Supporting Information

Materials and Methods

All reagents were purchased from Sigma-Aldrich and used without further purification unless otherwise noted. ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker Ascend 400 MHz spectrometer. Silica Gel 60 (40-63 μm) was used for flash column purification. High performance liquid chromatography-mass spectrometry analysis (HPLC-MS) was performed with on a Waters instrument equipped with a Waters 2424 ELS Detector, Waters 2998 UV-Vis Diode array Detector, Waters 2475 Multi-wavelength Fluorescence Detector, and a Waters 3100 Mass Detector. Separations employed Waters XTerra RP C18 5 μm or Waters XSelect CSH Fluoro-Phenyl 2.5 uM columns, with a water:acetonitrile solvent gradient (0.1 % formic acid added). Fluorescence measurements were conducted with a Perkin Elmer LS55B Luminescence Spectrometer, and UV-VIS absorption spectra on an Agilent Technologies Cary 100 UV-Vis Spectrophotometer.

Fluorescence assays

The fluorescence purity of all compounds was verified by LCMS prior to quantitative activation experiments, for which a fresh aliquot of the fluorophore collected from the analytical HPLC elution was used. Exceptionally pure material is required to obtain the peak measured turn-on ratios, as the presence of trace bright contaminants limits the maximum observable ratio. Stock solutions of the freshly-purified tetrazine dyes were prepared in MeCN and stored in the dark at 4°C during experiments. For fluorescence measurements, the probes diluted into 2 mL or 3 mL of phosphate buffered saline (PBS), pH 7.4 (Corning, cellgro) in a standard 10 mm quartz cuvette. Working at peak excitation and emission wavelengths for each probe, data were collected as a continuous time series to enable accurate measurement of the baseline intensity values and optimize signal to noise. Fluorescence experiments were conducted at a range of dye concentrations spanning 100nM-750nM, with 500nM being a typical working concentration. The time to peak turn on ratio (but not the final magnitude) is a function of the added TCOc concentration; for the time courses presented in the text, 10μM TCOc was used. Measurements of solvent and pre-activation emission intensity for baseline values were collected serially over at least 30 seconds, prior to addition of TCOc to initiate the fluorogenic reaction. After addition of TCOc (typically a 20-fold excess, as above), the fluorescence emission intensity was monitored until a plateau was reached. Activation ratios were calculated from the peak emission intensity of the dihydropyridazine product and the corresponding baseline intensity over background. Data were normalized to set the initial background fluorescence of the HELIOS probe to one unit over background, as plotted in Figure 1b in the main text.

Quantum yield determinations: quinine sulfate dihydrate (Fluorescence Reference Standard grade, AnaSpec, Inc) in 0.5 M H_2SO_4 was used as a reference, with an excitation wavelength of 370 nm; a value of 0.546 was used for the reference quantum

yield (Eaton, D. F., *Pure and Applied Chemistry*, **1988** *60*(7), 1107–1114). Calculations were made according to the methods described by Crosby and Demas (*Chemical Reviews*, **1971**, 75(8), 991–1024).

In Vitro Imaging

Microscope: Multichannel images were collected on an Olympus *Fluoview* FV1000 confocal laser microscope. Coumarin probes were excited with a 405nm laser, with alternate excitation sources used as relevant for reference channels, paired with appropriate emission filter sets.

Cell culture: A-431 cells (ATCC CRL-1555) and COS-1 cells (ATCC CRL-1650) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10 % fetal bovine serum and grown in standard culture conditions in 10 cm dishes. OVCA-429 cells were cultivated in RPMI-1640 supplemented with 10 % fetal bovine serum under standard culture conditions. For imaging experiments cells were plated on Millicell EZ slides (EMD Millipore, Inc, Billerica, MA).

Antibody reagents: Monoclonal antibody-TCO conjugates were prepared by incubation of commercially available monoclonal antibodies TCO-PEG4-NHS (Click Chemistry Tools, Scottsdale, AZ).

Anti EGFR (Cetuximab, Imclone)
Anti cytochrome c oxidase (COXIV, Cell signaling Technology, #4844, Danvers, MA)

An aliquot of antibody in the manufacturer-supplied storage solution was buffer-exchanged into PBS with 10mM sodium bicarbonate, pH 8.0, on a 40K ZebaSpin desalting column (0.5mL, Thermo Fisher Scientific, Rockford, IL). To this solution was added 20 equivalents of TCO-PEG4-NHS; the mixture was allowed to react at 25°C for 30 minutes, with continuous shaking. The reaction mix was loaded onto a 40K ZebaSpin column to remove organic solvent and small molecule fractions; this eluate was loaded onto a second 40K ZebaSpin column to ensure comprehensive removal of any excess TCO.

EGFR Imaging: Fixed A431 cells were prepared by treatment with 4 % paraformaldehyde solution (10 min, room temperature), followed by 3 washes with PBS. Fixed cells were stored at 4 °C until the time of imaging, when they were incubated for 20 minutes with 20 µg/mL cetuximab-TCO, then rinsed three times with PBS.

For optimal image quality, HELIOS 370H probe must be purified on the day of imaging by reversed phase HPLC-MS. The concentration of stock solutions in PBS were calculated by absorbance spectrometry, based on the measured extinction coefficient of 19000 $M^{-1}cm^{-1}$. Prior to imaging, the purified stock solutions were subjected to turn-on

testing, verifying a fluorogenic turn-on ratio of >1000-fold for HELIOS 370H. For imaging experiments, the acetonitrile stocks were diluted into PBS to yield a 100 nM solution.

Image acquisition: Immediately prior to imaging, buffer was replaced with a 100 nM solution of HELIOS 370H probe in PBS. Specific staining was evident within 10 seconds and reached maximum signal/background intensity over a time course of 3-5 minutes.

Mitochondria Imaging: At ~70% confluence, OVCA-429 cells were incubated with 3% v/v of CellLight Fluorescent mitochondria-targeted red fluorescent protein BacMam reagent, reconstituted according to the manufacturer's guidelines (C10601, Invitrogen, Carsbad, CA), in complete medium for 24 h. Following incubation, cells were washed in PBS and incubated in growth media a further 24 hrs before fixation.

The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X-100 in PBS. Cells were incubated with anti-COX IV-TCO (10μg/mL) for 40 minutes, then washed twice with PBS prior to imaging. HELIOS 388H was freshly purified as described above and added to cells at 100nM concentration. Mitochondrial labeling was evident within 2 minutes and stable target to background ratios were observed on serial images collected up to an hour from dye addition.

Actin Cytoskeleton Imaging: carried out per procedures developed by Mitchison and coworkers (e.g. Cramer, L., and Mitchison, T.J., *J Cell Biol.* 1993 Aug;122(4):833-43, http://mitchison.med.harvard.edu/protocols.html). In brief, phalloidin-TCO was dissolved in methanol to prepare a stock solution at 250-1000 μg/mL (stored at -80°C); this stock was diluted into the labeling buffer to give a final staining solution at 1μg/mL. Labeling buffer: 10mM Tris buffered saline, pH 7.4 (TBS), with 0.1% triton X-100 and 2% bovine serum albumin.

COS-1 cells were grown in standard culture conditions as described above and then fixed and permeabilized per the procedures of Mitchison and coworkers (*vide supra*). In brief, cells were fixed in 4% formaldehyde in cytoskeleton buffer for 20 minutes, and then permeabilized with 0.1% Triton-X-100 in TBS. Cytoskeleton buffer: 10mM MES, pH6.1, 138mM KCl, 3mM MgCl, 2mM EGTA, 0.32M sucrose. Nuclear staining was performed by incubating the fixed and permeabilized cells with DRAQ5 (Biostatus, DR50050) diluted to a final concentration of 1 μM for 3-5 minutes at room temperature. After 20-40 minutes incubation with phalloidin-TCO (1μg/mL), cells were washed once with PBS and then imaged after addition of 100nM HELIOS 388H or HELIOS 370H.

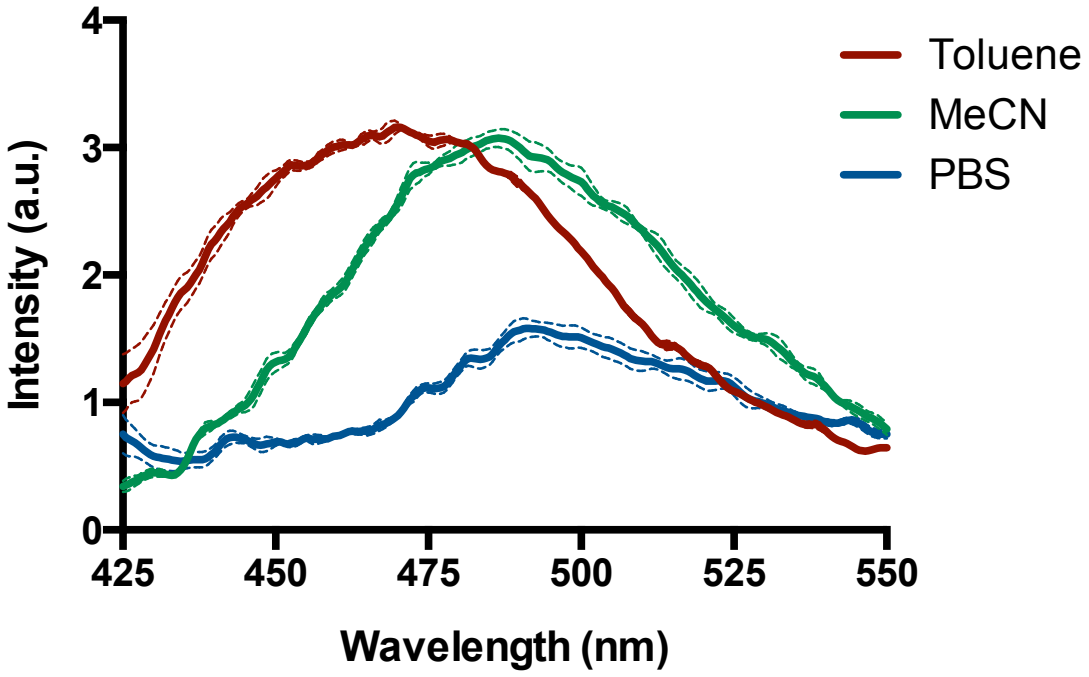


Figure S1: Solvent polarity effects. Fluorescence emission spectra of HELIOS 400Me in PBS (pH 7.4), acetonitrile (dielectric constant 37.5), and toluene (dielectric constant 2.4). Emission spectra are the mean of 2 or 3 scans and the dashed lines represent +/- SEM. Instrument settings were adjusted to optimize sensitivity given the minimal fluorescence of the native HELIOS probe, and samples were prepared by matched dilution of a concentrated stock solution of HELIOS 400Me into the respective solvents. Redox-based quenching, such as through photoinduced electron transfer (PET) from the excited coumarin to the relatively electron-poor tetrazine ring, was judged unlikely to contribute significantly, because the fluorescence emission intensity was largely independent of solvent polarity, with less than a two fold change between PBS and the organic solvents, and no intensity difference between toluene ($\varepsilon = 2.4$) and acetonitrile ($\varepsilon = 37.5$). PET is characteristically enhanced by the relative stabilization of charge-separated states in polar solvents [E. E. Neuteboom, S. C. J. Meskers, E. H. A. Beckers, S. Chopin, R. A. J. Janssen, J. Phys. Chem. A 2006, 110, 12363].

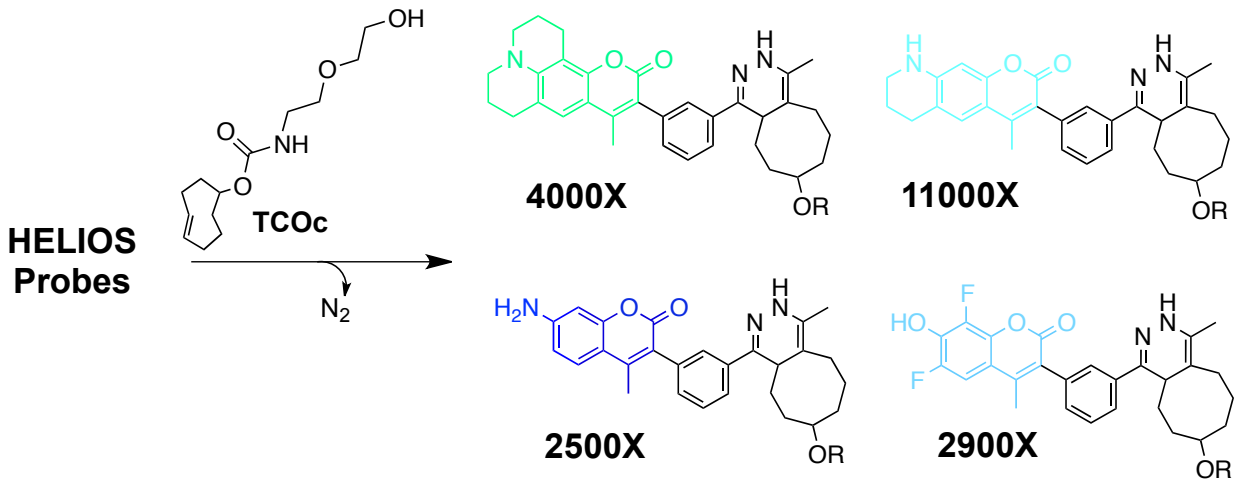


Figure S2: Click reaction between TCOc and various HELIOS probes. The fluorogenic turn on ratio of each probe in phosphate buffered saline is indicated below the structure.

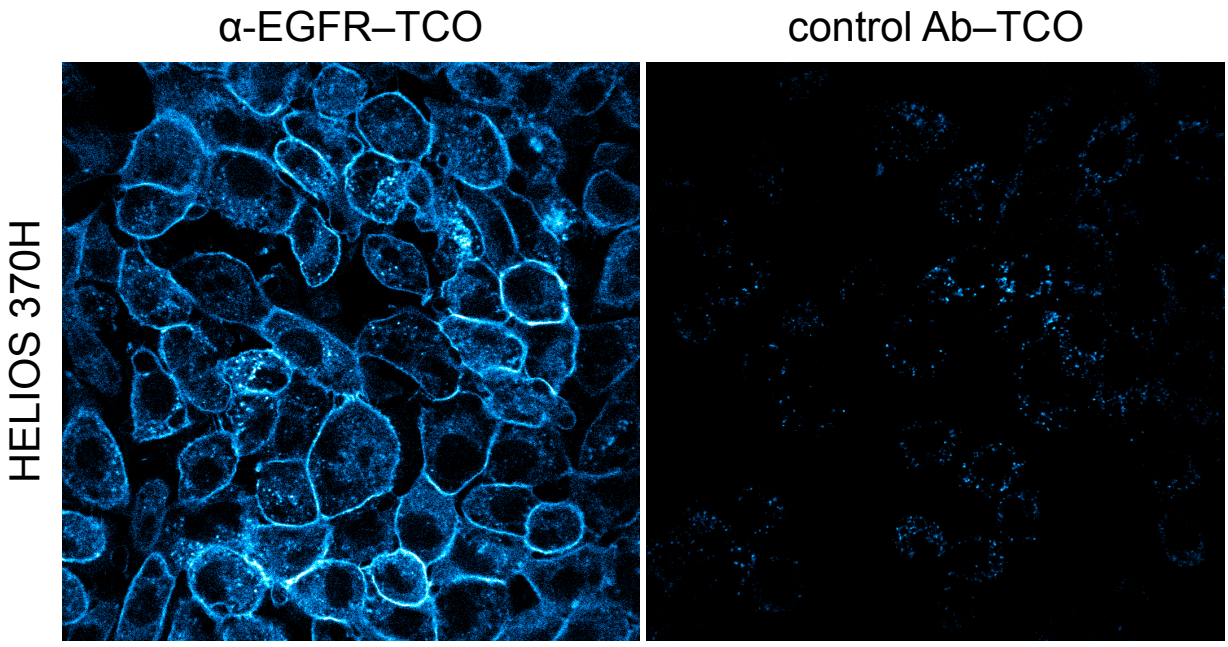
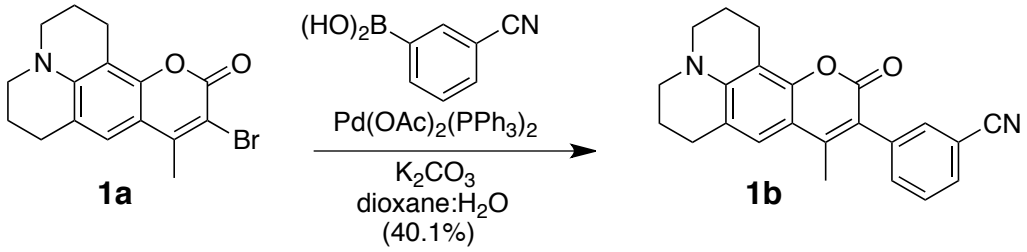


Figure S3. No wash fluorogenic imaging of EGFR expression on fixed A431 cells. Cells were incubated with TCO-conjugated monoclonal antibodies as indicated, washed briefly with PBS, and then imaged immediately after the addition of 100 nM HELIOS 370H in PBS. The control cells exhibited punctate autofluorescence at baseline, prior to addition of dye, as also seen in the t = 0 images in Figure 2c of the main text.

