Electronic Supplementary Information (ESI):

# Green- to Far-Red-Emitting Fluorogenic Tetrazine Probes – Synthetic Access and No-Wash Protein Imaging inside Living Cells.**

*Achim Wieczorek†, Philipp Werther†, Jonas Euchner† and Richard Wombacher**

# Content

# 1 General Information

Chemicals were purchased from Sigma-Aldrich Co. LLC, abcr GmbH & Co. KG, acros Organics, Alfa Aesar, TCI Europe N.V. and VWR International GmbH and used as received unless otherwise noted. TCO-PEG4-NHS ester and TCO-PEG3-maleimide was supplied by Jena Bioscience GmbH and Phalloidin amine by AAT Bioquest. NMR solvents were purchased from euriso-top SAS. Polygram Sil G/UV254 TLC plates from Macherey-Nagel GmbH & Co KG and Sigma Aldrich were used for thin layer chromatography. Normal phase column chromatography was performed using silica gel from Fluka with a pore size of 60 Å and a particle size range of 40-63 µm. Solvents were p.a. quality.

HPLC analytics and semi-preparative purifications were done on an Agilent 1100 series HPLC system. Phenomenex Luna 3µ and 5µ C18 reversed-phase columns were used for these purposes (Solvent A: $H_2O$ containing 0.1% trifluoroacetic acid (TFA); Solvent B: MeCN containing 0.1% TFA). Collected HPLC fractions were dried by lyophilization. Mass spectrometry was performed on a Bruker microTOF-QII mass spectrometer. NMR spectra were recorded on a Varian Mercury Plus 300 MHz spectrometer or a Varian 500 MHz NMR System. Peak shifts were reported relative to solvent peaks according to Fulmer *et al*.[1] Fluorescence measurements were performed on a JASCO Spectrofluorometer FP-6500. Absorption spectra were recorded on a Cary 100 Bio UV-Visible or a JASCO V660 UV-Vis Spectrophotometer.

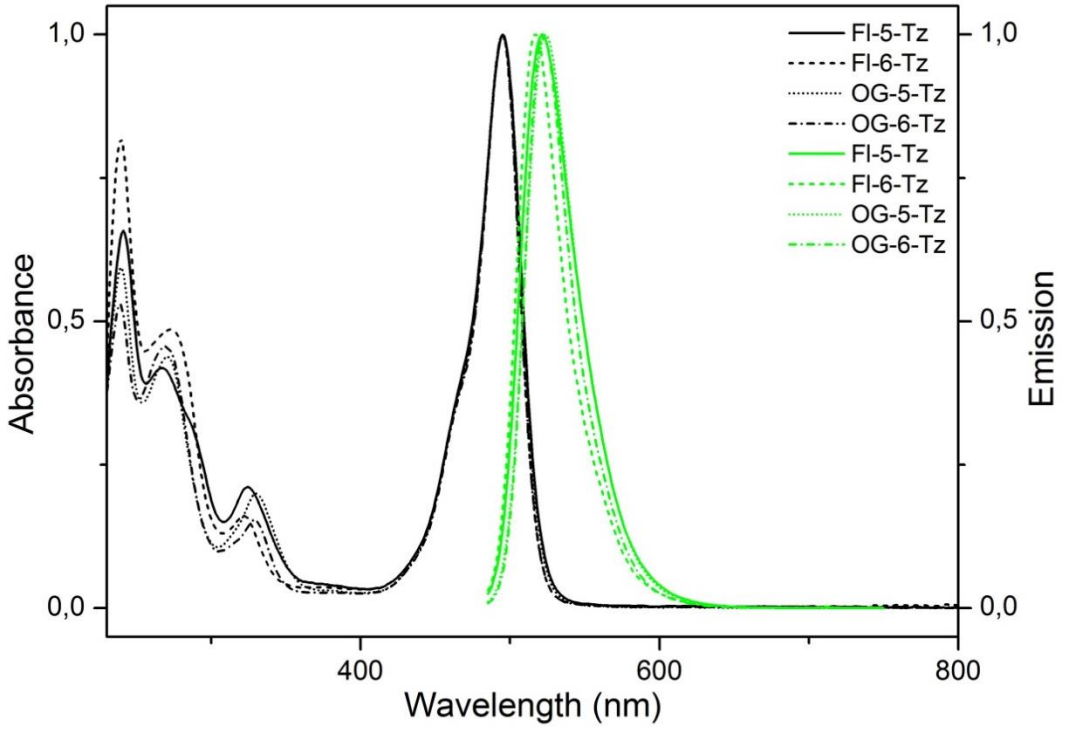
# 2 Photophysical Measurements

Samples for absorption and emission measurements were prepared from 10 mM stock solutions of the dyes in DMSO. The dyes were diluted to various concentrations in aqueous PBS.

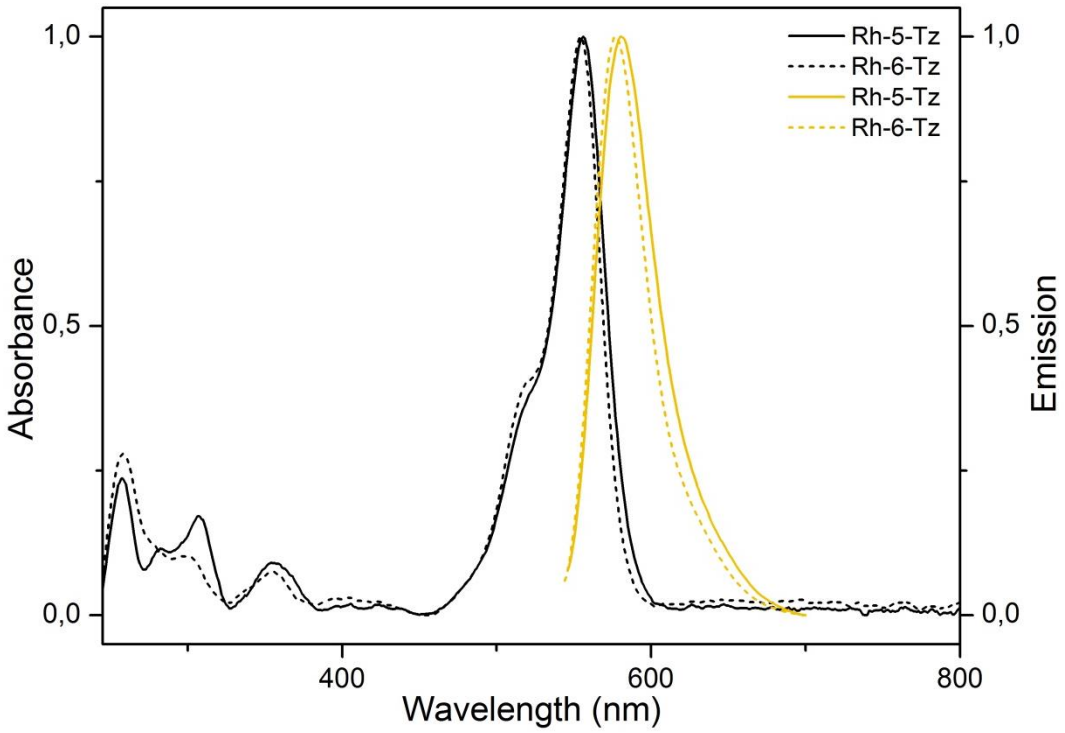
Relative fluorescence quantum yields were determined as described by Demas and Crosby.[2] Fluorescein in borate buffer (pH = 9.1) was used as standard ($\Phi_f$ = 0.92,[3] $\lambda_{ex}$ = nm) for fluorescein and Oregon Green dyes, sulforhodamine 101 in EtOH ($\Phi_f$ = 0.95,[3] $\lambda_{ex}$ = 535 nm) for rhodamine dyes and cresyl violet in EtOH ($\Phi_f$ = 0.56,[4] $\lambda_{ex}$ = 590 nm) as standard for Si-rhodamines. Kinetic measurements involving fluorescence readout were performed on Tecan Safire 2 multimode microplate reader. Therefor black 96-well plates were filled with 500 nM solutions of the dyes and the fluorescence intensity start value was determined. A stock solution of *trans*-cyclooct-4-en-1-ol (TCO) was added to each well (final concentration: 500 nM) using a multi-pipette, the measurement was started and the fluorescence intensity read out every ten seconds. Temperature was kept constant between 31 °C and 32 °C.

## 2.1 Normalized absorption and emission spectra

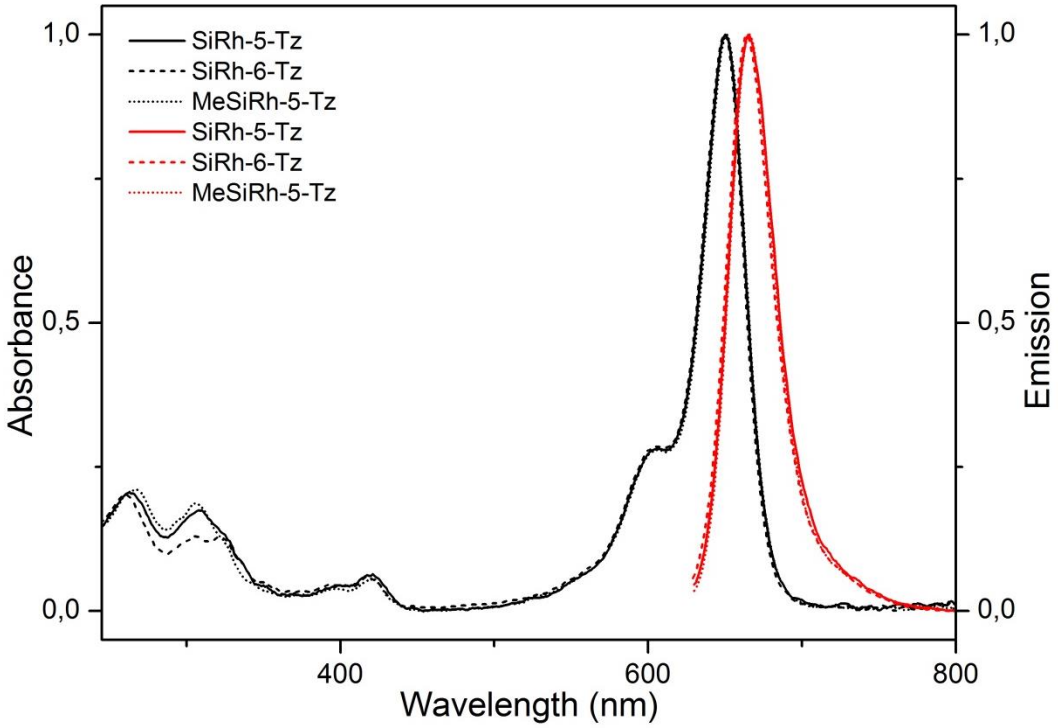
### 2.1.1 OG- and Fl-Tz dyes




### 2.1.2 Rh-Tz dyes



### 2.1.3 SiRh-Tz dyes




## 2.2 Stability of OG-5-Tz

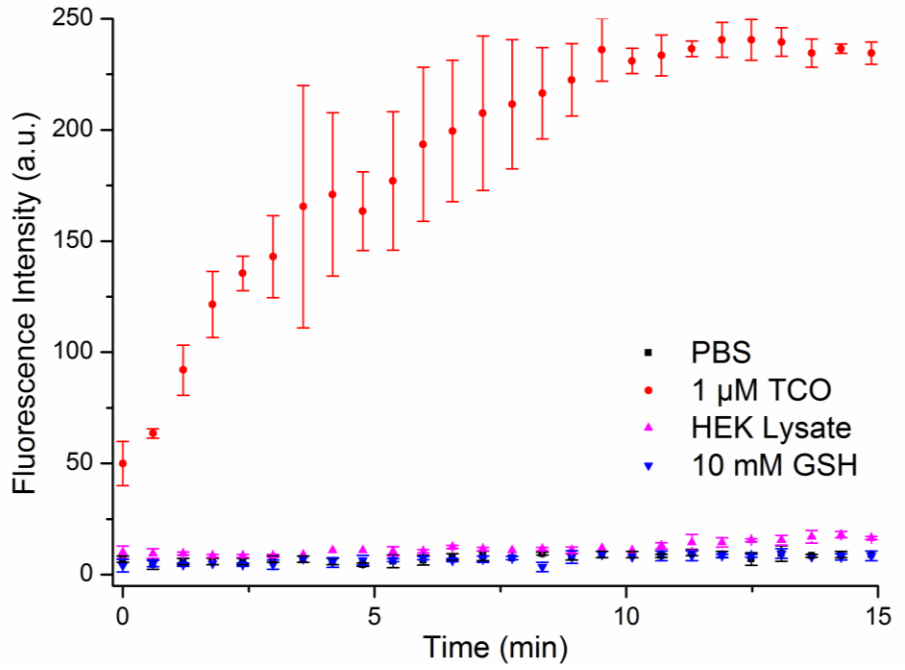



**Fig. S1:** A solution of OG-5-Tz (final concentration: 500 nM) was added to 1 µM TCO, 10 mM GSH (both in PBS) and HEK lysate at 37 °C and the change in fluorescence intensity monitored over time in the Tecan plate reader (for details see above) while keeping the temperature constant.

## 3 In vitro protein labeling

The model protein eDHFR-E17C-C85A-C152S (containing a Biotin acceptor peptide sequence which was not vital for the experiment described here) was expressed in E. coli (BL21-DE3 strain) using a pET-27b-derived plasmid (kind gift from Aaron Hoskins) through induction with isopropyl β-D-thiogalactoside and purified according to literature.[5] An aliquot of the purified protein was subjected to buffer exchange from its storage buffer (50 mM Na-phosphate, pH 7, 1 mM DTT, 50% glycerol) to PBS by gel filtration on Sephadex G-25 spin columns. A 3.6 µM solution of the protein in PBS was added a 20-fold molar excess of TCO-PEG3-maleimide (72 µM) and incubated for 2.5 h at rt. Excess TCO-PEG3-Maleimide was removed by gel filtration on Sephadex G-25 spin colums. This step was performed twice. The TCO-labeled protein was then reacted with **SiRh-5-Tz** and **MeSiRh-5-Tz** at 0.6 µM each in PBS. The fluorescence increase (Table 1, main text) was determined 10 min after addition of the dyes in the spectrofluorometer, when a plateau was reached. In order to verify the covalent attachment of the dyes, the solutions from the fluorescence study were analyzed by SDS-PAGE (15%) with subsequent fluorescence readout on a Typhoon FLA9500 laser scanner (Fig. S1).

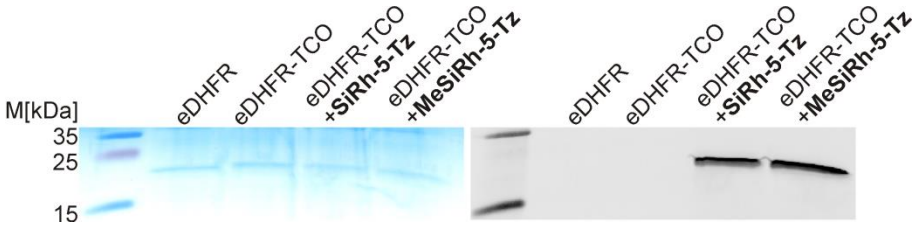



**Figure S2:** SDS-PAGE (15%) analysis of eDHFR model protein labeling with TCO-PEG3-maleimide and the Si-rhodamine tetrazines **SiRh-5-Tz** and **MeSiRh-5-Tz**. Left: coomassie stained; right: fluorescence scan (Cy5-scan setting).

# 4 Cell Culture & Microscopy

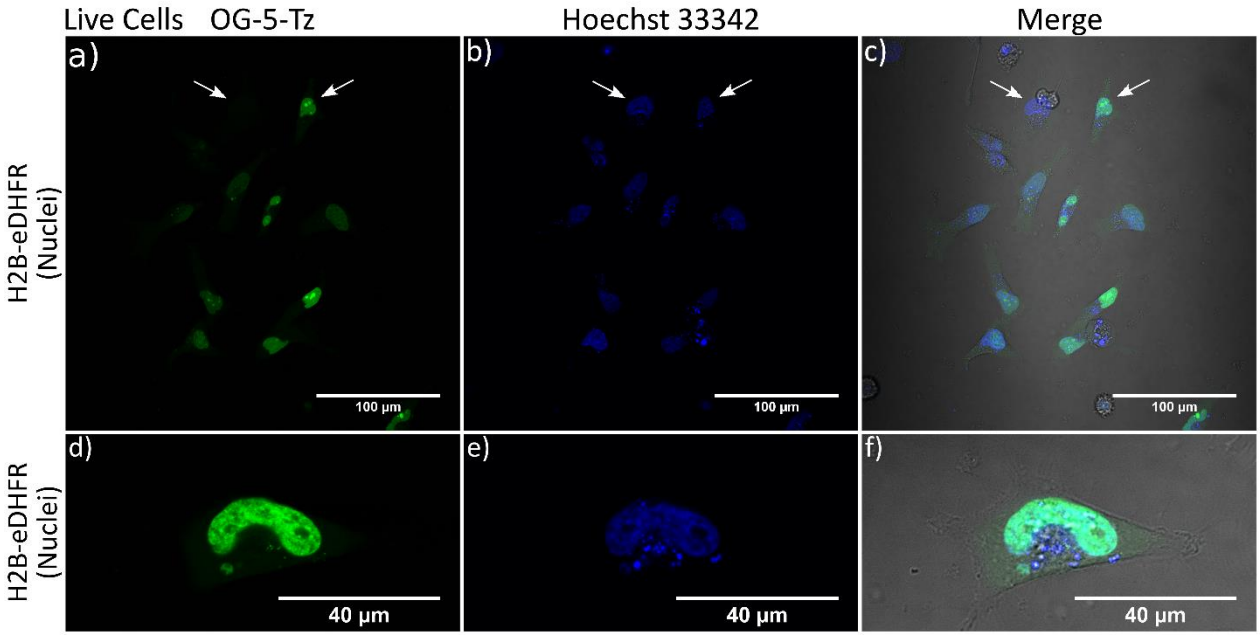



**Figure S3:** Live cell no-wash confocal imaging of nuclei. HeLa cells expressing H2B-eDHFR (a-f) were incubated with **TMP-TCO*** (10 µM) and washed. **OG-5-Tz(piv)$_2$** (10 µM) was added and cells were imaged after 30 min without removal of excess dye (a, d). Hoechst 33342 was used as nuclear colocalization control (b, e). The merged channels (c, f) show colocalization of **OG-5-Tz** and the Hoechst colocalization control dye. Note that only transfected cells show nuclear staining in the green channel (**OG-5-Tz)** (white arrows, a-c).

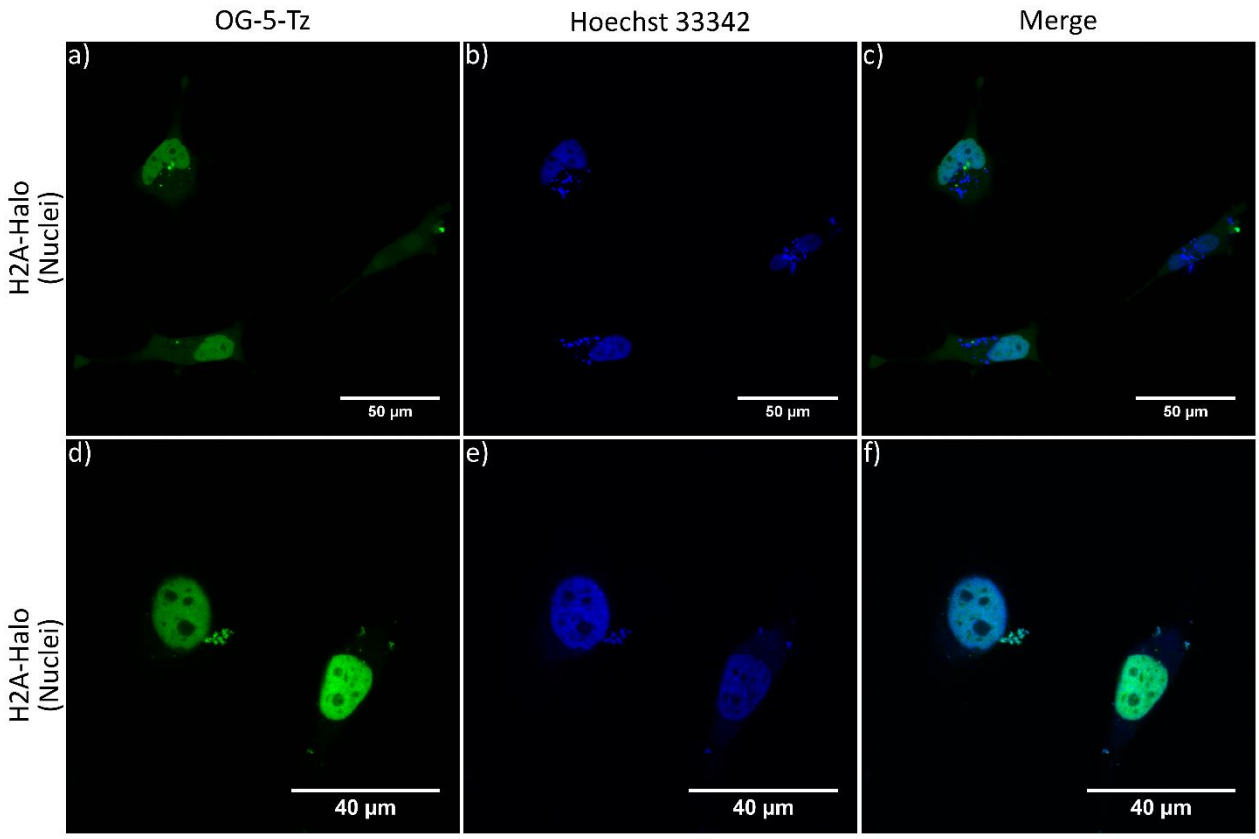



**Figure S4**: No-wash confocal imaging of nuclei inside living (a-c) and fixed cells (d-f) using covalent Halo domain tag. Live HeLa cells expressing H2A-Halo (a-c) were incubated with **Halo-TCO** (10 µM), washed and then a 10 µM solution of **OG-5-Tz(piv)$_2$** was added (a). Hoechst 33342 was used as nuclear stain (b). The merged channels (c) show colocalization of **OG-5-Tz** and the Hoechst 33342 colocalization control. Images were taken 30 min *post* addition of the dye without previous dye washout. HeLa cells expressing H2A-Halo (d-f) were fixed and permeabilized. The cells were then incubated with **Halo-TCO** (10 µM), washed and a 10 µM solution of **OG-5-Tz** was added (d). Hoechst 33342 was used as nuclear stain (e). The merged channel (f) shows colocalization of **OG-5-Tz** and the Hoechst 33342 colocalization control. Images were taken 30 min *post* addition of the dyes without previous dye washout.

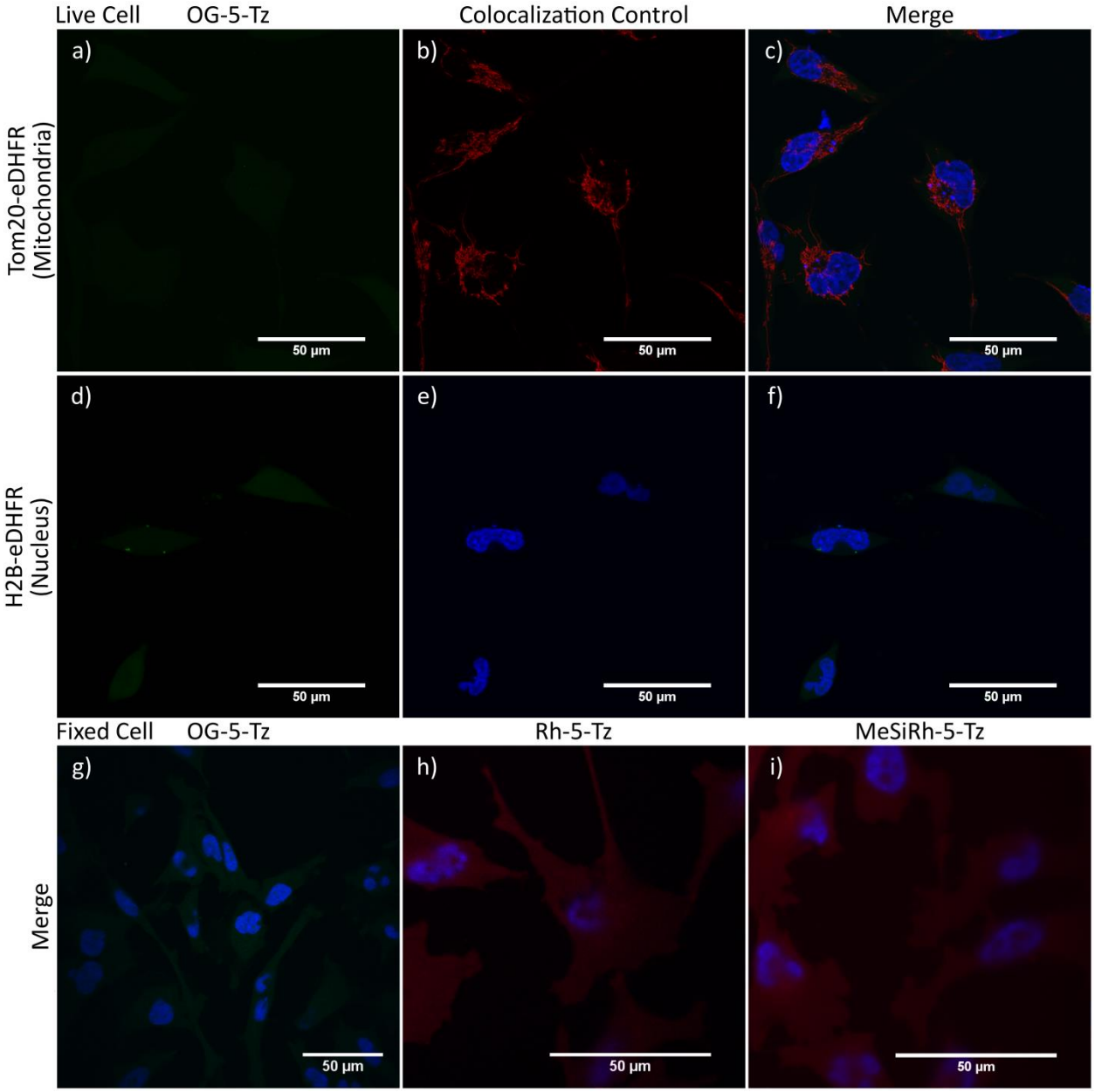



**Figure S5**: Negative control images were taken with the same microscope settings and dye concentrations using the respective protocol in the absence of **TMP-TCO*** and **phalloidin–TCO** respectively. No-wash confocal imaging for mitochondria (a-c) and nuclei (d-f) staining inside living cells and actin cytoskeleton staining in fixed cells (g-i). Treatment with 10 µM **OG-5-Tz(piv)$_2$** (a, d). MitoTracker Red CMXRos was used as mitochondrial colocalization control (b), Hoechst 33342 as nuclear stain (e). The merged channels (c, f) show only minimal signal and no colocalization for **OG-5-Tz** with the colocalization control. Images (a-c) were taken 60 min *post* addition of the dye without previous dye washout using microscope settings as in Fig. 4 b). Images (d-f) were taken 30 min *post* addition of the dye without previous dye washout using microscope settings as in Fig. S2 d). Treatment with 10 µM **OG-5-Tz** (g), **Rh-5-Tz** (h) or **MeSiRh-5-Tz** (i). Hoechst 33342 was used as nuclear stain. Images (g-i) were taken 30 min *post* addition of the dyes without previous dye-washout using microscope settings as in Fig. 3 a-c).

HeLa cells (HeLa ATCC CCL-2) were cultured in DMEM-GlutaMAX-I (6 mL) (containing 10% FCS and 1% penicillin-streptomycin (10.000 U/mL)) in 50 mL CELLSTAR cell culture flasks. The cells were incubated at 37 °C, 5% $CO_2$ and high humidity. Cells were passaged at 80% confluence using standard 0.05% trypsin-EDTA solution (1 mL). 2.5 x $10^5$ cells were used for the next cell culture flask.

For imaging experiments, 3.000 HeLa cells were transferred into each channel of the Ibidi µSlide VI$^{0.4}$. 100 µL of DMEM-GlutaMAX was added 30 min *post* incubation of the cells. The cells were incubated at 37 °C and 5% $CO_2$ for 24 h and washed once with PBS. For transfection, OptiMEM (60 µL) was added to each channel. The transfection mixture was prepared separately for each channel (according to the manufacturer's protocol - FuGENE® HD Transfection Reagent). 200 ng of total plasmid DNA (Tom20-eDHFR, H2B-eDHFR, H2A-Halo) was mixed with OptiMEM (10 µL). FuGENE HD Transfection Reagent (0.6 µL) was added and the transfection mixture was incubated for 15 min at room temperature. The transfection mixture was added to each channel. Then, OptiMEM (60 µL) was added to the channels and the cells were incubated for 24 h at 37 °C and 5% $CO_2$.

For live cell mitochondria imaging, **TMP-TCO*** (10 mM stock in DMSO) was diluted in OptiMEM to a final concentration of 10 µM and incubated in combination with 100 nM MitoTracker Red CMXRos with the cells for 1 h. If nuclear staining was applied, cells were washed 1x with PBS and were incubated with 2 µg/mL Hoechst 33342 in OptiMEM for 15 min followed by 1x washing with PBS. If no nuclear staining was applied, cells were washed 3x with FluoroBrite DMEM Media over a period of 20 min. 10 µM **OG-5-Tz(piv)$_2$** (lyophilized stock) was added to the cells in FluoroBrite DMEM Media. Cells were imaged 1 h *post* addition of the dye.

For live cell nuclei imaging, **TMP-TCO*** or **Halo-TCO** (10 mM and 5 mM stock in DMSO and DMSO/MeCN respectively) were diluted in OptiMEM to a final concentration of 10 µM and incubated with the cells for 1 h. Cells were washed 1x with PBS and were incubated with 2 µg/mL Hoechst 33342 in OptiMEM for 15 min followed by 1x washing with PBS. 10 µM **OG-5-Tz-(piv)$_2$** (lyophilized stock) was added to the cells in FluoroBrite DMEM Media. Cells were imaged 30 min *post* addition of the dye. For timecourse recording, cells were directly imaged after addition of the dye once every minute using exposure times of four seconds for each channel.

For fixed cell nuclei imaging, cells were fixed using 4% PFA in PBS for 20 min, followed by 2x wash with PBS. Cells were permeabilized with 0.2% Triton-X100 in PBS for 5 min, followed by 2x wash with PBS. **Halo-TCO** (5 mM stock in DMSO/MeCN) was diluted in labeling buffer to a final concentration of 10 µM and incubated with the cells for 1 h. Cells were washed 1x with PBS and were incubated with 2 µg/mL Hoechst 33342 in OptiMEM for 15 min. Cells were washed 1x with PBS and 10 µM **OG-5-Tz** (lyophilized stock) was added to the cells in PBS. Cells were imaged 30 min *post* addition of the dye.

For fixed cell actin cytoskeleton imaging, the procedure used by Cramer *et al.* and Meimetis *et al.* was applied.[6],[7] Cells were fixed using 4% PFA in Cytoskeleton buffer (10 mM MES, pH = 6.1, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA and 0.32 M sucrose) for 20 min, followed by 2x wash with PBS. Cells were permeabilized with 0.1% Triton-X100 in 10 mM TBS (pH = 7.4) for 5 min, followed by 1x wash with PBS. **Phalloidin-TCO** (43 µg/mL stock in methanol) was diluted in labeling buffer (10mM TBS (pH 7.4), 0.1% Triton X-100 and 2% BSA) to a final concentration of 1 µg/mL and incubated with the cells for 40 min. Cells were washed 1x with PBS and were incubated with 2 µg/mL Hoechst 33342 in OptiMEM for 15 min. Cells were washed 1x with PBS and 10 µM fluorogenic dye (lyophilized stock or 10 mM DMSO stock) was added to the cells in PBS. Cells were imaged 30 min *post* addition of the dye.

Channels were recorded using an A1R point scanning confocal microscope mounted on a fully automated Nikon Ti-E inverted microscope with 40x or 60x objective magnification and a Galvano scanner unit. For Hoechst dyes a setup using a 405 nm laser in combination with a 450/50 nm emission filter was applied. For fluorescein and Oregon Green derivatives a setup using a 491nm laser in combination with a 525/50 nm emission filter was applied. For tetramethylrhodamine derivatives and MitoTracker Red CMXRos a setup using a 561 nm laser in combination with a 595/50 nm emission filter was applied. For silicon rhodamine derivatives a setup using a 640 nm laser in combination with a 700/75 nm emission filter was applied.

For computer-aided image enhancement, the LUT was auto-scaled between the minimal and maximal value using Fiji ImageJ (Version 1.50e).[8] For displayed images, following LUTs were used: Hoechst dye was shown in a “Blue” LUT using Fiji. Fluorescein derivatives were shown in a “Green” LUT using Fiji. Tetramethylrhodamine derivatives were shown in a “glow” LUT using Fiji. Silicon rhodamine derivatives were shown in an “ironred” LUT using NIS-Elements 4.5.

# 5 Syntheses

## 5.1 General procedure (1) for the Preparation of Tetrazines

This procedure was adapted from the literature.[9],[10]

The hydroxymethyl benzonitrile (1.0 eq), $Ni(OTf)_2$ (0.5 eq), acetonitrile (50 eq) and hydrazine monohydrate (10 eq) were combined in a microwave reaction tube. The tube was sealed and the reaction mixture was stirred for 24 hours at 60 °C. After cooling the reaction mixture to 0 °C, it was diluted with water and extracted with dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was dissolved in dichloromethane and $PhI(OAc)_2$ (1.5 eq) was added and the mixture was stirred for two hours at rt. The reaction mixture was subsequently subjected to flash chromatography (silica, EtOAc/cyclohexane).

## 5.2 General Procedure (2) for the Preparation of Tetrazinyl Benzaldehydes

Dess-Martin periodinane (1.1 eq) was added to the solution of a tetrazinyl benzylic alcohol (1.0 eq) in dichloromethane. The mixture was stirred for one to two hours at rt until TLC analysis indicated complete conversion. The reaction mixture was filtered and the filtrate was concentrated and subsequently purified by flash column chromatography (silica, EtOAc/cyclohexane).

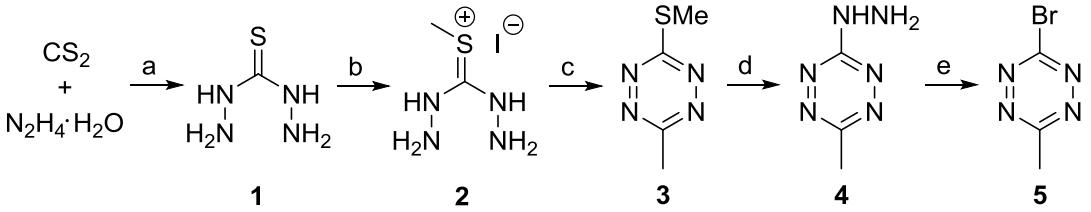
## 5.3 General Procedure (3) for the Preparation of Rh-Tz Derivatives

The diarylether (1.0 eq), tetrazinyl benzaldehyde (2.0 eq) and $CuBr_2$ (0.1 eq) were dissolved in dry 1,2-dichloroethane in a microwave tube and activated molecular sieves (4 Å) was added. The tube was sealed and the mixture stirred at 80 °C for 18 hours. After cooling down to rt, *p*-chloranil (1.0 eq) was added and stirred for one to four hours. Subsequently, the solvent was removed *in vacuo* and the crude mixture was subjected to flash chromatography (silica, $MeOH/CH_2Cl_2$). For yield determination, bromide was assumed as counterion. HPLC purification (Solvent B, Solvent A) yielded TFA salts of the dyes and was performed prior to photophysical measurements.

## 5.4 General Procedure (4) for the Preparation of SiRh-Tz Derivatives

The diarylsilane (1.0 eq), tetrazinyl benzaldehyde (2.0 eq) and anhydrous $AlBr_3$ (1.0 eq) were dissolved in dry 1,2-dichloroethane in a microwave tube and activated molecular sieves (4 Å) was added. The tube was sealed and the mixture stirred at rt for 18 hours. After cooling down to rt, *p*-chloranil (1.0 eq) was added and stirred for one to two hours. Subsequently, the solvent was removed *in vacuo* and the crude mixture was subjected to flash chromatography (silica, MeOH/$CH_2Cl_2$ respectively EtOAc/$CH_2Cl_2$). For yield determination, bromide was assumed as counterion. HPLC purification (Solvent B, Solvent A) yielded TFA salts of the dyes and was performed prior to photophysical measurements.

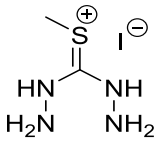
## 5.5 Synthesis of 3-Bromo-6-methyl-1,2,4,5-tetrazine (5)




**Scheme S1.** Synthesis of 3-Bromo-6-methyl-1,2,4,5-tetrazine (**5**). a: $H_2O$, 50%; b: MeI, EtOH, 58%; c: 1) $CH_3C(OMe)_3$, $NEt_3$, EtOH, 2) $NaNO_2$, TFA, 23%; d: $N_2H_4 \cdot H_2O$, EtOH, 39%; e: $Br_2$, AcOH, 40%.

**Thiocarbohydrazide (1)** was synthesized according to literature.[11]

**S-Methylisothiocarbohydrazide hydroiodide (2)**

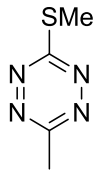


This procedure was adapted from the literature.[12]

**1**[11] (9.07 g, 85.4 mmol) was suspended in EtOH (300 mL) and heated to reflux temperature. During refluxing iodomethane (5.96 mL, 95.7 mmol) was added within 30 min and stirred for another 30 min under reflux. The solution was filtered while still hot and then cooled to rt. A crystalline precipitate formed which was collected by suction and dried in a desiccator to afford **2** (12.32 g, 58%).

$^1$H-NMR (500 MHz, DMSO-$d_6$) δ 9.53 (bs, 2H), 5.35 (bs, 4H), 2.38 (s, 3H). $^{13}$C-NMR (126 MHz, DMSO-$d_6$) δ 170.6, 12.3.

**3-(Methylthio)-6-methyl-1,2,4,5-tetrazine (3)**

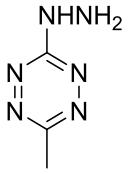



This procedure has been described in the literature for triethyl orthoacetate instead of trimethyl orthoacetate as starting material.[13]

**2** (8.00 g, 32.2 mmol) was suspended in absolute EtOH (200 mL) and heated to 50 °C. At 50 °C trimethyl orthoacetate (4.26 mL, 33.9 mmol) was added, stirred for 5 min when triethylamine (4.50 mL, 32.2 mmol). The mixture was then refluxed for 30 min. The heating bath was removed and sodium nitrite (4.45 g, 64.5 mmol) and trifluoroacetic acid (2.40 mL, 32.2 mmol) were added successively, the latter dropwise. After another 45 min under reflux the reaction mixture was cooled to rt, poured in water (400 mL) and extracted with diethyl ether. The combined organic extracts were 2 times washed with water and dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, 5% $Et_2O$/pentane) afforded **3** (1.046 g, 23%) as a red oil.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 2.97 (s, 3H), 2.73 (s, 3H).

