Supporting Information

A Photoactivable Formaldehyde Donor with Fluorescence Monitoring Reveals Threshold to Arrest Cell Migration

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1. Materials

Dimethylsulfide was purchased from Acros Organics. 3',4'-(Methylenedioxy)acetophenone was purchased from AK-Scientific. Nitromethane was purchased from Alfa Aesar. 4-Bromo-3-methylanisole was purchased from Combi-Blocks. Dimethylformamide was purchased from EMD Millipore. Acetic acid, dichloromethane, diethyl ether, ethyl acetate, hexanes and tetrahydrofuran were purchased from Fisher Chemical. Sodium chloride was purchased from Home Depot. Dimethylsulfoxide, hydrochloric acid, methanol, nitric acid and sodium hydroxide were purchased from Macron Fine Chemicals. 1-bromo-2-(trifluoromethyl)benzene, 3-bromophenol, diisopropylethylamine, dimethylcarbamoyl chloride, imidazole, *N*-bromosuccinimide, *N*-chlorosuccinimide, sodium bicarbonate, sodium borohydride, sodium sulfate, *tert*-butyldimethylsilyl chloride and tetrabutylammonium fluoride (1 M in THF) were purchased from Oakwood Chemical. Acetonitrile (anhydrous), benzoylperoxide (Luperox® A90), boric acid, dichloromethane (anhydrous), dichlorodimethylsilane, methyl 2-methylbenzoate, *n*-butyllithium (1.6 M in hexanes), phosphoric acid, *sec*-butyllithium (1.4 M in cyclohexane), sulfuryl chloride, *tert*-butyllithium (1.7 M in pentane) and ^{13}C-labeled dimethylsulfide were purchased from Sigma-Aldrich. 2,2',4,4'-tetrahydroxybenzophenone was purchased from 3B Scientific. Anhydrous diisopropylethylamine and tetrahydrofuran obtained through drying over activated 3 Å molecular sieves. All other chemicals were used as purchased without further purification. 3,6-dihydroxy-9*H*-xanthen-9-one was prepared from 2,2',4,4'-tetrahydroxybenzophenone following a reported procedure.[1] Phosphate buffered saline (PBS, 1x, pH 7.4) without calcium and magnesium (Corning) was used throughout all experiments.

Thin-layer chromatography (TLC) was performed using glass-backed TLC plates coated with silica gel containing an UV254 fluorescent indicator (Macherey-Nagel). Flash chromatography was performed with 60M silica gel with a particle size of 0.04-0.063 mm (Macherey-Nagel).

HEK 293 cells were purchased from ATCC. Cells were cultured in phenol-red free Dulbecco's modified eagle medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 1 mM sodium pyruvate and 1% penicillin/streptomycin (Corning). Serum free DMEM contained all the above-mentioned except for FBS. Cells were generally incubated at 37 °C under 5% CO_2. Experiments were performed in 8-well chambered cover glasses (Lab-Tek, Thermo Scientific) or 6-well plates (Nunclon Delta Surface, Thermo Scientific). Plates were pretreated with poly-L-lysine (Cultrex). Cell detachment throughout experiments was induced using 0.25% trypsin with 2.21 mM EDTA, 1x [-] sodium bicarbonate (Corning). Britton-Robinson buffer was freshly prepared from acetic acid, phosphoric acid and boric acid following a literature protocol.[2] This buffer was adjusted with concentrated sodium hydroxide solution to obtain the desired pH values. Tracker dyes for colocalization experiments (ER-Tracker Green, LysoTracker Green and MitoTracker Green) were purchased from Invitrogen, Thermo Fisher Scientific.

2. Instrumentation

NMR spectra were taken using Bruker or Varian 500 MHz spectrometers at 25 °C. The following abbreviations are used to describe coupling constants: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), doublet of triplets (dt), doublet of doublets of doublets (ddd), quartet of doublets (dq) and multiplet (m).

High-resolution mass spectra were acquired with a Waters Q-TOF Ultima ESI mass spectrometer and a Waters Synapt G2-Si ESI/LC-MS mass spectrometer.

UV-visible spectra were recorded on a Cary 60 spectrometer (Agilent Technologies).

Fluorescence spectra were recorded on a QuantaMaster-400 scanning spectrofluorometer (Photon Technology International) using micro fluorescence quartz cuvettes (Science Outlet).

Cell counting and cell viability evaluation was performed using a Countess II FL automated cell counter (Invitrogen, Thermo Fisher Scientific). Cell diameter evaluation for cell volume determination was performed using a TC20 automated cell counter (BIO-RAD).

An IVIS Spectrum in vivo imaging system (PerkinElmer) was used to acquire fluorescence images of cells cultured in 6-well plates. In our experiments the 570 nm excitation filter set and the 620 nm emission filter set was used together with auto exposure settings. Fluorescence cell imaging for the calibration curve assay was performed on an EVOS Cell Imaging System (Advanced Microscopy Group) equipped with a EVOS Cy5 Light Cube (Life Technologies). Confocal microscopy was performed on a Zeiss LSM 700 Confocal Microscope.

A 5810R (Eppendorf) and an accuSpin Micro 17R (Fisher Scientific) centrifuge were used for cell-related work.

The measurement of pH values in the context of buffer adjustments was performed on a SevenCompact pH-meter (Mettler Toledo).

Activation of photoFADs was accomplished using a high power LED Chip 30W Purple Ultraviolet UV 395nm/900mA/DC 30V-34V/30 Watt (Chanzon) in combination with a PS-305 D DC power supply (UniSource). For the determination of irradiation power a PM100D power meter (Thorlabs) in combination with a S121C photodiode power sensor (Thorlabs) was used. Photobleaching experiments were performed using a F30K LED flood light lamp (Techno Earth).

Data quantification, processing and representation was performed using ImageJ 1.52n, Perkin Elmer Living Image 4.5, Carl Zeiss ZEN 2.3 (black) 14.0, Microsoft Office 365 ProPlus and OriginPro 2017 b9.4.0.220.

3. Photophysical characterization

Absorbance and emission spectra were measured for 2 µM solutions in PBS buffer (pH 7.4, containing 0.1% DMSO). To obtain emission spectra, the photoFADs were excited 20 nm hypsochromic to the absorbance maximum of their corresponding dye products (excitation in the region of the absorbance maximum of photoFADs led to unwanted activation). All other compounds were excited 20 nm hypsochromic to their absorbance maximum.

Extinction coefficients (ε) were determined through measurement of the absorbance values of samples of known concentration in PBS buffer at six different concentrations with absorbance *A* values below $A = 1$. Three different stock solutions were prepared for each compound ($n = 3$).

Fluorescence quantum yields (Φ_F) were determined through comparison with standard samples following an established protocol.[3] Cresyl violet in ethanol was used as the standard.

4. Photobleaching assay

A quartz cuvette equipped with a stir bar was charged with 3 mL of 2 µM solutions of compounds **16**, **18** or **4** in PBS buffer (pH 7.4, containing 0.1% DMSO). The cuvette was placed at a distance of 6.5 cm in front of a white LED flood lamp. It was then irradiated while stirring (500 rpm) and fluorescence spectra were recorded at 0, 5, 10, 15, 20, 25 and 30 min of total irradiation time. The spectra were

integrated for quantification. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained values were used for error evaluation.

5. Kinetic assay

A quartz cuvette equipped with a stir bar was charged with 3 mL of 2 µM solutions of photoFAD-1, photoFAD-2 or photoFAD-3 in PBS buffer (pH 7.4, containing 0.1% DMSO). The cuvette was placed at a distance of 1 cm in front of a 395 nm LED array. The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the cuvette. The solution was irradiated while stirring (500 rpm) and fluorescence spectra were recorded every minute for 10 min of total irradiation time. In order to achieve full turn over photoFAD-1, photoFAD-2 and photoFAD-3 were irradiated for an additional 10, 30 and 40 min, respectively and fluorescence spectra were recorded. The obtained fluorescence spectra were integrated for quantification. In a separate experiment 2 µM solutions of photoFAD-3 in PBS buffer (pH 7.4, containing 0.1% DMSO) were photoactivated with different light intensities to obtain different initial release rates. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

6. Turn-over assay

A quartz cuvette equipped with a stir bar was charged with 3 mL of 2 µM solutions of photoFAD-1, photoFAD-2 or photoFAD-3 in PBS buffer (pH 7.4, containing 0.1% DMSO). The cuvette was placed at a distance of 1 cm in front of a 395 nm LED array. The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the cuvette. The solution was irradiated while stirring (500 rpm) and fluorescence spectra were recorded at various time points between 0 and 60 min of total irradiation time. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

7. Mass spectrometry evaluation of photoFAD activation

To confirm the formation of **4** upon irradiation of photoFAD-3, 200 µL of a 50 µM solution of photoFAD-3 in deionized water (containing 2.5% DMSO) was added to a glass vial with stir bar. The vial was placed at a distance of 1 cm in front of a 395 nm LED array (~10 mW). The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the vial. The solution was irradiated for 1 h while stirring (500 rpm) and the resultant magenta-colored solution was subjected to mass spectrometric analysis.

8. Confirmation of formaldehyde formation

To confirm the formation of FA from photoFAD-3 upon irradiation with light, a solution of ^{13}C-photoFAD-3 deuterated methanol was irradiated in an NMR tube using a 395 nm LED array. The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the solution. ^{13}C NMR spectra were recorded at 0, 30 and 180 min of total irradiation time. A FA reference sample was prepared by heating a suspension of paraformaldehyde in deuterated methanol

to 80 °C for 1 h. It was allowed to cool to room temperature before the supernatant was taken up and filled into an NMR tube upon filtration through cotton.

9. Demonstration of spatial control of photoFAD activation

HEK 293 cells were incubated with a solution of 0.5 µM Calcein AM and 5 µM photoFAD-3 in DPBS buffer (pH 7.4) for 30 min at 37 °C. The solution was replaced with fresh DPBS buffer and cells were imaged via fluorescence microscopy. The activation of photoFAD-3 was performed through irradiation of a selected field of cells with the DAPI channel in combination with a high magnification objective (100x). Fluorescence microscopy images were acquired at low magnification (4x).

10. Evaluation of stability under ambient conditions

To confirm the stability of photoFAD-3 under ambient laboratory conditions, an 8 mL scintillation vial was charged with 3 mL of 2 µM photoFAD-3 in PBS (pH 7.4, 0.1% DMSO). 1 mL of this solution was transferred into a 1 mL quartz cuvette and the fluorescence spectrum was recorded. The residual 2 mL were stored in the capped vial either on the bench top under ambient light or in the dark (control). After a storage period of 2 h 1 mL of the solution was transferred into a 1 mL quartz cuvette and the fluorescence spectrum was recorded. The cuvette was subsequently placed in 1.0 cm distance in front of a 395 nm LED array. The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the cuvette. The solution was irradiated for 30 min. After completion of the irradiation, the fluorescence spectrum was recorded to confirm functionality of photoFAD-3 after storage. Fluorescence spectra were integrated for quantification. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

11. Evaluation of stability under biological conditions

To confirm the stability of photoFAD-3 under biological conditions, a 2 µM solution of photoFAD-3 in DMEM (10% FBS, 0.1% DMSO, 7 mL) was added into 8 mL a scintillation vial. The solution was incubated at 37 °C in a water bath under exclusion of light. At 0, 1, 3, 8 and 24 h of total incubation time, 1 mL was transferred into a quartz cuvette and the fluorescence spectrum was recorded. After 24 h of incubation, the cuvette was placed at a distance of 1 cm in front of a 395 nm LED array. The LED array was operated at 0.05 A and 29.1-29.3 V to obtain an irradiation power of ~10 mW at the position of the cuvette. The solution was irradiated for 30 min and the fluorescence spectrum was recorded to confirm that photoFAD-3 could still be activated. The solutions were excited at 580 nm and the emission was recorded between 590 nm and 720 nm. Fluorescence spectra were integrated for quantification. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

12. Evaluation of chemostability

Initial emission spectra of 2 µM photoFAD-3 solutions in PBS buffer (pH 7.4) were recorded. 100 mM stock solutions of hydrogen peroxide, *tert*-butyl hydroperoxide, cysteine, glutathione and sodium hypochlorite were prepared. A 100 mM stock solution of potassium superoxide in DMSO was prepared.

A 100 mM stock solution of Mohr's salt in 0.1 N aqueous and degassed hydrochloric acid was prepared. Test solutions for radical species were obtained by addition of appropriate amounts of either hydrogen peroxide or *tert*-butyl hydroperoxide together with 10 µL of the Mohr's salt stock solution to 2 µM photoFAD-3 in degassed PBS buffer (pH 7.4) under nitrogen atmosphere. All other test solutions were prepared by addition of the appropriate amounts of the stock solutions to the previously measured solutions of photoFAD-3. All test solutions were incubated for 30 min at 37 °C. The emission spectra were recorded and normalized to the initial recording of the corresponding sample. A 2 µM solution of photoFAD-3 in Britton-Robinson buffer at pH 7, that was activated with light for 30 min before the solution was incubated for 30 min at 37 °C, was used as a positive control (*hv*). The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

13. Evaluation of stability at different pH

2 µM solutions of photoFAD-3 in Britton-Robinson buffer across a pH range of 4 to 9 were prepared. The solutions were incubated for 30 min at 37 °C and the emission curves were recorded. A 2 µM solution of photoFAD-3 in Britton-Robinson buffer at pH 7, that was activated with light for 30 min before the solution was incubated for 30 min at 37 °C, was used as a positive control (pH 7 + *hv*). The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

14. Cytotoxicity assay

6-well plates were seeded with 3.0 x 10^5 cells/well and incubated at 37 °C for 48 h. The medium was removed and photoFAD-3, turn-over dye, or DMSO control was applied as a 4 µM solution in 2 mL DMEM (10% FBS). After 4 and 8 h, cells were suspended via trypsinization, pelleted via centrifugation at 1000 × g for 5 min and the supernatant was discarded. The pellet was re-suspended in PBS (300 µL) and mixed 1:1 with trypan blue (0.4 % w/v stock in PBS). The mixture was allowed to incubate for 1-3 min at room temperature and viability was evaluated with an automated cell counter. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

15. Co-localization imaging

HEK 293 cells were seeded in an 8-well chambered cover glass and grown to ~50% confluency. The medium was removed and cells were treated with 400 µL of 4 µM photoFAD-3 or compound **4** in serum-free DMEM for 30 min at 37 °C. The medium was replaced with 400 µL of serum-free DMEM containing MitoTracker Green (50 nM), LysoTracker Green (50 nM), ER Tracker Green (100 nM). Cells were imaged at 63x magnification with an oil immersion objective and Pearson correlation factors were determined with ImageJ to evaluate co-localization.

16. Evaluation of cell lysate derived spectroscopy samples

Confluent HEK 293 cells were treated with trypsin (2.0 mL per T75 flask) and incubated at 37 °C until detachment was observed. Trypsin was quenched by addition of DMEM containing 10% FBS (10 mL).

Cells were counted to determine concentration of the suspension and it was transferred into 15 mL conical tubes. The cells were spun down (200 x g, 5 min, 4 °C) and the supernatant was removed. The obtained cell pellets were incubated in the presence of DMSO (300 µL per 11.2 million cells) under sonication (sonication bath) for 30 min. PBS buffer (1.2 mL per 11.2 million cells) was added to each sample and carefully mixed. The cell debris was removed via centrifugation (1800 x g, 15 min, 4 °C) and the obtained supernatant was transferred into 1.6 mL Eppendorf tubes for further purification via centrifugation (17,000 x g, 10 min, 20 °C). The supernatant was collected and used to prepare solutions of compound **4** with a concentration of 10 nM, 40 nM and 70 nM. The emission spectra of these solutions were recorded. Solutions of compound **4** in regular PBS with a concentration of 10 nM, 40 nM and 70 nM were prepared and their emission spectra were recorded. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

17. Formaldehyde quantification study

Five 6-well plates with confluent HEK 293 cells were used in this study. The cells were incubated with solutions of 5 µM photoFAD-3 in serum-free DMEM (2 mL) for 30 min at 37°C. The medium was replaced with serum-free DMEM (3 mL) and each well was irradiated using a 395 nm LED array that was placed at a distance of 1 cm from the cells (~10 mW). Irradiation times were varied between 0 and 180 s (one condition per plate) to obtain different degrees of photoactivation. 20 min after the beginning of the photoactivation, the cells were either imaged using an epi fluorescence microscope (at 20x magnification) or full plates were imaged using an IVIS imaging system. The cells were subsequently detached via treatment with trypsin (0.5 mL per well) and incubated at 37 °C until detachment was observed. Trypsin was quenched by addition of DMEM containing 10% FBS (1.5 mL) and the contents of two wells were transferred and combined in a 15 mL conical tube to obtain three sample per irradiation condition (n = 3). Each well was washed with PBS (2 mL) and the resultant suspensions were transferred to the corresponding conical tubes. The individual cell suspensions were carefully mixed and the cell concentration was determined using an automated cell counter. The cells were spun down (200 x g, 5 min, 4 °C) and the supernatant was removed. The obtained cell pellets were incubated in the presence of DMSO (300 µL) under sonication (sonication bath) for 30 min. PBS buffer (1.2 mL) was added to each sample and carefully mixed. The cell debris was removed via centrifugation (1800 x g, 15 min, 4 °C) and 1 mL of the obtained supernatant was transferred into a 1.6 mL Eppendorf tube for further purification via centrifugation (17,000 x g, 10 min, 20 °C). 900 µL of the supernatant were transferred into a quartz cuvette and the fluorescence spectrum of each sample was recorded.

To construct a reference curve, six solutions of photoFAD-3 in a range of 0 and 7 µM in 1:4 DMSO/PBS were prepared from 2 mM stock solutions in DMSO. A total of 1 mL of each solution was pre-mixed in a 1.6 mL Eppendorf tube. 900 µL of each sample were transferred into a quartz cuvette and the fluorescence spectrum of each sample was recorded. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

18. Wound healing assay

Confluent HEK 293 cells in 6-well plates were treated with 5 µM solutions of photoFAD-3 or Ctrl-photFAD-3 in serum-free DMEM (2 mL per well). The cells were incubated for 30 min at 37 °C and the medium was subsequently replaced with serum-free DMEM (3 mL). The photolabile compounds were

activated using a 395 nm LED array that was placed at a distance of 1 cm from the cells (~10 mW). Irradiation times were varied between 0 and 180 s to obtain different degrees of photoactivation. 20 min after the beginning of the photoactivation, the plates were imaged using an IVIS imaging system. After imaging was complete, the medium was replaced with DMEM containing 10% FBS (3 mL) and a wound was created by scratching the well with a 200 µL plastic pipette tip. The cells were incubated for 24 h at 37 °C and the wounds were monitored via brightfield microscopy (20x magnification) at various time points between 0 and 24 h after the wound was introduced. Total radiant efficiencies were used for emission quantification. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

19. AM-FAD-3 assay

HEK 293 cells in 6-well plates were cultured to confluency and treated with 5 µM solutions of photoFAD-3 or AM-FAD-3 in serum-free DMEM (2 mL per well). The cells were incubated for 30 min at 37 °C and the medium was subsequently replaced with serum-free DMEM (3 mL). Cells that have been treated with photoFAD-3 were irradiated for 180 s using a 395 nm LED array placed at a distance of 1 cm from the cells (~10 mW). 20 min after the beginning of the photoactivation the medium was replaced with DMEM containing 10% FBS (3 mL) and a wound was created by scratching the well with a 200 µL plastic pipette tip. The cells were incubated for 24 h at 37 °C and the wounds were monitored via brightfield microscopy (20x magnification) at various time points between 0 and 24 h. The experiment was performed in triplicate (n = 3) and the standard deviations of the obtained mean values were used for error evaluation.

20. Apoptosis assay

HEK 293 cells cultured to 70-80% confluency in a 6-well plate were treated with a 5 µM solution of photoFAD-3 in serum-free DMEM (2 mL per well). The cells were incubated for 30 min at 37 °C and the medium was subsequently replaced with serum-free DMEM (3 mL). Wells were irradiated using a 395 nm LED array that was placed at a distance of 1 cm from the cells (~10 mW). Irradiation times were varied between 0 and 180 s to obtain different degrees of photoactivation. The medium was subsequently replaced with DMEM containing 10% FBS (3 mL), for the positive control the medium contained 10 µM raptinal (apoptosis associated cytotoxin). The cells were incubated at 37 °C for 8 h. The medium was transferred into conical tubes and the cells were brought into suspension via treatment with trypsin and transferred into the corresponding conical tubes. The wells were washed with PBS (2 mL) and the suspensions were also transferred. The cells were spun down (1000 x g, 3 min, 4 °C) and the supernatant was discarded. The pellet was resuspended in PBS (900 µL), it was transferred to a 1.6 mL Eppendorf tube and the cells were spun down (1000 x g, 3 min, 4 °C). The supernatant was discarded and the pallets were stored at -80 °C overnight. The samples were thawed and subjected to SDS PAGE. The gels were blotted onto a membrane, which was subsequently incubated with PARP- and actin-specific antibodies. Bands were visualized through horseradish peroxidase and luminol induced chemiluminescence.

21. Supporting tables and figures

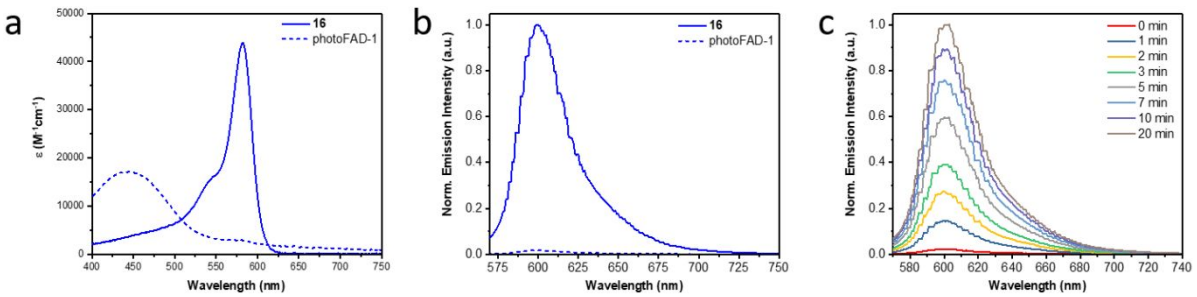


Figure S1: Photophysical properties of 2 µM photoFAD-1 and compound **16** in PBS buffer. (a) Absorbance spectra. (b) Emission spectra. (c) Emission spectra of photoFAD-1 upon photoactivation for different amounts of time between 0 min and 20 min.

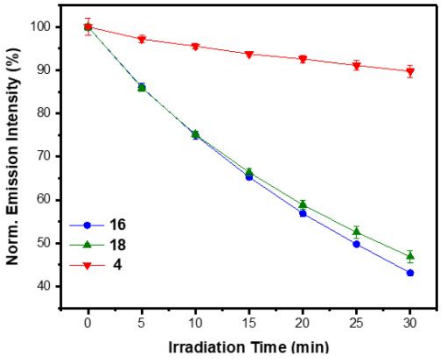


Figure S2: Photostability of 5 µM **16**, **18** and **4** in PBS buffer. Samples were irradiated for a total of 30 min with a white LED flood lamp. Data are represented as mean ± SD (*n* = 3).

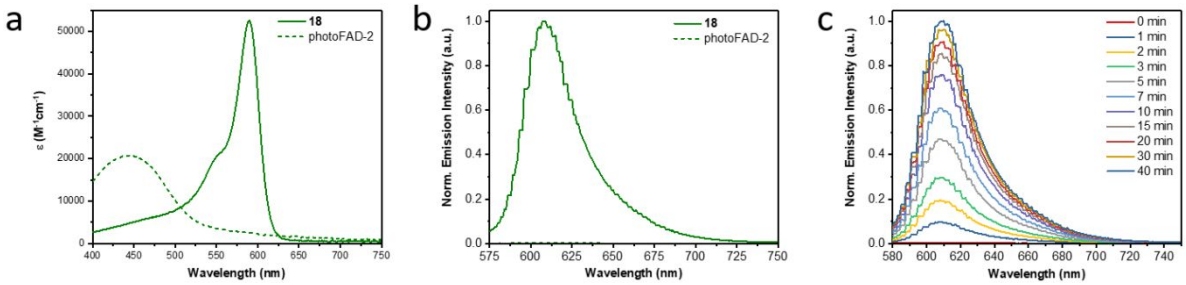


Figure S3: Photophysical properties of 2 µM photoFAD-2 and compound **18** in PBS buffer. (a) Absorbance spectra. (b) Emission spectra. (c) Emission spectra of photoFAD-2 upon photoactivation for up to 40 min.

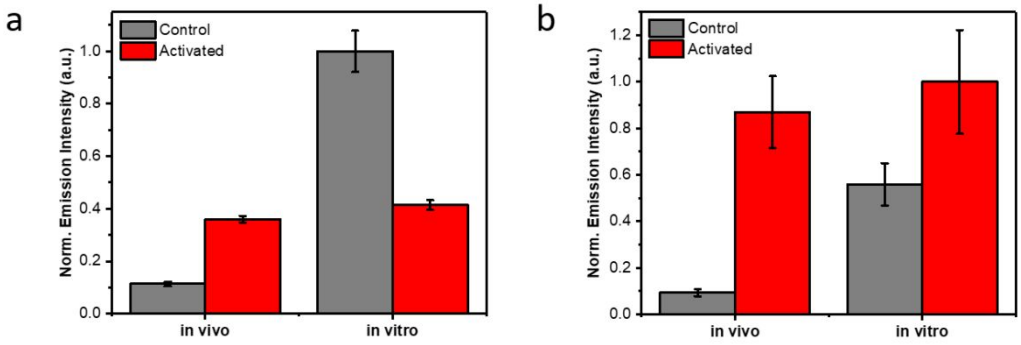


Figure S4: Assessment of dye retention upon photoactivation. HEK 293 cells were incubated with 5 µM (a) photoFAD-2 or (b) photoFAD-3. One set of cells was photoactivated for 3 min while the other (control) was not. The cells were lysed and the fluorescence signal of the lysate after in vivo photoactivation was evaluated. All samples (in vivo activated & control) were subsequently photoactivated in vitro until all photoFAD was fully converted into its dye product. Comparison of the fluorescence signals of control and in vivo activated cells indicates the retention of the dye in cells after the initial in vivo activation. While the dye product of photoFAD-2 (**18**) is leaking out of the cells (lower signal than control), the dye product of photoFAD-3 (**4**) is retained (higher signal than control). Data are represented as mean ± SD (*n* = 3).

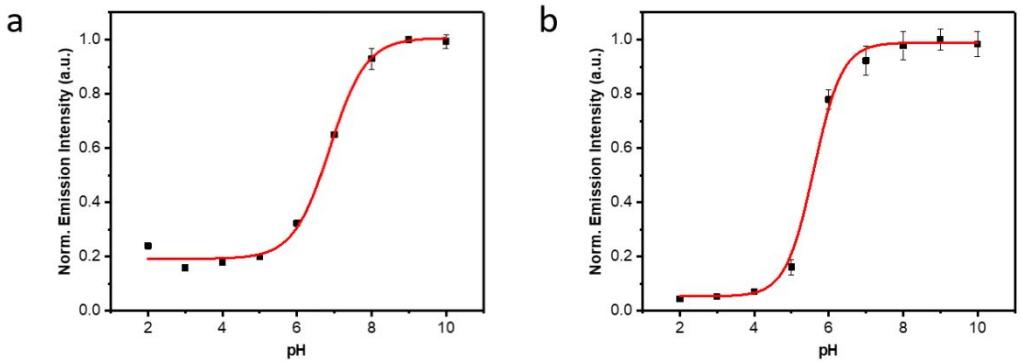


Figure S5: pH profiles of **18** and **4** in Britton-Robinson buffer based on fluorescence emission. A sigmoidal Boltzmann fit was used for curve fitting (red). The p*K*a value of **18** was determined to be 6.9, while the p*K*a value of **4** was 5.6. Data are represented as mean ± SD (*n* = 3).

Table S1: Summary of the photophysical properties of photoFAD-1, photoFAD-2, photoFAD-3, their corresponding dye products (**16**, **18** & **4**), Ctrl-photoFAD-3 and AM-FAD-3. For some of the listed compounds the fluorescence quantum yield Φ_F could not be determined (n.d.) due to photoactivation when exciting at their absorbance band or insignificant fluorescence intensity. Extinction coefficients ε and Φ_F are represented as mean ± SD (*n* = 3).

Compound	**λ_{Abs} (nm)**	**ε ($M^{-1}cm^{-1}$)**	**λ_{Em} (nm)**	**ϕ_F (%)**
photoFAD-1	442	16,800 ± 100	-	n.d.
16	582	43,600 ± 700	599	62 ± 3
photoFAD-2	445	20,300 ± 300	-	n.d.
18	590	52,500 ± 500	608	58 ± 1
photoFAD-3	451	22,300 ± 200	-	n.d.
4	601	53,000 ± 2,000	616	60 ± 1
Ctrl-photoFAD-3	440	22,400 ± 100	-	n.d.
AM-FAD-3	452	19,300 ± 200	564	n.d.

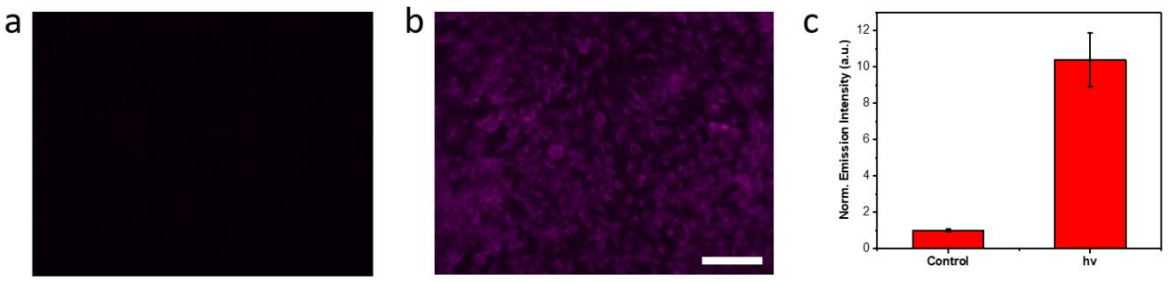


Figure S6: In vivo evaluation of photoFAD-3 via fluorescence microscopy. HEK 293 cells were incubated with 5 µM photoFAD-3 in serum free DMEM for 30 min at 37 °C. The comparison of fluorescence microscopy images (a) before and (b) after photoactivation shows a clear fluorescence increase. Scale bar represents 100 µm. (c) Quantification confirmed a 10-fold turn on in vivo. Data are represented as mean ± SD (*n* = 3).

