Electronic Supplementary Material (ESI) for ChemComm.

ELECTRONIC SUPPORTING INFORMATION

Dyeing Fungi: Amphotericin B Based Fluorescent Probes for Multiplexed Imaging

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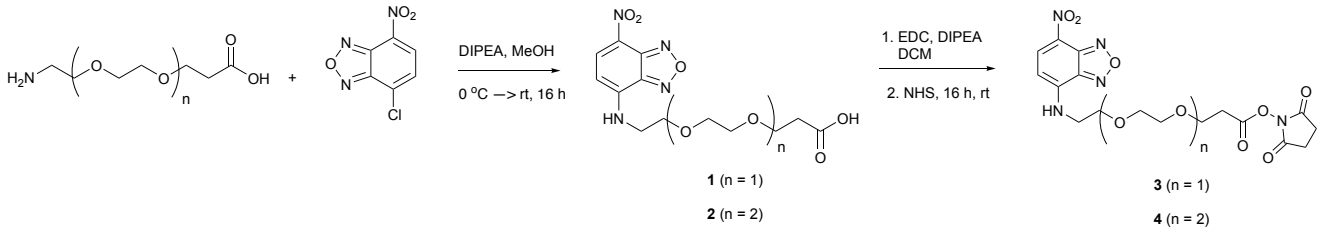
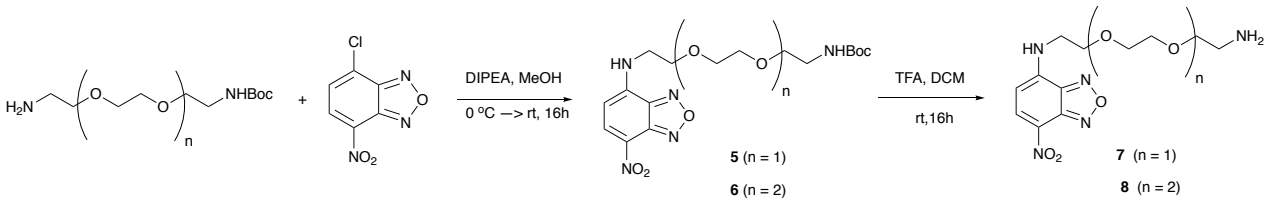
Table of Content

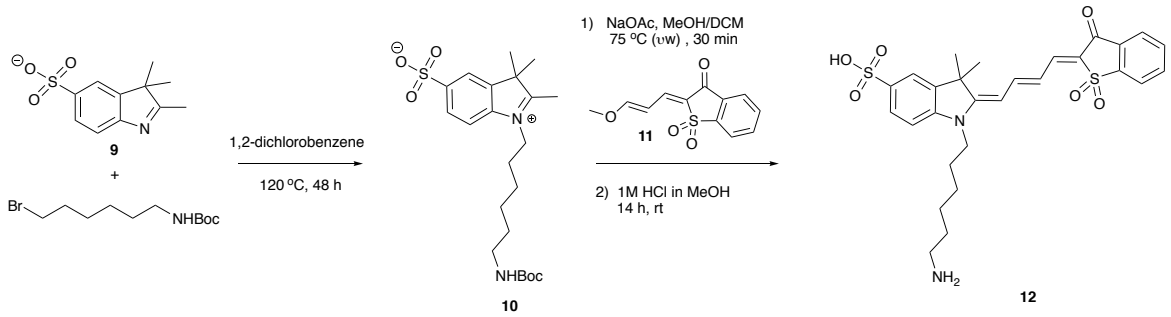
1. Supporting figures and Schemes



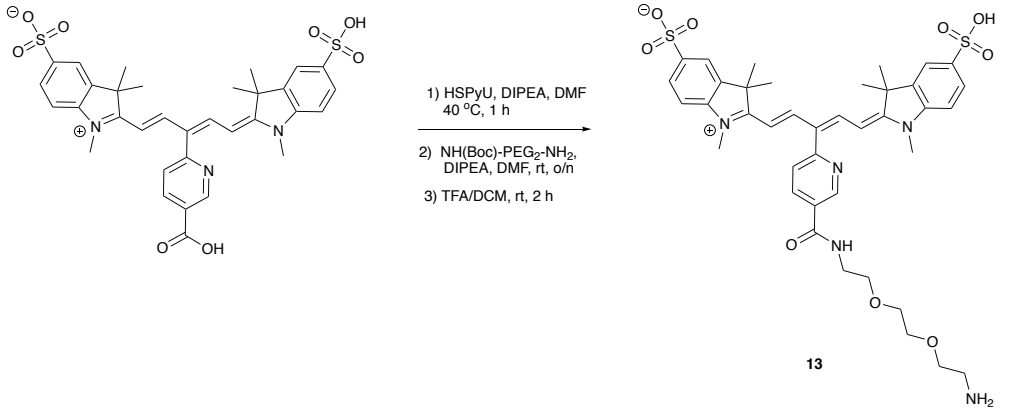
Scheme S1. Synthesis of NHS-ester functionalised NBD-PEG fluorophores.



Scheme S2. Synthesis of amino functionalised NBD-PEG fluorophores**.**



Scheme S3. Synthesis of Sulfo-MeroCy-NH_2 **12**.



Scheme S4. Synthesis of Sulfo-Cy5-NH_2 **13**.

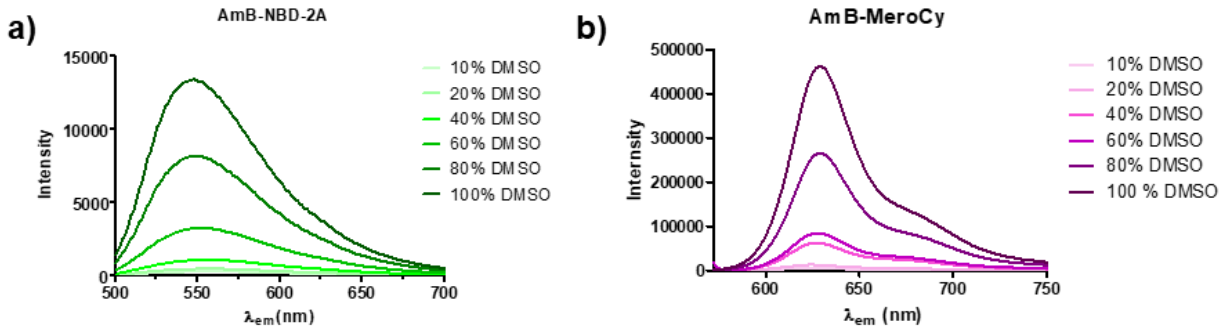


Figure S1. Increase of the fluorescence intensity with increase in DMSO concentration in H_2O for a) **AmB-NBD-2A** (46-fold, λ_{ex} = 488 nm) and b) **AmB-MeroCy** (73-fold, λ_{ex} = 561 nm).

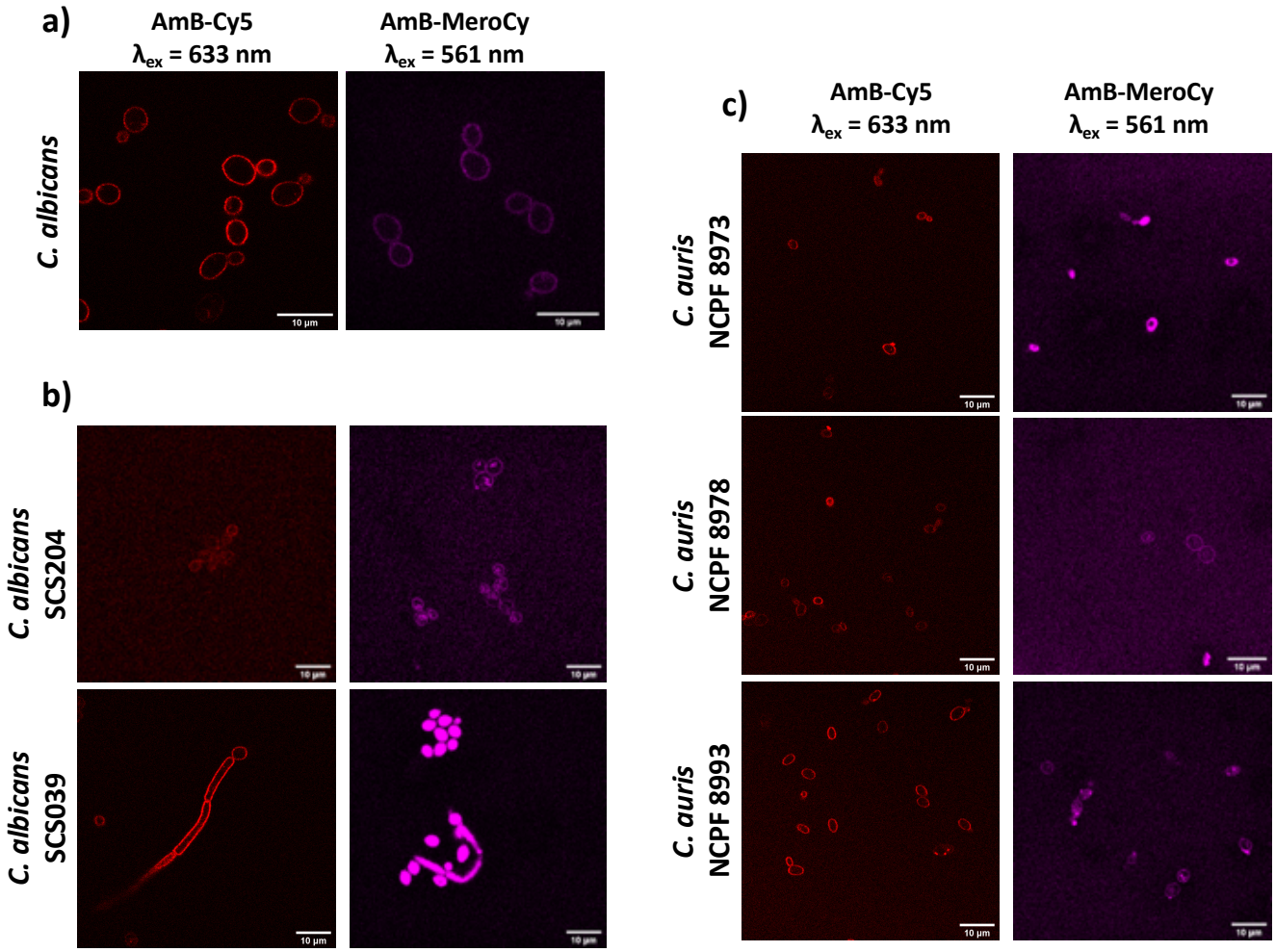
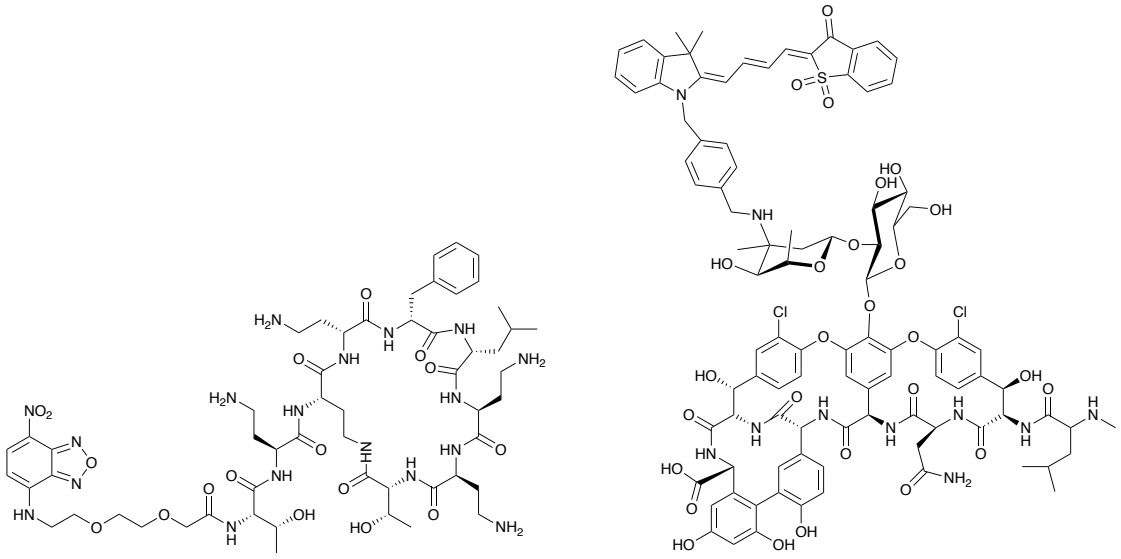


Figure S2. a) *Candida albicans* (laboratory strain ATCC MYA-2876) labelling by the far-red emitting AmB probes. Confocal fluorescence images of the fungi after incubation with AmB-MeroCy (10 µM, 2h at 37 °C) and AmB-Cy5 probes (10 µM, 1h at 37 °C). b)–c) Confocal fluorescence images of clinically isolated *Candida* strains labelled with **AmB-MeroCy** and **AmB-Cy5** (10 µM, 2 h at 37 °C). Scale bar 10 µm.

Table S1. Amphotericin B susceptibility profiles of *C. albicans, C. auris, A, fumigatus* and *F. solani.*

	C. auris			*C. albicans*		*A. fumigatus*	*F. solani*
Strain	**NCPF 8973**	**NCPF 8978**	**NCPF 8993**	**SCS039**	**SCS204**	**AF293**	**NCPF 2171**
MIC (μg/mL)	0.25	0.25	1	0.5	0.5	0.5	2
MFC (μg/mL)	32	32	>32	8	2	8	2



PMX-NBD **Van-MeroCy**

Scheme S5. Structures of bacterial probes **PMX-NBD**[1] and **Van-MeroCy**.[2]

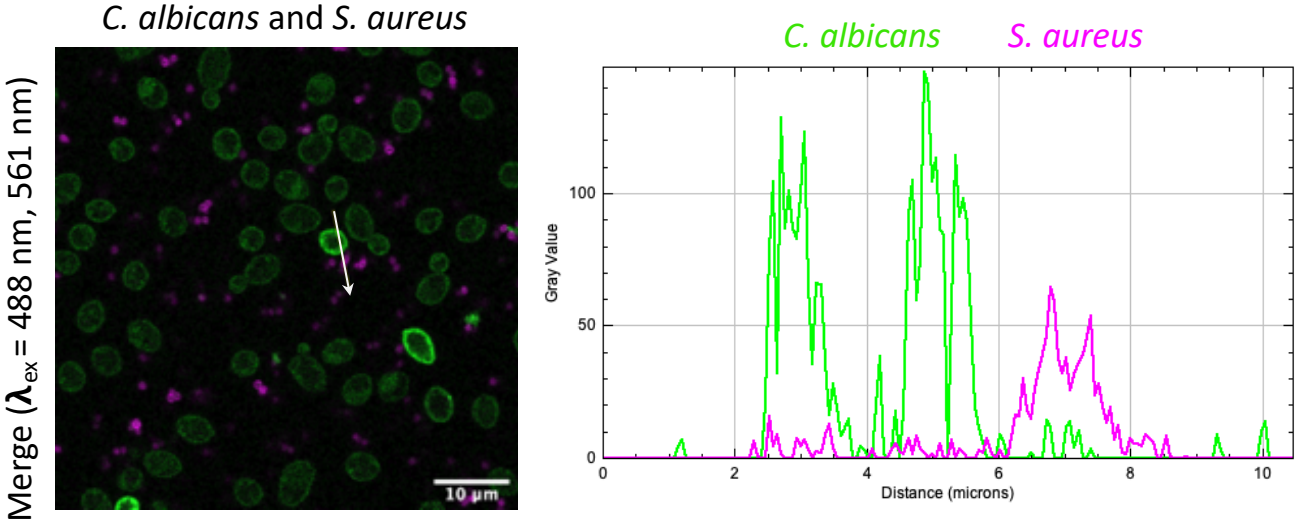


Figure S3. Selective labelling of Gram-positive bacteria using **Van-MeroCy**. Mixed culture of *C. albicans* and *S. aureus* were incubated with **AmB-NBD-2A** and **Van-MeroCy** for 1h at 37 °C. The media was removed, PBS added, and the cells were imaged at λ_{ex} = 488 nm (for NBD, showing bacteria) and λ_{ex} = 561 nm. (for MeroCy, showing fungi). Scale bar = 10μm.

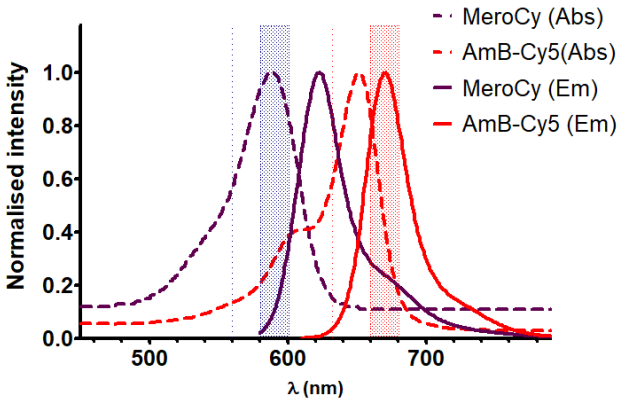


Figure S4. Absorbance and emission spectra of MeroCy flurophore and AmB-Cy5 probe. The measurements were performed at 1uM in DMSO. Doted lines and doted areas represent wavelengths of excitation laser and emission detector band settings of the microscope respectively.