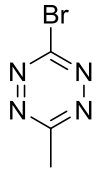
**3-(Hydrazinyl)-6-methyl-1,2,4,5-tetrazine (4)**




To a solution of **3** (972 mg, 6.84 mmol) in EtOH (70 mL) was added hydrazine monohydrate (398 µL, 8.20 mmol) at rt. The mixture was stirred for 24 h at rt, then poured in brine. The precipitating NaCl was filtered off by suction and the filtrate was extracted 5 times with EtOAc. The combined organic extracts were washed with brine, dried over $MgSO_4$ and evaporated. Filtration over a short plug of silica (elution with 2:1 cyclohexane/EtOAc) afforded crude product which was further purified by flash chromatography (silica, 1-2% MeOH/$CH_2Cl_2$) to obtain **4** (100 mg, 12%) as a red solid.

$^1$H-NMR (300 MHz, DMSO-$d_6$) δ 9.28 (s, 1H), 4.50 (s, 2H), 2.70 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 163.6, 163.3, 20.3. HRMS ($ESI^+$) m/z 127.0727 calcd for $[C_3H_6N_6Na]^+$ ($M+Na^+$), 127.0728 found.

**3-Bromo-6-methyl-1,2,4,5-tetrazine (5)**

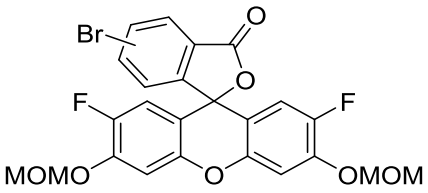



**4** (97 mg, 0.77 mmol) was dissolved in AcOH (2 mL) and bromine (160 mg, 1.0 mmol) was added at rt. The reaction mixture was stirred for 90 min at rt. Ice (4.5 g) and diethyl ether (20 mL) were added and the pH of the aqueous phase was set to 6 by addition of $Na_2CO_3$. The phases were separated and the organic phase was washed with 5% aqueous $NaHCO_3$ solution and brine, dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, 5% $Et_2O$/pentane) afforded **5** (54 mg, 40%) as magenta colored crystals.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 3.05 (s, 3H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 168.3, 161.4, 20.8.

## 5.6 Syntheses Tetrazinyl Oregon Green Derivatives and Precursors

**5/6-Bromo Oregon Green 3',6'-bis(methoxymethyl) ether (7)**

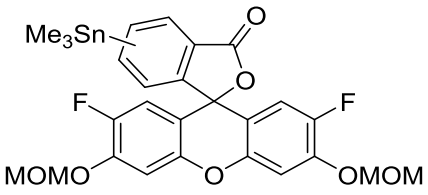



5/6-Bromo Oregon Green starting material (**6**) was synthesized according to literature.[14]
*N*,*N*-Diisopropylethylamine (3.87 mL, 22.4 mmol) and **6** (2.00 g, 4.47 mmol) were dissolved in anhydrous dichloromethane (50 mL) under argon. Chloromethyl methyl ether (1.70 mL, 22.4 mmol) was added and the mixture was stirred overnight at rt. After dilution with $CH_2Cl_2$ (150 mL) the organic phase was washed with 1 M aqueous HCl and brine, dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, first 3:1 then 1:2 cyclohexane/EtOAc) afforded the bis-MOM diether product **7** (420 mg, 18%) and the bis-MOM ester ether (1.532 g, 64%), respectively.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 8.17 (dd, J = 1.8, 0.6 Hz, 1H), 7.89 (dd, J = 8.1, 0.6 Hz, 1H), 7.80 (m, 2x1H), 7.31 (dd, J = 1.5, 0.6 Hz, 1H), 7.13 (d, $^4J_{H,F}$ = 7.1 Hz, 2H), 7.13 (d, $^4J_{H,F}$ = 7.0 Hz, 2H),

7.05 (dd, J = 8.2, 0.6 Hz, 1H), 6.49 (d, $^{3}J_{H,F}$ = 10.8 Hz, 2H), 6.47 (d, $^{3}J_{H,F}$ = 10.9 Hz, 2H), 5.31 – 5.23 (m, 2x4H), 3.53 (s, 6H), 3.52 (s, 6H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 167.9, 167.3, 154.2, 151.1, 151.1, 150.9, 147.9, 147.8, 147.8, 147.7, 147.7, 147.6, 147.5, 147.4, 147.4, 138.6, 134.1, 130.8, 128.5, 128.5, 127.3, 126.8, 125.5, 125.1, 124.6, 114.2, 114.2, 113.9, 113.9, 110.5, 110.4, 110.3, 105.7, 105.7, 95.6, 56.7, 27.1. HRMS (ESI$^+$) m/z 558.9999 calcd for $[C_{24}H_{17}BrF_2O_7Na]^+$ (M+Na$^+$), 559.0002 found.

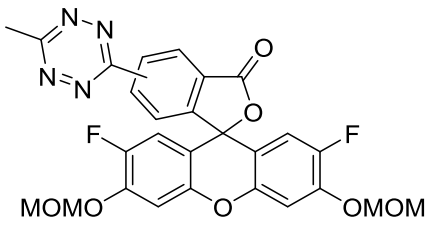
**5/6-Trimethylstannyl Oregon Green 3',6'-bis(methoxymethyl) ether (8)**




**7** (350 mg, 654 µmol) and $Pd(PPh_3)_4$ (38 mg, 33 µmol) were placed in a microwave tube. The tube was sealed and put under argon. Anhydrous toluene was degassed separately by freeze-pump-thawing and added. Hexamethyltin (203 µL, 980 µmol) was added and the reaction mixture was refluxed overnight. After filtration over Celite (coarse, 560) and evaporation the residue was purified by flash chromatography (silica, 4:1 cyclohexane/EtOAc) to yield **8** (207 mg, 51%) as an off-white solid.

$^{1}$H-NMR (500 MHz, $CDCl_3$) δ 8.21 – 8.12 (m, 1H), 7.98 – 7.94 (m, 1H), 7.85 – 7.73 (m, 2x1H), 7.31 – 7.19 (m, 1H), 7.15 – 7.10 (m, 5H), 6.48 (d, J = 11.0 Hz, 2H), 6.47 (d, J = 11.0 Hz, 2H), 5.29 – 5.24 (m, 2x4H), 3.54 (s, 6H), 3.53 (s, 6H), 0.48 – 0.32 (m, 9H), 0.36 – 0.24 (m, 9H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 169.4, 169.2, 154.2, 152.2, 151.2, 150.4, 150.4, 148.5, 148.4, 147.9, 147.9, 147.9, 147.3, 147.2, 147.2, 147.1, 146.2, 142.5, 137.5, 132.7, 130.7, 126.4, 125.7, 124.1, 123.1, 114.5, 114.4, 114.3, 114.2, 111.5, 111.5, 111.5, 111.5, 105.6, 95.6, 95.6, 82.5, 82.3, 56.7, 56.7, -9.0, -9.1. HRMS (ESI+) m/z 621.0746 calcd for $[C_{27}H_{27}F_2O_7Sn]^+$ (M+H$^+$), 621.0731 found.

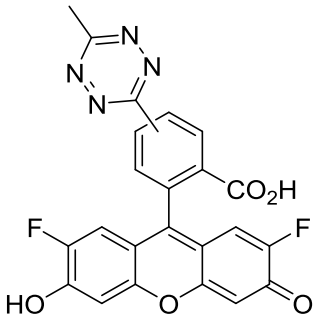
**5/6-(6-Methyl-1,2,4,5-tetrazin-3-yl) Oregon Green 3',6'-bis(methoxymethyl) ether (9)**




**8** (142 mg, 229 µmol) was dissolved in dry dimethylacetamide (2.5 mL) and transferred to a Schlenk tube under argon. The solution was degassed by freeze-pump-thawing and tris(dibenzylideneacetone)dipalladium(0) (11 mg, 12 µmol), triphenylarsine (14 mg, 46 µmol), CuI (4.4 mg, 23 µmol) and 3-bromo-6-methyl-1,2,4,5-tetrazine **(5)** (40 mg, 229 µmol) were added. The mixture was stirred for 20 h at 70 °C under argon. After dilution with EtOAc the organic phase was washed 3 times with water, dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, 17-20% EtOAc/cyclohexane) afforded **9** (27 mg, 21%) as a magenta colored solid.

$^1$H-NMR (500 MHz, $CDCl_3$) δ 9.27 (dd, J = 1.6, 0.8 Hz, 1H), 8.94 (dd, J = 8.1, 1.6 Hz, 1H), 8.91 (dd, J = 8.1, 1.4 Hz, 2H), 8.40 (m, 3H), 8.25 (dd, J = 8.1, 0.8 Hz, 2H), 7.40 (dd, J = 8.1, 0.8 Hz, 1H), 7.16 – 7.15 (m, 6H), 6.54 (d, J = 10.8 Hz, 1H), 6.53 (d, J = 10.9 Hz, 2H), 5.32 – 5.23 (m, 10H), 3.53 (s, 8H), 3.52 (s, 12H), 3.16 (s, 3H), 3.10 (s, 5H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 168.2, 168.2, 168.0, 167.8, 163.2, 163.2, 155.7, 153.5, 150.5, 150.4, 148.5, 148.5, 147.8, 147.8, 147.8, 147.8, 147.5, 147.5, 147.4, 147.4, 138.7, 134.7, 134.6, 129.8, 129.4, 127.6, 126.4, 125.3, 125.0, 123.5, 114.2, 114.0, 110.5, 110.5, 110.4, 110.4, 105.7, 105.7, 95.6, 95.5, 77.2, 56.7, 27.0, 21.4, 21.4. HRMS (ESI$^+$) m/z 573.1192 calcd for $[C_{27}H_{20}F_2N_4O_7Na]^+$ (M+$Na^+$), 573.1189 found.

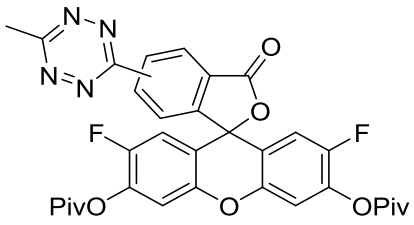
**5/6-(6-Methyl-1,2,4,5-tetrazin-3-yl) Oregon Green (OG-5/6-Tz)**



**9** (27 mg, 49 µmol) was dissolved in anhydrous 1,4-dioxane (2 mL). At 0 °C a 4 M solution of HCl in 1,4-dioxane (2 mL) was added dropwise. The cooling bath was removed and the reaction mixture was stirred for one hour at rt. The solvent was evaporated and the residue was purified by flash chromatography (silica, 2-3% MeOH/$CH_2Cl_2$ containing 0.5% AcOH) to yield **OG-5/6-Tz** (21 mg, 93%) as a red solid. Prior to spectroscopic measurements HPLC purification was performed (40-60% Solvent B/Solvent A over 35 min) providing isomerically pure **OG-5-Tz** and **OG-6-Tz**.

**OG-5/6-Tz**: HRMS ($ESI^-$) m/z 461.0703 calcd for $[C_{23}H_{11}F_2N_4O_5]^-$ ($M\text{-}H^+$), 461.0719 found.
**OG-5-Tz**: $^1$H-NMR (300 MHz, DMSO-$d_6$) δ 10.78 (s, 2H), 8.91 (s, 1H), 8.83 (dd, J = 8.1, 1.6 Hz, 1H), 7.58 (d, J = 8.1 Hz, 1H), 6.91 (d, J = 7.5 Hz, 2H), 6.71 (d, J = 11.4 Hz, 2H), 3.07 (s, 3H).
**OG-6-Tz**: $^1$H-NMR (300 MHz, DMSO-$d_6$) δ 10.76 (s, 2H), 8.78 (dd, J = 8.1, 1.4 Hz, 1H), 8.27 (d, J = 8.1 Hz, 1H), 8.21 (d, J = 1.3 Hz, 1H), 6.90 (d, J = 7.6 Hz, 2H), 6.69 (d, J = 11.3 Hz, 2H), 2.99 (s, 3H).

**5/6-(6-Methyl-1,2,4,5-tetrazin-3-yl) Oregon Green 3',6'-dipivalate (OG-5/6-Tz(piv)$_2$)**




**OG-5/6-Tz** (5.0 mg, 11 µmol) was dissolved in dry pyridine under argon. Pivalic anhydride (9.9 µL, 49 µmol) and one crystal of 4-(*N*,*N*-dimethylamino)pyridine were added and the mixture was stirred for three hours at rt. The solvent was evaporated and the residue dried under high vacuum. HPLC purification (80-90% Solvent B/Solvent A) yielded **OG-6-Tz(piv)$_2$** (1.8 mg, 26%) and **OG-5-Tz(piv)$_2$** (0.7 mg, 10%) containing 9% of the 6-isomer as magenta colored solids. Additionally a regioisomeric mixture of **OG-6-Tz(piv)$_2$** and **OG-5-Tz(piv)$_2$** (0.5 mg, 7%) was obtained.

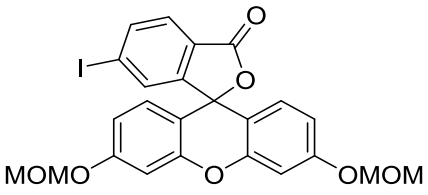
**OG-6-Tz(piv)$_2$**: $^1$H-NMR (500 MHz, DMSO-$d_6$) δ 8.81 (dd, J = 8.1, 1.4 Hz, 1H), 8.39 (dd, J = 1.4, 0.7 Hz, 1H), 8.32 (dd, J = 8.2, 0.7 Hz, 1H), 7.53 (d, J = 6.5 Hz, 2H), 7.13 (d, J = 10.3 Hz, 2H),

2.99 (s, 3H), 1.31 (s, 18H). HRMS ($ESI^+$) m/z 653.1818 calcd for $[C_{33}H_{28}F_2N_4O_7Na]^+$ ($M+Na^+$), 653.1799 found.

**OG-5-Tz(piv)$_2$**: $^1$H-NMR (500 MHz, DMSO-$d_6$) δ 8.95 (d, J = 1.5 Hz, 1H), 8.85 (dd, J = 8.1, 1.6 Hz, 1H), 7.72 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 6.5 Hz, 2H), 7.15 (d, J = 10.3 Hz, 2H), 3.07 (s, 3H), 1.32 (s, 18H). HRMS ($ESI^+$) m/z 653.1818 calcd for $[C_{33}H_{28}F_2N_4O_7Na]^+$ ($M+Na^+$), 653.1794 found.

## 5.7 Syntheses of Tetrazinyl Fluorescein Derivatives and Precursors

**6-Iodofluorescein 3',6'-bis(methoxymethyl) ether (11)**

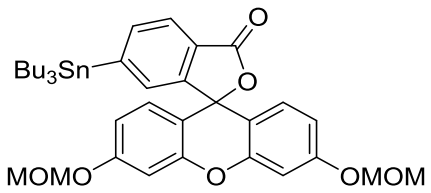



6-Iodofluorescein (**10**) starting material was synthesized according to literature.[15]

**10** (458 mg, 1.00 mmol) was suspended in anhydrous MeCN (10 mL) and $Ag_2O$ (579 mg, 2.50 mmol), activated molecular sieves (300 mg; 4 Å) and chloromethyl methyl ether (304 µL, 4.00 mmol) were added. The reaction mixture was stirred for four hours at rt, then diluted with dichloromethane, filtered over Celite (coarse, 560) and evaporated. Purification of the residue by flash chromatography (silica, 10% EtOAc/cyclohexane containing 1% $NEt_3$ to avoid partial deprotection of the MOM groups) afforded **11** (444 mg, 81%) as a colorless resin.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 7.95 (dd, J = 8.0, 1.4 Hz, 1H), 7.72 (dd, J = 8.0, 0.5 Hz, 1H), 7.52 (dd, J = 1.4, 0.6 Hz, 1H), 6.96 (d, J = 2.3 Hz, 2H), 6.76 (dd, J = 8.8, 2.3 Hz, 2H), 6.71 (d, J = 8.7 Hz, 2H), 5.21 (s, 4H), 3.49 (s, 7H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 168.8, 159.2, 154.9, 152.3, 139.3, 133.4, 129.2, 126.2, 113.3, 111.8, 103.9, 103.0, 94.5, 82.5, 56.4. HRMS ($ESI^+$) m/z 547.0248 calcd for $[C_{24}H_{20}IO_7]^+$ ($M+H^+$), 547.0248 found.

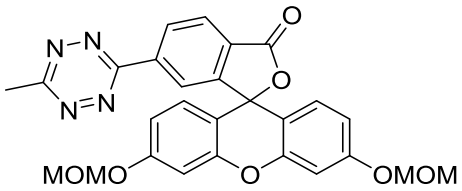
**6-(Tributylstannyl)fluorescein 3',6'-bis(methoxymethyl) ether (12)**




**11** (310 mg, 567 µmol) was dissolved in anhydrous toluene, the solution was degassed by freeze-pump-thawing and put under argon. $Pd(PPh_3)_4$ (33 mg, 28 µmol) and bis(tributyltin) (315 µL, 624 µmol) were added and the mixture was stirred overnight at 70 °C (incomplete conversion), then for 40 h at 100 °C. After filtration over silica (elution with 50-100% EtOAc/cyclohexane) and evaporation the residue was purified by flash chromatography (silica, 10:1 cyclohexane/EtOAc containing 1% $Net_3$) to afford **12** (198 mg, 49%) as a slightly yellowish oil.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 7.96 – 7.90 (m, 1H), 7.81 – 7.63 (m, 1H), 6.97 (dd, J = 2.1, 0.7 Hz, 2H), 6.73 – 6.69 (m, 2H), 6.69 – 6.65 (m, 2H), 5.25 – 5.18 (m, 4H), 3.48 (s, 6H), 1.54 – 1.32 (m, 6H), 1.31 – 1.17 (m, 6H), 1.14 – 0.91 (m, 6H), 0.80 (t, J = 7.2 Hz, 9H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 169.9, 158.9, 153.3, 152.7, 151.6, 137.4, 131.8, 129.3, 126.8, 123.7, 113.0, 113.0, 103.7, 94.5, 83.3, 56.3, 29.1, 27.3, 13.7, 10.0. HRMS ($ESI^+$) m/z 711.2346 calcd for $[C_{36}H_{47}O_7Sn]^+$ ($M+H^+$), 711.2317 found.

**6-(6-Methyl-1,2,4,5-tetrazin-3-yl)fluorescein 3',6'-bis(methoxymethyl) ether (13)**

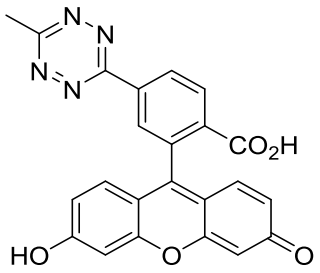



**12** (190 mg, 268 µmol) was dissolved in dry dimethylacetamide (1 mL) and transferred to a Schlenk tube under argon. The solution was degassed by freeze-pump-thawing and tris(dibenzylideneacetone)dipalladium(0) (12 mg, 13 µmol), triphenylarsine (16 mg, 54 µmol), CuI (5.1 mg, 27 µmol) and 3-bromo-6-methyl-1,2,4,5-tetrazine **5** (47 mg, 268 µmol) were added. The mixture was stirred overnight at 70 °C under argon. After dilution with EtOAc the organic

phase was washed 3 times with water and the aqueous phase was re-extracted with EtOAc; the combined organic phases were dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, 11-20% EtOAc/cyclohexane) and trituration of the obtained solid with cyclohexane afforded **13** (39 mg, 28%) as a magenta colored solid.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 8.86 (dd, J = 8.1, 1.4 Hz, 1H), 8.38 (dd, J = 1.4, 0.7 Hz, 1H), 8.22 (dd, J = 8.1, 0.8 Hz, 1H), 6.99 (dd, J = 2.2, 0.6 Hz, 2H), 6.76 (dd, J = 8.8, 0.6 Hz, 2H), 6.72 (dd, J = 8.8, 2.2 Hz, 2H), 5.20 (s, 4H), 3.47 (s, 6H), 3.08 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 168.6, 168.1, 163.3, 159.1, 154.4, 152.4, 138.3, 129.8, 129.3, 129.1, 126.0, 123.6, 113.2, 111.8, 103.9, 94.5, 83.5, 77.2, 56.3, 21.4. HRMS ($ESI^+$) m/z 515.1561 calcd for $[C_{27}H_{23}N_4O_7]^+$ ($M+H^+$), 515.1548 found.

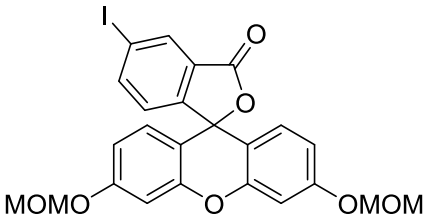
**6-(6-Methyl-1,2,4,5-tetrazin-3-yl)fluorescein (Fl-6-Tz)**




**13** (27 mg, 53 µmol) was dissolved in anhydrous 1,4-dioxane (2 mL). At 0 °C a 4 M solution of HCl in 1,4-dioxane (2 mL) was added dropwise. The cooling bath was removed and the reaction mixture was stirred for one hour at rt. The precipitate was filtered off, washed with anhydrous 1,4-dioxane and dried under high vacuum to afford **Fl-6-Tz** (21 mg, 92%) as a red solid. Prior to spectroscopic measurements HPLC purification was performed (30-70% Solvent A/Solvent B over 30 min).

$^1$H-NMR (500 MHz, DMSO-$d_6$) δ 10.18 (s, 2H), 8.76 (dd, J = 8.1, 1.4 Hz, 1H), 8.28 (dd, J = 8.1, 0.8 Hz, 1H), 8.16 (dd, J = 1.4, 0.7 Hz, 1H), 6.71 (d, J = 2.4 Hz, 2H), 6.69 (d, J = 8.7 Hz, 2H), 6.56 (dd, J = 8.7, 2.4 Hz, 2H), 2.98 (s, 3H). $^{13}$C-NMR (126 MHz, DMSO-$d_6$) δ 168.0, 167.4, 162.6, 159.7, 151.8, 138.6, 129.2, 129.1, 128.9, 126.0, 122.6, 112.7, 109.0, 102.3, 20.9. HRMS ($ESI^-$) m/z 425.0891 calcd for $[C_{23}H_{13}N_4O_5]^+$ ($M-H^+$), 425.0880 found.

**5-Iodofluorescein 3',6'-bis(methoxymethyl) ether (15)**

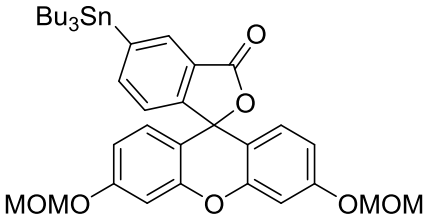



5-Iodofluorescein (**14**) starting material was synthesized according to literature.[15]

**14** (510 mg, 1.11 mmol) was suspended in anhydrous MeCN (5 mL) and $Ag_2O$ (645 mg, 2.78 mmol), activated molecular sieves (370 mg; 4 Å) and chloromethyl methyl ether (338 µL, 4.45 mmol) were added. The reaction mixture was stirred for twenty hours at rt, filtered over Celite (coarse, 560) eluting with MeCN and evaporated. Purification of the residue by flash chromatography (silica, 9-17% EtOAc/cyclohexane containing 1% $NEt_3$) afforded **15** (534 mg, 88%) as a slightly yellowish resin.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 8.35 (dd, J = 1.6, 0.6 Hz, 1H), 7.96 (dd, J = 8.1, 1.6 Hz, 1H), 6.96 (dd, J = 2.2, 0.6 Hz, 2H), 6.92 (dd, J = 8.1, 0.6 Hz, 1H), 6.73 (dd, J = 8.8, 2.2 Hz, 2H), 6.69 (dd, J = 8.8, 0.6 Hz, 2H), 5.20 (s, 4H), 3.48 (s, 6H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 167.7, 159.2, 152.6, 152.4, 143.9, 134.2, 129.1, 129.0, 125.8, 113.2, 111.8, 103.9, 95.0, 94.5, 83.4, 56.4. HRMS (ESI$^+$) m/z 569.0068 calcd for $[C_{24}H_{19}IO_7Na]^+$ ($M+Na^+$), 569.0077 found.

**5-(Tributylstannyl)fluorescein 3',6'-bis(methoxymethyl) ether (16)**

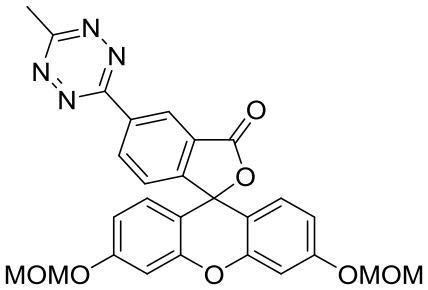



**15** (485 mg, 888 µmol) and bis(tributyltin) (536 µL, 1065 µmol) were dissolved in anhydrous toluene, the solution was degassed by freeze-pump-thawing and put under argon. $Pd(PPh_3)_4$ (51 mg, 44.3 µmol) was added and the mixture was stirred for 44 h at 110 °C. After filtration over silica (elution with EtOAc) and evaporation the residue was purified by flash chromatography

(silica, 10:1 cyclohexane/EtOAc containing 1% $NEt_3$) to afford **16** (373 mg, 59%) as a colorless oil.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 8.17 – 8.03 (m, 1H), 7.80 – 7.64 (m, 1H), 7.12 – 7.06 (m, 1H), 6.96 – 6.95 (m, 2H), 6.71 (m, 4H), 5.19 (s, 4H), 3.48 (s, 6H), 1.74 – 1.40 (m, 6H), 1.43 – 1.28 (m, 6H), 1.24 – 1.01 (m, 6H), 0.90 (t, J = 7.3 Hz, 9H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 170.2, 158.9, 153.0, 152.5, 145.2, 142.7, 132.8, 129.3, 126.0, 123.1, 113.0, 112.8, 103.8, 94.5, 82.9, 56.3, 29.2, 27.5, 13.8, 10.0. HRMS (ESI$^+$) m/z 711.2346 calcd for $[C_{36}H_{47}O_7Sn]^+$ (M+H$^+$), 711.2317 found.

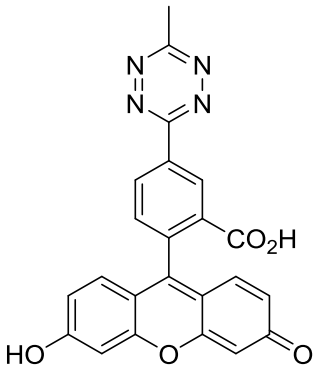
**5-(6-Methyl-1,2,4,5-tetrazin-3-yl)fluorescein 3',6'-bis(methoxymethyl) ether (17)**




**16** (360 mg, 507 µmol) was dissolved in dry dimethylacetamide (2.5 mL) and transferred to a Schlenk tube under argon. The solution was degassed by freeze-pump-thawing and tris(dibenzylideneacetone)dipalladium(0) (23 mg, 25.4 µmol), triphenylarsine (31 mg, 101 µmol), CuI (9.7 mg, 50.7 µmol) and 3-bromo-6-methyl-1,2,4,5-tetrazine (**5)** (89 mg, 507 µmol) were added. The mixture was stirred overnight at 70 °C under argon. After dilution with EtOAc the organic phase was washed 3 times with water; the combined organic phases were washed with brine, dried over $MgSO_4$ and evaporated. The residue was purified by flash chromatography (silica, 20% EtOAc/cyclohexane). The obtained solid was suspended in cyclohexane, filtered, washed excessively with cyclohexane and dried under high vacuum to afford **17** (65 mg, 25%) as a magenta colored foam.

$^1$H-NMR (500 MHz, $CDCl_3$) δ 9.25 (dd, J = 1.6, 0.7 Hz, 1H), 8.89 (dd, J = 8.1, 1.6 Hz, 1H), 7.39 (dd, J = 8.1, 0.8 Hz, 1H), 6.99 (dd, J = 2.2, 0.6 Hz, 2H), 6.77 (dd, J = 8.8, 0.6 Hz, 2H), 6.74 (dd, J = 8.8, 2.2 Hz, 2H), 5.21 (s, 4H), 3.49 (s, 7H), 3.16 (s, 3H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 168.5, 168.1, 163.4, 159.2, 156.8, 152.4, 134.3, 134.1, 129.1, 128.0, 125.2, 125.1, 113.3, 111.8, 103.9, 94.5, 83.3, 77.2, 56.4, 21.4. HRMS (ESI$^+$) m/z 515.1561 calcd for $[C_{27}H_{23}N_4O_7]^+$ (M+H$^+$), 515.1553 found.

**5-(6-Methyl-1,2,4,5-tetrazin-3-yl)fluorescein (Fl-5-Tz)**

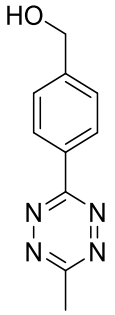



**17** (65 mg, 126 µmol) was dissolved in anhydrous 1,4-dioxane (2 mL). At 0 °C a 4 M solution of HCl in 1,4-dioxane (2 mL) was added dropwise. The cooling bath was removed and the reaction mixture was stirred for two hours at rt. The precipitate was filtered off, washed with anhydrous 1,4-dioxane and dried under high vacuum. Purification by flash chromatography (silica, 2% MeOH/$CH_2Cl_2$ containing 0.5% AcOH) afforded **Fl-5-Tz** (30 mg, 56%) as a red solid. Prior to spectroscopic measurements HPLC purification was performed (30-70% Solvent A/Solvent B over 30 min).

$^1$H-NMR (500 MHz, DMSO-$d_6$) δ 10.21 (s, 2H), 8.91 (dd, J = 1.6, 0.7 Hz, 1H), 8.82 (dd, J = 8.1, 1.6 Hz, 1H), 7.57 (dd, J = 8.1, 0.7 Hz, 1H), 6.71 (d, J = 2.6 Hz, 2H), 6.69 (d, J = 8.6 Hz, 2H), 6.57 (dd, J = 8.7, 2.4 Hz, 2H), 3.06 (s, 3H). $^{13}$C-NMR (126 MHz, DMSO-$d_6$) δ 167.9, 167.6, 162.6, 159.8, 151.8, 134.3, 134.1, 129.2, 127.4, 125.4, 123.5, 112.8, 108.9, 102.3, 20.9. HRMS (ESI$^+$) m/z 427.1037 calcd for $[C_{23}H_{15}N_4O_5]^+$ (M+H$^+$), 427.1038 found.

## 5.8 Syntheses of Tetrazinyl Benzaldehydes

### (4-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (18)

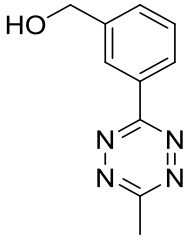



This compound has been described previously.[9]

Commercially available 4-(hydroxymethyl)benzonitrile (150 mg, 1.13 mmol) was treated according to general procedure 1. Purification by flash chromatography (silica, 20% EtOAc/cyclohexane) afforded **18** as red crystals. Yield: 129 mg, 57%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 8.56 (d, J = 8.3 Hz, 2H), 7.58 (d, J = 8.2 Hz, 2H), 4.83 (s, 2H), 3.09 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 167.3, 164.1, 145.8, 131.1, 128.3, 127.5, 64.9, 21.3. HRMS ($ESI^+$) m/z 225.0747calcd for $[C_{10}H_{10}N_4NaO]^+$ ($M+Na^+$), 225.0737 found.

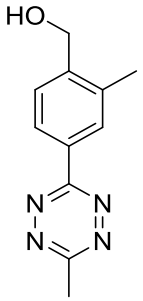
### (3-(6-Methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (19)




Commercially available 3-(hydroxymethyl)benzonitrile (100 mg, 751 µmol) was treated according to general procedure 1. The reaction time of the dihydrotetrazine synthesis differed to 48 hours. Purification by flash chromatography (silica, 20% EtOAc/cyclohexane) afforded **19** as a red solid. Yield: 73 mg, 48%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 8.56 (s, 1H), 8.48 (d, J = 7.5 Hz, 1H), 7.67 – 7.52 (m, 2H), 4.83 (s, 2H), 3.09 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 167.4, 164.1, 142.3, 132.1, 131.1, 129.6, 127.2, 126.4, 65.0, 21.3. HRMS ($ESI^+$) m/z 225.0747calcd for $[C_{10}H_{10}N_4NaO]^+$ ($M+Na^+$), 225.0745 found.

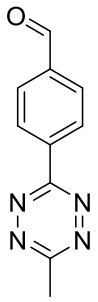
**(2-Methyl-4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (20)**



4-(Hydroxymethyl)-3-methylbenzonitrile (200 mg, 1.36 mmol) (prepared accordingly to a previously published procedure[16]) was treated according to general procedure 1. Purification by flash chromatography (silica, 0-15% EtOAc/cyclohexane) and afforded **20** as a red solid. Yield: 95 mg, 32%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 8.43 (d, J = 8.0 Hz, 1H), 8.41 (s, 1H), 7.63 (d, J = 7.9 Hz, 1H), 4.85 – 4.80 (m, 2H), 3.09 (s, 3H), 2.46 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 167.3, 164.2, 143.6, 137.0, 131.0, 129.6, 127.9, 125.9, 63.2, 21.3, 18.9. HRMS ($ESI^+$) m/z 239.0903 calcd for $[C_{11}H_{12}N_4NaO]^+$ ($M+Na^+$), 239.0910 found.

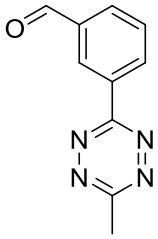
**4-(6-Methyl-1,2,4,5-tetrazin-3-yl)benzaldehyde (21)**



**18** (180 mg, 890 µmol) was treated according to general procedure 2. Purification by flash chromatography (silica, 10-15% EtOAc/cyclohexane) afforded **21** as red crystals. Yield: 172 mg, 96%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 10.16 (s, 1H), 8.79 (d, J = 8.3 Hz, 2H), 8.11 (d, J = 8.7 Hz, 2H), 3.14 (s, 3H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 191.7, 167.9, 163.6, 139.1, 137.2, 130.4, 128.6, 21.4. HRMS ($ESI^-$) m/z 199.0625 calcd for $[C_{10}H_7N_4O]^-$ ($M-H^+$), 199.0622 found.

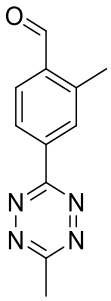
**3-(6-Methyl-1,2,4,5-tetrazin-3-yl)benzaldehyde (22)**



**19** (137 mg, 677 µmol) was treated according to general procedure 2. Purification by flash chromatography (silica, 10% EtOAc/cyclohexane) afforded **22** as a red solid. Yield: 99 mg, 73%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 10.17 (s, 1H), 9.11 (s, 1H), 8.87 (d, J = 7.8 Hz, 1H), 8.16 (d, J = 7.7 Hz, 1H), 7.79 (t, J = 7.7 Hz, 1H), 3.14 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 191.5, 168.0, 163.5, 137.4, 133.4, 132.6, 130.0, 21.4. HRMS ($ESI^+$) m/z 223.0590 calcd for $[C_{10}H_8N_4NaO]^+$ ($M+Na^+$), 223.0590 found.

**2-Methyl-4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzaldehyde (23)**

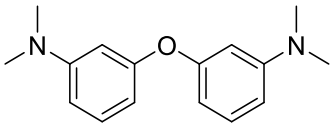


**20** (83 mg, 383 µmol) was treated according to general procedure 2. Purification by flash chromatography (silica, 10% EtOAc/cyclohexane) afforded **23** as red solid. Yield: 73 mg, 89%.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 10.40 (s, 1H), 8.58 (d, J = 8.1 Hz, 1H), 8.53 (s, 1H), 8.02 (d, J = 8.1 Hz, 1H), 3.14 (s, 3H), 2.82 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 192.3, 167.8, 163.7, 141.6, 136.9, 136.1, 132.6, 131.2, 125.9, 21.4, 19.8. HRMS ($ESI^-$) m/z 213.0782 calcd for $[C_{11}H_9N_4O]^-$ ($M-H^+$), 213.0792 found.

## 5.9 Syntheses of Tetrazinyl Rhodamines and Precursors

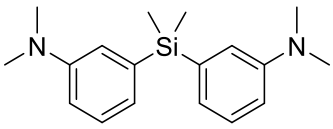
### 3,3'-Oxybis(*N*,*N*-dimethylaniline) (24)



The title compound was prepared following a literature procedure for the synthesis of diarylethers.[17] 3-Dimethylaminophenol (807 mg, 5.89 mmol), $K_3PO_4$ (2.08 g, 9.81 mmol), CuI (93 mg, 491 µmol) and 2-picolinic acid (121 mg, 981 µmol) were placed in a microwave tube, sealed, evacuated three times and backfilled with argon. 3-Iodo-*N,N*-dimethylaniline (prepared from the corresponding bromide by halogen exchange[18] was dissolved in dry dimethylsulfoxide (10 mL), added to the tube and the mixture was stirred at 85 °C for twenty hours. After cooling to rt the reaction mixture was diluted with $H_2O$ (50 mL), basified with saturated $NaHCO_3$ solution and extracted four times with EtOAc. The combined organic extracts were washed with brine, dried over $MgSO_4$ and evaporated. Purification of the residue by flash chromatography (silica, 15:1 cyclohexane/EtOAc) afforded **24** (1.185 g, 94%) as a white solid.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 7.20 – 7.11 (m, 2H), 6.50 – 6.42 (m, 4H), 6.35 (ddd, *J* = 8.0, 2.1, 1.0 Hz, 2H), 2.93 (s, 12H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 158.5, 152.2, 129.9, 107.5, 106.9, 103.5, 40.7. HRMS (ESI$^+$) m/z 257.1648 calcd for $[C_{16}H_{21}N_2O]^+$ ($M+H^+$), 257.1646 found.

### 3,3'-(Dimethylsilanediyl)bis(*N*,*N*-dimethylaniline) (25)

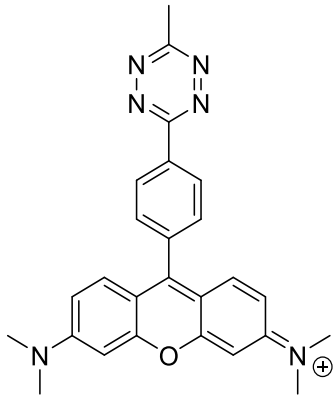


3-Bromo-*N*,*N*-dimethylaniline (1.47 mL, 10 mmol) was dissolved in dry tetrahydrofuran (20 mL) under argon. At -78 °C *sec*-butyllithium (1.4 M in cyclohexane; 10.5 mmol, 7.5 mL) was added dropwise and the mixture was stirred for 30 min keeping the temperature at -78 °C. Dichlorodimethylsilane (722 µL, 6.0 mmol) dissolved in tetrahydrofuran (3.3 mL) was added dropwise and stirring was continued for 10 min at -78 °C. The cooling bath was removed and the reaction mixture was allowed to reach rt and stirred overnight. After quenching with $H_2O$ (20 mL) and basification with saturated $NaHCO_3$ solution, the aqueous phase was extracted three times with $Et_2O$. The combined organic extracts were dried over $MgSO_4$ and evaporated. Purification

of the residue by flash chromatography (silica, 20:1 cyclohexane/EtOAc) afforded **25** (1.274 g, 85%) as a colorless oil.

