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Supporting Information

Bioorthogonal Turn-On BODIPY-Peptide Photosensitizers for Tailored Photodynamic Therapy

Greta Linden and Olalla Vázquez*[a]

Table of Contents

SUPPORTING INFORMATION

Abbreviations

°C	degree Celsius
µ	micro (10^{-6})
abs	absolute
Alloc	allyloxycarbonyl
AU	absorbance units
br	broadened (spectra)
calcd	calculated
cat	catalytic
CPB	citrate phosphate buffer
d	doublet (NMR spectra)
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyano-p-benzoquinone
DIC	*N,N'*-diisopropylcarbodiimide
DiI	dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine (perchlorate)
DIPEA	*N,N*-diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
DODT	2,2'-(ethylenedioxy)diethanethiol
DPBF	1,3-diphenylisobenzofuran
DPBS	Dulbecco's phosphate-buffered saline
EB	erythrosine B
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
eq	equivalent
ESI	electrospray ionization mass spectrometry
et al.	and others (Latin et alii)
FA	formic acid
FBS	fetal bovine serum
Fmoc	9-fluorenylmethoxycarbonyl
F_x	cyclohexyl-alanine
HeLa	cervial cancer cell line (Henrietta Lacks)
HMQC	heteronuclear multiple quantum correlation (NMR spectra)
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
Hz	hertz
IC_{50}	half maximal inhibitory concentration
iEDDA	inverse-electron-demand Diels-Alder
J	coupling constant (in NMR spectrometry)
K	Kelvin
LED	light-emitting diode
m	mili (10^{-3})
	multiplet (NMR spectra)
m/z	mass-to-charge ratio
M	molar (moles per liter)
M^+	molecular ion

MPP	mitochondria penetrating peptide
MS	mass spectrometry
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
PA	palmitic acid
PC-3	prostate cancer cell line
ppm	parts per million
PS	photosensitizer
RB	rose bengal
R_f	retention factor
RFU	relative fluorescence units
RP	reversed phase
rpm	revolutions per minute
RPMI	growth medium (Roswell Park Memorial Institute)
Rr	Pearson's correlation coefficient
s	singlet (NMR spectra)
t	triplet (NMR spectra)
TCO	(*E*)-cyclooct-4-enol
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIPS	triisopropylsilane
TLC	thin-layer chromatography
TMRE	tetramethylrhodamine ethyl ester (perchlorate)
TNBS	2,4,6-trinitrobenzenesulfonic acid
TPP	tetraphenylporphyrin
t_R	retention time
Tris	tris(hydroxymethyl)aminomethane
Tz	tetrazine
UV	ultraviolet
UV-vis	ultraviolet-visible
v/v	volume per volume
vol %	volume percent
δ	chemical shift in parts per million downfield from tetramethylsilane
ε	molar absorption coefficient
λ_{max}	wavelength of maximum absorption

SUPPORTING INFORMATION

Experimental Procedures

Materials

All commercially available reagents were used without further purification as delivered from the corresponding company. The respective reagents were purchased from the following companies: 4-hydroxy-benzaldehyde and zinc-trifluoro-methansulfonate from Alfa Aesar (USA); 2,4-dimethylpyrolle, boron trifluoride diethyl etherate, propagylamine and 4-cyanobenzoic acid from Acros Organics (Belgium); RPMI from Gibco Thermo Fisher Scientific (USA); 1,2-dibromoethane and bromoacetic anhydride from Sigma Aldrich (USA). TEA, THF and DCM were dried using standard procedures.[1]

Solid Phase Peptide Synthesis

Peptides were manually synthesized using Fmoc solid phase strategy in 2 mL polypropylen reactors with plunger and frit, (pore size 25 µm, Multi Syn Tech GmbH, Germany) TentaGel® S RAM resin (loading = 0.25 mmol/g) was used as solid support. The following synthesis protocol correspond to a 20 µmol scale. For shaking an Edmund Bühler Swip rocker was used.

Swelling: 80 mg of resin (20 µmol) were swollen in DMF (1.5 mL) for 30 min.

Deprotection of the Fmoc protecting group: Piperidine (500 µL, 20% in DMF, vol %) was added to the resin and shaken for 5 min. This step was repeated once, afterwards the resin was filtered off. Subsequently the resin was washed with DMF (5×1.5 mL), DCM (5× 1.5 mL) and DMF (5× 1.5 mL).

Coupling of amino acids: The Fmoc-amino acid (4.00 eq) was dissolved in Oxyma (0.5 M in DMF, 4.00 eq), and DIC (4.00 eq) was added. The resulting solution was pre-activated for 3 min and then added to the resin. This suspension was shaken for 45 min. Afterwards, the resin was filtered off, and washed with DMF (5× 1.5 mL), DCM (5× 1.5 mL) and DMF (5× 1.5 mL). Monitoring of the coupling completion was confirmed by the TNBS-test[2] on few resin beads.

Capping: A solution of Ac_2O/2,6-lutidine/DMF (1 mL, 5:6:89, v/v/v) was added to the resin and shaken for 5 min. The resin was filtered off, and washed with DMF (5× 1.5 mL), DCM (5× 1.5 mL) and DMF (5× 1.5 mL).

After the last coupling cycle, the resin was washed with DMF (5×1.5 mL), DCM (10×1.5 mL) and dried in vacuo for 15 min.

Cleavage deprotection step: The dried resin was treated with the TFA-based cleavage cocktail TFA/DODT/H_2O/TIPS, (94:2.5:2.5:1, v/v/v/v (1.0 mL for 20 mg of resin) and shaken for 3 h. Then the resin was filtered off and the filtrate was added to dry ice-cold Et_2O (10.0 mL of Et_2O for 1.0 mL cleavage cocktail). After 10 min, the precipitated peptide was centrifuged (4000 rpm, 10 min, 4 °C), the supernatant was discarded, the pellet washed with Et_2O and pelleted again, this step was repeated once. Afterwards, the peptide pellet was dried under N_2, dissolved in ultrapure H_2O, and purified.

Purification: The probes were purified at 40°C by preparative RP-HPLC, performed on a Varian (USA) ProStar Preparative HPLC system with a preparative Juptier 10 u C18 300 Å column (10 µm, 250 × 10 mm; Phenomenex) with a flow rate of 8 mL/min. Detection of the signals was achieved with a UV detector at the wavelength of 220 and 260 nm. The eluents were ultrapure H_2O (A) and MeCN (B) with addition of 0.1% TFA. a linear gradient over 30 min was used. The collected fractions, containing the desired peptides as their TFA salts, were lyophilized and stored at -20°C.

gradient A: 0% to 30% of solvent B
gradient B: 5% to 75% of solvent B
gradient C: 15% to 95% of solvent B

Characterization: The freeze-dried products were identified via analytical RP-HPLC-MS on an Agilent 1260 Infinity II HPLC-System (Agilent Technologies). If not stated differently, for all analytical RP-HPLC runs an Agilent eclipse XDB-C18 column (5 µm, 4.6 × 150 mm) or Macherey Nagel Nucleodur 100-C18 ec column (3 µm, 2 × 125 mm) using isocratic regime during the first 5 min, followed by a linear gradient over 30 min of B as stated below with a were used and detection was monitored at 220 nm. The eluents were ultrapure H_2O (A) with addition of 0.05% TFA and MeCN (B) with addition of 0.03% TFA.

gradient 1: 0% to 30% of solvent B
gradient 2: 5% to 75% of solvent B
gradient 3: 5% to 95% of solvent B

For NMR analysis of compounds **9** and **18-20** a Büchi (Switzerland) Pure C-810 flash chromatography system was used for additional purification using a EcoFlex C-18 cartridge (50 µm spherical, 12 g) with isocratic regime during the first 3 min, followed by a linear gradient of solvent B from 0% to 100% over 10 min with a flow rate of 30 mL/min. Peaks were UV-detected at 496 nm or 528 nm and automatically collected. The eluents were ultrapure H_2O (A) with addition of 0.1% TFA and MeCN (B) with addition of 0.1% TFA.

Nuclear Magnetic Resonance Spectroscopy (NMR)

All NMR spectra were automatically measured at 300 K either in a Bruker AV III HD 300 MHz spectrometer at a frequency of 300 MHz (^{1}H), 75 MHz (^{13}C) or 470 MHz (^{19}F) or on a Bruker AV III HD 500 MHz spectrometer at a frequency of 500 MHz (^{1}H), 125 MHz (^{13}C) or 160 MHz (^{11}B). The ^{1}H and ^{13}C NMR spectra were referenced to solvent residue peaks. As internal standards, deuterated chloroform ($CDCl_3$) was used. Solvent shifts (ppm): $\delta(CDCl_3)$ = 7.26 ppm (^{1}H), $\delta(CDCl_3)$ = 77.16 ppm (^{13}C).[3] ^{19}F and ^{11}B NMR spectra were referenced to $CFCl_3$ and $BF_3{\cdot}OEt_2$, respectively. The assignment of the signals was based on two-dimensional nuclear magnetic resonance spectroscopy (2D NMR), i.e. heteronuclear multiple quantum correlation spectroscopy (HMQC).

UV-vis and Fluorescence Spectroscopy

Concentration determinations, UV-vis and fluorescence measurements were performed on a Tecan (Switzerland) Spark 20M multimode microplate reader at room temperature. Concentration determinations and UV-vis measurements were performed in a 1400 µL quartz cuvette (Hellma Analytics, 104F-QS) with a pathlength of 1 cm in a volume of 800 µL. Fluorescence measurements were performed in black 96 well plates (Greiner Bio-one, Austria) in 200 µL in triplicate with the following settings: excitation wavelength: 475 nm, 5 nm bandwidth; emission wavelength scan: 500-600 nm, 10 nm bandwidth. Solvent blank values were subtracted for every measurement.

Molar Extinction Coefficients

For the accurate determination of concentrations, the molar extinction coefficients of compounds **7** and **8** were determined. Thus, a specific amount of the corresponding compound was weighted (~ 5.00 mg) on a Mettler Toledo XP6 micro balance to prepare a solution of known concentration. Increasing amounts of the corresponding stock solution were added without exceeding 10 % of the initial volume of the cuvette; absorbance was measured as explained before. In all the cases the recorded absorbance was between 0.1 and 1 AU to be in concordance with Lambert-Beer law:

$$A = \varepsilon \cdot c \cdot l$$

where A = absorbance; ε = molar extinction coefficient; c = concentration; l = path length.

Singlet Oxygen Measurements

Singlet oxygen quantum yields (Φ_Δ) were determined as described in the literature.[4] The procedure was alike the determination of $^1O_2$ described above. Measurements were performed in black clear bottom 96 well plates (µclear, Greiner Bio-one, Austria) in triplicates: 20 µL of a 10 µM stock of the conjugate of interest (1.00 µM final concentration) and 50 µL of 800 µM DPBF solution (200 µM final concentration) were mixed in a total of 200 µL solvent. For the reference molecules: erythrosine B (EB) and rose bengal (RB), MeOH was used as solvent. After an initial measurement, the decrease of the DPBF absorbance at 415 nm was monitored with a Spark 20M platereader (Tecan, Switzerland) at 25°C after every 10 s of irradiation. Values were obtained by the following equation:

$$\Phi_\Delta^x = \Phi_\Delta^{ref} \cdot \frac{k_x}{k_{ref}} \cdot \frac{I_a^{ref}}{I_a^x}$$

Where Φ_Δ stands for singlet oxygen quantum yield, *ref* for the standards (Φ_Δ = 0.76 for RB and 0.6 for EB),[5] k_x and k_{ref} for first order rate constants (10^{-3} s^{-1}) of DPBF consumption of the sample and the standard, respectively; I_a are the absorbance value of the sample (x) and the standard (ref) at the irradiation wavelength of 525 nm. From all values, the background signal was subtracted.

A custom-made aluminium based 96-LED-array (wavelength of maximum emission: 525 nm, luminous intensity: 16000-27000 mcd, viewing angle: 23°, AVAGO HLMP-CM2B-120DD, Broadcom Limited, United States) was developed for irradiation. A calibrated spectroradiometer PS–200 (Apogee Instruments, US) was acquired to measure the LEDs irradiance (detector integration: 10 ms, 100 measurements per average) the LEDs flux density is 69.4 ± 0.6 W m^{-2}.

SUPPORTING INFORMATION

Cell Culture

Step-Wise Protocol for the Cell-Based Assays

Cell Viability Assays for IC_{50} Determination

Seeding: The day before assessing cytotoxicity 200 µL of cells suspension (1× 10^5 cell/mL, DMEM supplemented with 2.5% FBS) was dispensed into black µclear F-bottom 96-well cell culture microplates (Greiner Bio-One, Austria). The plates were incubated for 21 h.

Addition of substrates: The next day 110 µL of cell culture media were removed from every well, and serial dilutions of the compounds (10 µL each; final concentration 0.03-4.00 µM in ultrapure H_2O with 12.5% MeCN (final MeCN concentration 1.25%) were added to the cells (final volume 100 µL) and mixed properly. The plate was then centrifuged in an Eppendorf 5810 R centrifuge (1 min, 37°C, 1000 rpm) and incubated for 2 h until being irradiated.

Irradiation: When it was required the plates were irradiated for 160 s with the custom made LED plate also used for $^1O_2$ measurements and the cells were afterwards incubated for another 18 h.

For assessing the dark toxicity the cells were not irradiate, and therefore, incubated for 20 h after addition of the compounds.

Data evaluation: After incubation, 20 µL of a 1.63 mM solution of resazurin in DPBS was added to each well and mixed appropriated. After addition, the plate was further shaken for 30 s using the SpectraMax M5 shaking function (Molecular Devices) and centrifuged with an Eppendorf 5810 R centrifuge (1 min, 37°C, 1000 rpm). The fluorescence was next measured using the SpectraMax M5 plate reader with the following settings: fluorescence bottom read, excitation wavelength: 560 nm, emission wavelength: 590 nm, cut-off filter: 590 nm, 6 readings per well in 30 min intervals until saturation in the untreated cells was reached. The slope of each individual well was calculated and percentage of cell survival in the untreated control was assumed as 100 %. The effect of the vehicle solution (12.5 % MeCN in ultrapure H_2O) was corroborated to be inert too. Relative viability = (experimental slope - background slope)/(slope of untreated control-background slope) × 100 %. All values were obtained in triplicates per sample/control. Always at least two independent measurements i.e. from different stock solutions, were performed.

Intracellular iEDDA and Cell Viability Assays

Seeding: Firstly, cells were seeded as explained before for the IC_{50} experiments (2× 10^4 cells/well, DMEM supplemented with 2.5% FBS) and incubated for 21 h.

Addition of substrates: Then 110 µL of the culture media were removed from each well and 10 µL of solutions of different concentrations of compounds **2**-**6A** in 12.5% MeCN ultrapure H_2O were added to the cells (final concentrations 1.00-0.13 µM), mixed properly, centrifuged and incubated for 1.5 h.

Washing: The medium was carefully aspirated and cells were washes twice with DPBS (2× 100 µL) and media (DMEM, 2.5% FBS, 1× 100 µL).

Addition of TCO: Next, fresh culture medium (90 µL/100µL, DMEM, supplemented with 2.5% FBS) was added, in the corresponding wells, 10 µL of TCO solution, containing 12.5 eq according to the concentration of compounds **2-6A** in ultrapure H_2O with 12.5% MeCN were added and incubated (100 µL final volume, 1.25% MeCN final content per well).

Irradiation: After 30 min of incubation, the plates were irradiated for 160 s with the custom made LED plate also used for $^1O_2$ measurements and the cells were afterwards incubated for another 18 h.

Data evaluation: Cell viability was evaluated as explained in the data evaluation step in the *Cell Viability Assays for IC_{50} Determination* section above. Cells treated only with solvent, and TCO were considered as negative controls.

Experiments with PC-3 cell line

PC-3 cells were cultured alike HeLa cells as described in the main manuscript but grown in RPMI media supplemented with FBS (10 % v/v), penicillin (100 units/mL), and streptomycin (100 µg/mL). The same protocols described above for the cell-based assays were followed, besides the DMEM media was replaced by RPMI with the same supplements.

Synthesis and Characterization of the Compounds

Chromatograms of the Synthesized Peptides

Figures S1-S5 show the analytical RP-HPLC chromatograms of the synthesized peptides **11-14** and the intermediate **15**.

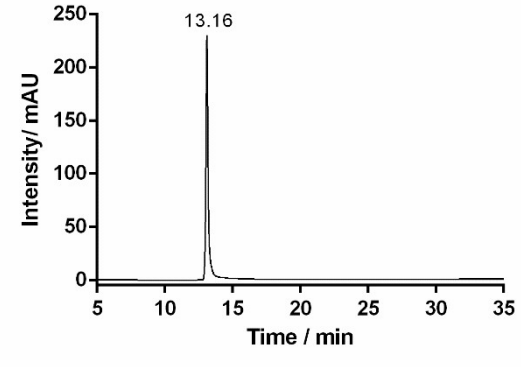


Figure S1: RP-HPLC chromatogram of the purified peptide H_2N-CRRRRR-$CONH_2$ (**11**), detected at 220 nm (gradient 1).

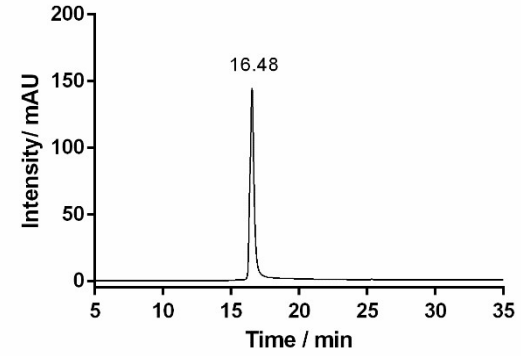


Figure S2: RP-HPLC chromatogram of purified peptide H_2N-CRRRRRRRR-$CONH_2$ (**12**), detected at 220 nm (gradient 1).

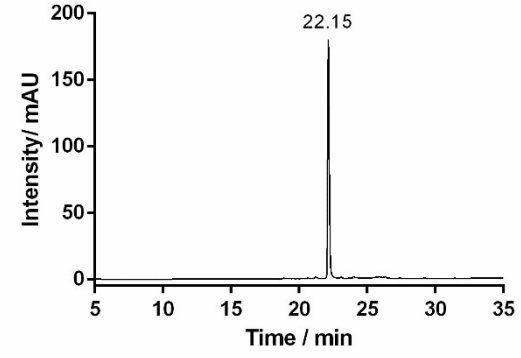


Figure S3: RP-HPLC chromatogram of purified peptide H_2N-$CF_xrF_xrF_xr$-$CONH_2$ (**13**), detected at 220 nm (gradient 2).

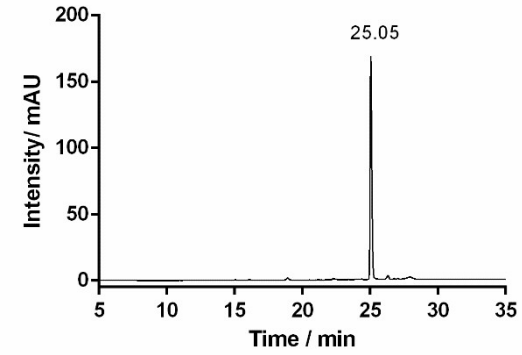


Figure S4: RP-HPLC chromatogram of purified peptide H_2N-CrF_xrF_xrF_xr-$CONH_2$ (**14**), detected at 220 nm (gradient 2).

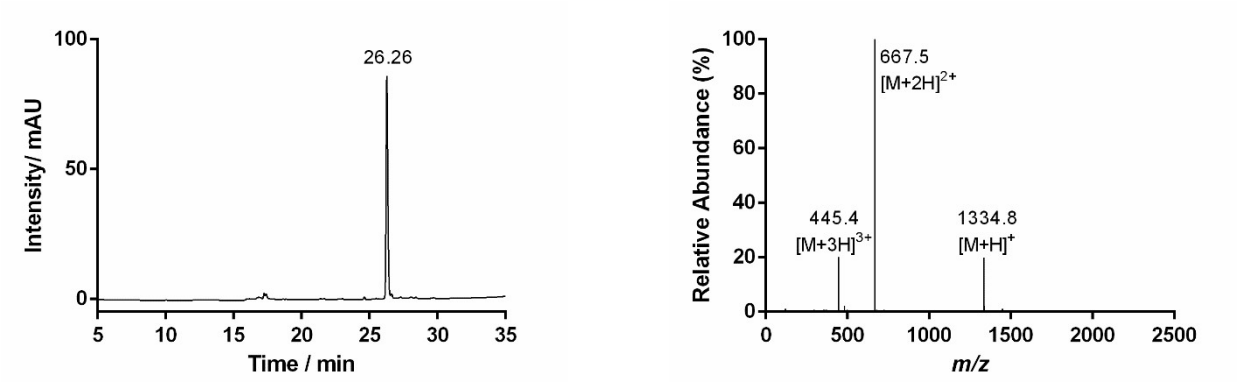
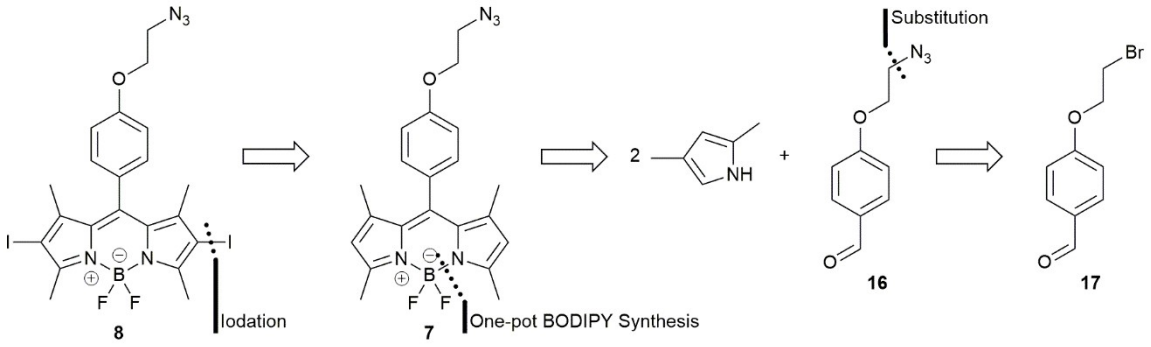


Figure S5: RP-HPLC chromatogram of test cleavage of peptide Fmoc-CK(PA)RRRR-$CONH_2$ (**15**), detected at 220 nm (left, gradient 3) and ESI-MS corresponding to the peak at t_R= 26.26 min (right). Chemical formula: $C_{64}H_{108}N_{20}O_9S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 667.4237; found: 667.4267.