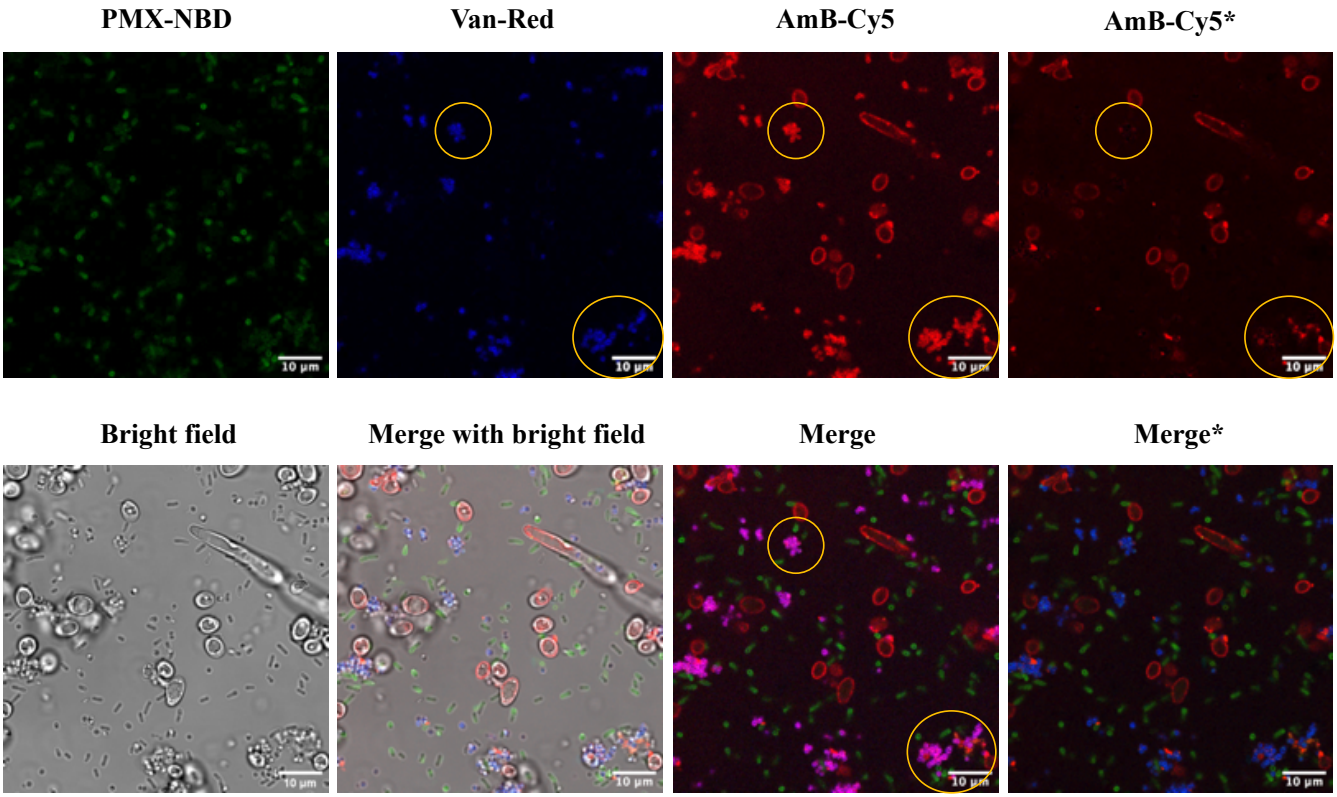


Figure S5. Selective labelling of fungi and bacteria in a mixed culture. Cells were co-stained with three different probes (1 h incubation) and three laser lines (488 nm, 561 nm, and 633 nm) were used for excitation simultaneously. Green = *E. coli l*abelled by Gram-negative **PMX-NBD**[1] (5 µM), Blue = *S. aureus* labelled by Gram-positive bacteria probe **Van-MeroCy**[2] (1 µM), and Red = *C. albicans* labelled by fungi-specific **AmB-Cy5** (10 µM). The Yellow circles show *S. aureus* clusters labelled by **Van-MeroCy**. *Due to overlap in emission spectra, some of the MeroCy signal is detected in the Cy5 channel (660–680 nm) and due to its high quantum yield and extinction coefficient in viscous environments this led to some signal 'leakage'. Nevertheless, the merged image can be used for signal differentiation. Otherwise, simple image processing of subtracting

'blue' signals from 'red' can be applied using open access software FIJI. *Protocol: Process → Image calculator→ 'Channel AmB-Cy5' – Subtract – 'Channel Van-Red' → Ok. Using same protocol can be applied to obtain 3D-image and Z-stack of mixed fungi-bacteria culture (See supporting movies 1 and 2).

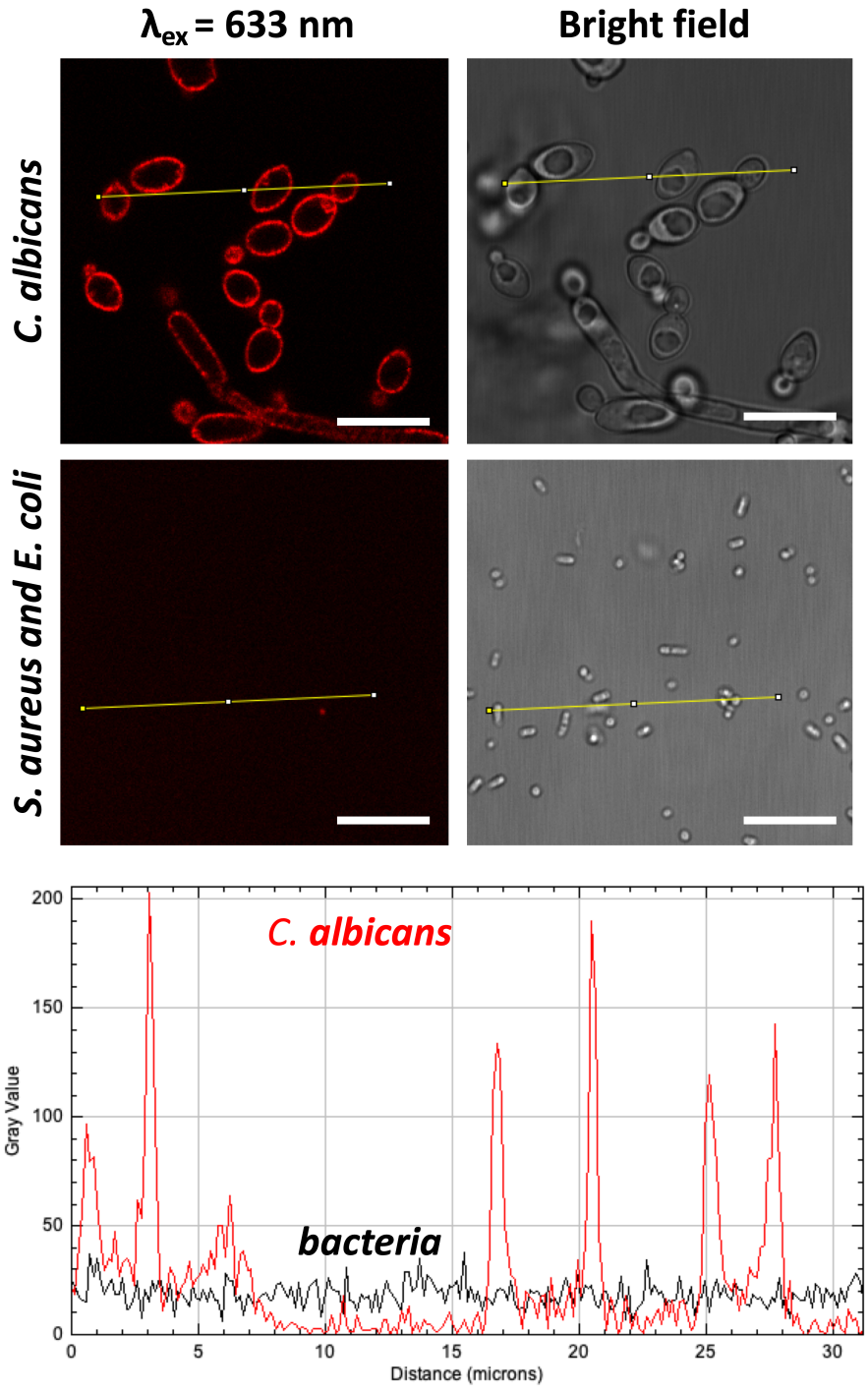


Figure S6. Multiplexed imaging of fungi-bacteria mixture. *C. albicans* and a mixed culture of *S. aureus* and *E. coli* were incubated with **AmB-Cy5** (10 μM) for 1 h at 37 °C. The media was removed, PBS added, and the cells were imaged at λ_{ex} = 633 nm. The fluorescence plot profiles corresponding to yellow line show selectivity of **AmB-Cy5**

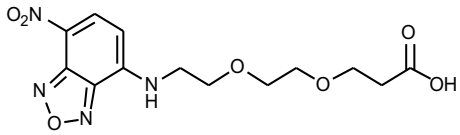
2. Experimental

2.1. General information

All chemicals were purchased from Sigma Aldrich, Merck, Acros, VWR, Fisher Scientific, or Carbosynth and used without further purification. NMR spectra were recorded at 298 K in deuterated solvents using Bruker AVA500 spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. Chemical shifts are reported in ppm and are referenced to residual non-deuterated solvent. High Resolution Mass Spectra (HRMS) were performed on a Bruker 3.0 T Apex II spectrometer. Microwave-assisted reactions were performed with a Biotage Initiator 2.0. Normal phase column chromatography was carried out on silica gel 60 (230–400 mesh). Reverse phase chromatography was carried out on a Biotage Isolera™ Spektra One system equipped with a Biotage® SNAP Ultra C18 column. Analytical RP–HPLC was done on an Agilent 1100 system equipped with a Phenomenex Kinetex® 5 µm XB-C18 100 Å LC Column (50 × 4.6 mm) with a flow rate of 1 mL/min, and eluting 6 min with a gradient of H_2O/CH_3CN (95/5) to H_2O/CH_3CN (5/95) with 0.1% HCOOH, followed by 3 min isocratic elution, with detection by evaporative light scattering and additionally at 600 nm for Merocyanine and Cy5, and 495 nm for NBD.

2.2. Syntheses

NBD-PEG$_2$-COOH (1)

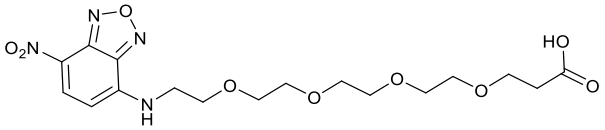


To NH_2-PEG_2-COOH (0.25 g, 1.4 mmol) in MeOH (3 mL), DIPEA (2.47 mL, 14 mmol) was added and the mixture stirred for 10 min at room temperature. A solution of 4-chloro-7-nitrobenzofuran (0.56 g, 2.8 mmol) in MeOH (5 mL) was cooled to 0 °C and the NH_2-PEG_2-COOH solution above was added dropwise over 30 min. The reaction mixture was stirred overnight at room temperature under N_2. The solvent was removed *in vacuo* and the crude product purified by column chromatography using 9:1 DCM/MeOH with 0.1% AcOH as an eluent to give **1** (0.43 g) in 90% yield.

HPLC (ELSD) t_R = 3.74 min, purity 100%.
HR ESI-MS: calculated for $C_{13}H_{16}N_4O_7$ $[M+H]^+$ m/z 341.1092; Found 341.1089.

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.54 (d, J = 8.9 Hz, 1H), 6.44 (d, J = 8.9 Hz, 1H), 3.81 (t, J = 5.1, 4.6 Hz, 2H), 3.79 – 3.67 (m, 4H), 3.67 – 3.60 (m, 4H), 2.49 (t, J = 6.4 Hz, 2H).
^{13}C NMR (126 MHz, Methanol-d_4) δ 175.5, 146.0, 145.6, 145.6, 138.5, 123.4, 100.2, 71.53, 71.46, 69.9, 67.9, 44.9, 35.9.

NBD-PEG$_4$-COOH (2)



DIPEA (2.47 mL, 14 mmol) was added to a solution of NH_2-PEG$_4$-COOH (374 mg, 1.41 mmol) in MeOH (5 mL), and the mixture was stirred 10 min at room temperature. 4-Chloro-7-nitrobenzofuran (563 mg, 2.82 mmol) in MeOH (5 mL) was cooled to 0 °C and the mixture of NH_2-PEG$_4$-NH(Boc) and DIPEA was added dropwise over 20 min. The reaction mixture was stirred at room temperature for 16 h under N_2. The solvent was removed *in vacuo* and the crude product purified by column chromatography using a gradient of 10% MeOH to 20% MeOH in DCM as an eluent to give **2** in 90% yield.

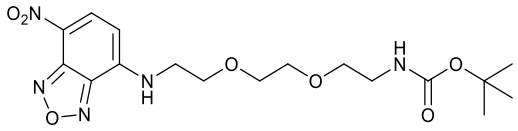
HPLC (ELSD) t_R = 3.80 min, purity 100 %

HR ESI-MS: calculated for $C_{17}H_{24}N_4O_9$ $[M+Na]^+$ m/z 451.1435; Found 451.1436

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.53 (d, J = 8.9 Hz, 1H), 6.44 (d, J = 8.9 Hz, 1H), 3.83 – 3.80 (m, 2H), 3.80 – 3.73 (m, 2H), 3.72 (t, J = 6.4 Hz, 2H), 3.68 – 3.63 (m, 4H), 3.62 – 3.59 (m, 4H), 3.59 – 3.56 (m, 4H), 2.51 (t, J = 6.4 Hz, 2H).

^{13}C NMR (126 MHz, Methanol-d_4) δ 175.8, 146.8, 145.9, 145.6, 138.4, 123.2, 100.3, 71.62, 71.59, 71.56, 71.54, 71.45, 71.35, 69.9, 68.0, 44.9, 36.2.

NBD-PEG$_2$-NH(Boc) (5)



Boc(NH)-PEG$_2$-NH_2 (71.2 µL, 0.30 mmol) in 1 mL of MeOH and DIPEA (255 µL, 1.83 mmol) were added, consecutively, to NBD-Cl (118 mg, 0.59 mmol) in MeOH (4 mL). The reaction mixture was protected from light and stirred at room temperature for 14 h. The solvent was evaporated *in vacuo* and the crude product purified by column chromatography (3:1 EtOAc/Hexane) to give **5** (117 mg) as a yellow solid in 95% yield.

TLC (EtOAc/Hexane 3:1) R_f = 0.27.

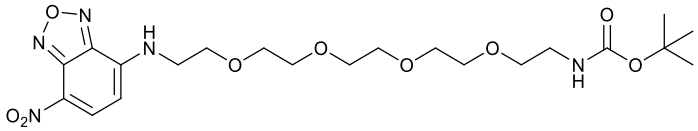
HPLC (ELSD) t_R = 4.86 min, purity 98 %.

HR ESI-MS: calculated for $C_{17}H_{26}N_5O_7$ $[M+H]^+$ m/z 412.1826; Found 412.1821

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.53 (d, J = 8.8 Hz, 1H), 6.43 (d, J = 8.9 Hz, 1H), 3.85 – 3.79 (m, 2H), 3.76 (s, 2H), 3.68 – 3.60 (m, 4H), 3.50 (t, J = 5.7 Hz, 2H), 3.19 (t, J = 5.7 Hz, 2H), 1.41 (s, 9H).

^{13}C NMR (126 MHz, Methanol-d_4) δ 158.4, 146.8, 145.9, 145.6, 138.4, 123.4, 100.1, 80.11, 71.6, 71.4, 71.1, 69.9, 44.8, 41.2, 28.7.

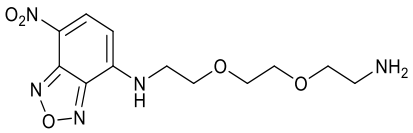
NBD-PEG$_4$-NH(Boc) (6)



DIPEA (52 µL, 0.3 mmol) was added to NH_2-PEG$_4$-NH(Boc) (0.01 g, 0.03 mmol) in MeOH (1 mL) and the mixture was stirred at room temperature for 10 min. A solution of 4-chloro-7-nitrobenzofuran in MeOH (0.9 mL) was cooled to 0 °C and the mixture of NH_2-PEG$_4$-NH(Boc) and DIPEA was added dropwise over 30 min. The reaction mixture was warmed to room temperature and stirred overnight under N_2. The solvent was removed *in vacuo* and the crude product purified by column chromatography using 3:2 Hexane/EtOAc with 0.1% TEA as an eluent to give **6** in 98% yield.

HPLC (ELSD) t_R = 4.81 min, purity 100 %.
HR ESI-MS: calculated for $C_{21}H_{33}N_5O_9$ $[M+H]^+$ m/z 500.2351; Found 500.2364
^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 8.52 (d, *J* = 8.9 Hz, 1H), 6.43 (d, *J* = 8.9 Hz, 1H), 5.84 – 5.76 (m, 1H), 5.04 – 4.83 (m, 1H), 4.21 – 3.88 (m, 1H), 3.82 (dd, *J* = 5.6, 4.8 Hz, 2H), 3.73 – 3.50 (m, 13H), 3.48 (t, *J* = 5.6 Hz, 2H), 3.20 (t, *J* = 5.6 Hz, 2H), 1.42 (s, 9H).
^{13}C NMR (126 MHz, Methanol-*d*$_4$) δ 158.4, 147.0, 146.0, 145.6, 138.5, 123.4 100.4, 80.1, 71.63, 71.62, 71.56, 71.5, 71.2, 71.0, 44.8, 41.3, 28.8.

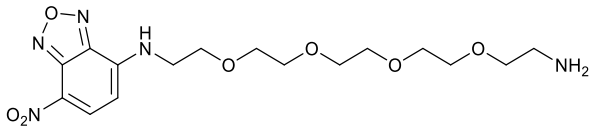
NBD-PEG$_2$-NH$_2$ (7)



NBD-PEG$_2$-NH(Boc) **5** (200 mg, 0.5 mmol) was dissolved in 3 mL of DCM/TFA (1:1) and the reaction mixture was stirred at room temperature for 2 h. DCM (100 mL) was added and the solvent was removed *in vacuo*. The crude product was dissolved in DCM (100 mL) and washed with sat. $NaHCO_3$ (100 mL) and the organic layer was dried over $MgSO_4$. After filtration, the solvent evaporated *in vacuo* giving **7** (152 mg) in 98% yield.

