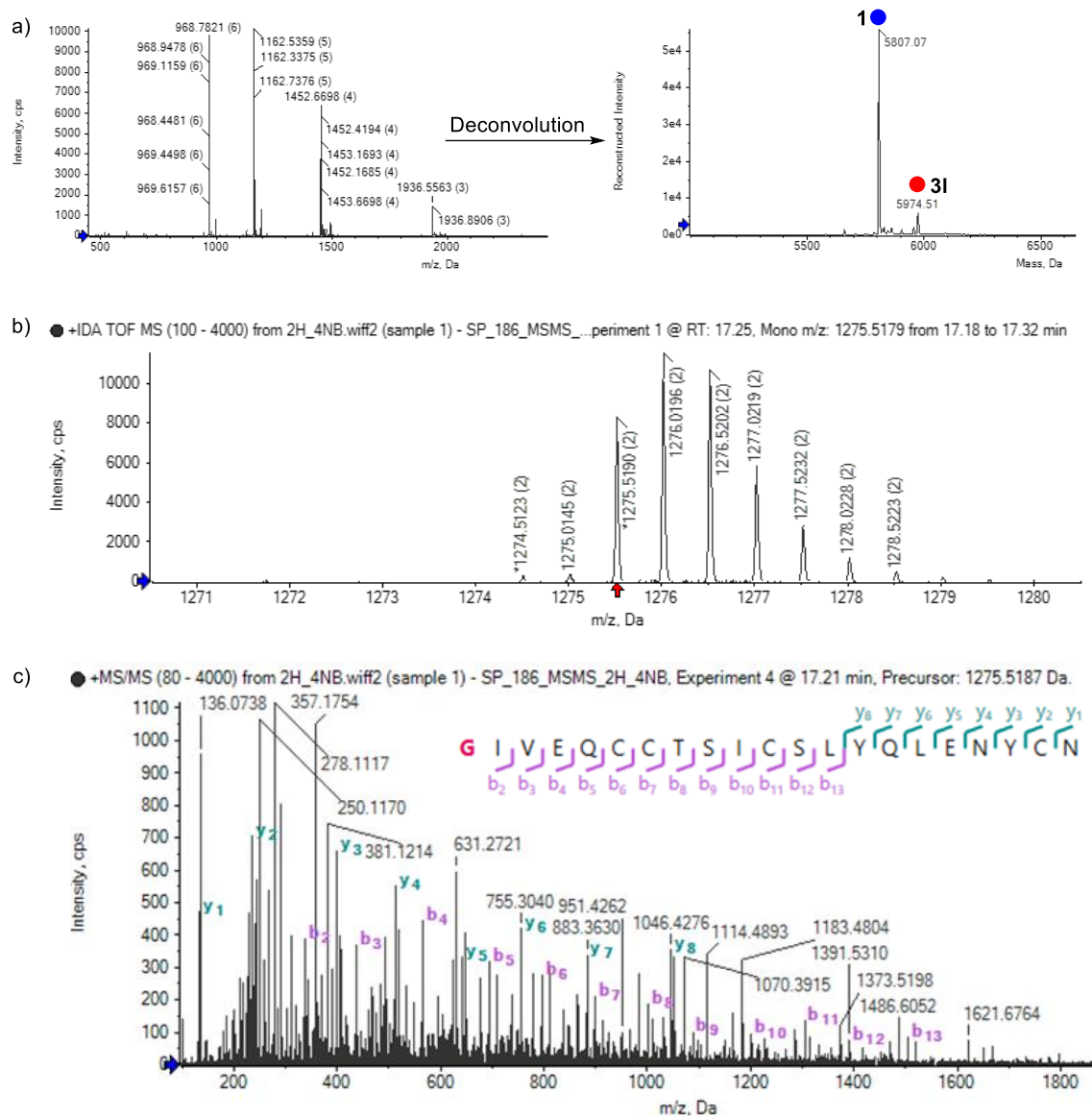


Supplementary Fig. 101 | The calculated intermediate **IN2** (R= -H, -Me) for the E- and Z-isomeric forms.

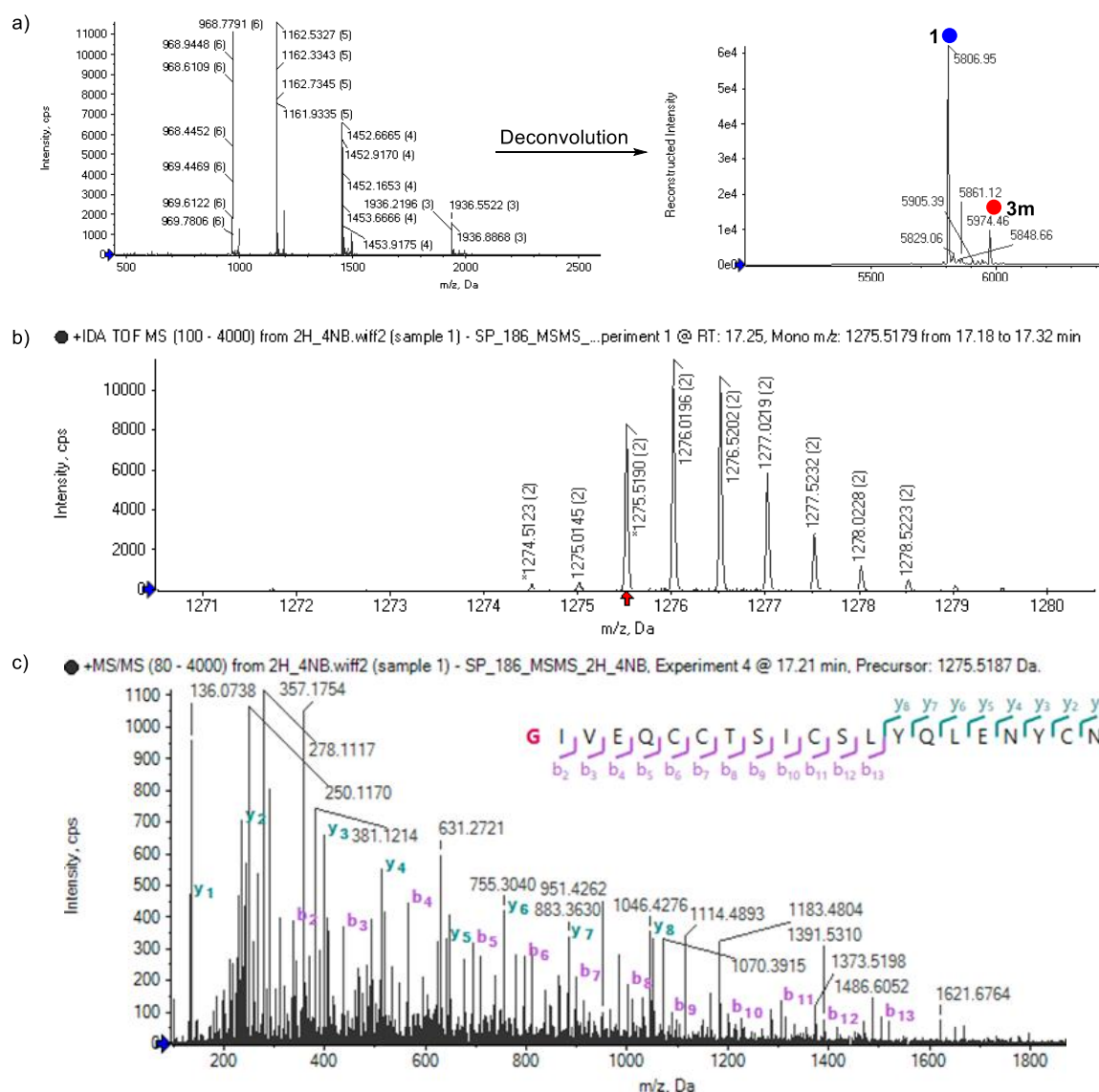
3.11 Reagent design flexibility for late-stage derivatization



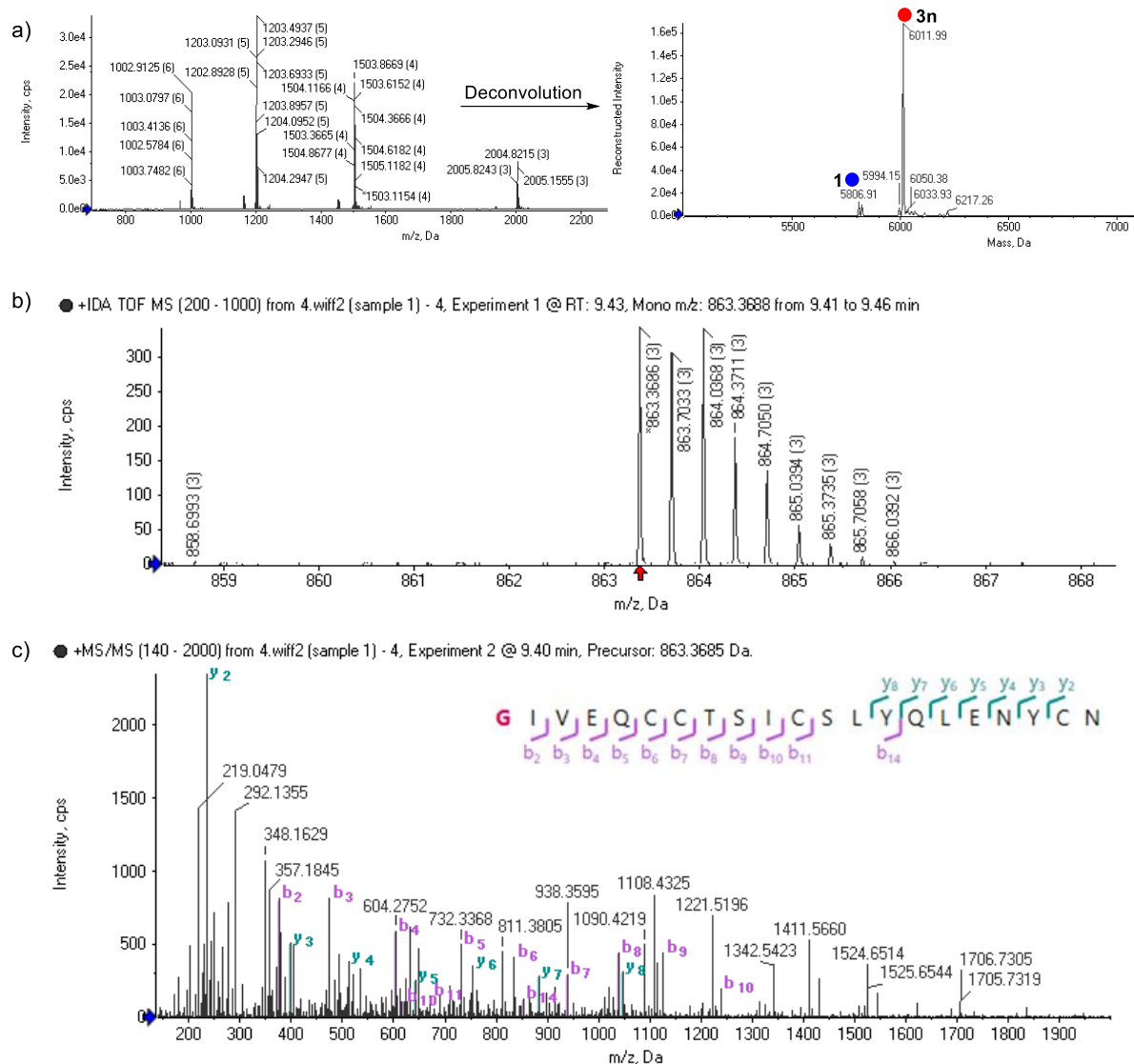
Supplementary Fig. 102. N-Gly modification of insulin with derivatized *rapi*-Gly tag reagents equipped with various biorthogonal handles.

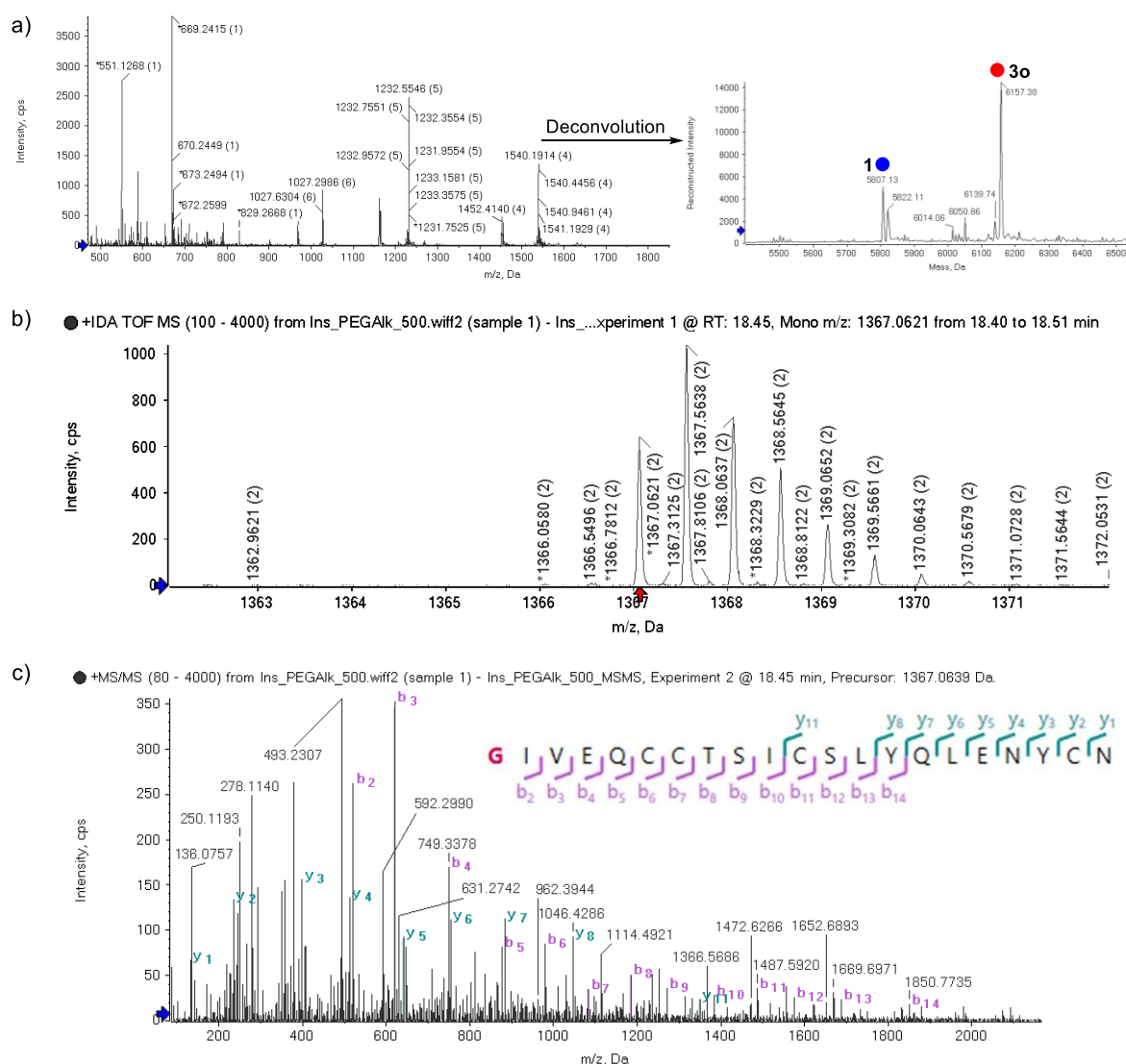


Supplementary Fig. 103. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2I** for N-Gly bioconjugation. (b) Peptide mapping of **3I** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI C SLYQLENYCN (G1-N21, m/z 1275.5187, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).

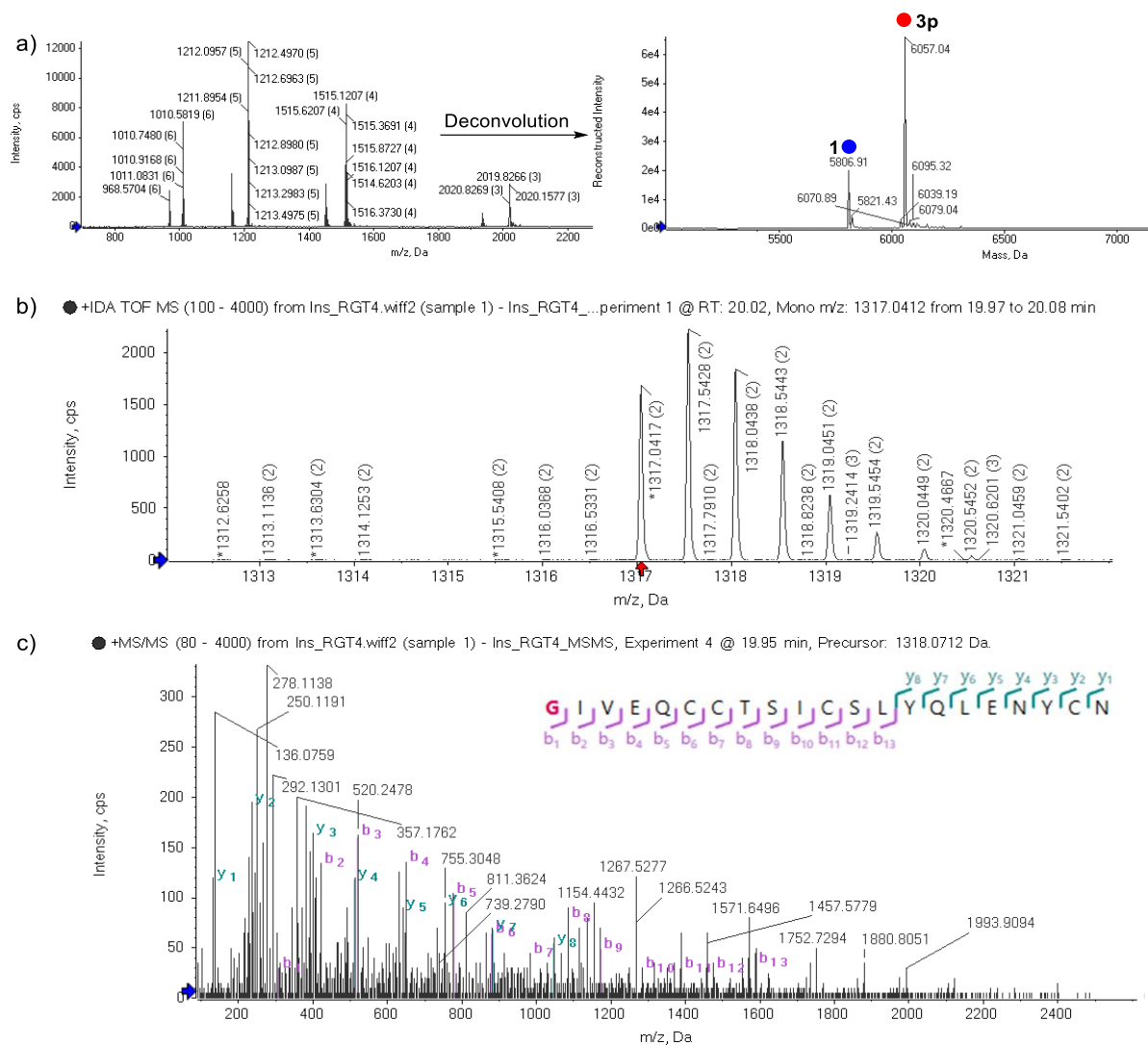


Supplementary Fig. 104. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2m** for N-Gly bioconjugation. (b) Peptide mapping of **3m** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTICSLSLYQLENYCN (G1-N21, m/z 1275.51, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).

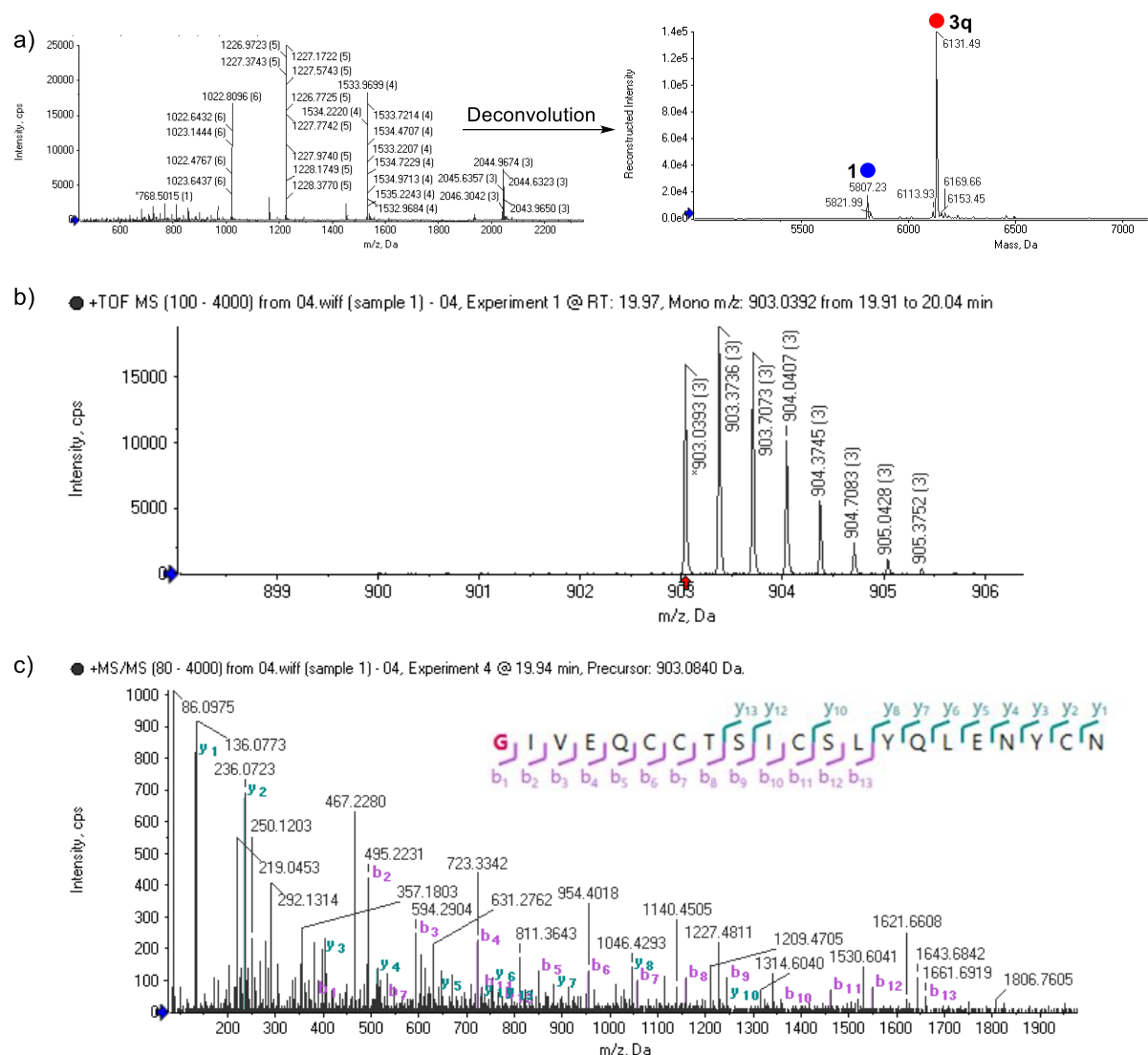




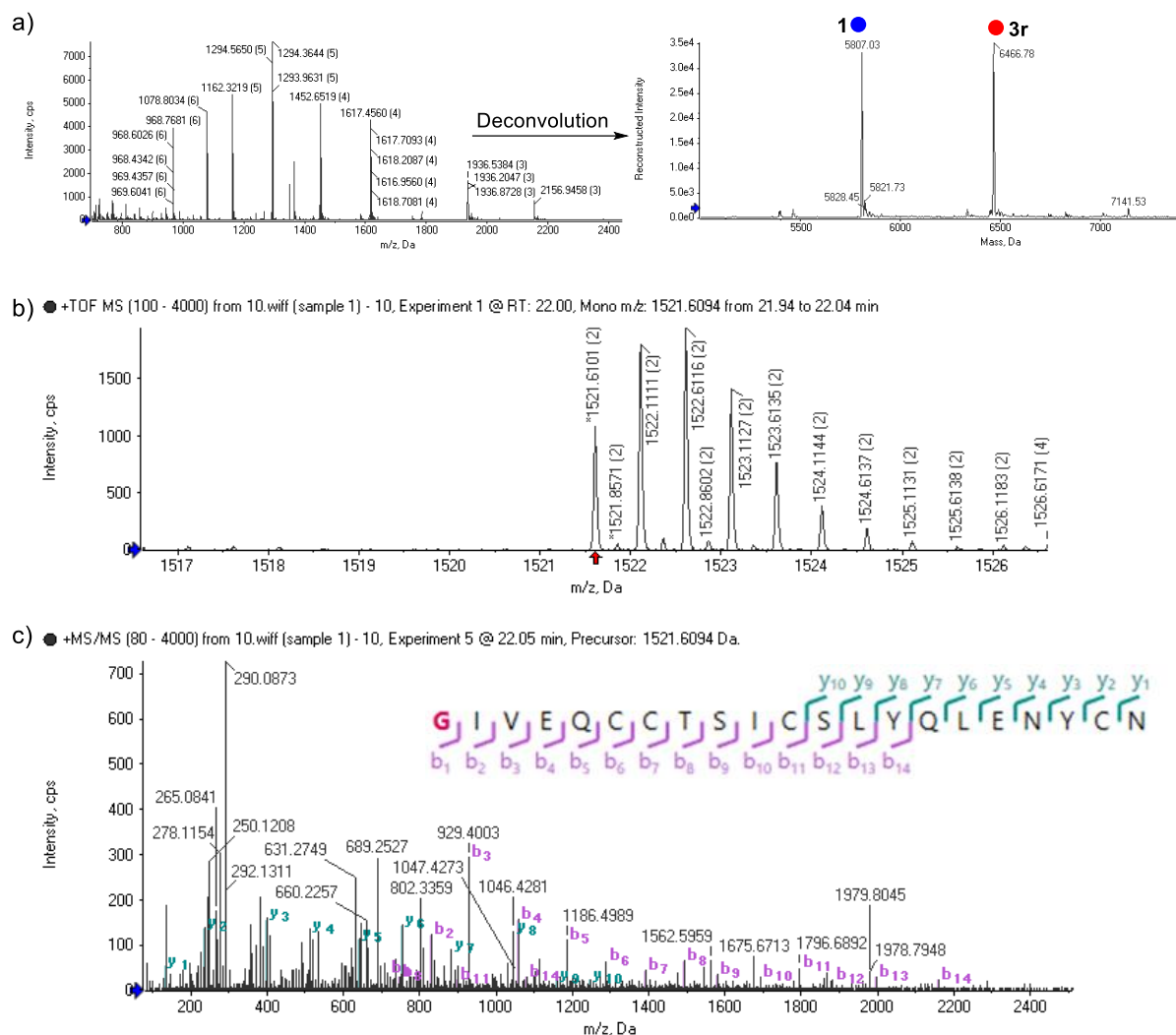
Supplementary Fig. 106. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2o** for N-Gly bioconjugation. (b) Peptide mapping of **3o** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSICSLYQLENYCN (G1-N21, m/z 1367.06, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



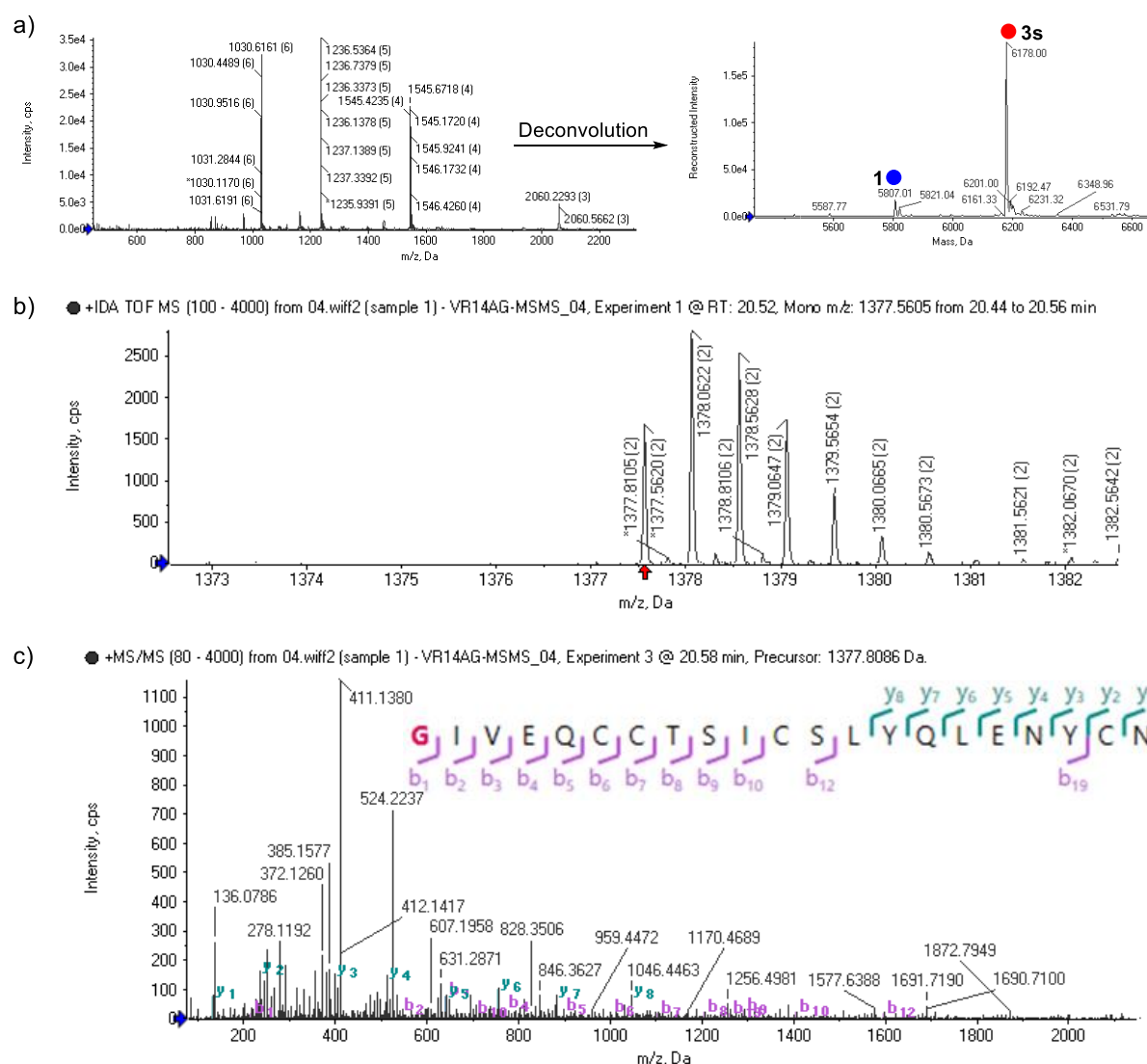
Supplementary Fig. 107. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2p** for N-Gly bioconjugation. (b) Peptide mapping of **3p** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI C S L Y Q L E N Y C N (G1-N21, m/z 1318.07, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).



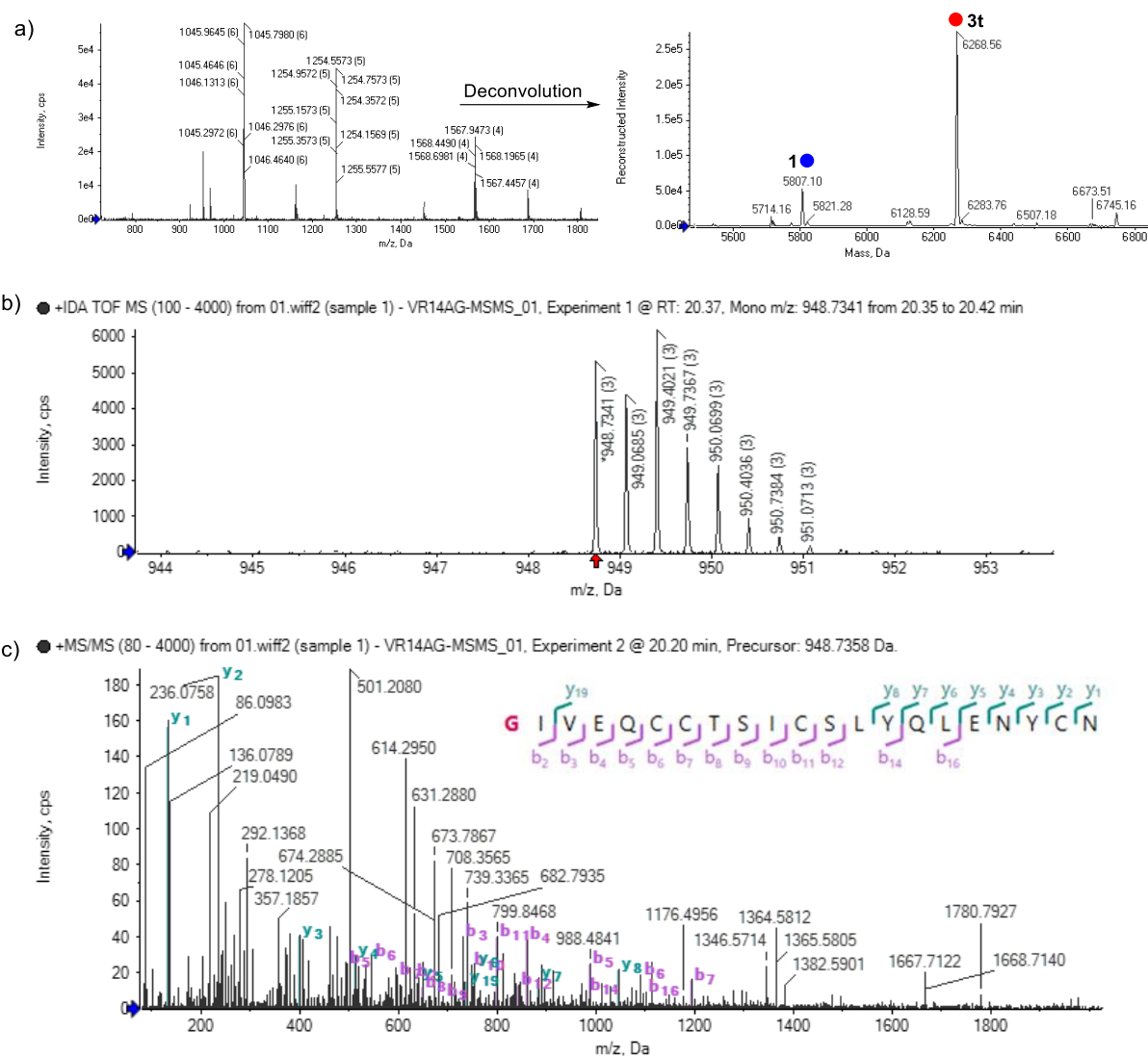
Supplementary Fig. 108. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2q** for N-Gly bioconjugation. (b) Peptide mapping of **3q** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI C S L Y Q L E N Y C N (G1-N21, m/z 903.08, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



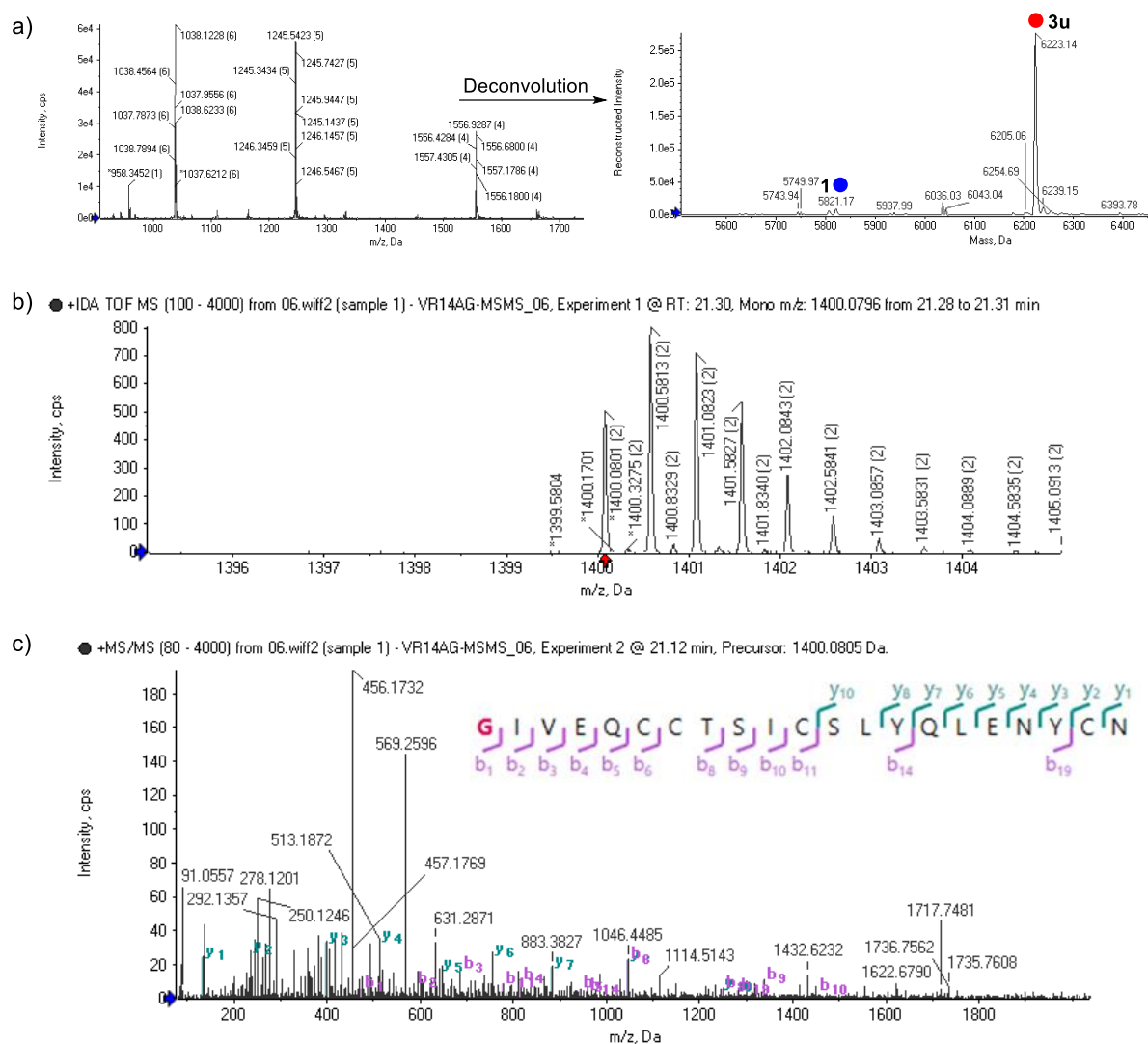
Supplementary Fig. 109. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2r** for N-Gly bioconjugation. (b) Peptide mapping of **3r** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSICSLYQLENYCN (G1-N21, m/z 1521.60, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).



Supplementary Fig. 110. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2s** for N-Gly bioconjugation. (b) Peptide mapping of **3u** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSICSLYQLENYCN (G1-N21, m/z 1377.80, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).

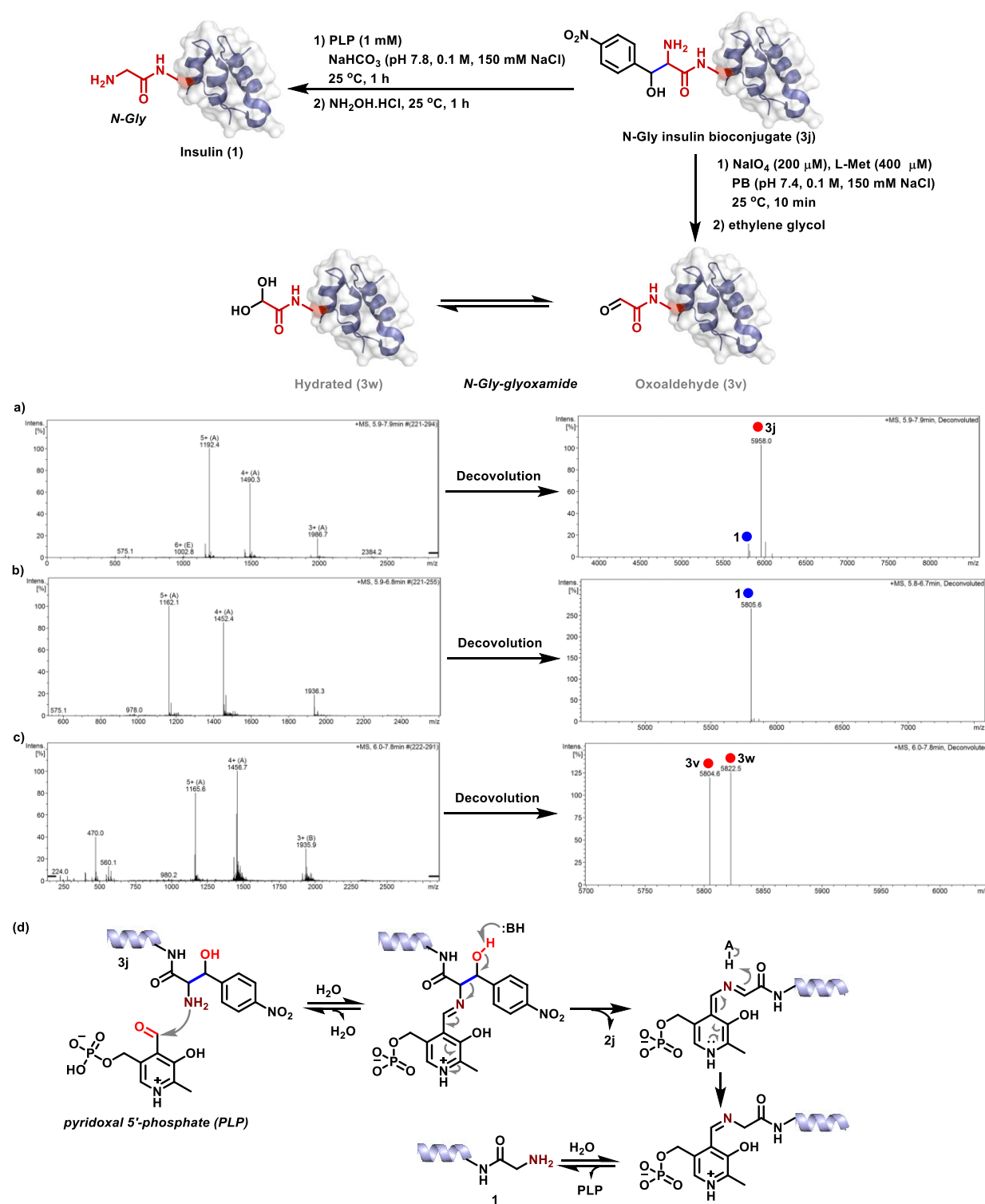


Supplementary Fig. 111. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2t** for N-Gly bioconjugation. (b) Peptide mapping of **3t** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSI CS L Y Q L E N Y C N (G1-N21, m/z 948.73, $[M+3H]^{3+}$) confirms the site of modification, N-Gly (G1).