Synthesis and Characterization Data

Preparation of **1b**:



1a **1b**

To bromocoumarin **1a** (245.0 mg, 0.733 mmol) in 8.0 mL of dioxane:water (3:1) was added 3-cyanophenylboronic acid (215.4 mg, 1.47 mmol), $Pd(OAc)_2(PPh_3)_2$ (27.4 mg, 0.037 mmol), and K_2CO_3 (202.6 mg, 1.46 mmol). The reaction mixture was refluxed for 7 hours after which it was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate gradient, 6:1 to 4:1) to give **1b** (105.0 mg, 0.29 mmol, 40.1%) as a yellow solid**.**

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61-7.47 (m, 4H), 7.04 (s, 1H), 3.25 (m, 4H), 2.89 (t, J = 6.4 Hz, 2H), 2.76 (t, J = 6.0 Hz, 2H), 2.16 (s, 3H), 1.97 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.9, 150.5, 149.5, 146.2, 137.4, 135.6, 134.5, 131.2, 129.2, 122.6, 118.9, 118.5, 118.1, 112.6, 109.0, 106.7, 50.1, 49.7, 27.9, 21.7, 20.8, 20.6, 16.6. ESIMS $[M+H]^+$ calcd for $C_{23}H_{21}N_2O_2$ 357.42, found 357.16.

Bromocoumarin **1a** was prepared from literature protocol (Gong, et al., PCT Int. Appl. (2006), WO 2006026368 A2 20060309).

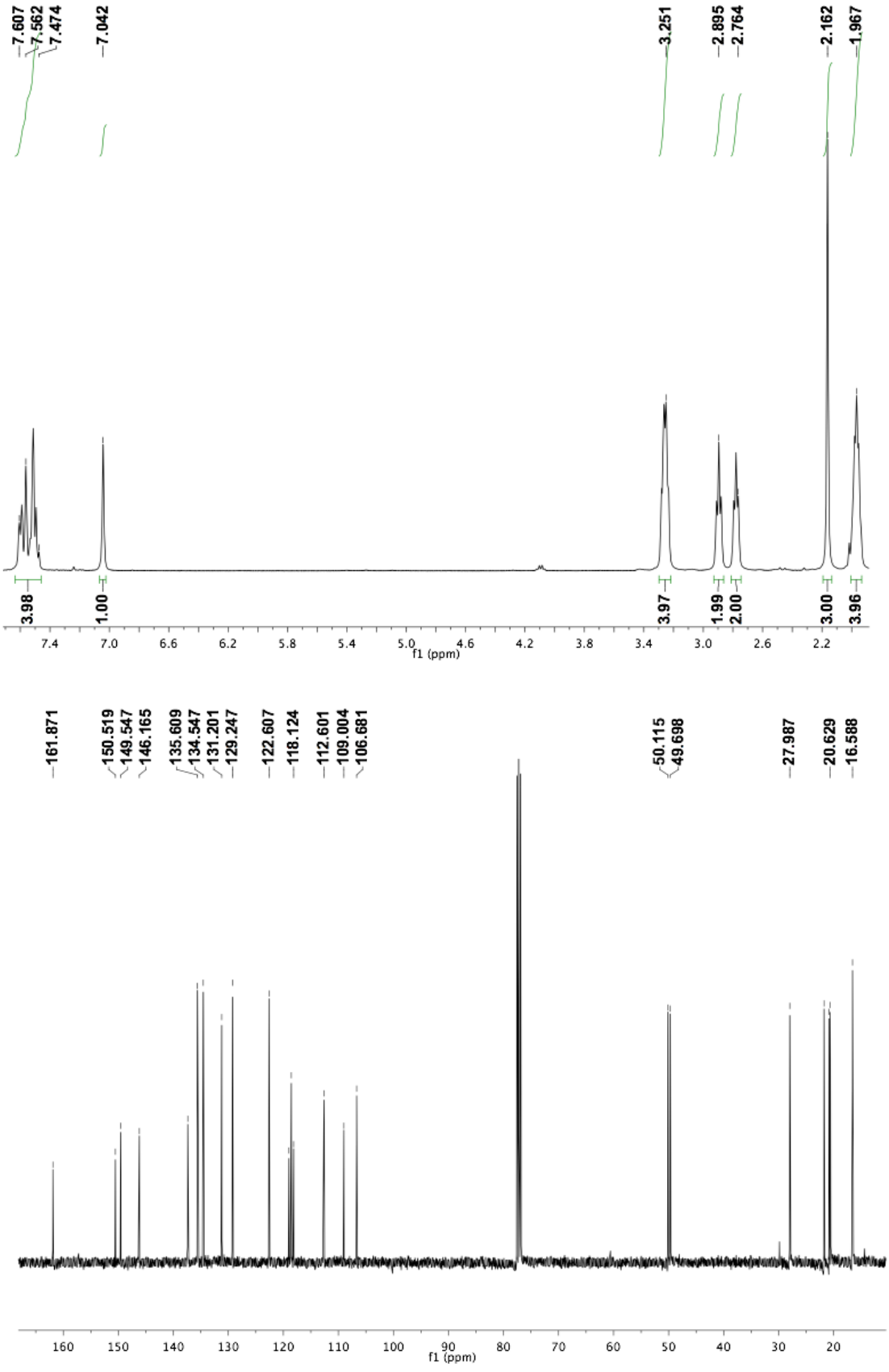
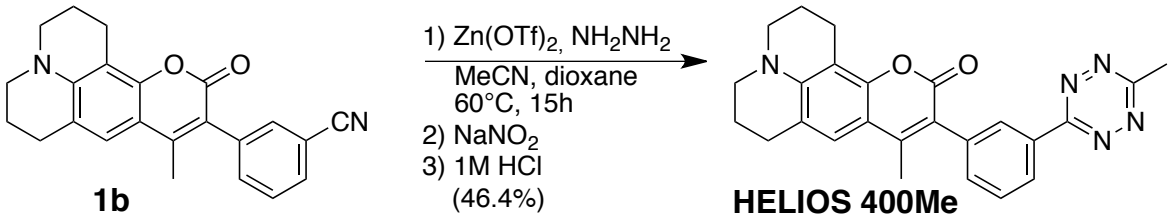


Figure 1.1 ^{1}H and ^{13}C NMR spectra of **1b** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 400Me**:



1b **HELIOS 400Me**

To nitrile **1b** (100.0 mg, 0.28 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (51.3 mg, 0.14 mmol), MeCN (0.15 mL, 2.80 mmol), dioxane (0.22 mL) and NH_2NH_2 (0.44 mL, 14.0 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (386.4 mg, 5.60 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 5:1 to 1:1) to give **HELIOS 400Me** (55.3 mg, 0.13 mmol, 46.4%) as an orange solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, J = 8.0 Hz, 1H), 8.50 (s, 1H), 7.63 (t, J = 8.0 Hz, 1H), 7.55 (d, J = 7.6 Hz, 1H), 7.06 (s, 1H), 3.26 (m, 4H), 3.07 (s, 3H), 2.93 (t, J = 6.4 Hz, 2H), 2.79 (t, J = 6.4 Hz, 2H), 2.23 (s, 3H), 1.99 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 164.3, 162.1, 150.4, 149.1, 145.6, 137.2, 135.2, 132.0, 130.3, 129.4, 127.2, 122.6, 119.9, 118.6, 109.7, 107.2, 50.2, 49.8, 27.9, 21.8, 21.3, 20.9, 20.7, 16.7. ESIMS $[M+H]^+$ calcd for $C_{25}H_{24}N_5O_2$ 426.19, found 426.24.

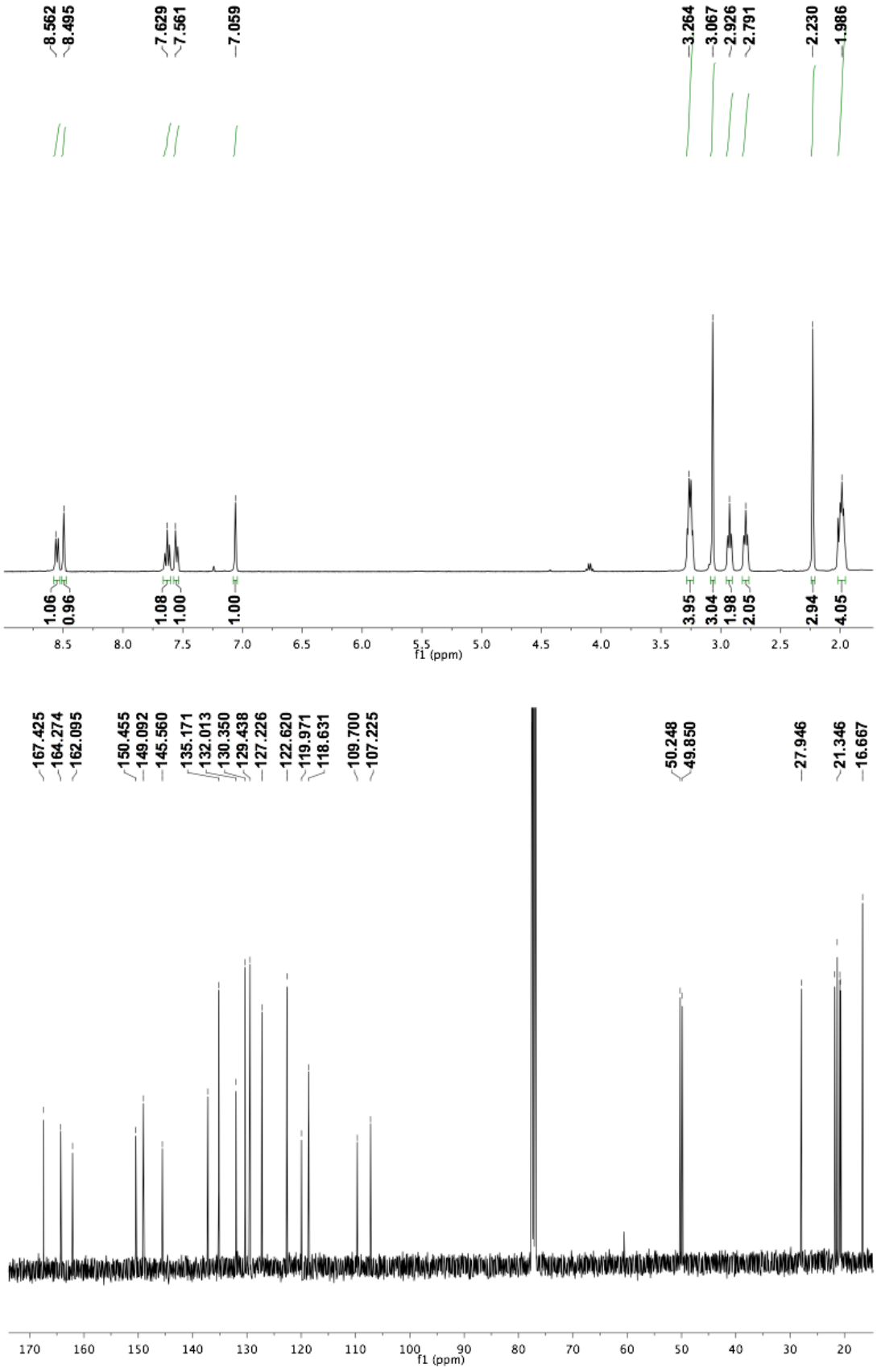
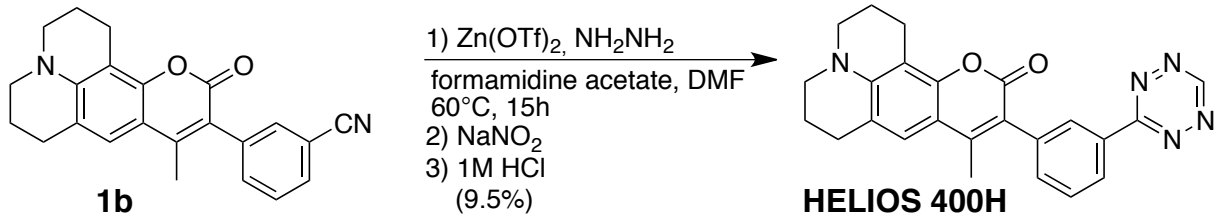


Figure 1.2 ^{1}H and ^{13}C NMR spectra of **HELIOS 400Me** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 400H**:



To nitrile **1b** (110.4 mg, 0.31 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (56.6 mg, 0.15 mmol), formamidine acetate (322.7 mg, 3.10 mmol), DMF (0.24 mL) and NH_2NH_2 (0.49 mL, 15.5 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (427.8 mg, 6.20 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 4:1 to 2:1) to give **HELIOS 400H** (12.1 mg, 0.029 mmol, 9.5%) as an orange solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.19 (s, 1H), 8.60 (d, J = 7.6 Hz, 1H), 8.54 (s, 1H), 7.66 (t, J = 7.6 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.08 (s, 1H), 3.28 (m, 4H), 2.94 (t, J = 6.4 Hz, 2H), 2.81 (t, J = 6.4 Hz, 2H), 2.24 (s, 3H), 2.01 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.5, 161.9, 157.8, 150.3, 148.9, 145.6, 137.2, 135.6, 131.6, 130.6, 129.4, 127.4, 122.4, 119.6, 118.4, 109.4, 106.9, 50.0, 49.6, 27.8, 21.6, 20.7, 20.5, 16.5. ESIMS $[M+H]^+$ calcd for $C_{24}H_{22}N_5O_2$ 412.17, found 412.18.

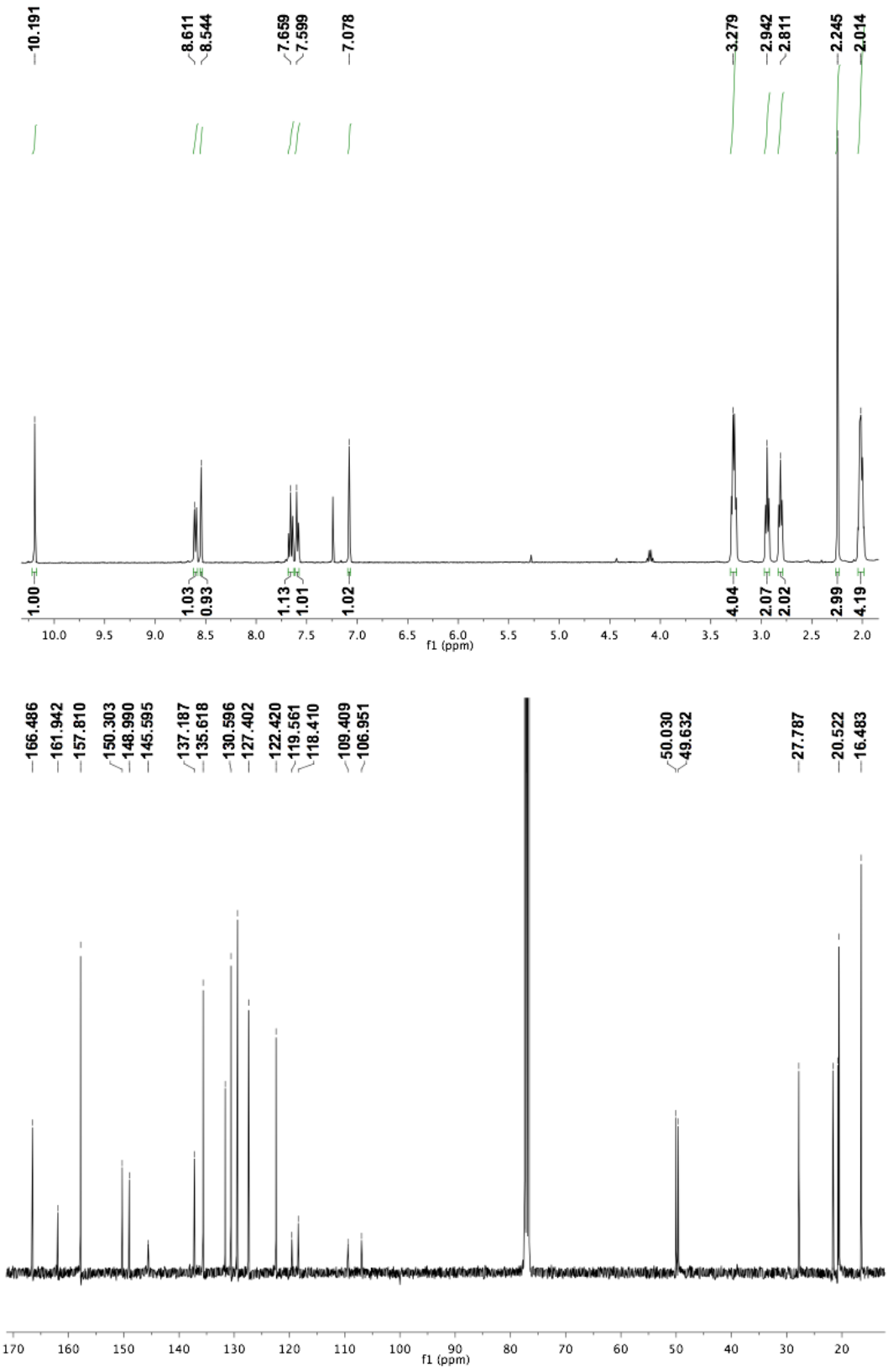
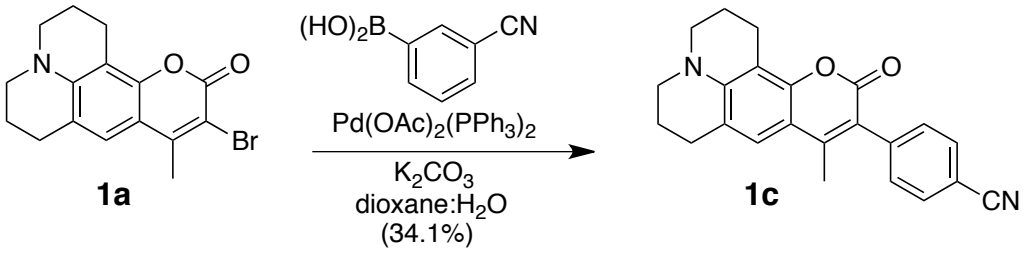