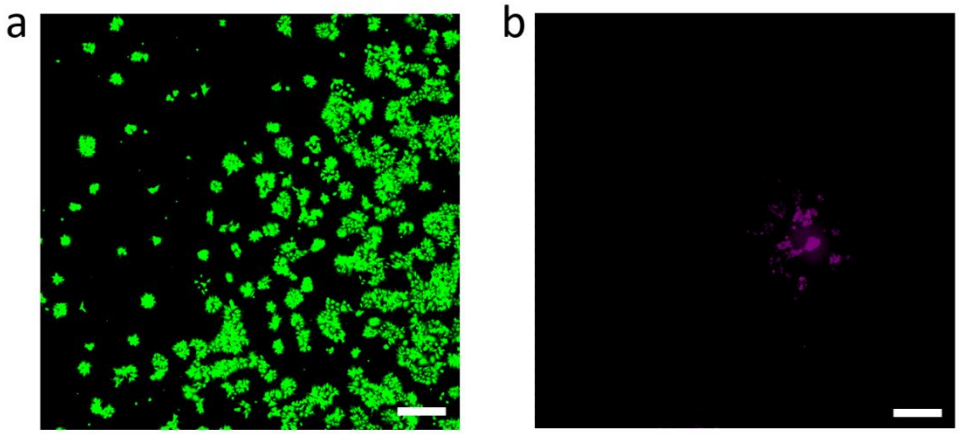


Figure S7: Demonstration of spatial control of FA release in HEK 293 cells via fluorescence microscopy. Cells were incubated with 0.5 µM Calcein AM and 5 µM photoFAD-3 in DPBS buffer (pH 7.4) for 30 min at 37 °C. A select set of cells was irradiated with the DAPI channel to activate photoFAD-3. (a) All live cells show emission in the FITC-channel. (b) Only the cells in the previously irradiated area show emission in the Cy5-channel due to formation of compound **4** and FA release. Scale bar represents 330 µm.

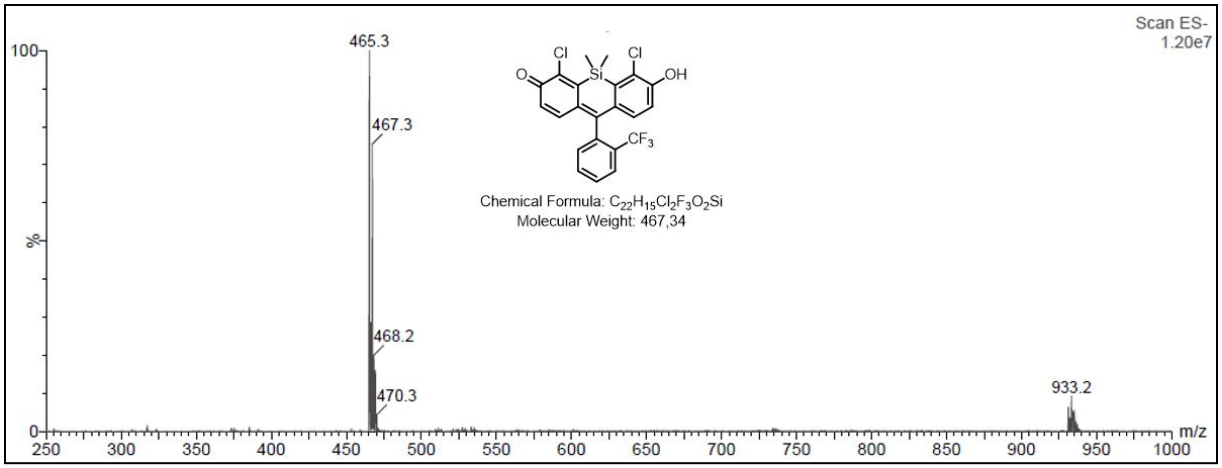


Figure S8: Confirmation of formation of **4** upon 1 h of photoactivation of 50 µM photoFAD-3 in water via mass spectrometry.

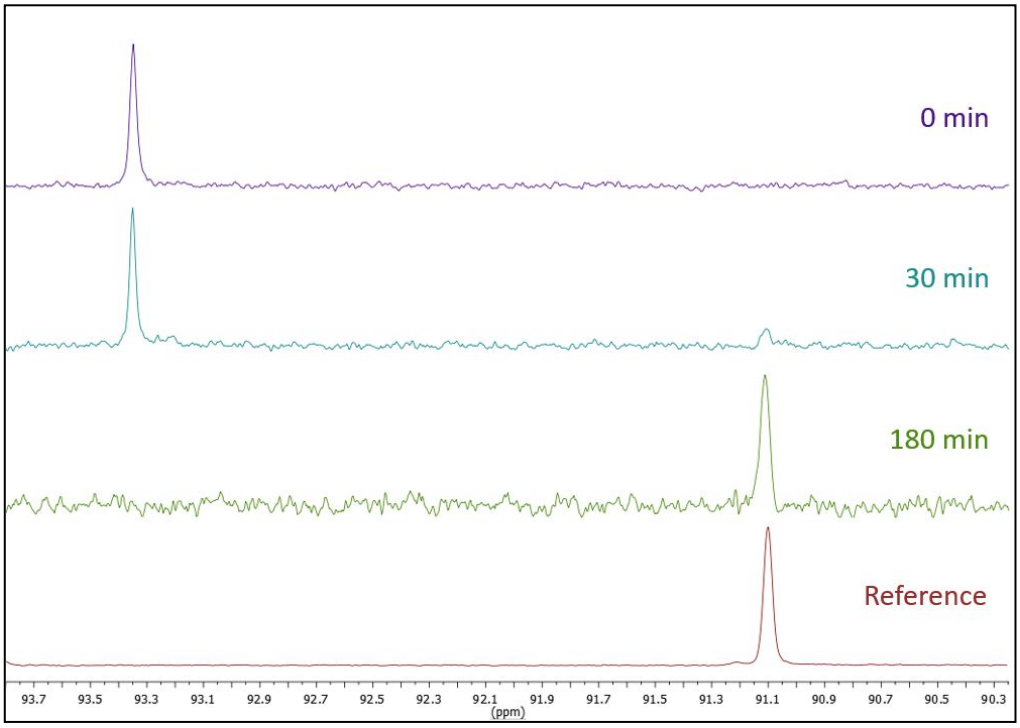


Figure S9: Confirmation of FA formation using ^{13}C-labeled photoFAD-3 via ^{13}C NMR spectroscopy. A solution of ^{13}C-photoFAD-3 in MeOD-d_4 was photoactivated for 0, 30, and 180 min. A decrease of the signal at 93.35 ppm and the formation of a signal at 91.10 ppm was observed. The comparison with a FA reference sample confirms the formation of the hemi-formal derived from FA in methanol upon photoactivation.

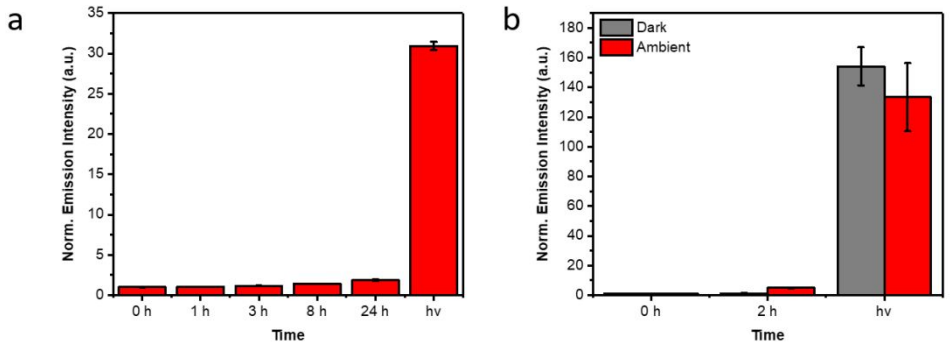


Figure S10: Stability evaluation of photoFAD-3. (a) Stability of 2 µM photoFAD-3 in DMEM containing 10% FBS upon incubation at 37 °C. After incubation for 24 h the solution was photoactivated for 30 min to show retention of functionality. (b) Stability of 2 µM photoFAD-3 in PBS buffer (pH 7.4) upon storage in the dark and under ambient light for 2 h. Photoactivation for 30 min shows retention of functionality. Data are represented as mean ± SD (*n* = 3).

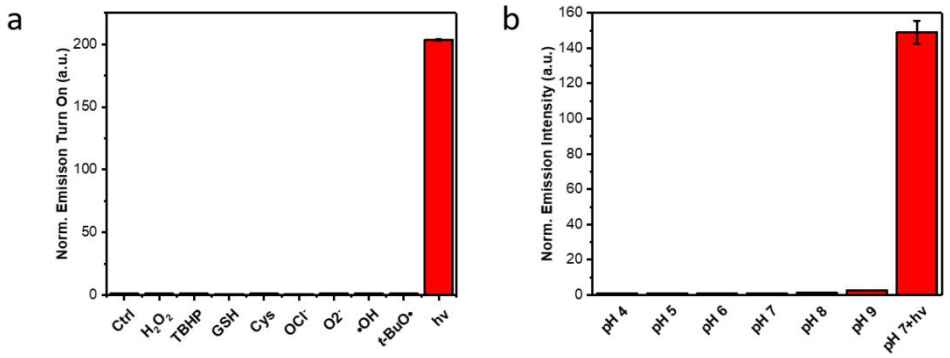


Figure S11: Evaluation of the chemostability of photoFAD-3. (a) Stability of 2 µM photoFAD-3 in PBS buffer (pH 7.4) after incubation for 30 min at 37 °C in the presence of various thiols (1 mM glutathione, 100 µM cysteine) and ROS (100 µM hydrogen peroxide, *tert*-butyl hydroperoxide, hypochlorite, superoxide, hydroxy radical, *tert*-butoxy radical). (b) Stability of 2 µM photoFAD-3 in Britton-Robinson buffer across various pH values after incubation for 30 min at 37 °C. Data are represented as mean ± SD (*n* = 3).

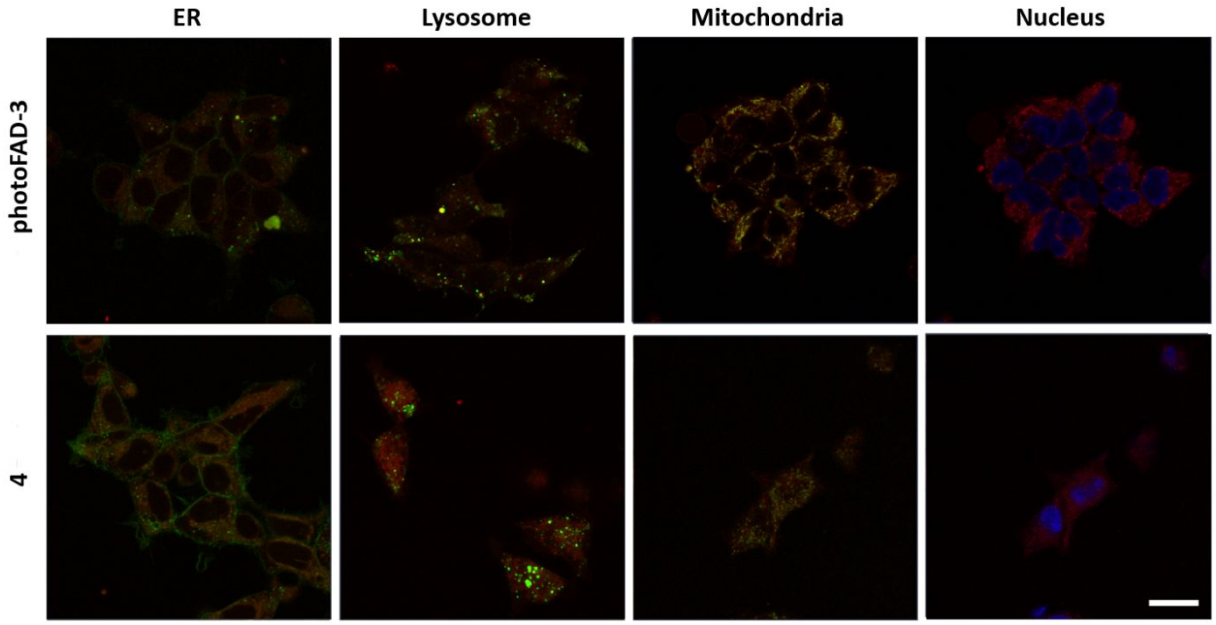


Figure S12: Co-localization assay in HEK 293 cells upon treatment with 4 µM photoFAD-3 and **4** (red) in combination with corresponding tracker dyes (green) and nuclear stain (blue). Scale bar represents 20 µM. The obtained Pearson coefficients for photoFAD-3 (ER: 0.55 ± 0.07; lysosome: 0.68 ± 0.11; mitochondria: 0.78 ± 0.06) and **4** (ER: 0.63 ± 0.04; lysosome: 0.51 ± 0.02; mitochondria: 0.11 ± 0.11). indicate a cytoplasmic localization of both compounds.

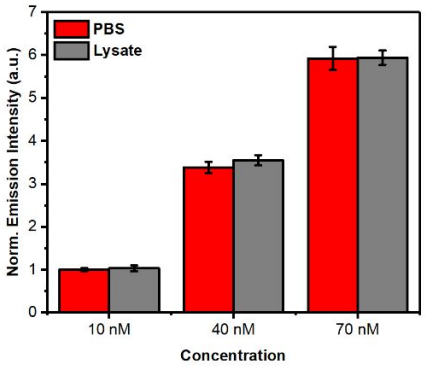


Figure S13: Test of purification efficiency of lysate-derived samples through centrifugation. The fluorescence emission signal of compound **4** in regular PBS buffer (20% DMSO, pH 7.4) was compared to the signals of compound **4** in PBS buffer (20% DMSO, pH 7.4) that was previously mixed with cell lysate and cell debris was subsequently removed via centrifugation. Concentrations of 10, 40 and 70 nM compound **4** were tested. The test was performed to prove that the data from the cell-derived samples can be compared to the reference samples. Data are represented as mean ± SD (*n* = 3).

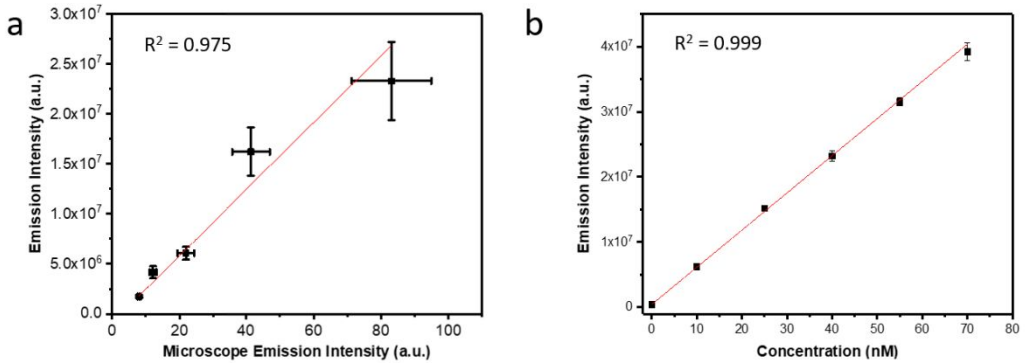


Figure S14: Quantification of FA release in vivo. (a) Calibration plot of in vitro cell lysate fluorescence versus mean fluorescence signal obtained from in vivo fluorescence microscopy (n = 3). (b) Reference plot of in vitro fluorescence versus concentration of **4**. Data are represented as mean ± SD (n = 3).

Equation S1: Calculation of intracellular concentration increase of FA through incubation with photoFAD-3 and photoactivation for 180 s through the slope of the calibration curve a_{cal}, intercept of the calibration curve b_{cal}, slope of the reference curve a_{ref}, intercept of the reference curve b_{ref}, mean emission intensity measured via fluorescence microscopy Iμ, mean emission intensity measured in vitro I_{em}, the volume of the lysate sample $V_{lysate,}$ cell count n_{cell} and the volume of HEK 293 cells V_{HEK293}.

a) Increase of intracellular FA concentration for all cells inside the well after 180 s of photoactivation Δc_{180}:

$$a_{cal}\, I_{\mu} + b_{cal} = I_{em} = a_{ref}\, \Delta c_{180} + b_{ref}$$

$$\Delta c_{180} = \frac{I_{\mu}\, a_{cal} + b_{cal} - b_{ref}}{a_{ref}} = 46.3 \text{ nM}$$

b) Amount of FA donated after 180 s of photoactivation n_{180}:

$$n_{180} = c_{cell}\, V_{lysate} = 69.5 \text{ pmol}$$

c) Amount of FA donated per cell n_{FA}:

$$n_{FA} = \frac{n_{180}}{n_{cell}} = 6.20 \text{ amol}$$

d) Intracellular FA concentration increase per cell Δc_{FA}:

$$\Delta c_{FA} = \frac{n_{cell}}{V_{HEK293}} = 4.0 \text{ µM}$$

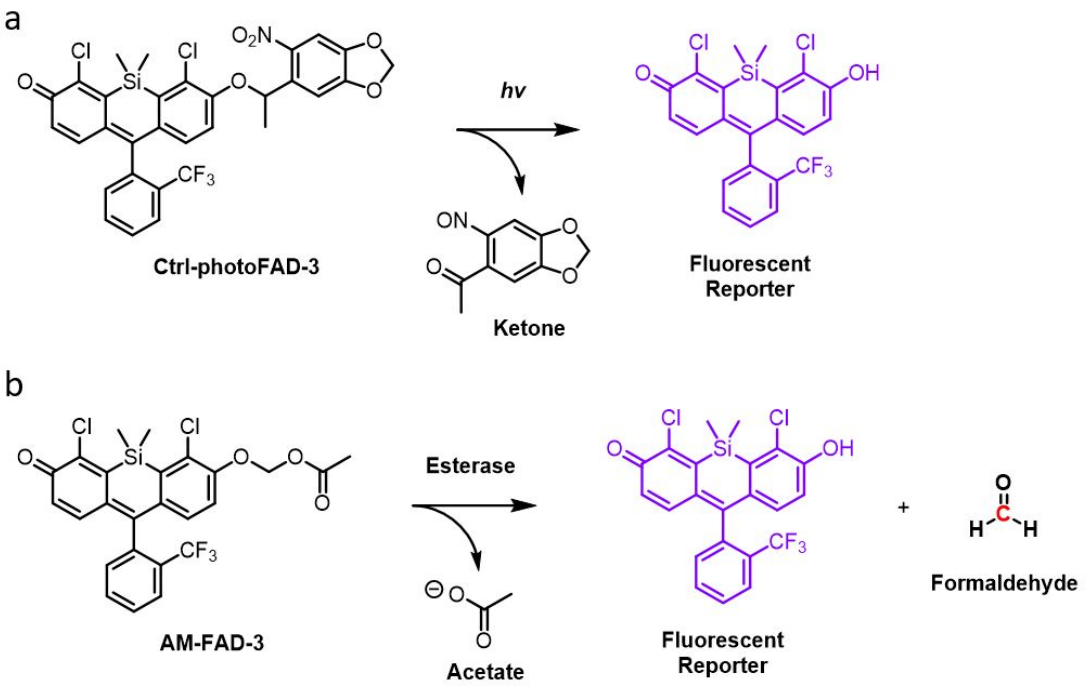


Figure S15: Structure and functional principle of (a) Ctrl-photoFAD-3 and (b) AM-FAD-3.

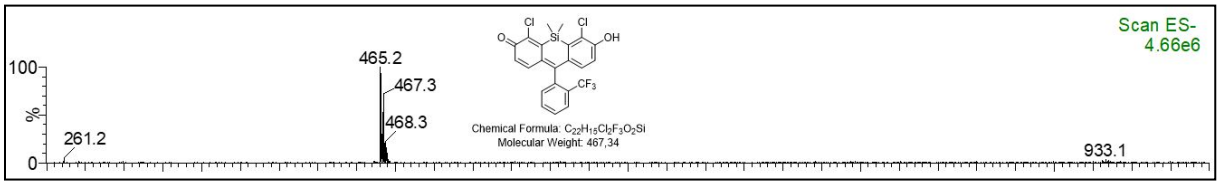


Figure S16: Confirmation of formation of **4** upon 1 h of photoactivation of 50 µM Ctrl-photoFAD-3 in water via mass spectrometry.

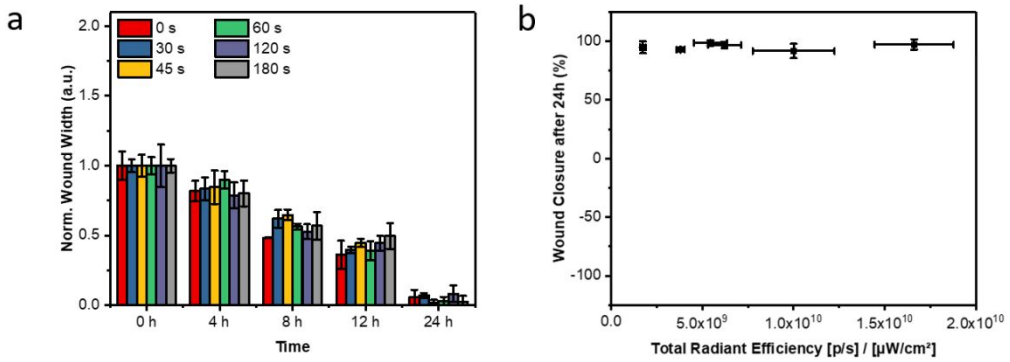


Figure S17: Wound healing assay of HEK 293 cells after treatment with 5 µM Ctrl-photoFAD-3 in serum-free DMEM. The compound was photoactivated for various durations between 0 and 180 s and the wound size was monitored for 24 h. (a) Wound widths throughout the experiment in dependence of time. Full wound closure was observed after 24h independent of the level of activation. (a) Correlation plot of total radiant efficiency versus wound closure after 24 h. Data are represented as mean ± SD (*n* = 3).

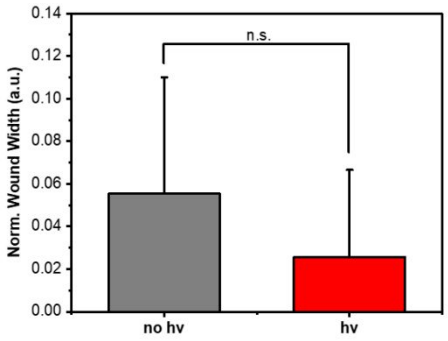


Figure S18: Comparison of wound healing of HEK 293 cells after treatment with 5 µM Ctrl-photoFAD-3 in serum-free DMEM and 24 h of incubation at 37 °C post wound induction. Ctrl-photoFAD-3 was either not photoactivated (0 s irradiation, grey) or fully photoactivated (180 s irradiation, red). No statistical significance was observed between these two conditions ($p > 0.05$), indicating that the release of nitrosoacetophenone did not interfere with cellular behavior. Data are represented as mean ± SD (*n* = 3).

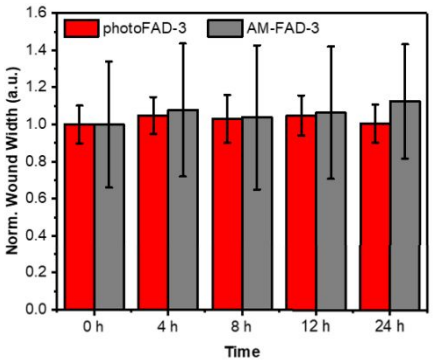


Figure S19: Wound healing assay of HEK 293 cells after treatment with 5 µM photoFAD-3 (+ 180 s of photoactivation, red) or 5 µM AM-FAD-3 (gray) in serum-free DMEM. The wound size was monitored for 24 h. Treatment with AM-FAD-3 resulted in the same cytostatic behavior that was observed with photoFAD-3 upon 180 s of photoactivation. There is no statistically significant difference for any of the time points. Data are represented as mean ± SD (*n* = 3).

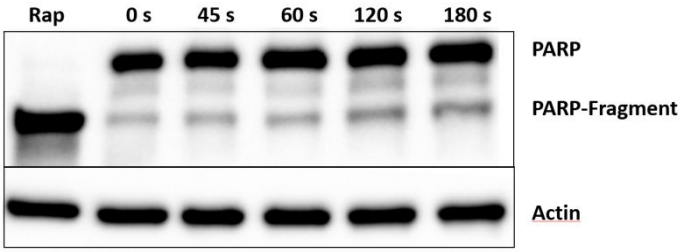


Figure S20: Apoptosis evaluation via Western blot. HEK 293 cells were treated with 10 µM raptinal (Rap, positive control) and 5 µM photoFAD-3 followed by photoactivation for various durations between 0 - 180 s. Cleavage of poly (ADP-ribose) polymerase (PARP) through caspases indicates initiation of apoptosis. Actin served as a loading control. No significant initiation of apoptosis through the intracellular release of FA could be observed.

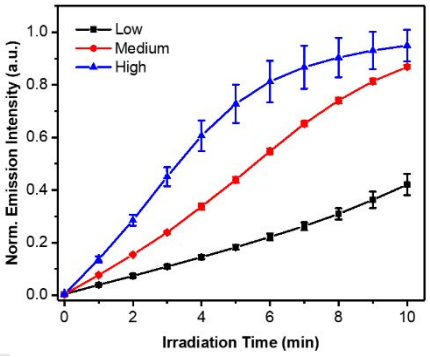
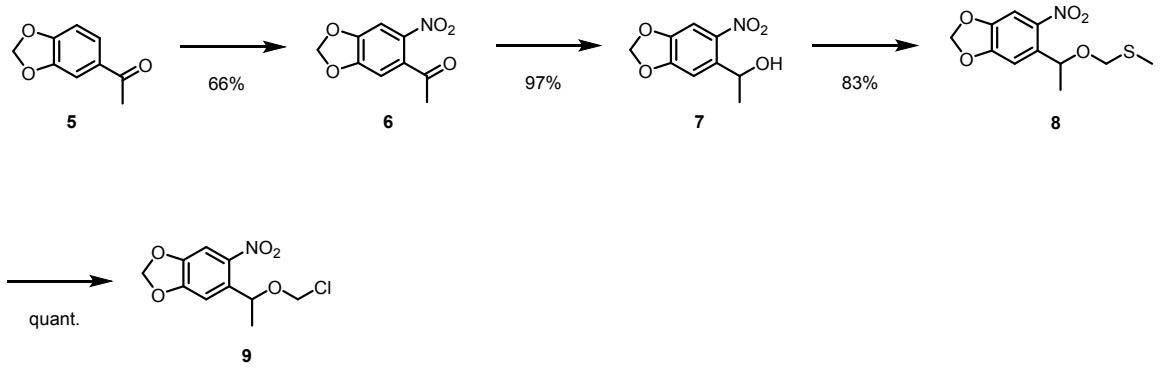
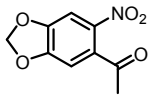


Figure S21: Release kinetics of 2 µM photoFAD-3 in PBS (pH 7.4) upon photoactivation with low (blue), medium (red) and high (black) light intensity. The following initial rates were determined for the chosen conditions: k_{low} = 69.9 nM/min; k_{medium} = 152 nM/min; k_{high} = 281 nM/min. Data are represented as mean ± SD (n = 3).

22. Synthetic methods

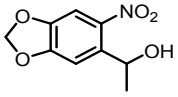


4',5'-Methylenedioxy-2'-nitroacetophenone (6):



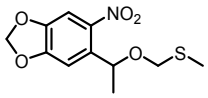
3',4'-(Methylenedioxy)acetophenone (3.830 g, 23.33 mmol, 1.0 eq.) was dissolved in nitromethane (42.8 mL, 793.3 mmol, 34 eq.) and conc. nitric acid (8.34 mL, 186.6 mmol, 8.0 eq.) was added dropwise over a period of 20 min while stirring. The reaction was stirred for an additional 6 h at room temperature before sat. $NaHCO_3$ aq. (120 mL) was added dropwise to quench the reaction. The mixture was transferred into a separatory funnel containing more sat. $NaHCO_3$ aq. and the organic components were extracted with CH_2Cl_2 (3x). The combined organic layers were washed with brine, dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 -> 1:1 EtOAc/hexanes) to give the desired product as a light yellow solid (3.216 g, 15.38 mmol, 66%). R_f = 0.31 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49 (s, 1H), 6.71 (s, 1H), 6.16 (s, 2H), 2.45 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 199.30, 152.80, 148.92, 140.10, 135.14, 106.22, 104.82, 103.78, 30.27. HR-MS: Calculated for $C_9H_7NO_5Na$ $[M+Na]^+$ m/z 232.0222, found 232.0231.

1-(6-Nitro-1,3-benzodioxol-5-yl)ethanol (7):



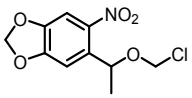
4',5'-Methylenedioxy-2'-nitroacetophenone (**6**) (3.216 g, 15.38 mmol, 1.0 eq.) was dissolved in THF (76 mL) and sodium borohydride (1.163 g, 30.753 mmol, 2.0 eq.) was added. The resultant suspension was stirred overnight at room temperature and then quenched with 2N HCl until the formation of gas had ceased. The mixture was poured into brine, the organic components were extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate before being concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product as a yellow solid (3.152 g, 14.93 mmol, 97%). R_f = 0.31 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.24 (s, 1H), 6.13 – 6.05 (m, 2H), 5.42 (q, *J* = 5.7 Hz, 1H), 2.52 (s, 1H), 1.50 (d, *J* = 6.3 Hz, 3H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 152.52, 147.01, 141.54, 139.15, 106.43, 105.20, 103.05, 65.78, 24.33. HR-MS: Calculated for $C_9H_9NO_5Na$ $[M+Na]^+$ m/z 234.0378, found 234.0378.

