ELECTRONIC SUPPLEMENTARY INFORMATION

A multi-valent polymyxin-based fluorescent probe for the detection of Gram-negative infections.

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1. Materials and Methods

1.1. General methods

Savinase (Protease from Bacillus sp., glycerol suspension at ≥16 U/g, P3111) was purchased from Sigma Aldrich. Polymyxin B sulphate was purchased from Apollo Scientific Biochemicals and used as a mixture of Polymyxin B1 and B2. All other commercially available reagents were purchased from Sigma Aldrich, Thermo Fisher or Fluorochem and used as received. Thin-layer chromatography was performed on aluminium sheets coated with silica gel containing the phosphor F254 and were visualised by UV-illumination (λ = 254 nm and 365 nm). Flash column chromatography was performed using an appropriately sized glass column filled with silica gel 60 (mesh 0.040-0.063). NMR spectra were recorded on an automated Bruker AV500 in the indicated deuterated solvents at 298 K. Chemical shifts are reported on the δ scale in parts per million (ppm) and are referenced to the residual non-deuterated solvent peak for ^{1}H NMR, and to the deuterated carbon of the solvent for ^{13}C NMR. Coupling constants (*J*) are given in Hertz. Analytical reverse-phase high-performance liquid chromatography (RP–HPLC) was performed on an Agilent 1100 system equipped with a Kinetex XB-C18 column (50 x 4.6 mm, 5 µm) with a flow rate of 1 mL/min and eluting with H_2O/Acetonitrile (95/5) to H_2O/Acetonitrile (5/95) all containing 0.1% formic acid, over 6 min, holding at 95% for 3 min, with detection at 254, 282, or 495 nm and by an evaporative light scattering (ELS) detector. Semi-preparative HPLC was performed on an Agilent 1100 system equipped with a Aeris XB-C18 column (250 x 10 mm, 5 µm) with a flow rate of 2 mL/min and eluting with H_2O/Acetonitrile (95/5) to H_2O/Acetonitrile (5/95) all containing 0.1% formic acid, over 35 min, holding at 95% for 3 min, with detection at 254, 282 and 495 nm. Electrospray ionization mass spectrometry (ESI–MS) analyses were carried out on an Agilent Technologies LC/MSD Series 1100 quadrupole mass spectrometer (QMS) in ESI mode. HRMS in ESI mode were obtained by the Mass Spectrometry department of the University of Edinburgh and were performed on a Finnigan MAT 900 XLP high resolution double-focussing mass spectrometer. HRMS in FT-MS mode were recorded on a Bruker SolariX Fourier transform ion cyclotron resonance mass spectrometer. MALDI spectra were acquired on a Bruker Ultraflextreme MALDI TOF/TOF with a matrix solution of sinapic acid (10 mg/mL) in H_2O/CH_3CN/TFA (50/50/0.1). Infrared spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer. Microwave-assisted reactions were performed on a Biotage Initiator 2.0 using 0.5 to 20 mL vials.

1.2. Probe structures

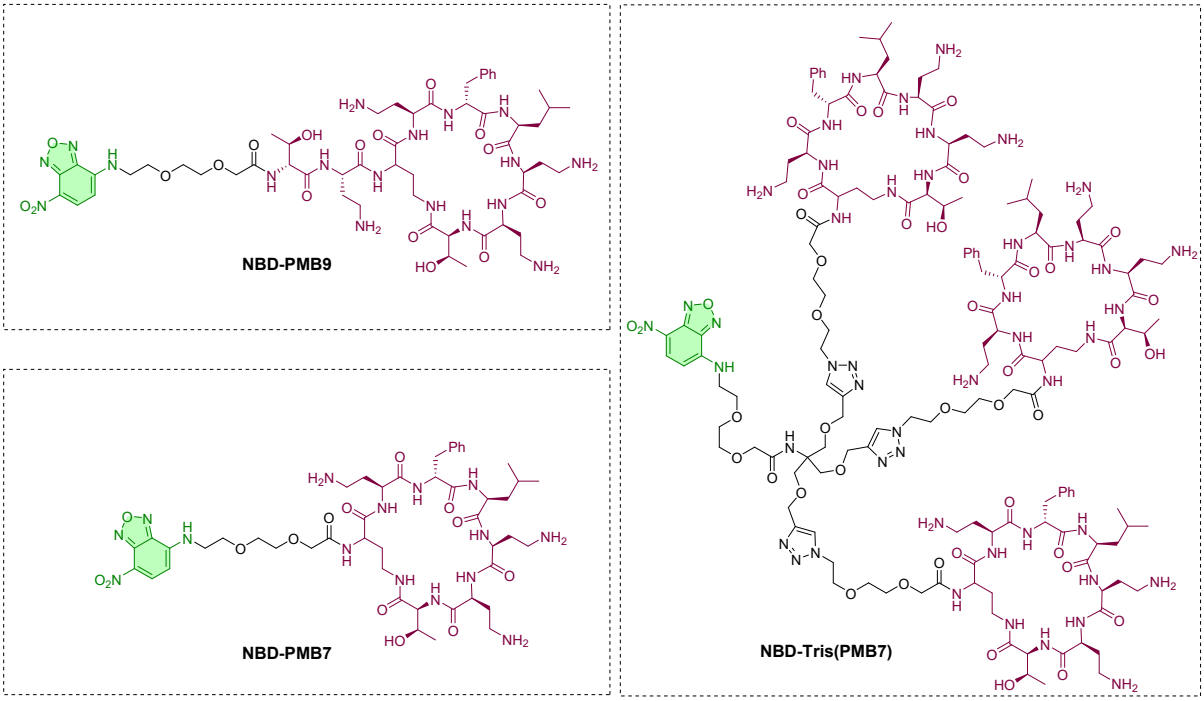


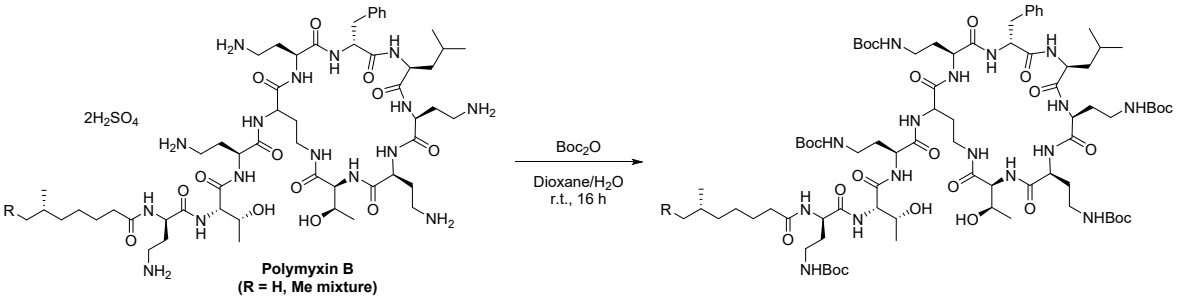
Figure S1. Chemical structures of the polymyxin B-derived fluorescent probes NBD-PMB9, NBD-PMB7 and NBD-Tris(PMB7). The polymyxin binding domains are represented in purple, NBD fluorophore in green, and spacers in black.

1.3. Synthesis and procedures

NBD-$(EG)_2$-CO_2H and its corresponding NHS-ester were synthesized according to literature procedures.[1]

1.3.1. Synthesis of the Boc-protected PMB7 derivative (**2**)

Boc-protected Polymyxin B (1)

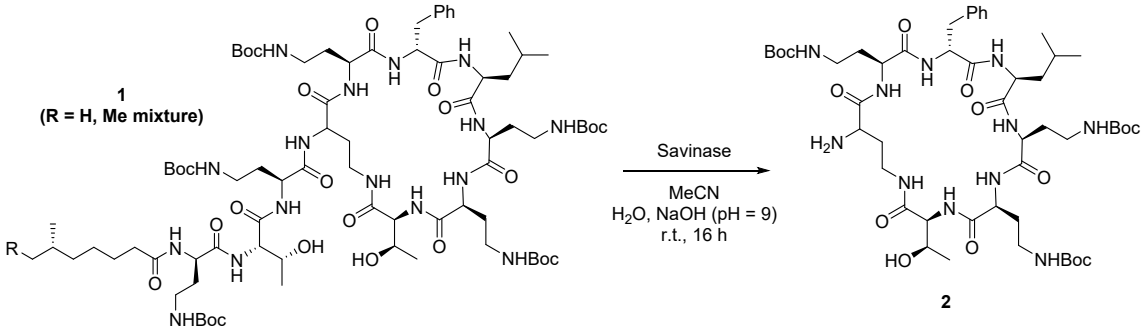


Polymyxin B sulphate (2.0 g, 1.43 mmol, 1.0 equiv.) was dissolved in water (20 mL) followed by the addition of 1,4-dioxane (40 mL) and left to stir for 10 minutes at room temperature. To the reaction mixture was added Boc anhydride (4.37 g, 20.0 mmol, 14 equiv.), and the reaction was stirred at room temperature and was monitored by RP-HPLC. The pH of the reaction mixture was then adjusted to 6

using 1 M HCl, the resulting precipitate was collected by filtration, washed with water (50 mL) and hexane (50 mL) then dried under vacuum to yield the Boc_5-protected Polymyxin as a white solid (2.4 g, quant.).

LC-MS (ESI): *m/z* = 1703.8 $[M+H]^+$. **HRMS (ESI):** *m/z* = 1704.0180 and 1725.9991; calcd for $C_{81}H_{139}N_{16}O_{23}$ $[M+H]^+$: 1704.0194 and $C_{81}H_{138}N_{16}O_{23}Na$ $[M+Na]^+$: 1726.0013. **HPLC (ELS detection):** t_R = 7.4 min

di-*tert*-butyl(((2*R*,5*R*,8*R*,11*R*,14*S*,17*S*)-22-amino-5-benzyl-11-(2-((*tert*-butoxycarbonyl)amino)ethyl)-17-((*S*)-1-hydroxyethyl)-8-isobutyl-3,6,9,12,15,18,23-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosane-2,14-diyl)bis(ethane-2,1-diyl))dicarbamate (2)

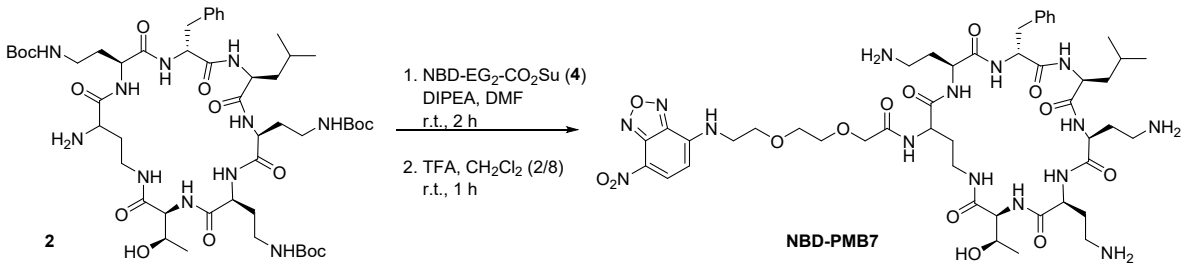


Boc-protected polymyxin B **1** (400 mg, 0.235 mmol, 1.0 equiv.) was dissolved in acetonitrile (66 mL) and water (34 mL), followed by addition of NaOH (2M) to give a pH = 9. Savinase (10 mL, ≥16 U/g, suspension in glycerol) was then added, and the mixture was stirred at r.t. overnight. The reaction was monitored by RP-HPLC and TLC, showing total conversion of starting material and MS confirmed the formation of the cleaved $(Boc)_3$heptapeptide. The reacting mixture was then diluted with AcOEt (60 mL), and the organic layer separated. The organic layer was washed with NaOH (0.1 M, 2 x 30 mL) and water (30 mL), then dried over Na_2SO_4, filtered and evaporated. The crude was precipitated in CH_2Cl_2/Pentane (1/9) and the supernatant was removed. The resulting solid was washed with Et_2O (2 x 15 mL) to give the cyclic peptide as an off-white powder (180 mg, 72%).

