Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

A Palette of Background-Free Tame Fluorescent Probes for Intracellular Multi-Color Labelling in Live Cells

*Samira Husen Alamudi, Dongdong Su, Kyung Jin Lee, Jung Yeol Lee, José Luis Belmonte-Vázquez, Hee-Sung Park, Eduardo Peña-Cabrera, Young-Tae Chang**

I. Supplemental Figures

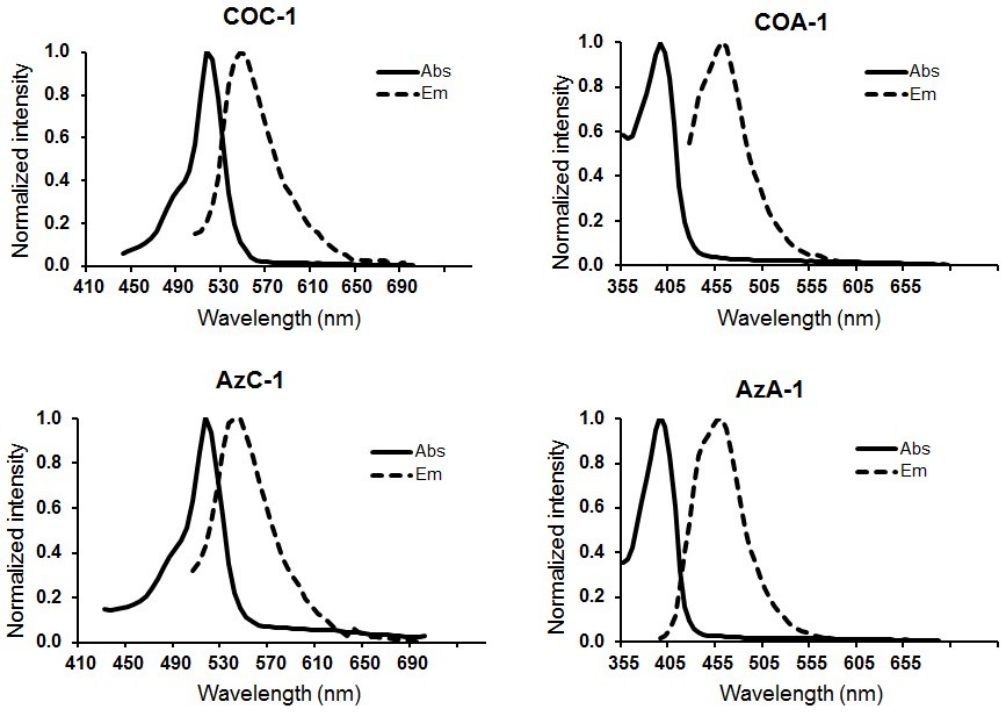
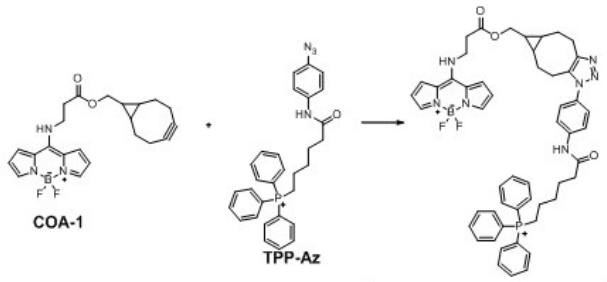


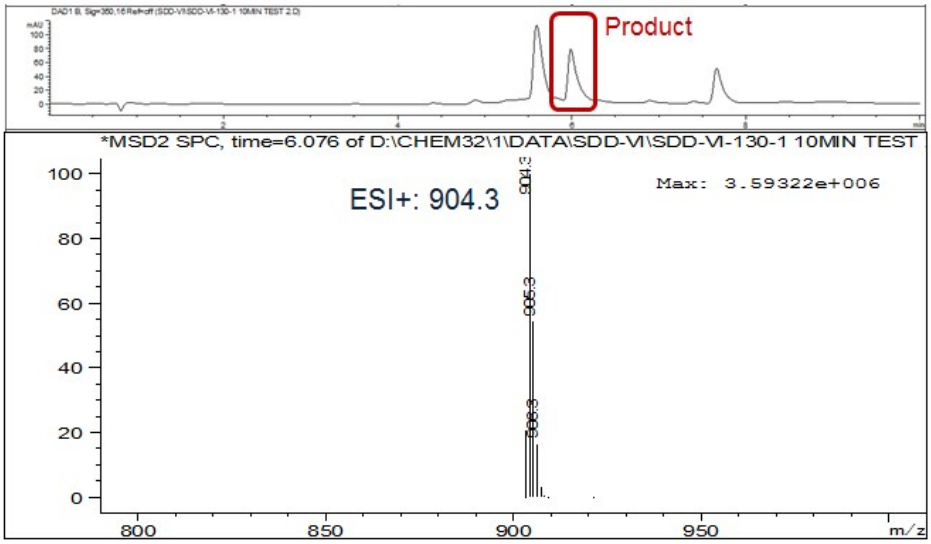
Figure S1. Absorption (Abs) and emission (Em) spectra for COC-1, COA-1, AzC-1 and AzA-1.

Absorbance and fluorescence emission were measured in DMSO at 10 μM of probe concentration.

A



Chemical Formula: $C_{52}H_{54}BF_2N_7O_3P^+$
Exact Mass: 904.41



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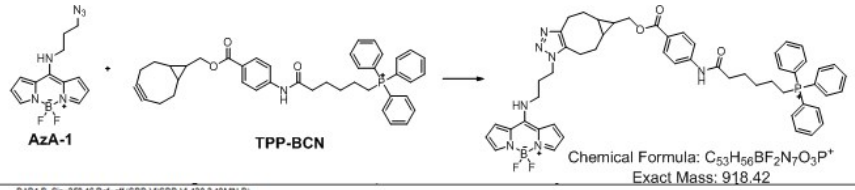

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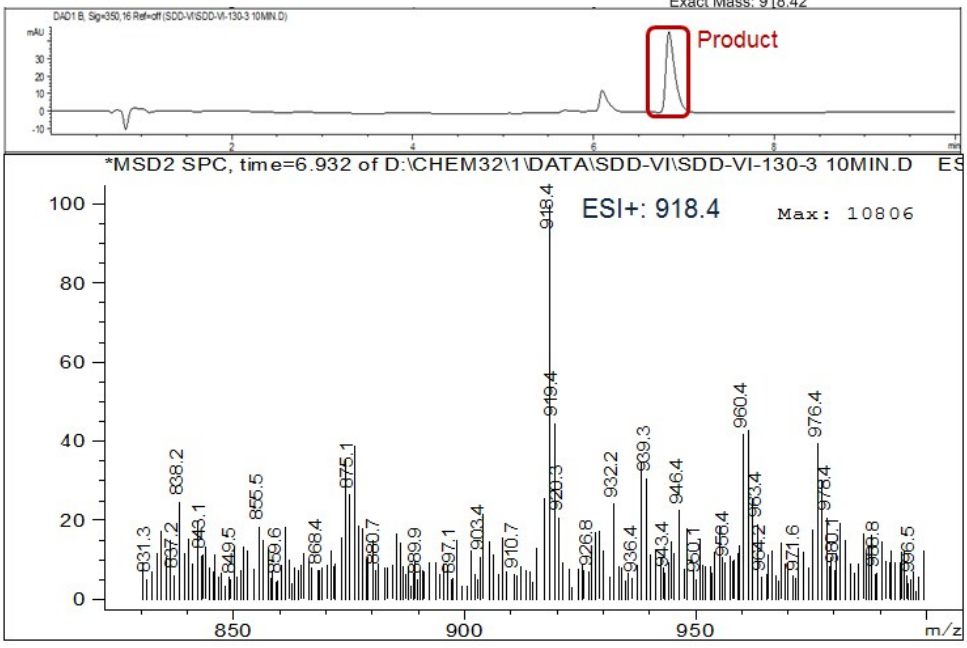

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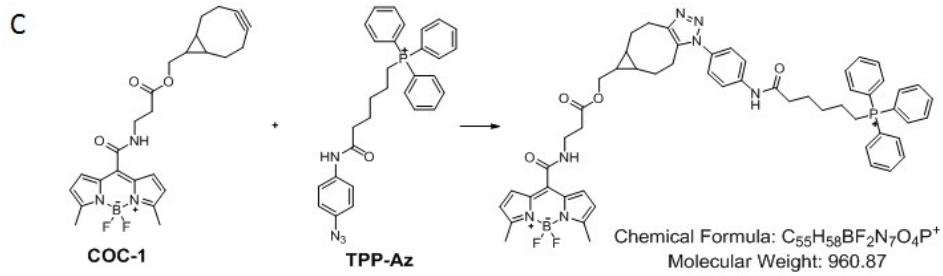
COC-1
TPP-Az
Chemical Formula: $C_{55}H_{58}BF_2N_7O_4P^+$
Molecular Weight: 960.87


Product
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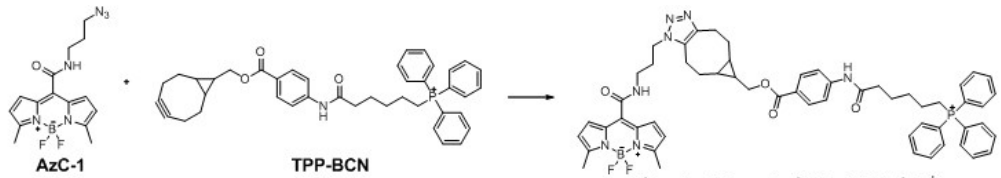
D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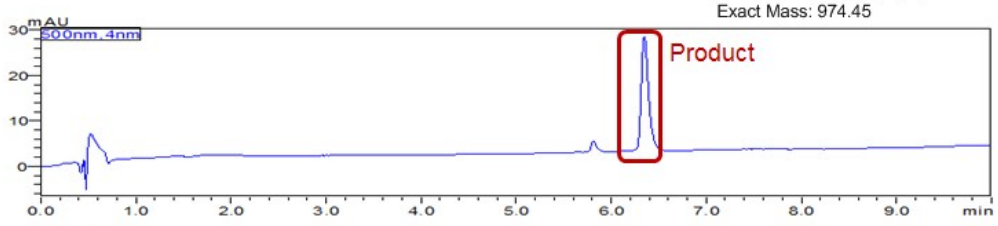
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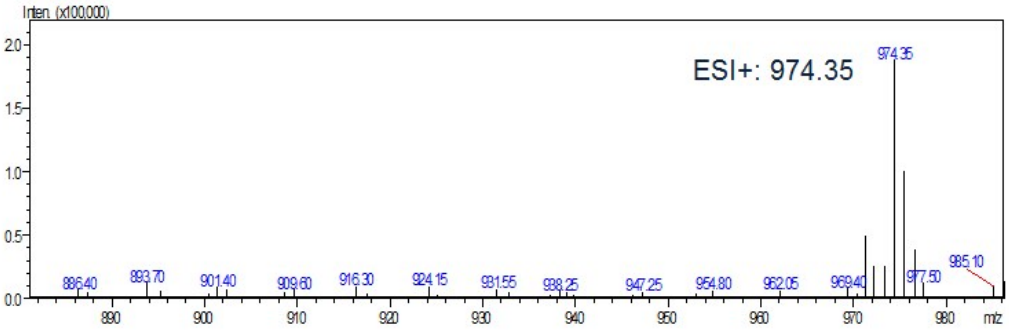
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m/z

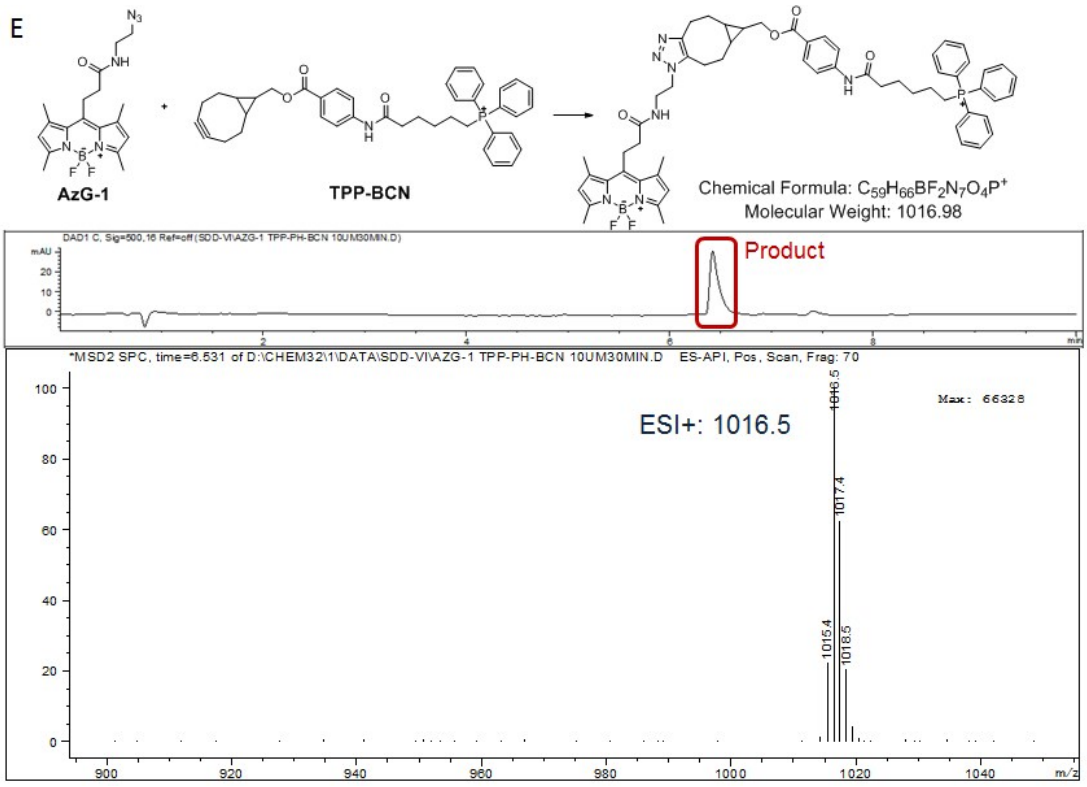


Figure S2. ***In vitro*** **reactions between probes and TPP-Az or TPP-BCN.**

The reactions proceeded at room temperature for 10 minutes in PBS (pH 7.4)/10% DMSO before were analyzed with LCMS. Probe (10 μM), **TPP-Az or TPP-BCN** (20 μM). The experiment was performed on HPLC-MS (model: Agilent-1200 with a DAD detector and a single quadrupole mass spectrometer 6130 series). The analytical method is mobile phase A: H2O (0.1% HCOOH), mobile phase B: CH3CN (0.1% HCOOH), gradient from 10 to 90% B in 10 minutes . Column: C18 (2) Luna column (250 × 4.6 mm, 5 μm particle size). Data was collected at 350 nm or 500 nm. Reaction between **CO-1** and **TPP-Az** see reference (*Nat. Commun*., 2016, **11**, 11964)