Synthesis of Organic Molecules

BODIPY Synthesis

The BODIPY fluorophore **7** was synthesized following the altered literature procedure of the Huang lab,[6] while the halogenation to obtain **8** was performed according to Li et al..[7]



Scheme S1: Retrosynthetic scheme for the molecules **16, 7** and **8**.

4-(2-Bromoethoxy)benzaldehyde (17): Altering the procedure of the Huang lab[6] 4-hydroxybenzaldehyde (5.00 g, 40.9 mmol, 1.00 eq) and potassium carbonate (11.3 g, 81.9 mmol, 2.00 eq) were suspended in EtOH (50.0 mL), 1,2-dibromoethane (35.4 mL, 409 mmol, 10.0 eq) was added and the flask was emerged into an 90°C oil bath. Reflux was maintained for 14 h. The slurry was cooled at room temperature and filtrated. The precipitate was washed with DCM and discarded while the filtrate was concentrated for purification. Using flash column chromatography on silica gel (DCM/pentane, 2:1, v/v) the product **17** (7.70 g, 33.6 mmol, 82%) was obtained as a white solid. The characterization is in agreement with the literature ref.[6] **TLC**: R_f= 0.41 (DCM). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 9.90 (s, 1H, C*H*O), 7.85 (d, 2H, ^3J= 8.7 Hz, 2×H_{arom}), 7.02 (d, 2H, ^3J= 8.8 Hz, 2×H_{arom}), 4.38 (t, ^3J = 6.3 Hz, 2H, O-CH_2), 3.67 (t, ^3J = 6.2 Hz, 2H, CH_2-Br) ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ = 190.7 (1C, *C*HO), 163.2 (1C, $C_{q.}$), 132.2 (2C, 2×C_{arom}),

130.7 (C, $C_{q.}$), 115.1 (2C, 2×C_{arom}), 68.2 (1C, CH_2), 28.5 (1C, CH_2) ppm. **HRMS-ESI⁺:** = $[M+H]^+$ calcd $C_9H_{10}O_2Br$: 228.9859, found 228.9860.

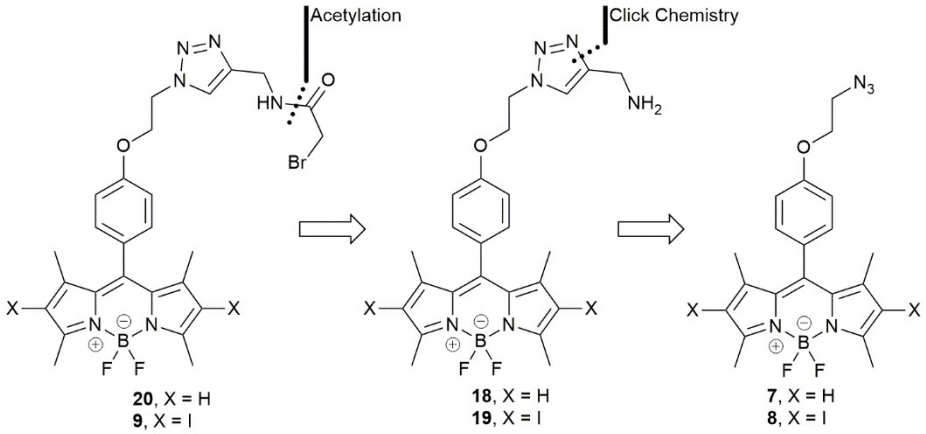
4-(2-Azidoethoxy)benzaldehyde (16): Compound **17** (3.50 g, 15.3 mmol, 1.00 eq) was dissolved in DMF (12.0 mL) and sodium azide (1.19 g, 18.3 mmol, 1.20 eq) was added gradually, then the mixture was heated to 165°C for 3 h. The reaction was cooled to room temperature, diluted with DCM (100 mL) and further washed with distilled H_2O (4× 150 mL) and brine (1× 150 mL). The organic layer was dried over anhydrous $MgSO_4$. After filtration the solution was concentrated under reduced pressure and the pale brown oil was purified by column flash chromatography on silica gel (DCM) yielding **16** as pale-yellow oil (2.73 g, 14.1 mmol, 93%). The characterization is in agreement with the literature ref.[8] **TLC:** R_f= 0.37 (DCM). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ= 9.91 (s, 1H, C*H*O), 7.86 (d, 2H, ^3J = 8.9 Hz, 2×H_{arom}), 7.03 (d, 2H, ^3J = 8.7 Hz, 2×H_{arom}), 4.37–4.07 (t, 2H, ^3J= 5.0°Hz, CH_2), 3.65 (t, 2H, ^3J=5.0°Hz, CH_2)ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ=190.6 (1C, *C*HO), 163.1 (1C, $C_{q.}$), 132.0 (2C, 2×C_{arom}), 130.5 (1C, $C_{q.}$), 114.8 (2C, 2×C_{arom}), 67.2 (1C, OCH_2), 50.0 (1C, CH_2N_3) ppm. **HRMS-ESI⁺ (m/z):** calcd for $[M+H]^+$ $C_9H_{10}N_3O_2$: 192.0768; found: 192.0769.

8-(4-(2-Azidoethoxy)phenyl)-1,3,5,7-tetra-methyl-4,4- difluoro-4-bora-3a,4a-diaza-*s*-indacene (7): Under inert atmosphere a mixture of 4-(2-bromoethoxy)benzaldehyde (928 mg, 4.85 mmol, 1.00 eq) and 2,4-dimethylpyrole (1.00 mL, 9.71 mmol, 2.00 eq) was dissolved in freshly distilled anhydrous DCM (85 mL). One drop of TFA (cat) was added to the mixture placed in an ice bath, and the reaction was stirred at room temperature for 14 h. DDQ (1.10 g, 4.85 mmol, 1.00 eq) was gradually added to the solution. After 30 min dry TEA (6.80 mL) was added to the mixture placed in an ice bath cooling, following the addition of boron trifluoride diethyl etherate (6.21 mL, 48.5 mmol, 10.0 eq). After 2 h, the mixture was concentrated and purified by column flash chromatography on silica gel (DCM/pentane (2:3, v/v) to DCM), giving **7** as orange solid (665 mg, 1.62 µmol, 33%). The characterization is in agreement with the literature ref.[6] **TLC:** R_f= 0. 73 (DCM); R_f= 0. 12 (DCM/pentane, 2:3, v/v). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 7.19 (d, ^3J = 8.7 Hz, 2H, 2×H_{arom}), 7.03 (d, ^3J = 8.7 Hz, 2H, 2×H_{arom}), 5.98 (s, 2H, 2× C*H*), 4.21 (t, ^3J = 5.0 Hz, 2H, CH_2), 3.66 (t, ^3J = 5.0 Hz, 2H, CH_2), 2.55 (s, 6H, 2× CH_3), 1.43 (s, 6H, 2× CH_3) ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ = 159.0 (1C, $C_{q.}$), 155.5 (2C, 2×$C_{q.}$), 143.3 (2C, 2×$C_{q.}$), 141.7 (1C, $C_{q.}$), 132.0 (2C, 2×$C_{q.}$), 129.6 (2C, 2×C_{arom}), 128.0 (1C, $C_{q.}$), 121.3 (2C, 2×C_{arom}), 115.3 (2C, 2×C_{arom}), 67.1 (1C, CH_2), 50.4 (1C, CH_2), 14.7 (4C, 4×CH_3) ppm. **^{19}F-NMR (470 MHz, 300 K, $CDCl_3$):** δ = -146.5 (q, 2F, ^1J = 33.0 Hz, BF_2) ppm. **^{11}B-NMR (160 MHz, 300 K, $CDCl_3$):** δ = 0.55 (t, 1B, ^1J = 33.0 Hz, BF_2) ppm. **HRMS-ESI⁺ (m/z):** calcd for $[M+Na]^+$ $C_{21}H_{22}BF_2N_5ONa$: 432.1781; found: 432.1780.

8-(4-(2-Azidoethoxy)phenyl)-2,6-diiodo-1,3,5,7-tetra-methyl-4,4- difluoro-4-bora-3a,4a-diaza-s-indacene (8): Compound **7** (350 mg, 855 µmol, 1.00 eq) was dissolved in degassed DCM (20.0 mL) and *N*-iodosuccinimide (827 mg, 3.67 mmol, 4.30 eq) was added successively. The dark red solution was stirred for 2 h, concentrated under reduced pressure and purified by column flash chromatography on silica gel (DCM/pentane, 1:1, v/v). **8** was obtained as red solid (545 mg, 824 µmol, 96%). The characterization is in agreement with the literature ref.[7] **TLC:** R_f= 0.38 (DCM/pentane, 1:1, v/v). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 7.16 (d, ^3J = 8.7 Hz, 2H, 2×$H_{arom.}$), 7.06 (d, ^3J = 8.7 Hz, 2H, 2×$H_{arom.}$), 4.23 (t, ^3J = 5.0 Hz, 2H,CH_2), 3.68 (t, ^3J = 4.9 Hz, 2H, CH_2), 2.64 (s, 6H, CH_3), 1.44 (s, 6H, CH_3) ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ = 159.3 (1C, $C_{q.}$), 156.9 (2C, 2×$C_{q.}$), 145.5 (2C, 2×$C_{q.}$), 141.4 (1C, $C_{q.}$), 131.8 (2C, 2×$C_{q.}$), 129.4 (2C, 2×C_{arom}), 127.6 (1C, $C_{q.}$), 115.6 (2C, 2×C_{arom}), 85.8 (2C, 2×C*I*), 67.2 (2C, CH_2), 50.4 (2C, CH_2), 17.4 (2C, 2×CH_3), 16.2 (2C, 2×CH_3) ppm. **^{19}F-NMR (470 MHz, 300 K, $CDCl_3$):** δ = -145.8 (q, 2F, ^1J = 32.2 Hz, BF_2) ppm. **^{11}B-NMR (160 MHz, 300 K, $CDCl_3$):** δ = 0.35 (t, 1B, ^1J= 32.1 Hz, BF_2) ppm. **HRMS-ESI⁺ (m/z):** calcd for $[M+Na]^+$ $C_{21}H_{20}BF_2I_2N_5ONa$: 683.9716; found: 683.9721.

Bromoacyl-BODIPY Synthesis

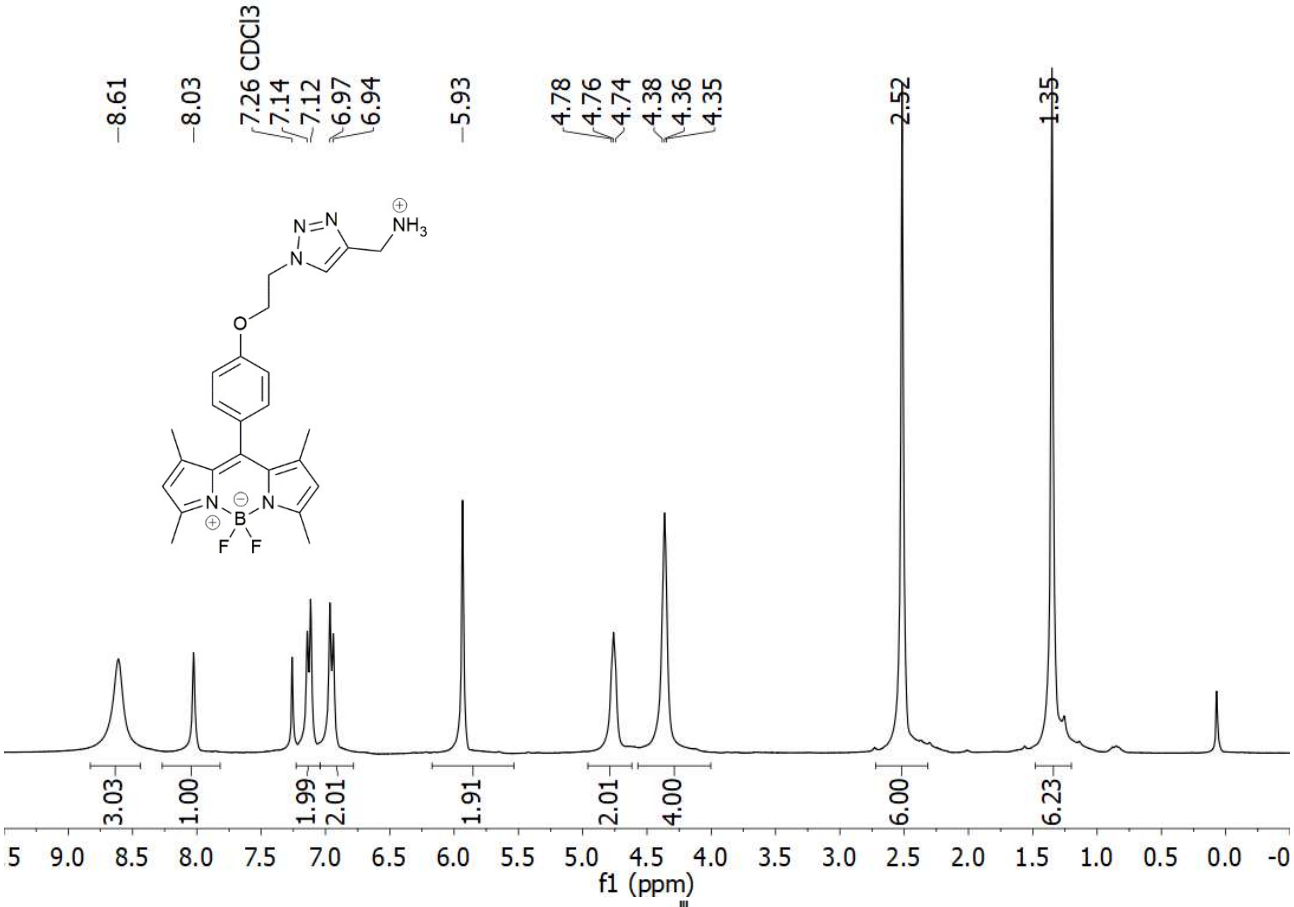
The bromoacyl group was added to BODIPY fluorophore (**7**) and 2I-BODIPY photosensitizer (**8**) to obtain **20** and **9** in two further steps:



Scheme S2: Retrosynthetic scheme for the molecules **20** and **9**.

SUPPORTING INFORMATION

8-(4-(2-((4-Aminomethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)-phenyl)-1,3,5,7-tetra-methyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (18): Under inert atmosphere **7** (150 mg, 367 µmol, 1.00 eq), CuI (14.0 mg, 73.4 µmol, 0.20 eq) and 2,6-lutidine (17.0 µL, 146 µmol, 0.40 eq) were dissolved in anhydrous THF (6.00 mL). Propargylamine (352 µL, 5.49 mmol, 15.0 eq) was added last and the amber solution was stirred for 3 h. The crude product was concentrated under reduced pressure and purified by flash column chromatography on silica gel (DCM to DCM/MeOH/TEA, 93:6:1, v/v/v). **18** was obtained as orange solid (132 mg, 285 µmol, 78%). **TLC:** R_f= 0.13 (DCM/MeOH/TEA, 93:6:1, v/v/v). **^{1}H-NMR (300 MHz, 300 K, CDCl$_3$):** δ = 8.61 (brs, 3H, NH_3), 8.03 (s, 1H, $H_{triazole}$), 7.13 (d, ^3J = 8.3 Hz, 2H, 2×H_{arom}), 6.96 (d, ^3J = 8.6 Hz, 2H, 2×H_{arom}), 5.93 (s, 2H, 2× CH), 4.76 (t, ^3J = 4.9 Hz, 2H, CH_2), 4.38-4.35 (m, 4H, 2×CH_2), 2.52 (s, 6H, 2×CH_3), 1.35 (s, 6H, 2×CH_3) ppm. **^{13}C-NMR (75 MHz, 300K, CDCl$_3$):** δ = 158.4 (1C, $C_{q.}$), 155.7 (2C, 2×$C_{q.}$), 143.1 (2C, 2×$C_{q.}$), 141.4 (1C, $C_{q.}$), 140.3 (1C, $C_{q.}$), 131.8 (2C, 2× $C_{q.}$), 129.6 (2C, 2×C_{arom}), 128.4 (1C, $C_{q.triazole}$), 125.4 (1C, H$C_{triazole}$), 121.4 (2C, 2×C_{arom}), 115.2 (2C, 2×C_{arom}), 66.1 (1C, CH$_2$), 50.1 (1C, CH$_2$), 34.8 (1C, CH$_2$NH$_2$), 14.7 (2C, 2×CH$_3$), 14.6 (2C, 2×CH$_3$) ppm. **^{19}F-NMR (470 MHz, 300K, CDCl$_3$):** δ =-146.2 (q, 2F, ^1J = 32.6 Hz, BF_2) ppm. **^{11}B-NMR (160 MHz, 300 K, CDCl$_3$):** δ = 0.47 (t, 1B, ^1J = 32.6 Hz, BF$_2$) ppm. **HRMS-ESI$^+$ (m/z):** calcd for [M+H-F]$^+$ $C_{24}H_{28}BFN_6O$:446.2401; found: 446.2403.



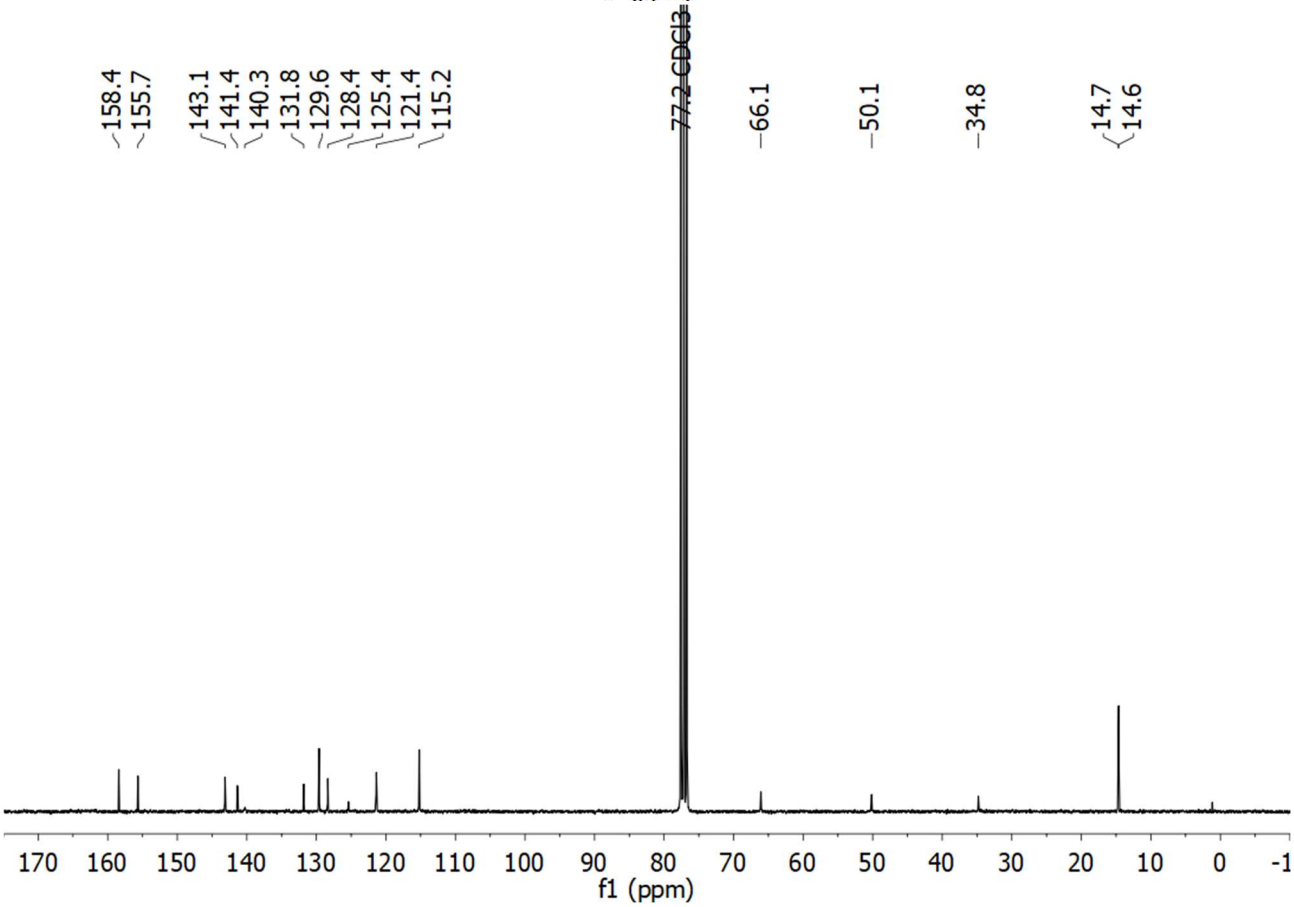


Figure S6: ^{1}H and ^{13}C NMR spectra of compound **18** in $CDCl_3$.