LC-MS (ESI): *m/z* = 1062.8 $[M+H]^+$. **HRMS (ESI):** *m/z* = 1062.6200 and 1084.6015; calcd for $C_{50}H_{84}N_{11}O_{14}$ $[M+H]^+$: 1062.6194 and $C_{50}H_{83}N_{11}O_{14}Na$ $[M+Na]^+$: 1084.6019. **HPLC (ELS detection):** t_R = 4.7 min.

1.3.2. Synthesis of the mono-branched NBD-PMB7 probe

2-(2-(2-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)ethoxy)ethoxy)-*N*-((3*S*,6*S*,9*R*,12*R*,15*R*,18*R*)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-((*S*)-1-hydroxyethyl)-12-isobutyl-2,5,8,11,14,17,20-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosan-21-yl)acetamide (NBD-PMB7)



A solution of *N*-(4-Nitrobenz-2-oxa-1,3-diazol-7-yl)amino-3,6-dioxaoctanoic acid succinimidyl ester (NBD-$(EG)_2$-CO_2Su **4**,[1] 14 mg, 32.9 µmol, 2.5 equiv.), DIPEA (7 µL, 40.0 µmol, 3 equiv.) and the Boc-protected polymyxin derivative **2** (14 mg, 13.2 µmol, 1 equiv.) in anhydrous DMF (1 mL) was stirred at room temperature for 2 h in the dark. After completion of the reaction (TLC), solvents were removed under vacuum. Analysis of the crude confirmed formation of the Boc-protected product.

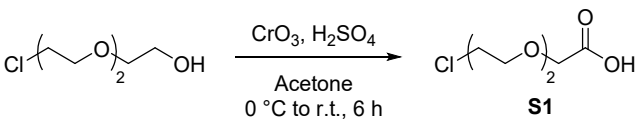
HPLC (495nm): t_R = 6.1 min. **LC-MS (ESI):** *m/z* = 1370.6 $[M+H]^+$

The crude was then dissolved in 20% TFA in DCM (2 mL) and vigorously stirred for 1 h at room temperature in the dark. The reaction mixture was evaporated, and to the product was precipitated with Et_2O and collected by centrifugation (3 × 1 mL washes). The resulting crude was purified by semi-preparative RP-HPLC eluting with a H_2O/MeCN mixture (0.1% formic acid buffer; see materials & methods). The combined fractions were freeze-dried to afford **NBD-PMB7** as an orange solid.

LC-MS (ESI): *m/z* = 1071.5 $[M+H]^+$; 535.9 $[M+2H]^{2+}$; 357.6 $[M+3H]^{3+}$. **HRMS (MALDI):** *m/z* = 1092.6721, calcd for $C_{47}H_{71}N_{15}O_{14}Na$ $[M+Na]^+$: 1092.5197. **HPLC (495 nm):** t_R = 2.7 min.

1.3.3. Synthesis of the clickable polymyxin-azide intermediate **5**

2-(2-(2-chloroethoxy)ethoxy)acetic acid (S1)[2]

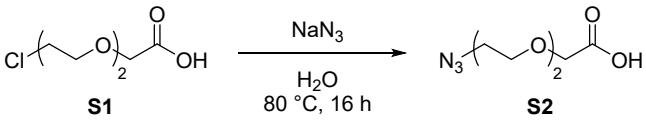


Chromium trioxide (11.9 g, 118 mmol, 5 equiv.) was slowly dissolved in H_2SO_4 (1.5 M, 140 mL, 214 mmol, 9 equiv.) at 0 °C. A solution of 2-[2-(2-Chloroethoxy)ethoxy]ethanol (4 g, 23 mmol, 1 equiv.) in acetone (79 mL) was then added dropwise at 0 °C, and the resulting mixture was stirred at r.t. for 6 h.

The mixture was then partially concentrated under vacuum to remove the acetone, then the aqueous layer was extracted with CH_2Cl_2 (4 x 40 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to give the crude acid as a clear oil which was used without further purification in the next step (3.0 g, 69%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.53 (s, 1H), 4.20 (s, 2H), 3.80 – 3.73 (m, 4H), 3.72 (dt, *J* = 5.9, 1.9 Hz, 2H), 3.63 (t, *J* = 5.9 Hz, 2H). **^{13}C NMR (126 MHz, $CDCl_3$) δ** 174.7, 71.5, 71.2, 70.6, 68.5, 42.7. **LC-MS (ESI):** *m/z* = 181.1 $[M-H]^-$. **HPLC (ELS detection):** t_R = 2.5 min.

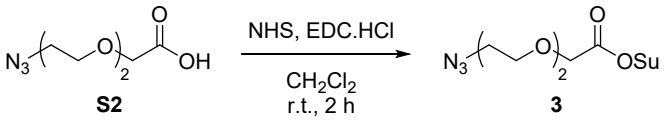
2-(2-(2-azidoethoxy)ethoxy)acetic acid (S2)[2]



To a stirred solution of **S1** (2.0 g, 11.0 mmol, 1 equiv.) in water (11 mL) was added NaN_3 (2.84 g, 43.8 mmol, 4 equiv.). The mixture was stirred for 16 h min at 80 °C, then it was cooled to r.t., and the reaction mixture was acidified with aq HCl (1M) and extracted with AcOEt (3 x 25 mL). The combined organic layers were dried over Na_2SO_4, filtered and evaporated to give **S2** as a light brown oil (1.73 g, 83%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.97 (s, 1H), 4.17 (s, 2H), 3.80 – 3.59 (m, 6H), 3.38 (t, *J* = 5.0 Hz, 2H). **^{13}C NMR (126 MHz, $CDCl_3$) δ** 174.5, 71.1, 70.5, 70.0, 68.4, 50.7. **HPLC (ELS detection):** t_R = 2.8 min.

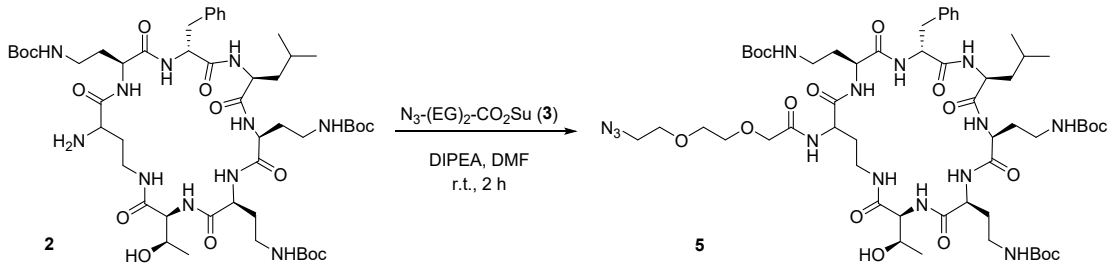
2,5-dioxopyrrolidin-1-yl 2-(2-(2-azidoethoxy)ethoxy)acetate (3)



To a stirred solution of the azido-EG_2 linker **S2** (200 mg, 1.06 mmol, 1 eq.) and NHS (122 mg, 1.06 mmol, 1 eq.) in CH_2Cl_2 (2.6 mL) was added EDC.HCl (203 mg, 1.06 mmol, 1 eq.). The reaction mixture was stirred at r.t. for 2 h, then it was diluted with CH_2Cl_2 (15 mL) and HCl (1M, 10 mL) was added. The organic layer was separated, then washed with more HCl 1M and with water, dried over Na_2SO_4. Filtration and concentration under reduced pressure yielded the activated ester **3** as a pale brown oil (246 mg, 81%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.52 (s, 2H), 3.83 – 3.79 (m, 2H), 3.73 – 3.69 (m, 2H), 3.67 (dd, *J* = 5.6, 4.6 Hz, 2H), 3.44 – 3.36 (m, 2H), 2.85 (s, 4H). **^{13}C NMR (126 MHz, $CDCl_3$) δ** 168.9, 166.1, 71.6, 70.8, 70.2, 66.8, 50.8, 25.7. **HPLC (ELS detection):** t_R = 3.8 min.

di-*tert*-butyl (((2*R*,5*R*,8*R*,11*R*,14*S*,17*S*)-22-(2-(2-(2-azidoethoxy)ethoxy)acetamido)-5-benzyl-11-(2-((*tert*-butoxycarbonyl)amino)ethyl)-17-((*S*)-1-hydroxyethyl)-8-isobutyl-3,6,9,12,15,18,23-heptaoxo-1,4,7,10,13,16,19-heptaazacyclotricosane-2,14-diyl)bis(ethane-2,1-diyl))dicarbamate (5)

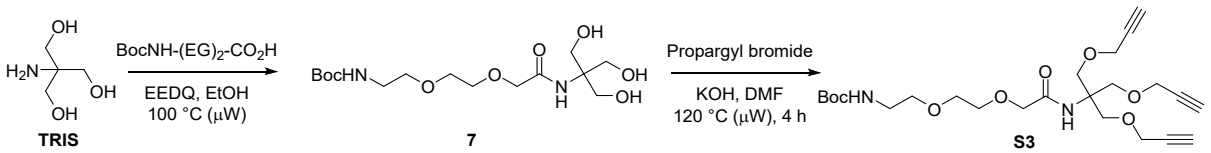


Succinimidyl-8-azido-3,6-dioxaoctanoate **3** (129 mg, 0.451 mmol, 1.1 equiv.), compound **2** (480 mg, 0.5 mmol, 1.0 equiv.) and anhydrous DIPEA (58 µL, 0.5 mmol, 1.1 equiv.) were dissolved in anhydrous DMF (4.5 mL) and stirred at room temperature for 16 h. The solvent was then removed under reduced pressure, and the mixture was dissolved in CH_2Cl_2 (30 mL). The solution was then washed successively with aqueous HCl (0.1M, 15 mL), with saturated aqueous $NaHCO_3$ (2 x 20 mL), and with water (20 mL), then dried over Na_2SO_4 and concentrated under reduced pressure to give compound **5** as a white solid (374 mg, 67%).

FT-IR *ν* (cm^{-1}): 3306 m, 2932 m, 2120 m, 1651 s, 1519 s, 1251 s; **HRMS (ESI):** *m/z* = 1255.6655, calcd for $C_{56}H_{92}N_{14}O_{17}Na$ $[M+Na]^+$: 1255.6657. **HPLC (ELS detection):** t_R = 5.9 min.

1.3.4. Synthesis of the NBD-Tris(PMB7) probe by CuAAC

***N*-Boc-2-[2-(2-aminoethoxy)ethoxy]ethanamide, *N*-[2-(2-propyn-1-yloxy)-1,1-bis[(2- propyn-1-yloxy)methyl]ethyl] (S3)**



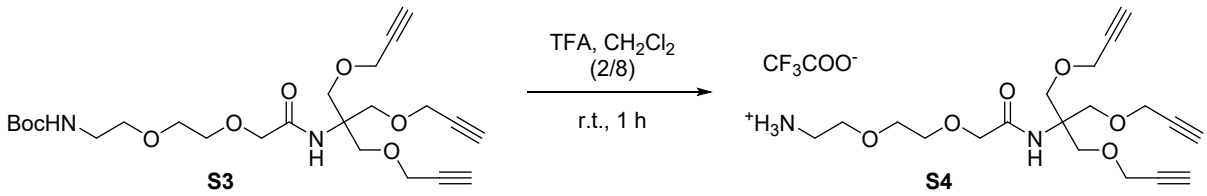
A solution of TRIS (500 mg, 4.13 mmol, 1.0 equiv.), *N*-Boc-3-[2-(2-aminoethoxy)ethoxy]propionic Acid (BocNH-$(EG)_2$-CO_2H, 1.09 g, 4.13 mmol, 1.0 equiv.) and EEDQ (1.02 g, 4.13 mmol, 1 equiv.) in ethanol (10 mL) was placed in a sealed microwave vial and heated to 100 °C in a μW reactor for 2 h. After cooling to r.t., the mixture was concentrated under high vacuum at 60 °C. The resulting clear oil was used directly in the following step.

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.13 (s, 1H), 4.11 – 3.89 (m, 4H), 3.81 – 3.60 (m, 11H), 3.58 – 3.50 (m, 2H), 3.31 (s, 2H), 1.45 (s, 9H). **LC-MS (ESI):** *m/z* = 367.3 $[M+H]^+$. **HPLC (ELS detection):** t_R = 3.0 min.

