Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

A bifunctional imidazolium-based cholesterol analog for the tracking of cellular cholesterol distributions and cholesterol-protein interactions

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1.1 General Information

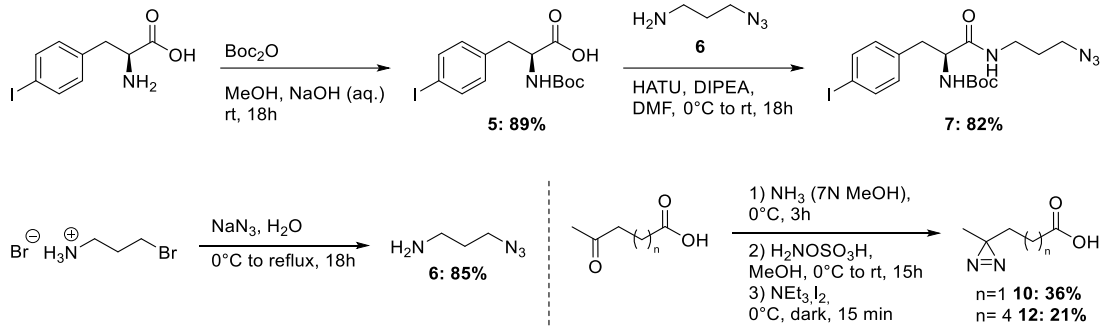
All reactions were performed in oven-dried glassware under argon. Reaction temperatures are referred to the ones of the heating/cooling media (heating block, cryogenic bath). Reagents were purchased from Sigma-Aldrich, Merck, ACROS Organics, Alfa Aesar, Fluorochem, Combiblocks, TCI and used without further purification. Reactions were stirred using PTFE-coated magnetic stirring bars at ~ 1000 rpm. Low boiling solvents (<110 °C) were removed by rotary evaporation under reduced pressure, heating the solution with a water bath at 50 °C. High boiling solvents (>110 °C) were removed in vacuo (<1 mbar) at room temperature or under mild heating (<60 °C). Identity and purity of tested compounds was verified by means of ^{1}H-NMR, ^{13}C-NMR and HR-ESI-MS. Literature known compounds were characterized by ^{1}H NMR and compared with the available analytical data to confirm their identity.

Thin layer chromatography (TLC) was performed using Merck silica gel 60 F254 aluminium plates. TLC plates were visualized by exposure to UV light (254 nm) and/or staining with basic $KMnO_4$ solution (4 g of $KMnO_4$, 10 g K_2CO_3, 1 g NaOH in 200 ml of distilled water) or CAM (Cerium Ammonium Molybdate) solution (100 g $(NH_4)_2MoO_4$, 4 g $Ce(SO_4)_2$, 100 mL conc. H_2SO_4 in 900 mL of distilled water). GC samples were filtered over a short plug of silica (length: 2-3 cm, diameter: 4 mm) eluting with EtOAc before analysis, if not stated otherwise. GC-MS spectra were recorded on an Agilent Technologies 7890A GC-system (Agilent 5975C VL MSD or an Agilent 5975 MSD) or Agilent Intuvo 9000 (Agilent 5977B MSD), and a HP-5MS column (0.25 mm · 30 m, film: 0.25 µm) and analyzed using MSD ChemStation E.02.02.1431. ^{1}H- and ^{13}C-NMR spectra were recorded at room temperature on a Bruker Avance 400 (^{1}H: 400.13 MHz; ^{13}C: 100.62 MHz), Avance Neo 400 (^{1}H: 400.23 MHz; ^{13}C: 100.65 MHz), Varian 500 MHz INOVA (^{1}H: 499.83 MHz; ^{13}C: 125.70 MHz) or Varian Unity plus 600 (^{1}H: 599.31 MHz; ^{13}C: 150.71 MHz) in deuterated solvent (> 99.5 Deuteration) purchased from Sigma-Aldrich ($CDCl_3$). Chemical shifts (δ) for ^{1}H- and ^{13}C-NMR spectra are given in parts per million (ppm) relative to TMS using the residual solvent signals as references for ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$: δ_H= 7.26 ppm, δ_C= 77.16 ppm). NMR signals multiplicities that can be analyzed as first order multiplets are reported using the following abbreviations (or combination thereof): s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal. All spectra were processed using MestReNova 14 using standard phase and baseline correction automations. ESI accurate mass spectra were recorded on a TQ Orbitap LTQ XL (Thermo-Fisher Scientific, Bremen) with loop injection or an Orbitrap Velos Pro (Thermo-Fisher Scientific, Bremen) with nanospray injection.

Unless otherwise noted, compounds were purified by flash column chromatography using ACROS Organics silica (0.035-0.070 mm, 60 Å) and the specified solvent system under 0.3-0.5 bar argon overpressure. Pentane, dichloromethane and ethyl acetate used for chromatography were purchased of technical grade and preliminary purified by atmospheric pressure distillation. Unless otherwise stated, dry (H_2O content < 50 ppm) reaction solvents were used to perform reactions. The following solvents (ACROS ExtraDry solvents with ACROSeal® cap) were purchased from ACROS Organics, stored

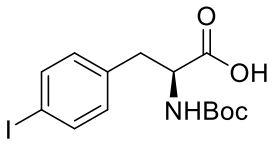
under 3 or 4 Å activated molecular sieves and collected under positive argon pressure: triethylamine (NEt_3), dimethylsulfoxide (DMSO), 1,4-dioxane. The following solvents were purchased from ACROS Organics, Sigma-Aldrich and Fischer Scientific (HPLC grade) and purified using a custom SPS with activated alumina columns (built by the "Feinmechanische Werkstatt des Organisch-Chemischen Instituts, WWU Münster") and collected under positive argon pressure: acetonitrile (MeCN), tetrahydrofuran (THF), diethyl ether (Et_2O), *N*,*N*-dimethylformamide (*N*,*N*-DMF), hexane, toluene, 1,2-dichloroethane (1,2-DCE) and dichloromethane (DCM).

1.2 Synthesis



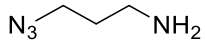
Supporting Scheme 1: Synthesis of the functional linker building blocks.

***N*-Boc-4-iodo-*L*-phenylalanine** (**5**)



The synthesis was performed according to a modified literature procedure.[1] 4-Iodo-*L*-phenylalanine (500 mg, 1.72 mmol, 1 eq.) was dissolved in methanol (1.3 mL) and NaOH (1 M; 2 mL). Boc_2O (0.39 g, 1.79 mmol, 1 eq.) was slowly added as a liquid and the mixture was stirred at room temperature overnight. Water was added to the mixture and the aqueous layer was extracted with pentane (2× 20 mL). The organic layer was extracted with saturated, aqueous $NaHCO_3$-solution. The combined aqueous fractions were acidified with $KHSO_4$-solution (1 M) to pH ≈ 2. The subsequently precipitated solid was dissolved with Et_2O and the aqueous layer was extracted with Et_2O (3×). The combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The resultant oil was triturated in *n*-pentane and the subsequently precipitating solid was collected by filtration, and dried in vacuum. The resulting white solid (355 mg, 0.91 mmol, 53%) was used for the next step without any further purification.

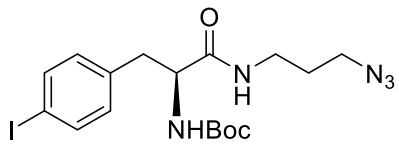
3-azidopropan-1-amine (6)



The synthesis was performed according to a literature procedure.[2] NaN_3 (3.3 g, 50 mmol, 3.3 eq.) was carefully added to a stirred solution of 3-Bromopropylamine hydrobromide (3.3 g, 15 mmol, 1 eq.) in dest. H_2O (10 mL) at 0 °C. After complete addition, the reaction mixture was stirred at 100 °C for 15 h. After cooling down to 0 °C Et_2O (10 mL) and KOH (~4g) were carefully added. The organic layer was separated and the aqueous phase was extracted with 3x 20 mL Et_2O. The combined organic layers were dried over $MgSO_4$. The solvent was removed under reduced pressure (≥800 mbar, volatile!) to yield compound **6** as a yellow oil (1.27 g, 12.7 mmol, 85%).

^{1}H-NMR (300 MHz, Chloroform-*d*): *δ* (ppm) = 3.29 (t, *J* = 6.7 Hz, 2H), 2.72 (t, *J* = 6.9 Hz, 2H), 1.65 (p, *J* = 6.7 Hz, 2H), 1.47 (s, 2H).

***tert*-Butyl (*S*)-(1-((3-azidopropyl)amino)-3-(4-iodophenyl)-1-oxopropan-2-yl)carbamate** (**7**)

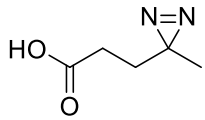


Compound **5** (100 mg, 0.26 mmol, 1 eq.), 3-azido-1-propanamine (25.6 mg, 0.256 mmol, 1 eq.) and DIPEA (223 µL, 1.28 mmol, 5 eq.) were dissolved in dry DMF (0.3 M; 1 mL). HATU (146 mg, 0.38 mmol, 1.5 eq.) was added to the mixture at 0 °C under stirring. The mixture was brought back to room temperature and stirred overnight. Water was added and the mixture was extracted with CH_2Cl_2 (3×). The combined organic fractions were washed with saturated, aqueous NaCl-solution, dried over $MgSO_4$, filtered and concentrated. Purification by column chromatography (*n*-pentane/ethylacetate = 10/0 → 7/3) afforded the product as a white solid (99.6 mg, 0.21 mmol, 82%).

HRMS (ESI)**:** *m/z* calculated $[C_{17}H_{24}IN_5O_3Na]^+$: 496.08160, found: 496.08086.

^{1}H-NMR (400 MHz, Chloroform-*d*): *δ* (ppm) = 7.64 – 7.58 (m, 2H), 6.97 – 6.92 (m, 2H), 6.16 (t, *J* = 6.1 Hz, 1H), 5.06 (d, *J* = 8.0 Hz, 1H), 4.25 (q, *J* = 7.5 Hz, 1H), 3.25 (dq, *J* = 12.4, 6.5 Hz, 4H), 2.98 (d, *J* = 7.1 Hz, 2H), 1.66 (td, *J* = 6.6, 2.0 Hz, 2H), 1.40 (s, 9H).
^{13}C{^{1}H}-NMR (400 MHz, Chloroform-*d*): *δ* (ppm) = 171.2, 155.6, 137.8 (2C), 136.5, 131.5 (2C), 92.5, 80.6, 55.9, 49.2, 38.1, 37.1, 28.7, 28.4 (3C).

4,4-Azo-pentanoic acid (**10**)

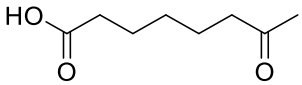


The synthesis was performed according to a literature procedure.[3] Ammonia (7 M in MeOH; 4.3 mL) was added to a flask with levulinic acid (500 mg, 4.3 mmol, 1 eq.) at 0 °C and stirred at 0 °C for 3 h. Hydroxylamine-O-sulfonic acid (560 mg, 4.945 mmol, 1.15 eq.) was dissolved in methanol (3.7 mL) and dropwise added to the mixture at 0 °C. The mixture was stirred overnight while allowing the reaction to warm up to room temperature. For removing the ammonia, argon was blown through the suspension for 1 h. Then, the mixture was filtrated and evaporated. During all subsequent steps, the diazirine was protected from light

by covering the flask in aluminum foil. Methanol (3 mL) was added to the mixture and cooled to 0 °C. After 5 min triethylamine (0.92 mL, 6.78 mmol, 1.58 eq.) was added and the mixture was stirred at 0 °C for additional 5 min. Iodine chips were gradually and slowly added to the stirring mixture until the solutions stays persistently red-brown. The solution was mixed with EtOAc, washed with hydrochloric acid (1 M), 10% $Na_2S_2O_3$ solution and saturated, aqueous NaCl solution sequentially, and the organic layer was dried with Na_2SO_4, filtered, and evaporated. The product was obtained as a light sensitive and viscous, yellow liquid (198 mg, 1.55 mmol, 36%).

^{1}H-NMR (400 MHz, Chloroform-*d*): *δ* (ppm) = 2.21 (t, J = 7.5 Hz, 2H), 1.69 (t, J = 7.2 Hz, 2H), 1.02 (s, 3H).

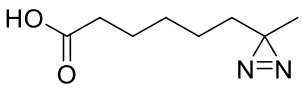
7-Oxooctanoic acid (13)



According to a literature procedure[4] 2-Acetylhexanone (0.78 ml, 6.0 mmol, 1.0 equiv.) and sodium hydroxide solution (5wt%, 6 mL) were added to a reaction flask equipped with a stir bar. The reaction mixture was heated to 120 °C and stirred for 2 h under air. It was cooled down to room temperature and aqueous hydrochloric acid (3 M, 6 mL) was added. The reaction mixture was extracted with dichloromethane (3x 25 mL) and the combined organic layers were subsequently washed with water (25 mL) and concentrated. Purification by column chromatography on silica gel (*n*-pentane/ethyl acetate = 1/1) afforded the product as a yellowish oil (470.9 mg, 3.0 mmol, 50%).

^{1}H-NMR (400 MHz, Chloroform-*d*): δ (ppm) = 2.44 (t, J = 7.4 Hz, 2H), 2.35 (t, J = 7.4 Hz, 2H), 2.13 (s, 3H), 1.69 – 1.54 (m, 4H), 1.39 – 1.29 (m, 2H).

7,7-Azo-octanoic acid (12)



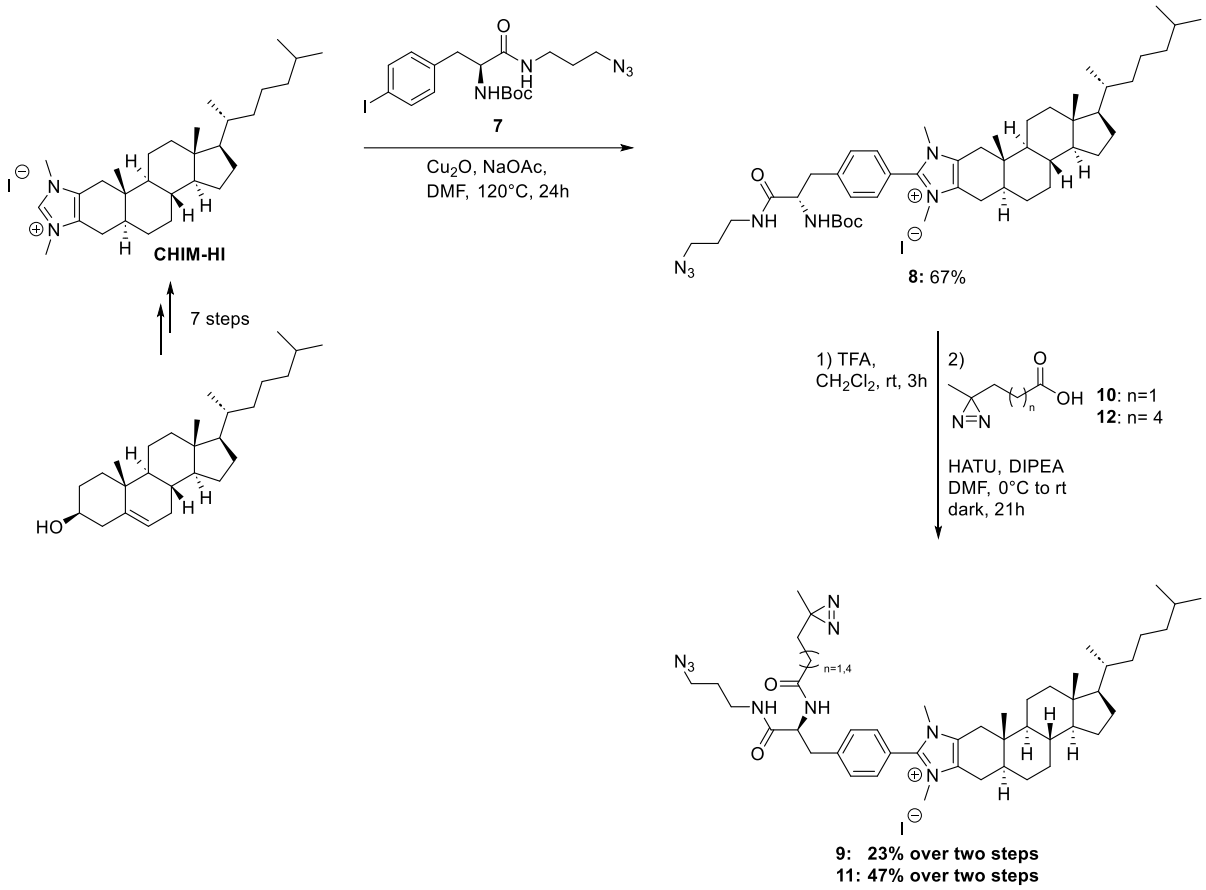
The synthesis was performed according to a literature procedure.[3] Ammonia (7 M in MeOH; 2.9 mL) was added to a flask with 7-oxooctanoic acid (460 mg, 2.9 mmol, 1.0 eq.) at 0 °C and stirred at 0 °C for 3 h. Hydroxylamine-O-sulfonic acid (378 mg, 3.3 mmol, 1.15 eq.) was dissolved in methanol (2.5 mL) and dropwise added to the mixture at 0 °C. The mixture for 15 h while allowing the reaction to warm up to room temperature. For removing the ammonia, argon was blown through the suspension for 1 h. Then, the mixture was filtrated and evaporated. During all subsequent steps, the diazirine was protected from light by covering the flask in aluminum foil. Methanol (2 mL) was added to the mixture and it was cooled to 0 °C. After 10 min triethylamine (0.64 mL, 4.59 mmol, 1 58 eq.) was added and the mixture was stirred at 0 °C for additional 5 min. Iodine chips were gradually and slowly added to the stirring mixture until the solutions stayed persistently red-brown. The solution was mixed with EtOAc, washed with hydrochloric acid (1 M), 10% $Na_2S_2O_3$-solution and saturated, aqueous NaCl-solution sequentially, and the organic layer was dried with $MgSO_4$, filtered, and evaporated. Purification by column

chromatography on silica gel (*n*-pentane/ethylacetate = 10/0 → 7/3) afforded the product as a light sensitive and viscous, yellow liquid (101.8 mg, 0.6 mmol, 21%).

