Electronic Supplementary Material (ESI) for Chemical Communications.

SUPPLEMENTARY INFORMATION

Intracellular Delivery of Therapeutic Proteins through *N*-terminal Site-specific Modification

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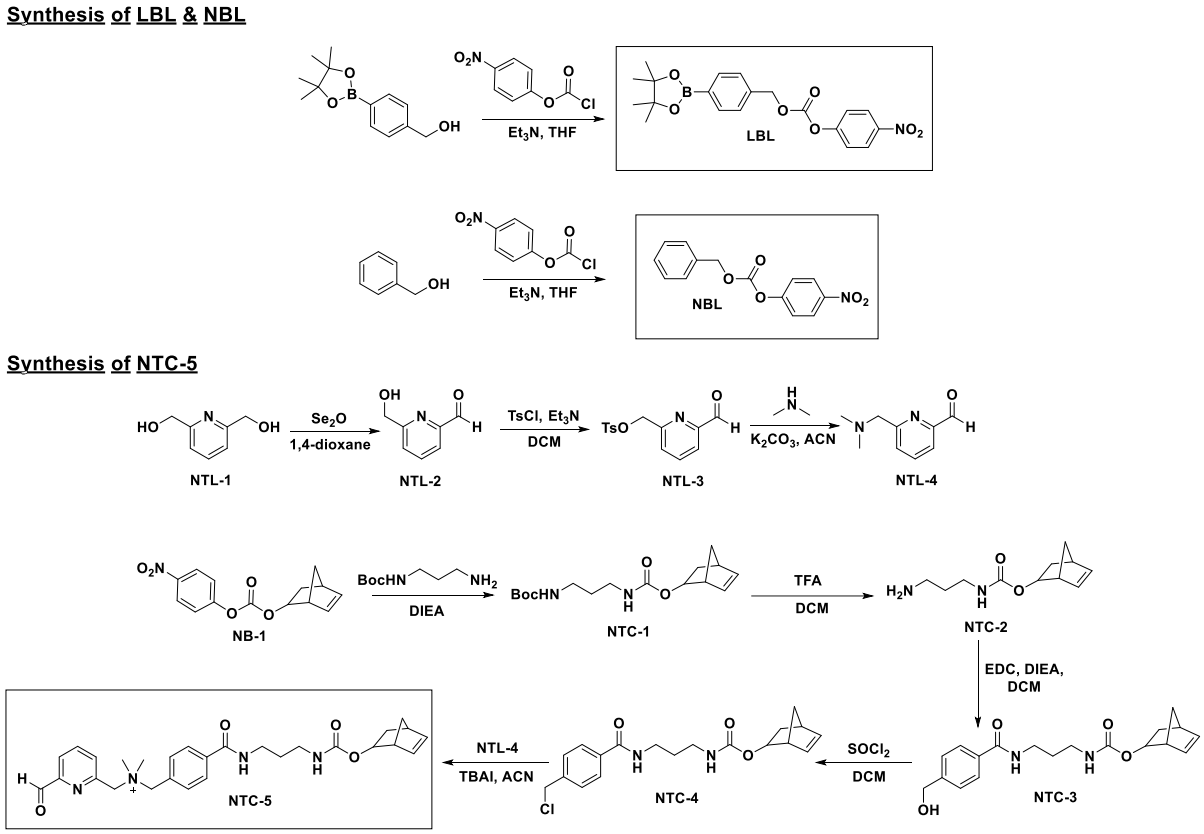
1. General Information

All commercial reagents were purchased from reputable vendors and used without further purification, unless indicated otherwise. All reactions were sealed with septa through which a nitrogen atmosphere was introduced unless otherwise stated. All non-aqueous reactions were carried out under a nitrogen/argon atmosphere in oven-dried glassware. Reactions were conducted in round-bottomed flasks containing Teflon-coated magnetic stir bars. Heating of reactions was accomplished with a silicon oil bath on top of a stirring hotplate equipped with an electronic contact thermometer to maintain the indicated temperatures. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254 nm, 250 μm thickness) and spots were visualized by UV light or appropriate staining (e.g ceric ammonium molybdate (CAM), basic $KMnO_4$). Flash column chromatography was carried out using 200 or 400 mesh silica gel. All ^1H NMR and ^{13}C NMR spectra were carried out on a Bruker ACF-300 or 500 MHz NMR spectrometer. Chemical shifts were reported in parts per million (ppm) relative to residual solvent peaks. ^1H and ^{13}C chemical shifts (δ) were referenced to TMS or residual solvent peaks ($CDCl_3$ = 7.26 ppm and Methanol-d_4 = 3.31 ppm) for ^1H NMR and ($CDCl_3$ = 77.16 ppm and Methanol-d_4 = 49.10 ppm) for ^{13}C NMR. The following abbreviations were used for reporting ^1H NMR spectra: chemical shift (δ ppm), multiplicity (*s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *dd* = doublet of doublets, *m* = multiplet), coupling constant (Hz), integration. Confocal microscopy was performed on an Olympus FV3000 confocal microscope equipped with a 60× water-immersed objective.

Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin-streptomycin (Thermo). All cell lines were grown in a humidified incubator at 37 ºC with 5% CO_2 atmosphere. Protein concentration was determined by Nanodrop™ 2000c spectrophotometer (Thermo) and Pierce™ BCA Protein Assay Kit (Thermo). Zeba™ Spin Desalting Columns used for protein purification was purchased from Thermo Fischer. Human immunoglobulin G (IgG, human plasma, 16-16-090707) was purchased from Athens Research & Technology. Bovine Serum Albumin (BSA) was purchased from Bio Basic Inc. GFP (Cat No. P7410) was purchased from Beyotime Biotechnology. RNase A (Cat No: 101076) was purchased from MP Biomedicals, LLC. Cytochrome C from equine heart was purchased from Sigma Aldrich. RNase A activity assay kit (RNaseAlert™ Substrate) was bought from Integrated DNA Technologies. Pierce™ Protein Transfection Reagent (Pro-Ject™) was purchased from Thermo Fischer (Cat No. 89850). Antibodies used for Western-blotting (WB) and Immunofluorescence assay (IF) were purchased from the following vendors: mouse *anti*-GFP antibody (Cat No: Ab1218, Abcam), goat *anti*-mouse IgG (H+L) secondary antibody, HRP (Cat No. 626520, Invitrogen), mouse *anti*-NPC primary antibody (Cat No. N8786, Sigma), FITC-conjugated goat *anti*-mouse IgG (H+L) secondary antibody (Cat No. 31569, Thermo). Flow cytometric analysis (FACS) was performed on a BD Accuri™ C6 cell analyzer. In-gel fluorescence scanning of the SDS-PAGE gels was carried out with Typhoon 9410 fluorescence gel scanner (Amersham Biosciences).

2. Chemical Synthesis

2.1 Synthetic Scheme



Scheme S1. *Synthetic routes of* ***LBL, NBL*** *and* ***NTC-5****.*

2.2 Synthetic Procedures

The synthesis of **NBL**, **LBL** and **NTL-4** was adapted from previously published protocols.[1, 2]

NMR of NTL-4:

^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.02 (s, 1H), 7.80-7.78 (m, 2H), 7.62-7.60 (m, 1H), 3.65 (s, 2H), 2.28 (s, 6H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 193.68, 159.76, 152.41, 137.46, 127.59, 120.27, 65.25, 45.56. HRMS (ESI) calculated for $[M+H]^+$: 165.1028, found 165.1018.

Synthesis of NTC-1:

To a solution of *N*-Boc-1,3-propanediamine[3] (180.0 mg, 1.03 mmol) in dry DCM (15.0 mL), DIEA (1.32 mL, 7.40 mmol) and **NB-1** (236 mg, 0.86 mmol) was added. The mixture was stirred at room temperature for 24 h. After which, the solvent was removed *in vacuo* and the residue was purified by chromatography using ethyl acetate : hexanes = 1:3 to give the desired product **NTC-1** (192.0 mg, 72.0%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 6.21-6.15 (m, 1H), 5.90-5.85 (m, 1H), 5.08-5.04 (m, 0.77H), 4.42 (d, J = 8.8 Hz, 0.27H), 3.03-2.93 (m, 5H), 2.74-2.70 (m, 1H), 2.03-1.97 (m, 0.79H), 1.59-1.48 (m, 3.52H), 1.33 (s, 9H), 1.25-1.23 (d, J = 8.8

Hz, 1H), 0.84-0.79 (m, 0.78H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 159.06, 158.99, 158.35, 142.15, 139.17, 133.72, 132.66, 79.84, 76.51, 76.39, 48.42, 47.21, 43.42, 38.95, 38.64, 35.48, 31.19, 28.92.

Synthesis of NTC-2:

NTC-1 (170 mg, 0.55 mmol) was dissolved in anhydrous DCM (15.0 mL), followed by the dropwise addition of trifluoroacetic acid (15.0 mL). The reaction mixture was left to stir at room temperature for 2 h. After which, the solvent was removed *in vacuo* to give the crude product **NTC-2** as a TFA salt (115.0 mg), which was used directly in the next step. ^{1}H NMR (400 MHz, Methanol-d_4) δ 6.33-6.30 (m, 1H), 6.02-5.96 (m, 1H), 5.20 (dd, J = 8.8, 4.4 Hz, 0.77H), 4.57 (d, J = 4.4Hz, 0.27H), 3.24-3.19 (m, 2H), 3.09-3.05 (m, 1H), 2.99-2.93 (m, 2H), 2.84 (d, J = 12 Hz, 1H), 2.16-2.12 (m, 1H), 1.86-1.83 (m, 2H), 1.78-1.70 (m, 1H), 1.68-1.60 (m, 1H), 1.48 (d, J = 4.4 Hz, 1H), 0.96-0.88 (m, 1H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 159.96, 159.95, 140.91, 137.93, 132.19, 131.13, 117.28, 114.41, 75.45, 45.85, 42.08, 40.37, 36.88, 36.77, 34.10, 34.05, 31.33, 27.84, 22.28. HRMS (ESI) calculated for $[M+H]^+$: 210.1368, found 211.1437.

Synthesis of NTC-3:

4-(Hydroxymethyl)benzoic acid (73.0 mg, 0.48 mmol) was dissolved in anhydrous THF (12.0 mL). Following which, EDC (112.9 mg) and DIEA (91.7 μL) were added. After 30 mins, **NTC-2** (100.0 mg, 0.48 mmol) was added. The reaction mixture was left to stir at room temperature overnight. Next, the solvent was removed *in vacuo* and the residue was purified by chromatography using ethyl acetates : hexanes = 4:1 to give the desired product **NTC-3** (99.1 mg, 60.0%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.82 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 6.32 – 6.26 (m, 1H), 6.02-5.97 (m, 1H), 5.21 – 5.18 (m, 0.77H), 4.68 (s, 2H), 4.55 (d, J = 8.0 Hz, 0.29H), 3.43 (q, J = 8.0 Hz, 2H), 3.23 – 3.16 (m, 2H), 3.11 (s, 1H), 2.86 (s, 1H), 2.15-2.09 (m, 0.77H), 1.82-1.74 (m, 2H), 1.71-1.67 (m, 0.70H), 1.58-1.56 (m, 0.34H), 1.47-1.43 (m, 1H), 1.30-1.40 (m, 1H), 0.96-0.90 (m, 1H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 168.68, 157.93, 145.38, 140.87, 137.91, 133.09, 133.05, 132.35, 131.30, 127.01, 126.37, 75.22, 63.24, 47.09, 45.92, 45.69, 42.13, 40.43, 37.88, 37.79, 37.01, 36.96, 34.22, 34.15, 29.42. HRMS (ESI) calculated for $[M+Na]^+$: 367.1634, found 367.1632.

Synthesis of NTC-4:

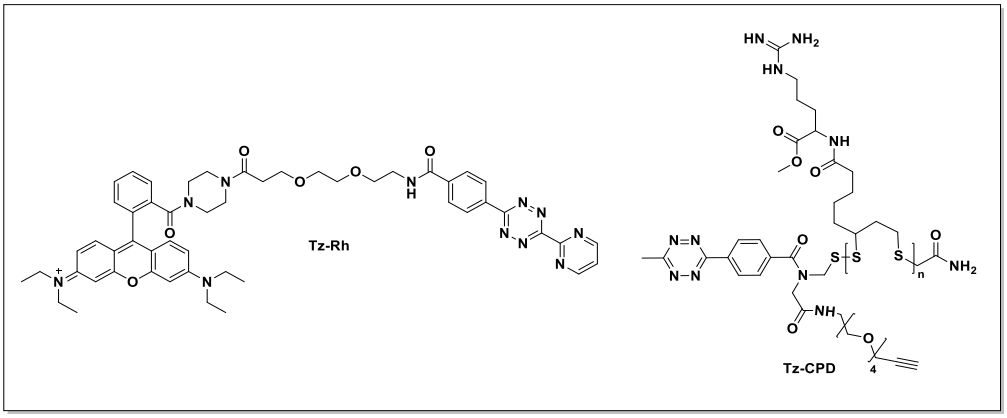
NTC-3 (170 mg, 0.48mmol) was dissolved in anhydrous DCM (15.0 mL). Anhydrous DMF (0.05 ml) was added followed by the dropwise addition of thionyl chloride (80.0 μL). After the mixture was stirred for 2 h at room temperature, the reaction was quenched by the addition of 10 mL water, and purified by flash chromatography ethyl acetates : hexanes = 1:10 to give the desired product **NTC-4** (177 mg, 99%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.83 (d, J = 8.0 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 6.32-6.26 (m, 1H), 6.02-5.96 (m, 1H), 5.20-5.18 (m, 0.71H), 4.71 (s, 2H), 4.55 (d, J = 8.0 Hz, 0.23H), 3.46-3.40 (m, 2H), 3.21-2.11 (m, 2H), 3.11 (s, 1 H), 2.85-2.82 (m, 1H), 2.15-2.09 (m, 0.78H), 1.84-1.80 (m, 2H), 1.79-1.70 (m, 0.69H), 1.69-1.65 (m, 0.40H), 1.47-1.41 (m, 1H), 1.36 (d, J = 8.0 Hz, 1H), 0.99-0.90 (m, 1H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 168.28, 163.45, 157.94, 141.46, 140.83, 137.85, 134.16, 132.31, 131.25, 128.62, 128.41, 127.27, 75.29, 75.18, 45.89, 45.61, 44.69, 42.10, 40.40, 37.82, 37.73, 37.00,

36.96, 35.57, 34.16, 34.08, 29.34. HRMS (ESI) calculated for $[M+Na]^+$: 385.1295, found 385.1291.