$^{1}$H-NMR (300 MHz, $CDCl_3$) δ 7.30 – 7.18 (m, 2H), 6.97 – 6.88 (m, 4H), 6.77 (ddd, *J* = 8.3, 2.8, 1.0 Hz, 2H), 2.93 (s, 12H), 0.54 (s, 6H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 150.1, 139.1, 128.6, 122.9, 118.5, 113.7, 40.8, -2.0. HRMS (ESI$^+$) m/z 299.1938 calcd for $[C_{18}H_{27}N_2Si]^+$ ($M+H^+$), 299.1935 found.

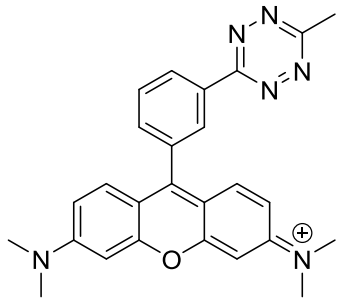
**5-(6-Methyl-1,2,4,5-tetrazin-3-yl)*N*,*N*,*N*',*N*'-tetramethylrhodamine (Rh-5-Tz)**



**24** (17.1 mg, 66.6 µmol) was treated according to general procedure 3. **Rh-5-Tz** was purified by flash chromatography (silica, 0-5% MeOH/ $CH_2Cl_2$) and afforded as a red solid. Yield: 18.0 mg, 51%.
Prior to photophysical measurements HPLC purification was performed (30-60% Solvent B/Solvent A).

$^{1}$H-NMR (500 MHz, $CD_3CN$) δ 8.79 (d, J = 8.6 Hz, 2H), 7.71 (d, J = 8.6 Hz, 2H), 7.40 (d, J = 9.5 Hz, 2H), 7.03 (dd, J = 9.5, 2.5 Hz, 2H), 6.88 (d, J = 2.5 Hz, 2H), 3.28 (s, 12H), 3.08 (s, 3H). $^{13}$C-NMR (126 MHz, $CD_3CN$) δ 169.0, 164.6, 158.9, 158.5, 157.8, 137.1, 135.0, 132.4, 131.5, 128.9, 115.5, 114.3, 97.4, 41.4, 21.5. HRMS (ESI$^+$) m/z 437.2084 calcd for $[C_{26}H_{25}N_6O]^+$ ($M^+$), 437.2081 found.

**6-(6-Methyl-1,2,4,5-tetrazin-3-yl)*N*,*N*,*N*',*N*'-tetramethylrhodamine (Rh-6-Tz)**



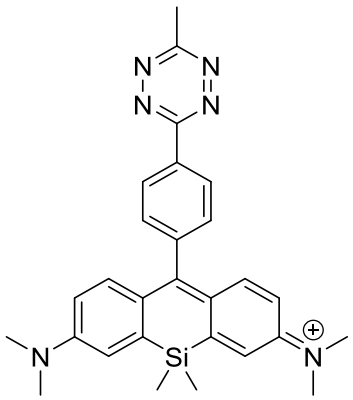
**24** (12.8 mg, 49.9 µmol) was treated according to general procedure 3. **Rh-6-Tz** was purified by flash chromatography (silica, 0-1% MeOH/ $CH_2Cl_2$) and afforded as a red solid. Yield: 7.0 mg, 27%.

Prior to photophysical measurements HPLC purification was performed (35-60% Solvent B/Solvent A).

$^1$H-NMR (500 MHz, $CD_3CN$) δ 8.80 (ddd, J = 8.0, 1.7, 1.2 Hz, 1H), 8.57 – 8.56 (m, 1H), 7.93 (t, J = 8.0 Hz, 1H), 7.73 (ddd, J = 7.6, 1.7, 1.2 Hz, 1H), 7.40 (d, J = 9.5 Hz, 2H), 7.01 (dd, J = 9.6, 2.5 Hz, 2H), 6.88 (d, J = 2.5 Hz, 2H), 3.27 (s, 12H), 3.05 (s, 3H). $^{13}$C-NMR (126 MHz, $CD_3CN$) δ 169.0, 164.5, 158.9, 158.5, 157.8, 134.4, 134.2, 134.1, 132.4, 131.0, 130.0, 129.3, 115.5, 114.6, 97.3, 41.4, 21.5. HRMS ($ESI^+$) m/z 437.2084 calcd for $[C_{26}H_{25}N_6O]^+$ ($M^+$), 437.078 found.

## 5.10 Syntheses of Tetrazinyl Si-Rhodamines

**5-(6-Methyl-1,2,4,5-tetrazin-3-yl)*N*,*N*,*N*',*N*'-tetramethyl-Si-rhodamine (SiRh-5-Tz)**

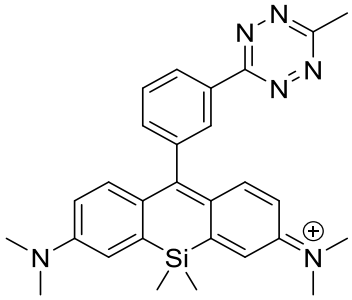


**25** (37.0 mg, 100 µmol) was treated according to general procedure 4. **SiRh-5-Tz** was purified by flash chromatography (silica, 0-5% MeOH/$CH_2Cl_2$) and afforded as a blue solid. Yield: 9.0 mg, 13%.

Prior to photophysical measurements HPLC purification was performed (35-60% Solvent B/Solvent A).

$^{1}$H-NMR (500 MHz, $CD_3CN$) δ 8.69 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 2.9 Hz, 2H), 7.15 (d, J = 9.7 Hz, 2H), 6.71 (dd, J = 9.7, 2.9 Hz, 2H), 3.29 (s, 12H), 3.07 (s, 3H), 0.60 (s, 6H). $^{13}$C-NMR (126 MHz, $CD_3CN$) δ 168.8, 168.7, 164.7, 155.2, 149.1, 144.4, 142.4, 133.6, 131.2, 128.4, 128.2, 122.4, 114.9, 41.3, 21.5, -1.0. HRMS ($ESI^+$) m/z 479.2374 calcd for $[C_{28}H_{31}N_6Si]^+$ ($M^+$), 479.2379 found.

**6-(6-Methyl-1,2,4,5-tetrazin-3-yl)*N*,*N*,*N*',*N*'-tetramethyl-Si-rhodamine (SiRh-6-Tz)**

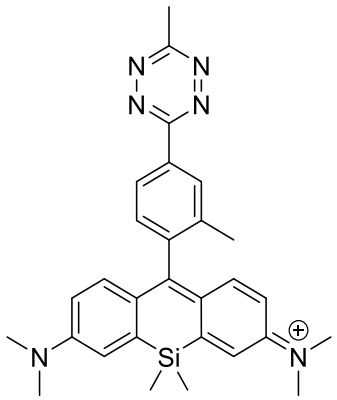


**25** (27.5 mg, 92.4 µmol) was treated according to general procedure 4. **SiRh-6-Tz** was purified by flash chromatography (silica, 0-7% MeOH/$CH_2Cl_2$) and afforded as a blue solid. Yield: 12 mg, 23%.

Prior to photophysical measurements HPLC purification was performed (35-60% Solvent B/Solvent A).

$^{1}$H-NMR (500 MHz, $CD_3CN$) δ 8.69 (ddd, J = 7.9, 1.7, 1.1 Hz, 1H), 8.41 – 8.39 (m, 1H), 7.85 – 7.81 (m, 1H), 7.57 (ddd, J = 7.6, 1.7, 1.2 Hz, 1H), 7.32 (d, J = 2.9 Hz, 2H), 7.15 (d, J = 9.7 Hz, 2H), 6.69 (dd, J = 9.7, 2.9 Hz, 2H), 3.29 (s, 12H), 3.03 (s, 1H), 0.61 (s, 6H). $^{13}$C-NMR (126 MHz, $CD_3CN$) δ 168.8, 168.6, 164.7, 155.1, 155.1, 149.2, 142.5, 141.5, 133.9, 133.6, 130.4, 129.0, 128.6, 128.5, 122.3, 114.9, 41.3, 21.5, -0.9, -1.0. HRMS ($ESI^+$) m/z 479.2374 calcd for $[C_{28}H_{31}N_6Si]^+$ ($M^+$), 479.2382 found.

**3-Methyl-5-(6-methyl-1,2,4,5-tetrazin-3-yl)*N*,*N*,*N*',*N*'-tetramethyl-Si-rhodamine (MeSiRh-5-Tz)**



**25** (25.1 mg, 84.0 µmol) was treated according to general procedure 4. **MeSiRh-5-Tz** was purified by flash chromatography (silica, 10-15% EtOAc/$CH_2Cl_2$, then 0-4% MeOH/$CH_2Cl_2$) and afforded as a blue solid. Yield: 6.1 mg, 6%.

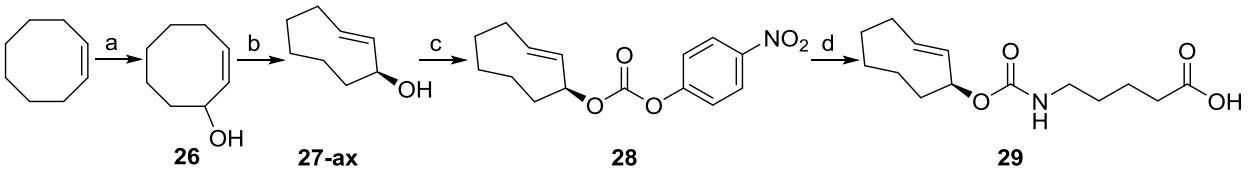
Prior to photophysical measurements HPLC purification was performed (30-60% Solvent B/Solvent A).

$^1$H-NMR (500 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.56 (d, J = 8.0 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.31 (d, J = 2.9 Hz, 2H), 7.10 (d, J = 9.6 Hz, 2H), 6.66 (dd, J = 9.7, 2.8 Hz, 2H), 3.44 (s, 12H), 3.15 (s, 3H), 2.18 (s, 3H), 0.69 (s, 3H), 0.68 (s, 3H). $^{13}$C-NMR (126 MHz, $CDCl_3$) δ 167.8, 167.7, 163.8, 154.4, 148.7, 143.2, 141.1, 137.5, 132.6, 130.2, 129.7, 127.2, 125.4, 121.5, 114.4, 41.5, 21.4, 19.8, -0.4, -0.7. HRMS (ESI$^+$) m/z 493.2530 calcd for $[C_{29}H_{33}N_6Si]^+$ ($M^+$), 493.2541 found.

## 5.11 Syntheses of TCO derivatives

**rel-(1R, 4E)-Cyclooct-4-en-1-ol** was prepared according to literature.[19]

**Phalloidin-TCO** was prepared according to literature.[7]

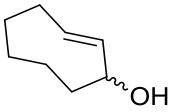



**Scheme S2.** TCO* syntheses. a: 1) NBS, AIBN, $CCl_4$, 2) $NaHCO_3$, acetone, $H_2O$, 48%; b: *hv*, $PhCOOCH_3$, $Et_2O$, petroleum ether, 29%; c: NVOC chloride, pyridine, 85%; d: 5-aminovaleric acid, DIPEA, DMF, 56%;

**(Z)-Cyclooct-2-en-1-ol (26)** was synthesized according to literature.[20]

**(*S,E*)-Cyclooct-2-en-1-yl (4-nitrophenyl) carbonate (28)** was synthesized according to literature.[21]

**(*E*)-Cyclooct-2-en-1-ol (27)** was synthesized following a modified literature procedure.[19]

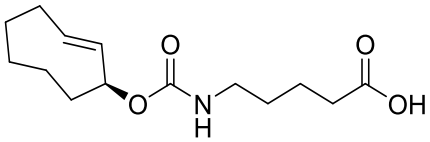



A column packed with silica gel (200 g) was impregnated with $AgNO_3$ (23.6 g). By passing $AgNO_3$ (25.0 g) dissolved in MeCN (1 L) through the column containing the silica, the column was impregnated. The column was then washed with cyclohexane (1 L).

**26** (15.6 g, 123 mmol) and methyl benzoate (15.6 mL, 123 mmol) were dissolved in $Et_2O$ (400 mL) and petroleum ether (800 mL). The reaction was irradiated at a wavelength of 253.7 nm by a Rayonet RMR-200 for 100 h, while constantly pumped through the $AgNO_3$ impregnated column (flow rate: 10 mL/min). After the reaction, the column was washed with DCM (1 L) and dried. The silica was collected and stirred in a 1:1 mixture of 25% ammonia water and DCM (2 L). The organic layer was separated and the aqueous layer was extracted two times with DCM (2 x 500 mL). The combined organic layers were dried over $Na_2SO_4$ and evaporated. The crude product was purified by flash chromatography (silica, 1-25% EtOAc/cyclohexane) to separate the axial and equatorial isomer. The axial isomer **27-ax** (4.49 g, 29%) was obtained as colorless clear oil. The equatorial isomer **27-eq** (3.72 g, 24%) was obtained as colorless clear oil.

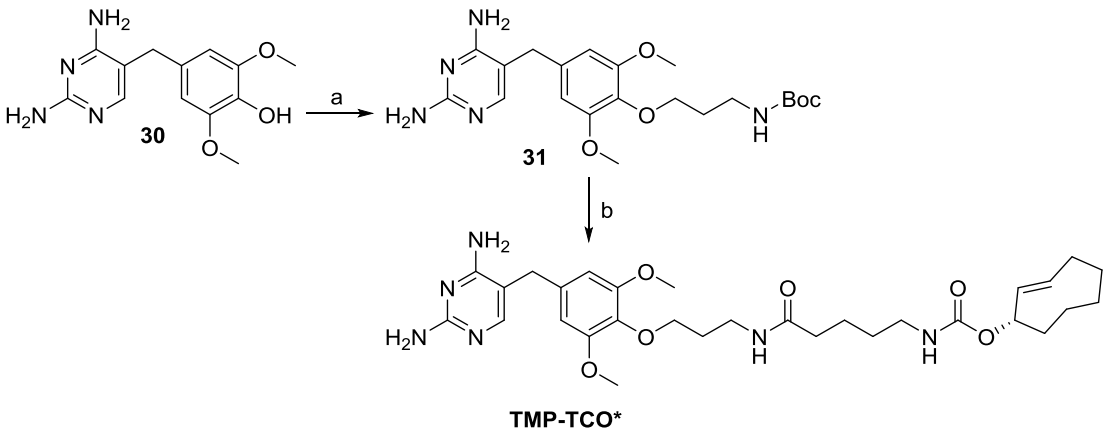
**27-ax**: $^{1}$H-NMR (300 MHz, $CDCl_3$) δ 6.05–5.87 (m, 1H), 5.57 (dd, J = 16.5, 2.3 Hz, 1H), 4.61 ("s", 1H), 2.54–2.42 (m, 1H), 2.10–1.78 (m, 4H), 1.74–1.36 (m, 4H), 1.18–1.03 (m, 1H), 0.83–0.69 (m, 1H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 135.27, 130.73, 71.43, 43.37, 36.23, 36.03, 29.36, 23.29. HRMS ($ESI^+$) m/z 149.0937 calcd for $[C_8H_{14}ONa]^+$ ($M+Na^+$), 149.0932 found.
**27-eq**: $^{1}$H-NMR (300 MHz, $CDCl_3$) δ 5.74–5.46 (m, 2H), 4.34–4.18 (m, 1H), 2.46–2.32 (m, 1H), 2.15 (m, 1H), 2.04–1.73 (m, 4H), 1.57–1.30 (m, 3H), 0.96–0.75 (m, 2H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 135.72, 132.16, 77.03, 44.51, 36.01, 35.56, 29.24, 27.79. HRMS ($ESI^+$) m/z 149.0937 calcd for $[C_8H_{14}ONa]^+$ ($M+Na^+$), 149.0929 found.

**(*S,E*)-5-(((cyclooct-2-en-1-yloxy)carbonyl)amino)pentanoic acid (29)**




5-Aminovaleric acid (117 mg, 1.00 mmol) was dissolved in DMF (2.5 mL) at 0 °C. **28** (200 mg, 0.69 mmol) and DIPEA (233 µL, 1.37 mmol) were added to the solution and the mixture was stirred overnight at room temperature in the dark. The solvent was evaporated and the crude product was purified by column chromatography (silica, 6% MeOH/DCM) to yield **29** (151 mg, 56%) as a white solid.

$^{1}$H-NMR (300 MHz, $CD_3OD$) δ 5.87 – 5.78 (m, 1H), 5.54 (dd, J = 16.4, 2.3 Hz, 1H), 5.23 (s, 1H), 4.80 (bs, 1H), 3.11 (t, J = 6.8 Hz, 2H), 2.50–2.38 (m, 1H), 2.22 (t, J = 7.2 Hz, 2H), 2.11–1.40 (m, 12H), 1.23–1.07 (m, 1H), 0.95–0.79 (m, 1H). $^{13}$C-NMR (75 MHz, $CD_3OD$) δ 177.35, 158.43, 132.93, 132.51, 75.01, 41.65, 41.25, 37.04, 36.77, 34.52, 30.38, 30.11, 25.19, 23.25. HRMS ($ESI^+$) m/z 292.1519 calcd for $[C_{14}H_{23}NO_4Na]^+$ ($M+Na^+$), 292.1516 found.

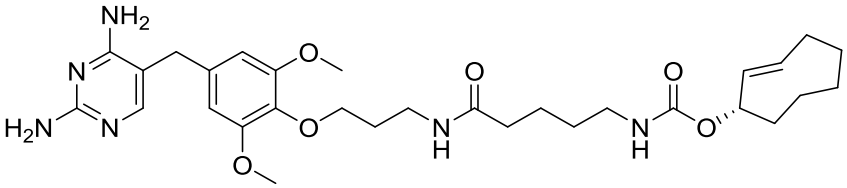



**Scheme S3.** Synthesis of TMP-TCO*. a: 48% HBr, $H_2O$, 60%; b: *tert*-butyl (3-iodopropyl)carbamate, $Cs_2CO_3$, DMF, 59%.

**4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenol (30)** was synthesized according to literature.[22]

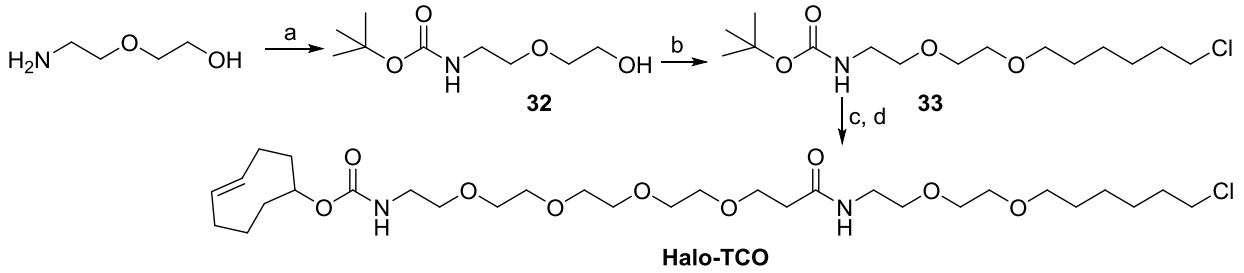
***tert*-Butyl (3-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)carbamate (31)** was synthesized according to literature.[22]

**(*S,E*)-cyclooct-2-en-1-yl (5-((3-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenoxy)propyl)amino)-5-oxopentyl)carbamate (TMP-TCO*)**




**31** (30 mg, 69 µmol) was dissolved in 4 M HCl/dioxane (1 mL) under argon at 0 °C. The reaction was stirred for 3 h. The completion of the deprotection was checked by TLC. The solvent was evaporated *in vacuo*. Without further purification, DIPEA (11.7 µL, 69 µmol) dissolved in DMF (1 mL) was added and stirred for 10 min. Then, **25** (19.7 mg, 69 µmol) mixed with DIPEA (11.7 µL, 69 µmol) and *O*-(6-Chlorobenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HCTU) (30 mg, 69 µmol) in DMF (1 mL) was added to the solution. The reaction was stirred for 2 h and the solvent was evaporated *in vacuo*. The crude product was purified by HPLC (30%-70% Solvent B/Solvent A in 45 min). HPLC purification yielded **TMP-TCO*** (20.4 mg, 51%) as a colorless resin.

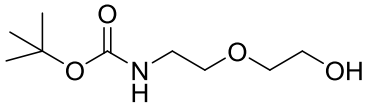
$^1$H-NMR (500 MHz, $CD_3OD$) δ 7.23 (s, 1H), 6.57 (s, 2H), 5.87 – 5.78 (m, 1H), 5.54 (dd, *J* = 16.4, 2.3 Hz, 1H), 5.21 (s, 1H), 3.97 (t, *J* = 6.1 Hz, 2H), 3.82 (s, 6H), 3.67 (s, 2H), 3.40 (t, *J* = 6.8 Hz, 2H), 3.09 (t, *J* = 6.8 Hz, 2H), 2.48 – 2.39 (m, 1H), 2.21 (t, *J* = 7.4 Hz, 2H), 2.07 – 1.93 (m, 3H), 1.91 – 1.82 (m, 3H), 1.76 – 1.69 (m, 1H), 1.63 (m, 3H), 1.50 (m, 3H), 1.20 – 1.10 (m, 1H), 0.92 – 0.81 (m, 1H). $^{13}$C-NMR (126 MHz, $CD_3OD$) δ 166.30, 156.14, 155.04, 140.38, 137.15, 133.79, 132.95, 132.54, 111.06, 107.29, 75.03, 72.47, 56,67, 49.85, 41.65, 41.19, 38.11, 37.04, 36.77, 33.93, 30.77, 30.40, 30.10, 25.20, 24.20. HRMS (ESI$^+$) m/z 585.3395 calcd for $[C_{30}H_{45}N_6O_6]^+$ (M+H$^+$), 585.3392 found.




**Scheme S4.** Synthesis of Halo-TCO. a: $Boc_2O$, EtOH, 99%; b: 1-chloro-6-iodohexane, NaH, THF, DMF, 37%; c: 4 M HCl/dioxane, 97%; d: TCO-PEG4-NHS ester, DCM, DIPEA, 65%.

***tert*-Butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate (33)** was synthesized according to literature.[23]

***tert*-butyl (2-(2-hydroxyethoxy)ethyl)carbamate (32)**

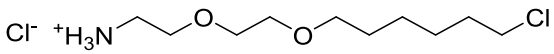


Di-*tert*-butyldicarbonate (57.3g, 263 mmol) was dissolved in ice cold isopropanol (100 mL) and $Et_3N$ (50 mL, 360 mmol) was added. The reaction was cooled to 0 °C and 2-(2-aminoethoxy)ethanol (24.4 g, 232 mmol) dissolved in 100 mL isopropanol was added. The reaction was stirred at rt for 16 h. The product **32** (47.1 g, 99%) was obtained as a colorless liquid by evaporation in high vacuum.

$^1$H-NMR (300 MHz, DMSO-$d_6$) δ 6.74 (t, *J* = 5.9 Hz, 1H), 4.56 (t, *J* = 5.5 Hz, 1H), 3.47 (m, 2H), 3.42 – 3.34 (m, 4H), 3.06 (q, *J* = 6.0 Hz, 2H), 1.37 (s, 9H). $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 156.03,

78.02, 72.49, 69.61, 60.64, 40.22, 28.67. HRMS (ESI$^+$) m/z 228.1206 calcd for $[C_9H_{19}NO_4Na]^+$ (M+Na $^+$), 228.1208 found.

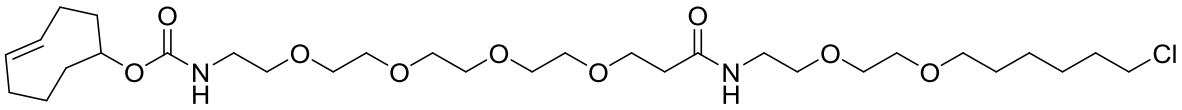
**2-(2-((6-chlorohexyl)oxy)ethoxy)ethan-1-amine (34)**



**33** (415 mg, 1.2 mmol) was dissolved in dioxane (250 µL) under argon at 0 °C. 4 M HCl/dioxane (1 mL) was slowly added at 0 °C and the mixture was stirred for 45 min at RT. The completion of the deprotection was checked by TLC. The solvent was evaporated *in vacuo* and the crude product was dissolved in $H_2O$ (pH = 5, 5 mL) and diethyl ether (5 mL) was added. The aqueous layer was separated and lyophilized yielding **34** (280 mg, 90%) as a pale-yellow solid.

$^1$H-NMR (300 MHz, $CDCl_3$) δ 8.35 (s, 3H), 3.82 (t, *J* = 4.9 Hz, 2H), 3.72 – 3.64 (m, 2H), 3.64 – 3.57 (m, 2H), 3.54 (t, *J* = 6.7 Hz, 2H), 3.47 (t, *J* = 6.7 Hz, 2H), 3.23 (t, *J* = 5.0 Hz, 2H), 1.78 (p, *J* = 6.8 Hz, 2H), 1.60 (p, *J* = 6.9 Hz, 2H), 1.52 – 1.30 (m, 4H).$^{13}$C-NMR (75 MHz, $CDCl_3$) δ 71.45, 70.58, 70.06, 66.77, 45.22, 39.84, 32.66, 29.50, 26.82, 25.51. HRMS (ESI$^+$) m/z 224.1412 calcd for $[C_{10}H_{23}ClNO_2]^+$ (M+H$^+$), 224.1418 found.

**(*E*)-cyclooct-4-en-1-yl (28-chloro-15-oxo-3,6,9,12,19,22-hexaoxa-16-azaoctacosyl)carbamate (Halo-TCO)**

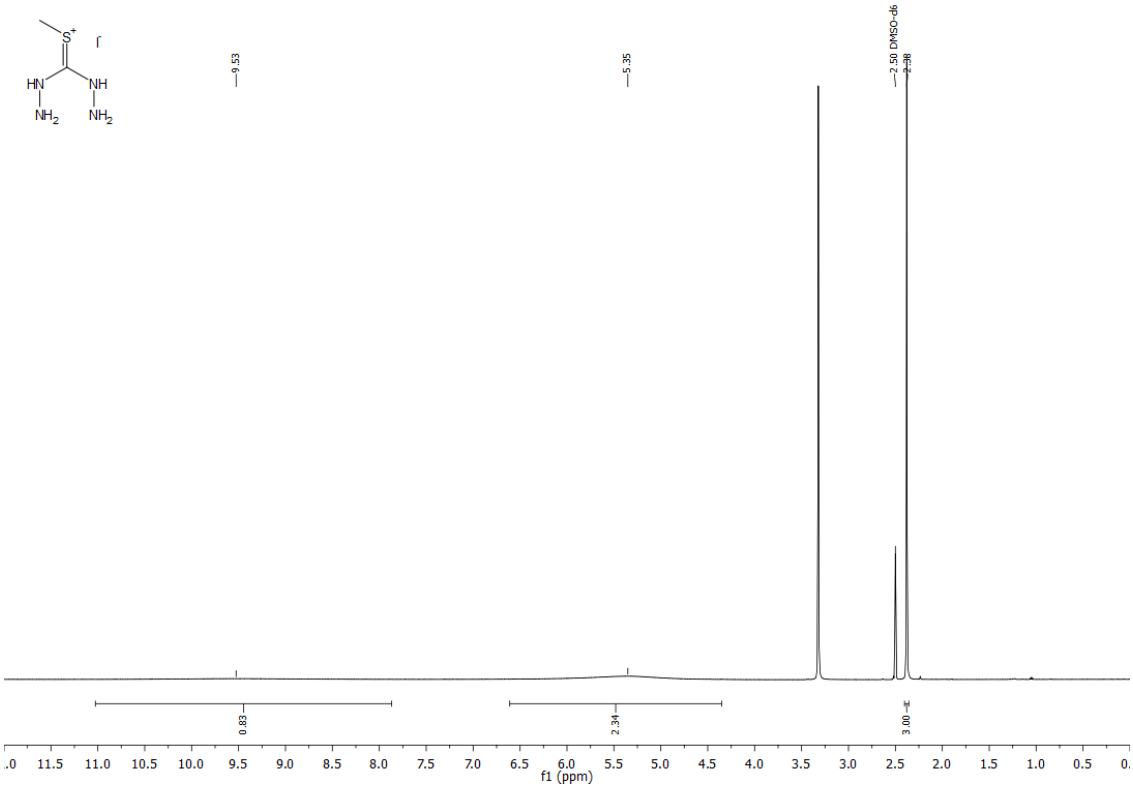


**31** (1.8 mg, 8.1 µmol) was dissolved in dry DCM (610 µL) and TCO-PEG-4-NHS ester (4 mg, 7.7µmol) dissolved in dry DCM (390 µL) were mixed and DIPEA (2.8 µL, 16.2 µmol) was added. The mixture was stirred for 2 h in the dark at RT. The solvent was evaporated and the crude product was purified by HPLC (10%-70% Solvent B/Solvent A in 50 min) yielding **Halo-TCO** (3.23 mg, 65%).

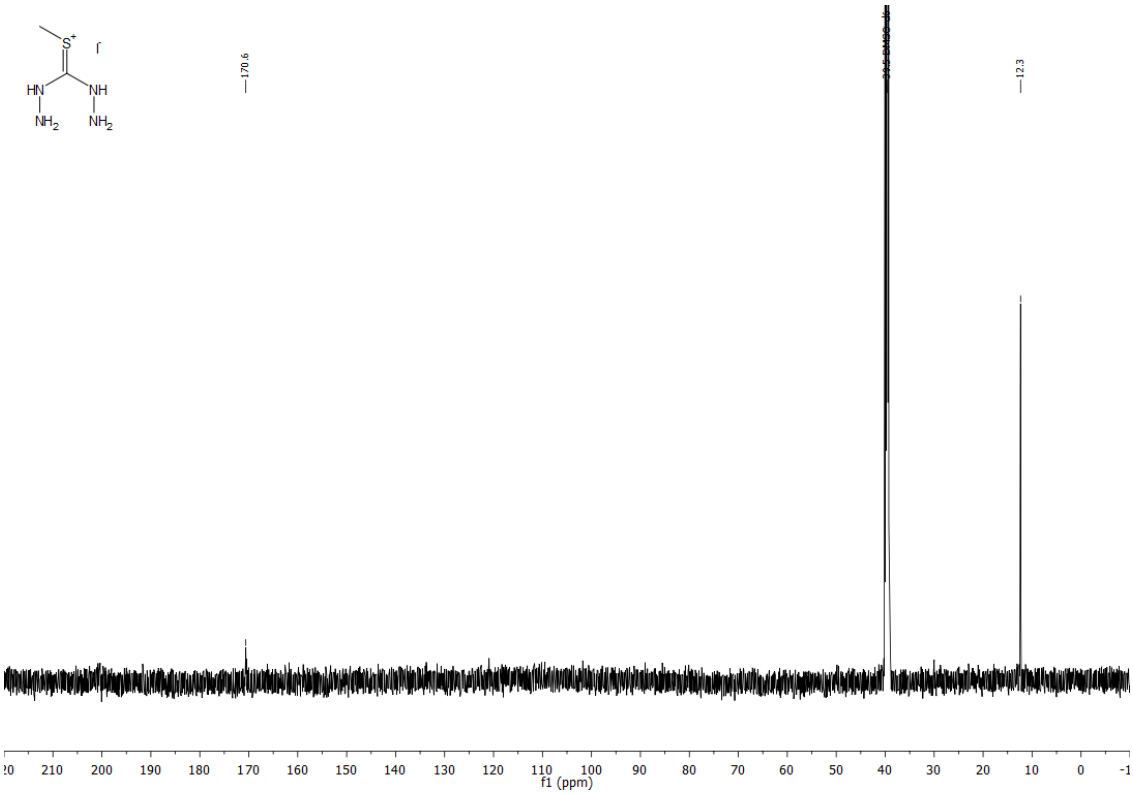
$^1$H-NMR (300 MHz, DMSO-$d_6$) δ 7.62 (s, 1H), 6.64 (s, 1H), 5.78 – 5.32 (m, 2H), 4.70 – 4.20 (m, 1H), 3.62 (q, *J* = 6.5 Hz, 4H), 3.57 – 3.48 (m, 16H), 3.48 – 3.38 (m, 6H), 3.25 (q, *J* = 5.8 Hz, 2H), 3.19 – 3.10 (m, 2H), 2.35 (t, *J* = 6.3 Hz, 2H), 2.3520 – 2.23 (m, 2H), 2.23 – 2.05 (m, 2H), 1.97 – 1.23 (m, 14H). HRMS (ESI$^+$) m/z 645.3488 calcd for $[C_{30}H_{55}ClN_2O_9Na]^+$ (M+Na $^+$), 645.3484 found.

# 6 NMR-Spectra

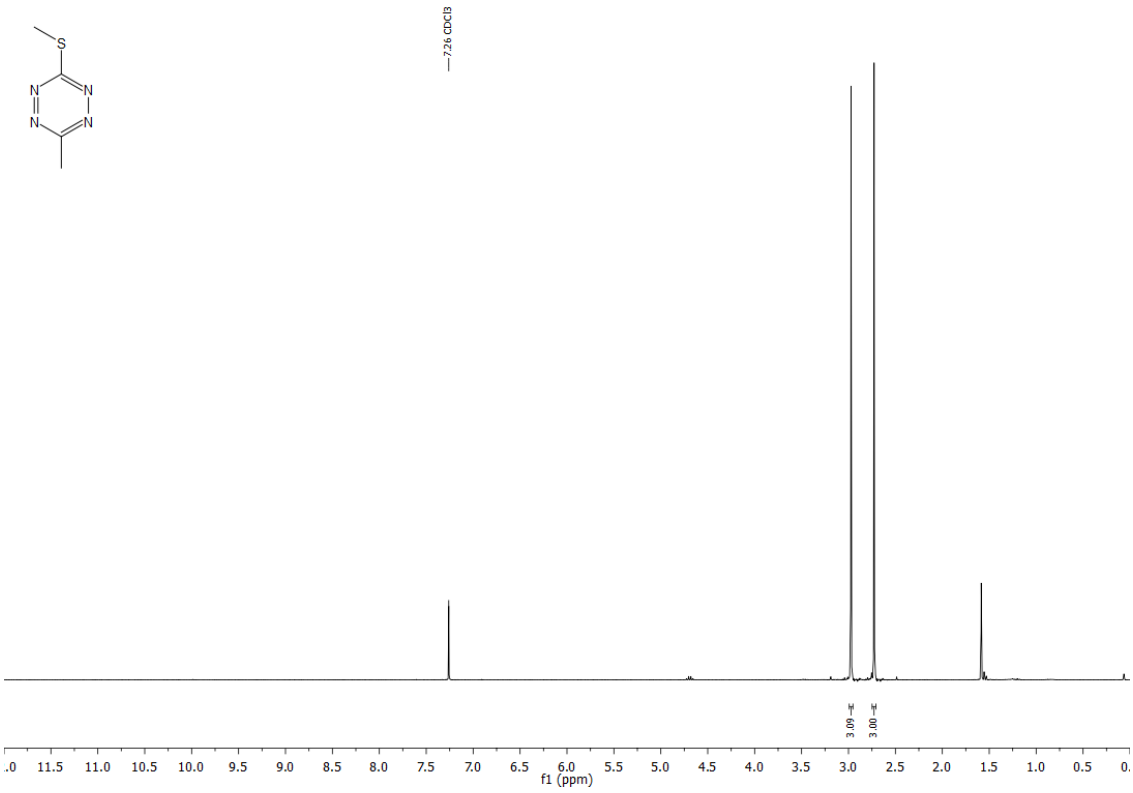
$^{1}$H-NMR of compound **2** (DMSO-$d_6$)



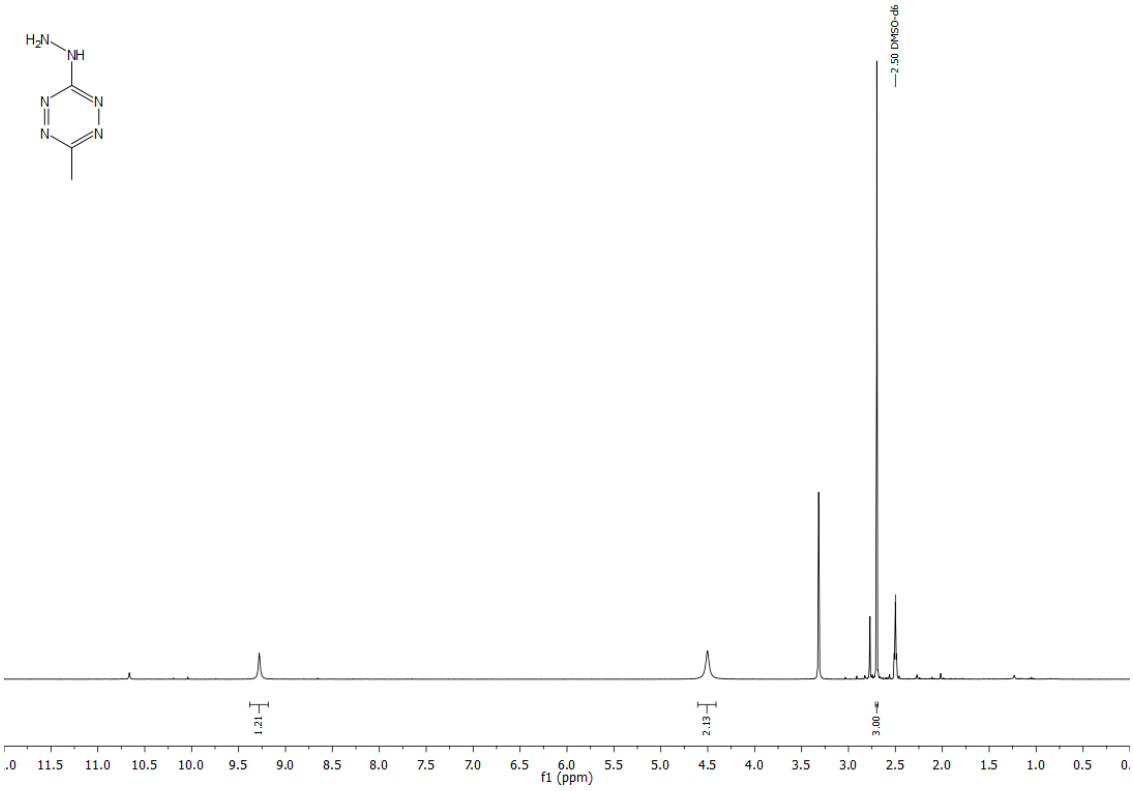

$^{13}$C-NMR of compound **2** (DMSO-$d_6$)