HRMS (ESI)**:** *m*/*z* calculated $[C_8H_{13}N_2O_2]^-$: 169.09825, found: 169.09822

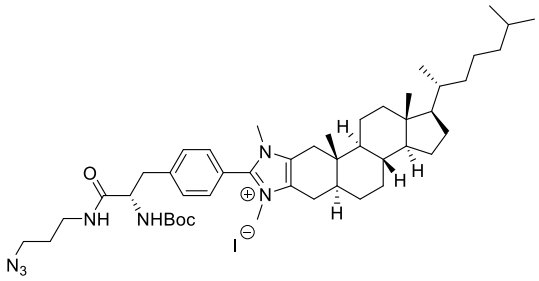
^{1}H-NMR (400 MHz, Chloroform-*d*): δ (ppm) = 2.34 (t, J = 7.5 Hz, 2H), 1.60 (p, J = 7.5 Hz, 2H), 1.38 – 1.27 (m, 4H), 1.24 – 1.13 (m, 2H), 0.99 (s, 3H).

^{13}C{^{1}H}-NMR (101 MHz, Chloroform-*d*): δ (ppm) = 179.3, 34.2, 33.8, 28.7, 25.9, 24.5, 23.8, 20.0.



Supporting Scheme 2: Synthetic route to access the bifunctional imidazolium-based lipid analogs.

(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)phenyl)-Chol-IMe iodide (8)



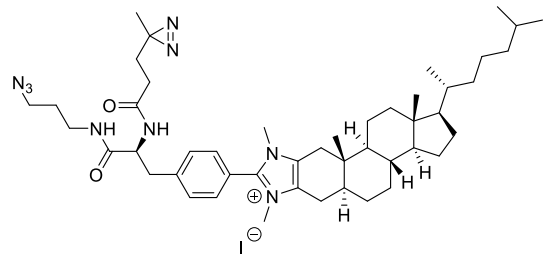
The synthesis was performed according to a modified literature procedure.[5] **CHIM·HI** (40 mg, 0.072 mmol, 1 eq.), compound **7** (68 mg, 0.144 mmol, 2 eq.), Cu_2O (2 mg, 0.014 mmol, 0.2 eq.) and NaOAc (6 mg, 0.072 mmol, 1 eq.) were added to an oven-dried Schlenk tube and dry DMF (0.5 mL) was added. The reaction mixture was stirred at 120 °C for 24 h. Afterwards the solvent was removed under reduced pressure and the crude was subjected to column chromatography (DCM/methanol = 100/0 → 95/5) to yield compound **8** (43 mg, 0.047 mmol, 67%).

HRMS (ESI)**:** *m*/*z* calculated $[C_{47}H_{74}N_7O_3]^+$: 784.5848, found: 784.5849

^{1}H-NMR (599 MHz, Chloroform-*d*): δ (ppm) = 7.66 (d, *J* = 7.8 Hz, 2H), 7.55 (d, *J* = 7.8 Hz, 2H), 4.44 (t, *J* = 7.0 Hz, 1H), 3.61 (d, *J* = 17.3 Hz, 6H), 3.30 (t, *J* = 6.8 Hz, 4H), 3.22 – 3.11 (m, 2H), 2.69 (dd, *J* = 16.2, 5.0 Hz, 1H), 2.60 (d, *J* = 15.8 Hz, 1H), 2.42 (d, *J* = 15.9 Hz, 1H), 2.31 – 2.22 (m, 1H), 2.05 – 2.00 (m, 1H), 1.87 – 1.67 (m, 7H), 1.63 – 1.44 (m, 5H), 1.40 (s, 9H), 1.38 – 0.95 (m, 16H), 0.93 – 0.90 (m, 6H), 0.86 (dd, *J* = 6.6, 2.8 Hz, 6H), 0.68 (s, 3H).

^{13}C{1H}-NMR (151 MHz, Chloroform-*d*): δ (ppm) = 171.2, 171.1, 143.3, 142.5, 131.3, 131.2, 129.8, 128.7, 119.8, 56.3, 56.2, 53.2, 49.4, 42.5, 41.5, 39.8, 39.6, 37.1, 37.0, 36.9, 36.3, 35.9, 35.6, 34.4, 33.9, 33.8, 31.3, 29.8, 28.9, 28.7, 28.5, 28.4, 28.3, 28.1, 25.0, 24.4, 24.0, 22.9, 22.7, 21.5, 18.8, 13.0, 12.1.
Note: Due to overlapping of the signals, *four carbon signals are missing.*

(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-(3-(3-methyl-3*H*-diazirin-3-yl)propanamido)-3-oxopropyl)phenyl)-Chol-IMe iodide (9)



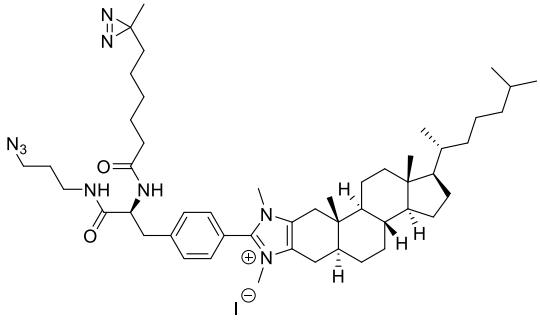
Compound **8** (28 mg, 0.031 mmol, 1 eq.) was dissolved in dry DCM (1 mL) and TFA (100 µL) was added at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure and the resulting deprotected product was directly used for the next step. Dry DMF (1.4 mL) was added and the resulting solution was cooled to 0 °C and the flask was wrapped with aluminium foil. 4,4-Azo-pentanoic acid **10** (7.9 mg, 0.061 mmol, 2 eq.); HATU (17.6 mg, 0.046 mmol, 1.5 eq.) and DIPEA (27 µL, 0.155 mmol, 5 eq.) were added subsequently and the reaction mixture was allowed to warm to room temperature. It was stirred for 21 h. Water and DCM were added. The layers were separated and the aqueous layer was extracted with DCM. The combined organic layers were washed with water and concentrated. Column chromatography on silica gel (DCM/methanol = 100/0 → 95/5) afforded compound **9** (**X-CHIM**) (6.5 mg, 0.007 mmol, 23% over two steps).

HRMS (ESI)**:** *m/z* calculated $[C_{47}H_{72}N_9O_2]^+$: 794.5803, found: 794.5813.

^{1}H-NMR (500 MHz, Chloroform-*d*): *δ* (ppm) =.51 (d, *J* = 8.3 Hz, 2H), 7.44 (d, *J* = 8.5 Hz, 2H), 6.75 (t, *J* = 5.9 Hz, 1H), 6.68 (d, *J* = 8.5 Hz, 1H), 4.84 – 4.74 (m, 1H), 3.53 (d, *J* = 6.4 Hz, 6H), 3.34 – 3.25 (m, 4H), 3.20 (h, *J* = 6.4 Hz, 2H), 2.61 – 2.53 (m, 2H), 2.32 – 2.21 (m, 3H), 2.19 – 2.01 (m, 3H), 1.90 – 1.80 (m, 1H), 1.79 – 1.65 (m, 8H), 1.63 – 0.95 (m, 21H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.89 – 0.87 (m, 6H), 0.86 (d, *J* = 2.3 Hz, 3H), 0.69 (s, 3H).

^{13}C{^{1}H}-NMR (126 MHz, Chloroform-*d*): *δ* (ppm) = 172.1, 171.1, 143.6, 142.9, 131.2, 130.4, 129.5, 128.5, 119.6, 56.3, 56.2, 54.6, 53.4, 49.2, 42.5, 41.6, 39.8, 39.6, 38.7, 38.0, 37.1, 37.0, 36.3, 35.9, 35.6, 34.2, 32.7, 30.4, 30.0, 29.8, 28.7, 28.7, 28.3, 28.1, 25.8, 24.6, 24.4, 24.0, 23.0, 22.7, 21.5, 19.8, 18.8, 12.1, 12.1.

(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-(6-(3-methyl-3*H*-diazirin-3-yl)hexanamido)-3-oxopropyl)phenyl-)-Chol-IMe iodide (11)



Compound **8** (39.7 mg, 0.044 mmol, 1 eq.) was dissolved in dry DCM (2.2 mL) and TFA (200 µL) was added at 0 °C. The reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the resulting deprotected product was directly used for the next step. Dry DMF (2.0 mL) was added and the resulting solution was cooled to 0 °C and the flask was wrapped with aluminium foil. 7,7-Azo-octanoic acid **12** (14.8 mg, 0.087 mmol, 2 eq.); HATU (24.8 mg, 0.065 mmol, 1.5 eq.) and DIPEA (38 µL, 0.218 mmol, 5 eq.) were added subsequently and the reaction mixture was allowed to warm to room temperature. It was stirred for 21 h. Water and DCM were added. The layers were separated and the aqueous layer was extracted with DCM. The combined organic layers were washed with water and concentrated. Column chromatography on silica gel (DCM/methanol = 100/0 → 95/5) afforded compound **11** (**X-CHIM-C8**) (6.5 mg, 0.007 mmol, 47% over two steps).

HRMS (ESI)**:** *m/z* calculated $[C_{50}H_{78}N_9O_2]^+$: 836.6273, found: 836.6279.

^{1}H-NMR (500 MHz, Chloroform-*d*): *δ* (ppm) = 7.53 – 7.39 (m, 4H), 6.99 – 6.81 (m, 2H), 4.78 (d, *J* = 7.5 Hz, 1H), 3.51 (d, *J* = 7.9 Hz, 6H), 3.32 – 3.23 (m, 3H), 3.18 (s, 2H), 2.61 – 2.51 (m, 2H), 2.39 – 2.16 (m, 5H), 2.04 (d, *J* = 12.7 Hz, 1H), 1.77 – 1.65 (m, 5H), 1.63 – 1.06 (m, 27H), 1.01 – 0.96 (m, 5H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.89 – 0.85 (m, 9H), 0.69 (s, 3H).

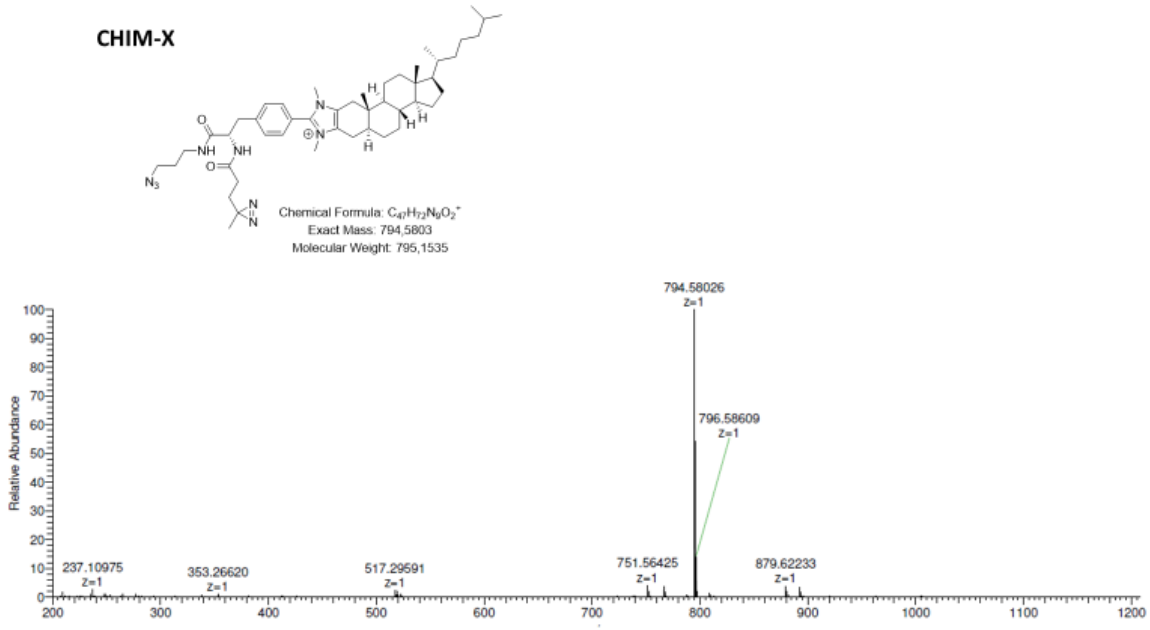
^{13}C{^{1}H}-NMR (101 MHz, Chloroform-*d*): *δ* (ppm) = 173.7, 171.2, 143.4, 142.4, 131.0, 130.5, 129.5, 128.5, 119.8, 56.3, 56.2, 54.3, 53.3, 49.1, 42.5, 41.6, 39.8, 39.6, 37.1, 36.9, 36.2, 36.1, 35.9, 35.5, 34.1, 32.6, 28.8, 28.7, 28.6, 28.3, 28.1, 26.0, 25.5, 24.5, 24.3, 24.0, 23.9, 23.0, 22.7, 21.4, 20.0, 18.8, 12.1.

Note: Due to overlapping of the signals, seven carbon signals are missing.

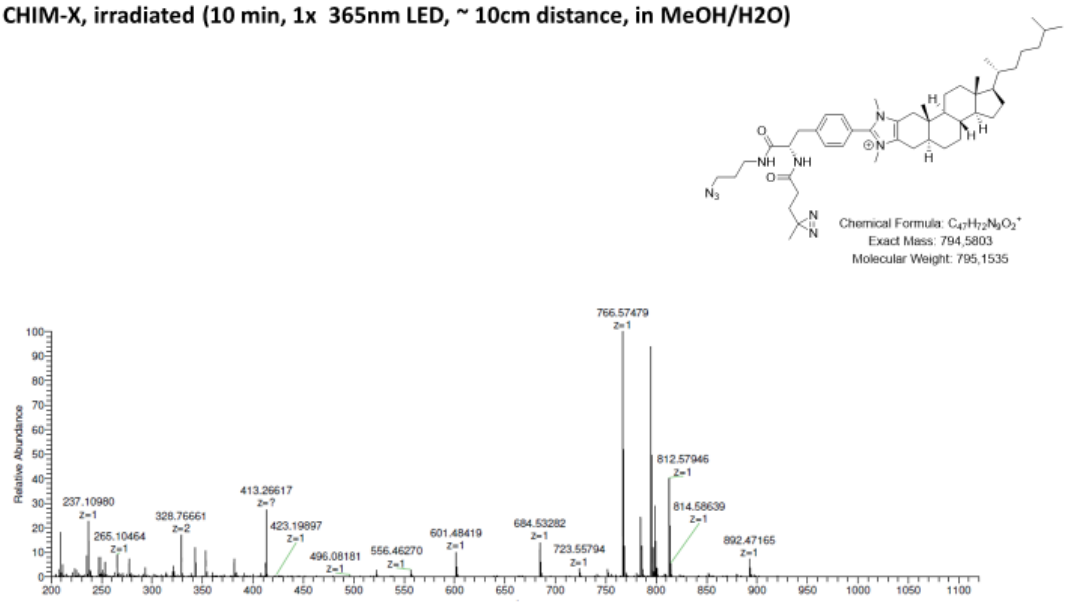
1.3 *In vitro* HR-ESI-MS evaluation

A few drops of X-CHIM dissolved in MeOH (HPLC grade) or DPPC-X-CHIM liposomes (weight ratio DPPC/X-CHIM 9:1.1) in H_2O were added to ~500 µL of MeOH/H_2O (HPLC grade) (70/30) + 0.1% formic acid. The mixture was irradiated with two 365 nm LEDs for 10-20 minutes and a sample of the resulting mixture was analyzed via HR-ESI-MS directly afterwards. In case of the dye-containing experiment, a few drops of a DBCO-Sulfo-Cye3-dye solution were added to the mixture directly after irradiation and before HR-ESI-MS sample submission.