Figure 1.3 ^{1}H and ^{13}C NMR spectra of **HELIOS 400H** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **1c**:



To bromocoumarin **1a** (105.5 mg, 0.31 mmol) in 4.0 mL of dioxane:water (3:1) was added 3-cyanophenylboronic acid (91.1 mg, 0.62 mmol), $Pd(OAc)_2(PPh_3)_2$ (11.6 mg, 0.015 mmol), and K_2CO_3 (85.6 mg, 0.62 mmol). The reaction mixture was refluxed for 7 hours after which it was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate gradient, 6:1 to 4:1) to give **1c** (37.7 mg, 0.10 mmol, 34.1%) as a yellow solid**.**

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.86 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 8.0 Hz, 2H), 7.20 (s, 1H), 3.25 (m, 4H), 2.75 (m, 4H), 2.14 (s, 3H), 1.89 (m, 4H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 160.2, 149.7, 149.3, 145.6, 140.8, 131.8 (2C), 122.8, 118.8, 118.0, 117.3, 109.9, 108.0, 105.2, 49.2, 48.6, 27.0, 20.9, 20.1, 19.9, 16.1. ESIMS $[M+H]^+$ calcd for $C_{23}H_{21}N_2O_2$ 357.16, found 357.16.

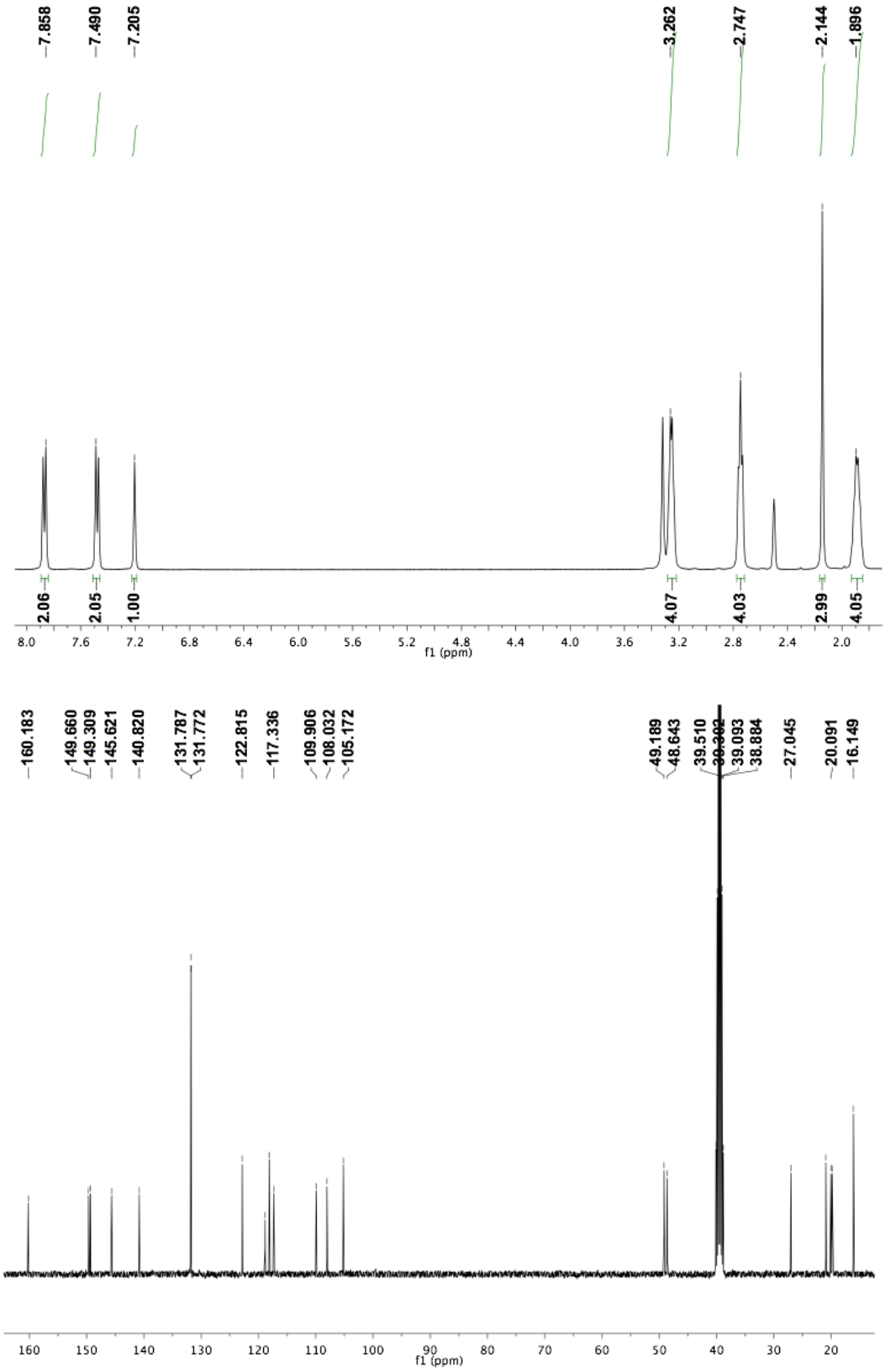
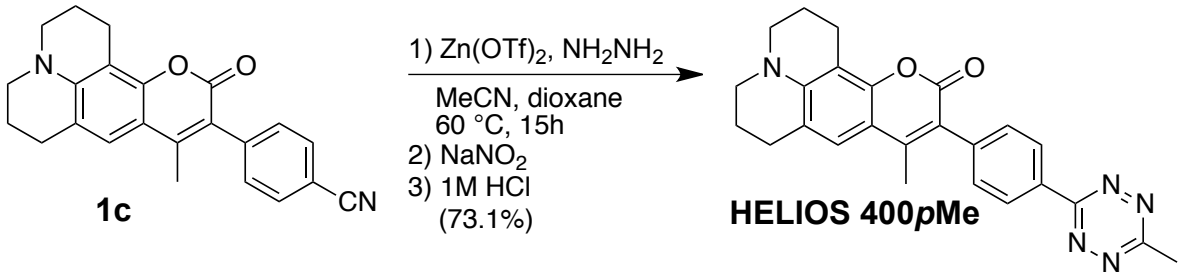


Figure 1.4 ^{1}H and ^{13}C NMR spectra of **1c** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 400*p*Me**:



To nitrile **1c** (37.7 mg, 0.10 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (19.3 mg, 0.52 mmol), MeCN (0.055 mL, 1.05 mmol), dioxane (0.083 mL) and NH_2NH_2 (0.16 mL, 5.28 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (145.9 mg, 2.11 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 4:1 to 2:1) to give **HELIOS 400*p*Me** (31.1 mg, 0.073 mmol, 73.1%) as an orange solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.06 (s, 1H), 3.26, (m, 4H), 3.08 (s, 3H), 2.93 (t, J = 6.0 Hz, 2H), 2.79 (t, J = 6.4 Hz, 2H), 2.34 (s, 3H), 1.99 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 164.2, 161.9, 150.5, 149.0, 145.8, 140.7, 131.9 (2C), 130.9, 127.9 (2C), 122.6 , 119.7, 118.5, 109.4, 106.9, 50.2, 49.8, 28.0, 21.8, 21.4, 20.9, 20.7, 16.7. ESIMS $[M+H]^+$ calcd for $C_{25}H_{24}N_5O_2$ 426.19, found 426.19.

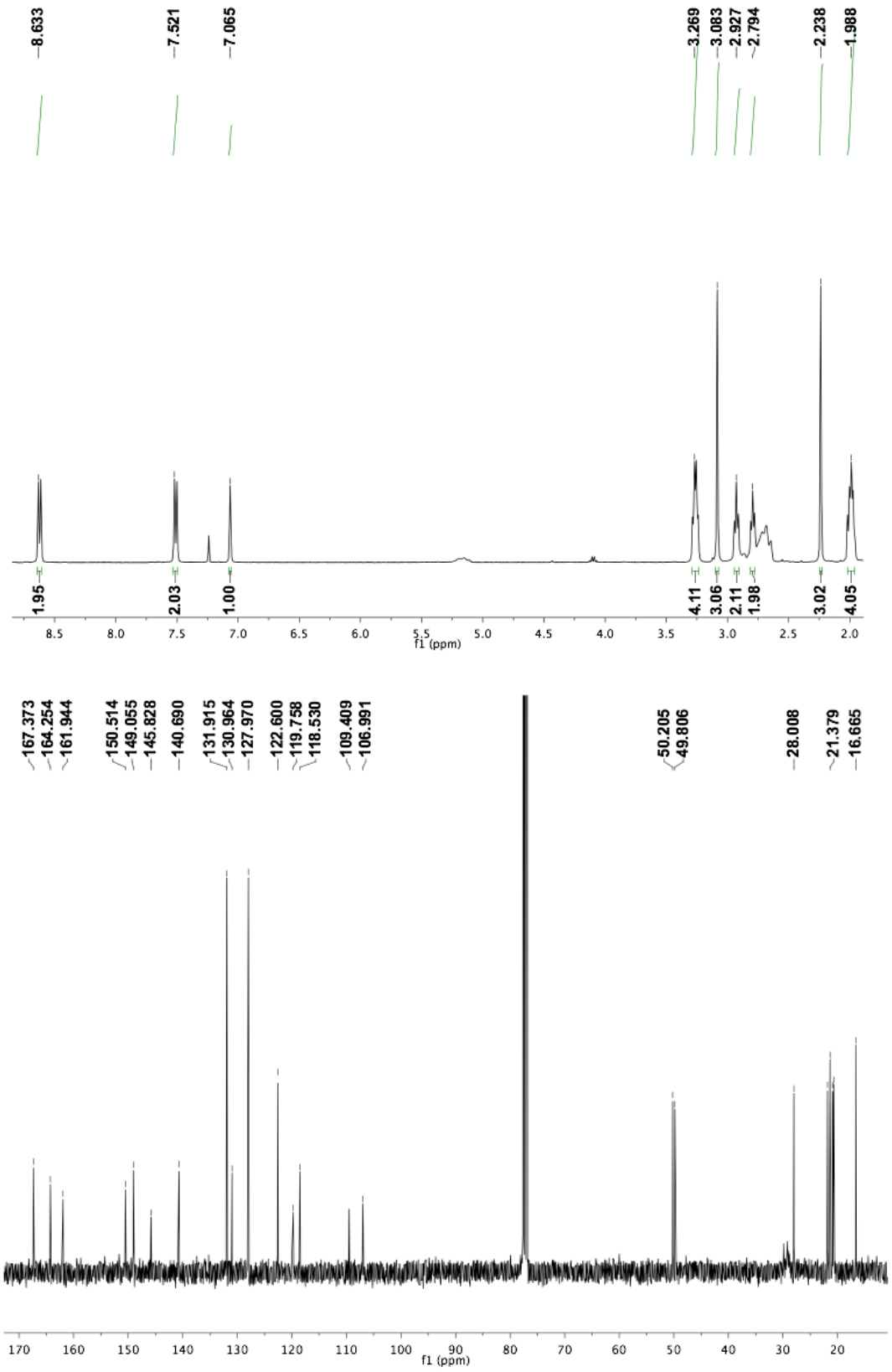
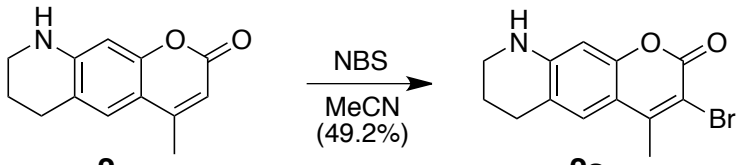


Figure 1.5 ^{1}H and ^{13}C NMR spectra of **HELIOS 400*p*Me** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **2a**:



2 **2a**

To coumarin 339 (**2**) (410.6 mg, 1.90 mmol) dissolved in 25 mL of acetonitrile was added NBS (373.7 mg, 2.10 mmol) and the reaction mixture allowed to stir for 2 hours. The crude mixture was concentrated using a rotary evaporator and purified using flash column chromatography (methylene chloride to methylene chloride:methanol 250:1) to give **2a** (276.4 mg, 0.94 mmol, 49.4%) as a yellow solid**.**

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.27 (s, 1H), 6.93 (bs, 1H), 6.29 (s, 1H), 3.24 (t, J = 4.8 Hz, 2H), 2.72 (t, J = 5.6 Hz, 2H), 2.44 (s, 3H), 1.79 (m, 2H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 156.9, 152.2, 152.1, 149.4, 125.6, 118.1, 107.9, 103.2, 97.0, 40.3, 26.3, 20.6, 18.9. ESIMS $[M+H]^+$ calcd for $C_{13}H_{13}BrNO_2$ 294.01, found 294.00.

Coumarin 339 (**2**) was prepared from literature protocol (R. L. Atkins, D. E. Bliss, *J. Org. Chem.* **1978**, *43*, 1975).