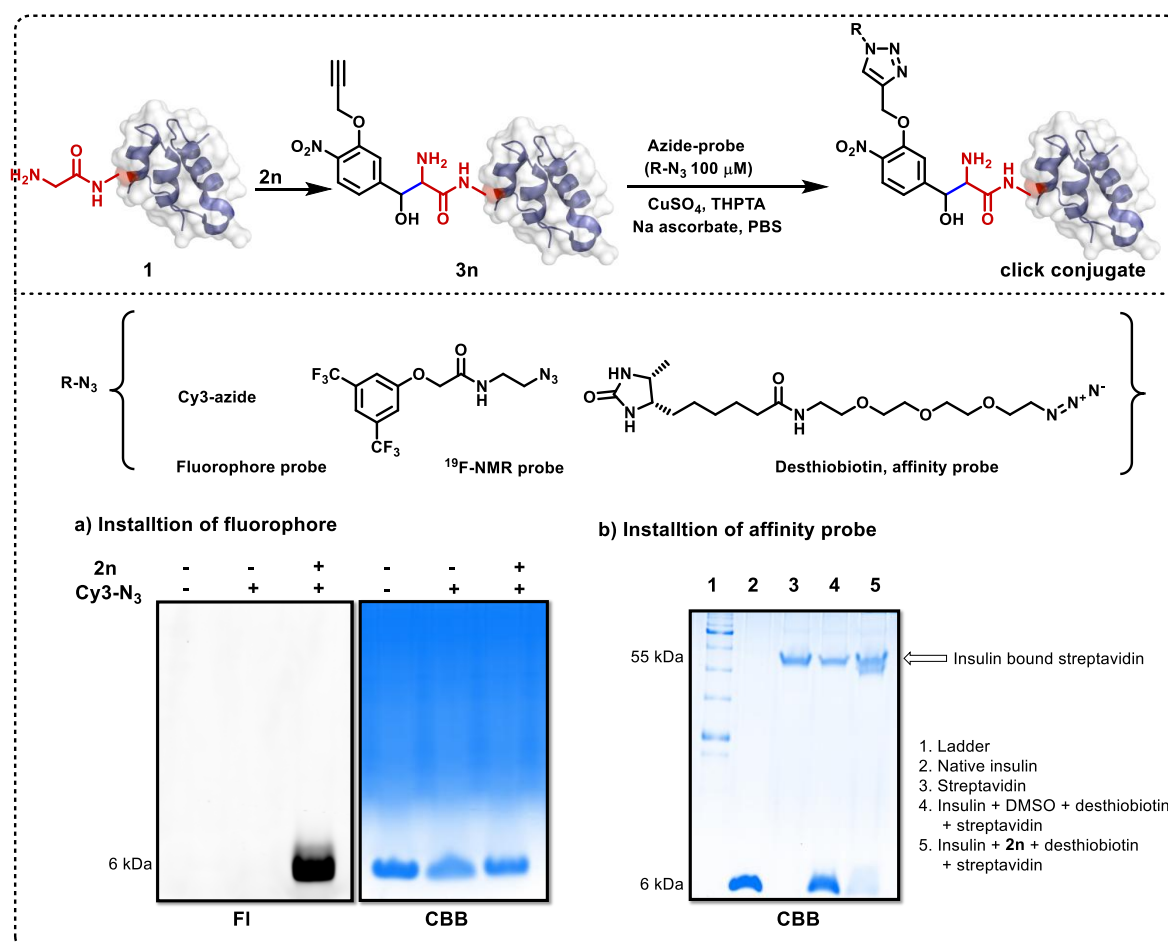
Supplementary Fig. 112. (a) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2u** for N-Gly bioconjugation. (b) Peptide mapping of **3u** after DTT cleavage. (c) MS-MS of labeled peptide GIVEQCCTSLYQLENYCN (G1-N21, m/z 1400.08, $[M+2H]^{2+}$) confirms the site of modification, N-Gly (G1).

3.12 Late-stage bond engineering of aminoalcohol

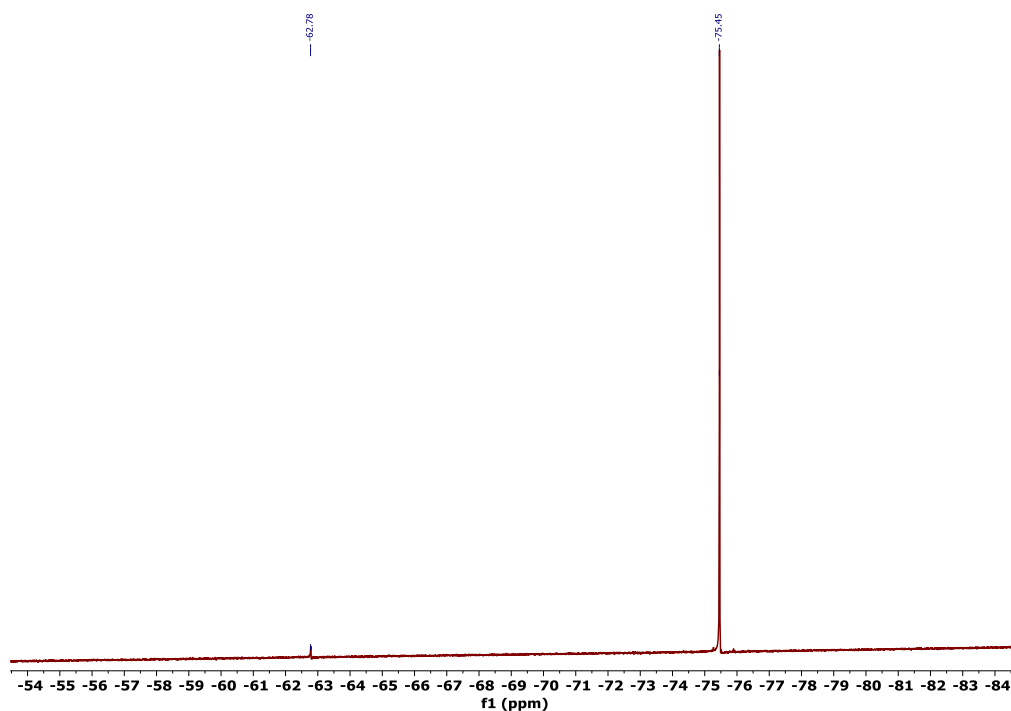


Supplementary Fig. 113. Late-stage bond engineering of amino alcohol for C-C bond dissociation and oxidation (a) Charged and deconvoluted MS spectra for insulin (1) after reaction with 2j for N-Gly bioconjugation. (b) Charged and deconvoluted MS spectra for N-Gly modified insulin (3j) after C-C bond dissociation with pyridoxal 5'-phosphate (PLP). (c) Charged and deconvoluted MS spectra for N-Gly modified insulin (3j) after oxidation of aminoalcohol with NaIO_4 . (d) Resonance-assisted electron density (RED) polarization mechanism for C-C bond dissociation of 3j using PLP.

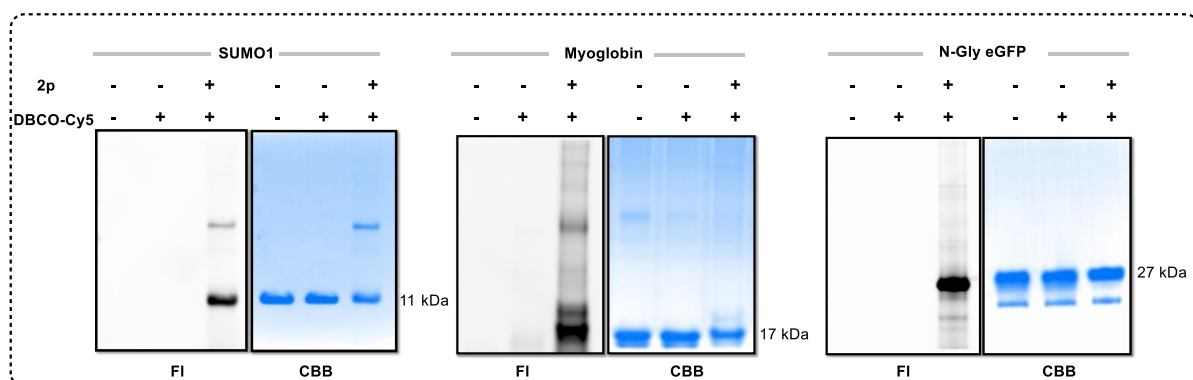
3.13 Late-stage probe installation through click chemistry



Supplementary Fig. 114. Cu-catalyzed click chemistry for installation of probe on insulin N-Gly bioconjugate **3n**. (a) The uncropped data for in-gel fluorescence analysis of Cy3 installation on insulin bioconjugate **3n** with SDS PAGE. (b) Desthiobiotin installation on insulin bioconjugate **3n** followed by binding with streptavidin. The binding of desthiobiotin-conjugated insulin with streptavidin is analyzed by SDS-PAGE. FI: In-gel-fluorescence, CBB: Coomassie brilliant blue.

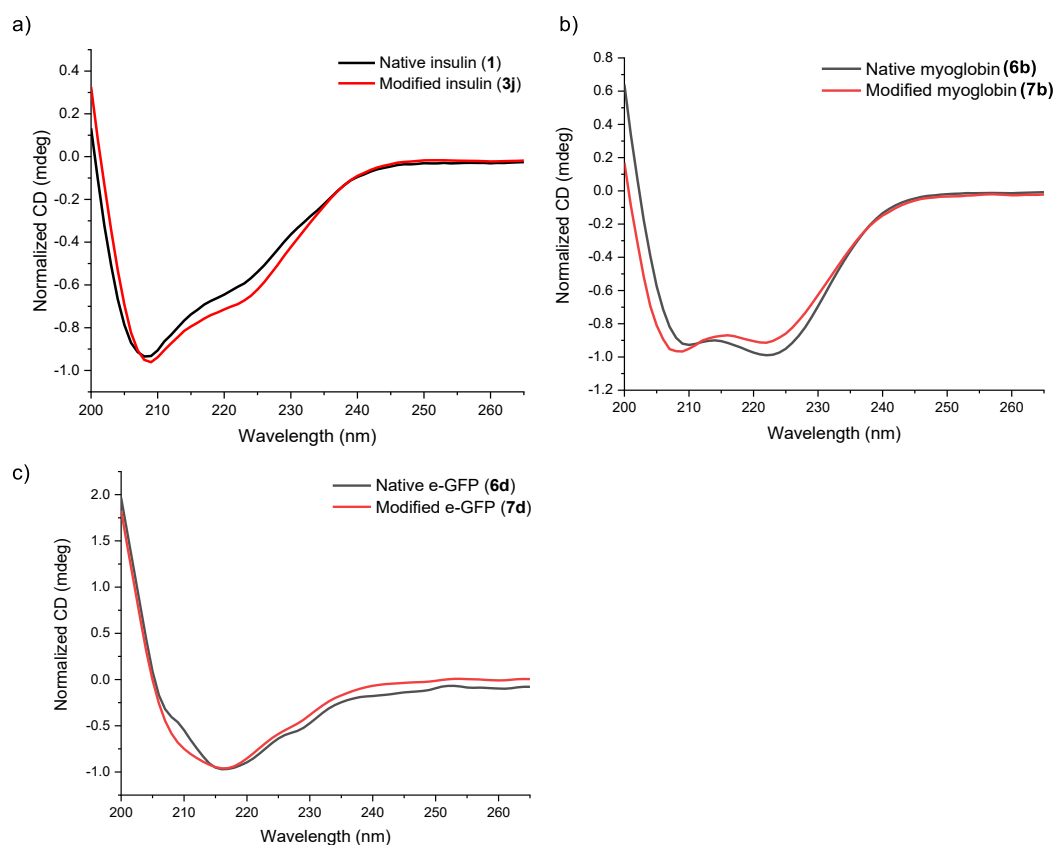


Supplementary Fig. 115. ^{19}F -NMR spectrum of labeled insulin **3n** post click reaction with ^{19}F -azide NMR probe. NMR was performed in phosphate buffer (0.1 M, pH 7.0): D_2O (9:1).



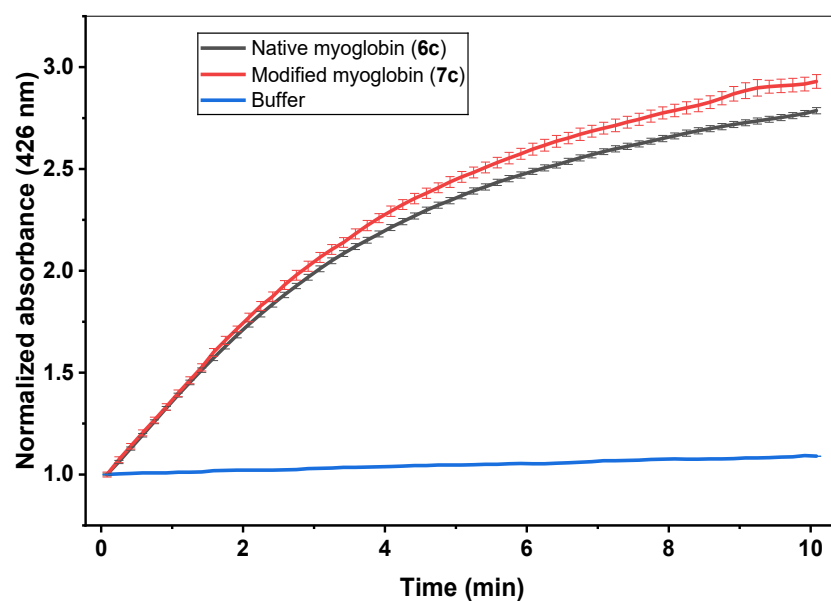
Supplementary Fig. 116. Cu-free click chemistry for installation of probe on N-Gly proteins: SUMO1 (**6b**), myoglobin (**6c**), and N-Gly eGFP (**6d**) after N-Gly bioconjugation with **2p**. The uncropped data for in-gel fluorescence analysis of Cy5 installation using SDS-PAGE. FI: In-gel-fluorescence, CBB: Coomassie brilliant blue.

3.14 Circular dichroism spectra of proteins

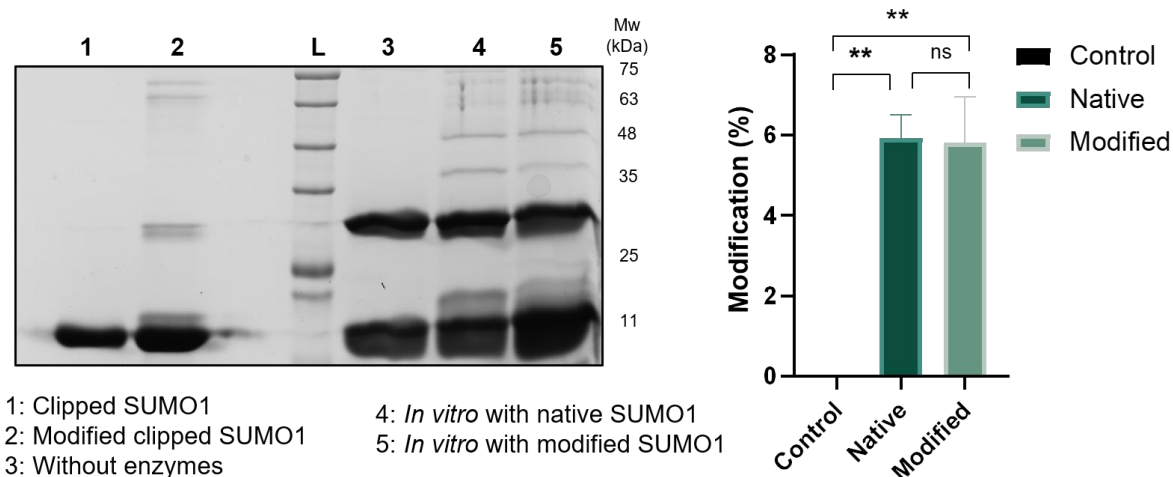


Supplementary Fig. 117. Effect of the labeling on the structure of proteins. (a) Circular Dichroism (CD) of insulin **1** (black line) and modified insulin **3j** (red line). (b) CD of myoglobin **6c** (black line) and modified myoglobin **7c** (red line). (c) CD of N-Gly eGFP **6d** (black line) and modified N-Gly eGFP **7d** (red line).

3.15 Activity of modified proteins



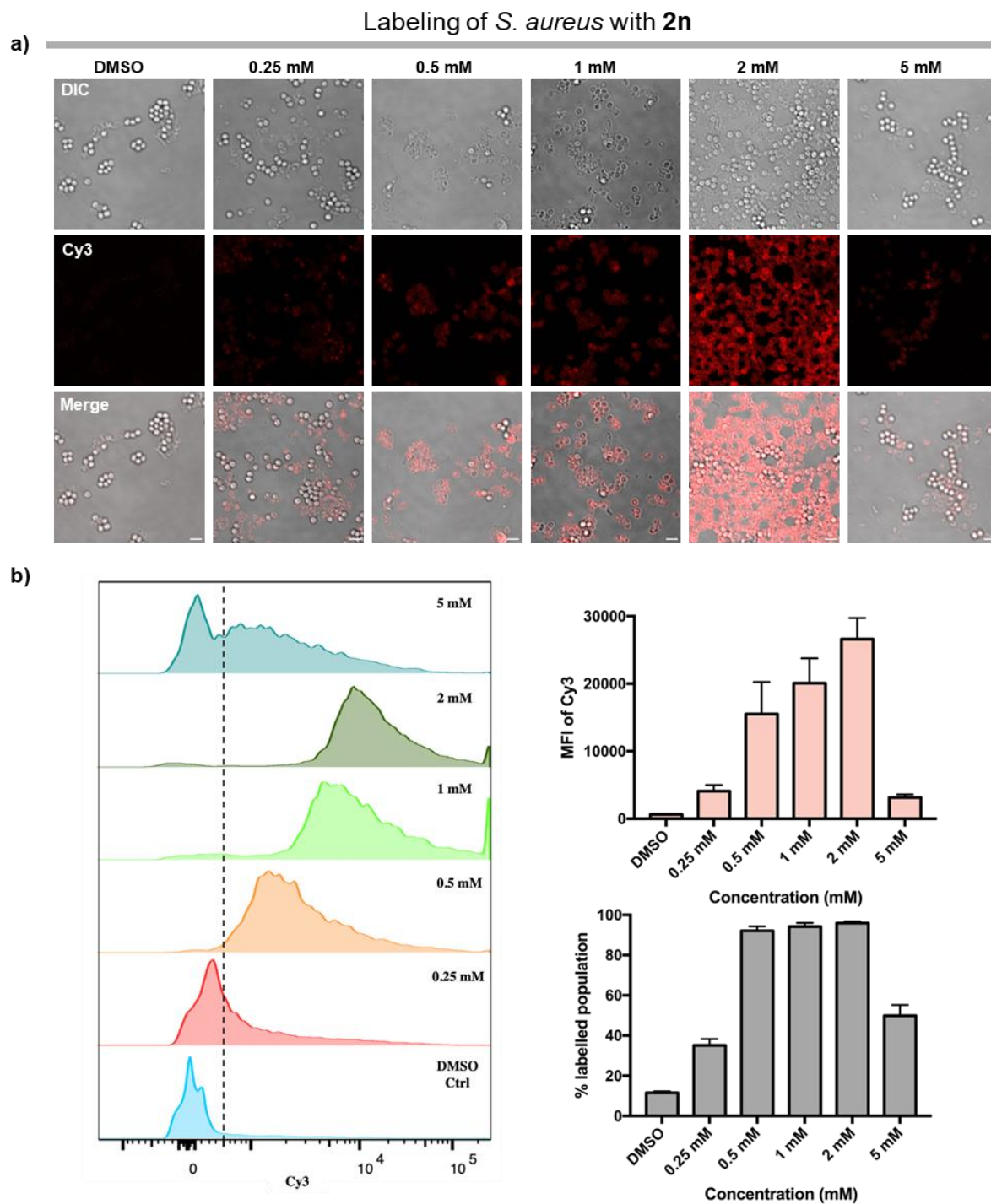
Supplementary Fig. 118. Effect of labeling on the enzymatic activity of myoglobin. Time-dependent UV-Vis spectra were recorded to compare the enzymatic activity of native myoglobin (**6b**) and labeled myoglobin (**7b**). Myoglobin activity before and after the labeling is examined by oxidation of *o*-phenylenediamine with hydrogen peroxide. The enzymatic activity of myoglobin remains unperturbed after the chemical modification. Experiments were performed *n* = 3.

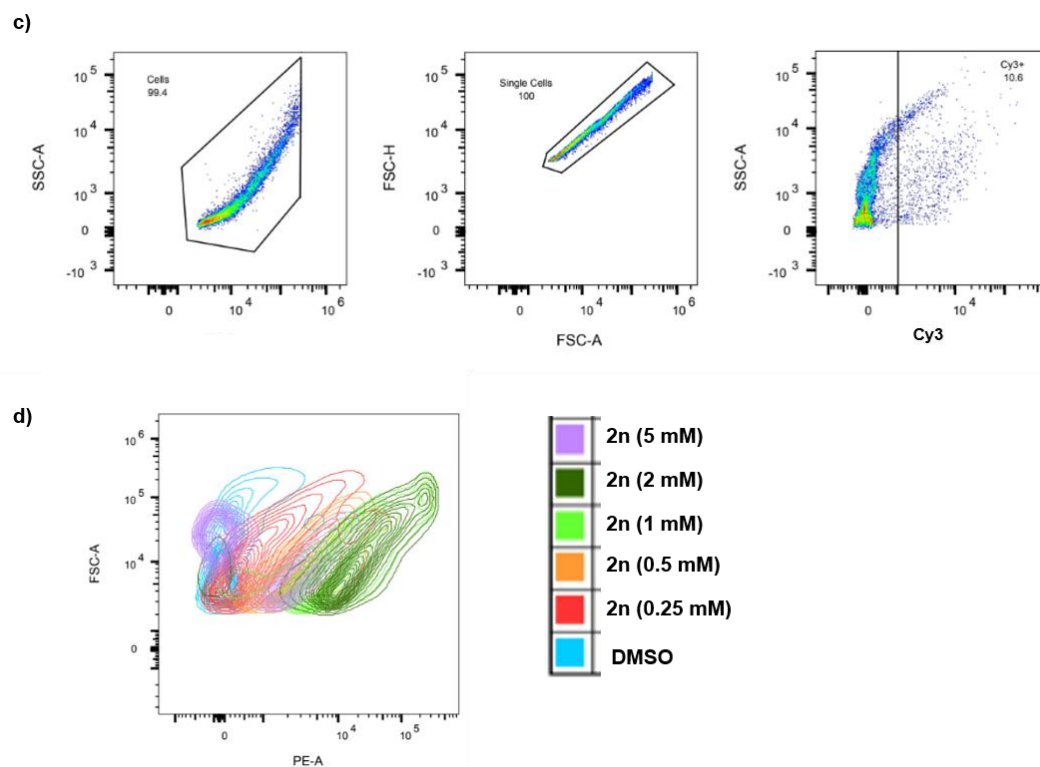


Supplementary Fig. 119. Activity analysis of modified SUMO1 (**7c**) in an in vitro SUMOylation reaction. SDS-PAGE of modified SUMO1 **7c** followed by Coomassie staining. SDS-PAGE: lane 1- SUMO1 (native), lane 2- SUMO1 (**7c**, modified), lane L- Ladder, lane 3- SUMO1 + substrate (no enzymes), lane 4- SUMOylation with native SUMO1 (12 h), lane 5- SUMOylation with modified SUMO1 **7c** (12 h). Experiments were performed *n* = 3.

4. N-Gly residue-specific modification in live cells

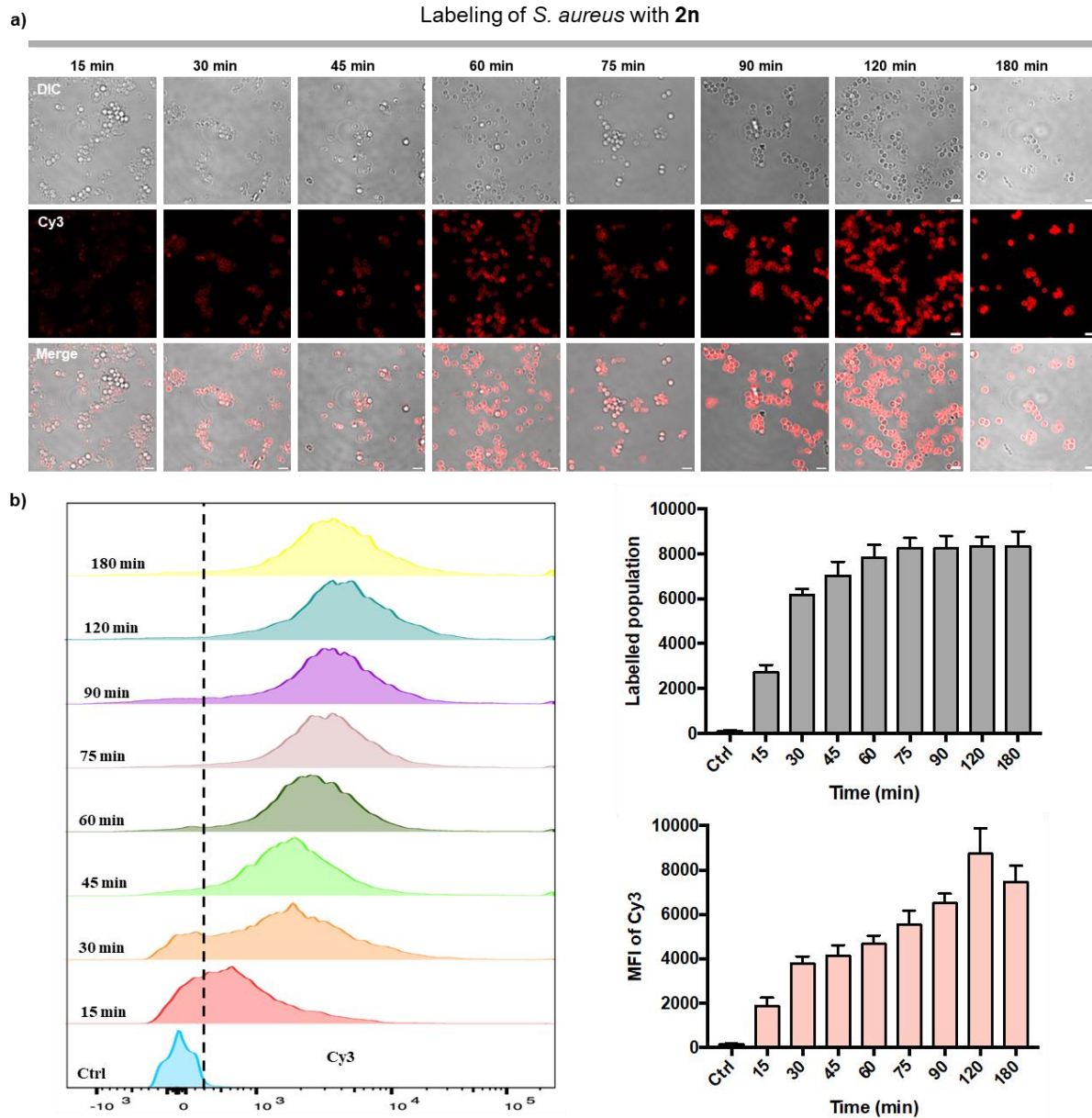
4.1 Concentration-dependent labeling of *S. aureus* with 2n





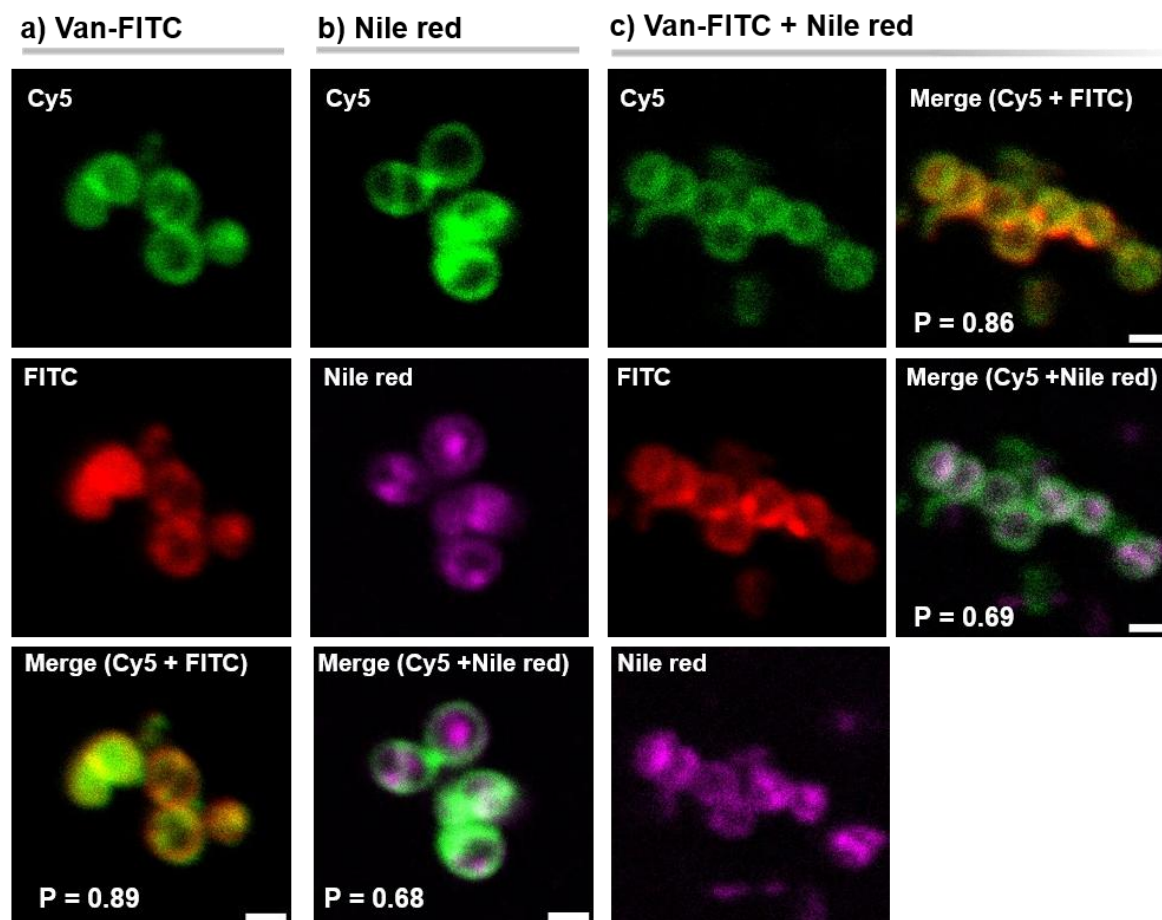
Supplementary Fig. 120. Concentration-dependent labeling of *S. aureus* peptidoglycan signature Gly₅ with **2n** (0.25-5 mM). (a) CLSM images after labeling. Scale bar, 2 μ m. (b) Flow cytometry analysis with a mean fluorescence intensity (MFI) plot of labeled *S. aureus* after treatment. (c) A general gating strategy for flow cytometry analysis. This strategy has been utilised in all the experiments in this manuscript. (d) Contour plot from FACS analysis for labeling of *S. aureus* with **2n**. Experiments were performed $n = 3$; data are presented as the mean $\pm$ SD.

4.2 Time-dependent labeling of *S. aureus* with 2n



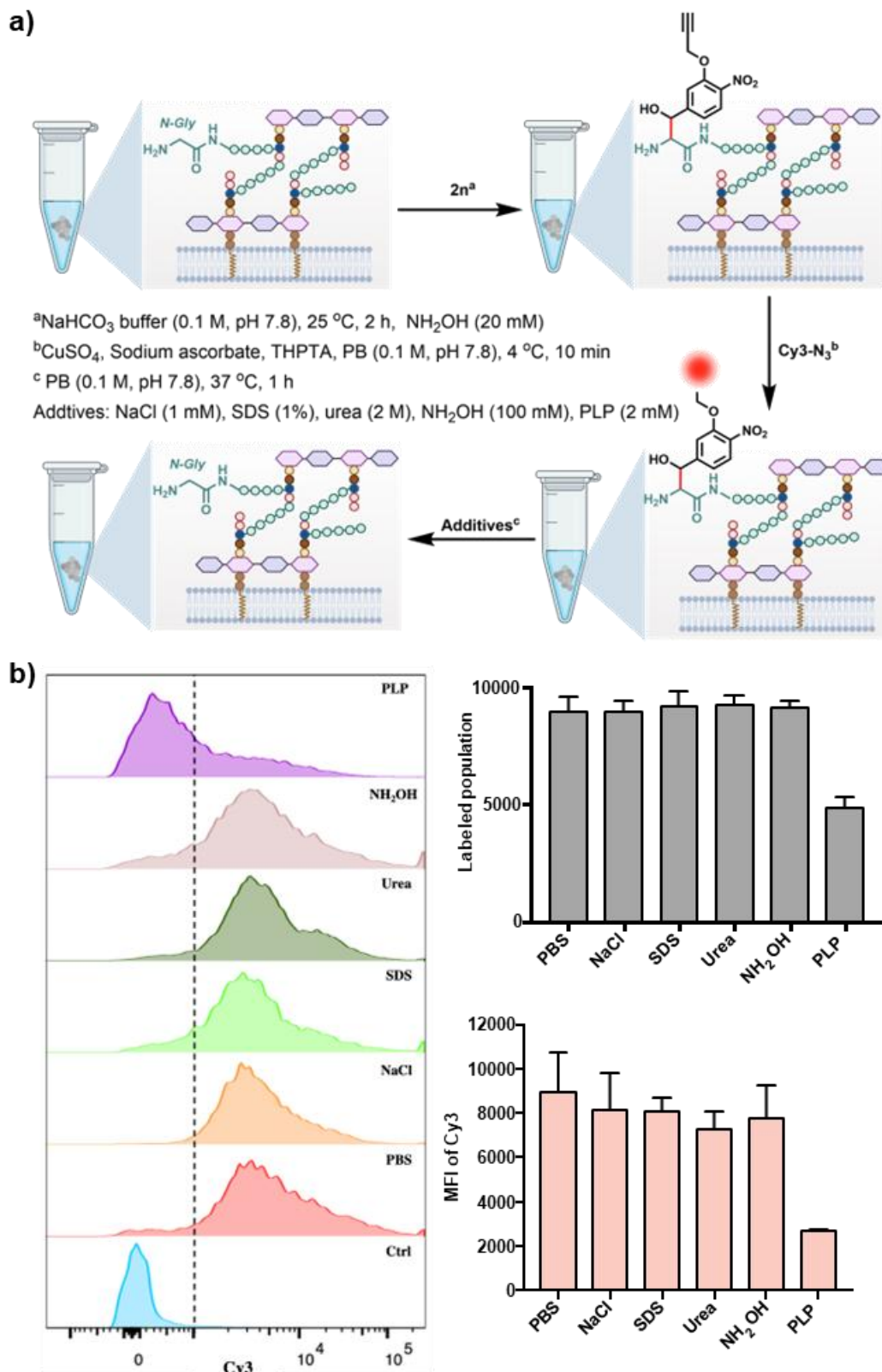
Supplementary Fig. 121. Time-dependent labeling of *S. aureus* peptidoglycan signature Gly₅ with 2n (2 mM). (a) CLSM images after labeling. Scale bar, 2 μ m. (b) Flow cytometry analysis with a mean fluorescence intensity (MFI) plot of labeled *S. aureus* after treatment. Experiments were performed n = 3; data are presented as the mean $\pm$ SD.

4.3 Cell wall co-localization experiments



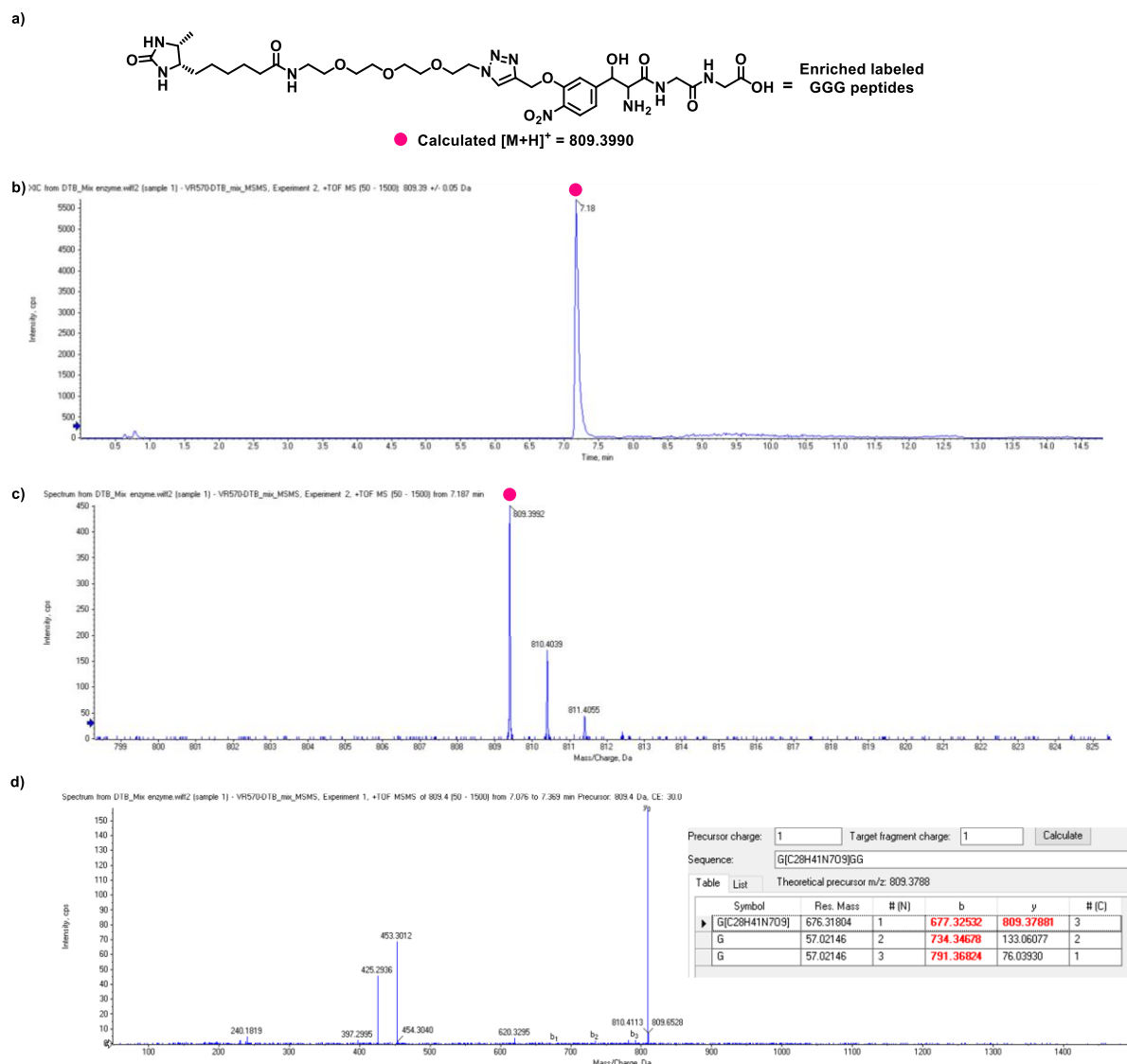
Supplementary Fig. 122 | Co-localization study of *rapi*-Gly-tag labeling. co-localization of **2n**-led Cy5 labeling with **a**, cell wall targeting Van-FITC labeling. **b**, membrane targeting Nile red labeling. **c**, co-localization of **2n**-led Cy5 labeling with both Van-FITC and Nile red. Scale bar, 1 μ m.

4.4 Confirmation of covalent modification in *S. aureus* and labeling with control reagent

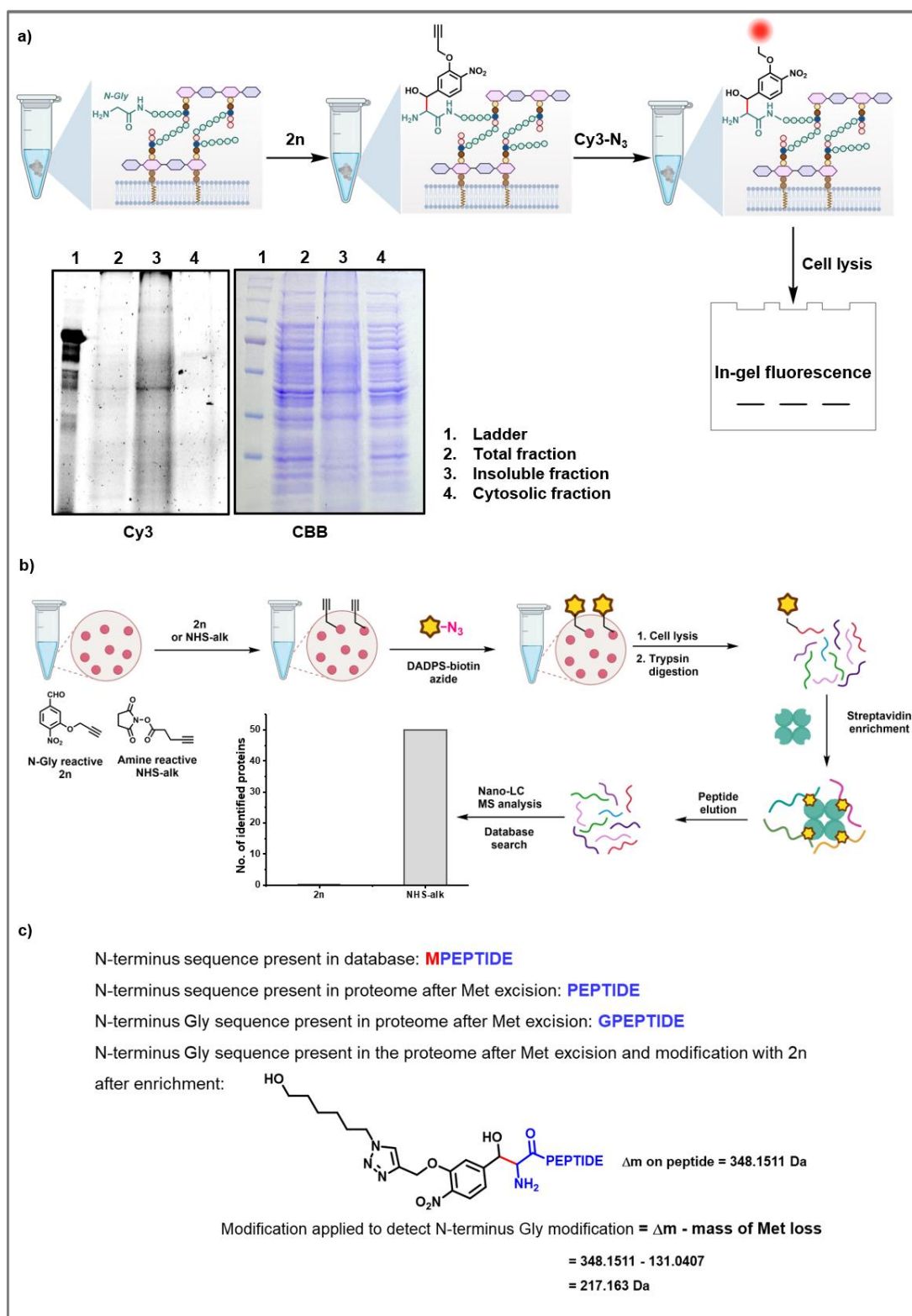


Supplementary Fig. 123. (a) Effect of additives on **2n**-treated *S. aureus*. (b) Flow cytometry analysis displayed a difference in labeled population and mean fluorescence intensity (MFI) with samples treated with the additive versus those treated with PBS. Experiments were performed $n = 3$; data are presented as the mean $\pm$ SD.

4.5 Molecular-level target validation and global proteomics

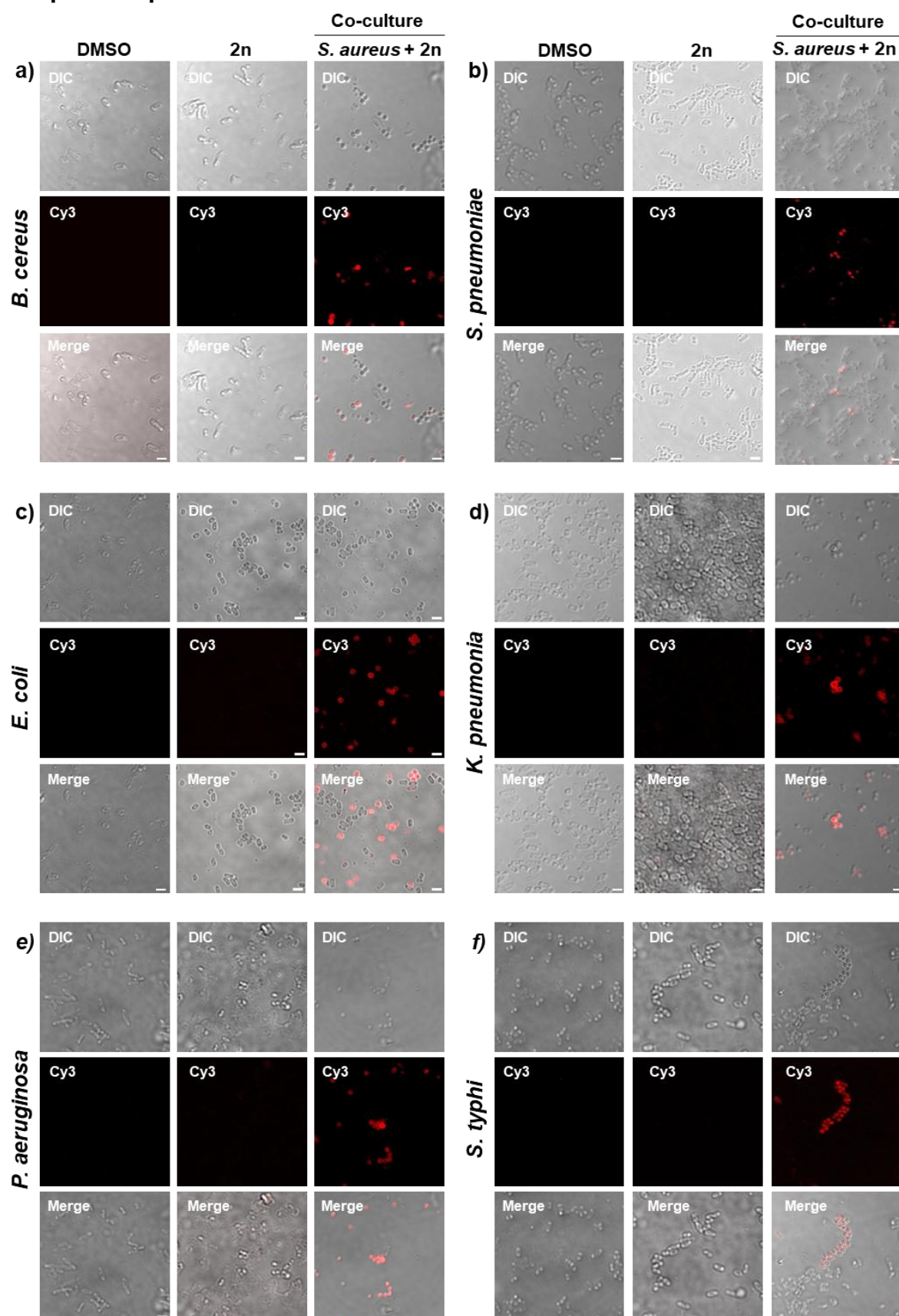


Supplementary Fig. 124. (a) Structure of enriched labeled peptides. (b) XIC data for m/z 809.39 Da from the labeled GGG peptide. (b) MS spectra of the labeled Gly₃ peptide (calculated, $[M+H]^+$ 809.3990 Da; observed, $[M+H]^+$ 809.3992 Da). (c) MS-MS of labeled GGG peptide (m/z 809.40, $[M+H]^+$) confirms the site of modification, N-Gly (G1).