HPLC (ELSD) t_R = 2.24 min, purity 98 %
HR ESI-MS: calculated for $C_{12}H_{18}N_5O_5$ $[M+H]^+$ m/z 312.1303; Found 312.1313
^{1}H NMR (500 MHz, Methanol-*d*$_4$) δ 8.52 (d, *J* = 8.4 Hz, 2H), 6.43 (d, *J* = 8.9 Hz, 1H), 3.83 (t, *J* = 5.0 Hz, 2H), 3.77 (s, 2H), 3.73 – 3.70 (m, 2H), 3.70 – 3.65 (m, 4H), 3.08 (t, *J* = 4.9 Hz, 2H).
^{13}C NMR (126 MHz, Methanol-*d*$_4$) δ 147.0, 145.9, 145.6, 138.4, 123.5, 100.3, 71.5, 71.4, 69.8, 68.1, 44.7, 40.7.

NBD-PEG$_4$-NH$_2$ (8)



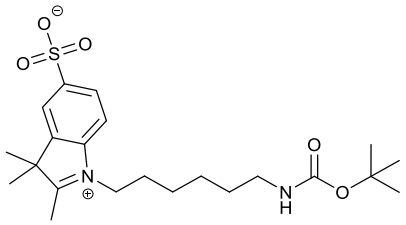
NBD-NH-PEG$_4$-NH(Boc) **6** (100 mg, 0.2 mmol) was stirred overnight in 50% TFA in DCM (4 mL). The solvent was evaporated *in vacuo,* the residue dissolved in DCM (50 mL) and washed with brine (2 × 50 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated to give **8** in 98 % yield.

HPLC (ELSD) t_R = 2.86 min, purity 99%.

HR ESI-MS: calculated for $C_{16}H_{26}N_5O_7$ $[M+H]^+$ m/z 400.1827; Found 400.1838

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.55(s,1H), 8.52 (d, J = 8.9 Hz, 1H), 6.44 (d, J = 8.9 Hz, 1H), 3.81 (td, J = 6.2, 5.8, 0.4 Hz, 2H), 3.79 – 3.69 (m, 1H), 3.68 – 3.65 (m, 2H), 3.66 – 3.64 (m, 2H), 3.64 – 3.62 (m, 2H), 3.62 – 3.60 (m, 4H), 3.60 – 3.56 (m, 4H), 3.06 – 3.03 (m, 2H).
^{13}C NMR (126 MHz, Methanol-d_4) δ 147.0, 145.9, 145.6, 138.4, 123.2, 100.4, 71.6, 71.5, 71.43, 71.41, 71.37, 71.2, 70.0, 68.2, 44.8, 40.7.

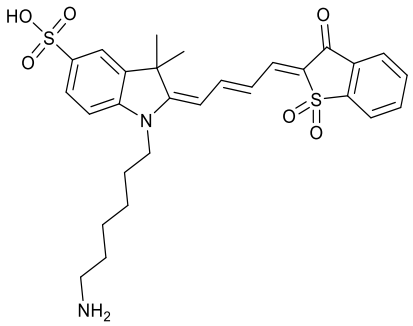
1-(3-(Boc-amino)-2,3,3-3*H*-indolium sulfonate (10)



6-(Boc)-aminohexyl bromide (556 mg, 1.99 mmol) and KI (479 mg, 2.89 mmol) in acetonitrile (5 mL) was heated to 40 °C for 1 h. Potassium 2,3,3-trimethyl-3*H*-indole-5-sulfonate **9** (500 mg, 1.80 mmol) was added and the reaction mixture was refluxed for 48 h. After cooling to room temperature, diethyl ether (50 mL) was added and the precipitate was collected. The solid was dissolved in water (0.5 mL), precipitated in acetone, filtered and dried *in vacuo* to give **10** in 71% yield (560 mg).

HPLC (ELSD) t_R = 3.43 min, purity 98%.
HR ESI-MS: calculated for $C_{22}H_{34}O_5N_2S$ $[M+H]^+$ m/z 439.2261; Found 439.2282
^{1}H NMR (500 MHz, Methanol-d_4) δ 8.11 (d, J = 8.1 Hz, 1H), 8.03 (dd, J = 8.0, 1.6 Hz, 1H), 7.91 (d, J = 8.3 Hz, 1H), 4.51 (t, J = 7.8 Hz, 2H), 3.04 (t, J = 6.8 Hz, 2H), 1.63 (s, 6H), 1.56 – 1.43 (m, 8H), 1.42 (s, 9H).
^{13}C NMR (126 MHz, DMSO-d_6) δ 197.6, 156.1, 150.2, 142.0, 141.3, 126.9, 121.3, 115.3, 77.8, 54.8, 48.1, 29.7, 28.8, 27.6, 26.3, 26.0, 22.4, 14.4.

Sulfo-MeroCy-NH$_2$ (12)



1-(3-(Boc-amino)-2,3,3-3*H*-indolium sulfonate **10** (550 mg, 1.25 mmol), sodium acetate (103 mg, 1.25 mmol), and 3-methoxyprop-[2-en-1-ylidene]-1-benzothiophen-3-one 1,1-dioxide **11**[3] (40 mg, 0.96 mmol) were mixed in 2.4 mL of MeOH:DCM (1:1) in a microwave vial and sealed. The reaction was heated at 75 °C for 30 min under microwave irradiation. The solvent was evaporated *in vacuo* and the residue was dissolved in 1 M HCl in MeOH (5 mL) and the mixture was stirred 24 h at room temperature. The solvent was evaporated *in vacuo* and the crude product was purified by reverse-phase column chromatography using a gradient of acetonitrile (5% to 95%) in water with 0.1% formic acid as an eluent to give **12** in 9% yield (48 mg).

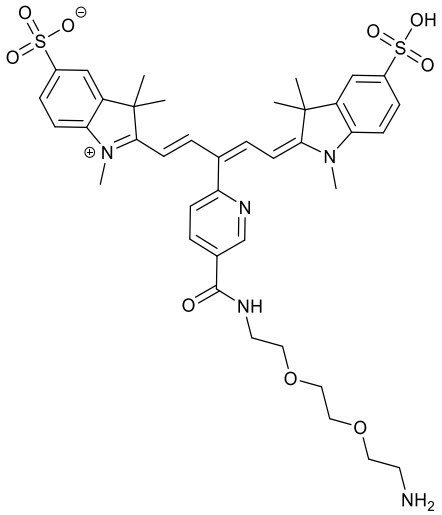
HPLC (550 nm) t_R = 3.43 min, purity 98%.

HR ESI-MS: calculated for $C_{28}H_{32}O_6N_2S_2$ $[M+H]^+$ m/z 557.1775; Found 557.1765

^{1}H NMR (500 MHz, Methanol-d_4) δ 8.14 (t, *J* = 12.9 Hz, 1H), 7.97 (dd, *J* = 22.9, 7.5 Hz, 2H), 7.93 – 7.84 (m, 3H), 7.84 (d, *J* = 1.7 Hz, 1H), 7.22 (d, *J* = 8.2 Hz, 1H), 6.82 (t, *J* = 13.0 Hz, 1H), 6.22 (d, *J* = 13.3 Hz, 1H), 4.07 (t, *J* = 7.2 Hz, 2H), 2.92 (t, *J* = 7.5 Hz, 1H), 1.84 (t, *J* = 6.9 Hz, 2H), 1.73 (s, 6H), 1.65 (t, *J* = 6.7 Hz, 2H), 1.54 – 1.43 (m, 4H).

^{13}C NMR (126 MHz, DMSO-d_6) δ 176.1, 172.0, 166.3, 155.8, 145.0, 144.3, 143.6, 142.8, 140.5, 135.2, 134.3, 133.7, 126.4, 123.7, 120.8, 120.2, 118.0, 116.5, 109.8, 101.7, 48.7, 43.4, 28.6, 27.8, 27.1, 26.11, 26.06.

SulfoCy5-PEG$_2$-NH$_2$ (13)



To sulfonated Cy5-carboxylic acid[4] (50 mg, 0.075 mmol) in DMF (6 mL), HSPyU (31 mg, 0.075 mmol) and DIPEA (40 µL, 0.230 mmol) were added, and the mixture was stirred 2 h at 40 ° covered from light. NH_2-PEG_2-NH(Boc) (89 µL, 0.375 mmol) and DIPEA (40 µL, 0.230 mmol) were added and the reaction mixture was stirred for 18 h at room temperature. The solvent was evaporated *in vacuo* and the residue was dissolved in 50% TFA in DCM (3 mL) and stirred 2 h at room temperature. The solvent was evaporated *in vacuo* and the crude product was purified by reverse-phase column chromatography, using a gradient of acetonitrile (2% to 75%) in water with 0.1% formic acid as an eluent, to give the product in 68% yield.

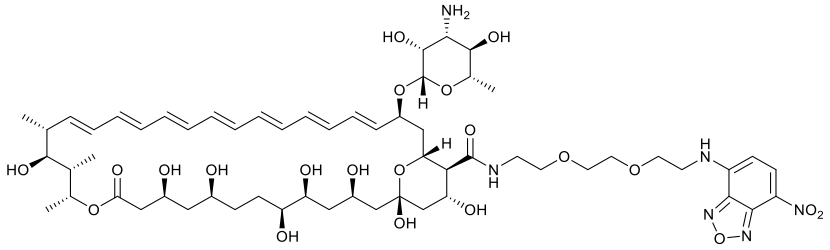
HPLC (600 nm) t_R = 2.53 min, purity 98 % (ELSD)

HR ESI-MS: calculated for $C_{39}H_{47}O_9N_5S_2$ $[M+H]^+$ m/z 794.2888; Found 794.2887

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.45 (d, *J* = 14.4 Hz, 2H), 8.35 (d, *J* = 8.1 Hz, 1H), 7.84 (s, 2H), 7.64 (d, *J* = 8.3 Hz, 2H), 7.62 (d, *J* = 8.0 Hz, 2H), 7.31 (d, *J* = 8.3 Hz, 2H), 5.84 (d, *J* = 14.3 Hz, 2H), 3.64 – 3.59 (m, 6H), 3.58 (d, *J* = 5.3 Hz, 2H), 3.51 (d, *J* = 5.8 Hz, 2H), 3.37 (s, 6H), 2.95 (t, *J* = 5.3 Hz, 2H), 1.76 (s, 12H).

^{13}C NMR (126 MHz, MeOD) δ 177.0, 168.4, 149.8, 149.8, 145.3, 142.6, 142.3, 138.41, 138.39, 127.8, 120.9, 119.2, 112.0, 102.1, 70.9, 70.8, 70.2, 67.6, 50.6, 40.6, 40.4, 31.9, 27.5.

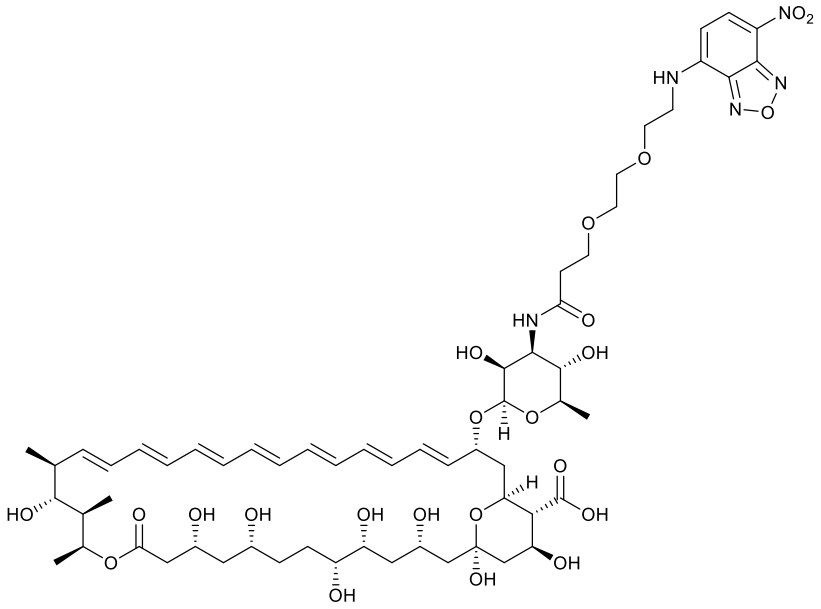
AmB-NBD-1A



To a solution of NBD-PEG$_2$-NH$_2$ **7** (19 mg, 0.06 mmol) and Amphotericin B (18 mg, 0.02 mmol) in anhydrous DMSO (0.3 mL), trimethylamine (20 µL, 0.14 mmol) was added. PyBOP (16 mg, 0.03 mmol) was added portion wise over 20 min and the reaction mixture was stirred at room temperature. The reaction was monitored by LC-MS and, after 1 h, fresh portion of PyBOP (5 mg, 0.01 mmol) was added and the reaction mixture was stirred for 14 h. Upon completion, 3 mL of diethyl ether was added and the mixture was stirred vigorously for 2 min, and the diethyl ether layer was removed. This process was repeated until the diethyl ether wash became colourless. To the oily residue, 1.5 mL of acetone was added and the mixture was stirred until a precipitate formed. The precipitate was collected by vacuum filtration and washed with acetone, and then with diethyl ether to give **AmB-NBD-1A** in 33% yield (8 mg).

HPLC (ELSD) t_R = 4.10 min, purity 97 %
HR ESI-MS: calculated for $C_{59}H_{88}N_6O_{21}$ $[M+Na]^+$ m/z 1239.5895; Found 1239.5885

AmB-NBD-1B



EDC hydrochloride (28.5 mg, 0.15 mmol) and DIPEA (26 µL, 0.15 mmol) were added to NBD-PEG$_2$-COOH **1** (46 mg, 0.14 mmol) in DCM (9 mL) and the mixture was stirred for 10 min at room temperature. *N*-Hydroxysuccinimide (17 mg, 0.15 mmol) was added and the reaction mixture was stirred at room temperature for 16 h covered from light. Upon completion, the reaction mixture was diluted with DCM (10 mL) and the organic

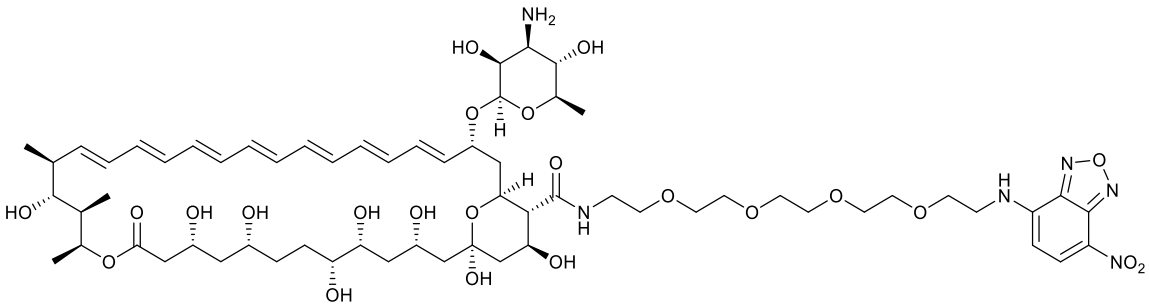
layer washed with 5% citric acid (aq. 10 mL) and then with sat. $NaHCO_3$ (10 mL). The Organic layer was dried over $MgSO_4$, filtered, and the solvent evaporated *in vacuo* to give the NHS-ester **3** that was used for the next step without any purification.

NBD-PEG_2-NHS **3** (56 mg, 0.13 mmol) in 0.5 mL of DMF/DCM (2:1) was added to a solution of Amphotericin B (55 mg, 0.06 mmol) and trimethylamine (20 µL, 0.14 mmol) in DMSO (0.5 mL), and the reaction mixture was stirred at room temperature for 16 h covered from light. The reaction mixture was washed with diethyl ether (50 mL) and evaporated under N_2 flow. The crude product was purified first by silica column using DCM/MeOH/HCOOH (10:2:0.2) as an eluent to give 77 mg of impure product, of which 28 mg was dissolved in DMSO (3 mL) and trimethylamine (0.1 mL) and stirred for 2 h. Solution was washed with diethyl ether (30 mL) and the remaining dark viscous residue was then precipitated with acetone (2 mL). The dark yellow precipitate was collected by vacuum filtration, and further purified by reverse-phase column chromatography using a gradient of acetonitrile (30% to 85%) in water with 0.1% TFA as an eluent to give **AmB-NBD-1B** in 19% yield (5 mg).

HPLC (ELDS) t_R = 5.20 min, purity 86 %.

HR ESI-MS: calculated for $C_{60}H_{87}N_5O_{23}$ $[M+Na]^+$ m/z 1268.5684; Found 1268.5671.

AmB-NBD-2A

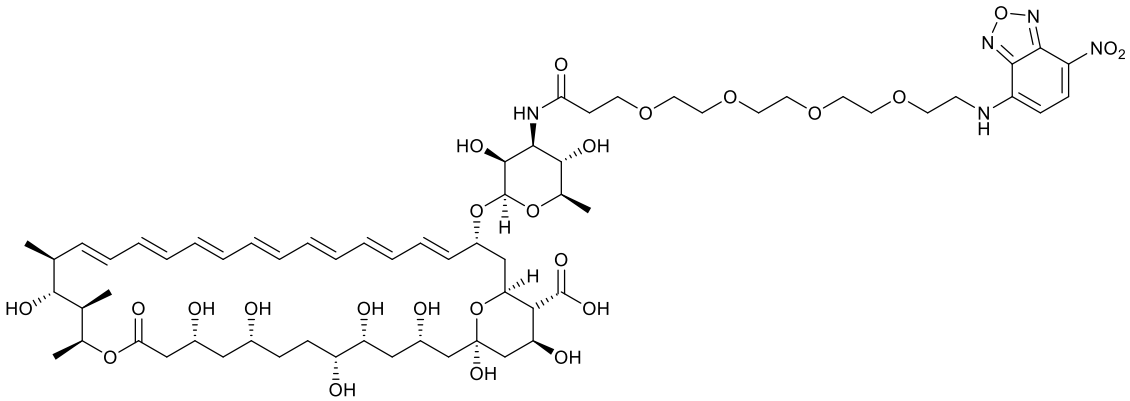


Trimethylamine (20 µL, 0.14 mmol) was added to NBD-PEG_4-NH_2 **8** (26 mg, 0.06 mmol) and Amphotericin B (20 mg, 0.02 mmol) in anhydrous DMSO (0.3 mL). PyBOP (16 mg, 0.03 mmol) was added portion wise over 15 min and reaction mixture was stirred at room temperature for 1 h (the reaction was monitored by LC-MS). After completion, diethyl ether was added (3 mL) and the mixture was stirred vigorously for 2 min, and diethyl ether layer was removed. This process was repeated four times until diethyl ether wash became colourless. Acetone (3 mL) was added to the oily residue and the mixture was stirred until a precipitate formed. The precipitate was collected by vacuum filtration and washed with acetone, and diethyl ether, to give **AmB-NBD-2A** in 40% yield.