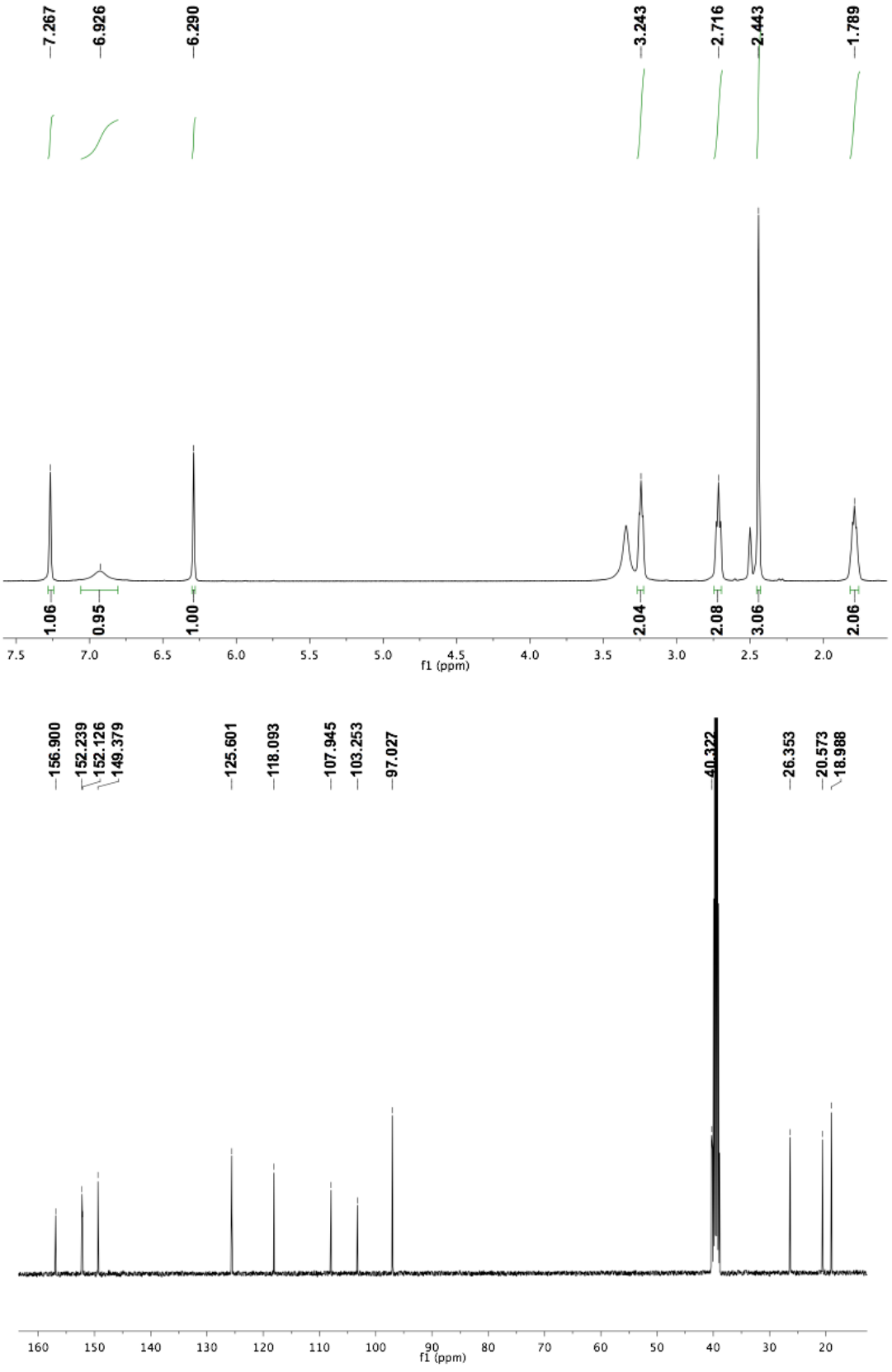
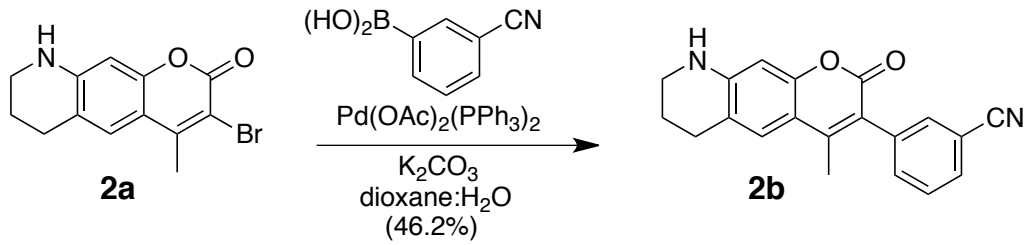


Figure 1.6 ^{1}H and ^{13}C NMR spectra of 2**a** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **2b**:



2a **2b**

To bromocoumarin **2a** (150 mg, 0.51 mmol) in 5.2 mL of dioxane:water (3:1) was added 3-cyanophenylboronic acid (151.6 mg, 1.01 mmol), $Pd(OAc)_2(PPh_3)_2$ (19.1 mg, 0.025 mmol), and K_2CO_3 (140.9 mg, 1.01 mmol). The reaction mixture was refluxed for 7 hours after which it was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate gradient, 4:1 to 2:1) to give **2b** (74.5 mg, 0.23 mmol, 46.2%) as a yellow solid**.**

^{1}H NMR (400 MHz, CD_3OD) δ 7.72 (m, 1H), 7.67 (s, 1H), 7.60 (m, 2H), 7.33 (s, 1H), 6.36 (s, 1H), 3.36 (t, J = 5.6 Hz, 2H), 2.83 (t, J = 6.4 Hz, 2H), 2.23 (s, 3H), 1.93 (quin, J = 5.6 Hz, 2H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 160.4, 152.9, 149.5, 149.1, 136.9, 135.7, 134.2, 131.0, 129.2, 125.7, 118.7, 117.7, 116.9, 111.1, 108.2, 97.1, 40.4, 26.4, 20.7, 16.1. ESIMS $[M+H]^+$ calcd for $C_{20}H_{17}N_2O_2$ 317.12, found 317.18.

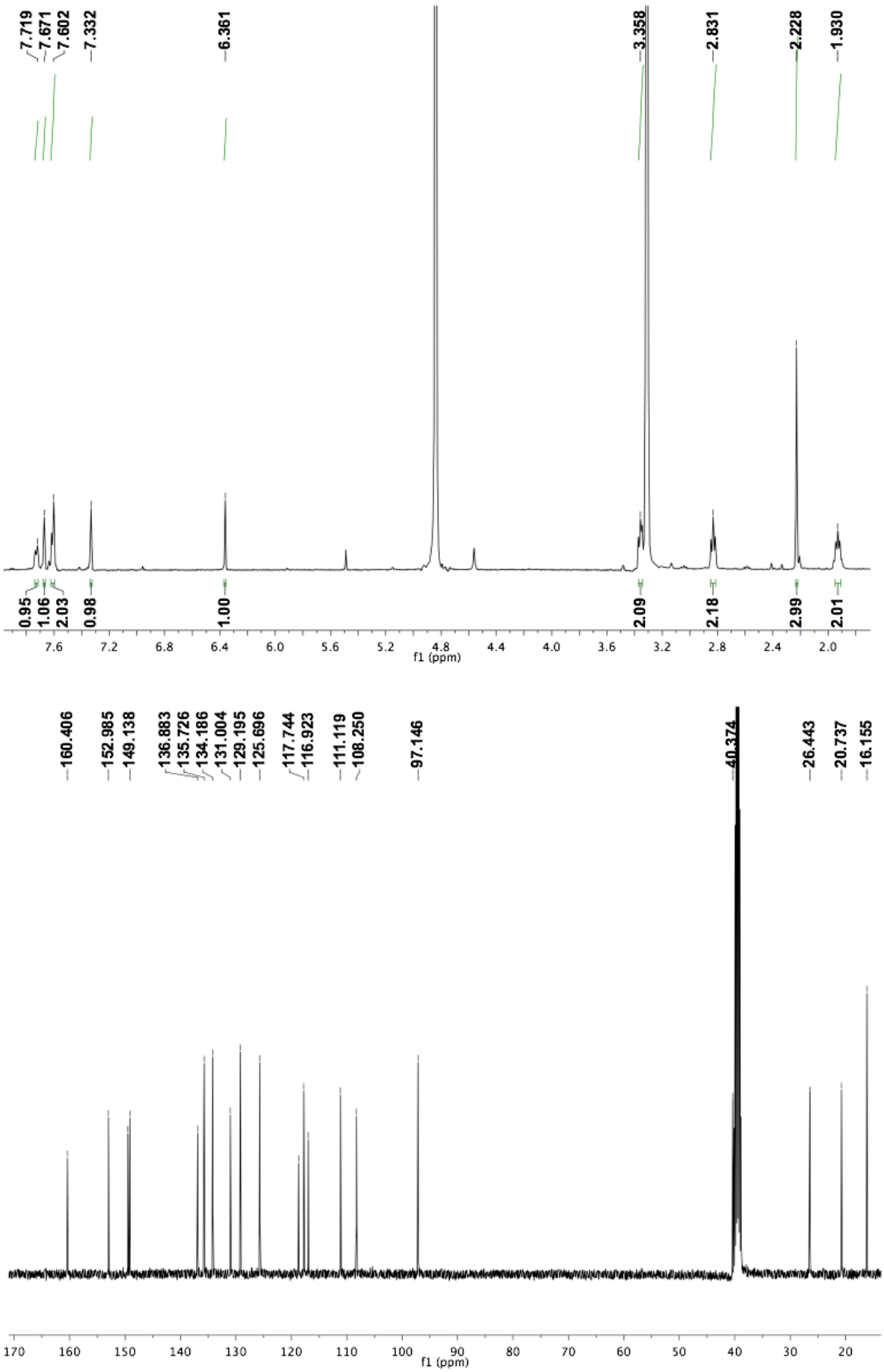
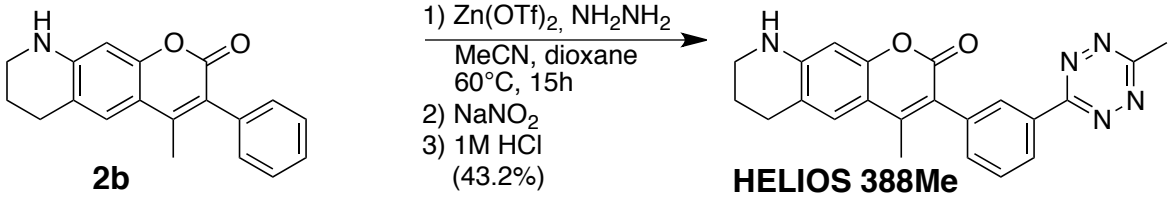


Figure 1.7 ^{1}H and ^{13}C NMR spectra of **2b** recorded in CD_3OD and $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 388Me**:



2b **HELIOS 388Me**

To nitrile **2b** (64.8 mg, 0.20 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (37.4 mg, 0.10 mmol), MeCN (0.11 mL, 2.10 mmol), dioxane (0.16 mL) and NH_2NH_2 (0.32 mL, 10.20 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (276.0 mg, 4.0 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 3:1 to 1:1) to give **HELIOS 388Me** (33.3 mg, 0.086 mmol, 43.2%) as an orange solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, J = 8.0 Hz, 1H), 8.50 (s, 1H), 7.64 (t, J = 7.6 Hz, 1H), 7.56 (d, J = 7.6 Hz, 1H), 7.18 (s, 1H), 6.37 (s, 1H), 4.55 (s, 1H), 3.37 (m, 2H), 3.07 (s, 3H), 2.80 (t, J = 6.0 Hz, 2H), 2.25 (s, 3H), 1.95 (quin, J = 5.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 164.3, 162.1, 153.6, 149.1, 148.3, 136.9, 135.1, 132.1, 130.3, 129.5, 127.3, 125.7, 120.5, 118.5, 110.6, 99.2, 41.8, 27.2, 21.7, 21.4, 16.7. ESIMS $[M+H]^+$ calcd for $C_{22}H_{20}N_5O_2$ 386.16, found 386.13.

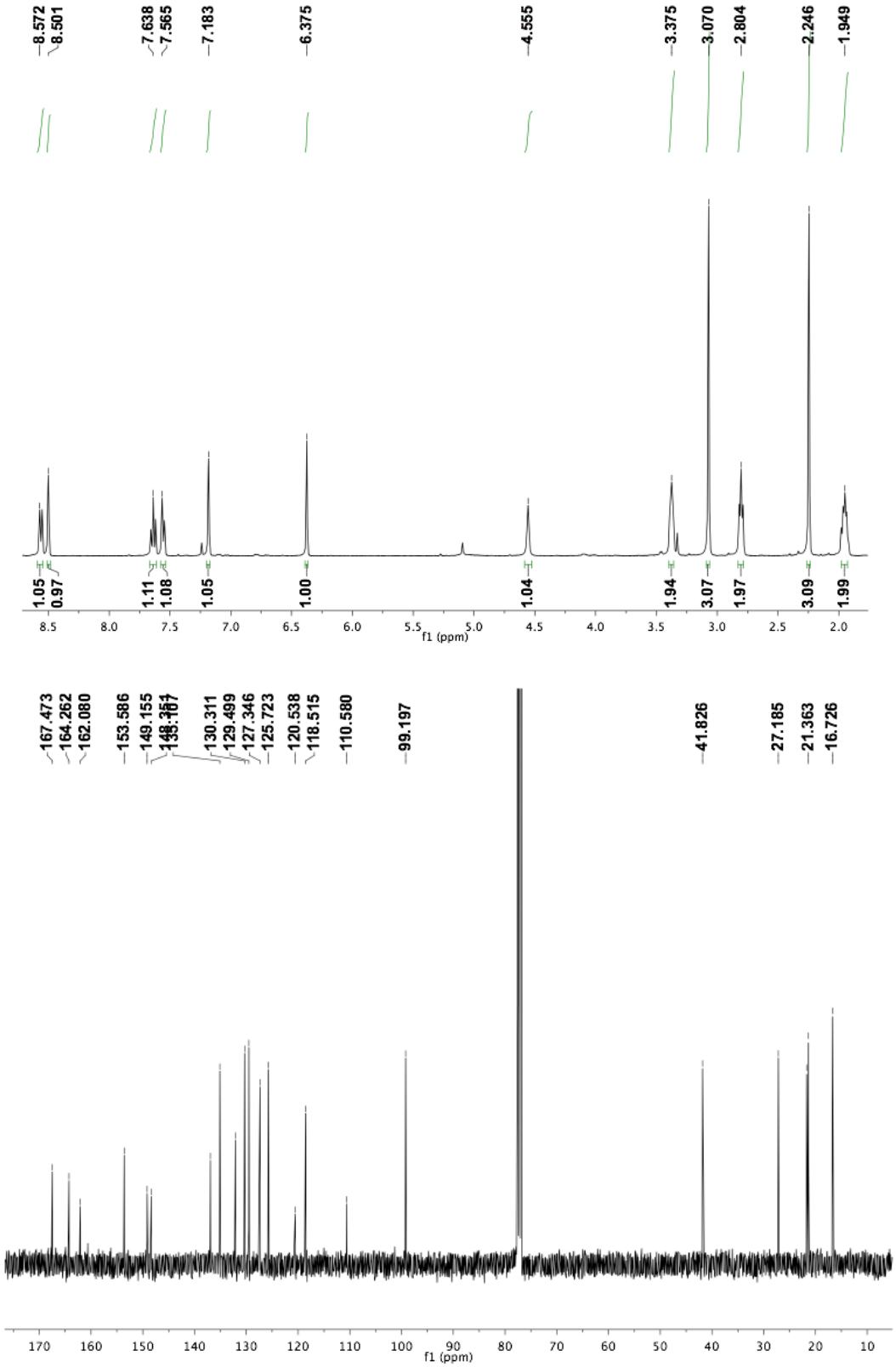
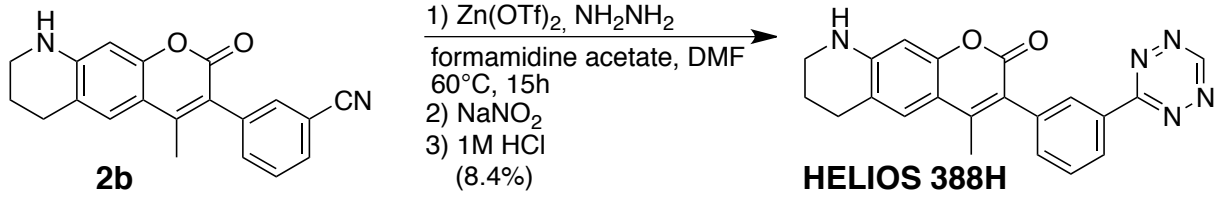


Figure 1.8 ^{1}H and ^{13}C NMR spectra of **HELIOS 388Me** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 388H**:



2b **HELIOS 388H**

To nitrile **2b** (76.7 mg, 0.24 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (44.3 mg, 0.12 mmol), formamidine acetate (251.9 mg, 2.42 mmol), DMF (0.19 mL) and NH_2NH_2 (0.38 mL, 12.1 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (333.9 mg, 4.84 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 3:1 to 1:1) to give **HELIOS 388H** (7.48 mg, 0.020 mmol, 8.4%) as an orange solid.

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 10.62 (s, 1H), 8.50 (d, J = 8.0 Hz, 1H), 8.38 (s, 1H), 7.74 (t, J = 7.6 Hz, 1H), 7.62 (d, J = 7.6 Hz, 1H), 7.33 (s, 1H), 6.86 (s, 1H), 6.36 (s, 1H), 3.28 (m, 2H), 2.76 (t, J = 6.0 Hz, 2H), 2.23 (s, 3H), 1.82 (m, 2H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 165.4, 160.6, 158.1, 152.9, 149.2, 149.0, 136.8, 135.1, 131.7, 129.9, 129.2, 126.7, 125.7, 118.1, 117.7, 108.4, 97.2, 40.4, 26.5, 20.8, 16.2. ESIMS $[M+H]^+$ calcd for $C_{21}H_{18}N_5O_2$ 372.14, found 372.12.

