Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

A Trp-BODIPY cyclic peptide for fluorescence labelling of apoptotic bodies

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Materials and Methods

Fmoc-amino acids were obtained from Iris Biotech GmbH (Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Phe-OH), Chem-Impex International [Fmoc-Asp-OAllyl, Fmoc-Gln-OH, Fmoc-His(Mmt)-OH] and Sigma-Aldrich [Fmoc-Arg(NO_2)-OH]. Resins and HBTU were obtained from Iris Biotech GmbH. HOBt was purchased from Carbosynth. Pd-based catalysts and bases were obtained from Sigma-Aldrich. All reagents were used without further purification unless otherwise stated. All microwave reactions were carried out in 10 mL sealed glass tubes in a focused mono-mode microwave oven ("Discover" by CEM Corporation) featured with a surface sensor for internal temperature determination. Cooling was provided by compressed air ventilating the microwave chamber during the reaction. Spectroscopic data and quantum yield data were measured on a Synergy HT spectrophotometer (Biotek), and the data analysis was performed using GraphPad Prism 5.0.

Reactions were monitored by HPLC-MS at 220 nm using a HPLC Waters Alliance HT comprising a pump (Edwards RV12) with degasser, an autosampler, a YMC-Pack ODS-AQ, 50 × 4.6 mm, S-3 µm column and a diode array detector. Eluents: H_2O (0.1% FA) and ACN (0.1% FA). Flow: 1.6 mL min^{-1}. The MS detector was configured with an electrospray ionization source (Micromass ZQ4000) and nitrogen was used as the nebulizer gas. Data acquisition was performed with MassLynx software. HRMS (ESI positive) were obtained in a LTQ-FT Ultra (Thermo Scientific) mass spectrometer. NMR spectra were recorded on Bruker Avance-III 600 MHz spectrometer in DMSO-*d6* at 308 K. Chemical shifts (δ) are reported in ppm. Multiplicities are referred by the following abbreviations: s = singlet, d = doublet, t = triplet, dd = double

doublet, ddd = double double doublet, dt = double triplet, q= quartet and m = multiplet.

General procedures for SPPS

All peptides were manually synthesized in polystyrene syringes fitted with a polyethylene porous disc using Fmoc-based SPPS. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine: DMF (1: 4) (1 × 1 min, 2 × 5 min). Peptide synthesis transformations and washings were performed at r.t.

Resin loading. Fmoc-Asp-OAllyl (1 eq.) was attached to the resin (1 eq.) with DIPEA (3 eq.) in DCM at r.t. for 10 min and then DIPEA (7 eq.) for 40 min. The remaining trityl groups were capped adding 0.8 µL MeOH mg^{-1} resin for 10 min. The resin was filtered and washed with DCM (4 × 1 min), DMF (4 × 1 min). The loading of the resin was determined by titration of the Fmoc group.

Peptide elongation. After the Fmoc group was removed with piperidine: DMF (1: 4) (1 × 1 min, 2 × 5 min), the resin was washed with DMF (4 × 1 min), DCM (3 × 1 min), DMF (4 × 1 min). Unless otherwise noted, standard coupling procedure with DIC (3 eq.) and OxymaPure (3 eq.) in DMF for 1 h and 5 min of pre-activation was carried out. The completion of the coupling was monitored with the Kaiser test. Then, the resin was filtered and washed with DCM (4 × 1 min) and DMF (4 × 1 min) and was ready for the elongation with the next Fmoc amino acid.

Final cleavage. The resin bound peptide was treated 5 times with TFA: DCM (1: 99) for 1 min in each treatment and washed with DCM. The combined filtered mixtures were poured over DCM and evaporated under vacuum. The residue was precipitated in Et_2O, dissolved in ACN: H_2O and liophilised.

Experimental Section

Chemical synthesis.

cLac-1. Starting from 750 mg of *2*-chlorotrityl PS resin (0.3 mmol g^{-1}). Amino acid coupling. Fmoc-AA-OH (3 eq.) were incorporated with a 5 min pre-activation with DIC (3 eq.) and OxymaPure (3 eq.) in DMF for 1 h. Fmoc-AA-OH: Fmoc-Asp-OAllyl, Fmoc-Gly-OH, Fmoc-Arg(NO_2)-OH, Fmoc-Gln-OH, Fmoc-Ile-OH, Fmoc-His(Mmt)-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH. Peptide cyclisation. The C-terminal allyl ester group of the aspartic acid was removed after addition of the last amino acid with $Pd(PPh_3)_4$ (26 mg, 0.023 mmol, 0.1 eq.) and N-methylmorpholine (244 µL, 2.25 mmol, 10 eq.) in THF for 1 h at r.t. (3 × 15 min). The head-to-tail cyclization was performed by removal of the *N*-terminal Fmoc group before addition of PyAOP (352 mg, 0.674 mmol, 3 eq.), HOAt (92 mg, 0.674 mmol, 3 eq.) and DIPEA (235 µL, 1.35 mmol, 6 eq.) in DMF at r.t for 1 h. Peptide cleavage. The resin bound peptide was treated repeated times with the TFA cocktail obtaining 210 mg of cyclic peptide crude (87% purity by HPLC-MS). Removal of nitro group. The crude protected peptide (210 mg, 0.153 mmol) and 20% $Pd(OH)_2$-C (105 mg) were dissolved in HCO_2H: DMF: H_2O (5: 47.5: 47.5) (10 mL) and the reaction flask was flushed with Ar, evacuated and filled with H_2. The reaction mixture was stirred under balloon pressure of H_2 for 48 h (H_2 was refilled periodically during the reaction along with re-addition of $Pd(OH)_2$-C (2 X). The catalyst was removed through filtration with Celite and the filtrate was evaporated *in vacuo* to afford 164 mg of the crude peptide (83% purity by HPLC-MS, 75% yield). Peptide purification. A highly pure fraction of the totally deprotected cyclic peptide was obtained by semi-preparative RP-HPLC (XBRIDGE^{TM}, C_{18}, 5 µM OBD 19 × 150 mm

column). The pure fractions were lyophilised rendering the pure peptide as a white solid (>99% purity by HPLC-MS).

^{1}H NMR (600 MHz, DMSO-d_6): δ 10.70 (s, 1H), 8.61 (m, 1H), 8.46 – 8.31 (m, 3H), 8.23 (s, 1H), 8.18 (s, 1H), 8.15 (s, 1H), 8.06 (s, 1H), 7.99 (s, 1H), 7.88 (s, 1H), 7.71 – 7.59 (m, 1H), 7.57 – 7.51 (m, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 7.31 – 7.27 (m, 3H), 7.25 – 7.20 (m, 4H), 7.07 (s, 1H), 7.03 (ddd, *J* = 8.1, 7.0, 1.2 Hz, 1H), 6.96 (ddd, *J* = 7.9, 6.9, 1.1 Hz, 1H), 6.83 (s, 1H), 6.74 (s, 1H), 4.57 (q, *J* = 7.1 Hz, 1H), 4.42 (q, *J* = 6.3 Hz, 2H), 4.33 (s, 1H), 4.27 (td, *J* = 8.3, 5.0 Hz, 1H), 4.18 (q, *J* = 7.8, 7.1 Hz, 1H), 4.10 (t, *J* = 7.4 Hz, 1H), 3.91 (dd, *J* = 16.9, 6.1 Hz, 1H), 3.79 – 3.68 (m, 9H), 3.65 – 3.61 (m, 1H), 3.55 – 3.50 (m, 1H), 3.11 – 3.03 (m, 2H), 2.99 (dd, *J* = 14.7, 4.6 Hz, 2H), 2.96 – 2.87 (m, 3H), 2.81 (dd, *J* = 15.0, 9.2 Hz, 1H), 2.76 – 2.65 (m, 1H), 2.46 (1H), 2.15 – 2.00 (m, 2H), 1.99 – 1.88 (m, 1H), 1.83 – 1.70 (m, 3H), 1.65 – 1.55 (m, 1H), 1.54 – 1.42 (m, 2H), 1.40 – 1.27 (m, 1H), 1.07 – 0.94 (m, 1H), 0.80 – 0.75 (m, 6H) ppm. **HRMS** (ESI) (m/z): $[M+H]^+$ calcd. for $C_{59}H_{80}O_{16}N_{20}$, 1324.6056; found, 1324.6046.

cLac-BODIPY. Starting from 750 mg of *2*-chlorotrityl PS resin (0.3 mmol g^{-1}). Amino acid coupling. Fmoc-Trp(BODIPY)-OH[1] (1.5 eq.) was incorporated with HBTU (1.2 eq.), HOBt (1.2 eq.) and DIEA (2.4 eq.) in DMF for 1 h. The other amino acids Fmoc-AA-OH (3 eq.) were incorporated with a 5 min pre-activation with DIC (3 eq.) and OxymaPure (3 eq.) in DMF for 1 h. Fmoc-AA-OH: Fmoc-Asp-OAllyl, Fmoc-Gly-OH, Fmoc-Arg(NO_2)-OH, Fmoc-Gln-OH, Fmoc-Ile-OH, Fmoc-His(Mmt)-OH, Fmoc-Phe-OH. Peptide cyclisation. The C-terminal allyl ester group of the aspartic acid was removed after addition of the last amino acid with $Pd(PPh_3)_4$ (26 mg, 0.023 mmol, 0.1 eq.) and N-methylmorpholine (244 µL, 2.25 mmol, 10 eq.) in THF for 1 h at r.t (3 x 15 min). The head-to-tail

cyclization was performed by removal of the *N*-terminal Fmoc group before addition of DIC (96 µL, 0.548 mmol, 3 eq.) and HOBt (75 mg, 0.548 mmol, 3 eq.) in DMF at r.t. for 5 h. Peptide cleavage. The resin bound peptide was treated repeated times with the TFA cocktail obtaining 69 mg of cyclic peptide crude (60% purity by HPLC-MS). Removal of nitro group. The crude protected peptide (69 mg, 0.041 mmol) and 20% $Pd(OH)_2$-C (34 mg) were dissolved in HCO_2H: DMF: H_2O (5: 47.5: 47.5) (10 mL) and the reaction flask was flushed with Ar, evacuated and filled with H_2. The reaction mixture was stirred under balloon pressure of H_2 for 32 h (H_2 was refilled periodically during the reaction along with re-addition of $Pd(OH)_2$-C (2 X). The catalyst was removed through filtration with Celite and the filtrate was evaporated *in vacuo* to afford 64 mg of the crude peptide (70% purity by HPLC-MS, 69% yield). Peptide purification. A highly pure fraction of the totally deprotected cyclic peptide was obtained by semi-preparative RP-HPLC (XBRIDGE^{TM}, C_{18}, 5 µM OBD 19 × 150 mm column. The pure fractions were lyophilised rendering the corresponding peptide as a red solid (>99% purity by HPLC-MS).

