Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting information for

Bioorthogonal Double-Fluorogenic Siliconrhodamine Probes for Intracellular Super-resolution Microscopy

by

E. Kozma,[a] G. Estrada Girona,[b] G. Paci,[b] E. A. Lemke[b] and P. Kele*[a]

[a]*Chemical Biology Research Group, Institute of Organic Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Magyar tudósok krt. 2, H-1117, Budapest, Hungary;*

e-mail: **kele.peter@ttk.mta.hu**

[b]*Structural and Computational Biology Unit, Cell Biology and Biophysics Unit*

European Molecular Biology Laboratory, D-69117, Heidelberg, Meyerhofstrasse 1.,Germany

Table of contents

1. Supplementary Methods

1.1. Spectral measurements

Photophysical measurements were performed on a JASCO FP 8300 spectrofluorometer. A stock solution in DMSO was prepared from SiR dyes (2.5 mM for **13** and **14** and 10 mM for **11**, **12**, **15** and **16**). Dyes were from DMSO stock solution. Due to precipitation from water or PBS, all experiments were conducted in water with 0.1 m/V% SDS.

Absorbance and fluorescence spectra of **18** were recorded in solvents with different dielectric constants (water:dioxane mixtures) after reacting **11** (10 µM) with 100 µM **17** (**OXTCO**, see [1]). Excitation and emission spectra were recorded after reacting the dyes **11**-**16** (10 µM) with 100 µM **17**. For time course measurements, dye **11**-**16** was reacted with 10 eq. **17** under continuous stirring. 0 min represents fluorescence without **17**. Fluorescence was normalized to 0 min value. Maximum excitation wavelength was used to excite each dye and fluorescence was detected at its emission maximum. Turn-on values in Table 1 were calculated dividing start and end fluorescence intensity values of time course measurements at the emission maxima of the products. Second order rate constants were calculated using OriginPro 9.0.0. Least squares exponential association fits were done by using the equation: $y = y0 + ymax \times (1 - e^{-kt})$ in order to determine pseudo first-order rate constants. Conversion to the second order rate constants was done by using the equation $k_2 = \frac{Kobs}{[Dye]o}$.[2] Since curves showed complex pattern, for single exponential fit only the dominant portions were used omitting initial and late phases. Fluorescence spectra for fluorogenicity measurements (Figure S2) were recorded with 10 µM **11**-**16** or **11**-**16** (10 µM) reacting with **17** (100 µM). Quantum yields were determined using cresyl violet in methanol as standard.[3]

1.2. Cell culture and transfection

COS-7 cells (Sigma 87021302) were maintained in Dulbecco's modified Eagle's medium (Life Technologies 41965-039) supplemented with 1% penicillin-streptomycin (Sigma P0781), 1% L-Glutamine (Sigma G7513), 1% sodium pyruvate

(Life Technologies 11360), and 10% FBS (Sigma F7524). Cells were cultured at 37°C in a 5 % CO_2 atmosphere and passaged every 2–3 days up to 15–20 passages. Transfections were performed with the JetPrime reagent (PeqLab) according to the manufacturer's recommendations. 35,000 cells were seeded 15-20 h prior to transfection on 24-well plates with glass bottom (Greiner Bio-One) or four-well chambered Lab-Tek #1.0 borosilicate coverglass (ThermoFisher). Cells were transfected with the vimentin^{TAG} and the tRNA^{Pyl}/NESPylRS^{AF} vectors (see [4] for full description) at a 1:1 ratio with total 1ug of DNA per well. Medium was changed 4 h after transfection and BCN^{endo} (SiChem) was added at a final concentration of 250 µM. 24 h after, medium was replaced for fresh one without unnatural amino acid, and cells were incubated overnight.

1.3. Labeling of vimentin^{BCNendo}-mOrange

After transfection, cells were rinsed and then incubated with the labeling mixture (dye stock in DMSO diluted to the desired concentration in complete medium). Cells were then incubated at 37°C for 30 min (or otherwise stated), then rinsed and left in fresh medium for 45 min. After, cells were rinsed again and left in fresh medium for 45 more min. Then, cells were rinsed a third time and left in fresh medium for 2 h, after which they were fixed with 2% paraformaldehyde in PBS at room temperature (RT) for 10 min. Cells were imaged on the same or the following day.

1.4. Confocal imaging

Confocal images were acquired on a Leica SP8 STED 3X microscope using the 548 nm (for mOrange reference channel), and 650 nm (for the labeling channel) laser lines for excitation. A 63x/1.40 Oil objective was used and a time gating was applied to the HyD detectors in order to reject unwanted background and reflection for both channels.

1.5. Super-resolution (SRM) imaging

SRM imaging of vimentin was performed on a commercial Leica GSD microscope, equipped with a Leica HCX PL APO 160x/1.43 Oil CORR TIRF PIFOC objective.

Transfected cells were identified with 532 nm (mOrange fusion) excitation laser. The SiR dyes were excited with a 642 nm laser and images were acquired in TIRF mode. For each image, around 20,000 frames were acquired with 30 ms exposure, for which we used an imaging buffer according to a published protocol.[5]

Super-resolution images were reconstructed using the Localizer Package for IgorPro (Wavemetrics, Portland, OR).[6] Firstly, a threshold based on the maximum likelihood ratio was applied, followed by fitting with a symmetrical 2D Gaussian function for localization of the spots. Final super-resolution images were reconstructed from binning all the detected events and convolving the resulting image with a Gaussian width according to the resolution determined by the Fourier ring correlation 0.143 criterion.[7]

1.6. Chemical syntheses

1.6.1. General methods

Unless otherwise indicated, all starting materials were obtained from commercial suppliers (Sigma-Aldrich, Fluka, Merck, Alfa Aesar, Reanal, Molar Chemicals, Fluorochem) and used without further purification.

Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} precoated aluminum TLC plates from Merck.

Preparative thin-layer chromatography was performed on Kieselgel 60 F_{254} preparative thin layer chromatography plates.

Flash column chromatography was performed on Teledyne Isco CombiFlash® Rf+ automated flash chromatographer with silica gel (25-40 μm) from Zeochem.

Fluorescence measurements were performed on Jasco FP 8300 spectrofluorometer.

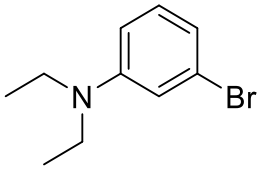
Microwave experiments were performed on Monowave 300 microwave reactor.

NMR spectra were recorded on a Varian Inova 500 MHz and Varian 600 MHz NMR spectrometers. Chemical shifts (δ) are given in parts per million (ppm) using solvent signals as the reference. Coupling constants (J) are reported in Hertz (Hz). Splitting patterns are designated as s (singlet), d (doublet), t (triplet), qr (quadruplet), qv (quintuplet), m (multiplet), h (heptett), dd (doublet of a doublet), td (triplet doublet), dt (doublet triplet), bs (broad singlet).

HRMS data were obtained using a Waters Q-TOF Premier high resolution mass spectrometer equipped with an electrospray ion source.

1.6.2. Synthesis of SiR derivatives

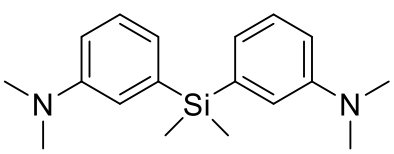
Synthesis of 3-bromo-N,N-dimethylaniline (**2**)



3-bromo-N,N-diethylaniline was synthesized according to previously reported method with minor modifications.[8] To a suspension of NaH (60% 10.0 g, 250 mmol, 2.5 eq) in abs. THF (200 mL) at 0 ˚C was added commercially available 3-bromoaniline (17.2 g, 100 mmol, 1.0 eq) under nitrogen atmosphere. The reaction mixture was stirred for 30 min at the same temperature. Iodoethane (39 g, 250 mmol, 2.5 eq) was added slowly, and the mixture was stirred at room temperature for 24 h. The reaction was quenched with water (100 mL) and the mixture was extracted with dichloromethane (3x100 mL). The combined organic phases were washed with brine and dried over MgSO4. After removal of the solvent in vacuo, the crude residue was purified with silica gel column chromatography (hexane/DCM 5/1) resulting 3-bromo-N,N-diethylaniline as a pale yellow oil (19.9 g, 87%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 – 7.04 (m, 1H), 6.82 (d, *J* = 1.7 Hz, 1H), 6.77 (d, *J* = 7.8 Hz, 1H), 6.61 (d, *J* = 8.5 Hz, 1H), 3.35 (q, *J* = 7.1 Hz, 4H), 1.19 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 148.79, 130.28, 123.46, 117.79, 114.07, 110.12, 44.12, 12.31. HRMS (ESI): m/z calc. for $C_{10}H_{15}NBr$ 228.0388; found: 228.0398 $[M+H]^+$

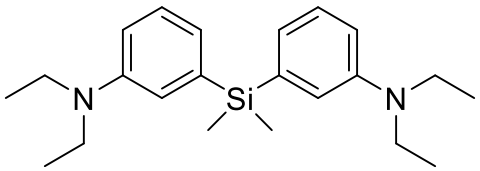
Synthesis of 3,3'-(dimethylsilanediyl)bis(N,N-dimethylaniline) (**3**)



To a 250 mL well-dried flask flushed with nitrogen, commercially available 3-bromo-N,N-dimethylaniline (5.0 g, 25 mmol, 1.0 eq) and abs. THF (100 mL) were added. The solution was cooled to -78 °C, n-butyllithium (17 mL 1.6 M solution in hexane, 27.5 mmol, 1.1 eq) was added dropwise over 15 min and the reaction mixture was stirred at -78 °C for 2 h. Dichlorodimethylsilane (1.8 mL, 15 mmol, 0.6 eq) was added dropwise, and the suspension reaction mixture was slowly warmed to room temperature, then stirred overnight. The reaction was quenched with water (30 mL) and extracted with EtOAc (3x30 mL). The organic layers were combined, washed with brine and dried over anhydrous $MgSO_4$. After filtration and removal of the solvent under reduced pressure, the residue was purified by flash column chromatography on silica gel (1% to 15% EtOAc in hexane over 25 min) resulting a light yellow oil (2.66 g, 71 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (t, *J* = 7.6 Hz, 2H), 6.96 (dd, *J* = 12.7, 4.3 Hz, 4H), 6.84 – 6.75 (m, 2H), 2.95 (s, 12H), 0.56 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 150.07, 139.11, 128.61, 122.94, 118.54, 113.75, 40.83, -2.01. HRMS (ESI): m/z calc. for $C_{18}H_{27}N_2Si$ 299.1944; found: 299.1942 $[M+H]^+$

Synthesis of 3,3'-(dimethylsilanediyl)bis(N,N-diethylaniline) (**4**)

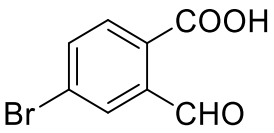


To a 250 mL well-dried flask flushed with nitrogen, compound **2** (3.68 g, 16.1 mmol, 1.0 eq) and abs. THF (35 mL) were added. The solution was cooled to -78 °C, n-butyllithium (11.1 mL 1.6 M solution in hexane, 17.7 mmol, 1.1 eq) was added dropwise over 15 min and the reaction mixture was stirred at -78 °C for 2 h. Dichlorodimethylsilane (1.2 mL, 9.7 mmol, 0.6 eq) was added dropwise, and the reaction mixture was slowly warmed to room temperature, then stirred overnight. The reaction was quenched with water (10 mL) and extracted with EtOAc (3x30 mL). The organic layers were combined, washed with brine and dried over anhydrous $MgSO_4$. After filtration and removal of the solvent under reduced pressure, the residue was

purified by flash column chromatography on silica gel (5% to 70% DCM in hexane over 50 min) resulting a yellow oil (1.29 g, 45 %).

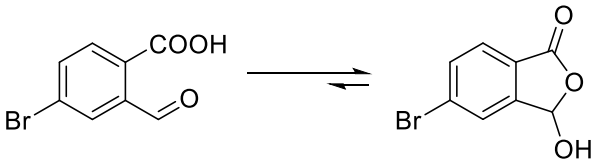
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 – 7.25 (m, 2H), 6.94 (d, *J* = 2.3 Hz, 2H), 6.92 (d, *J* = 7.1 Hz, 2H), 6.77 (dd, *J* = 8.3, 2.2 Hz, 2H), 3.39 (q, *J* = 7.1 Hz, 8H), 1.20 (t, *J* = 7.1 Hz, 12H), 0.60 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 147.16, 139.10, 128.74, 121.55, 117.89, 112.91, 44.47, 12.69, -2.12. HRMS (ESI): m/z calc. for $C_{22}H_{35}N_2Si$ 355.2570; found: 355.2564 $[M+H]^+$

Synthesis of 4-bromo-2-formylbenzoic acid (**5**)



Known compound **5** was synthesized according to literature with some modifications: to a 250 mL round bottom flask were added commercially available 5-bromophthalide (4.582 g, 21.5 mmol, 1.0 eq), *N*-bromosuccinimide (4.209 g, 23.7 mmol, 1.1 eq), azobisisobutyronitrile (177 mg, 1.1 mmol, 0.05 eq) and 100 mL 1,2-dichloroethane. The reaction mixture was stirred and refluxed for 2 h and then kept at -20 °C for 2h. After filtration and removal of the solvent under reduced pressure, water (50 mL) was added and the resulting mixture was refluxed for 2 h. The reaction mixture was then cooled to room temperature and kept at 4 °C overnight. The precipitate was collected, washed with ice-cold water (2x10 mL) and dried in *vacuo* to give the desired compound (white solid, 4.417 g, 90%).

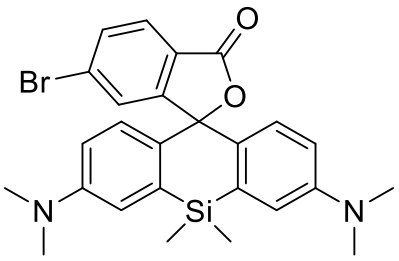
In DMSO, 95% of the compound can be found in a lactone form:



5% 95%

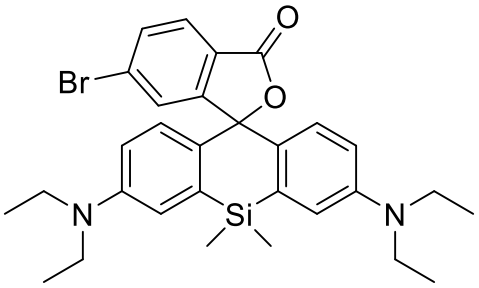
^{1}H NMR (400 MHz, DMSO-d6) δ 7.93 (d, *J* = 1.6 Hz, 1H), 7.85 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 6.65 (s, 1H). (lactone) HRMS (ESI): m/z calc. for $C_8H_4O_3BrNa_2$ 272.9139; found: 272.9144 $[M+2Na-H]^+$

Synthesis of 6'-bromo-3,7-bis(dimethylamino)-5,5-dimethyl-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**6**)



To a pressure tube charged with a magnetic stir bar were added compound **3** (167 mg, 0.56 mmol, 1.0 eq), compound **5** (641 mg, 2.8 mmol, 5.0 eq) and copper(II) bromide (13 mg, 0.056 mmol, 0.1 eq). The tube was sealed tightly and heated at 140 °C for overnight. After cooling to room temperature, the reaction mixture was dissolved in 3 mL dichloromethane and purified by column chromatography (Hexane/EtOAc 4/1 with 1% Et_3N) resulting in white solid product (59 mg, 21%).
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 – 7.78 (m, 1H), 7.64 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.38 (d, *J* = 1.0 Hz, 1H), 6.95 (d, *J* = 2.8 Hz, 2H), 6.82 (d, *J* = 8.9 Hz, 2H), 6.60 (dd, *J* = 8.9, 2.9 Hz, 2H), 2.98 (s, 12H), 0.64 (s, 3H), 0.59 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.05, 157.09, 149.52, 136.62, 132.36, 131.17, 129.17, 128.27, 127.66, 126.99, 125.60, 116.61, 113.73, 91.33, 40.35, 0.39, -0.95. HRMS (ESI): m/z calc. for $C_{26}H_{28}N_2O_2BrSi$ 507.1103; found: 507.1100 $[M+H]^+$

Synthesis of 6'-bromo-3,7-bis(diethylamino)-5,5-dimethyl-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**7**)

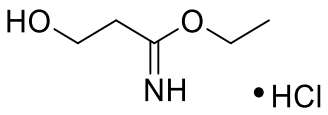


To a pressure tube charged with a magnetic stir bar were added compound **4** (1.291 g, 3.64 mmol, 1.0 eq), compound **5** (4.168 g, 18.2 mmol, 5.0 eq) and copper (II)

bromide (81 mg, 0.36 mmol, 0.1 eq). The tube was sealed tightly and heated at 140 °C overnight. After cooling to room temperature, the reaction mixture was dissolved in 5 mL dichloromethane and purified by column chromatography (Hexane/EtOAc 5/1 with 1% Et_3N) resulting in white solid product (492 mg, 24%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.1 Hz, 1H), 7.66 (d, *J* = 8.1 Hz, 1H), 7.44 (s, 1H), 6.92 (s, 2H), 6.76 (d, *J* = 8.9 Hz, 2H), 6.55 (d, *J* = 8.0 Hz, 2H), 3.38 (q, *J* = 6.9 Hz, 8H), 1.17 (t, *J* = 7.0 Hz, 12H), 0.64 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.95, 156.82, 146.75, 136.90, 132.27, 130.00, 128.99, 128.56, 127.86, 126.86, 126.01, 115.95, 112.92, 91.77, 44.41, 12.67, 0.33, -1.25. HRMS (ESI): m/z calc. for $C_{30}H_{36}N_2O_2BrSi$ 563.1729; found: 563.1738 $[M+H]^+$

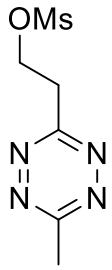
Synthesis of ethyl 3-hydroxypropionimidate hydrochloride (**19**)



In a 250 mL flask, commercially available 3-hydroxypropionitrile (1.4 mL, 20 mmol, 1 eq) was dissolved in abs. ethanol (14 mL, 240 mmol, 12 eq). Acetyl chloride (3 mL) was added dropwise to the stirring solution, then it was purged with HCl gas for 2 h at room temperature. Completition of the reaction was checked with TLC. The solvents were removed under reduced pressure then the crude material was kept at -20 ˚C overnight. The off-white crystals were collected and washed with diethyl-ether resulting in 2.5 g (83%) product.

^{1}H NMR (500 MHz, DMSO-d_6) δ 4.80 (broad s, 1H), 4.44 (q, *J* = 7.0 Hz, 2H), 3.70 (t, *J* = 5.9 Hz, 2H), 2.76 (t, *J* = 5.9 Hz, 2H), 1.34 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 177.92, 69.01, 56.75, 36.44, 13.36.

Synthesis of 2-(6-methyl-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate (**8**)

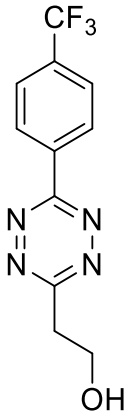


Known compound **8** was synthesized according to literature, with minor modifications. To a mixture of compound **19** (6.14 g, 40 mmol, 1.0 eq) and acetonitrile (24 mL, 200 mmol, 5.0 eq) was added hydrazine hydrate (40 mL) under nitrogen atmosphere, and the solution was stirred for 2 h at room temperature. Sodium nitrite (34.5 g, 500 mmol, 12.5 eq) was dissolved in water (50 mL) and was added to the reaction mixture. cc. HCl was added slowly until pH=3 and the gas evolution ceased. The mixture was extracted with EtOAc (5x50 mL), the combined organic phases were dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude material was dried under vacuum, then was purified by silica flash chromatography (0% to 70% EtOAc in hexane over 25 min) resulting in the OH-tetrazine as a pink oil (1.69 g, 30%).

OH-tetrazine (1.64 g, 11.7 mmol, 1.0 eq) was dissolved in 20 mL DCM and was cooled to 0 °C on an ice bath. Methanesulfonyl chloride (1.81 mL, 23.4 mmol, 2.0 eq) was slowly added, followed by the dropwise addition of Et_3N (3.26 mL, 23.4 mmol, 2.0 eq). The mixture was stirred for 10 min at room temperature, then washed with water (50 mL), extracted with DCM (3x30 mL), dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. Silica flash chromatography purification (5% to 50% EtOAc gradient in hexane over 20 min) resulted 2.32 g (91%) pink solid which was stored at -20 ˚C.

^{1}H NMR (500 MHz, $CDCl_3$) δ 4.82 (t, *J* = 6.2 Hz, 2H), 3.72 (t, *J* = 6.2 Hz, 2H), 3.02 (s, 3H), 2.98 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.16, 166.13, 66.19, 37.51, 34.57, 21.16. HRMS (ESI): m/z calc. for $C_6H_{11}N_4O_3S$ 219.0552; found: 219.0556 $[M+H]^+$

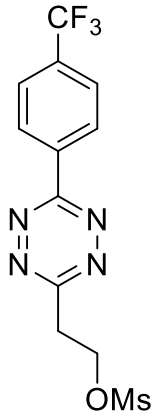
Synthesis of 2-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)ethan-1-ol (**20**)



Commercially available 4-(trifluormethyl)benzonitrile (1.0 g, 5.8 mmol, 1.0 eq), $Zn(OTf)_2$ (1.05 g, 2.9 mmol, 0.5 eq), 3-hydroxypropionitrile (1.189 mL, 17.4 mmol, 3.0 eq) and hydrazine hydrate (14.2 mL, 290 mmol, 50.0 eq) was suspended in 10 mL 1,4-dioxane. The mixture was stirred at 70 ˚C for 40 h, after which the the liquid components were evaporated under reduced pressure. The dry residue was redissolved in 15 mL EtOAc and $NaNO_2$ (8 g, 116 mmol, 20.0 eq) in 15 mL water was added. The mixture was stirred on ice-bath and 2 M HCl was added dropwise until the gas evolution ceased and pH=3. The mixture was extracted with EtOAc (5x15 mL). The combined organic phases were washed with brine, dried over $MgSO_4$, filtered and evaporated in vacuo. The product was purified with flash silica gel column chromatography (10% to 50% EtOAc in hexane over 40 min, product Rf=0.55 in hexane/EtOAc 1/1) resulting 208 mg pink crystals (13%).

^{1}H NMR (500 MHz, CD_3OD) δ 8.74 (d, *J* = 8.0 Hz, 2H), 7.93 (d, *J* = 8.1 Hz, 2H), 4.21 (t, *J* = 6.1 Hz, 2H), 3.58 (t, *J* = 6.1 Hz, 2H). ^{13}C NMR (126 MHz, CD_3OD) δ 170.14, 164.82, 137.28, 134.83 (q, *J* = 32.6 Hz), 129.46, 127.20 (q, *J* = 3.8 Hz), 125.34 (q, *J* = 271.6 Hz), 60.93, 39.17. ^{19}F NMR (235 MHz, $CDCl_3$) δ -63.15 (s). HRMS (ESI): m/z calc. for $C_{11}H_{10}N_4OF_3$ 271.0807; found: 271.0802 $[M+H]^+$

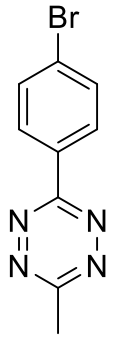
Synthesis of 2-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)ethyl methanesulfonate (**9**)



Compound **20** (282 mg, 1.04 mmol, 1.0 eq) and methanesulfonyl chloride (121 µL, 1.56 mmol, 1.5 eq) was dissolved in DCM (7 mL) and Et_3N (291 µL, 2.08 mmol, 2 eq) was added dropwise. The mixture was stirred for 10 min at room temperature then washed with water (15 mL). The organic phase was dried over anhydrous $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. Silica flash chromatography purification (0 % to 60% EtOAc gradient in hexane over 45 min) resulted 200 mg (55%) pink solid which was stored at -20 ˚C.