For propargylation, a solution of this crude (800 mg, 2.18 mmol, 1 equiv.) in anhydrous DMF (6 mL) was placed in a microwave vial at 0°C, and finely grounded KOH (858 mg, 15.2 mmol, 7.0 equiv.) was added. Propargyl bromide (80% wt. in toluene, 1.45 mL, 13.1 mmol, 6.0 equiv.) was added dropwise to the mixture. The vial was then flushed with nitrogen, sealed and heated to 120 °C in a microwave reactor for 4 h. After cooling to r.t., the mixture was diluted with water (30 mL), and extracted with ethyl acetate (20 mL). The mixture was separated and extracted with AcOEt (3 x 20 mL), then the combined organic layers were washed with water (3 × 30 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give a brown crude mixture. The crude brown oil was used without further purification in the following step. A sample of the crude was purified by column chromatography (DCM:MeOH 1:0 to 9:1) for analytical purposes.

^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.84 (br, 1H), 5.03 (br, 1H), 4.17 (d, 6H), 3.92 (s, 2H*),* 3.88 (s, 6H), 3.69 – 3.59 (m, 4H), 3.56 (t, *J* = 5.2 Hz, 2H), 3.34 (p, *J* = 5.4 Hz, 2H), 2.45 (t, *J* = 2.4 Hz, 3H), 1.46 (s, 9H). **^{13}C NMR** (126 MHz, Chloroform-*d*) δ 169.6, 156.0, 79.6, 79.3, 74.7, 71.0, 70.9, 70.5, 70.1, 68.5, 59.1, 58.7, 40.4, 28.4. **LC-MS (ESI)** *m/z* = 503.1 $[M+Na]^+$. **FT-HRMS:** *m/z* = 503.2380, calcd for $C_{24}H_{37}N_2O_8Na$ $[M+Na]^+$: 503.2364**; HPLC (ELS detection):** t_R = 5.1 min.

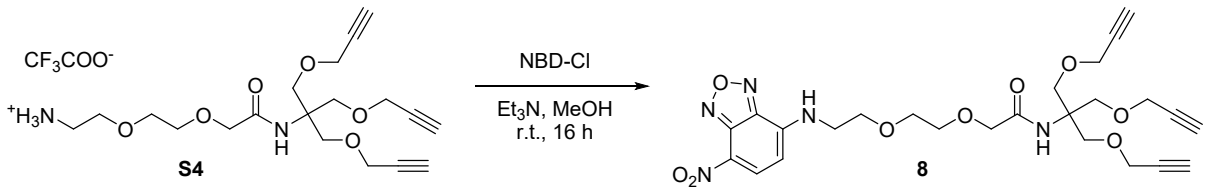
2-[2-(2-aminoethoxy)ethoxy]-ethanamide, *N*-[2-(2-propyn-1-yloxy)-1,1-bis[(2-propyn-1-yloxy)methyl]ethyl] (S4)



8-*tert*-butyloxycarbonylamino-3,6-dioxaoctanoic-tris(hydroxymethyl)propargyl **S3** (110 mg, mmol, 1 equiv.) was dissolved in 20% TFA in DCM (5 mL) and stirred at r.t. for 2 h. The solvent was evaporated under vacuum, then the residue was suspended in toluene and evaporated. The crude solid was then washed with Et_2O (3 x 10 mL) and dried under vacuum. The resulting powder was used without further purification in the following step (90 mg, quant.).

^{1}H NMR (500 MHz, CD_3OD) δ 4.19 (d, *J* = 2.4 Hz, 6H), 3.98 (s, 2H), 3.85 (s, 6H*)* 3.79 – 3.75 (m, 2H), 3.74 (d, 4H), 3.19 – 3.13 (m, 2H), 2.89 (t, *J* = 2.4 Hz, 3H), 1.31 (br, 3H). **^{13}C NMR** (126 MHz, CD_3OD) δ 170.7, 79.1, 74.8, 70.5, 70.0, 69.9, 67.9, 66.6, 59.3, 58.1, 39.3. **LC-MS (ESI):** $[M+Na]^+$ 403.1; **FT-HRMS:** *m/z* = 381.2022, calcd for $C_{19}H_{29}N_2O_6$ $[M+H]^+$: 381.20201; **HPLC (ELS detection):** t_R = 3.3 min.

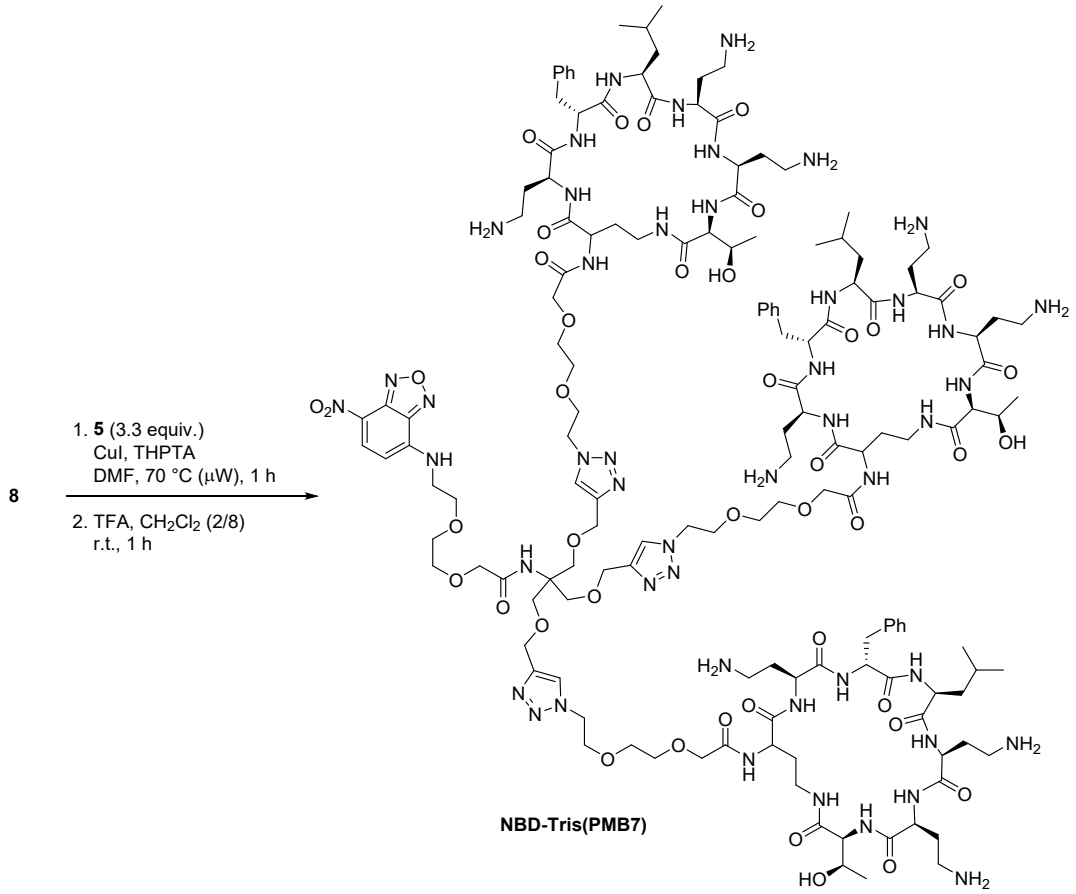
***N*-(1,3-bis(prop-2-yn-1-yloxy)-2-((prop-2-yn-1-yloxy)methyl)propan-2-yl)-2-(2-(2-((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl)amino)ethoxy)ethoxy)acetamide (8)**



4-Chloro-7-nitrobenzofurazan (182 mg, 0.910 mmol, 3 equiv.) was added portion-wise over 15 min to a stirred solution of crude compound **S4** (150 mg, 0.303 mmol, 1.0 equiv.) and Et_3N (200 µL, 1.52 mmol, 5.0 equiv.) in methanol (5 mL). The resulting dark brown reaction mixture was stirred overnight at room temperature. The solvent was then evaporated under reduced pressure, and the residue purified by column chromatography (DCM/MeOH, 1:0 to 9:1) to give **8** as a dark orange powder (yield over 4 steps: 34% starting from TRIS).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.48 (d, *J* = 8.6 Hz, 1H), 7.02 – 6.96 (m, 1H), 6.77 (s, 1H), 6.21 (d, *J* = 8.6 Hz, 1H), 4.14 (d, *J* = 2.4 Hz, 6H), 3.96 (s, 2H), 3.88 (dd, *J* = 5.6, 4.6 Hz, 1H), 3.86 (s, 6H), 3.76 – 3.69 (m, 6H), 2.42 (t, *J* = 2.4 Hz, 3H). **^{13}C NMR (126 MHz, $CDCl_3$) δ** 169.6, 144.4, 144.1, 136.4, 124.9, 99.0, 79.6, 74.8, 71.2, 71.0, 70.6, 68.7, 68.6, 59.3, 58.9, 29.8. **HR-MS (ESI):** *m/z* = 566.1861, calcd for $C_{25}H_{30}N_5O_9Na$ $[M+Na]^+$: 566.1857. **HPLC (495 nm detection):** t_R = 5.1 min.

Tri-branched probe NBD-Tris(PMB7)



A solution of tris-alkyne scaffold **8** (2.0 mg, 3.7 µmol, 1.0 equiv.), azide **5** (15.0 mg, 12.1 µmol, 3.3 equiv.), THPTA Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 0.5 mg, 1.1 µmol, 30 mol%) and CuI (0.2 mg, 1.1 µmol, 30 mol%) in anhydrous DMF (0.2 mL) was placed in a microwave vial and degassed with nitrogen for 10 min. The vial was then sealed with a PTFE cap and heated to 70 °C in a microwave reactor for 1 h. After cooling down to r.t., the mixture was concentrated under high vacuum and dissolved in 20% TFA in DCM (1 mL) and stirred at r.t. for 2 h. The solvent was evaporated under high vacuum, then the residue was dissolved in water (0.2 mL) and purified by semi-preparative HPLC eluting with a H_2O/MeCN mixture (0.1% formic acid buffer; see materials & methods). The combined fractions were freeze dried to afford **NBD-Tris(PMB7)** as an orange solid.

HR-MS (ESI): *m/z* = 1114.59059, calcd for $C_{148}H_{236}N_{47}O_{42}$ $[M+3H]^{3+}$: 1114.59198. **HPLC (495 nm detection):** t_R = 3.1 min.

2. Photophysical characterisation

2.1. Methods

All photophysical studies were performed with freshly-prepared air-equilibrated solutions at room temperature (298 K). UV/Vis absorption spectra of ~10^{-5} M solutions were recorded on an Agilent 8453 spectrophotometer. Steady-state fluorescence measurements were performed on solutions (ca. 10^{-6} M, optical density ≤ 0.1) contained in standard 1 cm thick cuvettes with excitation at the wavelength of the absorption maximum using a Shimadzu RF-6000 spectrofluorimeter.

2.2. Additional spectra

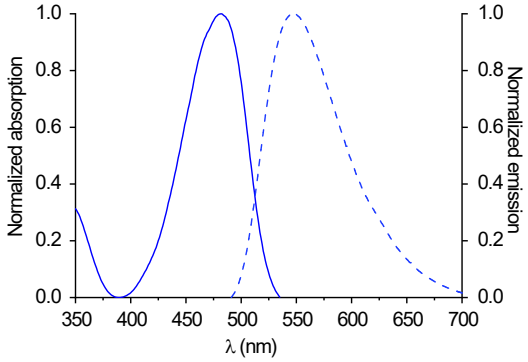


Figure S2. Normalised absorption (continuous) and emission (dashed) spectra of NBD-$(EG)_2$-CO_2H in DMSO.

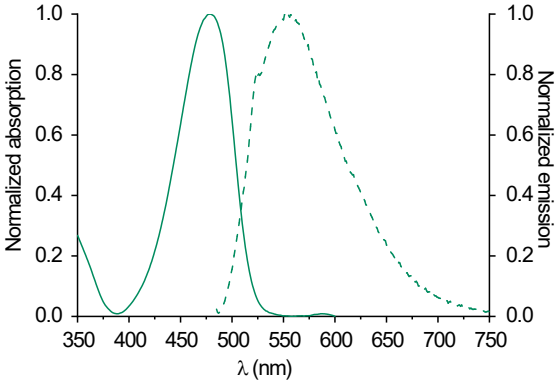


Figure S3. Normalised absorption (continuous) and emission (dashed) spectra of NBD-Tris(PMB7) in PBS.