HPLC (ELSD) t_R = 4.17 min, purity 95 %.

HR ESI-MS: calculated for $C_{63}H_{96}N_6O_{23}$ $[M+H]^+$ m/z 1305.6600; Found 1305.6599.

AmB-NBD-2B



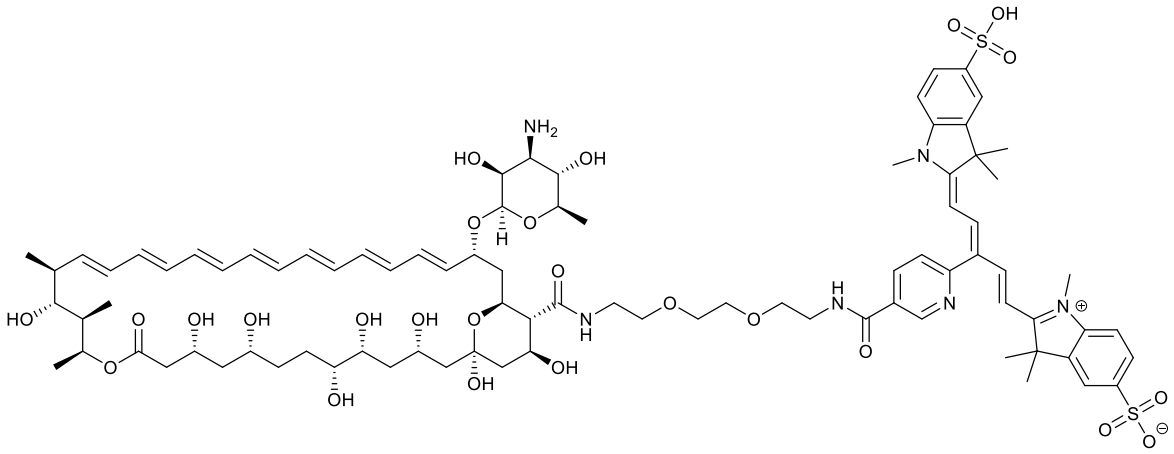
EDC hydrochloride (49 mg, 0.256 mmol) and DIPEA (43 µL, 0.256 mmol) were added to a solution of NBD-PEG$_4$-COOH **2** (100 mg, 0.234 mmol) in anhydrous DCM (16 mL), and the mixture was stirred for 10 min at room temperature. *N*-Hydroxysuccinimide (30 mg, 0.256 mmol) was added and the reaction mixture was stirred at room temperature for 16 h covered from light (the conversion was monitored by HPLC and LC-MS). The reaction mixture was diluted with DCM (10 mL) and washed with 5% citric acid (aq. 10 mL) and sat. $NaHCO_3$ (aq. 10 mL). The organic layer was collected, dried over $MgSO_4$, and filtered. The solvent was evaporated *in vacuo* to give the NHS-ester **4** that was used for the next step without purification.

NBD-PEG$_4$-CO-NHS **4** (74 mg, 0.14 mmol) in DMF/DCM (2:1, 0.5 mL) was added to a solution of Amphotericin B (44 mg, 0.048 mmol) and DIPEA (24 µL, 0.14 mmol) in DMSO (0.5 mL), and the reaction mixture was stirred at room temperature for 12 h. Upon completion, the reaction mixture washed several times with diethyl ether, after which the dark oily residue was mixed with 10 mL of *n*-butanol and water (1:1). The mixture was washed with water, the organic layer collected, and the solvent removed *in vacuo*. The crude product was purified by column chromatography using DCM/MeOH/H_2O/HCOOH (10:2:0.02:0.02) as an eluent to give **AmB-NBD-2B** in 11% yield (7 mg).

HPLC (ELSD) t_R = 5.09 min, purity 91 %.

HR ESI-MS: calculated for $C_{64}H_{95}N_5O_{25}$ $[M+Na]^{+-}$ m/z 1356.6208; Found 1356.6208.

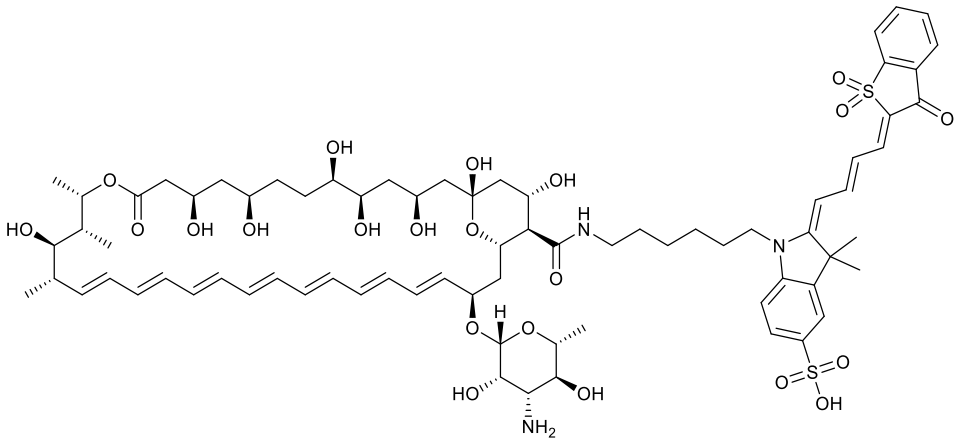
AmB-Cy5



Trimethylamine (20 µL, 0.14 mmol) was added to a solution of Cy5-PEG_2-NH_2 **13** (30 mg, 0.038 mmol) and Amphotericin B (12 mg, 0.013 mmol) in anhydrous DMSO (0.3 mL). PyBOP (20 mg, 0.038 mmol) was added in four portions over 15 min and the reaction mixture stirred at room temperature for 1 h (the reaction was monitored by LC-MS). Upon completion, the reaction mixture was vigorously stirred with 3 mL of diethyl ether for 2 min and diethyl ether layer was removed, with the process repeated twice. Acetone (3 mL) was added to oily residue and the mixture was stirred, and the formed precipitated collected by vacuum filtration. The crude product was purified by reverse-phase column chromatography using a gradient acetonitrile (2% to 75%) in water with 0.1% formic acid as an eluent, and **AmB-Cy5** was obtained in 20% yield after lyophilisation.

HPLC (600 nm) t_R = 3.78 min, purity 97 %.
HR ESI-MS: calculated for $C_{86}H_{116}O_{25}N_6S_2$ $[M+2Na]^{2+}$ m/z 872.3686; Found 872.3686.

AmB-MeroCy



To a solution of Sulfo-Mero-NH_2 **12** (20 mg, 0.036 mmol) and Amphotericin B (12 mg, 0.013mmol) in anhydrous DMSO (0.3 mL) was added trimethylamine (20 µL, 0.14 mmol). PyBOP (20 mg, 0.038 mmol) was added in 4 portions over 20 min and the reaction mixture was stirred at room temperature for 2 h (the reaction was monitored by LC-MS). The reaction mixture washed with diethyl ether (30 mL) to give an oily residue that was purified by

reverse phase column chromatography using gradient of acetonitrile (5% to 95%) in water with 0.1% formic acid as an eluent to give **AmB-MeroCy** in 11% (2.1 mg) yield after lyophilisation.

HPLC (550 nm) t_R = 4.59 min, purity 92 %.
HR ESI-MS: calculated for $C_{75}H_{103}N_3O_{22}Na$ $[M+Na]^+$ m/z 1484.6379; Found 1484.6379.

2.3. Fluorescence spectroscopy

Stock solutions of probes (1 mM in DMSO) were diluted to a final concentration of 1 μM in DMSO/PBS. The fluorescence emission spectra of **AmB-NBD2A** and **AmB-MeroCy** were measured with a RF-6000 spectrofluorophotometer (Shimadzu) upon excitation at 480 nm and 561 nm, respectively.

2.4. Biological Evaluation

Fungal growth and standardisation

Eight fungal and two bacterial strains were used in this study (microorganism details and growth conditions are summarised in Table S2). All strains were stored on beads in glycerol at –80˚C. Organisms were all grown on agar under the following conditions; bacteria were grown at 37˚C for 24h, yeasts were grown at 30˚C for 48h and spore-forming fungi were grown at 37˚C for 72h. Conidia were harvested from *A. fumigatus* and *F. solani* by flooding the plate with PBS with 0.025% Tween (ThermoFisher). Plates were gently agitated and the PBS was transferred to a 30 mL tube. Conidia were counted using a haemocytometer and stored at 4 °C. For all assays, conidia were used as the starting inoculum without need for overnight culturing.

Overnight cultures were centrifuged at 3000 rpm for 10 min and re-suspended in 1 mL of PBS. Cells were placed into Eppendorf vial (1.5 mL) and centrifuged at 13500 rpm for 2 min. The supernatant was removed and the cells were re-suspended in fresh PBS (1 mL) and diluted further with PBS to obtain OD_{600} of 0.5. For the bacterial and fungal co-culture, the cells were incubated together for 1.5 h in LB broth with 50% FBS.

Table S2. Summary of study organism growth conditions

Organism	Strain	Agar	Growth media	Broth Conditions
Candida albicans	ATCC MYA-2876	Sabourauds Dextrose	Potato Dextrose	37 ˚C with shaking
Candida albicans	SCS039	Sabourauds Dextrose	Yeast Peptone Dextrose	30 ˚C with shaking
Candida albicans	SCS204	Sabourauds Dextrose	Yeast Peptone Dextrose	30 ˚C with shaking
Candida auris	NCPF 8973	Sabourauds Dextrose	Yeast Peptone Dextrose	30 ˚C with shaking
Candida auris	NCPF 8978	Sabourauds Dextrose	Yeast Peptone Dextrose	30 ˚C with shaking
Candida auris	NCPF 8993	Sabourauds Dextrose	Yeast Peptone Dextrose	30 ˚C with shaking
Aspergillus fumigatus	Af293	Sabourauds Dextrose	N/A	N/A
Fusarium solani	NCPF 2171	Sabourauds Dextrose	N/A	N/A
Staphylococcus aureus	ATCC 25923	Lysogeny broth	Lysogeny broth	37˚C
Escherichia coli	ATCC 25922	Lysogeny broth	Lysogeny broth	37˚C

Fungal and Bacterial microscopy

μ-Slide 8-well confocal chambers (ibidi) were coated with poly-D-lysine (0.1 mg/mL in PBS) for 20 min at 37 °C, after which the wells were washed with PBS. *A. fumigatus* and *F. solani* conidia were diluted to $1x10^6$conidia/mL in RPMI for 16h to germinate. Fungi were incubated with the AmB-probes (10 μM in PBS with 1% DMSO) in Eppendorf (1.5 mL) vials in dark at 37 °C for 1–2 h, after which the media was removed, PBS added, and the cells imaged. For the multiplexed imaging of fungi–bacteria co-culture, *C. albicans*, *S. aureus* and *E. coli* were co-incubated 1 h with **AmB-Cy5** (10 μM in PBS), PMX-NBD[1] (5 μM) and Gram-positive bacteria probe (1 μM) in PBS with 1% DMSO.

Imaging was performed on a Leica TCS SP8 - laser scanning confocal microscope (λ_{ex} = 488 nm for NBD based probes, λ_{ex} = 561 nm for Merocyanine based probe, and λ_{ex} = 633 nm for Cy5 based probe). Images were analysed using ImageJ2 (National Institutes of Health).

Fungal biofilm formation

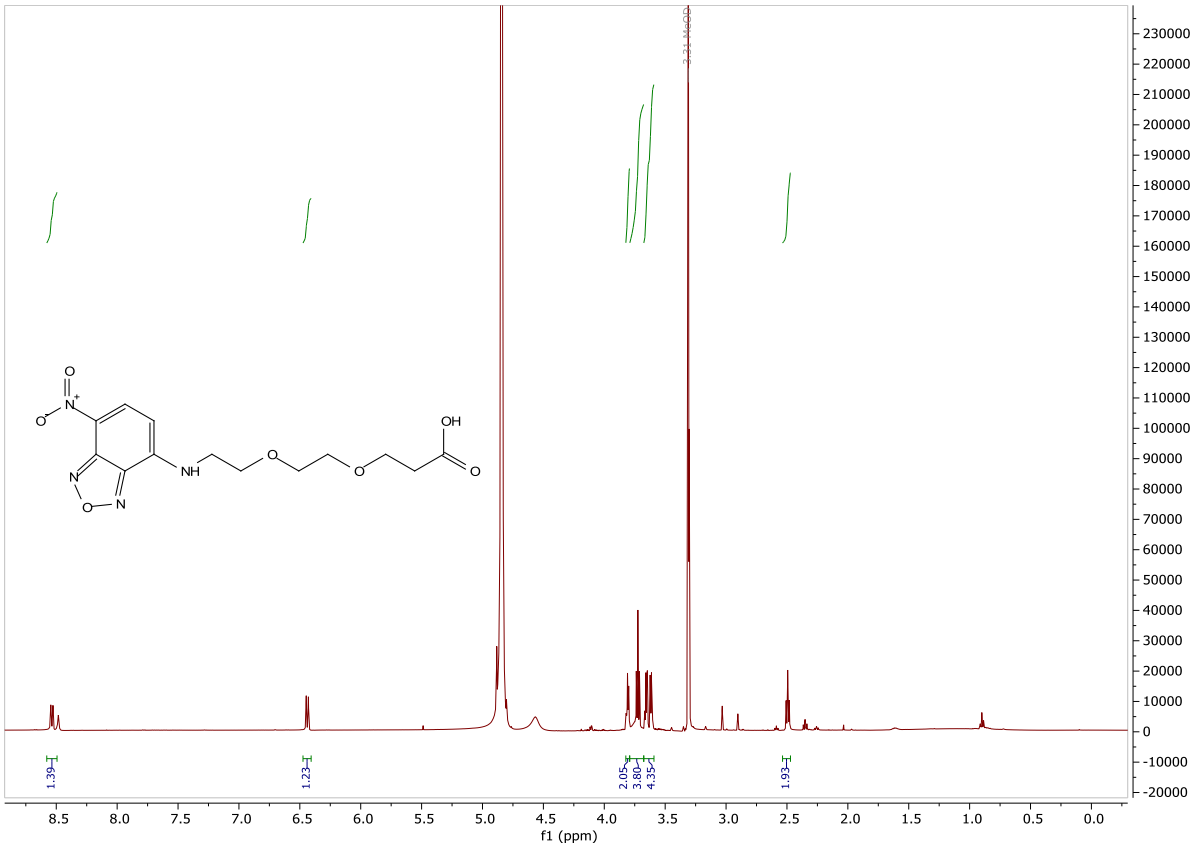
Candida cells from overnight broths were washed twice with PBS before counting in a haemocytometer. *Candida* cells were diluted to 1×10^6 cells/mL by diluting in Todd Hewitt broth (THB; Sigma Aldrich) supplemented with 10 μM menadione and 4 μg/mL Hemin and Roswell Parks Memorial Institute (RPMI; ThermoFisher) mixed together at a ratio of 1:1 (referred to at 1:1 broth from herein). Inoculated media was seeded into μ-Slide 8-well confocal chambers coated with poly-D-lysine, appropriate media controls were included and the plate was incubated at 37 °C for 24 h. After the incubation step, growth media was removed and each biofilm was washed with 200 μL PBS to remove any non-adherent cells.

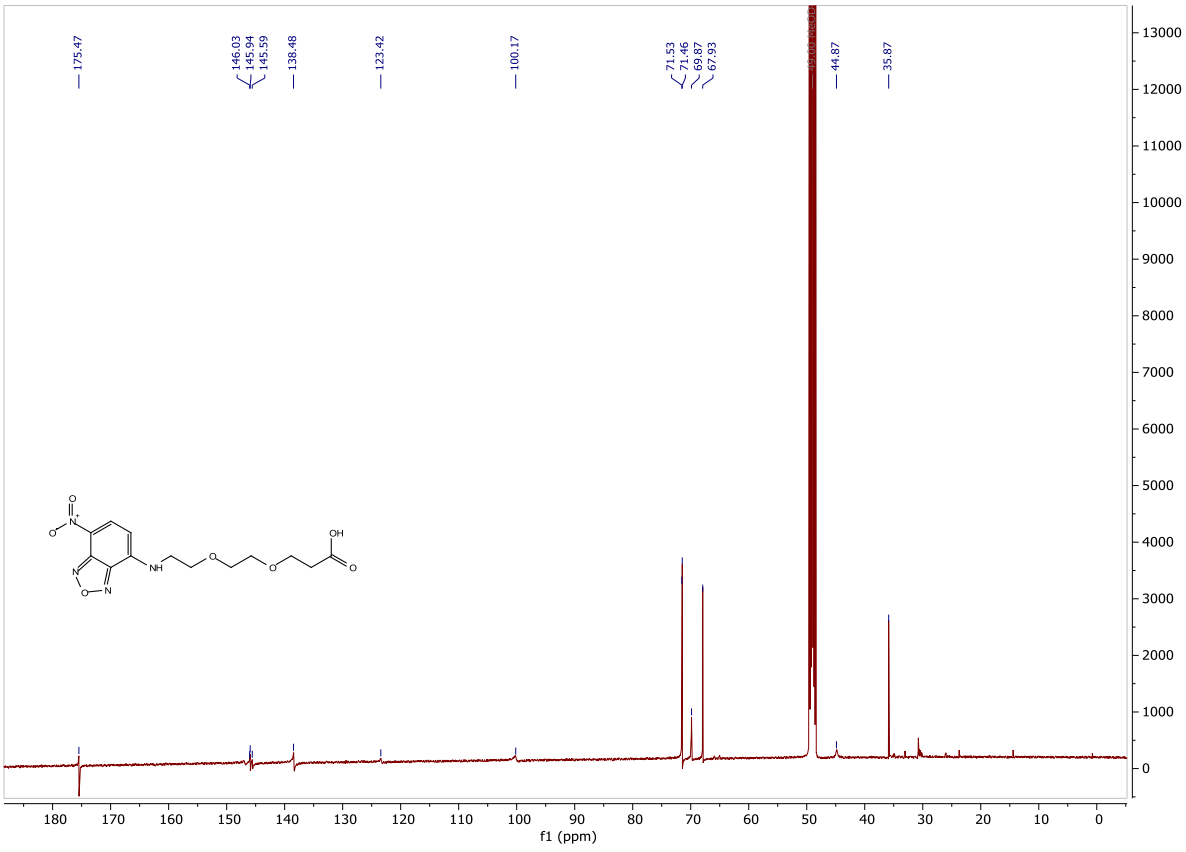
Fungal susceptibility testing

Planktonic minimum inhibitory concentration (PMIC) testing was carried out on single species inoculum in 1:1 broth following standard broth microdilution testing protocols.[5] The minimum fungicidal concentration (MFC) was determined by plating 20 μL of each concentration of antifungal onto Sabourauds Dextrose agar. Yeasts were incubated at 30 °C for 48 h whereas filamentous fungi were incubated at 37 °C for 72 h.