^{1}H NMR (600 MHz, DMSO-d_6): δ 11.27 (s, 1H), 8.37 – 7.99 (m, 7H), 7.90 – 7.83 (m, 1H), 7.66 – 7.59 (m, 2H), 7.43 – 7.36 (m, 1H), 7.34 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.32 – 7.26 (m, 2H), 7.26 – 7.18 (m, 3H), 7.15 (t, *J* = 7.2 Hz, 3H), 7.07 (t, *J* = 7.4 Hz, 1H), 6.92 (t, *J* = 8.3 Hz, 1H), 6.79 (s, 1H), 6.19 (d, *J* = 4.5 Hz, 2H), 4.63 – 4.44 (m, 2H), 4.40 – 4.29 (m, 1H), 4.28 – 4.18 (m, 3H), 4.17 – 4.05 (m, 1H), 3.87 – 3.51 (m, 14H), 3.18 – 3.11 (m, 2H), 3.09 – 3.04 (m, 2H), 2.96 – 2.89 (m, 1H), 2.56 (m, 1H), 2.47 – 2.45 (m, 6H), 2.18 – 2.06 (m, 2H), 1.95 (s, 1H), 1.86 – 1.65 (m, 3H), 1.60 – 1.47 (m, 3H), 1.43 (d, *J* = 9.4 Hz, 6H), 1.40 – 1.31 (m, 1H), 1.29 – 1.24 (m, 2H), 1.04 (m, 1H), 0.81 – 0.73 (m, 6H) ppm. **HRMS** (ESI) (m/z): $[M+H]^+$ calcd. for $C_{78}H_{97}O_{16}N_{22}BF_2$, 1646.7509; found, 1646.7509.

Spectral characterisation.

PS and PC stock solutions were prepared by solubilisation in EtOH at a concentration of 1 mg mL^{-1}. 96-well plates were filled with 100 µL of different PS/PC solutions to form films after evaporation of EtOH at r.t. overnight. PS/PC-coated plates were incubated with **cLac-BODIPY** (5 µM) for 1 h at 37 °C in the dark. Quantum yields were determined by measuring the integrated emission area of the fluorescence spectra and comparing it to the area measured for fluorescein in 0.1 M NaOH when excited at 450 nm (QY: 0.92).[2] Quantum yields were calculated using the equation:

$$\Phi_{sample} = \Phi_{reference}\left(\frac{F^{sample}}{F^{reference}}\right)\left(\frac{Abs^{reference}}{Abs^{sample}}\right)\left(\frac{\eta^{sample}}{\eta^{reference}}\right)^2$$

where F represents the area of fluorescent emission, η is the refractive index of the solvent, and Abs is absorbance at the excitation wavelength selected (i.e. 450 nm). Emission was integrated between 480 and 600 nm.

Surface pressure measurements.

DOPS, eggPG and Liss-Rho-DOPE were purchased from Avanti Polar Lipids (Alabaster, Alabama, U.S.A.). eggPC was purchased from Lipid Products (South Nutfield, UK). Surface pressure assays were performed with a DeltaPi-4 tensiometer (Kibron, Helsinki, Finland). Langmuir lipid monolayers at the air-water interface were used as a model membrane system to study lipid-peptide interaction. Surface pressure experiments were carried out with a DeltaPi-4 at 22°C with constant stirring. The aqueous phase consisted of 1.25 mL of 25 mM HEPES, 150 mM NaCl (pH 7.4). Lipids dissolved in chloroform: methanol (2: 1), eggPC, DOPS and eggPG were spread gently over the surface until the desired initial surface pressure was attained. The peptides were injected with a micropipette through a hole connected to the subphase. The increment in

surface pressure was recorded until a stable signal was obtained. Values of critical pressure (π_c) were determined by linear regression using the values over the saturation pressure (π_s).

Confocal microscopy.

Electroformation of Giant Unilamellar Vesicles. GUV formation took place with aid of a TG330 function generator (Thurlby Thandar Instruments, Huntingdon, UK). GUVs were prepared by electroformation on a pair of platinum (Pt) wires by a method first developed by Angelova and Dimitrov,[3,4] modified as described previously.[5] Lipid stock solutions were prepared in 2:1 (v/v) chloroform/methanol at 0.2 mg mL^{-1}. Labelling was carried out by pre-mixing the fluorescent probes with the lipids in organic solvent. We used Liss-Rho-DOPE as marker for lipid membranes. The average concentration of individual fluorescent probes in each sample was 0.2 mol%. 2.5 µL lipid mixtures containing the fluorescent probes were deposited on Pt wires. The Pt wires were placed under vacuum for 2 h to completely remove the organic solvent. The sample was covered to avoid light exposure and allowed to precipitate onto the Pt wires for 5 min. One side of the chamber was then sealed with a coverslip. 500 µL assay buffer, prepared with high-purity water (Millipore SuperQ) heated at 37ºC was added to the chamber until it covered the Pt wires and connected to a TG330 function generator. AC field was applied in three steps, all of them performed at 37ºC: 1), frequency 500 Hz, amplitude 220 mV (35 V/m) for 5 min; 2), frequency 500 Hz, amplitude 1900 mV (313 V/m) for 20 min; 3), frequency 500 Hz, amplitude 5.3 V (870 V/m) for 90 min. The temperatures used for GUV formation correspond to those at which the different membranes display a single fluid phase.

Confocal microscopy of GUVs. Nikon D-eclipse C1 confocal system (Nikon corporation, Tokyo, Japan) was used for GUV imaging, treating the images using the software EZ-C1 3.20 (Nikon Inc., Melville, N.Y.). The excitation wavelengths were 488 nm for **cLac-BODIPY** and 561 nm for Liss-Rho-DOPE. Fluorescence emission was retrieved at 500-530 for **cLac-BODIPY** and at 573-613 for Liss-Rho-PE. cLac-BODIPY was used at 3.3 μg mL^{-1} to study the labelling of the GUVs. All experiments were performed at 22 ºC.

Confocal microscopy of cells. Human lung A549 epithelial cells (ATCC CCL-185) were grown using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin) and 2 mM L-glutamine in a humidified atmosphere at 37 °C with 5% CO_2. A549 cells were regularly passaged in T-75 cell culture flasks and plated on glass chamber slides Lab-Tek™ II (Nunc) the day before imaging. For imaging experiments, cells were treated with TNF-α (10 ng mL^{-1}) and flavopiridol (500 nM) for 6 h to induce apoptosis as previously reported,[6] and incubated for 15 min with **cLac-BODIPY** (1 μM). Fluorescence and brightfield images were acquired under a Zeiss LSM 510 META confocal microscope equipped with a live cell imaging stage and a 63X oil objective, and analysed and processed with ImageJ.