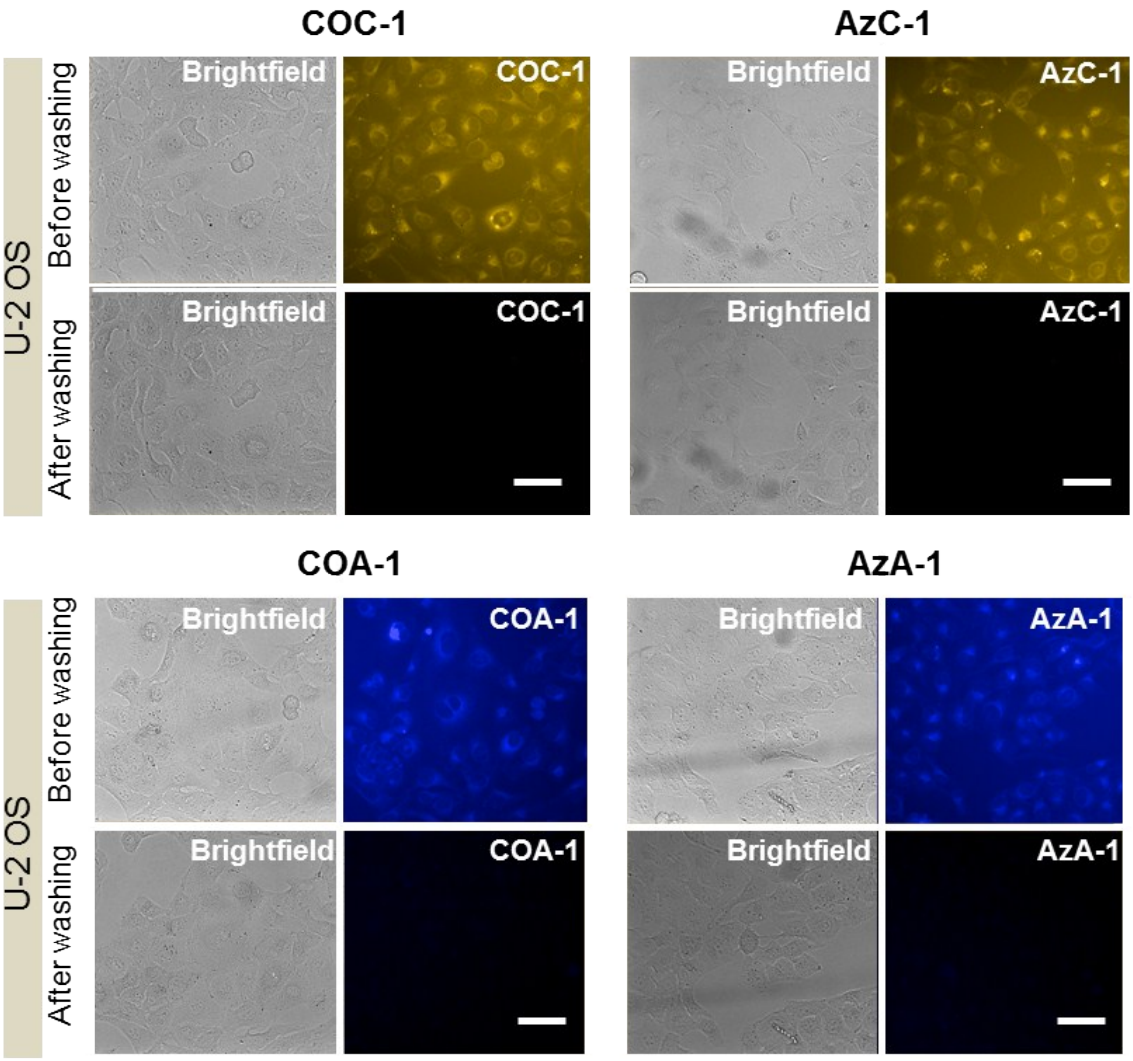


Figure S3. Cellular retention of COC-1, COA-1, AzC-1 and AzA-1 in U-2 OS cells.

Cells were stained with probes at 2 μM final concentration in growth medium at 37 °C. After 30 min, cells were imaged using Operetta imaging system (before washing image) and then were washed with fresh growth medium (3 × 5 min at 37 °C). Afterwards, cells were imaged again (after washing image). Probe signal is significantly decreased after washing for all probes (yellow signal from TRITC channel and blue signal from DAPI channel). Scale bar, 30 μm.

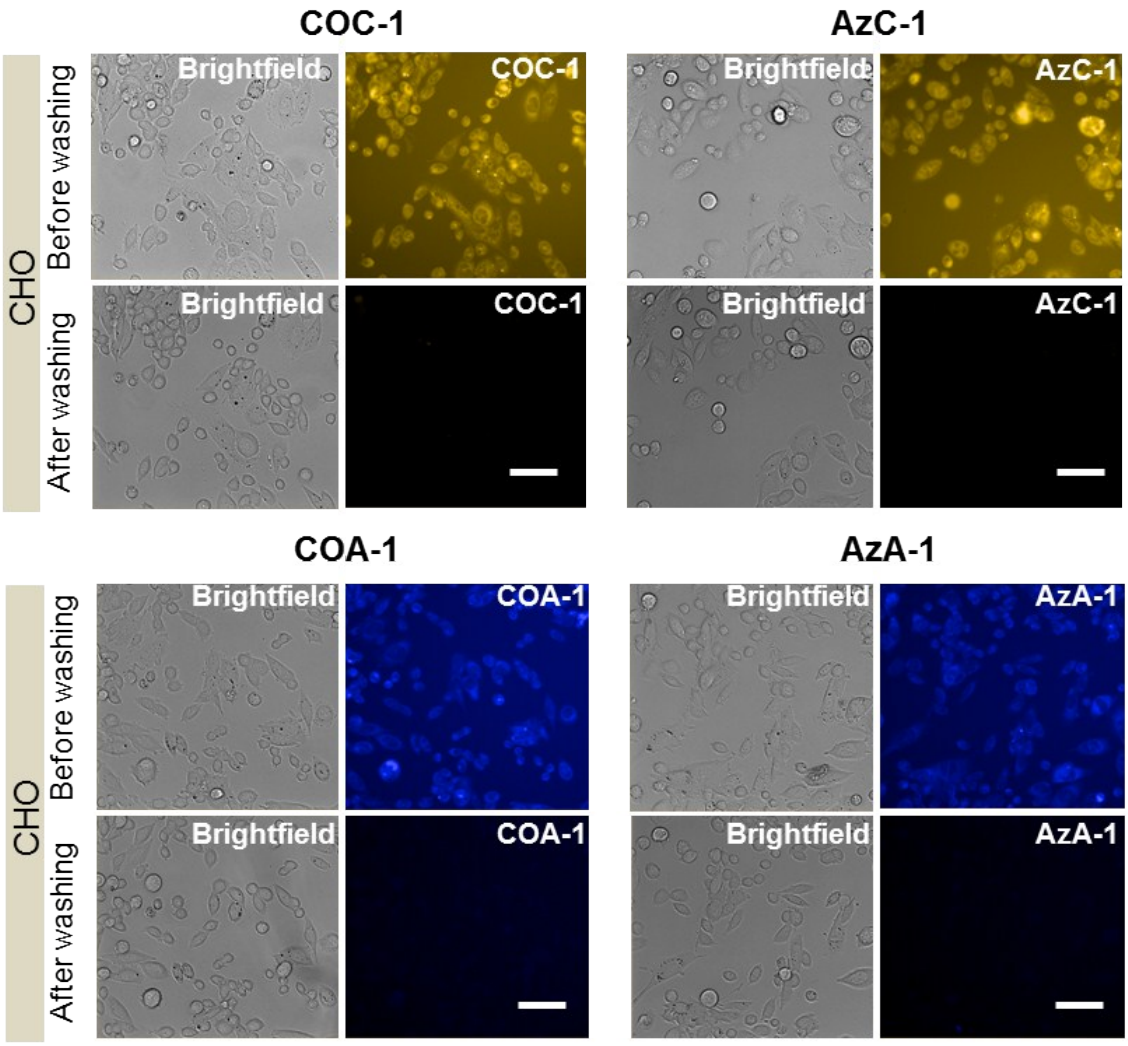


Figure S4. Cellular retention of COC-1, COA-1, AzC-1 and AzA-1 in CHO cells.

Cells were stained with probes at 2 μM final concentration in growth medium at 37 °C. After 30 min, cells were imaged using Operetta imaging system (before washing image) and then were washed with fresh growth medium (3 × 5 min at 37 °C). Afterwards, cells were imaged again (after washing image). Probe signal is significantly decreased after washing for all probes (yellow signal from TRITC channel and blue signal from DAPI channel). Scale bar, 30 μm.

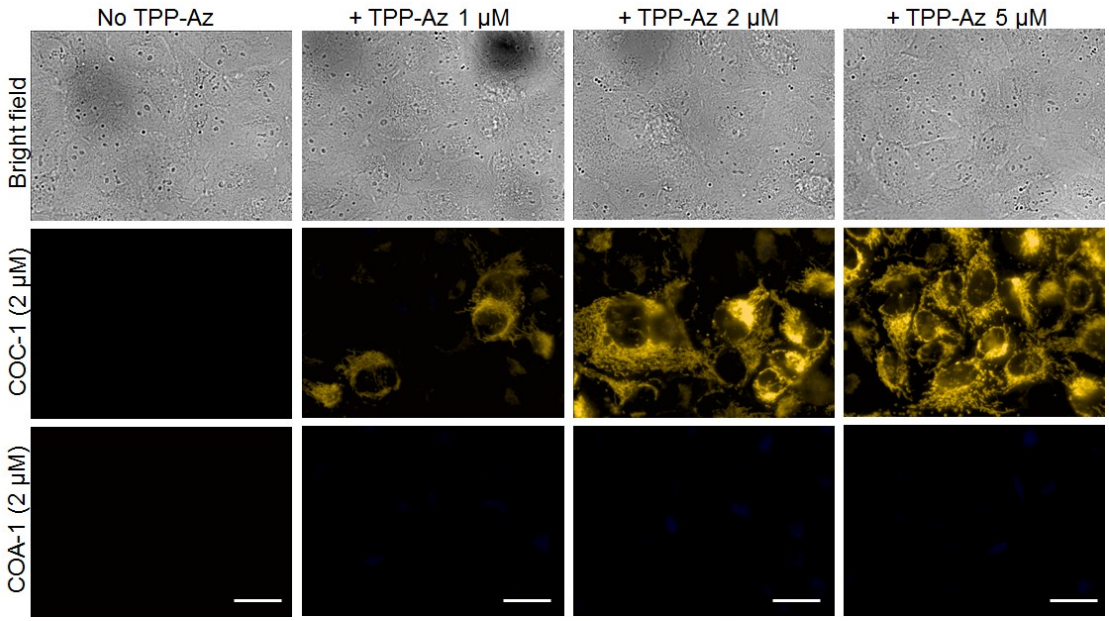


Figure S5. Sequential labelling of TPP-Az-treated U-2 OS with COC-1 and COA-1.

Live U-2 OS cells were treated with 0, 1, 2 or 5 μM of TPP-Az for 20 min at 37 °C. Cells were then firstly labelled with **COC-1** (2 μM, 30 min), briefly washed and followed by second labelling with **COA-1** (2 μM, 30 min). After brief washing, cells were the imaged with Operetta microscopy. Scale bar, 20 μm.

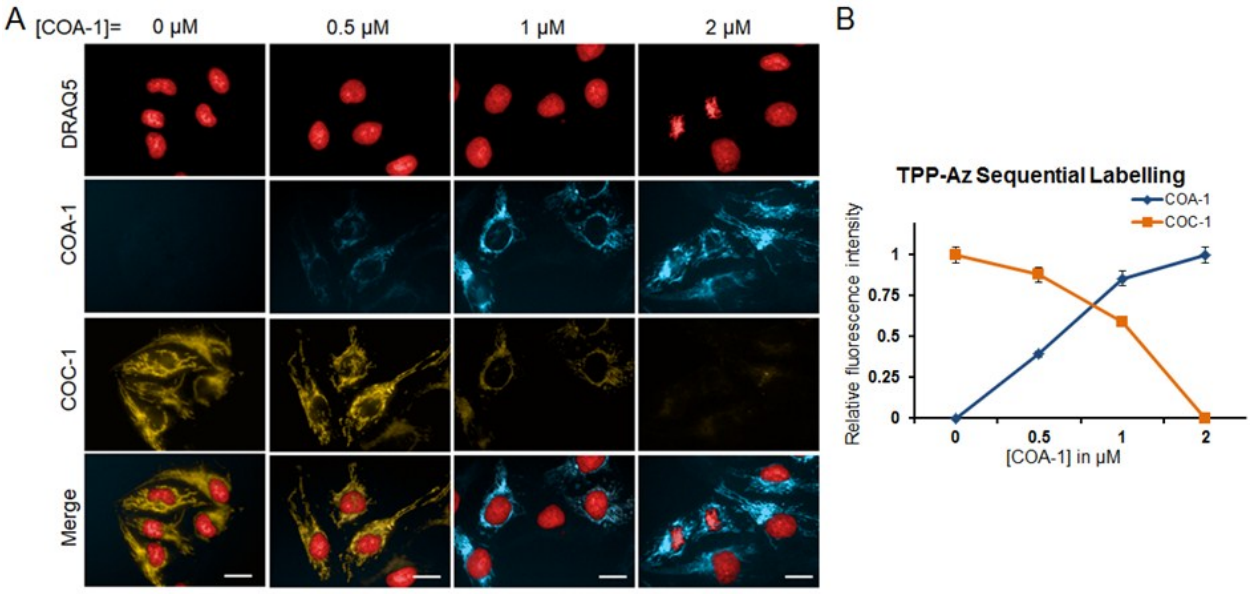


Figure S6. Sequential labelling of **TPP-Az**-treated U-2 OS with **COA-1** and **COC-1**. A) Live U-2 OS cells were treated with 5 µM of **TPP-Az** for 20 min at 37 °C. Cells were then firstly labelled with **COA-1** at respective different concentration (0, 0.25, 0.5, 1 or 2 µM for 30 min), briefly washed and followed by second labelling with **COC-1** (2 µM for 30 min). After brief washing, cells were the imaged with Operetta microscopy. Scale bar, 20 µm. B) Normalized mean fluorescence intensity of **COA-1** and **COC-1** in cells treated with **TPP-Az** (see panel A) relative to mean fluorescence intensity of cells treated with vehicle (n=3).