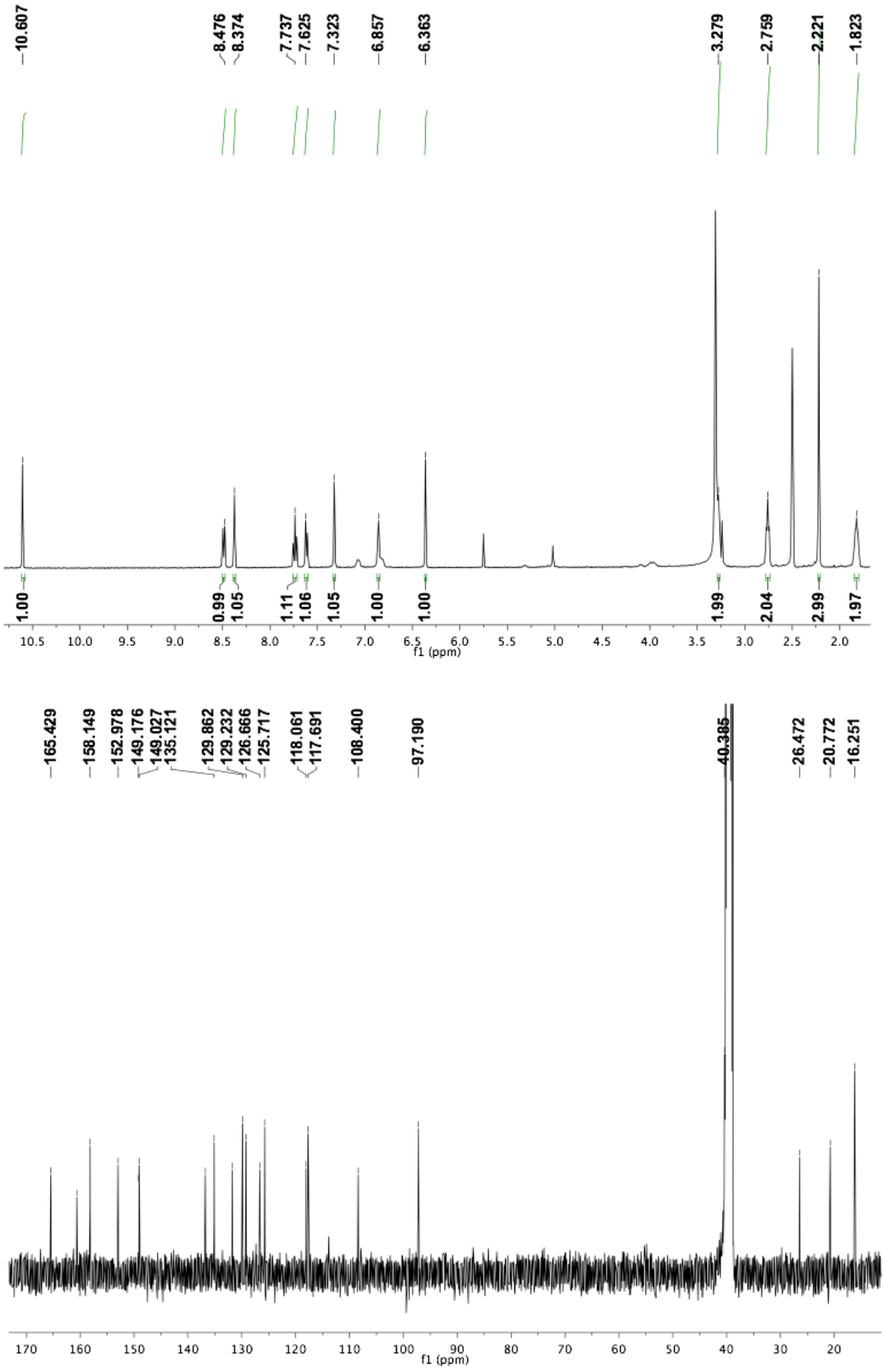
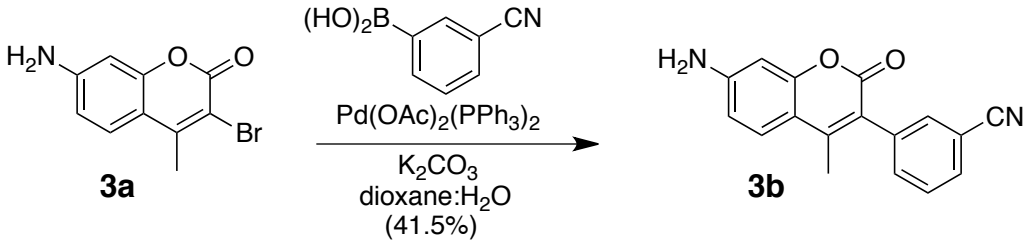


Figure 1.9 ^{1}H and ^{13}C NMR spectra of **HELIOS 388H** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **3b**:



To bromocoumarin **3a** (5.25 g, 20.7 mmol) in 133.0 mL of dioxane:water (3:1) was added 3-cyanophenylboronic acid (4.56 g, 31.0 mmol), $Pd(OAc)_2(PPh_3)_2$ (775.3 mg, 1.03 mmol), and K_2CO_3 (5.71 g, 41.4 mmol). The reaction mixture was refluxed for 2 hours after which it was concentrated using a rotary evaporator. The crude was partitioned between water and methylene chloride and extracted 3 times (250 mL), concentrated using a rotary evaporator and purified using flash column chromatography (methylene chloride:methanol, 10:0.05) to give **3b** (2.38 g, 8.6 mmol, 41.5%) as a white solid**.**

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.82 (m, 1H), 7.78 (s, 1H), 7.64 (m, 2H), 7.50 (d, J = 8.4 Hz, 1H) 6.63 (d, J = 8.8 Hz, 1H), 6.46 (s, 1H), 6.19 (s, 2H), 2.15 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 160.4, 154.6, 153.1, 149.6, 136.7, 135.7, 134.2, 131.1, 129.3, 126.9, 118.7, 117.6, 111.6, 111.2, 108.9, 99.3, 16.2. ESIMS $[M+H]^+$ calcd for $C_{17}H_{13}N_2O_2$ 277.09, found 277.06.

Bromocoumarin **3a** was prepared from literature protocol (M. S. Schiedel, C. A. Briehn, P. Bauerle, Angew*. Chem. Int. Ed.* **2001**, *40*, 4677-4680).

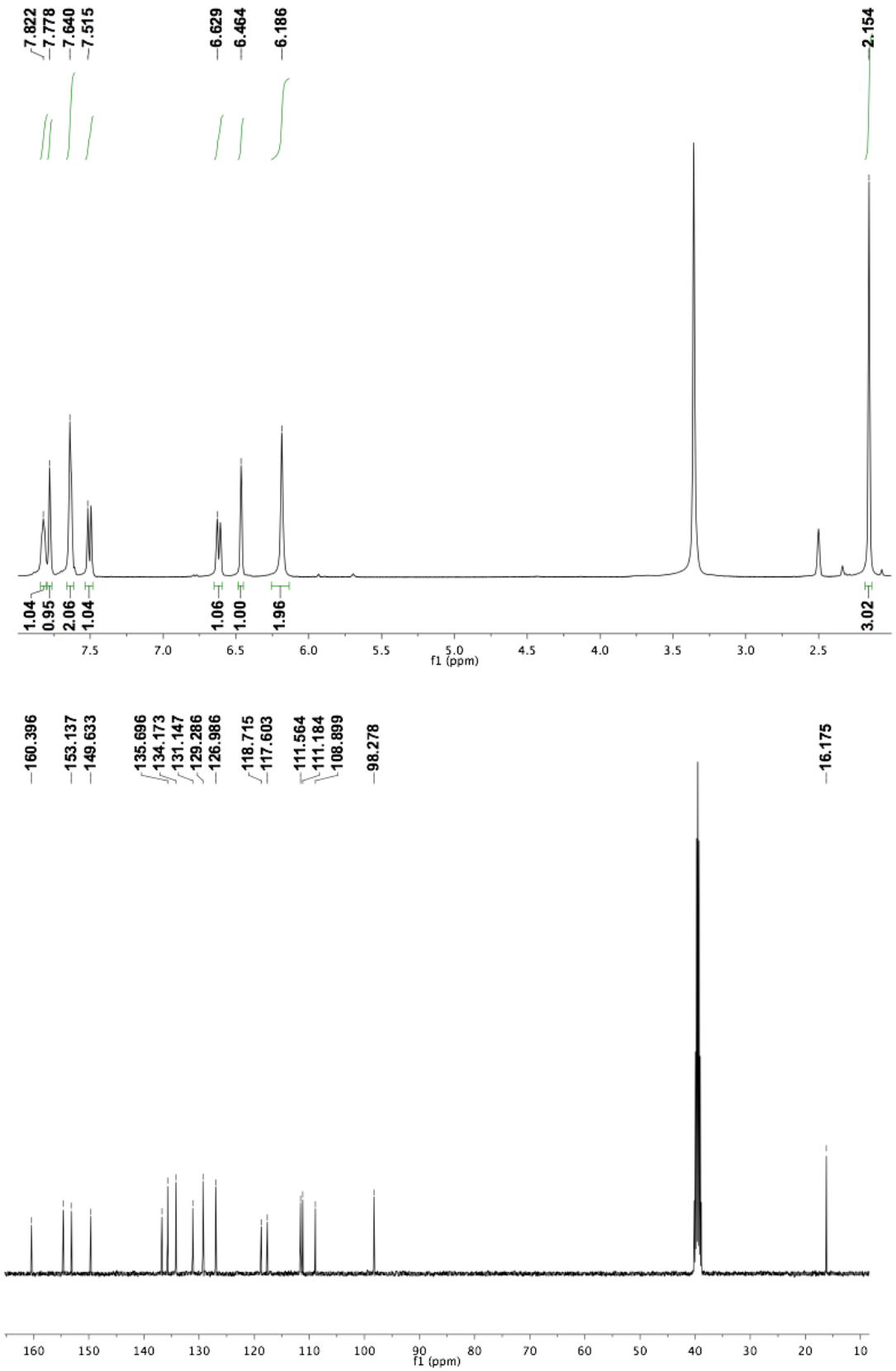
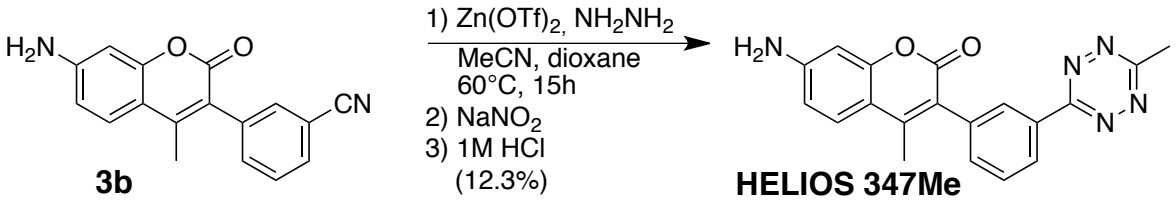


Figure 1.10 ^{1}H and ^{13}C NMR spectra of **3b** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 347Me**:



3b **HELIOS 347Me**

To nitrile **3b** (39.0 mg, 0.14 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (25.8 mg, 0.070 mmol), MeCN (0.073 mL, 1.40 mmol), dioxane (0.11 mL) and NH_2NH_2 (0.22 mL, 7.00 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (193.2 mg, 2.80 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 2:1 to 1:1) to give **HELIOS 347Me** (5.94 mg, 0.017 mmol, 12.3%) as a red solid.

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 8.46 (d, J = 7.6 Hz, 1H), 8.35 (s, 1H), 7.72 (t, J = 8.0 Hz, 1H), 7.60 (d, J = 7.6 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 6.62 (d, J = 8.4 Hz, 1H), 6.48 (s, 1H), 6.16 (s, 2H), 3.00 (s, 3H), 2.22 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 167.1, 163.2, 160.6, 154.6, 152.9, 149.2, 136.5, 134.6, 131.8, 129.5, 129.2, 126.9, 126.4, 118.7, 111.5, 109.0, 98.3, 20.8, 16.2. ESIMS $[M+H]^+$ calcd for $C_{19}H_{16}N_5O_2$ 346.13, found 346.11.

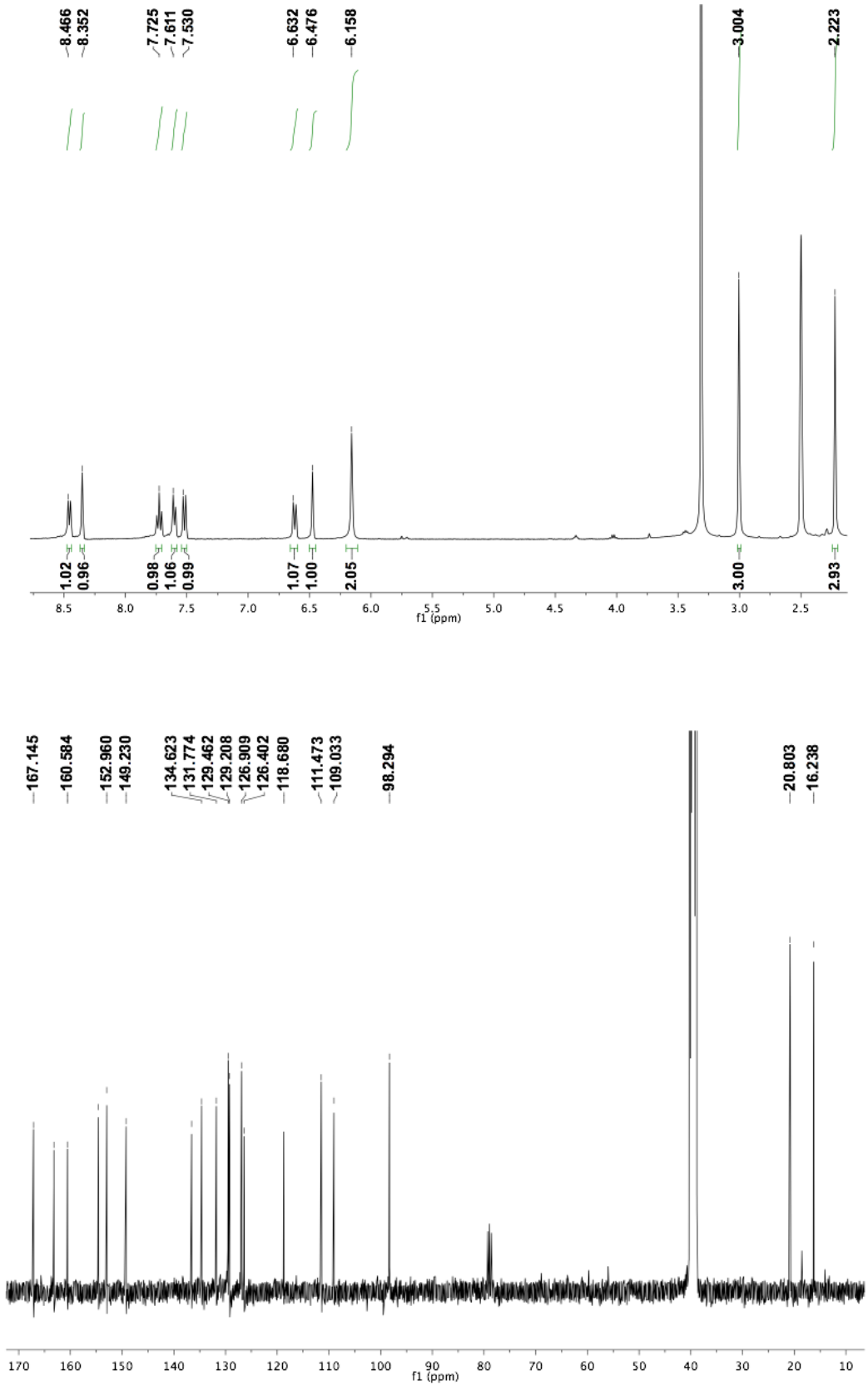
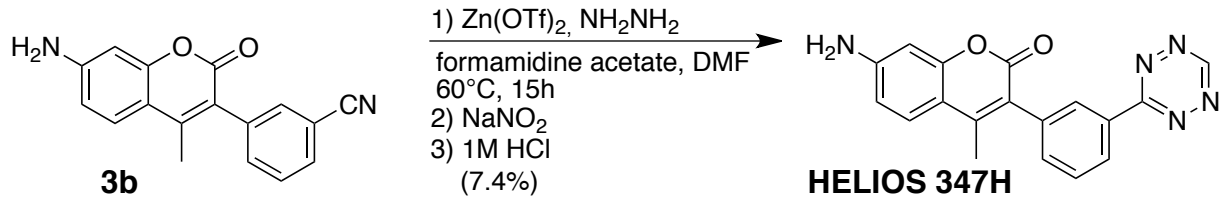


Figure 1.11 ^{1}H and ^{13}C NMR spectra of **HELIOS 347Me** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 347H**:



3b **HELIOS 347H**

To nitrile **3b** (200.0 mg, 0.72 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (132.2 mg, 0.36 mmol), formamidine acetate (749.6 mg, 7.2 mmol), DMF (0.56 mL) and NH_2NH_2 (1.13 mL, 36.0 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (993.6 mg, 14.4 mmol) in 15 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (150 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 2:1 to 1:1) to give **HELIOS 347H** (17.6 mg, 0.053 mmol, 7.4%) as a red solid.