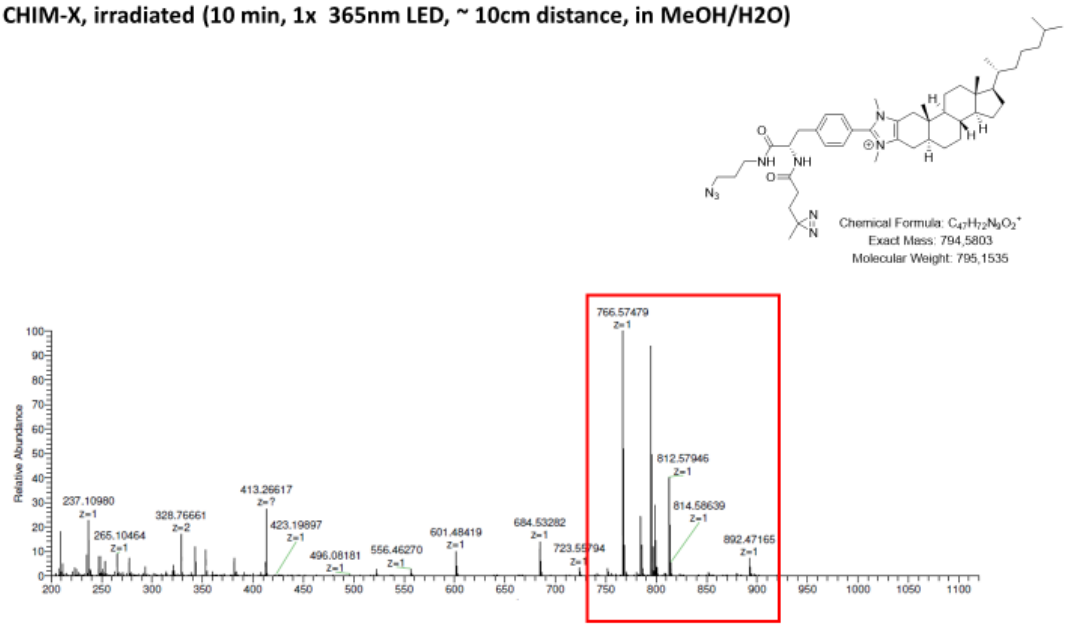
X-CHIM 10 minutes irradiation



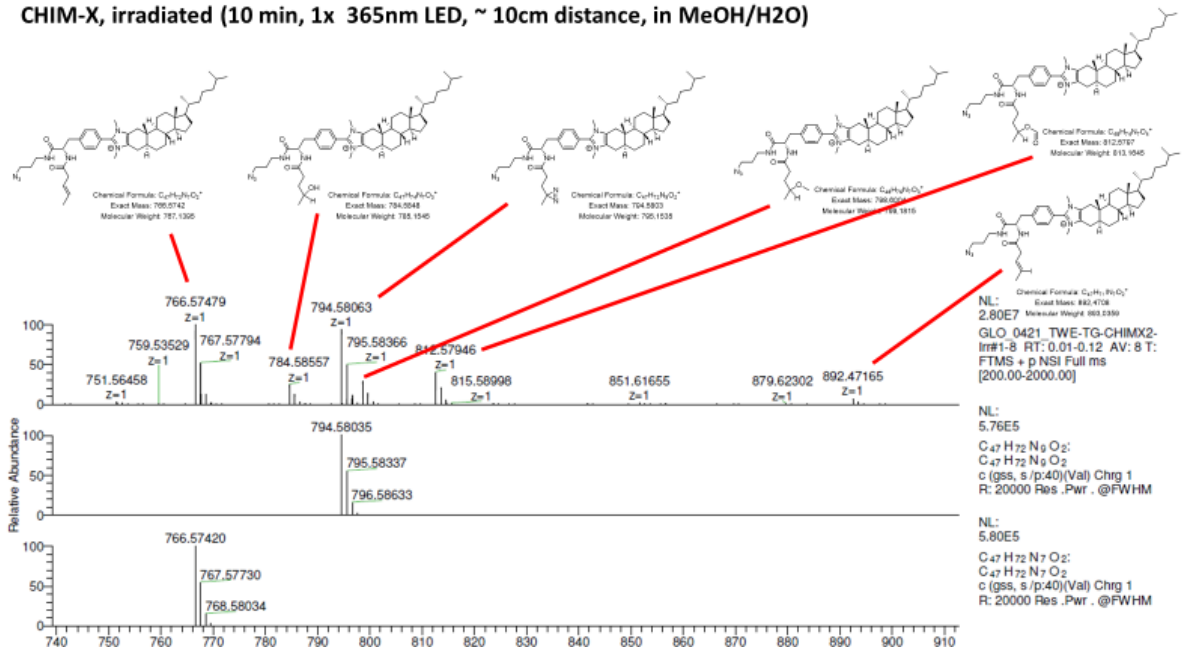
CHIM-X, irradiated (10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O)



CHIM-X, irradiated (10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O)

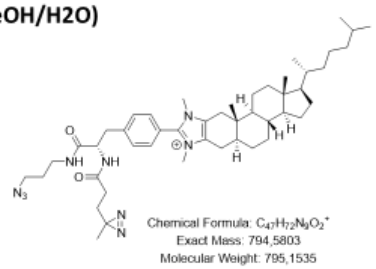


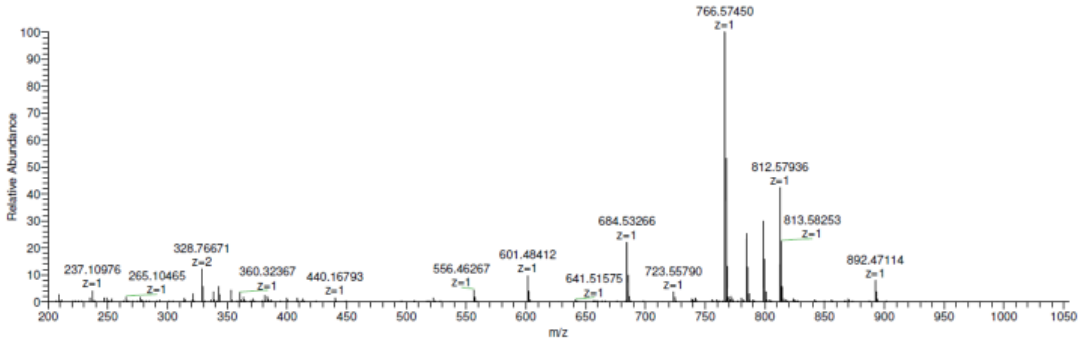
CHIM-X, irradiated (10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O)



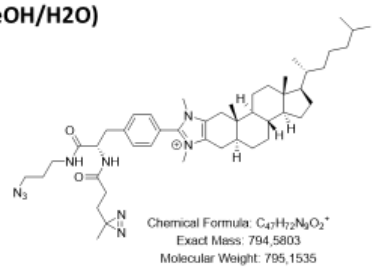
X-CHIM 20 minutes irradiation

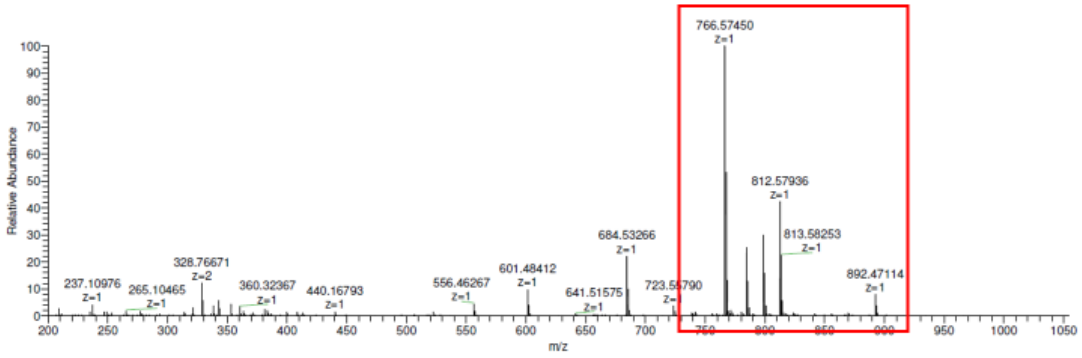
CHIM-X, irradiated (20 min, 2x 365nm LED, ~ 10cm distance, in MeOH/H2O)



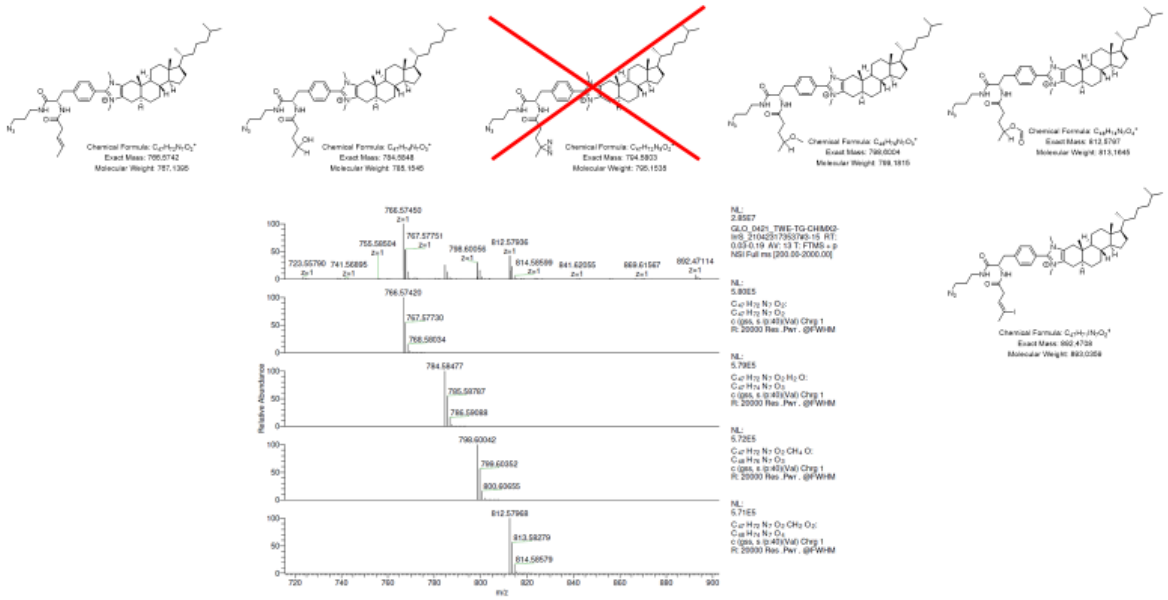


CHIM-X, irradiated (20 min, 2x 365nm LED, ~ 10cm distance, in MeOH/H2O)



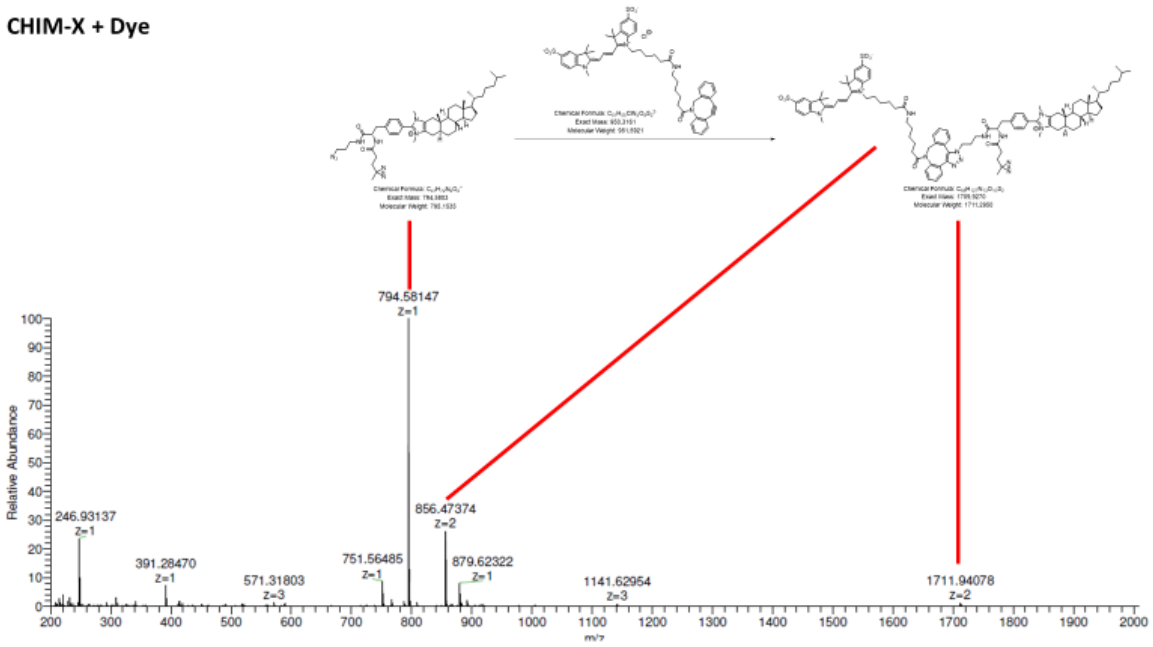


CHIM-X, irradiated (20 min, 2x 365nm LED, ~ 10cm distance, in MeOH/H2O)