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, *J* = 8.0 Hz, 2H), 7.87 (d, *J* = 8.1 Hz, 2H), 4.93 (t, *J* = 6.1 Hz, 2H), 3.86 (t, *J* = 6.1 Hz, 2H), 3.04 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.82, 163.98, 134.91, 134.49 (q, *J* = 32.8 Hz), 128.57, 126.38 (q, *J* = 3.8 Hz), 123.78 (q, *J* = 272.6 Hz), 66.00, 37.70, 34.91. ^{19}F NMR (235 MHz, $CDCl_3$) δ -63.16 (s). HRMS (ESI): m/z calc. for $C_{12}H_{12}N_4O_3SF_3$ 349.0582; found: 349.0584 $[M+H]^+$

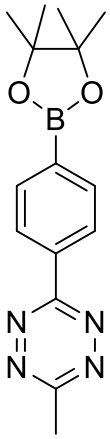
Synthesis of 3-(4-bromophenyl)-6-methyl-1,2,4,5-tetrazine (**21**)



Commercially available 4-bromobenzonitrile (1.0 g, 5.5 mmol, 1.0 eq) and $Zn(OTf)_2$ (1.0 g, 2.8 mmol, 0.5 eq) was dissolved in acetonitrile (8.6 mL, 165 mmol, 30.0 eq) and hydrazine hydrate (13.3 mL, 275 mmol, 50.0 eq). The mixture was stirred at 70 °C under nitrogen atmosphere overnight, after which volatile components were removed under reduced pressure. The dry residue was redissolved in DCM (20 mL) and tert-butyl nitrite (6.5 mL, 55 mmol, 10.0 eq) was added slowly. The solution was stirred for 90 min then the solvent was evaporated and the residue was dried *in vacuo*. The product was purified with flash silica gel column chromatography (0% to 55% DCM in hexane over 40 min) giving 445 mg (32%) of pink solid. (R_f=0.4 DCM/hexane 1/1)

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.42 (d, *J* = 8.3 Hz, 2H), 7.70 (d, *J* = 8.3 Hz, 2H), 3.08 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.52, 163.65, 132.66, 130.83, 129.40, 127.78, 21.29. HRMS (ESI): m/z calc. for $C_9H_8N_4Br$ 250.9932; found: 250.9935 $[M+H]^+$

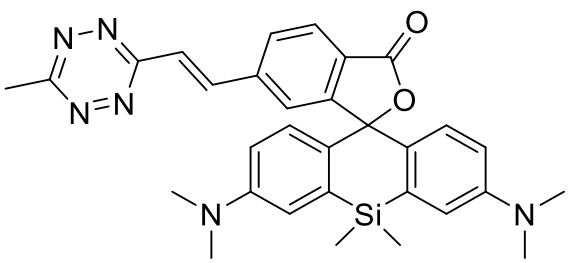
Synthesis of 3-methyl-6-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-1,2,4,5-tetrazine (**10**)



Compound **10** was synthesized according to our previously reported method with minor modifications.[9] Compound **21** (105 mg, 0.414 mmol, 1.0 eq), commercially available bis(pinacolato)diboron (117 mg, 0.456 mmol, 1.1 eq), $PdCl_2$dppf (15 mg, 0.021 mmol, 0.05 eq) and KOAc (61 mg, 0.622 mmol, 1.5 eq) was suspended in 5 mL abs. 1,4-dioxane. The reaction mixture was refluxed for 90 min, cooled to room temperature and the solvent was evaporated in vacuo. The crude product was purified with flash silica gel column chromatography (0% to 30% EtOAc in hexane over 20 min), ersulting in 120 mg pink crystals (98%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.52 (d, *J* = 8.0 Hz, 2H), 7.97 (d, *J* = 8.0 Hz, 2H), 3.04 (s, 3H), 1.35 (s, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.30, 164.17, 135.49, 134.09, 126.97, 84.24, 24.96, 21.18. HRMS (ESI): m/z calc. for $C_{15}H_{20}N_4O_2B$ 299.1679; found: 299.1682 $[M+H]^+$

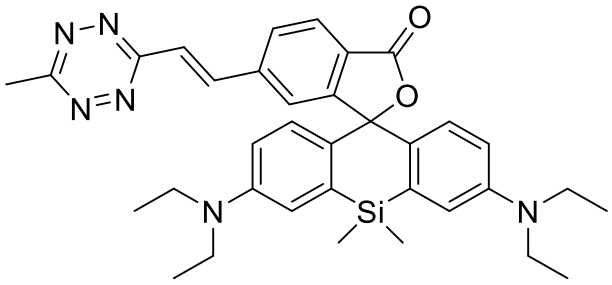
Synthesis of (E)-3,7-bis(dimethylamino)-5,5-dimethyl-6'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**11**)



To a dried microwave pressure tube with magnetic stir bar were added compound **6** (61 mg, 0.12 mmol, 1.1 eq) compound **8** (24 mg, 0.11 mmol, 1.0 eq), N,N-dicyclohexylmethylamine (94 µL, 0.44 mmol, 4.0 eq), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 10 mg, 0.011 mmol, 0.1 eq) and 1,2,3,4,5-pentaphenyl-1′-(di-tert-butylphosphino)ferrocene (QPhos, 32 mg, 0.044 mmol, 0.4 eq) in 3 mL abs. dimethylformamide. The tube was sealed tightly, and the mixture was microwaved at 50 ºC for 40 min. The solvent was evaporated in vacuo, the residue was redissolved in DCM (2 mL) and purified by preparative TLC (silica, hexane/EtOAc 2/1) resulting in 29 mg red crystals (48 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (d, *J* = 16.3 Hz, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.52 (s, 1H), 7.48 (d, *J* = 16.3 Hz, 1H), 7.01 (s, 2H), 6.86 (d, *J* = 8.9 Hz, 2H), 6.61 (d, *J* = 6.9 Hz, 2H), 3.06 (s, 3H), 2.98 (s, 12H), 0.70 (s, 3H), 0.62 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.13, 166.83, 164.42, 155.88, 140.66, 139.40, 136.97, 128.52, 128.35, 127.88, 127.12, 126.41, 124.14, 123.68, 40.61, 21.37, 0.47, -0.96. HRMS (ESI): m/z calc. for $C_{31}H_{33}N_6O_2Si$ 549.2434; found: 549.2440 $[M+H]^+$

Synthesis of (E)-3,7-bis(diethylamino)-5,5-dimethyl-6'-(2-(6-methyl-1,2,4,5-tetrazin-3-yl)vinyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**12**)

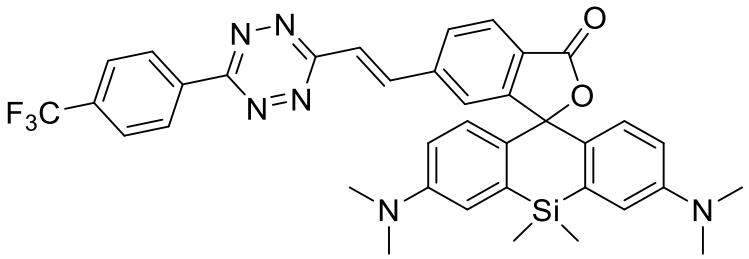


To a dried microwave pressure tube with magnetic stir bar were added compound **7** (65 mg, 0.12 mmol, 1.0 eq) compound **8** (30 mg, 0.14 mmol, 1.2 eq), N,N-dicyclohexylmethylamine (103 µL, 0.48 mmol, 4.0 eq), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 11 mg, 0.012 mmol, 0.1 eq) and 1,2,3,4,5-pentaphenyl-1′-(di-tert-butylphosphino)ferrocene (QPhos, 35 mg, 0.048 mmol, 0.4 eq) in 3 mL abs. dimethylformamide. The tube was sealed tightly, and the mixture was microwaved at 50 ºC for 40 min. The solvent was evaporated in vacuo, the residue was redissolved in DCM (2 mL) and purified by flash chromatography

(15% to 50% EtOAc gradient in hexane over 30 min) resulting in 26 mg purple crystals (36 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.31 (d, *J* = 16.3 Hz, 1H), 8.02 (d, *J* = 7.9 Hz, 1H), 7.84 (d, *J* = 7.8 Hz, 1H), 7.57 (s, 1H), 7.50 (d, *J* = 16.2 Hz, 1H), 6.93 (s, 2H), 6.78 (d, *J* = 8.1 Hz, 2H), 6.52 (d, *J* = 6.5 Hz, 2H), 3.36 (d, *J* = 6.8 Hz, 8H), 3.06 (s, 3H), 1.16 (t, *J* = 6.8 Hz, 12H), 0.67 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.12, 163.77, 146.16, 139.80, 138.85, 136.45, 129.74, 127.95, 127.79, 127.71, 125.60, 123.34, 123.28, 115.37, 112.17, 43.73, 20.68, 12.02, -1.96. HRMS (ESI): m/z calc. for $C_{35}H_{41}N_6O_2Si$ 605.3060; found: 605.3065 $[M+H]^+$

Synthesis of (E)-3,7-bis(dimethylamino)-5,5-dimethyl-6'-(2-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)vinyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**13**)

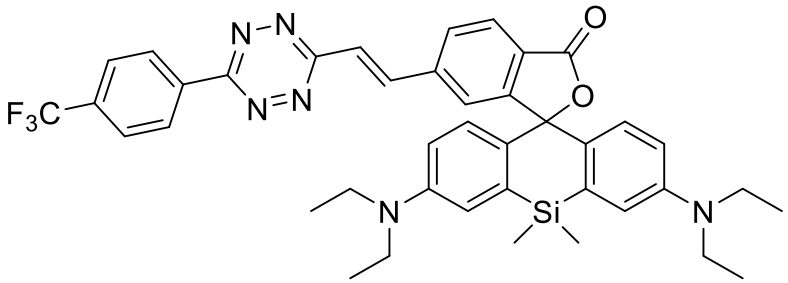


To a dried pressure tube with magnetic stir bar were added compound **6** (100 mg, 0.2 mmol, 1.0 eq) compound **9** (89 mg, 0.26 mmol, 1.3 eq), N,N-diisopropylethylamine (105 µL, 0.6 mmol, 3.0 eq), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 18 mg, 0.02 mmol, 0.1 eq) and 1,2,3,4,5-pentaphenyl-1′-(di-tert-butylphosphino)ferrocene (QPhos, 43 mg, 0.06 mmol, 0.3 eq) in 3 mL abs. 1,4-dioxane. The tube was sealed tightly and the mixture was heated at 90 ºC for 16 h on an oil bath. The solvent was evaporated in vacuo and purified with flash silica gel column chromatography (10% to 60% EtOAc gradient in hexane over 50 min) resulting in 44 mg purple crystals (32%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, *J* = 8.1 Hz, 2H), 8.39 (d, *J* = 16.2 Hz, 1H), 8.04 (d, *J* = 7.9 Hz, 1H), 7.87 (d, *J* = 8.1 Hz, 3H), 7.60 – 7.54 (m, 2H), 7.01 (d, *J* = 1.9 Hz, 2H), 6.88 (d, *J* = 8.9 Hz, 2H), 6.61 (dd, *J* = 8.9, 2.1 Hz, 2H), 2.98 (s, 12H), 0.71 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.46, 164.07, 161.79, 155.38,

148.76, 139.80, 139.79, 136.18, 134.49, 133.69 (q, *J* = 32.7 Hz), 131.02, 127.97, 127.77, 127.65, 127.46, 125.76, 125.72 (q, *J* =3.8 Hz), 123.18 (q, *J* = 272.4 Hz), 123.17, 123.15, 116.16, 113.13, 91.24, 39.76, -0.23, -1.61. ^{19}F NMR (235 MHz, $CDCl_3$) δ -63.12 (s). HRMS (ESI): m/z calc. for $C_{37}H_{34}N_6O_2SiF_3$ 679.2465; found: 679.2428 $[M+H]^+$

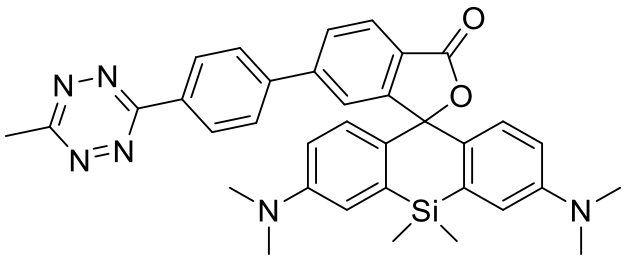
Synthesis of (E)-3,7-bis(diethylamino)-5,5-dimethyl-6'-(2-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)vinyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**14**)



To a dried pressure tube with magnetic stir bar were added compound **7** (60 mg, 0.106 mmol, 1.0 eq) compound **9** (41 mg, 0.11 mmol, 1.1 eq), N,N-diisopropylethylamine (55 µL, 0.318 mmol, 3.0 eq), tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 9 mg, 0.01 mmol, 0.1 eq) and 1,2,3,4,5-pentaphenyl-1′-(di-tert-butylphosphino)ferrocene (QPhos, 21 mg, 0.03 mmol, 0.3 eq) in 3 mL abs. 1,4-dioxane. The tube was sealed tightly and the mixture was heated at 110 ºC for 16 h on an oil bath. The solvent was evaporated in vacuo and purified with flash silica gel column chromatography (5% to 30% EtOAc gradient in hexane over 35 min) resulting in 20 mg purple crystals (26%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.75 (d, *J* = 8.2 Hz, 2H), 8.41 (d, *J* = 16.3 Hz, 1H), 8.04 (d, *J* = 7.8 Hz, 1H), 7.87 (d, *J* = 8.3 Hz, 3H), 7.59 (d, *J* = 16.7 Hz, 2H), 6.94 (s, 2H), 6.80 (d, *J* = 8.9 Hz, 2H), 6.53 (d, *J* = 7.8 Hz, 2H), 3.37 (q, *J* = 6.7 Hz, 8H), 1.17 (t, *J* = 6.7 Hz, 12H), 0.68 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.76, 162.48, 146.86, 140.52, 137.13, 135.16, 134.38 (q, *J* = 33.0 Hz), 131.77, 130.37, 128.64, 128.45, 127.76, 127.10, 126.48 – 126.33 (m), 124.09, 123.84 (q, *J* = 272.9 Hz), 116.07, 112.87, 44.42, 12.68, -1.27. ^{19}F NMR (235 MHz, $CDCl_3$) δ -63.12 (s). HRMS (ESI): m/z calc. for $C_{41}H_{42}N_6O_2F_3Si$ 735.3091; found: 735.3089 $[M+H]^+$

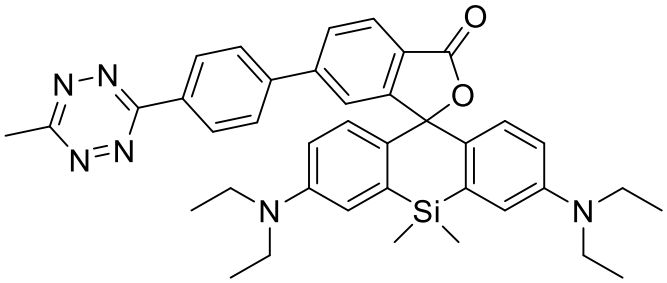
Synthesis of 3,7-bis(dimethylamino)-5,5-dimethyl-6'-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**15**)



To a dried pressure tube with magnetic stir bar were added compound **6** (145 mg, 0.29 mmol, 1.3 eq) compound **10** (65 mg, 0.22 mmol, 1.0 eq), Cs_2CO_3 (283 mg, 0.87 mmol, 3.0 eq) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) ($PdCl_2$(dppf), 47 mg, 0.06 mmol, 0.2 eq) in 6 mL abs. 1,4-dioxane. The tube was sealed tightly and the mixture was heated at 90 ºC for 16 h on an oil bath. The solvent was evaporated in vacuo and purified with flash silica gel column chromatography (5% to 60% EtOAc gradient in hexane over 50 min) resulting in 15 mg purple crystals (11%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.66 (d, *J* = 8.3 Hz, 2H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.86 (d, *J* = 8.0 Hz, 1H), 7.78 (d, *J* = 8.3 Hz, 2H), 7.56 (s, 1H), 7.06 (s, 2H), 6.94 (d, *J* = 8.9 Hz, 2H), 6.67 (d, *J* = 8.7 Hz, 2H), 3.11 (s, 3H), 2.99 (s, 12H), 0.68 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.27, 167.54, 163.88, 155.49, 148.21, 145.92, 137.44, 132.05, 128.69, 128.49, 128.46, 128.36, 126.57, 126.30, 123.04, 115.12, 41.48, 21.34, 0.45, -1.09. HRMS (ESI): m/z calc. for $C_{35}H_{35}N_6O_2Si$ 599.2591; found: 599.2579 $[M+H]^+$

Synthesis of 3,7-bis(diethylamino)-5,5-dimethyl-6'-(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-3'H,5H-spiro[dibenzo[b,e]siline-10,1'-isobenzofuran]-3'-one (**16**)

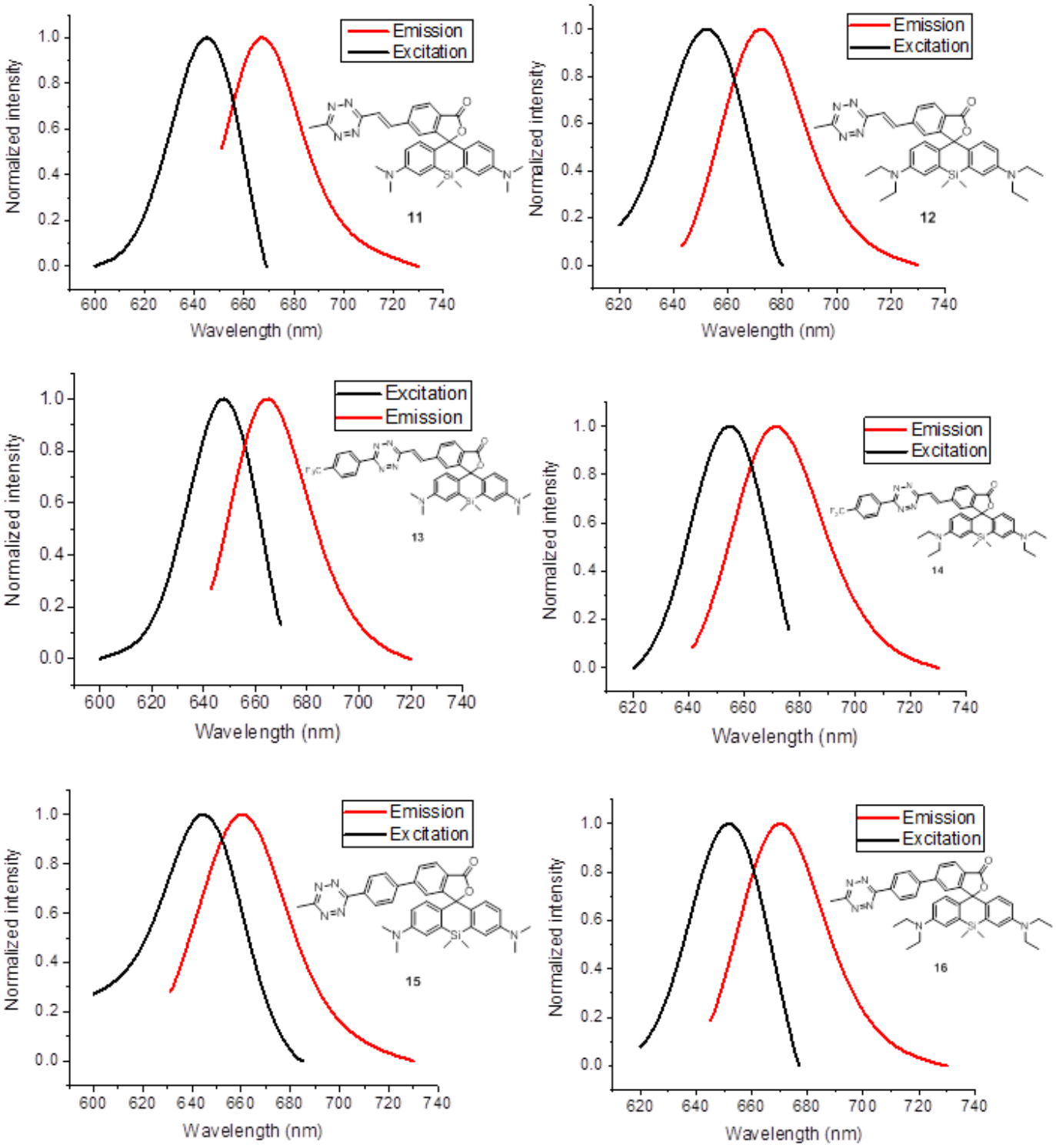


To a dried pressure tube with magnetic stir bar were added compound **7** (82 mg, 0.14 mmol, 1.0 eq) compound **10** (48 mg, 0.16 mmol, 1.1 eq), Cs_2CO_3 (137 mg, 0.42 mmol, 3.0 eq) and [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) ($PdCl_2$(dppf), 23 mg, 0.028 mmol, 0.2 eq) in 4 mL abs. 1,4-dioxane. The tube was sealed tightly and the mixture was heated at 90 ºC for 16 h on an oil bath. The solvent was evaporated in vacuo and purified with flash silica gel column chromatography (20% to 50% EtOAc gradient in hexane over 20 min) resulting in 52 mg purple crystals (56%).

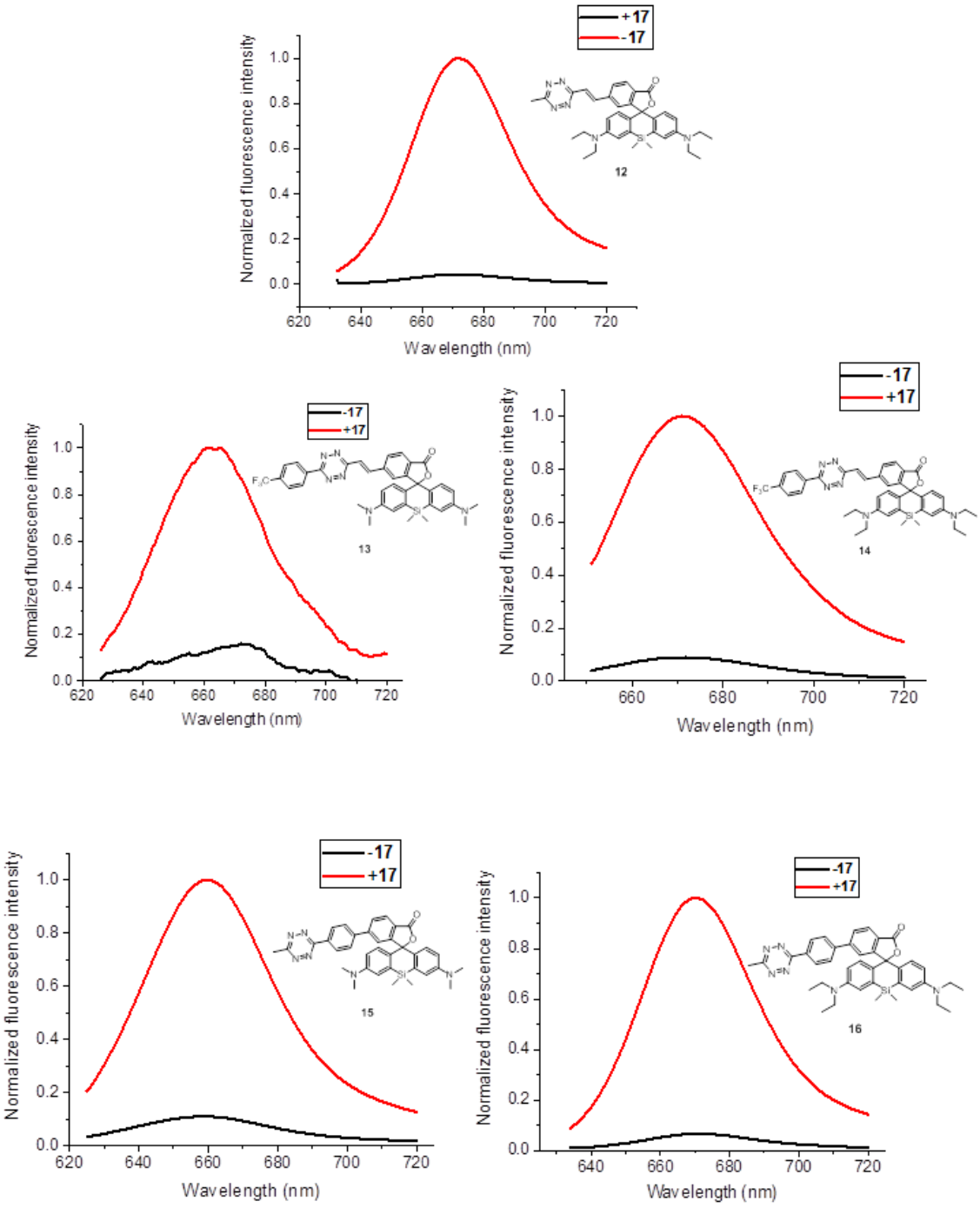
^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (d, *J* = 8.3 Hz, 2H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.87 (d, *J* = 8.0 Hz, 1H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.63 (s, 1H), 6.95 (s, 2H), 6.84 (d, *J* = 8.9 Hz, 2H), 6.54 (d, *J* = 8.0 Hz, 2H), 3.37 (q, *J* = 6.9 Hz, 8H), 3.10 (s, 3H), 1.16 (t, *J* = 6.9 Hz,12H), 0.66 (s, 3H), 0.63 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.43, 167.46, 163.89, 155.62, 146.78, 145.43, 143.93, 137.27, 132.83, 130.81, 128.61, 128.45, 128.00, 127.09, 126.21, 123.33, 116.10, 112.81, 44.40, 21.29, 12.69, 0.49, -1.42. HRMS (ESI): m/z calc. for $C_{39}H_{43}N_6O_2Si$ 655.3217; found: 655.3214 $[M+H]^+$

2. Supplementary Figures

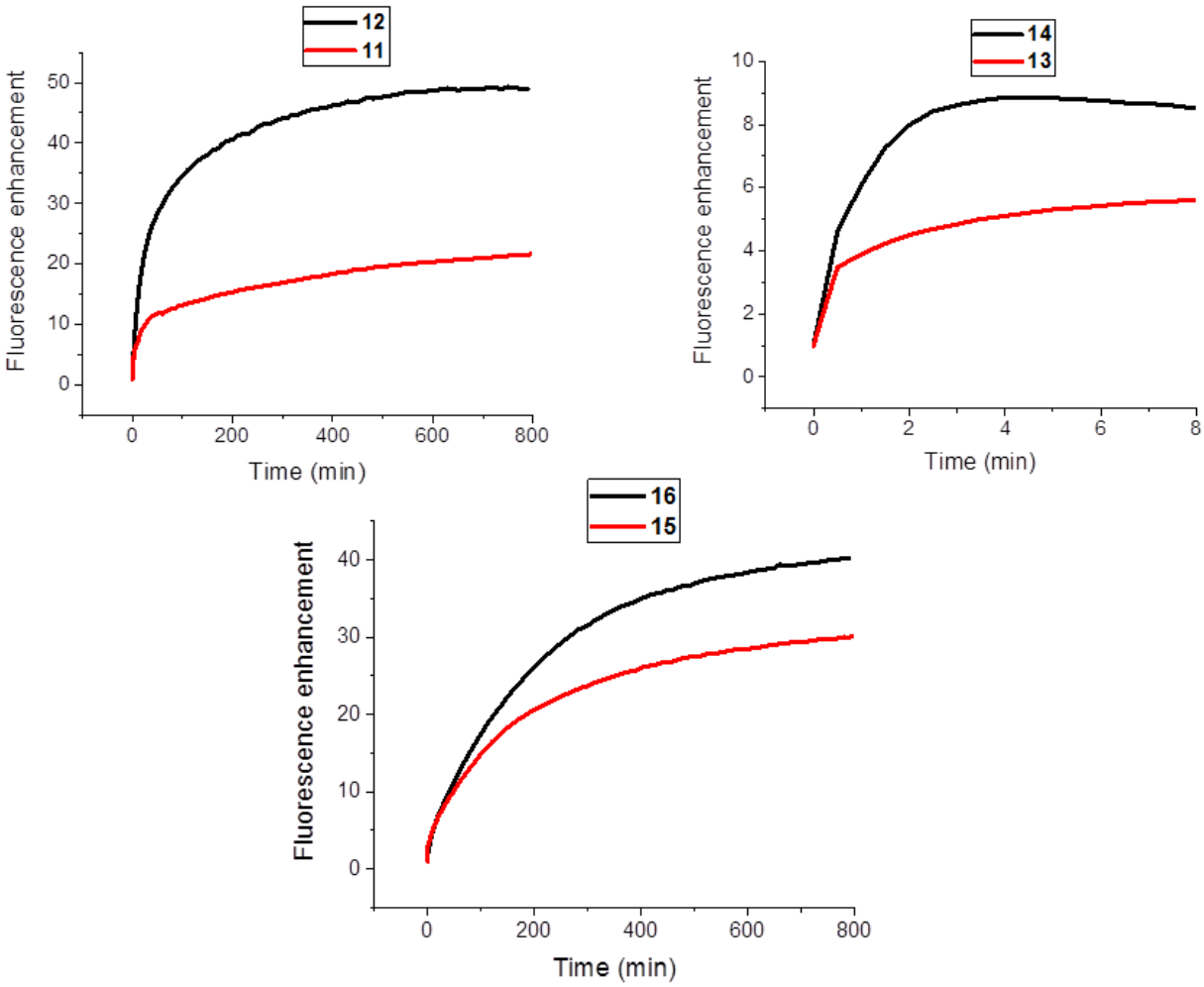
2.1. Figure S1. Excitation and emission spectra of SiR dyes