Supplementary Fig. 125. Selectivity of *rapi*-Gly-tag platform. **a**, in-gel fluorescence analysis of labeled cells with **2n** (2 mM, 2 h) followed by click conjugation of Cy3-azide. **b**, Global chemoproteomics analysis for identification of labeled proteins. Cells were incubated with **2n** (2 mM, 2 h) and NHS-alk (positive control, 200 μ M, 2 h) separately. Subsequently, click conjugation with DADPS-biotin azide. Next, cells were lysed, the proteome was trypsin digested, labeled peptides were enriched, and analysed by nano-LC Orbitrap. **c**, Description for modification applied for proteomics analysis for N-Gly modification with Proteome Discoverer 2.5. Experiments were performed $n = 3$.

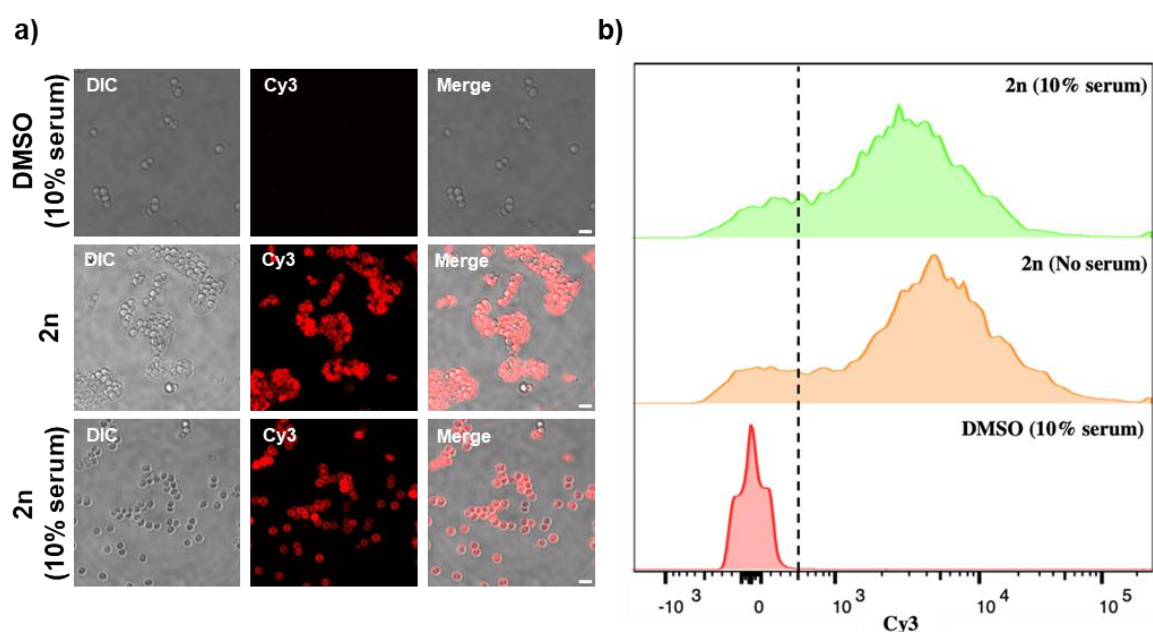
4.6 Species-specific detection of *S. aureus*



Supplementary Fig. 126. Species-specific detection of *S. aureus* when treated with DMSO (first panel), 2n (2 mM, second panel), or co-cultured with *S. aureus* and 2n (2 mM, third

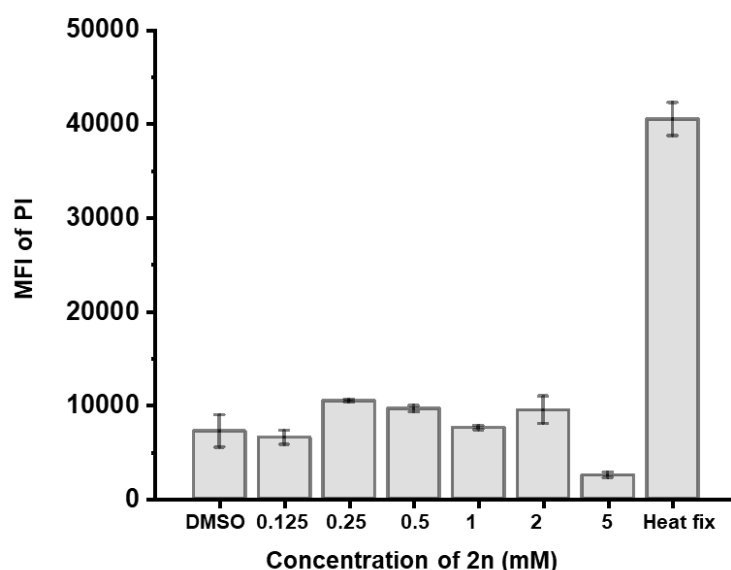
panel). The competing species include, (a) *B. cereus*, (b) *S. pneumoniae*, (c) *E. coli*, (d) *K. pneumoniae*, (e) *P. aeruginosa*, and (f) *S. typhi*. Scale bar, 2 μ m.

4.7 Labeling of *S. aureus* in human serum



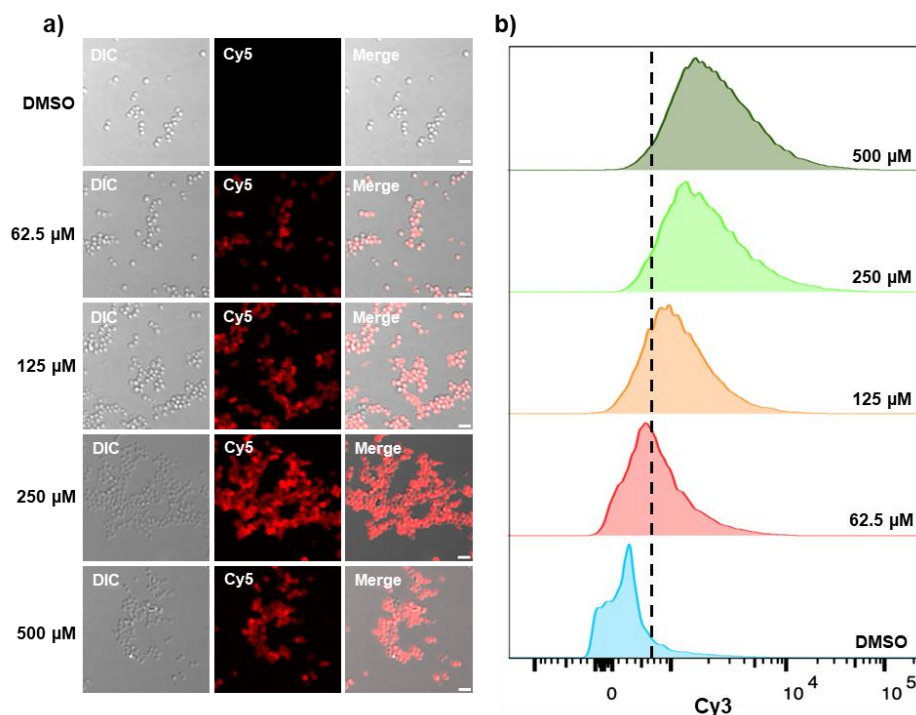
Supplementary Fig. 127. Detection of *S. aureus* in the presence of human serum when treated with DMSO (with 10% human serum), **2n** (2 mM, no human serum), or **2n** (2 mM, with 10% human serum). (a) CLSM images, scale bar, 2 μ m. (b) Flow cytometry analysis.

4.8 *S. aureus* viability upon treatment

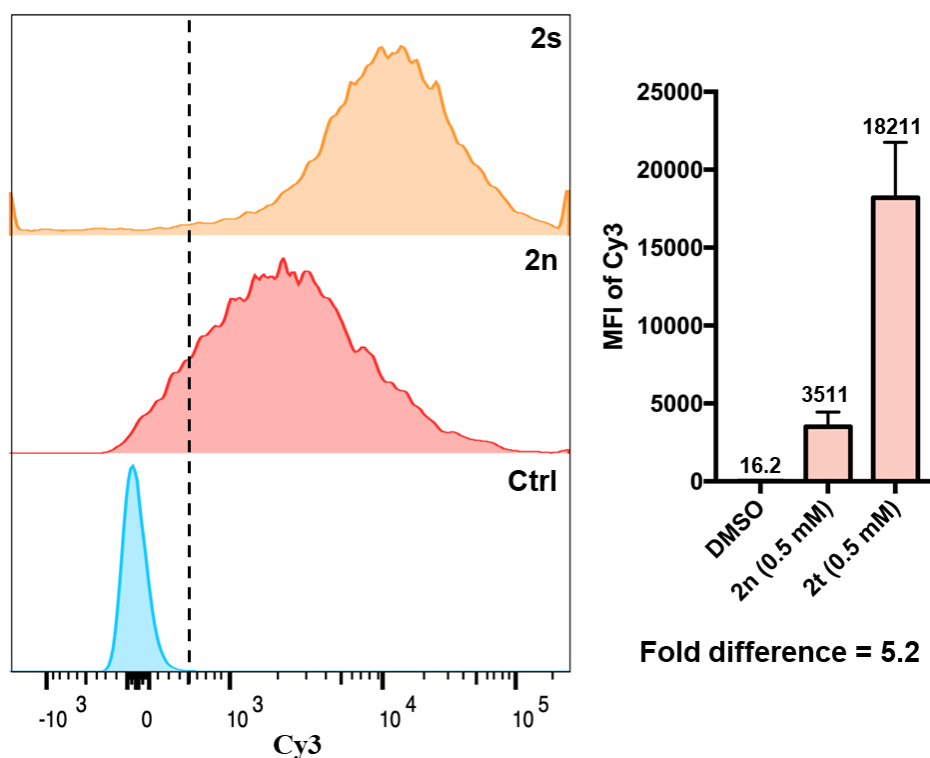


Supplementary Fig. 128. *S. aureus* viability analysis upon treatment with **2n**. Bacteria were incubated at different concentrations of **2n** (0.5-5 mM) for 2 h, followed by propidium iodide treatment (15 μ g/mL) for 15 min at 4 $^{\circ}$ C and subsequently analyzed by flow cytometry. MFI represents mean fluorescence intensity. Experiments were performed n = 3; data are presented as the mean $\pm$ SD.

4.9 Multiprobe installation on *S. aureus*

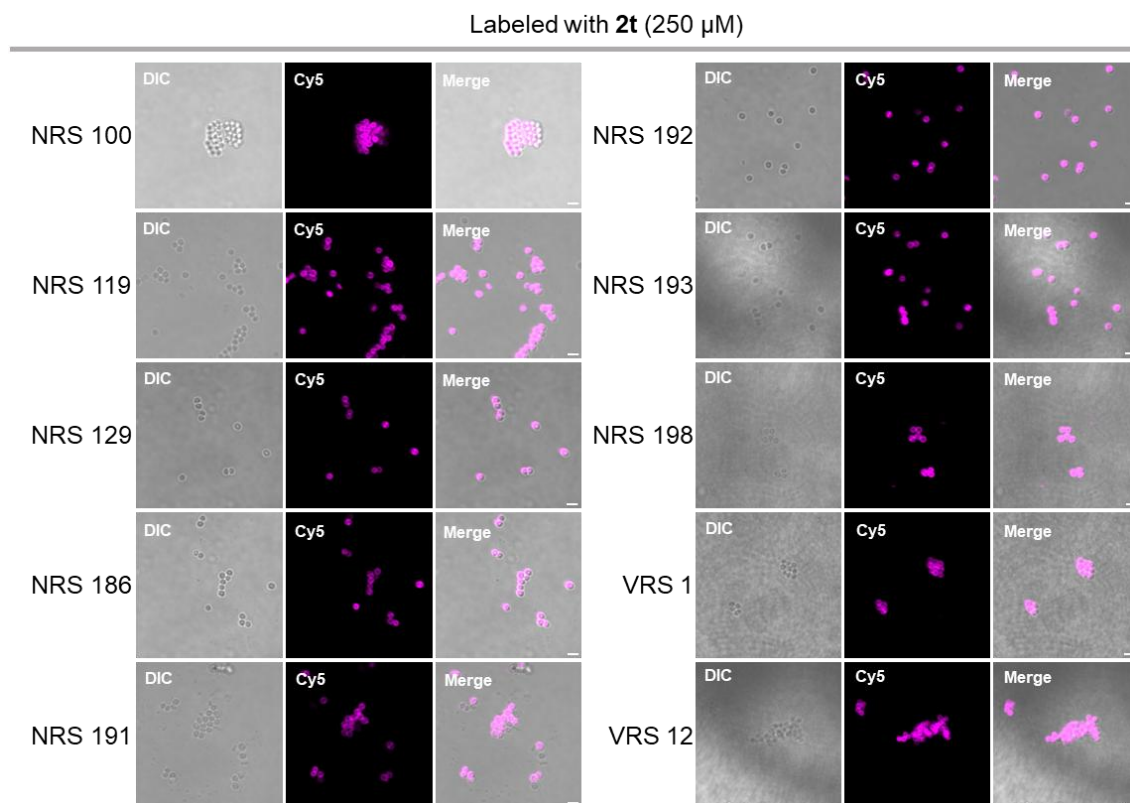


Supplementary Fig. 129. Concentration-dependent labeling of *S. aureus* with **2s** (62.5-500 μM) for installation of multiprobe. (a) CLSM images, scale bar, 2 μm . (b) Flow cytometry analysis.



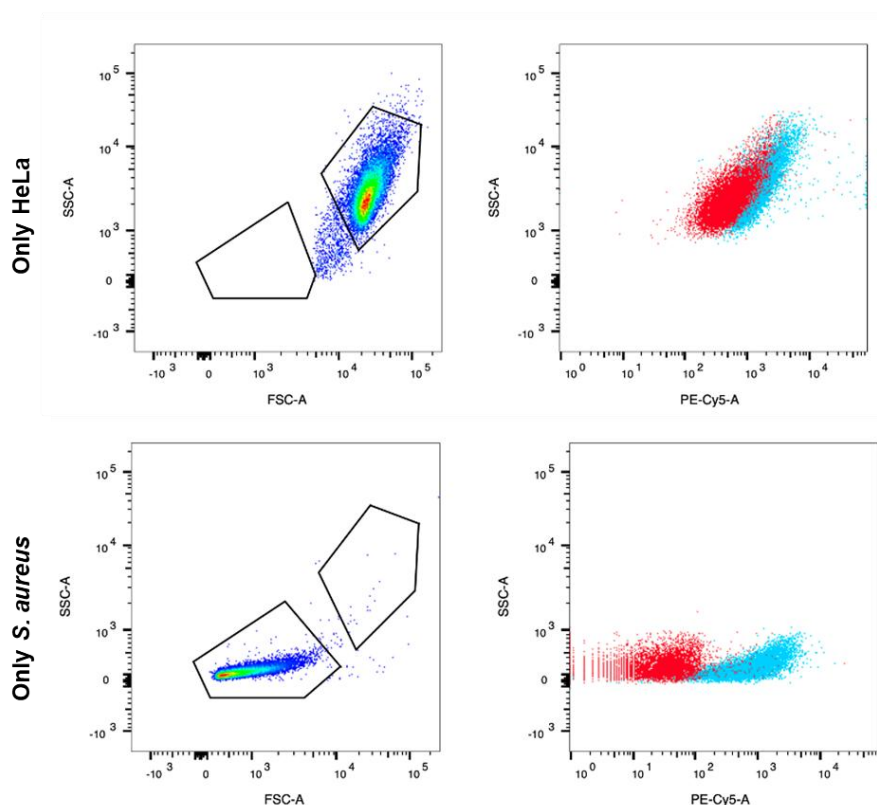
Supplementary Fig. 130. Comparison for enhanced detection of *S. aureus* with single probe **2n** (0.5 mM) and dual probe **2s** (0.5 mM) with flow cytometry analysis. Experiments were performed $n = 3$; data are presented as the mean $\pm$ SD.

4.10 Targeting clinical strains of *S. aureus*

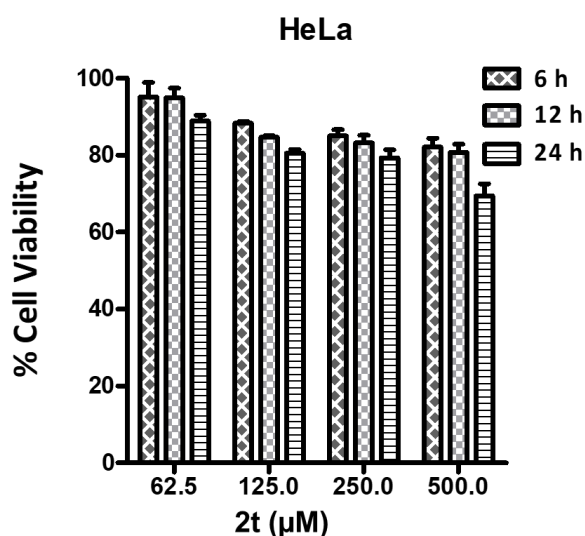


Supplementary Fig. 131. CLSM images for detection of *S. aureus* clinical isolates, including a series of MRSA and VRSA strains, with **2t** (250 μ M, 2 h); scale bar, 2 μ m.

4.11 Species-specific targeting of *S. aureus* in mammalian co-culture

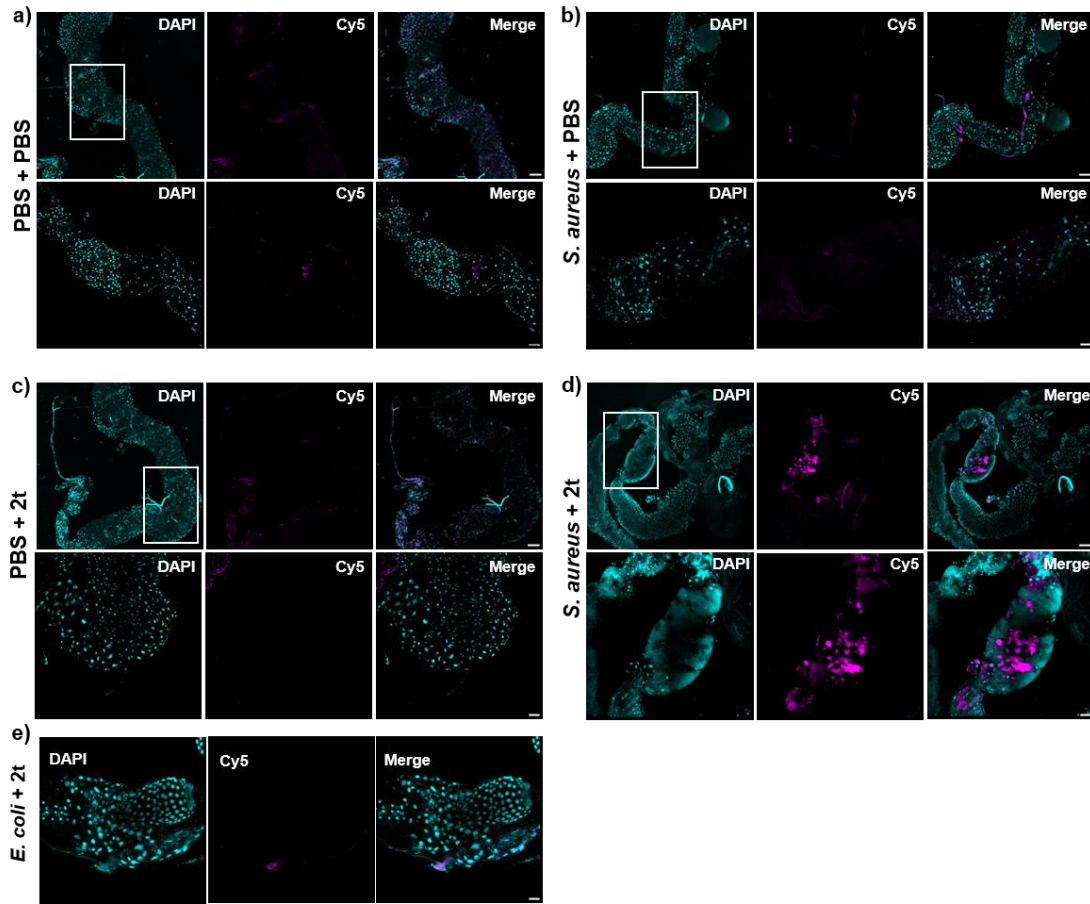


Supplementary Fig. 132. Flow cytometry analysis of a co-culture of HeLa and *S. aureus* when treated with either DMSO (red) or **2t** (250 μ M, 2 h, blue) followed by DBCO-Cy5. The HeLa and *S. aureus* populations were segregated based on size (left panel) and subsequently analyzed for enhanced Cy5 signal (right panels).



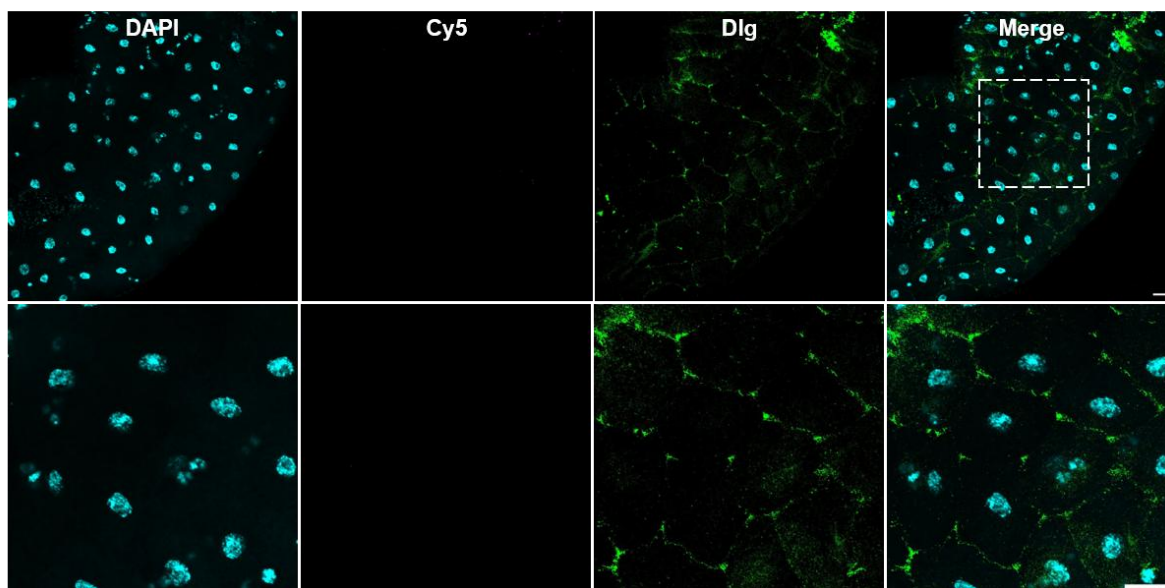
Supplementary Fig. 133. Cell viability analysis of **2t** using the MTT assay against the HeLa cell line after 6 h, 12 h, and 24 h incubation. Data represented as mean $\pm$ SD ($n = 3$). Statistical significance was evaluated using a one-sample t-test. *** $p < 0.001$.

5. *In vivo* *S. aureus* species-specific detection in *Drosophila melanogaster*

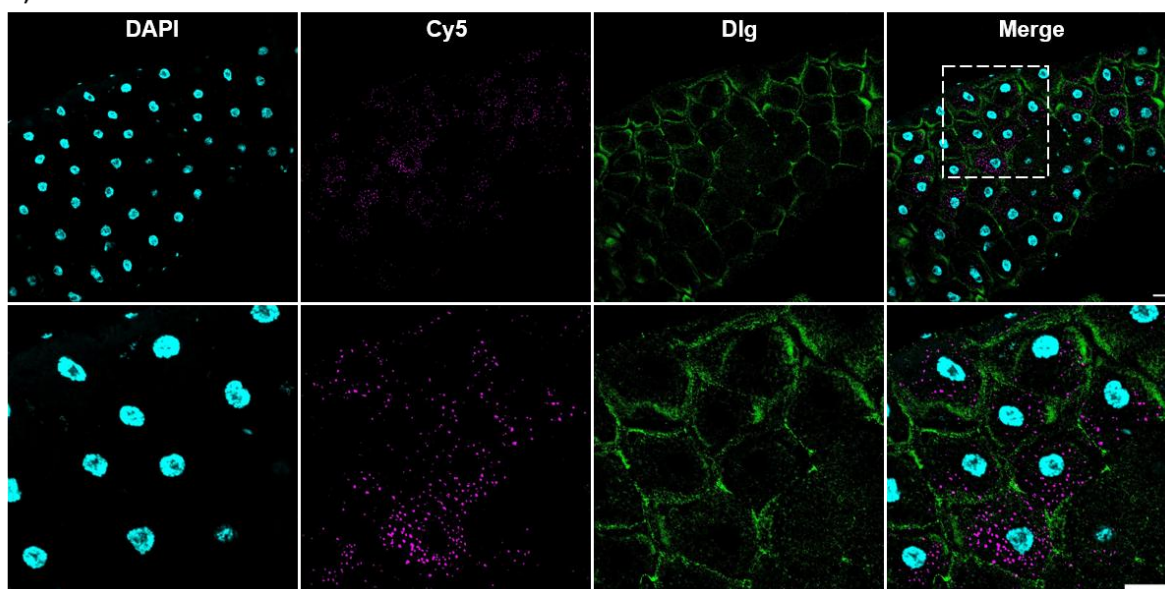


Supplementary Fig. 134. Confocal images of the dissected gut of *Drosophila* for *S. aureus* selective detection. The gut of infected larvae was dissected post-treatment with reagent **2t** for at least $n=10$ larvae. PBS is used as a negative control. Bottom panel (scale bar, 40 μm) represents the enlarged image of the top panel (scale bar, 100 μm). (a) Treated with PBS only (uninfected, no **2t**). (b) Infected with *S. aureus* but treated with PBS instead of the **2t**. (c) Treated with the probe **2t** but uninfected. (d) Infected with *S. aureus* and treated with **2t**. This panel clearly shows the specific targeting of *S. aureus* with **2t**. (e) Infected with *E. coli* and treated with **2t**. Experiments were performed $n = 3$.

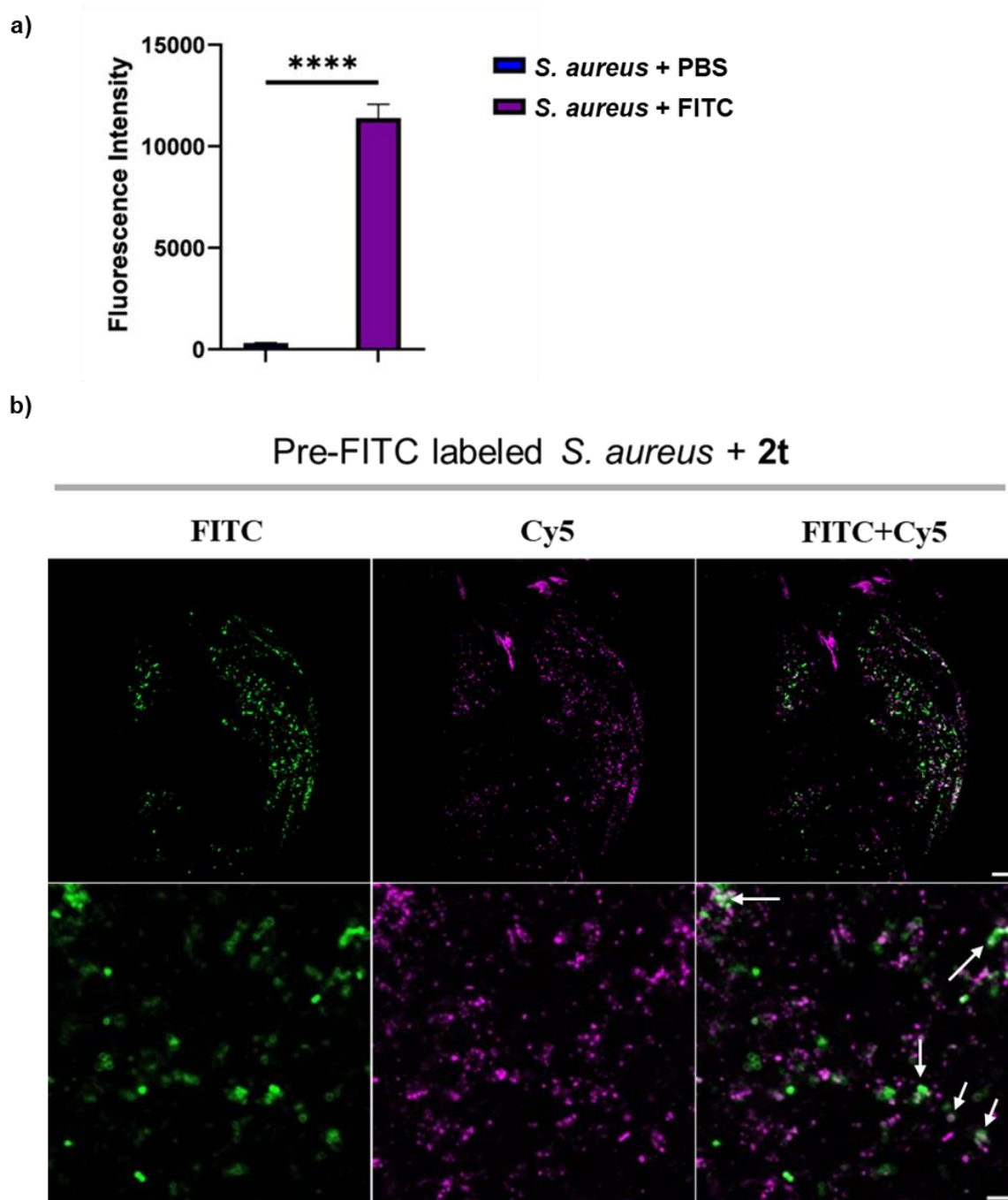
a) PBS + PBS



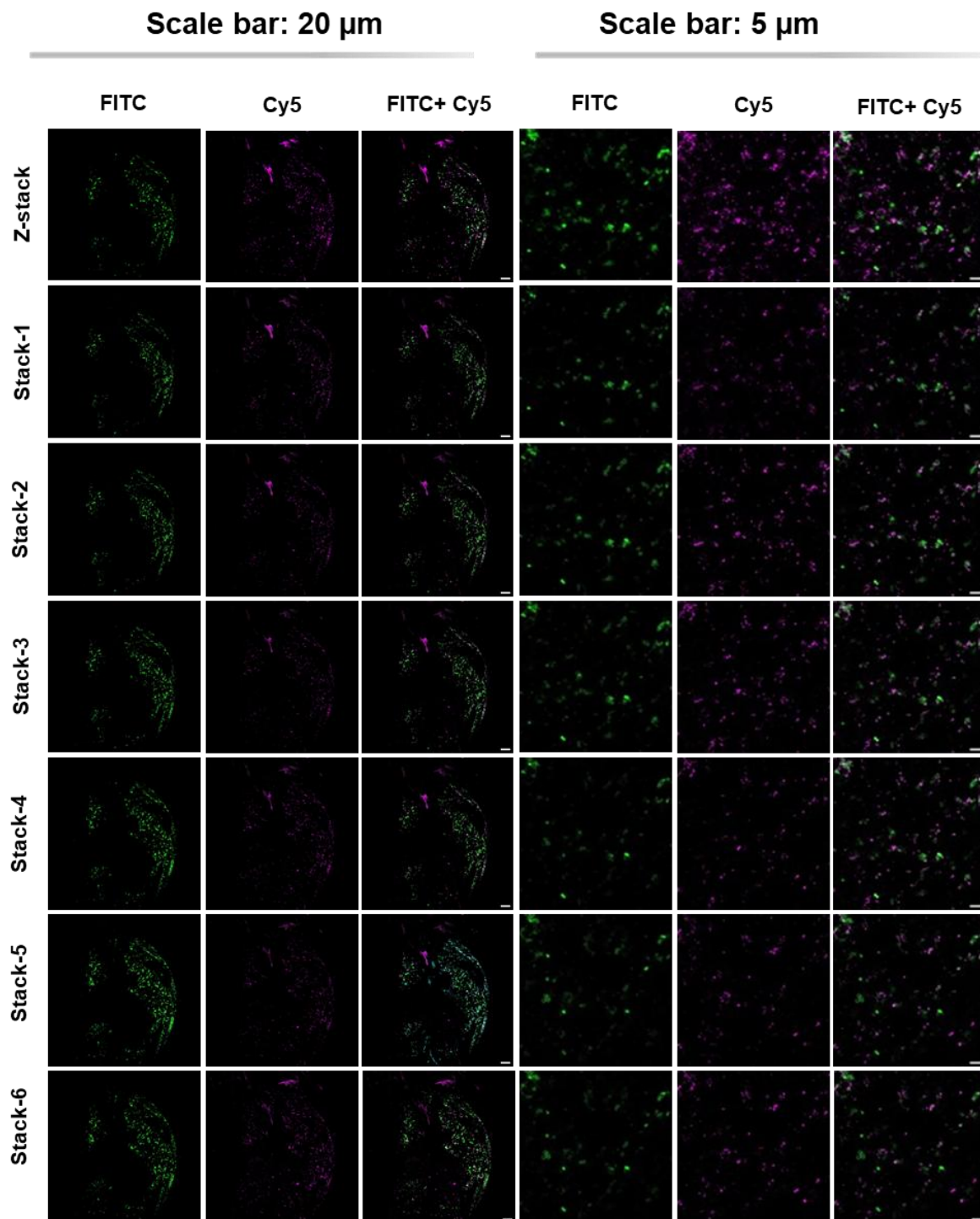
b) *S. aureus* + 2t



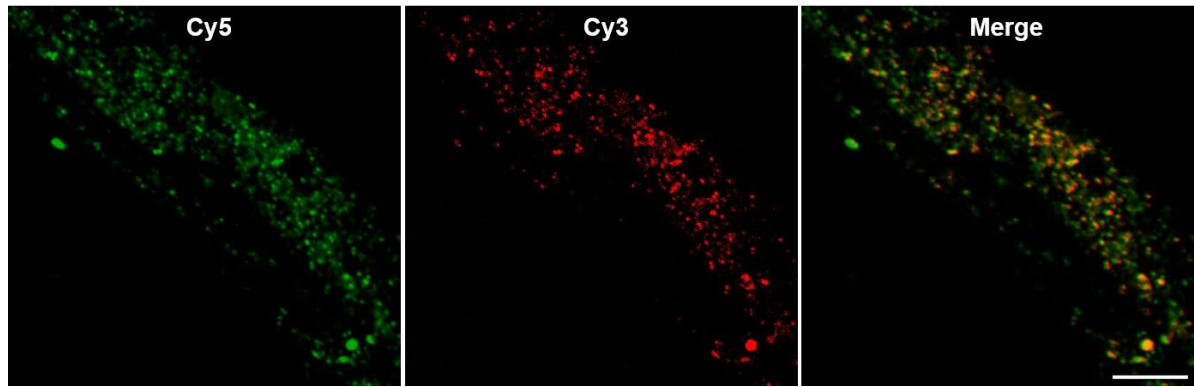
Supplementary Fig. 135. Specific detection of *S. aureus* in *Drosophila* gut with **2t**, co-stained with epithelial membrane protein marker. 2nd instar larvae were fed with either PBS or *S. aureus*, followed by treatment with PBS or **2t** (250 μ M, 2 h). The larval guts were dissected and subsequently treated with DBCO-Cy5. Next, immunofluorescence staining was performed for the epithelial membrane marker protein DLG. **a**, Treated with PBS only (uninfected, no **2t**). **b**, Infected with *S. aureus* and treated with **2t**. CLSM images show punctate labeling of *S. aureus* within the mid-gut region of *Drosophila* larvae, its characteristic location. Scale bar, 20 μ m. Experiments were performed n = 3.



Supplementary Fig. 136. (a) Fluorescence intensity of FITC-labeled *S. aureus*. Intensity was recorded after labeling the bacteria (O.D.=1.0) with FITC (200 μ M) for 2 h, followed by washing with PBS. Unlabeled bacteria were used as the control. (b) Revalidation of *in vivo* labeling of *S. aureus* in the gut of larvae, by infecting them with pre-FITC-labeled *S. aureus* and performing labeling experiments using similar conditions. Confocal images of dissected mid-gut after infection (pre-FITC-labeled *S. aureus*) and treatment with **2t**. Bottom panel (scale bar, 5 μ m) represents the enlarged image of the top panel (scale bar, 40 μ m). The white arrow highlights colocalization of FITC and Cy5 for representative cases. Experiments were performed n = 3.

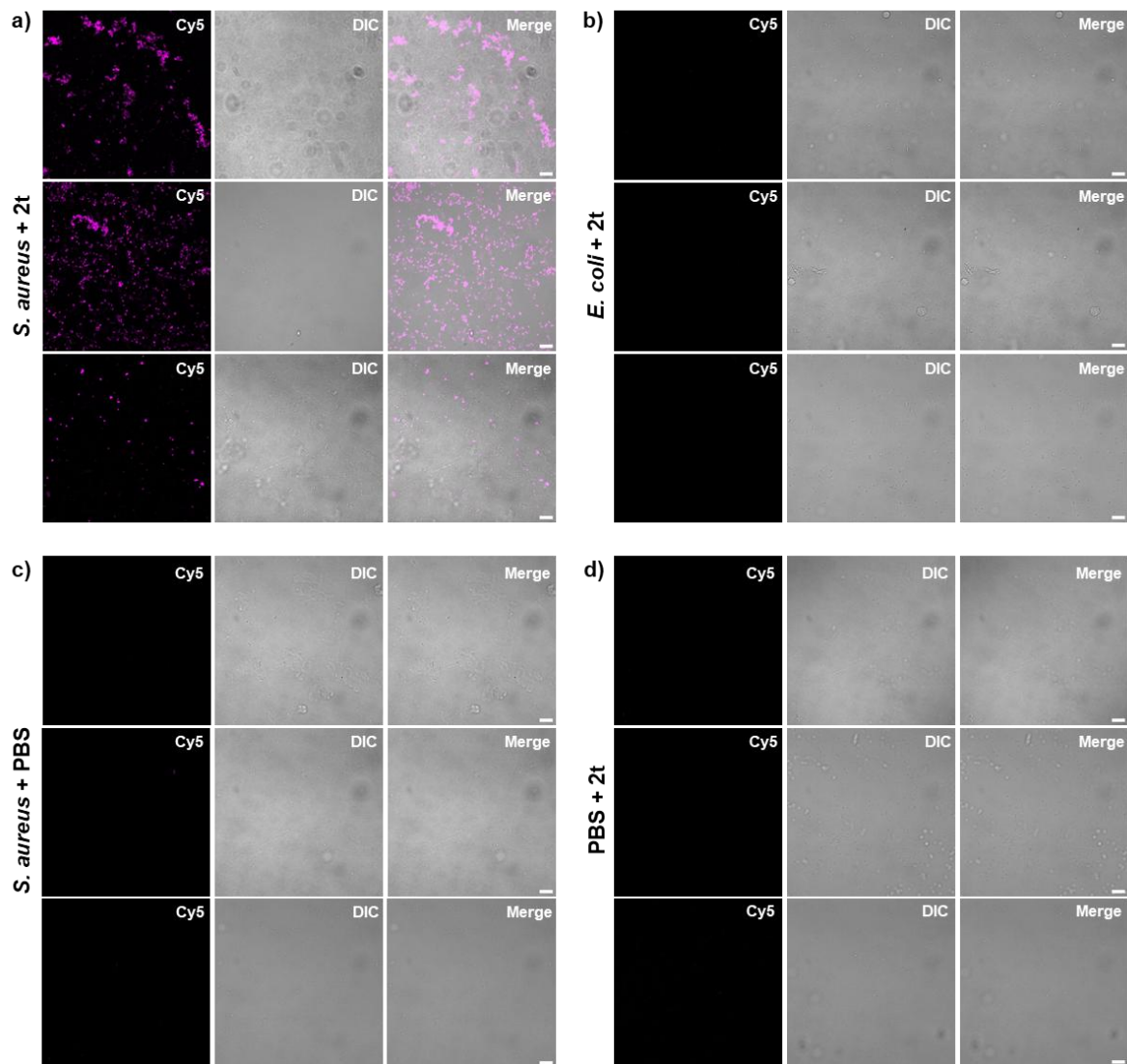


Supplementary Fig. 137. Stack analysis of pre- (FITC) and post-(Cy5) infection labeling of *S. aureus* in *Drosophila*.



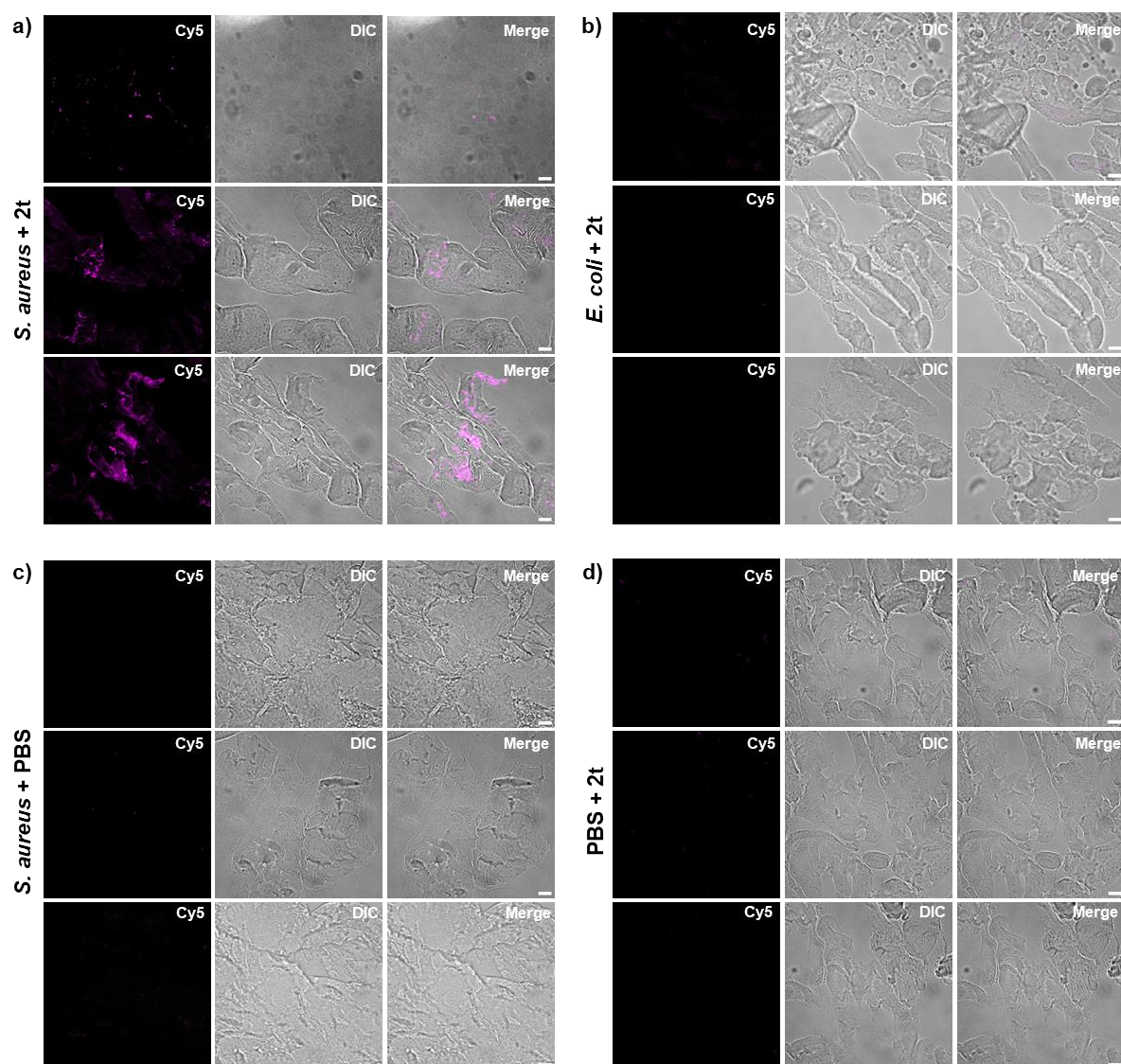
Supplementary Fig. 138. Sequential dual labeling of *S. aureus* with **2u**. At first, infected larvae were fed with pre-conjugated 2u-Cy5, and Cy3 was conjugated post-dissection. Scale bar, 20 μ m.