^1H NMR (400 MHz, $(CD_3)_2SO$) δ 10.61 (s, 1H), 8.50 (d, J = 8.0 Hz, 1H), 8.39 (s, 1H), 7.75 (t, J = 8.0 Hz, 1H), 7.63 (d, J = 7.6 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 6.62 (dd, J = 8.8, 2.4 Hz, 1H), 6.48 d (J = 2.0 Hz, 1H), 6.16 (s, 2H), 2.23 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 165.4, 160.6, 158.1, 154.6, 152.9, 149.2, 136.6, 135.0, 131.8, 129.8, 129.2, 126.9, 126.7, 118.6, 111.5, 109.0, 98.3, 16.2. ESIMS $[M+H]^+$ calcd for $C_{18}H_{14}N_5O_2$ 332.11, found 332.09.

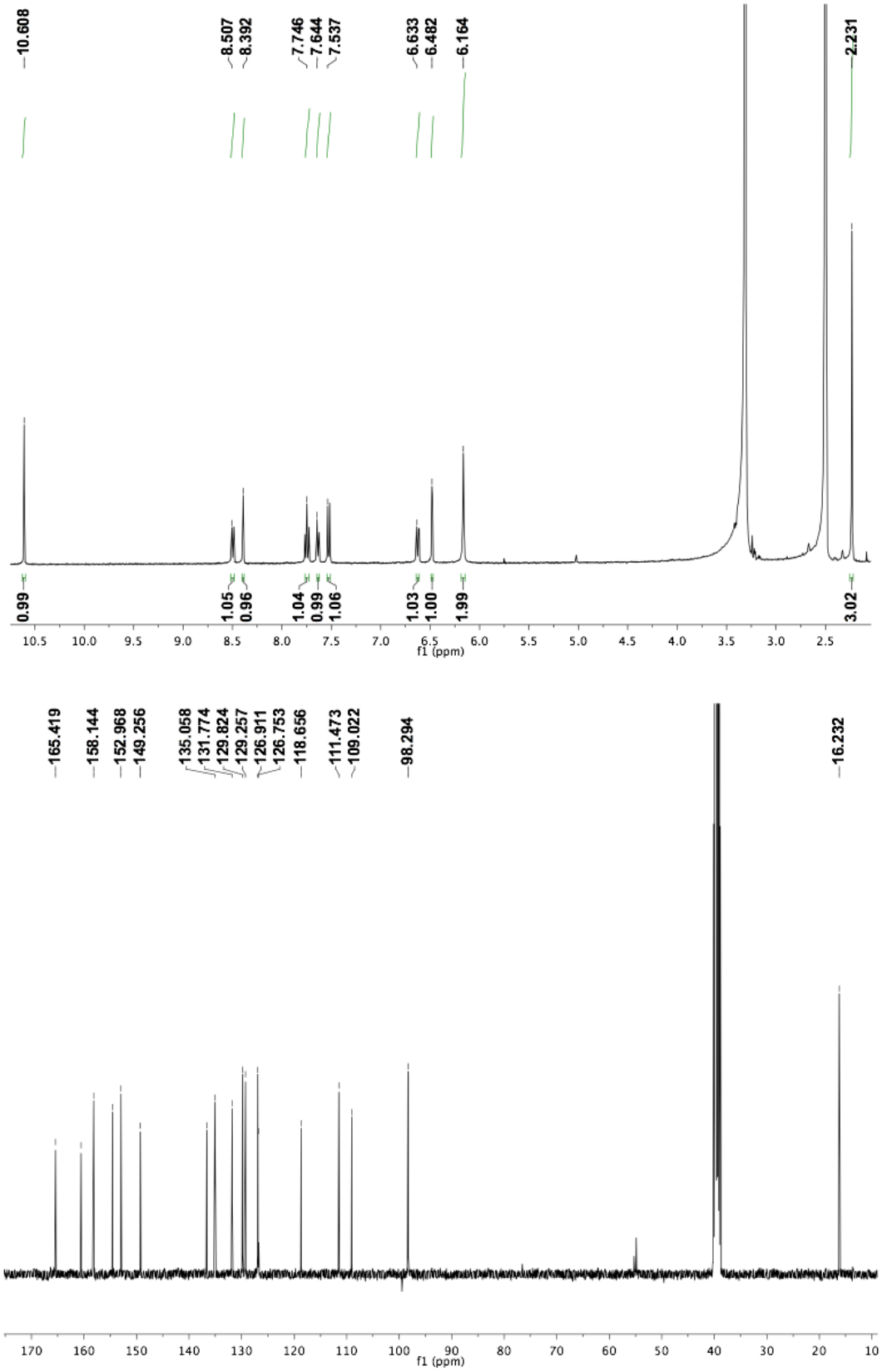
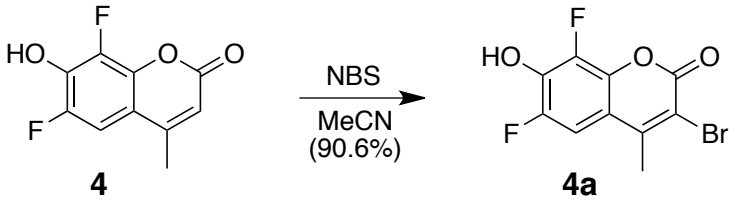


Figure 1.12 ^{1}H and ^{13}C NMR spectra of **HELIOS 347H** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **4a**:



4 **4a**

To difluorinated hydroxycoumarin (Marina Blue®) (**4**) (1.06 g, 4.99 mmol) dissolved in 50 mL of acetonitrile was added NBS (0.93 g, 5.24 mmol) and the reaction mixture allowed to stir for 1 hour. The crude mixture was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate, 2:1) to give **4a** (1.32 g, 4.52 mmol, 90.6%) as a white solid**.**

^{1}H NMR (400 MHz, MeOD) δ 7.30 (d, J = 11.2 Hz, 1H), 2.51 (s, 3H); ^{13}C NMR (100 MHz, MeOD) δ 147.9, 143.3 (t, J = 2.8 Hz), 141.0 (dd, J = 234.7, 5.0 Hz), 131.3 (dd, J = 216.0, 6.4 Hz), 129.9 (m, 2C), 102.7 (d, J = 9.1 Hz), 101.7, 97.7 (dd, J = 19.0, 3.3), 10.4. ESIMS $[M-H]^-$ calcd for $C_{10}H_4BrF_2O_3$ 288.93, found 288.88.

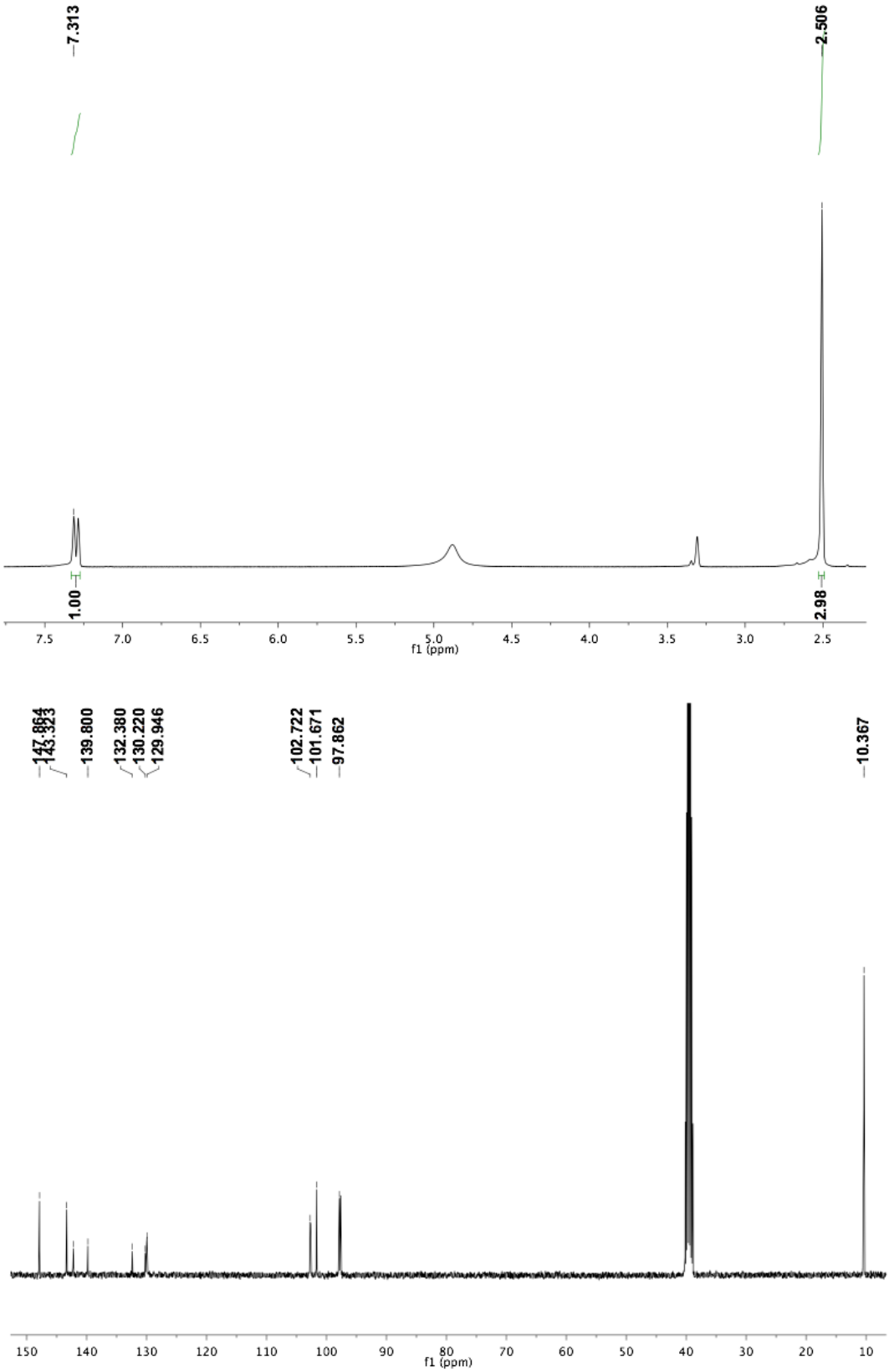
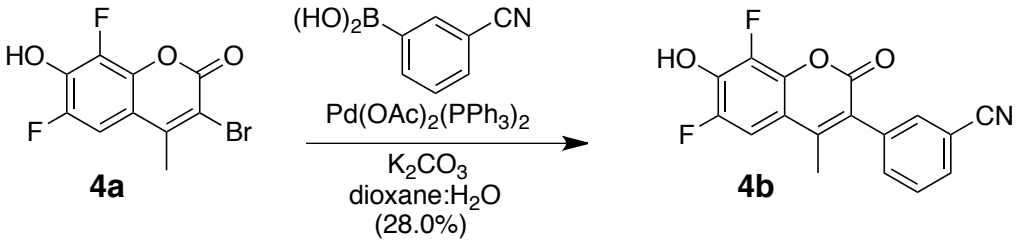


Figure 1.13 ^{1}H and ^{13}C NMR spectra of **4a** recorded in MeOD at 400 MHz and 100 MHz respectively.

Preparation of **4b**:



To bromocoumarin **4a** (475.9 mg, 1.63 mmol) in 15.0 mL of dioxane:water (3:1) was added 3-cyanophenylboronic acid (479.0 mg, 3.26 mmol), $Pd(OAc)_2(PPh_3)_2$ (61.2 mg, 0.082 mmol), and K_2CO_3 (225.3 mg, 3.26 mmol). The reaction mixture was refluxed for 7 hours after which it was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate gradient, 2:1 to 1:1) to give **4b** (142.9 mg, 0.45 mmol, 28.0%) as a white solid**.**

^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.89 (t, J = 3.6 hz, 1H), 7.83 (s, 1H), 7.69 (m, 2H), 7.60 (d, J = 11.6 Hz, 1H), 2.21 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 158.7, 148.8 (t, J = 2.6 Hz), 148.6 (dd, J = 232.6, 5.2 Hz), 139.1 (dd, J = 235.8, 6.8 Hz), 138.5 (dd, J = 7.6, 1.8 Hz), 137.6 (dd, J = 12.7, 5.3 Hz), 135.7, 135.3, 133.8, 131.8, 129.5, 122.6, 118.5, 111.4, 111.1 (d, J = 8.9 Hz), 107.0 (dd, J = 18.6, 2.9 Hz), 16.6. ESIMS $[M+H]^+$ calcd for $C_{17}H_{10}F_2NO_3$ 314.06, found 314.01.

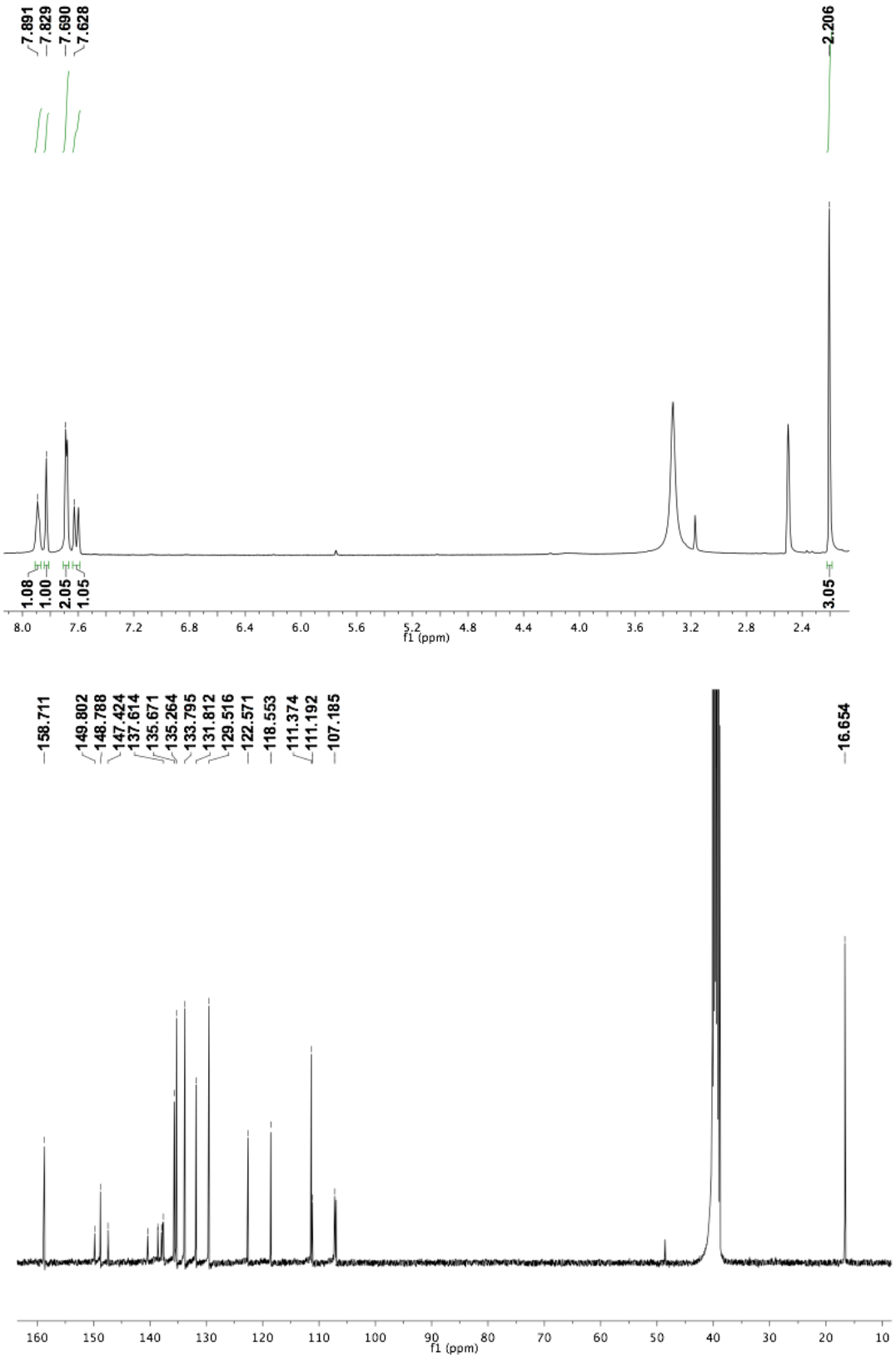
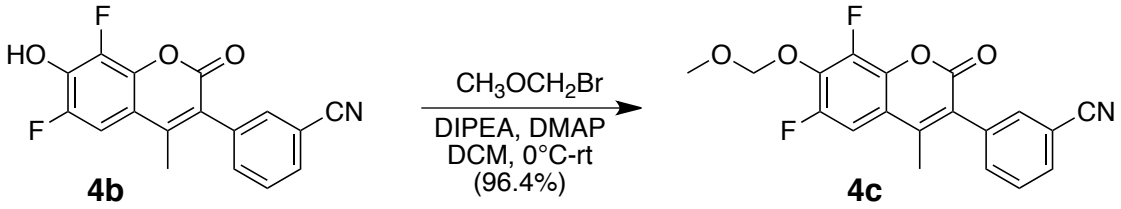


Figure 1.14 ^{1}H and ^{13}C NMR spectra of **4b** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **4c**



To nitrile **4b** (91.1 mg, 0.29 mmol) in 3.0 mL of methylene chloride was added *N,N*-Diisopropylethylamine (0.15 mL, 0.87 mmol) and DMAP (1.77 mg, 0.014 mmol). The mixture was then cooled to 0°C and bromomethyl methyl ether (0.059 mL,0.72 mmol) was added dropwise. The ice bath was removed and the reaction was allowed to stir at room temperature for one hour after which it was concentrated using a rotary evaporator and purified using flash column chromatography (hexanes:ethyl acetate 4:1) to give **4c** (99.9 mg, 0.28 mmol, 96.4%) as a white solid**.**

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, J = 7.6 Hz, 1H), 7.58 (s, 1H), 7.54 (m, 2H), 7.20 (dd, J = 8.8, 2.0 Hz, 1H), 5.28 (s, 2H), 3.59 (s, 3H), 2.25 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.0, 152.5 (dd, J = 241.0, 4.0 Hz), 147.8 (t, J = 2.6 Hz), 144.1 (dd, J = 246.7, 5.8 Hz), 139.1 (dd, J = 8.1, 2.4 Hz), 136.4 (dd, J = 11.2, 4.7 Hz), 135.4, 134.8, 133.9, 132.3, 129.7, 125.7, 118.5, 116.1 (d, J = 8.8 Hz), 113.2, 106.5 (dd, J = 18.6, 3.8 Hz), 99.3 (t, J = 3.8 Hz), 57.6, 17.1. ESIMS $[M+H]^+$ calcd for $C_{19}H_{14}F_2NO_4$ 358.08, found 358.04.