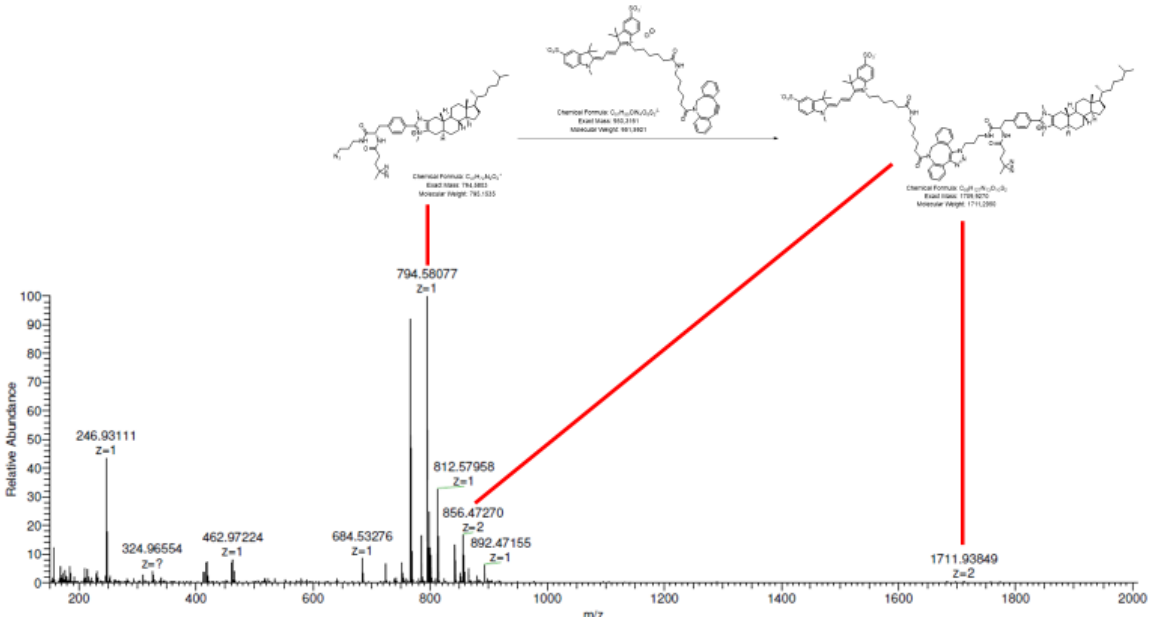


X-CHIM + dye

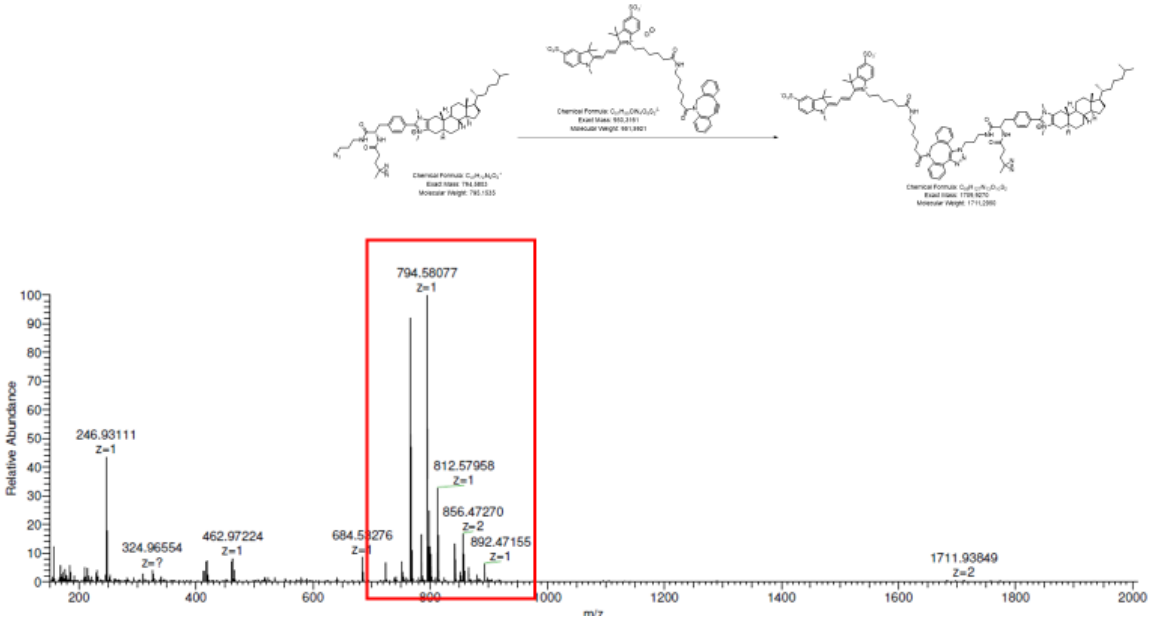
CHIM-X + Dye



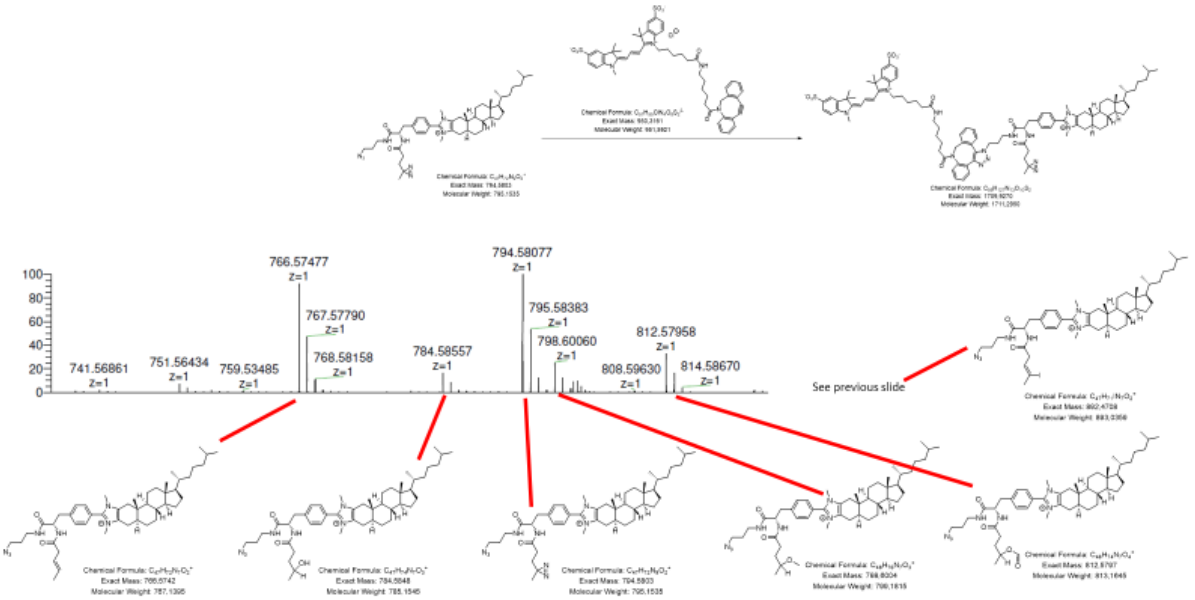
CHIM-X (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O) + Dye



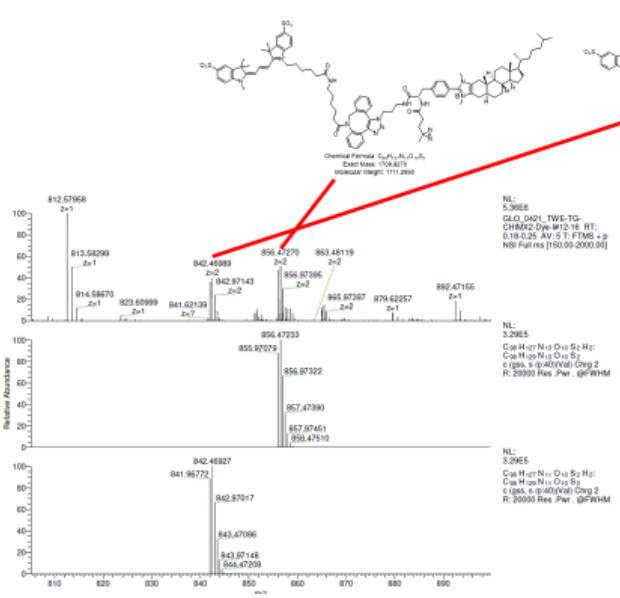
CHIM-X (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O) + Dye



CHIM-X (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O) + Dye

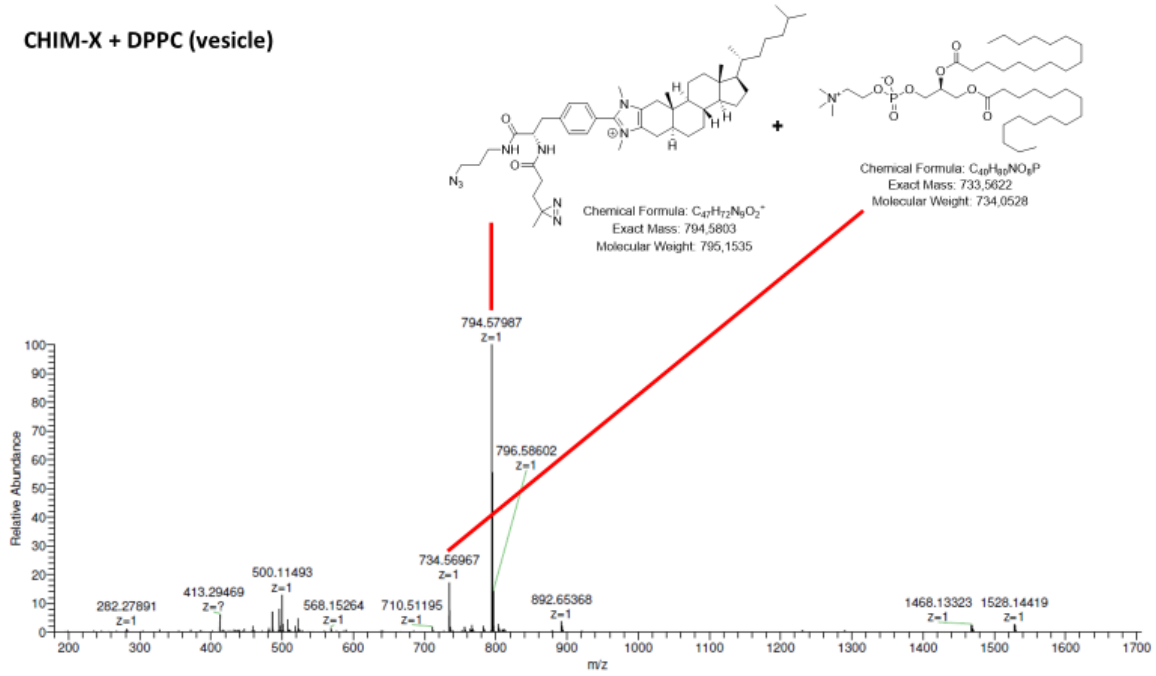


CHIM-X (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O) + Dye



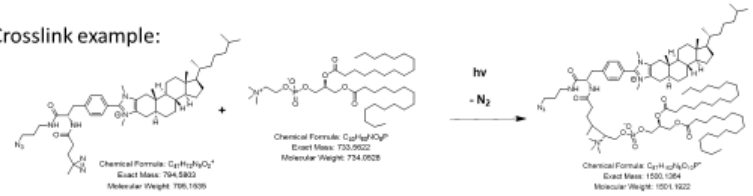
DPPC-X-CHIM liposomes

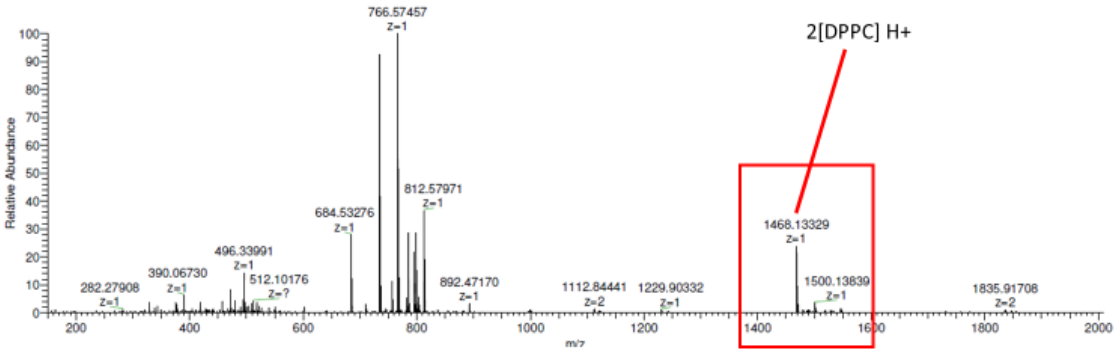
CHIM-X + DPPC (vesicle)



CHIM-X + DPPC (vesicle) (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O)

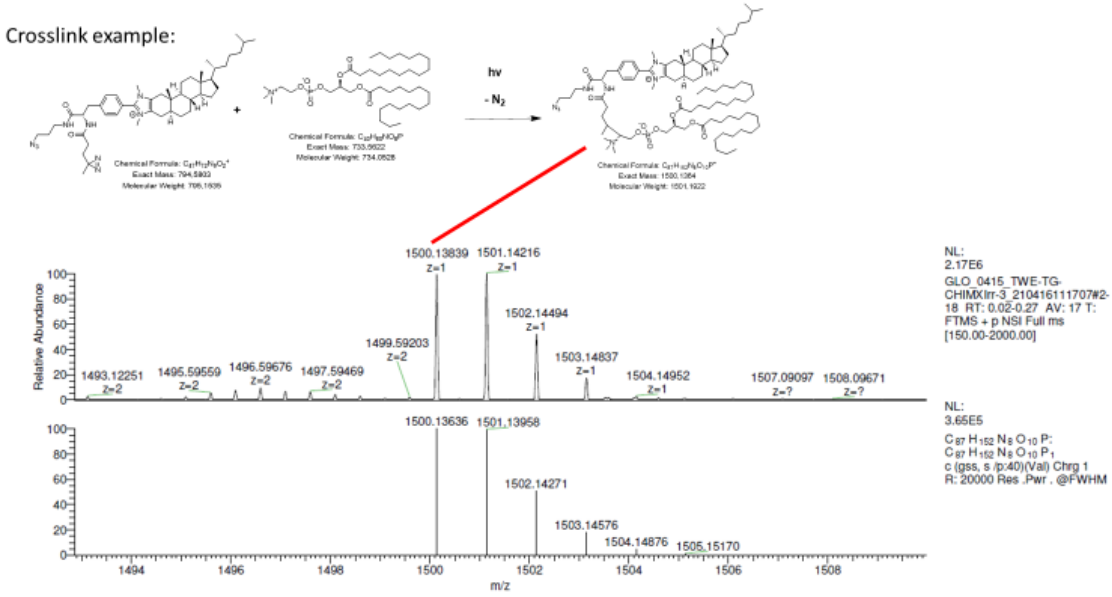
Crosslink example:





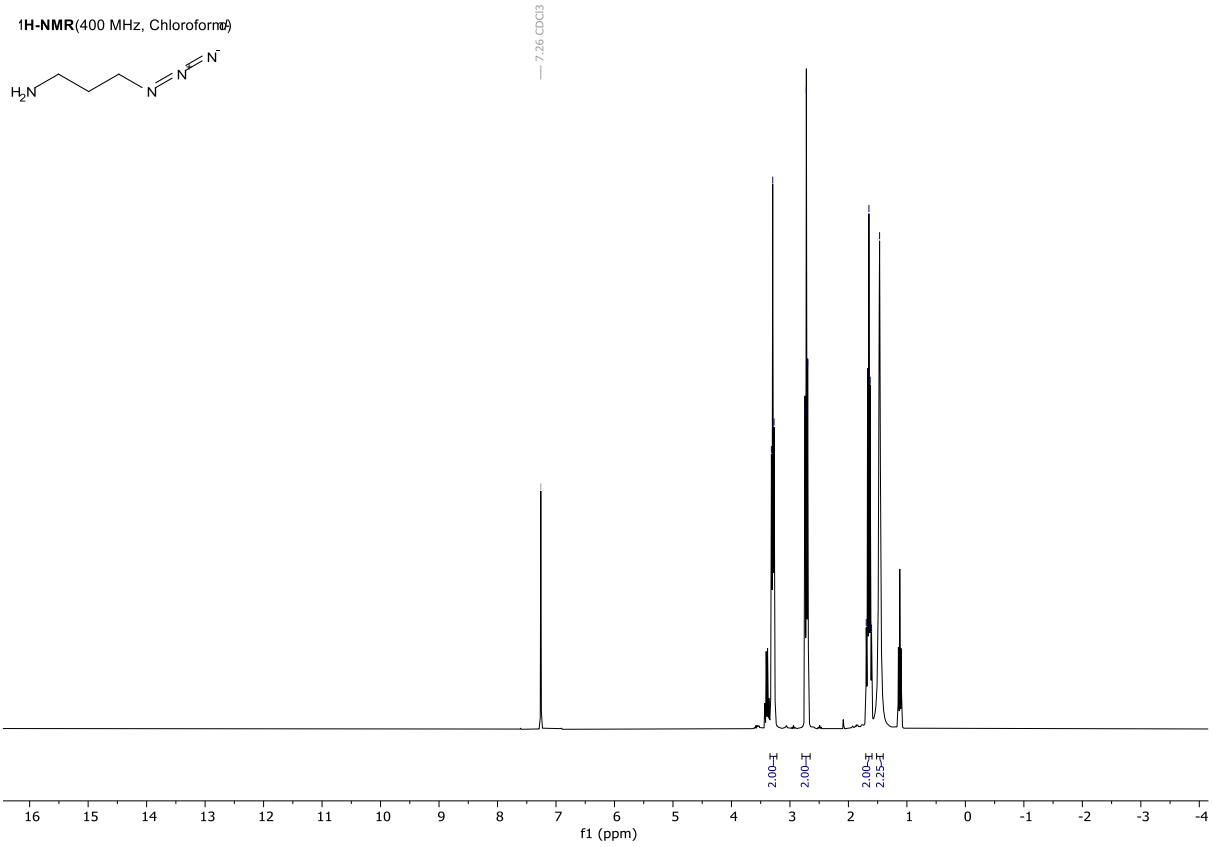
CHIM-X + DPPC (vesicle) (irradiated 10 min, 1x 365nm LED, ~ 10cm distance, in MeOH/H2O)

Crosslink example:

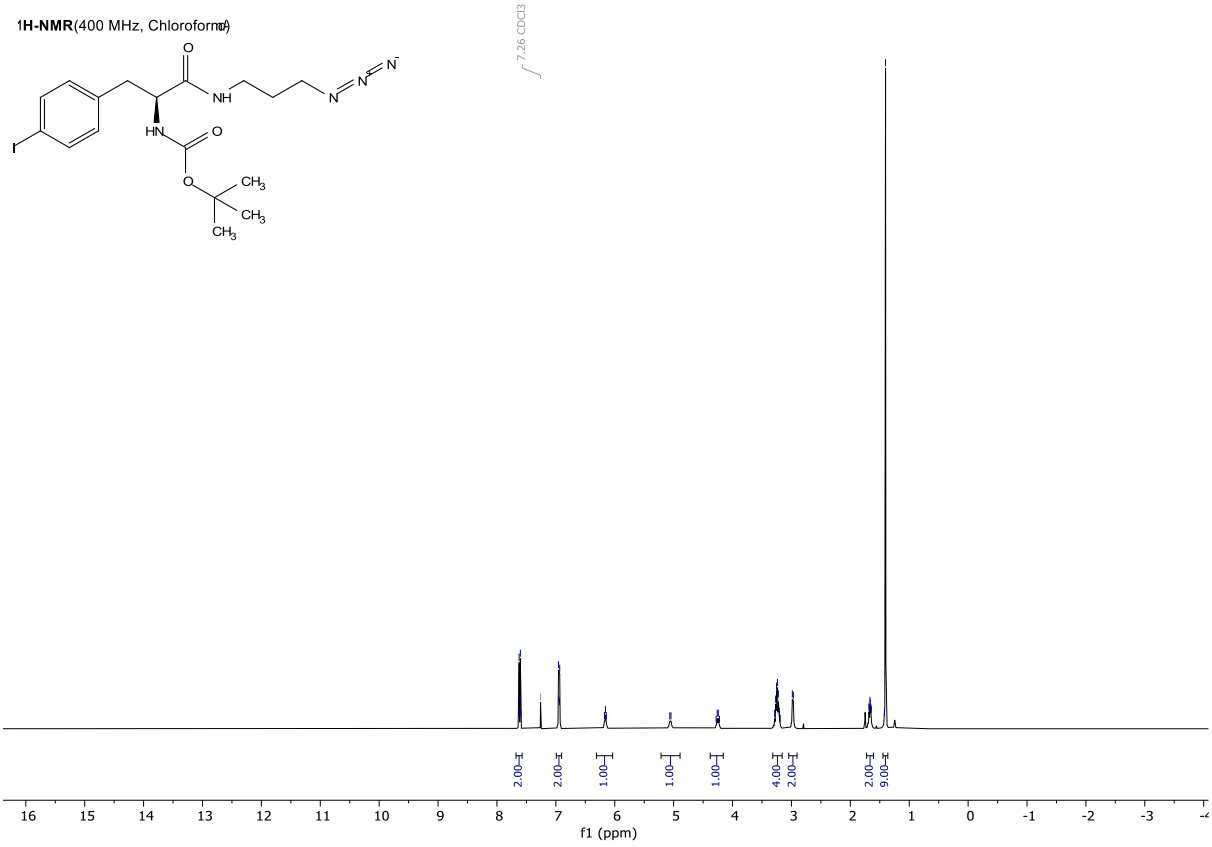


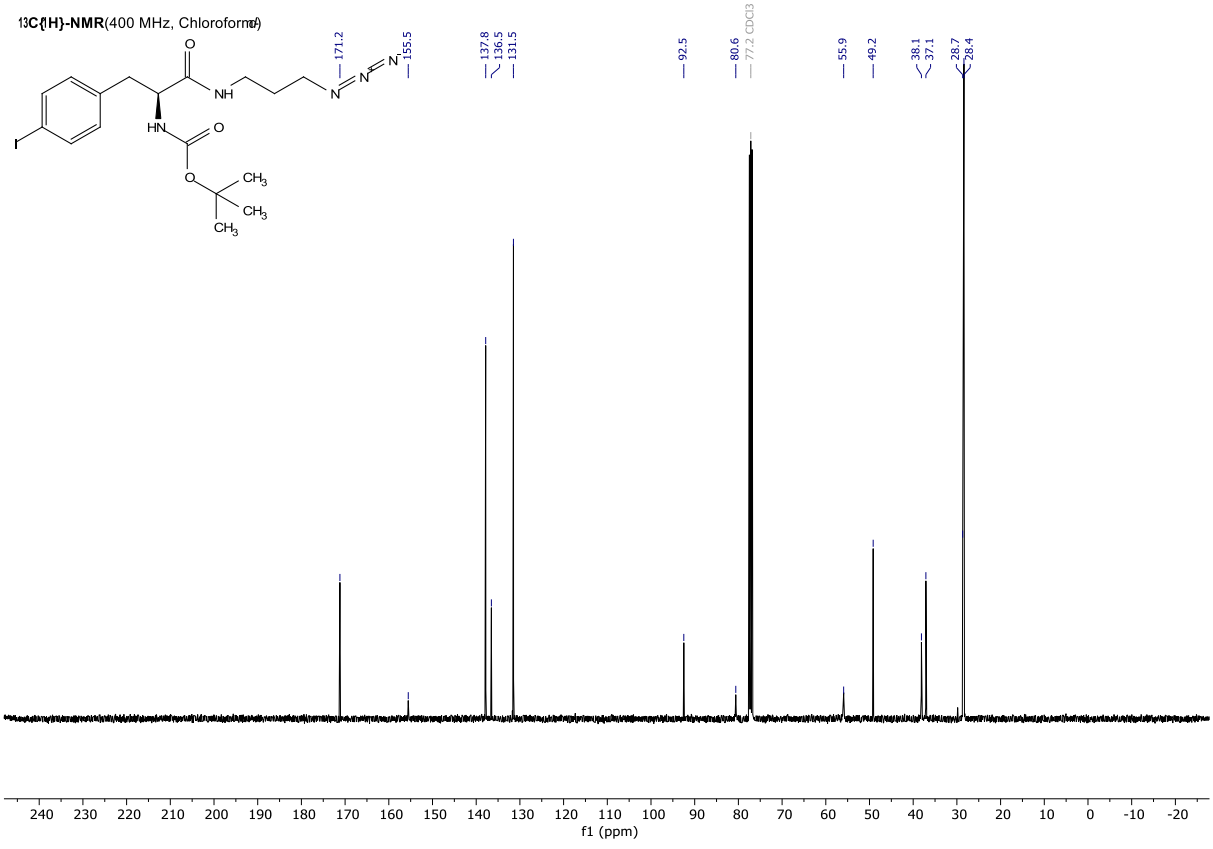
1.4 NMR Spectra

3-azidopropan-1-amine (6)

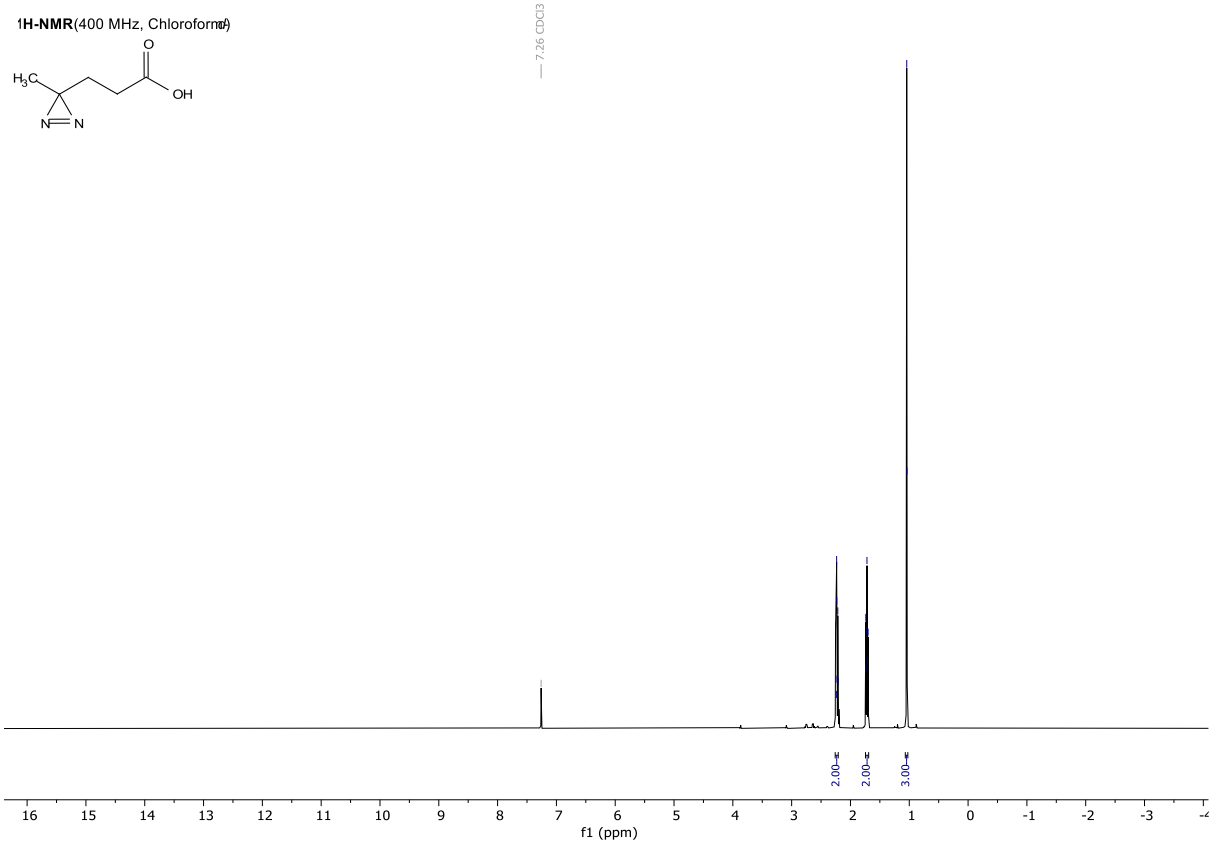


***tert*-Butyl (*S*)-(1-((3-azidopropyl)amino)-3-(4-iodophenyl)-1-oxopropan-2-yl)carbamate (7)**

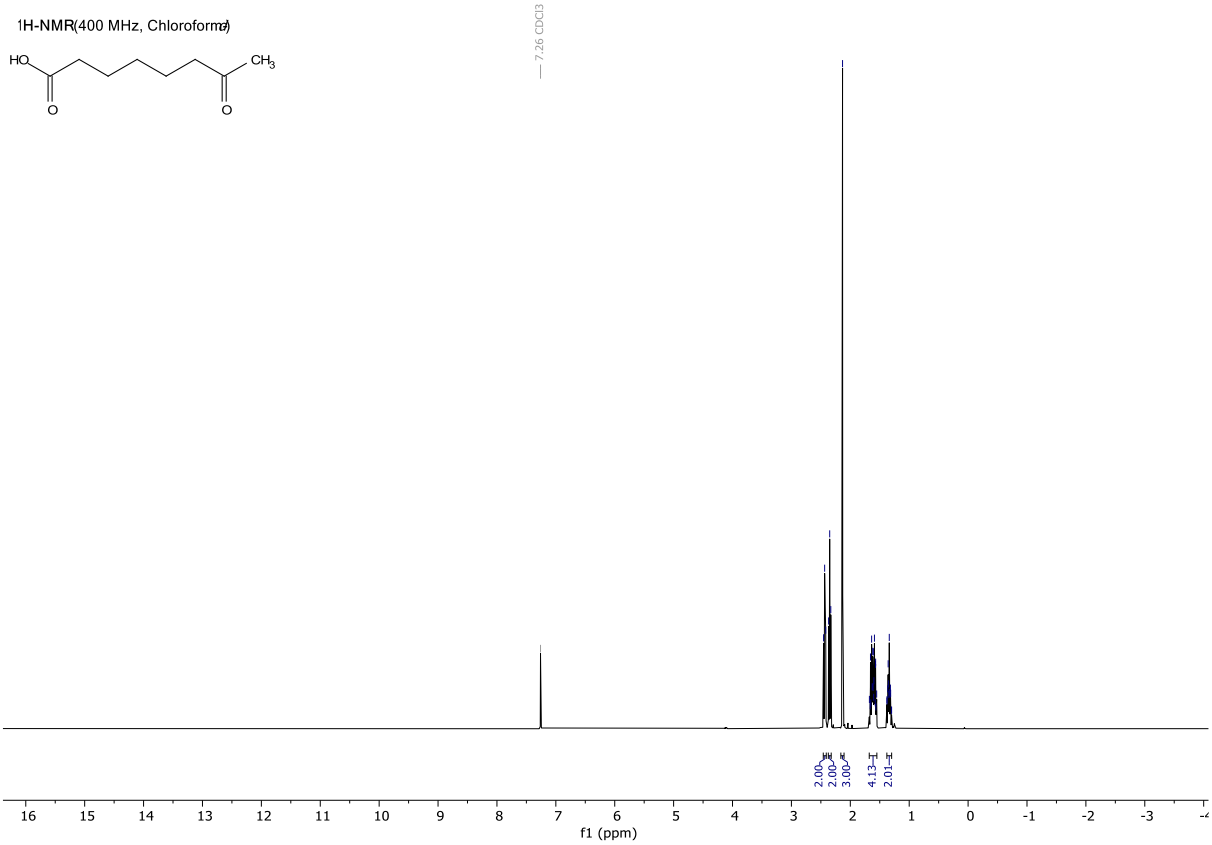




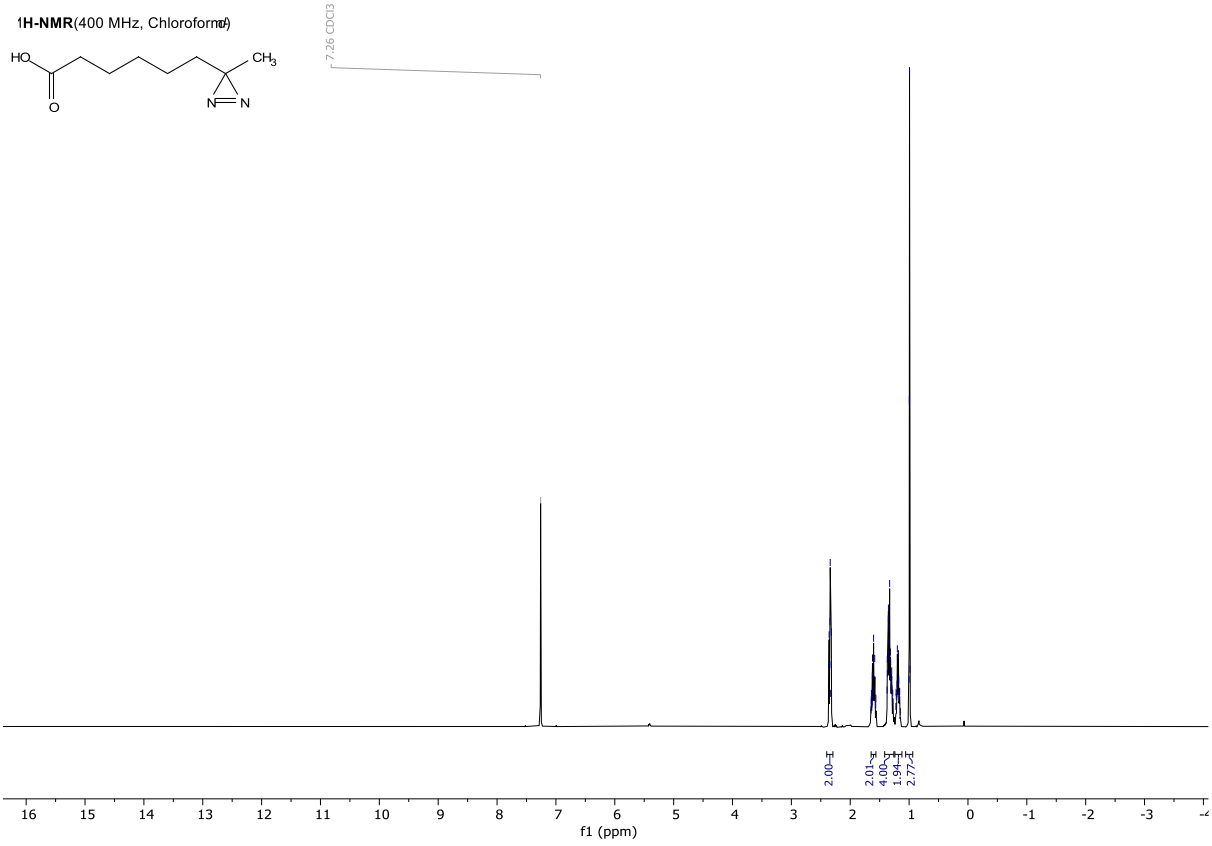
4,4-Azo-pentanoic acid (10)

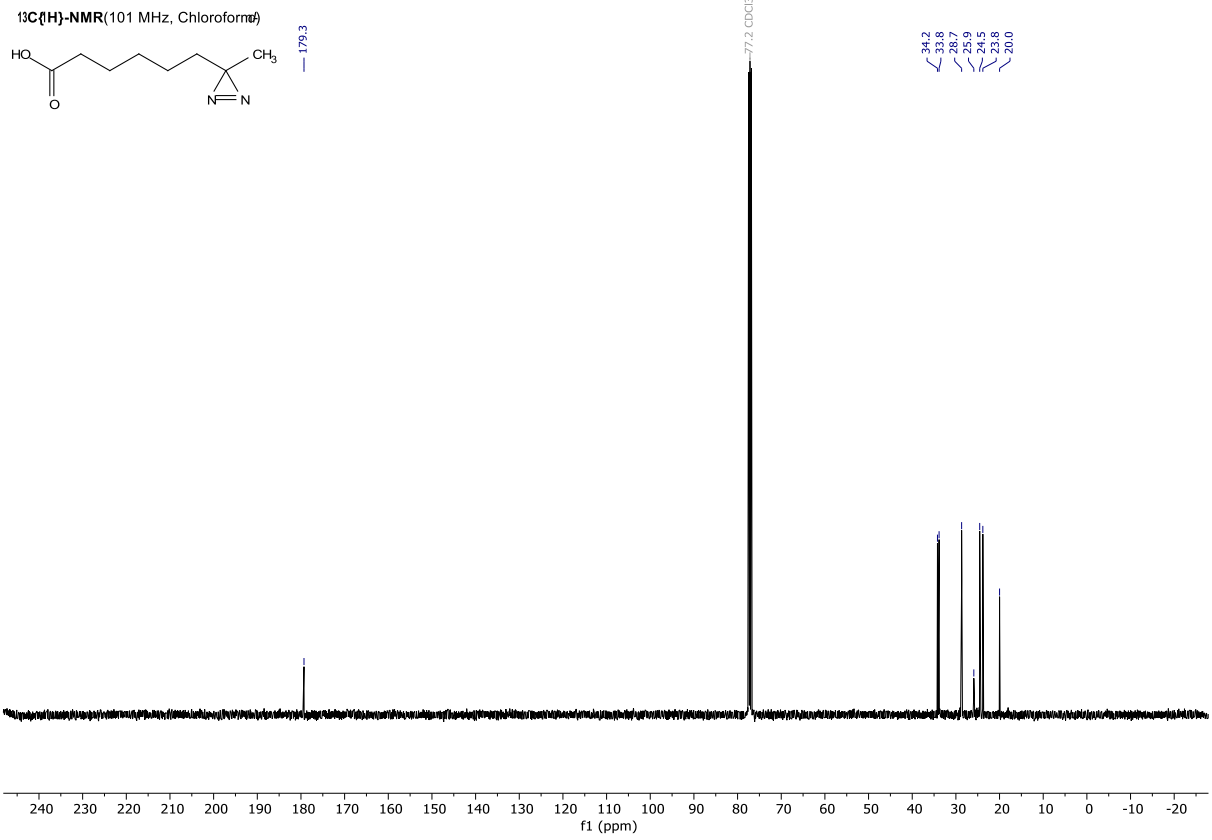


7-Oxooctanoic acid (13)