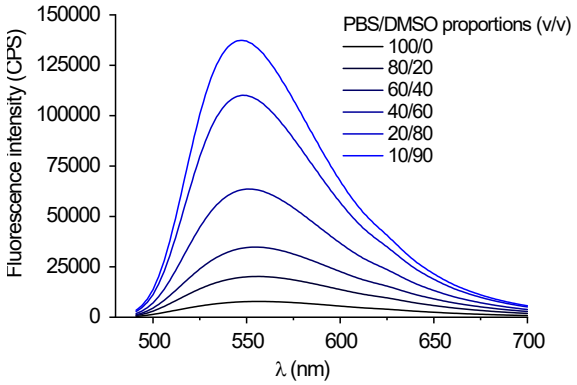


Figure S4. Evolution of the fluorescence intensity of solutions of NBD-$(EG)_2$-CO_2H (5 µM) with increasing percentages of DMSO in PBS upon excitation at 475 nm.

3. Bacterial imaging

3.1. Material and methods

Escherichia coli (DH5α), Micrococcus luteus (ATCC 4698), and *Bacillus subtilis* (ATCC 6051) were used as Gram-negative and Gram-positive test strains. Microbial culture broths and agar media (Invitrogen, UK; Fisher Scientific, UK; and Merck, , UK), buffers, and water were sterilized by autoclaving before use. All cultures were grown at 37 °C in a shaking incubator (200 rpm).

3.2 Bacteria labelling

Liquid cultures of *E. coli, M. luteus or B. subtilis* were washed with PBS and diluted to an OD600 of 0.5. Cells were attached to Ibidi 15 well glass bottom slides coated with poly-L-lysine. Different concentrations of probes were added to the bacteria and incubated at 37 °C for 1h. The solution was removed from the wells and the bacteria imaged under a confocal microscope without washing. Images were acquired using a Leica SP5 confocal spinning disk microscope (λ_{ex} = 480 nm, GFP filter settings, λ_{em} = 540 nm) with the LAS-AF software used to obtain the images which were then analysed by Fiji.

Bacteria were labelled with the commercial dye SYTO9 (Invitrogen, Paisly, UK) for comparison. A liquid culture of *E.coli* was prepared at a final density of 5 x 10^6 cfu/mL and the dye was addred to a concentration of 2.5 nM. The mixture was incubated for 30 minutes followed by adding to a glass slide and viewing under a Zeiss Axiovert 500M fluorescent microscope (60x objective) with λ_{ex} = 498 nm and λ_{em} = 505 nm.

3.3. Additional figures

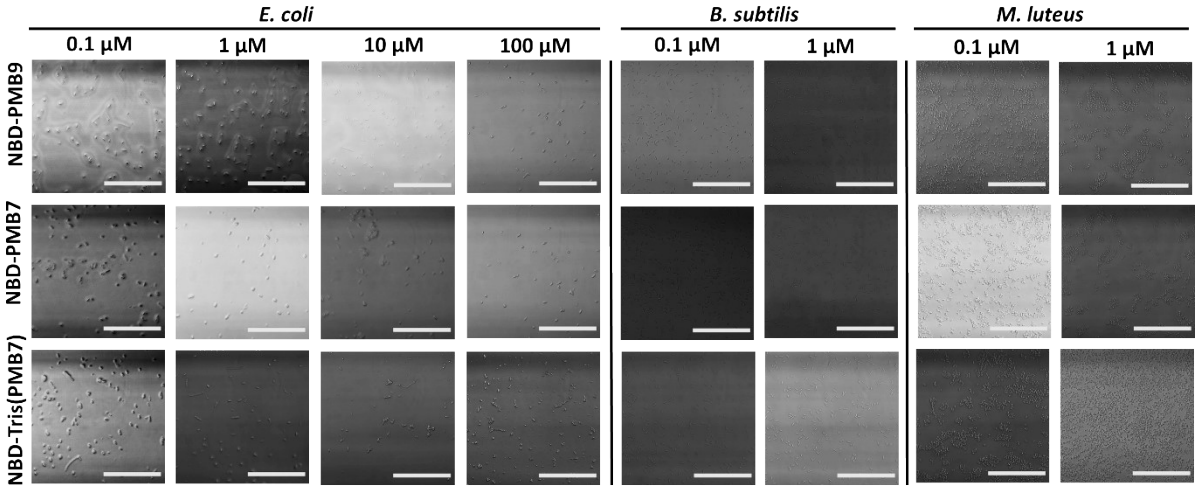


Figure S5: Brightfield images of *E. coli* (Gram-negative, target) at increasing concentrations of probes and *B. subtilis* and *M. luteus* (both Gram-positive, non-target) at 0.1 and 1 µM of NBD-PMB9, NBD-PMB7 and NBD-Tris(PMB7). These correspond to the fluorescence images in Figure 2 (λ_{ex} = 480 nm, GFP filter settings, λ_{em} = 540 nm). Scale bar is 100 µm. Bacterial concentration: 5 x 10^8 cfu/mL.

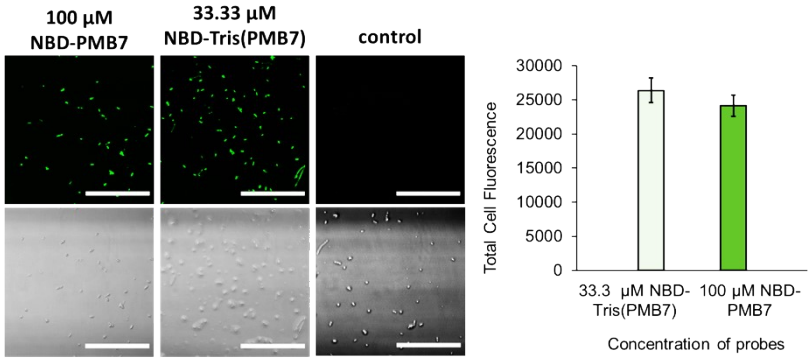


Figure S6. Comparison of saturation fluorescent labelling of *E. coli* with 33 µM of NBD-Tris(PMB7) and 100 µM of NBD-PMB7. Left panel: In each column, top: fluorescence (λ_{ex} = 480 nm, GFP filter settings, λ_{em} = 540 nm); bottom: brightfield. Scale bar, 100 µm. Right panel: Quantification of the fluorescence of the imaged bacteria as obtained using Fiji (ImageJ, NIH). Total cell fluorescence was calculated by subtracting the mean of the background (10 background feature intensities of the same size as bacterial cells) from the mean of the fluorescent bacteria (20 fluorescent labelled bacteria). Error bars represent the standard error calculated using the errors of fluorescent and background features.

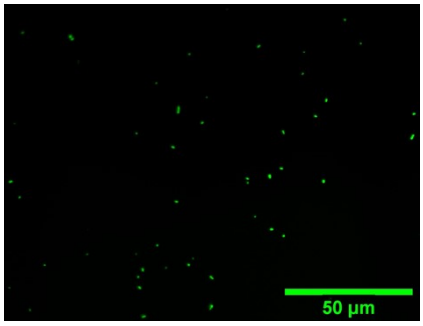


Figure S7. *E.coli* (5 x 10^6 cfu/mL) labelled with 2.5 nM of SYTO 9 dye as viewed through a 60x lens of a Zeiss Axiovert 500M fluorescent microscope with λ_{ex} = 498 nm and λ_{em} = 505 nm.

4. NMR, MS and HPLC data

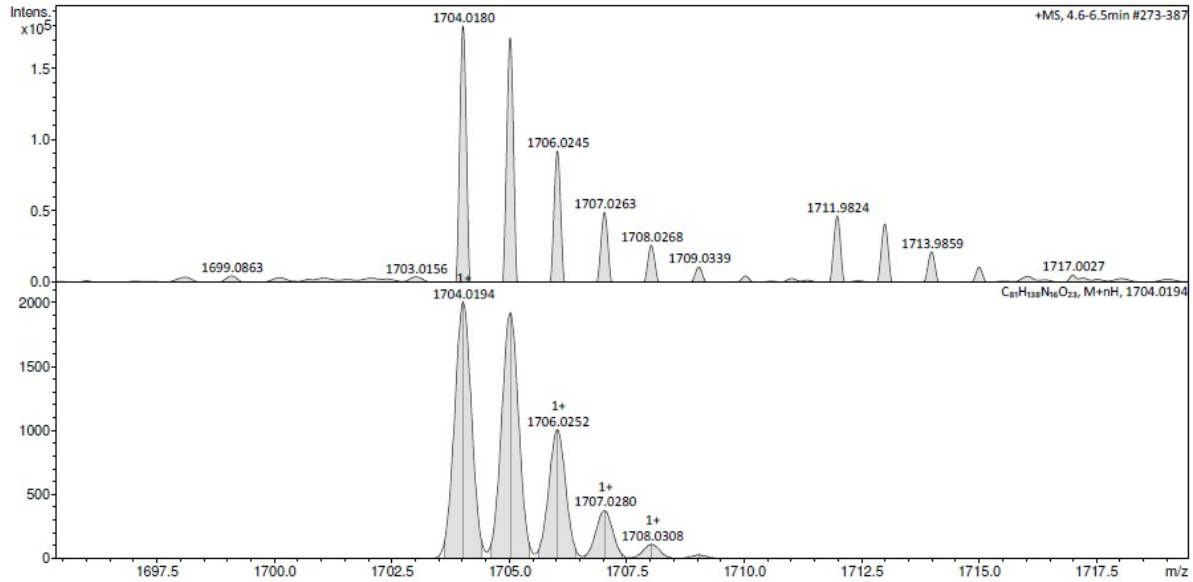


Figure S8 Experimental (top) and theoretical (bottom) HRMS (ESI) spectra for compound **1** ([M+H]$^+$).

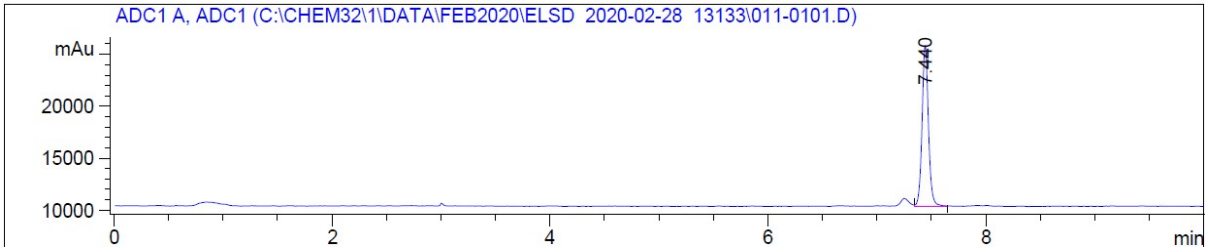


Figure S9. HPLC trace (ELS detection) for compound **1**.