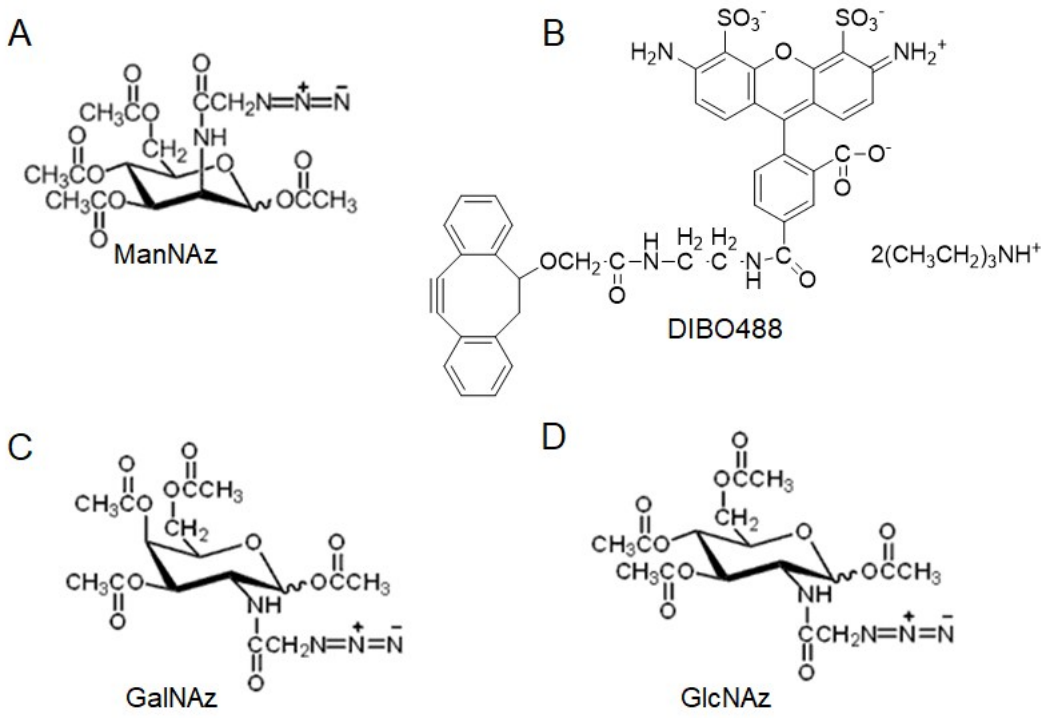


Figure S7. Structure of: A) Tetraacetylated N-azidoacetyl-D-mannosamine (ManNAz), B) Alexa Fluor 488 DIBO Alkyne (DIBO488), C) Tetraacetylated *N*-azidoacetylgalactosamine (GalNAz), and D) Tetraacetylated *N*-azidoacetylglucosamine (GlcNAz).

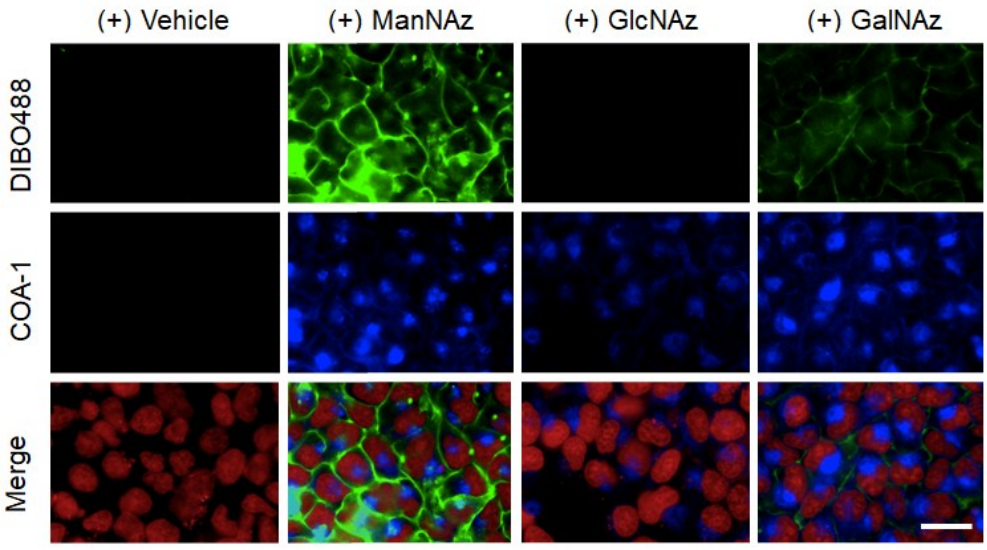


Figure S8. Multi labelling of both extracellular and intracellular azide sugar-tagged glycoconjugates in U-2 OS. Live U-2 OS were treated with vehicle or 50 μM ManNAz, GalNAz and GlcNAz individually for 48 h and were co-labelled with **DIBO488** and **COA-1.** Images shown are composed of signals from **DIBO488** (green)**, COA-1** (blue), and nuclear tracker, **DRAQ5** (red). Scale bar, 50 μm.

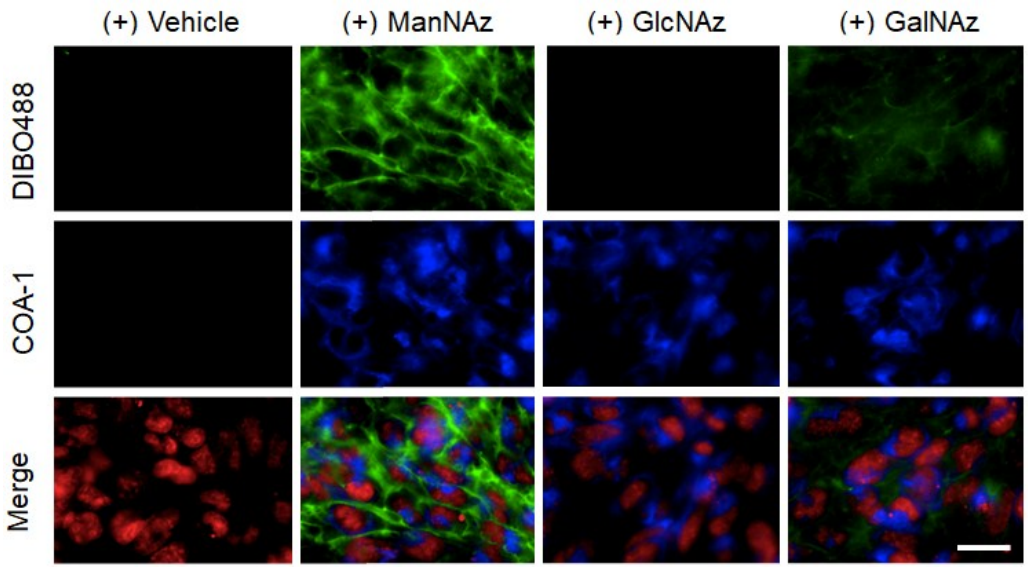


Figure S9. Multi labelling of both extracellular and intracellular azide sugar-tagged glycoconjugates in U251. Live U251 were treated with vehicle or 50 μM ManNAz, GalNAz and GlcNAz individually for 48 h and were co-labelled with **DIBO488** and **COA-1.** Images shown are composed of signals from **DIBO488** (green)**, COA-1** (blue), and nuclear tracker, **DRAQ5** (red). Scale bar, 50 μm.

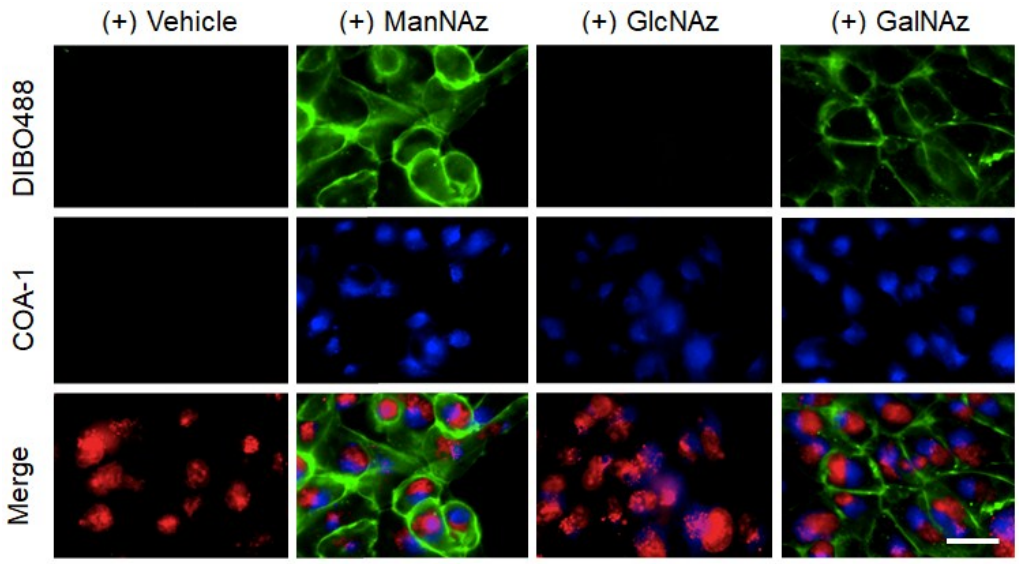


Figure S10. Multi labelling of both extracellular and intracellular azide sugar-tagged glycoconjugates in ACHN. Live ACHN were treated with vehicle or 50 μM ManNAz, GalNAz and GlcNAz individually for 48 h and were co-labelled with **DIBO488** and **COA-1.** Images shown are composed of signals from **DIBO488** (green)**, COA-1** (blue), and nuclear tracker, **DRAQ5** (red). Scale bar, 50 μm.

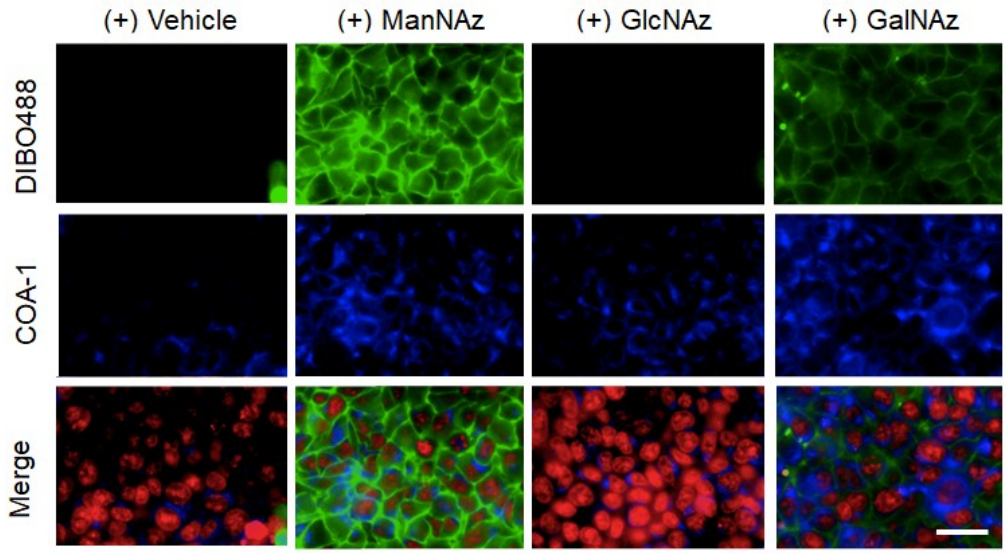


Figure S11. Multi labelling of both extracellular and intracellular azide sugar-tagged glycoconjugates in HCT-116. Live HCT-116 were treated with vehicle or 50 μM ManNAz, GalNAz and GlcNAz individually for 48 h and were co-labelled with **DIBO488** and **COA-1.** Images shown are composed of signals from **DIBO488** (green)**, COA-1** (blue), and nuclear tracker, **DRAQ5** (red). Scale bar, 50 μm.

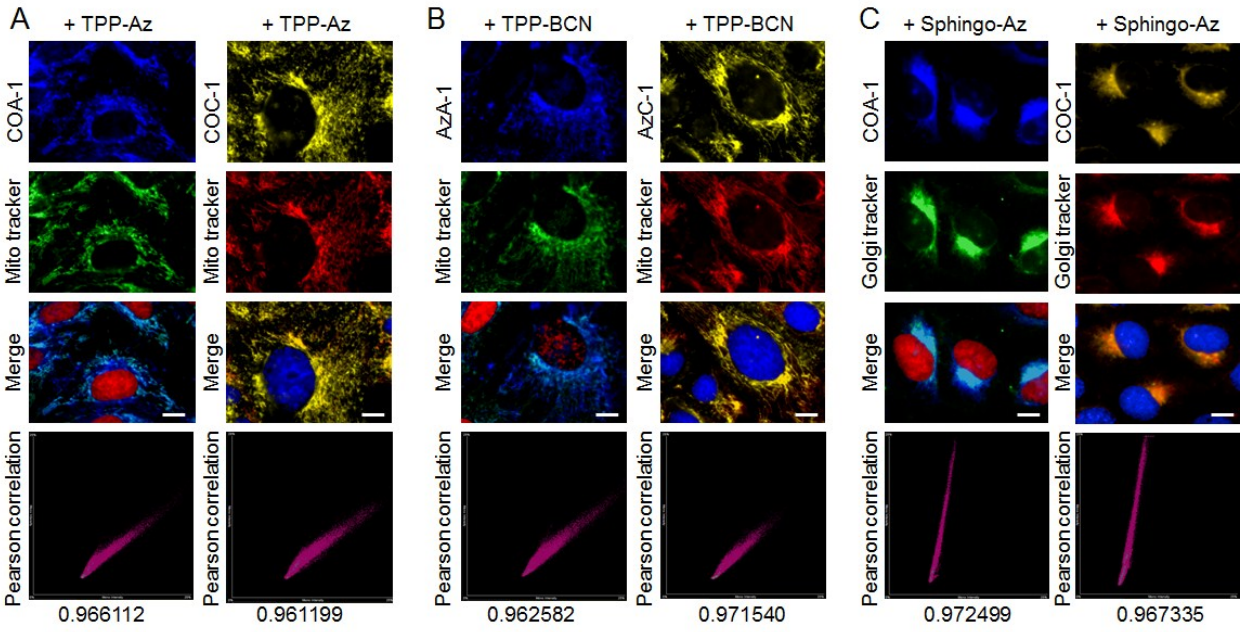


Figure S12. Intracellular colocalization of COA-1, COC-1, AzA-1 and AzC-1 in reporter-treated U-2 OS.