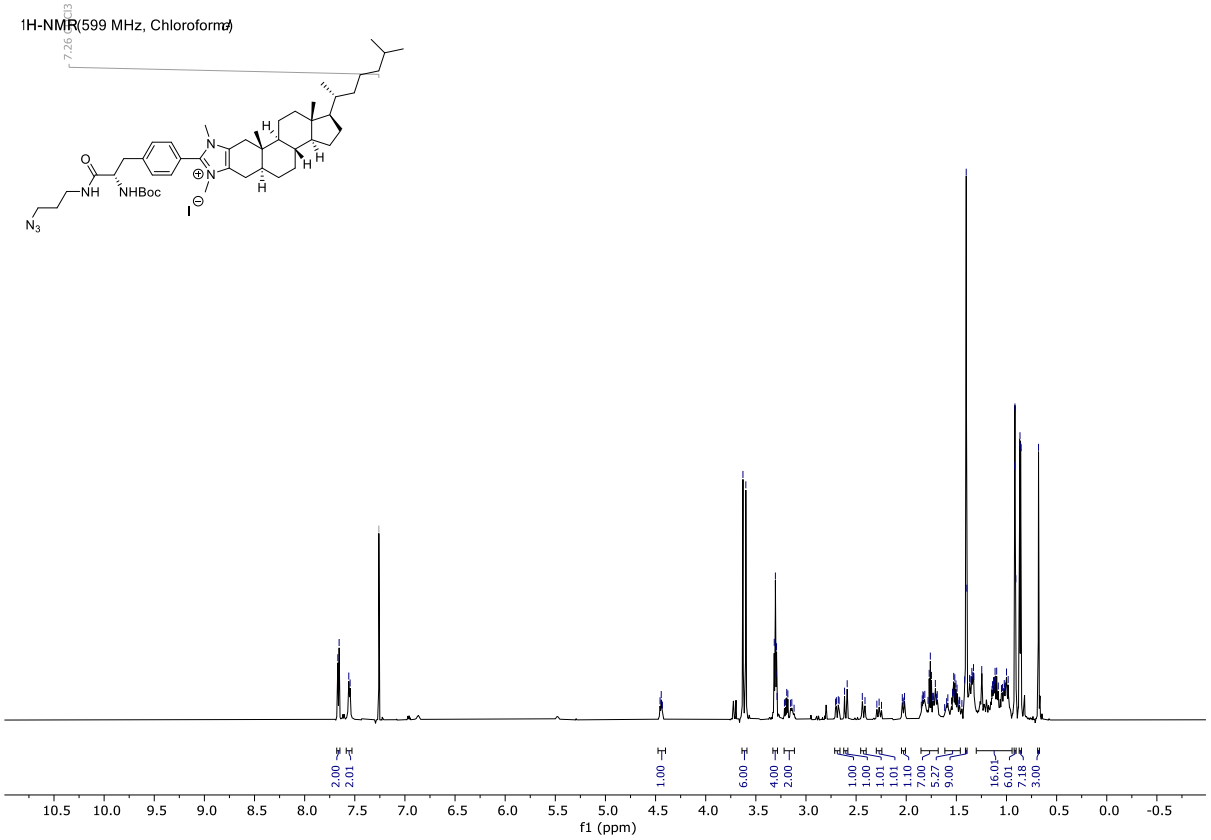


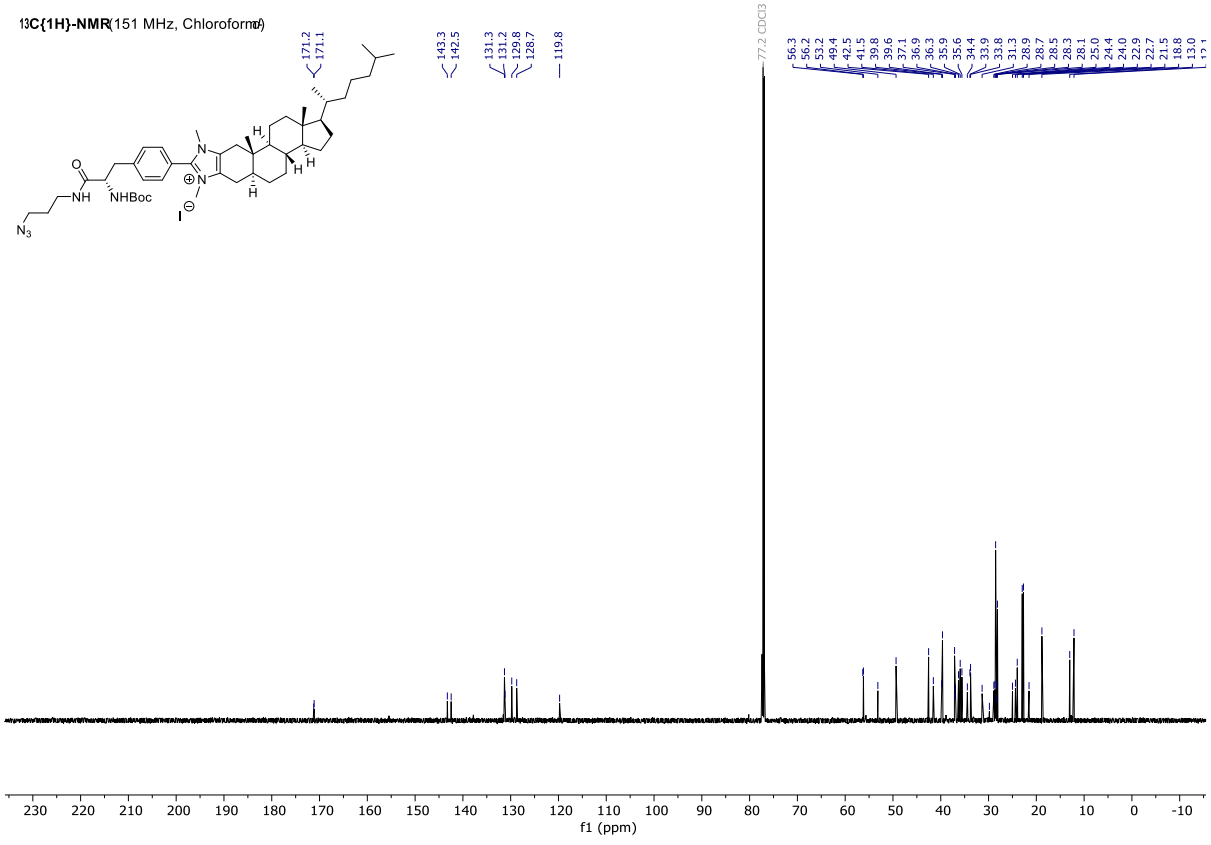
7,7-Azo-octanoic acid (12)



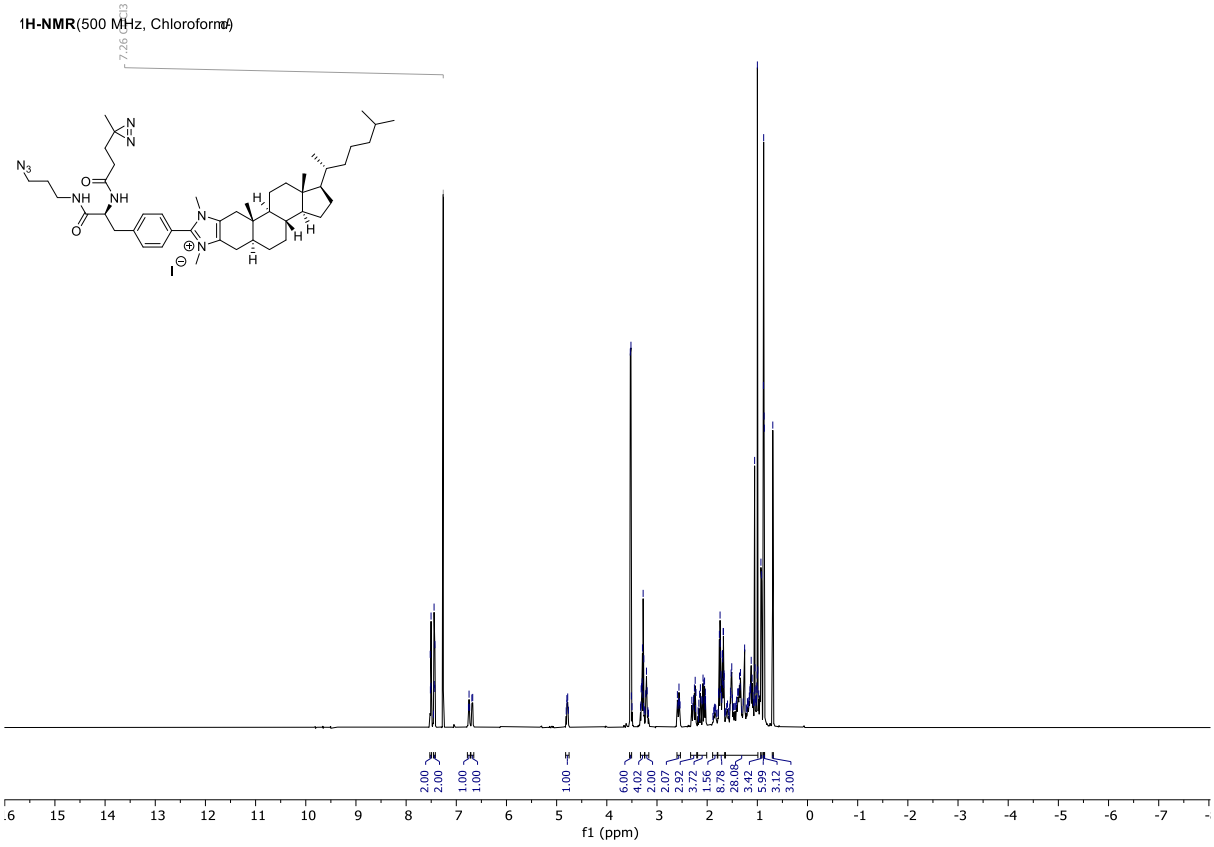


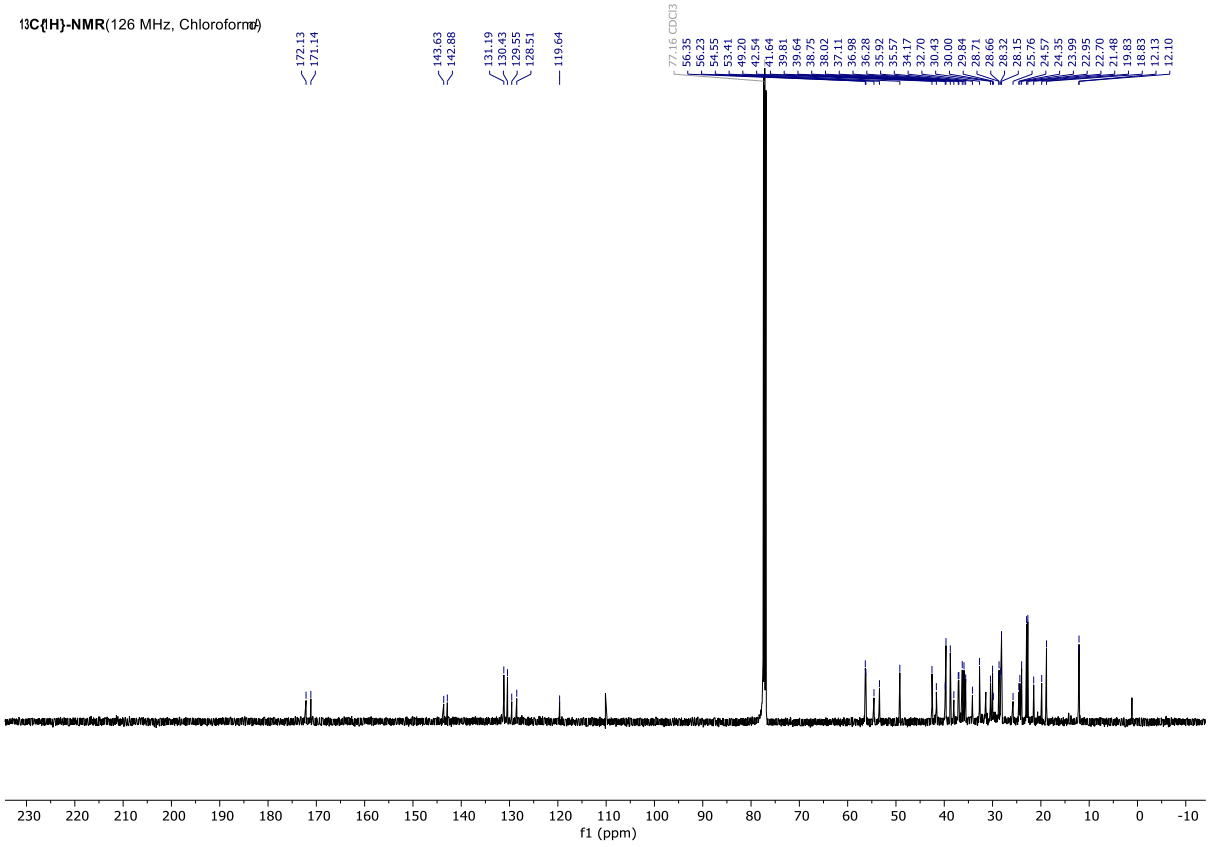
(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-((tert-butoxycarbonyl)amino)-3-oxopropyl)phenyl)-Chol-IMe iodide (8)