5-[1-[(Methylthio)methoxy]ethyl]-6-nitro-1,3-benzodioxole (8):

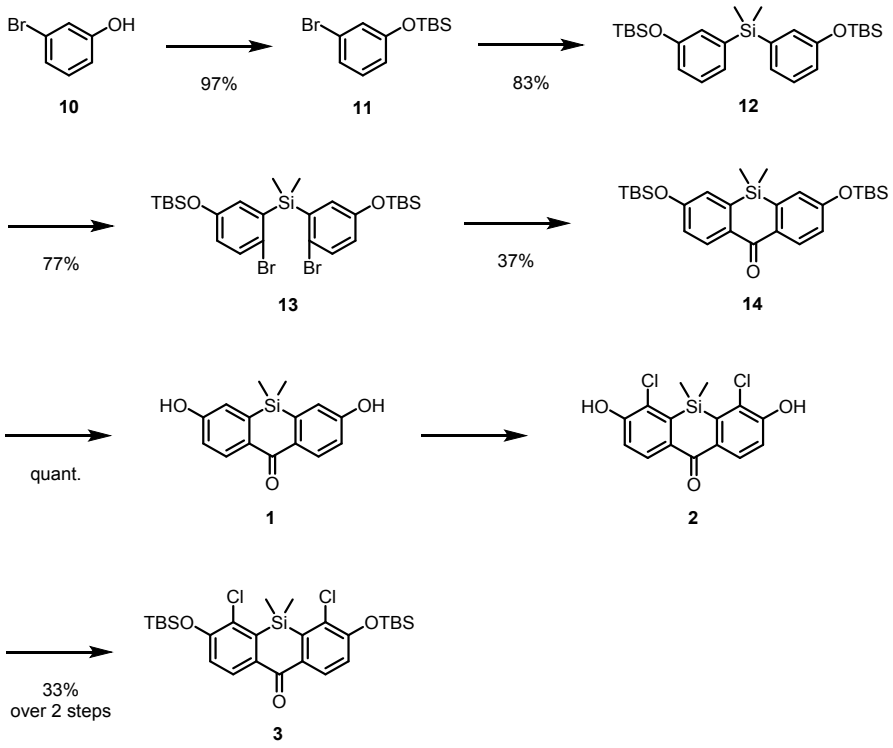


A flame-dried round-bottom flask under nitrogen atmosphere was charged with 1-(6-Nitro-1,3-benzodioxol-5-yl)ethanol (**7**) (727 mg, 3.44 mmol, 1.0 eq.). It was dissolved in anhydrous acetonitrile (18 mL) and the solution was cooled to 0 °C. Dimethylsulfide (2.0 mL, 27.5 mmol, 8.0 eq.) was added before benzoylperoxide (3.34 g, 13.8 mmol, 4.0 eq.) was added in four portions every 30 min. After completion of the addition the reaction was stirred for another 4 h at the same temperature. It was stirred for an additional 2 h at room temperature followed by addition of 1 M NaOH until a pH value of 9 was reached. It was stirred overnight at room temperature. The organic components were extracted with EtOAc (3x), the combined organic layers were dried over sodium sulfate, concentrated and purified via flash column chromatography (SiO_2, 1:9 EtOAc/hexanes) to give the desired product as a yellow solid (775 mg, 2.86 mmol, 83%). R_f = 0.25 (SiO_2, 1:9 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42 (s, 1H), 7.13 (s, 1H), 6.09 (d, *J* = 6.4 Hz, 2H), 5.41 (q, *J* = 6.3 Hz, 1H), 4.57 (d, *J* = 11.4 Hz, 1H), 4.26 (d, *J* = 11.5 Hz, 1H), 2.09 (s, 3H), 1.47 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.52, 147.06, 142.22, 137.02, 106.43, 105.08, 103.01, 73.10, 70.38, 23.34, 14.12. HR-MS: Calculated for $C_{11}H_{13}NO_5NaS$ $[M+Na]^+$ m/z 294.0412, found 294.0405.

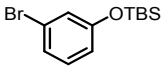
5-[1-(Chloromethoxy)ethyl]-6-nitro-1,3-benzodioxole (9):



A flame-dried round-bottom flask under nitrogen atmosphere was charged with 5-[1-[(methylthio) methoxy]ethyl]-6-nitro-1,3-benzodioxole (**A3**) (240 mg, 885 µmol, 1.0 eq.) and it was dissolved in anhydrous CH_2Cl_2 (1.2 mL). It was cooled to 0 °C and a solution of sulfuryl chloride in anhydrous CH_2Cl_2 (5.7 mL) was added slowly. The reaction was stirred for 4 h at room temperature before the volatiles were removed under reduced pressure. It was re-dissolved in chloroform and concentrated under reduced pressure. This sequence was repeated two more times to yield the desired product as a yellow oil (231 mg, 885 µmol, quant.) that was used directly for the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.50 (s, 1H), 7.08 (s, 1H), 6.13 (d, *J* = 4.5 Hz, 2H), 5.55 (q, *J* = 6.3 Hz, 1H), 5.51 (d, *J* = 5.9 Hz, 1H), 5.23 (d, *J* = 5.8 Hz, 1H), 1.56 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.62, 147.48, 142.14, 135.77, 106.62, 105.35, 103.20, 80.45, 73.22, 23.21.

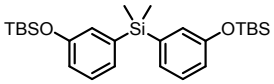


(3-bromophenoxy)(tert-butyl)dimethylsilane (11):



A flame-dried round-bottom flask under nitrogen atmosphere was charged with 3-bromophenol (5.050 g, 29.19 mmol, 1.0 eq.) and *tert*-butyldimethylsilyl chloride (4.839 g, 32.11 mmol, 1.1 eq.). The solids were dissolved in anhydrous CH_2Cl_2 (150 mL) and imidazole (2.981 g, 43.78 mmol, 1.5 eq.) was added to the solution. The reaction was stirred overnight at room temperature and was then poured into 1 M NaOH aq. The organic components were extracted with CH_2Cl_2 (1x), the organic layer was washed with 1 M NaOH aq. (2x) and it was dried over sodium sulfate. The volatiles were removed under reduced pressure to give the desired product as a colorless oil (8.171 g, 29.19 mmol, 97%). R_f = 0.68 (SiO_2, hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.09 (dd, *J* = 3.9, 1.2 Hz, 2H), 7.04 – 6.99 (m, 1H), 6.80 – 6.74 (m, 1H), 0.99 (s, 9H), 0.21 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.76, 130.66, 124.71, 123.76, 122.71, 119.06, 25.85, 18.43, -4.22. HR-MS: Calculated for $C_{12}H_{19}OSiBr$ $[M]^+$ m/z 286.03886, found 286.03974.

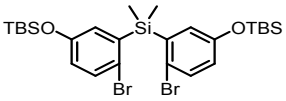
Bis(3-((tert-butyldimethylsilyl)oxy)phenyl)dimethylsilane (12):



A flame-dried round-bottom flask under nitrogen atmosphere was charged with (3-Bromophenoxy)(tert-butyl)dimethylsilane (**11**) (7.169 g, 24.96 mmol, 2.2 eq.). It was dissolved in anhydrous THF (74 mL) and the solution was cooled to -78 °C. A 1.6 M solution of *n*-butyllithium in hexanes (16 mL, 24.96 mmol, 2.2 eq.) was added dropwise and the reaction was stirred for 30 min at the same temperature. A solution of dichlorodimethylsilane (1.37 mL, 11.34 mmol, 1.0 eq.) in anhydrous THF (14 mL) was added dropwise and the reaction was allowed to warm to room

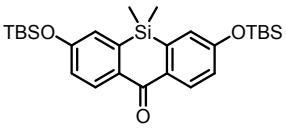
temperature. It was stirred for 3.5 h at room temperature and then was quenched via addition of water. The resulting mixture was poured into brine, extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate. It was concentrated under reduced pressure and the residue was purified via flash column chromatography (SiO2, 0:1 -> 1:19 EtOAc/hexanes) to give the desired product as colorless oil (4.455 g, 9.421 mmol, 83%). R_f = 0.31 (SiO_2, hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.23 (t, *J* = 7.6 Hz, 2H), 7.10 (dt, *J* = 7.2, 1.1 Hz, 2H), 6.98 (dd, *J* = 2.5, 1.0 Hz, 2H), 6.85 (ddd, *J* = 8.1, 2.6, 1.1 Hz, 2H), 0.98 (s, 18H), 0.52 (s, 6H), 0.17 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.23, 139.85, 129.09, 127.09, 125.70, 120.91, 25.88, 18.38, -2.32, -4.24. HR-MS: Calculated for $C_{25}H_{37}O_4Si_2$ $[M+H]^+$ m/z 473.2727, found 473.2731.

Bis(2-bromo-5-((tert-butyldimethylsilyl)oxy)phenyl)dimethylsilane (13):



Bis(3-((tert-butyldimethylsilyl)oxy)phenyl)dimethylsilane (**12**) (4.455 g, 9.421 mmol, 1.0 eq.) was dissolved in DMF (62 mL). *N*-bromosuccinimide (3.354 g, 18.84 mmol, 2.0 eq.) was added in portions over 5 min and the reaction was stirred at room temperature for 12 h. Another portion of *N*-bromosuccinimide (838 mg, 4.71 mmol, 0.5 eq.) was added and it was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was re-dissolved in EtOAc. The solution was washed with brine (3x), dried over sodium sulfate and concentrated. The residue was purified via flash column chromatography (SiO_2, 1:39 EtOAc/hexanes) to give the desired product as white solid (4.571 g, 7.247 mmol, 77%). R_f = 0.46 (SiO_2, 1:39 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 3.0 Hz, 2H), 6.71 (dd, *J* = 8.6, 3.0 Hz, 2H), 0.95 (s, 18H), 0.72 (s, 6H), 0.14 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 154.58, 140.02, 133.90, 128.75, 123.17, 121.41, 25.82, 18.38, -1.08, -4.28. HR-MS: Calculated for $C_{26}H_{42}O_2Si_3Br_2$ $[M]^+$ m/z 628.0859, found 628.0869.

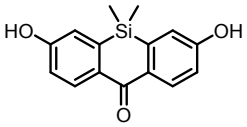
10,10-dimethyl-10-sila-3,6-bis((tert-butyldimethylsilyl)oxy)-9*H*-xanthen-9-one (14):



The reaction was performed with flame-dried equipment under nitrogen atmosphere. Bis(2-bromo-5-((tert-butyldimethylsilyl)oxy)phenyl)dimethylsilane (**13**) (4.571 g, 7.247 mmol, 1.0 eq.) was dissolved in anhydrous THF (26 mL) and the solution was cooled to -78 °C. A 1.4 M solution of *sec*-butyllithium in cyclohexane (10 mL, 14.49 mmol, 2.0 eq.) was added dropwise and the reaction was stirred for 30 min at the same temperature. A solution of dimethylcarbamoyl chloride (666 µL, 7.247 mmol, 1.0 eq.) in anhydrous THF (5.2 mL) was added dropwise and the reaction was allowed to warm to room temperature. It was stirred for 2.5 h at room temperature and then was quenched via addition of water. The resulting mixture was poured into brine, extracted with EtOAc (3x) and the combined organic layers were dried over sodium sulfate. It was concentrated under reduced pressure and the residue was purified via flash column chromatography (SiO_2, 1:39 -> 1:19 EtOAc/hexanes) to give the desired product as pale yellow solid (1.333 g, 2.672 mmol, 37%). R_f = 0.26 (SiO_2, 1:39 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.38 (d, *J* = 8.7 Hz, 2H), 7.05 (d, *J* = 2.5 Hz, 2H), 6.99 (dd, *J* = 8.7, 2.6 Hz, 2H), 1.01 (s, 18H), 0.46 (s, 6H), 0.27 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 186.10, 158.86,

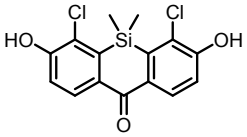
141.29, 134.69, 132.41, 123.85, 121.91, 25.80, 18.45, -1.39, -4.14. HR-MS: calculated for $C_{27}H_{43}O_3Si_3$ $[M+H]^+$ m/z 499.2520, found 499.2516.

3,6-Dihydroxy-10,10-dimethyl-10-sila-9*H*-xanthen-9-one (1):



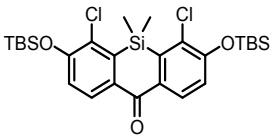
The reaction was performed with flame-dried equipment under nitrogen atmosphere. 10,10-Dimethyl-10-sila-3,6-bis((tert-butyldimethylsilyl)oxy)-9*H*-xanthen-9-one (**14**) (405 mg, 0.81 mmol, 1.0 eq.) was dissolved in anhydrous THF (7 mL) and a 1 M solution of tetrabutylammonium fluoride in THF (2.0 mL, 2.0 mmol, 2.5 eq.) was added. The reaction was stirred for 2.5 h and was then poured into slightly acidic brine (addition of a few drops of 2N HCl). It was extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate. It was concentrated under reduced pressure and the residue was purified via flash column chromatography (SiO_2, 1:39 MeOH/ CH_2Cl_2) to give the desired product as a white solid (220 mg, 0.81 mmol, quantitative). R_f = 0.39 (SiO_2, 1:39 v/v MeOH/ CH_2Cl_2). ^{1}H NMR (500 MHz, acetone-d_6): δ 9.24 (s, 2H), 8.38 (d, *J* = 8.7 Hz, 2H), 7.23 (d, *J* = 2.5 Hz, 2H), 7.10 (dd, *J* = 8.7, 2.7 Hz, 2H), 0.47 (s, 6H). ^{13}C-NMR (125 MHz, acetone-d_6): δ 185.35, 161.15, 142.17, 133.92, 132.94, 119.86, 118.18, -1.47. HR-MS: Calculated for $C_{15}H_{15}O_3Si$ $[M+H]^+$ m/z 271.0790, found 271.0797.

4,5-Dichloro-3,6-dihydroxy-10,10-dimethyl-10-sila-9*H*-xanthen-9-one (2):



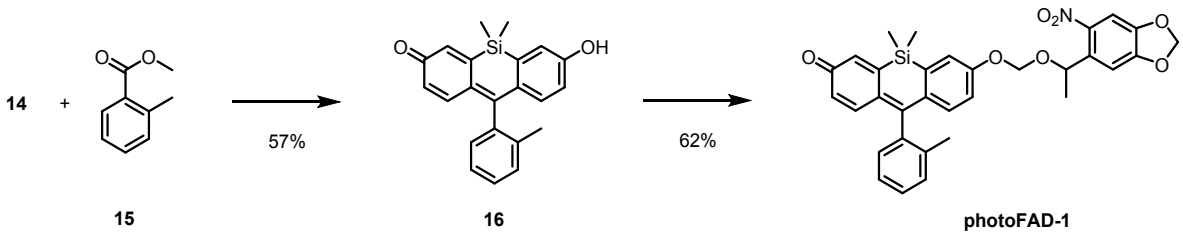
3,6-Dihydroxy-10,10-dimethyl-10-sila-9*H*-xanthen-9-one (**1**) (226 mg, 0.84 mmol, 1.0 eq.) was dissolved in DMF (11 mL) and *N*-chlorosuccinimide (257 mg, 1.92 mmol, 2.3 eq.) was added in portions over 5 min. The reaction was stirred at room temperature for 12 h and was then poured into slightly acidic brine (addition of a few drops of 2 N HCl). It was extracted with EtOAc (2x) and the combined organic layers were backwashed with brine (3x). It was dried over sodium sulfate, concentrated under reduced pressure and the residue was purified via flash column chromatography (SiO_2, 1:19 MeOH/ CH_2Cl_2) to remove by-products from the mixture of chlorination products. The obtained product mixture was directly used in the next step.

4,5-Dichloro-10,10-dimethyl-10-sila-3,6-bis((tert-butyldimethylsilyl)oxy)-9*H*-xanthen-9-one (3):

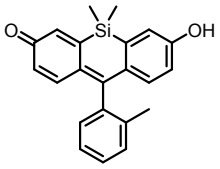


A flame-dried round-bottom flask under nitrogen atmosphere was charged with the product mixture from the previous step and *tert*-butyldimethylsilyl chloride (259 mg, 1.72 mmol, 2.1 eq.). The compounds were dissolved in anhydrous CH_2Cl_2 (14 mL) and imidazole (140 mg, 2.06 mmol, 2.5 eq.) was added to the solution. The reaction was stirred at room temperature for 12 h and was then poured

into brine. The organic components were extracted with CH_2Cl_2 (3x). The combined organic layers were dried over sodium sulfate and the volatiles were removed under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 CH_2Cl_2/hexanes) to give the desired product as a white solid (153 mg, 0.27 mmol, 33% over 2 steps). R_f = 0.25 (SiO_2, 1:39 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.39 (d, *J* = 8.7 Hz, 2H), 7.09 (d, *J* = 8.7 Hz, 2H), 1.09 (s, 18H), 0.84 (s, 6H), 0.32 (s, 12H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 184.96, 155.16, 140.60, 134.34, 131.12, 130.80, 121.68, 25.74, 18.52, -1.86, -4.11. HR-MS: Calculated for $C_{27}H_{41}O_3Cl_2Si_3$ $[M+H]^+$ m/z 567.1741, found 567.1741.

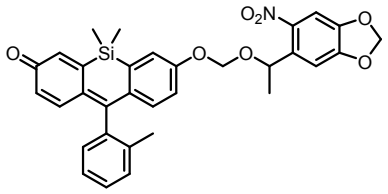


TokyoMagenta (16):

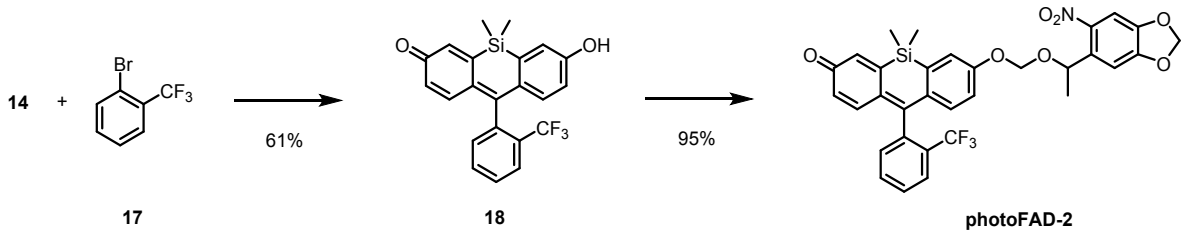


A flame-dried round-bottom flask under nitrogen atmosphere was charged with methyl 2-methylbenzoate (**18**) (262 mg, 1.74 mmol, 2.2 eq.) and it was dissolved in anhydrous THF (24.0 mL). The solution was cooled to -78 °C and a 1.7 M solution of *tert*-butyllithium in pentane (2.05 mL, 3.49 mmol, 4.4) was added dropwise. After completion of the addition the solution was stirred for 30 min at the same temperature followed by warming to -20 °C. A solution of Bis(2-bromo-5-((tert-butyldimethylsilyl)oxy)phenyl)dimethylsilane (**14**) (500 mg, 793 µmol, 1.0 eq.) in anhydrous THF (16.0 mL) was added dropwise at the same temperature. The reaction was stirred room temperature overnight before it was quenched by addition of 2N HCl (5.0 mL). It was stirred for another 30 min at room temperature and then poured into brine. It was washed, extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate. The residual solvent was removed under reduced pressure and the obtained residue was purified via flash column chromatography (SiO_2, 1:39 MeOH/ CH_2Cl_2) to give the desired product as a red solid (155 mg, 793 µmol, 57%). R_f = 0.14 (SiO_2, 1:39 v/v MeOH/ CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.73 (s, 1H), 7.37 (t, *J* = 7.5 Hz, 1H), 7.30 (d, *J* = 9.9 Hz, 2H), 7.11 – 7.04 (m, 3H), 6.94 (d, *J* = 9.5 Hz, 2H), 6.57 (dd, *J* = 9.5, 2.2 Hz, 2H), 2.03 (s, 3H), 0.42 (s, 3H), 0.41 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.56, 161.36, 145.99, 140.51, 139.29, 135.96, 130.23, 130.03, 129.21, 129.07, 128.52, 125.74, 122.15, 19.63, -1.17, -1.43. HR-MS: Calculated for $C_{22}H_{21}O_2Si$ $[M+H]^+$ m/z 345.1311, found 345.1295.

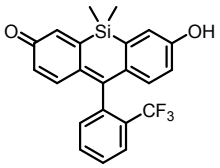
photoFAD-1:



A flame-dried round-bottom flask under nitrogen atmosphere was charged with TokyoMagenta (**16**) (109 mg, 316 µmol, 1.0 eq.) and freshly prepared 5-(1-(chloromethoxy)ethyl)-6-nitrobenzo[d][1,3]dioxole (113 mg, 437 µmol, 1.4 eq.). Anhydrous DMF (4.4 mL) was added followed by addition of anhydrous diisopropylethylamine (94 µL, 538 µmol, 1.7 eq.). The reaction was stirred at room temperature overnight under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product as yellow film (112 mg, 316 µmol, 62%). R_f = 0.19 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42 – 7.35 (m, 2H), 7.34 – 7.28 (m, 2H), 7.16 (dt, *J* = 3.1, 1.6 Hz, 1H), 7.08 (d, *J* = 7.4 Hz, 1H), 7.02 (d, *J* = 0.8 Hz, 1H), 6.94 (d, *J* = 10.1 Hz, 1H), 6.84 (d, *J* = 2.1 Hz, 1H), 6.83 – 6.79 (m, 2H), 6.23 (dd, *J* = 10.1, 2.2 Hz, 1H), 6.01 (ddd, *J* = 30.4, 2.8, 1.2 Hz, 2H), 5.46 (q, *J* = 6.3 Hz, 1H), 5.25 (d, *J* = 7.3 Hz, 1H), 5.13 (dd, *J* = 7.4, 0.9 Hz, 1H), 2.06 (s, 3H), 1.49 (d, *J* = 6.3 Hz, 3H), 0.49 (d, *J* = 2.9 Hz, 3H), 0.47 (d, *J* = 2.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 184.45, 157.83, 155.64, 152.25, 147.07, 147.07, 147.01, 141.78, 141.64, 140.42, 139.33, 139.31, 137.08, 137.07, 136.83, 136.78, 136.14, 136.11, 135.16, 134.52, 130.29, 129.39, 129.33, 128.88, 128.38, 127.92, 125.89, 122.20, 122.17, 116.73, 116.66, 106.77, 105.00, 104.99, 102.98, 102.96, 91.24, 72.18, 72.13, 23.55, 19.55, -1.00, -1.23, -1.34, -1.59. HR-MS: Calculated for $C_{32}H_{30}NO_7Si$ $[M+H]^+$ m/z 568.1792, found 568.1777.



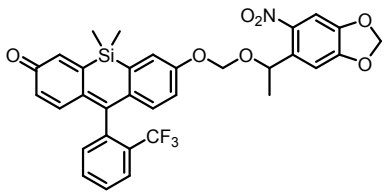
2-Trifluoromethyl-TokyoMagenta (18):



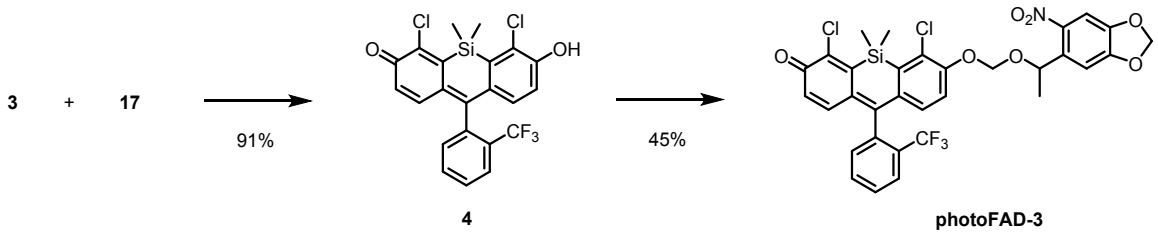
A flame-dried round-bottom flask under nitrogen atmosphere was charged with 1-bromo-2-(trifluoromethyl)benzene (**17**) (406 mg, 1.81 mmol, 5.0 eq.) and it was dissolved in anhydrous THF (4.2 mL). The solution was cooled to -78 °C and a 1.7 M solution of *tert*-butyllithium in pentane (1.06 mL, 1.81 mmol, 5.0) was added dropwise. After completion of the addition the solution was stirred for 10 min at the same temperature followed by dropwise addition of a solution of 10,10-

dimethyl-10-sila-3,6-bis((tert-butyldimethylsilyl)oxy)-9*H*-xanthen-9-one (**14**) (180 mg, 361 µmol, 1.0 eq.) in anhydrous THF (1.7 mL). The reaction was stirred for 30 min at room temperature before it was quenched by addition of 2N HCl (3.0 mL). It was stirred for another 20 min at room temperature and then poured into brine. It was washed, extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate. The residual solvent was removed under reduced pressure and the obtained residue was purified via flash column chromatography (SiO_2, 1:19 MeOH/ CH_2Cl_2) to give the desired product as a dark red solid (125 mg, 361 µmol, 87%). R_f = 0.31 (SiO_2, 1:39 v/v MeOH/ CH_2Cl_2). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82 (d, *J* = 7.6 Hz, 1H), 7.69 – 7.58 (m, 2H), 7.26 (d, *J* = 7.0 Hz, 2H), 7.04 (d, *J* = 2.4 Hz, 2H), 6.75 (d, *J* = 9.5 Hz, 2H), 6.52 (dd, *J* = 9.5, 2.4 Hz, 2H), 0.45 (s, 3H), 0.36 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.24, 156.41, 145.54, 140.23, 138.09, 138.08, 131.67, 131.39, 130.67, 129.64, 129.40, 129.16, 128.91, 128.87, 127.05, 126.81, 126.78, 126.74, 126.70, 124.86, 122.68, 122.00, 120.50, -0.69, -2.37. ^{19}F NMR (470 MHz, $CDCl_3$) δ -59.03. HR-MS: Calculated for $C_{22}H_{18}O_2F_3Si$ $[M+H]^+$ m/z 399.1028, found 399.1029.

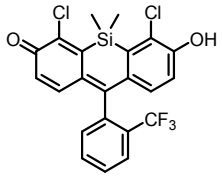
photoFAD-2:



A flame-dried round-bottom flask under nitrogen atmosphere was charged with 2-trifluoromethyl-TokyoMagenta (**18**) (170 mg, 427 µmol, 1.0 eq.) and freshly prepared 5-(1-(chloromethoxy)ethyl)-6-nitrobenzo[*d*][1,3]dioxole (153 mg, 0.59 mmol, 1.4 eq.). Anhydrous DMF (6.0 mL) was added followed by addition of anhydrous diisopropylethylamine (130 µL, 725 µmol, 1.7 eq.). The reaction was stirred at room temperature overnight under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product as yellow film (252 mg, 405 µmol, 95%). R_f = 0.17 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82 (d, *J* = 7.9 Hz, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.61 (t, *J* = 7.7 Hz, 1H), 7.38 (d, *J* = 2.4 Hz, 1H), 7.27 (s, 1H), 7.15 (dd, *J* = 9.9, 2.8 Hz, 1H), 7.00 (d, *J* = 14.4 Hz, 1H), 6.85 – 6.73 (m, 3H), 6.63 (dd, *J* = 9.0, 3.7 Hz, 1H), 6.19 (ddd, *J* = 10.2, 2.2, 1.0 Hz, 1H), 6.01 (dd, *J* = 6.0, 1.3 Hz, 1H), 5.95 (dd, *J* = 12.3, 1.3 Hz, 1H), 5.49 – 5.41 (m, 1H), 5.24 (d, *J* = 7.4 Hz, 1H), 5.13 (dd, *J* = 18.5, 7.3 Hz, 1H), 1.47 (dd, *J* = 6.3, 1.6 Hz, 3H), 0.52 (s, 3H), 0.41 (d, *J* = 9.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 184.26, 157.63, 157.56, 152.28, 152.20, 151.73, 151.72, 146.98, 146.95, 141.63, 141.59, 141.45, 139.86, 138.10, 138.08, 137.26, 137.24, 136.80, 135.28, 135.23, 134.88, 131.80, 131.54, 131.51, 129.37, 129.29, 129.26, 129.05, 129.02, 128.72, 127.66, 126.77, 126.74, 126.70, 126.66, 124.84, 122.66, 122.15, 121.65, 116.62, 116.16, 106.68, 104.88, 102.96, 91.27, 91.20, 72.33, 72.12, 23.52, 23.47, -0.52, -0.69, -2.43, -2.65. ^{19}F NMR (470 MHz, $CDCl_3$): δ -59.24. HR-MS: Calculated for $C_{32}H_{27}NO_7F_3Si$ $[M+H]^+$ m/z 622.1509, found 622.1516.

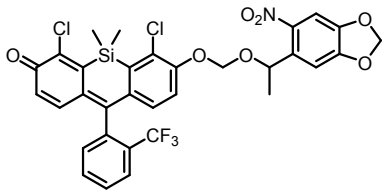


4',5'-Dichloro-2-trifluoromethyl-TokyoMagenta (4):



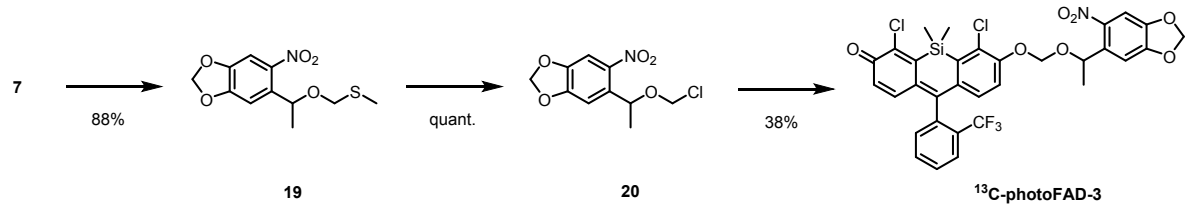
A flame-dried round-bottom flask under nitrogen atmosphere was charged with 1-bromo-2-(trifluoromethyl)benzene (1.08 g, 4.82 mmol, 7.0 eq.) and it was dissolved in anhydrous THF (15.0 mL). The solution was cooled to -78 °C and a 1.7 M solution of tert-butyllithium in pentane (2.8 mL, 4.82 mmol, 7.0) was added dropwise. After completion of the addition the solution was stirred for 10 min at the same temperature followed by dropwise addition of a solution of 10,10-dimethyl-10-sila-3,6-bis((tert-butyldimethylsilyl)oxy)-9*H*-xanthen-9-one (**3**) (391 mg, 689 µmol, 1.0 eq.) in anhydrous THF (7.0 mL). The reaction was stirred for 30 min at room temperature before it was quenched by addition of 2 N HCl (4 mL). It was stirred for another 20 min at room temperature and then poured into brine. It was washed, extracted with CH_2Cl_2 (3x) and the combined organic layers were dried over sodium sulfate. The residual solvent was removed under reduced pressure and the obtained residue was purified via flash column chromatography (SiO_2, 1:39 MeOH/ CH_2Cl_2) to give the desired product as a dark red solid (293 mg, 627 µmol, 91%). R_f = 0.42 (SiO_2, 1:39 v/v MeOH/ CH_2Cl_2). ^{1}H NMR (500 MHz, 1:1 MeOD/$CDCl_3$): δ 7.79 (d, *J* = 7.8 Hz, 1H), 7.65 (dt, *J* = 22.1, 7.5 Hz, 2H), 7.25 (d, *J* = 7.4 Hz, 1H), 6.65 (d, *J* = 9.4 Hz, 2H), 6.49 (d, *J* = 9.4 Hz, 2H), 0.82 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (125 MHz, 1:1 $CDCl_3$/MeOD): δ 167.36, 156.78, 141.94, 139.60, 138.60, 138.58, 136.55, 132.51, 131.72, 131.18, 130.01, 129.77, 129.73, 129.53, 129.28, 127.55, 127.35, 127.31, 127.27, 127.23, 125.37, 123.19, 121.01, -2.43, -2.97. ^{19}F NMR (470 MHz, 1:1 $CDCl_3$/MeOD) δ -59.93. HR-MS: Calculated for $C_{22}H_{16}O_2Cl_2F_3Si$ $[M+H]^+$ m/z 467.0249, found 467.0245.

photoFAD-3:

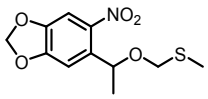


A flame-dried round-bottom flask under nitrogen atmosphere was charged with 4',5'-dichloro-2-trifluoromethyl-TokyoMagenta (**4**) (125 mg, 0.27 mmol, 1.0 eq.) and freshly prepared 5-(1-(chloromethoxy)ethyl)-6-nitrobenzo[d][1,3]dioxole (96 mg, 0.37 mmol, 1.4 eq.). Anhydrous DMF (4.0 mL) was added followed by addition of anhydrous diisopropylethylamine (79 µL, 0.45 mmol, 1.7 eq.). The reaction was stirred at room temperature for 12 h under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product

as yellow film (83 mg, 0.12 mmol, 45%). R_f = 0.23 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (d, *J* = 7.6 Hz, 1H), 7.68 (dt, *J* = 23.7, 7.4 Hz, 2H), 7.44 (s, 1H), 7.28 (d, *J* = 7.3 Hz, 1H), 7.00 (d, *J* = 9.0 Hz, 1H), 6.93 (s, 1H), 6.75 (d, *J* = 10.0 Hz, 1H), 6.61 (d, *J* = 9.0 Hz, 1H), 6.33 (d, *J* = 10.0 Hz, 1H), 6.05 – 6.01 (m, 1H), 6.00 – 5.93 (m, 1H), 5.51 (q, *J* = 6.2 Hz, 1H), 5.31 (d, *J* = 7.4 Hz, 1H), 5.24 (d, *J* = 7.4 Hz, 1H), 1.49 (d, *J* = 6.3 Hz, 3H), 0.86 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.32, 153.77, 152.73, 152.32, 147.10, 144.16, 142.08, 141.61, 141.50, 138.82, 138.24, 138.23, 136.74, 134.99, 134.18, 132.07, 131.35, 131.08, 129.74, 129.52, 129.49, 129.25, 129.09, 129.01, 127.01, 126.96, 126.92, 126.88, 124.81, 122.63, 120.45, 115.30, 106.57, 105.14, 103.00, 92.12, 73.50, 23.70, -2.45, -3.04. ^{19}F NMR (470 MHz, $CDCl_3$): δ -59.58. HR-MS: Calculated for $C_{32}H_{25}NO_7Cl_2F_3Si$ $[M+H]^+$ m/z 690.0729, found 690.0719.