6. *In vivo* *S. aureus* species-specific detection in mice

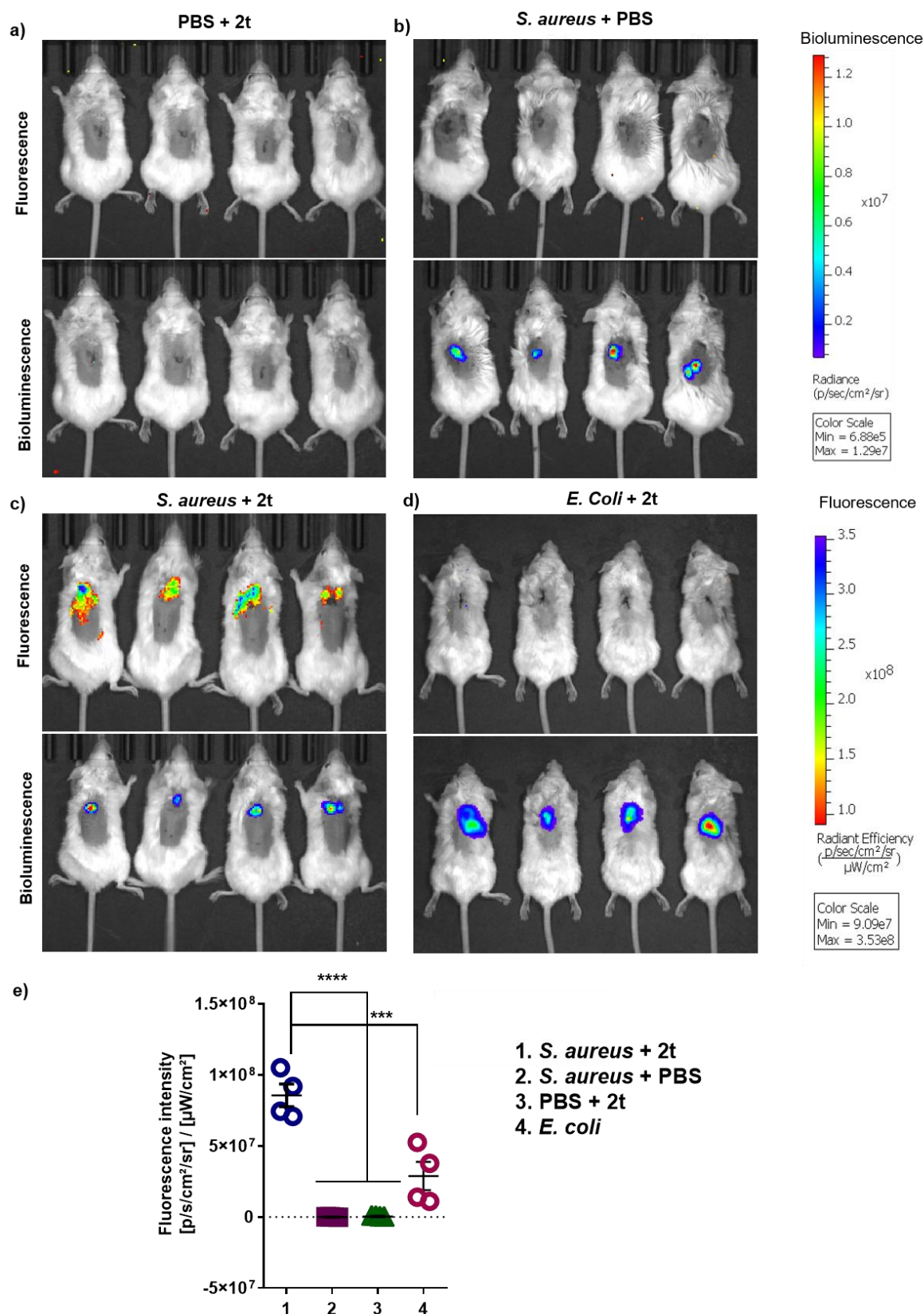


Supplementary Fig. 139. Species-specific detection of *S. aureus* in mice blood with **2t**. Blood samples were assayed in triplicate (top, middle, and bottom panels) for each condition. (a) *S. aureus* + **2t** (test), (b) *E. coli* + **2t** (non-specific binding control), (c) *S. aureus* + PBS

(label-omission control), and (d) PBS + **2t** (blank control); scale bar, 10 μ m. Experiments were performed n = 3.



Supplementary Fig. 140. Species-specific detection of *S. aureus* in mice muscle tissue with **2t**. Tissue samples were assayed in triplicate (top, middle, and bottom panels) for each condition. (a) *S. aureus* + **2t** (test), (b) *E. coli* + **2t** (non-specific binding control), (c) *S. aureus* + PBS (label-omission control), and (d) PBS + **2t** (blank control); scale bar, 10 μ m. Experiments were performed n = 3.



Supplementary Fig. 141. *In vivo* imaging for detection of *S. aureus* in mice skin infection model with 2t. Imaging was performed for n = 4 for each condition. The top images in each panel indicate fluorescence signals, and the bottom images indicate bioluminescence signals (a) PBS + 2t (control), (b) *S. aureus* + DMSO (control), (c) *S. aureus* + 2t (test), (d) *E. coli* + 2t (control). (e) Quantification of fluorescence intensity with ROI. Results obtained from

quantitative analysis are presented as a mean fluorescence intensity. Data means SD (n = 4) ; p values were calculated using one-way ANOVA with Tukey multiple comparison test; ****p< 0.0001, ***p < 0.001.

7. In vitro inhibition of *S. aureus*

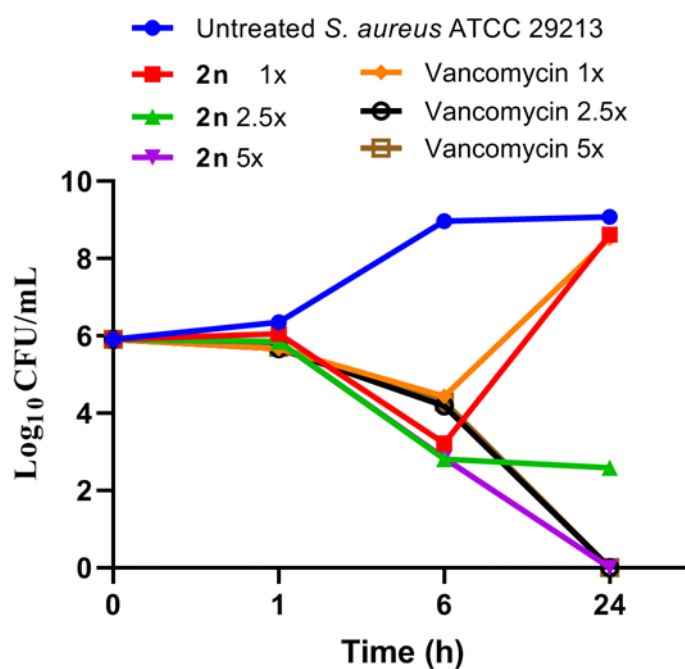
Supplementary Table 18: MIC, CC₅₀, and selectivity index (SI)

	MIC for 2n (µg/mL)	CC ₅₀ for 2n (µg/mL)
MSSA (ATCC 29213)	64	-
MRSA (NRS 191)	64	-
VRSA (VRS 4)	64	-
<i>E. coli</i> (ATCC 25922)	n.d. ^a	-
<i>K. pneumoniae</i> (BAA 1705)	n.d. ^a	-
<i>A. baumannii</i> (BAA 1605)	n.d. ^a	-
<i>P. aeruginosa</i> (ATCC 27853)	n.d. ^a	-
Vero cells ^b	-	710

^an.d.: not determined. No inhibition was observed till 256 µg/mL of **2n**.

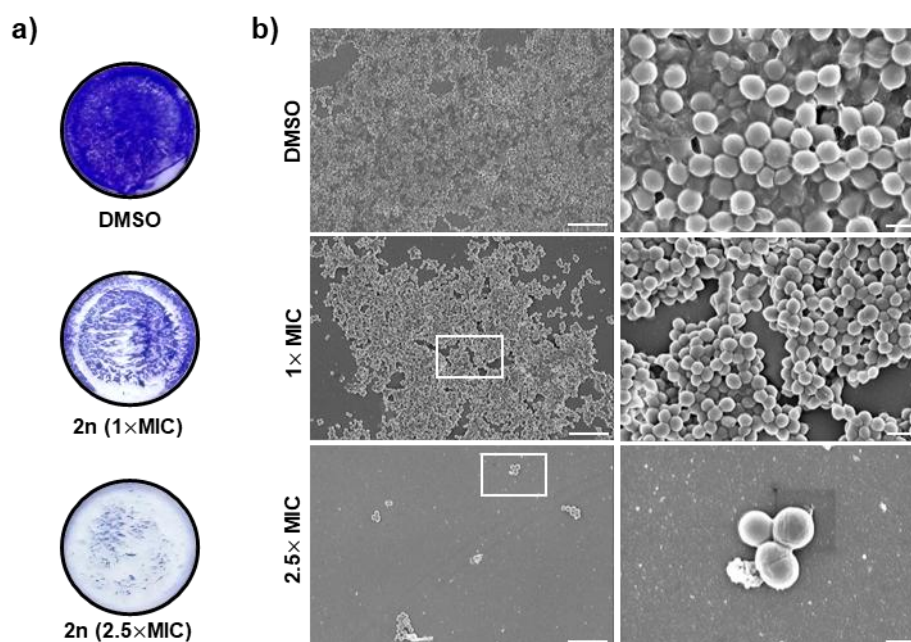
^bVero cells derived from kidney of the monkey were used to determine the selectivity index.

Selectivity index, SI = (CC₅₀, Vero Cells) / (MIC, *S. aureus*) = 710/64 = >11. Experiments were performed n = 3.

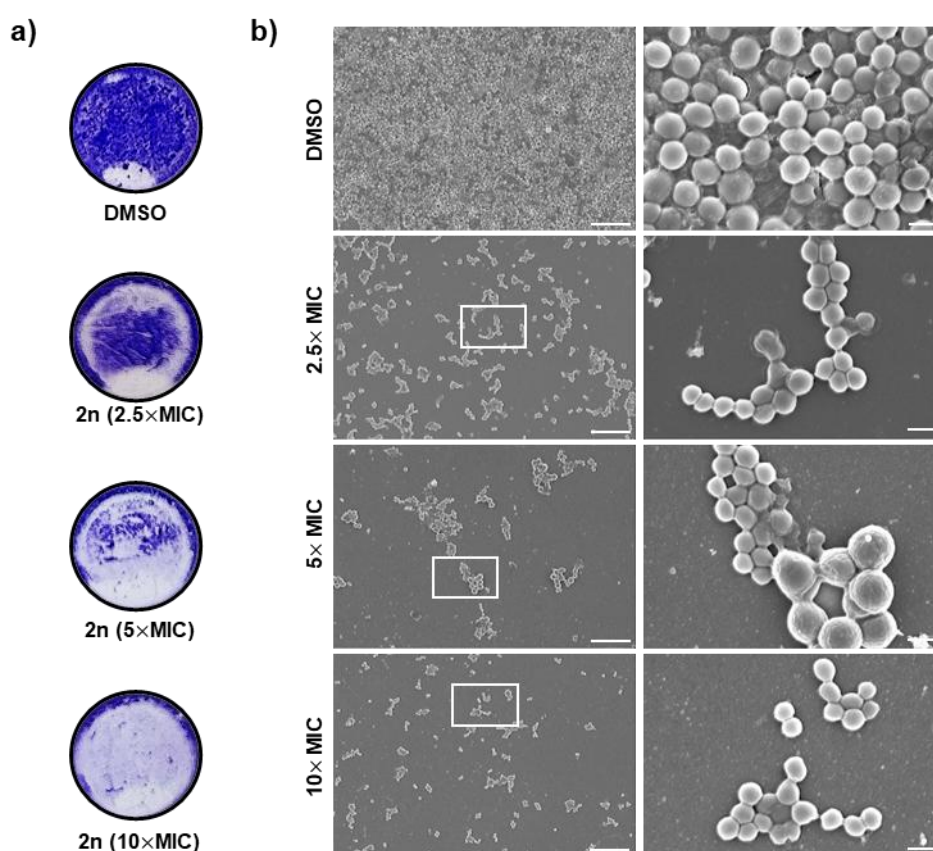


Supplementary Fig. 142. Time kill assay against *S. aureus* upon treatment of **2n** and vancomycin (positive control). Experiments were performed n = 3.

8. Biofilm inhibition and eradication



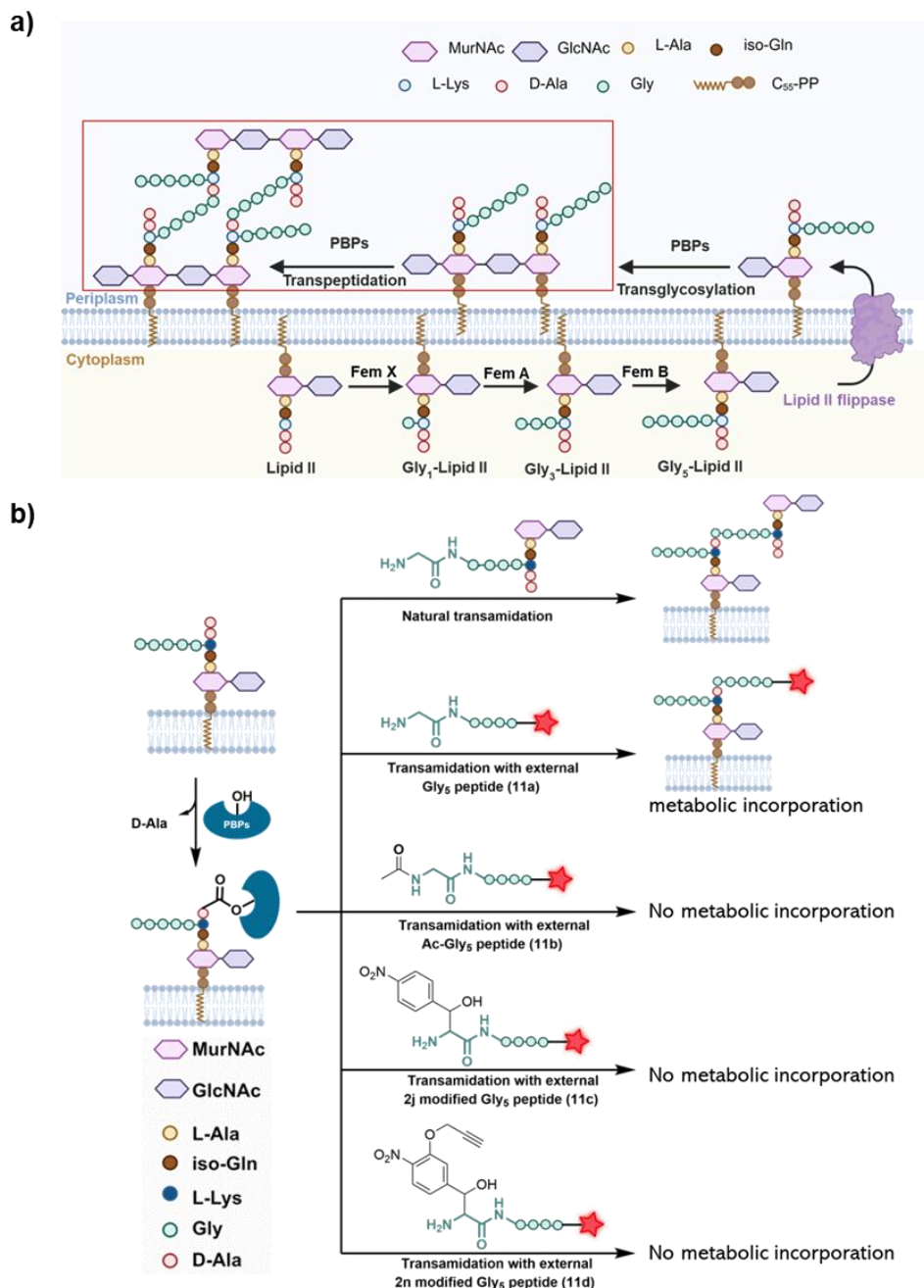
Supplementary Fig. 143. Inhibition of *S. aureus* biofilm formation with **2n**. (a) Crystal violet-stained biofilms with **2n** and DMSO (negative control) treatment. (b) FE-SEM analysis of biofilms showed reduced biofilm formation when treated with **2n**; scale bar, 10 μm (left), 1 μm (right). Experiments were performed $n = 3$.



Supplementary Fig. 144. Eradication of pre-formed *S. aureus* biofilm with **2n**. (a) Crystal violet-stained biofilms with **2n** and DMSO (negative control) treatment. (b) FE-SEM analysis

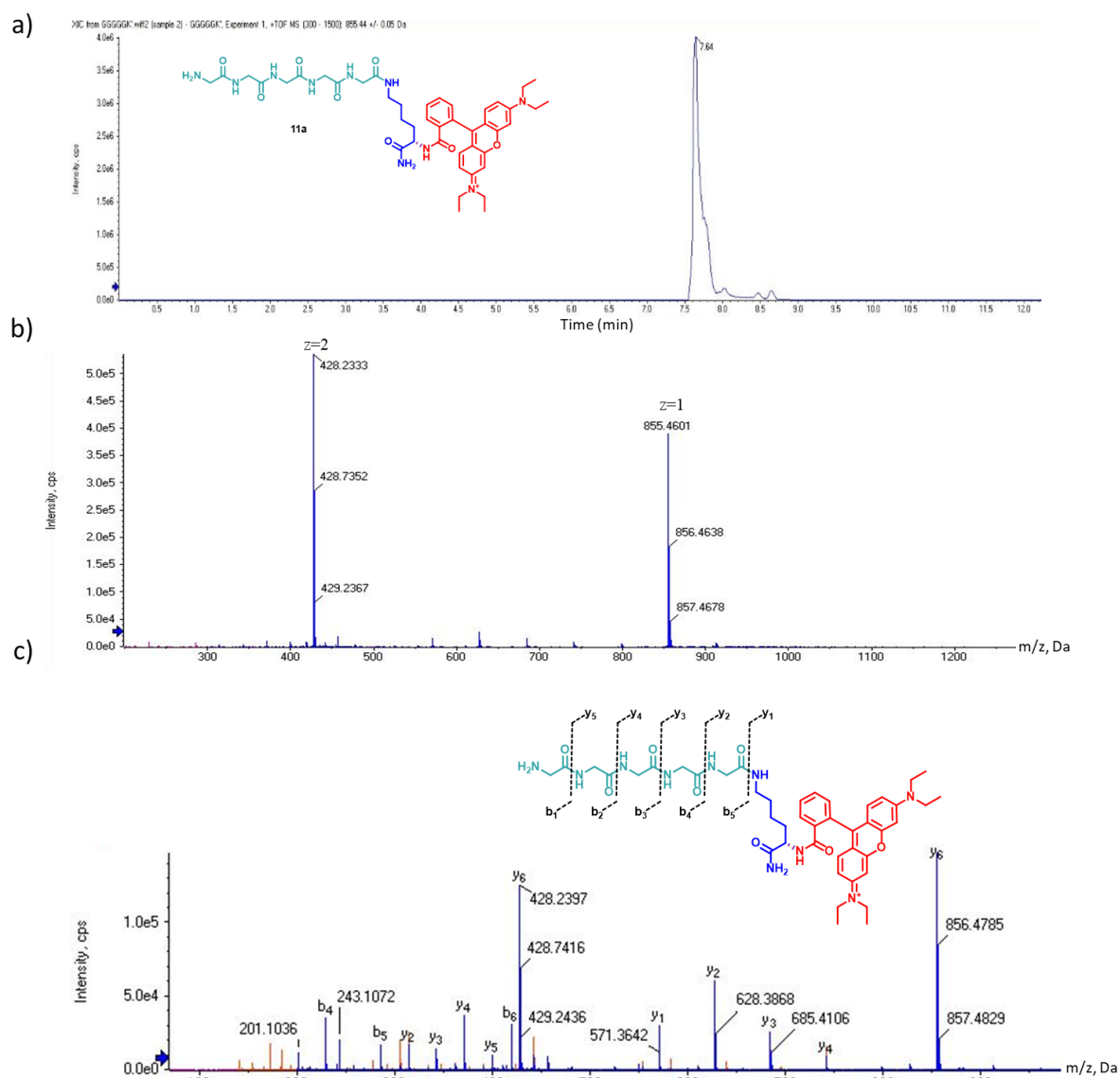
of biofilms showing reduction in pre-formed biofilms when treated with **2n**. Scale bar, 10 μm (left), 1 μm (right). Experiments were performed $n = 3$.

9. Mechanistic investigation for inhibition of *S. aureus*

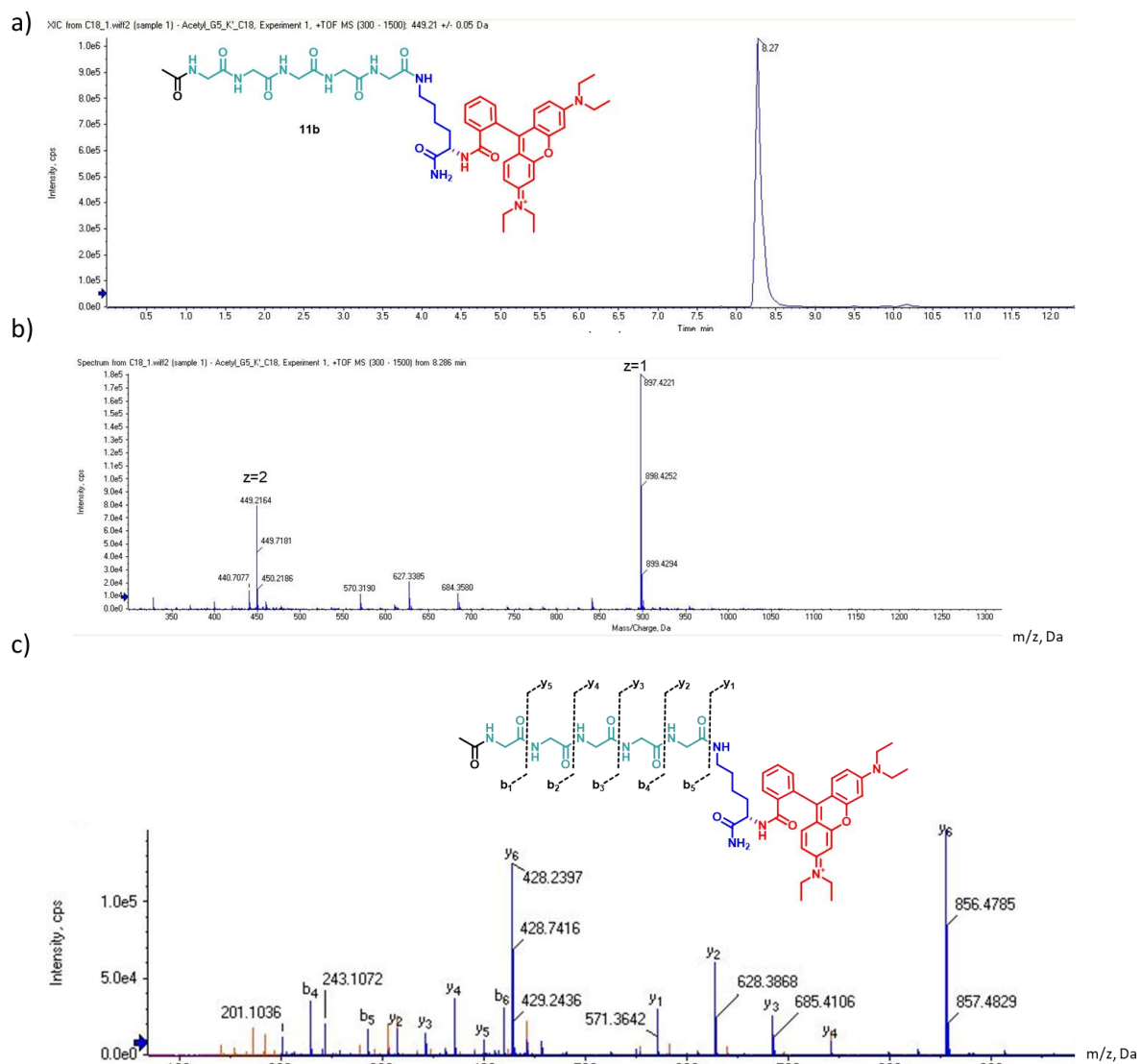


Supplementary Fig. 145. Mechanistic investigation of *S. aureus* growth inhibition with control experiments. (a) Cell wall biosynthesis pathways displaying synthesis of Gly₅ with a series of Fem enzymes, and its crosslinking with stem peptide by penicillin-binding proteins (PBPs).¹³ (b) Mechanism of transpeptidation by PBPs: at first, it removes one D-Ala from the stem peptide and forms an acyl complex; subsequently, the N-terminal amine of Gly₅ undergoes a transpeptidation reaction. Control experiments, with metabolic incorporation of **11a-d**, elucidate the critical role of free -NH₂ and unsubstituted α -carbon in N-Gly₅ for peptidoglycan crosslinking. Rhodamine-tagged Gly₅ (**11a**) is metabolically incorporated into native *S. aureus*.

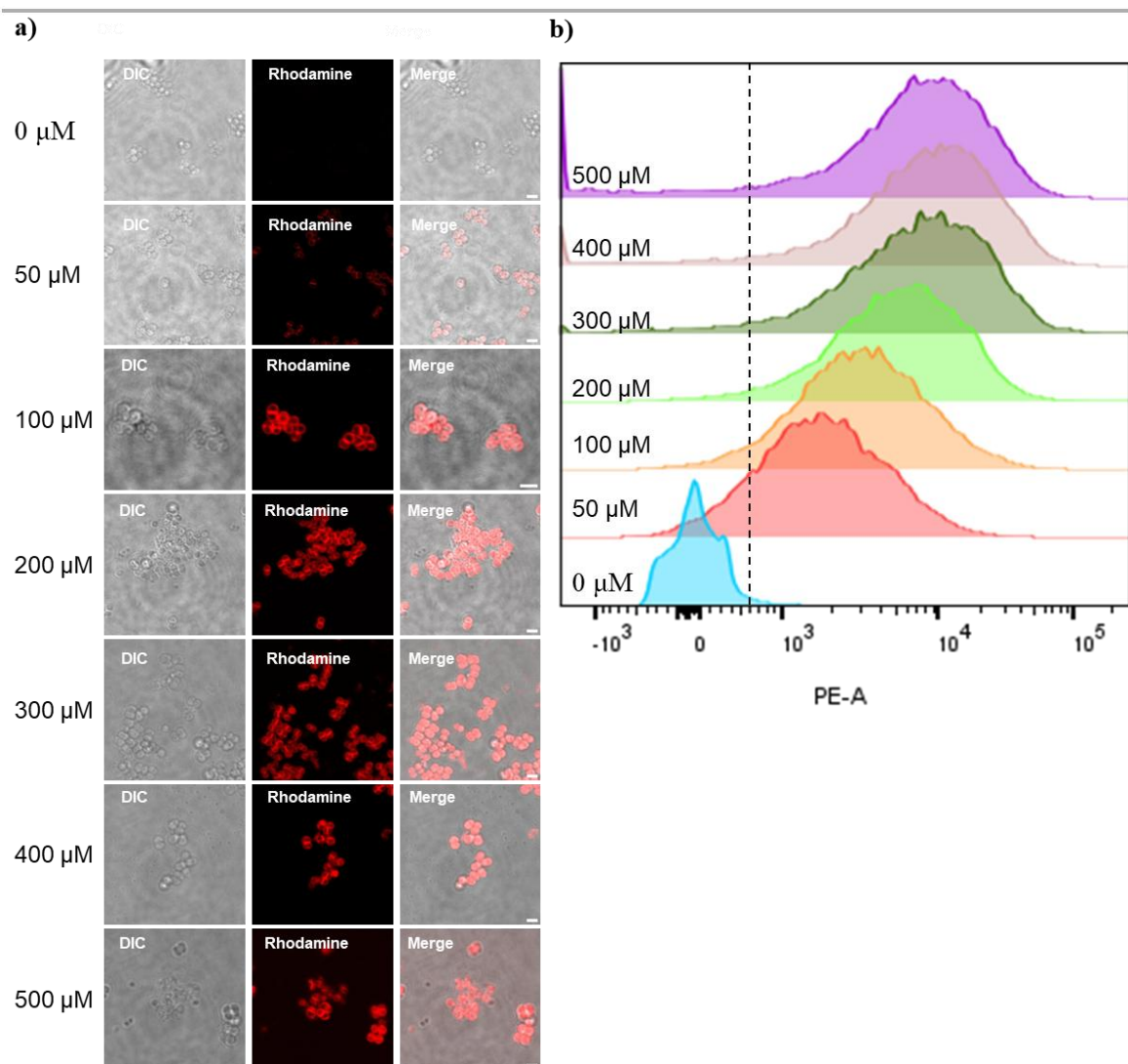
However, acylated (**11b**) or *rap*i-Gly-tag modified (**11c** or **11d**) rhodamine-tagged Gly₅ exhibits no metabolic incorporation.



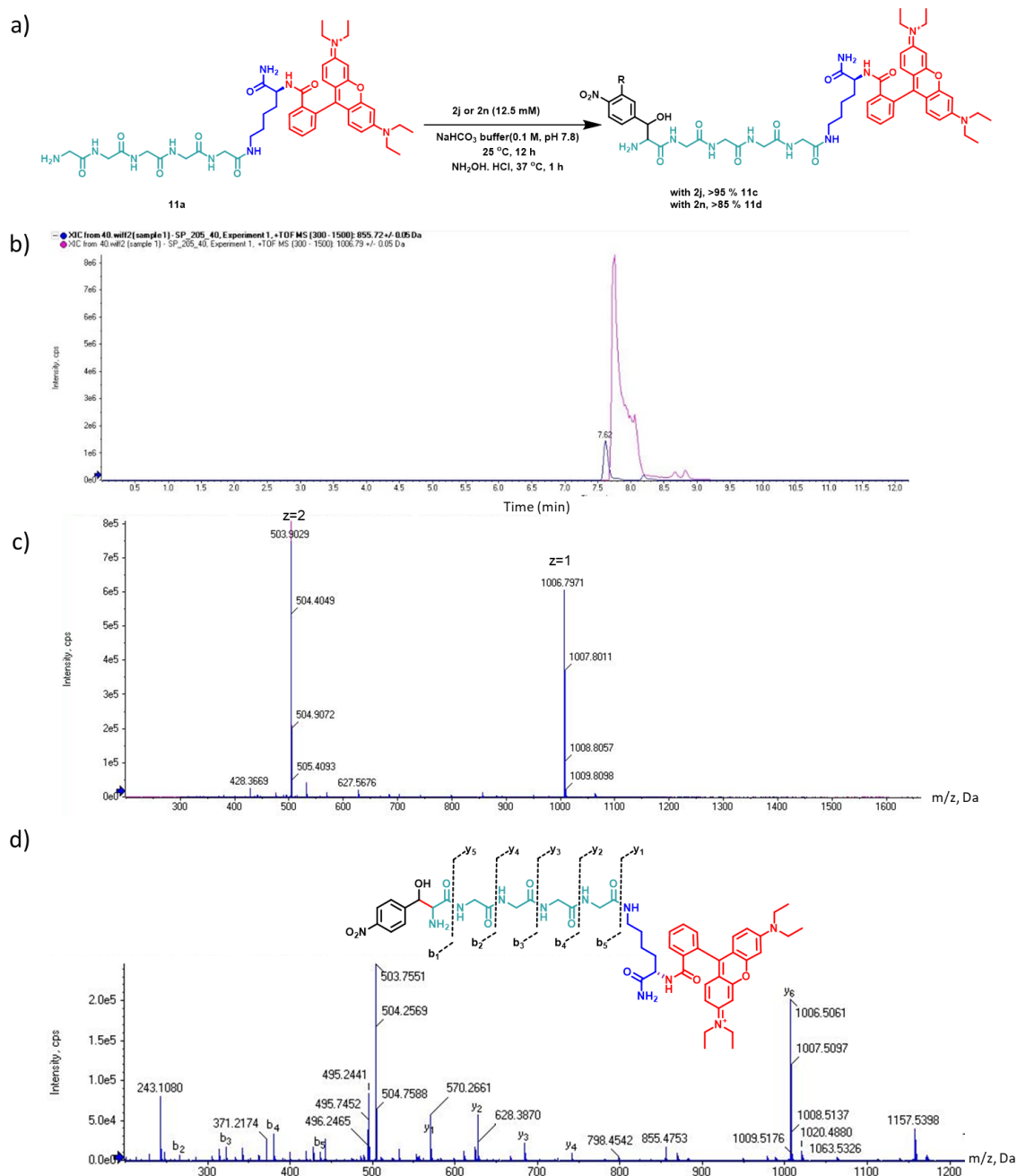
Supplementary Fig. 146. MS-based characterization of **11a**. (a) XIC data of **11a** with m/z 855.46 Da. (b) MS spectra of **11a** with m/z 855.46 [M+H]⁺, and m/z 428.23 [M+2H]²⁺. (c) MS-MS spectra of **11a** peptide.



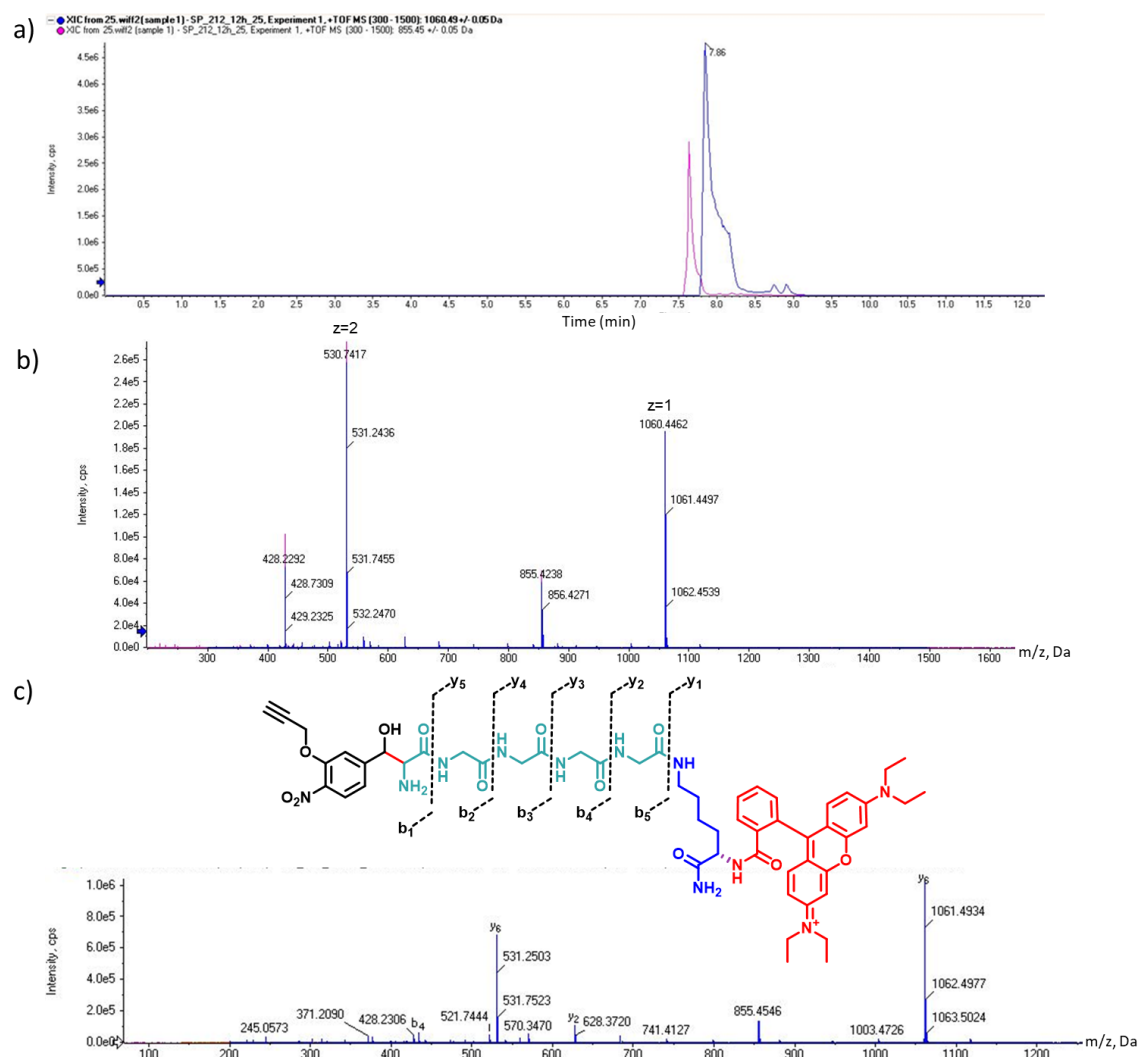
Supplementary Fig. 147. MS-based characterization of **11b**. (a) XIC data of **11b** with m/z 897.42 Da. (b) MS spectra of **11b** with m/z 897.42 $[M+H]^+$, and m/z 449.21 $[M+2H]^{2+}$. (c) MS-MS spectra of **11b** peptide.



Supplementary Fig. 148. Concentration-dependent metabolic incorporation of Gly₅-derived peptide **11a** into *S. aureus*. (a) CLSM images for metabolic incorporation, scale bar, 2 μm . (b) Flow cytometry analysis of metabolic incorporation. Experiments were performed $n = 3$.

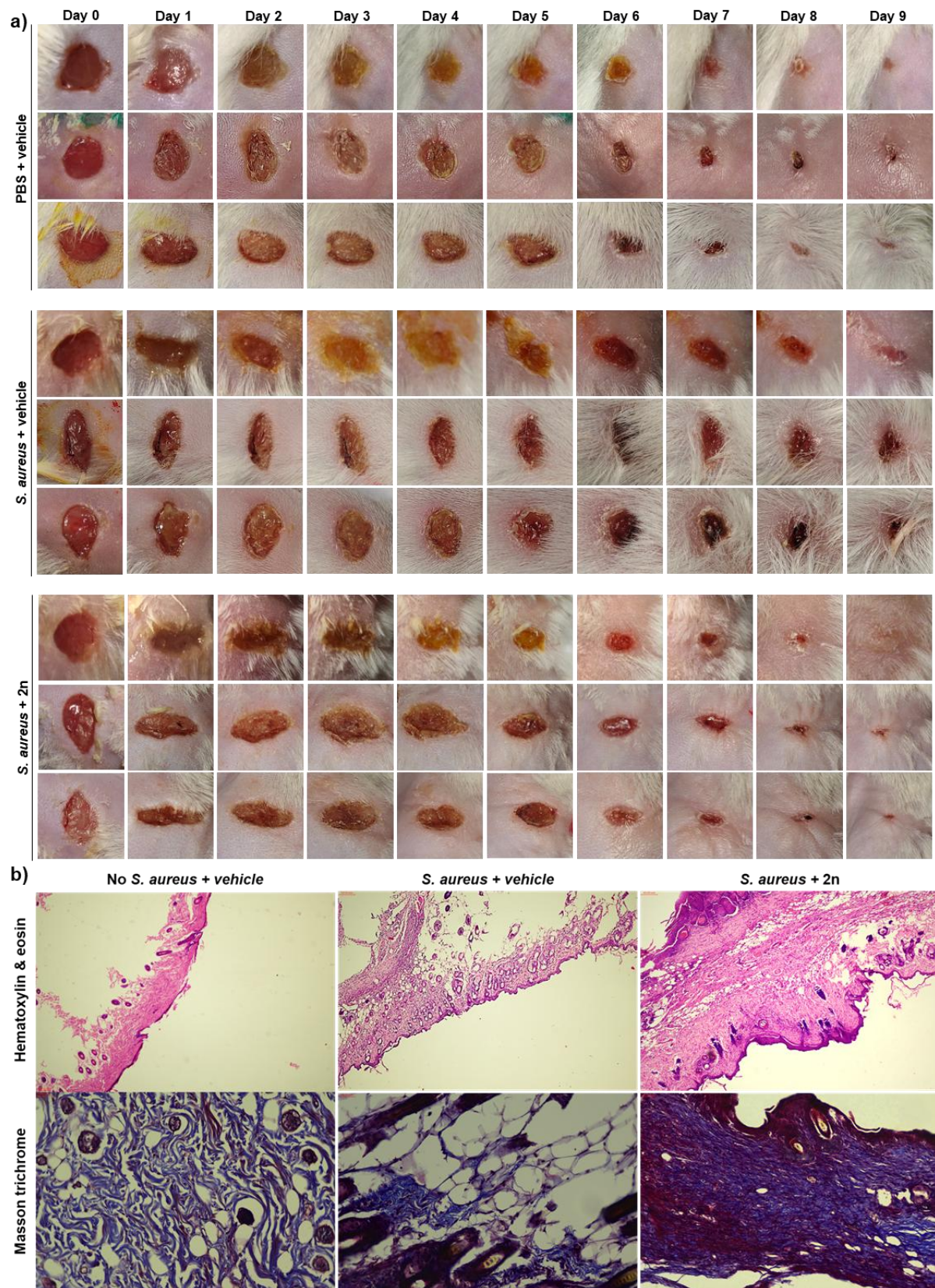


Supplementary Fig. 149. N-Gly modification of Gly₅ peptide with *rapi*-Gly-tag reagents. (a) Reaction of **11a** with **2j** and **2n**, yielding N-Gly modification to render **11c** (>95%) and **11d** (>85%), respectively. (b) XIC data of **11a** (m/z 855.42 Da) and **11c** (m/z 1006.79 Da). (c) MS spectra of **11c** with m/z 1006.79 [M+H]⁺ and m/z 503.90 [M+2H]²⁺. (c) MS-MS of labeled peptide **11c**.



Supplementary Fig. 150. (a) XIC data of **11a** (m/z 855.45 Da) and **11d** (m/z 1060.49 Da). (c) MS spectra of **11d** with m/z 1060.44 [M+H]⁺ and m/z 530.74 [M+2H]²⁺. (c) MS-MS of labeled peptide **11d**.