2.2. Figure S2. Tetrazine-fluorogenicity of dyes 12-16 upon reaction with OxTCO (17)

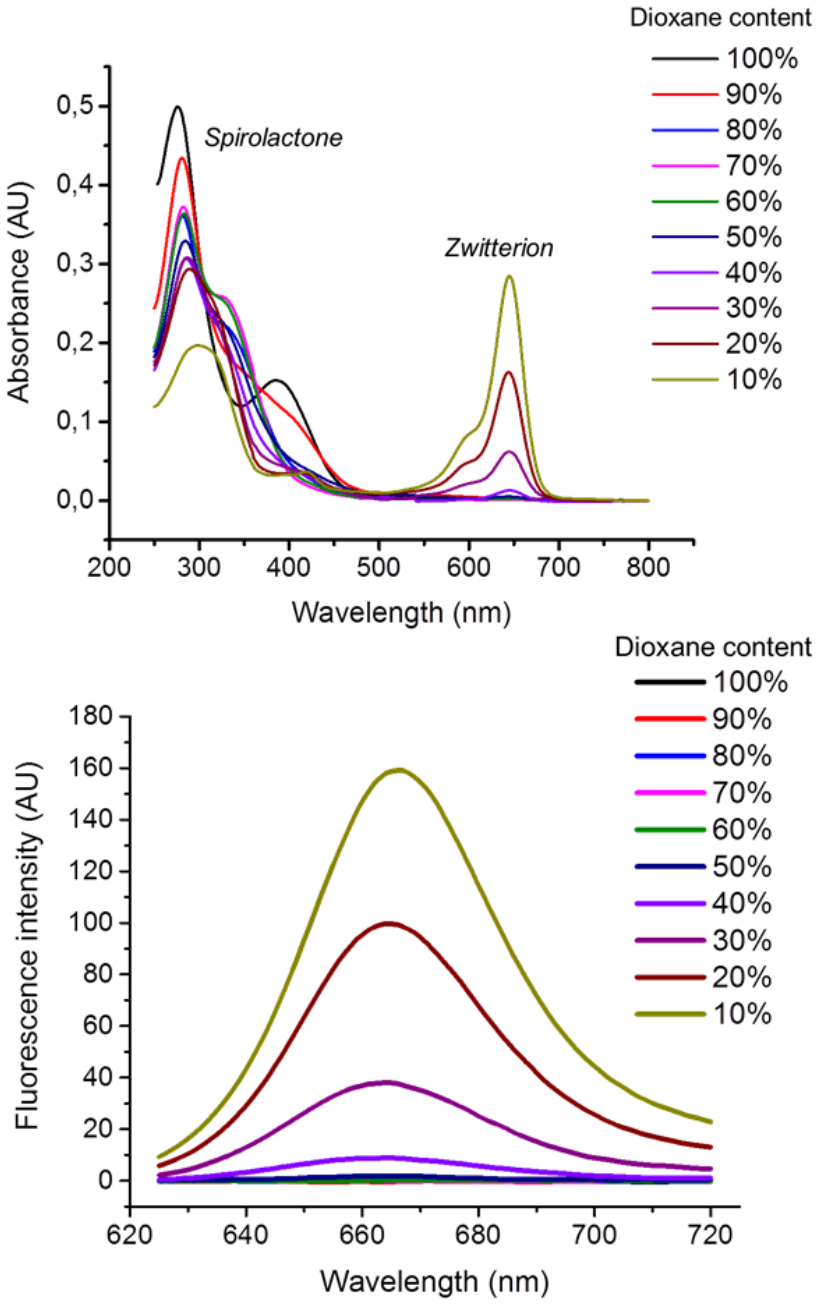


2.3. Figure S3. Time course measurements of IEDDA reaction with OxTCO (17)



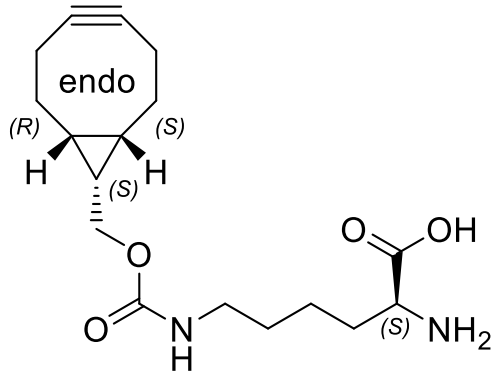
Compound	k_2 ($M^{-1}s^{-1}$)
11	333
12	98
13	1235
14	1923
15	12
16	20

2.4. Figure S4. Polarity dependence of absorbance and fluorescence spectra of conjugate 18

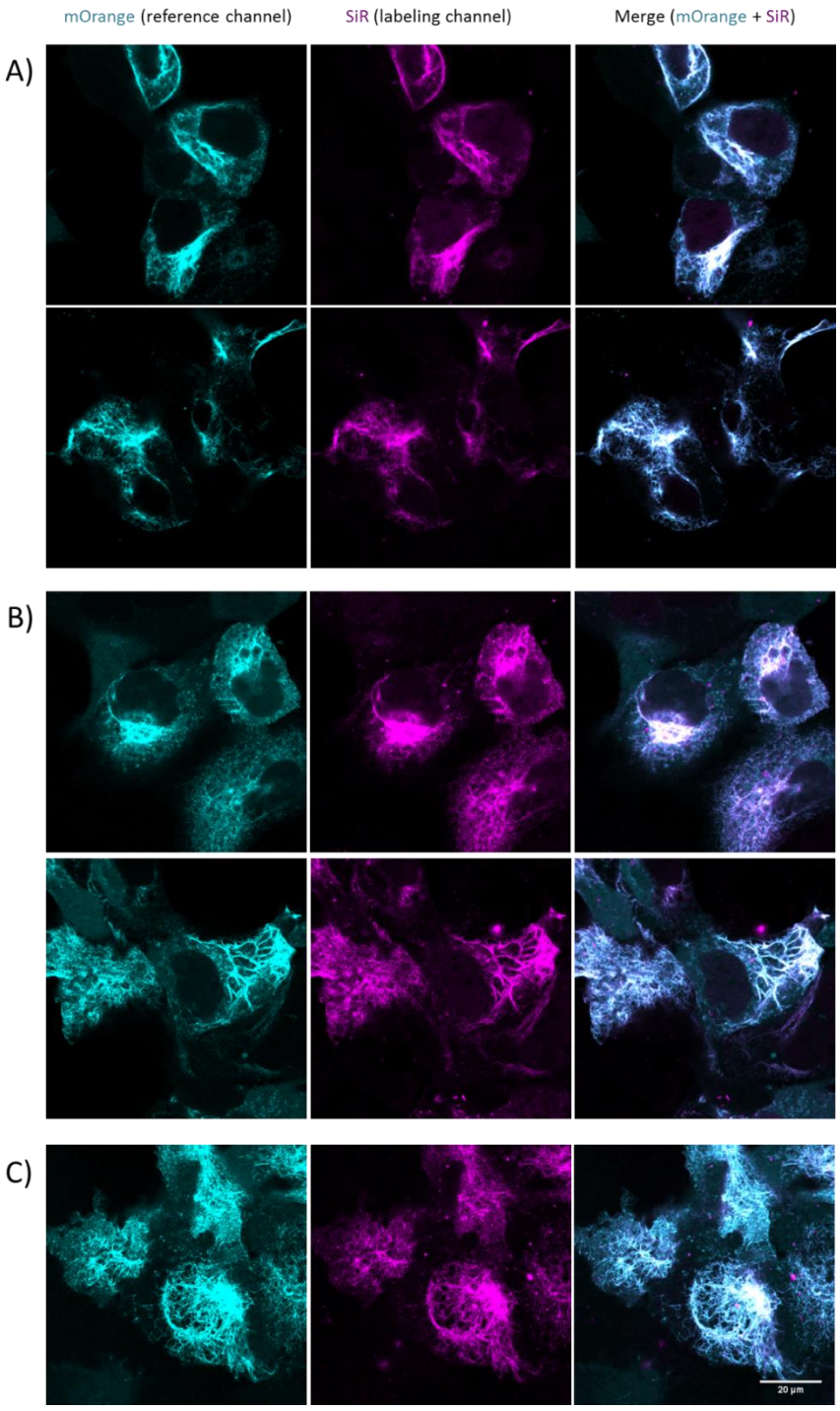


Changes of absorbance and fluorescence spectra of **11** (10 μM) reacted with **17** (100 μM) in dioxane/water mixtures. Absorbance of nonfluorescent spirolactone form (~300 nm) increased with increasing dioxane content, while absorbance of fluorescent zwitterion form (~650 nm) decreased. Spectra in 0% dioxane (100% water) was not included due to precipitation of the adduct. Fluorescence was detected between 624 nm and 720 nm with excitation at 615 nm.

2.5. Figure S5. Structure of BCN^{endo}

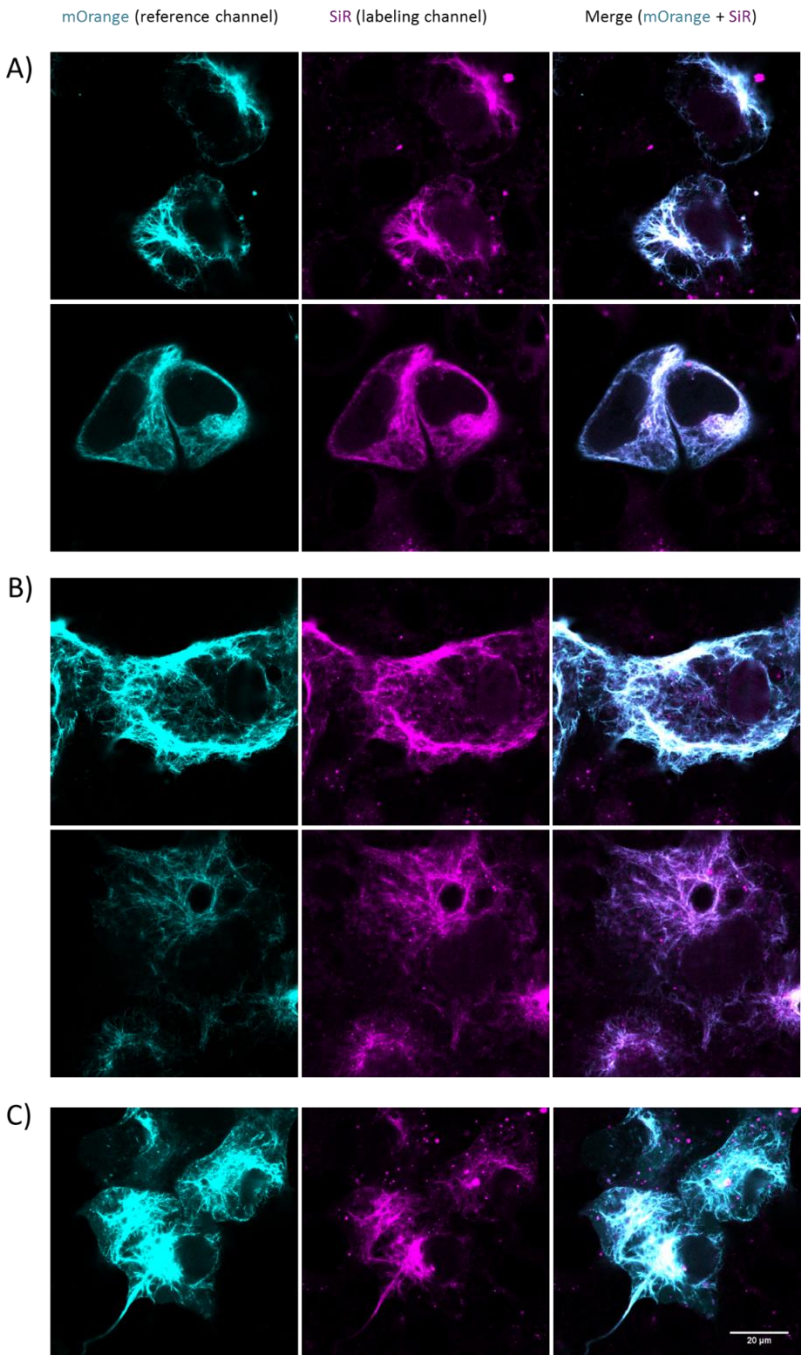


2.6. Figure S6. Additional confocal microscopy images of dye 11



Confocal images of vimentin^{BCNendo}-mOrange labeled live with dye **11** as described in Methods in different concentrations with different incubation times at 37 °C: a) 3 µM, 30 min, b) 3 µM, 10 min, c) 1.5 µM, 10 min.

2.7. Figure S7. Additional confocal microscopy images of dye 12



Confocal images of vimentin^{BCNendo}-mOrange labeled live with dye **12** as described in Methods in different concentrations with different incubation times at 37 °C: a) 3 µM, 30 min, b) 3 µM, 10 min, c) 1.5 µM, 10 min.

2.8. Figure S8. Confocal microscopy images of dye 11 and 12 with different post-labeling wash times

In order to asses for *in vivo* fluorogenicity and specificity of the labeling, an experiment was conducted in which cells were transfected and labeled identically but different washing times after the reaction were performed. See Figure S8.1 for experimental details. Confocal images of vimentin^{BCNendo}-mOrange with dye **11** (Figure S8.2) and dye **12** (Figure S8.3) were acquired as described in Methods.

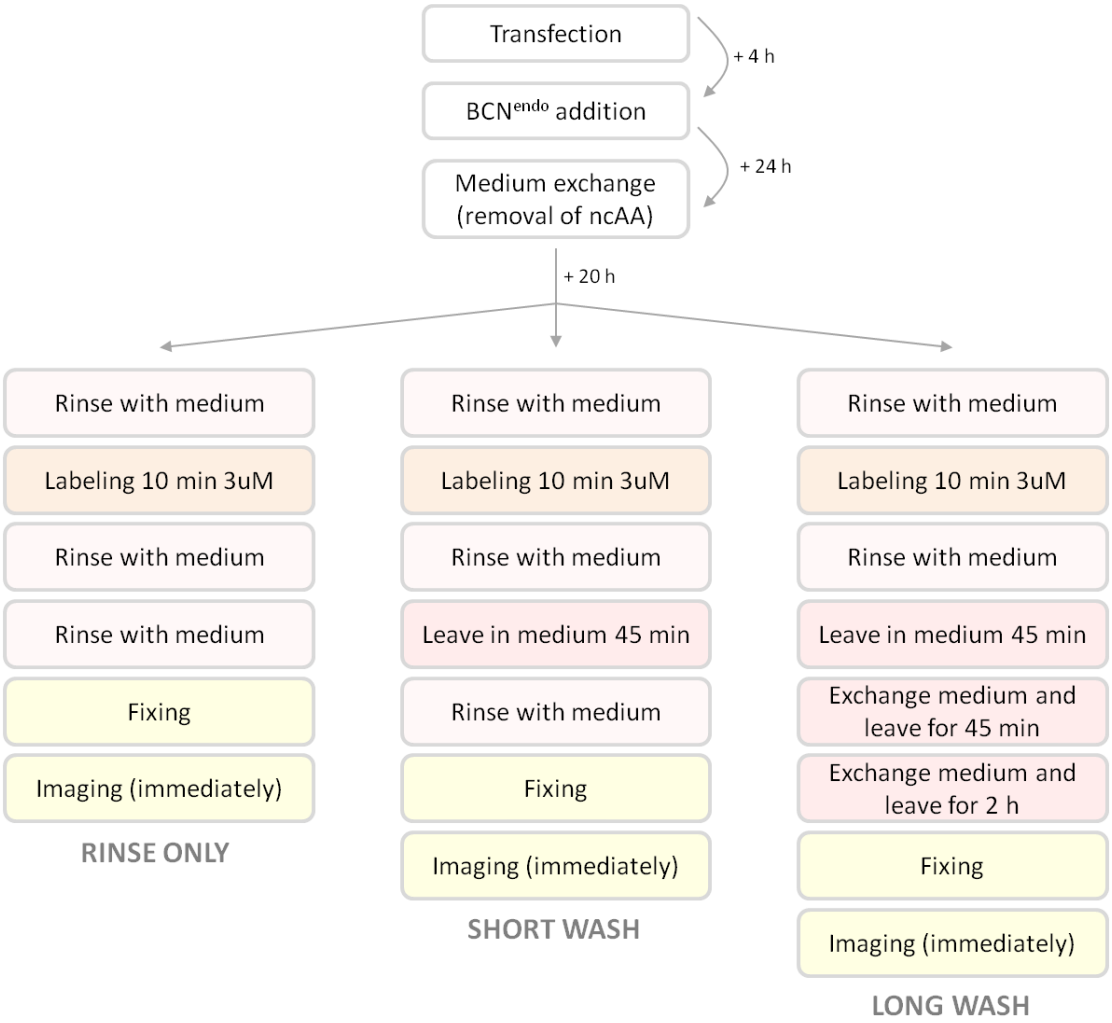


Figure S8.1. Scheme of the experimental setup for the comparison of different wash times.

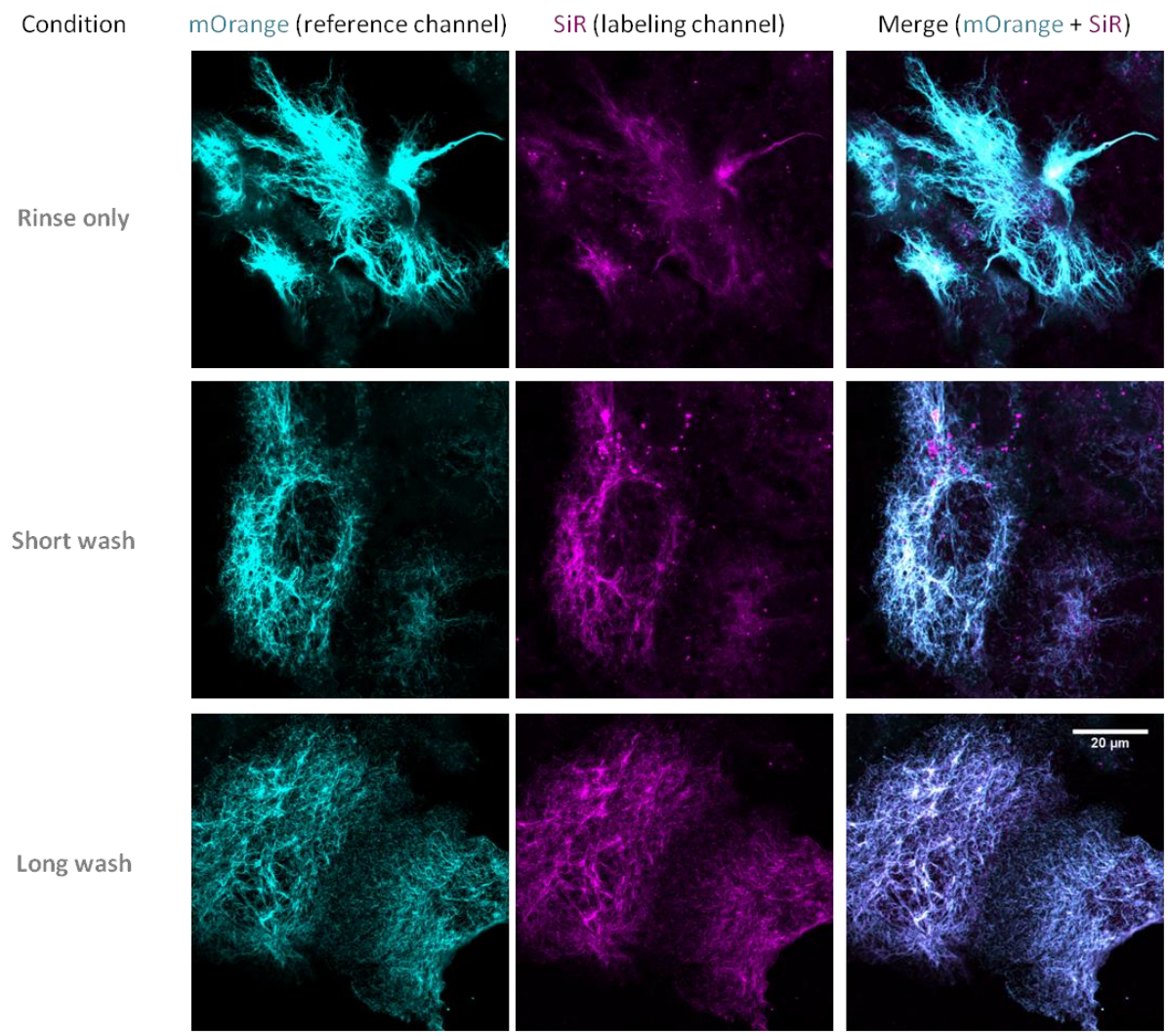


Figure S8.2. Confocal images of vimentin^{BCNendo}-mOrange labeled live with dye **11** as described in Methods in different wash conditions

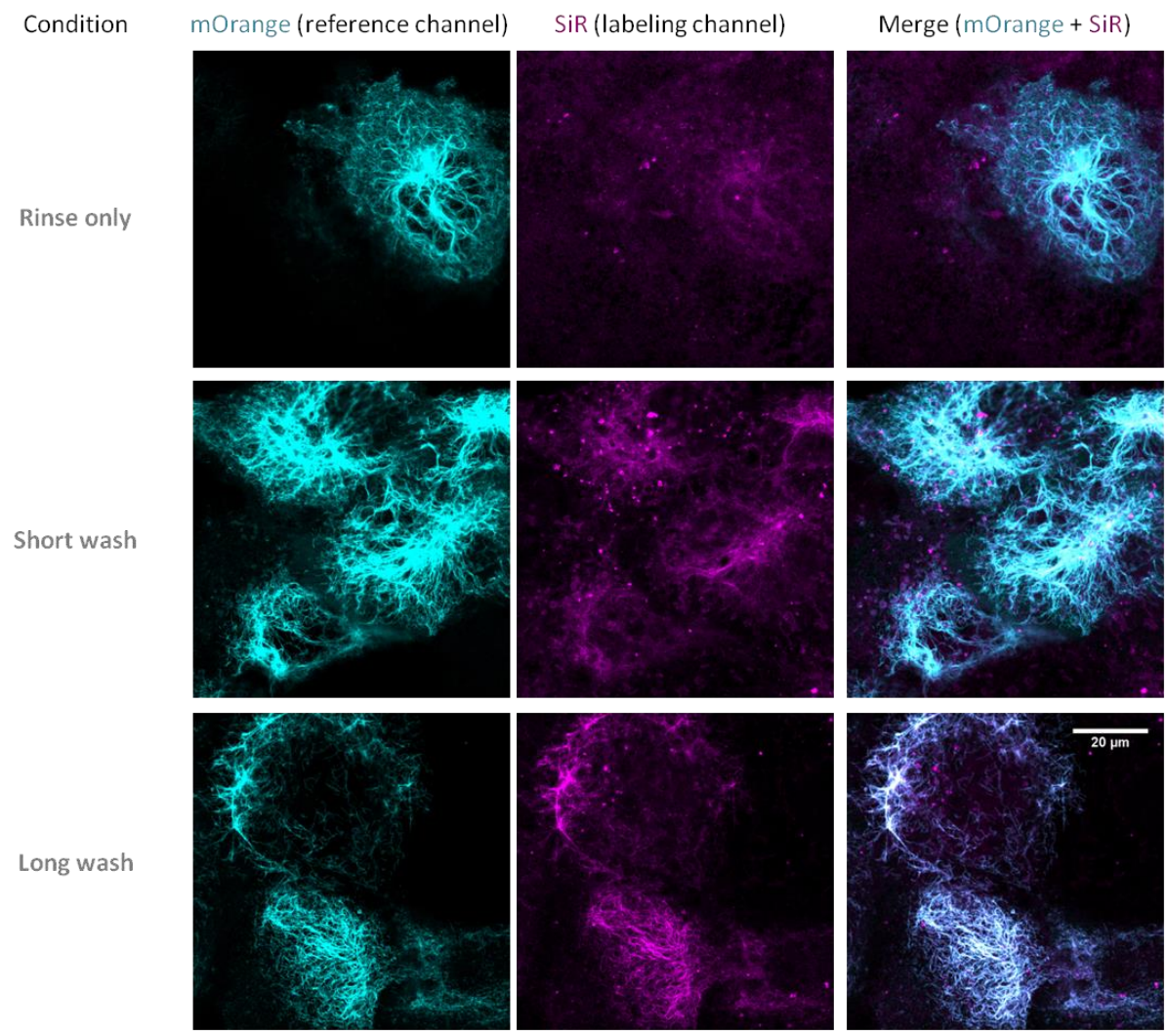
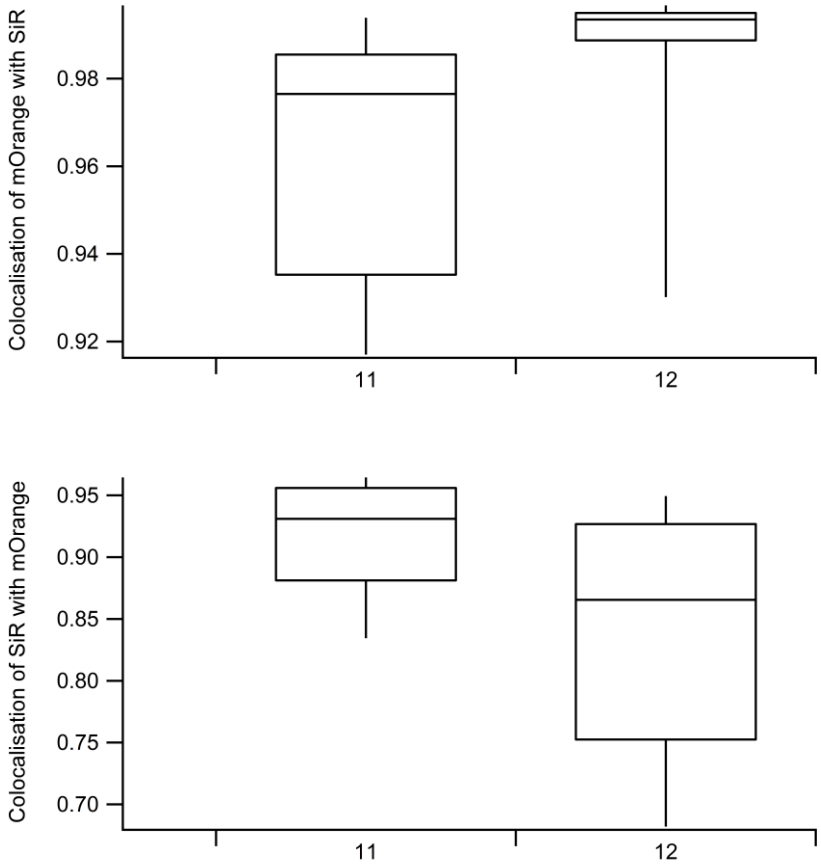