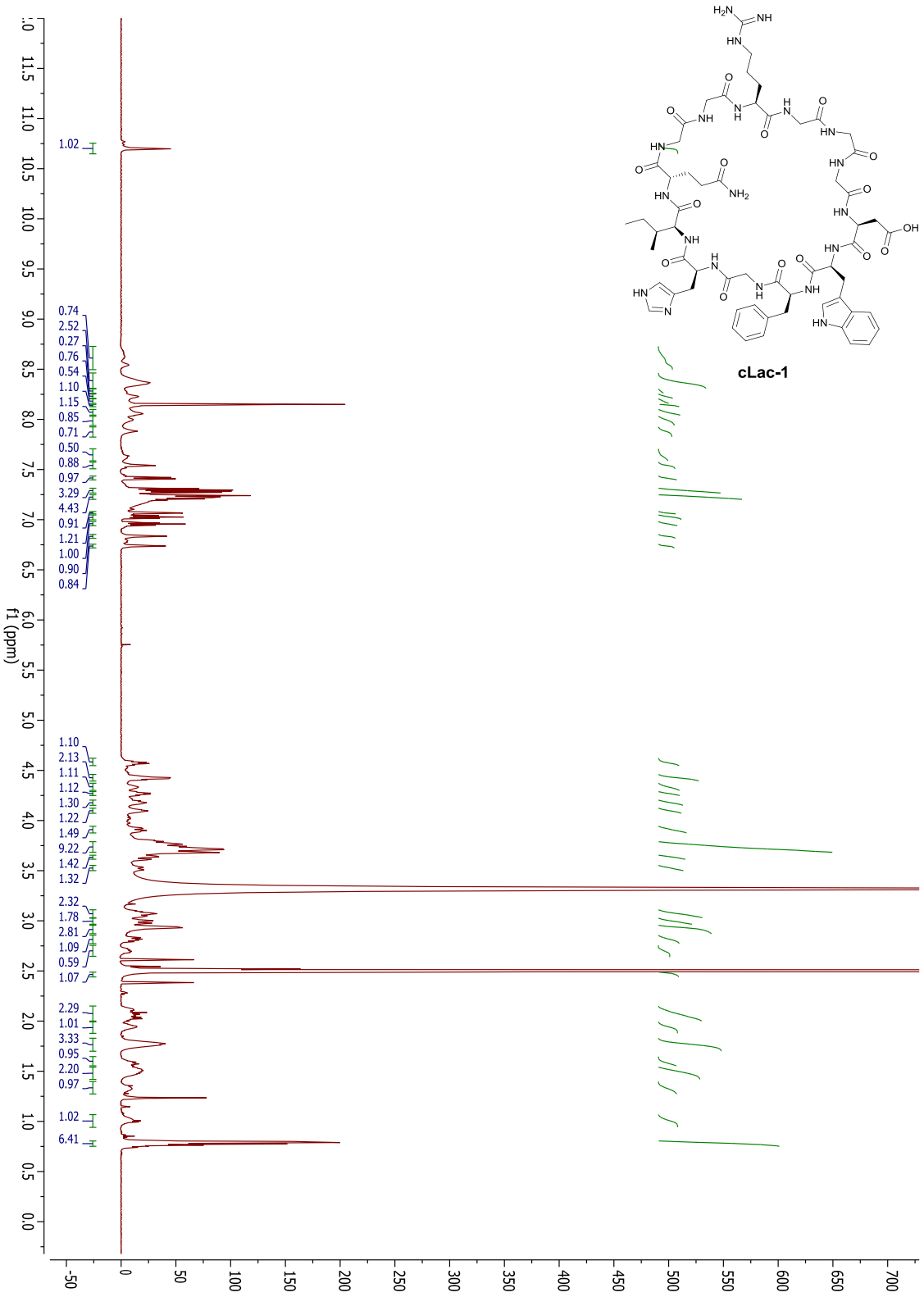
Flow cytometry.

Subcellular material was obtained from supernatants of the human Burkitt lymphoma cell line BL2 undergoing UV-induced apoptosis. Exponentially growing cells were centrifuged and resuspended in culture medium passed through a 0.1 μm PVDF filter (Millipore) then exposed to a UV-B dose of 300 mJ per cm^2. After reculture for 5 h, cells were centrifuged at 25 xg for 1 h at 4 ºC,

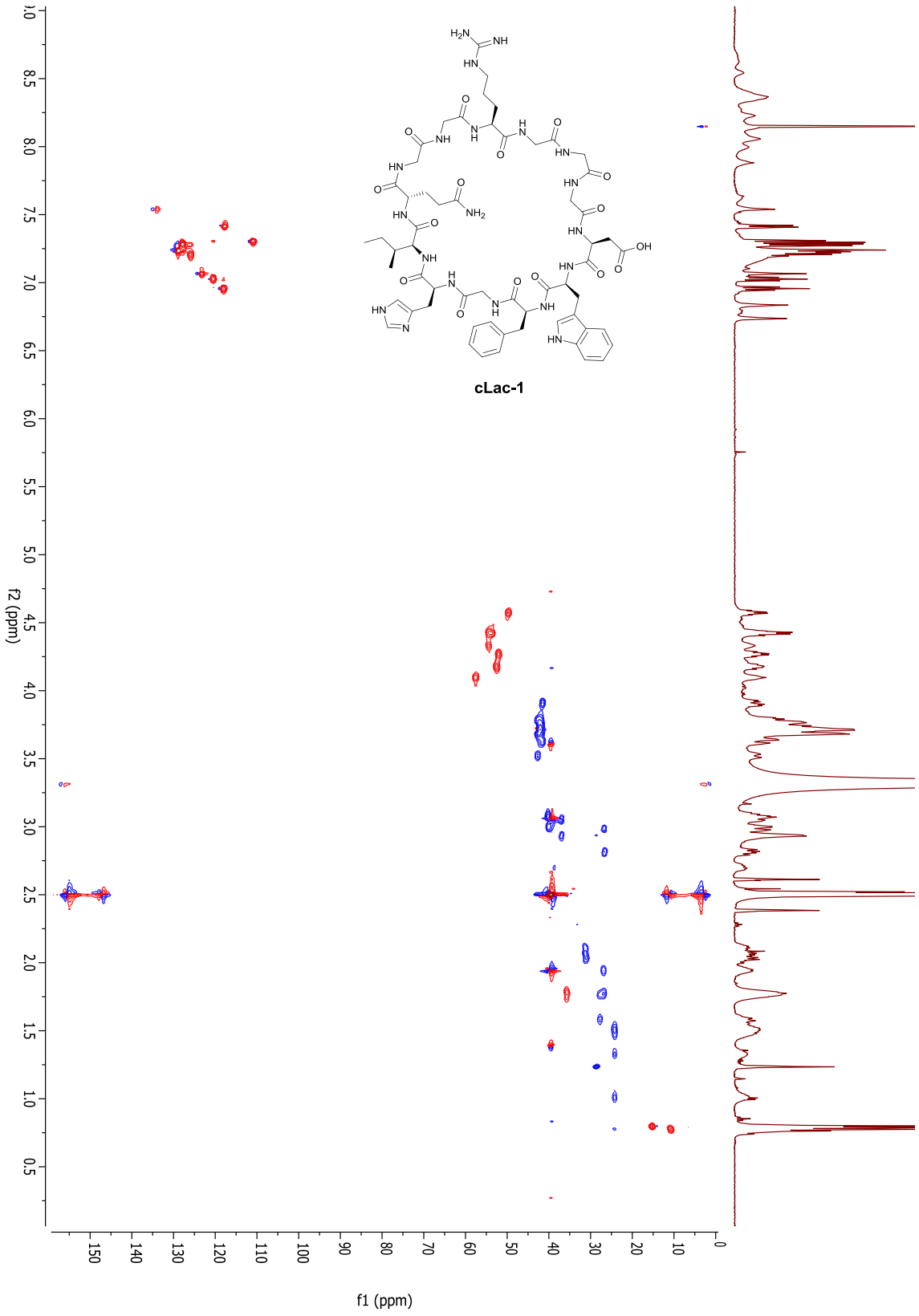
and the supernatant was filtered through a 5 μm mesh filter (PluriSelect Life Sciences, Liepzig, Germany) to exclude residual cells. Binding of **cLac-BODIPY** and PE-labelled Annexin V (Invitrogen) was analysed by flow cytometry after excitation with a 488 nm laser using emission filters at 530±30 nm and 574±26 nm respectively, with appropriate electronic compensation for channel spillover. Data acquisition was carried out using a dual laser Attune acoustic focusing cytometer (Thermo Fisher Scientific Inc, Waltham, MA, USA) with post-acquisition data analysis using Flowjo software (Flowjo, Ashland, OR, USA).