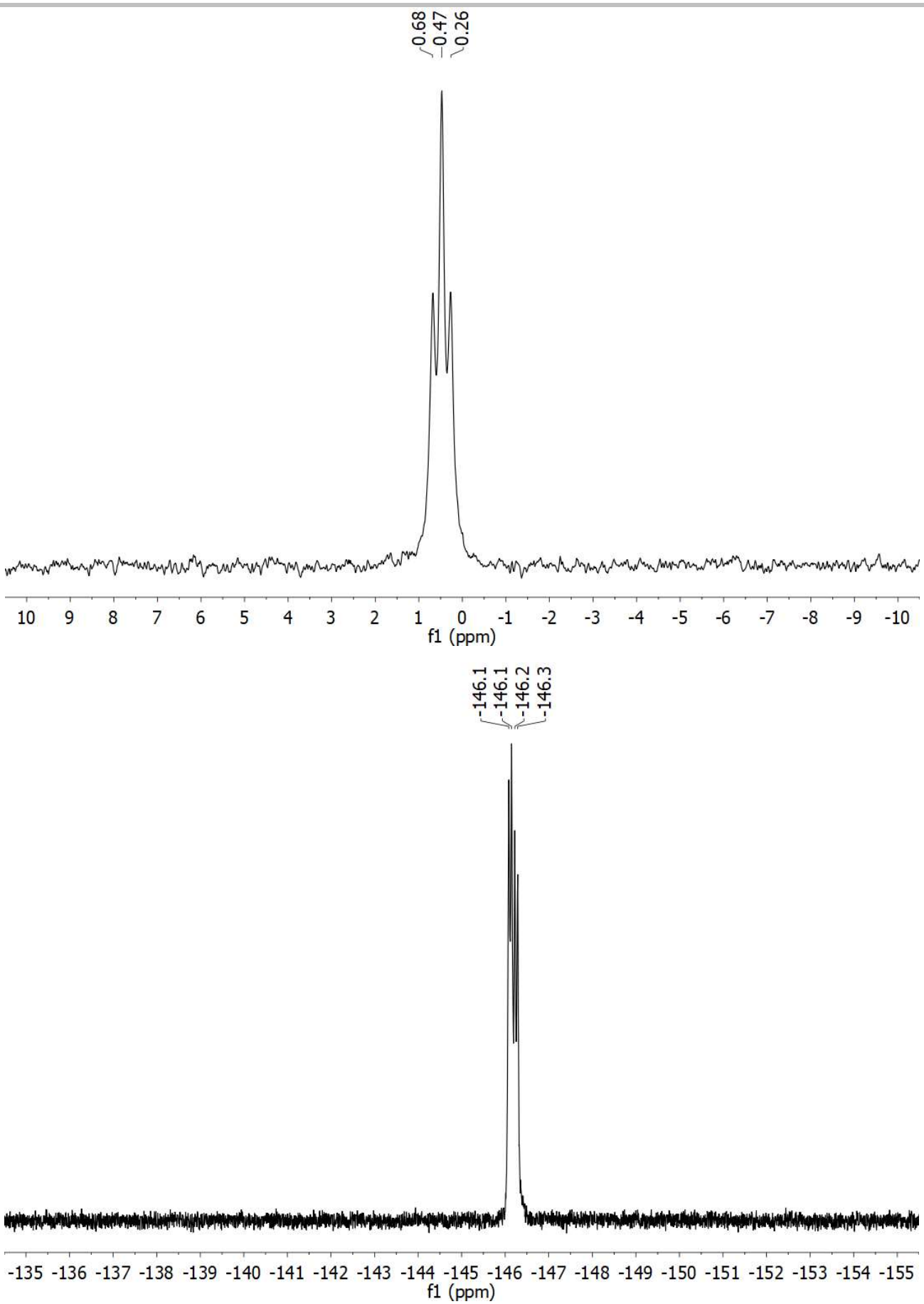


Figure S7: ^{11}B and ^{19}F NMR spectra of compound **18** in $CDCl_3$.

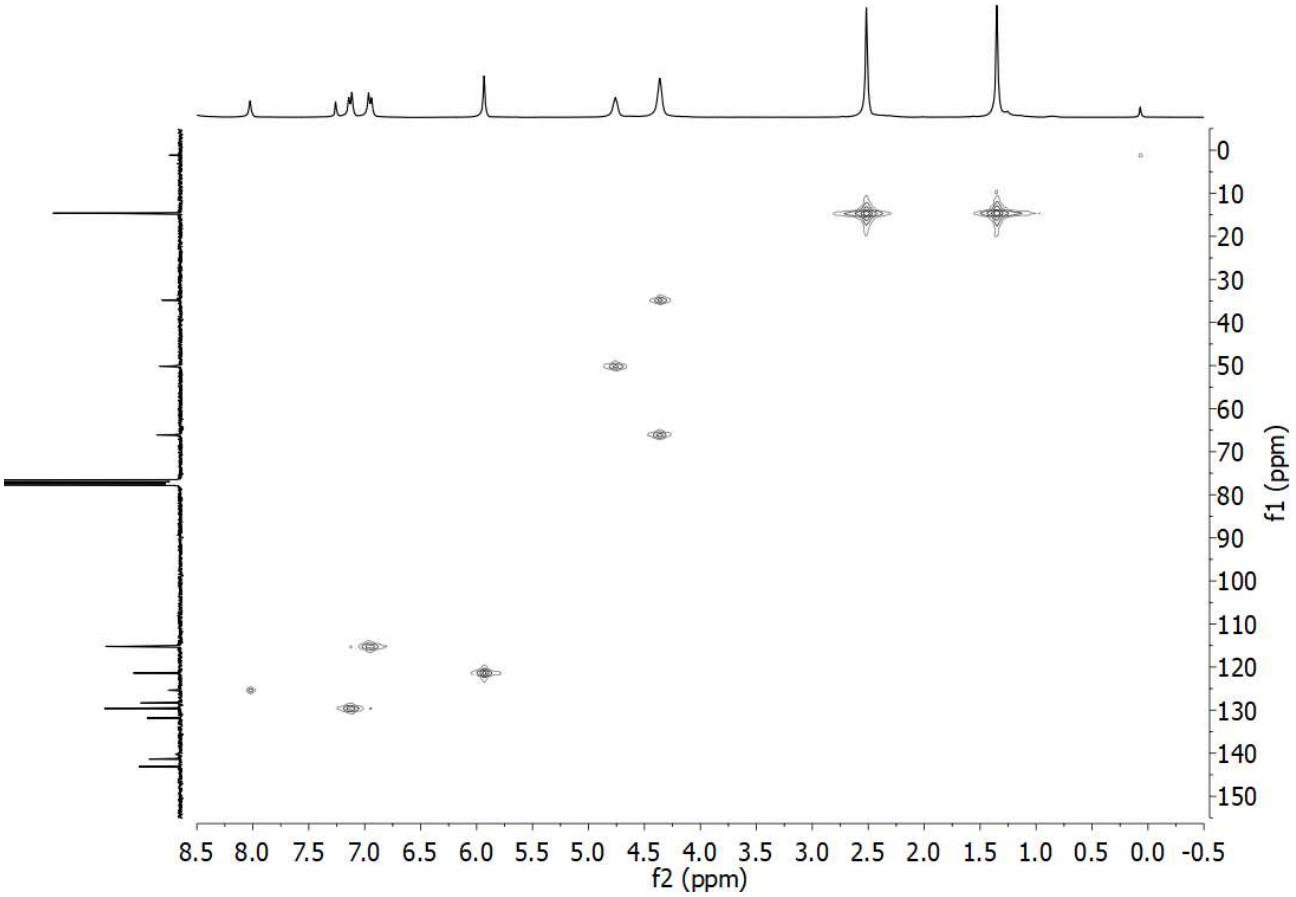


Figure S8: HMQC NMR spectrum of compound **18** in $CDCl_3$.

8-(4-(2-((4-(2-Bromo-*N*-ethylacetamido)methyl)-1*H*-1,2,3-triazol-1-yl)) 1,3,5,7-tetra-methyl-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (20): Under inert atmosphere **18** (23.2 mg, 50.0 µmol, 1.00 eq) and pyridine (4.0 µL, 50.0 µmol, 1.00 eq) were dissolved in degassed DMF (1.50 mL), and placed in an ice bath. Bromoacetic anhydride (40.0 mg, 150 µmmol, 3.00 eq) dissolved in degassed DMF (1.00 mL) was added dropwise. The orange solution was stirred for 3 h at room temperature. The mixture was concentrated under reduced pressure, diluted with EtOAc (25 mL), washed with distilled H_2O (2× 20 mL) and brine (1× 20 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered, concentrated under reduced pressure and purified by flash column chromatography on silica (EtOAc). **20** was obtained as bright orange compound (17.3 mg, 29.6 µmol, 59%). **TLC:** R_f = 0.26 (EtOAc). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 7.81 (s, 1H, $H_{triazole}$) 7.19 (d, ^3J = 8.7 Hz, 2H, 2×H_{arom}), 6.98 (d, ^3J = 8.7 Hz, 2H, 2×H_{arom}), 5.97 (s, 2H, CH_2), 4.80 (t, ^3J = 5.0 Hz, 2H, CH_2), 4.59 (d, ^3J = 5.7 Hz, 2H, CH_2), 4.42 (t, ^3J = 5.0 Hz, 2H, CH_2), 3.87 (s, 2H, CH_2), 2.54 (s, 6H, 2× CH_3), 1.40 (s, 6H, 2× CH_3).ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ =166.0 (1C, *C*O), 158.4 (1C, $C_{q.}$), 155.7 (2C, 2×$C_{q.}$), 144.0 (1C, *C*H$_{triazol}$), 143.1 (2C, 2×$C_{q.}$), 141.3 (1C, $C_{q.}$), 131.9 (2C, 2×$C_{q.}$), 129.7 (2C, 2×C_{arom}), 128.5 (1C, $C_{q.triazole}$), 124.0 (1C, H*C*$_{triazole}$), 121.4 (2C, 2×C_{arom}), 115.3 (2C, 2×C_{arom}), 66.3 (1C, *C*H$_2$), 50.1 (1C, *C*H$_2$), 35.5 (1C, *C*H$_2$-NH), 28.8 (1C, *C*H$_2$Br), 14.8 (2C, 2× CH$_3$), 14.7 (2C, 2× CH$_3$) ppm. **^{19}F-NMR (470 MHz, 300K, $CDCl_3$):** δ =-146.3 (q, 2F, ^1J = 33.0 Hz, BF_2) ppm. **^{11}B-NMR (160 MHz, 300K, $CDCl_3$):** δ = 0.49 (t, 1B, ^1J = 33.0 Hz, *B*F$_2$) ppm. **HRMS-ESI$^+$ (m/z):** calcd for $[M+Na]^+$ $C_{26}H_{28}BBrF_2N_6O_2Na$: 607.1416; found: 607.1419.

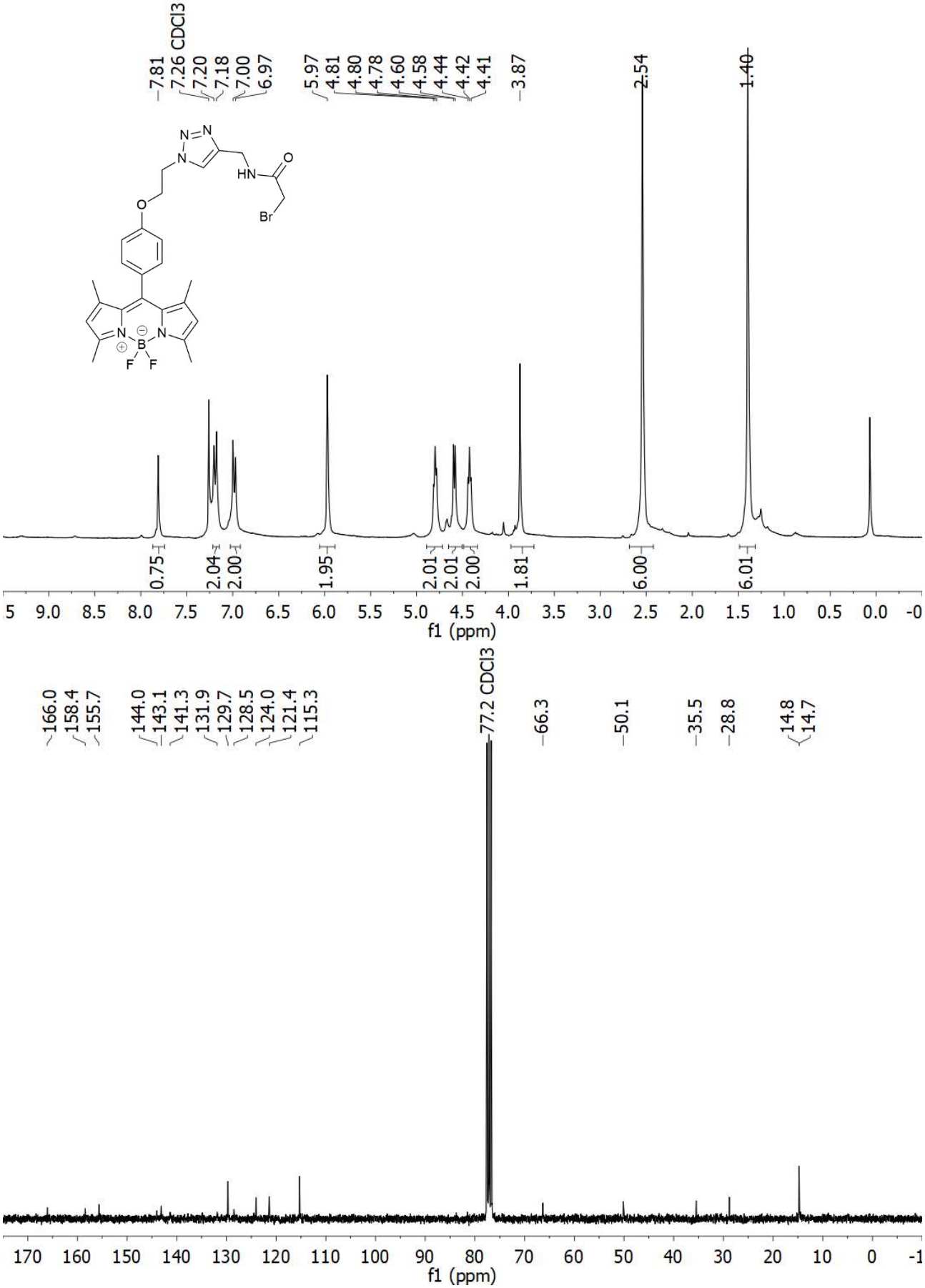


Figure S9: ^{1}H and ^{13}C NMR spectra of compound **20** in $CDCl_3$.

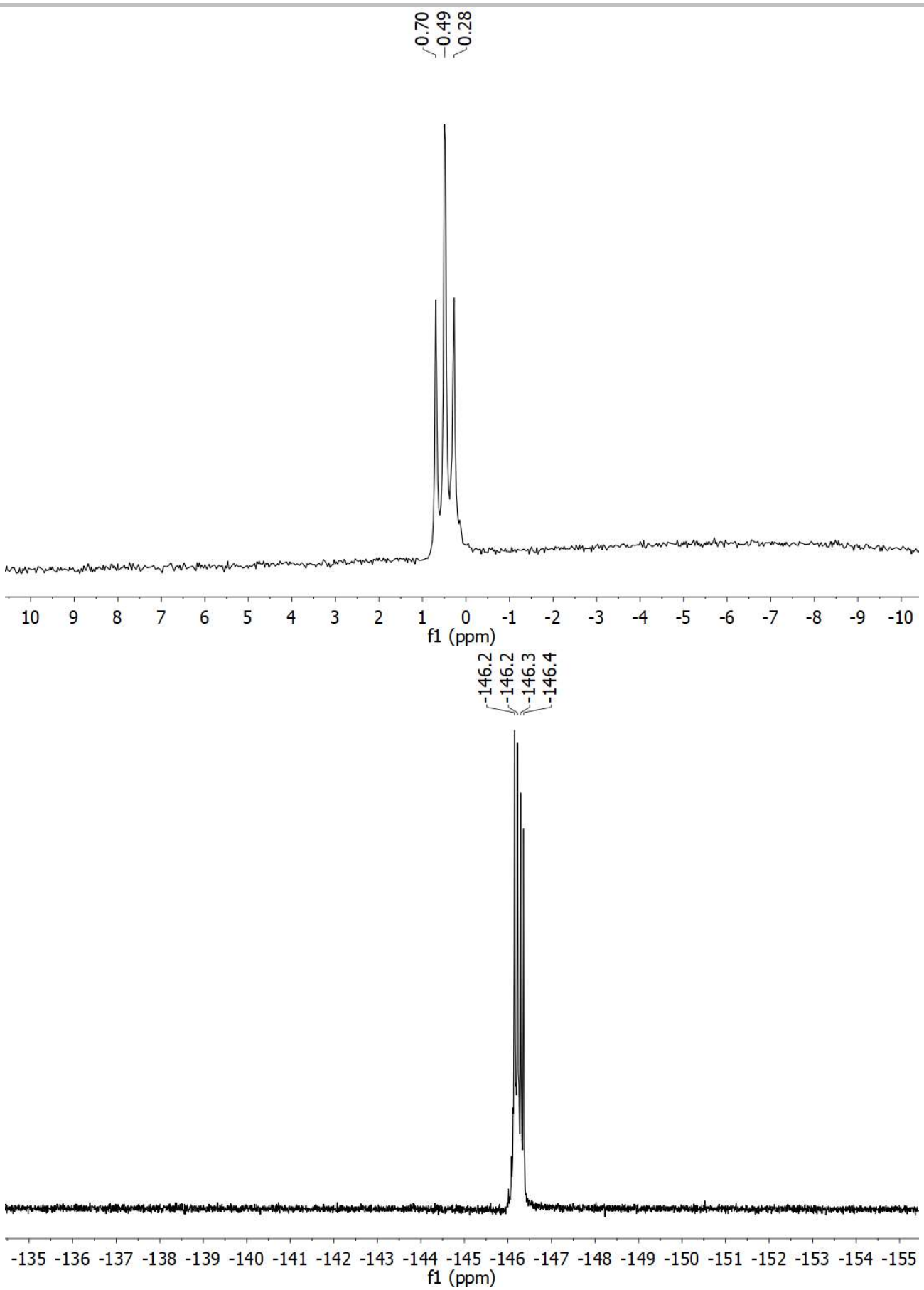


Figure S10: ^{11}B and ^{19}F NMR spectra of compound **20** in $CDCl_3$.

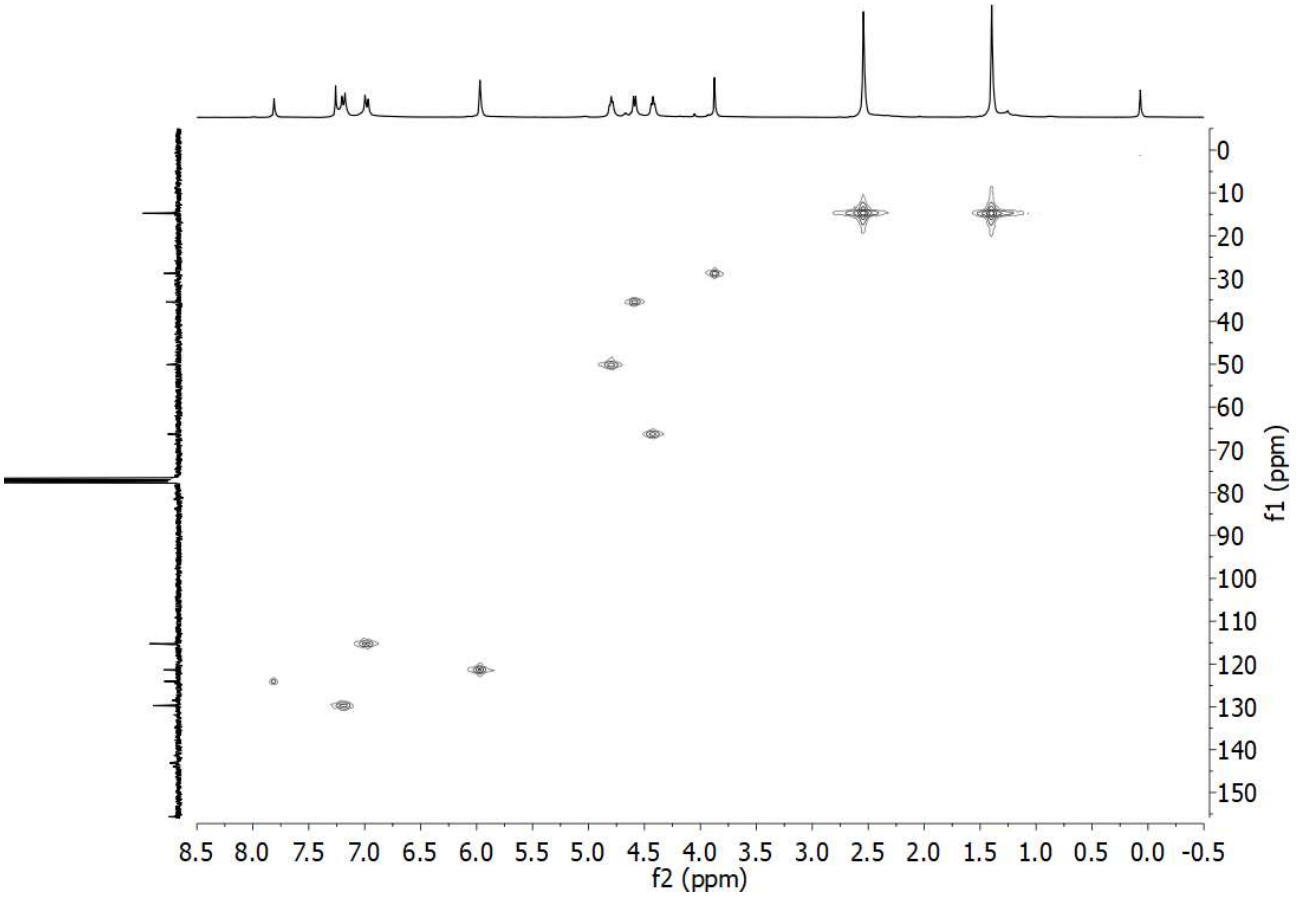


Figure S11: HMQC NMR spectrum of compound **20** in $CDCl_3$.

The synthesis of the iodinated analogues **19** and **9** are in the main text of the manuscript, Figures S12-S17 show the NMR spectra for characterization.