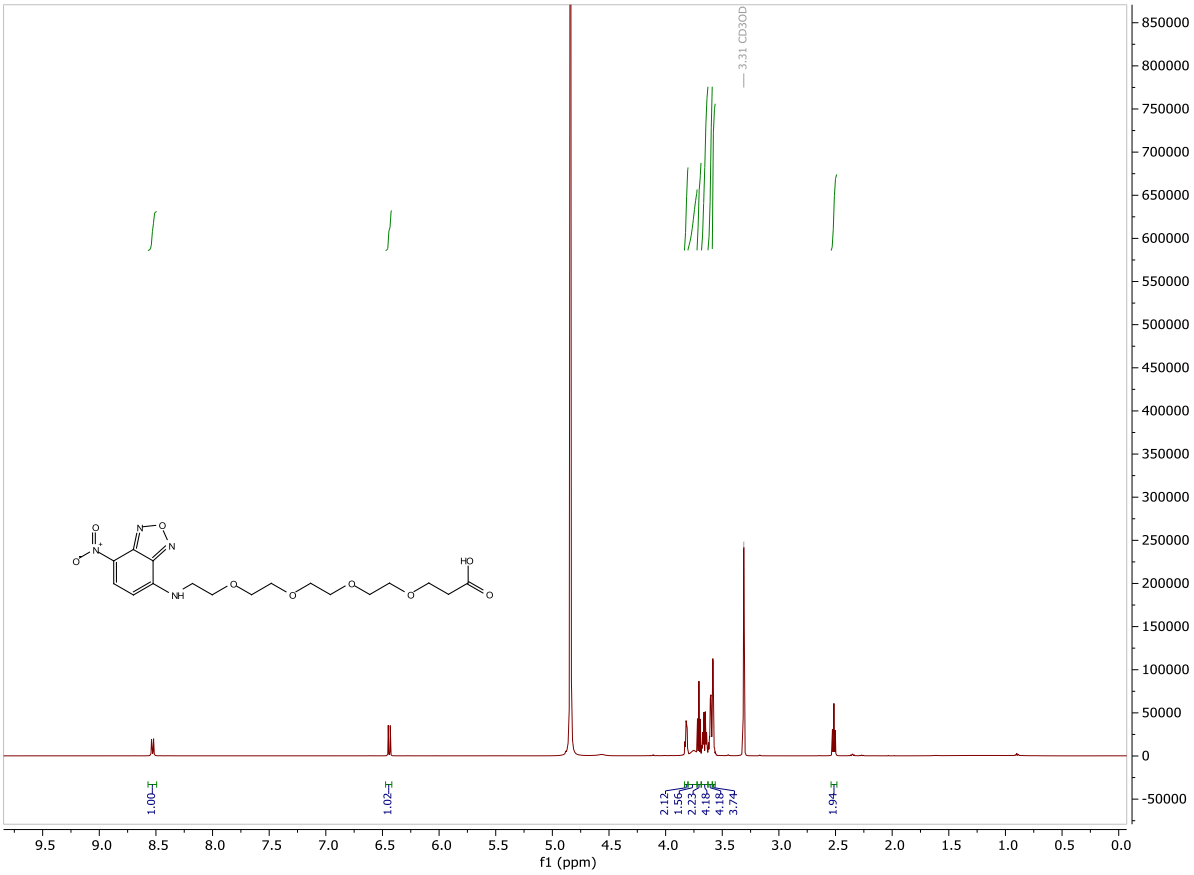
3. NMR spectra

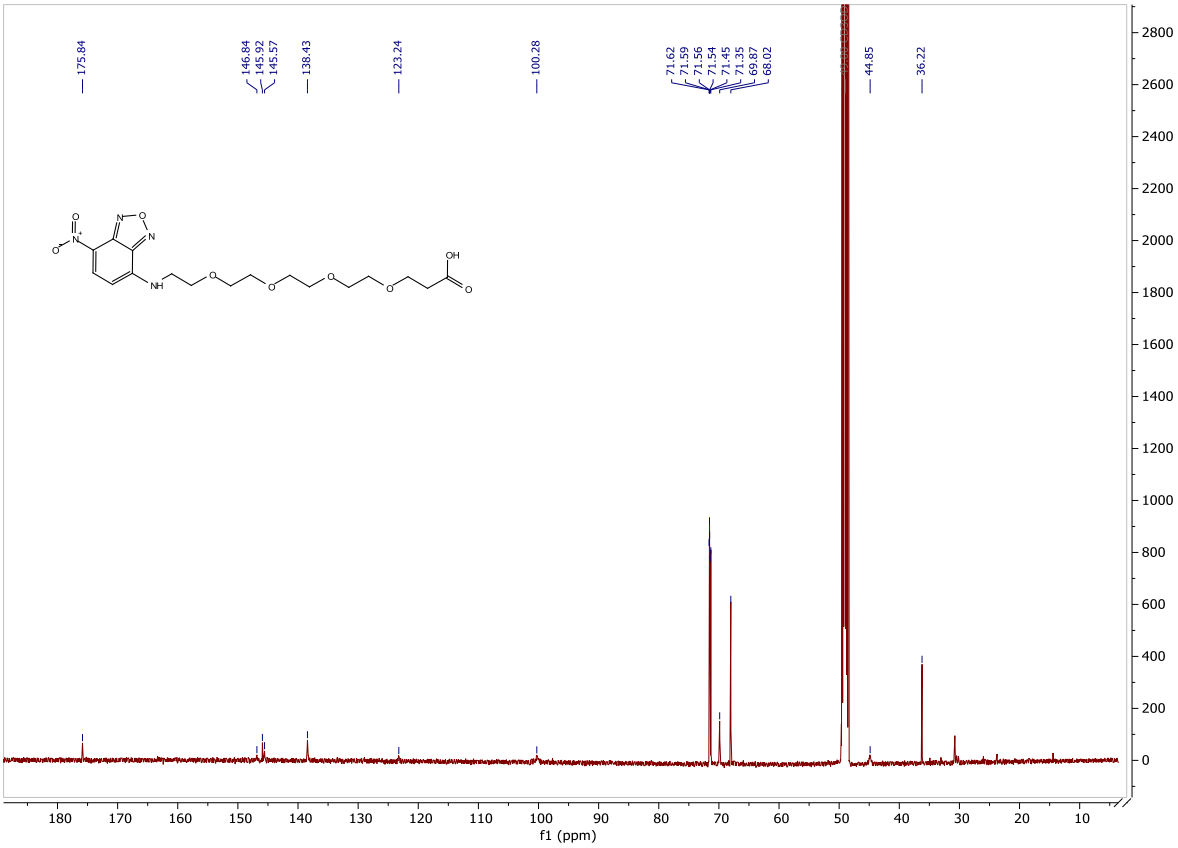
NBD-PEG$_2$-COOH (1)



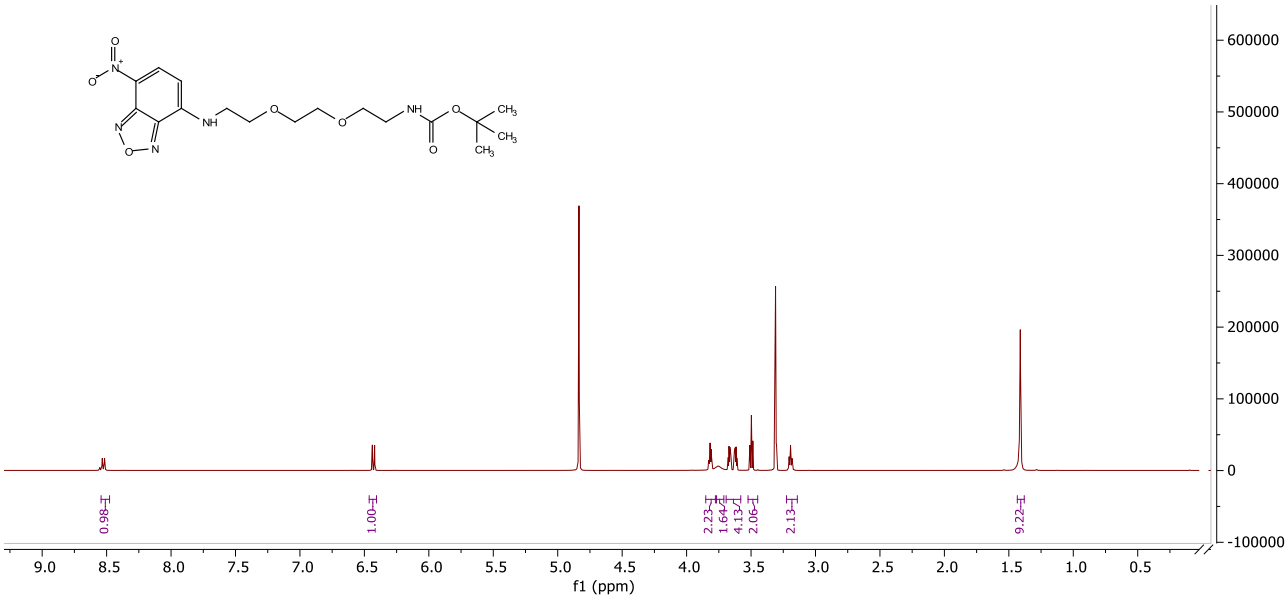


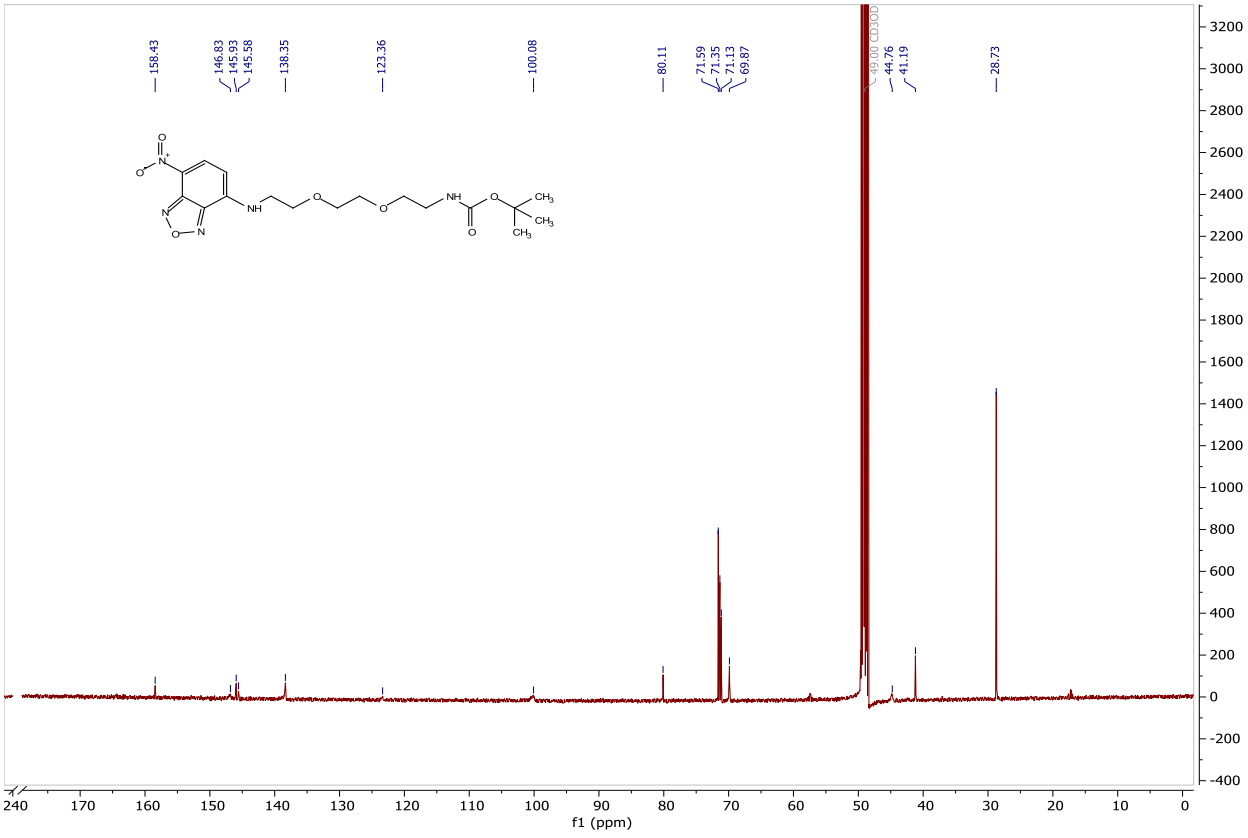
NBD-PEG$_4$-COOH (2)





NBD-PEG$_2$-NH(Boc) (5)