NMR spectra

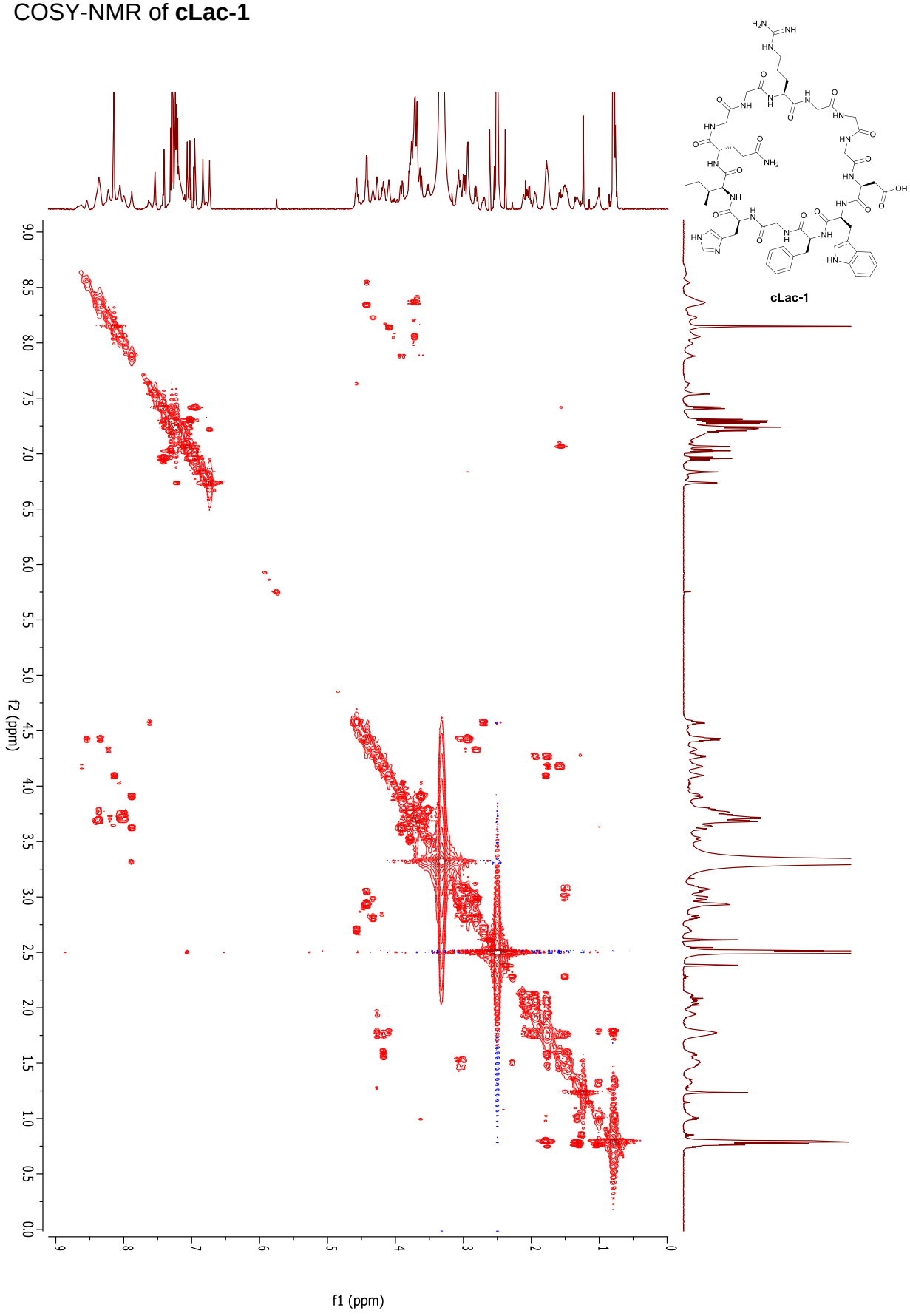
^{1}H-NMR of **cLac-1**



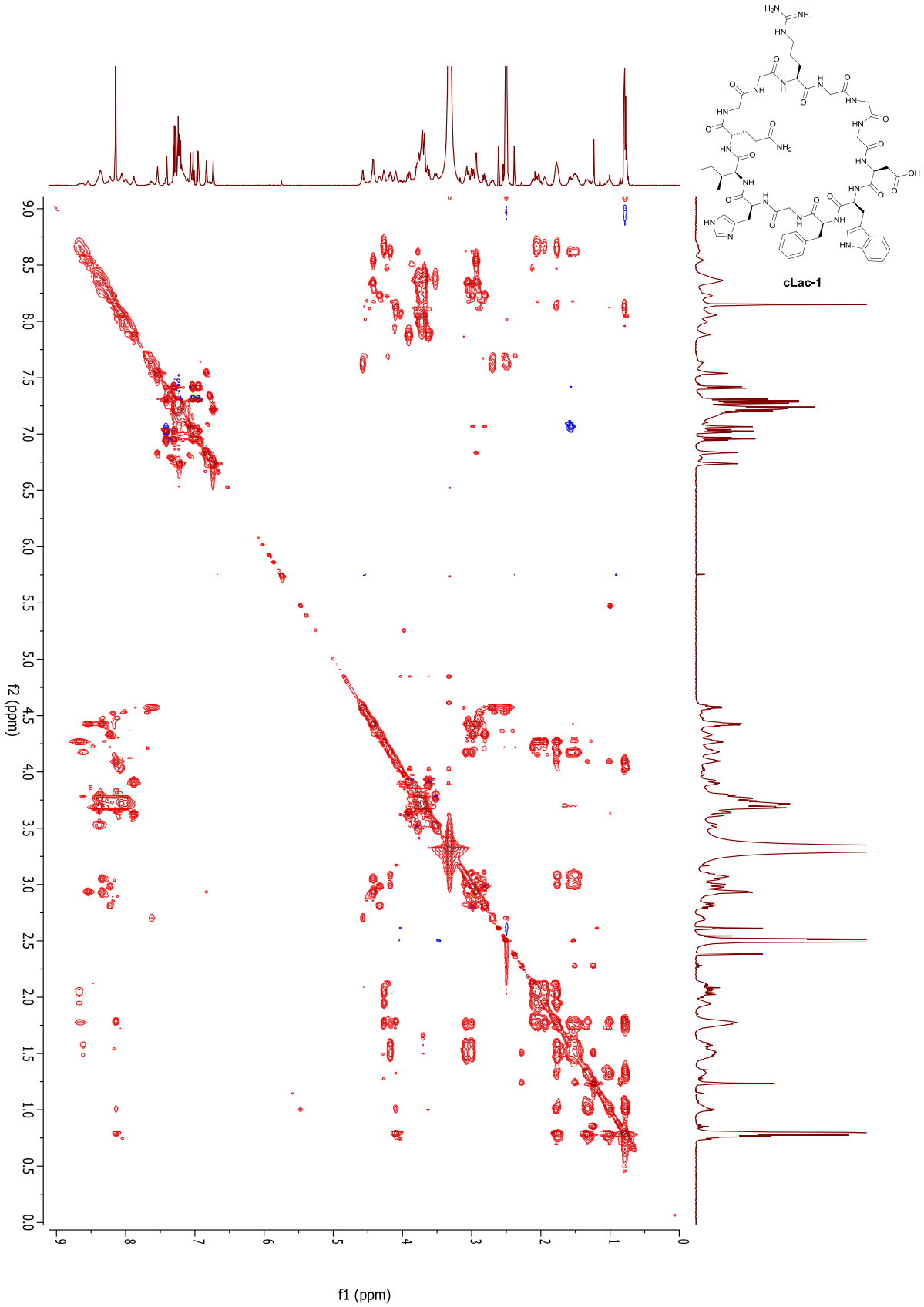
HSQC-NMR of **cLac-1**



COSY-NMR of **cLac-1**



TOCSY-NMR of **cLac-1**