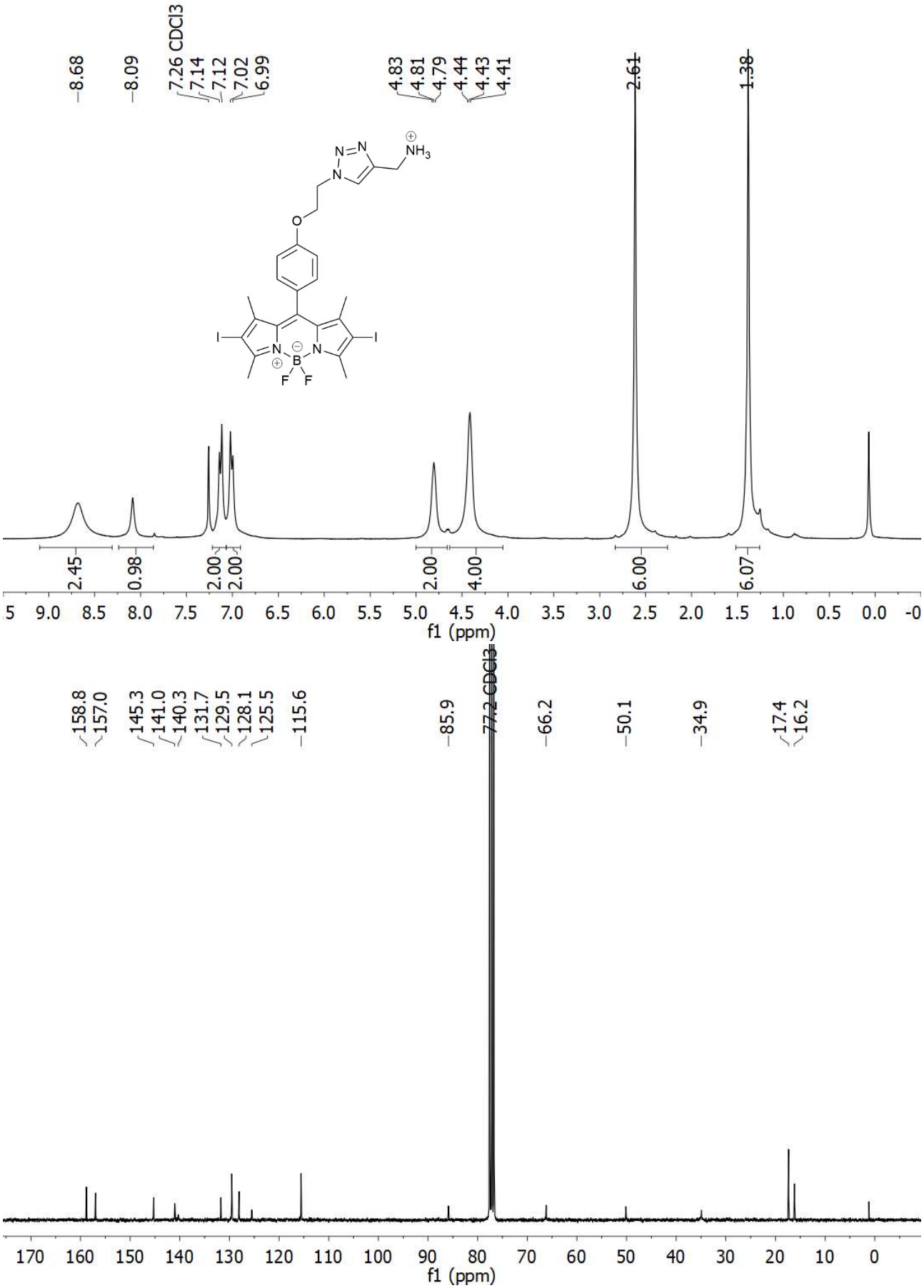


Figure S12: ^{1}H and ^{13}C NMR spectra of compound **19** in $CDCl_3$.

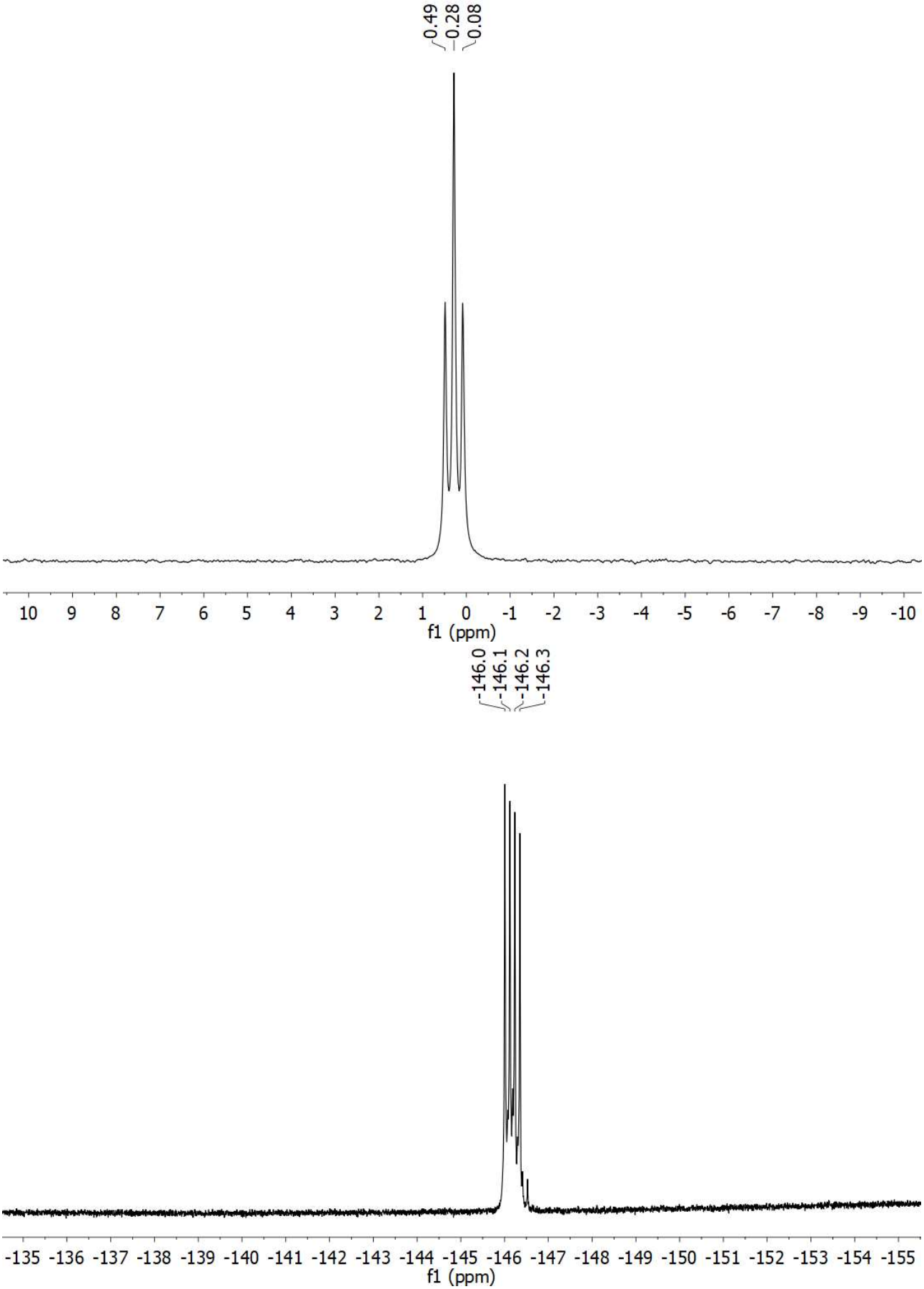


Figure S13: ^{11}B and ^{19}F NMR spectra of compound **19** in $CDCl_3$.

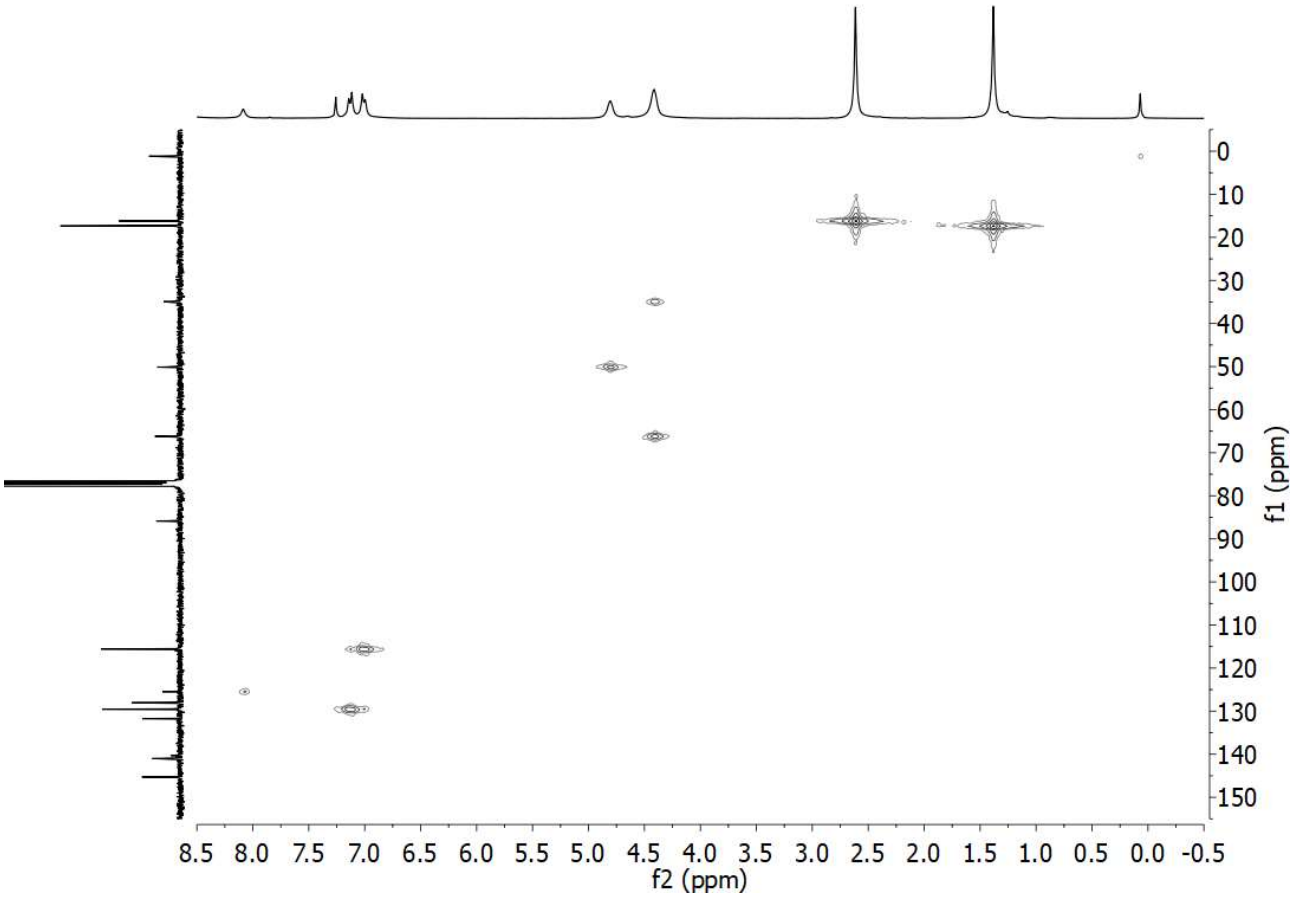


Figure S14: HMQC NMR spectrum of compound **19** in $CDCl_3$.

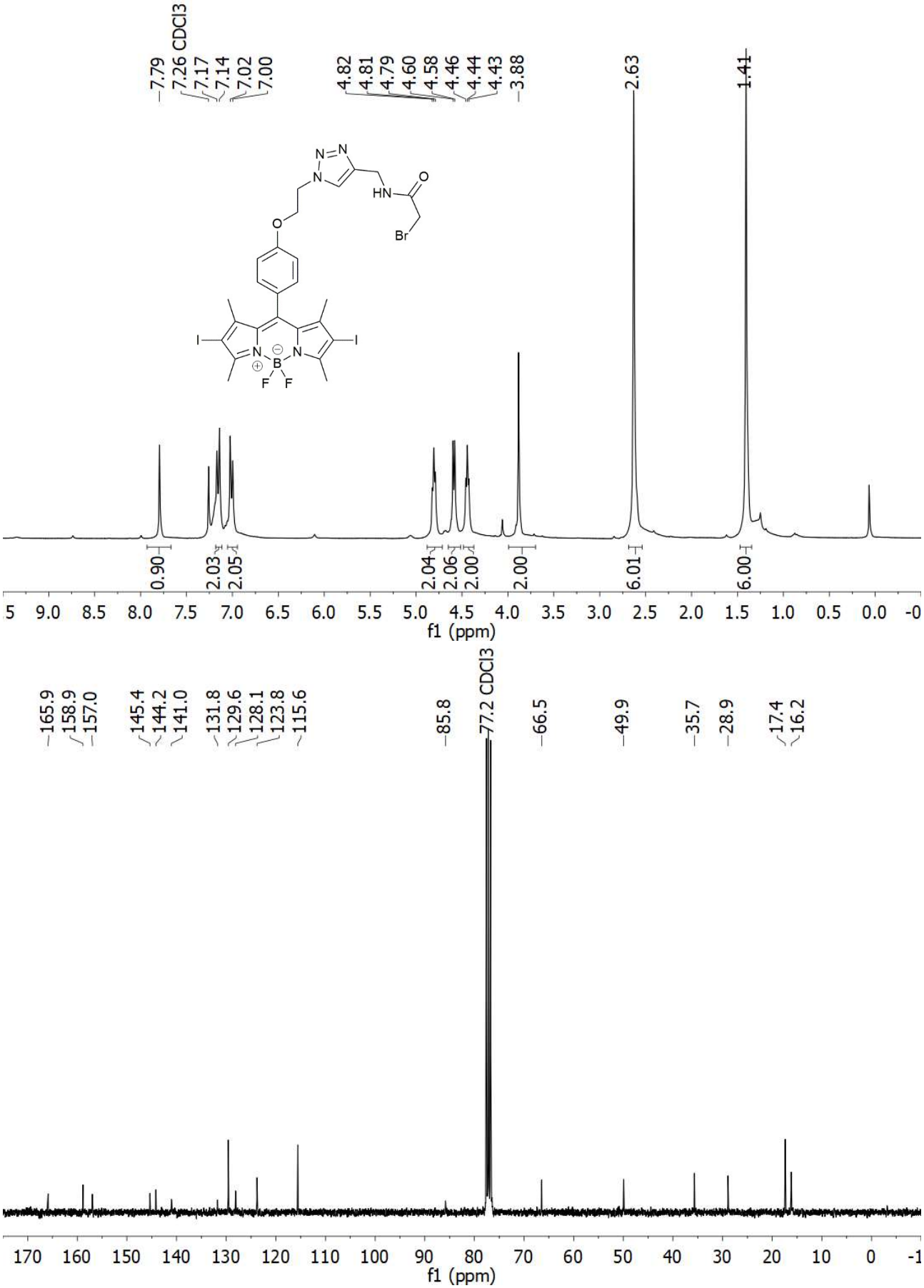


Figure S15: ^{1}H and ^{13}C NMR spectra of compound **9** in $CDCl_3$.

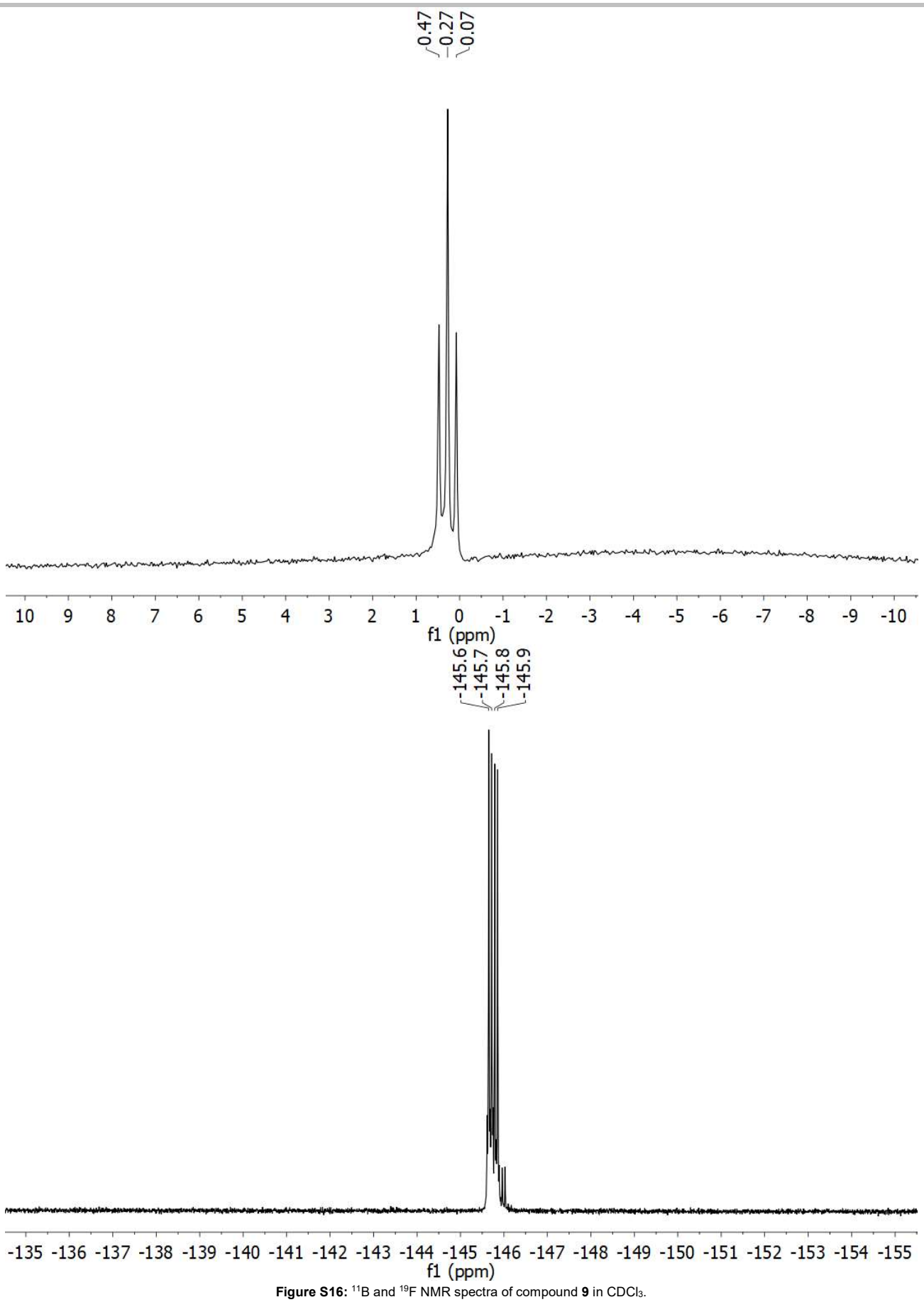


Figure S16: ^{11}B and ^{19}F NMR spectra of compound **9** in $CDCl_3$.

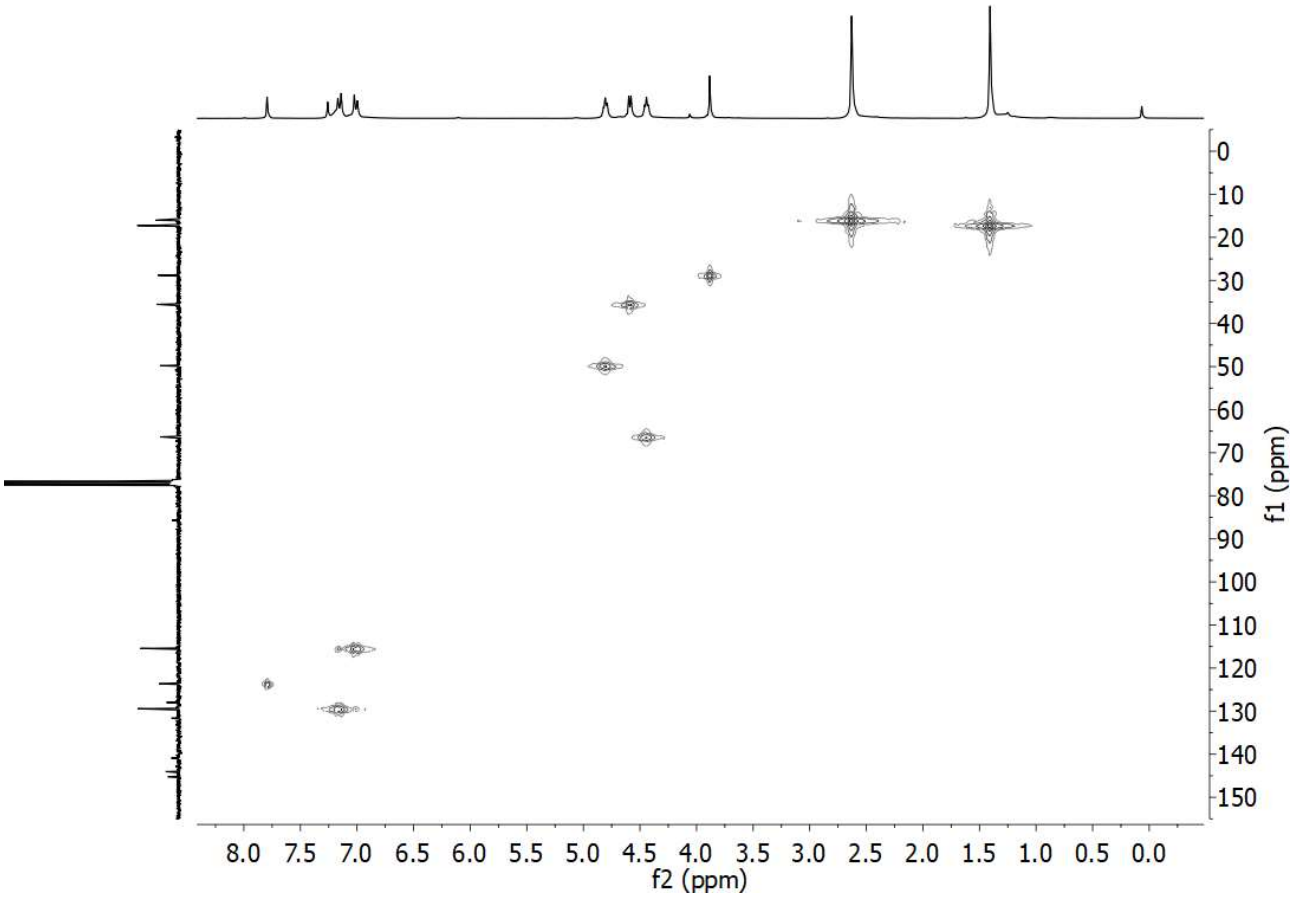
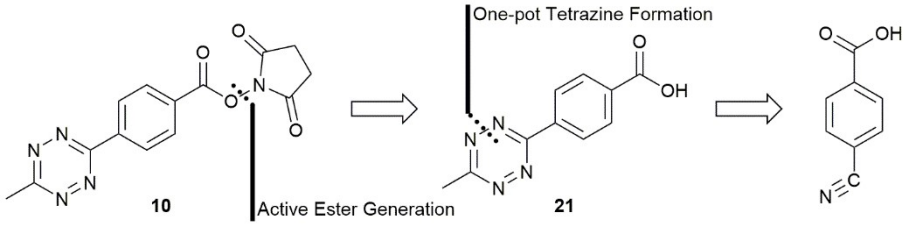


Figure S17: HMQC NMR spectrum of compound **9** in $CDCl_3$.