Live U-2 OS cells were treated with A) 2 μM **TPP-Az** for 20 min at 37 °C, B) 2 μM **TPP-BCN** for 20 min at 37 °C, or C) 5 μM **Sphingo-Az** for 30 min at 4 °C followed by 30 min at 37 °C. Cells were then labelled with respective probes (2 μM, 30 min) and counterstained with mitochondria tracker (MitoTracker Green and MitoTracker Red) or golgi tracker (BODIPY FL ceramide and BODIPY TR ceramide) and nuclear trackers. Pearson coefficients for each of the treatment (except nuclear tracker) are displayed at the bottom of merged images. Scale bar, 10 μm.

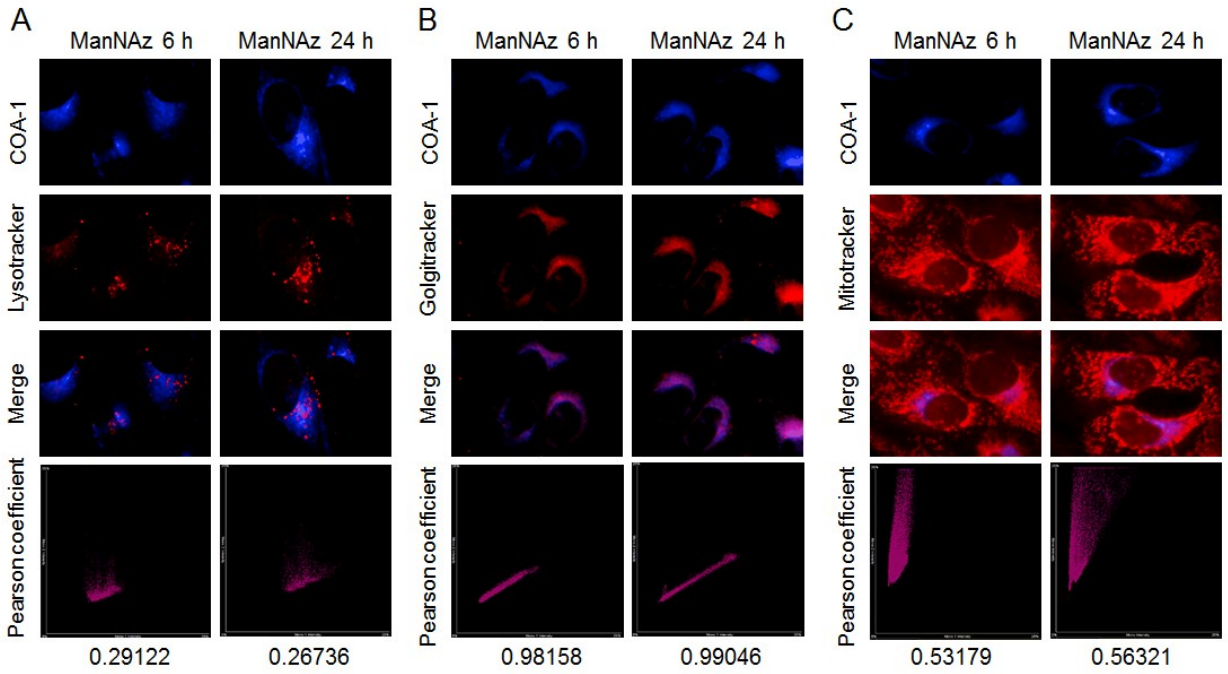


Figure S13. Intracellular colocalization of COA-1 in ManNAz-treated U-2 OS.

Live U-2 OS cells were treated with 50 μM ManNAz for 6 and 24 h at 37 °C. Cells were then labelled with **COA-1** (2 μM, 30 min) and counterstained with A) LysoTracker Red (lysosome), B) BODIPY TR ceramide (golgi apparatus) and C) MitoTracker Red (mitochondria). Pearson coefficients for each of the treatment are displayed at the bottom of merged images.

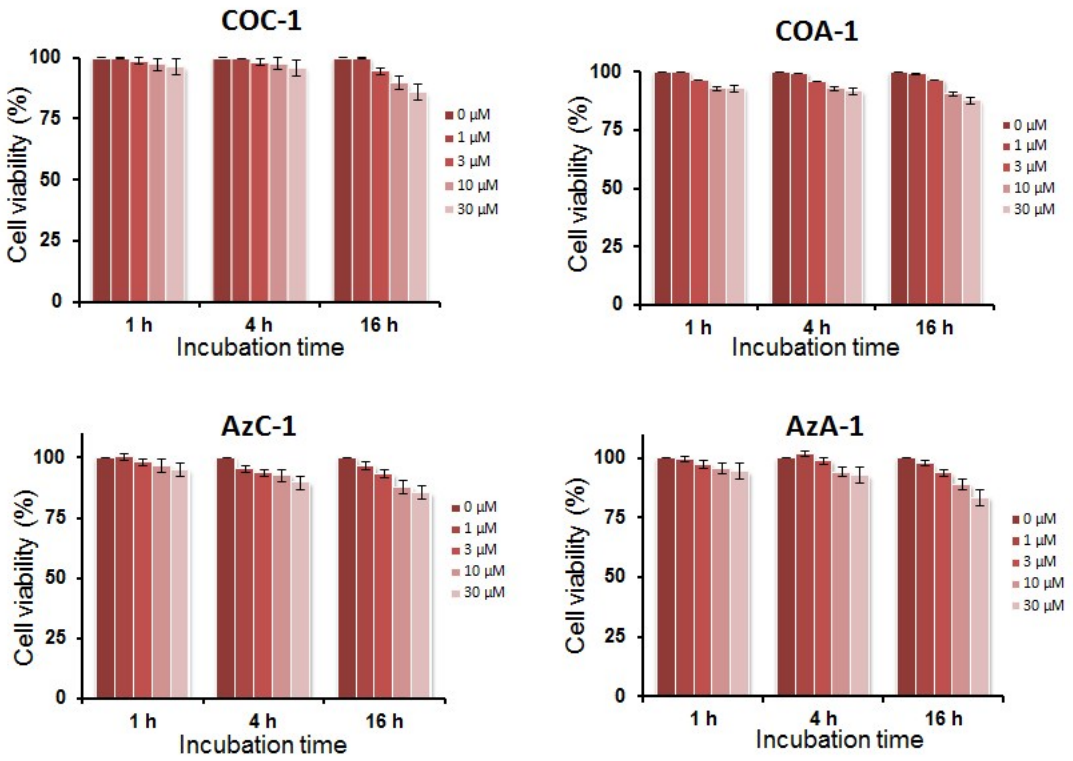


Figure S14. Cell viability test for COC-1, COA-1, AzC-1 and **AzA-1.**

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

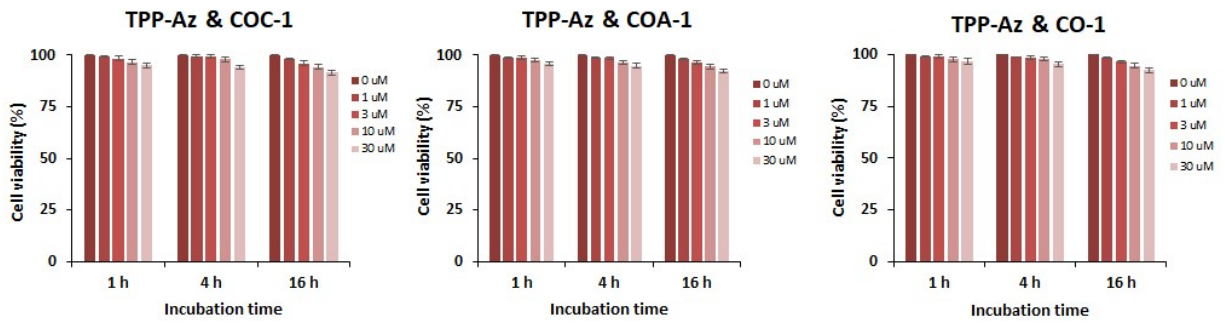


Figure S15. Cell viability test for TPP-Az pre-treated U-2 OS labelled with COC-1, COA-1, and CO-1.

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Before the addition of the probes, cells were treated with 5 μM of **TPP-Az** for 30 min at 37 °C. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

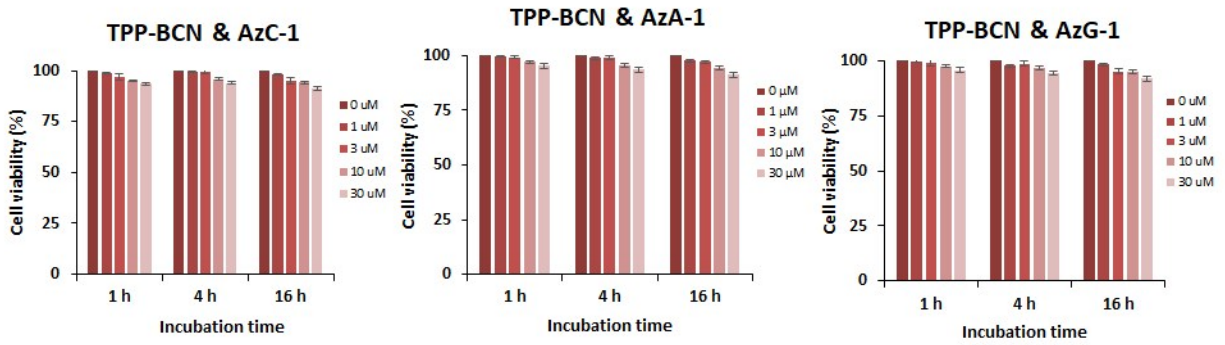


Figure S16. Cell viability test for TPP-BCN pre-treated U-2 OS labelled with AzC-1, AzA-1, and AzG-1.

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Before the addition of the probes, cells were treated with 5 μM of **TPP-BCN** for 30 min at 37 °C. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

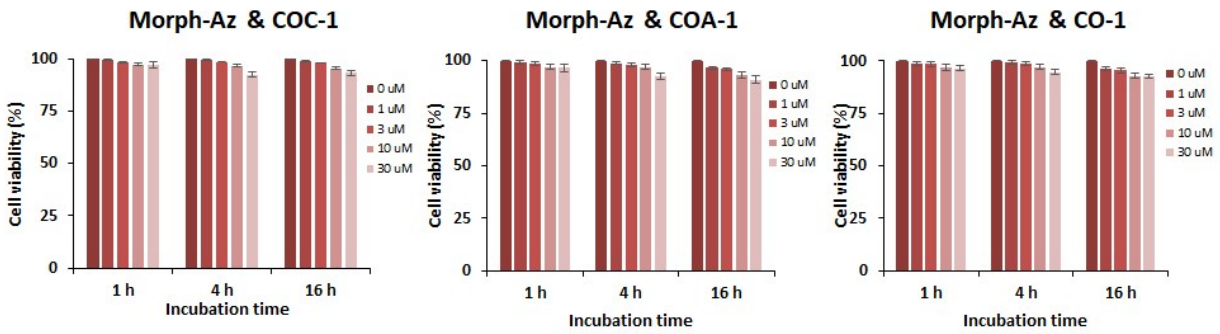


Figure S17. Cell viability test for Morph-Az pre-treated U-2 OS labelled with COC-1, COA-1, and **CO-1.**

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Before the addition of the probes, cells were treated with 5 μM of **Morph-Az** for 2 h at 37 °C. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

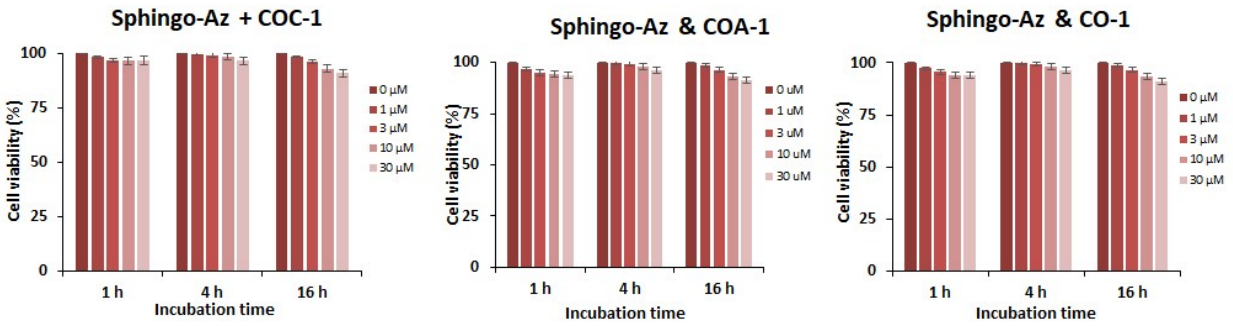


Figure S18. Cell viability test for Sphingo-Az pre-treated U-2 OS labelled with COC-1, COA-1, and **CO-1.**

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Before the addition of the probes, cells were treated with 5 μM of Sphingo-Az for 30 min at 4 °C followed by 30 min at 37 °C. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

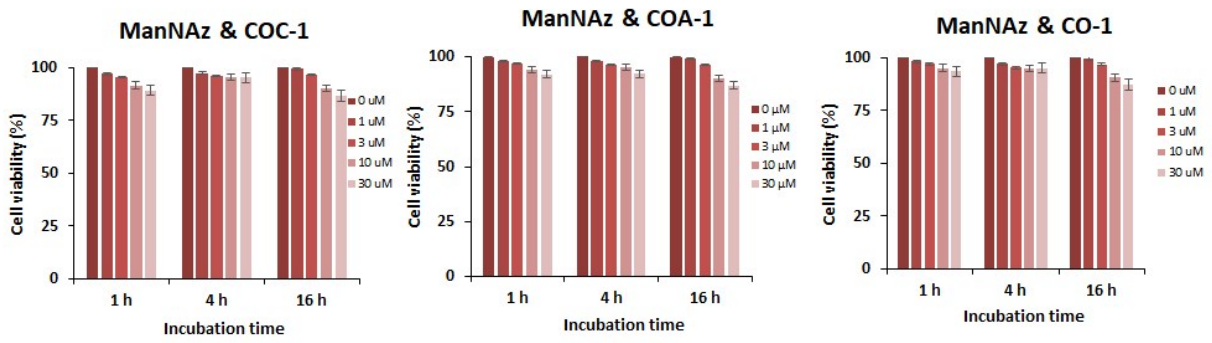


Figure S19. Cell viability test for ManNAz pre-treated U-2 OS labelled with COC-1, COA-1, and **CO-1.**

Cell viability was measured by MTS assay for concentration of probe at 0 μM, 1 μM, 3 μM, 10 μM and 30 μM in 1 hour, 4 hours and 16 hours incubation in U-2 OS cells. Before the addition of the probes, cells were treated with 50 μM of ManNAz for 24 h at 37 °C. Absorbance was determined at 490 nm. Each absorbance value was subtracted with blank sample (blank sample = cells containing compound at respective concentration without MTS reagent). Cell viability was calculated by: (viable cells)% = (OD of treated sample/OD of untreated sample)×100. Data are presented as the means ± SD obtained from triplicate experiments.