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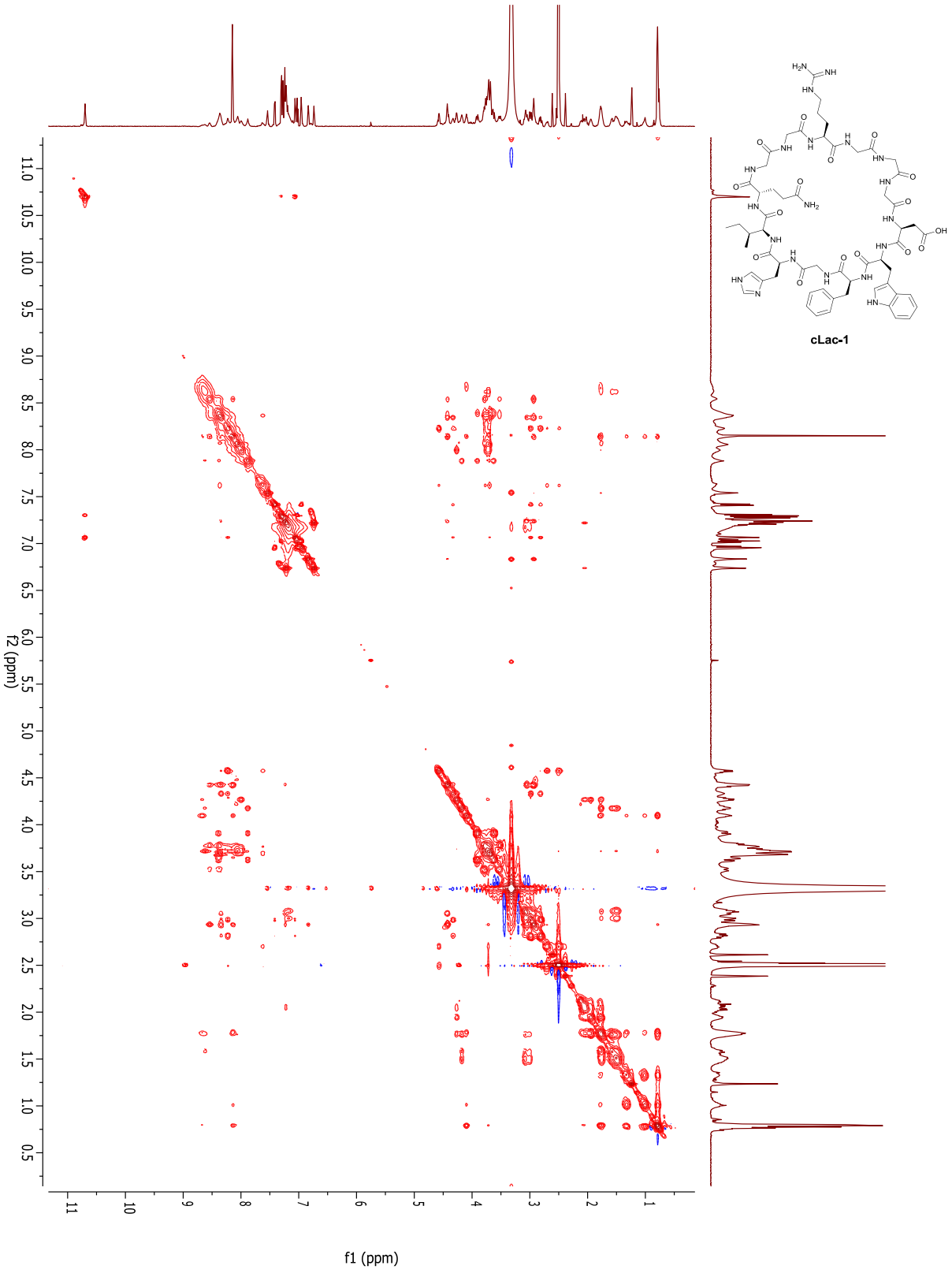
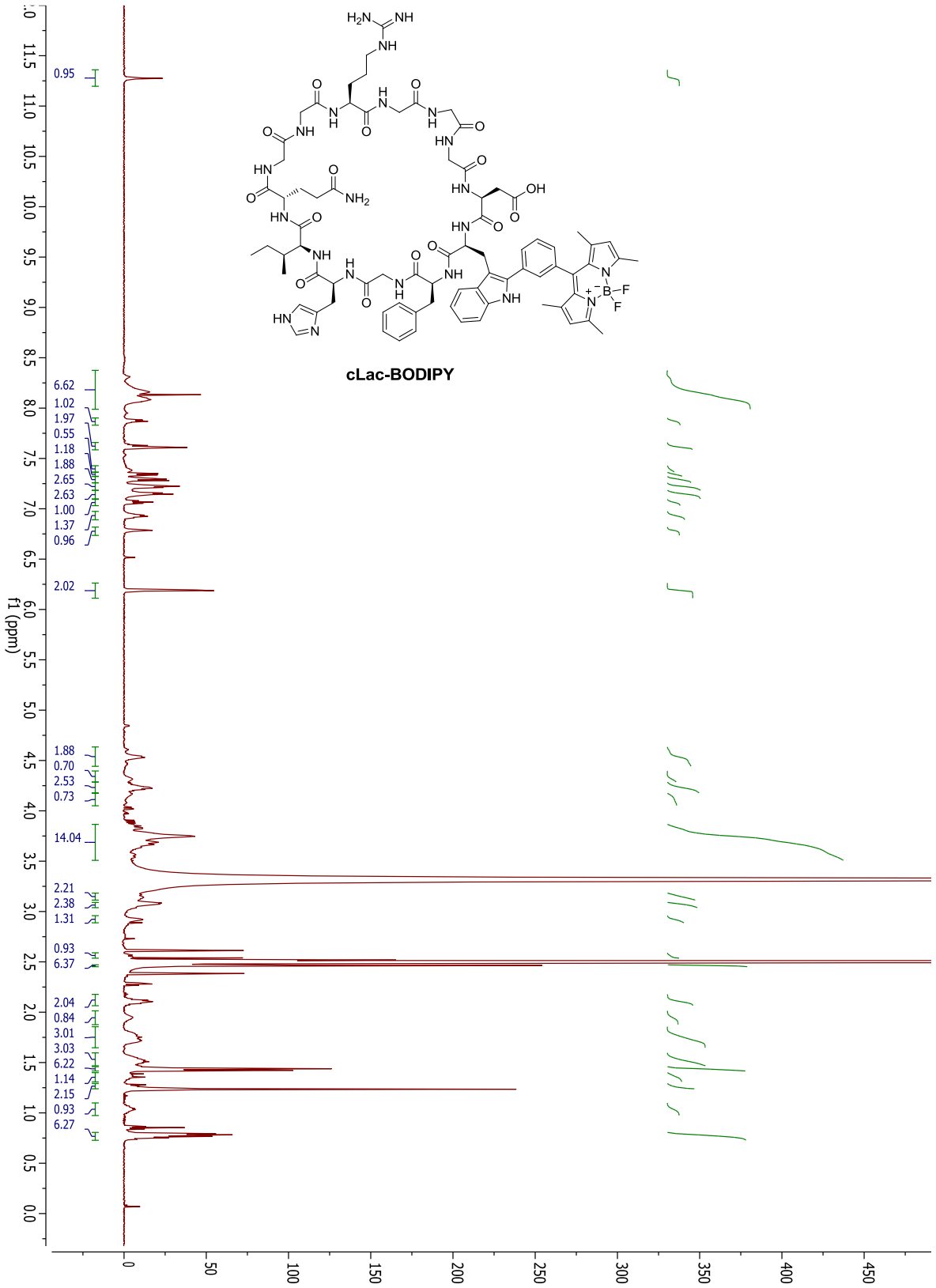
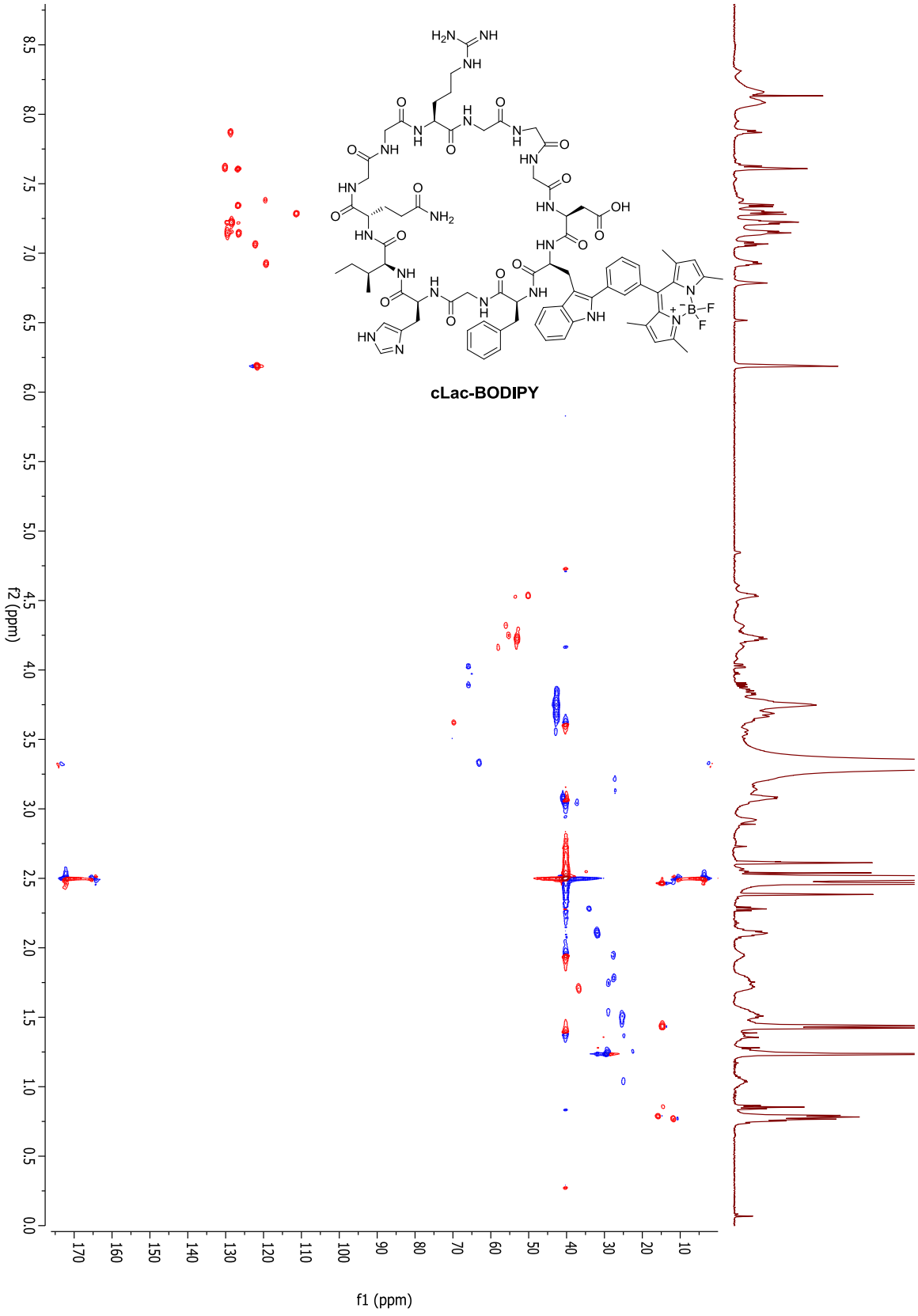