Synthesis of NTC-5:

To a solution of **NTC-4** (60.0 mg, 0.16 mmol) and **NTL-4** (32.5 mg, 0.20 mmol) in ACN (15.0 mL) was added TBAI (118.0 mg, 0.32 mmol) at room temperature. The mixture was stirred for 72 h. The solvent was removed in vacuo and the residue was purified by chromatography using DCM : methanol = 5:1 to give the desired product **NTC-5**. ^{1}H NMR (400 MHz, Methanol-d_4) δ 10.10 (s, 0.07H), 8.04 – 8.01 (m, 3H), 7.99 (d, J = 8.0 Hz, 2H), 7.86 (d, J = 8.0 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 6.33-6.26 (m, 1H), 5.99-5.97 (m, 1H), 5.62 (s, 1H), 5.21-5.18 (m, 0.76H), 4.84 (s, 2H), 4.65 (s, 2H), 4.56-4.55 (m, 0.29H), 3.37-3.32 (m, 2H), 3.20 (s, 1H), 3.19-3.17 (m, 2H), 3.13 (s, 6H), 2.84 (s, 1H), 2.15-2.09 (m, 0.86H), 1.82-1.70 (m, 2H), 1.69-1.65 (m, 0.78H), 1.58-1.56 (m, 0.37H), 1.45-1.44 (m, 1H), 1.37-1.35 (m, 1H), 0.99-0.95 (m, 1H). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.06 (s, 1H), 8.65 (d, J = 4.0 Hz, 1H), 8.28 (t, J = 8.0 Hz, 1H), 8.09 (d, J = 8.0 Hz, 1H), 8.02-7.98 (m, 3H), 7.79 (d, J = 8.0 Hz, 2H), 7.14-6.96 (m, 0.78H), 6.33-6.25 (m, 1H), 6.00-5.94 (m, 1H), 5.12-5.10 (m, 0.83H), 4.80 (s, 4H), 4.63 (d, J = 4.0 Hz, 0.37H), 4.45 (d, J = 8.0 Hz, 0.27H), 4.05 (s, 0.47H), 3.06 (s, 6H), 3.01 (s, 2H), 2.79 (s, 1H), 2.08-2.02 (m, 1H), 1.66-1.57 (m, 3H), 1.32 (s, 2H), 0.86-0.84 (m, 1H). ^{13}C NMR (100 MHz, Methanol-d_4) δ 192.21, 167.67, 160.24, 157.91, 147.92, 140.87, 138.50, 137.87, 136.52, 133.36, 131.27, 130.86, 127.76, 127.71, 127.35, 121.73, 97.40, 75.16, 67.21, 66.86, 53.52, 49.78, 45.90, 42.11, 40.41, 37.79, 37.72, 37.03, 34.17, 34.10, 29.39, 29.34, 29.07, 22.35. HRMS (ESI) calculated for $[M+ H]^+$: 491.2653, found 491.2662. *Note: Aldehyde proton for **NTC-5** was not visible in ^{1}H NMR when Methanol-d_4 was used, hence DMSO-d_6 ^{1}H NMR was also included.

2.3 Chemical Structures of Compounds Used in Click Conjugation

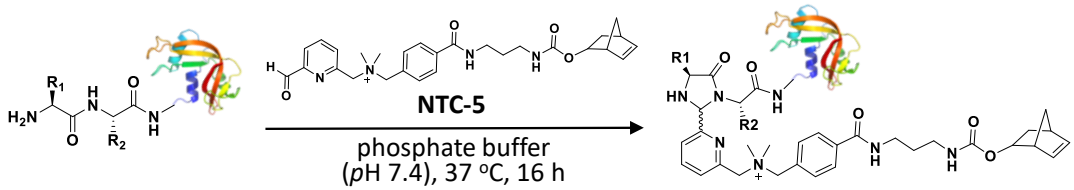


Scheme S2. *Chemical structures of probes used in protein labeling and click conjugation reactions. Tz-Rh[4] and Tz-CPD[5] were synthesized according to reported procedures.*

General procedure for Tz-CPD synthesis and characterization. The Tz-CPD polymers were synthesized and characterized according to published procedures and modified where applicable.[5] Briefly, stock solutions of the monomer (2M in DMF), Tz initiator (50 mM in DMF), terminator (Iodoacetamide, 0.5 M in H_2O, fresh) and TEOA buffer (1M, pH = 7.0) were prepared. 10 μL of Tz initiator was added to 80 μL of TEOA buffer and 10 μL of the monomer

stock solution. After 60 min of agitation at room temperature, the polymerization reaction was quenched by addition of 1.9 mL of the terminator stock solution. The resulting polymer was purified *via* dialysis using a Slide-A-Lyzer Dialysis Cassettes, 10K MWCO (Thermo Fisher) against H_2O in the same day, by following protocols provided by the vendor. The elution was lyophilized and kept in -20 ºC. HPLC analysis was carried out to ensure the removal of unreacted monomers and initiator. Quantification of Tz-CPD was achieved by UV-Vis measurements of the tetrazine absorbance at the wavelength of 520 nm. Tz initiators of different concentrations (50, 100, 200, 400 and 500 μM) was used to generate a corresponding calibration curve. The Tz-CPD polymer concentration of the obtained stock solution was estimated to be ~250 μM.

3. Protein Modifications



General procedure for N-terminal site-specific modification: 1 equiv. of protein (1 mg/mL) was treated with 200 equiv. of **NTC-5** (50 mM stock solution in DMSO). The reaction was carried out in 50 mM phosphate buffer (*p*H 7.4) at 37 ºC for 16 h. Following which, the reaction was purified using a Zeba™ Spin Desalting Column (7K MWCO). The eluates were collected, and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo). The success of *N*-terminal labeling was verified using SDS-PAGE gel analysis and in-gel fluorescence scanning. The labeled protein was first clicked with Tz-Rh (e.g. 1 μM of **NTC-5** modified BSA was reacted with 10 μM Tz-Rh) for 1 h at room temperature. 1× SDS loading buffer was added to the click reaction, boiled at 95 ºC for 15 min and resolved on a 10% or 15% SDS-PAGE followed by in-gel fluorescence scanning. All other proteins (RNase A, GFP, IgG) used in this work were modified under similar conditions.

General procedure for Tz-Rh click with NTC-5 labeled proteins: For BSA-**NTC-5**-Rh conjugate used in Rh dye:protein ratio determination, excessive Tz-Rh was removed by Zeba™ Spin Desalting Columns (7K MWCO) after the click reaction (as described above). The eluates were collected, and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo). The absorbance of BSA-**NTC-5**-Rh conjugate was measured at λ_{280nm} and λ_{max} = 550 nm. Ultraviolet-visible (UV-vis) absorption was measured using a Shimadzu UV-Vis spectrometer.

***General procedure for fluorescent labeling of proteins*:** BSA was labeled using Cy5-maleimide. 1 mg of BSA was dissolved in 100 μL of PBS (*p*H = 7.4) and reacted with 2.5 μL of Cy5-maleimide (GE Healthcare Life Science; 2 mM in DMSO) at room temperature for 4 h. Subsequently, excessive Cy5-maleimide was removed by Zeba™ Spin Desalting Columns

(7K MWCO). The eluates were collected, and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo). Fluorescein Isothiocyanate (FITC) labeling of RNase A and IgG were carried out after *N*-terminal modification. The reaction mixture was adjusted to *p*H 9.0 by the addition of 1 M $NaHCO_3$ solution (100 mM $NaHCO_3$ final concentration). 40 equiv. of FITC stock solution (10 mg/mL in DMSO) was added and the reaction was left to proceed at room temperature for 1 h. Excessive FITC was removed by Zeba™ Spin Desalting Columns (7K MWCO). The eluates were collected, and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo).

General procedure for LBL labeling of RNase A as pro-drug: **NTC-5** labeling of RNase A (RNase A-NTC) was prepared and purified as described above. RNase A-NTC was subsequently reacted with **LBL** to further label surface lysine on RNase A. Briefly, RNase A-NTC was dissolved in 100 μL $NaHCO_3$ buffer solution (100 mM, *p*H = 8.5) at a protein concentration of 400 μM. To the solution, 50 equiv. of **LBL** (100 mM stock solution in DMSO) was added and reacted at room temperature for 16 h. The double-labeled product (RNase-NTC-**LBL**) was purified by Zeba™ Spin Desalting Columns (7K MWCO). After purification, protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo). **NBL** labeling of RNase A-NTC was also carried out as stated above.

Fluorometric Alizarin Red S(ARS) Assay. To confirm successful boronic acid moiety labeling on RNase A-LBL, 0.0025% w/v ARS solution was incubated with 0.25 mg/mL of varied proteins, including native RNase A, **NTC-5** modified RNase A, RNase A-LBL and RNase A-NBL with or without H_2O_2 pre-treatment for 15 minutes, followed by ARS emission measurement. Emission was monitored at λ_{ex} = 490 ± 20 nm and λ_{em} = 600 ± 20nm using a BioTek Synergy 4 microplate reader.

4. Intracellular Delivery of Proteins

General Procedure For CLSM experiments**:** HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100.0 mg/L streptomycin and 100 IU/mL penicillin in a humidified atmosphere of 5% CO_2 at 37 °C, seeded in an 8-well μclear-bottom dish (80826, Ibidi GmbH) and grown until 50~60% confluency. Upon medium removal, cells were treated with 100 nM **NTC-5** modified RNase A^{FITC}, GFP, BSA^{Cy5} or IgG^{FITC} protein-CPD conjugates in 200 μL DMEM. Protein-CPD conjugates were obtained by reacting 5 μL of 4 uM **NTC-5** modified proteins with 5 μL of 4 μM Tz-CPD (1:1 ratio) for 1 h at room temperature. The resulting protein-CPD conjugates were directly used for intracellular delivery. HeLa cells incubated with the **NTC-5** modified proteins alone were done concurrently as negative controls. After 2 h incubation at 37 °C, cells were washed with PBS (200 μL) twice and co-stained with Hoechst and LysoTracker™ DND-99 Red followed by live-cell imaging using an Olympus FV3000 confocal microscope equipped with a 60× water-immersed objective.

General Procedure for FACS experiments**:** HeLa cells were seeded in a 12-well plate

(Greiner CELLSTAR®) and cultured overnight. Upon medium removal, the cells were treated with 100 nM of the protein-CPD conjugates (**NTC-5** modified RNase A^{FITC}, GFP, BSA^{Cy5} or IgG^{FITC} reacted with Tz-CPD) in 0.5 mL DMEM. At the same time, the same amounts of **NTC-5** modified RNase A^{FITC}, GFP, BSA^{Cy5} or IgG^{FITC} were also delivered by using Pro-Ject™[6] for comparison. The cells were incubated for 2 h at 37 °C and subsequently washed three times with PBS. The resulting cells were detached from the plate by treatment with 200 μL of 0.1% trypsin-EDTA at 37 °C for 2 min. The detached cells were collected by centrifugation. Upon further washing with cold PBS (500 μL) thrice, the cells were resuspended in PBS (1 mL). Experiments with HeLa cells treated with NTC-labeled proteins only were concurrently carried out as negative controls. Cells were analyzed on a BD Accuri™ C6 cell analyzer (10,000 cells were counted for each event; in triplicates) using λ_{ex} = 488 nm, λ_{em} = 525 ± 40 nm (FL1) for FITC/GFP; λ_{ex} = 640 nm, λ_{em} = 675 ± 25 nm for Cy5 (FL4). Lysates of treated cells were also collected, resolved on a 10% or 15% SDS-PAGE and visualized by in-gel fluorescence scanning.

General Procedure for Immunofluorescence (IF) staining experiments: HeLa cells were seeded in 8-well Ibidi™ plates and cultured overnight. To confirm *anti*-NPC antibody retains its function after intracellular delivery, immunofluorescence (IF) was performed on cells treated with the *anti*-NPC antibody-CPD conjugate. Briefly, cells were first treated with *anti*-NPC antibody-CPD conjugate (500 nM, 12 h). Upon medium removal, the cells were washed thrice with PBS, fixed with 3.7% formaldehyde (30 mins, rt), permeabilized with 0.02% Triton (15 mins, rt) and blocked with 5% BSA in TBST (1 h, rt). The cells were then incubated with FITC-conjugated goat *anti*-mouse IgG (H+L) secondary antibody (1:300) overnight at 4 ºC. After thoroughly washing with TBST buffer, cells were incubated with Hoechst for 10 min prior to image acquisition using an Olympus FV3000 confocal microscope equipped with a 60× water-immersed objective. For the positive controls, cells without any pretreatment were fixed, TBST-treated and blocked as above procedures, followed by incubation with *anti-NPC* antibody (1:300) at 4 ºC overnight.

Intracellular ROS measurement: The intracellular ROS level of HeLa cells were determined using 2',7'–dichlorofluorescin diacetate (DCFDA) staining, followed by flow cytometry analysis. Briefly, HeLa were seeded in a 12-well plate at a density of 150,000 cells per well one day before the experiment. Cells were washed once with PBS (500 μL), followed by 30 min. of incubation with 10 μM DCFDA in DMEM. The cells were harvested and washed with PBS before flow cytometry analysis. Cells were analyzed on a BD Accuri™ C6 cell analyzer (10,000 cells were counted for each event in triplicates) using λ_{ex} = 488 nm, λ_{em} = 525 ± 40 nm (FL1).

In Vitro Activity Assay for LBL-labeled RNase A: The enzymatic activity of dual-labeled RNase A-NTC-LBL was studied *in vitro* first to evaluate whether RNase A function could be inhibited by **LBL/NBL** conjugation and restored by subsequent H_2O_2 release. Briefly, 0.5 μL of 0.26 μg/μL native RNase A, RNase A-NTC and RNase A-NTC with **LBL/NBL** conjugation was treated with or without 5 mM H_2O_2 for 3 h at 37 ºC. The above mixture was diluted to

0.025 ng/mL and reacted with the RNaseAlert™ Substrate (IDT). Kinetic readings of the green-fluorescence substrate (λ_{ex} = 485 ± 20 nm, λ_{em} = 528 ± 20nm) were recorded at 37 ºC by using a BioTek Synergy 4 microplate reader.