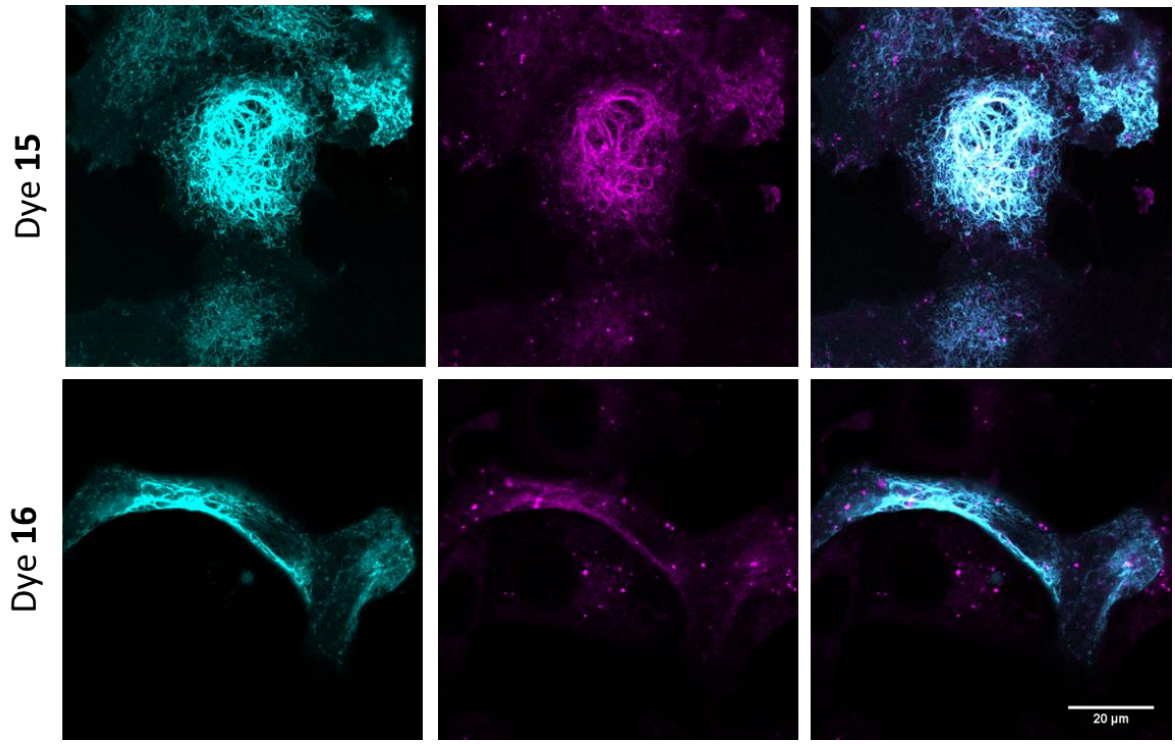
Figure S8.3. Confocal images of vimentin^{BCNendo}-mOrange labeled live with dye **12** as described in Methods in different wash conditions

2.9. Figure S9. Quantitative mOrange-SiR colocalisation analysis



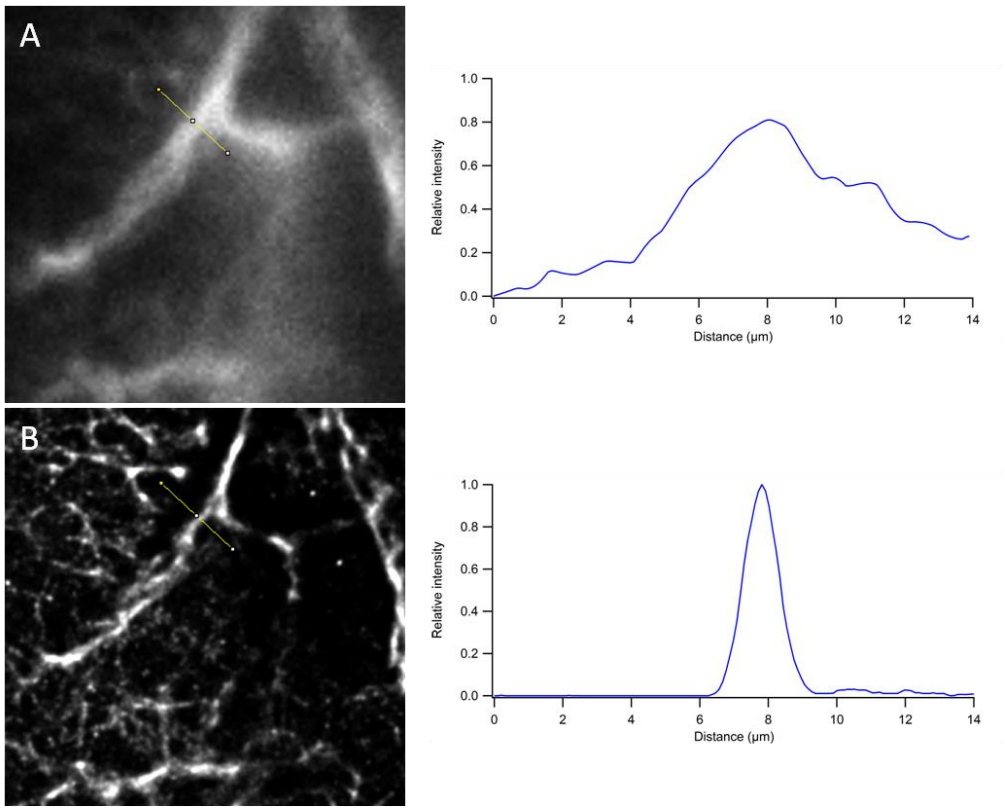
Quantitative estimation of the colocalisation between the mOrange and SiR signals from confocal images in the main text of vimentin^{BCNendo}–mOrange labeled with dye **11** or **12** (3 μM, 10 min). The values displayed in the boxplot correspond to the Mander's coefficient[10], calculated over 12 images for each dye using the JACoP plugin in ImageJ.

2.10. Figure S10. Confocal microscopy images of vimentin^{BCNendo}-mOrange labeled with dye 15 and 16



Vimentin^{BCNendo}-mOrange was expressed in COS-7 cells and labeled with SiR dyes **15** or **16** (1.5 µM, 15 min, 37 °C) as described in Methods. Reference fluorescence of mOrange is shown in the left panels (cyan), SiR-fluorescence in the middle panels (magenta) and overlay images on the right.

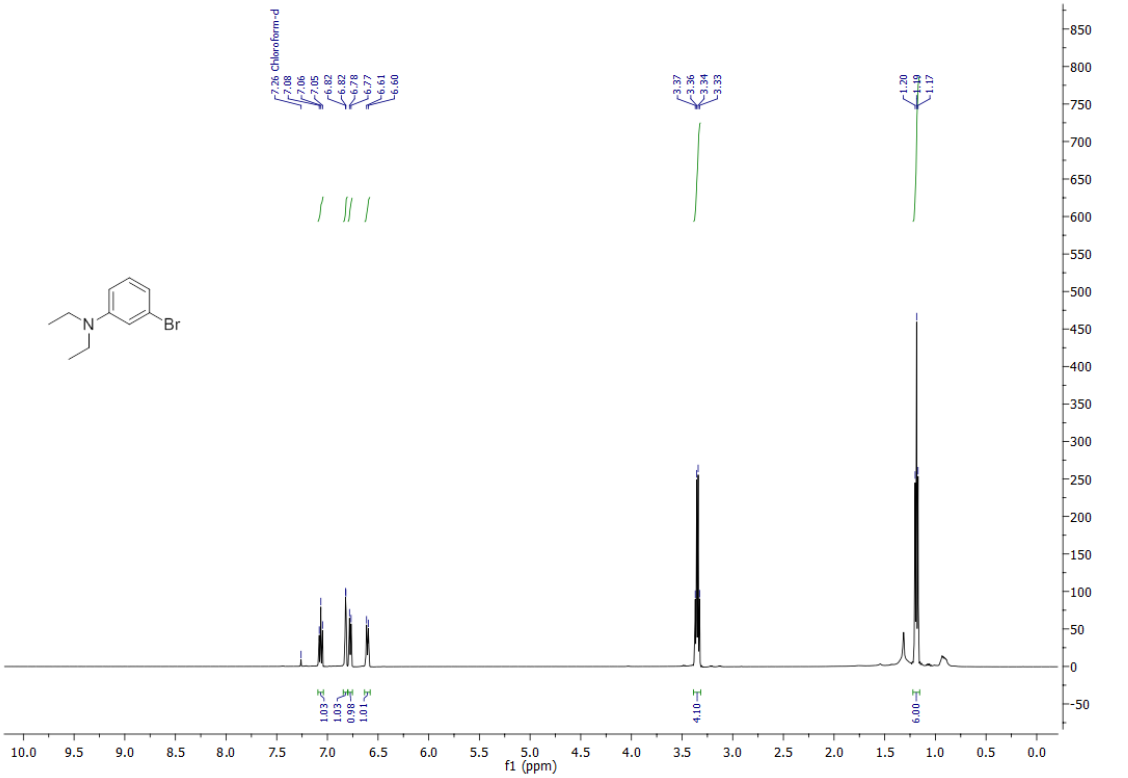
2.11. Figure S11. Comparison of conventional and SRM imaging of vimentin^{BCNendo}-mOrange fibers labeled with dye 11

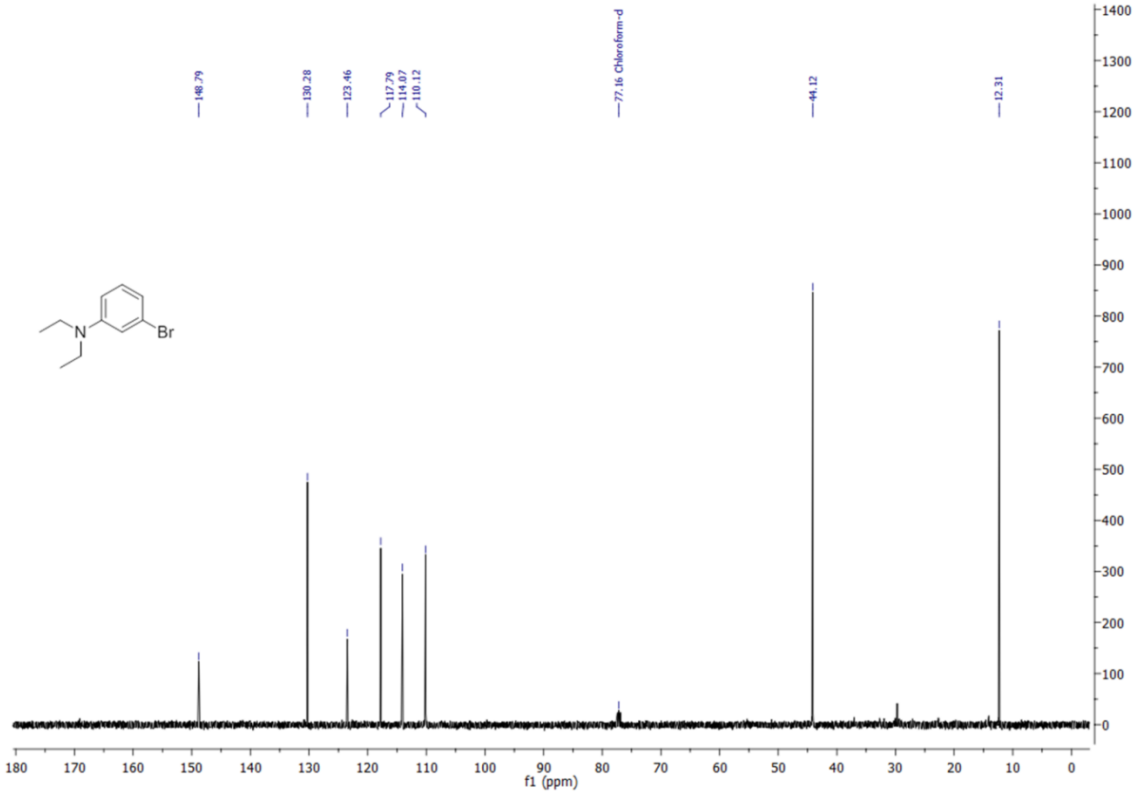


Comparison of conventional (A) and SRM (B) images of vimentin^{BCNendo}–mOrange labeled with dye **11**: the line scan through a selected fiber (drawn in yellow) highlights the increased resolution achieved in (B).