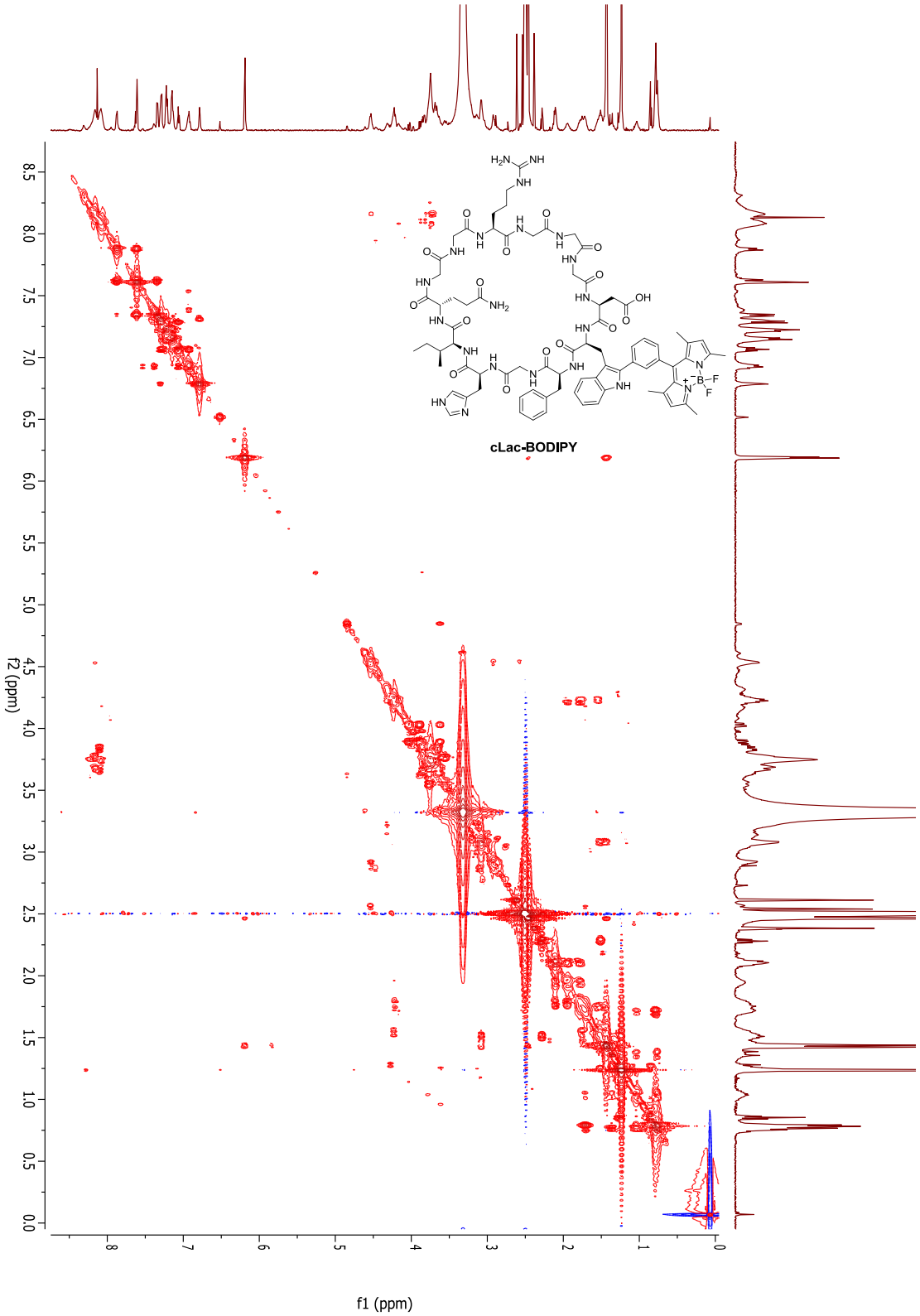
^{1}H-NMR of **cLac-BODIPY**



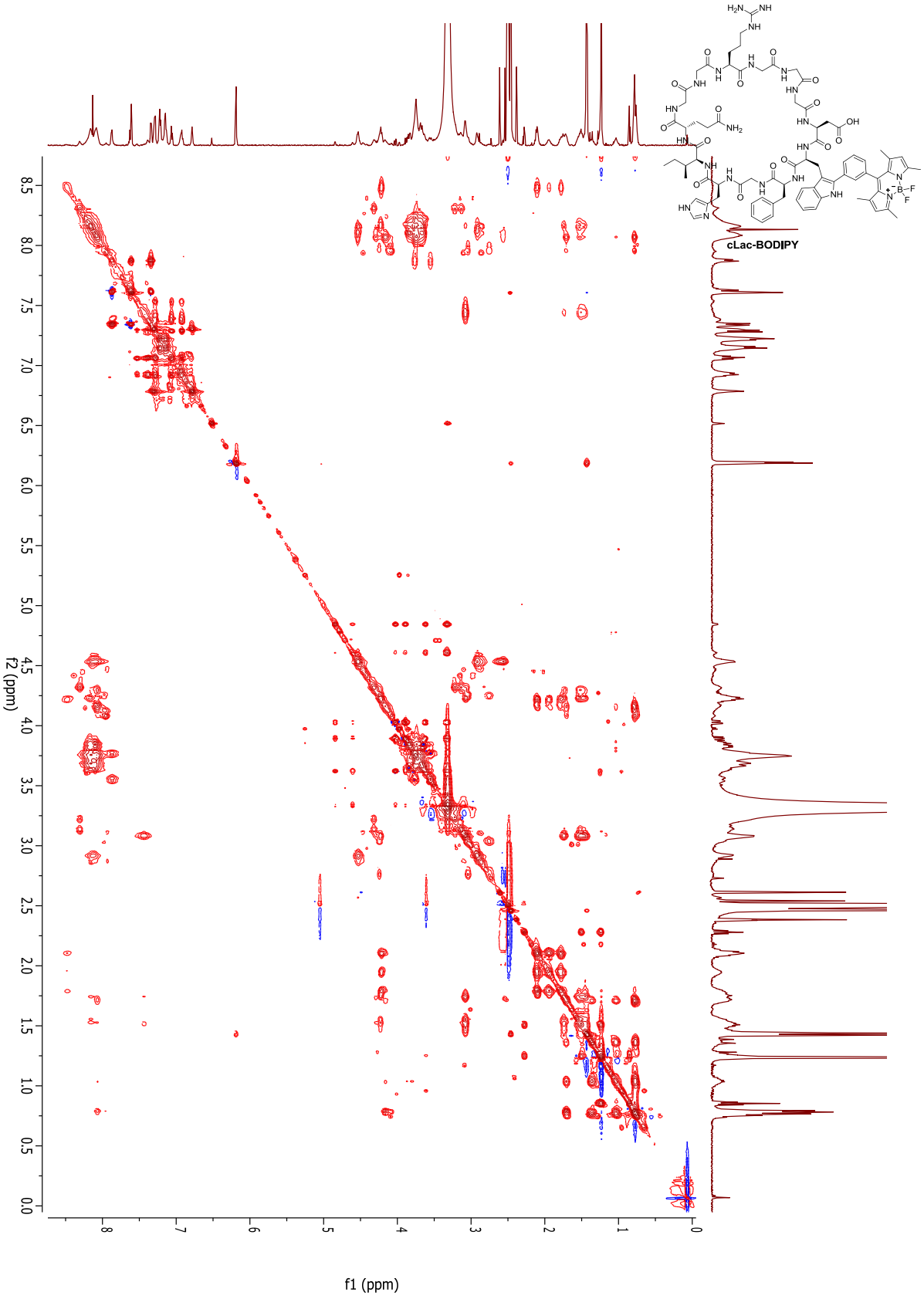
HSQC-NMR of **cLac-BODIPY**



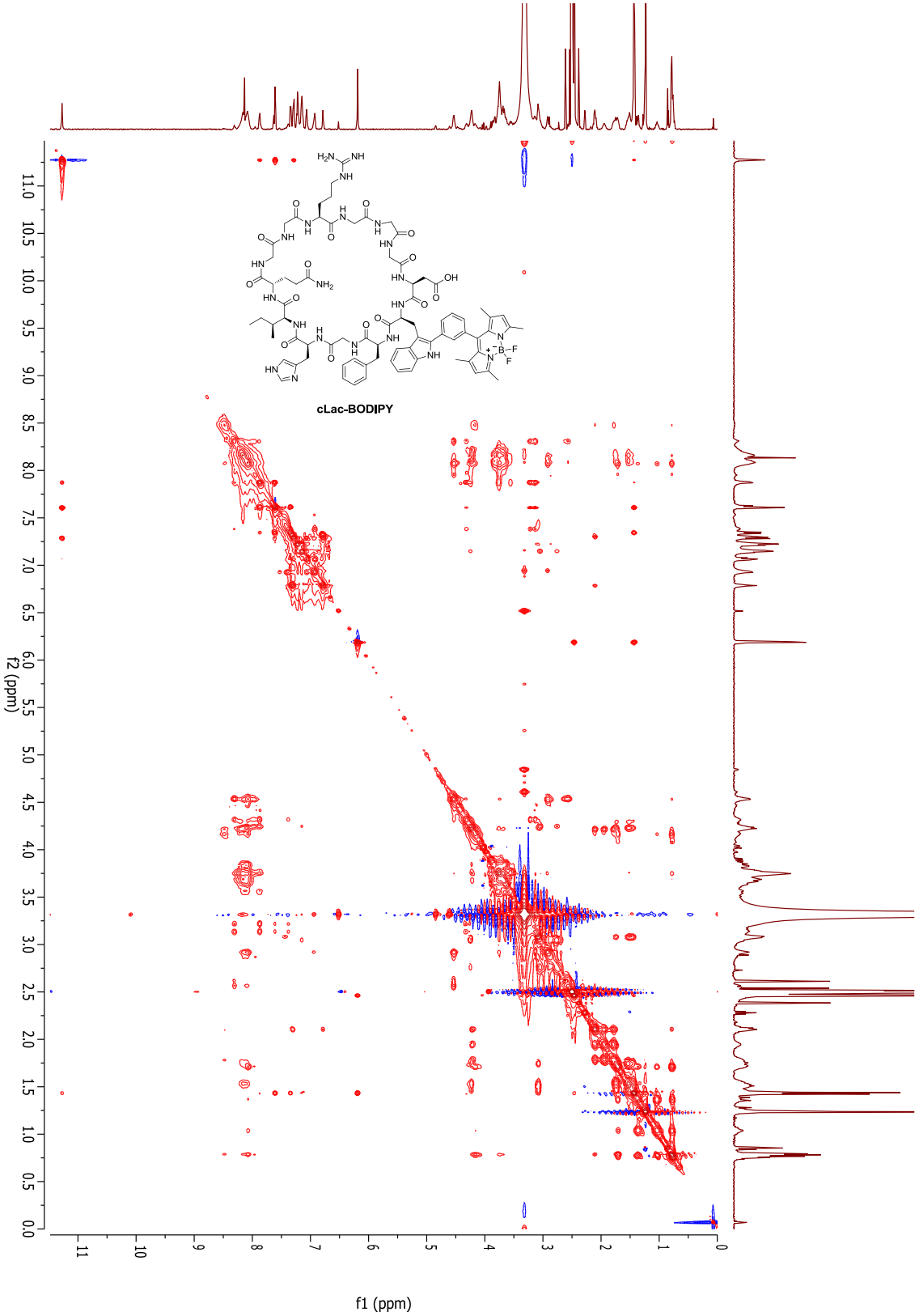
COSY-NMR of **cLac-BODIPY**



TOCSY-NMR of **cLac-BODIPY**



NOESY-NMR of **cLac-BODIPY**



Supplementary Figures

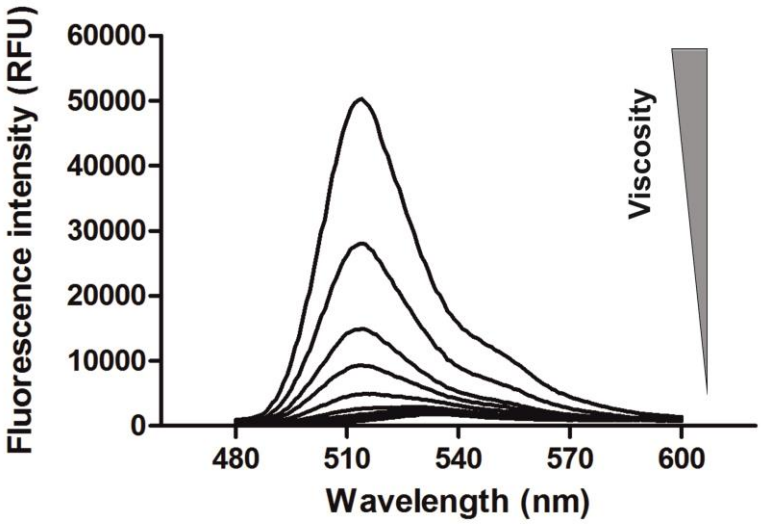


Fig. S1. Fluorogenic behaviour of the Trp-BODIPY amino acid. Fluorescence spectra of the Trp-BODIPY amino acid showing enhanced emission upon fluorophore rigidification in mixtures of glycerol: H_2O (10% steps) with increased viscosity. $\lambda_{exc.}$: 450 nm.

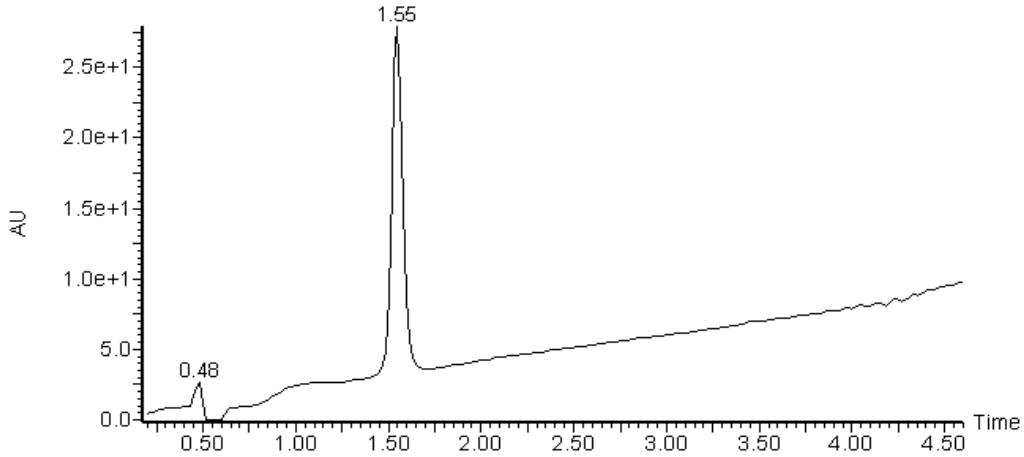


Fig. S2. HPLC trace of cLac-1. Eluents: H_2O (0.1% FA) and ACN (0.1% FA). Flow: 1.6 mL min^{-1}. Linear gradient from 5% to 100% ACN (0.1% FA) over 3.5 min. The plot corresponds to the integrated absorbance signal from 210 to 400 nm.