10. Inhibition of *S. aureus*: *in vivo*

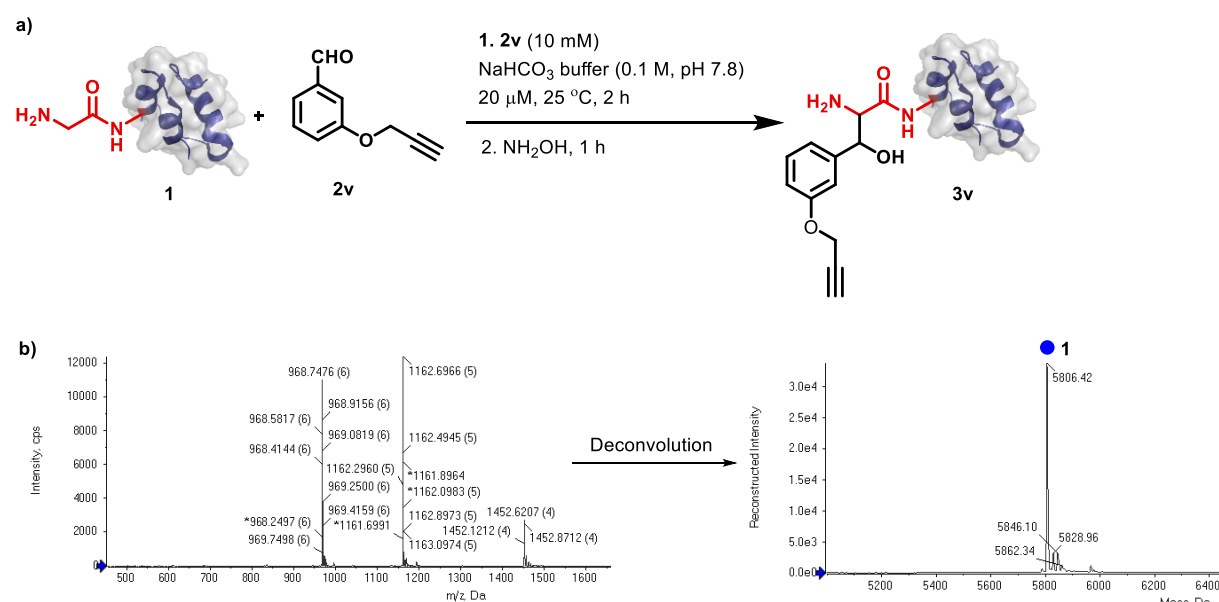


Supplementary Fig. 151. Inhibition of *S. aureus* in mice using a wound infection model. (a) Mice skin wounds from three treatment groups: control (PBS + vehicle), *S. aureus*-infected

treated with vehicle, and *S. aureus*-infected treated with **2n**. Photographs were taken daily from day 0 to day 9 to monitor wound progression and represent biologically independent triplicates per group. (b) Histological evaluation of wound healing on day 9 by Hematoxylin and Eosin (H&E) staining (upper panel) and Masson's Trichrome staining (MT) of wound sections. Uninfected wounds show normal healing progression with restored epithelial layers, whereas *S. aureus*-infected and vehicle-treated wounds exhibit pronounced inflammation, tissue disruption, and delayed epithelial closure. The **2n**-treatment of *S. aureus*-infected mice results in reduced inflammatory infiltration and improved tissue organization. Additionally, MT staining demonstrates extracellular matrix remodeling of wounds. Uninfected wounds display organized collagen deposition, while infected and vehicle-treated wounds show disrupted and sparse matrix. Treatment with **2n** enhances collagen density and alignment. Experiments were performed n = 3.

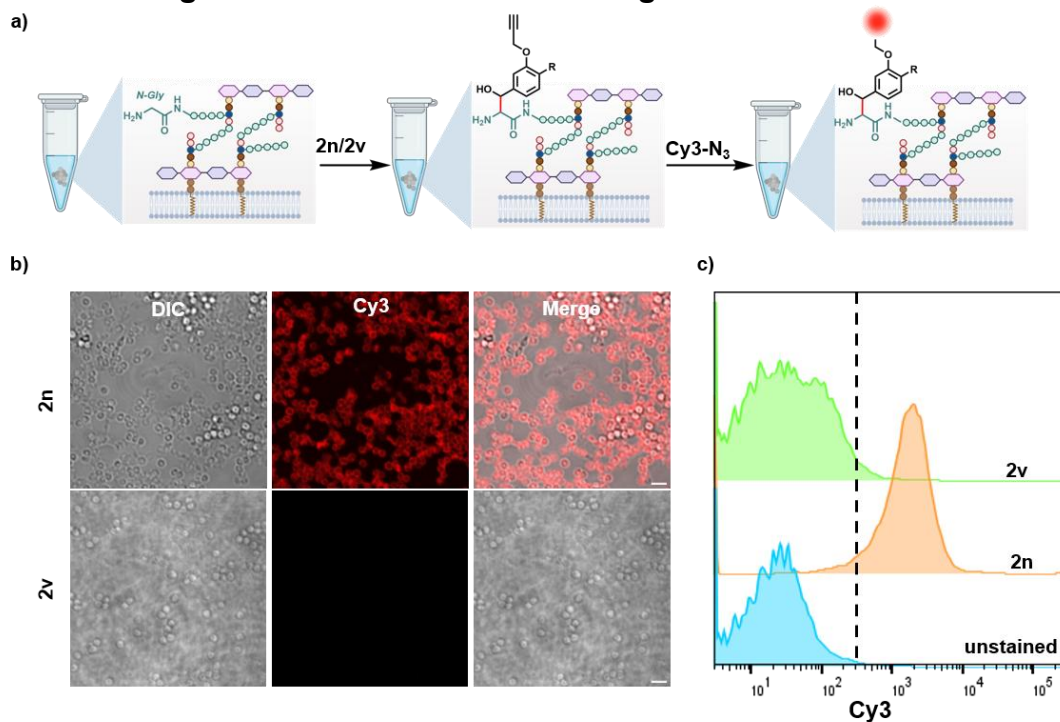
11. Additional results and discussion

11.1 Reactivity of control reagent 2v with model protein



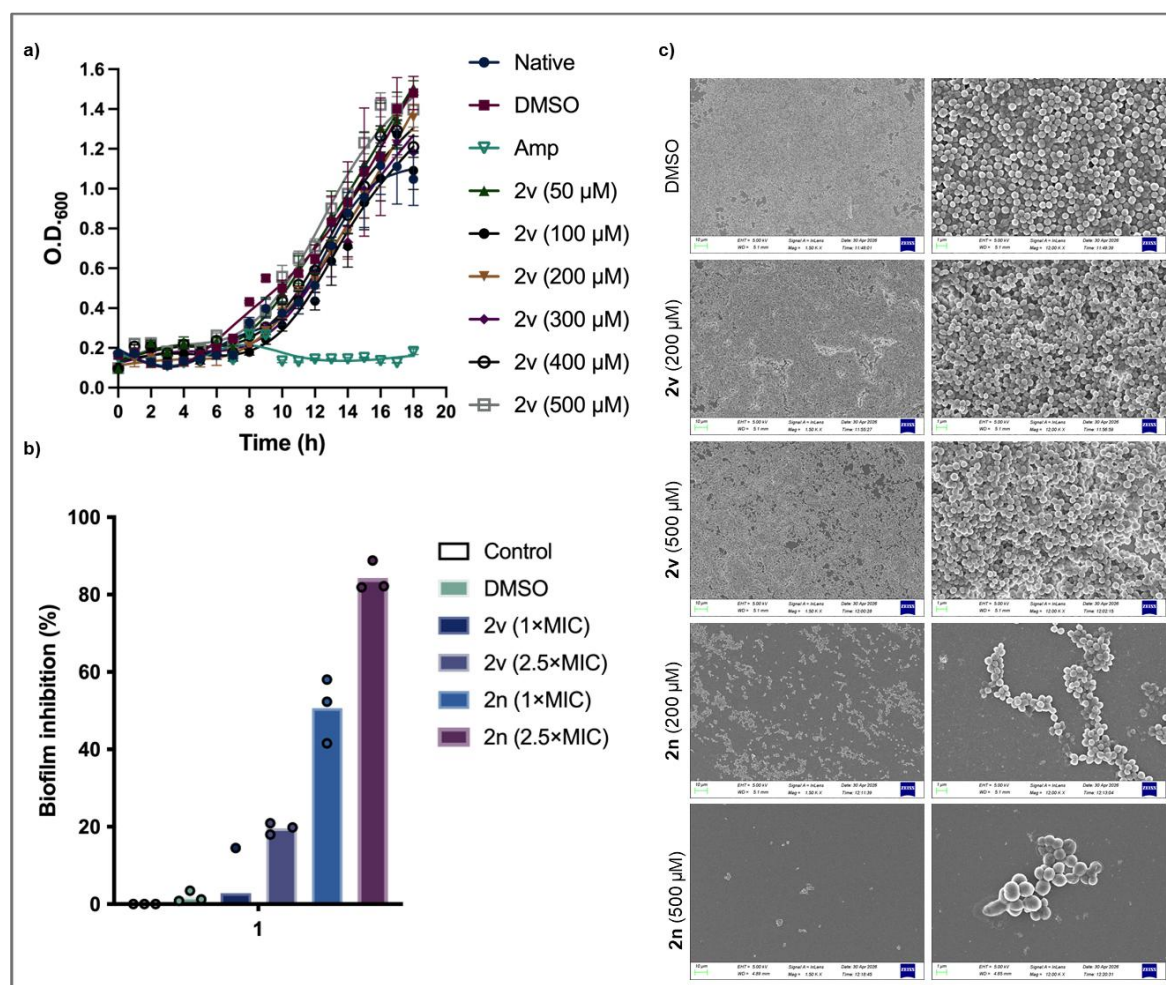
Supplementary Fig. 152. (a) Labeling of insulin (**1**) with control reagent **2v** (lacking -NO₂). (b) Charged and deconvoluted MS spectra for insulin (**1**) after reaction with **2v**.

11.2 Labeling of *S. aureus* with control reagent 2v

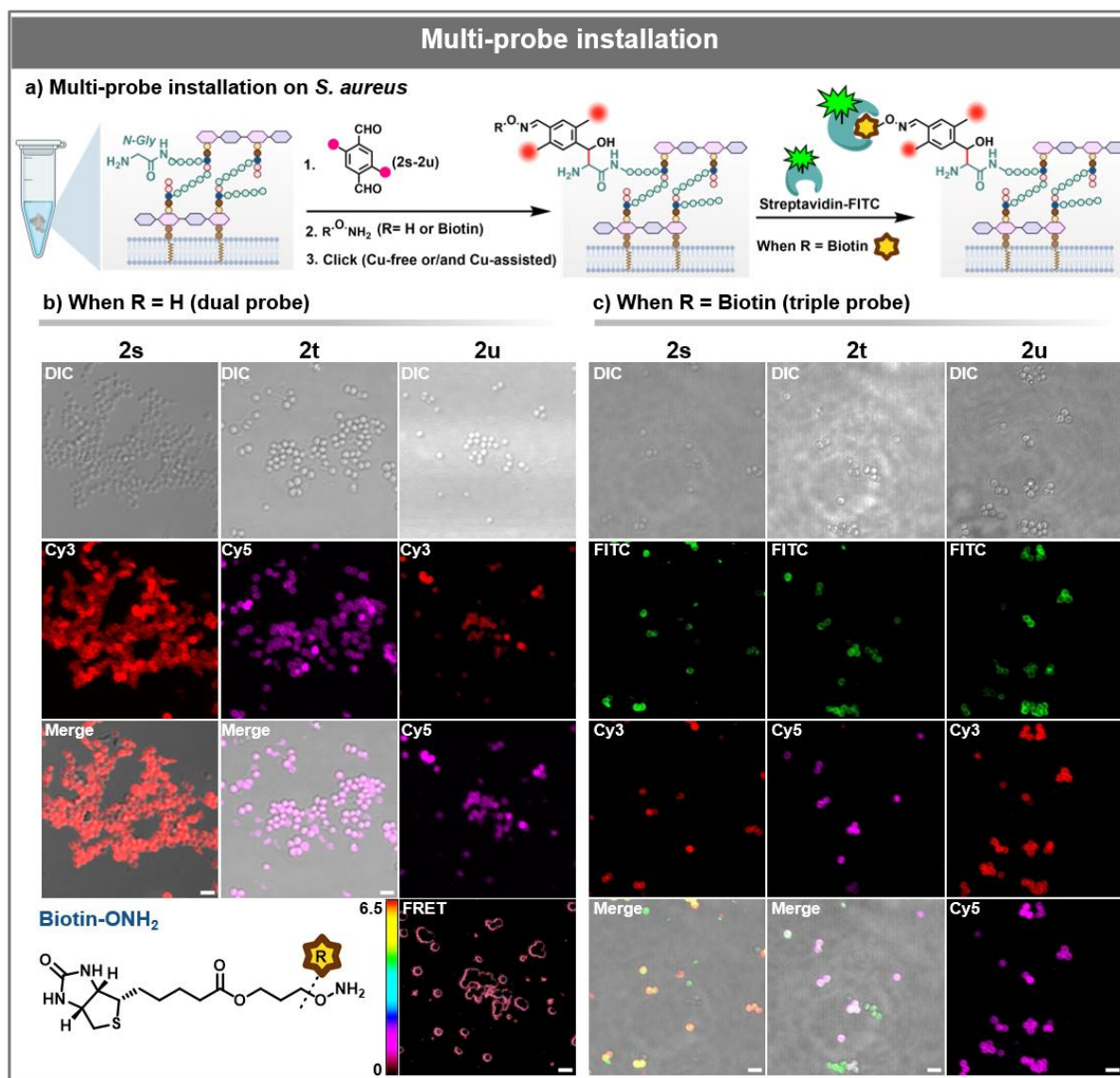


Supplementary Fig. 153. Negative control for selectivity of *rap*i-Gly-tag platform. **a**, labeling of insulin with *rap*i-Gly-tag reagent **2n** and **2v** (control reagent). **b**, Workflow for labeling of *S. aureus* with **2n/2v** followed by click conjugation of Cy3-azide. **c**, CLSM analysis, scale bar 2 μ m. **d**, flow cytometry analysis for labeling of *S. aureus* with **2n/2v**. Experiments were performed $n = 3$.

11.3 Functional effect of control reagent 2v on growth and biofilm inhibition

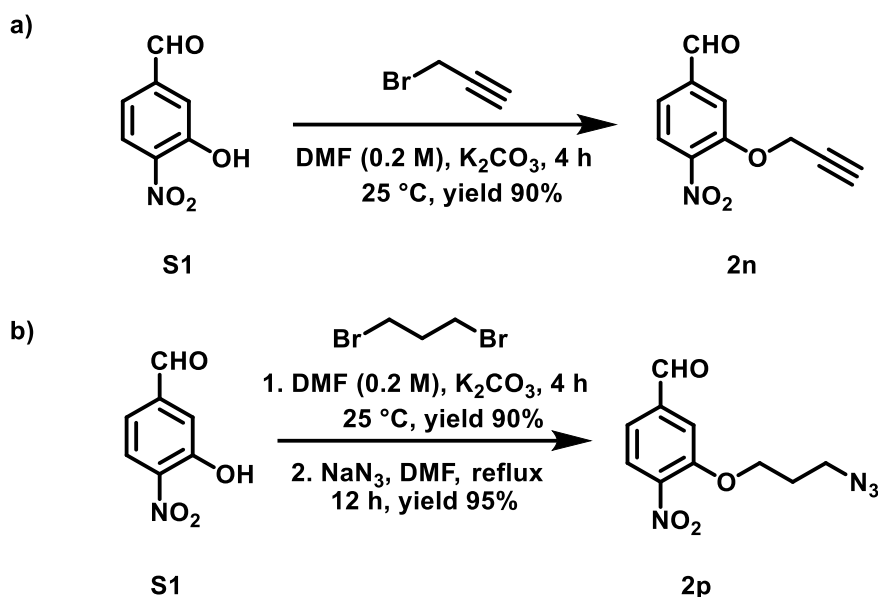


Supplementary Fig. 154. Functional studies of negative control reagent **2v**. **a**, Growth curves of *S. aureus* upon treatment with different concentrations of **2v** (50-500 μ M). **b**, **c**, Inhibition of *S. aureus* biofilm formation: Crystal violet assay and FE-SEM analysis showing reduction in biofilm formation when treated with different concentrations of **2n/2v** at 1 $\times$ MIC and 2.5 $\times$ MIC. Scale bar, 10 μ m (left panel), 1 μ m (Right panel). Experiments were performed n = 3; data are presented as the mean $\pm$ SD.



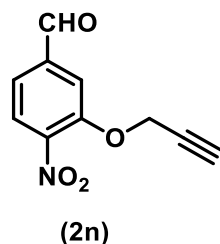
Supplementary Fig. 155 | Multi-probe installation on *S. aureus*. **a**, Multifunctional probes (**2s-2u**) installation on the *S. aureus* surface. Subsequently, RONH_2 is utilized for oxime formation. When $\text{R} = \text{H}$, dual probe installation. When $\text{R} = \text{Biotin}$, triple probe installation. Post oxime formation, Cy5 (for **2t**) was installed with Cu-free click, Cy3 (for **2s**) was installed with Cu-assisted click. For **2u**, Cy5 (Cu-free) and Cy3 (Cu-assisted) were installed sequentially. For triple probe installation, cells post click conjugation were incubated with streptavidin-FITC. **b**, CLSM images show *S. aureus* treated with **2s** (dual-Cy3), **2t** (dual-Cy5), and **2u** (complementary probes, Cy3 and Cy5) at $250\ \mu\text{M}$ for 2 h. FRET analysis using the Cy3 (donor) / Cy5 (acceptor) pair; pixel intensities are presented as a pseudocolour map, and a colour-coded scale bar maps pixel-value to display colour. **c**, CLSM images show simultaneous triple probe installation. Scale bar, $2\ \mu\text{m}$.

12. Synthesis of reagents



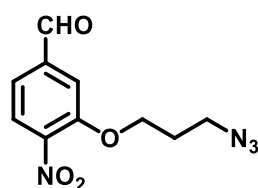
Supplementary Fig. 156. (a) Synthesis of 4-nitro-3-(prop-2-yn-1-yloxy)benzaldehyde (**2n**). (b) Synthesis of 3-(3-azidopropoxy)-4-nitrobenzaldehyde (**2p**).

Synthesis of 4-nitro-3-(prop-2-yn-1-yloxy)benzaldehyde (**2n**)



In a 50 mL round-bottom flask, a solution of 3-hydroxy 4-nitro benzaldehyde (**S1**, 500 mg, 2.99 mmol) in DMF (0.2 M) and K_2CO_3 (826 mg, 5.98 mmol) was added, followed by the addition of propargyl bromide (250 μ L, 3.29 mmol). The reaction mixture was stirred at 25 °C for 4 h. The progress of the reaction was monitored by TLC and LC-MS. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in ethyl acetate, and salts were removed by solvent extraction with ethyl acetate: water (1:1, 50 ml). The organic layers were dried with anhydrous Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (15:85) to afford 4-nitro-3-(prop-2-yn-1-yloxy)benzaldehyde (**2n**, 552 mg, 90% yield; R_f = 0.5, ethyl acetate:n-hexane 20:80; white, brown solid). 1H NMR (500 MHz, $CDCl_3$): δ 10.06 (s, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.75 (d, J = 1.1 Hz, 1H), 7.60 (dd, J = 8.1, 1.1 Hz, 1H), 4.93 (d, J = 2.3 Hz, 2H), 2.62 (t, J = 2.3 Hz, 1H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 190.1, 150.9, 143.9, 139.4, 126.1, 123.2, 114.7, 77.9, 76.4, 57.4 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_{10}H_8NO_4$ 206.0453, found 206.0441.

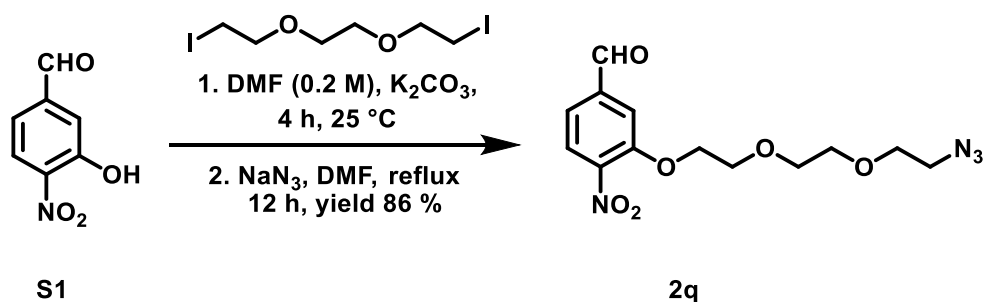
Synthesis of 3-(3-azidopropoxy)-4-nitrobenzaldehyde (2p)



(2p)

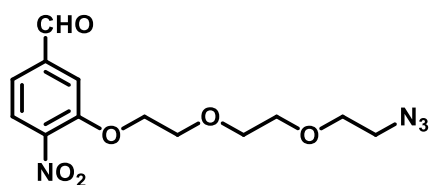
In a 50 mL round-bottom flask, a solution of 3-hydroxy 4-nitrobenzaldehyde (**S1**, 500 mg, 2.99 mmol) in DMF (0.2 M) and K_2CO_3 (826 mg, 5.98 mmol) was added, followed by the addition of 1,3-dibromopropane (430 μ L, 4.19 mmol). The reaction mixture was stirred at 25 °C for 4 h. The progress of the reaction was monitored by TLC and LC-MS. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in ethyl acetate, and salts were removed by solvent extraction with ethyl acetate: water (1:1, 50 mL). The organic layers were dried with anh. Na_2SO_4 and concentrated using a rotary evaporator. Then the crude mixture was taken to the next step to get the final compound.

Next, in a 50 mL round-bottom flask, a crude mixture of 3-(3-bromopropoxy)-4-nitrobenzaldehyde (200 mg, 0.69 mmol) in DMF (0.2 M) and NaN_3 (140 mg, 2.07 mmol) was added. The reaction mixture was refluxed for 12 h. The progress of the reaction was monitored by TLC. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in ethyl acetate, and salts were removed by solvent extraction with ethyl acetate: water (1:1, 50 mL). The organic layers were dried with anh. Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (10:90) to afford 3-(3-azidopropoxy)-4-nitrobenzaldehyde (**2p**, 164 mg, 95% yield; R_f = 0.6, ethyl acetate:n-hexane 20:80; yellow viscous liquid). 1H NMR (500 MHz, $CDCl_3$): δ 10.04 (s, 1H), 7.93 (d, J = 8.1 Hz, 1H), 7.99 (s, 1H), 7.55 (d, J = 8.1 Hz, 1H), 4.28 (t, J = 5.8 Hz, 2H), 3.57 (t, J = 6.4 Hz, 2H), 2.11 (m, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 190.1, 152.0, 143.3, 139.6, 125.9, 122.7, 113.3, 66.3, 47.6, 28.3 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_{10}H_{11}N_4O_4$ 251.0780, found 251.0766.



Supplementary Fig. 157. Synthesis of 3-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-4-nitrobenzaldehyde (**2q**).

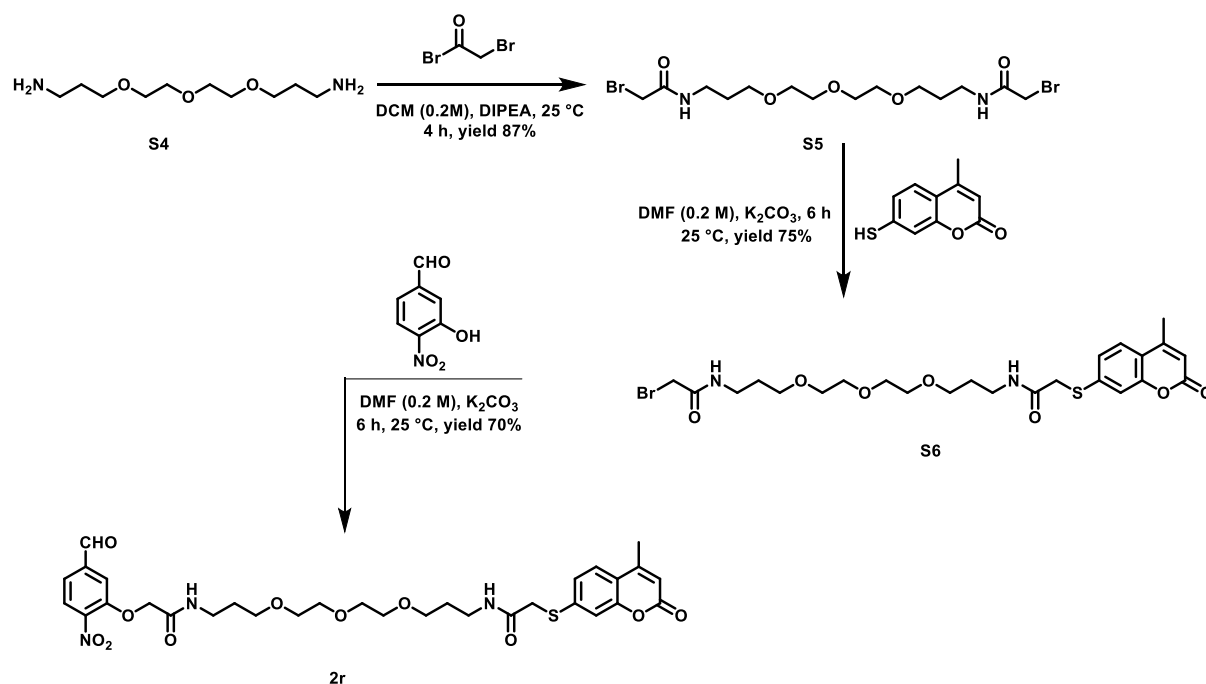
Synthesis of 3-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)-4-nitrobenzaldehyde (2q)



(2q)

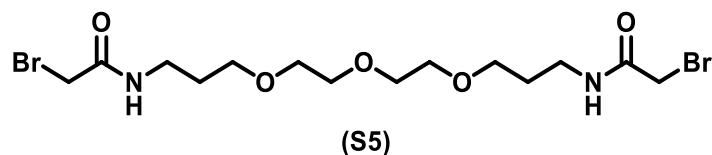
In a 100 mL round-bottom flask, a solution of 3-hydroxy 4-nitrobenzaldehyde (**S1**, 1.0 g, 5.98 mmol) in DMF (0.2 M) and K_2CO_3 (1.6 g, 11.97 mmol) was added, followed by the addition of 1,2-bis(2-iodoethoxy)ethane (1.53 mL, 8.37 mmol). The reaction mixture was stirred at 25 °C for 4 h. The progress of the reaction was monitored by TLC and LC-MS. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in dichloromethane (DCM), and salts were removed by solvent extraction with DCM: water (1:1, 50 mL). The organic layers were dried with anhydrous Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was then taken to the next step to get the final compound.

Next, in a 50 mL round-bottom flask, a crude mixture of 3-(2-(2-(2-iodoethoxy)ethoxy)ethoxy)-4-nitrobenzaldehyde (300 mg, 0.73 mmol) in DMF (0.2 M) and NaN_3 (140 mg, 2.19 mmol) was added. The reaction mixture was refluxed for 12 h. The progress of the reaction was monitored by TLC. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in DCM, and salts were removed by solvent extraction with DCM: water (1:1, 50 mL). The organic layers were dried with anhydrous Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (50:50) to afford 3-(3-azidopropoxy)-4-nitrobenzaldehyde (**2q**, 225 mg, 95% yield; R_f = 0.3, ethyl acetate:n-hexane 60:40; brown solid). 1H NMR (500 MHz, $CDCl_3$): δ 10.01 (d, J = 1.2 Hz, 1H), 7.89 (dd, J = 8.0, 1.2 Hz, 1H), 7.62 (d, J = 1.2 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 4.34 (t, J = 4.6 Hz, 2H), 3.91 (t, J = 4.7 Hz, 2H), 3.73 (m, 2H), 3.67-3.64 (m, 4H), 3.36 (t, J = 5.0 Hz, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 190.1, 152.2, 143.4, 139.4, 125.7, 122.4, 114.1, 70.9, 70.5, 69.9, 69.8, 69.1, 50.5 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_{13}H_{17}N_4O_6$ 325.1148, found 325.1127.



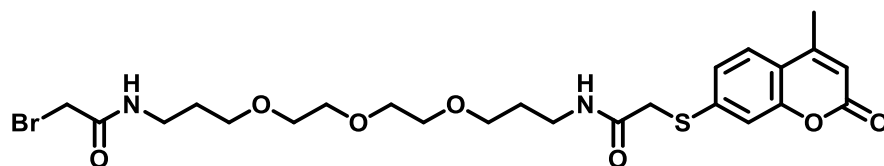
Supplementary Fig. 158. Synthesis of 2-(5-formyl-2-nitrophenoxy)-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)oxy)-2-oxo-7,10,13-trioxa-3-azaheptadecan-16-yl)acetamide (**2r**).

Synthesis of N,N'-(((oxybis(ethane-2,1-diyl))bis(oxy))bis(propane-3,1-diyl))bis(2-bromoacetamide) (**S5**)



In a 25 mL round-bottom flask, 3,3'-((oxybis(ethane-2,1-diyl))bis(oxy))bis(propan-1-amine) (**S4**, 500 mg, 2.27 mmol) in DCM, followed by the addition of DIPEA (1.2 mL, 6.81 mmol). Next, dropwise into the reaction mixture under ice-cold conditions, bromo-acetyl bromide (440 μ L, 5.0 mmol) was added. After 30 mins, the reaction mixture was brought to 25 $^{\circ}$ C and stirred for 4 h. The reaction progress was monitored with TLC and LC-MS. After completion, the reaction mixture was diluted with DCM and extracted with water three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl methanol:DCM (2:98) to afford N,N'-(((oxybis(ethane-2,1-diyl))bis(oxy))bis(propane-3,1-diyl))bis(2-bromoacetamide) (**S5**, 913 mg, 87% yield; R_f = 0.6, methanol:DCM (10:90); white solid). ^1H NMR (500 MHz, CDCl_3): δ 7.17 (bs, 2H), 3.84 (s, 4H), 3.65 (m, 4H), 3.61 (m, 4H), 3.58 (m, 4H), 3.39 (m, 4H), 1.80 (m, 4H) ppm. ^{13}C NMR (126 MHz, CDCl_3): δ 166.6, 70.6, 70.4, 70.3, 39.0, 29.4, 28.6 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_{14}\text{H}_{26}\text{Br}_2\text{N}_2\text{NaO}_5$ 483.0106, found 483.0094.

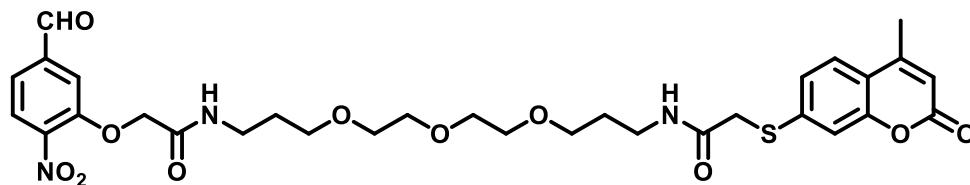
Synthesis of 2-bromo-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)thio)-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)acetamide (S6)



S6

In a 50 mL round bottom flask, N,N'-(((oxybis(ethane-2,1-diyl))bis(oxy))bis(propane-3,1-diyl))bis(2-bromoacetamide) (**S5**, 700 mg, 1.51 mmol), 7-mercapto-4-methyl-2H-chromen-2-one (290 mg, 1.51 mmol), and K₂CO₃ (210 mg, 1.51 mmol) were dissolved in DMF (0.2 M). The reaction mixture was stirred at 25 °C for 4 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 50 mL) three times. The organic layer was dried with Na₂SO₄ and concentrated using a rotary evaporator. The crude mixture was purified by silica gel gravity column chromatography using methanol:DCM (1:99) to afford 2-bromo-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)thio)-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)acetamide (**S6**, 649 mg, 75% yield; R_f = 0.4, methanol:DCM, 5:95; viscous liquid). ¹H NMR (500 MHz, CDCl₃): δ 7.53 (m, 1H), 7.34-7.29 (m, 2H), 7.23-7.20 (m, 2H), 6.26 (m, 1H), 3.89 (m, 2H), 3.73 (s, 2H), 3.71-3.61 (m, 8H), 3.58 (m, 2H), 3.53 (m, 2H), 3.47-3.39 (m, 4H), 2.44 (m, 3H), 1.85 (m, 2H), 1.76 (m, 2H) ppm. ¹³C NMR (126 MHz, CDCl₃): δ 167.2, 165.7, 160.5, 153.8, 152.2, 141.2, 125.2, 122.8, 118.1, 114.9, 114.6, 70.6, 70.5, 70.4, 70.3 (2C), 70.2, 39.0, 38.7, 36.5, 29.4, 28.9, 28.7, 18.7 ppm. HRMS (ESI) [M+H]⁺ calcd. C₂₄H₃₄BrN₂O₇S 573.1270, found 573.1279.

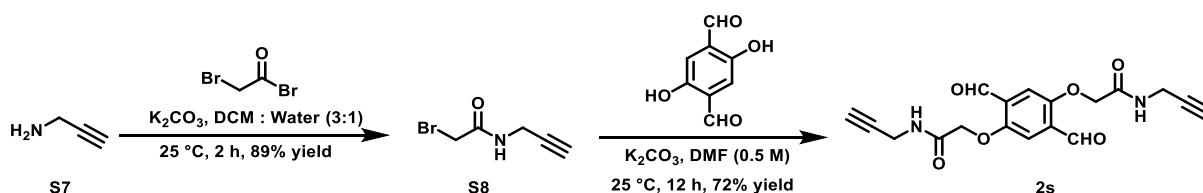
Synthesis of 2-(5-formyl-2-nitrophenoxy)-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)thio)-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)acetamide (2r)



2r

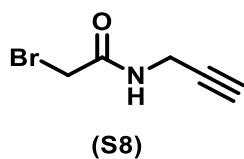
In a 25 mL round-bottom flask, 2-bromo-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)thio)-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)acetamide (**S6**, 310 mg, 0.55 mmol), 3-hydroxy-4-nitrobenzaldehyde (140 mg, 0.83 mmol), and K₂CO₃ (152 mg, 1.1 mmol) were dissolved in DMF (0.2 M). The reaction mixture was stirred at 25 °C for 6 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of

DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 60 mL) three times. The organic layer was dried with Na₂SO₄ and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using methanol:DCM (2:98) to afford 2-(5-formyl-2-nitrophenoxy)-N-(1-((4-methyl-2-oxo-2H-chromen-7-yl)thio)-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)acetamide (**2r**, 254 mg, 70% yield; R_f = 0.5, methanol:DCM, 5:95; viscous liquid). ¹H NMR (500 MHz, CDCl₃): δ 10.06 (s, 1H), 8.10 (d, *J* = 8.2 Hz, 1H), 7.64 (d, *J* = 8.2 Hz, 1H), 7.57 (s, 1H), 7.49 (d, *J* = 8.2 Hz, 1H), 7.29 (m, 1H), 7.21 (s, 1H), 7.19-7.17 (m, 2H), 6.21 (s, 1H), 3.68 (s, 2H), 3.61-3.58 (m, 7H), 3.56-3.44 (m, 9H), 3.38-3.34 (m, 2H), 2.39 (s, 3H), 1.84 (m, 2H), 1.71 (m, 2H) ppm. ¹³C NMR (126 MHz, CDCl₃): δ 189.8, 167.2, 166.2, 160.5, 153.8, 152.2, 151.3, 142.5, 141.2, 140.5, 127.2, 125.2, 123.4, 122.8, 118.0, 114.9, 114.5, 114.4, 70.6, 70.5, 70.4, 70.3, 70.2, 69.0, 68.3, 38.8, 37.0, 36.5, 29.4, 28.8, 18.7 ppm. HRMS (ESI) [M+H]⁺ calcd. C₃₁H₃₈N₃O₁₁S 660.2227, found 660.2226.



Supplementary Fig. 159. Synthesis of 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(prop-2-yn-1-yl)acetamide) (**2s**).

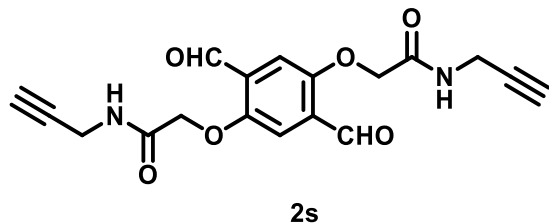
Synthesis of 2-bromo-N-(prop-2-yn-1-yl)acetamide (**S8**)



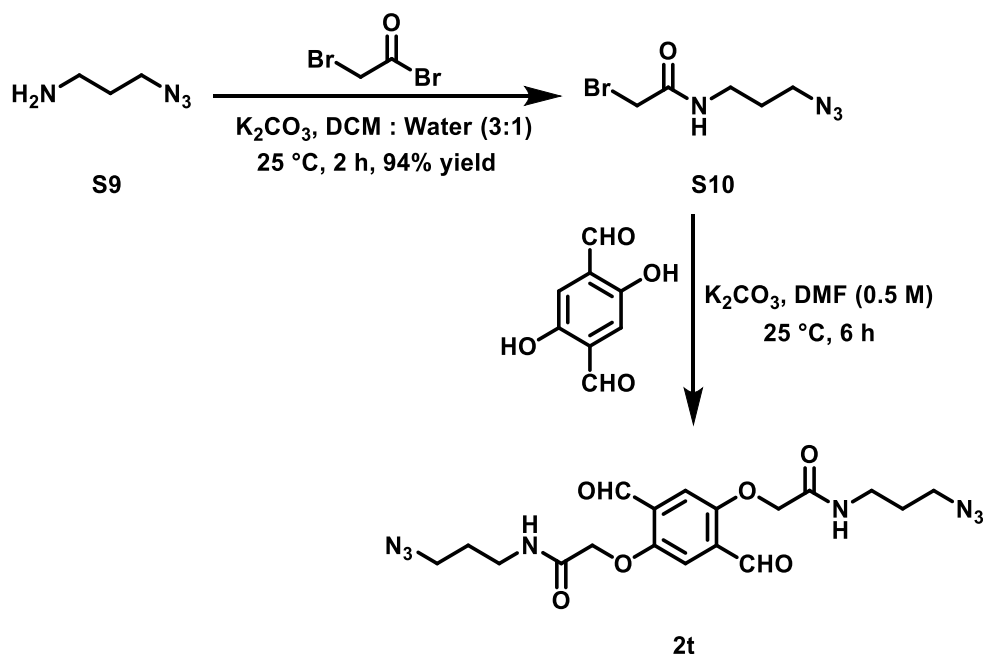
In a 25 mL round-bottom flask, prop-2-yn-1-amine (**S7**, 460 μL, 7.26 mmol) in DCM and water (3:1), followed by the addition of K₂CO₃ (2.7 mL, 14.52 mmol). Next, bromoacetyl bromide (1.9 mL, 21.78 mmol) was added dropwise into the reaction mixture under ice-cold conditions. After 30 mins, the reaction mixture was brought to 25 °C and stirred for 2 h. The reaction progress was monitored with TLC and LC-MS. After completion, the reaction mixture was diluted with DCM and extracted with water (1:1, 50 mL) three times. The organic layer was dried with Na₂SO₄ and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (40:60) to afford 2-bromo-N-(prop-2-yn-1-yl)acetamide (**S8**, 1.14 gm, 89% yield; R_f = 0.4, ethyl acetate:n-hexane (50:50); white solid). ¹H NMR (500 MHz, CDCl₃): δ 6.66 (bs, 1H), 4.09 (dd, *J* = 5.3, 2.6 Hz,

2H), 3.90 (s, 2H), 2.28 (t, $J = 2.6$ Hz, 1H) ppm. ^{13}C NMR (126 MHz, CDCl_3): δ 165.1, 78.5, 72.3, 29.9, 28.7 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_5\text{H}_7\text{BrNO}$ 175.9711, found 175.9701.

Synthesis of 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(prop-2-yn-1-yl)acetamide) (2s)

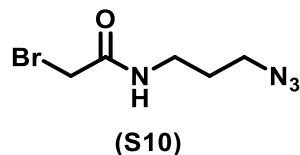


In a 50 mL round-bottom flask, 2-bromo-N-(prop-2-yn-1-yl)acetamide (**S8**, 630 mg, 3.6 mmol), 2,5-dihydroxyterephthalaldehyde (200 mg, 1.2 mmol), and K_2CO_3 (498 mg, 3.6 mmol) were dissolved in DMF (0.2 M). The reaction mixture was stirred at 25 °C for 12 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 50 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (80:20) to afford 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(prop-2-yn-1-yl)acetamide) (**2s**, 308 mg, 72% yield; $R_f = 0.5$, ethyl acetate:n-hexane, 80:20; yellow solid). ^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 10.49 (s, 2H), 8.63 (t, $J = 6.0$ Hz, 2H), 7.39 (s, 2H), 4.76 (s, 4H), 3.92 (m, 4H), 3.10 (m, 2H) ppm. ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$): δ 189.5, 167.0, 153.9, 128.9, 112.4, 80.9, 73.0, 67.6, 27.7 ppm. HRMS (ESI) $[\text{M}+\text{Na}]^+$ calcd. $\text{C}_{18}\text{H}_{16}\text{N}_2\text{NaO}_6$ 379.0906, found 379.0890.



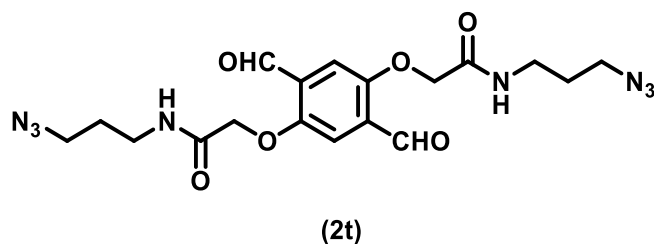
Supplementary Fig. 160. Synthesis of 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(3-azidopropyl)acetamide) (**2t**).

Synthesis of N-(3-azidopropyl)-2-bromoacetamide (**S10**)



In a 25 mL round-bottom flask, 3-azidopropan-1-amine (**S9**, 500 mg, 4.99 mmol) in DCM and water (3:1), followed by the addition of K_2CO_3 (2.1 g, 14.97 mmol). Next, bromo-acetyl bromide (1.3 mL, 14.97 mmol) was added dropwise into the reaction mixture under ice-cold conditions. After 30 mins, the reaction mixture was brought to 25 °C and stirred for 2 h. The reaction progress was monitored with TLC and LC-MS. After completion, the reaction mixture was diluted with DCM and extracted with water (1:1, 70 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (30:70) to afford N-(3-azidopropyl)-2-bromoacetamide (**S10**, 1.04 gm, 94% yield; R_f = 0.3, ethyl acetate:n-hexane (40:60); yellow liquid). 1H NMR (500 MHz, $CDCl_3$): δ 7.06 (bs, 1H), 3.82 (s, 2H), 3.35-3.30 (m, 4H), 1.77 (m, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 165.7, 49.2, 37.9, 29.1, 28.4 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_5H_9BrN_4NaO$ 242.9857, found 242.9847.

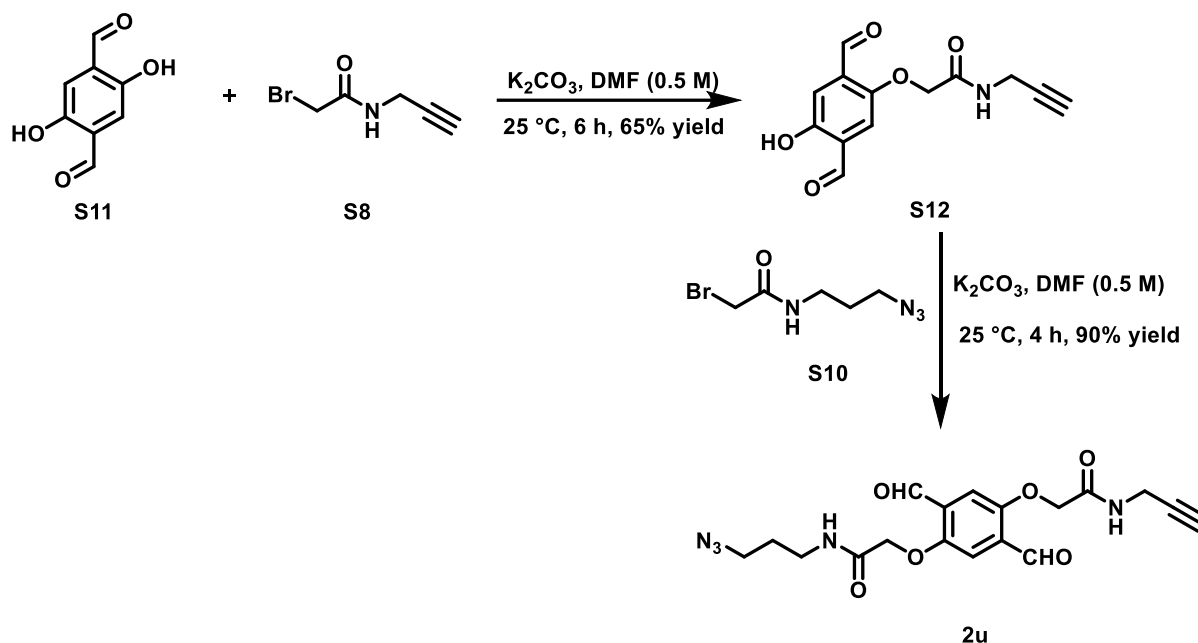
Synthesis of 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(3-azidopropyl)acetamide) (**2t**)



In a 50 mL round-bottom flask, N-(3-azidopropyl)-2-bromoacetamide (**S10**, 995 mg, 4.5 mmol), 2,5-dihydroxyterephthalaldehyde (250 mg, 1.5 mmol), and K_2CO_3 (622 mg, 4.5 mmol) were dissolved in DMF (0.5 M). The reaction mixture was stirred at 25 °C for 6 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 60 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (80:20) to afford 2,2'-((2,5-diformyl-1,4-phenylene)bis(oxy))bis(N-(3-azidopropyl)acetamide) (**2t**, 406

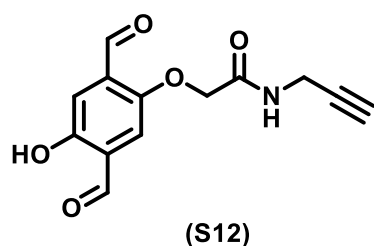
mg, 72% yield; R_f = 0.3, ethyl acetate; brown solid). ^1H NMR (500 MHz, CDCl_3): δ 10.34 (s, 2H), 7.41 (s, 2H), 7.28 (bs, 2H), 4.64 (s, 4H), 3.50 (m, 4H), 3.44 (t, J = 6.5 Hz, 4H), 1.89 (m, 4H) ppm. ^{13}C NMR (126 MHz, CDCl_3): δ 188.3, 166.9, 152.9, 129.2, 116.4, 68.3, 49.5, 37.0, 28.6 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_{18}\text{H}_{23}\text{N}_8\text{O}_6$ 447.1741, found 447.1720.