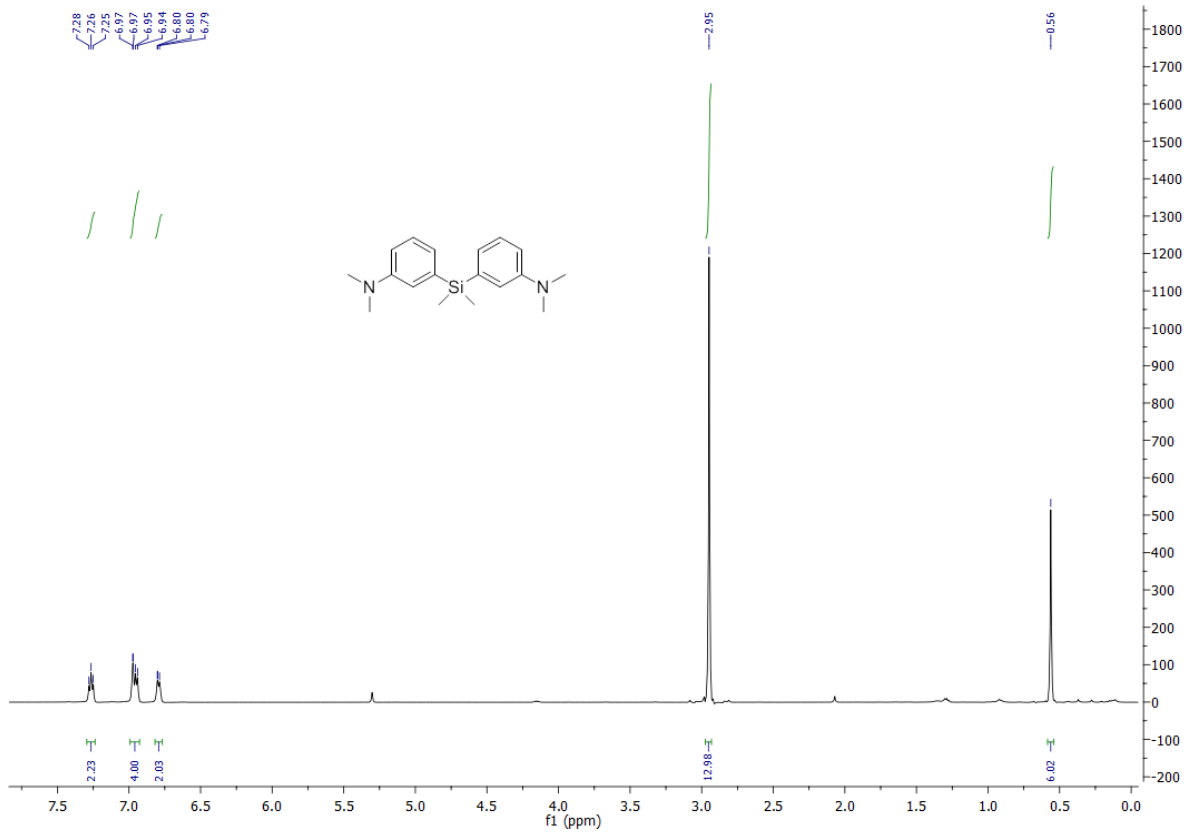
3. NMR spectra

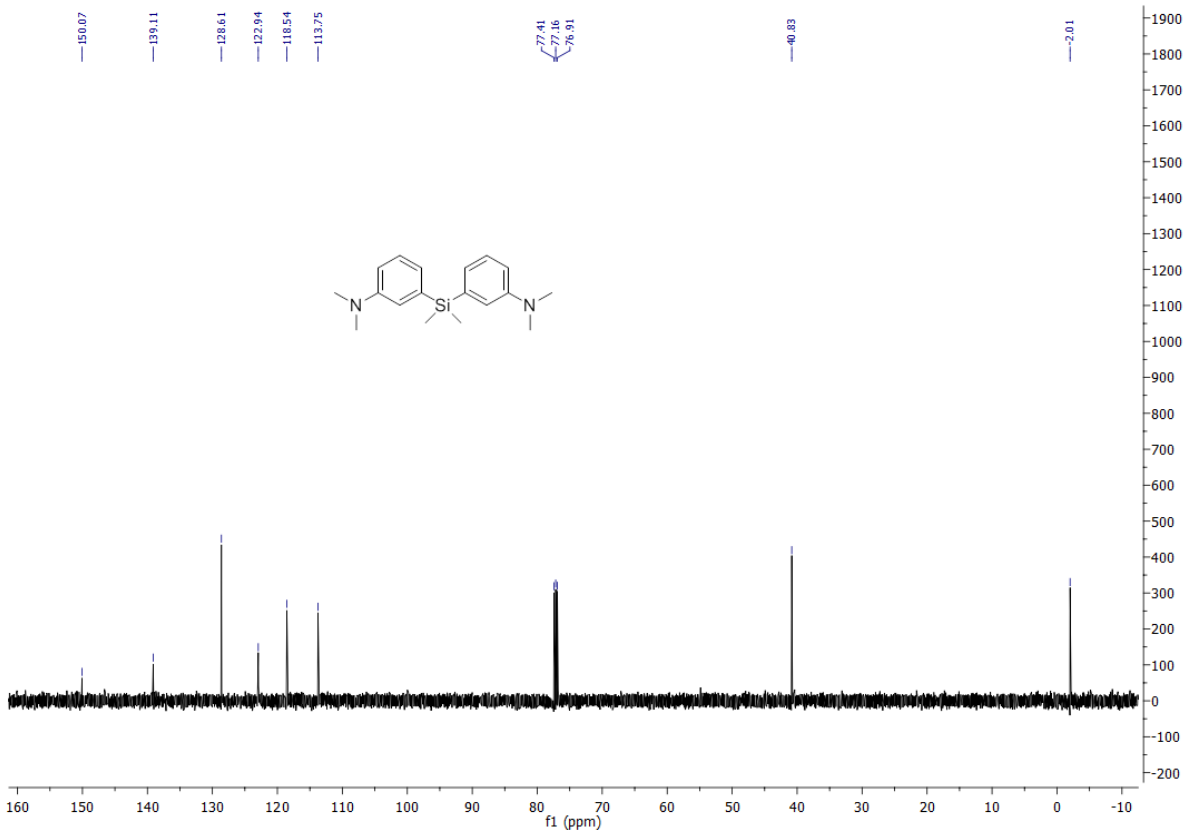
NMR spectra of compound 2



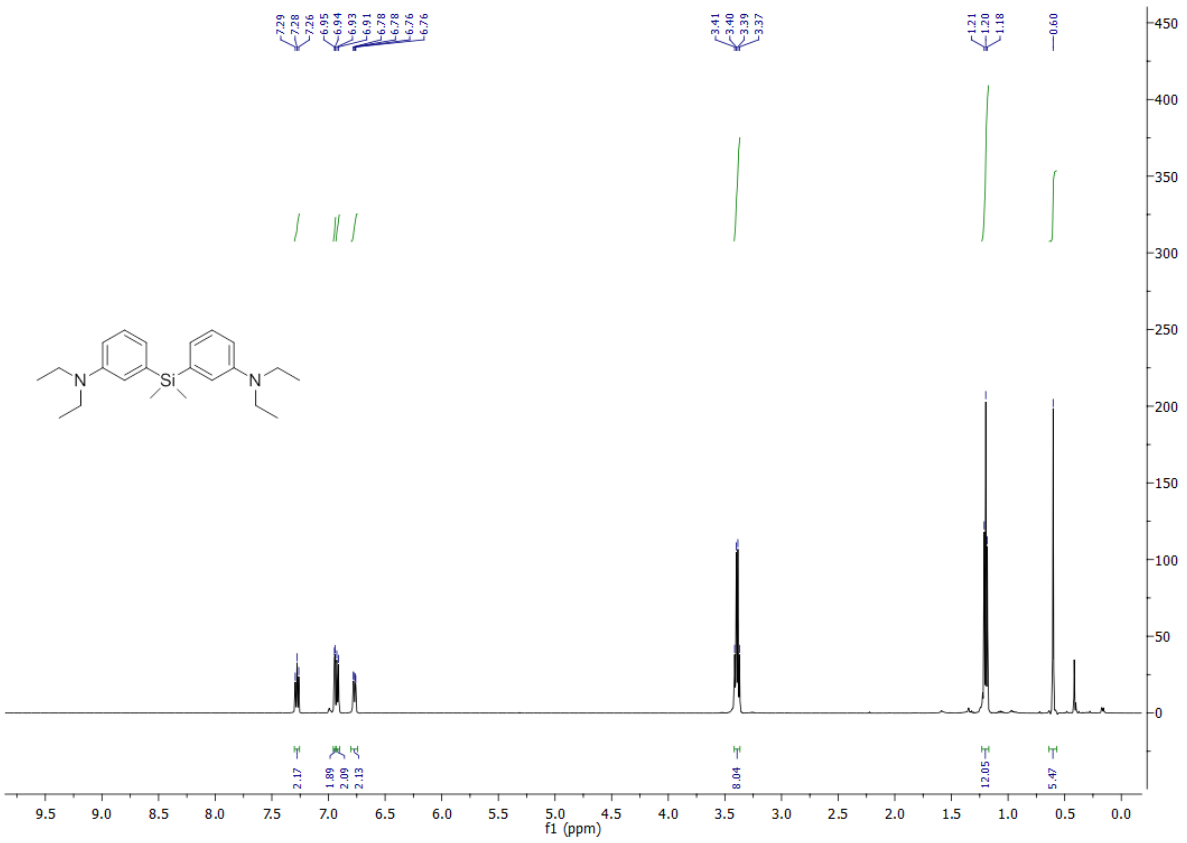


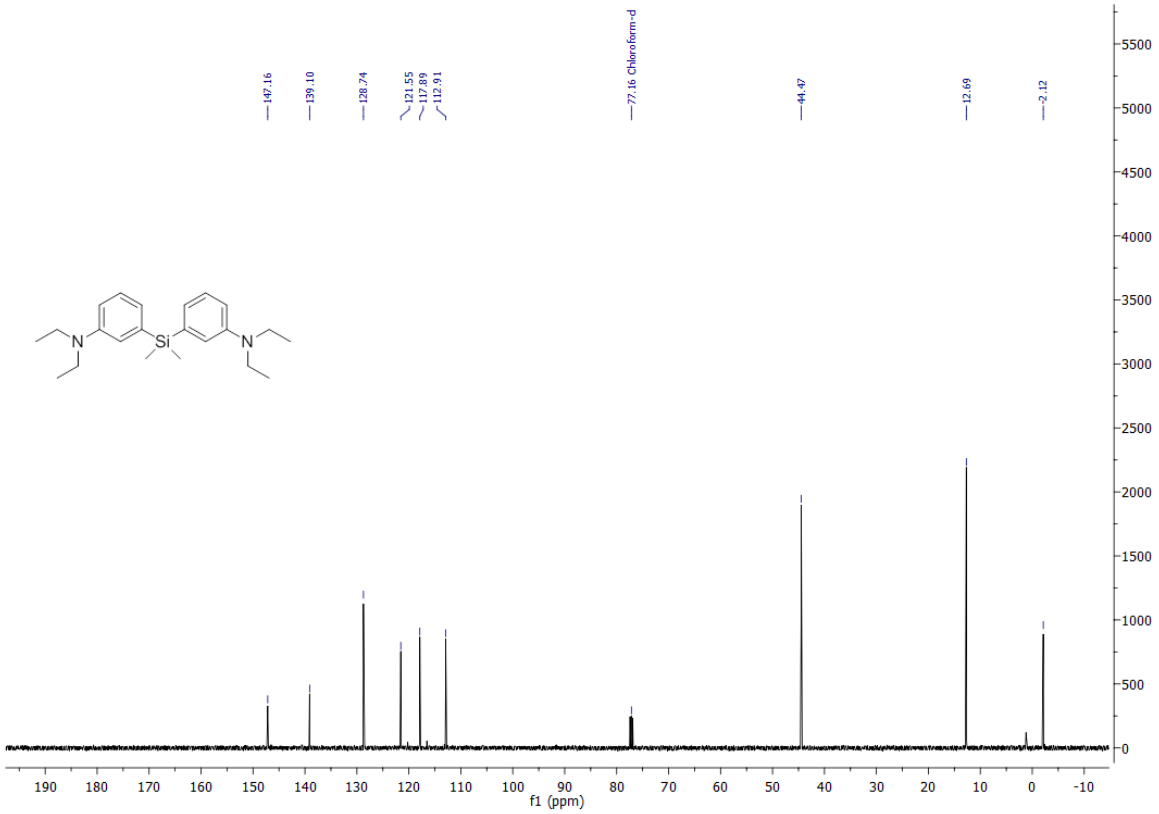
NMR spectra of compound 3



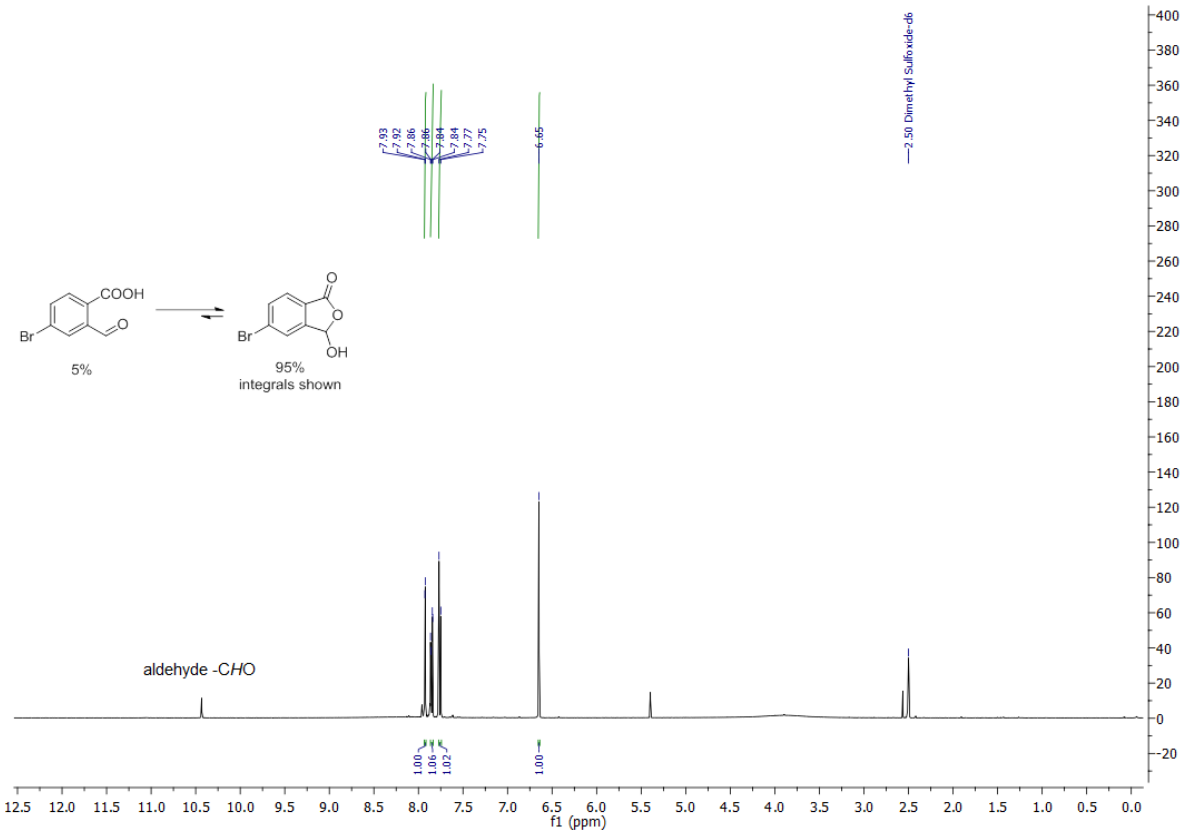


NMR spectra of compound 4

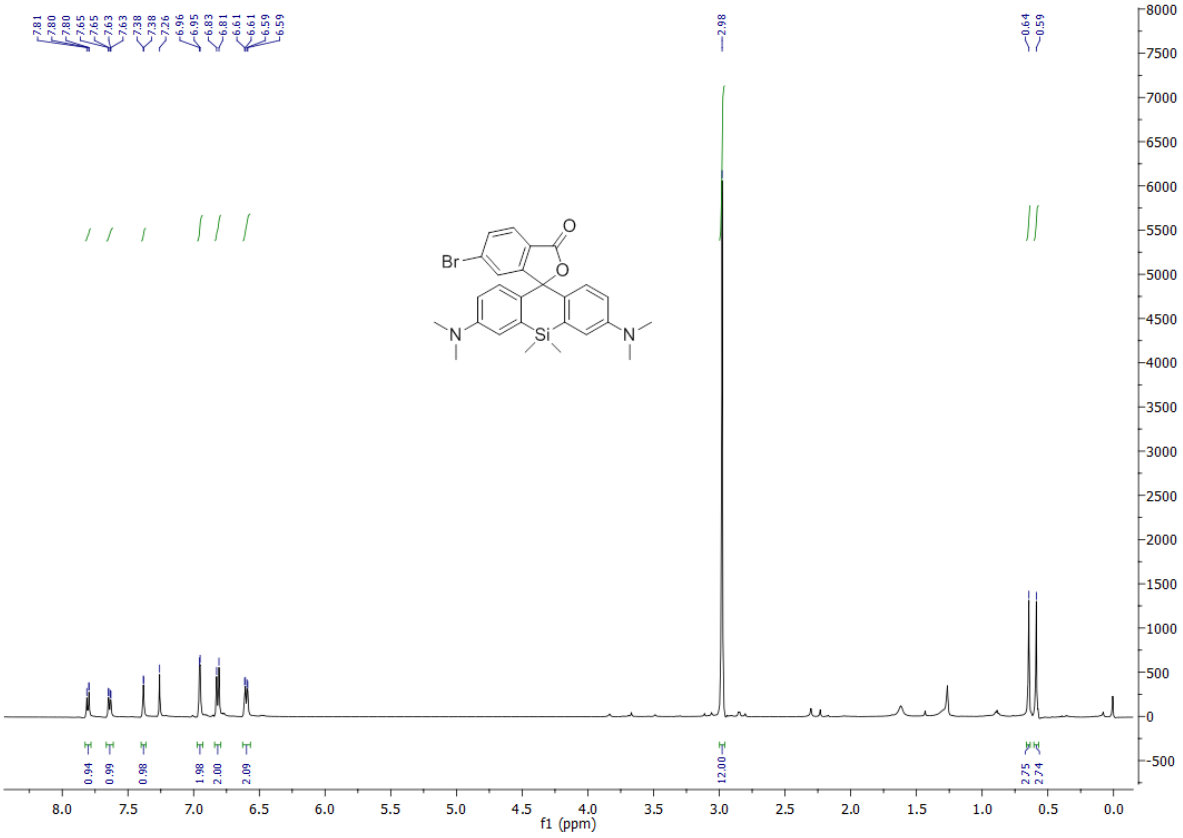


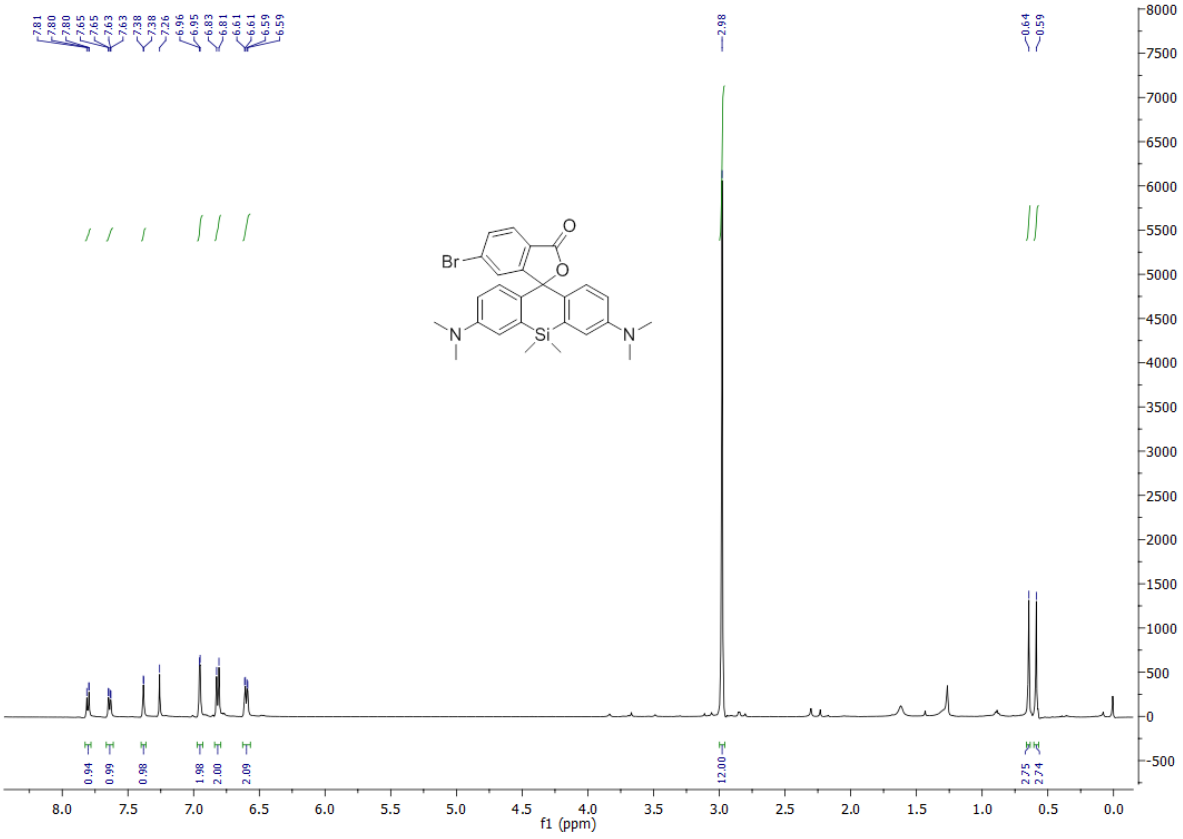


NMR spectra of compound 5

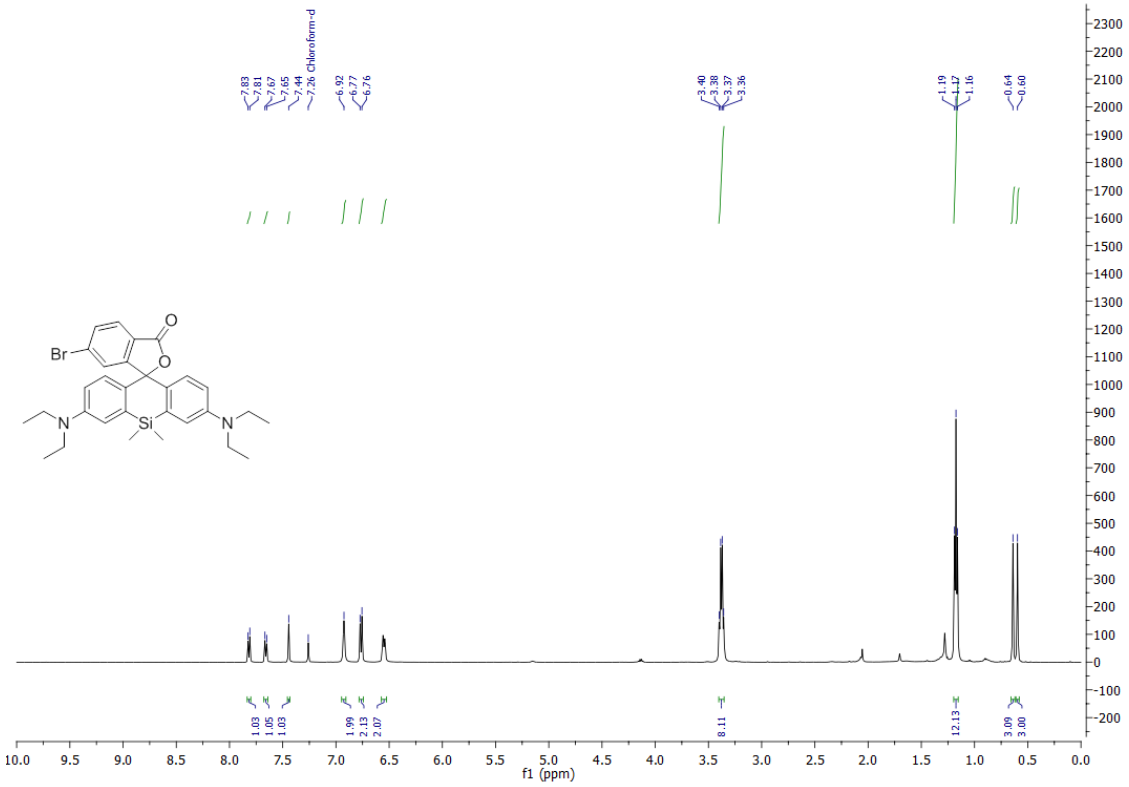


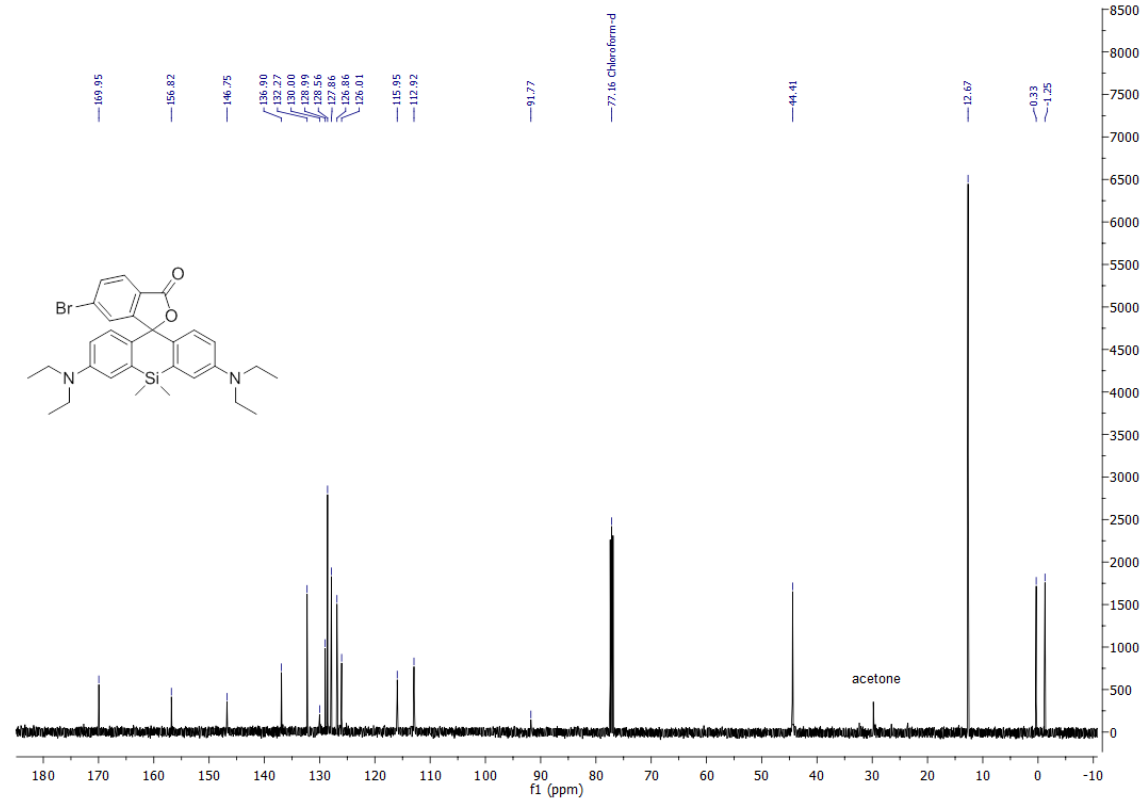
NMR spectra of compound 6



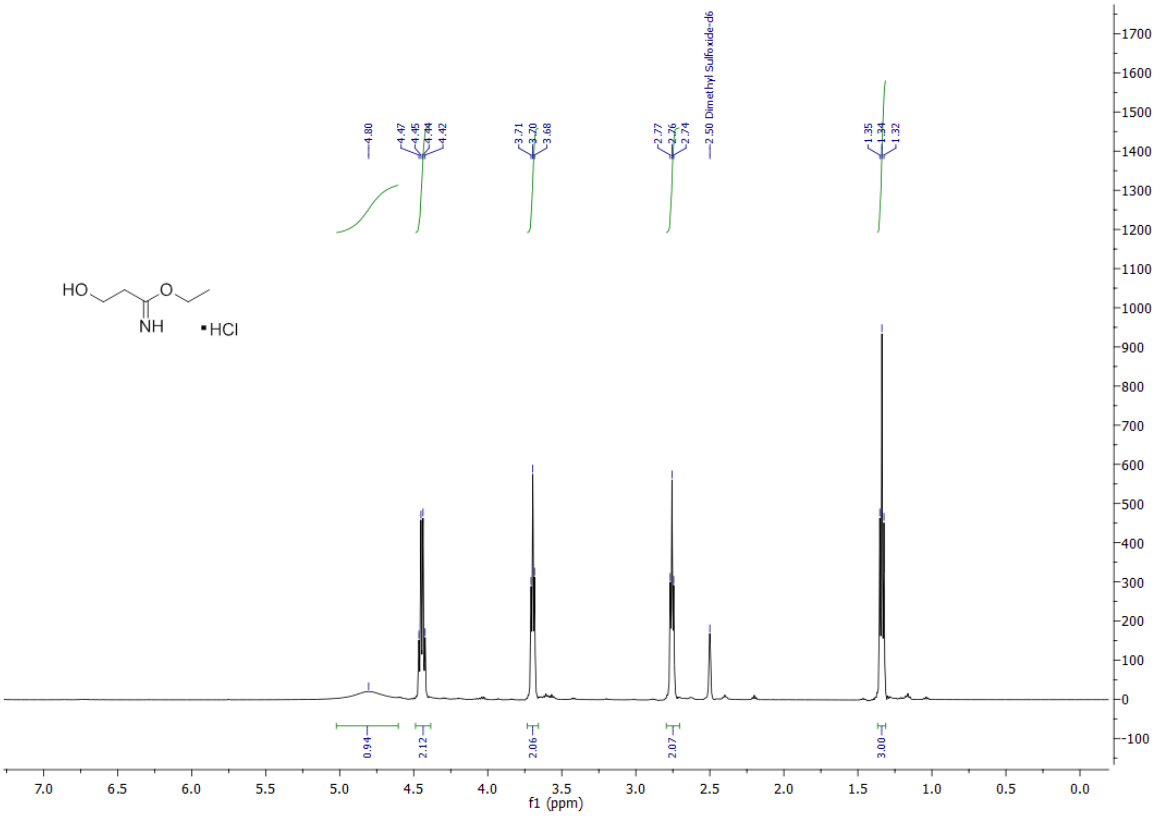


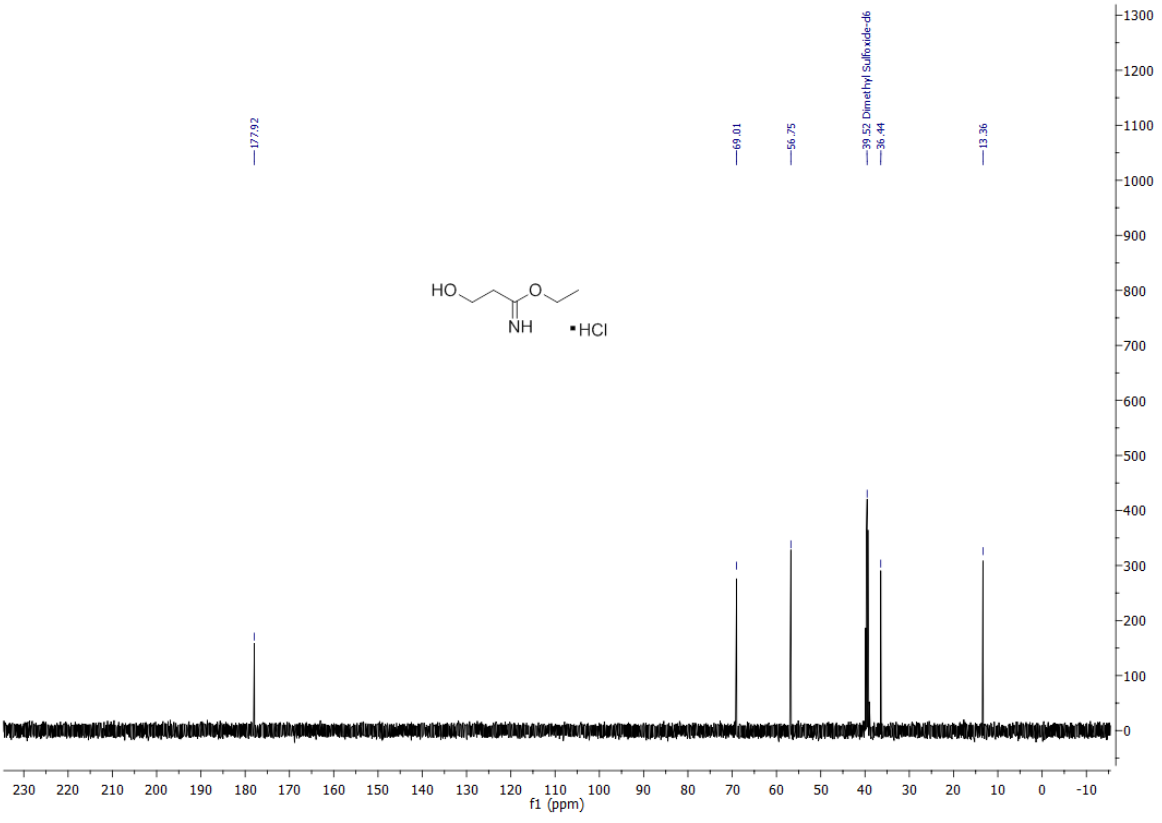
NMR spectra of compound 7



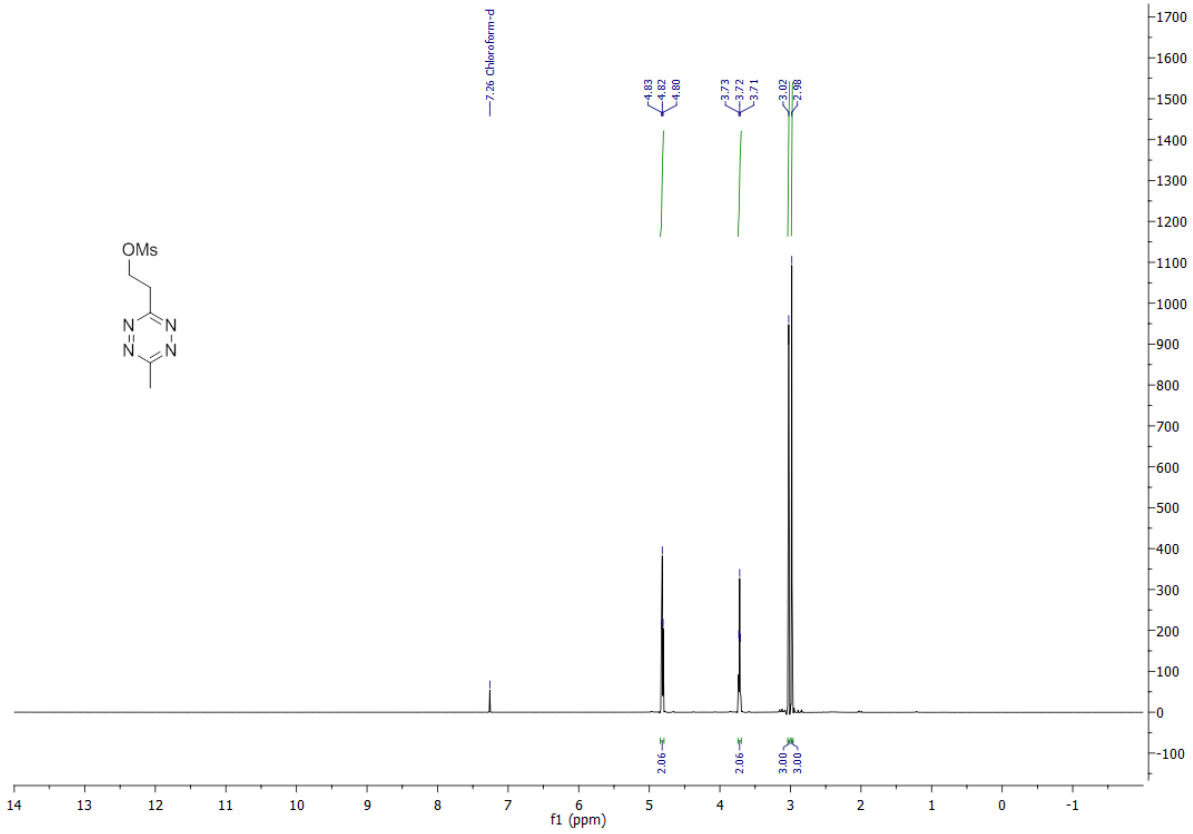