Tetrazine Synthesis

The tetrazine moiety **21** was synthesized starting from 4-cyanobenzoic acid according to our previous procedure.[9] The active ester was obtained by reaction with NHS in the presence of EDC·HCl and DIPEA.

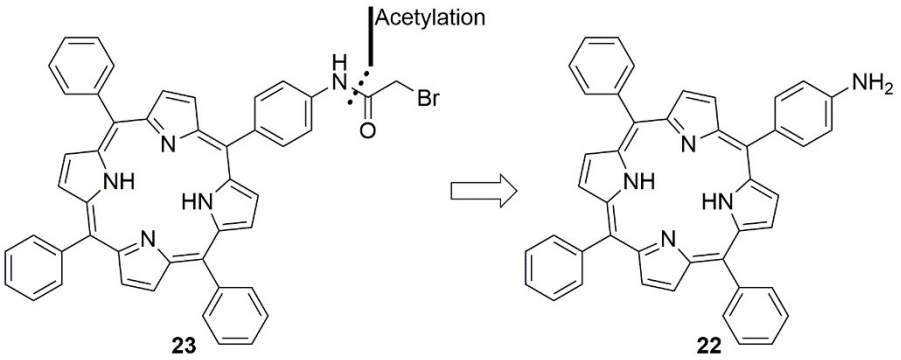


Scheme S3: Retrosynthetic scheme for tetrazine **10**.

2,5-Dioxopyrrolidin-1-yl 4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzoate (10): In a microcentrifuge tube (1.50 mL volume) **21** (30.0 mg, 139 µmol, 1.00 eq) was dissolved in degassed DCM (580 µL) upon DIPEA (118 µL, 694 µmol, 5.00 eq) addition. Subsequently EDC·HCl (106 mg, 555 µmol, 4.00 eq) and NHS (31.9 mg, 278 µmol, 2.00 eq) were added and the dark pink solution was shaken at room temperature for 3 h. The mixture was concentrated under reduced pressure and the crude product was purified by column flash chromatography on silica gel (DCM/MeOH/FA, 100:2:0.5, v/v/v). The product **10** was obtained as pink solid (38.6 mg, 117 µmol, 85%). The characterization is in agreement with the literature ref.[10] **TLC:** R_f = 0.18 (DCM). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 8.70 (d, ^3J = 8.4 Hz, 2H, 2×H_{arom}), 8.29 (d, ^3J = 8.4 Hz, 2H, 2×H_{arom}), 3.08 (s, 3H, CH_3), 2.88 (s, 4H, 2×CH_2) ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ =168.9 (2C, 2×*C*O), 167.9 (1C, $C_{q.}$), 163.3 (1C, $C_{q.}$), 161.3 (1C, *C*OO), 137.6 (1C, $C_{q.}$), 131.3 (2C, 2×C_{arom}), 128.6 (1C, $C_{q.}$), 128.1 (2C, 2×C_{arom}), 25.7 (2C, 2×CH_2), 21.3 (1C, CH_3) ppm. **HRMS-ESI$^+$ (m/z):** calcd for $[M+Na]^+$ $C_{14}H_{11}N_5ONa$: 336.0703, found: 336.0706.

Tetraphenylporphyrin Synthesis

The tetraphenylporphyrin (TPP) amine **22** photosensitizer was prepared according to our previous described procedure,[9] and the bromoacyl group was introduced following a procedure by the Másson lab.[11]



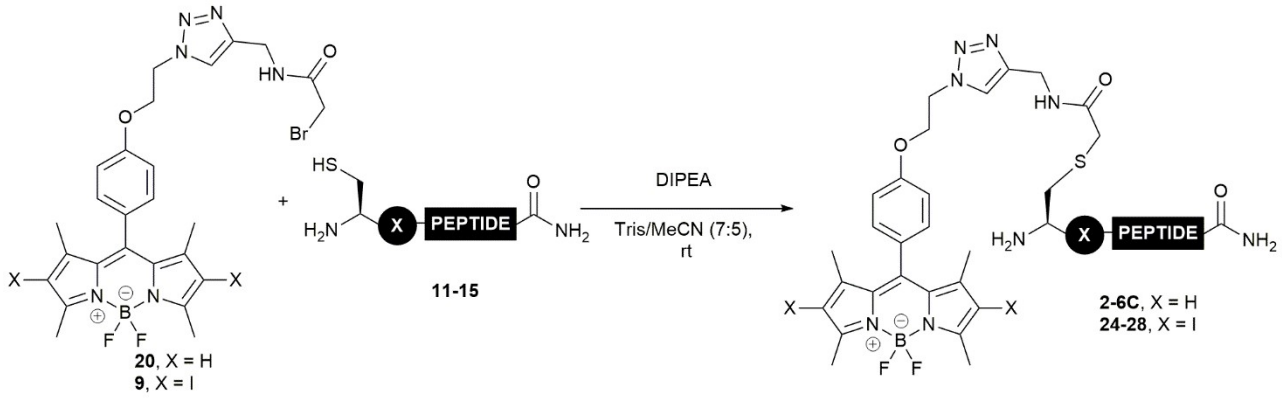
Scheme S4: Retrosynthetic scheme for the molecule **23**.

5-(4α-Bromoacetylamidophenyl)-10,15,20-triphenylporphyrin (23): In a microcentrifuge tube (1.50 mL volume) **22** (13.2 mg, 20.0 µmol, 1.00 eq) was dissolved in 400 µL degassed DCM and triethylamine (6.1 µL, 44.0 µmol, 2.20 eq) was added. After addition of bromoacetylbromide (2.5 µL, 28.0 µmol, 1.40 eq) the dark solution was shaken at room temperature for 1 h. Full conversion was confirmed by tlc analysis and the mixture was concentrated under reduced pressure. The crude product was purified by column flash chromatography on silica gel (DCM). The product **23** was obtained as purple solid (9.5 mg, 12.7 µmol, 63%). The characterization is in agreement with the literature ref.[11] **TLC:** R_f = 0.30 (DCM). **^{1}H-NMR (300 MHz, 300 K, $CDCl_3$):** δ = 8.85 (s, 8H; C$H_{pyrrole}$), 8.46 (bs, 1H, N*H*CO), 8.24-8.21 (m, 8H, CH_{phenyl}), 7.93 (d, 2H, ^3J=8.5 Hz, CH_{phenyl}), 7.77-7.75 (m,9, CH_{phenyl}), 4.19 (s, 2H, CH_2), -2.76 (bs, 2H, N$H_{pyrrole}$) ppm. **^{13}C-NMR (75 MHz, 300K, $CDCl_3$):** δ =163.8 (1C, *C*O), 142.3 (8C, $C_{pyrrole}$), 139.3 (4C, C_{phenyl}), 136.8 (1C, C_{phenyl}), 135.3 (6C, *C*H$_{phenyl}$),134.7 (2C, *C*H$_{phenyl}$), 131.2 (8C, *C*H$_{pyrrole}$), 127.9 (3C, *C*H$_{phenyl}$), 126.6 (6C, *C*H$_{phenyl}$), 120.4 (4C, C_{arom}), 118.3 (2C, *C*H$_{phenyl}$), 29.6 (1C, *C*H$_2$) ppm. **HRMS-ESI$^+$ (m/z):** calcd for $[M+H]^+$ $C_{46}H_{33}BrN_5O$: 750.1863, found: 750.1844.

Conjugation Reactions

The conjugation reactions were performed in microcentrifuge tubes (1.50 mL volume, Sarstedt) minimizing exposure to oxygen. Solvents were degassed by N_2 passing through for 30 min. Reactions were monitored by RP-HPLC-MS analysis.

General Procedure for the BODIPY-Peptide Synthesis:



Scheme S5: General reactionscheme for the synthesis of fluorescent BODIPY-peptides **2-6FL** and 2I-BODIPY peptides **24-28**.

To a 0.12 M solution of the corresponding peptide (1.20 eq) in Tris buffer (0.1 M, pH= 8.5), DIPEA (2.20 eq) was added. Afterwards either the 2-bromoacyl photosensitizer **9** or fluorescent dye **20** (1.00 eq, 0.10 M in MeCN) was added. The reaction was mixed on an Eppendorf Thermoshaker and followed by analytical RP-HPLC-MS until full conversion of the BODIPY starting material was observed. The compound was purified by semipreparative RP-HPLC using gradient B for compounds **2-5FL**, **24-27** and gradient C for compounds **6FL** and **28**. For compounds **6FL** and **28** the reaction was performed in DMSO as solvent (due to micelle formation of peptide **15** in the

Tris buffer/MeCN solvent mixture), further the reaction mixture was diluted with ultrapure H_2O/MeCN (7:3) to have a DMSO concentration ≤5% before semipreparative RP-HPLC purification.

H_2N-C(BODIPY)-RRRRR-$CONH_2$ (**2FL**): obtained from peptide **11** and compound **20**; reaction time: 2 h; orange-yellow solid; yield: 83%; t_R = 21.85 min (gradient 2). Chemical formula: $C_{59}H_{95}BF_2N_{28}O_8S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 703.3911; found: 703.3904.

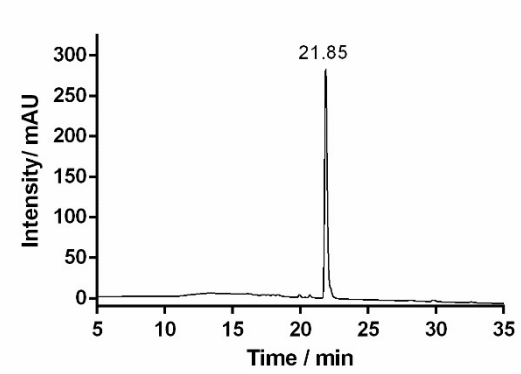


Figure S18: RP-HPLC chromatogram of the purified compound **2FL**, detected at 220 nm.

H_2N-C(BODIPY)-RRRRRRRR-$CONH_2$ (**3FL**): obtained from peptide **12** and compound **20;** reaction time: 6 h; orange-yellow solid; yield: 56%; t_R= 21.23 min (gradient 2). Chemical formula: $C_{77}H_{131}BF_2N_{40}O_{11}S$. HRMS-ESI$^+$ (m/z): $[M+3H]^{3+}$ calcd: 625.3644; found: 625.3639.

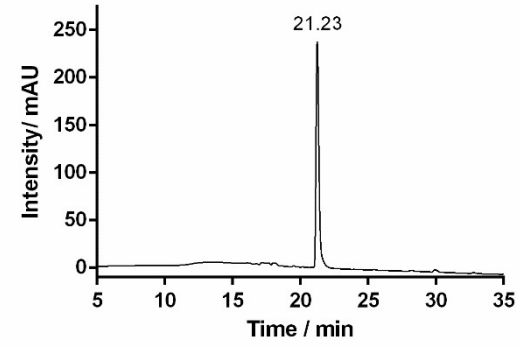


Figure S19: RP-HPLC chromatogram of the purified compound **3FL**, detected at 220 nm.

H_2N-C(BODIPY)-$F_xrF_xrF_xr$-$CONH_2$ (**4FL**): obtained from peptide **13** and compound **20**; reaction time: 3 h; orange-yellow solid; yield: 50%. t_R= 26.01 min (gradient 2). Chemical formula: $C_{74}H_{116}BF_2N_{23}O_9S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 776.9633; found: 776,9659.

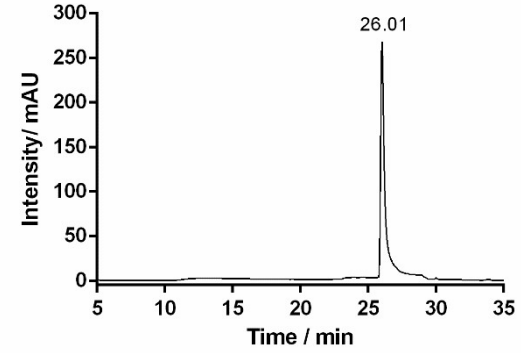


Figure S20: RP-HPLC chromatogram of the purified compound **4FL**, detected at 220 nm.

H_2N-C(BODIPY)-rF_xrF_xrF_xr-$CONH_2$ (**5FL**): obtained from peptide **14** and compound **20**; reaction time: 3.5 h; orange-yellow solid; yield: 38%. t_R= 24.55 min (gradient 2). Chemical formula: $C_{80}H_{128}BF_2N_{27}O_{10}S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 855.0139; found: 855.0170.

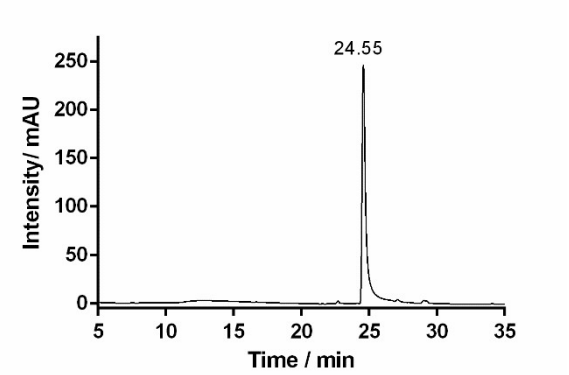


Figure S21: RP-HPLC chromatogram of the purified compound **5FL**, detected at 220 nm.

H_2N-C(BODIPY)-K(PA)RRRR-$CONH_2$ (**6FL**): obtained from peptide **15** and compound **20**; reaction time: 3 h; orange-yellow solid; yield: 47%. t_R= 25.22 min (gradient 3). Chemical formula: $C_{75}H_{125}BF_2N_{26}O_9S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 808.5031; found: 808.5064.

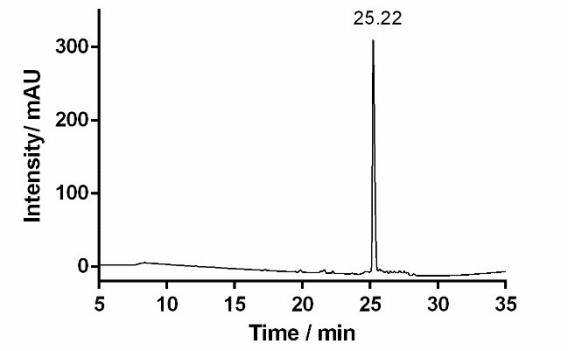


Figure S22: RP-HPLC chromatogram of the purified compound **6FL**, detected at 220 nm.

H_2N-C(2I-BODIPY)-RRRRR-$CONH_2$ (**24**): obtained from peptide **11** and compound **9**; reaction time: 4 h; pink solid; yield: 63%; t_R= 22.78 min (gradient 2). Chemical formula: $C_{59}H_{93}BF_2I_2N_{28}O_8S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 829.2879; found: 829.2888.

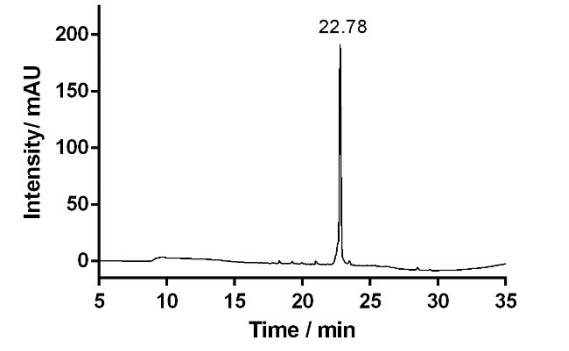


Figure S23: RP-HPLC chromatogram of the purified compound **24**, detected at 220 nm.

H_2N-C(2I-BODIPY)-RRRRRRRR-$CONH_2$ (**25**): starting from peptide **12** and compound **9**; reaction time: 3 h; pink solid; yield: 48%; t_R= 21.45 min (gradient 2). Chemical formula: $C_{77}H_{129}BF_2I_2N_{40}O_{11}S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 1063.4397; found:1063.4401.

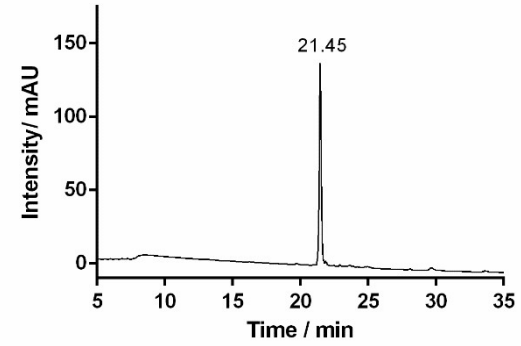


Figure S24: RP-HPLC chromatogram of purified compound **25**, detected at 220 nm.

H_2N-C(2I-BODIPY)-$F_xrF_xrF_xrF_x$r-$CONH_2$ (**26**): obtained from peptide **13** and compound **9**; reaction time: 3 h; pink solid; yield: 32%. t_R= 25.99 min (gradient 2). Chemical formula: $C_{74}H_{114}BF_2I_2N_{23}O_9S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 902.8599; found: 902.8635.

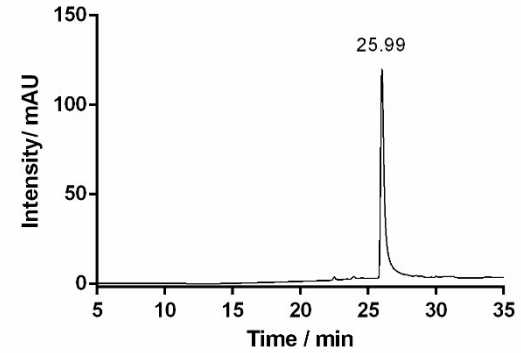


Figure 25: RP-HPLC chromatogram of purified compound **26**, detected at 220 nm.

H_2N-C(2I-BODIPY)-r$F_xrF_xrF_xrF_x$r-$CONH_2$ (**27**): obtained from peptide **14** and compound **9**; reaction time: 4 h; pink solid; yield: 40%. t_R= 24.55 min (gradient 2). Chemical formula: $C_{80}H_{126}BF_2I_2N_{27}O_{10}S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 980.9105; found: 980.9144.

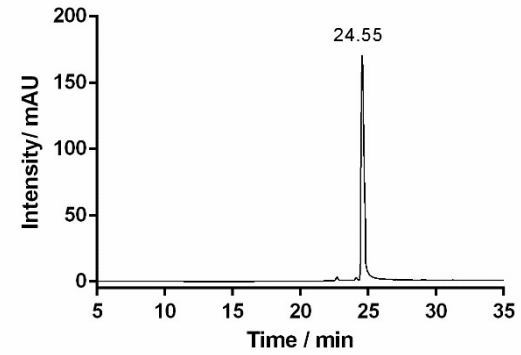


Figure S26: RP-HPLC chromatogram of purified compound **27**, detected at 220 nm.

H_2N-C(2I-BODIPY)-K(PA)RRRR-$CONH_2$ (**28**): obtained from peptide **15** and compound **9**; reaction time: 3 h; pink solid; yield: 38%. t_R= 25.81 min (gradient 3). Chemical formula: $C_{7t5}H_{123}BF_2I_2N_{26}O_9S$. HRMS-ESI$^+$ (m/z): $[M+2H]^{2+}$ calcd: 934.3998; found:934.4027.

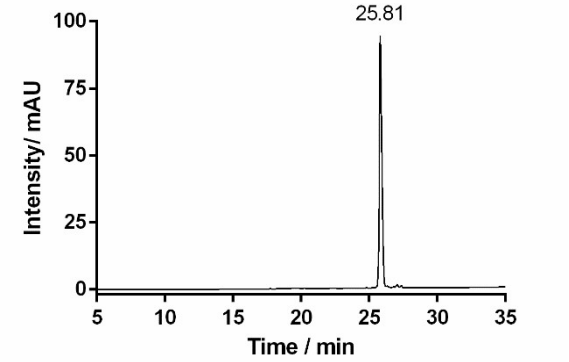


Figure S27: RP-HPLC chromatogram of purified compound **28**, detected at 220 nm.

Chromatograms of the Synthesized Turn-Off Photosensitizers

Figures S28-S32 show the analytical RP-HPLC chromatograms of the synthesized turn-off photosensitizers **2-6OFF**.

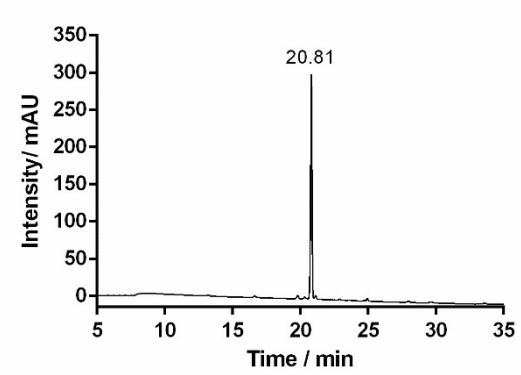


Figure S28: RP-HPLC chromatogram of purified turn-off photosensitizer Tz-C(2I-BODIPY)-RRRRR-$CONH_2$ (**2OFF**), detected at 220 nm (gradient 2).

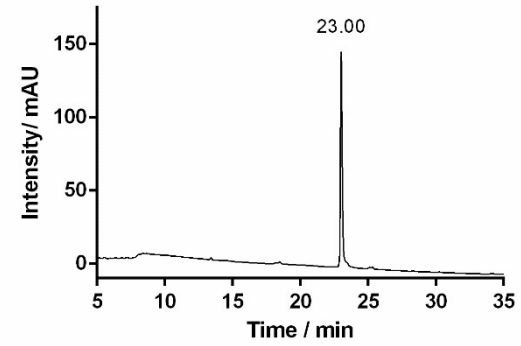


Figure S29: RP-HPLC chromatogram of purified turn-off photosensitizer Tz-C(2I-BODIPY)-RRRRRRRR-$CONH_2$ (**3OFF**), detected at 220 nm (gradient 2).