Synthesis of 2u



Supplementary Fig. 161. Synthesis of N-(3-azidopropyl)-2-(2,5-diformyl-4-(2-oxo-2-(prop-2-yn-1-ylamino)ethoxy)phenoxy)acetamide (2u).

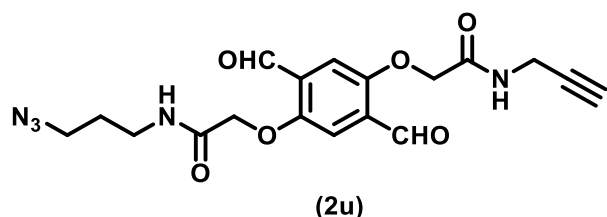
Synthesis of 2-(2,5-diformyl-4-hydroxyphenoxy)-N-(prop-2-yn-1-yl)acetamide (S12):



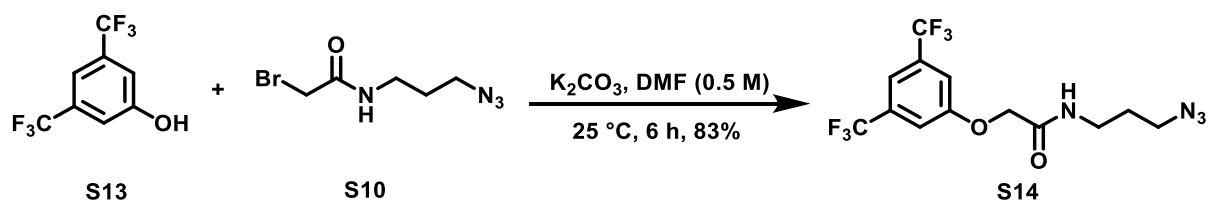
In a 50 mL round-bottom flask, 2-bromo-N-(prop-2-yn-1-yl)acetamide (**S8**, 500 mg, 2.84 mmol), 2,5-dihydroxyterephthalaldehyde (**S11**, 380 mg, 2.27 mmol), and K_2CO_3 (390 mg, 2.84 mmol) were dissolved in DMF (0.2 M). The reaction mixture was stirred at 25 °C for 6 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 50 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl

acetate:n-hexane (60:40) to afford 2-(2,5-diformyl-4-hydroxyphenoxy)-N-(prop-2-yn-1-yl)acetamide (**S12**, 482 mg, 65% yield; R_f = 0.3, ethyl acetate:n-hexane, 80:20; pale yellow solid). ^1H NMR (500 MHz, DMSO- d_6): δ 10.46 (s, 1H), 10.32 (s, 1H), 8.59 (t, J = 5.2 Hz, 1H), 7.33 (s, 1H), 7.28 (s, 1H), 4.68 (s, 2H), 3.92 (d, J = 5.2 Hz, 2H), 3.09 (s, 1H) ppm. ^{13}C NMR (126 MHz, DMSO- d_6): δ 189.9, 189.8, 167.2, 154.8, 152.2, 129.8, 126.7, 115.7, 112.0, 80.9, 72.9, 67.6, 27.8 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_{13}\text{H}_{11}\text{NNaO}_5$ 284.0535, found 284.0523.

Synthesis of N-(3-azidopropyl)-2-(2,5-diformyl-4-(2-oxo-2-(prop-2-yn-1-ylamino)ethoxy)phenoxy)acetamide (**2u**)

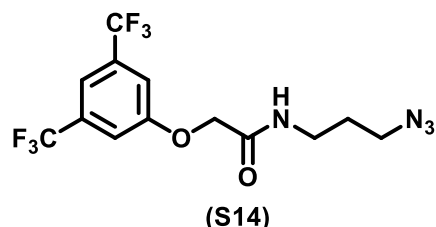


In a 50 mL round-bottom flask, 2-(2,5-diformyl-4-hydroxyphenoxy)-N-(prop-2-yn-1-yl)acetamide (**S12**, 130 mg, 0.49 mmol), N-(3-azidopropyl)-2-bromoacetamide (**S10**, 210 mg, 0.98 mmol), and K_2CO_3 (135 mg, 0.98 mmol) were dissolved in DMF (0.5 M). The reaction mixture was stirred at 25 °C for 4 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 40 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using methanol:DCM (2:98) to afford N-(3-azidopropyl)-2-(2,5-diformyl-4-(2-oxo-2-(prop-2-yn-1-ylamino)ethoxy)phenoxy)acetamide (**2u**, 177 mg, 90% yield; R_f = 0.3, methanol:DCM, 5:95; pale yellow solid). ^1H NMR (500 MHz, DMSO- d_6): δ 10.50 (s, 1H), 10.48 (s, 1H), 8.63 (t, J = 5.7 Hz, 1H), 8.25 (t, J = 5.9 Hz, 1H), 7.40 (s, 1H), 7.37 (s, 1H), 4.76 (s, 2H), 4.72 (s, 2H), 3.93 (dd, J = 5.7, 2.5 Hz, 2H), 3.32 (m, 2H), 3.19 (m, 2H), 3.11 (t, J = 2.5 Hz, 1H), 1.67 (m, 2H) ppm. ^{13}C NMR (126 MHz, DMSO- d_6): δ 189.5, 189.4, 167.1, 166.9, 154.0, 153.9, 128.9 (2C), 112.4, 112.2, 80.9, 73.0, 67.8, 67.6, 48.3, 35.7, 28.4, 27.7 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_{18}\text{H}_{19}\text{N}_5\text{NaO}_6$ 424.1233, found 424.1217.



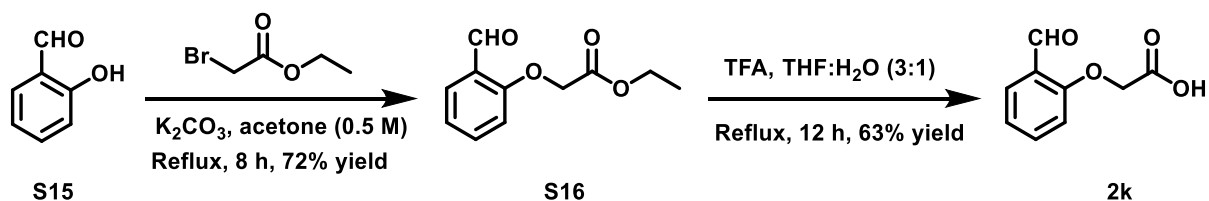
Supplementary Fig. 162. Synthesis of N-(3-azidopropyl)-2-(3,5-bis(trifluoromethyl)phenoxy)acetamide (**S14**).

Synthesis of N-(3-azidopropyl)-2-(3,5-bis(trifluoromethyl)phenoxy)acetamide (S14**)**



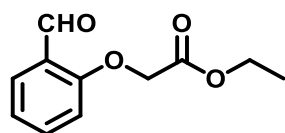
In a 50 mL round-bottom flask, 3,5-bis(trifluoromethyl)phenol (**S13**, 200 mg, 0.87 mmol), N-(3-azidopropyl)-2-bromoacetamide (**S10**, 230 mg, 1.04 mmol), and K_2CO_3 (240 mg, 1.74 mmol) were dissolved in DMF (0.5 M). The reaction mixture was stirred at 25 °C for 6 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of DMF to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 30 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using methanol:DCM (2:98) to afford N-(3-azidopropyl)-2-(3,5-bis(trifluoromethyl)phenoxy)acetamide (**S14**, 267 mg, 83% yield; R_f = 0.4, methanol:DCM, 5:95; pale yellow solid). 1H NMR (500 MHz, $CDCl_3$): δ 7.56 (s, 1H), 7.36 (s, 2H), 6.74 (bs, 1H), 4.58 (s, 2H), 3.47 (m, 2H), 3.40 (t, J = 6.5 Hz, 2H), 1.85 (m, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 166.9, 157.7, 133.5 (q, J = 33.7 Hz), 123 (q, J = 272.9 Hz), 116.0 (m), 115.3 (m), 67.8, 49.5, 37.1, 28.8 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_{13}H_{12}F_6N_4NaO_2$ 393.0762, found 393.0740.

Note: The synthesis of reagents in Supplementary Figs. 171 and 172 was performed as per our pre-established methods.¹⁴



Supplementary Fig. 163. Synthesis of 2-(2-formylphenoxy)acetic acid (**2k**)¹³.

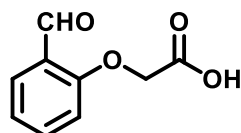
Synthesis of ethyl 2-(2-formylphenoxy)acetate (**S16**)



(**S16**)

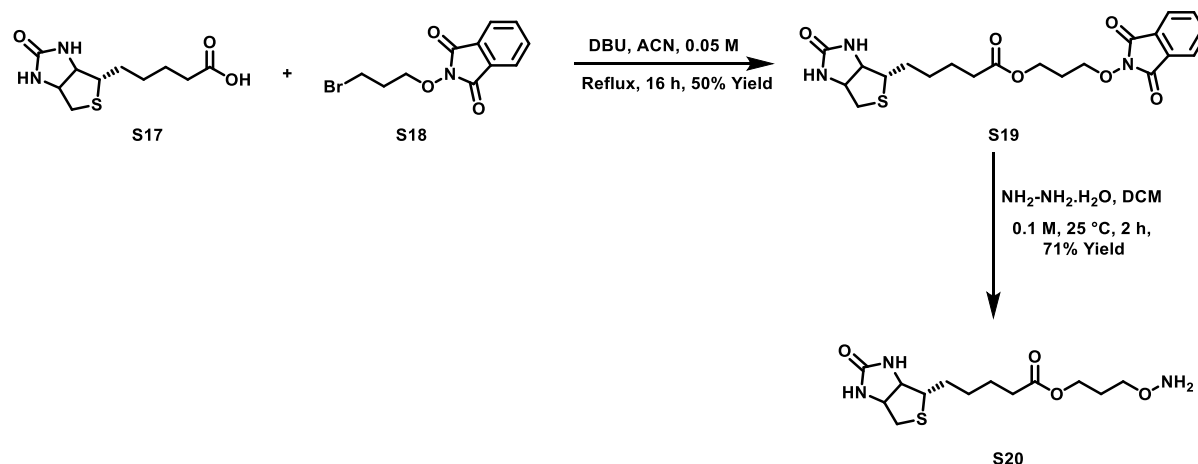
In a 25 mL round-bottom flask, 2-hydroxy benzaldehyde (**S15**, 366 mg, 3 mmol), ethyl 2-bromoacetate (1.0 g, 6 mmol), and K_2CO_3 (828 mg, 6 mmol) were dissolved in acetone (6 mL). The reaction mixture was allowed to reflux for 8 h. The reaction was monitored using TLC and LC-MS. Upon completion, the reaction mixture was filtered to remove potassium carbonate. The solution was concentrated under vacuum, and the product was purified using flash column chromatography using ethyl acetate:n-hexane (10:90) to afford ethyl 2-(2-formylphenoxy)acetate (**S16**, 177 mg, 72% yield; R_f = 0.3, ethyl acetate:n-hexane, 20:80; pale yellow solid). 1H NMR (500 MHz, $CDCl_3$): δ 10.55 (s, 1H), 7.85 (d, J = 7.7, 1.8 Hz, 1H), 7.52 (t, J = 7.9 Hz, 1H), 7.07 (t, J = 7.5 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 4.74 (s, 2H), 4.26 (q, J = 7.1 Hz, 2H), 1.28 (t, J = 7.1 Hz, 3H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 189.6, 168.2, 160.2, 135.8, 128.6, 125.5, 121.9, 112.7, 65.7, 61.7, 14.2 ppm. HRMS (ESI) $[M+H]^+$ calcd. $C_{11}H_{12}NaO_4$ 231.0633, found 231.0656.

Synthesis of 2-(2-formylphenoxy)acetic acid (**2k**)



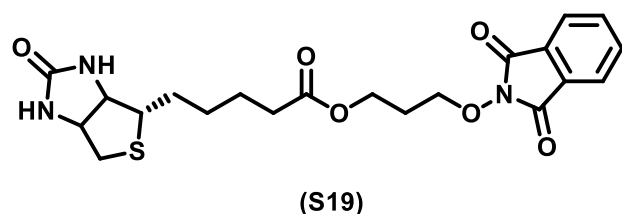
(**2k**)

In a 25 mL round-bottom flask, ethyl 2-(2-formylphenoxy)acetate (**S16**, 440 mg, 2.1 mmol) was mixed with THF: H_2O - 3:1 (0.2 M). To this solution, trifluoroacetic acid (964 mg, 8.4 mmol) was added dropwise at 0-5 $^{\circ}C$. The reaction mixture was allowed to reflux for 12 h. The reaction was monitored using TLC and LC-MS, upon completion of the reaction, the solution was concentrated under vacuum and the product was purified using flash column chromatography using ethyl acetate:n-hexane (50:50) to afford 2-(2-formylphenoxy)acetic acid (**2k**, 238 mg, 63% yield; R_f = 0.2, ethyl acetate:n-hexane, 40:60; pale yellow solid). 1H NMR (500 MHz, $CDCl_3$): δ 10.39 (s, 1H), 9.15 (bs, 1H), 7.85 (d, J = 7.6 Hz, 1H), 7.58 (t, J = 7.8 Hz, 1H), 6.92 (d, J = 8.3 Hz, 1H), 4.81 (s, 2H) ppm. ^{13}C NMR (126 MHz, $CDCl_3$): δ 190.7, 171.9, 159.3, 136.2, 131.0, 125.4, 122.6, 113.4, 65.7 ppm. HRMS (ESI) $[M+Na]^+$ calcd. $C_9H_8NaO_4$ 203.0320, found 203.0330.



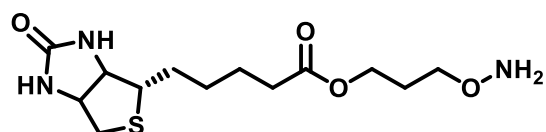
Supplementary Fig. 164. Synthesis of 3-(aminooxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**S20**).¹³

Synthesis of 3-((1,3-dioxoisindolin-2-yl)oxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (S19**)**



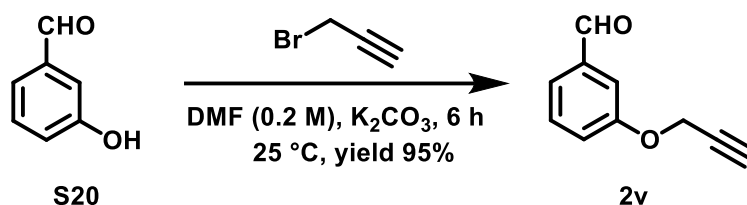
In a 50 mL round-bottom flask, biotin (**S17**, 250 mg, 1 mmol), 2-(3-bromopropoxy)isoindoline-1,3-dione (**S18**, 568 mg, 2 mmol), and DBU (304 μL , 2 mmol) were dissolved in acetonitrile (20 mL). The reaction mixture was refluxed for 16 h. The reaction progress was monitored by TLC and LC-MS. The reaction mixture was filtered, followed by evaporation of acetonitrile to get the crude reaction mixture. After completion, the reaction mixture was diluted with ethyl acetate and extracted with water (1:1, 50 mL) three times. The organic layer was dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using methanol:DCM (5:95) to afford 3-((1,3-dioxoisindolin-2-yl)oxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**S19**, 224 mg, 50% yield; R_f = 0.4, methanol:DCM, 10:90; pale yellow solid). ^1H NMR (500 MHz, CDCl_3): δ 7.84 (dd, J = 5.4, 3.1 Hz, 2H), 7.74 (dd, J = 5.4, 3.1 Hz, 2H), 5.95 (s, 1H), 5.46 (s, 1H), 4.49 (m, 1H), 4.38-4.23 (m, 5H), 3.10 (m, 1H), 2.89 (dd, J = 12.8, 5.0 Hz, 1H), 2.72 (d, J = 12.8 Hz, 1H), 2.34 (t, J = 7.5 Hz, 2H), 2.16-2.05 (m, 2H), 1.80-1.60 (m, 4H), 1.53-1.37 (m, 2H) ppm. ^{13}C NMR (126 MHz, CDCl_3): δ 173.7, 163.7, 163.7, 134.7, 128.9, 123.7, 75.1, 62.0, 60.7, 60.2, 55.5, 40.7, 33.9, 28.4, 28.3, 27.8, 24.9 ppm. HRMS (ESI) $[\text{M}+\text{H}]^+$ calcd. $\text{C}_{21}\text{H}_{26}\text{N}_3\text{O}_6\text{S}$ 448.1542, found 448.1548.

Synthesis of 3-(aminooxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**S20**)



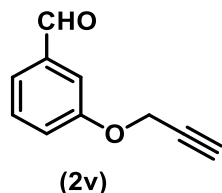
(**S20**)

In a 5 mL round-bottom flask, 3-((1,3-dioxoisindolin-2-yl)oxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**S19**, 134 mg, 0.3 mmol) in DCM (3 mL) and hydrazine monohydrate (80%, 37 μ L, 0.75 mmol) were stirred at 25 °C. The progress of the reaction was followed by TLC and LC-MS. After 2 h, the reaction mixture was filtered, and the solution was concentrated under vacuum to afford 3-(aminooxy)propyl 5-((4S)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**S20**, 68 mg, 71% yield). ^1H NMR (500 MHz, D_2O): 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), 3.38-3.34 (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), 2.00 (m, 2H), 1.77-1.61 (m, 4H), 1.46 (m, 2H) ppm. ^{13}C NMR (126 MHz, D_2O): δ 176.9, 165.3, 72.4, 62.1, 62.0, 60.3, 55.3, 39.7, 33.7, 27.9, 27.6, 26.8, 24.1 ppm. HRMS (ESI)[$\text{M}+\text{H}$] $^+$ calcd. $\text{C}_{13}\text{H}_{23}\text{N}_3\text{NaO}_4\text{S}$ 340.1307, found 340.1297.



Supplementary Fig. 165. Synthesis of 3-(prop-2-yn-1-yloxy)benzaldehyde (**2v**).

Synthesis of 3-(prop-2-yn-1-yloxy)benzaldehyde (**2v**)



In a 50 mL round-bottom flask, a solution of 3-hydroxy benzaldehyde (**S20**, 500 mg, 4.09 mmol) in DMF (0.2 M) and K_2CO_3 (1131 mg, 8.19 mmol) was added, followed by the addition of propargyl bromide (250 μ L, 3.29 mmol). The reaction mixture was stirred at 25 °C for 6 h. The progress of the reaction was monitored by TLC and LC-MS. After completion, the DMF was concentrated under an air purge. The crude mixture was re-suspended in ethyl acetate, and salts were removed by solvent extraction with ethyl acetate: water (1:1, 50 ml). The

organic layers were dried with anh. Na₂SO₄ and concentrated using a rotary evaporator. The crude mixture was purified by silica gel flash column chromatography using ethyl acetate:n-hexane (15:85) to afford 4-nitro-3-(prop-2-yn-1-yloxy)benzaldehyde (**2v**, 620 mg, 95% yield; R_f = 0.6, ethyl acetate:n-hexane 10:90; white, brown solid). ¹H NMR (500 MHz, CDCl₃): δ 9.96 (s, 1H), 7.50 (d, *J* = 7.6 Hz, 1H), 7.46 (m, 2H), 7.24 (d, *J* = 8.1 Hz, 1H), 4.74 (m, 2H), 2.55 (m, 1H) ppm. ¹³C NMR (126 MHz, CDCl₃): δ 191.9, 158.2, 137.9, 130.3, 124.2, 122.2, 113.7, 77.9, 76.2, 56.1 ppm. HRMS (ESI) [M+H]⁺ calcd. C₁₀H₈NO₄ 206.0453, found 206.0441.

12. Protein sequence

A. Insulin

PDB ID: 1A7F

amino acid sequence:

Chain A: GIVEQCCTSICSLYQLENYCN

Chain B: FVNQHLCGSHLVEALYLVCGERGFFYTPKT

B. Melittin

PDB ID: 1BH1

amino acid sequence:

GIGAVLKVLTTGLPALISWIKRKRQQ

C. Myoglobin from equine skeletal muscle

PDB ID: 1WLA

amino acid sequence:

GLSDGEWQQVLNVWGKVEADIAGHGQEVLRFTGHPETLEKFDKFKHLKTEAEMKASED
LKKHGTVVLTALGGILKKKGHHEALKPLAQSHATKHKIPKYLEFISDAIIHVLHSHKHPGDFG
ADAQGAMTKALELFRNDIAAKYKELGFQG

The SUMO1 and eGFP protein with N-terminus glycine was expressed as per the procedure in section 2.12.

D. SUMO1

amino acid sequence:

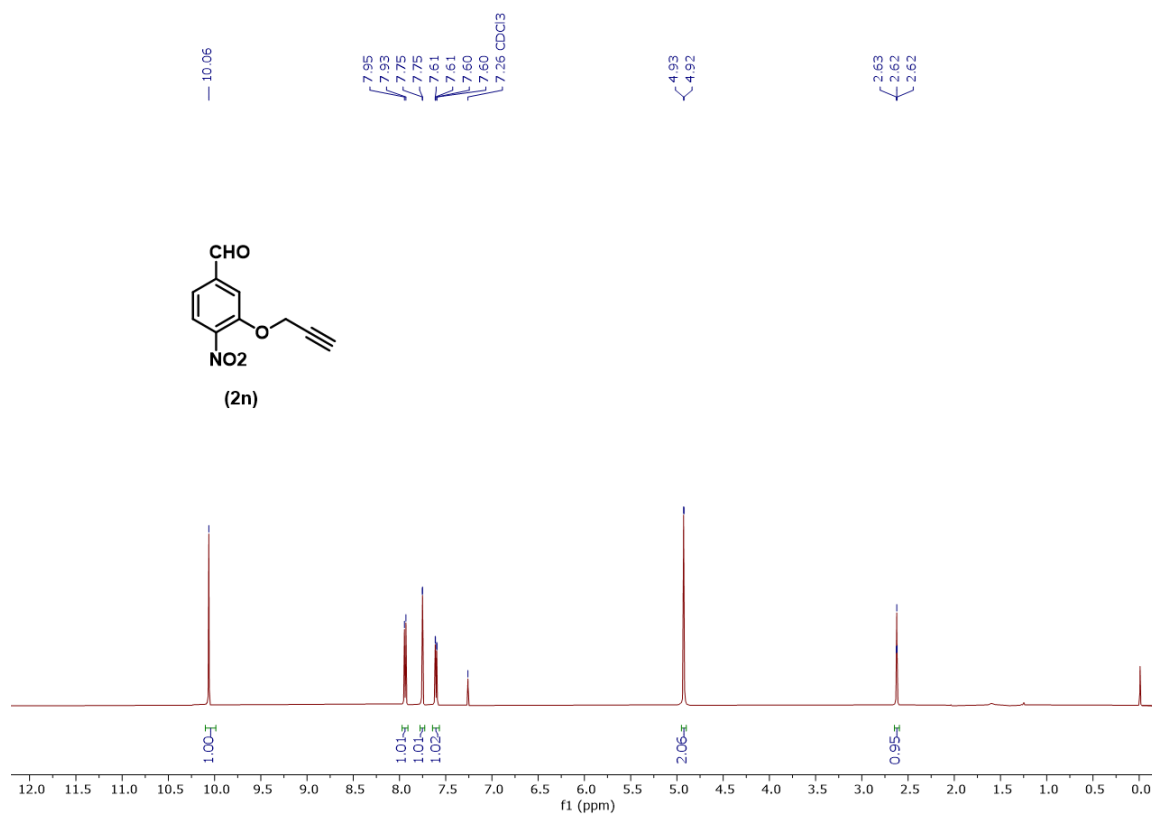
GPLGSMDSQEAKPSTEDLGDKKEGEYIKLVIGQDSSEIHFKVKMTTHLKKLKESYCQRQG
VPMNSLRFLFEGQRIADNHTPKELGMEEEDVIEVYQEQTGG

D. N-Gly eGFP

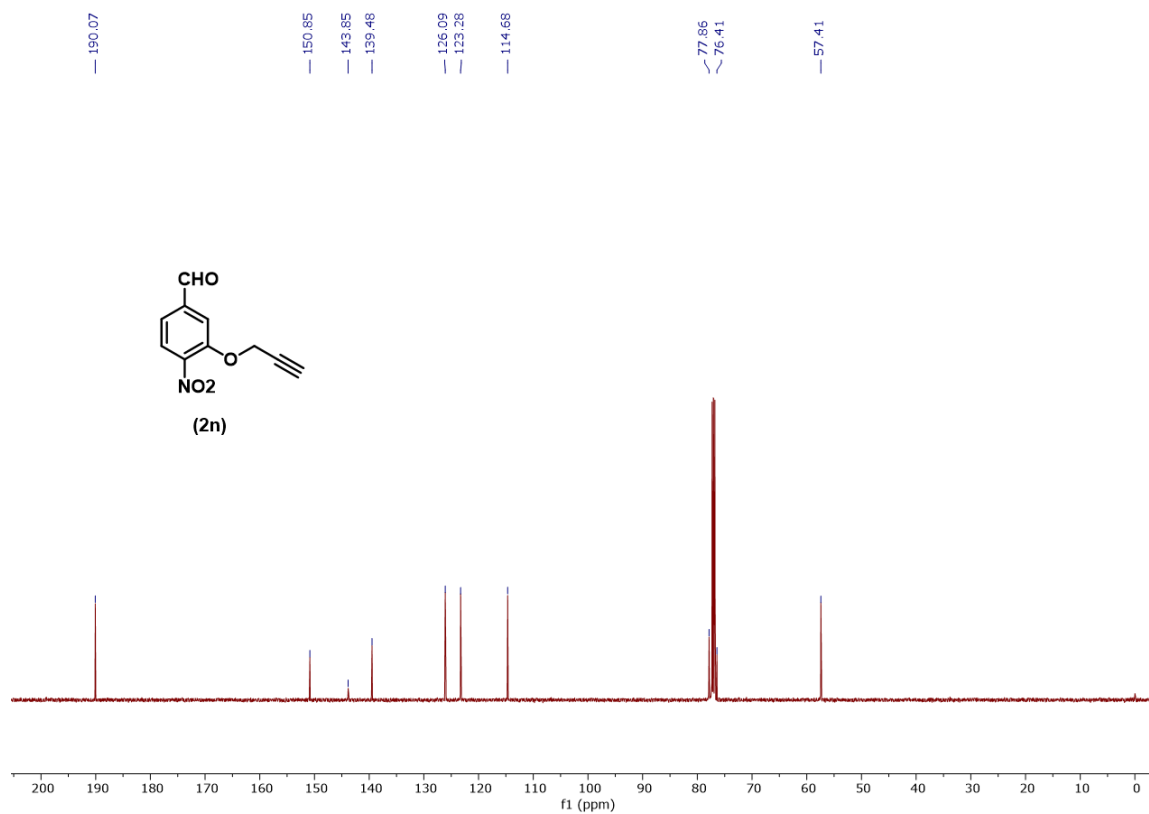
amino acid sequence:

GSSMVSKEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPW
PTLVTTLTLYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLAD
HYQQNTPIGDGPVLLPDNHYLSTQSALSKD PNEKRDHMLLEFVTAAGITLGMDELYK

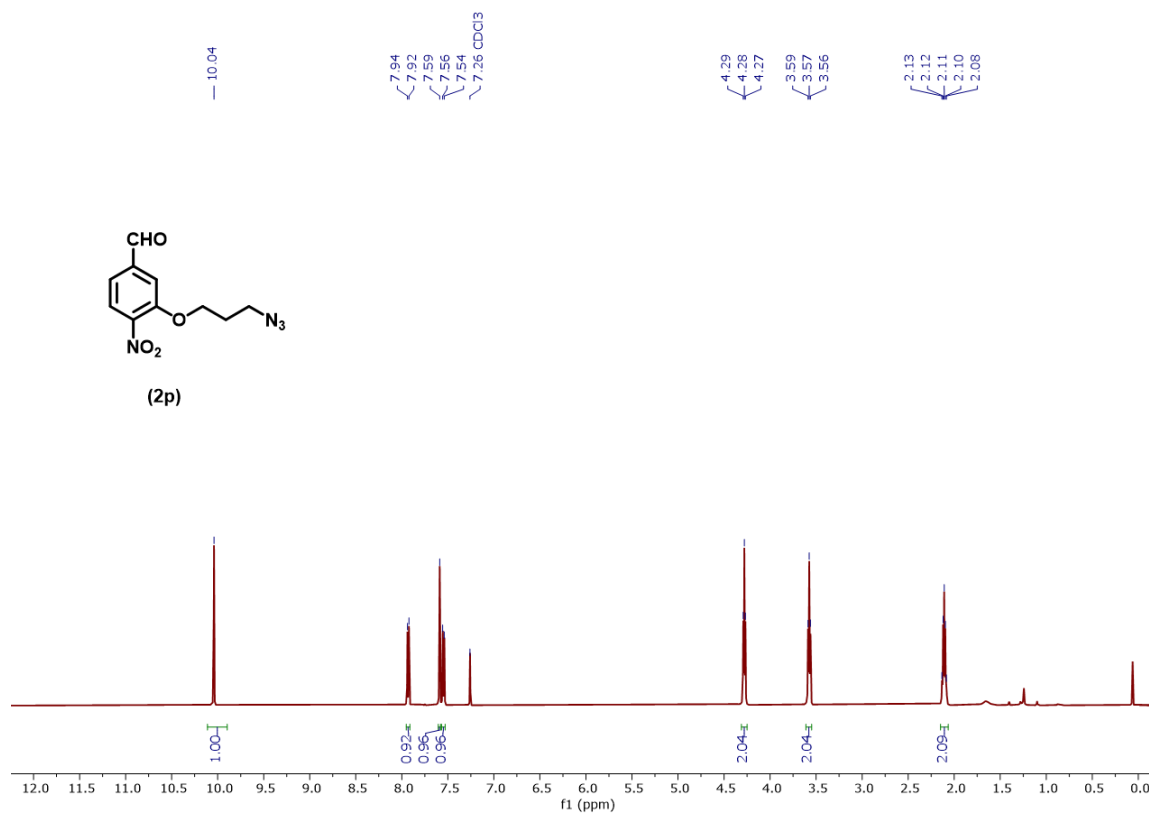
13. NMR data



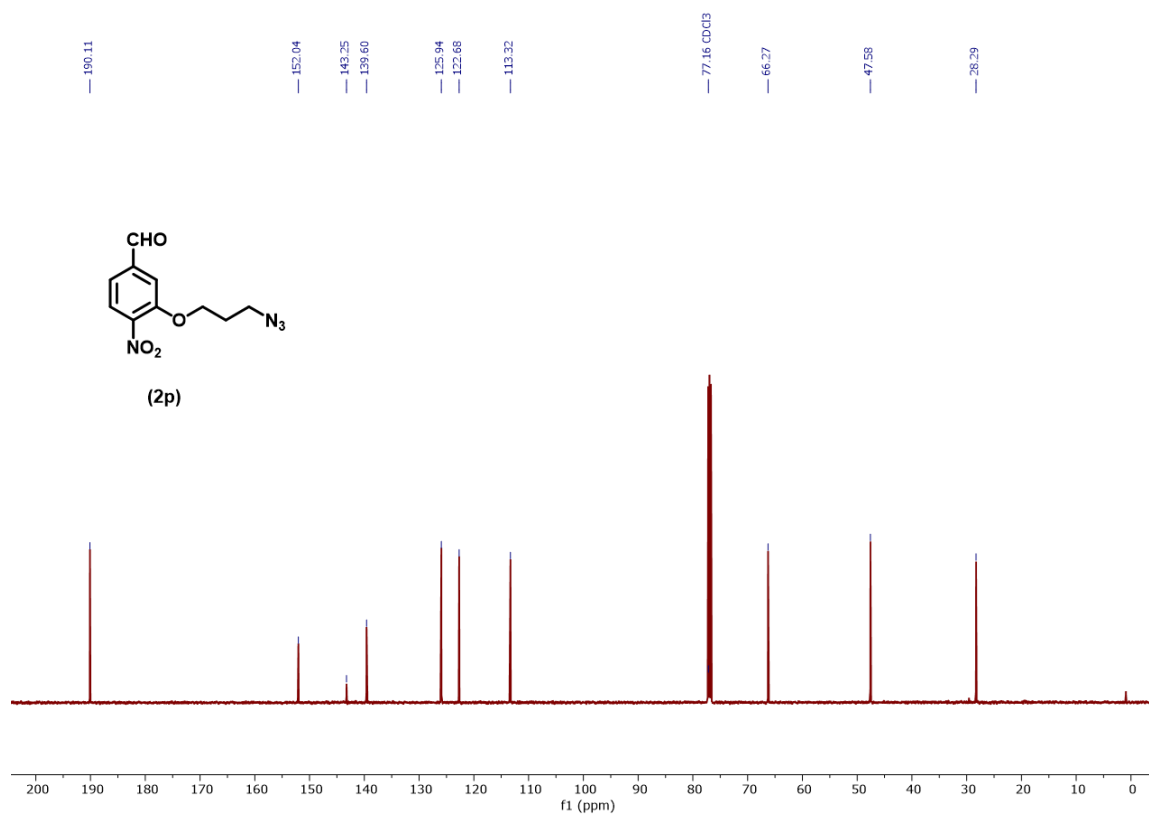
Supplementary Fig. 166. ¹H-NMR spectrum in CDCl₃ of compound **2n**.



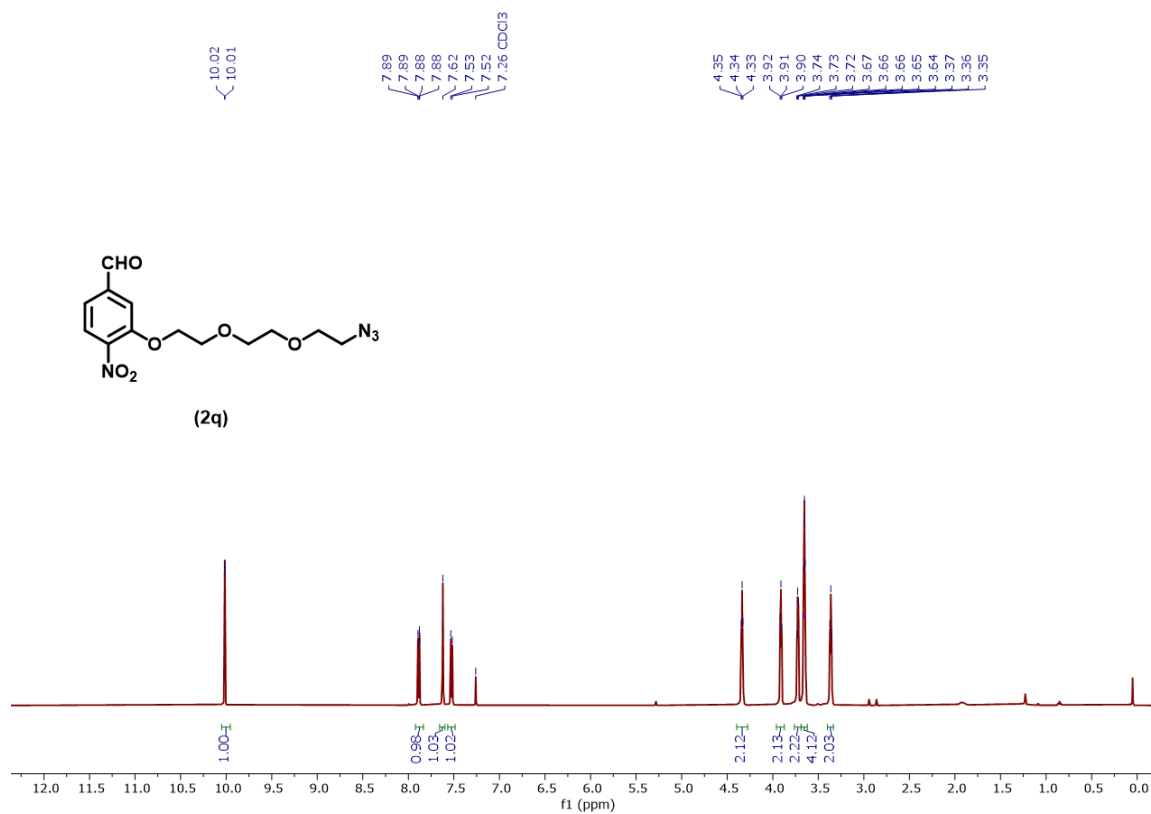
Supplementary Fig. 167. ¹³C-NMR spectrum in CDCl₃ of compound **2n**.



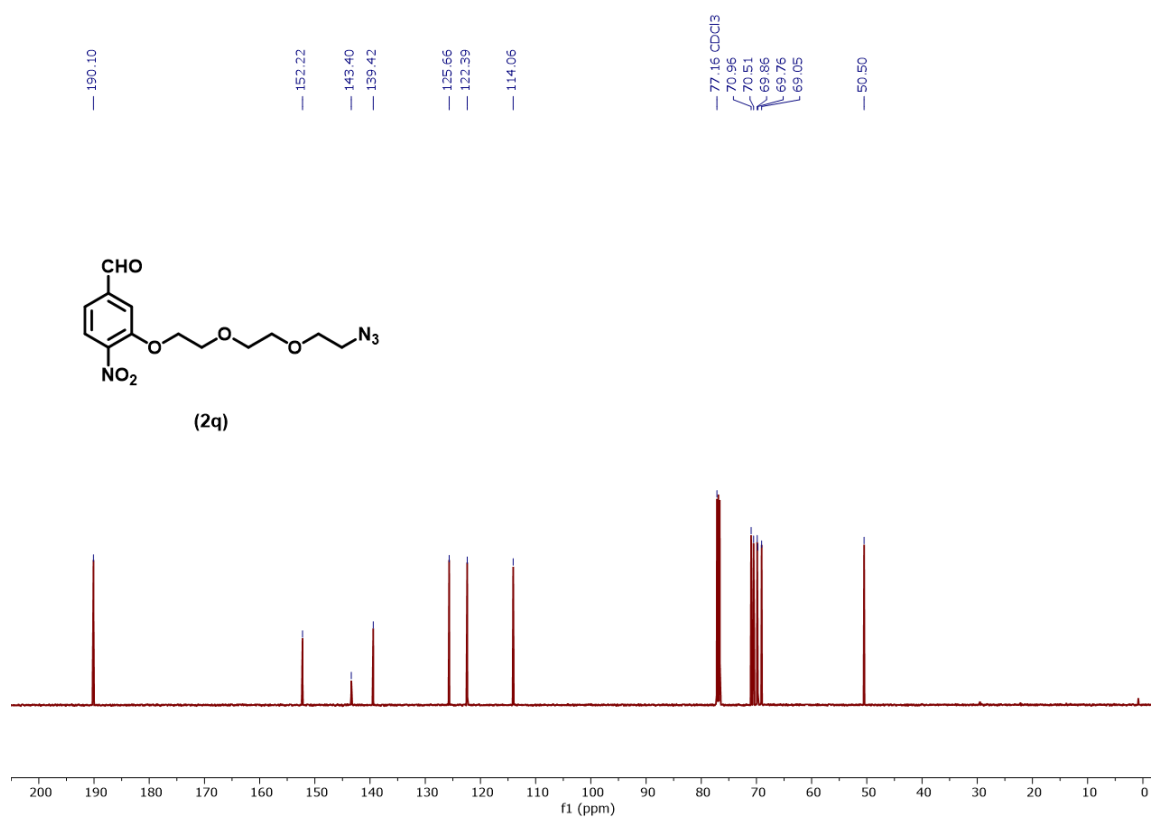
Supplementary Fig. 168. ¹H-NMR spectrum in CDCl₃ of compound **2p**.



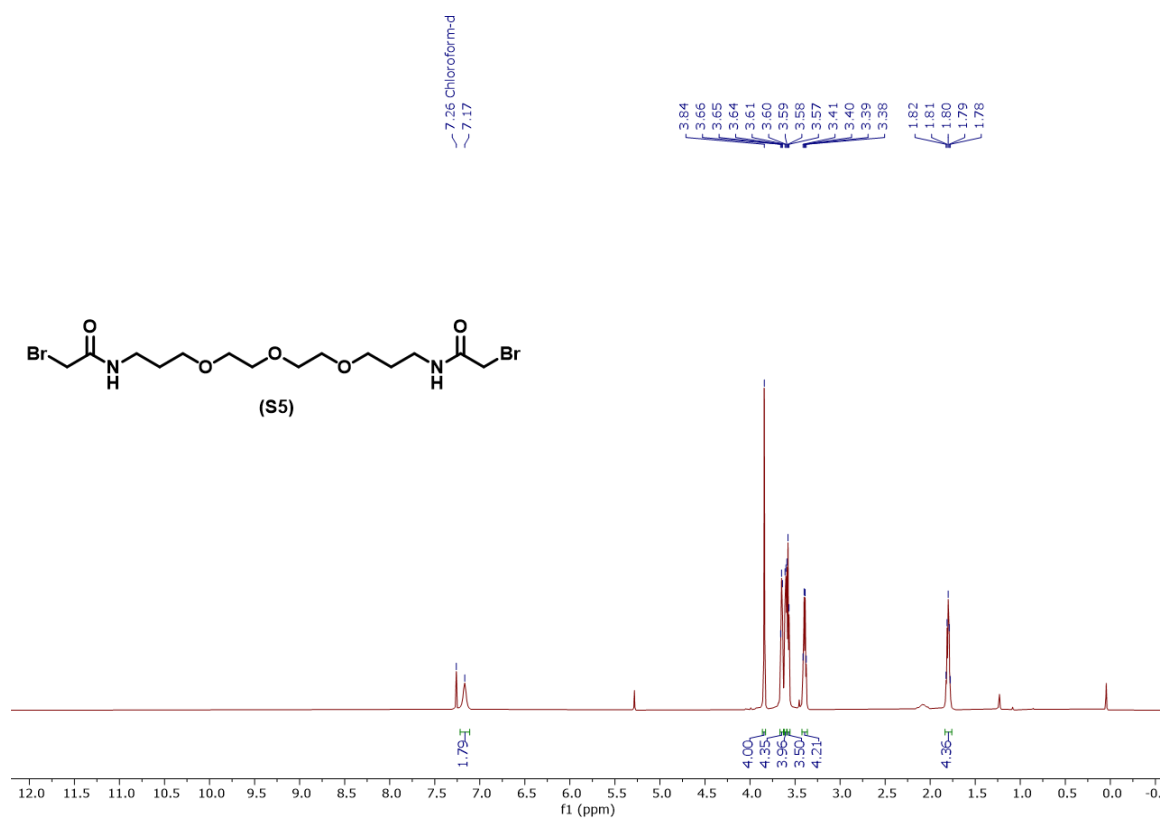
Supplementary Fig. 169. ¹³C-NMR spectrum in CDCl₃ of compound **2p**.