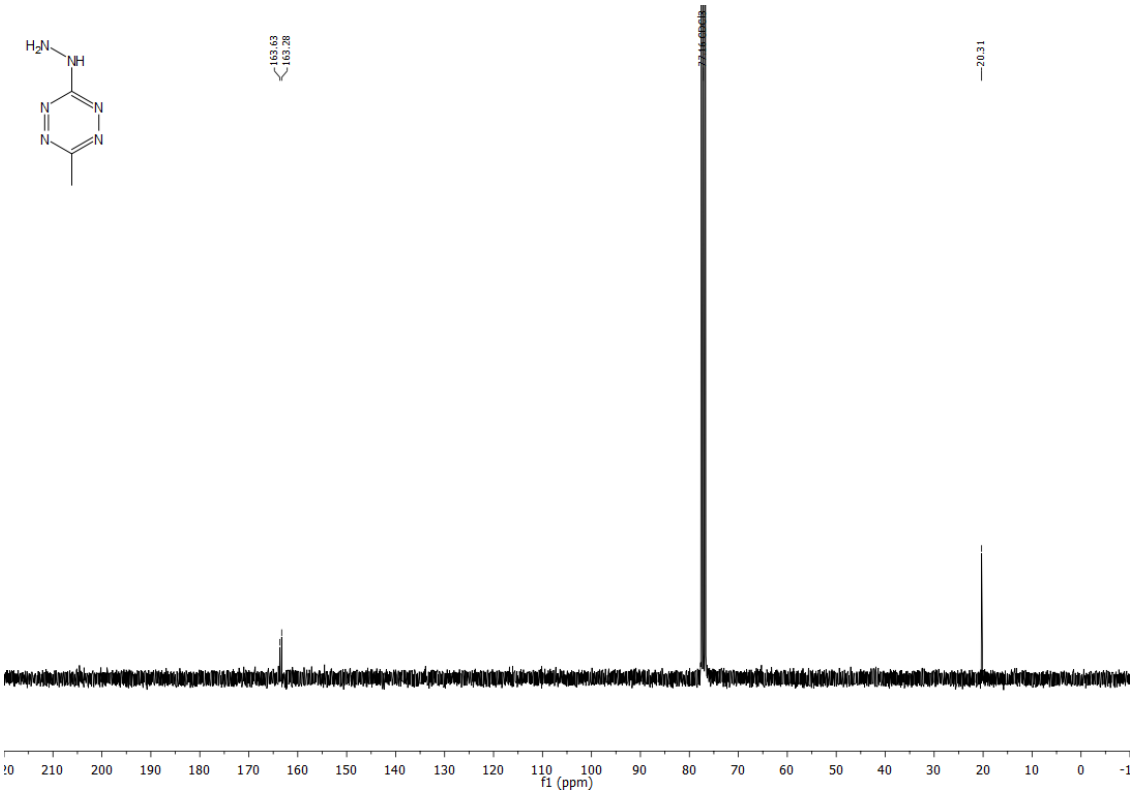
$^{1}$H-NMR of compound **3** ($CDCl_3$)



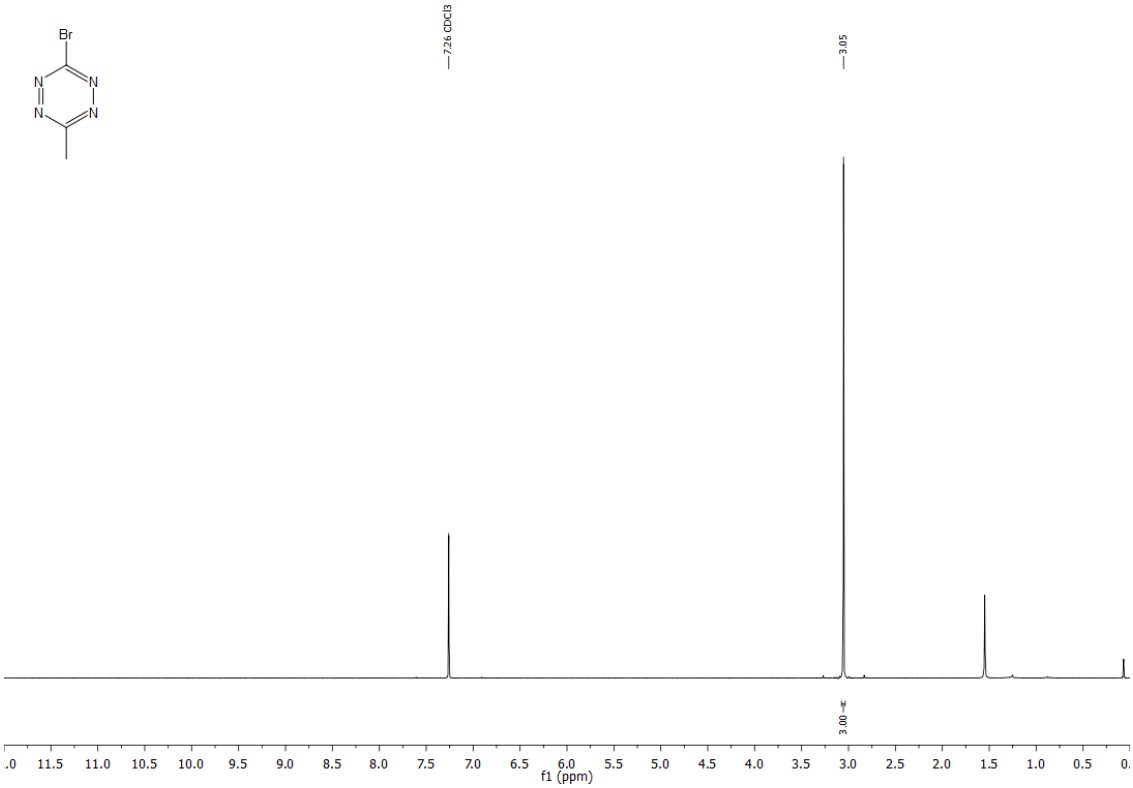
$^{1}$H-NMR of compound **4** (DMSO-$d_6$)



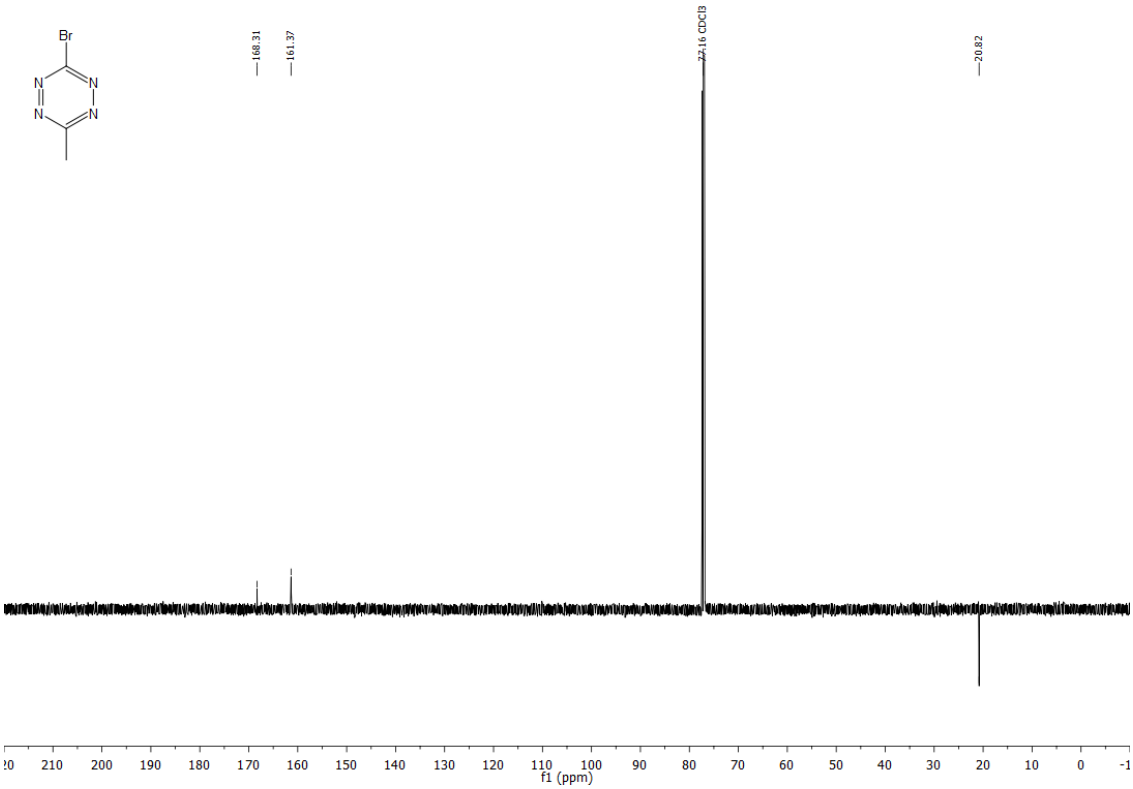

$^{13}$C-NMR of compound **4** ($CDCl_3$)



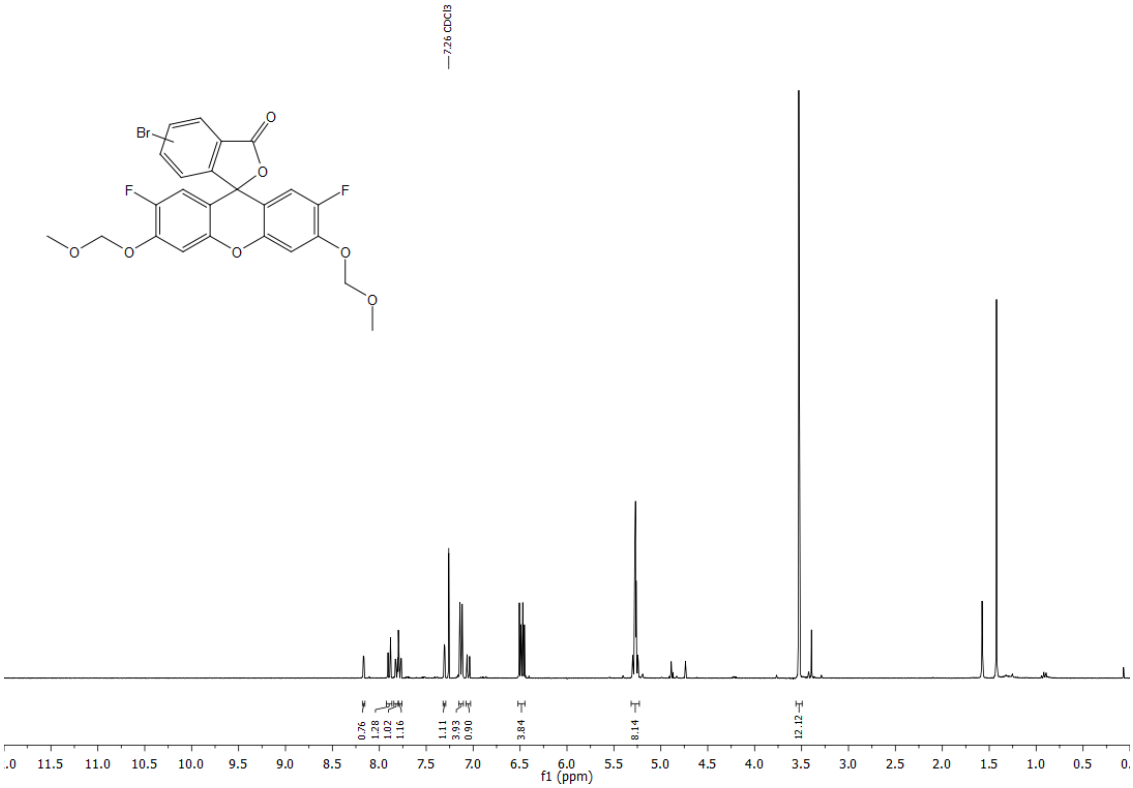
$^{1}$H-NMR of compound **5** ($CDCl_3$)



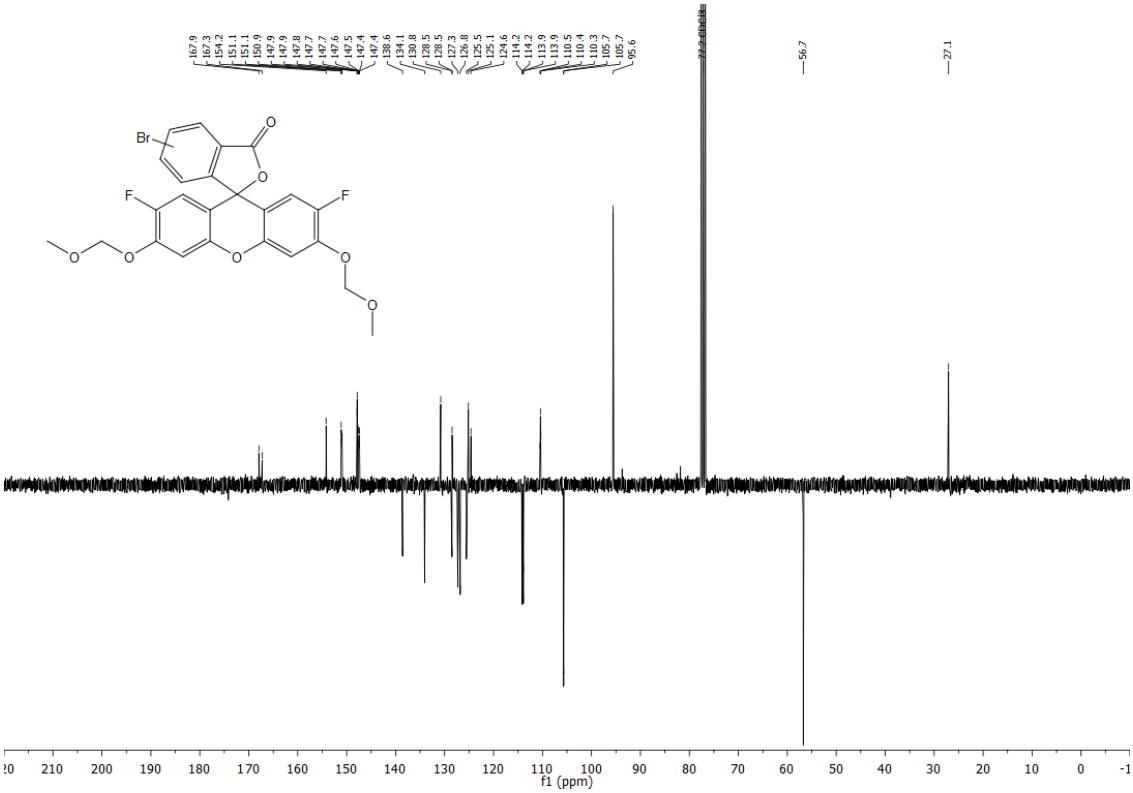

$^{13}$C-NMR of compound **5** ($CDCl_3$)



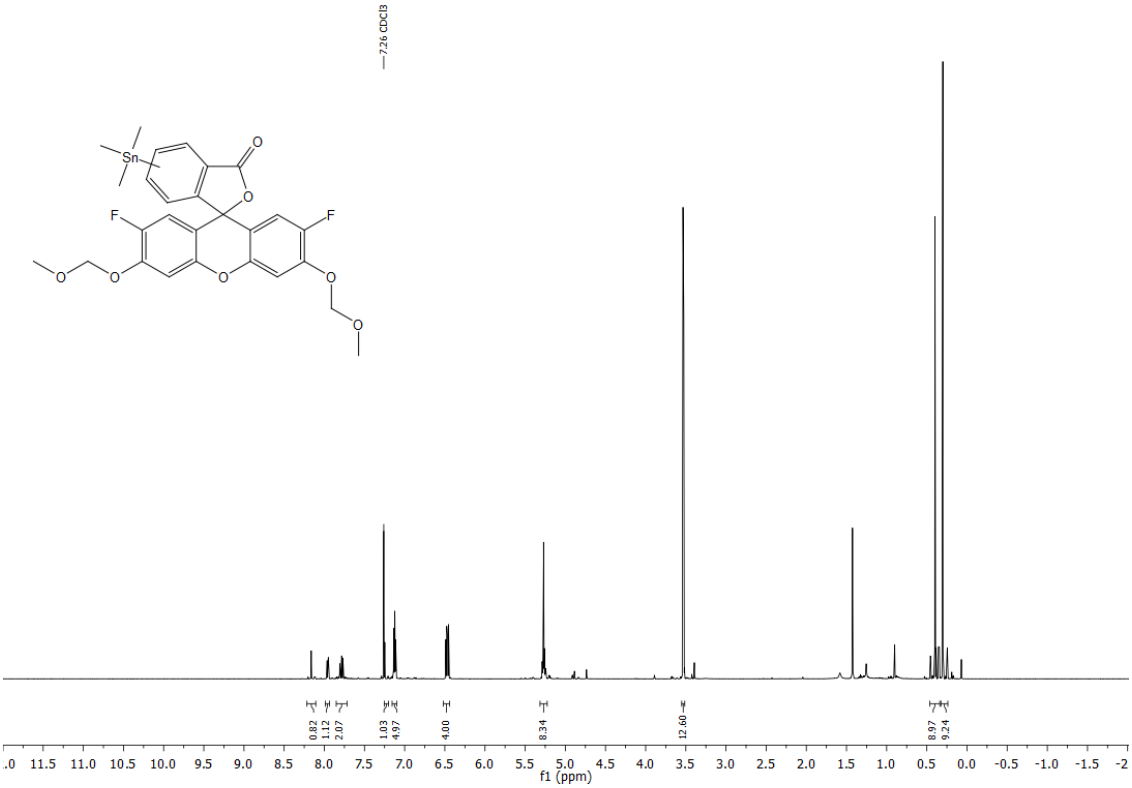
$^{1}$H-NMR of compound **7** ($CDCl_3$)



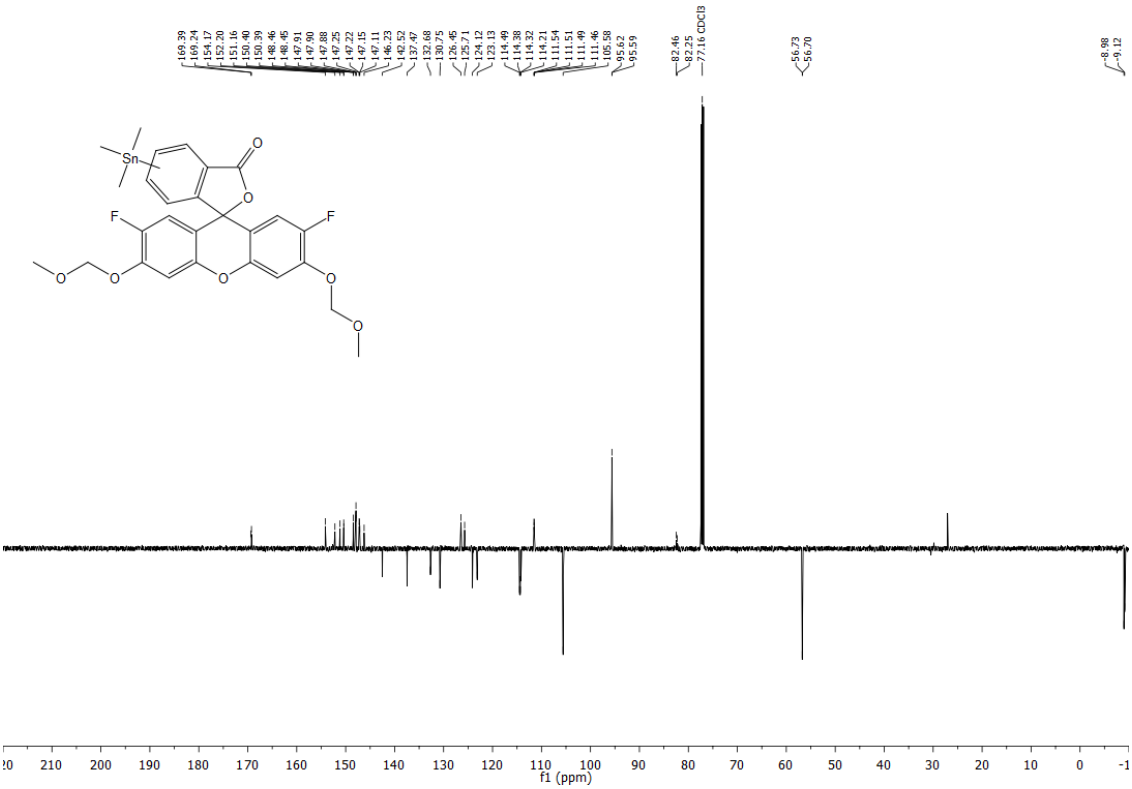

$^{13}$C-NMR of compound **7** ($CDCl_3$)



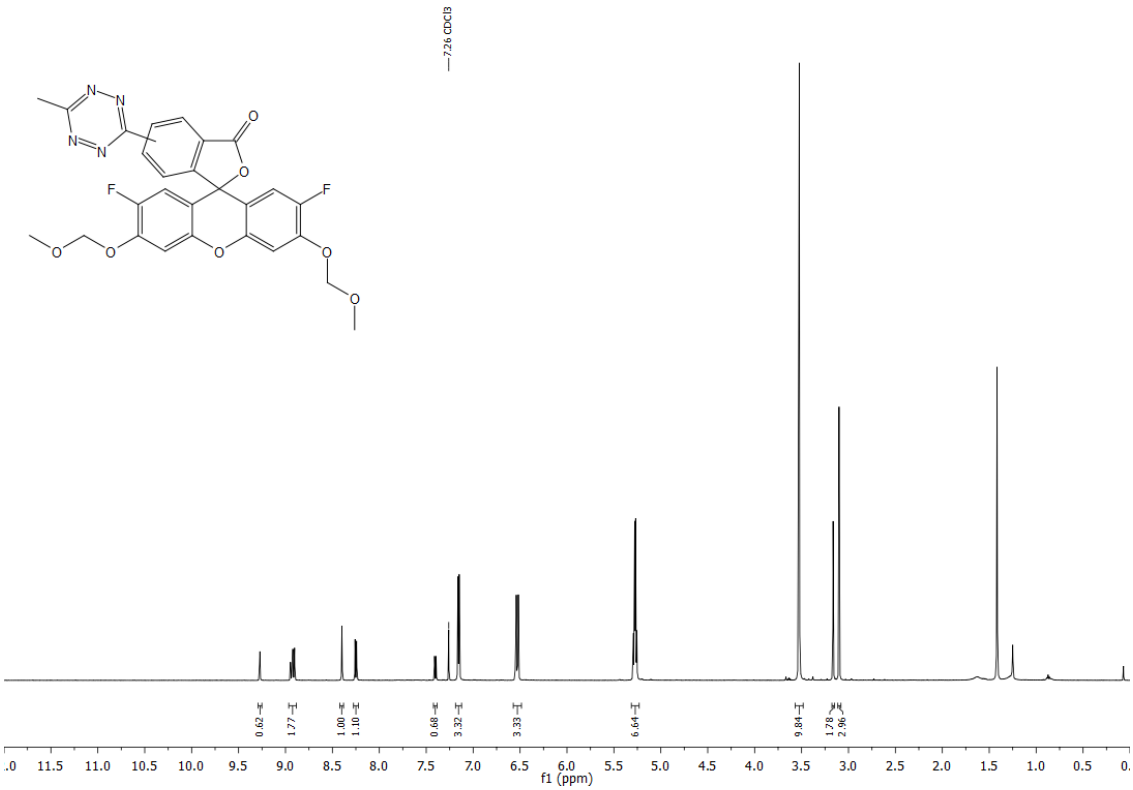
$^{1}$H-NMR of compound **8** ($CDCl_3$)



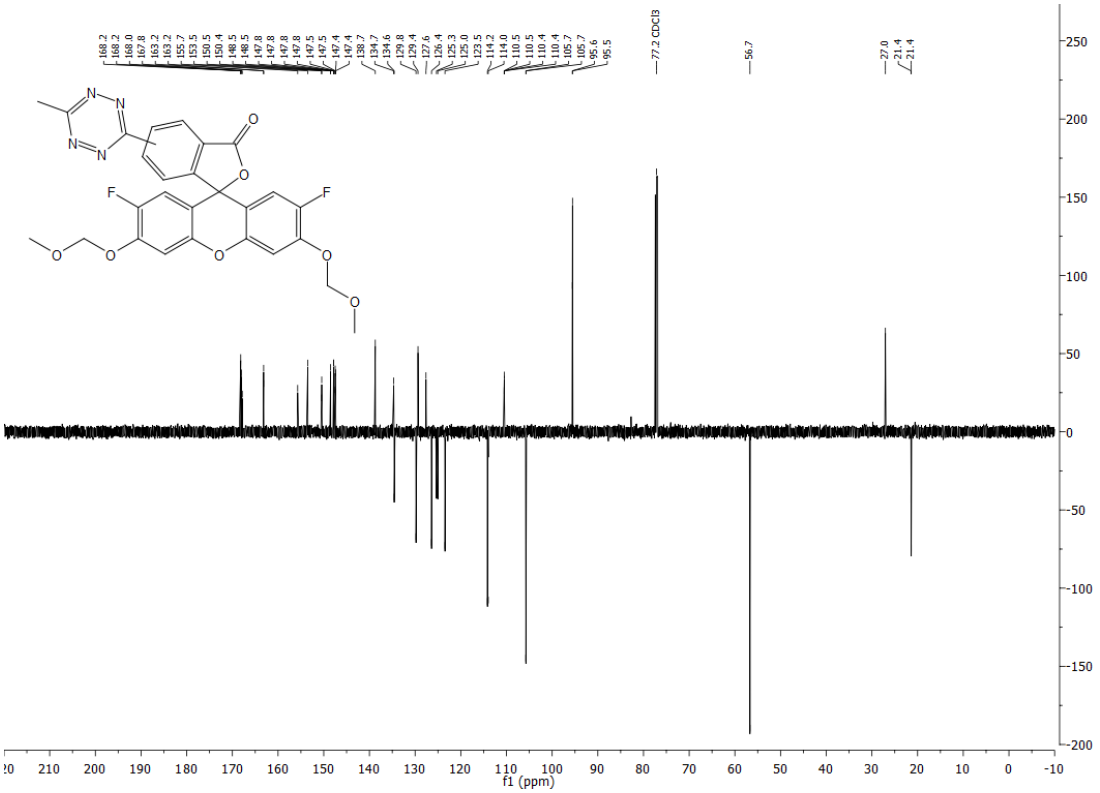

$^{13}$C-NMR of compound **8** ($CDCl_3$)



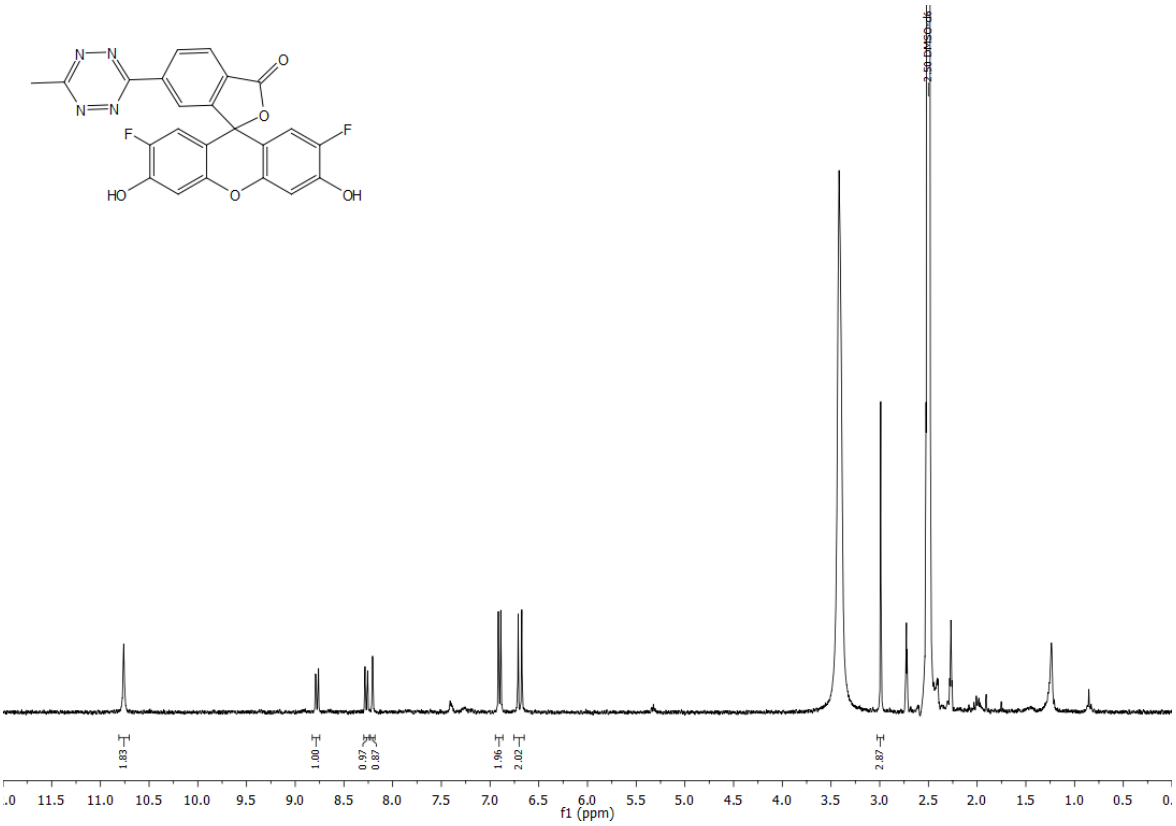
$^{1}$H-NMR of compound **9** ($CDCl_3$)



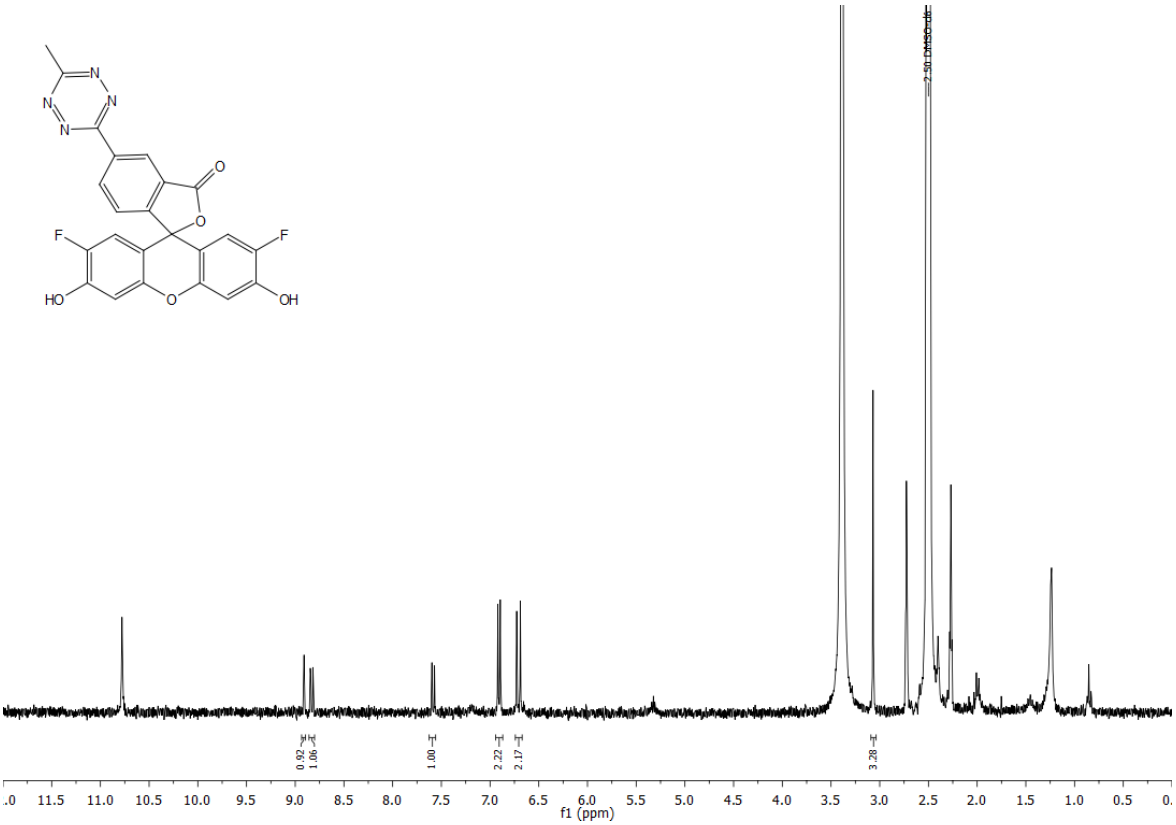

$^{13}$C-NMR of compound **9** ($CDCl_3$)



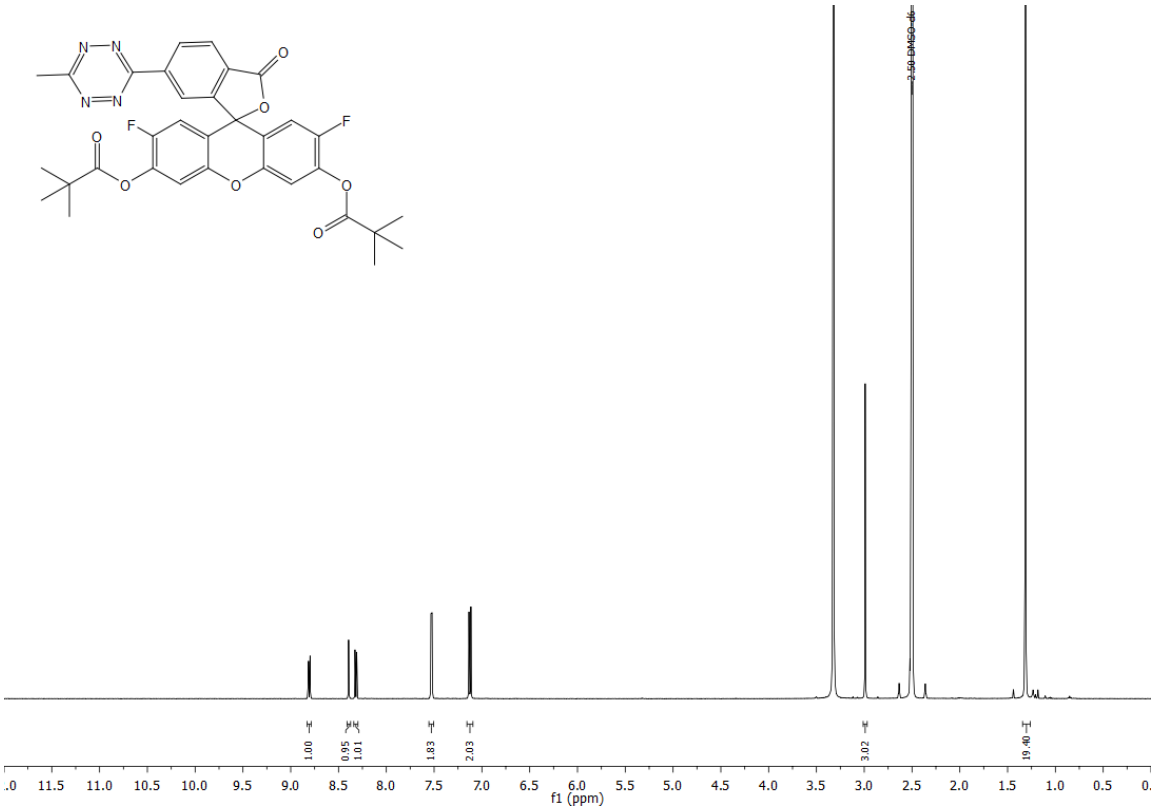
$^{1}$H-NMR of compound **OG-6-Tz** (DMSO-$d_6$)



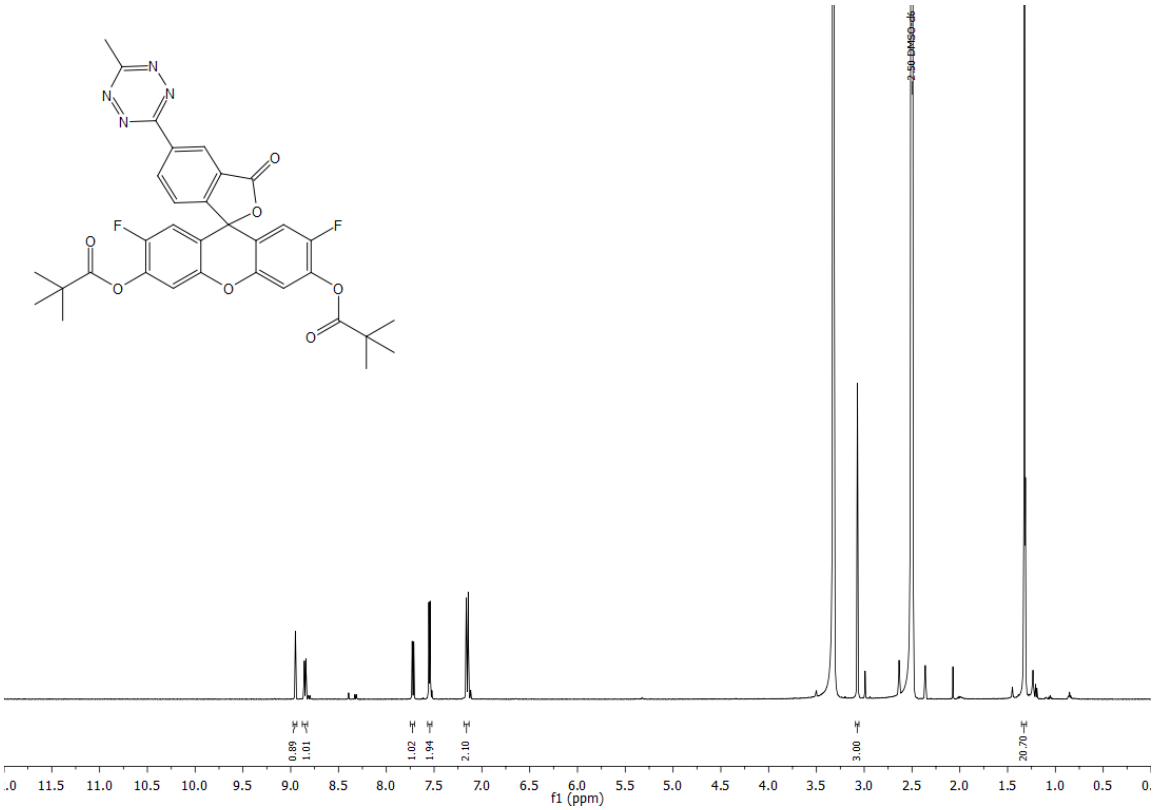

$^{1}$H-NMR of compound **OG-5-Tz** (DMSO-$d_6$)