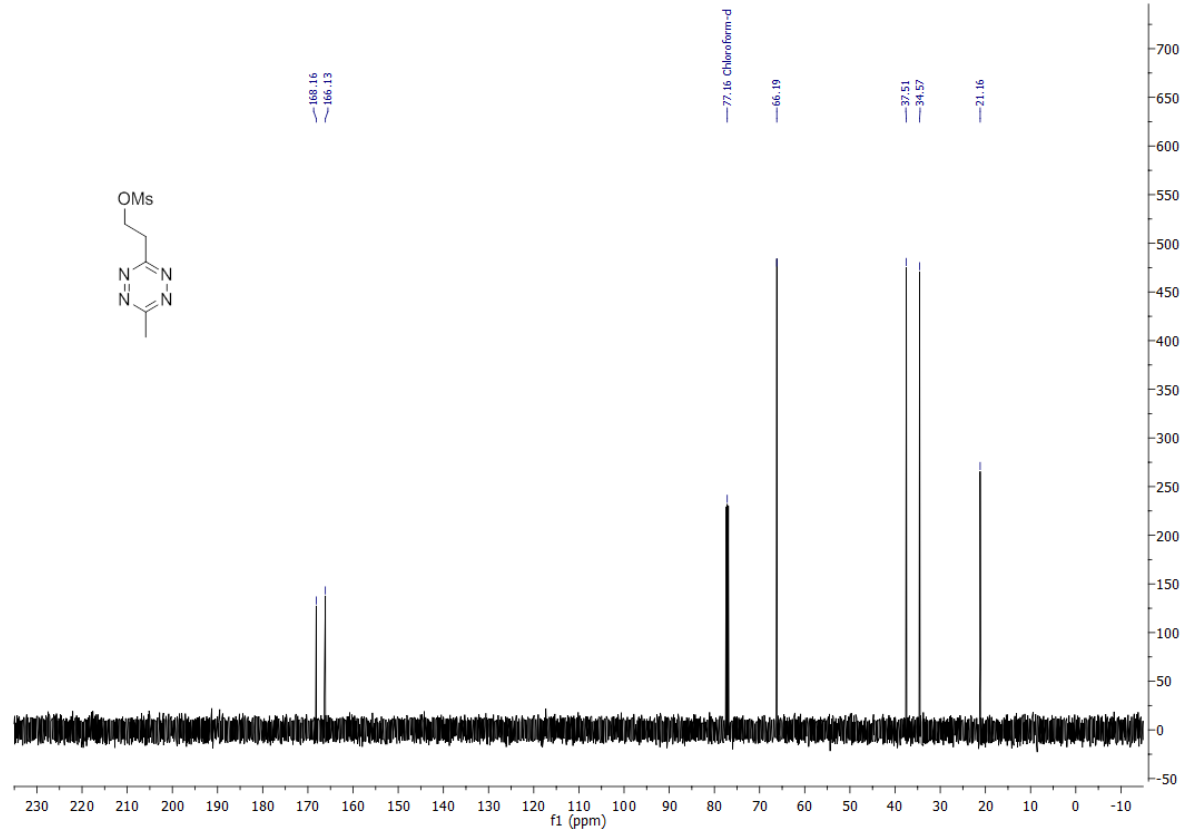
NMR spectra of compound 19



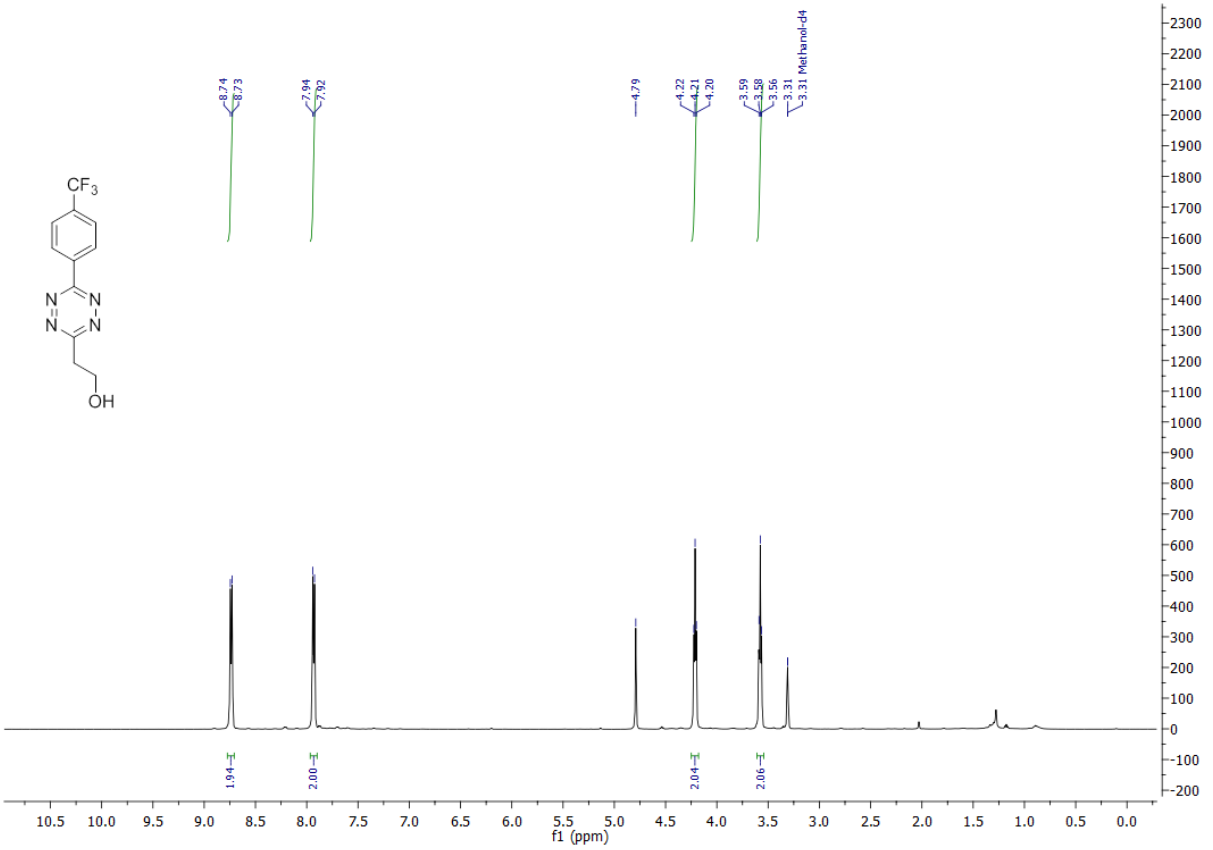


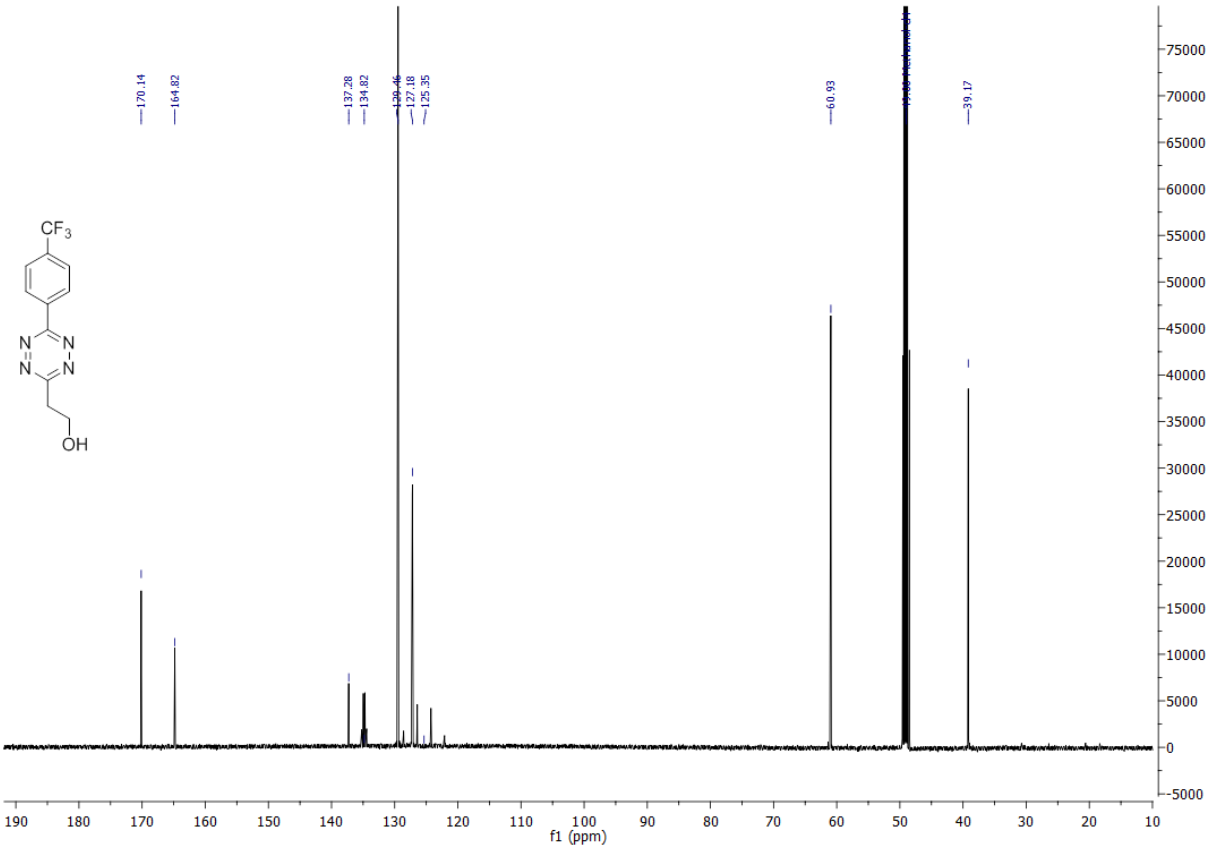
NMR spectra of compound 8

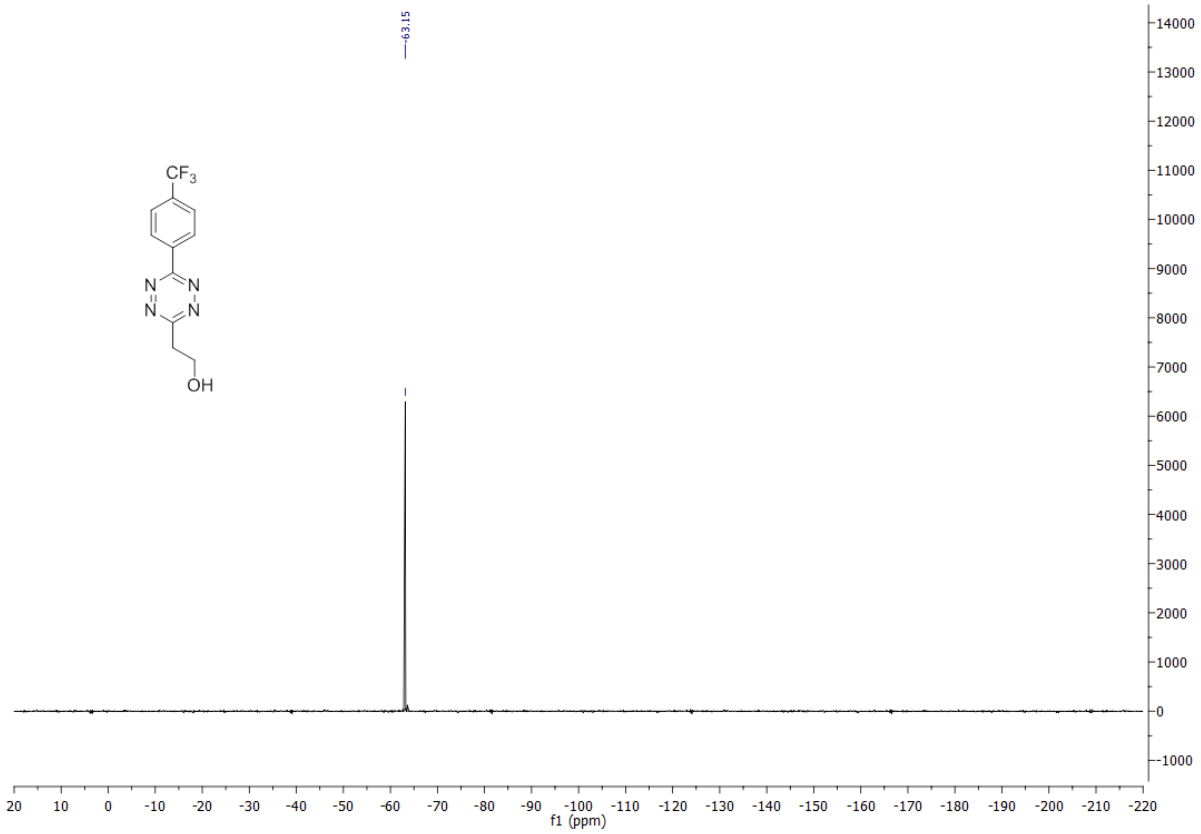




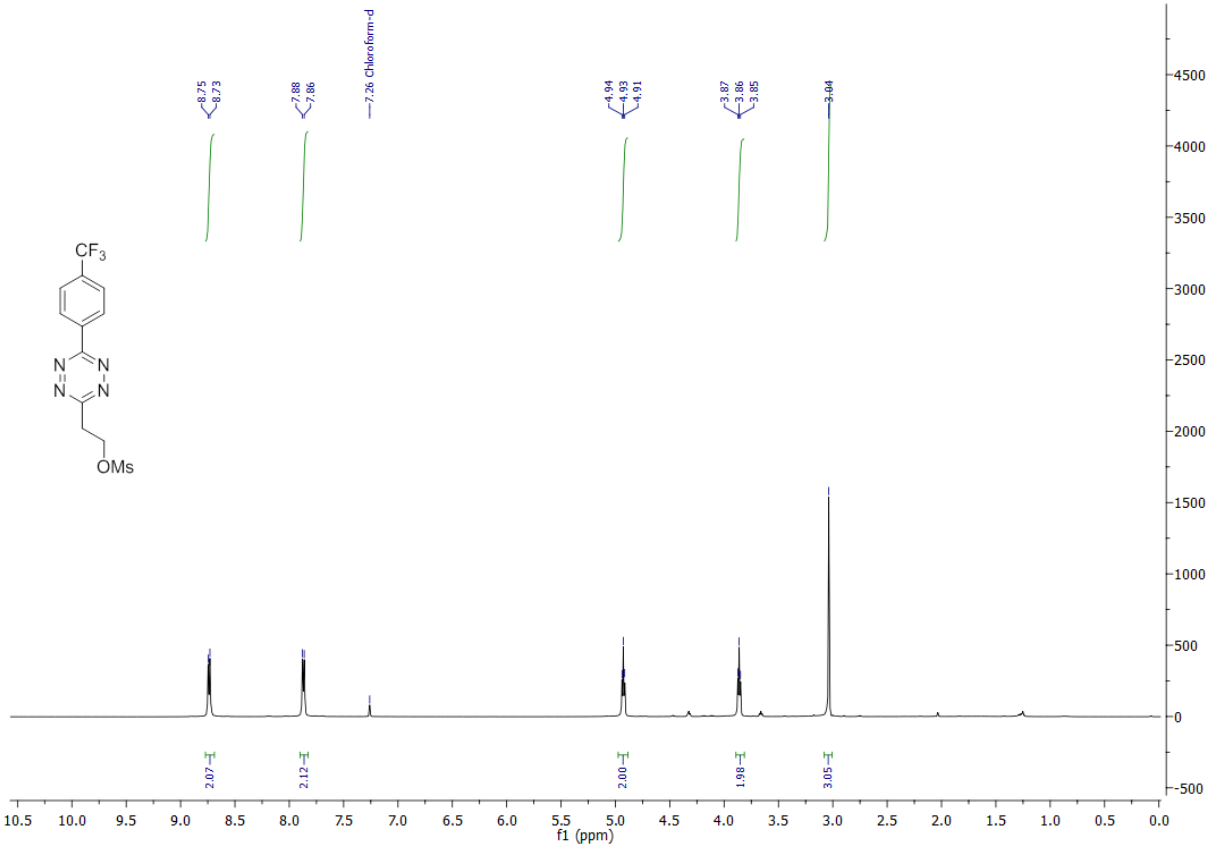
NMR spectra of compound 20

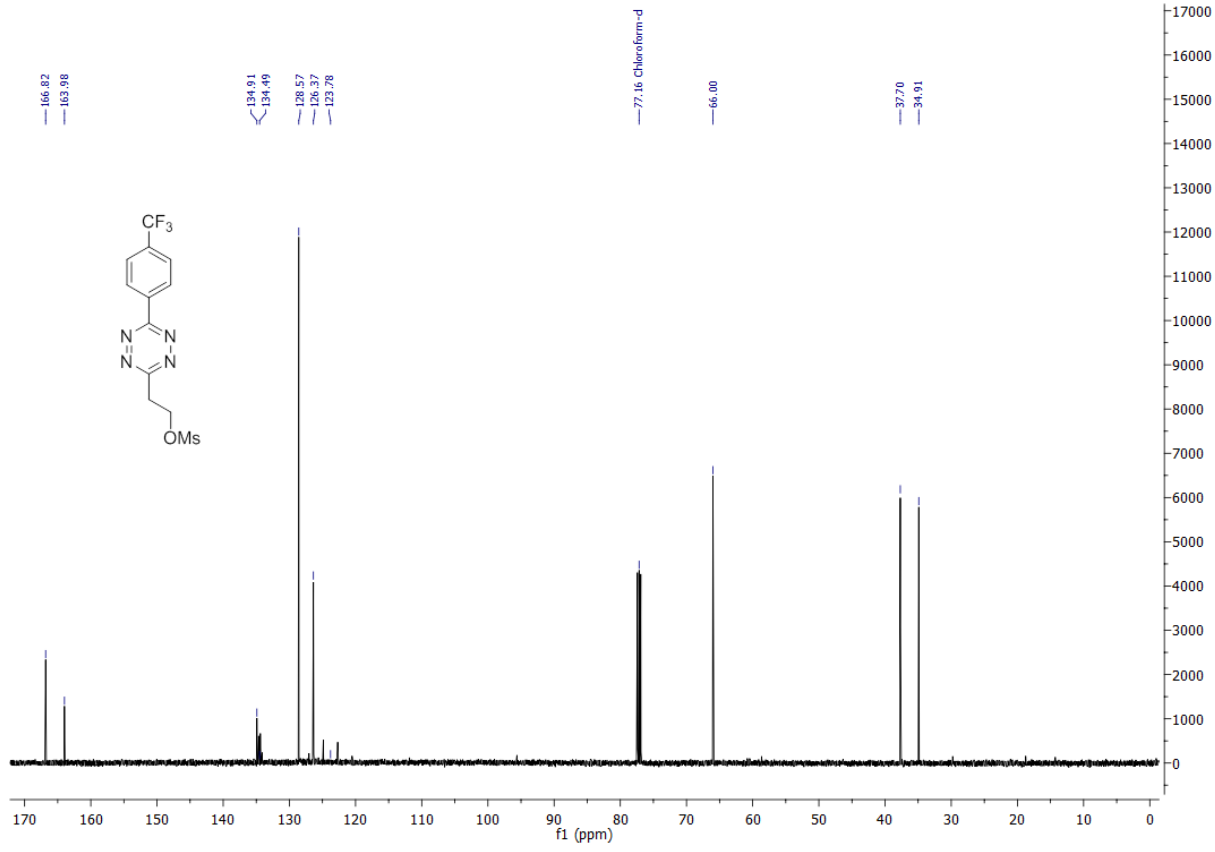


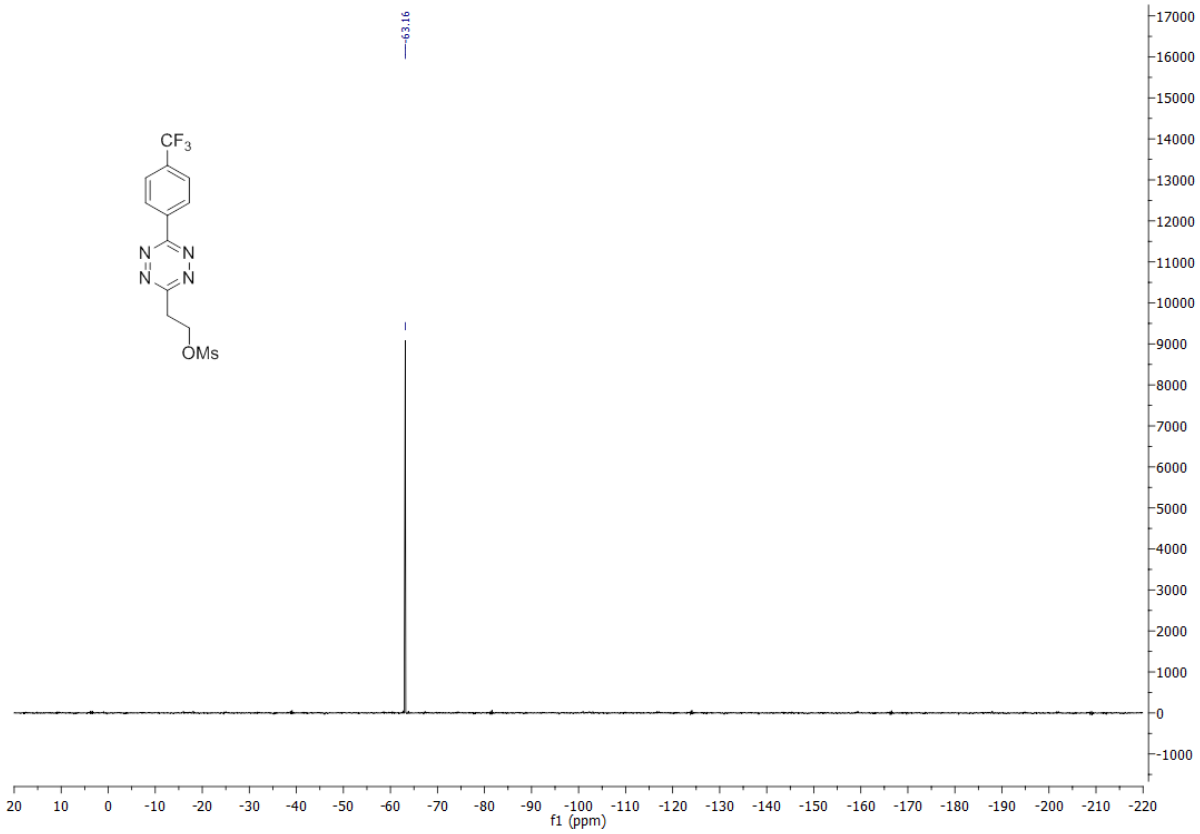



-63.15
CF3
N
N
N
N
OH
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220
f1 (ppm)

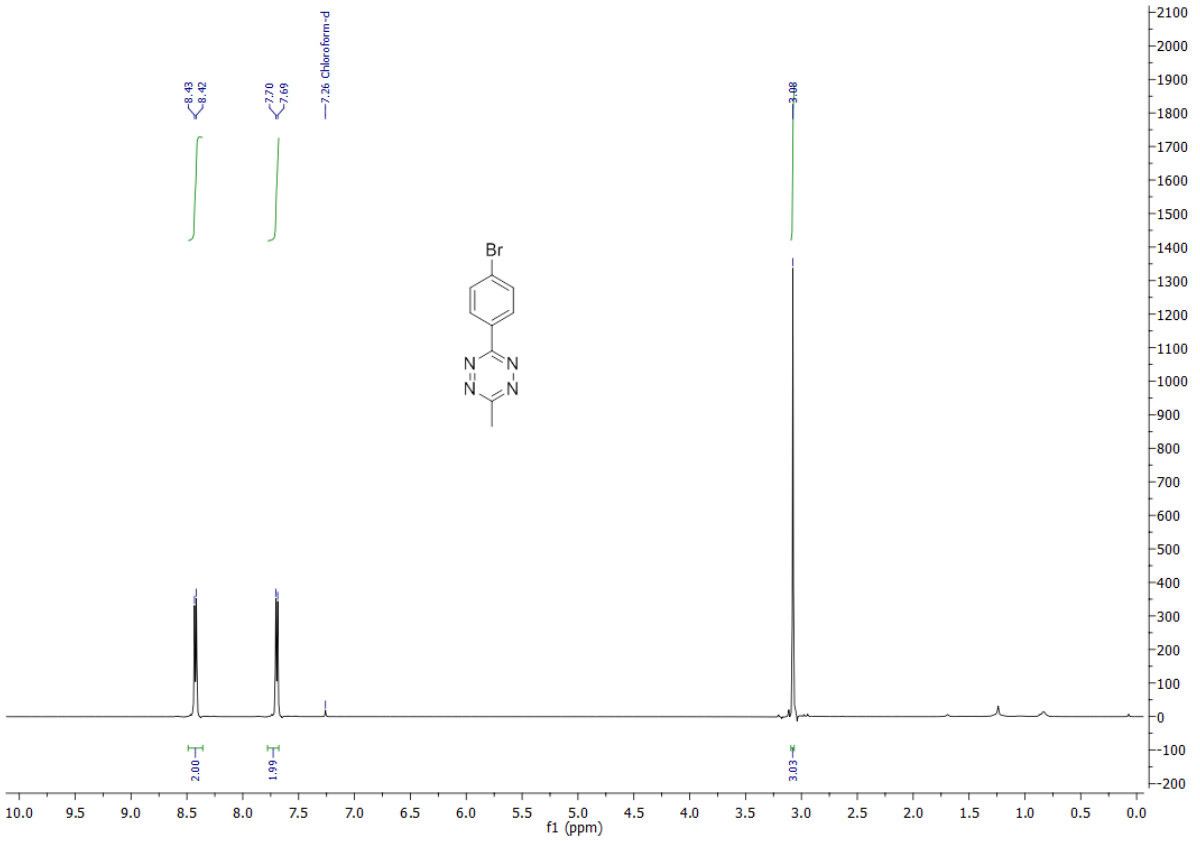
NMR spectra of compound 9

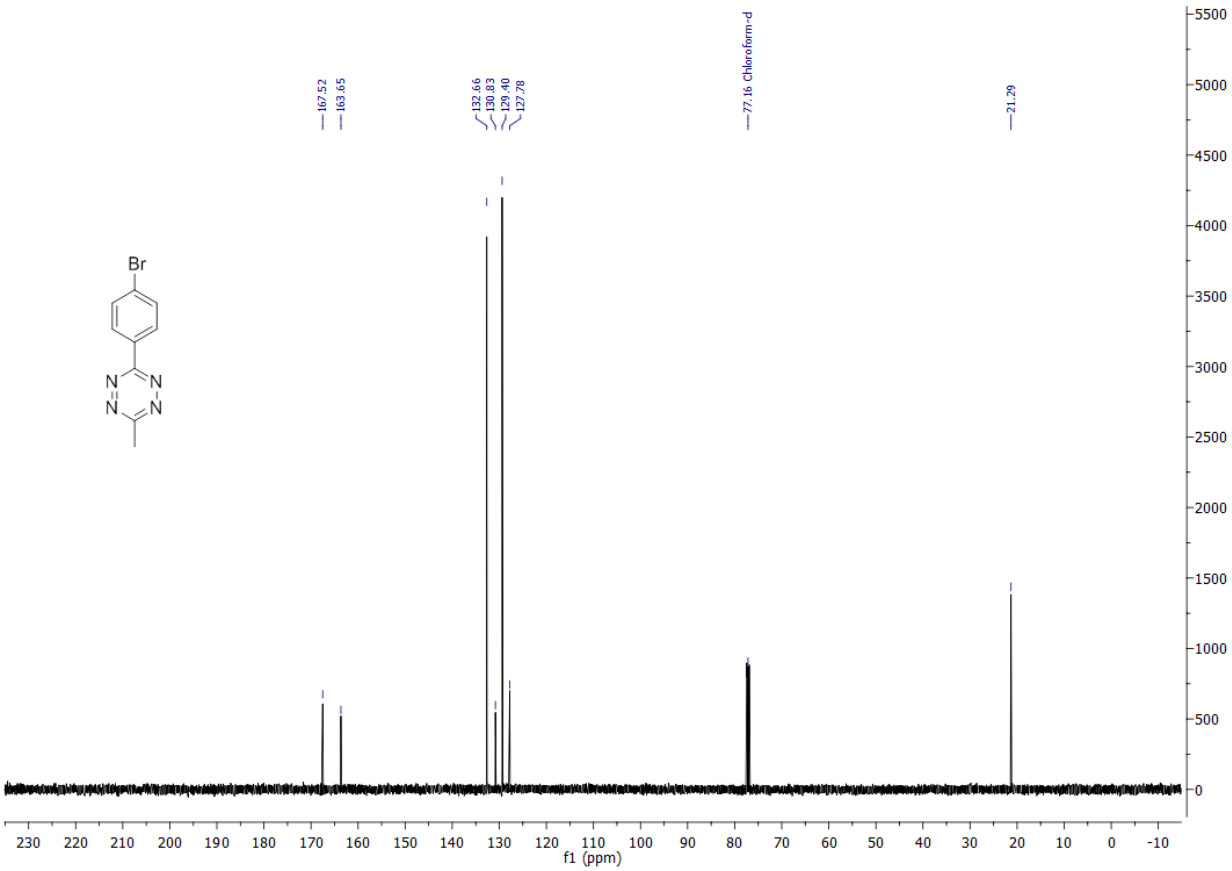




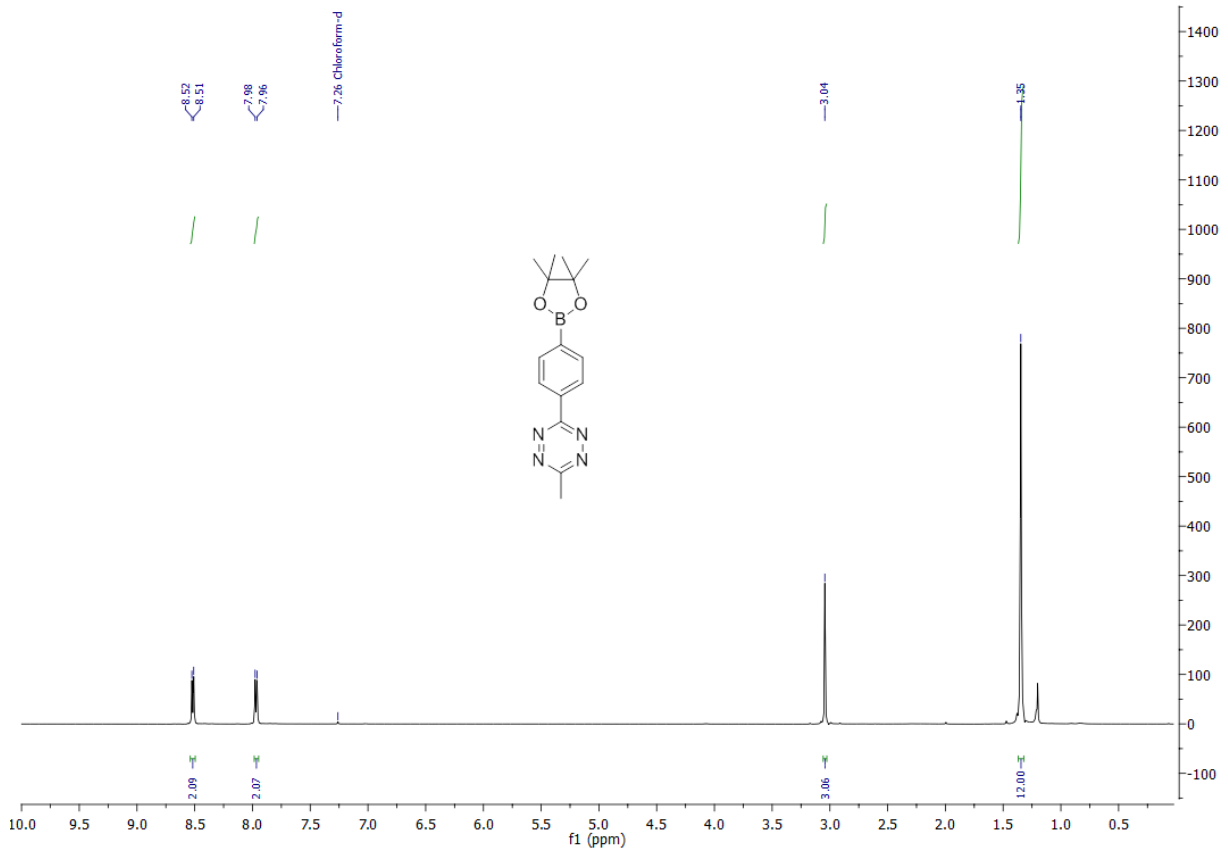

-63.16
CF3
N
N
N
N
OMs
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220
f1 (ppm)