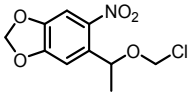


13C - 5-[1-[(Methylthio)methoxy]ethyl]-6-nitro-1,3-benzodioxole (19):



The reaction was performed with flame-dried equipment under nitrogen atmosphere. 1-(6-Nitro-1,3-benzodioxol-5-yl)ethanol (**7**) (150 mg, 0.71 mmol, 1.0 eq.) was dissolved in anhydrous acetonitrile (4 mL) and the solution was cooled to 0 °C. ^{13}C-labeled dimethylsulfide (0.42 mL, 5.7 mmol, 8.0 eq.) was added before benzoylperoxide (688 mg, 2.8 mmol, 4.0 eq.) was added in four portions every 30 min. After completion of the addition the reaction was stirred for another 4 h at the same temperature. It was stirred for an additional 2 h at room temperature followed by addition of 1 M NaOH until a pH 9 was reached. It was stirred overnight at room temperature. The reaction was poured into brine and the organic components were extracted with EtOAc (3x). The combined organic layers were dried over sodium sulfate, concentrated and purified via flash column chromatography (SiO_2, 1:9 EtOAc/hexanes) to give the desired product as a yellow solid (170 mg, 0.63 mmol, 88%). R_f = 0.25 (SiO_2, 1:9 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.45 (s, 1H), 7.15 (s, 1H), 6.11 (dd, *J* = 6.6, 1.2 Hz, 2H), 5.44 (qd, *J* = 6.3, 4.6 Hz, 1H), 4.43 (tdd, *J* = 154.6, 11.4, 5.4 Hz, 2H), 2.12 (dd, *J* = 139.1, 4.3 Hz, 3H), 1.50 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 152.59, 147.13, 142.33, 137.11, 106.53, 105.20, 103.04, 73.19, 70.49, 23.46, 23.43, 14.23. HR-MS: Calculated for $^{12}C_9{}^{13}C_2H_{13}NO_5NaS$ $[M+Na]^+$ m/z 296.0479, found 296.0489.

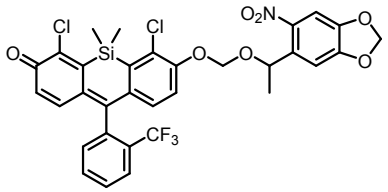
13C - 5-[1-(Chloromethoxy)ethyl]-6-nitro-1,3-benzodioxole (20):



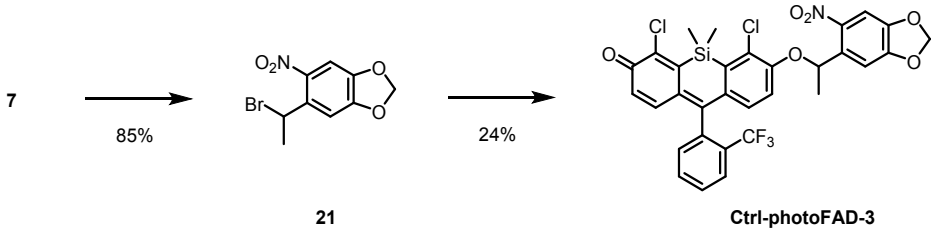
A flame-dried round-bottom flask under nitrogen atmosphere was charged with ^{13}C-labeled 5-[1-[(methylthio)methoxy]ethyl]-6-nitro-1,3-benzodioxole (**19**) (25 mg, 91 µmol, 1.0 eq.) and it was

dissolved in anhydrous CH_2Cl_2 (0.4 mL). It was cooled to 0 °C and a solution of sulfuryl chloride in anhydrous CH_2Cl_2 (0.6 mL) was added slowly. The reaction was stirred for 4 h at room temperature before the volatiles were removed under reduced pressure. It was re-dissolved in chloroform and concentrated under reduced pressure. This sequence was repeated two more times to yield the desired product as a yellow oil (24 mg, 91 µmol, quant.) that was used directly for the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49 (s, 1H), 7.08 (s, 1H), 6.12 (d, J = 3.9 Hz, 2H), 5.70 – 5.03 (m, 3H), 1.55 (d, J = 6.2 Hz, 3H).^{13}C NMR (125 MHz, $CDCl_3$, 298 K): δ 80.45. All non-labeled peaks are equivalent to the reported peaks for compound **9**.

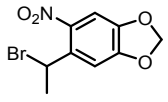
^{13}C-photoFAD-3:



A flame-dried round-bottom flask under nitrogen atmosphere was charged with 4',5'-dichloro-2-trifluoromethyl-TokyoMagenta (**4**) (37 mg, 80 µmol, 1.0 eq.) and freshly prepared ^{13}C-labeled 5-(1-(chloromethoxy)ethyl)-6-nitrobenzo[d][1,3]dioxole (**20**) (29 mg, 0.11 mmol, 1.4 eq.). Anhydrous DMF (1.1 mL) was added followed by addition of anhydrous diisopropylethylamine (24 µL, 0.14 mmol, 1.7 eq.). The reaction was stirred at room temperature overnight under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product as yellow film (21 mg, 30 µmol, 38%). R_f = 0.23 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 (d, *J* = 7.7 Hz, 1H), 7.68 (dt, *J* = 23.1, 7.5 Hz, 2H), 7.45 (s, 1H), 7.31 – 7.27 (m, 1H), 7.00 (d, *J* = 9.0 Hz, 1H), 6.76 (d, *J* = 10.0 Hz, 1H), 6.61 (d, *J* = 9.0 Hz, 1H), 6.35 (d, *J* = 10.1 Hz, 1H), 6.01 (d, *J* = 29.2 Hz, 2H), 5.51 (dt, *J* = 10.7, 5.3 Hz, 1H), 5.49 – 5.39 (m, 1H), 5.17 – 5.05 (m, 1H), 1.50 (d, *J* = 6.3 Hz, 3H), 0.87 (s, 3H), 0.82 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 92.12. All non-labeled peaks are equivalent to the reported peaks for photoFAD-3. ^{19}F NMR (470 MHz, $CDCl_3$): δ -59.22. HR-MS: calculated for $^{12}C_{31}{}^{13}CH_{25}NO_7Cl_2F_3Si$ $[M+H]^+$ m/z 691.0763, found 691.0761.



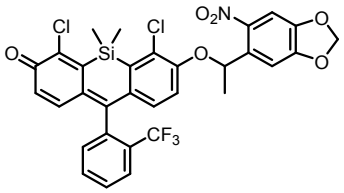
5-(1-bromoethyl)-6-nitrobenzo[*d*][1,3]dioxole (21):



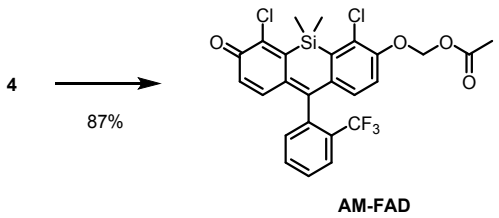
A flame dried flask under nitrogen atmosphere was charged with **7** (175 mg, 0.83 mmol, 1.0 eq.) and it was dissolved in anhydrous CH_2Cl_2 (4.1 mL). The solution was cooled to 0 °C and phosphorus tribromide (31 µL, 0.33 mmol, 0.4 eq.) was added dropwise. It was stirred at room temperature for 3 h

before it was quenched with ice and poured into brine. It was extracted with CH_2Cl_2 (3x), dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:1 CH_2Cl_2/hexanes) to give the desired product as yellow solid (193 mg, 0.70 mmol, 85%). The obtained analytical data matched the literature.[4]

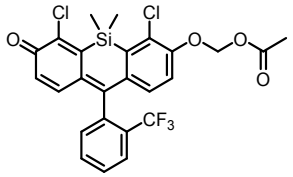
Ctrl-photoFAD-3:



A flame-dried round-bottom flask under nitrogen atmosphere was charged with 4',5'-dichloro-2-trifluoromethyl-TokyoMagenta (**4**) (48 mg, 0.10 mmol, 1.0 eq.) and 5-(1-Bromoethyl)-6-nitro-1,3-benzodioxole (39 mg, 0.14 mmol, 1.4 eq.). Anhydrous DMF (2.0 mL) was added followed by addition of anhydrous diisopropylethylamine (30 µL, 0.17 mmol, 1.7 eq.). The reaction was stirred at room temperature overnight under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 EtOAc/hexanes) to give the desired product as yellow film (17 mg, 0.03 mmol, 24%). R_f = 0.24 (SiO_2, 1:3 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 – 7.78 (m, 1H), 7.67 – 7.58 (m, 2H), 7.52 (s, 1H), 7.20 (d, *J* = 8.9 Hz, 2H), 6.74 (d, *J* = 10.0 Hz, 1H), 6.49 (d, *J* = 1.1 Hz, 2H), 6.33 (d, *J* = 10.0 Hz, 1H), 6.14 (q, *J* = 6.2 Hz, 1H), 6.10 (dd, *J* = 10.6, 1.1 Hz, 2H), 1.74 (d, *J* = 6.2 Hz, 3H), 0.90 (s, 3H), 0.87 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$, 298 K): δ 177.37, 153.95, 153.22, 152.76, 147.72, 144.14, 142.03, 141.52, 141.19, 139.02, 138.15, 135.69, 134.49, 134.44, 131.99, 131.20, 131.11, 129.56, 129.48, 129.32, 129.11, 127.05, 127.01, 126.93, 124.74, 122.56, 120.38, 114.26, 106.17, 105.59, 103.34, 72.99, 29.85, 23.38, -2.29, -2.98. ^{19}F NMR (470 MHz, $CDCl_3$, 298 K): δ -59.28. HR-MS: calculated for $C_{31}H_{23}NO_6Cl_2F_3Si$ $[M+H]^+$ m/z 660.0624, found 660.0632.



AM-FAD-3:

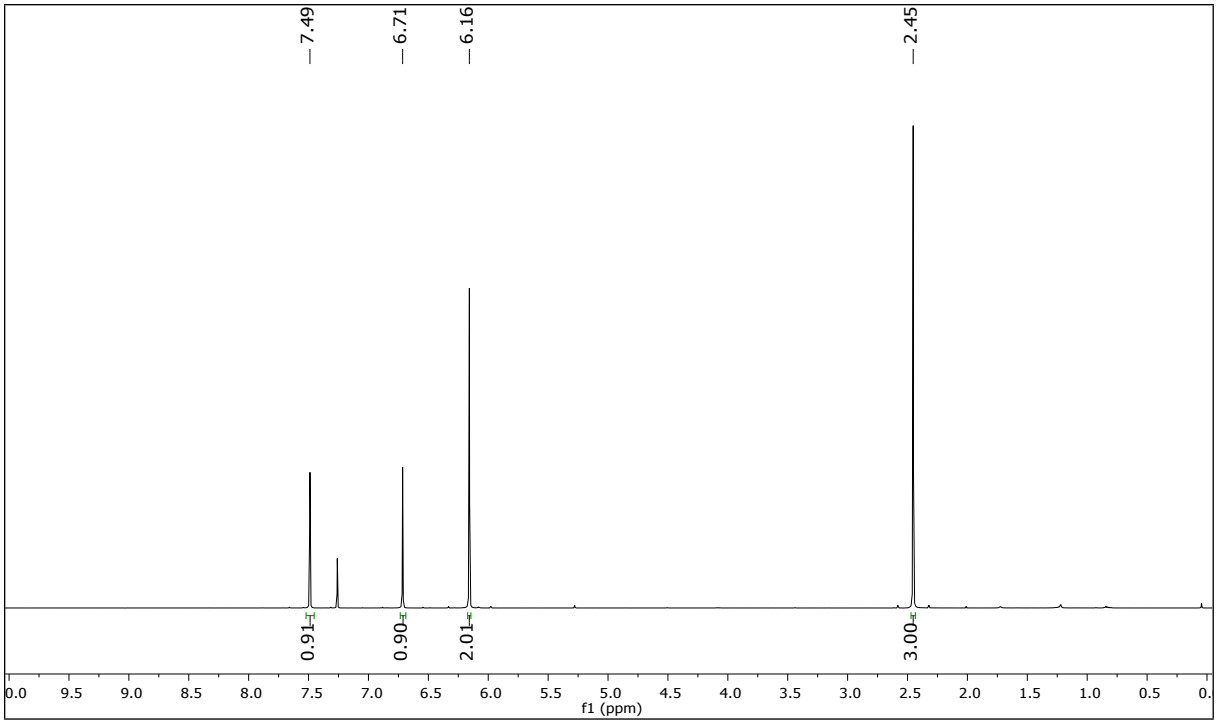


A flame-dried round-bottom flask under nitrogen atmosphere was charged with 4',5'-dichloro-2-trifluoromethyl-TokyoMagenta (**4**) (100 mg, 0.21 µmol, 1.0 eq.) and it was dissolved in anhydrous DMF (5.0 mL). Bromoethyl acetate (53 µL, 0.54 mmol, 2.5 eq.) was added, followed by addition of anhydrous diisopropylethylamine (0.11 mL, 0.64 mmol, 3.0 eq.). The reaction was stirred at room

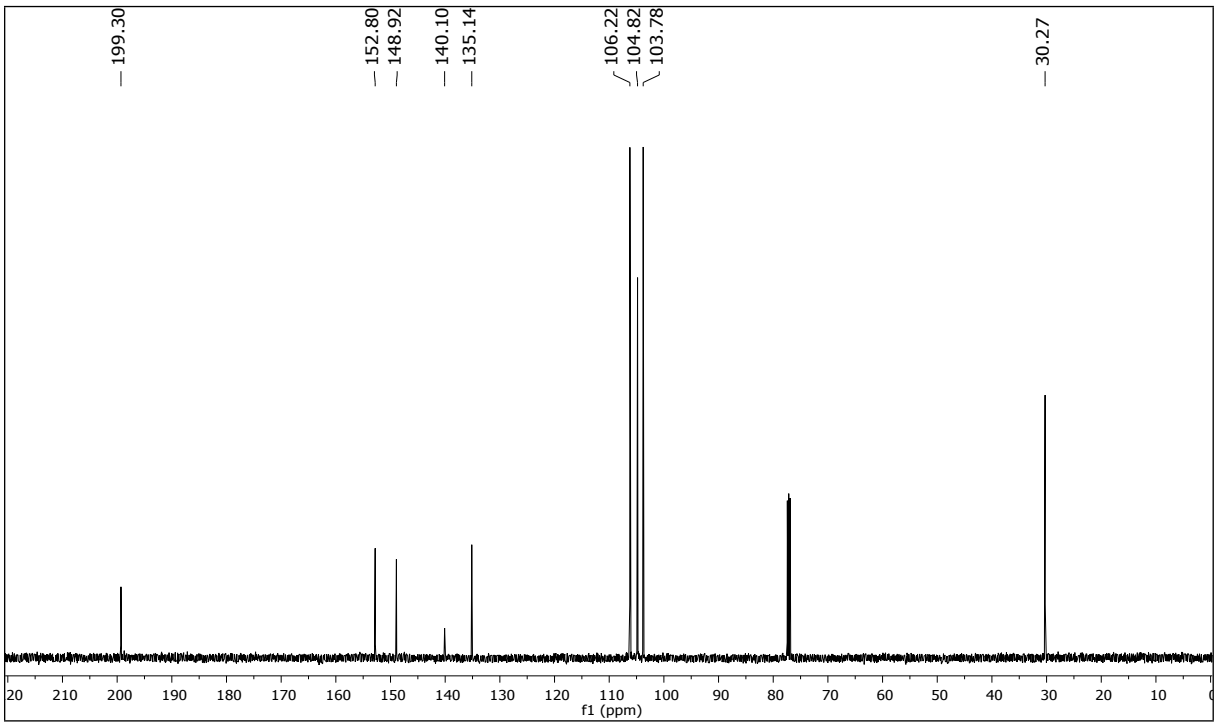
temperature overnight under exclusion of light. It was poured into brine, extracted with EtOAc (2x) and the combined organic layers were washed with brine (3x). The organic layer was dried over sodium sulfate and concentrated under reduced pressure. The residue was purified via flash column chromatography (SiO_2, 1:3 to 1:1 EtOAc/hexanes) to give the desired product as yellow solid (100 mg, 0.19 mmol, 87%). R_f = 0.49 (SiO_2, 1:1 v/v EtOAc/hexanes). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.82 (d, *J* = 7.7 Hz, 1H), 7.65 (dt, *J* = 24.4, 7.2 Hz, 2H), 7.27 (d, *J* = 6.8 Hz, 1H), 6.75 (d, *J* = 10.1 Hz, 1H), 6.64 – 6.57 (m, 2H), 6.32 (d, *J* = 10.0 Hz, 1H), 4.72 (d, *J* = 2.0 Hz, 2H), 3.77 (s, 3H), 0.88 (s, 3H), 0.85 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.19, 168.06, 154.46, 152.52, 144.03, 141.95, 141.40, 138.98, 138.04, 138.02, 134.94, 134.17, 131.94, 131.22, 130.74, 129.63, 129.48, 129.47, 129.39, 129.14, 129.01, 128.90, 126.91, 126.88, 126.84, 126.81, 126.77, 124.64, 122.46, 120.28, 113.21, 65.53, 52.53, -2.52, -3.08. ^{19}F NMR (470 MHz, $CDCl_3$, 298 K): δ -59.23. HR-MS: calculated for $C_{25}H_{20}O_4Cl_2F_3Si$ $[M+H]^+$ m/z 539.0460, found 539.0469.

23. NMR Spectra

Compound **6**:

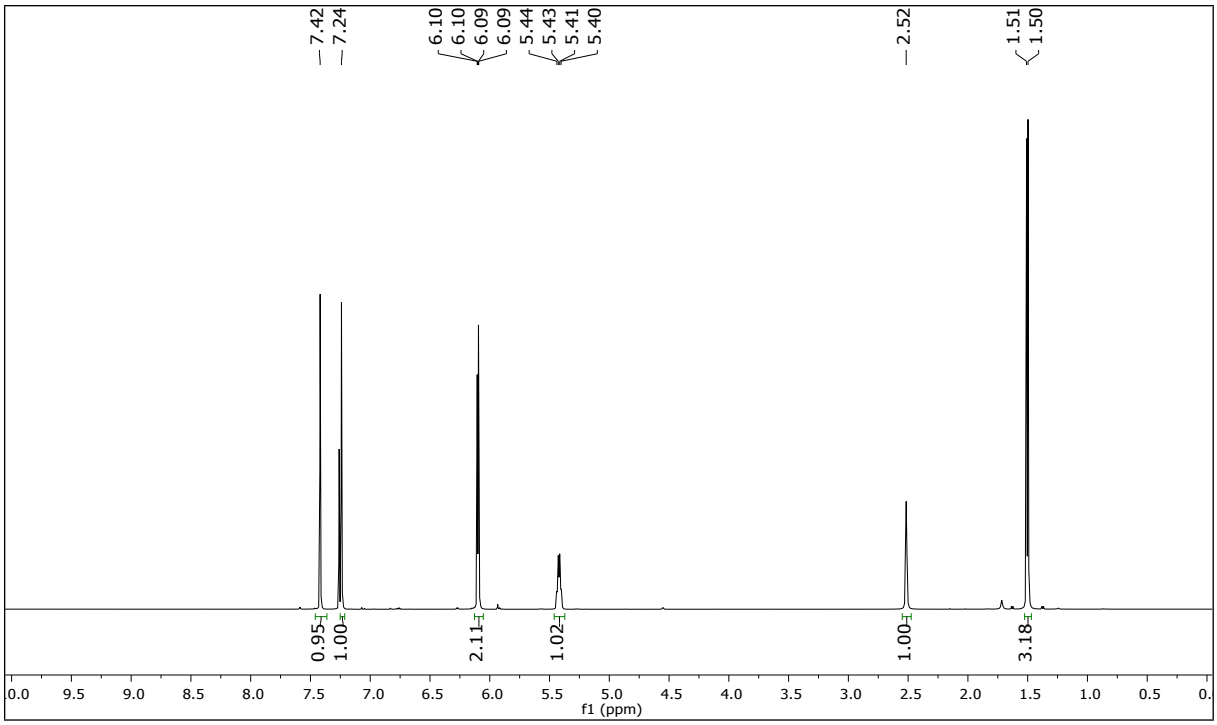


^{1}H NMR (500 MHz, $CDCl_3$)

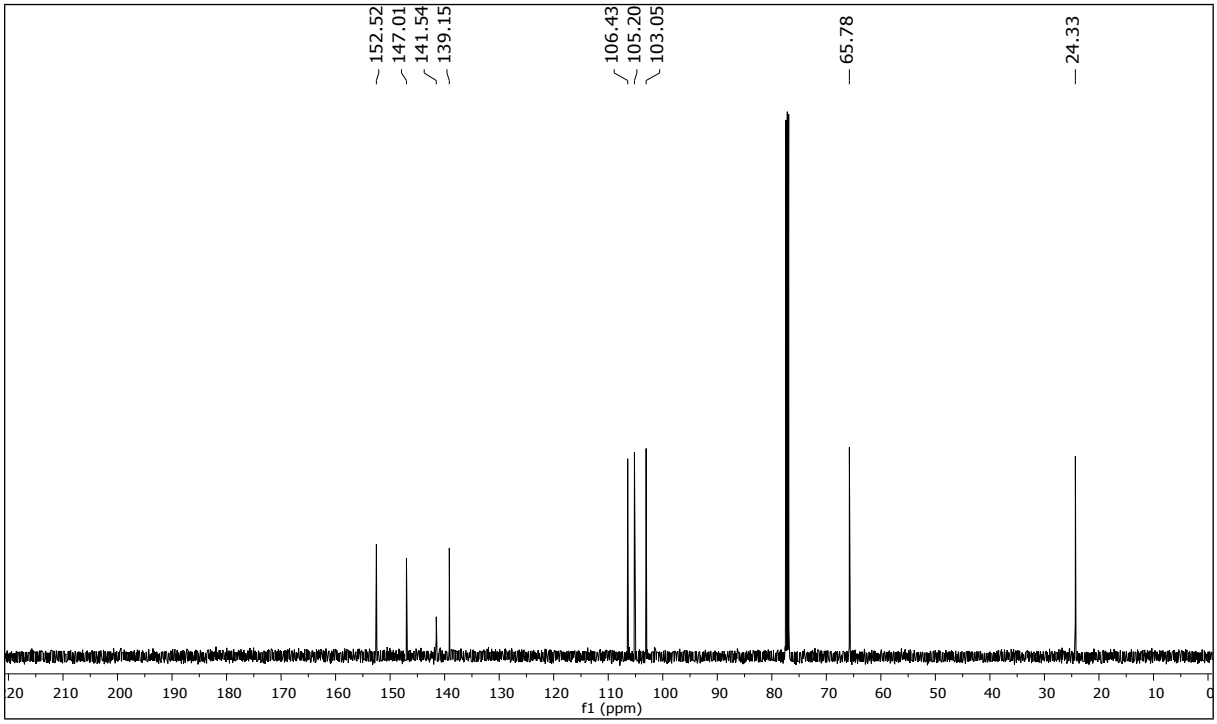


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **7**:

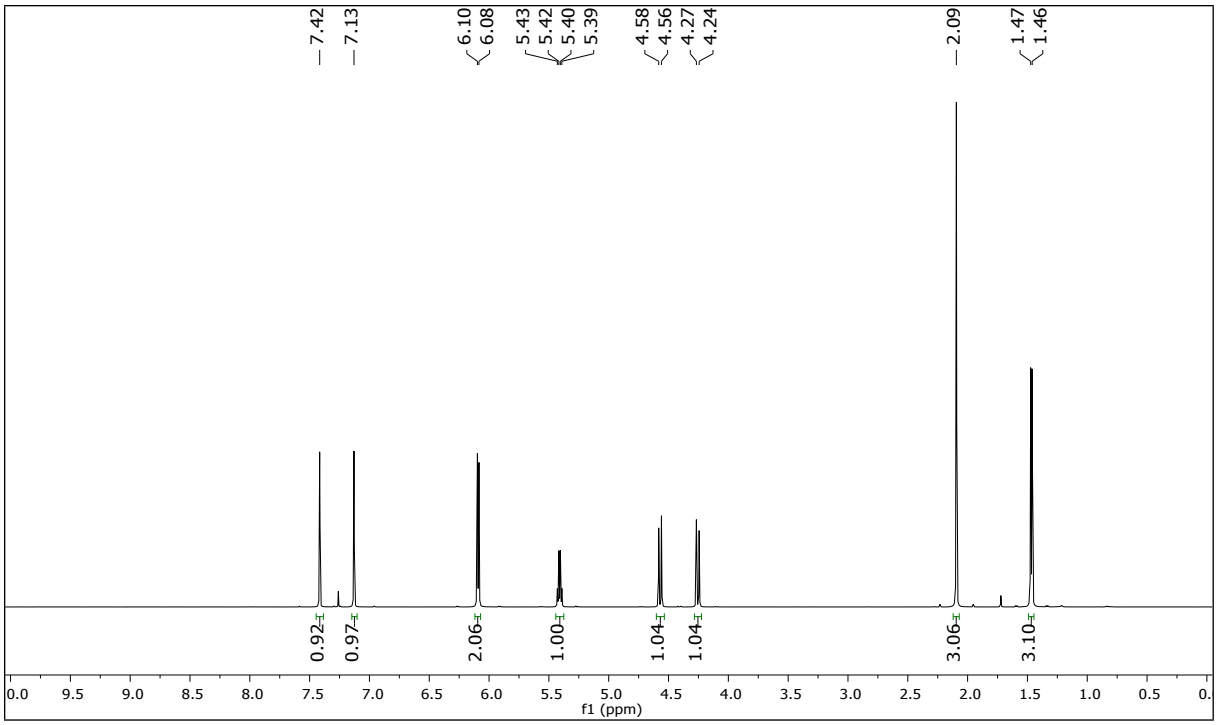


^{1}H NMR (500 MHz, $CDCl_3$)

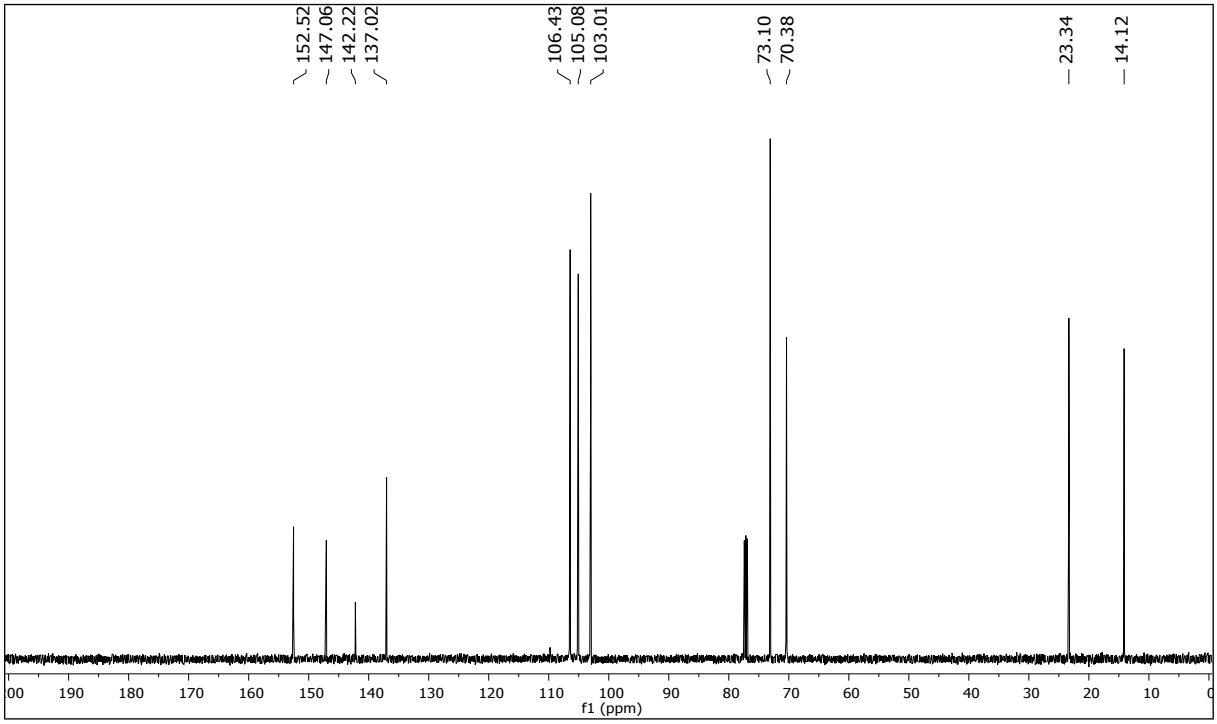


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **8**:

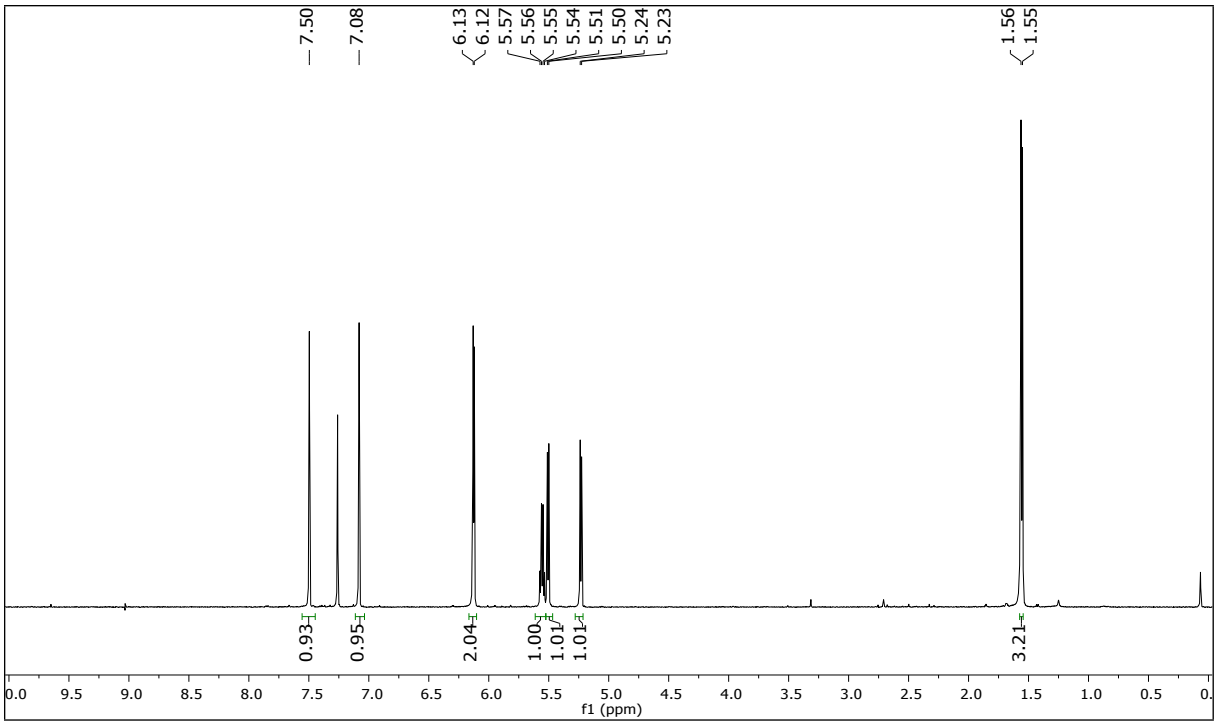


^{1}H NMR (500 MHz, $CDCl_3$)

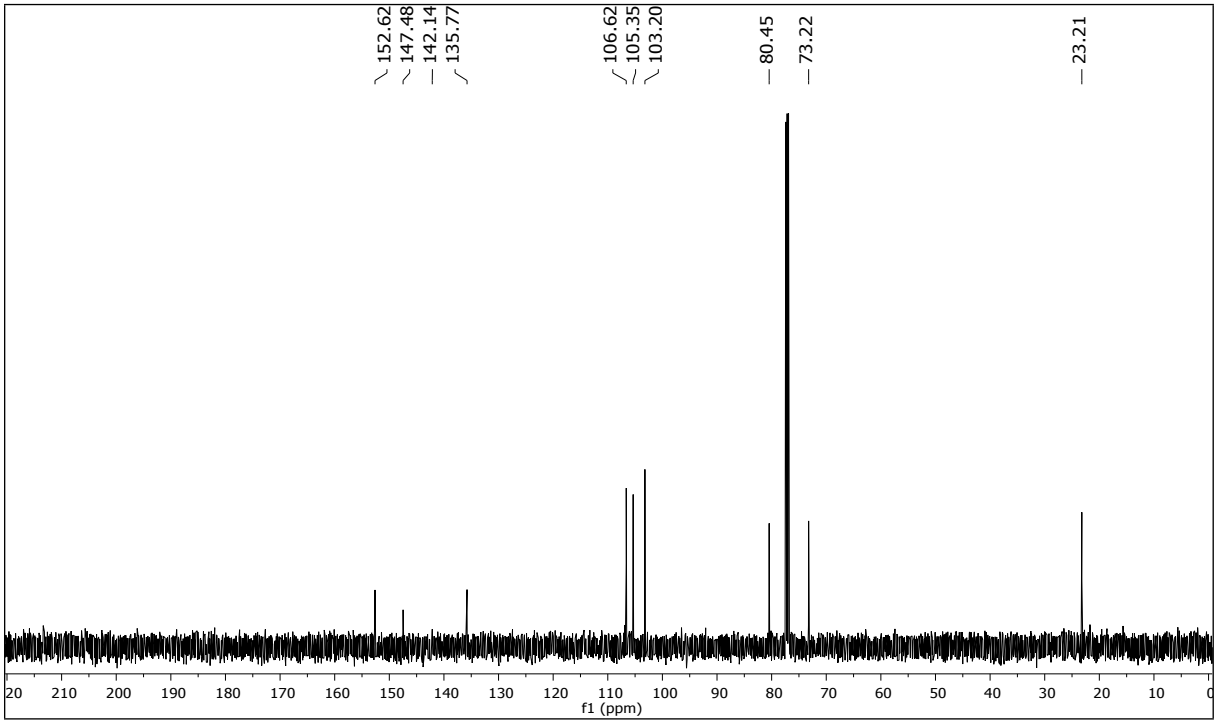


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **9**:

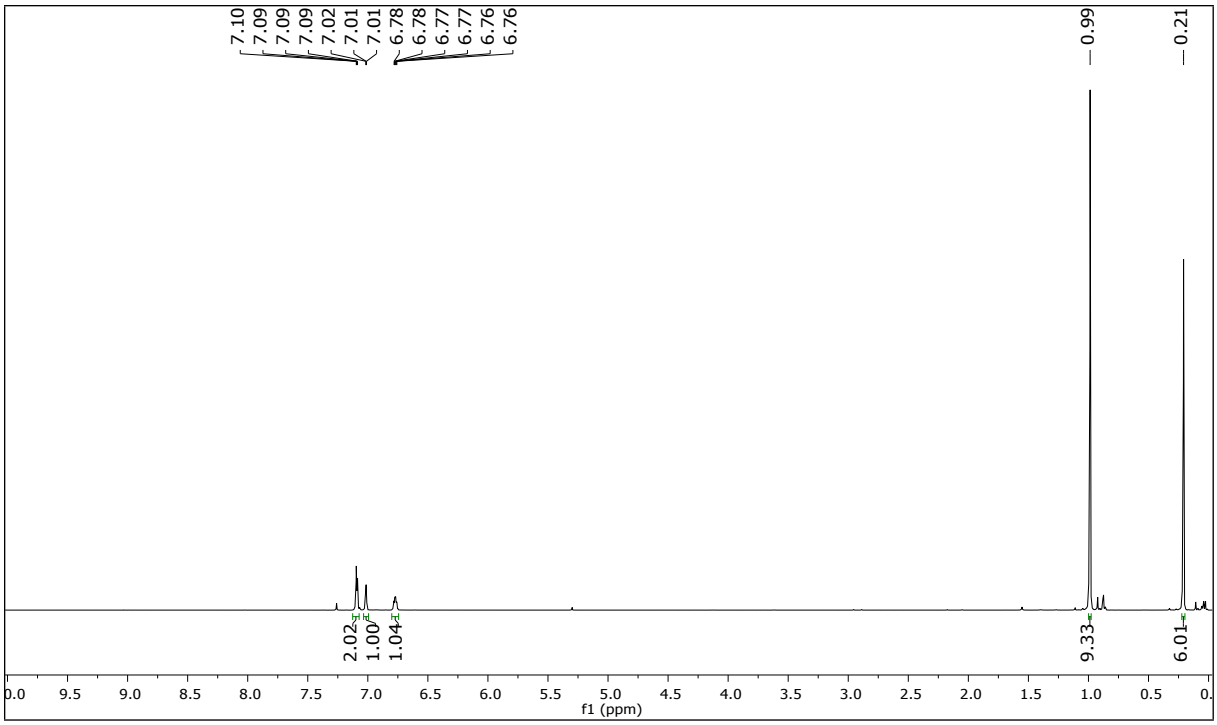


^{1}H NMR (500 MHz, $CDCl_3$)

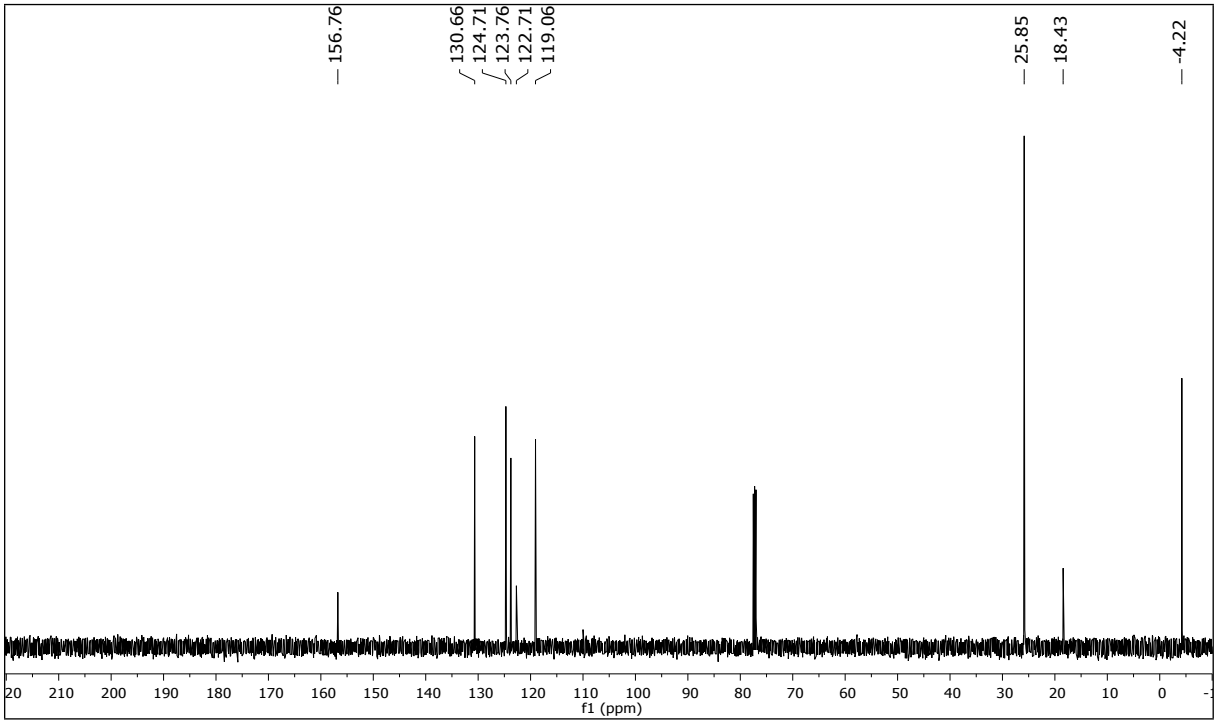


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **11**:

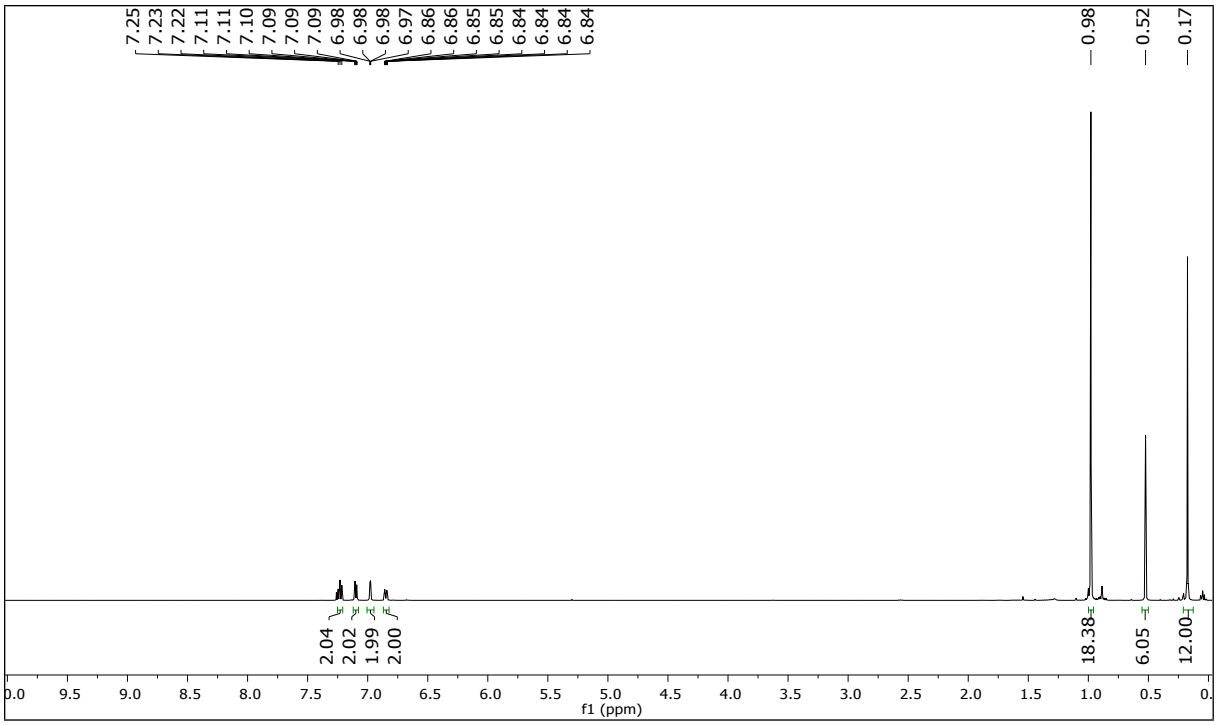


^{1}H NMR (500 MHz, $CDCl_3$)

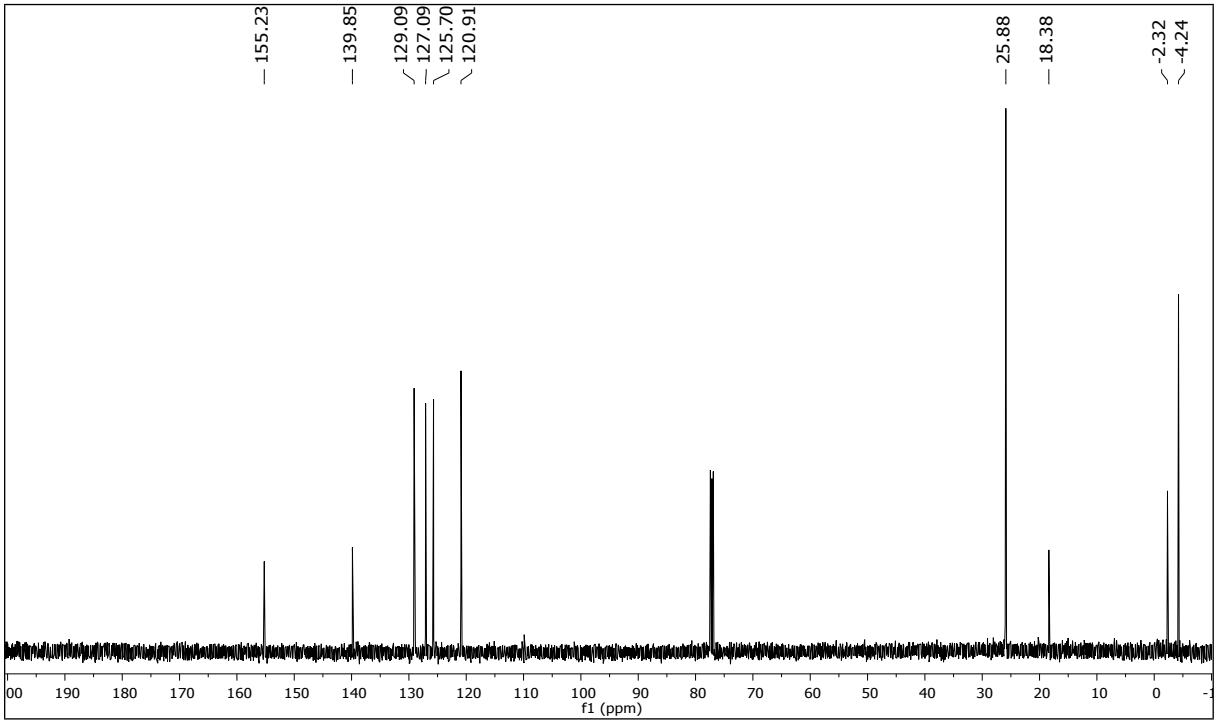


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **12**:

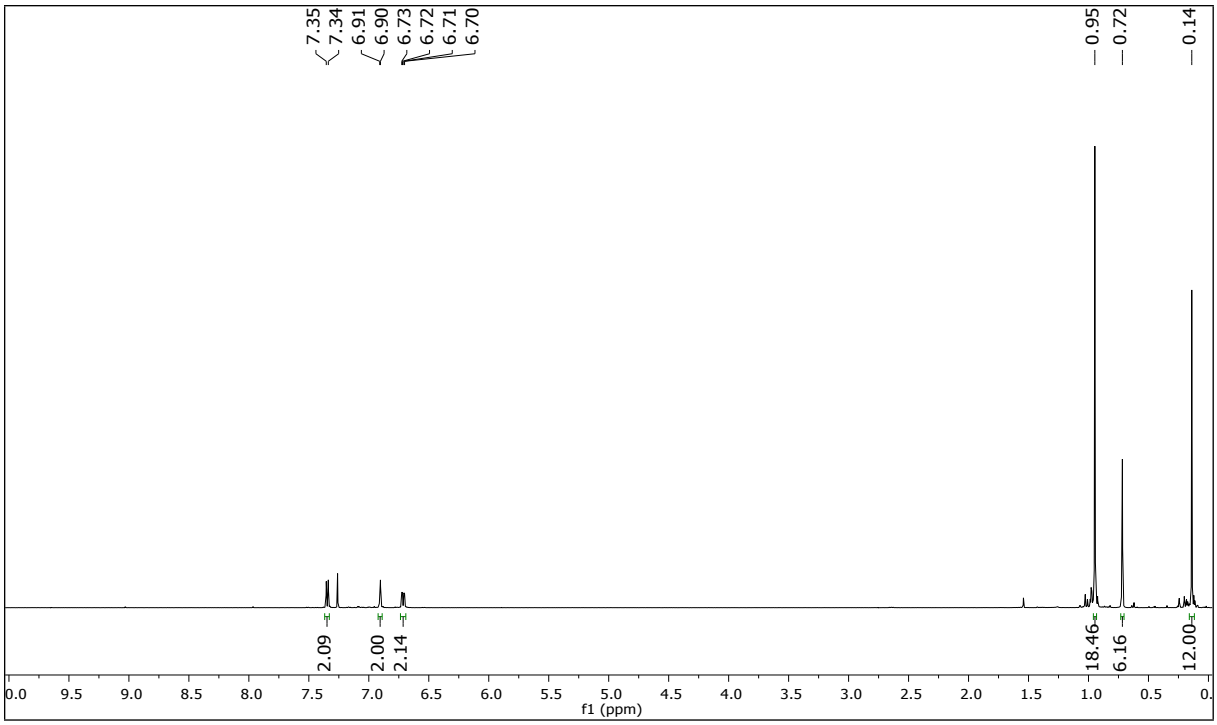


^{1}H NMR (500 MHz, $CDCl_3$)

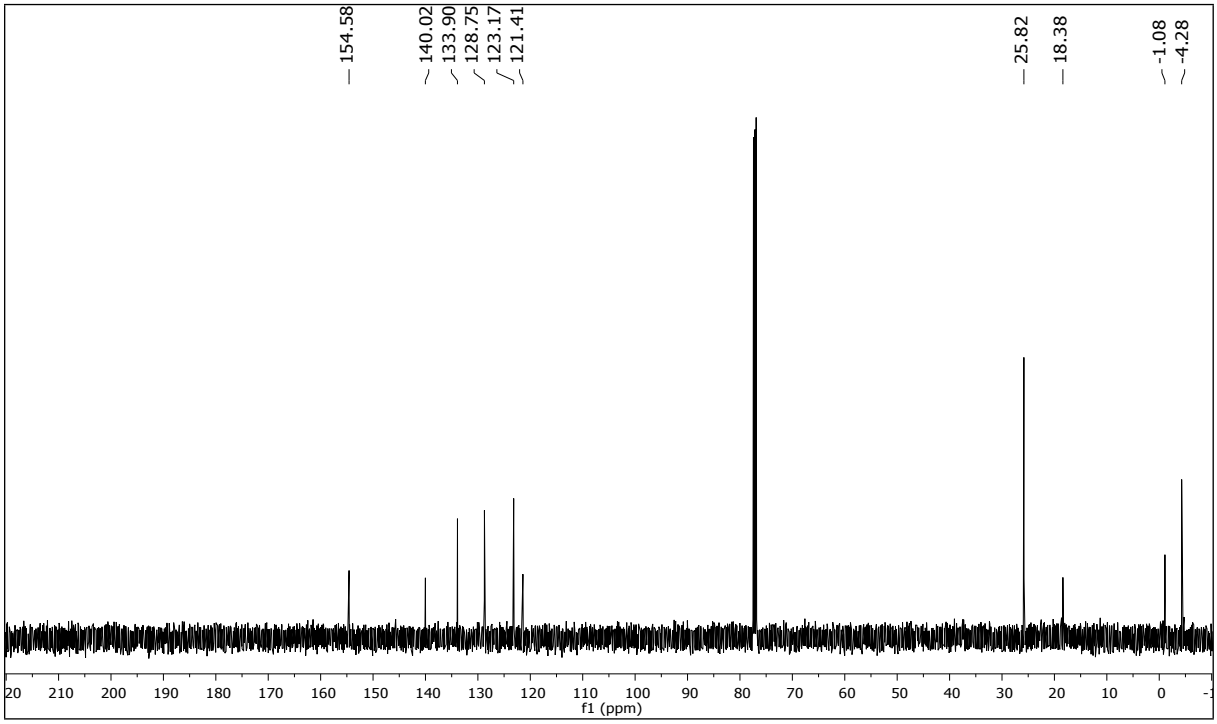


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **13**:

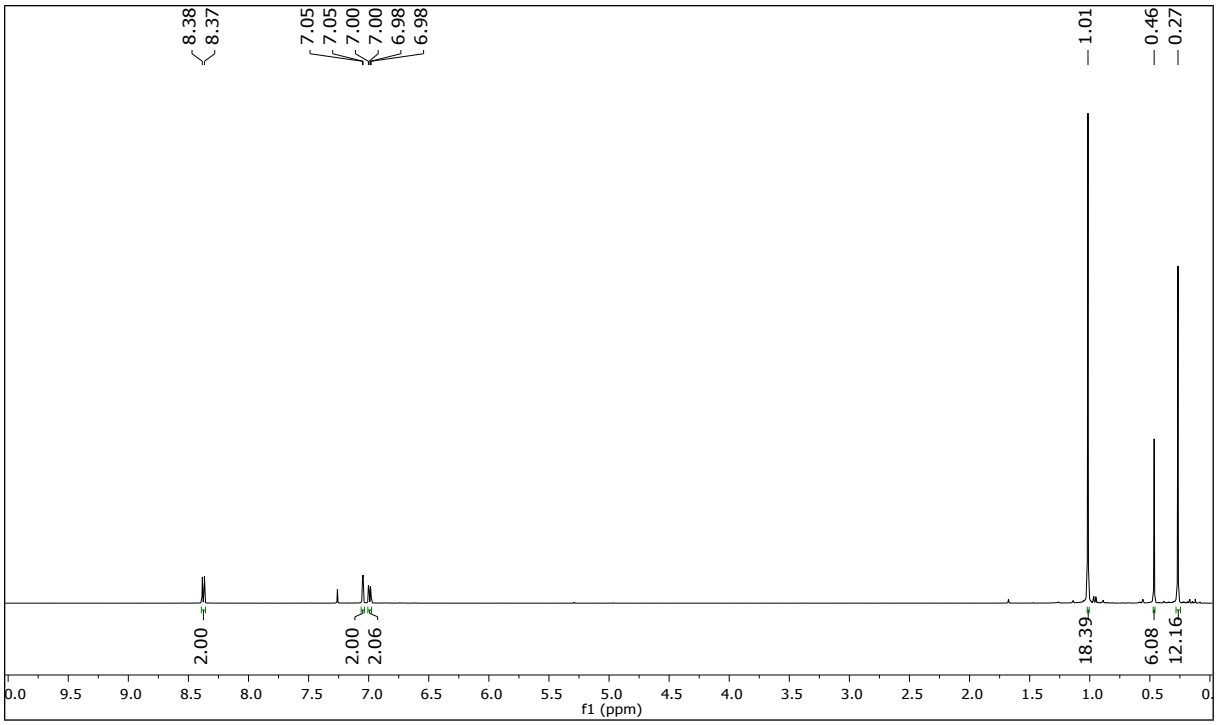


^{1}H NMR (500 MHz, $CDCl_3$)

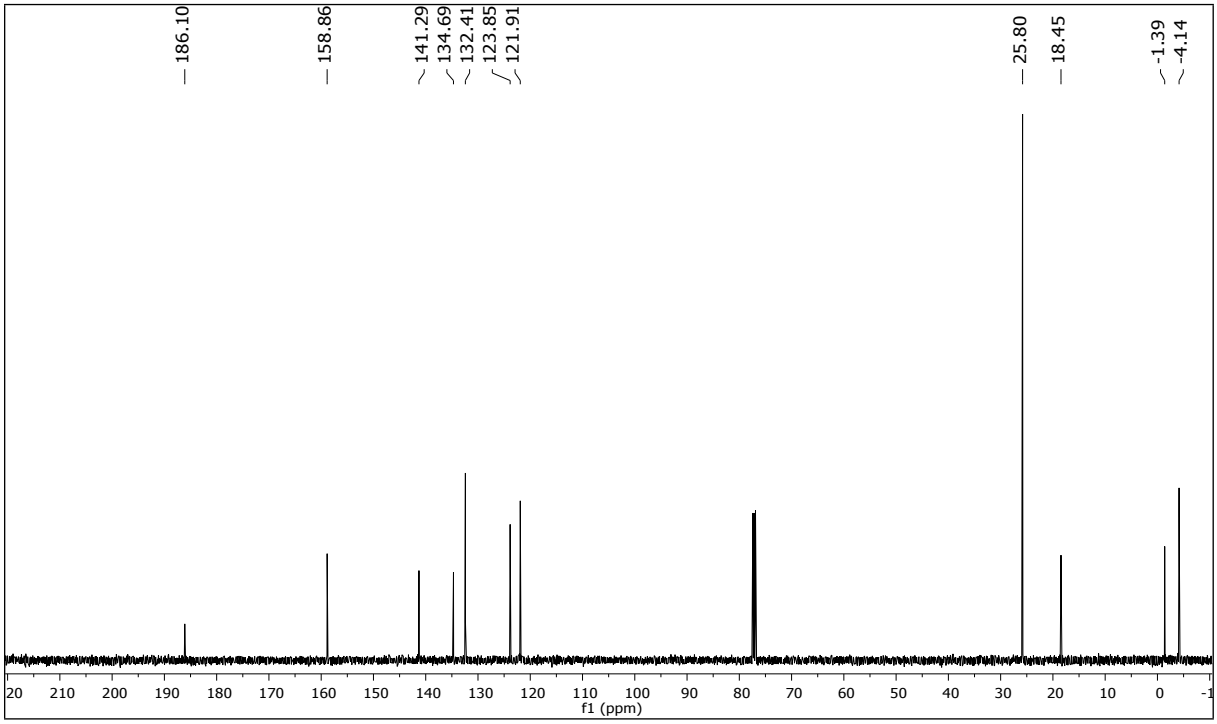


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **14**:

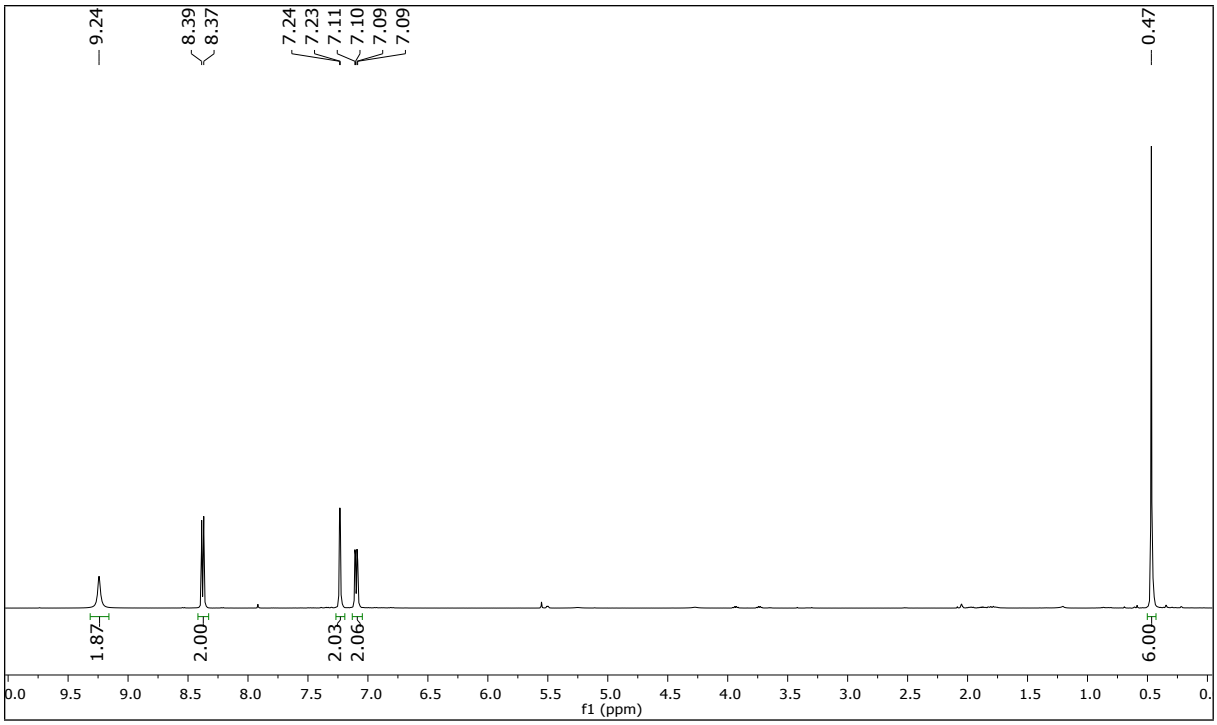


^{1}H NMR (500 MHz, $CDCl_3$)