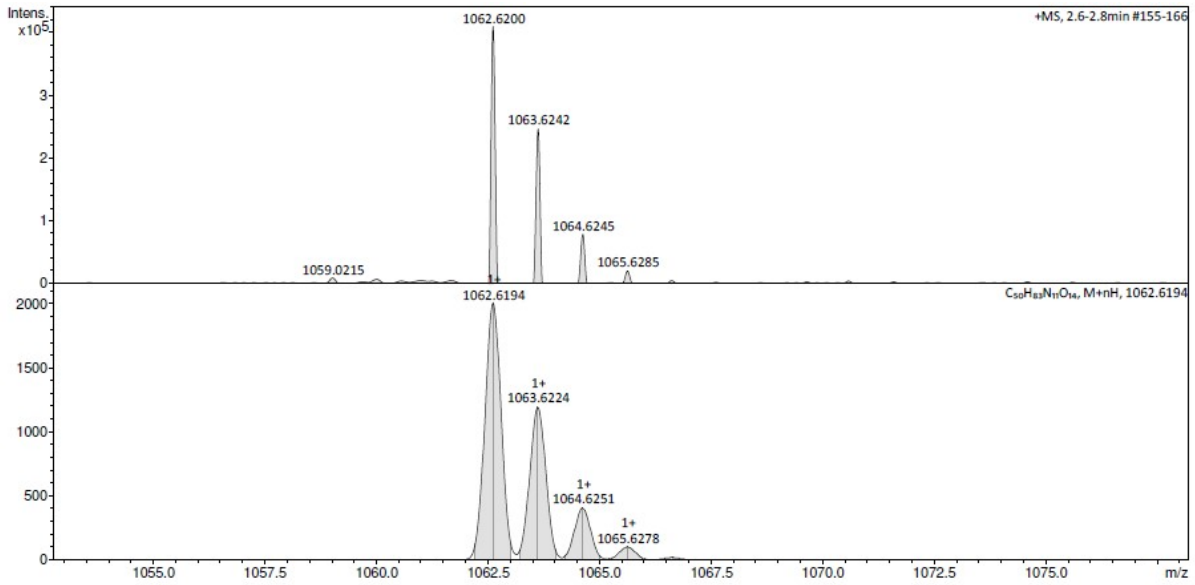


Figure S10. Experimental (top) and theoretical (bottom) HRMS (ESI) spectra for compound **2** ($[M+H]^+$).

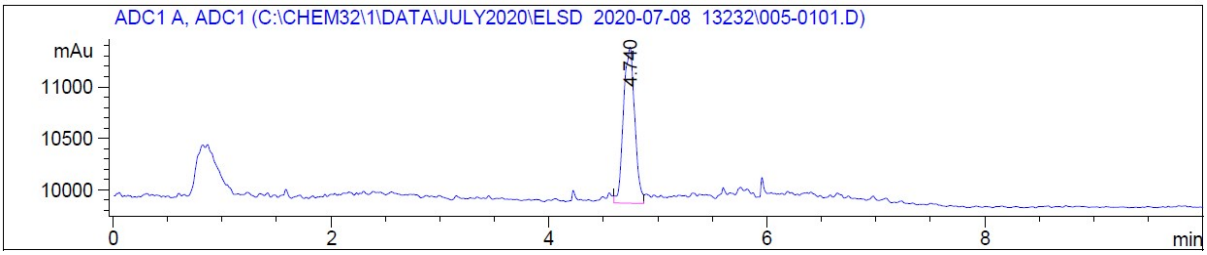


Figure S11. HPLC trace (ELS detection) for compound **2**.

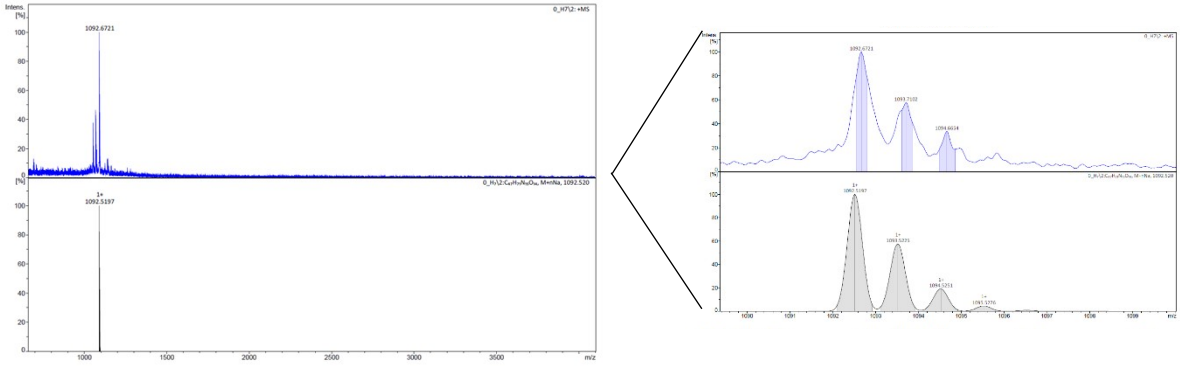


Figure S12. HRMS (MALDI) spectrum for compound **NBD-PMB7**. Top shows experimental spectrum, bottom shows theoretical ($[M+Na]^+$).

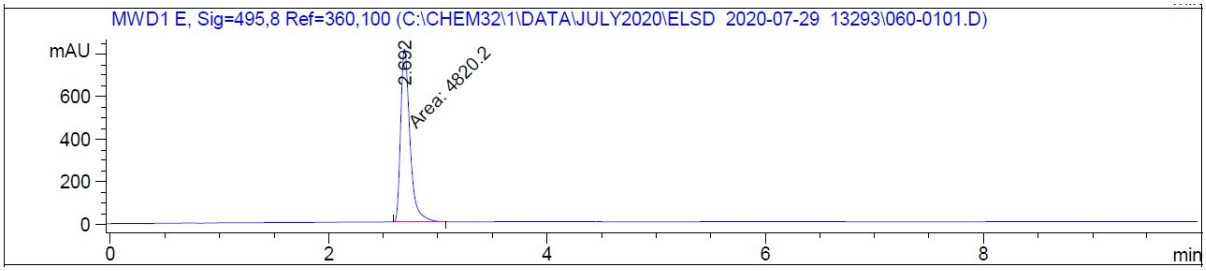


Figure S13. HPLC trace (495 nm detection) for **NBD-PMB7**.

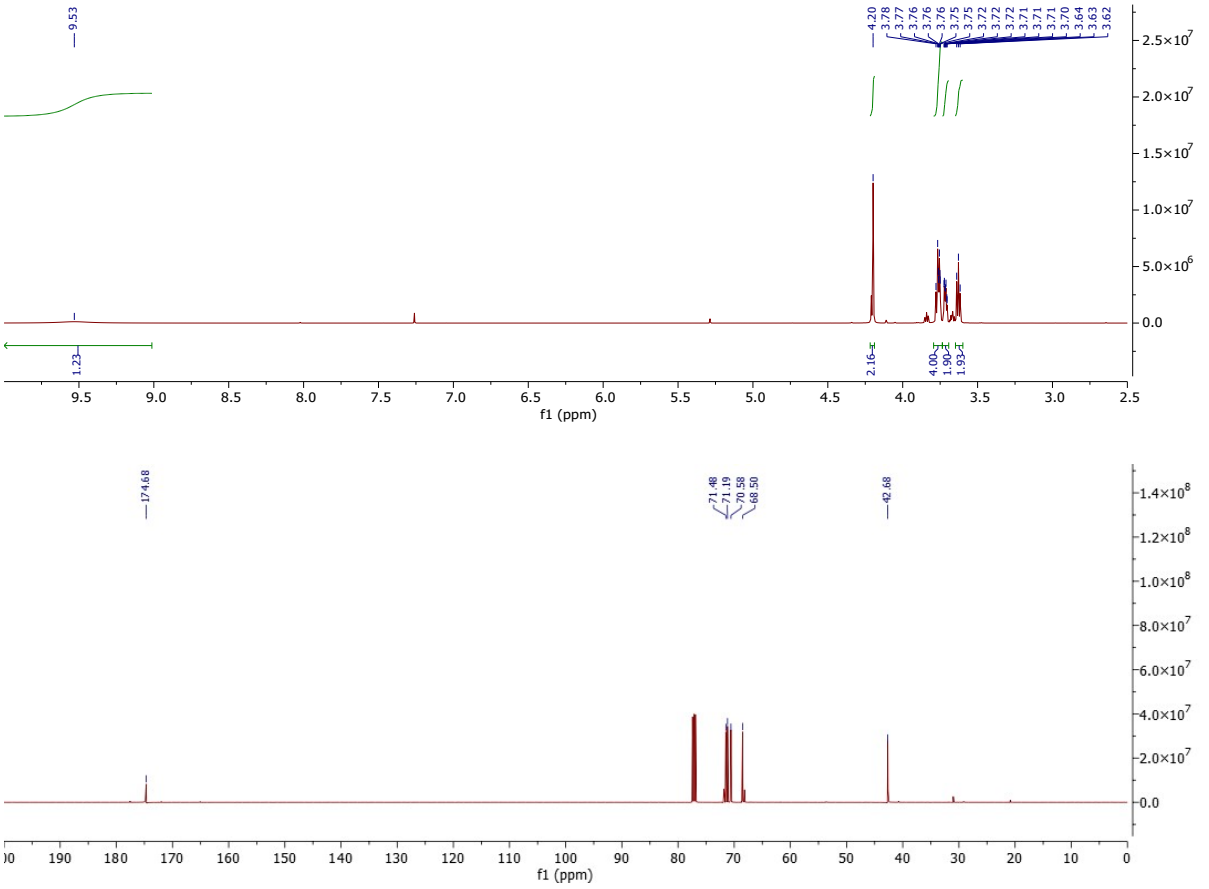


Figure S14. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **S1**, recorded at 500 MHz and 126 MHz respectively, in $CDCl_3$.

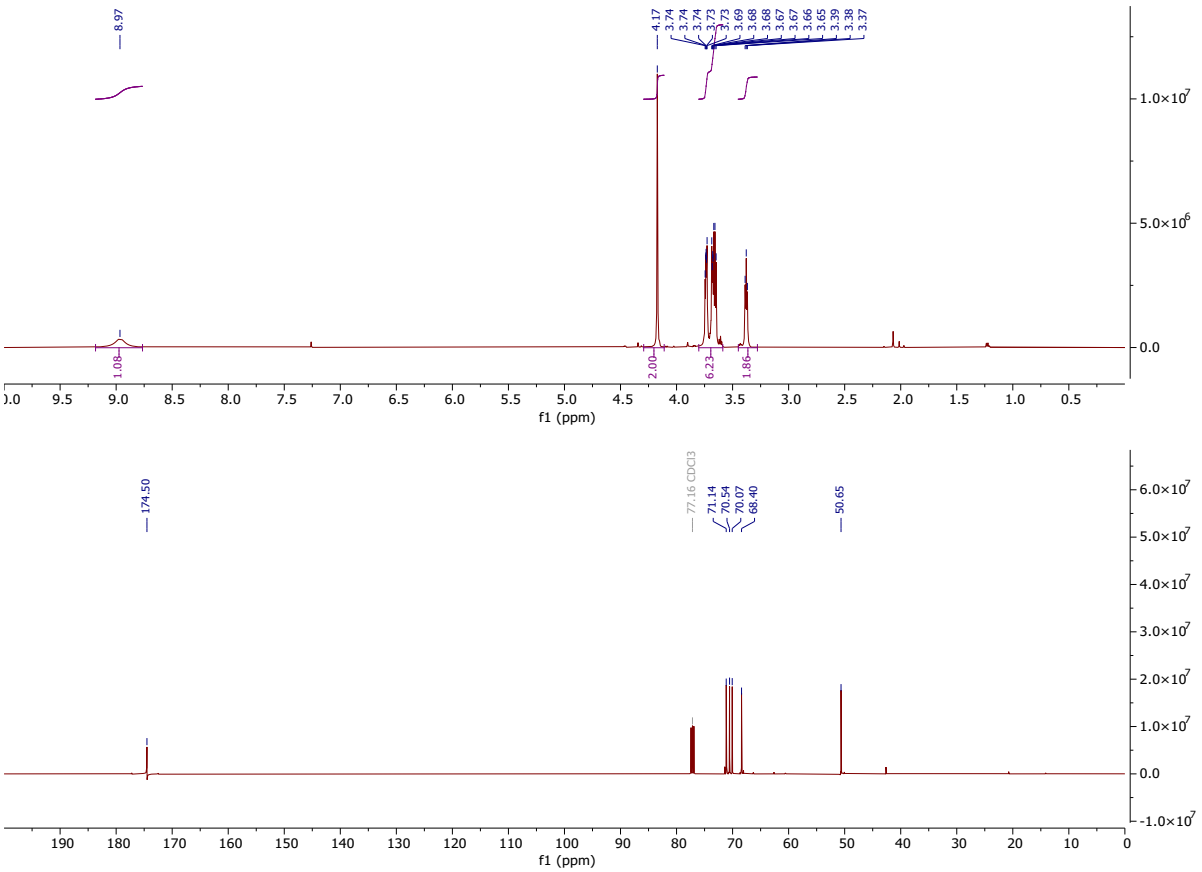
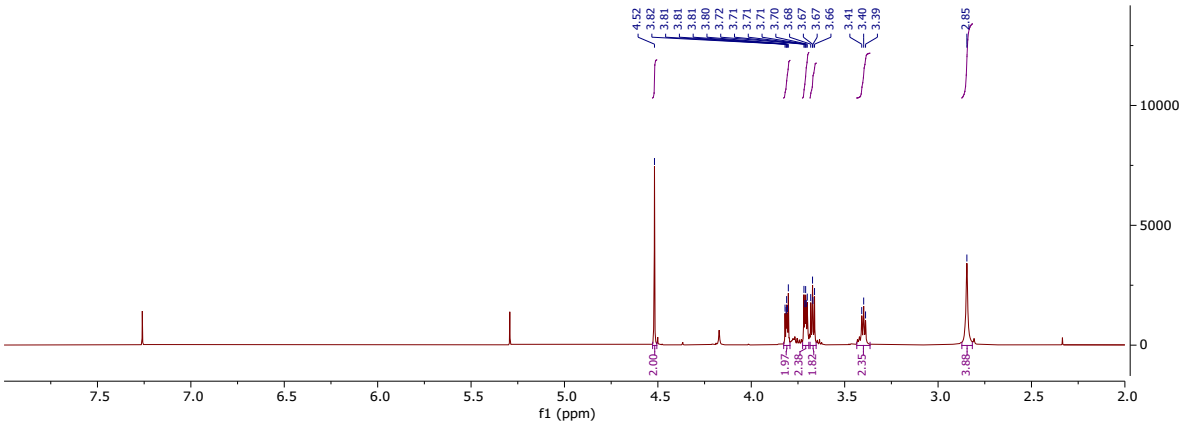


Figure S15. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **S2**, recorded at 500 MHz and 126 MHz respectively, in $CDCl_3$.