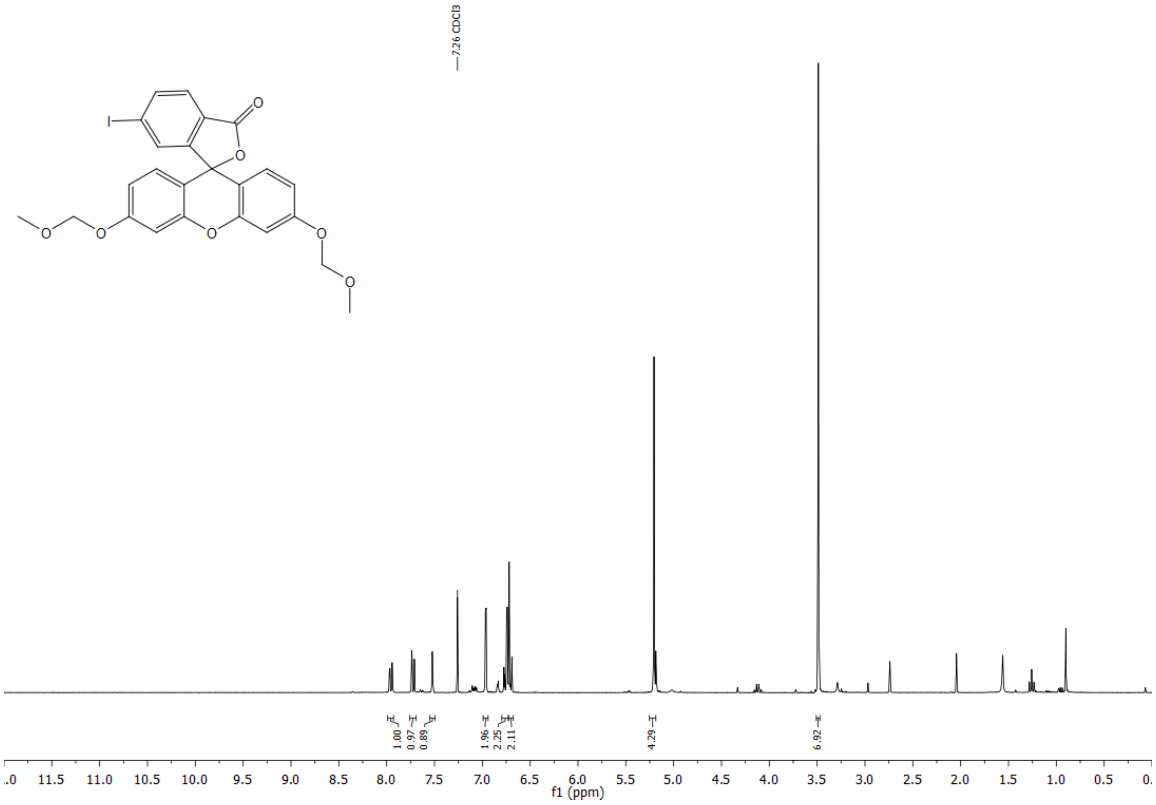
$^{1}$H-NMR of compound **OG-6-Tz(piv)$_2$** ($CDCl_3$)



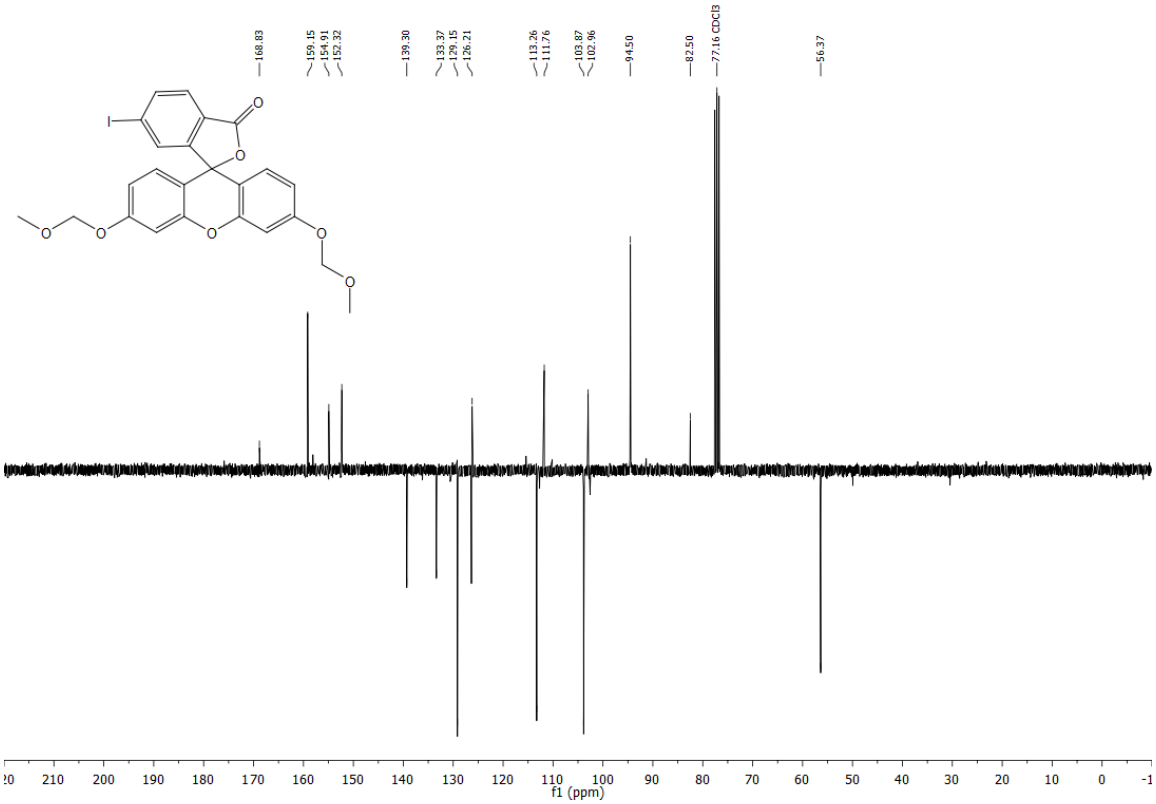

$^{1}$H-NMR of compound **OG-5-Tz(piv)$_2$** ($CDCl_3$)



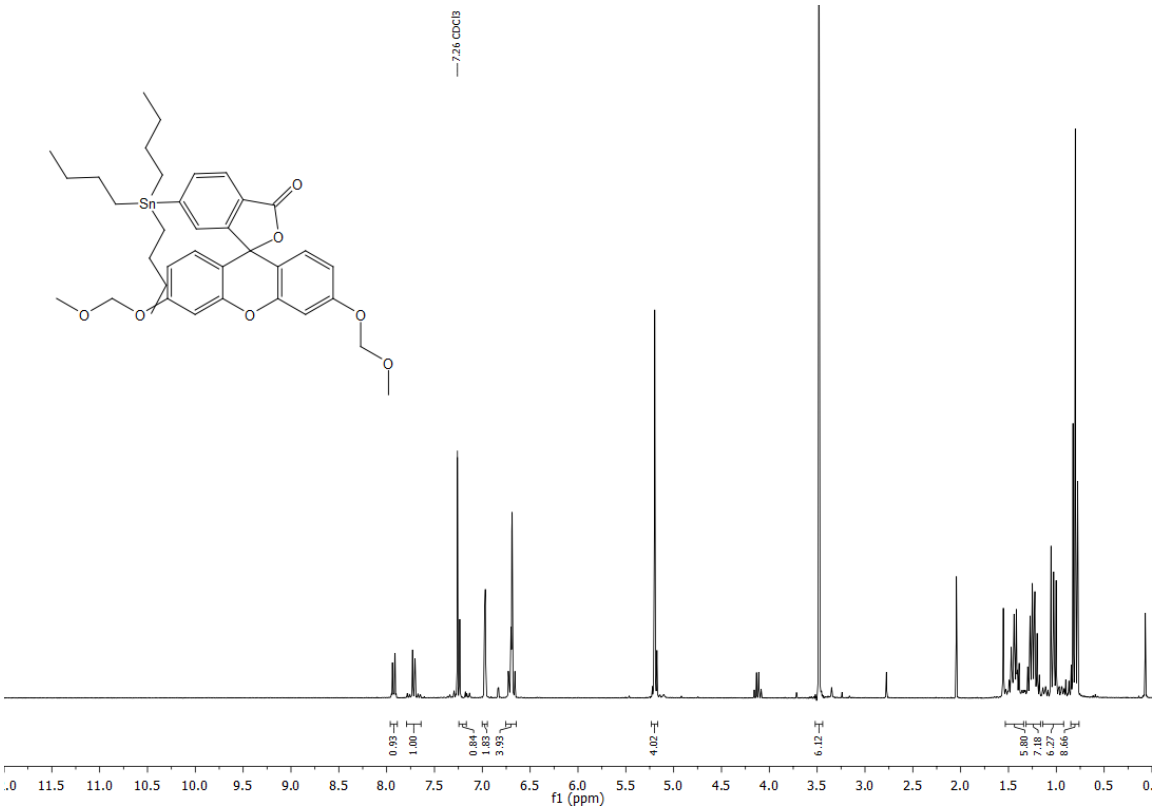
$^{1}$H-NMR of compound **11** ($CDCl_3$)



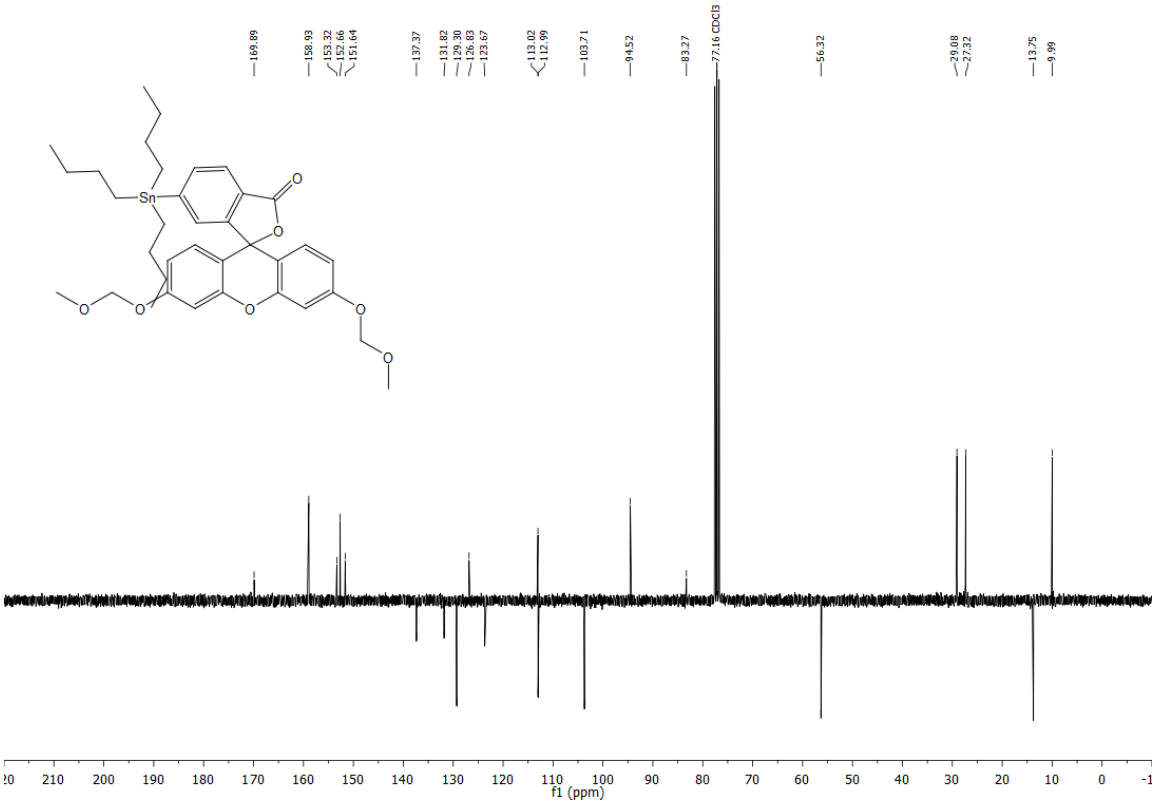

$^{13}$C-NMR of compound **11** ($CDCl_3$)



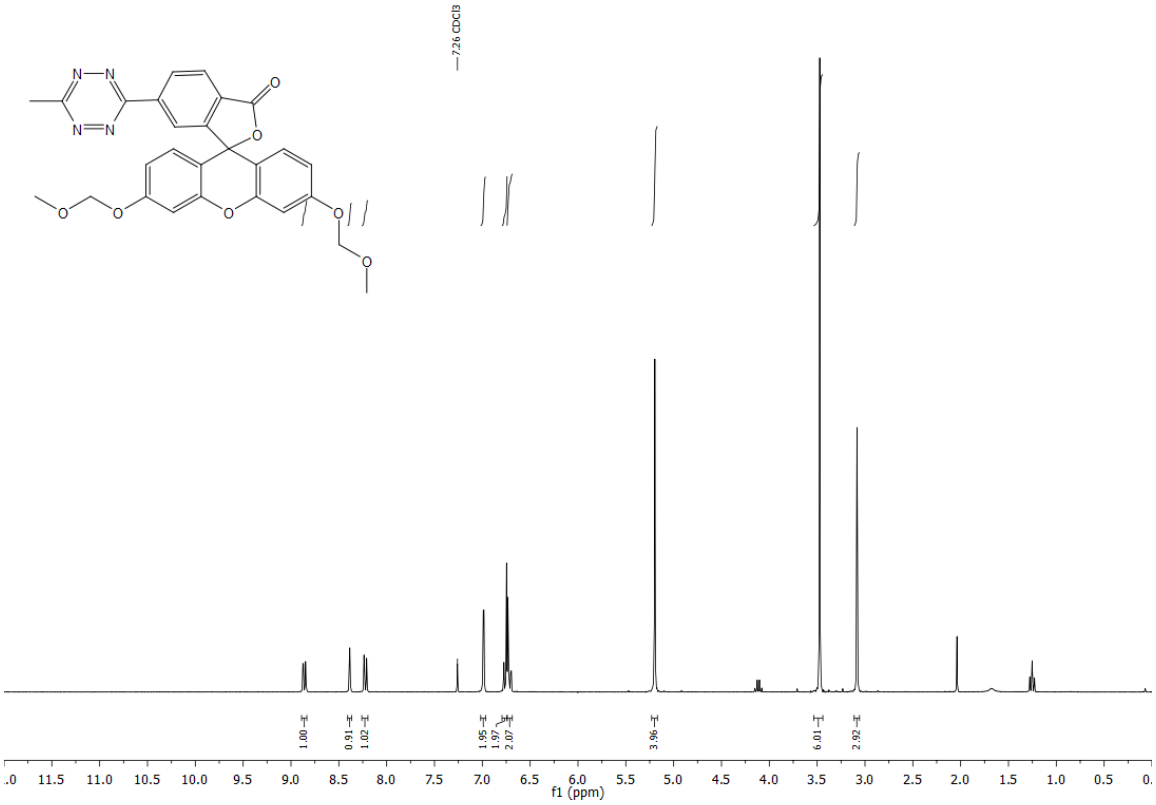
$^{1}$H-NMR of compound **12** ($CDCl_3$)



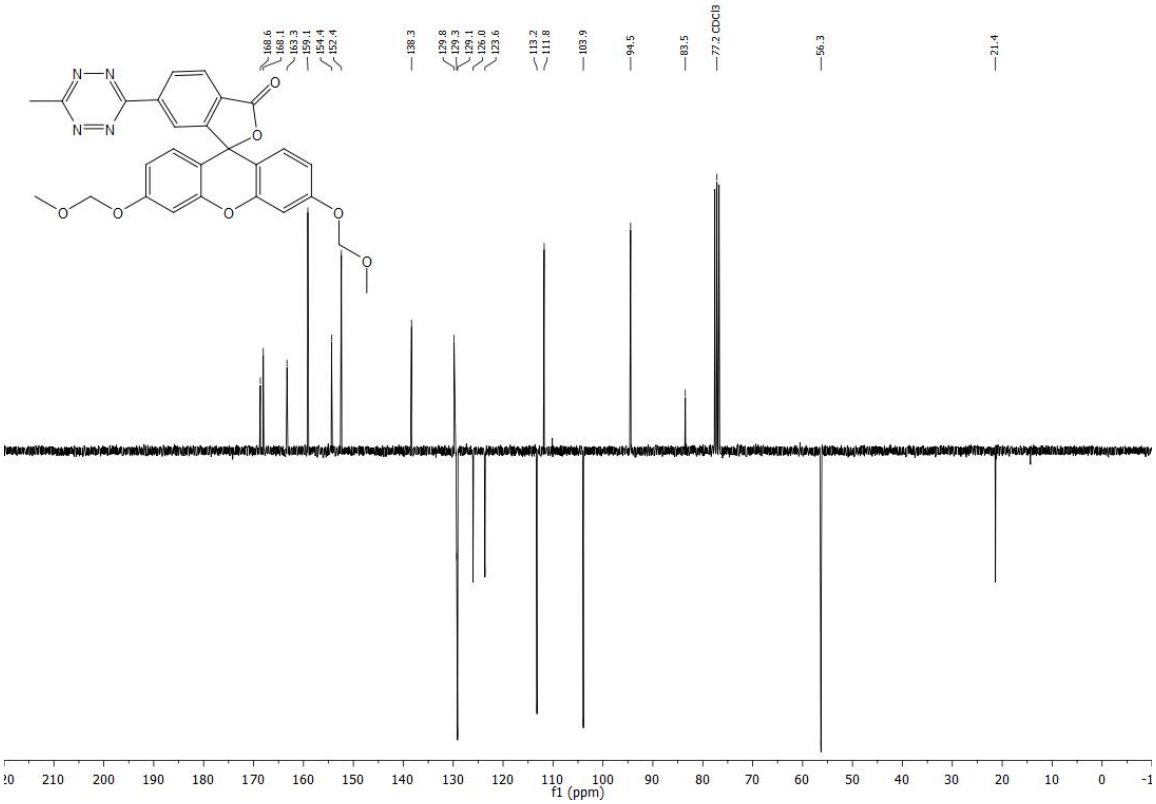

$^{13}$C-NMR of compound **12** ($CDCl_3$)



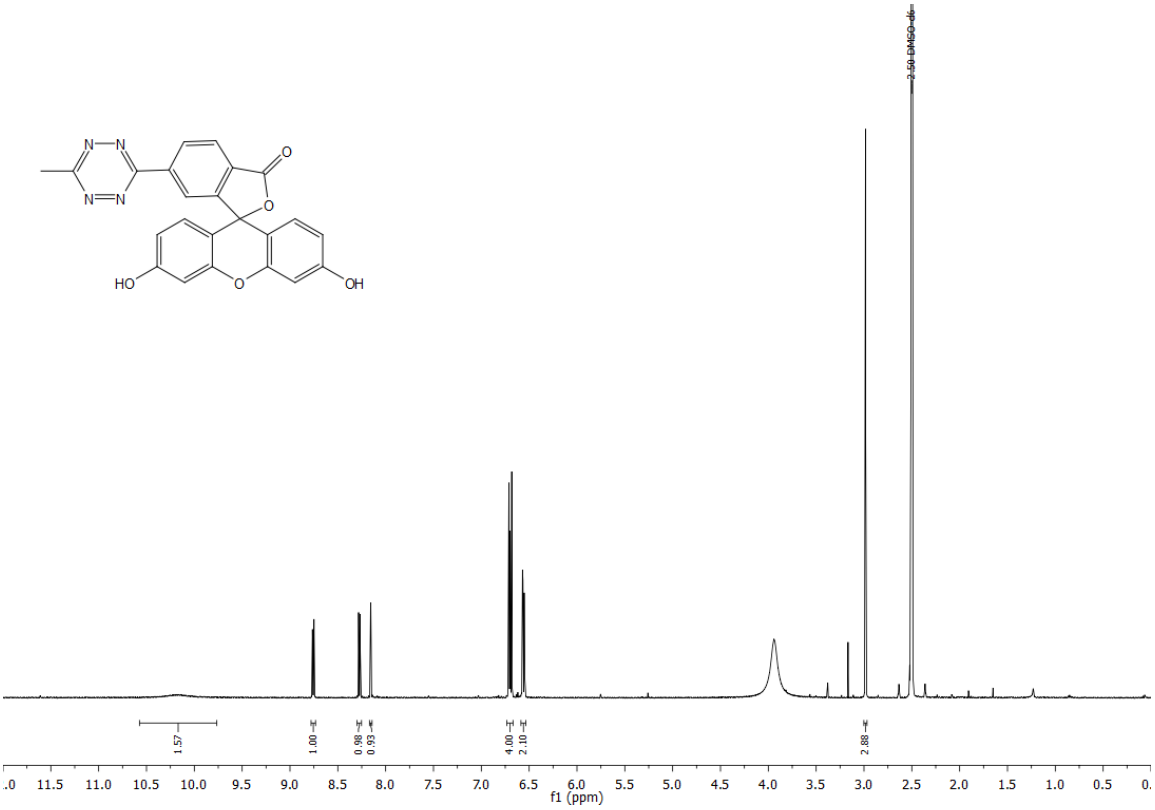
$^{1}$H-NMR of compound **13** ($CDCl_3$)



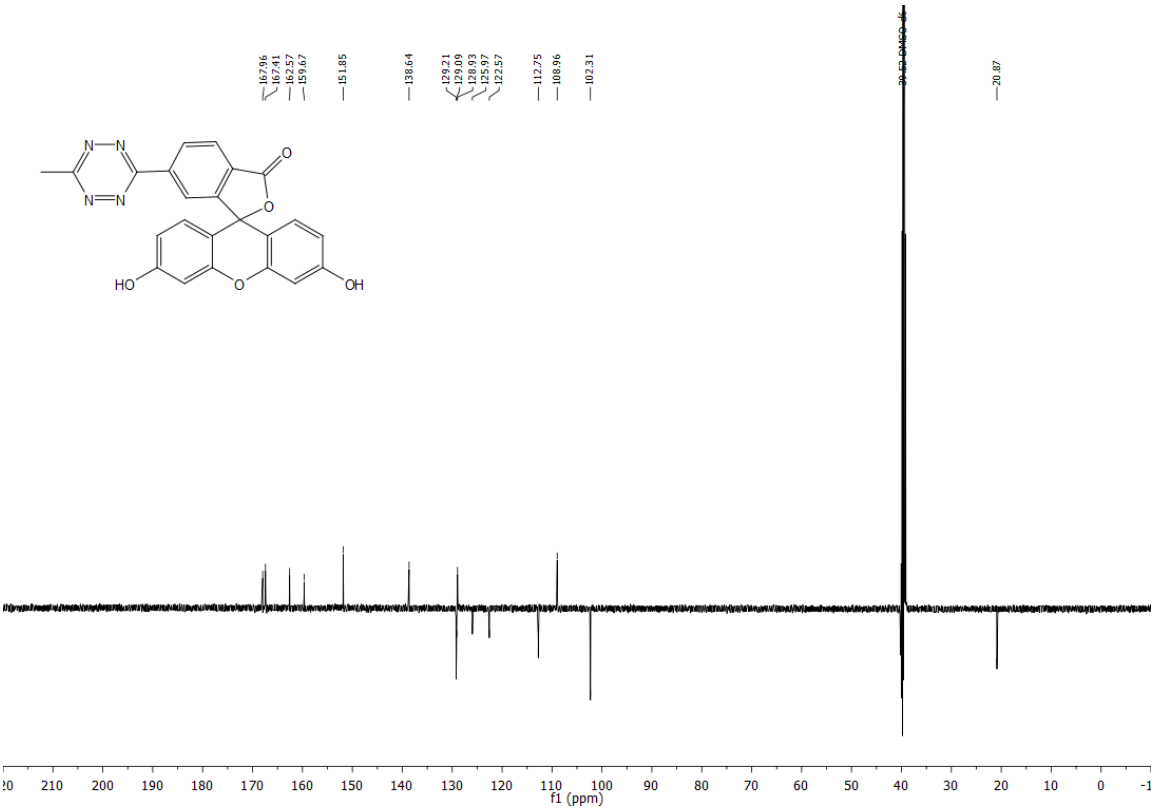

$^{13}$C-NMR of compound **13** ($CDCl_3$)



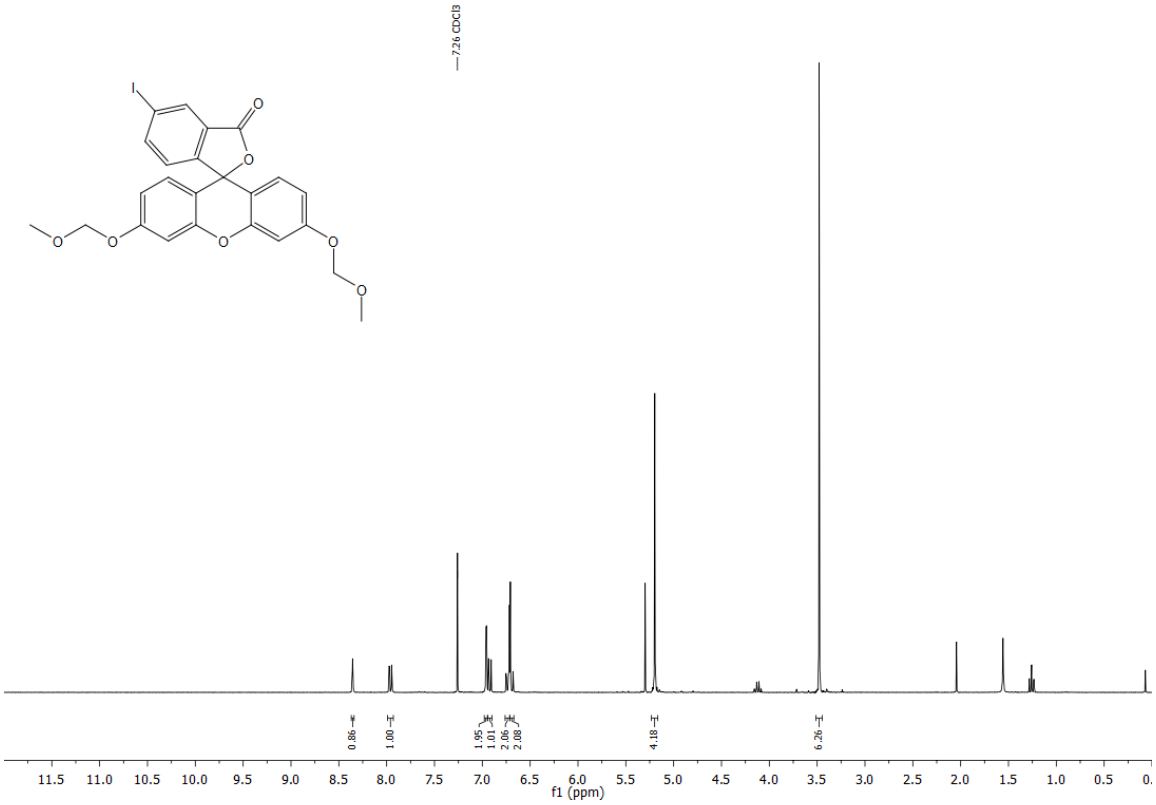
$^{1}$H-NMR of compound **Fl-6-Tz** (DMSO-$d_6$)



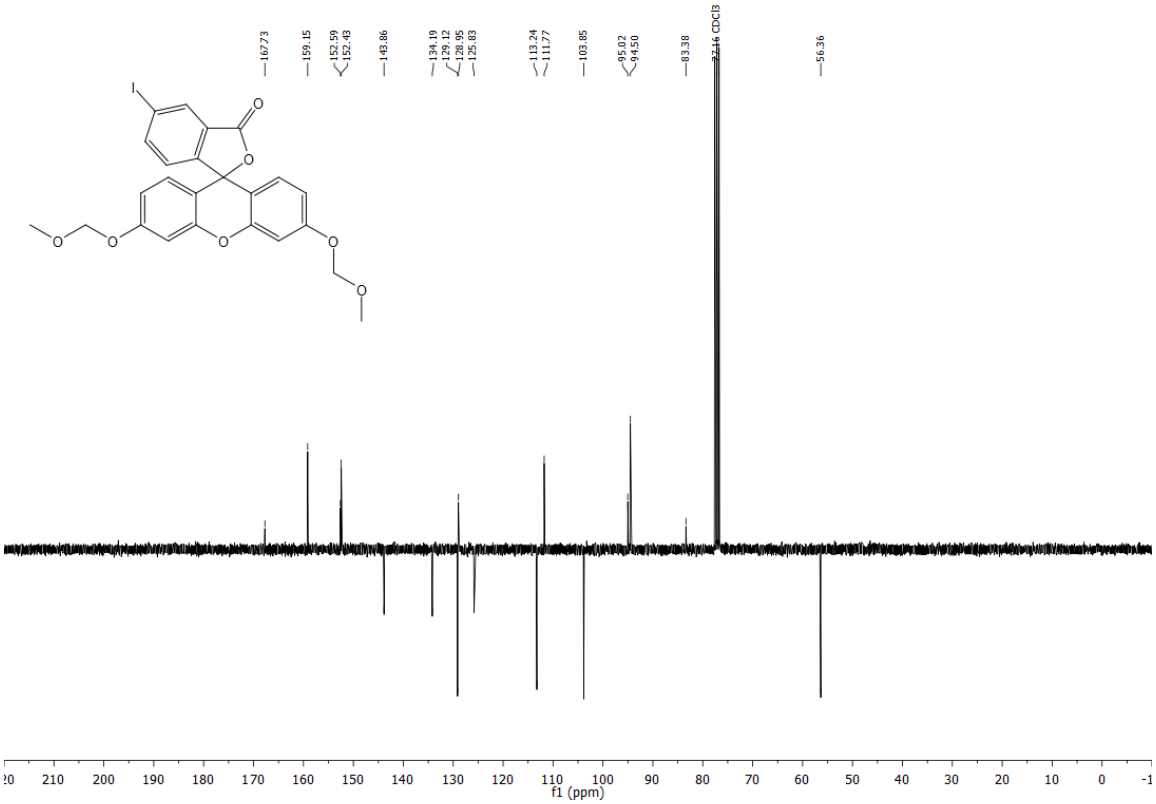

$^{13}$C-NMR of compound **Fl-6-Tz** (DMSO-$d_6$)



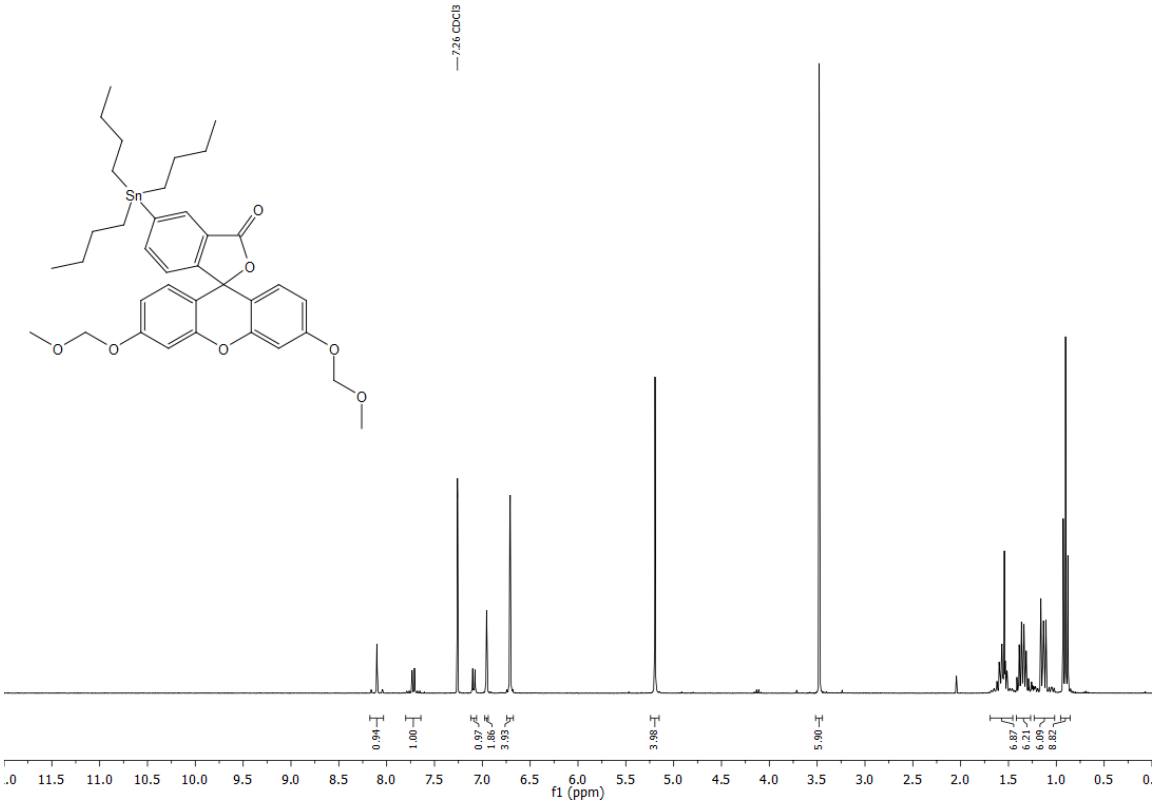
$^{1}$H-NMR of compound **15** ($CDCl_3$)



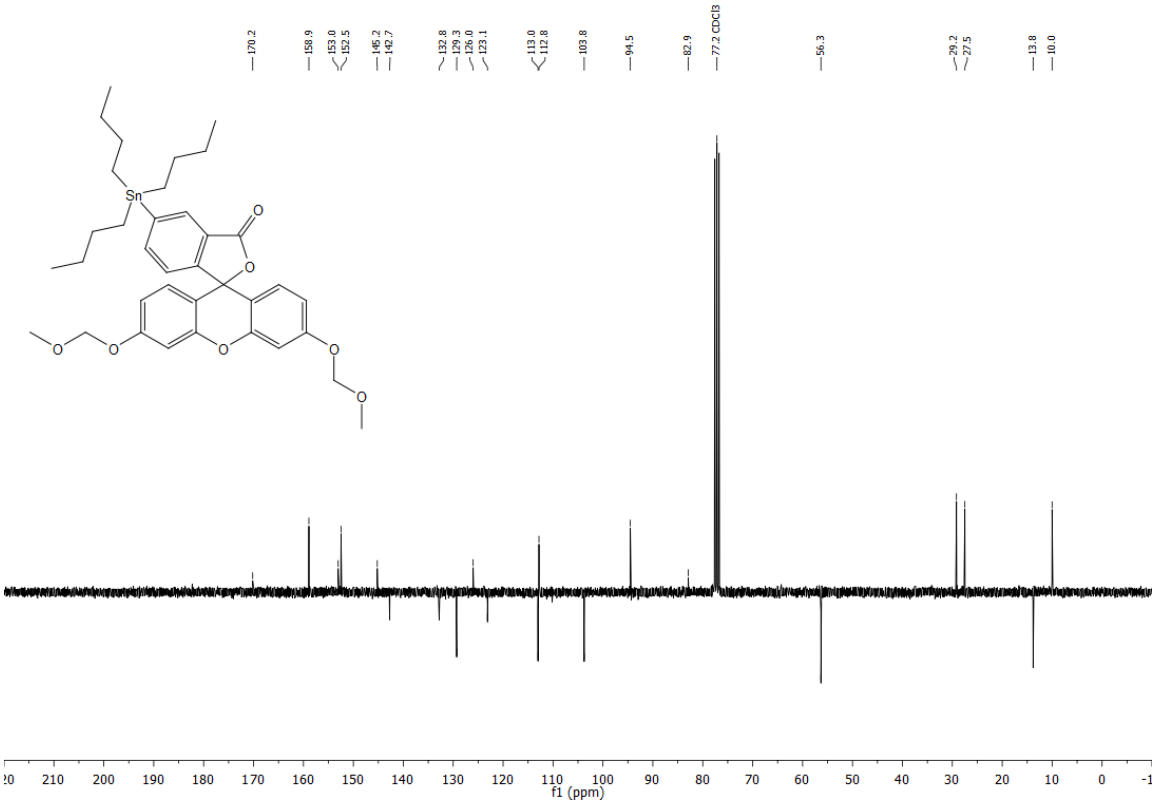

$^{13}$C-NMR of compound **15** ($CDCl_3$)



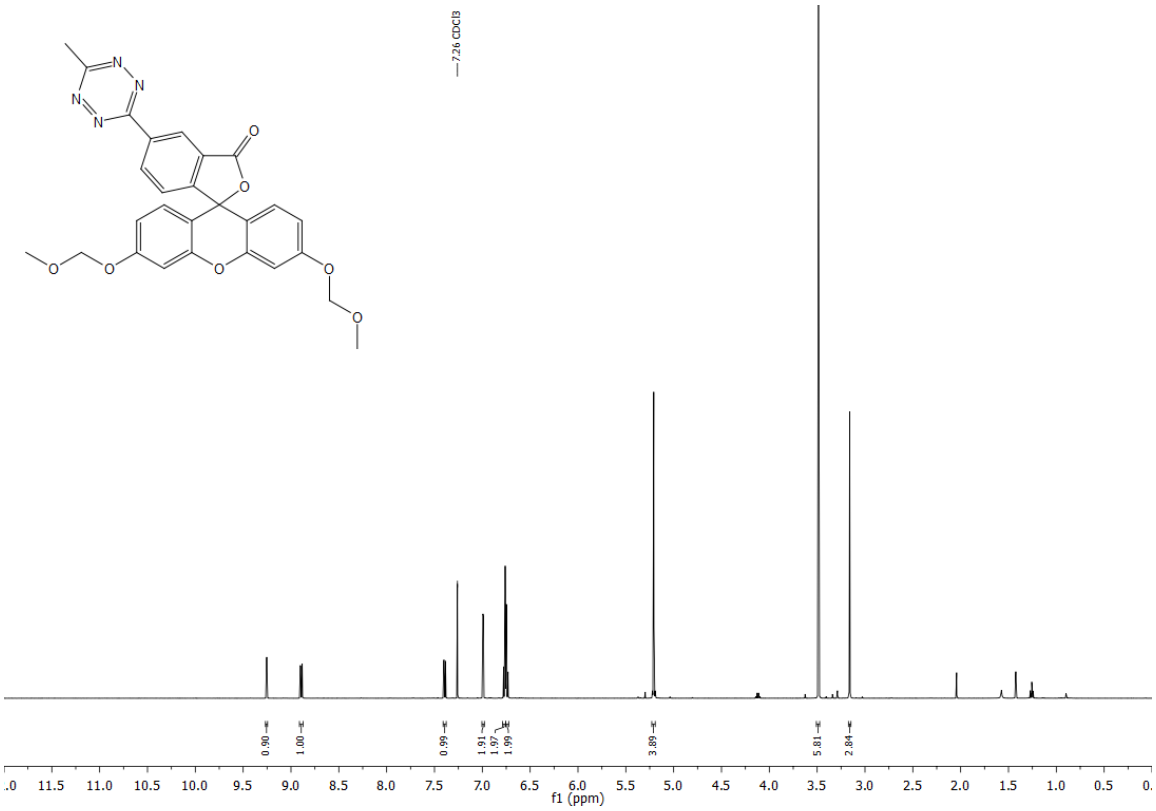
$^{1}$H-NMR of compound **16** ($CDCl_3$)