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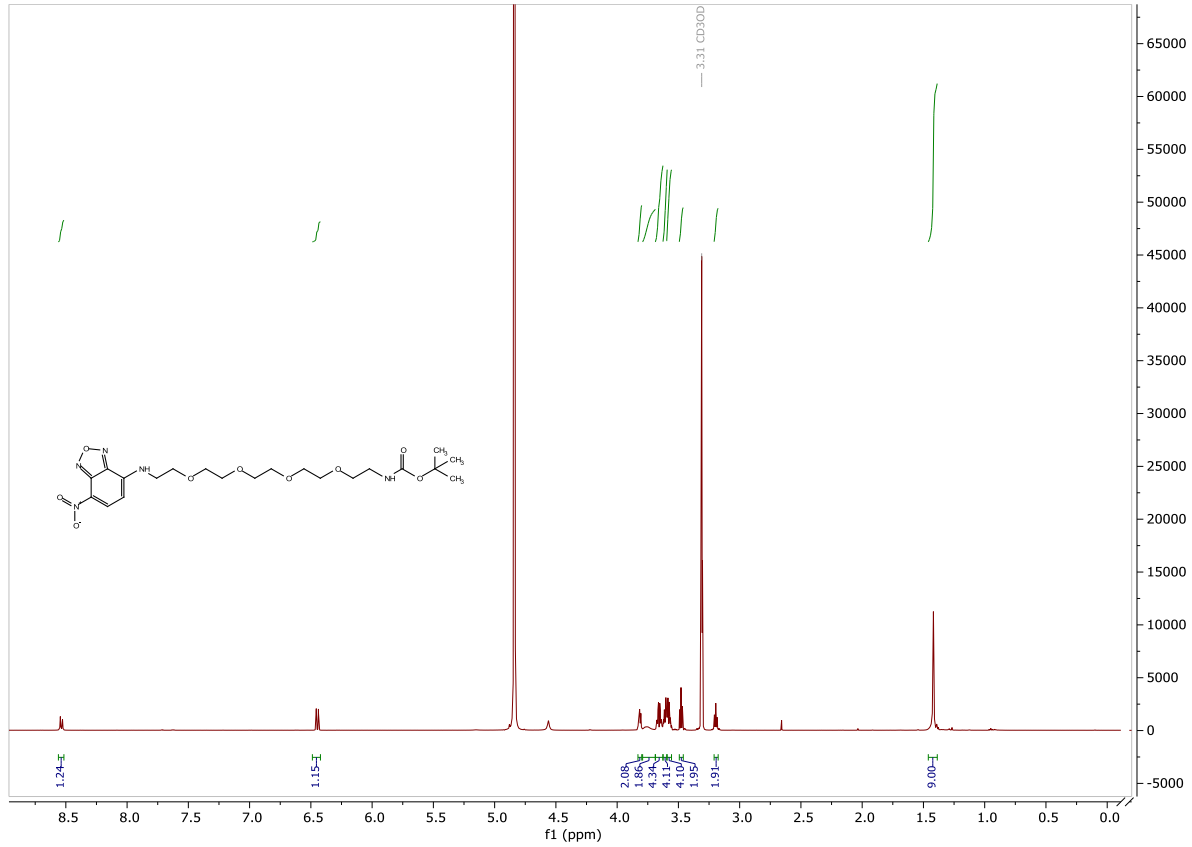
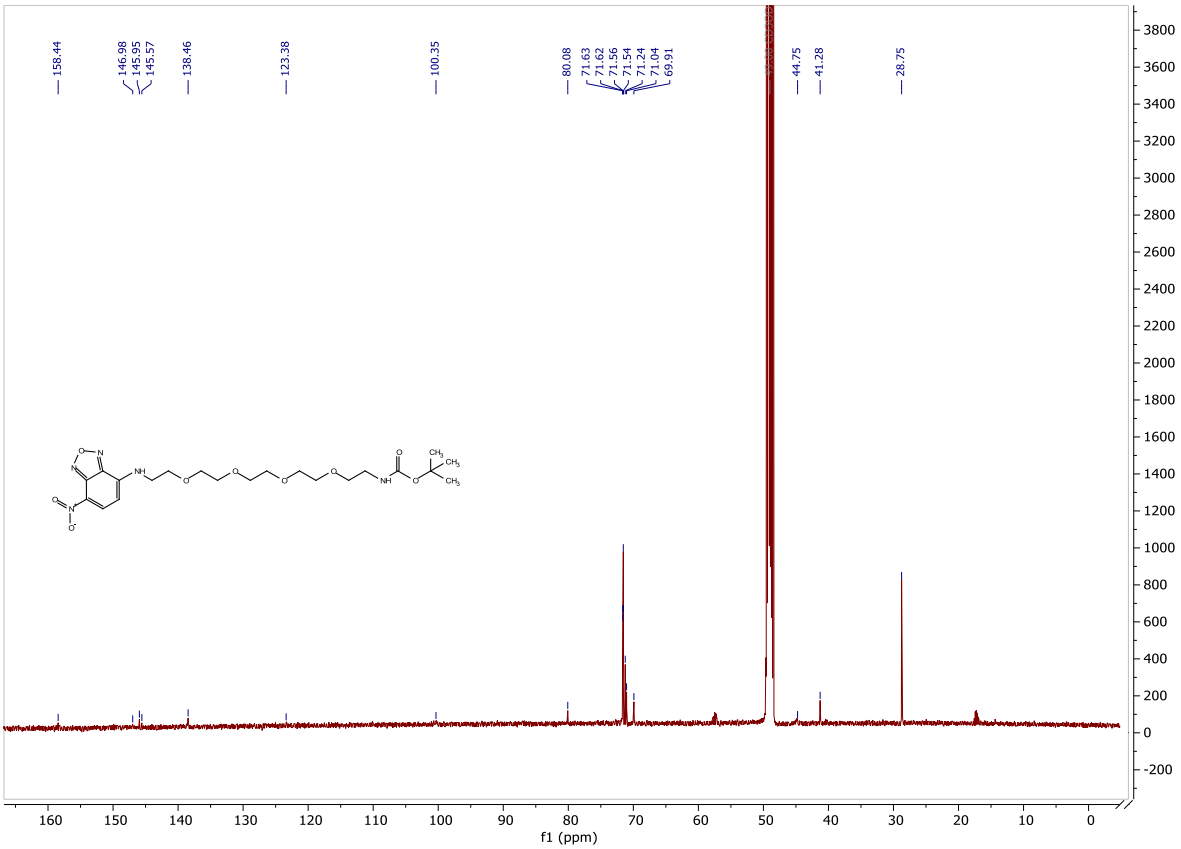
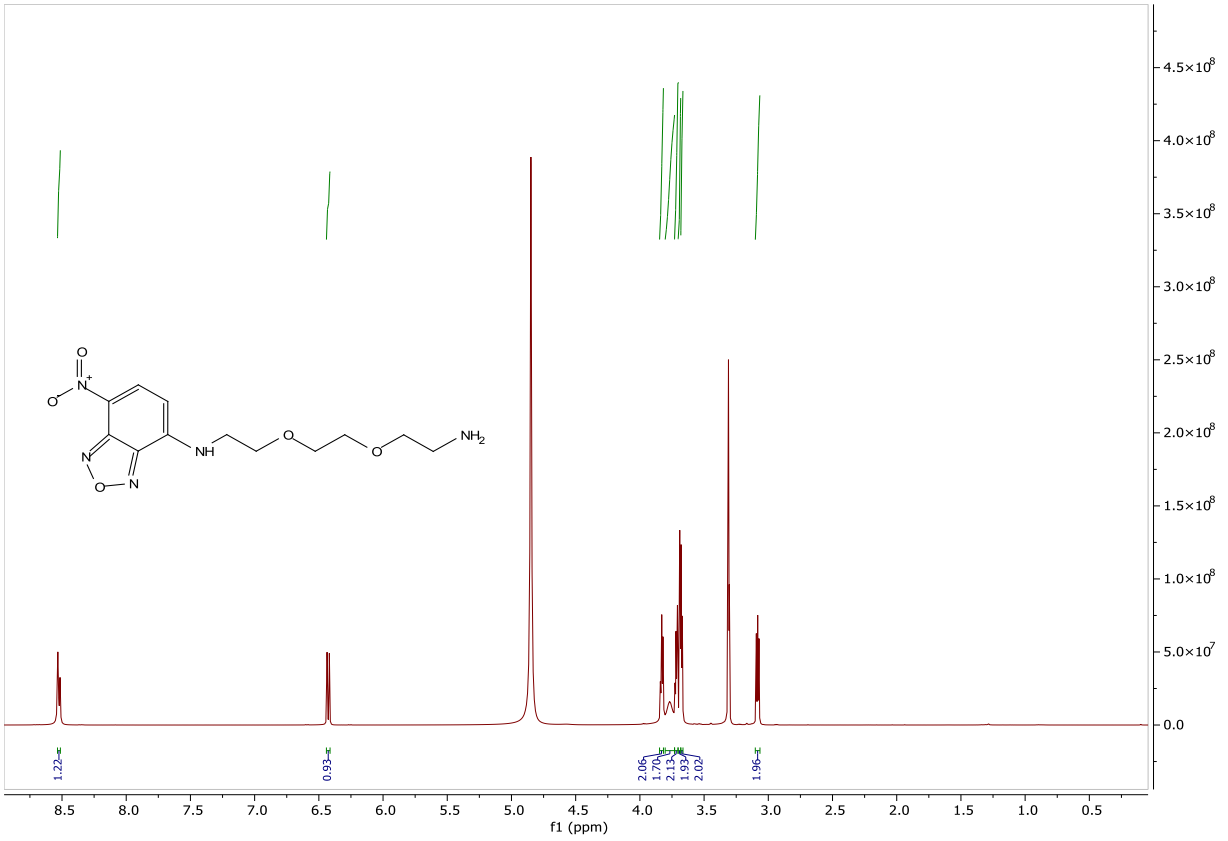
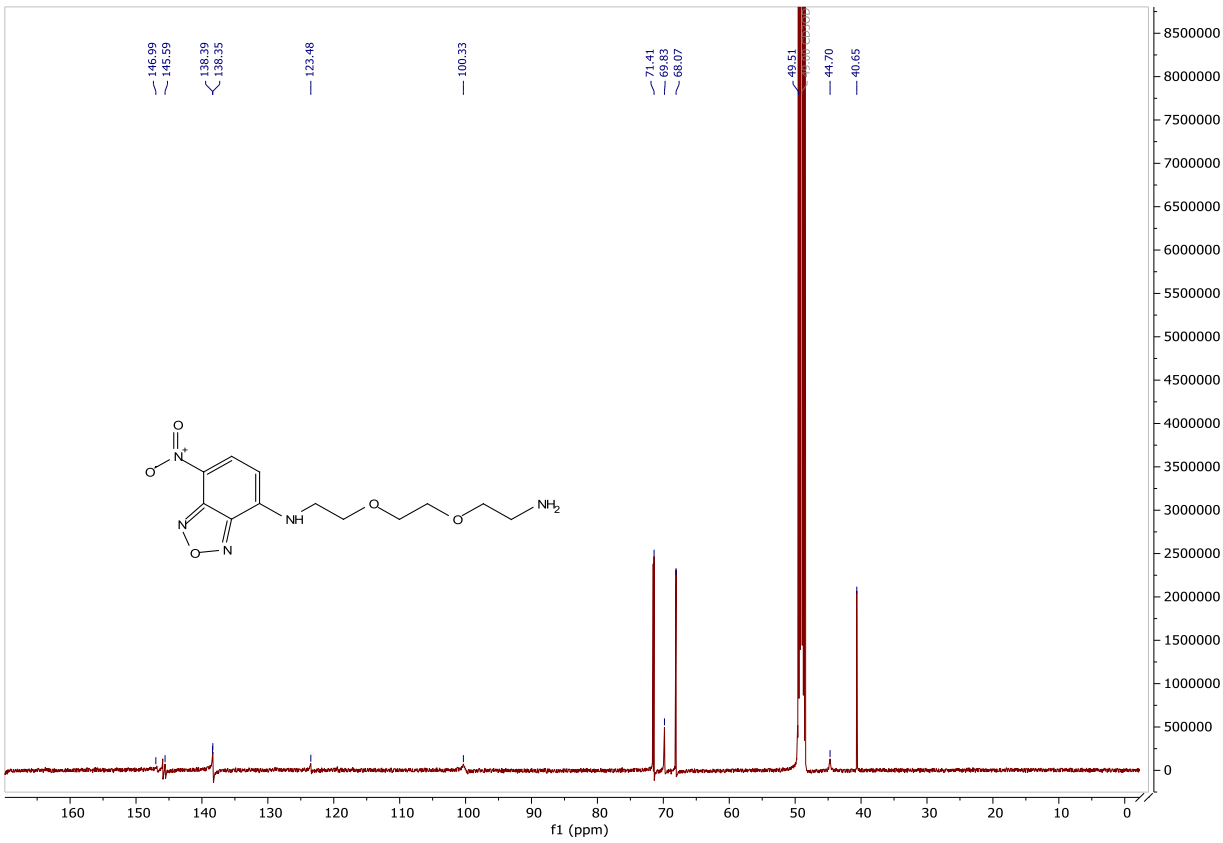


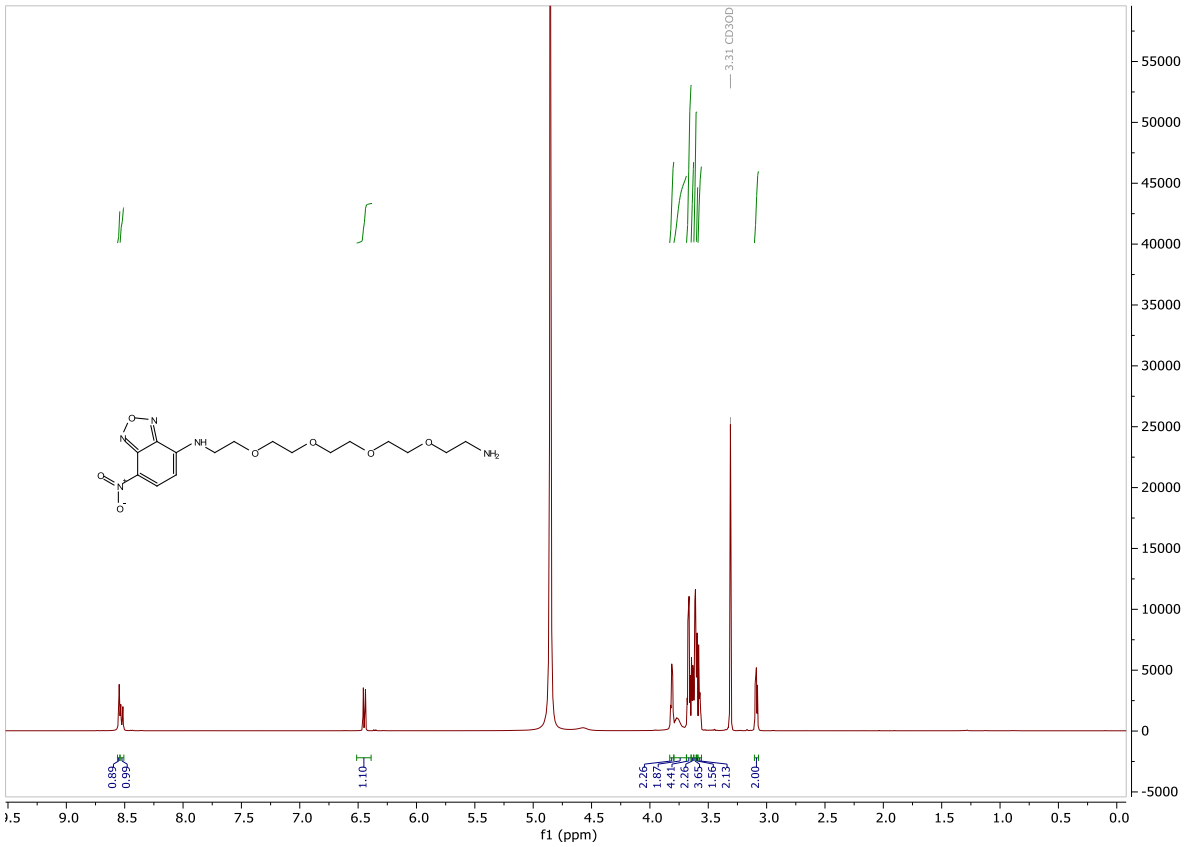


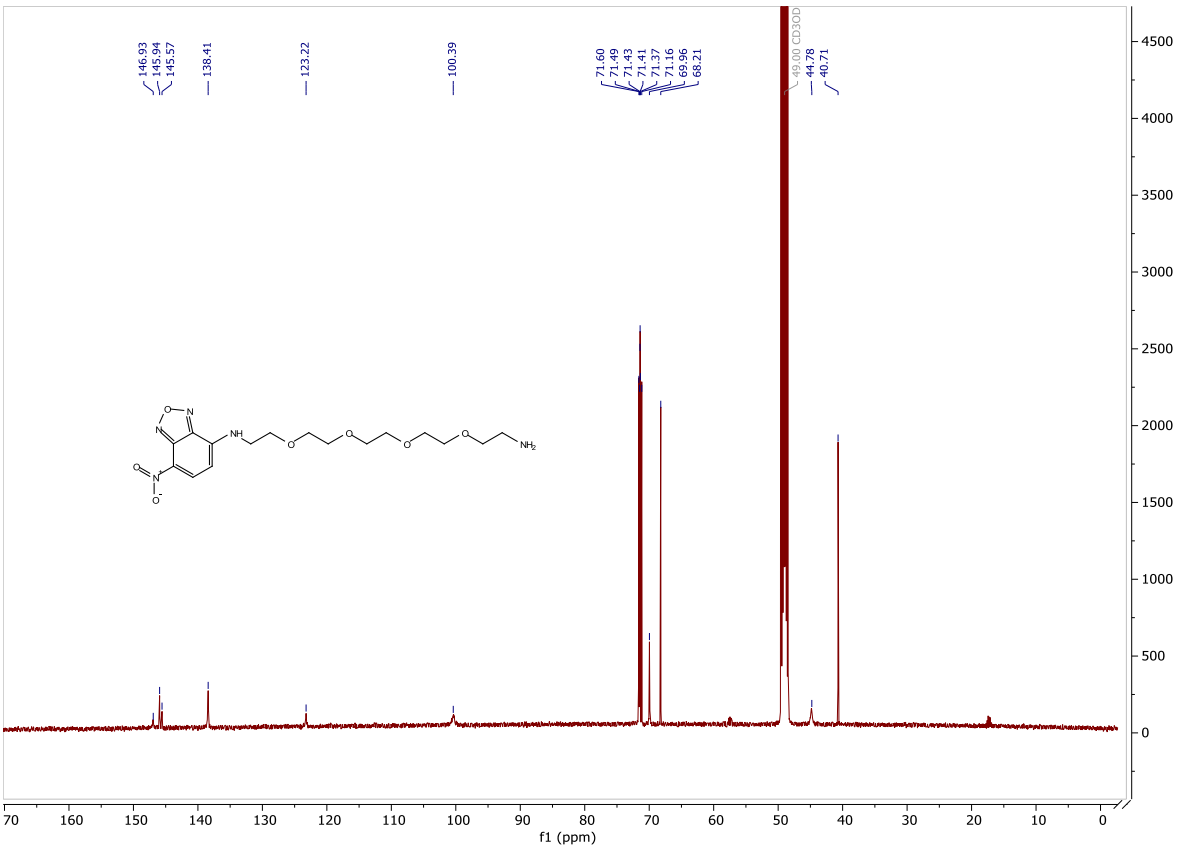
NBD-PEG$_2$-NH$_2$ (7)