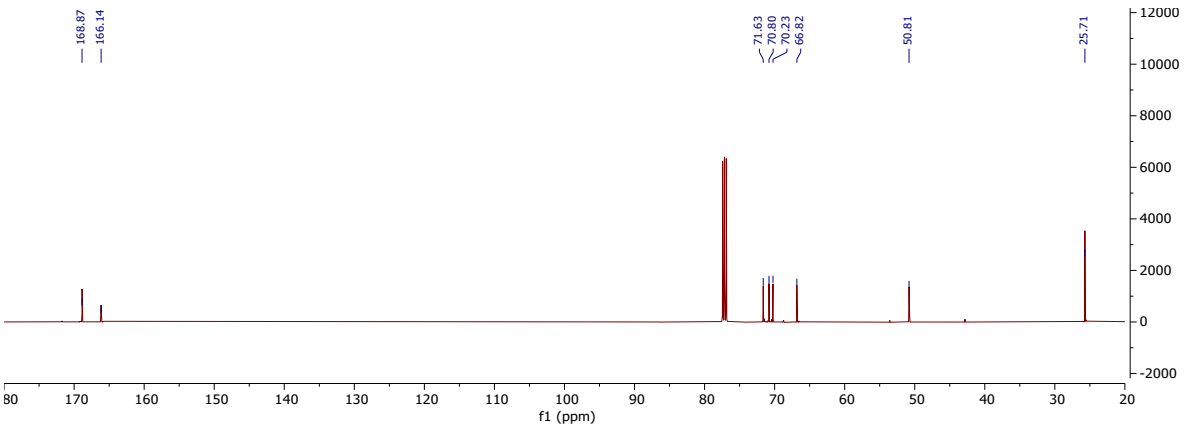


Figure S16. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **3**, recorded at 500 MHz and 126 MHz respectively, in $CDCl_3$.

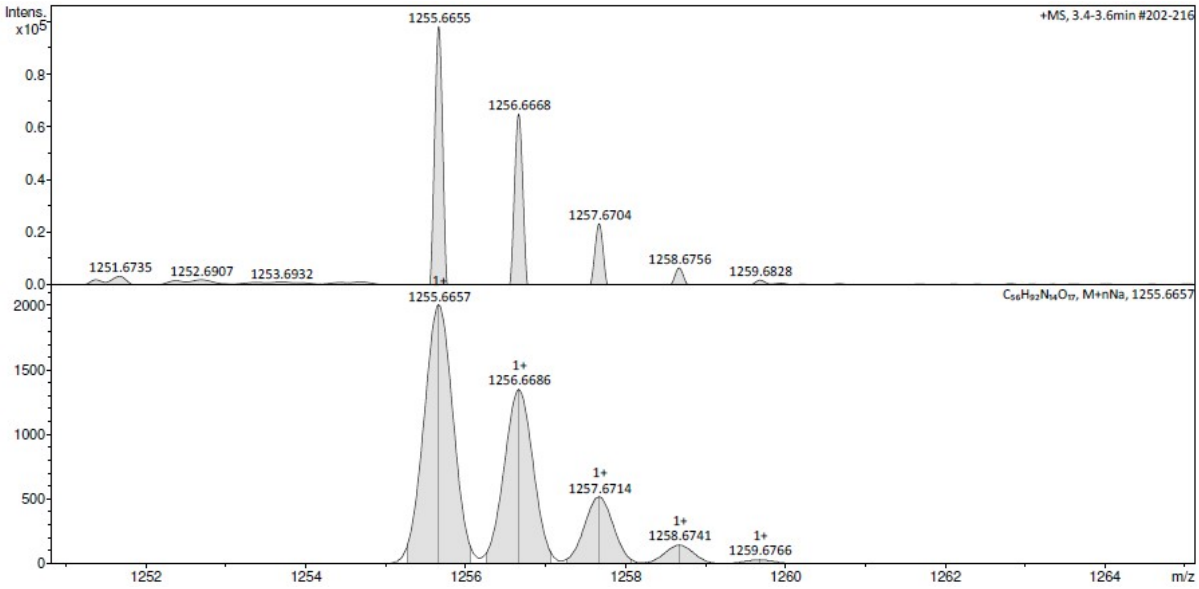


Figure S17. Experimental (top) and theoretical (bottom) HRMS (ESI) spectra for compound **5** ($[M+Na]^+$).

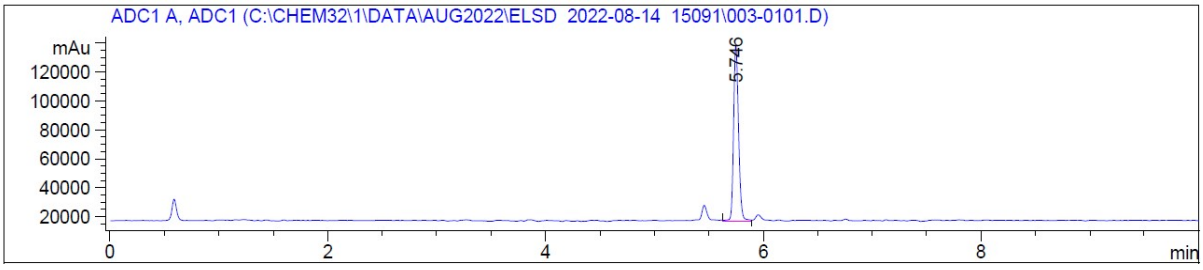


Figure S18. HPLC trace (ELS detection) for compound **5**.

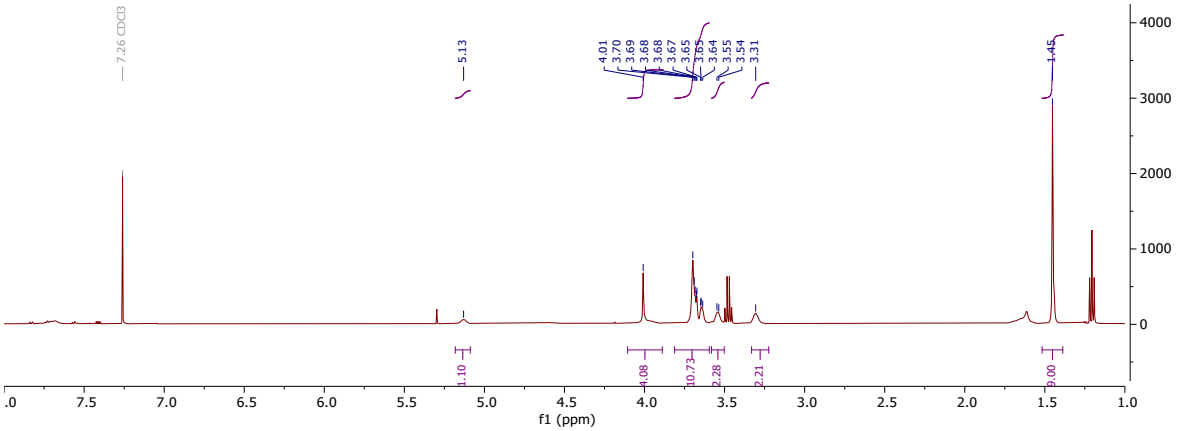


Figure S19. ^{1}H spectrum of crude compound **7**, recorded at 500 MHz in $CDCl_3$.

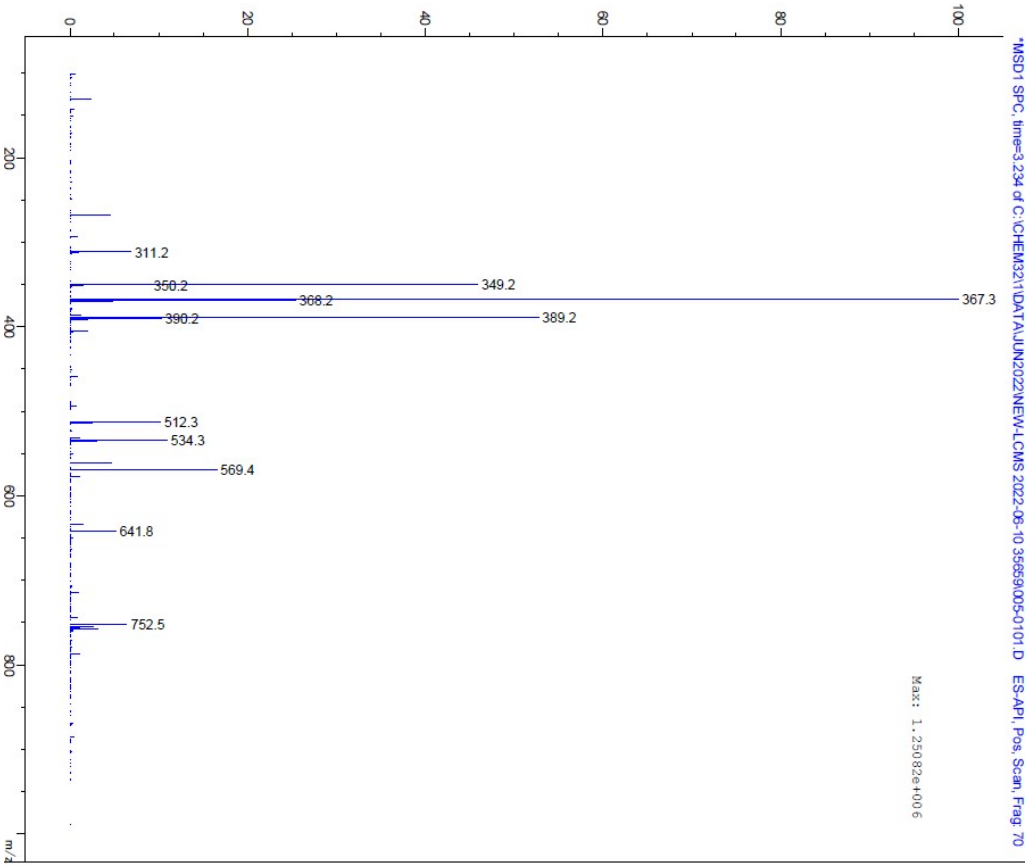


Figure S20. LC-MS (ESI) spectrum for compound 7 ($[M+H]^+$ = 367.3).