Note: A methoxymethyl ether protecting group was utilized en route to **HELIOS 370Me** and **HELIOS 370H** in order to achieve maximum yields. However, Both probes can be accessed using protecting group free chemistry directly from nitrile **4b** albeit it at lower yields (<10%).

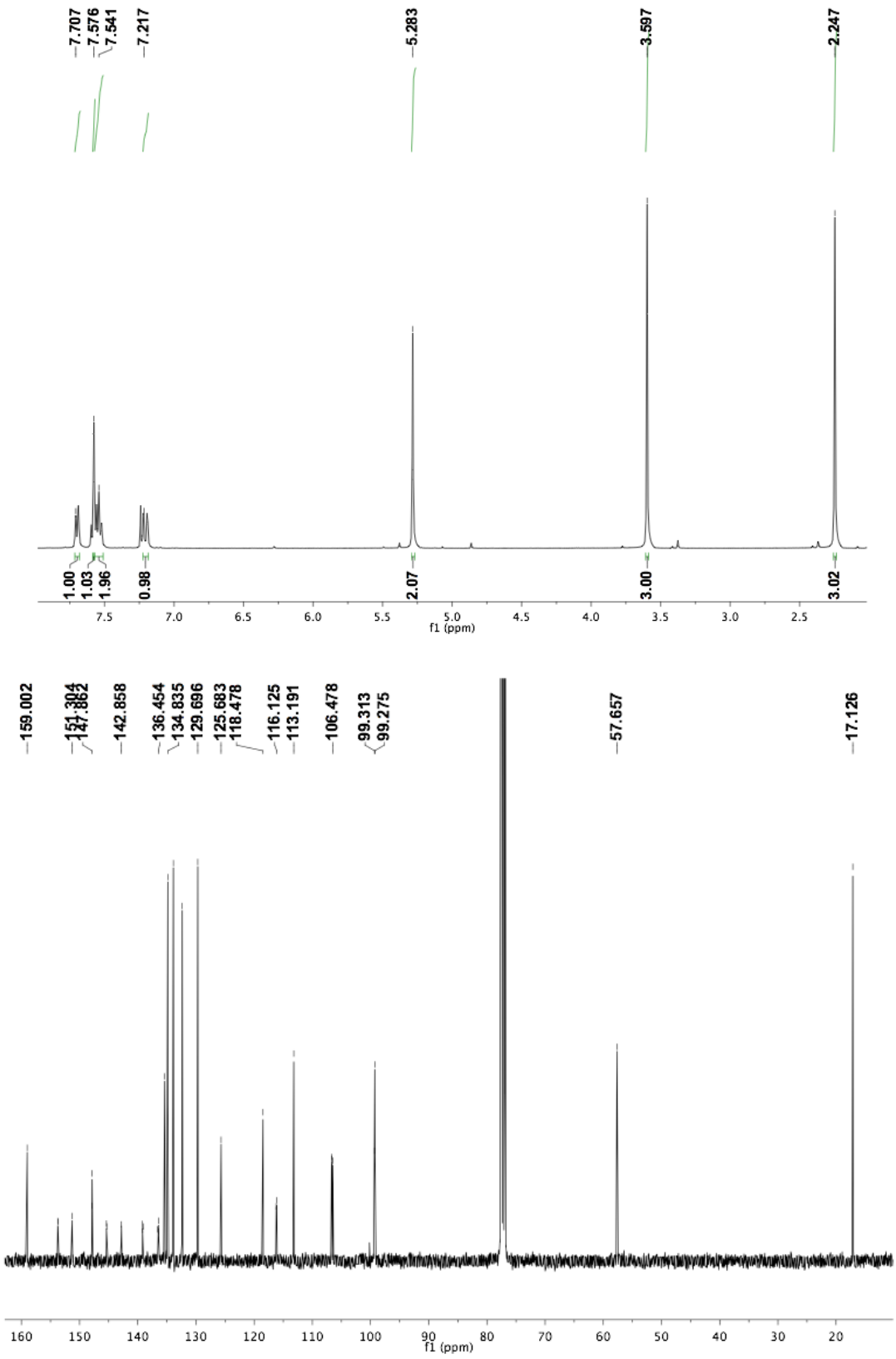
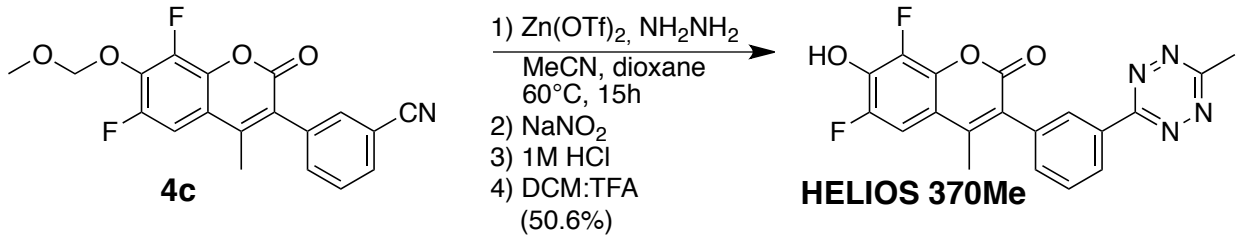


Figure 1.15 ^{1}H and ^{13}C NMR spectra of **4c** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 370Me**:



To nitrile **4c** (144.0 mg, 0.40 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (73.6 mg, 0.201 mmol), MeCN (0.21 mL, 4.03 mmol), dioxane (0.32 mL) and NH_2NH_2 (0.63 mL, 20.1 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (556.1 mg, 8.06 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was filtered through 10g of silica (methylene chloride:methanol, 100:0.5, 100mL) and concentrated using a rotary evaporator. This mixture was then dissolved in 8 mL of methylene chloride and TFA (1 mL) was added and the reaction was allowed to stir at room temperature for 30 minutes. after which it was concentrated under a stream of nitrogen. The crude mixture was purified using flash column chromatography (methylene chloride:methanol, 10:0.1) to give **HELIOS 370Me** (77.4 mg, 0.20 mmol, 50.6%) as a red solid.

^1H NMR (400 MHz, $(CD_3)_2SO$) δ 8.50 (d, J = 7.60 Hz, 1H), 8.40 (s, 1H), 7.76 (t, J = 7.6 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.55 (m, 1H), 3.01 (s, 3H), 2.26 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 167.2, 163.1, 158.9, 148.6 (dd, J = 233.0, 5.3 Hz), 148.3 (t, J = 2.6 Hz), 139.1 (dd, J = 233.1, 9.3 Hz), 138.5 (dd, J = 7.5, 2.0 Hz), 137.4 (m), 135.5, 134.2, 131.9, 129.4, 129.1, 126.9, 123.6, 111.3 (d, J = 9.3 Hz), 107.0 (dd, J = 19.0, 2.7 Hz), 20.8, 16.7. ESIMS $[M-H]^-$ calcd for $C_{19}H_{11}F_2N_4O_3$ 381.08, found 381.06.

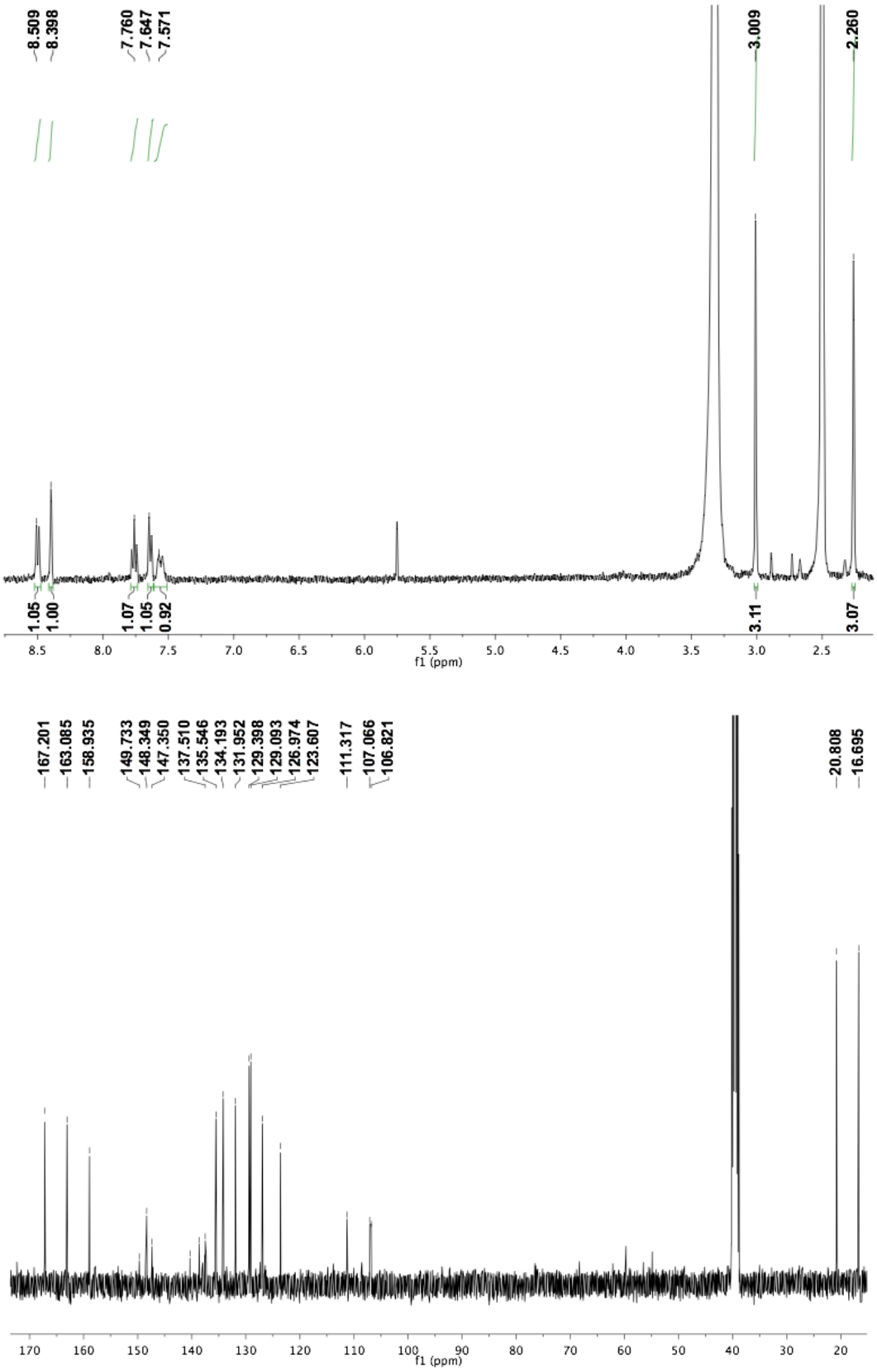
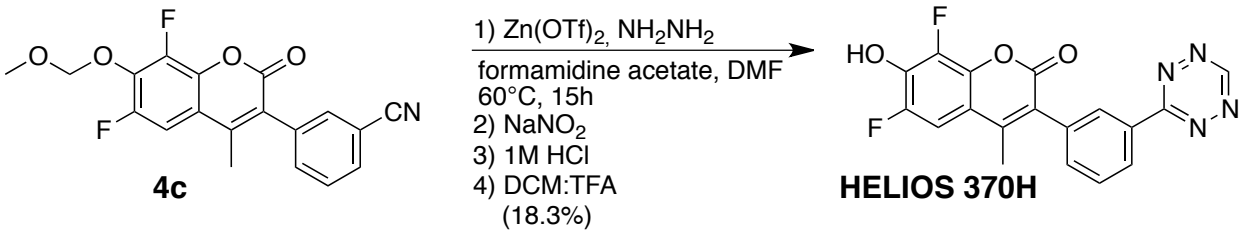


Figure 1.16 ^{1}H and ^{13}C NMR spectra of **HELIOS 370Me** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **HELIOS 370H**:



To nitrile **4c** (144.0 mg, 0.40 mmol) in a microwave reaction tube under a stream of argon was added $Zn(OTf)_2$ (73.6 mg, 0.201 mmol), formamidine acetate (419.6 mg mL, 4.03 mmol), DMF (0.32 mL) and NH_2NH_2 (0.63 mL, 20.1 mmol). The vessel was sealed and allowed to stir at 60°C for 15 hours after which it was allowed to cool and the septum removed. To the reaction mixture was added $NaNO_2$ (556.1 mg, 8.06 mmol) in 10 mL of water followed by 1 M HCl until the pH = 3. The aqueous phase was extracted three times with methylene chloride (100 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated using a rotary evaporator. The crude mixture was filtered through 10 g of silica (methylene chloride:methanol, 100:0.5, 100mL) and concentrated using a rotary evaporator. This mixture was then dissolved in 8 mL of methylene chloride and TFA (1 mL) was added and the reaction was allowed to stir at room temperature for 30 minutes, after which it was concentrated under a stream of nitrogen. The crude mixture was purified using flash column chromatography (methylene chloride:methanol, 10:0.1) to give **HELIOS 370H** (26.9 mg, 0.073 mmol, 18.3%) as a red solid.

^1H NMR (400 MHz, $(CD_3)_2SO$) δ 10.61 (s, 1H), 8.53 (d, J = 8.0 Hz, 1H), 8.44 (s, 1H), 7.78 (t, J = 7.6 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.60 (d, J = 11.6 Hz, 1H), 2.27 (s, 3H); ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 165.3, 158.9, 158.2, 148.5 (dd, J = 232.0, 5.1 Hz), 148.5 (t, J = 2.4 Hz), 139.1 (dd, J = 235.6, 7.2 Hz), 138.5 (dd, J = 9.4), 137.4 (m), 135.5, 134.6, 131.9, 129.5 (2C), 127.3, 123.6, 111.3 (d, J = 8.9 Hz), 107.0 (dd, J = 21.8, 2.8 Hz), 16.7. ESIMS $[M-H]^-$ calcd for $C_{18}H_9F_2N_4O_3$ 367.06, found 366.96.