Cellular Uptake Efficiency of Various Cell Lines: Cells were seeded in a 12-well plate (Greiner CELLSTAR®) and cultured overnight. Upon medium removal, the cells were treated with **NTC-5** modified RNase A^{FITC} protein-CPD conjugates in 0.5 mL DMEM (100 nM, 2 h). After 2 h, the cells were subsequently washed three times with PBS. The resulting cells were detached from the plate by treatment with 200 µL of 0.1% trypsin-EDTA at 37 °C for 2 min. The detached cells were collected by centrifugation. Upon further washing with cold PBS (500 µL) thrice, the cells were resuspended in PBS (1 mL). Cells treated with **NTC-5** modified RNase A^{FITC} protein only were carried out as negative controls. Cells were analyzed on a BD Accuri™ C6 cell analyzer (10,000 cells for each event in triplicates) using λ_{ex} = 488 nm, λ_{em} = 525 ± 40 nm (FL1).

MTT Assay for Dual Labeled RNase A after Intracellular Delivery: To determine the viability of cells treated with dual-labeled RNase A-LBL/RNase A-**NBL**-CPD conjugates, HeLa cells (10,000 cells per well) were incubated with each sample at the desired concentrations in a 96 well plate at 37 °C. Negative control experiments were done with CPD only and **NTC-5** modified RNase A only. For intracellular delivery of dual-labeled RNase A-LBL/RNase A-**NBL**-CPD conjugates to cancer/non-cancerous cell lines, 500 nM of above dual-labeled RNase A-CPD conjugates (final concentration) in 100 µL of DMEM was incubated with HeLa cells grown in a 96-well plate (~30% confluency) in triplicates. After 24 h incubation, the cells were washed with PBS thrice, replaced with fresh culture medium before viability measurement using the MTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines.

5. Results and Discussion

5.1 NTC-5 Protein Labeling Optimisation

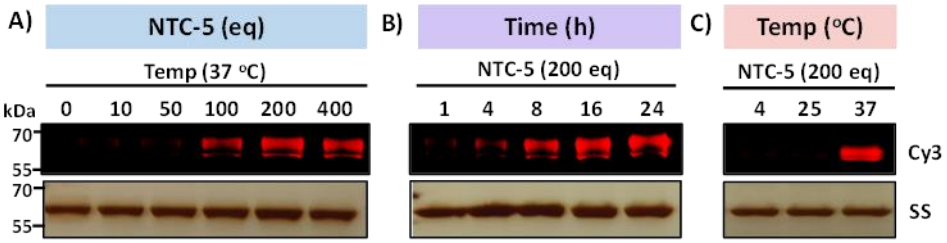


Figure S1. BSA was used as model protein to optimize **NTC-5** labeling conditions. **(A)** Effect of probe concentration on labeling efficiency. BSA was reacted with **NTC-5** at the indicated ratios of 1:10 to 1:400 for 16 h at 37 °C (final concentration of BSA was 15 µM). The reaction was purified by Zeba™ Spin Desalting Columns (7K MWCO). The **NTC-5** labeled BSA was clicked with the indicated ratio of Tz-TER (10 equiv.) for 1 h at room temperature. After which, the reaction was analysed by SDS-PAGE gel, visualized by in-gel fluorescence scanning (Cy3 channel) as well as silver staining (SS). **(B)** Effect of reaction time on labeling efficiency. BSA (15 µM) was reacted with **NTC-5** (200 equiv.) for 1 h, 4 h, 8 h, 16 h and 24 h at 37 °C. **(C)** Effect of incubation time on labeling efficiency. BSA (15 µM) was reacted with **NTC-5** (200 eq) at room temperature for 16 h at 4 °C, 25 °C and 37 °C. Optimal labeling condition was achieved when 200 equiv. of **NTC-5** was used and the reaction was allowed to proceed at 37 °C for 16 h.

5.2 Characterisation of NTC-5 Labeled BSA

A) ESI-TOF MS

Protein	Molecular Weight
Unmodified BSA	**66, 432.90**
NTC-5 modified BSA	**66, 910.58**

B) Quantitative Analysis of NTC-5 Labeling on BSA[7]

- The degree of **NTC-5** labeling on BSA can be estimated *by* determining the dye:protein ratio of BSA-**NTC-5**-Rh conjugate.

1. Calculate Molarity of Protein in BSA-**NTC-5**-Rh Conjugate
   - ε = BSA molar extinction coefficient
   - A_{max} = Absorbance of BSA-**NTC-5**-Rh conjugate at the λ_{max} of the dye (λ_{max} = 550nm)
   - CF = Correction Factor adjusts for the amount of absorbance at 280 nm caused by the dye (CF = 0.3400 for Rh dye) [7]

$$\textbf{Protein Concentration in M} = A_{280} - (A_{max} \times CF) / \varepsilon$$

2. Calculate Rh Dye:Protein Ratio in BSA-**NTC-5**-Rh Conjugate

Moles of dye per mole of protein = A_{max} of protein-dye conjugate / (ε_{Rh} x protein

concentration in M).

	BSA-NTC-5-Rh Conjugate
A_{280nm}	1.468
ε_{BSA}	43,824 $M^{-1}cm^{-1}$
BSA Protein Concentration	26.88 µM
A_{max}	0.856
ε_{Rh}	65,000 $M^{-1}cm^{-1}$
Rh Dye Concentration	13.0 µM
Rh Dye:Protein Ratio	0.48
% of NTC-5 labelled BSA	**~ 48%**

Figure S2. (A) BSA (control) and **NTC-5** modified BSA characterized by ESI-TOF MS. Spectra provided in Section 7.3. **(B)** Determination of Rh dye:protein ratio in BSA-NTC-5-Rh conjugate.[7]

5.3 Fluorescent Gel Characterisation

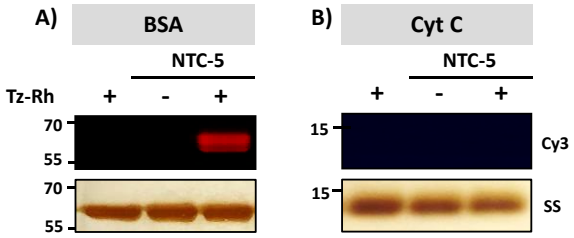


Figure S3. Labeling of **(A)** BSA and **(B)** *N*-terminal acetylated Cytochrome C (Cyt C) with **NTC-5** (200 equiv., 16 h, 37 °C) was verified with Tz-Rh click, resolved on a 15% SDS-PAGE followed by in-gel fluorescence (Cy3) scanning as well as silver staining (SS).

5.4 Cell Lysate Analysis

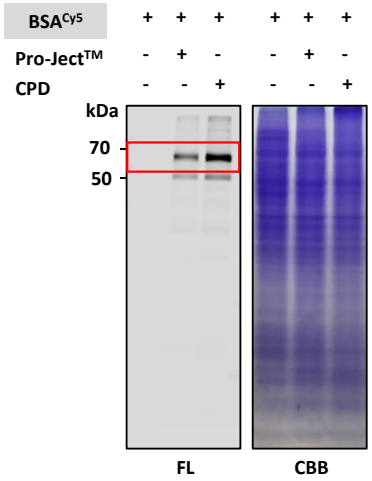


Figure S4. SDS-PAGE/In-gel fluorescence scanning of lysates from HeLa cells treated with CPD-conjugated BSA^{Cy5} (100 nM, 2 h incubation) showing successful cellular uptake. Cells treated with BSA^{Cy5} only (100 nM; 2 h incubation) and Pro-Ject™ assisted BSA^{Cy5} delivery were run concurrently as controls. BSA^{Cy5} used in above experiments were **NTC-5** modified (unless otherwise stated), but for simplicity, they were abbreviated as BSA^{Cy5}.

Note: In the **NTC-5** modified BSA^{Cy5} protein-CPD conjugate samples (used for delivery experiments), unmodified BSA^{Cy5} protein is present due to the intrinsic labeling efficiency of Francis's approach (~50%).[2] Since the unmodified BSA^{Cy5} will not be conjugated to CPD, they cannot be delivered into cells, hence they were not removed from the sample. This was confirmed by our results in Figure 2 and Figure 3 (i.e. unlabeled protein did not affect our overall results and conclusions).

5.5 NTC-5 Modification of Various Proteins

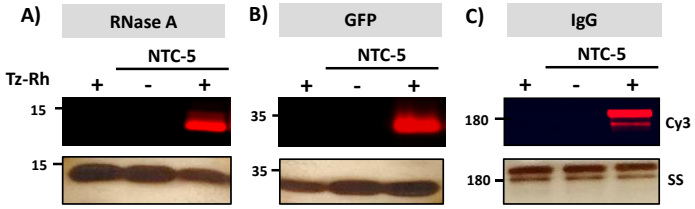


Figure S5. Labeling of **(A)** RNase A **(B)** GFP and **(C)** IgG with **NTC-5** (200 equiv., 16 h, 37 °C) was verified with Tz-Rh click, resolved on a 10% or 15% SDS-PAGE followed by in-gel fluorescence (Cy3) scanning as well as silver staining (SS).

5.6 CPD vs Pro-Ject Intracellular Protein Delivery

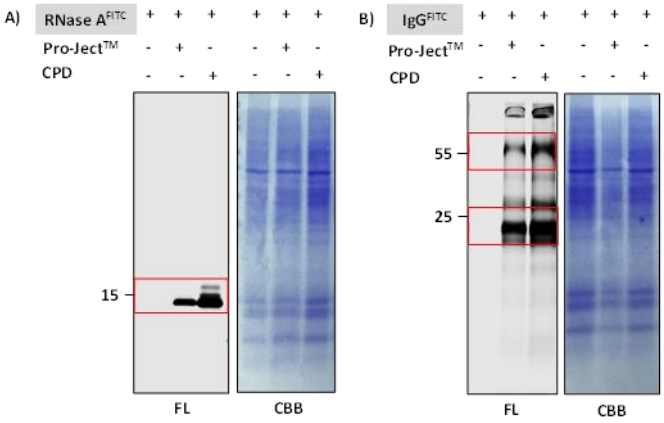


Figure S6. SDS-PAGE/In-gel fluorescence scanning and Coomassie Brilliant Blue (CBB) stained-gel of HeLa cell lysates incubated with **(A)** RNase A$^{\mathbf{FITC}}$ and **(B)** IgG$^{\mathbf{FITC}}$ protein-CPD conjugates as well as Pro-Ject^{TM} assisted intracellular protein delivery. During the treatment of cell lysate, DTT added in loading dye results in the reduction of IgG (150 kDa) to form heavy chain (50 kDa) and light chain (25 kDa) bands. Only the heavy chain 50 kDa band was represented in Figure 3B. Part of the fluorescence gels from **(A)** and **(B)** were reproduced in Figure 3B. All proteins used in above experiments were **NTC-5** modified, but for simplicity, they were abbreviated by their protein names, i.e. RNase A^{FITC} and IgG^{FITC}.

Note: In the **NTC-5** modified RNase A^{FITC} and IgG^{FITC} protein-CPD conjugate samples (used for delivery experiments), unmodified protein is present due to the intrinsic labeling efficiency of Francis's approach (~50%).[2] Since the unmodified proteins will not be conjugated to CPD, they cannot be delivered into cells, hence they were not removed from the sample. This was confirmed by our results in Figure 2 and Figure 3 (i.e. unlabeled protein did not affect our overall results and conclusions).

5.7 Standard Immunofluorescence Using Anti-NPC Antibody

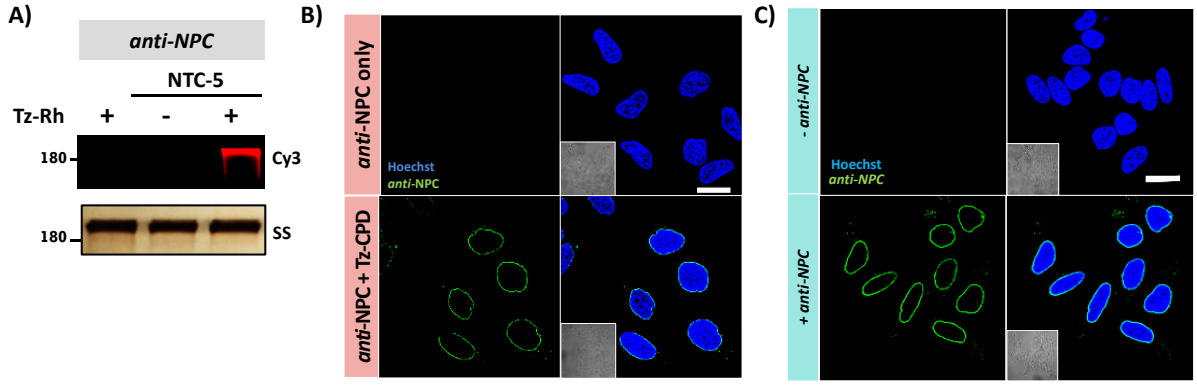


Figure S7. (A) Labeling of *anti*-NPC antibody with **NTC-5** (200 equiv., 16 h, 37 °C) was verified with Tz-Rh click, resolved on a 10% SDS-PAGE followed by in-gel fluorescence scanning (Cy3) as well as silver staining (SS). **(B)** CLSM images of live HeLa cells treated with *anti*-NPC antibody-CPD conjugate (500 nM, 12 h) prior to fixation and incubation with FITC-conjugated goat anti-mouse IgG (H+L) secondary antibody. Cells incubated with *anti*-NPC only (no CPD) were carried out as controls. Scale bar = 20 µm. **(C)** CLSM images showing immunofluorescence (IF) staining of fixed HeLa cells with *anti*-NPC antibody (lower panels) and negative controls (upper panels; no *anti-NPC*). Scale bar =

20 μm.