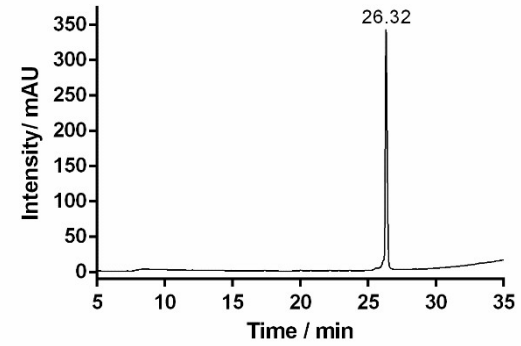


Figure S30: RP-HPLC chromatogram of purified turn-off photosensitizer Tz-C(2I-BODIPY)-$F_xrF_xrF_xr$-$CONH_2$ (**4OFF**), detected at 220 nm (gradient 3).

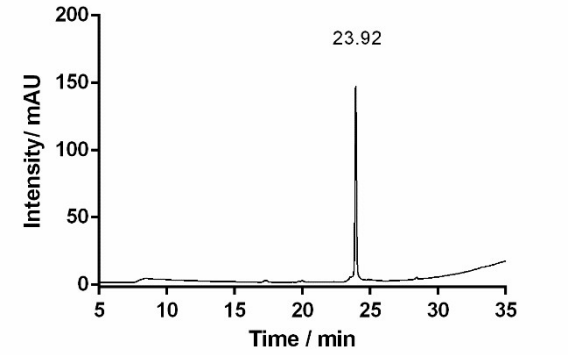


Figure S31: RP-HPLC chromatogram of purified turn-off photosensitizer Tz-C(2I-BODIPY)-rF$_x$rF$_x$rF$_x$r-CONH$_2$ (**5OFF**), detected at 220 nm (gradient 3).

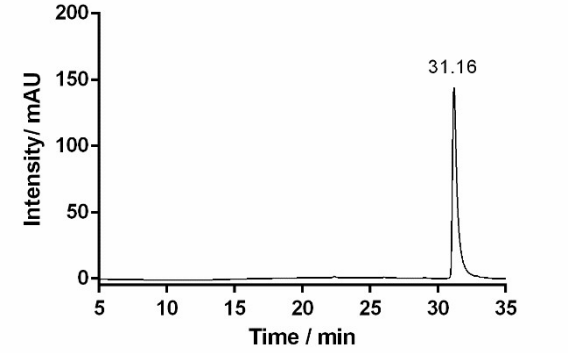


Figure 32: RP-HPLC chromatogram of purified turn-off photosensitizer Tz-C(2I-BODIPY)-K(PA)RRRR-CONH$_2$ (**6OFF**), detected at 220 nm (gradient 3).

Chromatograms for the Synthesized Turn-On Photosensitizers

Figures S33-S37 show the analytical RP-HPLC chromatograms of the synthesized turn-on photosensitizers **2-6ON**, different isomers could be detected.

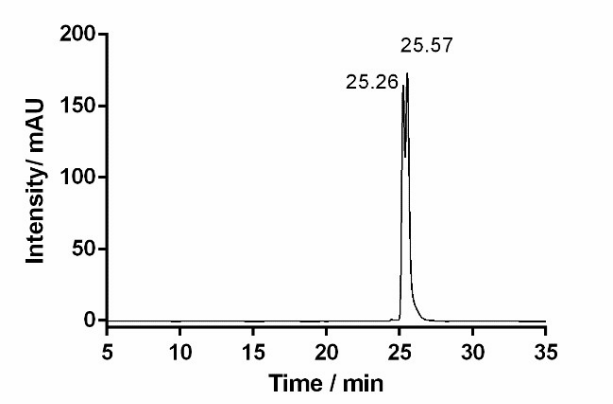


Figure S33: RP-HPLC chromatogram of purified turn-on photosensitizer [TCO:Tz]-C(2I-BODIPY)-RRRRR-CONH$_2$ (**2ON**), detected at 220 nm (gradient 2).

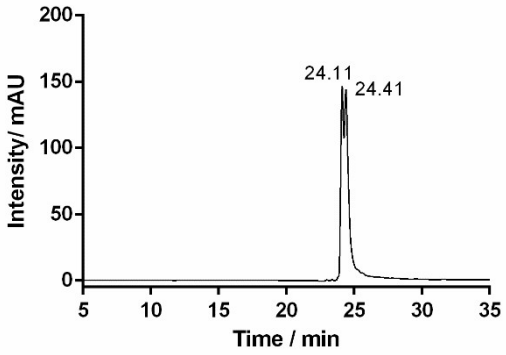


Figure S34: RP-HPLC chromatogram of purified turn-on photosensitizer [TCO:Tz]-C(2I-BODIPY)-RRRRRRRR-$CONH_2$ (**3ON**), detected at 220 nm (gradient 2).

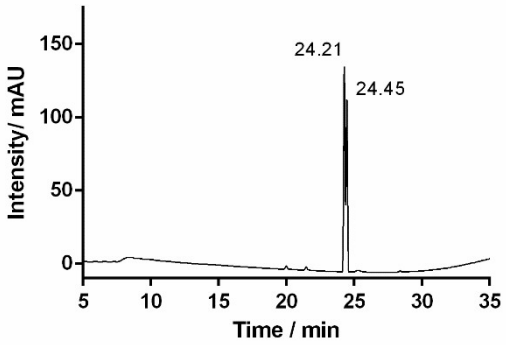


Figure S35: RP-HPLC chromatogram of purified turn-on photosensitizer [TCO:Tz]-C(2I-BODIPY)-$F_xrF_xrF_xr$-$CONH_2$ (**4ON**), detected at 220 nm (gradient 3).

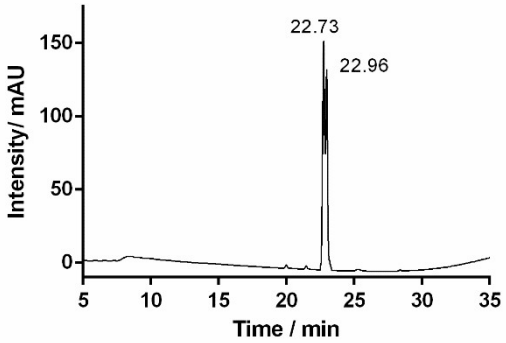


Figure S36: RP-HPLC chromatogram of purified turn-on photosensitizer [TCO:Tz]-C(2I-BODIPY)-$rF_xrF_xrF_xr$-$CONH_2$ (**5ON**), detected at 220 nm (gradient 3).

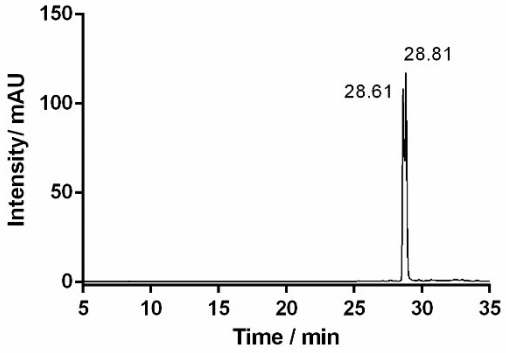
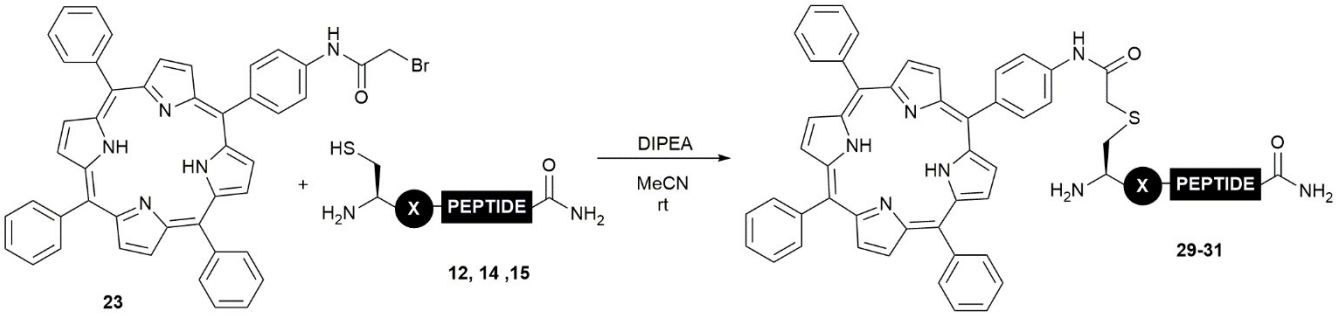


Figure S37: RP-HPLC chromatogram of purified turn-on photosensitizer [TCO:Tz]-C(2I-BODIPY)-K(PA)RRRR-$CONH_2$ (**6ON**), detected at 220 nm (gradient 3).

General Procedure for the TPP-Peptide Synthesis:

To a 0.12 M solution of the corresponding peptide (1.20 eq) in MeCN, DIPEA (2.20 eq) was added. Afterwards the 2-bromoacyl photosensitizer **23** (1.00 eq, 0.10 M in MeCN) was added. The reaction was mixed on an Eppendorf Thermoshaker and followed by analytical RP-HPLC-MS until full conversion of the TPP starting material was observed. The compound was purified by semipreparative RP-HPLC using gradient C. For conjugation to peptide **15** the reaction was performed in DMSO as solvent, further the reaction mixture was diluted with ultrapure H_2O/MeCN (7:3) to have a DMSO concentration ≤5% before semipreparative RP-HPLC purification.



Scheme S6: General reactionscheme for the synthesis of TPP-peptides **29-31**.

H_2N-C(TPP)-RRRRRRRR-$CONH_2$ (**29**): obtained from peptide **12** and compound **23**; reaction time: 3 h; brown solid; yield: 63%. t_R= 23.44 min (gradient 3). Chemical formula: $C_{97}H_{135}N_{39}O_{10}S$. HRMS-ESI$^+$ (m/z): $[M+5H]^{5+}$ calcd: 408.8301; found: 408.8273.

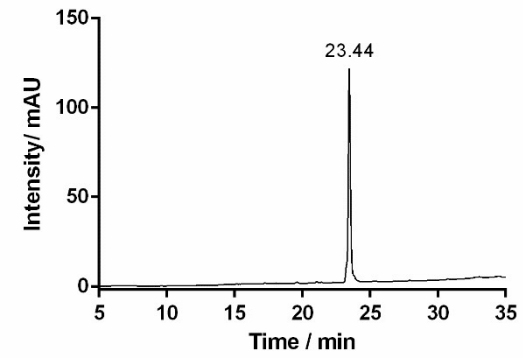


Figure S38: RP-HPLC chromatogram of purified H_2N-C(TPP)-RRRRRRRR-$CONH_2$ (**29**), detected at 220 nm (gradient 3).

H_2N-C(TPP)-rF_xrF_xrF_xr-$CONH_2$ (**30**): obtained from peptide **14** and compound **23**; reaction time: 3 h; brown solid; yield: 55%. t_R= 26.73 min (gradient 3). Chemical formula: $C_{100}H_{132}N_{26}O_9S$. HRMS-ESI$^+$ (m/z): $[M+4H]^{4+}$ calcd: 469.5177; found: 469.5194.

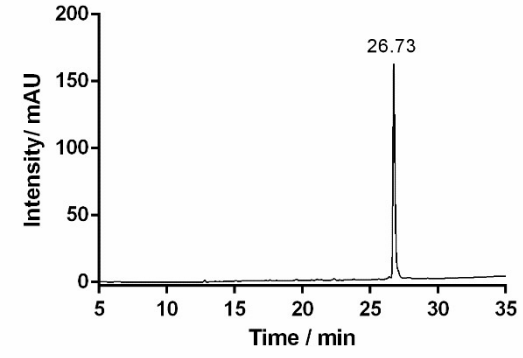


Figure S39: RP-HPLC chromatogram of purified H_2N-C(TPP)-rF_xrF_xrF_xr-$CONH_2$ (**30**) detected at 220 nm (gradient 3).

H_2N-C(TPP)-K(PA)RRRR-$CONH_2$ (**31**): obtained from peptide **15** and compound **23**; reaction time: 3 h; brown solid; yield: 48%. t_R= 33.74 min (gradient 3). Chemical formula: $C_{95}H_{129}N_{25}O_8S$. HRMS-ESI$^+$ (m/z): $[M+4H]^{4+}$ calcd: 46.2624; found: 466,2639.

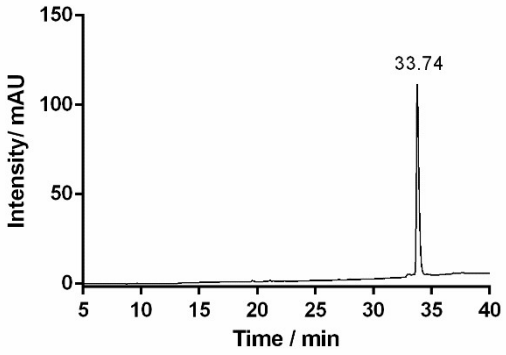


Figure S40: RP-HPLC chromatogram of purified H_2N-C(TPP)-K(PA)RRRR-$CONH_2$ (**31**) detected at 220 nm (gradient 3).

Results and Discussion

UV-vis Measurements

Molar Extinction Coefficients

For the compounds **7** and **8** the extinction coefficients were measured in acetonitrile. Linear regression of the obtained absorbance measurements versus the concentration of the corresponding compound gave the molar extinction coefficient and regression value (R^2). All determinations of each compound were repeated three times independently i.e. from three different stock solutions. For all values, the corresponding background signal was subtracted.

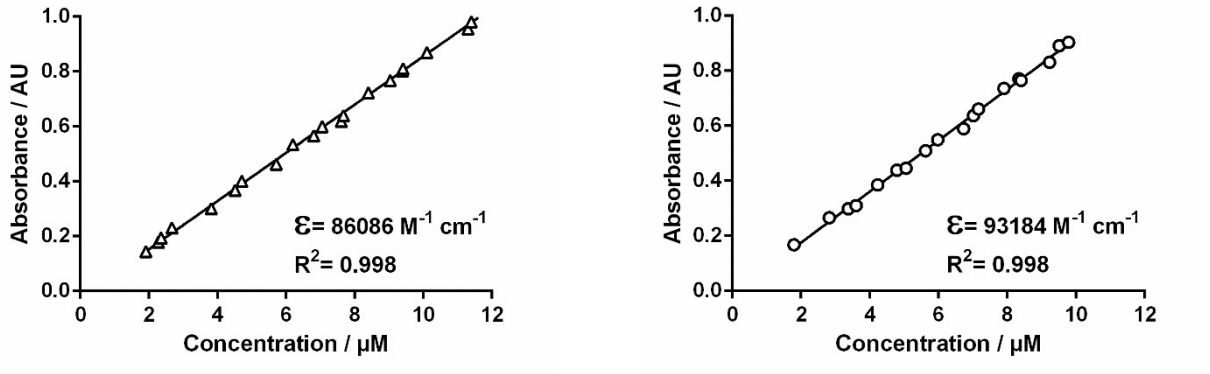


Figure S41: Determination of the extinction coefficient of compounds **7** (triangles, left) and **8** (circles, right).

Table S1 summarizes the obtained extinction coefficients in MeCN.

Table S1: Extinction coefficients obtained in MeCN for BODIPYs **7** and **8**.

compound	λ_{abs} [nm]	ε [M^{-1} cm^{-1}]
7	496	86086
8	528	93184

For the fluorescent probes **2-6FL,** which neither have the Tz moiety, nor iodine substituents, we assumed the same extinction coefficient as the one of compound **7** : ε =86086 M^{-1} cm^{-1} in MeCN at 496 nm.

For the turn-off probes **2-6OFF**, which bear both the Tz and the 2I-BODIPY moiety, we assumed the same extinction coefficient as the one of compound **1OFF**: ε = 78636 M^{-1} cm^{-1} in MeCN, at 528 nm.

For the turn-on probes **2-6ON,** which bear both the pyrazine and the 2I-BODIPY moiety, we assumed the same extinction coefficient as the one of compound **1ON**: ε = 63956 M^{-1} cm^{-1} in MeCN, at 528 nm.[9]

For TPP probes **29-31**, which bear the TPP moiety, we assumed the same extinction coefficient as the one of TPP: ε = 14084 M^{-1} cm^{-1} in DMSO, at 516 nm.[12]

Fluorescence Measurements

To evaluate if the fluorescence emission of fluorescent BODIPY **8** is pH dependent fluorescence emission spectra at 1 μM concentration in a solution containing Dulbecco's phosphate buffered saline (DPBS) at physiological pH 7.4 or containing acidic citrate phosphate buffer (CPB) at pH 5.0 mimicking the lysosomal environment.[13] were recorded.

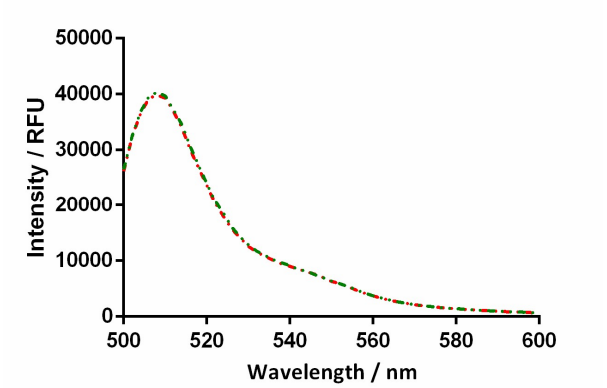


Figure S42: Fluorescence emission spectra of 1 μM solutions of **8** in MeCN/DPBS (pH 7.4, 1:1, v/v, green dotted line) and MeCN/CPB (pH 5.0, 1:1, v/v, red dotted line).

Singlet Oxygen Measurements

Figure S43 shows the DPBF consumption upon irradiation due to $^1O_2$ generation of pairs **2OFF/ON** and **4OFF/ON**; additional compounds to these shown in Figure 2 of the manuscript.

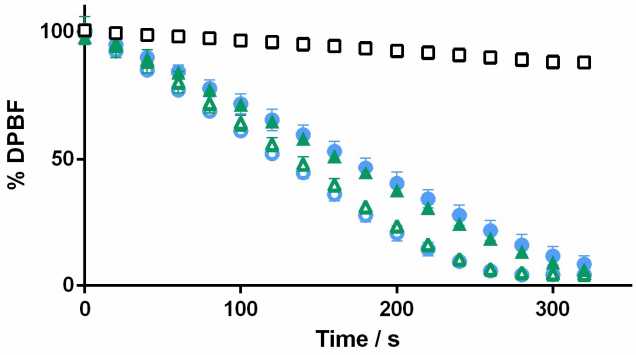


Figure S43: Consumption of DPBF in MeCN (200 µM) over time due to $^1O_2$ generation in the presence of 0.1 µM of **2OFF** (light blue filled circles); **2ON** (light blue clear circles); **4OFF** (green filled triangles) and **4ON** (green clear triangles), as well as DPBF control (white squares).

Further we evaluated if the $^1O_2$ production ability of the employed BODIPY photosensitizer **8** is pH dependent and performed the experiment shown in Figure 2 of the main manuscript and Figure S43 additionally in systems containing DPBS at physiological pH 7.4 or containing CPB at pH 5.0.

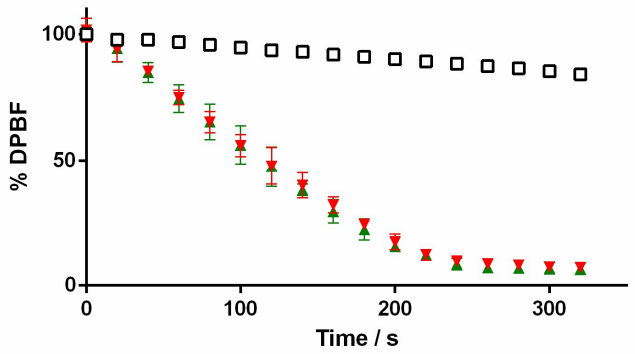


Figure S44: Consumption of DPBF (200 µM) in MeCN/DPBS (pH 7.4, 1:1, v/v, green triangles) and MeCN/CPB (pH 5.0, 1:1, v/v, red triangles) over time due to $^1O_2$ generation in the presence of 0.1 µM of **8.** DPBF alone (white squares).

For the determination of the reaction constants of $^1O_2$ production (*k*), a linear plot of ln[DPBF] against the irradiation time was employed, giving the rate constant as reciprocal of the slope. The decrease of DPBF absorbance by $^1O_2$ follows strictly first order kinetics.[14] The slope i.e. the DPBF consumption rate, was determined using GraphPad Prism 6 software; the values are in the main manuscript (Table1)

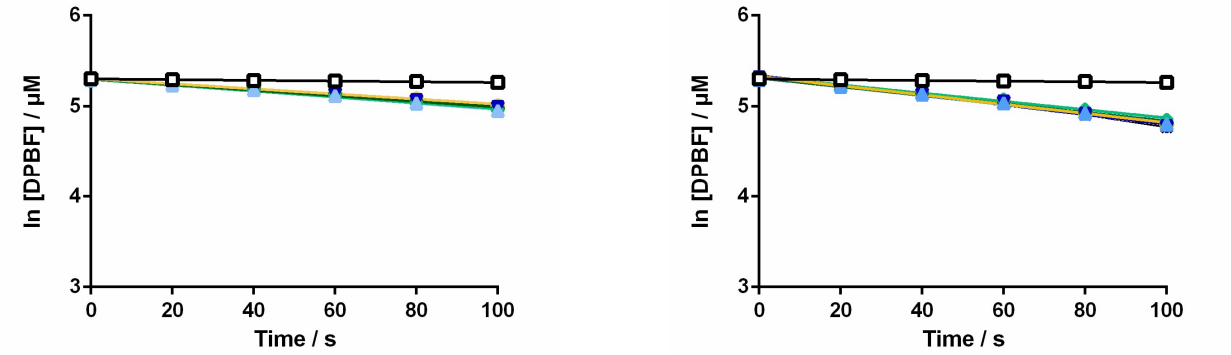


Figure S45: Plots of compounds **2-6OFF** and DPBF (left; **2OFF** light blue triangles pointing up, **3OFF** blue triangles pointing down, **4OFF** light green diamonds, **5OFF** dark green hexagons, **6OFF** yellow circles and DPBF white squares) and **2-6ON**, **8** and DPBF (right; **2ON** light blue triangles pointing up, **3ON** blue triangles pointing down, **4ON** light green diamonds, **5ON** dark green hexagons, **6ON** yellow circles, **8** black crossed and DPBF white squares), to determine *k*.