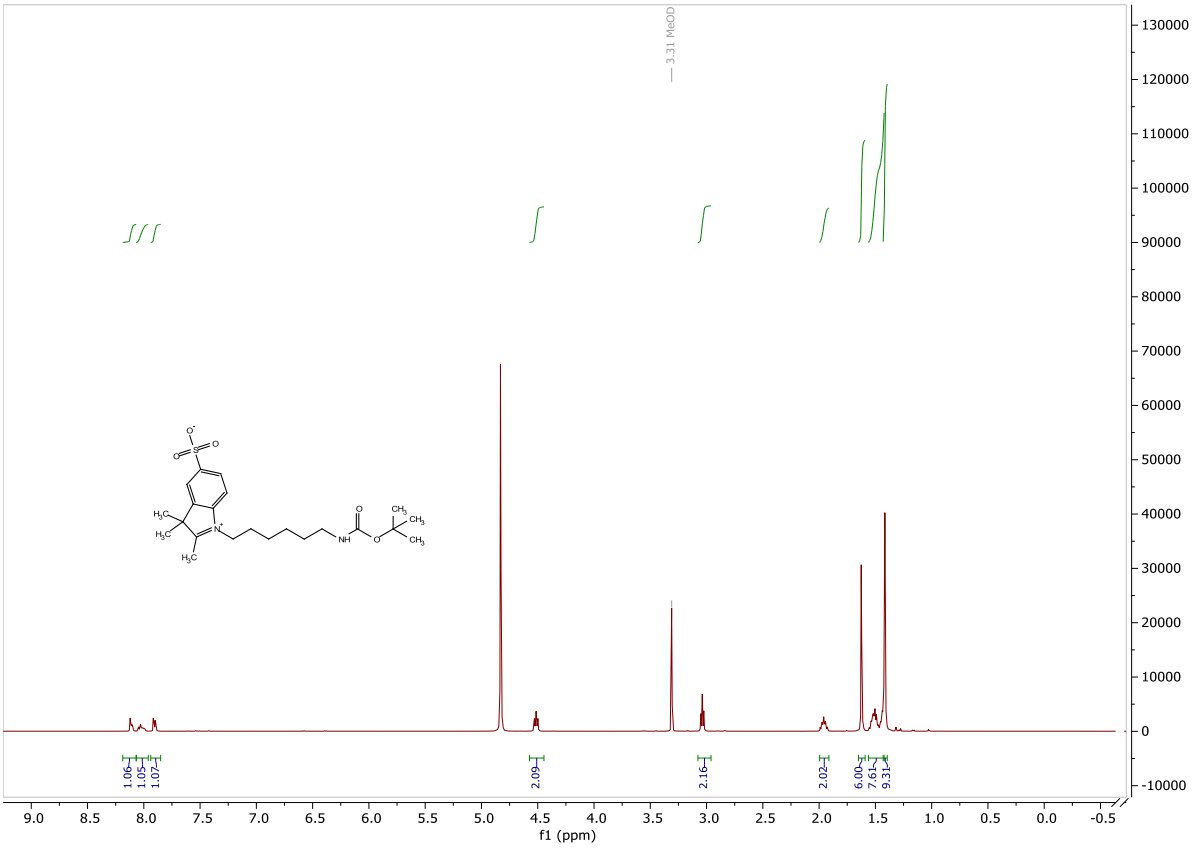


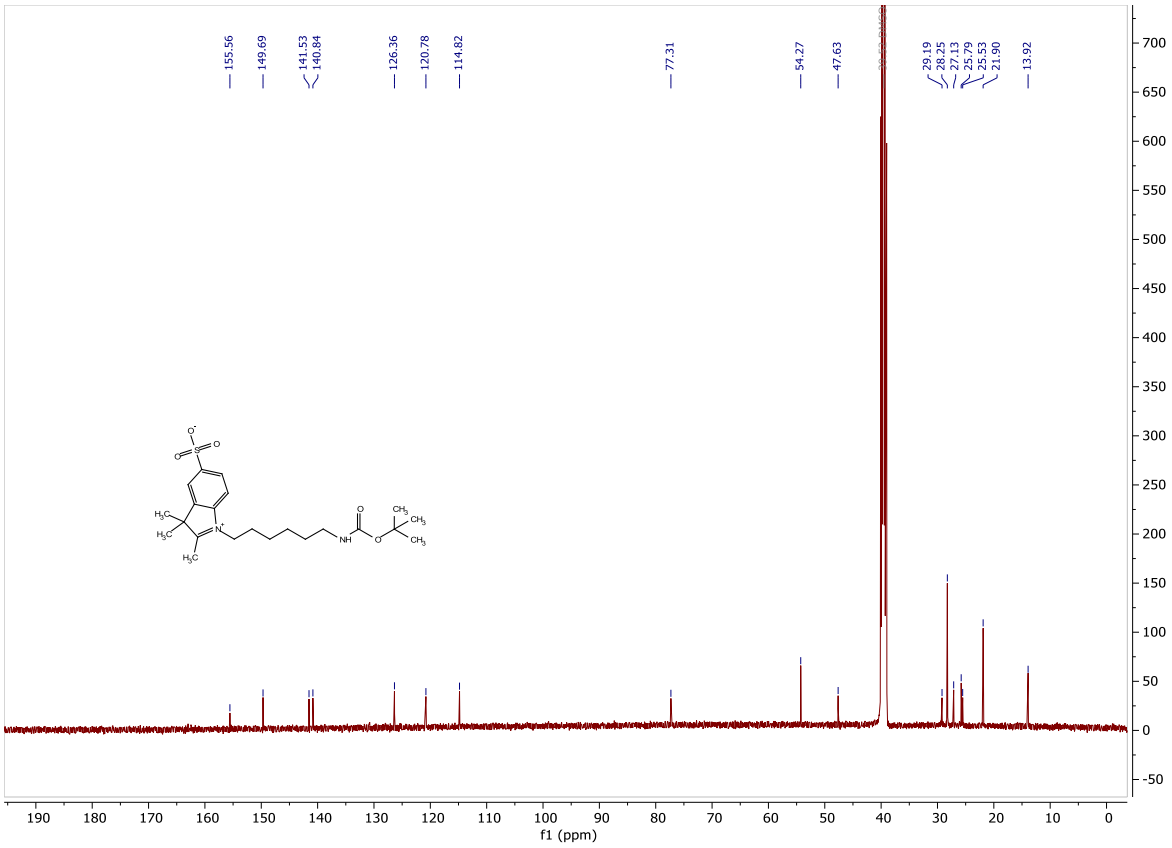
NBD-PEG$_4$-NH$_2$ (8)



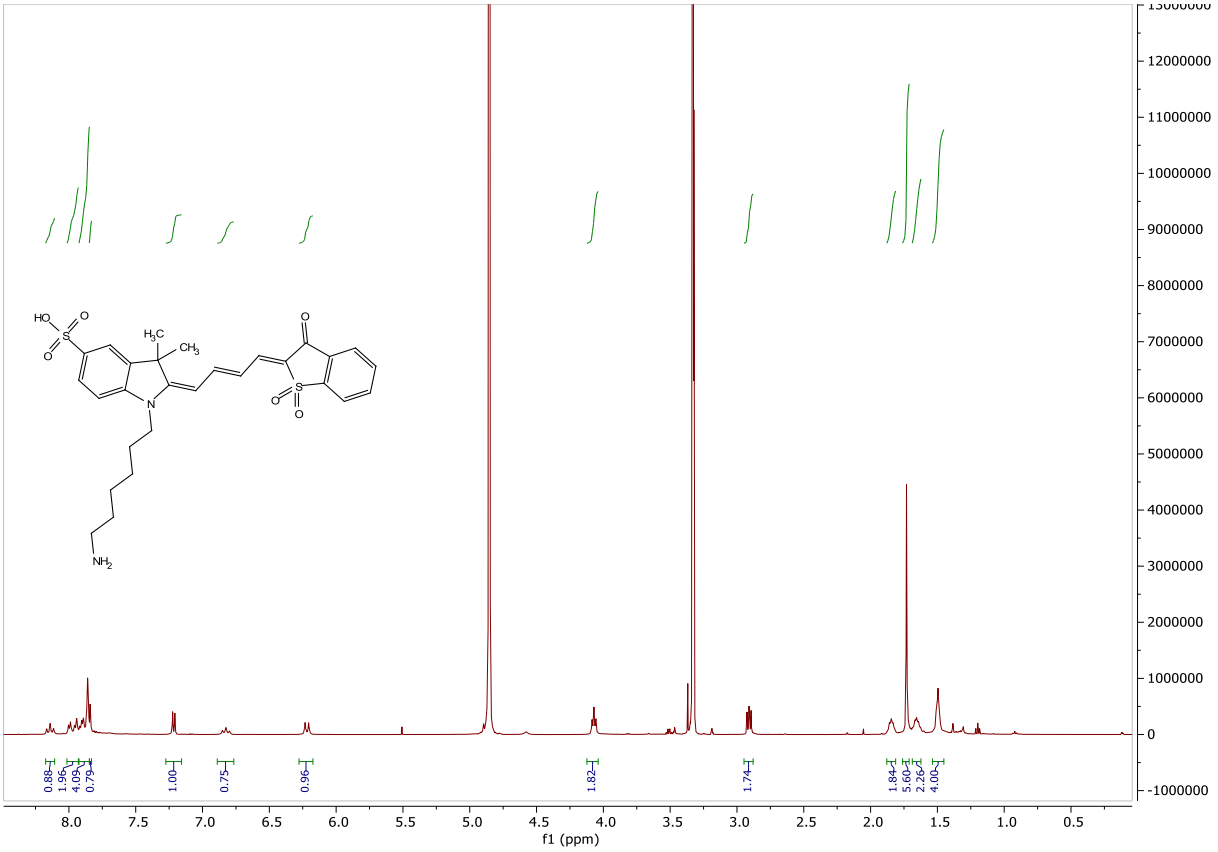


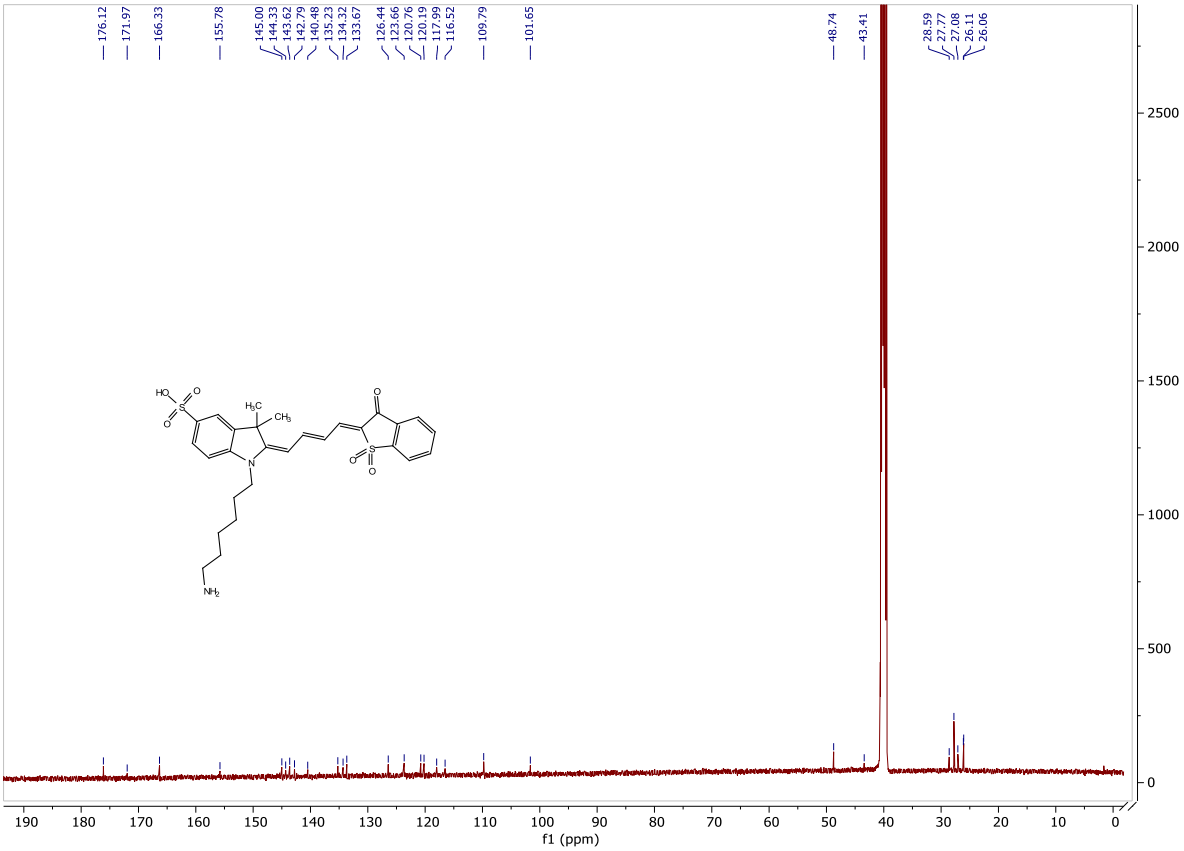
1-(3-(Boc-amino)-2,3,3-3H-indolium sulfonate (10)



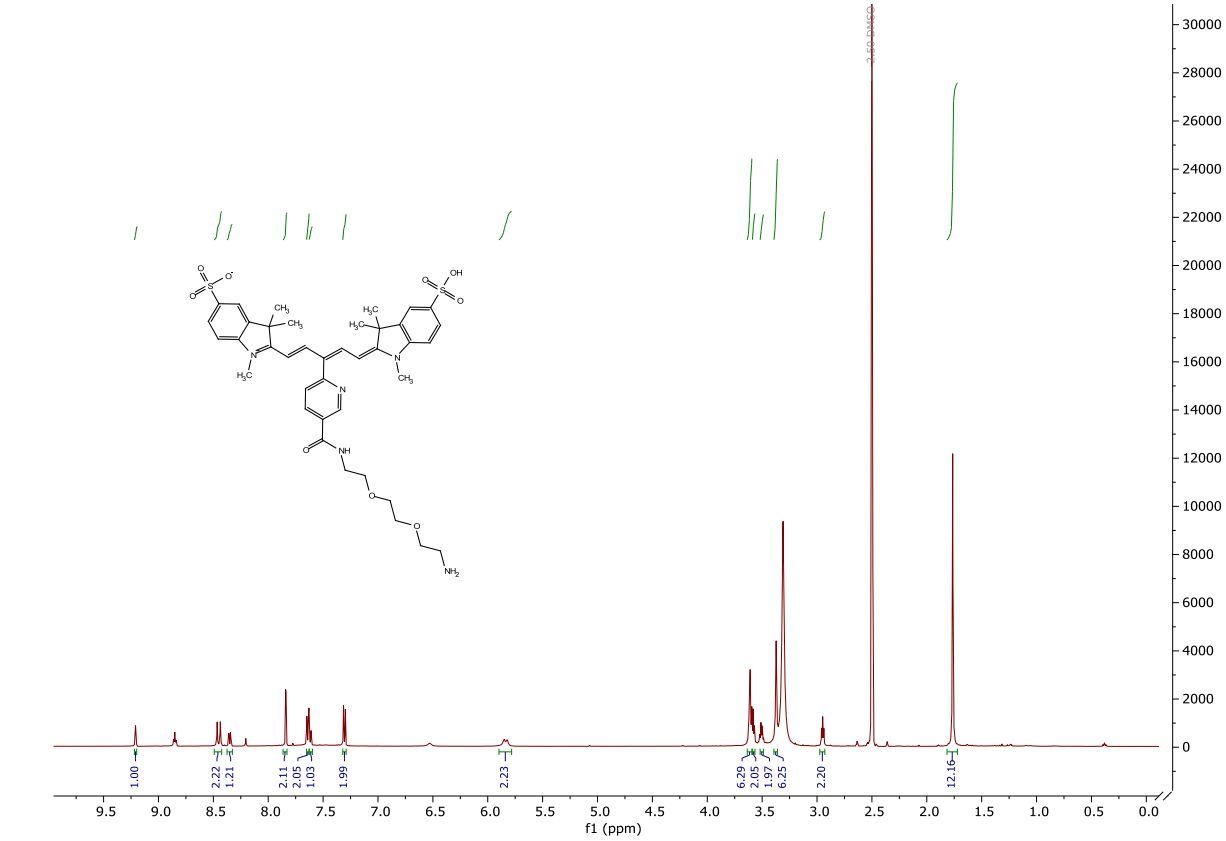


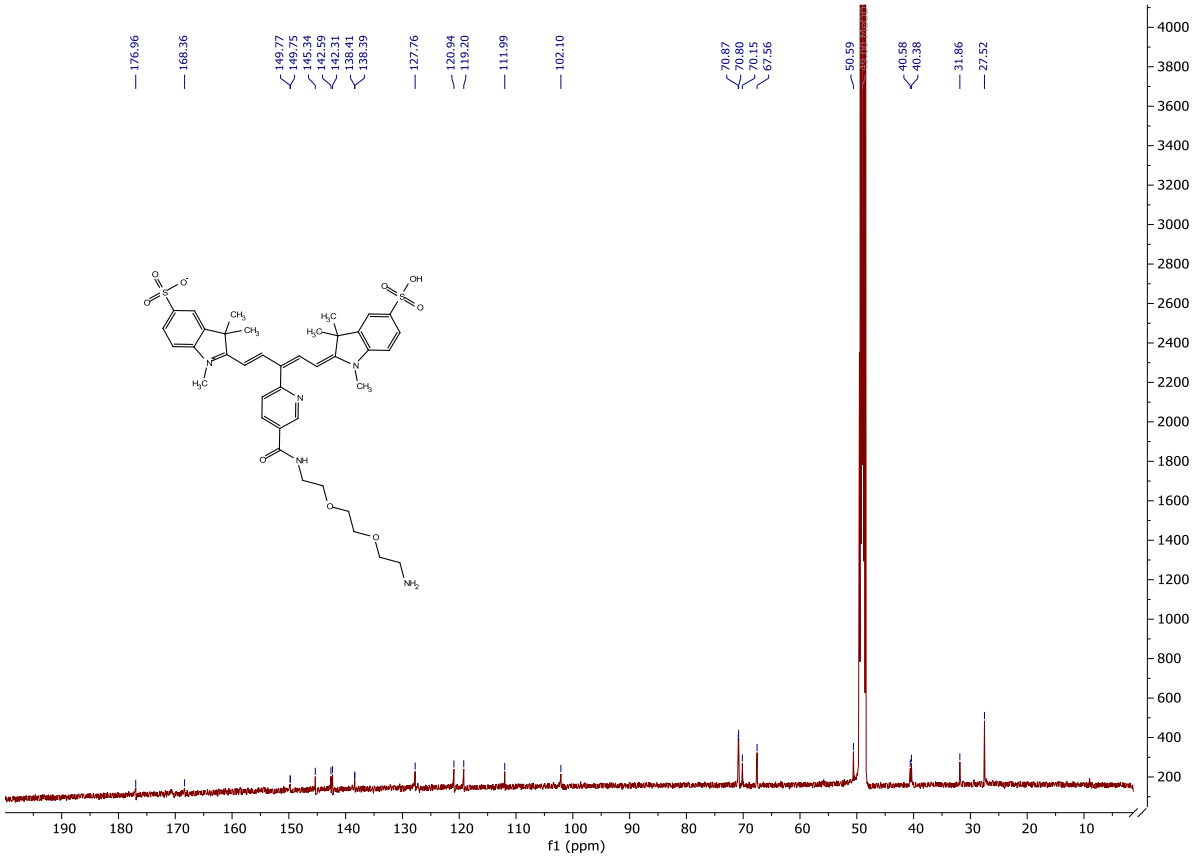
Sulfo-MeroCy-NH_2 (12)