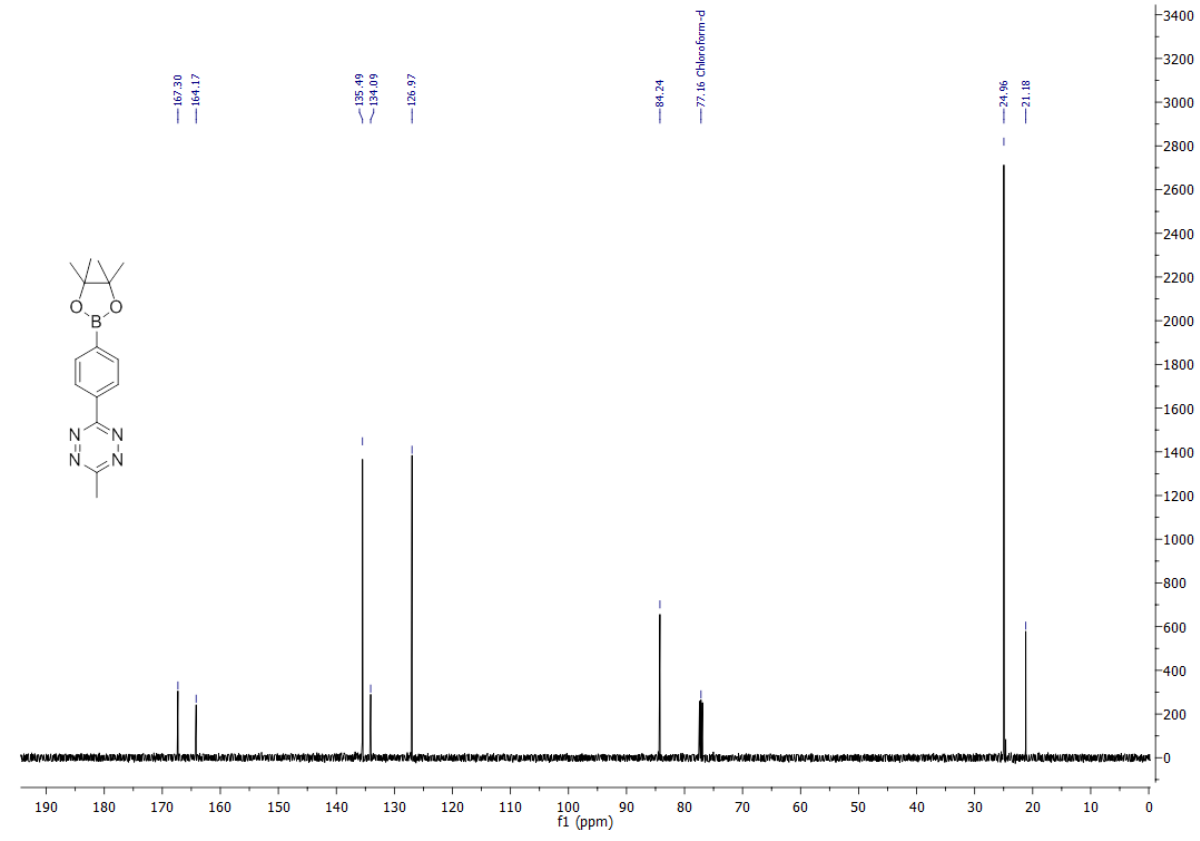
NMR spectra of compound 21



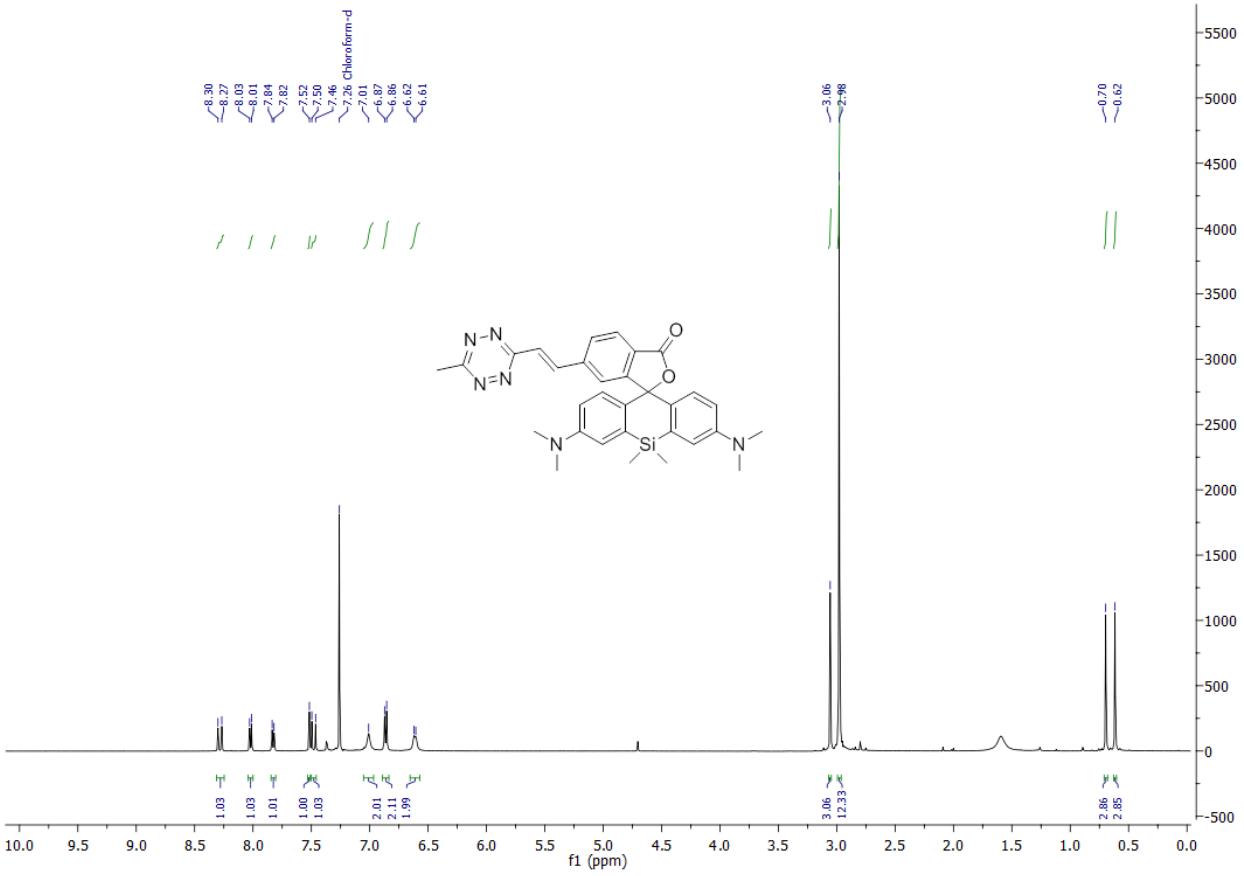


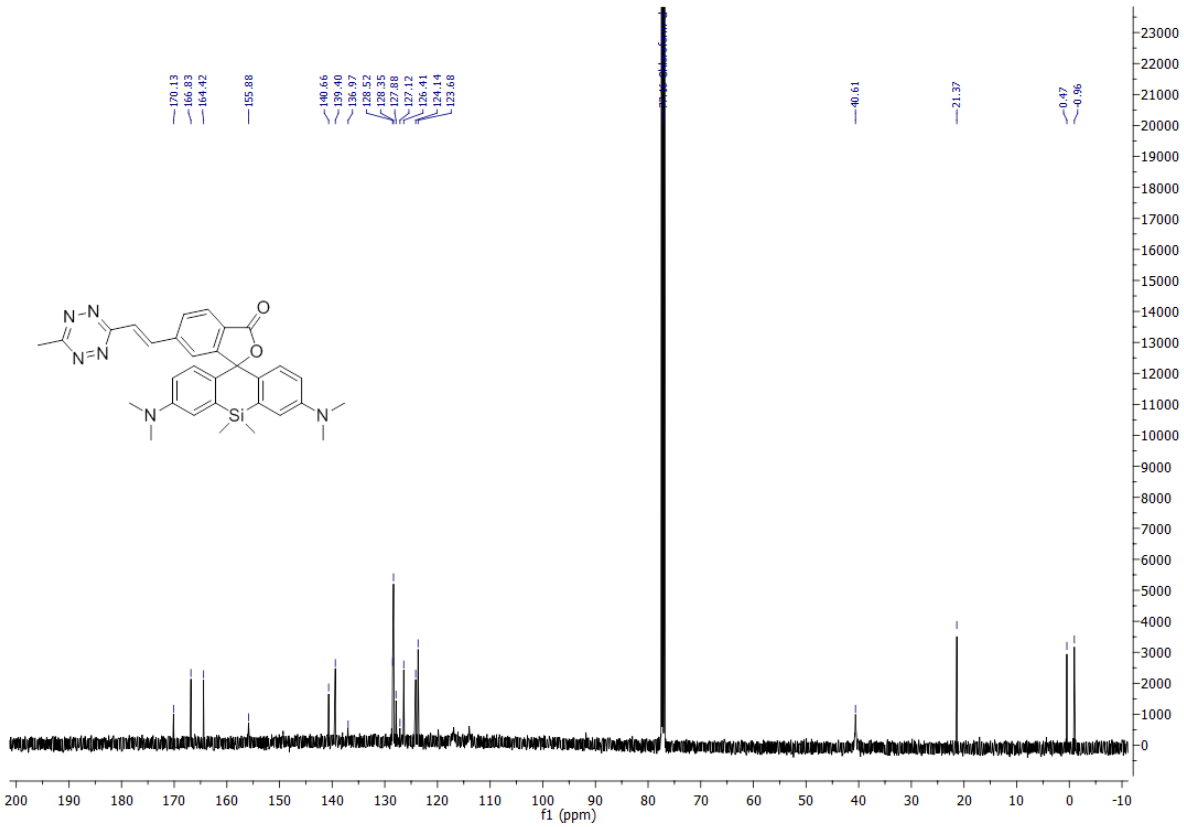
NMR spectra of compound 10



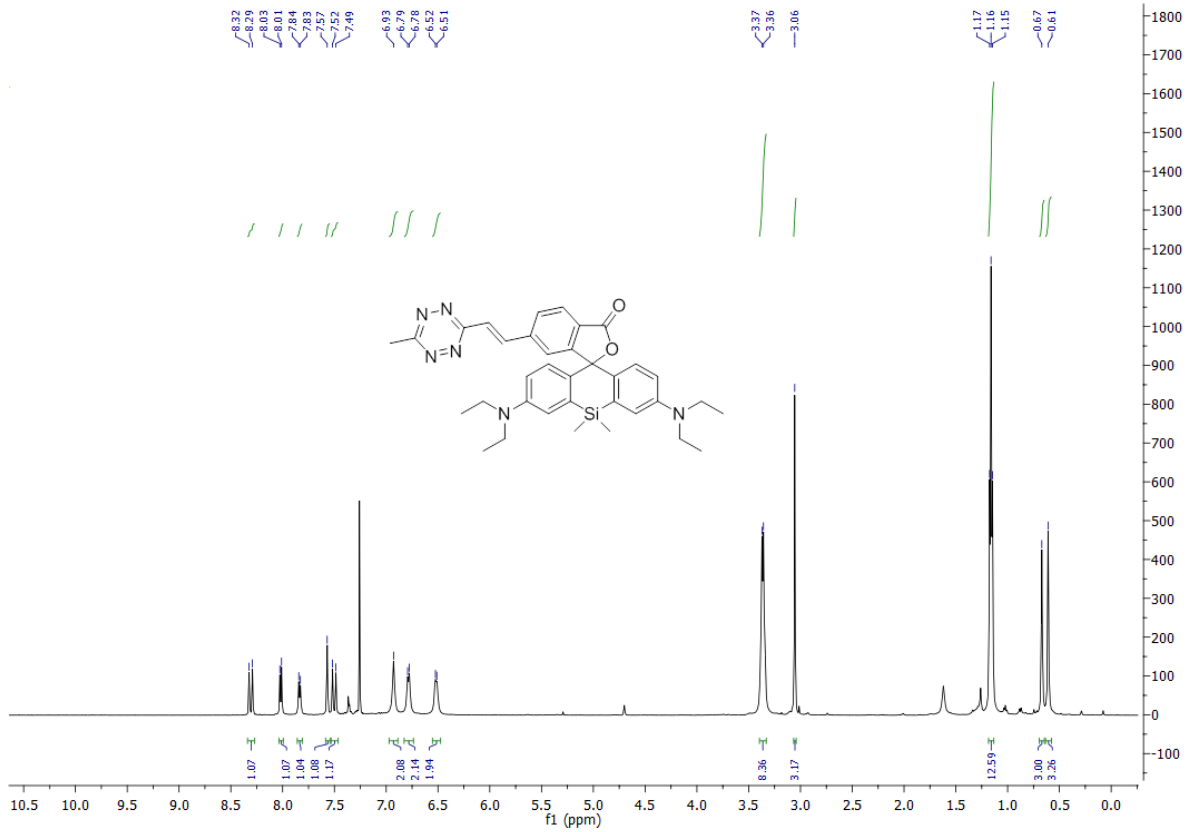


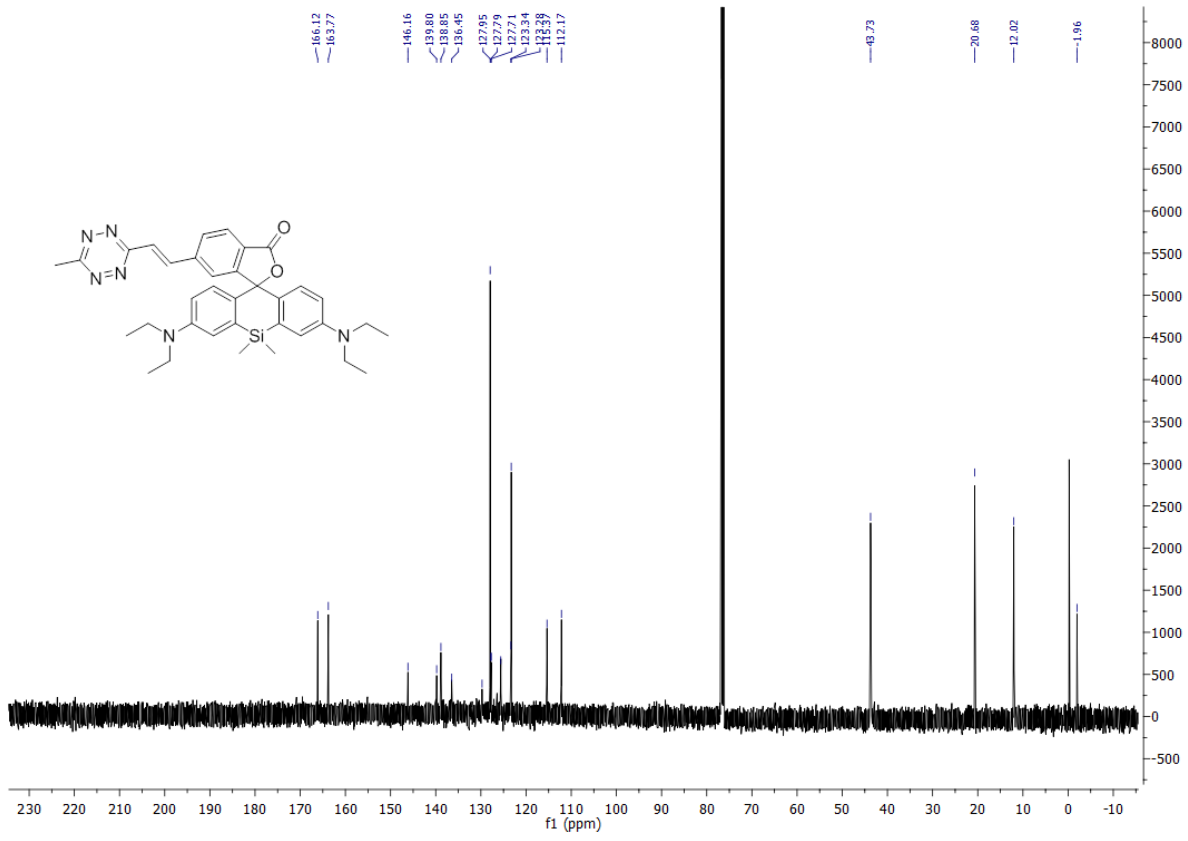
NMR spectra of compound 11



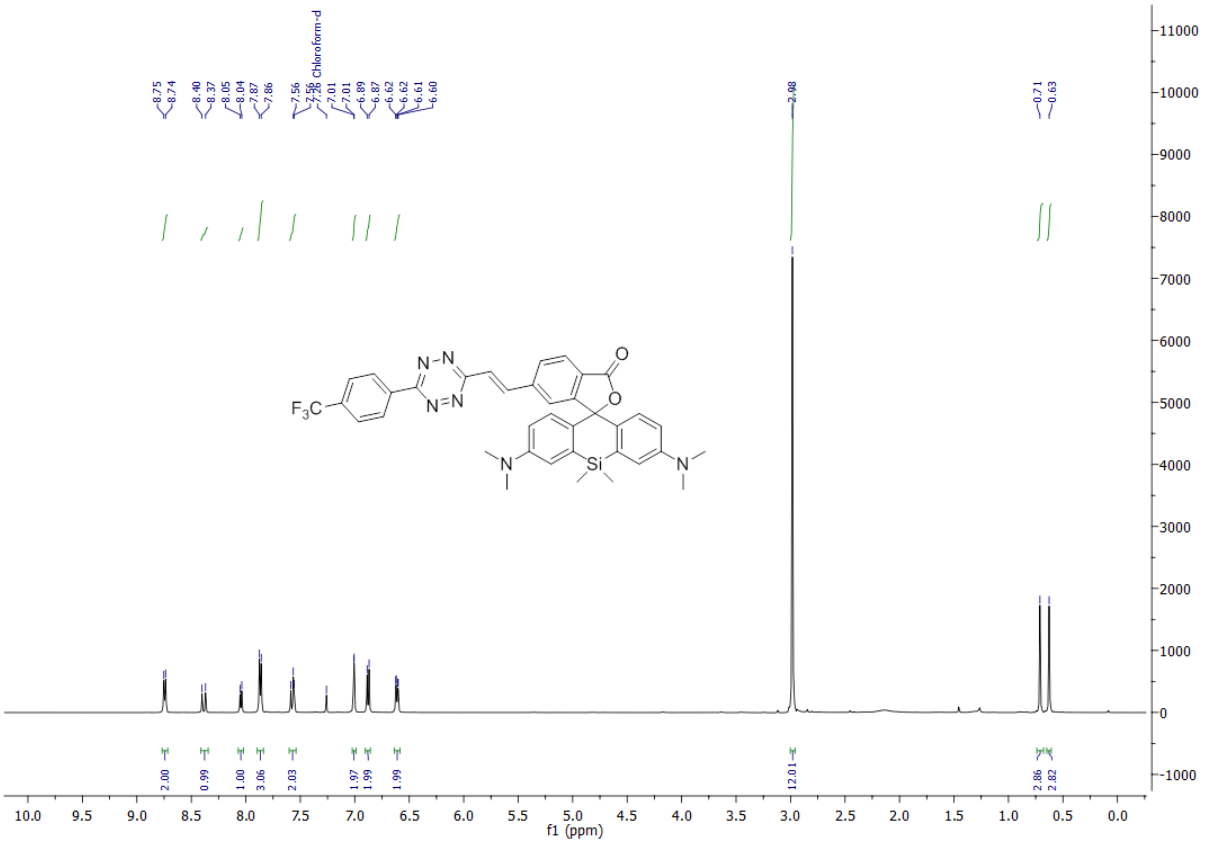


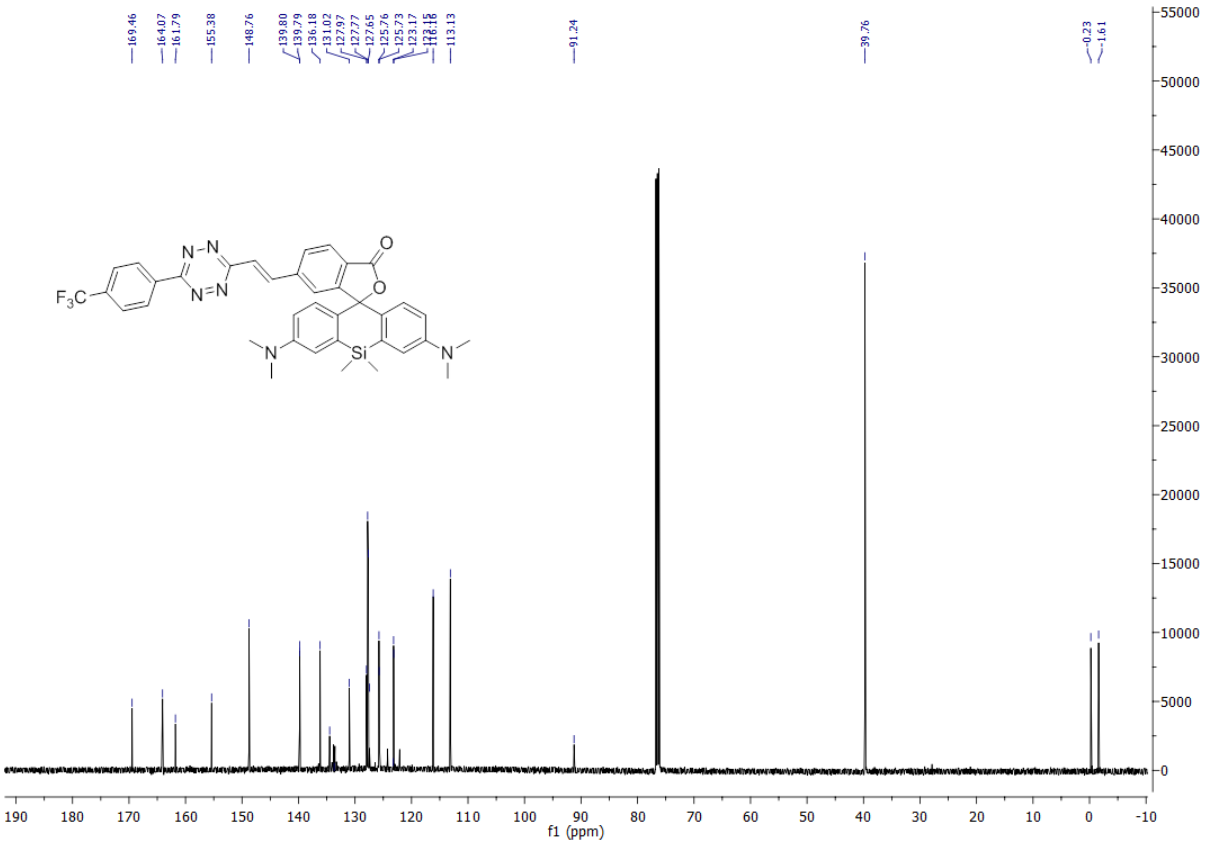
NMR spectra of compound 12

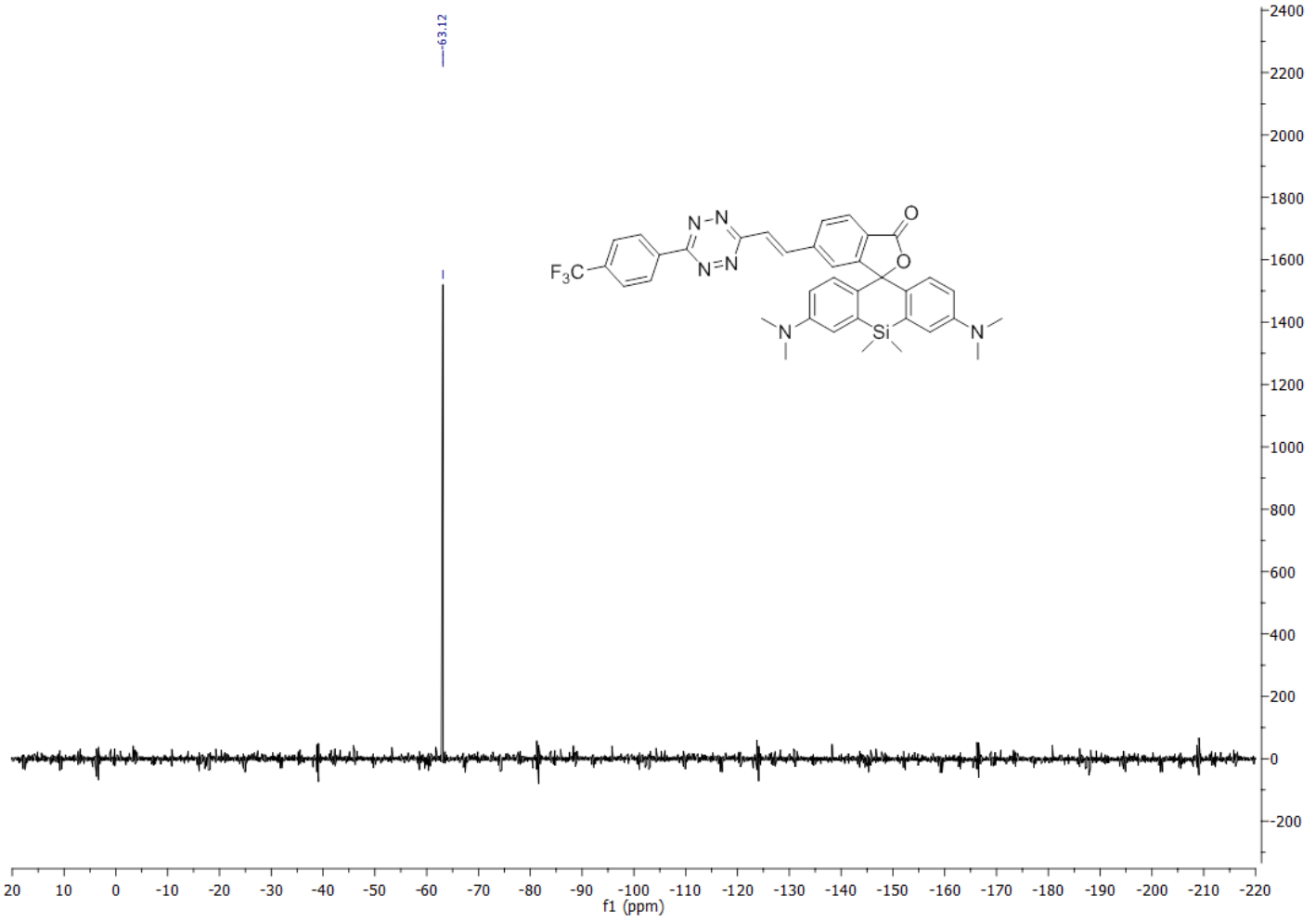




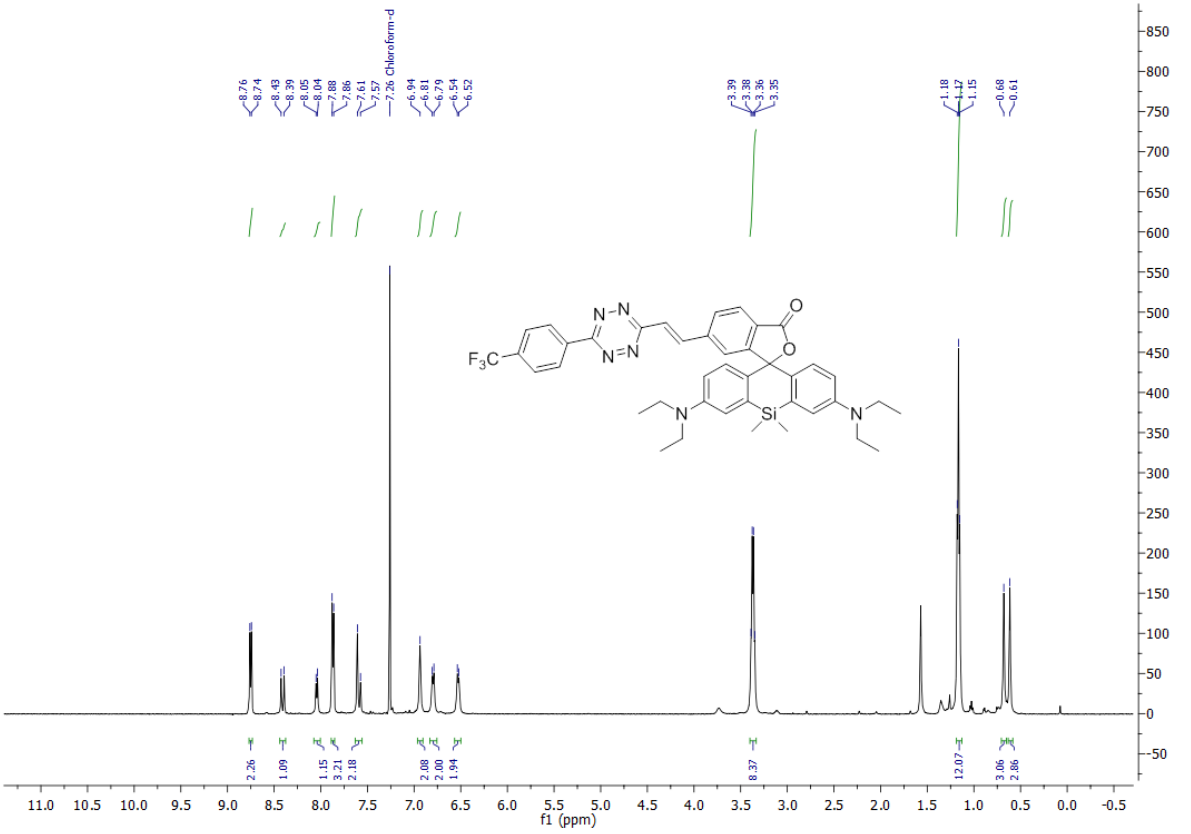
NMR spectra of compound 13

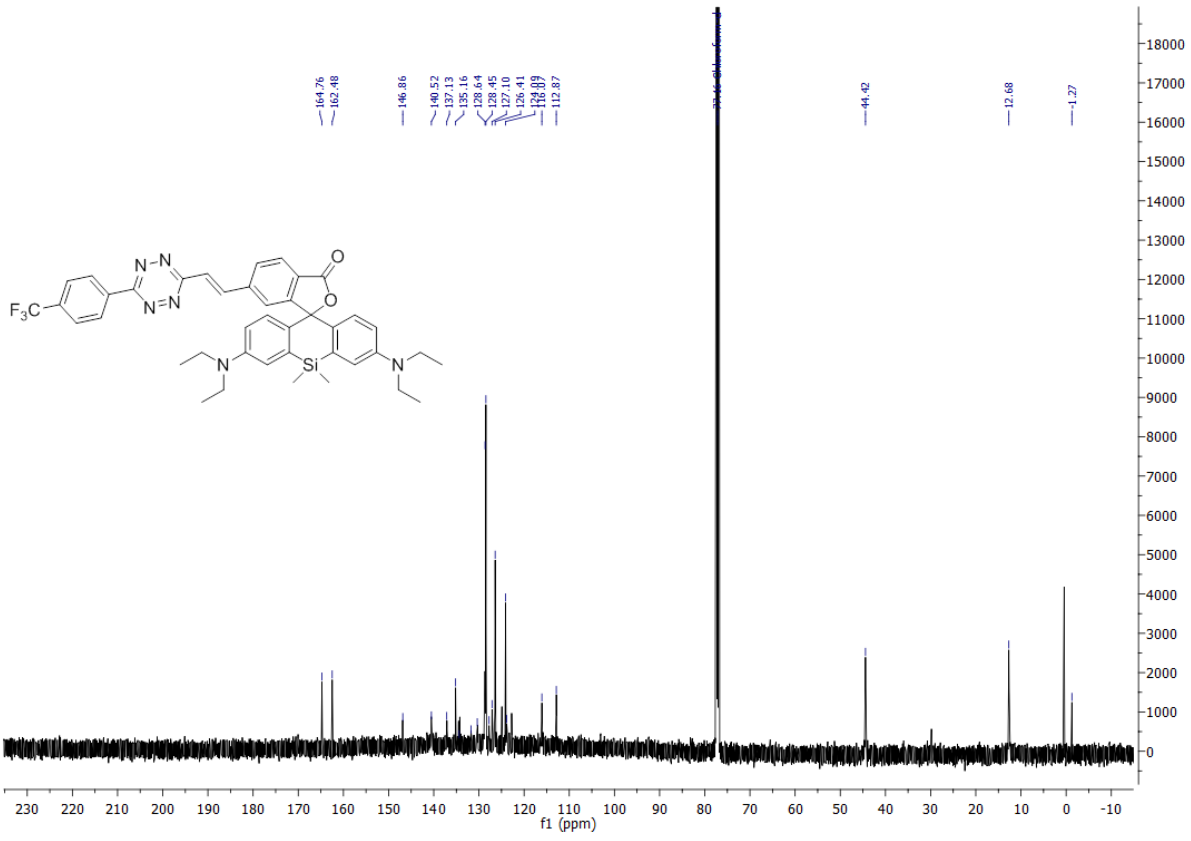


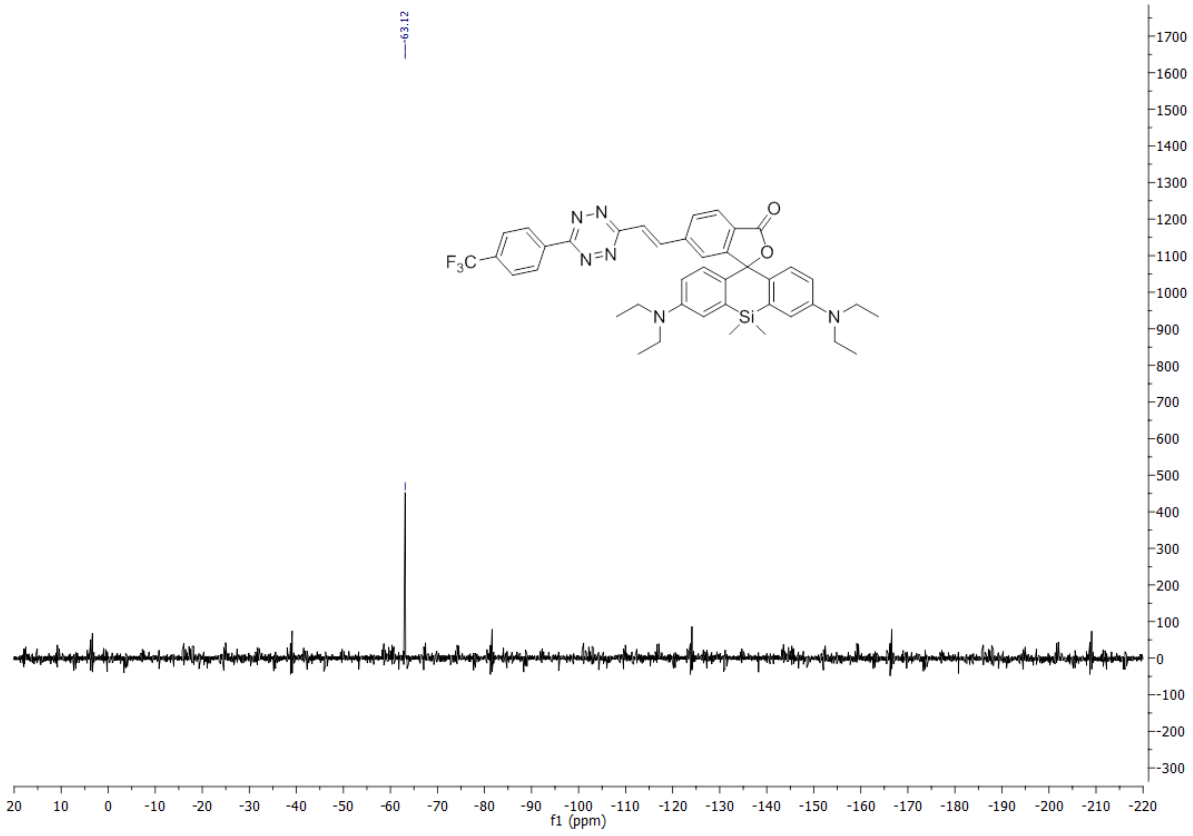



63.12
F3C
N
N
N
N
O
O
Si
N
N
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220
f1 (ppm)

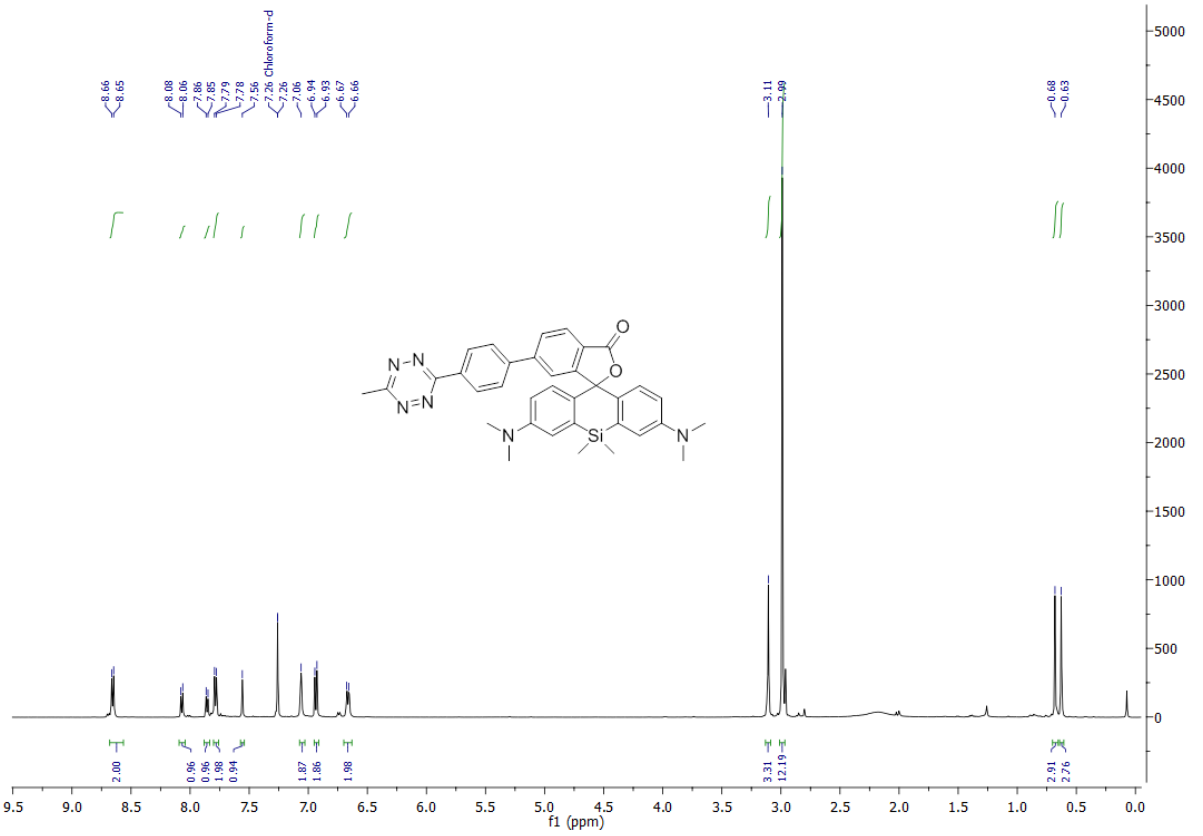
NMR spectra of compound 14

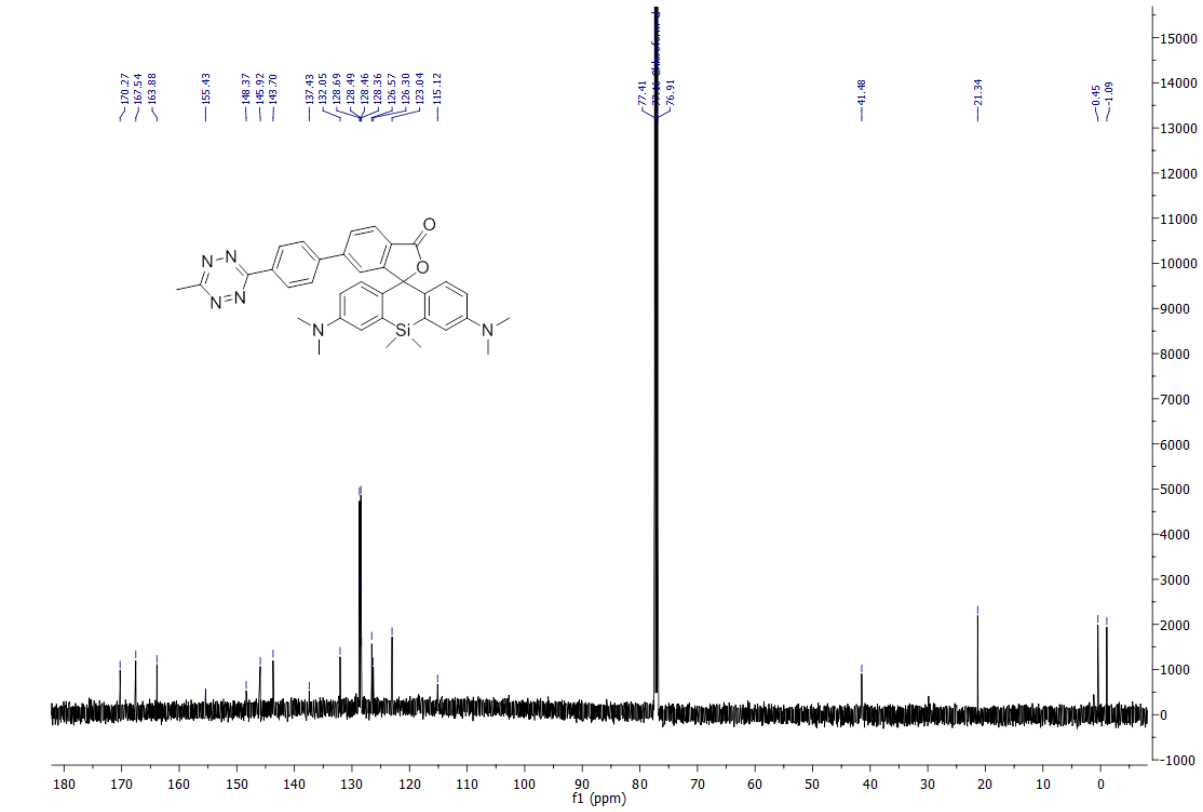




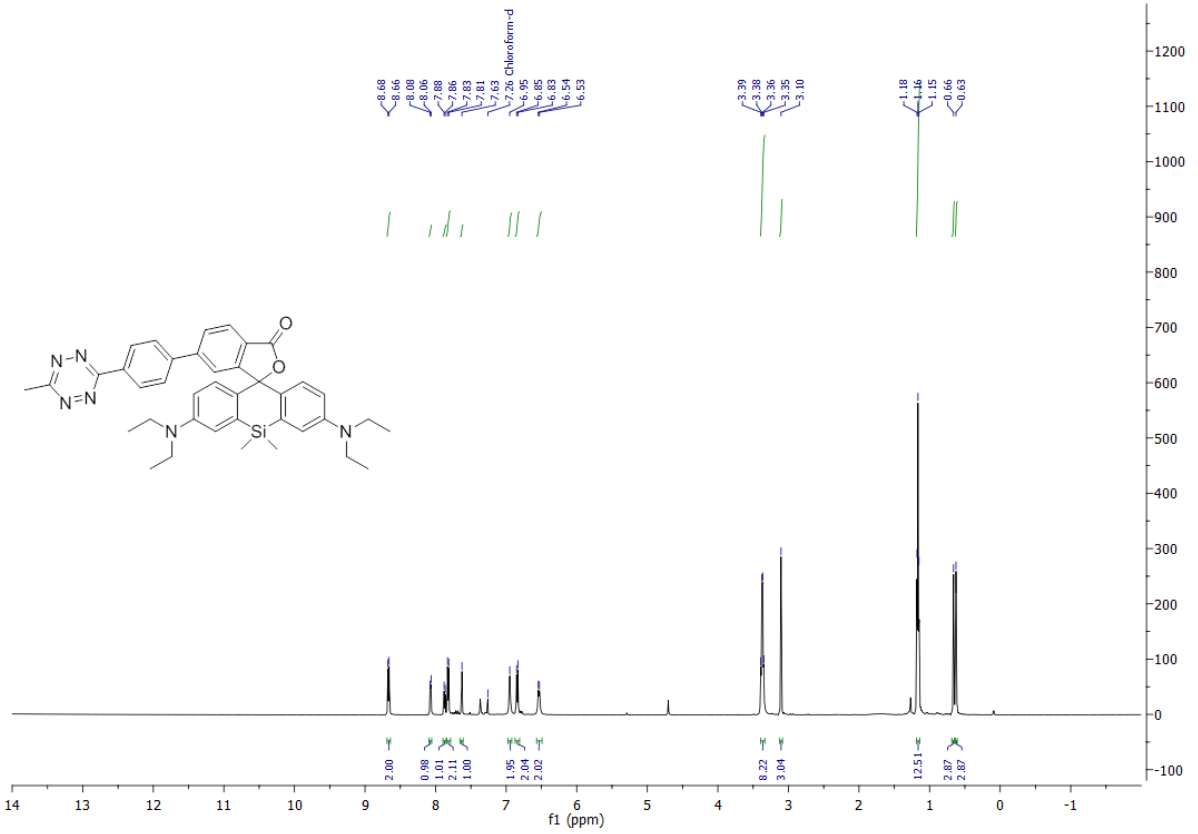

-63.12
F3C
N
N
N
N
O
O
Si
N
N
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
-200
-300
20 10 0 -10 -20 -30 -40 -50 -60 -70 -80 -90 -100 -110 -120 -130 -140 -150 -160 -170 -180 -190 -200 -210 -220
f1 (ppm)

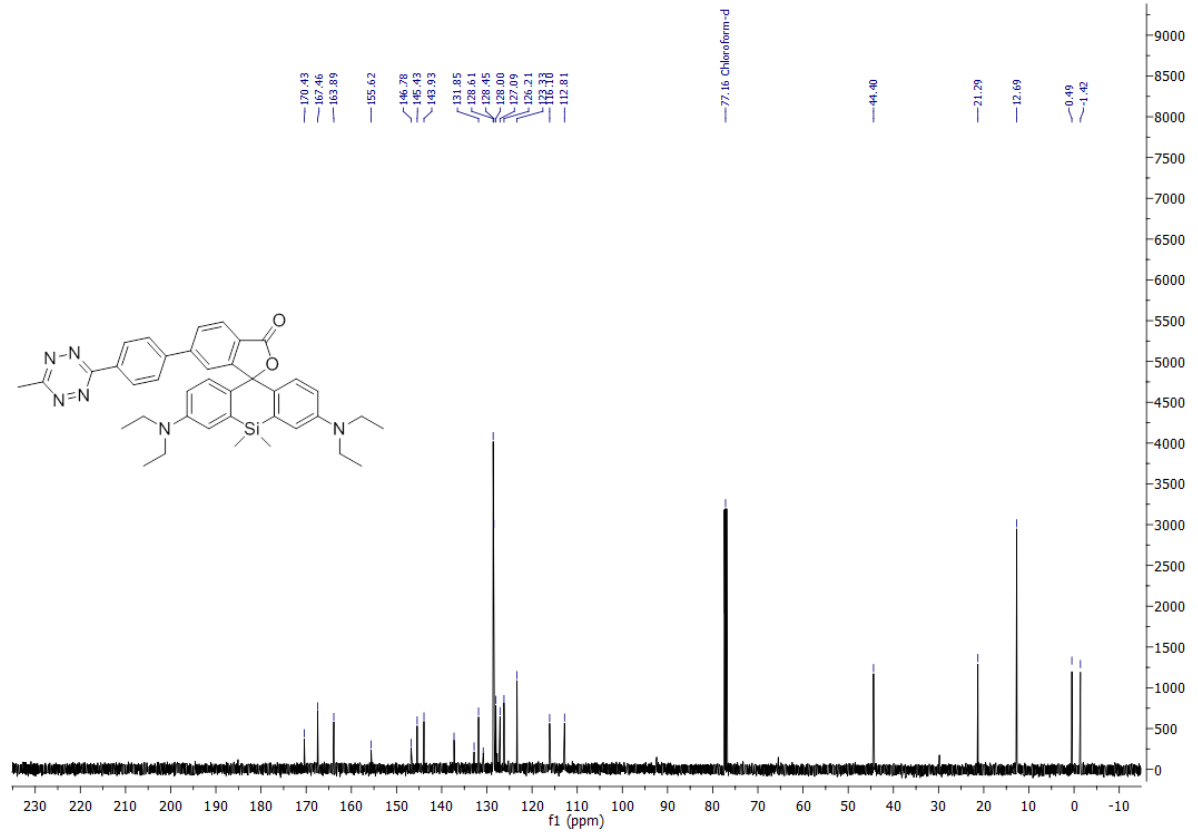
NMR spectra of compound 15





NMR spectra of compound 16





4. Supplementary References

[1] S. Hilderbrand, B. R. Varga, R. Weissleder, *Cyclooctenes for Bioorthogonal Reactions*, WO2016025480 (A1), 2016.

[2] H. Wu, J. Yang, J. Šečkutė, N. K. Devaraj, *Angew. Chem. Int. Ed.*, 2014, **53**, 5805.

[3] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy 3rd ed*, Springer, New York, 2006.

[4] I. Nikić, G. E. Girona, J. H. Kang, G. Paci, S. Mikhaleva, C. Koehler, N. V. Shymanska, C. V. Santos, D. Spitz and E. Lemke, *Angew. Chem. Int. Ed.*, 2016, **55**, 16172.

[5] G. T. Dempsey, J. C. Vaughan, K. H. Chen, M. Bates and X. Zhuang, *Nat. Methods*, 2011, **8**, 1027.

[6] P. Dedecker, S. Duwé, R. K. Neely and J. Zhang, *J. Biomed. Opt.*, 2012, **17**, 126008.