Additional reaction constants were calculated for the pH dependent experiment depicted in Figure S44, the plot is show in Figure S46 and values are given in Table S2.

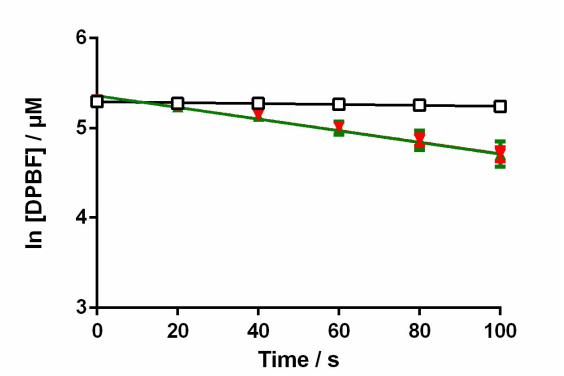


Figure S46: Plots to determine *k* of compound **8** at different pH (pH 5.0 red triangles, pH 7.4 green triangles, DPBF white squares)

Table S2: Rate constant of the DPBF consumption at different pH values.

	k [10^{-3} s^{-1}]	
compound	pH 5.0	pH 7.4
8	4.79	4.64

Cell Culture

Cell-Based Assays

In Figures S47-S49 the plots for IC_{50} determination in HeLa cells of compounds **2-6OFF** and **2-6ON** in Table 1 of the main manuscript are shown, as well as of TPP-peptides **29-31**. The IC_{50} values were obtained in GraphPad Prism version 6 (GraphPad Software, USA) applying the log(inhibitor) vs. response – variable slope (four parameters) fit. Data derived from at least two independent experiments, each concentration in triplicate.

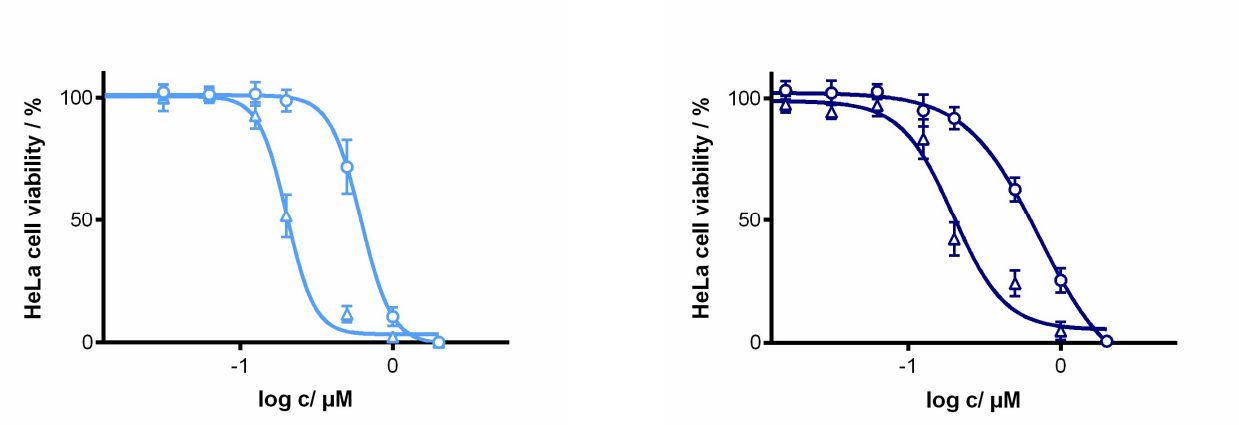


Figure S47: Dose-dependent curves for left: **2OFF** (IC_{50} = 0.617 ± 0.024 µM, light blue circles) and **2ON** (IC_{50} = 0.201 ± 0.012 µM, light blue triangles) and right: **3OFF** (IC_{50} = 0.716 ± 0.078 µM, blue circles) and **3ON** (IC_{50} = 0.196 ± 0.017 µM, blue triangles).

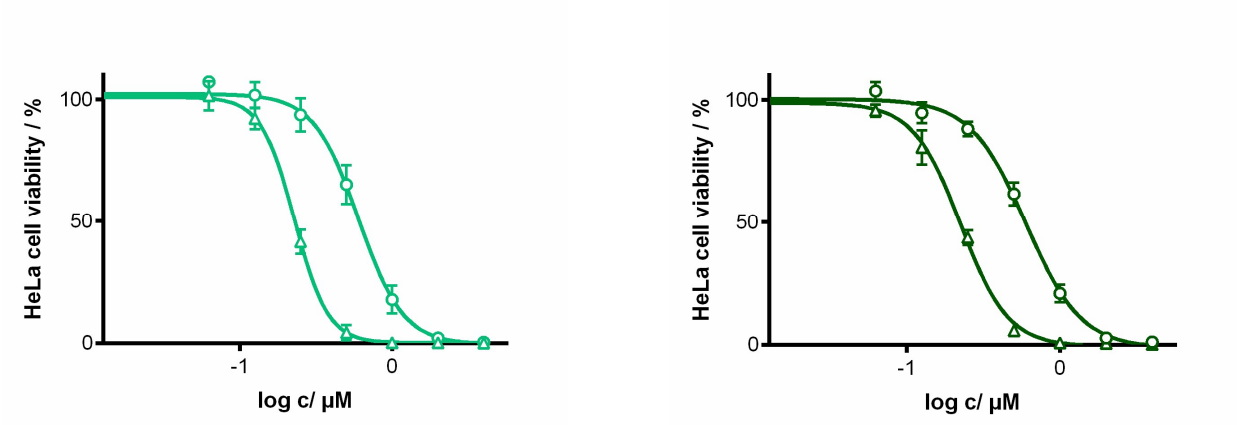


Figure S48: Dose-dependent curves for left: **4OFF** (IC_{50} = 0.599 ± 0.043 µM, light green circles) and **4ON** (IC_{50} = 0.228 ± 0.008 µM, light green triangles) and right: **5OFF** (IC_{50} = 0.598 ± 0.037 µM, dark green circles) and **5ON** (IC_{50} = 0.225 ± 0.011 µM, dark green triangles).

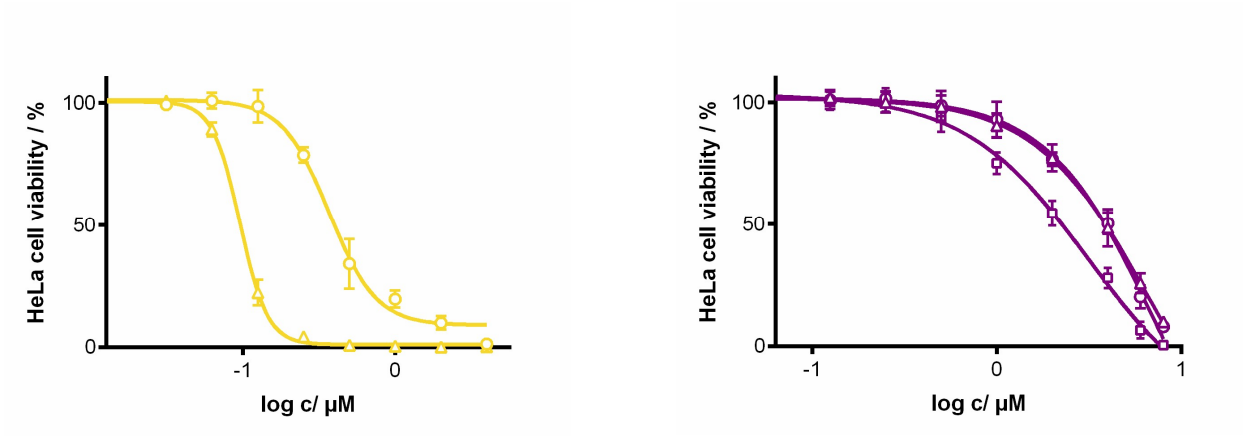


Figure S49: Dose-dependent curves for left **6OFF** (IC_{50} = 0.369 ± 0.030 µM, yellow circles) and **6ON** (IC_{50} = 0.096 ± 0.003 µM, yellow triangles) and right TPP-peptides **29** (IC_{50} = 3.894 ± 0.453 µM), **30** (IC_{50} = 3.923 ± 0.469 µM) and **31** (IC_{50} = 2.310 ± 0.301 µM).

Cell viability assays were additionally performed without the irradiation step but under the same conditions as for the IC_{50} determination (Fig S47-S49). Figure S50 shows the negligible dark toxicity of compounds **2-6OFF** and **2-6ON** at the highest concentrations used for IC_{50} determination. Data derived from two independent experiments, each concentration in triplicate.

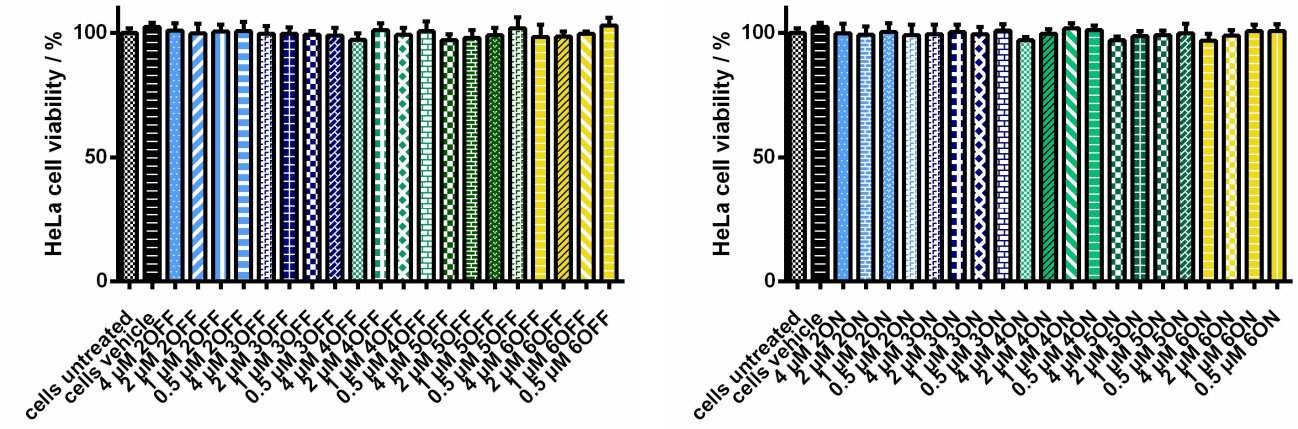


Figure S50: Effect of compounds **2-6OFF** (left) and **2-6ON** (right) at 4.00-0.50 µM concentration without irradiation; and cell vehicle control (8.75% ultrapure H_2O, 1.25% MeCN) without irradiation.

Also, the peptides **11-15** were shown to not affect cell viability at the employed concentrations under the same conditions. Data displayed in Figure S51 derived from two independent experiments, each concentration in triplicate.

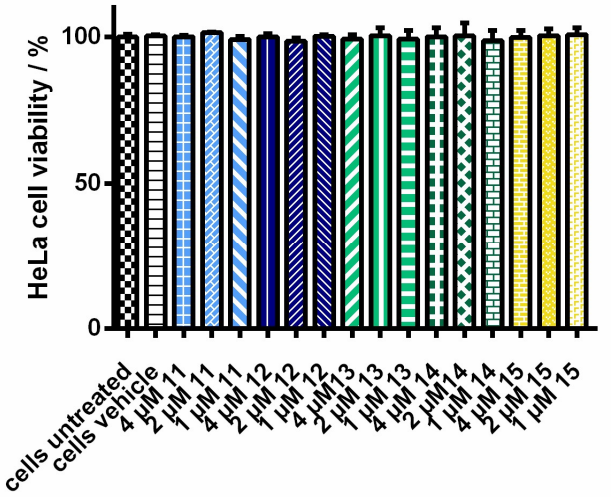


Figure S51: Effect of peptides **11-15** at 4.00-1.00 µM concentration and cell vehicle control (8.75% ultrapure H_2O, 1.25% MeCN) on HeLa cell viability.

Additionally, IC_{50} values for compounds **3OFF/ON**, **5OFF/ON** and **6OFF/ON** were determined PC-3 cells. Assay conditions were equal to these of HeLa cells, but RPMI media was used instead of DMEM. Figure S52 show plots for IC_{50} determination obtained for **3OFF/ON**, Figure S53 for compounds **5OFF/ON** and Figure S54 for compounds **6OFF/ON**. Data displayed derived from two independent experiments, each concentration in triplicate.

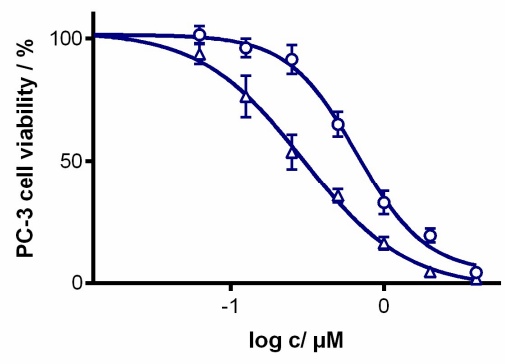


Figure S52: Dose-dependent curves for **3OFF** (IC_{50} = 0.667 ± 0.063 µM, dark blue circles) and **3ON** (IC_{50} = 0.297 ± 0.037 µM, dark blue triangles) in PC-3 cells.

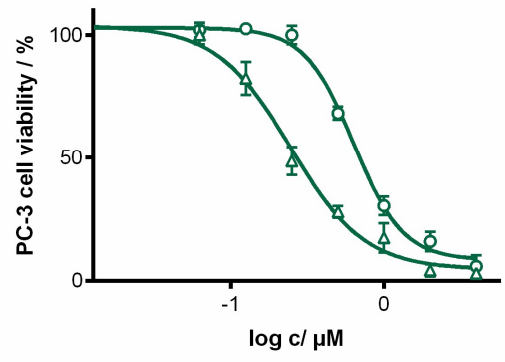


Figure S53: Dose-dependent curves for **5OFF** (IC_{50} = 0.679 ± 0.056 µM, dark green circles) and **5ON** (IC_{50} = 0.277 ± 0.037 µM, dark green triangles) in PC-3 cells.

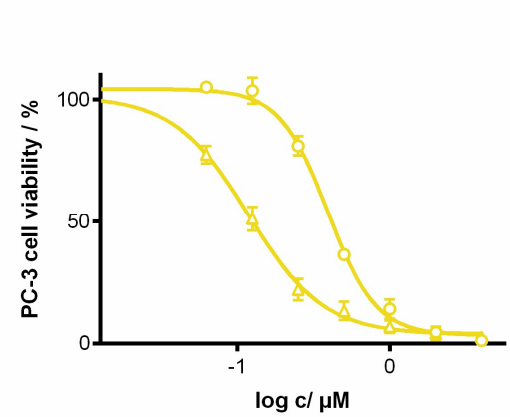


Figure S54: Dose-dependent curves for **6OFF** (IC_{50} = 0.392 ± 0.028 µM, dark green circles) and **6ON** (IC_{50} = 0.119 ± 0.012 µM, dark green triangles) in PC-3 cells.

Table S3 summarizes the IC_{50} values obtained in PC-3 cells (Fig S52-54).

Table S3: Half maximal inhibitory concentration obtained from resazurin cell viability assays in PC-3 cells.

compound	IC_{50} [µM]
3OFF	0.667 ± 0.063
5OFF	0.679 ± 0.056
6OFF	0.392 ± 0.028
3ON	0.297 ± 0.037
5ON	0.277 ± 0.037
6ON	0.119 ± 0.012

Further the effect of only 30 min incubation time before irradiation was examined. Figure S55 shows the plots for IC_{50} determination of compounds **5OFF/ON** when an incubation time of only 30 min before irradiation was employed.

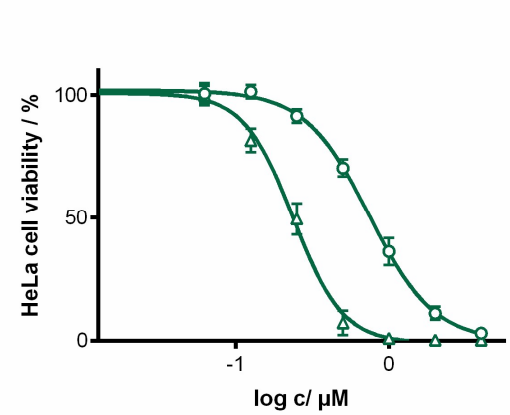


Figure S55: Dose-dependent curves for left: **5OFF** (IC_{50} = 0.744 ± 0.046 µM, dark green circles) and **5ON** (IC_{50} = 0.237 ± 0.026 µM, dark green triangles) obtained when irradiation was employed 30 min instead of 2 h after compound addition.

SUPPORTING INFORMATION

Figures S56-S58 show the cell viability observed after the intracellular iEDDA reactions at different concentrations, additional to those shown in Figure 5 of the manuscript.

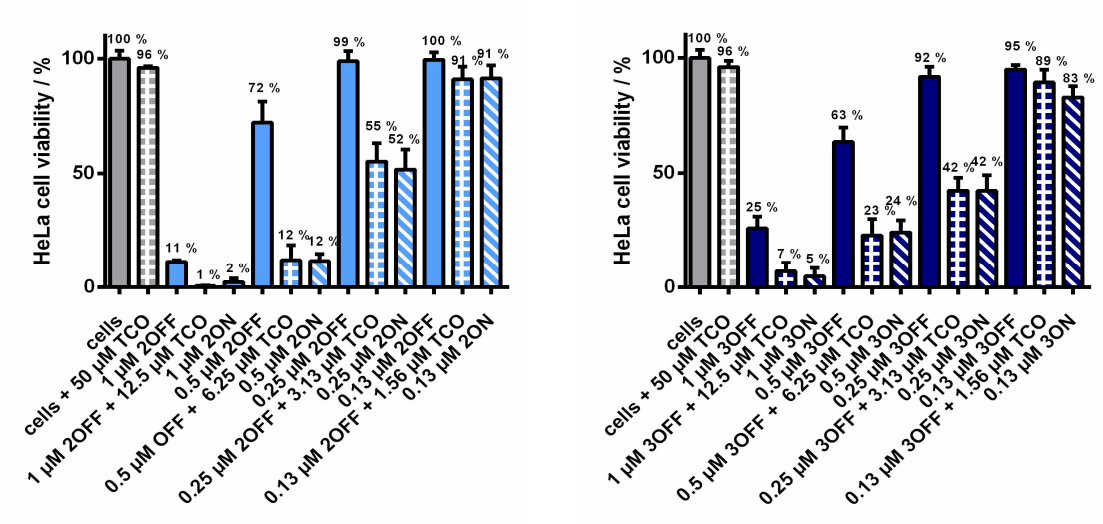


Figure S56: Intracellular iEDDA of **2OFF** (left) and **3OFF** (right) at different concentrations with 12.5 eq TCO. Effect of the turn-on probes **2ON** and **3ON** on cell viability at the same concentrations is included for comparison.

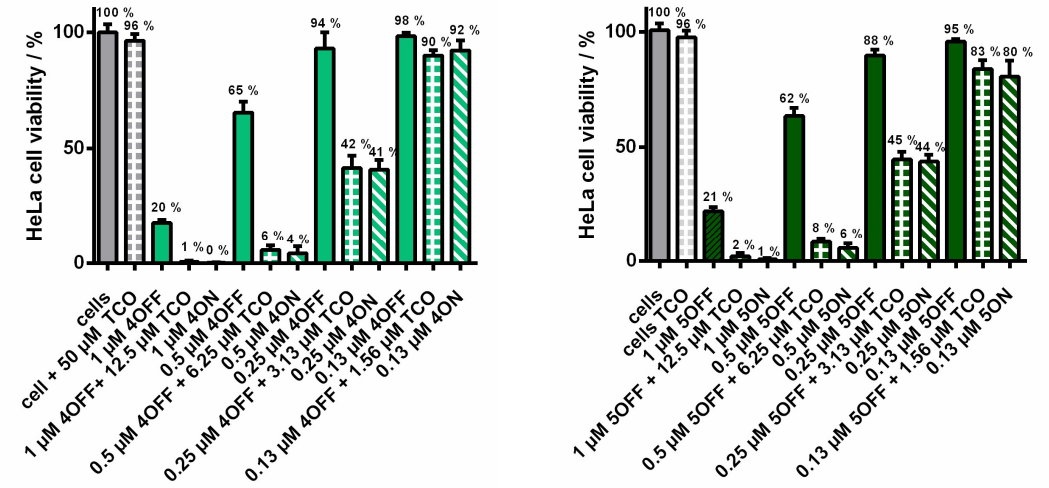


Figure S57: Intracellular iEDDA of **4OFF** (left) and **5OFF** (right) at different concentrations with 12.5 eq TCO. Effect of the turn-on probes **4ON** and **5ON** on cell viability at the same concentrations is included for comparison.

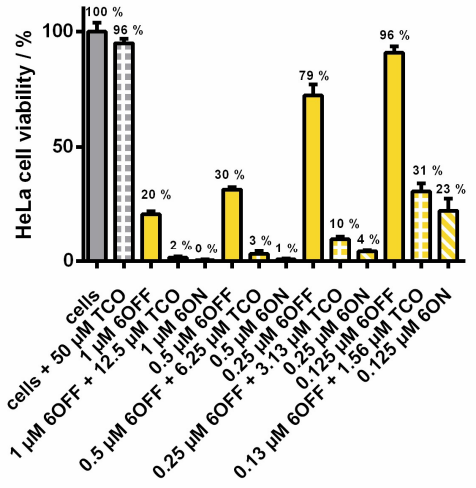


Figure S58: Intracellular iEDDA of **6OFF** at different concentrations with 12.5 eq TCO. Effect of turn-on probe **6ON** on cell viability at the same concentrations is included for comparison.

Fluorescence Microscopy

Fluorescence microscopy z-stack images (1 µm steps) for the images shown in Figure 4 of the manuscript are shown in Figures S59-S63.