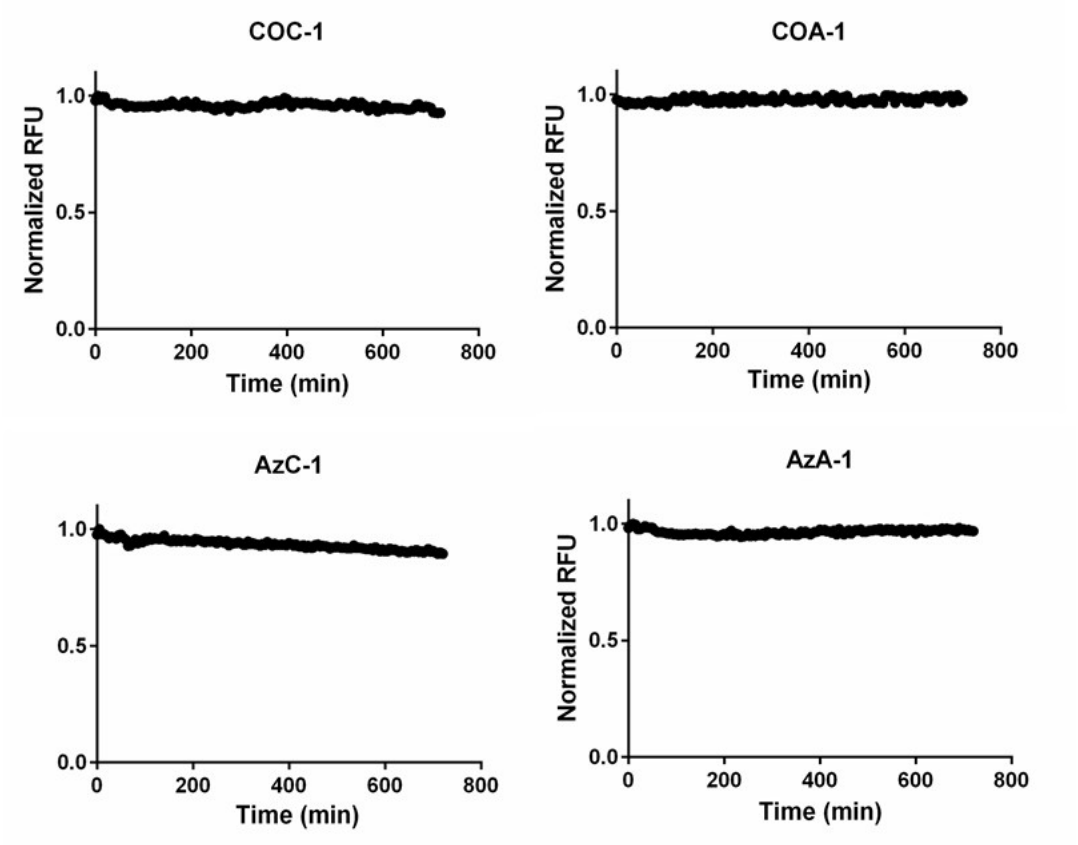


Figure S20. Photostability analysis for COC-1, COA-1, AzC-1 and **AzA-1.**

10 μM of the probes solution in PBS buffer (pH 7.4) containing 1% DMSO were placed in a 96-well plates. Fluorescence measurement were recorded every 30 seconds interval for a total period of 12 hours under a xenon flashlamp.

II. Supporting Tables

Table S1. Molecular descriptor values for COC-1, COA-1, AzC-1 and AzA-1

No	Probe name	SlogP	logS	Q_VSA_FNEG
1	**COC-1**	3.65	-5.26	0.34
2	**COA-1**	3.01	-4.94	0.36
3	**AzC-1**	2.98	-2.46	0.39
4	**AzA-1**	2.33	-2.14	0.43

Table S2. Spectroscopic properties of probes in DMSO (10 μM; **AzA-1** and **COA-1:** λ_{ex}= 370 nm; **AzG-1** and **CO-1:** λ_{ex}= 470 nm; **AzC-1** and **COC-1:** λ_{ex}= 500 nm)

	λ_{abs} (nm)	*Log* ε_{max}	λ_{em} (nm)	ϕ	Stokes shift (nm)
AzA-1	396	4.41	456	0.11	60
COA-1	394	4.3	458	0.21	64
AzG-1	498	4.31	513	0.75	15
CO-1	492	4.36	511	0.58	19
AzC-1	516	4.3	545	0.60	29
COC-1	516	4.42	545	0.39	29

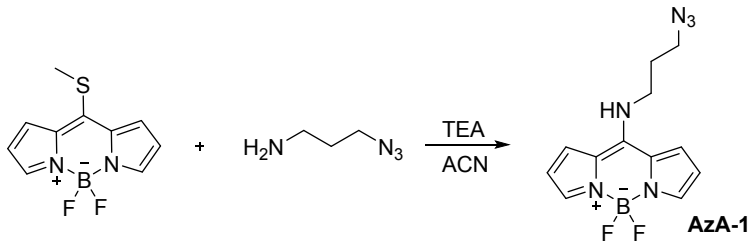
III. Materials and Methods

Reagent and general method

All chemical reagents for the probe synthesis were obtained from ThermoFisher, Sigma Aldrich, Alfa Aesar, or MERCK, and used without further purification unless otherwise specified. Column chromatography was carried out on Merck Silica Gel 60 (0.040-0.064 mm, 230–400 mesh). Synthetic reactions and analytical characterization were monitored by HPLC-MS (Agilient-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. NMR spectra (600 MHz and ^{13}C-151 MHz) were recorded on Bruker Avance 600 NMR spectrometers. The high resolution electron spray ionization (HR-ESI) mass spectra were obtained on a Bruker micrOTOFQII. Spectroscopic and quantum yield data were measured on spectroscopic measurements, performed on a fluorometer and UV/VIS instrument, Spectra Max M2 by Molecular Device.

Synthesis and Characterization

Synthesis of AzA-1



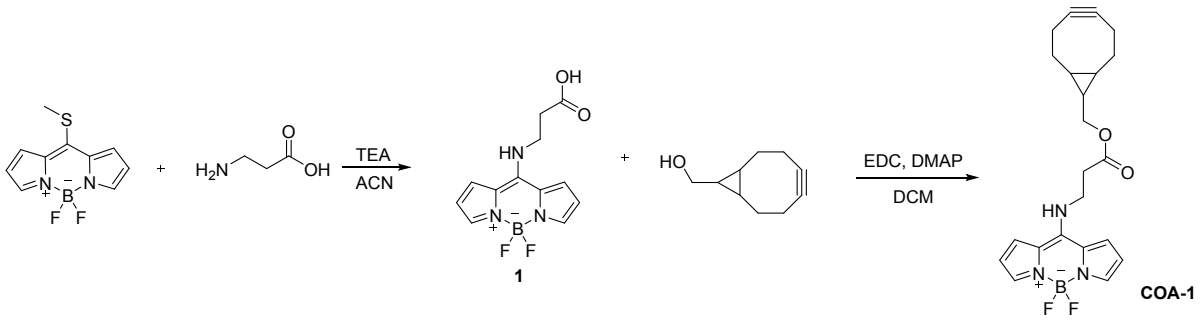
*Synthesis of **AzA-1**:* 5,5-difluoro-10-(methylthio)-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine[1] (3 mg, 0.013 mmol), 3-azidopropan-1-amine[2] (1.4 mg, 0.014 mmol) and TEA were dissolved together in acetonitrile (1 mL) and stirred for 2 h at room temperature. After removing the solvent, the crude material was purified by column chromatography (DCM). The product was obtained as yellow solid, yield: 2.8 mg (76 %).

HRMS m/z ($C_{12}H_{13}BF_2N_6$) calculated: 313.1161 (M+Na), found: 313.1157 (M+Na).

^{1}H NMR (600 MHz, CD_3CN) δ 7.96 (s, 1H), 7.60 (s, 1H), 7.39 (s, 1H), 7.35 (s, 1H), 7.24 (s, 1H), 6.57 (s, 1H), 6.42 (s, 1H), 3.84 (t, J = 7.0 Hz, 2H), 3.56 (t, J = 6.5 Hz, 2H), 2.10 (p, J = 6.7 Hz, 2H).

^{13}C NMR (151 MHz, CD_3CN) δ 149.71, 135.17, 131.99, 126.17, 124.82, 123.02, 116.97, 115.52, 114.21, 49.85, 45.67, 27.93.

Synthesis of COA-1



*Synthesis of **1***: 5,5-difluoro-10-(methylthio)-5H-4λ^4,5λ^4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (5 mg, 0.02 mmol), 3-aminopropanoic acid (2.7 mg, 0.03 mmol) and TEA were dissolved together in acetonitrile: H_2O (9:1, 1 mL) and stirred for 2 h at room temperature. After removing the solvent, the crude material was purified by column chromatography (DCM: MeOH = 20:1). The product was obtained as yellow solid, yield: 4.4 mg (53 %).

ESI-MS m/z ($C_{12}H_{12}BF_2N_3O_2$) calculated: 280.1 (M+H), found: 280.0 (M+H).

^{1}H NMR (600 MHz, MeOD) δ 7.58 (s, 1H), 7.40 – 7.30 (m, 3H), 6.56 (s, 1H), 6.38 (s, 1H), 4.05 (t, J = 6.8 Hz, 2H), 2.91 (t, J = 6.8 Hz, 2H).

^{13}C NMR (151 MHz, MeOD) δ 174.41, 150.22, 135.29, 132.25, 126.61, 124.67, 123.57, 117.34, 115.45, 114.09, 43.90, 33.12.

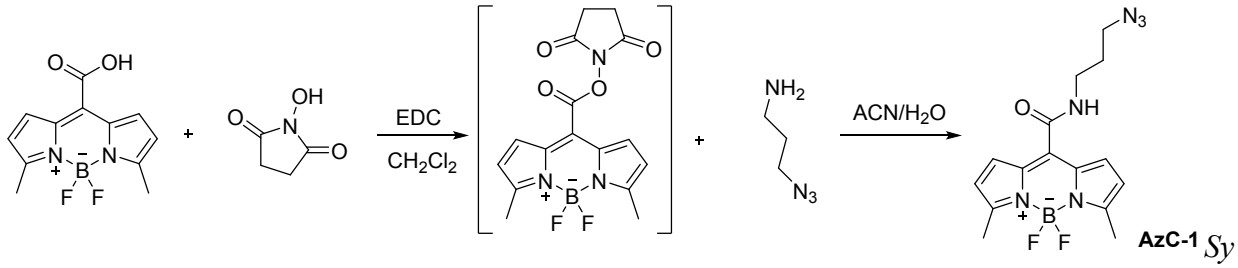
Synthesis of ***COA-1***: Compound **1** (5 mg, 0.018 mmol), bicyclo[6.1.0]non-4-yn-9-ylmethanol (3.3 mg, 0.022 mmol), EDC (5.2 mg, 0.027 mmol) and DMAP (1 mg, 0.009 mmol) were dissolved together in DCM (2 mL) and stirred for 8 h at room temperature. Water (0.5 mL) was added to the reaction mixture and the organic layer was extracted in DCM. The crude was purified by column chromatography (DCM: MeOH= 99:1). Product was obtained as yellow solid, yield: 3.3 mg (44 %).

HRMS m/z ($C_{22}H_{24}BF_2N_3O_2$) calculated: 434.1830 (M+Na), found: 434.1826 (M+Na).

^{1}H NMR (600 MHz, CD_3CN) δ 8.02 (s, 1H), 7.60 (s, 1H), 7.39 (s, 1H), 7.35 (s, 1H), 7.21 (s, 1H), 6.58 (s, 1H), 6.42 (s, 1H), 4.21 (d, J = 8.2 Hz, 2H), 4.05 (dd, J = 12.4, 6.2 Hz, 2H), 2.90 (t, J = 6.6 Hz, 2H), 2.26 – 2.08 (m, 4H), 1.54 (dd, J = 22.5, 11.0 Hz, 4H), 0.95 – 0.85 (m, 3H).

^{13}C NMR (151 MHz, CD_3CN) δ 171.86, 149.60, 135.33, 132.18, 126.09, 124.80, 122.98, 117.01, 115.61, 114.28, 99.60, 63.78, 43.79, 33.27, 29.71, 21.67, 20.88, 18.11.

Synthesis of AzC-1



Synthesis of ***AzC-1****:* To meso-BODIPY acid[3] (10 mg, 0.038 mmol) in DCM (10 mL) was added N-hydrosuccinimide (6.5 mg, 0.057 mmol) at room temperature. The reaction mixture was cooled to 0 °C, then EDC (24 mg, 0.095 mmol) was added. The reaction mixture was warmed to room temperature and stirred for 2 h. Water was added to the reaction mixture and the organic layerwas extracted in DCM. The crude was used for next step without further purification.

The crude solid and 3-azidopropan-1-amine (5.7 mg, 0.057 mmol) were dissolved together in acetonitrile: H_2O (9:1, 10 mL) and stirred for 6 h at room temperature. After removing the

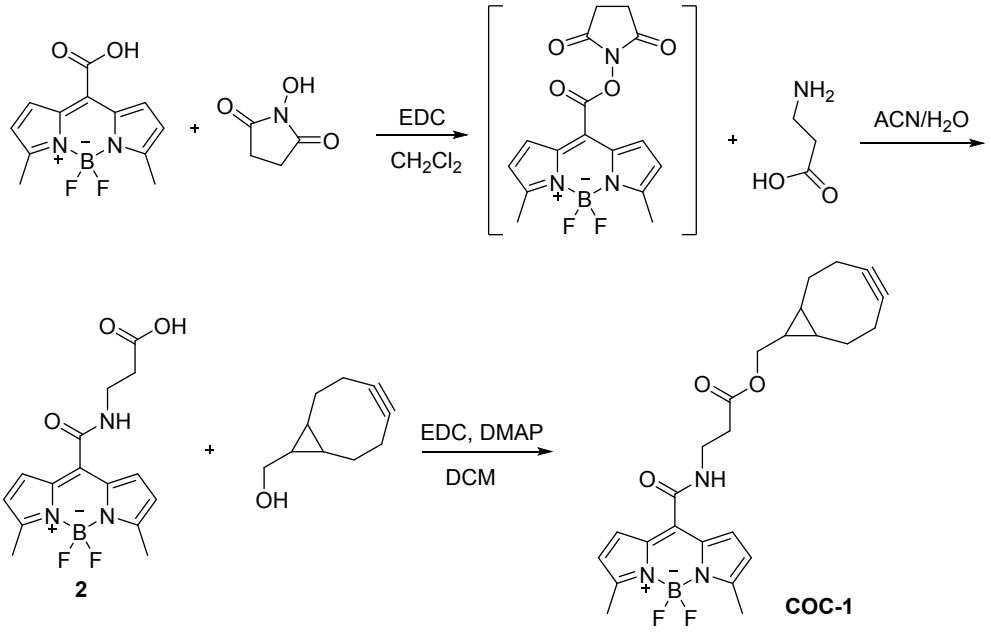
solvent, the crude was purified by column chromatography (DCM: MeOH = 20:1). Product was obtained as orange solid, yield: 4.2 mg (32 %).