5.8 Fluorometric Alizarin Red S Assay

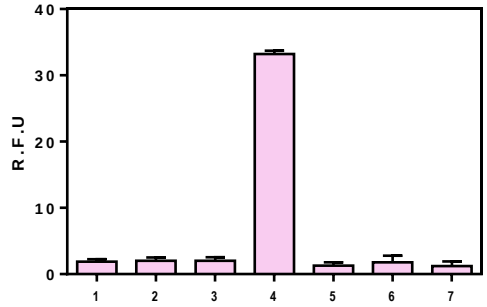


Figure S8. Fluorescence of Alizarin Red S dye (ARS, 0.0025% w/v) in the presence of 1) PBS, 2) native RNase A, 3) RNase A, 4) RNase A-LBL, 5) RNase A-LBL + 5 mM H_2O_2, 6) RNase A-NBL, 7) RNase A-NBL + 5 mM H_2O_2. Note: All RNase A used in above experiments was **NTC-5** modified RNase A (unless otherwise stated), but for simplicity, they were abbreviated as RNase A.

5.9 Uptake Efficiency of RNase A^{FITC} protein-CPD conjugate in Different Cell Lines

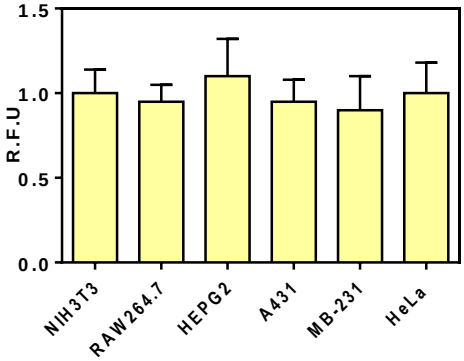


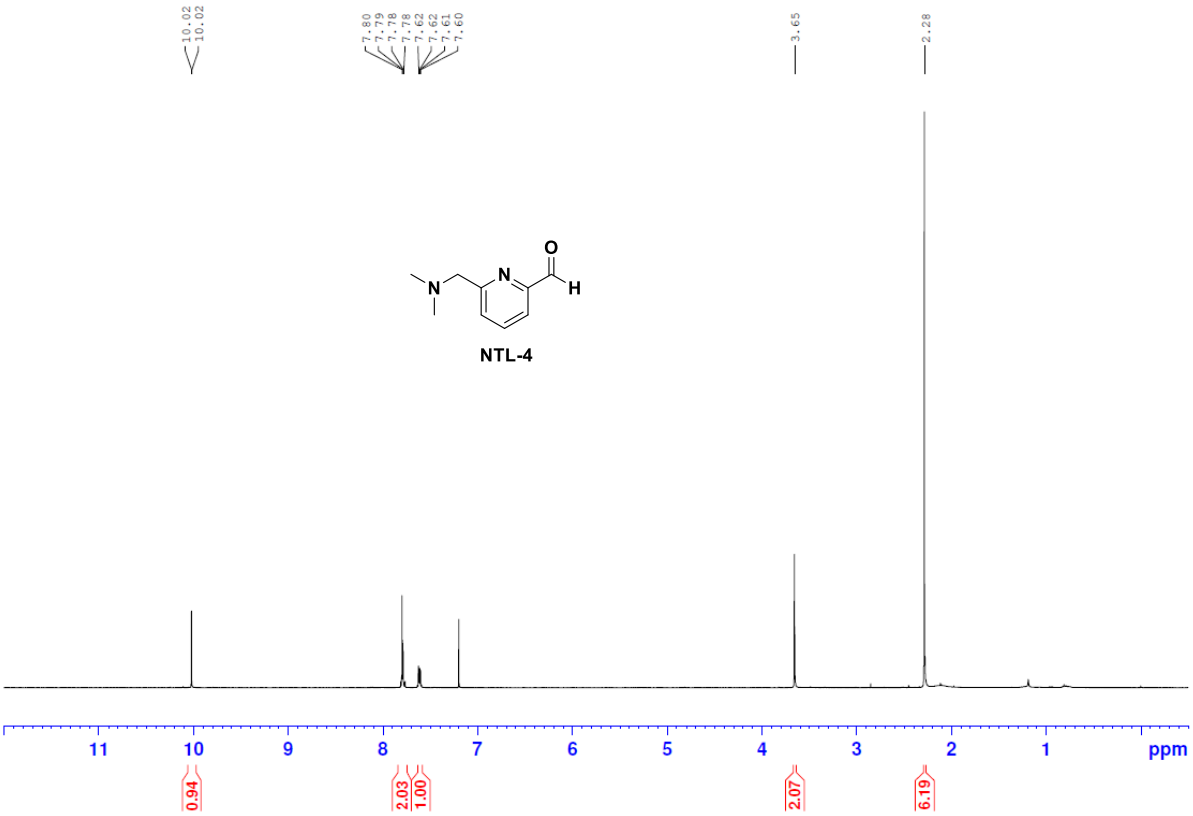
Figure S9. Quantitative analysis of RNase A^{FITC} protein-CPD conjugate uptake efficiency in various cell lines (100 nM, 2h) by flow cytometry. The RFU of each sample was normalized against HeLa cells (set as 1). Error bars were obtained from triplicates.

6. References

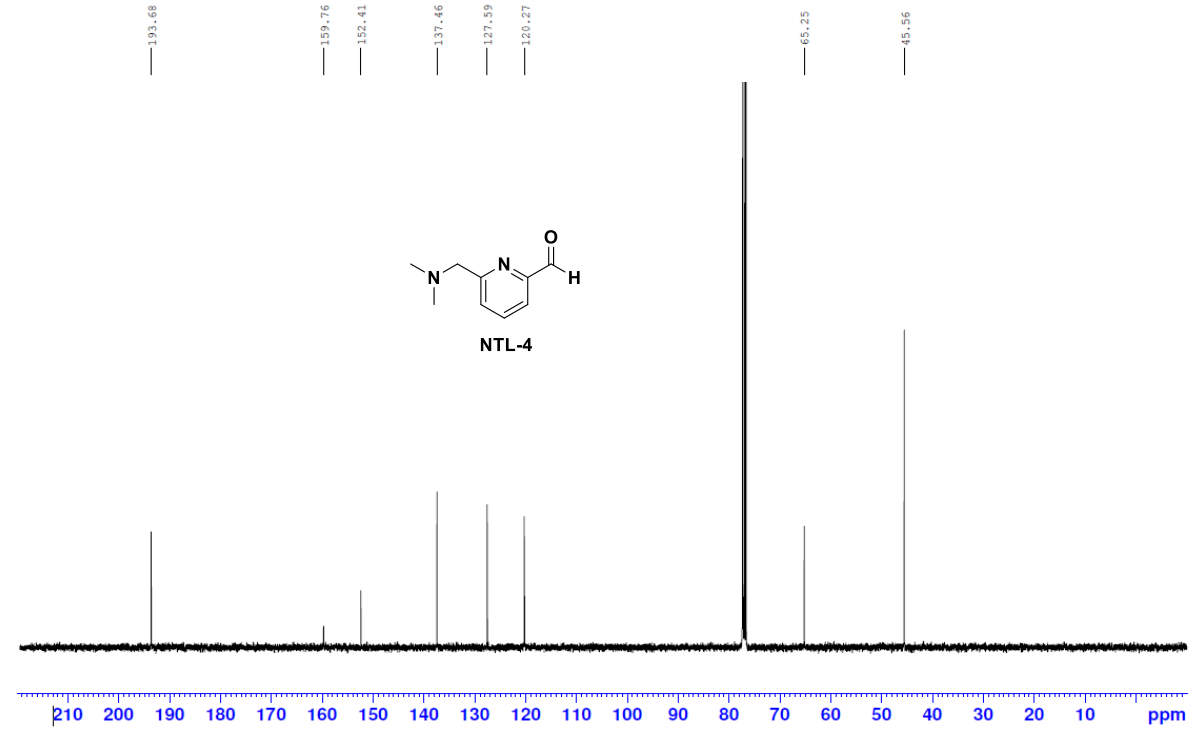
1. J. L. Major Jourden and S. M. Cohen, *Angew. Chem. Int. Ed.*, 2010, **49**, 6795-6797.
2. J. I. MacDonald, H. K. Munch, T. Moore and M. B. Francis, *Nat. Chem. Biol.*, 2015, **11**, 326-331.
3. C. Pieri, D. Borselli, C. Di Giorgio, M. De Meo, J. M. Bolla, N. Vidal, S. Combes and J. M. Brunel, *J. Med. Chem.*, 2014, **57**, 4263-4272.
4. S. Pan, S. Y. Jang, D. Wang, S. S. Liew, Z. Li, J. S. Lee and S. Q. Yao, *Angew. Chem. Int. Ed.*, 2017, **56**, 11816-11821.
5. J. Fu, C. Yu, L. Li and S. Q. Yao, *J. Am. Chem. Soc.*, 2015, **137**, 12153-12160.
6. ThermoFisher Home Page, http://www.thermofisher.com/order/catalog/product/89850, (accessed Mar 3, 2020).
7. ThermoFisher Home Page, https://assets.thermofisher.com/TFS-Assets/LSG/brochures/TR0031-Calc-FP-ratios.pdf, (accessed Aug 15, 2020).

7. Characterisation

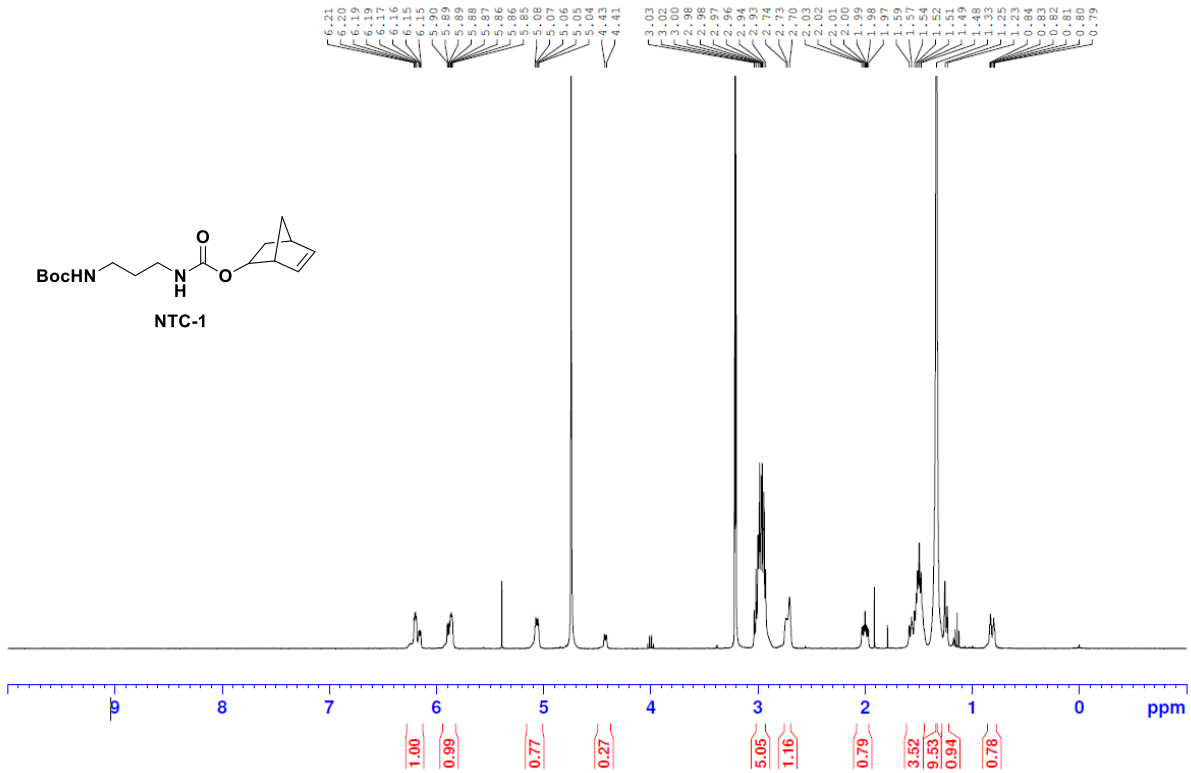
7.1 ^{1}H and ^{13}C NMR



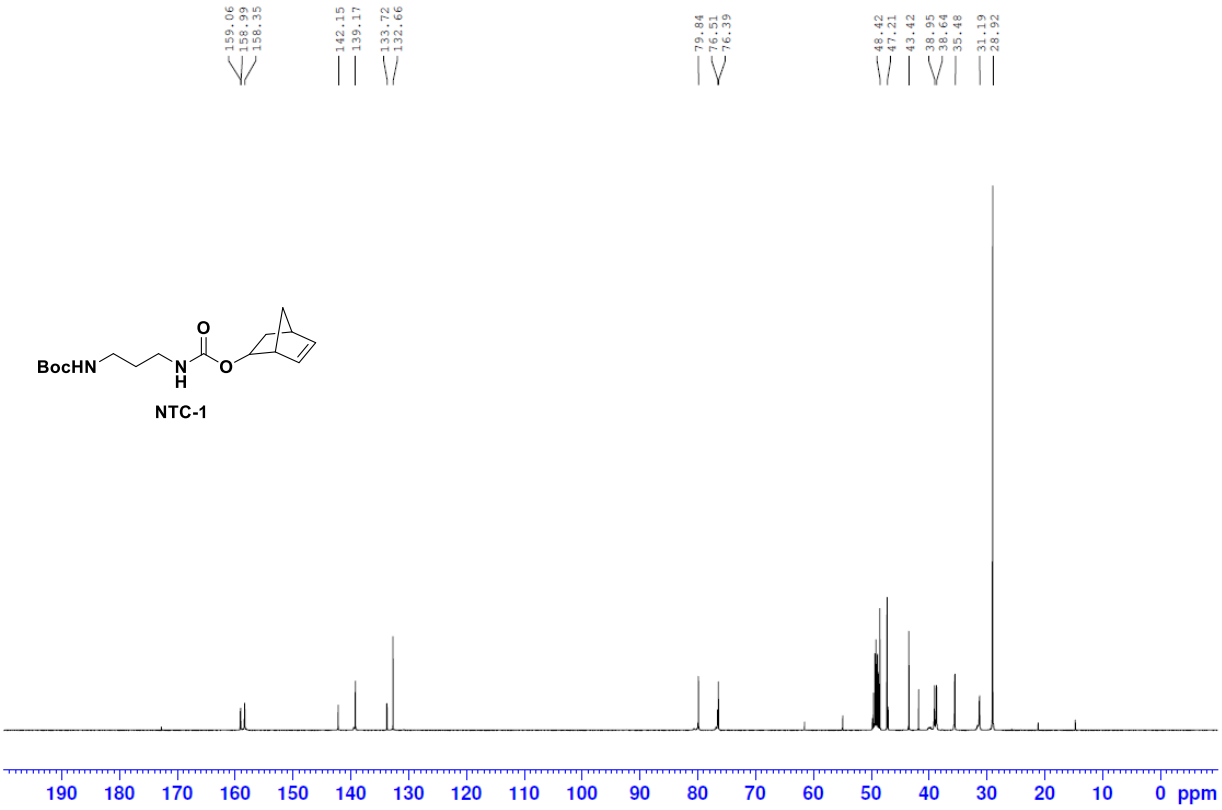
^{1}H NMR spectrum of **NTL-4** in Chloroform-*d*.