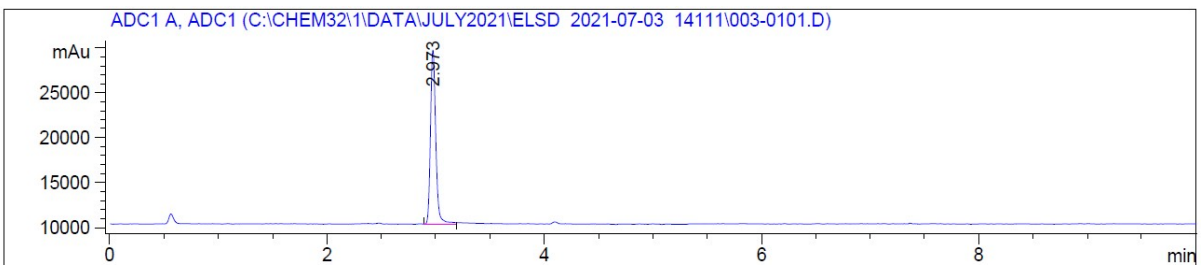


Figure S21. HPLC trace (ELS detection) for compound **7**.

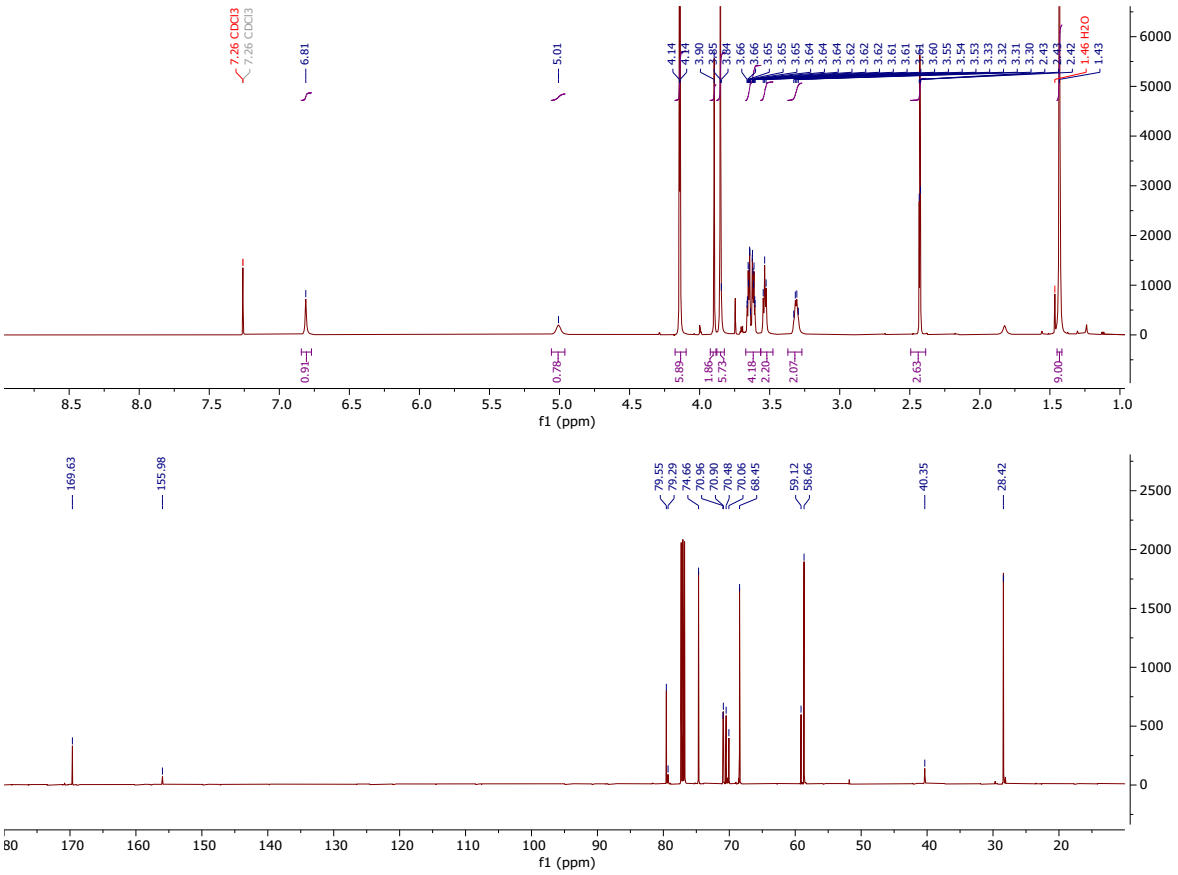


Figure S22. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **S3**, recorded at 500 MHz and 126 MHz respectively, in $CDCl_3$.

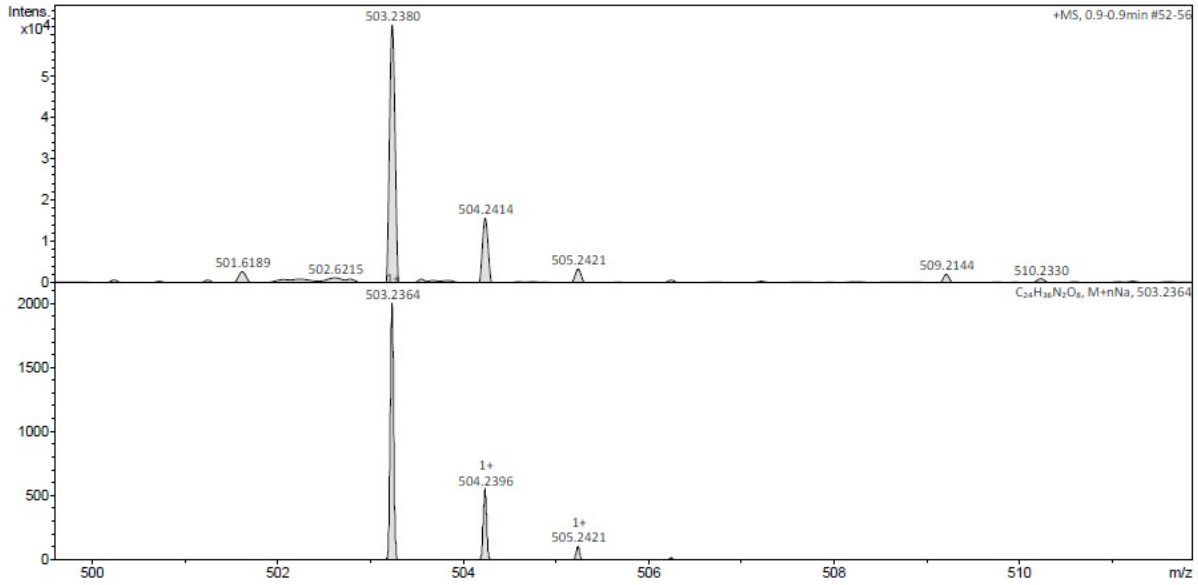


Figure S23. Experimental (top) and theoretical (bottom) HRMS (FT) spectra for compound **S3** ([M+Na]$^+$).

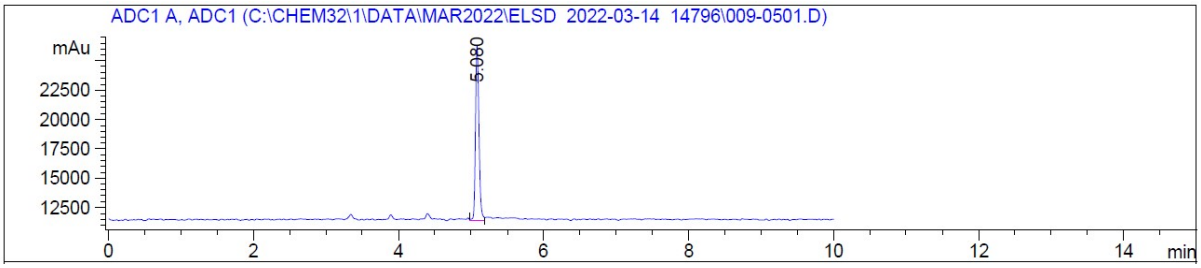
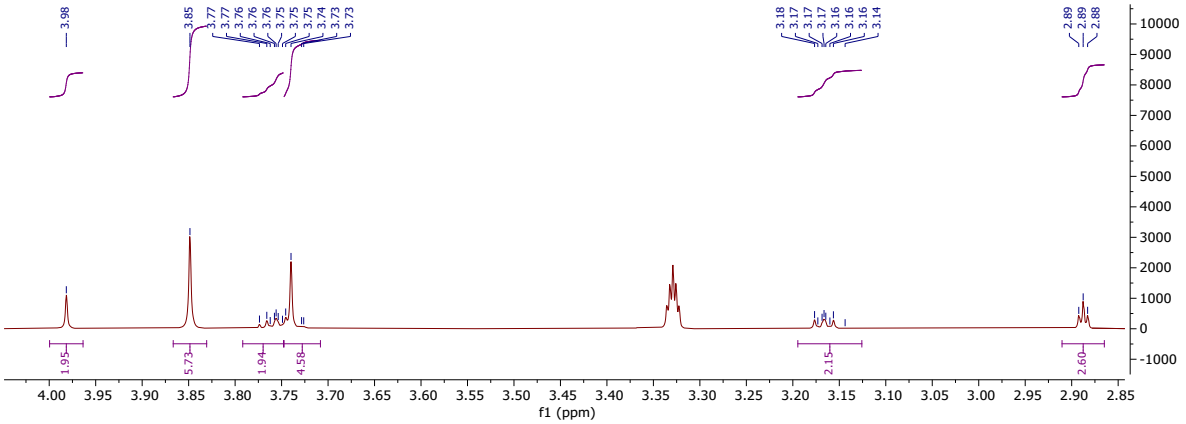


Figure S24. HPLC trace (ELS detection) for compound **S3**.



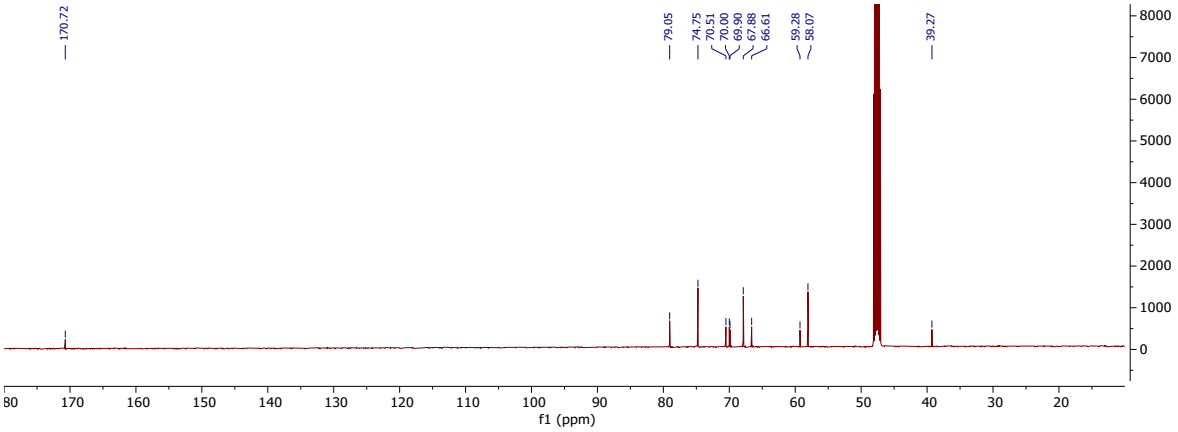


Figure S25. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **S4**, recorded at 500 MHz and 126 MHz respectively, in CD_3OD.