[7] N. Banterle, K. H. Bui, E. A. Lemke and M. Beck, *J. Struct. Biol.*, 2013, **183**, 363.

[8] B. Wang, X. Chai, W. Zhu, T. Wang and Q. Wu, *Chem. Commun.*, 2014, **50**, 14374.

[9] G. Knorr, E. Kozma, A. Herner, E. A. Lemke and P. Kele, *Chem. Eur. J.,* 2016, **22**, 8972.

[10] S. Bolte, F. P. Cordelieres, *J. Microsc*., 2006, **224.3**, 213.

Full references from main text:

[9f] J. C. T. Carlson, L. G. Meimetis, S. A. Hildebrand and R. Weissleder, *Angew. Chem. Int. Ed.*, 2013, **52**, 6917.

[10a] G. Lukinavičius, K. Umezawa, N. Olivier, A. Honigmann, G. Yang, T. Plass, V. Mueller, L. Reymond, I. R. Corrêa, Z.-G. Luo, C. Schultz, E. A. Lemke, P. Heppenstall, C. Eggeling, S. Manley and K. Johnsson, *Nat. Chem.*, 2013, **5**, 132.

[10b] S. Uno, M. Kamiya, T. Yoshihara, K. Sugawara, K. Okabe, M. C. Tarhan, H. Fujita, T. Funatsu, Y. Okada, S. Tobita and Y. Urano, *Nat. Chem*., 2014, **6**, 681.

[13] C. Uttamapinant, J. D. Howe, K. Lang, V. Beránek, L. Davis, M. Mahesh, N. P. Barry and J. W. Chin, *J. Am. Chem. Soc.*, 2015, **137**, 4602.

[14a] T. G. Kim, J. C. Castro, A. Loudet, J. G. Jiao, R. M. Hochstrasser, K. Burgess and M. R. Topp, *J. Phys. Chem. A*, 2006, **110**, 20.

[18a] T. Plass, S. Milles, C. Koehler, J. Szymanski, R. Mueller, M. Wiessler, C. Schultz and E. A. Lemke, *Angew. Chem. Int. Ed.*, 2012, **51**, 4166.

[18e] I. Nikić, G. Estrada Girona, J. H. Kang, G. Paci, S. Mikhaleva, C. Koehler, N. V. Shymanska, C. Ventura Santos, D. Spitz and E. A. Lemke, *Angew. Chem. Int. Ed.*, 2016, **55**, 16172.

[19] R. S. Erdmann, H. Takakura, A. D. Thompson, F. Rivera-Molina, E. S. Allgeyer, J. Bewersdorf, D. K. Toomre and A. Schepartz, *Angew. Chem. Int. Ed.*, 2014, **53**, 10242.

[20] N. Banterle, K. H. Bui, E. A. Lemke and M. Beck, *J. Struct. Biol.*, 2013, **183**, 363.