HRMS m/z ($C_{15}H_{17}BF_2N_6O$) calculated: 347.1598 (M+H), found: 347.1600 (M+H).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.45 (d, J = 0.8 Hz, 1H), 7.26 (d, J = 3.5 Hz, 1H), 6.52 (s, 1H), 6.50 (d, J = 3.8 Hz, 1H), 3.77 (dd, J = 12.3, 5.9 Hz, 2H), 3.66 (t, J = 6.0 Hz, 2H), 2.81 (s, 6H), 2.10 (dt, J = 12.3, 6.1 Hz, 2H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 163.68, 160.34, 133.95, 132.47, 129.43, 120.59, 49.72, 38.30, 28.73, 15.29.

Synthesis of COC-1



*Synthesis of **2***: The crude solid and 3-aminopropanoic acid (4.9 mg, 0.055 mmol) were dissolved together in acetonitrile: H_2O (9:1, 10 mL) and stirred for 6 h at room temperature. After removing the solvent, the crude was purified by column chromatography (DCM: MeOH =9:1). Product was obtained as orange solid, yield: 4.3 mg (34 %).

ESI-MS m/z ($C_{15}H_{16}BF_2N_3O_3$) calculated: 334.1 (M−H), found: 334.0 (M−H).

^{1}H NMR (500 MHz, MeOD) δ 7.13 (d, J = 4.1 Hz, 2H), 6.39 (d, J = 4.2 Hz, 2H), 3.66 (t, J = 6.5 Hz, 2H), 2.66 (t, J = 6.5 Hz, 3H), 2.57 (s, 6H).

^{13}C NMR (126 MHz, MeOD) δ 175.38, 165.82, 160.88, 136.09, 133.57, 130.67, 121.19, 37.32, 34.51, 14.97.

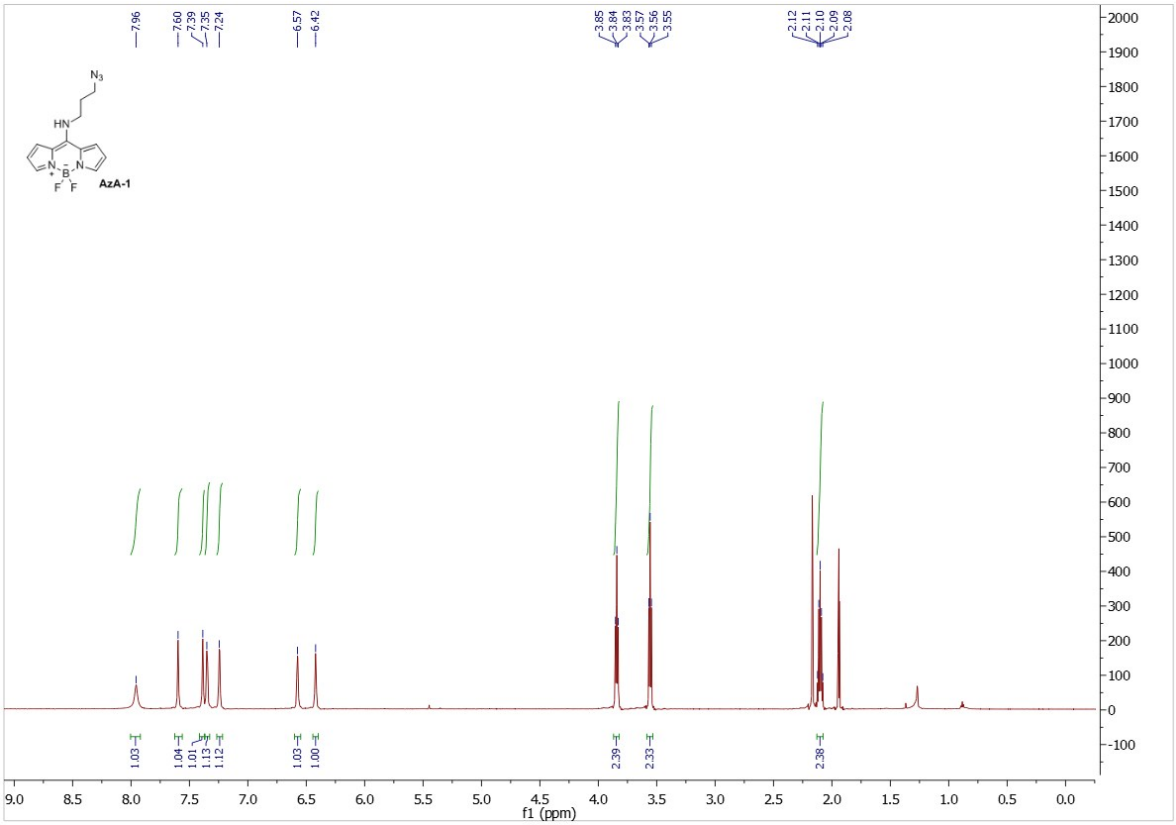
Compound **2** (4 mg, 0.012 mmol), bicyclo[6.1.0]non-4-yn-9-ylmethanol (2.1 mg, 0.014 mmol), EDC (3.5 mg, 0.018 mmol) and DMAP (0.37 mg, 0.003 mmol) were dissolved together in DCM (2 mL) and stirred for 8 h at room temperature. Water was added to the reaction mixture and the organic layer was extracted in DCM. The crude was purified by column chromatography (DCM: MeOH= 99:1). Product was obtained as orange solid, yield: 3.4 mg (61 %).

HRMS m/z ($C_{25}H_{28}BF_2NO_3$) calculated: 490.2089 (M+Na), found: 490.2088 (M+Na).

^{1}H NMR (600 MHz, CD_3CN) δ 7.21 (s, 1H), 7.02 (d, J = 17.1 Hz, 2H), 6.30 (d, J = 16.9 Hz, 2H), 4.08 (dd, J = 17.7, 8.0 Hz, 2H), 3.53 (dd, J = 11.6, 6.0 Hz, 2H), 2.54 (dd, J = 11.4, 6.4 Hz, 2H), 2.46 (d, J = 18.0 Hz, 6H), 2.15 – 2.04 (m, 4H), 1.45 (s, 4H), 0.80 (s, 3H).

^{13}C NMR (151 MHz, CD_3CN) δ 172.63, 163.80, 160.43, 136.13, 133.04, 130.58, 121.11, 99.56, 63.35, 36.67, 34.48, 29.64, 21.62, 20.79, 18.04, 15.10.