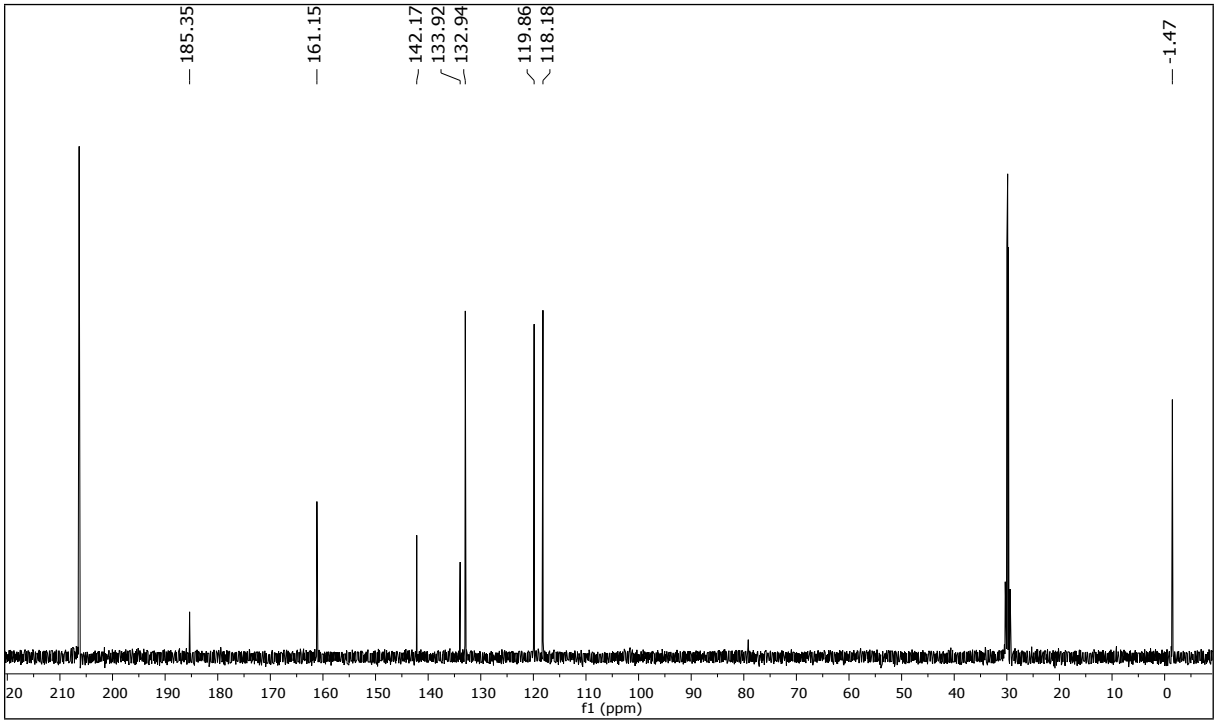


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **1**: 1H-NMR (500 MHz, acetone-d_6, 298K); 13C-NMR (125 MHz, acetone-d_6, 298K)

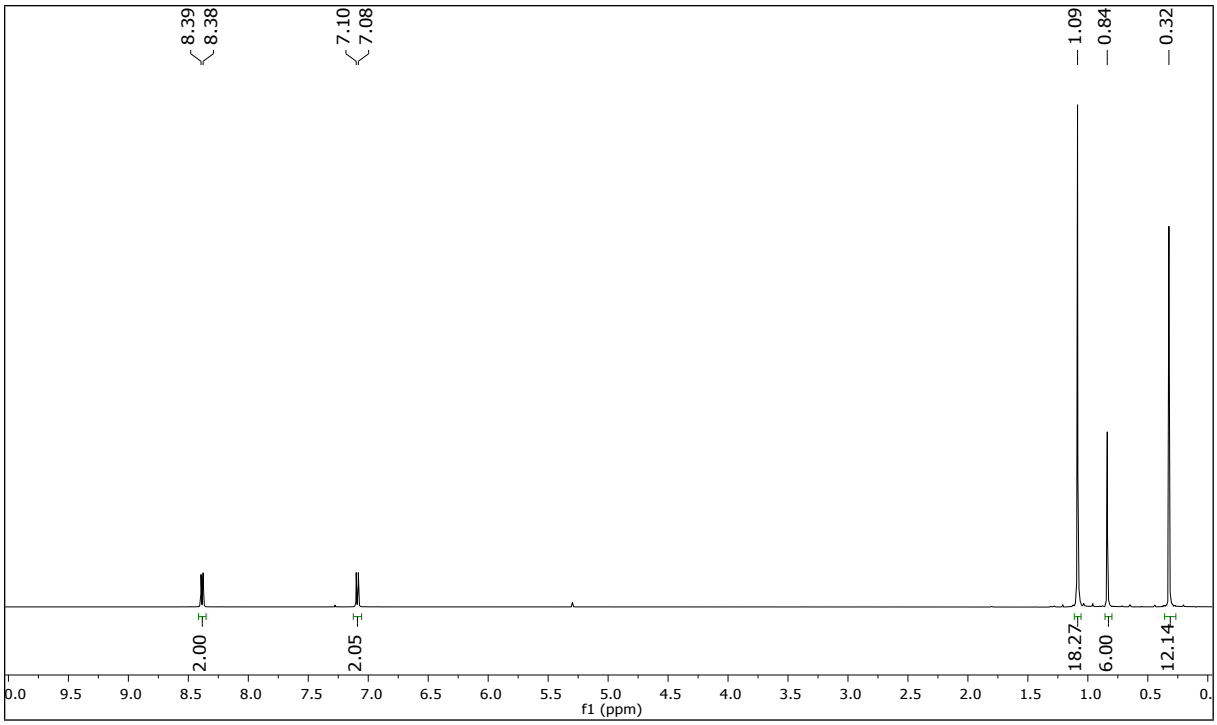


^{13}C NMR (125 MHz, acetone-d_6)

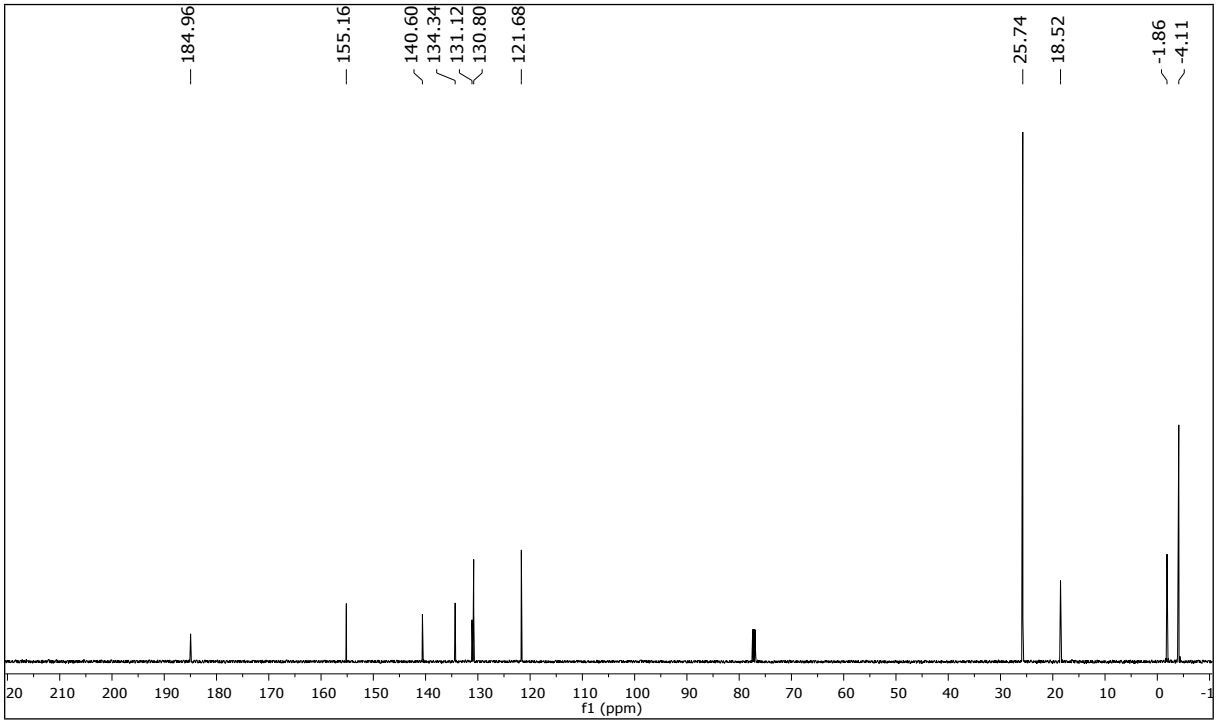


^{13}C NMR (125 MHz, acetone-d_6)

Compound **3**: 1H-NMR (500 MHz, $CDCl_3$, 298K); 13C-NMR (125 MHz, $CDCl_3$, 298K)

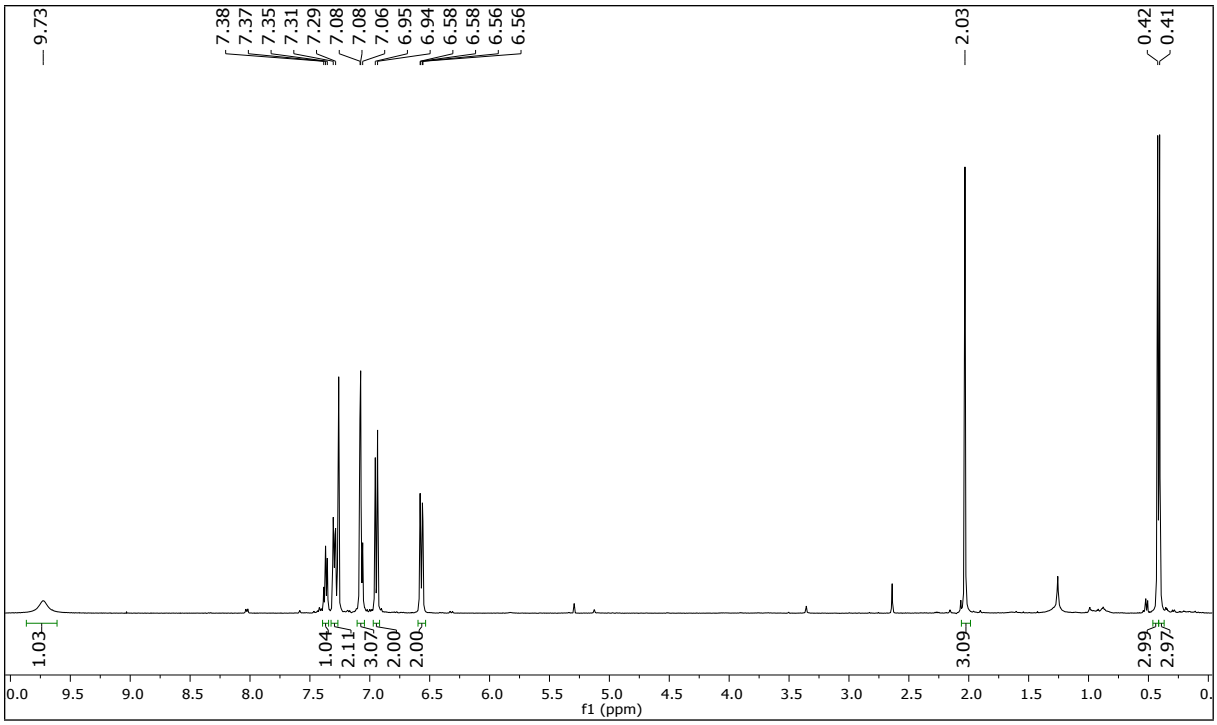


^{1}H NMR (500 MHz, $CDCl_3$)

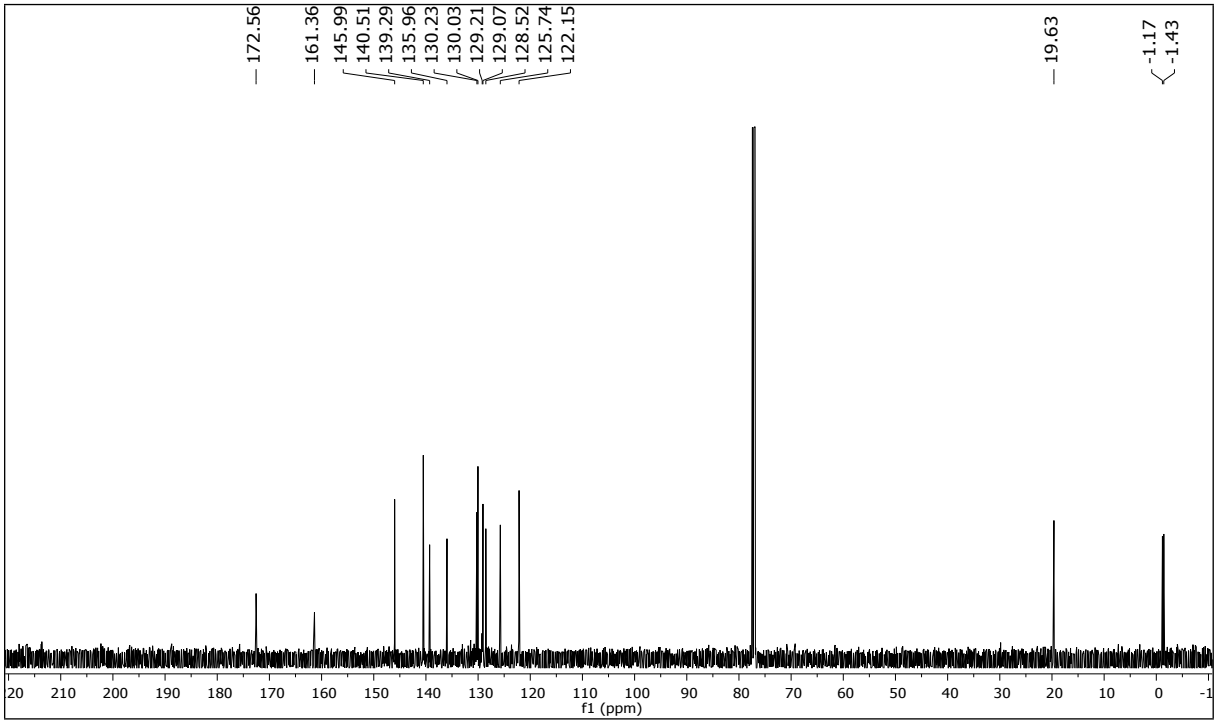


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **16**:

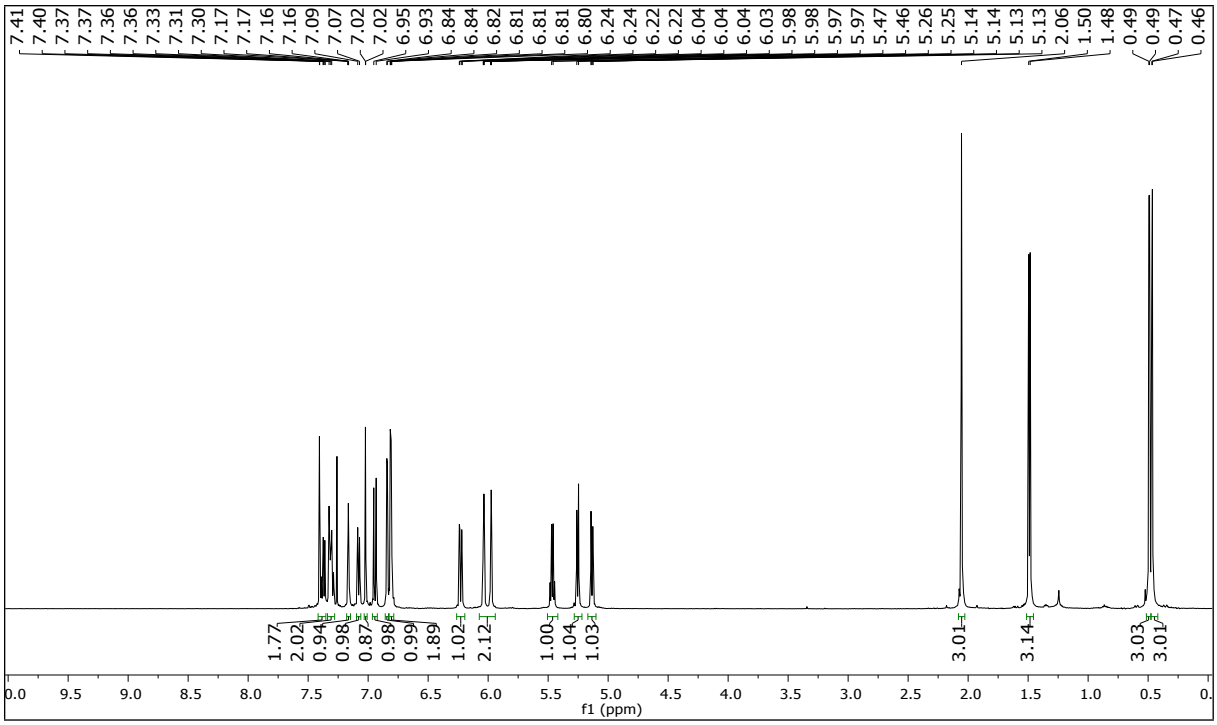


^{1}H NMR (500 MHz, $CDCl_3$)

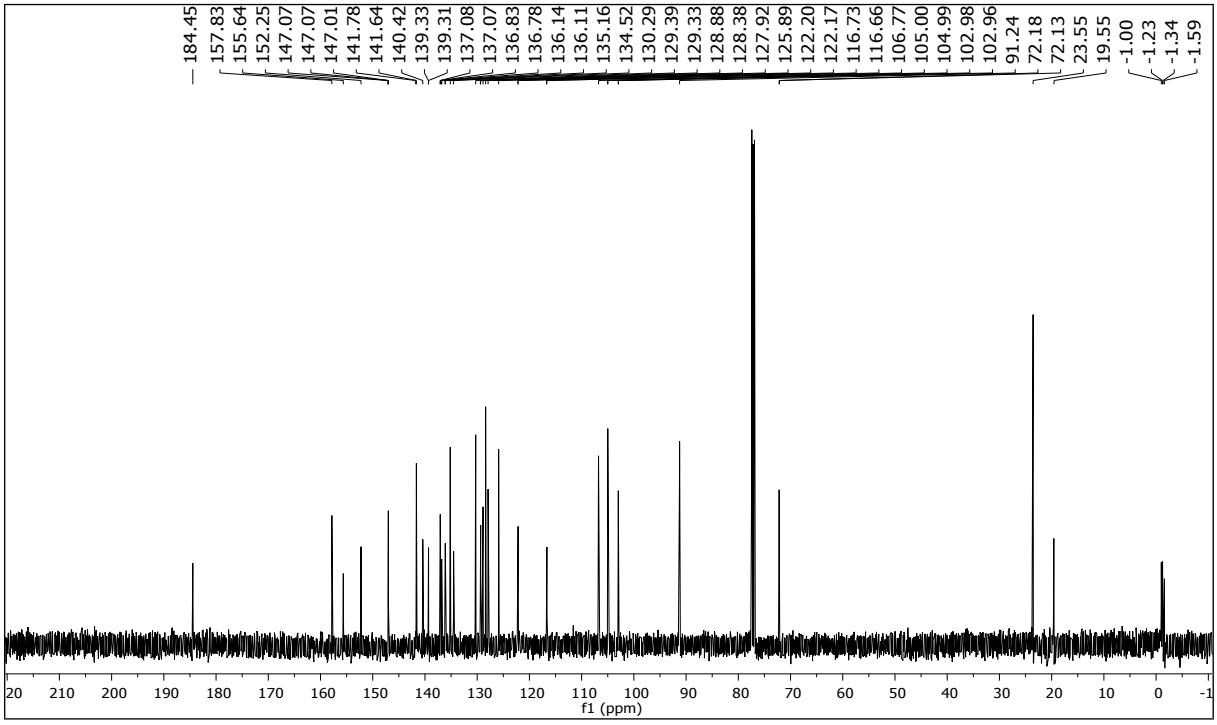


^{13}C NMR (125 MHz, $CDCl_3$)

photoFAD-1:

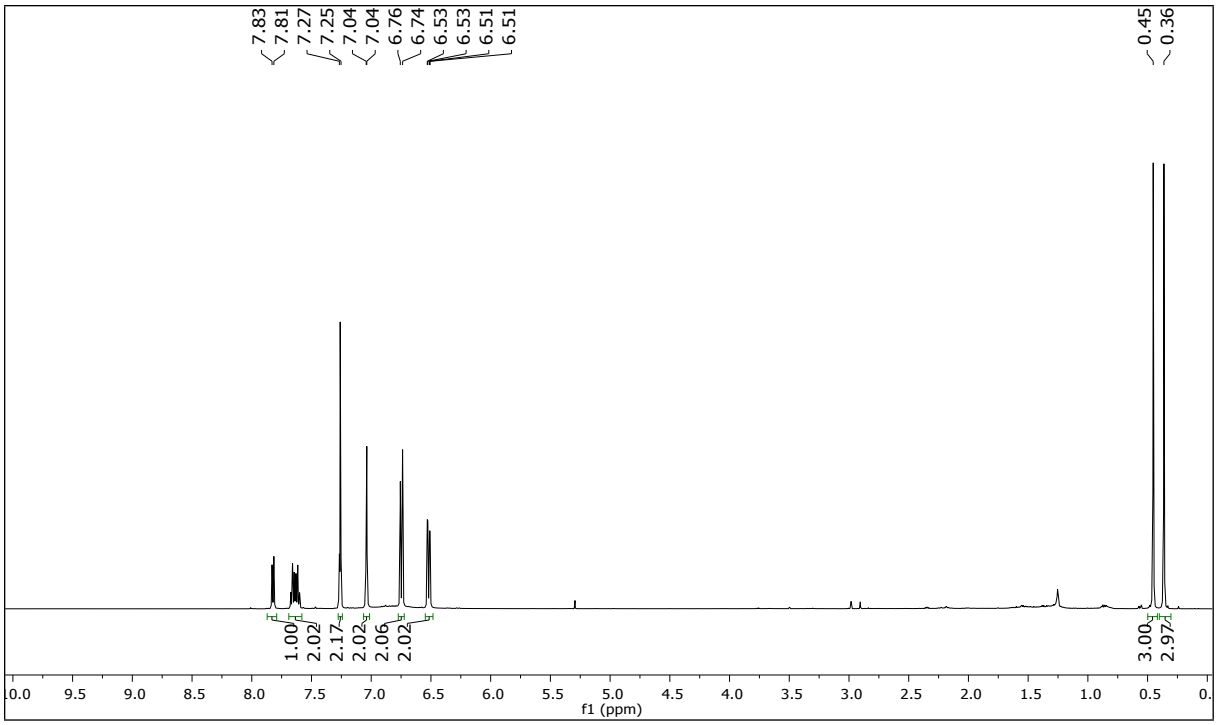


^{1}H NMR (500 MHz, $CDCl_3$)

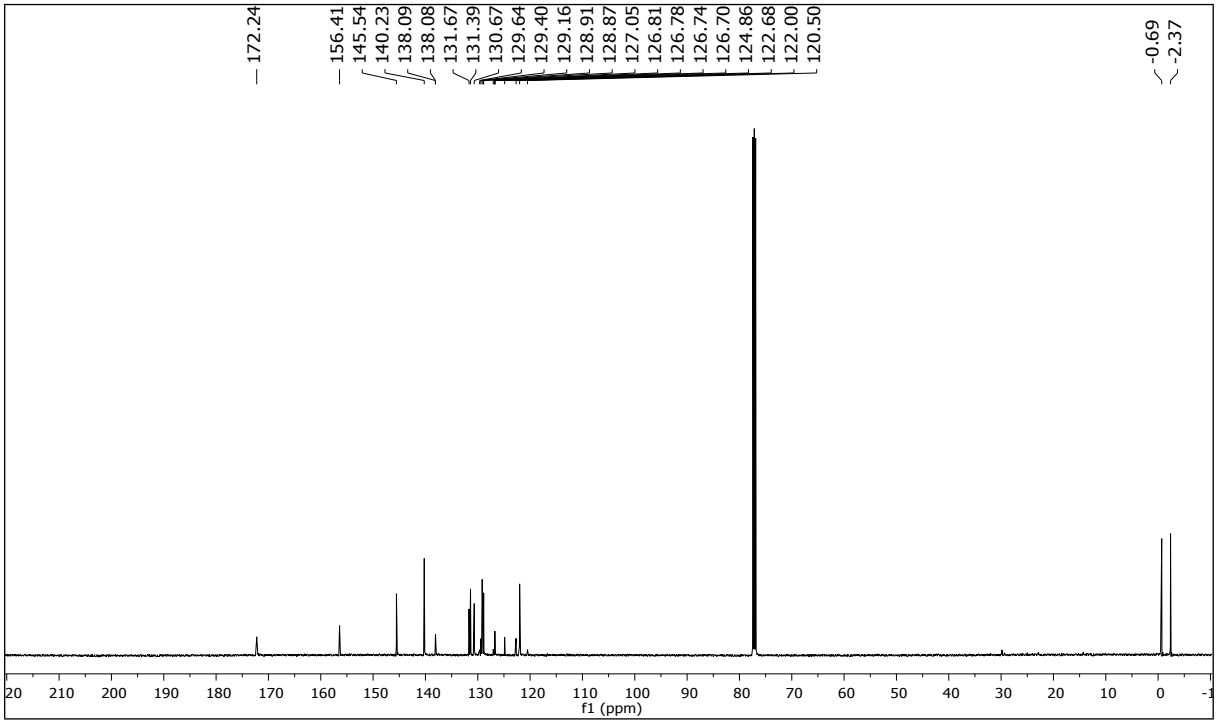


^{13}C NMR (125 MHz, $CDCl_3$)

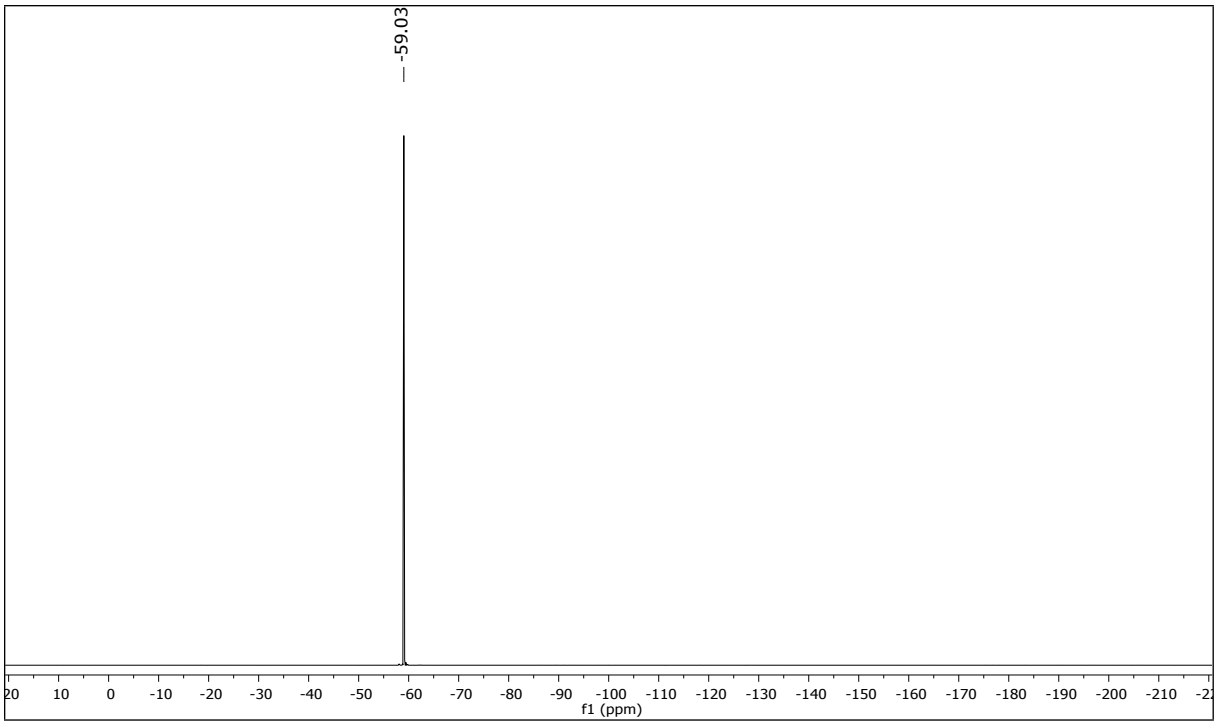
Compound **18**:



^{1}H NMR (500 MHz, $CDCl_3$)

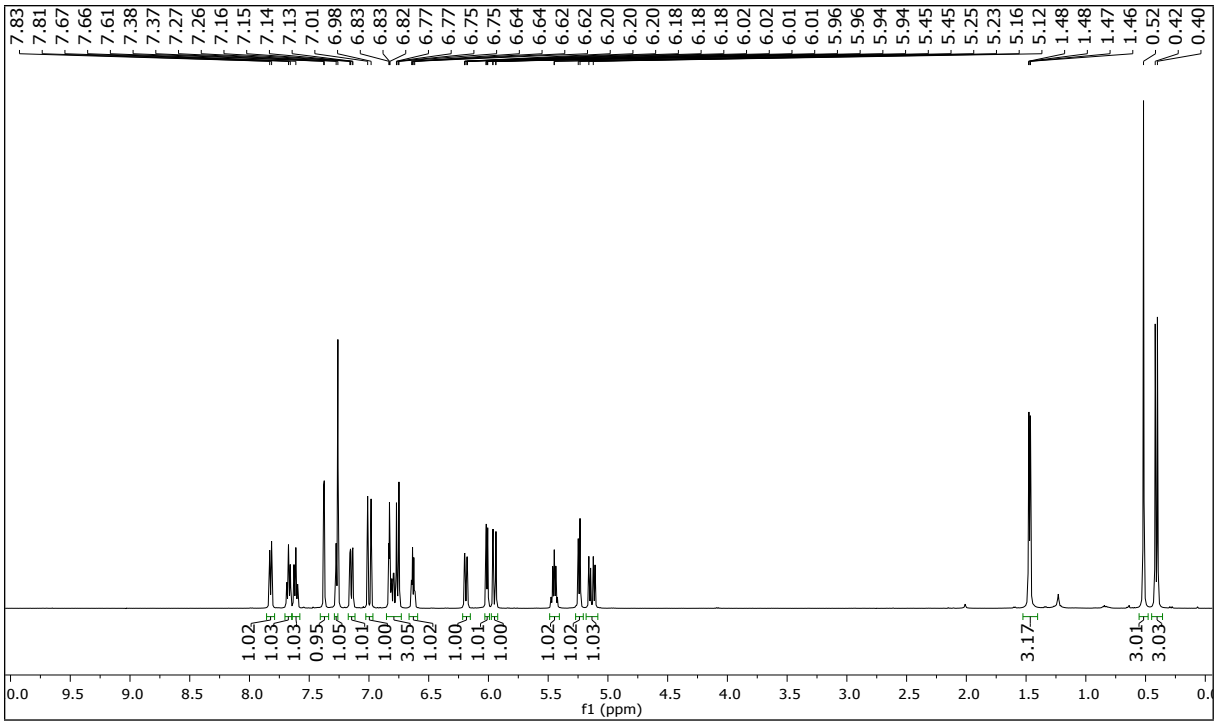


^{13}C NMR (125 MHz, $CDCl_3$)