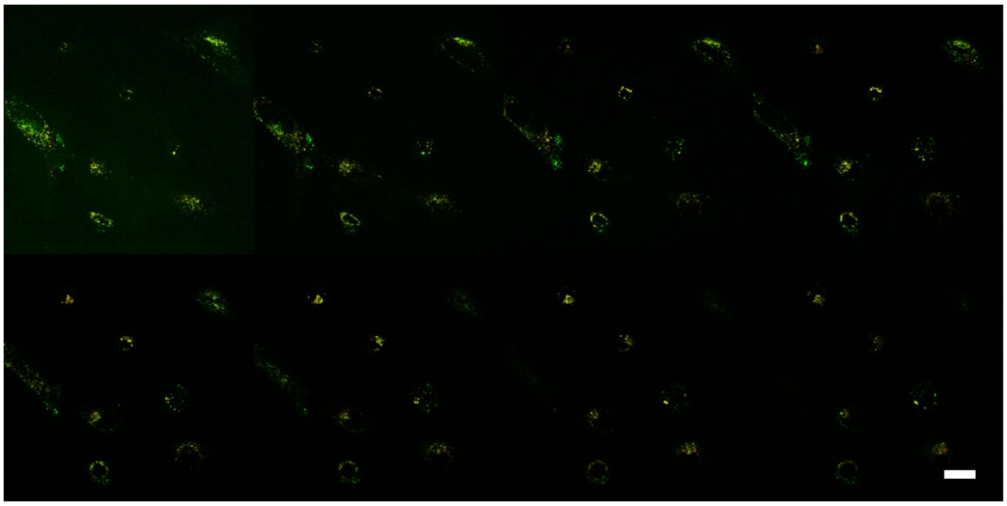


Figure S59: Z-stack images (1 µm steps) of HeLa cells treated with 2 µM of **2FL** and 100 nM LysoTracker® Red DND-99, corresponding to Figure 4 A (top). Scale bar: 20 µm. (Lasers: 488 nm: 0.25%, 633 nm: 2.0%; Filters: ChS1: 491-535 nm, Ch2: 635-700 nm; Master gain: ChS1: 650, Ch2: 780; Digital gain: ChS1: 1.00, Ch2: 2.00).

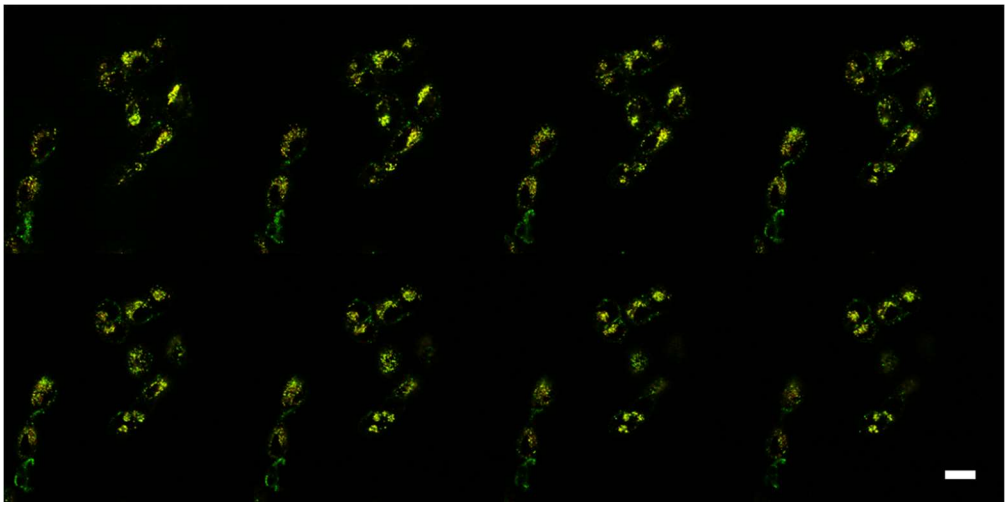


Figure S60: Z-stack images (1 µm steps) of HeLa cells treated with 2 µM of **3FL** and 100 nM LysoTracker® Red, corresponding to Figure 4 A (bottom). Scale bar: 20 µm. (Lasers: 488 nm: 0.25%, 633 nm: 2.0%; Filters: ChS1: 491-535 nm, Ch2: 635-700 nm; Master gain: ChS1: 650, Ch2: 780; Digital gain: ChS1: 1.00, Ch2: 2.00).

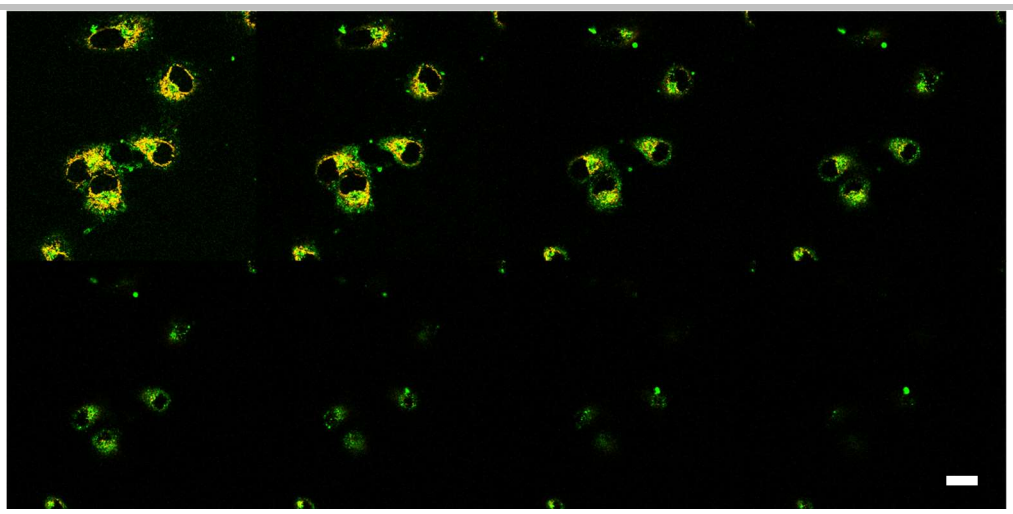


Figure S61: Z-stack images (1 µm steps) of HeLa cells treated with 2 µM of **4FL** and 50 nM TMRE, corresponding to Figure 4 B (top). Scale bar: 20 µm. (Lasers: 488 nm: 0.25%, 543 nm: 2.0%; Filters: ChS1: 491-535 nm, ChS2: 545-589 nm; Master gain: ChS1: 650, ChS2: 650; Digital gain: ChS1: 1.00, ChS2: 2.00).

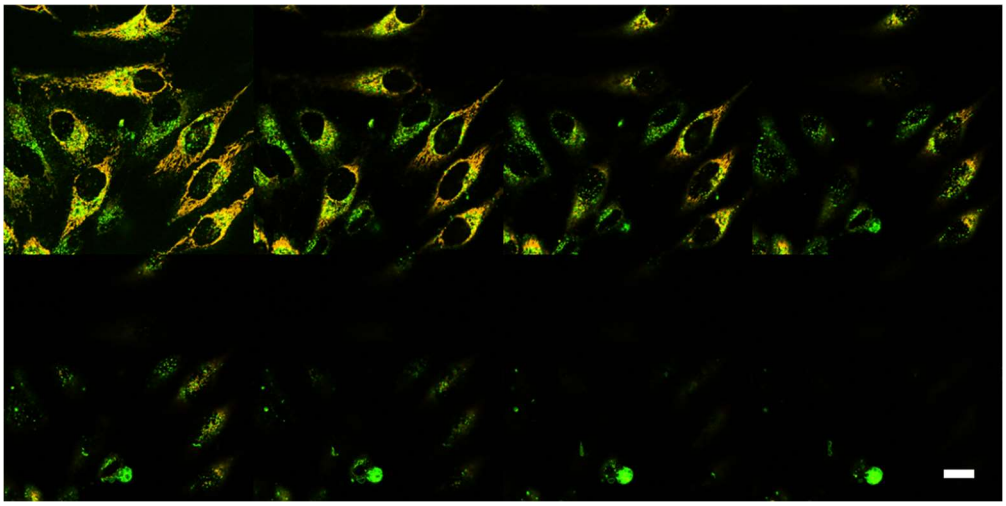


Figure S62: Z-stack images (1 µm steps) of HeLa cells treated with 2 µM of **5FL** and 50 nM TMRE, corresponding to Figure 4 B (bottom). Scale bar: 20 µm. (Lasers: 488 nm: 0.25%, 543 nm: 2.0%; Filters: ChS1: 491-535 nm, ChS2: 544-589 nm; Master gain: ChS1: 650, ChS2: 650; Digital gain: ChS1: 1.00, ChS2: 2.00).

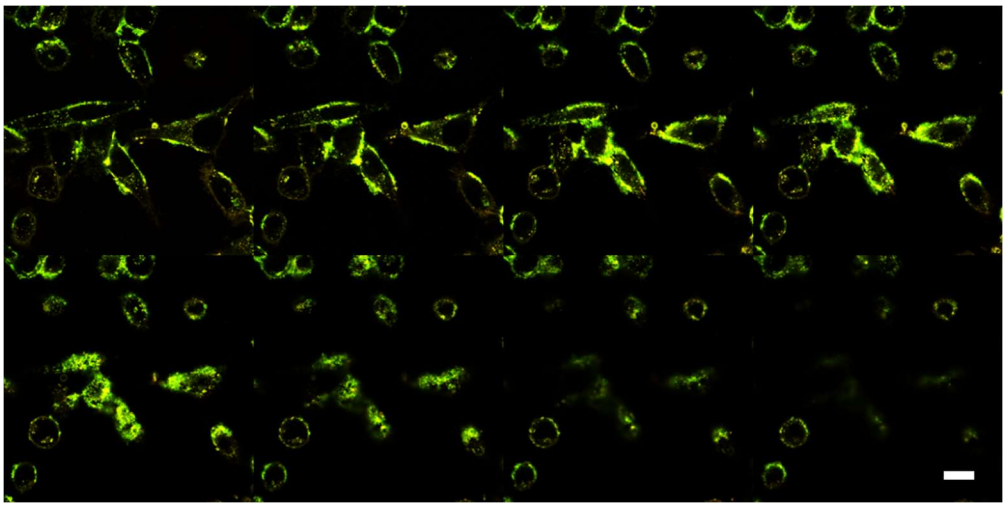


Figure S63: Z-stack images (1 µm steps) of HeLa cells treated with 2 µM of **6FL** and 10 µM DiI, corresponding to Figure 4 C. Scale bar: 20 µm. (Lasers: 488 nm: 0.40%, 543 nm: 1.0%; Filters: Ch1: 491-535 nm, ChS1: 544-589 nm; Master gain: Ch1: 500, ChS1: 650; Digital gain: CS1: 1.50, ChS1: 1.50)

Cells treated with mitochondrial-penetrating peptide (MPP) dye constructs **4FL** and **5FL** were likewise treated with LysoTracker® Red DND-99 and the Pearson's correlation coefficient (Rr) was determined using the JACoP plugin[15] in ImageJ software[16]. Figure S64 shows the zoom and results obtained for colocalization analysis of compounds **4FL** and **5FL** with the LysoTracker® Red DND-99.

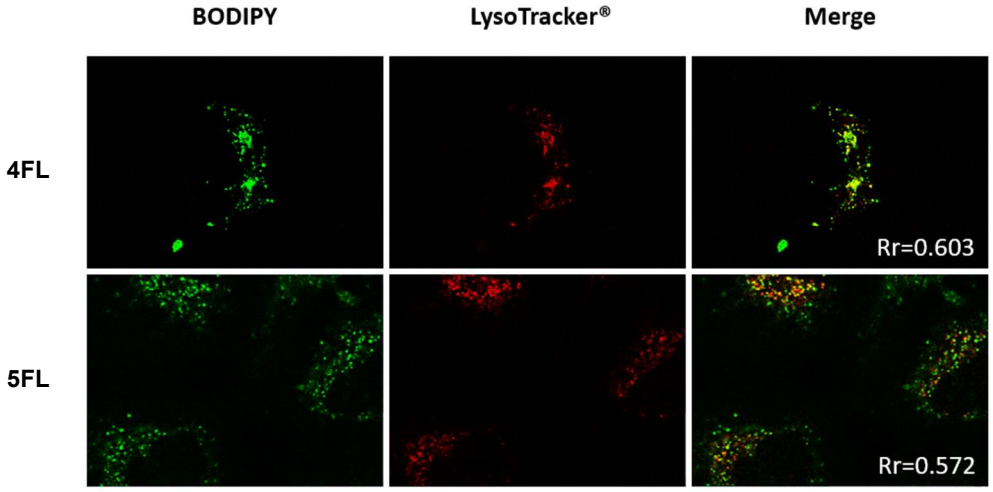


Figure S64: Colocalization analysis of compounds **4FL** and **5FL** with LysoTracker® Red DND-99.

For MPP peptides also the distribution within the cell after an incubation time of only 30 min instead of 2 h was exanimated. Figure S65 shows microscopy pictures and results for colocalization analysis of compound **5FL** with LysoTracker® Red DND-99 (up) and TMRE (below) after the shorter 30 min incubation time.

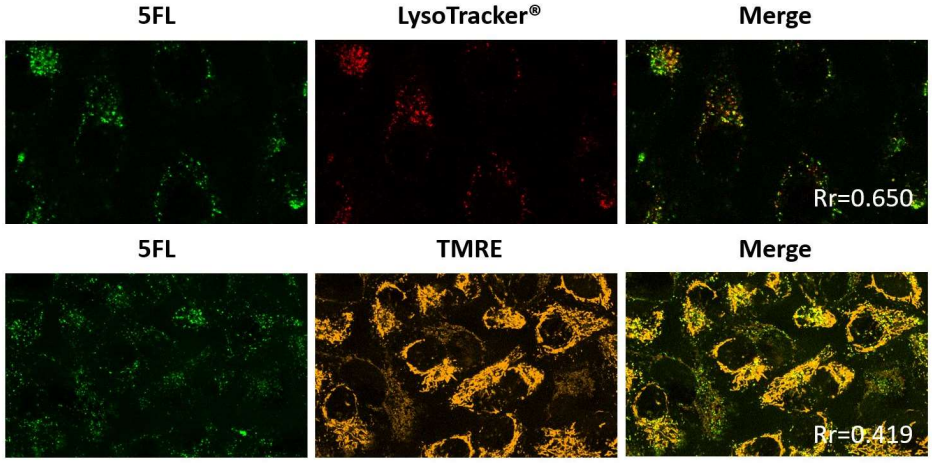


Figure S65: Colocalization analysis of compound **5FL** with LysoTracker® Red DND-99 and TMRE. Cells were incubated with **5FL** for 30 min.

Flow Cytometry Measurements

Upon flow cytometry analysis the cell populations were manual gated based on forward scatter-area (FSA-A) and side scatter-area (SSC-A) characteristics as shown in Figure S66 (left) for the vehicle control (12.5% MeCN in ultrapure H_2O). Quadrants were manually selected to illustrate fluorescence plots (Figure S66, right).

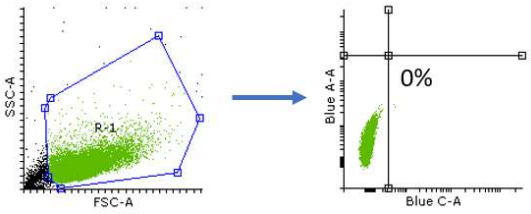


Figure S66: Manual gating (left) and quadrant selection (right) applied for flow cytometry analysis, example shows the vehicle control.

Figure S67 further shows the dot plots and values obtained for percentage of labelled cells for the samples treated with 0.2 µM of peptide-dye conjugates **2**-**6FL** corresponding to these shown in Figure 2B of the manuscript.

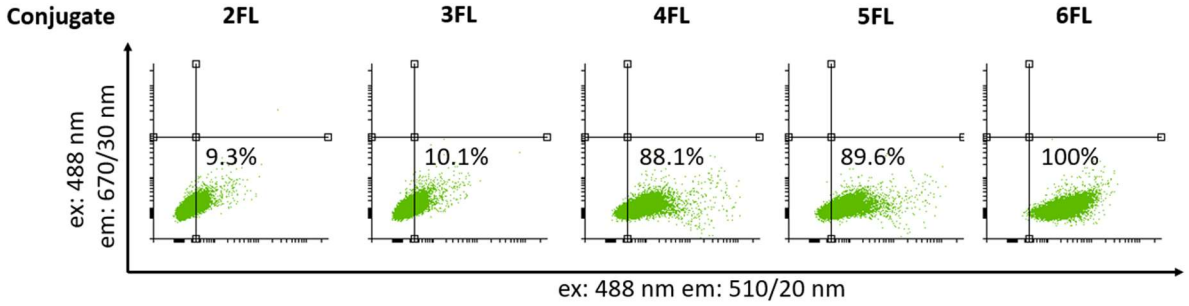


Figure S67: 0.2 µM results of measurements shown in Figure 2B.

In Figure S68 the duplicate of the measurements shown in Figure 2B of the manuscript and Figure S67 as well as values obtained for percentage of labelled cells are depicted.

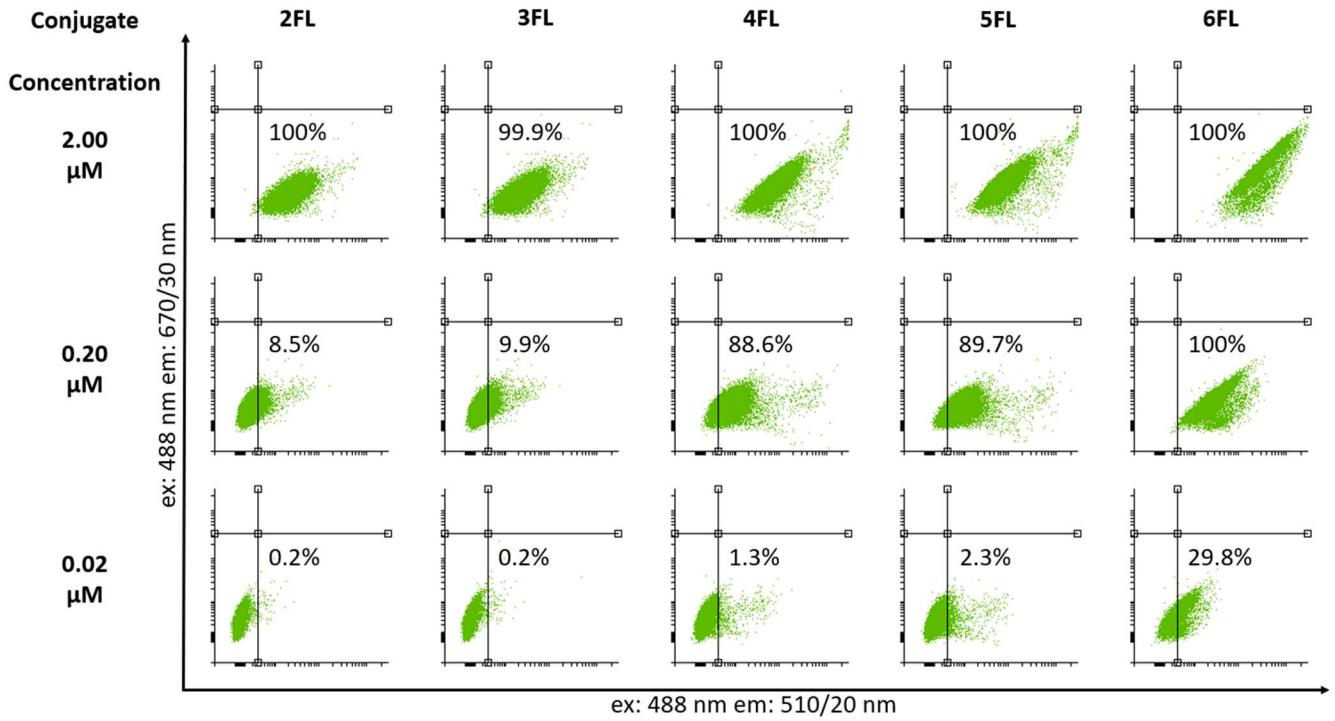


Figure S68: Duplicate data of flow cytometry measurements from Figure 2B and Figure S65.

SUPPORTING INFORMATION

Table S4 further summarizes the values for median fluorescence intensity in the Blue C-A channel (ex: 488 nm em 510/20 nm) acquired in the two individual experiments shown in Figure 2 of the manuscript and Figures S65-S66.

Table S4: Values for median fluorescence intensity obtained.

	2FL	3FL	4FL	5FL	6FL	untreated
2μM	2217	2284	8231	8856	34485	
	2486	2556	7257	7857	38300	23
0.2μM	158	163	588	614	2455	21
	145	150	539	556	2203	
0.02μM	37	38	79	93	249	
	42	42	78	92	223	

Figure S69 depicts the overlaid histogram profiles of representative flow cytometry data at 0.2 μM (left) and 0.02 μM (right) conjugate concentration, additional to the histogram shown in Figure 3C of the manuscript.

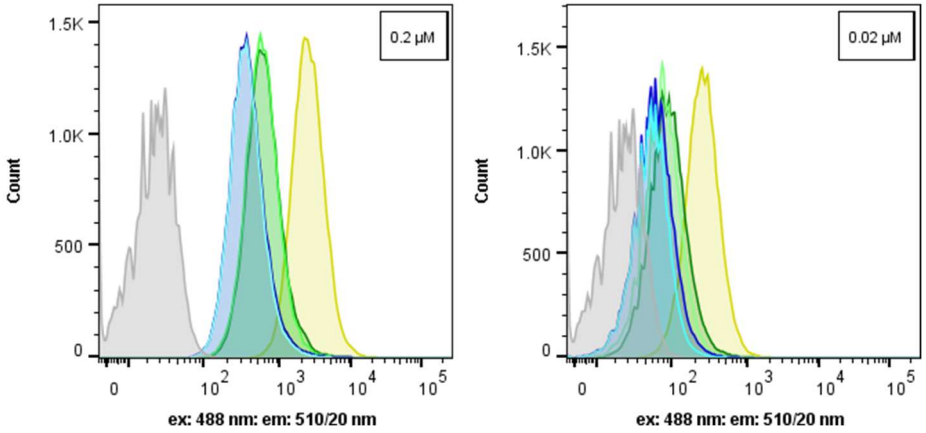


Figure S69: Overlaid histograms showing the shift of flow cytometry fluorescence intensity signal for conjugate-untreated (grey), **2FL** (light blue), **3FL** (dark blue), **4FL** (light green), **5FL** (dark green) and **6FL** (yellow) treated cells at 0.2 μM (left) and 0.02 μM (right) concentration.

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