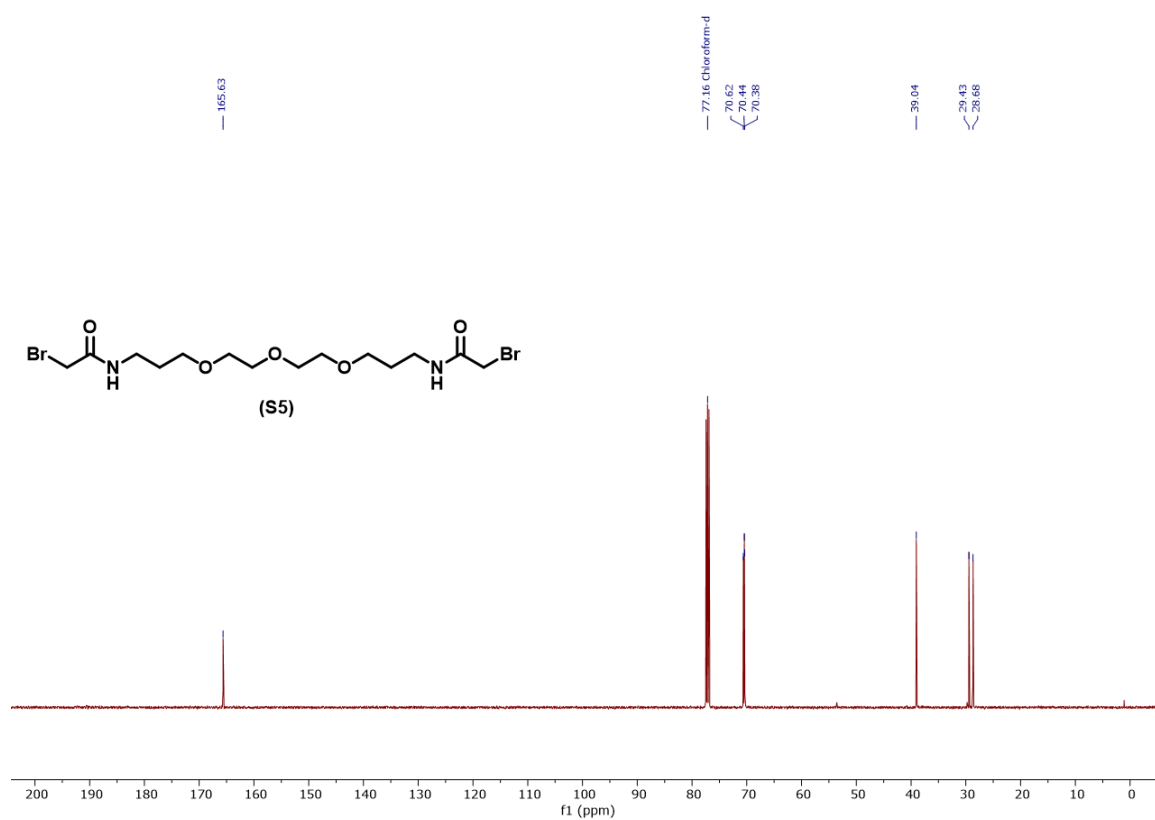
Supplementary Fig. 170. ^1H -NMR spectrum in CDCl₃ of compound **2q**.



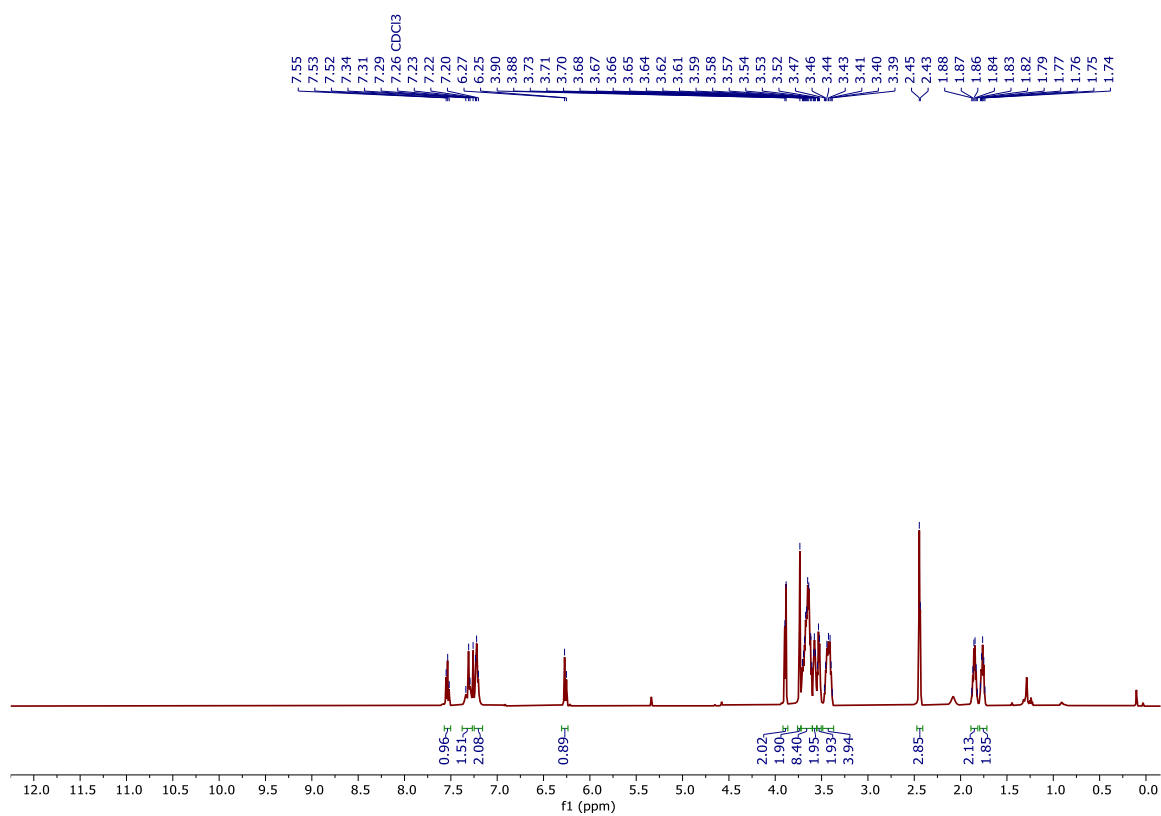
Supplementary Fig. 171. ^{13}C -NMR spectrum in CDCl₃ of compound **2q**.



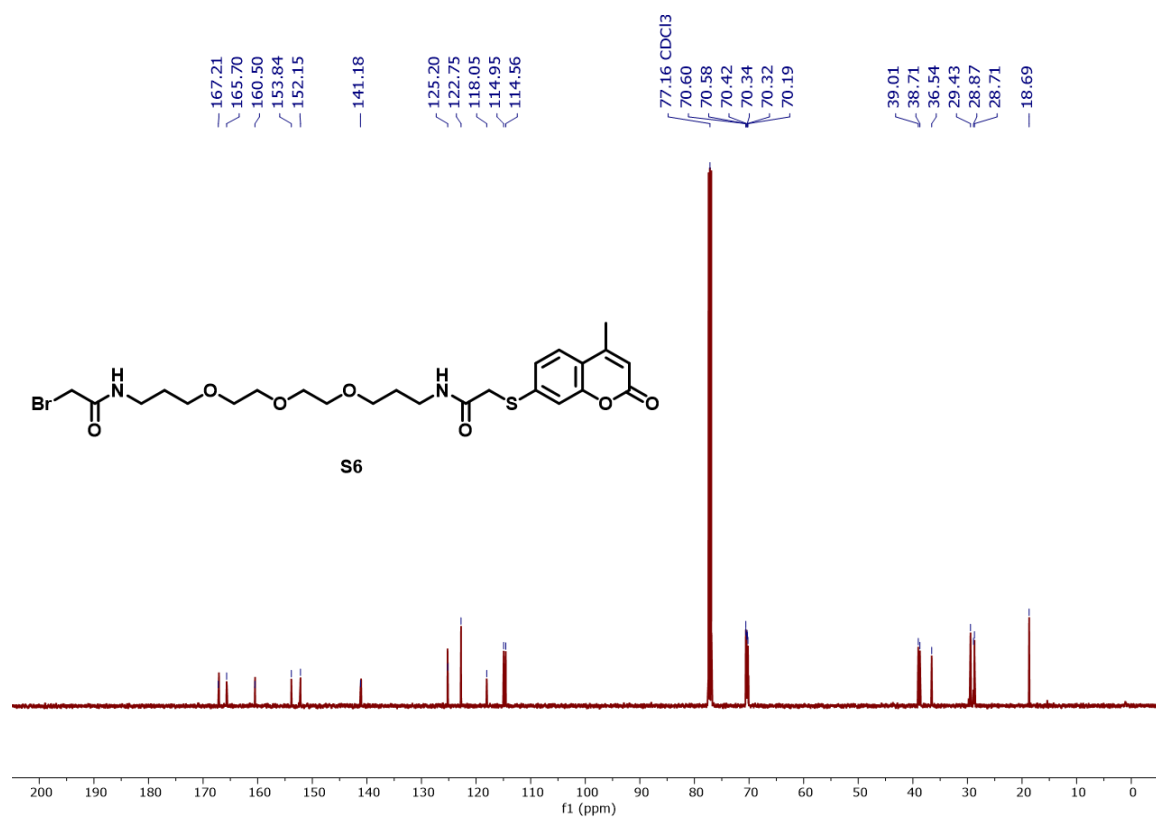
Supplementary Fig. 172. ^1H -NMR spectrum in CDCl_3 of compound **S5**.



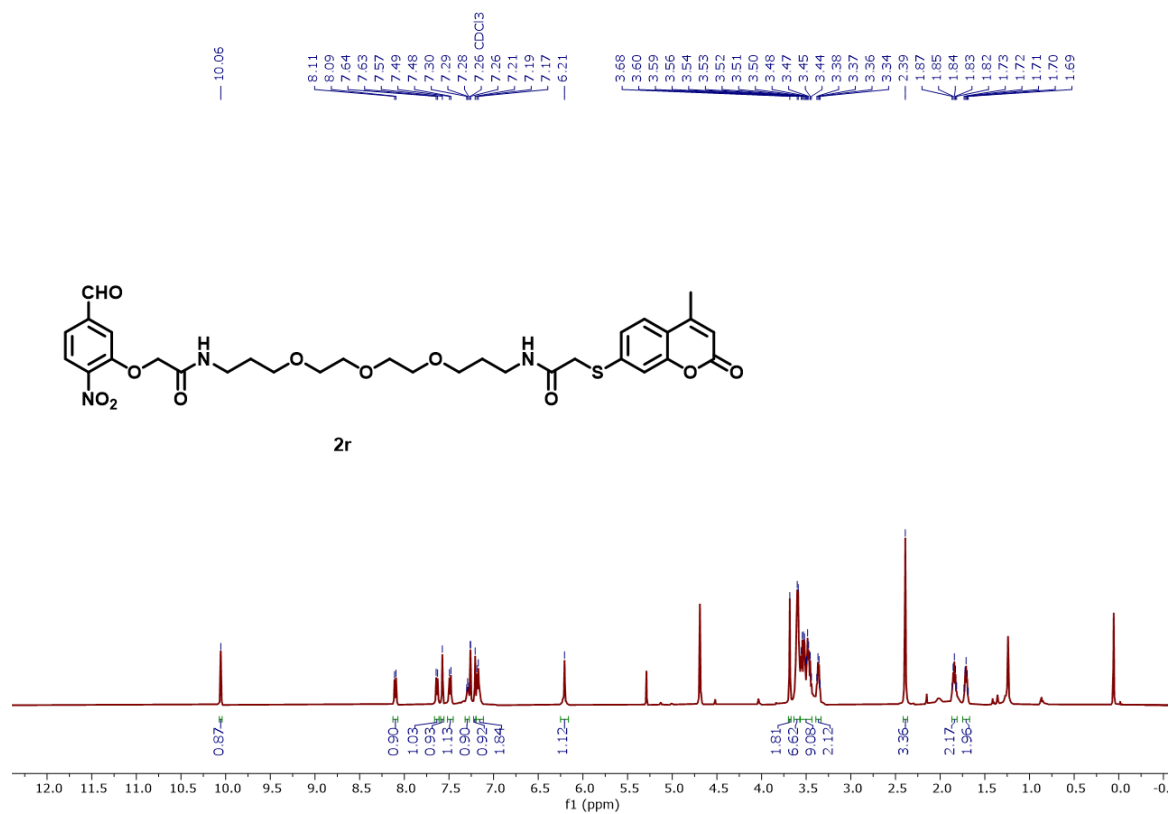
Supplementary Fig. 173. ^{13}C -NMR spectrum in CDCl_3 of compound **S5**.



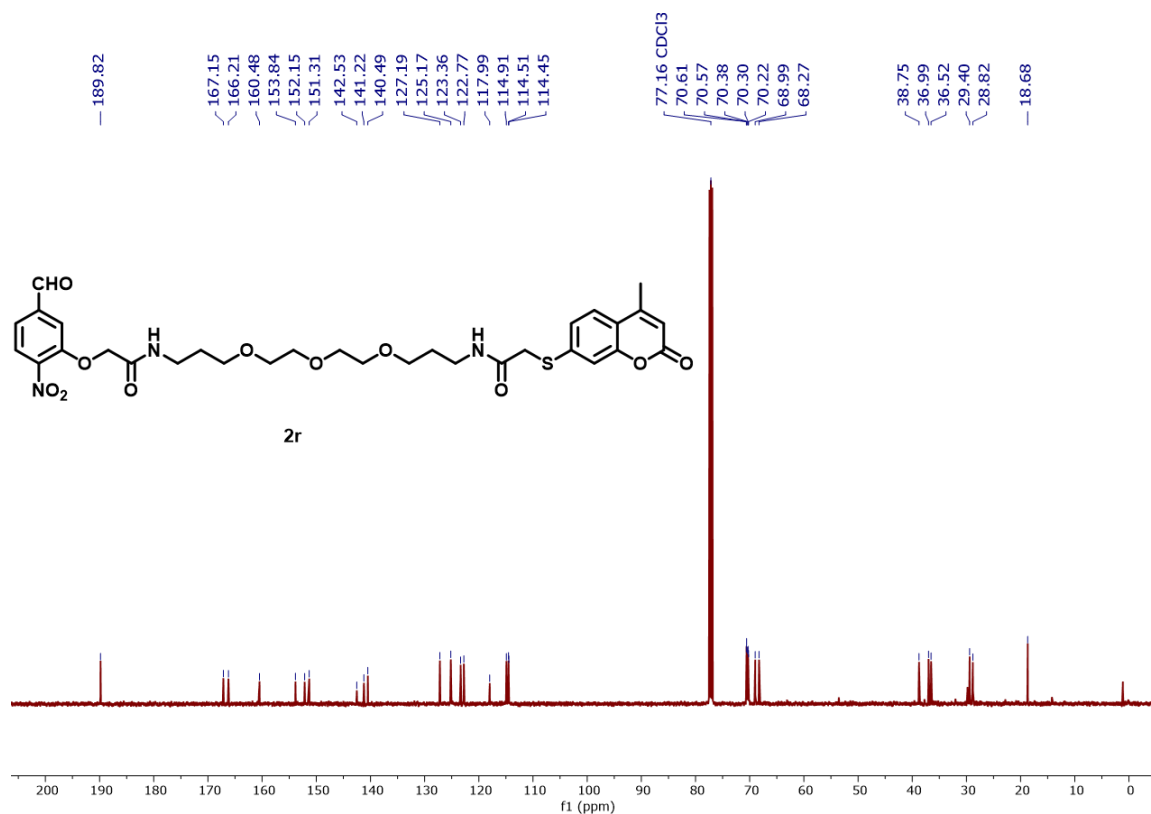
Supplementary Fig. 174. ¹H-NMR spectrum in CDCl₃ of compound S6.



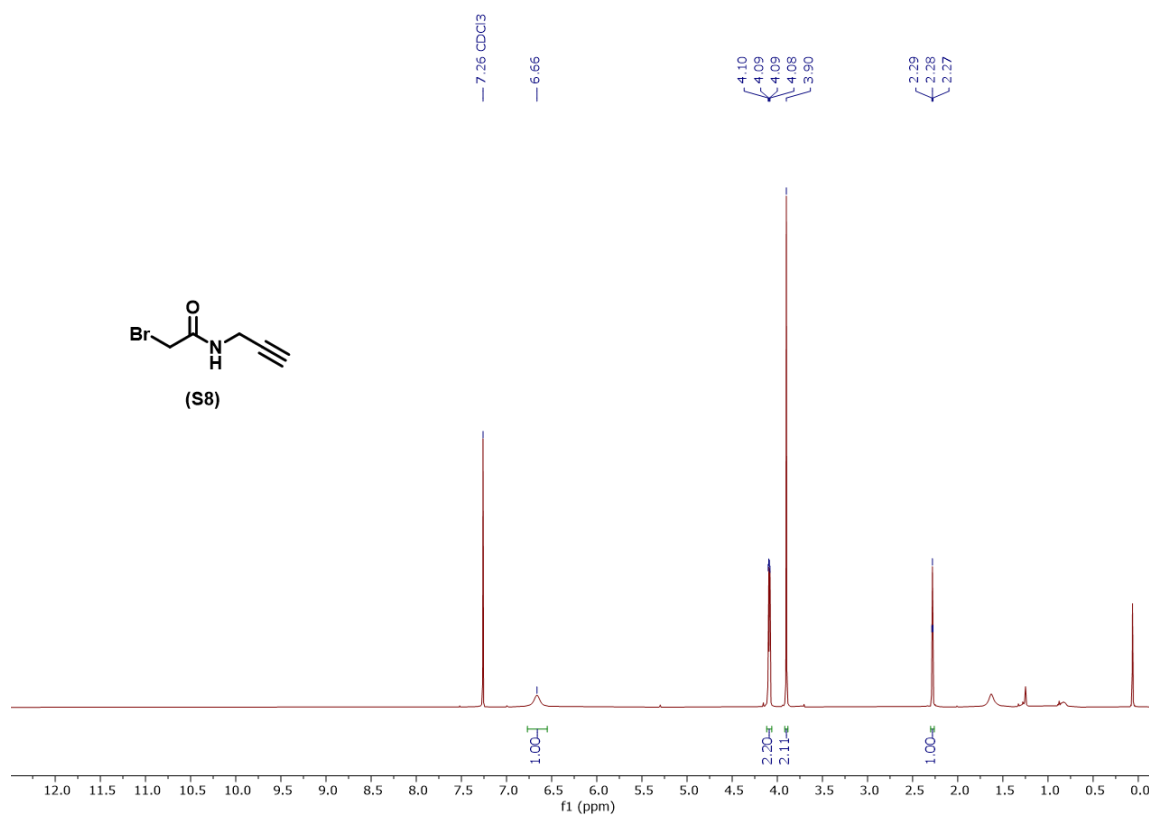
Supplementary Fig. 175. ¹³C-NMR spectrum in CDCl₃ of compound S6.



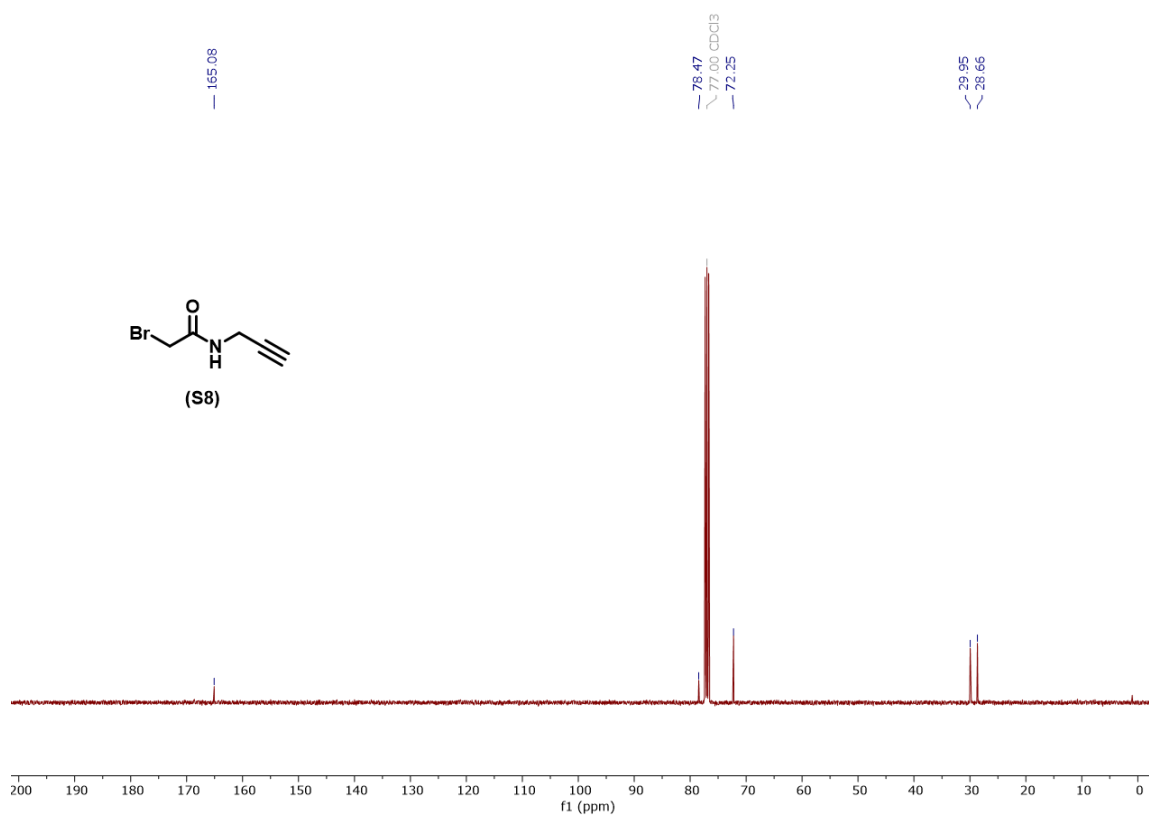
Supplementary Fig. 176. ^1H -NMR spectrum in CDCl_3 of compound **2r**.



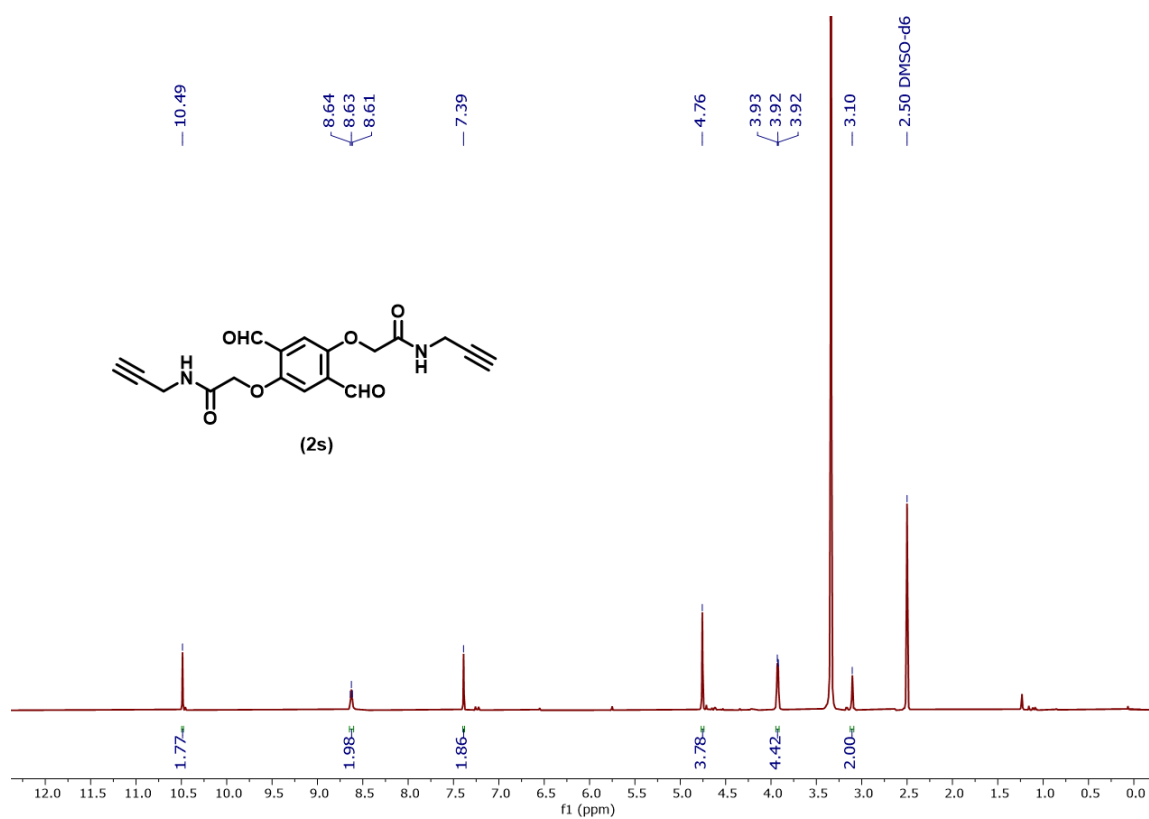
Supplementary Fig. 177. ^{13}C -NMR spectrum in CDCl_3 of compound **2r**.



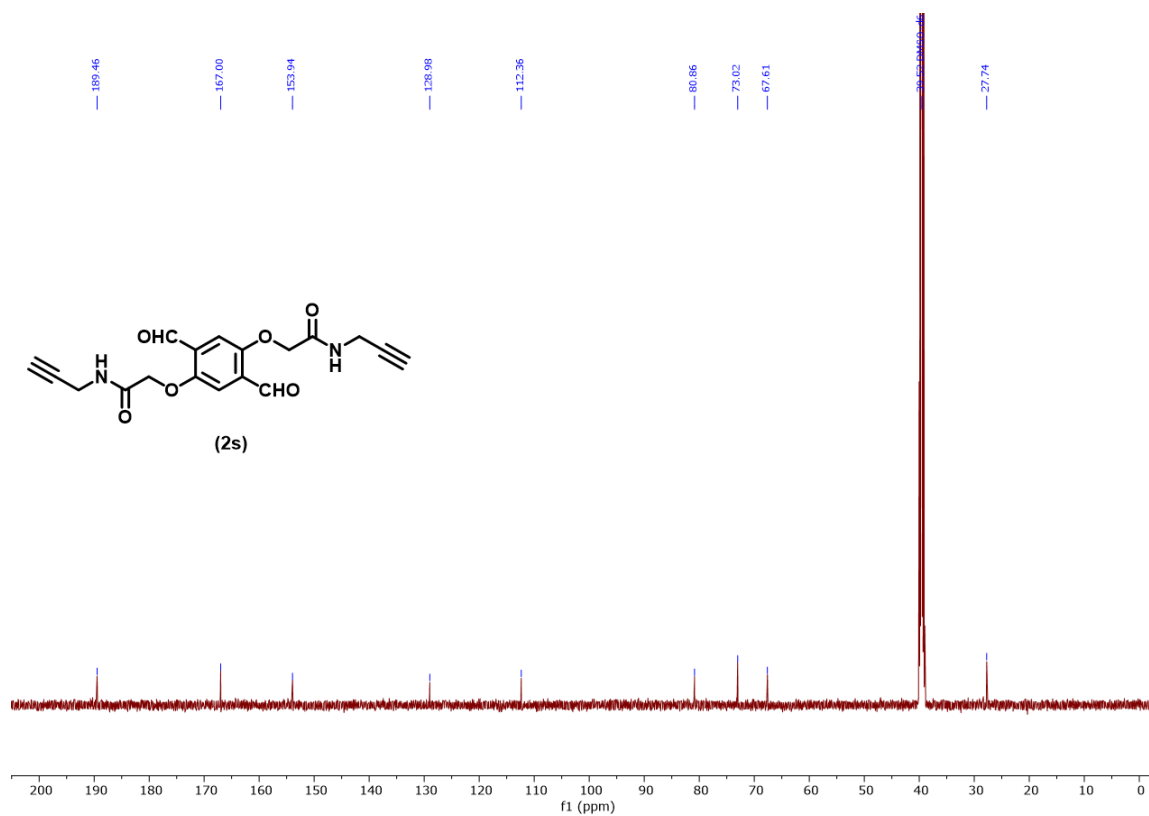
Supplementary Fig. 178. ¹H-NMR spectrum in CDCl₃ of compound **S8**.



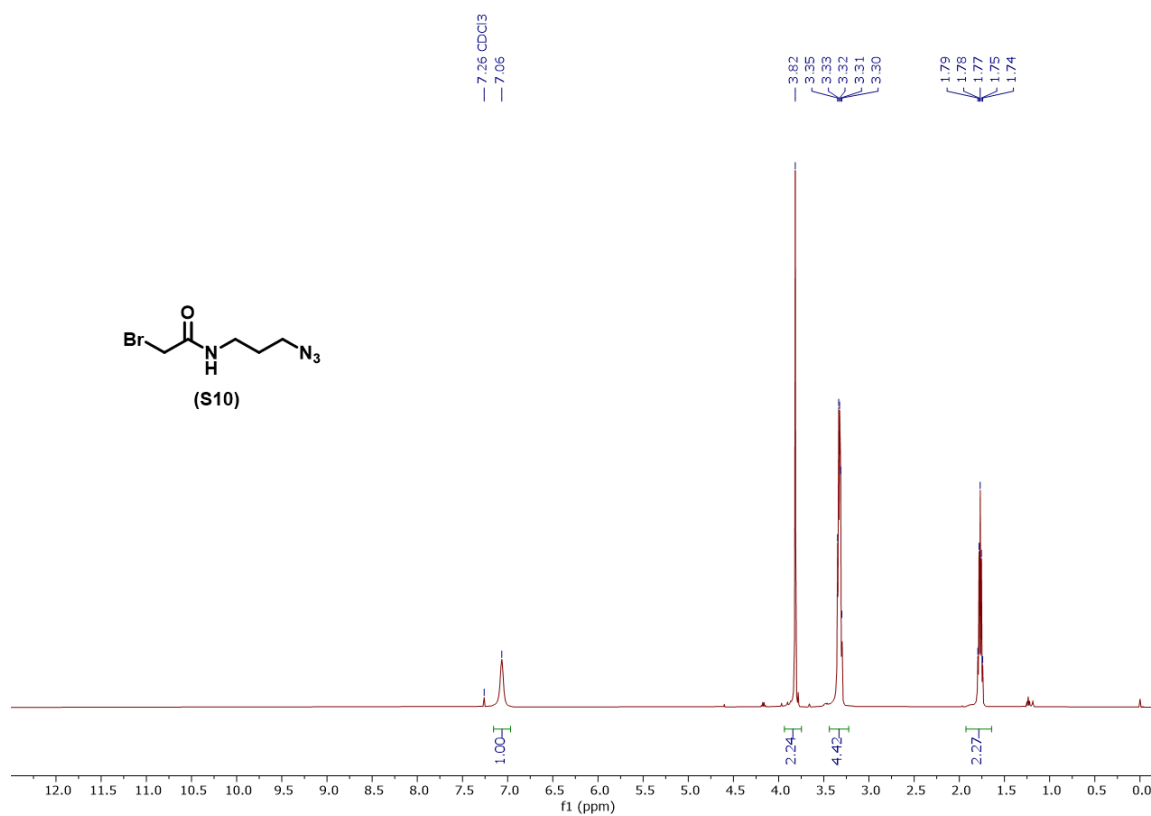
Supplementary Fig. 179. ¹³C-NMR spectrum in CDCl₃ of compound **S8**.



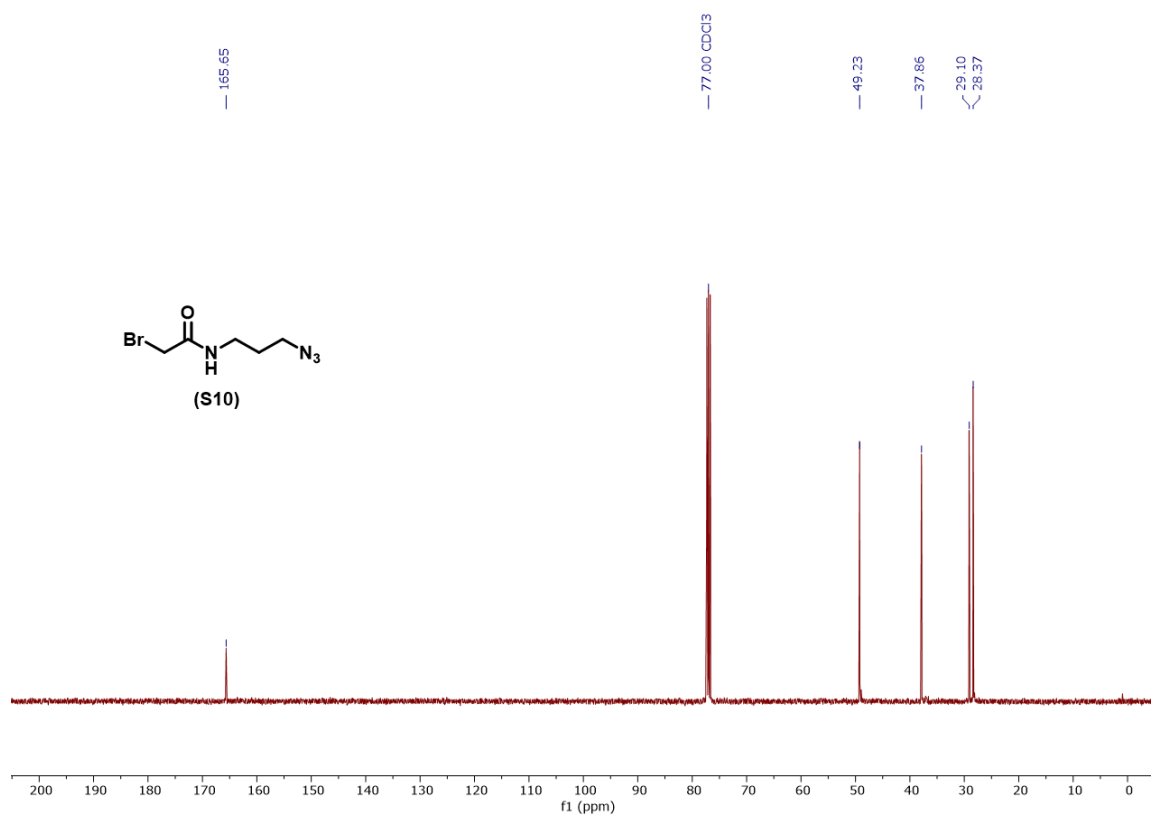
Supplementary Fig. 180. ¹H-NMR spectrum in DMSO-*d*₆ of compound **2s**.



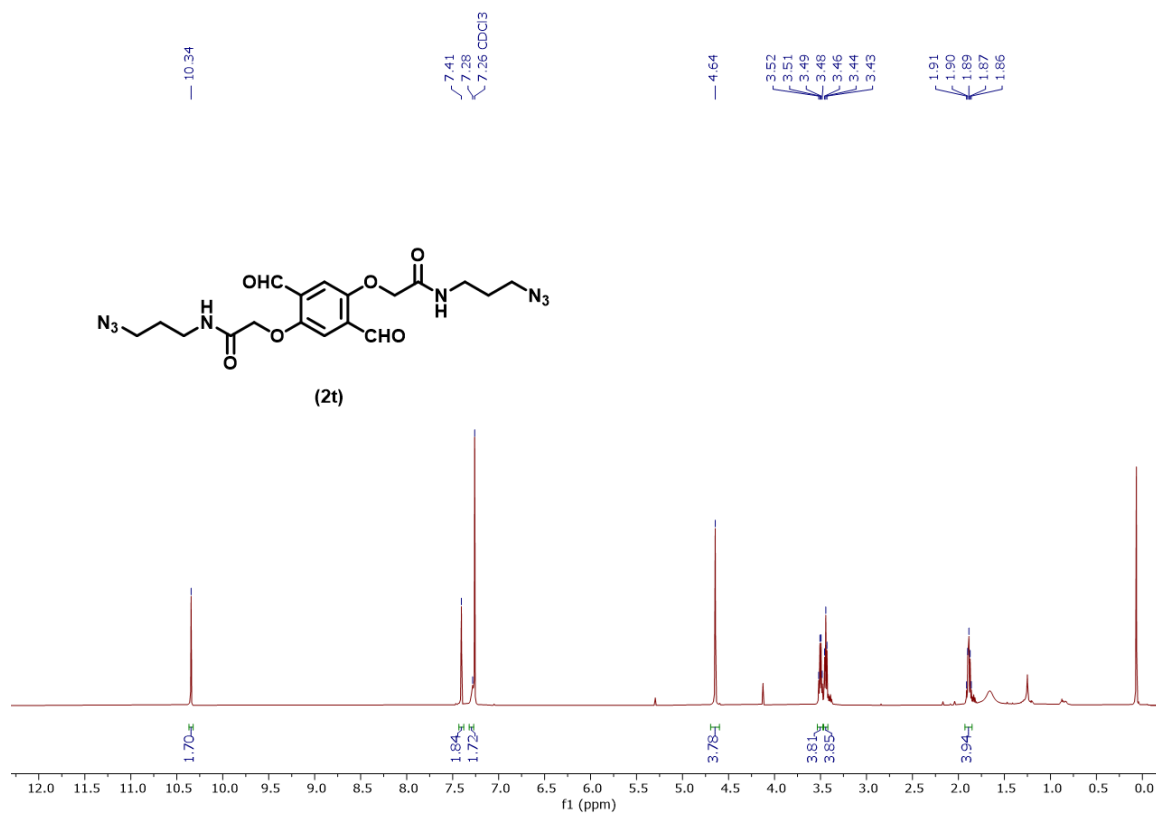
Supplementary Fig. 181. ¹³C-NMR spectrum in DMSO-*d*₆ of compound **2s**.



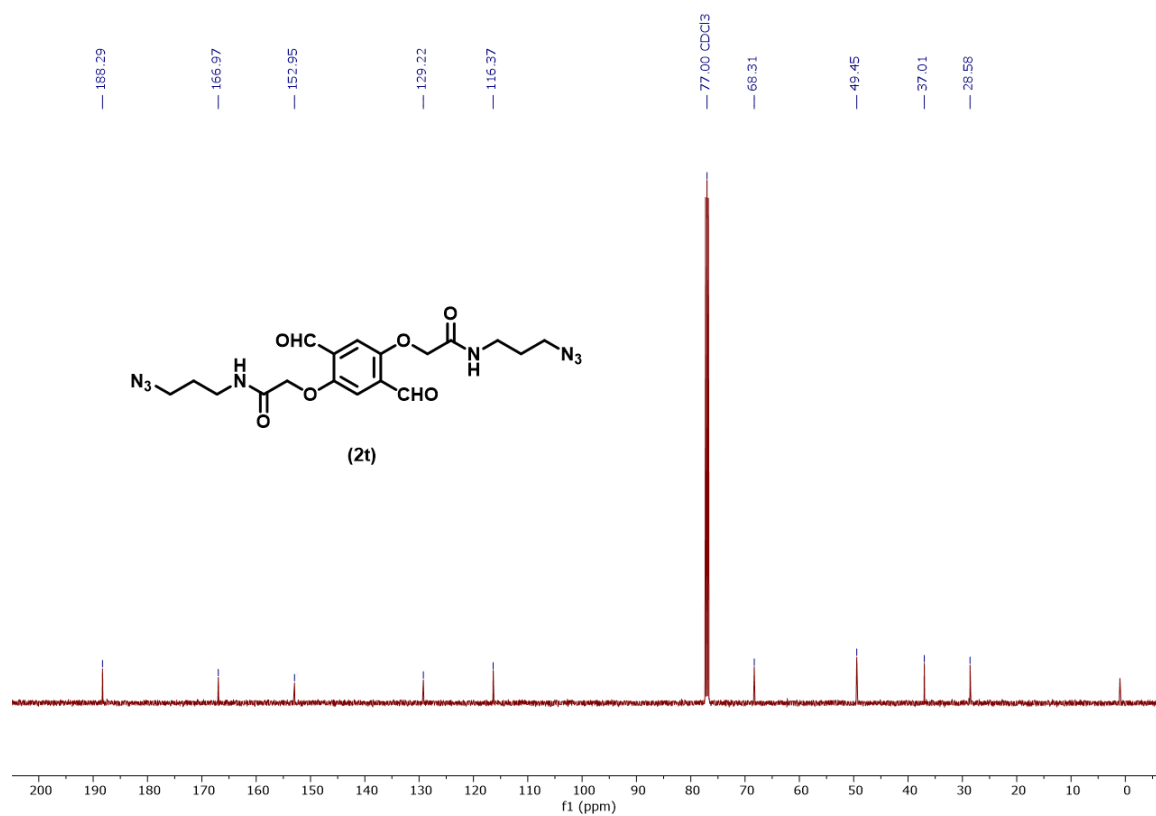
Supplementary Fig. 182. ¹H-NMR spectrum in CDCl₃ of compound **S10**.



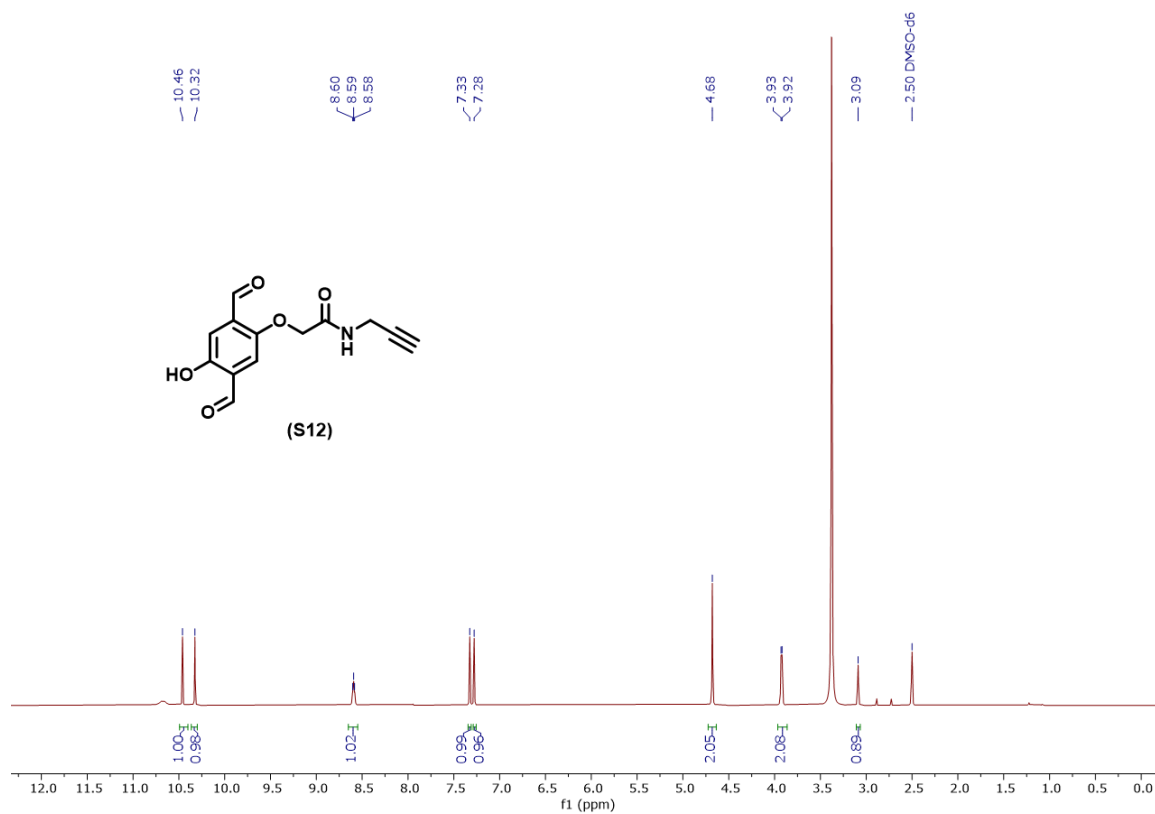
Supplementary Fig. 183. ¹³C-NMR spectrum in CDCl₃ of compound **S10**.



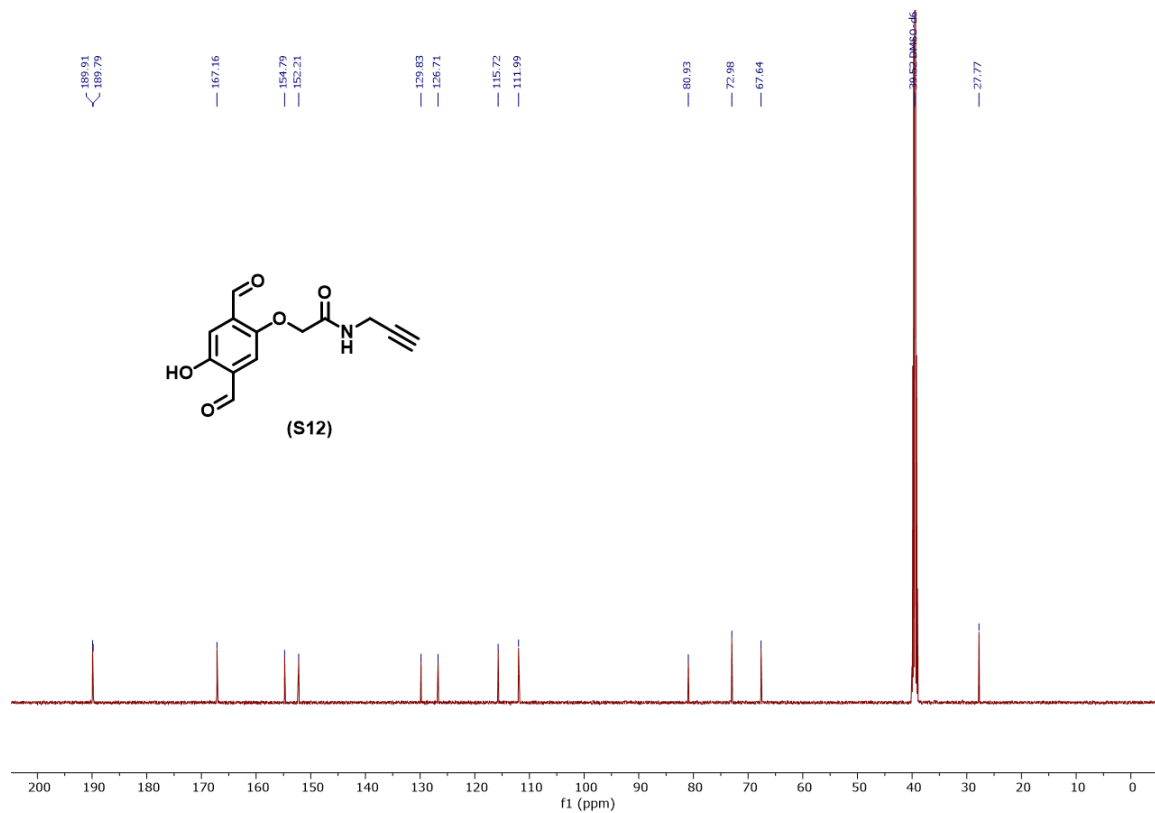
Supplementary Fig. 184. ^1H -NMR spectrum in CDCl₃ of compound **2t**.