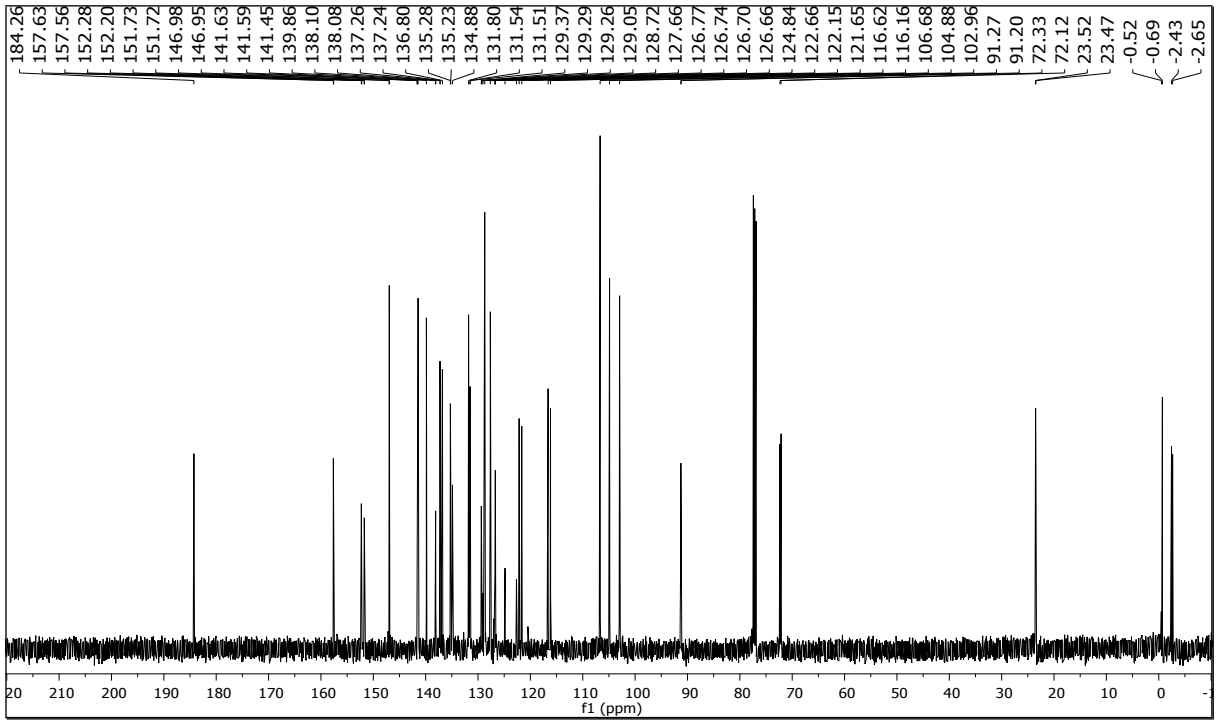


^{19}F NMR (470 MHz, $CDCl_3$)

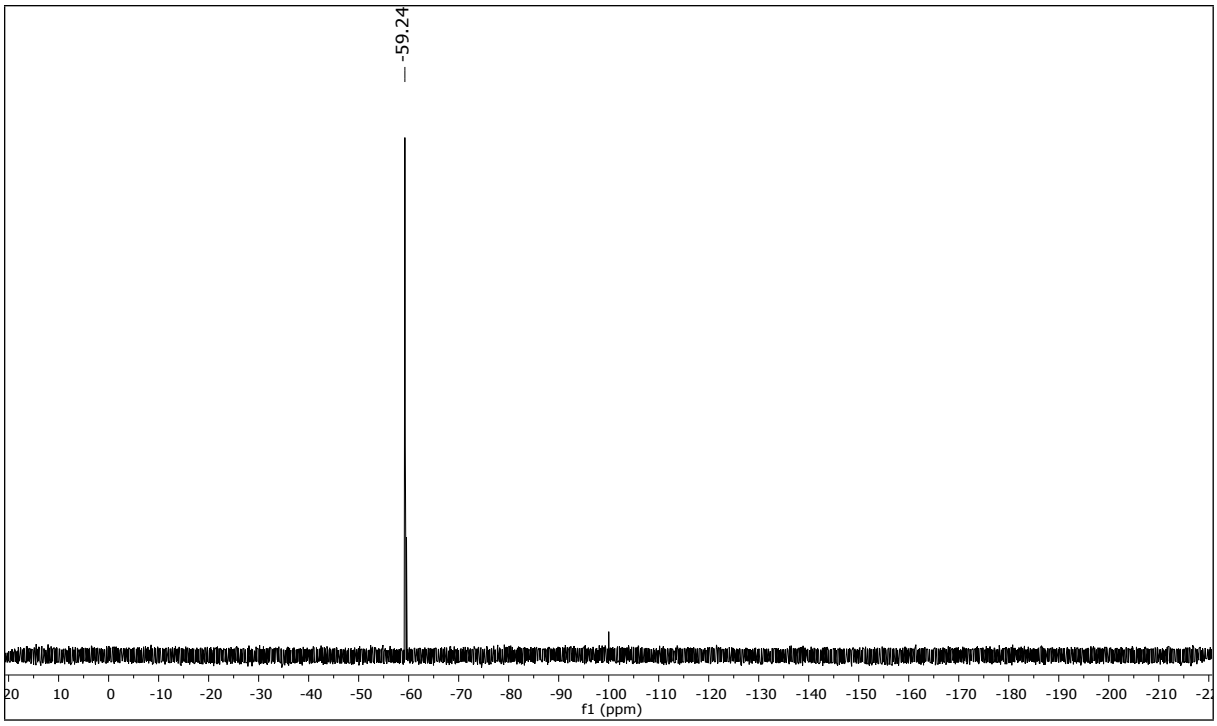
photoFAD-2:



^{1}H NMR (500 MHz, $CDCl_3$)

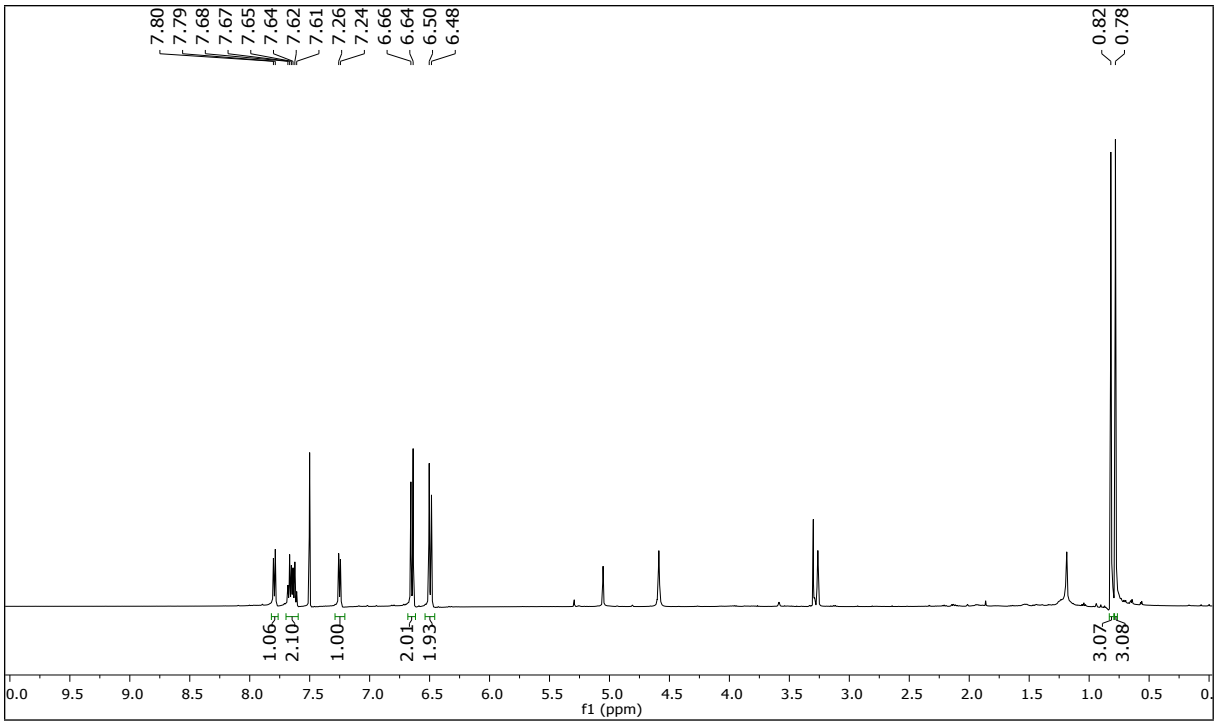


^{13}C NMR (125 MHz, $CDCl_3$)

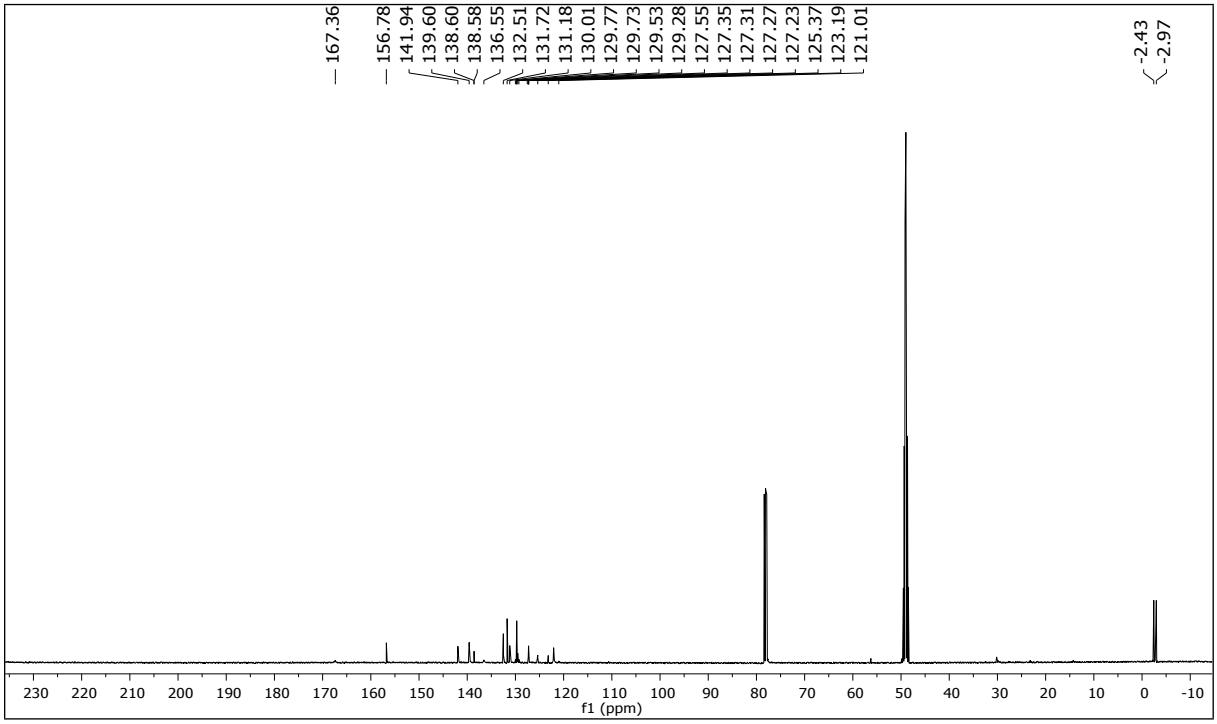


^{19}F NMR (470 MHz, $CDCl_3$)

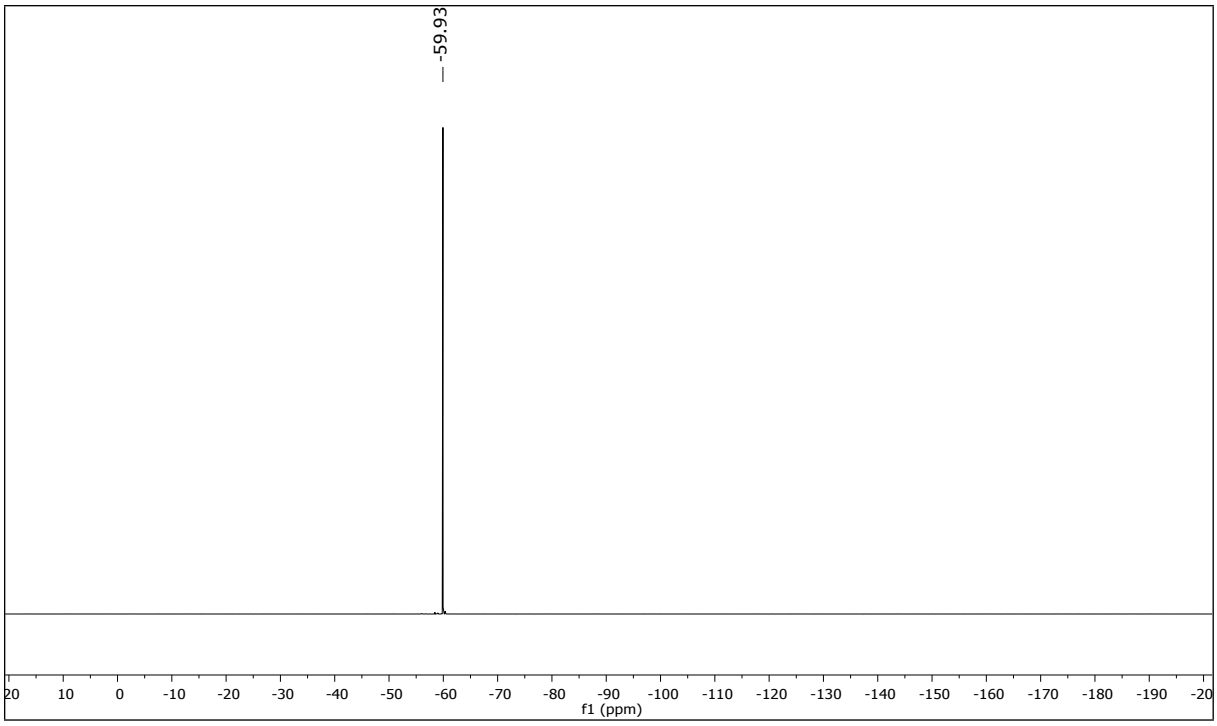
Compound **4**:



^{1}H NMR (500 MHz, 1:1 $CDCl_3$/MeOD)

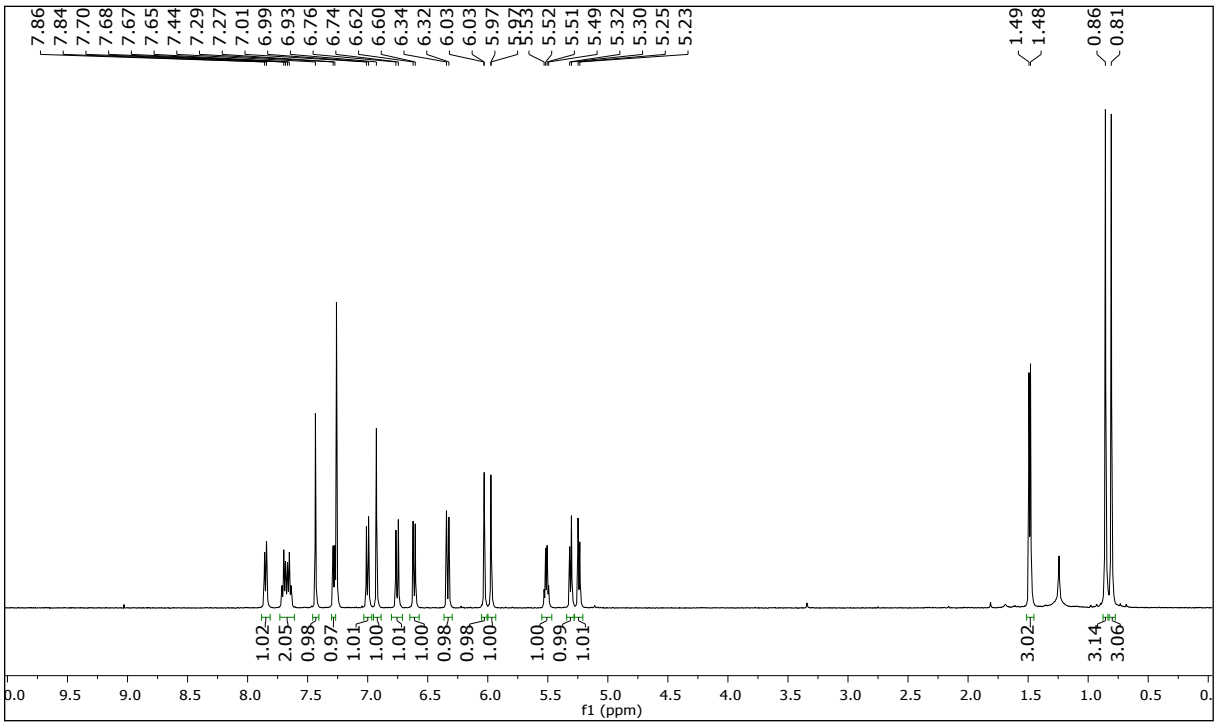


^{13}C NMR (125 MHz, 1:1 $CDCl_3$/MeOD)

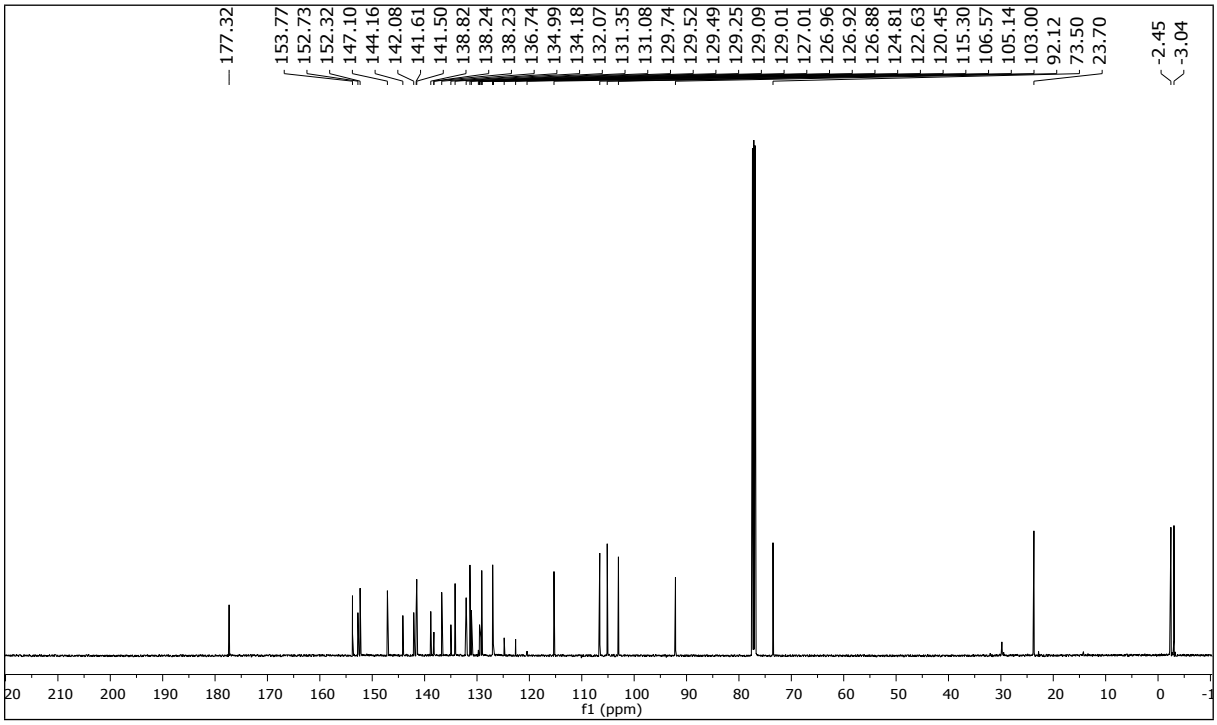


^{19}F NMR (470 MHz, 1:1 $CDCl_3$/MeOD)

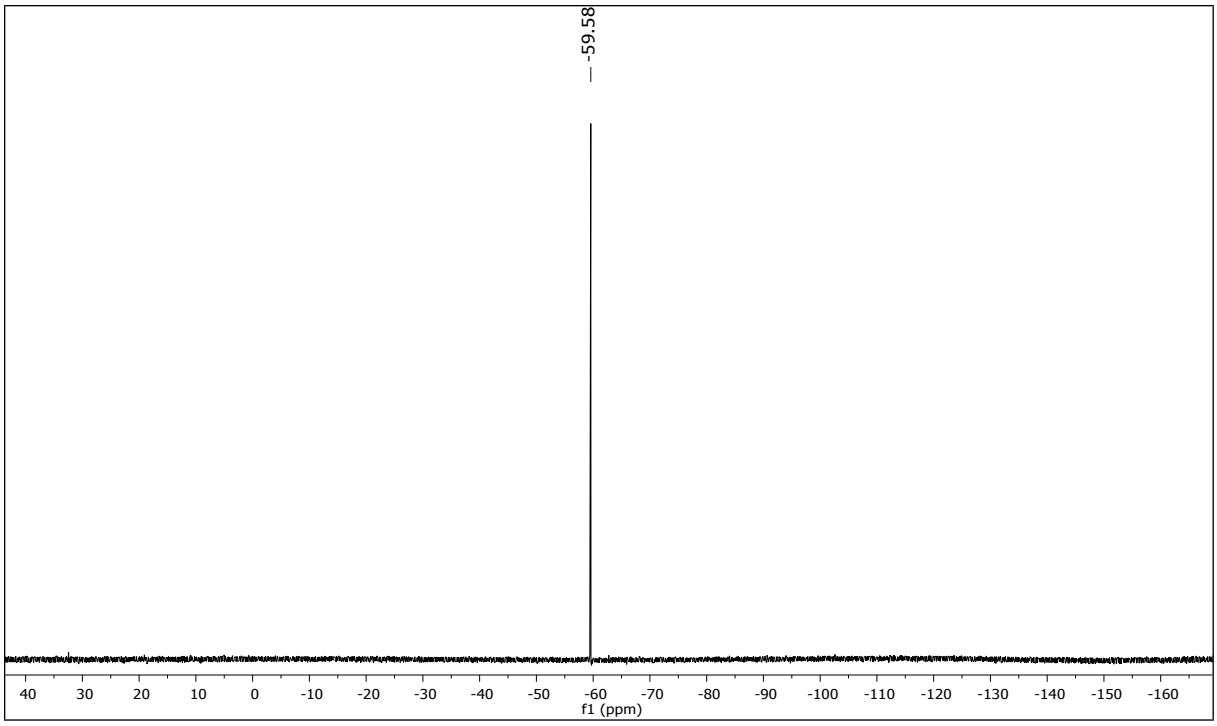
photoFAD-3: 1H-NMR (500 MHz, $CDCl_3$, 298K); 13C-NMR (125 MHz, $CDCl_3$, 298K); 19F-NMR (470 MHz, $CDCl_3$, 298K)



^{1}H NMR (500 MHz, $CDCl_3$)

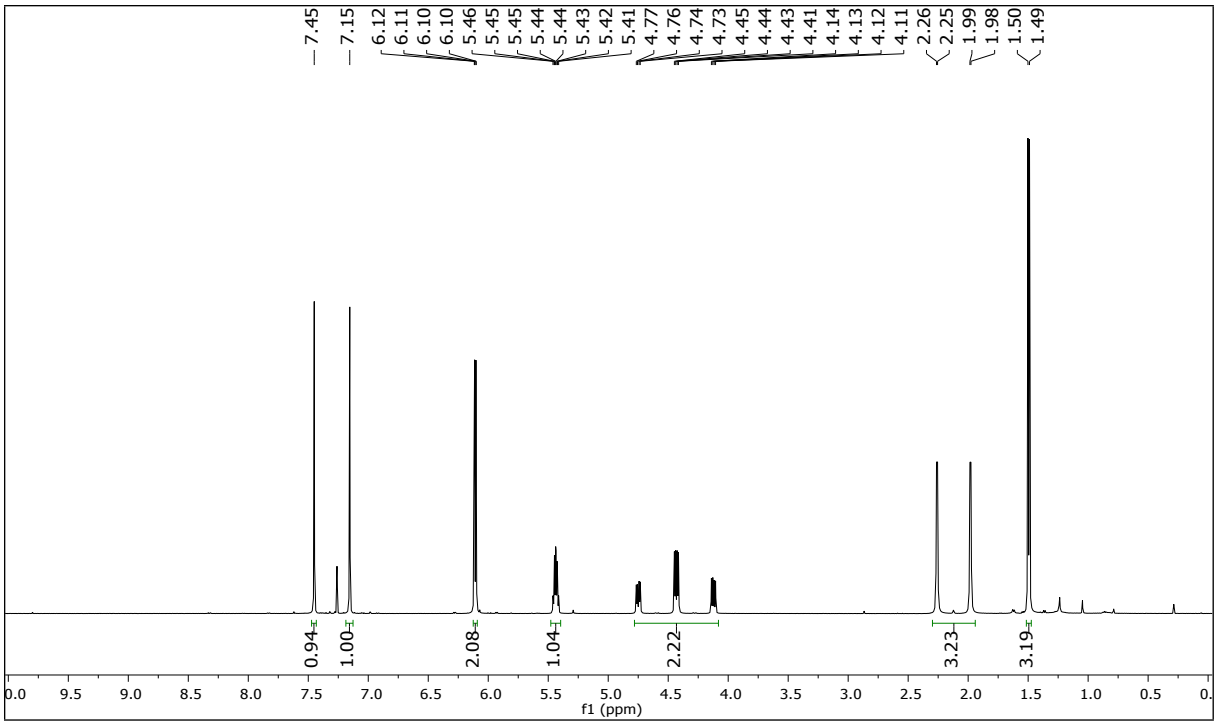


^{13}C NMR (125 MHz, $CDCl_3$)

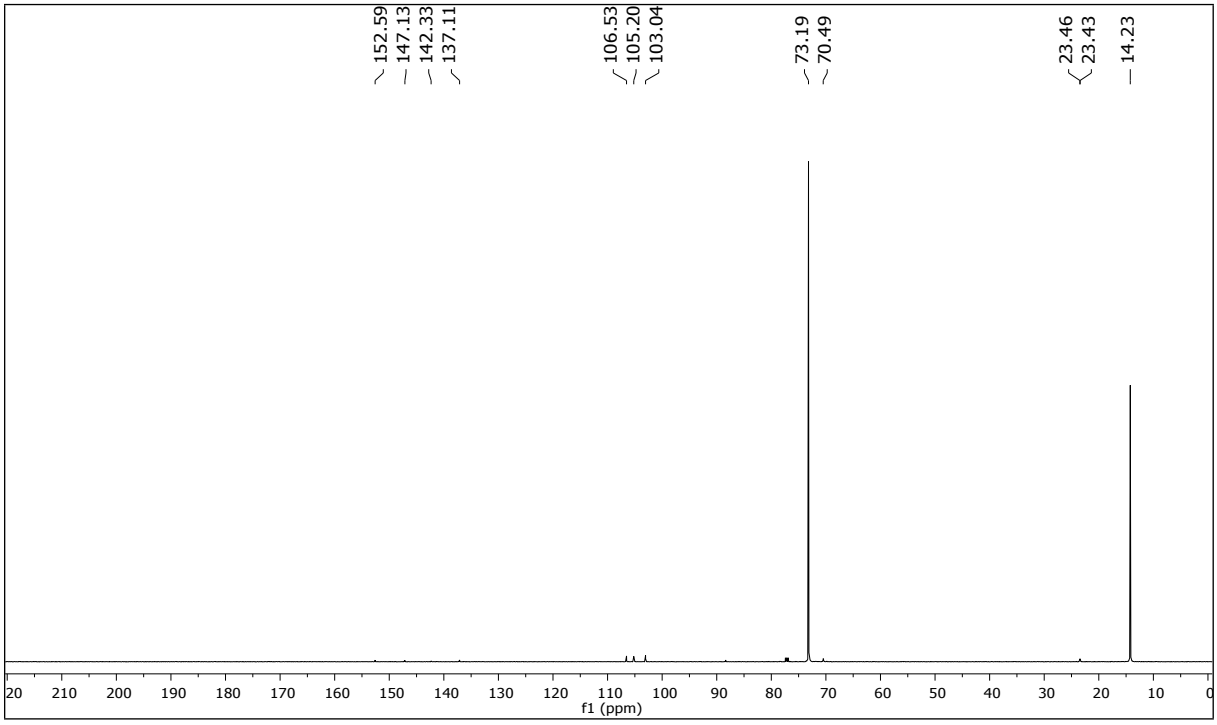


^{19}F NMR (470 MHz, $CDCl_3$)

Compound **19**:

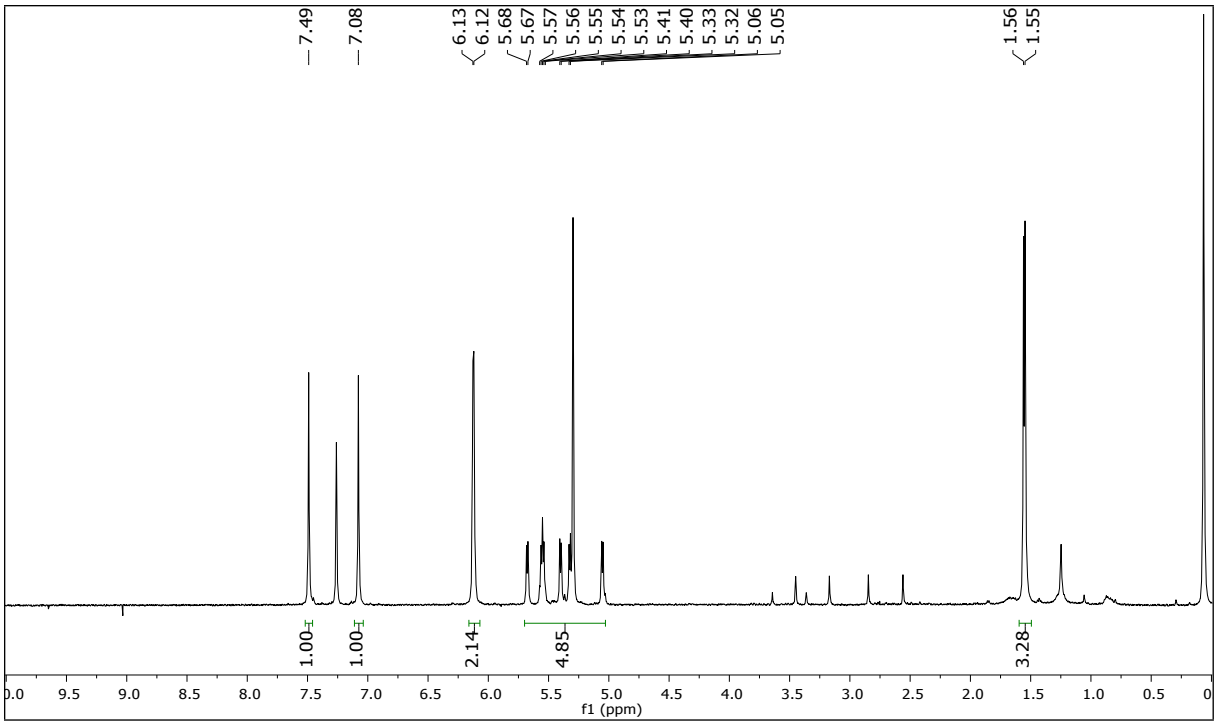


^{1}H NMR (500 MHz, $CDCl_3$)

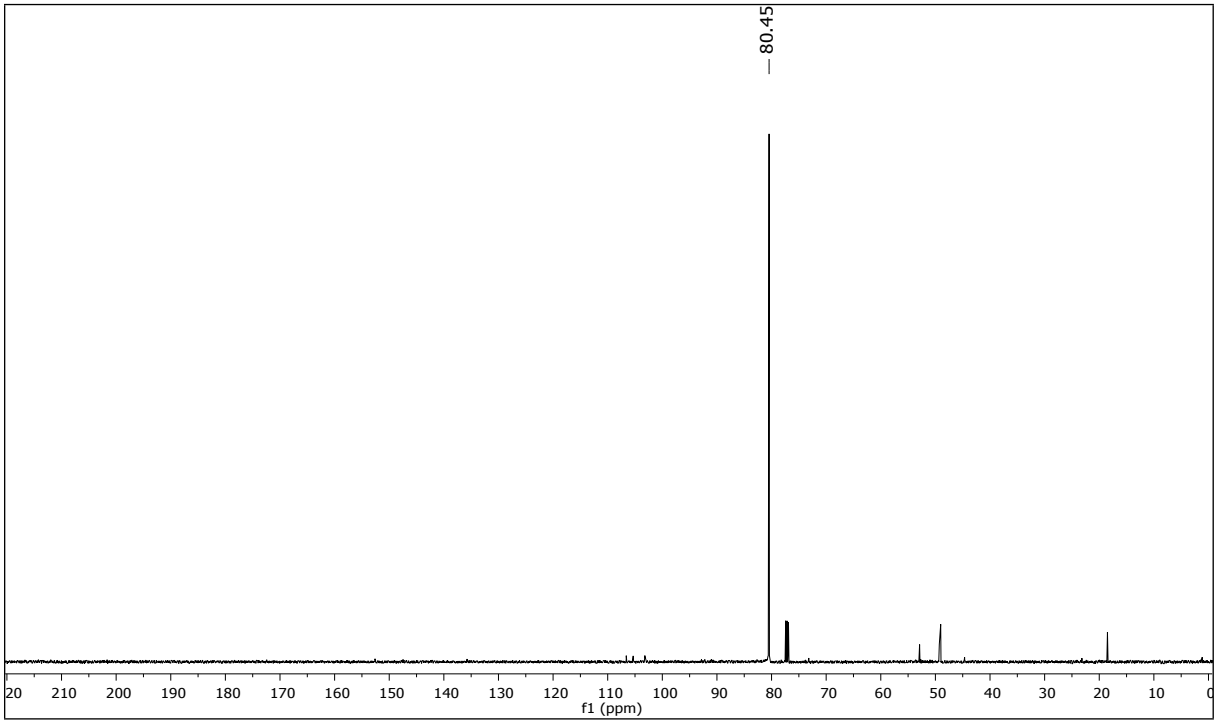


^{13}C NMR (125 MHz, $CDCl_3$)

Compound **20**:

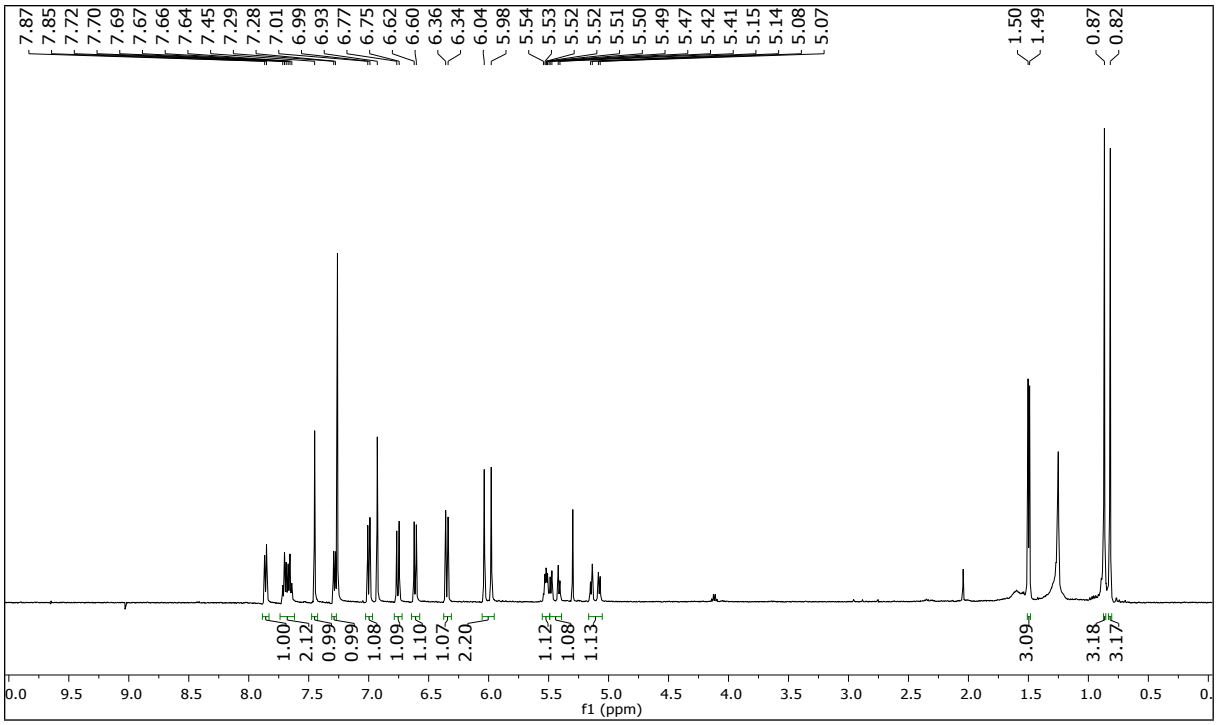


^{1}H NMR (500 MHz, $CDCl_3$)

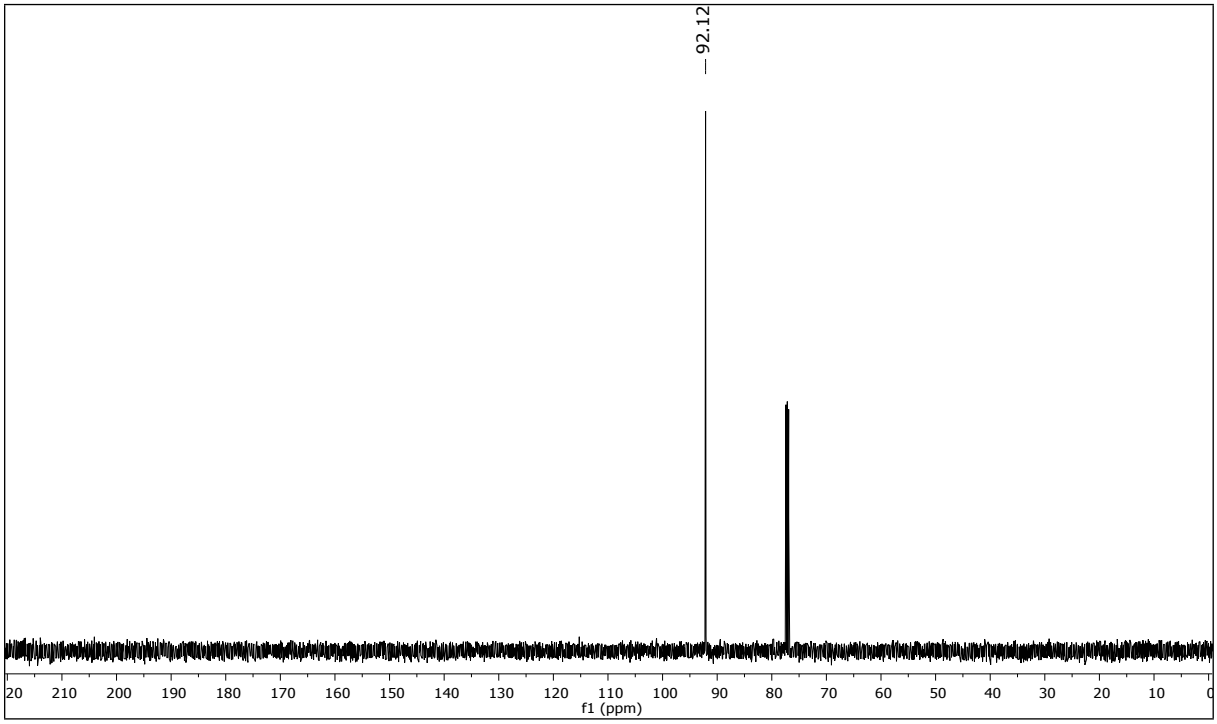


^{13}C NMR (125 MHz, $CDCl_3$)

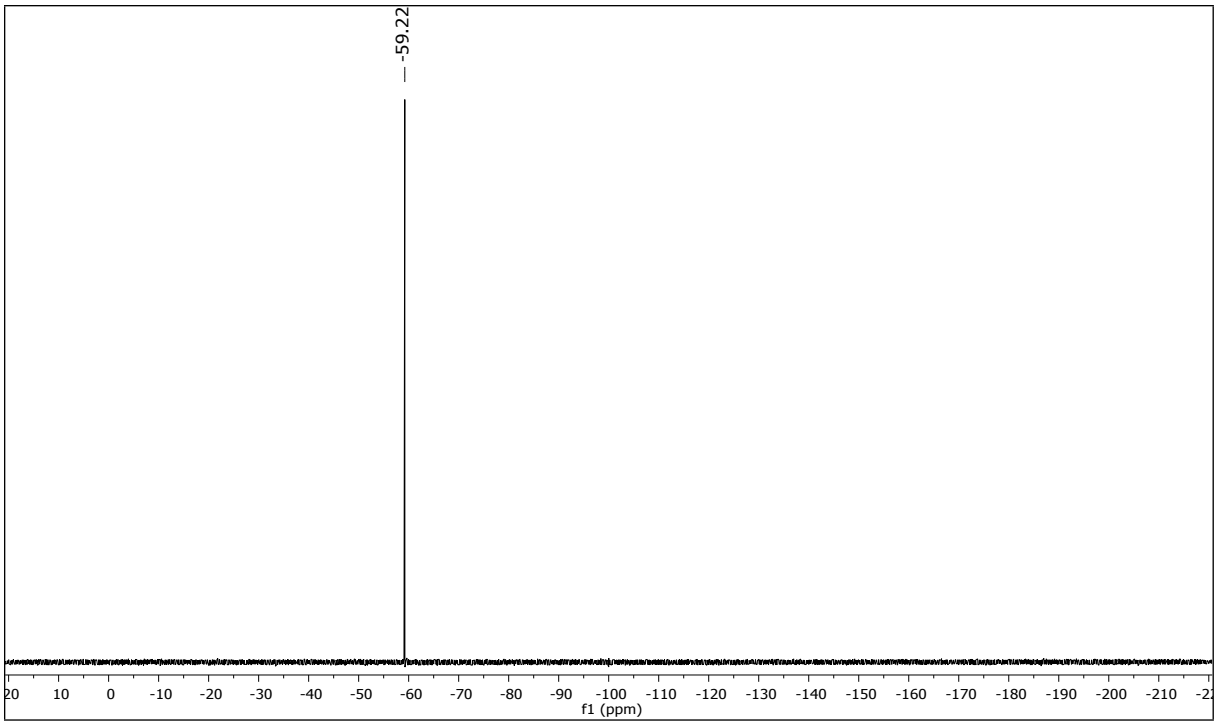
^{13}C-photoFAD-3: 1H-NMR (500 MHz, $CDCl_3$, 298K); 13C-NMR (125 MHz, $CDCl_3$, 298K)



^{1}H NMR (500 MHz, $CDCl_3$)

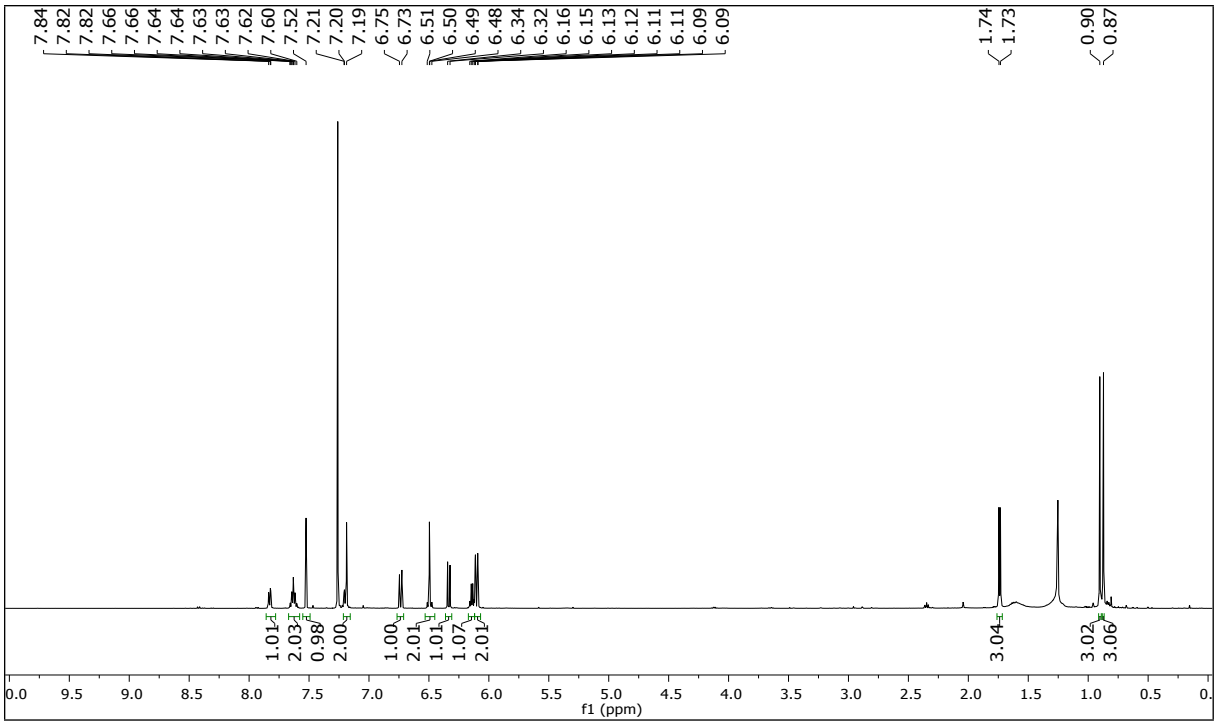


^{13}C NMR (125 MHz, $CDCl_3$)

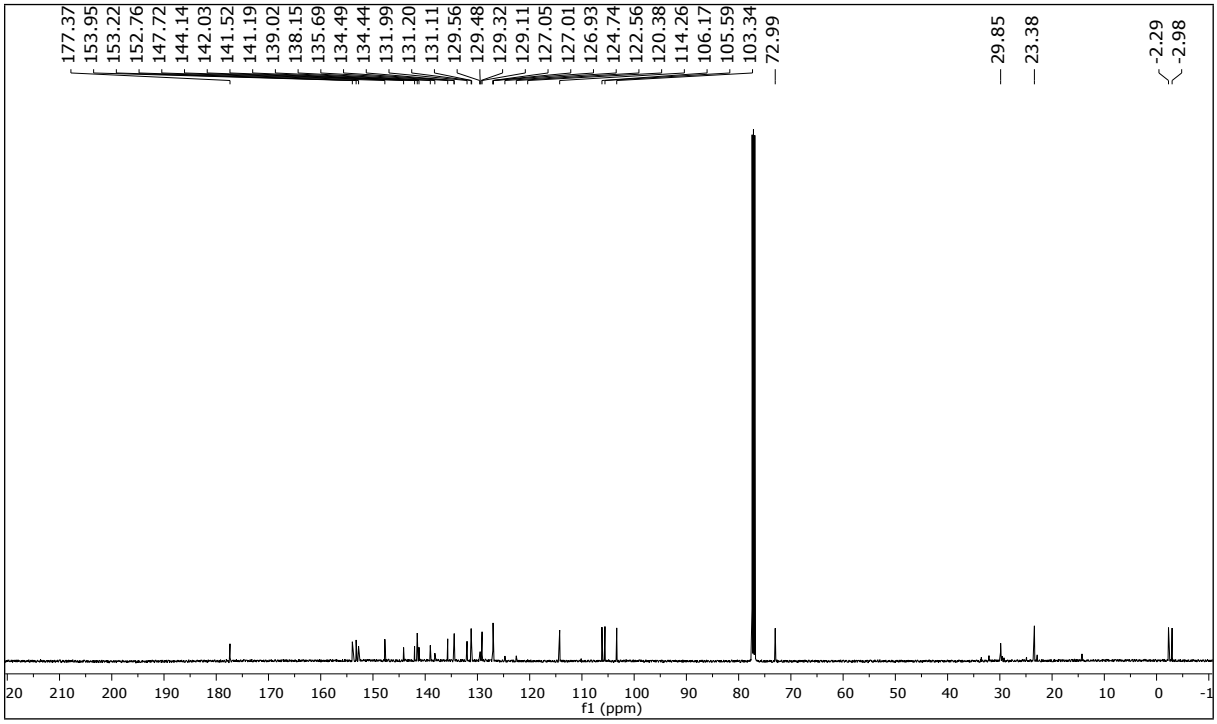


^{19}F NMR (470 MHz, $CDCl_3$)

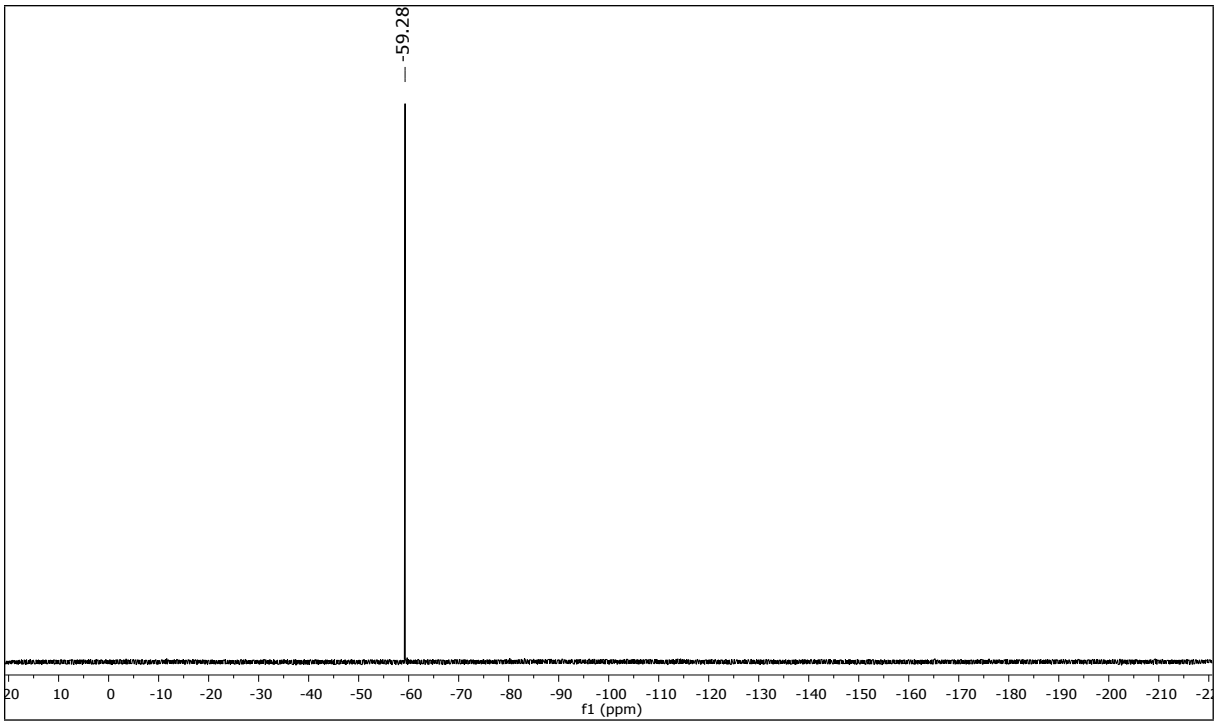
Ctrl-photoFAD-3:



^{1}H NMR (500 MHz, $CDCl_3$)

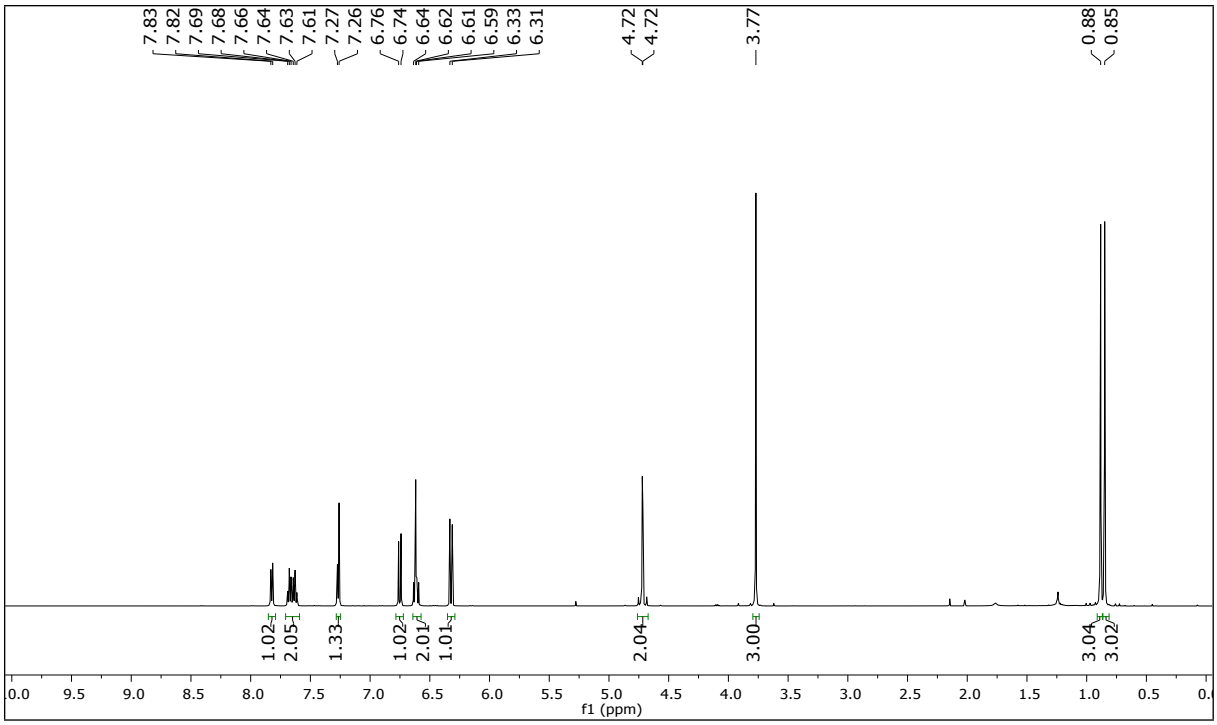


^{13}C NMR (125 MHz, $CDCl_3$)

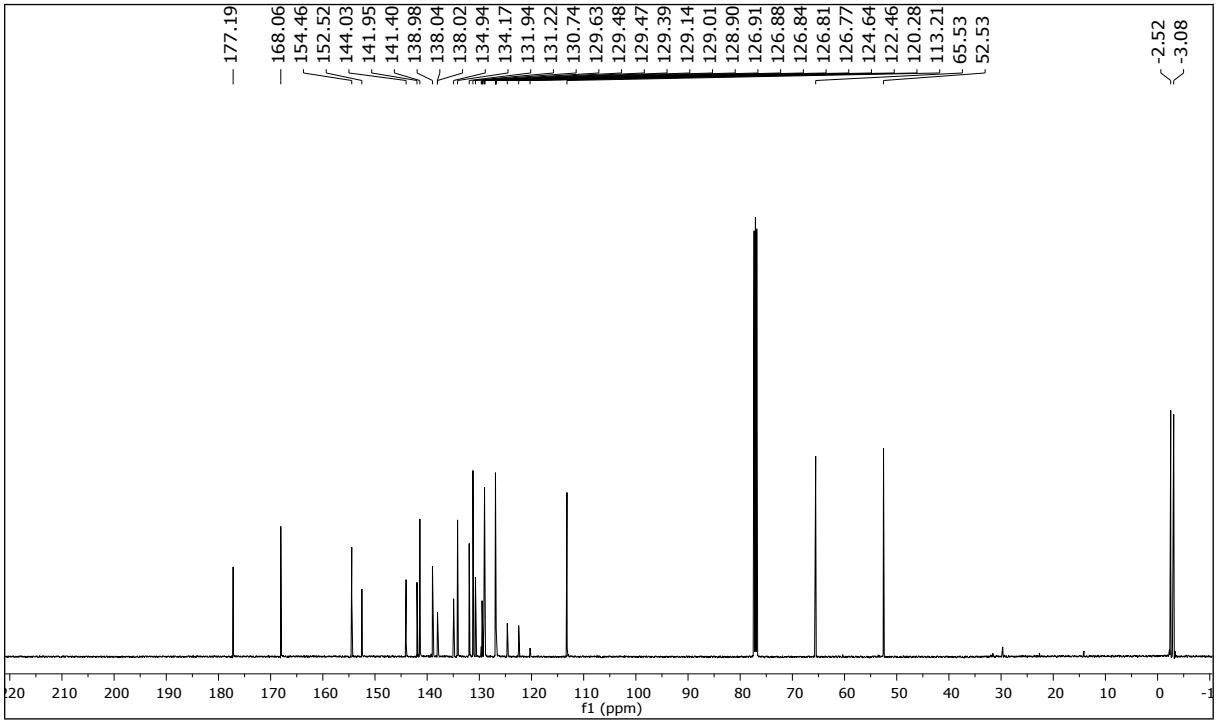


^{19}F NMR (470 MHz, $CDCl_3$)

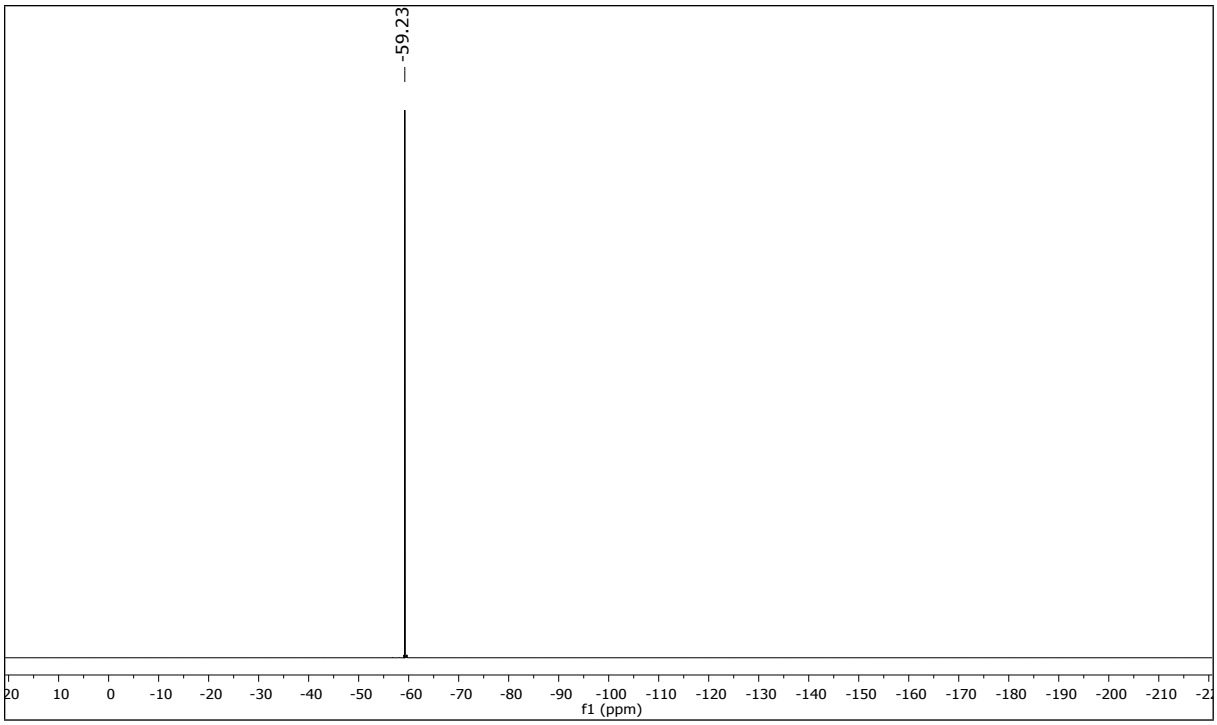
AM-FAD-3: 1H-NMR (500 MHz, $CDCl_3$, 298K); 13C-NMR (125 MHz, $CDCl_3$, 298K)



^{1}H NMR (500 MHz, $CDCl_3$)



^{13}C NMR (125 MHz, $CDCl_3$)



^{19}F NMR (470 MHz, $CDCl_3$)

24. References

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(4) Huguenin-Dezot, N.; Alonzo, D. A.; Heberlig, G. W.; Mahesh, M.; Nguyen, D. P.; Dornan, M. H.; Boddy, C. N.; Schmeing, T. M.; Chin, J. W. Trapping Biosynthetic Acyl-Enzyme Intermediates with Encoded 2,3-Diaminopropionic Acid. *Nature* **2019**, *565* (7737), 112–117.