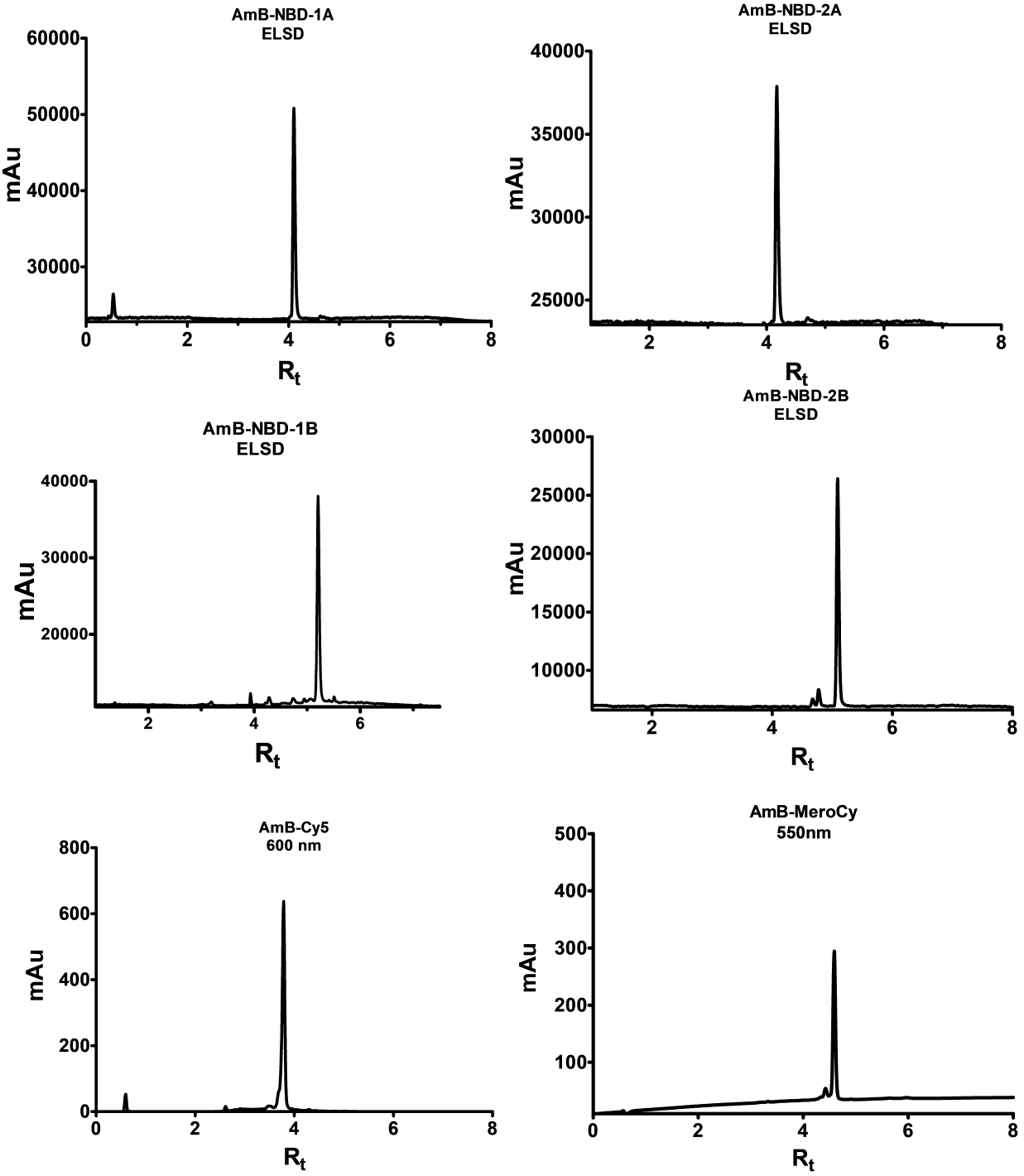


SulfoCy5-PEG$_2$-NH$_2$ (13)



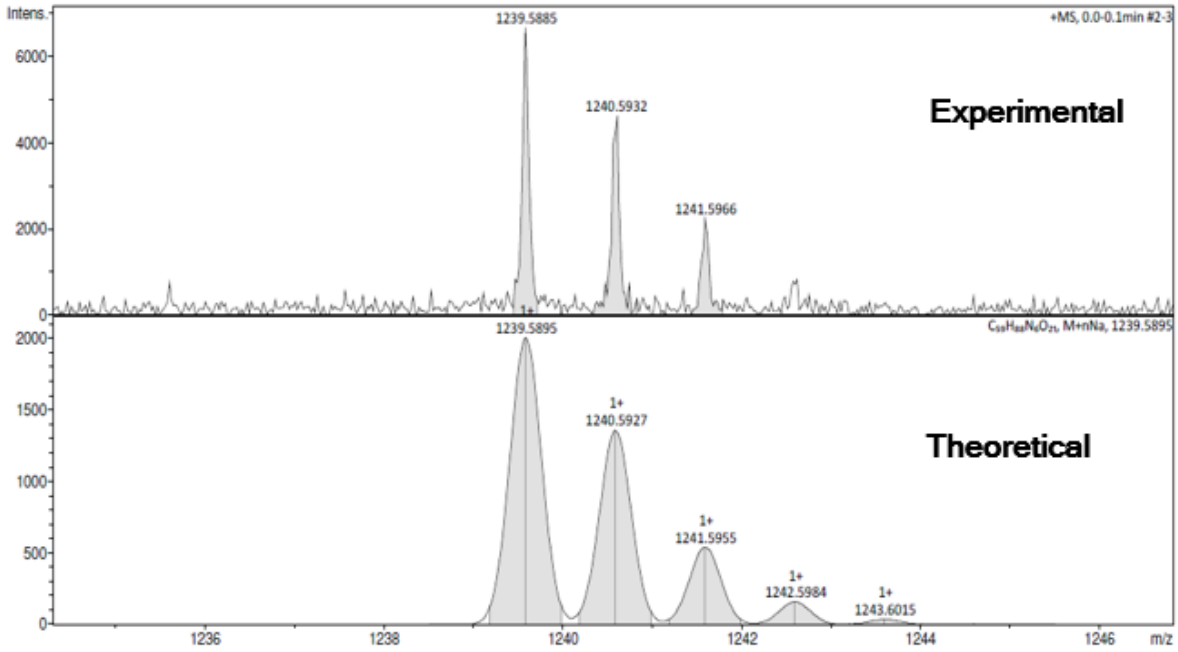


4. HPLC traces

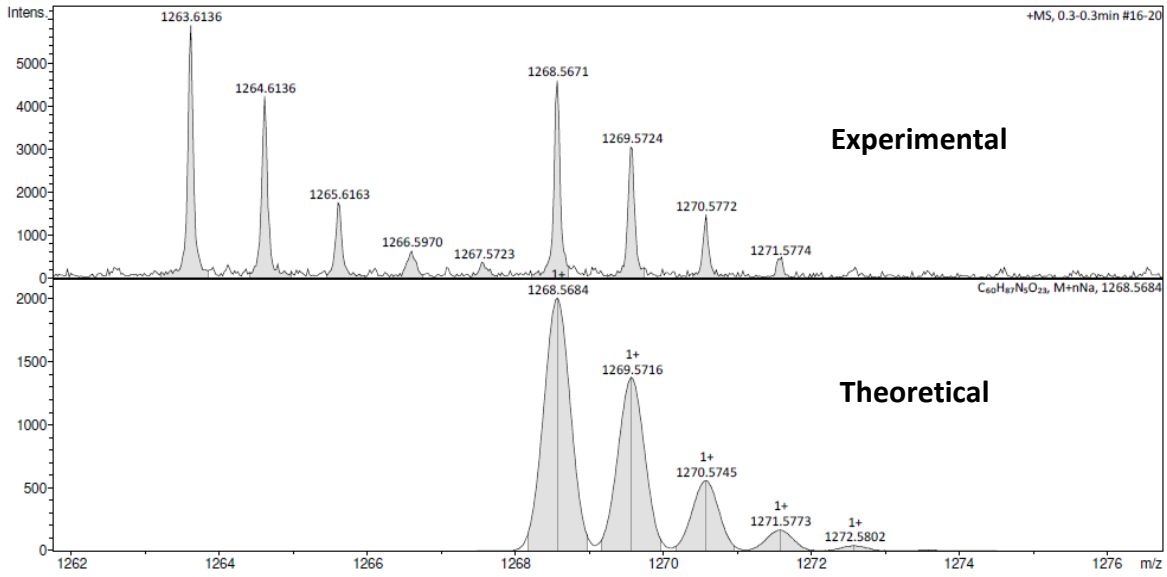


5. High resolution mass spectra

AmB-NBD-1A



AmB-NBD-1B



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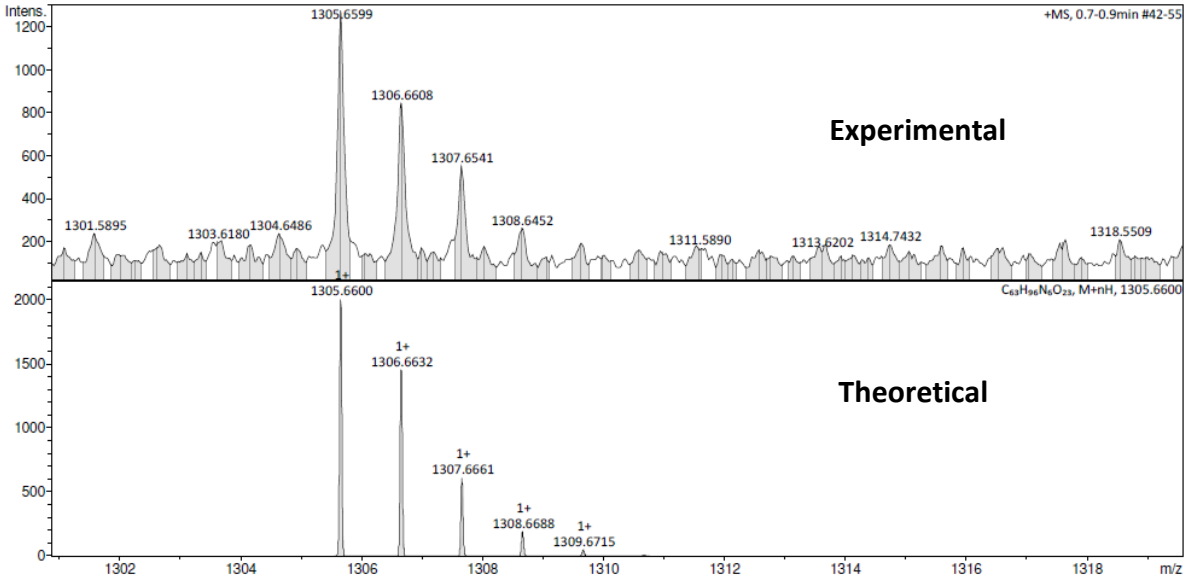

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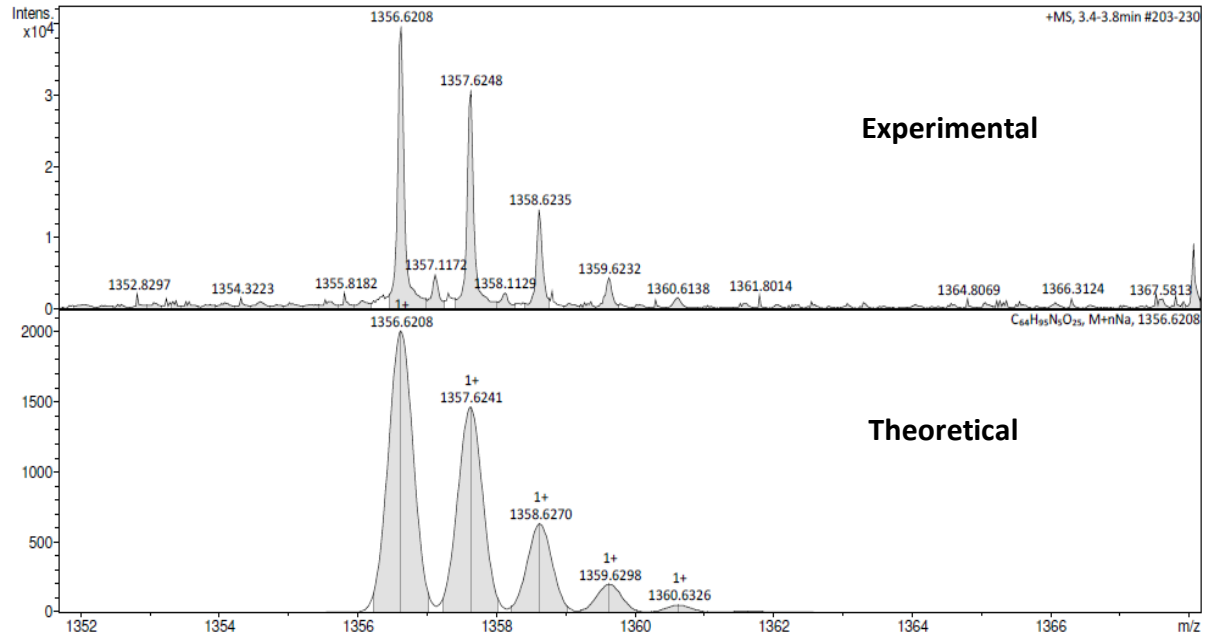
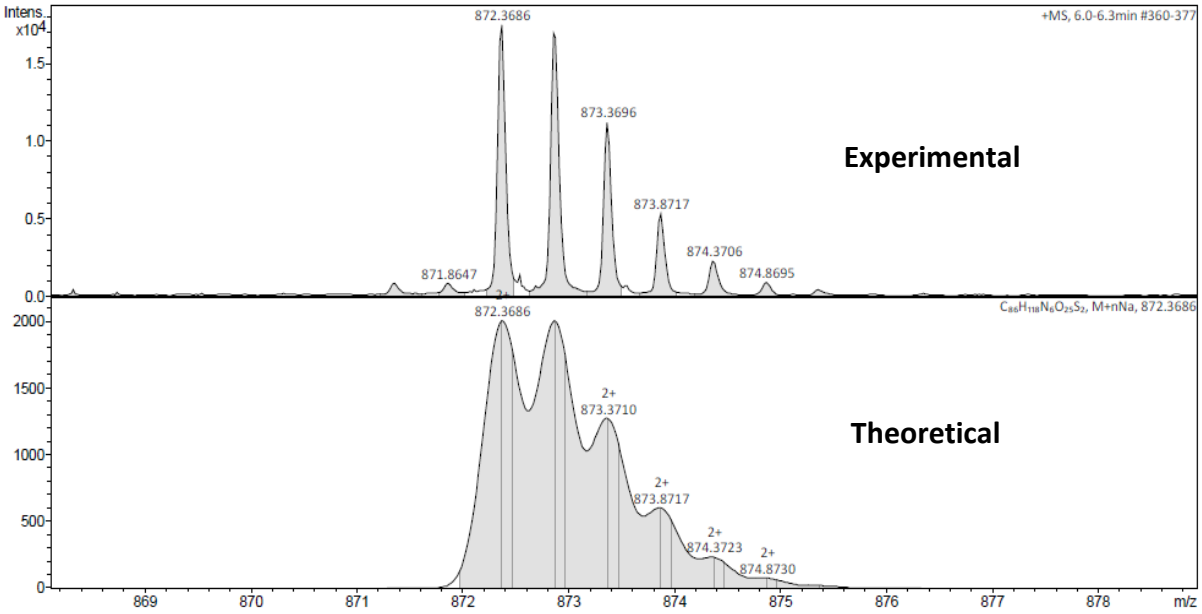
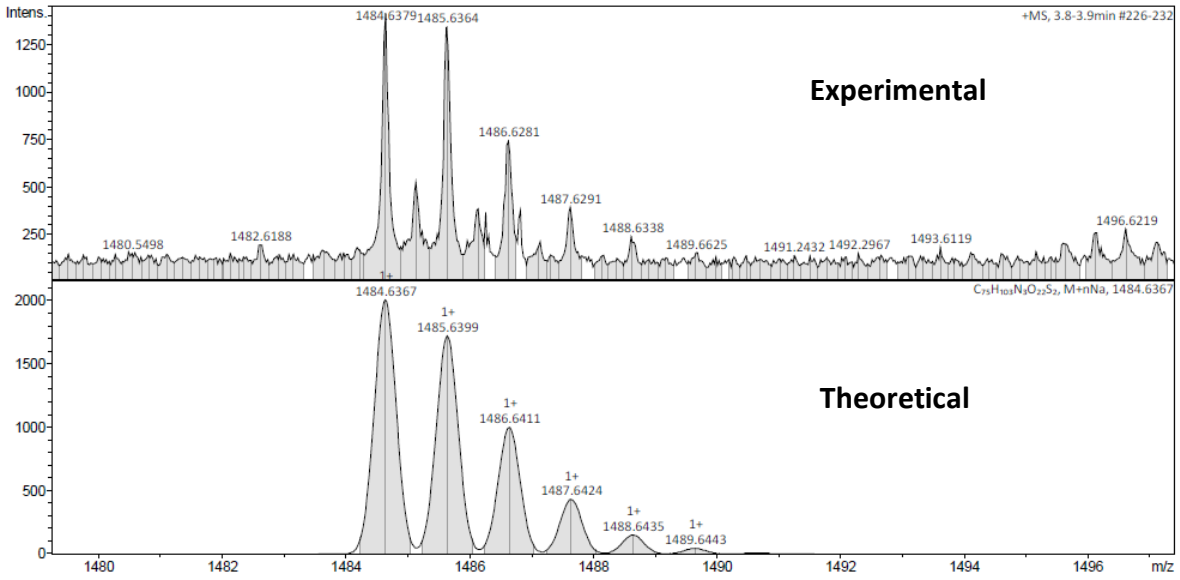


AmB-Cy5



AmB-MeroCy



6. References.

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