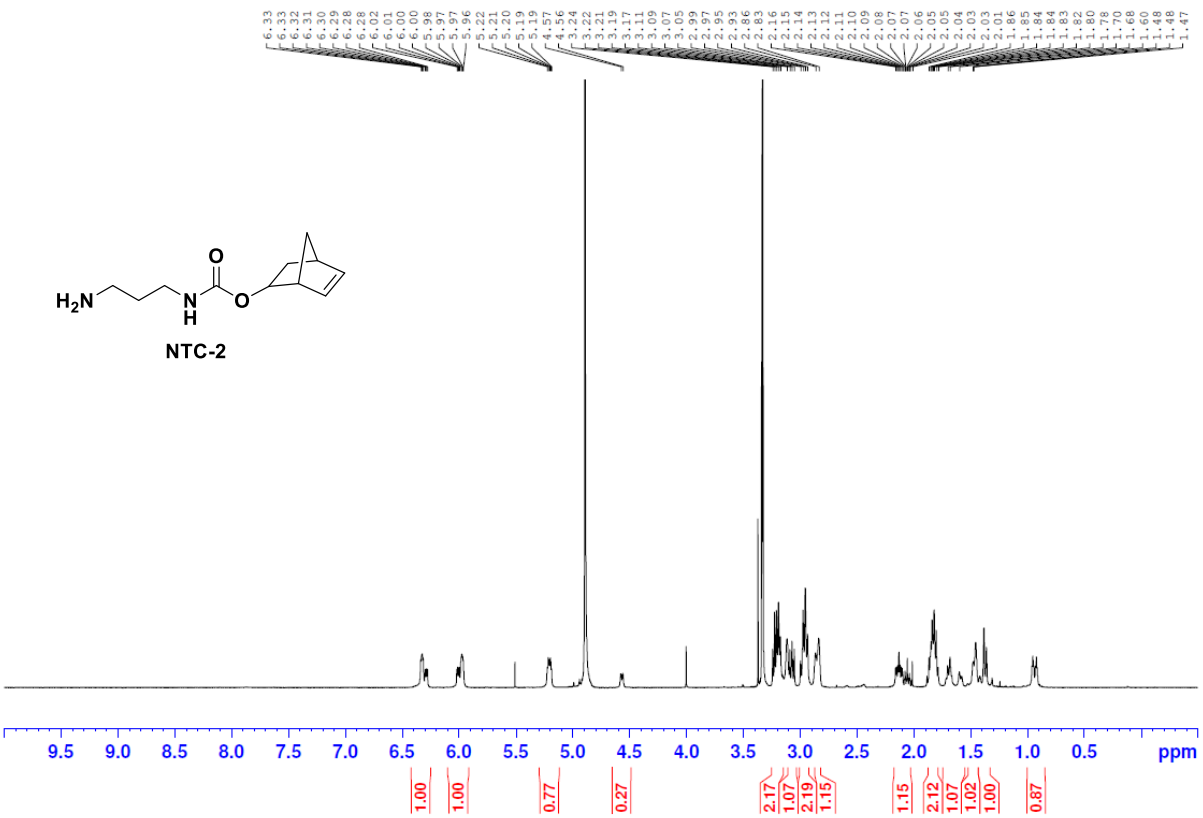
^{13}C NMR spectrum of **NTL-4** in Chloroform-*d*



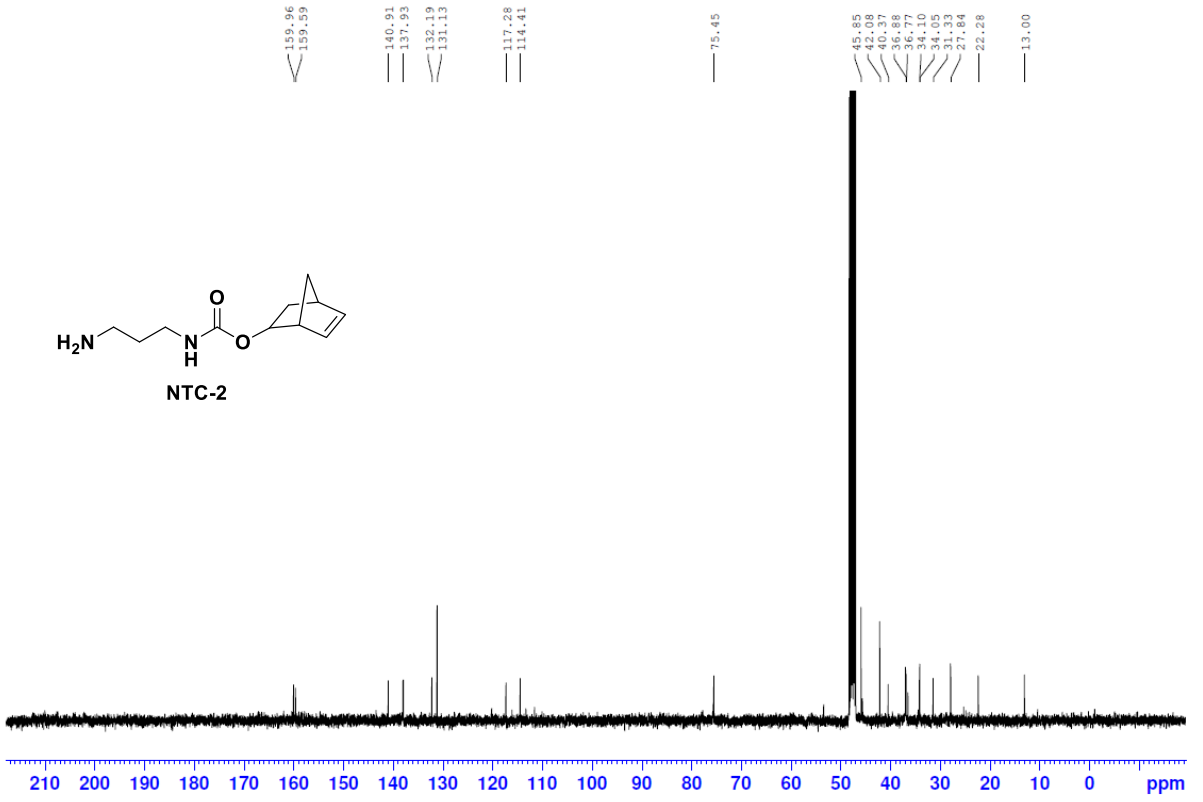
^{1}H NMR spectrum of **NTC-1** in Methanol-d_4.



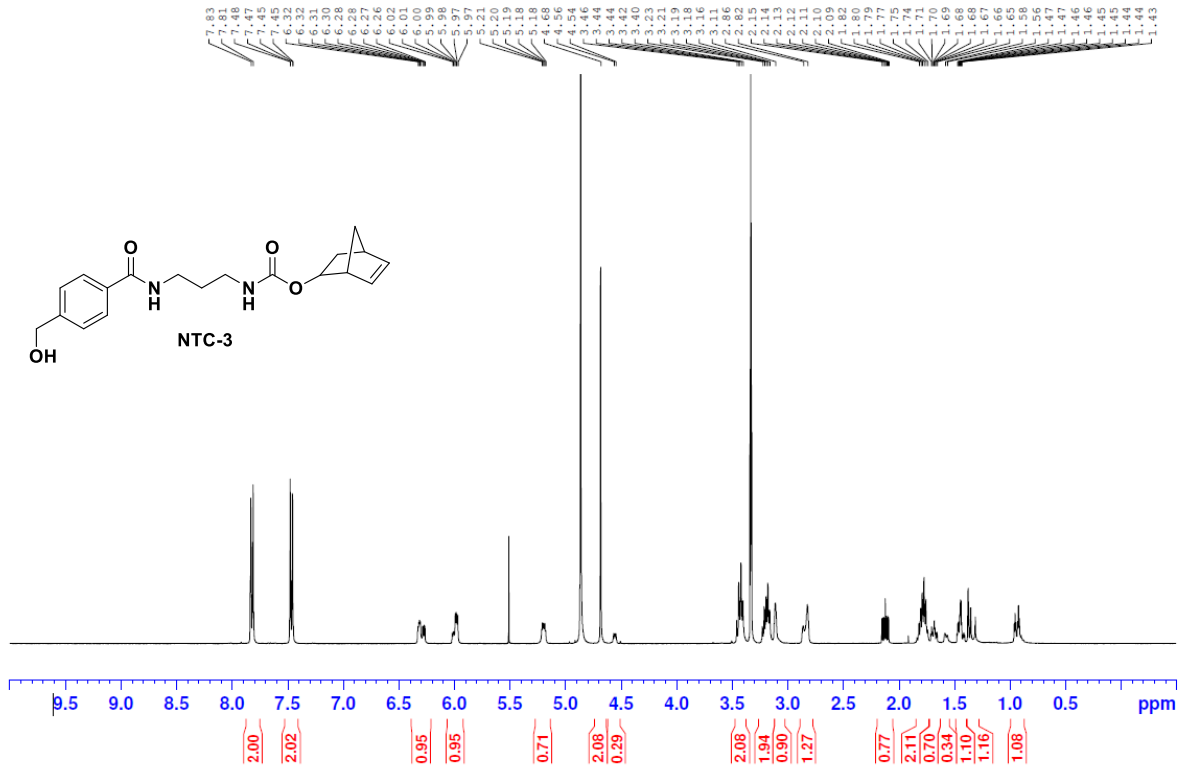
^{13}C NMR spectrum of **NTC-1** in Methanol-d_4.



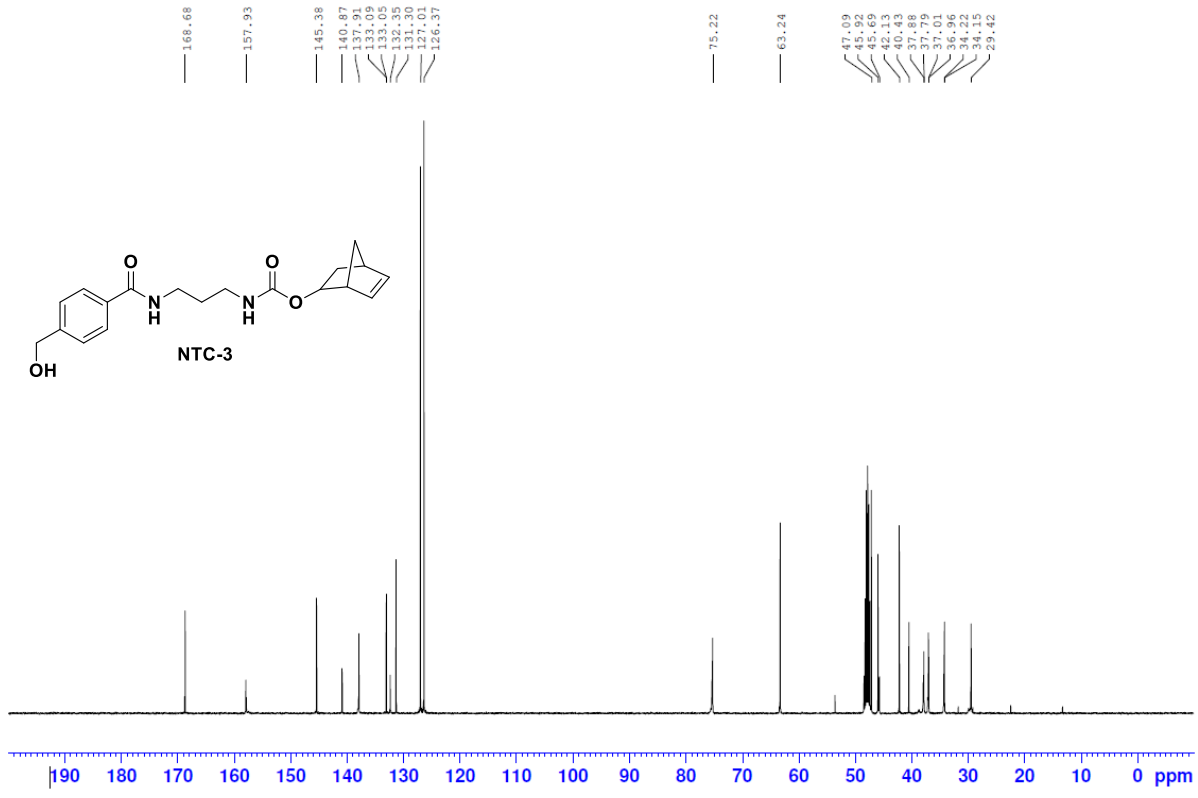
^{1}H NMR spectrum of **NTC-2** in Methanol-d_4.