NMR Spectra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2.38
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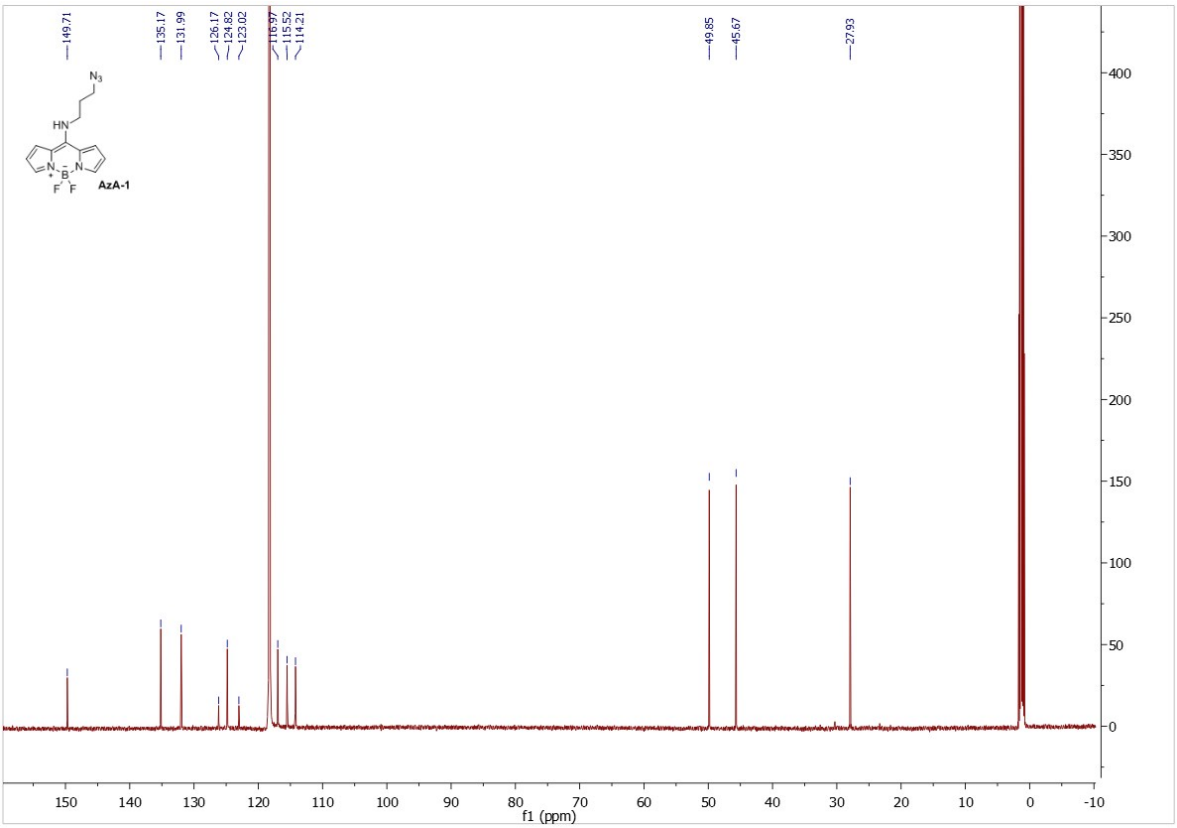
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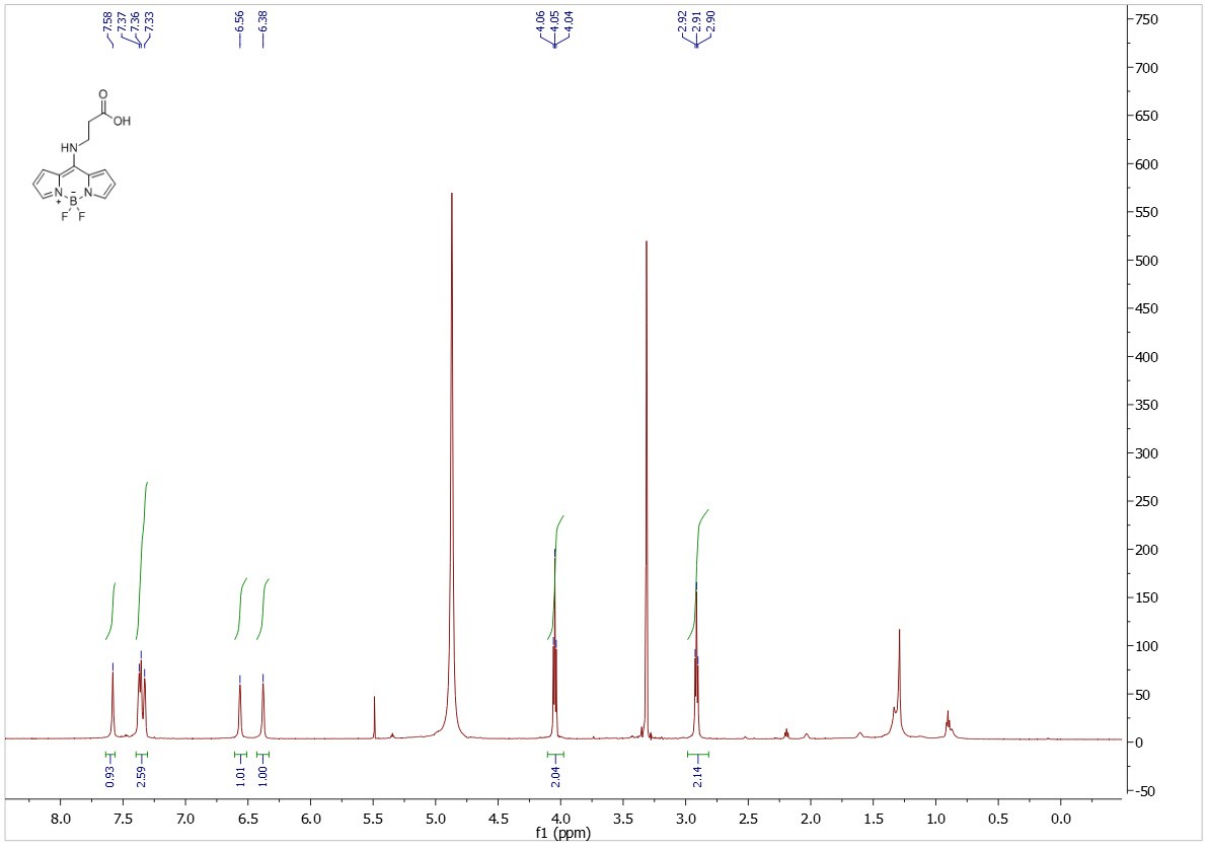
f1 (ppm)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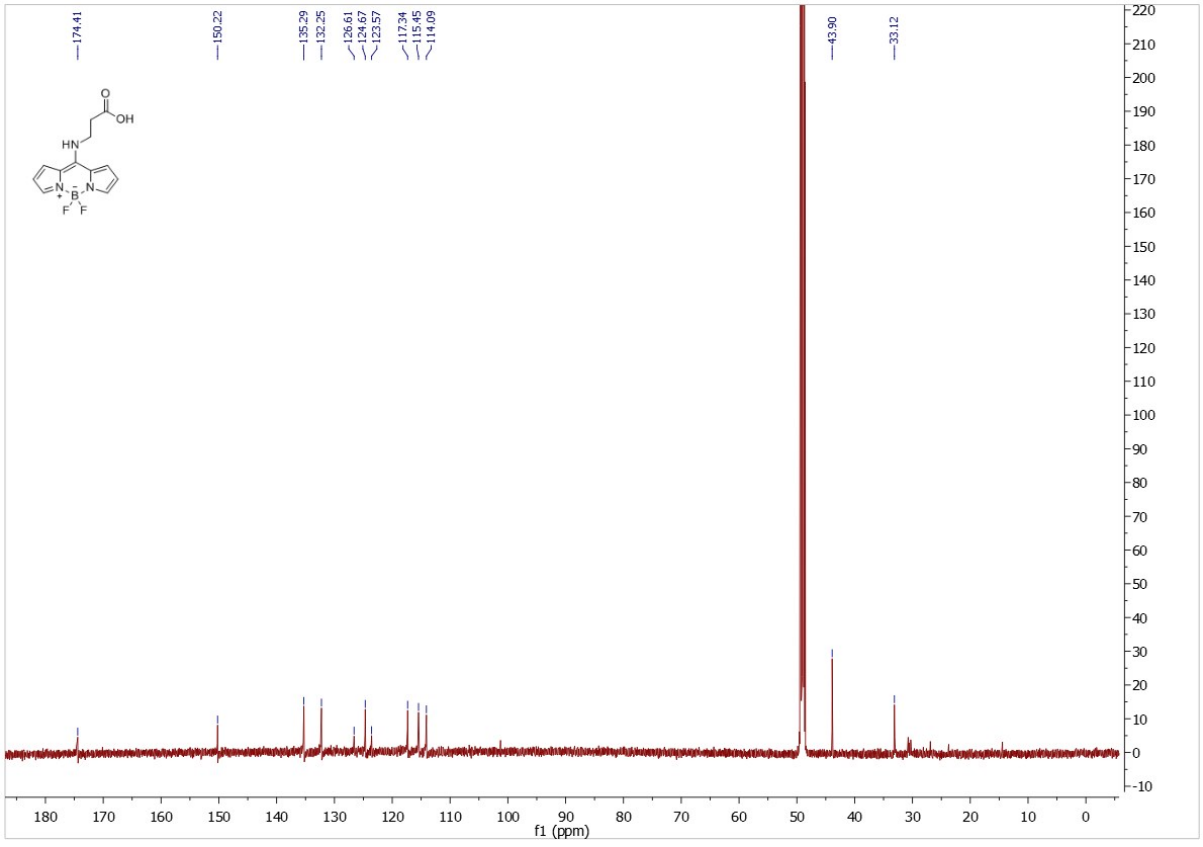
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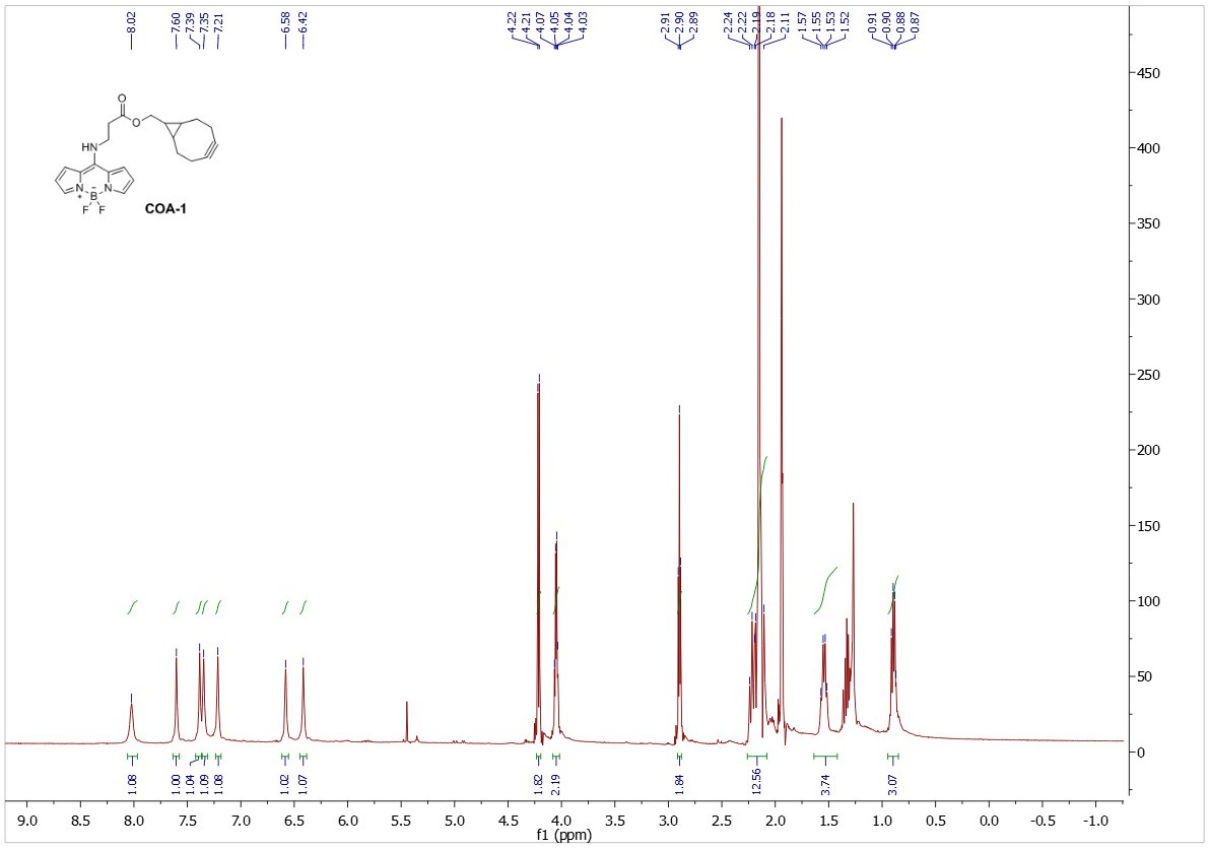
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2.90
2.89
2.24
2.22
2.19
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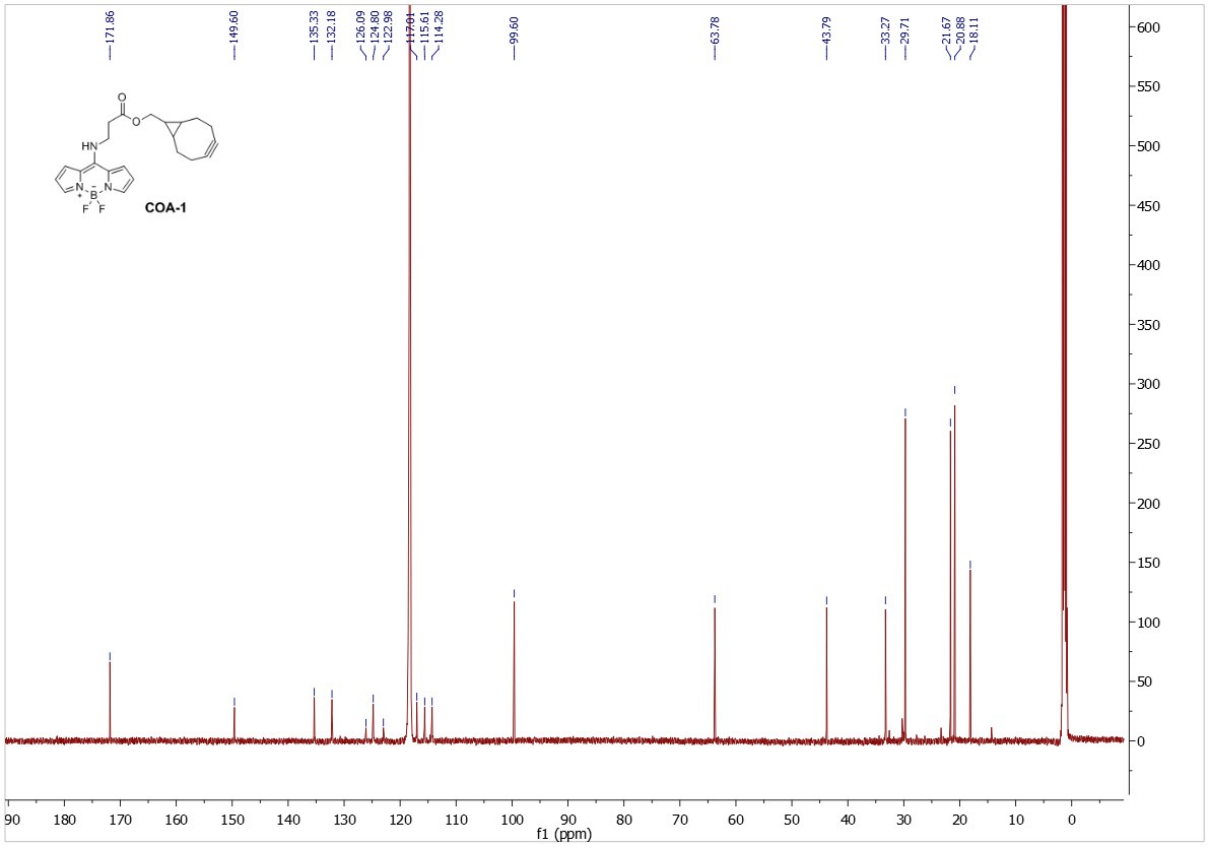
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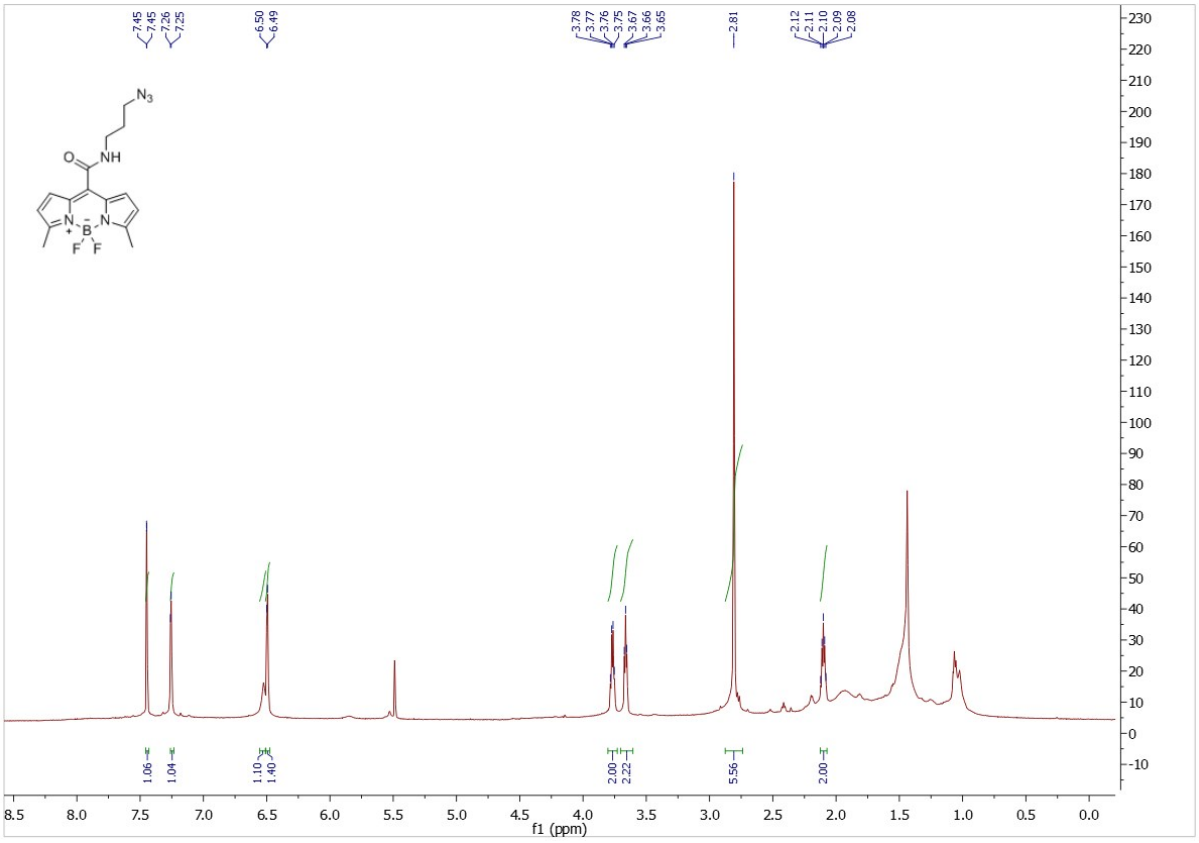
20.88
18.11
COA-1
f1 (ppm)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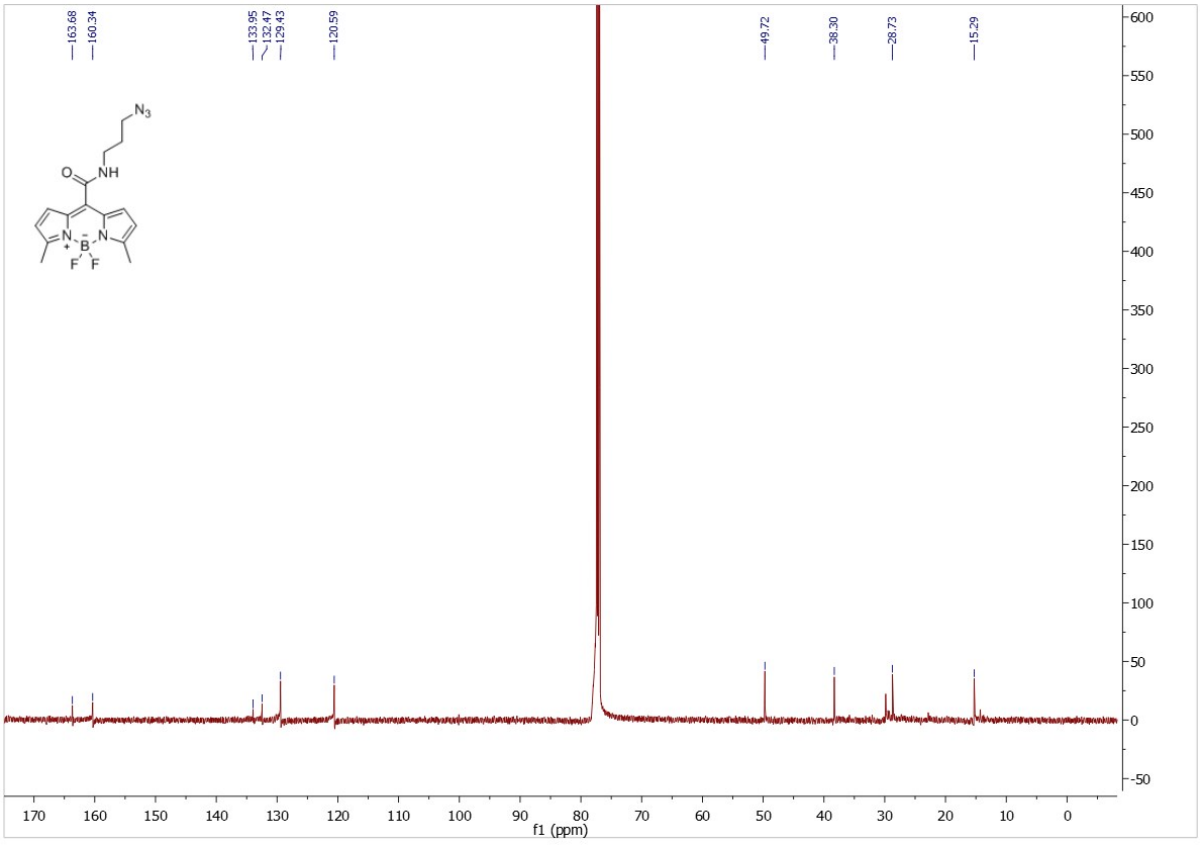
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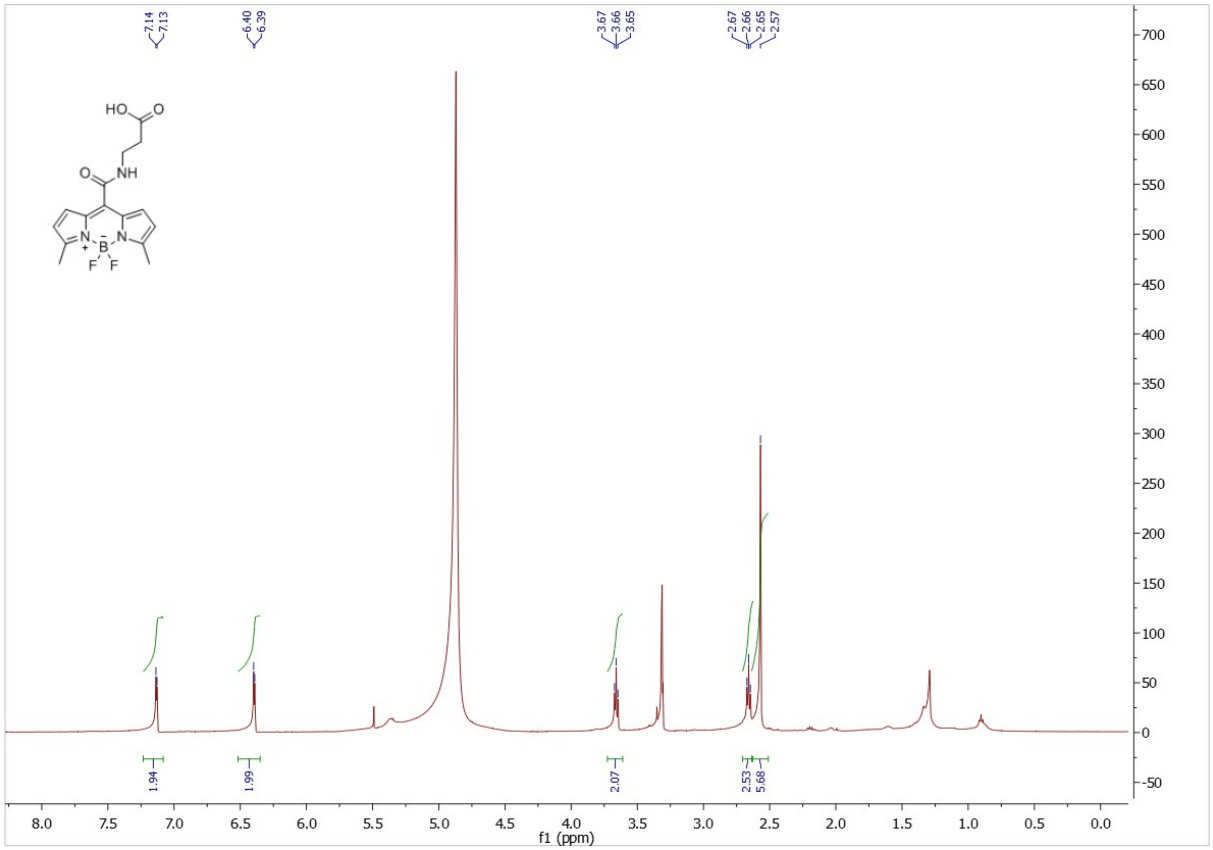