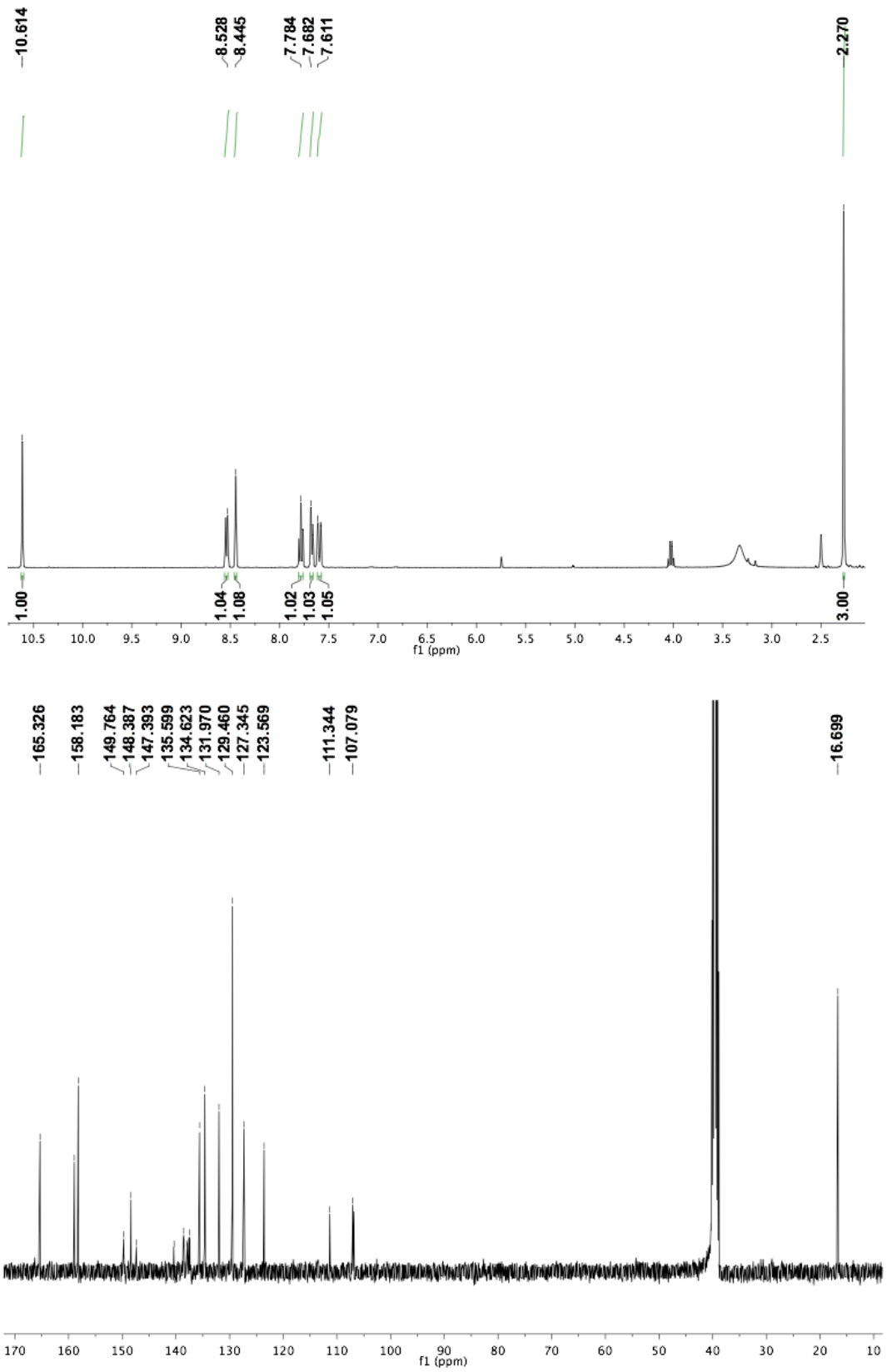
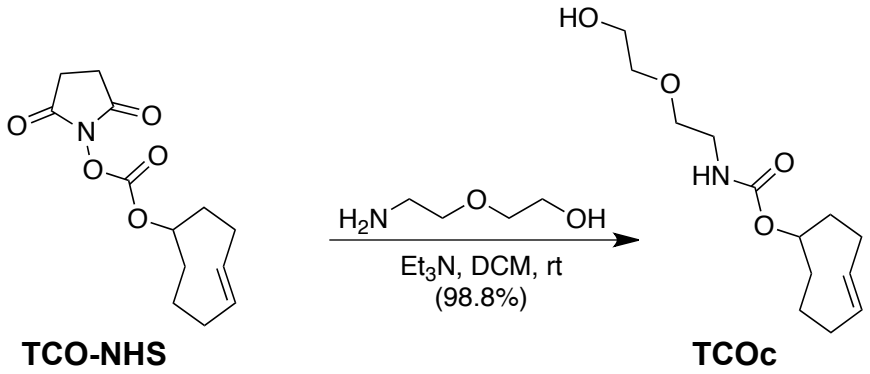


Figure 1.17 ^{1}H and ^{13}C NMR spectra of **HELIOS 370H** recorded in $(CD_3)_2SO$ at 400 MHz and 100 MHz respectively.

Preparation of **TCOc**:



TCO-NHS **TCOc**

To **TCO-NHS** (45.4 mg, 0.17 mmol) dissolved in 2 mL of methylene chloride was added triethylamine (0.05 mL, 0.35 mmol), and 2-(2-Aminoethoxy)ethanol (0.07 mL, 0.71 mmol). The mixture was allowed to stir at room temperature for thirty minutes after which it was concentrated under a stream of nitrogen. The crude mixture was purified using flash column chromatography (hexanes:ethyl acetate gradient, 1:1 to 100% ethyl acetate) to give **TCOc** (43.3 mg, 0.073 mmol, 98.8%) as a clear oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 (m, 2H), 5.03 (m, 1H), 4.31 (dd, J = 9.6, 6.0 Hz, 1H), 3.71 (m, 2H), 3.53 (m, 4H), 3.33 (m, 2H), 2.31 (m, 4H), 2.01-1.85 (m, 4H), 1.77-166 (m, 2H), 1.55-1.51 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.6, 135.1, 133.2, 80.9, 72.4, 70.4, 61.9, 41.3, 40.9, 38.8, 34.5, 32.7, 31.1. ESIMS $[M+H]^+$ calcd for $C_{13}H_{24}NO_4$ 258.17, found 258.37.

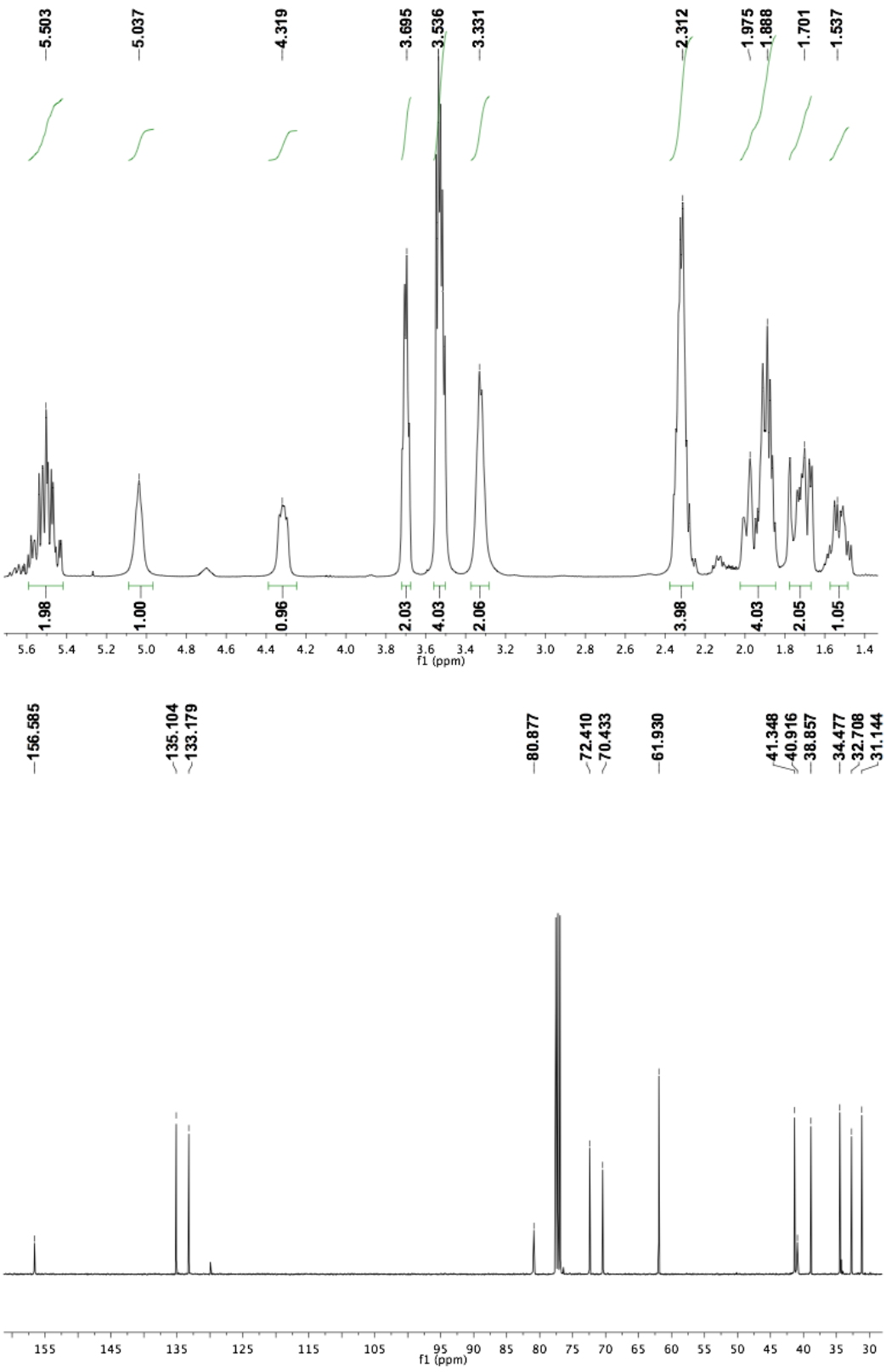
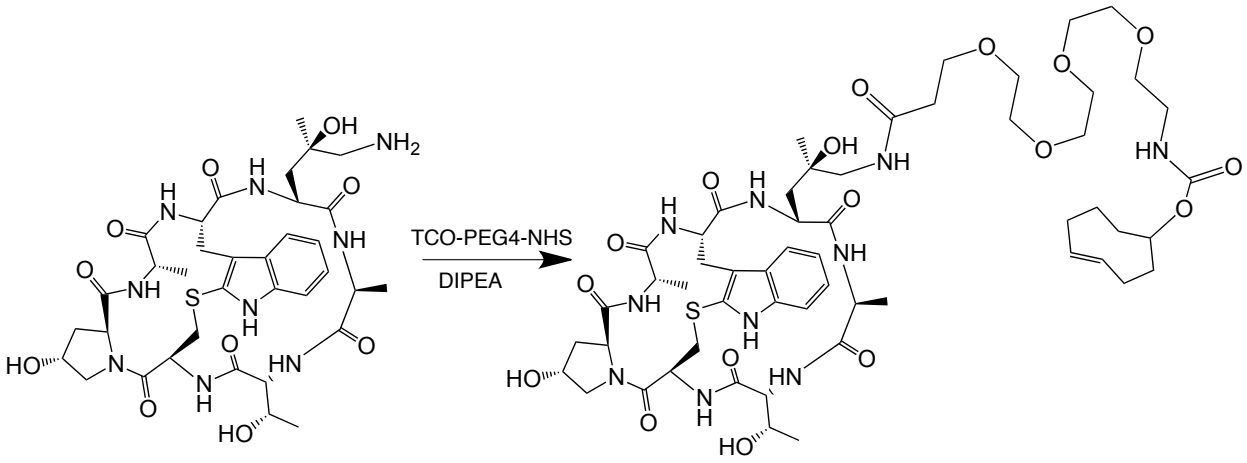
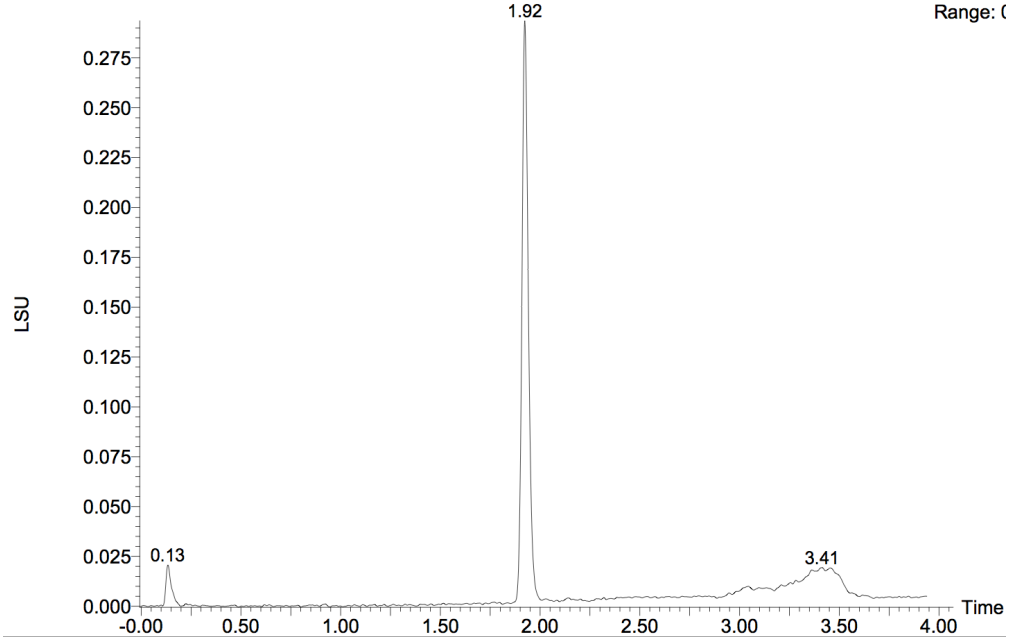


Figure 1.18 ^{1}H and ^{13}C NMR spectra of **TCOc** recorded in $CDCl_3$ at 400 MHz and 100 MHz respectively.

Preparation of **Phalloidin-TCO:**

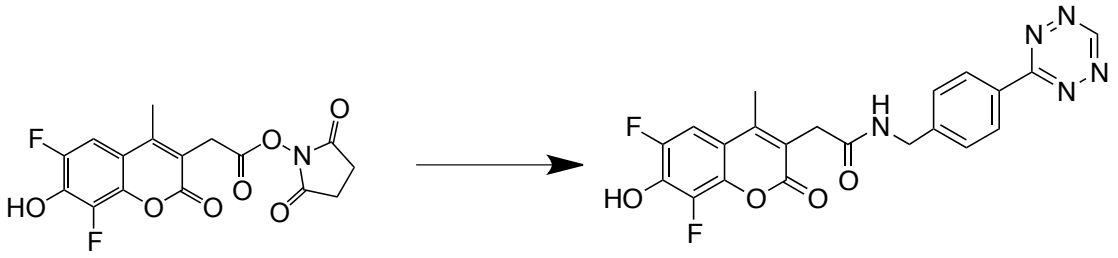


To a 10 mM solution of TCO-PEG4-NHS (70 μL, 0.7 μmoles, Click Chemistry Tools, Scottsdale, AZ) in DMF in a microvial was added amino-phalloidin (60 mcg, 0.07 μmoles, American Peptide Company, Sunnyvale, CA) and diisopropylethylamine (DIPEA, 0.2 uL, 1.1 μmole). After 30 minutes at room temperature with occasional vortex agitation, the reaction mixture was purified by reverse phase chromatography on a Waters Xterra C18, 2.5 μm, 10 mm x 50 mm, column (water:acetonitrile, both with 0.1% formic acid; gradient elution from 5% to 75% acetonitrile) to give phalloidin TCO (58 mcg, 0.05 μmoles, 70%). The amount of aminophalloidin and phalloidin-TCO product were determined spectrophotometrically, based on the known extinction coefficient of phalloidin at 291nm (13,500). Reverse phase LCMS characterization of the purified material: ESI-MS [M+H]+ calculated for $C_{55}H_{82}N_{10}O_{17}S$ 1187.56, found 1187.43.



Elution gradient ramp 5% to 100% acetonitrile:water (0.1% formic acid) from 0 to 3.5 minutes; Waters Xterra C18 5 μm 4.6 x 50 mm column; evaporative light scattering detection.

Preparation of **Marina Blue - Tz:**



To a solution of Marina Blue - succcinimidyl ester (LifeTechnologies, M10165, Grand Island, NY) at 10mM in DMF (50uL, 0.5µmoles) was added 1uL of diisopropylethylamine, followed by a small aliquot of dry benzylaminotetrazine-HCl (MW 223.06). After 30 minutes at room temperature with occasional vortex agitation, the reaction mixture was purified by reverse phase chromatography on a Waters Xterra C18, 2.5 μm, 10 mm x 50 mm, column (water:acetonitrile, both with 0.1% formic acid; gradient elution from 5% to 75% acetonitrile) to give Marina Blue - Tz (yield not determined). Reverse phase LCMS characterization of the purified material: ESI-MS $[M-H]^-$ calculated for $C_{21}H_{15}F_2N_5O_4$ 438.11, found 438.01.