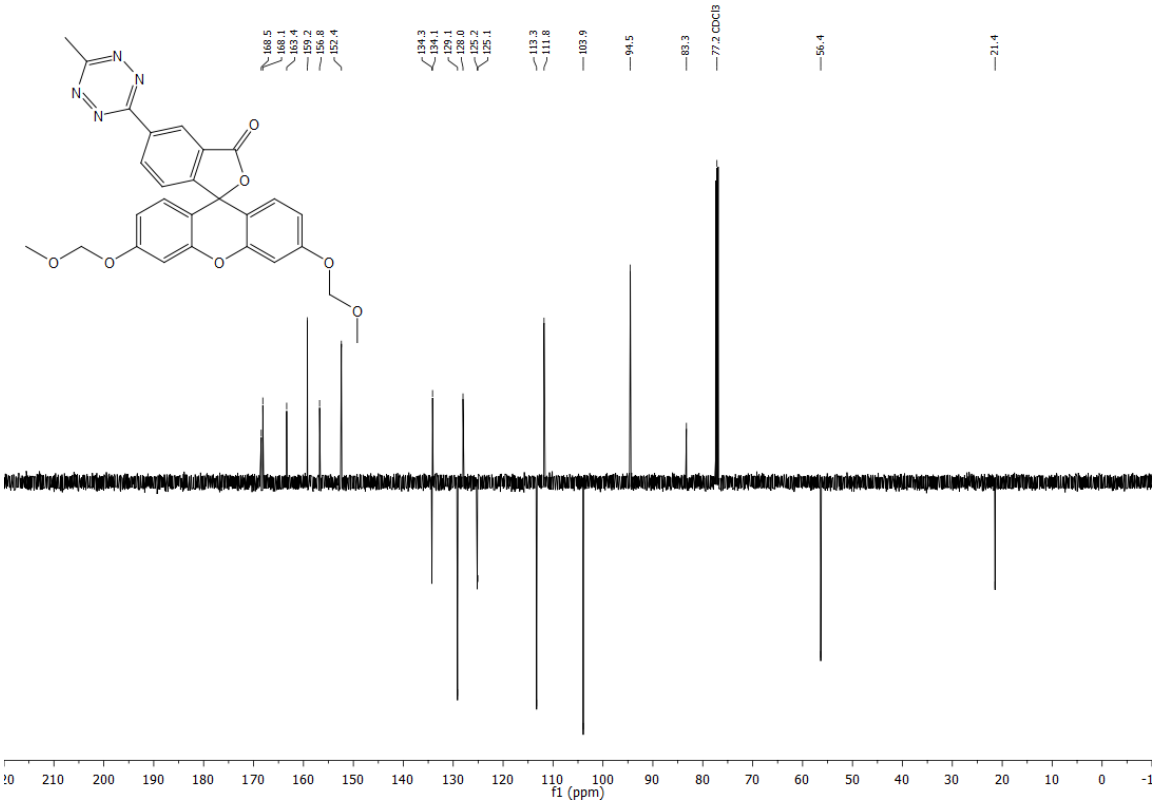

$^{13}$C-NMR of compound **16** ($CDCl_3$)



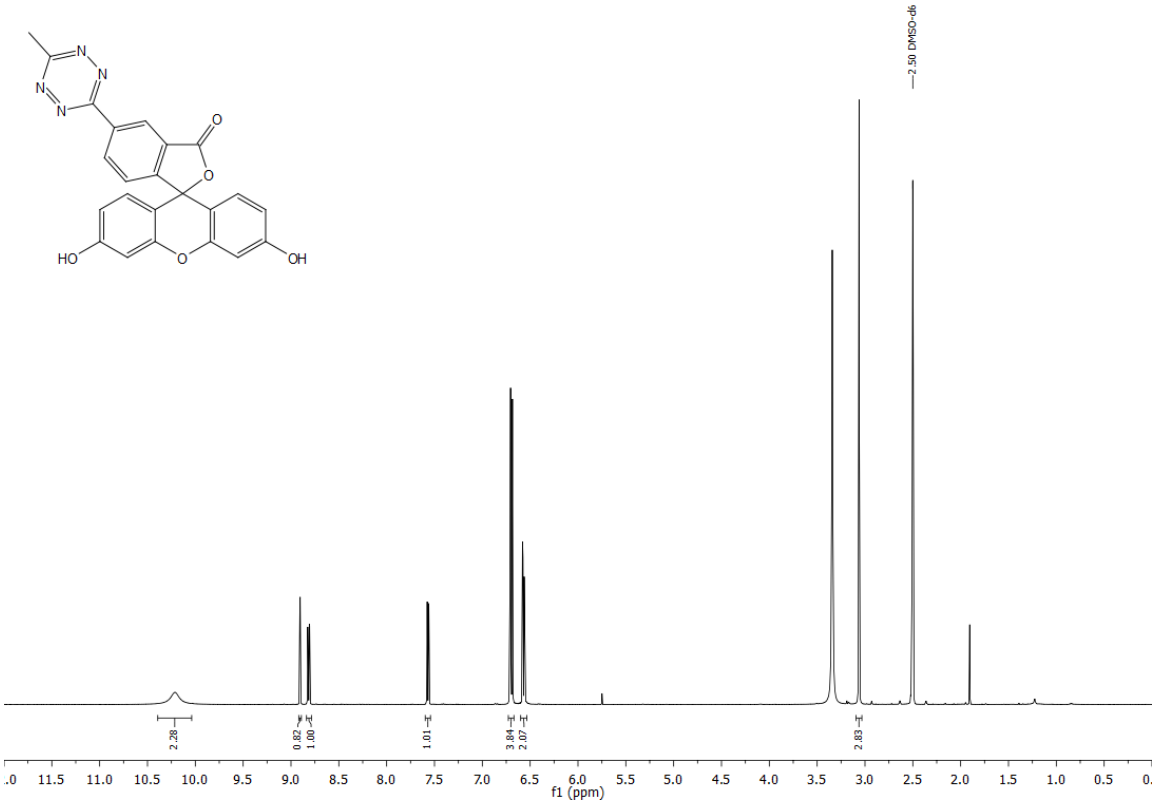
$^{1}$H-NMR of compound **17** ($CDCl_3$)



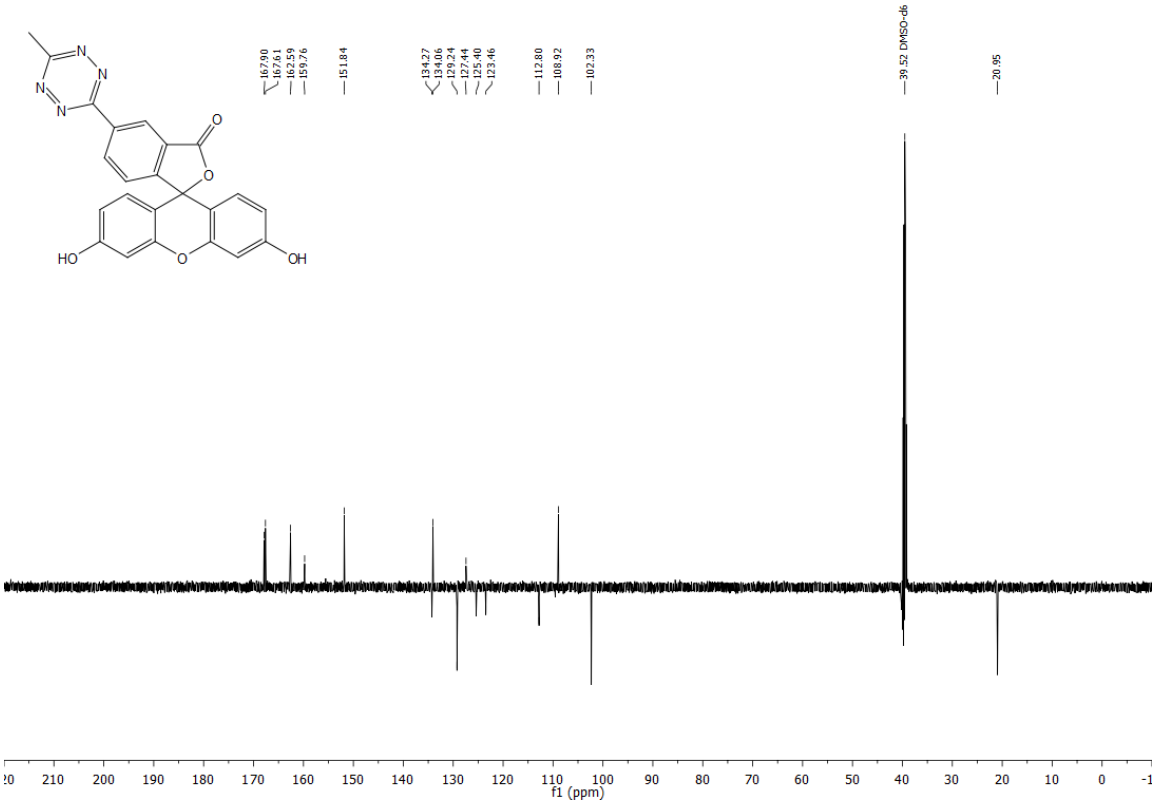

$^{13}$C-NMR of compound **17** ($CDCl_3$)



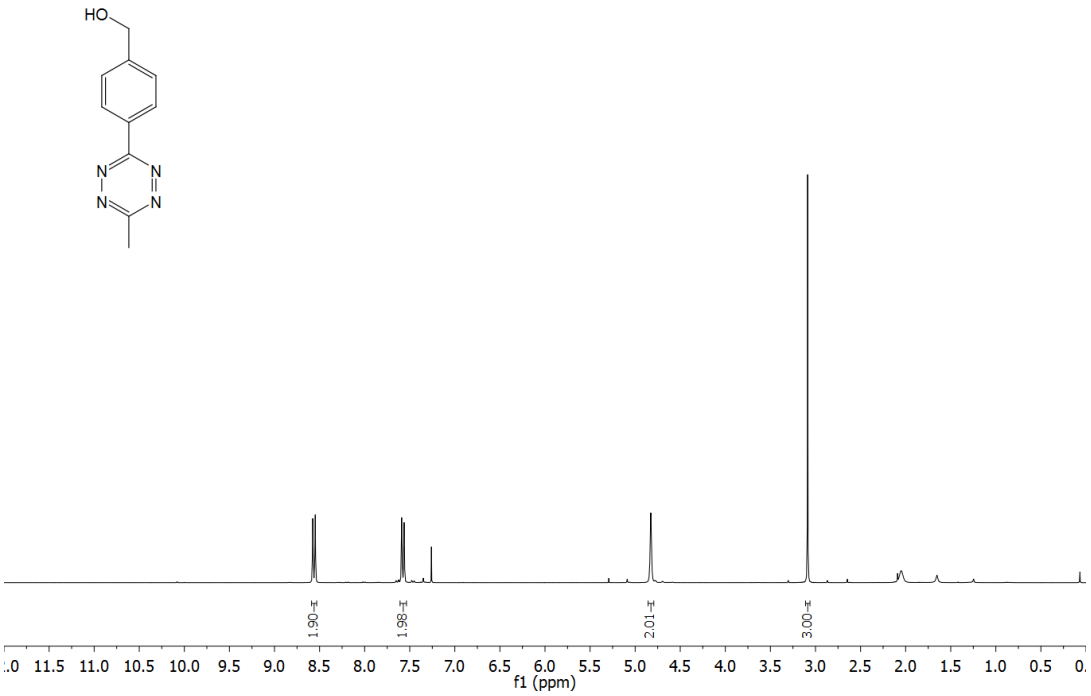
$^{1}$H-NMR of compound **Fl-5-Tz** (DMSO-$d_6$)



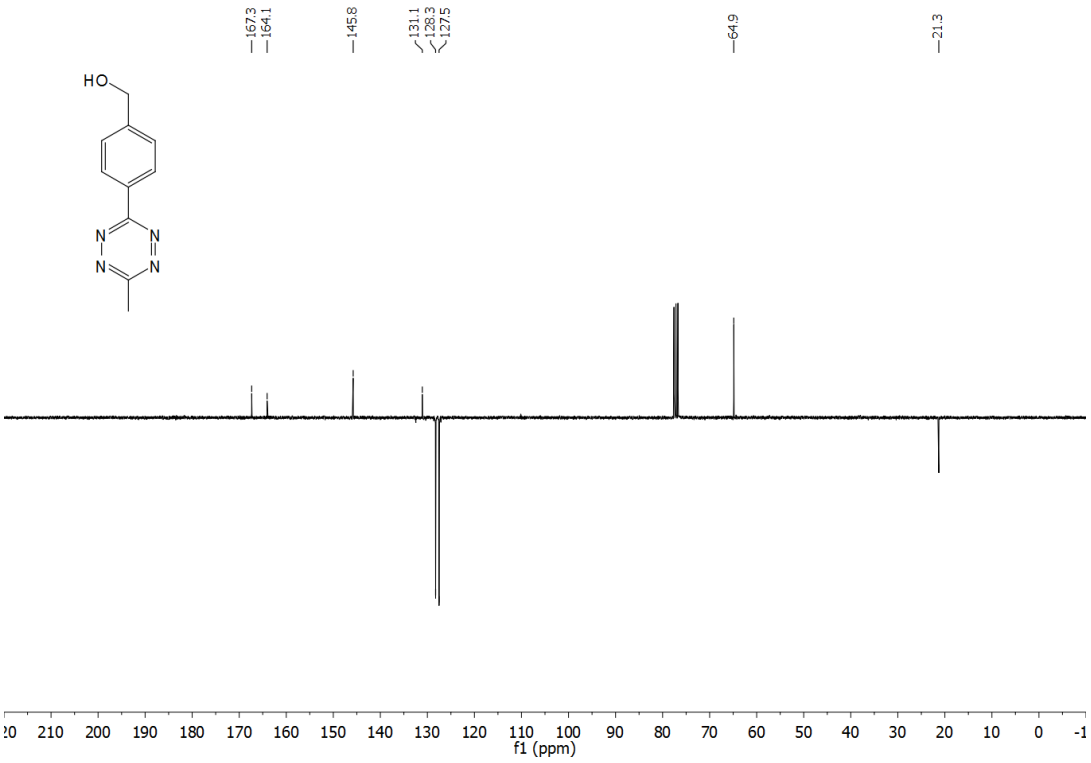

$^{13}$C-NMR of compound **Fl-5-Tz** (DMSO-$d_6$)



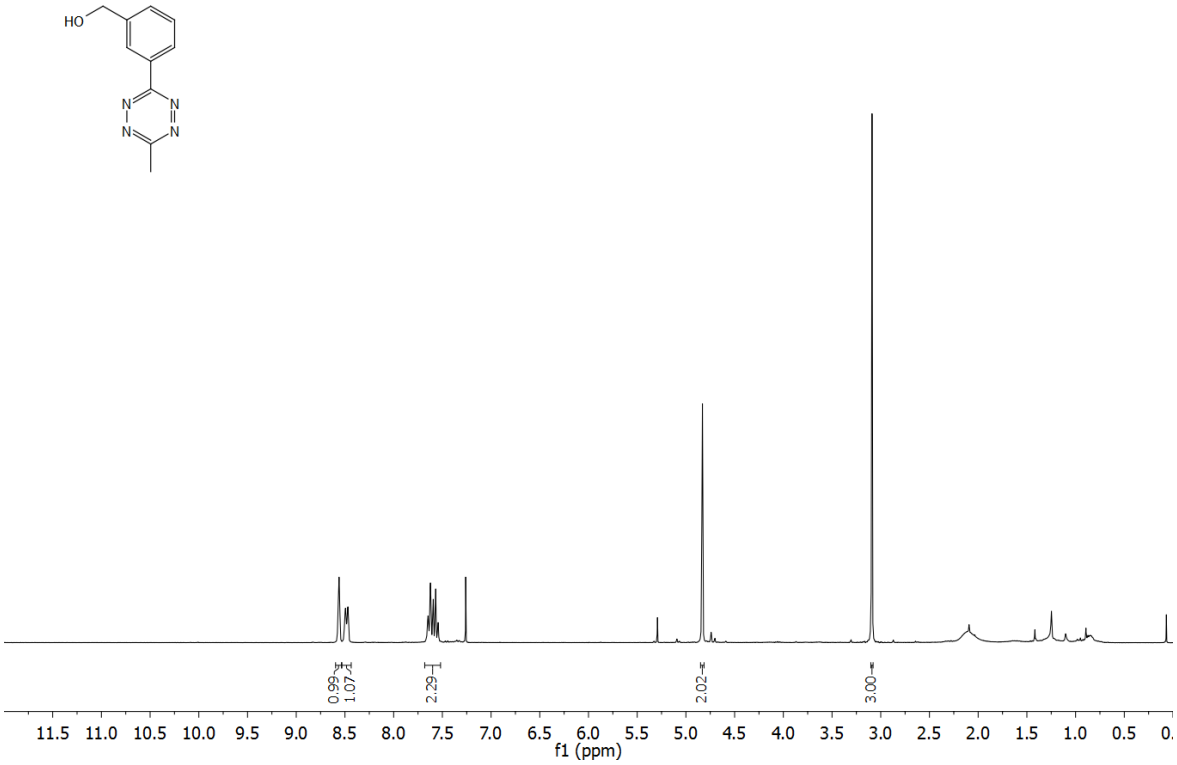
$^{1}$H-NMR of compound **18** ($CDCl_3$)



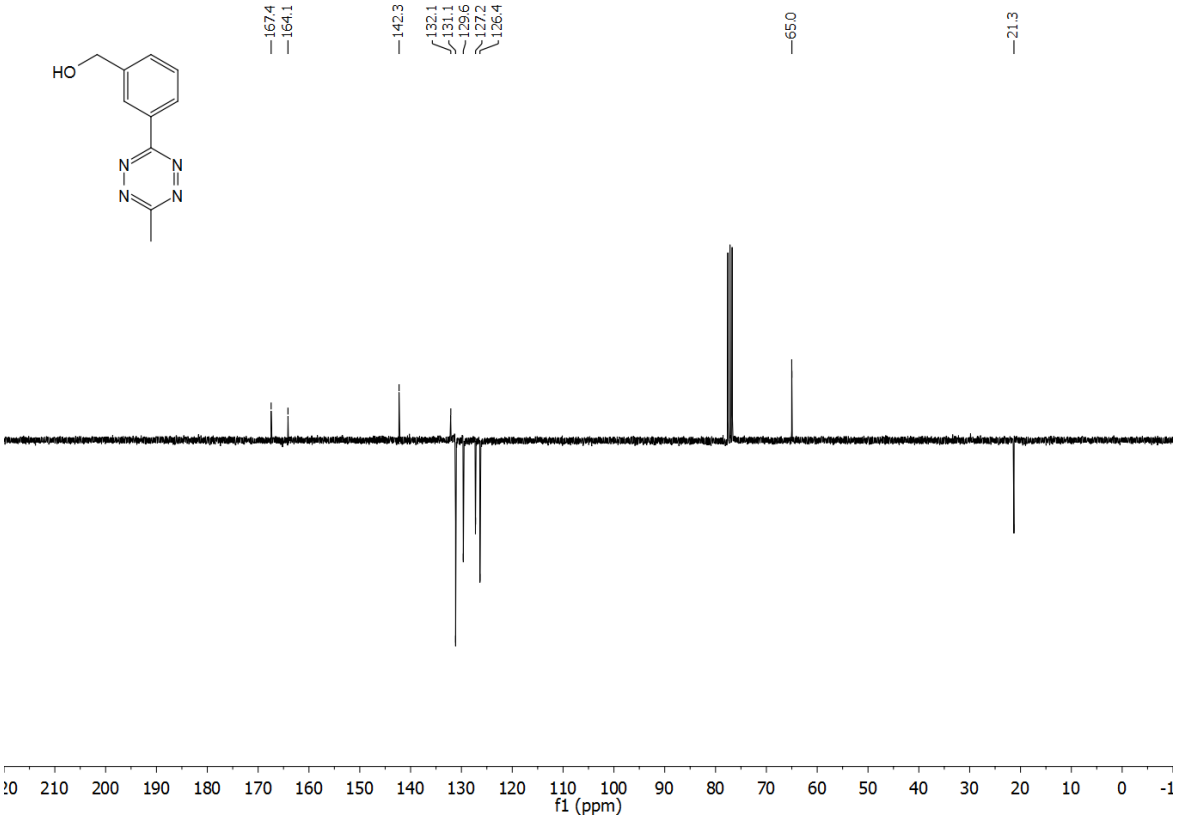

$^{13}$C-NMR of compound **18** ($CDCl_3$)



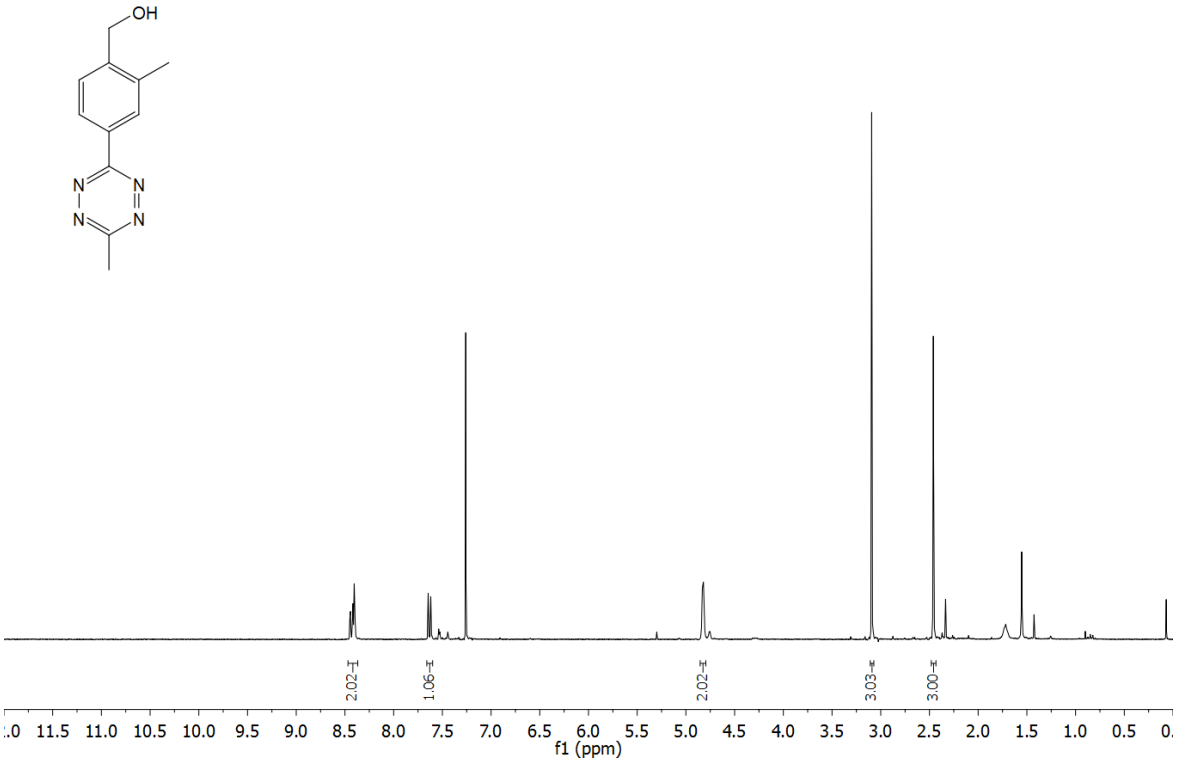
$^{1}$H-NMR of compound **19** ($CDCl_3$)



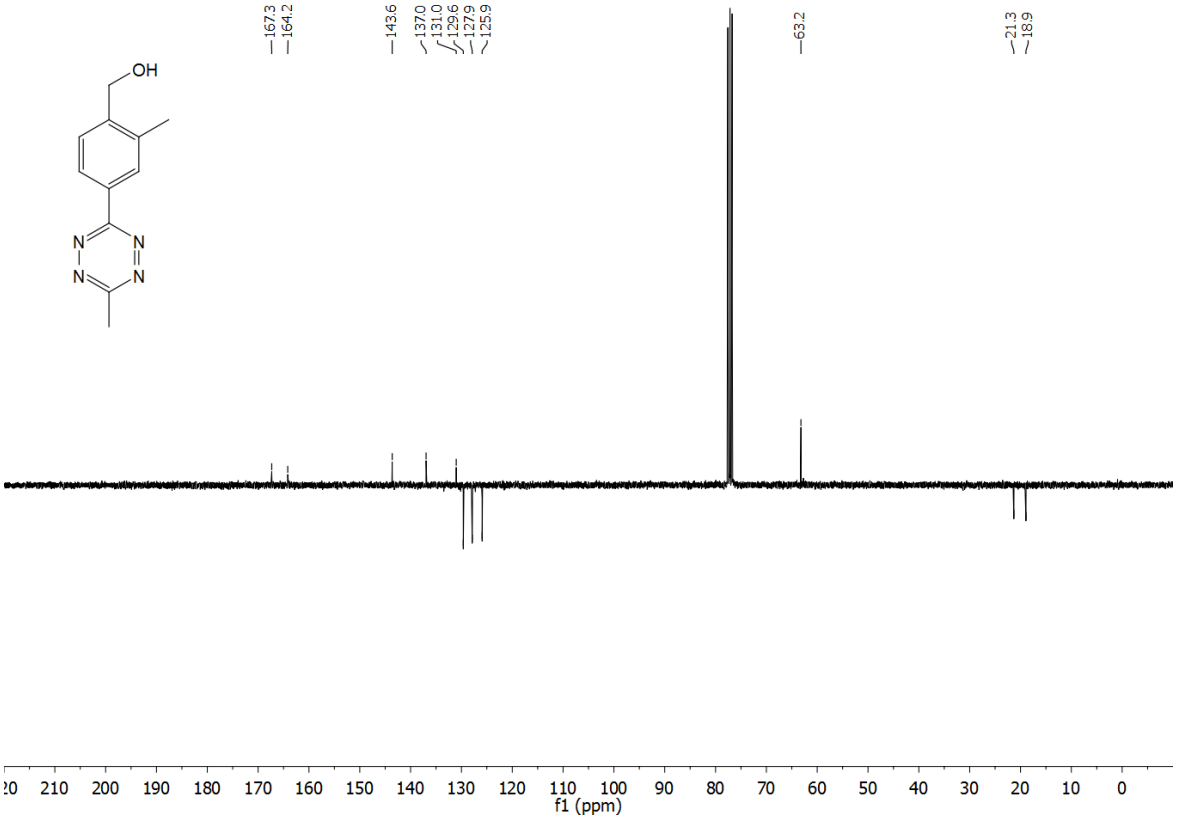

$^{13}$C-NMR of compound **19** ($CDCl_3$)



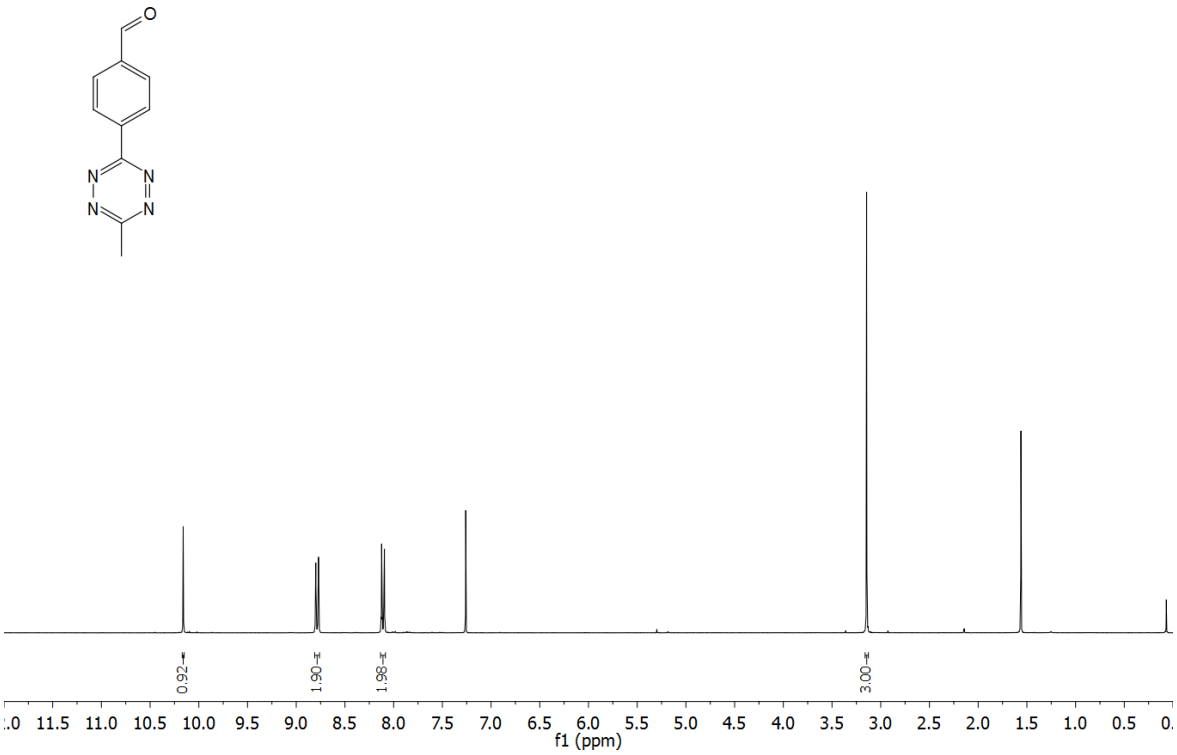
$^{1}$H-NMR of compound **20** ($CDCl_3$)



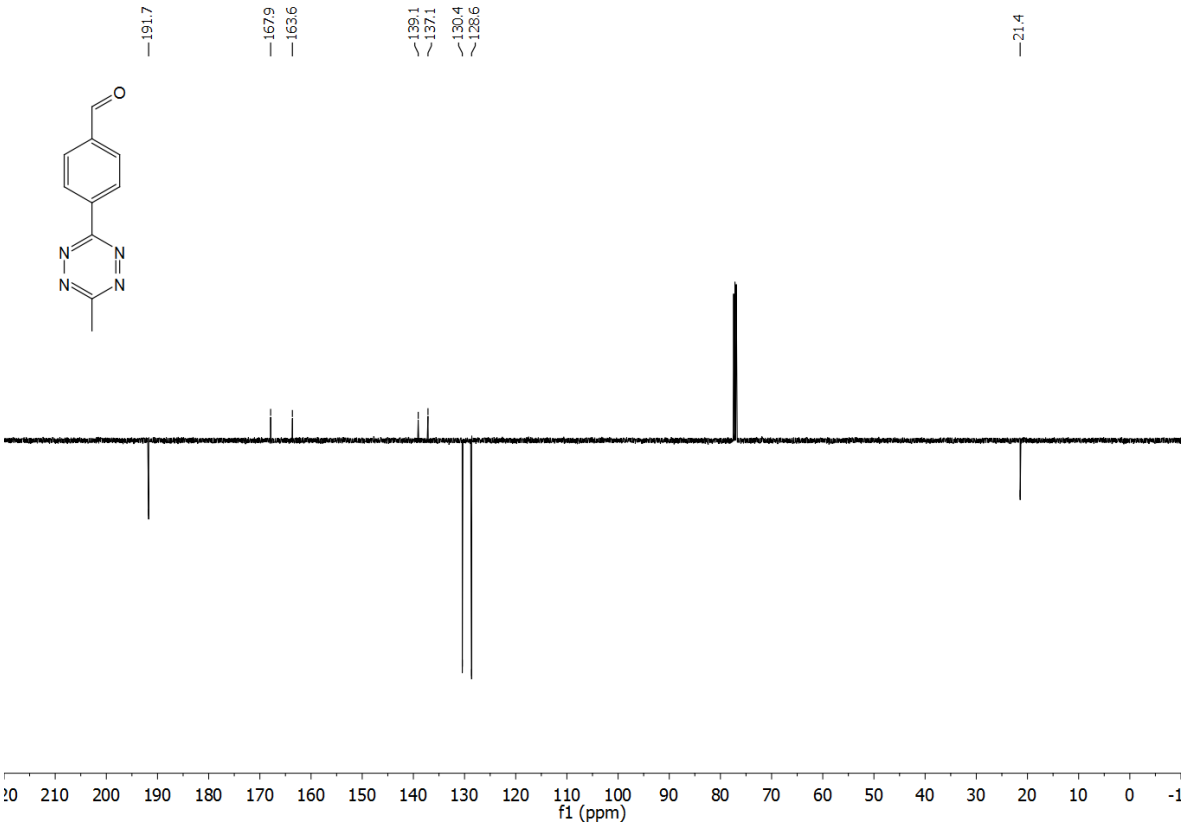

$^{13}$C-NMR of compound **20** ($CDCl_3$)



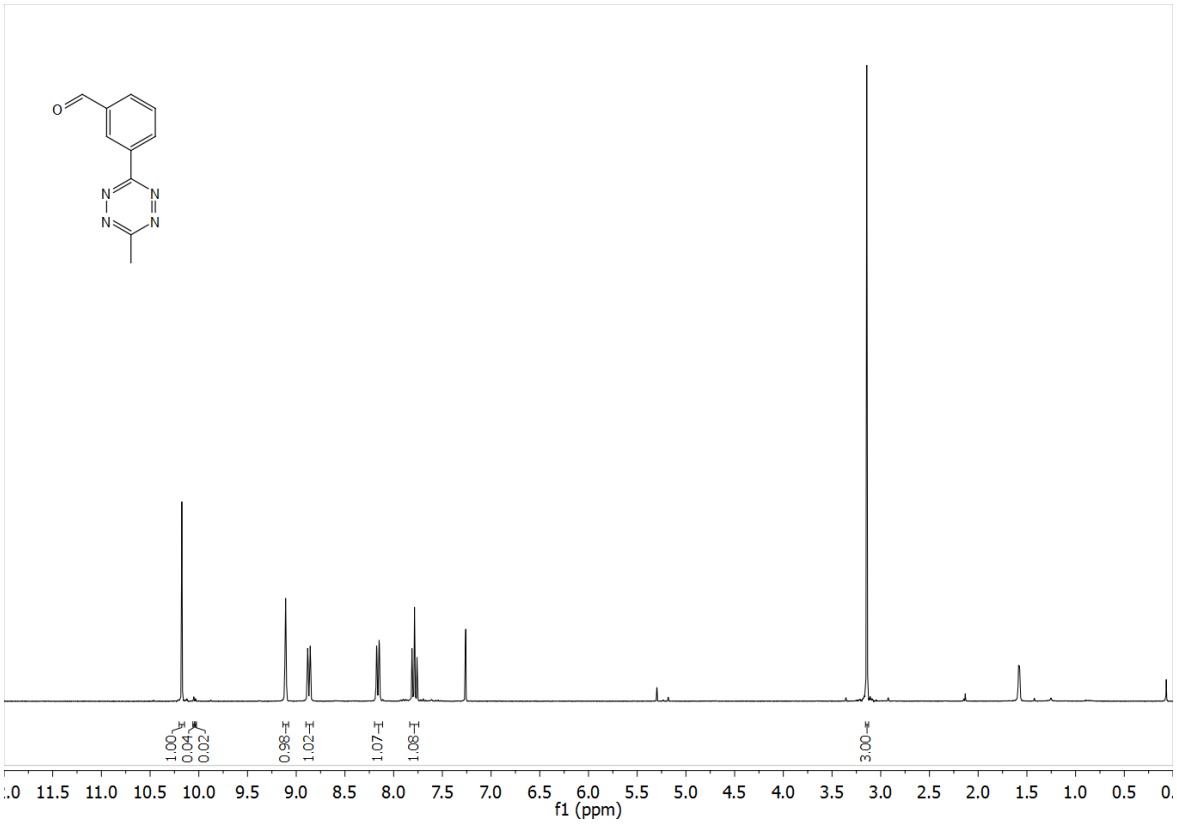
$^{1}$H-NMR of compound **21** ($CDCl_3$)



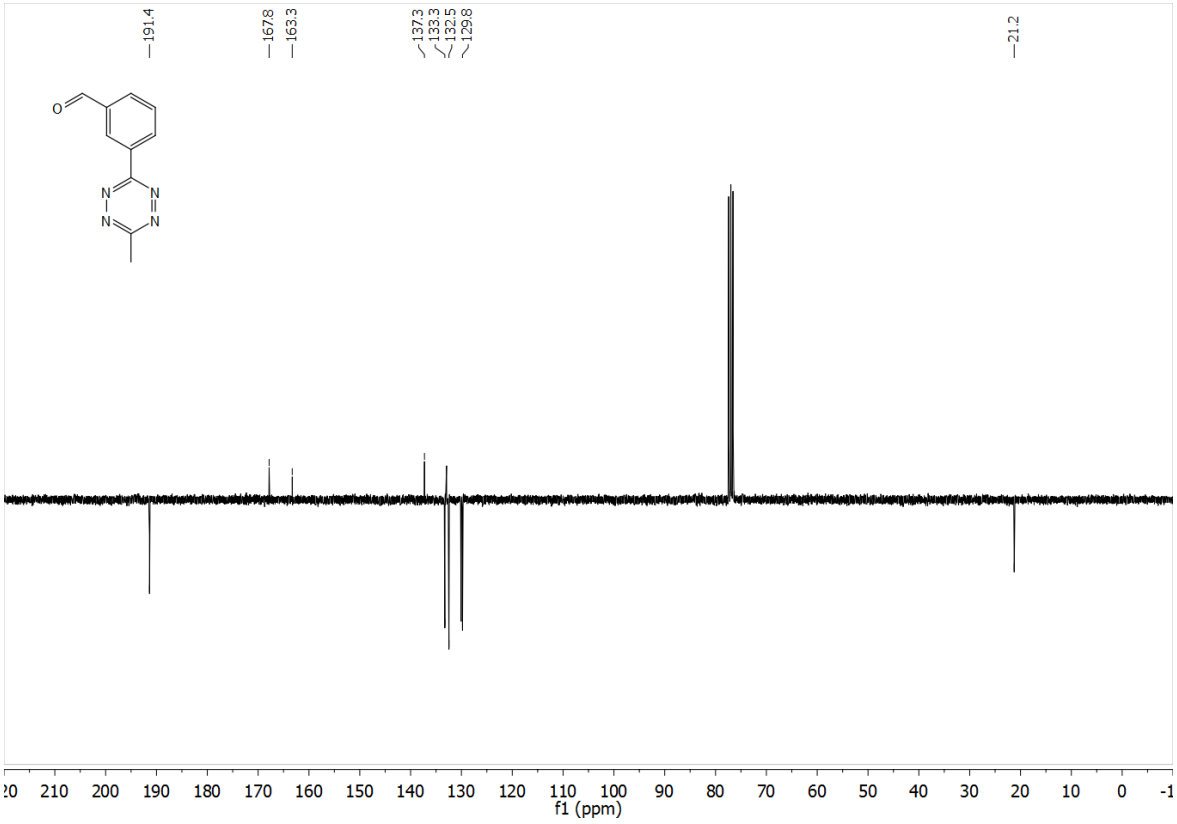

$^{13}$C-NMR of compound **21** ($CDCl_3$)



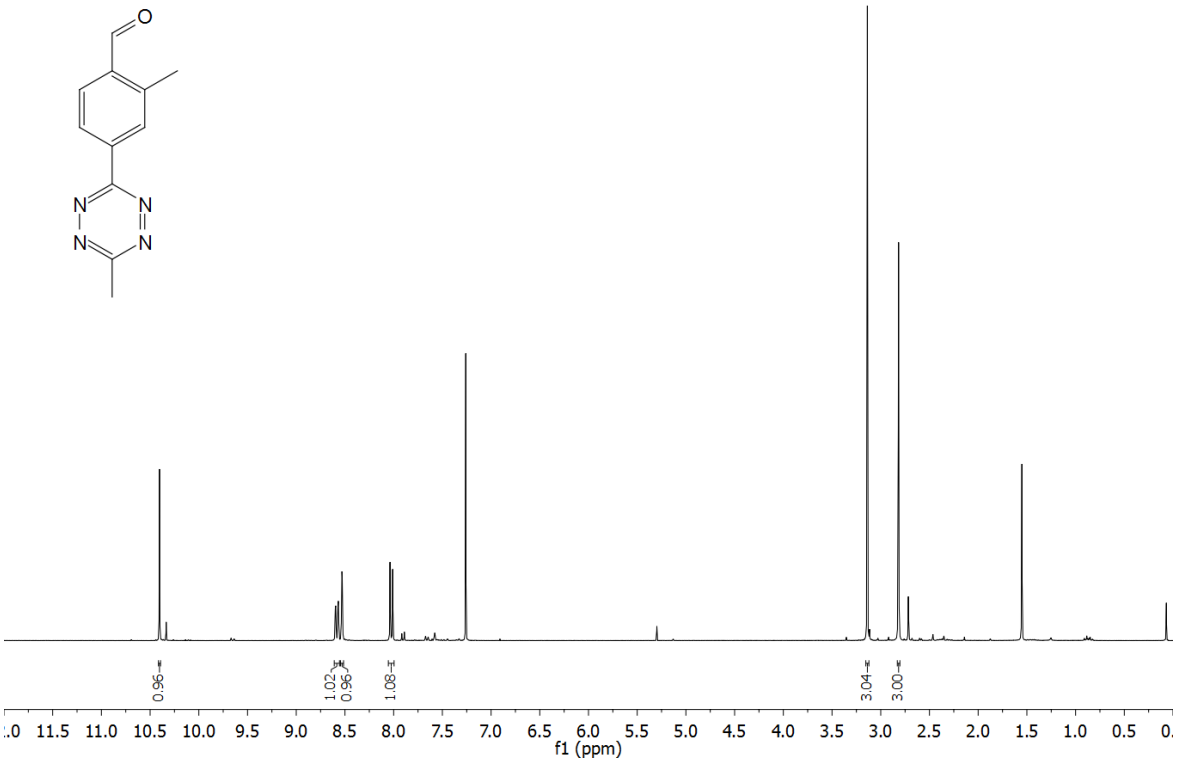
$^{1}$H-NMR of compound **22** ($CDCl_3$)