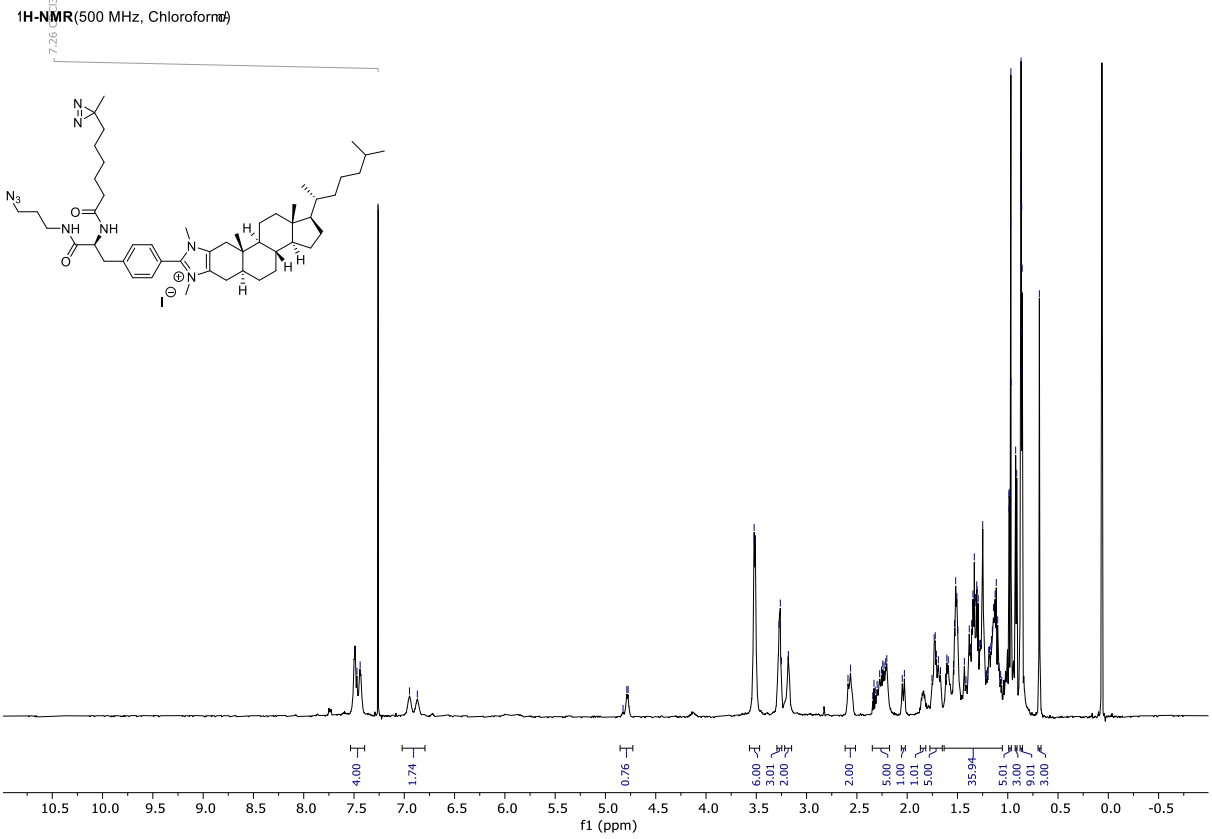


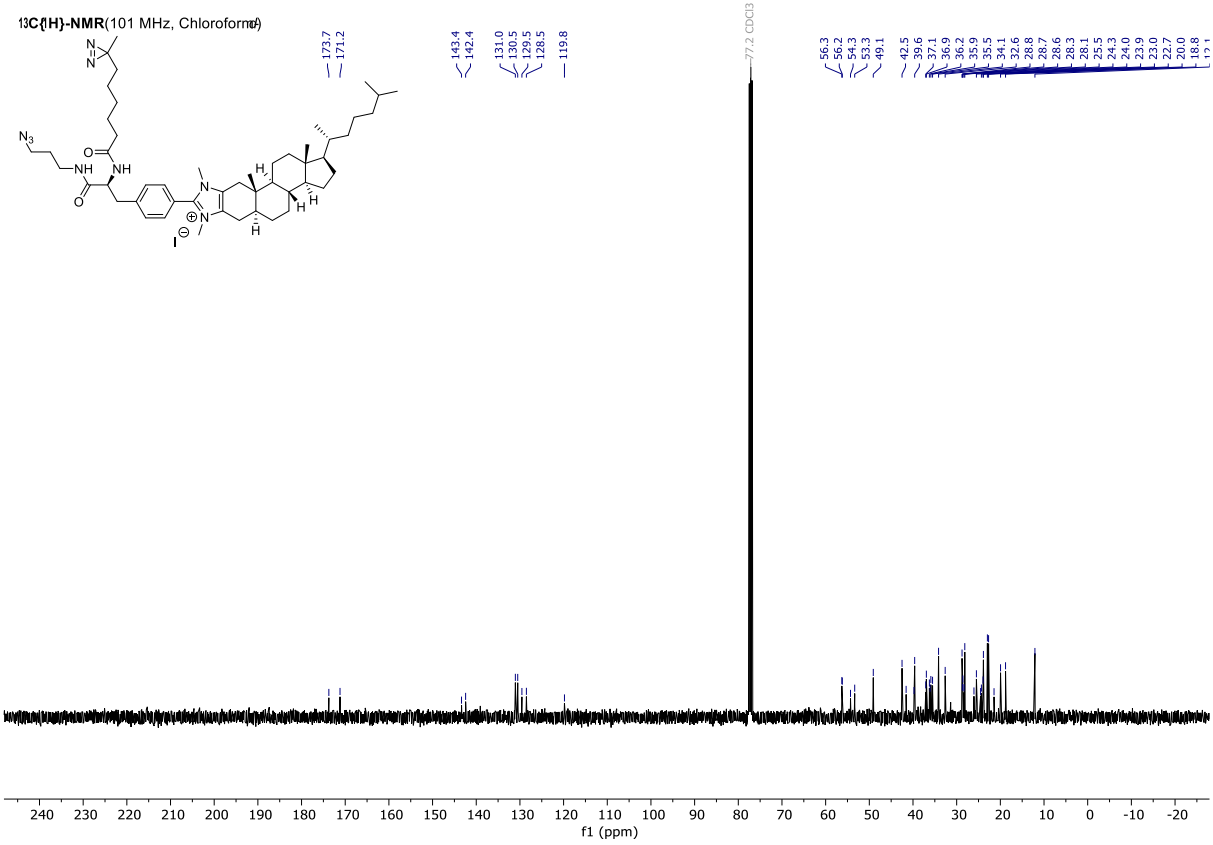
(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-(3-(3-methyl-3*H*-diazirin-3-yl)propanamido)-3-oxopropyl)phenyl)-Chol-IMe iodide (9)





(*S*)-2-(4-(3-((3-azidopropyl)amino)-2-(6-(3-methyl-3*H*-diazirin-3-yl)hexanamido)-3-oxopropyl)phenyl)-)-Chol-IMe iodide (11)





2. Biological experiments

2.1. Materials and Methods

Lipids and chemicals

The lipids cholesterol (Chol), 1-palmitoyl-2-oleoyl-sn-glycero-3-phoshocholine (POPC), 1-palmitoyl-2-oleoylsn-glycero-3-phosho-L-serine (sodium salt) (POPS), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLiPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-choline (DPPC) were purchased from Avanti Polar Lipids (Alabaster, USA). Lipids and cholesterol were dissolved in chloroform. Click-iT-DIBO Alexa Fluor® 647, click-iT DIBO Alexa Fluor® 488, sDIBO-biotin (Thermo Fischer Scientific), AFDye 405 DBCO (Click Chemistry Tools), and IRDye 800CW DBCO (LI-COR). Other chemicals were purchased from Applichem (Darmstadt, Germany), Merck KGaA (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany) and Sigma-Aldrich (Munich, Germany). Water was purified and deionized with a cartridge system from Millipore (18.2 MΩ). For all the SDS-PAGE and Western blots the marker PageRuler Plus Prestained Protein Ladder from Thermo Scientific was used (Waltham, MA, United States).

Protein expression and purification

Human AnxA2 encoding cDNA that carries a substitution for antibody detection (glutamate for alanine) at amino acid position 66 was cloned into the pSE420 expression vector (psE420-AnxA2A66E37).[6,7] For protein expression, E. coli cells transformed with the respective pSE420 plasmid were grown at 37 °C in LB medium supplemented with ampicillin to an optical density of 0.6 at 600 nm (OD600). Protein expression was then induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. After expression for 4 h, cells were harvested by centrifugation at 5000 x g for 10 min at 4°C. To disrupt the cells, a french press apparatus was employed. The cell pellet was thawed and filled up to 40 mL total with lysis buffer (50 mM imidazole, 300 mM NaCl, 10 mM MgCl2, 2 mM DTT and a tablet of Complete Protease Inhibitor EDTA-free (Roche), pH set to 7.4). After 3 rounds of french pressing the sample was centrifuged at 70400 x g for 1 h at 4 °C. The supernatant was dialyzed against DE52 Buffer (10 mM imidazole, 10 mM NaCl, 1 mM EGTA, 2 mM DTT, pH 7.4) overnight. DE52 material was equilibrated to the DE52 buffer, afterwards the protein solution was incubated rotating with the material for 1 h at 4 °C. Afterwards, the material was loaded into a glass column and allowed to settle. The flow through and a wash (35 mL of DE52 buffer) were collected. Both fractions were centrifuged at 70400 x g for 1 h at 4 °C and the supernatant was dialyzed against CM buffer (20 mM sodium acetate, 10 mM NaCl, 1 mM EGTA, 1 mM DTT, pH 5.6) overnight. Next day, 5 g of CM material was equilibrated to CM buffer and afterwards incubated rotating with the dialyzed protein solution for 2 h at 4 °C. The material was then transferred to a glass column and, after settling, the flow through was captured. The material was washed two times with CM buffer and the protein elution was performed by an increase

of the salt concentration. After two elution steps with 50 mM NaCl (15 mL), two elution steps with 100 mM NaCl (15 mL), finally the remaining bound protein was eluted by three times 10 mL buffer with 600 mM NaCl. The ion exchange chromatography steps (diethylamioethyl- and carboxymethyl-cellulose) were performed using Diethylaminoethyl Cellulose (DE52, Whatman, GE Healthcare, Fairfield, CT) and CM Sepharose™ Fast Flow (GE Healthcare, Sweden, now Cytiva) was used. To prevent disulfide mediated protein crosslink, AnxA2 was alkylated specifically at Cys-8 by 2-iodoacetamide treatment as previously described.[8] See Figure S6 for steps AnxA2 purification.

Model Membranes (GUVs and LUVs) and liposome binding assay

Giant unilamellar vesicles (GUVs) were generated employing the gentle agarose swelling method.[9,10] Briefly, lipid mixtures containing DPPC:DLiPC:Chol (60:40:20) were prepared from 5 mM stock solutions and added to a thin film of agarose on a 2-well ibidi slide. The film was prepared by addition of a 1% w/v solution of ultra-low gelling agarose in double distilled water at 60°C. The lipid mixture was allowed to dry for 30 min in vacuum, then equilibrated in HEPES buffer for 1 h, followed by labeling with FAST DiI (5 µM) and staining ~~and~~ with CHIM-L or CHIM-X ~~or CHIM-X-C8~~ pre-clicked to AF405 (5 µM).

Large unilamellar vesicles (LUVs) composed of POPS/POPC/CHOL/X-CHIM (20:60:5:15) were used in liposome binding assay to ensure efficient co-pelleting by preventing rupture, often observed with high curvature vesicles. LUVs were prepared via extrusion using membranes with a 200 nm pore size, and final concentration of 1 mg/mL. Liposomes were incubated with sDIBO-biotin for 30 min at 4 °C to allow the binding to X-CHIM via click-chemistry. Next, 15 µg AnxA2 was added and let rotating for 1 h at 4 °C in HEPES-buffer containing 1 mM $CaCl_2$. Afterwards one sample was irradiated for 10 min at a wavelength of 365 nm to activate the crosslinking, while another sample was excluded from irradiation. A first fraction (I) was kept, and the remaining liposome-protein mixture was ultracentrifuged (UC) to pellet the liposomes (96600 g, 4 °C for 20 min), the supernatant (fraction II) was collected and the pellet resuspended in 200 µL of buffer with 5 mM EGTA to elute Ca^{2+}-dependently and non-crosslinked AnxA2 and incubated for 20 min at 4 °C. After a second UC, the supernatant (fraction III) was collected and the pellet (fraction IV) was resuspended in 200 µL buffer. All fractions were analyzed via SDS-PAGE, followed by immunoblotting with AnxA2-specific antibody.[11]

Western Blot

Equal amounts of fraction I to IV were mixed with Laemmli sample buffer were heated at 95 °C for 10 min, cooled down and subjected to 15% SDS–PAGE and transferred to nitrocellulose membranes (GE Healthcare Life Science). Membranes were blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 (TBST) (1 h, RT) and incubated with primary antibody mouse monoclonal anti-AnxA2 directed against an N-terminal peptide of the protein (HH7) or fluorophore-conjugated Streptavidin (IRDye® 800CW Streptavidin) overnight at 4 °C. After washing and treatment with goat anti-mouse IgGs conjugated with IRDye800CW (LI-COR Biosciences, Lincoln, USA) secondary antibodies for 1 h

at RT. After washing, immunoreactive bands were visualized using the Odyssey Infrared Imaging System (Li-COR Bioscience).

LDH-cytotoxicity assay

Determination of the cytotoxicity of various CHIM molecules was performed on MDCK type II using the LDH Cytotoxicity Detection Kit and according to the manufacturer's instructions (TaKaRa Bio Inc). The cell treatment was performed with CHIM-L, CHIM-X, CHIM-X8 or DMSO for 1 h, 5 h or 24 h at 37 °C. Percent cytotoxicity was calculated using the manufacturer's equation, with 1% Triton added to the cells for the high control.

Cell culture and live cell microscopy

HUVECs (Promocell) was cultured in growth medium (1:1 mixture of ECGM2 and M199) +100 i.u. heparin + 10% FCS (Sigma-Aldrich), supplemented with 30 µg/ml gentamycin and 0.015 µg/ml amphotericin B, cultivated at 37 °C and 5% CO_2. HeLa, A549, VERO, CaLu 3 and MDCK type II cells were cultured in DMEM supplemented with 10% standardized fetal bovine serum (FBS Superior, Biochrom), 100 U/mL penicillin and 0.1 mg/mL streptomycin cultivated at 37 °C and 5% (A549/VERO/CaLu 3) or 7% (MDCK/HeLa) CO_2 atmosphere.

For microscopy, HeLa, MDCK type II, HUVEC A549, CaLu 3 and VERO were seeded in 8 well-chambered glass bottom coverslips (ibidi). In the case of HUVEC, all dishes or slide surfaces were coated with collagen. Cell density was adjusted to reach approx. 70% confluency at the time of imaging. Live imaging was performed utilizing an LSM 780 confocal laser scanning microscope (CLSM, Carl Zeiss) equipped with a heating and CO_2 chamber. CLSM possesses an Argon-Ion laser (LASOS) and a Plan-Apochromat x 63 /1.4 oil immersion differential interference contrast objective lens (Carl Zeiss). All cell lines were regularly tested for mycoplasma contamination.

Cell surface click-reaction and uptake of pre-clicked X-CHIM or X-CHIM-C8

Bioorthogonal click reactions to label CHIM-L or CHIM-X were performed in a copper free manner with Click-iT-DIBO Alexa Fluor® 647, click-iT DIBO Alexa Fluor® 488 (Thermo Fischer Scientific), or AFDye 405 DBCO (Click Chemistry Tools). Therefore, HeLa, MDCK type II, HUVEC, A549, CaLu 3 and VERO cells were incubated with X-CHIM (prepared in full medium, DMEM for HeLa, MDCK type II, A549, CaLu 3 and VERO or mixed ECGM2 and M199 medium for HUVEC) either at room temperature or on ice (in the latter case the medium also contained 20 mM HEPES, pH 7.4) and then washed 4 times at the respective temperature. Labeling via click reaction was then performed by adding the respective Click-iT DIBO-dye in the same medium on ice, followed by washing the cells four times with PBS+/+ (containing Ca2+ and Mg2+) and two times with the respective growth medium containing 20 mM HEPES. For incorporation of pre-clicked (dye-labeled) CHIM; HeLa, MDCK type II, HUVEC, A549, CaLu

3 and VERO were cooled down on ice for 20 min and then treated for an additional 20 min on ice with 3 or 5 µM of a pre-clicked 1:1 mixture of X-CHIM and the alkyne-fluorophore, followed by four times washing with PBS+/+ and two times with the respective growth medium containing 20 mM HEPES. For specific concentrations and incubation time see figure legends.

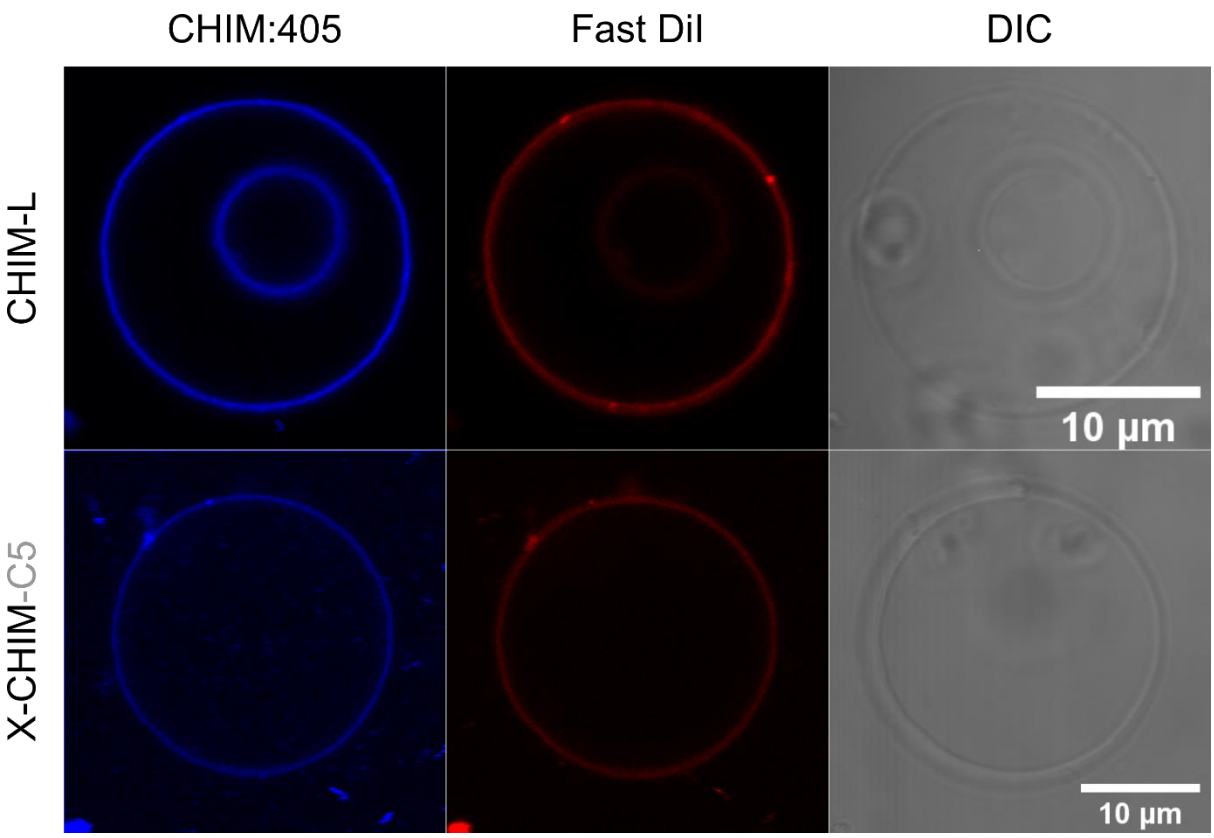
For BODIPY-cholesterol and/or CHIM-X (AF405) labelling HeLa cells were cooled to room temperature for 20 min in full growth medium containing 20 mM HEPES. AF405-labeled CHIM-X and BODIPY-cholesterol were diluted to 5 µM in full growth medium containing 20 mM HEPES, and then given to the for 60 min which were then subjected to live cell imaging.