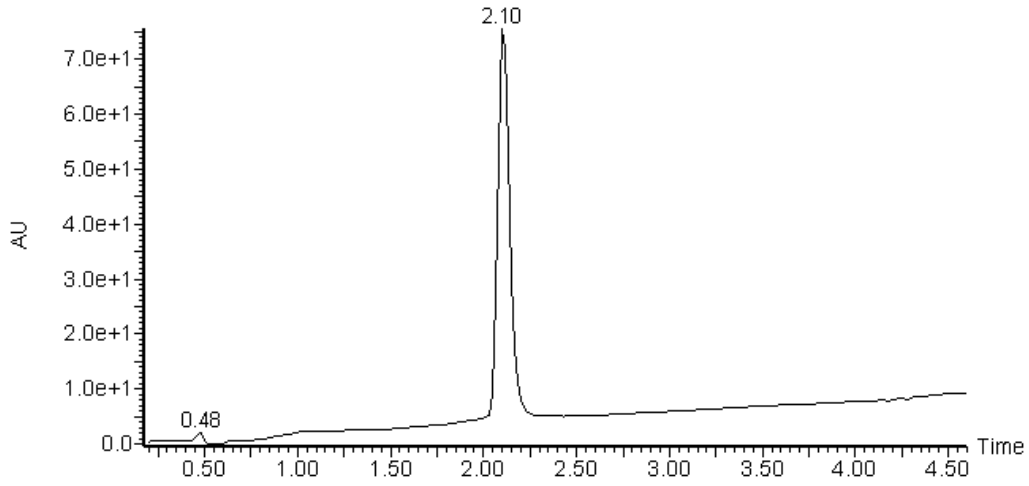


Fig. S3. HPLC trace of cLac-BODIPY. Eluents: H_2O (0.1% FA) and ACN (0.1% FA). Flow: 1.6 mL min^{-1}. Linear gradient from 5% to 100% ACN (0.1% FA) over 3.5 min. The plot corresponds to the integrated absorbance signal from 210 to 400 nm.

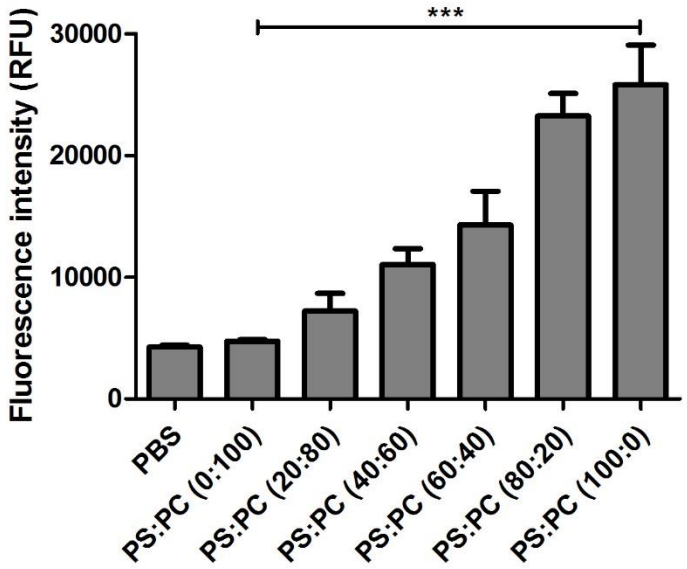


Fig. S4. Fluorescence intensity values of **cLac-BODIPY** (5 µM) after incubation with different PS: PC films. Values are represented as means and error bars as SD (n = 3). *** for $p < 0.001$.

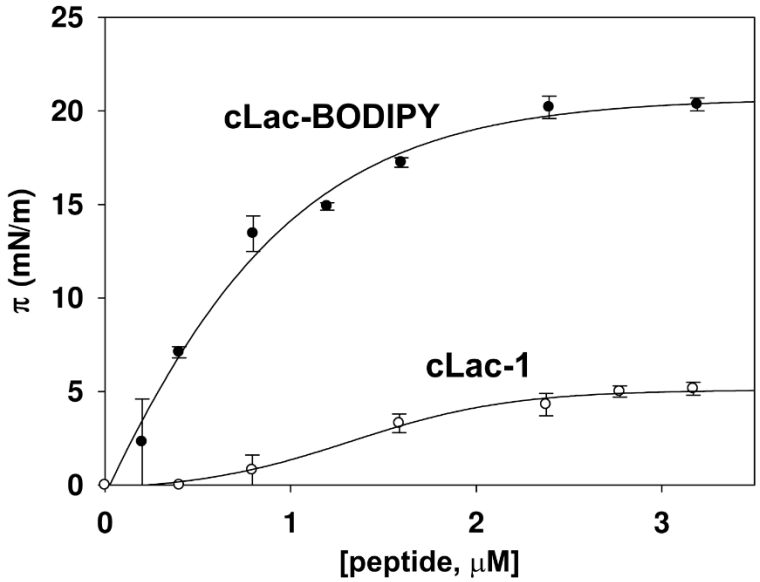


Fig. S5. Determination of the tensioactivity of **cLac-1** and **cLac-BODIPY**. **cLac-BODIPY** shows high tensioactivity with a saturation pressure (π_s) of 20.4 mN m^{-1} and a saturation concentration (C_s) of 3 μM. The π_s for **cLac-1** is 5 mN m^{-1}. Values represented as means and error bars as SD (n = 3).