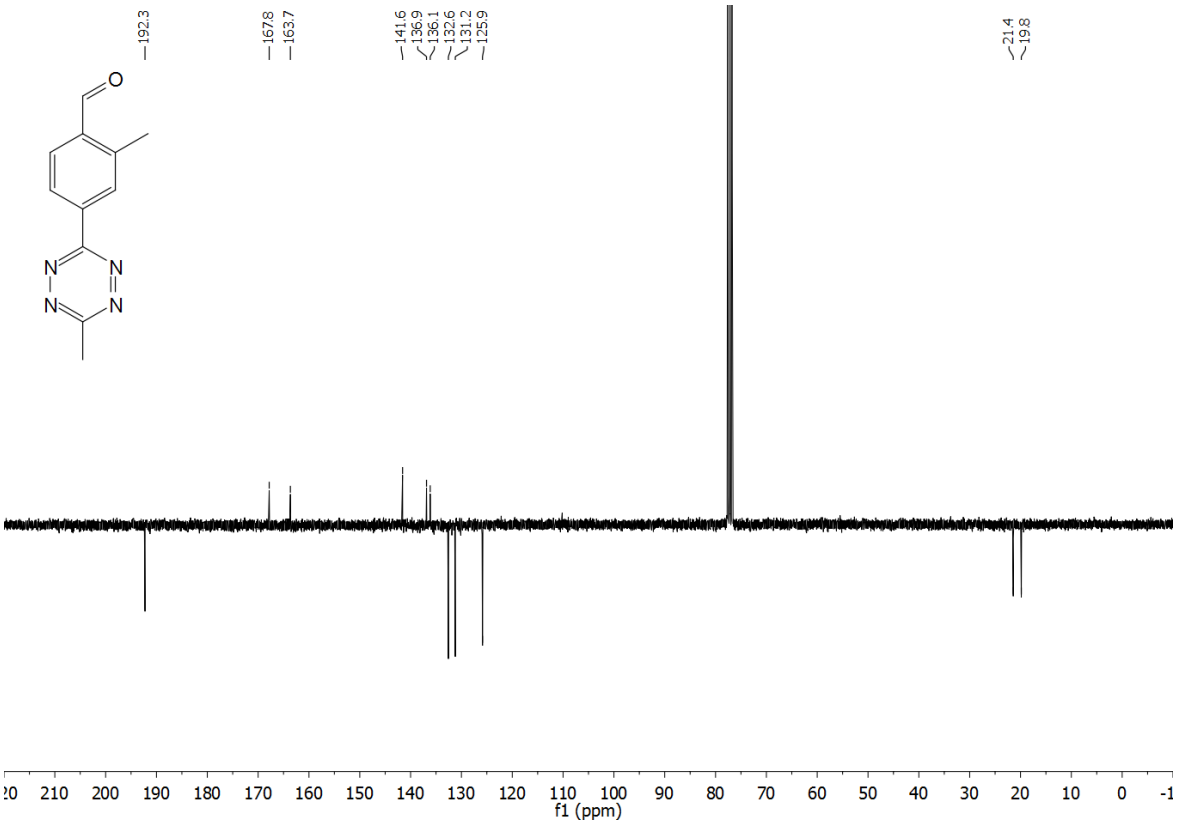

$^{13}$C-NMR of compound **22** ($CDCl_3$)



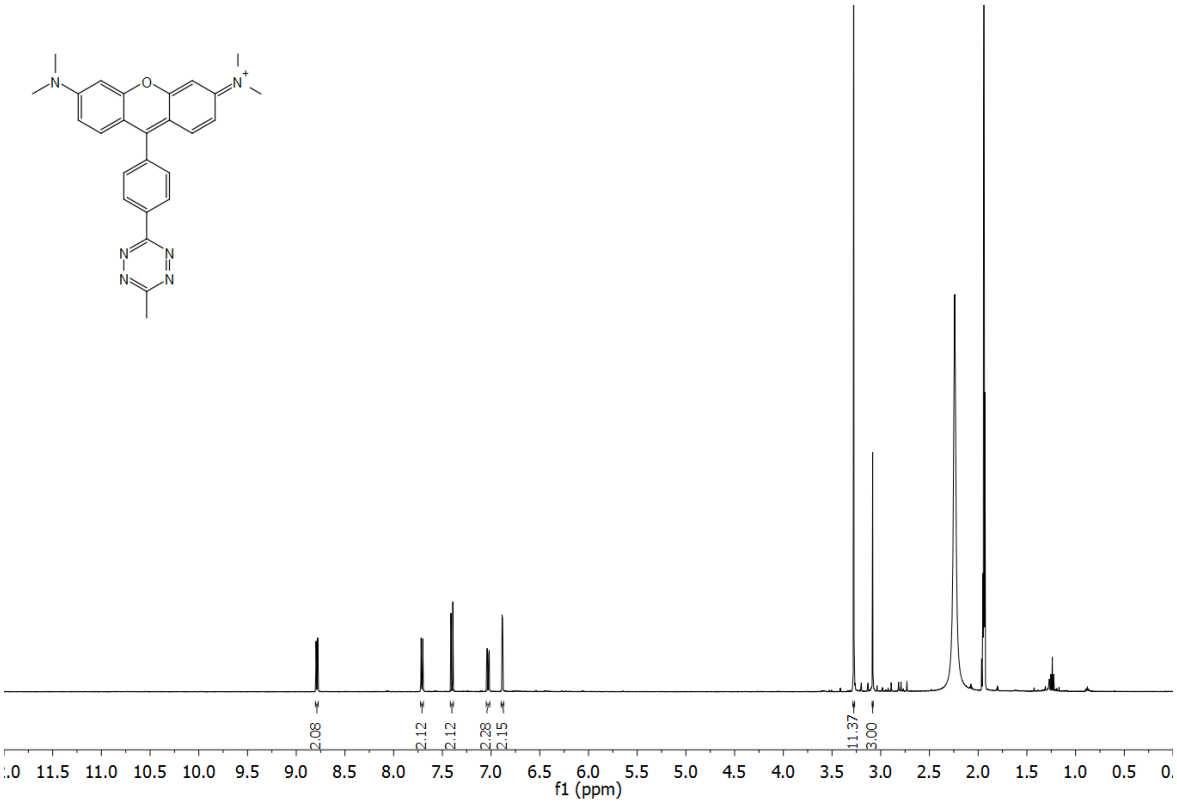
$^{1}$H-NMR of compound **23** ($CDCl_3$)



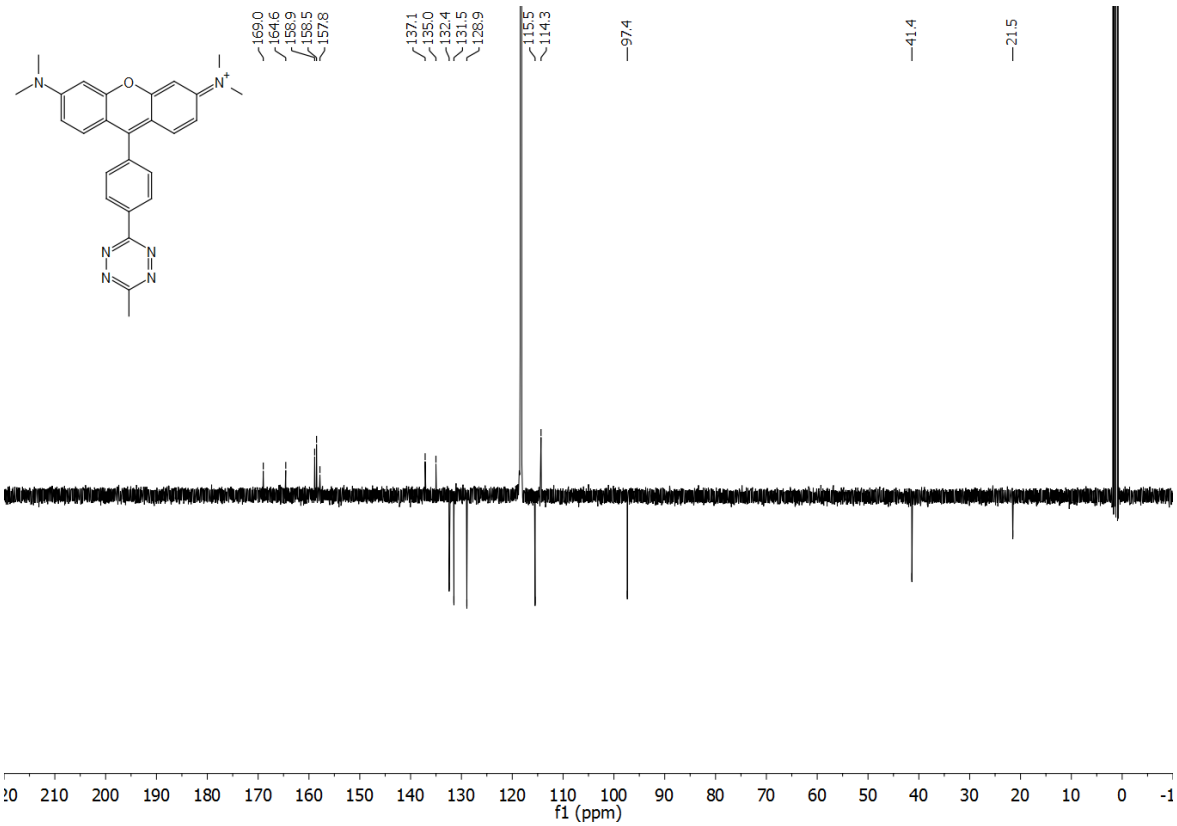

$^{13}$C-NMR of compound **23** ($CDCl_3$)



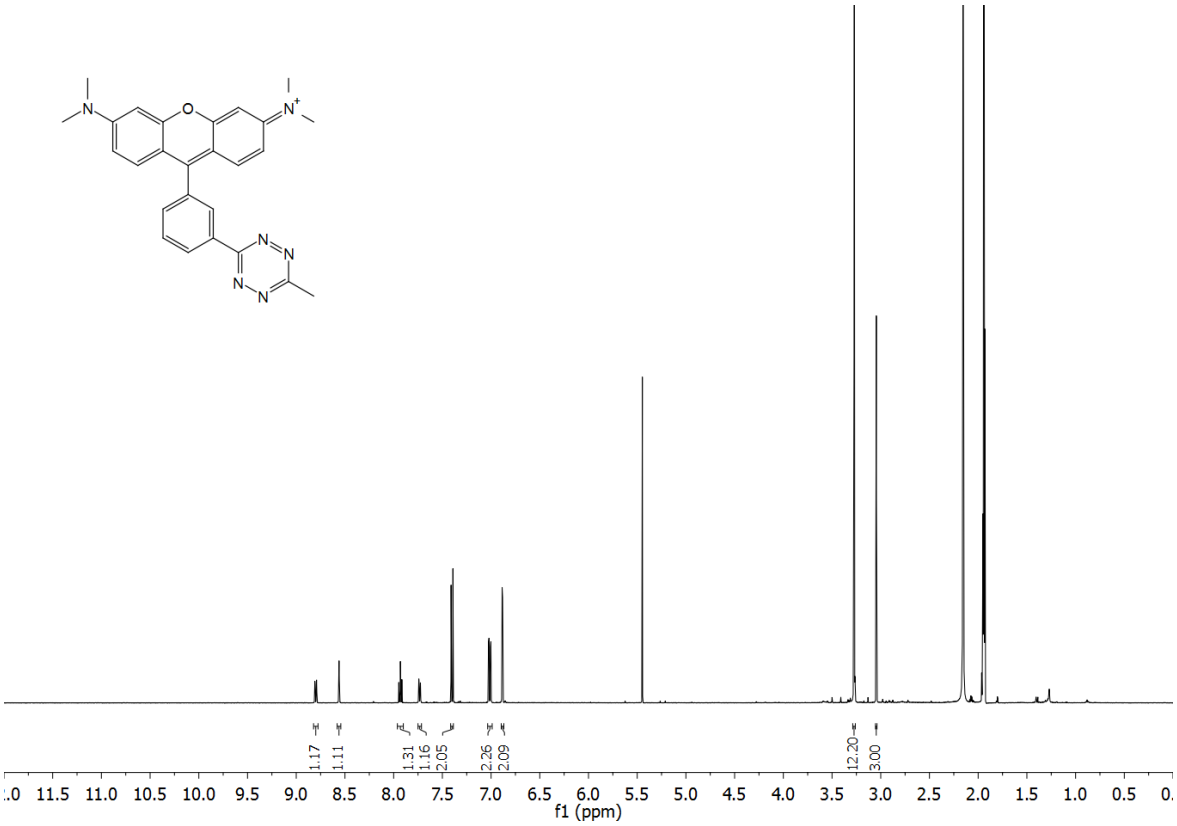
$^{1}$H-NMR of compound **Rh-5-Tz** ($CD_3CN$)



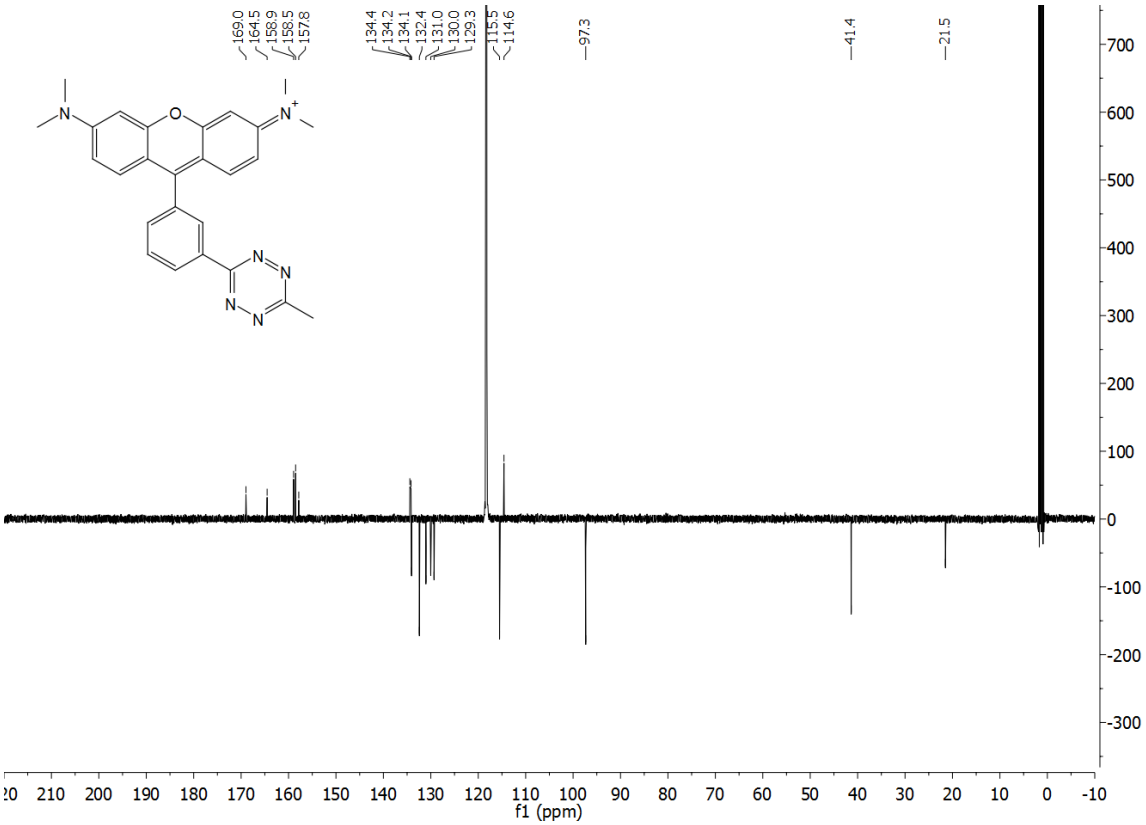

$^{13}$C-NMR of compound **Rh-5-Tz** ($CD_3CN$)



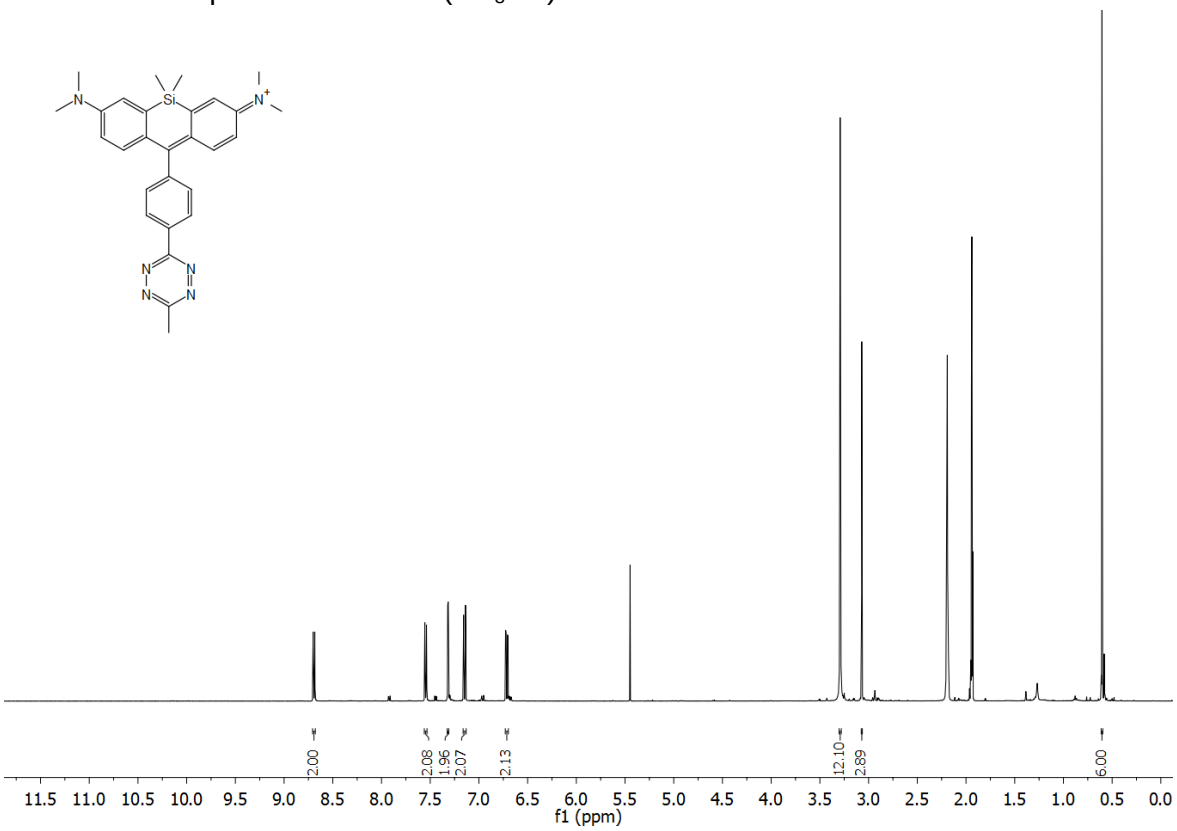
$^{1}$H-NMR of compound **Rh-6-Tz** ($CD_3CN$)



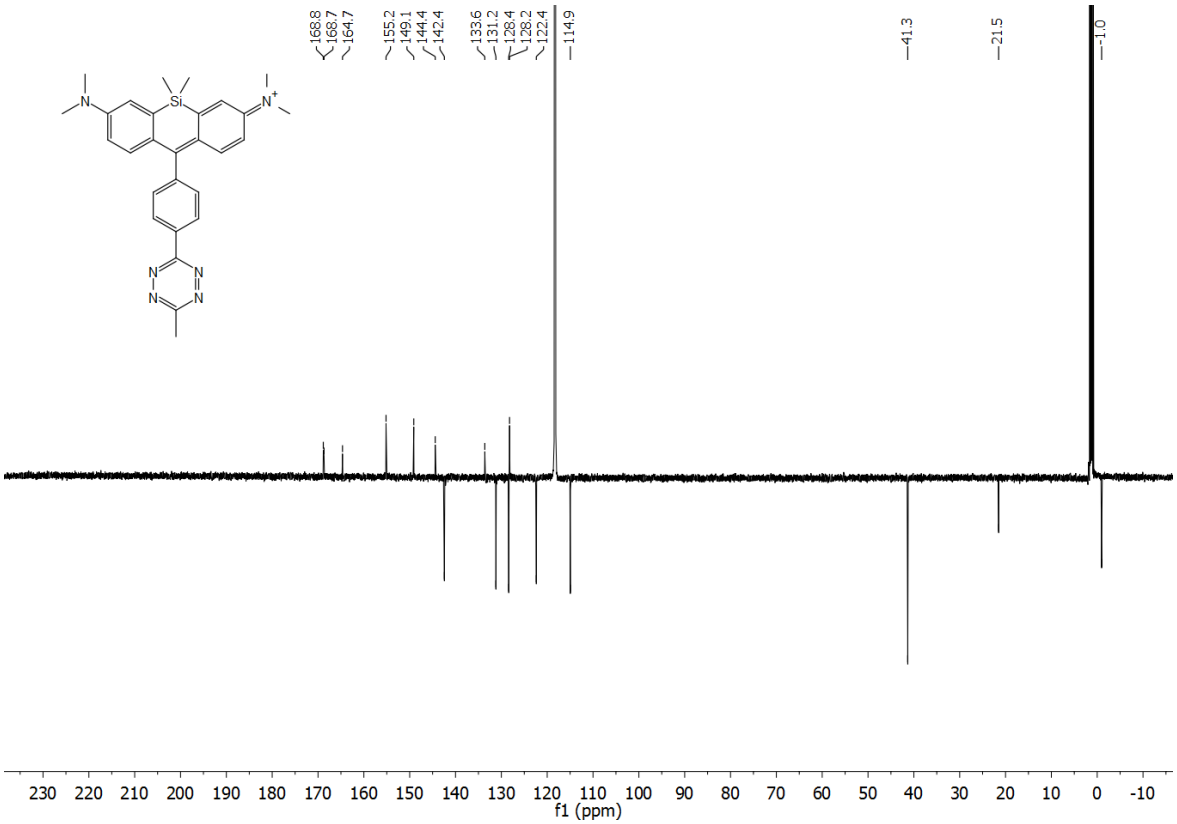

$^{13}$C-NMR of compound **Rh-6-Tz** ($CD_3CN$)



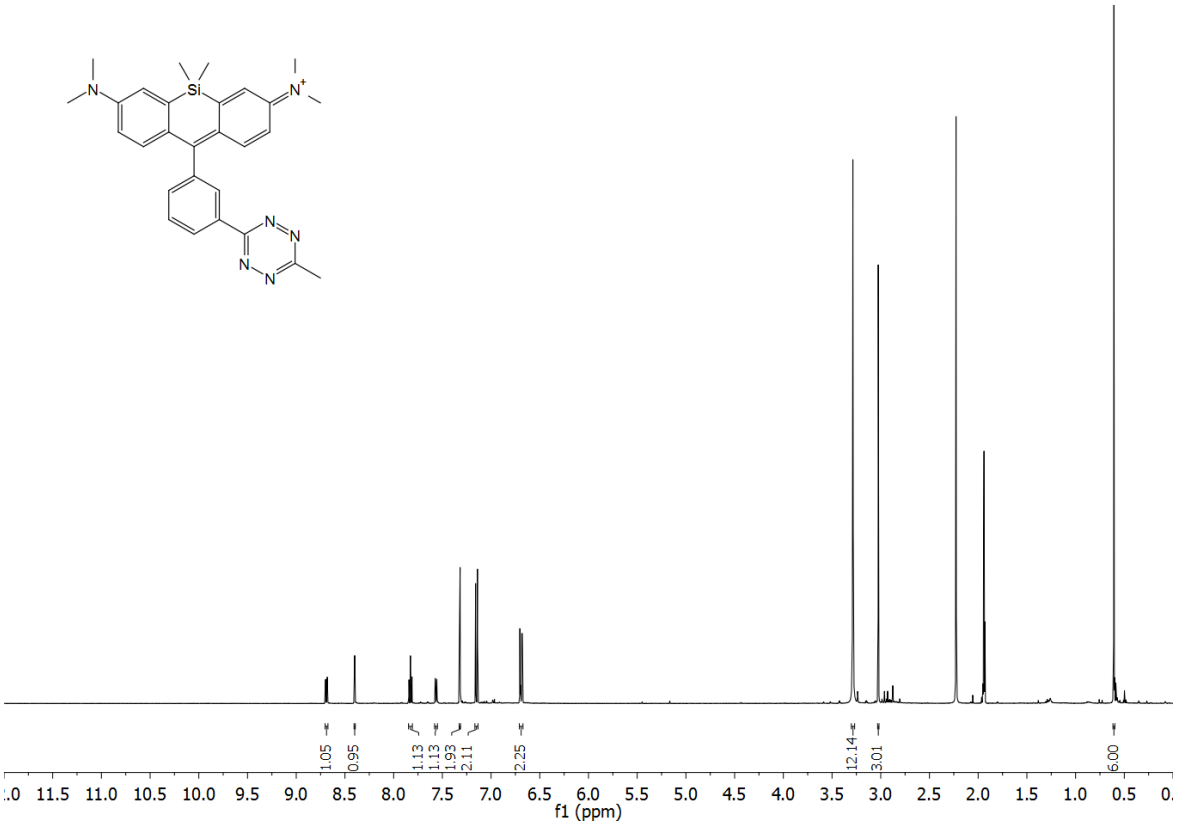
$^{1}$H-NMR of compound **SiRh-5-Tz** ($CD_3CN$)



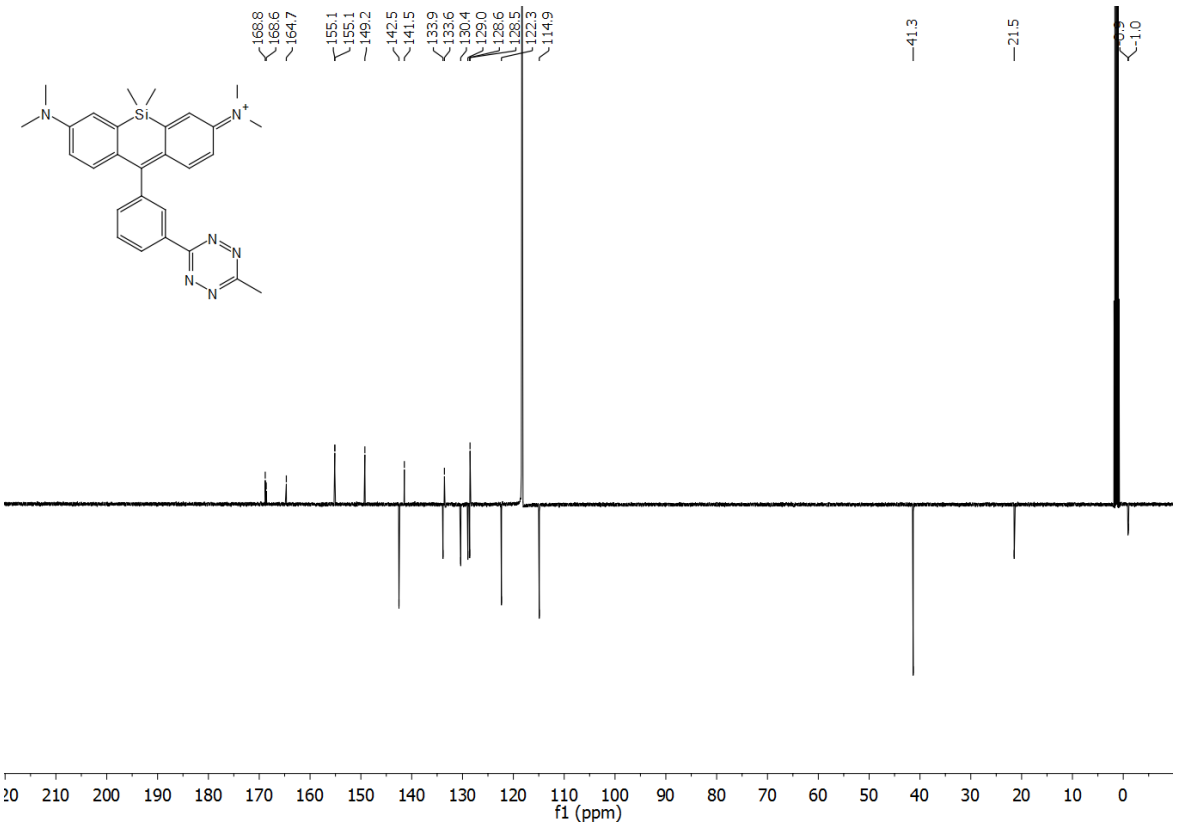

$^{13}$C-NMR of compound **SiRh-5-Tz** ($CD_3CN$)



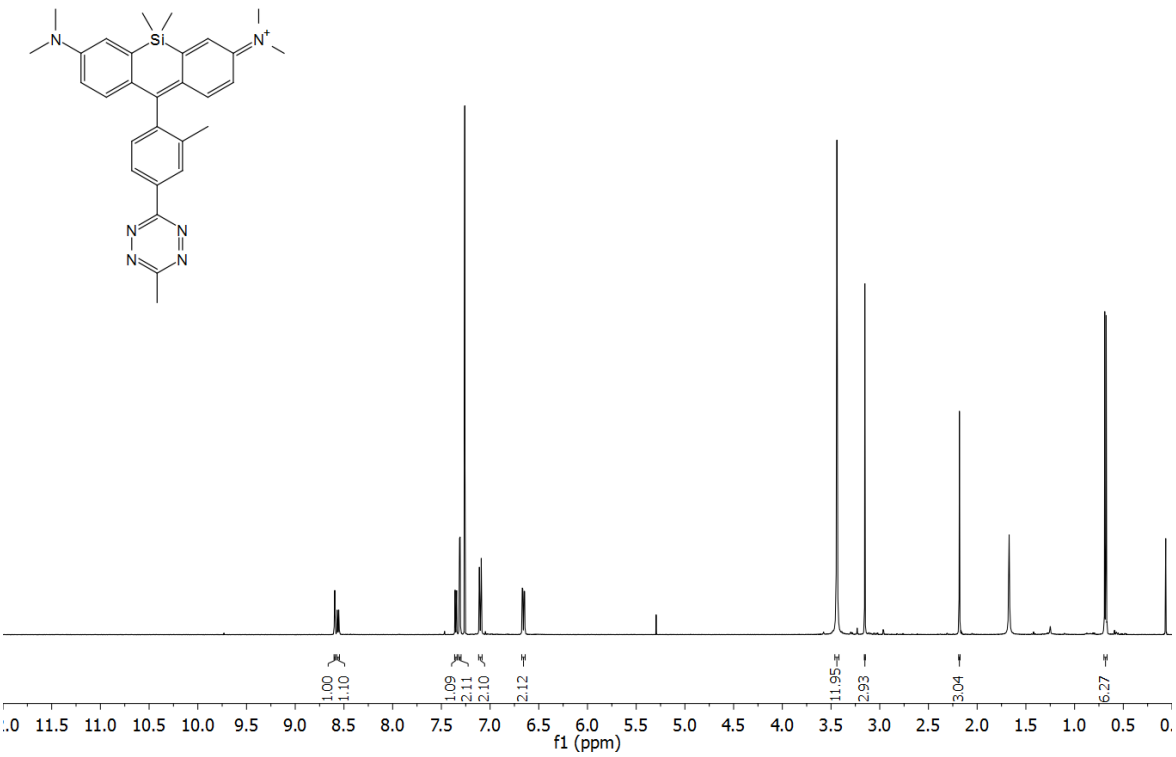
$^{1}$H-NMR of compound **SiRh-6-Tz** ($CD_3CN$)



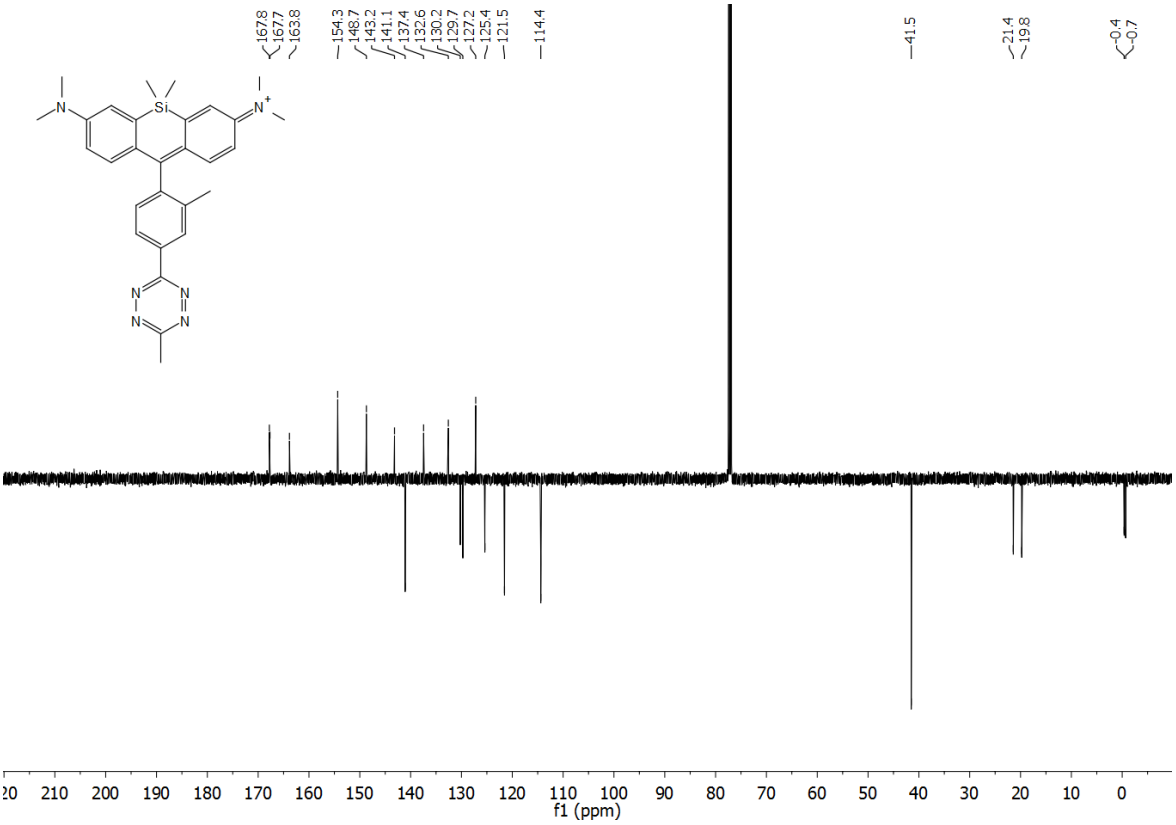

$^{13}$C-NMR of compound **SiRh-6-Tz** ($CD_3CN$)



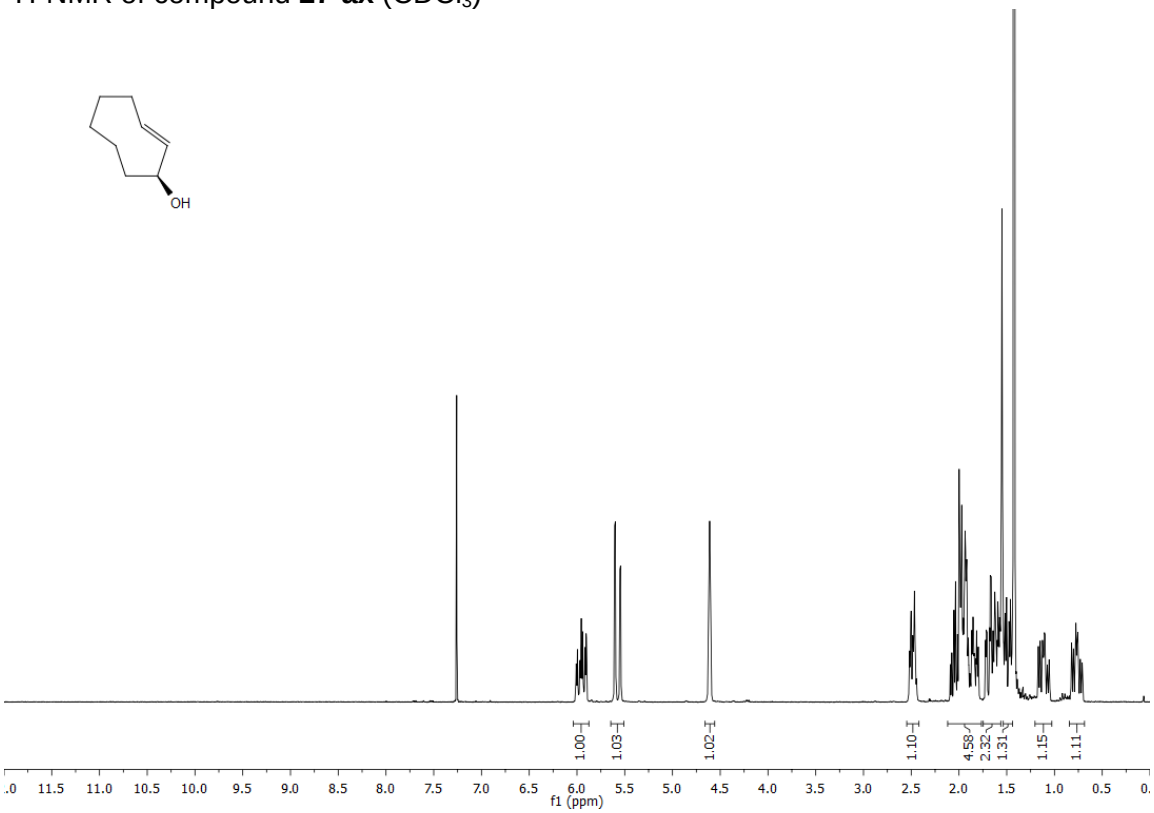
$^{1}$H-NMR of compound **MeSiRh-5-Tz** ($CDCl_3$)