f1 (ppm)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B
F
F
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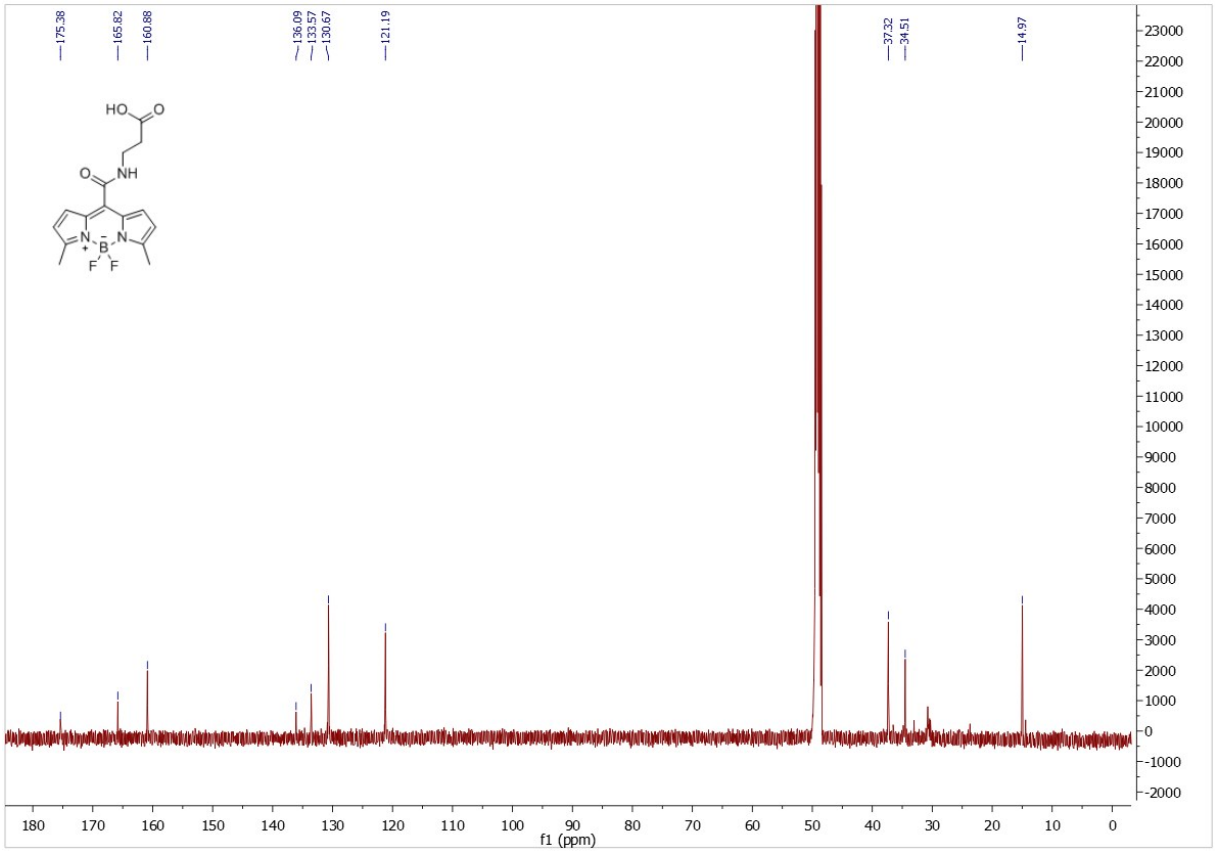
5.68
f1 (ppm)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F
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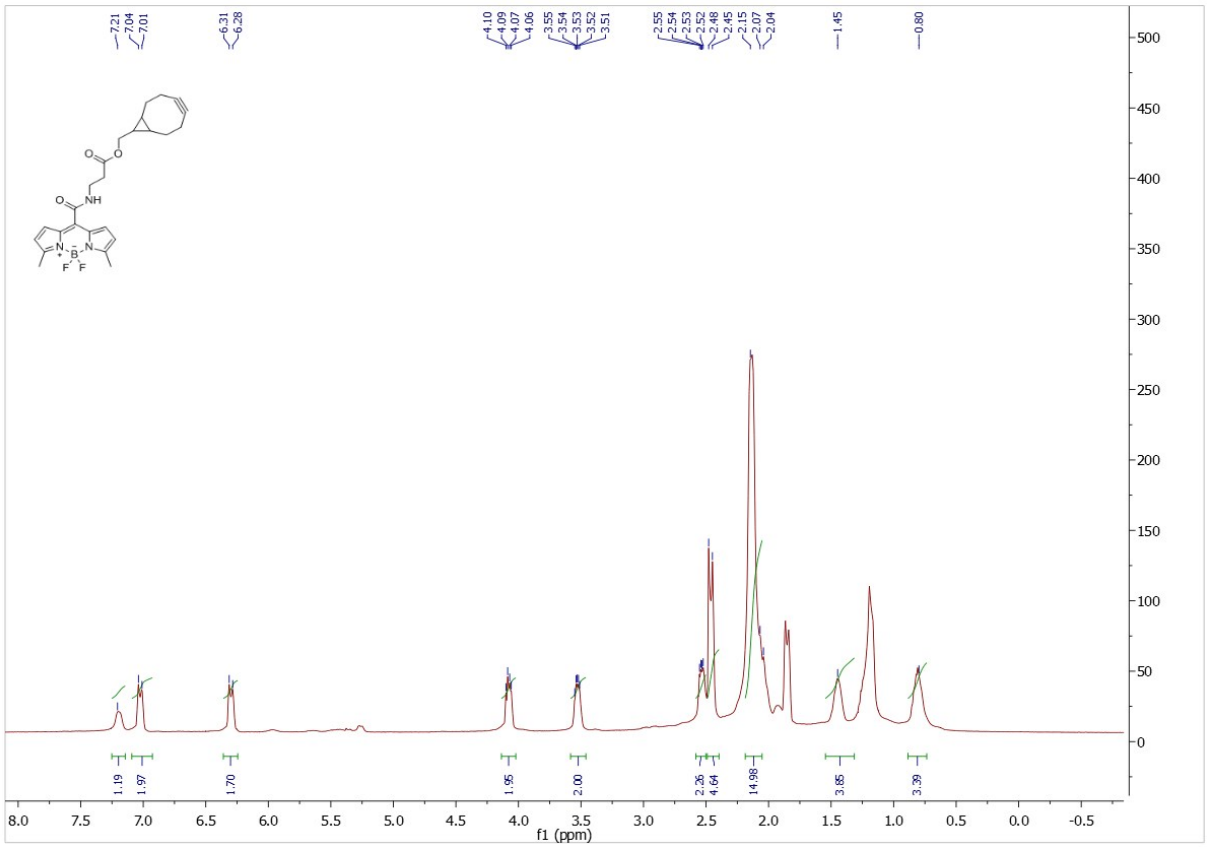
f1 (ppm)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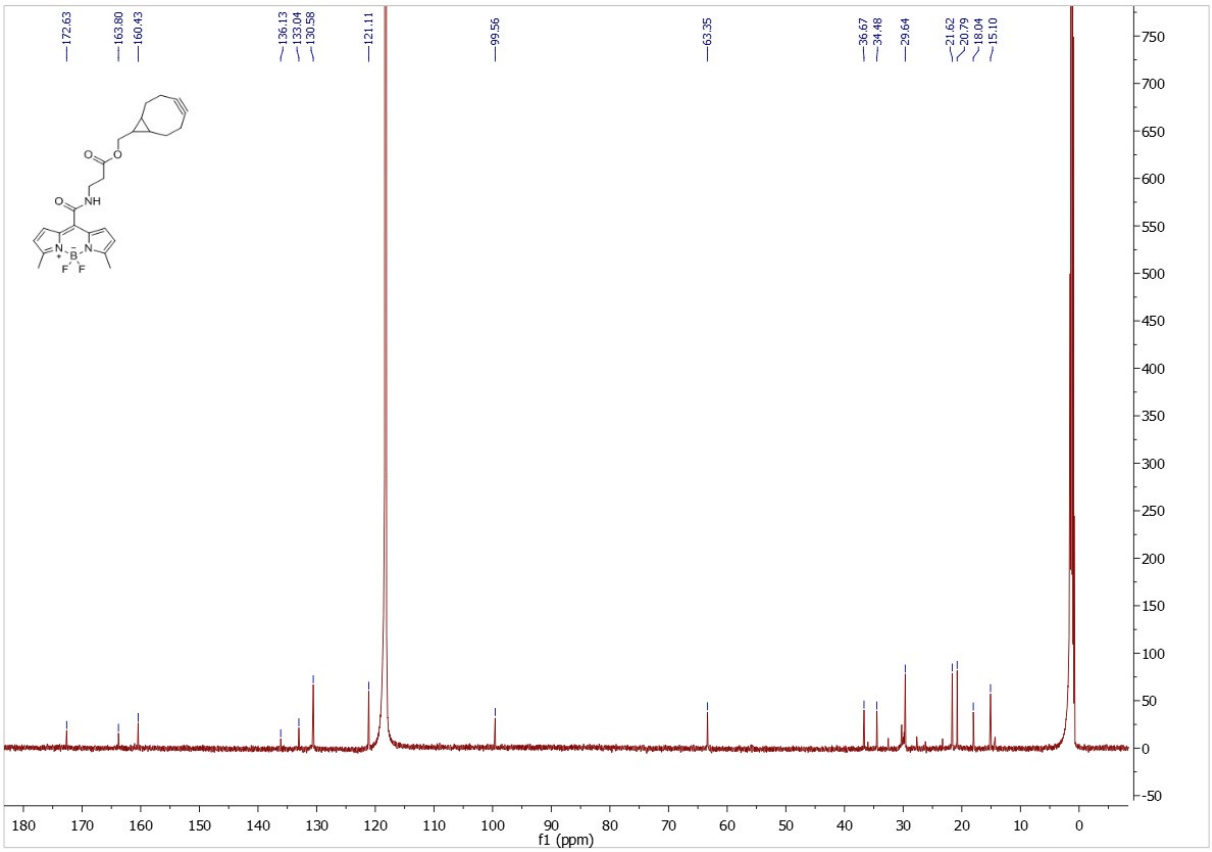
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21.62
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15.10
f1 (ppm)

IV. Supplementary References.

1. Banuelos, J.; Martin, V.; Gomez-Duran, C. F.; Arroyo Cordoba, I. J.; Pena-Cabrera, E.; Garcia-Moreno, I.; Costela, A.; Perez-Ojeda, M. E.; Arbeloa, T.; Lopez Arbeloa, I. *Chemistry* **2011**, *17*, 7261.
2. Shi, Y.; Pierce, J. G. *Org. Lett.* **2015**, *17*, 968.
3. Palao, E.; Slanina, T.; Muchova, L.; Solomek, T.; Vitek, L.; Klan, P. *J. Am. Chem. Soc.* **2016**, *138*, 126.