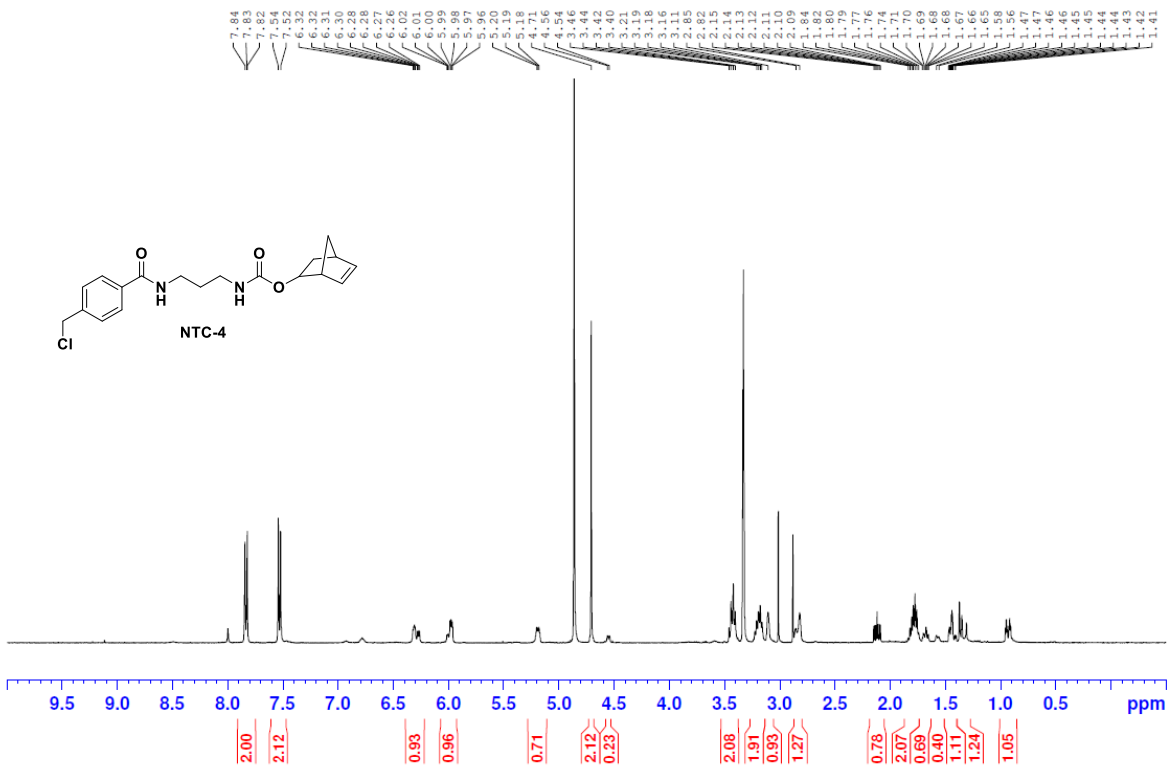
^{13}C NMR spectrum of **NTC-2** in Methanol-d_4.



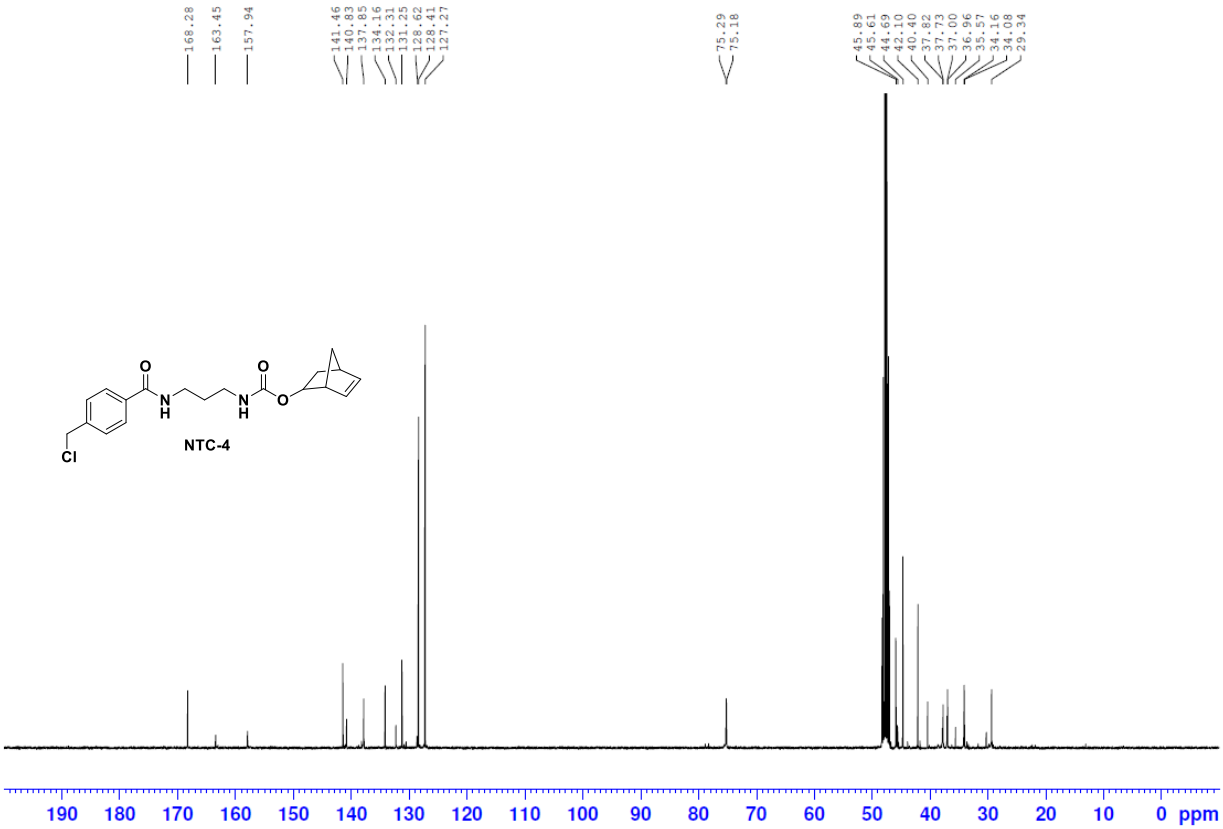
^{1}H NMR spectrum of **NTC-3** in Methanol-d_4.



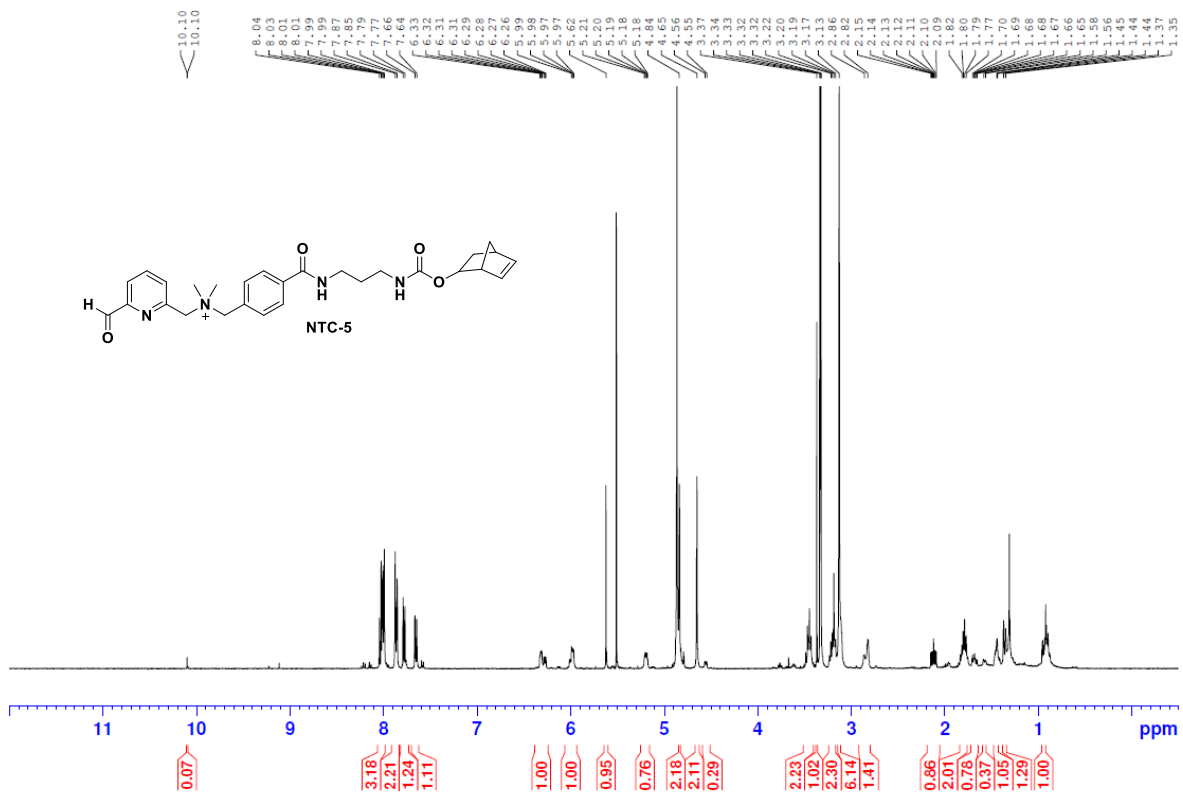
^{13}C NMR spectrum of **NTC-3** in Methanol-d_4.



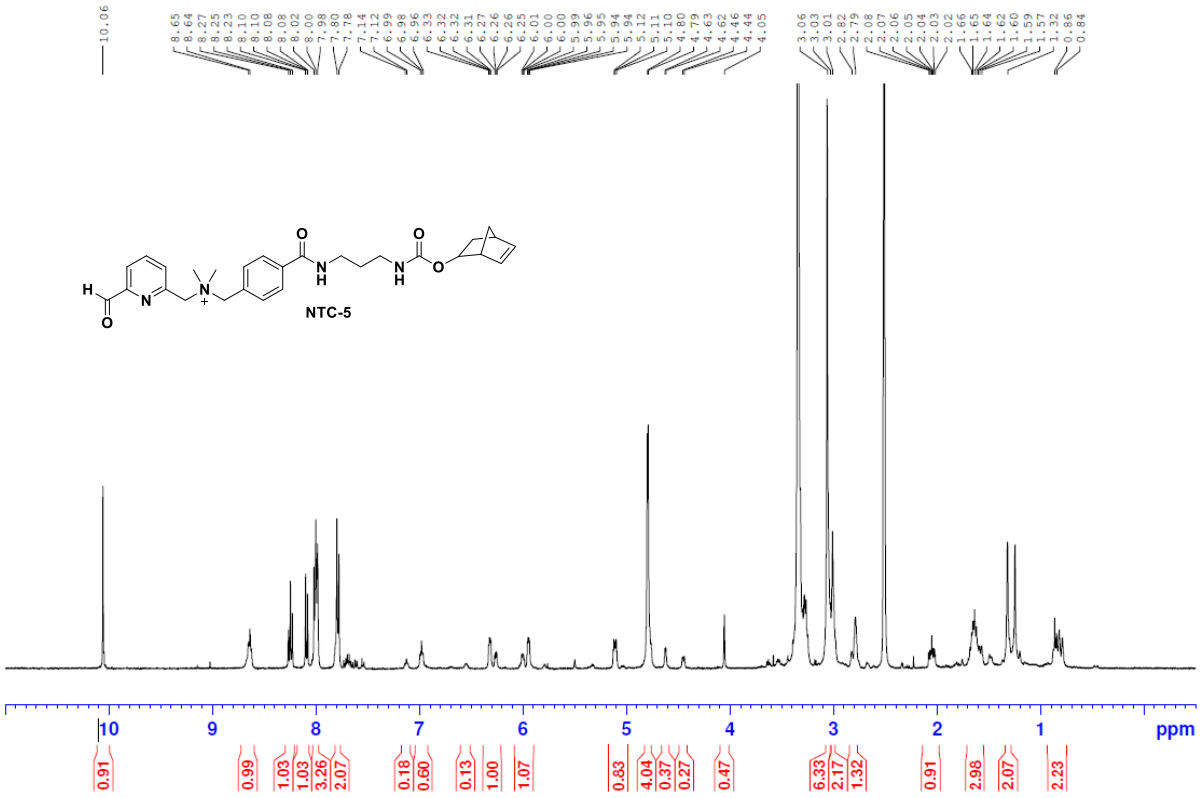
^{1}H NMR spectrum of **NTC-4** in Chloroform-*d*.



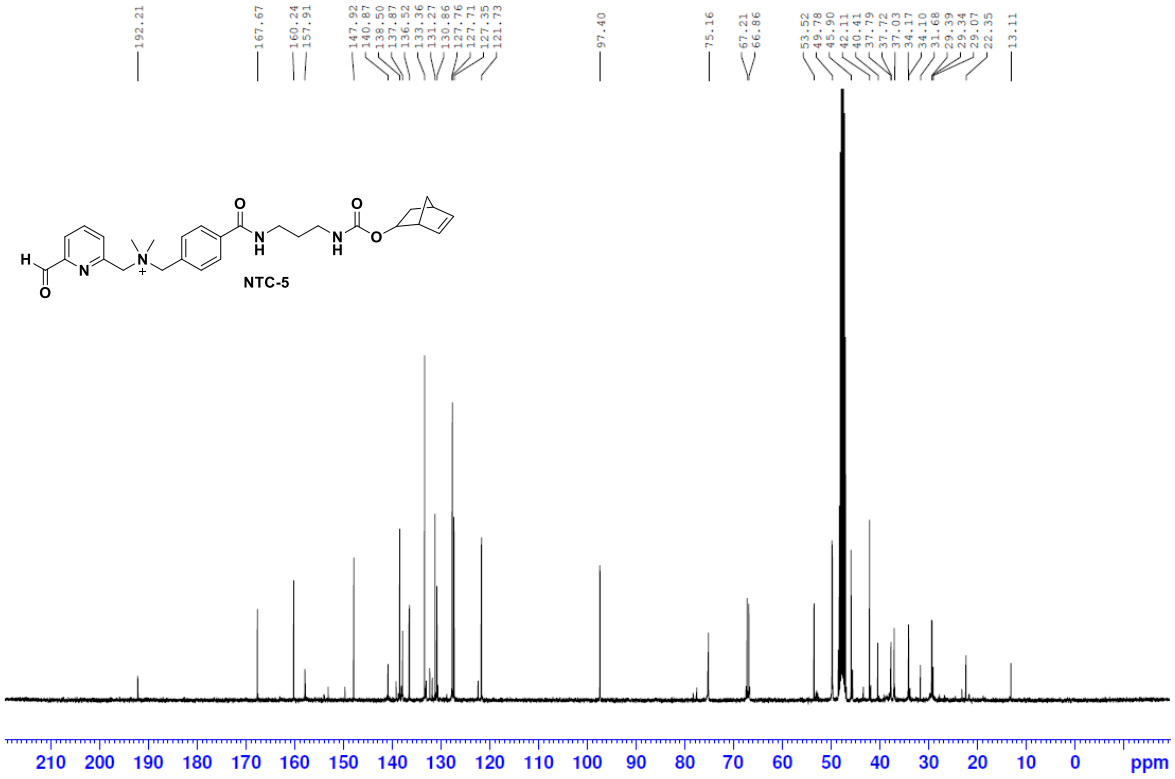
^{13}C NMR spectrum of **NTC-4** in Chloroform-*d*.