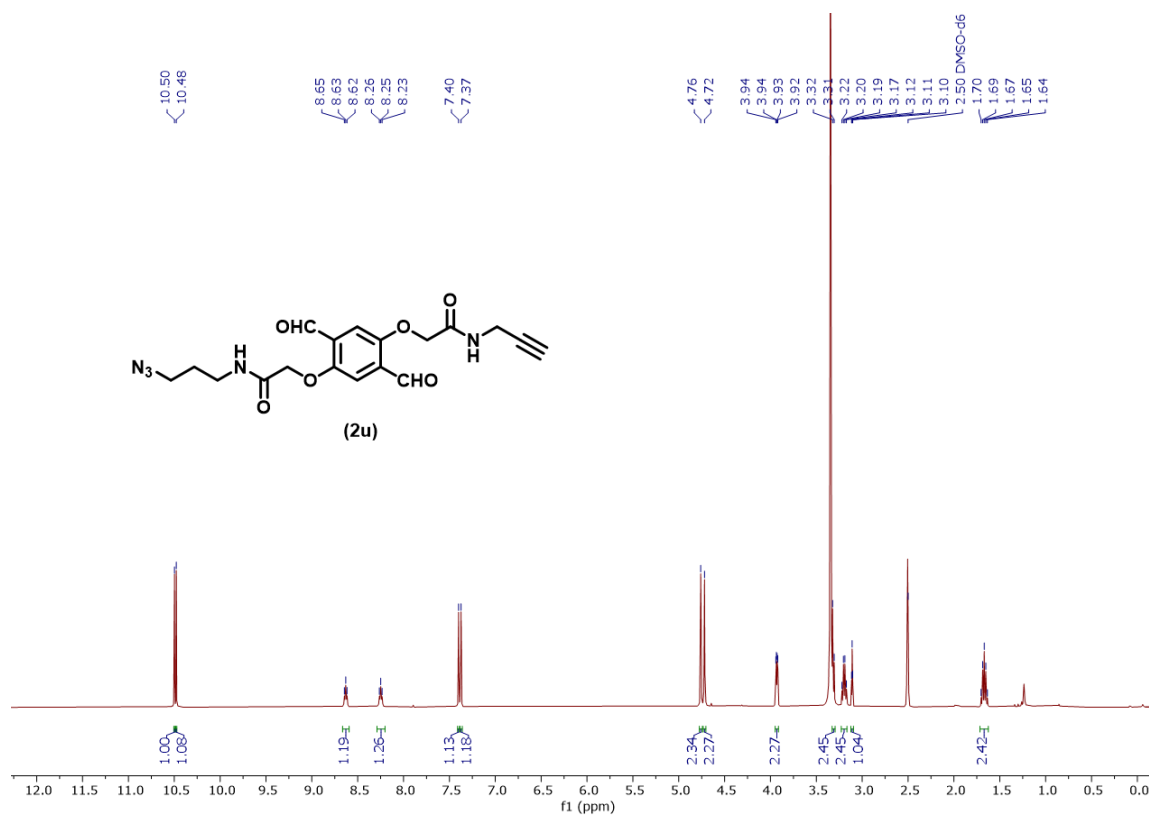
Supplementary Fig. 185. ^{13}C -NMR spectrum in CDCl₃ of compound **2t**.



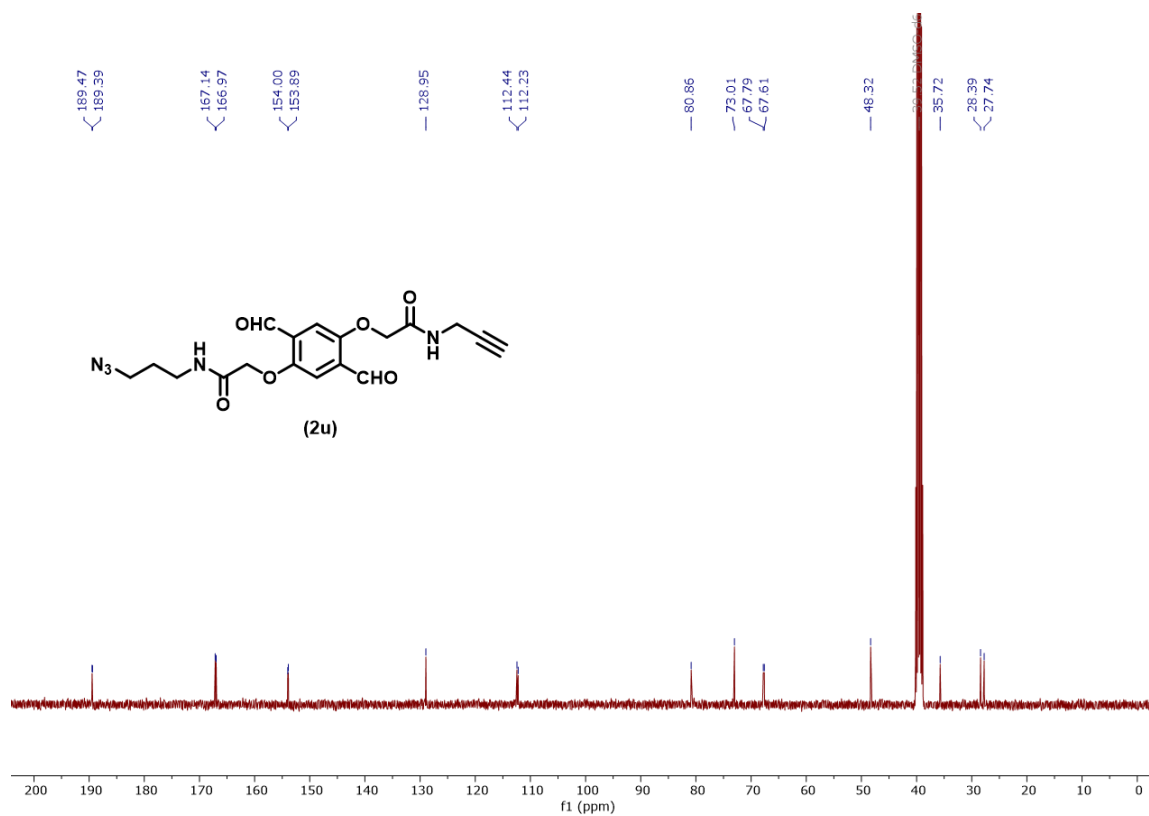
Supplementary Fig. 186. ¹H-NMR spectrum in DMSO-*d*₆ of compound **S12**.



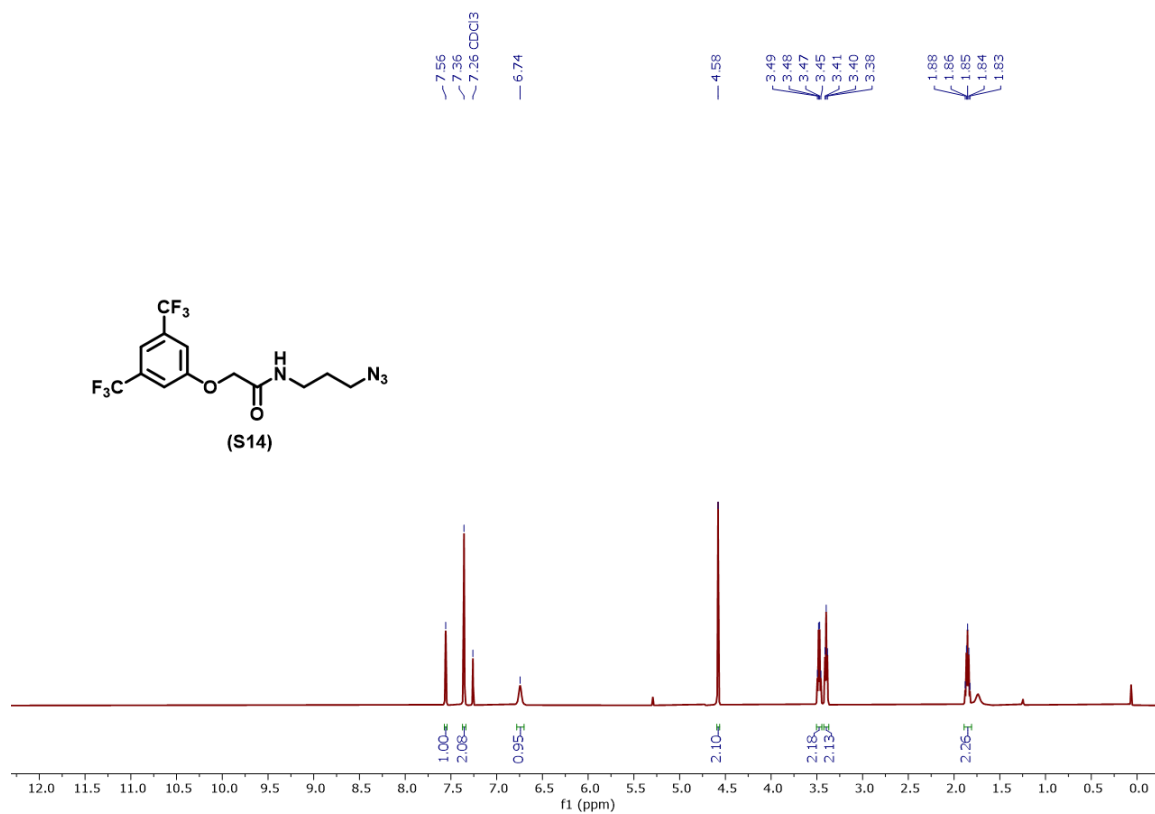
Supplementary Fig. 187. ¹³C-NMR spectrum in DMSO-*d*₆ of compound **S12**.



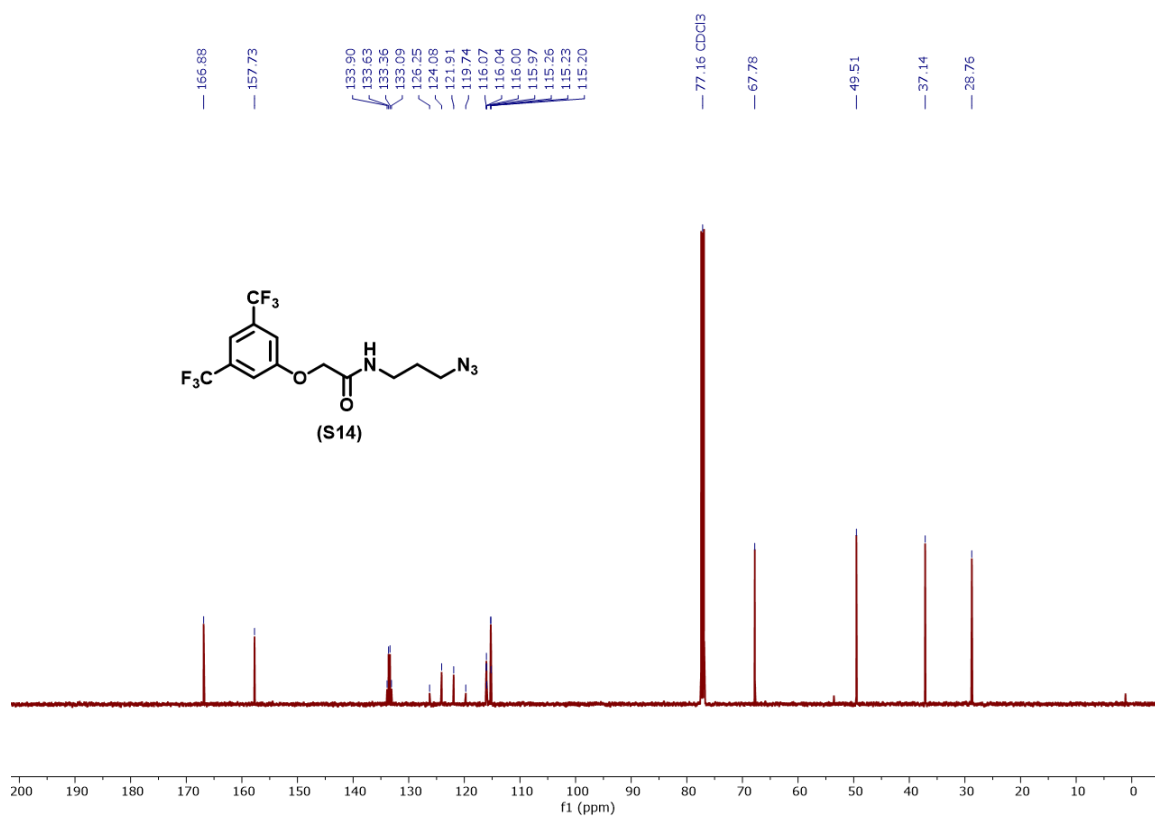
Supplementary Fig. 188. ¹H-NMR spectrum in DMSO-*d*₆ of compound 2u.



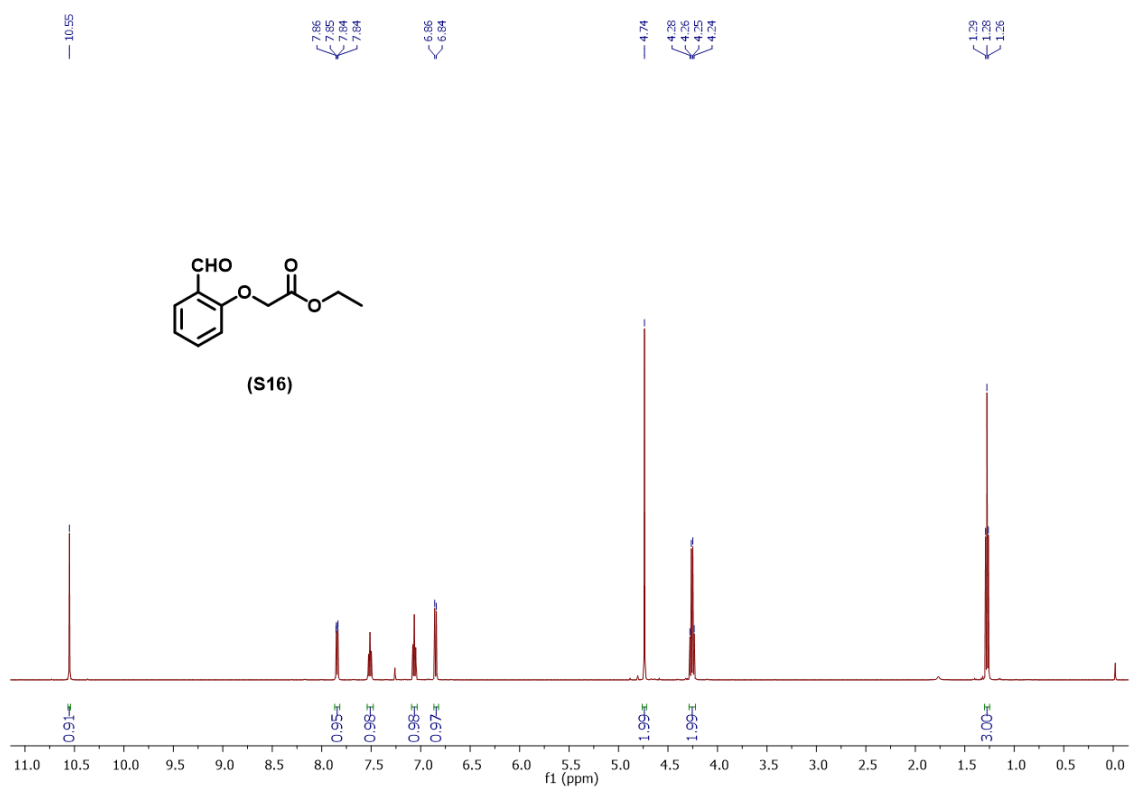
Supplementary Fig. 189. ¹³C-NMR spectrum in DMSO-*d*₆ of compound 2u.



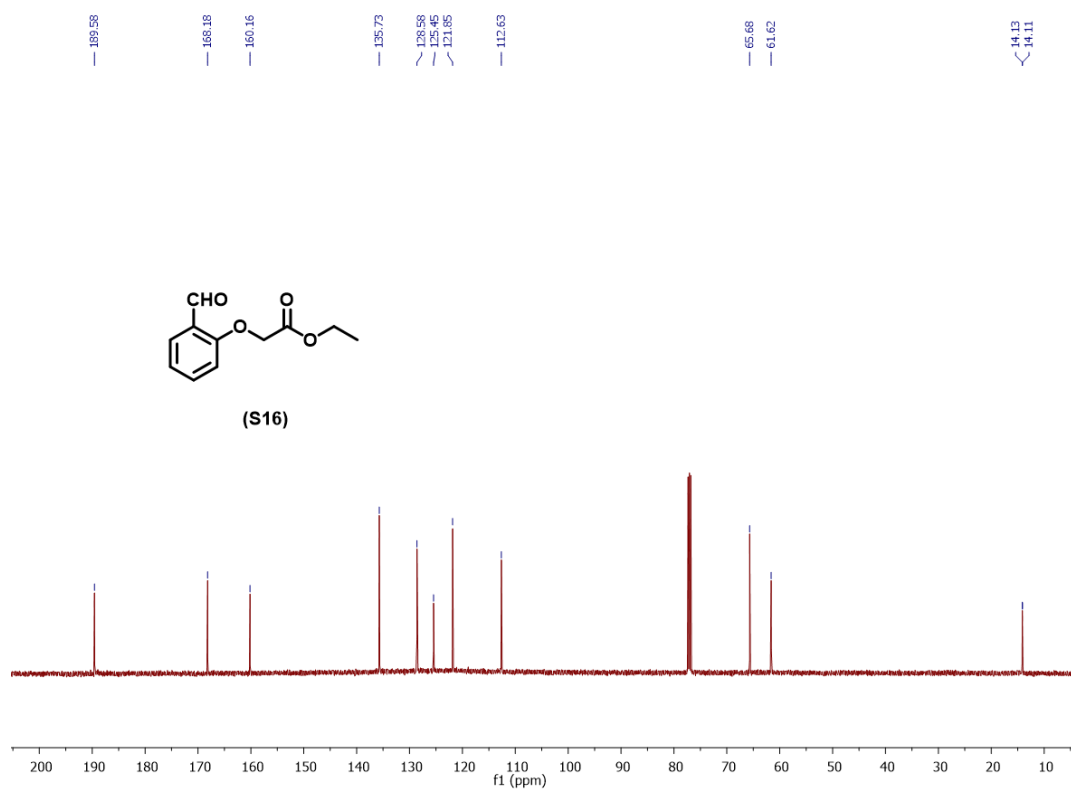
Supplementary Fig. 190. ¹H-NMR spectrum in CDCl₃ of compound **S14**.



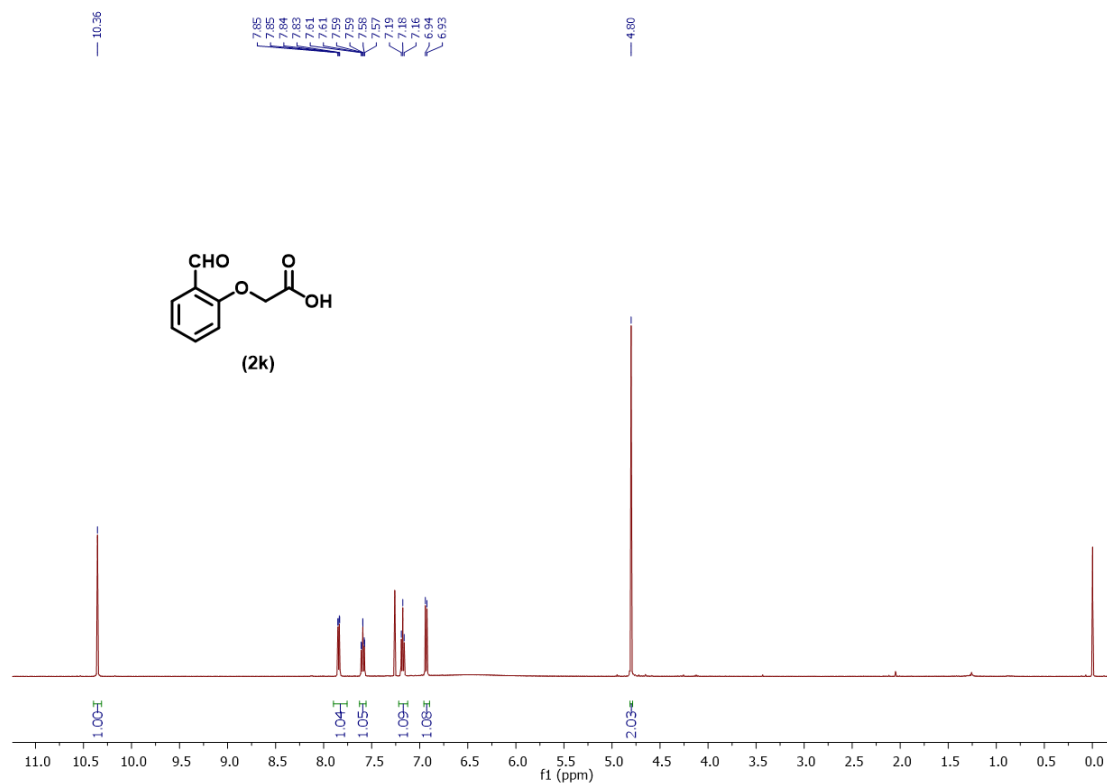
Supplementary Fig. 191. ¹³C-NMR spectrum in CDCl₃ of compound **S14**.



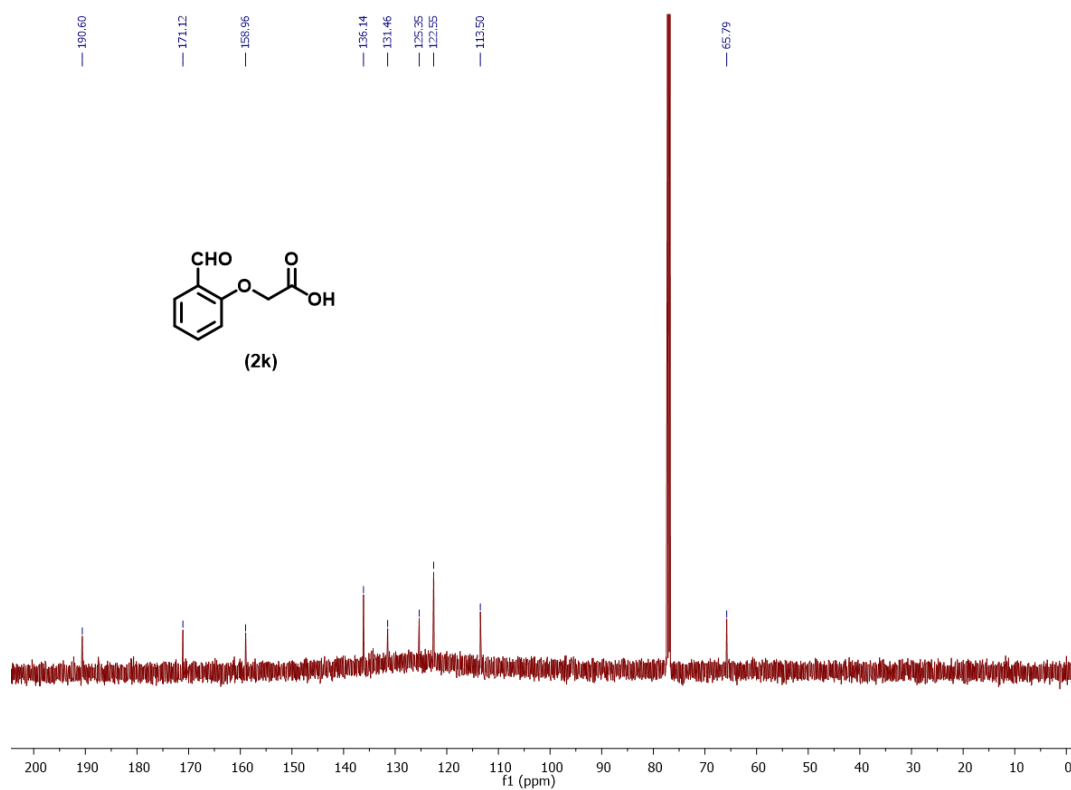
Supplementary Fig. 192. ¹H-NMR spectrum in CDCl₃ of compound **S16**.



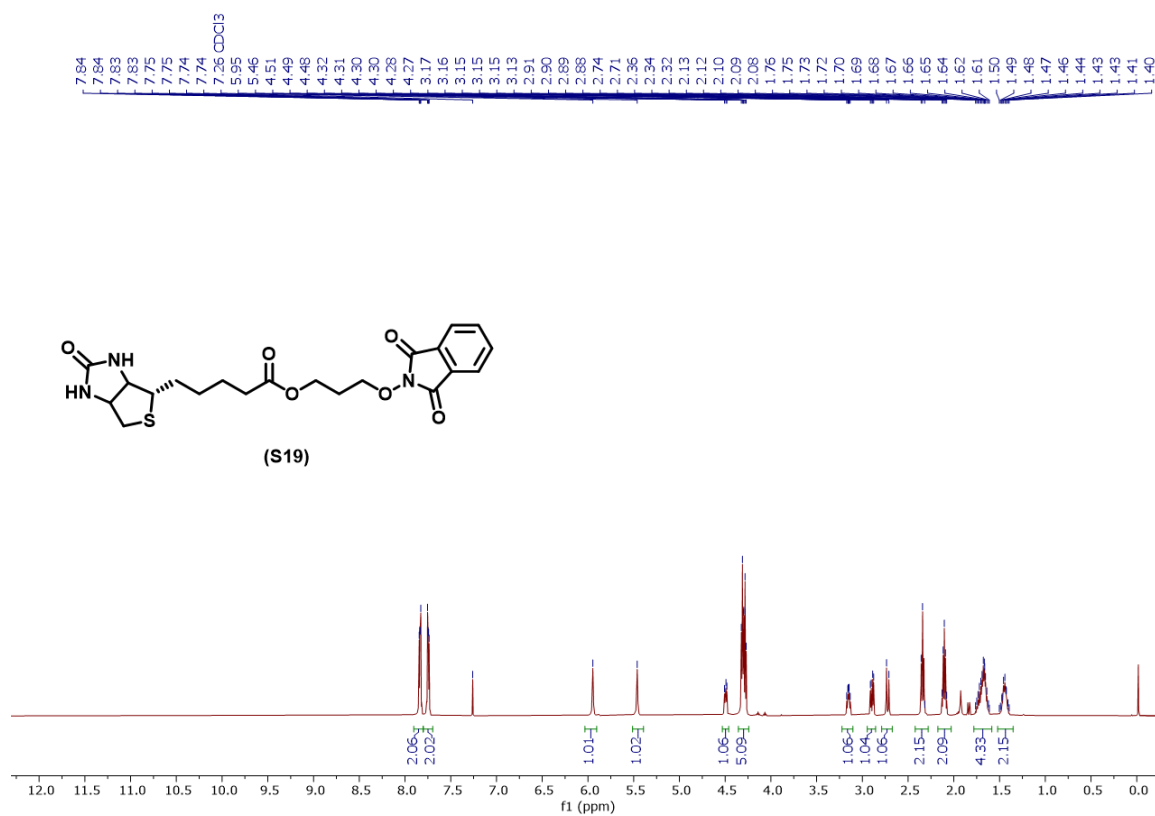
Supplementary Fig. 193. ¹³C-NMR spectrum in CDCl₃ of compound **S16**.



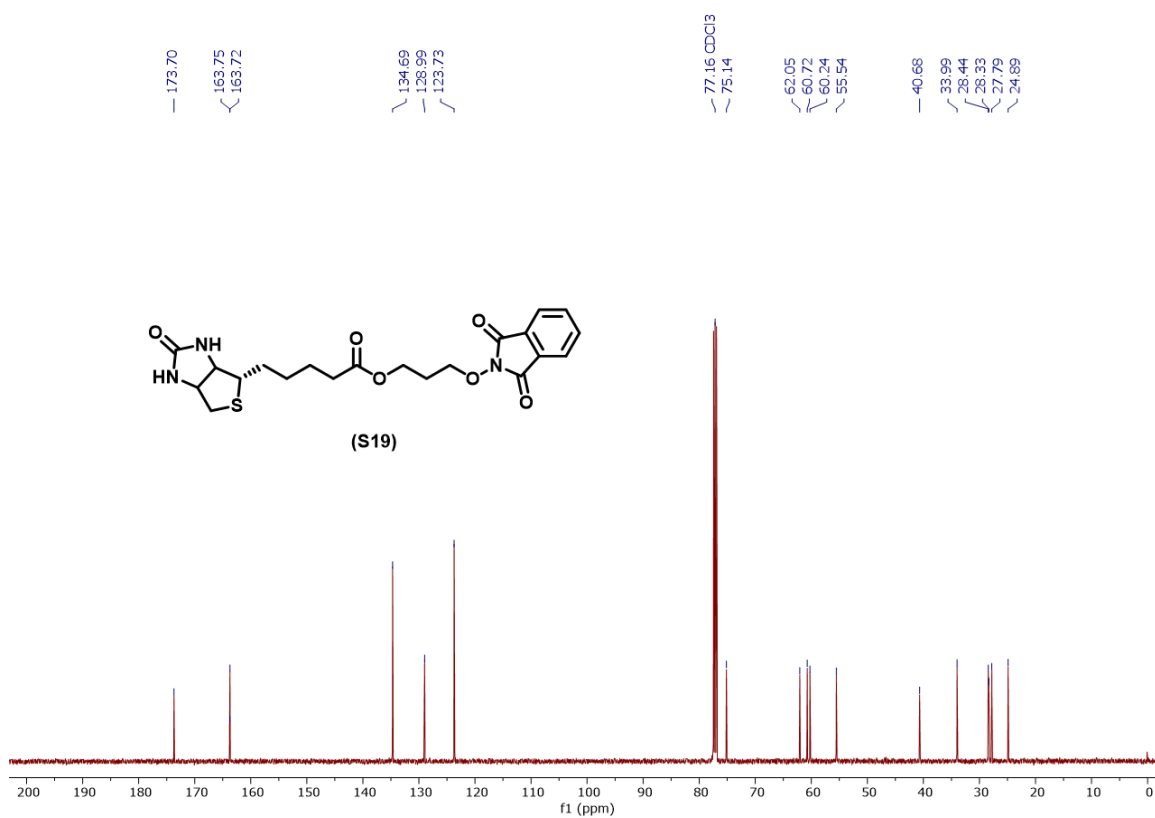
Supplementary Fig. 194. ¹H-NMR spectrum in CDCl₃ of compound **2k**.



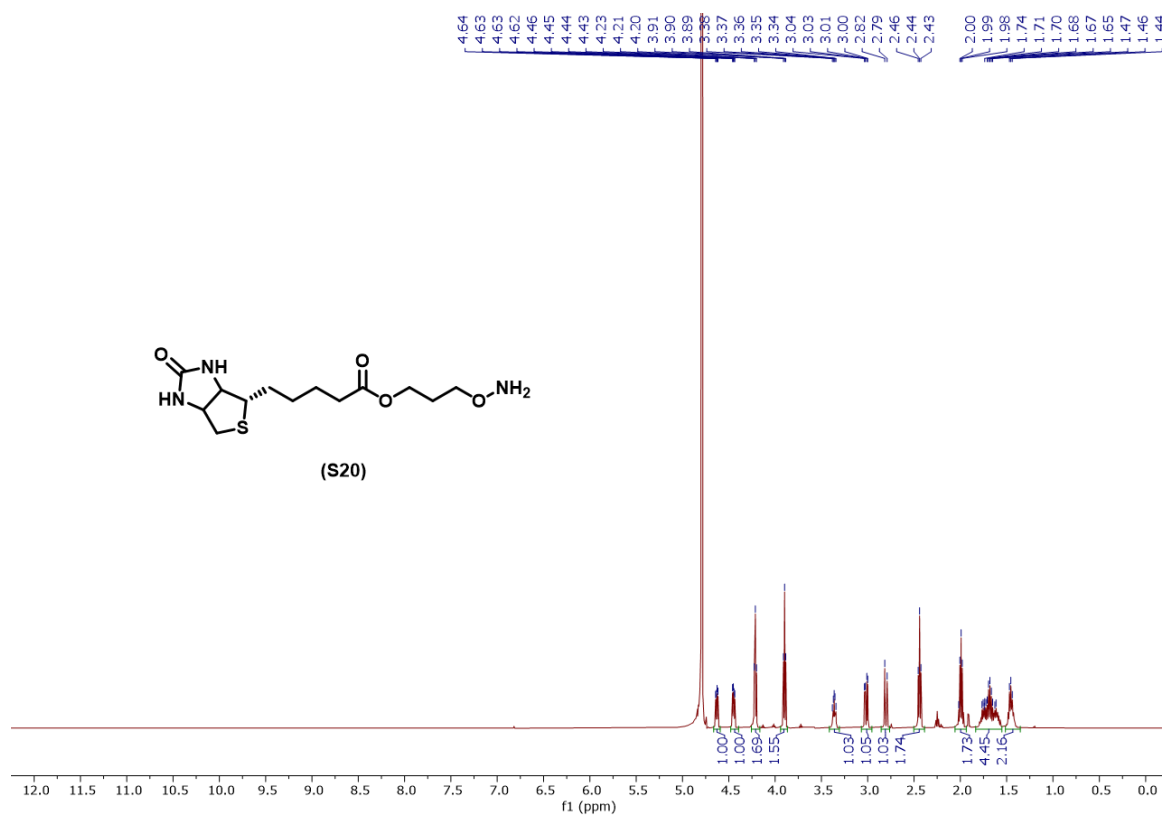
Supplementary Fig. 195. ¹³C-NMR spectrum in CDCl₃ of compound **2k**.



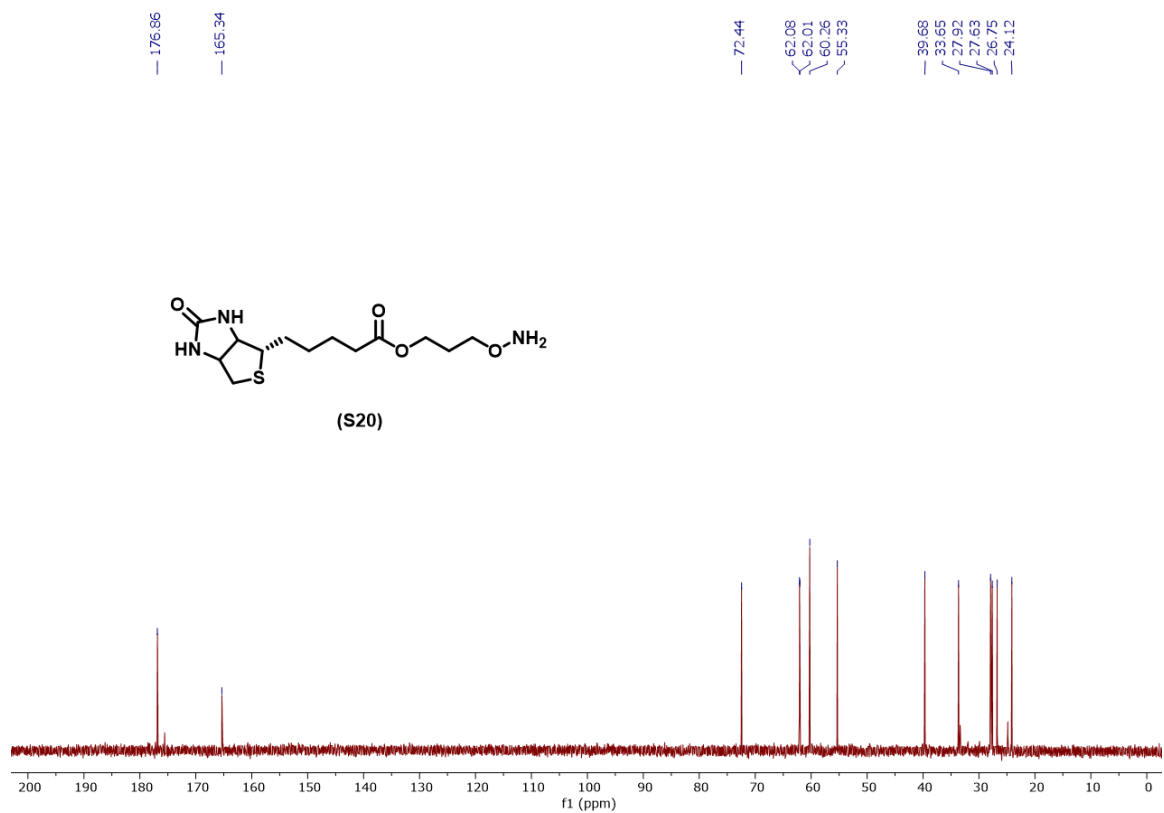
Supplementary Fig. 196. ¹H-NMR spectrum in CDCl₃ of compound **S19**.



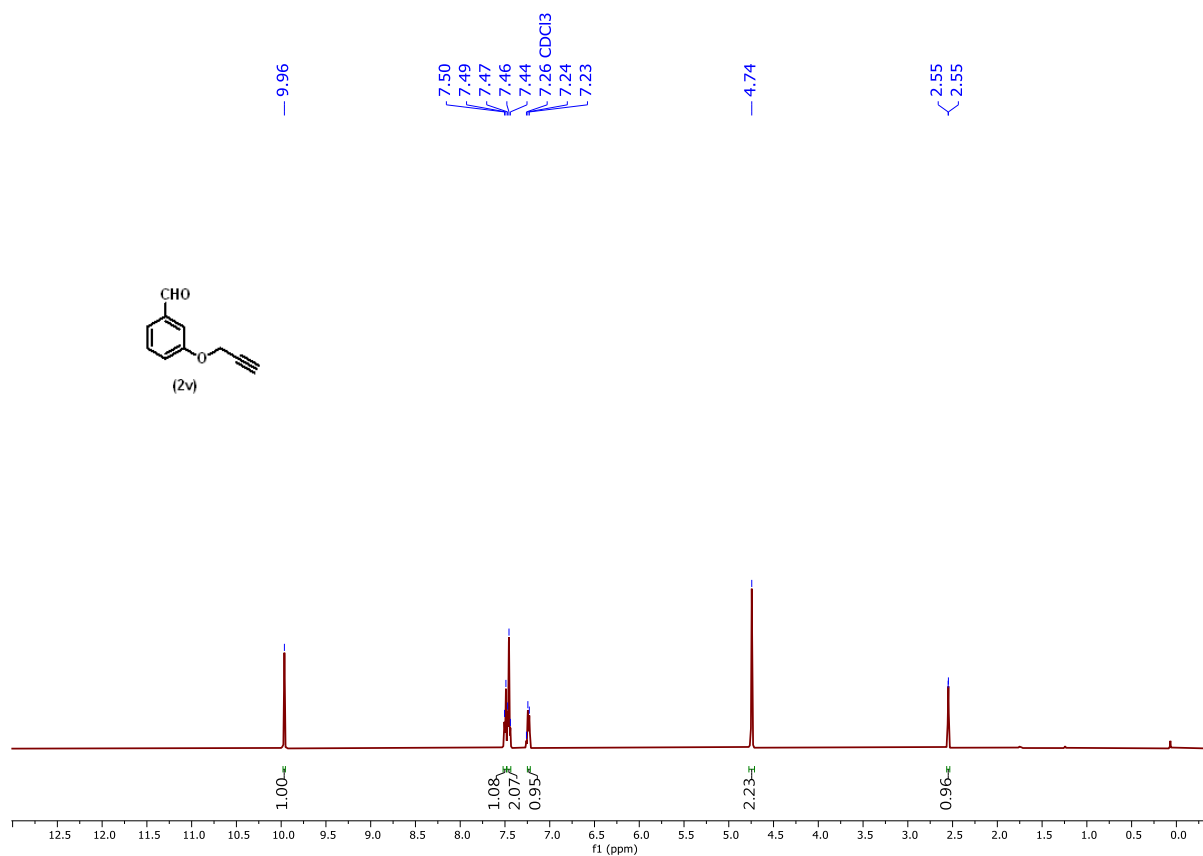
Supplementary Fig. 197. ¹³C-NMR spectrum in CDCl₃ of compound **S19**.



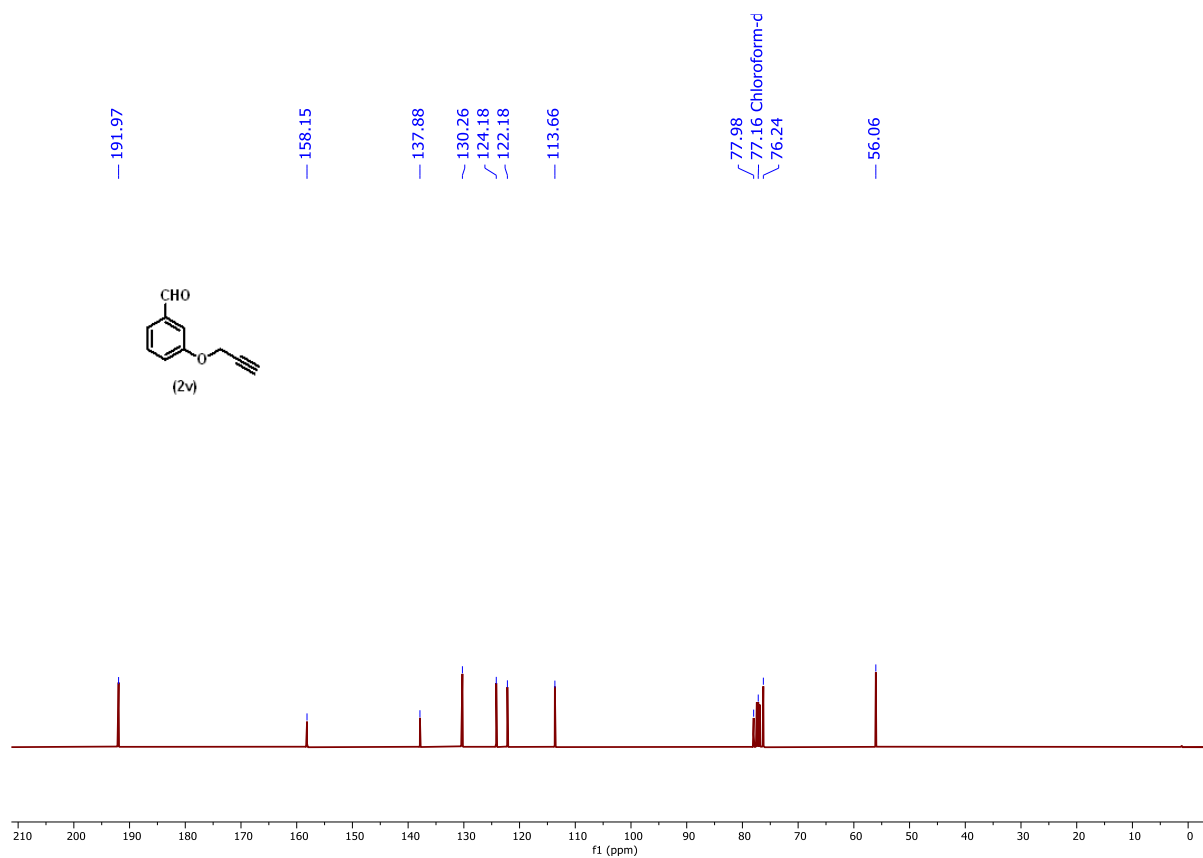
Supplementary Fig. 198. ¹H-NMR spectrum in D₂O of compound **S20**.



Supplementary Fig. 199. ¹³C-NMR spectrum in D₂O of compound **S20**.



Supplementary Fig. 200. ^1H -NMR spectrum in CDCl₃ of compound **2v**.



Supplementary Fig. 201. ^{13}C -NMR spectrum in CDCl₃ of compound **2v**.

14. Supplementary References

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