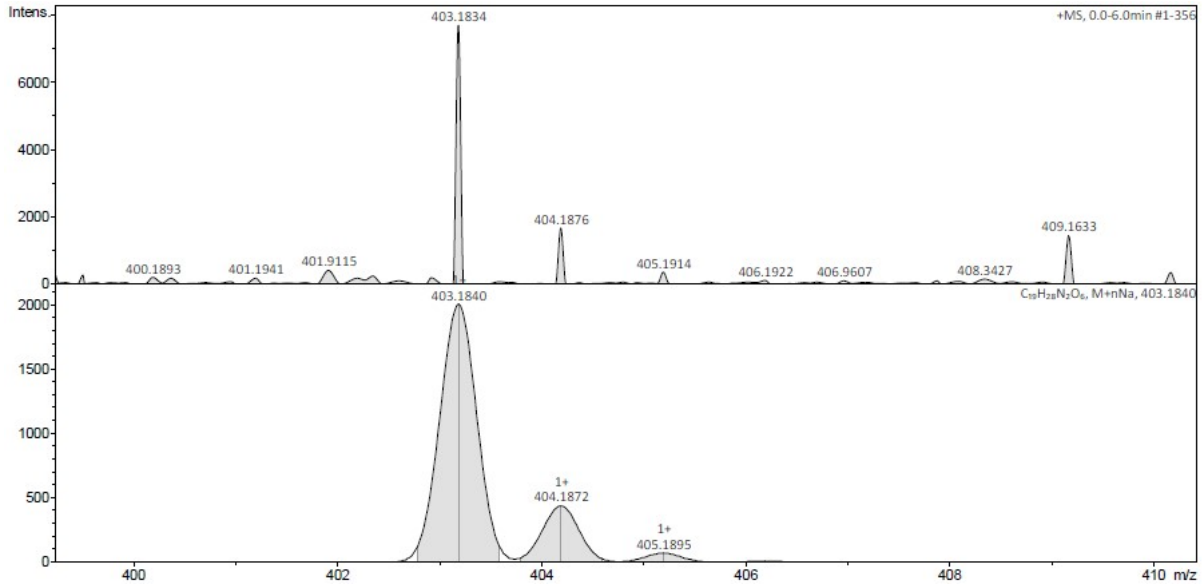


Figure S26. Experimental (top) and theoretical (bottom) HRMS (FT) spectra for compound **S4** ($[M+Na]^+$).

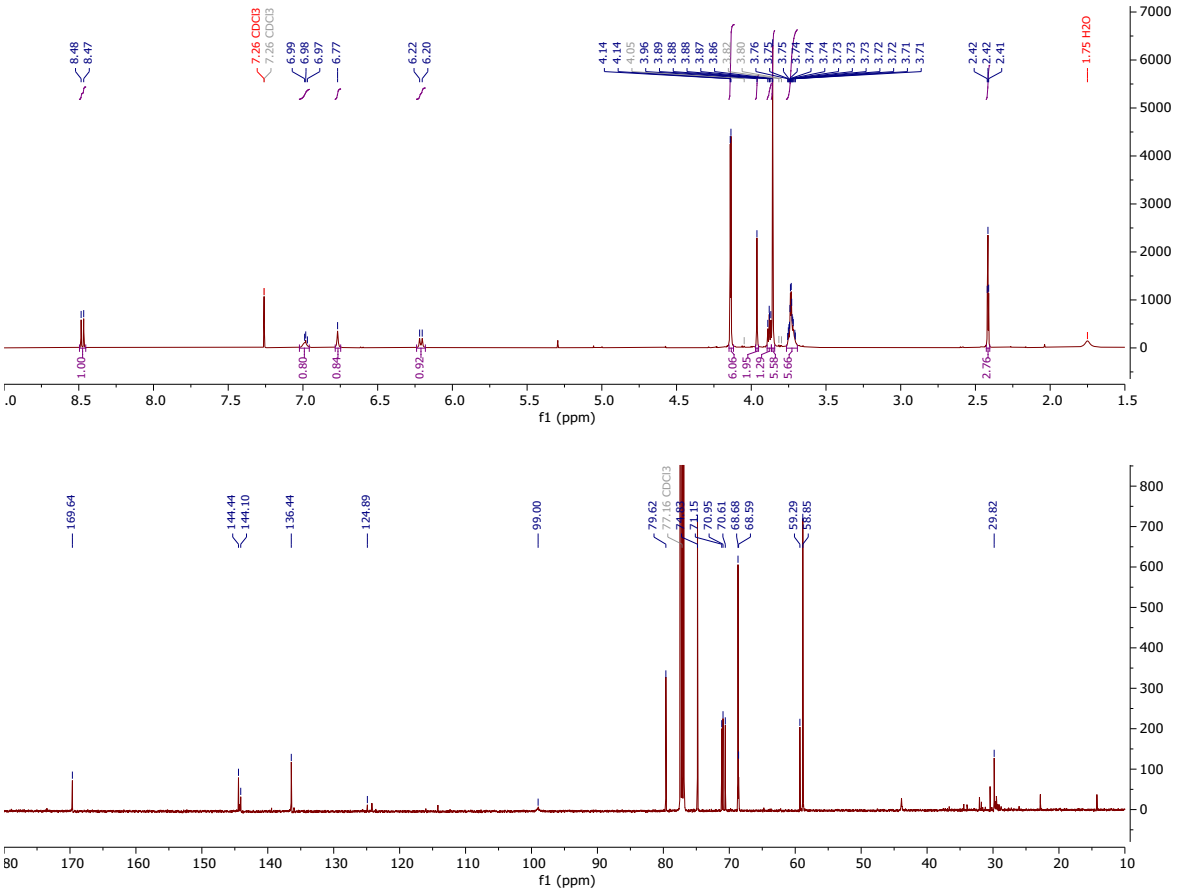


Figure S27. ^{1}H (top) and ^{13}C NMR (bottom) spectra of compound **8**, recorded at 500 MHz and 126 MHz respectively, in $CDCl_3$.

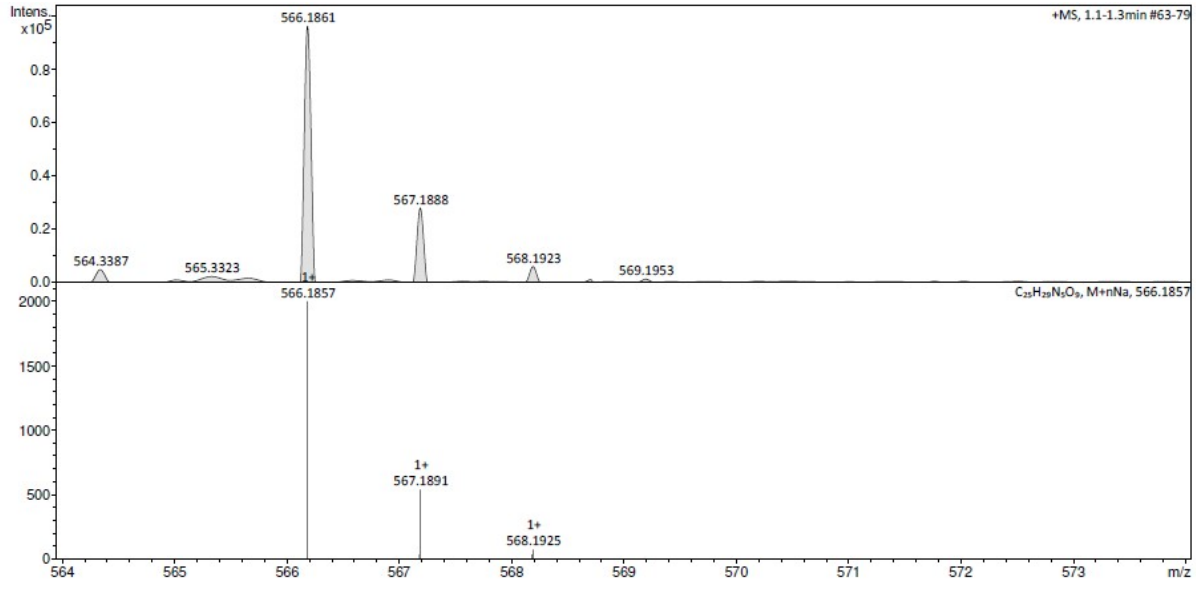


Figure S28. Experimental (top) and theoretical (bottom) HRMS (ESI) spectra for compound **8** ($[M+Na]^+$).

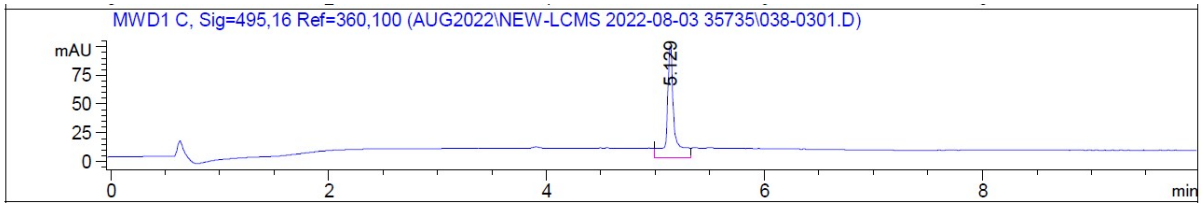


Figure S29. HPLC trace (495 nm detection) for compound **8**.

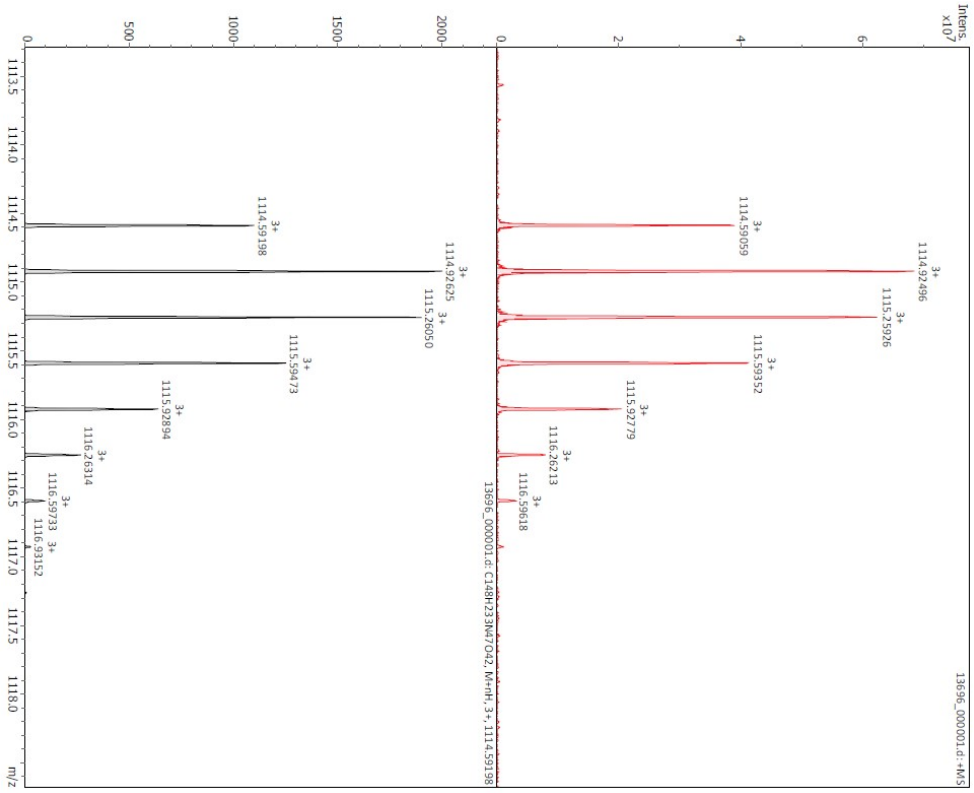


Figure S30. Experimental (red) and theoretical (black) HRMS (ESI) spectra for **NBD-Tris(PMB7)** ($[M+3H]^{3+}$).

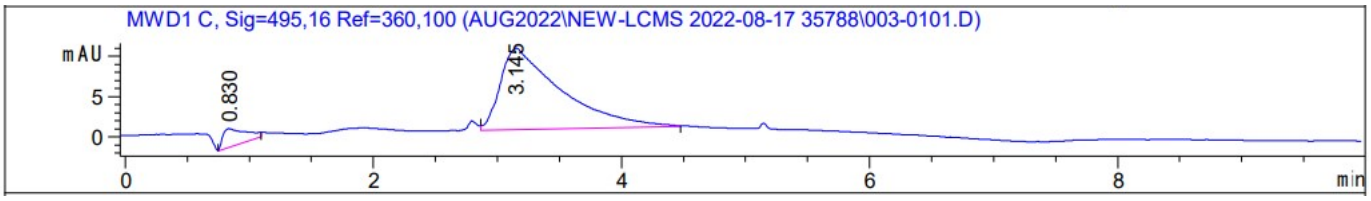


Figure S31. HPLC trace (495 nm detection) for **NBD-Tris(PMB7)**.

5. Supplementary References

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