^{1}H NMR spectrum of **NTC-5** in Methanol-d_4.



^{1}H NMR spectrum of **NTC-5** in DMSO-d_6.



^{13}C NMR spectrum of **NTC-5** in Methanol-d_4.

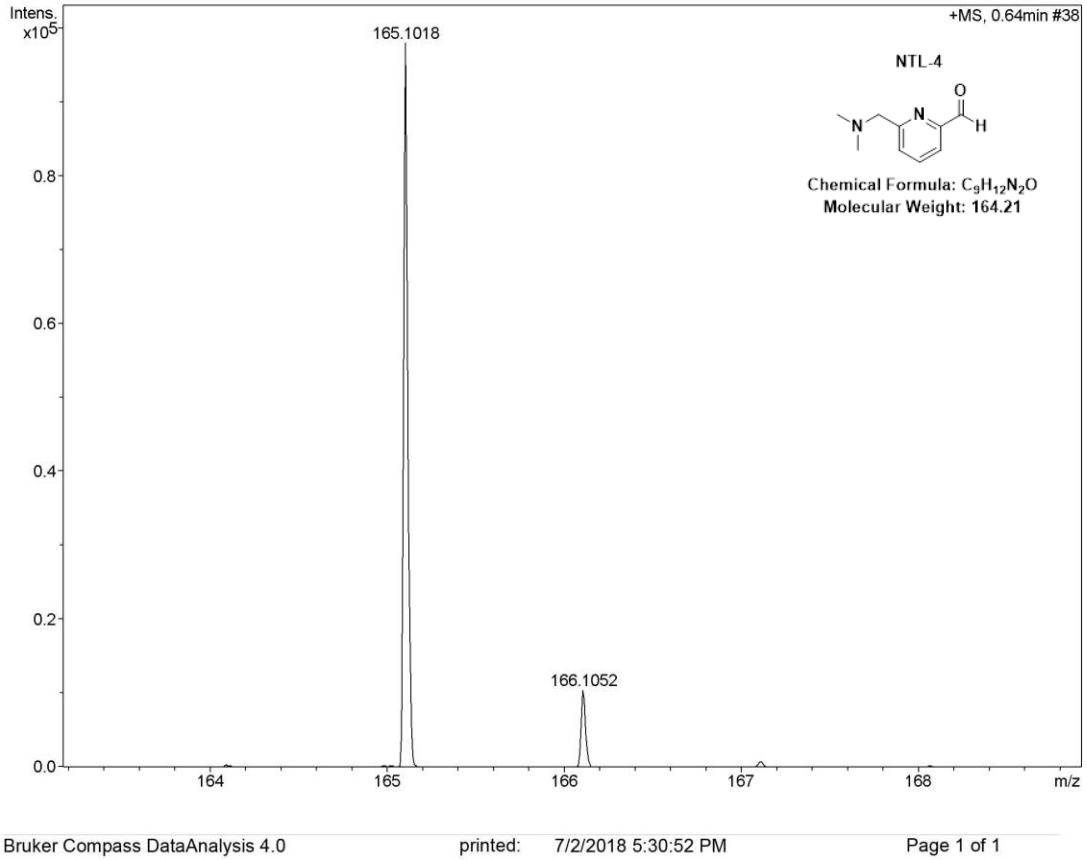
7.2 High Resolution Mass Spectrometry (HR-MS) Analysis

ESI-HRMS: m/z $[M+H]^+$ calcd, 165.1028; found, 165.1018.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	2.0 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	6.0 l/min
Scan End	800 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Formula	m/z	err [ppm]	rdb	e^- Conf	N-Rule
165.1018	1	C 9 H 13 N 2 O	165.1022	2.7	4.5	even	ok



Bruker Compass DataAnalysis 4.0 printed: 7/2/2018 5:30:52 PM Page 1 of 1

ESI-HRMS: m/z $[M+H]^+$ calcd, 210.1368; found, 211.1437.

Acquisition Parameter

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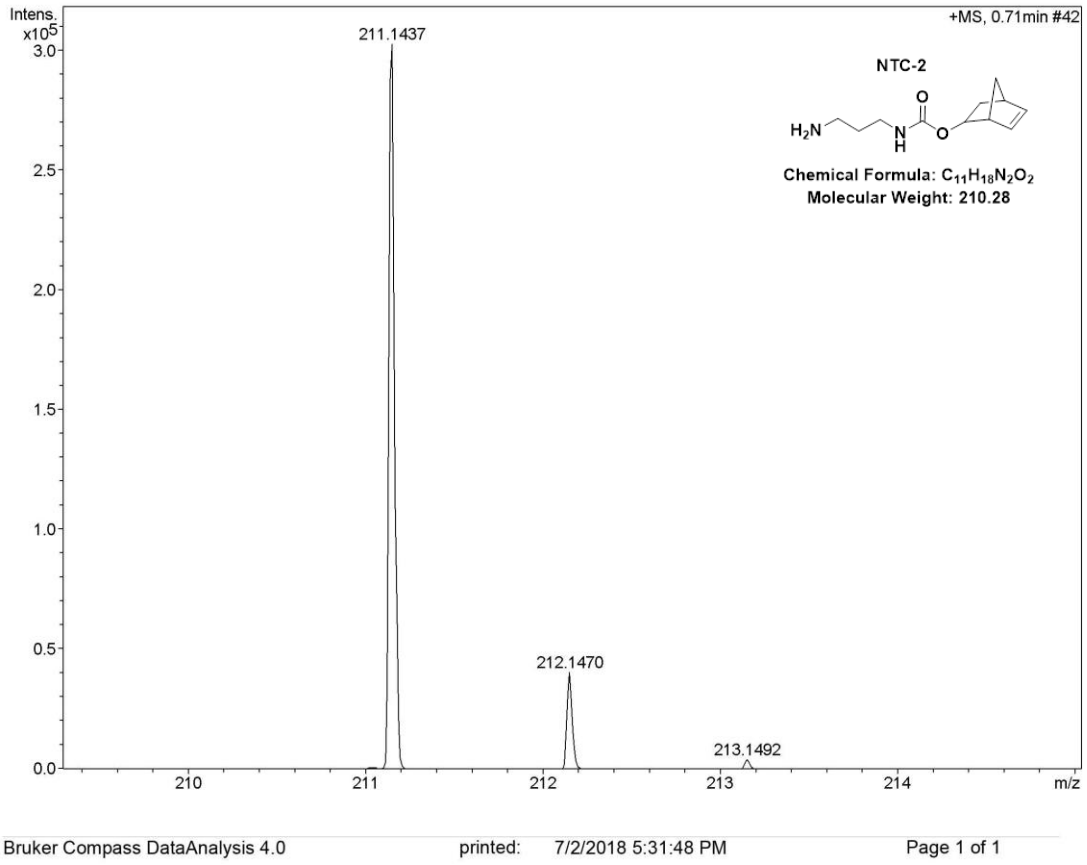
Scan End	800 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Formula	m/z	err [ppm]	rdb	e⁻ Conf	N-Rule
211.1437	1	C 11 H 19 N 2 O 2	211.1441	1.7	3.5	even	ok



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ESI-HRMS: m/z $[M+Na]^+$ calcd, 367.1634; found, 367.1632.

Acquisition Parameter

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Scan End	800 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Formula	m/z	err [ppm]	rdb	e⁻ Conf	N-Rule
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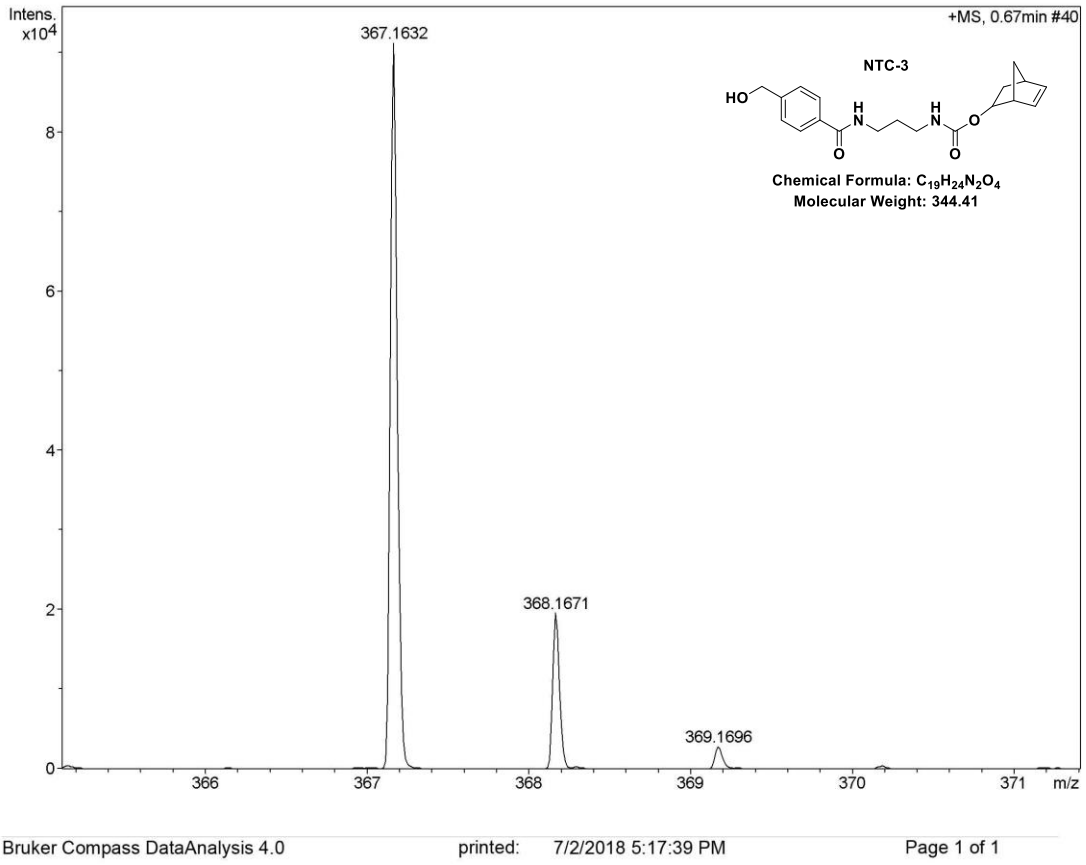

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ESI-HRMS: m/z $[M+Na]^+$ calcd, 385.1295; found, 385.1291.

Acquisition Parameter

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Scan End	800 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Formula	m/z	err [ppm]	rdb	e⁻ Conf	N-Rule
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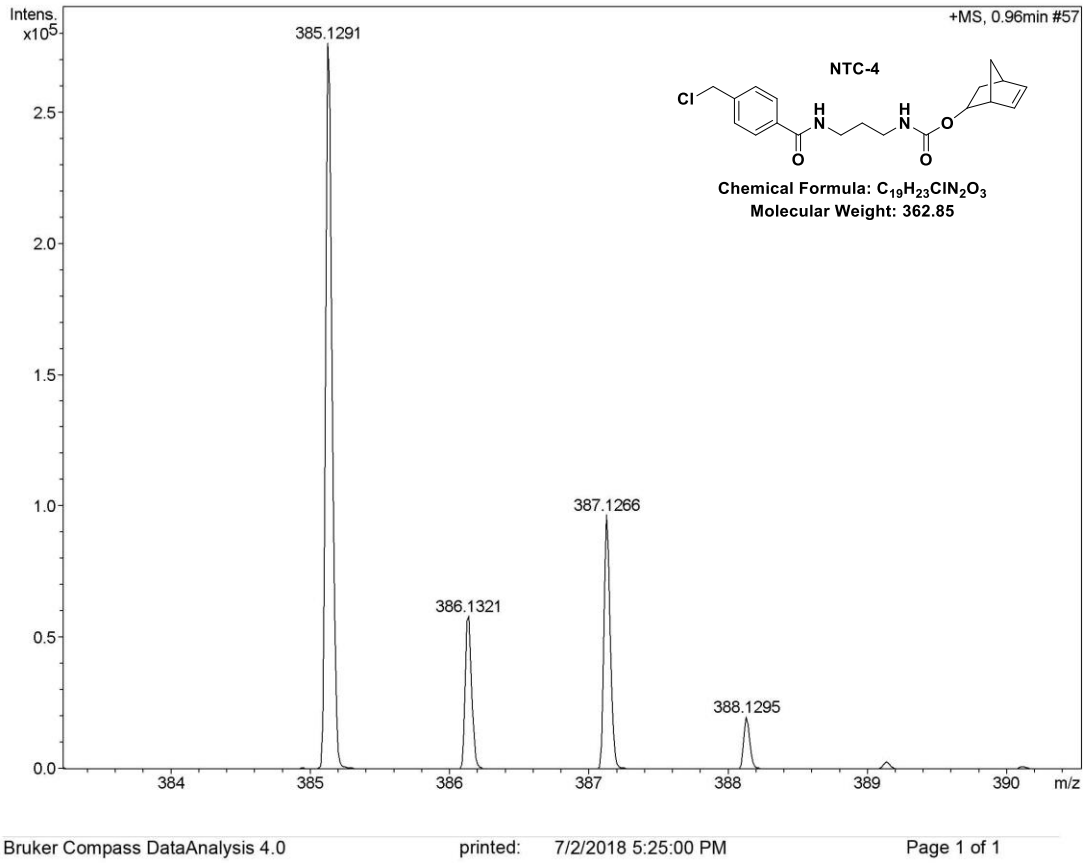

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ESI-HRMS: m/z $[M]^+$ calcd, 491.2653; found, 491.2662.