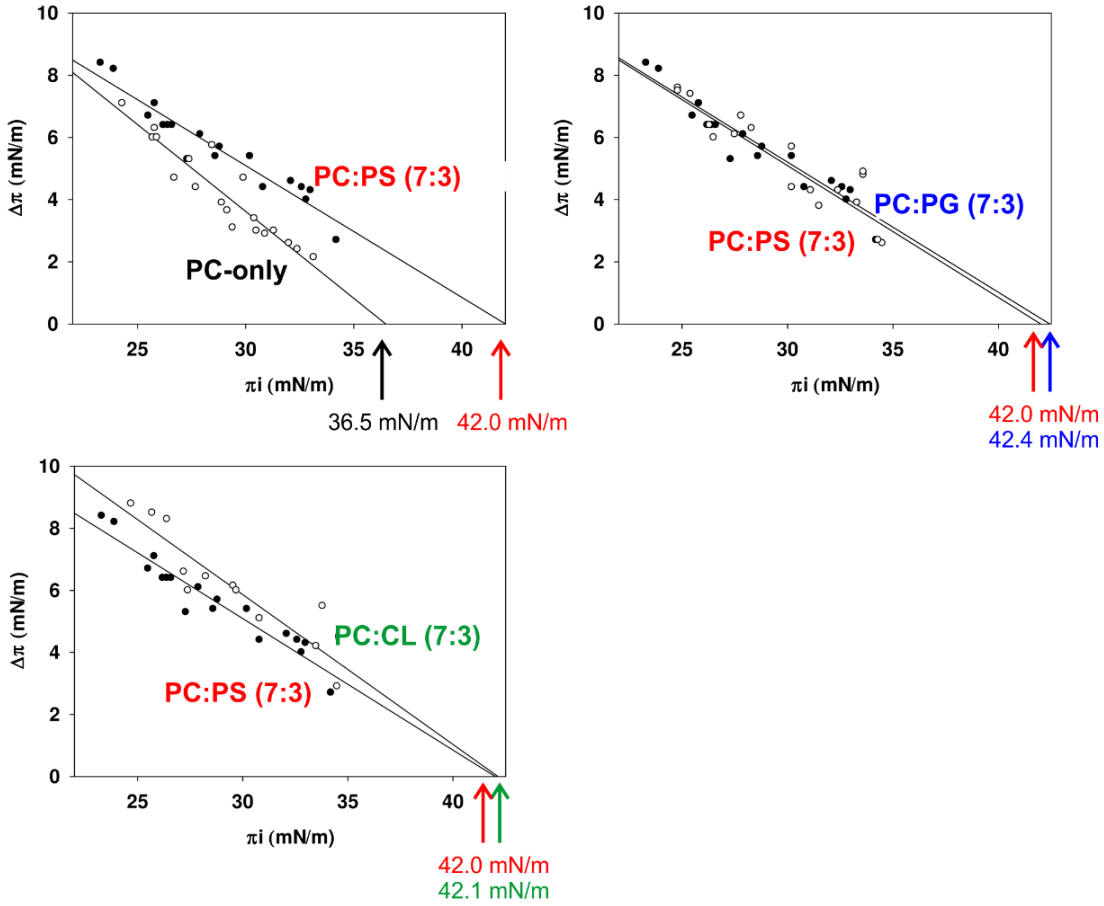


Fig. S6. Quantitative binding assays of **cLac-BODIPY** to monolayers with variable lipid composition. Arrows point at the π_c values for every lipid composition. PC: phosphatidylcholine, PS: phosphatidylserine, PG: phosphatidylglicine, CL: cardiolipin. Values represented as means from n = 3.

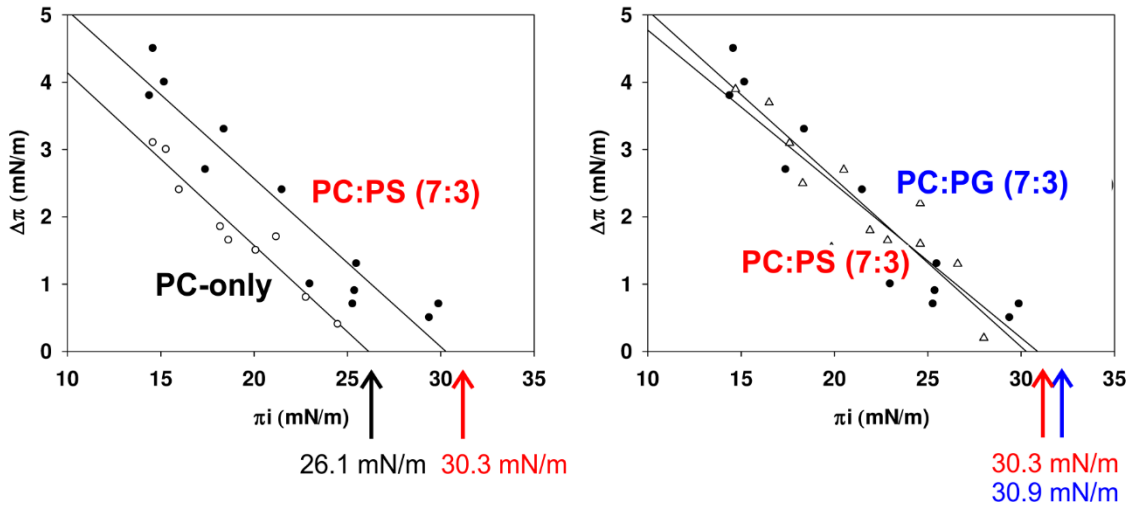


Fig. S7. Quantitative binding assays of **cLac-1** to monolayers with variable lipid composition. Arrows point at the π_c values for every lipid composition. PC: phosphatidylcholine, PS: phosphatidylserine, PG: phosphatidylglicine. Values represented as means from n = 3.

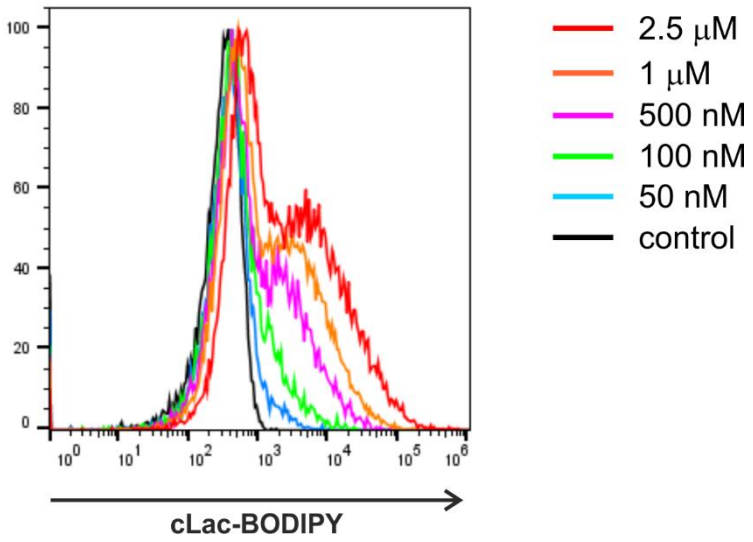


Fig. S8. Flow cytometry analysis of the fluorescence labelling of apoptotic bodies after incubation with different concentrations of **cLac-BODIPY**.

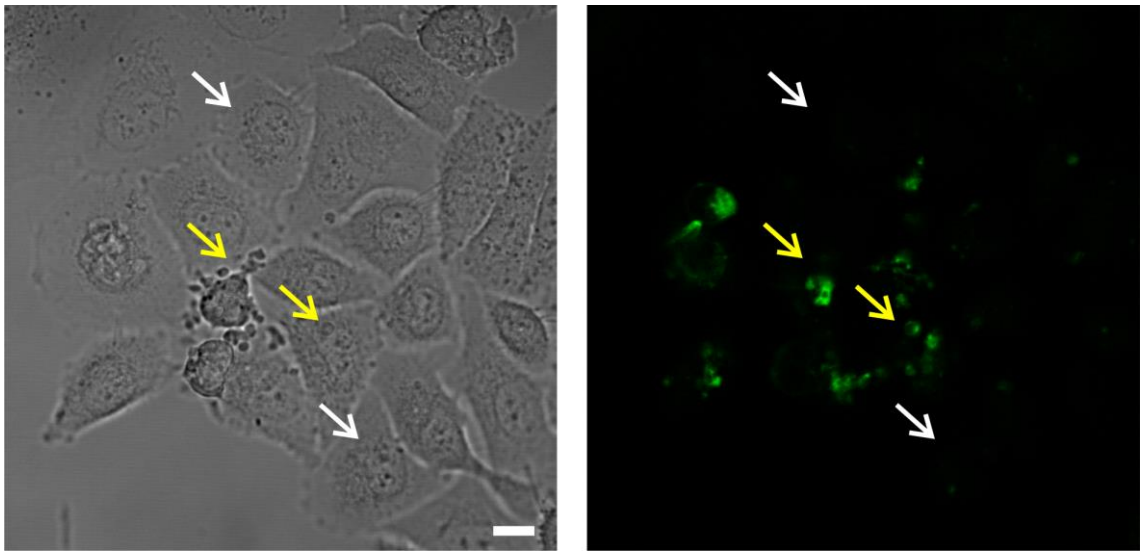


Fig S9. Brightfield and fluorescence microscopy images of human epithelial A549 cells upon induction of apoptosis and treatment with **cLac-BODIPY** (1 mM). Subcellular bodies derived from apoptotic cells (yellow arrows) are brightly stained whereas viable cells (white arrows) are not stained by **cLac-BODIPY**. Scale bar: 10 μm.

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