Cell treatment with CHIM-X for SDS PAGE

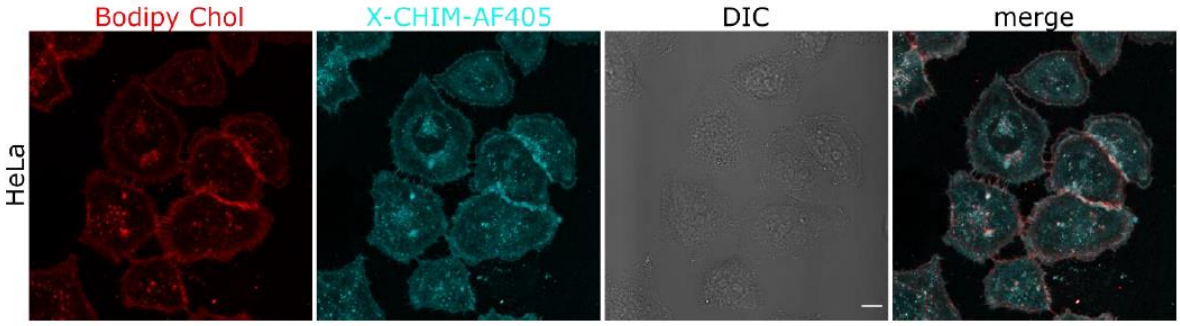
Cells were grown in 10 cm dishes to full confluency and incubated with CHIM-X, (2 µM) treatment in full medium supplemented with HEPES (20 mM) for 1 h also on ice or in full medium overnight. Thereafter, cells were washed twice with cold PBS (-/-) and either illuminated with UV light (365 nm, 3 W) for 10 min (+UV) while cooled on ice or directly scraped without UV irradiation (-UV) and lysed by sonification in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 1× Complete EDTA-free Proteinase Inhibitor Cocktail) on ice and the cell suspension was sonicated for 1 minute (amplitude 100, 0.5 cycle). Incubation of the cells with the fluorophore IRDye 800CW DBCO (2 µM) for the click reaction with the CHIM-X was performed after scraping and before lysis.

After centrifugation (15,000g, 10 min, 4°C), the protein concentration of the cell lysates was measured using BCA protein assay reagent (Thermo Fisher Scientific). Equal amounts of total protein in Laemmli sample buffer were subjected to 15% SDS–PAGE and scanned for the IRDye signal using the Odyssey Infrared Imaging System (Li-COR Bioscience). Lastly, stained with Coomassie brilliant blue staining for bands identification.

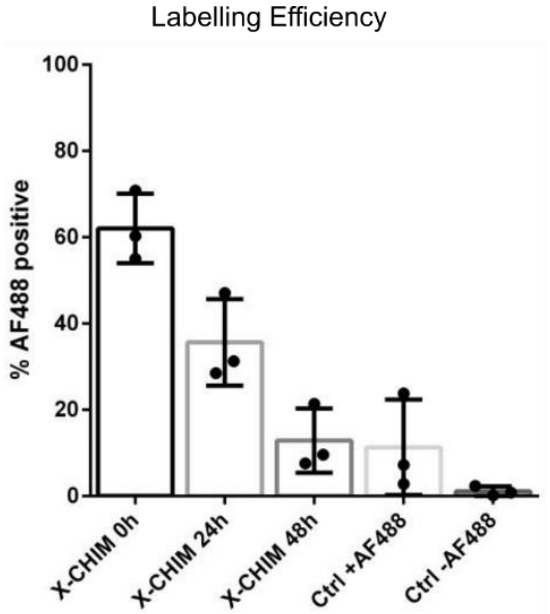
2.2 Incorporation of X-CHIM in cell and model membranes



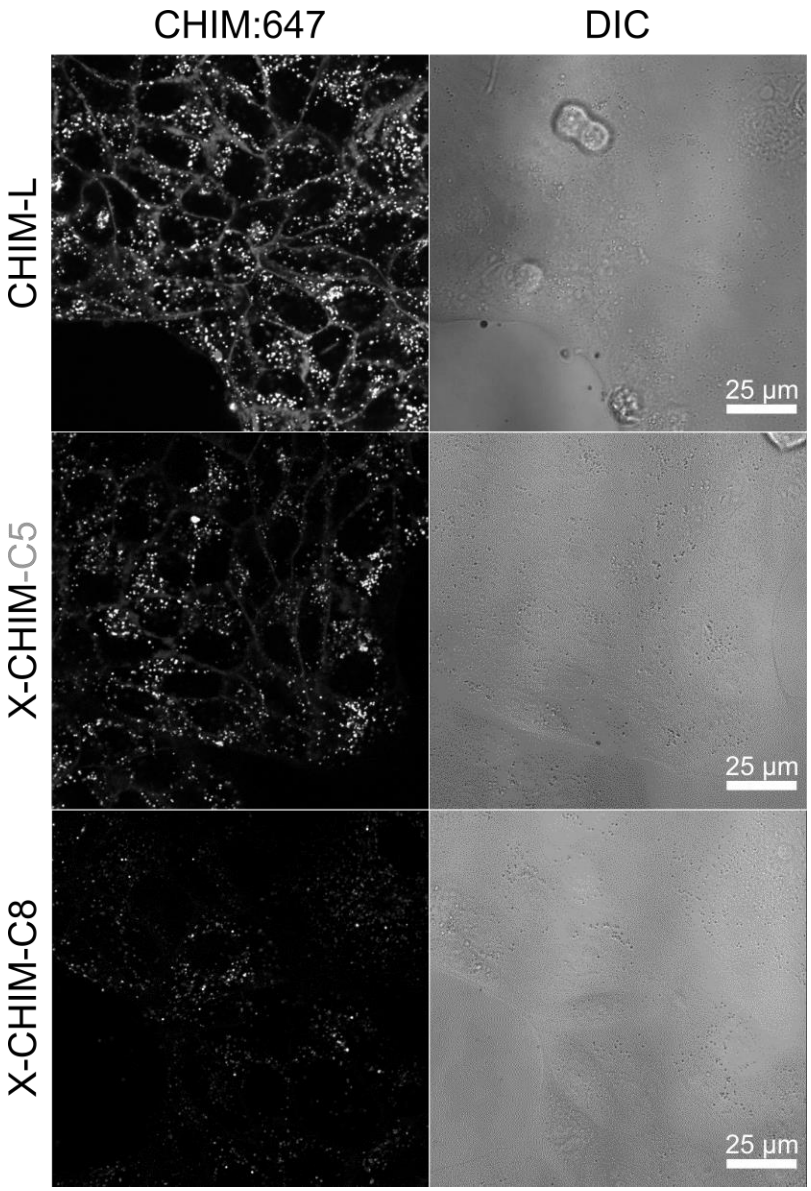
S1. Visualization of membrane incorporation into model membranes for CHIM derivatives pre-clicked to AF405. DPPC:DLiPC:Chol (60:40:20) based GUVs were formed by agarose swelling, treated with the dye Fast DiL for membrane staining and with CHIM-L or CHIM-X-C5 pre-clicked to AF405.



S2. HeLa cells co-incubated with X-CHIM (click labeled with AF405) and BODIPY-cholesterol. Cells were cooled down to room temperature and treated with medium containing AF405-labeled X-CHIM and BODIPY-cholesterol at 5 µM for 60 min. Cells were washed two times with PBS++ and imaged in the respective medium containing 20 mM HEPES. Scale bar, 10 µm.

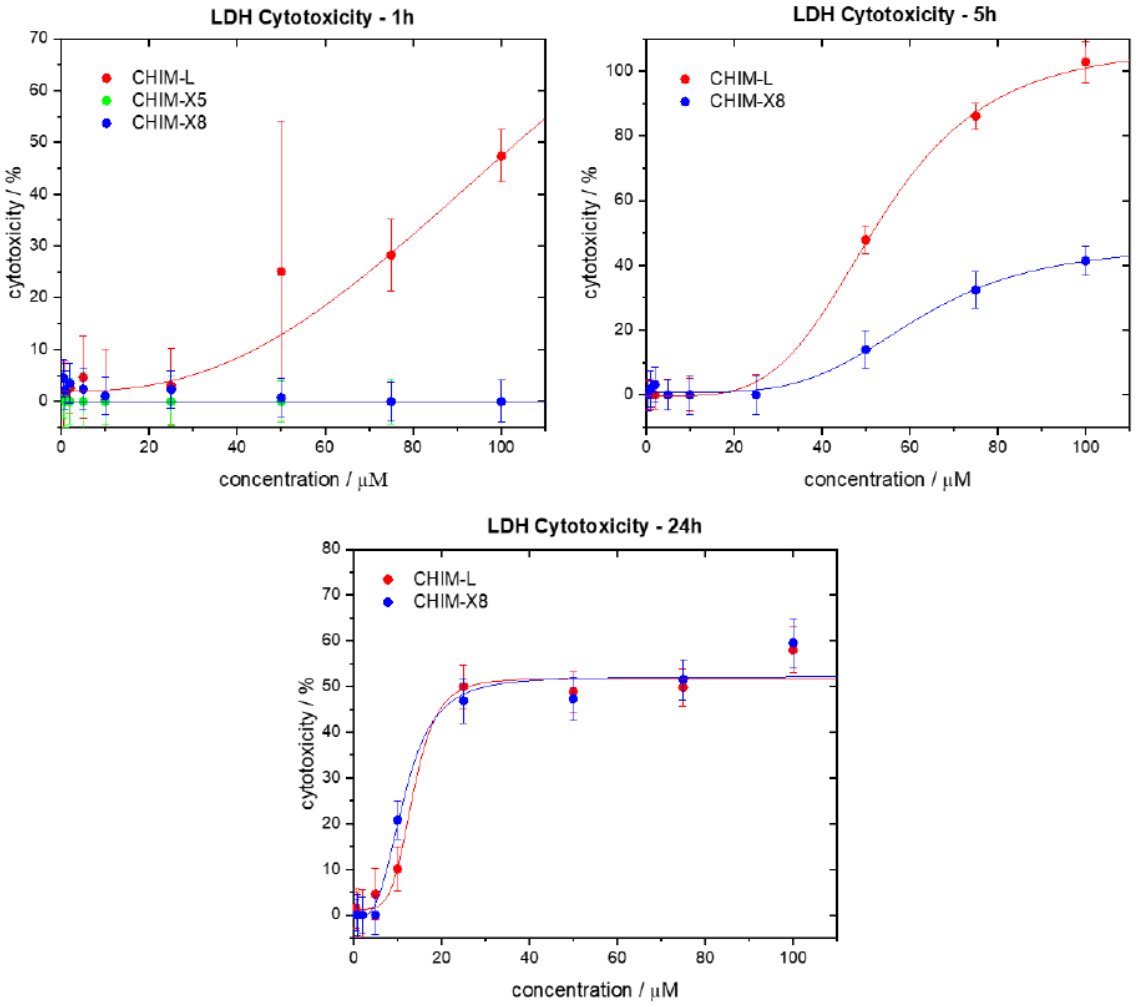


S3. Quantification of labeling efficiency. The percentage of DBCO-Alexa Fluor 488 positive cells was measured for HeLa cells subjected to click-chemistry 0, 24 or 48 h after incubation with X-CHIM. In a control (Ctrl), non-X-CHIM-treated cells were incubated with the Alexa fluorophore. The efficiency of the cell surface labeling was measured by performing FACS. Cells were seeded in 6 well dishes and cell surface click-reaction was performed. Cells were then detached by adding 0.5 mL Accutase (GE healthcare, Little Chalfont, UK) per well, centrifuged for 4 min, at 4 °C, 200 x g, washed once with PBS + 5 % BSA and centrifuged again. Finally, cells were resuspended in PBS + 5 % BSA. Flow cytometry and data analysis were performed using a Guava easyCyte12 Cytometer and the InCyte Software (MilliporeSigma). Mean and standard deviations were obtained from three (n=3) individual experiments.



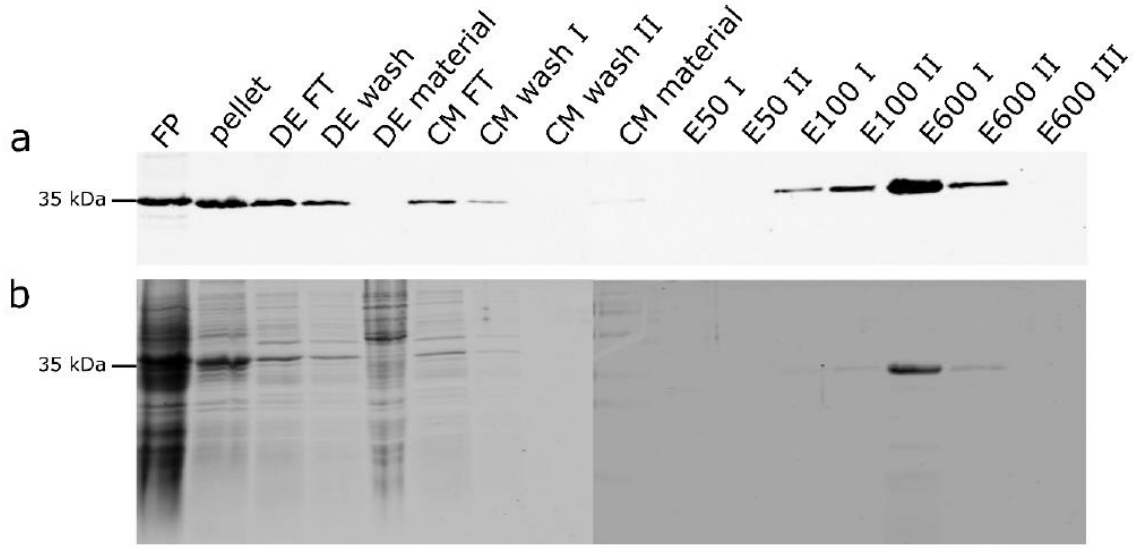
S4. Visualization of membrane incorporation and distribution for CHIM molecules pre-clicked with AF647 in MDCK cells. MDCK cells were incubated with 5 µM pre-clicked CHIM:AF647 molecules for 1 h at 37 °C and after washing, live cell images were taken at 37 °C with the same laser power and settings for the CHIM-L, X-CHIM-C5 and X-CHIM-C8 treated cells.

2.3 LDH-Cytotoxicity Assay



S5. LDH-Cytotoxicity-Assay for CHIM-L, CHIM-X-C5 and CHIM-X-C8 on MDCK cells. MDCK cells were treated with different concentrations (between 0.5 – 100 µM) of CHIM-L (red), CHIM-X-C5 (green) or CHIM-X-C8 (blue) for 1 h, 5 h or 24 h and percent cytotoxicity was determined using the LDH cytotoxicity assay. 1% Triton-treatment served as a reference for 100% cytotoxicity.

2.4 Annexin A2 Purification



S6. Steps of Annexin A2 (AnxA2) purification. Bacteria expressing recombinant AnxA2 were lysed and the soluble fraction passed over a DE52 ion exchange column. The non-bound fraction (DE FT) containing the majority of AnxA2 was dialyzed and subjected to CM ion exchange chromatography. AnxA2 bound to the column (hardly any AnxA2 in the CM FT and CM wash fractions) and was eluted in a NaCl step gradient in CM buffer. a) Western Blot stained with mouse monoclonal anti-AnxA2 antibodies (HH7) as primary antibodies and IRDye800CW (anti mouse) as secondary antibodies. b) Coomassie staining of a 15 % SDS-PAGE gel of aliquots taken of the different purification steps. Fraction E600 I which contains the majority of purified AnxA2 was dialyzed against 20 mM HEPES and used for further experiments. FT = Flow through, E = Elution with 50/100/600 mM NaCl.

3. Supporting References

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