Acquisition Parameter

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Scan End	800 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Formula	m/z	err [ppm]	rdb	e⁻ Conf	N-Rule
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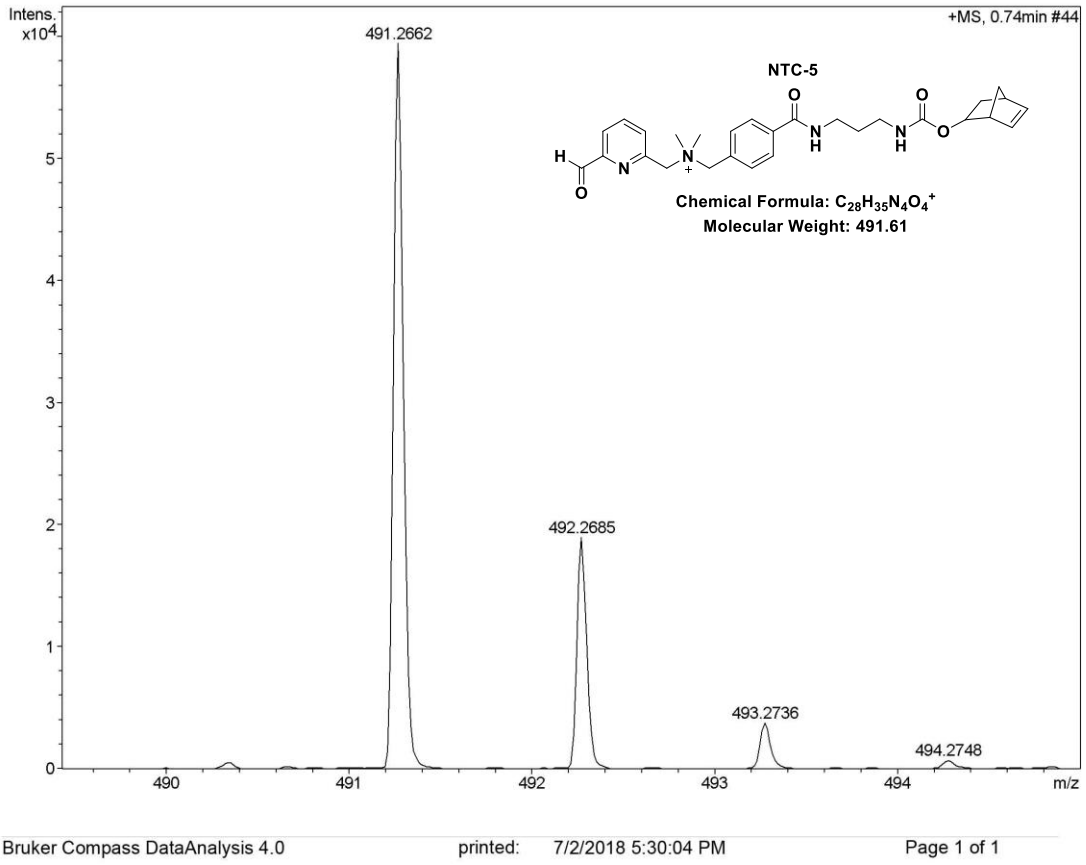

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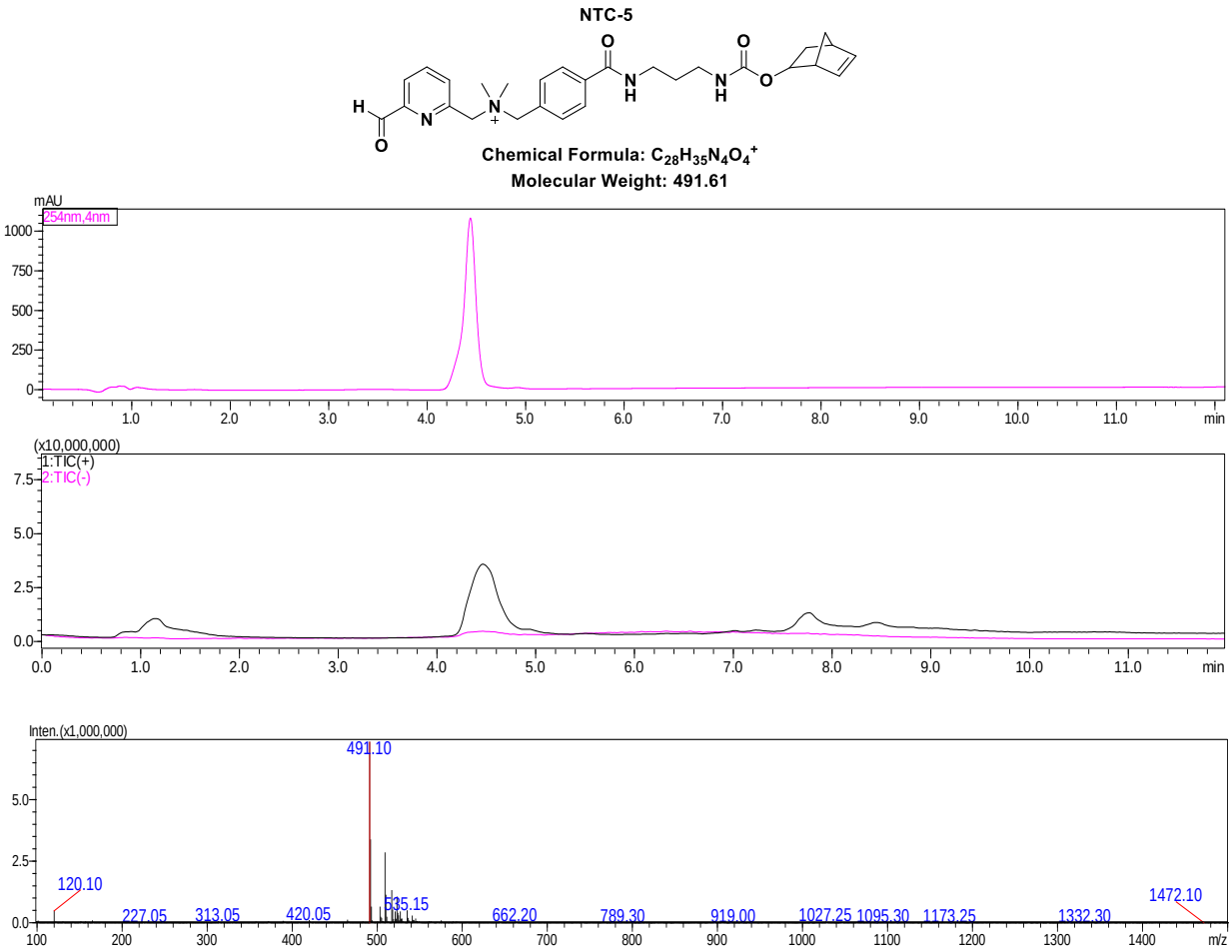
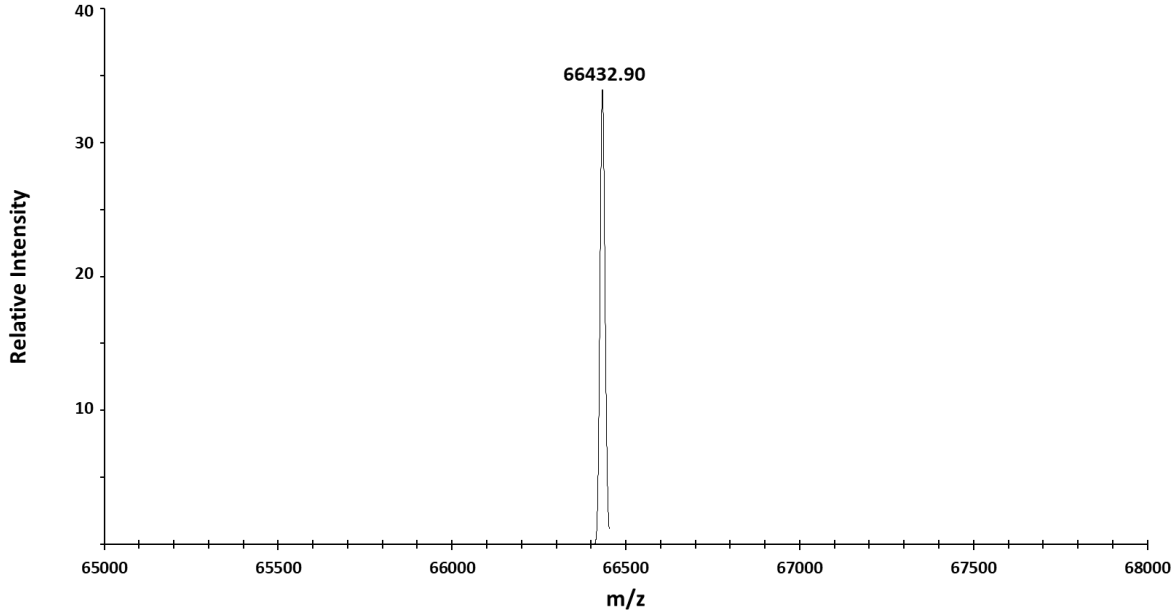
LCMS of NTC-5 (Purity Check)

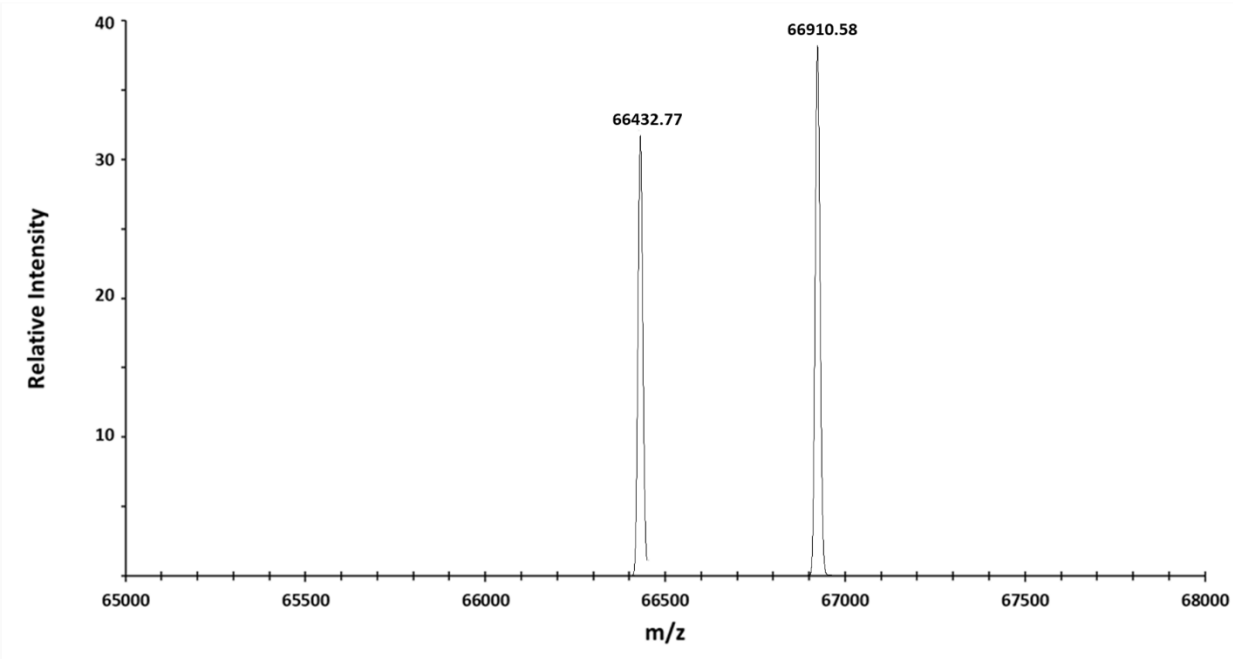


7.3 Protein ESI Mass Spectrometry

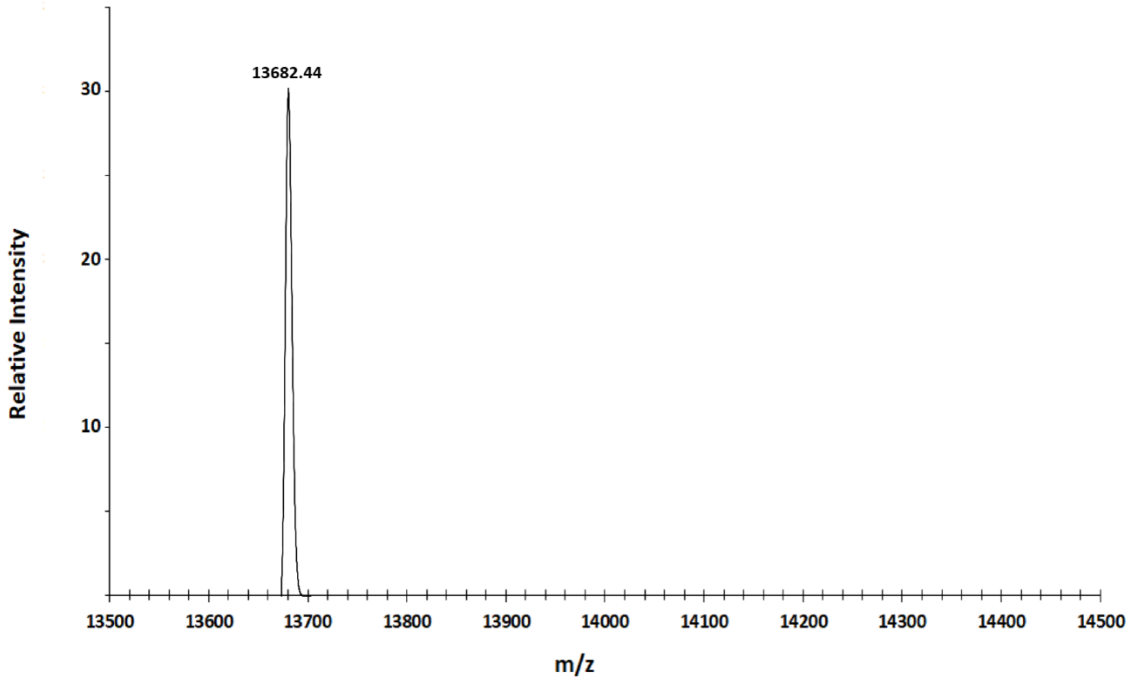
A) ESI mass spectrum for BSA (control)



ESI mass spectrum for NTC-5 labeled BSA



B) ESI mass spectrum for RNase A (control)



ESI mass spectrum for NTC-5 labeled RNase A