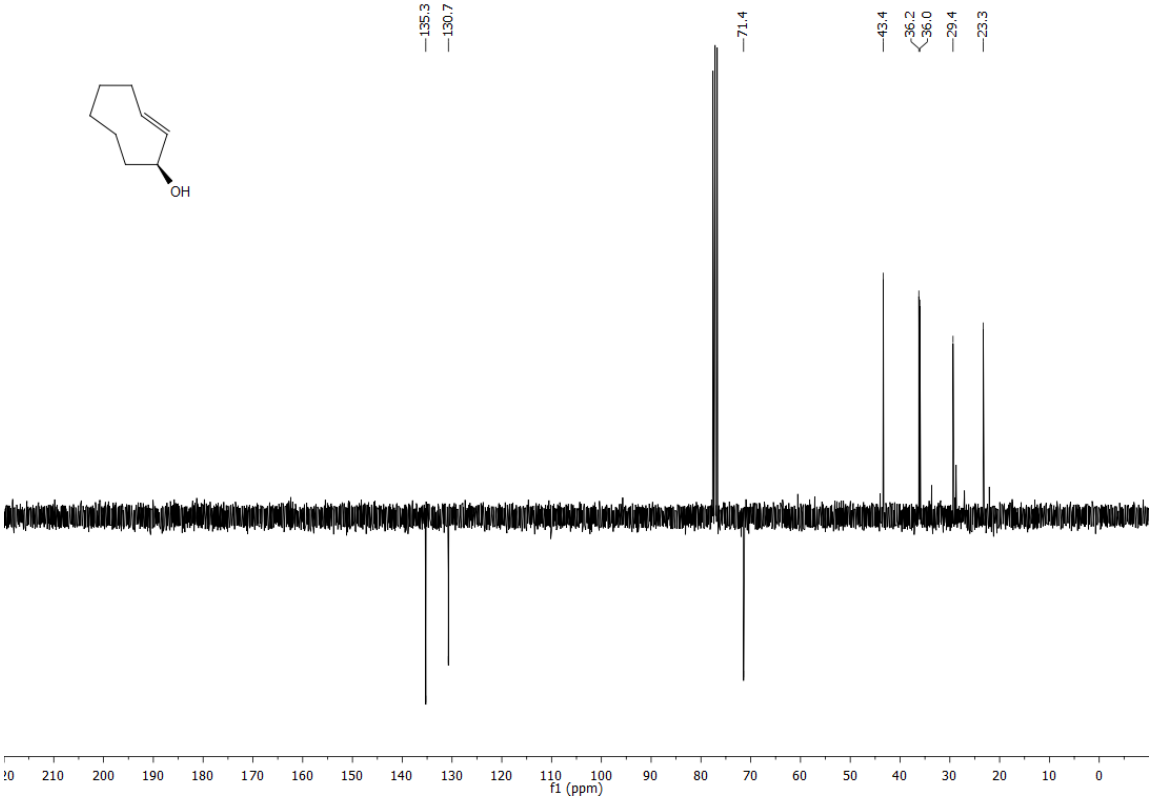

$^{13}$C-NMR of compound **MeSiRh-5-Tz** ($CDCl_3$)



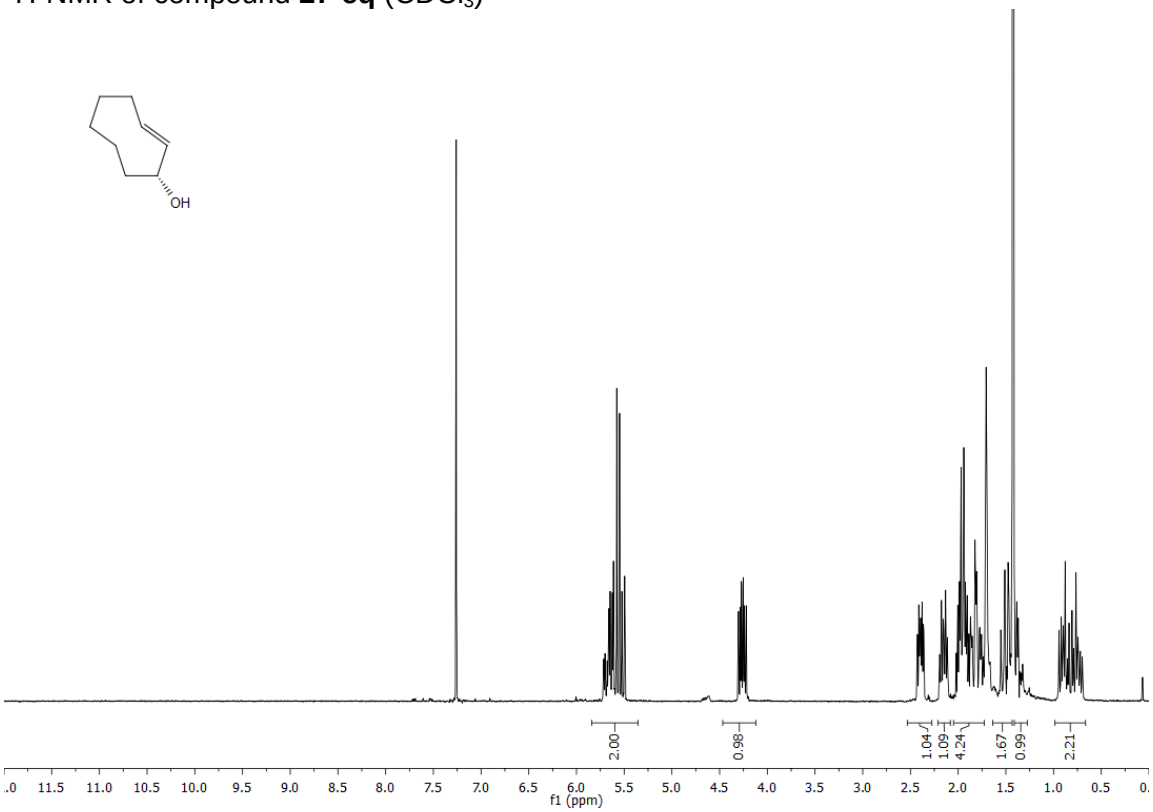
$^{1}$H-NMR of compound **27-ax** ($CDCl_3$)



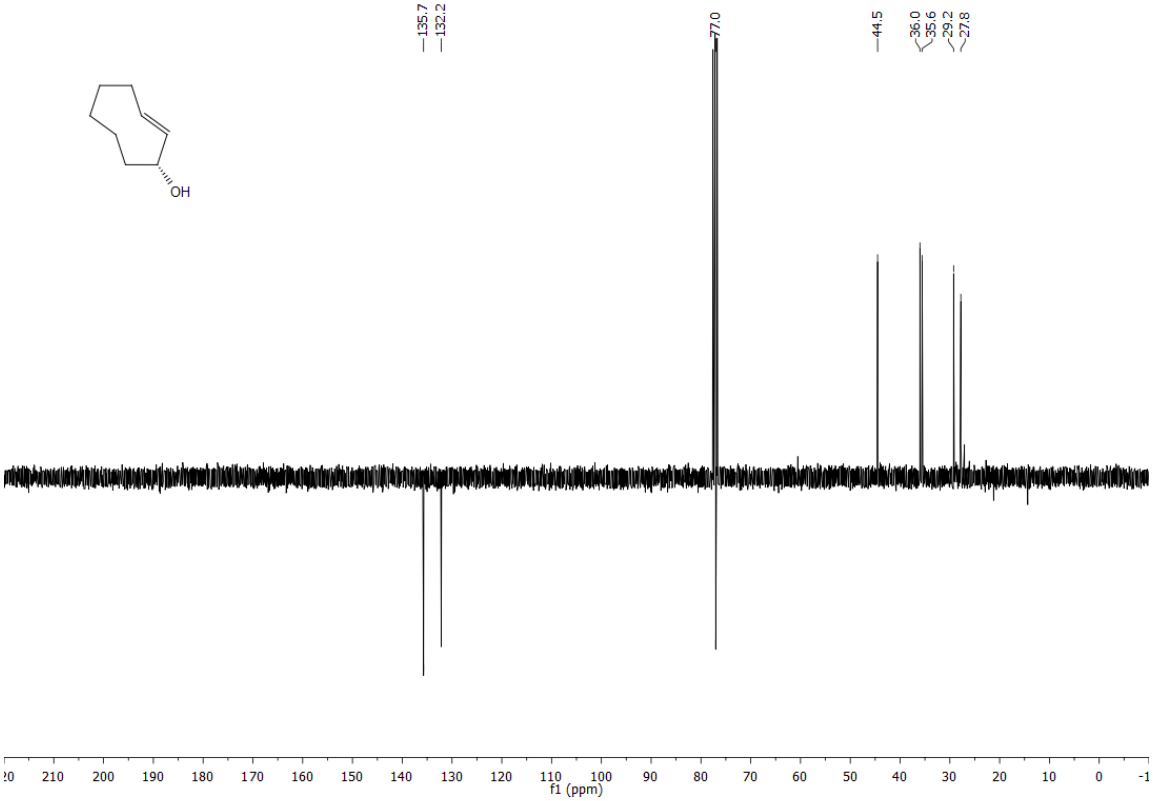

$^{13}$C-NMR of compound **27-ax** ($CDCl_3$)



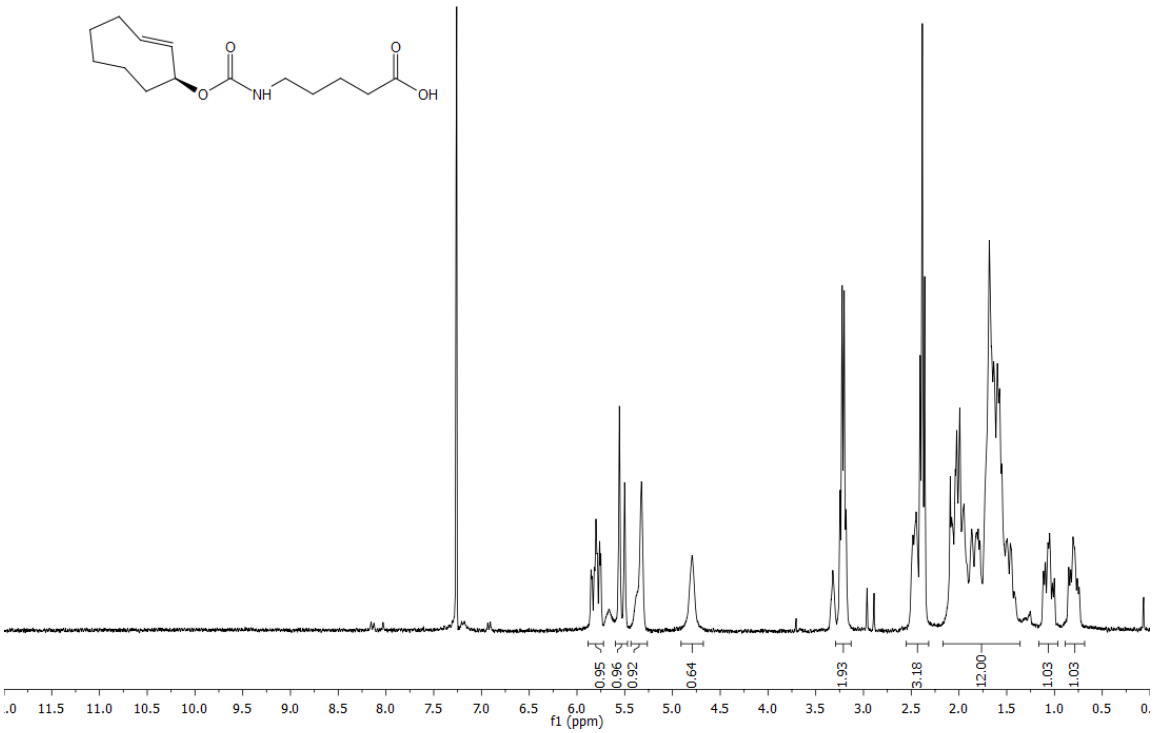
$^{1}$H-NMR of compound **27-eq** ($CDCl_3$)



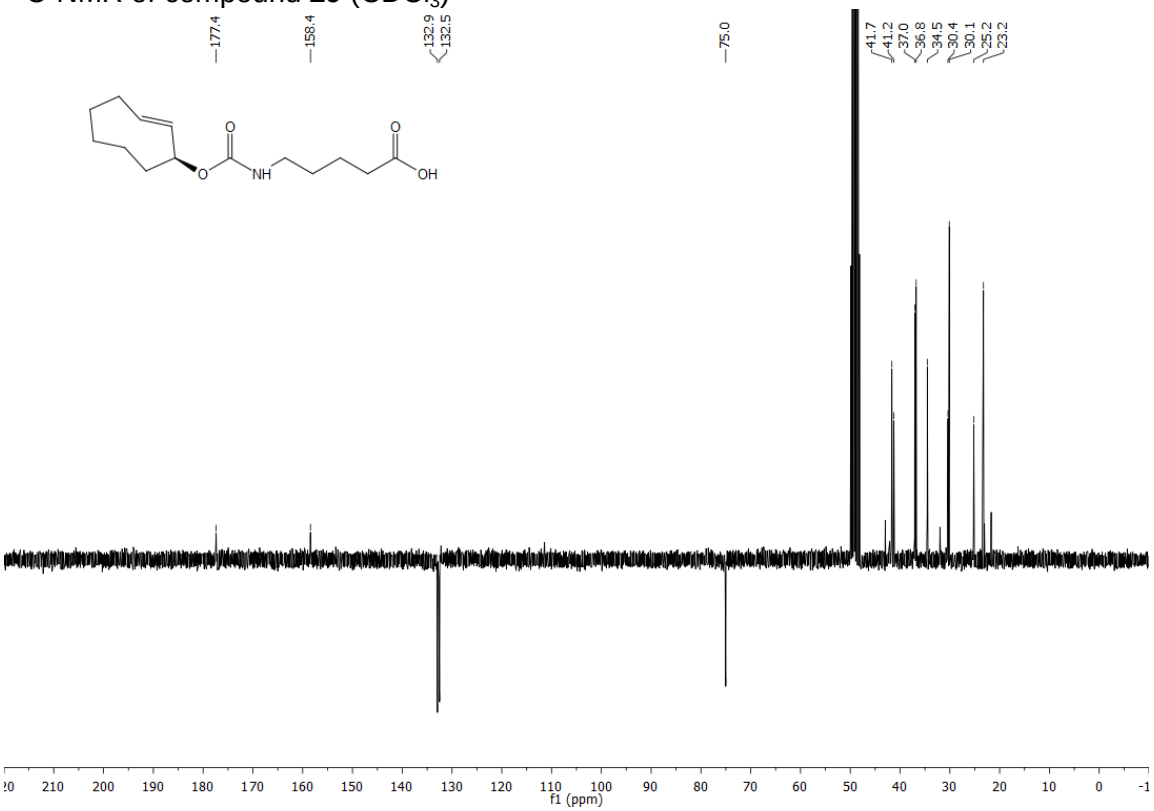

$^{13}$C-NMR of compound **27-eq** ($CDCl_3$)



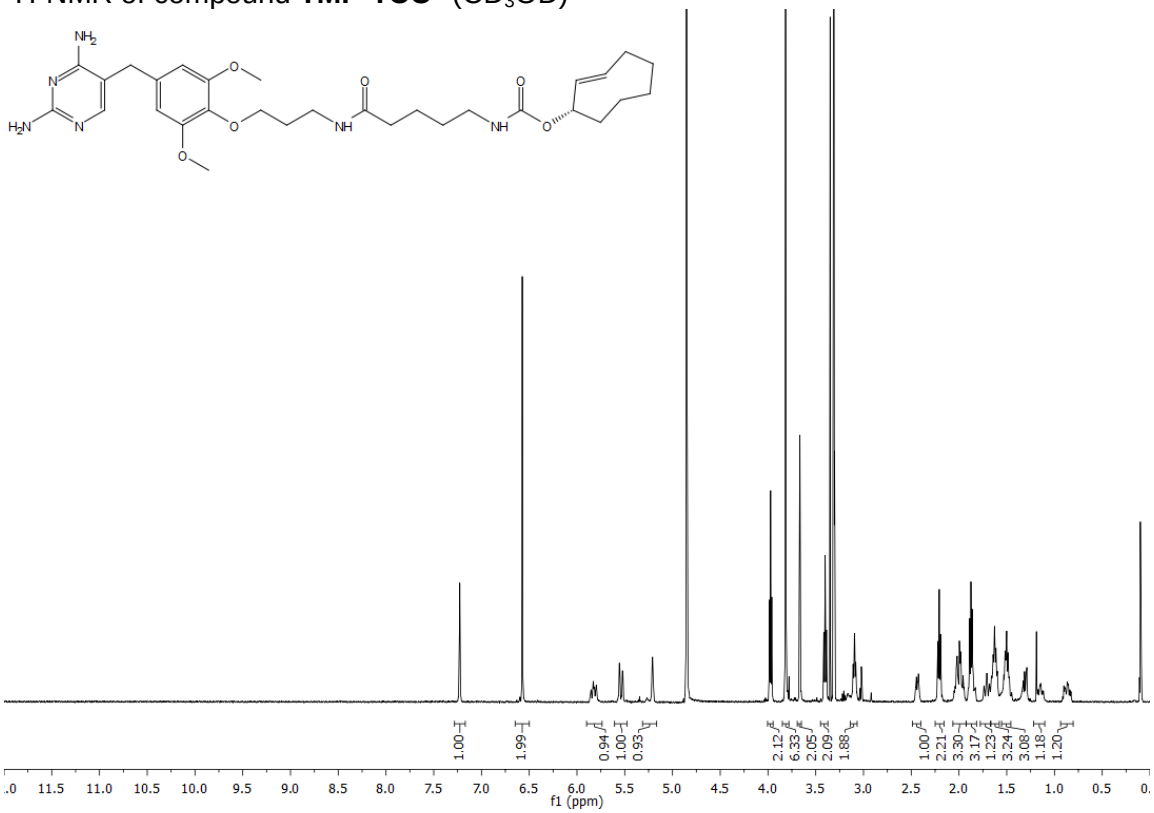
$^{1}$H-NMR of compound **29** ($CDCl_3$)



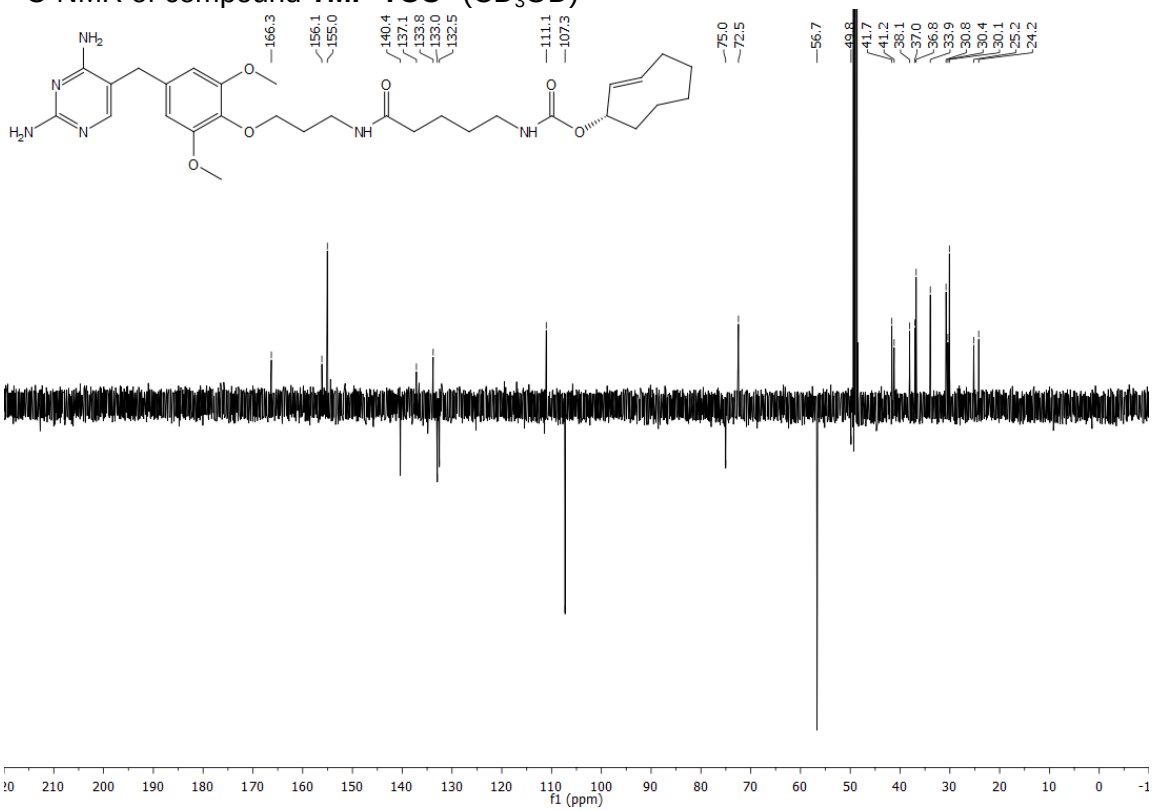

$^{13}$C-NMR of compound **29** ($CDCl_3$)



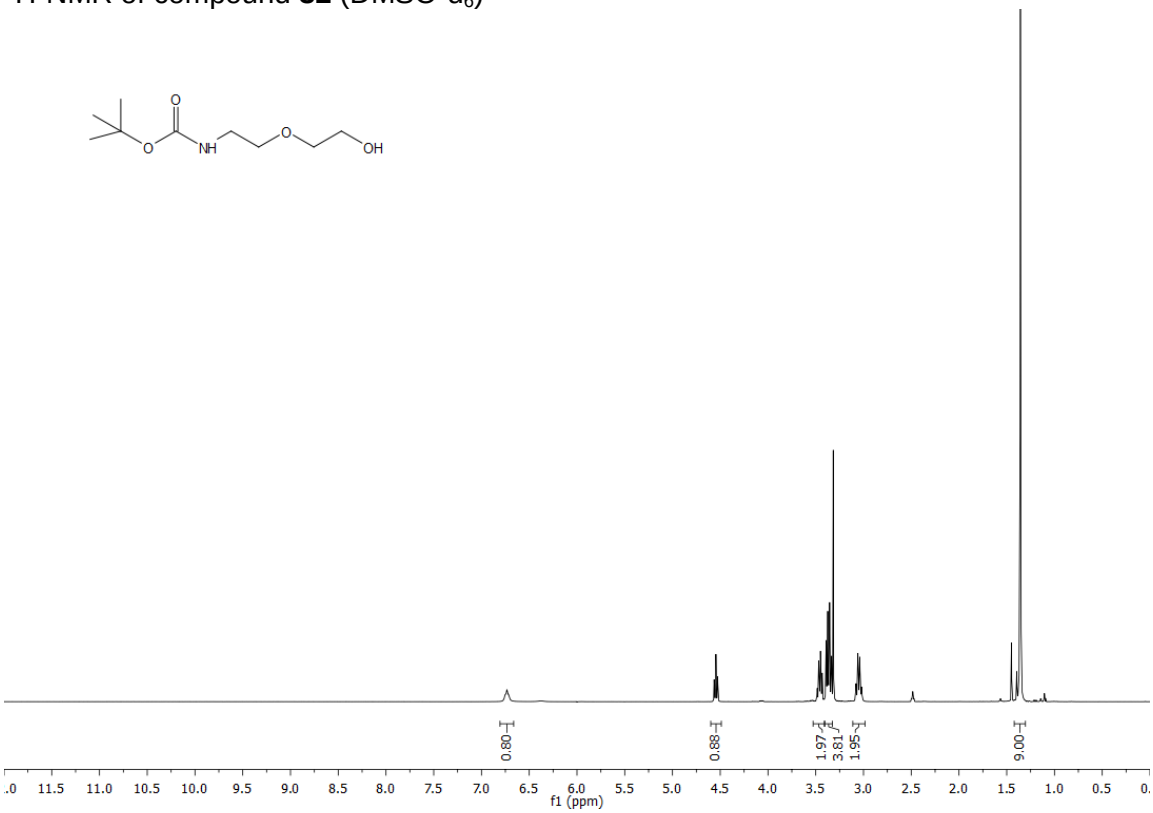
$^{1}$H-NMR of compound **TMP-TCO*** ($CD_3OD$)



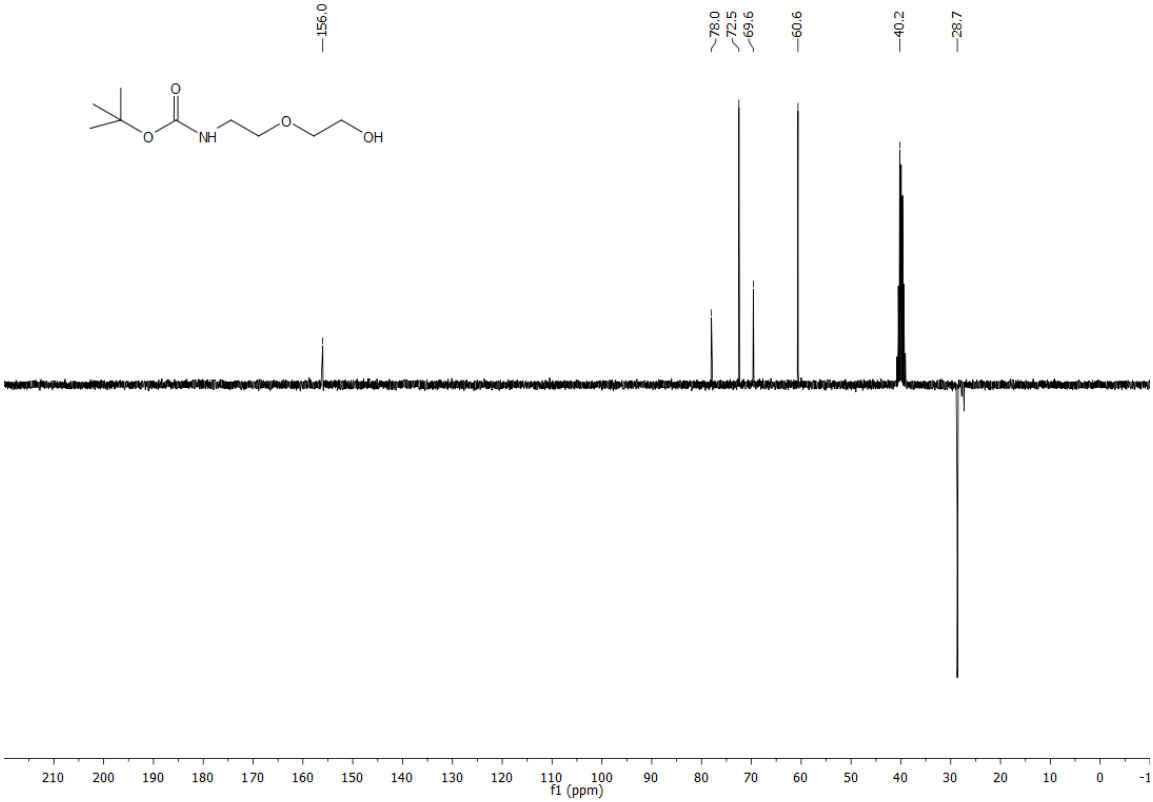

$^{13}$C-NMR of compound **TMP-TCO*** ($CD_3OD$)



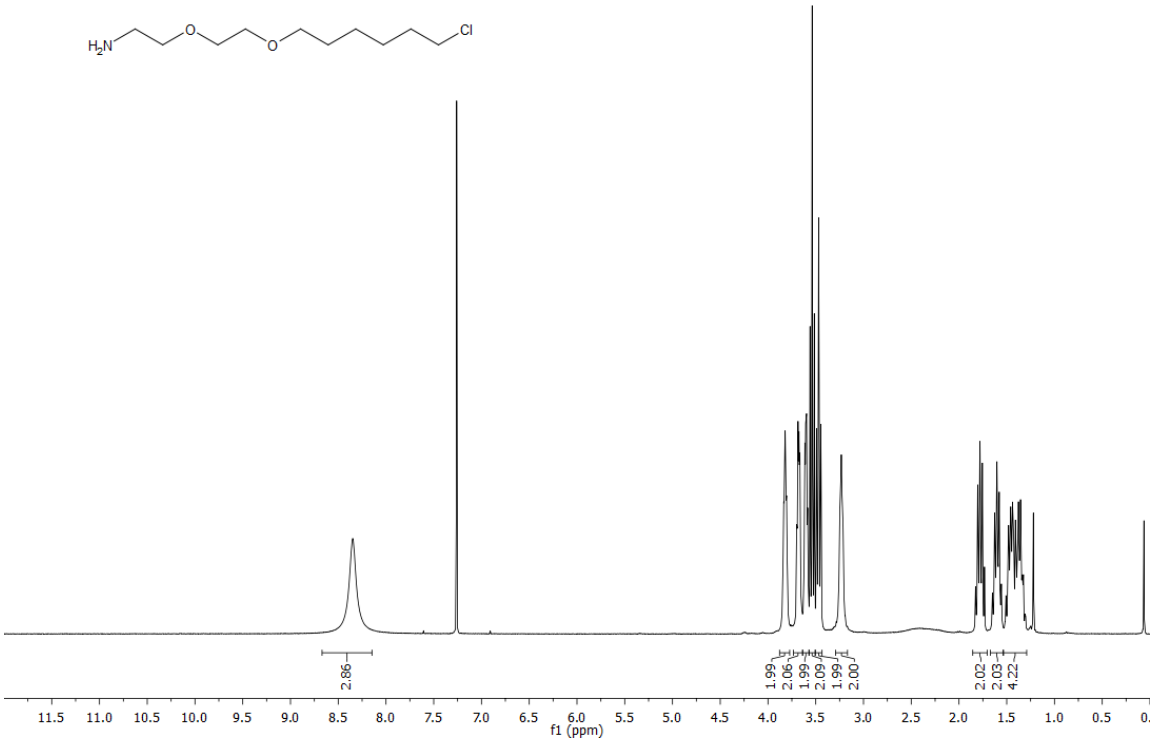
$^{1}$H-NMR of compound **32** (DMSO-$d_6$)



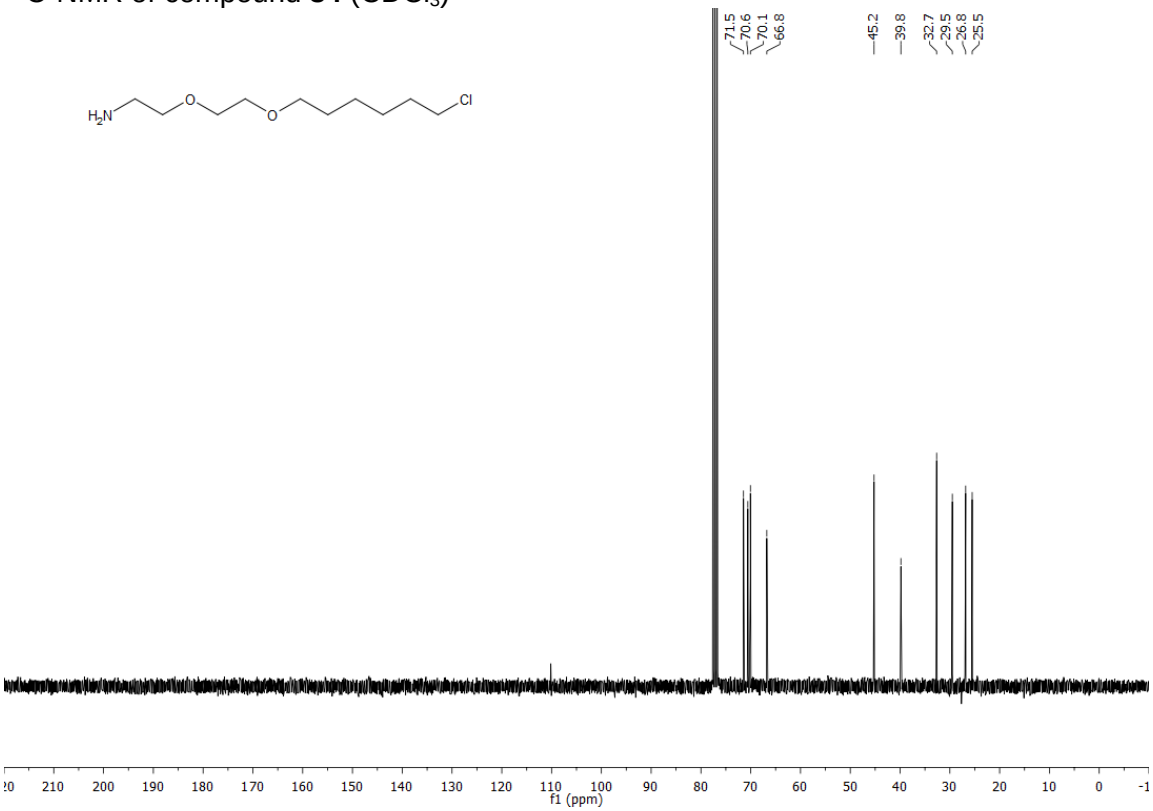

$^{13}$C-NMR of compound **32** (DMSO-$d_6$)



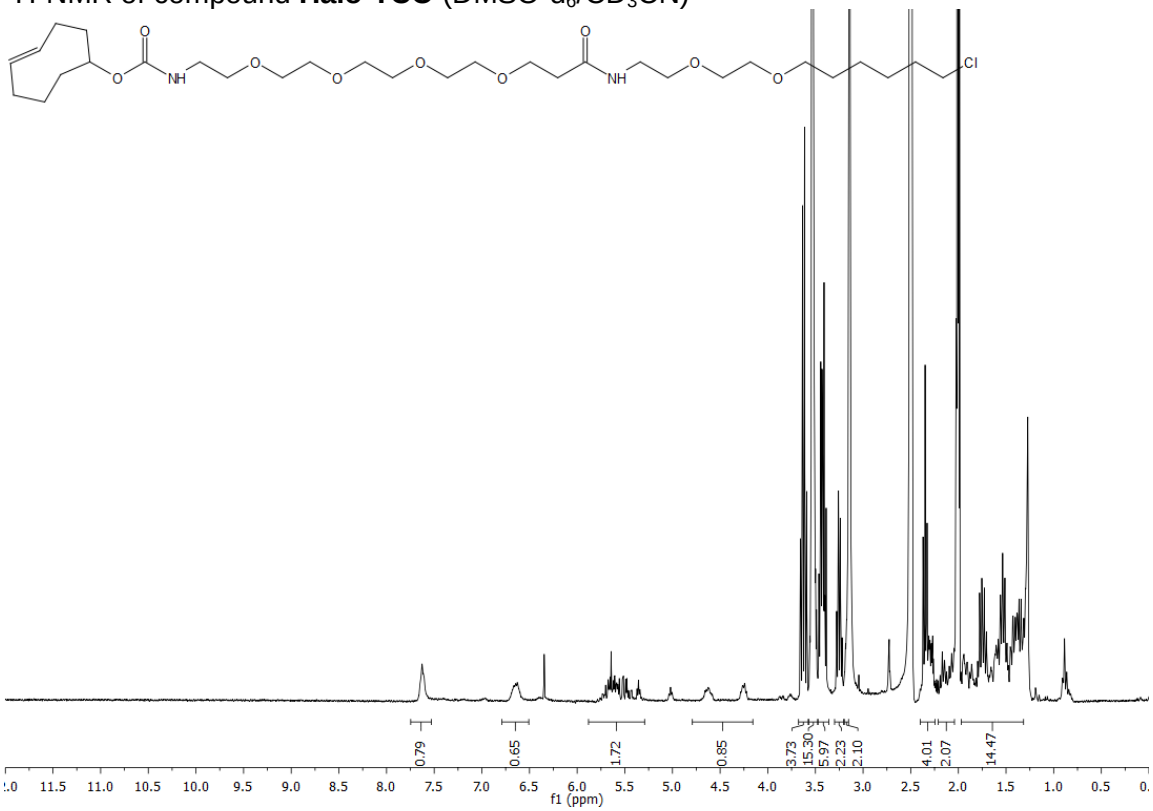
$^{1}$H-NMR of compound **34** ($CDCl_3$)




$^{13}$C-NMR of compound **34** ($CDCl_3$)



$^{1}$H-NMR of compound **Halo-TCO** (DMSO-$d_6$/$CD_3CN$)



# 7 References


[1] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg, *Organometallics* **2010**, *29*, 2176-2179.
[2] G. A. Crosby, J. N. Demas, *J. Phys. Chem.* **1971**, *75*, 991-1024.
[3] R. A. Velapoldi, H. H. Tønnesen, *J. Fluoresc.*, *14*, 465-472.
[4] R. Sens, K. H. Drexhage, *J. Lumin.* **1981**, *24*, 709-712.
[5] G. P. Miller, S. J. Benkovic, *Biochemistry* **1998**, *37*, 6327-6335.
[6] L. Cramer, T. Mitchison, *J. Cell Biol.* **1993**, *122*, 833-843.
[7] L. G. Meimetis, J. C. T. Carlson, R. J. Giedt, R. H. Kohler, R. Weissleder, *Angew. Chem., Int. Ed.* **2014**, *53*, 7531-7534.
[8] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat. Methods* **2012**, *9*, 676-682.
[9] J. Yang, M. R. Karver, W. Li, S. Sahu, N. K. Devaraj, *Angew. Chem., Int. Ed.* **2012**, *51*, 5222-5225.
[10] R. Selvaraj, J. M. Fox, *Tetrahedron Lett.* **2014**, *55*, 4795-4797.
[11] J. L. Liu, B. Yan, *J. Phys. Chem. C* **2008**, *112*, 14168-14178.
[12] E. S. Scott, L. F. Audrieth, *J. Org. Chem.* **1954**, *19*, 1231-1237.
[13] S. C. Fields, M. H. Parker, W. R. Erickson, *J. Org. Chem.* **1994**, *59*, 8284-8287.
[14] H. Wu, J. Yang, J. Seckute, N. K. Devaraj, *Angew. Chem., Int. Ed.* **2014**, *53*, 5805-5809.
[15] G. S. Jiao, J. W. Han, K. Burgess, *J. Org. Chem.* **2003**, *68*, 8264-8267.
[16] S. Lévesque, Y. St-Denis, B. Bachand, P. Préville, L. Leblond, P. D. Winocour, J. J. Edmunds, J. R. Rubin, M. A. Siddiqui, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3161-3164.
[17] D. Maiti, S. L. Buchwald, *J. Am. Chem. Soc.* **2009**, *131*, 17423-17429.
[18] A. Klapars, S. L. Buchwald, *J. Am. Chem. Soc.* **2002**, *124*, 14844-14845.
[19] M. Royzen, G. P. A. Yap, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 3760-3761.
[20] J. Zhang, M. E. Matta, H. Martinez, M. A. Hillmyer, *Macromolecules* **2013**, *46*, 2535-2543.
[21] I. Nikić, T. Plass, O. Schraidt, J. Szymański, J. A. G. Briggs, C. Schultz, E. A. Lemke, *Angew. Chem., Int. Ed.* **2014**, *53*, 2245-2249.
[22] C. Jing, V. W. Cornish, *ACS Chem. Biol.* **2013**, *8*, 1704-1712.
[23] V. Singh, S. Wang, E. T. Kool, *J. Am. Chem. Soc.* **2013**, *135*, 6184-6191.